Role of platelets on liver regeneration after 90% hepatectomy in mice

（マウス 90%肝切除後の肝再生における血小板の役割）

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List of Abbreviations:

PH: partial hepatectomy

LR: liver regeneration

HGF: hepatocyte growth factor

IGF-1: insulin-like growth factor-1

TGFβ1: transforming growth factor beta 1

EGF: epidermal growth factor

PDGF: platelet derived growth factor

IL-6: interleukin 6

ERK1/2, extracellular signal-regulated kinase 1/2

TNF-α: tumor necrosis factor-α

PCNA: proliferating cell nuclear antigen

PEG-rHuMGDF: pegylated recombinant human megacaryocyte growth and development factor

ALT: alanine transaminase

STAT3: signal transducer and activator of transcription3

c-Met: mesenchymal epithelial transition factor

EGFR: epidermal growth factor receptor

cRNA: complimentary RNA

IGFBP-1: insulin-like growth factor binding protein 1

IL-1: interleukin 1

VEGF: vascular endothelial growth factor

FGF-2: fibroblast growth factor-2

BAD: Bcl-2-associated death promoter

ASK-1: apoptosis signal-regulating kinase 1

MLK3: mixed lineage kinase 3

GSK3β: glycogen synthase kinase 3 beta

mTOR: mammalian target of rapamycin

JAKs: Janus kinase

Tnfaip3: tumor necrosis factor, alpha-induced protein 3

NF-κB: nuclear factor κB

p21: cyclin-dependent kinase inhibitor 1A

PI3K: phosphatidylinositol 3-kinase

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

1. Introduction

1.1 Research History of the Relationship Between Liver Regeneration and Platelets

Among all organs the liver has a unique regeneration capacity after injury. It occurs due to the hyperplasia of the residual lobes and mitosis of the hepatocytes which are quiescent under normal conditions [1-3]. Liver regeneration (LR) has been a subject of scientific research for a long time, focusing on the replication mechanism, promoting, inhibiting factors and expressed genes [3, 4]. Surgical or chemical liver injury experimental models were used for the stimulation of regeneration. The most commonly used surgical experimental model of LR in mice is 70% partial hepatectomy (PH) which was introduced by Higgins and Anderson in 1931 and considered to be the most optimal for LR study in mice [5].

It is well known that in addition to substances responsible for haemostasis platelets contain in their α-granules various growth factors like HGF, IGF-1, TGFβ1, EGF, serotonin and PDGF which does not contribute to LR [6, 7]. Pioneering studies which connected LR with platelet growth factors were performed by Paul and Piasecki [8]. They established that platelets contain heat stabile substances which promote DNA synthesis in primary adult rat hepatocyte cultures [8]. Recently it was reported that platelet-derived serotonin mediates liver regeneration. By using thrombocytopenic mice it was suggested that platelet-derived serotonin may influence the proliferation of hepatocytes directly or may be involved in the release of growth factors, such as IL-6, at the site of liver injury [9, 10].

Our laboratory has relatively old and rich research history of the relationship between liver regeneration and platelets. In the in vivo study performed by Murata et al.,

where 70% PH model was used, it was discovered that platelets promote liver regeneration in early period after hepatectomy in mice. Platelets accumulated significantly in the livers of thrombocytotic mice [11]. By the means of electron microscopy it was revealed that platelets migrated from the sinusoidal space through the porosities of the endothelial cells to the space of Disse and were in direct contact with hepatocytes at 5 minutes after hepatectomy in thrombocytotic group. Platelet accumulation in the liver induced strong activation of the Akt pathway in hepatocytes which resulted in the increase of LR markers such as PCNA, Ki-67 labeling indices and liver/body weight ratios [11]. In the in vitro study performed by Matsuo et al. using primary hepatocyte cell cultures and immortalized hepatocytes (TLR2), it was revealed that platelets strongly induce hepatocyte proliferation by the release of the growth factors such as IGF-1 and HGF [12]. Corresponding with the in vivo study it was shown that direct contact between platelets and hepatocytes was necessary for the proliferative effect. After the addition of platelets to the hepatocyte cultures Akt and ERK1/2 signaling pathways were activated [12]. Furthermore, it was proved that platelets promote LR even under conditions of Kupffer cell depletion after 70% PH. In Kupffer cell depleted group hepatocyte proliferation was delayed due to the decreased level of TNF-α which is produced by these cells and acts as an initiator of the priming phase of LR [13]. However, the regeneration failure was improved significantly under the condition of combined Kupffer cell depletion and thrombocytosis. Platelets caused stimulation of HGF and IGF-1 expression. As a result Akt signaling pathway was activated and sufficient LR occurred [13].

In our laboratory in vivo research of the relationship between platelets and LR was not limited only to surgical hepatectomy model. Several studies were performed

which used a model of chemically induced liver fibrosis to clarify the role of platelets on LR in this case as well as their influence on fibrosis itself. Carbone tetrachloride (CCl_4) or dimethylnitrosamine were used as inducers of liver fibrosis. In one study performed by Watanabe et al. it was revealed that platelets contribute to the reduction of liver fibrosis and promote LR even under cirrhotic condition in mice [14]. The proof of more significant LR were increased liver volume, PCNA labeling index, and mitotic index in fibrotic liver mice with combined thrombocytosis compared to mice without platelet increment. Platelets suppressed mRNA of profibrotic factor TGFβ and increased expression of antifibrotic metalloproteinase-9 in the liver. In addition to that various genes were overexpressed under the condition of thrombocytosis. Many of these genes were involved in cell proliferation [14]. Another research investigated LR in combined liver fibrosis and 70% PH model under thrombocytotic condition in rats. In fibrotic livers regeneration was promoted significantly under the influence of platelets, which was proved by the increased mitotic index, PCNA labeling index and liver/body weight ratio, compared to the regeneration of fibrotic livers without any treatment [15]. Additionally, in platelet increment group the area of fibrosis was significantly reduced compared to platelet normal group. However, administration of the platelet antibody inhibited the antifibrotic effect and proliferation of liver, providing one more proof that only platelets but not thrombopoietin itself possess stimulatory effect on LR [15]. Therefore, it was summarized that, induced by thrombopoietin administration, increment and accumulation of platelets in the cirrhotic liver reduces liver fibrosis and stimulates LR after hepatectomy [15].

Moreover, the method of platelet freeze-drying was developed in our laboratory for the purpose of platelets successful preservation with the minimal decrease of their

properties as well as increase of their storage period [61]. Freeze-dried platelets kept their morphological countenance and responded to the agonist thrombin. They stored adenine nucleotides, PDGF and IGF-1 in almost same amounts as fresh platelets. And, what is more important in the case of our research, freeze-dried platelets preserved their proliferative effect on hepatocytes identical to that of fresh platelets [65].

1.2 Preliminary Studies of 90% Partial Hepatectomy

At first 90% PH in rats was associated with 100% mortality, until the introduction of improved operation technique in 1984 by Gaub and Iversen. In their study the mortality rate was only 14%. Measurements of total liver DNA, 3[H] thymidine labeling index and mitotic index suggested rapid cell proliferation [16]. After that the model of 90% PH was widely used in rat LR studies. For example, in one study by the means of 90% PH there was made an attempt to activate bipotent hepatic progenitor cells in rats [17]. Although the LR was substantially slower after 90% PH [18], it was established that only 95% PH was lethal for rat [19, 20]. However, regenerative reply to this kind of surgical stress in mouse was different compared to rat. Makino et al. in their study, which aimed to find a safe limit of experimental liver resection, revealed that an extra massive 90% PH was lethal for mice. The cause of death was associated with severe acute liver failure induced by small residual liver [21]. All 90% hepatectomized mice in that study died within 24 hours while all 70% hepatectomized mice survived until one week. Liver histology revealed more prominent vacuolar degeneration; serum aminotransferase and total bilirubin levels were significantly higher in 90% hepatectomized mice. Until now, 90% PH has been described as a perfect experimental model of acute liver failure in mice [21, 22]. The reason of safe liver resection limit difference between mouse and rat remains to be

unknown but probably it occurs because of earlier LR induction in rat [3].

In the present study we continued the investigation of platelets' role in LR using a thrombocytosis model in mice after 90% PH which was previously considered as lethal. It was challenging by the use of stimulatory substance such as thrombopoietin to induce platelet increment which would promote sufficient LR, prevent acute liver failure and save the animals. Mice were divided into two groups as follows; normal group: mice without platelet number elevation and thrombocytotic group: mice with raised platelet count. Serum parameters, biological markers, signal transduction and overexpressed genes were investigated and compared in the proliferating livers of both groups. This is the first report which clarifies that platelet increment (thrombocytosis) has a stimulatory effect on LR in mice even after extra massive 90 % hepatectomy.

2. Materials and Methods

2.1 Animals

Eight week old male BALB/C mice weighting 24-28 g were purchased from Clea (Japan) and utilized in the experiment. All mice were maintained in a temperature-controlled room on a 12-hour light-dark cycle, with free access to water and standard chow during whole experiment. After one week of acclimation period mice were divided into two groups as follows; normal group: mice without platelet number elevation, and thrombocytotic group: mice with a raised platelet count (n=3-7 in each group, depending on the time point of sacrifice). All animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba and in accordance with the Regulation for Animal Experiments in our university and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology.

2.2. Induction of Thrombocytosis

To induce thrombocytosis we used pegylated recombinant human megacaryocyte growth and development factor (PEG-rHuMGDF) [23, 24] which was kindly donated by Kirin Brewery Co (Takasaki, Japan). To investigate the dose effect of PEG-rHuMGDF on platelet count elevation, it was injected intraperitoneally in the doses of 4, 20, 40, 60 μg/kg body weight into five mice per each group 5 days before measuring. Mice in the thrombocytotic group received intraperitoneal injection of PEG-rHuMGDF at a dose of 20 μg/kg five days before operation [25].

2.3 Surgical Procedure and Anesthesia

Ninety percent hepatectomy was performed in both groups. This procedure is a modification of Higgins-Anderson operation [5] – removing the left lateral, left median and right median lobes using a single ligature (70% PH), afterwards resecting the right lateral lobe (20%) and leaving only the caudate lobe. Hepatectomy was carried out under ether anesthesia.

2.4 Liver Tissue Collection

Five mice from each group were sacrificed and liver tissue was collected without performing PH to investigate the effect of PEG-rHuMGDF administration. Resected liver tissue was collected and used in the experiments as 0 hour sample. At 2, 6, 12, 18 and 24 hours after PH mice were sacrificed. Regenerated liver samples were collected and wet remnant liver weight was measured. Liver weight/body weight ratio was verified (LW/BW × 100%) for each mouse. Mean value was calculated for each group at each time point. Tissue was divided into three specimens. The first was immediately frozen in the liquid nitrogen. The second was immersed into the OCT compound (Sakura Fine technical, Tokyo, Japan) and quick-frozen in the liquid nitrogen. Both were kept at -80°C until use. The third specimen was fixed in 10% buffered formalin for the following histological and immunohistological analyses.

2.5 Platelet Count and Serum Parameters

Blood was collected from the heart of sacrificed mice in the quantity of 0.5-0.8ml. The number of platelets was checked by a platelet count analyzer (Horiba Ltd., Micros abc, France). Blood was centrifuged for 10 minutes at 4°C at 3,500 rpm. Supernatants were collected and stored at -80°C until tested by a serum multiple biochemical analyzer (Fuji Drichem; Fuji Film Inc., Tokyo, Japan) for measuring total

bilirubin, serum albumin, ALT, glucose, cholesterol and triglyceride levels.

2.6 Histology and Immunohistochemistry

Liver tissue, fixed in 10% buffered formalin, was used for histological and immunohistological analyses. All samples were stained in hematoxylin-eosin. Proliferating cell nuclear antigen (PCNA) staining kit was purchased from Zymed Laboratories Inc. (San Francisco, CA). Liver sections were incubated with PCNA antibody and the ratio of PCNA positive/total hepatocytes was calculated. Liver sections were also incubated with Ki-67 antibody (YLEM, Rome, Italy). During the evaluation of PCNA the number of cells was counted in five different viewpoints per each slide. The detection of apoptosis was performed using terminal transferase and biotin-16-dUTP (TUNEL-Enzyme Method) (TaKaRa Bio Inc, Otsu, Japan). OCT compound immersed tissue samples were used for the detection of platelets in the residual liver 2 hours after PH. 5 μm cryostat sections were fixed in acetone. To detect platelets frozen sections were stained with anti-mouse platelet monoclonal antibody Pm-1 which was prepared as previously described [26].

2.7 Enzyme-Linked Immunosorbent Assay for HGF

Immunoreactive HGF was measured by rat specific HGF ELISA kit (Institute of Immunology, Tokyo, Japan) which crossreacts with mouse HGF as well. 18 hour samples of both groups were utilized in the experiment. Procedure was carried on according to the manufacturer's instruction.

2.8 Immunoblotting

Liver tissue extracts were prepared from the liquid nitrogen snap-frozen specimens in such a way: 20 to 30 mg of liver tissue was homogenized in a buffer consisting of 150 mmol/L NaCl, 50mM TrisCl, 1% NP-40, 10 mmol/L NaF, 1 mmol/L

Na_3 VO_4 and proteinase inhibitors. The samples were kept at 4°C for 2 minutes, and then centrifuged for 10 minutes at 4°C at 15,000 rpm. Supernatants were stored at -80°C until analyzed. Liver lysates of 0, 2, 6, 12, 18 and 24 hour timepoints in the quantity of 2.5 μg per lane were subjected to SDS-PAGE gels. Proteins were transferred to a nitrocellulose membrane (Millipore, Bedford, MA). Immunoblots were developed using polyclonal antibodies against phosphoserine 473 Akt (9271), total Akt (9272), phosphotyrosine 705 STAT3 (9131), total STAT3 (9132), phospho ERK1/2 (9101), total ERK1/2 (9102), phosphotyrosine c-Met (3135), total c-Met (3127), phosphotyrosine EGFR (2236) and total EGFR (2232) (Cell Signaling Technology, Beverly, MA). Immunoblots were analyzed by enhanced chemiluminescence.

2.9 Microarray Analysis of Gene Expression

Liver tissue was extracted out of three mice from each group at 2, 6, 12 and 24 hours after 90% PH. Total RNA was isolated from 50-100mg of snap-frozen liver tissue using the Isogen method (Nippon gene, Tokyo, Japan). Quality of total RNA was evaluated by spectrophotometry and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Agilent low RNA input fluorescent linear amplification kit (Cat. No. 5184-3523, Agilent) in the presence of Cy3- and Cy5-CTP was applied to perform in vitro transcription. Oligo microarray hybridization was performed by using fluorescent-labeled cRNA according to the manufacturer's guidelines. In situ hybridization kit plus (Cat. No. 5184-3568, Agilent) was used for the preparation of hybridization solution. Hybridization was performed at 60°C for 17 hours in the hybridization incubator by using Agilent 44K mouse oligo microarray slides (G2534-60003, Agilent). After hybridization slides were washed, dried and scanned by means of Agilent DNA microarray system (Agilent). Feature extraction 7.1 software

(Agilent) was used for data analysis. Schematically the procedure of mRNA extraction and microarray analysis are shown in Figure 1.

2.10 Measurement of Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) Gene Expression by Real-time PCR

An aliquot of each RNA sample was mixed with an amount of RNA fragment synthesized from pEGFP-C1 vector (Invitrogen Co, Carlsbad, CA). The resulting mixture was subjected to synthesis of the first-strand cDNA. The procedure of RNA synthesis from EGFP and Real-time PCR were the same as previously reported [27]. The Real-time PCR was performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using 15 μl of reaction mixture containing 3 μl of cDNA, 1x SYBR Green Real-time PCR Master Mix Plus (Toyobo Co, Osaka, Japan), 0.4 μM IGFBP-1 primer pair (forward: 5'-ATCTGCCAAACTGCAACAAG-3', reverse: 5'-GACCCAGGGATTTTCTTTC-3'), or 0.4 μM EGFP primer pair (forward: 5'-CAGCAGAACACCCCCATC-3', reverse: 5'-GAACTCCAGCAGGACCATGT-3').

2.11 Statistical Analysis

All data are expressed as the mean ± standard deviation of the samples. Unpaired t-test was used for the comparison between two groups. Comparisons between various time points were performed using one-way ANOVA. Significant data were examined by Bonferroni-Dunn multiple comparisons post hoc test. *P* values less than 0.05 were considered to be significant. Kaplan-Meier estimator was used for the survival rate evaluation.

3. Results

3.1 Platelet Count

PEG-rHuMGDF exhibited a significant dose dependent effect on platelet count elevation (Fig. 2A). The number of platelets was significantly higher in thrombocytotic group compared to normal group in all time points, except 2 hour timepoint where only tendency was observed. In thrombocytotic group the highest platelet number appeared to be at 12 hour timepoint, slightly decreasing afterwards but still remaining to be significantly higher compared to normal group (Fig. 2B). Platelet count remained to be significantly elevated in thrombocytotic group until 48 hours after the operation.

3.2 Survival Rate

Eleven mice from each group were operated for verification of the survival rate. Mice in which acute portal vein congestion occurred immediately after the operation were not included in the evaluation of the results. All mice from normal group died within 30 hours after PH. Three mice from thrombocytotic group survived one week after the operation. According to the obtained results, the survival rate of thrombocytotic mice after 90% PH was 54.5% and 27.3% ($P<0.05$), at 30 hours and one week, respectively (Fig. 3).

3.3 Liver Regeneration

Liver weight increased gradually after the operation in thrombocytotic group. Starting from 6 hours after PH liver weight/body weight ratio was significantly higher in thrombocytotic group compared to normal group (Fig. 4).

3.4 Histological Findings

Hydropic and necrotic changes were observed in zone 1 and neutrophils were

visible in sinusoids of normal group livers. On the other hand, in thrombocytotic group nuclei of hepatocytes appeared to be almost normal. In both groups congestion was not observed (data not shown).

The number of immunohistochemically stained platelets in the liver tissue 2 hours after 90% PH was much higher in thrombocytotic group ($p<0.05$) (Fig. 5A and B).

Mitotic activity was not observed in the livers of nonoperated mice which received PEG-rHuMGDF injections 5 days before sacrifice. There were no mitotic and PCNA positive cells (data not shown) and the result indicates that PEG-rHuMGDF does not stimulate hepatocyte proliferation in the intact liver.

The number of PCNA positively stained cells was much higher in thrombocytotic group 24 hours post hepatectomy (Fig 6A). Some areas in the livers obtained from the normal group contained no PCNA positively stained cells. PCNA labeling index was significantly higher in thrombocytotic group in contrast to normal group 24 hours after PH ($p<0.01$) (Fig. 6B). All liver tissue samples were negative for PCNA at 18 hour and for Ki-67 antigen at 18 and 24 hour time points after the operation (data not shown). The apoptotic cells were not detected neither in thrombocytotic, nor in normal group by TUNEL-Enzyme staining.

3.5 Enzyme-linked Immunosorbent Assay for Hepatocyte Growth Factor

The levels of HGF in the liver tissue 18 hours after the operation were nearly on the same level in both groups (data not shown).

3.6 Serum Parameters

Serum albumin levels were decreasing in both groups after the operation, but more rapidly in normal group. Starting from 18 hours its level was higher in

thrombocytotic group and at 24 hours there was a significant difference with higher levels in thrombocytotic group ($p<0.01$) (Fig. 7A). Serum cholesterol levels were higher in thrombocytotic group at all time points with significant differences at 2 hours and 24 hours after the operation ($p<0.01$) (Fig. 7B). There was a tendency of a more rapid decrease of total bilirubin levels in thrombocytotic group, though no significant difference between the groups was observed ($p<0.133$) (data not shown). The levels of glucose, ALT and triglycerides were almost the same in both groups (data not shown).

3.7 Phosphorylation of Akt, STAT3, ERK1/2, c-Met and EGFR

In thrombocytotic group, Akt was phosphorylated at 2 hours and became strongly phosphorylated at 6, 12 and 18 hours after PH. In normal group phosphorylation started at 6 hours and was visible at 12 and 18 hours after the operation, but was much weaker compared to thrombocytotic group. Phosphorylation of STAT3 started much earlier in thrombocytotic group beginning at 2 hours and continuing at 6 hours after the operation. In normal group STAT3 phosphorylation started at 12 hours and was still proceeding at 24 hours after PH. Phosphorylation of c-Met was stronger and maintained longer in thrombocytotic group. (Fig. 8). We did not observe any difference in the phosphorylation of ERK1/2 and EGFR in both groups.

3.8 Microarray Analysis of Gene Expression

In total, 240 genes were overexpressed more than 3-fold in thrombocytotic group compared to normal group. All overexpressed genes were divided into groups according to their functions. The most important genes for LR are shown in Table 1. Several genes were overexpressed at two different time points. There was no big difference between gene expression at 2 and 6 hours after the operation; however, at 12

and 24 hours a variety of transcription factors, cell cycle, signal transduction, metabolism and transport genes were significantly overexpressed.

3.9 Real-time PCR Quantification of IGFBP-1

IGFBP-1 was induced in both groups after PH. In normal group IGFBP-1 level was almost the same from 2 to 6 hours and had a peak value at 12 hours after PH. In thrombocytotic group the peak value was observed even at 2 hours and moderately decreased afterwards (Fig. 9).

4. Discussion

4.1 Extra Massive 90% Partial Hepatectomy and Trials of Rescue

Extra massive 90% PH in mice in contrast to widely used 70% PH has been described as an acute liver failure model. Few studies which tried to develop the methods of rescue focused on transplantation of specially engineered hepatic tissue or antiapoptotic pentapeptide V5 treated monkey hepatocytes on the surface of the spleen [22, 28] and subcutaneous implantation of liver-assist device which contained embryonic stem cells derived hepatocytes [29]. In the mentioned above studies it was reported about improving of the metabolism and detoxication ability of the liver and increasing of the animals' survival rate. However, no studies were conducted which tried to promote LR and rescue mice using stimulatory substances or growth factors. Only one recent study relative to our own concern is reporting on a positive regenerating effect after the administration of IL-6 into mice which underwent 87% PH [30]. Our previous studies showed that platelets promote liver regeneration and strongly induce hepatocyte proliferation [11, 12]. In our current study we are focusing on the role of platelets in the LR after extra massive hepatectomy. Platelets accumulated in a large quantity in the residual liver of thrombocytotic mice, and the survival rate was significantly higher. Signal pathways were activated earlier and stimulatory genes were overexpressed. These results clearly indicate that platelets are strongly contributing into LR.

4.2 Albumin and Cholesterol as Prognosis Factors

Albumin is a marker of a synthetic function of the liver. It increases the ability of serum to bind endo and exogenous toxins, improves wound healing and bowel

function [31, 32]. Albumin is considered to be an important independent prognostic factor of morbidity and mortality in patients with the acute and chronic illness, including patients in postoperative period. Its serum concentration is inversely related to the mortality risk [33-35]. In another research it was reported that not only albumin but cholesterol level as well exhibited the inverse relationship in the risk of dying [36]. The rapid decrease of albumin after PH during first 24 hours could be explained mostly by its increased transcapillary leak into the interstitial space induced by cytokines such as TNF and IL-1 during the injury and partly by decreasing of its synthesis in the liver as it starts to produce acute phase proteins in high quantities [33]. The greater rate of vascular permeability is associated with higher mortality [35]. Some of the platelet's growth factors such as VEGF and FGF-2 have a trophic effect on the endothelial cells [37] preserving them from injury and maintaining their function thus preventing increased vascular permeability and loss of albumin. Ishii et al. reported that HGF, which is also present in platelets, not only stimulates LR but also elevates albumin level in hepatectomized rats due to its stimulatory effect on the protein production in the hepatocytes, finding increased mRNA content of albumin in the liver in vivo [38]. In our study both albumin and cholesterol levels were significantly higher in thrombocytotic group 24 hours after the operation. These results and previous reports indicate that survival prognosis is better in thrombocytotic mice and growth factors of platelets are preventing the decrease of albumin after the operation.

4.3 Glucose and Lipid Metabolism and Metabolite Transport Stimulation in the Regenerating Liver After 90% Partial Hepatectomy

Metabolic networks are linking liver function with cell growth and proliferation and it is well known that metabolic demands are greatly increased in hepatocytes of

regenerating liver [39]. Overexpression of genes which are responsible for metabolism is an indefeasible process during LR [40]. Schematically glucose metabolism stimulation after 90% PH is shown in Figure 10. Gene of glycolysis enzyme glucokinase (Gck) was overexpressed as early as 6 hours after PH. The pyruvate dehydrogenase kinase 4 (Pdk4), which was overexpressed 24 hours after the operation, is preventing catabolism of glucose and gluconeogenesis precursors by phosphorylation of pyruvate dehydrogenase, sparing glucose for vital organs, while metabolism is shifting towards fat utilization [41]. Therefore, according to the obtained results, glucose metabolism is highly activated during the first hours after PH. However, at 24 hours after the operation, when the reserve of glycogen is already utilized, the process of glycolysis is inhibited and lipids are becoming the main source of energy.

Lipid metabolism stimulation after 90% PH is shown in Figure 11. Acyl-CoA thioesterase 5 (Acot5) which hydrolyzes acyl-CoA to the free fatty acid and coenzyme A [42] was significantly overexpressed at 12 and 24 hours after PH, indicating that starting from 12 hours after the operation lipids are highly utilized in the metabolic pathways. At 24 hours, the most critical period for the surviving, when majority of normal group mice died, genes of glucose but predominantly genes of lipid energy metabolism were significantly overexpressed. Enolase (Eno3) is a gene of glycolysis metabolic pathway. Triglyceride lipase takes part in the catabolism of stored fat in adipose and nonadipose tissues. Abhydrolase domain containing 5 (Abhd5 or CGI-58), which was overexpressed at 24 hours in our study, was reported to increase the activity of this triglyceride lipase twenty times. This enzyme also facilitates lipolysis cooperating with other factors and lipases [43]. Triacylglycerol hydrolase 2 (TGH-2

or AU018778) takes part in adipocyte lipolysis with free fatty acids released during the period of increased energy demand [44]. All genes responsible for the lipid metabolism were significantly overexpressed in thrombocytotic group at 12 and 24 hours after the hepatectomy. These findings confirm the predominance of the lipid utilization over glucose metabolism during the later post-hepatectomy period.

Metabolite transport genes like very low density lipoprotein receptor (Vldlr), glycerol-3-phosphate transporter (Slc37a1) and facilitated glucose transporter 1 (Slc2a1), which transports glucose through the cell membrane by the energy independent facilitated diffusion mechanism [45], were also overexpressed, contributing to the complex process of metabolism (Fig. 12).

According to our results, it is suggested that energy supply is vital for LR after 90% hepatectomy starting from glucose catabolism, shifting to lipid utilization at 12 hours which riches the peak at 24 hours after the operation during the extreme energy demand.

4.4 Signaling Pathway Activation and its Impact on Liver Regeneration Stimulation Under the Condition of Thrombocytosis

Akt signal transduction pathway, which is a downstream of growth factor receptors like HGF and IGF-1, is very important in the promotion of cell survival. The mechanism of its acting is multidirectional. It was reported to inhibit apoptosis by phosphorylation and inactivation of apoptosis stimulators BAD, ASK-1 and MLK3 [46 - 48]. On the other hand it stimulates cell replication through the phosphorylation of GSK3β, which causes nuclear accumulation of cyclin D1, a regulator of G1/S phase transition [49] and further DNA synthesis. Akt is increasing cell size via the mTOR-dependent and -independent pathways which also inhibits protein degradation

[50]. Finally, Akt is promoting cell survival, seize and metabolism maintaining cell surface transporters of glucose, amino acids, low-density lipoprotein and iron through the mTOR-dependent mechanism [51]. In our study phosphorylation of Akt was visible at 2, 6, 12, and 18 hours after the operation in thrombocytotic group and was much earlier and stronger compared to normal group, where this pathway was activated only at 6 hours after PH. These results suggest that platelet's growth factors are responsible for earlier and stronger activation of Akt pathway and this leads to earlier promotion of mechanisms which stimulate proliferation, prevent apoptosis, increase seize and metabolism of remaining hepatic cells.

STAT3 is a member of STAT protein family. It is activated by cytokine receptor associated kinases JAKs or growth factor receptor tyrosine kinases through tyrosine phosphorylation. STAT3 is responsible for the promotion of cell cycle progression, cellular transformation and prevention of apoptosis [52]. Conditional STAT3 knockout mice showed a significantly higher mortality rate after 70% PH at the early time points and slightly decreased hepatocyte DNA synthesis which is recovering later in survived mice [53]. This data is showing that STAT3 signaling is important in the early stages of LR. In our previous study with 70% hepatectomized mice we did not find that platelets strongly contributed to STAT3 phosphorylation in the early period after PH [11]. However, in the present study STAT3 was strongly phosphorylated at 2 and 6 hours after PH in thrombocytotic group, while in normal group its activation started only at 12 hours after the operation. These results indicate that in the case of 90% PH activated STAT3 signaling pathway additionally contributes along with Akt pathway to the promotion of DNA synthesis and suppression of apoptosis in thrombocytotic mice in immediate early stages of LR and thus facilitating their survival.

Activation of HGF receptor c-Met which is important for the induction of LR was proved to occur early even in 30-60 minutes after PH [12]. Its phosphorylation was observed in both groups but it was stronger and maintained longer in thrombocytotic group proving that platelets induce more prominent activation of c-Met.

4.5 Contribution of Transcription, Growth Factor and Cell Cycle Control Genes into the Liver Regeneration After 90% Partial Hepatectomy

Besides IGF transport IGFBP-1 regulates cell activity, influence cell adhesion and migration [54]. It is one of the most rapidly and highly induced genes after PH [56]. IGFBP-1 knockout mice have impaired LR after PH characterized by liver necrosis and reduced and delayed hepatocyte DNA synthesis [55]. IGFBP-1 is stimulated through the IL-6-STAT3 pathway [56]. In our study STAT3 was activated at 2 hours in thrombocytotic group and at 12 hours after PH in normal group. These results completely correlate with the peak overexpression time of IGFBP-1 in each group. From previous reports and our results it is suggested that early and strong expression of IGFBP-1 has an important role for prompt LR after extra massive PH. Simplified mechanism of platelets' growth factors release, IGFBP-1 and c-Met stimulation, Akt signaling pathway activation and, as a result of this, hepatocyte proliferation is depicted in Figure 13.

The importance of transcription factor c-myc was shown in the study with its inhibition by antisense oligomers. This resulted in the reduction of PCNA and cell arrest in the G0/G1 phase of the cell cycle with overall LR slowdown [57]. The other cell cycle related gene tumor necrosis factor, alpha-induced protein 3 (Tnfaip3 or A20), was reported by Longo and coworkers to be up-regulated after lethal radical hepatectomy

and increased the survival rate of the operated mice. It acts as an antiapoptotic and anti-inflammatory agent through the reciprocal inhibition of NF-κB and additionally stimulates hepatocyte proliferation by decreased expression of cyclin-dependent kinase inhibitor $p21^{waf1}$. Tnfaip3 also limits hepatocellular damage maintaining bilirubin clearance and synthetic function of hepatocytes [58]. In our study c-myc and Tnfaip3 were significantly overexpressed under the thrombocytotic condition compared to normal group. Activation of phosphatidylinositol 3-kinase (PI3K) is stimulated by several growth factors receptors. PI3K in its turn activates Akt forming PI3K/Akt signaling pathway which transduces mitogenic signals to the cell cycle machinery [59]. This fact one more time proves the importance of Akt signaling pathway. PI3K/Akt pathway activation plays a critical role in the early regenerative response of the liver after resection. Inhibition of this pathway markedly abrogates the normal hepatic regenerative response [60]. In our study PI3K mRNA was overexpressed significantly by two independent genes (NM_011085 8.76-fold and NM_001024955 3.43-fold) shown in Table 1) under the condition of thrombocytosis indicating great importance of PI3K/Akt pathway for the efficient hepatocyte proliferation. These results support previous reports and indicate that platelets stimulate LR through the overexpression of transcription, cell cycle related and signal transduction genes.

In our previous study with 70% PH mouse model we proved the importance of Akt signaling pathway stimulation by platelets' growth factors for the adequate hepatocyte enlargement and proliferation [11]. In the in vitro study we discovered that platelet IGF-1 containing fraction had the most significant mitogenic activity, while HGF containing fraction was on the second place [12]. In the current study we revealed that under the thrombocytotic condition hepatocyte IGF-1 binding protein

(IGFBP-1) was strongly overexpressed as early as 2 hours post hepatectomy and HGF receptor c-Met was activated since the beginning until 18 hours after the operation, supporting our previous reports. Both IGF-1-IGFBP-1 and HGF-c-Met are capable of Akt signaling pathway activation [12]. However, 90% PH model has its own specificity characterized by earlier and stronger stimulation of STAT3 signaling pathway in thrombocytotic group. Stimulation of this pathway supposed to be inevitable for the earlier overexpression of IGFBP-1 which could induce stronger influence of IGF-1 on liver regeneration. The process of LR after 90% PH under the thrombocytotic condition in mice is a very complex and multifactorial, involving early signaling pathway activation and various genes' overexpression. This finally results in the cell survival, growth and sufficient proliferation, restitution of the lost tissue and restoration of the liver function (Fig. 14).

4.6 Clinical Applications

After living donor liver transplantation a challenging task in recipients is to prevent a hyperacute rejection and small-for-size syndrome which is a dysfunction or failure of liver graft with portal hyperperfusion [61, 62]. In living donors from which liver grafts were taken the morbidity and mortality rates were reported to be 29.1% and 0.23% respectively [63].Therefore, platelet infusion could be applied in patients with small-for-size transplants and living donors for the liver growth acceleration after the operation.

Usage of platelets for LR stimulation until the recent times had some restriction due to the limited 3-5 day storage life of extracted platelets in blood banks. However, recently developed in our and other laboratories freeze-drying method of effectively preserving platelets is definitely helping to solve this storage problem and allow wide

usage of the platelets for blood transfusion purposes and treatment of hepatic failure [64-66]. Freeze-drying method makes platelet transfusion affordable and easy to perform way of treatment.

4.7 Future Research Plans

In our previous investigations and current study we clarified the effect of platelet on hepatocyte, however, the influence of platelet on nonparenchymal liver cells i.e., liver sinusoidal endothelial, Kupffer and stellate cells was still poorly understood. In our recent study which is currently under submission we are elucidating the role of platelets on activation and proliferation of liver sinusoidal endothelial cells and its connection with hepatocyte proliferation.

In the clinical practice in many cases liver resection is performed in patients with hepatic tumor or metastatic invasion of the liver. In the previous studies it was proved that platelets have promotional effect on hepatocyte proliferation [9-15], though their influence on cancer remains to be unknown. Recently, we started a new research which is aimed to reveal the effect of platelet administration to the murine hepatoma cell line Hepa 1-6.

Recently it was discovered that small size particles, i.e. microvesicles and exosomes, are present in the peripheral blood. Microvesicles which are derived from platelets comprise 2/3 out of their total amount in blood [65]. Microvesicles and exosomes contain functional mRNAs and microRNAs which can be delivered by the blood flow to various cells and facilitate their communication: growth, differentiation, cancer progression [67, 68]. Therefore, according to these results it is hypothesized that besides the growth factors platelet contain microvesicles and exosomes with functional mRNAs and/or microRNAs. Their possible impact on hepatic cells is our next goal in

the investigation of platelet's and its components' ifluence on LR. We already clarified that mRNAs of diverse transcription, growth factor, cell cycle control, signal transduction, metabolism and transport genes are present in blood. Our next step is examination of gene expression and their function particularly in platelets under the normal condition and after thrombopoietin stimulation. Following this platelet microvesicles and exosomes will be isolated, their RNA will be extracted and their gene expression, function and possible impact on hepatocytes will be elucidated as well.

Platelet infusion for the promotion of LR in patients with liver cirrhosis just started the first phase of clinical trial in the University of Tsukuba Hospital, however the amount of patients applied is too small to make any conclusion so far. Futher investigation is needed. Administration of platelet infusion for the purpose of LR acceleration in case of major liver resection i.e., segmentectomy or lobectomy will take place in the University of Tsukuba Hospital or elsewhere in Japan or abroad.

Conclusion

In this study it was confirmed that platelet number is thrombopoietin dose dependent. Platelet count was significantly higher in thrombocytotic group mice, in which platelets accumulated in large quantities in their remnant livers after PH. Survival rate was drastically higher and LR was much more efficient under the condition of thrombocytosis which was proved by such markers of regeneration as liver/body weight ratio and PCNA. Besides this, survival prognosis appeared to be better in platelet increment mice due to the higher amount of serum albumin and cholesterol compared to the platelet normal mice. Akt and STAT3 signaling pathways were stimulated much earlier and with HGF-c-Met signaling pathway they were much more activated in thrombocytotic compared to platelet normal group. This resulted in effective prevention of apoptosis, earlier stimulation of metabolism, cell growth and development and finally sufficient proliferation of the remaining hepatocytes. Metabolism was radically triggered in mice with platelet increment by the overexpression of metabolism and metabolite transport genes. At the beginning of the regeneration process glucose utilization was predominant though at the later stages lipids were used as the main source of energy. Besides metabolism, various transcription, cell cycle related and signal transduction genes were significantly overexpressed in thrombocytotic group contributing in their particular way to the complex mechanism of LR after extra massive hepatectomy.

In conclusion, this is the first study which proved that under the condition of thrombocytosis liver regeneration occurred even in 90% hepatectomized mice. Platelets contribute to the cell cycle progression and metabolic pathways and are able

to maintain liver function. As a result, platelets prevent acute liver failure. Platelet infusion could be applied in patients with small-for-size livers and for the liver growth acceleration after the transplantation.

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References:

1. Fausto N. Liver regeneration. J Hepatol 2000;32: 19-31.
2. Michalopoulos GK, DeFrances MC. Liver regeneration. Science 1997;276: 60-66.
3. Fausto N, Riehle KJ. Mechanisms of liver regeneration and their clinical implications. J Hepatobiliary Pancreat Surg 2005;12: 181-189.
4. Su AI, Guidotti LG, Pezacki JP, Chisari FV, Shultz PG. Gene expression during the priming phase of liver regeneration after partial hepatectomy in mice. Proc Natl Acad Sci USA 2002;99: 11181-11186.
5. Higgins GM, Anderson RM. Experimental pathology of the liver. Arch Pathol 1931;12: 186-202.
6. Nakamura T, Teramoto H, Ichihara A. Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. Proc Natl Acad Sci USA 1986;83: 6489-6493.
7. Michalopoulos GK. Liver regeneration. J Cell Physiol. 2007;213: 286-300.
8. Paul D, Piasecki A. Rat platelets contain growth factor(s) distinct from PDGF which stimulate DNA synthesis in primary adult rat hepatocytes cultures. Exp Cell Res 1984;154: 95-100.
9. Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA. Platelet-derived serotonin mediates liver regeneration. Science 2006;312: 104-107.
10. Nocito A, Georgiev P, Dahm F, Jochum W, Bader M, Graf R, Clavien PA. Platelets and platelet-derived serotonin promote tissue repair after normothermic hepatic ischemia in mice. Hepatology 2007;45: 369-376.

11. Murata S, Ohkohchi N, Matsuo R, Ikeda O, Myronovych A, Hoshi R. Platelets promote liver regeneration in early period after hepatectomy in mice. World J Surg 2007;31: 808-816.
12. Matsuo R, Ohkohchi N, Murata S, Ikeda O, Nakano Y, Watanabe M, Hisakura K, Myronovych A, Kubota T, Narimatsu H, Ozaki M. Platelets strongly induce hepatocyte proliferation with IGF-1 and HGF in vitro. J of Surg Res 2008;145: 279-286.
13. Murata S, Matsuo R, Ikeda O, Myronovych A, Watanabe M, Hisakura K, Nakano Y, Hashimoto I, Ohkohchi N. Platelets promote liver regeneration under conditions of Kupffer cell depletion after hepatectomy in mice. World J Surg 2008;32: 1088-1096.
14. Watanabe M, Murata S, Hashimoto I, Nakano Y, Ikeda O, Aoyagi Y, Matsuo R, Fukunaga K, Yasue H, Ohkohchi N. Platelets contribute to the reduction of liver fibrosis in mice. J Gastroenterol Hepatol 2009;24: 78-89.
15. Murata S, Hashimoto I, Nakano Y, Myronovych A, Watanabe M, Ohkohchi N. Single administration of thrombopoietin prevents progression of liver fibrosis and promotes liver regeneration after partial hepatectomy in cirrhotic rats. Ann Surg 2008;248: 821-828.
16. Gaub J, Iversen J. Rat liver regeneration after 90% partial hepatectomy. Hepatology 1984; 4: 902-904.
17. Yamamoto H, Togo S, Zheng YW, Kubota T, Taniguchi H, Shimada H. Adult rat hepatic bipotent progenitor cells remain dormant even after extensive hepatectomy. Wound Repair Regen 2007;15: 422-429.

18. Columbano A, Simbula M, Pibiri M, Perra A, Deidda M, Locker J, Pisanu A, Uccheddu A, Ledda-Columbano GM. Triiodothyronine stimulates hepatocyte proliferation in two models of impaired liver regeneration. Cell Prolif. 2008;41: 521-531.
19. Kamimukai N, Togo S, Hasegawa S, Kubota T, Kurosawa H, Li XK, Suzuki S, Shimada H. Expression of Bcl-2 family reduces apoptotic hepatocytes after excessive hepatectomy. Eur Surg Res 2001; 33: 8-15.
20. Morita T, Togo S, Kubota T, Kamimukai N, Nishizuka I, Kobayashi T,. Ichikawa Y, Ishikawa T, Takahashi S, Matsuo K, Tomaru Y, Okazaki Y, Hayashizaki Y, Shimada H. Mechanism of postoperative liver failure after excessive hepatectomy investigated using a cDNA microarray. J Hepatobiliary Pancreat Surg 2002; 9: 352-359.
21. Makino H, Togo S, Kubota T, Morioka D, Morita T, Kobayashi T, Tanaka K, Shimizu T, Matsuo K, Nagashima Y, Shimada H. A good model of hepatic failure after excessive hepatectomy in mice. J of Surg Research 2005;127: 171-176.
22. Soto-Gutierrez A, Navarro-Alvarez N, Rivas-Carrillo JD, Tanaka K, Chen Y, Misawa H, Okitsu T, Noguchi H, Tanaka N, Kobayashi N. Construction and transplantation of an engineered hepatic tissue using a polyaminourethane-coated nonwoven polytethrafluoroethylene fabric. Transplantation 2007;83: 129-137.
23. Ulich TR, del Castillo J, Senaldi G, Kinstler O, Yin S, Kaufman S, Tarpley J, Choi E, Kirley T, Hunt P, Sheridan WP. Systemic hematologic effects of PEG-rHuMGDF-induced megakaryocyte hyperplasia in mice. Blood 1996;87: 5006-5015.

24. Shibuya K, Akahori H, Takahashi K, Tahara E, Kato T, Miyazaki H. Multilineage hematopoietic recovery by a single injection of pegylated recombinant human megacaryocyte growth and development factor in myelosuppressed mice. Blood 1998;91: 37-45.
25. Skomorovski K, Harpak H, Ianovski A, Vardi M, Visser TP, Hartong SC, van Vliet HH, Wagemaker G, Agur Z. New TPO treatment schedules of increased safety and efficacy: preclinical validation of a thrombopoiesis stimulation model. Br J Haematol 2003;123: 683-691.
26. Nagata Y, Nagahisa H, Aida Y, Okutomi K, Nagasawa T, Todokoro K. Thrombopoietin induces megakaryocyte differentiation in hematopoietic progenitor FDC-P2 cells. J Biol Chem. 1995;270: 19673-19675.
27. Kiuchi S, Yamada T, Kiyokawa N, Saito T, Fujimoto J, Yasue H. Genomic structure of swine taste receptor family 1 member 3, TAS1R3, and its expression in tissues. Cytogenet Genome Res 2006;115: 51-61.
28. Tanaka K, Kobayashi N, Gutierrez AS, Rivas-Carrillo JD, Navarro-Alvarez N, Chen Y, Narushima M, Miki A, Okitsu T, Noguchi H, Tanaka N. Prolonged survival of mice with acute liver failure with transplantation of monkey hepatocytes cultured with an antiapoptotic pentapeptide V5. Transplantation 2006;81: 427-437.
29. Soto-Gutierrez A, Kobayashi N, Rivas-Carrillo JD, Navarro-Alvarez N, Zhao D, Okitsu T, Noguchi H, Basma H, Tabata Y, Chen Y, Tanaka K, Narushima M, Miki A, Ueda T, Jun HS, Yoon JW, Lebkowski J, Tanaka N, Fox IJ. Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes. Nat Biotechnol 2006; 24: 1412-1419.

30. Jin X, Zhang Z, Beer-Stolz D, Zimmers TA, Koniaris LG. Interleukin-6 inhibits oxidative injury and necrosis after extreme liver resection. Hepatology 2007;46: 802-812.
31. Chuang VT, Otagiri M. Stereoselective binding of human serum albumin. Chirality. 2006; 18: 159-166.
32. Doweiko JP, Nompleggi DJ. The role of albumin in human physiology and pathophysiology, Part III: Albumin and disease states. JPEN J Parenter Enteral Nutr. 1991; 15: 476-483.
33. Goldwasser P, Feldman J. Association of serum albumin and mortality risk. J Clin Epidemiol 1997;50: 693-703.
34. Bar-Or D, Rael LT, Bar-Or R, Slone DS, Craun ML. The formation and rapid clearance of a truncated albumin species in a critically ill patient. Clin Chim Acta. 2006; 365: 346-349.
35. Ryan AM, Hearty A, Prichard RS, Cunningham A, Rowley SP, Reynolds JV. Association of hypoalbuminemia on the first postoperative day and complications following esophagectomy. J Gastrointest Surg 2007;11: 1355-1360.
36. Delgado-Rodriguez M, Medina-Cuadros M, Gomez-Ortega A, Martinez-Gallego G, Mariscal-Ortiz M, Martinez-Gonzalez MA, Sillero-Arenas M. Cholesterol and serum albumin levels as predictors of cross infection, death, and length of hospital stay. Arch Surg 2002;137: 805-812.
37. Pintucci G, Froum S, Pinnell J, Mignatti P, Rafii S, Green D. Trophic effects of platelets on cultured endothelial cells are mediated by platelet-associated fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF). Thromb Haemost. 2002; 88: 834-842.

38. Ishii T, Sato M, Sudo K, Suzuki M, Nakai H, Hishida T, Niwa T, Umezu K, Yuasa S. Hepatocyte growth factor stimulates liver regeneration and elevates blood protein level in normal and partially hepatectomized rats. J Biochem (Tokyo). 1995; 117: 1105-1112.
39. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology. 2006; 43: S45-53.
40. Arai M, Yokosuka O, Chiba T, Imazeki F, Kato M, Hashida J, Ueda Y, Sugano S, Hashimoto K, Saisho H, Takiguchi M, Seki N. Gene expression profiling reveals the mechanism and pathophysiology of mouse liver regeneration. J Biol Chem 2003;278: 29813-29818.
41. Savkur RS, Bramlett KS, Michael LF, Burris TP. Regulation of pyruvate dehydrogenase kinase expression by the farnesoid X receptor. Biochem Biophys Res Commun 2005;329: 391-396.
42. Hunt MC, Rautanen A, Westin MA, Svensson LT, Alexson SE. Analysis of the mouse and human acyl-CoA thioesterase (ACOT) gene clusters shows that convergent, functional evolution results in a reduced number of human peroxisomal ACOTs. FASEB J 2006;20: 1855-1864.
43. Lass A, Zimmermann R, Haemmerle G, Riederer M, Schoiswohl G, Schweiger M, Kienesberger P, Strauss JG, Gorkiewicz G, Zechner R. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. Cell Metab 2006;3: 309-319.
44. Okazaki H, Igarashi M, Nishi M, Tajima M, Sekiya M, Okazaki S, Yahagi N, Ohashi K, Tsukamoto K, Amemiya-Kudo M, Matsuzaka T, Shimano H, Yamada N, Aoki J, Morikawa R, Takanezawa Y, Arai H, Nagai R, Kadowaki T, Osuga J,

Ishibashi S. Identification of a novel member of the carboxylesterase family that hydrolyzes triacylglycerol: a potential role in adipocyte lipolysis. Diabetes 2006;55: 2091-2097.

45. Mueckler M, Weng W, Kruse M. Glutamine 161 of Glut1 glucose transporter is critical for transport activity and exofacial ligand binding. J Biol Chem 1994;269: 20533-20538.
46. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997; 91: 231-241.
47. Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol Cell Biol. 2001; 21: 893-901.
48. Barthwal MK, Sathyanarayana P, Kundu CN, Rana B, Pradeep A, Sharma C, Woodgett JR, Rana A. Negative regulation of mixed lineage kinase 3 by protein kinase B/AKT leads to cell survival. J Biol Chem. 2003; 278: 3897-3902.
49. Boylan JM, Gruppuso PA. D-type cyclins and G1 progression during liver development in the rat. Biochem Biophys Res Commun. 2005;330: 722-730.
50. Faridi J, Fawcett J, Wang L, Roth RA. Akt promotes increased mammalian cell size by stimulating protein synthesis and inhibiting protein degradation. Am J Physiol Endocrinol Metab. 2003; 285: E964-972.
51. Edinger AL, Thompson CB. Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. Mol Biol Cell. 2002; 13: 2276-2288.
52. Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 2000;19: 2468-2473.

53. Moh A, Iwamoto Y, Chai GX, Zhang SS, Kano A, Yang DD, Zhang W, Wang J, Jacoby JJ, Gao B, Flavell RA, Fu XY. Role of STAT3 in liver regeneration: survival, DNA synthesis, inflammatory reaction and liver mass recovery. Lab Invest 2007;87: 1018-1028.
54. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 2002;23: 824-854.
55. Leu JI, Crissey MA, Craig LE, Taub R. Impaired hepatocyte DNA synthetic response posthepatectomy in insulin-like growth factor binding protein 1-deficient mice with defects in C/EBP beta and mitogen-activated protein kinase/extracellular signal-regulated kinase regulation. Mol Cell Biol 2003;23: 1251-1259.
56. Leu JI, Crissey MA, Leu JP, Ciliberto G, Taub R. Interleukin-6-induced STAT3 and AP-1 amplify hepatocyte nuclear factor 1-mediated transactivation of hepatic genes, an adaptive response to liver injury. Mol Cell Biol 2001;21: 414-424.
57. Arora V, Knapp DC, Smith BL, Statdfield ML, Stein DA, Reddy MT, Weller DD, Iversen PL. c-Myc antisense limits rat liver regeneration and indicates role for c-Myc in regulating cytochrome P-450 3A activity. J Pharmacol Exp Ther 2000;292: 921-928.
58. Longo CR, Patel VI, Shrikhande GV, Scali ST, Csizmadia E, Daniel S, Sun DW, Grey ST, Arvelo MB, Ferran C. A20 protects mice from lethal radical hepatectomy by promoting hepatocyte proliferation via a p21waf1-dependent mechanism. Hepatology 2005;42: 156-164.
59. Sheng H, Shao J, Townsend CM Jr, Evers BM. Phosphatidylinositol 3-kinase mediates proliferative signals in intestinal epithelial cells. Gut 2003;52: 1472-1478.

60. Jackson LN, Larson SD, Silva SR, Rychahou PG, Chen LA, Qiu S, Rajaraman S, Evers BM. PI3K/Akt activation is critical for early hepatic regeneration after partial hepatectomy. Am J Physiol Gastrointest Liver Physiol 2008;294: 1401-1410.
61. Dahm F, Georgiev P, Clavien PA. Small-for-size syndrome after partial liver transplantation: definition, mechanisms of disease and clinical implications. Am J Transplant. 2005;5: 2605-2610.
62. Wang H, Ohkohchi N, Enomoto Y, Usuda M, Miyagi S, Masuoka H, Sekiguchi S, Kawagishi N, Fujimori K, Sato A, Satomi S. Effect of portocaval shunt on residual extreme small liver after extended hepatectomy in porcine. World J Surg. 2006;30: 2014-2022.
63. Patel S, Orloff M, Tsoulfas G, Kashyap R, Jain A, Bozorgzadeh A, Abt P. Living-donor liver transplantation in the United States: identifying donors at risk for perioperative complications. Am J Transplant. 2007;7: 2344-2349.
64. Read MS, Reddick RL, Bode AP, Bellinger DA, Nichols TC, Taylor K, Smith SV, McMahon DK, Griggs TR, Brinkhous KM. Preservation of hemostatic and structural proteins of rehydrated lyophilized platelets: potential for long-term storage of dried platelets for transfusion. Proc. Natl. Acad. Sci. USA 1995;92: 397-401.
65. Hoshi R, Murata S, Matsuo R, Myronovych A, Hashimoto I, Ikeda H, Ohkohchi N. Freeze-dried platelets promote hepatocyte proliferation in mice. Cryobiology 2007;55: 255-260.
66. Wolkers WF, Walker NJ, Tablin F, Crowe JH. Human platelets loaded with trehalose survive freeze-drying. Cryobiology 2001; 4279-4287.

67. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee ML, Schmittgen TD, Nana-Sinkam SP, Jarjoura D, Marsh CB. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One 2008;3: 1-11.

68. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9: 654-659.

Figure Legends

Figure 1

Scheme of the mRNA extraction and microarray gene expression analysis. mRNA (shown in blue) was extracted from the liver tissue samples using the Isogen method. Complimentary cDNA (shown in orange) was synthesized from the mRNA. Fluorescent marked nucleotides were used for the synthesis of the following fluorescent labeled cDNA (shown in red) from the unmarked cDNA. Hybridization of the fluorescent labeled cDNAs to their corresponding oligonucleotide spots on the microarray plate. Fluorescent labeled cDNAs are shown in red and their corresponding spot oligonucleotides in green.

Figure 2

(A) Platelet count in groups without and with 4, 20, 40, 60 μg/kg body weight injection of thrombopoietin. Thrombopoietin (PEG-rHuMGDF) was administered intraperitoneally 5 days before platelet number calculation. PEG-rHuMGDF had a significant dose dependent effect on the platelet count elevation. We used 20 μg/kg body weight of PEG-rHuMGDF for the induction of thrombocytosis in the thrombocytotic group. Data are expressed as mean ± SD. n = 5 in each group. *$P<0.05$ versus normal group.

(B) Platelet count in normal and thrombocytotic groups at 0, 2, 6, 12, 18 and 24 hours post hepatectomy. The number of platelets was significantly higher in thrombocytotic group compared to normal group in almost all time points. Significant thrombocytosis in thrombocytotic group remained until 48 hours after partial

hepatectomy. Data are expressed as mean ± SD. n = 3-7 in each group. *P<0.05 versus normal group, **P<0.01 versus normal group.

Figure 3

Survival rate in normal and thrombocytotic groups. Kaplan-Meier method. Survival rate was significantly higher in thrombocytotic group indicating more sufficient liver regeneration and restoration of the liver functions under the influence of platelets. All mice from normal group died within 30 hours after hepatectomy, predominantly between 20 and 30 hours. In contrast, in thrombocytotic group 6 (54.5%) and 3 (27.3%) mice were alive 30 hours and one week after the operation, respectively. n = 11 in each group. *P<0.05 versus normal group.

Figure 4

Increasing of liver weight/body weight ratio in normal and thrombocytotic groups at 2, 6, 12, 18 and 24 hours post hepatectomy. Ratio started to be significantly higher in thrombocytotic compared to normal group since 12 hours after hepatectomy. Liver weight/body weight ratio is a direct feature of liver mass restitution and regeneration, which proved to be more prominent under the thrombocytotic condition. Data are expressed as mean ± SD. n = 3-7 in each group. *P<0.05, **P<0.01 versus normal group.

Figure 5

Accumulation of platelets in the liver tissue.

(A) Representative immunohistochemistry samples of liver frozen sections

obtained 2 hours after 90% partial hepatectomy. A much larger amount of stained platelets can be seen in the livers obtained from thrombocytotic group indicating accumulation of the platelets in the sinusoidal and Disse spaces which is necessary for the direct platelet-hepatocyte contact and stimulation of liver regeneration.

(B) The number of immunohistochemically stained platelets in the liver was significantly higher in thrombocytotic compared to normal group proving the importance of platelet count elevation for the increased accumulation of platelets in the liver. Platelets were calculated in five different viewpoints per slide. Data are expressed as mean ± SD. n = 5 in each group. *P<0.05 versus normal group.

Figure 6

Effect of platelets on liver regeneration 24 hours post hepatectomy.

(A) Representative PCNA (proliferating cell nuclear antigen) staining of both groups. PCNA is a marker antigen which can be detected only in the cells undergoing mitosis. It is a direct feature of proliferative process and therefore liver regeneration. A much larger amount of PCNA positively stained cells can be seen in thrombocytotic group (indicated by arrows) which is a proof of more significant liver regeneration under the platelet increment influence compared to the normal group.

(B) PCNA labeling index 24 hours post hepatectomy. PCNA labeling index reflects the number of hepatocytes positive for PCNA as a percentage of total cells per microscopic field. Index was significantly higher in thrombocytotic group indicating that much more hepatocytes were undergoing mitosis under the condition of thrombocytosis compared to the platelet normal condition. Data are expressed as mean ± SD. n = 7 in each group. *P<0.01 versus normal group.

Figure 7

Serum parameters of normal and thrombocytotic groups.

(A) Serum albumin levels at 0, 12, 18 and 24 hours post hepatectomy. A more rapid decline was observed in normal group compared to thrombocytotic group. n = 3-7 in each group. *P<0.01 versus normal group.

(B) Serum cholesterol levels at 2 and 24 hours post hepatectomy. Serum levels of albumin and cholesterol are inversely related to the mortality risk. Both albumin and cholesterol levels were significantly higher in thrombocytotic group indicating more favorable survival prognosis compared to the normal group. n = 4-7 in each group. *P<0.05 versus normal group.

Figure 8

Phosphorylation of Akt, STAT3, ERK1/2, c-Met and EGFR in normal and thrombocytotic groups at the indicated time points. Liver lysates of 0, 2, 6, 12, 18 and 24 hour time points in the quantity of 2.5 μg per lane were subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and then incubated with specific antibodies. The stronger phosphorylation of Akt at 6 and 12 hour time points and earlier phosphorylation of STAT3 at 2 and 6 hour time points compared to the normal group. Phosphorylation of c-Met was stronger and lasted longer in thrombocytotic group. Akt signaling pathway is considered to be the most important in the surgical liver regeneration model. It is necessary for the hepatocyte survival and proliferation. STAT3 signaling pathway is responsible for the cell cycle progression, cellular development and prevention of apoptosis. c-Met is a receptor of hepatocyte growth factor (HGF). It is important for the induction of liver regeneration. Earlier and

stronger activation of these pathways in thrombocytotic group indicated more adequate liver regeneration initiation and progression under thrombocytosis compared to the platelet normal condition. STAT3, signal transducer and activator of transcription 3; ERK1/2, extracellular signal-regulated protein kinase 1/2; c-Met, mesenchymal epithelial transition factor; EGFR, epidermal growth factor receptor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Figure 9

Overexpression of IGFBP-1 gene verified by Real-time PCR. In normal group IGFBP-1 level reached its peak value at 12 hours after partial hepatectomy. In thrombocytotic group IGFBP-1 reached its peak value even at 2 hours, moderately decreasing afterwards. IGFBP-1 as a binding protein of insulin-like growth factor 1 (IGF-1) is important for the prevention of liver necrosis and proper hepatocyte DNA synthesis. Therefore, its much earlier overexpression in thrombocytotic group protects liver from the destruction and induces earlier regenerative response compared to normal group. n = 3 in each group. IGFBP-1, insulin-like growth factor binding protein 1; PCR, polymerase chain reaction; EGFP, enhanced green fluorescent protein.

Figure 10

Glucose metabolism stimulation in thrombocytotic group after 90% PH. Glucokinase (Gck), which was overexpressed 3.42-fold in thrombocytotic mice livers at 6 hours after hepatectomy facilitates the beginning of the metabolic process of glycolysis. Pyruvate dehydrogenase kinase (Pdk4), which was overexpressed 10.93-fold at 24 hours after the operation prevents glucose catabolism which is spared

for the vital organs. In conclusion, metabolism of glucose is stimulated at the beginning of the regeneration process. Though, at its later stages, when the reserve of glycogen is already utilized, the process of glycolysis is inhibited.

Figure 11

Lipid metabolism stimulation in thrombocytotic group after 90% PH. Thriacylglycerol hydrolase 2 (TGH-2) which was overexpressed 7.32-fold in the livers of thrombocytotic mice at 24 hours after hepatectomy takes part in adipocyte lipolysis during increased energy demand. Abhydrolase domain containing 5 (Abhd5) which was overexpressed 4.63-fold at 24 hours after the operation increases the activity of adipose triglyceride lipase up to 20-fold, facilitating adipocyte lipolysis. Acyl-CoA thioesterase 5 (Acot5) which was overexpressed 18.05-fold at 12 hours and 7.15-fold at 24 hours after PH hydrolyzes acyl-CoA to the free fatty acid and coenzyme A. According to these results it is clear that lipid metabolism is significantly stimulated starting from 12 hours and continuing at 24 hours after PH in mice with platelet count elevation.

Figure 12

Glucose transport stimulation in thrombocytotic group after 90% PH. Solute carrier family 2 facilitated glucose transport gene (Slc2a1) which was overexpressed 6.2-fold 12 hours after hepatectomy codes facilitated glucose transporter protein (Glut1) This protein transports glucose through the cell membrane by the energy independent facilitated diffusion mechanism. Several transport genes were significantly overexpressed in the liver under the thrombocytotic condition

contributing to the metabolism promotion after 90% PH.

Figure 13

Platelet-hepatocyte interaction after 90% PH. Following the contact with hepatocytes platelets release their growth factors i.e., IGF-1, VEGF, HGF, etc. These growth factors are binding their corresponding receptors on the membrane of hepatocyte i.e., c-Met, IGFBP-1. Stimulation of the growth factor receptors causes activation of the Akt signaling pathway. Hepatocyte enters the cell cycle and accumulation of cyclin D1 in the nucleus occurs. At the M-phase of the cell cycle hepatocyte undergoes its replication.

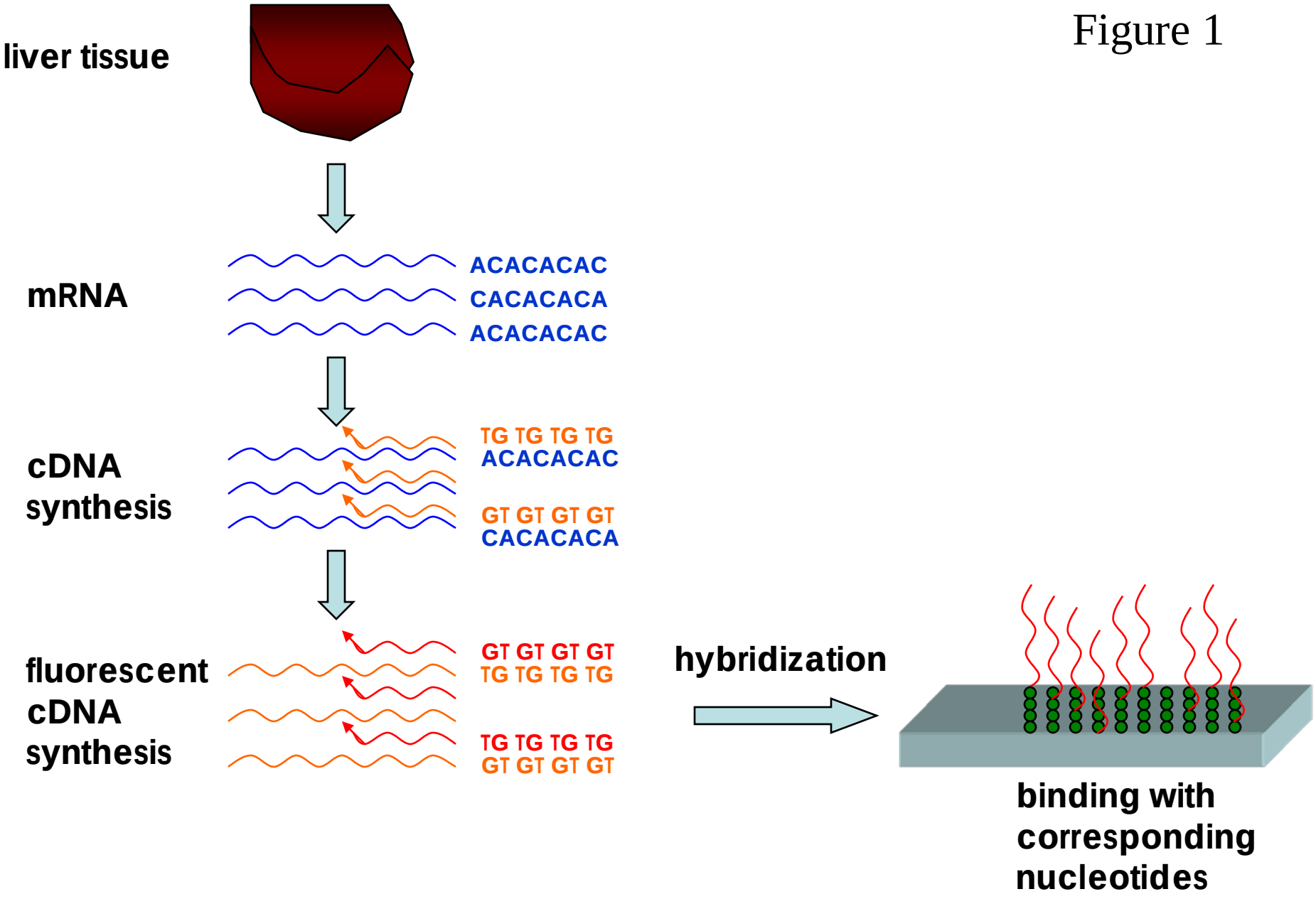
Figure 14

Mechanism of liver regeneration after 90% PH under condition of thrombocytosis. Platelets strongly accumulate in the remnant liver during the first two hours after the operation. Following the contact with hepatocytes, platelets release IGF-1 and HGF growth factors, which bind their corresponding receptors IGFBP-1 and c-Met on hepatocytes. Stimulation of these receptors leads to the activation of Akt-signaling pathway. In the same time platelets influence non-parenchymal cells, i.e., Kupffer and sinusoidal endothelial cells. Both cells are known to secrete interleukin-6 (IL-6) which is capable of the STAT3 signaling pathway activation. STAT3 stimulates IGFBP-1 gene overexpression and therefore enhances the effect of IGF-1 on liver regeneration. Activation of the Akt and STAT3 signaling pathways leads to the hepatocytes' gene overexpression and induction of the cell cycle and metabolism stimulation. As a result hepatocytes enter the cell cycle, replicate their DNA, undergo

cell division and liver regeneration occurs. Black arrows symbolize pathways related to hepatocyte. Red arrows show pathways associated with nonparenchymal liver cells. IGF-1, insulin-like growth factor, HGF, hepatocyte growth factor; IGFBP-1, insulin-like growth factor binding protein 1; c-Met, mesenchymal epithelial transition factor; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3.

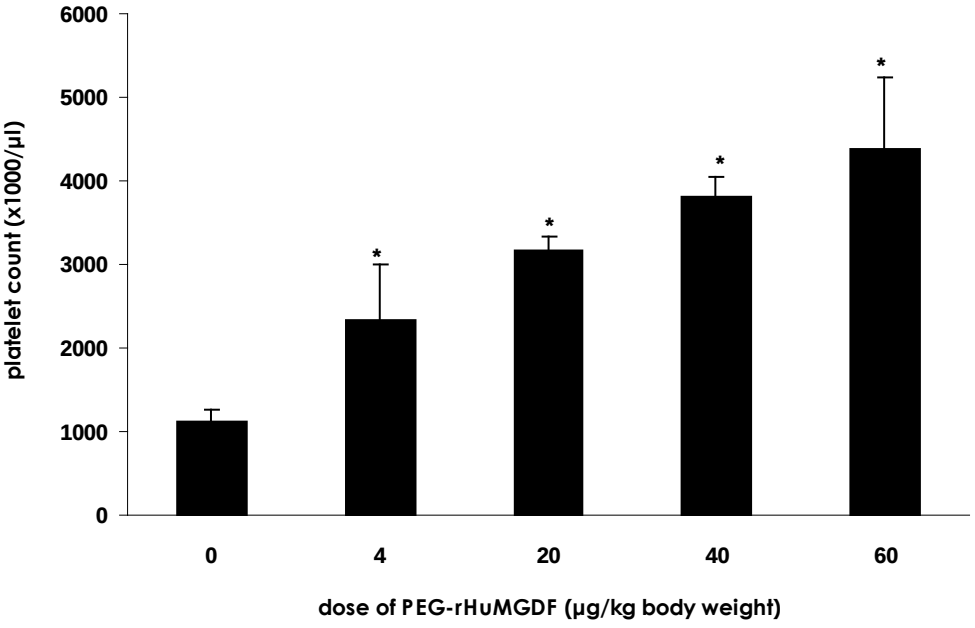
参　考　論　文

Figure 1


liver tissue
mRNA
ACACACAC
CACACACA
ACACACAC
cDNA synthesis
TG TG TG TG
ACACACAC
GT GT GT GT
CACACACA
fluorescent cDNA synthesis
GT GT GT GT
TG TG TG TG
TG TG TG TG
GT GT GT GT
hybridization
binding with corresponding nucleotides

A

Figure 2


6000
5000
4000
3000
2000
1000
0
platelet count (x1000/µl)
*
*
*
*
0
4
20
40
60
dose of PEG-rHuMGDF (µg/kg body weight)

B

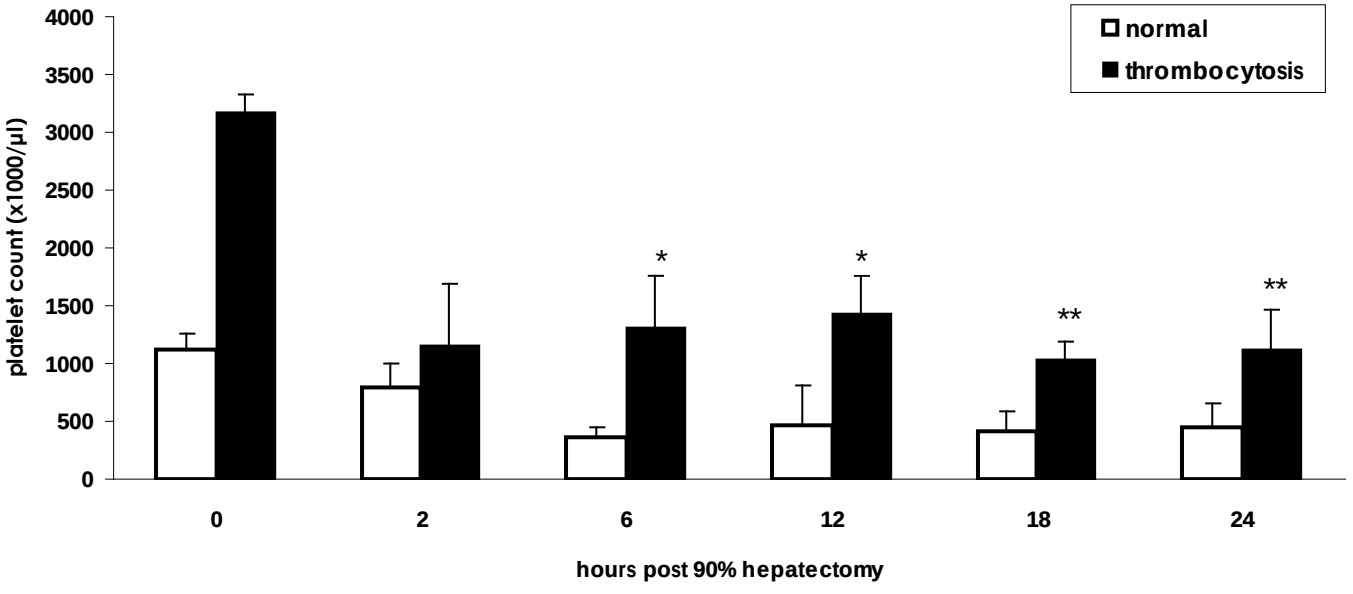

4000
3500
3000
2500
2000
1500
1000
500
0
platelet count (x1000/µl)
normal
thrombocytosis
*
*
**
**
0
2
6
12
18
24
hours post 90% hepatectomy

Figure 3

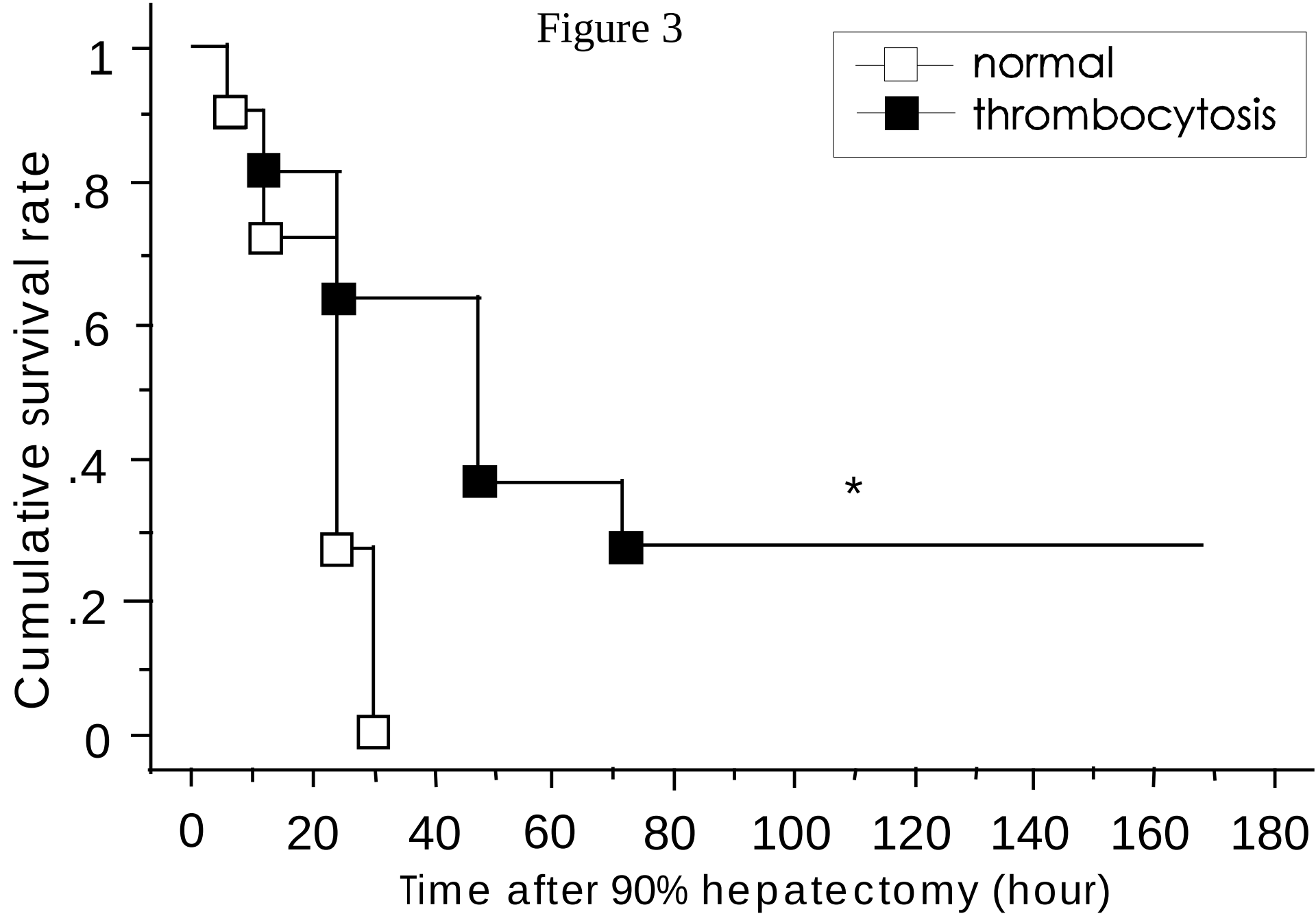

normal
thrombocytosis
Cumulative survival rate
1
.8
.6
.4
.2
0
*
0
20
40
60
80
100
120
140
160
180
Time after 90% hepatectomy (hour)

Figure 4

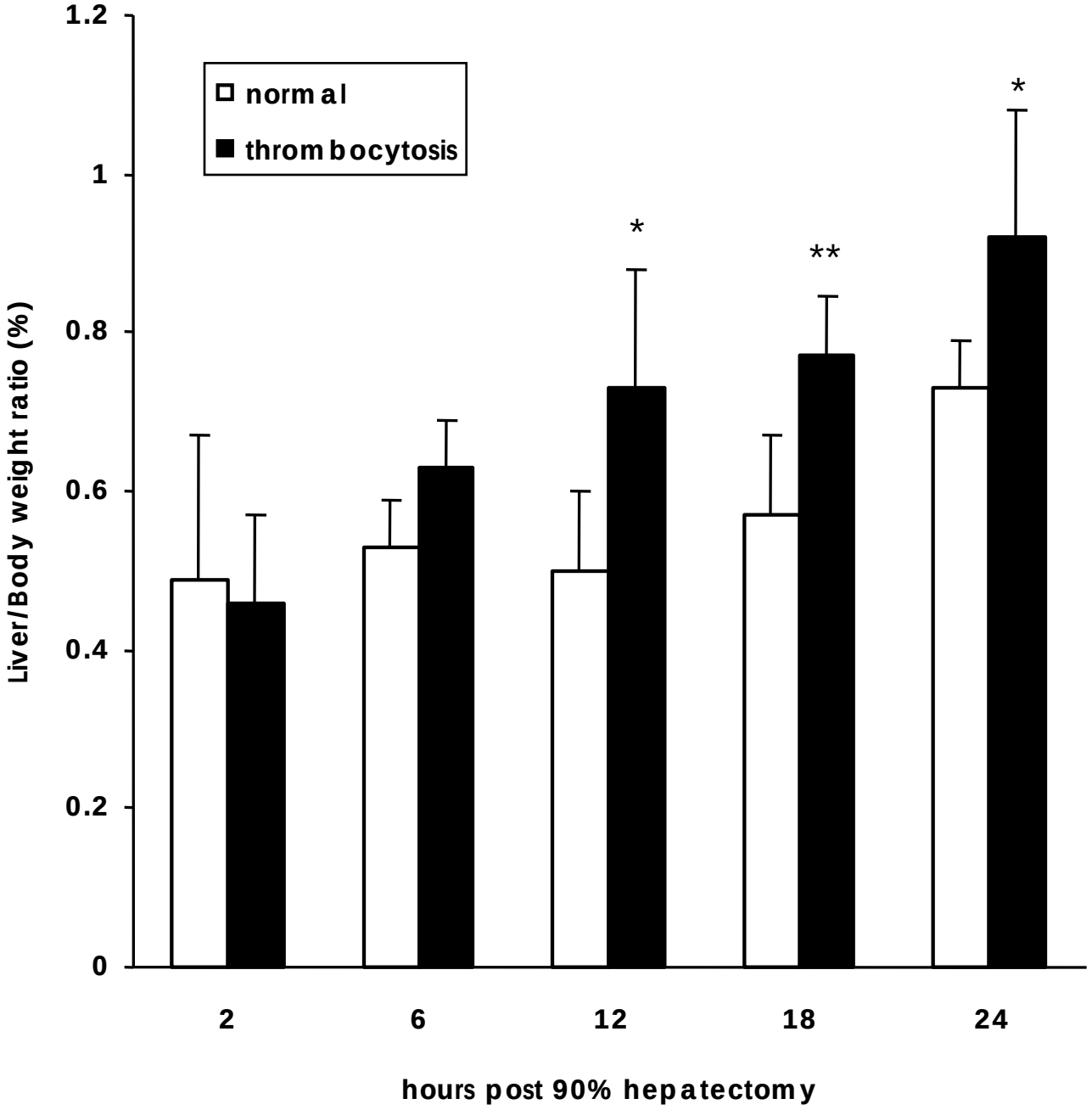
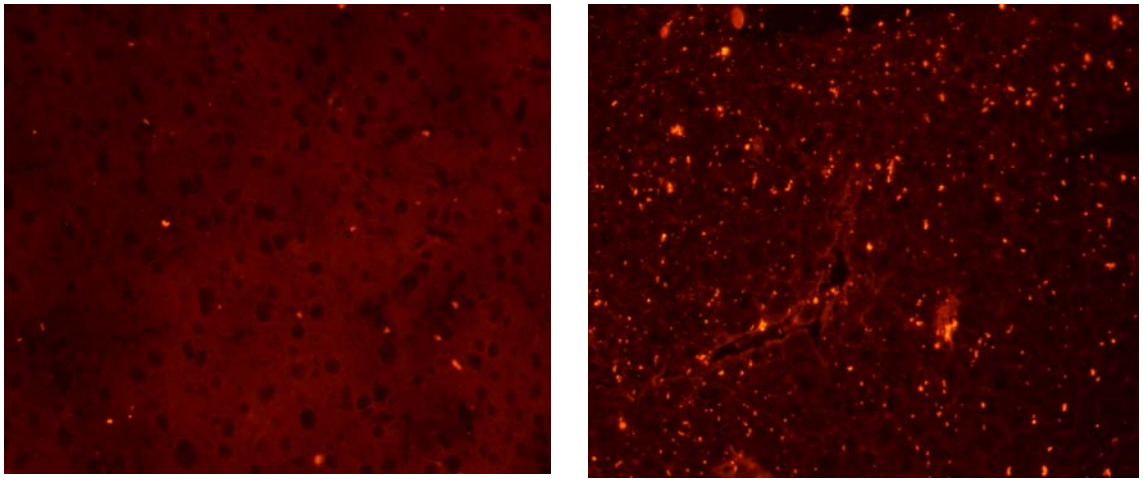

normal
thrombocytosis
Liver/Body weight ratio (%)
1.2
1
0.8
0.6
0.4
0.2
0
*
**
*
2
6
12
18
24
hours post 90% hepatectomy

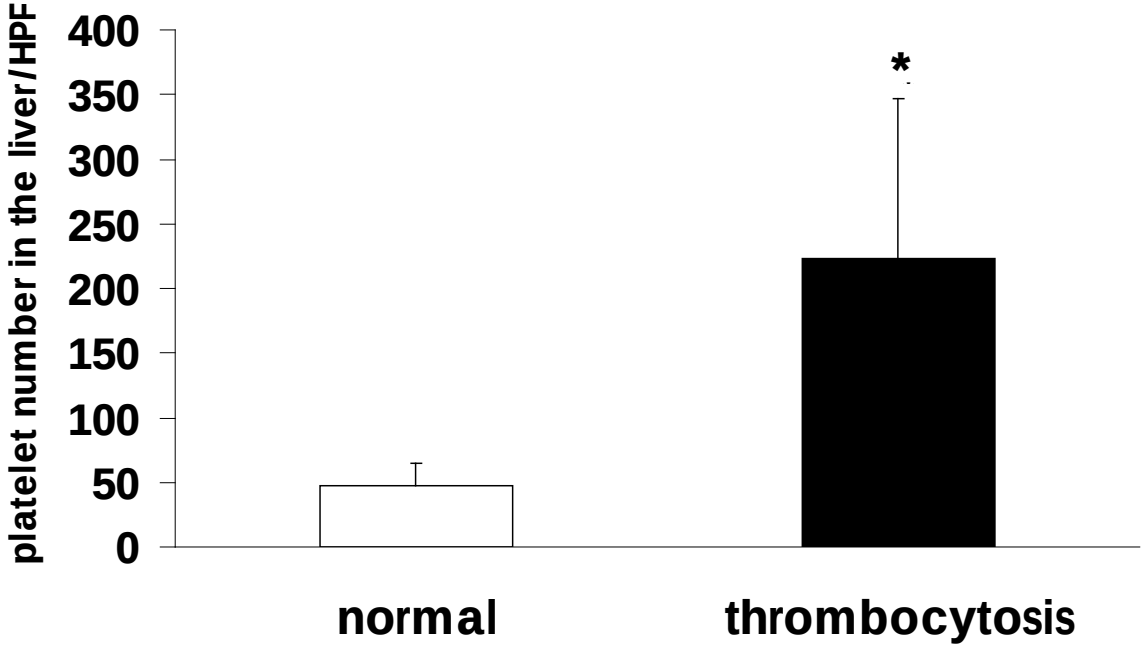
Figure 5

A



normal thrombocytosis

B



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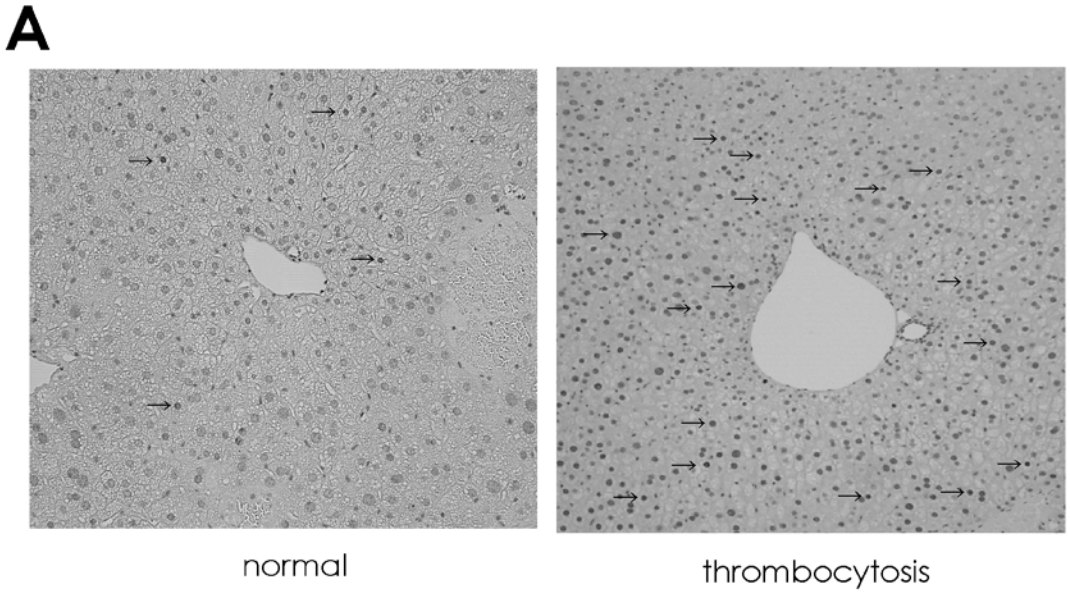

A
normal
thrombocytosis
B
PCNA labeling index (%)
40
35
30
25
20
15
10
5
0
*
normal
thrombocytosis

Figure 7

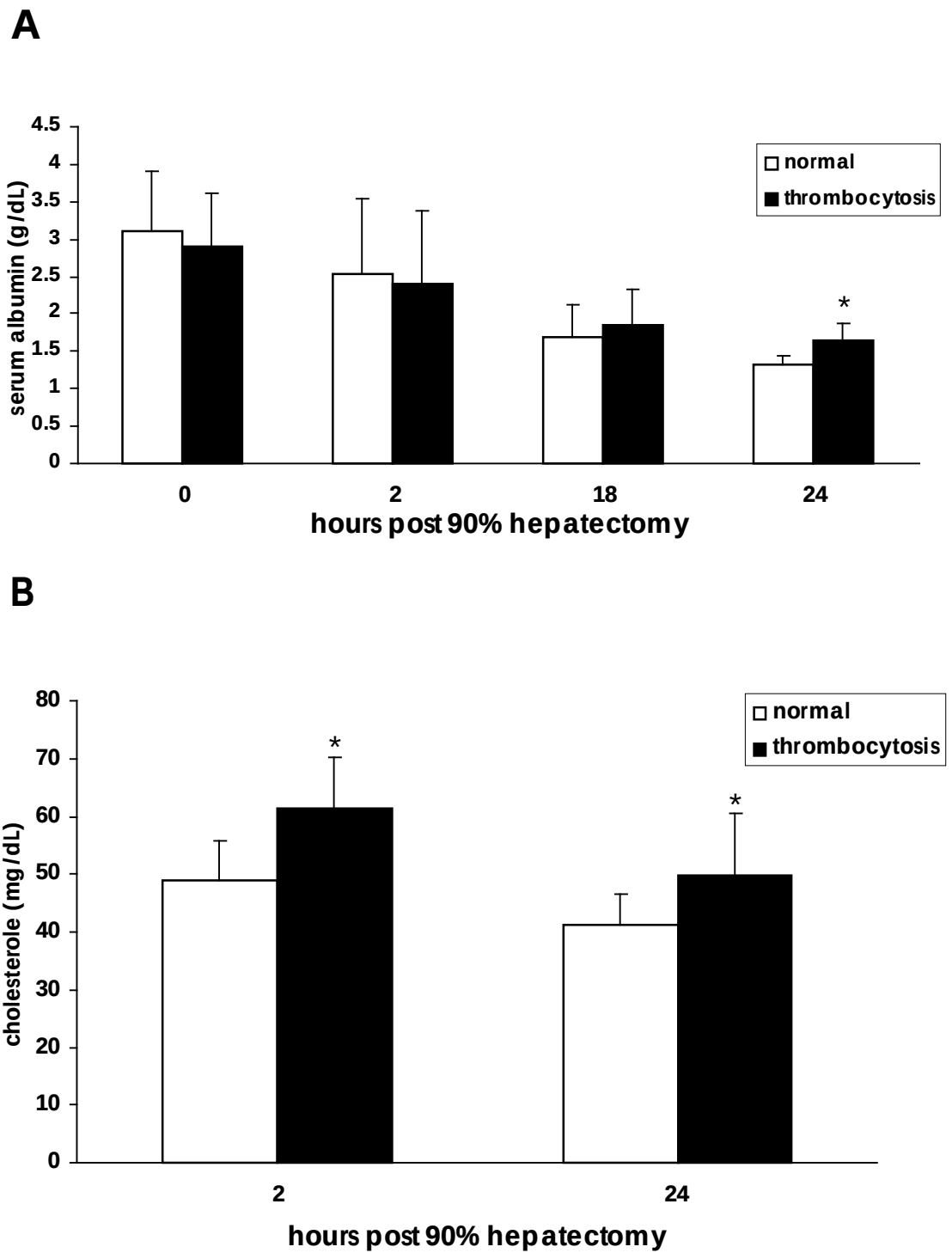

A
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
serum albumin (g/dL)
normal
thrombocytosis
*
0
2
18
24
hours post 90% hepatectomy
B
80
70
60
50
40
30
20
10
0
cholesterole (mg/dL)
normal
thrombocytosis
*
*
2
24
hours post 90% hepatectomy

Figure 8

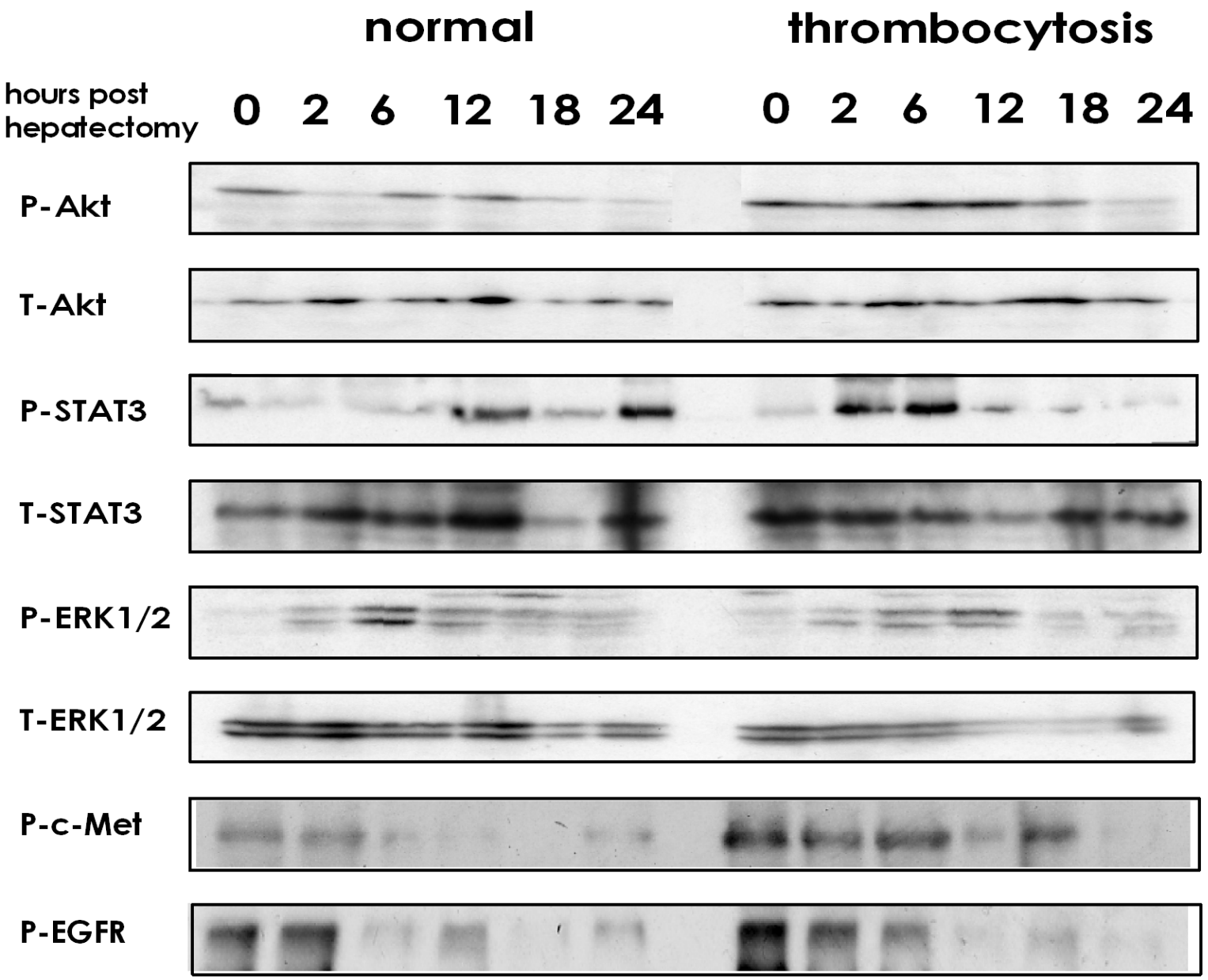

normal
thrombocytosis
hours post hepatectomy
0 2 6 12 18 24
0 2 6 12 18 24
P-Akt
T-Akt
P-STAT3
T-STAT3
P-ERK1/2
T-ERK1/2
P-c-Met
P-EGFR

Figure 9

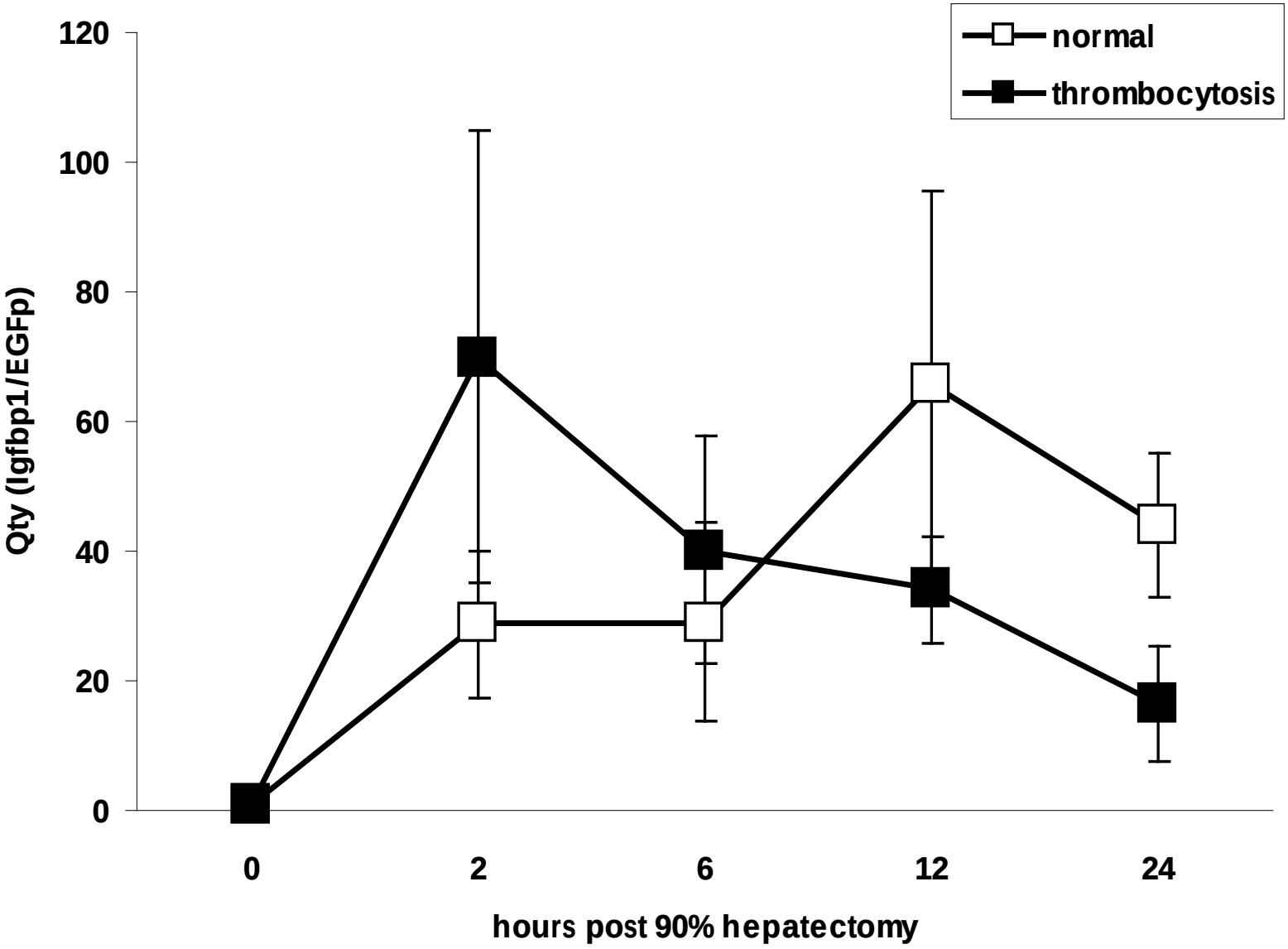
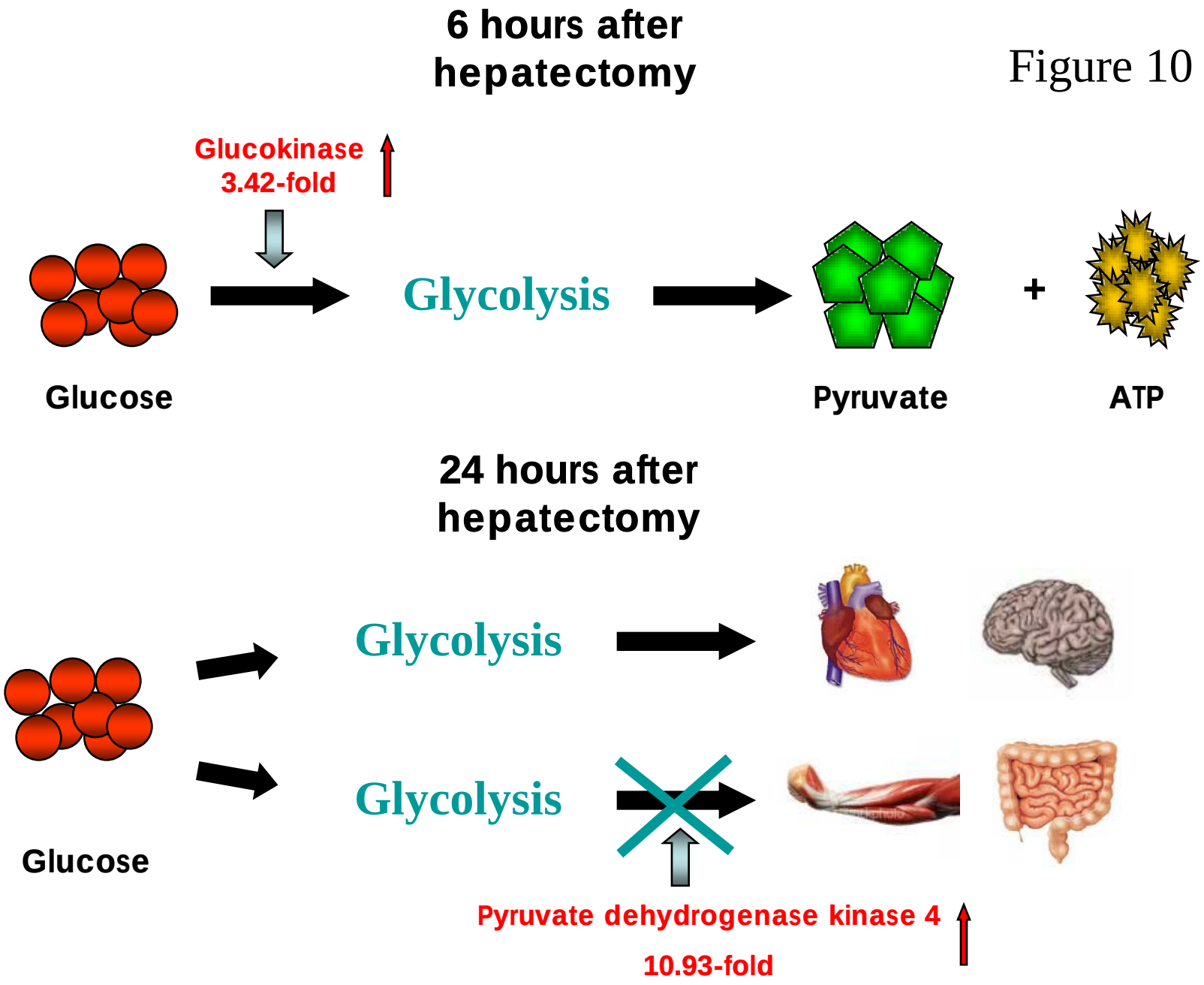
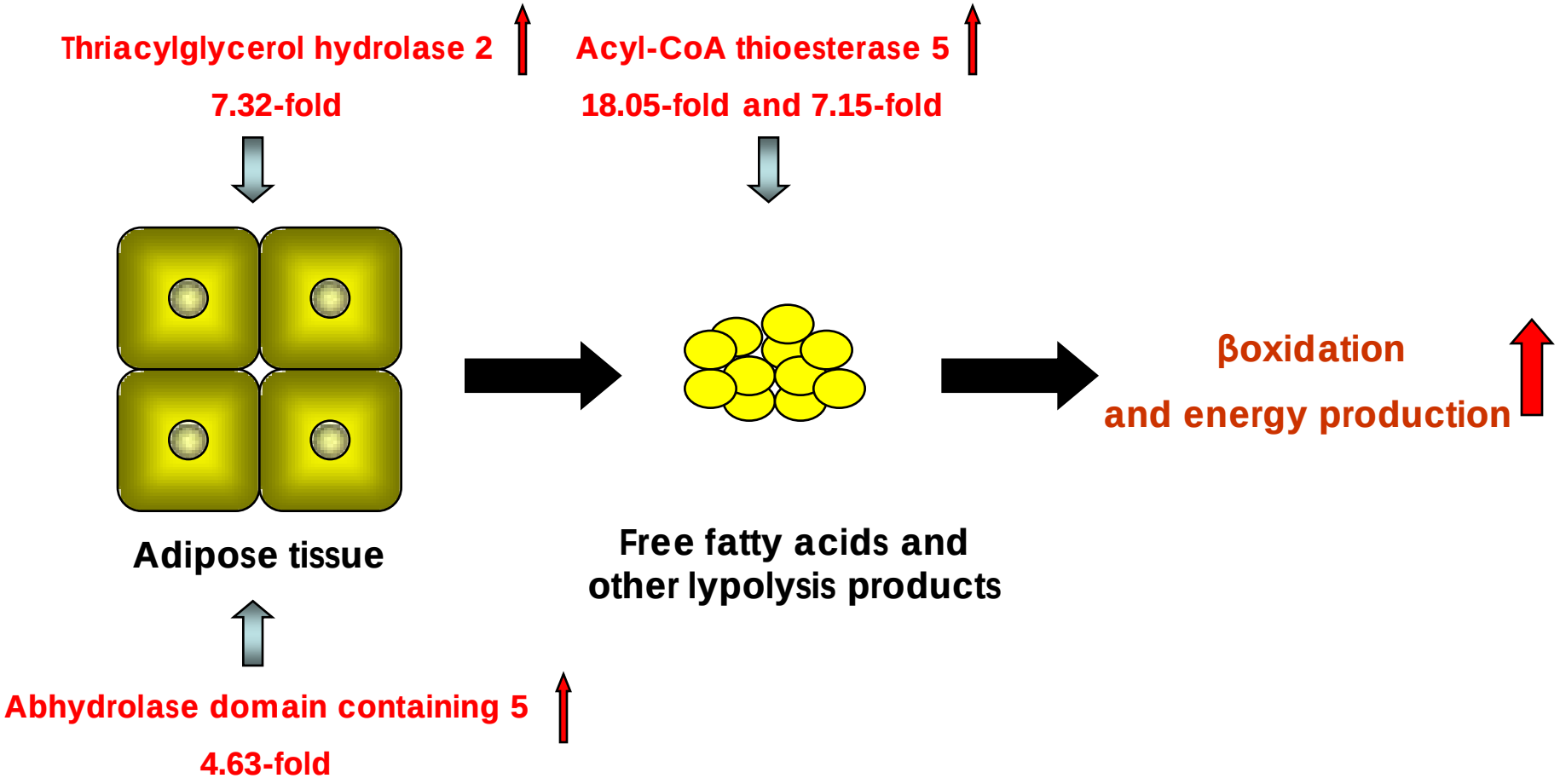

normal
thrombocytosis
120
100
80
60
40
20
0
Qty (Igfbp1/EGFp)
0
2
6
12
24
hours post 90% hepatectomy

Figure 10



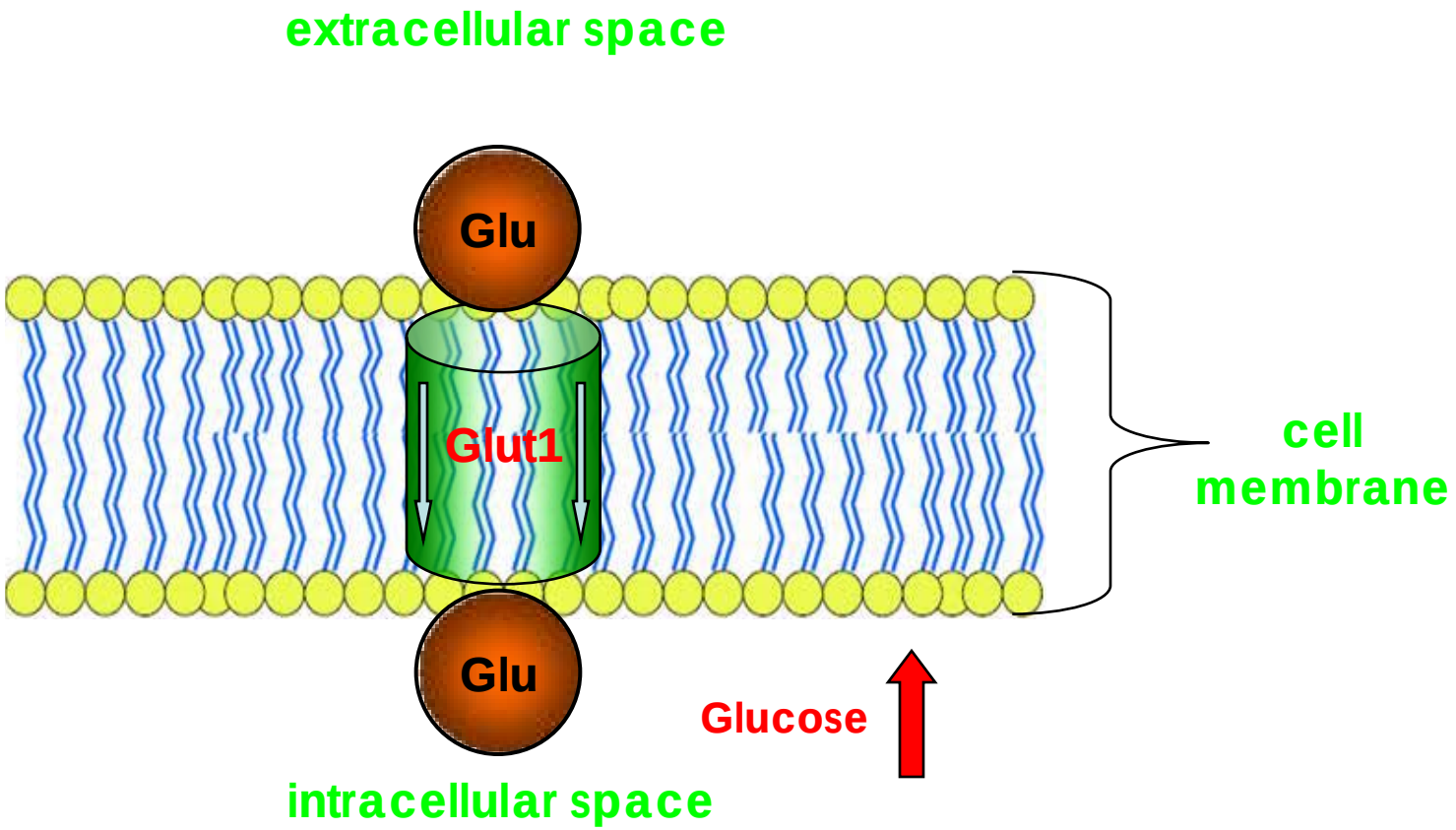
Shown in red are the genes overexpressed in thrombocytotic group.

Figure 11



Shown in red are the genes overexpressed in thrombocytotic group.

Figure 12

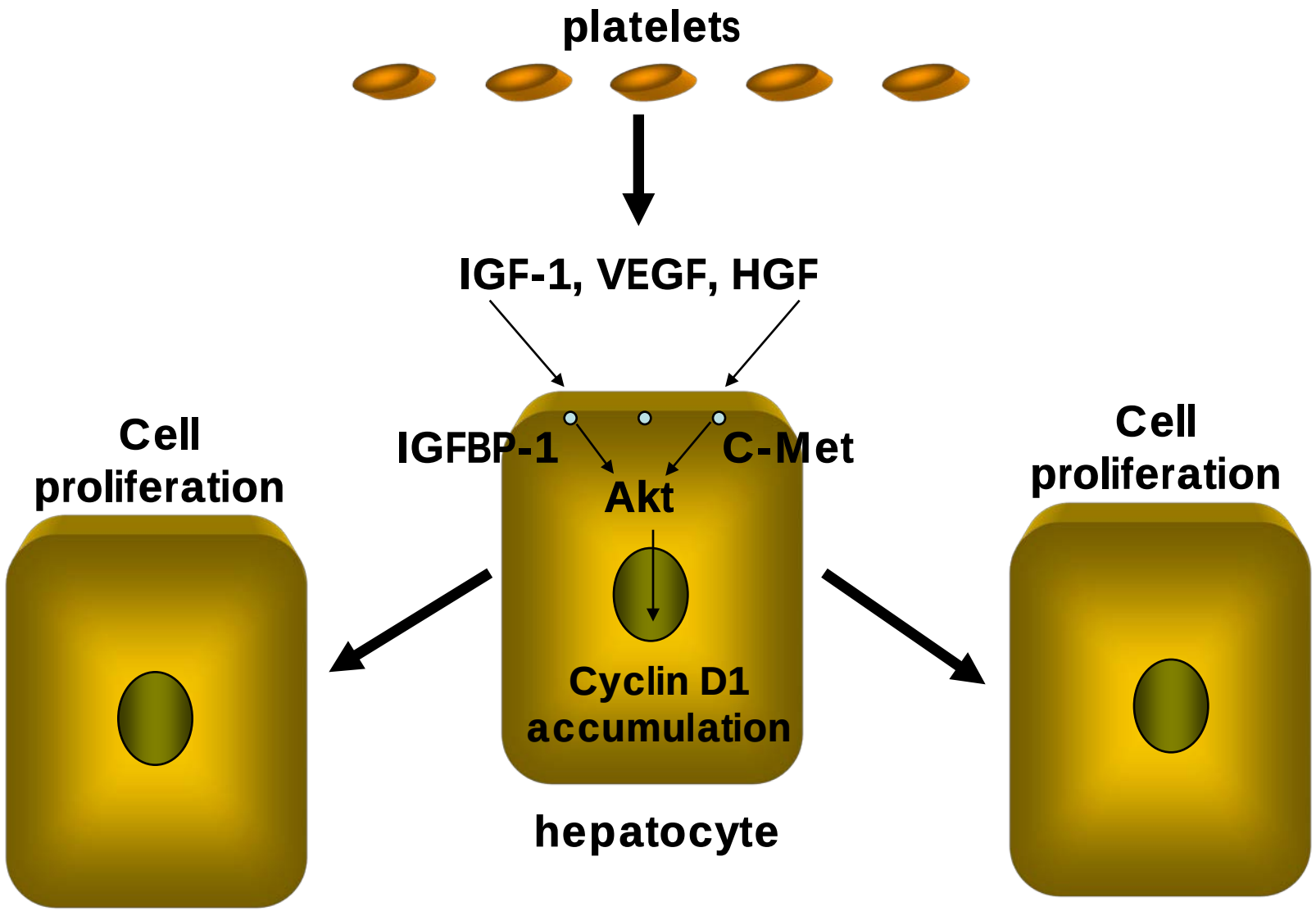


Glu — Glucose molecule

Glut1 — Facilitated glucose transporter, 6.2-fold

Glut1 gene was overexpressed in thrombocytotic group.

Figure 13


platelets
IGF-1, VEGF, HGF
IGFBP-1
C-Met
Akt
Cell proliferation
Cyclin D1 accumulation
Cell proliferation
hepatocyte

Mechanism of hepatocyte proliferation after 90% PH

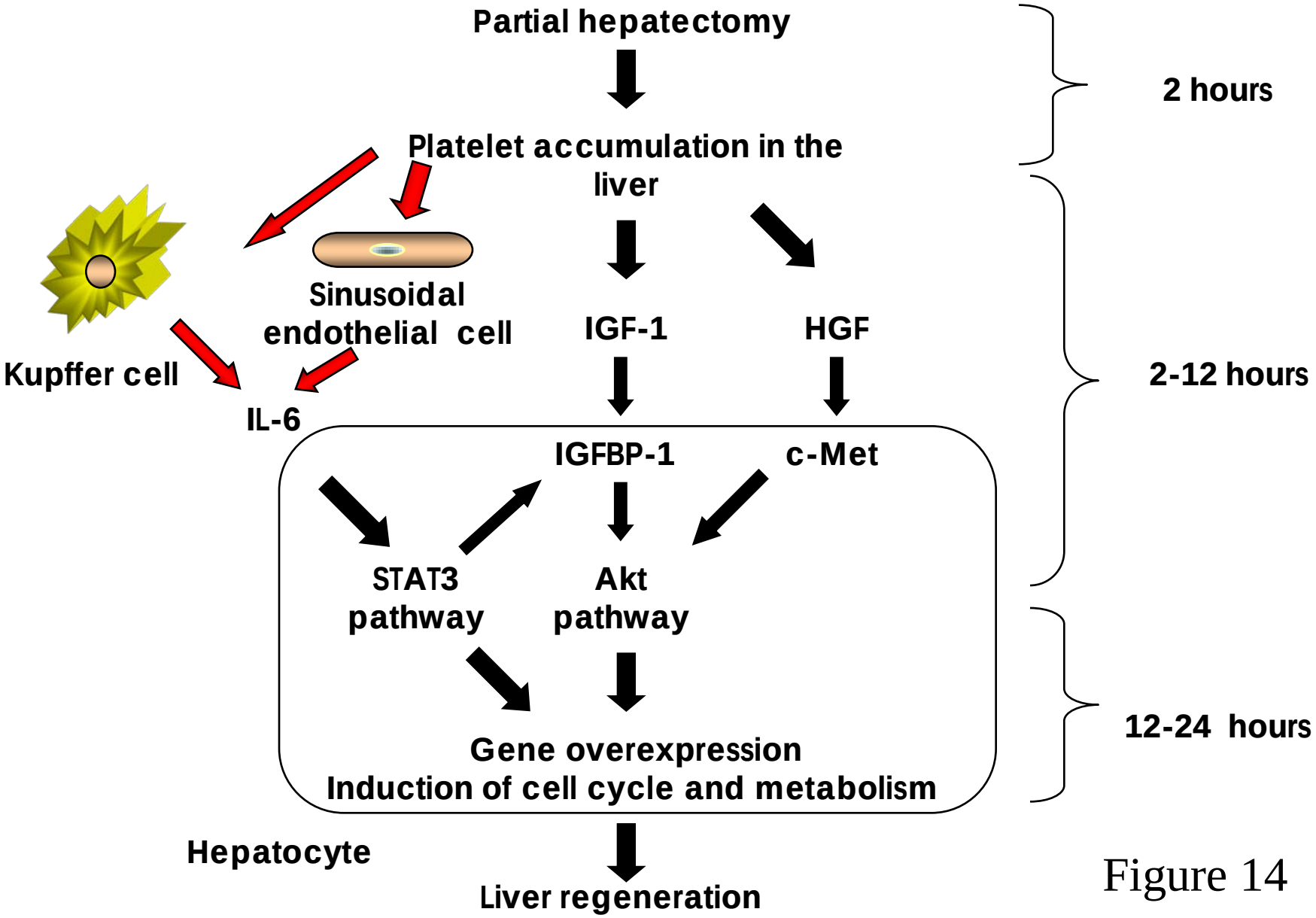


Figure 14

Table 1. Overexpressed genes in thrombocytotic group compared to normal group.

Accession number	Gene abbreviation	Gene name	Expression time (hr)	Expression fold
Transcription factors				
NM_007913	Egr1	Early growth response 1	12 24	7.66 5.41
NM_011448	Sox9	SRY-box containing gene 9	12	4.87
NM_007498	Atf3	activating transcription factor 3	12	4.03
NM_007678	Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	12	3.74
NM_010849	C-myc	myelocytomatosis oncogene	24	3.24
NM_008453	Klf3	Kruppel-like factor 3	24	3.14
Growth factors and cell cycle control				
NM_008655	Gadd45β	growth arrest and DNA-damage-inducible 45β	12	6.05
NM_007836	Gadd45α	growth arrest and DNA-damage-inducible 45α	12	5.42
NM_007569	Btg1	B-cell translocation gene 1, anti-proliferative	12	4.9
NM_009344	Phlda1	pleckstrin homology-like domain	12	4.02
NM_007570	Btg2	B-cell translocation gene 2, anti-proliferative	12	3.08
NM_008341	IGFBP-1	insulin-like growth factor binding protein 1	24	4.24
NM_009770	Btg3	B-cell translocation gene 3	24	4.88
NM_032002	Nrg4	neuregulin 4	24	3.31
NM_009397	Tnfaip3	tumor necrosis factor, alpha-induced protein 3	24	3.17
Signal Transduction				
NM_145133	T2bp	Traf binding protein	12 24	4.79 6.54
NM_011363	Sh2bp1	Sh2B adaptor protein 1	12	3.88
NM_011085	Pik3r1	phosphatidylinositol 3-kinase	24	8.76
NM_010704	Lepr	lepton receptor	24	4.07
NM_009895	Cish	cytokine inducible SH2-containing protein	24	3.8
NM_016693	Map3k6	mitogen-activated protein kinase kinase kinase 6	24	3.7
NM_001024955	Pik3r1	phosphatidylinositol 3-kinase	24	3.43
Receptors and transport				
NM_011400	Slc2a1	solute carrier family2 facilitated glucose transport	12	6.2
NM_153062	Slc37a1	solute carrier family37 glycerol-3-phosphate trans.	12	4.65
NM_007812	Cyp2a5	cytochrome P450, family 2, polypeptide 5	24	9.84
NM_009997	Cyp2a4	cytochrome P450, family 2, polypeptide 4	24	8.32
NM_008630	Mt2	metallothionein 2	24	3.15
NM_013602	Mt1	metallothionein 1	24	3.21
NM_013703	Vldlr	very low density lipoprotein receptor	24	3.64
Metabolism				
NM_010292	Gck	glucokinase	6	3.42
NM_145444	Acot5	acyl-CoA thioesterase 5	12 24	18.05 7.15
NM_009731	Akr1b7	aldo-keto reductase	12 24	7.1 16.4
NM_013743	Pdk4	pyruvate dehydrogenase kinase	24	10.93
NM_144930	TGH-2	thriacylglycerol hydrolase 2	24	7.32
NM_007933	Eno3	enolase 3, beta muscle	24	6.76
NM_026179	Abhd5	abhydrolase domain containing 5	24	4.63
NM_007606	Car3	carbonic anhydrase 3	24	3.68
NM_010442	Hmox1	heme oxygenase (decycling) 1	24	3.77

Note: In total, 240 genes were overexpressed more than 3-fold. The most important 38 genes contribute to the liver regeneration.