

STUDYING LIVER REGENERATION BY MEANS OF MOLECULAR BIOLOGY: HOW FAR WE ARE IN INTERPRETING THE FINDINGS?

David Rychtrmoc^{1,2}, Antonin Libra², Martin Bunčec², Tomáš Garnol¹, Zuzana Červinková¹

Charles University in Prague, Faculty of Medicine in Hradec Králové, Czech Republic: Department of Physiology¹; GENERI BIOTECH Ltd., Hradec Králové, Czech Republic²

Summary: Liver regeneration in mammals is a unique phenomenon attracting scientific interest for decades. It is a valuable model for basic biology research of cell cycle control as well as for clinically oriented studies of wide and heterogeneous group of liver diseases. This article provides a concise review of current knowledge about the liver regeneration, focusing mainly on rat partial hepatectomy model. The three main recognized phases of the regenerative response are described. The article also summarizes history of molecular biology approaches to the topic and finally comments on obstacles in interpreting the data obtained from large scale microarray-based gene expression analyses.

Key words: Liver Regeneration; Hepatectomy; Gene Expression; Microarray; Hepatocytes; Rat

Introduction

The liver regeneration attracts scientific attention for decades as a unique phenomenon in mammalian physiology. The interest does not decline in recent years. It rather grows as its relevancy to clinical situations and discovery potential for basic research are well recognised.

New methods employed in this field yield important and useful knowledge which in turn shapes design of further studies. The literature dealing with liver regeneration is replete and arises from various scientific branches including but not limited to physiology, genomics, radiology and imaging disciplines, tissue engineering and liver surgery and transplantation fields. The aim of this article is to provide a brief review of current understanding of the mechanisms of liver regeneration, list molecular biology approaches employed in this field and to bring a discussion of challenges arising when interpreting the results of recent microarray gene expression analyses.

On the normal architecture and function of the liver

The liver is a vital organ performing plenty of essential body functions. It synthesizes and secretes plasma proteins and majority of clotting factors; maintains blood glucose and ammonia levels critical for central nervous system function. Creation of bile and urea and many more roles in intermediate metabolism and elimination of both endogenous and exogenous substances make liver the main detoxifying organ of the body.

Concerning the structure the liver is an organ with a complex architecture, with several cell populations taking part. The parenchymal cells - hepatocytes build up sixty per cent of the total cell count and eighty per cent of the overall organ volume. The remaining twenty per cent is shared by lining cells of liver sinusoids and several types of immune system cells, Ito cells and epithelial cells of intrahepatic bile ducts. Kupffer cells and dendritic cells belong among the resident immune system cells, while various subpopulations of lymphocytes rank among the migrating ones. Ito cells, called also stellate (or fat-storing) can produce several paracrine factors and thus play a regulatory role in extracellular matrix remodelling and eventual development of liver fibrosis (28, 37).

Basically, the liver parenchyma is organised into hexagonal lobules each having a centrally located hepatic vein, terminal branch of systemic venous vasculature draining blood into the inferior vena cava. Each structural lobule is surrounded by usually six peripherally located portal spaces, containing mainly triads of following structures: terminal branch of portal vein, terminal branch of hepatic artery and interlobular bile duct. Besides these lymphatic vessels, nerves and small amount of interstitial connective tissue can be found in portal spaces (Fig. 1). Only 3 % of liver volume is constituted by extracellular matrix (ECM), making the liver highly cell dense organ.

Despite its small proportion the ECM can importantly influence the signal transduction by binding or release of regulatory substances either in active or inactive form. ECM compounds can even perform cleavage of the humoral factors to increase or limit their function (54).

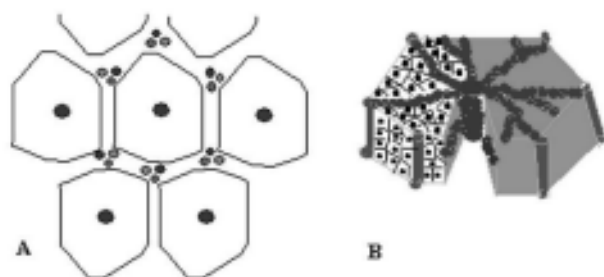


Fig. 1: Simplified representation of liver parenchyma microarchitecture. A) View of several neighbouring structural lobules, each having a centrally located vein. Triads of structures stand for portal triads in each corner of the approximately hexagonal lobule. B) Closer look at the single lobule structure. Portal triads are again simply represented in its corners. We see also several sinusoids, spanning from the terminal branches of portal vein to the central vein. Individual hepatocytes are shown on the left, while the right part of the picture implies the spatial organisation of the lobule. For details see text.

Functional division of the liver parenchyma follows different principles, main factor being the quality of blood supply to the hepatocytes. From this point of view portal triads are central structures to the functional hepatic acini. Each acinus has three zones numbered I to III from the centre to the periphery with oxygen and nutrients levels decreasing with the distance from the portal triad (48, 55). The differences in the characteristics of supplied blood are responsible for so called functional heterogeneity of hepatocytes among the zones of acini, impacting the scope of metabolic activities and susceptibility or resistance to oxygen or nutrient deprivation or chemical intoxication.

Importance and description of the liver regenerative capacity

Referring to liver regenerative capacity would be incomplete without mentioning the ancient Greek myth of Prometheus, who brought people the secret of fire from the divine seat mount Olympus and was punished by chaining to a rock. Every day an eagle arrived to batten on his liver, which amazingly regrew its volume again.

The liver is a central organ in above mentioned inevitable functions and at the same time it is due to its localisation first exposed to antigenic and toxic substances ingested by an organism. These enter the liver via its functional blood supply by portal vein. Portal vein collects blood from all other azygous abdominal organs, thus presenting vast entrance gateway of exogenous substances (and endogenous catabolites as well) into the internal environment of an organism. It is hypothesized that the unique regenerative capacity of the liver is an adaptation to this enormous load with an intention to enable survival of a being, which for instance tried a previously unknown feed. The liver regenera-

tion feature is seen in all vertebrates from fish to humans (38).

Liver functions can not be omitted during the regeneration. Hepatocytes do not dedifferentiate during the regeneration and are capable of readily bearing increased functional demands after loss of part of the organ volume (5, 13). It is truly amazing that the regenerating liver despite the reduced mass serve the rest of the body by all of its homeostatic functions (38).

The minimal volume of the liver remnant capable of such functioning and simultaneous recovery from an insult has been subject to research and both in animals and humans survivable 90 per cent liver resections were reported (16, 49). In such a large scale hepatectomies additional measures are employed to increase the survival rate. These include high energy diets or portal vein arterialization in experimental and clinical settings (9, 40, 42).

The liver regeneration after partial hepatectomy is not only a biological model for study of tissue homeostasis but it may also bring better insight into the regeneration of the donor liver remnant in the living donor liver transplantation. Surgeons could then provide safer care for donors who are preoperatively healthy volunteers and harm to them should be avoided or minimised (24). Donor complications are nowadays reported with varying methodology, which leads to largely different results among the transplantation centres (1). Other clinically relevant settings are liver resections due to tumour metastases or liver injury. Developers of artificial liver support devices also seek knowledge about the mechanisms of regeneration in effort to increase their efficiency (5, 43).

It is worth emphasizing that unless specifically prevented, the liver volume restoration is accomplished by proliferation of mature parenchymal cells - hepatocytes, unlike wound repair seen in skin or small intestine (38, 56). A record number of 12 sequential hepatectomies performed in rats without failure of the regenerative potential of the liver was reported (51). Overturf and colleagues (1997) have shown even more striking results with adult mice hepatocytes. Increase in telomerase activity may contribute to this almost endless capacity of hepatocytes to divide *in vivo*. Moderate (up to twofold) increase in telomerase activity was found in regenerating rat hepatocytes, but there is a detectable telomerase activity in quiescent rodent livers which is not the case in humans (17). A study in pig model, which better fits human telomerase biology with no activity in quiescent livers, revealed even fivefold increase in telomerase activity after two thirds partial hepatectomy (63).

In vivo models of liver regeneration

The need of *in vivo* models of liver regeneration is emphasized by complexity of the process. Significance of the interactions among all liver cell populations is nowadays undoubted and the action of circulating modulators of extrahepatic origin can not be simulated *in vitro*. Moreover

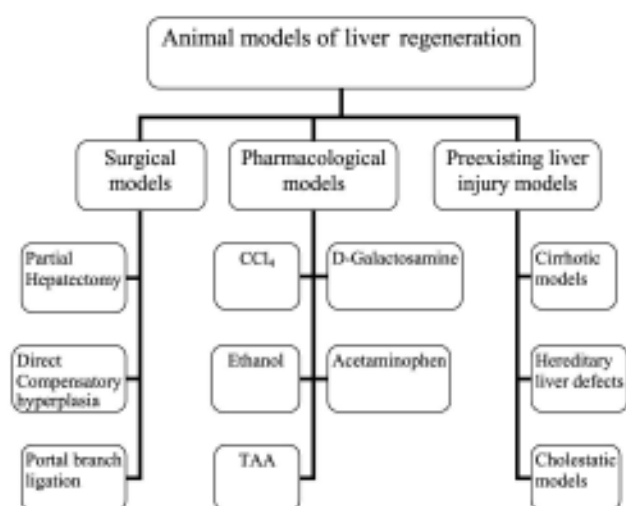


Fig. 2: Animal models of liver regeneration. Adapted with kind permission from Ref. 43. TAA = thioacetamide; CCl_4 = tetrachlormethane.

the regulation by body functional demands is only possible in the whole organism (43). Studies in humans present ethical obstacles and also the heterogeneity of conditions leading to liver injury in clinical settings complicates evaluation of such studies (44).

Animal *in vivo* studies are a reasonable compromise in terms of availability, affordability, reproducibility and standardised conducting and evaluation. But of course evidence exist about differences in physiology of regeneration among smaller and higher animals and situation in humans (43). The already established animal models are summarised in Fig. 2.

The time frame of liver regeneration depends on the kind of organ damage and its severity and extent. Rats or mice are the most frequently used model organisms because the liver regeneration is especially rapid in small rodents. In both species two thirds partial hepatectomy first introduced by Higgins and Anderson in 1931 (19) is the prevalent intervention. The anatomical differences necessitate adjustment of surgical procedure for the actual species, but both techniques are widely spread. While rat model gives more reproducible results, mice are valuable for the possible use of genetically engineered strains (13). The full organ size restoration is reported within 5 to 10 days in most of the rodent studies.

In humans reported times necessary for liver regeneration vary greatly among the articles. It is of course due to the different insults studied and varied methods used for measuring the organ size. But there is even no consensus about the completeness of the response. While Michalopoulos (2007) states that normal liver weight is re-established in 8–15 days in humans, followed by a slow lobular reorganization taking place for several weeks (62), Court et al., (2002) states, that under normal circumstances, the liver regeneration in humans is initiated within 3 days and by 6

months the recovery of original mass is complete. Transplant clinician Manzarbeitia (2002) in clinical setting of living donor liver transplantations witnesses almost complete restoration of both the donors' and recipients' livers in 6–8 weeks, with most of the recovery occurring in the first two weeks. Older report by Jansen (1990) found out maximal 75 ± 2 % size recovery after a post partial hepatectomy follow-up period of 50 days. Taken together it is not simple to determine the time needed for ultimate recovery in humans and the comprehensive discussion of this issue is above the frame of this article.

Overview of liver regeneration principles

Response of the liver to a damaging insult which is not immediately lethal to the organism is commonly called "regeneration" although it does not fulfil criteria for doing so. The principle is different from what is seen in amphibians regrowing lost limbs. It is not a new creation of lost tissue in its original localisation. In fact, the remaining mature cells undergo replication, and thus enlargement of unaffected organ remnant underlies the volume recovery of the liver. Therefore the phenomenon should be named compensatory hyperplasia (12, 56). Nevertheless the term liver regeneration is already well established and is commonly used even in scholarly literature. That is why we keep the term also in this article.

The liver regeneration occurs after a variety of different insults including the mechanic tissue loss (injury, resection) or necrosis of some portion of the liver cells without reduction in organ volume (leading to functional deficiency) caused e.g. by ischemia, acute or chronic intoxication eventually by infection (typically viral hepatitis).

It is generally accepted, that the liver regeneration process after the loss of functional mass consists of three fundamental phases: a) initiation or priming, progression of the quiescent hepatocytes to repeated division, b) proliferative phase, restoring the liver tissue to its volume before an insult and c) termination of growth and balancing the resultant size of the liver to just comply with functional demands of the organism (14).

Priming phase

Hepatocytes the most numerous liver cells are long lived elements which rarely divide. Their replication rate is one in twenty thousand (50). Under normal conditions hepatocytes are irresponsive to growth stimuli. This changes amazingly after an appropriate triggering event, partial hepatectomy being among the most efficient ones.

Other triggering events are necrosis after blunt injury, metabolic stress imposed e.g. by toxins, or any phenomenon leading to disruption of intercellular contacts (10, 25, 43) or digestion of the extracellular matrix (31).

This change from quiescent to cycling state is called priming and is generally ascribed to cytokine-dependent sig-

nalling pathways action in short time after an insult. It is concluded that the triggering factors must be in place faster than de novo proteosynthesis could occur and that they are substances already present in the body. One of the suggested early initiators is lipopolysaccharide (LPS), produced by the gut flora and released from the intestines as a consequence of deteriorated intestinal barrier due to surgical stress. LPS reaches liver with the portal blood and can readily influence its cells. The LPS activates the non-parenchymal cells (including Kupffer cells and stellate cells) and increases their production of tumour necrosis factor alpha (TNF α) and interleukin 6 (IL-6) (56). Another substances acting as rapidly as LPS are activated complement compound C3a and C5a, showing that the response is not isolated to the liver itself, but that the circulating factors take part as well (35, 53).

Cytokine signals including TNF α and IL-6 cause transcription factors NF κ B, STAT3, AP-1 and CEBP β to bind DNA rapidly by means of their posttranslational modifications (6, 12). Subsequently, in 20 to 30 minutes after the PH, derepressed transcription factors increase expression of so called immediate early genes. These include the protooncogenes c-fos, c-jun, c-myc and c-met, the latter being the HGF receptor (52).

Cytokine mediated pathway is responsible for G0/G1 transition, after which the action of growth factors is inevitable. Complete hepatic mitogens recognised in vivo and also in primary hepatocyte cultures are HGF, TGF α , and EGF. These allow cells to overcome the G1 restriction point and continue towards mitosis (32).

Extrahepatic body organs also have a role to play in reaction to liver injury. Cooperative priming signals come from the pancreas (insulin), duodenum or salivary glands (EGF; epidermal growth factor), thyroid gland (T $_3$, triiodothyronine) and adrenal glands (norepinephrine). These comitogens help initiate the recovery of the liver (7, 8, 30, 56).

Proliferative phase

Proliferative phase is characterised by mitotic waves of hepatic cells. The well obvious marker of the cell cycle progression is the rate of DNA synthesis observed to appear in a coordinated fashion in individual liver cell subpopulations. Hepatocytes reach the S phase first, with the DNA synthesis beginning to rise 12 hours after the PH and peaking at about 24 hours after the operation. The S phase in the nonparenchymal cells occurs later; at 48 hours for Kupffer and biliary cells and around 96 hours for endothelial cells (12, 56). After an insult causing necrosis or apoptosis of hepatocytes, the course of the cell priming is similar as after the PH, but the replicative waves are less coordinated.

Functional heterogeneity of hepatocytes applies also to the onset of DNA synthesis within the liver acini, starting around the portal vein (zone I) and gradually proceeding towards the central vein (zone III).

During the proliferative phase almost all of the hepatocytes undergo mitosis. This percentage is approximately 95 % in young rats and decreases to about 70 % in old animals (3, 46). The mechanism of replicative senescence of the hepatocytes is not clarified so far. In humans there are less observations of such kind available, but in liver transplantation grafts from younger donors generally carry better outcome (67).

Not all the hepatocytes that passed the S phase finally replicate. The proportion of binucleate cells within the liver increases, and some hepatocytes become polyploid but undivided. The first mitotic wave concerning the vast majority of parenchymal cells is followed by a second one, about 48 hours after PH, involving smaller percentage of hepatocytes. In total there is about 1.6 cycles of replication in all cells to fully restore the liver (56).

Termination phase

Cessation of the regenerative response is the least elucidated phase of the process. It is still not clear whether it is onset of inhibitory agents or withdrawal of stimulatory substances that finally stops regeneration. The interplay among the liver cell subpopulations and probable participation of the recovered extracellular matrix complicate revealing the mechanisms of observed precise liver size regulation. Nonparenchymal cells may mediate the inhibition of regrowth during structural reorganisation of the liver which comes after the volume regain is finished. Reappearing extracellular matrix may play an important role by renewed binding of pro-HGF (35).

Mechanisms that link the replicative activity of hepatocytes in the regenerating liver with body functional demands, have been sought and mammalian target of rapamycin (mTOR) and its downstream effectors belong to proposed candidates (11).

Factors with known inhibitory effects on hepatocyte proliferation in cultures such as TGF- β 1 (in vivo normally synthesized by Ito cells) have been subject of research. Some observations suggest that disappearance of (detectable) tissue TGF- β 1 from periportal to pericentral region of lobule enables progression of hepatocyte mitotic wave in the same direction at the onset of regeneration. TGF- β 1 released in the plasma shortly after PH is probably inactivated by binding to α 2 macroglobulin. Hepatocytes themselves are transiently resistant to mito-inhibitory effects of TGF- β 1 during the proliferative phase. After this refractory period, TGF- β 1 could play a role in ending the regeneration, however in transgenic mice overexpressing TGF- β 1 regeneration is slowed down but it finally completes (5, 37). Related TGF β family members, such as activin, act similarly.

Several negative feedback loop interactions related to priming phase stimuli were recognised. To name some of these: plasminogen activator inhibitor (PAI) induced by IL-6, blocks the HGF action by preventing the cleavage of

pro-HGF into an active HGF. Suppressor of cytokine signalling-3 (SOCS3) upregulated downstream to IL-6, causes downregulation of phosphorylated signal transducer and activator of transcription 3 (STAT3) ultimately terminating the original IL-6 signal (56).

Apoptosis, as a possible mechanism correcting the resultant size of the liver was studied in normal and IL-6^{-/-} mice, by Sakamoto et al (1999). This work suggested greater role of apoptosis in normal than in IL-6 deficient liver in eliminating the superfluous hepatocytes generated by regeneration.

Molecular biology methods to study mechanisms of the liver regeneration

Given the long history of research interest in the liver regeneration, it is not surprising, that it has been studied by molecular biology methods as well. Early works, in the 1980's, assessed the presence and relative abundance of the specific mRNAs by autoradiography measurements following membrane-based hybridisation techniques like Northern blot and Dot blot (21, 29, 58). These approaches were eventually complemented by histochemistry and cytogenetic methods (36). These works mostly dealt with several genes of interest focusing on narrowly specified aspects of hepatocyte growth. Experiments by Taub and co-workers outstand by the number of genes analysed and the time intervals studied (18).

Experiments using the genetically engineered mice strains gained wider use in 1990's. The ability to permanently or transiently overexpress or suppress (knock out) a gene of choice has enabled much progress in studying the involvement of individual factors or whole transcription pathways in the process (2, 13).

Advent of microarray platforms, introduced in 1995, allowed for wider scope of gene expression analyses. Simultaneous gene expression analyses of thousands of genes raised a new challenge. It is now demanding to sort out genes truly related to the observed functional or morphological changes among many detected by high throughput microarray experiments. Usually time course of expression alterations after the intervention is used as a criterion for creating clusters, subgroups of genes with similar expres-

sion patterns, which is based on assumption that common activation or suppression indicates functional relationships among transcripts. Much database searching necessarily follows when interpreting the findings (15, 39, 59, 64). Extensive use of public data repositories and bioinformatic platforms fosters their rapid development.

Real time PCR gene expression analyses usually complement high throughput methods. Either as a confirmative approach for selected genes or as a preliminary method for more precise focusing of laborious and expensive advanced techniques. Moreover the real time PCR provides wider linear dynamic range of transcript quantitation and thus refines the results. Work by Cimica et al. (2007), who used serial analysis of gene expression (SAGE) for one post partial hepatectomy interval based on previous real time PCR and immunostaining results, is a recent example of such combination of methods.

Interesting novel approach was used by Juskeviciute et al. (2008), who were able to identify previously unknown transcription factors participating in liver regeneration, thanks to bioinformatic analysis of shared transcription factor binding sites in the clustered microarray data.

Interpreting recent microarray data

Majority of so far published papers is focused on early changes after the liver insult, based on the notion, that the reactivation from quiescence and subsequent entry into the S phase of the cell cycle is the most exceptional feature of liver regeneration model. Propagation of the proliferative response seems to follow the same principles as seen in other extrahepatic cells or tissues, making later phases of regeneration less scientifically attractive. This general fact applies to microarray-based analyses as well.

As seen in Tab. 1, summarising several features of seven recently published studies using microarrays, the sample acquisition is usually denser in shorter intervals and becomes notably looser after 24 hours after the resection. Thus it would seem logical; that the molecular mechanisms underlying the first 12 or 24 hours of liver regeneration should be well described and understood in good agreement among the researchers. Unfortunately the situation is more complicated. Anyone trying to interpret the findings of large

Tab. 1: Recently published microarray-based works on liver regeneration and their sampling intervals.

First author	PY	Model organism	Post partial hepatectomy sampling intervals																						
			1 m	30 m	90 m	3 h	4 h	6 h																	
Mortensen	2008	pig																							
Fukuhara	2003	rat						6 h	12 h	18 h	24 h		48 h		72 h		168 h								
Togo	2004	mouse		60 m		3 h	6 h	12 h		24 h															
Xu	2005	rat				2 h	4 h	8 h	12 h	16 h	24 h	36 h	48 h		72 h	96 h	144 h								
Shao	2007	rat		30 m	60 m	2 h	4 h	6 h	8 h	12 h	24 h	36 h	54 h	66 h	72 h	120 h	144 h	168 h							
Yokoya	2004	rat		60 m				6 h			24 h		48 h					168 h							
Xu	2007	rat		30 m	60 m	2 h	4 h	6 h	8 h	12 h	16 h	18 h	24 h	30 h	36 h	42 h	48 h	54 h	60 h	66 h	72 h	96 h	120 h	144 h	168 h

PY = year of publication; m = minute(s); h = hours(s)

Tab. 3: Genes involved in acute phase response and their coverage in the seven compared microarray-based studies.

Gene Name	RGD ID	GENE SYMBOL	ENTREZ GENE ID	UNIGENE	Ref No.							
					64	47	66	39	59	15	65	
BILE ACID-COENZYME A: AMINO ACID N-ACYLTRANSFERASE	2190	BAAT	29725	RN.11129		X	X				X	
LIPOPOLYSACCHARIDE BINDING PROTEIN	61865	LBP	29469	RN.48863								
TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL, SUBFAMILY V, MEMBER 1	628841	TRPV1	83810	RN.3073								
TRANSFERRIN	3845	TF	24825	RN.91296								
ENDOTHELIN RECEPTOR TYPE B	2536	EDNRB	50672	RN.11412								
PROSTAGLANDIN E RECEPTOR 3 (SUBTYPE EP3)	3435	PTGER3	24929	RN.10361		X						
INTERLEUKIN 1 BETA	2891	IL1B	24494	RN.9869								
SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3	3772	STAT3	25125	RN.10247			X					
ALPHA-2-MACROGLOBULIN	2004	A2M	24153	RN.780	X	X	X					X
TUBEROUS SCLEROSIS 2	3908	TSC2	24855	RN.5875								
PHOSPHOLIPASE A2, GROUP IVA (CYTOSOLIC, CALCIUM-DEPENDENT)	67366	PLA2G4A	24653	RN.10162								
TRANSFERRIN RECEPTOR	70488	TFRC	64678	RN.98672								
SIMILAR TO SINGLE IG IL-1 RECEPTOR RELATED PROTEIN	1306732	SIGIRR	309106	RN.16525								
INSULIN 2	2916	INS2	24506	RN.989								
CCAAT/ENHANCER BINDING PROTEIN (C/EBP), ALPHA	2326	CEBPA	24252	RN.22163								
EPH RECEPTOR A3	68389	EPHA3	29210	RN.10713								
ARGININOSUCCINATE SYNTHETASE	2163	ASS	25698	RN.5078								
CHEMOKINE (C-C) RECEPTOR 5	620596	CCR5	117029	RN.10736								
INTERLEUKIN 1 RECEPTOR ANTAGONIST	621159	IL1RN	60582	RN.85806		X						
MACROPHAGE INFLAMMATORY PROTEIN-1 ALPHA RECEPTOR GENE	708446	CCR1	57301	RN.34673								
ALPHA-2-HS-GLYCOPROTEIN	2075	AHSG	25373	RN.32083								
SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 5B	3774	STAT5B	25126	RN.54486								
CHEMOKINE (C-C MOTIF) LIGAND 5	69069	CCL5	81780	RN.8019								

Thanks to this prerequisite, searching for the genes involved in the acute phase response among all the seven large scale studies became significantly more efficient. Resultant findings are also in the Tab. 3. Although there are again only scarce matches to the gene products we were looking for, confirming small overlap between independent papers, we want to emphasize the benefit of such interpreting procedure. It lies mainly in removing uncertainty from the gene specifications and thus allowing comparison of the results originally presented using different ways, e.g. only gene names or its symbols vs. giving the database descriptors which unfortunately may turn obsolete from the time of publication. The latter being frequent impediment given the perpetually ongoing refinements and updates to the internet-based genomic databases. Finally, the similar approach may be used for

any of the Gene Ontology functional categories, allowing anyone interested to focus on the field of their study.

Conclusion

This article deals with liver regeneration, the unique ability of the liver to recover from serious damages. The article describes significance of this interdisciplinary study topic and summarizes current knowledge in the field. Particular attention is paid to molecular biology methods utilized in liver regeneration studies. Finally, authors provide a discussion of possible obstacles in interpreting the recent findings from microarray-based studies and propose their way of circumventing them as a solution for others experiencing similar difficulties.

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List of abbreviations

AP-1, activator protein 1 (= c-jun); CEBP, CCAAT/enhancer binding protein; EGF, epidermal growth factor; HGF, hepatocyte growth factor; NF κ B, nuclear factor of kappa light polypeptide gene enhancer in B-cells; PAI, plasminogen activator inhibitor; SOCS3, suppressor of cytokine signalling-3; STAT3, signal transducer and activator of transcription 3; TGF, transforming growth factor

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Corresponding author:

David Rychtrmoc, MD, Charles University in Prague, Faculty of Medicine in Hradec Králové, Department of Physiology, Šimkova 870, 500 38 Hradec Králové 1, Czech Republic; e-mail: rychtrmoc@lfhk.cuni.cz
