



Role of death receptors in the regulation of hepatocyte proliferation and apoptosis during rat liver regeneration

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ABSTRACT. The balance between hepatocyte proliferation and apoptosis is critical for liver homeostasis during liver regeneration. We created a rat liver regeneration model by partial hepatectomy (PH) to investigate the overall mechanism that regulates the proliferation and apoptosis of hepatocytes. The Rat Genome 230 2.0 Array was used to investigate changes in the expression levels of genes associated with the known proliferation or apoptosis signaling pathways. Ingenuity Pathway Analysis 9.0 was used to determine interactions among these signaling pathways. The results revealed that the expression levels of multiple key genes in three death receptor (DR) pathways, Fas/FasL, TNFR/TNF α , and DR6, were significantly altered in hepatocytes after PH. The expression level of the gene encoding DR6 increased by over 100-fold, whereas the levels of the genes encoding Fas, FasL, and TNF α were increased by 2-4-fold 12 h after PH. Fas/FasL, TNFR/TNF α , and DR6 are known to participate in numerous cellular events including cell proliferation and apoptosis. Our results suggest that the DR6 pathway plays a major role in the regulation of hepatocyte apoptosis, whereas Fas/FasL and TNFR/

TNF α pathways may have roles in coordinating signaling activities between proliferation and apoptosis.

Key words: Liver regeneration; Hepatocyte proliferation; Death receptors; Hepatocyte apoptosis;

INTRODUCTION

Liver regeneration (LR) is an important process that allows the organs to recover from various pathological conditions as well as from surgery and transplantation. Hepatocytes are parenchymal cells and make a major contribution to LR. Under physiological conditions, less than 0.01% of hepatocytes in adult rat livers undergo mitosis (Zimmermann, 2004). However, when injured, the remaining hepatocytes can rapidly enter the cell cycle, together with other types of liver cell, to compensate for the lost tissue and restore the function of the liver, which is referred as LR. Numerous cellular events are involved in LR including cell activation, de-differentiation, proliferation, re-differentiation, and remodeling of tissue structure (Fausto, 2000; Hortelano et al., 2007). However, how these signaling pathways are integrated to ensure LR within the boundary of homeostasis is not well understood.

It is widely accepted that the process of rat LR can be divided into three stages: the priming phase (2-6 h after PH), the progressing phase (12-72 h after PH), and the terminal phase (120-168 h after PH). Multiple signaling pathways have been shown to participate in LR. A complex signaling network has been proposed to orchestrate the physiological activities during the process of LR. Members of the tumor necrosis factor receptor super family (TNFRSF) play important roles in the regulation of LR (Yamada et al., 1998; Kang et al., 2012).

The TNFRSF includes FAS, TNFR, TRAIL, CD27, DR4, DR5, and DR6 receptors (Wang and Xu, 2010). All members of the TNFRSF consist of cysteine-rich extracellular subdomains that indicate ligand binding specificity. Binding to the ligand leads the receptor towards trimerization and activation (Naismith and Sprang, 1998). This initiates the recruitment of adaptor proteins such as Fas-associated death domain (FADD) or TNF-receptor-associated death domain (TRADD). Recruiting FADD initiates the assembly of the death-inducing signaling complex, thereby facilitating apoptosis (Ru et al., 2012). Recruiting TRADD results in the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), JNK, and p38 MAP kinase pathways, which regulate several essential cellular activities such as proliferation, differentiation, and apoptosis. Enormous effort has been put into understanding the death receptor-mediated signaling pathways in the regulation of hepatocyte activities. It has been found that loss of TNF or FAS function delays LR in mice. Overexpression of the TNF/TNF receptor (TNFR) causes hepatic damage in animal models (Webber et al., 1998; Yamada et al., 1998; Mohammed et al., 2004). However, owing to the complexity of the signaling network, the molecular mechanism coordinating the proliferation and apoptosis of hepatocytes is not well understood.

In the current study, we used rat partial hepatectomy to create a LR model and employed systemic biological methods to examine the expression of genes in rat hepatocytes and their potential functions. The gene expression profile at nine time-points after PH was determined by rat gene microarray. The results were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and analyzed using the Ingenuity Pathway Analysis (IPA) software. IPA includes a comprehensive database and is a powerful tool for the systematical analysis of biological data in the context of current research publications. Here we describe the potential role of three members of the TNFRSF and their downstream components in the regulation of hepatocyte proliferation and apoptosis.

MATERIAL AND METHODS

Isolation and identification of hepatocytes from regenerating rat liver

Male adult Sprague Dawley rats weighing 190 ± 20 g were provided by the Animal Center of Henan Normal University. The rats were kept at $21 \pm 2^\circ\text{C}$ and $60 \pm 10\%$ relative humidity with free access to water and food. A total of 114 rats were randomly divided into 19 groups with six rats per group: nine partial hepatectomy (PH) groups, nine sham operation (SO) groups, and one control group. PH rats were subjected to two-thirds hepatectomy by the Higgins and Anderson method (Higgins and Anderson, 1931). After PH, the abdominal cavity was reopened at 2, 6, 12, 24, 30, 36, 72, 120, and 168 h to collect liver tissues. The hepatocytes were then isolated using a two-step collagenase perfusion and Percoll density gradient centrifugation (Grisham, 1983; Vondran et al., 2008). The isolated hepatocytes were identified by two marker proteins, ALB and G6P (Wang et al., 2008), by immunocytochemical staining. The purity of the isolated hepatocytes was $\geq 95\%$. All experiments were carried out in compliance with the Current Animal Protection Law of China.

Rat Genome 230 2.0 microarray detection and data analysis

The total RNA was extracted from hepatocytes and purified following the protocol previously described (Antonov et al., 2010). Briefly, biotin-labeled complementary DNA (cDNA) was prepared using a GeneChip IVT kit according to the manufacturer instructions (Affymetrix Inc., Santa Clara, CA, USA) (Eisen et al., 1998). The hybridization process was carried out automatically using a GeneChip Fluidics Station 450 (Affymetrix Inc., Santa Clara, CA). The results were scanned and converted into signal values using a GeneChip Scanner 3000 (Affymetrix Inc.). The signal values were normalized according to manufacturer instructions. The P values were determined using the Affymetrix GCOS 2.0 software (Knepp et al., 2003). If the P-value of a gene is < 0.05 , the gene is defined as present, if the P value is < 0.065 the gene is defined as marginal, and if the P value is > 0.065 the gene is defined as absent. A gene was considered significantly changed if its PH-to-control ratio was ≥ 3 or ≤ 0.33 . The F-test was used to analyze the significance of gene expression difference between the PH and SO groups. If $P < 0.05$ at any PH time-point, the gene was defined as an LR-associated gene. To minimize experimental operation and microarray errors, assays of the samples at each time-point were repeated three times and the average value of the three independent assays was used for statistical analysis.

RT-PCR

To validate the reliability of the microarray data, the expression levels of four genes: *Ppp2r1b* (NM_001025418), *Pdgfrb* (NM_031525), *Myh9l1* (NM_013194), and *Sirpa* (NM_013016), were examined by RT-PCR. The β -actin gene *Actb* (NM_031144) was included as an internal control. The primers were designed using the Primer Express 5.0 software. mRNA was prepared from the purified rat hepatocytes and the first-strand cDNA was obtained by reverse transcription (Promega, USA). The RT-PCR was performed under the following conditions: 2 min at 95°C , followed by 40 cycles of 15 s at 95°C , 15 s at 60°C , and 30 s at 72°C . Each sample was examined by performing the procedure in triplicate.

Construction of the apoptotic networks using the IPA software

To analyze the microarray data with respect to HC apoptosis, we input the ratio values of the genes associated with LR into the IPA software and searched for “apoptosis”. The apoptotic genes were defined based on their participation in apoptosis-related signaling transduction. The signaling network generated from these apoptotic genes was named “small net”. To put “small net” in the context of the current understanding of hepatocyte apoptosis, we searched for hepatocyte apoptosis-related signaling pathways in IPA. The signaling network generated from this search was named “middle net”. We also researched for apoptosis signaling pathways reported for all cell types in IPA. A comprehensive signaling network consisting of a total of 30 apoptotic signaling pathways was obtained. This network was named “big net”. We compared these three networks to analyze the potential mechanisms underlying the process of hepatocyte apoptosis.

Analysis of the relationship between the expression of the apoptotic genes and signaling transduction activities

Based on the ratio values of the apoptotic genes at each PH time-point, IPA calculated the corresponding signaling transduction activity values [$-\log(P \text{ value})$] related to apoptosis. If the activity value is above the baseline [$-\log(P \text{ value}) = 1.3$], it indicates that the participation of this pathway in cell apoptosis is increased. The F-test was used to determine the significance of differences between the PH and SO groups. $P \leq 0.05$ was considered to be statistically significant. If the $-\log(P \text{ value})$ of a PH group was greater than the corresponding SO group, apoptosis activity was enhanced in the PH group. Otherwise, the apoptosis activity was weakened in the PH group.

RESULTS

Confirmation of the microarray results by RT-PCR

The Rat Genome 230 2.0 Array contained 24,618 rat genes. The microarray showed that the expression of 9366 genes in hepatocytes were significantly changed during rat LR. To validate the microarray results, we randomly selected 30 genes for quantitative RT-PCR analysis. The results of RT-PCR and microarray were not exactly matched but the trend was consistent in general. Figure 1 shows the expression patterns of four genes, *Ppp2r1b*, *Pdgfrb*, *Myh9l1*, and *Sirpa*, as detected by the two methods. The RT-PCR results for other genes have been published elsewhere (Wang and Xu, 2010).

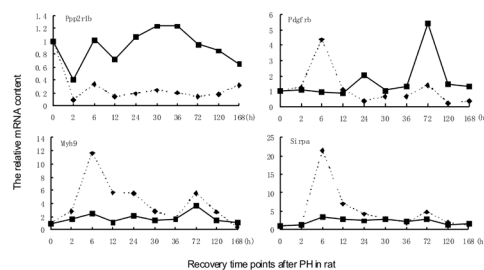


Figure 1. Comparison of relative mRNA levels detected by Affymetrix Rat Genome 230 2.0 arrays (solid lines) and reverse transcription polymerase chain reaction RT-PCR (dotted lines).

Expression changes in hepatocyte death receptors during rat LR

The F-test was used to analyze the microarray data for signaling factors related to hepatocyte proliferation and apoptosis. The results indicated that the expression levels of the genes encoding death receptors including Fas, TNF α , and DR6 was significantly increased. The genes encoding FasL and DR6 were upregulated as early as 2 h, and the expression level of the gene encoding TNFR1 increased during the progressing and terminal phases of LR (Table 1). Particular consideration was given to DR6, which is a relevant new member of the death receptor family. The increase in the level of DR6 was extremely rapid and dramatic. It reached 30-fold at PH 2 h, continued to increase to over 100-fold at PH 12 h, then gradually decreased but was still 4-fold higher than the control by the end (PH 168 h). This expression pattern suggests that DR6 plays a major role in the regulation of liver homeostasis, and may actively participate in both hepatocyte proliferation and apoptosis.

Table 1. Expression changes in death receptor genes in hepatocytes during rat liver regeneration (LR).

Protein	Gene	Recovery time (h) after partial hepatectomy									
		0	2	6	12	24	30	36	72	120	168
Fas	<i>Fas</i>	1.00	1.84	0.66	1.26	3.39	4.27	2.07	3.07	5.00	3.69
FasL	<i>FasL</i>	1.00	3.94	4.72	3.81	3.80	2.79	3.27	4.09	1.40	3.96
TNF α	<i>Tnf</i>	1.00	1.71	4.98	2.26	1.96	2.80	3.65	3.40	2.03	1.39
TNFR1	<i>Ngfr</i>	1.00	3.39	7.09	3.12	1.08	8.34	4.07	3.33	5.70	1.22
	<i>Tnfrsf11b</i>	1.00	0.95	1.18	0.97	12.04	0.99	7.04	27.41	4.87	2.06
DR6	<i>Tnfrsf21</i>	1.00	29.29	64.65	108.81	32.68	7.99	14.55	14.73	7.44	4.09

Bold numbers represent fold-change value of upregulated and downregulated genes between PH and SO groups. Gene with fold change >3 and F-test P value <0.05 is defined as upregulated gene; gene with fold <1/3 and F-test P value <0.05 is defined as downregulated gene at least on time point during LR.

Expression of genes related to death receptor-mediated cell proliferation during rat LR

To gain further insight into the death receptor-mediated signaling in relation to hepatocyte proliferation and apoptosis, the downstream genes were identified using the IPA software. The expression patterns of the downstream death receptor genes associated with cell proliferation are summarized in Table 2.

Table 2. Expression changes in genes related to death receptor-mediated cell proliferation.

Protein	Gene	Recovery time (h) after partial hepatectomy									
		0	2	6	12	24	30	36	72	120	168
JNK	<i>Mapk8</i>	1.00	2.77	1.28	2.34	1.66	2.04	1.32	3.52	1.56	2.27
	<i>Mapk12</i>	1.00	1.09	2.19	1.40	2.82	0.59	3.03	6.59	2.26	2.44
	<i>Mapk10</i>	1.00	3.52	2.16	3.99	3.02	1.68	3.73	4.66	1.33	6.31
MEKK	<i>Map3k8</i>	1.00	14.44	2.28	2.21	1.55	1.18	1.08	1.89	3.04	1.91
	<i>Map3k6</i>	1.00	9.77	18.63	25.45	6.30	4.46	4.24	8.77	0.73	0.40
NIK	<i>Map4k4</i>	1.00	1.05	2.03	1.52	6.31	5.07	6.67	7.27	2.87	2.81
AKT	<i>Akt3</i>	1.00	0.86	1.26	2.59	1.44	1.45	6.80	4.42	3.66	1.58

Bold numbers represent fold-change value of upregulated and downregulated genes between PH and SO groups. Gene with fold change >3 and F-test P value <0.05 is defined as upregulated gene; gene with fold <1/3 and F-test P value <0.05 is defined as downregulated gene at least on time point during LR.

The results show that *Mapk8*, *Mapk10*, and *Mapk12* were upregulated in the late progressing phase and *Map3k8* and *Map3k6* were upregulated during the entire course of rat LR. The *Map4k4* gene encoding NIK (NF- κ B inducing kinase) was mainly upregulated during the progressing phase.

Based on these data, we speculate that *Map3k8* and *Map3k6* are critical for initiating the proliferation of hepatocytes immediately after PH. *Map3k6* might also promote hepatocyte proliferation during the progressing phase as the expression of the *Map3k6* gene stayed high throughout LR. The genes encoding NIK and AKT mainly increased at late PH time-points, suggesting that NIK and AKT might not be critical for triggering hepatocyte proliferation but might contribute to hepatocyte proliferation at the later stages of LR. The increased gene expression levels at different PH time-points for *Mapk10*, *Mapk12*, and *Mapk8* might assist hepatocyte proliferation during the course of rat LR.

Expression of genes related to hepatocyte survival and apoptosis during rat LR

Activation of Fas/FasL and TNF/TNFR initiates multiple signaling pathways and leads to a variety of cell physiological activities including cell proliferation, survival, and apoptosis. Proliferation of hepatocytes is essential for LR and apoptosis is critical for limiting liver volume. Apoptosis is a tightly regulated process that is controlled by both anti-apoptosis and pro-apoptosis factors. Hepatocyte genes with significant changes related to death receptor-mediated apoptosis are listed in Table 3. Bcl-2 and Bcl-XL are powerful anti-apoptotic factors. Our data showed that the expression of the *Bcl2* gene was significantly upregulated during the course of rat LR, suggesting that *Bcl2* plays an important role in the protection of hepatocytes from apoptosis. In line with this observation, two genes encoding pro-apoptosis proteins, *Gas2* and *Dffb*, were downregulated during rat LR. *Gas2* was downregulated during the progressing phase and deoxyribonuclease CAD (caspase-activated deoxyribonuclease) was downregulated in the terminal phase. GAS2 is a caspase-3 substrate that plays a role in regulating microfilament and cell shape changes during apoptosis (Sgorbissa et al., 1999). It can also modulate cell susceptibility to p53-dependent apoptosis by inhibiting calpain activity (Benetti et al., 2001). Reduced expression levels of *Gas2* and *Dffb* in hepatocytes suggest that their mediated apoptosis processes are attenuated during rat LR.

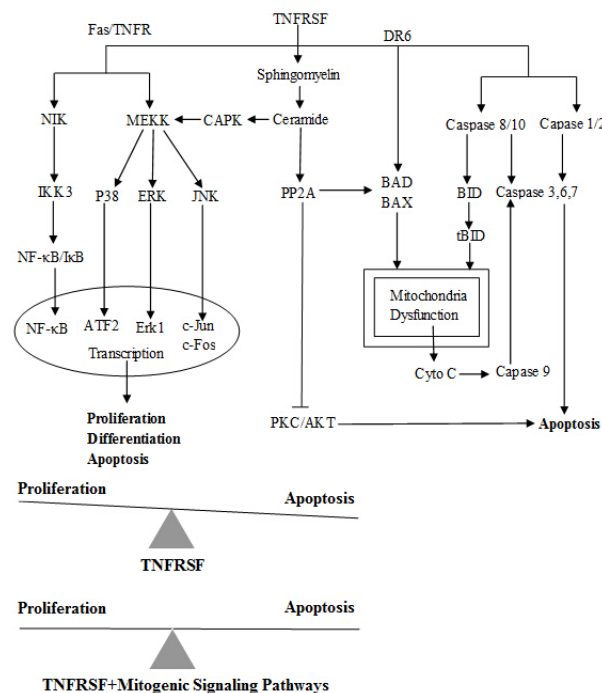
As LR requires the correct balance between proliferation and apoptosis, we also analyzed the genes related to apoptosis. Our microarray data showed that the expression of *Ppp2r1b* and *Ppp2r2a* was consistently upregulated during the course of rat LR. *Ppp2r1b* and *Ppp2r2a* encode protein phosphatase 2A (PP2A), which is a serine/threonine-protein phosphatase with broad substrate specificity (Zhang and Claret, 2012). Among the targets of PP2A are proteins of oncogenic signaling cascades, such as Raf, MEK, AKT, and apoptotic proteins, such as Bcl-2, BAD, and BAX. Dephosphorylation of the oncogenic proteins decreases proliferation, whereas dephosphorylation of apoptotic proteins promotes apoptosis. Furthermore, the expression of the *Xiap* gene encoding anti-apoptotic protein cIAP (cellular inhibitor of apoptosis protein) was significantly decreased during the terminal phase. Finally, genes encoding the transcription factors c-Fos and c-Jun were upregulated during rat LR. c-Fos and c-Jun regulate the expression of a broad range of proteins that in turn regulate cell proliferation, differentiation, and apoptosis. Based on the above results, a schematic representation of the death receptor-mediated signaling network is provided to illustrate the key signaling cross-talk points and the overall outcome (Figure 2).

Fas, TNFR, and DR6-mediated signaling pathways play an important role in the regulation of both hepatocyte proliferation and apoptosis. Their downstream components constitute a complex signaling network with multiple nodes of interaction and cross-regulation. Overall, our data indicate that Fas/TNFR/DR6-mediated signaling networks may favor hepatocyte apoptosis. With the participation of multiple growth factor signaling pathways, the balance between proliferation and death receptor-mediated apoptosis is tightly regulated and maintained in hepatocytes.

Table 3. Expression changes in genes related to hepatocyte cell survival and apoptosis.

Protein	Gene	Recovery time (h) after partial hepatectomy									
		0	2	6	12	24	30	36	72	120	168
Bcl-2	<i>Bcl2</i>	1.00	3.07	1.85	4.39	3.60	5.06	4.98	6.04	3.06	3.15
Bcl-XL	<i>Bcl2l1</i>	1.00	3.46	1.38	1.91	1.13	1.46	1.19	1.23	1.30	0.96
PP2A	<i>Ppp2r1b</i>	1.00	6.23	5.07	9.81	5.08	10.37	11.63	5.91	4.36	4.87
	<i>Ppp2r2a</i>	1.00	6.40	6.94	4.12	6.64	3.35	0.74	6.40	5.28	7.79
IKK	<i>Ikkkb</i>	1.00	2.31	2.71	1.93	1.68	1.47	1.23	3.21	3.04	1.91
cIAP	<i>Xiap</i>	1.00	0.99	0.56	1.63	0.98	3.81	1.03	0.43	0.31	1.06
Gas2	<i>Gas2</i>	1.00	0.68	0.19	0.15	0.58	0.79	0.73	0.70	0.76	0.91
CAD	<i>Dffb</i>	1.00	0.87	1.25	1.31	1.31	0.65	1.42	0.86	0.92	0.32
ICAD	<i>Dffa</i>	1.00	1.16	3.49	2.81	1.10	1.87	1.20	2.04	1.66	1.56
c-Fos	<i>Fos</i>	1.00	5.02	1.12	4.73	14.96	2.31	1.04	5.21	2.76	4.23
c-JUN	<i>Jun</i>	1.00	3.29	2.68	1.97	3.97	1.54	1.91	2.14	1.75	2.18

Bold numbers represent fold-change value of upregulated and downregulated genes between PH and SO groups. Gene with fold change >3 and F-test P value <0.05 is defined as upregulated gene; gene with fold <1/3 and F-test P value <0.05 is defined as downregulated gene at least on time point during LR.

**Figure 2.** A schematic representation of death receptor-mediated hepatocyte proliferation and apoptosis.

DISCUSSION

Hepatocyte proliferation is essential for the reconstitution of damaged tissue, whereas apoptosis eliminates unwanted DNA-damaged cells. Using systemic biological approaches and comprehensive analysis, the current study provides a broader view for understanding the role of the three death receptors in coordinating hepatocyte proliferation and apoptosis. The results of this study revealed that multiple key players in death receptor-mediated signaling coordinate the

balance between hepatocyte proliferation and apoptosis (Table 1). With regards to genes promoting cell survival and proliferation, *Map3k8* and *Map3k6* were significantly upregulated in hepatocytes during the early phase of LR, whereas *Akt3*, *Mapk8*, *Mapk10*, and *Mapk12* increased moderately towards the late phase (Table 2). This timeframe supports the theory that hepatocytes proliferate primarily during the early stages of LR.

In addition to death receptor-mediated cell proliferation, a large number of cytokines, growth factors, and hormones have been reported to promote hepatocyte proliferation during LR (Fulda and Debatin, 2006). Many mitogenic signaling pathways have an influence on death receptor-mediated cellular activities. For example, in the presence of activated Akt and NF- κ B, Fas stimulates cell proliferation, whereas in their absence it promotes apoptosis (Hortelano et al., 2007). Cytokines and growth factors produced during LR might influence TNF/TNFR- or FASL/FAS-mediated hepatocyte apoptosis or proliferation in a similarly way. As discussed in the introduction, the activation of death receptors can lead to different cellular activities including cell survival, proliferation, and apoptosis. The final outcome may depend on the downstream factors and cross interactions among the signaling pathways.

There are two major apoptotic pathways: extrinsic and intrinsic. Death receptors mediate the extrinsic pathway and mitochondria mediate the intrinsic pathway. Mitochondria-mediated apoptosis is the result of the loss of integrity of the outer mitochondrial membrane that leads to the release of intermembrane space proteins including cytochrome c, which promotes caspase activation in the cytosol. The two pathways have distinct subsets of caspase factors, but the signaling pathways also cross-interact. Death receptor-mediated apoptosis is executed by two types of signal route. The Type I pathway mediates apoptosis through the activation of caspase-8, caspase-7, or caspase-3. The Type II pathway is initiated when the amount of activated caspase-8 is not sufficient to activate caspase-7/3, and is coupled with the mitochondria-mediated signaling pathway. In the Type II pathway, caspase-8 cleaves Bid to its truncated form (tBid), which is translocated to the mitochondrial membrane and triggers cytochrome c release followed by caspase activation leading to apoptosis.

DR6 promotes apoptosis via a unique mitochondria-dependent pathway (Zeng et al., 2012). DR6-induced apoptosis can bypass the Type I and Type II pathways and directly activates BAX to trigger mitochondria-mediated apoptosis. We found that the gene expression of DR6 was dramatically upregulated after PH (Table 1). It reached a level of almost 30 times that of the control at PH 2 h and attained a peak level of over 100-fold at PH 12 h, then gradually decreased to 4-fold towards the end of LR. The initial increase may have been partially due to apoptosis in response to surgical damage. The function of DR6 has not been well characterized. It would be very interesting to see if DR6, like FAS and TNFR, is involved in promoting cell proliferation. Based on the current knowledge, it is reasonable to speculate that DR6 plays a major role in hepatocyte apoptosis. DR6 may promote apoptosis throughout the course of rat LR to eliminate any unhealthy or unneeded cells.

In addition to DR6-mediated apoptosis, *Ppp2r2a* and *Ppp1r1b* genes for PP2A were also significantly upregulated during LR (Table 3). The activity of PP2A is stimulated by ceramide, which is an important second messenger for both cell proliferation and apoptosis (Hannun, 1994; Kolesnick and Fuks, 1995; Jarvis et al., 1996; Smyth et al., 1997). It has been shown that the activation of death receptors including Fas and TNF promote the hydrolysis of sphingolipids to ceramide. In addition to stimulating PP2A, ceramide also activates ceramide-activated protein kinase, which in turn stimulates MEKK-mediated cell proliferation. The increase in the expression of the gene that encodes cIAP (*Xiap*) was modest during the end of LR, whereas the expression of the gene that encodes PP2A (*Ppp2r1b*) was upregulated during the entire course of rat LR,

suggesting that the ceramide-mediated signaling pathway may favor hepatocyte apoptosis over proliferation. This supports the theory that apoptosis is an ongoing process during the course of LR. The increased expression of *Bcl2* and *Bcl2l1* may counterbalance apoptosis signaling activities in general.

In summary, three death receptors, Fas, TNFR, and DR6, play an important role in both the proliferation and apoptosis of hepatocytes, and their downstream components constitute a complex signaling network with multiple nodes of interaction and cross-regulation.

Conflicts of interest

The authors declare no conflict of interest.

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