RESEARCH ARTICLE

Eight paths of ERK1/2 signalling pathway regulating hepatocyte proliferation in rat liver regeneration

J. W. LI[1], G. P. WANG[1,2], J. Y. FAN[2], C. F. CHANG[1,2] and C. S. XU[1,2*]

[1]*College of Life Sciences, Henan Normal University and* [2]*Key Laboratory for Cell Differentiation Regulation, Xinxiang 453007, Henan Province, People's Republic of China*

Abstract

Although it is known that hormones, growth factors and integrin promote hepatocyte proliferation in liver regeneration (LR) through ERK1/2 signalling pathway, reports about regulating processes of its intracellular paths in hepatocytes of LR are limited. This study aims at exploring which paths of ERK1/2 signalling pathway participate in the regulation of rat LR, especially in hepatocyte proliferation, and how they do so. In all, 14 paths and 165 genes are known to be involved in ERK1/2 signalling pathway. Of them, 161 genes are included in Rat Genome 230 2.0 Array. This array was used to detect expression changes of genes related to ERK1/2 signalling pathway in isolated hepatocytes of rat LR, showing that 60 genes were related to hepatocytes of LR. In addition, bioinformatics and systems biology methods were used to analyse the roles of 14 above paths in regenerating hepatocytes. We found that three paths, RTK→SHC→GRB2/SOS→RAS→RAF, Integrinβ→FAK→RAC→PAK→RAF and Gβγ→PI3Kβγ→RAC→PAK→RAF, promoted the G_1 phase progression of hepatocytes by activating ERK1/2. A further four paths, Gq→PLCβ→PKC→SRC/PYK2→GRB2/SOS→RAS→RAF, RTK→PLCγ→PKC→SRC/PYK2→GRB2/SOS→RAS→RAF, Integrinβ→FAK/SRC→GRB2/SOS→RAS→RAF and Integrinβ→FAK→RAC→PAK→RAF, advanced the cell progression of S phase and G_2/M checkpoint by activating ERK1/2, and so did PP1/2→Mek1/2 by decreasing the negative influence on ERK1/2. At the late phase of LR, Gαs→AC→EPAC→Rap1→Raf blocked hepatocyte proliferation by decreasing the activity of ERK1/2 and so did PP1/2→Mek1/2. In summary, 60 genes and 8 paths of ERK1/2 signalling pathway regulated hepatocyte proliferation in rat LR.

[Li J. W., Wang G. P., Fan J. Y., Chang C. F. and Xu C. S. 2011 Eight paths of ERK1/2 signalling pathway regulating hepatocyte proliferation in rat liver regeneration. *J. Genet.* **90**, 435–442]

Introduction

The liver has important physiological functions and a strong ability to regenerate (Yokoyama *et al.* 2007). After injury or partial hepatectomy (PH), it can rapidly restore to the normal mass via proliferation and growth of the remnant liver, which is called liver regeneration (LR) (Taub 2004). Usually, rat LR is divided into four phases: immediate early phase (0–6 h after PH), early phase (6–12 h after PH), middle phase (12–72 h after PH) and late phase (72–168 h after PH), and involves many physiological activities including activation of growth factors and cytokines, synthesis and activation of transcription factors and enzymes, and synthesis and reconstruction of extracellular matrix (Kountouras *et al.* 2001). PH does little damage to liver and is widely applied in mechanistic research on LR (Koniaris *et al.* 2003). It is well known that hepatocytes are the main cells of liver, accounting for 70–80% of hepatic mass and 65% of total hepatic cells, and have more physiological functions including material storage, substance metabolism, bile secretion, oxidation protection, detoxification, biotransformation, immune response, etc. (Xu *et al.* 2010). Under normal condition, only 0.0012–0.01% hepatocytes of adult rat liver undergo mitosis (Koniaris *et al.* 2003). Nevertheless, a large number of residual hepatic cells rapidly enter into the cell cycle to compensate for the lost liver mass, following PH, which is regulated by many signalling pathways including ERK1/2 signalling pathway (Michalopoulos 2010).

ERK1/2 signalling pathway is a complex net containing many paths. We collated all the 14 paths which have been reported more than once and can be searched on the web of signalling pathways. According to the ERK1/2 activating factors, these paths can be divided into four types, including

*For correspondence. E-mail: xucs@x263.net.

Keywords. liver regeneration; hepatocytes; ERK1/2 signalling pathway; proliferation; Rat Genome 230 2.0 Array; gene expression profile.

activation of G-protein by hormones and neurotransmitters, the increased concentration of intracellular calcium, the combination of receptor tyrosine kinase (RTK) with growth factors and cytokines, and stimulation of extracellular matrix to integrin. It was reported that G-protein regulated MAPK by Ras-dependent and Rap1-dependent pathway (paths 1, 7–9 and 11) (Goldsmith and Dhanasekaran 2007; May and Hill 2008). Characteristic intracellular Ca^{2+} oscillations also activated MAPK cascade in hepatocyte proliferation after rat 2/3 hepatectomy (path 2) (Kitamura *et al.* 1995). It has been generally accepted that classical RTK-induced activation of ERK1/2 depended on the Ras/Raf/MEK/ERK1/2-signalling (paths 3–4) (Murata *et al.* 2007; Lorenz *et al.* 2009). Fibronectin and integrinβ could influence cell survival and proliferation via ERK1/2 signalling pathway (paths 5–6) (Rescan *et al.* 2001). What is more, the activity of ERK1/2 was also regulated by other kinases (paths 10, and 12–14) (Khare *et al.* 1999; Yuen *et al.* 2001; Datta *et al.* 2010; Lee *et al.* 2010). Activated ERK1/2 entered into nuclear to phosphorylate multiple transcription factors and nuclear proteins, further to promote hepatocyte proliferation, etc. Several groups had reported that PH could trigger activation of ERK1/2 (Fausto 2000; Leu *et al.* 2003; Huh *et al.* 2004), and ERK1/2 signalling pathway could promote cell proliferation by regulating cell cycle progression in LR (Talarmin *et al.* 1999; Rescan *et al.* 2001; Murata *et al.* 2007). However, it is unclear as which paths of ERK1/2 signalling pathway participate in the regulation of rat LR, especially in hepatocyte proliferation and how.

In this study, using isolated hepatocytes, Rat Genome 230 2.0 Array, bioinformatics and systems biology methods, we evaluated expression changes of genes related to ERK1/2 signalling pathway and its paths at the transcriptional level, which is helpful to explore the relevance of ERK1/2 signalling pathway to rat LR.

Materials and methods

Isolation and identification of hepatocytes of rat regenerating liver

A total of 114 healthy Sprague-Dawley rats, 12 weeks old, each weighting 230 ± 20 g, were provided by the Animal Center of Henan Normal University, China. They were randomly divided into 19 groups consisting of 9 2/3 hepatectomy (PH) groups, −9 sham operation (SO) groups and one control group. Each group contained six rats. PH was performed following Higgins and Anderson (1931), and hepatocytes were taken from regenerating livers at 0, 2, 6, 12, 24, 30, 36, 72, 120 and 168 h after PH, respectively. Briefly, the rats were anesthetized by ether and sterilized with 75% alcohol before opening the abdominal cavity. After the liver was exposed, the vena cava below and above the liver was ligated and a tube was inserted into liver portal vein. Following which liver was perfused with calcium-free perfusate at 37°C till the liver surface turned slightly yellow, then with 15 mL of 0.05% collagenase IV solution to disperse liver cells. The cells were collected and washed thrice with PBS at 37°C, and the cell concentration was adjusted to 1×10^8 cells/mL. Six mL of mixed cell suspension was spread onto the surface of 4 mL 60% percoll in a 10 mL tube for a single 200 g centrifugation at 4°C for 5 min. Pellet was harvested and hepatocytes were obtained. Finally, hepatocyte marker proteins ALB and G6P were identified by immunocytochemistry for identification of hepatocytes (Grisham 1983; Heo *et al.* 2006). The handling process went in compliance with the laws of animal protection of the People's Republic of China.

Rat Genome 230 2.0 microarray detection and date analysis

The total RNA of 1×10^6 hepatocytes was extracted according to the manual of Trizol reagent (Invitrogen, Carlsbad, USA) and purified following the RNeasy mini protocol (Qiagen, Valencia, USA). The quality of total RNA was assessed by optical density measurement at 260/280 nm and agarose electrophoresis (180 V, 0.5 h), the sample with a 2 : 1 ratio of 28S RNA to 18S RNA was regarded as qualified. T7-oligo DT (24) (Keck Foundation, New Haven, USA), SuperScript II RT (Invitrogen, Carlsbad, USA) and 5 μg of total RNA was used to synthesize the first strand of cDNA. The second strand was synthesized using the Affymetrix cDNA single-stranded cDNA synthesis kit (Affymetrix, Santa Clara, USA). The cDNA product was purified following the cDNA purify protocol of the manufacturer. The 12 μL purified cDNA and the reagents in the GeneChip *in vitro* transcript labeling kit (ENZO Biochemical, New York, USA) were used to synthesize biotin-labelled cRNA. The labelled cRNA was purified using the RNeasy Mini kit columns (Qiagen, Valencia, USA). Their concentration, purity and quality were assessed as above RNA extraction. 15 μL cRNA (1 μg/μL) was incubated with 6 μL 5× fragmentation buffer and 9 μL RNase free water for 35 min at 94°C, and digested with 35–200 bp cRNA fragments. The prehybridized Rat Genome 230 2.0 microarray was put into a hybridization buffer which was prepared following the Affymetrix protocol, and hybridized in a rotating chamber, by 60 rpm, for 16 h, at 45°C. The hybridized arrays were washed by wash buffer to remove the hybridization buffer, and stained in GeneChip Fluidics Station 450 (Affymetrix, Santa Clara, USA). Then the arrays were scanned and imaged with a GeneChip Scanner 3000. The images showing gene expression abundance were converted into signal values, signal detection values (P, A, M) and experiment/control values (Ri) using Affymetrix GCOS 2.0. The data of each array was initially normalized by scaling all signals to a target intensity of 200. When P value is < 0.05, it means that the gene is present (P), when $P < 0.065$, means marginal (M), and when $P > 0.065$, means marked absent (A). On the other hand, the normalized signal value in PH to that in control were used to calculate the relative value, i.e., ratio value of gene expression abundance. When ratio value is ≥3, it means that the gene expression

was significantly upregulated, when ≤ 0.33, means significantly downregulated, and when 0.33–2.99, means biologically insignificant. To minimize the technical error from array analysis, each sample was tested at least thrice using Rat Genome 230 2.0 microarray. Their average value was calculated as the corrective value. Finally, these values were analysed with GeneMath, GeneSpring, Microsoft Excel software and pathway studio 5.0 (Twigger *et al.* 2006; Wang *et al.* 2007; Guo *et al.* 2008).

Quantitative real-time polymerase chain reaction

To verify the chip data, three genes were selected for quantitative real-time polymerase chain reaction (RT-PCR) analysis. Firstly, their total mRNA was isolated. Of them, 2 μg RNA was reverse transcribed using random primers and reverse transcription kit (Promega, Beijing, China). The primer was designed with Primer Express 2.0 software according to GenBank number of three target genes *myc*, *jun*, *trim24* that are NM_012603, NM_021835, NM_001044266 and synthesized by Shanghai Generay Biotech, Shanghai, China. The gene-specific primers were the following; forward primer: 5′-GAGGAGAAACGAGCTGAAGCG-3′ and reverse primer: 5′-TGAACGGACAGGATGTAGGC-3′ for *myc*, forward primer: 5′-TGCAAAGATGGAAACGACCTT-3′ and reverse primer: 5′-GCCGTAGGCGCCACTCT-3′ for *jun*, forward primer: 5′-CAGTGGGAGGGTCTTACAATC-3′ and reverse primer: 5′-CTGGCCAGGGTCTACACTTG-3′ for *trim24*. Total RNA (2 μg) was reverse transcribed using random primers and reverse transcription kit (Promega, Beijing, China). First-strand cDNA samples were subjected to quantitative PCR amplification by using SYBR Green I on the Rotor-Gene 3000 (Corbett Robotics, San Francisco, USA). Every sample was analysed in triplicate. Standard curves were generated from five repeated 10-fold serial dilutions of cDNA, and the copy numbers of target genes in every millilitre of the sample were calculated according to standard curve (Wang and Xu 2010).

Confirmation of hepatocytes associated with ERK1/2 signalling pathway and genes related to liver regeneration in rat

In confirming rat hepatocyte associated with ERK1/2 signalling pathway, the phrase 'ERK1/2 signalling pathway' was input into NCBI (http://www.ncbi.nlm.nih.gov) and RGD (http://rgd.mcw.edu) to collect genes that are associated with rat, mouse and human. Then, these genes were further obtained according to the biological pathway maps at the databases including BIOCARTA (http://www.biocarta.com/genes/index.asp), KEGG (http://www.genome.jp/kegg/pathway.html) and GENMAPP (http://www.genmapp.org), etc. and reconfirmed with related articles (Osband and Cashon 1990; Ogata *et al.* 1999; Doniger *et al.* 2003). To confirm the genes related to LR, gene was considered as the significantly upregulated gene when its expression changes were ≥3 compared with control, as the significantly downregulated, when ≤0.33, as the significant upregulated/downregulated, when it was not only upregulated but also downregulated in LR (Chalmers *et al.* 1998).

Synergy analysis of genes in ERK1/2 signalling pathway

As Wang *et al.* (2009) described, a mathematical model (E_t) was established to describe how the physiological activities are governed by gene synergy (Vera and Wolkenhauer 2008), according to the gene expression abundance detected by Rat Genome 230 2.0 Array in rat AHF, gene functions and interactions identified by Pathway Studio 7.0 software of ResnetCore1.2 database, the principle that gene synergy governs physiological activity, the multivariate statistics, time series analysis (McGuigan 2006) and correlation analysis (Chen *et al.* 2010).

$$E_t = \frac{\sum_{i=1}^{n}\sum_{k=1}^{s_i} r\left(X_i^{(t)}, X_k^{(t)}\right)}{\sum_{i}^{n} s_i}.$$

Where t, time series, $X_i^{(t)}$ and $X_k^{(t)}$ abundances of gene at t, $r(X_i, X_k)$ presents correlation coefficient of genes X_i and X_k, n the number of X_i, and s_i refers to the number of gene X_k. When gene X_k promotes the function of gene X_i, the $r(X_i, X_k)$ is $X_i \times X_k$. When gene X_k inhibits the action of gene X_i, the $r(X_i, X_k)$ is $X_i \times (-X_k)$. When gene X_k binds to gene X_i, the $r(X_i, X_k)$ is $(X_i + X_k)/2$. When gene X_k counteracts the action of gene X_i, the $r(X_i, X_k)$ is $X_i - X_k$. If synergy value (E_t) of genes participating in physiological activity was significantly greater than control (E_c), it means that the physiological activities were in PH stronger than in control. E_t is remarkably less than E_c, meaning that the physiological activities in PH were weaker. And E_t has no remarkable difference to E_c, suggesting that the physiological activities in both are similar.

Results

The expression profiles of genes related to ERK1/2 signalling pathway of hepatocytes in liver regeneration

The data from NCBI, RGD etc. and biological pathway maps in GENMAPP, KEGG etc. showed that 165 genes were involved in ERK1/2 signalling pathway. Of them, 161 genes were in Rat Genome 230 2.0 Array. The detection of RT-PCR and Rat Genome 230 2.0 Array showed that the expression trends of three genes including *myc*, *jun* and *trim24* detected by the two methods were generally consistent (figure 1). Moreover, the expression trends of two markers of liver regeneration including *pcna* and *mki67* (Selzner *et al.* 1999) were detected by the array in hepatocytes of LR

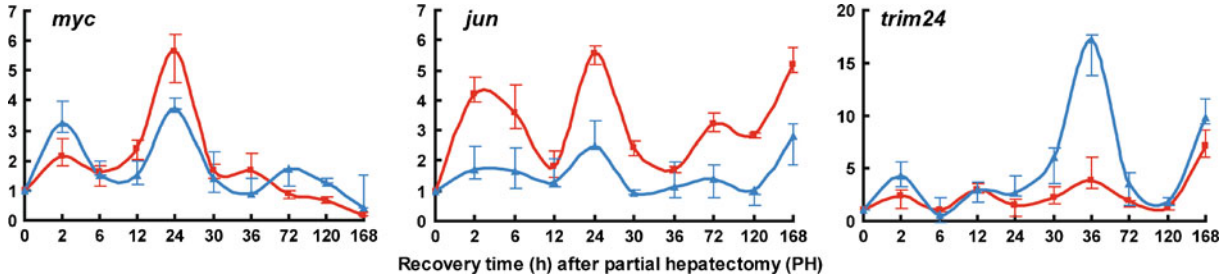

Figure 1. Comparison of results obtained with microarray and RT-PCR analysis for changes in mRNA levels in hepactocytes during LR at 10 time points after PH relative to the level of control sample in microarray analysis (blue lines) and RT-PCR analysis (red lines) are shown for the proliferation-related genes: *myc*, *jun*, *trim24*.

(figure 2), which were accord with time kinetics of DNA synthesis of hepatocytes of rat regenerating liver (Michalopoulos and DeFrances 1997). These figures of the two studies suggested that the array check results were reliable. Moreover, the array analysis showed that 60 genes were significantly changed in hepatocytes of rat regenerating liver. In detail, ERK1/2-activating genes included 43 upregulated and 4 downregulated, and ERK1/2-inhibiting genes contained 12 upregulated and 5 downregulated (table 1). In this study, we divided ERK1/2 signalling pathway into 14 paths, which contained 165 genes above. Of them, paths 1–10 activated ERK1/2, and paths 11–14 weakened it (figure 3).

The physiological activities governed by ERK1/2 signalling pathway of hepatocytes in liver regeneration

The gene synergy and the physiological activities in ERK1/2 signalling pathway of hepatocytes in LR were analysed using the mathematical model (E_t), showing that eight paths of the pathway were related to hepatocytes of rat regenerating liver. In detail, in early phase of LR, E_t of genes related to paths 3, 6 and 7 which promoted the activity of ERK1/2 were higher than E_c. In middle phase, E_t of genes related to paths 1, 3, 5 and 6 which activated ERK1/2 were higher, and path 12 which inhibited the activity of ERK1/2 was smaller. In late phase, E_t of genes related to path 8 which inhibited the activity of ERK1/2 was lower and path 12 was higher. The other six paths including paths 2, 9–11 and 13–14 were not found to be involved in LR (figure 4).

Discussion

Studies showed that LR is completed within seven days after partial hepatectomy (PH). During LR, the first proliferation of hepatocytes is divided into G_0 phase (0–6 h after PH), G_1 phase (6–12 h after PH), S phase (12–24 h after PH), G_2 phase (24–30 h after PH) and M phase (30–36 h after PH), and the second is during 36–66 h after PH. Cell differentiation and tissue reconstruction occurred during 120–168 h after PH (Xu *et al.* 2010). In this study, the expression profiles of genes related to the 14 paths of ERK1/2 signalling pathway was analysed using E_t. It was found that it was at 6 h after PH that the earliest remarkable changes occurred to paths 4 and 7, which meant that hepatocyte entered into the early G_1 phase. This result agreed with study by Talarmin *et al.* (1999). Moreover, Talarmin *et al.* found that ERK1/2 cascade was activated at two time points of the G_1 phase progression of hepatocytes early G_1 and mid–late G_1 phase in LR, which further confirmed that ERK1/2 signalling pathway was the crucial one regulating the G_1 phase progression of hepatocytes. Rescan *et al.* (2001) reported that growth factors, fibronectin and integrinβ could promote the entering of hepatocytes into S phase via ERK1/2 signalling pathway. Systems biology methods were applied in this study. It was found that E_t of genes related to the three paths including the

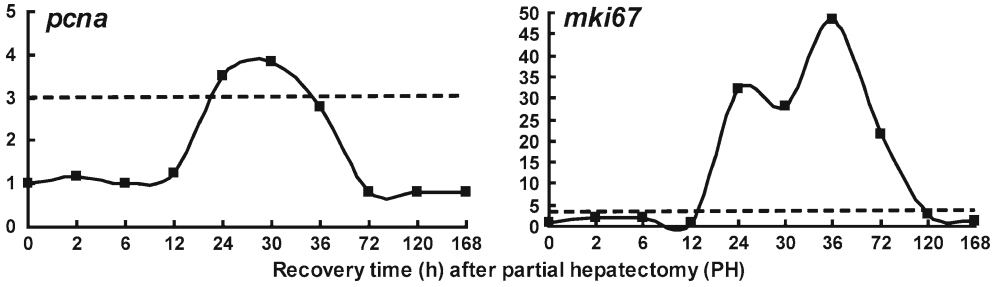

Figure 2. The expression trends of two markers of LR in the array in hepatocytes of LR at 10 time points after PH relative to the level of control sample in microarray analysis are provided. The dotted line is as the standard line above which the gene at a certain time-point means the gene upregulated.

Table 1. The expression changes of genes associated with ERK1/2 signalling pathway of hepatocytes in regenerating liver.

Protein symbol	Gene symbol	Related paths	Recovery time (h) after partial hepatectomy (PH)								
			2	6	12	24	30	36	72	120	168
1.Gq	Gna15	1	1.00	2.85	**3.09**	3.66	**3.48**	1.55	**5.96**	2.00	**4.21**
	Gna14	1	*0.31*	0.51	1.25	0.48	0.82	0.37	1.28	0.72	0.68
	Gng2	1,7	1.27	0.89	1.62	0.94	**3.93**	1.44	2.87	3.03	1.50
	Gngt2	1,7	0.83	0.98	1.16	1.40	2.17	1.47	**3.81**	2.32	2.66
	Gng8	1,7	**3.21**	1.96	1.89	**3.49**	2.38	2.65	2.83	2.22	2.44
	Gnb3	1,7	1.39	2.40	1.71	2.72	**3.66**	2.88	**3.94**	2.64	2.67
	Gng11	1,7	1.65	1.08	**3.15**	**3.32**	**3.14**	**3.44**	**13.57**	**7.25**	3.12
	Gng3	1,7	**4.92**	**4.66**	**3.95**	**3.98**	**3.88**	**4.48**	**7.95**	2.31	**4.61**
	Gnb5	1,7	2.86	**4.47**	**10.17**	**3.31**	**4.58**	**3.40**	2.22	**4.85**	**3.18**
3.PKC	Prkcz	1,2,3	4.11	0.71	2.78	2.88	2.42	0.70	2.43	**6.83**	1.70
	Prkch	1,2,3	1.38	0.94	1.40	**3.54**	1.00	2.41	**4.45**	1.86	**4.21**
	Prkcc	1,2,3	2.11	**4.84**	**3.17**	**3.56**	2.50	2.51	**5.87**	**3.88**	3.88
	Prkcq	1,2,3	**3.14**	1.07	**6.94**	4.63	**5.33**	3.36	1.81	**3.30**	0.98
4.SRC	Src	1,2,3,5	1.33	1.77	2.40	2.06	**3.11**	1.89	**4.78**	2.04	1.48
14.CaCn	Cacna1b	2	1.08	2.56	2.41	1.31	**3.18**	2.43	2.20	1.29	1.79
	Cacna2d1	2	2.44	1.57	2.12	2.71	2.53	2.07	**3.12**	1.12	2.58
	Cacng2	2	0.78	1.38	1.22	1.87	2.09	0.84	1.73	0.93	**3.00**
	Cacna2d3	2	2.23	2.26	0.94	**5.47**	**10.19**	2.65	**5.15**	3.45	7.22
	Cacnb3	2	2.07	2.68	2.70	**3.07**	2.16	2.39	2.36	1.43	**3.49**
	Cacng1	2	2.19	1.30	1.34	**3.91**	1.87	0.90	**3.38**	0.91	**4.65**
	Cacnb1	2	2.82	**5.51**	**3.89**	**4.53**	**3.72**	**4.08**	**4.11**	**3.22**	2.94
	Cacnb2	2	**4.16**	2.42	1.08	**3.65**	**3.41**	1.90	**3.16**	0.79	**3.96**
	Cacnb4	2	**3.14**	**19.05**	**9.97**	**5.36**	**3.38**	**11.35**	1.35	0.86	**6.46**
	Cacng8	2	**5.42**	**6.30**	**5.19**	**4.57**	**9.71**	**6.95**	**4.76**	1.15	**3.95**
	Cacng3	2	0.71	0.43	0.49	*0.25*	0.56	0.42	0.56	*0.26*	0.86
15.RTK	Pdgfrb	3,4	1.10	0.97	0.84	2.02	1.07	1.32	**5.41**	1.48	1.33
	Tek	3,4	0.54	0.94	0.97	1.71	1.53	1.53	**5.65**	1.41	1.41
	Ntrk1	3,4	1.30	**3.76**	2.52	2.37	2.42	1.73	**3.45**	2.26	2.13
	Fgfr1	3,4	0.67	2.55	0.92	**3.74**	2.29	2.35	**6.36**	**4.81**	2.36
	Pdgfra	3,4	2.39	**3.70**	**5.12**	**6.44**	2.30	2.15	**32.45**	**8.41**	0.85
	Met	3,4	1.43	0.36	0.58	0.64	0.75	0.51	*0.23*	0.36	0.70
17.SHC	Shc2	4	1.87	**3.14**	**4.55**	**5.08**	**4.19**	**4.79**	**6.84**	2.05	1.42
18.Integrins	Itgam	5,6	1.19	0.44	0.46	1.69	**5.41**	1.54	1.88	1.63	1.50
	Itgb6	5,6	1.44	0.65	2.14	1.46	1.41	2.29	**5.80**	2.57	1.94
	Itgb7	5,6	1.37	1.88	1.84	1.93	2.49	2.13	**4.41**	1.71	2.14
21.PAK	Pak1	6.7	0.62	0.68	1.16	1.01	**3.07**	1.10	1.34	1.11	1.30
	Pak3	6.7	1.05	1.25	1.17	1.80	1.15	1.55	2.94	**3.48**	1.22
	Pak4	6.7	1.89	1.73	**4.10**	**4.60**	**5.85**	**3.40**	**3.61**	2.46	**4.35**
23.PI3K	Pik3r3	7	2.31	1.17	**3.21**	1.38	2.33	2.61	2.61	1.18	2.81
	Pik3c2g	7	0.80	0.77	*0.26*	*0.19*	0.35	*0.33*	0.38	0.76	0.92
25.AC	Adcy5	8,9,11	1.97	2.29	2.00	2.33	6.56	5.37	2.59	2.44	1.69
	Adcy2	8,9,11	1.04	2.35	2.80	2.53	2.90	2.70	**3.44**	2.08	2.18
	Adcy4	8,9,11	1.15	1.79	1.36	**3.26**	1.76	2.38	**6.06**	2.53	2.26
	Adcy8	8,9,11	2.30	2.02	2.70	**6.58**	**9.12**	1.77	**11.37**	1.24	**8.85**
26.EPAC	Rapgef3	8	2.03	**4.83**	2.69	**3.97**	2.62	2.65	**9.04**	**3.06**	2.91
30.MOS	Mos	10	0.69	1.12	1.56	0.83	**3.45**	2.01	3.65	0.91	1.47
31.NF1	Nf1	13	**4.33**	**4.50**	**4.93**	1.90	**3.65**	**3.95**	**3.57**	**4.86**	**3.82**
33.PP1/2	Ppp2r2c	12	1.19	1.33	**3.99**	1.42	**3.08**	1.29	1.72	1.18	1.46
	Ppp1r1b	12	0.89	1.16	1.04	2.71	**8.91**	0.96	5.32	0.90	1.00
	Ppp1r3d	12	2.17	1.97	2.44	2.78	2.39	2.49	**5.57**	**3.00**	2.99
	Ppp1r3a	12	1.92	**3.53**	1.31	1.32	**4.69**	1.10	2.73	1.23	3.74
	Ppp1r12b	12	1.77	1.74	2.83	**4.10**	1.18	2.32	**3.21**	2.32	2.73
	Ppp2r2a	12	**6.40**	**6.94**	**4.12**	**6.64**	**3.35**	0.74	**6.40**	**5.28**	**7.79**
	Ppp2r1b	12	**6.23**	**5.07**	**9.81**	**5.08**	**10.37**	**11.63**	**5.91**	**4.36**	**4.87**
	Ppp1r3c	12	*0.12*	0.61	0.94	0.59	0.40	0.37	0.60	0.74	1.29
	Ppp1r14a	12	0.88	*0.29*	0.77	0.97	1.27	1.08	0.75	1.21	0.97
	Ppp2r2b	12	1.34	0.43	*0.27*	0.44	0.89	1.45	0.86	1.74	2.48
	Ppp1r3b	12	*0.20*	*0.24*	*0.23*	*0.15*	*0.26*	*0.21*	*0.18*	0.47	1.20
34.MKP-1	Dusp1	14	*0.33*	0.50	*0.23*	0.72	0.36	*0.19*	0.47	0.53	0.96

*The values that are in bold represent the expression abundance of upregulated genes, those in italics are of the downregulated and the other value are insignificantly changed.

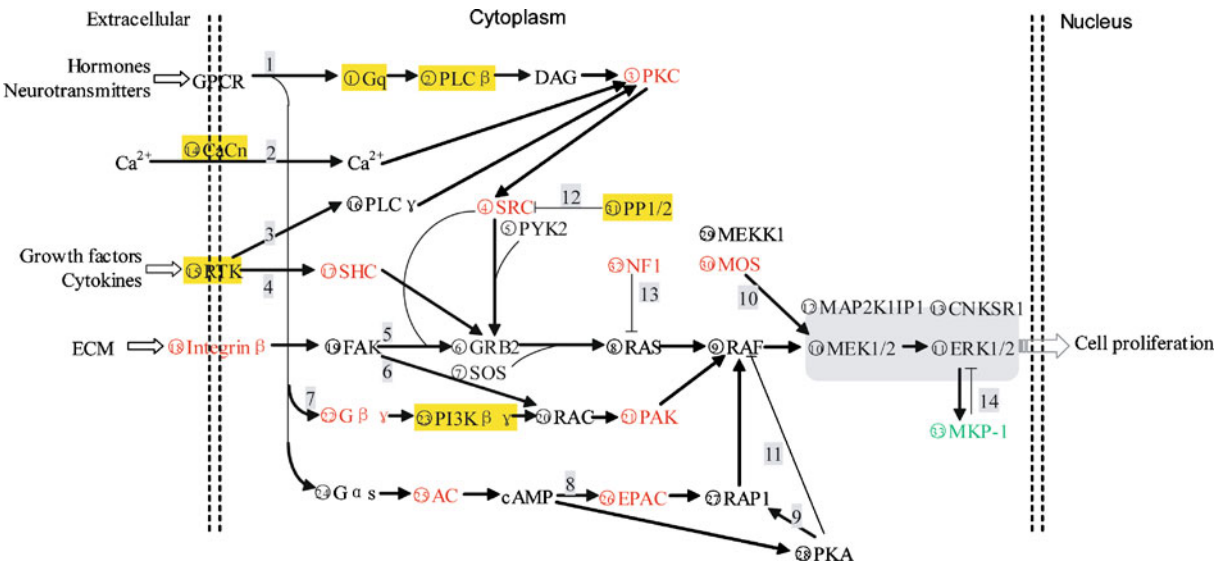

Figure 3. Fourteen paths of ERK1/2 signalling pathway and their relationship were shown with the expression changes of the genes above. Symbols in red mean the upregulated proteins, those in green the downregulated, and those under yellow ground the upregulated/downregulated. The figures under grey ground mean the ordinal numbers of the paths, and those in circle the ordinal numbers of proteins which share the same ordinal numbers in the table above.

growth factor-induced path (path 4), the integrinβ-mediated path (path 6) and path 7 promoting hepatocyte proliferation were greater than E_c at the two time points above, which confirmed previous results (Talarmin *et al.* 1999; Rescan *et al.* 2001) at the transcriptional level. Hence, it was inferred that path 7 probably had a positive effect on hepatocyte proliferation in LR.

It has been reported that leptin upregulated the proportion of smooth muscle cells in the S phase and at G_2/M checkpoint via ERK1/2 signalling pathway in rat LR (Huang *et al.* 2010). Factor *et al.* (2010) found that HGF/c-met-mediated ERK1/2 activation was required by G_2/M progression during mice LR. The analysis using systems biology methods in this study indicated that E_t of genes related to four

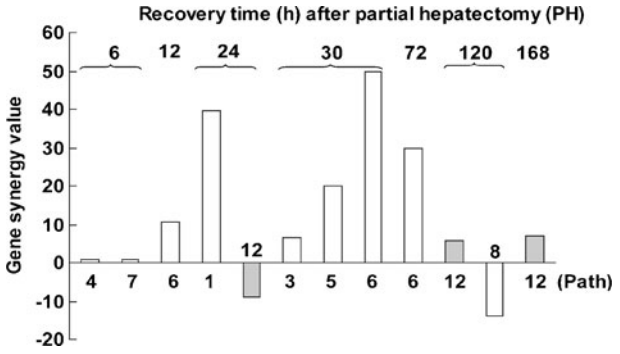

Figure 4. The physiological activities were regulated by ERK1/2 signalling pathway of hepatocytes in liver regeneration. White columns mean activating ERK1/2, grey columns inhibiting ERK1/2. X-axis represents the numbers of the paths to which remarkable changes occurred; Y-axis represents the value of $E_t - E_c$.

cell–proliferation-promoting paths including the hormone-induced path (path 1), the growth factor-induced path (path 3), paths 5 and 6 were greater in PH than E_c, and E_t of genes related to path 12 inhibiting proliferation was smaller, at the two time points above. It was indicated that five paths above advanced the hepatocyte proliferation progression in S phase and at G_2/M checkpoint.

Taub (2004) pointed out that proliferation of hepatic cells occurred mainly within three days after PH. Gene synergy analysis was carried out in this study. It was found that E_t of genes related to path 6 which promoted hepatocyte proliferation was greater than E_c at 12, 30 and 72 h after PH, which meant that path 6 might promote hepatocyte proliferation during twice cell cycles, and was a probably novel and key path activating ERK1/2. E_t of genes related to path 12 which promoted hepatocyte proliferation was higher at 72 h and 120 h after PH, which suggested that path 12 might weaken hepatocyte proliferation in the late LR, and it was lower at 24 h after PH, which further indicated that path 12 was of importance for weakening hepatocyte proliferation. E_t of genes related to path 8 was smaller at 120 h after PH, which implied that path 8 decreased the promotion of hepatocyte proliferation in late phase of LR.

In summary, this study analysed gene expression profiles of ERK1/2 signalling pathway of hepatocytes using Rat Genome 230 2.0 Array, and the regulation of rat LR by ERK1/2 pathway using systems biology methods during LR. And it was concluded that paths 4, 6 and 7 promoted the G_1 phase progression of hepatocyte by activating ERK1/2, that paths 1, 3, 5 and 6 advanced the cell progression of S phase and G_2/M by activating ERK1/2, so did path 12 by weakening the inhibition to ERK1/2, and that path 8 by

decreasing the activation of ERK1/2 and path 12 by inhibiting the activity of ERK1/2 blocked hepatocyte proliferation at the late LR. We will further confirm the above results using gene addition and RNA interference *in vivo* and *in vitro* in future.

Acknowledgements

Many thanks to Dr Wang Gaiping for fruitful suggestions and crucial help. This work was supported by the National 973 Pre-research Programme of China (no. 2010CB534905).

References

Chalmers J. J., Zborowski M., Sun L. and Moore L. 1998 Flow through, immunomagnetic cell separation. *Biotechnol. Prog.* **14**, 141–148.

Chen X., Xu C., Zhang F. and Ma J. 2010 Comparative analysis of expression profiles of chemokines, chemokine receptors, and components of signalling pathways mediated by chemokines in eight cell types during rat liver regeneration. *Genome* **53**, 608–618.

Datta N. S., Kolailat R., Fite A., Pettway G. and Abou-Samra A. B. 2010 Distinct roles for mitogen-activated protein kinase phosphatase-1 (mkp-1) and erk-mapk in pth1r signalling during osteoblast proliferation and differentiation. *Cell Signal* **22**, 457–466.

Doniger S. W., Salomonis N., Dahlquist K. D., Vranizan K., Lawlor S. C. and Conklin B. R. 2003 Mappfinder: using gene ontology and genmapp to create a global gene-expression profile from microarray data. *Genome Biol.* **4**, R7.

Factor V. M., Seo D., Ishikawa T., Kaposi-Novak P., Marquardt J. U., Andersen J. B. *et al.* 2010 Loss of C-Met disrupts gene expression program required for g2/m progression during liver regeneration in mice. *PLoS ONE* **5**, e12739.

Fausto N. 2000 Liver regeneration. *J. Hepatol.* **32**, 19–31.

Goldsmith Z. G. and Dhanasekaran D. N. 2007 G Protein regulation of mapk networks. *Oncogene* **26**, 3122–3142.

Grisham J. W. 1983 Cell types in rat liver cultures: their identification and isolation. *Mol. Cell Biochem.* **53–54**, 23–33.

Guo W., Cai C., Wang C., Zhao L., Wang L. and Zhang T. 2008 A preliminary analysis of genome structure and composition in gossypium hirsutum. *BMC Genomics* **9**, 314.

Heo J., Factor V. M., Uren T., Takahama Y., Lee J. S., Major M. *et al.* 2006 Hepatic precursors derived from murine embryonic stem cells contribute to regeneration of injured liver. *Hepatology* **44**, 1478–1486.

Higgins G. M. and Anderson R. M. 1931 Experimental pathology of the liver: restoration of the liver of the white rat following partial surgical removal. *Arch. Pathol.* **12**, 186–202.

Huang F., Xiong X., Wang H., You S. and Zeng H. 2010 Leptin-induced vascular smooth muscle cell proliferation via regulating cell cycle, activating Erk1/2 and Nf-Kappab. *Acta Biochim. Biophys. Sin.* **42**, 325–331.

Huh C. G., Factor V. M., Sanchez A., Uchida K., Conner E. A. and Thorgeirsson S. S. 2004 Hepatocyte growth factor/c-met signalling pathway is required for efficient liver regeneration and repair. *Proc. Natl. Acad. Sci. USA* **101**, 4477–4482.

Khare S., Bissonnette M., Wali R., Skarosi S., Boss G. R., von Lintig F. C. *et al.* 1999 1,25-dihydroxyvitamin D3 but not Tpa activates Pld in Caco-2 cells via Pp60(C-Src) and Rhoa. *Am. J. Physiol.* **276**, G1005–G1015.

Kitamura T., Watanabe S., Ikejima K., Hirose M., Miyazaki A., Yumoto A. *et al.* 1995 Different features of Ca2+ oscillations in differentiated and undifferentiated hepatocyte doublets. *Hepatology* **21**, 1395–1404.

Koniaris L. G., McKillop I. H., Schwartz S. I. and Zimmers T. A. 2003 Liver regeneration. *J. Am. Coll. Surg.* **197**, 634–659.

Kountouras J., Boura P. and Lygidakis N. J. 2001 Liver regeneration after hepatectomy. *Hepatogastroenterology* **48**, 556–562.

Lee J. S., Padmanabhan A., Shin J., Zhu S., Guo F., Kanki J. P. *et al.* 2010 Oligodendrocyte progenitor cell numbers and migration are regulated by the zebrafish orthologs of the nf1 tumor suppressor gene. *Hum. Mol. Genet.* **19**, 4643–4653.

Leu J. I., Crissey M. A., Craig L. E. and Taub R. 2003 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.* **23**, 1251–1259.

Lorenz K., Schmitt J. P., Vidal M. and Lohse M. J. 2009 Cardiac hypertrophy: targeting Raf/Mek/Erk1/2-signalling. *Int. J. Biochem. Cell Biol.* **41**, 2351–2355.

May L. T. and Hill S. J. 2008 Erk phosphorylation: spatial and temporal regulation by G protein-coupled receptors. *Int. J. Biochem. Cell Biol.* **40**, 2013–2017.

McGuigan K. 2006 Studying phenotypic evolution using multivariate quantitative genetics. *Mol. Ecol.* **15**, 883–896.

Michalopoulos G. K. 2010 Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am. J. Pathol.* **176**, 2–13.

Michalopoulos G. K. and DeFrances M. C. 1997 Liver regeneration. *Science* **276**, 60–66.

Murata S., Ohkohchi N., Matsuo R., Ikeda O., Myronovych A. and Hoshi R. 2007 Platelets promote liver regeneration in early period after hepatectomy in mice. *World J. Surg.* **31**, 808–816.

Ogata H., Goto S., Sato K., Fujibuchi W., Bono H. and Kanehisa M. 1999 Kegg: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **27**, 29–34.

Osband M. E. and Cashon G. W. 1990 Biocare: biotechnology in the clinical practice of medicine. *Clin. Res.* **38**, 5–9.

Rescan C., Coutant A., Talarmin H., Theret N., Glaise D., Guguen-Guillouzo C. *et al.* 2001 Mechanism in the sequential control of cell morphology and s phase entry by epidermal growth factor involves distinct Mek/Erk activations. *Mol. Biol. Cell* **12**, 725–738.

Selzner M., Camargo C. A. and Clavien P. A. 1999 Ischemia impairs liver regeneration after major tissue loss in rodents: protective effects of interleukin-6. *Hepatology* **30**, 469–475.

Talarmin H., Rescan C., Cariou S., Glaise D., Zanninelli G., Bilodeau M. *et al.* 1999 The mitogen-activated protein kinase kinase/extracellular signal-regulated kinase cascade activation is a key signalling pathway involved in the regulation of g(1) phase progression in proliferating hepatocytes. *Mol. Cell Biol.* **19**, 6003–6011.

Taub R. 2004 Liver regeneration: from myth to mechanism. *Nat. Rev. Mol. Cell Biol.* **5**, 836–847.

Twigger S. N., Smith S. J., Zuniga-Meyer A. and Bromberg S. K. 2006 Exploring phenotypic data at the rat genome database. *Curr. Protoc. Bioinformatics* Chapter 1, unit 1. 14, doi:10.1002/0471250953.bio114s14.

Vera J. and Wolkenhauer O. 2008 A system biology approach to understand functional activity of cell communication systems. *Methods Cell Biol.* **90**, 399–415.

Wang G. P. and Xu C. S. 2010 Reference gene selection for real-time Rt-Pcr in eight kinds of rat regenerating hepatic cells. *Mol. Biotechnol.* **46**, 49–57.

Wang J. Z., Du Z., Payattakool R., Yu P. S. and Chen C. F. 2007 A New method to measure the semantic similarity of go terms. *Bioinformatics* **23**, 1274–1281.

Wang W. B., Fan J. M., Zhang X. L., Xu J. and Yao W. 2009 Serial expression analysis of liver regeneration-related genes in rat regenerating liver. *Mol. Biotechnol.* **43**, 221–231.

Xu C., Chen X., Chang C., Wang G., Wang W., Zhang L. *et al.* 2010 Transcriptome analysis of hepatocytes after partial hepatectomy in rats. *Dev. Genes Evol.* **220**, 263–274.

Yokoyama Y., Nagino M. and Nimura Y. 2007 Mechanisms of hepatic regeneration following portal vein embolization and partial hepatectomy: a review. *World J. Surg.* **31**, 367–374.

Yuen P. H., Ryan E. A., Devroe E. and Wong P. K. 2001 A single Glu(62)-to-Lys(62) mutation in the mos residues of the R7delta447gag-Tmos protein causes the mutant virus to induce brain lesions. *Oncogene* **20**, 692–703.

Received 9 April 2011, in final revised form 12 July 2011; accepted 3 August 2011
Published on the Web: 5 December 2011