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Involvement of ERK1/2 signaling in proliferation of eight liver cell types during hepatic regeneration in rats

X.G. Chen[1], C.S. Xu[2,3] and Y.M. Liu[1]

[1]Animal Science and Technology School,
Henan University of Science and Technology, Luoyang, China
[2]Key Laboratory for Cell Differentiation Regulation, Xinxiang, China
[3]College of Life Science, Henan Normal University, Xinxiang, China

Corresponding author: C.S. Xu
E-mail: cellkeylab@126.com

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ABSTRACT. It has been well established that ERK1/2 signaling, often subdivided into nine types of pathways, can regulate the hepatocyte proliferative response during liver regeneration. However, the effect of ERK1/2 signaling on the proliferation of other hepatic cell types remains unclear. We isolated and purified 8 liver cell types at 10 time points after 2/3 hepatectomy in adult rats. For each cell type, mRNA expression changes for ERK1/2 signaling-involved genes were monitored up to 168 h, using microarrays. Real-time PCR assays were performed for array data verification. The expression levels of these genes varied considerably between different cell types. Integrating microarray results with gene synergical analysis, at the priming phase, activation of integrin/Grb2/Ras pathway in hepatocytes apparently contributed to G0/G1 transition. Two other pathways, G-protein/EPAC/Rap1 and G-protein/PKA/Rap1, were stimulated in hepatic stellate cells, while RTK/PKC/Ras and RTK/Grb2/Ras were stimulated in Kupffer cells. At the progressive phase, the ERK1/2 pathway is involved in hepatocyte replication; three pathways, namely Ca^{2+}/PKC/Ras, RTK/Grb2/Ras and

G-protein/EPAC/Rap1, were found to play roles in biliary epithelial cell proliferation, while RTK/PKC/Ras and RTK/Grb2/Ras were involved in Kupffer cell proliferation, and G-protein/PKC/Ras in pit cell proliferation. At the terminal phase, the promotive effect of the ERK1/2 pathway on replication of hepatocytes, biliary epithelial cells, oval cells, hepatic stellate cells, Kupffer cells, and dendritic cells was considerably reduced, possibly due to their differentiation at the end of regeneration. G-protein/PKC/Ras, integrin/Grb2/Ras and G-protein/PKA/Rap1 pathways were active in sinusoidal endothelial cells, perhaps to aid in their proliferation. We conclude that ERK1/2 has a signaling role in the regulation of proliferation of 8 cell types during liver regeneration process.

Key words: Liver restoration; Liver cell types; Gene synergy; ERK1/2 signaling pathway; Gene expression profiling

INTRODUCTION

The liver performs many crucial functions required for survival and has a relatively unique regenerative capability (Xu et al., 2010). After injury or partial hepatectomy (PH), the liver can rapidly restore itself to normal mass through hyperplasia of the remnant liver, a process called liver regeneration (LR). LR is usually segmented into a priming phase (0-6 h after PH), a progressing phase (6-72 h after PH), and a terminal phase (120-168 h after PH) involving the activation of growth factors and cytokines, synthesis of transcription factors, reconstruction of extracellular matrix, and other processes (Ma et al., 2009). Hepatocytes (HCs) are well known to constitute the chief functional cells of the liver, responsible for delivering most of the hepatic functions important for body homeostasis, such as material storage, substance metabolism, bile secretion, oxidation protection, detoxification, biotransformation, and immune response (Xu et al., 2010). Normally, mitotic activity occurs in only 0.0012-0.01% of HCs in adult rat liver. However, under certain physiopathological stress situations such as 2/3 hepatectomy or toxic injury, a large number of residual HCs can divide in response to a loss in liver mass. This process is regulated by many signaling pathways, including that of extracellular signal-related kinases 1 and 2 (ERK1/2) (Michalopoulo, 2010). For instance, Talarmin et al. (1999) have reported that partial liver resection can lead to ERK1/2 activation, which in turn enhances LR by stimulating HC proliferation. However, it is unclear which of the 9 pathways of ERK1/2 signaling (Figure 1) is involved in HC proliferation. Aside from HCs, cellular components such as biliary epithelial cells (BECs), oval cells (OCs), hepatic stellate cells (HSCs), sinusoidal endothelial cells (SECs), Kupffer cells (KCs), pit cells (PCs), and dendritic cells (DCs) compose the liver. Whether ERK1/2 signaling is involved in the proliferation of these liver cells during LR and its mechanism of action if it is involved have been poorly investigated up to date.

Therefore, we used a Rat Genome 230 2.0 array to detect the expression alterations of genes involved in the ERK1/2 signaling pathway in 8 liver cell types isolated from rat regenerating liver, and then analyzed the relevance of ERK1/2 signaling pathways to rat LR at the mRNA level using bioinformatics and systems biology methods, which are helpful in exploring the relevance of ERK1/2 signaling to rat LR.

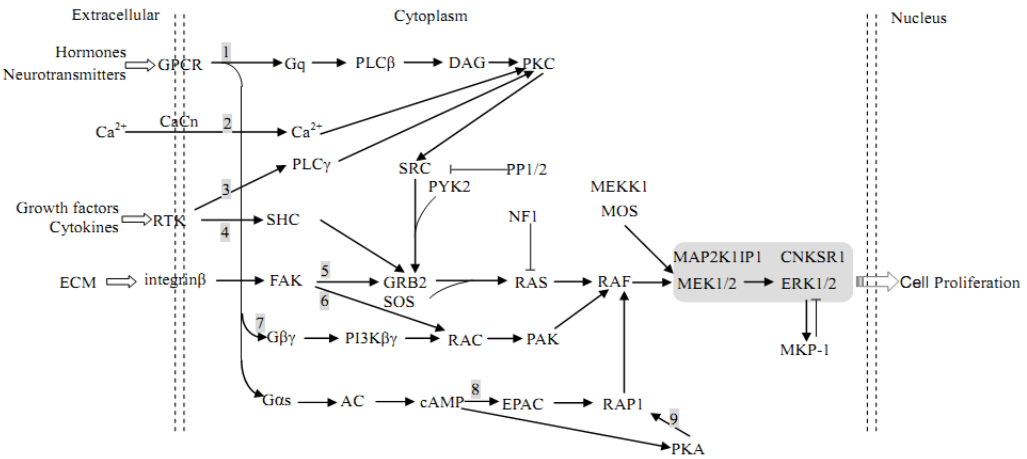

Figure 1. Diagram shows the major components of ERK1/2 signaling pathway.

MATERIAL AND METHODS

Rat 2/3 hepatectomy and LR

Sprague-Dawley rats were obtained from Henan Normal University (Animal Center of Henan Normal University, Xinxiang, Henan) in compliance with current animal protection laws in China. A total of 114 cleaning-grade Sprague-Dawley adult rats weighing 230 ± 20 g were randomly divided into 9 PH groups, 9 operation control groups, and one normal control group. Six rats were placed in each group with a ratio of males to females of 1:1. Rats in the PH groups underwent 2/3 PH surgery following a method described by Higgins and Anderson (1931). Briefly, the left and median lateral liver lobes were surgically removed, and the rats were killed 0, 2, 6, 12, 24, 30, 36, 72, 120, and 168 h after PH. Rats in the operational control groups underwent the operation process, but no liver lobes were removed.

Isolation and identification of liver cell types

Rats were subjected to abdominal skin disinfection with alcohol after being anesthetized with inhaled diethyl ether. The abdominal cavity was opened to expose the liver, and the inferior vena cava was ligated below and above followed by portal vein cannulation. A conventional 2-step perfusion method was used to separate a liver cell suspension. Briefly, the liver was perfused with calcium-free perfusate preheated to 37°C until it turned gray. Then, 15 mL 0.05% collagenase IV solution instead of perfusate was used to perfuse the liver at a flow rate of 1 mL/min. After the liver capsule was removed, the perfused liver was cut into small pieces and digested with 0.05% collagenase IV for 15 min at 37°C. After filtration through 200-well nylon netting, the liquid was centrifuged at 500 *g* for 3 min. The pellet at the bottom was collected and washed 3 times in a 4°C phosphate-buffered saline (PBS) solution to adjust the cell concentration to 1 x 10^8 cells/mL. Six milliliters of the mixed cell suspension

was spread onto the surface of 4 mL 60% Percoll (Pharmacia, Biotech AB, Uppsala, Sweden) in a 10-mL tube for a single centrifugation at 200 *g* for 5 min at 4°C. The centrifuged pellets and supernatant were the purified HCs and nonparenchymal cell-enriched supernatant fractions, respectively. The supernatant was mixed with an equal volume of PBS, centrifuged at 400 *g* twice for 2 min each time at 4°C. The mixed nonparenchymal cell-rich pellet collected was adjusted to a concentration of 1 x 10^8 cells/mL with PBS and mixed with 10 µL/mL rat anti-thymidylate synthase complementing protein, anti-glial fibrillary acidic protein, anti-cytokeratin (CK) 31, anti-cluster of differentiation (CD) 68, anti-CD161a, and anti-CD11c PE antibodies. Hepatic OCs, HSCs, SECs, KCs, PCs, and DCs were selected using a method described elsewhere (Chalmers et al., 1998). Furthermore, white intrahepatic bile duct fractions left on the nylon netting were added to the digestive solution containing 0.25% trypsin and 0.05% collagenase IV, incubated at 37°C for 50 min, and filtered through 200-well nylon netting. The filtered solution was centrifuged at 300 *g* for 5 min. The resulting sediment was the pellet enriched with BECs. These BECs were isolated with rat anti-CK19 PE (phycoerythrin) antibody as described above. Finally, anti-albumin and glucose-6-phosphate, CK18 and gamma-glutamyltransferase 1, OC2 and OV6, CD14 and endothelin 1, lisozyme and extracellular domain 2, desmin and vimentin, CD8 and CD56, and CD86 and CD103 antibodies were used to identify HCs, BECs, hepatic OCs, HSCs, SECs, KCs, PCs, and DCs, respectively, as described elsewhere (Grisham, 1983).

Microarray detection and data analysis

Total RNA from above liver cell samples was extracted following the manual of the Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer instructions and purified following the RNeasy Mini Protocol (Qiagen, Valencia, CA, USA). The quality of total RNA samples was assessed by measuring the optical density at 260/280 nm and using agarose electrophoresis (180 V, 0.5 h) with a 2:1 ratio of 28S to 18S ribosomal RNA intensity. As a template, 5 µg total RNA was used to synthesize the first strand of cDNA using SuperScript II RT (Invitrogen) and T7-oligo dT(24) (W.M. Keck Foundation, New Haven, CT, USA) as the primer. Second-strand synthesis was performed with an Affymetrix cDNA Single-Stranded cDNA Synthesis Kit (Affymetrix Inc., Santa Clara, CA, USA). The cDNA product was purified following a cDNA purification protocol.

Twelve microliters purified cDNA served as a template for the production of a biotin-labeled cRNA transcript using the GeneChip *In Vitro* Transcript Labeling Kit (ENZO Biochemical, New York, NY, USA). The labeled cRNA was purified using the RNeasy Mini Kit columns (Qiagen). The concentration, purity, and quality of cDNA and cRNA were assessed as above. Fifteen microliters 1 µg/µL cRNA was incubated with 6 µL 5X fragmentation buffer and 9 µL RNase-free water for 35 min at 94°C and digested into 35- to 200-bp cRNA fragments. The prehybridized Rat Genome 230 2.0 microarray was put into a hybridization buffer prepared following the Affymetrix protocol and hybridized in a rotating chamber (60 rpm, 16 h, 45°C). Arrays were washed to remove the superfluous hybridization buffer, stained in a GeneChip Fluidics Station 450 (Affymetrix Inc.), and scanned with a GeneChip Scanner 3000 (Affymetrix Inc.) to obtain images. The images were converted to signal values using the Affymetrix GCOS 1.4 software (Affymetrix Inc.).

The probe signal values were scaled to evaluate gene expression ($P < 0.05$), marginal

expression ($P < 0.065$), and no expression ($P > 0.065$). Then, the signal value of each chip was normalized, and the relative values of genes were assessed with ratios comparing the normalized P value in the PH groups to that in controls. For example, gene expression with a relative value of ≥3 was considered to be upregulated, gene expression with a relative value of ≤0.33 was considered to be downregulated, and genes with a relative expression value of 0.33-2.99 were considered to be meaningless genes (Vardhanabhuti et al., 2006). To minimize technical errors from microarray analysis, we analyzed each sample at least 3 times with the Rat Genome 230 2.0 microarray. Average values were calculated as a corrective value. Finally, these values were analyzed using GeneMath, GeneSpring (Silicon Genetics, San Carlos, CA, USA) and other programs (Mulrane et al., 2008).

Real-time polymerase chain reaction (RT-PCR)

The RNA samples used for RT-PCR were prepared from 8 kinds of liver cells isolated at 10 recovery time points. cDNA synthesis was performed according to the manufacturer protocol. Primer and probe sequences were designed using Primer Express 2.0 according to mRNA sequences of HC marker glucose-6-phosphatase-α, BEC marker gamma-glutamyltransferase 1, OC marker *OC2*, HSC marker glial fibrillary acidic protein, SEC marker *CD14*, KC marker lisozyme, PC marker *CD56*, and DC marker *CD86* and synthesized by Shanghai GeneCore BioTechnologies Co., Ltd. (Shanghai). Target genes were subjected to amplification and PCR testing on a PRISM 7900 Sequence Detector (ABI Company, USA) according to the operational guideline manual with a QuantiTect SYBR Green RT-PCR Kit (Qiagen). The copies of target genes in the sample per milliliter were calculated according to a standard curve, and the relative expression content was computed according to *β-actin* copy number (Wang and Xu, 2010).

RESULTS

Identification of ERK1/2 signaling-involved genes

According to receptor types of ERK1/2, such as G-protein, calcium ion channel, receptor tyrosine kinase (RTK) and integrin, ERK1/2 signaling was roughly divided into 4 pathways: G-protein-mediated ERK1/2, Ca^{2+}-mediated ERK1/2, RTK-mediated ERK1/2, and integrin-mediated ERK1/2. Despite the variations in their specific extracellular signals, these pathways had some common components, including a set of adaptors (Shc, GRB2, Crk, etc.) linking the receptor to a guanine nucleotide exchange factor (SOS, C3G, etc.) for transducing the signals to small guanosine triphospate binding proteins (Ras, Ras-proximate-1), which in turn stimulated mitogen-activated protein kinase (MAPK) kinase kinase (Raf), MAPK kinase (MEK1/2) and MAPK (RK1/2). Genes involved in these pathways were obtained by checking available signaling pathways in databases including the Rat Genome Database, Gene MicroArray Pathway Profiler, Kyoto Encyclopedia of Genes and Genomes, BIOCARTA, and Biocompare. These genes were then reconfirmed through retrieval of pertinent articles. We found that the number of genes involved in these 4 signaling pathways were 79, 50, 44, and 30, respectively (Table 1).

Table 1. Overview of ERK1/2 signaling pathway-involved genes.

Pathways	Involved genes	Genes on arrays	Liver regeneration-related genes							
			HC	BEC	OC	HSC	SEC	KC	PC	DC
G-protein-mediated ERK1/2 pathway	79	77	24	27	12	34	21	17	20	30
Ca^{2+}-mediated ERK1/2 pathway	50	48	16	16	10	19	13	14	13	20
RTK-mediated ERK1/2 pathway	44	41	13	11	6	19	12	12	7	15
Integrin-mediated ERK1/2 pathway	30	29	6	6	2	6	9	5	4	6
Total*	165	161	59	55	32	64	41	39	44	69

HC = hepatocytes; BEC = biliary epithelial cells; OC = oval cells; HSC = hepatic stellate cells; SEC = sinusoidal endothelial cells; KC = Kupffer cells; PC = pit cells; DC = dendritic cells. *Two or more signal pathways shared one gene.

Reliability of chip results by quantitative RT-PCR

To test the reliability of the chip data, we measured the expression levels of 8 marker genes corresponding to the 8 selected liver cell types using RT-PCR. The comparison analysis of the RT-PCR results and the microarray results showed no significant difference in abundances or trends of gene expression obtained from the 2 methods, demonstrating the reliability of the chip results (Figure 2).

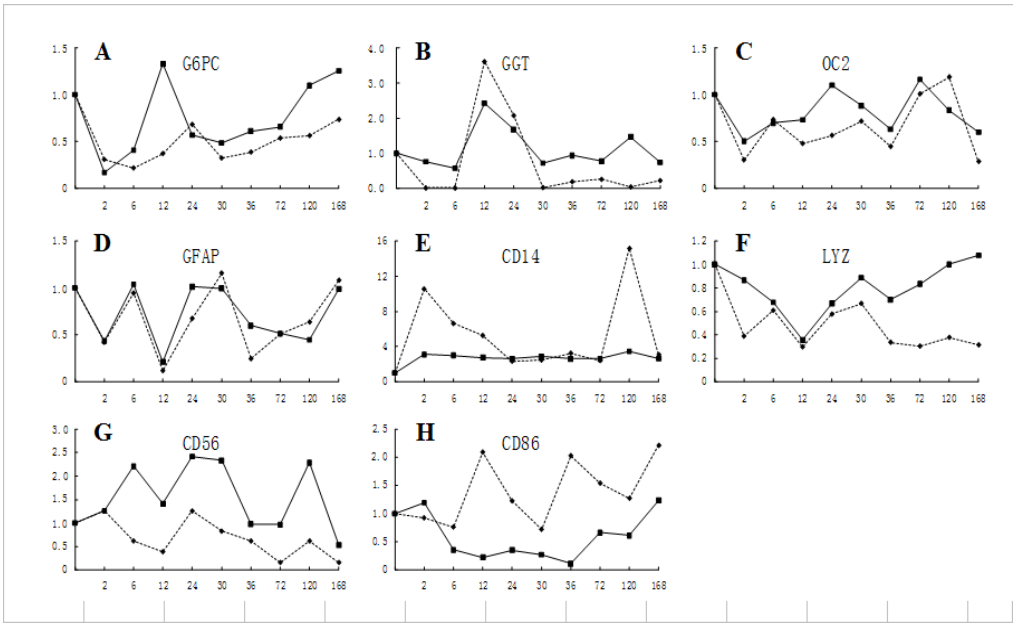

Figure 2. Comparison of mRNA levels of cell marker genes measured by microarray (solid lines) and RT-PCR (broken lines) in 8 liver cell types including hepatocytes (**A**), biliary epithelial cells (**B**), oval cells (**C**), hepatic stellate cells (**D**), sinusoidal endothelial cells (**E**), Kupffer cells (**F**), pit cells (**G**), and dendritic cells (**H**). Vertical axis indicates the relative mRNA level; horizontal axis indicates the recovery time points after 2/3 hepatectomy.

Global characteristics of transcriptional profiles of ERK1/2 signaling pathway genes in 8 cell types during rat LR

The Rat Genome 230 2.0 array used in this study encompassed the entire rat genome. Array hybridizations were performed in triplicate for each sample. The relevant data retrieved from signaling pathway databases and scientific publications were compared with chip results, showing that 77, 48, 41, and 29 genes were on chip that were separately involved in G-protein-, Ca^{2+}-, RTK-, and integrin-mediated ERK1/2 pathways, respectively (see Table 1). Among these genes, those showing 1) the same expression patterns at the same time point in 3 independent analyses, 2) at least a 3-fold change in expression level compared to that in the control for at least one time point, and 3) significant ($P \leq 0.05$) or extremely significant ($P \leq 0.01$) differences between the PH and sham-operated groups, were identified based on stringent standards as LR-related genes. By interrogating the microarray results of 8 rat liver cell types, we found that many genes did not conform to the criteria for defining LR-related genes and that a large difference in expression changes in these genes occurred from one cell type to another. The 4 pathway-involved genes conforming to the above standard in HCs, BECs, OCs, HSCs, SECs, KCs, PCs, and DCs are shown in Table 1.

To visualize the temporal expression patterns of genes across the 8 rat liver cell types after PH, we performed H-clustering analysis. The results suggested that ERK1/2 signaling-involved genes were clustered in the confined regions in the heat map (Figure 3). For example, a majority of genes involved in the G-protein-mediated ERK1/2 pathway were upregulated during LR in HCs, SECs, and PCs (Figure 3A); Ca^{2+}-mediated ERK1/2 pathway-involved genes were largely upregulated in HCs, BECs, PCs, and DCs but downregulated in HSCs (Figure 3B). RTK-mediated ERK1/2 pathway-involved genes showed markedly reduced expression in HSCs (Figure 3C). Integrin-mediated ERK1/2 pathway-involved genes showed significantly increased expression in HCs and BECs (Figure 3D).

Differential expressions of gene involved in 4 ERK1/2 signaling pathways in 8 rat liver cell types

An overall analysis of various online databases showed that the above 4 ERK1/2 pathways acted through 9 different specific pathways. As shown in Figure 3, the G-protein-mediated ERK1/2 pathway can be subdivided into the G-protein/protein kinase C (PKC)/Ras (pathway 1), G-protein/phosphoinositide-3-kinase (PI3K)/Rac (pathway 7), G-protein/EPAC (exchange protein directly activated by cyclic AMP)/Ras-proximate-1 (Rap1) (pathway 8), and G-protein/PKA (protein kinase A)/Rap1 (pathway 9) pathways. The Ca^{2+}-mediated ERK1/2 pathway comprises only Ca^{2+}/PKC/Ras (pathway 2); the RTK-mediated ERK1/2 pathway contains RTK/PKC/Ras (pathway 3) and RTK/growth factor receptor-bound protein 2 (Grb2)/Ras (pathway 4), and the integrin-mediated ERK1/2 pathway is classified into integrin/Grb2/Ras (pathway 5) and integrin/focal adhesion kinase/Rac (pathway 6). The number of genes (which were presented in array) involved in the nine signaling pathways were 47, 48, 38, 30, 24, 21, 31, 20, 25, 2, 19, 1, 42, and 1, respectively (Figure 4). However, different cell types varied in expression changes and the number of genes involved in the above signal pathways.

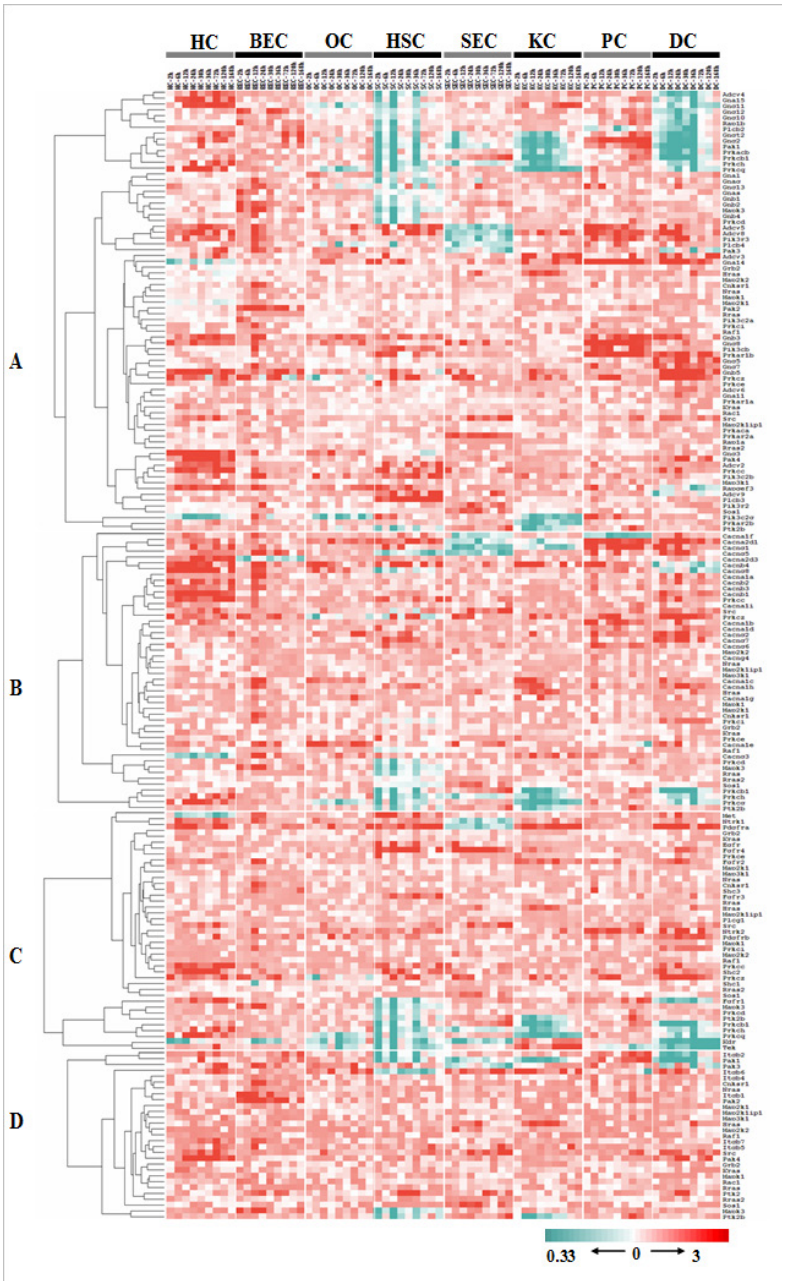

Figure 3. Hierarchical clustering of the genes of ERK1/2 signaling pathways in 8 rat liver cell types after partial hepatectomy. Two heat maps A, B, C, D, and E represented the gene clusters related to G-protein mediated, Ca^{2+}-mediated, RTK-mediated, intergrin-mediated ERK1/2 cascade, and regulatory pathway. Numbers oriented horizontally at the top indicate the recovery time points post-surgery. The increase, decrease and meaningless changes in expression level were colored in red, blue and white, respectively. For abbreviations, see legend to Table 1.

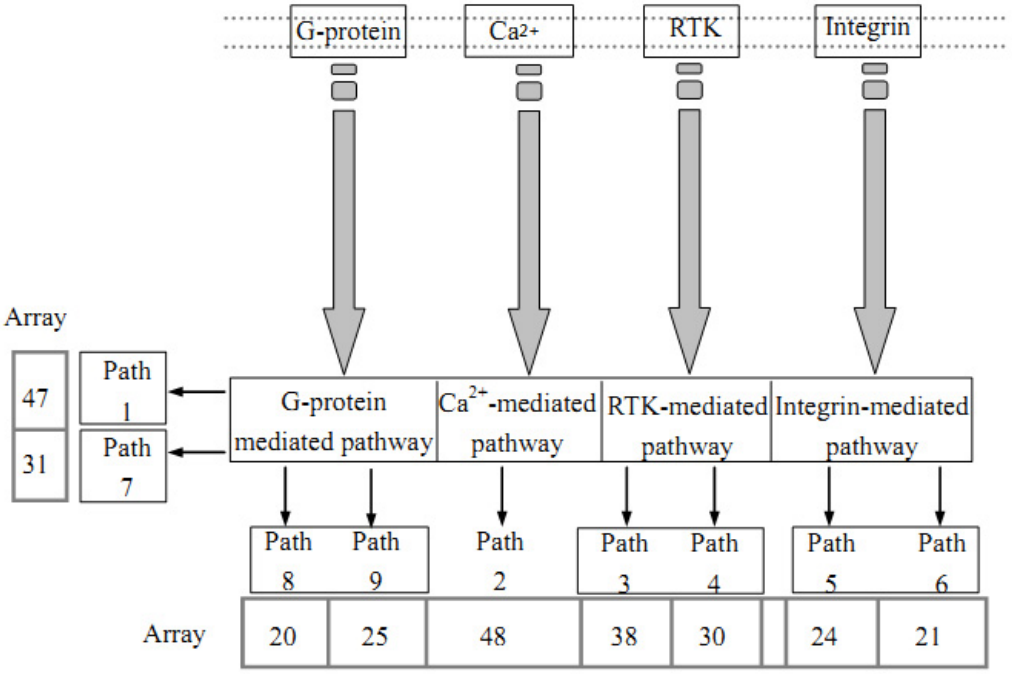

Figure 4. Schematic representations of 9 ERK1/2 signaling pathways and number of the related genes detected by microarray. Path 1 = G-protein→PKC→Ras; Path 2 = Ca^{2+}→PKC→Ras; Path 3 = RTK→PKC→Ras; Path 4 = RTK→Grb2→Ras; Path 5 = integrins→Grb2→Ras; Path 6 = integrins→FAK→Rac; Path 7 = G-protein→PI3K→Rac; Path 8 = G-protein→EPAC→Rap1; Path 9 = G-protein→PKA→Rap1.

Physiological activities governed by ERK1/2 signaling in 8 liver cell types from hepatectomized rats

Based on gene ontology categories, transcript abundance, expression dynamics, and the correlation (Nikitin et al., 2003) among genes in certain specific biological process during LR, a mathematical model based on multivariate statistical analyses (Wang and Xu, 2010) was established for depicting the timing, occurrence, and intensity of biological activities in LR using time sequence analysis theory and systematic biology methodologies (Wolkenhauer, 2001). Depending on this model, the synergy among the components involved in ERK1/2 signaling in each cell type was calculated. The results showed that, as a whole, ERK1/2 signaling was only slightly affected at the priming phase (2-6 h after PH), whereas the G-protein-mediated, RTK-mediated, and integrin-mediated pathways were strikingly enhanced in HCs, and the G-protein-, Ca^{2+}-, and RTK-mediated pathways were dominated by activation in BECs, SECs, KCs, and PCs at the progressing phase (6-72 h after PH). Most of the ERK1/2 signaling pathway activations gradually decreased and returned to normal or became even weaker than those in the control - e.g., G-protein-, RTK-, and integrin-mediated ERK1/2 pathways in DCs (Table 2).

Table 2. Proliferative activities of 8 liver cell types governed by nine ERK1/2 signaling pathways during rat liver regeneration.

Liver cell types	ERK1/2 signaling pathways	Initial phase		Proliferative phase		Terminal phase	
		E_t	Effects	E_t	Effects	E_t	Effects
HC	1	52.5	-	66.0	↑*	72.8	-
	3	50.2	-	67.8	↑**	76.8	-
	5	25.7	-	34.1	↑**	36.0	-
	6	16.3	-	22.6	↑**	25.3	-
	7	28.3	-	37.0	↑**	40.4	-
	8	24.3	-	34.8	↑**	37.2	-
	9	24.3	-	32.0	↑**	33.0	-
BEC	2	26.9	-	34.5	↑**	25.6	-
	4	58.4	-	67.8	↑*	57.1	-
	8	21.6	-	25.6	↑*	19.0	-
	9	26.4	-	29.5	-	23.5	↓*
OC	1	70.5	↓*	74.7	-	77.6	-
HSC	7	23.6	-	25.6	↓*	26.8	-
	8	17.7	↑*	17.5	-	18.3	-
	9	17.0	↑*	17.1	-	18.0	-
SEC	1	47.4	↓*	52.6	-	54.3	↑*
	5	29.6	-	35.8	-	38.2	↑*
	7	27.6	-	30.3	↓*	31.7	-
	9	20.3	-	22.9	↑*	24.4	↑*
KC	3	53.5	↑*	54.6	↑*	56.9	-
	4	44.5	↑*	45.0	↑*	44.9	-
PC	1	49.4	-	55.6	↑*	56.6	↑*
	7	33.4	-	32.7	-	32.9	↑*
DC	1	58.1	-	57.6	-	42.7	↓*
	4	47.1	-	44.8	-	33.2	↓*
	5	19.2	-	20.4	-	19.7	↓*
	6	17.6	-	18.7	-	19.0	↓*
	7	29.2	-	28.8	-	25.1	↓*
	8	18.2	-	16.9	-	16.0	↓*
	9	23.0	-	21.5	-	19.9	↓*

*Significant difference; **extremely significant difference; (↑) = activity is stronger than control; (↓) = activity is weaker than control. 1. G-protein→PKC→Ras; 2. Ca^{2+}→PKC→Ras; 3. RTK→PKC→Ras; 4. RTK→Grb2→Ras; 5. integrins→Grb2→Ras; 6. integrins→FAK→Rac; 7. G-protein→PI3K→Rac; 8. G-protein→EPAC→Rap1; 9. G-protein→PKA→Rap1. For liver cell type abbreviations, see legend to Table 1.

DISCUSSION

This study detected the expression profiles of genes involved in ERK1/2 signaling in 8 liver cell types after rat PH and then comparatively analyzed the proliferation activities predicted by expression changes using bioinformatics and systems biology methods. We found that a total of 9 ERK1/2 signaling pathways have roles in the regulation of cell proliferation.

The early period of 2-6 h after PH is often called the priming phase, and the main events occurring in this stage are the activation of HCs, which enter G1 from G0 during which the active nuclear factor kappa B signaling pathway plays a major role (Liu and Qian, 2006). Whether other signal pathways such as ERK1/2 contribute to cell activation remains poorly studied. Talarmin et al. (1999) have reported that ERK1/2 activation at 0.5-4 h after PH is responsible for the priming of quiescent HCs, with transition from G0 to G1. In the present study, gene synergy analysis showed that the proliferation-promoting integrin/Grb2/Ras pathway in HCs was more active than that in controls, and at the same time, Src in this pathway showed increased expression with a peak 4.7-fold higher than that in the control 6 h after PH, implying that Src may play a key role in this event. In addition, the proliferation-promoting G-

protein/EPAC/Rap1 and G-protein/PKA/Rap1 pathways in HSCs were significantly enhanced in our study. Balabaud et al. (2004) have suggested that HSCs are activated at the early phase, and activation lasts until at least 24 h post-PH. However, these authors do not point to the specific signaling pathway responsible for HSC stimulation. In the context of our results, we speculate that ERK1/2 may participate in HSC activation via the above-mentioned G-protein/EPAC/Rap1 pathway and G-protein/PKA/Rap1 pathway. Also, gene synergy analysis showed that the proliferation-promoting RTK/PKC/Ras and RTK/Grb2/Ras pathways in KCs were activated, and theoretically, the stimulation of the 2 pathways was implicated in KC activation. Despite a lack of data addressing the timing of KC activation, Desbois-Mouthon et al. (2006) reported that KCs activated after hepatectomy can secrete and release a bulk of cytokines and growth factors (including tumor necrosis factor α, hepatocyte growth factor, epidermal growth factor) promoting G0/G1 transition of HCs suggests that the timing of KC activation is close to that of HC. Based on our analysis, the enhancement of RTK/PKC/Ras and RTK/Grb2/Ras pathway activities was helpful for KC activation after PH.

The period of 6-72 h after PH is defined as the progressing phase of LR, during which HCs enter the G1 phase of the cell cycle in response to growth factors and progress. Desbois-Mouthon et al. (2006) have reported that ERK1/2 signaling is associated with the induction of cyclins A, D1, E, and B1 between 10 and 11 h in the G1/S transition phase. In this study, almost all of the proliferation-promoting REK1/2 pathways showed increased HC activities during the progressing phase, inferring the promotion of the cell cycle of HCs through this pathway, which is consistent with the results of Desbois-Mouthon et al. (2006).

Notably, various liver cell populations displayed great differences in peak DNA replication, which occurred 36 h after PH in BECs but 24 h after PH in HCs (Magami et al., 2002). Our study showed that the proliferation-promoting Ca^{2+}/PKC/Ras, RTK/Grb2/Ras, and G-protein/EPAC/Rap1 pathways in BECs augmented activation - all 3 pathways may be involved in BEC replication. As mentioned above, the activities of the G-protein/EPAC/Rap1 and G-protein/PKA/Rap1 pathways in HSCs during the priming phase showed insignificant difference compared to that of the control during the progressing phase, whereas the proliferation-promoting G-protein/PI3K/Rac pathway was enhanced at this time. Michalopoulos (2010) have found that DNA replication of HSCs after PH peaks 44-48 h after surgery. Obviously, our result was inconsistent with theirs, which infers that ERK1/2 signaling may not be involved in HSC proliferation. SECs, one type of nonparenchymal cells, displayed delayed replication. DNA synthesis in SECs is completed within 96 h after PH (Clavien et al., 2007), which is beyond the usual definition of the progressing phase (6-72 h after PH). Therefore, we propose that ERK1/2 signaling has a very small role in SEC proliferation. According to our data, the proliferation-promoting G-protein/PI3K/Rac pathway in SECs was decreased, whereas the G-protein/PKA/Rap1 pathway increased, and the regulatory effects of the 2 pathways on SEC proliferation are opposed, consistent with a minor role for ERK1/2. Regarding the proliferation dynamics of 3 additional liver immune cells (KCs, PCs, and DCs), as far as we know, DNA replication reaches a peak 48 h post-PH in KCs; for PCs, the peak occurs 24-72 h after PH (Xu et al., 2011), and the timing for DCs is unclear. In this study, the RTK/PKC/Ras and RTK/Grb2/Ras pathways in KCs were activated during the progressing phase, and these 2 pathways likely contribute to KC repopulation at this time. Gene synergy of the G-protein/PKC/Ras pathway in PCs was significantly higher than that of the control, and the consistency between the activation time of this pathway and the timing of PC proliferation clearly showed that these pathways are involved in PC proliferation.

The 120- to 168-h period after PH is considered the terminal phase, during which HCs cease growth and start differentiation for the rebuilding of tissue structure (Liu and Qian, 2006). BECs, HSCs, and KCs also begin to differentiate at this stage, implying that the ERK1/2 signal pathway no longer has a positive effect on the proliferation of these cells. Our analysis showed that the synergistic effect of ERK1/2 signaling-involved genes in these liver cell types was approximately equal to or even lower than that in the control - a result concordant with the hypothesis of decreased ERK1/2 effect. As for stem cells in the liver, or OCs, the activity of ERK1/2 signaling had no noticeable effect in comparison with controls at the terminal phase. Previously, we observed the impairment of OC proliferation 72 h after surgery. The consistency between that result and those of the present study reinforces the conclusion that the growth and proliferation of OCs are blocked at the end of LR. As described above, SEC proliferation peaks 96 h after PH (Clavien et al., 2007), which occurs within the terminal phase and logically assumes the involvement of ERK1/2 signaling in SEC replication. Unanimously, proliferation-promoting pathways including G-protein/PKC/Ras, integrins/Grb2/Ras, and G-protein/PKA/Rap1 were enhanced at this phase, inferring the positive regulation of SEC proliferation by ERK1/2 signaling via the 3 pathways. Of note, only the adenylate cyclase type 3 gene in pathway 9 displayed a markedly increased expression with a peak 3-fold higher than that in controls 6 h after PH, perhaps implying the importance of the adenylate cyclase type 3 in the activity of the G-protein/PKA/Rap1 pathway. Based on the observation that PC proliferation occurs between 24 and 74 h after hepatectomy, we propose that the major events occurring in PCs is differentiation, during which the effect of ERK1/2 on PC proliferation would be obviously weakened. However, our results showed a striking increase in synergistic effects of the proliferation-promoting G-protein/PKC/Ras and G-protein/PI3K/Rac pathways during the terminal phase compared to that in the control, which does not support our hypothesis. According to above analysis, these 2 signaling pathway likely have no promotive effect on cell proliferation but rather influence other biological effects. Finally, the synergistic effect of Erk1/2 signaling in DCs was much lower than that in the control, which was helpful to DC differentiation at the end of LR.

In conclusion, this study measured the transcriptional profiles of ERK1/2 signaling-involved genes using a Rat Genome 230 2.0 array and obtained many detailed but accurate data concerning ERK1/2 signaling in 8 liver cell types. These data suggest the involvement of 9 pathways of ERK1/2 signaling in the proliferation of these cell types after PH in rats. However, a Rat Genome 230 2.0 array can measure only gene transcription profiles and cannot reflect protein synthesis. Therefore, we would use methods such as gene addition, RNA interference, and protein interaction in future studies that test the above results.

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