

Short communication

ANALYSIS OF THE ROLE OF THE INTEGRIN SIGNALING PATHWAY IN HEPATOCYTES DURING RAT LIVER REGENERATION

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Abstract: To explore the role of the integrin signaling pathway in hepatocytes during rat liver regeneration, the integrin signaling pathway-related gene expression profile in hepatocytes of regenerative liver was detected using Rat Genome 230 2.0 array. The chip data showed that 265 genes of the integrin signaling pathway were included by Rat Genome 230 2.0 array and 132 genes showed significant expression changes in hepatocytes of regenerative liver. The numbers of up-, down- and up/down- regulated genes were 110, 15 and 7 respectively. In addition, bioinformatics and systems biology methods were used to analyze the role of the integrin signaling pathway in hepatocytes. The analysis of gene synergy value indicated that paths 1, 8, 12, and 15 promoted hepatocyte proliferation at the priming phase of liver regeneration; paths 1, 3, 8, and 12-15 enhanced hepatocyte proliferation at the progressing phase; paths 11 and 14 promoted hepatocyte proliferation, while paths 12 and 13 reduced hepatocyte proliferation at the terminal phase. Additionally, the other 8 paths (2, 4, 5-7, 9-10 and 16) were not found to be related to liver regeneration. In conclusion, 132 genes and 8 cascades of the integrin signaling pathway participated in regulating hepatocyte proliferation during rat liver regeneration.

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Abbreviations used: AC – adenylyl cyclase; Akt – v-Akt murine thymoma viral oncogene; ECM – extracellular matrix; ERK – extracellular regulated protein kinases; FAK – focal adhesion kinase; GSK3 – glycogen synthase kinase-3; ILK – integrin-linked kinase; JNK – c-Jun NH₂-terminal kinase; MAPK – mitogen-activated protein kinase; mTOR – mammalian target of rapamycin; PAK-p21 – activated kinase; PH – partial hepatectomy; PI3K – phosphoinositide 3-kinase; PKA – protein kinase-A; PKB – protein kinase B; PKC – protein kinase C; ROCK – Rho-associated kinase

Key words: Integrin signaling pathway, Rat, Liver regeneration, Partial hepatectomy, Immunohistochemistry, Hepatocyte proliferation, Rat Genome 230 2.0 array, RT-PCR, Gene expression profile, Gene synergy value

INTRODUCTION

It is well known that the rat liver has an enormous capacity to regenerate in response to partial hepatectomy (PH) or liver injury [1]. After liver injury or PH, the residual liver cells are rapidly activated and enter the cell cycle to restore the hepatic mass, which is called liver regeneration [1]. Liver regeneration after PH is a very complex and well-orchestrated phenomenon. It is carried out with the participation of all mature liver cell types, and involves numerous physiological activities including activation/expression of a series of cytokines and growth factors, accompanied by cell proliferation, cell differentiation and dedifferentiation, and tissue structure reconstruction, etc. [2]. As the main functional cell populations in the liver, hepatocytes make up 70-80% of hepatic mass and 65% of total hepatic cell number [3-4], and perform many important physiological functions, including energy storage and metabolism, bile secretion, detoxification, immune response, as well as modification and excretion of substances [5]. Additionally, hepatocytes are the first cells to begin DNA synthesis and the cell cycle after PH [2]. Therefore, study of hepatocyte proliferation will provide new insights into liver regeneration. Previous studies showed that hepatocyte differentiation and proliferation are greatly affected by extracellular matrix (ECM) through the integrin signaling pathway [6]. Recent research demonstrated that disruption of ECM/integrin signaling via elimination of integrin-linked kinase (ILK) in hepatocytes interferes with signals, leading to termination of liver regeneration [7].

However, the ECM/integrin signaling pathway is a complex signaling transduction network which is made up of many branches. In this study, the branches are named "paths". According to the related websites and references, there are mainly 16 paths in the integrin signaling pathway (as Fig. 3 depicts). In paths 1-2, integrins bind to the membrane protein caveolin-1 (Cav-1) through their external and transmembrane domains, then recruitment of Fyn and Shc to Cav-1 results in phosphorylation of Shc Tyr³¹⁷, leading to recruitment of the Grb2/Sos complex for Ras→ERK (extracellular regulated protein kinase) cascade activation [8]. Equally, the residue Tyr⁹²⁵ of focal adhesion kinase (FAK) recruits Grb2/Sos complex and then activates the ERK cascade [9]. In paths 3-4, integrins bind to ILK, and then trigger the downstream signaling cascades such as ERKs, v-Akt murine thymoma viral oncogene (Akt) / protein kinase-B (PKB) and glycogen synthase kinase-3 (GSK3) [8, 10]. In paths 5-13, FAK has many phosphorylation sites and combines with numerous intracellular signaling molecules including Src, PI3K (phosphoinositide 3-kinase), RhoGAP, RhoGEF, and p130CAS (Crk-associated substrate), then activates their downstream signaling pathways, such as mitogen-activated protein kinase

(MAPK), c-Jun NH₂-terminal kinase (JNK), and p21-activated kinase (PAK) signaling pathway [9, 11-12]. α -actinin links tails of integrins to actin fibrils, and also binds to several cytoplasmic molecules including vinculin, zyxin, and ERK1/2, forming path 14 [13]. In addition, CD47 associates with integrin heterodimer to form a protein complex; the complex stimulates Gs-mediated up-regulation of the cAMP cascade through adenylyl cyclase (AC), resulting in nuclear translocation of the catalytic subunit of protein kinase-A (PKA), and then enters into PKA→GSK3 and PKA→ERK cascade forming paths 15-16 [14]. Studies on regulation of the integrin signaling pathway in relation to liver regeneration and hepatic cell differentiation and proliferation have been reported. However, the integrin signaling pathway is a complex signaling transduction network including 16 paths. It is unclear which path of the integrin signaling pathway participates in liver regeneration and how it does, especially the roles of every path in regulation of hepatocyte proliferation. The purpose of this study is to explore which paths of the integrin signaling pathway are involved in regulation of hepatocyte proliferation during rat liver regeneration at the transcriptional level. Firstly, Rat Genome 230 2.0 Array was used to detect expression profiles of integrin signaling pathway-related genes of hepatocytes, which were isolated by Percoll density gradient centrifugation from rat regenerative liver, and then the role of the integrin signaling pathway in hepatocytes during rat liver regeneration was analyzed using bioinformatics and systems biology methods.

MATERIALS AND METHODS

Preparation of rat partial hepatectomy model

Sprague-Dawley rats were provided by the Animal Center of Henan Normal University in compliance with the Laws of Animal Protection of China. A total of 114 SD healthy adult rats, weighing 190 ± 20 g, were randomly divided into 19 groups consisting of nine PH groups, nine sham-operation (SO) groups and one control group with six rats per group. The PH operation was performed according to the classical model of Higgins and Anderson [15]. SO was performed by mid-ventral laparectomy. The rats in the control group, serving as a 0-hour sample of both SO groups and PH groups, were sacrificed immediately after the surgical removal of the left and median lateral lobes. The Laws of Animal Protection of China were enforced strictly.

Isolation of hepatocytes

The rats from the SO groups and PH groups were anesthetized by ether and sterilized with 75% alcohol after PH 2, 6, 12, 24, 30, 36, 72, 120 and 168 h respectively, and then the abdominal cavity was opened. Rats were subjected to ligation of the inferior vena cava both below and above the liver, followed by catheterization of the liver portal vein. Conventional two-step perfusion method was used to disperse liver cells. Briefly, the liver was perfused via the portal vein with calcium-free perfusate preheated at 37°C until the liver surface turned gray, and then perfused with 0.05% type IV collagenase at a flow rate of 1 ml/min.

The perfused liver was cut into small pieces and digested with 0.05% type IV collagenase for 15 min at 37°C. The digested sample was filtered through 400-mesh nylon net. The filtrate was centrifuged at 200g for 5 min. The pellet at the bottom was washed thrice by phosphate buffered saline buffer at 4°C. After adjusting cell concentration to 1×10^8 cells/ml, 6 ml of the mixed cell suspension was spread on the surface of 4 ml 60% Percoll in a 10 ml tube, and then centrifuged at 200g at 4°C for 15 min. The sediment was enriched with hepatocytes [3, 16].

Immunochemical analysis

A few purified hepatocytes were taken and individually fixed with 10% formaldehyde for 30 min, and then smeared onto glass slides. When the cell suspension dried on glass slides, microwave antigen retrieval was performed. The sections were incubated separately with a 1:200 dilution (V/V) of ALB and G6PC antibodies overnight at 4°C and then with a 1:5,000 (V/V) diluted biotin-labeled secondary antibody at 37°C for 60 min. The system was hybridized with streptavidin–biotin complex (SABC) at 37°C for 30 min. All steps were performed at room temperature. Meanwhile, the paraffinized sections of liver tissue at the corresponding time points were prepared for immunohistochemical performance. The results were observed and analyzed under an optical microscope.

Detection of rat genome 230 2.0 microarray and data analysis

The total RNA of hepatocytes was extracted according to the manual of Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA) and purified following the RNeasy Mini protocol (Qiagen, Inc, Valencia, CA, 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 [17]. T7-oligo DT (24) (Keck Foundation, New Haven, CT), SuperScript II RT (Invitrogen Corporation, Carlsbad, CA) and 5 µg of total RNA were used to synthesize the first strand of cDNA. The second strand was synthesized using the Affymetrix cDNA single-stranded cDNA synthesis kit. The cDNA product was purified following the cDNA purify protocol. 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-labeled cRNA. The labeled cRNA was purified using the RNeasy Mini Kit columns (Qiagen, Valencia, CA). Their concentration, purity and quality were assessed as above for 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 into 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 (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 Inc., 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 were initially normalized by scaling all signals to a target intensity of 200. When the *P* value is < 0.05, it means that the gene is present (P), a value < 0.065 means marginal (M), and a value > 0.065 is marked absent (A). On the other hand, the normalized signal value in PH to that in the control was used to calculate the relative value, that is, the ratio value of gene expression abundance. To minimize the technical error from array analysis, each sample was tested at least three times using Rat Genome 230 2.0 microarray. Their average value was calculated as the corrective value. Finally, these values were analyzed with GeneMath, GeneSpring, Microsoft Excel Software and Pathway Studio 7.0 [18-20].

RT-PCR

To verify the chip data, six genes were selected for quantitative RT-PCR analysis. Firstly their total mRNA was isolated. Of them, 2 µg of RNA was reverse-transcribed using random primers and a reverse transcription kit (Promega). The primer was designed with Primer Express 2.0 software according to the GenBank number of six target genes including *alb*, *g6pc*, *apoe*, *jun*, *trim24*, and *myc*, and synthesized by Shanghai Generay Biotech Co, Ltd. Total RNA (2 µg) was reverse-transcribed using random primers and a reverse transcription kit (Promega). First-strand cDNA samples were subjected to quantitative PCR amplification by using SYBR[®] Green I on the Rotor-Gene 3000 (Corbett Robotics, San Francisco, CA). Every sample was analyzed in triplicate. Standard curves were generated from five repeated ten-fold serial dilutions of cDNA, and the copy numbers of target genes in every milliliter of the sample were calculated according to the standard curve [21].

Identification of genes associated with integrin signaling pathway of hepatocytes and their relation to liver regeneration

To identify genes associated with integrin signaling pathways of hepatocytes in rat regenerating liver, the words “integrin” and “integrin signaling pathway” were entered into NCBI (www.ncbi.nlm.nih.gov) and RGD (www.rgd.mcg.edu) to identify its genes associated with rat, mouse and human. Then, the influential genes were collated according to the biological pathways embodied by GENEMAP (www.genmapp.org), KEGG (www.genome.jp/kegg/pathway.html), and BIOCARTA (www.biocarta.com/genes/index.asp), etc., and the genes associated with the integrin signaling pathway were collated and reconfirmed through the literature by searching pertinent articles [22-24]. To confirm the genes related to liver regeneration, a gene was considered as significantly up-regulated when its expression change was ≥ 3 as compared with the control, as significantly down-regulated when the change was ≤ 0.33 , and as significantly up/down-regulated when it has been up-regulated and down-regulated in liver regeneration [25].

Gene synergy analysis of integrin signaling pathway

According to the gene expression, abundance of the integrin signaling pathway of hepatocytes in regenerating liver detected by Rat Genome 230 2.0 Array, and interactions of genes curated from the ResnetCore7.01 database, which is attached in Pathway Studio 7.0 software, and integrating the rationale that the role of a gene relies on its interactions with others, a mathematical model (E_t) was established to measure gene synergy between the related genes with time series analysis [26] and correlation analysis [27] based on the multivariate statistical method. This mathematical model describes how physiological activities are governed by gene synergy [25].

$$E_t = \frac{\sum_{i=1}^n \sum_{k=1}^n [(X_i^{(t)}, X_k^{(t)}) * r_{ik}]}{n(n+1)}$$

In the formula, the gene correlation coefficient (r_{ik}) was defined as:

$$r_{ik} = \frac{m(\sum_{t=1}^m X_i^{(t)} X_k^{(t)}) - (\sum_{t=1}^m X_i^{(t)}) (\sum_{t=1}^m X_k^{(t)})}{\sqrt{\left[m \sum_{t=1}^m (X_i^{(t)})^2 - (\sum_{t=1}^m X_i^{(t)})^2 \right] \left[m \sum_{t=1}^m (X_k^{(t)})^2 - (\sum_{t=1}^m X_k^{(t)})^2 \right]}}$$

“E” means gene synergy value which refers to physiological activity. “t” represents order time series. “ $X_i^{(t)}$ ” and “ $X_k^{(t)}$ ” are abundances of gene at t. “n” is the total number of genes. Physiological activities in liver regeneration are more strengthened than control groups when $E_t > 2E_0$; the activities are suppressed or lowered when $E_t - E_0 < 0$, and the activities are similar in two groups when $E_0 < E_t < 2E_0$.

RESULTS

Isolation and identification of hepatocytes

As an example, here we present the morphology and vitality of hepatocytes isolated from a PH 0 h liver sample. Immunohistochemistry data showed that ALB and G6PC were distributed throughout the cytoplasm of liver tissue and hepatocytes. After isolation, the percentage of ALB- and G6PC-positive cells among the purified hepatocytes was for both over 96% at any time point after PH (Fig. 1).

Consistency of the quantitative RT-PCR results and the microarray results

In order to determine the validity of results obtained by the microarray analysis, six randomly selected genes were subjected to quantitative RT-PCR analysis. The detection of RT-PCR and Rat Genome 230 2.0 Array showed that the expression trends of six genes including *alb* detected by the two methods were generally consistent, suggesting that the array check results were reliable (Fig. 2).

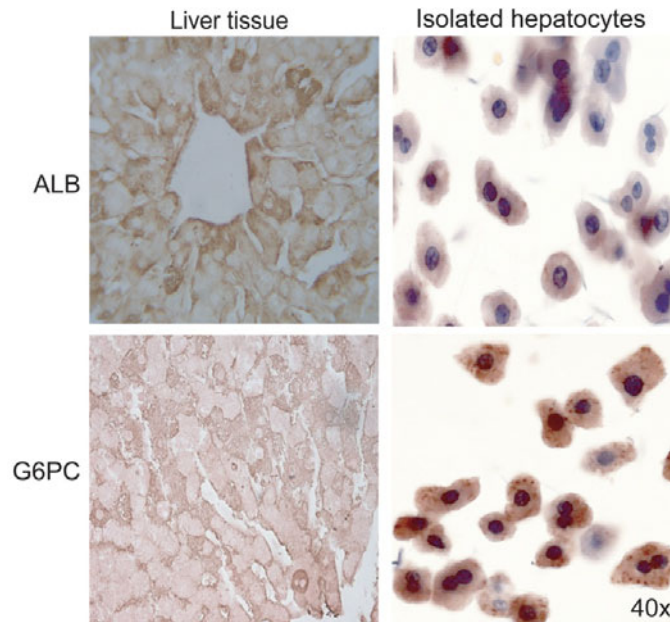


Fig. 1. ALB and G6PC immunostaining of hepatocytes isolated from regenerative liver after PH 0 h.

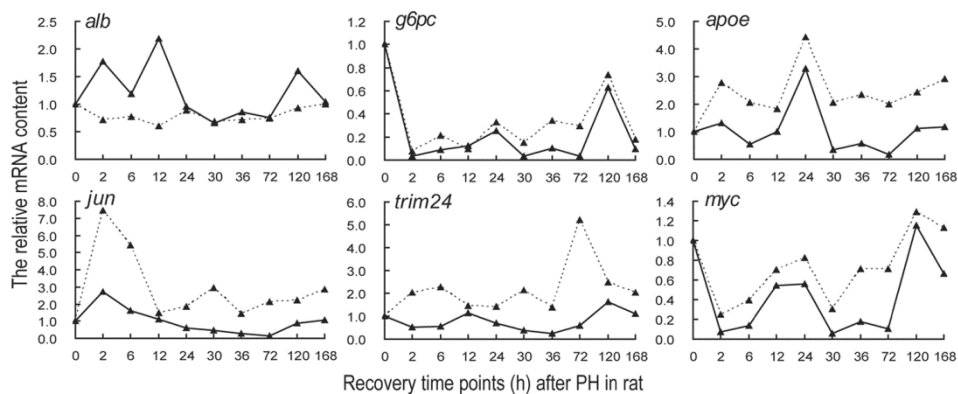


Fig. 2. Comparison of relative mRNA levels in regenerating liver detected by Affymetrix Rat Genome 230 2.0 arrays (real lines) and real-time PCR analysis (dotted lines).

The expression profiles of integrin signaling pathway-related genes in hepatocytes

The data from NCBI, RGD, etc. and biological pathway maps in GENMAPP, KEGG and BIOCARTA, etc., showed that there were 16 paths including 305 genes in the integrin signaling pathway. Of them, 265 genes were detected by Rat Genome 230 2.0 Array of hepatocytes. The array analysis showed that 132 genes showed significant expression in hepatocytes during rat liver regeneration. In these significant expression genes, 110 genes showed up-regulation, 15 genes

showed down-regulation and 7 genes showed up/down-regulation in hepatocytes during liver regeneration (Table 1 and Fig. 3).

Table 1. the expression change of 132 significant genes in hepatocytes during liver regeneration.

Protein/Gene	Recovery time points of rat partial hepatectomy										Protein / Gene	Recovery time points of rat partial hepatectomy									
	2 h	6 h	12h	24 h	30 h	36 h	72 h	120 h	168 h	2 h		6 h	12 h	24 h	30 h	36 h	72 h	120 h	168 h		
Path 1-2 ECM→CAV1→RAS→ELK1→CCND1																					
① ECM																					
COL/ COL1A1	0.95	0.95	0.91	2.36	1.34	1.51	17.9	2.22	1.52	/ ARHGAP11A	3.14	2.51	2.42	35.4	30.7	36.5	16.9	5.55	0.75		
/ COL1A2	0.52	0.95	0.67	1.95	0.61	0.98	20.5	3.28	0.83	/ ARHGAP4	3.8	4.1	6.92	4.18	8.11	6.9	5.06	9.46	2.57		
/ COL3A1	0.41	0.6	0.4	3.19	0.49	1.35	20.3	6.18	0.78	/ ARHGAP9	1.27	3.29	1.71	3.56	4.15	5.23	4.88	3.64	1.15		
/ COL4A1	0.65	0.6	0.62	1.77	0.89	1.87	8.21	2.6	1.23	/ ARHGAP27	1.36	3.24	2.08	4.47	3.6	3.13	3.48	3.77	0.38		
/ COL5A1	1.56	1.95	1.57	3.21	2.06	3.38	11.2	4.14	1.8	/ ARHGAP28	1.11	1.85	2.09	1.22	2.57	1.26	3.91	2.28	1.3		
/ COL5A2	0.74	1.97	1.33	3.66	2.07	3.14	16.8	3.16	1.48	/ ARHGAP22	1.89	5.32	9.3	1.87	4.77	3.51	2.67	5.08	2.73		
/ COL5A3	1.47	3.79	1.26	1.13	1.79	1.57	3.11	1.13	1.2	/ ARHGAP15	1.36	2.73	1.39	1.34	1.84	1.91	3.42	1.29	1.32		
/ COL6A1	1.03	0.74	0.63	1.35	0.74	0.95	3.59	1.69	0.74	/ ARHGAP20	3.69	4.45	1.26	1.22	1.34	1.83	5.47	1	0.88		
/ COL6A2	1.1	1.23	1.08	5.23	1.55	1.62	16.4	1.22	1.25	/ ARHGAP10	1.48	3.11	2.53	1.51	3.1	3.1	1.54	2.5	2.6		
/ COL6A3	1.04	0.98	0.58	1.32	1.17	2.46	5.07	1.84	0.94	② ARHGEF											
/ COL8A1	2.68	3.57	1.46	5.9	3.28	7.52	17.1	3.07	5.39	/ ARHGEF4	1.88	4.13	2.79	4.11	1.35	1.52	1.01	1.34	1.07		
/ COL9A3	1.93	4.08	4.52	12.3	15.3	24.2	7.19	12.3	10.1	/ NET1	3.08	2.4	2.27	1.18	0.72	0.67	1.25	1.58	1.25		
/ COL10A1	3.26	5.6	5.25	2.05	4.9	4.11	4.05	4.19	5.18	/ ARHGEF9	1.26	1.36	1.36	1.75	3.33	1.89	2.8	3.05	2.31		
/ COL11A1	5.84	2.93	2.15	8.12	1.57	1.23	3.49	1.97	3.76	③ RHOA/ RHOA	1.11	0.78	1.06	0.67	1.11	0.94	0.52	0.85	3.47		
/ COL13A1	3.4	4.1	3.24	6.02	5.32	6.28	18.9	6.33	7.49	④ PKC/ PRKCH	1.38	0.94	1.39	3.54	1.01	2.41	4.46	1.86	4.21		
/ COL14A1	1.12	0.72	0.44	1.55	1.08	1.06	5.69	2.28	1.07	⑤ NF-KB/ NFKB1	3.92	5.22	3.49	4.27	1.06	1.28	5.69	0.14	1.24		
/ COL15A1	1.8	1.8	2.02	2.58	3.2	3.13	6.06	1.78	2.27	/ NFKB2	3.82	4.11	4.11	3.58	5.11	1.21	3.72	0.25	0.27		
/ COL17A1	3.29	2.59	1.9	4.13	3.03	1.06	1.31	0.96	3.14	⑥ ROCK1/ ROCK2	0.83	2.27	2.93	2.98	3.69	3.08	4.46	1.86	3.21		
/ COL24A1	5.25	9.09	4.56	4.39	5.01	10.1	15.2	4.4	3.12	⑦ PPTASE/ PPP1R1B	0.89	1.16	1.04	2.71	8.91	0.96	5.32	0.9	1.1		
CAN/ VCAN	2.04	3.28	3.45	9.12	6.62	4.8	18.2	3.69	4.51	/ PPP1R3A	1.92	3.53	1.31	1.32	4.69	1.1	2.73	1.23	3.74		
/ NCAN	2.43	2.85	1.35	2.12	4.29	1.55	1.91	1.43	1.02	/ PPP1R3B	0.2	0.24	0.23	0.15	0.26	0.21	0.18	0.47	1.2		
/ ECM1	0.97	1.13	0.95	2.73	0.82	1.61	3.82	1.31	0.93	/ PPP1R3C	0.12	0.61	0.94	0.59	0.4	0.37	0.59	0.74	1.29		
EFEMP1/ EFEMP1	4.15	1.52	0.81	1.29	2.12	3.5	17.8	1.15	1.22	/ PPP1R3D	2.17	1.97	2.44	2.78	2.39	2.49	5.57	3.21	2.99		
/ EFEMP2	1.44	3.76	5.15	7.2	2.71	4.62	8.34	4.13	3.52	Path 11 PI3K→AKT→GSK3→CCND1											
FN1/ FBN1	0.26	0.39	0.62	2.11	0.64	1.73	7.63	3.17	4.14	⑧ PI3K/ PIK3R1	1.34	0.92	0.77	0.59	0.85	0.69	0.23	0.19	0.54		
/ FBN2	1.15	1.57	0.84	2.43	0.85	1.78	3.64	0.72	1.72	/ PIK3R3	2.31	1.17	3.21	1.38	4.33	3.61	2.61	1.18	2.81		
/ FN1	1.26	1.62	1.4	1.32	0.93	1.09	1.26	3.16	4.03	⑨ AKT/ AKT2	0.86	1.26	2.59	1.44	1.45	6.8	4.42	3.66	1.58		
/ FRAS1	3.88	4.3	1.53	2.32	13	3.74	2.52	6.6	9.43	Path 12 PI3K→VAW→RAC→JNK→CCND1 / NF-KB											
/ FREM2	1.31	1.41	1.81	1.29	3.47	1.65	1.27	1.26	1.39	⑩ VAV/ VAV1	0.25	0.74	0.84	0.75	1.09	0.32	1.26	5.52	1.28		
/ HAPLN3	2.89	3.17	2.83	2.77	5.06	4.19	2.65	2.32	1.02	/ VAV2	0.89	0.26	0.32	1.79	1.38	0.95	1.21	1.21	1.43		
/ IMPG1	1.84	1.47	1.47	10	5.3	1.19	6.43	5.97	1.78	/ VAV3	0.56	0.33	1.08	2.29	1.28	1.18	0.83	2.26	1.64		
/ IMPG2	0.77	1.12	0.95	0.77	0.74	1.86	3.01	0.73	0.72	⑪ PAK/ PAK1	0.62	0.68	1.16	1.73	1.01	3.07	1.15	1.34	1.11		
LAM/ LAMA1	3.25	6.83	3.97	3.73	3.52	4.19	9.78	3.07	2.57	/ PAK3	1.05	1.25	1.17	2.38	1.82	1.15	1.55	2.94	3.48		
/ LAMA4	0.65	0.22	0.65	0.74	1.28	1.08	2.97	1.28	0.96	/ PAK4	1.89	1.73	4.1	5.37	4.64	5.85	3.42	3.61	2.46		
/ LAMA5	3.03	2.68	3.47	6	3.81	3.74	4.73	3.92	3.09	⑫ MAPKS/ MAPK11	1.09	3.43	1.48	1.33	5.12	2.92	4.06	4.83	2.54		
/ LAMB1	3.37	0.88	4.46	9.77	5.34	10.7	22.7	7.76	4.51	/ MAPK12	1.09	2.19	1.4	1.24	2.83	0.59	3.03	6.59	2.26		
/ LAMB2	0.2	0.21	0.32	0.16	0.15	0.28	0.29	0.28	0.32	/ MAPK13	3.31	2.52	1.5	2.25	2.86	6.28	2.6	1.59	6.65		
/ LAMC1	3.3	1.43	1.63	1.24	0.82	1.13	1.44	0.97	0.93	/ PPP1R12B	1.77	1.74	2.83	4.12	1.18	2.32	3.21	2.32	2.73		
/ LAMC2	0.93	3.07	1.52	2.28	1.62	1.79	3.65	1.85	1.84	/ PPP1R14A	0.88	0.29	0.77	0.97	1.27	1.08	0.75	1.21	0.97		
MEPE/ MEPE	5.12	2.54	7.95	7.04	5.33	3.71	5.69	2.27	5.24	/ MAPK4	2.94	1.33	3.21	3.57	1.49	1.21	0.87	2.26	1.96		
MMRN/ MMRN2	3.64	1.04	1.77	2.14	4.61	2.26	14.4	5.34	2.68	⑬ JNK/ MAPK8	2.77	1.28	2.34	1.66	2.04	1.32	3.52	1.56	2.27		
SPOC/ SPOCK1	1.18	1.97	0.94	4.29	0.89	1.16	2.57	2.77	1.73	/ MAPK10	3.52	2.16	3.99	3.02	1.68	3.75	4.66	1.33	6.31		
/ SPOCK3	0.97	18.5	14.7	2.63	2.97	5.78	3.12	11.9	14.6	⑭ C-JUN/ JUN	3.29	2.68	1.97	3.97	1.54	1.91	2.14	1.75	2.18		
SPP/ SPP1	5.17	3.25	2.85	3.16	22.1	2.97	10.3	1.56	4.49	Path 13 SRC→CAS→RAC→PAK→CCND1 / NF-KB											
TNC/ TNC	1.77	1.32	1.29	3.23	7.52	1.56	11.1	1.47	5.75	⑮ SRC/ SRC	1.33	3.77	4.41	2.06	3.11	1.89	4.78	2.04	1.48		
② ITG/ ITGA11	4.36	1.37	1.79	4.16	1.55	4.45	3.35	6.95	1.50	⑯ p130CAS/ BCAR1	0.75	0.32	0.21	0.75	0.61	0.76	0.95	1.17	1.56		
/ ITGA9	3.89	4.21	3.38	7.12	5.24	6.10	4.79	3.37	3.88	⑰ CRK/ CRK	3.2	1.41	4	1.16	2.52	0.76	0.98	0.84	1.35		
/ ITGAD	1.26	1.44	1.79	1.17	5.83	1.16	3.92	4.85	1.94	⑱ DOCK1/ DOCK1	0.97	0.86	0.3	1.14	1.08	1.09	1.16	1.05	0.81		
/ ITGAM	7.19	0.44	0.46	4.69	5.41	1.54	1.88	1.63	1.05	Path 14 ACTN→ZYX→DOCK1→CCND1 / NF-KB											
/ ITGAV	1.44	1.69	4.98	1.75	3.96	5.44	3.27	1.29	1.42	⑲ α-ACTIN/ ACTN2	6.29	2.02	3.71	2.73	4.25	2.16	1.48	2.74	2.14		
/ ITGB1	1.34	0.95	1.58	3.47	1.25	1.47	1.32	1.31	1.29	/ ACTN1	0.68	0.65	1.36	2.73	2.32	3.04	3.32	1.79	1.72		
/ ITGB6	1.44	0.65	2.14	1.53	1.41	2.29	5.80	2.57	1.36	/ ACTN3	1.15	0.85	0.44	0.21	0.65	0.65	0.72	0.94	0.46		
/ ITGB7	1.37	1.88	1.84	1.46	2.49	2.13	4.41	1.71	1.02	/ ACTN4	0.96	0.28	0.49	1.05	0.15	1.19	1.41	1.1	0.92		
③ CAV/ CAV1	0.14	0.14	0.20	1.93	0.23	0.18	0.27	0.17	0.28	⑳ ZYX/ ZYX	3.05	4.27	3.83	1.42	5.16	1.22	1.58	1.82	1.14		
④ FYN/ FYN	0.61	2.53	1.47	2.95	2.63	3.16	3.88	0.21	0.14	Path 15-16 CD47→AC→PKA→NF-KB											
⑤ SHC/ SHC	1.87	3.14	4.55	5.08	4.19	4.79	6.84	2.05	1.42	㉑ Gα/ GNAT1	4.17	3.95	6.12	5.96	3.12	2.03	2.82	3.04	1.01		
⑥ ELK/ ELK1	3.80	4.15	1.39	2.30	2.59	1.88	0.19	0.21	1.13	/ GNAT2	1.24	0.98	5.3	5.64	1.58	1.39	1.54	1.33	2.67		
/ ELK2	4.03	3.29	1.21	1.25	0.76	1.39	1.36	1.30	1.06	/ GNAZ	3.9	3.04	2.85	3.78	1.92	5.79	5.05	3.71	3.18		
⑦ CCND1/ CCND1	5.75	3.24	3.28	4.14	8.92	6.57	2.11	0.15	0.27	㉒ Gβ/ GNB2	0.97	0									

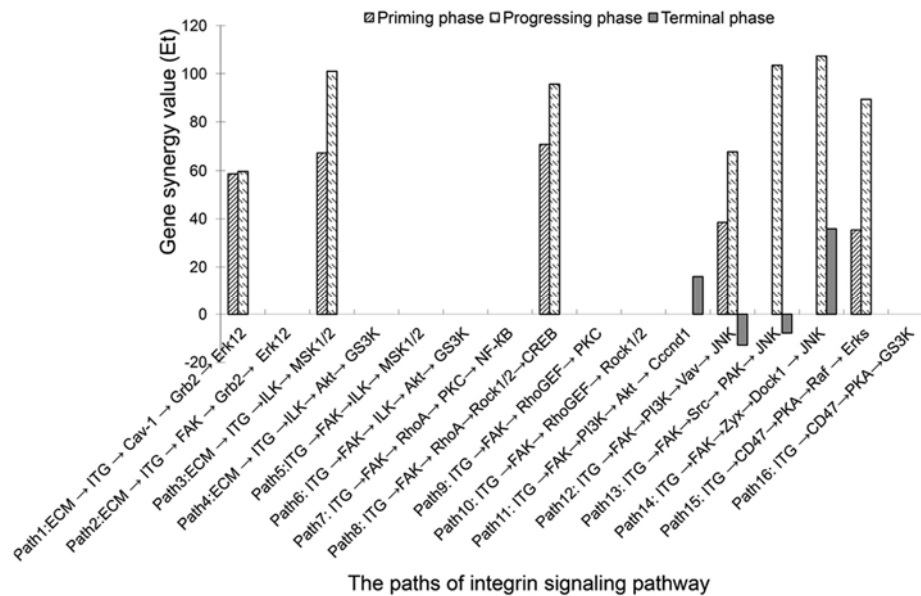


Fig. 4. Proliferation of hepatocyte cells regulated by integrin signaling pathway.

DISCUSSION

Liver regeneration involves a sequence of orchestrated events including activation/expression of a series of cytokines and growth factors, accompanied by cell proliferation, cell differentiation and dedifferentiation, and tissue structure reconstruction, etc. [28]. Hepatocytes are the chief functional cells of the liver, making up roughly 80% of the mass of the liver, and play an important role in liver regeneration [29]. Therefore, research into the underlying mechanism of hepatocyte proliferation would provide new insights into liver regeneration. Firstly, we collected high-purity (over 96%) hepatocytes isolated from six PH rats per group together using two-step perfusion and Percoll density gradient centrifugation. Meanwhile, we established a mathematical model (E_i) to measure gene synergy of every path in the integrin signaling pathway.

At the priming phase (0.5-6 h after PH) of rat liver regeneration, expression of numerous genes including cell proliferation- and apoptosis-related genes promoted the conversion of hepatocytes from G0 phase into G1 phase, completing the launching of liver regeneration [30]. Analysis of the integrin signaling pathway related gene expression profile and the gene synergy value of paths showed that some genes and paths were significantly different compared with control groups. The gene synergistic effect (E_i) of path 1 was higher than the control at the priming phase, meaning that this path possibly takes part in hepatocyte G0/G1 transition of the cell cycle. Meanwhile, Cav-1 in path 1 is down-regulated at 2 h and 6 h after PH. The genes of Elk1, Elk2, and Ccnd1 are up-regulated. A recent study showed that integrins bind to the membrane protein

caveolin and activate ERK1/2 via the Grb2/Sos→Ras → Raf →MEKs cascade reaction (path 1), and finally regulate cyclin D1 gene expression [31]. Williams *et al.* reported that decrease of Cav1 expression resulted in activation of the MEK/ERK signaling pathway, and thus accelerated the cell cycle process and promoted cell proliferation [32]. Additionally, FAK is primarily recruited to sites of integrins via interaction between its C-terminal domain and integrin-associated proteins. The cytoplasmic tail of β -integrins (β 1, β 3 and β 5) facilitates FAK activation, then activates the Rho GAPase → PIP2→PKC (path 8) or PI3K →Vav→PAK→MAPK (path 12) signaling pathway, and then regulates the cell cycle process and cell proliferation [33]. A previous study showed that cells injected with GST-C-term of FAK had decreased DNA synthesis compared with the control. These findings indicated that FAK functions in the regulation of cell migration and cell proliferation [34]. Increased FAK expression facilitates transcription factor NF-KB activation, thereby promoting cell proliferation [35]. In addition, activated RhoGAPase can also control the cell cycle process through regulating expression of cyclin D in G1 phase [33, 34]. In our study, we have found that gene synergistic effects (E_t) of both path 8 and path 12 were also higher than the control, meaning that these paths possibly regulated hepatocyte proliferation in the primary phase of liver regeneration. Moreover, many genes involved in these paths (i.e. ptk2, ptk2b encoding FAK, Arhgap11a, Arhgap4, Arhgap20, Arhgap9, Arhgap10, Arhgap22, Arhgap27 encoding RhoGAPase) were up-regulated in the primary phase of liver regeneration, indicating that these genes play an important role in hepatocyte proliferation. In addition, CD47 associated with integrin heterodimer to form a protein complex, which stimulates Gs-mediated up-regulation of the cAMP cascade through AC; cAMP may also activate PKA, which may further activate the Rap pathway, finally activating ERK 1/2 (path 15). G proteins regulate adenylate cyclase activity by adjusting the level of intracellular cAMP, and play an important role in regulation of the cell cycle and cell proliferation [14]. Studies have proved that G-protein-coupled receptor signaling and small GTPase signal transduction can promote the growth of remnant liver at the primary phase [36]. In our study, we found that gene synergistic effects (E_t) of path 15 were also higher than the control; meanwhile, the genes (Gna15, Gnai1, Gnat1, Gnat2, Gnaz; Gnb3, Gnb5; and Gng3, Gng8, Gng11) encoding $G\alpha$, $G\beta$ and $G\gamma$ subunits, respectively, were up-regulated in hepatocytes at the primary phase, and the gene which encodes adenyl cyclase was also up-regulated, indicating that these genes play an important role in hepatocyte proliferation. To summarize, four paths (paths 1, 8, 12 and 15) mediated by integrin may promote hepatocyte proliferation at the primary phase, based on the analysis of gene expression and gene synergy value. Previous studies have demonstrated that the proliferation of hepatocytes occurred mainly at the progressing phase [37]. In the present study, analysis of the gene synergistic effect (E_t) of the four paths mentioned above showed that their E_t s were also higher than the control, indicating that these paths were promoting hepatocyte proliferation not only at the primary phase, but also at the

progressing phase. In addition, the E_t of path 3 was higher than the control, too. Integrins combined with ILK, and then regulated expression of cyclin D1 by activation of the ERK→MSK1/2→CREB pathway (path 3) [7]. Transfer of ILK-overexpressing hepatocytes resulted in continued high levels of expression of cyclin D1 and cyclin A proteins, indicating that, when overexpressed, ILK induces signaling pathways resulting in the stimulation of G1/S cyclin-Cdk activities, finally leading to hepatocytes entering S phase [8, 10]. This study showed the genes *Ilk*, *Rps6ka4*, *Rps6ka5*, *Creb1*, *Creb3*, *Creb4*, and *Ccnd1* involving path 3 were up-regulated at 24-30 h, 24-72 h, 12-72 h, 72 h, 72 h, 12 h and 30 h, and 30 h and 72 h, respectively, indicating that these genes probably were related to an increase of hepatocyte proliferation activities at the progressing phase. P130Cas interacts with FAK through its SH3 domain and is then phosphorylated by Src, leading to recruitment of Crk, and enters the Rac→PAK→MAPK→JNK pathway through Dock1 (path 13) [9]. α -actin links tails of integrins and binds to zyxin, then also enter the Rac→PAK→MAPK→JNK pathway (path 14) [13]. Previous studies indicated that Src protein expression and activity increased significantly in cells which had strong proliferation activity [38]. When Src gene expression was inhibited, the cells during division stopped at G2 phase, and stopped at conversion to the M phase [39]. We found that the gene synergistic effects (E_t) of paths 13 and 14 were higher than the control, and what is more, the genes involved in these paths (i.e. Src, Actn1, Actn2, Ccnd1, Nfkb1, Nfkb2) were up-regulated at the progressing phase, meaning that these paths and genes possibly regulated hepatocyte proliferation at the progressing phase. Finally, seven paths (1, 3, 8, and paths 12-15) mediated by integrin may promote hepatocyte proliferation at the progressing phase of liver regeneration.

In the terminal phase (120-168 h after PH), cell differentiation, adhesion and tissue structure reconstruction were the main physical activity. Cell proliferation and apoptosis were weak at this phase [40]. The report showed that hepatocyte proliferation-related genes were down-regulated and cell proliferation was inhibited in the terminal phase [41]. The genes *Fn1* and *Fbn1* encoding fibronectin, the genes *Col1a2*, *Col3a1*, *Col5a1*, *Col5a2*, *Col8a1*, *Col9a3*, *Col10a1*, *Col11a1*, *Col13a1*, *Col17a*, and *Col24a1* encoding collagen, and the genes *Lama5* and *Lamb1* encoding laminin, which participate in cell adhesion and tissue structure reconstruction, were up-regulated in hepatocytes at the terminal phase. Our finding was consistent with the results above. Moreover, the gene synergistic effects (E_t) of paths 11 and 14 were higher than the control; conversely the E_t of paths 12 and 13 were lower than the control, indicating that hepatocyte proliferation was reduced at the terminal phase of liver regeneration.

In conclusion, 132 genes and 8 paths of the integrin signaling pathway participated in regulation of hepatocyte proliferation during rat liver regeneration. The above results will be confirmed using gene knockout and addition or RNA interference in vivo and in vitro in future.

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