Short communication

EXPRESSION PROFILES UNCOVER THE RELATIONSHIP BETWEEN ERYTHROPOIETIN AND CELL PROLIFERATION IN RAT HEPATOCYTES AFTER A PARTIAL HEPATECTOMY

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Abstract: Erythropoietin (EPO) has a beneficial effect on hepatic cell proliferation during liver regeneration. However, the underlying mechanism has not yet been elucidated. To uncover the proliferation response of EPO in rat liver regeneration after partial hepatectomy (PH) at the cellular level, hepatocytes (HCs) were isolated using Percoll density gradient centrifugation. The genes of the EPO-mediated signaling pathway and the target genes of the transcription factor (TF) in the pathway were identified in a pathway and TF database search. Their expression profiles were then detected using Rat Genome 230 2.0 Microarray. The results indicated that the EPO-mediated signaling pathway is involved in 19 paths and that 124 genes participate, of which 32 showed significant changes and could be identified as liver regeneration-related genes. In addition, 443 targets regulated by the TFs of the pathway and 60 genes associated with cell proliferation were contained in the array. Subsequently, the synergetic effect of

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these genes in liver regeneration was analyzed using the *E(t)* mathematical model based on their expression profiles. The results demonstrated that the *E(t)* values of paths 3, 8, 12 and 14–17 were significantly strengthened in the progressing phase of liver regeneration through the RAS/MEK/ERK or PI3K/AκT pathways. The synergetic effect of the target genes, in parallel with target-related cell proliferation, was also enhanced 12–72 h after PH, suggesting a potential positive effect of EPO on HC proliferation during rat liver regeneration. These data imply that the EPO receptor may allow EPO to promote HC proliferation through paths 3, 8, 12 and 14–17, mediating the RAS/MEK/ERK and PI3K/AκT pathways in rat liver regeneration after PH.

Keywords: Rat liver regeneration, Erythropoietin, Rat Genome 230 2.0 Microarray, Gene expression profiles, Gene synergetic effect

INTRODUCTION

The liver has the unique capacity to regain its original and optimal mass after damage due to various events, such as partial hepatectomy (PH), viral infection, and intoxication [1, 2]. Liver regeneration can be regulated by various signaling pathways that are woven into a complex network through crosstalk among them, governing a variety of biological processes, such as cell proliferation, differentiation, apoptosis, and immunity [3, 4].
Currently, the treatment option for many primary and secondary liver tumors is regular or irregular resection. Despite the regenerative capabilities of the liver, excessive resections may lead to liver insufficiency and hepatic failure within a few days of surgery, especially when performed on patients with a diseased liver, which dramatically increases the postoperative mortality [5–7]. It is very important to identify substances that maximize HC proliferation and liver regeneration.
Erythropoietin (EPO) is a glycoprotein hormone stimulator of erythropoiesis. It is produced in the fetal liver and subsequently in the adult kidney, and is an essential cell factor in the regulation of the transformation of erythroid progenitors to mature erythrocytes, where it binds to its special cell surface receptors in the bone marrow [8]. Previous studies have shown that EPO and its receptor are expressed on many different cells, especially hepatic, endothelial, central nervous system, and uterine cells [9]. Recently, EPO has also been recognized as a pleiotropic cytokine associated with cell proliferation, differentiation, anti-apoptosis, and tissue protection [10–13].
The beneficial effect of EPO treatment on liver resection and living donor liver transplantation in rats has been demonstrated [14–20]. Indeed, increased synthesis of EPO has been found in liver regeneration, and enhanced EPO serum levels correlate with the peak of liver regeneration after PH [21]. However, Klemm et al. [22] reported a conflicting finding to that of Vassiliou et al. [20], suggesting that multiple doses of EPO after PH, even at a relatively low dosage (500 IU/kg), increased hepatocellular apoptosis and impaired liver regeneration in rats.

There are still no reports on the precise mechanism of action of EPO and the EPO receptor on liver regeneration at the cellular level. In this study, we aimed to clarify this effect by determining the expression profiles of genes related to the EPO signaling pathway in regenerating HCs using the Rat Genome 230 2.0 Microarray. We also assessed their synergetic effect using the mathematical model *E(t)*.

MATERIALS AND METHODS

Preparation of the rat partial hepatectomy (PH) model
Adult male Sprague-Dawley rats (6 weeks old), weighing 230 ± 20 g, were obtained from the animal center of Henan Normal University. The rats were kept at 21 ± 2ºC, with a relative humidity of 60 ± 10%, 12 h illumination per day (8:00-20:00), and free access to chow and water. A total of 114 rats were randomly divided into 19 groups with 6 rats per group: 9 groups underwent a two-thirds hepatectomy (PH groups), 9 groups underwent a sham operation (SO groups), and one group had no operation (control group). The operation on the rats in the PH groups followed the method of Higgins and Anderson [23] to remove the left and median lateral liver lobes. The rats in the SO groups underwent the same operation without removal of the liver lobes. Rats were killed 0, 2, 6, 12, 24, 30, 36, 72, 120 and 168 h after the operations. The study complied with the Animal Protection Law of China.

Isolation and identification of hepatocytes
Rats were anesthetized with ether and sterilized with 75% ethanol (analytically pure, AR). The abdominal cavity was opened and the vena cava below and above the liver were ligated following the catheterization of the liver portal vein. HCs were isolated using conventional two-step perfusion and Percoll density gradient centrifugation according to the method previously described by Xu et al. [24].
HCs were identified with their marker proteins ALB and G6P using immunocytochemistry [24]. Briefly, purified hepatocytes were taken and fixed with 10% formaldehyde for 30 min, and then smeared onto polylysine-coated glass slides. After the cell suspension had dried, a peroxidase block step was performed. The sections were incubated separately with a 1:200 dilution (V/V) of ALB and G6PC antibodies (GeneTex Inc.) at 4ºC overnight, and then with a 1:5,000 (V/V) diluted biotin-labeled secondary antibody at 37ºC for 1 h. The system was hybridized with streptavidin-biotin complex (SABC) at 37ºC for 30 min. The viability of the HCs was measured using trypan blue staining. The purity and viability of the HCs used in this study were both over 95%.

Rat Genome 230 2.0 Microarray detection and data analysis
Total RNA was extracted and purified using the previously described protocol [25]. The cDNA first chain was synthesized using the SuperScript II RT reverse transcription system (Life Technologies), and the second chain was synthesized according to the guidelines for the Affymetrix cDNA kit. Biotin-labeled cRNA was prepared using GeneChip IVT kit according to the manufacturer's

instructions. cRNA fragments of 35-200 bp were prepared using fragmentation reagent treatment. The Rat Genome 230 2.0 Microarray was hybridized with the cRNA fragments, which had been pretreated. They were then stained, washed automatically using a GeneChip Fluidics Station 450 and scanned using a GeneChip Scanner 3000 (Affymetrix Inc.). The spots were converted into signal values using Affymetrix GCOS 2.0 software [26]. The signal values were normalized according to the manufacturer's instructions.

The p-values were determined based on the probe signal. When the p-value of a gene is < 0.05, the gene is defined as present (P), while $p < 0.065$ is marginal (M), and $p > 0.065$ is absent (A). To minimize the experimental operation and microarray test differences, each sample was repeated three times, and the average value was used for statistical analysis [27–28].

Quantitative real-time PCR
mRNA was prepared from the purified hepatocytes as described above. Primers for *β-actin, Coq10a, Tat, Sirpa, Trfr2, Ywhaq, mapk3, Ccnd1, Rap1a* and *Xbp1* were designed using Primer Express 5.0 software. Their first chain of cDNA was synthesized using the SuperScript II RT reverse transcription system. PCR was performed under the following conditions with SYBR Green I: 2 min at 95ºC, followed with 40 cycles for 15 s at 95ºC, 15 s at 60ºC, and 30 s at 72ºC. Each sample was performed in triplicate. The PCR products were analyzed using a melting curve to confirm the specificity of amplification. *β-actin* was used as an internal control [29].

Confirmation of the significant expression genes and liver regeneration-related genes
In this paper, the genes with ratio values of PH/SO ≥ 3 or ≤ 0.33 were considered significantly expressed [30]. In addition, genes were considered to be liver regeneration-related genes if the samples were in liver regeneration at least at one of the PH time points and that had an *F*-test difference of $0.01 \leq P < 0.05$ or $P \leq 0.01$ between PH and SO.

Identification of EPO signaling pathway-related genes and target genes of TF
The term "EPO signaling pathway" was entered in the NCBI (www.ncbi.nlm.nih.gov) and RGD (www.rgd.mcw.edu) databases to find the genes to the related EPO signaling pathway in rat, mouse and human [31-32]. To search for the target genes of TF in the pathway, TF was input into TRED (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=searchTF GeneForm) and Lymph TF databases (http://www.iupui.edu/~tfinterx/activity.php). These targets were then matched against Gene Ontology (http://www.ncbi.nlm.nih.gov/gene) to select the targets only associated with cell proliferation. The literature review was performed to confirm the function of the genes identified using the NCBI database.

Gene synergy analysis

Xu et al. [24] established the mathematical model *E(t)* to describe how physiological activities are governed by gene synergy according to the expression levels of genes detected by the Rat Genome 230 2.0 Microarray. Based on the methods of multivariate statistics and time series analysis, and following the knowledge that physical activity is regulated by the synergy of one lot of genes to another, the spectrum function *E(t)* was described as follows.

$$E(t) = \frac{\sum_{i=1}^{n-1}\sum_{k=i+1}^{n}[(X_i(t)+X_k(t))\cdot|r_{ik}|]}{C_n^2} = \frac{\sum_{i=1}^{n-1}\sum_{k=i+1}^{n}[(X_i(t)+X_k(t))\cdot|r_{ik}|]}{\frac{n(n-1)}{2}}$$

In the formula, the gene correlation coefficient (r_{ik}) is defined by the Pearson correlation coefficient:

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

Here, n is the number of all genes participating in a physiological activity at the time point *t*. The spectral function *E(t)* describes the effectiveness of the gene synergy dominating a physiological activity at one point. By comparing with the control, the strength of the physiological activities at that time can be predicted. At *t*, assuming that the reference value is *E(0)*, the corresponding physical activity is more strengthened than in control when $E(t) - E(0) \geq E(0)$, more weakened when *E(t) – E(0) < 0*, and similar with the control when *E(0) < E(t) < 2E(0)*.

RESULTS

Isolation and identification of HCs

In this study, we demonstrated the purity and activity of HCs isolated from liver samples. The results showed that the positive regions of ALB and G6PC were distributed throughout the cytoplasm of HCs. The activity of HCs was over 95%. At the same time, the percentages of the ALB- and G6PC-positive cells among the isolated cells were greater than 95% at any recovery time after PH (Fig. 1).
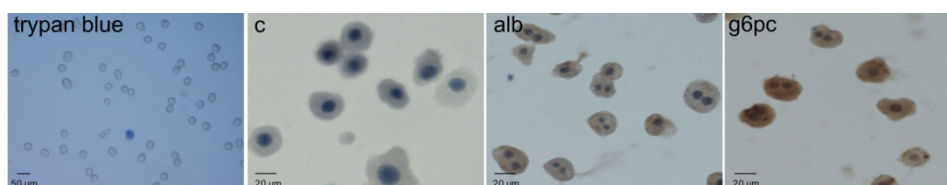

Fig. 1. ALB, G6P immunostaining and Trypan blue exclusion test of HCs isolated from rat liver. "C" was the negative control that was incubated with PBS instead of anti-ALB or anti-G6PC antibody. HCs were stained using 0.4% trypan blue.

Confirmation of the Rat Genome 230 2.0 Microarray results
In order to confirm the validity of the results tested with the Rat Genome 230 2.0 Microarray, 30 randomly selected genes were subjected to quantitative RT-PCR (qRT-PCR). The results showed that the expression trends detected by the two methods were generally consistent, suggesting that the microarray results were reliable. The results for *Coq10a, Tat, Sirpa, Trfr2, Ywhaq, mapk3, Ccnd1, Rap1a* and *Xbp1* are provided (Fig. 2). The RT-PCR results for the other genes were previously published [24, 31, 35–41].
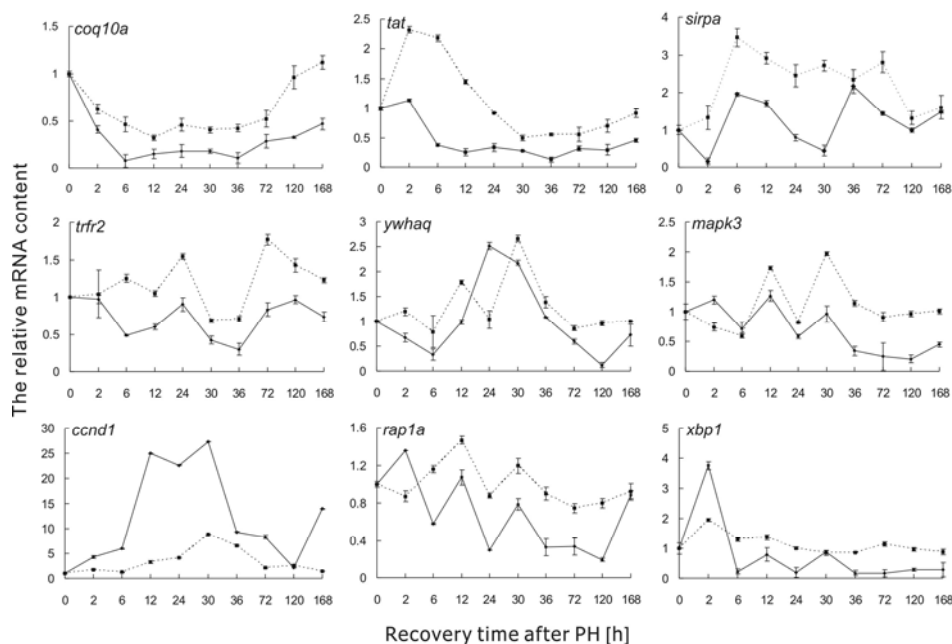

Fig. 2. A comparison of the relative mRNA levels in regenerating HCs detected using the Rat Genome 230 2.0 Microarray (dotted lines) and qRT-PCR (solid lines) during liver regeneration.

The expression profiles of genes related to the EPO-mediated signaling pathway
According to the data from NCBI, RGD, etc. and biological pathway maps in GENMAPP, KEGG, BIOCARTA, etc., there are 19 branches with 124 genes involved in the EPO-mediated signaling pathway. Of these, 112 genes are present on the Rat Genome 230 2.0 Microarray. The array analysis showed that 32 genes were significantly changed in regenerating HCs and identified as liver regeneration-related genes by comparing the gene differential expression in PH and SO group. In these genes, 25 genes were upregulated, and 7 genes were downregulated during rat liver regeneration (Table 1 and Fig. 3).

Table 1. Expression changes of genes related to the EPO signaling pathway in HCs during liver regeneration.

Protein/Gene	Recovery time [h] after partial hepatectomy (PH)								
	2	6	12	24	30	36	72	120	168
Pathway 1–19: EPO→EpoR									
Epo/*Epo*	0.86	0.86	1.09	0.79	3.76	2.66	4.99	0.64	0.92
Pathway 1: EPO→EpoR→JAK2→STAT5→BclXL									
BclXL/*Bcl2l1*	3.46	1.38	1.91	1.13	1.46	1.19	1.23	1.30	0.96
Pathway 3–8, 11, 18, 19: Ras→Raf1→MEKs→ERK→CREB/CcnD1/c-Jun/c-Fos									
Gα1/*Gnai1*	2.10	5.32	8.20	3.72	5.76	1.55	3.09	3.44	3.57
Gβ/*Gnb3*	1.39	2.40	1.71	2.72	3.66	2.88	3.94	2.64	2.67
/*Gnb5*	2.86	4.47	10.17	3.31	4.58	3.40	2.22	4.85	3.18
Gγ/*Gng11*	1.65	1.08	3.15	3.32	3.14	3.44	13.57	7.25	3.12
/*Gng2*	1.27	0.89	1.62	0.94	3.93	1.44	2.87	3.03	1.50
/*Gng3*	4.92	4.66	3.95	3.98	3.88	4.48	7.95	2.31	4.61
/*Gng8*	3.21	1.96	1.89	3.49	2.38	2.65	2.83	2.22	2.44
/*Gngt2*	0.83	0.98	1.16	1.40	2.17	1.47	3.81	2.32	2.66
MEKs/*Map2k6*	0.13	0.30	0.14	0.29	0.57	0.38	1.05	0.73	0.40
Ras/*Mras*	0.32	0.88	0.76	0.71	0.66	0.50	0.80	0.66	0.63
CREB/*Creb1*	1.76	6.02	1.94	1.55	2.42	4.55	3.29	1.15	2.47
/*Creb3l4*	2.03	2.04	1.42	1.06	2.79	1.56	5.58	1.11	1.36
CcnD1/*Ccnd1*	1.75	1.24	3.28	4.14	8.92	6.57	2.11	2.61	1.40
c-Fos/*Fos*	5.02	1.12	4.73	14.96	2.31	1.04	5.21	2.76	4.23
c-Jun/*Jun*	3.29	2.68	1.97	3.97	1.54	1.91	2.14	1.75	2.18
JNK1/*Mapk8*	2.77	1.28	2.34	1.66	2.04	1.32	3.52	1.56	2.27
PPtase/*Ppp1r1b*	0.89	1.16	1.04	2.71	8.91	0.96	5.32	0.90	1.00
/*Ppp1r3a*	1.92	3.53	1.31	1.32	4.69	1.10	2.73	1.23	3.74
/*Ppp1r3b*	0.20	0.24	0.23	0.15	0.26	0.21	0.18	0.47	1.20
/*Ppp1r3c*	0.12	0.61	0.94	0.59	0.40	0.37	0.60	0.74	1.29
/*Ppp1r3d*	2.17	1.97	2.44	2.78	2.39	2.49	5.57	3.00	2.99
/*Ppp1r12b*	1.77	1.74	2.83	4.10	1.18	2.32	3.21	2.32	2.73
/*Ppp1r14a*	0.88	0.29	0.77	0.97	1.27	1.08	0.75	1.21	0.97
Pathway 15–17: PI3K→PIP3→Aκt/PKB→GATA1									
C3G/*Rapgef1*	5.13	2.23	2.01	2.42	3.24	2.61	2.40	1.33	2.78
Src/*Src*	1.33	1.77	2.40	2.06	3.11	1.89	4.78	2.04	1.48
PI3K/*Pik3r1*	1.34	0.92	0.77	0.59	0.85	0.69	0.23	0.39	0.54
/*Pik3c2g*	0.80	0.77	0.26	0.19	0.35	0.33	0.38	0.76	0.92
GATA1/*Gata1*	2.94	0.55	3.59	3.32	3.16	3.90	4.00	1.19	1.89
/*Pik3r3*	2.31	1.17	3.21	1.38	2.33	2.61	2.61	1.18	2.81
TIMP1/*Timp1*	1.88	4.32	4.90	14.94	6.13	8.45	33.89	2.66	1.33

* Dark gray fields represent the expression levels of upregulated genes, light gray fields are for the downregulated genes, and the other values are insignificantly changed.
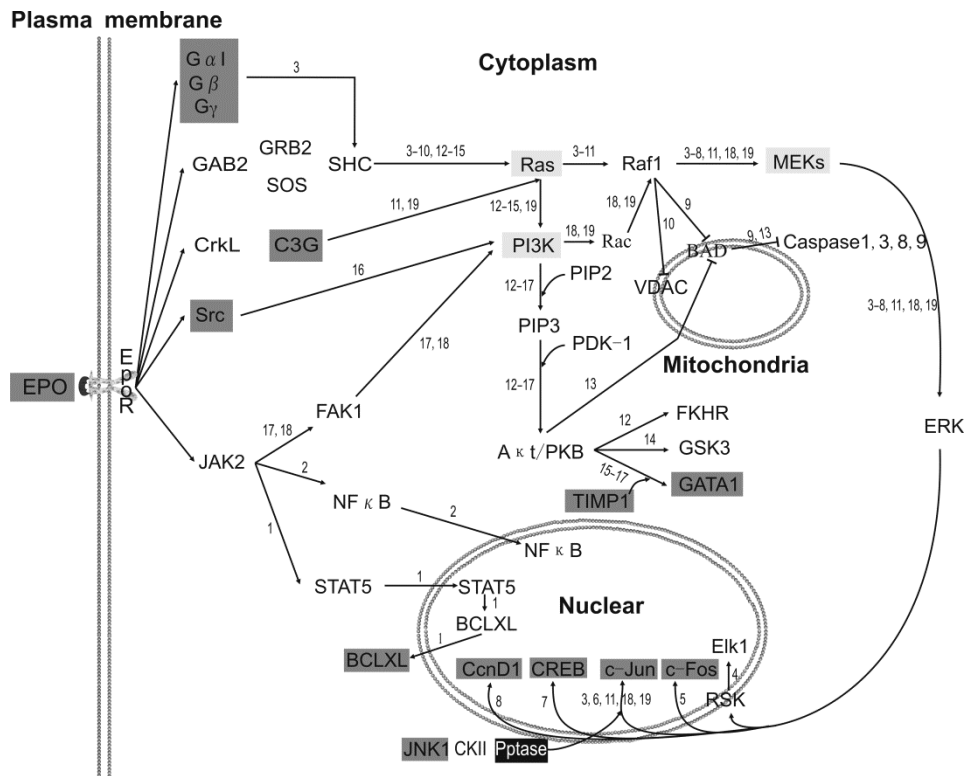
Fig. 3. The pathways of the EPO signaling pathway and their inter-relationships. Gene symbols with dark gray backgrounds represent upregulated genes, while those in light gray are downregulated. Black backgrounds represent the up- and downregulated genes. Figures beside the arows mean the ordinal numbers of the pathway.

EPO-mediated signaling transduction activities in HCs during liver regeneration

To clarify whether the expression pattern of the genes can affect the true biological activities in HCs during rat liver regeneration, the synergetic effects of genes participating in EPO-induced signaling pathway were analyzed using the mathematical model $E(t)$. The results indicated that in the priming and terminal phases, the $E(t)$ values of 19 branches of EPO-induced signaling pathway were similar to those for the control, suggesting that the biological activities of these branches were inactive during rat liver regeneration after PH. However, the $E(t)$ values of paths 3, 8, 12, and 14–17 were enhanced significantly compared to the control in the progressing phase (belonging to the proliferating phase) during rat liver regeneration. Of them, the $E(t)$ value of path 3 was significantly higher than the control at 30 and 72 h, that of path 8 at 30 h, that of path 12 and 14 at 72 h, and those of paths 15–17 at 12–72 h after PH. That implied that the 7 paths were related to HC proliferation at these phases (Table 2).

Table 2. The physiological activities regulated by the EPO-mediated signaling pathway of hepatocytes in liver regeneration.

Pathway	Recovery time [h] after partial hepatectomy (PH)									
	0	2	6	12	24	30	36	72	120	168
Path 1	0.37	0.64	0.46	0.47	0.35	0.58	0.46	0.63	0.38	0.39
Path 2	0.49	0.86	0.56	0.63	0.61	0.71	0.66	0.95	0.70	0.58
Path 3	0.25	0.31	0.32	0.45	0.32	0.55	0.39	0.60	0.43	0.37
Path 4	0.17	0.17	0.13	0.18	0.14	0.25	0.18	0.22	0.16	0.16
Path 5	0.17	0.16	0.14	0.17	0.09	0.27	0.20	0.20	0.15	0.15
Path 6	0.16	0.15	0.11	0.17	0.10	0.26	0.17	0.19	0.14	0.14
Path 7	0.15	0.17	0.15	0.19	0.14	0.29	0.20	0.27	0.14	0.15
Path 8	0.22	0.24	0.19	0.29	0.24	0.49	0.35	0.30	0.23	0.22
Path 9	0.31	0.33	0.33	0.40	0.32	0.51	0.37	0.46	0.32	0.35
Path 10	0.26	0.30	0.31	0.37	0.26	0.45	0.36	0.33	0.25	0.26
Path 11	0.19	0.22	0.19	0.25	0.17	0.33	0.23	0.23	0.17	0.19
Path 12	0.10	0.11	0.13	0.16	0.12	0.17	0.15	0.23	0.11	0.15
Path 13	0.19	0.14	0.14	0.19	0.15	0.22	0.18	0.24	0.15	0.18
Path 14	0.12	0.12	0.15	0.18	0.15	0.20	0.17	0.25	0.14	0.16
Path 15	0.10	0.13	0.15	0.22	0.30	0.24	0.26	0.61	0.13	0.15
Path 16	0.18	0.23	0.32	0.46	0.72	0.54	0.58	1.54	0.28	0.29
Path 17	0.12	0.17	0.20	0.30	0.46	0.33	0.39	0.98	0.16	0.19
Path 18	0.08	0.08	0.06	0.09	0.07	0.13	0.11	0.15	0.07	0.09
Path 19	0.07	0.07	0.07	0.10	0.07	0.13	0.10	0.13	0.06	0.08

* Dark gray fields mean the physiological activities of the experimental group were enhanced compared to the control. White fields mean there was no significant difference between the experimental group and the control.

Analysis of the correlation between the EPO signaling pathway and HC proliferation

In this study, the expression changes of TFs related to the EPO-mediated signaling pathway, CREB (coded by *Creb1* and *Creb3l4*), c-JUN (coded by *Jun*), c-FOS (coded by *Fos*), and TIMP1 (coded by *Timp1*), were significantly upregulated at the transcriptional level during rat liver regeneration after PH. Based on the information from TRED and the Lymph TF DB, a total of 507 targets can be regulated by these TFs (CREB, c-JUN, c-FOS, and TIMP1). The Rat Genome 230 2.0 Microarray contained 443 of these genes (Suppl. Table 1 in Supplementary material at http://dx.doi.org/10.2478/s11658-014-0198-0), 60 of which were related to cell proliferation (Suppl. Table 2).

To analyze the correlation between the EPO-mediated signaling pathway and HC proliferation in rat liver regeneration, the spectrum function $E(t)$ was used to analyze the gene synergetic effect of total target genes and cell proliferation-related target genes regulated by the TFs of this pathway. The results demonstrated that the $E(t)$ values of the target genes were significantly higher than the control during the progressing phase (6–72 h after PH). Similarly, the

E(t) values of the target genes related to cell proliferation were also strengthened compared to the control at 12–72 h after PH. Their *E(t)* values were elevated at 24–30 h and reached a peak at 72 h after PH. The results indicated that the synergetic effects of the total target genes and targets related to cell proliferation of the EPO-induced signaling pathway have a similar pattern in general during liver regeneration after PH (Fig. 4).
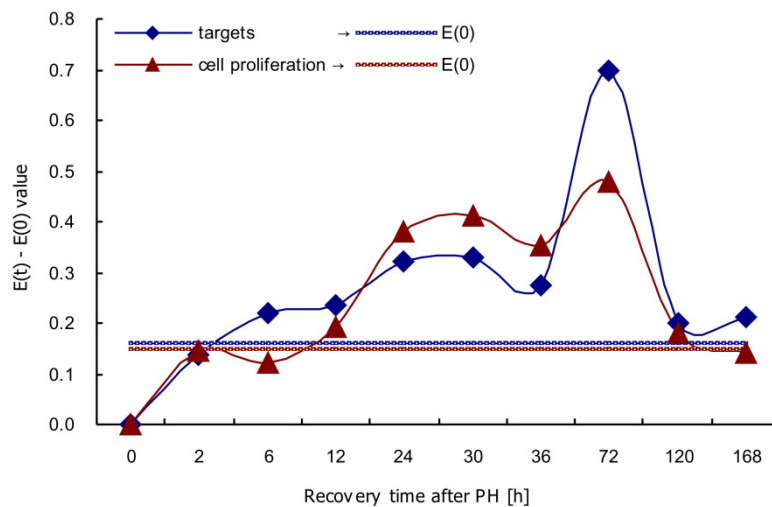

Fig. 4. The correlation between the EPO-mediated signaling pathway and HC proliferation in regenerating HCs after PH.

DISCUSSION

Liver cells, which are normally in a quiescent state, have a unique capability to enter the cell cycle in response to cell loss caused by surgical hepatectomy, chemical stimuli, or ischemic damage [42]. Liver restoration, which is critical for liver mass recovery, is a stringently regulated process that can mainly be segmented into the priming phase (2–6 h after PH), progressing phase (6–72 h after PH), and terminal phase (72–168 h after PH) based on cellular physiological activities [43–44]. HCs are the major component of the liver parenchyma cells, and are closely related to the increase in liver cell number in liver regeneration.

In addition, liver regeneration is an orchestrated process that is regulated by many signaling pathways and involved in various cytokines. Among these cytokines, EPO is considered to play an important role in rat liver regeneration. EPO has been shown to have a powerful cell proliferation, cell differentiation, anti-apoptotic, and tissue protective effect in animal models of a wide variety of tissues [11–13], including the liver [15, 19]. During development, the physiological source of EPO switches from the fetal liver to the kidney in adults. However, HCs are the primary cell type responsible for *Epo* expression in the liver and the liver maintains the capacity to express EPO [45]. Rankin et al. found

that Hif-2 regulated hepatic EPO expression in the early postnatal, *Vhlh*-deficient, and anemic adult liver [46]. Most recently, a growing body of evidence suggests that the beneficial effect of EPO treatment on liver resection and living donor liver transplantation in rats [14, 17–18]. Moreover, microarray analysis is a powerful approach for monitoring molecular events during liver regeneration after PH, which has been demonstrated in previous studies [24, 25, 33-39, 45–48].

To better understand the effect of the EPO-mediated signaling pathway in rat liver regeneration at the cellular level, the gene expression profiles of target genes of transcription factor (TF) involved in EPO-mediated signaling pathway were detected using Rat Genome 230 2.0 Microarray. In this study, the biological activities of target genes and targets related to cell proliferation were enhanced in the progressing phases (belonging to the cell proliferation phase) during rat liver regeneration, suggesting that there is a positive correlation between the signaling transduction activity induced by EPO and HC proliferation.

As previously stated, the C intracellular cytoplasmic domain combination of the EPO receptor can combine with G protein following EPO simulation. Subsequently, Gi is released from the receptor with its concomitant dissociation into the Gα, Gβ, and Gγ subunit followed by their corresponding activations. Then, the MAPK pathway is activated by Gα subunit or Gβ–Gγ subunit mediating tryosine phosphorylation of SHC, and proceeds through the RAF/MEK/ERK signaling cascade leading to the activation of c-JUN, c-Fos, CREB, and CCND1, which enhances the transcription of growth-related proteins and promotes cell proliferation [49-50].

To clarify how EPO impact the regeneration process at the cellular level, we performed high-throughput analysis to detect the expression profiles of EPO-mediated signaling pathway genes in rat HCs following PH. The results showed that the genes *Gnai1* (coding the Gα subunit), *Gnb3* and *Gnb5* (coding the Gβ subunit), and *Gng11*, *Gng2*, *Gng3*, *Gng8* and *Gngt2* (coding the Gγ subunit) were significantly upregulated in regenerating HCs after rat PH. Moreover, the expression changes in the RAF/MEK/ERK downstream genes, *Jun* (coding c-JUN), *Fos* (coding c-FOS), *Ccnd1* (coding CCND1), and *Creb1* and *Creb3l4* (coding CREB), were also upregulated, which was consistent with the above results. In path 3 (EPO→EPOR→Gi→GAB2→RAS→RAF1 →MEKs→ERK→c-JUN), *Jun* was upregulated at 2 h and 30 h after PH, while the synergetic effect of genes related to the path was enhanced at 30 h and 72 h during rat liver regeneration, suggesting that HC proliferation was active in the branch at this phase. In path 8 (EPO→EPOR→Gi→GAB2→RAS→RAF1→ MEKs→ERK→CCND1), the expression of *Ccnd1* was upregulated from 12 to 36 h after PH, reaching a peak at 30 h. The biological activity of the path was strengthened dramatically 30 h after PH. These results show that EPO may be involved in HC proliferation through the RAF/MEK/ERK pathway during rat liver regeneration after PH.

Zhao et al. demonstrated [51] that a low concentration of EPO can efficiently mediate a moderate rate of cell proliferation following the activation of PI3K via tyrosine kinase SRC. PI3K then phosphorylates membrane-bound PIP2 to generate PIP3 acting as a negative watchdog of this process, which triggers the phosphorylation of AκT with the help of PDK-1 protecting cells from apoptosis. Phosphorylation of the GATA1 signaling molecule by AκT is required for the transcriptional activation of the TIMP1, which plays an important role in promoting cell survival. The microarray data showed that, in path 16, *Rapgef1* (coding *C3G*), *Src* (coding *SRC*), *Pik3r* (coding *PI3K*) and *Gata1* (coding *GATA1*) rose in mRNA abundance in HCs at the progressing phase of rat liver regeneration. Meanwhile, the signaling transduction activity was also enhanced at this phase (12–72 h after PH).

In summary, we performed a large-scale analysis of gene expression profiles, and obtained some detailed data on rat liver regeneration at cellular level. These data indicated the potential positive relevance between EPO and HC proliferation through the RAS/MEK/ERK and PI3K/AκT pathways at the transcriptional level in rat liver regeneration after PH. These results are mainly drawn from gene expression changes detected using Rat Genome 230 2.0 Microarray.

Obviously, microarray data can only lead to the discovery of the changes in gene transcription, but not reflect the protein synthesis process, and they are even less able to accurately reflect EPO-mediated signaling pathway activities and the final biological effect. Therefore, we further need to prove the above results through experimental investigation, such as proteomics analysis, gene addition, RNA interference, and protein interaction.

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