DOI 10.1007/s10517-020-05000-7

MICROBIOLOGY AND IMMUNOLOGY

Liver Macrophages Stimulate the Expression of Hepatocyte Nuclear Factor-6 and Promote Hepatocyte Proliferation at the Early Stage of Liver Regeneration

Y. Ch. Peng[1], T. H. Lv[2], Zh. K. Du[1], X. N. Cun[3], and K. M. Yang[1]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 170, No. 7, pp. 50-56, July, 2020
Original article submitted January 17, 2020

Hepatocyte nuclear factor (HNF-6) is a liver-specific protein and a key component in the differentiation process during the development of mature liver. The immunohistochemical staining and RT-PCR techniques were employed to examine the expression of HNF-6 and proliferation of Ki-67$^+$ cells during the early regeneration of the liver on postsurgery in 3, 6, 12, and 24 h in original model of partial hepatectomy in rats. The earliest proliferating (Ki-67$^+$) cells were observed in 3 h after surgery in liver sinusoids (liver macrophages) and then in liver parenchyma. Expression of HNF-6 in hepatocytes and epithelial cells of the bile ducts attained maximum in 6 h after surgery. At later terms, this parameter somewhat decreased, but still surpassed the control level.

Key Words: *hepatocyte nuclear factor 6; liver regeneration; partial hepatectomy; cell proliferation*

Liver diseases seriously threaten human health. End-stage liver diseases, especially liver cirrhosis, represent a worldwide health problem [15]. Liver cancer is the fifth most common malignancy and the third most common cause of cancer-related deaths. Five hundred million individuals are infected with hepatitis B or C viruses and a considerable part will progress to liver failure. In China, the incidence of hepatitis C and liver cancer-related mortality are high. Globally, about 53% new liver cancer cases are recorded in mainland China. In China, liver cancer is the second cancer-related cause of deaths. Along with the development of the liver transplant technology, stem cell-based strategies are intensively studied as an attractive alternative approach to liver repair and regeneration. Liver regeneration is a complex process, and many genes and cell factors are involved in the dynamic control of liver regeneration [2,12]. Despite intensive molecular and cellular biology research, including hormone, growth factors and cytokine genes, the molecular mechanisms of liver regeneration, especially regulation mechanisms of this process remain poorly understood.

The hepatocyte nuclear factor 6 (HNF-6) is a cell-specific transcription factor regulating gene expression in the liver. It was demonstrated using the chromatin immunoprecipitation technique that HNF-6 is involved in the regulation of cell proliferation and expression of cell-type specific genes. At present, most studies in this field are focused on its function in the embryonic development; published report suggest that HNF-6 is involved in the development of bile ducts inside and outside the liver [1]. However, the function of HNF-6

[1]Department of Anatomy, School of Basic Medical Science, Dali University, Dali, PR China; [2]Department of Surgery of Dali Prefecture People's Hospital, Dali, PR China; [3]Department of Surgery of Affiliated Hospital of Dali University, Dali, PR China. ***Address for correspondence:*** 176501584@qq.com. K. M. Yang

in the liver regeneration is still unclear. In study [13], injection of adenovirus expressing the HNF-6 cDNA (*AdHNF*-6) into the caudal vein was used to increase hepatic levels of HNF-6 during mouse liver regeneration induced by partial hepatectomy. The result showed that elevated expression of HNF-6 during mouse liver regeneration significantly increased the number of hepatocytes entering DNA replication (S-phase) through stimulating TGF-α, cyclin-dependent kinase CDK2, cyclin D1, and FOXM1 protein, *i.e*. the same factors that are necessary for stimulation of stem cell reproduction during liver regeneration [3]. Further research showed that increased HNF-6 levels stimulate hepatocytes proliferation through transcriptional induction of cell cycle regulatory genes.

In our study, we analyzed the expression of HNF-6 and cell proliferation at the early stage of liver regeneration after partial hepatectomy in rats to discuss the role of these processes in liver regeneration.

MATERALS AND METHODS

The study was performed on mature male and female Sprague-Dawley rats (n=30) weighing 250±10 g (provided by Dali University Laboratory Animal Center). The experiments were approved by the Local Ethics Commission and performed in accordance with the accepted international rules for the use of experimental animals for research purposes. Before surgery, the animals were deprived of food and water and randomly divided into 4 experimental and one control group (6 rats per group). Liver resection (70%) was performed using the method of Higgins. After surgery, the animals were placed in warm and clean cages and had free access to water and food. The rats of the experimental groups were euthanized in 3, 6, 12, and 24 h after surgery. The liver was divided into two parts, one part was fixed in 10% neutral formaldehyde, and the other part was stored in liquid nitrogen.

Liver regeneration degree (LRD) and liver regeneration index (LRI). Liver regeneration was assessed by using the LRD and LRI. During surgery, the rat body weight (G) and weight of resected (A) and remaining (B) parts of the liver were determined. LRD and LRI were calculated by the formulas [14]:

$$LRD=[B-(A/0.684-A)]/A;$$

$$LRI=B/G\times 100\%.$$

Immunohistochemical analysis. The samples of regenerating liver were fixed in 10% neutral formaldehyde overnight and embedded in paraffin. Serial 5-μ slices were stained by immunohistochemical methods (SABC protocol). To this end, the sections were incubated overnight at 4°C with polyclonal rabbit antibodies to HNF-6 (1:200, Santa Cruz) and Ki-67 (1:200, Santa Cruz). Then, goat anti-rabbit IgG (1:200, Bei Jing ZhongShan Golden Bridge Co., Ltd) were added to the slices and incubated for 2 h at 37°C, followed by DAB visualization (BeiJing ZhongShan Golden Bridge Co., Ltd); then, the slices were poststained with hematoxylin. The sections incubated with phosphate buffer instead of primary antibodies to HNF-6 served as the control. The images were obtained using an Olympus Image Analysis Collection System and processed using a JIEDA-80 graphic analysis system (NanJing JieDa Science and Technology Co., Ltd); the morphometric parameters and the mean optical density of fragments were analyzed.

Isolation of total RNA. The total RNA was isolated from the liver using Trizol (Invitrogen) according to manufacturer instruction. Dissolved RNA into 20 μl water with DEPC was stored at -20°C. The purity of total RNA was assessed by UV spectrophotometery at A260/280 nm. The samples with A260/280<1.8 were not used in the experiments. The integrity of the total RNA was evaluated by agarose gel electrophoresis followed by analysis of gels using a GDS8000Gel imaging system. Clear-cut electrophoretic band corresponding to 28S, 18S, and 5S attested to preserved integrity of the isolated RNA.

Primers. The primer for HNF-6 was synthesized by BeiJing SanBo YuanZhi Biotechnology Co. Ltd on the basis of mRNA sequence of this protein (Table 1). Then, the primer was dissolved in water without RNase (20 M) and stored at -20°C until use.

RT-PCR. RT-PCR of the total RNA from rat liver was carried out. The reaction system included Prime Script 1 Step Enzyme Mix consisting of reverse transcriptase, DNA polymerase, RNase inhibitor (2 μl); 1 Step Buffer 2× consisting of a buffer, dNTP mixture (400 μM), and One Step Enhancer Solution (25 μl); upstream primer (20 μmol, 1 μl); downstream primer (20 μmol, 1 μl), template RNA (1 μl), RNase free dH2O (20 μl). The reaction protocol was as follows: 30 min at 50°C (1 cycle); 2 min at 94°C (1 cycle); 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C (30 cycles).

TABLE 1. Oligonucleotide Primers Used for Gene Expression Analysis by RT-PCR

Gene	Sense strand (5'→3')	Antisense strand (3'→5')	Annealing temperature, °C	Product size, bp
HNF6	TGACTTGTGCCCAGGTGAGAG	GCAAACGCAAAGAGCAAGAACC	55	677
ACTB	GTAAAGACCTCTATGCCAACA	GAGTGACAGGTGGAAGGTC	52	191

The product was cooled on ice and stored at -20°C. RNase free dH2O was used as the negative control.

Electrophoresis of amplification product of RT-PCR. The products (2 μl) was mixed with the buffer and applied to agarose gel (20 g/liter) and electrophoresis was performed at 100 V for 10-20 min. The gels were scanned and analyzed using a GDS8000 AGAR image analysis system and Grab2IT219 software.

Statistical analysis. Five slices and 10 fields of view in each slice were randomly selected. The mean optical density was measured using Grab2IT219 software. The data were processed using SPSS16.0 software. The variance homogeneity examination was carried out, and the data was variance together, so we carried out variance inspection for the data. The differences were significant at $p<0.05$.

RESULTS

Judging from LRD and LRI, the weight of the liver started to increase in 24 h after partial resection (Table 2).

Immunohistochemical analysis. In the liver of control rats, weak expression of HNF-6 was found, but expression in normal bile duct epithelial is obvious (Fig. 1, *a*). After liver resection, HNF-6 expression markedly increased at all terms of observation: it peaked in 6 h after surgery and then gradually decreased but remained above the control. The expression of HNF-6 was primarily located in the nucleus and it's expressing reached at 6 h after PH, then gradually dropped, but it was still higher than normal group (Fig. 1, *b*, *c*). When we detected which cells proliferation by Ki-67 immunoreactions in early stage of liver regeneration (at 3h), the results display the early positive reactive cells were liver macrophages where located in hepatic blood sinus, and then (at 6 h) spread to hepatocytes (Fig. 2, *a*, *b*).

RT-PCR. RT-PCR assay demonstrated the expression of HNF-6 in the control and after liver resection (Table 3). The expression of HNF-6 in the control group was weak, but increased shortly after partial hepatectomy peaking in 6 h after partial hepatectomy; then, the expression decreased, but remained higher than in the control (Table 3, Fig. 3).

It is known that mammalian liver has a unique capacity to regenerate following resection or injury mainly through proliferation of remaining mature hepatocytes. Partial hepatectomy is a surgical procedure in

TABLE 2. Indexes of Liver Regeneration LRD and LRI after Hepatectomy (*M*±*SEM*, *n*=6)

Time after hepatectomy	LRD	LRI
3 h	0.17±0.07*	1.110±0
6 h	0.23±0.04	0.310±0.001
12 h	0.30±0.12	1.520±0.003
24 h	0.34±0.08*	1.590±0.003*

Note. *$p<0.05$.

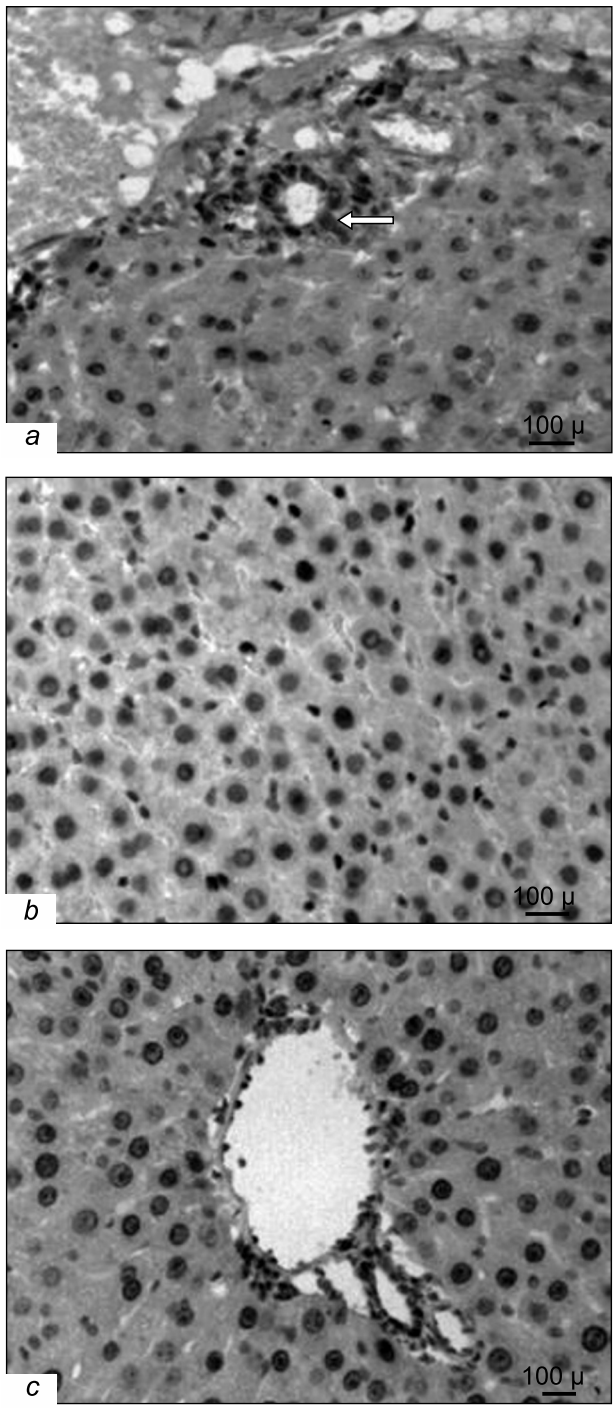

Fig. 1. The expression of NHF-6 in rat liver tissue, immunochemical SABC staining, ×400 (*a*), ×200 (*b*, *c*). *a*) Control (arrow shows epithelium of interlobular bile duct); *b*) 6 h after hepatectomy; *c*) 24 h after hepatectomy.

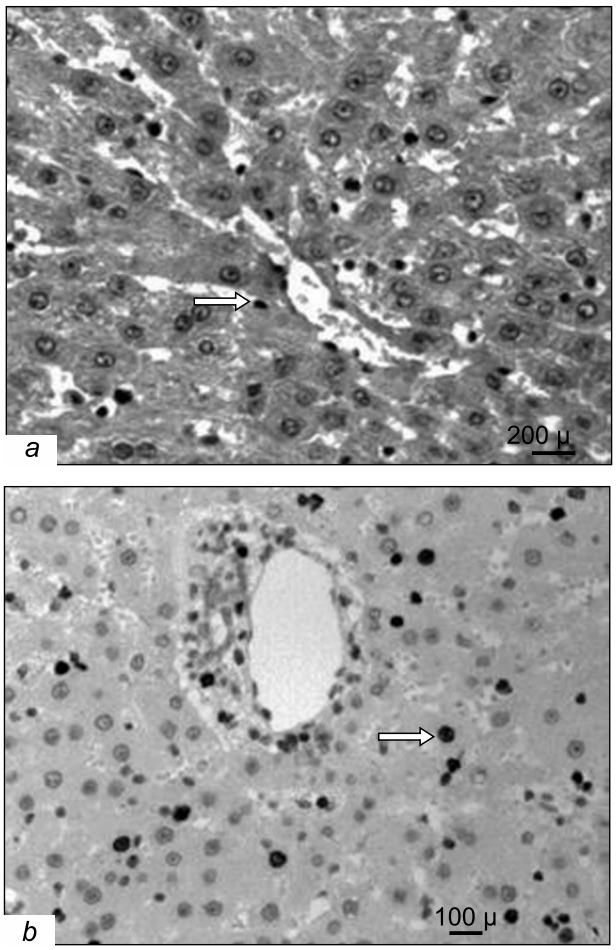

Fig. 2. Immunohistochemical staining of Ki-67 cells in rat liver in 3 (*a*) and 6 (*b*) h after hepatectomy, ×100. Arrow shows macrophages (*a*) and hepatocytes (*b*).

which specific liver lobes are removed without damage to the remaining lobes [7]. The survival rate in mice subjected to resection of $^{2}/_{3}$ of the liver weight is near 100%. It should be taken into account that the weight of the residual liver shortly after partial hepatectomy is affected by tissue edema. Two days after surgery, active proliferation of liver cells leads to an increase in the weight of liver residue, due to which its weight can exceed 90% weight of normal liver. Then, its growth speed decreased. This suggests that the regenerating liver has already adapted to the metabolic need of the body and started to rebuild small bile ducts and blood vessel. The liver residue can reach its normal weight in 7-14 day after surgery [6]. In our study, changes in LRD and LRI also showed that liver regeneration at the early stage was not so obvious; this can indicate that liver weight increased due to edema, rather than cell proliferation. The significant increase in liver weight was observed only in 24 h after surgery (Table 2).

Normally, regeneration of human liver starts in 3 days after resection (or trauma) and original weight of the liver can be attained in 5-6 months. Rat liver can reach its original weight within 1 week after partial hepatectomy, and liver functions also recovered at the same time. Previous study identified all kinds of cells proliferated orderly during regeneration, including hepatocytes (the peak of proliferation is observed in 24 h after partial hepatectomy), bile duct epithelial cells (proliferation starts in 48 h after partial hepatectomy), Kupffer cells and epithelial cells of hepatic sinusoids (proliferation starts in 72 h after liver resection). It is now accepted that 3 types of cells participate in liver regeneration: mature hepatocytes characterized by strong proliferation potential, stem cells of the bile ducts that could differentiate into hepatocytes and bile duct cells, and stem cells of bile canaliculi that originate from the bone marrow and has the potential function of long-term proliferation. Among these 3 types of cells, the hepatocytes play the leading part in regeneration. However, our results are at controversy to this picture. We found that Kupffer cells rather than hepatocytes first contributed to regeneration at the early stages of this process (Fig. 2, *a*). It can be hypothesized that proliferation Kupffer cells are essential for the start of massive proliferation of hepatocytes. It cannot be excluded that proliferation of Kupffer cells plays a trigger/stimulatory role for induction of hepatocyte proliferation.

Many factors affected liver regeneration, including growth factors, circulating hormones, and kinases. All they affect the cell cycle, especially the stage of DNA replication. This complex network of mechanisms involved in the maintenance of liver regeneration includes many transcriptional regulatory elements for genes expressed in hepatocytes, *e.g.* hepatocyte nuclear factors HNF-1, HNF-3, HNF-4, and HNF-6 essential for both hepatocyte differentiation during liver development and regulation of liver regeneration [8].

In our experiment, significant increase in the liver weight was observed in 24 h after partial hepatectomy. This indicates completion of DNA replication in the liver and the start of cell division. According to

TABLE 3. Expression of HNF-6 in the Control Group and at Different Terms after Hepatectomy (*M*±*SEM*, *n*=6)

Group	HNF-6 expression
Control	0.590±0.011
Hepatectomy	
3 h	0.643±0.012*
6 h	0.725±0.01*
12 h	0.673±0.005*
24 h	0.623±0.013*

Note. F=21.965, *p<0.05

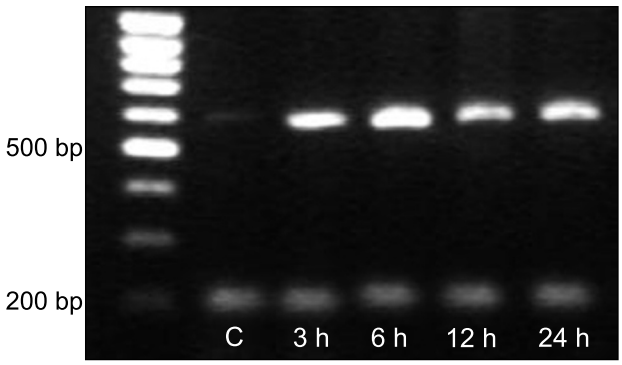

Fig. 3. RT-PCR products of HNF-6 in the control (C) and in 3, 6, 12, and 24 h after hepatectomy.

some published reports, liver regeneration begins in 6 h after partial hepatectomy [4]. Our findings agree with these data: HNF-6 expression peaked in 6 h after liver resection and remained elevated at latter terms. This result indicates the expression HNF-6 gene is vital for hepatocyte proliferation at early terms of liver regeneration.

The cell cycle includes G0/G1, S, and G2/M phases. In eukaryotes, the cell proceeds through G1-S-G2-M phases during which DNA is replicated (S phase) and the cell enters mitosis (G2/M phase). The proliferation ratio at this time could be the proliferation index. Normally, hepatocytes are in the G0 stage. Successfully regeneration of the requires reactivation of mature hepatocyte and induction of their proliferative activity. And then started the following DNA proliferation and cell division process [10]. From our observation, this proliferative activity of mature hepatocytes was probably a result of reactivation of liver macrophages. Which factors come from Kupffer cells to switch to HNF-6 expression facilitating hepatocytes proliferation is good for research.

During liver regeneration, the increase in HNF-6 level leads to an increase in the number of S-phase cells via stimulation of TGF-α, CDK2, cyclin D1, and FOXM1 protein. The experiment indicated HNF-6 could stimulate transcription activity of FOXM1, and the complex of HNF-6 and FOXM1b could further stimulate transcription of the promoter of TGF-α. Increased level of HNF-6 during liver regeneration could stimulate the expression of genes encoding FOXM1, CDK2, cyclin D1, and TGF-α in liver cell, and these genes were very important for cell proliferation during liver regeneration.

The early expression of cyclin D1 is related to stimulation of DNA replication in liver cells. Studies of the DNA-binding domain of HNF-6 showed that HNF-6 CUT domain folds into a topology homologous to the Oct-1 POU DNA binding domain, even though there is no sequence homology between the CUT and POU domain sequences [11]. At the same time, the POU homeodomain of GHF1/Pit1, Brn1, Oct-2 and Oct-3 transcription factors plays an essential role in stimulating cell proliferation and regulation of the expression of cell-type specific genes [5]. Published hepatocyte chromatin immunoprecipitation assays demonstrate that HNF-6 transcription factor occupied endogenous promoters of the cell cycle regulatory genes *Cdc25A*, *Cdk2*, and *E2F1* [9], suggesting that HNF-6 regulates hepatocyte proliferation during early liver regeneration. All the above and our observation suggested that HNF-6 takes part in the process of liver regeneration and can be involved in the cell cycle regulation. It should be noted that, HNF-6$^{-/-}$ embryos fail to develop functional endocrine pancreas, gallbladder, and extrahepatic bile ducts. Similar to the FOXM1 transcription factor, HNF-6 is also required for the development of intrahepatic bile ducts in the developing liver. In adult mouse liver, HNF-6 protein is expressed in hepatocytes and especially in epithelial cells of bile ducts. This suggests that HNF-6 is involved in the development of bile duct and in normal renewal of bile duct epithelial cells.

We found that HNF-6 was weakly expressed in normal hepatocytes and intensively expressed in epithelial cells of bile duct, which suggests that HNF-6 is necessary for renewal and proliferation of normal liver cells and can be involved in normal renewal of hepatocytes and epithelial cells of bile ducts. The results of immunohistochemical analysis and RT-PCR both indicated that HNF-6 expression immediately increased in 3 h and reached its peak in 6 h after partial hepatectomy, then its expression decreased, but remained above the control level. This can be related with its trigger function in cell division and DNA replication in hepatocyte and bile duct epithelial cells. In addition, Kupffer cells in hepatic blood sinus, but not hepatocytes or bile duct epithelium, were the first cells exhibiting proliferation characteristic and then the expression of HNF-6 as well as hepatocytes proliferation appeared. These findings suggest Kupffer cells to switch to HNF-6 expression facilitating hepatocytes proliferation. The mechanism of this process worth comprehensive studies.

REFERENCES

1. Conforto TL, Steinhardt GF 4th, Waxman DJ. Cross talk between GH-regulated transcription factors HNF6 and CUX2 in adult mouse liver. Mol. Endocrinol. 2015;29(9):1286-1302.
2. Duncan AW, Dorrell C, Grompe M. Stem cells and liver regeneration. Gastroenterology. 2009;137(2):466-481.
3. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. J. Hepatol. 2012;57(3):692-694.
4. Geng X, Chang C, Zang X, Sun J, Li P, Guo J, Xu C. Integrative proteomic and microRNA analysis of the priming phase

during rat liver regeneration. Gene. 2016;575(2, Pt 1):224-232.
5. Goodall J, Martinozzi S, Dexter TJ, Champeval D, Carreira S, Larue L, Goding CR. Brn-2 expression controls melanoma proliferation and is directly regulated by beta-catenin. Mol. Cell. Biol. 2004;24(7):2915-2922.
6. Köhler C, Bell AW, Bowen WC, Monga SP, Fleig W, Michalopoulos G.K. Expression of Notch-1 and its ligand Jagged-1 in rat liver during liver regeneration. Hepatology. 2004; 39(4):1056-1065.
7. Michalopoulos GK. Advances in liver regeneration. Expert Rev. Gastroenterol. Hepatol. 2014;8(8):897-907.
8. Nagaki M, Moriwaki H. Transcription factor HNF and hepatocyte differentiation. Hepatol. Res. 2008;38(10):961-969.
9. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, Young RA. Control of pancreas and liver gene expression by HNF transcription factor. Science. 2004;303:1378-1381.
10. Pahlavan PS, Feldmann RE Jr, Zavos C, Kountouras J. Prometheus' challenge: molecular, cellular and systemic aspects of liver regeneration. J. Surg. Res. 2006;134(2):238-251.
11. Sheng W, Yan H, Rausa FM 3rd, Costa RH, Liao X. Structure of the hepatocyte nuclear factor 6alpha and its interaction with DNA. J. Biol. Chem. 2004;279(32):33,928-33,936.
12. Su AI, Guidotti LG, Pezacki JP, Chisari FV, Schultz PG. Gene expression during the priming phase of liver regeneration after partial hepatectomy in mice. Proc. Natl Acad. Sci. USA. 2002;99(17):11 181-11 186.
13. Tan Y, Yoshida Y, Hughes DE, Costa RH. Increased expression of hepatocyte nuclear factor 6 stimulates hepatocyte proliferation during mouse liver regeneration. Gastroenterology. 2006;130(4):1283-1300.
14. Yang S, Leow CK, Tan TM. Expression patterns of cytokine, growth factor and cell cycle-related genes after partial hepatectomy in rats with thioacetamide-induced cirrhosis. World J. Gastroenterol. 2006;12(7):1063-1070.
15. Zabaleta N, Hommel M, Salas D, Gonzalez-Aseguinolaza G. Genetic-based approaches to inherited metabolic liver diseases. Hum. Gene Ther. 2019;30(10):1190-1203.