FEBS Letters 579 (2005) 4562–4566 FEBS 29807

Expression of neuritin during liver maturation and regeneration

Nobuhiko Kojima[a,b,*], Nobuyoshi Shiojiri[c], Yasuyuki Sakai[a,d], Atsushi Miyajima[b,e,f]

[a] Institute of Industrial Science, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan
[b] Stem Cell Regulation Project, Kanagawa Academy of Science and Technology (KAST), 907 Nogawa, Miyamae-ku, Kawasaki, Kanagawa 216-0001, Japan
[c] Department of Biology, Faculty of Science, Shizuoka University, 836 Oya, Shizuoka 422-8529, Japan
[d] Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[e] Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
[f] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi 332-0012, Japan

Received 18 May 2005; revised 26 June 2005; accepted 5 July 2005

Available online 26 July 2005

Edited by Robert Barouki

Abstract **Cell surface molecules are not only important for cell–cell interactions but also useful for a marker to define cell types and differentiation stages. Unlike hematopoietic system in which numerous such antigens have been identified, only a few cell surface molecules have been used to define differentiation stage of hepatocytes. In order to identify such cell surface molecules, we performed DNA microarray analysis using mRNA from fetal hepatocytes in E12.5 and E17.5 mice and cDNAs encoding a membrane protein were selected. Northern blot analysis was employed to confirm the genes upregulated during maturation of fetal hepatocytes and neuritin, a GPI-anchored protein, was found as a membrane protein expressed in hepatocytes, but not in nonparenchymal cells. Its expression increased along with liver development and the maximum expression was achieved from the neonatal to adult stage. The neuritin protein was localized in sinusoidal lumen of hepatocytes in adult liver. Partial hepatectomy transiently downregulated the expression of neuritin. The expression of neuritin mRNA in C/EBPα deficient liver was reduced to about 50% of that of wild type mice. Thus, neuritin expression is well correlated to the maturation of hepatocytes and can be a useful tool to define the differentiation stage of hepatocytes.**

Keywords: Neuritin; Liver development; Liver regeneration; C/EBPα deficient mouse

1. Introduction

The liver development can be divided into several distinct stages depending on its morphological and functional properties. Fetal liver is a major hematopoietic tissue in embryo and supports massive production of blood cells. However, fetal liver lacks most of the metabolic functions and liver acquires such functions in the perinatal stage. After birth, liver exhibits numerous metabolic functions and gradually reduces their proliferation potential. Interestingly, while fully matured liver is a quiescent organ, it has an extraordinary capacity to regenerate upon liver damage. Thus, there are dramatic changes in the characteristics of the liver during the transition from fetal to adult stages and also during liver injury and regeneration. Hepatocytes in each stage express a set of specific proteins and the earliest hepatocytes, i.e., hepatoblasts, which are precursors of hepatocytes and biliary epithelial cells, are known to express α-feto protein (AFP) and albumin [1,2]. Suzuki et al. [3] described prospective isolation of liver progenitors using a combination of mAbs against c-Met, c-Kit, CD49f, CD29, CD45 and Ter 119. We demonstrated that delta-like (Dlk), a transmembrane protein with EGF repeats, also known as Pref-1, is highly expressed in hepatoblasts. Its expression is downregulated along with differentiation and completely disappears after birth [4].

After the perinatal stage, hepatocytes express a number of liver specific genes, such as carbamoylphosphate synthetase-1 (CPS-1), tyrosine amino transferase, glucose 6-phosphatase, haptoglobin and so on [5,6]. Several genes including tryptophan oxygenase and serine dehydratase are expressed by terminally differentiated hepatocytes and they are downregulated in regenerating liver [7,8]. However, almost all of these inducible marker genes in liver maturation are enzymes involved in intracellular metabolic reactions or production of serum proteins. Therefore, identification of cell surface proteins, which are upregulated during liver maturation may provide not only insight into the understanding of the liver maturation, but also useful markers for analysis and isolation of specific cell populations with cell sorters.

In this study, we show that neuritin, which was previously reported as a GPI-anchored membrane protein expressed in neural cells, is expressed in mature hepatocytes in a C/EBPα dependent manner.

2. Materials and methods

2.1. Mice

All experiments were performed using wildtype C57BL/6CrSlc mice (Nihon SLC, Hamamatsu, Japan) and C/EBPα deficient mice [9]. For tissue blots, brain, heart, lung, liver, kidney, spleen, skeletal muscle (s. muscle) and placenta were taken from adult mice at the age of 8–12 weeks. Livers were also obtained from E12.5, E14.5, E17.5,

*Corresponding author. Fax.: +81 3 5452 6348.
E-mail addresses: kojima@iis.u-tokyo.ac.jp (N. Kojima), miyajima@iam.u-tokyo.ac.jp (A. Miyajima).

0014-5793/$30.00
doi:10.1016/j.febslet.2005.07.015

neonatal (days 0–1) and adult (10–12 weeks) mice for Northern blot analysis. For partial hepatectomy, mice were subjected to conventional 70% partial hepatectomy under anesthesia [10]. C/EBPα deficient fetal livers were obtained from E16.5–E18.5 fetuses.

2.2. Microarray analysis

Fetal hepatocytes from 200 livers of E12.5 mice and 50 livers of E17.5 mice were isolated by anti-Dlk antibody and autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) [4]. Total RNA extracted by using TRIZOL (Invitrogen, Carlsbad, CA) was used for microarray analysis with GEM2 Microarray (Incyte Genomics, Wilmington, DE) carrying 9514 clones.

2.3. Northern blot analysis

Total RNA was prepared from various organs using TRIZOL (Invitrogen). Ten micrograms of total RNA from each samples were separated by electrophoresis on a 1.5% agarose gel containing 2% formaldehyde. RNA was transferred to a positively-charged Nylon Membrane (Roche Diagnostics, Basel, Switzerland) and was hybridized with digoxigenin (DIG)-labeled cDNA probes for neuritin, CPS-1, tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), cyclin D1 and GAPDH. Membrane was further incubated with alkaline phosphatase-labeled anti-DIG antibody (Roche Diagnostics). Blots were developed with CDP-star (New England Biolabs, Beverly, MA). For quantitative analysis, NIH image 1.62 (National Institute of Health, MD) was used to measure densities of developed bands. The intensity of the bands for neuritin, CPS-1 or cyclin D1 was normalized by that of GAPDH in the same sample or that of ethidium bromide staining of ribosomal RNA.

2.4. Immunohistochemistry

Frozen sections (8 μm) of adult liver were incubated with anti-neuritin polyclonal antibody (AF283, R&D systems, Minneapolis, MN) followed by biotin-conjugated anti-rabbit IgG. Anti-intercellular adhesion molecule-1 (ICAM-1) monoclonal antibody (KAT-1, Caltag laboratories, Burlingame, CA) and biotin-conjugated anti-mouse IgG were also used for ICAM staining. Immunoreactive proteins were visualized by using Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). Counter staining was performed with Mayer's hematoxylin solution (Wako Pure Chemicals, Osaka, Japan).

3. Results

3.1. Expression of neuritin in liver

To identify genes encoding a membrane protein, which are regulated in liver development, we performed DNA microarray analysis of fetal hepatocytes in E12.5 and E17.5 livers. Fetal hepatocytes from each embryonic day were positively selected by using anti-Dlk mAb and mRNA from each population was used for microarray analysis. Comparison of the gene expression profiles of the two cell populations led us to find 218 genes that were differentially expressed. Among those genes, we focused our attention on neuritin because it is a GPI-anchored membrane protein.

To confirm the expression of this protein in liver, we extracted total RNA from adult mouse tissues and examined neuritin expression by Northern blot analysis (Fig. 1). Neuritin was highly expressed in brain and liver and weak expression was observed in lung and heart.

3.2. Neuritin expression increases along with liver maturation

To address whether the expression level of neuritin is altered in liver maturation, we performed Northern blot analysis using total RNA extracted from livers at several stages, E12.5, E14.5, E17.5, neonatal and adult. Neuritin was hardly detected in E12.5 liver and weakly expressed in E14.5 liver. The expression level increased significantly along with liver development

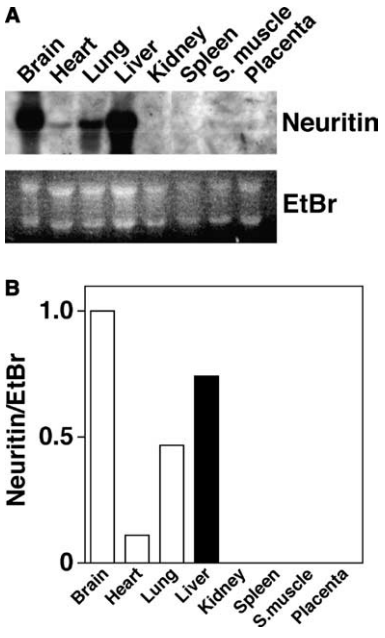

Fig. 1. Liver is a neuritin expressing tissue. (A) Neuritin expression in adult mouse tissues was confirmed by Northern blot analysis. Total RNA of adult tissues was extracted from brain, heart, lung, liver, kidney, spleen, skeletal muscle and placenta. Ten micrograms of total RNA was blotted and probed with DNA probe for neuritin. Expression level of liver was comparable with that of brain. Neuritin expression in lung and heat was also detected. (B) The intensity of the neuritin bands was normalized by ethidium bromide staining of ribosomal RNA.

and became the maximum in the neonatal to adult stage (Fig. 2), indicating that neuritin is gradually upregulated in liver development.

Recently, it has been shown that neuritin expression in mouse is regulated by male hormones [11]. We therefore compared the neuritin expression levels in male and female mouse, by Northern blot analysis. The degree of neuritin expression in female liver was almost equal to male one (Fig. 2), suggesting that male hormones do not affect neuritin expression in normal adult liver.

3.3. Expression of neuritin in hepatocytes

Liver consists of hepatocytes and various nonparenchymal cells. To identify the cell type that expressed mRNA for neuritin in adult liver, hepatocytes and nonparenchymal cells were separated by two-step collagenase perfusion method [12]. Total RNA from each population was subjected to Northern blot analysis (Fig. 3). CPS-1 is a key enzyme of ornithine cycle and known to be specifically expressed in hepatocytes but not in nonparenchymal cells [13], whereas TIMP-2 is expressed in nonparenchymal cells in liver [14]. Expression of CPS-1 and TIMP-2 indicated that hepatocytes and nonparenchymal cells were well separated. Because neuritin was detected only in RNA from hepatocytes, neuritin is specifically expressed in hepatocytes.

3.4. Localization of neuritin at the sinusoidal lumen of hepatocytes

Frozen section of adult liver was stained immunohistochemically with anti-neuritin antibody to confirm the expression in adult liver (Fig. 4). Interestingly, the staining signal was

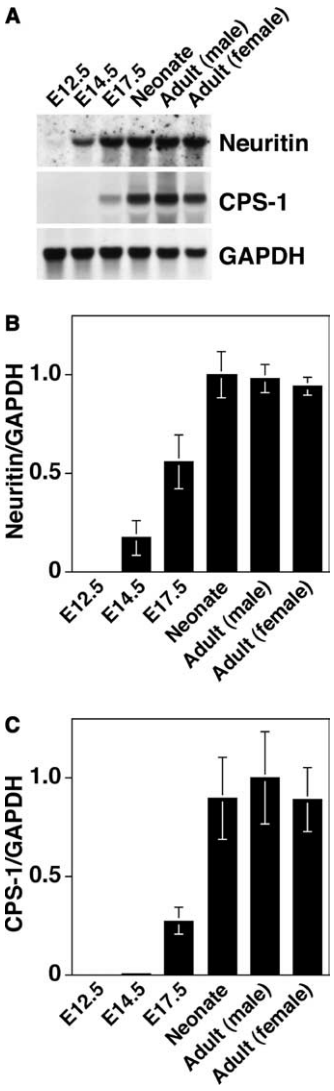

Fig. 2. Neuritin expression in liver is increased along with liver development. (A) Total RNA from various stages of developing livers was subjected to Northern blot analysis to detect neuritin expression. DNA probe for neuritin was hybridized against membrane transferred RNA. The amount of neuritin and CPS-1 RNA induced development dependent manner. (B,C) Signal intensity of neuritin or CPS-1 in Northern blot analysis was normalized by that of GAPDH. In each condition, three livers were used. Data are expressed as means ± S.D.

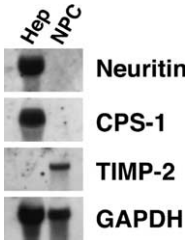

Fig. 3. Neuritin was expressed by hepatocytes but not by nonparenchymal cells. Hepatocytes (Hep) and nonparenchymal cells (NPC) of mouse adult liver were separated by two-step collagenase perfusion method. Ten micrograms of each total RNA was blotted and neuritin, CPS-1, TIMP-2 and GAPDH expression were probed. CPS-1 and TIMP-2 were used as markers for hepatocytes and nonparenchymal cells, respectively.

observed along with sinusoidal vessels. This staining pattern was similar to that of anti-ICAM-1, which is mainly expressed by sinusoidal endothelial cells but is only weakly expressed in hepatocytes [15]. As the neuritin expression was restricted in hepatocytes as shown in Fig. 3, anti-neuritin antibody was likely to bind the sinusoidal lumen of hepatocytes, but not sinusoidal endothelial cells. In addition to the sinusoidal lumen, a weak signal was also detected in intracellular area of hepatocytes.

3.5. Downregulation of neuritin in regenerating liver

Adult liver is a unique organ that can regenerate when it is partially removed by a surgical method, and regeneration processes are reminiscent of developmental processes. It is known that hepatocytes in regenerating liver express AFP, which is usually expressed only in fetal hepatocytes but not in adult liver [1]. Likewise, we recently found a subset of oval cell that appear in regenerating rat liver express Dlk/Pref1, a specific marker of hepatoblasts [4]. We examined neuritin expression in regenerating liver in which 70% partial hepatectomy was performed. Alteration of CPS-1 and cyclin D1 levels indicated that liver regeneration was induced by partial hepatectomy. Neuritin was downregulated at around 24 h after partial hepatectomy (Fig. 5) when DNA synthesis reached to the peak [16], and the neuritin expression was gradually recovered. Injured liver by CCl_4 injection also downregulated the neuritin expression at around 24 h after CCl_4 administration (data not shown). These results suggest that neuritin is downregulated when hepatocytes enter the S phase.

3.6. Neuritin expression requires C/EBPα

C/EBPα is essential for functional maturation of liver, because C/EBPα deficient mice die soon after birth with hypoglycemia and hyperammonemia [17]. If neuritin expression is coordinated with liver maturation, its expression is not expected in C/EBPα deficient mice. In fact, there are some putative C/EBP binding sites in the neuritin promoter region (Fig. 6A). To confirm the relation between the expression level of neuritin and maturation of hepatocytes, E16.5–E18.5 fetal liver from C/EBPα deficient mice was analyzed by Northern blot (Fig. 6B), and the intensity of the signals was quantified by the NIH-image (Fig. 6C). The expression level of neuritin mRNA in the knockout liver was reduced to about 50%, indicating that neuritin expression in liver requires C/EBPα and is developmentally regulated.

4. Discussion

The aim of this report is to find a membrane protein, of which expression level changes depending on liver differentiation stages. DNA microarray analysis of fetal hepatocytes on E12.5 and E17.5 revealed the expression of a GPI-anchored protein called neuritin, which previously cloned as a regulator of neurite outgrowth or brain development [18]. Indeed, expression of neuritin was the highest in the brain, however the liver also expressed it at a level comparable to the brain. Neuritin is mainly expressed by matured hepatocytes and the neuritin protein localized to the sinusoidal space, i.e., basolateral area of hepatocytes.

Neuritin expression is gradually upregulated as of the liver development progresses. Quiescent adult hepatocytes enter cell

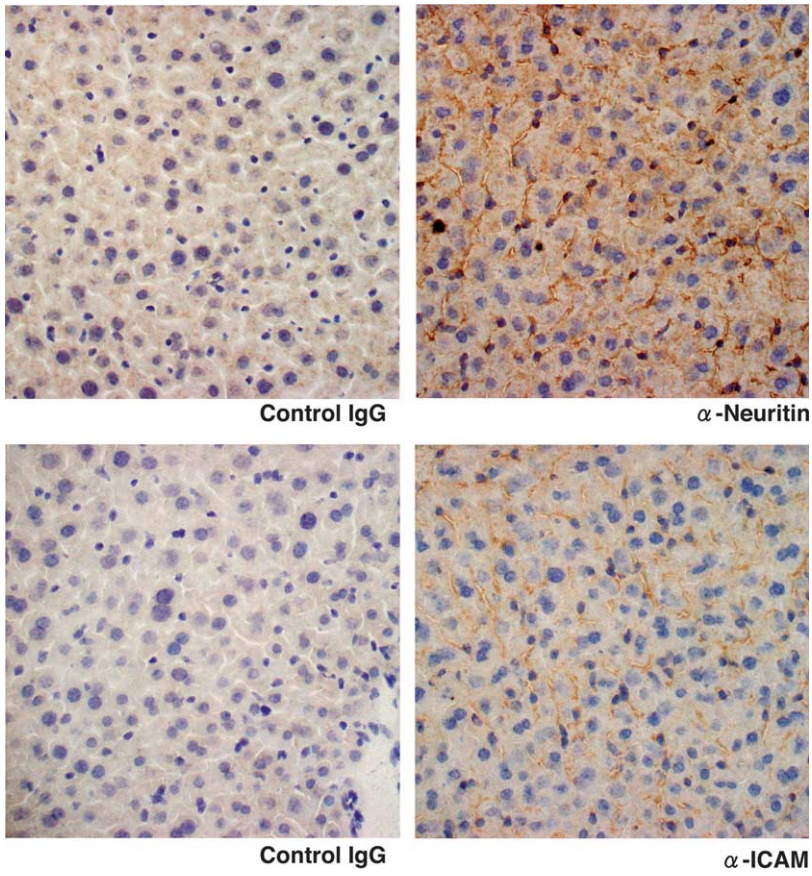

Fig. 4. Neuritin protein was concentrated in sinusoidal lumen of hepatocytes. Frozen sections of adult mouse liver were stained with anti-neuritin polyclonal antibody or anti-ICAM monoclonal antibody, and counter stained with hematoxylin. Anti-ICAM antibody reacted to surface of sinusoidal endothelial cells. Anti-neuritin antibody stained sinusoidal area like anti-ICAM antibody. Because neuritin expression was restricted in hepatocytes (Fig. 3), the expression thought to be concentrated in sinusoidal area of hepatocytes. Intracellular area of hepatocytes also stained weakly. Original magnifications, 100×.

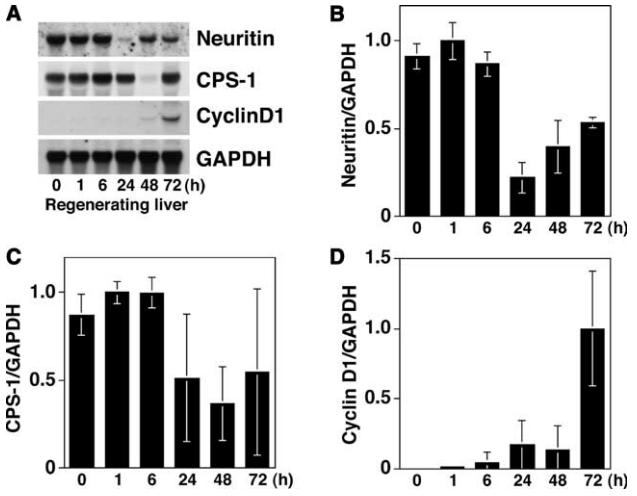

Fig. 5. Regenerating liver transiently decrease the expression of neuritin. (A) Liver regeneration was induced by 70% partial hepatectomy. At 0, 1, 6, 24, 48, 72 h after hepatectomy, livers were sampled and total RNA was extracted. Neuritin CPS-1 and cyclin D1 expressions were examined by Northern blot analysis. Downregulation of CPS-1 and induction of Cyclin D1 indicate that liver regeneration occurred. Neuritin was reduced after 24 h, transiently. (B,C,D) Quantitative analysis of neuritin, CPS-1 and cyclin D1, respectively. In each condition, three livers were used. Data are expressed as means ± S.D.

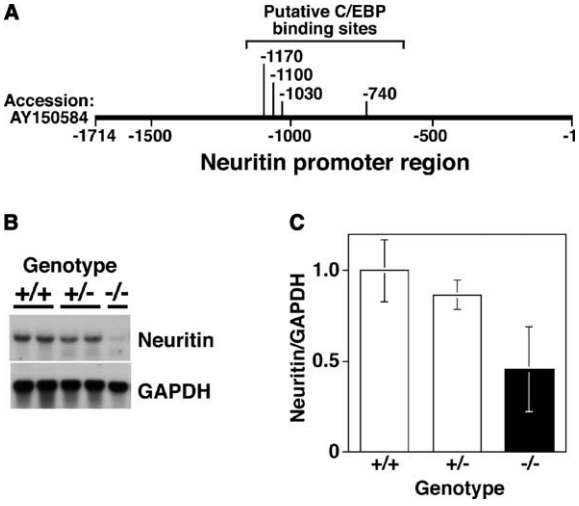

Fig. 6. Expression of neuritin requires C/EBPα. (A) Schematic representation of the neuritin promoter region. There are some putative binding sites for the C/EBP family in the promoter region (identified with "TFSEARCH"; http://mbs.cbrc.jp/research/db/TFSEARCH.html). (B) Ten micrograms of total RNA from liver of E16.5 C/EBPα deficient mouse was subjected to Northern blot analysis. (C) The intensity of the neuritin signal was quantified and normalized by that of GAPDH signal. The expression level of neuritin mRNA was reduced about 50% in C/EBPα knockout liver. At least three fetal livers (E16.5–E18.5) were used in each condition. Data are expressed as means ± S.D.

cycle upon liver injury and various liver gene expression changes in this process [16]. Neuritin expression was also transiently downregulated in parallel with the changes in gene expression of liver enzymes during liver regeneration. Moreover, neuritin expression was significantly reduced in C/EBPα deficient liver. These results indicate that neuritin is an excellent differentiation marker for hepatocytes.

It was previously reported that a soluble form of neuritin induced neurite outgrowth, while the mechanism remains unknown [18]. In liver, it is possible that neuritin is involved in proliferation and/or functional maturation of hepatocytes in liver development or regeneration. Furthermore, as neuritin protein is localized at the sinusoidal lumen of hepatocytes, it may play a role for regulation of cells in sinusoid such as stellate cells or sinusoidal endothelial cells. It would be interesting to test such a possibility if the culture systems representing the normal development of these cells are established.

As neuritin is a cell surface protein, it will provide a means to isolate mature hepatocytes. Recently, hepatocyte-like cells have been developed from various sources such as bone marrow, cord blood or ES cells. Neuritin may also be useful as a marker for identification of such hepatocyte-like cells.

Acknowledgments: We are grateful to Drs. K. Nakamura, M. Tanaka, H. Yanai, N. Tanimizu, H. Anzai, H. Saito, H. Nonaka, T. Atake and H. Yamasaki for technical assistance and helpful discussion. This work was supported in part by Grants-in-Aid for Scientific Research and the Special Coordination Fund for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, a research grant from the Ministry of Health, Labour and Welfare, the Japan Government and CREST of Japan Science and Technology Agency.

References

[1] Shiojiri, N., Lemire, J.M. and Fausto, N. (1991) Cell lineages and oval cell progenitors in rat liver development. Cancer Res. 51, 2611–2620.
[2] Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R. and Zaret, K.S. (1996) Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev. 10, 1670–1682.
[3] Suzuki, A., Zheng, Y.W., Kaneko, S., Onodera, M., Fukao, K., Nakauchi, H. and Taniguchi, H. (2002) Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. J. Cell Biol. 156, 173–184.
[4] Tanimizu, N., Nishikawa, M., Saito, H., Tsujimura, T. and Miyajima, A. (2003) Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J. Cell Sci. 116, 1775–1786.
[5] Kamiya, A., Kinoshita, T., Ito, Y., Matsui, T., Morikawa, Y., Senba, E., Nakashima, K., Taga, T., Yoshida, K., Kishimoto, T. and Miyajima, A. (1999) Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J. 18, 2127–2136.
[6] Kojima, N., Kinoshita, T., Kamiya, A., Nakamura, K., Nakashima, K., Taga, T. and Miyajima, A. (2000) Cell density-dependent regulation of hepatic development by a gp130-independent pathway. Biochem. Biophys. Res. Commun. 277, 152–158.
[7] Nagao, M., Nakamura, T. and Ichihara, A. (1986) Developmental control of gene expression of tryptophan 2,3-dioxygenase in neonatal rat liver. Biochim. Biophys. Acta 867, 179–186.
[8] Noda, C., Ohguri, M. and Ichihara, A. (1990) Developmental and growth-related regulation of expression of serine dehydratase mRNA in rat liver. Biochem. Biophys. Res. Commun. 168, 335–342.
[9] Tomizawa, M., Garfield, S., Factor, V. and Xanthopoulos, K.G. (1998) Hepatocytes deficient in CCAAT/enhancer binding protein alpha (C/EBP alpha) exhibit both hepatocyte and biliary epithelial cell character. Biochem. Biophys. Res. Commun. 249, 1–5.
[10] Higgins, G.M. and Anderson, R.M. (1931) Experimental pathology of the liver. I. Restoration of the liver of the white rat following surgical removal. Arch. Pathol. 12, 186–202.
[11] Marron, T.U., Guerini, V., Rusmini, P., Sau, D., Brevini, T.A.L., Martini, L. and Poletti, A. (2005) Androgen-induced neurite outgrowth is mediated by neuritin in motor neurones. J. Neurochem. 92, 10–20.
[12] Seglen, P.O. (1976) Preparation of isolated rat liver cells. Methods Cell Biol. 13, 29–83.
[13] Gaasbeek Janzen, J.W., Lamers, W.H., Moorman, A.F., de Graaf, A., Los, J.A. and Charles, R. (1984) Immunohistochemical localization of carbamoyl-phosphate synthetase (ammonia) in adult rat liver; evidence for a heterogeneous distribution. J. Histochem. Cytochem. 32, 557–564.
[14] Rudolph, K.L., Trautwein, C., Kubicka, S., Rakemann, T., Bahr, M.J., Sedlaczek, N., Schuppan, D. and Manns, M.P. (1999) Differential regulation of extracellular matrix synthesis during liver regeneration after partial hepatectomy in rats. Hepatology 30, 1159–1166.
[15] Volpes, R., van den Oord, J.J. and Desmet, V.J. (1990) Immunohistochemical study of adhesion molecules in liver inflammation. Hepatology 12, 59–65.
[16] Taub, R. (1996) Liver regeneration 4: transcriptional control of liver regeneration. FASEB J. 10, 413–427.
[17] Wang, N.D., Finegold, M.J., Bradley, A., Ou, C.N., Abdelsayed, S.V., Wilde, M.D., Taylor, L.R., Wilson, D.R. and Darlington, G.J. (1995) Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269, 1108–1112.
[18] Naeve, G.S., Ramakrishnan, M., Kramer, R., Hevroni, D., Citri, Y. and Theill, L.E. (1997) Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. Proc. Natl. Acad. Sci. USA 94, 2648–2653.