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Investigation on Hepatopoietin and Other Novel Genes from Human Fetal Liver*

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The aim of this study is to discover the molecular mechanism of the 22-week gestated human fetal liver (HFL) which rarely displays both hematopoietic and hepatic functions. Based on large-scale cDNA library sequencing and bioinformatic analysis, the largest gene expression profile of human fetal liver in the world was successfully established. A set of gene clusters functionally related to the liver development, hepatocarcinogenesis and hematopoiesis have been identified. This is for the first time that we could panoramically understand the molecular mechanism of the dual functions of human fetal liver. Moreover, 201 unrecorded human homologous genes and 609 novel genes have been identified and annotated, which accounting for more than 7% of the known human genes in 2001. In the recent human genome annotation map (human genome build 35.1), 45 genes were nominated based on this study. In addition, we have characterized a set of gene families represented by hepatopoietin (HPO), Semaphorin, LSECtin and ARFGAP. Two distinctive novel pathways, "extracellular HPO→HPO receptor→EGF receptor→Raf→MEK→MAPK" for autocrine and "intracellular HPO→JAB1→c-JUN(AP-1)" for intracrine of HPO, an unusual cytokine functioned in the regeneration of liver, has been reported for the first time, which have shed new lights on the study of the signal transduction of the entire HPO family. We have also demonstrated that HPO could act as a FAD thioloxidase and that only its intracrine pathway is dependent on the enzymatic activity. It is also known for the first time that the enzyme activity is critically important for the cytokine HPO. Regarding the regulation of the gene expression of HPO, it was demonstrated that HPO promoter includes a negative regulatory element and a core promoter (comprises an initiator and its flanking three tandem IFE elements). Furthermore, two novel members of Semaphorin family, SEMA6C and SEMA6D, were cloned and shown to be able to determine the orientation of the cell growth. We have also discovered and characterized a novel lectin family including LSECtin, CD23, DC-SIGN and DC-SIGNR. The function of LSECtin was also defined to be important in adhesion of the cells. In addition, the first human member of ARFGAP family was cloned and shown to regulate protein secretion. The publications based on this study have been cited for 145 times by SCI journals before 2005. This study has provided important original data for the annotation of human genome and establishment of human transcriptome. It also played an important role in Chinese national achievement of cloning and annotation of the 10% human cDNAs project and set up the corner-stone for the leading role of China in the international "Human Liver Proteome Project".

Key words hepatopoietin, human fetal liver, gene expression profile, signal transduction, functional genomics

To have normal and stable hematopoietic function is obviously important for the body to maintain various physiological activities. At the same time, it is one of key steps for the patient to recover the hematopoietic function after irradiation injury because of radiation disease or using radiotherapy to treat the malignant tumors. It is widely known that the human fetal liver (HFL) during 12 to 26 weeks (HFL22W) of pregnancy is the main source of stem/progenitor cells of hematopoietic and immune system. From the development to maturity of the body, the intact hematopoietic system will firstly immigrate into

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fetal liver from the yolk sac, and then emigrate to the bone marrow. During that process, the 22-week is the critical turning point for the development and immigration/emigration of hematopoietic system, but its molecular mechanism is still unclear. In the 1970 to 80's, HFL was widely used to treat various diseases in China, such as haematopoietic disorder and fulminant hepatic failure, *etc*, and its remarkable therapeutic effect suggested that there might be a large amount of effective components in HFL with clinical significance which need further exploration.

In 1989, we firstly verified a component which could effectively cure acute fulminant hepatitis through stimulating hepatocyte proliferation, called hepatopoietin (HPO). Then we applied the US invention patent in 1992 and were awarded in 1995. Hagiya M *et al* cloned rat augmenter of liver regeneration (ALR) in 1994, and pointed out that its biological activity was identical to human HPO. We cloned the HPO gene in 1995 and proved that it is the orthologue of rat ALR. Then, we prepared the recombinant HPO and demonstrated its activity is protective on liver damage, specific stimulation of hepatocyte proliferation and effective treatment to acute fulminant hepatitis. HPO is the only human cytokine that specifically stimulates the proliferation of hepatocytes. However, its molecular mechanism is still unclear. HPO family includes human HPO, mouse/rat ALR, yeast ERV1/2, and virus E10R. So far, the research for this family focuses mostly on their augmenting function in liver regeneration and their activity as disulfide isomerase in oxidative phosphorylation. However, there are still lots of key questions need to be answered, especially the cellular signal transduction mechanism of the entire HPO family.

In order to illustrate the above questions, a strategy of large-scale sequencing analysis of the HFL22W cDNA library followed with experimental validation was performed in this project. By using automatically DNA sequencing method coupled with bioinformatic approaches based on internet resources and public biological databases, the gene expression profile of human fetal liver at 22-week stage has been established. A comparative analysis of the gene expression profile was also performed among those of different liver-related tissues and cells and hematopoietic tissues and cells. A lot of novel genes were identified based on the gene expression profile of HFL22W. Furthermore, functional characterization were performed on those closely related with dual functions of both hematopoiesis and liver-specific physiology of HFL guided by bioinformatic analysis. Furthermore, the HPO gene was considered to be an example for the functional annotation in this study. Some key events regarding the signal transduction of human HPO were addressed in this project. On one hand, the project explored the cellular signaling pathway of the proliferation of hepatocytes stimulated by extracellular HPO (including its receptor, signaling transduction pathway and the downstream transcription factor) based on the model of hepatoma cell lines. On the other hand, some other proteins which may regulate the function of intracellular HPO were identified by using yeast two-hybrid system and then revealed in COS cells.

1 Establishment of gene expression profile of HFL22W and identification and annotation of previously unknown genes at large-scale level

In the present project, the largest gene expression profile of HFL was established at first through large-scale sequencing of the cDNA library of HFL22W and systematic bioinformatic analysis. Totally about 20,000 individual clones were successfully sequenced from the cDNA library of HFL22W. The gene expression profile of HFL22W reported here was based on 1660 known human genes that could be categorized into 15 clusters based on their functions. Genes related to hematopoiesis, liver metabolism and housekeeping functions were highly expressed in HFL22W. Genes for signal transduction and those associated with transcription regulation were noticeably activated in HFL22W. Six gene groups were identified with the functions associated with different developmental stages of human fetal liver, tumorigenesis, different physiological functions of Itoh cells (different to the liver parenchymal cells-hepatocytes) and fetal hematopoiesis. The gene expression profile therefore remarkably revealed the unique dual functional characteristics (hepatic and hematopoietic) of HFL22W. The discovered and annotated genes make up of 7% of the total human protein coding genes (about 12,000 genes) when this project was being executed in 2001[1]. In the recent version of human genome map (human genome build 35.1), 45 genes were annotated based on this project. The results provided fruitful original data for the annotation of

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human genome project and the establishment of human transcriptome (the research article has been cited by recent paper on human transcriptome-A transcript finishing initiative for closing gaps in the human transcriptome, *Genome Research* (14: 1413—23, 2004), and contributed a lot to the complement of 10% human cDNAs project in China.

Before this project, study on gene expression profiling in HFL and hematopoietic system were published by Choi SS *et al*. (Mammalian Genome 6: 653—7, 1995), where they has built gene expression profiling of HFL at the age of 22 wk of gestation (HFL22W). However, the data obtained by Choi SS can not reflect full view of gene expression profiling of fetal liver actually and were not published in the international core journal because only 1231 individual clones were determined(< 1/15 of our data). In addition, there were gene expression profiles of other types of liver such as HFL19w, 40w, adult liver, Itoh cell, HepG2 cell (unpublished, see http://bodymap.ims.u-tokyo.ac.jp) and gene expression profiling of $CD34^+$ cells related with haematopoiesis (PNAS 95: 8175—80, 1998). As previously described, HFL22w was at the critical point of hematopoietic formation and migration. The hematopoietic function of HFL40w has completely declined, therefore, its gene expression profiling was not comparable with that of HFL22w, so as other tissue and cells. Actually, by comparing with above gene expression profiling, results showed that gene expression profiling of HFL22W has its specificity (Genome Research 11: 1392—403, 2001), and obviously indicated that its gene expression profile display the molecular features of its unique functional characteristics such as both hematopoietic and hepatic functions corresponding to the turning point between immigration and emigration of the hematopoietic system.

2 Systematic researches on the regulation of HPO gene expression and signal transduction mechanism

As stated above, the HPO family contains human HPO, rat ALR, mouse ALR, yeast ERVI, ERV2, and virus E1OR. Human HPO, the first member of the family, was identified by our laboratory in 1980's. And then, rat ALR, mouse ALR, yeast ERVI, ERV2, virus E1OR were identified and cloned one after another. HPO was mainly studied by Chinese scientists. Before this project, molecular cloning, preparation of recombinant protein, characterization of its anti-hepatic fibrosis effect and preclinical effect of serious hepatitis remedy have been accomplished. The studies of ALR in rat and mouse have revealed the significant enhancement property in liver regeneration. In recent years, HPO was reported to function as liver immunomodulator and was extensively used in organ transplantation. However, it hasn't been proved whether it could stimulate directly the proliferation of hepatocytes. Researches about the yeast ERV focused on its function and mechanism in oxidative phosphorylation of mitochondrion. The studies on virus E10R found that it have disulfide bond isomerase activity. Until 2001, all members of HPO family have been shown to have isomerase activity except human HPO. Considering that there is no evidence of mouse and rat ALRs directly on hepatocyte and that yeast ERV is localized in mitochondrion, the facts that there are no proof of existence of their membrane receptors and no example of cytokine with enzyme activity, and the discovery of its disulfide bond isomerase activity all together confused researchers: is HPO family really cytokine? Are there the receptors of HPO? Is there transmembrane signal transduction of HPO? What is its intracellular signal transduction? So it is a real black-box on HPO family signal transduction before this study. So far, the extra- and intra-cellular signal transduction pathways we found are the only ones for all the members of HPO family. Among members of this family, HPO is the only one whose receptor was identified hitherto. We have obtained the following results concerning those questions.

2.1 Regulation of the gene expression of HPO

It was revealed that the 5'-flanking region at the initiation codon of HPO has promoter activity in HepG2, HeLa and HEK293 cells. The negative regulatory element spans from the positions −416 to −236 relative to the transcriptional start site. The core promoter spans from the positions −54 to +42, which contains an initiator and three tandem repeats of IFE element surrounding the transcriptional start site. Any mutation in each element resulted in dramatic decrease of the transcriptional activity. This is the first report on the regulation of gene expression of HPO.

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2.2 An autocrine loop of HPO and HPO receptor exists in hepatoma cells

We demonstrated that an autocrine loop of HPO and HPO receptor exists in hepatoma cell lines and that the autocrined HPO was critically important for the autonomous proliferation of hepatoma cells.

2.3 Identification and characterization of the receptor for HPO

Through labeling with radioactive isotope (^{125}I), we demonstrated that there were specific receptors for HPO with high affinity on the cellular membrane of rat hepatocytes, cells of human fetal liver and human hepatoma cells. The density of HPO receptor was about 10, 000 per cell in rat hepatocytes, 20, 000 in human liver cells and 55, 000 in human hepatoma cells. The kinetic dissociation constant (K_d) was 2.0 pM, 1.4 pM and 0.7 pM respectively. The number and binding affinity of HPO receptors among these three types of cells above are also different. The binding could not be inhibited by EGF, TGF-β and insulin. The tissue distribution and subcellular localization of HPO receptor were specific to liver tissues or liver-derived hepatoma cells. Affinity cross-linking of the receptor displayed a polypeptide of molecular mass approximately 75 kD by SDS-PAGE analysis. These results confirmed the existence of a specific HPO receptor in hepatocytes, which may account for the potentiation of HPO to hepatocytes proliferation[2]. Our findings implicated its functional mechanism of receptor-based signal transduction. Since HPO is a novel and specific cytokine to stimulate the proliferation of hepatocytes, the identification and characterization of the receptor is critically important for understanding the whole family of HPO.

2.4 Extracellular HPO stimulates hepatocyte proliferation *via* MAPK pathway

We further demonstrated that extracellular HPO could stimulate proliferation of hepatocytes *via* the mitogen-activated protein kinase (MAPK) pathway, whereas HPO has no effect on the activation of STAT3 and STAT4. HPO could activate EGF receptor (EGFR) in the manner different from that of EGF, following with activation of MAPK pathway *via* EGFR to stimulate cell proliferation. HPO could synergize with EGF to stimulate the proliferation of primarily-cultured hepatocytes. The results also showed that the tyrosine kinase activity of EGFR was necessary for the biological effect of HPO and that the activation of EGFR and MAPK by HPO was depended on the HPO receptor. Regarding the Genistein sensitivity, there was significant difference in the activation of EGFR between HPO and EGF. In summary, our data clearly showed that the transmembrane signal transduction pathway of HPO is "extracellular HPO→HPO receptor→EGF receptor→Raf→MEK → MAPK"[3]. Some previous reports have shown that EGFR not only played a key role in transmembrane signal transduction of EGF and TGF-α as their receptor, but also took part in other signal transduction pathway in ligands- (EGF/TGF-α) independent mode, such as G protein coupled receptor. The fact that EGFR took part in HPO transmembrane signal transduction as cytokine or growth factor receptor in EGF-independent mode have not been found before.

2.5 Intracellular HPO activates transcription factor AP-1 through interaction with JAB1

At first, we showed HPO interacts specifically with JAB1 (Jun activation domain-binding protein 1) both *in vitro* and *in vivo*. The interaction region of HPO with JAB1 was mapped to the 63 amino acids at N-terminal (Full length of the 15 kDa isoform HPO contains 125 amino acids). HPO could trigger the signal transduction pathway "HPO→JAB1→AP-1" *in vivo*. The potentiation effect of HPO on AP-1 activity *via* interaction with JAB1 was depended on intracrine way of HPO. The physical interaction of HPO and JAB1 enhanced AP-1 activity through increase of the phosphorylation level of c-Jun. However, neither HPO nor JAB1 had any effect on the expression of transfected c-Jun, endogenous JNK, or its phosphorylation, indicating that the intracrined HPO potentiates AP-1 activity through JAB1 independent of MAPK (ERK, JNK) pathway. Our data showed that intracrined HPO interacts with JAB1 *in vivo* to regulate AP-1 transcriptional activity, indicating a novel mechanism for AP-1 activation by cytokines and growth factors[4]. To our knowledge, HPO is the first intracrined growth factor triggering AP-1 pathway through intracellular interaction with a transcriptional coactivator. Intracrined HPO with JAB1 represents for a novel intracellular immediately responsed signaling pathway in triggering transcription factors during initiation of liver regeneration. More interestingly, Richard N Re, the founder of "Intracrine hypothesis", cited our result of HPO intracellular signal pathway in his review twice. However, the similar MIF reported in *Nature* was not mentioned at all probably because the concentration the author used

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was extremely higher than the physiological level.

2.6 Cytokine HPO is a flavin-linked sulfhydryl oxidase, while the enzymatic activity is essential for its intracellular biological function

Two intramolecular disulfides were identified in HPO in our study. One is formed by the cysteine residues within the motif redox CXXC and functions as the enzymatic center. It was demonstrated that HPO was a flavin-linked sulfhydryl oxidase, while the CXXC motif is essential for its enzymatic activity. The signal transduction of intracellular HPO was depended on its sulfhydryl oxidase activity, whereas the signaling of extracellular HPO did not[5]. This is the first report bridging the function of cytokine and the enzymatic activity, and was immediately confirmed in other molecules shortly after our publication.

According to which, we found that HPO functions in both autocrine and intracrine pathways. The pathways could be described as: extracellular HPO→HPO receptor→EGF receptor→Raf→MEK→MAPK and intracellular HPO→JAB1→c-JUN (AP-1), respectively. The autocrine pathway elucidated for the first time the mechanism for the synergic effects of HPO and EGF in stimulating the proliferation of hepatocytes, whereas the intracrine pathway provide the shortest signal pathway and the most immediate response for liver regeneration. To date, both the two HPO signal transduction pathways are the only knowledge about the signal transduction for the whole HPO family (including human HPO, rat/mouse ALRs, yeast ERV1 and ERV2 and virus E10R). These findings will be significant in guiding the studies on the orthologs of HPO.

3 Characterization and functional annotations of the Semaphorin, LSECtin and ARFGAP family genes

3.1 Cloning and functional studies of two novel members of the semaphorin gene family, SEMA6C and SEMA6D

It was found that both SEMA6C and SEMA6D belong to class VI of the semaphorin family with a typical transmembrane domain. Three isoforms of SEMA6C and five isoforms of SEMA6D were identified respectively. Among the adult tissues, SEMA6C is expressed only in skeletal muscle, whereas SEMA6D is expressed abundantly in kidney, brain, and placenta. Deletion analysis indicated that the SEMA domain and the PSI domain are integrally necessary for correct post-translation modification and subcellular localization. The extracellular domain of SEMA6C could inhibit axonal extension of differentiated PC12 cells induced by nerve growth factor and could cause the growth cone collapse of chicken dorsal root ganglion, rat hippocampal neurons, and rat cortical neurons in a dose-dependent manner. SEMA6D acted like SEMA6C except that it had no significant effect on the growth cones of rat cortical neurons. These results suggested that SEMA6C and SEMA6D play important roles in development of nervous system[6]. Semaphorin is one kind of important gene family, and the research on its members has been reported frequently in top international journals. Previous studies demonstrated two members, Sema6A and Sema6B in the class VI subgroup of this family. In this study, we cloned two novel members of the subgroup, Sema6C and Sema6D which splicing form, tissue expressing profile and biological function are quite different from Sema6A and Sema6B and have contribute a lot in this field[7,8].

3.2 Cloning, characterization, and functional study of a novel C-type lectin LSECtin/CD23L and discovery of a novel LSECtin gene family

LSECtin, a novel adhesion molecule, was mapped to chromosome 19p13.3 adjacent to the previously described C-type lectins, CD23, DC-SIGN and DC-SIGNR. The LSECtin gene was localized between the CD23 gene and DC-SIGN gene, which was followed by the DC-SIGNR gene. From the direction of centromere to telomere, the four genes for DC-SIGNR, DC-SIGN, LSECtin and CD23 are perfectly arrayed in tandem repeat and formed a compact cluster in an insert size of 105 kb. The LSECtin protein shows 32%, 31% and 31% identity to DC-SIGNR, DC-SIGN and CD23 at amino acid levels, respectively. LSECtin was previously designated by us as CD23L (CD23-like protein) because of being homologous to CD23. Considering its specific expression in sinusoidal cells of liver and lymph node, it was renamed as LSECtin representing for a novel C-type lectin. LSECtin is a type II integral C-type lectin with a short N-terminal domain in cytoplasm, a transmembrane domain, a neck region and a single carbohydrate recognition domain (CRD). LSECtin was only expressed in liver and lymph node among 15 human tissues tested, intriguingly neither expressed on hematopoietic cell lines nor on monocyte-derived

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dendritic cells. Moreover, LSECtin is expressed predominantly in sinusoidal endothelial cells of human liver and lymph node and co-expressed with DC-SIGNR. LSECtin could bind to mannose, N-acetylglucosamine (GlcNAc) and fucose in a Ca^{2+}-dependent manner but not to galactose. LSECtin is an adhesion molecule and preferentially binds to activated T cells. This binding activity is inhibited by both EDTA and mannose, which indicating that the adherence of LSECtin to activated T cells is mediated by Ca^{2+} and mannose-like glycogroups. Taken together, LSECtin belongs to the adhesion molecule family and plays an important role in cell-cell or cell-molecule recognition[9]. Before the family of LSECtin was found, CD23, DC-SIGN and DC-SINGR had been reported. Besides as a receptor of IgE, CD23 plays an important role in various biological and pathological activities. DC-SIGN and DC-SIGNR were specially expressed on the surface of dendritic cells and acted as the famous trans-receptors of HIV and HCV, which is quite important in this field of adhesion molecule[10].

3.3 Cloning and identification of ARFGAP1 (renamed as ARFGAP3 later), the first human member of ARF GAP (ADP-ribosylation factor GTPase activating protein) family

We found that the ARFGAP1 protein contains a typical ARFGAP domain at N-terminus that might play a vital role for the function, in which for the first time a zinc finger motif ($CXXCX_{16}CXXC$) was found. Moreover, we found a repeat motif $SISSX_3FG$ at its C-terminus. This motif was conserved in other ARFGAP proteins and might function in localization to some intracellular membranes. ARFGAP1 is highly expressed in glands and testis. It is localized in the cytoplasm and concentrated in perinuclear region, particularly the Golgi body when overexpressed. ARFGAP3 had GAP activity *in vitro* and the GAP activity of ARFGAP1 was regulated by phospholipids, i.e. phosphatidylinositol 4, 5-diphosphate as agonist and phosphatidylcholine as antagonist. Its transiently ectopic overexpression in cultured mammalian cells reduced the constitutive secretion of secreted alkaline phosphatase, indicating that its ectopic overexpression inhibits the early secretory pathway of proteins *in vivo*. These results demonstrated that ARFGAP1 is a novel GAP for ARF1 and might be involved in intracellular traffic of proteins and vesicular transport as predicted.

4 Conclusion remarks

4.1 Establishment of gene expression profiling of HFL, and cloning and identification of a series of novel genes: The molecular basis of the multiple physiological functions (liver, hematopoiesis, tissue immigration/emigration and development) of HFL22W was panoramically revealed by the most comprehensive gene expression profile in this study. There are 201 unrecorded human homologous genes (only found in other species) and 669 novel genes have been identified and annotated, totally accounts for ~7% of the human protein-coding genes (about 12000) in 2001.

4.2 Mechanisms of signal transduction of HPO: There are two signal transduction pathways of HPO through autocrine or intracrine way have been revealed: (1) extracellular HPO→HPO receptor→EGF receptor→Raf→MEK→MAPK); and (2) intracellular HPO→JAB1→c-JUN (AP-1). HPO has an FAD-linked sulfhydryl oxidase activity, where the CXXC motif is the enzymatic activity center. Only its intracrine pathway is dependent on the enzymatic activity, building a bridge between the cytokine function and enzymatic activity for the first time. The promoter of HPO includes an inhibitory element and a core promoter comprising an initiator and its flanking three tandem IFE elements.

4.3 The novel LSECtin family and its founder member LSECtin, and novel members SEMA6C and SEMA6D of Semaphorin family: Cloning and systematical elucidation of their gene structures and functions, which act as the functional representatives of cell migration, adhesion (recognition) in the fetal liver, finding of the LSECtin family (including CD23, LSECtin, DC-SIGN, DC-SIGNR) whose genes form a tight cluster on chromosome 19p13.3 and are highly homologous to each other.

4.4 The first human member of ARFGAP family—ARFGAP1: Cloning and systematical elucidation of its gene structure and functional domains as an important regulator of protein secretion of fetal liver.

Taken together, we have established the largest gene expression profile of fetal liver in the world, identified a set of gene clusters functionally related to the liver development, hepatocarcinogenesis and hematopoiesis, and for the first time panoramically revealed the molecular mechanism of its dual functions. After comparing the related data obtained by

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others on the stages including HFL19W when the hematopoietic system immigrated and hematopoiesis initiated; HFL22W which reflects the turning point of both the blooming and fade of liver hematopoiesis and the immigration/emigration of the hematopoietic system; HFL40W when hematopoiesis is faded and the hematopoietic system emigrated; and adult human liver which lacks hematopoietic function under normal conditions and some related cells including Itoh cells in the liver, hepatoma cells, bone marrow cells, $CD34^{+}$ hematopoietic progenitor cells, ES cells, neutrophile granular cells, leukemia cells and so on, a cluster of genes associated with the development of human liver, hepatocellular tumorigenesis as well as hematopoiesis were successfully identified in our study. Thus, our project, for the first time, has systematically discovered the dual functions of HFL in both hematopoiesis and hepatic metabolism and explored the molecular mechanisms for the blooming/decay of hematopoiesis and immigration/emigration of hematopoietic system. Moreover, the identification of four gene families including HPO, Semaphorin, LSECtin and ARFGAP identified in this project also provide new insights on the molecular mechanism of the dual functions of human fetal liver. The publications based on this project have been cited 145 times by SCI journals before 2005. The project has also provided important original data for the annotation of human genome and establishment of human transcriptome. It played a role in Chinese national achievement of cloning and annotation of the 10% human cDNAs project and set up the corner-stone for the leading role of China in the international "Human Liver Proteome Project".

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