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HNF factors form a network to regulate liver-enriched genes in zebrafish

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Abstract

Defects in some of liver-enriched genes in mammals will cause liver- and/or blood-related diseases. However, due to the fact that embryogenesis happens intrauterinally in the mammals, the function of these liver-enriched genes during liver organogenesis is poorly studied. We report here the identification of 129 genuine liver-enriched genes in adult zebrafish and show that, through in situ hybridization, 69 of these genes are also enriched in the embryonic liver. External embryogenesis coupled with the well-established morpholino-mediated gene knock-down technique in zebrafish offers us a unique opportunity to study if this group of genes plays any role during liver organogenesis in the future. As an example, preliminary study using morpholino-mediated gene knock-down method revealed that a novel liver-enriched gene *leg1* is crucial for the liver expansion growth. We also report the analysis of promoter regions of 51 liver-enriched genes by searching putative binding sites for Hnf1, Hnf3, Hnf4 and Hnf6, four key transcription factors enriched in the liver. We found that promoter regions of majority of liver-enriched genes contain putative binding sites for more than one HNF factors, suggesting that most of liver-enriched genes are likely co-regulated by different combination of HNF factors. This observation supports the hypothesis that these four liver-enriched transcription factors form a network in controlling the expression of liver-specific or -enriched genes in the liver.

Keywords: Microarray; Liver-enriched genes; HNF factors; Liver development; Promoter analysis; Functional genomics

Introduction

Adult liver is one of the largest organs in the body and consists of two lobes. Approximately 60% of cells in the adult rat liver are hepatocytes, while the remaining cells consist largely of cholangiocytes (bile duct cells), Kuppfer cells, stellate cells and a variety of endothelial cells including those lining the sinusoids (sinusoidal endothelial cells) (Blouin et al., 1977). Liver is an essential organ that plays a number of vital functions in the body. The functions of the liver include processing nutrients from ingested food, forming and maintaining metabolite and serum protein concentrations in the blood and serving as a site for detoxification and for hematopoiesis during gestation. In response to the metabolic demand of the body, the liver expresses vast varieties of genes to encode enzymes and blood components to comply with these diverse functions (Kawamoto et al., 1996). Among the vast number of genes expressed in a mature liver, expression of many genes, including genes for transcription factors and metabolic enzymes, has been found to be specific to or enriched in the liver (Cereghini, 1996; Kawamoto et al., 1996).

The liver is originated from the definitive endoderm. Based on extensive studies (cell lineage tracing, anatomic, molecular and genetic studies) in chick and mouse, the process of liver organogenesis is arbitrarily divided into the following stages: (1) the ventral foregut endodermal cells proximal to cardiogenic mesoderm to gain competence to become hepatoblasts; (2) specification of hepatoblast; (3) formation of liver bud; (4) fast proliferation of hepatoblasts and (5) differentiation of hepatoblast cells into hepatocytes and billiary lineages (Duncan, 2003; Zaret, 2002). The accomplishment of liver organogenesis is under the control of a genetic network that relies on the precise coordinate actions of many genes that are essential for normal development. Gene expression profiling, for example, has revealed that gene expression patterns in the liver change accordingly during the different stage of liver development (Jochheim et al., 2003; Petkov et al., 2004). Although genes enriched in a mature liver have been well-established, genes

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enriched in the developing liver have been less systematically studied. Only a handful of such genes were obtained in *Xenopus* through screening the expression cDNA libraries (Chen et al., 2003; Zorn and Mason, 2001). In addition, it is an intriguing question if mature liver-enriched genes are also enriched in the embryonic liver since this group of genes might play a crucial role for maintaining the liver structure or the status of liver stem cells that are important for liver regeneration after hepatectomy (Su et al., 2002).

Due to some of its unique advantages for genetic studies, zebrafish (*Denrio rerio*) has been adopted as a genetic model system to study the vertebrate development in recent years (Driever et al., 1996; Haffter et al., 1996). Zebrafish is particularly amenable for the study of liver organogenesis. External development and optical clarity of embryo facilitate histological studies of liver development during early embryogenesis. In mammals, since the embryonic liver is an early hematopoietic organ, mutations affecting liver development will cause anemia that will lead to early lethality (Reimold et al., 2000), thus making in vivo studies of the progress of mouse liver morphogenesis difficult. Hematopoiesis in zebrafish takes place in the intermediate cell mass (ICM) and subsequently in the kidney, not the liver (Thisse and Zon, 2002), thus liver defects do not lead to anemia. Meanwhile, zebrafish embryos receive enough oxygen through diffusion to allow embryonic development to proceed relatively normally for several days even without blood circulation (Stainier, 2001), eliminating some of the problems encountered with mammalian model organisms. There have been a few reports describing the early liver organogenesis in zebrafish (Field et al., 2003; Ober et al., 2003; Wallace and Pack, 2003). By using a gut GFP line, Field et al. reveals two phases of liver morphogenesis: budding and growth. The budding period, which can be further subdivided into three stages, starts when hepatocytes first aggregate, shortly after 24 h post-fertilization (hpf), and ends with the formation of a hepatic duct at 50 hpf. The growth phase immediately follows and is responsible for a dramatic alteration of liver size and shape (Field et al., 2003). The adult zebrafish liver, as in mouse and human, consists of two lobes and is surrounded by blood vessels. Recently, through screening for hepatomegaly in 297 zebrafish lines bearing mutations in genes that are essential for embryogenesis, seven genes have been identified to encode factors important for regulating liver expansion growth, including the class C vacuolar sorting protein Vps18, transcription factor Sox9a, novel factor foie gras (Fgr), etc. (Sadler et al., 2005). On the other hand, loss-of-function of *def*, a novel pan-endoderm gene, and of *nil per os* (*npo*) that encodes an RNA-binding protein, caused hypoplastic liver, pancreas and gut (Chen et al., 2005; Mayer and Fishman, 2003). Other studies have shown that *gata5* (Reiter et al., 1999), *hhex* (Wallace et al., 2001), *hnf1* (Sun and Hopkins, 2001), *hnf6* (Matthews et al., 2004) and *prox1* (Liu et al., 2003), as known in mammals and other vertebrates, play crucial roles in controlling liver organogenesis and cell differentiation in zebrafish.

Our primary interest is to identify genes that are important for liver initiation and development in zebrafish. For this purpose, we have been taking both genomic and genetic approaches. In this report, we present our work on identification of 129 genuine adult liver-enriched genes in zebrafish via the genomic approach. Further analysis based on database mining revealed that majority of these liver-enriched genes in zebrafish share high homology with their mammalian counterparts, suggesting that the profile of liver-enriched genes in an adult zebrafish liver is similar to that identified in mammals and other vertebrates. Based on analysis of the zebrafish genome sequence available in both NCBI and ESEMBLE database, promoter regions for 51 genes were obtained and used for identification of putative binding sites for Hnf1, Hnf3, Hnf4 and Hnf6, four well-known liver-enriched transcription factors (Cereghini, 1996; Schrem et al., 2002). We found that, only in very rare cases a liver-enriched gene is solely regulated by a single of these four transcription factors. Majority of liver-enriched genes are most likely co-regulated by different combination of these transcription factors. We also examined the expression of all 129 adult liver-enriched genes during embryonic stages from 3–5 days post-fertilization (dpf) and found that more than half of these genes are also enriched in the embryonic liver. As an initial effort to study the function of these liver-enriched genes during the early stage of liver development, a novel gene, *liver-enriched gene 1* (*leg1*), was chosen from the list and was subjected to preliminary functional study via morpholino-mediated gene knock-down method. We found that *leg1* morphant conferred a smaller liver phenotype, demonstrating that *leg1* is essential for the expansion growth of the embryonic liver in zebrafish. Extensive efforts will be made to study if and how this group of genes is involved in controlling liver development in zebrafish in the future.

Materials and methods

Affymetrix zebrafish Genechip hybridization

Total RNA from the adult liver and the liver-free remaining body from three independent batches of zebrafish (wild type AB strain) was extracted using TRIzol (Gibco-BRL, USA), treated with DNaseI and purified through Qiagen RNeasy kit (Qiagen, Germany). cDNA synthesis, RNA probe labeling, GeneChip hybridization, washing and staining were performed following the manufacturer's instructions (Affymetrix, Santa Clara, California, USA). GeneChip arrays were scanned on an Affymetrix probe array scanner. Data were analyzed using a statistics software MAS5.0 from Affymetrix.

cDNA microarray hybridization

Total RNA from the adult liver and the liver-free remaining body (wild type AB strain) was extracted using TRIzol (Gibco-BRL, USA) followed by mRNA purification (PolyA Tract, *Promega*). The mRNA samples were labeled, reciprocally, with fluorescent dye Cy3 and Cy5. Generation of zebrafish cDNA microarray, microarray hybridization and microrray data analysis were performed as described (Lo et al., 2003; Wen et al., 2005).

RNA analysis

Ten micrograms of total RNA was separated on a formaldehyde gel and then transferred to nylon membrane (Hybond N+, Amersham). Probes for candidate genes were DIG-labeled (Roche Molecular Biochemicals) through PCR amplification using vector primer pairs T3/T7, respectively. For candidate genes that have matches in our own EST collection, the corresponding EST clones were retrieved and used for probe labeling. For 22 candidate genes