Research Article

JOURNAL OF HEPATOLOGY

Liver X receptor α (LXRα/NR1H3) regulates differentiation of hepatocyte-like cells via reciprocal regulation of HNF4α

Kai-Ting Chen[1,2,3], Kelig Pernelle[4], Yuan-Hau Tsai[2], Yu-Hsuan Wu[2], Jui-Yu Hsieh[2], Ko-Hsun Liao[2], Christiane Guguen-Guillouzo[4,5], Hsei-Wei Wang[1,2,6,7,*]

[1]*Taiwan International Graduate Program in Molecular Medicine, National Yang-Ming University and Academia Sinica, Taipei, Taiwan;* [2]*Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan;* [3]*Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan;* [4]*Inserm UMR 991, Université de Rennes 1, Faculté de médecine, F-35043 Rennes cedex, France;* [5]*Biopredic international, Parc d'activité Bretèche batA4, 35760 Saint-Grégoire, France;* [6]*YM-VGH Genome Research Center, National Yang-Ming University, Taipei, Taiwan;* [7]*Department of Education and Research, Taipei City Hospital, Taipei, Taiwan*

Background & Aims: Hepatocyte-like cells, differentiated from different stem cell sources, are considered to have a range of possible therapeutic applications, including drug discovery, metabolic disease modelling, and cell transplantation. However, little is known about how stem cells differentiate into mature and functional hepatocytes.

Methods: Using transcriptomic screening, a transcription factor, liver X receptor α (NR1H3), was identified as increased during HepaRG cell hepatogenesis; this protein was also upregulated during embryonic stem cell and induced pluripotent stem cell differentiation.

Results: Overexpressing NR1H3 in human HepaRG cells promoted hepatic maturation; the hepatocyte-like cells exhibited various functions associated with mature hepatocytes, including cytochrome P450 (CYP) enzyme activity, secretion of urea and albumin, upregulation of hepatic-specific transcripts and an increase in glycogen storage. Importantly, the NR1H3-derived hepatocyte-like cells were able to rescue lethal fulminant hepatic failure using a non-obese diabetic/severe combined immunodeficient mouse model.

Conclusions: In this study, we found that NR1H3 accelerates hepatic differentiation through an HNF4α-dependent reciprocal network. This contributes to hepatogenesis and is therapeutically beneficial to liver disease.

Keywords: Hepatogenesis; LXRα/NR1H3; HNF4A; HepaRG progenitor cell.
Received 19 December 2013; received in revised form 10 July 2014; accepted 14 July 2014; available online 27 July 2014

* Corresponding author. Address: National Yang-Ming University, No. 155, Sec. 2, Linong Street, Taipei 112, Taiwan. Tel.: +886 2 2826 7109.
E-mail address: hwwang@ym.edu.tw (H.-W. Wang).

Abbreviations: NR1H3, liver X receptor α; HNF, hepatocyte nuclear factor; MSCs, mesenchymal stem cells; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; RT, reverse-transcription; ChIP, chromatin immunoprecipitation.

Introduction

Liver development depends on a complex network, requiring several growth factors, genetic homeostasis and cell-extracellular matrix interactions [1]. Identifying the hepatogenesis-promoting factors, favouring liver development, is thought to be essential for liver regeneration and could improve hepatocyte transplantation for end-stage liver disease [2]. Different sources of progenitor cells have been investigated as potential sources for hepatic differentiation, including human mesenchymal stem cells (MSCs) [3,4], embryonic stem cells (ESCs) [5] and induced pluripotent stem cells (iPSCs) [6], all of which hold the potential to differentiate into hepatocyte-like cells. However, unstable hepatic function and atypical morphology have limited the usefulness of present cell treatments. Thus, improved progenitor cells are needed in order to carry out further studies of the treatments of liver disease.

The human bipotent liver progenitor cell line HepaRG is an alternative source of cells that is used for toxicity screening during drug discovery [7,8], viral hepatitis research, hepatocyte differentiation and transplantation testing [9]. This cell line has several potential advantages. First, during differentiation, HepaRG cells evolve from a homogeneous depolarized epithelial phenotype showing no specific organization into a structurally well-defined and polarized monolayer that closely resembles those formed by primary human hepatocytes in culture [10]. Secondly, these cells show long-term stability in terms of various mature hepatocyte functions, including iron storage, cytochrome P450 enzyme activities and albumin secretion [11]; Thirdly, polarized hepatocyte-like cells differentiated from HepaRG cells mimic mature primary human hepatocytes (PHHs) [12], as evidenced by the fact that they can support the *in vitro* infection and replication of human hepatitis B virus (HBV) [13], a feature that has not been observed in hepatocyte-like cells generated from other stem cell systems. Thus, the HepaRG cell line has become a promising *in vitro* model to determine not only the basic regulation of hepatic cell fate but also the maturation into hepatocytes.

In order to screen the hepatic differentiation-inducing factors, genome-wide studies have been conducted in other stem cell systems to identify the regulatory circuitry involved in liver development and hepatic cell specification. For example, by genome-wide chromatin immunoprecipitation (ChIP) combined with promoter microarray analysis in liver and pancreas tissues, the transcriptional regulators HNF1α, HNF4α and HNF6α were found to occupy the promoters of various tissue-specific genes and were also shown to be involved in the control of regulatory circuits that are necessary for normal hepatocyte function [14]. In addition, HNF4α was found to be essential for the specification of hepatic progenitors from human pluripotent stem cells (iPSCs) [15]. The homeobox transcription factor Prox1 (prospero-related homeobox 1), an early specific marker during the development of liver and pancreas from the foregut endoderm, is a co-regulator of HNF4α and human liver receptor homolog-1 (hLRH-1) [16,17]. Furthermore, combining genome-wide location and microarray analysis has also helped to reveal the targets of C/EBP beta (CCAAT enhancer-binding protein beta), a basic-leucine zipper transcription factor; these targets are critical to the regulation of numerous biological processes, including liver development [18]. These and other findings have demonstrated that a genomic approach is one of the key ways of screening for important candidates that are likely to control hepatocyte differentiation and liver development [19,20].

In this study we have identified the function of a novel transcription factor, liver X receptor α (NR1H3), and show that it is involved in the induction of the hepatic differentiation. This was done by establishing the genomic profile associated with the development of functional hepatocyte-like cells from undifferentiated HepaRG cells. Hepatocytes overexpressing NR1H3 showed significant increases in several adult liver cell markers and in the process gained a range of liver cell functions; moreover, these cells were able to rescue hepatic failure when transplanted into CCl_4-injured severe combined immunodeficiency (SCID) mice.

Materials and methods

HepaRG cell culture

HepaRG cells were cultured in William's E medium (Gibco) supplemented with 10% FCS, 100 mM glutamine, 5 mg/ml insulin, 50 μM hydrocortisone hemisuccinate and 1% penicillin. The differentiated HepaRG cells were obtained as described [10].

RNA isolation, reverse-transcription PCR (RT-PCR) and quantitative RT-PCR

RNA was extracted using the RNeasy kit (Qiagen). 1 μg RNA was reverse-transcribed into cDNA using the Moloney murine leukemia virus reverse-transcriptase (M-MLV Reverse Transcriptase, Promega). For the reverse-transcription polymerase chain reaction (RT-PCR), the following conditions were used: 94 °C for 40 s, 56 °C for 50 s, 72 °C for 60 s for 30 cycles, after an initial denaturation at 94 °C for 5 min. The primers used are listed in Table 1. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems), and normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to quantify the messenger RNA levels.

Periodic acid-Schiff (PAS) staining

HepaRG cells were fixed in 4% formaldehyde for 30 min and then permeabilized with 0.1% Triton X-100 for 10 min. The cells were next either treated or not treated with diastase (Sigma–Aldrich) for 40 min, 37 °C. The samples were then oxidized in 1% periodic acid for 5 min, rinsed three times in deionized (d) H_2O, treated with Schiff's reagent (Sigma–Aldrich) for 15 min in the dark, and rinsed in dH_2O for 5 min. Finally, cells were visualized by light microscopy.

Table 1. Primers for RT-qPCR.

Primer	Sequence
Albumin	F: GGC ACA ATG AAG TGG GTA AC
	R: AGG CAA TCA ACA CCA AGG
CK-18	F: CTA CAT CAA CAA CCT TAG GC
	R: TCC ACA TCC TTC TTG ATG
HNF4A	F: CCA AGT ACA TCC CAG CTT TC
	R: TTG GCA TCT GGG TCA AAG
TDO2	F: GGA ACT ACC TGC ATT TGG
	R: TCT CTG AAG TCA TTG AAG TCC
TAT	F: ACT GTG TTT GGA AAC CTG CC
	R: GCA GCC ACT TGT CAG AAT GA
CYP3A4	F: CCT TAC ATA TAC ACA CCC TTT G
	R: GGT TGA AGA AGT CCT CCT AAG CT
CYP2B6	F: ATG GGG CAC TGA AAA AGA CTG A
	R: AGA GGC GGG GAC ACT GAA TGA C
NR1H3	F: GCT GCA AGT GGA ATT CAT CAA CC
	R: ATA TGT GTG CTG CAG CCT CTC CA
ETV-1	F: GTG CCT GTA CAA TGT CAG TG
	R: GGT AGC TGC TAT CTG GTA TG
HHEX	F: GGT AAG CCT CTG CTG TGG TC
	R: TCT TCT CCA GCT CGA TGG TT
XBP-1	F: ACA CGC TTG GGA ATG GAC AC
	R: CCA TGG GAA GAT GTT CTG GG
GAPDH	F: GAG TCC ACT GGC GTC TTC
	R: GAC TGT GGT CAT GAG TCC TTC

Measurement of cytochrome P450 enzyme activity

CYP3A4 activity was measured using a P450-Glo Assays kit (Promega) according the manufacturer's instructions. Briefly, cells were washed with phosphate-buffered saline (PBS), which was then replaced with fresh medium containing luminogenic CYP3A4 substrate luciferin-PFBE, followed by a 3 h incubation at 37 °C. To determine CYP P450 enzyme activity, 50 μl of medium was transferred and 50 μl luciferin detection reagent was added to initiate the luminescent reaction for 30 min. The luminescence of the mixture was then read using an Infinite M1000 (TECAN Group Ltd.) luminometer. Cytochrome activity was stated as relative light units (RLU)/10^6 cells/ml.

Gene expression microarray

Total RNA was isolated from HepaRG cells by RNeasy Mini kit (Qiagen). The expression profiles were performed by the National Research Progress for Genomic Medicine Microarray and Gene expression analysis Core Facility, National Yang-Ming University, Taiwan. Gene expression analysis was done as described in our previous publication [21].

Animal model and HepaRG cell transplantation

Five-week-old NOD-SCID mice were injected intrasplenically with 2.5×10^7 viable NR1H3-derived hepatocytes and HepaRG cells per kilogram body weight 24 h after administration of carbon tetrachloride (CCl_4), the procedure was modified based on previously publications [22,23]. Primary human hepatocytes were purchased from Life Technologies (HEP10, Gibco®) and cultured according the manufacturer's instructions.