Forced Expression of *Hnf1b/Foxa3* Promotes Hepatic Fate of Embryonic Stem Cells

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Abstract

Embryonic stem (ES) cell-derived hepatocytes have the potential to be used for basic research, regenerative medicine, and drug discovery. Recent reports demonstrated that in addition to conventional differentiation inducers such as chemical compounds and cytokines, overexpression of lineage-specific transcription factors could induce ES cells to differentiate to a hepatic fate. Here, we hypothesized that lentivirus-mediated inducible expression of hepatic lineage transcription factors could enhance mouse ES cells to hepatocyte-like cells. We screened the effects of candidate transcription factors *Hnf1b*, *Hnf1a*, *Hnf4a*, *Foxa1*, *Foxa3* and *Hex*, and determined that the combination of *Hnf1b*/*Foxa3* promoted expression of several hepatic lineage-specific markers and proteins, in addition to glycogen storage, ICG uptake, and secretion of albumin and urea. The differentiated cells were engraftable and expressed albumin when transplanted into a carbon tetrachloride-injured mouse model. These results demonstrated the crucial role of *Hnf1b* and *Foxa3* in hepatogenesis *in vitro* and provided a valuable tool for the efficient differentiation of HLCs from ES cells.

Keywords: Embryonic stem cells; Forced expression; Hepatic-lineage induction; Hepatic transcription factors.

1. Introduction

Embryonic stem (ES) cells, due to their unlimited self-renewal capacities and diverse differentiation abilities [1], are expected to provide an unlimited source of cells for various applications. ES cell-derived hepatocyte-like cells (HLCs) can be used for basic research, regenerative medicine, and drug discovery [2, 3]. Numerous studies have substantially contributed to the differentiation of ES cells into hepatocytes by reporting continual improvement of growth factors, cell-matrix reconstruction, cell-cell interactions, and genetic or epigenetic modifications (for review see [4]). Another approach for hepatocyte generation *in vitro* is the direct conversion or trans-differentiation of somatic cells to induced hepatocyte-like cells (HLCs) with combinations of different transcription factors [5-10] under hepatic conditions. Most of these differentiation protocols attempt to recapitulate liver development within a few days of the *in vitro* culture.

Over the last few years a number of transcription factors have been identified that have pivotal roles in liver development [11, 12]. Therefore, it can be speculated that certain specific transcription factors or a combination of them have the capability to facilitate differentiation or direct reprogramming of ES cells into HLCs. Recently, it has been demonstrated that transcription factors or micRNAs which constitutively or transiently express in ES cells could induce hepatic differentiation in addition to conventional differentiation methods [13-19].

We hypothesized that the transient expression of hepatic lineage transcription factors could positively influence and expedite directed differentiation of ES cells into HLCs. In order to prove our hypotheses, we first constructed mouse ES cells that inducibly expressed hepatocyte nuclear factors (*Hnf1b*, *Hnf1a*, *Hnf4a*), forkhead box (*Foxa1*, *Foxa3*) and *Hex* via lentivirus expression vectors. Next, we screened for effective transcription factors and determined that the forced expression of combined *Hnf1b/Foxa3* led to hepatocyte

progenitors (HP) in hepatic medium. The results indicated that this forced expression of *Hnf1b/Foxa3* enhanced hepatic marker expressions, improved functionality of HLCs, and engraftment after transplantation of HPs into mice with carbon tetrachloride-induced acute liver damage.

2. Materials and methods

2.1. Mouse embryonic stem cell culture, differentiation and viral transduction

We cultured mouse embryonic stem (ES) cells (RB20) [20] under standard conditions in 2i [21]. Differentiation was induced by exogenous gene expression in presence of 5 µg/ml doxycycline (Dox) to the basal medium for 7 days. Later, the basal medium was supplemented with 40 ng/ml fibroblast growth factor 4 (FGF4) and 40 ng/ml bone morphogenetic protein 4 (BMP4) for 3 days, followed by 25 ng/ml hepatocyte growth factor (HGF), 10^{-7} M dexamethasone (Dex), 1% insulin-transferrin-selenium (ITS), and 5 mM nicotinamide for an additional 3 days. Finally, the resultant cells were replated on Matrigel-coated dishes in hepatocyte culture medium (HCM) supplemented with 25 ng/ml HGF, 10^{-7} M Dex, and 50 ng/ml oncostatin M (OSM) for 10 days.

LR reactions between the entry clones of the genes of interest and the pLIX403 destination vector were performed in order to obtain the lentiviral expression vectors. The resultant vectors were transfected to 293T cells in conjunction with packaging plasmid psPAX2 and envelop plasmid pMD2.G to produce lentiviruses. Viral supernatant was collected at 24 and 48 h after transfection. For transduction we transfected the ES cells in ES medium with lentiviruses. At 48 h post-transfection, the cells were subjected to puromycin (0.2 µg/ml) to select the transduced cells.

All the procedures including cell cultures, viral transduction, immunostaining, quantitative RT-PCR (qRT-PCR), and functional analyses have been described in details in supplementary Materials and methods.

3. Results

3.1. Screening for hepatic lineage-inducing factors in ES cells led to finding *Hnf1b* and *Foxa3*

In order to transfer transcription factor genes to mouse ES cells, we used a tetracycline (tet) inducible-lentiviral system. Initially, we performed the LR recombination reaction by Gateway to generate an expression plasmid that contained the gene of interest for each factor. Using this system, we have constructed pTRE-GOI-PGK-PuroR-2A-rtTA, in which the PGK promoter constitutively drives reverse tetracycline-controlled transactivator (rtTA) expression. In the Tet-On system, expression of gene of interest (GOI) is correlated with a Tet analog, Dox. In the presence of Dox, rtTA binds to the tetracycline response element (TRE) promoter and induces expression of GOI. In the absence of Dox, rtTA is unable to activate the system (Fig. 1A). Lentivirus particles were produced by co-transfection of 293T cells with the expression construct and the packaging plasmid mix pMD2.G that consisted of VSV-G and psPAX2 which contained *gag/pol*. We transduced ES cells with lentiviruses to generate cells that expressed individual transcription factors under Dox culture conditions. Six transcription factors (*Hnf1b*, *Hnf1a*, *Hnf4a*, *Foxa1*, *Foxa3* and *Hex*) were transduced into mouse ES cells. The genes were selected based on their critical role for embryonic liver development and function - *Foxa1* and *Foxa3* as pioneer factors [22]; *Hex* and *Hnf1b* as hepatic specification factors [23, 24]; and *Hnf1a* and *Hnf4a* as mature hepatic regulators [25, 26]. After 48 h, we applied puromycin to the ES cell medium in order to select successfully transduced cells. Puromycin selected colonies were expanded by passaging. The

immunofluorescence assay for Oct4 confirmed that transduced ES cells maintained their pluripotency state after transduction (Supplementary Fig. 1A). Then RT-PCR analysis was performed to confirm the exogenous gene expressions in transduced cells which cultured in Dox-containing medium for 7 days (Supplementary Fig. 1B).

To examine the effect of ectopic expression of transcription factor on hepatic gene activation, the transcription factors were overexpressed for 7 days in basal medium in the presence of Dox in an adherent culture model which was feeder free and contained low serum, followed by 6 days by withdrawing Dox in the same medium. RT-PCR analysis at day 7 indicated that forced expression of *Hnf1b* induced expression of *Foxa2*, a potent early hepatic regulator (Fig. 1B). Expression analysis at day 13 also showed a 5-20 fold upregulation of *Alb* with *Foxa3* cells which has demonstrated a higher effect (Fig. 1C). *Foxa3* is the most expressed *Foxa* family member in the adult liver [27].

We analyzed the effects of inducible expression of the factors on hepatic differentiation of ES cells in hepatic/inductive medium by culturing the transduced cells for 7 days in basal medium in the presence of Dox. Then, the enforced cells were treated with BMP4 and FGF4 for three days, followed by HGF, ITS, Dex, and nicotinamide for another three days to induce hepatic specification (Fig. 1D). *Hnf1a* and *Hnf1b* overexpressing cells showed a significant higher *Alb* expression in hepatic/inductive medium (Fig. 1E). Under these induction conditions, the morphology of mouse ES cells gradually changed in the two types of *Hnf1a* or *Hnf1b* cells into an extension pattern of short-sided polygons with prominent nuclei (Fig. 1F). Interestingly, a portion of these cells in the *Hnf1a* or *Hnf1b* cells were Alb- and cytokeratin 19 (Ck19)-positive (Fig. 1G).

In order to examine the cooperative effect of *Hnf1b*, *Hnf1a*, and *Foxa3* on the expression of hepatic markers, we constructed double lines in which two transcription factors (*Hnf1b/Hnf1a*, *Hnf1b/Foxa3*, or *Hnf1a/Foxa3*) overexpressed for one week in basal medium

in the presence of Dox. At day 13, the expressions of *Alb*, as well as endogenous *Hnf1a* and *Hnf4a* were evaluated. RT-PCR analysis indicated that *Hnf1b/Foxa3* had the most upregulation of the liver specific transcription factors (Fig. 1H). Therefore, the combination of enforced *Hnf1b/Foxa3* induced hepatic lineage specification from ES cells even in basal medium.

3.2. Overexpression of *Hnf1b/Foxa3* in ES cells under hepatic medium generated a hepatic fate

In the next step, we evaluated the effect of forced expression of *Hnf1b/Foxa3* on hepatic differentiation in the presence of hepatic medium. *Hnf1b/Foxa3* ES cells were induced to forced expression by Dox treatment for one week and culture in hepatic medium for 6 days as previously described (Fig. 1D). At day 13, the cells exhibited morphologic changes to form colonies that contained epithelial like cells with an extension pattern of short-sided polygons and prominent nuclei (Fig. 2A). Gene expression analysis showed that HP markers *Alb*, *alpha-fetoprotein (Afp)*, *transferrin (Ttr)*, *Hnf4a*, and *delta-like 1 homolog (Dlk1)* significantly upregulated, but not in the Dox negative control cells (Fig. 2B). We observed co-expression of *Alb/Ck19* and *Afp/Hnf4a* at the protein level using immunofluorescence staining (Fig. 2C). Flow cytometry analysis demonstrated that $35.5\pm6.0\%$ of the overexpressed cells expressed *Alb* whereas the control group only expressed $12.5\pm2.0\%$ (Fig. 2D, $P<0.01$). Taken together, these findings indicated that overexpression of *Hnf1b/Foxa3* promoted hepatic specification with assistance of extrinsic inducers.

We next investigated whether the HPs could differentiate to HLCs. The HPs were partially digested by trypsin-EDTA, replated on Matrigel-coated dishes, and cultured in HCM that contained OSM, Dex, and HGF for 10 days. Morphologically, the cell population transformed gradually from an extension pattern of short-sided polygons to large polygonal

flattened cells, which in some cases contained double nuclei (Fig. 3A). In this step we tested the expressions of mature hepatocyte markers by RT-PCR and found that the day 23 HLCs expressed *asialoglycoprotein receptor (Asgpr)*, *tyrosine aminotransferase (Tat)*, *glucose-6-phosphatase (G6p)*, *tryptophan dioxygenase (Tdo)*, *cytochrome P450 (Cyp) 1A1*, and *Cyp2B9*. The expressions of these genes were not detected in HPs (Fig. 3B). Protein expressions of Alb and Cyp1A1 were confirmed by immunofluorescence staining (Fig. 3C). In an attempt to further characterize the functionality of the induced cells, we evaluated glycogen storage by PAS staining (Fig. 3D). We also observed a biotransforming system in our hepatocyte cells with a test of ICG uptake and release (Fig. 3E). Further, we collected culture medium supernatant at days 13 and 23 and measured Albumin and urea levels by the ELISA and calorimetric assays, respectively. The results showed that HLCs could secrete higher Albumin levels and produce more urea compared to HPs which served as the negative control (Fig. 3F). To show homing and *in vivo* differentiation, we transplanted HPs intrasplenically into CCl₄-treated mice. Prior to transplantation, we labeled the progenitor cells with a fluorescent dye, DiI. Two weeks later, liver samples were examined by immunohistofluorescence staining with antibody against Alb. The presence of double positive cells for DiI and Alb indicated that transplanted cells could integrate and differentiate to hepatic cells *in vivo* (Fig. 3G). Together, these results supported the result that overexpression of *Hnf1b/Foxa3* in ES cells under hepatic medium led to the generation of a hepatic fate.

4. Discussion

In this study, we have observed that transient expressions of *Hnf1b*, *Hnf1a*, *Hnf4a*, *Foxa1*, *Foxa3*, and *Hex* in mouse ES cells that upregulated *Alb* mRNA independent of other intrinsic and extrinsic factors. These liver-specific genes are important in endoderm differentiation,

control of early hepatic growth, coordination of hepatic and cell differentiation necessary to generate a functional liver (for review see [28, 29]). These factors have been used in direct conversion or trans-differentiation of mouse and human fibroblasts to induced HLCs in different studies - *Hnf4a* plus *Foxa1*, *Foxa2* or *Foxa3* [5]; *Hnf1a* plus *Gata4* and *Foxa3*, along with inactivation of p19^{Arf} [6]; *HNF1α*, *HNF4α*, *HNF6*, *ATF5*, *PROX1*, *C/EBPβ* and p53 shRNA [7]; *FOXA3*, *HNF1α*, and *HNF4α* [8]; or *FOXA2*, *HNF4α*, *C/EBPβ* and *c-Myc* [9] or *Hnf4a*, *Cebpa* and *Nr1i2* [10]. Forced expressions of both *Foxa3* and *Hnf4a* converted rat bone marrow-mesenchymal stem cells to functional HLCs [30].

Interestingly, both *Hnf1b/Foxa3* out of the six factors in ES cells under hepatic medium enhanced the hepatic fate. *Hnf1b/Foxa3*-ES cells transformed gradually to polygonal flattened cells with prominent nuclei and expressed higher levels of HP markers *Alb*, *Afp*, *Ttr*, *Hnf4a*, and *Dlk1* after two weeks compared with the absence of forced expression. Additionally, the HPs further differentiated to HLCs that expressed *Asgpr*, *Tat*, *G6p*, *Tdo*, *Cyp1A1* and *Cyp2B9*. They showed glycogen storage, ICG uptake and secretion of albumin and urea. The *Hnf1b/Foxa3*-HPs were engraftable and expressed Alb when transplanted into CCl₄-treated mice.

Hnf1b has an important role in hepatic specification. It has been demonstrated that the expression of *Foxa* factors is strongly reduced or absent in the presumptive hepatic endoderm of *Hnf1b*^{-/-} embryos which indicated a requirement of *Hnf1b* for maintaining the expressions of *Foxa1* and *Foxa2*, specifically in the ventral endoderm, as well as for *Foxa3* induction [24]. We observed significant upregulation of *Foxa2* after overexpression of *Hnf1b*. The functional *Hnf1b* binding sites in the regulatory sequences of *Foxa1* [24], *Foxa2* [31], and *Foxa3* [32] have been identified. More importantly, a comprehensive analysis of promoter occupancy during mouse hepatic development demonstrated that *Hnf1b* recruited to the promoter regions of a core group of transcription factors -*Foxa2*, *Hnf1a*, *Hnf4a*, *Hnf6* and

Gata6, which comprise autoregulatory and cross-regulatory circuits [31]. *Hnf1b* has a preeminent role in a transcriptional circuit by inducing *Hnf4a*, *Hnf6* [33], *Hnf1a* [31], and *Foxa3*, [24, 32] and by maintaining *Foxa2* expression [31]. On the other hand, the winged helix (FOX) proteins regulate both liver formation and function (for review see [34]). It has been shown that neither *Foxa1* nor *Foxa3* is required during early mouse embryogenesis. Both *Foxa1*^{-/-} and *Foxa3*^{-/-} embryos appear normal and develop to birth, however, *Foxa2*^{-/-} has been shown to have a defective hepatic specification [35, 36]. In contrast, by E10.5, *Foxa3* expression is seen in the liver primordium and persists throughout development into the adult liver [27]. Recently, it has been reported that both *Hnf1b/Foxa3* are sufficient to reprogram mouse fibroblasts into induced hepatic stem cells [37]. Therefore, regulation of liver transcription appears to be controlled by multiple transcription factors acting in concert, [38] where *Hnf1b/Foxa3* are the “pioneer” factors that have sequence-recognition capability and the ability to bind to a sequence inaccessible to other factors, thus initiating the opening of chromatin at specific sites by recruiting chromatin remodeling factors to their target loci [39].

Taken together, forced expression of both *Hnf1b/Foxa3* under hepatic conditions enhanced a hepatic lineage fate. Our results have suggested another evidence for the crucial role of these factors in mouse hepatogenesis but research should determine whether these factors could induce human ES cells to a hepatic fate.

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Figure legends

Fig. 1. Hepatic lineage specification in transcription factor-inducible embryonic stem (ES) cells.

(A) Schematic structure of the lentivirus construct. The reverse Tet repressor (rtTA) is encoded by a PGK promoter, binds TRE, and activates transcription in the presence of doxycycline (Dox).

(B) RT-PCR analysis of transduced cells for expressions of *Foxa2* at day 7 and (C) *Alb* at day 13 of induction in basal medium.

(D) Schematic represents the procedure of embryonic stem (ES) cell induction in hepatic/inductive medium. The transduced cells were cultured for 7 days in basal medium in the presence of Dox. Then, enforced cells were treated by bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 4 (FGF4) for three days, followed by hepatocyte growth factor (HGF), insulin-transferrin-selenium (ITS), dexamethasone (Dex), and nicotinamide for another three days to induce hepatic specification.

(E) Relative expression level of *Alb* mRNA analyzed by RT-PCR in ES cells that overexpressed *Hnf1b*, *Hnf1a*, *Hnf4a*, *Foxa1*, *Foxa3*, and *Hex* gene in hepatic medium.

(F) Phase contrast images of *Hnf1b* and *Hnf1a* overexpressing ES cells at day 13.

(G) Co-immunofluorescent staining of albumin (Alb) with Ck19 for *Hnf1a* and *Hnf1b* ES cells in hepatic medium. The nucleus was stained with DAPI.

Scale bar represents 50 μm in G (upper panel) and 100 μm in F and G (lower panel)

(H) Forced expressions of *Hnf1b* and *Foxa3* together induced hepatic gene expression in basal-cultured condition. Quantitative RT-PCR analyses of *Alb* and endogenous *Hnf1a* and *Hnf4a* expressions at day 13.

The data were normalized to *Gapdh* and original ES cells. The data are presented as mean $\pm$ SD (n=3; ANOVA, Tukey post-hoc test, * $P<0.05$, *** $P<0.001$).

Fig. 2. Generation of hepatic progenitors (HPs) by co-forced expression of *Hnf1b/Foxa3* in hepatic medium at day 13.

(A) Phase contrast morphology of *Hnf1b/Foxa3* embryonic stem (ES) cells. The cells had an epithelial-like morphology with an extension pattern of short-sided polygons and prominent nuclei. The right panel shows morphology at higher magnification.

(B) The expressions of early hepatic markers in the presence (+) or absence (-) of Dox by qRT-PCR. The data were normalized to *Gapdh* and original ES cells.

(C) Co-expressions of Alb and Ck19 (upper panel), as well as Hnf4a and Afp (lower panel) in *Hnf1b/Foxa3* overexpressing ES cells. Nuclei were counterstained with DAPI.

(D) Flow cytometry analysis for Alb expression.

The data are presented as mean $\pm$ SD (n=3; ** $P<0.01$, **** $P<0.0001$, Student *t*-test). Scale bars: 50 μ m in A (right panel) and 100 μ m in other pictures.

Fig. 3. Differentiation of hepatic progenitors (HPs) to hepatocyte-like cells (HLCs).

Hepatic progenitors (HPs) were further differentiated to mature hepatocyte-like cells (HLCs) in hepatocyte culture medium (HCM) that contained oncostatin M (OSM), dexamethasone (Dex), and hepatocyte growth factor (HGF) for 10 days and evaluated at day 23.

(A) Phase contrast microscopy of differentiated cells that showed large polygonal flattened cells.

(B) Relative expressions of asialoglycoprotein receptor (*Asgpr*), tyrosine aminotransferase (*Tat*), glucose-6-phosphatase (*G6p*), tryptophan dioxygenase (*Tdo*), cytochrome P450 (*Cyp1A1*, and *Cyp2B9*) genes. The data were normalized to *Gapdh* and original ES cells.

(C) Immunofluorescence staining for Alb; (upper panel) and Cyp1A1 (lower panel). Nucleus was counterstained with DAPI.

(D) Glycogen storage by periodic acid-Schiff (PAS) staining.

(E) Indocyanine green (ICG) uptake and release.

(F) Albumin secretion levels and urea synthesis were measured by ELISA and calorimetric assays, respectively.

(G) Homing of transplanted cells was detected by DiI and Alb double positive cells in the same section two weeks after transplantation.

HPs were labeled with DiI prior to injection to assess homing of the transplanted cells in carbon tetrachloride (CCL₄) acute injury liver model. Double DiI/Alb-positive cells were markers of functional activity of the transplanted HPs. The nuclei were counterstained with DAPI.

The data are presented as mean $\pm$ SD (n=3; * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001, Student t -test.). Scale bars: 100 μ m in D and 50 μ m in A, C, E and G.

Figure 1

Figure 2

Figure 3