Forced Expression of Hnf4a induces hepatic gene activation through directed differentiation

Neda Yahoo¹, Behshad Pournasr^{2*}, Jalal Rostamzadeh³, Fardin Fathi^{1*}

1. Cellular and Molecular Research Center, Kurdistan University of Medical Sciences, Sanandaj, Iran.
2. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran.
3. Department of Biological Sciences and Biotechnology, School of Science, University of Kurdistan, Sanandaj, Iran

***Correspondence:**

farfath@gmail.com (F.F.) or Pournasr@RoyanInstitute.org (B.P.)

Running title: Overexpression of Hnf4a during directed differentiation induces hepatic genes activation in ES cells

Competing financial interests: The authors declare they have no competing financial interests.

Abstract

Embryonic stem (ES) cells are capable of unlimited self-renewal and have a diverse differentiation potential. These unique features make ES cells as an attractive source for developmental biology studies. Having the mature hepatocyte in the lab with functional activities is valuable in drug discovery studies. Overexpression of hepatocyte lineage-specific transcription factors (TFs) becomes a promising approach in pluripotent cell differentiation toward liver cells. Many studies generate transgenic ES cell lines to examine the effects of specific TFs overexpression in cell differentiation. In the present report, we have addressed whether a suspension or adherent model of differentiation is an appropriate way to study the role of Hnf4a overexpression. We generated ES cells that carried a doxycycline (Dox)-inducible Hnf4a using lentiviral vectors. The transduced cells were subjected to induced Hnf4a overexpression through both spontaneous and directed differentiation methods. Gene expression analysis showed substantially increased expression of hepatic gene markers, particularly *Ttr* and endogenous *Hnf4a*, in transduced cells differentiated by the directed approach. These results demonstrated that forced expression of TFs during directed differentiation would be an appropriate way to study relevant gene activation and the effects of overexpression in the context of hepatic differentiation.

Keywords: Embryonic stem cells; Overexpression; Hnf4a ; Directed differentiation; Hepatic genes

1. Introduction

Pluripotent embryonic stem (ES) cells are derived from the inner cell mass of mouse blastocysts and have the potential to give rise to all cell types within the body. In standard culture medium, ES cells can be maintained in an undifferentiated state with indefinite proliferation, a feature known as self-renewal. These properties make ES cells a potential source for embryonic and *in vitro* developmental studies [1, 2]. Many groups have utilized various protocols to induce differentiation of ES cells toward organ-specific cells (for review see [3]).

In spontaneous differentiation, ES cells aggregate in suspension culture and form spheroid colonies called embryoid bodies (EBs). Similar to early embryos, differentiated EBs are composed of all three germ layer derivative cells [4-7]. Directed differentiation of ES cells involves the step-wise use of a cocktail of growth factors and cytokines in an adherent culture [8, 9]. In addition, forced expression of lineage-specific transcription factors (TFs) can guide ES cells to direct cellular differentiation [10-13]. TFs, by binding to target regulatory elements, can drive expression of cell-type specific genes which lead to the establishment of a cellular identity [14]. Numerous studies generate transgenic ES cell lines to study the effects of TFs overexpression in cell differentiation [15, 16]. Although these studies provide clear evidence to produce desired cell types, some have addressed whether spontaneous or directed differentiation is an appropriate method during forced expression.

In this study we intended to evaluate the associations of gene overexpression with the differentiation protocol. We selected Hnf4a as a well-known TF for its critical role in

embryonic liver development [17]. Previously, trans-differentiation studies demonstrated the capability of Hnf4a in hepatic gene activation [18-20]. Our primary hypothesis was that ectopic expression of Hnf4a in a monolayer culture of ES cells would be a good opportunity to study gene expression outcomes. To prove our hypothesis, we first attempted to resolve the optimal promoter system in mouse ES cells. Next, we created a tet-inducible ES line with a lentivirus vector which expressed Hnf4a in a controllable manner.

2. Materials and Methods

2.1. Mouse embryonic stem (ES) cell culture and differentiation

Mouse ES cells (RB20) [21] were maintained on 0.1% gelatin-coated culture dishes in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 15% knockout serum replacement, 1x GlutaMAX™, 1x non-essential amino-acids (NEAAs), 0.1 mM beta-mercaptoethanol (all from Invitrogen), and 1000 units/ml leukemia inhibitory factor (Royan BioTech). We used a combination of the following two inhibitors - 1 μ M PD0325901 (MEK inhibitor; Cayman Chemical) and 10 μ M SB431542 (TGF- β inhibitor; Cayman Chemical) to block mouse ES cell differentiation [22].

In order to induce differentiation, ES cells were dissociated with 0.05% trypsin-EDTA and resuspended in DMEM/F12 that contained 15% fetal bovine serum (FBS; Invitrogen), 1x GlutaMAX™, 1x NEAAs, and 0.1 mM beta-mercaptoethanol. The cells were incubated by the hanging drop culture method (1000 ES cells per 30 μ l per drop) to form EBs. After four days of culture in an atmosphere of 5% CO₂ at 37°C, the EBs were transferred onto gelatin coated dishes and allowed to attach in differentiation medium. We added hepatic growth factors at the following concentrations: 25 ng/ml hepatocyte growth factor (HGF; R&D Systems), 10⁻⁷ M dexamethasone (Dex; Sigma), 1% insulin-transferrin-selenium (ITS), and

10 mM nicotinamide (all from Invitrogen). To induce ectopic expression of Hnf4a , 5 µg/ml doxycycline (Dox; Clontech) was added to the hepatic medium for 7 days.

Directed differentiation was performed as previously described [13]. Dissociated cells were seeded in gelatin coated dishes and differentiated in basal medium that consisted of advanced DMEM supplemented with 3% FBS, 1x GlutaMAX™, 1x NEAAs, 0.1 mM beta-mercaptoethanol, 1x B27 (Invitrogen), and 0.5% bovine serum albumin (BSA; Merck). In the first step, Dox was added to the basal medium for 7 days to induce Hnf4a expression. Later on, the basal medium was supplemented with 40 ng/ml fibroblast growth factor 4 (FGF4; R&D Systems), and 40 ng/ml bone morphogenetic protein 4 (BMP4; Stemfactor) for 3 days, followed by 25 ng/ml HGF, 10^{-7} M Dex, 5 mM nicotinamide, and 1 % ITS for the next 3 days.

3.2. Plasmid construction, virus production, and transduction

In this study, we used the pLenti6.3/TO/V5 expression backbone (Invitrogen), pLVX-tight-puro (Addgene), and FUW-M2rtTA plasmids (Addgene, 20342). The pLenti-TRE vector was generated by removing a Cla1/Ahl1 fragment that contained the cytomegalovirus (CMV) promoter from pLenti6.3/TO/V5 and replacing it with a Cla1/Xba1 fragment of pLVX-tight-puro that contained the tet-responsive element (TRE), which changed the CMV promoter. To create FUW-M2rtTA-neo, the neomycin cassette of the pLenti3.3/TR plasmid was isolated by digestion with Kpn1 and cloned into FUW-M2rtTA.

Hek-293T packaging cells were grown in DMEM/F12 supplemented with 10% FBS, 1x GlutaMAX™, 1x NEAAs, and 0.1 mM beta-mercaptoethanol, at 37°C and 5% CO₂. For virus production, 293T cells were plated at 5×10^6 per each 10 cm culture dish in growth medium. The next day, we used the lentiviral expression vector and third-generation packaging plasmids pMDLg/pRRE, pMD2.G (VSV-G), and pRSV-Rev (Addgene plasmids

#12251, 12259, and 12253, respectively) for viral transfection. After 8 hours of culture, the medium was replaced with DMEM/F12 that contained 2% FBS and 1% ITS. The lentivector containing supernatant was collected 24 and 48 hours following transfection, passed through 0.45 µm pore-sized filters and concentrated by ultracentrifugation (20000 g for 120 minutes at 4°C). The pellet was resuspended in DMEM and stored at -70°C.

For transduction, ES cells were dissociated to single cells and incubated in the presence of lentivirus vectors in ESC medium at 37°C and 5% CO₂ for 4 hours. The cells were then transferred to a gelatin-coated dish. After 2 days, the cells were subjected to neomycin and blasticidin in order to select transduced cells.

3.3. RNA isolation and reverse transcription polymerase chain reaction

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the PrimeScriptTM RT Reagent Kit (Takara). Real-time PCR was performed using a SYBR Green Real-time PCR Master Mix (Takara) for quantitative analysis of the gene expressions. qRT-PCR was carried out on a Rotor Gene 6000 (Corbett Life Science) with cycling conditions at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 20 seconds, and 70°C for 20 seconds. Specific primers for *Oct3/4*, forkhead box a2 (*Foxa2*), alpha-fetoprotein (*Afp*), albumin (*Alb*), hepatocyte nuclear factor 4a (*Hnf4a*), transthyretin (*Ttr*), exogenous *Hnf4a* (Ex-*Hnf4a*), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were used. The primer sequences are listed in Table 1. The gene expression levels were normalized by *GAPDH* expression as a housekeeping gene and original ES cells.

3.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. Results were presented as mean $\pm$ SD. We used the student's t-test to determine differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of two different promoter systems for transgene expression in mouse embryonic stem cells

In order to optimize exogenous gene expression in mouse ES cells, we compared two promoter candidates (CMV and TRE) in lentiviral vectors context. Green fluorescent protein (GFP) reporter expression was used for characterization.

GFP expressed under control of the CMV promoter in lentiviral-mediated constitutive expression (Fig. 1A), while TRE promoter regulated GFP expression in the tet-inducible lentiviral system. In this system, two vector components contributed to express the gene of interest (Fig. 1B). The regulatory vector which constitutively encoded the reverse tetracycline-controlled transactivator (rtTA) and the response vector that carried the gene of interest downstream of a tet response element (TRE). In the Tet-On system, rtTA bound to the TRE promoter and activated transcription in the presence of the tetracycline analogue, Dox (Fig. 1C).

We transfected suspended ES cells in 2i medium with lentiviruses and analyzed GFP expression after 48 hours. In the case of the TRE system, we co-transduced ES cells simultaneously with inducible transgene expression and transactivator vectors. The two vectors contained different antibiotic resistance genes, therefore we applied both blasticidin and neomycin to select double transduced cells. The selected colonies were expanded and

incubated in the presence of Dox for 2 days, then analyzed for GFP expression by fluorescent microscopy.

We observed markedly different GFP expression between the two promoter candidates. The CMV promoter was obviously inactive in mouse ES cells (Fig. 1D), while the TRE promoter could robustly drive GFP expression (Fig. 1E). As shown in Fig. 1F, the GFP positive colonies grew after 7 days of induction. Therefore, we chose the TRE promoter system for the next experiments.

3.2. Evaluation of exogenous gene expression in mouse embryonic stem (ES) cells

Next, we decided to confirm the transgene expression in ES cells more quantitatively. To this end, we transduced cells with the lentiviral *Hnf4a* expression vector along with the rtTA encoding plasmid. After antibiotic selection, the transduced cells were cultured in basal medium that contained 5µg/ml Dox for transgene expression. After 3 days of induction, we observed altered cell proliferation with morphological changes (Fig. 2A).

RT-PCR analysis showed a 300-fold upregulation of *Hnf4a* mRNA in Dox-treated cells but not in the untreated group. The results also showed that forced expression of *Hnf4a* led to reduced expression of *Oct3/4* as an ES cell marker and increased expression of *Foxa2* as an early marker of endoderm differentiation (Fig. 2B). These observations suggested that *Hnf4a* underwent successful forced expression and could modulate the undifferentiated phenotype of ES cells.

3.3. The effect of forced expression of *Hnf4a* during embryonic stem (ES) cell differentiation

In general, two current in vitro methods are used for differentiation of ES cells - spontaneous and directed. In this step, we have sought to determine whether spontaneous or directed

differentiation was appropriate to evaluate the effects of exogenous gene expression on ES cells. For this purpose, we dissociated transduced *Hnf4a* ES cells and allowed them to reaggregate in hanging drops in order to form EBs. The EBs were then plated on gelatin coated dishes and grown in the presence of Dox under hepatic conditions.

The hepatic medium that contained HGF, Dex, ITS, and nicotinamide was utilized to supply extrinsic stimuli for hepatic gene activation. We collected cells at day 13 and analyzed hepatic mRNA expressions of *Alb*, *Afp*, *Ttr*, and *Hnf4a* by qRT-PCR. As shown in Fig. 3, we did not observe any significant changes in hepatic gene expressions in the presence or absence of exogenous *Hnf4a*. These finding indicated that overexpression of *Hnf4a* during autonomous differentiation did not affect hepatic gene activation.

For directed differentiation, we applied a two-step protocol in which ES cells that carried *Hnf4a* were initially induced to express transgene by Dox treatment for 7 days. Then, the enforced cells were exposed to BMP4 and FGF4 for 3 days, followed by HGF, Dex, ITS, and nicotinamide for the next 3 days. At day 13, the cell morphology changed into epithelial-like cells with a short-sided polygonal pattern (Fig. 4A, left panel). Gene expression analysis showed obvious upregulation of hepatic markers, in particular *Ttr* and *Hnf4a*, which significantly differed from the control group (Fig. 4B). Taken together these results indicated that overexpression of *Hnf4a* in adherent ES culture followed by step-wise differentiation method was a compelling one to study the effects of gene overexpression on target gene activation.

4. Discussion

By taking into consideration the developmental potential of ES cells and their convenience for genetic manipulation, engineered ES cells are a valuable source to study the molecular mechanism of *in vitro* differentiation [15]. In this research, we have generated transgenic ES

lines using a lentivirus system to study Hnf4a overexpression during hepatic differentiation. One of the first issues to be addressed in such experiments is an evaluation of the promoter activities in ES cells. Functional analyses of different promoters in undifferentiated mouse ES cells show that the CMV promoter fails to drive gene expression in ES cell lines [23-25]. Due to conflicting results from other studies [26, 27], we have decided to compare two different promoter systems in the current experiment. The results indicated that the TRE promoter was superior to the CMV promoter for gene expression in mouse ES cells.

For further confirmation, we created a tet-inducible ES line which carried the *Hnf4a* gene. This ES line showed high expression of exogenous *Hnf4a* in the presence of Dox. In addition, we observed remarkably increased expression of *Foxa2* mRNA compared to the control group, which did not induce transgene overexpression. In addition, there was a significant decline in mRNA expression that encoded *Oct3/4* as a key factor of the ES cell transcriptional network [28]. Together these findings have demonstrated the functionality of Hnf4a overexpression in ES cells. Hnf4a is wide acting TF in hepatocytes that occupy a large number of promoter regions of hepatic genes [29]. The basic role of Hnf4a in liver development has been previously demonstrated. In addition, it could induce a hepatic program in ES cells [30]. Therefore we choose Hnf4a factor for analysis of hepatic gene expression upon its overexpression in two differentiation methods.

In the current experiment, overexpression of Hnf4a during the second step of spontaneous differentiation did not show a substantial effect on hepatic gene expression. This suggested that forced expression of Hnf4a did not alter hepatic differentiation of EBs cultured in hepatic medium. Possibly, factors such as cell-cell contact and cell-matrix interaction caused by the spheroid structure might be more important for expression of liver specific genes rather than Hnf4a overexpression [31, 32]. The results from microarray data supported this hypothesis since the HGF and FGF receptor signaling considered crucial in embryonic liver

development showed upregulation in the developing EBs [33]. A recent study reported that ectopic expression of *Hnf4a* during suspension differentiation did not activate hepatocyte-related genes in the absence of extrinsic inducers [34].

In addition to the spontaneous culture, we examined *Hnf4a* overexpression during directed differentiation of the cells. Although we did not observe any compelling differences in *Afp* and *Alb* mRNA expressions, there was significant upregulation of *Ttr* and endogenous *Hnf4a* in the test group. These findings appeared to be consistent with previous reports that showed enforced expression of *Hnf4a* in hepatic medium that contained a number of assistant factors which could have positively influenced hepatic gene expressions [34, 35].

Taken together, forced expression of *Hnf4a* during directed differentiation would be an appropriate method to study the effects of overexpression in the context of hepatic differentiation.

Acknowledgments

This study, as a Ph.D thesis was funded by grants provided from Royan Institute, the Iranian Council of Stem Cell Research and Technology, the Iran National Science Foundation (INSF), Iran Science Elites Federation and Kurdistan University of Medical Sciences. We express our appreciation to all members of the Hepatocyte Program at Royan Institute for their helpful deliberation and consultation during this work. We express our appreciation to Dr. Mohsen Basiri for his assistance in performing the cloning studies.

References

1. Murry, C.E. and G. Keller, Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell*, 2008. **132**(4): p. 661-80.
2. Nichols, J. and A. Smith, Pluripotency in the embryo and in culture. *Cold Spring Harb Perspect Biol.* **4**(8): p. a008128.
3. Keller, G., Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev*, 2005. **19**(10): p. 1129-55.
4. Asahina, K., et al., Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells. *Genes Cells*, 2004. **9**(12): p. 1297-308.
5. Dolmazon, V., et al., Forced expression of LIM homeodomain transcription factor 1b enhances differentiation of mouse embryonic stem cells into serotonergic neurons. *Stem Cells Dev.* **20**(2): p. 301-11.
6. Guddati, A.K., et al., Embryonic stem cells overexpressing Pitx2c engraft in infarcted myocardium and improve cardiac function. *Int Heart J*, 2009. **50**(6): p. 783-99.
7. Hamazaki, T., et al., Hepatic maturation in differentiating embryonic stem cells in vitro. *FEBS Lett*, 2001. **497**(1): p. 15-9.
8. Teratani, T., et al., Direct hepatic fate specification from mouse embryonic stem cells. *Hepatology*, 2005. **41**(4): p. 836-46.
9. Zhou, M., et al., Differentiation of mouse embryonic stem cells into hepatocytes induced by a combination of cytokines and sodium butyrate. *J Cell Biochem.* **109**(3): p. 606-14.
10. Bai, F., et al., Directed Differentiation of Embryonic Stem Cells Into Cardiomyocytes by Bacterial Injection of Defined Transcription Factors. *Sci Rep.* **5**: p. 15014.
11. Fujikura, J., et al., Differentiation of embryonic stem cells is induced by GATA factors. *Genes Dev*, 2002. **16**(7): p. 784-9.
12. Mong, J., et al., Transcription factor-induced lineage programming of noradrenaline and motor neurons from embryonic stem cells. *Stem Cells.* **32**(3): p. 609-22.
13. Yahoo, N., et al., Forced expression of Hnf1b/Foxa3 promotes hepatic fate of embryonic stem cells. *Biochem Biophys Res Commun.*
14. Hochedlinger, K. and R. Jaenisch, Induced Pluripotency and Epigenetic Reprogramming. *Cold Spring Harb Perspect Biol.* **7**(12).

15. Correa-Cerro, L.S., et al., Generation of mouse ES cell lines engineered for the forced induction of transcription factors. *Sci Rep.* **1**: p. 167.
16. Yamamizu, K., D. Schlessinger, and M.S. Ko, SOX9 accelerates ESC differentiation to three germ layer lineages by repressing SOX2 expression through P21 (WAF1/CIP1). *Development.* **141**(22): p. 4254-66.
17. Si-Tayeb, K., F.P. Lemaigre, and S.A. Duncan, Organogenesis and development of the liver. *Dev Cell.* **18**(2): p. 175-89.
18. Huang, P., et al., Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. *Cell Stem Cell.* **14**(3): p. 370-84.
19. Pournasr, B., M.H. Asghari-Vostikolaee, and H. Baharvand, Transcription factor-mediated reprogramming of fibroblasts to hepatocyte-like cells. *Eur J Cell Biol.* **94**(12): p. 603-10.
20. Sekiya, S. and A. Suzuki, Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *Nature.* **475**(7356): p. 390-3.
21. Hassani, S.N., et al., Simultaneous suppression of TGF-beta and ERK signaling contributes to the highly efficient and reproducible generation of mouse embryonic stem cells from previously considered refractory and non-permissive strains. *Stem Cell Rev.* **8**(2): p. 472-81.
22. Ying, Q.L., et al., The ground state of embryonic stem cell self-renewal. *Nature*, 2008. **453**(7194): p. 519-23.
23. Chung, S., et al., Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines. *Stem Cells*, 2002. **20**(2): p. 139-45.
24. Hong, S., et al., Functional analysis of various promoters in lentiviral vectors at different stages of in vitro differentiation of mouse embryonic stem cells. *Mol Ther*, 2007. **15**(9): p. 1630-9.
25. Suter, D.M., et al., Rapid generation of stable transgenic embryonic stem cell lines using modular lentivectors. *Stem Cells*, 2006. **24**(3): p. 615-23.
26. Ward, C.M. and P.L. Stern, The human cytomegalovirus immediate-early promoter is transcriptionally active in undifferentiated mouse embryonic stem cells. *Stem Cells*, 2002. **20**(5): p. 472-5.
27. Fathi, F., et al., Transfection of CCE mouse embryonic stem cells with EGFP and BDNF genes by the electroporation method. *Rejuvenation Res*, 2006. **9**(1): p. 26-30.
28. Teo, A.K., et al., Pluripotency factors regulate definitive endoderm specification through eomesodermin. *Genes Dev.* **25**(3): p. 238-50.
29. Odom, D.T., et al., Control of pancreas and liver gene expression by HNF transcription factors. *Science*, 2004. **303**(5662): p. 1378-81.

30. Kuai, X.L., et al., Treatment of surgically induced acute liver failure by transplantation of HNF4-overexpressing embryonic stem cells. *Chin J Dig Dis*, 2006. **7**(2): p. 109-16.
31. Asahina, K., K. Teramoto, and H. Teraoka, Embryonic stem cells: hepatic differentiation and regenerative medicine for the treatment of liver disease. *Curr Stem Cell Res Ther*, 2006. **1**(2): p. 139-56.
32. Bratt-Leal, A.M., R.L. Carpenedo, and T.C. McDevitt, Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. *Biotechnol Prog*, 2009. **25**(1): p. 43-51.
33. Novik, E.I., et al., Embryoid body-mediated differentiation of mouse embryonic stem cells along a hepatocyte lineage: insights from gene expression profiles. *Tissue Eng*, 2006. **12**(6): p. 1515-25.
34. Liu, T., et al., Induction of hepatocyte-like cells from mouse embryonic stem cells by lentivirus-mediated constitutive expression of Foxa2/Hnf4a. *J Cell Biochem*. **114**(11): p. 2531-41.
35. Yamamizu, K., et al., Identification of transcription factors for lineage-specific ESC differentiation. *Stem Cell Reports*. **1**(6): p. 545-59.

Figure legends

Fig. 1. Comparison of two different promoter activities in mouse embryonic stem (ES) cells

(A) Construct of the lentiviral vector that contained the cytomegalovirus (CMV) promoter.

(B) Tet-inducible two-component lentivirus vector system. The regulatory vector that contained rtTA expressed by a constitutive promoter (upper panel) and the response vector that contained the TRE-regulated transgene (lower panel). Both vectors had a central polypurine tract of HIV-1 (ppt), a woodchuck hepatitis virus post-transcriptional control element (WPRE), and self-inactivating mutations in the LTR.

(C) Schematic representation of the tet-On working process. rtTA is expressed under the control of a human ubiquitin C (hUbC) promoter, binds to TRE, and activates transgene expression only in the presence of doxycycline (Dox).

(D-F) Phase contrast and fluorescent imaging of transduced cells related to CMV (upper panel) and TRE (lower panel) promoters. Scale bars represent 200 μm in D and 100 μm in E and F.

Fig. 2. Analysis of enforced *Hnf4a* in expression changes of *OCT4* and *FOXA2*

(A) Morphological changes after 3 days of *Hnf4a* overexpression (left panel). Right panel shows cell morphology without *Hnf4a* overexpression. Scale bars represent 500 μm .

(B) Quantitative RT-PCR for exogenous *Hnf4a* (*Ex-Hnf4a*), *FOXA2*, and *OCT4* in embryonic stem (ES) cells that carry *Hnf4a* in the presence (Dox+) or absence (Dox-) of doxycycline (Dox) during 3 days of differentiation. Data are expressed as mean $\pm$ SD (n=3; ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ versus Dox-, student's t-test).

Fig. 3. The effects of *Hnf4a* overexpression during spontaneous differentiation at day 13.

Relative expression levels of hepatic genes *AFP*, *TTR*, *Hnf4a*, and *ALB* in spontaneous differentiation. Data are mean $\pm$ SD (n=3). The data were normalized to *GAPDH* and original embryonic stem (ES) cells.

Fig. 4. The effects of *Hnf4a* overexpression during directed differentiation at day 13.

(A) Phase contrast morphology of *Hnf4a*-embryonic stem (ES) cells that showed an epithelial structure with short-sided polygon-like cells. Right panel shows cell morphology without *Hnf4a* overexpression. Scale bars represent 100 μm .

(B) Gene expression levels of hepatic markers that included *AFP*, *TTR*, *Hnf4a*, and *ALB* were examined by RT-PCR. Data are mean $\pm$ SD (n=3; * $P<0.05$, student's t-test). The data were normalized to *GAPDH* and original embryonic stem (ES) cells.

Table 1. Primer sequences used in this research.

Gene symbol	Sequences (5'-3')	Accession no.	Amplicon size (bp)
<i>FOXA2</i>	F: GCAGACACTTCCTACTACCAA R: CTCCACTCAGCCTCTCATT	NM_010446	123
<i>TTR</i>	F: TCCTCTGATGGTCAAAGTCC R: TCCTTCTACAACTTCTCATCTG	NM_013697.5	172
<i>Hnf4a</i>	F: ATGACAATGAATATGCCTGCC R: GGTCGTTGATGTAATCCTCC	NM_001312907.1	131
<i>ALB</i>	F: AGACATCCTTATTTCTATGCC R: GACCAATGCTTTCTCCTTCAC	NM_009654.3	141
<i>OCT4</i>	F: GAACTAGCATTGAGAACCGT R: CATACTCGAACCACATCCTTC	NM_013633	129
<i>AFP</i>	F: ACAAGAGCTTCTCATTACCTG R: CAACTTTGGACCCTCTTCTG	NM_007423.4	151
<i>GAPDH</i>	F: GACTTCAACAGCAACTCCCAC R: TCCACCACCCTGTTGCTGTA	NM_008084	125
<i>Ex-Hnf4a</i>	F: CAAGCAAGAGGCCATCTGAG R: ATCCTTAGTGGTGGTGGTGG	<i>Hnf4a</i> - expression vector	137

A

B

D

C

E

F

A

B