AdhHNF6-infected iPSHCs (condition 4; see above differentiation of human iPSCs), HepG2 cells, and hepatocytes were incubated in culture medium containing 300 μM testosterone for 6 h. To terminate the 6β-hydroxylase activity, a double volume of 0.05% phosphoric acid containing acetonitrile was mixed with collected medium. The mixtures were centrifuged at 15,000 rpm for 15 min and the supernatants were subjected to HPLC analysis. A HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with Capcell Pak C18 UG120 column (4.6 × 250; particle size 5.0; Shiseido, Tokyo, Japan) was used. The flow rate was set at 1.0 mL/min and analyses were performed at 40°C. The mobile phase was solvent A (distilled water) and solvent B (0.05% phosphoric acid/acetonitrile). Typical conditions for elution were as follows: 25% B (0–15 min); 25–50% B (15–17 min); 50% B (17–21 min); 50–25% B (21–22 min); and 25% B (22–35 min). A linear gradient was used for all solvent changes. UV absorption intensity was monitored at 254 nm.

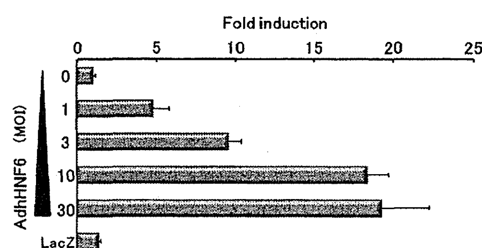

Fig. 1. Effect of HNF6 on CYP3A4 reporter activity in HepG2 cells
(A) HepG2 cells were seeded in 48-well tissue culture plates at 6.0×10^4 cells per well and were then transfected with pCYP3A4-362-7.7k (0.4 μg) and pGL4.82 (0.1 μg) as an internal control. The next day, the cell medium was exchanged for culture medium and cells were infected with recombinant adenovirus AdLacZ (MOI of 30) or AdhHNF6 (MOI of 1, 3, 10, or 30). After 72 h, reporter activities were measured. The reporter activity in non-infected HepG2 cells was set equal to 1.0. The data represent mean ± SD ($n = 3$).

Results

Transcriptional activation of the *CYP3A4* reporter gene by HNF6: To investigate the effect of HNF6 on *CYP3A4* transcriptional activation, a reporter assay was performed in HepG2 cells using a *CYP3A4*-luciferase reporter plasmid, pCYP3A4-362-7.7k, which contained a proximal promoter (+11 bp to 362 bp) and a distal xenobiotic-responsive enhancer module (XREM; −7.2 kb to −7.8 kb). HepG2 cells that had been infected with various amounts (1, 3, 10, and 30 MOI) of AdhHNF6 showed an increase in luciferase activity in an MOI-dependent manner, with an approximately 20-fold increase compared with the activity in non-infected cells (**Fig. 1**).

Expression of *CYP* and liver-specific genes in HNF6-overexpressed HepG2 cells: To confirm bioavailability of AdhHNF6 on the expression of *CYP* and liver-specific genes in HepG2 cells, cells were infected with AdhHNF6. *HNF6* mRNA levels showed a significant increase in an AdhHNF6 MOI-dependent manner. Protein expression of HNF6 was also confirmed in the cells (data not shown). The mRNA levels of *CYPs*, albumin (*ALB*), and α-fetoprotein (*AFP*) in AdhHNF6-infected HepG2 cells were measured by real-time PCR (**Fig. 2**). A previous study has shown that increase in hepatic HNF6 expression, caused by infection with AdHNF6, led to marked upregulation of *CYP7A1* mRNA.[29] In this study, HNF6 overexpression also resulted in an increase in *CYP7A1* mRNA expression. Of all the *CYPs*, the mRNA levels of *CYP3A4* and *CYP3A7* were significantly upregulated in HepG2 cells in an AdhHNF6 MOI-dependent manner, with an approximately 20-fold and 30-fold increase, respectively, as compared with the levels of these mRNAs in non-infected HepG2 cells. Similar increases were also observed for *CYP2C9* and *CYP2C19*, but not for *CYP1A1*, *CYP1A2*, or *CYP2D6*. A typical mature liver-specific gene, *ALB*, was significantly transactivated by the AdhHNF6 infection; however, a typical immature liver-specific gene, *AFP*, was not activated.

Expression of *CYP* and liver-specific genes in HNF6-overexpressed HFL cells and hepatocytes: To confirm the effect of HNF6 overexpression on the expression of *CYP* and liver-specific genes in HFL cells, cells were infected with AdhHNF6. *CYP3A4* mRNA levels showed the greater increase (120-fold increase compared to the levels in non-infected HFL cells) among the *CYPs* tested (**Fig. 3A**). mRNA levels of *CYP1A1*, *CYP1A2*, *CYP2C19*, and *CYP3A7* also significantly increased in HFL cells in an AdhHNF6 MOI-dependent manner, while the AdhHNF6-mediated induction of *AFP* and *ALB* gene transcription reached a peak at 10 MOI, above which the levels decreased. *CYP7A1* mRNA expression was below the detection limit of quantitative analysis by real-time PCR.

A developmental switch occurs between *CYP3A4* and *CYP3A7*, wherein hepatic *CYP3A7* expression dominates in the fetus, and hepatic *CYP3A4* expression dominates in the adult.[31] In contrast, *HNF6* is expressed in both fetal and adult hepatocytes.[44] To confirm the effect of HNF6 on human adult hepatocytes, we infected hepatocytes with AdhHNF6. Consequently, we found that AdhHNF6 infection increased *CYP3A7* mRNA expression, while *CYP3A4* mRNA expression did not change much (**Fig. 3B**). The increase in *CYP3A7* mRNA expression in hepatocytes peaked at 30 MOI, and the levels decreased thereafter in an AdhHNF6 MOI-dependent manner.

Effects of HNF6 on expression of *CYP* and liver-specific genes in iPSC-derived hepatocyte-like cells: Our protocol for differentiating iPSCs into iPSHCs is illustrated in **Figure 4**. This protocol comprised 3 stages: endoderm induction, hepatic specification, and hepatic maturation. In the hepatic maturation stage, we investigated whether HNF6 overexpression affects the expression of *CYP*, *ALB*, and *AFP*. iPSHCs were infected with AdhHNF6 at 3 or 6 d before the end of the hepatic differentiation process and were collected after a total culture period of 18, 22, or 25 d (see Materials and Methods for details). Similar to HepG2 cells, these cells demonstrated a significant increase in *HNF6* mRNA and protein expression after AdhHNF6 infection (**Figs. 5A** and **5B**). AdLacZ infection had no effect on *HNF6* mRNA or on other targeted transcripts in this study (data not shown).

CYP7A1 mRNA levels in iPSHCs at day 18 was similar to those in hepatocytes, but decreased depending on the culture period. However, *CYP7A1* mRNA levels in AdhHNF6-infected iPSHCs under condition 2 (14-fold), 3 (4-fold), or 4 (3-fold) were higher than those in non-infected iPSHCs after each culture period (22 d or 25 d; **Fig. 6A**).

Although *CYP3A4* mRNA was observed in iPSHCs, the expression level was very low as compared with that in HepG2 cells or hepatocytes. However, these levels increased markedly upon AdhHNF6 infection and were enhanced in a culture period-dependent manner (**Fig. 6A**). Maximum expression was observed in iPSHCs infected with AdhHNF6 for 5 d. The *CYP3A4* mRNA level was 1,450-fold higher than that in non-infected iPSHCs, and

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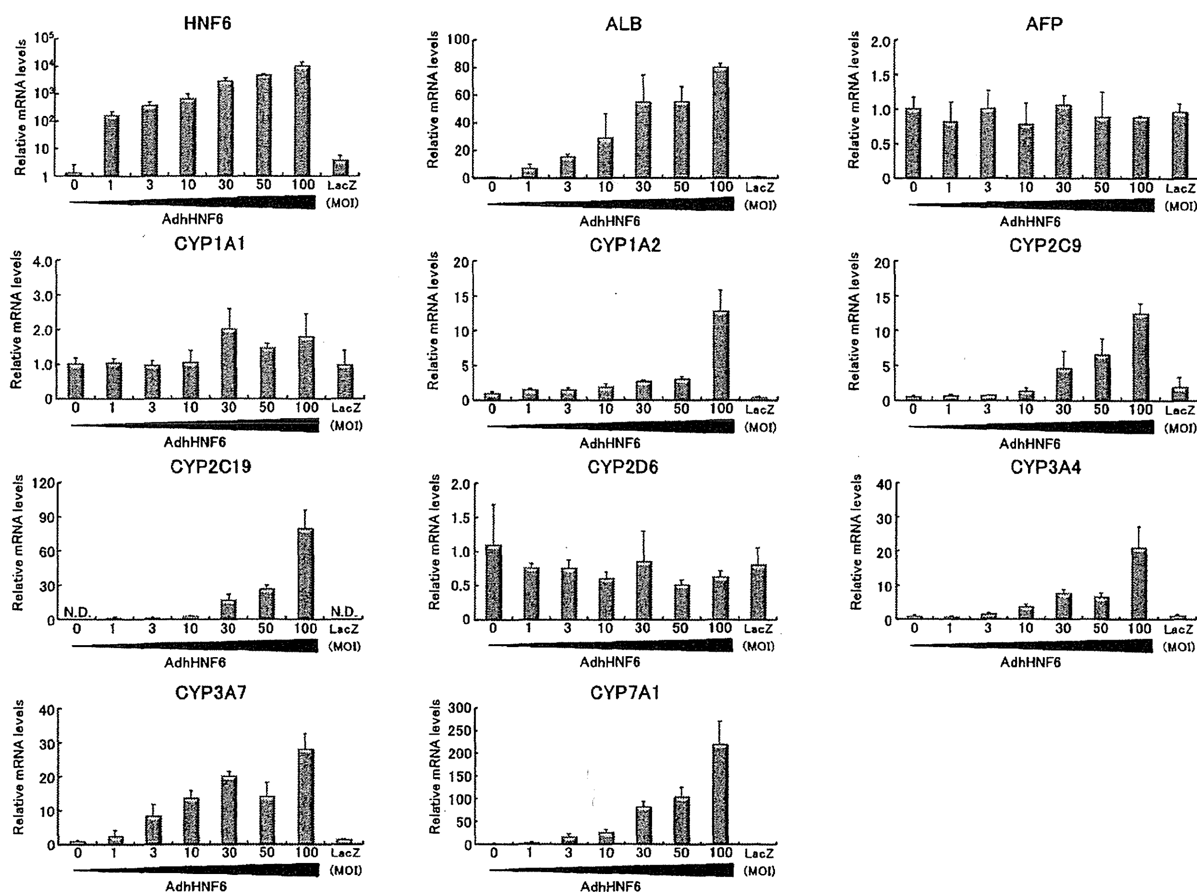

Fig. 2. Effect of HNF6 on *CYP*, *ALB*, and *AFP* mRNA levels in HepG2 cells
HepG2 cells were seeded in 24-well tissue culture plates at 1.0×10^5 cells per well a day before adenovirus infection. The cells were infected with AdLacZ (MOI of 100) or AdhHNF6 (MOI of 1, 3, 10, 30, 50, or 100). After 72 h, total RNA was extracted and real-time PCR was carried out as described in Materials and Methods. *CYP*, *ALB*, and *AFP* mRNA levels were normalized to those of *GAPDH*; the values for each of these mRNAs in non-infected HepG2 cells were set equal to 1.0. The data represent mean ± SD ($n = 3$).

nearly one-tenth of that in hepatocytes. Testosterone 6β-hydroxylase activity in AdhHNF6-infected iPSHCs was also measured. This metabolite was detected in AdhHNF6-infected iPSHCs and hepatocytes, but not in iPSCs, AdLacZ-infected iPSHCs, or HepG2 cells (**Fig. 6B**). Its activity in AdhHNF6-infected iPSHCs was equal to nearly one-fiftieth of that in hepatocytes.

We also observed some increase in *CYP1A2* and *CYP3A7* mRNA levels, but not in those of *CYP1A1*, *CYP2C9*, or *CYP2D6*. In contrast, *CYP2C19* mRNA levels in iPSHCs significantly increased in a culture period-dependent manner, but decreased upon AdhHNF6 infection.

AFP mRNA levels in iPSHCs reached the same levels as those in HepG2 cells and were significantly higher than those in hepatocytes. The *ALB* mRNA levels in iPSHCs at days 22 and 25 were 16-fold higher than those in HepG2 cells. Introducing AdhHNF6 did not affect the *ALB* or *AFP* mRNA levels in iPSHCs.

Discussion

In this study, we investigated whether HNF6 could modulate *CYP* gene transactivation in hepatocyte-like cells differentiated from iPSCs (**Fig. 6A**). Expression levels of the *CYP3A4* gene in iPSHCs were extremely low as compared with those in hepatocytes. Other *CYP* genes, excluding *CYP1A1*, were also expressed at levels significantly lower than those in hepatocytes. Furthermore, *AFP* mRNA levels in iPSHCs were similar to those in HepG2 cells, and were significantly higher than those in hepatocytes. Thus, iPSHCs differentiate into more fetal, rather than adult, hepatocyte-like cells. However, when HNF6 was introduced into iPSHCs, *CYP3A4* expression markedly increased and the cells gained the ability to metabolize testosterone (**Fig. 6B**). To our knowledge, our study is the first to show that HNF6 transactivates *CYP3A4* in iPSHCs.

CYP gene expression changes markedly during liver development, which is regulated by a dynamic network of LETFs. In particular, *CYP3A* genes show unique expression changes, influenced by development, and this developmental switch in gene expression between *CYP3A4* and *CYP3A7* occurs during the first 1–2 years after birth.[45] Previous studies have shown that c/EBPα, HNF3α, HNF3β, HNF3γ, and NF I interact with nucleotide sequences in the *CYP3A4* and *CYP3A7* proximal promoter regions, resulting in their transcriptional activation.[17,18,46] Saito *et al.*[47] indicated that NF I and HNF3β control the basal expression of *CYP3A7*, but not of *CYP3A4*. Conversely, c/EBPα and HNF3γ cooperatively enhance the transcription of *CYP3A4*. These tran-

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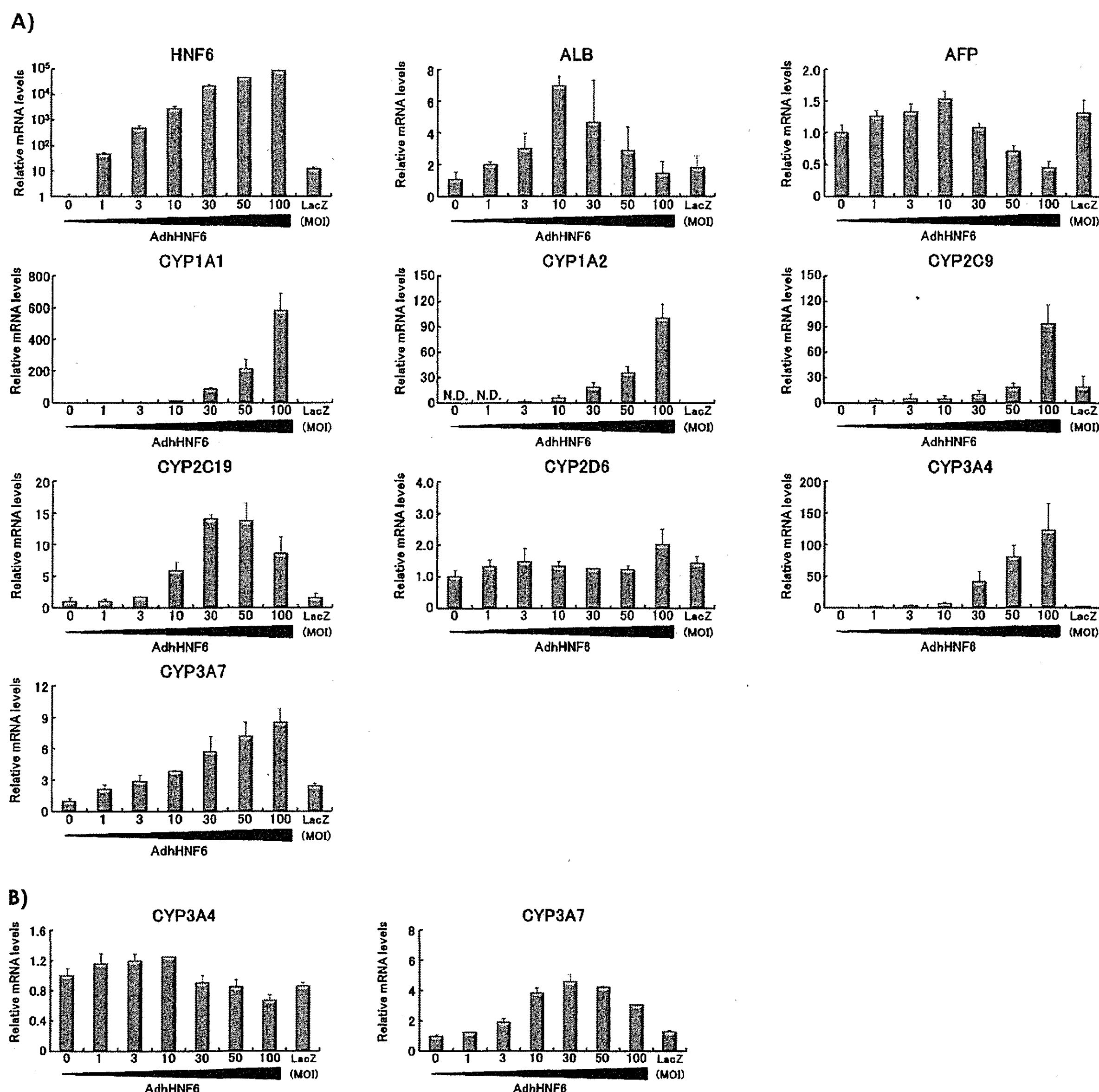

Fig. 3. Effect of HNF6 on *CYP*, *ALB*, and *AFP* mRNA levels in HFL cells and hepatocytes

HFL cells and hepatocytes were seeded in type I collagen-coated 24-well tissue culture plates at 5.0×10^4 and 1.0×10^5 cells per well, respectively, at 1 d before adenovirus infection. These cells were infected with AdLacZ (MOI of 100) or AdhHNF6 (MOI of 1, 3, 10, 30, 50, or 100). After 72 h, total RNA was extracted and real-time PCR was carried out as described in Materials and Methods (A: HFL cells, B: hepatocytes). *CYP3A4* and *CYP3A7* mRNA levels were normalized to those of *GAPDH*; the values for each of these mRNAs in non-infected hepatocytes or HFL cells were set equal to 1.0. The data represent mean ± SD ($n = 3$).

scription factors also upregulate transcription of *CYP3A7*, but to a lesser extent than that of *CYP3A4*. Although the 5′-flanking regions of these 2 genes are more than 90% identical over approximately 9 kb, distinct transcription factor complexes may contribute to developmental regulation by binding to *CYP3A4* and *CYP3A7* proximal promoters. Odom *et al.* showed that HNF6 binds to the promoter region of the genes encoding multidrug resistance-associated protein 2, CYP3A43, CYP51, and uridine diphosphate glucuronosyltransferase, as well as nuclear receptor genes, such as those encoding the pregnane X receptor and HNF4α.[48] These findings support the theory that HNF6 plays important roles in the regulation of pharmacokinetics-related genes. We demonstrated that HNF6 introduction resulted in increased *CYP3A4* gene transactivation in HFL cells and HepG2 cells, which have features similar to those of fetal hepatocyte-like cells, in terms of expressing *CYP3A7* and *AFP* (**Figs. 2** and **3A**). Our results also indicated that HNF6 significantly increased *CYP3A4* transcription in the iPSHCs generated, which showed features similar to those of fetal hepatocyte-like cells (**Fig. 6A**). In contrast, in hepatocytes, HNF6 introduction did not affect *CYP3A4* mRNA expression (**Fig. 3B**). These results suggest that HNF6 may play an important role in the transcriptional regulation of *CYP3A4* during hepatocyte maturation. This concept was supported by the observed changes in *HNF6* mRNA levels between fetal and adult hepatocytes.[49]

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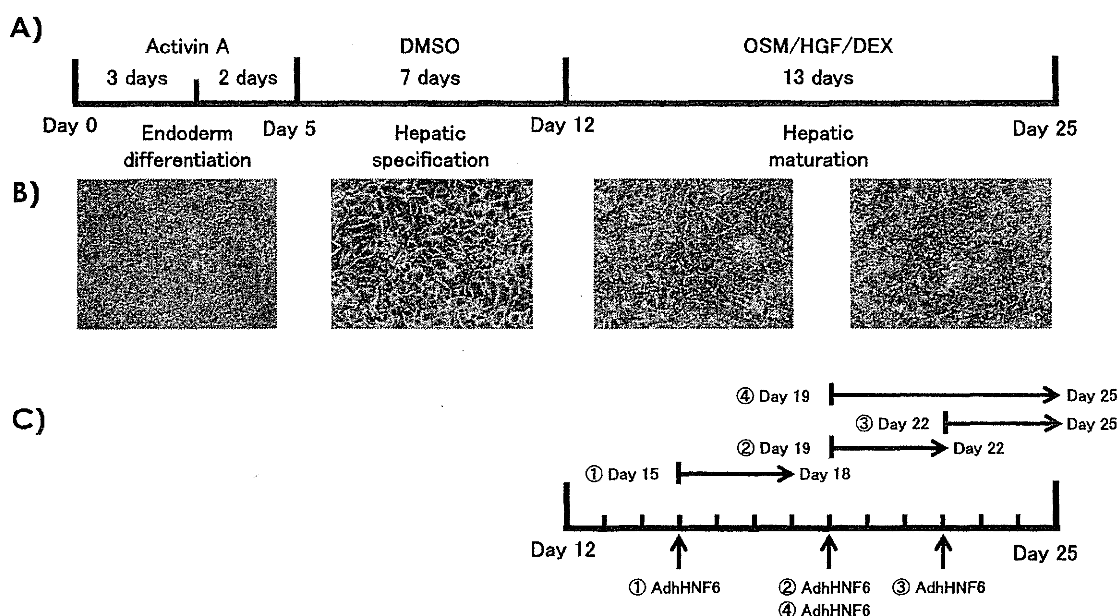

Fig. 4. (Color online) Strategy for the differentiation of iPSCs into hepatocyte-like cells
(A) iPSCs were cultured in RPMI 1640 supplemented with 0.5% FBS, GlutaMAX, 1 mM sodium butyrate, and 100 ng/mL activin for 3 d, and in RPMI 1640 supplemented with 2% KSR, GlutaMAX, 0.5 mM sodium butyrate, and 100 ng/mL activin for 2 d, to induce definitive endoderm formation. Next, the cells were plated onto Matrigel™-coated 24-well tissue culture plates in KnockOut™ DMEM supplemented with 20% KSR, GlutaMAX, 0.1 mM NEAA, 0.1 mM 2-ME, and 1% dimethyl sulfoxide for 7 d to induce hepatoblast formation. Subsequently, the iPSC-derived hepatoblasts were induced to differentiate into hepatocytes by switching to modified Lanford medium supplemented with 10 ng/mL HGF, 20 ng/mL OSM, and 100 nM DEX; these cells were then cultured for a further 14 d. (B) Morphological changes in iPSCs at different stages of differentiation. (C) A schematic representation of the AdhHNF6 infection schedule. Cells were cultured and infected with AdhHNF6 (100 MOI) using 4 conditions. For condition 1, the cells were infected with AdhHNF6 on day 15. After 3 d, the cells were recovered; thus, under condition 1, the culture period was 18 d. Under condition 2, the cells were infected with AdhHNF6 on day 19. After 3 d, the cells were recovered, totaling a culture period of 22 d. Under condition 3, the cells were infected with AdhHNF6 on day 22. After 3 d, the cells were recovered, resulting in a culture period of 25 d. Under condition 4, the cells were infected with AdhHNF6 on both days 19 and 22. Six days after the first infection, the cells were recovered, resulting in a culture period of 25 d.

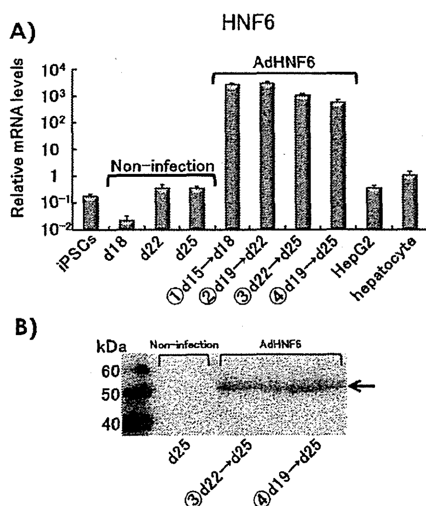

Fig. 5. *HNF6* mRNA and protein expression levels in iPSHCs infected with AdhHNF6
iPSHCs were infected with AdhHNF6 (100 MOI) at days 15, 19, and 22 or at days 19 and 22 (conditions 1, 2, 3, and 4, respectively). The cells were recovered after another 3 or 6 d, that is, following a total culture period of 18 d, 22 d, 25 d, and 25 d, respectively. After these cells were harvested, total RNA extracted and whole cell lysates prepared were used for real-time PCR (A) and immunoblot analyses (B), respectively, as described in Materials and Methods. Hepatocytes were cultured 48 h after plating the cells. *HNF6* mRNA levels were normalized to those of *GAPDH*; the value for HNF6 mRNA in hepatocytes, which had been cultured for 48 h, were set equal to 1.0. The data represent mean ± SD ($n = 3$).

Several studies have reported the successful differentiation of human iPSCs into hepatocyte-like cells. However, Song *et al.* [6] have shown that liver-specific genes such as *CYP2A6*, *CYP3A4*, *A1AT* (encoding α1-antitrypsin), and *ALB* were expressed at a lower level in differentiated iPSCs than in hepatocytes. Another study has also demonstrated that mRNA levels of most phase I and phase II enzymes were lower in iPSCs than in adult liver.[7] However, Takayama *et al.*[50] established a method to induce efficient hepatic differentiation of iPSCs by *HEX*, *SOX17*, and *HNF4α* transduction. *CYP3A4* mRNA levels in these hepatocyte-like cells were similar to those observed in primary hepatocytes. A previous study also showed that HNF1α, HNF4α, and HNF6 form part of a core transcriptional regulatory circuitry in hepatocytes.[51] Thus, HNF6, like HNF4α, may play a relatively important role in hepatic differentiation of iPSCs.

In this study, we observed transcriptional activation of the *CYP3A4* reporter gene, constructed with a proximal promoter and XREM regions, by introduction of HNF6 in HepG2 cells (**Fig. 1**). This result raises the possibility that HNF6 activates *CYP3A4* transcription through the proximal promoter and/or XREM regions. Previous studies have indicated that HNF6 directly controls *HNF1β* expression, cooperates with HNF1α to regulate *HNF4α* expression, and interacts with HNF3β without binding to DNA to stimulate *HNF3β*.[21,52,53] Several researchers have also reported that HNF6 participates in the recruitment of a coactivator, such as peroxisome proliferator-activated receptor gamma coactivator-1α,

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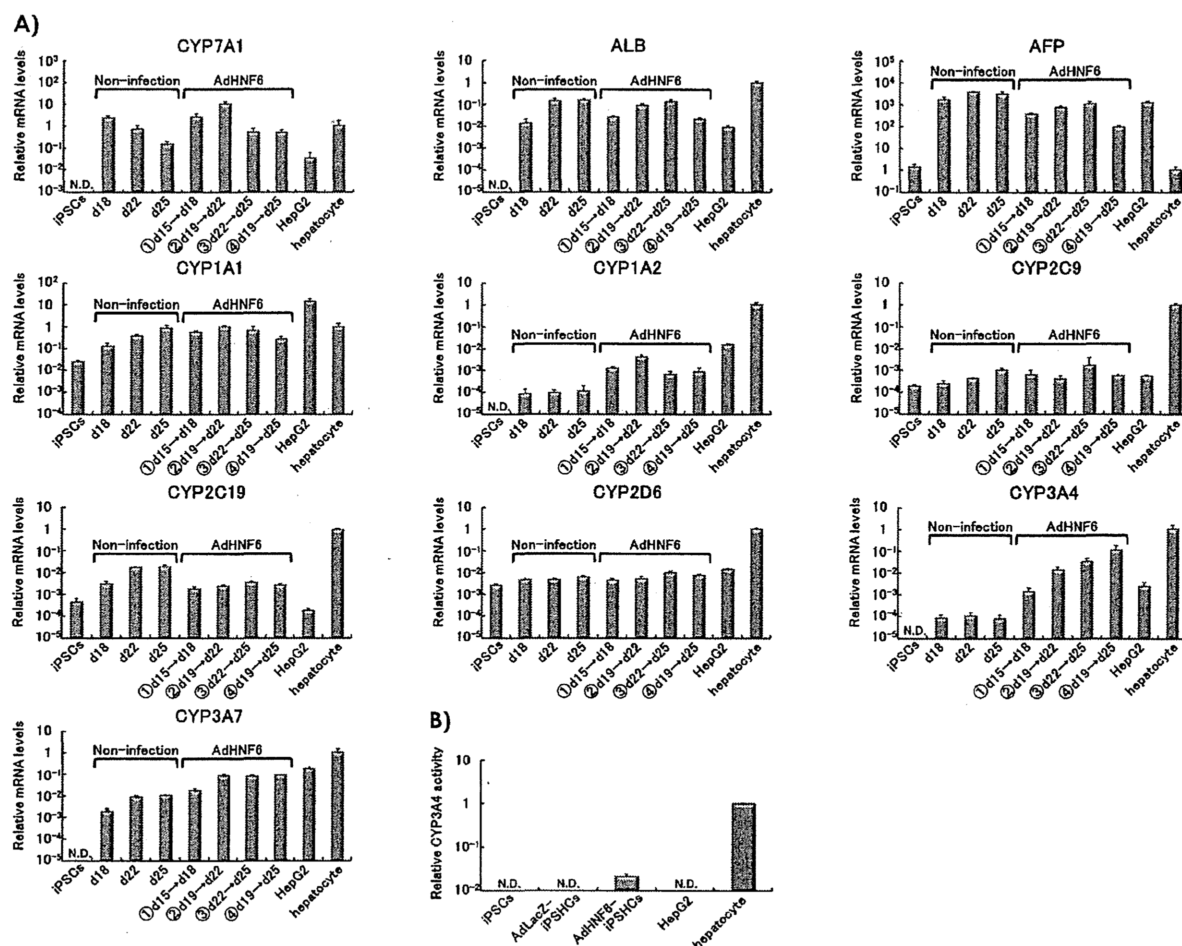

Fig. 6. Effect of HNF6 overexpression on *CYP*, *ALB*, and *AFP* mRNA levels and CYP3A4 activity in iPSHCs
(A) iPSHCs were infected with AdhHNF6 (100 MOI) on days 15, 19, and 22 or on days 19 and 22 (conditions 1, 2, 3, and 4, respectively). The cells were recovered after another 3 or 6 d, that is, following a total culture period of 18 d, 22 d, 25 d, and 25 d, respectively. After these cells were harvested, total RNA was extracted, and real-time PCR was carried out as described in Materials and Methods. *CYP*, *ALB*, and *AFP* mRNA levels were normalized to those of *GAPDH*; the values for each of these mRNAs in hepatocytes, which had been cultured for 48 h, were set equal to 1.0. The data represent mean ± SD ($n = 3$). (B) 6β-Hydroxytestosterone was measured with HPLC as described in Materials and Methods. The amounts of 6β-hydroxytestosterone in hepatocytes, which had been cultured for 48 h, were set equal to 1.0. The data represent mean ± SD ($n = 3$).

CREB-binding protein, and p300/CBP-associated factor, which is involved in *CYP* gene expression.[20,28] Thus, cross-regulatory cascades between HNF6 and other LETFs may be involved in the expression of *CYP3A4* in fetal hepatocyte-like cells, iPSHCs, HepG2 cells, and HFL cells. Further studies are needed to clarify whether the HNF6 directly or indirectly controls the expression of *CYP3A4* gene. Expression of other *CYP* genes was different in the effect of HNF6 introduction among HepG2 cells, HFL cells, and iPSHCs. This phenomenon may be caused by the different pattern of individual LETF expression relating to *CYP* gene expression in these cells.

In this study, we examined the role of HNF6 in the transactivation of *CYP* and liver-specific genes in iPSHCs. Our results showed that HNF6 strongly increases *CYP3A4* mRNA levels in iPSHCs, as well as in HepG2 cells and HFL cells. Furthermore, these cells had the ability to metabolize testosterone. The hepatic differentiation method using AdhHNF6 may contribute to the efficient development of hepatocytes from iPSCs. However, our AdhHNF6-infected iPSHCs still retained characteristics of immature hepatocytes. Future studies are required to further investigate the combinatorial effects of HNF6 and other transcriptional factors.

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