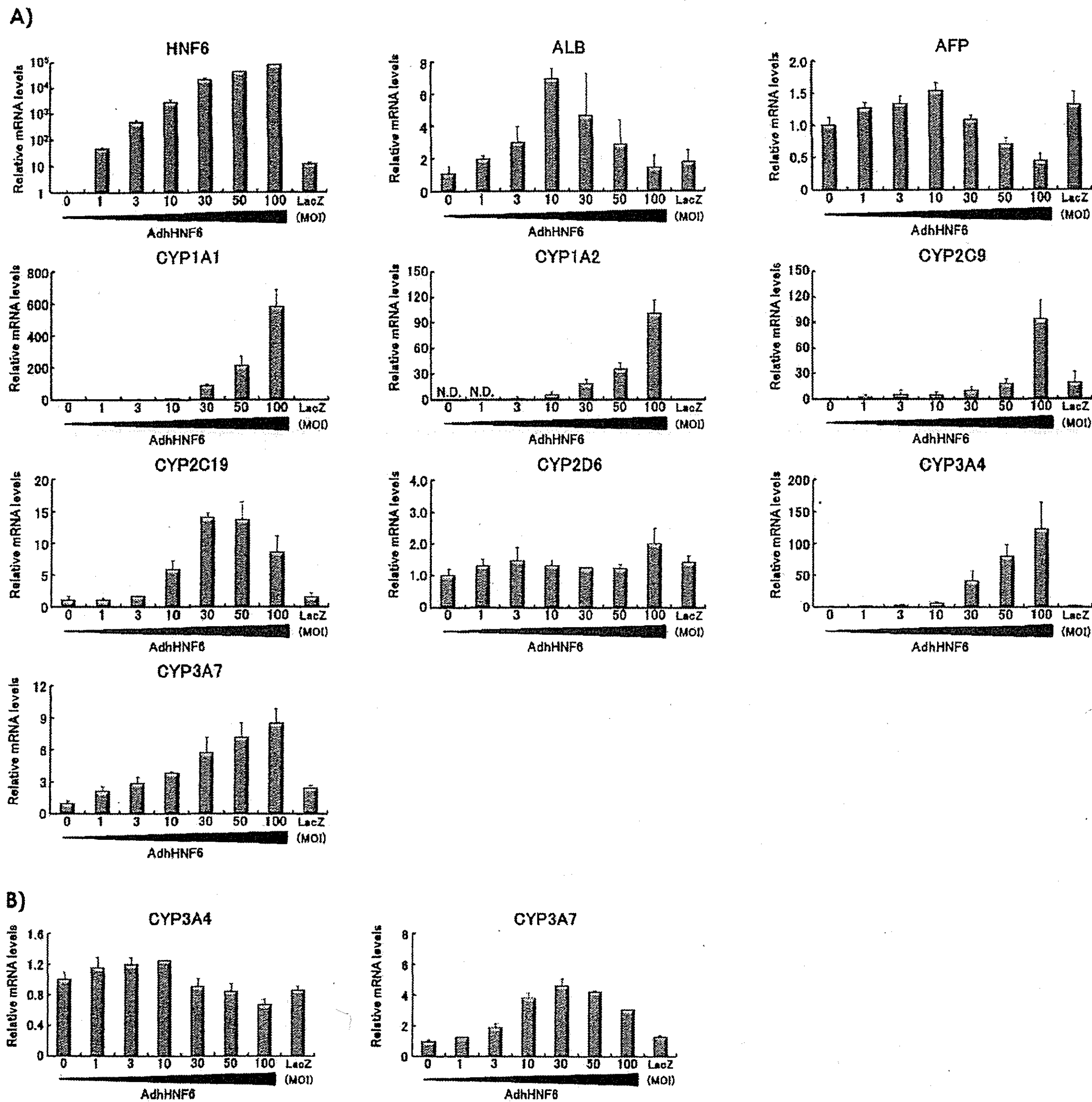

Fig. 3. Effect of HNF6 on *CYP*, *ALB*, and *AFP* mRNA levels in HFL cells and hepatocytes
HFL cells and hepatocytes were seeded in type I collagen-coated 24-well tissue culture plates at 5.0×10^4 and 1.0×10^5 cells per well, respectively, at 1 d before adenovirus infection. These cells were infected 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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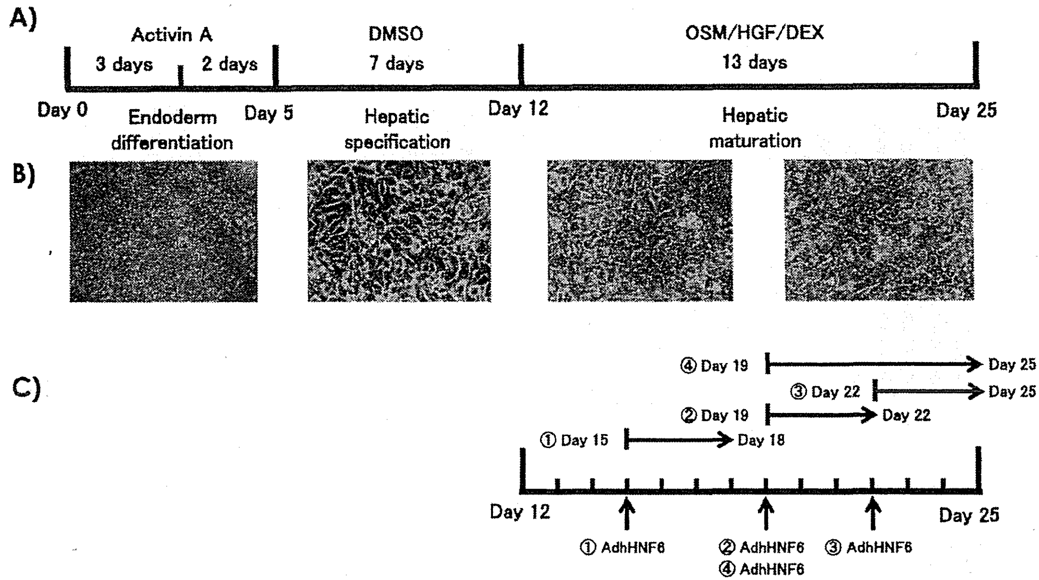

Fig. 4. (Color online) Strategy for the differentiation of iPSCs into hepatocyte-like cells
(A) iPSCs were cultured in RPMI 1640 supplemented with 0.5% FBS, GlutaMAX, 1 mM sodium butyrate, and 100 ng/mL activin for 3 d, and in RPMI 1640 supplemented with 2% KSR, GlutaMAX, 0.5 mM sodium butyrate, and 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.

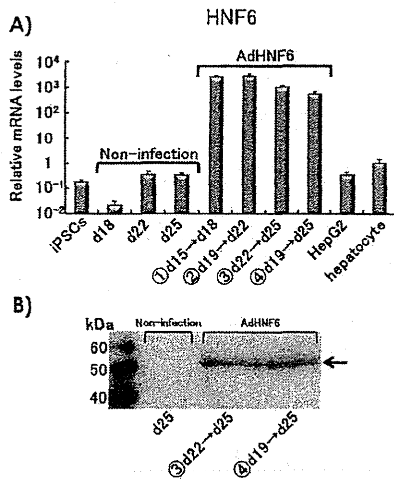

Fig. 5. ***HNF6*** **mRNA and protein expression levels in iPSHCs infected with AdhHNF6**
iPSHCs were infected with AdhHNF6 (100 MOI) at days 15, 19, and 22 or at days 19 and 22 (conditions 1, 2, 3, and 4, respectively). The cells were recovered after another 3 or 6 d, that is, following a total culture 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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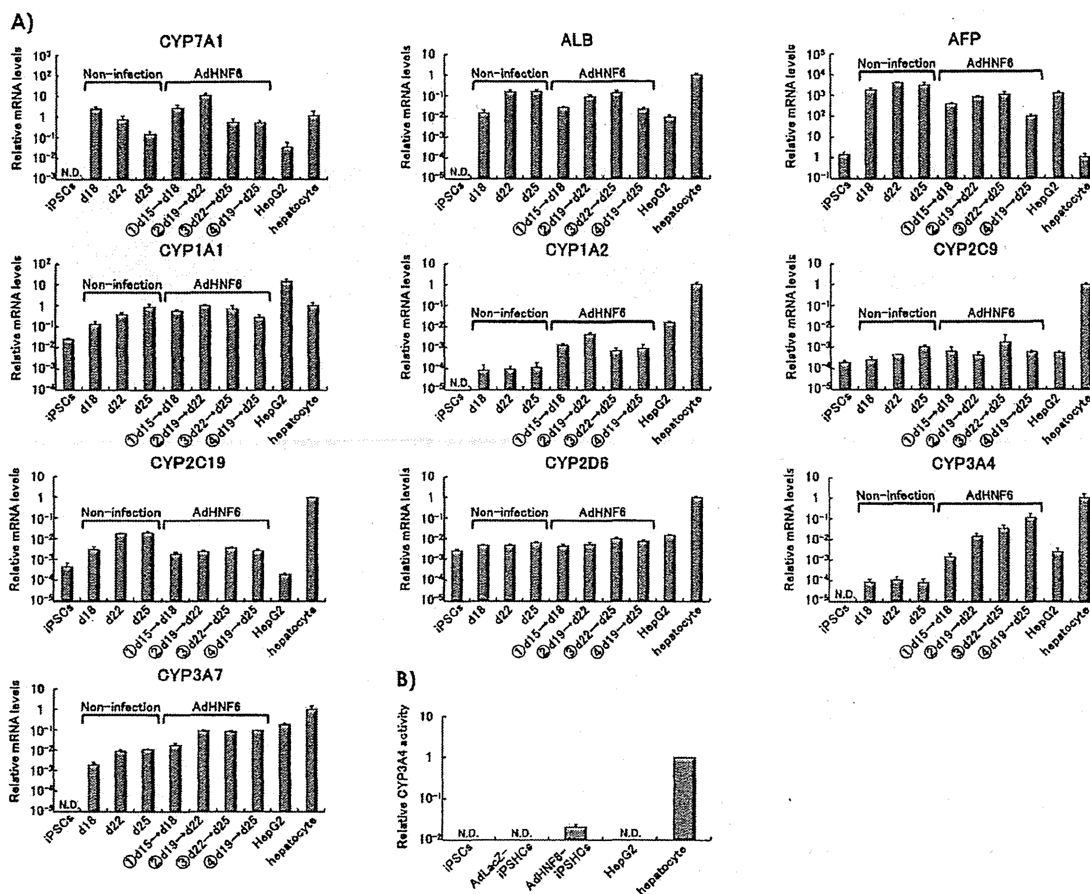

Fig. 6. Effect of HNF6 overexpression on *CYP*, *ALB*, and *AFP* mRNA levels and CYP3A4 activity in iPSHCs
(A) iPSHCs were infected with AdhHNF6 (100 MOI) on days 15, 19, and 22 or on days 19 and 22 (conditions 1, 2, 3, and 4, respectively). The cells were recovered after another 3 or 6 d, that is, following 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 $\pm$ 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 $\pm$ 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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