Direct Conversion of Mouse Fibroblasts into Cholangiocyte Progenitor Cells

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SUMMARY

Disorders of the biliary epithelium, known as cholangiopathies, cause severe and irreversible liver diseases. The limited accessibility of bile duct precludes modeling of several cholangiocyte-mediated diseases. Therefore, novel approaches for obtaining functional cholangiocytes with high purity are needed. Previous work has shown that the combination of Hnf1β and Foxa3 could directly convert mouse fibroblasts into bipotential hepatic stem-cell-like cells, termed iHepSCs. However, the efficiency of converting fibroblasts into iHepSCs is low, and these iHepSCs exhibit extremely low differentiation potential into cholangiocytes, thus hindering the translation of iHepSCs to the clinic. Here, we describe that the expression of Hnf1α and Foxa3 dramatically facilitates the robust generation of iHepSCs. Notably, prolonged in vitro culture of Hnf1α- and Foxa3-derived iHepSCs induces a Notch signaling-mediated secondary conversion into cholangiocyte progenitor-like cells that display dramatically enhanced differentiation capacity into mature cholangiocytes. Our study provides a robust two-step approach for obtaining cholangiocyte progenitor-like cells using defined factors.

INTRODUCTION

Cell therapy using hepatocytes has been highlighted as a promising treatment for repairing the irreversible liver diseases as an alternative to liver transplantation (Dhawan et al., 2010; Forbes et al., 2015). However, due to the limited accessibility and non-expandable nature of primary hepatocytes, a number of studies have attempted to generate hepatocyte-like cells from distinct types of stem cells, such as pluripotent stem cells (PSCs) (Hay et al., 2008; He et al., 2014; Si-Tayeb et al., 2010; Zhang et al., 2012). Indeed, hepatocyte-like cells generated from PSCs clearly share the key cellular features of primary hepatocytes (Hay et al., 2008; Si-Tayeb et al., 2010), but ethical and safety concerns abound on the use of PSC-derived hepatocyte-like cells in the clinic (Tang et al., 2011). To obviate the issues associated with using PSC-derived hepatocyte-like cells, recent studies directly generated hepatocyte-like cells, namely iHeps (induced hepatocyte-like cells), from both mouse and human somatic cells with defined factors without the cells having to first pass through a PSC state (Du et al., 2014; Huang et al., 2011, 2014; Kim et al., 2015; Lim et al., 2016; Sekiya and Suzuki, 2011).

Recent studies have demonstrated that the relatively small population of non-parenchymal cell types, such as cholangiocytes, plays also an important role in liver regeneration (Alvaro et al., 2007; Walter et al., 2014). Recently, direct conversion technology has been applied for generating self-renewing and bipotential HepSCs, so-called induced hepatic stem-like cells (iHepSCs), using the defined factors Hnf1β and Foxa3 (Yu et al., 2013). But prior to the translation of iHepSC technology to the clinic, a few issues need clear resolution. First, the final factor combination for iHepSC generation was determined without considering the actual conversion efficiency using authentic HepSC-specific markers. Second, the efficiency of converting somatic cells into iHepSCs is very low, less than 0.5%, and needs to be improved. Third, and most importantly, iHepSCs exhibit very low differentiation potential into mature cholangiocytes, which strongly necessitates further optimization of the combination of factors used for obtaining either iHepSCs with enhanced cholangiocyte differentiation potential or cholangiocyte progenitor cells (CPCs).

In the current study, we revisited the roles of several HepSC-specific candidate factors in reprogramming and found that the combination of Hnf1α and Foxa3 dramatically facilitates the generation of iHepSCs that are transcriptionally closer to the endogenous hepatic progenitor cells than are iHepSCs from previous study. Moreover, the prolonged culture of Hnf1α and Foxa3-derived iHepSCs...
could induce further reprogramming that is mediated by Notch signaling into cholangiocyte progenitor-like cells, so-called induced CPCs (iCPCs), which display dramatically enhanced differentiation potential into mature cholangiocytes. Our study provides a strategy for generating iCPCs using defined factors.

RESULTS

Hnf1α and Foxa3 Robustly Induce Hepatic Stemness in Fibroblasts

To define the combination of factors that is required for inducing either HepSC or CPC identities in somatic cells, we selected five candidate factors based on their roles in liver development (Foxa1, Foxa2, and Hnf1α) and regeneration (Foxa3 and Hnf1α). Mouse embryonic fibroblasts (MEFs), which are devoid of epithelial cells, were transduced with all candidate factors as described previously (Lim et al., 2016) (Figure 1A). After 2 weeks of transduction, we observed the growth of epithelial colonies of a compact and three-dimensional (3D) shape that was morphologically distinct from that typical of iHep colonies (Figure S1A). Notably, a significant number of colonies expressed both the fetal hepatocyte marker α-fetoprotein (AFP) and the cholangiocyte marker cytokeratin 19 (CK19) (67.5% ± 13.5%; Figures 1B and 1C). Moreover, these colonies were strongly positive for other cholangiocyte and HepSC markers, such as CK7 and TROP2, respectively (Figure 1B). However, partially reprogrammed cells with epithelial morphology failed to activate both AFP and CK19 (Figure S1B), indicating that our unbiased approach could accurately measure the conversion efficiency. Taken together, the epithelial colonies that had emerged from the five factor-transduced MEFs might have a cellular identity similar to HepSCs and distinct from iHeps.

We next attempted to minimize the number of factors required for HepSC conversion. For this, we removed the factors from the cocktail one by one and found that removing any of the three factors Foxa2, Foxa3, and Hnf1α drastically reduced the number of AFP+/CK19+ iHepSC colonies (Figure 1C). The removal of either Hnf1β or Foxa1 did not negatively influence both iHepSC conversion and hepatic gene activation (figures 1C and 1D). In contrast, iHepSCs generated in the absence of Hnf1α displayed poor activation of endogenous HepSC markers (Figure 1D). However, the gene expression pattern of iHepSCs generated in the absence of either Foxa2 or Foxa3 was comparable with that of iHepSCs generated with all five factors together (Figure 1D). Thus, we hypothesized that Hnf1α might play a key role in the transcriptional activation of the endogenous hepatic program and that Foxa2 and Foxa3 might rather play assistant roles that would enhance the conversion efficiency (Figures 1C and 1D). To test our hypothesis, we introduced Hnf1α with either Foxa2 (1a2) or Foxa3 (1a3) in MEFs. Interestingly, 1a3-transduced MEFs exhibited the more mature expression patterns of both cholangiocyte (CK7 and CK19) and HepSC (Epcam and Trop2) markers (Figure 1E) with significantly higher numbers of AFP+/CK19+ iHepSC colonies (Figure 1F) than did 1a2-transduced MEFs. The majority of 1a3-derived iHepSC colonies were strongly double-positive for other hepatocyte and cholangiocyte markers (Figure 1G). Notably, 1a3-transduced MEFs produced a significantly higher number of EPCAM+ cells strongly expressing hepatocyte-, cholangiocyte-, and HepSC-specific markers (Figures 1H and 1I). All the clonal lines derived from 1a3-transduced MEFs displayed the features typical of HepSCs as determined by a series of gene expression analyses (Figures S1C–S1E). Finally, iHepSCs could be generated from mouse adult tail-tip fibroblasts and were stably expanded for more than 10 passages without losing features typical of HepSCs (Figures S1F and S1G). Notably, three-factor (Hnf1α, Foxa2, Foxa3) transduced MEFs exhibited the most mature expression patterns of all markers investigated (Figures S1H and S1I).

Figure 1. Direct Conversion of Fibroblasts into iHepSCs by Hnf1α and Foxa3
(A) Schematic depicting the procedure for the direct conversion of fibroblasts into iHepSCs.
(B) Immunofluorescence of HepSC colony after 2 weeks of transducing all five transcription factors. Nuclei were stained with DAPI. Scale bars, 100 μm.
(C) The number of AFP+/CK19+ colonies was counted after 2 weeks of transduction. Data are presented as mean ± SD from three independent experiments. Two-tailed Student’s t test: *p < 0.05, **p < 0.01.
(D and E) Expression patterns of hepatocyte-, cholangiocyte-, and HepSC-specific markers were analyzed by RT-PCR after transduction of MEFs with different combinations of factors.
(F) The number of AFP+/CK19+ colonies was counted on day 4 after transduction of MEFs with Hnf1α together with Foxa2 (1a2) or Foxa3 (1a3). Data are presented as mean ± SD from three independent experiments. Two-tailed Student’s t test: *p < 0.05.
(G) Immunofluorescence of 1a3-transduced iHepSC colony. The nuclei were stained with DAPI. Scale bars, 100 μm.
(H) Percentage of EPCAM+ cells was evaluated by flow cytometry 2 weeks after transduction of MEFs with either 1a2 or 1a3. MEFs, i.e., non-transduced cells, were used as a negative control. Data are presented as mean ± SD from three independent experiments. Two-tailed Student’s t test: *p < 0.05.
(I) Expression of hepatocyte-, cholangiocyte-, and HepSC-specific markers in EPCAM+ or EPCAM− cells was measured by qPCR. The levels were normalized to those of EPCAM− cells and are presented as mean ± SD from triplicate values.
Figure 2. Differential Potential of 1a3-iHepSCs into Mature Hepatocytes and Cholangiocytes In Vitro
(A) Immunofluorescence of established 1a3-iHepSC-derived hepatocytes. The nuclei were stained with DAPI. Scale bar, 100 μm.
(B) In vitro functional analyses of 1a3-iHepSC-derived hepatocytes by periodic acid-Schiff (PAS) staining and indocyanine green (ICG) uptake assay. Scale bars, 100 μm.
(C) Serum albumin secreted from 1a3-iHepSC-derived hepatocytes was measured by ELISA. MEFs and primary hepatocytes were used as negative and positive controls, respectively. Data are presented as mean ± SD from triplicate values.
(D) Morphology of 1a3-iHepSC-derived cholangiocytes in branches and ductal cysts was analyzed under bright-field (upper panel) and immunofluorescence (lower panel) microscopy. Antibody directed against CK19 was used, and the nuclei were stained with DAPI. Scale bar, 100 μm.
(E) Expression of mature cholangiocyte markers was evaluated by qPCR upon cholangiocyte differentiation. All the values were normalized to those of undifferentiated 1a3-iHepSCs. Bile duct tissues were used as a positive control. Data are presented as mean ± SD of triplicate values from three individual cell lines. Two-tailed Student’s t test: *p < 0.05, **p < 0.01.
(F) Transport of rhodamine-123 (Rho123) into the central lumen of a ductal cyst. Treatment with the MDR inhibitor verapamil (Ver) to block the transport activity of 1a3-iHepSC-derived ductal cysts. Scale bar, 100 μm.
(G) Quantification of degree of cyst swelling of 1a3-iHepSC-derived cholangiocytes after 24 hr of stimulation with forskolin (FSK) and IBMX in the absence or presence of the CFTR inhibitor CFTRinh-172. The degree of swelling after 24 hr of stimulation was quantified based on the unstimulated cyst size. Five cysts in each group from three independent experiments were analyzed. Data are presented as mean ± SD. Paired t test: *p < 0.05.
and Foxa3; 1a23) could not further enhance reprogramming efficiency compared with 1a3 (Figure S1H). Taken together, our data clearly show that 1a3 is the best combination for a robust induction of hepatic stemness on somatic cells but is not enough for inducing a CPC cell identity.

1a3-iHepSCs Can Differentiate into Mature Hepatocytes and Cholangiocytes In Vitro

We next assessed the in vitro differentiation potential of 1a3-derived iHepSCs (hereafter referred to as 1a3-iHepSCs) to determine whether they had acquired hepatic stemness. Within 24 hr of hepatic differentiation (Li et al., 2006; Yu et al., 2013), aggregates typical of differentiated cells were readily observed (Figure S2A). After 7 days, we were able to identify mature aggregates with strong activation of albumin (ALB) and complete inactivation of CK19 (Figure 2A). RT-PCR analysis also showed that the expression of hepatocyte markers was strongly upregulated, whereas both cholangiocyte and HepSC markers were dramatically suppressed (Figure S2B). Moreover, 1a3-iHepSCs were found to display glycogen storage, xenobiotic metabolic activity, and albumin secretion upon hepatic differentiation, indicating that they have the potential to differentiate into mature hepatocytes in vitro (Figures 2B and 2C).

After 7 days of differentiation into cholangiocytes (Li et al., 2010), 1a3-iHepSCs had differentiated into CK19+ cholangiocytes that exhibited a typical branching structure (Figure 2D, left). Under 3D differentiation conditions containing 40% Matrigel (Tanimizu et al., 2007), we observed CK19+ mature cystic structures (Figure 2D, right). The cysts strongly expressed cholangiocyte-associated genes (Figure 2E) and exhibited apicobasal polarity as shown by the localization of F-actin in the inner layer of the lumen (Figure S2C), demonstrating that cholangiocytes that had differentiated from 1a3-iHepSCs share molecular and structural characteristics with primary cholangiocytes. A major physiological function of cholangiocytes is the secretion of substances such as water and ions for modulating bile composition, and this process is mediated by transmembrane channel proteins such as multidrug resistance protein 1 (MDR1) and cystic fibrosis transmembrane regulator (CFTR). Thus, we examined the transporter activity of MDR1 in the cholangiocytes derived from 1a3-iHepSCs by evaluating the efflux of rhodamine-123 (Rho123), and found that the cholangiocytes could transport Rho123 into the luminal space (Figure S2D). However, in the presence of the MDR1 inhibitor verapamil, Rho123 did not accumulate in the lumen, indicating that the differentiated cholangiocytes in cysts behave like their in vivo counterpart (Figure 2F). We also performed a forskolin-induced swelling assay to monitor CFTR-mediated fluid transport and cyst swelling in the cholangiocytes. After 24 hr of forskolin treatment, the size of the cysts had increased by 3.31 ± 0.44-fold (Figures 2G and S2E). In contrast, the forskolin/IBMX (3-isobutyl-1-methylxanthine)-induced cyst swelling was abolished in the presence of CFTRinh-172, a CFTR inhibitor, showing that the size of the cysts that had differentiated from 1a3-iHepSCs is regulated by CFTR, as in in vivo cholangiocytes (Figure 2G). Notably, iHepSCs from adult tail-tip fibroblasts also differentiated into both hepatocytes and cholangiocytes (Figures S2F and S2G). Taken together, our data clearly indicate that 1a3-iHepSCs possess an in vitro differentiation potential into both functionally mature hepatocytes and cholangiocytes.

Hnf1α Plays a Critical Role in the Induction Phase of Hepatic Stemness Acquisition

As the previous study (Yu et al., 2013) had used Hnf1β and Foxa3 (1b3) for converting fibroblasts into iHepSCs, we decided to compare the roles of the two reprogramming cocktails 1a3 and 1b3 during the induction phase of hepatic stemness acquisition (Figure 3A). In line with our

Figure 3. Hnf1α Plays a Critical Role in the Induction Phase of Hepatic Stemness Acquisition

(A) Schematic comparing the direct conversion process of fibroblasts into iHepSCs driven by Foxa3 together with Hnf1α (1a3) or Hnf1β (1b3).

(B) Conversion efficiency (%) into iHepSCs from the MEFs transduced with either 1a3 or 1b3 was determined by flow-cytometric analysis using an antibody directed against E-cadherin (left) or EPCAM (right) on day 14 of transduction.

(C) Expression of hepatocyte-, cholangiocyte-, and HepSC-specific markers was analyzed by qPCR in a time course manner after transduction of MEFs with each reprogramming cocktail. The levels were normalized to those of MEFs and are presented as mean ± SD of triplicate values.

(D) Heatmaps representing the expression patterns of markers related to mesenchymal-epithelial transition (MET) process, fibroblasts, cell cycle, hepatocytes, cholangiocytes, and hepatic progenitors after 12 days of introducing 1a3 or 1b3 into MEFs. Color bar at the bottom indicates gene expression in log2 scale. Red and green colors represent higher and lower expression levels, respectively.

(E) TRNs in the MEFs after 12 days of transducing MEFs with 1a3 or 1b3. Subnetwork modules were extracted using DEGs between LEPCs and MEFs by CellNet. Yellow and red dashed circles represent MEF- and LEPC-specific modules, respectively.

(F) Heatmaps describing gene expression profiles in LEPC- and MEF-specific modules in MEFs 12 days post infection with 1a3 or 1b3. Hierarchical clustering analysis based on the gene expression profiles from the heatmap is shown at the top. MEFs and LEPCs were used as negative and positive controls, respectively.
Figure 4. 1a3-iHepSCs Are Transcriptionally Closer Than 1b3-iHepSCs to LEPCs

(A) RT-PCR analysis comparing the expression pattern of hepatocyte-, cholangiocyte-, and HepSC-specific markers in three independent clonal lines from 1a3- and 1b3-iHepSCs, which were generated by fluorescence-activated cell sorting with EPCAM.

(B) Heatmap analysis describing whole-genome expression profiles of 1a3- and 1b3-iHepSCs at passage 10. Hierarchical clustering analysis based on the expression profiles from the heatmap is depicted at the top. MEFs and LEPCs were used as negative and positive controls, respectively.

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screening assay results (Figure 1), 1a3-transduced MEFs produced significantly increased numbers of either E-cadherin* or EPCAM* populations compared with the 1b3-transduced MEFs (Figure 3B). This increased yield of 1a3 was due to neither distinct transduction levels of individual transgenes nor distinct proliferation rates of the MEFs transduced with distinct combinations of factors (Figures S3A and S3B). Notably, 1a3-transduced MEFs exhibited dramatically accelerated conversion kinetics toward a HepSC state, as evidenced by the enhanced activation of genes associated with hepatic stemness compared with 1b3-transduced MEFs (Figures 3C, 3D, and S3C). Taken together, these data suggest that a conversion cocktail 1a3 is more potent than 1b3 for the robust induction of hepatic stemness.

To explore the mechanism underlying 1a3-mediated enhanced iHepSC generation, we monitored the early transcriptional changes taking place following the introduction of 1a3 or 1b3 to the cells. Whole-genome transcription analysis revealed that a similar number of genes was either up- or downregulated (Figure S3D), and that both combinations activated similar pathways involved in various metabolic functions of the liver (Figure S3E). Despite this high similarity (figure S3F), we found that 441 and 357 genes were highly enriched in 1a3- and 1b3-transduced MEFs, respectively (Figure S3G). Gene ontology (GO) analysis of differentially expressed genes (DEGs) from 1a3- and 1b3-transduced MEFs (1a3-DEGs and 1b3-DEGs) show that various metabolic processes were highly ranked in 1a3-DEGs, whereas the genes involved in cell adhesion and neuronal development were top-ranked in 1b3-DEGs (Figure S3H). These data underscore the notion that 1b3 could not sufficiently activate the HepSC program and has off-target effects, activating genes associated with ectoderm lineage.

We next investigated the transcriptional signatures of 1a3-and 1b3-transduced MEFs by comparing the cells’ transcriptional regulatory network (TRN). To this end, we employed CellNet (Cahan et al., 2014), a computational network biology platform providing a comprehensive description of transcriptional networks constructed by numerous interactions among highly ranked transcription factors. We first generated a HepSC-TRN using both mouse liver epithelial progenitor cells (LEPCs)-specific DEGs (Li et al., 2006) and MEF-specific DEGs (Figure S3H). We found two major distinct subnetwork modules in the HepSC-TRN that are specific to LEPCs and MEFs, respectively (Figures S3I and S3J). To evaluate the early reprogramming status of both 1a3- and 1b3-transduced MEFs, we applied the DEGs (1a3 versus MEFs and 1b3 versus MEFs) on the HepSC-TRN (Figure 3E). In line with our kinetic analysis results (Figures 3C and 3D), 1a3-transduced MEFs displayed faster activation of genes involved in the LEPC-specific module compared with 1b3-transduced MEFs (Figure 3F). These data support the notion that Hnf1α is the authentic driving force for inducing hepatic stemness.

**1a3-iHepSCs Are Transcriptionally Closer Than 1b3-iHepSCs to LEPCs**

To compare the molecular and cellular characteristics of the established 1a3-iHepSCs with those of 1b3-iHepSCs, we generated three clonal iHepSC lines from each combination. During clonal expansion, we were unable to observe any significant difference in the proliferation rate and clonal efficiency of the resultant iHepSCs, indicating that the self-renewal capacities of both 1a3- and 1b3-iHepSCs are comparable (Figures S4A and S4B). Notably, the majority of stem cell markers were strongly activated in all 1a3-iHepSC clones, whereas some epithelial and HepSC markers were relatively less activated in most 1b3-iHepSC lines (Figure 4A). Heatmap analysis also showed that 1a3-iHepSCs displayed the relatively well-reprogrammed pattern of gene transcription compared with the expression pattern of 1b3-iHepSCs, although these cells clustered together (Figure 4B). Particularly, the fibroblast-specific transcriptional signature was largely maintained in the established 1b3-iHepSCs but nearly erased in 1a3-iHepSCs (Figure 4B), indicating that Hnf1α plays a critical role not only in the induction phase but also in the maturation phase of the process of acquiring hepatic stemness.

We also performed TRN analysis by applying both the 1a3- and 1b3-specific DEGs defined from the established iHepSC lines onto the HepSC-TRN (Figure 4C). Consistent with our expression profiling (Figures 4A and 4B), the expression levels of genes associated with LEPC-specific modules were significantly higher in 1a3-iHepSCs than in 1b3-iHepSCs, resulting in the relatively closer clustering of LEPCs and 1a3-iHepSCs (Figure 4D). Principal component analysis also showed that the expression pattern of the LEPC-specific module in 1a3-iHepSCs is very similar to that in LEPCs (Figure 4E). To our surprise, GO analysis showed that 1a3-specific DEGs were all associated with typical hepatic features, such as distinct metabolic processes (Figure 4F). However, non-hepatic events were highly ranked in 1b3-DEGs (Figure 4G), supporting the notion that 1b3 activates subsets of genes that do not

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(C) TRNs in 1a3- and 1b3-iHepSCs. Yellow and red dashed circles represent MEF- and LEPC-specific modules, respectively.
(D and E) Heatmap (D) and PCA (E) describing the gene expression profiles of the LEPC-specific module in iHepSCs. Hierarchical clustering results are displayed on top of the heatmap. MEFs and LEPCs were used as negative and positive controls, respectively.
(F and G) GO enrichment analysis of each iHepSC-specific DEGs.
Figure 5. 1a3-iHepSCs Exhibit Enhanced Differentiation Potential into Cholangiocytes

(A) Expression of hepatocyte- and cholangiocyte-specific markers was analyzed by qPCR after 7 days of differentiation of iHepSCs. All the values were normalized to those of MEFs and are presented as mean ± SD of triplicate values from three biological replicates. Paired t test: *p < 0.05, **p < 0.01.

(B and C) In vitro functional analyses of iHepSC-derived hepatocytes by PAS staining (B) and ICG uptake assay (C). Scale bars, 100 μm.

(D and E) Comparison of secretion levels of serum albumin (D) and urea (E) from iHepSC-derived hepatocytes. MEFs and primary hepatocytes were used as negative and positive controls, respectively. Data are presented as mean ± SD of triplicate values.

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belong to the hepatic lineage. Taken together, our data indicate that Hnf1α is indeed a master factor for inducing hepatic stemness.

**Hnf1α Dramatically Enhances In Vitro Differentiation Potential of iHepSCs into Cholangiocytes**

To investigate the effect of Hnf1α and Hnf1β on the functionality of iHepSCs, we compared the *in vitro* differentiation potential of the two iHepSC lines. Following 7 days of differentiation into hepatocytes, both iHepSC lines had formed typical aggregates and exhibited increased expression of mature hepatic genes but reduced expression of both cholangiocyte- and stem cell-specific markers (Figures 5A and S5A–S5D). Moreover, the genes associated with drug transport activity as well as the CYP450 genes were significantly increased to a similar extent (Figures 5E and 5F). The additional set of *in vitro* functional studies clearly suggests that the iHepSC lines from two distinct combinations are comparable in their hepatic differentiation capacity (Figures 5B–5E).

Next, we compared the differentiation potential of these two iHepSC lines into cholangiocytes. Although both 1α3- and 1β3-iHepSCs had formed cysts with typical apicobasal polarity under the 3D culture conditions (Figure 5F), their differentiation efficiency into cholangiocytes was dramatically different (Figures 5G and 5H). Indeed, 1α3-iHepSCs exhibited significantly increased numbers of Rho123-transporting mature cysts (8.6-fold higher) compared with 1β3-iHepSCs (Figure 5I). Furthermore, 1α3-iHepSC-derived cholangiocytes strongly expressed cholangiocyte markers to the level similar to that of bile duct tissues (Figure 5J). However, 1β3-iHepSC-derived cholangiocytes displayed relatively weak or no expression of those markers (Figure 5J). These data indicate that Hnf1α plays a crucial role as a determinant of hepatic stemness, resulting in the generation of iHepSCs with a dramatically enhanced cholangiocyte differentiation potential.

**Notch Signaling-Mediated Secondary Conversion into Cholangiocyte Progenitor-like Cells**

Interestingly, 1α3-iHepSCs from different passages (P10 and P30) displayed distinct differentiation efficiency toward mature cholangiocytes (Figure 6A). In contrast, their hepatic differentiation was dramatically reduced upon further passaging, and completely abolished in 1α3-iHepSCs from P30 (Figure 6B). To understand these distinct passage-dependent differentiation patterns of 1α3-iHepSCs toward cholangiocytes and hepatocytes, we determined the expression levels of hepatocyte and cholangiocyte markers. Although 1α3-iHepSCs from later passages (P20 and P30) displayed dramatically reduced levels of hepatocyte markers (Figures 6A and 6B), expression levels of cholangiocyte markers including CK19, Aqp1, Cftr, Ggt, and Hnf β were stably maintained or further enhanced even after long-term passaging (Figures 6A and 6C), indicating that prolonged *in vitro* culture could induce further commitment of 1α3-iHepSCs into iCPCs, which predominantly differentiate into mature cholangiocytes (Figures 6A and 6B). However, 1β3-iHepSCs maintained their hepatic stemness even after long-term culture (Figure 6A), suggesting that the secondary conversion of iHepSCs into iCPCs is mediated by 1α3 but not 1β3. This unique property of 1α3-iHepSCs offers a two-step strategy for generating iCPCs.

Recent studies have described Notch signaling-mediated differentiation of human PSCs into CPCs or mature cholangiocytes (Geisler et al., 2008; Ogawa et al., 2015; Sampaziotis et al., 2015). Thus we next investigated whether the secondary conversion of 1α3-iHepSCs into iCPCs is also governed by Notch signaling. As a result, we found that the expression levels of Notch and its targets (Notch2, Jag1, and Hes1) were increased upon serial passaging of 1α3-iHepSCs (Figure 6C). However, the expression levels of all the hepatic markers were stably maintained in 1α3-iHepSCs in the presence of DAPT, a γ-secretase inhibitor that blocks Notch signaling *in vitro* (Figures 6C–6E). Furthermore, the differentiation of 1α3-iCPCs (at P20) into mature cholangiocytes was completely blocked by DAPT treatment (Figures 6D–6F). Taken together, our data indicate that the secondary conversion process of iHepSCs into iCPCs as well as the unipotential differentiation potential of iCPCs into mature cholangiocytes are determined by Notch signaling (Figure 6F).

To further investigate the *in vivo* functionality of 1α3-iCPCs, we used 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-fed mice, a well-known model for activation of liver progenitor cells and bile duct proliferation (Preisegger et al., 1999), which allow for the spontaneous differentiation of...
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(A) Relative expression levels for Afp, Alb, Hnf4a, and CK19 in different conditions.

(B) Immunofluorescence images showing AFP/CK19/DAPI and HNF4A/SOX9/DAPI staining.

(C) Relative expression levels for Ttr, Hnf4a, and Ggt in different conditions.

(D) Relative expression levels for CK19 in different conditions.

(E) Immunofluorescence images showing AFP/CK19/DAPI staining for Mock and -DAPT conditions.

(F) Schematic diagram illustrating the transition from fibroblasts to HepSCs, followed by bipotential HepSCs and unipotent ICPCs, with Notch signaling-mediated secondary conversion to Cholangiocytes. The diagram also highlights Foxa3/Hnf1a factors.

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liver progenitor cells into cholangiocytes. $1 \times 10^6$ GFP-labeled 1a3-iCPCs (P20) with normal karyotype (Figure S7A) were transplanted into DDC-fed mice via intrasplenic injection. After 4 weeks of transplantation, the 1a3-iCPCs had not only incorporated into pre-existing bile ducts but also newly formed a significant number of GFP+ ductal structures through bile ductular proliferation without forming tumor (Figures 7A and S7B). However, only a few GFP+ ductal structures were observed in the liver sections of mice transplanted with 1b3-iHepSCs that had been maintained under the same culture conditions as the 1a3-iCPCs (Figure 7A). We compared the differentiation efficiency of both cell lines by counting the number of GFP+ cholangiocytes per total number of cholangiocytes. The number of GFP+ ductal structures derived from 1a3-iCPCs was significantly higher than that from 1b3-iHepSCs (Figure 7B), although the in vivo hepatic differentiation potential of 1b3-iHepSCs overwhelmingly exceeds that of 1a3-iCPCs (Figures S7C and S7D). Furthermore, a larger number of most GFP+ 1a3-iCPC-derived cells strongly expressed CK19 and the functional cholangiocyte-specific marker osteopontin (OPN), compared with 1b3-iHepSC-derived cells (Figures 7C–7F). Collectively, these results clearly demonstrate that iCPCs that had converted from 1a3-iHepSCs via Notch signaling pathway are functionally mature.

**DISCUSSION**

To maintain several metabolic functions, the liver consists of a few cell types that play distinct roles in hepatic homeostasis (Altin and Bygrave, 1988). Although parenchymal hepatocytes make up more than 85% of the liver, non-parenchymal cell types are known to be involved in liver regeneration (Michalopoulos, 2007). Indeed, several cholangiocyte-related diseases such as cystic fibrosis, Alagille syndrome, and primary sclerosing cholangitis exhibit severe liver dysfunction caused by abnormality in the bile ducts (Kobelska-Dubiel et al., 2014; Lindor et al., 2015; Turnpenny and Ellard, 2012), indicating the need for a novel approach for obtaining functional cholangiocytes with high purity. For this, Yu et al. (2013) demonstrated the direct generation of iHepSCs from mouse fibroblasts using Hnf1β and Foxa3. Although the previous study clearly characterized the bipotency of the iHepSCs, the conversion efficiency into iHepSCs was too low (less than 0.5%), with unusually extremely low differentiation potential into cholangiocytes (less than 2%). In the current study, we found that the combination of Hnf1α and Foxa3 is sufficient for the robust generation of bipotential iHepSCs with 3.2- to 6.8-fold increased conversion efficiency compared with the previously defined combination (Figure 3). Throughout our mechanistic and functional studies, we observed that 1a3-iHepSCs are superior to 1b3-derived iHepSCs in their gene expression profile (Figures 4A, 4B, and 4D), conversion kinetics (Figures 3C and 3D), and both in vitro and in vivo differentiation potential into cholangiocytes (figures 5 and 7), indicating that Hnf1α is a master factor for inducing hepatic stemness in somatic cells.

Previous in vivo studies might indirectly suggest a distinct role for each transcription factor (Hnf1α, Hnf1β, and Foxa3) in the generation of iHepSCs. Foxa3 is a pioneer factor that belongs to the Foxa subfamily of winged helix/forkhead box transcription factors, which are known to guide many transcription factors in accessing their targets during liver development and regeneration (Kaestner et al., 1994; Lee et al., 2005; Wangensteen et al., 2015), suggesting its potential and essential role in iHepSC generation. However, Hnf1β is essential for the earliest step of hepatic bud formation, as its depletion leads the embryonic lethality (Coffinier et al., 2002; Lokmane et al., 2008), indicating that Hnf1β plays a critical role in early liver development. In contrast, Hnf1α is known to play a critical role in liver regeneration (Fausto, 2004; Leu et al., 2001; Nagy et al., 1994), despite its dispensable role in liver development (Pontoglio et al., 1996). Upon liver injury, HNF1A is known to directly bind to STAT3 and AP-1, resulting in the activation of several regeneration-associated hepatic genes (Leu...
et al., 2001), suggesting that Hnf1α plays a key role in liver regeneration. Importantly, Hnf1α, together with Foxa3, is activated specifically during oval cell proliferation; oval cells are the prototype adult liver progenitor cell population (Fausto, 2004; Nagy et al., 1994). Indeed, in the adult livers undergoing rapid regeneration process after 70%
partial hepatectomy (PHx), the expression of Hnf1α preceded that of Hnf1β; Hnf1α was immediately activated after PHx, while Hnf1β was slowly and progressively upregulated (Figure S7E). Considering that Hnf1α and Foxa3 together lead the robust induction of hepatic stemness (Figure 3), 1α3-mediated direct conversion process toward an iHepSC state may share a similar pathway with the in vivo liver regeneration event that is orchestrated by both Hnf1α and Foxa3.

Furthermore, prolonged in vitro culture of 1α3-iHepSCs could induce the Notch signaling-mediated secondary conversion of 1α3-iHepSCs into unipotent iCPCs. Upon in vitro differentiation of 1α3-iCPCs into mature cholangiocytes, we observed bile duct proliferation (Figure S7F), a typical regenerative response to liver injury (Verdonk et al., 2016), suggesting that 1α3-iCPCs hold clinical potential for modeling various forms of liver diseases caused by cholangiocyte dysfunction. Further efforts in generating human iHepSCs/iCPCs and applying this technology for modeling genetic cholangiocyte-related diseases would essentially be required for successfully translating iHepSC/iCPC technology to the clinic.

EXPERIMENTAL PROCEDURES

Mice and Derivation of Fibroblasts
All mice used were bred and housed at the mouse facility of Konkuk University (KU) or at the Chinese Academy of Science (CAS). Animal handling was in accordance with both the KU and CAS animal protection guidelines. MEFs were derived on embryonic day 13.5 after removing the head and all internal organs, including the liver, from C57/B6 mouse strain embryos, and were cultured in DMEM (Hyclone) containing 10% fetal bovine serum and 5 mL of penicillin/streptomycin/glutamine (Invitrogen).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.03.002.

AUTHOR CONTRIBUTIONS

K.T.L., J.K., and S.I.H. performed the experiments and analyzed and interpreted the results. L.Z. and L.H. performed cell transplantation and analyzed the data. H.H., D.B., and I.L. analyzed the RNA sequencing data. K.-P.K. and H.R.S. analyzed and interpreted the results. Y.-P.H. kindly provided the LEPCs. D.W.H. conceived and supervised the study and wrote the manuscript.

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REFERENCES

Altin, J.G., and Bygrave, F.L. (1988). Non-parenchymal cells as mediators of physiological responses in liver. Mol. Cell. Biochem. 83, 3–14.

Alvaro, D., Mancino, M.G., Glaser, S., Gaudio, E., Marzioni, M., Francis, H., and Alpini, G. (2007). Proliferating cholangiocytes: a neuroendocrine compartment in the diseased liver. Gastroenterology 132, 415–431.

Cahan, P., Li, H., Morris, S.A., Lummertz da Rocha, E., Daley, G.Q., and Collins, J.J. (2014). CellNet: network biology applied to stem cell engineering. Cell 158, 903–915.

Coiffier, C., Gresh, L., Fiette, L., Tronche, F., Schütz, G., Babinet, C., Pontoglio, M., Yaniv, M., and Barra, J. (2002). Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 129, 1829–1838.

Dhawan, A., Puppi, J., Hughes, R.D., and Mitry, R.R. (2010). Human hepatocyte transplantation: current experience and future challenges. Nat. Rev. Gastroenterol. Hepatol. 7, 288–298.

Du, Y., Wang, J., Jia, J., Song, N., Xiang, C., Xu, J., Hou, Z., Su, X., Liu, B., Jiang, T., et al. (2014). Human hepatocytes with drug metabolic function induced from fibroblasts by lineage reprogramming. Cell Stem Cell 14, 394–403.

Fausto, N. (2004). Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 39, 1477–1487.

Forbes, S.J., Gupta, S., and Dhawan, A. (2015). Cell therapy for liver disease: from liver transplantation to cell factory. J. Hepatol. 62, S157–S169.

Geisler, F., Nagl, F., Mazur, P.K., Lee, M., Zimmer-Strobl, U., Strobl, L.J., Radtke, F., Schmid, R.M., and Siveke, J.T. (2008). Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 48, 607–616.

Hay, D.C., Zhao, D., Fletcher, J., Hewitt, Z.A., McLean, D., Urruticoechea-Uriguen, A., Black, J.R., Elcombe, C., Ross, J.A., Wolf, R., and Cui, W. (2008). Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. Stem Cells 26, 894–902.

He, J., Lu, H., Zou, Q., and Luo, L. (2014). Regeneration of liver after extreme hepatocyte loss occurs mainly via biliary transdifferentiation in zebrafish. Gastroenterology 146, 789–800.e8.

Huang, P., He, Z., Ji, S., Sun, H., Xiang, D., Liu, C., Hu, Y., Wang, X., and Hui, L. (2011). Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475, 386–389.

Huang, P., Zhang, L., Gao, Y., He, Z., Yao, D., Wu, Z., Cen, J., Chen, X., Liu, C., Hu, Y., et al. (2014). Direct reprogramming of human
fibroblasts to functional and expandable hepatocytes. Cell Stem Cell 14, 370–384.

Kaestner, K.H., Hiemisch, H., Luckow, B., and Schutz, G. (1994). The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. Genomics 20, 377–385.

Kim, J., Kim, K.P., Lim, K.T., Lee, S.C., Yoon, J., Song, G., Hwang, S.I., Scholer, H.R., Cantz, T., and Han, D.W. (2015). Generation of integration-free induced hepatocyte-like cells from mouse fibroblasts. Sci. Rep. 5, 15706.

Kobelska-Dubiel, N., Klimczewicz, B., and Cichy, W. (2014). Liver disease in cystic fibrosis. Prz. Gastroenterol. 9, 136–141.

Lee, C.S., Friedman, J.R., Fulmer, J.T., and Kaestner, K.H. (2005). The initiation of liver development is dependent on Foxa transcription factors. Nature 435, 944–947.

Leu, J.I., Crissey, M.A., Leu, J.P., Caliberto, G., and Taub, R. (2001). Interleukin-6-induced STAT3 and AP-1 amplify hepatocyte nuclear factor 1-mediated transactivation of hepatic genes, an adaptive response to liver injury. Mol. Cell. Biol. 21, 414–424.

Li, F., Liu, P., Liu, C., Xiang, D., Deng, L., Li, W., Wangensteen, K., Song, J., Ma, Y., Hui, L., et al. (2010). Hepatoblast-like progenitor cells derived from embryonic stem cells can repopulate livers of mice. Gastroenterology 139, 2158–2169.e8.

Li, W.L., Su, J., Yao, Y.C., Tao, X.R., Yan, Y.B., Yu, H.Y., Wang, X.M., Li, J.X., Yang, Y.J., Lau, J.T., et al. (2006). Isolation and characterization of bipotent liver progenitor cells from adult mouse. Stem Cells 24, 322–332.

Lim, K.T., Lee, S.C., Gao, Y., Kim, K.P., Song, G., An, S.Y., Adachi, K., Jang, Y.J., Kim, J., Oh, K.J., et al. (2016). Small molecules facilitate single factor-mediated hepatic reprogramming. Cell Rep. 15, 814–829.

Lindor, K.D., Kowdley, K.V., and Harrison, M.E.; American College of Gastroenterology (2015). ACG clinical guideline: primary sclerosing cholangitis. Am. J. Gastroenterol. 110, 646–659, quiz 660.

Lokmane, L., Haumaitre, C., Garcia-Villalba, P., Anselme, I., Schneider-Maunoury, S., and Cereghini, S. (2008). Crucial role of vHNF1 in vertebrate hepatic specification. Development 135, 2777–2786.

Michalopoulos, G.K. (2007). Liver regeneration. J. Cell. Physiol. 213, 286–300.

Nagy, P., Bisgaard, H.C., and Thorgeirsson, S.S. (1994). Expression of hepatic transcription factors during liver development and oval cell differentiation. J. Cell Biol. 126, 223–233.

Ogawa, M., Ogawa, S., Bear, C.E., Ahmadi, S., Chin, S., Li, B., Grompe, M., Keller, G., Kamath, B.M., and Ghanekar, A. (2015). Directed differentiation of cholangiocytes from human pluripotent stem cells. Nat. Biotechnol. 33, 853–861.

Pontoglio, M., Barra, J., Hadchouel, M., Doyen, A., Kress, C., Bach, J.P., Babinet, C., and Yaniv, M. (1996). Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. Cell 84, 575–585.

Preissinger, K.H., Factor, V.M., Fuchsblicher, A., Stumpner, C., Denk, H., and Thorgeirsson, S.S. (1999). Atypical ductular proliferation and its inhibition by transforming growth factor beta1 in the 3,5-diethoxycarbonyl-1,4-dihydrocollidine mouse model for chronic alcoholic liver disease. Lab. Invest. 79, 103–109.

Sampaziotis, F., de Brito, M.C., Madrigal, P., Bertero, A., Saeb-Parsy, K., Soares, F.A., Schrumpf, E., Melum, E., Karlsen, T.H., Bradley, J.A., et al. (2015). Cholangiocytes derived from human induced pluripotent stem cells for disease modeling and drug validation. Nat. Biotechnol. 33, 845–852.

Sekiya, S., and Suzuki, A. (2011). Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 475, 390–393.

Si-Tayeb, K., Noto, F.K., Nagaoka, M., Li, J., Battle, M.A., Duris, C., North, P.E., Dalton, S., and Duncan, S.A. (2010). Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology 51, 297–305.

Tang, C., Lee, A.S., Volkmer, J.P., Sahoo, D., Nag, D., Mosley, A.R., Inlay, M.A., Ardehali, R., Chavez, S.L., Pera, R.R., et al. (2011). An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. Nat. Biotechnol. 29, 829–834.

Tanimizu, N., Miyajima, A., and Mostov, K.E. (2007). Liver progenitor cells develop cholangiocyte-type epithelial polarity in three-dimensional culture. Mol. Biol. Cell 18, 1472–1479.

Tumpenny, P.D., and Illard, S. (2012). Alagille syndrome: pathogenesis, diagnosis and management. Eur. J. Hum. Genet. 20, 251–257.

Verdonk, R.C., Lozano, M.F., van den Berg, A.P., and Gouw, A.S. (2016). Bile ductual injury and ductular reaction are frequent phenomena with different significance in autoimmune hepatitis. Liver Int. 36, 1362–1369.

Walter, T.J., Vanderpool, C., Cast, A.E., and Huppert, S.S. (2014). Intrahepatic bile duct regeneration in mice does not require Hnf6 or Notch signaling through Rbpj. Am. J. Pathol. 184, 1479–1488.

Wangensteen, K.J., Zhang, S., Greenbaum, L.E., and Kaestner, K.H. (2015). A genetic screen reveals Foxa3 and TNFR1 as key regulators of liver repopulation. Genes Dev. 29, 904–909.

Yu, B., He, Z.Y., You, P., Han, Q.W., Xiang, D., Chen, F., Wang, M.J., Liu, C.C., Lin, X.W., Borjigin, U., et al. (2013). Reprogramming fibroblasts into bipotential hepatic stem cells by defined factors. Cell Stem Cell 13, 328–340.

Zhang, W., Li, W., Liu, B., Wang, P., Li, W., and Zhang, H. (2012). Efficient generation of functional hepatocyte-like cells from human fetal hepatic progenitor cells in vitro. J. Cell. Physiol. 227, 2051–2058.