



Differentiation and isolation of iPSC-derived remodeling ductal plate-like cells by use of an *AQP1-GFP* reporter human iPSC line

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ARTICLE INFO

Keywords:

iPSC
Differentiation
Cholangiocyte progenitor
Remodeling ductal plate cell
Isolation
AQP1

ABSTRACT

Cholangiocytes are the epithelial cells that line bile ducts, and ductal plate malformation is a developmental anomaly of bile ducts that causes severe congenital biliary disorders. However, because of a lack of specific marker genes, methods for the stepwise differentiation and isolation of human induced pluripotent stem cell (hiPSC)-derived cholangiocyte progenitors at ductal plate stages have not been established. We herein generated an *AQP1-GFP* reporter hiPSC line and developed a combination treatment with transforming growth factor (TGF) β 2 and epidermal growth factor (EGF) to induce hiPSC-derived hepatoblasts into *AQP1*⁺ cells *in vitro*. By confirming that the isolated *AQP1*⁺ cells showed similar gene expression patterns to cholangiocyte progenitors at the remodeling ductal plate stage around gestational week (GW) 20, we established a differentiation protocol from hiPSCs through *SOX9*⁺*CK19*⁺*AQP1*⁻ ductal plate-like cells into *SOX9*⁺*CK19*⁺*AQP1*⁺ remodeling ductal plate-like cells. We further generated 3D bile duct-like structures from the induced ductal plate-like cells. These results suggest that *AQP1* is a useful marker for the generation of remodeling ductal plate cells from hiPSCs. Our methods may contribute to elucidating the differentiation mechanisms of ductal plate cells and the pathogenesis of ductal plate malformation.

1. Introduction

Cholangiocytes are the epithelial cells that line bile ducts and play a crucial role in bile transportation from the liver to duodenum. Cholangiocytes, especially those in intrahepatic bile ducts, first emerge as a sheet of ductal plate cells around periportal mesenchyme at gestational week (GW) 6. Ductal plates are bilayered and start to form primitive ductal structures at GW12 (Vestentoft et al., 2011). This process is known as ductal plate remodeling, whose failure, called ductal plate malformation, causes several congenital disorders, such as autosomal recessive polycystic kidney disease (ARPKD) and Caroli's syndrome, and is also associated with some types of biliary atresia (Awasthi et al., 2004; Raynaud et al., 2011; Vuković et al., 2012). Although studying human cholangiocytes at developmental stages is necessary to understand the detailed mechanisms of ductal plate malformation and develop curative therapies for these biliary disorders, it is technically and ethically difficult to obtain a sufficient number of human remodeling ductal plate cells for research use. Moreover, the gene expression pattern of human cholangiocyte lineages is different from that of rodents (Glaser et al., 2006), which makes it difficult to use

rodent models for studies on human cholangiocyte lineages. Therefore, a novel resource to supply human cholangiocyte lineage cells, such as remodeling ductal plate cells, is required.

Human induced pluripotent stem cells (hiPSCs) and embryonic stem cells (hESCs) have made substantial contributions to research of developmental biology, regenerative therapy, disease modeling and drug screening (Kamiya and Chikada, 2015). However, because specific markers for cholangiocyte lineages have not been fully identified, it has been challenging to differentiate hiPSC/ESC-derived cholangiocytes from hepatoblasts through ductal plate and remodeling ductal plate. Although some studies have reported the generation of hiPSC/ESC-derived cholangiocyte-like cells (Dianat et al., 2014; Ogawa et al., 2015; Sampaziotis et al., 2015), the directed differentiation of hiPSC/ESC-derived ductal plate or remodeling ductal plate cells has not been achieved.

In this study, we generated an *AQP1-GFP* reporter hiPSC line and developed a combination treatment of transforming growth factor (TGF) β 2 and epidermal growth factor (EGF) that can induce the differentiation of hiPSC-derived hepatoblasts through *SOX9*⁺*CK19*⁺*AQP1*⁻ ductal plate-like cells into *SOX9*⁺*CK19*⁺*AQP1*⁺

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<https://doi.org/10.1016/j.scr.2019.101400>

Received 25 April 2018; Received in revised form 19 December 2018; Accepted 30 January 2019

Available online 31 January 2019

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remodeling ductal plate-like cells. Furthermore, the induced ductal plate-like cells develop CK19⁺ bile duct-like structures in a three-dimensional (3D) culture system under the presence of hepatocyte growth factor (HGF), EGF, Jagged-1 (JAG1) and CHIR99021. These results suggest that *AQP1* is a useful marker for the stepwise generation and isolation of hiPSC-derived cholangiocyte progenitors. Our results may provide a novel research tool for analyzing biliary development and modeling congenital biliary diseases.

2. Materials and methods

2.1. Cell culture

All the experiments using hiPSCs and hESCs were approved by the Ethics Committee of Kyoto University and performed in accordance with the relevant guidelines. Informed consent was obtained from all donor subjects from which hiPSCs were generated. Three hiPSC lines, 585A1 (Okita et al., 2013), 23C27, which is an *AQP1-GFP* reporter hiPSC line generated from 585A1, and 692D2 (Okita et al., 2013), and an hESC line, KhES3 (Suemori et al., 2006), were cultured on feeder layers of mitomycin C-treated mouse SNL feeder cells in Primate ES medium (ReproCELL) with 500 U/ml penicillin/streptomycin (P/S; Thermo Fisher Scientific) and 4 ng/ml recombinant human basic fibroblast growth factor (bFGF; Wako). Cells were split at a ratio of 1:4 every six days using CTK dissociation solution consisting of 0.25% trypsin (Thermo Fisher Scientific), 0.1% collagenase IV (Thermo Fisher Scientific), 20% Knockout serum replacement (KSR; Thermo Fisher Scientific) and 1 mM CaCl₂ in PBS. All hiPSC and hESC lines used in this study were routinely examined for mycoplasma contamination.

2.2. Differentiation protocols

After the removal of feeder cells by treatment with CTK dissociation solution, we dissociated hiPSCs/ESCs by Accutase (Innovative Cell Technologies) treatment and gentle pipetting into single cells, seeded them on Matrigel (BD Biosciences)-coated 12-well plates at a density of 7.7×10^4 cells/cm² and differentiated them into definitive endoderm cells for 5 days in RPMI1640 (Nacalai Tesque) supplemented with 1 × B27 supplement (Thermo Fisher Scientific), 500 U/ml P/S, 100 ng/ml recombinant human/mouse/rat activin A (R&D Systems), 1–3 μM CHIR99021 (StemRD; 3 μM for the first day and 1 μM for the following 2 days) and 10 μM Y-27632 (Wako; 10 μM for the first day). We then used a previously reported differentiation method to generate hepatoblasts (Kajiwara et al., 2012; Si-Tayeb et al., 2010). In short, hiPSC/ESC-derived definitive endoderm cells were cultured for 6 days in Knock Out-DMEM (KO-DMEM; Thermo Fisher Scientific) supplemented with 10% KSR, 1 mM L-glutamine (Thermo Fisher Scientific), 1% (vol/vol) nonessential amino acids (Thermo Fisher Scientific), 500 U/ml PS, 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific), 1% (vol/vol) DMSO (Sigma), 20 ng/ml recombinant human bone morphogenetic protein (BMP)4 (Peprotech) and 10 ng/ml FGF2 (Wako). For hepatocyte-like cell differentiation, hiPSC/ESC-derived hepatoblasts were treated with Hepatocyte Culture Medium (Lonza) containing 20 ng/ml recombinant human HGF (Peprotech) and 20 ng/ml of recombinant human Oncostatin M (Peprotech) for 7 days.

To induce cholangiocyte progenitors, we cultured hiPSC/ESC-derived hepatoblasts for 3–7 days in Hepatocyte Basal Medium (Lonza) supplemented with 10% KSR, 10 ng/ml recombinant human TGFβ2 (Peprotech) and 25 ng/ml recombinant human EGF (R&D Systems).

2.3. 3D cultures

hiPSC/ESC-derived hepatoblasts, ductal plate-like cells or remodeling ductal plate-like cells (1.0×10^6) were seeded into 100 μl of a gel composed of 60% Collagen Type I (Thermo Fisher Scientific) and 40% Matrigel in a culture insert. After cell seeding, the gel was

solidified by incubating at 37 °C for 2 h. After solidification, 200 and 500 μl of culture medium was added into the insert and the well under it, respectively. Culture medium was composed of Hepatocyte Basal Medium supplemented with 10% KSR, 20 ng/ml recombinant human HGF, 50 ng/ml recombinant human EGF, 50 ng/ml recombinant human JAG1 (R&D Systems) and 3 μM CHIR99021. We cultured the cells for 10 days, and medium change was done every two days.

2.4. Generation of an *AQP1-GFP* reporter hiPSC line

The human BAC clone RP11-956B2, which contained all the exons of *AQP1* gene and extended from 50 kb upstream to 50 kb downstream of the gene locus, was purchased from BACPAC Resource Center at Children's Hospital, Oakland Research Institute. The *AQP1-GFP* vector was generated by insertion of a *GFP-PGK-Neo* construct. By electroporating the *AQP1-GFP* vector into 585A1 cells, we generated an *AQP1-GFP* reporter hiPSC line, 23C27.

Taqman quantitative PCR (qPCR) was performed with 100 ng genomic DNA, 250 nM Taqman probes and 500 nM primers to detect the loss of the *AQP1*-coding region, which was replaced by the GFP-Neo cassette (Fig. S1B). The sequences of the primers were as follows: AQP1F, 5'-CCAGCGAGTTCAAGAAGAAGC-3'; AQP1R, 5'-CCGATGCTGATGAAGACAAGA-3' and AQP1 probe, 5'-(FAM)-CAGTGGTGGCCGAGTT(MGB)-3'.

2.5. Statistical analysis

The statistical analysis of multiple group comparison was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test. *P* values < .05 were considered to be statistically significant.

3. Results

3.1. Generation of an *AQP1-GFP* reporter hiPSC line

It was reported that *AQP1* is not expressed in hepatoblasts, hepatocytes or ductal plate cells in human fetal or adult liver, but is in remodeling ductal plate cells beginning around GW15 (Fig. S1A) (Vestentoft et al., 2011). Therefore, we hypothesized that *AQP1* might be a useful marker gene for the *in vitro* induction of remodeling ductal plate cells. We aimed to generate *AQP1-GFP* reporter hiPSC lines by our previously reported strategy using bacterial artificial chromosome (BAC)-based vectors (Fig. S1B) (Mae et al., 2013). 585A1 cells were transfected with the BAC-based vector, in which the *AQP1*-coding region was replaced with the *GFP-pgk-Neo* cassette. Although a homologous recombinant was not found by genomic PCR, we obtained about 50 candidate *AQP1-GFP* transgenic hiPSC lines (data not shown). Of these cell lines, we selected one line, 23C27, which could be easily and stably maintained and had normal karyotype (Figs. S1C and D).

3.2. Induction of *AQP1*⁺ cholangiocyte lineage cells from hiPSCs

We next generated hepatoblasts from 23C27 cells using previously reported hepatic differentiation protocols (Kajiwara et al., 2012; Si-Tayeb et al., 2010). We first induced SOX17⁺ definitive endoderm cells on culture day 5 and subsequently differentiated them into AFP⁺ hepatoblasts on day 11 (Fig. S2A). qRT-PCR analyses showed that these cells on day 11 significantly upregulated the expression of other hepatoblast markers, *CK19* and *HNF1B*, compared with definitive endoderm cells on day 5. Although not statistically significant, the expressions of *AFP*, *ALBUMIN (ALB)*, *HNF4A* and *DLK1* were also higher on day 11 than on day 5. Biliary marker genes, such as *AQP1*, *SOX9*, *CFTR*, *OPN*, *TGFBR2* and *EGFR*, were slightly upregulated on day 11, but the expression level of *AQP1* was significantly lower than in GW20 fetal liver (Fig. S2B). Other endoderm lineage markers, such as *SOX2* (esophagus or stomach), *NKX2.1* (lung or thyroid), *PDX1* (pancreas or

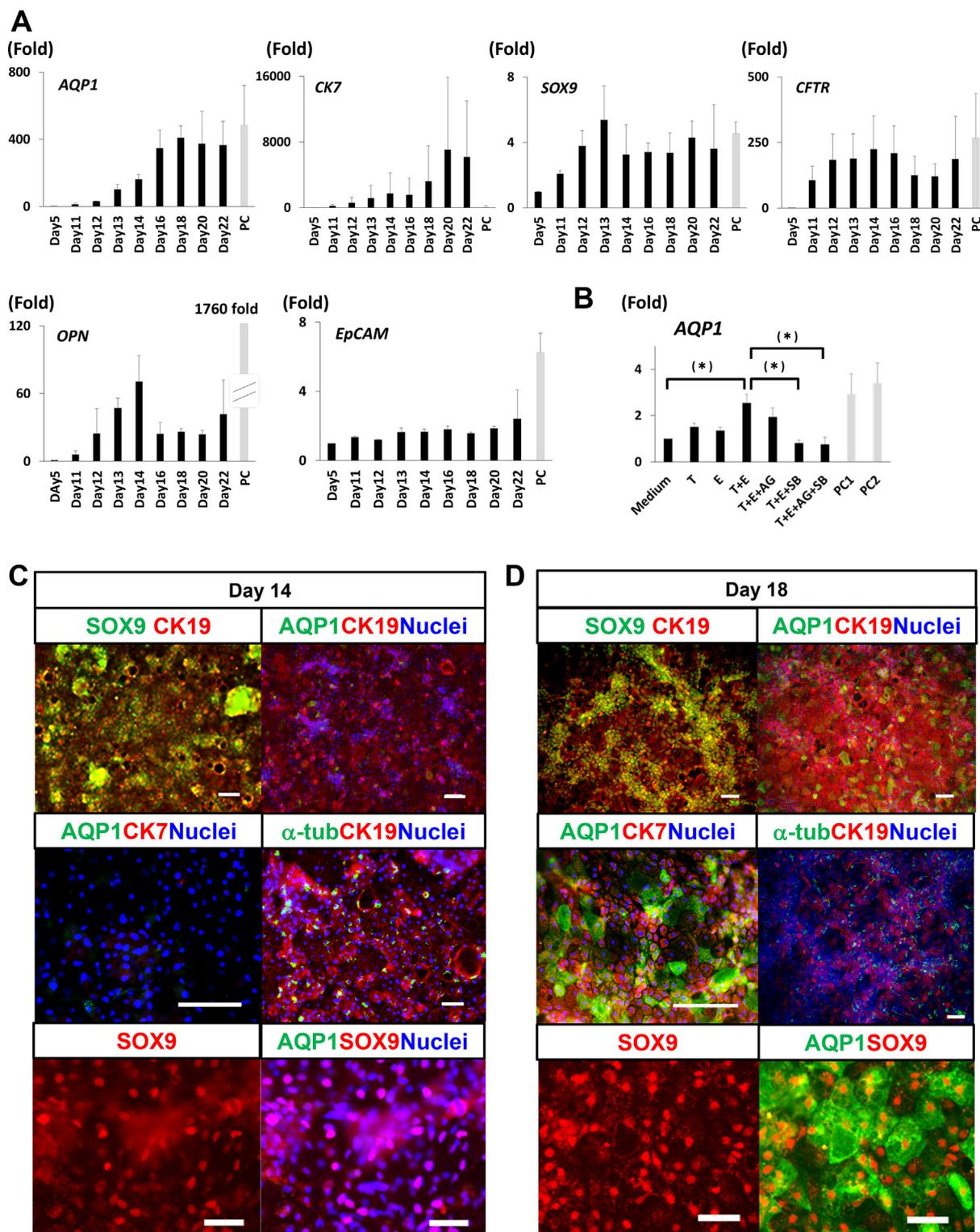


Fig. 1. Differentiation of AQP1⁺ cholangiocyte lineage cells from hiPSCs.

(A) Temporal expression of biliary marker genes from culture days 5 to 22 ($n = 3$). Each value was normalized to the samples on day 5. The mRNA of GW20 human fetal liver was used as a positive control (PC). (B) The expression levels of AQP1 under various combinations of growth factors or chemical inhibitors. Each value was normalized to the samples on day 18 cultured without TGF β 2 or EGF (Medium). * $P < .05$, one-way ANOVA with Bonferroni's test ($n = 3$). T, TGF β 2; E, EGF; SB, SB431542; AG, AG1478. The mRNAs of GW20 human fetal liver and adult liver were used as positive control 1 (PC1) and positive control 2 (PC2), respectively. (C, D) Double immunostaining of biliary markers on day 14 (C) and 18 cells (D). Scale bars, 100 μ m for SOX9/CK19, AQP1/CK19 and acetylated α -tubulin (α -tub)/CK19, and 50 μ m for AQP1/CK7 and AQP1/SOX9.

extrahepatic bile duct) and *CDX2* (intestine) were not detected by RT-PCR analyses (Fig. S2C). We then confirmed that the hiPSC-derived AFP⁺ hepatoblasts could be further differentiated into hepatocyte-like cells with glycogen synthesis capability by using previously reported protocols (Fig. S2D) (Kajiwara et al., 2012; Si-Tayeb et al., 2010).

It has been reported that TGFβ signaling is one of the most crucial pathways for biliary differentiation from hepatoblasts (Clotman et al., 2005; Clotman and Lemaigre, 2006; Weinstein et al., 2001). In mice, the differentiation of hepatoblasts into hepatocytes or cholangiocyte progenitors starts around embryonic day (E) 13. At this stage, TGFβ1 is ubiquitously expressed in liver, but TGFβ2 expression is localized in the portal mesenchyme adjacent to emerging ductal plates (Antoniou et al., 2009). EGF is also needed for the formation of biliary epithelia through stimulation by Wnt/β catenin signaling (Michalopoulos et al., 2001, 2003; Tan et al., 2005). With reference to these developmental findings, we examined various combination treatments of TGFβ2 and EGF and found that 7-day treatment with 10 ng/ml TGFβ2 and 25 ng/ml EGF induced the hiPSC-derived hepatoblasts to most efficiently express *AQP1* (data not shown, Fig. 1A). The expressions of other cholangiocyte lineage markers, *CK7*, *SOX9*, *CFTR* and *OPN*, were also increased, although their peak expressions were not at the same time (Fig. 1A). Another biliary lineage marker, *EpCAM*, was not upregulated throughout the differentiation process, which is consistent with the finding that it is also expressed in other cell types in developing livers, such as hepatoblasts (Vestentoft et al., 2011). We also confirmed that *AQP1* mRNA expression was significantly reduced under a TGFβ signaling inhibitor, SB431542 (10 μM), alone or with an EGF signaling inhibitor, AG1478 (6 μM; Fig. 1B).

Based on immunostaining analyses, most cells on day 14 of our differentiation protocol became SOX9⁺CK19⁺AQP1⁻CK7⁻ (Fig. 1C). On day 18, some cells expressed *AQP1* and *CK7*, two markers of remodeling ductal plate cells, in addition to *SOX9* and *CK19* (Vestentoft et al., 2011) (Fig. 1D). Primary cilia stained by an acetylated α-tubulin (α-tub) antibody were also identified in day 14 and 18 cells (Fig. 1C, D and S3).

3.3. hiPSC-derived cholangiocyte lineage cells on day 18 correspond to remodeling ductal plate cells around GW20

In order to confirm differences between hiPSC-derived cholangiocyte lineage cells on days 14 and 18, which were induced with 3 and 7 days of combination treatment with TGFβ2 and EGF, respectively, we examined the gene expression profiles of multiple biliary marker genes by qRT-PCR analysis (Fig. 2A and Fig. S4A). We found that the expression levels of *AQP1* and *CK7* were significantly higher in hiPSC-derived cells on day 18 than on day 14. The gene expression levels of other biliary markers, *SOX9*, *CFTR*, *CK19*, *OPN*, *EpCAM*, *JAG1*, *TGFBR2*, *EGFR*, *HNF1B*, *DLK1*, *HHEX* and *ASBT*, and *ALB* were not significantly different between hiPSC-derived cells on day 14 and day 18. The gene expression of a mature biliary marker, *SLC10A2*, was very low compared with GW20 fetal liver.

Next, to further characterize hiPSC-derived AQP1⁺ cells on day 18, we isolated AQP1⁺ cells by using 23C27 cells (Fig. 2B). GFP⁺ cells induced from 23C27 cells using 7-day treatment with TGFβ2 and EGF were isolated by flow cytometry for RT-PCR and immunostaining analyses. We found that the isolated GFP⁺ cells, but not GFP⁻ cells, expressed *AQP1* mRNA (Fig. S4B) and were positively stained with anti-AQP1 immunostaining (Fig. S4C), indicating that 23C27 cells can be used to monitor AQP1⁺ cell differentiation. The induction rate of AQP1⁺ cells by our differentiation protocol was 33.8 ± 3.3% on day 18 (*n* = 3; Fig. 2B). RT-PCR analyses showed that the isolated AQP1⁺ cells expressed multiple biliary marker genes, such as *CK7*, *SOX9*, *CFTR*, *CK19*, *OPN*, *JAG1*, *TGFBR2*, *EGFR*, *HNF1β*, *DLK1*, *HHEX* and *TGR5*, and a mature biliary marker, *SLC10A2*, but not other mature biliary markers, *AE2* or *GGT*, hepatocyte markers, *ALB* or *HNF4a*, or markers for other endoderm lineages, such as *SOX2*, *NKX2.1*, *PDX1* and *CDX2* (Fig.

S4D). Furthermore, principal component analysis (PCA) of the microarray data showed that the isolated hiPSC-derived AQP1⁺ cells have distinct expression patterns from undifferentiated hiPSCs, definitive endoderm (hiPSC-DE), hepatoblasts (hiPSC-HB) and an immortalized human cholangiocyte cell line, MMNK-1 (Fig. 2C). Taken together with the findings that the expressions of *AQP1* and *CK7* start during the remodeling ductal plate stage around GW15 and 20, respectively (Desmet et al., 1990; Vestentoft et al., 2011), these data indicate that hiPSC-derived AQP1⁺ cells on day 18 correspond to remodeling ductal plate cells around GW20 and that our combination treatment induces hiPSC/ESC-derived hepatoblasts through ductal plate-like cells into remodeling ductal plate-like cells (Fig. 2D).

3.4. Reproducibility and robustness of our differentiation protocol for remodeling ductal plate-like cells

hiPSC/ESC lines differ in differentiation potentials among cell lines (Osafune et al., 2008; Takayama et al., 2010). We thus examined whether our differentiation protocol for remodeling ductal plate-like cells is applicable to multiple hiPSC/ESC lines (Fig. 3). qRT-PCR analyses showed that the expressions of several biliary markers, *AQP1*, *CK7*, *SOX9* and *CK19*, but not *OPN* or *ALB*, were significantly upregulated from another hiPSC line, 692D2, and an hESC line, KhES3, in addition to 23C27 (Fig. 3A). Immunostaining analyses confirmed that SOX9⁺CK19⁺AQP1⁺CK7⁺ remodeling ductal plate-like cells were also generated from the other two cell lines (Figs. 3B and C). These results indicate that our differentiation protocol for remodeling ductal plate-like cells has broad application for multiple hiPSC/ESC lines.

3.5. hiPSC-derived ductal plate-like cells form functional bile duct-like structures

Next, to confirm the developmental capability of hiPSC-derived hepatoblasts, ductal plate-like cells and remodeling ductal plate-like cells to form functional bile duct-like structures, we tested the day 11, 14 and 18 cells by modifying previously reported 3D culture systems (Fig. 4A) (Kido et al., 2015; Ogawa et al., 2015). We found that a Wnt/β catenin pathway activator, CHIR99021, and *JAG1* facilitates 3D structure formation (Figs. 4B, S5A and B). After 10 days of 3D culture under treatment with 20 ng/ml HGF, 50 ng/ml EGF, 50 ng/ml *JAG1* and 3 μM CHIR99021, CK19⁺ duct-like structures were formed from hiPSC-derived hepatoblasts on day 11 and ductal plate-like cells on day 14, while remodeling ductal plate-like cells on day 18 formed few duct-like structures (Fig. 4B and C). However, we confirmed that isolated AQP1(GFP)⁺ cells differentiated from 23C27 cells on day 18, but not AQP1(GFP)⁻ cells, formed duct-like structures (Fig. 4B). These duct-like structures showed the uptake and transportation of rhodamine 123, a substrate for P-glycoprotein encoded by *multidrug resistance (MDR) 1* gene, into the luminal spaces, and the addition of a P-glycoprotein inhibitor, verapamil, inhibited the transportation (Fig. 4D). These results suggest that hiPSC-derived hepatoblasts and ductal plate-like cells have developmental potential to form bile duct-like structures with functional MDR1 transporters.

4. Discussion

In this study, we developed methods for the stepwise differentiation and isolation of hiPSC-derived AQP1⁺ remodeling ductal plate-like cells. Although some studies have reported the differentiation of cholangiocyte-like cells from hiPSCs/ESCs (Ogawa et al., 2015; Sampaziotis et al., 2015; Dianat et al., 2014), the stepwise differentiation and isolation of remodeling ductal plate-like cells had not been reported. Sampaziotis et al. differentiated cholangiocyte progenitors that did not elevate *AQP1* mRNA expression levels compared to hepatoblasts, indicating that these cells may correspond to SOX9⁺CK19⁺AQP⁻ ductal plate-like cells before GW15, but not

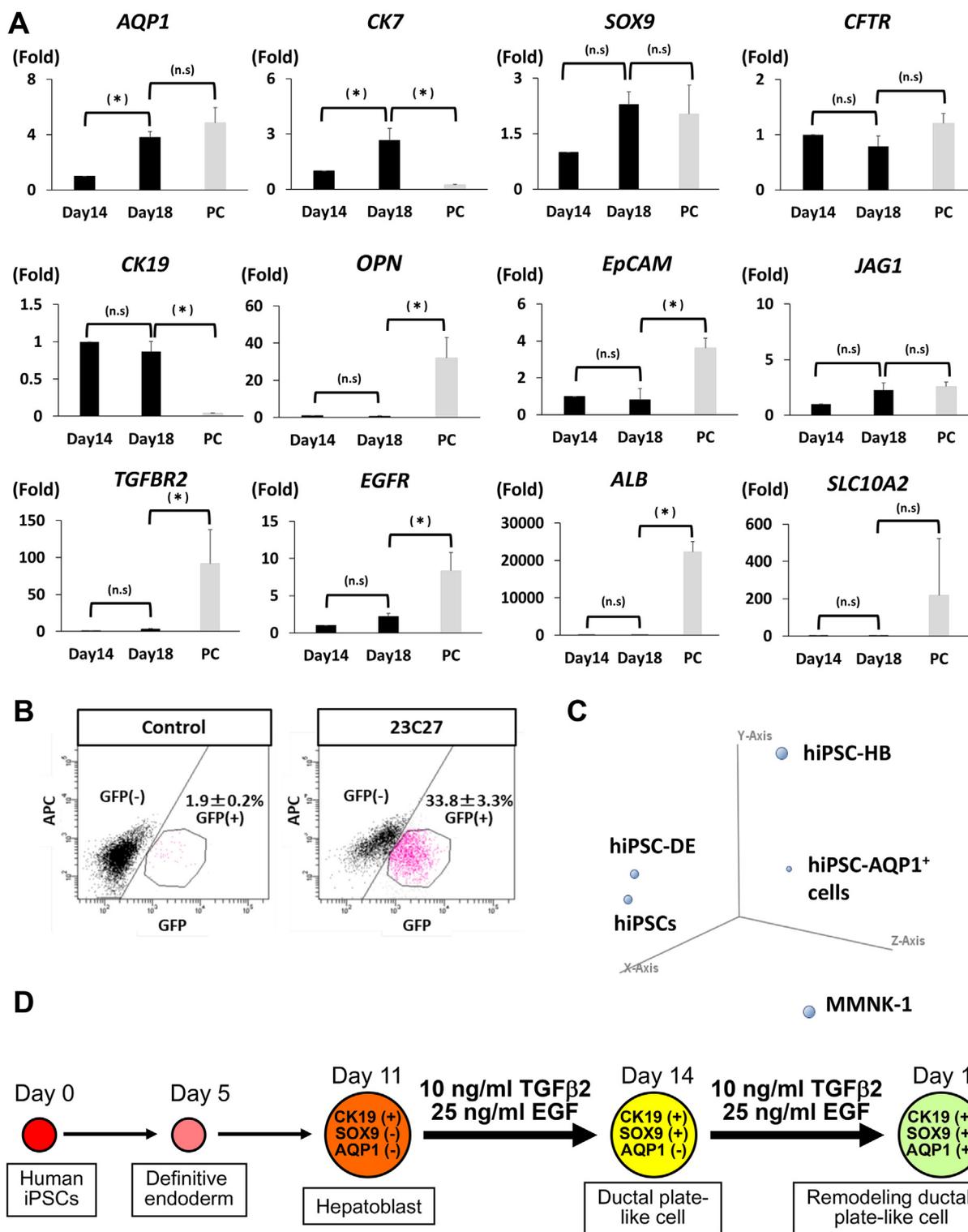


Fig. 2. Characterization of hiPSC-derived ductal plate-like cells on day 14 and remodeling ductal plate-like cells on day 18. (A) The gene expression of biliary markers on days 14 and 18 (n = 3). Each value was normalized to the samples on day 14. The mRNA of GW20 human fetal liver was used as a positive control (PC). (B) Flow cytometric analysis of GFP⁺ cells on day 18 generated from 23C27 cells. 585A1 cells, from which 23C27 was generated, were used as a control. (C) Microarray analysis of undifferentiated hiPSCs (hiPSCs), hiPSC-derived definitive endoderm (hiPSC-DE), hiPSC-derived hepatoblasts (hiPSC-HB), isolated hiPSC-derived AQP1⁺ cells and an immortalized human cholangiocyte cell line, MMNK-1. The gene expression profiles were analyzed using principal component analysis. (D) A schematic showing the stepwise differentiation into remodeling ductal plate-like cells.

SOX9⁺CK19⁺AQP1⁺ remodeling ductal plate-like cells around GW20 (Sampaziotis et al., 2015).

In qRT-PCR analyses, the expression levels of only two biliary marker genes, AQP1 and CK7, were significantly higher in the hiPSC-

derived remodeling ductal plate-like cells on day 18 than those on day 14 (Figs. 2A and S4A). Marker genes expressed in remodeling ductal plate cells but not in ductal plate cells are poorly studied. To the best of our knowledge, these markers include only AQP1 and CK7 (Desmet

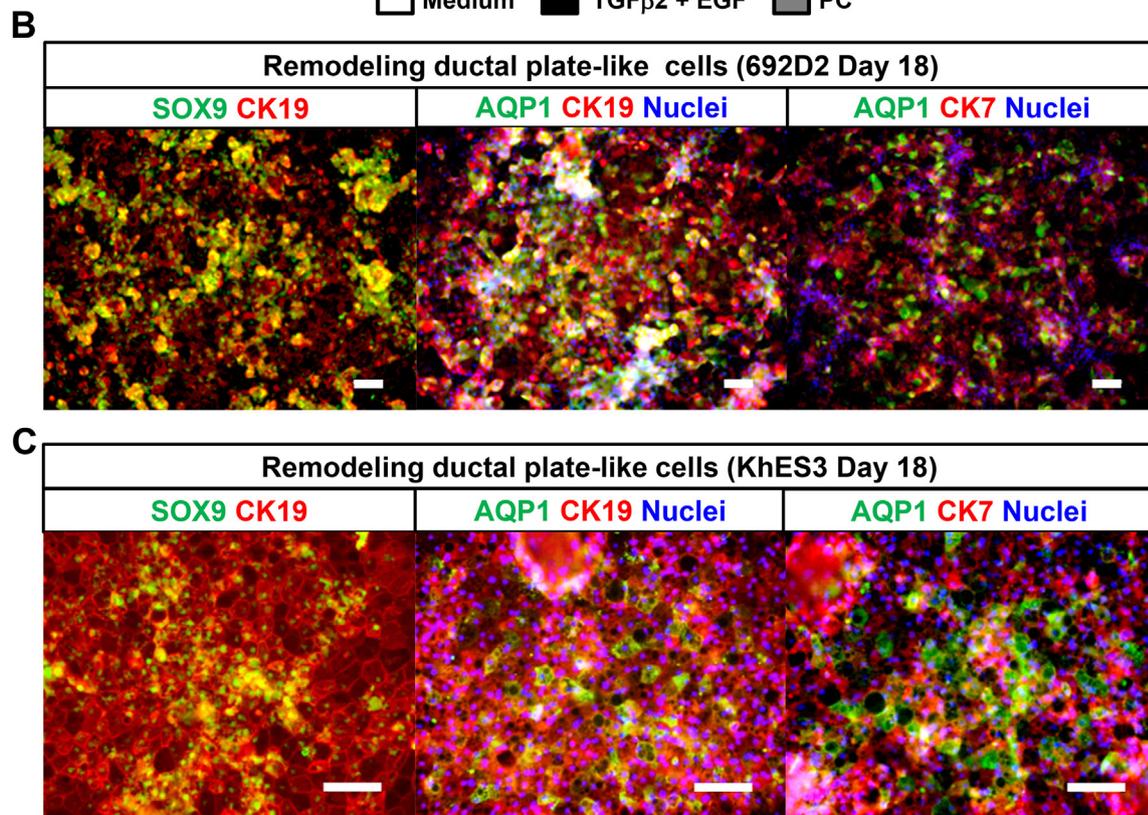
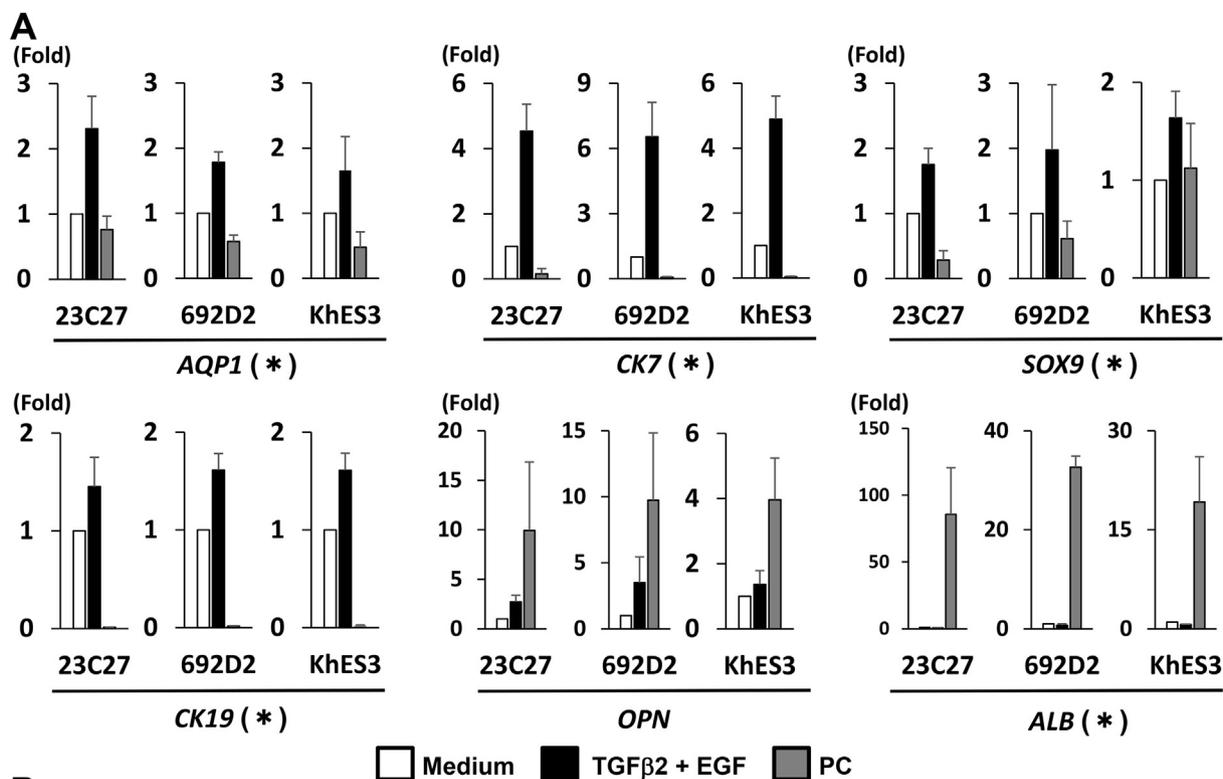


Fig. 3. Differentiation of remodeling ductal plate-like cells from multiple hiPSC/ESC lines. (A) The expression levels of biliary marker genes in three different hiPSC/ESC lines (n = 3). White bars, day 18 cells cultured without TGFβ2 or EGF (Medium); black bars, day18 cells treated with TGFβ2 and EGF; grey bars, GW20 human fetal liver. Each value was normalized to samples on day 18 cultured without TGFβ2 or EGF. * P < .05 between Medium and TGFβ2 + EGF, one-way ANOVA with Bonferroni's test. (B, C) Immunostaining of remodeling ductal plate-like cells on day 18 generated from an hiPSC line, 692D2, (B) and an hESC line, KhES3, (C) for cholangiocyte progenitor markers SOX9, CK19, AQP1 and CK7. Scale bars, 100 μm.

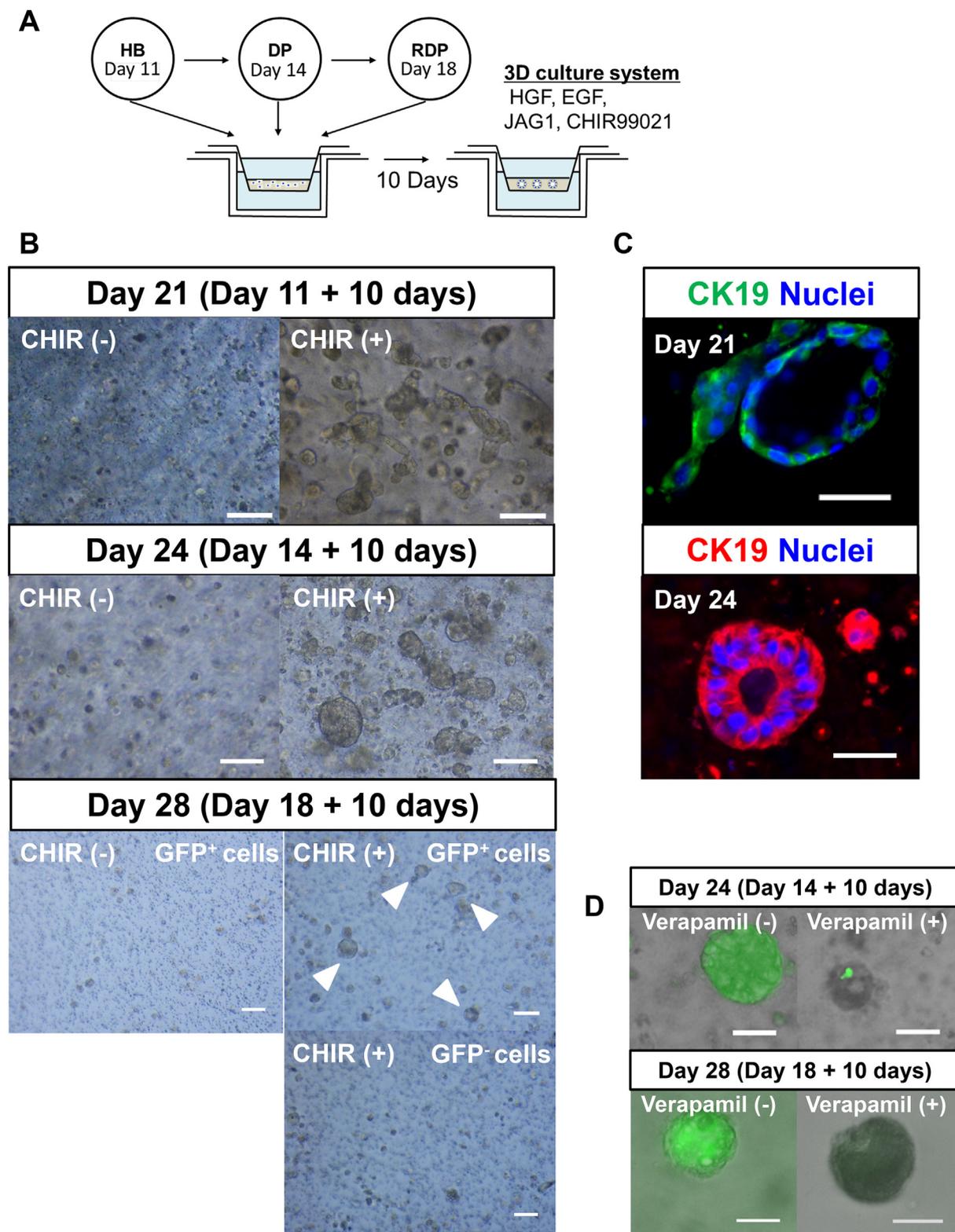


Fig. 4. Formation of functional bile duct-like structures from hiPSC-derived cholangiocyte progenitors.

(A) The 3D culture system used to generate functional bile duct-like structures. HB, hepatoblast; DP, ductal plate-like cell; RDP, remodeling ductal plate-like cell. (B) Bright field images of duct-like structures generated from day 11, 14 and 18 cells after 10 days of 3D culture under HGF, EGF and JAG1 without (left panels) or with CHIR99021 (right panels). AQP1(GFP)⁺ and AQP1(GFP)⁻ cells generated from 23C27 cells on day 18 and separated by flow cytometry sorting were used. AQP1(GFP)⁺ cells formed a larger number of small bile duct-like structures (arrowheads) than AQP1(GFP)⁻ cells. (C) Anti-CK19 immunostaining images of duct-like structures generated from day 11 (upper panel) and 14 cells (lower panel) after 10 days of 3D culture under HGF, EGF, JAG1 and CHIR99021. (D) A rhodamine123 transportation assay for duct-like structures generated from day 14 cells (upper panels) and isolated 23C27-derived AQP1(GFP)⁺ cells on day 18 (lower panels) without (left panels) or with verapamil treatment (right panels). Representative images from at least three independent experiments are shown in (B) - (D). Scale bars, 100 μ m in (B), 50 μ m in (C) and 20 μ m in (D).

et al., 1990; Vestentoft et al., 2011). We confirmed that hiPSC-derived cells on day 14 of our differentiation protocol are AQP1⁻CK7⁻ by immunostaining (Fig. 1C), although the mRNA expression of AQP1 and CK7 started to elevate at this time (Fig. 1A). Thus, the AQP1⁺CK7⁺ cells induced with our protocol correspond to fetal cholangiocyte progenitors in the remodeling ductal plate around GW20. Future studies should identify more markers that can distinguish ductal plate cells and remodeling ductal plate cells.

Although the induction efficiency of our protocol to generate remodeling ductal plate-like cells was not high, we developed a method to isolate these cells by use of a biliary marker gene, AQP1. AQP1 is not specific for cholangiocytes but is expressed in biliary lineage cells and portal vein endothelia of developing livers (Fig. S1A). AQP1 is also expressed in other endoderm lineage cells, such as alveolar and bronchial cells, pancreatic ductal cells, extrahepatic bile duct cells and intestinal cells (Burghardt et al., 2003; Song et al., 2017; Zhu et al., 2017). Therefore, to use AQP1 as an isolation marker for the cholangiocyte progenitors differentiated *in vitro* from hiPSCs/ESCs risks the coexistence of these endoderm lineage cells in the differentiation cultures. However, we confirmed no expression of marker genes for these other lineages, such as *SOX2*, *NKX2.1*, *PDX1* and *CDX2*, in the differentiation culture at the hepatoblast stage or in isolated AQP1⁺ cells, indicating the successful isolation of cholangiocyte lineage cells (Figs. S2C and S4D).

We also confirmed that hiPSC-derived SOX9⁺CK19⁺AQP⁻ ductal plate-like cells on day 14 of our differentiation protocol had the capability to form functional 3D bile duct-like structures under treatment with HGF, EGF, JAG1 and CHIR99021 (Fig. 4). This result is consistent with previously reported findings that Wnt/ β catenin signaling contributes to the formation of biliary structures in mouse embryonic livers in part by stimulating the expression of EGF (Decaens et al., 2008; Tan et al., 2005). However, the efficiency for generating bile duct-like structures was low with hiPSC-derived ductal plate-like cells and even lower with hiPSC-derived AQP1⁺ remodeling ductal plate-like cells on day 18. It is unknown whether these isolated AQP1⁺ cells have enough potential to proliferate and form functional 3D structures or rather they have already lost the potential and are in the state of a remodeled ductal plate after bile duct formation. Future studies should optimize the culture conditions that induce 3D bile duct structures from hiPSC/ESC-derived fetal cholangiocyte progenitors.

In ARPKD, the initial abnormality in bile duct development, known as ductal plate malformation, occurs by GW20 (Awasthi et al., 2004). The current study is the first to identify remodeling ductal plate-like cells around GW20 in the *in vitro* differentiation of hiPSCs/ESCs to cholangiocyte lineages and might contribute to understanding the mechanisms of ductal plate malformation in ARPKD. However, to reliably elucidate the mechanisms underlying ductal plate malformation, the interactions between cholangiocyte lineage cells and other cell types, such as periportal mesenchymal cells, hepatocytes, endothelial cells and the cells in sinusoidal space, should also be considered. Co-culture systems that incorporate these cell types might create better disease models. Nevertheless, the methods to generate and isolate the hiPSC-derived remodeling ductal plate-like cells established in this study provide a model to study human embryonic ductal plate cells, which cannot be easily accessed because of technical and ethical problems.

In conclusion, we established methods for the stepwise differentiation of hiPSCs/ESCs into ductal plate-like and remodeling ductal plate-like cells and the isolation of hiPSC-derived AQP1⁺ remodeling ductal plate-like cells. Our methods may contribute to understanding the differentiation mechanisms of ductal plate cells and the pathogenesis of ductal plate malformation by use of disease-specific hiPSCs.

Acknowledgments

The authors thank Drs. Kanae Mitsunaga, Tomoko Kasahara, Elena Yuki Yoshitoshi-Uebayashi and Tomonaga Ameku for their technical

assistance in flow cytometry and microarray analyses, Dr. Akira Watanabe for helpful suggestions, Dr. Hirofumi Hitomi for statistical analysis and Dr. Peter Karagiannis for reading the manuscript. This work was partially supported by Otsuka Pharmaceutical Co., Ltd. and by the Japan Agency for Medical Research and Development (AMED) through its research grant “Core Center for iPS Cell Research, Research Center Network for Realization of Regenerative Medicine” and “Practical Research Project for Rare/Intractable Diseases” to K.O.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2019.101400>.

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