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An *in vitro* model of polycystic liver disease using genome-edited human inducible pluripotent stem cells

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ABSTRACT

In the developing liver, bile duct structure is formed through differentiation of hepatic progenitor cells (HPC) into cholangiocytes. A subtype of polycystic liver diseases characterized by uncontrolled expansion of bile ductal cells is caused by genetic abnormalities such as in that of protein kinase C substrate 80 K-H (PRKCSH). In this study, we aimed to mimic the disease process *in vitro* by genome editing of the *PRKCSH* locus in human inducible pluripotent stem (iPS) cells. A proportion of cultured human iPS cell-derived $CD13^+CD133^+$ HPC differentiated into $CD13^-$ cells. During the subsequent gel embedding culture, $CD13^-$ cells formed bile ductal marker-positive cystic structures with the polarity of epithelial cells. A deletion of *PRKCSH* gene increased expression of cholangiocytic transcription factors in $CD13^-$ cells and the number of cholangiocytic cyst structure. These results suggest that PRKCSH deficiency promotes the differentiation of HPC-derived cholangiocytes, providing a good *in vitro* model to analyze the molecular mechanisms underlying polycystic diseases.

1. Introduction

The liver is the largest organ in the body, and it is an important organ related to maintaining homeostasis of living bodies. Hepatocytes (liver parenchymal cells) are important for liver function and express various metabolic enzymes. On the other hand, several non-parenchymal cells (cholangiocytes, sinusoidal endothelial cells, stellate cells, Kupffer cells, *etc*.) exist in the liver, and they perform various functions while interacting with hepatocytes. Hepatocytes and bile duct cells both originate from hepatic progenitor cells (Kamiya and Inagaki, 2015). Differentiation from hepatic progenitor cells into cholangiocytes is controlled by soluble factors such as tumour growth factor β (TGF-β) and intercellular factors such as the Notch-Jagged system. By the stimulation from fibroblasts around the portal vein in E13 fetal mouse livers, a proportion of hepatic progenitor cells near the portal vein differentiate into special structures called ductal plates (Antoniou et al., 2009; Clotman et al., 2005; Hofmann et al., 2010). Ductal plates are differentiated into mature bile ducts in perinatal livers (Carpentier et al., 2011, Raynaud et al., 2011a, 2011b).

Polycystic liver diseases that produce multiple cysts derived from bile ductal cells in the liver are known (Everson et al., 2004). Autosomal dominant polycystic kidney disease (ADPKD) accompanied by multiple kidney cysts and autosomal dominant polycystic liver disease (ADPLD) that produces liver-specific cysts are major polycystic liver diseases (Wills et al., 2014). These are thought to be genetic diseases, and polycystic kidney disease 1 (*PKD1*) and *PKD2* have been identified as the causative genes of ADPKD (Tahvanainen et al., 2005). Linkage analysis of polycystic liver disease families showed mutation of the protein kinase C substrate 80 K-H (*PRKCSH*) gene and SEC63 as a factor of ADPLD (Davila et al., 2004; Drenth et al., 2003). The *PRKCSH* gene is a subunit of glucosidase II, which acts on the sugar chains of the endoplasmic reticulum and encodes a protein called hepatocystin. SEC63 localises in the endoplasmic reticulum membrane and is involved in protein transport. Although an association between these endoplasmic reticulum functions and cilia has been observed, details of the molecular mechanism of cyst formation in ADPLD patients remain unknown.

Recently, human inducible pluripotent stem cells (iPS cells) with high proliferative ability and pluripotency, capable of differentiating into various tissue cells, have been developed (Takahashi et al., 2007). These cells have made it possible to construct an *in vitro* pathological condition analysis system of various diseases. In this study, for the

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purpose of modelling the pathogenesis of human bile duct diseases, we established (1) an induction culture of cholangiocytic cysts *via* hepatic progenitor cell differentiation from human iPS cells and (2) a pathogenic model of human polycystic liver diseases using genome editing. We previously isolated and cultured hepatic progenitor cells derived from human iPS cells through continuous addition of cytokines and fractionating using specific cell surface antigens and flow cytometry (Tsuruya et al., 2015; Yanagida et al., 2013). The hepatic progenitor cell marker $CD13^+$ fraction decreased during the subculture of human iPS cell-derived hepatic progenitor cells. Moreover, as a result of analysing the expression of CD13 and the differentiation potential of cells in the passage culture, we found that $CD13^-$ cells can efficiently differentiate into cholangiocytic cyst structures in the matrix-embedded culture. Next, by using CRISPR/Cas9 genomic editing, human iPS cells showing the *PRKCSH* mutation reported as the cause of ADPLD were established. It was found that PRKCSH deficiency promotes differentiation into cholangiocytic cyst structures. We constructed an efficient cholangiocytic cyst culture differentiation system using human iPS cells and established an *in vitro* system of human polycystic liver disease using this system.

2. Materials and methods

2.1. Differentiation of human iPS cells towards hepatic lineage cells in vitro and flow cytometry analyses

The differentiation protocol for induction of hepatocytes was based on our previous report (Yanagida et al., 2013) with some modifications. The 3-day-addition of activin A (PeproTech, Rocky, NJ, USA) induced the differentiation of iPS cells into endodermal cells. Cells were incubated with 100 ng/ml recombinant human activin A (PeproTech) in RPMI 1640 supplemented with increasing concentrations of B27 (0%, 0–1 day; 0.2%, 1–2 days; 2%, 2–3 days). Next, the 3-day-addition of 10 ng/ml basic fibroblast growth factor (bFGF, PeproTech) and 20 ng/ml recombinant human Bone morphogenic protein 4 (PeproTech) induced the specification of hepatocytic lineage cells. Finally, the 3-day-addition of 40 ng/ml recombinant human hepatocyte growth factor (HGF, PeproTech) induced the differentiation of iPS cells into hepatocytic progenitor cells. In the several experiments, we used the Cellartis iPS Cell to Hepatocyte Differentiation System (Takara Bio., Shiga, Japan) for the differentiation of human iPS cells into hepatic progenitor cells, according to the manufacturer's protocol.

Hepatocytic progenitor cells derived from human iPS cells were trypsinized using 0.05% trypsin-EDTA (Sigma, St Louis, MO). Trypsinized cells were washed with phosphate-buffered saline (PBS) containing 3% Fetal bovine serum (FBS), and then incubated with antibodies against cell surface proteins (shown in Supplementary Table 1) for 1 h at 4 °C. After washing with PBS containing 3% FBS, the cells were analyzed and sorted by fluorescence-activated cell sorting using a FACS Aria I and III (BD Biosciences, San Jose, CA, USA). Dead cells were eliminated with propidium iodide staining. Data analysis was performed using FlowJo (Tree Star Inc., Ashland, OR, USA).

2.2. Induction of cholangiocytic cyst formation by progenitor cells derived from human iPS cells

The differentiation protocol for induction of hepatocytes was based on our previous report (Yanagida et al., 2013) with some modifications. Colonies derived from colony formation assays were trypsinized and sorted as described above. Purified progenitor cells were then combined with an extracellular matrix gel consisting of a mixture of collagen type-I and Matrigel (BD Biosciences, Bedford, MA), and inoculated on 24-well culture plates (1500–2000 cells/50 μl extracellular matrix gel/well). The culture medium was a 1:1 mixture of H-CFU-C medium and DMEM/F-12 supplemented with 2% B27, 0.25 μM A-83-01, 10 μM Y-27632, 20 ng/ml epidermal growth factor (EGF, PeproTech), 40 ng/ml HGF, 40 ng/ml recombinant human Wnt-3a (R&D Systems, Minneapolis, MN), and 100 ng/ml recombinant human R-spondin 1 (Rsp-1, PeproTech). After 37 °C incubation for 10 min, culture medium was added, followed by incubation for 10–12 days with medium changes every 3 days. Cysts in gels were stained according to previously described methods (Tanimizu et al., 2007), and analyzed under a LSM700 confocal microscope (Carl Zeiss, Oberkochen, Germany). The antibodies used are listed in Supplementary Table 1.

Other methods are shown in Supplementary Materials and Methods.

3. Results

3.1. Purification of cholangiocytic progenitor cells derived from human iPS cells

Differentiation into liver cells is possible by subjecting human iPS cells to the continuous addition of cytokines (Si-Tayeb et al., 2010). We previously reported that hepatic progenitor cell marker $CD13^+CD133^+$ cells can proliferate on mouse embryonic fibroblast (MEF) feeder cells (Yanagida et al., 2013). These cells have a high proliferation ability to form colonies derived from a single cell in a low density culture and can be cultured for a long period by passaging. We found that a portion of the cultured cells lost expression of CD13 during the subculture step (Figs. 1A and S1). Thus, we sorted both $CD13^+$ and $CD13^-$ cells in P1 culture and inoculated on new feeder cells. In P2 culture, both $CD13^+$ and $CD13^-$ cells had high proliferative ability and formed several colonies derived from a single cell. $CD13^+$ cells mainly proliferated while maintaining CD13 expression. In contrast, both $CD13^+$ and $CD13^-$ cells were detected in P2 culture derived from $CD13^-$ cells (Fig. 1A). The phenotype of each $CD13^+$ and $CD13^-$ progenitor cell was analyzed in this study. Cholangiocytic cyst structures can be induced by embedding hepatic progenitor cells in Matrigel/collagen-mixed gel and performing cytokine-stimulated culture (Tanimizu et al., 2007; Yanagida et al., 2013). Therefore, using this system, the differentiation abilities of $CD13^+$ and $CD13^-$ cells in P2 culture were analyzed (Fig. 1B and C). A large number of cholangiocytic cysts were formed from the $CD13^-CD13^-$ cell fraction which were shown in Fig. S1, whereas few cystic structures were formed from $CD13^+$ cells. These structures were AFP-negative and cholangiocyte marker cytokeratin (CK) 7- and CK19-positive (Fig. 2A). In addition, the cysts maintained the polarity of the epithelial system as shown by the expression of β-catenin and protein kinase Cζ (Fig. 2B and C). The expression of Sox9, another cholangiocytic progenitor marker (Dianat et al., 2014), was also detected in the cysts (Fig. 2D). Expression of cholangiocytic functional genes such as Aquaporin 1 (AQP1) and cystic fibrosis transmembrane conductance regulator (CFTR) was also detected in the cysts (Fig. S2). These results suggested that cholangiocyte progenitor cells were enriched in the $CD13^-CD13^-$ cell fraction.

Next, gene expression was compared between $CD13^+$ and $CD13^-$ cells. After human iPS cells were induced to differentiate into hepatocytic cells, $CD13^+CD133^+$ primary hepatic progenitor cells were fractionated by flow cytometry and cultured. The obtained colony cells in P1 culture were fractionated by the expression of CD13 and inoculated. After P2 culture, the $CD13^+CD13^+$ and $CD13^-CD13^-$ cell fractions were purified. The cells used for the microarray analyzes and quantitative PCR are shown in Supplementary Fig. S1. We analyzed the expression of liver-enriched transcription factors and other marker genes in these cells (Figs. S3 and S4). The fetal hepatocytic marker α-feto protein (AFP) was expressed in the $CD13^+CD13^+$ cell fraction but tended to be reduced in the $CD13^-CD13^-$ fraction (Fig. S4A). In contrast, cholangiocytic functional genes, *SLC12A2* and *SLC4A4* (Sampaziotis et al., 2015), were upregulated in the $CD13^-CD13^-$ fraction (Fig. S4B). In addition, the expression of *Sox9* and *ONECUT1* was induced in the $CD13^-CD13^-$ fraction. ONECUT1 has been reported to be an important transcription factor involved in the differentiation of the bile duct (Clotman et al., 2005, Matthews et al., 2004,

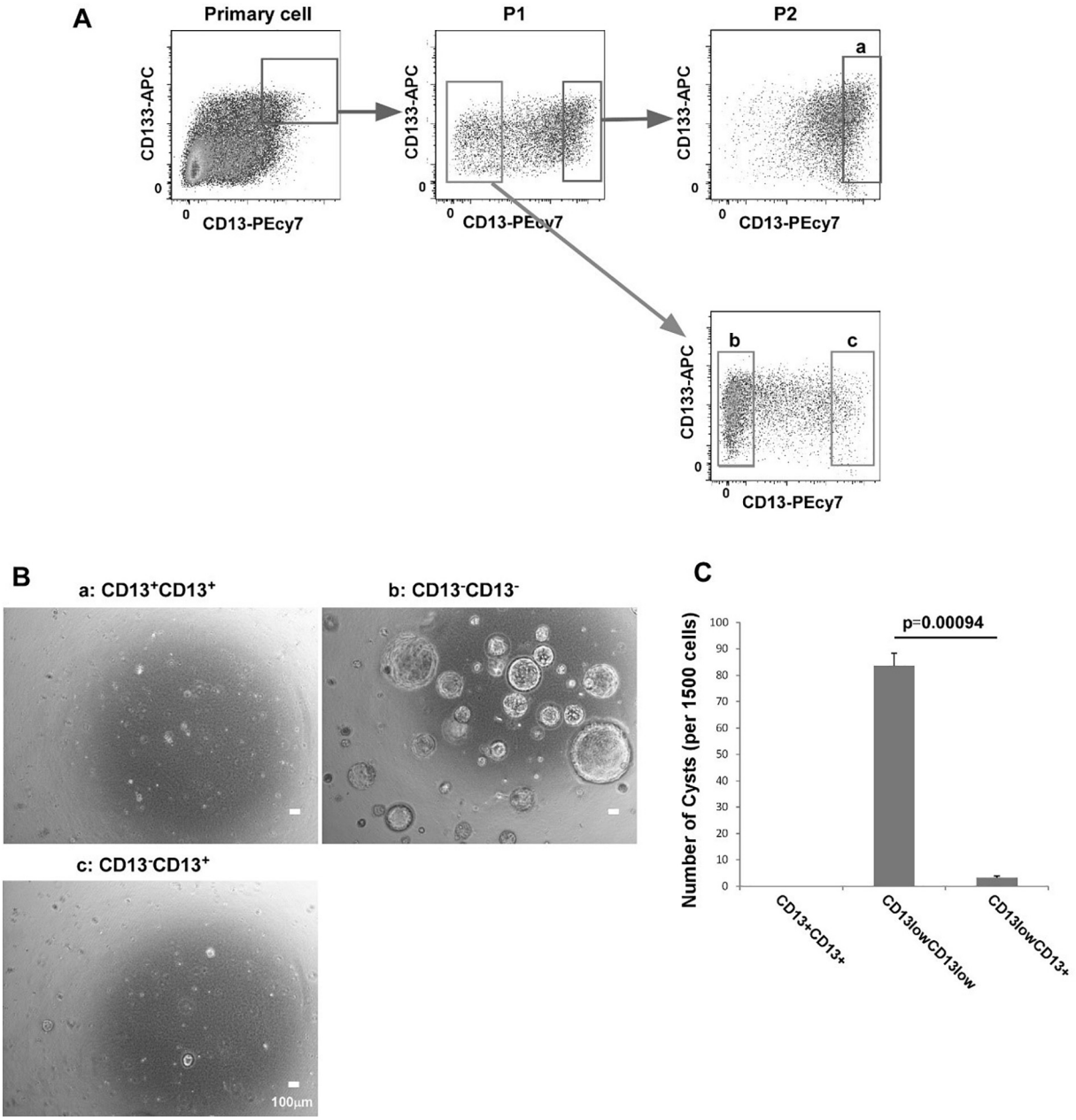

Fig. 1. Sequential culture of hepatic progenitor cells derived from human iPS cells induced cholangiocyte progenitor cells. (A) Flow cytometry analyses of the passage of $CD13^+$ and $CD13^-$ progenitor cells derived from human iPS cells. Primary $CD13^+CD133^+$ cells differentiated into both $CD13^+$ and $CD13^-$ cells upon primary culture (P1). $CD13^+$ cells derived from the P1 culture mainly proliferated as $CD13^+$ cells at the P2 culture (a). In contrast, $CD13^-$ cells derived from the P1 culture mainly proliferated as $CD13^-$ cells, (b) but a proportion of the cells expressed CD13 (c). The experimental procedure is shown in Supplemental Fig. S1. (B) Cholangiocytic cyst formation derived from human iPS cells. $CD13^+CD13^+$ cells (a), $CD13^-CD13^-$ cells (b), and $CD13^-CD13^+$ cells (c) at P2 were inoculated into an extracellular matrix gel and stimulated with cytokines for 13 days. Representative photographs are shown. White bar, 100 μm. (C) Count of cyst formation in the culture shown in (B). Cysts with a diameter of 100 μm or more were counted (1500 cells per 1 well). Results are presented the mean colony count ± SD (n = 3).

Raynaud et al., 2011a, 2011b). In contrast, TBX3 is an important factor for hepatoblast differentiation, and the downregulation of *TBX3* expression is required for cholangiocytic development (Ludtke et al., 2009). HNF4α and C/EBPα are also a hepatocyte-related transcription factor. The expression of these hepatic transcription factors was significantly downregulated in the $CD13^-CD13^-$ fraction (Fig. S4A). These results show that the $CD13^+CD13^+$ cell fraction has the characteristics of hepatic progenitor cells, while the $CD13^-CD13^-$ fraction has the characteristics of cholangiocytic progenitor cells. It is possible that the difference in these transcription factors expression may be involved in the differentiation of human iPS cells into cholangiocytic cyst structures.

3.2. Requirement of cytokines and signal inhibitors in cholangiocytic cyst formation derived from human iPS cells

In order to analyze the importance of cell signals regulating cyst formation of iPS cell-derived cholangiocyte progenitor cells, cytokines and cell signal inhibitors were removed from our culture medium during cyst formation gel culture. As shown in Fig. 3A, we typically added both Y-27632 (Rock inhibitor) and A83–01 (TGF-β signal inhibitor). Thus, we removed these 2 inhibitors for last 4 days of culture (−2i condition). In addition, we removed one of four cytokines which are used for the stimulation of cyst formation (Figs. 3 and S5). It was found that cholangiocytic cyst formation was markedly inhibited under

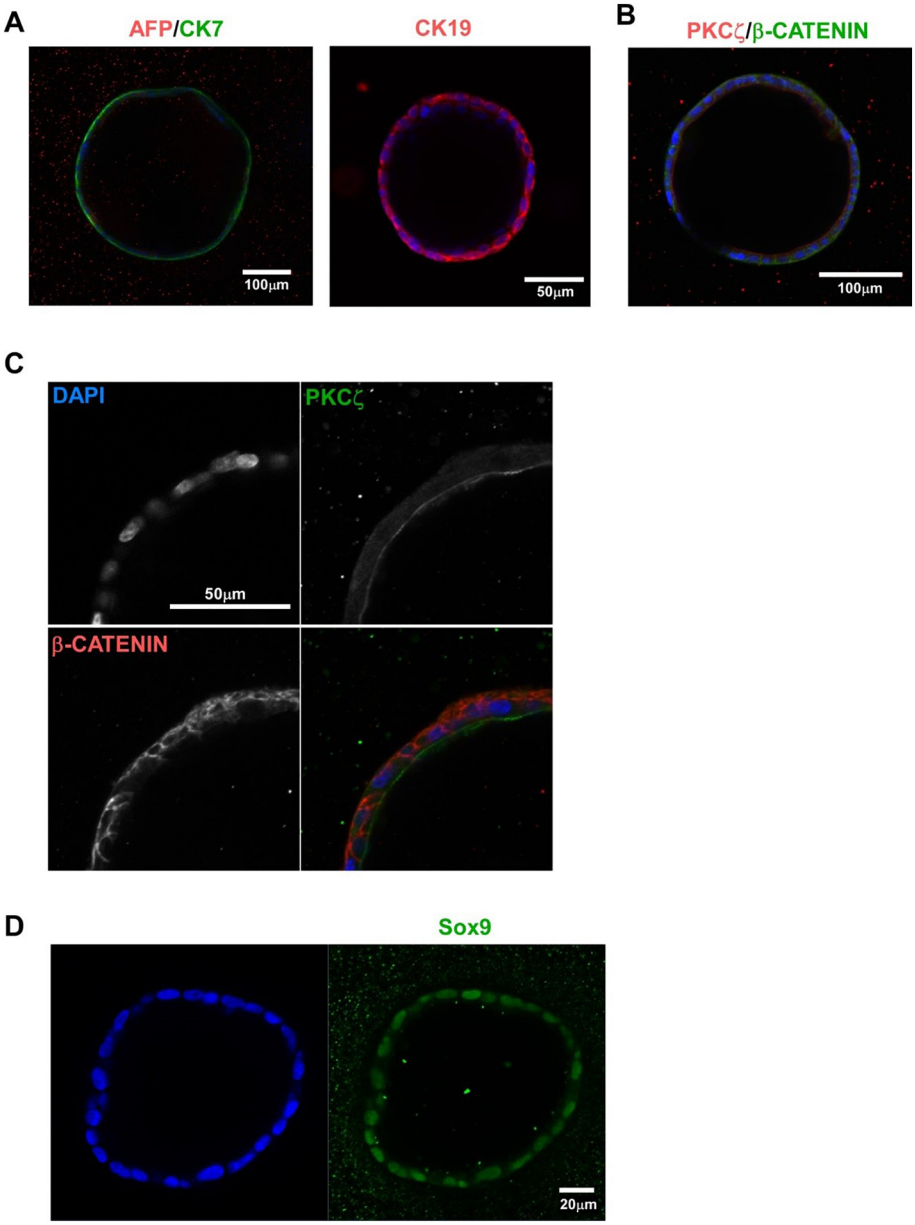

Fig. 2. Immunostaining of cholangiocytic cysts derived from human iPS cells. Human iPS cell-derived $CD13^{-}CD13^{-}$ cells were cultured in an extracellular matrix gel. After 10–12 days of culture, several epithelial cysts were formed. Expression of AFP, CK7 (A), β-catenin, PKCζ (B and C), and Sox9 (D) was observed by immunostaining. Nuclei were counterstained with DAPI (blue). White bar, scale bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the condition without either EGF or Rsp-1 (Fig. 3B, +2i). Furthermore, cyst formation was suppressed by the removal of 2 inhibitors over the course of the culture period (Fig. 3B, −2i). These results suggest that the activity of the signal downstream of the EGF Rsp-1 receptors is important, and that continuous inhibition of Rock and TGF-β downstream signals is required for cholangiocyte differentiation.

3.3. Establishment of polycystic liver disease-specific human iPS cells using genome editing

Polycystic liver disease is thought to be a result of abnormal amplification of the intrahepatic bile ducts and it is considered important to elucidate the molecular mechanisms of hepatic progenitor cells in cholangiocyte differentiation and subsequent proliferation. We tried to mimic genetic mutations found in patients with ADPLD pathology using the CRISPR/Cas9 genomic editing enzyme (Doudna and Charpentier, 2014) and a homologous recombination vector. In one of the ADPLD families reported in the previous report, pathological condition-specific mutations of the *PRKCSH* gene were observed in which protein translation was stopped due to point mutation at the splicing sequences between exons 15 and 16 (Drenth et al., 2003). Therefore, by inserting a polyA signal sequence between exons 15 and 16 through recombination with a homologous recombination vector, human iPS cells with termination of *PRKCSH* at this point were established (Figs. S6 and S7A). PRKCSH is expressed in undifferentiated human iPS cells. Human iPS cells with hetero- (+/−) and homozygous-mutated (−/−) allyls of the *PRKCSH* gene could proliferate with no morphological changes. Expression of PRKCSH protein in these cells was analyzed using an antibody targeted to the N-terminal peptide of PRKCSH. Expression of full-length PRKCSH protein was not seen while the faint band of truncated-PRKCSH protein was detected in PRKCSH −/− iPS cells (Fig. S7B). Therefore, it was possible to prepare and analyze human iPS cells

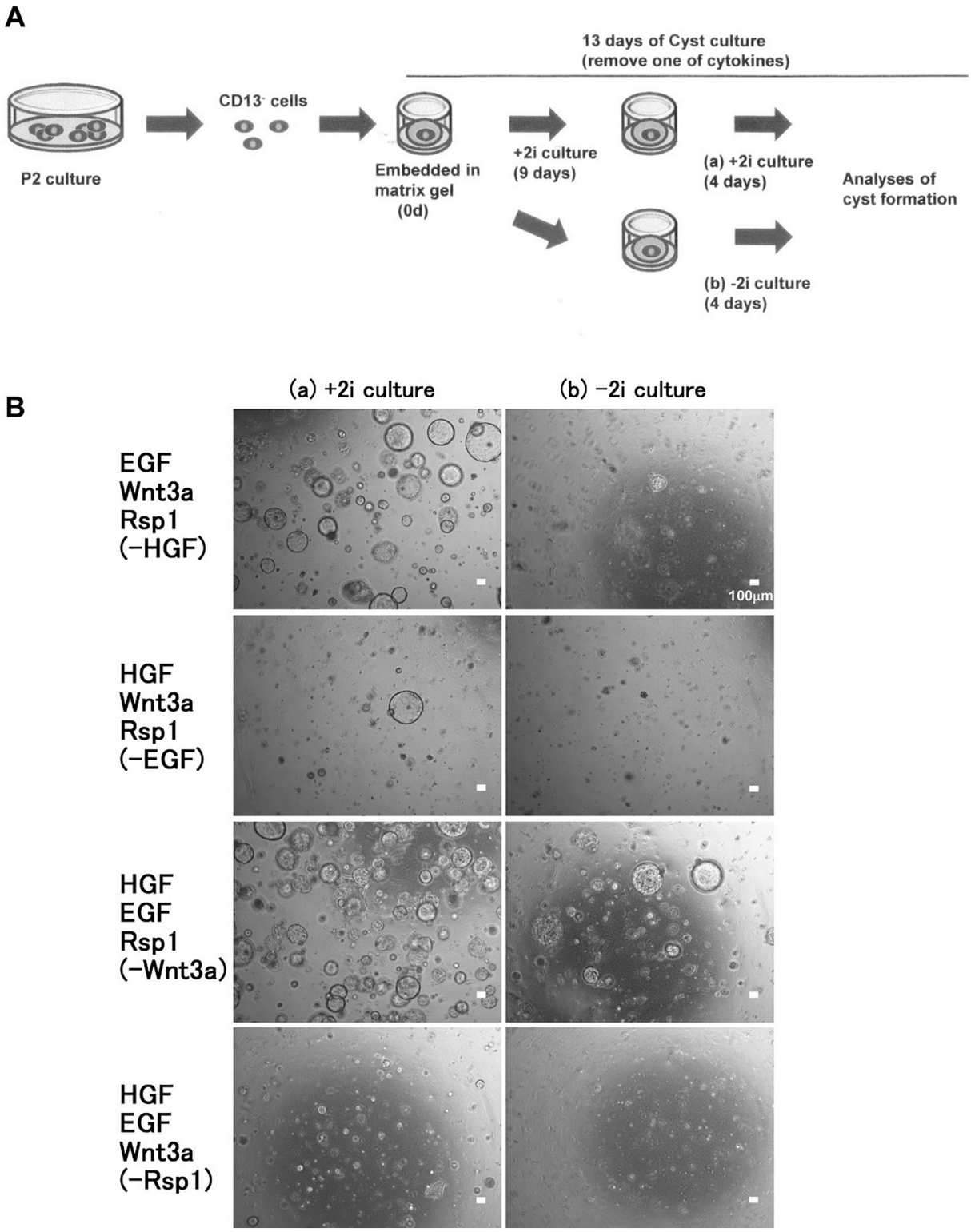

Fig. 3. Cell signalling pathways regulating cholangiocytic cyst formation of progenitor cells derived from human iPS cells. (A) Schematic of the experimental procedure. Sorted CD13^{-} cells were inoculated in extracellular matrix gels (**0 d**) and supplied with cytokines and 2 inhibitors (2i, Y27632, and A-83-01). Culture condition (a) added 2 inhibitors for 13 days. In contrast, culture condition (b) added 2 inhibitors removed inhibitors for last 4 days. (B) Requirement of cytokines in cholangiocytic cyst formation. Cholangiocyte progenitor cells were culture with a combination of several cytokines with or without the long-term addition of 2 inhibitors. The culture condition without EGF or Rsp1 suppressed cystic formation. In addition, the long-term addition of 2 inhibitors is required for cystic formation. White bar, 100 μm.

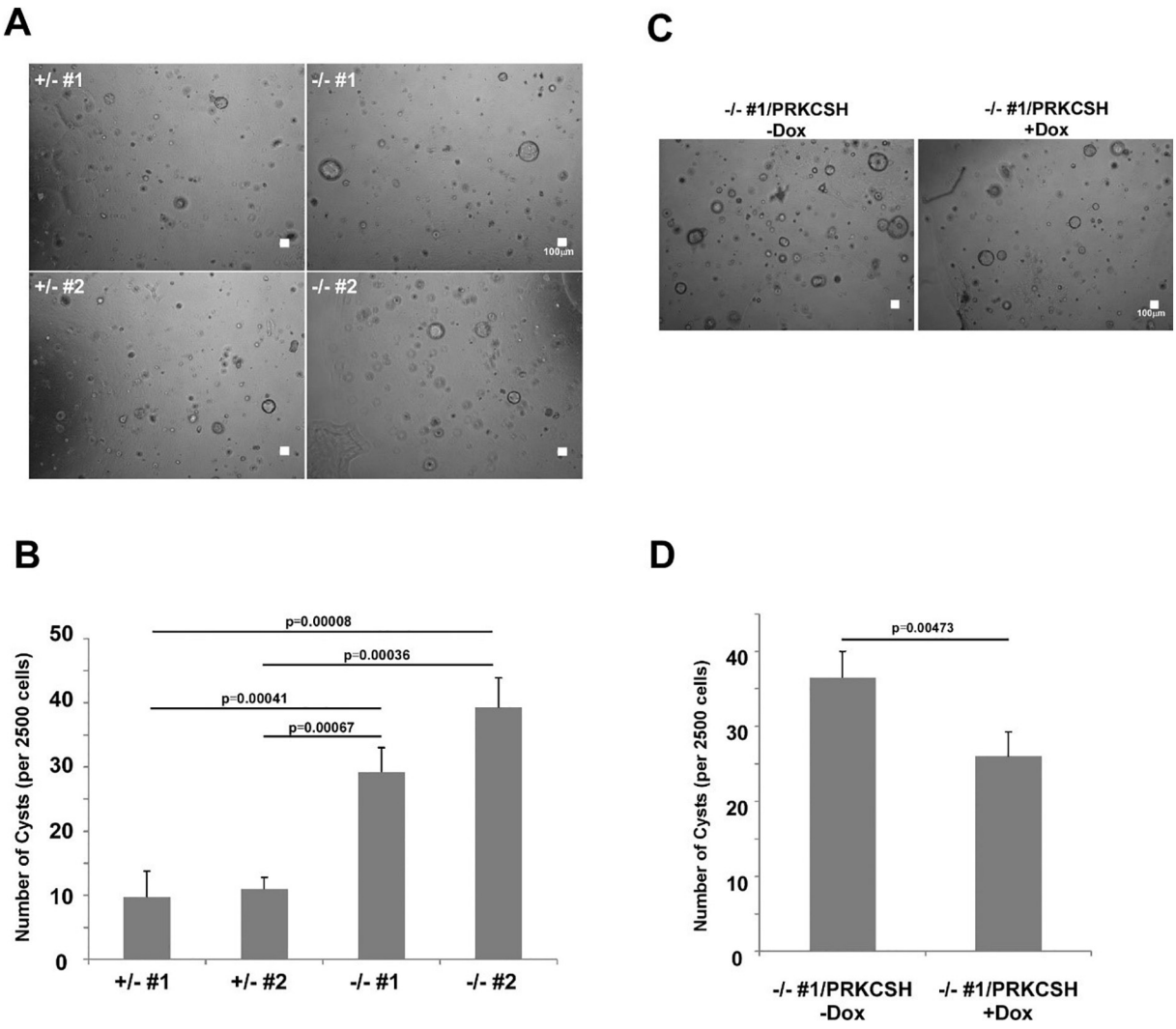

Fig. 4. Formation of bile ductal cysts derived from *PRKCSH*-mutated human iPS cells. (A) Representative view of bile ductal cysts derived from iPS cell-derived progenitor cells. *PRKCSH*-mutated hetero- and homo-iPS cells were differentiated into $CD13^-CD13^-$ cholangiocytic progenitor cells. These cells were cultured in extracellular matrices gel for 14 days. White bar, 100 μm. (B) Number of bile ductal cysts (1 well per 2000 cells) in the extracellular matrices gel culture. Cysts with a diameter of 100 μm or more were counted. (C) Representative view of bile ductal cysts derived from iPS cell-derived progenitor cells. *PRKCSH*-mutated homo iPS cells transfected with the *PRKCSH*-inducible vector were differentiated into $CD13^-CD13^-$ cholangiocyte progenitor cells. Expression of PRKCSH was induced by the addition of doxycycline. These cells were cultured in extracellular matrices gel for 14 days. (D) Number of bile ductal cysts (1 well per 2000 cells) in the extracellular matrices gel culture. Cysts with a diameter of 100 μm or more were counted. (B and D)Results are presented the mean colony count ± SD ($n = 4$). White bar, 100 μm.

derived from ADPLD pathology using CRISPR/Cas9.

3.4. Analyses of ADPLD in vitro using disease-specific human iPS cells

Functions of PRKCSH in hepatocyte and bile duct differentiation were analyzed using disease-specific human iPS cells. First, PRKCSH-mutated iPS cells were differentiated into hepatocytes using a 2D-culture method with soluble factors and matrices (Fig. S8A). The expression of albumin, a mature hepatocyte marker, was detected in cells derived from PRKCSH +/+, +/−, and −/− cells. In addition, we analyzed the $CD13^-CD13^-$ cell fractions in the colony formation culture derived from PRKCSH-mutated iPS cells. The expression of cholangiocytic marker genes (ONECUT1, 2, and SOX9) in the $CD13^-CD13^-$ cholangiocytic progenitor cell fraction was increased compared to the $CD13^+CD13^+$ fraction derived from the PRKCSH +/+ cell culture. Interestingly, the PRKCSH −/− $CD13^-CD13^-$ cholangiocytic progenitor cell fraction expressed higher levels of these markers than the PRKCSH +/+ cells (Fig. S8B). These results suggest that the deletion of PRKCSH induces the differentiation of human iPS cells into cholangiocytic progenitor cells.

As shown above, we induced differentiation of human iPS cells into $CD13^-$ cholangiocyte progenitor cells and embedded them into extracellular mixed gel. These cells were differentiated into cholangiocytic cyst structures for 15 days of culture (Fig. 4A). It was found that the number of cholangiocytic cyst structures derived from PRKCSH −/− human iPS cells was increased compared to that of PRKCSH +/− human iPS cell (Fig. 4B). In addition, we compared the number of cholangiocytic cyst structures in PRKCSH +/+ and −/− human iPS cells in culture (Fig. S9).

The cholangiocytic cysts derived from PRKCSH −/− human iPS cells were similar to those derived from PRKCSH +/+ human iPS cells (Fig. S10A and B). The cysts expressed the cholangiocytic markers CK19 and Sox9 and had a normal epithelial polarity, as shown by the expression of β-catenin and PKCζ. Primary cilia are solitary organelles on the apical surface of cells, and are specific to cholangiocytes in the liver parenchyma tissues. We compared the effect of PRKCSH deletion on primary cilia formation by staining for acetylated tubulin, and found no difference between PRKCSH −/− and PRKCSH +/+ cells (Fig. S10B). Furthermore, there was no difference in the proliferative activity of cells in the cysts derived from the PRKCSH-mutated cells (Figs. S10C

and S11).

In order to confirm the ability of *PRKCSH* deficiency to induce cholangiocytic cyst structures, the following experiment was conducted. A PRKCSH expression vector was introduced into PRKCSH$^{-/-}$ human iPS cells using the PiggyBac transposon system. This vector contains the Tet operon (Tanaka et al., 2013), and expression of PRKCSH can be induced by the addition of doxycycline at suitable timing. Thus, we established PRKCSH$^{-/-}$ human iPS cells carrying the PRKCSH doxycycline-induced expression vector. These cells were differentiated and purified into CD13^{+}CD133^{+} primary progenitor cells. Progenitor cells were inoculated and cultured with or without doxycycline (P1 culture in Fig. S1). Then, CD13^{-} cells were purified and cultured with or without the continuous addition of doxycycline. CD13^{-}CD13^{-} cells derived from P2 culture were purified and cultured in an extracellular mixed gel. As a result, it was observed that by restoring expression of PRKCSH in PRKCSH$^{-/-}$ human iPS cell-derived progenitor cells, the increase in cholangiocytic cyst structures can be suppressed (Fig. 4C and D).

Expression of PRKCSH is detected in the several types of cells including human iPS cells and hepatoblasts. The effect of PRKCSH overexpression in normal hepatoblasts was analyzed using a mouse fetal liver cell culture system (Kamiya et al., 1999). We previously showed that hepatoblasts can differentiate into hepatocytic and cholangiocytic cells in culture (Anzai et al., 2016; Kamiya et al., 2002). Overexpression of PRKCSH was induced *via* retroviral infection (Fig. S12A) and the expression of hepatocytic and cholangiocytic cell markers was analyzed by real-time PCR. The expression of hepatic marker genes *Tat* and *Cps1* was induced by the addition of hepatic maturation factors Oncostatin M (OSM) and extra cellular matrices (EHS). In contrast, the expression of cholangiocytic marker genes *Ck19*, *Ck7*, *Sox9*, and *Grhl2* (Tanimizu et al., 2014) was suppressed by OSM and EHS. We found that PRKCSH overexpression does not alter the expression of either hepatocytic or cholangiocytic markers (Fig. S12B-D).

It was clarified that the induction into cholangiocytic cyst structures by PRKCSH deficiency occurs in the differentiation process of human iPS cells, and it is possible to reproduce phenotypes of ADPLD in an *in vitro* culture system.

4. Discussion

In this study, induction of CD13^{-} cholangiocytic progenitor cells and culture of cholangiocytic cyst structures were performed using hepatic progenitor cells derived from human iPS cells. Furthermore, genetic modification using a genomic editing enzyme could successfully reproduce phenotypes of human polycystic liver diseases *in vitro*. The liver is composed of various non-parenchymal cells in addition to hepatocytes, liver parenchymal cells. Non-parenchymal cells such as stellate and sinusoidal endothelial cells are important for regulation of hepatocytic functions, and it is efficient to mix and culture these cells and hepatocytes when constructing liver tissue *in vitro*. These liver tissues are known to function as a metabolic organ by keeping the blood flow after transplantation into mice (Takebe et al., 2013). On the other hand, currently prepared *in vitro* artificial liver tissue has a vascular system but no bile ductal network inside. When expansion of liver tissue is attempted, self-generated bile acids are able to induce liver tissue damages. Therefore, the construction of a system to induce cholangiocyte progenitor cells capable of forming a bile ductal network is important to the progression of an *in vitro* artificial liver tissue system. Several groups have reported that human iPS cells can differentiate into bile duct-like cells through further treatment with chemical components, cytokines, *etc*. (Dianat et al., 2014; Sampaziotis et al., 2015). In this study, we found that a portion of the hepatic progenitor cells derived from human iPS cells acquire the ability to differentiate into cholangiocyte cells during the subculture period. In *in vivo* liver development, hepatic progenitor cells proliferate after the specification of foregut endoderm into the liver bud to form the fetal liver. In this stage, a proportion of the proliferating hepatic progenitor cells differentiate into a ductal plate, and then further into a mature bile duct by the stimulation with extracellular signals derived from periportal fibroblasts (Tanimizu and Miyajima, 2004, Tchorz et al., 2009). We previously showed that mouse hepatic progenitor cells derived from mid-fetal livers do not have the ability to differentiate into bile duct-like structures *in vitro* after purification, but we were able to perform 2D pre-culture on plastic dishes to acquire the ability to differentiate into cholangiocytic structures (Anzai et al., 2016). It seems that there is a mechanism by which hepatic progenitor cells acquire the function of cholangiocyte progenitor cells in the expansion process both *in vivo* and *in vitro*. Induction and purification methods of cholangiocyte progenitor cells from human iPS cells constructed in this study are considered to be useful for analyses of these mechanisms.

Development of genome editing enzymes such as Zinc Finger, TALEN, and CRISPR/Cas9 enables genetic modification in human pluripotent stem cells and reproduces various genetic diseases using human iPS cell-derived somatic tissue cells. These disease-related human iPS cells are expected to be useful for treatment and drug discovery. In this study, we focused on *PRKCSH*, a causative gene of polycystic liver diseases. In ADPLD, many cysts derived from biliary duct cells are generated only in the liver. Recent analysis of familial ADPLD patients has found mutations in genes important for cilia functions, including *PRKCSH* and *SEC63*. However, the molecular mechanism of how these genetic mutations induce multiple occurrences of liver cysts and the signalling pathway through which they exert their function is still unknown. One of the reasons for this is the lack of an experimental system for analysing ADPLD mutations such as in that of *PRKCSH* in human cells. Therefore, in this study, human iPS cells which harbour the genetically altered *PRKCSH* mutation found in diseased patients were prepared to induce bile duct differentiation using genome editing technology. We found that differentiation into cholangiocytic cyst structures was induced by the *PRKCSH* mutation, and we succeeded in establishing a system reproducing liver cystic symptoms by *PRKCSH* mutation *in vitro*. The mutant iPS cells clearly expressed no wild-type PRKCSH protein; however, some expression of a C-terminal truncated protein was seen. A previous linkage analysis of polycystic liver disease families showed that both exon 4 and 15 stop mutations were correlated with the onset of PCLD (Davila et al., 2004; Drenth et al., 2003), suggesting that the loss of function of PRKCSH due to C-terminal deletion was important for the pathology of PCLD. However, it is possible that the C-terminal truncated PRKCSH has some inhibitory function leading to biliary diseases. The correlation between cholangiocytic formation and PRKCSH mutation remains unknown. We found that PRKCSH mutation induced the expression of several cholangiocytic genes in the iPS cell-derived hepatic progenitor cells. However, the proliferative ability of cholangiocytic cysts was not changed by the PRKCSH mutation. In addition, the cell survival and death due to oxidative and ER stress might be regulated by PRKCSH mutation. These investigations about the PRKCSH-mutated proteins are important to be included in the future studies.

It was recently reported that other biogenesis pathway-related genes in the endoplasmic reticulum such as ALG8, GANAB, and SEC61B were also involved in the onset of PCLD (Besse et al., 2017). Other new polymorphism in patients might also be related to the PCLD pathology. The patient-derived human iPS cells are very useful for the analyses of the onset and pathology of several diseases. These cells might reveal gene abnormalities related to pathological conditions other than PRKCSH. Polycystic liver diseases are rare diseases, and effective therapeutic agents have yet to be discovered. Thus, the pathology of PCLD can be further understood by a combination of the established technology of patient-derived iPS cells and genome editing technology. The discovery of a new drug might not be very far, using the system established in this study. It might be possible to use our system to analyze each of these signals to elucidate the mechanism of human bile duct diseases.

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Author contributions

A.K., H.C., K.I., E.A, and K.T. performed experiments and prepared the data. A.K., T.K., and Y.I. analyzed the data. A.K. and Y.I. prepared the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

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