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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,