Establishment of Inducible *SMAD4* Knockout PSC-Derived Cholangiocyte Committed Cells

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Abstract

Cholangiocarcinoma (CCA), malignancies of the biliary duct system, is a major public health problem, especially in the region of northeastern Thailand. The previous study demonstrated SMAD4, which is a crucial mediator of TGF-β and BMP4 signaling pathway was high mutation frequency in CCA. The mechanism of *SMAD4* mutation drive cholangiocarcinogenesis remains unknown. With ethical and reproducible limitation, adult human cholangiocyte still lacks the reliable in *vitro* model to study the pathogenesis. Pluripotent stem cells (PSCs) have the potential to bridge the gap with unlimited cell source. Recently, PSC-derived cholangiocyte differentiation protocols have been reported. The constitutive activation of conventional CRISPR-Cas9 system is not suitable for study the role of *SMAD4* by affecting the cell differentiation. To dissecting *SMAD4* gene function with precise and less interfere differentiation procedure, an inducible knockout system is indispensable for *SMAD4* disruption in this lineage. In this study, we developed an inducible *SMAD4* knockout PSC for *SMAD4* knockout, which is stage-specific inducible gene knockout during cholangiocyte differentiation. The disruption of the *SMAD4* gene was succeeded in PSC and cholangiocyte lineage-committed cells after doxycycline treatment. T7EI analysis revealed a ~20% Indel rate in PSC. Indel rate was succeeded in a differentiated cell; hepatoblast (10.81%) and cholangiocyte progenitor (6.31%). Three indel mutation patterns were confirmed by DNA sequencing with frameshift mutation. These results suggest that inducible *SMAD4* knockout cassette has the potential to generate DSB in several stages of cholangiocyte lineage-committed cells. This study will be applied for developing the model and dissecting *SMAD4* gene function in any developmental stage in further study.

Keywords**:** *Cholangiocyte, Cholangiocarcinoma, Pluripotent stem cells, SMAD4, CRISPR-Cas9, Inducible Cas9*

1. Introduction

Cholangiocarcinoma (CCA), malignancies of the biliary duct system, is a major public health problem, especially in the region of northeastern Thailand and increased in several parts of the world. An early point of CCA development mostly associated with chronic inflammation such as primary sclerosing cholangitis, recurrent cholangitis, liver cirrhosis and infection with a parasite (ex. *Opisthorchis viverrini*). It has been suggested that biliary disease involved signaling pathways is linking inflammation to carcinogenesis. TGF-β is a key inflammatory cytokine which plays an important role in various cancers. Abrogation of SMAD4, a crucial mediator of TGF-β and BMP4 signaling pathway, causes the alteration of TGF-β target gene expression and promote cancer progression. The high *SMAD4* mutation frequency has been reported in CCA with *Opisthorchis viverrini* infection (Chan-On et al., 2013). To study the effect of mutations in *SMAD4*, in *vitro* model that recapitulates human disease is still needed.

In the past, cell lines are widely used as a tool for studying pathogenesis, drug screening and development of new therapies. These cell lines, however, loss of the natural heterogeneity of cancer and showed genotypic and phenotypic drift after continuous culture. The immortalized cell line is not a good in *vitro* model due to genomic instability, and most cell lines are poorly characterized (Zabron, Edwards and Khan, 2013). An ethical issue is a major limitation of using isolated primary cells as a model. Despite the best in *vitro* model, human cholangiocytes are cannot be long-term culture *in vitro* environment. The advance of pluripotent stem cells (PSCs) has provided the unlimited cell source which contains self-renew and differentiation. Based-on developmental study, several PSC-derived cholangiocyte protocols have been established. (Si-Tayeb, Lemaigre and Duncan, 2010; Gordillo, Evans & Gouon-Evans, 2015; Dianat et al.,

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2014; Ogawa et al., 2015; De Assuncao et al., 2015; Sampaziotis et al., 2015; Sampaziotis et al., 2017). Additionally, Sampaziotis (2017) has reported that PSC-derived cholangiocyte model has the potential to use as cystic fibrosis modelling with depending on 3-dimensional differentiation culture technique. Due to self-organized capacity, it is indicated that 3-dimensional condition may fulfill the human cholangiocyte in *vitro* model in term of functionality and polarization than monolayer condition (Liu, & Chen, 2018). Therefore, the use of PSC-derived cholangiocyte models will allow the possibility of pathogenesis studies and the pathological mechanisms in term of in *vitro* model.

CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated genes) is the type II CRISPR nuclease system which consists of Cas9, gRNA, crRNA and trRNA. In the fundamental mechanism of CRISPR-Cas9, complex CAS9-gRNA will be generated by trRNA. This complex can bind and cleave target gene. Interestingly, it has been widely used for gene manipulation in PSC with precisely genome editing at a specified site (Zhang et al., 2018). *SMAD4* is necessary for both of early and late developmental stages of cholangiocyte lineage. In order to study the role of *SMAD4*, the constitutive activation of conventional CRISPR-Cas9 was restricted by uncontrollable activation. An inducible Cas9 gene knockout system has been established (Gonzalez et al., 2014) with controllable in the Cas9 activation at a specific time and precise genomic location. In this study, inducible *SMAD4* knockout (KO) PSC was generated by using TALENs to target inducible *SMAD4* knockout cassette into *AAVS1*, safe harbor site. An inducible *SMAD4* knockout cassette which consists of SMAD4 guide RNA (gRNA) and Cas9 protein induce *SMAD4* gene disruption by generating non-homologous enjoining (NHEJ) on *SMAD4* gRNA target site. By inducible knockout cassette, this system does not interfere during differentiation and provide gene disruption in stage-specific. Additionally, an inducible *SMAD4* knockout-PSC can be used for dissecting *SMAD4* gene at any differentiation stages. Thus, this study will be useful for developing the model of cholangiocyte disease for understanding the role of *SMAD4* in CCA.

2. Objectives

To generate an inducible *SMAD4* knockout cassette for disruption of *SMAD4* gene in cholangiocyte lineage-committed PSC

3. Materials and Methods

3.1 PSC-derived hepatoblast, cholangiocyte progenitor and cholangiocyte differentiation

Cholangiocyte differentiation protocol was performed as described in Sampaziotis (2017). PSCs were dissociated by using 1ml of CTK enzyme (consist of collagenase, trypsin, and knockout serum replacement.) when the confluency of the cell is up to 80%-90%. Cells were incubated for 7 min at 37°C. Then cells were washed by 1ml of 1x phosphate buffer saline (PBS) for two times. After that, cells were maintained on a gelatin-coated plate in chemically defined medium-polyvinyl alcohol (CDM-PVA) medium supplemented with 10 ng/ml activin A and 12 ng/ml FGF2. This day referred to as Day 0. Day 1, the medium was replaced into CDM–PVA supplemented with 100 ng/ml activin A, 80 ng/ml FGF2, 10 ng/ml BMP-4, 10μM LY294002 and 3μM CHIR99021. Day 2, the medium was replaced into CDM–PVA supplemented with 100 ng/ml activin A, 80 ng/ml FGF2, 10 ng/ml BMP-4 and 10μM LY294002. Day 3, the medium was replaced into Roswell Park Memorial Institute (RPMI/B27) medium supplemented with 100 ng/ml activin A and 80 ng/ml FGF2. Day 4-8, the medium was replaced into RPMI/B27 medium supplemented with 50 ng/ml activin A. Day 9-12, the medium was replaced into RPMI/B27 medium supplemented with 10 μM SB-431542 and 50 ng/ml BMP-4. At the end of day 12, differentiated cells were referred to as hepatoblast stage. To generate cholangiocyte progenitor, the hepatoblast cell at day 12 were cultured into RPMI/B27 medium supplemented with 50 ng/ml, activin A, 50 ng/ml FGF10 and 3 μM retinoic acid for three days (day 13-16). At the end of day 16, differentiated cells were referred to as cholangiocyte progenitor stage. In order to differentiate into a cholangiocyte-like cell, cholangiocyte progenitor was dissociated and cultured in 3D with Matrigel. 2 x 10^5 cells were mixed with 50% (vol/vol) Matrigel supplemented with 20 ng/ml EGF and 10 μM Rho kinase inhibitor (Y-27632) and this mixture was dropped into 24-well plate for 50 μl. Cells were

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incubated for 30 min at 37 °C. Day 17–26, the medium was replaced into WE medium supplemented with 20 ng/ml EGF every two days. Cells at day 26 were referred to as cholangiocyte-like cells.

3.2 Generation of Inducible SMAD4 knockout cassette

SMAD4 gRNA was used in this study obtained from Sato (2015). To generate U6 promoter with *SMAD4* gRNA Cassette, *SMAD4* gRNA was cloned into BPK1520 vector followed Joung lab gRNA cloning protocol. A pair of primer which contains homology arm of Puro-Cas9 donor at *SacI* restriction site was designed for amplifying U6-*SMAD4* gRNA PCR product. This PCR product was cloned into Puro-Cas9 donor by using In-Fusion HD Cloning Kits (Clontech). In order to validate vector, an inducible *SMAD4* gRNA cassette was analyzed by DNA sequencing.

3.3 PSC Transfection and Selection

PSCs were pre-treated with 10 µM Y27632 one day before electroporation. $1x10^6$ cells of PSCs were dissociated into single cells by using accutase. Cells were resuspended in 100 µl of Nucleofection solution P3 which contains 5 µg of AAVS1-TALEN-L, 5 µg of AAVS1-TALEN-R,10 µg of AAVS1-Neo-M2rtTA and 10 µg of SMAD4 Puro-Cas9 donor. This cell mixture was transferred into nucleofection cuvette and electroporated by 4D-Nucleofector™ X (Lonza) with CB-150 program. Pre-warm mTesR1 medium was added into the cell mixture in the nucleofection cuvette. The cell mixture was gently mixed and transferred to a new Matrigel-coated plate with mTeSR1 medium and 10 µM Y27632. After electroporation, transfected cells were treated with 0.5 µg/mL of Puromycin for three days, followed by 70 µg/mL of Geneticin treatment for five days. After that, transfected cells were treated with 0.5 µg/mL of Puromycin and 70 µg/mL of Geneticin combination for six days.

3.4 AAVS1-targeted site validation

To determine *AAVS1*-target site of the vector, CAS9-AAVS1 primer and m2rtTA-AAVS1 primer were used for analysis by thermocycler. PCR product was separated by gel electrophoresis.

3.5 Doxycycline treatment

To activate Cas9 function in PSC stage, inducible *SMAD4* KO PSCs were treated with doxycycline (2 µg/ml, 5 µg/ml) for three days. The medium was replaced daily. Cells were dissociated for genomic DNA or RNA extraction.

3.6 T7 endonuclease assay

To detect the double-strand break of genomic DNA, 10 ng of genomic DNA was amplified by PCR. PCR products were reannealed by using the following protocol: 95°C, 5 min; 95°C–85°C at −2°C/s; 85°C–25°C at −0.1°C/s; hold at 4°C. The hybridized PCR products were incubated with 5U of T7EI (NEB) at 37°C for 15 min and analyzed with gel electrophoresis. The intensity of the product band was determined by ImageJ.

3.7 RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted RNA by using RNeasy Mini Kit (QIAGEN). qRT-PCR primers of cholangiocyte differentiation were obtained from Sampaziotis (2015). qRT-PCR primer of Cas9 fragment was obtained from Gonzalez (2014). GAPDH was used as an internal control housekeeping gene.

3.8 Genomic DNA extraction

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). Briefly, pellet cells were resuspended in 200 µl of 1xPBS. 20 µl of Proteinase K solution and 200 µl of AL solution was added into cell mixture. Then the cell mixture was incubated in a heating block at 56°C for 10 min. Absolute Ethanol was added into the cell mixture. The cell mixture was transferred to the column and centrifuged at 8,000 rpm for 3 min. It was washed by 500 µl of AW1 solution and 500 µl of AW2 solution. After centrifugation,

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genomic DNA was eluted with nuclease-free water. The concentration of genomic DNA was measured by spectrophotometer.

3.9 Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were permeabilized and blocked with blocking buffer solution (5% goat serum, 0.3% Triton X-100 in PBS or 5% donkey serum, 0.3% Triton X-100 in PBS) for 1 hour at room temperature. Next, the cells were incubated at 4 C for overnight with the following primary antibodies diluted in antibody diluent solution. Cells were washed three times with 0.05% Tween 20 in PBS for 5 min and incubated for 2 hours at room temperature with secondary antibodies in antibody diluent solution. Cells were washed three times, with 0.05% Tween 20 in PBS for 5 min. Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) (1:1000) for 5 min. Cells were washed three times with PBS for 5 min each and then imaged using a fluorescence microscope.

3.10 pGEM-T easy vector cloning

The amplified *SMAD4* fragment was cloned into a pGEM-T easy vector (Promega). This plasmid DNA was transformed into competent cells and extracted for genomic DNA sequencing analysis.

4. Results and Discussion

4.1 Characterization of PSC-derived cholangiocyte-like cell or cholangiocyte organoid

Recently, there were many reported of PSC-derived cholangiocyte differentiation protocol. This study was based on a previously published protocol (Sampaziotis et al., 2017). To cholangiocyte induction, PSC was exposed to temporal specific growth factors which are necessary for liver development (Si-Tayeb et al., 2010). However, the variation of differentiation capacity in PSC still challenge on the fields. To investigate the PSC of our study for cholangiocyte differentiation, colonies of the cell at an early stage of differentiation was packed colonies. Then differentiated cells were migrated and formed cystic-like structure at the end of differentiation (Figure 1A). The immunofluorescence staining indicated that cells were expressed definitive endoderm marker SRY-box transcription factor 17 (SOX17) (Figure 1A). Following the endodermal stage, cells were positive for the foregut progenitor marker forkhead box protein A2 (FOXA2). Immunostaining at day 12 revealed the expression of hepatocyte nuclear factor 4 alpha (HNF4A) and Cytokeratin 19 (CK19). Cells were expressed cholangiocyte progenitor SRY-box transcription factor 9 (SOX9) and CK19 at day 16. With the 3D organoid system, PSC-derived cholangiocyte-like cell or cholangiocyte organoid (CO) could recapitulate the tissue of origin. PSC-derived cholangiocyte organoid (CO) was revealed the expansion capacity with cystic-like structure (Figure 1B). Immunofluorescence staining indicated the origin cell characteristic which expression of biliary marker Cytokeratin 7 (CK7), CK19 and SOX9. However, fetal gene marker AFP still expressed in this culture system (Figure 1B). The qRT-PCR analysis revealed that the expression level of cholangiocyte markers of our PSC-derived cholangiocyte organoid was expressed as in primary cholangiocyte organoid (PCO) (Figure 1C). Thus, these findings indicated the primary cholangiocyte similarity of our PSC-derived cholangiocyte organoid, which can use as a tool for disease modeling.

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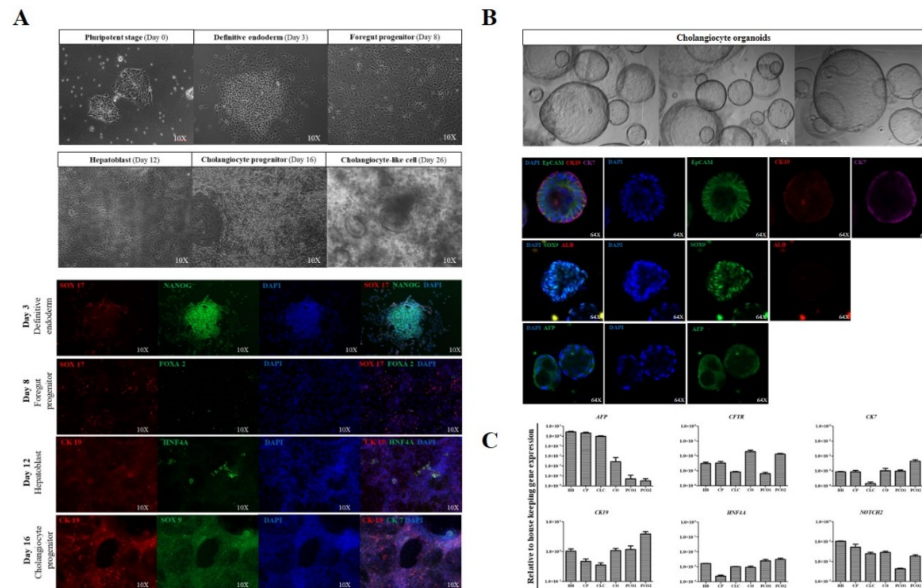

Figure 1 Characterization of PSC-derived cholangiocyte-like cell or CO (A) Light microscopy and immunofluorescence images of cholangiocyte differentiation. (B) Light microscopy and immunofluorescence images of PSC-derived cholangiocyte organoid. (C) Quantitative real-time RT-PCR (qRT-PCR) analysis of cholangiocyte differentiation and cholangiocyte organoid. The relative expression levels were compared to the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). HB, hepatoblast; CP, cholangiocyte progenitor; CLC, cholangiocyte-like cell; CO, cholangiocyte organoid; PCO, primary cholangiocyte organoid. n = 3 biological replicates for each sample.

4.2 Transfection and generation of inducible SMAD4 knockout (KO) PSC

Previously, an inducible Cas9 vector system with sgRNA which targeting into "safe harbor" locus (known as *AAVS1*) was generated by Gonzalez (2014). This system was suitable for genetic manipulation model which induce stage-specific inducible gene knockout during differentiation. By co-transfecting inducible *SMAD4* knockout cassette and M2rtTA donor, TALEN-mediated gene targeting was performed (Figure 2A). 2 clones of inducible *SMAD4* KO-PSC were obtained after antibiotic selection. In order to investigate *AAVS1*-targeted site of inducible *SMAD4* cassette and Neo-M2rtTA vector, CAS9-AAVS1 and m2rtTA-AAVS1 primer pair were used. The amplified PCR product of CAS9-AAVS1 primer revealed that two clones of inducible *SMAD4* KO PSC containing an inducible *SMAD4* knockout cassette (1,573 bp). For amplified PCR product of m2rtTA-AAVS1 primer indicated that clone 1 of inducible *SMAD4* KO PSC containing Neo-M2rtTA donor (1,353 bp). However, clone 2 of inducible *SMAD4* KO PSC demonstrated an incomplete of Neo-M2rtTA donor fragment (Figure 2B). This finding revealed only one clone of inducible *SMAD4* KO PSC was obtained. To determine the doxycycline dose for activate *Cas9* expression, inducible *SMAD4* KO PSC was treated with 1 and 5 µg/mL of doxycycline concentrations for 72 h doxycycline treatment. The qRT-PCR analysis revealed that doxycycline treatment was capable of inducing *Cas9* expression in a dose-dependent manner (Figure 2C). Besides, compact morphology was observed with a high nucleus to cytoplasmic ratio to examine the self-renewal and pluripotency of inducible *SMAD4* knockout PSC. Immunofluorescence staining revealed both of pluripotency markers OCT4 and NANOG were expressed in our inducible *SMAD4* KO PSC (Figure 2D).

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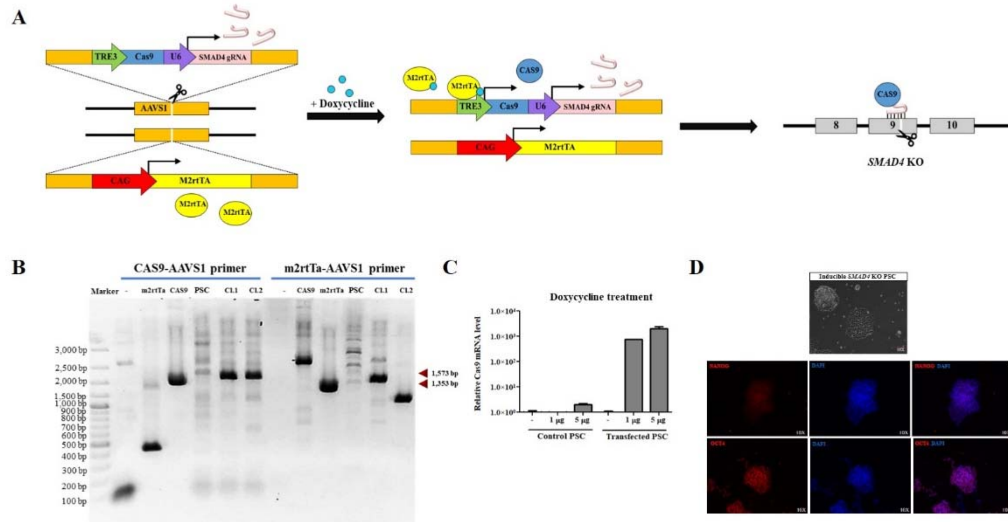

Figure 2 Strategy and validation of an inducible *SMAD4* knockout cassette in PSC (A) TALEN-mediated gene targeting of an inducible *SMAD4* knockout cassette and Neo-m2rtTA into *AAVS1* locus. Induced *Cas9* expression with doxycycline treatment results in DNA double-strand break (DSB) in the *SMAD4* target gene. (B) PCR analysis of *AAVS1*-target site of inducible *SMAD4* knockout cassette and m2rtTA vector. (C) Quantitative real-time RT-PCR (qRT-PCR) analysis of *Cas9* expression with or without doxycycline treatment in PSC. (D) Light microscopy image and immunofluorescence of inducible *SMAD4* KO PSC. m2rtTa, Neo-M2rtTA vector; Cas9, inducible *SMAD4* knockout cassette; Cl.1, clone 1; Cl.2, clone 2.

4.3 Validation of inducible SMAD4 KO PSC

In order to validate for *SMAD4* gene knockout in PSC stage, two clones of inducible *SMAD4* KO PSC were treated with 2 µg/mL doxycycline for 72 h (Figure 3A). The T7 endonuclease I (T7EI) mismatch detection assay was performed. The amplified PCR product revealed ~20% mutation rates in clone 1 of inducible *SMAD4* KO-PSC (Figure 3B). However, clone 2 of inducible *SMAD4* KO PSC was not detected. The result revealed that only clone 1 was capable of generating *SMAD4* gene knockout with complete an expression of *SMAD4* target gRNA, Cas9 and M2rtTA. This result was corresponding with *AAVS1*-targeted site validation of vector donor in our previous result. To confirm *SMAD4* knockout with indel mutations, the amplified *SMAD4* fragment was cloned into the pGEM-T easy vector. Plasmid DNA was isolated from bacterial clones and analyzed by genomic DNA sequencing to determine out-of-frame indel mutation. The results indicated that three patterns of *SMAD4* gene out-of-frame deletion mutation were detected in this study (Figure 3C).

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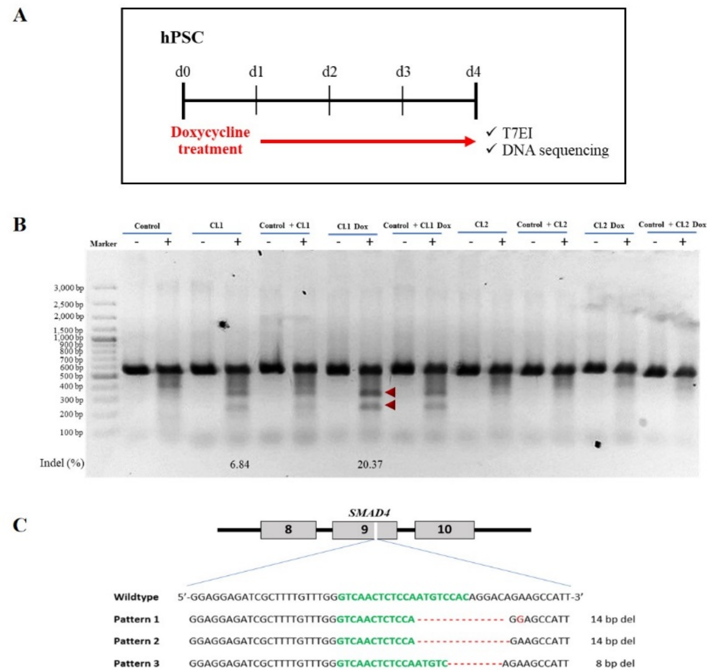

Figure 3 *SMAD4* KO in PSC (A) Timeline of doxycycline treatment in inducible *SMAD4* knockout PSC (B) T7 endonuclease I mismatch detection of *SMAD4* gene knockout at PSC stage. In this study, red arrowhead indicates the expected T7EI-specific fragments used to quantify indel frequency. (C) Representative sequence patterns of various knockout mutant clones.

4.4 Validation of inducible SMAD4 KO PSC-derived hepatoblast and cholangiocyte progenitor cells

To determine for *SMAD4* gene knockout in cholangiocyte lineage-committed cells, inducible *SMAD4* KO PSC was differentiated into the cholangiocyte-like cell as described above. Cells were treated with 2 and 5 µg/mL doxycycline for 72 h (Figure 4A). Inducible *SMAD4* KO PSC revealed the potential to differentiate into cholangiocyte-like cells (Figure 4B). The T7 endonuclease I (T7EI) mismatch detection result demonstrated that the indel rate was increased in a dose-dependent manner. The amplified PCR product revealed ~10.81% and 6.31% mutation rates in PSC-derived hepatoblast and PSC-derived cholangiocyte progenitor at 5 µg/mL doxycycline treatment (Figure 4C, 4D). Therefore, our result indicated that an inducible *SMAD4* knockout vector could generate indel mutation in cholangiocyte lineage-committed cells.

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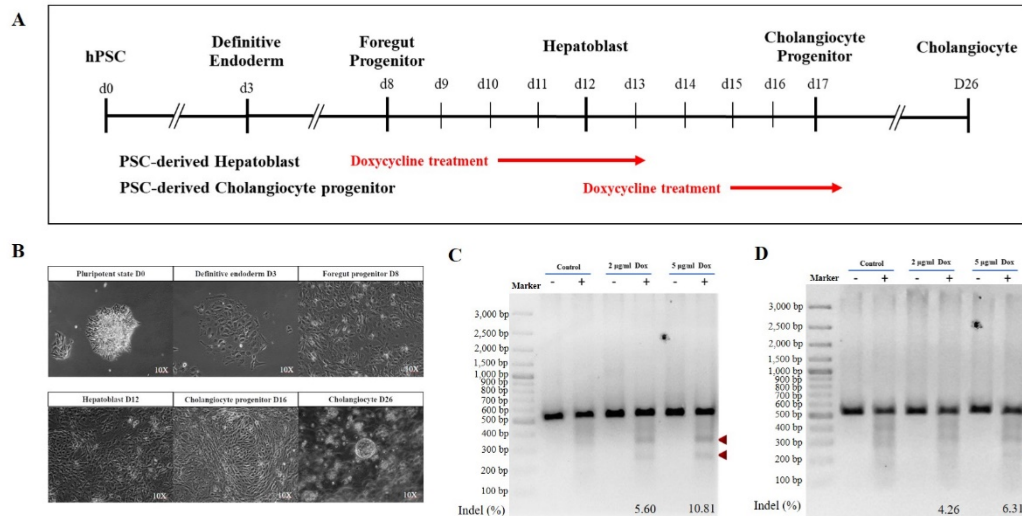

Figure 4 *SMAD4* KO in inducible *SMAD4* knockout PSC-derived cholangiocyte lineage-committed cells (A) Timeline of doxycycline treatment in cholangiocyte lineage-committed cells; hepatoblast, cholangiocyte progenitor. (B) Light microscopy images of cholangiocyte differentiation of inducible *SMAD4* knockout PSC. (C) T7 endonuclease I mismatch detection of *SMAD4* gene knockout at hepatoblast stage. (D) T7 endonuclease I mismatch detection of *SMAD4* gene knockout at cholangiocyte progenitor stage. In this study, red arrowhead indicates the expected T7EI-specific fragments used to quantify Indel frequency.

We have established an inducible *SMAD4* knockout PSC which has the potential to differentiate into cholangiocyte lineage-committed cells and generate DSB on the *SMAD4* gene. The ethical issue is a major limitation of using isolated primary cells as a cholangiocyte model. Remarkably, pluripotent stem cells (PSC) has provided the unlimited cell source which contains self-renew and differentiation. PSC-derived cholangiocyte-like cells protocol was reported. Based-on study of Sampaziotis's differentiation protocol in 2017, our PSC line revealed the differentiation potential to the cholangiocyte-like cell with cystic structure. Our finding demonstrated that PSC-derived cholangiocyte organoid revealed the capable of propagating in long-term culture and also expressing the key cholangiocyte markers (CK7, CK19, SOX9, and CFTR) as reported in several PSC-derived cholangiocyte protocols (Dianat et al., 2014; Ogawa et al., 2015; De Assuncao et al., 2015; Sampaziotis et al., 2015; Sampaziotis et al., 2017). Thus, this PSC-derived cholangiocyte organoid will be applied as a tool for the cholangiocyte model. Current in *vitro* PSC-derived models have reported the immaturity in several cell type (Baxter et al., 2015; Sampaziotis et al., 2017; Hartman, Dai & Laflamme, 2016). Future study will be defined as cholangiocyte organoid characteristic, maturation, function and gene expression include developing efficient differentiation protocol for this cell type.

CRISPR-Cas9 is an efficient genome manipulation tool for precisely specified DNA site. Due to convenient than other nuclease systems, CRISPR/Cas9 has been widely used in stem cell research for gene knockout, gene knockin, gene correction and disease modeling (Zhang et al., 2018). The previous study was reported the CRISPR-Cas9 based vector (Mali et al., 2013). With uncontrollable of constitutive Cas9 expression, it may affect to self-renewal of cell differentiation of PSC. The developmental study indicated that SMAD4 is necessary for both of early and late stage of liver development (Si-Tayeb et al., 2010). Additionally, *SMAD4* disruption has an impact on definitive endoderm in mice (Chu, Dunn, Anderson, Oxburgh, & Robertson, 2004). To achieve this, an inducible Cas9 knockout cassette with *AAVS1* homology arm was pioneered by Gonzalez (2014). An inducible knockout cassette allows convenient, controllable, less interfere for stem cell development and applicable to any cell type. It was delivered precisely into *AAVS1* site

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by TALENs. Based-on single-cell selection, the homogeneous population of inducible knockout PSC lines was expressing the construct of target gRNA and Cas9 protein. Additionally, gene knockout controllable was depended on the presence of doxycycline to induce Cas9 expression. Our finding indicated that our inducible *SMAD4* KO-PSC line was achieved to generating *SMAD4* disruption in PSC involve PSC-derived hepatoblast and cholangiocyte progenitor cells. Indel mutation efficiency was depended on the variation of the knockout system (Gonzalez et al., 2014). To increase the efficiency of bi-allelic mutation, a pair of two gRNA may provide gene knockout efficiency. In a further study, we will be determining *SMAD4* inactivation in protein expression level and investigated the effect of *SMAD4* knockout in cholangiocyte-lineage committed cells include PSC-derived cholangiocyte. In addition to generating inducible *SMAD4* knockout PSC, our inducible *SMAD4* KO-PSC can be applied not only cholangiocyte lineage model but also PSC-based model in all stage of several cell type for studying the role of *SMAD4* gene function.

5. Conclusion

In this study, our findings demonstrated the potential of an inducible *SMAD4* KO-PSC, which inducing *SMAD4* gene knockout in every stage of cholangiocyte differentiation. This inducible *SMAD4* KO-PSC may apply for disease modeling in cholangiocyte lineage including any cell types that related to *SMAD4* dysfunction.

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7. References

Baxter, M., Withey, S., Harrison, S., Segeritz, C. P., Zhang, F., Atkinson-Dell, R., . . . Hanley, N. A. (2015). Phenotypic and functional analyses show stem cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes. *J Hepatol, 62*(3), 581-589. doi:10.1016/j.jhep.2014.10.016

Chan-On, W., Nairismagi, M. L., Ong, C. K., Lim, W. K., Dima, S., Pairojkul, C., . . . Teh, B. T. (2013). Exome sequencing identifies distinct mutational patterns in liver fluke-related and non-infection-related bile duct cancers. *Nat Genet, 45*(12), 1474-1478. doi:10.1038/ng.2806

Chu, G. C., Dunn, N. R., Anderson, D. C., Oxburgh, L., & Robertson, E. J. (2004). Differential requirements for Smad4 in TGFbeta-dependent patterning of the early mouse embryo. *Development, 131*(15), 3501-3512. doi:10.1242/dev.01248

De Assuncao, T. M., Sun, Y., Jalan-Sakrikar, N., Drinane, M. C., Huang, B. Q., Li, Y., . . . Huebert, R. C. (2015). Development and characterization of human-induced pluripotent stem cell-derived cholangiocytes. *Lab Invest, 95*(6), 684-696. doi:10.1038/labinvest.2015.51

Dianat, N., Dubois-Pot-Schneider, H., Steichen, C., Desterke, C., Leclerc, P., Raveux, A., . . . Dubart-Kupperschmitt, A. (2014). Generation of functional cholangiocyte-like cells from human pluripotent stem cells and HepaRG cells. *Hepatology, 60*(2), 700-714. doi:10.1002/hep.27165

Gonzalez, F., Zhu, Z., Shi, Z. D., Lelli, K., Verma, N., Li, Q. V., & Huangfu, D. (2014). An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. *Cell Stem Cell, 15*(2), 215-226. doi:10.1016/j.stem.2014.05.018

Gordillo, M., Evans, T., & Gouon-Evans, V. (2015). Orchestrating liver development. *Development, 142*(12), 2094-2108. doi:10.1242/dev.114215

Hartman, M. E., Dai, D. F., & Laflamme, M. A. (2016). Human pluripotent stem cells: Prospects and challenges as a source of cardiomyocytes for in vitro modeling and cell-based cardiac repair. *Advanced drug delivery reviews*, *96*, 3–17. https://doi.org/10.1016/j.addr.2015.05.004

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Liu, Y., & Chen, Y. G. (2018). 2D- and 3D-Based Intestinal Stem Cell Cultures for Personalized Medicine. *Cells*, *7*(12), 225. https://doi.org/10.3390/cells7120225

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., . . . Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science, 339*(6121), 823-826. doi:10.1126/science.1232033

Ogawa, M., Ogawa, S., Bear, C. E., Ahmadi, S., Chin, S., Li, B., . . . Ghanekar, A. (2015). Directed differentiation of cholangiocytes from human pluripotent stem cells. *Nat Biotechnol, 33*(8), 853-861. doi:10.1038/nbt.3294

Sampaziotis, F., de Brito, M. C., Madrigal, P., Bertero, A., Saeb-Parsy, K., Soares, F. A. C., . . . Vallier, L. (2015). Cholangiocytes derived from human induced pluripotent stem cells for disease modeling and drug validation. *Nat Biotechnol, 33*(8), 845-852. doi:10.1038/nbt.3275

Sampaziotis, F., de Brito, M. C., Geti, I., Bertero, A., Hannan, N. R., & Vallier, L. (2017). Directed differentiation of human induced pluripotent stem cells into functional cholangiocyte-like cells. *Nature protocols*, *12*(4), 814–827. https://doi.org/10.1038/nprot.2017.011

Si-Tayeb, K., Lemaigre, F. P., & Duncan, S. A. (2010). Organogenesis and development of the liver. *Dev Cell, 18*(2), 175-189. doi:10.1016/j.devcel.2010.01.011

Zabron, A., Edwards, R. J., & Khan, S. A. (2013). The challenge of cholangiocarcinoma: dissecting the molecular mechanisms of an insidious cancer. *Dis Model Mech, 6*(2), 281-292. doi:10.1242/dmm.010561

Zhang, Z., Zhang, Y., Gao, F., Han, S., Cheah, K. S., Tse, H.-F., & Lian, Q. (2017). CRISPR/Cas9 Genome-Editing System in Human Stem Cells: Current Status and Future Prospects. *Molecular Therapy - Nucleic Acids, 9*, 230-241. doi:10.1016/j.omtn.2017.09.009

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