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Brca2 결손 마우스 유래 3-D 췌장 오거노이드의 염색체 불안정성 분석

Analysis of chromosome instability in 3-D pancreatic organoid in mice disrupted of tumor suppressor Brca2

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Abstracts

Pancreatic cancer remains to be one of the most lethal diseases despite extensive researches. While essential genes responsible for driving pancreatic cancer were revealed, such as *K-Ras* or *Brca2,* molecular mechanism to drive pancreatic cancer is to be elucidated. To unveil molecular mechanism about how *Brca2* deficiency is related to the development of pancreatic cancer, I have utilized mouse models and 3-D pancreatic organoid culture throughout this study.

Brca2 is tumor-suppressor gene that functions in DNA repair, telomere homeostasis and mitotic checkpoint. During mitosis, it mediates the acetylation of BubR1 with PCAF, thus reinforces spindle assembly checkpoint and prevents chromosome instability. In this study, $BubR1^{K243R/+}$ acetylation deficient mouse model were crossed with $K\text{-}Ras^{G12D/+}$*; Pdx1-CRE* mouse, to assess how chromosome instability can affect the development of pancreatic cancer. Surprisingly, $BubR1^{K243R/+}$*;* $K\text{-}Ras^{G12D/+}$*; Pdx1-CRE* mice exhibited expedited tumor growth with shortened life span compared to $K\text{-}Ras^{G12D/+}$*; Pdx1-CRE* mice. Analysis of mitotic progression with Mouse embryonic fibroblast implied that mitotic progression was aberrated in the

existence of BubR1 acetylation deficiency and oncogenic K-Ras mutation.

As well-known tumor-suppressor gene, *Brca2* mutation is the highest risk factor of familiar pancreatic cancer and also frequently found in spontaneous pancreatic as well. In order to assess how Brca2 depletion can influence on the integrity of telomere in the context of pancreatic ductal epithelium, I have utilized 3-D organoid culture from *Brca2*$^{f11/f11}$*; mTerc*$^{-/-}$ mouse model. Unlike other genotypes, *Brca2*$^{f11/f11}$*; mTerc*$^{-/-}$ showed higher rate of telomere fragility, heterogeneity of length with increased aneuploidy rate.

Lastly, I have established pancreatic cancer organoid culture method from patient-derived biopsy sample. This method is envisioned to be the most faithful avatar that reflects individual cancers. A tiny tissue from Endoscopic Ultrasound (EUS)-guided Fine-needle Biopsy (FNB) could be successfully generate pancreatic ductal epithelium, presumably from pancreatic ductal adenocarcinoma and normal ductal tissue. This methodology will be utilized to analyze the patterns of chromosome instability including telomere instability to scrutinize the molecular mechanism of human pancreatic cancer development.

Keywords: Pancreatic cancer, 3-D organoid, Brca2, K-Ras, Chromosome instability, Spindle assembly checkpoint

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I. Introduction

I-1. Maintenance of chromosome instability by mitotic function of Brca2

Genome of the cell should be strictly protected as its aberrations can directly result in various diseases. In order to develop surveillance mechanism to ensure genomic integrity, proliferating eukaryotic cells have evolved distinct molecular machinery, so called cell cycle checkpoint. Mitosis, a phase that duplicated chromosomes should be properly segregated into two daughter cells, is not the exception with the checkpoint named spindle assembly checkpoint. Spindle assembly checkpoint (SAC) is a surveillance mechanism during the mitosis to assure that all chromosomes are properly attached to bipolar spindles with tensions and thus be ready to be segregated. SAC can halt the progression of mitosis even if a single chromosome is not attached with microtubules and its effect is realized through a complex of molecules, Mitotic checkpoint complex (MCC).

BubR1 is a core component of MCC and has been known to be associated with the checkpoint activity along with other molecules such as Mad2, Bub3 etc. Our lab has previously reported acetylation of human BubR1 at K250

residue can work as a molecular switch which can decide the progression of mitotic checkpoint. When BubR1 is acetylated, APC/C (Anaphase promoting complex / Cyclosome), E3 ubiquitin ligase, is inhibited and thus cell cycle is arrested in metaphase until BubR1 is deacetylated. Further studies have suggested that this acetylation by PCAF, an acetyltransferase, is mediated through Brca2. It has been shown that Brca2, previously known to function in DNA repair mainly, specifically localize at kinetochore as cells enter mitosis. Brca2 can interact with BubR1 with its C-terminal while N-terminal is interacting with PCAF and it mediates BubR1 acetylation in mitosis. During mitosis, Brca2 is thought to react with other mitotic key players including Plk1, HDAC along with BubR1 and assumed to function as scaffold.

To assess the impact of Brca2 scaffold in the maintenance of genomic integrity, we can abrogate BubR1 acetylation as it is a direct consequence of Brca2 function. *BubR1* ^{K243R} allele was generated to impair acetylation in mitosis as K243 residue in mouse corresponds to K250 residue in human. While *BubR1*$^{K243/K243}$ mouse couldn't survive due to early embryonic lethality, *BubR1* $^{K243R/+}$ mice showed spontaneous tumorigenesis with massive chromosome instability. This implies not only that mitotic function of Brca2 is crucial to the genomic instability, also that chromosome missegregation

itself can lead to spontaneous tumorigenesis,

Aneuploidy, a state of a cell harboring erroneous number of chromosomes, is a long-known hallmark of cancer. Although it is of controversy that aneuploidy alone can be tumorigenic, malfunctioning condition which results in chromosome missegregation is tumorigenic, proven by *BubR1* $^{K243R/+}$mouse model. As a model to mimic chromosome instability, I have adopted this mouse model to assess how chromosome instability can impede the development of pancreatic cancer. This experiment will also assess how Brca2 deficiency can result in chromosome instability, and further, development of pancreatic cancer as well.

I-2. Telomere maintenance by Brca2 and induction of Alternative lengthening of telomere by Brca2 depletion.

As the structure of chromosome revealed, one of mysterious question on the biology of nucleic acid was end-replication problem: How chromosome end is protected from shortening after rounds of replication? Telomere is a short, repetitive sequence located at the end of linear chromosome, which protects chromosome from being shorten and detected as a damage site.

Telomere functions as parameter to let cells stop after several rounds of replication so that it doesn't become malignant. In other words, cancer cells have adopted unique mechanism to escape from telomere shortening to become malignant. Majority of cancers are reported to harbor mutations to activate telomerase, a reverse transcriptase that can elongate telomere, so that it can elongate telomere enough to divide infinitely. However, strikingly, some cancers don't depend on reactivation of telomerase.

Scientists have defined Alternative Lengthening of Telomere (ALT) to address such cells that maintain telomere independently from telomerase activity. Yet detailed molecular mechanism is to be investigated, there have been suggested common characteristics of ALT cells: High heterogeneity in

telomere length, existence of APB, telomere sister chromatid exchange (T-SCE) and frequent recombination in telomere region. Unveiling molecular mechanism of ALT induction would grant a clue how normal cell inhibits such illegitimate recombination at telomere, and more, potential therapeutic strategy to treat ALT cancers

From our previous study, BRCA2 is suggested to be required for telomere homeostasis as its depletion resulted in telomere shortening, erosion and end fusions. Unlike to normal cells, *Brca2*$^{f11/f11}$ mouse embryonic fibroblast, which expresses Brca2 protein with in-frame deleted exon11, have exhibited telomere attrition. Further studies on Brca2 depletion to telomere maintenance have suggested that Brca2 depletion may lead to induction of Alternative lengthening of telomere. Recalling illegitimate elongation of telomere is linked to the development of cancer, how Brca2 depletion may contribute to develop cancer in the context of telomere is interesting question at a stake.

To scrutinize how Brca2 depletion may lead to development of pancreatic cancer with telomere aberrations, I have utilized *Brca2* $^{f11/f11}$ mice model that

are crossed with *mTerc*$^{-/-}$ mice model to rule out the activity of telomerase. As Brca2 mutation is highly associated with pancreatic cancer, pancreatic ductal epithelium was selected as a model to study telomere aberrations. Knowing that mouse has uniquely long telomere compared to the ones in human, this study deals with cells from higher generations inbred from *mTerc*$^{-/-}$.

I-3. 3-D organoid culture as a model to study *in vivo* organ biology

Although *in vivo* mouse models were introduced to better understand and faithfully model the human diseases, lack of appropriate experimental tool to study physiological aspects in *in vivo* organ have impeded our research. One of the obstacles was that it is impossible to maintain primary cells out of the body for longer periods without spontaneous transformation. As I have designed experiments to assess how chromosome instability can lead to tumorigenesis, conventional 2-D cell culture couldn't fulfill the experimental aim. Thus, 3-D organoid culture which mimic the proliferation of adult stem cell residing in a specific organ were selected to as an experimental platform.

Lgr5 has received tremendous interests currently as it is renowned as an adult stem cell marker in the majority of epithelia of mammalian tissue. Based on that Lgr5 is a Wnt signaling receptor, Hans Clevers group have invented three dimensional cell culture method which specifically proliferates Lgr5-positive adult stem cells. This method, called organoid culture method, is the most advanced tool that is able to reflect physiologies in mammalian organs. In pancreas, among three different types of cells are mainly composing the organ, acinar, islet and ductal cells, ductal epithelial cells were reportedly

known to be Lgr5 positive cells. Interestingly, more than 80% of pancreatic cancers are exhibiting the characteristics of epithelial cell, as called pancreatic ductal adenocarcinoma. Taken together, establishing robust method to maintain pancreatic ductal cells in three dimensions will be the best model to recapitulate both organ biology and pancreatic cancer biology.

Adding a note that organoid culture method is capable of maintaining cells without spontaneous transformation more than a couple of months, I thought it will be valuable to study the induction of ALT. Albeit short understanding on ALT induction, it is believed that critical shortening of telomere is required for the uptake of ALT. Several rounds of cell division *ex vivo* in organoid is assumed to mimic proliferation of ductal epithelial cells of adult human pancreas.

Furthermore, I have struggled to apply pancreatic organoid culture method to patient-derived fine needle biopsy samples. One of the characteristics of pancreatic cancer is that only 30~40% of patients are capable of surgery due to late diagnosis. To circumvent this limit, it is of importance to establish a methodology that can model tumors from every individual patient. Conceding

that endoscopic biopsy can be applied to any pancreatic cancer patient, we concluded that setting up organoid culture method from endoscopic biopsy could extend our research to all populations of pancreatic cancer. Pancreatic cancer organoid from patient biopsy is envisioned to be a great source of research and grounds to realize precision medicine.

II. Materials and methods

II-1. Maintenance of mouse models and genotyping

All mice were crossed and maintained in special pathogen-free facility and taken care of under the approval of IACUC of Seoul National University.

To confirm genotype of every pups, tiny piece of tail were dissected from pup and lysed with Tail Lysis Direct PCR reagent (VIAGEN) upon manufacturer's protocol.

Primer sequences for BubR1$^{K243R/+}$ allele were as follows: forward primer 5'-ACC CAG TCG TGC TGT TCT TT; reverse primer 5'-CAT CTC ACC AGC CCA GAA GA.

Primer sequences for K-Ras$^{G12D/+}$ allele were as follows: mutant allele primer 5'-CTA GCC ACC ATG GCT TGA GT ; wildtype allele primer 5'-ATG TCT TTC CCC AGC ACA GT; common allele primer 5'- TCC GAA TTC AGT GAC TAC AGA TG.

Primer sequences for Pdx1-CRE transgene were as follows: forward primer 5'-GCG GTC TGG CAG TAA AAA CTA TC ; reverse primer 5'- GTG AAA

CAG CAT TGC TGT CAC TT .

PCR protocol for every reaction was started with initial denaturation at 95℃ for 10 minutes, 35 cycles of 95℃ for 30 sec, 58℃ for 30sec and 72℃ for 30sec, and final extension at 72℃ for 10min at the end.

II-2. Mouse Embryonic Fibroblast culture

MEF were derived from embryonic day 13.5 (E13.5) embryos from respective genotypes of mice. After harvest, MEF cells were cryopreserved into vials and thawed at the moment of experiment. MEF cells were cultured under 10% CO2 37℃ chamber with DMEM/High glucose media (Lonza) supplemented with 16.6 v/v Fetal bovine serum (FBS), 100 units/ml of penicillin, 100ug/ml of streptomycin and 0.1mM of β-mercaptoethanol.

II-3. 3-D organoid culture from mouse and human biopsy

Mouse pancreas was dissected from mice body and quickly washed with cold Hank's Buffered Saline solution (HBSS). Then, it was lysed with 1mg/ml Collagenase P (Roche), 0.1mg/ml DNase 1 (Worthington) solution dissolved in HBSS at 37℃ shaking incubator until organ is dissociated properly. Lysed

organ was flowed through 100um Cell strainer (BD). Remnants of pancreatic lysate at the strainer were reconstituted with HBSS. With microscope, ductal structured cell were collected manually and embedded in Matrigel (Corning). After solidification of matrigel, liquid media supplemented with growth factors were added.

Human biopsy sample was lysed with 0.3mg/ml Collagenase P (Roche), 0.1mg/ml DNase 1 (Worthington), 100x Dispase (Corning) solution dissolved in HBSS at 37℃ shaking incubator until no visible clump is found. Lysed sample were washed with HBSS properly and embedded in Growth Factor reduced Matrigel (Corning). After solidification of matrigel, liquid media supplemented with growth factors were added.

To make liquid media for mouse organoid, Advanced DMEM/F12 (Gibco) was supplemented with 10mM HEPES (Gibco), 1x Glutamax (Gibco), 100units/ml Penicillin and 100ug/ml Streptomycin (Lonza), 1x B27 supplement (Gibco), 1.25mM N-acetylcysteine (Sigma), 10nM Gastrin (Tocris), 50ng/ml EGF (Peprotech), 1000ng/ml Rspondin1 (R&D) or 10% v/v Rspondin1 conditioned media (gift from Calvin Kuo's lab), 100ng/ml Noggin (Peprotech), 100ng/ml Fgf10 (R&D), 10mM Nicotinamid (Sigma).

To make liquid media for human organoid, 100ug/ml Wnt3a (R&D), 0.5uM Prostaglandin E2 (tocris), 1uM A83-01 (tocris) and 1x primocin (invivogen) was added to mouse organoid media.

Liquid media was changed with every 2~3 days to ensure proper supply of growth factors.

With every week, organoid embedded in matrigel was minced with rigorous pipetting and replated in fresh matrigel at the volume of 8~10 times for passaging.

II-4. 2-D and 3-D immunofluorescence assay

MEFs were fixed in 4% Paraformaldehyde solution 10minutes and washed with Phosphate Buffered Saline (PBS). Then 0.5% PBS-Triton X-100 were used for permeablization for 15min. 3% BSA in 0.1% PBS-triton X-100 was used for blocking solution. 1:1000 of FITC-Tubulin (sigma), 1:2000 of anti-CREST (Cortex biochem) was incubated at 4℃ overnight, then 1:200 of Alexa 568-goat anti Human antibody was incubated at RT 1hr. Mounting media with DAPI (Vectashield) was used for mounting.

Organoid growing in matrigel was fixed in 4% paraformaldehyde for

30min and washed with PBS. Then 0.2% PBS-Triton X-100 were used for permeablization for 30min. 5% normal Goat serum with 3% BSA in 0.1% PBS-Triton X-100 solution was used for blocking reagent.

II-5. Chromosome orientation Fluorescence *in situ* hybridization (CO-FISH)

Cells were treated with BrdU/BrdC in final concentration of 7.5uM/2.5uM respectively for 10 hours. Then, colcemid was added in final concentration of 0.1ug/ml for 6 hours. Dispase was added to fully digest matrigel and 0.25x Trypsin was further treated to make sure organoid are broken down into single cells. After that, single cells were incubated in 0.075M KCl hypotonic solution for 20min. Finally, methanol:acetic acid 3:1 solution was added for fixative and stored in -20℃

Chromosome spread was done in regular protocol by dropping fixed cells into humidified slide glasses and enzymatic digestion with Exonuclease III was followed. TelC-FAM and TelG-Cy3 probe was used to label two different newly synthesized telomere strand, respectively.

III. Results

III-1. Generation and cooperative tumorigenecity of *BubR1$^{K243R/+}$; K-Ras$^{G12D/+}$; Pdx1-CRE* mouse

To assess how chromosome instability derived from chromosome missegregation can affect the development of pancreatic cancer, I crossed BubR1 acetylation deficient mouse (*BubR1$^{K243R/+}$*) with pancreatic cancer mouse model (*K-Ras$^{G12D/+}$; Pdx1-CRE)* .

K-Ras$^{G12D/+}$; Pdx1-CRE is well-known pancreatic mouse model which can mimic the development of human pancreatic cancer. K-Ras is a oncogene which acts as upstream regulator of EGF Receptor signaling. This protein can transmit proliferative signals to downstream pathways such as MAPK pathway, PI3K/Akt pathway, Ral GDS pathway and so on only when it is GTP bound form. By its innate GTPase activity, signals can be transiently activated and turned down in a short time. However, its oncogenic mutation – for instances G12D or G13D form – possess lessen GTPase activity and thus can stimulate proliferative signals in a great extent compared to the wildtype form.

G12D mutation of K-Ras is one of frequently found mutations in pancreatic

cancer, to the extent of 40% of human pancreatic cancer, and thought to be a initial driver of this disease. To specifically target the expression of the point mutation, LSL allele (LoxP-Stop-LoxP) is flanked in front of the mutation allele (LSL-KRas^{G12D}). By crossing this mouse with *Pdx1-Cre* model which expresses CRE recombinase in response to pancreatic specific promoter, Pdx1, human pancreatic cancer can be modeled.

It is surprising that pancreatic cancer mouse harboring BubR1 acetylation deficiency were dead in a short life span as 7 weeks in average. This shortened life span implies that chromosome instability could expedite the development of pancreatic cancer seriously. Thinking that BubR1 acetylation deficiency reflects deficient mitotic function of Brca2, it is assumed Brca2 deficiency can cooperate in developing pancreatic cancer with K-Ras mutation as well.

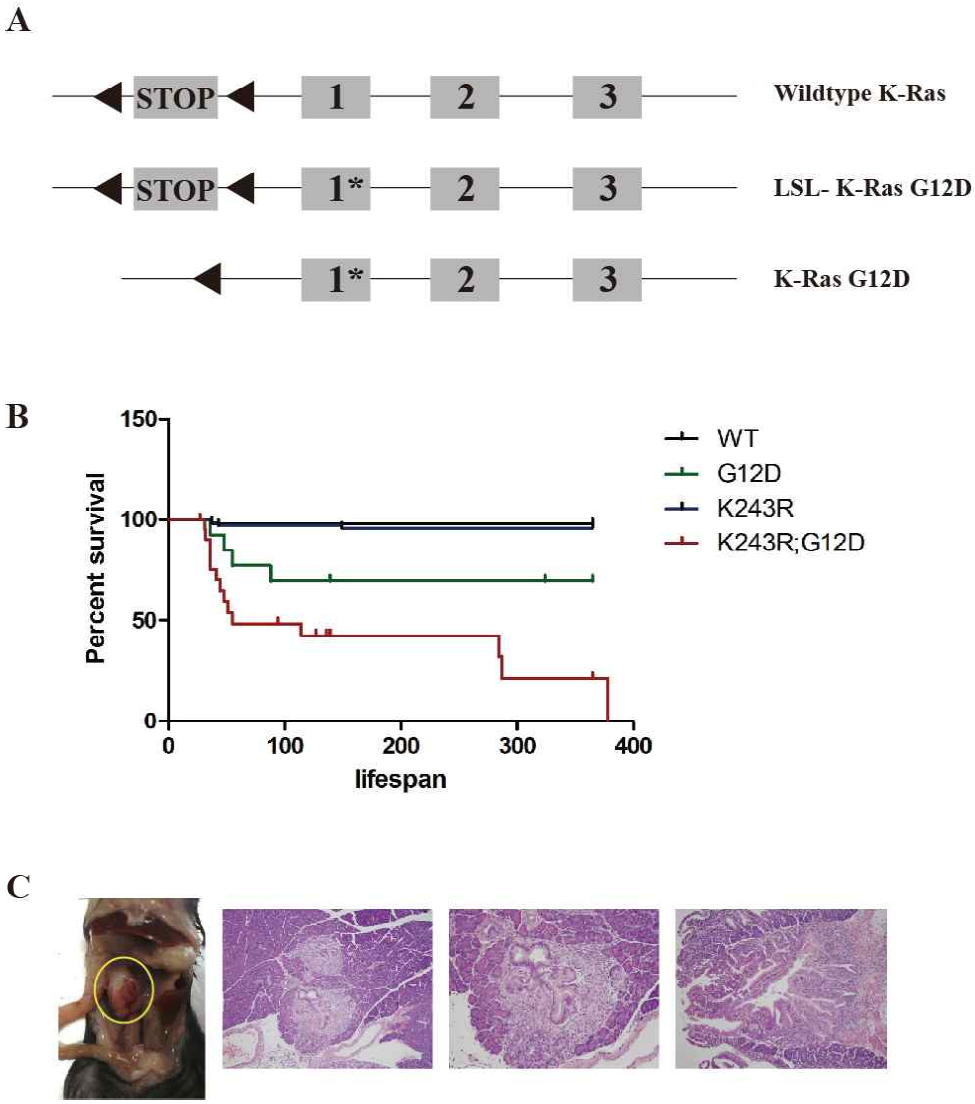
Figure 1. Generation of *BubR1*$^{K243R/+}$; *K-Ras*$^{G12D/+}$; *Pdx1-CRE* mouse and its phenotype

(A) Graphical illustration of wildtype Kras and LSL-Kras^{G12D} allele

(B) Life span of mice with respective genotype. Red line states clearly shortened life span of *BubR1*$^{K243R/+}$; *K-Ras*$^{G12D/+}$; *Pdx1-CRE* mouse model. (n=21)

(C) Histological feature of *BubR1*$^{K243R/+}$; *K-Ras*$^{G12D/+}$; *Pdx1-CRE* mice with 6.7 weeks old.

Figure 1. Generation of *BubR1$^{K243R/+}$; K-Ras$^{G12D/+}$; Pdx1-CRE* mouse and its phenotype



III-2. Synergistic mitotic aberration in *BubR1$^{K243R/+}$; K-Ras$^{G12D/+}$* MEFs

As BubR1 acetylation is a specific alteration during mitosis, I hypothesized that synergistic tumorigenesis of BubR1 and KRas is based on cooperative aberration in mitotic progression. To assess mitotic progression, embryonic fibroblast (MEF) cells were harvested with respective genotype and Immunofluorescence assay was conducted. To activate *K-Ras$^{G12D/+}$* allele in the context of MEF, adeno-CRE was administrated instread of endogenous CRE recombinase.

As a meaning of assessing proper chromosome alignment, MG132 was administrated to MEFs with 10uM concentration in 2hrs. This chemical is known to inhibit Ubiquitin mediated proteolysis, thus arrests cells in metaphase. While normal cells with MG132 exhibits higher rate of metaphase aligned chromosomes, cells with erroneous mitotic progression is expected to exhibit lesser rate of metaphase chromosomes due to impaired KT-MT attachments.

From Immunofluorescence assays, MEF expressing oncogenic K-Ras showed decreased ratio of metaphase arrest compared to Wildtype cells.

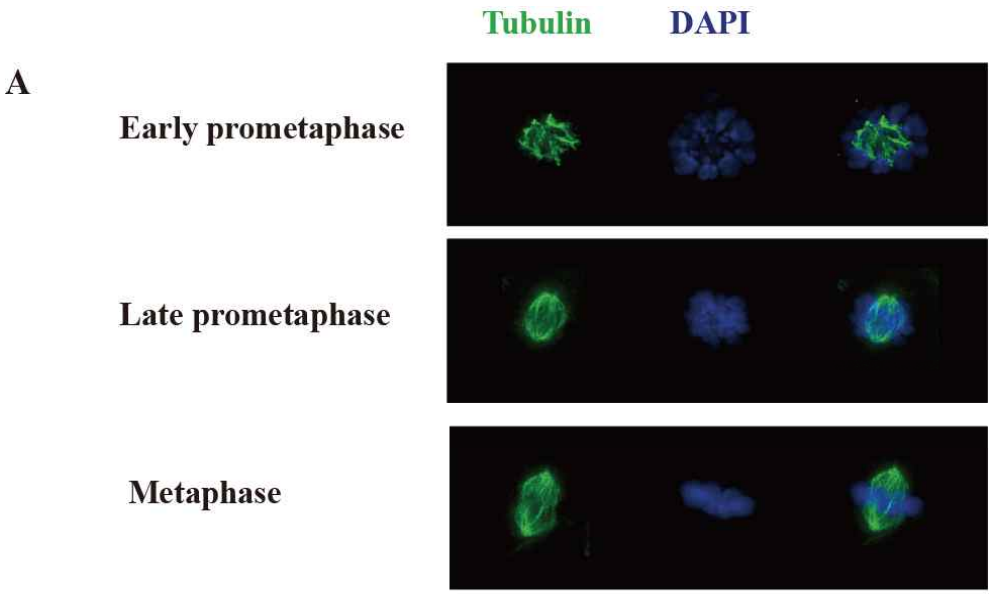
Furthermore, MEF with BubR1 acetylation deficiency showed synergistic aberrations in mitotic progression, demonstrated by decreased metaphase ratio.

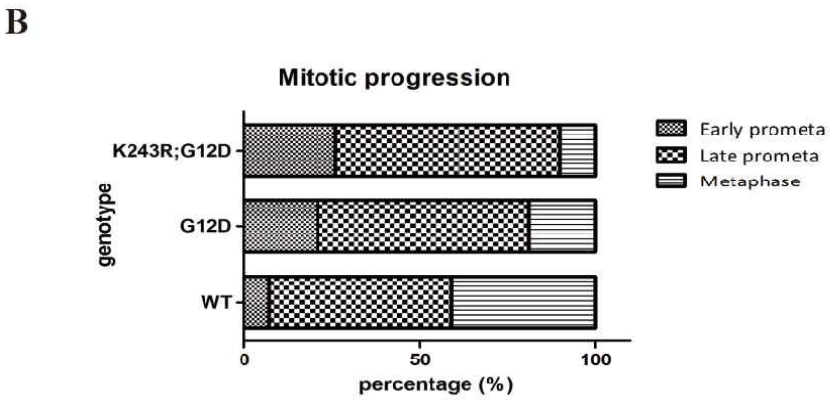
Figure 2. Mitotic progression in *BubR1*$^{K243R/+}$; *K-Ras*$^{G12D/+}$ MEFs

(A) Representative images of Early, late prometaphase and normal metaphase aligned MEF

(B) Metaphase alignment of MG132 arrested MEF were synergistically decreased with BubR1 acetylation deficiency and oncogenic K-Ras mutation.

Figure 2. Mitotic progression in *BubR1*$^{K243R/+}$; *K-Ras*$^{G12D/+}$ MEFs





III-3. Establishment of mouse pancreatic organoid

In order to circumvent limitations of conventional cell culture method that exerts spontaneous tumorigenesis and to faithfully model pancreatic cancer development, 3-D pancreatic organoid culture is established.

To confirm that growing cells are derived from ductal epithelium, not from acinar or islet cells residing in the pancreas, immunofluorescence assay was performed with cell type specific markers. Positivity to pan-cytokeratin, Sox9 and DBA-lectin confirms that origin of spherically growing cells are from ductal epithelium.

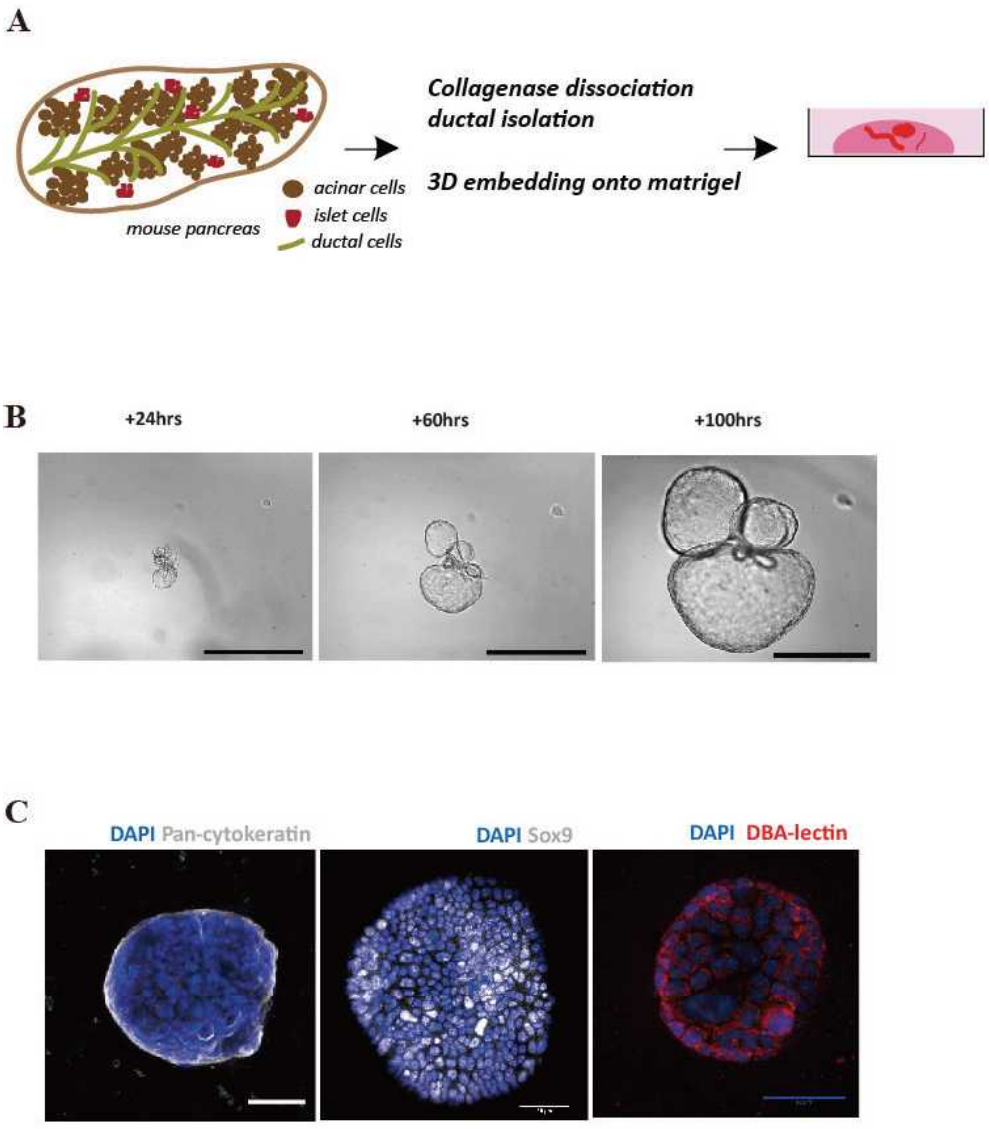
Figure 3. Establishment of mouse pancreatic organoid

(A) Schematic illustration of mouse pancreatic organoid culture. Whole organ from the body is digested with Collagenase and ductal cells are selectively embedded in matrigel

(B) Representative growth images of mouse pancreatic ductal organoid. Scale bar is 500um.

(C) 3-D immunofluorescence imaging of mouse pancreatic organoid. Pan-cytokeratin, Sox9 and DBA-lectin is known to be a specific marker represent ductal cell population in pancreas Scale bar is 50um.

Figure 3. Establishment of mouse pancreatic organoid



III-4. Depletion of *Brca2* impedes the growth of pancreatic organoid

Brca2 deficiency is highly associated with the emergence of pancreatic cancer, yet detailed molecular basis is still hidden. To assess the impact of Brca2 deficiency in the context of pancreas, mouse pancreatic organoid of four genotype was generated: $Brca2^{+/+}$; $mTerc^{+/+}$, $Brca2^{F11/F11}$; $mTerc^{+/+}$, $Brca2^{+/+}$; $mTerc^{-/-}$, $Brca2^{F11/F11}$; $mTerc^{-/-}$.

It is noteworthy that $mTerc^{-/-}$ mice is a direct descendant of $mTerc^{+/-}$,named Generation 1. Mouse has comparably long telomere to the ones of human and thus, phenotype is assumed to be influenced by how *mTerc* was descended through the generation.

While there has not been observed distinct difference with $mTerc^{+/+}$ (G0) and $mTerc^{-/-}$ (G1), Brca2 depletion produced clear retardation in the growth of pancreatic ductal organoid. It will be further studied what is the basis of this growth retardation.

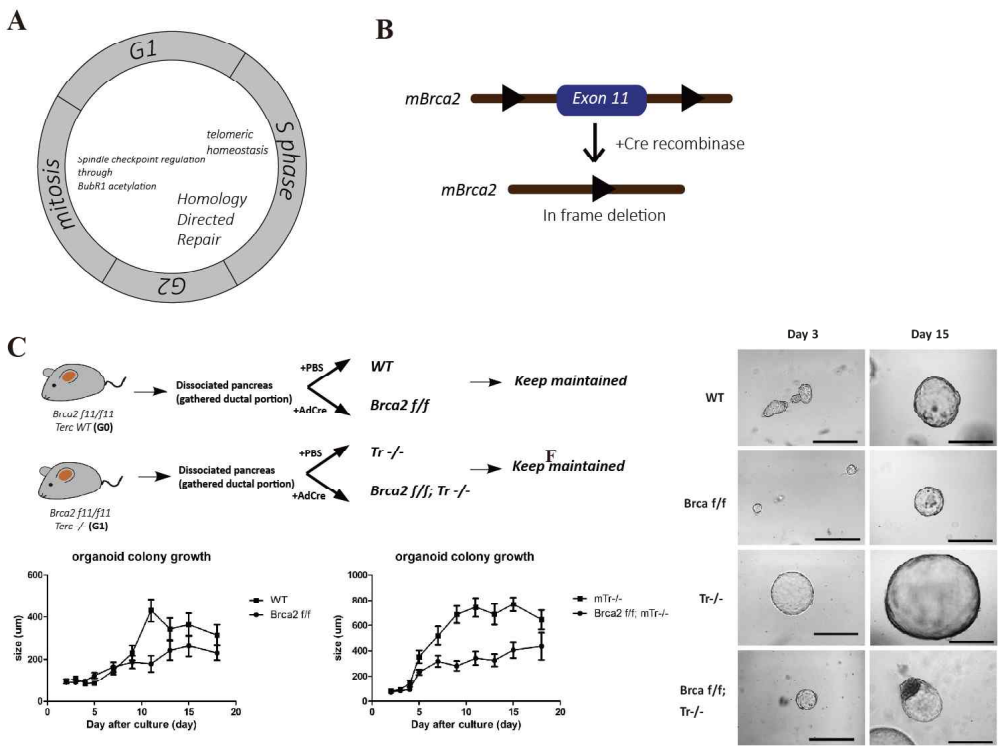
Figure 4. Growth pattern of *Brca2*$^{fl1/fl1}$; *mTerc*$^{-/-}$ pancreatic organoid

(A) Schematic illustration of function of BRCA2 throughout cell cycle.

(B) *Brca2*$^{fl1/fl1}$ allele is designed to delete Exon 11 in frame in response to CRE recombinase.

(C) *mTerc*$^{+/+}$ and *mTerc*$^{-/-}$ mice were treated with either PBS or Adeno-CRE to deplete BRCA2. Regardless of *mTerc* genotype, Brca2 depletion resulted in growth retardation of pancreatic ductal organoid

Figure 4. Growth pattern of *Brca2*$^{f11/f11}$; *mTerc*$^{-/-}$ pancreatic organoid



III-5. Depletion of Brca2 can diminish telomere integrity in pancreatic organoid

To further analyze the impact of Brca2 depletion to telomere integrity, I conducted Chromosome-Orientation Fluorescence *in situ* Hybridization (CO-FISH). This experiment shows how newly-synthesized telomere is arranged in the chromosome. Normal chromosome should express clear Red (Tel-C) and Green (Tel-G) foci in each ends of chromosomes. However when telomere is fragile, due to replication error in S-phase, foci shapes with multiple dots or lined manners instead of circular dot. Furthermore, this experiment enables to quantify the length of telomere with regard to the intensity of fluorescence intensity.

In this experiement, organoid with Generation 2 *mTerc*$^{-/-}$ was used to emphasize the impact of telomere shortening. This sample was harvested after 10 days of Brca2 depletion by administration of 4-OHT (1uM)

Figure 5. Analysis of telomere integrity from *Brca2*$^{f11/f11}$; *mTerc*$^{-/-}$ pancreatic organoid

(A) Representative CO-FISH image from wildtype pancreatic organoid

(B) Representative CO-FISH image from *Brca2*$^{f11/f11}$ pancreatic organoid

(C) Representative telomere foci showing fragile telomere

(D) Telomere length assessment using the intensity of telomere fluorescence foci. While Brca2 depletion resulted in the decrease of average length of telomere in *mTerc* Wildtype background, Brca2 depletion with

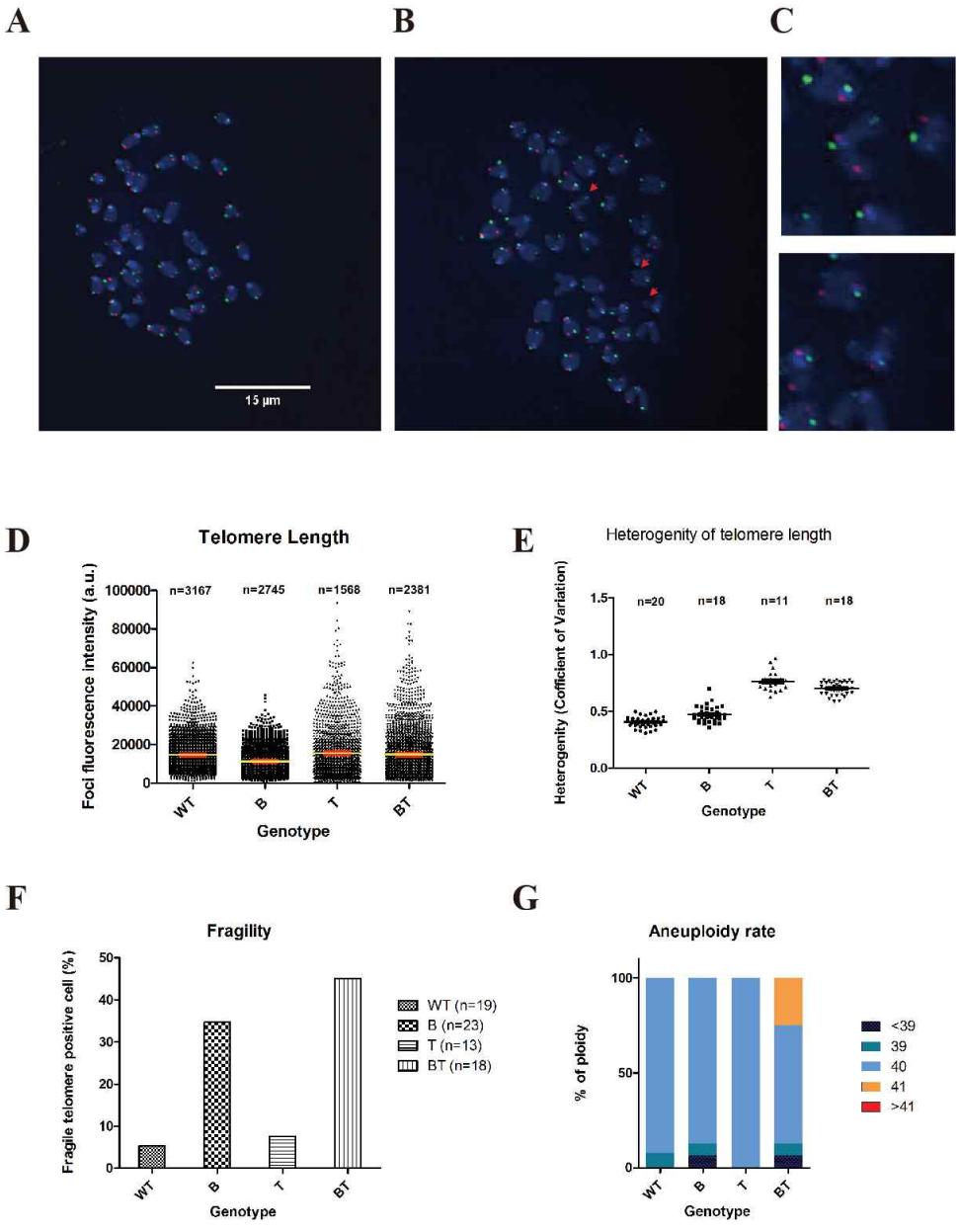
mTerc **Knockout didn't result in same outcome. This implies that Brca2 depletion in the absence of telomerase activity could lead to the illegitimate elongation of telomere length.**

(E) Telomere length heterogeneity calculated from the coefficient of variation (mean intensity of fluorescence divided by standard deviation of intensity).

(F) Telomere fragility was increased in the absence of Brca2.

(G) Aneuploidy rate was increased only in the absence of both Brca2 and mTerc

Figure 5. Analysis of telomere integrity from *Brca2*$^{fl1/fl1}$; *mTerc*$^{-/-}$ pancreatic organoid



III-6. Establishment of patient-derived pancreatic organoid

It can't be emphasized enough that experimental tool should represent human disease faithfully. While mouse model is valuable with clear genomic background and versatility to manipulate genes, it also carries limitation in modeling human disease.

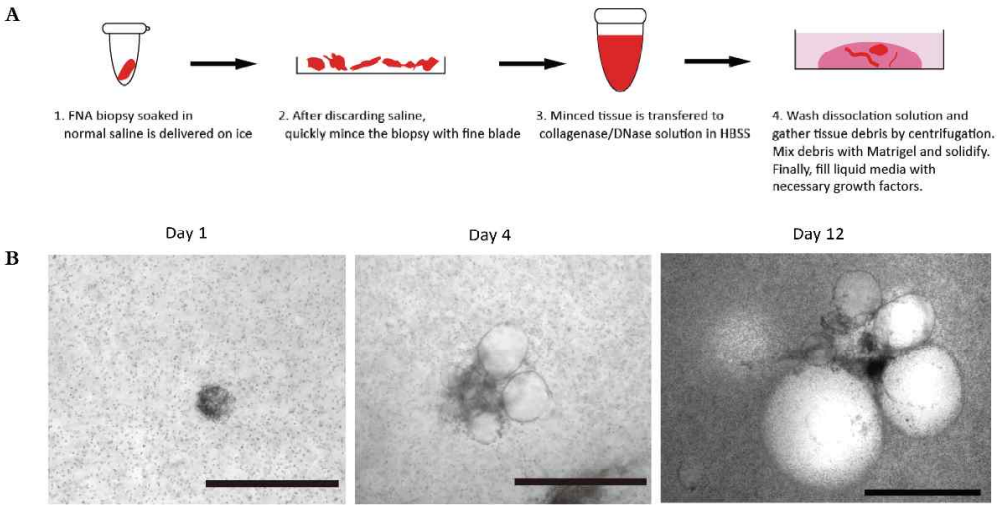
In order to study human pancreatic cancer *ex vivo,* we adopted pancreatic organoid culture method to patient derived biopsy samples. Although this biopsy is tiny in the amount of cells, it bears numerous advantages in the research. This biopsy sample can be acquired from nearly all patients, more beneficial than surgical samples, and can be acquired as soon as patient gets diagnosed.

Figure 6. Establishment of patient-derived pancreatic organoid

(A) Schematic illustration of organoid culture from Fine-needle biopsy (FNB) sample from pancreatic cancer patient

(B) Representative images of human pancreatic cancer organoid growth

Figure 6. Establishment of patient-derived pancreatic organoid



IV. Discussion

Deciphering molecular mechanism of the disease is crucial since it not only offers a clue to interpret the illness, also suggests possible therapeutic strategy. Pancreatic cancer is the most lethal malignancy with extremely low 5-year survival rate and it is due to limited therapeutic choices. Thus, it is of great importance to unveil the molecular basis of this disease.

I have started to scrutinize molecular basis of chromosome instability with Brca2 deficiency. To do so, multiple experiments including mouse phenotype and karyotypic analysis from pancreatic organoid implies that Brca2 deficiency can aggravate the development of pancreatic cancer with incresing extent of chromosome instability.

Aneuploidy is a hallmark of cancer and pancreatic cancer is not the exception as well. From previous studies, it has been noted that genomic instability is frequently found in human pancreatic cancer genome. Massive genomic analysis and researches on pancreatic cancer related mutations concluded that only Brca2 is the gene with correlated with massive genomic instability. Conceding the function of Brca2 throughout the cell cycle, it is logical to start assessing how chromosome instability can be accumulated in

pancreatic organoid in association with Brca2 deficiency.

To dissect molecular pathways related to the function of Brca2, I approached with two different mouse models. *BubR1*$^{K243R/+}$ model will represent how mitotic error can contribute, while *Brca2*$^{fl1/fl1}$*; mTerc*$^{-/-}$ model will demonstrate how telomere instability can contribute to the development of pancreatic cancer, respectively.

Further analysis to scrutinize patterns of chromosome instability in *BubR1*$^{K243R/+}$ pancreatic organoid will be performed. In addition, organoid culture from mouse pancreatic cancer will be conducted to reinforce overall mouse phenotype.

ALT phenotype can be found some human cancers including mesenchymal origin cancers. However, its molecular mechanism is far from being understood. Unlike other studies linking common characteristics of ALT with cancer cells, our study can define the induction of ALT in a molecular level, depletion of Brca2.

To induce ALT in *in vivo* pancreas, it bears tremendous obstacles that we should cross multiple transgenes. Furthermore, as Brca2 depletion is critical starting from the development of embryo, targeting Brca2 in animal model is

problematic. To circumvent such obstacles, I utilized pancreatic organoid model, which represent *ex vivo* pancreas. It will be interesting to experiment how organoid can be tumorigenic in the absence of both Brca2 and Terc. This result can decide whether we can create ALT dependent cancers with defined molecular pathway, which has never done before.

So far, it has not been well stated about telomere integrity in pancreatic cancer. However, there exist no doubts on that telomere elongation is necessary in developing cancers. Recent studies have only focused on the mutation pattern of telomerase and concluded that ALT is hardly found in pancreatic ductal adenocarcinoma. However, to define ALT phenotype, chromosome analysis should be performed and our approach using patient-derived organoid will be utilized to assess existence of ALT in pancreatic ductal adenocarcinoma.

V. References

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국문 초록

오랜 기간의 연구에도 불구하고 췌장암은 여전히 완치율이 극히 낮은 질병으로 알려져 있다. 이를 극복하기 위해선 췌장암의 발병기작을 이해하고 분자기작에 근거한 치료법을 제시하는 것이 시급한 실정이다. *Kras, Brca2*와 같은 췌장암 발병 연관유전자가 규명되었음에도 불구하고, 이들의 돌연변이에 근거한 발병기작은 연구가 부족한 상황이다. *Brca2* 결손에 따른 췌장암 발병기작을 세포, 분자적 수준에서 이해하기 위해 본 연구는 *Brca2* 결손 모사 마우스 모델과 3차원 췌장 오거노이드 배양 방법을 구축하고 이를 이용한 분석결과를 제시하고자 한다.

본 연구는 인간 췌장암 마우스 모델인 $K\text{-}Ras^{G12D/+}$; *Pdx1-CRE* 를 도입하고 *Brca2* 결손에 따른 염색체 비분리를 모사할 수 있는 $BubR1^{K243R/+}$ 마우스 모델을 교배해 염색체 비분리가 췌장암 발병에 미치는 영향을 살펴보았다. 또한 *Brca2 f11/f11* 모델을 이용해 *Brca2* 결손에 따른 텔로미어 이상과 염색체 불안정성을 췌장 오거노이드 세포에서 확인하였다. 3차원 췌장 오거노이드는 기존의 세포배양법과 달리 충실히 모사한 췌장내 미세환경을 기반으로 하여 췌장 내에 존재하는 췌관상피세포를 선택적으로 배양하는 방법

으로 알려져 있다. 줄기세포능과 관련된 유용성과 더불어서 조직적으로 췌관상피세포의 특징을 보이는 대부분의 췌장암을 모사하고 연구함에 있어서도 최적의 모델로 알려져 있다.

또한 본 연구를 통해 인간 췌장암을 충실히 모사하여 발병기작을 이해하고 맞춤형 치료구현에 핵심적인 인간 췌장암 유래 3차원 오거노이드 배양법을 확립하였다. 기존의 수술시료를 이용한 방법이 아닌 초음파내시경 유래 미세조직을 이용한 본 실험방법은 수술이 불가능한 췌장암 환자에 대해서도 적용가능하고 진단 직후 배양을 시작할 수 있어 폭넓은 연구가 가능한 방법으로 생각된다.