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Use of CRISPR/Cas9 in Canine Liver Organoids to Model the Wild-Type P-glycoprotein Mutation

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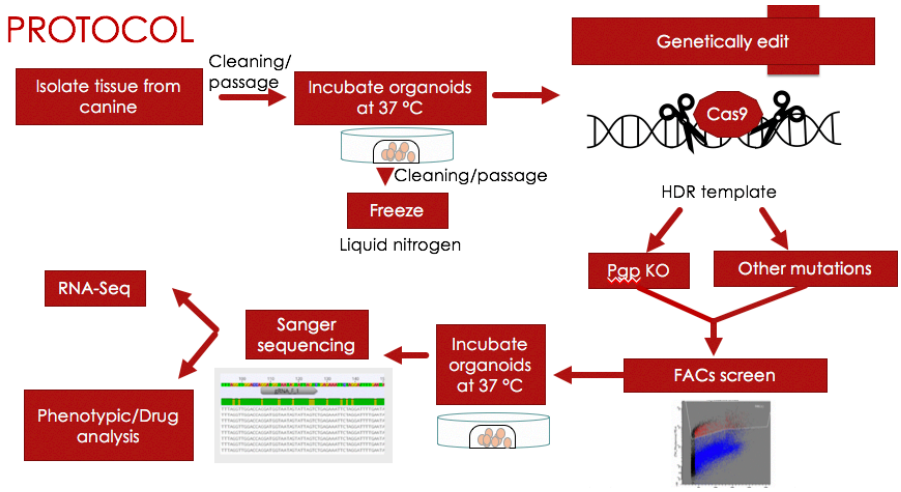
Abstract

The use of CRISPR/Cas is a boom in the news regarding curing genetic diseases. It seems science fiction that someone can cut a mutation and paste the same gene without errors. Many animal studies are using this mechanism. In this case, we explore the use of CRISPR/Cas in organoids extracted from dogs. We present the use of Homology Directed Repair (HDR) to create a WT four base deletion, a PGP mutation. Also, humans have the P-gp protein transcribed by the MDR-1 gene. Its mutations affect P-gp activity. Editing this mutation in dogs could better understand how CRISPR/Cas could help eliminate mutations that affect drug-resistance development and possibly apply them in future human phenotypic and drug analysis.

Introduction

Emmanuelle Charpentier and Jennifer A. Doudna won the 2020 Nobel prize in chemistry for finding a new gene technology: CRISPR/Cas9. Bacteria and archaea have this gene-editing tool as an immunogenic adaptive system. In this way, bacteria and archaea introduce a part of the virus's genomic material, the infectious agent, into its genome using this system (Chen et al., 2016). Different applications use CRISPR/Cas9 as the DNA target-binding sequence can be manipulated. It has become a versatile and cheaper option for genome editing (Andersson-Rolf et al., 2016; Wierson et al., n.d.). Organoids could be used as a 3D in-vitro model with valuable properties that can give us comparable results to the model organism or in vivo (Gaebler et al., 2020). In the study of pharmacological components, the use of this tool has increased. P-glycoprotein is an efflux transporter involved in the absorption of drugs (Pawłowski et al., 2013). More specifically, it has a function in cell detoxification and resistance to antimicrobial and chemotherapeutic drugs. The deletion of the P-gp gene can lead to an alteration of drug-drug interactions in dogs and humans. The bioavailability of the drugs can decrease when alteration of P-gp happens, affecting the drug transport pathway. The multi-drug resistance gene (ABCB1, Mdr-1) codes for P-gp production (Hodges, 2011; Q. Xia et al., 2005). Therefore, P-gp inhibition can be of tremendous importance in pharmaceuticals, specifically drug candidates' clinical evaluation. Herding dogs can have this P-gp mutation (Mealey & Fidel, 2015; Sajid et al., 2018). We took a healthy liver organoid culture and apply the methodology found here to convert the healthy organoids into diseased organoids with the exact P-gp mutation. In this case, we attempted to use two gRNA to cleave approximately 300 bp of the canine liver organoid's DNA via Homology direct repair (HDR), leaving blunt ends which will be paired up with the HDR template (KO Pgp) when introduced with the RNP's complex into the organoid. HDR does not

have the best efficiency, but in a previous study, HDR increased efficiency when used after on gRNA and Cas9 complex (Eun et al., 2019; Zhang et al., 2017).



Basic streamline of the study

Materials and Methods

gRNA

The gRNAs were designed using Genescript and CRISPOR tools (Concordet & Haeussler, 2018). Four gRNAs were ordered from IDT, as seen in Table 1. Additionally, Label-It or GFP plasmid. The cutting percentages were measured using TIDE analysis. The two most efficient gRNAs are still being determined, and the HDR template modeled for Dog 00 will be ordered after that. The selected gRNAs will determine the size in bp of The HDR template with 4bp WT deletion. The HDR template will contain a specific number of base pairs homology arms that extended past the two gRNA cut sites. Again, this will depend on the gRNA selected.

The gRNA was made based on previous gRNA study steps (Jost et al., 2020).

Labeled gRNA was made by mixing 1. Ul of cRNA with 1. Ul of tracr RNA into a single PCR tube. It was incubated for 5 minutes at 95°C. It was put on a bench to return to room temperature slowly. Then 400 pmol labeled gRNA was added to 400 pmol Cas9.

PCR Primers	5' Sequence 3'	
MDR1_P4_F1	TGTCCCATTCCTCTCATCAAAAC	
MDR1_P4_R1	TTTACCTCTTCCTGAAACTTCCT	
MDR1_P5_F1	CCCATTCCTCTCATCAAAACTCA	
MDR1_P5_R1	TCCTTTACCTCTTCCTGAAACTT	
MDR1_P6_F1	TCCCAGAATGTCCCATTCCT	
MDR1_P6_R1	CAAGGTCTAGATAAGGTGGTTCC	
gRNAs	5' Sequence 3'	PAM
PGP_F_1	GACTAATACTATTACCATCC	TGG
PGP_F_2	GCTTGATAGGTTGTATATGT	TGG
PGP_R_1	GTCTGAGCTACAGTTAATAT	TGG
PGP_R_2	ATTTGTGGCACTGATCTTAG	AGG

Table 1: The primer and gRNA sequences used.

Organoid Isolation and Culture

Liver organoids were extracted from three years old canines. After Isolation, organoids were passage into 24 wells to try the four guides and chose the best ones. 6 wells of organoids were used per guide. When guides are chosen, only six wells will be needed for transfection if the well looks well-populated. If there are not as many organoids on each well, 8 to 9 wells can be used (Fig. 1A and 1B).

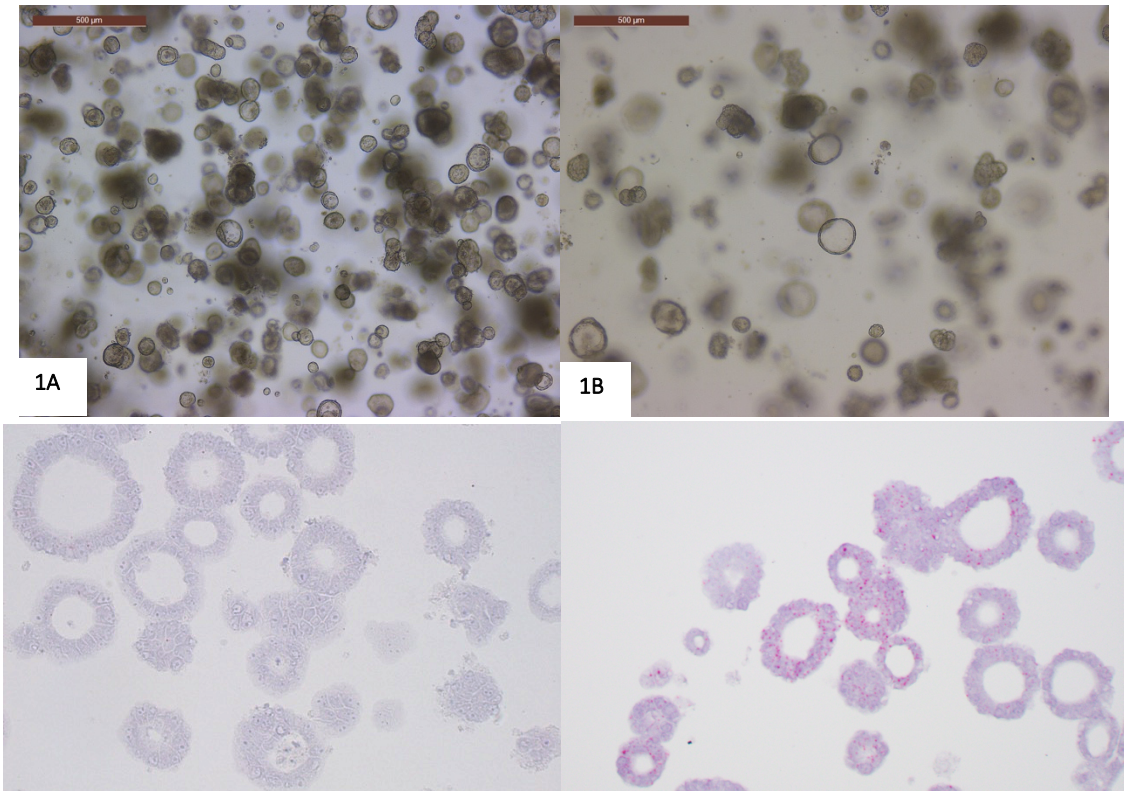


Fig. 1A, Very populated liver organoids on a well at 5x after passaging and growing for two days used for first trial (only 6 wells used). Fig. 1B, Liver organoids after two days of growing post-passaging (9 wells used). Fig. 1C and 1D, Liver organoids with RNA scope staining 40X.

Plasmid prep

Plasmids (GFP+) were used to verify the electroporation step. The heat bath was set to 42°C. Then 0.6 g of LB Invitrogen Broth Base was added to 30 mL of distilled water in a flask. The flask was covered with aluminum foil and autoclaved. Right after, it was swirled to mix for 3 to 5 minutes or until the powder disappeared. Again, it was autoclaved for 20 minutes at 121°C. It was allowed to cool at room temperature.

Meanwhile, thaw one vial of competent cells (One Shot™ TOP10 Chemically Competent E. coli) from -80°C on ice and warm SOC medium from the fridge the heat bath. 1 to 5 µL of plasmid was pipetted into a vial. Then, the vial was incubated on ice for 30 minutes. Subsequently, the vial was placed in the 42°C water bath for 30 seconds. 250 µL of SOC medium was added to the vial. The mixture was shaken at 37°C for one hour at 225 rpm. Kanamycin was added to LB broth. 1 µL of antibiotic per mL of broth was used. Transformed cells were added into the LB broth and incubated overnight at 37°C at 250 rpm in a 50 mL vial. The next day, 50 mL was collected from the shaker. Qiagen EndoFree Plasmid Mega Kit was followed to get transfection grade plasmid until the final step, where the pellet was reconstituted and aliquoted into ten tubes. Also, the concentration was checked using Nanodrop. The aliquots were stored at -20°C.

Labeled gRNA

Labeled gRNA was made by mixing 1.5 µL of cRNA with 1.5 µL of tracr RNA into a single PCR tube. It was incubated for 5 minutes at 95°C. After, it was put on a bench to return to room temperature slowly. About 10 to 13 minutes, 4.92 µL of Cas9 was added to labeled gRNA. The mixture was incubated for 10 minutes at room temperature. 150 pmol/L of concentration for tracrRNA and cRNA 300 was used. A 2:1 ratio was measured for the concentration of Cas9 (300 pmol/L).

Electroporation

Nine wells of organoids were chosen instead of 6 wells as the wells had fewer organoids than the average. Media (CMFG+, Rocki, and Gski) was removed from each well. 500 µL of Cold DMEM F12 was added per well and pipette up and down to de-attach the Matrigel from the plate. All the content was transferred to a 15 mL tube centrifuged at 700g at 4°C for 5 min to form a pellet. The supernatant was removed down to 500 µL. 500 µL of Tryple Express was added and pipette 15 times to break down the organoids into cells. Then, it was placed in a hot water bath for 10 minutes. It was vortex two times for 2 seconds at the halfway point, and at the end of the time, the mixture was pipetted up and down ten times. Immediately after, cold 6 mL of DMEM was added to stop the chemical dissociation reaction of Tryple Express. Again, the mixture with organoids was centrifuged at 700g at 4°C for 5 min, and the supernatant was discarded. Even though in another study, PBS was used to resuspend (Fujii et al., 2015; Potter & Heller, 2018), in this case, pellet (organoids) was resuspended in 90 µL of BTX (electroporation solution) in a 1.5 mL tube. The gRNA mixtures were added as well and mixed gently. All the mixture was added into a previously chilled cuvette (10 minutes in ice prior).

The cuvette was checked for no bubbles and wiped dry. It is placed on the Nepagene 21 electroporator with the settings from Table 2 (Artegiani et al., 2019; Merenda et al., 2017). It is essential to check the impotence before doing the electroporation. A good impotence value is between 30 to 40 Ω. After electroporation, 400 µL of room temperature media was added to the cuvette and transferred into a 1.5 mL tube. It was incubated for 30 minutes at room temperature. Then, it was centrifuged at 700g for 5 min. There was a pellet formed, so the supernatant was removed. Matrigel was added to resuspend the pellet, and 30 µL of the new mixture (Matrigel and organoids) was plated in each well. Once finished incubation for 15 minutes at 37°C and 5% CO_2, 500 µL of warm Media (CMFG+, Rocki, and Gski) was added in each well.

	Poring Pulse	Transfer Pulse
Voltage (V)	175	20
Pulse Length (ms)	5.0	50.0
Number of Pulses	2	5
Pulse Interval (ms)	50.0	50.0
Decay Rate (%)	10	40
Cuvette (mm)	2	2

Table 2: The electroporation settings used on the Nepagene 21 electroporator (Artegiani et al., 2019; Merenda et al., 2017)

DNA extraction

To ensure the gRNA has cut on the right places and choose the correct one for reverse and forward position, DNA extraction was done two days after electroporation from one of the trials.

Mutation Screening and FACS sorting

Wells were assessed for successful knockin through a ZEISS SteREO fluorescent endoscope. The organoids were harvested 48 hours after transfection by adding 500 µl of DMEM and transferred into a 15 mL tube. It was centrifuged at 700 g at 4 ° C for 5 min to form a pellet. The supernatant was removed to the 500 µl mark, and 500 µl of TrypLe Express was added. The pellet was resuspended and mechanically dissociated by pipetting up and down forcefully 10 to 20 times. Then the mixture was put in the hot water bath for 12 minutes at 37°C. The mixture vigorously mechanically dissociated again after removing it from the water bath. The solution was then filtered through a 40 µm cell filter to separate the remaining pieces of organoids. This cell solution was taken directly to the FACS sorting facility at Iowa State University and processed on a FACSAria III cell sorter.

In vitro culture of single cells without sorting

The best environment to grow the single cell into an organoid after FACS sorting was uncertain. Therefore, a PCR plate was used with different contents in each well (Table 3) based on

previous research data on cell sorting (Fujimichi et al., 2019). One well of electroporated organoids was dissociated into single cells and filtered with a 40 µm cell filter. Then, they were added to each PCR plate well.

	1	2	3	4	5	6	7	8	9	10	11
A	1% M	20% M	37% M	50% M		1% M	20% M	37% M	50% M		1% M
B	1% M	20% M	37% M	50% M		1% M	20% M	37% M	50% M		1% M
C	1% M	20% M	37% M	50% M		1% M	20% M	37% M	50% M		1% M
D	1% M	20% M	37% M	50% M		1% M	20% M	37% M	50% M		1% M
E	1% M	20% M	37% M	50% M							
F	1% M	20% M	37% M	50% M							
G	1% M	20% M	37% M	50% M							
H	1% M	20% M	37% M	50% M							

M = matrigel
CMFG+, Rocki, GSKi
DMEM
DMEM with % matrigel and 100 µl CMFG+, Rocki, GSKi on top
DMEM with % matrigel and 20 µl CMFG+, Rocki, GSKi on top

Table 3: The 96-well experiment with different concentrations of Matrigel, Media (CMFG+, Rocki, GSKi), and DMEM/F12 (12)

Results

Electroporation with Bio-Rad

Electroporation is possible in liver organoids. Later, rhodamine labeling of gRNAs in ribonucleoprotein was used in another trial of electroporation in liver organoids. There was an extra fluorescent label/dye in the media, making it difficult to tell which organoids are transfected. Therefore, we proceed with FACs sorting.

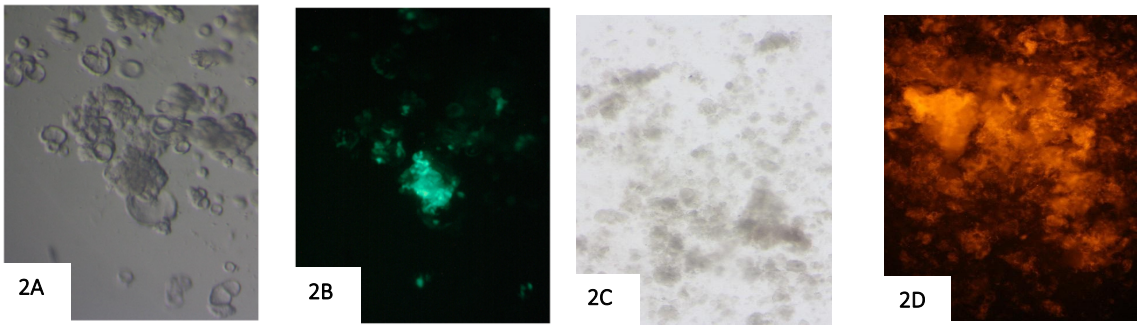


Fig. 2A Electroporated liver organoids GFP+ in a standard microscope. Fig. 2B, The same electroporated liver organoids GFP+, seen in a fluorescent scope. Fig. 2C Electroporated liver organoids with rhodamine labeling gRNAs

in a standard microscope. Fig. 2D The same electroporated liver organoids with rhodamine labeling gRNAs seen in a fluorescent scope.

FACS analysis

FACS sorting with GFP+ confirmed that electroporation worked, but organoids' survival and efficiency of electroporation rate was low in GFP+ and rhodamine labeling due to the Bio-Rad electroporator.

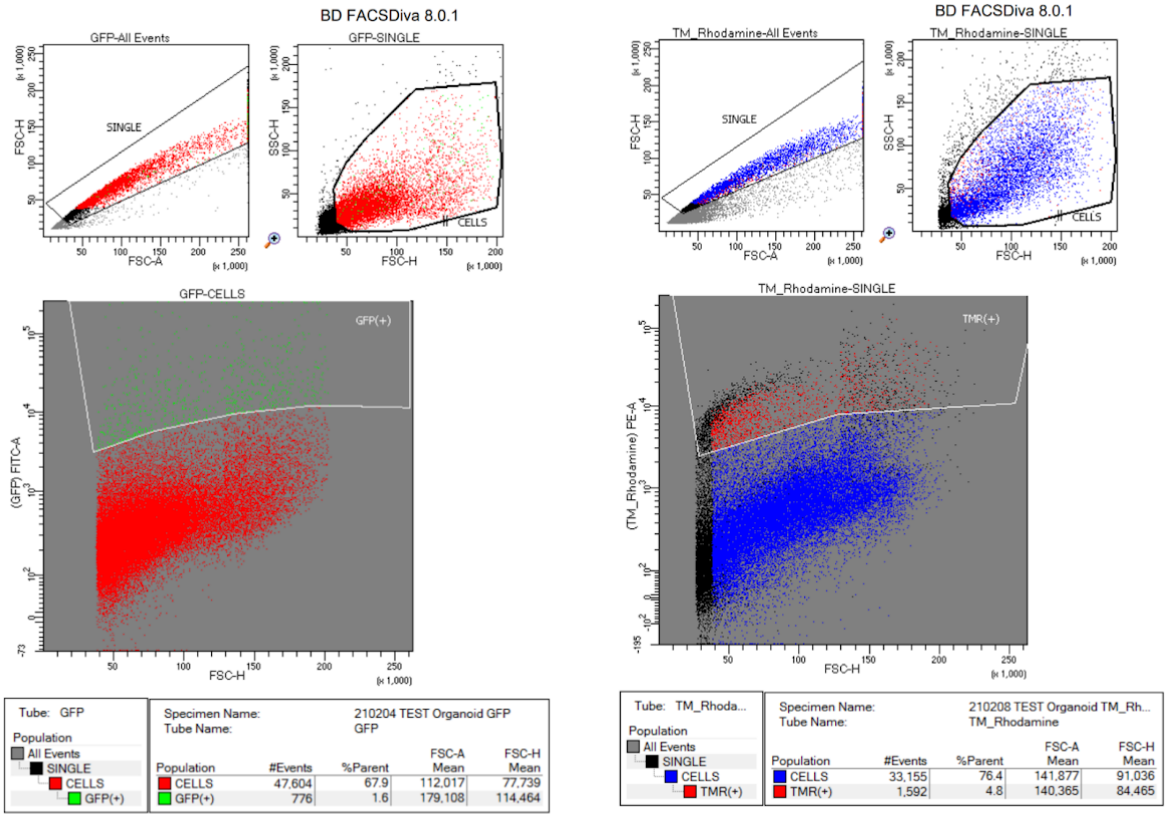


Fig. 3A FACs results on electroporated liver organoids with GFP+. Fig. 3B FACs results on electroporated liver organoids with rhodamine labeling gRNA.

DNA extraction

DNA extraction showed that the cut did not happen for gRNA one nor gRNA 2.

Much noise should be seen at this point (cut), but the mutant matches the original gene sequence. Review on troubleshooting issues needs to be reviewed.

In vitro culture of single cells without sorting

We tried to replicate the optimal conditions for the growth of organoids for one cell after FACs sorting. The best condition from this one trial was CMFG+, Rocki, GSKi with Matrigel. However, the percentage of Matrigel needed is still unclear as the success happens with 1% and 50% concentration. The experiment will be retried with a plate with a conical base instead of a PCR plate. PCR plate was sealed to prevent contamination to other plates in the incubator. The sealing could have been a factor in limiting the organoid's growth.

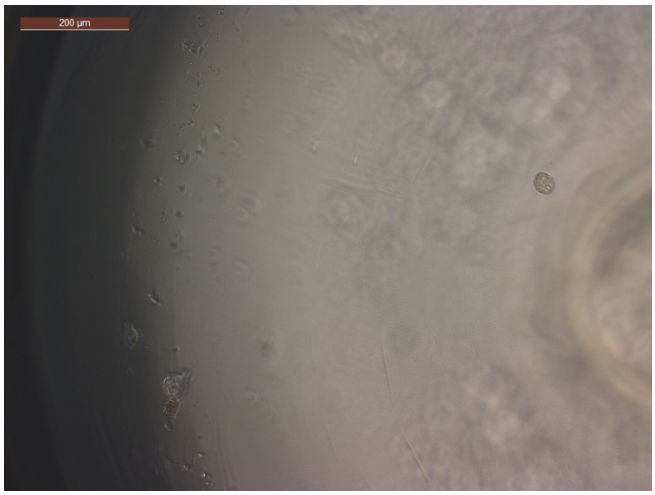


Figure 3. Picture of the organoid growing on 4A well from table 3.

Discussion

Methods to be done in the future are confirming mutation and in vitro culture of the electroporated cells after sorting. When finalized the protocol and successfully mutated the gene, the protocol could be applied to other organoids from different tissues. In this way, we could target specific drug studies deleting the possibility of low drug bioavailability due to an Mdr-1 mutation leading to unbalanced levels of Pgp protein.

However, the use of CRISPR is limited when discussing its use in embryonic human cell lines. It is undeniable that this tool is a massive advancement for finding a cure for different genetic

diseases. In the future, it could be possible to erase a mutation that can cause cancer while the embryo has not been born. However, the question depends on whether this restoration to a "healthy" genotype could lead to deleterious genome modifications. The scientific community does not even know how some genes work, specifically in diseases that involve multiple genes.

In the case of disorders with multiple genes involved, off-targeting editing is a real problem. It could lead to harmful mutations causing different splicing sites or silencing a gene in charge of targeting cancerous cells. Therefore, the National Cancer Institute recommends CRISPR use in ex-vivo treatments: higher safety for the patient.

Moreover, social and economic consequences should be considered. Will society change its perception of people that could not receive this treatment because of lack of insurance or monetary issues? Will CRISPR only be used to treat diseases, or could it improve genetic traits not desirable by our society? Perhaps, a bioethics committee is imperative to avoid crossing the ethical barrier.

Nevertheless, this novel technology can be good for gene therapy in the treatment of diseases like cancer. One of the first trials of CRISPR for patients was cancer treatment. The trial used CRISPR/Cas9 to modify T cells to be more effective at localizing the cancer cell and eliminate it (NIH, n.d.; Wierson et al., n.d.). Without a doubt, there will be more studies and treatments using CRISPR to come.

Troubleshooting

Change from intestines to the liver

We had problems with defrosting the duodenum organoids. We defrosted them, and they would not grow. This issue happened with three samples previously frozen with liquid nitrogen after spending time on -80 C. We need to improve the protocol for defrosting duodenum organoids. Liver organoids grew after defrosting and have better and more consistent results when passaging and cleaning. Liver organoids seemed to survive after freezing them.

Electroporators

Nepagene 21 electroporator is highly recommended to reproduce this experiment. Previous tries without as much success with Bio-Rad possibly due to too high voltage and duration of the voltage. One study used a Bio-Rad electroporator for mice zygotes (Chen et al., 2016). However, NEPA 21 electroporator targets in vivo, in vitro, in Utero, in Ovo, and ex-vivo

transfections, leading to better results and efficiency, especially in more complex organoids. We needed an electroporator that would poring pulse mode and transfer pulse mode to obtain the organoids' minimum damage. Unfortunately, Bio-Rad electroporators on campus lack those options.

gRNA

Unfortunately, the protocol did not include the 95°C for 5 minutes to make sgRNA (tracrRNA, crRNA with cas9). This step is crucial because the tracrRNA has hairpin loops that will prevent its binding to the crRNA. Therefore, the cutting of the DNA would not happen even when the electroporation is correct.

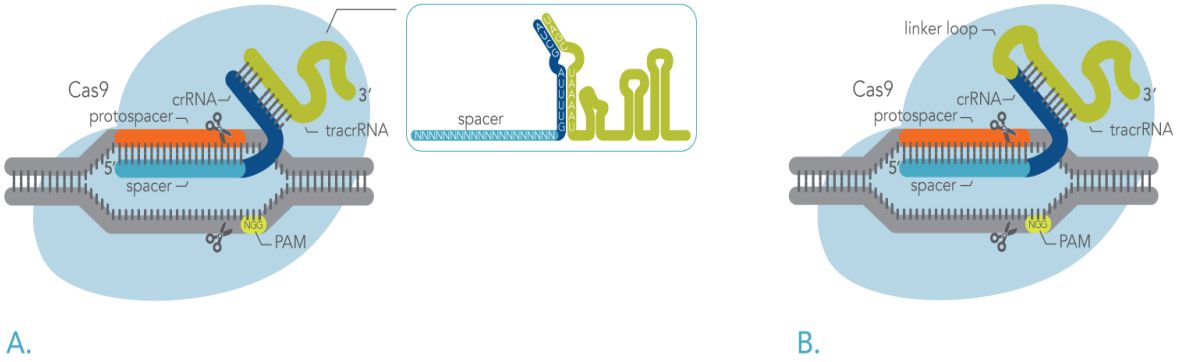


Fig 2. DNA cleavage by the CRISPR-Cas9 system. (A) Cas9 with two-part guide RNA "The Alt-R CRISPR-Cas9 Genome Editing System: IDT." *Integrated DNA Technologies*, www.idtdna.com/pages/technology/crispr/crispr-genome-editing/Alt-R-systems/cas9.

Conclusion

These sets of experiments continue to be refined to obtain optimal results. We discovered troubleshooting issues that we corrected during the development of the methods, such as the change from duodenum organoids to liver organoids, increased survival of the organoids by the electroporator, and the correct preparation of gRNA. The results of the FACS sorting confirm that organoids can be electroporated. After figuring out the problem with the gRNA and successfully cutting the dsDNA, we will order an HDR template with the mutation paired with the cut ends.

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