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Investigating the Localization and Function of PKHD1L1 as a Possible Tumor Suppressor

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Abstract

Polycystic Kidney and Hepatic Disease 1-like 1 (PKHD1L1) has been found to be mutated in many different types of cancers. Its mutation and downregulation have led to the belief that it is a tumor suppressor gene. Preliminary research suggests that this gene may be involved with regulating cell growth or movement. The objective of this study is to determine the function of PKHD1L1 and its localization within mammalian cells. A variety of human cells were utilized: bladder cells (UM-UC-3), kidney cells (HEK 293), and breast cells (AG11138). Experiments done with mutant knockout UM-UC-3 cells indicate that loss of PKHD1L1 may increase cell proliferation and that its regulation may be density dependent. Localization was unable to be determined due to significant cell death after puromycin selection, but an examination of PKHD1L1's expression demonstrated that the gene is expressed within HEK 293 and AG11138 cells. While this research lays the groundwork for investigation into PKHD1L1's function and location, more studies are required in order to draw solid conclusions on its role within the cell and in relation to tumor suppression.

Introduction

Cancer and tumor suppressors

Cancer is a common diagnosis in today's society. In the United States alone, approximately 39.5% of the population will receive a cancer diagnosis at some point in their lives (National Cancer Institute, 2015). Cancer comes in many different forms and can affect different tissues. It begins as a tumor, which occurs when cells divide out of control. Normally, cells have genes that regulate their growth and division so this does not occur. Some of these genes act as barriers against irregular growth and are able to slow or pause cell division, induce apoptosis (programmed cell death), and repair damaged DNA. These genes are known as tumor suppressors (Cooper, 2000). When these genes are damaged or mutated, the likelihood of tumors, and cancer, increases. One example of a well-known tumor suppressor occurs in retinoblastoma. In order to develop retinoblastoma, two mutations of the Rb gene are necessary. If there is one functional Rb copy, the gene is able to suppress the growth of tumors and retinoblastoma will not develop. For inherited retinoblastoma, one nonfunctional copy of Rb is inherited and the other must be mutated for tumor development (American Cancer Society, 2021). This revelation on the relationship between Rb and retinoblastoma gave much insight into tumor suppressors and their existence.

Due to their great influence, tumor suppressor genes are a point of interest for many researchers as they indicate functions that without which the cells become more prone to tumor formation. Knowing which functions suppress tumor formation gives more insight into how cancer may be prevented and also what the underlying causes of tumor and cancer formation are.

One method of identifying genes that may be possible tumor suppressors is by looking at the mutation rates of genes in different types of cancers. If a gene has a high mutation rate that results in lower gene expression or a nonfunctional protein for a specific cancer, it stands to reason that the mutation of this gene allows for tumor formation. In other words, tumors are more likely to form when the gene is not functioning normally. In this way, possible tumor suppressor genes can be identified using databases such as that of The Cancer Genome Atlas Program by the National Cancer Institute.

PKHD1L1 and relevant research

One gene we identified as being a possible tumor suppressor is Polycystic Kidney and Hepatic Disease 1-like 1 (PKHD1L1). This specific gene had a high mutation rate in multiple epithelial cancers (Shi and Yoo, 2018). This led us to believe that for these types of cancers, PKHD1L1 may play an important role in tumor suppression. Unfortunately, not much is known about PKHD1L1. Originally believed to be a mouse gene, PKHD1L1 is located on chromosome 8 of the human genome. Most knowledge of PKHD1L1 comes from its role as a coat protein in hair stereocilia. In mice, PKHD1L1 has been shown to make up the surface coat of hair cell stereocilia (Ivanchenko et al., 2020). It also seems to be a large, mainly extracellular protein that is required for normal hearing in mice (Wu et al., 2019). Although this gives us some information about PKHD1L1's possible function, it does not answer any questions about how it may function as a tumor suppressor.

The research done on PKHD1L1 as it relates to cancer is very limited. Much of this research stems from seeing a correlation between the mutation rate of PKHD1L1 and

the occurrence of cancer. One group of researchers, while examining patterns in metastatic triple-negative breast cancer, found that PKHD1L1 was one of the genes whose mutation correlated with tumor occurrence (Saravia et al., 2019). Another research group, while examining lung adenocarcinoma (LUAD), found that PKHD1L1 was associated with LUAD and that some of its mutation sites were related to its gene expression (Wang et al., 2020). While these associations further support that PKHD1L1 is a tumor suppressor, they do not tell us what its function is or how it relates to cancer formation.

There is some literature that pertains to how PKHD1L1 functions within a cell and how its loss of function relates to tumor formation. One paper examined PKHD1L1's possible role in thyroid cancer. The researchers noticed that this gene was highly associated with papillary thyroid cancer. Using RT-q PCR, the researchers were able to compare PKHD1L1 expression levels between cancerous thyroid tissues and normal thyroid tissues. They found that PKHD1L1 was significantly downregulated in the tumor cells compared to the noncancerous cells (Zheng et al., 2019). This further suggests that PKHD1L1 is a tumor suppressor. The same researchers also showed that when PKHD1L1 was knocked out in cancerous thyroid cells, the migratory and invasive abilities of these cells were increased (Zheng et al., 2019). Not only does this provide increased evidence that PKHD1L1 functions in some way to prevent tumor formation, but it also suggests that without PKHD1L1, tumor metastasis may be more likely.

Although the aforementioned research does present evidence that PKHD1L1 is a tumor suppressor and even suggests what factors it may influence, there is still a large knowledge gap. The specific functions of PKHD1L1 are not known, nor is its localization

within the cell. This important information can then guide research on ways tumors can be prevented. It could also reveal what functions are most important for tumor suppression.

Research questions and objectives

Due to PKHD1L1's position as a possible tumor suppressor and the lack of knowledge about it, our lab chose to investigate further into PKHD1L1's function and role in tumor suppression. Our main question relates to function: what does PKHD1L1 do? To answer this, we began by investigating what functions would be affected by loss of the gene. We also began attempting to determine the localization of PKHD1L1 within human cells, believing that this would give us some clue as to its possible function. As another way of looking at function, we also examined expression levels of the gene in normal human cells.

Several methods were utilized to achieve these goals. The first was to create a knockout using CRISPR-Cas9 in a bladder cell line, UM-UC-3. From there, antibodies could be used to determine protein location. While a knockout was created by another researcher in the Yoo lab, Katherine Lindsay, in a human bladder cell line, this was only confirmed on the DNA level and could not be confirmed on the protein level. This knockout was also used in a cell proliferation assay and a colony formation assay, although the results are only moderately useful due to the unconfirmed nature of the knockout.

The next method was used to determine localization. Using the Mendenhall and Myers CRISPR-Cas9 system, we wanted to add a FLAG-tag to the gene. The FLAG-tag

could then be transfected into two human cell lines, AG11138, a breast cell line, and 293, a kidney cell line.

Our lab also sought to learn more about PKHD1L1 by looking at mRNA expression levels in human cell lines. This would allow us to establish a baseline of expression for PKHD1L1 in normal human cell lines. Additionally, this would allow for verification of knockouts created.

The overarching goal of this project was to investigate PKHD1L1's function as a possible tumor suppressor in order to gain knowledge about what prevents and causes tumors and cancer. Our lab hopes that this research will be taken further and that more experiments can be run to either confirm the knockout line or to create a more successful one. This would allow for assays- such as cell proliferation and death assays- to test for different reactions of cells with and without PKHD1L1. It would further our advancements in our knowledge about tumor suppressors and PKHD1L1's role in cancer.

Methods

Cell Culture

Three different human cell types were used throughout this project: the bladder cell line UM-UC-3, the kidney cell line HEK 293, and the breast cells AG11138. All mammalian cells were thawed in a 37° water bath, spun down in 10mL of proper growth medium, resuspended in 10mL of growth medium and plated on a 10cm dish.

UM-UC-3 and HEK 293 cells were cultured in DMEM (Thermofisher) and MEM/EBSS (Cytiva Hyclone) media respectively. Media was supplemented with 1% penicillin streptomycin antibiotic (Pen-strep) and 10% fetal calf serum (FCS). Cells were

maintained in a 37° C incubator with 5% CO_2 and humidity. They were split at about 70-90% confluency by aspirating the media, washing with 5mL 1x phosphate buffered saline (1x PBS), adding 500µL 0.25% Trypsin, then resuspending in complete growth medium.

AG11138 cells were cultured in MEBM media (Lonza) supplemented with 0.5mL hydrocortisone, 50µl of cholera toxin, 0.5mL human Epidermal Growth Factor, 0.5mL insulin, 2.0 mL bovine pituitary extract, and 5mL of pen-strep per 500mL of MEBM (Lonza).

MTS assay on PKHD1L1 knockouts in UM-UC-3 cells

A cell proliferation assay (MTS) was performed upon the PKHD1L1 knockouts created by another member of the lab. Experiment was done using the mutant knockout UM-UC-3 cells and the parental UM-UC-3 cells. Cells were plated after trypsinization on 96 well plates at two different densities: 1.0×10^4 cells and 1.3×10^4 cells. Seven replicates were done for both parental and mutant cells for each density. 48 hours after plating, 20µl of a 5% PMS, 95% MTS solution were added to each well. Readings were taken with an ELISA plate reader at 490nm absorbance at 1 hour and 2 hours, and 3 hours post addition. Readings were saved and analyzed using JMP Pro to run an ANOVA and Tukey-Kramer post-hoc test.

Colony formation assay

Multiple colony formation assays were performed to compare the parental and knockout UM-UC-3 cells. A cell count was performed on trypsinized cells and 100 or 200 cells were plated in six wells of a 6-well plate, three wells of parental cells and three

wells of mutant cells. Cells were allowed to grow in 2.5mL of complete DMEM media for 8 days before being stained with crystal violet.

Staining was done by placing the plates on ice and washing twice with 4°C PBS. They were then fixed for 10 minutes with ice cold 100% methanol. A crystal violet solution of 25% methanol and 0.5% crystal violet in deionized water was created. The fixed cells were then incubated in this solution for ten minutes, and then washed until dye stopped running. After being allowed to dry, plates were counted.

Tagging PKHD1L1

In order to insert a FLAG-tag into PKHD1L1, a target sequence must be cloned into an sgRNA scaffold. We used Benchling to identify appropriate oligos following the Zhang Lab general cloning protocol (Zhang lab). gRNA primers were created following this protocol, using PAM sequence NGG and searching within -30 and +50 base pairs of the stop codon.

Table 1. gRNA primers for PKHD1L1

Oligo pair	Sequence (5' to 3')
Forward 1	CACCGAAAGTGCTGTTCCGAAGAAT
Reverse 1	AAACATTCTTCGGAACAGCACTTTC
Forward 2	CACCGTTTTGTTTCAGCCTATTCTT
Reverse 2	AAACAAGAATAGGCTGAAACAAAAC
Forward 3	CACCGTTTAGTAGCTTCCTGAAAAA
Reverse 3	AAACTTTTTCAGGAAGCTACTAAAC

pSpCas9(BB)-2A-Puro (PX459) V2.0 was the plasmid used (AddGene). For each pair of primers, the following procedure was used, modified from the Zhang lab's target sequence cloning protocol (Zhang lab):

1µg of plasmid was digested for 4 hours at 37°C with 1µL BbsI, 1µg of plasmid, 2µL of 2.1 buffer, and ddH_2O to achieve a 20µL reaction. The digest was gel purified using a Thermoscientific GeneJET Gel Extraction kit and its accompanying protocol (ThermoScientific). Meanwhile, 1µL of forward oligo (100µM) and 1µL of reverse oligo (100µM) were annealed in a thermocycler with 1µL 10X T4 Ligation Buffer (NEB), 6.5µL ddH_2O, and 0.5µL PNK (NEB). The thermocycler was run with the following parameters: 37°C for 30 minutes followed by 95°C for 5 minutes and then ramp down to 25°C at 5°C per minute. Once both plasmid DNA and annealed oligos are ready, the following ligation reaction was set up and incubated overnight: 50ng of digested plasmid, 1µL of a 1:200 dilution of the oligo duplex, 1µL 10X T4 DNA ligase buffer, 1µL T4 DNA ligase, and ddH_2O to a 10µL reaction. This was followed by a transformation into competent cells, first with normal E. coli and then with NEB 10-beta E. coli. The transformed cells were selected for on ampicillin plates and colonies were plucked and grown in LB with 100µg/mL of ampicillin. A miniprep was done using a Promega Wizard Plus SV Miniprep kit and its accompanying protocol and purified DNA was digested with SacI and BbsI to determine identity, as well as sequenced (ProMega).

Transformations

Escherichia coli (E. coli) were transformed with various plasmids and used to create plasmid DNA preparations. E. coli cells were taken from the -80°C freezer and thawed on ice. After thawing, 75µL of thawed cells were added to each tube of plasmid DNA. This was incubated on ice for 10 minutes and then heat shocked at 42°C in a water bath for 45 seconds. Tubes were then placed on ice for 2 minutes. 900µL of luria broth was added to each tube using sterile technique. The tubes were then placed in a 37°C shaking incubator for 35 minutes before being spun down at 13,000 rpm for 2 minutes. All but 100µL of supernatant were removed and cells were resuspended in remaining supernatant. This was then pipetted and spread onto agar plates with 100µg/mL of ampicillin. These were then incubated at 37℃.

NEB 10-beta E. coli cells were also obtained for transformation with large plasmids. Cells were thawed on ice and 50µL were transferred to a separate tube for each different transformation. 1-5µL containing 1pg-100ng of plasmid DNA was added to each tube and flicked to mix. Tubes were placed on ice for 30 minutes before being heat shocked in a 42°C water bath for 30 seconds. They were then incubated on ice for five minutes without mixing. 950µL of room temperature NEB 10-beta/Stable Outgrowth Medium (New England Biolabs) was added to each tube and shaken at 37°C for 60 minutes. Agar plates containing 100µg/mL of ampicillin were warmed to 37°C and 50-100µL of each tube were spread onto the plates and incubated at 37°C. Colonies were then plucked and miniprepped.

Transfection

Transfections were done as part of the project attempting to localize PKHD1L1 by adding a FLAG-tag into the gene. The plasmid containing Cas9 and the generated target sequence oligos would need to be transfected into the desired cells along with the homology directed repair donor plasmid with proper homology arms and selection marker. Transfection was done on HEK 293 kidney cells using the Invitrogen Lipofectamine 3000 Reagent protocol (ThermoFisher Scientific). The protocol was scaled for 12 well plates. Additionally, puromycin was decided upon for antibiotic selection. Approximately 1.8 x 10^5 cells were plated for 70-90% confluency the next day.

Three experiments were set-up with three different conditions: 1.24µL of lipofectamine 3000 per well and 4µg/mL of puromycin, 2.47µL of lipofectamine 3000 per well and 2µg/mL of puromycin, and 2.47µL of lipofectamine 3000 and 4µg/mL of puromycin. The conditions were selected based upon the Invitrogen procedure and a preliminary experiment which showed early cell death that was thought to be due to either the lipofectamine or the puromycin. Each experiment contained a well of cells that would not be transfected but would be selected with antibiotic. Each experiment also contained one well of cells that received the original vectors and one well of cells that received the engineered sgRNA scaffold and pFETCH with the appropriate HOM arms. The experiment with the greater amount of Lipofectamine 3000 also contained a well of cells transfected with green fluorescent protein (GFP) to determine transfection efficiency of the lower amount. The greater amount of Lipofectamine 3000 was shown to have a transfection efficiency of 45% from an earlier experiment done by another member of the lab, Bella Kohrs. Invitrogen procedure was followed and 48 hours after transfection,

complete growth medium containing the appropriate concentration of puromycin was added to the wells that underwent transfection except for the well transfected with GFP. The well transfected with GFP was visualized with blue light. Antibiotic selection lasted three days. Due to cell death, experiments were unable to continue and visualization did not occur.

mRNA quantification

To determine the relative amounts of PKHD1L1 within normal human cells, we needed to isolate the PKHD1L1 mRNA and do qPCR. RNA was isolated from HEK 293 cells and AG11138 cells using a ThermoScientific GeneJET RNA Purification Kit and its accompanying protocol (ThermoScientific). RNA concentration was determined using a the nanodrop feature of a BioTek synergy multi-mode reader and then diluted to create a 50ng solution of each RNA extraction.

B-actin was used as a control and primers were created for B-actin and for PKHD1L1. PKHD1L1 primers were created using Primer3 (Primer3, bioinfo.ut.ee). The parameters used were between 18 and 30 base pairs (bp's) in length, a melting point between 60°C and 64°C, and a GC content between 35-65%. Amplicon length should be less than 500bp's in length.

Table 2. Genes and respective primers for qPCR.

Gene	Primer	Sequence (5' to 3')	Melting Point	GC content (%)
B-actin	Forward	AGTTGAAGGGAATAATGTCACACTGG	59.1°C	54.55
B-actin	Reverse	AGTTGAAGGGAATAATGTCACACTGG	60.6°C	54.55
PKHD1L1	Forward	CACCATTGGCAATGAGCGGTTC	61.25°	42.31
PKHD1L1	Reverse	AGGTCTTTGCGGATGTCCACGT	61.64°	50.00

For qPCR, primers were diluted to 10μM concentrations and master-mixes were created for B-actin and PKHD1L1, respectively, using AzuraQuant 1-Step Green LoRox and AzuraSpring RTase (Azura Genomics).

Table 3. Master-mix amounts and products for 12 reactions (six of each gene with one extra reaction's worth of materials).

B-actin	PKHD1L1
• 70ul one-step qPCR mix • 5.6ul Forward B-actin primer (10uM) • 5.6ul Reverse B-actin primer (10uM) • 7ul 20x RTase	• 70ul one-step qPCR mix • 5.6ul Forward PKHD1L1 primer (10uM) • 5.6ul Reverse PKHD1L1 primer (10uM) • 7ul 20x RTase

12 tubes were created, with three replicates of B-actin and PKHD1L1 for each cell line. For each tube, 12.6μL of the appropriate master-mix, 50ng of the appropriate RNA, and PCR grade H_2O as needed for a 20μL reaction was added to each tube. qPCR was run using a MyGo Mini S Real-Time PCR machine by Azura Genomics with the following program:

1. 50°C for ten minutes
2. 95°C for two minutes
3. 95°C for 5 seconds then62°C for 25 seconds
4. Repeat step three for 39 more cycles

Data was collected and Delta Ct numbers calculated by subtracting average B-actin Ct values from average PKHD1L1 Ct values for each cell type.

Data analysis

Data analysis was done using JMP Pro Version 15 to run ANOVA's and post-hoc tests if necessary. JMP Pro was also used to create graphs shown. Delta Ct numbers were calculated using Microsoft Excel Version 16.4821041102.

Results

MTS and Colony Formation Assay on PKHD1L1 knockouts in UM-UC-3 cells

An MTS assay was done on two different densities of parental and mutant cells ($1.0x10^4$ and $1.3x10^4$ per well in a 96 well plate). The cells at the lower density showed no significant difference between parental and mutant cells at the same time points, although there was a significant difference between time points within cell type

($p<0.001$) (Figure 1). The cells at the greater density ($1.3x10^4$) did show a significant difference between parental and mutant cells at the same time points, with the mutant cells showing significantly greater absorbance at each time period than the parental cells ($p<0.0001$) (Figure 2). This shows that that the PKHD1L1 mutant cells proliferate at a greater rate than the parental cells, although there may be some dependence on density.

A colony formation assay was also performed on the parental and mutant UM-UC-3 cells at either 100 or 200 cells per well in a six well plate. Colony counts varied greatly and there were no significant differences between mutant and parental cells at either density. Although neither are significant, at 100 cells per well there were fewer colonies of the mutant cells while at 200 cells per well there were fewer colonies of the parental cells (Figure 3).

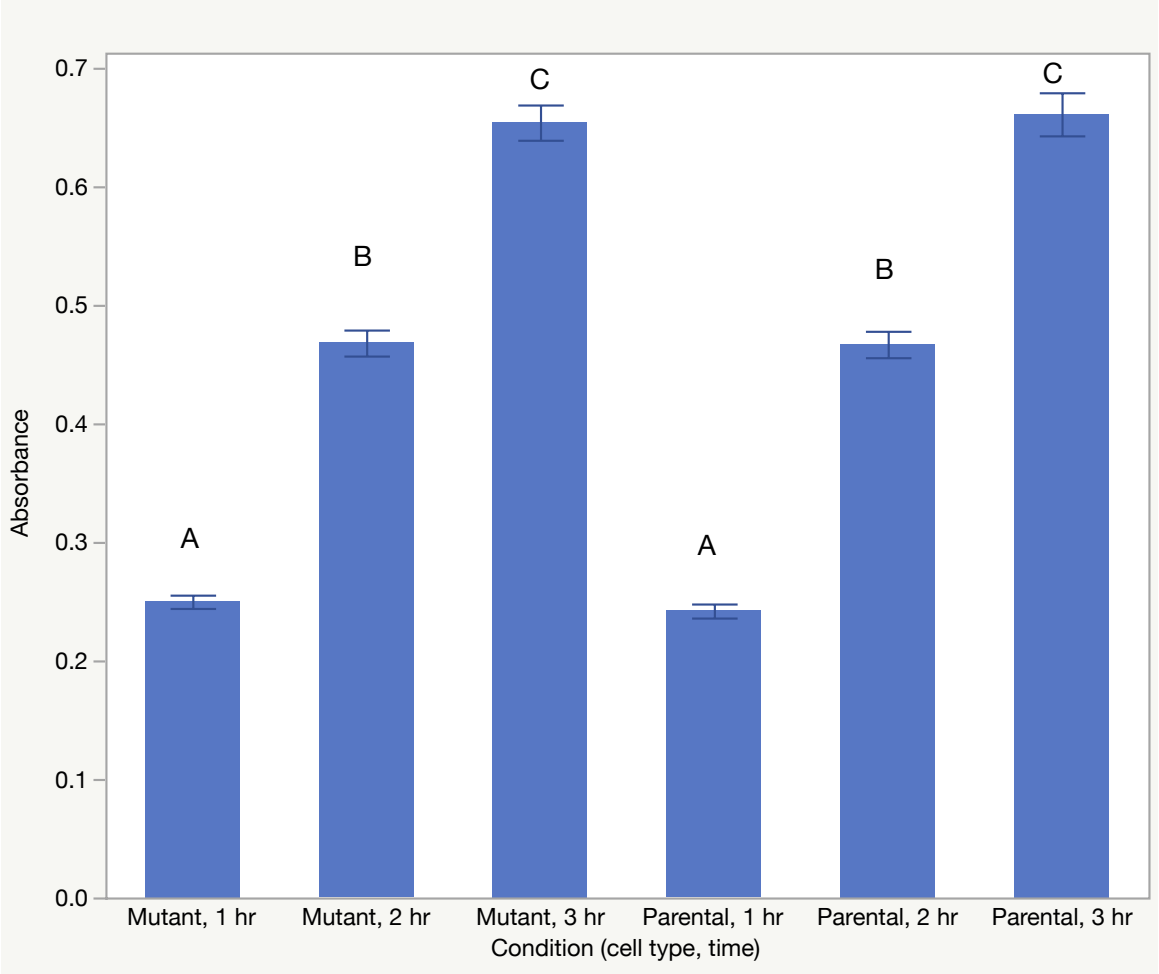

Figure 1. Normalized mean absorbance values of UM-UC-3 mutant and parental cells with MTS solution added. Cell density for both parentals and mutants was 1.0 x 10^4 cells per well. Absorbance measured at three different times (1 hour, 2 hours, and 3 hours). Differing letters show significant difference ($p<0.001$). Error bars are standard error.

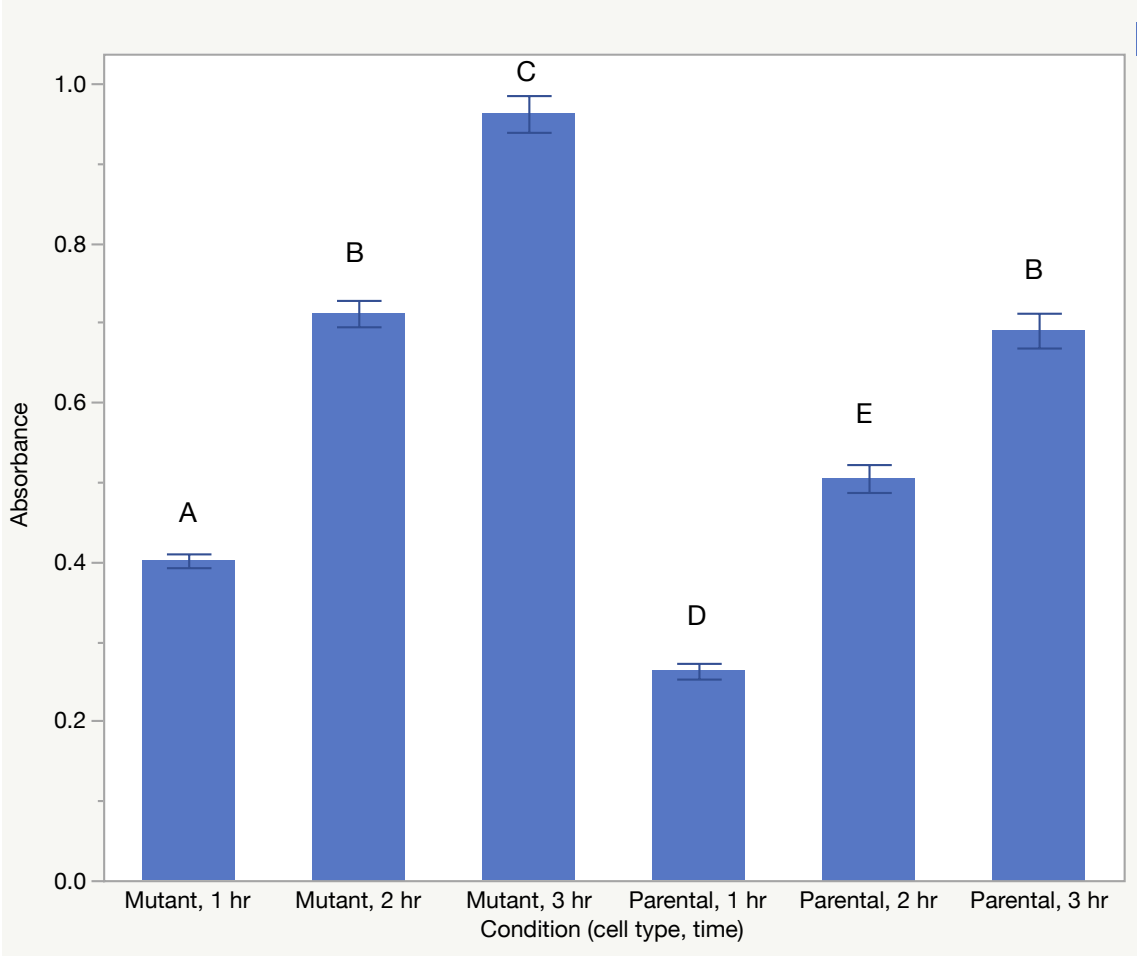

Figure 2. Normalized mean absorbance values of UM-UC-3 mutant and parental cells with MTS solution added. Cell density for both parentals and mutants was 1.3 x 10^4 cells per well. Absorbance measured at three different times (1 hour, 2 hours, and 3 hours). Differing letters show significant difference ($p<0.001$, $p= 0.007$ for A and E). Error bars are standard error.

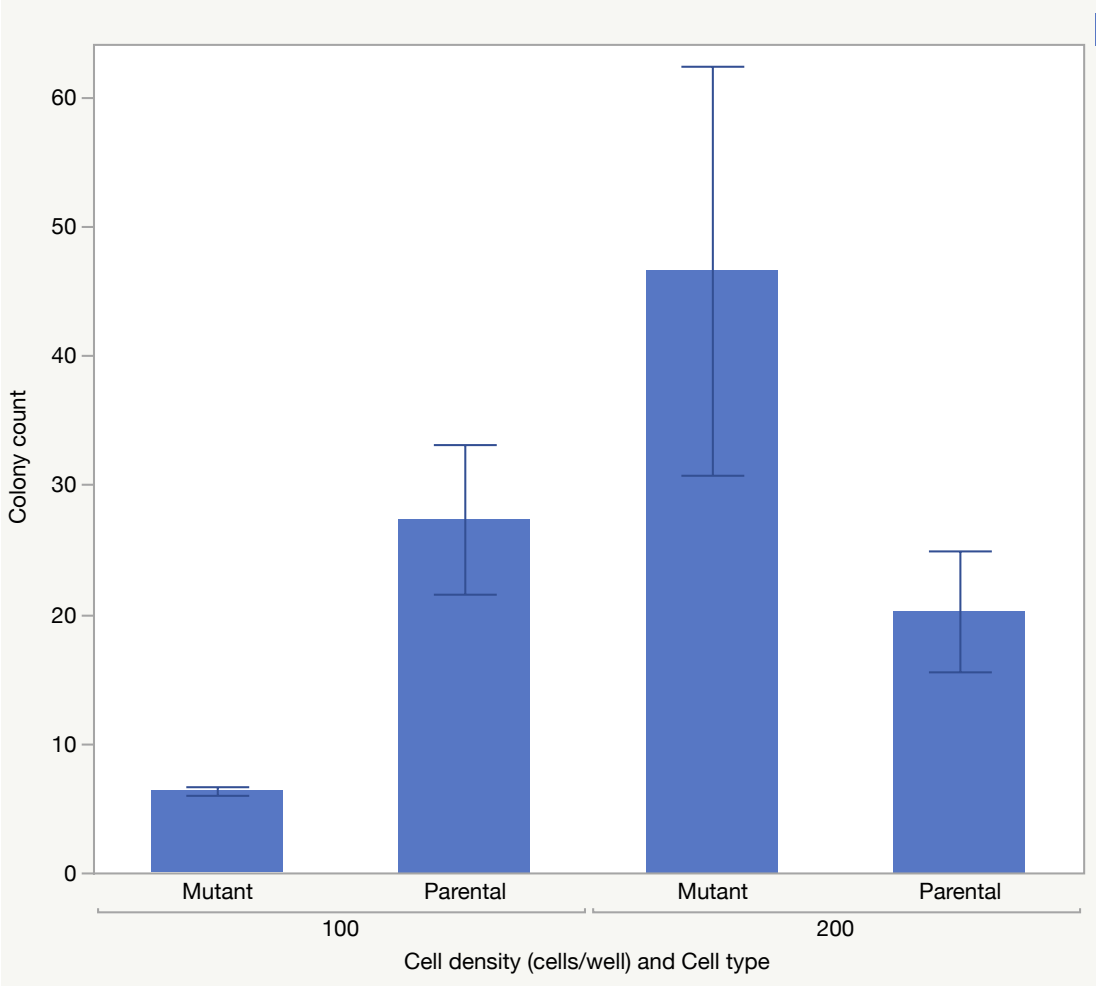

Figure 3. Colony count for mutant and parental UM-UC-3 cells plated at different densities. There was no significant different between mutant and parental cells at each plating density. Error bars are standard error.

Creating sgRNA scaffold for PKHD1L1

The target sequence was successfully cloned into an sgRNA scaffold. Primers were cloned into plasmid pSpCas9(BB)-2A-Puro (PX459) V2.0 (AddGene). The plasmid was then transformed into E. coli and purified DNA digested with SacI and BbsI to confirm identity. BbsI and SacI cut sites are 245, 267 and 3606, 6204 respectively

(Figure3). Oligos 1 and 2 were successfully cloned in twice and oligo 3 was successfully confirmed three times, as shown by lanes 3, 4, 7, 8, 10, 11, and 12 (Figure 4).

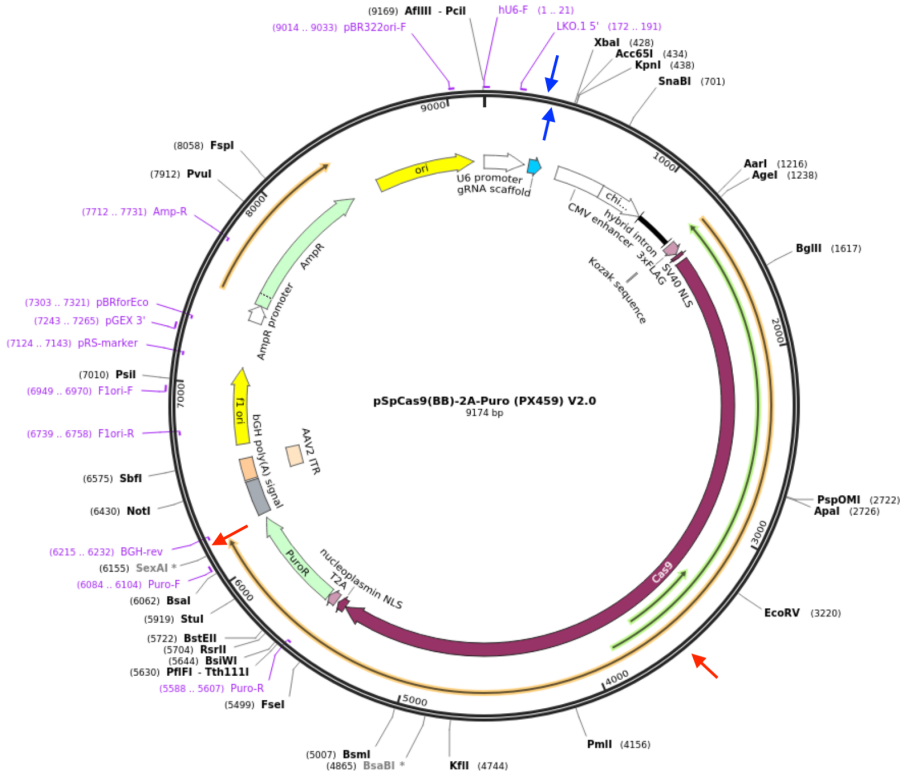

Figure 3. Plasmid map for pSpCas9(BB)-2A-Puro (PX459) V 2.0 (AddGene). Red arrows show approximate BbsI cut sites (245, 267). Blue arrows show approximate SacI cut sites (3606, 6204).

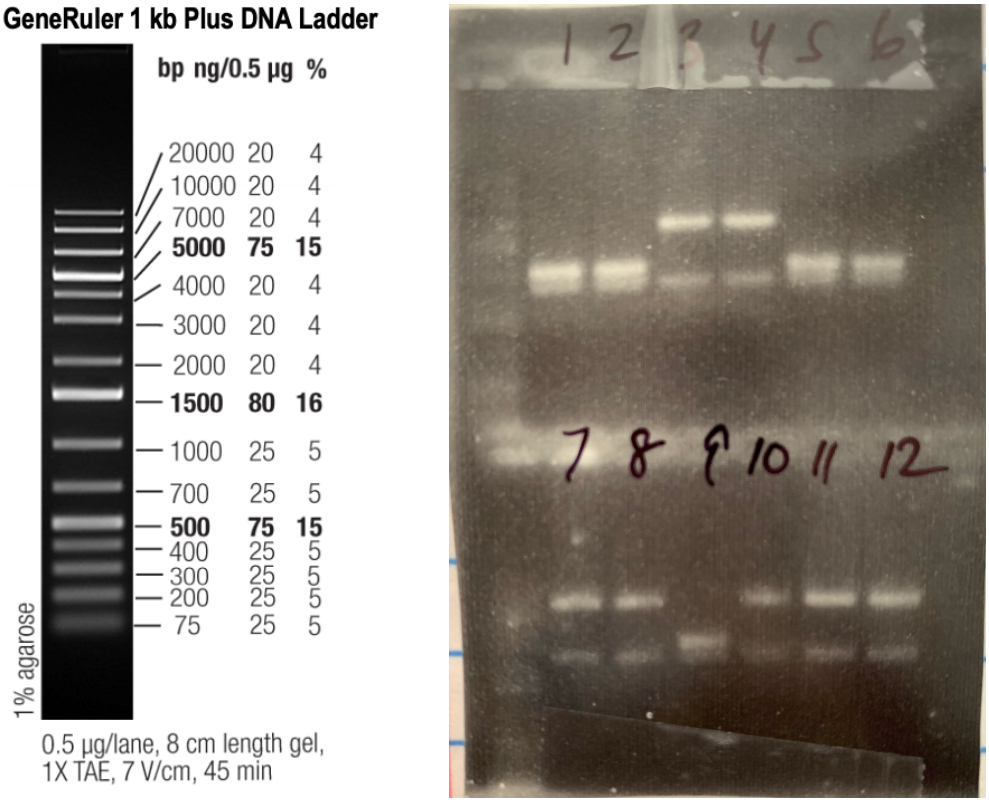

Figure 4. Gel of modified pspCas9 PX459 plasmid digested with SacI and BbsI enzyme along with accompanying ladder. Lanes 1-4 are Oligo 1, lanes 5-8 are Oligo 2, and lanes 9-12 are Oligo 3 (Table 1).

Transfection

For all transfection conditions done, cells were unable to recover after three days of antibiotic selection with puromycin. Cells at the lower concentration of puromycin seemed to have greater survival than at the higher concentration but were still unable to recover enough for antibody staining and visualization. Due to this, localization of PKHD1L1 has yet to be determined.

Quantifying PKHD1L1 Expression in HEK 293 and AG11138 cells

RT-qPCR was done using RNA from HEK 293 kidney cells and AG11138 breast cells. Resulting Delta Ct numbers show relative PKHD1L1 expression for each cell type. Delta Ct shows the difference in the number of cycles needed to reach threshold (Ct) between the gene of interest (PKHD1L1) and the reference gene (beta-actin). A greater Delta Ct means that there were lower amounts of RNA present. Delta Ct values are very consistent across both cell type and the amount of RNA used in the reaction. This means the relative expression of beta-actin was similar across both amounts of RNA. Relative expression of PKHD1L1 was also similar between the differing RNA amounts. This shows that expression of PKHD1L1 is similar across the two cell types and does not depend greatly on the amount of RNA used (Table 3, Figure 5). The s-curves showing intensity over time also depict consistency within both B-actin (control gene) and PKHD1L1 (gene of interest) for both amounts of RNA used (Figure 6, Figure 7).

Table 4. Amount of PKHD1L1 RNA input into the reaction, cell type, and the resulting Delta Ct from qPCR.

RNA amount	Cells	Delta Ct
40ng	AG11138	16.292
40ng	HEK 293	16.872
100ng	AG11138	18.254
100ng	HEK 293	16.935

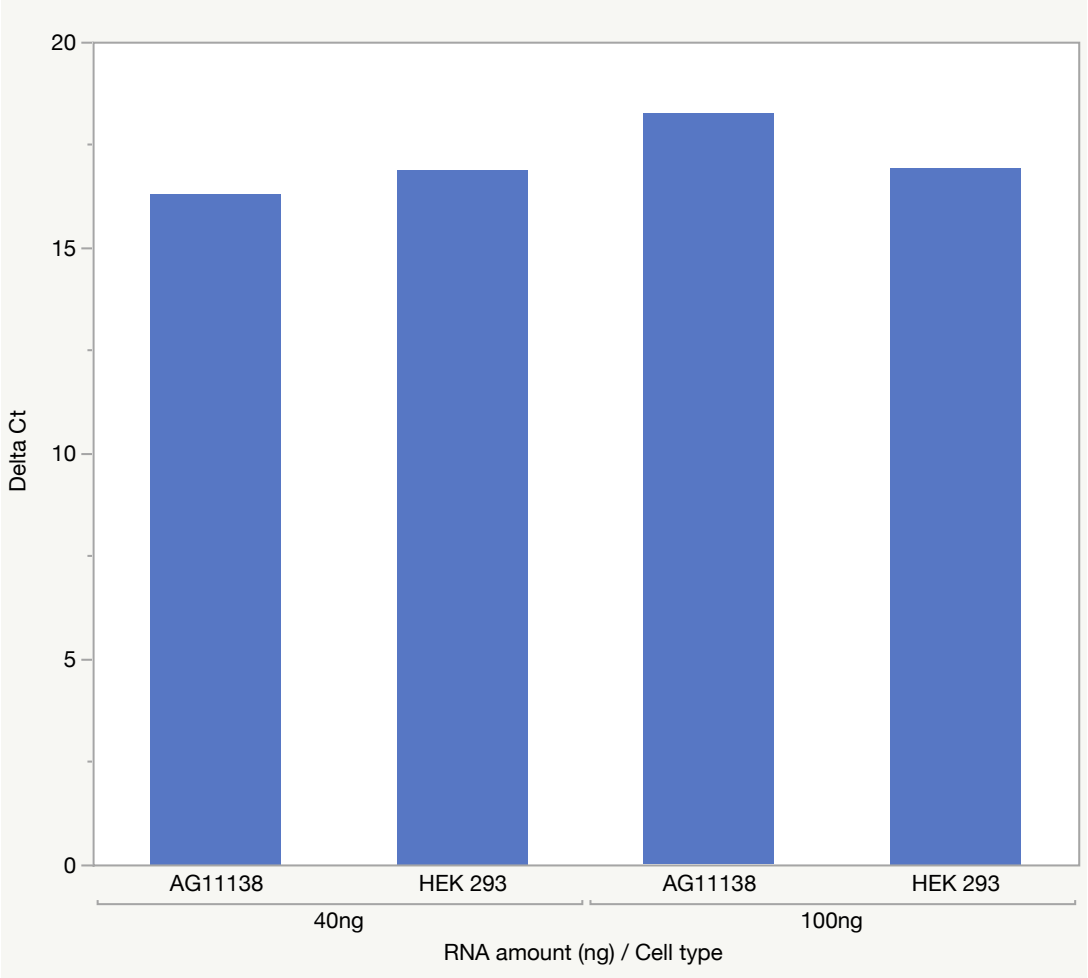

Figure 5. Delta Ct values for different amounts of RNA and different cell types.

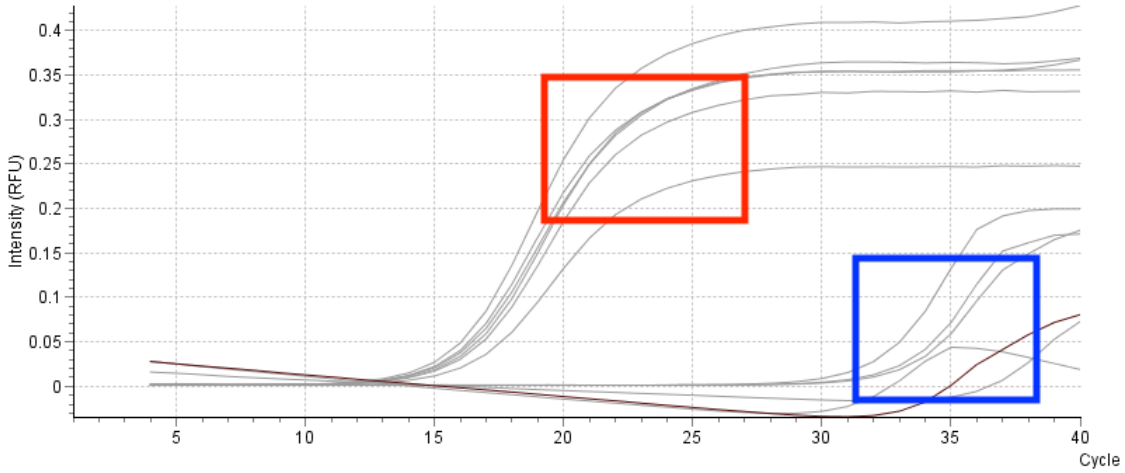

Figure 6. S-curves for B-actin and PKHD1L1 when using 40ng of RNA. Graph depicts intensity over number of cycles. The red box encloses the lines showing the samples using B-actin primers while the blue box encloses the lines representing the samples using PKHD1L1 primers.

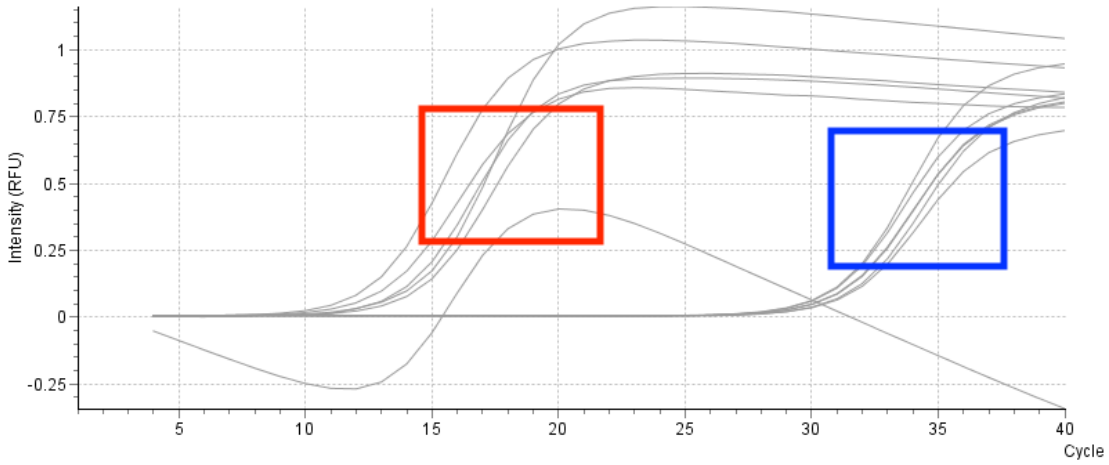

Figure 7. S-curves for B-actin and PKHD1L1 when using 100ng of RNA. Graph depicts intensity over cycle number. The red box encloses the lines showing the samples using B-actin primers while the blue box encloses the lines representing the samples using PKHD1L1 primers.

Discussion

PKHD1L1 is a gene found to be highly mutated in different types of cancer, including epithelial and breast cancers. This connection suggests that PKHD1L1 is a tumor suppressor and that when the gene's normal function is disrupted, there is a greater likelihood of tumor occurrence. As such, it is important to learn about PKHD1L1 and its role in tumors and tumor suppression. To do this, our lab conducted tests and examined the data to begin to gain an understanding of how PKHD1L1 is related to tumors and what its normal function is.

The Role of PKHD1L1 in Cell Proliferation

Preliminary knockouts of PKHD1L1 suggest that it plays a role in regulating cell growth and proliferation. The MTS assay indicates that at a proper density, UM-UC-3 cells with PKHD1L1 knocked out proliferate at a greater rate than the normal UM-UC-3 cells (Figure 2). This suggests that PKHD1L1's normal function may have to do with limiting or regulating cell growth.

The colony formation assay results support this hypothesis, as at the greater plating density, significantly more colonies were formed (Figure 3). This was not seen at the 100 cells per six well density. This assay also seems to suggest that PKHD1L1 plays an important role in limiting cell growth or regulating the cell cycle. It is important to note, however, that there was much variation in the colony counts and thus the analysis was done using averages.

Interestingly, both assays suggest that density is important for the effects of mutant PKHD1L1 to be shown. Significant difference is only seen for the higher density

conditions in each experiment. This leads us to believe that UM-UC-3 cells depend on growth signals from other cells in order to grow properly. One paper found that in BSC-1 cells, epithelial cells from African green monkeys, that epithelial growth hormone (EGF) receptors decrease at higher densities (Holley et al., 1977). This indicates that gene expression does change based upon cell density and that they can sense cell density. Keeping in line with this finding, if PKHD1L1 is believed to be involved in cell growth regulation by limiting overgrowth, it would also make sense that the protein is mostly needed at greater densities when cell growth should be limited. This would explain why we see a correlation between cell density and a difference in cell growth between parental and mutant cells. If this is true, it could mean that PKHD1L1's expression is linked to cell density.

While these findings indicate that the knockout of PKHD1L1 changes the rate of cell growth, the knockout could only be confirmed on the DNA level. Although sequencing confirmed that the desired target had been knocked out, we were unable to determine whether or not this led to the protein being created at all. Antibody staining for the protein was inconclusive, meaning that although our results show significant differences between the mutant and parental cells, we cannot be sure that this is actually due to the successful knock-out of PKHD1L1.

Tagging PKHD1L1 Using CRISPR-Cas9

In order to determine where PKHD1L1 is located within a cell, we decided to engineer a FLAG-tag into the gene. In order to begin, an sgRNA scaffold must be created with the proper target sequence cloned in. This was done with the PKHD1L1 primers used shown in Table 1. After cloning the oligos in, plasmids were transformed into E. coli. The E. coli did not grow the first few times, so the target sequence cloning protocol was modified. Plasmid was digested with BbsI for up to four hours to ensure full cutting and NEB 10-beta E. coli optimized for larger plasmids were used. The plasmid contained ampicillin resistance, and one of the surviving cells was selected, grown, and purified DNA was then digested with SacI and BbsI to determine success. Successful cloning was found at least once for all three oligos. Success was determined by running an electrophoresis gel. Because the primers were cloned into the plasmid using the BbsI cut site, if cloning was successful then the BbsI cut site will no longer exist. Thus, during the diagnostic digest the plasmid will only cut at the SacI site. This will result in two differently sized pieces of DNA, one about 2600 bp's long and the other about 6600 bp's long. Unsuccessful plasmid creation will result in two or three visible bands of DNA segments of about 2600, 3200, and 3300 bp's long. The plasmid created using "Oligo 1" was selected for use in the transfection of HEK 293 and AG11138 cells.

Transfection of CRISPR/Cas plasmid system and immunofluorescence

Another student in the lab, Bella Kohrs, created a plasmid with the proper homology arms and selection marker. This, along with the synthesized gRNA and Cas9 vector, would be transfected into HEK 293 cells. Puromycin was used to select for

successfully transfected cells, with the amount determined by a kill curve done on normal HEK 293 cells. The selection with puromycin was found to be too harsh when combined with transfection and so staining was not possible within the time frame of this project. Kohrs, however, was able to stain and image a transfection done with G418. The staining was inconclusive. Combined with the earlier results implicating cell density as being an important factor in PKHD1L1 expression, it may be worthwhile to do another transfection with a greater number of cells at time of staining.

mRNA Expression of PKHD1L1 within HEK 293 and AG11138 cells

Purification and amplification of PKHD1L1 RNA through qPCR revealed that there are quantifiable levels of PKHD1L1 expressed in both HEK 293 and AG11138 cells. Expression was low compared to the housekeeping gene used, beta-actin. Corrected delta-Ct values show that PKHD1L1 expression does not significantly differ between cell lines nor between the amount of RNA put into the reaction. This shows that PKHD1L1 is expressed in normal human cells. Given the possibility of PKHD1L1 being density dependent, it would be interesting to isolate RNA from cells at differing levels of confluency and then run RT-qPCR to determine how this affects expression levels. This could provide further information on PKHD1L1's role regarding cell proliferation.

Future projects and areas of interest

These results demonstrate only the beginnings of knowledge about PKHD1L1's function and localization. The results, when taken collectively, suggest that PKHD1L1 may play a role in limiting cell growth. This role may also be correlated to cell density. This raises the question of whether or not PKHD1L1 is actually affected by cell density. More experiments done with different densities of cells could help confirm or deny this. Creating successful knockouts of PKHD1L1 that could be verified on the protein level would also lead to better conclusions on the role of PKHD1L1.

Additionally, information on the localization of PKHD1L1 is still quite sparse. Additional transfections should be run using the FLAG-tag CRISPR/Cas9 system in order to achieve more clear staining. Once localization can be discovered, assays can be tailored more closely to probably function.

The preliminary results found during this project are promising and show potential for PKHD1L1 being an important gene in tumor suppression. Determining its localization and function could not only increase knowledge on contributing factors of tumor formation but also help reveal more about cell growth regulation. Hopefully, the findings of this project can help guide further research on PKHD1L1 and tumor suppression in general.

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