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ROLE OF METHYLTRANSFERASE LIKE-3 (METTL3) IN PANCREATIC DUCTAL ADENOCARCINOMA (PDAC) PROGRESSION

Bhargavi Brahmendra Barathi

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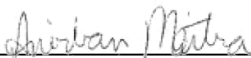
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**ROLE OF METHYLTRANSFERASE LIKE-3 (METTL3) IN
PANCREATIC DUCTAL ADENOCARCINOMA (PDAC)
PROGRESSION**


By

Bhargavi Brahmdra Barathi, B. Tech

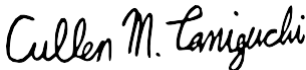
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ADENOCARCINOMA (PDAC) PROGRESSION**

A

THESIS

Presented to the faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

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In Partial Fulfilment

of the Requirements

of the Degree of

MASTER OF SCIENCE

By

Bhargavi Brahmendra Barathi, B. Tech

Houston, Texas

May 2021

Dedicated to Paati

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Abstract:

Role of Methyltransferase Like-3 (METTL3) in Pancreatic Ductal Adenocarcinoma (PDAC) Progression

Bhargavi Brahmendra Barathi, B. Tech; Advisor: Dr. Anirban Maitra, MBBS

Pancreatic Ductal Adenocarcinoma (PDAC) is an aggressive cancer with about a 10% five-year survival rate. The grim prognostic situation of pancreatic cancer patients underlines the need to identify novel molecular targets. Recent studies have brought to attention, the need to therapeutically exploit epigenetic pathways, apart from only targeting genetic mutations to effectively combat PDAC. To that effect, METTL3-mediated post-transcriptional methylation of RNA transcripts have been shown to contribute to cancer progression in multiple cancer types.

METTL3 deposits methyl groups onto adenosine bases within specific consensus sequences in RNA, resulting in the formation of N-6 methyl adenosine (m6A). m6A is the most abundant internal modification in mRNA and affects gene expression while also playing crucial roles in early-stage development, organogenesis, and cell-fate determination.

To investigate the effect of METTL3 in PDAC, we analyzed TCGA RNA-Seq expression profile data for the PAAD patient cohort and found that METTL3 expression is positively correlated with patient survival. The difference in median survival between patients expressing METTL3 at a higher level than average compared to patients expressing low levels of METTL3 was 6 months. We generated METTL3 knockdown clones of human pancreatic cancer cell lines (Panc-1 and MiaPaca-2) using shRNA targeting and performed in vitro assays to determine differences in growth and migratory characteristics upon METTL3 knockdown. METTL3 loss decreased proliferation and migration rates in MiaPaca-2 cells and decreased proliferation rate in Panc-1 cells when compared to control cell lines. As METTL3 is physiologically important in inducing differentiation and repressing pluripotent identity, we investigated the effect METTL3 knockdown exerts on EMT-associated markers in these pancreatic cancer cell lines. We found that in the epithelial cell line, Panc-1 upon METTL3 knockdown there was a reduction in E-Cadherin expression, along with a concordant increase in N-Cadherin and Vimentin expression. Our results indicate that expression of METTL3 positively regulates patient survival in PDAC, reduces proliferation and migration rates of pancreatic cancer cell lines, and induces an EMT-like phenotype.

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1. Introduction:

1.1 Pancreatic Ductal Adenocarcinoma:

Pancreatic Ductal Adenocarcinoma (PDAC) is an aggressive cancer of the exocrine section of the pancreas with a grave 5-year survival rate of about 10%.(1) Even though the Surveillance, Epidemiology, and End results (SEER) program of the National Cancer Institute only estimated PDAC to be the eleventh most common cancer in the US in 2020, which accounted for 3.2% of total new cancer cases, it was the third most leading cause of cancer-related deaths, that year. PDAC is also set to become the second leading cause of cancer-related deaths, only behind lung cancers by 2030.(2) Effective combative strategies to fight PDAC have been hindered due to a variety of factors. Lack of symptoms in the early stages of the disease leads to it being detected at a later stage, contributing to the bleak survival rate.(3) Progression of the disease occurs in distinct histopathological stages involving the sequential activation or suppression of different oncogenes and tumor suppressors, respectively. The most common pathological stage considered to be a precursor to adenocarcinoma of the pancreas is the Pancreatic Intraepithelial neoplasia (PanIN) stage. PanINs are lesions occurring in small pancreatic ducts that can only be observed under the microscope. Pathologists had originally adopted three degrees of grading the PanINs (grade 1-3), but are now moving to a two-tiered system being, low grade and high grade PanIN.(4)(5) Treating PDAC as a standard involves resecting the tumor surgically, along with administering adjuvant chemotherapy.(5) One of the key impeding factors to successful treatment has also been the development of therapeutic resistance to chemotherapy.(6) Disease recurrence can occur by way of undetected micrometastasis, also leading to a roadblock in treatment.(7)

1.2 Causes and risk factors of Pancreatic Cancer:

1.2.1 Age

Though one singular cause of pancreatic cancer is unknown, there are a plethora of risk factors that contribute to the development of the disease. Age is a known non-modifiable risk factor in the pancreatic cancer narrative. The proportion of risk involved in developing pancreatic cancer is in direct correlation with aging. Patients presenting with pancreatic cancer are usually older individuals. Diagnosing the cancer in patients under thirty years of age is uncommon.(5)(8). 65 to 74 years of age is reported to be the most frequent window when pancreatic cancer is diagnosed in in the United States, with a median age of 70.(1) The highest mortality rates are also reported within the same 65-74 years window, with the median age at death being 72.(1)

1.2.2 Sex

Some of the demographic factors that contribute to an increased risk of pancreatic cancer are sex and ethnicity. Men are, on average more likely to develop pancreatic cancer compared to women.(1) According to the Global Cancer Observatory, in 2020, males across the globe had an age-standardized rate (ASR) of incidence of pancreatic cancer of 5.7, compared to women that had an ASR of 4.1.(9) Although some studies suggest that this variation may be brought about by environmental risk factors such as a higher prevalence of smoking among men, it is unlikely to be the sole cause. Other host genetic factors that could potentially be contributing to this higher incidence in men are yet to be uncovered.(10)

1.2.3 Ethnicity

Historically, racial differences have been attributed to display significant variation in pancreatic cancer incidence and mortality rates. In the United States, patients of African American origin report higher numbers when compared to Caucasians and Asian Americans.(8) Ashkenazi Jews

are also known to possess a higher susceptibility to developing pancreatic cancer as a result of their genetic make-up.(11)(12)

1.2.4 Smoking

Several studies have been conducted to determine the effects various environmental risk factors exert on pancreatic cancer incidence. A conclusive detrimental relationship between smoking and about two-fold increase in risk of developing pancreatic cancer has been established.(13)(14) Independent studies on both animals and epidemiological data have confirmed that cigarette smoking and tobacco are carcinogens, in the pancreatic context.(11) Some groups have reported a dose-dependent effect to the number of cigarettes smoked and length of exposure, while some have claimed an inconclusive correlation.(13) A 2012 study revealed that nicotine, one of the major constituents of a cigarette, activates Src kinase promoting pancreatic cancer progression and metastasis.(15) Alcohol consumption, on the other hand has not revealed a convincing correlation to be a risk factor for pancreatic cancer incidence.(13)

1.2.5 Alcohol

The results from various studies determining the degree of risk alcohol poses to pancreatic cancer incidence have been mixed and the field seems to be split on the verdict. However, alcoholism or chronic overindulgence of alcohol is a major contributor to chronic pancreatitis, which is a well-documented risk factor for PDAC incidence.(16) This warrants us to treat alcohol as a potential risk factor for pancreatic cancer incidence and advocate for decreased alcohol consumption, as part of a holistic lifestyle change.

1.2.6 Obesity

Obesity is an important pancreatic cancer risk factor that has a bearing on millions of people across the globe, ever more so in a developed nation, such as the United States. Individuals with a Body Mass Index (BMI) of greater than or equal to 30, are considered obese and possess a less than

fivefold increase in risk of PDAC incidence.(12) A World Cancer Research Fund pancreatic cancer report in 2012, referenced several studies that demonstrated a higher risk of pancreatic cancer in obese individuals and showed that for increases in every 5 BMI units, the risk increased by 10%, regardless of gender of the patient.(5) Obesity has also been associated with the onset of Type 2 diabetes, which is a known risk factor for pancreatic cancer.

1.2.7 Chronic Pancreatitis

Chronic pancreatitis (CP), as the name suggests, is an inflammation of the pancreas that progresses over time. It is accompanied by acinar cell destruction, fibrosis, and an eventual inability of the patient to digest food and secrete pancreatic hormones.(8)(17) CP is considered a risk factor that predates pancreatic cancer in patients. Although it is a risk factor, there is considerable overlap between most symptoms of the two conditions, making the diagnosis of pancreatic cancer difficult.(13) Chronic pancreatitis can be caused by hereditary (somatic mutations), environmental (alcohol or cigarette smoking), or idiopathic factors. It has been reported that when comparing alcohol related chronic pancreatitis with genetic idiopathic chronic pancreatitis, the latter poses a higher risk of pancreatic cancer.(18) Of all the chronic pancreatitis patients, when we monitor them over a 20-year period, about 5% of them develop pancreatic cancer.(5)(19)

1.2.8 Genetic Factors

Pancreatic cancer, at the root of the disease is genetic and can be caused by inherited and acquired mutations. Family history also plays a significant role as a risk factor contributing to pancreatic cancer incidence, wherein it can be classified as familial incidence of the cancer. Familial pancreatic cancer is defined as when two or more first-degree relatives of a patient currently have or previously have been diagnosed with pancreatic cancer, in the absence of other known genetic conditions.(5)(12) There is an exponential increase in risk with the number of first-degree relatives involved that have pancreatic cancer. Specific familial genetic syndromes also contribute to an

increased risk of developing pancreatic cancer, through germline mutations in certain genes. Some of them include hereditary breast cancer (mutations in BRCA1, BRCA2), hereditary pancreatitis (PRSS1 gene is affected), familial atypical multiple-mole melanoma (p16/CDKN2A is mutated), and Peutz-Jeghers syndrome (STK11/LKB1 mutations).(5)(8)¹³(20)(21)

A majority of the pancreatic cancer cases arise due to mutations that occur randomly, as opposed to inherited familial cancer.(8) Though many hundreds of genes are mutated in a pancreatic cancer tumor, variable across patients, a select list of genes have been identified to be common across the patient population, believed to be driving this malignancy. They include mutations in well-known cancer associated genes and other relevant signaling pathways. Collectively known as the genetic drivers of PDAC, their expression patterns have been shown to denote the sequential histopathological stages of the disease.(22)

1.3 Common genetic drivers of PDAC:

Mutations in the K-RAS oncogene were the first to be discovered as playing a role in pancreatic cancer initiation.(23) Point mutations arising in codon 12 of the oncogene, activate it to express a mutated form of the Ras protein. These mutations, substituting the glycine with now, valine, aspartate, or arginine are detectable in about 30% of early neoplastic lesions and are almost ubiquitous in advanced tumors.(22)(24) K-RAS mutations have been shown to be the earliest genetic perturbation in the progression of PanINs in humans.(22) Activating oncogenic mutations in K-RAS bring about downstream cellular effects promoting tumor growth by engaging different signaling pathways including RAF-MAPK and PI3K.(22)(25)(26)

Loss of the CDKN2A gene and mutations in the tumor-suppressor P53 are also both commonly observed in the later stage of PDAC progression.(25) Mutations in the P53 gene occur as missense alterations in later stage PanINs. CDKN2A gene encodes two tumor suppressor genes at the 9q21

locus, INK4A and ARF. With the loss of CDKN2A, both INK4A and ARF are lost in most pancreatic tumors.(23)

SMAD4 is also a frequently altered gene in pancreatic cancers, which is an important regulator of the TGF- β signaling pathway. This mutation is observed in approximately half of the pancreatic cancer population.(23)

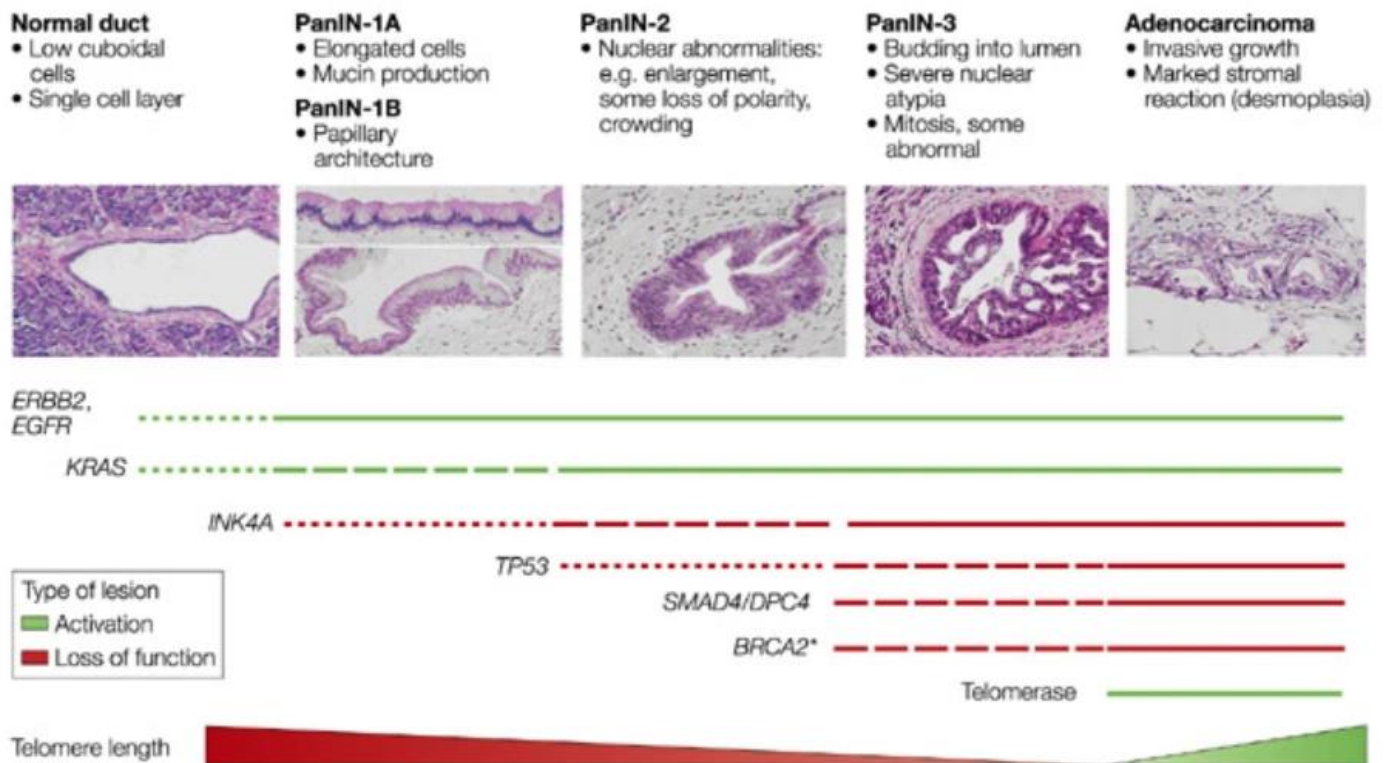


Figure 1: Histopathological progression of pancreatic cancer with temporal expression of genetic drivers. (Bardeesy, N. & DePinho, R. A. Pancreatic cancer biology and genetics. *Nat. Rev. Cancer* 2, 897–909 (2002). Used with permission from Copyright Clearance Center. License number – 5038411365197)

1.4 METTL3 and N-6 methyl adenosine (m6A):

Methyltransferase-like 3 (METTL3) is a 70kDa protein that is encoded by the METTL3 gene and methylates RNAs, including but not limited to mRNA, tRNA, lncRNA.(27) METTL3 along with METTL14 and WTAP proteins forms the methyltransferase writer complex or the m6A-METTL complex (MAC complex).(28) METTL3 acts as the methylator in the complex, and hence referred to as the writer. METTL14 provides structural support to METTL3 but does not methylate RNA transcripts.(29) METTL3 catalyzes the addition of a methyl group onto specific adenosine residues in the RNA, forming N-6 methyladenosine. This methylation is not a stochastic process but occurs within identified consensus motifs in the RNA. In the mRNA, the methylation is also preferentially enriched close to the stop codon, 3' UTR and long internal exons.(30) There are other key protein players in the methylation cascade known as readers and erasers. Erasers reverse the methylation, reverting the molecule to an adenosine once again. Readers, like the YTHDF and IGF2BP family of proteins recognize and bind to the methyl mark to bring about downstream effects on gene expression. ALKBH5 and FTO are two key demethylases considered erasers of this modification.(31),(32)

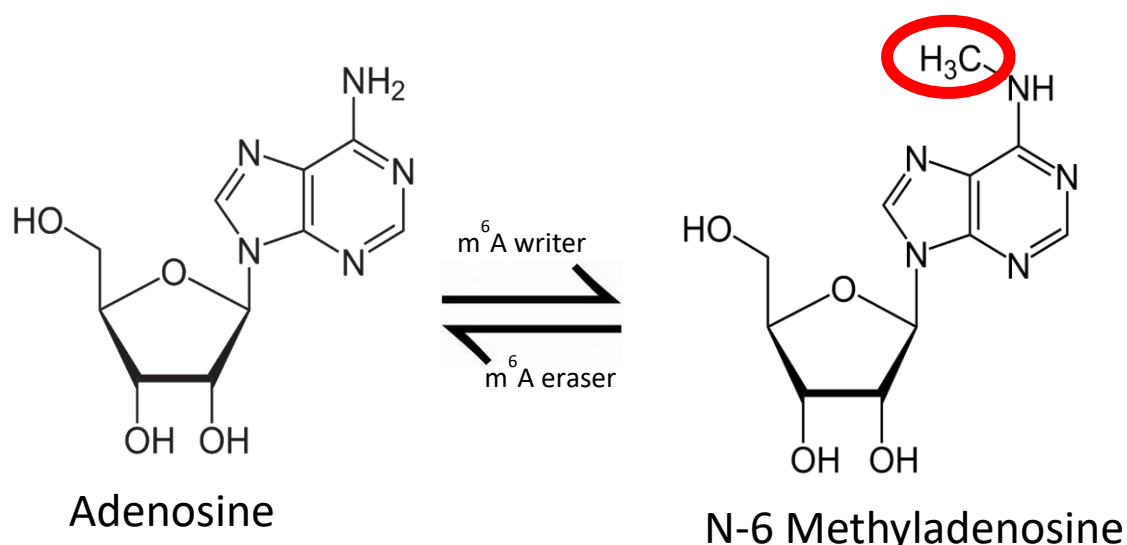


Figure 2: Chemical structures of METTL3-mediated m6A RNA methylation

1.5 Physiological effects and functions of METTL3 mediated m6A RNA methylation

m6A RNA methylation leads to a variety of effects on gene expression. Reader proteins are recruited to the methylation site that can then recognize and bind to effect these downstream changes. mRNA structure, mRNA splicing, mRNA half-life, mRNA export, and mRNA translational efficiency are influenced by the deposition of m6A marks.(32) YTHDF1 increases translational efficiency of methylated RNA transcripts while YTHDF2 reduces half-life of methylated transcripts. These are two key downstream effects of the m6A RNA methylation that have the most significant bearing on the human transcriptome.

From previously published studies understanding normal physiology, we know that METTL3 plays a key role in regulating early embryogenesis,(33) circadian rhythm of metabolic processes,(34) innate immunity,(35) and long-term memory(36) among a whole host of other functions. An important contribution of METTL3 and its associated methylation is the role it plays in early development and cellular differentiation. Geula et al. showed that METTL3 is important in regulating the transition of naïve pluripotent cells to differentiating towards a specific lineage. They cultured mouse embryonic stem cells (mESC) from a mouse with a METTL3 knockout (KO) and outlined that when conditioned to develop into a specific lineage, the cells still showed sustained expression of pluripotent factors, failing to differentiate.(37)

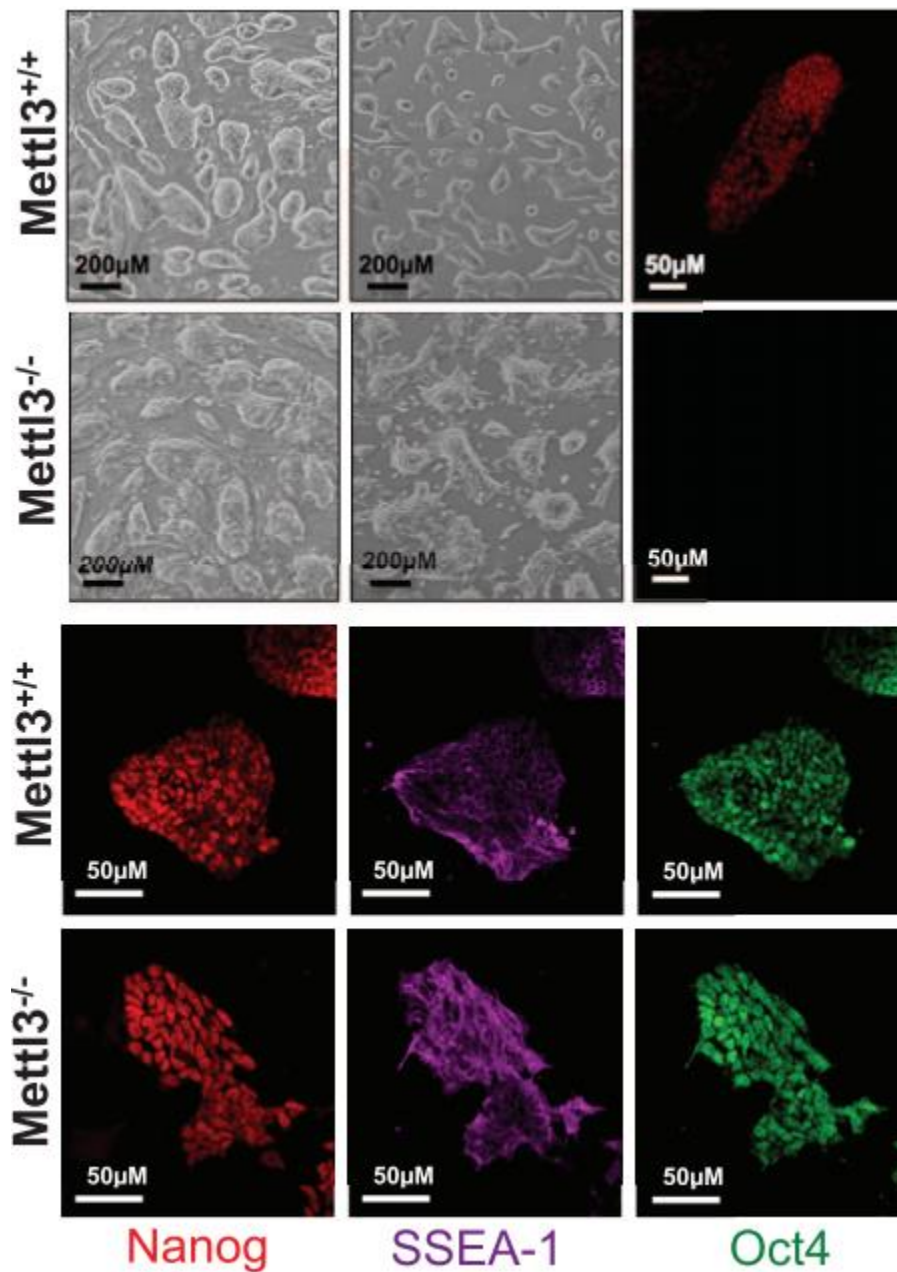


Figure 3: Embryonic stem cells with METTL3 knockout maintain pluripotent identity and fail to differentiate into a specific lineage.

(Geula, S. *et al.* m6A RNA methylation facilitates resolution of naive pluripotency toward differentiation. *Science* (80-.). 347, 1002–1006 (2015). Used with permission from Copyright Clearance Center. License – 5035930693527)

1.6 METTL3 and m6A methylation in cancer:

METTL3 expression and METTL3-mediated m6A RNA methylation have been recently uncovered to be playing critical roles in a variety of different cancers. METTL3, according to previous work by other groups can affect malignancies by playing tumorigenic or tumor suppressive roles, both directly by expression of the protein and/or deposition of the methyl mark on specific RNA transcripts.

In most of the cancers where researchers have elucidated the mechanism as to how METTL3 affects the pathogenesis of that cancer, we can note that METTL3 methylates specific RNAs, modifying them post-transcriptionally to now contribute to the carcinoma. For example, in lung adenocarcinoma, METTL3 mediated m6A methylation on YAP, TAZ genes increase their translational efficiency, leading to an elevated expression. This elevation in expression increases cell proliferation and invasion. The normal physiological downstream effect of increase in translation when m6A methylation occurs is exploited by cancer cells to further their cause.(38) Similarly, in acute myeloid leukemia, there occurs a preferential increase in methylation in genes regulating apoptosis and differentiation, like MYC, BCL2, and PTEN.(39) As these examples showcase, in both these cases METTL3 plays a tumor promoting role and aids in the progression of the cancer.

On the contrary, the overall survival of renal cell carcinoma (RCC) patients showed a positive correlation to METTL3 expression levels. Additionally, there was an increase in proliferation and migration in RCC cell lines when METTL3 was knocked down. Xiao et al noted that METTL3 acts as a tumor suppressor in renal cell carcinoma by potentially affecting the PI3K/Akt/mTOR pathway.(40) In PDAC, however METTL3 and/or METTL3 mediated m6A methylation affects the progression of the cancer is not known.

1.7 METTL3 and Epithelial to Mesenchymal Transition in cancer:

One of the noteworthy ways in which METTL3 affects cancer progression is by methylating specific transcripts involved in key pathways, contributing to cellular proliferation and survival of the tumor. In Hela cells, the increased abundance of m⁶A marks on Snail mRNA induces translation and drives the cells towards epithelial to mesenchymal transition (EMT).(41) EMT is a phenomenon of cancer cells wherein which they lose epithelial characteristics while acquiring mesenchymal characteristics, allowing them to migrate and form secondary tumors or metastasis.(42) This is induced by the repression of specific proteins conferring an epithelial state (E-Cadherin, Occludin, Claudin, Cytokeratin) and the activation of proteins associated with the mesenchymal state (N-Cadherin, Vimentin, Fibronectin).(42) While cancer cells undergo EMT, they also acquire stem-cell like features. METTL3 expression as detailed above, is important in the repression of pluripotent identity. These factors present the need to explore the role METTL3 and m6A methylation play in the induction of EMT in cancer.

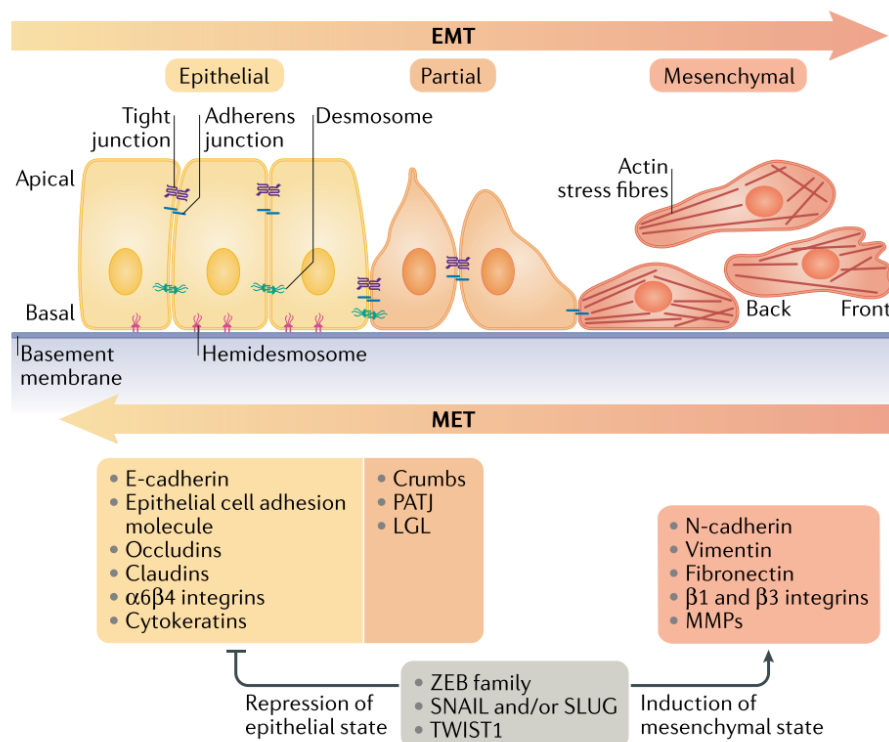


Figure 4: Epithelial to Mesenchymal Transition in Cancer

(Dongre A, Weinberg RA. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. *Nat Rev Mol Cell Biol.* 2019;20(2):69-84. doi:10.1038/s41580-018-0080-4. Used with permission from Copyright Clearance Center. License – 5058391407002)

2. Hypothesis and aims:

The proportion of pancreatic cancer incidence rates to the mortality rates every year in the US is almost close to 1, showcasing the aggressive nature of the cancer. The disease is invariably fatal with only 9.3% of patients having survived beyond the 5-year mark between 2009 and 2015 according to the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute. Treatment options, though have continuously evolved, over the last few decades, there has only been a marginal increase in survival rates. Given the heterogeneity in treatment outcomes among pancreatic cancer patients, there is a timely clinical need to investigate novel genes and molecular signaling pathways.

In understanding the influence of epigenetic modifications on cancer initiation and progression, various groups have implicated RNA methyltransferase, METTL3 in a slew of different cancers. METTL3 adds a methyl molecule onto specific adenosine residues in the RNA, giving rise to the modification, N-6 methyl adenosine (m6A). Both METTL3 and m6A, depending on the cancer context have been shown to play tumorigenic and tumor-suppressive roles. In the case of PDAC, the role of METTL3/m6A methylation and the mechanisms of how it affects pancreatic cancer are not known. We sought to answer the above question to address this gap of knowledge.

Based on our findings from investigating the relationship between METTL3 expression and overall pancreatic patient survival from TCGA, we hypothesized that METTL3 expression impacts pancreatic cancer progression. The specific aims designed to test this hypothesis were as follows:

Aim 1: To investigate the effect of METTL3 on proliferative and migratory characteristics of pancreatic cancer cells

Aim 2: To assess the effect of METTL3 loss on EMT-associated markers in pancreatic cancer cells

3. Materials and Methods:

3.1 Analyzing TCGA dataset:

METTL3 FPKM values of 156 patients in the TCGA PAAD cohort were analyzed against the clinical information from UC Xena. Based on average METTL3 expression, the patient cohort was divided into two groups: high METTL3 expressing and low METTL3 expressing. Kaplan Meier curves were plotted to determine the correlation between METTL3 expression and patient survival, with available clinical data. Patients with neuroendocrine carcinoma, mucinous adenocarcinoma, or any other categorization of the disease were removed from consideration in the cohort, as we strictly looked at PDAC patients. The METTL3 expression levels were also correlated with other relevant clinical information using statistical tools.

3.2 Cell lines/cell culture:

Human pancreatic cancer cell lines Panc-1, MiaPaca-2 and mouse PKCY cell lines were used in this study. The human lines were obtained from ATCC and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1x Penicillin-Strep (P/S) solution. Mouse PKCY cell lines were cultured from harvested pancreases of PKCY mice maintained as part of the lab's animal protocol. These 2D mouse cell lines were also cultured in DMEM supplemented with 10% FBS and 1x P/S solution.

3.3 Plasmid Production:

Bacterial stocks of MISSION shRNA targeting human and mouse METTL3 were purchased from Sigma. The bacterial stock was streaked onto an agar plate using sterile techniques and allowed to incubate at 37 degree Celsius for 12-16 hours. Bacterial colonies that grew on the plate were picked and inoculated in LB broth of 5ml volume and allowed to shake in a temperature-controlled shaker

for 12-16 hours overnight at 37 degree Celsius. Plasmid was isolated from a portion of the starter culture using a mini-prep kit and sent for sanger sequencing to confirm the identity of the genetic sequence. The remaining starter culture was used to inoculate a bigger volume of LB broth to obtain more plasmid DNA using the maxi-prep kit as was appropriate according to the initial volume of the culture. Plasmid DNA that was obtained was stored at -20 degree Celsius and used for lentivirus production.

3.4 Lentivirus Production:

To produce lentivirus carrying the appropriate transfer plasmid (targeting human or mouse METTL3 in this context), we used HEK293T cells. A low passage number of HEK293T cells were plated and cultured in complete media containing DMEM, 10% FBS, 1x P/S, and 1mM sodium pyruvate for about 20-24 hours. The media was then changed to antibiotic-free media, followed by addition of the transfection mix to the plate. The transfection mix included the lentiviral packaging plasmid (psPAX2), the lentiviral envelope expressing plasmid (pMD2.G - VSV-G) and our desired transfer plasmid in a solution containing Opti-MEM and lipofectamine 3000. The cells were allowed to incubate with the transfection mix overnight, before changing the media back to complete media. The supernatant produced by the cells, which constituted the lentivirus produced was centrifuged, filtered, and flash frozen 48h later for further use.

3.5 Transfection:

The transfection protocol was adapted from Addgene to generate stable cell lines expressing our gene of interest (a knocked down version of the METTL3 gene in this case). Five different lentiviral dilutions were created in a 6-well plate with DMEM complete media containing 10ug/ml polybrene, along with a no-virus control. DMEM complete media constituted of DMEM with 10% FBS, 1x P/S, and 4mM L-alanyl-L-glutamine. A reverse transduction was performed by seeding 50,000 cells into each

well of the 6-well plate, that already contained 0.5ml of the different lentiviral dilutions. The cells were incubated with the virus for 48-72h and monitored by observing under the microscope each day. Post the incubation, the media was changed to contain media containing puromycin to start the selection process. The no-virus control well was used to monitor the death of the cells and gauge the rate of selection in other wells. A particular well with the appropriate dilution of virus was selected based on the degree of cell killing using Poisson statistics principles to ensure that only one copy of the virus is incorporated into majority of the cells transfected. Once confluent, the growing polyclonal population of resistant cells in the selected well was expanded into a 10cm dish and further on in bigger plates. The polyclonal population obtained was tested for protein expression using Western Blotting and single-cell clones were created from them using a limiting dilution technique.

Panc-1 and MiaPaca-2 cell lines were transfected with lentivirus containing shRNA sequence targeting the human METTL3 gene. Polyclonal and single-cell clones from them were obtained using the above-described protocol. This work was carried out by Dr. Fredrik Thege in the Andrew Rhim lab. Mouse PKCY lines were transfected with lentivirus containing shRNA sequence targeting the mouse METTL3 gene. Polyclonal populations from different mouse lines were created by the same protocol by this author.

Sigma MISSION shRNA	Sequences of shRNA used	Species targeted by shRNA
TRCN0000039110 (referred to as shRNA 110)	CCGGGCAGAGCAAGAGACGAATTATCTCGAGATAATTCGTCTCTTGCTCTGCTTTTTG	Mouse
TRCN0000039111 (referred to as shRNA 111)	CCGGCCTCAGTGGATCTGTTGTGATCTCGAGATCACAACAGATCCACTGAGGTTTTTG	Mouse
TRCN0000039112 (referred to as shRNA 112)	CCGGCGTCAGTATCTTGGGCAAATTCTCGAGAATTTGCCCAAGATACTGACGTTTTTG	Mouse
TRCN0000039113 (referred to as shRNA 113)	CCGGGCACCCGCAAGATTGAGTTATCTCGAGATAACTCAATCTTGCGGGTGCTTTTTG	Mouse
TRCN0000289812	CCGGCGTCAGTATCTTGGGCAAGTTCTCGAGAACTTGCCCAAGATACTGACGTTTTTG	Human
SHC002	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT	Non- targeted control

Table 1: Description of different test shRNA plasmid sequences targeting METTL3

3.6 Western Blotting:

Panc-1 and MiaPaca-2 METTL3 knockdown cell lines were plated in 6-well plates 48h prior to protein collection and allowed to grow to confluency. Protein lysates were collected from the cells by cell scraping upon addition of RIPA buffer. RIPA buffer was prepared by adding 1x volume of protease inhibitor. 60ul of RIPA buffer was used per well of a 6-well plate. Protein lysate obtained was shaken and mixed using a rotor at 4 degree Celsius for 45 minutes. The lysate was then centrifuged at 15000g for 10 minutes to remove debris. Protein supernatant obtained was quantified using Bio Rad DC Protein Quantification Assay.

Total protein of 15ug was loaded into each well and a 4-15% BioRad gradient gel was used. We ran the gel at 60V for the first ten minutes and at 90V for the remainder of the time. Once the gel was separated enough (indicated by the Bio Rad Precision Plus Dual Color Standards Ladder), transfer to a PVDF membrane was carried out through the Bio Rad Trans-Blot Turbo Transfer system at a mixed setting of 25V, 7 min. Prior to transfer, the PVDF membrane was equilibrated in 1x Trans-Blot Turbo Transfer buffer and methanol for activation.

Post transfer, the membrane was blocked for 2 hours at room temperature using 3% BSA in TBST. 1:2000 concentration of the primary anti-rabbit Abcam METTL3 antibody (EPR 18810; ab195352) was used, and the membrane was incubated overnight at 4 degree Celsius. The membrane was washed the next day thrice with TBST and incubated for 50 minutes at room temperature in 1:2000 concentration of dilute HRP secondary antibodies. Post incubation, the membrane was washed thrice again in TBST and imaged using Bio Rad's Clarity ECL chemiluminescent substrate. The gel images obtained on the Chemi Doc were processed using image analysis software, Fiji - Image J. Primary Antibodies used to determine EMT expression levels were Cell signalling systems anti-rabbit E-Cadherin at 1:1000 concentration, Cell Signalling systems anti-rabbit Vimentin at 1:1000

concentration, and BD Biosciences anti-mouse N-Cadherin at 1:1000 concentration. B-actin or Hsp90 were used as internal loading controls, based on the size of the desired protein product.

The same western blotting protocol was used on the PKCY mouse METTL3 knockdown cell lines as well.

Antibody	Clone	Host species	Concentration	Manufacturer
METTL3	EPR 18810; ab 195352	Rabbit	1:2000	Abcam
E-Cadherin	24E10 mAb #3195	Rabbit	1:1000	Cell Signaling systems
N-Cadherin	32/N-Cadherin RUO Catalog no. - 610920	Mouse	1:1000	BD Biosciences
Vimentin	D21H3 mAb # 5741	Rabbit	1:1000	Cell Signaling systems

Table 2: Description of the various antibodies used in this study

3.7 Quantitative Polymerase Chain Reaction:

Panc-1 and MiaPaca-2 METTL3 knockdown cells were plated in 6-well plates 48h prior to RNA extraction and allowed to grow to confluency. RNA lysates were collected from the cells by adding RLT buffer followed by cell scraping. The lysates were collected with the samples and buffers being kept on ice, to minimize RNA degradation. RLT buffer was prepared by adding 10ul B-mercaptoethanol for every 1000ul volume of buffer. The collected lysates were passed through a 25–27-gauge syringe about ten times to thoroughly homogenize the sample. The lysate was then passed through the Qiagen RNeasy mini kit column to isolate purified RNA. Isolated RNA was quantified using the Nanodrop 2000 to determine purity and concentration.

2ug of isolated RNA was used to create cDNA using the Invitrogen Super Script Reverse Transcriptase III. 25-50ng/ul of generated cDNA was used in setting up the qPCR assay. Thermo Fisher's Power SYBR Green Master Mix was used to make the working solution with cDNA along with appropriate primers. The reaction was set up in a 96-well plate and run according to manufacturer's instructions. Relative gene expression was measured using the standard ($\Delta(\Delta C_t)$) comparison. Human beta-2-microglobulin was used as housekeeping internal control gene.

3.8 Proliferation Assay:

3000 cells/well were plated in 96-well plates in to assess the proliferative characteristics of Panc-1 and MiaPaca-2 METTL3 knockdown cell lines. 1x concentration of Incucyte NucRed was also added to the cells while plating as a nuclear stain to aid in the cell count. The plate was set up on an imaging schedule and the cells were allowed to grow in the Incucyte. Phase confluence and red object count images were taken every 4 hours for 5 days. After the completion of the 5 days, data collected was used to plot a growth curve of time vs cell count in knockdown and control conditions of these cell lines. Doubling times calculated from the growth curve statistics was used as a measure of

proliferative ability. Three independent experiments were conducted reproducibly, making it a sample size of n=3.

3.9 Transwell Migration Assay:

Transwell migration assay was performed to determine the migration rates of Panc-1 and MiaPaca-2 cell lines upon METTL3 knockdown. This assay measures the chemotactic ability of the cells migrating towards a chemo attractive gradient. Falcon Permeable Support for 6-well plate with 8.0um Transparent PET membrane sterile inserts from Corning were used in this study. 300000 cells/well were plated on top of the transwells in serum-free DMEM media (containing 1x P/S antibiotic) and allowed to migrate to the other side of the transwell. A chemo attractive gradient of serum was created by adding DMEM with 10% FBS (also containing 1x P/S antibiotic) on the other side of the transwell, making sure the media touches the membrane on the transwell on top of the well. Panc-1 and MiaPaca-2 cells were tested and optimized to migrate for 24h and 16h, respectively prior to the experiment. Once the appropriate incubation times for each cell line were elapsed, media on top of the inserts were aspirated and the transwell was moved to a new plate. A cotton-tipped applicator was used to remove any remaining media and cells that may not have migrated from the top of the transwell. Fixing/staining solution was added to the underside of the transwell insert, now placed in a new plate, and allowed to incubate at room temperature for 15 minutes. Post incubation, the transwells were washed and allowed to dry, followed by imaging them on a microscope and counting the fraction of migrated cells. Composition of the fixing/staining solution is as follows:

0.5g Crystal Violet (0.05% w/v)

27ml 37% Formaldehyde (1%)

100ml 10x PBS (1x)

10ml Methanol (1%)

863ml Distilled water to make up volume to 1L

3.10 Statistical Analysis:

GraphPad Prism version 8 was used to statistically analyze the results obtained. Error bars represent standard error of the means. P-value ≤ 0.05 were considered statistically significant. N=3 was the sample size for experiments unless specified otherwise.

4. Results:

4.1 METTL3 expression is positively correlated with survival in PDAC patients:

To determine the correlation between overall survival and METTL3 expression levels in PDAC patients, METTL3 FPKM values from the TCGA-PAAD cohort and clinical information from USC Xena were used. The patient cohort, based on the average level of METTL3 expression was split into two groups – high METTL3 expressing, low METTL3 expressing. Based on the clinical survival data available, a Mantel-Cox (log rank) test was performed to analyze overall survival as a function of METTL3 gene expression levels. A Kaplan-Meier curve was plotted to visualize the correlation between survival and METTL3 expression levels. We found that the patient cohort expressing higher levels of METTL3 than average, tended to survive longer than the patient cohort lower than average levels of METTL3. The difference in median survival was 6 months significantly longer in the patient group that showed higher than average levels of METTL3. P-value for the log rank test was 0.04, which rejects the null hypothesis that both survival curves are not significantly different. We thus found that METTL3 expression positively correlated with survival. Median survival in days for each patient group is indicated in table 3.

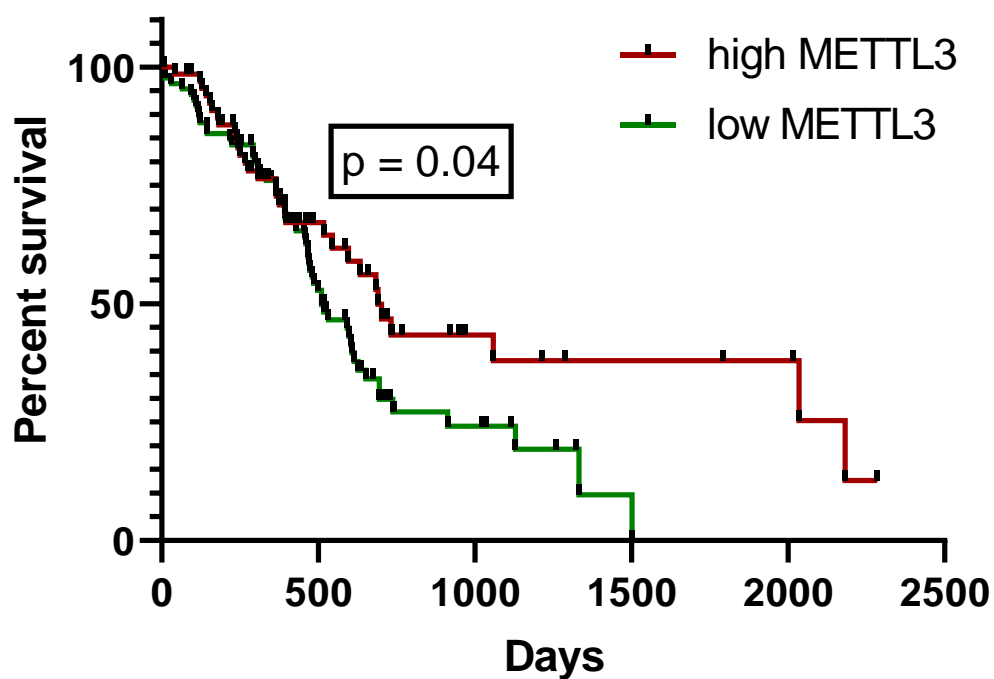


Figure 5: Analyzing TCGA PAAD Cohort Data - Kaplan-Meier curve depicting the correlation between overall survival rates and METTL3 expression in PDAC patients. Patients expressing high and low levels of METTL3 are shown in red and green, respectively.

Patient group	Median Survival (days)
High METTL3 expressing	691
Low METTL3 expressing	511

Table 3: Median survival of high METTL3 expressing group is higher than the low METTL3 expressing group by 6 months

4.2 Generating METTL3 knockdown cell lines:

To investigate the effect of METTL3 on the proliferative and migratory properties of human pancreatic cancer cells, we set out to generate a cellular system devoid of METTL3 expression. Panc-1, MiaPaca-2 and PKCY mouse cell lines were lentivirally transfected with the appropriate shRNA targeting human or mouse METTL3 gene using the adapted Addgene transfection protocol as described in the materials section. Dr. Fredrik Thege created single-cell clones from the transfected polyclonal populations of Panc-1 and MiaPaca-2 cell lines. The single-cell clones generated were assigned numbers and would from hereon be described as follows:

Clone generated	Nomenclature assigned
Panc-1 METTL3 KD Clone 2	PC2
Panc-1 METTL3 KD Clone 4	PC4
Panc-1 METTL3 KD Clone 5	PC5
Panc-1 METTL3 KD Clone 6	PC6
Panc-1 Non-targeted control	PNT
MiaPaca-2 METTL3 KD Clone 5	MC5
MiaPaca-2 METTL3 KD Clone 6	MC6
MiaPaca-2 METTL3 KD Clone 7	MC7
MiaPaca-2 METTL3 KD Clone 11	MC11
MiaPaca-2 Non-targeted control	MNT

Table 4: Nomenclature assigned to the different knockdown clones generated

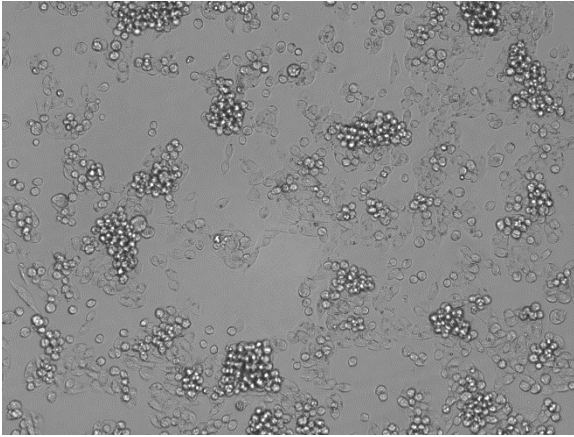
Pancreases of mice with genotype p53 L/L Kras G12D p48Cre Rosa Y/Y (PKCY) were harvested and cultured to generate mouse METTL3 knockdown pancreatic cancer cell lines. In these mice, cre

expression (C) is driven under the pancreas-specific p48 promoter that allows for the expression of mutant G12D Kras (K), loss of the P53 allele (P), and expression of the lineage label marker YFP (Y). Mice containing these four alleles are hence termed PKCY mice. Mice with identifiers as described in Table 5 were utilized. Single-cell clones were not generated from these murine METTL3 knockdown polyclonal populations.

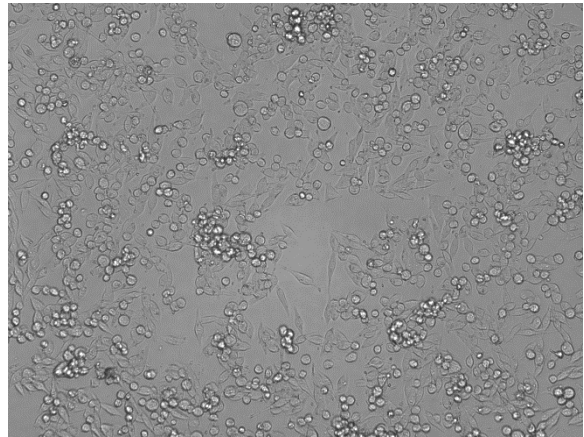
Mouse number	Genotype
14292	p53 L/L Kras G12D p48Cre Rosa Y/Y (PKCY)
16686	p53 L/L Kras G12D p48Cre Rosa Y/Y (PKCY)
17315	p53 L/L Kras G12D p48Cre Rosa Y/Y (PKCY)

Table 5: Identifier and genotype of the mice used to generate PKCY METTL3 knockdown cells

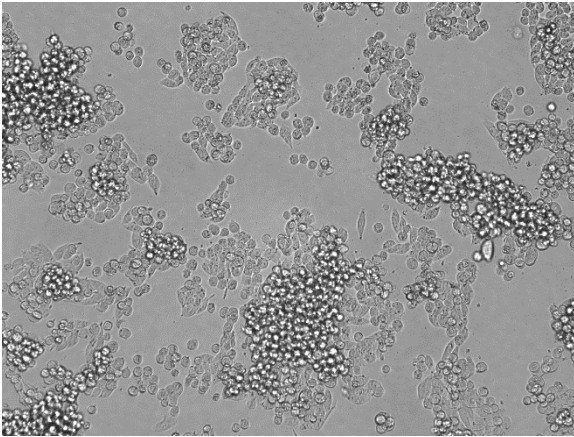
Figure 5 and 6 as follows showcase the morphology of the MiaPaca-2 and Panc-1 METTL3 KD single cell clones respectively. MiaPaca-2 cells are mesenchymal-like cells with an elongated morphology. Panc-1 cells on the other hand are epithelial-like cells that are polygonal, adherent and exhibit a cobblestone like morphology. Panc-1 cells upon METTL3 KD do not exhibit a characteristic change in morphology, compared to the non-targeted control. Whereas MiaPaca-2 cells seem to become less elongated and more clustered, almost exhibiting an epithelial-like morphology compared to control, when METTL3 is knocked down.



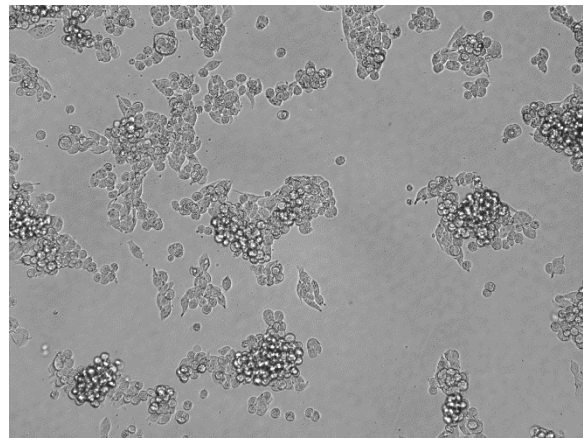
MC5



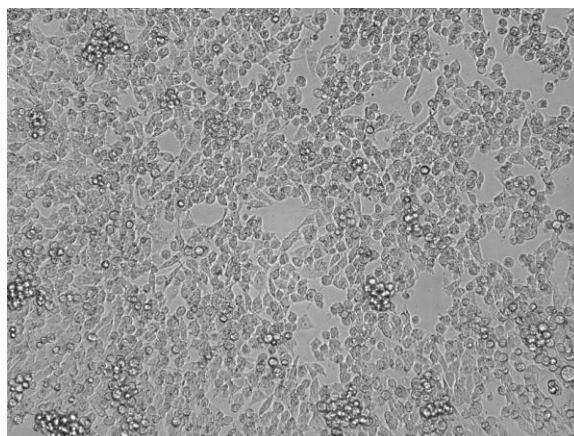
MC6



MC7

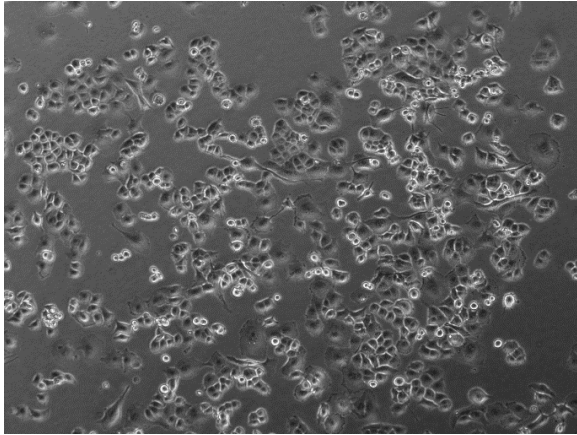


MC11

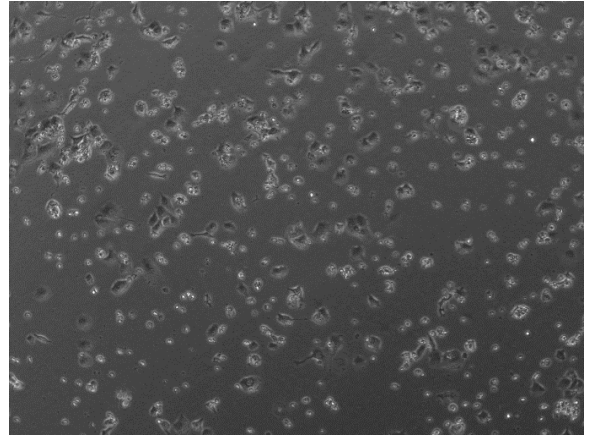


NT Control

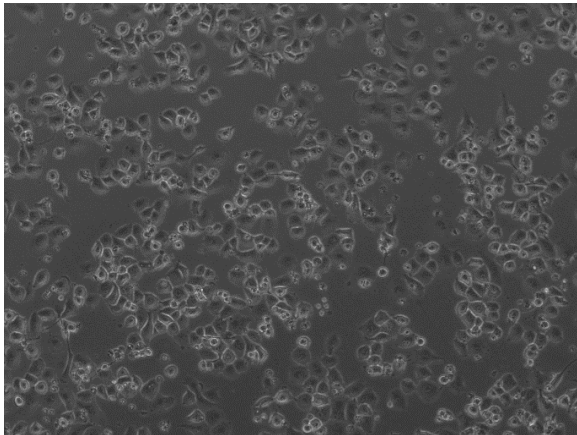
Figure 6: Morphology of MiaPaca-2 METTL3 knockdown single cell clones



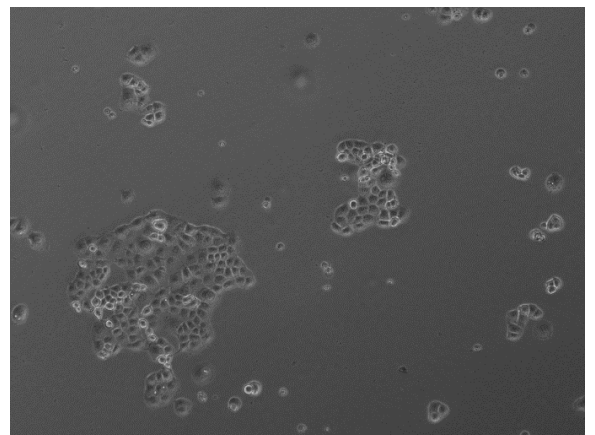
PC2



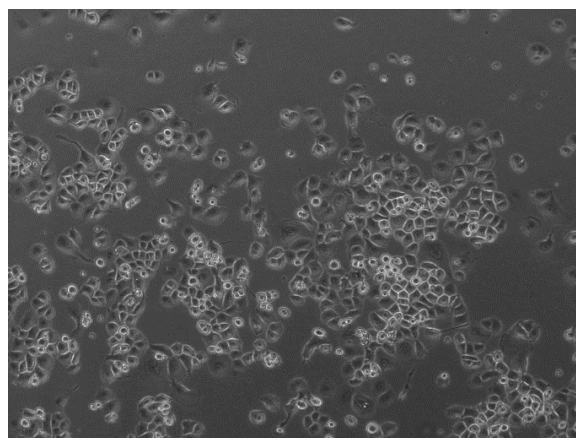
PC4



PC5



PC6



NT Control

Figure 7: Morphology of Panc-1 METTL3 knockdown single cell clones

4.3 Confirmation of METTL3 knockdown:

4.3.1 Western Blotting:

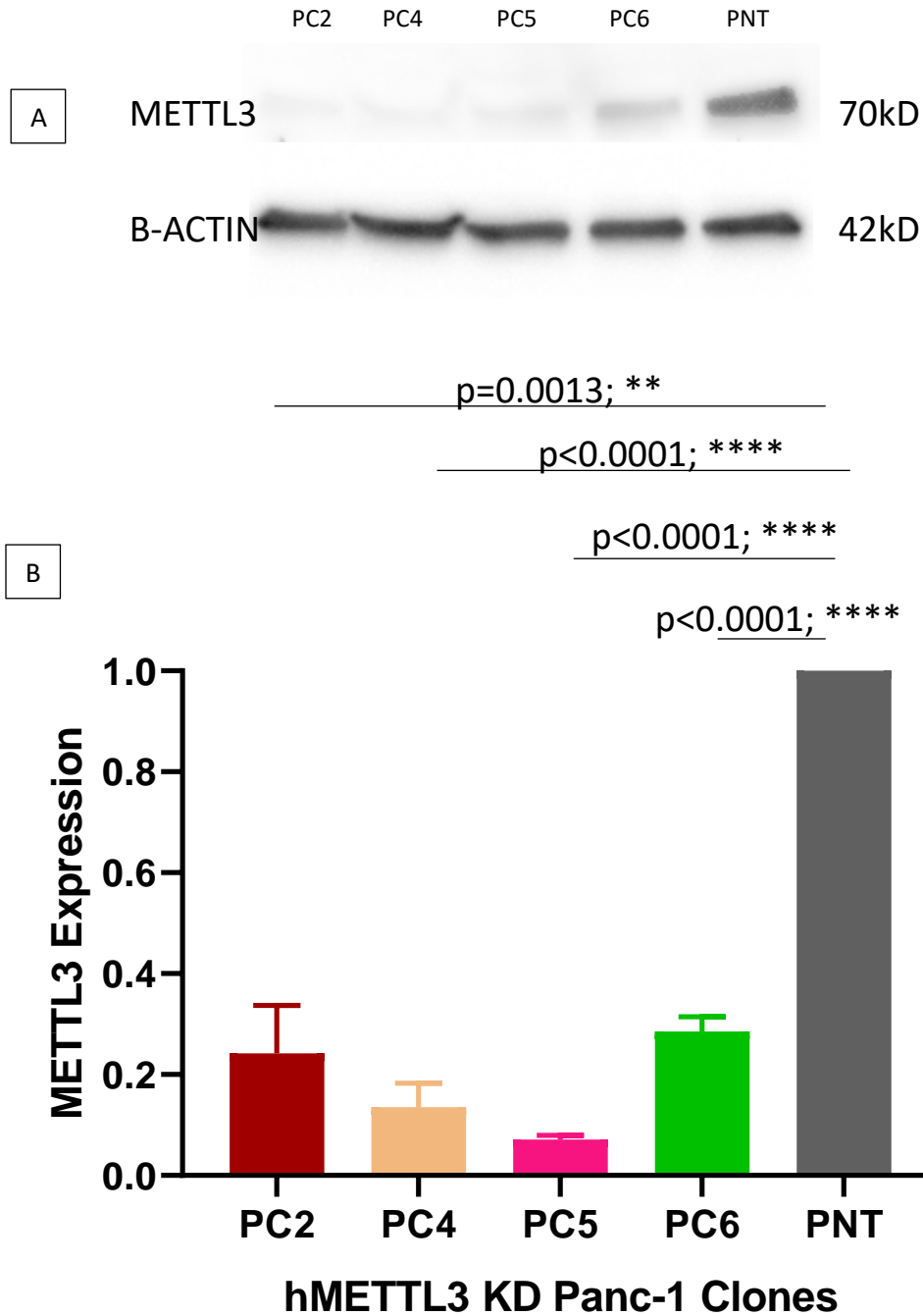


Figure 8: Confirmation of METTL3 knockdown in Panc-1 cells using Western Blotting

- A) Western blot denotes a decreased level of METTL3 expression in the Panc-1 single cell clones compared to the non-targeted control
- B) Bar graph indicates the level of METTL3 expression in the different Panc-1 single cell clones upon METTL3 knockdown

To test the level of METTL3 expression in the cellular system generated, we performed western blotting. Single-cell clones generated from the Panc-1 METTL3 knockdown polyclonal population were tested for the knockdown in expression levels of METTL3 using Western Blotting. Western blots imaged were quantified to determine expression levels of METTL3 using Fiji Image J. The bar graphs indicate level of METTL3 expression the clones express post the knockdown. Average knockdown levels of METTL3 expression determined by obtaining protein from three different passages (n=3) are as follows. Average knockdown levels here indicate the level by which METTL3 expression was reduced in these clones, as shown:

	PC2	PC4	PC5	PC6
AVG METTL3 KD%	75%	86%	92%	71%

All Panc-1 METTL3 knockdown single cell clones show significantly reduced expression of METTL3 when compared to non-targeted control using a t-test.

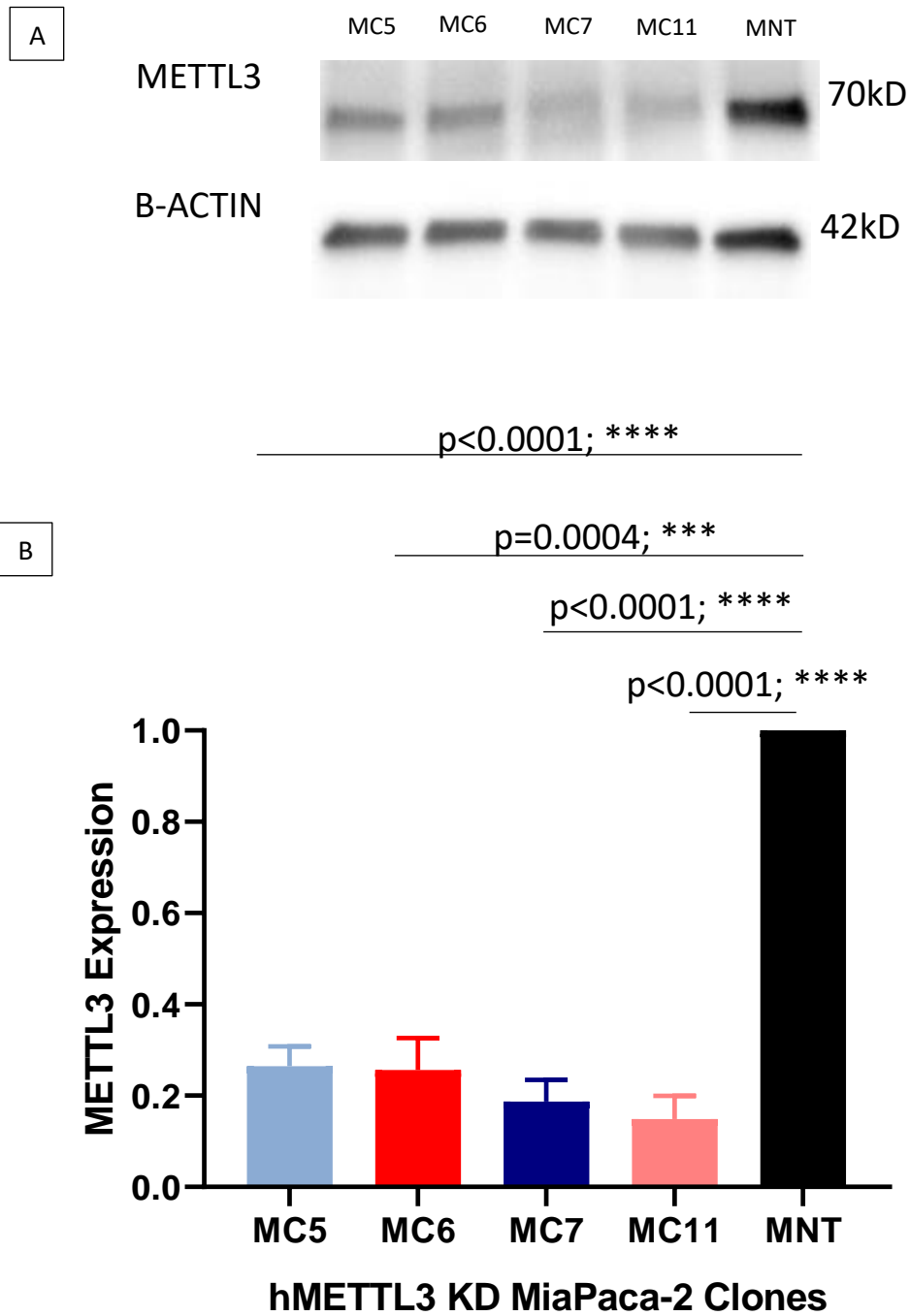


Figure 9: Confirmation of METTL3 knockdown in MiaPaca-2 cells using Western Blotting

- A) Western blot denotes a decreased level of METTL3 expression in the MiaPaca-2 single cell clones compared to the non-targeted control
- B) Bar graph indicates the level of METTL3 expression in the different MiaPaca-2 single cell clones upon METTL3 knockdown

To test the level of METTL3 expression in the cellular system generated, we performed western blotting. Single-cell clones generated from the Panc-1 METTL3 knockdown polyclonal population were tested for the knockdown in expression levels of METTL3 using Western Blotting. Western blots imaged were quantified to determine expression levels of METTL3 using Fiji Image J. The bar graphs indicate level of METTL3 expression the clones express post the knockdown. Average knockdown levels of METTL3 expression determined by obtaining protein from three different passages (n=3) are as follows. Average knockdown levels here indicate the level by which METTL3 expression was reduced in these clones, as shown:

	MC5	MC6	MC7	MC11
AVG METTL3 KD%	73%	74%	81%	85%

All MiaPaca-2 METTL3 knockdown single cell clones show significantly reduced expression of METTL3 when compared to non-targeted control using a t-test.

4.3.2 Quantitative Polymerase Chain Reaction:

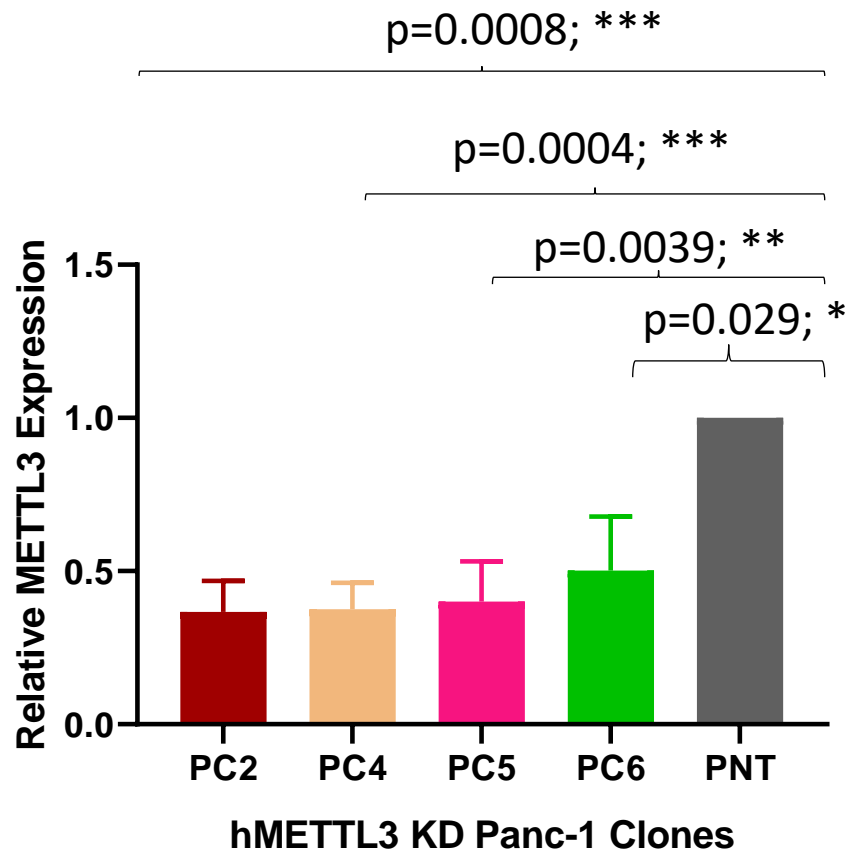


Figure 10: Confirmation of METTL3 knockdown in Panc-1 cells using qPCR

Bar graph indicates the level of METTL3 expressed by the Panc-1 single cell clones post shRNA knockdown of METTL3

Single-cell clones generated from the Panc-1 METTL3 knockdown polyclonal population were tested for the knockdown in expression levels of METTL3 using quantitative polymerase chain reaction (qPCR). Average knockdown levels determined by obtaining protein from three different passages (n=3) are as follows:

	PC2	PC4	PC5	PC6
AVG METTL3 KD%	63%	62%	60%	50%

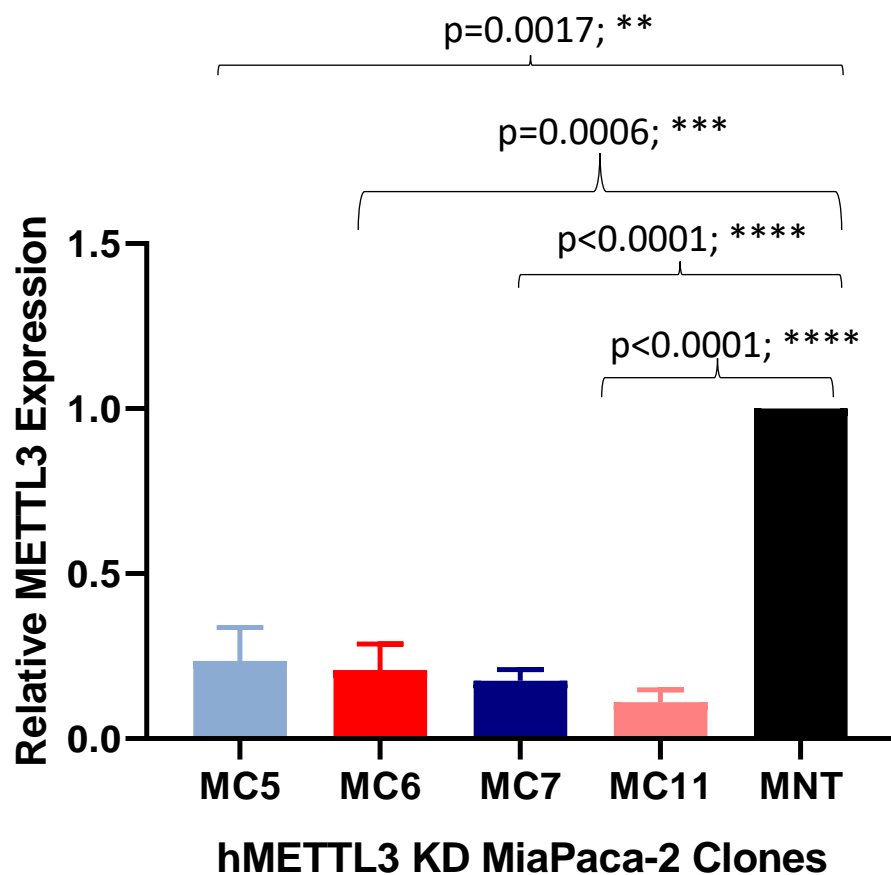


Figure 11: Confirmation of METTL3 knockdown in MiaPaca-2 cells using qPCR

Bar graph indicates the level of METTL3 expressed by the MiaPaca-2 single cell clones post shRNA knockdown of METTL3

Single-cell clones generated from the MiaPaca-2 METTL3 knockdown polyclonal population were tested for the knockdown in expression levels of METTL3 using quantitative polymerase chain reaction (qPCR). Average knockdown levels determined by obtaining protein from three different passages (n=3) are as follows:

	MC5	MC6	MC7	MC11
Avg METTL3 KD%	76%	80%	83%	90%

4.4 Proliferation assay

4.4.1 METTL3 knockdown reduces proliferation rates in some Panc-1 clones

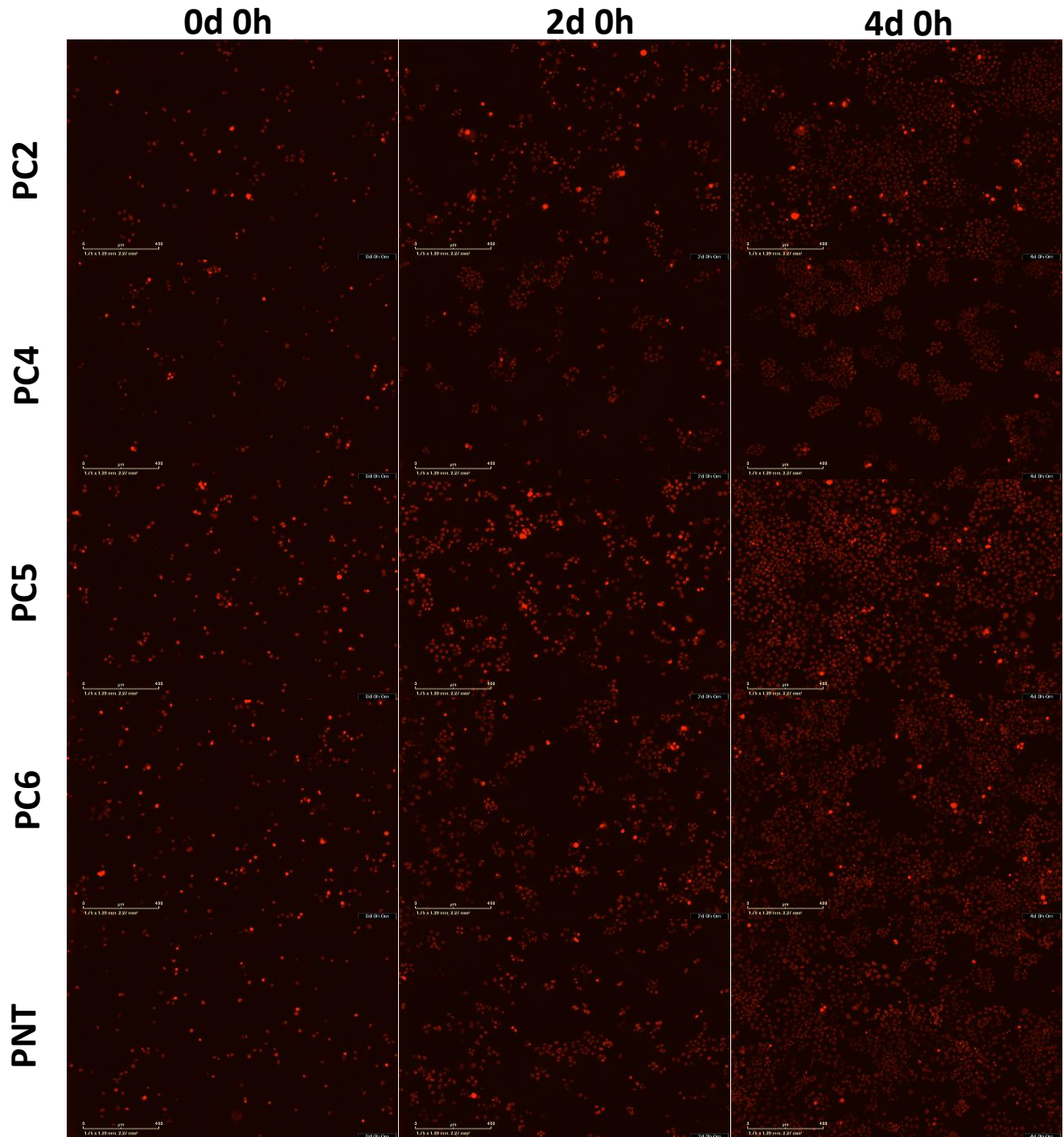


Figure 12: Proliferation assay in Panc-1 cells - This figure shows the growth of Panc-1 METTL3 knockdown clones imaged on a time course. The cells appear red upon incorporation of the Incucyte NucRed dye to aid in cellular counts.

Panc-1 METTL3 knockdown clones were plated in 96-well plates in duplicates in three independent experiments and allowed to grow in the Incucyte, while being imaged every 4 hours for 5 days. Incucyte NucRed fluorescent dye was added to aid in cellular count. Using the NucRed dye as a marker to label nuclei, cellular counts over 5 days were used to plot growth curves. Population doubling times were extracted from the growth curves, using the exponential growth curve equation. The population doubling times as compared against the non-targeted control are as follows:

	PC2	PC4	PC5	PC6	PNT
Population Doubling Time (h)	28.7	27.3	31.3	30.1	26.5

While population doubling time of clones 5 and 6 (PC5, PC6) were significantly slower than the non-targeted control, doubling times of clones 2 and 4 were not significantly different from the non-targeted control. We observed that METTL3 knockdown, hence reduces proliferation rates in some METTL3 knockdown clones but not the others in Panc-1 cells.

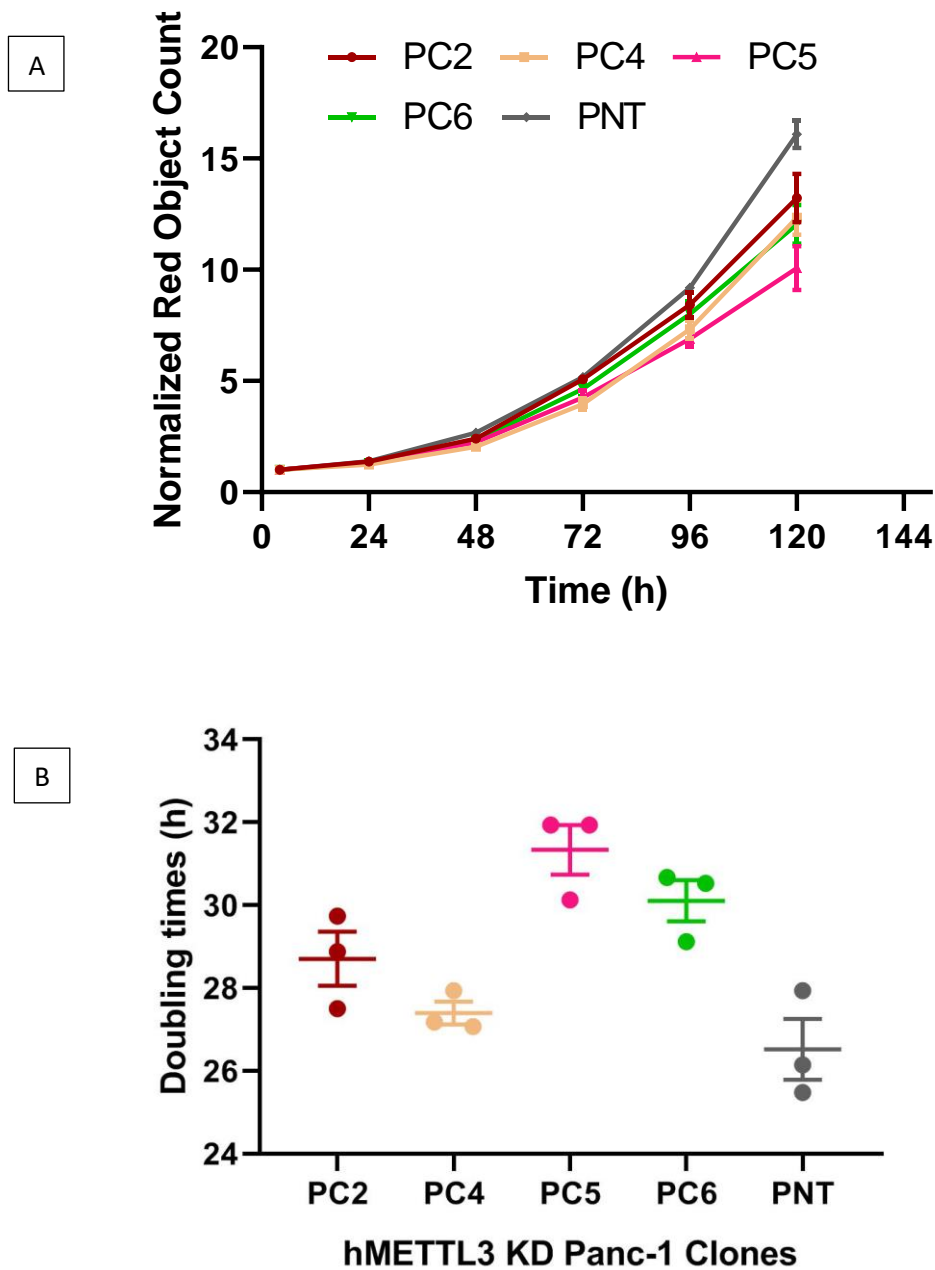


Figure 13: METTL3 knockdown reduces proliferation rates in some Panc-1 single cell clones

- A) Growth curve denotes the rate of proliferation in Panc-1 clones upon METTL3 knockdown compared to non-targeted control
- B) Doubling times of Panc-1 METTL3 knockdown clones extracted from the growth curve are depicted in this graph

4.4.2 METTL3 knockdown reduces proliferation rates in MiaPaca-2 cells

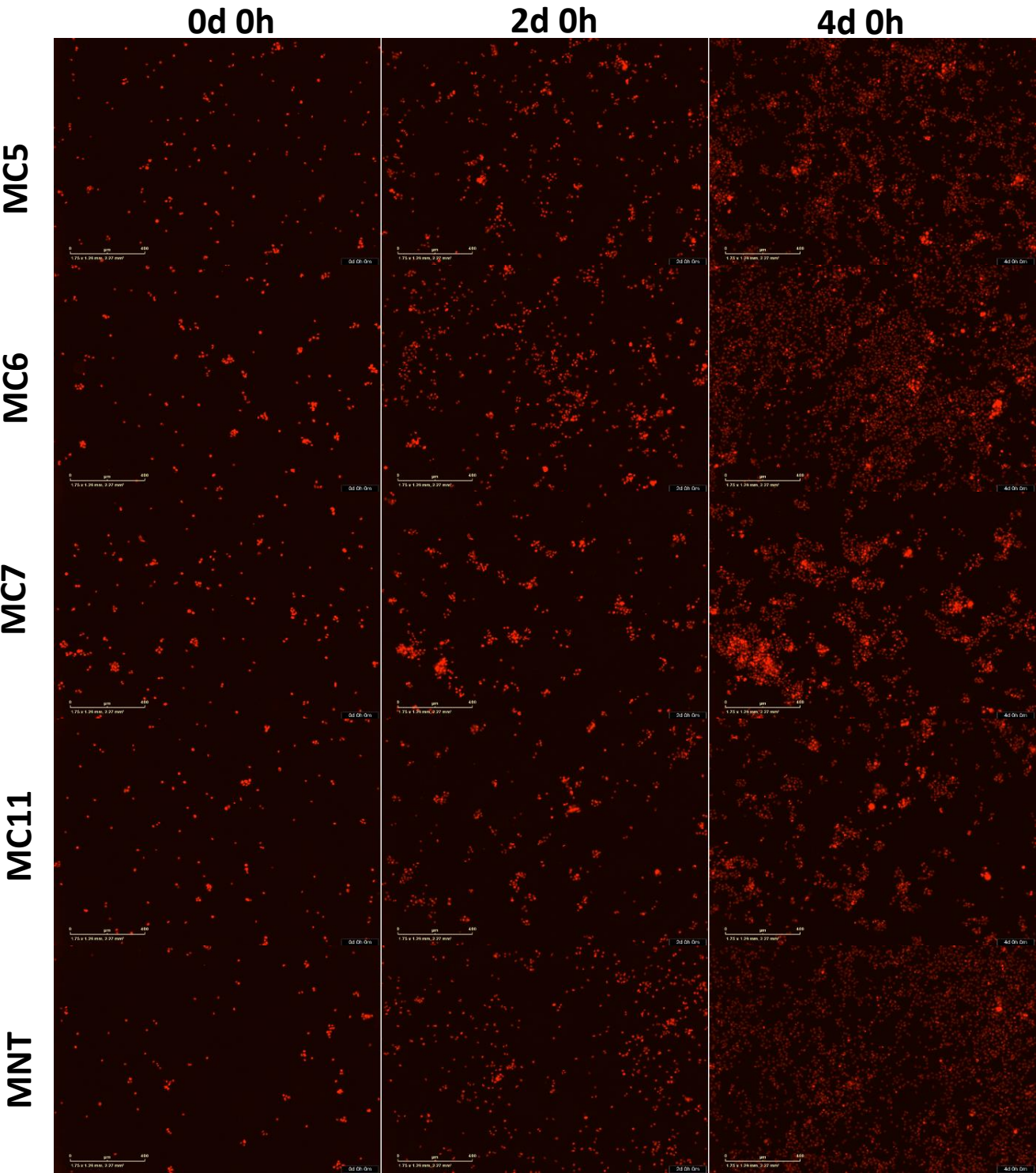


Figure 14: Proliferation assay in MiaPaca-2 cells - This figure shows the growth of METTL3 knockdown clones imaged on a time course. The cells appear red upon incorporation of the Incucyte NucRed dye to aid in cellular counts.

MiaPaca-2 METTL3 knockdown clones were plated in 96-well plates in duplicates in three independent experiments and allowed to grow in the Incucyte, while being imaged every 4 hours for 5 days. Incucyte NucRed fluorescent dye was added to aid in cellular count. Using the NucRed dye as a marker to label nuclei, cellular counts over 5 days were used to plot growth curves. Population doubling times were extracted from the growth curves, using the exponential growth curve equation. The population doubling times as compared against the non-targeted control are as follows:

	MC5	MC6	MC7	MC11	MNT
Population	26.0	27.5	32.5	32.4	21.6
Doubling Time (h)					

All MiaPaca-2 METTL3 knockdown clones except clone 6 (MC6) showed a significantly slower proliferation rate compared to the non-targeted control. The population doubling time of the knockdown clones being higher than the non-targeted denotes that it takes longer for the clones to double their population, due to their slower growth rate. We observed that METTL3 knockdown, hence reduces proliferation rates in most METTL3 knockdown clones, save for one in MiaPaca-2 cells.

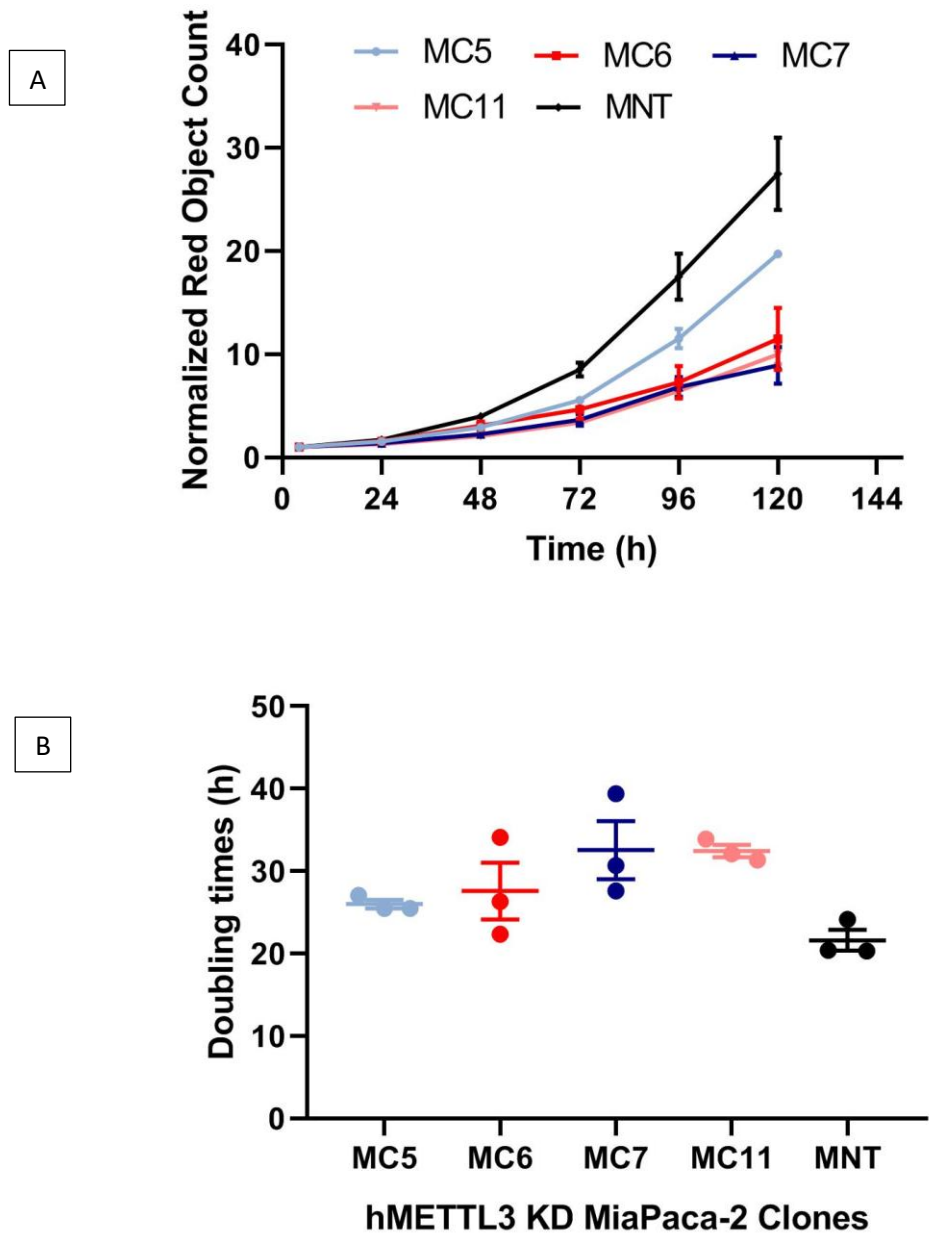


Figure 15: METTL3 knockdown reduces proliferation rates in MiaPaca-2 single cell clones

- A) Growth curve denotes the rate of proliferation in MiaPaca-2 clones upon METTL3 knockdown compared to non-targeted control
- B) Doubling times of MiaPaca-2 METTL3 knockdown clones extracted from the growth curve are depicted in this graph

4.5 Migration assay

4.5.1 METTL3 knockdown does not affect the migration rates of Panc-1 cells

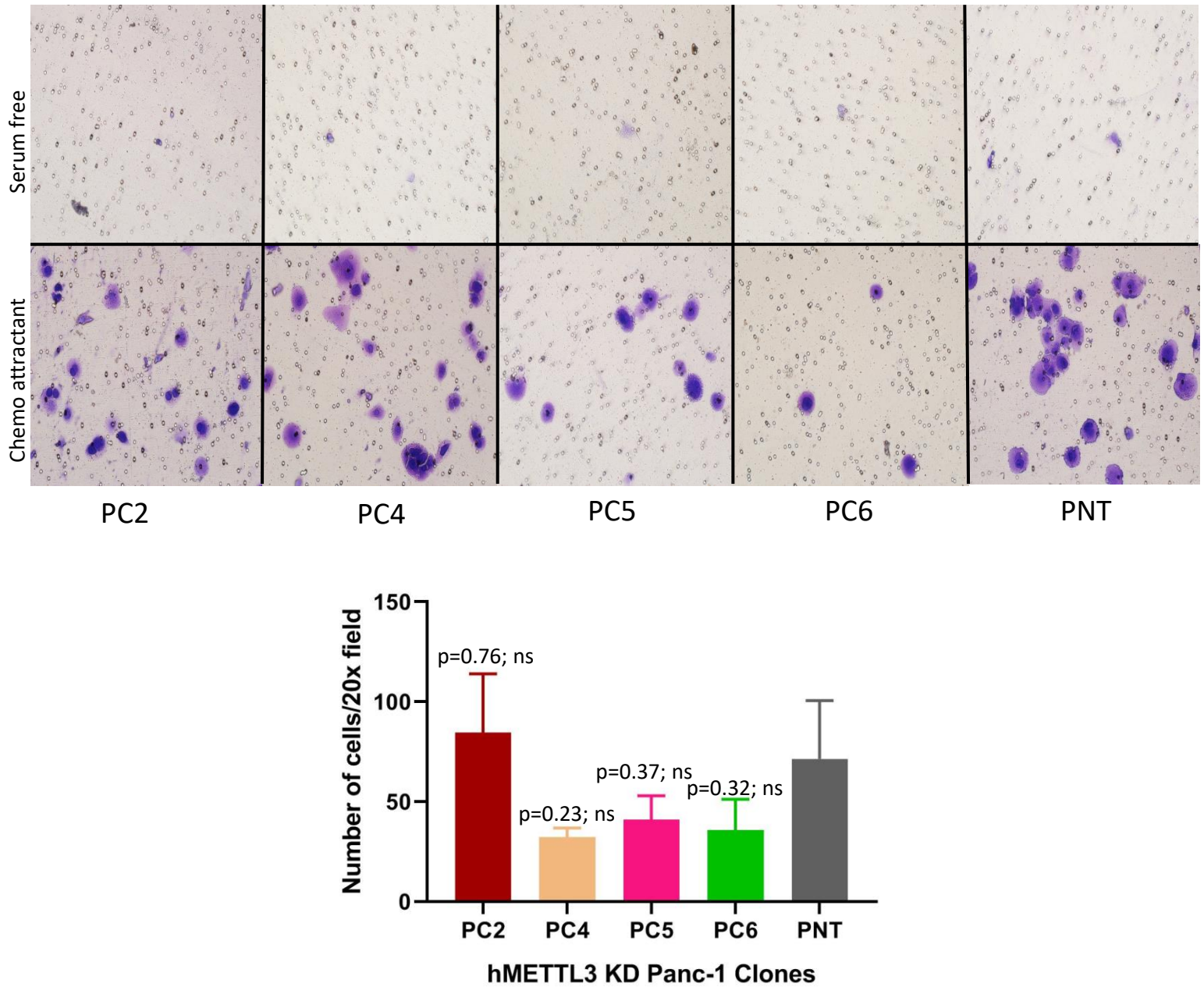


Figure 16: METTL3 knockdown does not affect migration rates of Panc-1 clones

- A) Panc-1 clones that migrated to the bottom of the transwell are shown here post fixing and staining with crystal violet
- B) Bar graph indicates the number of cells that migrated in each of the METTL3 knockdown clones compared to non-targeted control

To determine how METTL3 loss affects migration rates of pancreatic cancer cells, we conducted a transwell migration assay on Panc-1 METTL3 knockdown clones. 10% fetal bovine serum was used as chemotactic gradient. Panc-1 cells were allowed to migrate through the transwell for 24 hours, post which they were fixed and stained using crystal violet dye. The fixed and stained cells were observed under the microscope and images were captured. Automated cell counter as part of Fiji – Image J was used to count the number of cells that migrated to the bottom of the transwell. We observed no significant difference in the migration rates of the knockdown clones when compared to the non-targeted control in Panc-1 cells. Though we observed no significant difference in the migration rates of these cells, three out of the four clones exhibited a slower migratory trend compared to the control.

4.5.2 METTL3 knockdown reduces the migration rates of MiaPaca-2 cells

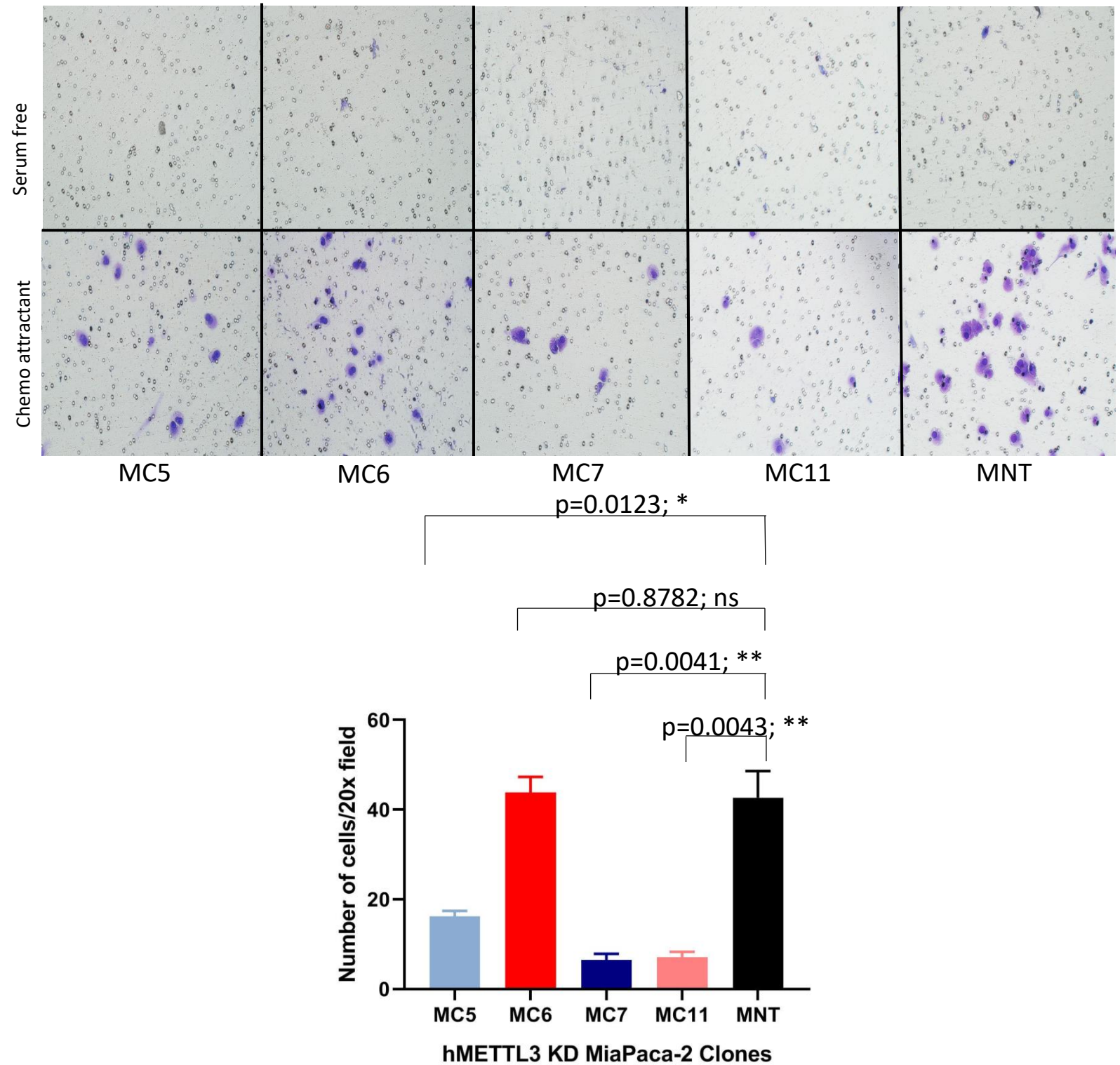


Figure 17: METTL3 knockdown reduces affect migration rates of MiaPaca-2 clones

- A) MiaPaca-2 clones that migrated to the bottom of the transwell are shown here post fixing and staining with crystal violet
- B) Bar graph indicates the number of cells that migrated in each of the METTL3 knockdown clones compared to non-targeted control

To determine how METTL3 loss affects migration rates of pancreatic cancer cells, we conducted a transwell migration assay on MiaPaca-2 METTL3 knockdown clones. 10% fetal bovine serum was used as chemotactic gradient. MiaPaca-2 cells were allowed to migrate through the transwell for 17 hours, post which they were fixed and stained using crystal violet dye. The fixed and stained cells were observed under the microscope and images were captured. Automated cell counter as part of Fiji – Image J was used to count the number of cells that migrated to the bottom of the transwell. We counted the total number of cells present at the bottom of the transwell and used that as a measure of the migration rate, since the knockdown clones as well as the non-targeted control were allowed to migrate for the same duration of time. We observed that the migration rates of three of the METTL3 knockdown clones were significantly slower when compared to the non-targeted control in MiaPaca-2 cells. Further investigation is needed to determine the variability in clone 6 and why the migration rate of that particular clone is higher than the NT control.

4.6 Sanger sequencing confirms sequences of shRNAs used to target murine METTL3 gene

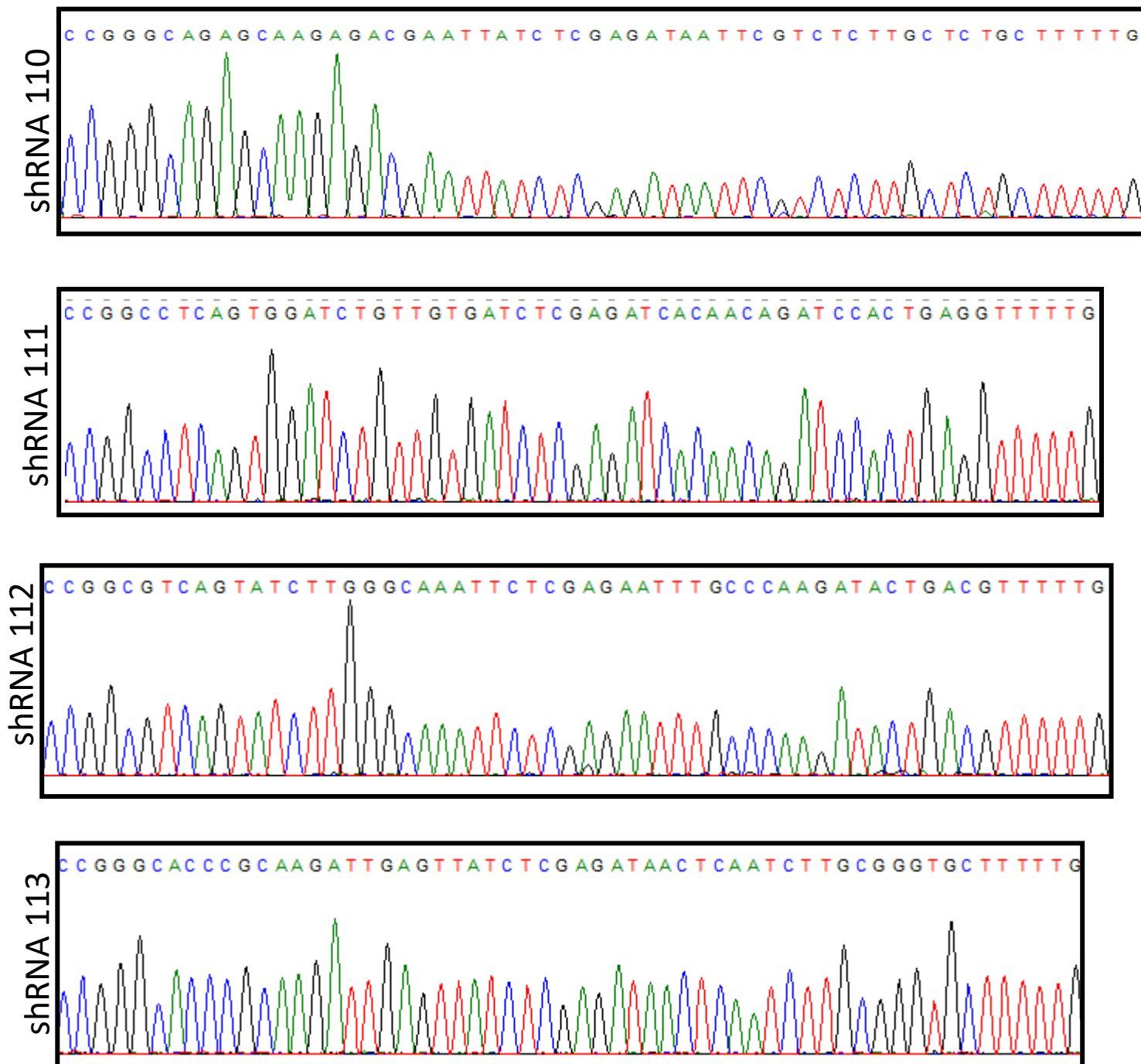


Figure 18: Confirming different shRNA plasmid sequences targeting murine METTL3 – Sanger sequencing results showing the nucleotide sequences of different shRNA plasmid constructs tested

To investigate the effects METTL3 loss may have on murine pancreatic cancer cells, we sought to generate a METTL3 knockdown in PKCY cell lines. We purchased four different shRNA plasmid constructs that target the murine METTL3 gene and tested them to identify the plasmid sequence that yields in the most genetic knockdown of METTL3 expression. The plasmids were isolated from bacterial stocks purchased, through a miniprep protocol. The purified plasmid sequences were verified by performing Sanger Sequencing at the MD Anderson Sequencing Core.

4.7 Targeting murine METTL3 gene using shRNA 112 yields the least METTL3 expression

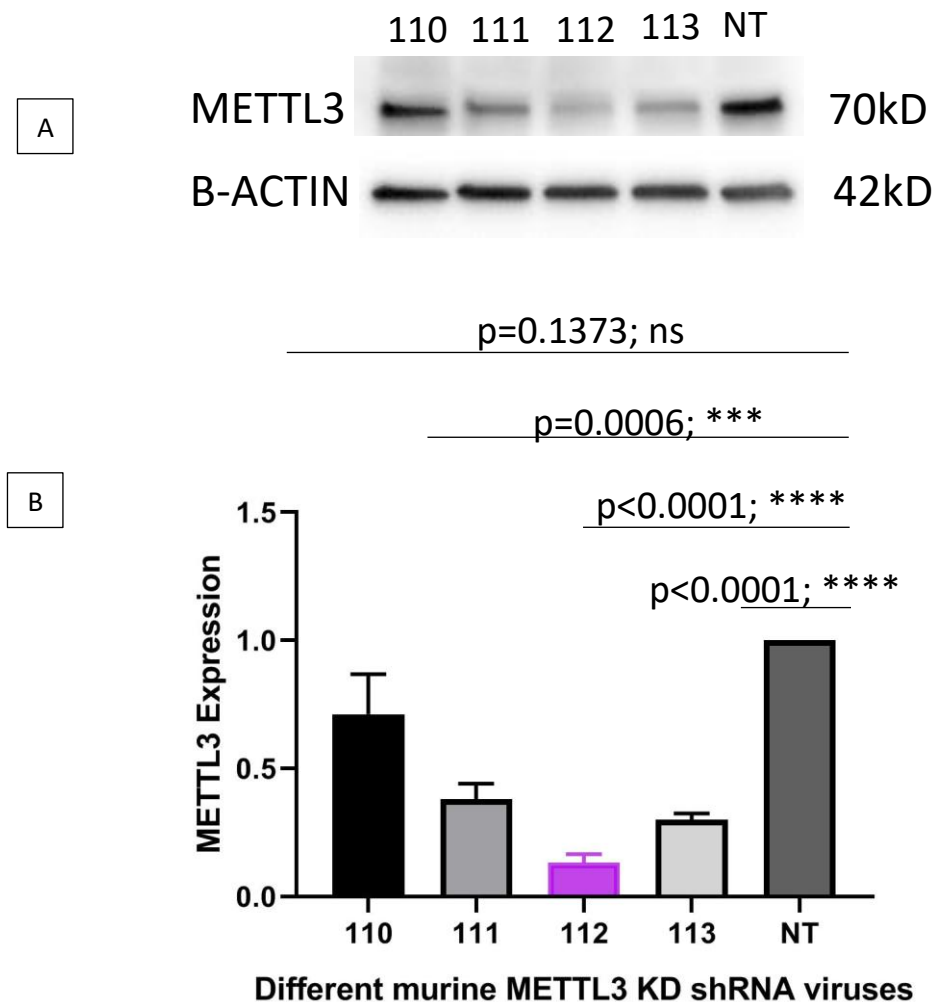


Figure 19: Testing knockdown efficacy of different shRNA lentivirus targeting murine METTL3

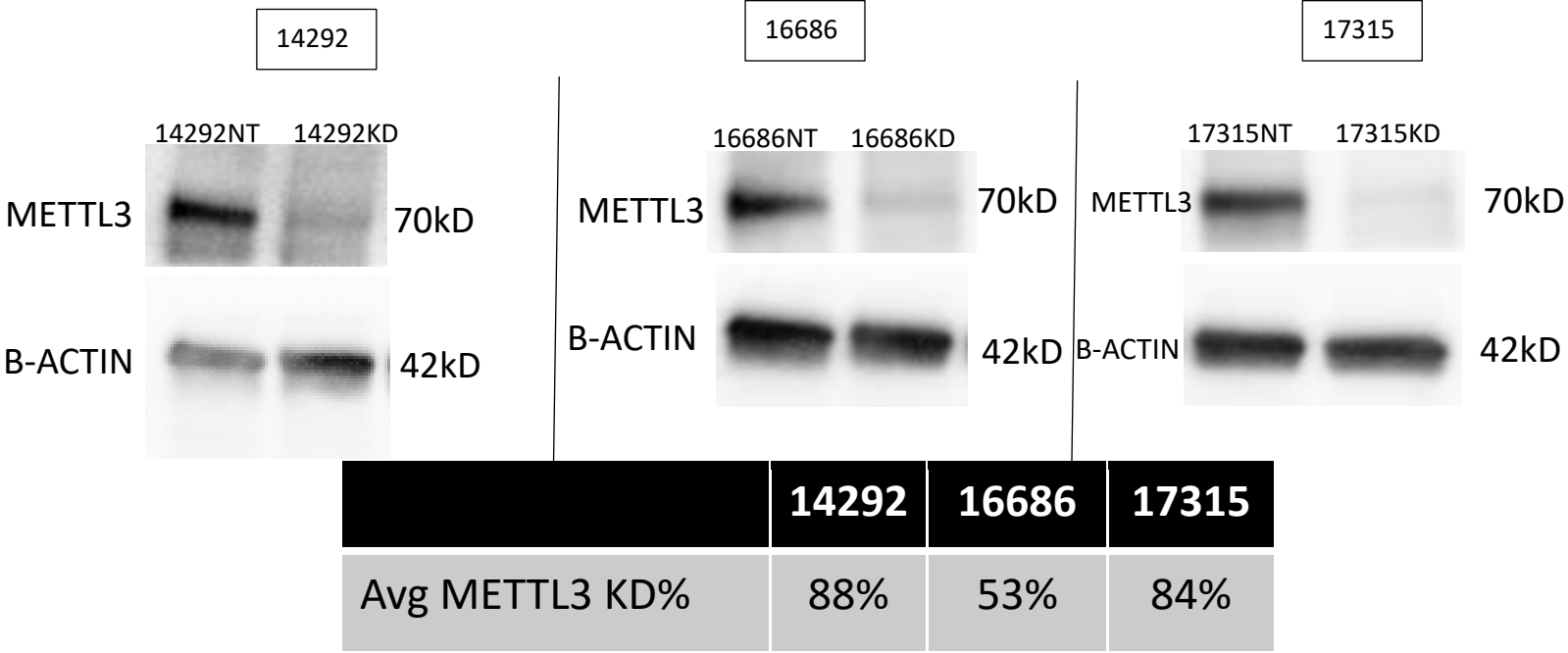
- A) Western blot showing METTL3 expression levels in PKCY cells when transfected with each virus
- B) Bar graph denotes the level of METTL3 expression in PKCY cells when transfected with each virus tested. This allows us to identify the lentiviral construct that produces the most knockdown in METTL3 expression

Four different shRNA plasmids (shRNA110, shRNA111, shRNA 112, shRNA 113) that target murine METTL3 were tested to determine their knockdown efficacies. We generated lentivirus from each of the plasmids and transfected one PKCY cell line, 14292 with each of the viruses. Protein from the

transfected PKCY cells were then collected, and a western blot was run to determine the level of METTL3 knockdown in the bulk population. We noted that when the 14292 PKCY cells were transfected with shRNA 112, the level of METTL3 expression was lowest, as shown below:

shRNA virus	Average METTL3 expression	Average METTL3 KD
110	70%	30%
111	37%	63%
112	12%	88%
113	29%	71%

This implies that the shRNA 112 plasmid produces the most METTL3 knockdown. To add more biological replicates, we used the shRNA 112 virus and transfected two more PKCY cell lines, namely – 16686 and 17315 (genotypes described in Table 5). The METTL3 knockdown bulk cell population of all the PKCY cells were tested for efficient knockdown. Results of the western blot are as follows:



Further experiments in the murine context were performed on these PKCY METTL3 knockdown bulk population of cells for these three cell lines.

4.8 Determining levels of expression of EMT markers:

4.8.1 METTL3 knockdown causes a decrease in E-Cadherin, increase in Vimentin, and does not affect N-Cadherin in Panc-1 cells

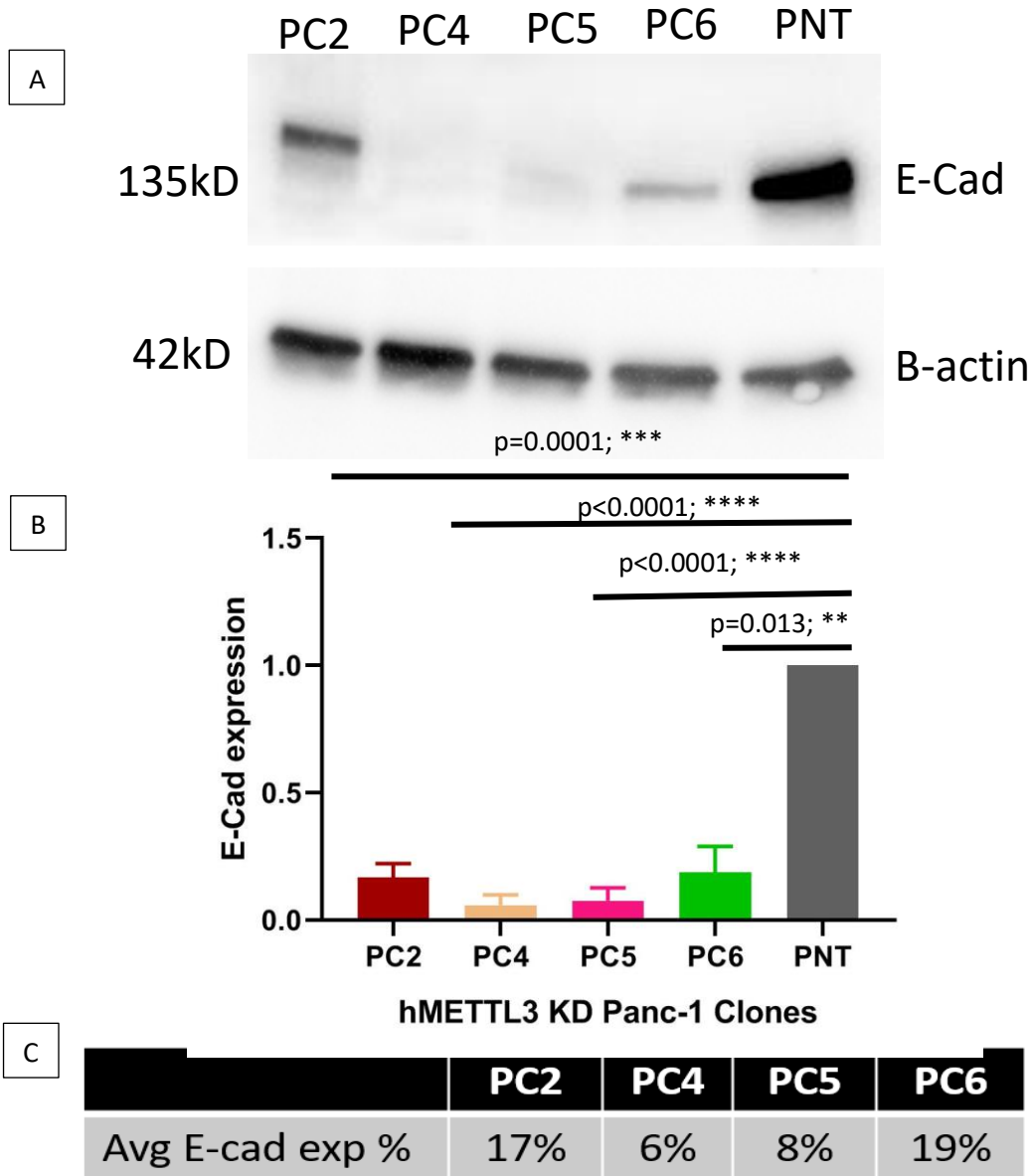
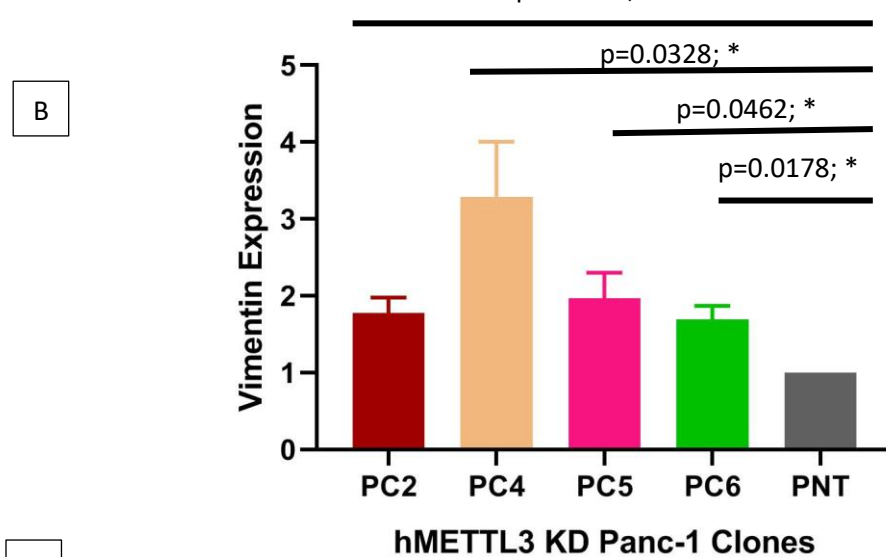
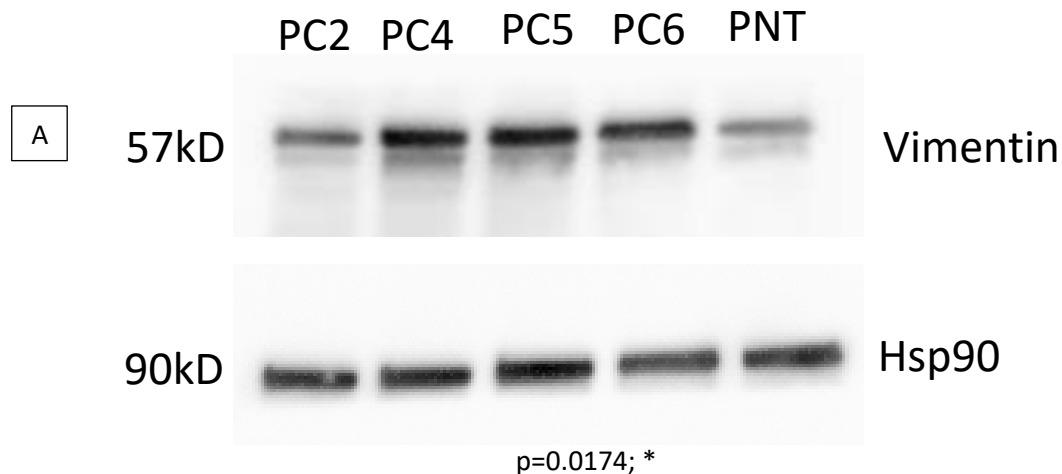


Figure 20: E-cadherin expression is reduced in Panc-1 cells upon METTL3 knockdown

- A) Western blot denotes a decreased level of E-cadherin expression in the Panc-1 single cell clones compared to the non-targeted control
- B) Bar graph indicates the level of E-cadherin expression in the different Panc-1 single cell clones upon METTL3 knockdown
- C) E-cadherin expression levels are quantified in Table C

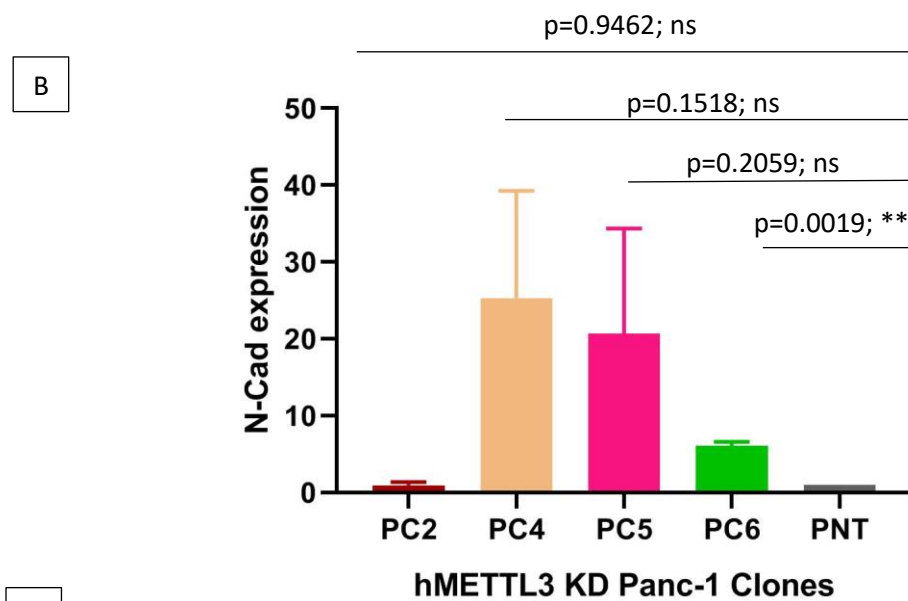
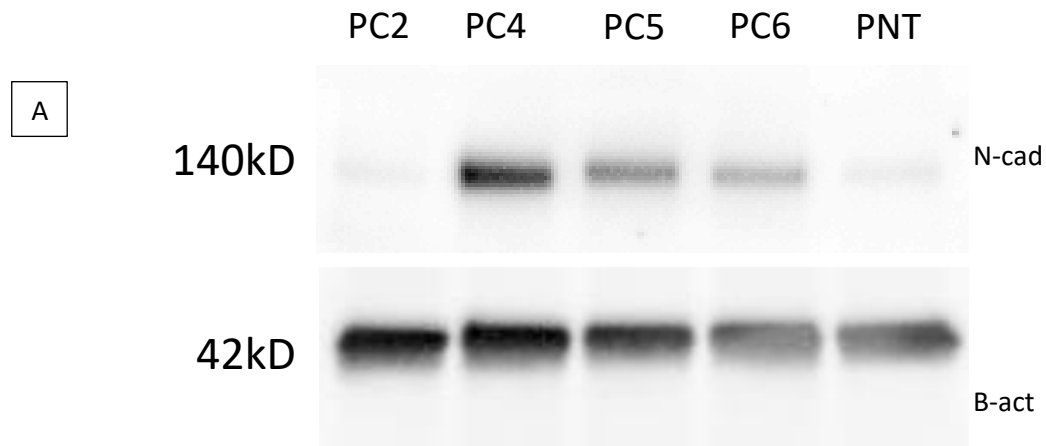


C

	PC2	PC4	PC5	PC6
Avg Vimentin exp %	178%	329%	197%	169%

Figure 21: Vimentin expression is increased in Panc-1 cells upon METTL3 knockdown

- A) Western blot denotes an increased level of Vimentin expression in the Panc-1 single cell clones compared to the non-targeted control
- B) Bar graph indicates the level of Vimentin expression in the different Panc-1 single cell clones upon METTL3 knockdown
- C) Vimentin expression levels are quantified in Table C



C

	PC2	PC4	PC5	PC6
Avg N-cad exp %	96%	3184%	2692%	611%

Figure 22: N-cadherin expression is not affected in Panc-1 cells upon METTL3 knockdown

- A) Western blot denotes an increased level of N-cadherin expression in some Panc-1 single cell clones compared to the non-targeted control
- B) Bar graph indicates the level of N-cadherin expression in the different Panc-1 single cell clones upon METTL3 knockdown
- C) N-Cadherin expression levels are quantified in Table C

In Panc-1 METTL3 knockdown single cell clones, there is a significant decrease in E-cadherin expression across all clones and a significant increase in Vimentin expression across all clones when compared to the non-targeted control.

N-cadherin expression on the other hand, seems to not be influenced by METTL3 loss. One of the clones, PC6 shows a significant increase in N-cadherin expression upon METTL3 knockdown. Two of the other clones PC4 and PC5 also show a marked increase in N-cadherin expression, but due to high variability in the replicates, the expression is not significantly different from the NT control. Even though the difference is not statistically significant, the overall trend in Panc-1 clones is for an increase in the expression level of N-cadherin compared to control, in three out of four clones.

4.8.2 METTL3 knockdown does not affect Vimentin expression in MiaPaca-2 cells

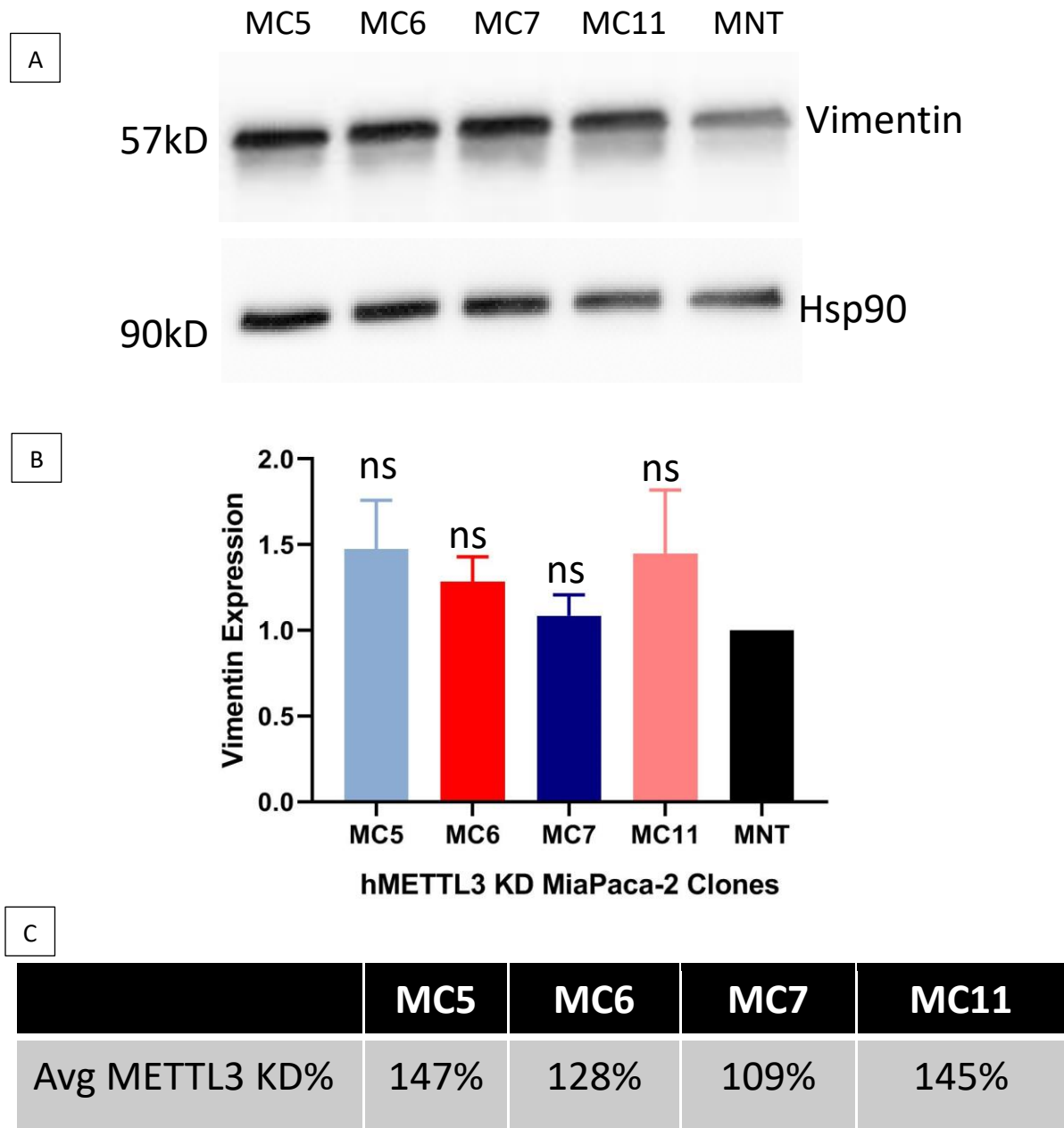
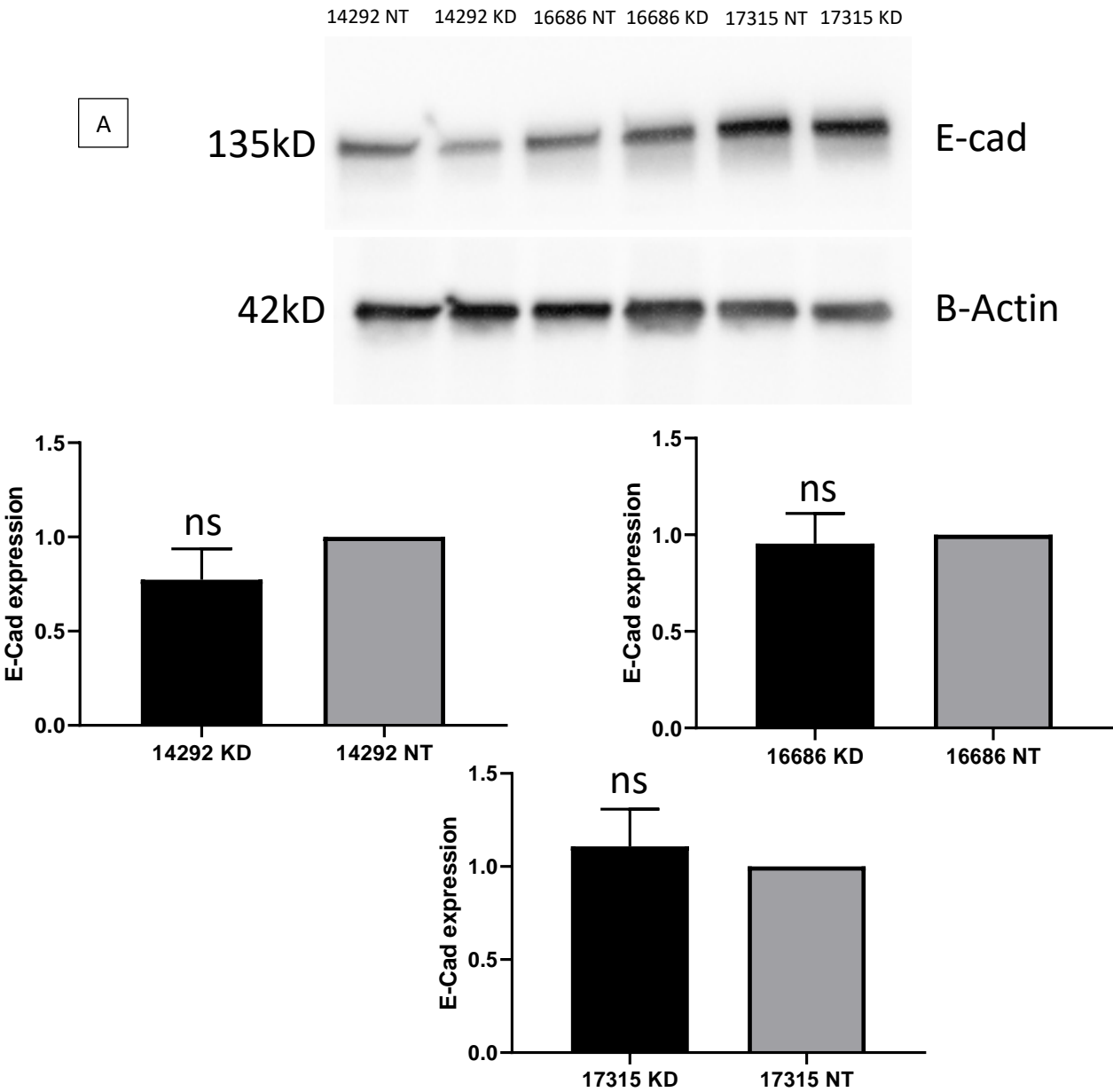


Figure 23: Vimentin expression is seemingly increased in MiaPaca-2 cells upon METTL3 knockdown

- A) Western blot denotes a seemingly increased level of Vimentin expression in the MiaPaca-2 single cell clones compared to the non-targeted control
- B) Bar graph indicates the level of Vimentin expression in the different MiaPaca-2 single cell clones upon METTL3 knockdown
- C) Vimentin expression levels are quantified in Table C

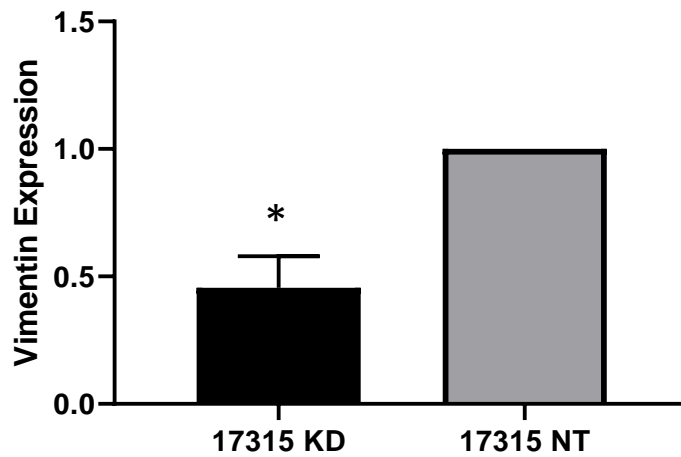
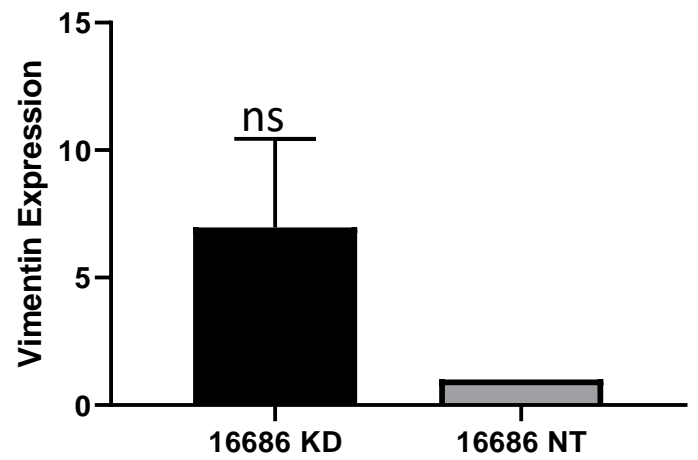
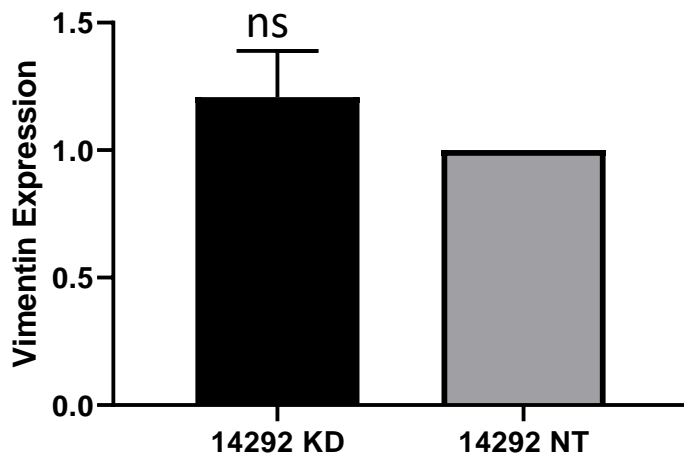
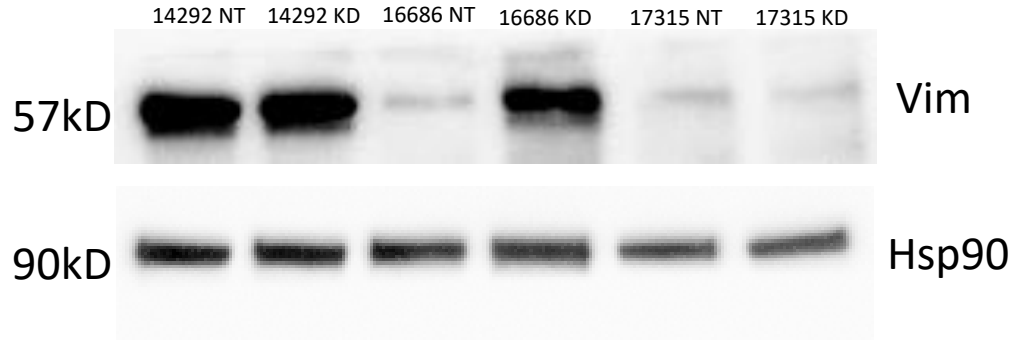
Vimentin expression in MiaPaca-2 METTL3 knockdown single cell clones, seems to not be affected. All the clones show a marginal increase in Vimentin expression, but the expression is not significantly different from the NT control. We can conclude that Vimentin expression is not affected by METTL3 knockdown.

4.8.3 Effect of METTL3 knockdown on E-Cadherin, Vimentin, and N-Cadherin expression in PKCY cells is inconclusive



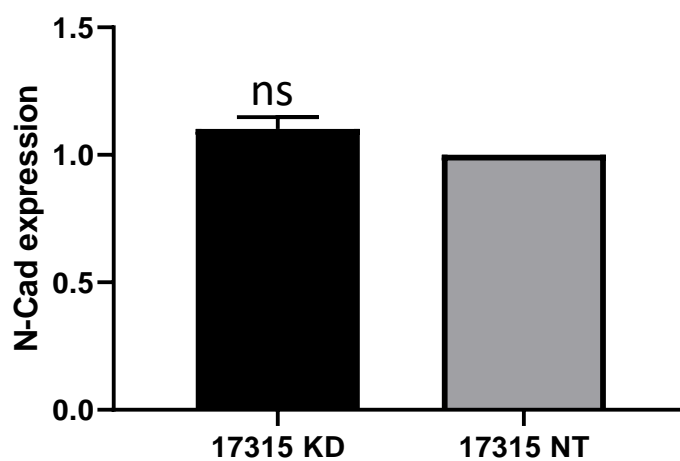
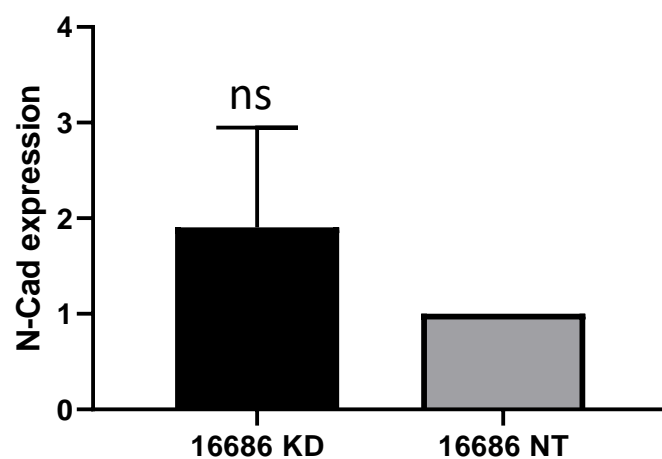
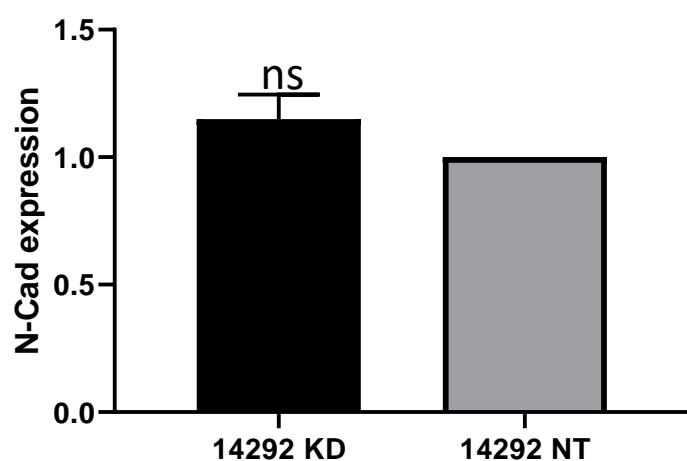
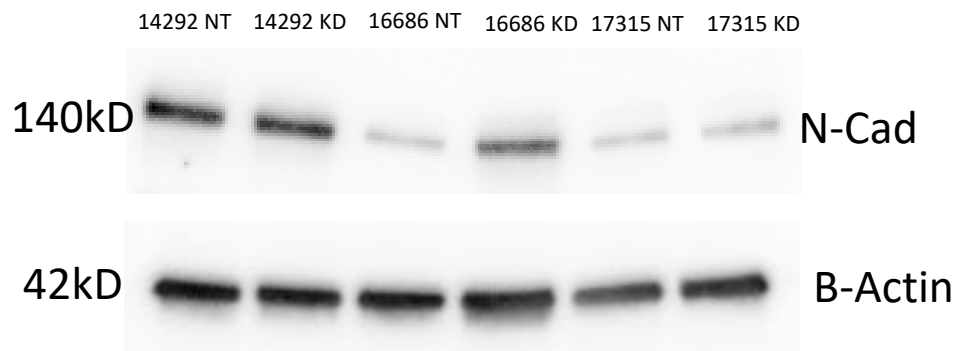
	14292	16686	17315
Avg E-cad exp %	77%	95%	110%

B



	14292	16686	17315
Avg Vimentin exp %	120%	697%	45%

C



	14292	16686	17315
Avg N-cad exp %	114%	190%	110%

Figure 24: Effect of METTL3 knockdown on EMT-associated proteins in PKCY cells is inconclusive

- A) Panel A shows the level of E-cadherin expression in METTL3 knockdown PKCY cells and their respective non-targeted controls
- B) Panel B shows the level of Vimentin expression in METTL3 knockdown PKCY cells and their respective non-targeted controls
- C) Panel C shows the level of N-cadherin expression in METTL3 knockdown PKCY cells and their respective non-targeted controls

We observe that the level of expression of E-cadherin, Vimentin, and N-cadherin remain uninfluenced by METTL3 loss in PKCY cells. Increase or decrease in expression of EMT-associated proteins compared to non-targeted control is extensively variable across the different PKCY METTL3 knockdown cell lines. This may be a function of the nature of the PKCY cell line. Be it an epithelial or mesenchymal-like cell line, having undergone malignant transformation, the way the cell line responds to METTL3 loss may be highly variable. Moreover, the increase or decrease in expression levels upon METTL3 knockdown are not significantly different from their respective non-targeted controls. We must bear in mind that these are METTL3 knockdown cell populations in bulk, not having had single cell clones cultured from each of them. This result could also be due to the heterogeneity in knockdown levels in a bulk population. These results cannot hence conclude that METTL3 loss has absolutely no effect on expression levels of E-cadherin, Vimentin, and N-cadherin. Further studies with single cell clones of PKCY METTL3 knockdown are needed to observe any potential effects.

5. Discussion:

Pancreatic Ductal Adenocarcinoma is one of the most aggressive solid cancers, whose treatment is made more challenging by compounding factors like late diagnosis, rapid metastatic spread, and chemoresistance. Genetic perturbations are observed in well characterized sets of genes, known as genetic drivers of the disease. These signature mutational landscapes are commonly observed in high frequencies across the PDAC patient population. Despite the uniform mutational profiles, treatment outcomes vary drastically among patients.

Recent studies have brought to attention, the need to therapeutically exploit epigenetic pathways, apart from only targeting genetic mutations to effectively combat PDAC. Dysregulation of epigenetic proteins and associated mechanisms have been implicated to contribute to the progression of the disease. DNA methylation, histone modifications, aberrant microRNA expression are some avenues of keen therapeutic interest due to the reversible nature of their effects.(43)-(44)

METTL3 being an important epigenetic regulator of RNA in the human body, we sought to understand the broader question of the role METTL3 plays in PDAC progression. We found that METTL3 expression positively correlates with survival in PDAC patients by analyzing data from the TCGA-PAAD cohort. Expression of METTL3 can potentially be used as a molecular prognostic factor in the diagnosis of PDAC. This survival correlation, however, begs the question, is METTL3 playing the role of a tumor suppressor and being protective in PDAC? We hypothesized that since higher METTL3 expression significantly extends survival by 6 months, presence of METTL3 does impact PDAC progression. From our *in vitro* loss of function studies, we find that METTL3 knockdown reduces proliferation and migration in MiaPaca-2 cells, but only reduces proliferation in Panc-1 cells. These results are in contradiction to the TCGA survival study, where METTL3 seems to be playing a protective role.

METTL3 is crucial in early development and differentiation. It represses pluripotent identity and induces cell fate specification.(37)(45) In a malignant context, when a tumor undergoes epithelial to mesenchymal transition (EMT), the restoration of early embryonic transcriptional programs occurs. Hence, we wanted to understand the role of METTL3 in effecting EMT-associated proteins in PDAC. We found that in Panc-1 cells, there was a significant reduction in E-cadherin and a concordant increase in Vimentin upon METTL3 knockdown. The effect was inconclusive in MiaPaca-2 cells. But in Panc-1 cells, this is consistent with the EMT phenotype, where expression of an epithelial marker is reduced while expression of a mesenchymal marker is increased. If knocking down METTL3 induces an EMT-like phenotype, it can be argued that METTL3 plays a potentially protective role in PDAC. METTL3 has been previously shown to effect pathogenesis in cervical cancer by enriching abundance of m6A in Snail mRNA, thus inducing EMT in cancer.(41) Given our preliminary results, the mechanism of how METTL3 affects EMT induction in PDAC requires further investigation.

We must consider that there may also be differences arising due to the nature of the cell lines. Panc-1 is an epithelial-like cell line whereas MiaPaca-2 is a mesenchymal-like cell line. Incorporating more biological replicates of the two types of cell lines can help answer the question of if the effect is dependent on the nature of the cell line. Another consideration to note is that this study used shRNA knockdown of METTL3 which is not a complete knockout of expression. Since m6A RNA methylation is highly crucial, the cells may have devised a strategy to compensate for the reduction in methylation without adverse outcomes. Utilizing METTL3 knockout CRISPR clones of pancreatic cancer cell lines can be an effective strategy to understand the behaviors of tumors completely devoid of METTL3. When it comes to the TCGA expression data, patient tissue samples like most pancreatic tumors are, may have been heterogenous with varied kinds of cell populations. We know that islet cells in the pancreas express high levels of METTL3. Hence, distinguishing the malignant compartment from normal pancreas, while performing RNA-Seq expression profiling is key to making an assertion.

Methylation is highly dynamic, reversible, and differs based on the tissue type, disease state, and other pathologies. Studies from other groups have indicated a dual role for METTL3/m6A methylation in tumors. METTL3 has been shown to be playing tumor promoting roles in lung cancer,(38) gastric cancer,(46) and acute myeloid leukemia.(47) Whereas in glioblastoma(48), renal cell carcinoma(40), and breast cancer(49) there is evidence to conclude that METTL3 plays the role of a tumor suppressor. A 2018 study found that knockdown of METTL3 increased the susceptibility of human pancreatic cancer cell lines to anti-cancer agents like cisplatin, gemcitabine, and 5-fluorouracil. They concluded that METTL3 promotes chemoresistance in pancreatic cancer cells.(50) Contradictory to their results, our study highlights an important clinical finding of patient overall survival positively correlating with METTL3 in PDAC. This can mean that the effect METTL3/m6A methylation exerts of PDAC must be examined at different temporal snapshots in the pathogenesis of the disease (initiation versus progression) to conclude a role with certainty.

Interestingly, independent of the potential effect the m6A writer has on tumor behavior, reader, and eraser proteins of the m6A methylation cascade have also been implicated in cancers. FTO and ALKBH5 have both been implicated to be playing tumorigenic roles in glioblastoma.(48) (51) IGF2BP proteins have been shown to target MYC and promote cellular migration, invasion in liver cancers.(52) It has also been shown to not always be that the effect an m6A writer and an m6A eraser exert be counteractive. While METTL3 (writer) acts as an oncogene in AML,(39) FTO (eraser) also acts as an oncogene in AML.(53) This dynamic interplay between the writers, readers, and erasers should be further explored for a better mechanistic insight in PDAC. Regardless of the primary site of origin of the cancer, the volume of implication METTL3 possesses in pathophysiology of cancer, and the therapeutic potential of understanding its role in the disease are unmistakable.

Methylated RNA Immunoprecipitation sequencing (MeRIP-Seq) is an antibody-dependent technique to help map m6A sites across the transcriptome.(30) Since METTL3 post-transcriptionally

affects RNA transcripts, MeRIP-Seq can be performed as future work to delineate the specific transcripts METTL3 targets. This can give us an insight into what effector pathways METTL3 employs to bring about the phenotypical effects observed.

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