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PVT1 exon 9 a regulator of claudin expression in triple negative breast cancer

Fayola Levine
CUNY Hunter College

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Fayola A. Levine

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May 15th 2020	Olorunseun O. Ogunwobi, MD, PhD
Date	Thesis Sponsor
May 15th 2020	Jill Bargonetti, PhD
Date	Second Reader

Dedication

בָּרוּךְ יְיָ הַמְּבָרֵךְ לְעוֹלָם וָעֶד

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1. Abstract

Breast cancer (BC) is a heterogeneous disease that is classically driven by the estrogen receptor (ER), progesterone receptor (PR) and human epithelial growth factor receptor 2 (EGFR2/HER2) signaling pathways. Triple negative breast cancer (TNBC), is a lethal subtype of invasive BC tumors that are ER-, PR- and HER2-. A subtype of TNBC is claudin low (CL). Dysregulation of claudin proteins disrupts tight junctions, consequently inducing the epithelial-to-mesenchymal transition (EMT) in cancers. This leads to enhanced motility and metastasis. Patients with CL TNBC have worse prognosis than patients with other BC subtypes.

PVT1 is a long noncoding RNA (lncRNA) transcribed from the 8q24 genomic locus that has been implicated in multiple cancers including BC. Amplification of the 8q24 gene locus is a common event in many malignant diseases and is associated with poor clinical outcomes. Although previous research has implicated PVT1 as an important player in BC, the underlying molecular mechanisms of PVT1 in CL TNBC were previously unknown.

We assessed PVT1 expression in BC, and we observed that PVT1 exon 9 is significantly upregulated in MDA MB 231 cells (claudin low) and significantly downregulated in MDA MB 468 cells (claudin high), in comparison to T47D (ER+). We have confirmed that claudin expression, specifically, claudins 1, 3, 4 and 7, are significantly higher in MDA MB 468 cells and significantly lower in MDA MB 231 cells. Knockdown of PVT1 exon 9 expression in the MDA MB 231 cell line, led to a significant reduction in migration when compared to cells transfected with a control scramble siRNA, indicating that PVT1 exon 9 regulates migration in CL TNBC.

Interestingly, we observed that claudin 4 expression, and not claudins 1, 3 and 7, was increased in cells where PVT1 exon 9 was knocked down when compared to the control cells. This indicates that PVT1 exon 9 regulates claudin 4 protein stability in CL TNBC. We also assessed the

expression of EMT markers (vimentin, fibronectin, and E-cadherin) in MDA MB 231 cells. We observed no changes in EMT markers when PVT1 exon 9 is knocked down; however, our data suggests that EMT markers are more highly expressed in MDA MB 231 cells in comparison to MDA MB 468 cells. Taken together, our data indicate that PVT1 exon 9 regulates claudin expression and migration in CL TNBC, and may have implications for clinical outcomes in TNBC.

2. List of abbreviations

PVT1	Plasmacytoma variant translocation 1
ER	Estrogen receptor
PR	Progesterone receptor
EGFR2/HER2	Human epithelial growth factor receptor 2
TNBC	Triple negative breast cancer
CL	Claudin low
CH	Claudin high
CLDN	Claudin
EMT	Epithelial to mesenchymal transition
VM	Vasculogenic mimicry
moAA	Men of African Ancestry
RT-qPCR	Real-time quantitative polymerase chain reaction

3. List of figures and tables

4. Introduction

4.1. Background

Breast cancer is the most commonly diagnosed non-skin cancer, and second leading cause of cancer mortality, in U.S. women (1, 2). The lifetime risk of a breast cancer diagnosis for a woman in the U.S. is 13%, while the risk of death is approximately 2.6%. According to the American Cancer Society, in 2020, an estimated 276,480 new cases of invasive breast cancer will be diagnosed in women, of which close to 42,170 women will die from the disease. Presently, it has been reported that there are 3.5 million breast cancer survivors in the U.S. (1).

Breast cancer is classified according to the expression of three specific molecular markers; estrogen receptor (ER), progesterone receptor (PR) and human epithelial growth factor receptor 2 (EGFR2/HER2) (3). Approximately 80% of breast cancers are ER+ (4), while 55-65% are PR+ (5). The ER and PR, which both exist as 2 different isoforms, are critical predictive and prognostic markers for breast cancer management (4, 5). Tumors that are ER+/PR+ respond better to endocrine therapy than tumors that are ER-/PR+, or ER+/PR- and especially ER-/PR- (5).

ER and PR status are closely associated with clinical outcomes. Patients with ER-/PR+, ER+/PR-, or ER-/PR- status experience higher risk of mortality (6) and higher histological grade than patients who are ER+/PR+ (7). EGFR2/HER2 amplification is observed in 25-30% of breast cancers and acts as a driver for aggressive tumors (8). Although overexpression of EGFR2/HER2 is used as a prognostic marker in breast cancer management, it is also associated with a poor prognosis and drug resistance (9). Moreover, breast cancer molecular subtypes are classified into intrinsic subtypes, each with a distinct gene expression pattern that can have

important implications for therapeutic strategies and treatment efficacy (10).

The most commonly categorized subgroups are luminal A, which accounts for 30-40% of all invasive breast cancer, luminal B, or HER2- which accounts for 20-30% of all invasive breast cancer, and HER2+, which accounts for 12-20% of all invasive breast cancer (5). Patients with luminal A subtype experience a more favorable prognosis, while prognosis of patients with a luminal B subtype is more intermediary (5).

Luminal subtypes are ER+ and are therefore more responsive to hormonal therapy, less aggressive and associated with better prognosis. HER2+ subtypes experience the opposite outcomes (3, 5, 10). Another intrinsic subtype of breast cancer, which accounts for 10-15% of all breast cancer cases (11), is triple negative breast cancer (TNBC). TNBC describes invasive tumors that are ER-, PR- and HER2- (12) and can further be subdivided into additional subtypes: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like/claudin low (MSL/CL) and luminal androgen receptor (LAR) (13).

TNBC disproportionately affects premenopausal African American (AA) women. These women are more likely to experience higher grade characterization, higher mitotic index, later stage diagnosis and higher mortality rate compared to white women (3, 14, 15). The molecular complexity of TNBC contributes to the difficulties associated with successful treatment. The loss of target receptors is the primary reason why hormonal therapy is not beneficial for TNBC patients.

Current approaches for managing TNBC include surgery and chemotherapy either individually, or in combination (16). Claudin low TNBC has the worst clinical outcomes when compared to other breast cancer subtypes (13). Furthermore, the underlying molecular

mechanism regulating claudin status is not fully understood. An investigation into the factors that mediate negative clinical outcomes in claudin low TNBC may provide insight into how they can be exploited for better prognostication and therapeutic strategies, consequently leading towards better clinical outcomes.

4.2. PVT1

Protein coding genes are estimated to account for approximately 2% of the human genome (17), while the remaining 98% were thought to be "junk DNA". As it turns out, these non-protein coding DNA can be transcribed into RNA, or non-coding RNAs (ncRNAs) (18). ncRNAs can be categorized according to their size: small ncRNAs and long ncRNAs (lncRNAs). Small ncRNAS, such as small nucleolar RNAs (snoRNAs), Piwi-associated RNAs (piRNAs) and microRNAs (miRNAs), are transcripts that are less than 200 bp, while lncRNAs are transcripts that are greater than 200 bp (19).

LncRNAs, which makes up the majority of ncRNAs, have been reported to play a significant regulatory role in various biological processes including transcription (20), translation (21), DNA repair (22) and chromatin remodeling (23). Aberrant expression of lncRNAs have been observed in multiple diseases (24-26), including cancer (27), and may serve as an important biological marker for diagnosis and targeted therapeutic strategies. Previous studies have demonstrated that amplification of the 8q24 gene locus is a common event in many malignant diseases (28).

Plasmacytoma variant translocation 1 (PVT1), a long noncoding RNA (lncRNA) that is transcribed at the 8q24 gene locus, has been demonstrated to play an oncogenic role in multiple cancers including breast cancer (29). Spanning from 128806779 to 129113499 (30), the

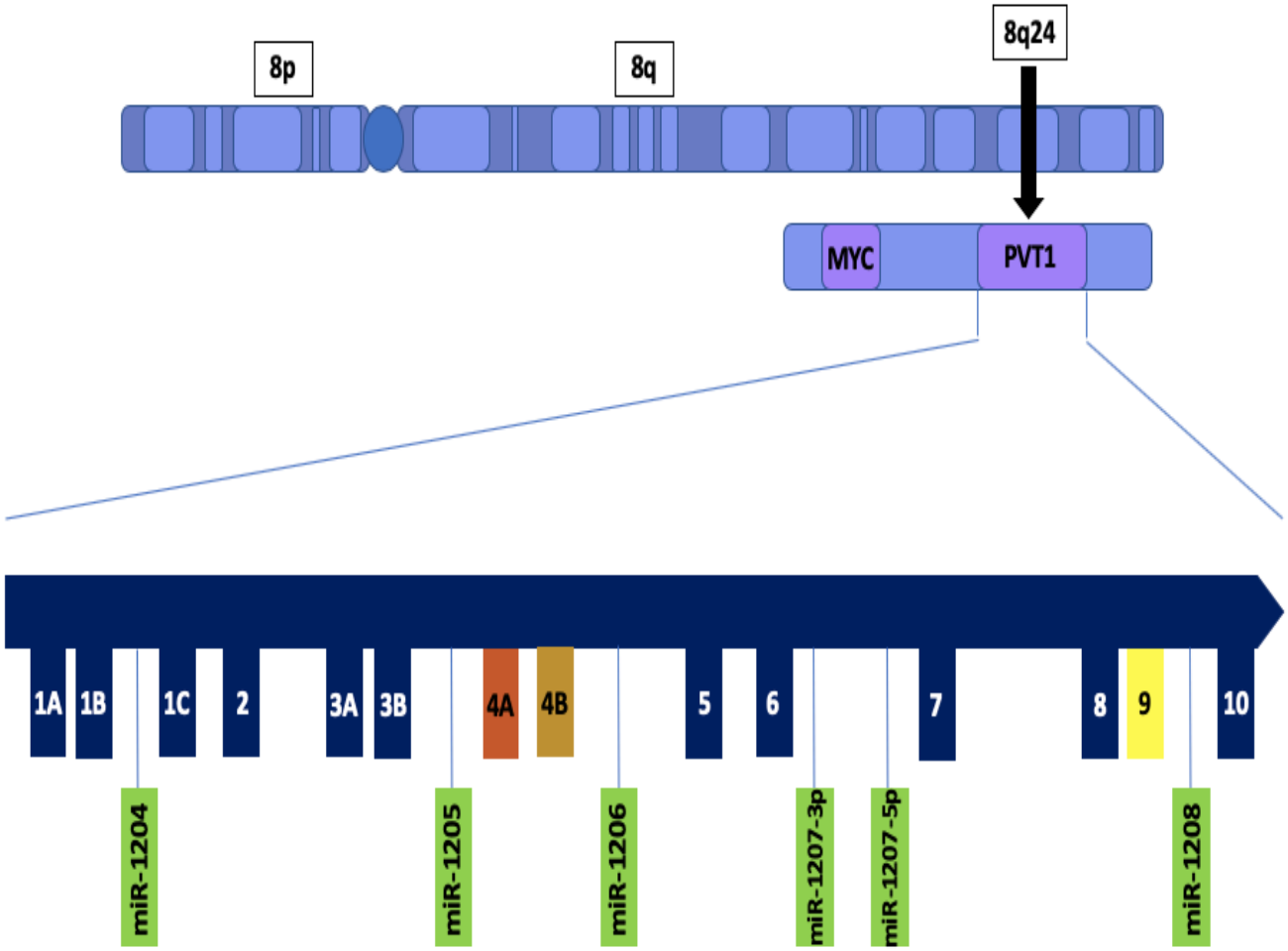

Figure 1: **PVT1 structure and transcripts.** PVT1 is located downstream of the MYC gene on chromosome 8q24. The PVT1 exon involved in this project is highlighted yellow: PVT1 exon 9. PVT1 also encodes 6 microRNAs: miR-1204, miR-1205, mir-1206, miR-1207-3p, miR-1207-

PVT1 gene is a large locus that is greater than 30 kb (20), and contains several exons, namely exons 1a, 1b, 1c, 2, 3a, 3b, 4a, 4b, 5, 6, 7, 8, 9 and 10 (28). Additionally, PVT1 encodes multiple alternatively spliced lncRNA as well as six annotated microRNAs: miR-1204, miR-1205, miR-1206, miR-1207-3p, miR-1207-5p and miR-1208 (31). Approximately 53 kb upstream of PVT1 is the oncogene MYC (32).

4.3. PVT1 and breast cancer

LncRNAs have been reported to play a significant role in various biological processes. The involvement of lncRNAs in tumorigenesis has been well recorded. More specifically, studies have demonstrated that lncRNAs are involved in the regulatory mechanisms of various processes by acting as critical regulators of gene expression (33). There is much evidence that demonstrates lncRNA, PVT1, involvement in regulating different cellular processes, which can ultimately promote malignancies, including angiogenesis (34), cell cycle progression (35), proliferation and apoptosis (36).

PVT1 amplification is associated with many clinicopathological characteristics in breast cancer (29). Studies have demonstrated that PVT1 promotes proliferation and tumorigenesis. Tang et. al. reported that PVT1 promotes proliferation in TNBC via a PVT1/KLF5/β-catenin pathway. PVT1 binds to, and stabilizes transcription factor KLF5. This then leads to β-catenin upregulation, which is associated with poor prognosis in breast cancer patients. Conversely, down regulation of PVT1 led to the rapid degradation of KLF5, which resulted in an inhibition of proliferation and tumor growth (37). Wang et. al. reported that upregulation of transcription factor SOX2 is correlated with PVT1 expression. SOX2 binds to the promoter of PVT1, activating its transcription, which then promotes cell growth and invasion in breast cancer (38).

PVT1 also regulates apoptosis (29) and metastasis (39) in breast cancer. One of the most vital processes in metastatic cancer is the epithelial-to-mesenchymal transition (EMT). In one study, PVT1 has been shown to affect EMT in triple negative breast cancer. E-cadherin and vimentin are 2 major EMT markers. Knockdown of PVT1 expression, using siRNAs, resulted in the upregulation of E-cadherin, and the down regulation of vimentin and N-cadherin.

This result suggests that downregulation of PVT1 inhibits EMT (40). Wang et. al. reported that PVT1 was overexpressed in both breast cancer tissue and cells via a SOX2 facilitated manner. Downregulation of PVT1 inhibited EMT, therefore blocking cancer progression (38).

PVT1-derived transcripts have been demonstrated to promote breast tumorigenesis as a well. miR-1207 has been shown to be distinctly upregulated in breast cancer in young women (41). Aberrant expression of miR-1207-5p has been shown to promote proliferation, colony formation and cell cycle progression in breast cancer, by targeting transcription factor, STAT-6, and downregulating its expression. miR-1207-5p has also been shown to promote chemoresistance in TNBC by targeting Leucine zipper tumor suppressor gene 1 (LZTS1). Consequently, this led to a decrease in apoptosis and cell growth arrest, which is induced by Taxol. Conversely, combined treatment of Taxol with antagomir-1207-5p upregulated expression of pro-apoptotic gene Bax, and downregulated expression of anti-apoptotic genes Bcl-2 and p-Akt (42). Liu et. al. reported that miR-1204 promotes proliferation and EMT by targeting the vitamin D receptor gene (VDR) and modulating its expression (39). VDR is a transcription factor that is involved in cell growth and differentiation in both normal, and tumor, breast tissue (43). Future research should be conducted to further explore the involvement of PVT1 transcripts in breast cancer, and even other malignancies.

4.4. Tight Junctions (TJs) and tumorigenesis

Epithelial and endothelial cell-cell adhesion are mediated through multi-protein junctional complexes, which among other things, are responsible for maintaining a cells' architectural structure. There are four main types of intercellular junctions; gap junctions, adherens junctions (AJs) and tight junctions (TJs), which are located in the apical most region of the

cell, and desmosomes, which are located towards the basal region of the cell (44, 45). AJs and desmosomes primarily contribute to the mechanical adhesions between neighboring cells, while TJs are linked to maintaining cellular homeostasis, as well as many other cellular functions (46).

TJs are multifunctional complexes composed of both transmembrane and peripheral proteins that connect cells via homophilic and heterophilic interaction (44, 45). They are comprised of a network of strands that encircle a cell creating a boundary that separates the apical domain of the cell from the basal domain. Consequently, this separation forms a "gate", or para-cellular diffusion barrier, which regulates permeability and a "fence", or intramembrane diffusion barrier, which limits apical-basolateral diffusion (47, 48). In addition to their role in intercellular adhesion and acting as a semipermeable barrier, TJs functions as a barrier to cellular motility (49), can recruit adaptor proteins which offer anchorage, and cytosolic partners that assemble into signaling complexes (44). TJ signaling occurs in a bidirectional manner. Signals transduced from inside the cell towards TJs guide their assembly and regulate their functions. Signals transmitted from TJs into the cell regulate gene expression, including cell proliferation and cell differentiation (47). TJ complexes are involved in various other cellular functions including maintaining cellular polarity, cell cycle and vesicle trafficking (44, 45, 47, 48, 50, 51). Currently, there are over 40 TJ proteins that has been topologically characterized into at least four major transmembrane proteins that constitutes epithelial TJs; occludin, claudins, the coxsackievirus and adenovirus receptor (CAR), and

members of the junctional adhesion molecule family (JAM) (44, 45).

The involvement of TJs in cancer biology is associated with dysfunctional signal transduction pathways that regulate cell-cell interactions, in other words, loss of cell polarity and growth control (52). Aberrant expression of TJ proteins disrupts normal physiological function, which could lead to pathological consequences. Many studies have demonstrated the dysregulation of TJ proteins in cancer (53-57). JAM and CAR, for example, are proteins are type I transmembrane proteins, and members of the immunoglobulin superfamily (58, 59) with various functionalities including cell adhesion including platelet activation (60, 61), angiogenesis (62) and cell migration (63, 64).

Previous work has demonstrated a novel link between JAM-A overexpression and reduced survival of breast cancer patients (65), contrastingly, it was reported that downregulation of JAM-A occurs early in renal cancer and increases renal cancer cell migration (66). CAR overexpression is associated with a lower survival rate in oral squamous cell carcinoma (67) and is associated with a higher grade of malignancy in breast cancer (68). Contrastingly, CAR has been reported to act as a metastatic suppressor in lung cancer (69) and a tumor suppressor in gastric cancer (70), therefore downregulation of CAR expression can have serious clinical implications.

4.5. Claudins in cancer

Named after the Latin word 'claudere' which means to close, claudins (CLDNs) are a family of TJ proteins that consists of 27 members (71). Ranging in size between 20-34 kDa, these tetraspan proteins contains an amino and carboxyl-terminal cytoplasmic domain (72), as well as 2 extracellular loops which are critical for maintaining TJ function (73). CLDNs, via

the PDZ-binding motifs along their carboxyl-terminus, can interact directly with multiple signaling pathways and PDZ domain-containing cytoplasmic TJ-associated proteins such as ZO-1, ZO-2, ZO-3, PATJ and MUPPI (45, 74, 75). CLDNs are extremely tissue or cell specific. Most tissues or cells express CLDNs in various combinations or ubiquitously, while others may express a single CLDN (76).

Arguably the most important constituent of TJs, CLDN functions primarily involves maintaining cellular polarity, signaling (77), as well as regulating and maintaining paracellular permeability via a paracellular barrier (47, 48) or paracellular channel (71). CLDNs, through use of both mechanisms, regulate the transport of solutes between cells, including charged and uncharged ions, and small molecules (71). Various studies demonstrate compelling evidence of CLDNs and their role in tumorigenicity. Aberrant expression of CLDNs have been well documented whereby both loss and gain of function have been associated with multiple cancers including prostate (78), colorectal (79, 80), liver (78, 80), pancreatic (80), lung (81), cervical (82), gastric (83), ovarian (84, 85) and breast (86, 87). In particular, CLDNs 1, 3, 4 and 7 are among the most frequently dysregulated of the CLDN family members (52, 88).

The discrepancy in the expression of CLDNs, in combination or individually, can vary according to tumor type. In one study, it was reported that epithelial tumors can be distinguished from other tumors, including lymphoid and soft tissue tumors, or tumors of a higher grade, due to the specific differential expression of CLDNs 1, 2, 3, 4, 5 and 7 (89). Downregulation of CLDNs impede barrier functions by amplifying the influx of nutrients and growth factors needed by cancerous cells (71). CLDN7 under expression have been reported

in invasive breast (90), colon (91) and prostate cancer (92), as well as esophageal (93) and renal cancer (94) Similarly, under expression of CLDN1 have been reported in invasive breast (95), prostate (92), liver (96) and colon cancer (91).

Altered expression of CLDN1 has been demonstrated to be associated with higher tumor grade (97), recurrence (87) and metastasis and invasion (98). Contrastingly, many studies have demonstrated that overexpression of CLDNs are also associated with cancer development and progression. Upregulation of CLDNs also affect barrier function and increase paracellular permeability (71). Overexpression of CLDN3 and CLDN4, for example, is a common process in several malignancies including pancreatic, uterus, thyroid, stomach, and breast (78). In ovarian cancer, CLDN3 and CLDN4 are among the most highly upregulated genes (99). Moreover, studies showed that increased expression of these CLDNs were found in advanced staged tumors (100), and may cause increased cell survival, motility and invasion (101).

Overall, the altered expression of CLDNs is dichotomous. This further supports that these TJs are indeed tissue and cell specific. There are many studies that demonstrate the role they play in tumorigenicity, however the underlying mechanisms and their biological significance in cancer remains unclear.

4.6. Claudin 4 and breast cancer

Claudin 4 (CLDN4), is one of the most frequently dysregulated of the CLDN family members (52, 88), and has been reported to be involved in various biological processes including EMT (102), metastasis and invasion (103), and epigenetic modulation (104). The clinicopathological associations of CLDN4 in breast cancer is well documented. Patients with CLDN4 overexpression experience high tumor grade and poor prognosis, as well as shorter disease-free survival. Additionally, it was reported that there is an association between ER

status and CLDN4 expression. CLDN4 expression was significantly higher in tumors that were ER- (105, 106).

Some studies have demonstrated how CLDN4 can be a useful prognostic marker in breast cancer (107, 108). Kulka et. al. reported that CLDN4 expression was significantly higher in basal-like carcinomas, as opposed to the other tumor groups (grades 1-3). In fact, tumors of lower grades, specifically grades 1 and 2, had decreased or absent expression of CLDN4 (109). Contrastingly, Kolokytha et. al. reported that enhanced expression of CLDN4 in luminal breast cancers were linked to poor outcomes, while overexpression of CLDN4 in TNBC was associated with favorable outcomes. According to the study, tumors which overexpressed CLDN4 displayed a less aggressive phenotype (105). The conflicting results concerning CLDN4 expression in breast cancer is a clear indication that future studies are warranted. In an *in vivo* study carried out by Ma et. al., mice that were implanted with MCF-7 cells where CLDN4 was knocked down had significantly smaller tumors than tumors from the control group. Additionally, it was reported that CLDN4 upregulation, via methylation status, increased cell proliferation and migration of MCF-7 cells, while inhibiting apoptosis. (110). Taken together, these results support that CLDN4 promotes breast tumorigenesis.

Vasculogenic mimicry (VM) refers to an important de novo process in which aggressive tumors undergo in the absence of endothelial cells. This process can occur independent of angiogenesis to establish an oxygen and nutrient supply via the formation of vessel-like channels (111). Cui et. al. reported that CLDN4 is required for VM formation. More specifically, upregulation of CLDN4 induced VM formation, while downregulation of CLDN4 inhibited VM formation in breast cancer cells. Additionally, it was observed that VM formation

was more prevalent in more aggressive breast cancer cells than cells that were less aggressive (112).

4.7. Project rationale

We've previously reported that alternatively spliced transcripts of PVT1 were differentially expressed in prostate cancer. More specifically, PVT1 exon 9 were overexpressed in prostate cancer tissue taken from men of African Ancestry (moAA) (113). This suggests that PVT1-derived transcripts in prostate cancer tend to include one or more of these 3 exons in moAA. PVT1 exon 9 expression was significantly higher in prostate cancer cell lines derived from moAA when compared to cell lines derived from Caucasian men. PVT1 exon 9 expression was elevated in more aggressive cell lines, in comparison to cell lines that were less aggressive (114). Taken together, these results suggest that PVT1 exon 9 is potentially associated with a more aggressive prostate cancer phenotype. Moreover, an *in vivo* study carried out by our group demonstrated that overexpression of PVT1 exon 9 plays a significant role in prostate cancer initiation and progression (115), thus establishing an oncogenic role for PVT1 exon 9 in prostate cancer.

PVT1 amplification is a common occurrence in many malignancies including breast cancer, and is associated with poor survival rate among patients (29, 37, 114). Many studies have demonstrated a critical functional role for PVT1 in breast cancer (29, 40), however the underlying molecular mechanisms associated with PVT1 transcripts in breast cancer are not well understood. In this study, we were interested in investigating the role of PVT1 exon 9 play in breast cancer. We hypothesized that one or more of these transcripts may be correlated

with PVT1 function in breast cancer.

4.8. Experimental workflow

A panel of 5 breast cancer cell lines, with distinct molecular subtyping (ie: luminal A, luminal B, HER2+ and triple negative), were used to assess the expression levels of PVT1 exon 9. Total RNA was isolated from each cell line, followed by cDNA synthesis. Using custom-designed primers, all mRNA expressions for PVT1 exon 9 were determined via RT-qPCR. PVT1 exon 9 was significantly overexpressed in claudin low (CL) triple negative breast cancer (TNBC) cell line, MDA MB 231, and significantly under expressed in claudin high (CH) TNBC cell line MDA MB 468.

PVT1 exon 9 expression was knocked down in CL TNBC cell line MDA MB 231, following transfection with a highly efficient patent-protected siRNA (siPVT1 exon 9). A corresponding scramble siRNA (siScramble) was used as a control. Verification of mRNA

Table 1: Characterization of 5 breast cancer cell lines

Cell line	**Tissue of origin**	**Tumor pathology**	**Receptor status**	**BRCA1 mutation status**	**Claudin status**	**References**
T47D	Mammary gland	Ductal Carcinoma	ER+, PR+, HER2-	Wildtype	Claudin high (luminal A)	(116-120)
MDA MB 468	Mammary gland	Adenocarcinoma	ER-, PR-, HER2-	Wildtype	Claudin high (basal)	(116-120)
MDA MB 231	Mammary gland	Adenocarcinoma	ER-, PR-, HER2-	Wildtype	Claudin-low	(116-120)
MCF-7	Mammary gland	Invasive Ductal Carcinoma	ER+, PR+, HER2-	Wildtype	Claudin high (luminal B)	(116-121)
BT474	Mammary gland	Invasive Ductal Carcinoma	ER+, PR+, HER2+	Wildtype	Claudin high (luminal A)	(116-120)

expression was done via total RNA isolation, cDNA synthesis and RT- qPCR. A functional assay was carried out to determine the regulatory role this transcript plays in TNBC.

PVT1 exon 9 was overexpressed in CH TNBC cell line MDA MB 468, following transfection with a plasmid vector expressing PVT1 exon 9. A corresponding empty vector was used as a control. Verification of mRNA expression was done via total RNA.

Table 2: Primer sequences used for RT-qPCR

Primer Name	Primer Sequence 5'-3'
PVT1 exon 4A-F	GGGTTCAAGTGATCCTCCTG
PVT1 exon 4A-R	TGTAATCCCAGCACGTTGAA
PVT1 exon 4B-F	CACCTGGGATTTAGGCACTT
PVT1 exon 4B-R	CCAATCTCAAAATACTCCAGCTTT
PVT1 exon 9-F	CATGACTCCACCTGGACCTT
PVT1 exon 9-R	GTGGGCGATGAAGTTCGTA
CLDN1-F	CTGCTGCTTCTCTCTGCCTT
CLDN1-R	GCAGGTTTTGGATAGGGCCT
CLDN3-F	GGACTTCTACAACCCCGTGG
CLDN3-R	TGGTGGCCGTGTACTTCTTC
CLDN4-F	TGGGAGGGCCTATGGATGAA
CLDN4-R	GCTTTCATCCTCCAGGCAGT
CLDN7-F	GTCTTGCCACCTTGGTAGCT
CLDN7-R	CCCTGCCCAGCCAATAAAGA
GAPDH-F	GAGTCAACGGATTTGGTCGT
GAPDH-R	TTGATTTTGGAGGGATCTCG

isolation, cDNA synthesis and RT-qPCR.

CLDNs 1, 3, 4 and 7 are among the most frequently dysregulated of the CLDN family members (52, 88). The discrepancy in the expression of CLDNs, in combination or individually, can vary according to tumor type. Following knock down, and overexpression, of PVT1 exon 9 in CL TNBC cell line MDA MB 231 and CH TNBC MDA MB 468, respectively, mRNA expression for

CLDNs 1, 3, 4 and 7 were determined using RT-qPCR. Western blotting was done to examine the protein expression CLDNs.

Studies show that dysregulation of CLDN proteins disrupts tight junctions consequently inducing the epithelial-to-mesenchymal transition (EMT) in cancers, resulting in enhanced motility (122). Following knock down, and overexpression, of PVT1 exon 9 in CL TNBC cell line MDA MB 231 and CH TNBC MDA MB 468, respectively, western blotting was carried out to assess the expression of EMT markers.

5. Materials and Methods:

5.1. Cell Lines and Cell Culture

A panel of 5 breast cancer cell lines were used to assess the expression of PVT1 exon 9. MDA MB 231, MDA MB 468, T47D and MCF-7 cells were kind gifts from Dr. Jill Bargonetti (City University of New York, Hunter College) and BT474 from Dr. Brian Zeglis (City University of New York, Hunter College). MDA MB 231, MDA MB 468, T47D and MCF-7 were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% FBS and 0.5% penicillin/streptomycin. Trypsinization of cells occurred using 0.05% trypsin when cells were 70-80% confluent. BT474 was maintained in F12/DMEM (GIBCO), supplemented with 10% FBS and 1% penicillin/streptomycin. Trypsinization of cells occurred using 0.25% trypsin when cells were 70-80% confluent. All cell lines were cultured in a 5% CO_2, 37 °C atmosphere.

5.2. Transfection of siRNAs

MDA MB 231 cells were grown in 6-well plates until they have reached 90-100% confluency. According to the manufacturer's instructions, cells were transfected with 10 nM of PVT1 exon 9 siRNA (siPVT1 exon 9) (Forward: 5' ACCUAUGAGCUUUGAAUAA 3'; Reverse: 5' UUAUUCAAAGCUCAUAGGU 3') (Sigma, St. Louis, MO, USA), or a non-targeting scramble control (siScramble) (Forward: 5' CUCACUACCGUCGACCCCA 3'; Reverse: 5' UGGGGUCGACGGUAGUGAG 3') (Sigma, St. Louis, MO, USA) using Lipofectamine RNAiMAX (Thermo Fisher Scientific Inc.; Wilmington, DE, USA). siRNAs and Lipofectamine were diluted in Opti-MEM(ThermoFisher Scientific Inc.; Wilmington, DE, USA). Following transfection, cells were incubated for 24 h in a 5% CO_2, 37 °C atmosphere

before being harvested, or migration assay.

5.3. RNA extraction and RT-q PCR

At 70-75% confluency, total RNA was extracted from cells in a 60 x 15 mm tissue culture dish, using the RNeasy Mini Kit (QIAGEN, Hilden Germany, cat#: 74104) according to the manufacturers' instructions. RNA concentration was measured using the spectrophotometer NanoDropTM 2000 (Thermo Fisher Scientific, Inc.). cDNA was synthesized from 1 μg of RNA using QuantiTect reverse transcription kit (Qiagen, Germany, cat# 205311). Amplification reactions were performed in 25 μL reaction volume using SYBR Green PCR master mix (Life Technologies, Grand Island, NY, USA cat# 4309155), cDNA template and 0.4 μM final concentration for primers. Primers used in this study were composed of the following oligonucleotide sequences listed in Table 2. Using the Quantifect Studio System (Applied Biosystems), relative expression of messenger RNA (mRNA) for each sample was assessed in quadruplicates in at least 3 independent experiments, and quantified via the comparative cycle threshold (ΔΔ Ct) method and normalized to GAPDH mRNA expression.

5.4. Protein extraction and Immunoblotting

Whole cell extracts were obtained using a cocktail of RIPA lysis buffer (VWR Life Science, cat# N653-100ML) supplemented with 10 x protease inhibitor and 100 mM of phenyl methylsulfonyl fluoride (PMSF) (Amresco, cat# M145-5G). Protein concentration was quantified via the Bradford Assay using the Bio-Rad Protein Assay Dye Reagent Concentrate (Biorad, cat# 500-0205). For Western blot analysis, 50 μg of protein were resolved by 15% SDS-PAGE gels and subsequently transferred onto nitrocellulose membranes. Membranes were blocked in 5% BSA or 5% milk in TBS-T for 1 hour at room temperature,

incubated overnight at 4 °C in primary antibodies, washed in 1 x TBS-T, incubated with secondary antibodies for 2 hours, washes with 1 x TBS-T and imaged using the LI-COR Odyssey CLx imager with infrared fluorescence. The primary antibodies used were directed against Claudin 1 (13050-1-AP), Claudin 4 (16195-1-AP) and Claudin 7 (10118-1-AP) (Proteintech, Rosemont, IL, USA), Claudin 3 (341700; Invitrogen, Waltham, MA, USA), GAPDH (5174S; 1:1000; Cell Signaling, MA, USA), alpha-tubulin (sc-32293; 1:1000; Santa Cruz Biotechnology, TX, USA). The secondary antibodies used were anti-mouse (925-32210; 1:15,000; LI-COR, Lincoln, NE, USA) and anti-rabbit (925-32211; 1:15,000; LI-COR, Lincoln, NE, USA).

5.5. Migration Assay

1 x 10^5 MDA MB 231 cells were grown in a 6-well tissue culture plate until 90-100% confluency. Cells were transfected with siRNA, or siScramble, and incubated at 5% CO_2, 37 °C for 24 h. Following transfection, and using a sterile 200 μL pipet tip, wounds were made to cell monolayer in each well. Wounded cells were washed with 1X PBS and incubated with media. Images of scratched areas were taken at 10X magnification using the Motic AE30 Inverted microscope.

5.6. Statistical Analysis

Data from at least 3 independent experiments were presented as mean ± standard error of the mean (S.E.M). Statistical significance was assessed using a two-tailed Student's *t* test. *p* values less than 0.05 were deemed significant.

6. Results:

6.1. Expression of claudins 1, 3, 4 and 7 in TNBC.

To assess the expression of claudins 1, 3, 4 and 7 in each cell line, we performed total RNA extraction, cDNA synthesis and real-time quantitative polymerase chain reaction (RT-qPCR).

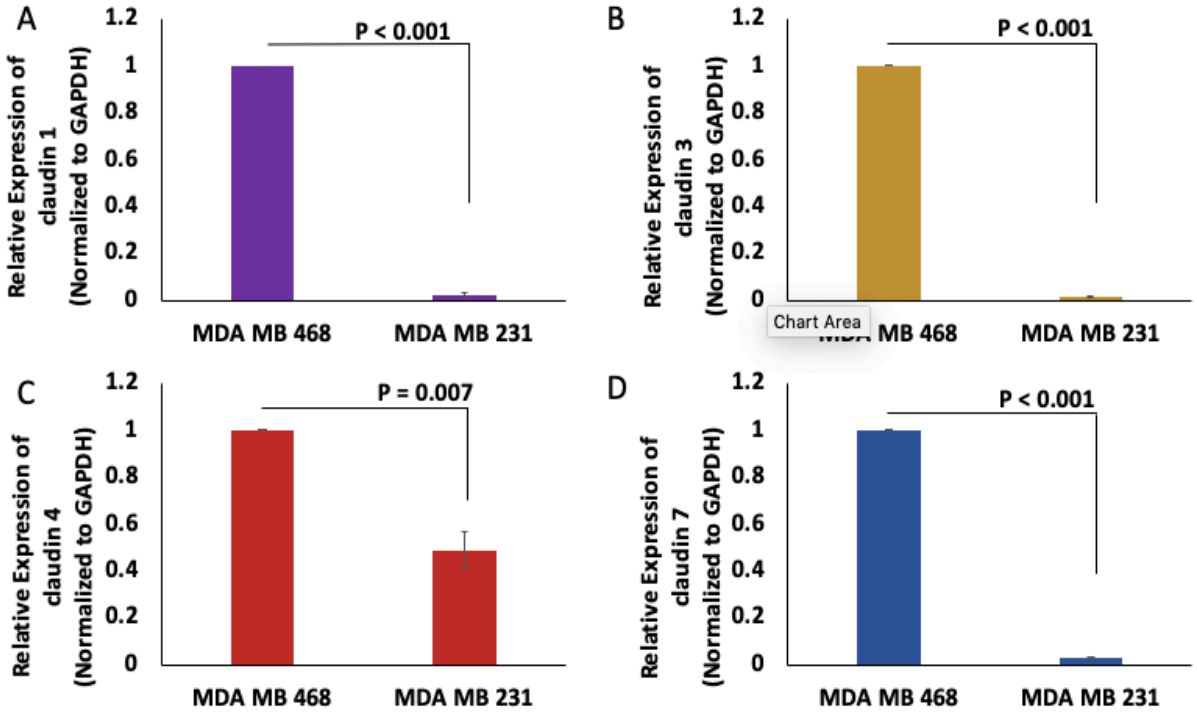

Figure 2: **Claudins 1, 3, 4 and 7 expression in MDA MB 468 and MDA MB 231 cells**. Claudin 1 **(A)**, claudin 3 **(B)**, claudin 4 **(C)** and claudin 7 **(D)** expression were assessed using qPCR in MDA MB 231 and MDA MB 468 cell lines; (n=4).

We observed that claudins 1, 3, 4 and 7 are downregulated in the MDA MB 231 TNBC cell line, when compared to the MDA MB 468 TNBC cell line. (Fig. 2).

6.2. Expression of PVT1 exon 9 is upregulated in claudin low TNBC

To assess the expression of PVT1 exon 9 in each cell line, we performed total RNA extraction, cDNA synthesis and real-time quantitative polymerase chain reaction (RT-qPCR). We

observed that PVT1 exon 9 is overexpressed in CL MDA MB 231 TNBC cell line when compared to CH MDA MB 468 TNBC cell line (Fig. 3).

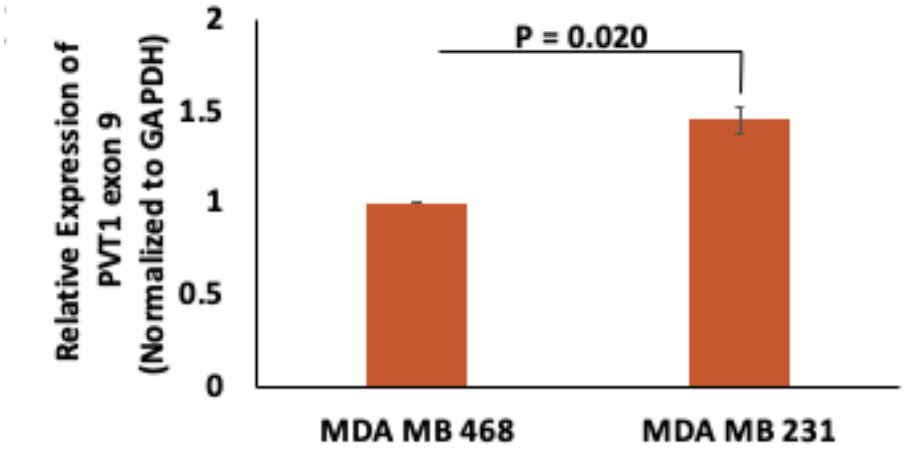

Figure 3: **PVT1 exon 9 expression in MDA MB 468 and MDA MB 231 cells.** PVT1 exon and 9 expression was assessed using qPCR in MDA MB 231 and MDA MB 468. Expression was normalized against GAPDH. Experiment was carried out at least 3 times.

6.3. PVT1 exon 9 regulates migration in claudin low TNBC

To determine the regulatory roles of PVT1 exon 9 in TNBC, an siRNA study was conducted. We knocked down the expression of PVT1 exon 9 in MDA MB 231 cells and performed wound healing assays (Fig. 2). The relative expression of PVT1 exon 9 was verified by qPCR. Assessment of the migratory capabilities of cells are based on the rate in which the wound closes. Pictures were taken at 0h, 4h, 24h and 28h. We observed that cells in which PVT1 exon 9 was knocked down were less migratory than their scramble control counterpart. siPVT1 exon 9 cells exhibited a 20% decrease in migration in comparison with siScramble cells (Fig 4). The decrease of migration in MDA MB 231 cells is statistically significant (p-

value: 0.01). This suggests that PVT1 exon 9 may be involved in regulating cell motility in TNBC.

6.4. PVT1 exon 9 regulates claudin 4 expression in claudin low TNBC

To determine if PVT1 exon 9 was regulating claudin expression, total RNA was isolated to assess CLDN expression, at a transcriptional level, following knockdown of PVT1 exon 9 in MDA MB 231 cells. RT-qPCR was used to qualitatively assess CLDN mRNA expression.

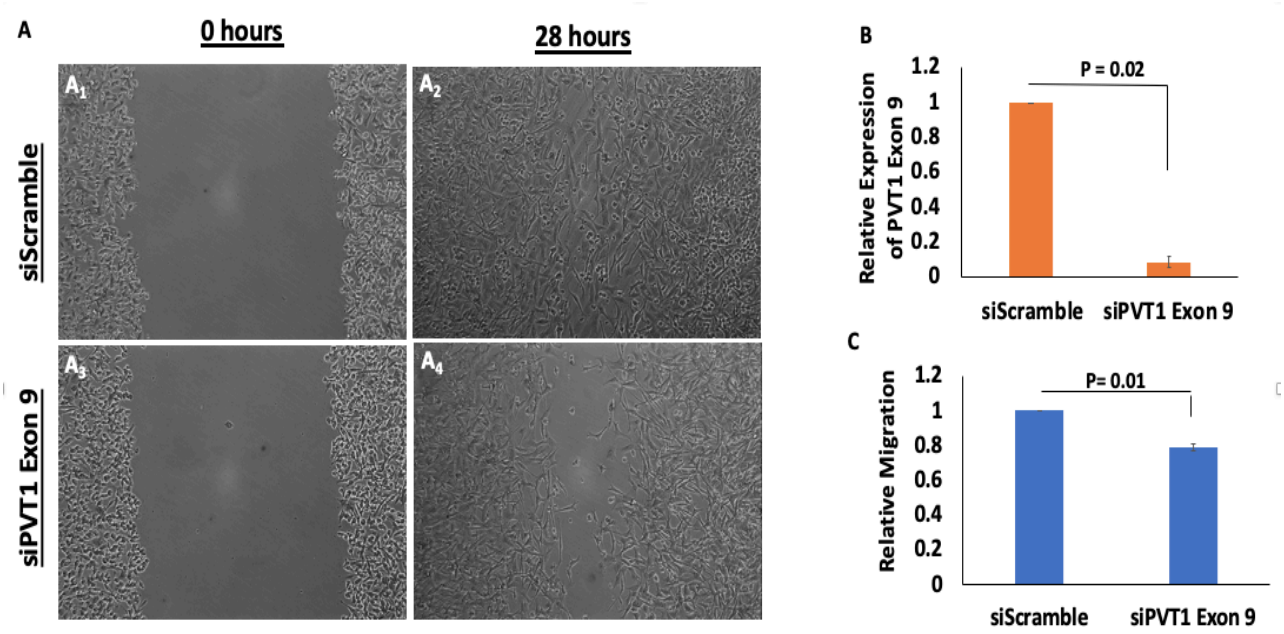

Figure 4: **PVT1 exon 9 regulates migration in MDA MB 231 cells. (A)** Wound healing migration assays were performed on MDA MB 231 cell line. Cells were transfected once confluent. After 24 hours wounds were made and measured between 0 hour and 28 hours. **(B)** siRNA Transfection. Relative expression of PVT1 Exon 9 in MDA MB 231 based on 2 independent experiments. **(C)** Migration quantification; n=3

We observed that knockdown of PVT1 exon 9 in MDA MB 231 does not significantly affect CLDN mRNA expression when compared to the control (Fig 5). Next, proteins were isolated to assess CLDN expression at a translational level. A western blot was used to assess CLDN protein expression when PVT1 exon 9 is knocked down in MDA MB 231 cells. We did not

observe any significant change in expression for CLDNs 1, 3 and 7 when PVT1 exon 9 is knocked down. Interestingly, we observed a re-expression of CLDN4 in MDA MB 231 when PVT1 exon 9 expression is knocked down (Fig 6). These results suggest that PVT1 exon 9 is regulating claudin 4 expression in TNBC.

6.5. PVT1 exon 9 does not regulate EMT in TNBC

EMT is a critical process that occurs in many malignancies (102), and PVT1 has been shown to be involved in EMT induction (40). To investigate if PVT1 exon 9 transcript may be regulating EMT, a western blot was used to assess the expression of EMT markers; vimentin,

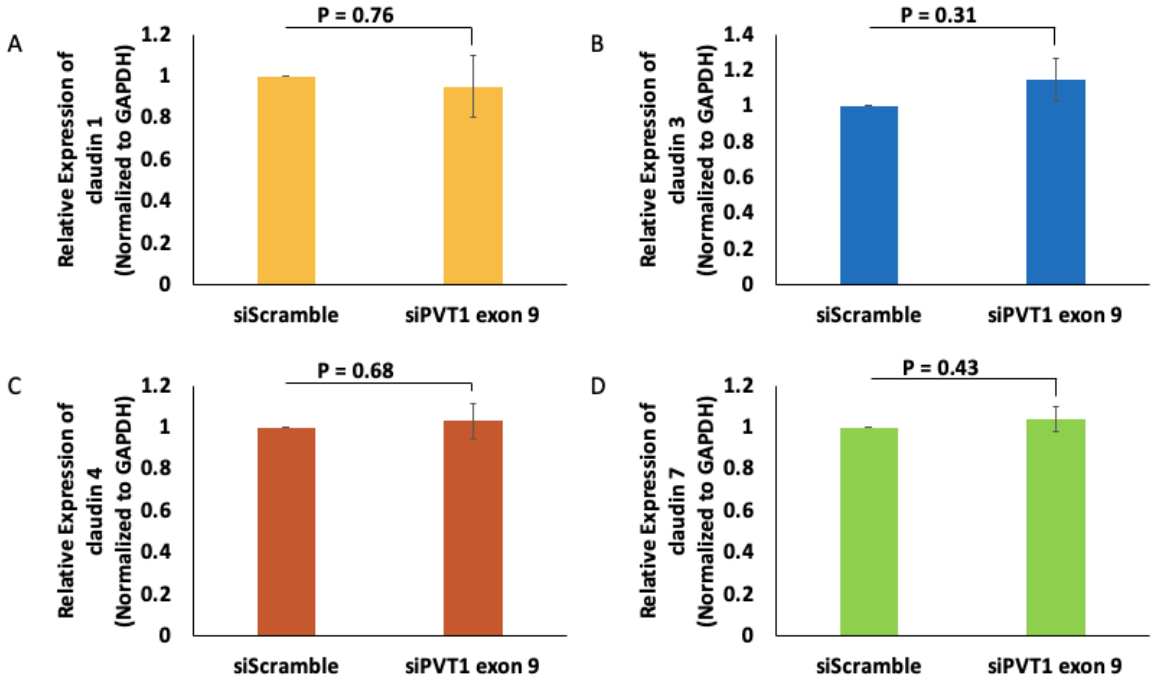

Figure 5: **PVT1 exon 9 does not regulate claudin transcription in MDA MB 231 cells.** Claudin 1 **(A),** claudin 3 **(B),** claudin 4 **(C)** and claudin 7 **(D)** expression were assessed following knock down of PVT1 exon 9 in MDA MB 231 cell line. Normalized against GAPDH; (n=3).

E-cadherin and fibronectin, in MDA MB 231 cells. We observed no changes in EMT markers when PVT1 exon 9 is knocked down; however, our data suggests that EMT markers are more

highly expressed in MDA MB 231 cells in comparison to claudin high MDA MB 468 TNBC cells (data not shown).

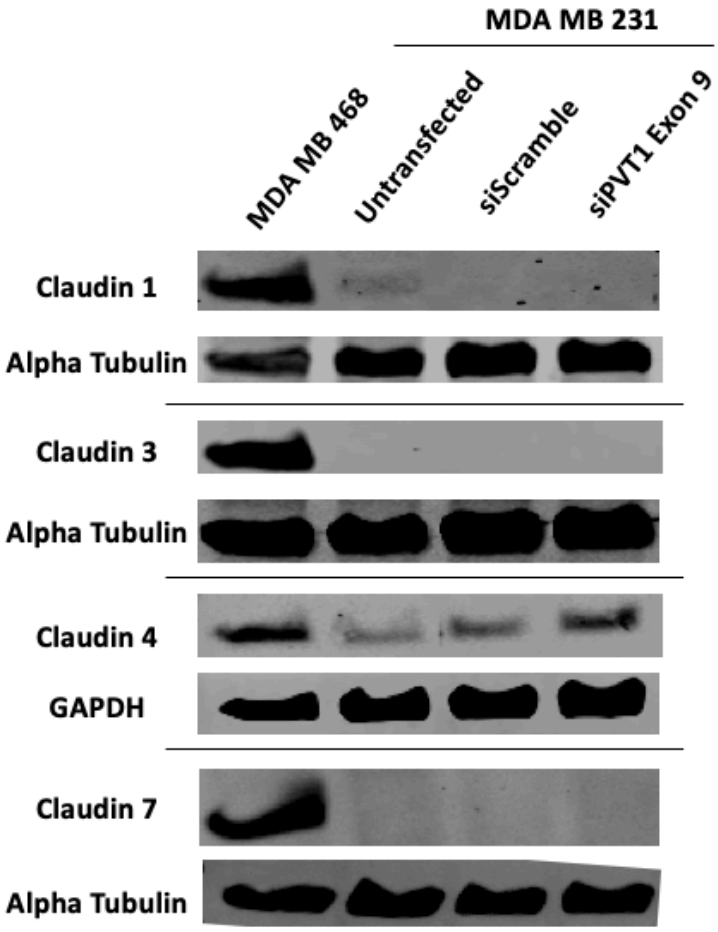

Figure 6: **PVT1 exon 9 regulates claudin 4 expression in MDA MB 231 cells**. MDA MB 231 were transfected with siPVT1 exon 9 specific siRNAs for 24 hours. Western blotting was performed using antibodies against claudin 1, claudin 3, claudin 4 and claudin 7. Loss of PVT1 exon 9 increased claudin 4 expression when compared to siScramble.

7. Discussion:

Breast cancer remains the second leading cause of cancer related death in U.S. women (1, 2). Although much progress has been made in breast cancer treatment, patients with TNBC continue to have poor prognosis (14). CL TNBC has the worst prognosis. Additionally, there are no effective treatments presently available to manage this breast cancer subtype (13). Therefore, it is imperative that efforts are made towards understanding the molecular mechanisms which regulate CLDN expression as it would facilitate the development of novel therapeutic strategies. PVT1 has already been demonstrated to play a role in breast tumorigenesis (38, 39). However, the specific long non-coding transcripts that are important in breast cancer are unclear. Moreover, to our knowledge, PVT1 regulation of CLDN expression has not been previously studied

With a total of approximately 12 exons, the PVT1 gene encodes several alternatively spliced lncRNA transcripts. Differential expression of these transcripts in breast cancer has not been previously investigated. The purpose of this study was to investigate the association between breast tumorigenesis and PVT1 transcripts containing exon 9. In this study, we demonstrated that PVT1 exon 9 may play an important regulatory role in TNBC. Our group has previously reported that the transcript from exon 9, PVT1 exon 9, was differentially expressed in prostate cancer. More specifically, PVT1 exon 9 was overexpressed in prostate cancer tissue taken from men of African Ancestry (moAA) (113). This finding suggests that alternatively spliced transcripts of PVT1, including transcript containing PVT1 exon 9, may be associated with increased risk of cancer among moAA. Additionally, PVT1 exon 9 expression was significantly higher in prostate cancer cell lines derived from moAA when compared to cell lines derived from Caucasian men, and the elevated expression was associated with an aggressive phenotype (114). In an *in vivo* study,

overexpression of PVT1 exon 9 induced malignant transformation of prostate epithelial cells and increased proliferation and migration in these cells (115). The aforementioned studies established an oncogenic role for PVT1 exon 9 in prostate cancer; however, the significance of PVT1 exon 9 in breast cancer was not previously investigated.

In breast cancer cell lines, each varying based on molecular characteristics, we observed that PVT1 exon 9 was significantly overexpressed in CL MDA MB 231 TNBC cells, and significantly under-expressed in CH MDA MB 468 TNBC cells, when compared to T47D cells. Consequently, we used claudin low MDA MB 231 as a model for PVT1 exon 9 overexpression, and claudin high MDA MB 468 as a model for PVT1 exon 9 under-expression.

We observed that MDA MB 231 cells transfected with siPVT1 exon 9 were less migratory when compared to cells that were transfected using siScramble. These results suggest that overexpression of PVT1 exon 9 increases the migratory capacity of TNBC cells, and that loss of PVT1 exon 9 expression may have a protective role by making cells less migratory. Cancer cell migration is a characteristic of metastasis in cancer patients and is associated with poor prognosis (39). Further studies are necessary in order to elucidate the underlying mechanisms by which this regulatory process occurs. Moreover, additional *in vitro* and *in vivo* functional assays, and molecular analysis, should be carried out in order to better understand the role of PVT1 exon 9 in breast tumorigenesis.

CLDNs 1, 3, 4 and 7 are among the most frequently dysregulated of the CLDN family members (52, 88). The difference in the expression of CLDNs is tissue and cell specific, which could signify a difference in their function based on their localization and expression. Previous studies reported that lncRNAs act as critical regulators of gene expression (33).

This is in keeping with our observation that PVT1 exon 9 is overexpressed in CL MDA MB 231 TNBC cells and under expressed in CH MDA MB 468 TNBC cells. Consequently, we speculate that PVT1 exon 9 may be regulating claudin expression either at a post-transcriptional level, a post-translational level, or indirectly.

In keeping with previous reports, we have confirmed the CLDN expression profile. Our results showed no significant change in CLDN mRNA expression. Consequently, this result suggests that PVT1 exon 9 may not be regulating claudin expression at a transcriptional level in claudin low MDA MB 231. It may be that PVT1 exon 9 may be interacting with CLDN proteins directly, or indirectly, to regulate their expression.

Our data demonstrate that PVT1 regulates CLDN expression. We observed that when PVT1 exon 9 is knocked down there is no change in protein expression for CLDNs 1, 3 and 7, however, we did observe a re-expression of CLDN4 in CL MDA MB 231 TNBC cells. LncRNAs are known to interact with proteins to regulate their stability, or ubiquitination (123). Since knockdown of PVT1 expression led to an increase in CLDN4 protein expression, it is plausible that PVT1 exon 9 regulates CLDN4 protein expression by regulating its ubiquitination. Consequently, PVT1 overexpression may regulate CLDN4 downregulation by regulating its ubiquitination. Overexpression of CLDN4 is reported to have unfavorable clinical outcomes (109), interestingly, the results suggest that re-expression of CLDN4 in CL MDA MB 231 TNBC cells is associated with a reduction in migration.

Evidence supports that abnormal expression of CLDNs and PVT1 induce the epithelial-to-mesenchymal transition (EMT) in many cancers, resulting in enhanced cellular motility (122). We assessed the expression of EMT markers E-cadherin, vimentin and fibronectin (data not

shown). We observed no changes in the expression of EMT markers when PVT1 exon 9 is knocked down. However, our data suggests that EMT markers are more highly expressed in CL MDA MB 231 TNBC cells in comparison to CH MDA MB 468 TNBC cells. Additionally, it also supports that MDA MB 231 cells are more mesenchymal than MDA MB 468 cells. Overall, this result suggests that PVT1 exon 9 may not regulate EMT in TNBC.

In conclusion, this study demonstrated that PVT1 exon 9 may have a regulatory role in claudin low TNBC. Knock down of PVT1 exon 9 resulted in the re-expression of CLDN4 protein expression, without any significant change in CLDN4 mRNA expression. This suggests that PVT1 exon 9 regulates CLDN4 expression at a translational, or post-translational level. Additionally, we demonstrated that over expression of PVT1 exon 9 is associated with increased migration in CL MDA MB 231 cells. Consequently, PVT1 exon 9 may regulate CLDN4 in TNBC. These data may have implications in prognostication and better clinical outcomes.

8. Future Directions:

Claudin low tumors have much worse outcomes than other subtypes (13) therefore future studies are critical to further understand the underlying molecular mechanism of PVT1 exon 9 in TNBC. Our results suggest that PVT1 exon 9 is regulating CLDN4 expression, however the mechanisms remain unclear. One strategy is to understand how PVT1 exon 9 may be interacting with CLND4. For example, is PVT1 exon 9 directly binding to CLDN4 to regulate its expression, or is PVT1 exon 9 regulating CLDN4 expression via downstream targets? Furthermore, it is important to investigate the overexpression of PVT1 exon 9 in MDA MB 468. PVT1 exon 9 is significantly under-expressed in MDA MB 468. *In vitro* studies to assess the significance of the transient overexpression of PVT1 exon 9 in a claudin high model should be carried out. These potential future experiments may provide additional insight into the role of PVT1 exon 9 in TNBC.

9. References

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