PTEN-LONG, A TRANSLATIONAL VARIANT OF THE TUMOR SUPPRESSOR PTEN

Benjamin David Hopkins

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Abstract

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Phosphatase and Tensin Homologue on chromosome Ten (PTEN) is a tumor suppressor and an antagonist of the phosphotidylinositol-3 kinase (PI3K) pathway. We identified a 576-amino acid translational variant of PTEN, termed PTEN-Long, that arises from an alternative translation start site 519 base-pairs upstream of the ATG initiation sequence, adding 173 N-terminal amino acids to the normal PTEN open reading frame. We demonstrate that PTEN-Long is able to act as a lipid phosphatase and that it is able to down regulate PI3K signaling when transiently transfected into cells. We observe that PTEN-Long is down regulated in the tumor cells of breast cancer cases, and that in some cases it is highly expressed by immune cells in the tumor microenvironment indicating that PTEN-Long may play a role in the endogenous response to tumor formation. We demonstrate that PTEN-Long is secreted from cells and can enter other cells. As an exogenous agent, PTEN-Long antagonized PI3K signaling and induced tumor cell death in vitro and in vivo. Furthermore we were able to use the PTEN-Long alternately translated region (ATR) to shuttle fused RFP and p53 into cells, thus opening the possibility that ATR-fusion proteins may be capable of restoring proteins such as lost tumor suppressors back into cells.

Using pancreatic ductal adenocarcinoma (PDAC) as a case study in order to expand upon our initial observations, we observed loss/down-regulation of PTEN-Long in human PDAC tumor samples as well as in tumor derived cell lines. We demonstrate that this
feature of the human disease is recapitulated in a Kras$^{G12D}$ p53$^{H172R}$ Pdx1-Cre (KPC) mouse model. We examined the efficacy of PTEN-Long treatment alone or in combination with gemcitabine in the KPC model. To our surprise PTEN-Long preformed equivalently to gemcitabine as a monotherapy, and the animals had a significantly increased survival when treated with the PTEN-Long/gemcitabine combination. Further studies are needed to understand the mechanism by which PTEN-Long is cooperating with gemcitabine in this model. The present data strongly supports further exploration of PTEN-Long’s utility and potential as a therapeutic agent.
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<tr>
<td>3d</td>
<td>3 Dimensional</td>
</tr>
<tr>
<td>4EBP1</td>
<td>Eukaryotic Initiation Factor 4E Binding Protein</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ATR</td>
<td>Alternately Translated Region</td>
</tr>
<tr>
<td>CDKN2</td>
<td>Cyclin dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell Permeable Peptide</td>
</tr>
<tr>
<td>CTRL</td>
<td>Control</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERBB2</td>
<td>Erythroblastic Leukemia viral Oncogene Homolog2</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O proteins</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPas activating Protein</td>
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<tr>
<td>GRP78</td>
<td>Glucose Regulated Protein 78</td>
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<tr>
<td>GSK</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HCK</td>
<td>hemopoietic cell kinase</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IM</td>
<td>Intramuscular</td>
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IP Intraperitoneal
IT Intratumoral
IV Intraveinous
KP(fl)C Kras G12D p53fl/fl Pdx1-Cre
KPC Kras G12D p53H172R Pdx1-Cre
Kras Kirsten Rat Sarcoma
LTK Leukocyte receptor tyrosine kinase
MDM2 Mouse double minute 2
miR Micro RNA
MMAC1 Mutated in multiple advanced cancers
mTOR Mamalian target of rapamycin
NFkB Nuclear factor kappa light-chain enhancer of activated B cells
PAR-4 Prostate Apoptosis Response 4
PDAC Pancreatic Ductal Adenocarcinoma
PDAC Pancreatic Ductal Adenocarcinoma
PDK-1 Phosphotinositide dependent kinase 1
PDZ Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (zo-1)
PH Plextrin Homology
PHTS PTEN Hereditary Tumor Syndrome
PI3K Phosphotidylinositol-3 Kinase
PIP2/3 Phosphotidylinositol 2/3 Phosphate
pRAS-40 AKT1 Substrate
<table>
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<tr>
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<tr>
<td>PTD</td>
<td>Protein Transduction Domains</td>
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<tr>
<td>Ptdins</td>
<td>Phosphoinositides</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homologue on chromosome Ten</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Florescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immuno Deficiency</td>
</tr>
<tr>
<td>SMAD4</td>
<td>Mothers Against Decapentaplegic Homolog 4</td>
</tr>
<tr>
<td>TAT</td>
<td>Transactivator of Transcription</td>
</tr>
<tr>
<td>TEP1</td>
<td>TGF-B regulated and epithelial cell enriched phosphatase</td>
</tr>
<tr>
<td>TGF-B</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue Microarray</td>
</tr>
<tr>
<td>TPTE 1/2</td>
<td>Transmembrane phosphoinositide 3-phosphatase and tensin homolog ½</td>
</tr>
<tr>
<td>TSC2</td>
<td>Tuberous sclerosis protein 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilms Tumor 1</td>
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For Smitho
no hay balas de plata
Chapter 1

INTRODUCTION
It is estimated that 580,000 Americans will die of cancer in 2013 (American Cancer Society, Surveillance Research 2013). Cancer is a complex set of disease states characterized by the aberrant and pathological growth of cells. Cancer is the result of an evolutionary process in which genetic events lead to the progressive transformation of normal cells into tumor cells. Through this process normal cells turn on pathways that drive proliferation, activate migration and invasion, induce angiogenesis, and allow for limitless replication while suppressing signals that activate cell death, inhibit growth (5, 6). The Phosphatase and Tensi̇n Homologue on Chromosome TEN (PTEN) and Phosphatidylinositol 3 Kinase (PI3K) network effects many of these processes and is frequently deregulated in cancer(7). Gaining knowledge about each of the individual components of this pathway, their normal functions as well as the ways in which they contribute to oncogenic transformation may inform future therapeutic efforts(8). Many alterations have been identified in cancer genomes and these alterations are central to our understanding of disease progression(9). Alterations that effect tumor progression are segregated into two categories, activating mutations which promote tumor development, and suppressing mutations which promote transformation through the inactivation of proteins that normally maintain cellular homeostasis(10).

**Oncogenes**

Under normal conditions proto-oncogenes control a variety of normal cellular functions through a variety of mechanisms. Proto-oncogenes have been identified which are growth factors, receptors, transcription factors, as well as regulators of apoptosis. In cancer these genes are altered to promote tumorigenesis through a gain in function. These alterations can occur as amplifications, activating mutations, or translocations and
result in the conversion of the proto-oncogene into an oncogene. The identification of the first oncogenes came from the observation that the genomic elements of RNA tumor viruses could induce oncogenic phenotypes(11, 12).

**Tumor Suppressors**

Alterations to tumor suppressor genes are inactivating mutations and as such promote tumor formation and progression through the removal of their normal activities. Similar to oncogenes tumor suppressors can have a variety of cellular functions, they act as transcription factors, and regulate signaling, or induce apoptosis, they control metabolism, the cell cycle and help to maintain genomic stability. Tumor suppressor genes were originally identified due to their loss in cancer (13). Functionally loss of tumor suppressors can occur in many ways, there can be gross genetic deletion of the chromosomal region, mutations can inactivate the gene product, they can be epigenetically silenced, or alterations to other genes can result in the instability or the incorrect localization of the protein. The end result of these alterations is the failure of the gene product to act and therefore a loss of control that leads to oncogenic transformation(14).

As a critical signaling pathway for cellular growth and proliferation the PI3K pathway contains numerous proto-oncogenes and tumor suppressors, which have been shown to be frequently altered in cancer. In this thesis we identify a novel component of this pathway, PTEN-Long a translational variant of the canonical tumor suppressor PTEN, and demonstrate that it can act as a negative regulator of PI3K signaling. We explore the some of the functions of PTEN-Long and its potential therapeutic utility.
Phosphotidylinositol 3 Kinase

Phosphoinositides (PtdIns) are lipids that are anionic derivatives of phosphatidic acid with an ester linkage to an inositol ring that can be phosphorylated at the 3, 4 and 5 positions. The permutations of the phosphorylation of these three positions allows for the generation of seven distinct PtdIns in the cell (15). PtdIns are the target of numerous kinases and phosphatases in cells, which affect the ratio of the various PtdIns(16). Of the phosphorylated PtdIns in the cell, phosphatidylinositol 4,5 bisphosphate (PIP2) is the most abundant. PIP2 is found at the cell membrane and within vesicles(17). It binds with low affinity to pleckstrin homology (PH) domains and is involved in cytoskeletal rearrangements and membrane invagination through interactions with PH domain containing proteins such as dynamin(18-20). PIP2 is a substrate of PI3K which it adds a phosphate to in order to convert it into the critical second messenger phosphatidylinositol (3,4,5) phosphate (PIP3)(16). PTEN antagonizes this activity cleaving a phosphate from PIP3, and thereby reducing it back to PIP2(21). Maintaining the balance of these interactions is critical to retaining appropriate levels of these signaling molecules and thereby controlling appropriate cellular signaling and homeostasis.

PI3K phosphorylate PtdIns at the 3 position of the inositol ring and can act on unphosphorylated PtdIns, PI(4)P, PI(4,5)P(16, 22-24). The kinases are made up of two subunits, a catalytic subunit and a regulatory subunit, and various forms exist which are segregated into three classes of PI3Ks, I, II, and III(25). Class IA PI3K has three catalytic subunits p110α,β, and δ that are activated in the context of receptor tyrosine kinase signaling(16, 26-29). These catalytic subunits have lipid kinase, helical, calcium
binding (C2), and Ras binding domains\(^{(30)}\). They interact with the regulatory subunit of p85\(\alpha,\beta\) and p55\(\gamma\) to form functional heterodimeric kinases\(^{(31-33)}\).

Only one member of Class IB has been identified p110\(\gamma\) which is regulated by p101 and is activated downstream of GTP-binding protein coupled receptors (GPCRs) through the direct association with the disassociated G protein G\(\beta\gamma\) subunit\(^{(34-36)}\). Class II is comprised of three catalytic subunits and is activated by growth factor and integrin signaling\(^{(37-40)}\). No regulatory subunits have been identified for class II kinases. Class III PI3Ks are highly homologous to yeast proteins. In yeast they function as members of three distinct complexes however in humans there interactions are more complicated and like their functions remain only partially defined\(^{(26)}\).

**AKT**

The generation of PIP3 by PI3K leads to the trafficking of PH domain containing proteins to the cell membrane. Protein Kinase B (AKT) has an N-terminal PH domain, a kinase domain, and a C-terminal region. The PH domain interacts with PIP3 at the cell membrane where it is activated by phosphorylation in the c-terminal extension at multiple sites\(^{(41-44)}\). AKT is phosphorylated within the activation loop of the protein at TH-308 by PDK-1 which is also recruited to the membrane via its PH domain interactions\(^{(45)}\). A second phosphorylation event at Ser473 is required for full activation of AKT, which is regulated by the mammalian target of rapamycin complexes (mTOR)\(^{(46, 47)}\). Protein phosphatase 2A (PP-2A) and PH domain and leucine rich repeat protein phosphatases (PHLPP) dephosphorylate AKT thereby deactivating the protein\(^{(48-50)}\).
Upon activation AKT functions to regulate a number of cellular processes. It regulates cell death by acting on several targets which include Bcl-2 associated death promoter (BAD), the forkhead box transcription factors (FOXOs) both of which are direct targets of AKT kinase activity and become mislocalized upon phosphorylation\(^{51, 52}\). AKT also inhibits cell death through activation of pro-survival transcription factor nuclear factor-κB (NF-κB) by its phosphorylation of inhibitor κB (IkB), which normally acts to sequester NF-κB in the cytoplasm\(^{53}\). Mouse double minute2 (MDM2), which is a critical negative regulator of p53, is a target of AKT which by phosphorylation and leads to the subsequent degradation of p53\(^{54, 55}\).

Tuberous sclerosis protein 2 (TSC2) is phosphorylated by AKT and inhibited from forming the TSC1/2 complex. This complex is a Rheb GTPase activating protein (GAP), and deactivates Rheb GTPase activity which leads to the downstream activation of mTOR \(^{56-59}\). mTOR is the kinase component of the mTORC1 and mTORC2 complexes which it forms with raptor and rictor respectively \(^{60-62}\). mTORC1 stimulates protein translation by phosphorylating p70 S6 kinase which in turn activates ribosomal protein S6 and eIF4B\(^{63-66}\). The complex also inactivates 4E-BP1 by phosphorylating it preventing it from inhibiting eIF4B\(^{63}\). mTORC2 has been shown to phosphorylate AKT at Ser473 creating a feed back loop in which AKT activates mTOR which in turn further activates leads to \(^{46, 67}\).

AKT also targets several proteins involved in cell cycle progression and proliferation. AKT phosphorylates glycogen synthase kinase 3-β (GSK3β) which prevents it from inhibiting the cyclins and thereby promotes cell cycle progression and cellular proliferation\(^{68, 69}\). By phosphorylating p21 and p27 AKT displacing them from the
nucleus and preventing them from inhibiting the cell cycle through their interactions with nuclear cyclins and cyclin dependent kinase (70-72).

The effects of AKT activation are summarized (Figure 1.1). Since these effects are all downstream of AKT activation, which occurs via its recruitment to the cell membrane through its N-terminal PH domain and PIP3, the generation of PIP3 can play a critical part in oncogenic transformation. While we have already described PI3Ks which generate PIP3, we will examine a PI3K antagonist, the dual specificity phosphatase, PTEN.

![PTEN/PI3K pathway](image)

**Figure 1.1 PTEN/PI3K pathway.** This figure depicts many of the known interactions of the pathway and highlights the roles of AKT in promoting protein synthesis, cell cycle progression, proliferation, and evasion of apoptosis (3).

**PTEN**

Phosphatase and tensin homologue deleted on chromosome ten (PTEN), also termed mutated in multiple advanced cancers (MMAC1), or TGF-β regulated and epithelial cell-enriched phosphatase (TEP-1) was originally identified by three independent groups.
Before the PTEN gene was identified it was shown that 10q, the chromosomal region where PTEN resides in the human genome was identified as under going loss of heterozygosity in tumors of the brain, kidney and prostate(73-75). Through analysis of this genetic analysis region in breast and brain cancer samples, Li et al and Steck et al identified the PTEN gene (76, 77). Li and Sun identified the gene through a screen of human cDNA clones for novel protein tyrosine phosphatases that were regulated by transforming growth factor β (TGFβ)(78).

**PTEN Structure**

PTEN is a dual specificity phosphatase and a member of the protein tyrosine phosphatase family. The N-terminus encodes a 179 amino acid phosphatase domain, which is connected to a C2 domain and a tail region that contains a PDZ—binding domain at the C-terminus (Figure 1.2)(79, 80). The N-terminus contains a basic region that binds to PIP2 and is important for lipid binding and phosphatase activity. The catalytic pocket of the phosphatase has an elliptical opening that distinguishes it from other tyrosine phosphatases in that it is both deeper and wider, and therefore capable of accommodating lipid substrates. The C-terminal region of the phosphatase domain contains a basic region just before the C2 domain. The C2 domain (amino acids 186-351) lacks the classic Ca$^{2+}$ chelating residues which bind to phospholipids in the absence of calcium(80). The tail region (amino acids 353-403) is dispensable for the catalytic activity of PTEN in vitro, however it has been demonstrated to be important for a variety of protein interactions, and it is thought that the tail region is critical for both the regulation and stability of the protein(80-82). The extreme C-terminus of the tail
contains a PSD-95, Discs-Large, Z0-1 (PDZ) binding domain, which is involved in protein-protein interactions.

![Cartoon of PTEN](image)

Figure 1.2 **Cartoon of PTEN.** PTEN with phosphatase, C2, and Tail domains. Motifs are indicated with lines PIP2 binding region (PIP2), Catalytic pocket of the phosphatase (P), Basic region (BR), Phosphorylation sites (PhS), and the PDZ binding motif (PDZ).

**PTEN Function**

PTEN is a dual specificity phosphatase however its ability to dephosphorylate phosphotyrosine, phosphoserine, and phosphothreonine peptides *in vitro* was not very good\(^{(78, 83)}\). It was reported that focal adhesion kinase (FAK) could be dephosphorylated by PTEN *in vitro*, however it is not clear if this interaction plays a significant role *in vivo*\(^{(84, 85)}\). Though no specific protein target has been identified the protein phosphatase activity of PTEN has been demonstrated to be important for its functions controlling cell cycle arrest and inhibition of cellular invasion\(^{(86-91)}\). Subsequently PIP3 was identified as the physiological substrate of PTEN and that PTEN was able to regulate signaling downstream of PI3K \(^{(21, 92)}\). Through its phosphatase activity PTEN is able to act as an antagonist of PI3K signaling and prevent the activation of AKT with its pro-survival, pro-growth, and pro-proliferation roles described above and thus sits at a critical node to prevent oncogenic transformation.

There is mounting evidence that PTEN is also playing a role in the nucleus\(^{(93, 94)}\). Nedd4 was identified as the E3 ubiquitin ligase for PTEN. Ubiquitination of PTEN
results in protein degradation and nuclear localization\(^{(95, 96)}\). It has been shown that PTEN interacts with the APC-CDH1 tumor suppressor complex as well as CENPC-C1 at the centrosome to promote genomic stability \(^{(97-99)}\).

**PTEN Loss in Cancer**

PTEN is a bona fide tumor suppressor. It is the second most frequently altered gene in human cancer behind p53, and it is estimated that it is altered in >10% of tumors \(^{(100)}\). The catalogue of somatic mutations in cancer database currently lists >2700 mutations throughout the length of the protein from 28 different tumor types (Figure 1.3, Table 1.1). The missense mutations in PTEN are spread across the gene with a hotspot in the catalytic pocket at position R130\(^{(3)}\).

![Histogram of missense mutations in PTEN](image)

**Figure 1.3 Histogram of missense mutations in PTEN.** Histogram depicting the frequency of substitutions at each amino acid with in the PTEN protein. Data is curated by the Catalogue of Somatic Mutations in Cancer (COSMIC) database July 2013 (www.sanger.ac.uk/genetics/CGP/cosmic).
The importance of PTEN in tumor suppression is further demonstrated by the existence of the PTEN Hereditary Tumor Syndromes (PHTS): Cowden disease, Bannayan-Riley-Ruvalcaba Syndrome, Proteus syndrome, and Proteus like syndrome. 80% of people with Cowden Disease have germline mutations in PTEN(101-103). These patients develop benign hamartomas in a variety of organs including the breast and skin and are more likely to develop thyroid or breast cancer(104-107). Proteus and Proteus like syndromes present in a manner similar to Cowden disease(108). It has been demonstrated that the changes to PTEN in these patients causes dis-regulation of PI3K.

Table 1.1  **Frequency of PTEN mutations by tissue type.** Data is curated by the Catalogue of Somatic Mutations in Cancer (COSMIC) database July 2013 ([www.sanger.ac.uk/genetics/CGP/cosmic](http://www.sanger.ac.uk/genetics/CGP/cosmic)).
signaling and activation of AKT\((109, 110)\). Mutations to PTEN are not the only mechanism by which tumors reduce levels of the protein. A variety of studies have shown that PTEN is down regulated by promoter methylation in thyroid, breast, lung, endometrial, ovarian, gastric, and brain tumors\((111-117)\). PTEN has also been shown to be silenced by the expression of micro RNAs\((118-128)\). It has been demonstrated that subtle changes in the dose of PTEN can still effect tumor susceptibility\((129, 130)\).

Many of the phenotypes seen as a result of PTEN loss in patients have been recapitulated in mice. PTEN\(^{−/−}\) mice die during embryonic development however PTEN\(^{+/−}\) are viable and recapitulate some of the phenotypes seen in patients with PHTS, displaying neoplasms of the breast and endometrium as well as intestinal polyps\((131-135)\). Tissue specific deletion of PTEN in mouse models have demonstrated that removal of PTEN by itself is sufficient to cause cancer in the breast, prostate, lung, bladder, and pancreas as well as cause lymphoma when deleted from T-cells\((3)\). In more complex models PTEN was shown to cooperate with other oncogenic events. In the breast PTEN deletion was shown to cooperate to enhance tumor formation with Erbb2\((136)\). In the brain PTEN deletion was shown to cooperate with loss of p53\((137)\). In the lung and pancreas of PTEN was shown to cooperate with Kras activation\((138, 139)\). In sum the data from analysis of human cancers and mouse modeling of tumor development demonstrate the importance of PTEN function in suppressing tumor development.

It is not currently feasible to replace lost PTEN in human tumors. However since its discovery countless experiments have examined the various functions of PTEN by re-introducing it in cell lines where it has been deleted. Using PTEN\(^{−/−}\) mouse embryonic fibroblasts one study demonstrated that re-introduction of PTEN restored normal
apoptotic responses to these cells (92). Studies using a PTEN null glioblastoma cell line (U87MG) demonstrated that expression of PTEN induces G1 cell cycle arrest, and decreases proliferation and reduces anchorage independent growth in culture and slowed tumor progression in xenografts(140-142). Studies from the Parsons lab indicated that expression of PTEN in PTEN null breast cancer cells induced apoptosis(143). Given its central role in the development of such a wide array of tumor types, and the multiple mechanisms through which it is believed to act as a tumor suppressor it has been suggested the clinical restoration of PTEN would be of significant benefit (144).

Figure 1.4  **PTEN-Long (PTEN-β) is concomitantly expressed with PTEN.** (A) Breast cancer cell lines in which the PTEN gene has been mutated BT-549 and HC1937 or remains intact MCF10A and ZR-75-1 and PTEN wt HEK293 cells blotted with commercial antibodies that recognize the C-Terminus of PTEN. (B) Immunoblot of cell lines showing varying expression of PTEN and PTEN-Long bands. (C) Immunoblot of WT, PTEN shRNA Knockdown and PTEN-/- mouse embryonic stem cells. (D) siRNA knockdown of PTEN and PTEN-Long in HEK293 cells. (Figure taken from Fine et al 2007) PTEN-β was a short lived name for the 75 kD translational variant that we now call PTEN-Long(I).
**PTEN-Long**

In work done in for his thesis in 2007, Barry Fine et. al. described preliminary data regarding a potential translational variant of PTEN, which we have since termed PTEN-Long(1). The following is an overview of the findings. The initial observation that led to the postulation of the PTEN-Long hypothesis, that there might be a larger alternative form of PTEN, was the finding that a ~75 kD protein was detected with multiple PTEN antibodies. When the PTEN gene was missing from a cell or had been silenced via either shRNA or siRNA the 75 kD band was concomitantly absent or knocked down along with the 55 kD PTEN(Figure 1.4). They went on to demonstrate that PTEN-Long could be expressed using expression vectors containing the 5’ region upstream of the start site of PTEN, but not when this region was omitted. Further they showed that they could enhance expression of the 75 kD band by mutating the putative start site ‘CTG’ at position -519 to an ‘ATG.’ They observed that this region was evolutionarily conserved and demonstrated that this 5’ region had homology to TPTE1 and 2, testis specific, membrane bound phosphatases.
Excited by the potential importance of the discovery of a translational variant of PTEN, they examined the PTEN-Long alternatively translated region (ATR) and discovered that it contained an N-terminal signal sequence with a putative cleavage site at position 22 (Figure 1.5). This led them to formulate the hypothesis that PTEN-Long was secreted. In support of this they demonstrated that PTEN-Long could be concentrated using Conconavalin A sepherose indicating that the protein might be glycosylated a hallmark of the secretory pathway (Figure 1.5). To corroborate this finding they also demonstrated that PTEN-Long could be detected in human serum.

Figure 1.5 Signal Peptide Prediction PTEN 5’UTR sequence was translated and inputed into SignalIP3.0. Hidden markov model for eukaryotic signal peptides was used for prediction. The N-region denotes the positively charged N-terminal sequence of the signal peptide. The H-region is the hydrophobic core of the signal peptide. The C-region is mildly polar region marked by a proline which usually breaks the helix of the hydrophobic core. The cleavage probability is predictive of a cleavage site to release the signal peptide, allowing the protein to be released into the lumen of the ER. (4) (I)
In sum these preliminary regarding the existence and possible functions of a translational variant of PTEN were extremely exciting. The published literature had clearly established the importance of the PTEN gene via its frequent alterations in a variety of pathologic states. This thesis focuses on the existence of PTEN-Long, demonstrating how it functions both endogenously and as therapeutic agent.

Figure 1.5 **Concanavalin A pulldown.** HEK293 cells were lysed and concanavalin sepharose was used to pulldown glycosylated proteins. Eluates were resolved by SDS-PAGE and immunoblotted for PTEN. An enrichment in the PTEN-Long can be observed in the pulldown versus input (1).
Chapter 2

PTEN-Long: Existence and Function

Benjamin Hopkins\textsuperscript{1,2,3,4}, Barry Fine\textsuperscript{2,3}, Nicole Steinbach\textsuperscript{1,4}, Zachary Rapp\textsuperscript{1}, Kyrie Pappas\textsuperscript{1,6}, Hanina Hibshoosh\textsuperscript{2,3,7}, and Ramon Parsons\textsuperscript{1}*

\textsuperscript{1}Department of Oncological Sciences, Mount Sinai School of Medicine, 1470 Madison Ave. New York, NY 10029. \textsuperscript{2}Institute for Cancer Genetics, \textsuperscript{3}Herbert Irving Comprehensive Cancer Center, \textsuperscript{4}Department of Cellular Molecular and Biophysical Studies, and \textsuperscript{5}Department of Medicine, \textsuperscript{6}Department of Pharmacology, \textsuperscript{7}Department of Pathology, Columbia University, 1130 Saint Nicholas Ave. New York, NY 10032

*To Whom Correspondence Should be Addressed: ramon.parsons@mssm.edu
Abstract

PTEN was identified through the mapping of chromosome ten in a region that undergoes frequent loss of heterozygosity (76, 77). The canonical PTEN gene product is a 403 amino acid dual specificity phosphatase. Examination of the PTEN mRNA revealed an evolutionarily conserved, in frame alternative start site, which is 519 base pairs upstream of the canonical start site. We show that from this alternate site a 576 amino acid translational variant of PTEN, which we have termed PTEN-Long, is generated. PTEN-Long is frequently down regulated in breast cancer as compared to normal breast epithelium from the same patient. PTEN-Long can act as a phosphatase against soluble PIP3 and is capable of down regulating the PI3K pathway in culture. The alternately translated region of PTEN-Long contains a signal sequence with a predicted cleavage site at amino acid 22. We show that PTEN-Long is secreted into culture medium and that it is present in human plasma and serum. As an exogenous agent, PTEN-Long can permeate the cell membrane and is detectable in the cytoplasm and nucleus of cells. The discovery of PTEN-Long has many implications for our understanding of the overarching significance of the PTEN gene, as it opens up new potential functions in normal as well as pathologic states.
Introduction

Cancer is a diverse set of disease states brought about by genomic alterations in cells that allow for aberrant cellular proliferation and tumor formation (5, 6). Alterations driving tumor formation can occur in proto-oncogenes, which then activate cell proliferation/survival enabling tumor growth, as well as in tumor suppressor genes, whose loss is thought to remove road blocks that the tumor must overcome in order to get through normal checks on growth and proliferation. The phosphatidylinositol 3 kinase (PI3K) pathway is a critical regulator of cell signaling and survival. It is responsible for the propagation of signals from numerous receptor tyrosine kinases and g-coupled protein receptors through the conversion of phosphatidylinositol 3,4 bisphosphate (PIP2) to phosphatidylinositol 3,4,5 trisphosphate (PIP3) (145). Downstream of this catalytic activity are a number of proteins such as phosphotidylinositol dependent kinase -1 (PDK-1), protein kinase B (AKT), and mammalian target of rapamycin (mTOR), which are critical for tumorigenesis as they control cellular metabolism, proliferation and survival. As a result, mutations and amplifications of components of the PI3K pathway are frequently found in cancer (7).

PTEN

Phosphatase and Tensin homologue deleted on chromosome Ten (PTEN) is a dual specificity phosphatase that dephosphorylates the critical second messenger PIP3 (21). Through this activity, PTEN directly opposes the function of PI3K and thereby prevents the activation of its downstream effectors which promote cell growth, proliferation and survival (146, 147). PTEN’s critical role as a tumor suppressor is supported by its
alteration in tumors of the brain, prostate, ovary, endometrium, breast, skin, lung, kidney, digestive tract, liver, thyroid, and lymphoid tissues and by its germline mutations in cancer predisposition syndromes such as Cowden disease and Lhermitte-Duclos disease (76, 101, 148-151).

Pten knockout mice have validated Pten’s role as a tumor suppressor. While Pten -/- mice are embryonic lethal, the heterozygous mice, as well as a myriad of tissue specific knockout mice, have been generated that develop an array of tumors (3). While PTEN’s phosphatase activity has been shown to have a tumor suppressive effect, this is not the only mechanism by which this effect is occurring. PTEN has been shown to suppress cell migration in a phosphatase independent manner (91, 152-154) It has also been shown to migrate to the nucleus where it can stimulate p53 and E2F transcriptional activities(7, 54, 55, 94, 96).

**PTEN-Long**

Previously work in our laboratory has shown by immunoblot that there is a PTEN specific 75 kD band recognized by multiple antibodies that are targeted to the C-terminus of PTEN and that this band is dependent upon the evolutionarily conserved 5’ region of a PTEN mRNA directly upstream of the canonical PTEN initiation site in exon 1(1). Here we provide evidence that this higher molecular weight protein is a translational variant of PTEN which we have termed PTEN-Long. We build on the previous observations that the PTEN-Long alternately translated region (ATR) contains a predicted signal sequence and cleavage site by testing the hypothesis that PTEN-Long is secreted. Through these studies we seek to identify some of the functions of PTEN-Long.
Alternate Initiation Codons

Translation of protein is the result of a complex series of events that requires a ribosome to scan a transcript and identify an initiation codon, where the ribosome begins to translate. The canonical initiation codon is AUG, but initiation of translation is dependent upon other factors as well, such as the immediate context (base-pairs immediately proximal to the initiation codon), the secondary structure of the mRNA (e.g. the presence of an IRES), the relative abundance of certain nutrients (such as methionine or oxygen), the cellular density, and the amount of stress that the cells are under (155-161). Although the canonical AUG initiation makes up the vast majority of start sites, there are many examples of translation initiation occurring from alternate codons such as WT-1, Osteopontin, ETS-R, BAG-1, VEGF, FGF2/3, c-Myc, N-Myc, L-MYC, HIF, RAR-β4, Pim-1, hck, and LTK (157, 161-164) (155, 157, 161, 165-170). These examples illustrate that CUG alternate initiation sites are utilized by an array of different genes. Many of the proteins represented in this group have been shown to play critical roles in cancer. For some of these proteins translation from the alternate start site results in a change in the protein’s localization.

Secretion

Secretion is a complex series of events that results in the export of proteins out of the cell. In the classical model, proteins targeted for secretion contain a signal sequence that is recognized by the translational machinery, leading to protein translation by ribosomes docked to the endoplasmic reticulum (ER). The resulting peptide product translocates into the lumen of the ER. From the lumen of the ER, the protein moves to the golgi and
finally to secretory vesicles where secreted proteins are cleaved to release them from the vesicular membrane and the proteins undergo glycosylation as well as other post-translational modifications. When the vesicles fuse with the cell, membrane cleaved proteins are released into the extracellular space while un-cleaved proteins remain tethered to the membrane (171, 172). Interruption of the vesicular trafficking between these subcellular compartments, such as inhibition of vesicular retrograde transport, can perturb the secretory pathway preventing secretion. Secreted factors play critical roles in tumor initiation and progression and can act as anti or pro-oncogenic (173-175). The idea of secreted tumor suppressors has been around for approximately two decades, during which time a set of proteins has been identified that perform a diverse array of functions. Conceptually, the challenge of secreted tumor suppressors is that they may be functioning in a non-cell autonomous manner, and therefore may be difficult to identify using traditional tissue culture based approaches where effects on the extra cellular components of tumors cannot be observed, or where incomplete gene silencing would prevent the observations of tumor suppressor functions (RNAi Screens) (176, 177). A concrete early example of a secreted tumor suppressors are the thrombospondins, which are secreted into the extracellular matrix and inhibit angiogenesis, a key component of tumor development(178, 179). Similarly, secreted Maspin exerts an antitumor effect by preventing the degradation of the extracellular matrix which is required for continued tumor growth(180-183). Secreted tumor suppressors such as Ecrg4 and MDA7/IL-24 are involved in the immune response to tumor development(184, 185). Still other secreted tumor suppressors act to dampen the signals of known oncogenic pathways by antagonizing signaling of oncogenic receptors(175, 186, 187). One recent example of
such a mechanism is EphA7TR, which was identified as a secreted tumor suppressor in lymphoma, and functions by blocking activation of the Eph-Receptor (175). Conversely, other secreted tumor suppressors function by interacting with pro-apoptotic receptors on the cell surface. Prostate apoptosis response-4 (PAR-4), which has known functions in the nucleus and cytoplasm, was reported to be spontaneously secreted upon induction of ER stress and was then shown to activate a caspase dependent apoptosis mechanism through a Glucose regulated protein 78 (GRP78) on the cell surface(188). Other secreted tumor suppressors such as transcription factor NKx3.1, have been reported to act in a paracrine fashion by exerting growth inhibitory effects on prostate epithelial cells. In this manner, NKx3.1 can act much like the drosophila homeobox transcription factor from which the penetrin cell permeable peptide was identified in that it can be secreted from its cell of origin and then translocate into another cell where it acts as a transcription factor(189).

**Cell permeable Peptides**

Protein transduction domains (PTDs), or Cell permeable peptides (CPPs), are peptides that are capable of entering cells through their plasma membranes. The HIV protein Transactivator of Transcription (TAT) and the Drosophila homeobox protein Penetratin were the first proteins to be observed with this function(190-192). Since their discovery, other peptides from an array of sources have been identified as having PTDs, including endogenous proteins such as dynorphins, FGF-1 and 2, and a myriad of engineered peptides based around chimeric or fully synthetic peptides (eg. pVEC, MAP, Transportan, R7, MPG, Pep-1)(193-201). Much of the therapeutic excitement
surrounding PTDs involves their ability to shuttle macromolecules into cells. This idea opens a set of therapeutic possibilities, as the potential for delivering macromolecules such as oligonucleotides, drugs, or fused proteins (e.g., phosphatases, tumor driver inhibitory peptides, or lost tumor suppressors) into cells opens the possibility of using agents therapeutically that cannot by themselves gain entrance into cells (202-204). Many PTDs share poly-basic motifs, epitomized by the synthetic poly-arginine PTD R7 (RRRRRRRR), that are required for their ability to cross the plasma membrane. There are many theories about the mechanism by which PTDs are taken up by cells, including direct pore formation, macropinocytosis, and endocytosis, each of which has support in the literature (205-208). Most of these theories focus around the poly-basic residues present the PTDs and their cationic interactions at the cell surface (either directly with the lipids of the membrane or with cell surface receptors such as heparin sulfate glycoproteins) (209, 210). Since there is no clear mechanism of cellular entry, studies of novel PTDs rely heavily upon functional effects (e.g., transactivation by the TAT protein) (211). The efficiency of cellular transduction is closely linked to the cargo being shuttled into the cells (207, 212). In studying PTDs and the cargo that they carry, it is important to be aware that the proteins are present in numerous compartments in and around the cell as they migrate from the extracellular space, into various compartments of the cell (211). As is the case with the FGF proteins endogenous PTD containing proteins may be acting in different ways in the various compartments that they pass through along the way (4, 13).

Several groups have shown promising results by generating fusion proteins using CPPs and known tumor suppressors in order to create cancer therapeutics (213-215). One group
showed that a TAT-PTEN fusion protein had anti tumor activity in a model of kidney cancer.
Materials

Reagents

HiTrap Heparin and HisTrap columns were purchased from GE Life Sciences (Piscataway, NJ). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Protein A/G Plus agarose, rabbit and mouse IgG agarose were from Santa Cruz (Santa Cruz, CA). Human Serum, Concanavalin-A and imidazole were from Sigma (St. Louis, MO). PIP-Strips were procured from Echelon (Salt Lake City, UT).

Antibodies

PTEN-Long (Gly-2) poly-clonal rabbit antibody to amino acids 153-173. A peptide with an amino terminal Cysteine followed by the sequence PRHQQLPSLSSFFSHRLPD of PTEN-Long was conjugated to keyhole limpet hemocyanin, immunized in rabbits and affinity purified using an affinity resin generated with the same peptide (Zymed Laboratories, San Francisco, CA). Antibody was termed Gly-2 as it was eluted from the affinity column using Glycine pH 2 elution buffer. PTEN (138G6), pAKT308, pAKT473, pGSK3, Total AKT, pFoxo, Foxo3, Cleaved Caspase 3, Cleaved Caspase 7, PARP, PRAS40 antibodies were from Cell Signaling (Danvers, MA). PTEN (6H2.1) was from Cascade (Winchester, MA). V5 and His6 antibodies came from Invitrogen (Carlsbad, CA). BAF-180 antibody was previously described(216). Tubulin and Actin antibodies were procured from Sigma (St. Louis, MO). Secondary antibodies were purchased from Pierce (Rockford, IL).

Cell Lines
Tumor cell lines were obtained from ATCC (Manassas, VA). Mouse embryonic stem cells were generated in the lab as previously described\(217, 218\).

**Plasmids and Constructs**

pCEP4 *PTEN*, which encodes the entirety of the canonical PTEN open reading frame and a portion of PTEN-Long up to the *Not I* site. To create expression vectors for PTEN-Long, the 5’ region upstream was extended from the *Not I* site by ligation of an adaptor encoding the 5’ region to the *Not I* site originally used for cloning\(76\). Alternate adaptors were made to encode an ATG at the *PTEN-Long* start site. Site directed mutagenesis was performed upon the canonical start site, converting it to ATA. pCDNA3.1 constructs were made using the pcDNA3.1 TA cloning kit from Invitrogen (Carlsbad, CA), using pCEP4 *PTEN-Long* as a PCR template and by ligating the purified product into the vector as per the manufacturers’ protocol. The mutant PCDNA3.1 constructs *PTEN-Long G302R*, *PTEN-LongΔA\(^6\)*, and *PTEN-LongΔR\(^6\)*, as well as the canonical *PTEN* and C-terminal truncation mutant *PL-RFP* (PTEN-Long Unique Region) were made using the site directed mutagenesis kit from Stratagene/Agilent (Santa Clara, CA). jpExpress404 *PTEN-Long* bacterial constructs including PTEN-Long-Luciferase and TAT-PTEN, were ordered from DNA2.0 (Menlo Park, CA) to match the pcDNA3.1 constructs described above. These constructs encode a truncated form of PTEN-Long that begins with an ATG in front of amino acid 22 (M\(_{\text{SES}}\)), thereby removing the signal sequence and cleavage site. Site directed mutagenesis was utilized to create matching Δ\(R\)^\(^6\), and somatic mutation variants of *PTEN-Long* and PTEN-Long-RFP (Stratagene).
Patient samples

Patient samples were acquired from the Columbia University Department of Pathology Breast Cancer Tumor Bank in accordance with the IRB guidelines. Matched breast tumor, normal breast pairs came from an unaffected portion of the same breast. Samples used for immunohistochemistry came from paraffin blocks. Samples used for immunoblotting came from frozen tissue.

Methods

Immunohistochemistry (IHC)

IHC for PTEN (138G6) and pAKT Serine473 antibodies used sodium citrate antigen retrieval buffer as previously described (219). PTEN-Long (Gly-2) immunohistochemistry was performed using the Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA), detected with VECTASTAIN Elite ABC Reagent and 3, 3’-diaminobenzidine substrate as per the manufactures protocol. Tissues were embedded in paraffin and cut as previously described(137).

Transfections

All DNA complexes were generated in serum-free media. Transfected cell lines were grown to 80% confluency and transfected with Lipofecamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Protein Purification
For bacterial purifications, BL21 (DE3) pLysE (Invitrogen) *E. Coli* were transformed with plasmids encoding *jpExpress PTEN-Long* constructs. Overnight cultures were used to inoculate 400 ml cultures of LB until OD595= 0.3. Protein expression was induced with 0.1 mM IPTG (Sigma) for 4.5 hours at 21 degrees C. Protein was extracted from bacteria by sonication in lysis buffer (150 mM NaCl, 50 mM Tris). For mammalian purifications, HEK293 cells were transfected with the ATG/ATG PTEN-Long construct and harvested after 24 hours and lysed on ice in TRIPA Buffer (50 mM Tris, 150 mM NaCl, 0.1% Triton X). Both HEK293 and E.Coli lysates were clarified by centrifugation at 40,000g for 30 minutes and filtered through a 0.22 micron filter. Lysates were then run sequentially over the following columns using an AKTA-FPLC: HisTrap (Running Buffer: 500 mM NaCl 25 mM Tris pH 7.6, 20 mM Imidazole, Elution Buffer: 500 mM NaCl 25 mM Tris pH7.5, 500 mM Imidazole), Desalting (Buffer 50 mM NaCl, 25 mM Tris pH 7.5) and HiTrap Heparin (Running Buffer: 25 mM Tris pH 7.5 25 mM NaCl, Elution Buffer: 25 mM Tris pH 7.5 1 M NaCl ). The resulting eluates were concentrated using Amicon Ultra 50KDa exclusion columns (Millipore, Billerica, MA). The protein was subsequently resolved by SDS-PAGE electrophoresis and quantified by Coomassie staining by comparison with a BSA standard (Pierce, Rockford, IL). The resulting protein was then utilized for experimentation.

**Immunoprecipitation**

Cells were lysed on ice in 150 mM NaCl, 25 mM Tris pH 7.5, 0.1% Triton X-100 with protease inhibitor cocktail (Sigma). The lysate was vortexed and centrifuged at 40,000g for one hour. The resulting supernatant was then filtered using a .22 micron filter.
Conditioned media and serum were similarly filtered before immunoprecipitation was performed. The samples were precleared by incubation with protein A/G agarose (Santa Cruz) and rabbit IgG rotating for one hour at 4 degrees. Immunocomplexes were then precipitated overnight with 4 micrograms antibody and 40 ul of Protein A/G-agarose.

**Conditioned Media**

Serum free conditioned media was generated on two 15 cm dishes of HEK293 cells that had been transfected with Lipofectomine 2000 to transiently overexpress the indicated constructs. To ensure that protein in the media was not the result of cell death, the media was collected after 12 hours for the Brefeldin A treated experiments, and 18 hours for the experiments with overexpression of the various PTEN/PTEN-Long constructs. Cells were observed at the time of harvest by light microscopy for signs of cell death. Media was filtered through a .22 micron filter before being used for immunoprecipitation or concentration over a heparin column.

**Concentration from Serum and Plasma**

Human serum and plasma were procured from Sigma (St. Louis, MO). A 1 ml sample of each was used for the purification. The samples were precleared by rotating with Protein A/G agarose (50 ul) for 1 hour. They were then run over a heparin column (GE) and eluted in 25 mM Tris,1 M NaCl pH 7.6 using an AKTA purifier (GE). Serum concentration of PTEN-Long was perturbed by pre-incubation of the sample with PTEN (138G6) for 1 hour, before being run over the heparin column. Peak fractions were then
mixed with Laemmli sample buffer, and resolved by electrophoresis and detected by immunoblotting with indicated antibodies. Immunoblot for secondary only was used as a control for heavy chain contamination.

**Phosphatase Assays**

Soluble di-C₈-D-myo-Phosphatidylinositol 3,4,5 trisphosphate (PIP3) was purchased from Echelon (Salt Lake City, UT) and diluted to indicated concentrations in phosphate free buffer (25 mM Tris, 100 mM NaCl pH 7.6). Purified proteins were added and the reaction was allowed to run for 30 minutes at 37 degrees C. To detect released phosphate, 50 ul of Malachite Green Reagent (Biomol) were added to the samples which were incubated for 15 minutes at room temperature. Absorption at 620 nm was quantified in a Micro-QUANT microplate spectrophotometer (Biotek-Instrument Inc., Winooski, VT). Phosphate standards were utilized to quantify the phosphate released by each sample. Samples were run in triplicate and results were normalized to a water control (220).

**Immunoblotting**

Lysates were resolved on 4-12% or 4-20% polyacrylamide gradient gels (Invitrogen) and blotted on PVDF using semi-dry transfer technique (Biorad). Membranes were blocked with 5% Milk-TBST and washed in TBST before being incubated from one to eighteen hours in either 5% BSA-TBST or 5% Milk-TBST with primary antibodies followed by one hour in horse radish peroxidase conjugated secondary antibodies in 5% Milk-TBST.
Blots were visualized using chemiluminescence (Pierce) according to the manufacturer’s protocol. Autoradiography film was obtained from Worldwide Scientific (USA).

**Cell viability assay**

Cells per well were seeded in 6 well culture dishes in media with 10% serum. Cells were allowed to adhere overnight and were treated with indicated amounts of PTEN-Long using serial dilutions from a common stock in 1% serum. Cells were allowed to grow for 24 hours in the presence of protein, at which point they were trypsinized and stained 1:1 with Trypan blue for quantification of cell number.

**PTEN-Long treatments in culture**

Cells were grown to 85% confluence in media with 10% serum. They were then washed 1x in serum free media and allowed to incubate for 30 minutes at 37 degrees. They were then re-fed with serum free media containing PTEN-Long related proteins from bacterial protein preparations. Cells were treated with quantities indicated and harvested at 15 minutes, 2 hours, or 24 hours after they were treated with the protein.

**Measurement of fluorescence**

Cells were plated on gelatin coated glass coverslips and treated with purified protein. Cells were allowed to incubate for 30 minutes with protein before they were washed with PBS and mounted on slides with Prolong Gold Antifade with DAPI (Invitrogen). They were then imaged on a Nikon Eclipse 80i with a CoolSnap HQ2 CCD camera.
Subcellular fractionation

Cells were treated with the indicated constructs where an untagged RFP overexpressing control vector was utilized for a mock purification of bacterial protein from the jpExpress404 vector. Cells were treated with indicated concentrations of purified protein or equal volume of a control and allowed to incubate at 37 degrees with the purified proteins. The plates were then washed three times in cold PBS with protease inhibitors and scraped from the plate with a rubber spatula. The cells were then pelleted by centrifugation at 700g for 5 minutes, and swelled in 300 ul of buffer (10 mM Hepes, 10 mM KCl, 100 uM EDTA with 0.01 M DTT) for 15 minutes on ice. A 1:20 dilution of 10% NP40 alternative was then added to the suspension, which was vortexed for 5 seconds and mixed by inversion for 2 minutes. The sample was then centrifuged at 1000g for 5 minutes, and the resulting supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in 100 ul of 20 mM Hepes, 400 mM NaCl, 1 mM EDTA with protease inhibitor and 0.01 M DTT. It was vortexed for 15 minutes at 4 degrees, followed by centrifugation at 15,000g for 15 minutes. The resulting supernatant was used as the nuclear fraction. The fractions for each sample were visualized after electrophoresis and immunoblotting (221).
Results

The 5’ region of PTEN contains an evolutionarily conserved alternate translation initiation site that gives rise to a translational variant of PTEN, PTEN-Long

PTEN is an essential gene in murine development. Sufficient levels of PTEN are critical for maintenance of normal cellular signaling and tissue homeostasis. We therefore investigated the existence of a high molecular weight band that consistently was recognized with PTEN antibodies at 75 kD. We termed this hypothetical, PTEN protein PTEN-Long. Inspection of the PTEN mRNA (NM_000314.4) revealed that there are 770 base-pairs within the open reading frame (ORF) upstream of the canonical PTEN start site at position 1032 (Figure 2.1).

![Figure 2.1: Cartoon of PTEN mRNA. PTEN mRNA (NM_000314.4) with annotations for PTEN-Long start site (CTG at position 513) the canonical PTEN start site (ATG at position 1032) and their shared stop codon (TGA at position 2243).](image)

This region is evolutionarily conserved and contains an alternate translation initiation site within the context of a weak Kozak sequence 519 base-pairs upstream of the canonical start site (Figure 2.2). Translation from this alternate start site is predicted to add 173 amino acids to the N-terminus of PTEN and thereby append ~21 kD to the protein. Since the alternate start site is within PTEN open reading frame, it is predicted to give rise to a protein with a unique alternately translated region (ATR) followed by the phosphatase, C2 and tail domains of canonical PTEN (Figure 2.3).
Figure 2.2 PTEN-Long is evolutionarily conserved. Clustal-W alignment of the N-Terminal sequences of Human PTEN (NM_000314.4) with genomic sequence from the Cow (98%) (Cow Oct. 2011 (Baylor Btau 4.6.1/bosTau7)), Horse (98%) (Sept 2007 (Broad/equCab2)), Elephant (97%) (Elephant Jul. 2009 (Broad/loxAfr3)), Mouse (92%) (Mouse Dec. 2011 (GRCm38/mm10)), Frog (24%) ( X. tropicalis Nov. 2009 (JGI 4.2/xenTro3)) and Lizard (35%) ( Lizard May 2010 (Broad AnoCar2.0/anoCar2)) obtained from the UCSC genome browser (http://genome.ucsc.edu/) where percentages represent pairwise alignment score for each species against the Human sequence as calculated by the software. (www.ebi.ac.uk/Tools/msa/clustalw2/). * indicate residues that were conserved across all tested species. Colors indicate similar amino acids. ":." means that conserved substitutions have been observed. ":." means that semi-conserved substitutions are observed.
To test whether this alternate start site could give rise to the observed 75 kD protein, we transiently transfected the PTEN/PTEN-Long PCDNA3.1 expression vectors into PTEN null cell lines and observed that PTEN-Long expression was dependent upon the presence of the 5’ region of PTEN, as neither (a) transfection of a vector containing the canonical PTEN start site but not the 5’ CTG (-/ATG) nor (b) a vector into which a single base-pair frameshift had been inserted between the alternate start site and the canonical start site (ATG-FS-ATG) yielded the PTEN-Long translational variant at 75 kD.

Figure 2.3 Cartoon of PTEN-Long Protein. Cartoon depicting the alternately translated region (ATR) of PTEN-Long with phosphatase, C2, and Tail domains that it shares with canonical PTEN.

Figure 2.4 PTEN-Long is expressed from PTEN message. Transient transfection of PTEN-Long constructs into BT549 (top panel) and U87MG (lower panel) cells where:(-/ATG) the 5’ alternative CTG translation start site is not present and the canonical ATG initiation site is intact, (CTG/ATG) the 5’ CTG and the canonical ATG are present (wildtype), (CTG/ATA) the 5’ CTG is present and the canonical start site has been mutated to ATA, (ATG/ATG) the 5’ start site has been altered to ATG, (ATG/ATA) the 5’ initiation site has been mutated to ATG and the canonical initiation site has been mutated to ATA, ATG-FS-ATG where an extra base has been inserted to create a frame shift between the 5’ and 3’ ATGs of the ATG/ATG construct. Immunoblotted as indicated.
Conversely, vectors containing intact 5’ region (CTG/ATG) expressed the PTEN-Long band, and this expression was increased by mutating the endogenous start site “CTG” to a classical translation initiation codon “ATG” (ATG/ATG) (FIGURE 2.4).

Having seen that PTEN-Long could be exogenously expressed, we proceeded to show that it could be detected endogenously in cells. In western blot analysis on mouse embryonic stem cells and human cell lines with known PTEN status, we observed the PTEN-Long band present in the wildtype but not the PTEN null cells using an antibody that recognizes the tail domain (138G6) which is shared by PTEN and PTEN-Long, as well as an antibody that is specific for the PTEN-Long ATR (Gly-2) (Figure 2.5).

Figure 2.5 PTEN-Long is endogenously expressed. Immunoblot of whole cell lysates from wildtype and Pten null embryonic stem cells and cancer cell lines of known PTEN status. PTEN-Long (Gly-2) recognizes an epitope specific to the PTEN-Long unique region. PTEN (138G6) recognizes an epitope that is common to both PTEN and PTEN-Long.

To confirm these findings we performed reciprocal immunoprecipitations using both the PTEN-Long specific and classical PTEN antibodies and showed that PTEN-Long could be immunoprecipitated (Figure 2.6).
PTEN-Long is a lipid phosphatase capable of down regulating the PI3K pathway in vitro.

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<td>95% CI</td>
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Figure 2.7 **PTEN-Long is a lipid phosphatase.** 40 nM PTEN and PTEN-Long, assayed with di-C₈-PIP₃ for lipid phosphatase activity, error bars indicate SEM of three replicates (p≤0.0007, ANOVA), Regression lines to Michaelis-Menten kinetics with Vₘₐₓ and 95% Confidence interval (CI) shown in inset moles PO₄/min/moles enzyme.
Having identified a translational variant of PTEN we next sought to test whether PTEN-Long was capable of acting like a lipid phosphatase and agonist of the PI3K pathway in a manner similar to that of classical PTEN. To do this we ran phosphatase activity assays using di-C8-D-myo-Phosphatidylinositol 3,4,5 trisphosphate as a substrate in a malachite green assay and observed that like PTEN, PTEN-Long was an active phosphatase (Figure 2.7). We observed that by creating a mutation analogous to a PTEN(G129R) tumor derived phosphatase deficient mutant, PTEN-Long(G302R), we could significantly reduce the phosphatase activity of PTEN-Long (Figure 2.8).

**Figure 2.8 Phosphatase activity of PTEN, PTEN-Long, and PTEN-Long(G302R).** PTEN, PTEN-Long, and phosphatase mutant PTEN-Long-G302R (40 nM) produced in E. coli, and assayed with 20 μM di-C8-PIP3 for lipid phosphatase activity. Error bars indicate standard deviation of three replicates (p≤0.001, t-test PTEN compared to PTEN-Long). Inset Coomassie and immunoblot with V5 antibody of purified proteins used for this assay which we estimate to be 80% purity including degradation products.

We assessed the ability of PTEN-Long to down regulate PI3K signaling by transiently transfecting PCDNA3.1 expression vectors into PTEN null U87MG cells (Figure 2.9). In this manner we were able to determine that PTEN-Long down regulates PI3K signaling as indicated by down regulation of pAKT, pGSK3B, pFOXO, and pPRAS40 all of which are downstream components of the PI3K pathway. The observed effects of PI3K signaling down regulation were similar for both PTEN and PTEN-Long. Also like
PTEN, PTEN-Long’s regulation of PI3K signaling is dependent upon an intact phosphatase domain.

Figure 2.9 **PTEN-Long reduces PI3K signaling in culture.** Transient transfection of PTEN-Long, PTEN, and phosphatase inactive mutants PTEN-LongG302R and analog PTENG129R differentially regulate PI3K signaling in U87MG cells, shown by immunoblot analysis with indicated antibodies.

**PTEN-Long protein levels in Human Breast Tumors and their microenvironment**

Since PTEN was identified as a tumor suppressor, we next asked how PTEN-Long expression might be changing in breast cancer by comparing samples of normal breast with breast tumor samples from the same patients via immunohistochemistry and Western blot analysis (Figure 2.10).
Through this analysis we were able to see a distinct reduction of PTEN-Long in the tumor samples. These samples were stained for pS6, pAKT(473), PTEN, and PTEN-Long and the levels of each of these proteins was scored by a pathologist (H.H.) (Table 2.1). This table indicates that PTEN-Long is consistently down-regulated in these breast tumors and that this is concordant with up-regulation of the PI3K pathway as indicated by pS6 positivity.

In addition to these tumor/normal pairs, we stained 50 more cases of human breast cancer for PTEN and PTEN-Long expression. Within this set we observed a strong stromal staining pattern of the histiocytes/macrophages immediately proximal to the tumor that was not observed in regions outside of the tumors immediate microenvironment (Figure 2.10).
2.11). This finding may indicate that PTEN-Long is playing a role in the innate response to tumor development in these cases, though more experiments are required to make any claims about this potential function of PTEN-Long.

Using a publically available database of somatic mutations in cancer we identified 6 missense mutations in the PTEN-Long ATR (Figure 2.12). This evidence further supports the idea that PTEN-Long plays a tumor suppressive role and we will explore some of the impact of these mutations on protein function in chapter three.

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Table 2.1 Scoring of matched normal breast-breast cancer biopsies taken from same case. Paraffin sections of the four breast tumors with wildtype PTEN and matched normal breast samples from Figure 2.10 were stained for pAKT(473), pS6, PTEN, and PTEN-Long and scored by a pathologist (H.H.). PTEN-Long levels are reduced in the tumor sample in 4 of 4 cases while phospho-S6 (an indicator of PI3K pathway activity) increases in 4 of 4 cases. PTEN staining was reduced in 3 of 4 cases, and pAKT was elevated in 1 of 4 cases.

**PTEN-Long is mutated in Human Cancer**

Using a publically available database of somatic mutations in cancer we identified 6 missense mutations in the PTEN-Long ATR (Figure 2.12). This evidence further supports the idea that PTEN-Long plays a tumor suppressive role and we will explore some of the impact of these mutations on protein function in chapter three.
Figure 2.11 **PTEN-Long in the tumor stroma of Human Breast Cancer.** To test the frequency with which this stromal PTEN-Long is occurring we stained 50 cases of Human breast cancer and observed the strong stromal staining pattern of the histiocytes/macrophages in 4 of the cases. Image from a representative case is shown (T denotes tumor). Slides were stained using the PTEN-Long (Gly-2) antibody as in Fig. S9. Hematoxylin was used as counter stain.
Having seen that PTEN-Long is capable of behaving in a manner similar to PTEN in tissue culture, we hypothesized that the PTEN-Long ATR might bequeath novel functions onto the translational variant and we attempted to identify such potential functions. As described in chapter one, a signal sequence prediction algorithm, SignalP (website), predicted that the PTEN-Long ATR contains a signal sequence followed by a cleavage site at amino acid 22 (Figure 1.3). Our previous studies also indicated that PTEN-Long could be concentrated using a Concanavalin A resin indicating that it could be glycosylated, a hallmark of secretory proteins (Figure 1.5, Figure 2.12). This lent credence to a previous observation from our laboratory in which several heparinated glycoproteins were identified as PTEN binding partners (Figure 2.13)(220). We mapped the interaction between PTEN-Long and heparin and observed that PTEN-Long has two regions that bind heparin, the ATR and the Phosphatase domain (Figure 2.14). To further examine PTEN-Long’s interaction with cell surface components, we utilized an in vitro binding assay and incubated PTEN-Long purified protein with strips spotted with several membrane components (Figure 2.15). In this manner we showed that PTEN-Long bound to phosphatidic Acid, phosphatidylserine, cardiolipin, 3-sulfagalactosylseramide,
Figure 2.13 **Mass spectrometry identified several heparinated glycoproteins as PTEN binding partners.** Red lines indicate peptides identified in the screen, percentages indicate the percent of the full length protein that were identified in the screen.
and to lesser degrees bound to phosphatidylinositol and phosphatidylglycerol. In sum, these binding interactions demonstrate that PTEN-Long may be interacting with a variety of different components on the outside of the cell membrane and thus support the hypothesis that PTEN-Long is secreted.

Figure 2.14 **Mapping of Heparin Binding regions of PTEN-Long.** PTEN-Long constructs were in vitro translated using the rabbit reticulocyte lysate which initiate translation methionines throughout the constructs. The resulting proteins were passed over heparin columns and eluted using a 2M salt gradient. The input, flowthrough (FT) and eluate (E) fractions are shown via western blot on the right. Cartoons of the protein and translated protein products that bound (blue) and did not bind (red) are depicted on the left. In this manner two distinct heparin binding regions were identified in PTEN-Long as indicated (black).
Figure 2.15 **PTEN-Long binding to cell surface moieties.** Purified PTEN-Long was incubated with membranes prebound with indicated cell surface moieties (echelon). The membrane was then washed and blotted using the epitope tag V5 antibody to visualize PTEN-Long binding to the membrane.
To test this hypothesis, we generated a PTEN-LongΔA₆ expression vector from which the predicted signal sequence has been ablated through the deletion of six tandem alanines from within the extreme N-terminus of the protein (Figure 2.16).

We transiently transfected PTEN, PTEN-Long, and PTEN-LongΔA₆ into HEK293 cells and were able to concentrate PTEN-Long, but not the other proteins, from the conditioned media of these cells (Figure 2.17).
To corroborate this finding we transiently transfected PTEN-Long into HEK293 cells again, this time treating them with an inhibitor of retrograde transport in the endoplasmic reticulum, Brefeldin-A. We observed that the treatment effectively blocked the accumulation of PTEN-Long in the media (Figure 2.18).

![Figure 2.18 Brefeldin A inhibits secretion of PTEN-Long.](image)

To test whether this secretion was occurring endogenously in humans, we concentrated heparin binding proteins from human plasma and serum and observed the presence of PTEN-Long via Western blot analysis. We confirmed the specificity of these findings by blocking the concentration of PTEN-Long by pre-incubating the serum with a PTEN antibody (Figure 2.19). Taken together, these findings show that PTEN-Long is secreted and that this secretion can be inhibited chemically as well as genetically in culture.
The PTEN-Long ATD has a poly-arginine sequence that enables cell penetration.

Examination of the PTEN-Long ATR revealed that it contains a poly-basic stretch, six tandem arginines that are reminiscent of the poly-basic residues of cell permeable peptides such as the HIV-Tat and Antennapedia(191, 192). We therefore hypothesized that the PTEN-Long ATR could enable cellular permeability. To test this hypothesis we generated a PTEN-Long construct from which the poly-arginine stretch had been removed, PTEN-LongΔR6. We also generated a set of PTEN-Long-RFP constructs in which the red fluorescent protein (RFP) had been appended onto the C-terminus of the constructs so that they could be visualized by fluorescent microscopy (Figure 2.20). We treated U87MG cells with purified PTEN-Long-RFP, PTEN-LongΔR6-RFP, or RFP and observed that there was a much larger accumulation of PTEN-Long-RFP than of either PTEN-LongΔR6–RFP or RFP proteins (Figure 2.21).
Figure 2.20 V5/His and RFP/V5/His PTEN-Long constructs in bacterial expression vector jPexpress 404. The first 21 amino acids of PTEN-Long were omitted to remove the signal sequence. Red fluorescent protein (RFP) was added in frame to the C-terminal tag. Site directed mutagenesis was utilized to remove the poly-arginine sequence in the PTEN-Long unique region (yellow). Blue indicates V5/His tag. Red indicates RFP/V5/His Tag. Purified protein lysates were visualized with Coomassie blue, representative protein preparations used in Figure 3A and B and related supplements are shown. We estimate these protein preparations to be of ~85% purity including degradation products.
To confirm that PTEN-Long was entering cells, we performed subcellular fractionation assays in which we treated MDA-MB-468 cells, which are null for PTEN, with PTEN-Long, PTEN-LongΔR⁶, PTEN, or RFP purified proteins and then accessed the fractions for the presence of the proteins. PTEN-Long was detected in the cytoplasmic and nuclear fractions of these cells, but the other proteins were not (Figure 2.22).
Figure 2.22 Subcellular fractionation of cells treated with PTEN-Long. Subcellular fractionation of MDA-MB-468 cells 1 hour after 25 nM treatment with indicated purified proteins. C and N indicate cytoplasmic and nuclear fractions, respectively. Offset, immunoblot of input protein samples used to treat the cells.
Discussion

PI3K signaling is a critical part of homeostasis. Defective PI3K signaling can lead to aberrant cellular growth, proliferation, and survival. As an antagonist of the PI3K pathway, PTEN plays a critical role in maintaining appropriate levels of PIP3 in the cell. Here we identify PTEN-Long, an alternatively translated form of the PTEN, and demonstrate that it is expressed from an alternate start site within the 5’ region of the PTEN mRNA. We demonstrate that PTEN-Long is expressed in cell lines that contain an intact PTEN gene but not in those where it has been deleted. Further, we show that we can express PTEN-Long in cells by transfecting them with constructs that contain the PTEN gene including the alternatively translated 5’ region, but not when this region has been omitted nor when a frameshift has been introduced between 5’ alternate start site and the canonical PTEN start site. This clearly identifies the 5’ alternatively translated region of PTEN as the source of PTEN-Long.

Using reciprocal immunoprecipitations as well as westernblots of isogenic and tumor cell lines, we show that the 75 kDa PTEN-Long band that we consistently observed in westernblots is in fact a translational variant of PTEN. Multiple sequence alignments of PTEN transcripts from a variety of animals indicate that PTEN-Long is evolutionarily conserved, implicating it as a significant factor in vertebrates. We show that, similar to canonical PTEN, PTEN-Long is an active phosphatase and that it is capable of down regulating the PI3K pathway when transiently expressed in mammalian cells. While it has been shown that PTEN is both a lipid and protein phosphatase in vitro, protein targets of PTEN have been difficult to confirm. The discovery of PTEN-Long with its ATR might explain why PTEN protein targets have remained elusive as the ATR
may be required for some of these interactions. Future studies of the potential protein targets of PTEN-Long could identify novel targets and explicate additional functions of the protein, thereby giving us a more complete sense of the impact of PTEN gene loss.

The finding that PTEN-Long is secreted opens a variety of potential interactions that have yet to be explored. As described in the introduction, there are several secreted tumor suppressors which have been identified. Here we show that PTEN is interacting with a series of cell surface lipids as well as heparinated glycoproteins. Since canonical PTEN is seen as a predominantly cytoplasmic protein that is also present in the nucleus, considerations of potential targets outside of the cell and on the cell surface have not been widely considered. The discovery of PTEN-Long as a secreted protein enables us to re-interpret previous studies showing that the PTEN gene played a tumor suppressive role in the tumor stroma as well as in the tumor cells themselves. It will be interesting to explore the role of PTEN-Long in the tumor microenvironment and potentially to test ways in which it might be acting as an extracellular agent and whether these extracellular functions may require its enzymatic activity.

We observed that PTEN-Long protein levels were reduced in breast cancer samples as compared to normal breast tissue from the same patient, sometimes in cases where PTEN levels were not significantly altered. This argues that PTEN-Long might be playing a tumor suppressive role in some of these breast cancer cases. We also observed pronounced PTEN-Long staining in histocytes immediately proximal to tumors in a subset of breast cancer samples as well as in the microglia around a mouse model of brain cancer, findings that implicate a potential role for the endogenous PTEN-Long in the host response to tumor development. Is this up-regulation of PTEN-Long an endogenous
response to tumor development? Further experiments are required to understand the potential role of endogenous PTEN-Long in host response to tumor development, but this is one potentially exciting area for future study.

The most surprising feature of PTEN-Long is that it is capable of entering cells from the extracellular space. In the context of its potential role as a tumor suppressor, this idea brings forth the intriguing idea of innate tumor response from neighboring cells. As tumors develop neighboring cells could potentially secrete PTEN-Long in response and thereby provide a “next cell” check on tumor development. The data supports a model of this “next cell hypothesis” in which one cell translates and secretes PTEN-Long in response to some external cue(s), so that the PTEN-Long can effect changes in neighboring cells (Figure 2.23)

![Figure 2.23 Model of PTEN-Long trafficking.](image)

This same line of logic also allows for the potential therapeutic use of PTEN-Long which could potentially be delivered as an exogenous agent. As we move forward into chapter three we will examine the effects of PTEN-Long when it enters cells and some of its potential effects as a therapeutic agent in vivo. This function opens up the possibility that
PTEN-Long could be used to restore PTEN functions back into cells in which they have been lost.
Chapter 3

PTEN-Long as a therapeutic agent

Benjamin Hopkins\textsuperscript{1,2,3,4}, Nicole Steinbach\textsuperscript{1,2,3,4}, Zachary Rapp\textsuperscript{1}, Hannah Goldstein\textsuperscript{3}, Peter Canoll\textsuperscript{3} and Ramon Parsons\textsuperscript{1*}

\textsuperscript{1}Department of Oncological Sciences, Mount Sinai School of Medicine, 1470 Madison Ave. New York, NY 10029.  \textsuperscript{2}Institute for Cancer Genetics, \textsuperscript{3}Herbert Irving Comprehensive Cancer Center, \textsuperscript{4}Department of Cellular Molecular and Biophysical Studies, 1130 Saint Nicholas Ave. New York, NY 10032

*To Whom Correspondence Should be Addressed: ramon.parsons@mssm.edu
Abstract

PTEN-Long is a translational variant of the tumor suppressor PTEN which is secreted and can enter cells as an exogenous agent. Here we test the hypothesis that PTEN-Long is capable of acting as a therapeutic agent, affecting tumor cell signaling and survival in vitro and in vivo. We demonstrate that purified recombinant PTEN-Long protein enters the blood stream and is detectable in the liver, lung, brain, and tumor xenografts of mice after treatment. We show that treatment with PTEN-Long alters PI3K signaling and can have profound impacts on tumor growth, and causes regressions in some tumor models. We demonstrate that the PTEN-Long ATR can be utilized as a carrier to deliver fused peptides across the cell membrane by showing that ATR-RFP as well as ATR-p53 are capable of cellular entry. Further, we show that these peptides traffic to the cytoplasm and nucleus respectively, and that the latter is capable of inducing known p53 targets. Taken together these results indicate that PTEN-Long as well as ATR-fusion proteins may have therapeutic potential.
Introduction

Our ability to treat disease is largely predicated on our understanding of the disease pathogenesis. As such, our ability to treat patients tends to evolve with our understanding of the underlying causes of their specific disease state. Cancer broadly speaking, was originally recognized as a set of disease states in which cells proliferated aberrantly so that tumors grew in normal tissues. Cancer was thereby categorized largely by the tissue in which it occurred and the agents employed against cancer were largely “targeted” to act as cytotoxic agents in highly proliferative tissues. As the field of oncology has progressed, cancer has been recognized to be a heterogeneous set of distinct malignant diseases. While we still tend to think about cancer as originating in a specific tissue we more and more also identify cancer through the genetic alterations that are driving tumor growth and disease progression. Since each tumor has its own underlying set of mutations that give rise to the malignant state, oncology is beginning to incorporate our understanding of specific genetic alterations into the therapy of patients. Understanding the alterations that are occurring in different tumors is critical to the development of targeted therapies. Over the last several decades, many therapeutic agents have entered the clinic which act through the targeting and inhibition of oncogenic proteins. This approach has been reasonably successful in some instances, such as drugs targeting the catalytic activity of HER-2 amplifications in breast cancer, the BCR-ABL translocation in myelogenous leukemia, and B-RAF mutations in melanoma (222-224). However, many tumors contain or develop resistance to these targeted agents so that over time even patients who initially respond to these agents have a propensity to relapse.
Another set of alterations that are known to drive cancer initiation and progression are those occurring in tumor suppressor genes. Mutations in tumor suppressors are thought to promote tumor growth through the removal of critical “check points.” While many tumor suppressors have been identified as being frequently altered in cancer, such as Rb, Trp53, CDKN2A, and PTEN, restoring their wildtype functions, has been difficult to achieve in the clinic. Experimental studies where groups have engineered ways to restore tumor suppressors have been promising, causing inhibition of tumor growth and in some cases tumor regression, but it has been difficult to translate these findings to the clinic. As such, the ability to deliver functional tumor suppressor proteins could have a profound impact on the way we treat cancer.

Typically, therapeutic attempts to compensate for the loss of PTEN have focused upon inhibition PI3K or downstream components of the PI3K pathway such as AKT or mTOR. A problem with these approaches is that they work with a fairly limited concept of the function of PTEN, which has been shown to function as a tumor suppressor through mechanisms that go beyond its inhibition of PI3K signaling. Therefore, pharmacologically compensating for the loss of PTEN would require using agents to target more than just the PI3K pathway.

Several studies have used cell permeable peptides (CPPs) in order to deliver functional proteins and other cargoes into cells. The use of CPPs to restore lost tumor suppressors is one potential therapeutic function for this class of peptide. There are many pathologic states caused by deficiency or loss of wildtype proteins and patients with those conditions might also benefit from technology capable of efficiently delivering functional proteins to these cells.
In chapter two we observed that PTEN-Long was secreted from cells, could be purified from human blood, and that purified recombinant PTEN-Long protein could enter cells as an exogenous agent. Using these ideas as a starting point, we hypothesized that PTEN-Long could function as a therapeutic agent capable of restoring the functions of PTEN altering PI3K signaling and tumor cell survival *in vitro* as well as *in vivo*. 
Materials

The HiTrap Heparin and HisTrap columns came from GE Life Sciences (Piscataway, NJ). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Protein A/G agarose, as well as the agarose conjugated mouse and rabbit IgGs were from Santa Cruz (Santa Cruz, CA). The Human Plasma, Serum, Concanavalin-A and imidazole were purchased from Sigma (St. Louis, MO).

Antibodies

PTEN-Long (Gly-2) is a poly-clonal rabbit antibody to amino acids 153-173 of the PTEN-Long ATR. It was generated using a peptide with an amino terminal cysteine followed by the sequence PRHQQLPSLSSFFSHRLPD of the PTEN-Long ATR conjugated to keyhole limpet hemocyanin, immunized in rabbits and purified using an affinity resin generated with the PRHQQLPSLSSFFSHRLPD peptide (Zymed Laboratories, San Francisco, CA). The antibody was termed Gly-2 because it was eluted from the affinity column using Glycine pH 2 elution buffer. PTEN (138G6), pAKT308, pAKT473, pGSK3, Total AKT, pFoxo, Foxo3, Cleaved Caspase 3, Cleaved Caspase 7, PARP, PRAS40 antibodies were from Cell Signaling (Danvers, MA). PTEN (6H2.1) was from Cascade (Winchester, MA). V5 and His6 antibodies came from Invitrogen (Carlsbad, CA). Tubulin and Actin antibodies were purchased from Sigma (St. Louis, MO). Secondary antibodies came from Pierce (Rockford, IL).

Cell Lines

Cell lines were obtained from ATCC (Manassas, VA).
Plasmids

jpExpress404 bacterial constructs including PTEN-Long, PTEN-Long-Luciferase, PL-p53, PL-RFP and TAT-PTEN were optimized for bacterial expression and generated by DNA2.0 (Menlo Park, CA). The PTEN-Long constructs encode a truncated form of PTEN-Long that begins with an ATG in front of amino acid 22 (MSES), thereby removing the signal sequence and cleavage site. Site directed mutagenesis was utilized to create matching $\Delta R^6$, and somatic mutation variants of PTEN-Long and PTEN-Long-RFP (Stratagene).

Methods

Immunohistochemistry

Tissues were embedded in paraffin and cut as previously described (137). Staining for His6 and pAKT Serine473 antibodies was performed using sodium citrate antigen retrieval buffer as previously described (219).

Protein Purification from Bacteria

BL21 (DE3) pLysE (Invitrogen) E. Coli were transformed with plasmids encoding jpExpress404 constructs encoding desired proteins with V5/His6 peptides at their extreme C-terminus. Cultures were established overnight in the presence of ampicillin and were used to inoculate 400 ml cultures of LB with ampicillin until OD595= 0.3, at which point protein expression was induced with 0.1 mM IPTG (Sigma) for 4.5 hours at 21 degrees C. Protein was extracted from bacteria by sonication in lysis buffer (150 mM NaCl, 50 mM Tris). Lysates were centrifuged at 40,000g for 30 minutes and filtered through a 0.22 micron filter. They were then passed sequentially over HisTrap (Running
Buffer: 500 mM NaCl 25 mM Tris pH 7.6, 20 mM Imidazole, Elution Buffer: 500 mM NaCl 25 mM Tris pH 7.5, 500 mM Imidazole), Desalting (Buffer 50 mM NaCl, 25 mM Tris pH 7.5) and HiTrap Heparin (Running Buffer: 25 mM Tris pH 7.5 25 mM NaCl, Elution Buffer: 25 mM Tris pH 7.5 1 M NaCl) columns using an AKTA-purifier. The desired fractions were then concentrated with Amicon Ultra 50KDa exclusion columns (Millipore, Billerica, MA). The protein was subsequently resolved by SDS-PAGE electrophoresis and quantified via Coomassie staining and comparison to BSA standards (Pierce, Rockford, IL). The resulting protein was then utilized for the experiments depicted in this chapter. All exogenous protein used in this chapter was purified from bacteria with the exception of the “method of injection” and HCC70, HCT116, and SUM149 curves of tumor volumes which were treated with proteins expressed in HEK293 cells as previously described(225).

**Immunoprecipitation**

Cells were lysed on ice in 150 mM NaCl, 25 mM Tris pH 7.5, 0.1% Triton X-100 with protease inhibitor cocktail (Sigma). The lysate was vortexed and centrifuged at 40,000g for one hour. The resulting supernatant was then filtered using a 0.22 micron filter. Conditioned media and serum were similarly filtered before immunoprecipitation was performed. The samples were pre-cleared with protein A/G agarose (Santa Cruz) and rabbit IgG rotating for one hour at 4 degrees. Immunocomplexes were then precipitated overnight with 4 micrograms antibody and 40 ul of Protein A/G-agarose.

**Immunoblotting**
Lysates were resolved on 4-12% or 4-20% polyacrylamide gradient gels (Invitrogen) and blotted on PVDF membrane using the semi-dry transfer technique (Biorad). Membranes were blocked with 5% Milk-TBST and washed in TBST before being incubated (from one to eighteen hours) in either 5% BSA-TBST or 5% Milk-TBST with primary antibodies followed by one hour in horse radish peroxidase conjugated secondary antibodies in 5% Milk-TBST. Blots were visualized using chemiluminescence (Pierce) according to the manufacturer’s protocol. Autoradiography film was obtained from Worldwide Scientific (USA).

**Xenografting**

Mice were housed in the mouse facility of the Irving Cancer Research Center and were treated under a protocol approved with the Columbia IACUC. Nude mice were obtained from Harlan Laboratories (NJ), and SCID mice were obtained from NCI (MD). Mice were treated daily via IP injection or as indicated. No detrimental effects were observed in the mice after protein treatments, as would have been indicated by their behavior and grooming, nor were there any gross abnormalities observed at the time of autopsy. Xenograft experiments were performed by the subcutaneous injection of 1x10⁶ cells in matrigel (BD) and were monitored daily. Tumor progression was monitored by imaging with Xenogen Spectrum small animal imaging system, 15 minutes after injection of 100 ul Luciferin Substrate (Caliper, Hopkinton, MA) IP into the mice or by caliper measurements as indicated. Day 0 indicates the first day of treatment. Mice were euthanized via induction with CO₂ at which point the desired tissues were snap frozen. Tissues were homogenized in 25 mM Tris pH 7.5, 500 mM NaCl and centrifuged at 20,000g prior to being mixed with Laemmlli buffer for immunoblotting.
Blood Glucose measurements in mice

Blood glucose measurements were taken after IP injection of the proteins at indicated times using OneTouch Ultra Mini glucometer (LifeScan, Milpitas, CA).
Results

Exogenous PTEN-Long alters PI3K signaling

To better understand the functions of PTEN-Long, we sought to understand what impact it had on cells after it entered. For this purpose nine cell lines were grown to 70% confluence, and then switched into serum free media for 16 hours, at which point they were treated with 25 nM PTEN-Long purified protein or an equal volume of a mock protein preparation for fifteen minutes. The samples were harvested in sample buffer and their relative levels of pAKT(308) were assessed by Westernblot analysis (Figure 3.1). We observed that treatment with PTEN-Long caused reductions in pAKT levels in the majority of these cell lines. In order to further assess the ability of PTEN-Long to act as an antagonist of PI3K signaling we pre-treated cells for ten minutes with 20 nM PTEN-Long or cell penetration deficient mutant PTEN-LongΔR6. We then stimulated pathway activation with either insulin or EGF for five minutes at which point the cells were harvested for Westernblot analysis. In this manner we observed that PTEN-Long was able to inhibit pAKT activation in both starved and stimulated settings and were able to demonstrate that this inhibition is at least in part dependent upon the ability of PTEN-Long to enter cells.
Figure 3.1 **PTEN-Long antagonizes PI3K signaling as an exogenous agent.** (A) Coomassie stained gels and Western blots of representative PTEN-Long PTEN-Long(~80%) /PTEN-LongΔR6 (~80%) protein preparations are shown. Estimates of purity including degradation products are given in parentheses. (B) Cell lines were starved for 16 hours and then treated with 25nM PTEN-Long or equal volume of mock protein preparation for 15 minutes. pAKT levels are normalized to actin levels for each sample. Within each cell line the pAKT level was then normalized to the corresponding Mock. Tissue of origin and status of the PTEN gene are noted for each cell line. (C) Cells were pretreated with 20nM PTEN-Long or PTEN-LongΔR6 for 10 minutes prior to stimulation with EGF or Insulin. Cells were harvested 5 minutes after stimulation and assessed via immunoblot with indicated antibodies.
**PTEN-Long induces cell death in a dose dependent manner**- To further elucidate the impact of PTEN-Long on tumor cell lines in culture, PTEN null U87MG and MDA-MB-468 cells were treated with increasing doses of PTEN-Long for 24 hours. Western blot analysis and trypan-blue staining showed that PTEN-Long alters PI3K signaling as indicated by pAKT(473), pFoxo, and pPRAS40, and impacts cell viability as indicated by cleavage of caspase 3-a marker of apoptosis, and trypan blue staining (Figure 3.2). Similar to the effect on PI3K signaling shown in Figure 3.1, the effect on apoptosis and cell viability is dependent on the presence of the poly arginine residues in the PTEN-Long ATR indicating that the cell permeable activity conferred by this motif is required for these effects.

**Figure 3.2 PTEN-Long impacts PI3K signaling and Cell survival in a dose dependent manner.** (A) Immunoblots of U87MG and (B) MDA-MB-468 cell lysates 24 hours after treatment with indicated doses of PTEN-Long. (C) Cell viability assay. U87MG and MDA-MB-468 cells were treated with the indicated doses of PTEN-Long or PTEN-LongΔR for 24 hours before scoring of the fraction of cells with trypan blue staining. Assayed in quadruplicate, error bars indicate SEM.
**PTEN-long enters the blood stream**

Having established that PTEN-Long was capable of restoring PTEN activity in culture, we hypothesized that PTEN-Long might be active *in vivo*. To test this hypothesis, we treated nude mice with recombinant PTEN-Long protein and took blood samples over time in order to determine if PTEN-Long could enter the blood stream and thus behave like a therapeutic agent. In this manner we were able to observe a transient increase of PTEN-Long in the blood of the mice after treatment that increased through the four hour time point and was no longer detectable 24 hours after treatment (Figure 3.3). Having observed PTEN-Long in the blood stream, we asked whether PTEN-Long might be able to down regulate insulin signaling in the mice as it was able to reduce the activation of pAKT downstream of insulin in vitro, and we know that PI3K inhibitors, have been shown to have transient impacts on blood glucose levels. To test this, we treated mice with PTEN-Long, phosphatase deficient mutant PTEN-Long(G302R), or a mock protein preparation. In this manner we were able to determine that PTEN-Long, like the PI3K inhibitors, causes a transient increase in blood glucose levels while the other treatments did not cause similar changes (Figure 3.4). Together these findings indicate that PTEN-Long is active in vivo and is able to affect insulin signaling in mice in a phosphatase dependent manner.
PTEN-Long impacts PI3K signaling in tissues and tumor xenografts

Having observed that PTEN-Long was able to affect blood glucose levels, we wanted to assess its ability to affect PI3K signaling in tissues and tumor xenografts. Wildtype mice were treated with 20mg/kg of PTEN-Long-RFP, PTEN-LongΔRΔR6-RFP, or RFP purified proteins and their brains were harvested 24 hours later. Western blots were performed to assess the impact of these treatments (Figure 3.5). We observed PTEN-Long-RFP in the brain tissues of these mice, and that the presence of the protein was concomitant with lower levels of pAKT and pFOXO in these samples. Similar changes were seen in the
Lungs, Livers, Brains, and MDA-MB-468 xenografted tumors in nude mice treated twice with 10mg/kg of PTEN-Long or equal volume of a mock protein preparation 24 hours apart and harvested 4 hours after the second injection (Figure 3.6). These changes in signaling were also observed in the tumor tissue of U87MG

![Graph showing blood glucose levels over time](image)

**Figure 3.4 PTEN-Long causes a transient increase in Blood Glucose Levels.** Mice were treated by IP injection with 10mg/kg of PTEN-Long, PTEN-Long(G302R), PTEN-LongΔR6, or equal volume of a mock protein preparation. Their blood sugar was monitored over time as indicated (P<0.0001 by ANOVA).

![Western blot analysis](image)

**Figure 3.5 PTEN-Long affects PI3K signaling in the brains of wildtype mice.** Western blot analysis of pAKT, pFOXO, pPRAS40 in the brain tissue from wildtype FVB mice 24 hours after a single 20mg/kg treatment with RFP, PTEN-Long-RFP, or PTEN-LongΔR6-RFP.
glioblastoma cells xenografted into nude mice treated with PTEN-Long for five days but not the tumors treated with the phosphatase deficient mutant PTEN-Long(G302R) (Figure 3.7). The signaling changes within tumor xenografts could also be observed as a loss of pAKT positive staining at the cell membrane via immunohistochemistry for pAKT473. The presence of exogenous PTEN-Long in the treated tumor cells was confirmed with an anti- His6 antibody to recognize the epitope tag (Figure 3.8). These results indicate that PTEN-Long can enter a variety of different tissues and tumor xenografts. With in these tissues it can down-regulate PI3K signaling, as indicated by reductions in pAKT, pFoxo, as well as induce apoptosis in tumor xenografts as indicated by cleaved caspases 3 in a phosphatase dependent manner.
Treatment of tumor xenografts

That PTEN-Long could be used to dose mice and effectively down regulate PI3K signaling brought us to test its efficacy as an anti tumor agent. Given the observations from our signaling experiments, we hypothesized that PTEN-Long therapy would be able to restore the lost functions of PTEN in tumor models from which PTEN had been deleted. Testing U87MG xenografts, which are a PTEN null cell line originally derived from a human glioblastoma, we observed that 4 mg/kg PTEN-Long treatment via intraperitoneal injection was capable of regressing these xenografted tumors (Figure 3.9A). Having observed these changes, we used a four day trial of PTEN-Long to identify the optimal mode of delivery. We tested intra-muscular (IM),

Figure 3.7 IHC of PTEN-Long Treated Tumors (40x). Tumor samples from mice shown in Fig S28 were stained with anti-His6 antibody to detect the PTEN-Long epitope tag and anti phospho serine 473 AKT to monitor change in AKT signaling.
intraperitoneal (IP), intratumoral (IT), Subcutaneous (SC), and intravenous (IV), methods of injections side by side and observed that IP, IT, SC and IV treatments all produced statistically similar tumor regressions over the observation period. Based upon this data and technical concerns, we chose to utilize IP treatments for our modeling (Figure 3.9B). We treated MDA-MB468, SUM-149, HCT116, and HCC70 tumor xenografts in nude mice with PTEN-Long or controls and observed that PTEN-Long slowed tumor growth or even caused regressions in all of the tumor types except HCT116 (Figure 10). To further test PTEN-Long therapy, we utilized a syngenic allograft model in which cells derived from a p53 -/- PTEN-/- glioblastoma over expressing PDGF and carrying a luciferase reporter were allografted into syngeneic mice and treated with PTEN-Long or PTEN-LongΔR^6 for five days, at which point the PTEN-Long treated tumors were no longer detectable by palpation nor utilizing the xenogen system to image the luciferase reporter. The mice originally treated with PTEN-LongΔR^6 were then switched to treatment with PTEN-Long for five days, causing regressions in the tumors that had initially been treated with PTEN-LongΔR^6 (Figure 3.11). These studies clearly demonstrate that PTEN-Long has anti-tumor activity and that this activity is dependent upon its ability to enter cells.

**Effects of Tumor derived mutations to the PTEN-Long ATR**

Having observed that PTEN-Long affected signaling in vitro and in vivo, we hypothesized that the mutations we identified as occurring within the PTEN-Long ATR (Figure 2.12) might impact the functions of PTEN-Long cell penetration, and PI3K signaling *in vitro* and *in vivo*. To do this, we used site directed mutagenesis to clone three tumor derived mutants, PTEN-Long (A98T), PTEN-Long(H121Y), and PTEN-
Long(R169G) into the PTEN-Long jpExpress 404 vector. We used this new vector to purify proteins harboring these mutations and compared their function to that of the parental PTEN-Long protein. To test cell permeability and to assess any potential gross defects in sub-cellular localization, we performed sub-cellular fractionation on MDA-MB-468 cells that had been treated with these proteins (Figure 3.12A). We observed, that while PTEN-Long was readily detectable in the cytoplasmic fraction, the amount of PTEN-Long(H121Y) inside the cells appeared to be reduced as compared to the wildtype protein. We next tested the ability of these proteins to down regulate the PI3K pathway in vitro and observed that MDA-MB-468 cells treated with the mutant proteins had higher levels of pAKT308 and pFOXO than did the cells treated with the wildtype protein (Figure 3.12B-C). Treating wildtype mice with these proteins revealed a similar defect, as pAKT signaling in the liver was turned off by the wildtype PTEN-Long protein but not by the mutant proteins (Figure 3.12D). From this data we can conclude that in at least some instances tumors are selecting for mutations that suppress PTEN-Long activity by decreasing its cellular uptake or inhibiting its catalytic activity.

Figure 3.8 **PTEN-Long effects signaling and survival in U87MG xenografts.** Immunoblots of markers of PI3K signaling in U87MG xenografts treated for 5 days with PTEN-Long or phosphatase mutant PTEN-Long(G302R).
Figure 3.9  PTEN-Long causes regression of U87MG tumor xenografts. (A) Graph of U87MG tumor volumes as measured by calipers and treated with either PTEN-Long (4 mg/kg) or an equal volume of mock purified protein (N= 5 mice/treatment, error bars indicate +/-SD). (B) Methods of drug delivery were tested on U87mg xenografts receiving 4mg/kg of PTEN-Long daily for 5 days. Null – Not Treated, IM – Intramuscular, IP – Intraperitoneal, IT – Intra tumoral, SC- Subcutaneous, and IV- Intra veinous. (N = 4 mice/group). Mice were treated with PTEN-Long purified from HEK293 cells.
Figure 3.10 Impact of PTEN-Long treatment on four tumor xenografts. (A) MDA-MB-468 xenografts were treated with 4mg/kg of PTEN-Long, PTEN-Long (G302R), or equal volume of mock protein preparation and their tumor growth was monitored with calipers. Error bars indicate standard error (N = 4 mice/treatment group) (B-D) Indicated cell lines were xenografted into immune compromised mice and treated with protein purified from HEK293 cells as indicated. Mock preparations were prepared using empty vector preparations. Graphs denote mean tumor surface area as measured by calipers. For HCC70 and HCT116 N = 3 in PTEN-Long treated group and 2 in respective control groups. For SUM149, N = 2 in PTEN-Long treated group and 3 in Vehicle group.
Figure 3.11 Treatment of PTEN-/- syngeneic allografts with PTEN-Long. Pten -/- p53 -/- PDGF overexpressing cells derived from the genetically engineered mouse model of GBM were allografted into the flank of syngeneic hosts and treated with 4 mg/kg of PTEN-Long or PTEN-LongΔR^6 for 5 days. Tumor volume was assessed daily by caliper measurements and imaged on days 0 and 5 with xenogen imaging system as shown. After 5 days of treatment the mice in the PTEN-LongΔR^6 (red arrows) cohort were then treated with PTEN-Long (green arrows) followed with caliper measurements. Blue arrows indicate initial PTEN-Long treatment (days 0-4).
Fusion of the PTEN-Long ATR confers cell permeability onto peptides

Based upon the findings that PTEN-Long could be used as a therapeutic agent in these models, we hypothesized that the PTEN-Long ATR was capable of delivering other fusion proteins into cells. To test this hypothesis we, made ATR-RFP and ATR-p53 constructs in which the PTEN-Long ATR (ATR) was fused to RFP and p53 respectively. Subcellular fractionation confirmed that both of these heterologous proteins were capable of entering cells (Figure 3.13-14). Interestingly, ATR-RFP, was detected almost exclusively in the cytoplasm, while ATR-p53 was detected in the nucleus, indicating that the ATR peptide was not interfering with these proteins normal localization. ATR-p53 treatment was further assessed for its ability to induce known p53 targets PUMA and p21 in a time course study (Figure 3.14A-B). Building upon these observations we asked whether MDA-MB-468 xenografts, which are know to harbor a point mutation in p53, would respond to restoration of p53, using ATR-p53. Over a ten day trial period the
ATR-p53 treated tumors did not progress and on average showed minimal signs of regression, while the control treated xenografts more than doubled in size (Figure 3.14C). These results are very exciting to us as they indicate that the ATR is capable of acting as a shuttle for fused peptides without inhibiting their normal localization or function. We hope that, based on these findings, more ATR-fusions will be examined for their ability to restore lost proteins back to cells.

Figure 3.13 ATR-RFP can enter cells. (A) Cartoon of ATR-RFP (PTEN-Long Unique region (1-173) with an RFP/V5/His tag) in jpExpress404. (B) Coomassie and immunoblot (using V5 antibody) of ATR-RFP protein preparation. (C) Subcellular fractionation of MDA-MB-468 cells 1 hour after 25 nM treatment with ATR-RFP. Lanes were loaded as ~2% total protein from whole cell lysate, nuclear, and cytoplasmic fractions. Fractionation showed that the ATR region allowed for efficient cellular entry as with full length PTEN-Long, but not entry into the nucleus (This indicated that the PL-domain was capable of shuttling other proteins into cells). We estimate this protein to be of >95% purity including degradation products.
Figure 3.14 **PL-p53 can enter cells and induce p53 targets.** (A) Subcellular fraction of H1299 cells after treatment with ATR-p53 indicating that ATR-p53 is able to enter cells and end up in the nucleus. (B) Time course experiment showing the induction of p53 targets by exogenous ATR-p53 protein. (C) Graph of Tumor volumes of xenografted human breast cell line MDA-MB-468 upon daily treatment with 10 mg/kg ATR-p53.
Discussion

The observation that exogenous PTEN-Long can induce changes in PI3K signaling and affect cell survival strongly supports the conclusions of chapter two that PTEN-Long is capable of subcellular entry. The observations that these effects are dependent upon the presence of an active catalytic pocket as well as the ability to enter cells utilizing the poly-arginine motif further support a model in which PTEN-Long is causing these changes by entering cells and then acting in a manner that is similar to PTEN, although other mechanisms cannot be definitively discounted as it is possible that these effects could be driven through PTEN-Long's activity upon an unidentified target.

The ability of PTEN-Long to enter the blood stream and to be distributed throughout a variety of tissues where it is still capable of down-regulating PI3K signaling is of interest as it demonstrates that PTEN-Long therapy may be capable of affecting signaling in a variety of different sights throughout the body and there for might be utilized in these sites. It is exemplified through the demonstration that PTEN-Long is able to down regulate endogenous insulin signaling creating a transient increase in blood glucose levels. These findings indicated that PTEN-Long had therapeutic potential and given frequent alterations of PTEN/PI3K pathway in cancer it made sense to more rigorously test the effects of PTEN-Long treatments in models of cancer.

We observed that exogenous PTEN-Long could be detected within tumors by both IHC and western blot analysis after treatment, and that PTEN-Long was capable of downregulating PI3K signaling and inducing apoptosis in some of our tumors. Our xenograft experiments show as one would expect given the heterogeneous nature of cancer that PTEN-Long does not cause uniform responses in all tumors. PTEN-Long had the
most pronounced effect on tumor progression in xenograft models where the PTEN gene is known to be mutated. In order to optimize efficacy of PTEN-Long in pre-clinical settings it would be of great utility to screen an array of other tumors in order to identify sets that do and do not respond to PTEN-Long. Gene expression studies of cells treated with PTEN-Long may also be useful in identifying the pathways that are affected by PTEN-Long therapy and could thus potentially lead to the identification of patient populations in which these effects might be beneficial.

By showing how PTEN-Long is functioning, we were able to specifically test the effects of PTEN-Long specific mutations upon cellular entry as well as cell signaling in vitro and in vivo. That tumor derived mutations in the ATR are impacting the ability of the protein to enter cells and affect signaling imply that these mutations could be more than just passenger mutations and thus imply that endogenous PTEN-Long may be playing a tumor suppressive role as was argued in chapter 2.

We observed that the PTEN-Long ATR was capable of shuttling fused RFP and p53 proteins into cells. The demonstration that these fusion products could not only enter but also function in a manner consistent with the parent proteins from which they were derived is exciting as it is a proof of concept, which implies a potential broader application of this technology as a means by which restore a variety of functional peptides across the cell membrane where they be of therapeutic benefit. In the specific context of cancer therapy we are particularly excited that this technology may represent a means by which to address mutations to tumor suppressors and thus may radically alter the way treat patients.
Chapter 4

PTEN-Long and Pancreatic Ductal Adenocarcinoma

Benjamin Hopkins, Zachary Rapp, Carmine Palemero, Meaghan Dendy, Steve Sastra, Kenneth Olive, and Ramon Parsons

1Department of Oncological Sciences, Mount Sinai School of Medicine, 1470 Madison Ave. New York, NY 10029. 2Institute for Cancer Genetics, 3Herbert Irving Comprehensive Cancer Center, 4Department of Cellular Molecular and Biophysical Studies, and 5Department of Pathology, Columbia University, 1130 Saint Nicholas Ave. New York, NY 10032

*To Whom Correspondence Should be Addressed: ramon.parsons@mssm.edu
Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) is an aggressive form of cancer. It is the 4th leading cause of cancer related deaths in the United States with a rate of incidence that almost matches its mortality rate each year. The high mortality rate is due to both late detection of the disease, at a point when it has already disseminated and patients are no longer candidates for curative surgery, and a lack of effective therapeutic agents to treat patients with advanced PDAC. In the present study, we demonstrate that PTEN-Long protein levels are down in multiple primary tumor samples as well as tumor derived PDAC cell lines. *In vitro*, we demonstrate that exogenous PTEN-Long is capable of down regulating AKT signaling in two PDAC cell lines. Further, we demonstrate that PTEN-Long is capable of entering the pancreatic tumors of two genetically engineered mouse models of PDAC and affecting PI3K signaling and cell survival in vivo. Using a clinically relevant genetically engineered mouse model of PDAC, we observed that treatment with PTEN-Long performs in a manner comparable to gemcitabine, the current standard of care. Furthermore, with combination therapy of PTEN-Long and gemcitabine, we see a significant benefit in the median overall survival of the mice. Taken together, these findings indicate that endogenous PTEN-Long may constrain PDAC progression in both the KPC model as well as in the human disease, and support further investigation into PTEN-Long’s potential use as a therapeutic agent for PDAC in the clinic.
Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) is an aggressive malignancy. ~80% of PDAC patients present with metastatic or locally advanced disease and are therefore not candidates for curative surgery (Table 5).

<table>
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<th>Stage at Diagnosis</th>
<th>Stage Distribution (%)</th>
<th>5-year Relative Survival (%)</th>
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</thead>
<tbody>
<tr>
<td>Localized (confined to primary site)</td>
<td>9</td>
<td>24.1</td>
</tr>
<tr>
<td>Regional (spread to regional lymphnodes)</td>
<td>27</td>
<td>9.0</td>
</tr>
<tr>
<td>Distant (cancer has metastasized)</td>
<td>53</td>
<td>2.0</td>
</tr>
<tr>
<td>Unknown (unstaged)</td>
<td>11</td>
<td>4.1</td>
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The median survival for metastatic patients is only 3-5 months, while patients with locally advanced disease have a median survival of only 6-10 months. The standard of care for non-resectable patients has evolved over time without any huge increases in overall survival. Until it was supplanted by a study in 1997, 5-Fluorouracil, an inhibitor of thymidylate synthetase was the standard of care. It was replaced by gemcitabine, a nucleoside analogue, based upon a minimal survival benefit (4.4-5.7 months) and a significant increase in the quality of life of these patients. Since then a variety of different therapeutic regimens have been tested in combination with gemcitabine in clinical trials, including gemcitabine plus fluorouracil (5-FU, Adrucil), gemcitabine plus capecitabine (Xeloda), gemcitabine plus cisplatin (Platinol), gemcitabine plus oxaliplatin (Eloxatin), gemcitabine plus nanoparticle albumin-bound paclitaxel (Abraxane), also called nab-paclitaxel, and gemcitabine plus erlotinib. Results from these studies varied but none showed more than a modest increase in the overall survival of the
patients. Recently, a drug regimen called FOLFIRINOX, consisting of 5-FU, leucovorin, irinotecan, and oxaliplatin has been shown to increase survival in patients with pancreatic cancer, but due to side effects is only recommended for patients who are otherwise healthy (233-235). At the present the five-year survival rates for people diagnosed with PDAC remain unacceptably low, so there is a desperate need for further advances.

Genetically, the major alterations that occur in human PDAC have been identified and models have been proposed for the sequential progression of transformation from normal tissue into cancer (Figure 4.1). While this model of progression from normal tissue through PANin lesions and into full PDAC has been recapitulated in numerous

![Figure 4.1 Progression model of pancreatic adenocarcinoma. Originally published (http://www.nature.com/nrc/journal/v2/n12/fig_tab/nrc949_F2.html)](2)
genetically engineered mouse models, there is still significant debate as to the cell of origin for PDAC. Four genes have been identified as altered in the majority of PDAC cases. Alterations in KRAS occur in >90% of PDAC, while the INK4a locus is altered between 80-95%. Mutations in Trp53 occur slightly less frequently in ~75% of cases, while deletions of SMAD4 are found in about 50% of PDAC(2, 226, 240).

**PI3K Pathway components in PDAC**

Though no single gene from the PI3K pathway is represented in the set of highly penetrant alterations in PDAC, components of the pathway are frequently altered. ERBB2 and EGFR are both cell surface receptor tyrosine kinases that are up regulated in the early stages of PDAC development. It has also been reported that AKT2, a downstream component of the PI3K pathway, is altered in up to 20% of cases(241). Furthermore, activation of the PI3K pathway, as indicated by immunohistochemistry for pAKT, pS6, and p4EBP1, are markers of poor prognosis in PDAC(242, 243). Alterations to the PI3K pathway have been shown to cooperate with RAS mutations in order to enable the tumor cells to meet their metabolic demands and inhibit oncogene induced senescence (236, 244-246).

**PTEN in PDAC**

In several small scale studies of human PDAC, PTEN has been shown to be lost or reduced via IHC in up to 82% of tumors(236, 247). Several mechanisms have been identified for the down regulation of PTEN in PDAC, including methylation of the promoter and up-regulation of MiR21, which is known to target PTEN(245, 246, 248). Loss of PTEN is seen with concomitant activation of PI3K signaling including pS6,
pAKT. In pancreas specific Pten knockout mice, <10% of the mice develop tumors, and
the majority have a defect in maintaining the proper balance between different cell types
in the pancreas, as centroacinar cells and islets are over represented (249). Pten was
identified as the first and second hits in two screens using the sleeping beauty transposon
system in KrasG12V driven models of PDAC(138, 250). Targeted disruption of Pten in
the setting of mutant Kras in the murine pancreas greatly accelerates the onset of PDAC
in the mice and shown to be haploinsufficient(247). Pten was also shown to cooperate
with loss of Smad4 as co-deletion allows high penetrance tumor initiation even though
Smad4 loss by itself is insufficient for PDAC initiation in mice(251). In one small study
of an EGFR inhibitor (Getfitinib) in combination with gemcitabine, PTEN status of the
tumor was shown to correlate with progression free as well as overall survival of the
patients(236). The patients who did not have PTEN via IHC in these studies did not
benefit from the addition of the EGFR inhibitor, while the cohort that were PTEN
positive had a significantly greater median progression free survival.

**Therapeutic Models of PDAC**

Pancreatic ductal adenocarcinoma is a complicated disease to model because it typically
presents late and in the context of a desmoplastic stroma(252, 253). Conceptually, these
features make it challenging to model PDAC using conventional xenografts as they fail to
recapitulate the complexity of the microenvironment of the human condition. Evidence
of these issues was highlighted in a 2009 paper where response rates were compared
between gemcitabine treatment of tumor xenograft responses and a genetically
engineered model of PDAC. In this study they observed that, similar to human PDAC
only, ~10% of the genetically engineered KPC tumors responded to gemcitabine, as is the
case in human PDAC, while a much higher percentage of the xenografts responded. The specifics of the tumor microenvironment in PDAC have led to a series of experiments and subsequent trials that have looked into treating the tumor microenvironment as well as the tumor cells themselves.

In order to model PDAC progression and create more effective preclinical models, many groups have generated conditional mouse models in which alterations associated with the human condition are modeled in mice (254, 255). These models have many benefits but also considerable limitations that must be taken into account in designing preclinical studies (256). While the same models, such as pdx1-cre driven KRas model, produce tumors that resemble human PDAC, their extended tumor latency period makes them difficult to utilize for preclinical studies that require sizeable cohorts of animals (256, 257). In contrast the Kras (G12D) p53 (fl/fl) pdx1-cre (KP(fl)C) model provides reproducible tumor initiation in a short time frame but fails to accurately recapitulate the histologic elements or the heterogeneity of the human disease, as the entire pancreata of these mice become uniform fibrotic tumors in a very short time frame. The KP(fl)C model in the present work was utilized to demonstrate the feasibility of these studies in a less intricate model system, and was thus shown as part of a logical progression that justified use of more rigorous model. Mice harboring lox-stop-lox alleles of Kras G12D and p53 R172H activated under the pdx1-cre promoter (KPC) succumb to PDAC that is histologically similar to the human disease while taking significantly less time to develop than the mice that only carry mutant Kras. One drawback to this model is that it can produce multiple sites of tumor initiation, a feature that is rarely seen in human patients (256). This model recapitulates many of the critical features of PDAC including
its complex stroma and thereby represents a good faith effort on the part of the authors to accurately model PTEN-Long therapy in PDAC. In order to recapitulate the late stage of diagnosis in humans, the tumors in the present study were allowed to grow until they measured 6 mm before treatment was initiated.

In this chapter we examine the expression of endogenous PTEN-Long in PDAC and assess the ability of exogenous PTEN-Long to act as a therapeutic agent in preclinical models of the disease.
Materials and Methods-

Reagents

HiTrap Heparin and HisTrap columns were purchased from GE Life Sciences (Piscataway, NJ). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Protein A/G Plus agarose, rabbit and mouse IgG agarose were from Santa Cruz (Santa Cruz, CA). Human Serum, Concanavalin-A and imidazole were from Sigma (St. Louis, MO).

Antibodies

PTEN-Long (Gly-2) poly-clonal rabbit antibody to amino acids 153-173. A peptide with an amino terminal Cysteine followed by the sequence PRHQPLLPSLSSFFFHRLPD of PTEN-Long was conjugated to keyhole limpet hemocyanin, immunized in rabbits and affinity purified using an affinity resin generated with the same peptide (Zymed Laboratories, San Francisco, CA). Antibody was termed Gly-2 as it was eluted from the affinity column using Glycine pH 2 elution buffer. PTEN (138G6), pAKT308, pAKT473, pGSK3, Total AKT, pFoxo, Foxo3, Cleaved Caspase 3 antibodies were from Cell Signaling (Danvers, MA). PTEN (6H2.1) was from Cascade (Winchester, MA). V5 antibody came from Invitrogen (Carlsbad, CA). Tubulin and Actin antibodies were procured from Sigma (St. Louis, MO). Secondary antibodies were purchased from Pierce (Rockford, IL).

Cell Lines

Tumor cell lines were obtained from ATCC (Manassas, VA). Mouse KPC cell lines generated as previously described(253).
Patient samples

Patient samples were acquired from the Columbia University Department of Pathology Tumor Bank in accordance with the IRB guidelines. Samples used for immunohistochemistry came from paraffin blocks. Samples used for immunoblotting came from frozen tissue.

Therapeutic agents

Gemcitabine, Gemzar™ was procured from Eli Lilly as a powder (a ~48% preparation of difluoro-deoxyxycytidine, dFdC) (Hannas, Delaware) and resuspended in sterile normal saline at 5mg/mL. GDC 0941 came from Chem Express (Hayuan, China) and was resuspended at 10mg/ml.

Immunohistochemistry

Immunohistochemistry for PTEN (138G6) and pS6 antibodies used sodium citrate antigen retrieval buffer as previously described (225). PTEN-Long (Gly-2) immunohistochemistry was performed using the Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA), detected with VECTASTAIN Elite ABC Reagent and 3, 3’-diaminobenzidine substrate as per the manufacturers protocol. Tissues were embedded in paraffin and cut as previously described (137).

Protein purification

BL21 (DE3) pLysE (Invitrogen) E. Coli were transformed with plasmids encoding jpExpress PTEN-Long constructs. Overnight cultures were used to inoculate 400 ml
cultures of LB until OD595= 0.3. Protein expression was induced with 0.1 mM IPTG (Sigma) for 4.5 hours at 21 degrees C. Protein was extracted from bacteria by sonication in lysis buffer (150 mM NaCl, 50 mM Tris). Lysates were clarified by centrifugation at 40,000g for 30 minutes and filtered through a .22 micron filter. Lysates were then run sequentially over the following columns using an AKTA-FPLC: HisTrap (Running Buffer: 500 mM NaCl 25 mM Tris pH 7.6, 20 mM Imidazole, Elution Buffer: 500 mM NaCl 25 mM Tris pH7.5, 500 mM Imidazole), Desalting (Buffer 50 mM NaCl, 25 mM Tris pH 7.5) and HiTrap Heparin (Running Buffer: 25 mM Tris pH 7.5 25 mM NaCl, Elution Buffer: 25 mM Tris pH 7.5 1 M NaCl ). The resulting eluates were concentrated using Amicon Ultra 50KDa exclusion columns (Millipore, Billerica, MA). The protein was subsequently resolved by SDS-PAGE electrophoresis and quantified by Coomassie staining by comparison with a BSA standard (Pierce, Rockford, IL).

**Immunoblotting**

Lysates were resolved on 4-12% or 4-20% polyacrylamide gradient gels (Invitrogen) and blotted on PVDF using semi-dry transfer technique (Biorad). Membranes were blocked with 5% Milk-TBST and washed in TBST before being incubated from one to eighteen hours in either 5% BSA-TBST or 5% Milk-TBST with primary antibodies followed by one hour in horse radish peroxidase conjugated secondary antibodies in 5% Milk-TBST. Blots were visualized using chemiluminescence (Pierce) according to the manufacturer’s protocol. Autoradiography film was obtained from Worldwide Scientific (USA).

**PTEN-Long treatments in culture**
Cells were grown to 85% confluence in media with 10% serum. They were then washed 1x in serum free media and allowed to incubate for 30 minutes at 37 degrees. They were then re-fed with serum free media containing PTEN-Long related proteins from bacterial protein preparations as indicated. Cells were treated with quantities indicated and harvested after 24 hours after they were treated with the protein.

**KP(fl)C Model**

The Kras\textsuperscript{LSL.G12D} p53\textsuperscript{fl/fl} Pdx1Cre\textsuperscript{tg/+} model (KP(fl)C) has been previously described, in brief the KP(fl)C model uses the pancreas specific Pdx1 promoter to drive the expression of Cre recombinase only in cells that normall express Pdx1 and thereby activate the expression of mutant Kras and the ablation of both copies of p53 (253). The mice in this model develop tumors within ~90 days of birth and typically succumb to their disease in ~10 days.

**KPC Model**

The Kras\textsuperscript{LSL.G12D} p53\textsuperscript{LSL.R172H} Pdx1Cre\textsuperscript{tg/+} (KPC)mice have been previously described(258). The KPC mice have heterozygous conditional alleles of mutant Kras and p53 that are activated through the excision of the lox-stop-lox (LSL) cassette via the activity of Cre recombinase under the control of the pancreas specific Pdx1 promoter. The mice typically survive for 3-11 months with a mean survival of 4.5 months.

**Enrollment Criteria for survival studies**
Enrollment of the KPC mice into our survival studies was based on tumor diameter as measured by ultrasound. Mice were palpated weekly until tumors were identified, at which point the tumor volumes were monitored via ultrasound until the average of the long and short axes of the tumor was >6mm. Once a mouse had a tumor that met this criteria the mouse was deemed enrollable. Enrollable mice were dosed as indicated and were imaged by 3D ultrasonography biweekly until endpoint criteria were met. Endpoint criteria included the development of abdominal ascites, severe cachexia, significant weight loss, exceeding 20% of initial weight or extreme weakness or inactivity.

**3D Ultrasonography of KPC Tumors**

Ultrasonography was performed as previously described using the Vevo 770 System with a 35MHz RMV scanhead (Visual Sonics, Inc.) (259). The 3D motor arm was utilized to collect images at 0.25mm intervals through the entirety of the KPC tumors. The integrated Vevo 770 software was used to reconstruct and quantify the resulting 2D images into 3D volumes.

**Dosing and Delivery of Therapeutic Agents**

10 mg/kg of PTEN-Long or related purified proteins were delivered via intra-peritoneal injection to the animals while they were on study. 100 mg/kg GDC0941 was given via oral gavage daily. 100 mg/kg Gemcitabine was given twice weekly via intra-peritoneal injection. Dextrose was delivered subcutaneously on the days when mice received Gemcitabine as a 10x mass (ul) injection of 10% dextrose.
Biopsy Protocol

Mice with tumors in the tail of the pancreas, as identified by the weekly ultrasonography of KPC mice with palpable tumors, were used for biopsy studies. The mice were anesthetized with isoflourine and abdominal hair around the incision site was removed and the region was sterilized with iodine wash. Small incisions were made to expose the pancreas and a 2mm core biopsy was taken. The hole left from the biopsy was filled with gel foam and sutures were used to repair the incisions. Upon completion of the procedure mice were given buprenorphine to alleviate any pain.

Blood Glucose measurements in mice

Blood glucose measurements were taken after IP injection of the proteins at indicated times using OneTouch Ultra Mini glucometer (LifeScan, Milpitas, CA).

Mouse Protocol

All mouse work was done in accordance with the Columbia University IACUC, New York, City, State and Federal regulations.
Results-

PTEN and PTEN-Long are down regulated in human PDAC. It has previously been shown that PTEN is down regulated in human PDAC. To confirm these reports, we stained a Tissue Microarray (TMA) containing samples from 19 patients (two tumor and one normal core for each patient) and observed extensive loss of PTEN in the tumor cells of 31% of the patients. In the same set we observed an increase in pS6 positive tumor cells in 43% of the cases. Four of the cases had both loss of PTEN and an increase in pS6 (Figure 4.2A&B). We hypothesized that PTEN-Long might be down regulated in PDAC as well. Because PTEN and PTEN-Long arise from the same transcript it is not possible to determine if PTEN-Long is altered independently of PTEN using message based technologies, so to test the hypothesis we used protein based approaches. In order to determine if PTEN-Long levels were changing, we performed Western blot analysis on Human PDAC samples and observed that PTEN-Long was lost independently of PTEN at the protein level in some of these samples (Figure 4.2C). We confirmed this finding by running western blots on four pancreatic tumor cell lines using two commercially available PTEN antibodies and observed that only MiaPaCa2 cells retained expression of PTEN-Long while all four cell cell lines still expressed canonical PTEN (Figure 4.2D). In this manner we also observed PI3K pathway activation in 2 of the 4 cell lines by probing for pS6, pFoxo, and pAKT.
Figure 4.2 PTEN and PTEN-Long in human PDAC. (A-B) Scoring of tissue microarray immunohistochemistry for PTEN and pS6. 2 tumor and 1 normal tissue core for 19 cases of PDAC were stained for PTEN or pS6. pS6 (gain) and PTEN (Loss) was scored by a pathologist (MS) in the tumor cells as compared to matched normal tissue. Tumors were counted as having lost PTEN if ≥50% of the tumor cells failed to stain. Inversely, tumors were counted as having activated pS6 if ≥50% of the cells showed positive staining. (C) Western blots of six PDAC cases taken from frozen sections probed with PTEN and PTEN-Long antibodies as indicated. (D) Western blots of lysates from four PDAC derived cell lines and probed with antibodies as indicated.
PTEN and PTEN-Long in KPC Tumors.

We next wanted to establish whether the KPC model recapitulated the expression of PTEN and the activation of the PI3K pathway that is seen in human PDAC. To this end we performed immunohistochemistry on a TMA containing some normal pancreas as well as ten KPC tumors. We observed significant loss of PTEN in 4 of 10 tumors, and loss of PTEN-Long in 6 of 9 tumors (Figure 4.3A-B). We recapitulated this finding by running western blots on samples of frozen tissue from 6 independent mice (Figure 4.3C).

![Figure 4.3 PTEN and PTEN-Long Expression in KPC Model.](image)

(A) Scoring of PTEN/PTEN-Long loss in KPC tumors. PTEN-Long/PTEN loss was scored as above, Tumors were counted as having lost PTEN if ≥50% of the tumor cells were negative. (B) Representative images from the TMAs. (C) Western blot analysis showing abundance of PTEN, PTEN-Long, and Total AKT as indicated.
Within the “normal” pancreas included within the KPC TMA, we observed an interesting staining pattern in the acinar tissue undergoing acinar to ductal metaplasia. In tissue from two separate cases, we observed that PTEN-Long expression was turned off as the cells undergo transition. The reactive stroma was frequently devoid of PTEN-Long expression around early lesions, a feature which was not observed in the PTEN stained sections (Figure 4.4). We also observed that ductal epithelium from normal pancreas did not express PTEN-Long (data not shown).

Figure 4.4 PTEN/PTEN-Long staining of “Normal” pancreas from mouse K8616. (A-C) H&E, PTEN-Long, and PTEN staining of the same region of the pancreas from mouse K8616 in a region with normal acinar tissue as well as a region of desmoplasia. (D) PTEN-Long staining of a region of acinar tissue undergoing metaplasia (^). (E-F) PTEN-Long and PTEN staining of the tumor from the same animal.
**PTEN-Long affects PI3K signaling in PDAC cells in culture.** We have previously observed in cell lines derived from both breast and brain tumors that exogenous PTEN-Long was capable of down regulating PI3K signaling and cell survival in culture (Chapter 3). In order to see if PTEN-Long could affect PI3K signaling in cell lines derived from pancreatic tumors, we treated MiaPaCa2 and Panc-1 cell lines with 25 uM PTEN-Long or PTEN-LongΔR^6 (a cell penetration deficient mutant) for 24 hours and performed Western blot analysis (Figure 4.5). We were able to determine that PTEN-Long was capable of down regulating the PI3K pathway in pancreatic tumor cells as indicated by reductions in pAKT in the cells treated with the wildtype PTEN-Long protein as compared to the cell penetration deficient mutant.

![Western blot analysis of PTEN-Long and PTEN-LongΔR^6](image)

**Figure 4.5 PTEN-Long treatment leads to inhibition of pAKT signaling.** MiaPaCa2 and Panc-1 cells were treated with 25 uM of PTEN-Long or PTEN-LongΔR^6 for 24 hours. The cells were harvested and Western blot analysis was performed with the indicated antibodies.
PTEN-Long affects signaling and induces apoptosis in KP(fl)C tumors. Having observed that PTEN-Long is capable of altering AKT signaling in PDAC tumor cell lines in culture, we next sought to determine if it could perform similarly in vivo. For this study, we treated KP(fl)C mice with PTEN-Long (10mg/kg) or equal volume of vehicle for four days and then assessed them via Western blot (Figure 4.6). Exogenous PTEN-Long was detectable in the pancreatic tumors of the treated animals (V5), and a markedly higher level of total PTEN-Long was observed using a C-terminal PTEN/PTEN-Long antibody (PTEN 138G6). We also observed that the PTEN-Long treated tumors had lower levels of pAKT and pERK, indicating that the PI3K and MAPK signaling pathways had been affected by PTEN-Long treatment. Finally, we observed higher levels of cleaved caspase 3 in the PTEN-Long treated tumors indicating that the treatment was inducing a greater level of apoptosis than was seen in the vehicle treated animals.

Figure 4.6 Effects of PTEN-Long monotherapy on KP(Fl)C mice. Four KP(fl)C mice were treated with 10 mg/kg PTEN-Long or equal volume of vehicle control once daily for four days. The mice were harvested four hours after the final treatment and were assessed via Western blot as indicated.
PTEN-Long as monotherapy in KPC mice

Having observed that PTEN-Long could enter cells and act as a therapeutic agent in the KP(fl)C model, we next wanted to assess if it would also work in the KPC model which recapitulates the poor vascularization, and hence poor drug delivery which are common features of human pancreatic tumors. First, we demonstrated that PTEN-Long treatment could enter these tumors by staining for the V5-epitope tag that is present on our exogenous protein and stained tumors that had been treated with PTEN-Long or Gemcitabine (as a control) (Figure 4.7).

![PTEN-Long Treated vs. Gemcitabine Treated](image)

Figure 4.7 **Immunohistochemistry of exogenous PTEN-Long within KPC tumors after treatment.** Tumors from the KPC animals treated as indicated were stained with anti-V5 antibody in order to visualize the presence of exogenous PTEN-Long in the tumors.

To confirm that PTEN-Long was an active agent in KPC tumors we utilized a biopsy protocol and compared a pre-treatment biopsy sample of a KPC tumor (#K1690) with the tumor tissues that harvested after one week of treatment with PTEN-Long from the same mouse. We could directly assess the effects of PTEN-Long therapy with in a single
tumor. In this manner, we saw that PTEN-Long had a profound effect upon pAKT308 signaling (Figure 4.8).

Figure 4.8 Response of KPC Tumor 1690 to PTEN-Long Monotherapy. (A) Cartoon of biopsy Protocol. 3D ultrasound sounds were taken on days -1, 3, and prior to harvest on day 6. On day -1 a 2mm core biopsy is collected from the tumor, which had previously met enrollment criteria. The mouse was treated daily with 10 mg/kg of PTEN-Long days 0-6. The tumor was harvested four hours after the final PTEN-Long treatment and (B) western blots were run as indicated. (C) Graphical representation of the 3D tumor volumes over time.
To further understand the impact of PTEN-Long therapy upon the KPC tumors, we treated a cohort of six late stage KPC mice with PTEN-Long (10mg/kg) in a survival study and compared their overall survival to the survival of historical sets of vehicle and gemcitabine treated KPC animals (Figure 4.8). While this data is not statistically significant in this small cohort, we observed an increase in the median survival in the PTEN-Long treated KPC mice that was comparable to the survival advantage observed when the mice were treated with gemcitabine. Scoring of the tumor volumes from 3 dimensional ultrasounds revealed this increase in survival with PTEN-Long monotherapy was not the result of primary tumor regression, nor the induction of stable disease, as all of the tumors treated with PTEN-Long continued to grow despite treatment (Figure 4.9)

![Survival of PTEN-Long Treated vs Historic Controls](Image)

**Figure 4.9** Survival study of KPC mice treated with PTEN-Long. Mice that reached enrollment criteria were treated daily with 10 mg/kg of PTEN-Long. Their overall survival was then compared to historic controls of the vehicle and gemcitabine treated mice (p = 0.093, Mantel-Cox Log rank test). Median survival for the Vehicle, Gemcitabine, and PTEN-Long cohorts are 11, 13.5, 15.5 days respectively.
Figure 4.10 Tumor volumes of PTEN-Long treated tumors. Tumor volumes (blue dots) are shown for each of the six mice with their enrollment (Green dotted line) and final (red dotted lines) demarcated.
Gemcitabine and PTEN-Long combination therapy

Having observed this subtle benefit in the overall survival of KPC mice treated with PTEN-Long alone, we hypothesized that there may be a benefit to combining PTEN-Long and gemcitabine treatments. The first mice enrolled in this combination study were to be treated with PTEN-Long (10mg/kg daily) and gemcitabine (100mg/kg biweekly). However, the mice in our initial treatment group showed external signs of acute hypoglycemia in response to the combination treatment. Given the compromised state of the pancreata in these mice we tested a set of non-tumor bearing mice in order to see if this effect was specific to the KPC mice. We have previously shown in chapter three that PTEN-Long alone creates a transient increase in blood sugar in wildtype mice. Furthermore, the PI3K inhibitor GDC0941 has also been reported to cause a similar transient increase in blood sugar. Therefore, we compared the effect of gemcitabine alone or in combination with either PTEN-Long or GDC0941 on blood glucose in wildtype mice. In this study we observed that gemcitabine alone as well as in combination with GDC0941 caused a transient increase in blood glucose levels; however, in combination with PTEN-Long it caused the opposite effect (Figure 4.11), indicating that the PTEN-Long in the combination treatment is doing something distinct from the PI3K inhibitor.
In order to work around this global metabolic effect, we dosed the mice with a bolus of dextrose when they were to receive the combination therapy and in this way suppressed the deleterious effects of the acute hypoglycemia.

We performed these combination studies by treating with several different control arms to help us focus upon the critical elements of PTEN-Long functions. As our primary treatment group, we treated mice with 10 mg/kg of PTEN-Long daily and 100 mg/kg of gemcitabine plus dextrose twice weekly. For comparison, we also treated cohorts with gemcitabine plus dextrose alone as well as in combination with control proteins PTEN-Long (G302R), a phosphatase dead mutant, and PTEN-LongΔR6, a mutant that is deficient in its cell permeability (Figure 4.12). In this manner we observed that PTEN-Long in combination with gemcitabine provided a significant survival advantage over gemcitabine alone (p = 0.035 Gehan-Breslow-Wilcoxon test), and that while PTEN-Long (G302R) in combination with gemcitabine is comparable to gemcitabine alone (p = 0.75 Gehan-Breslow-Wilcoxon test), that PTEN-LongΔR6 in combination with gemcitabine
Figure 4.12 **Survival study of PTEN-Long and gemcitabine combination in KPC mice.**
Once mice reached enrollment criteria the were treated daily with PTEN-Long related protein or Vehicle as indicated in combination with bi-weekly injections of gemcitabine and Dextrose. Plots indicate the percent survival of the mice in each group. N = 6 for all groups except the PTEN-Long + Gemcitabine combination which was doubled to 12 in order verify initial observations (p = 0.0004, Log-rank (Mantel-Cox) test comparing all of the curves).

has a significantly reduced overall survival when compared to combination treatment with either of the other proteins (p = 0.029 and 0.0001 by Gehan-Breslow-Wilcoxon test) indicating that PTEN-LongΔR⁶ may be having a dominant negative effect in the context of the combination treatment.
We also treated the mice with GDC0941 in combination with gemcitabine in order to observe the effects of PI3K inhibition in this setting without the restoration of PTEN’s other functions (Figure 4.13). In this manner we observed no significant benefit from the GDC0941 combination treatment as compared to gemcitabine alone, while the gemcitabine and PTEN-Long combination significantly improved survival over the gemcitabine and GDC0941 combination ($p = 0.042$, Gehan-Breslow-Wilcoxon test).

![Figure 4.13 Survival study using PI3K inhibitor GDC0941 in combination with gemcitabine in the KPC model.](image)

The three dimensional reconstructions of tumor volumes show that the PTEN-Long/gemcitabine combination is inducing tumor regressions and causing stable disease in several of the cases, while the GDC-0941/gemcitabine arm only shows a few instances of stable disease (Figure 4.14). Thus the combination studies provide evidence that PTEN-Long therapy is doing more than just inhibiting PI3K but they do not prove what that critical effect is.
Figure 4.15  **Quantification of tumor volumes.** Graphical representations of the quantification of the tumor volumes from KPC tumors treated as indicated.
Disscussion-

Pancreatic ductal adenocarcinoma is a lethal disease with an abysmal 5 year survival rate of ~4%. While the primary genetic events driving PDAC progression have been well categorized, this understanding has yet to yield effective targeted therapy. In this chapter we recapitulate the findings of previous groups that PTEN is down regulated and PI3K signaling is activated in human PDAC. Furthermore, we demonstrate that PTEN-Long levels are frequently reduced in human PDAC tumors and cell lines. This preliminary data supports the hypothesis that PTEN-Long is acting as a tumor suppressor in PDAC. This idea is supported in the literature, as PTEN loss has been shown to accelerate tumor progression in genetically engineered mouse models. It is further supported by the seemingly negative impact of the PTEN-LongΔR\textsuperscript{6} protein (both alone and in combination with gemcitabine), which may be playing a dominant negative role and inhibiting the functions of endogenous PTEN-Long in this system. PTEN-Long expression was also observed to be altered as cells undergo acinar to ductal metaplasia and might thus play a critical role in the crosstalk between pancreatic tumors and their microenvironments, although more experiments would be required to demonstrate this potential function of endogenous PTEN-Long.

Through the examination of PDAC tumors derived from the KPC genetically engineered mouse model of pancreatic cancer, we were able to establish that similar changes to the expression of PTEN and PTEN-Long are occurring in the model as those we observed in human patient samples. This data supports the notion that the KPC model is recapitulating the PI3K/PTEN pathway features of the human disease and is thus reasonable to use as a model for preclinical evaluation of PTEN-Long therapy in PDAC.
Due to the complexity of the KPC model, preliminary experiments using tissue culture and the less complex KP(fl)C model were performed to demonstrate that active PTEN-Long protein could reach the pancreas and affect signaling and cell survival. The demonstration that PTEN-Long therapy was also capable of entering the KPC tumors and altering PI3K signaling was also significant as it showed that the protein was able to reach tumor cells in the context of poorly vascularized tumors where drug delivery is known to be an issue. Together the signaling data from the preliminary studies of this chapter demonstrate that in these specific cell lines and tumors PTEN-Long is still able to function to down regulate the PI3K pathway and the tumors have not developed resistance to PTEN-Long during their evolution. Future studies using the biopsy protocol could be helpful in determining characteristics of the tumors that are likely to respond to PTEN-Long therapy. In particular, it would be interesting to ascertain whether either PTEN loss or PI3K activation predict responsiveness to PTEN-Long therapy. These findings could be of particular clinical import given that PI3K activation is a prognostic marker of poorer outcomes and thus this therapy may be of particular benefit to these patients poor prognosis category. It may also be of interest to utilize sequential biopsies in order to understand how tumors that respond to PTEN-Long therapy eventually develop resistance.

By using PTEN-Long in the KPC model of PDAC, we sought a stringent test to confirm the efficacy of PTEN-Long as a therapeutic agent. We observed that, in the context of this heterogeneous model in which neither PTEN nor PI3K is directly targeted, response to PTEN-Long as a single agent was modest at best although the survival curve for the PTEN-Long monotherapy study did overlay with historic curves for mice treated with the
current standard of care in PDAC, gemcitabine, providing a ~40% increase in median overall survival as compared to vehicle controls. That this treatment was insufficient to cause tumor regressions by itself was not surprising. Unexpectedly we observed that in combination with gemcitabine, PTEN-Long was able to significantly extend the median survival of the cohort, causing a transition to stable disease in some animals and frank regressions in others. One potential explanation for the variability in response to the combination is the variability of the KPC model. The model is heterogeneous with a significant tumor latency period that allows the tumors to acquire an array of different mutations. These differences are thought to account for some of the variety that exists between the tumors, and it is possible that induction of stable disease or frank regressions observed in the combination arm of our study are the result of the different genetics of the tumors with in the study. If this is the case, further biopsy studies may enable us to observe which genetic characteristics are present in the tumors that respond as compared to the tumors that did not respond. Such experiments could be informative for determining how to improve the combination by identifying other potential targets and could allow us to study how the tumors evolve in response to the treatment and eventually develop resistance.

We demonstrated that the PTEN-Long/gemcitabine combination has an acute effect upon glucose metabolism, an effect that was not expected given the effects of each drug alone, and which was not recapitulated by combination treatment of gemcitabine with a PI3K inhibitor. It is possible, that while gemcitabine and PTEN-Long each play independent roles inhibiting tumor growth and marginally increasing survival in the KPC model, the
pronounced affect observed in the PTEN-Long with gemcitabine combination is
dependent upon this metabolic effect and is therefore highly specific to this combination.

It would be interesting to determine the specificity of this effect and to test other
chemotherapeutic agents in combination with PTEN-Long in order to determine if the
survival benefit seen with PTEN-Long/gemcitabine can be improved upon. In a small
study of Greek patients, researchers demonstrated that PTEN status correlated with
response to gemcitabine/erlotinib combination (Reference). Based on this data, it may be
worthwhile to see if we can sensitize more tumors to the erlotinib/gemcitabine
combination through the restoration of PTEN activity via PTEN-Long.

Together this data suggests that PTEN-Long is likely to be playing a tumor suppressive
role in PDAC. Based on the data in our preclinical studies, we believe that there may be
therapeutic utility for PTEN-Long, but to optimize its impact, more studies are needed in
order to understand how best to use this agent.
Chapter 5

Significance
In our analysis of PTEN, we consistently observed a 75 kDa band that was detected by multiple commercially available antibodies raised against different epitopes within the 403 amino acid PTEN protein. We hypothesized that this higher molecular weight protein could be a longer variant of PTEN. This hypothesis was supported by the presence of an evolutionarily conserved alternate start site within the 5’ region of the open reading frame, which had been previously reported within the PTEN mRNA (76).

PTEN is a tumor suppressor that is critical for the maintenance of cellular homeostasis, so we thought that this translational variant, named PTEN-Long, may be important in human cancer. This idea was supported by our studies of the endogenous expression of PTEN-Long in cell lines and in human tumor samples, where we observed down regulation of PTEN-Long even in the context of normal expression of PTEN. While PTEN-Long is expressed at only ~10% of the level of PTEN, it seems that this protein is still playing a significant role in tumor suppression, as indicated by its frequent down regulation in tumors. This idea is further supported by our identification of somatic mutations in in the PTEN-Long ATR. Although this data indicates that PTEN-Long may be playing a role in tumor suppression, more experiments are needed in order to make the definitive claim that PTEN-Long is a bone fide tumor suppressor. Such evidence could come from the generation of PTEN-Long specific knockout mice. If PTEN-Long were genetically ablated without affecting PTEN, one could observe whether this genetic change affected tumor progression or initiation either by itself or in the context of a known oncogenic setting such as Myc or Erbb2 overexpression in the mammary tissue of these mice. These studies would allow researchers to determine what, if any, tumor suppressive effect endogenous PTEN-Long has, independent of the canonical PTEN
protein. Using bone marrow transplant studies, one could also use the bone marrow from these PTEN-Long knockout animals to examine the role of PTEN-Long in immune response to tumor formation, as it might be expected to play a role in this setting based on our data showing its up-regulation microglia surrounding brain tumors as well as in the histocytes in the tumor microenvironment of breast tumors.

While we observed PTEN-Long expression changing in tumor cells and in the tumor microenvironment, the present studies have not demonstrated the mechanism(s) by which PTEN-Long is being regulated. Expression of other translational variants that utilize CTG initiation sequences has been shown to be dependent on the abundance of specific metabolic factors such as glucose, amino acids and oxygen. Given the observation that PTEN-Long can be strongly up-regulated in the tumor microenvironment, studies to explore the regulation of PTEN-Long expression could be informative for our understanding of its endogenous functions.

To understand the functions of PTEN-Long, we began by assessing its ability to act in a fashion similar to PTEN. Using this approach, we observed that PTEN-Long acts as lipid phosphatase and that in vitro it was more efficient at enzyme lower concentrations of substrate than was PTEN. However, under the conditions presented in chapter 2 (in the context of overexpression of PTEN and PTEN-Long related proteins), no significant difference in PI3K regulation was detected between PTEN and PTEN-Long. Through the use of more sensitive assays using physiologic levels of expression of PTEN and PTEN-Long, it may be possible to identify functional differences in the activity of these proteins as they reduce PIP3 back to PIP2 under starved or stimulated conditions.
We next wanted to determine whether there was any functionality that was unique to PTEN-Long. This led us to examine the PTEN-Long ATR. Through our examination of the ATR we identified a signal sequence and cleavage site at the extreme N-terminus of PTEN-Long, indicating that the protein might be secreted. Validating this hypothesis allowed us to consider a variety of potential interactions for PTEN-Long outside of the cell. We observed that PTEN-Long bound to a variety of cell membrane components. While the present studies did not pursue what impact these extracellular interactions might have in vivo, these results support the idea that PTEN-Long is exiting the cell and may interact with proteins or entities at the cell surface. It is possible that through some of these interactions, cells may inhibit PTEN-Long by isolating it in the extracellular space. It is also possible that it has important functions outside of the cell. We did not determine whether PTEN-Long acts as a phosphatase on any targets outside of the cell, but recent reports indicate that there are secreted phosphatases with extracellular, so this is a possibility for such future studies (260). Such studies could be initiated by comparing treatment of cells with cell permeability deficient mutant PTEN-LongΔR⁶ and a double mutant protein PTEN-LongΔR⁶(G302R), which would be expected to be both cell permeability and phosphatase deficient. In this manner one could isolate the phosphatase dependent roles of PTEN-Long that are occurring outside of the cell.

The second functional motif that we recognized in the ATR was a poly-arginine stretch, reminiscent of the poly-basic residues seen in cell permeable peptides. Having already observed that PTEN-Long was being secreted from cells the presence of this motif led us to ask if PTEN-Long was also able to enter cells. In chapter 2 we demonstrated that exogenous PTEN-Long could be detected in cells which had been treated with the
purified protein and we validated these observations in chapter 3 by demonstrating that treatment with PTEN-Long was affecting cell signaling and survival in a dose dependent manner. Future studies aimed at dissecting the mechanism through which PTEN-Long enters cell may elucidate more of its endogenous functions. In chapter 2 we describe a model of the path that PTEN-Long takes from the time it is translated through its entry into a neighboring cell. At each point along the path PTEN-Long has the potential to interact with other proteins, and various components of both the extra- and intra-cellular compartments. Since many processes that occur within mammalian systems dependent upon PI3K signaling, not to mention other roles of PTEN/PTEN-Long, understanding these functions may enable us to identify situations where modulation of PTEN-Long may be of clinical benefit. Though PTEN-Long only represents a small fraction of the total PTEN within most cells, it is possible that modulating the activity of the PTEN-Long through the use of compounds or antibodies that inhibit its functions may be useful in some clinical settings such as increasing neovascularization, insulin sensitivity, or ovarian follicle activation.

The observations that PTEN-Long enters cells and affects cell signaling and survival as an exogenous agent gave us the rationale to pursue the use of PTEN-Long as a therapeutic agent. In chapter 3 we demonstrated that PTEN-Long had activity \textit{in vivo} by showing that it affects insulin signaling in cell culture. Based on these findings we tested the effect of PTEN-Long treatment on the blood sugar levels of mice. We first established that purified recombinant PTEN-Long protein was active in mice and we were then able to recapitulate previous reports which show that blood glucose levels transiently increase in response to inhibition of the PI3K pathway.
The notion of a secreted tumor suppressor is not novel. As reported in chapter 2 many secreted proteins have been reported to have tumor suppressive functions. The present data does not preclude the idea that PTEN-Long may be acting on extracellular targets, however we can infer from our results using PTEN-LongΔR^6 presented in chapters 3 and 4 that these potential extracellular targets are not responsible for the anti-tumor effects of PTEN-Long, as PTEN-LongΔR^6 did not affect tumor growth. Within the last decade several recombinant peptides have been identified that have therapeutic utility in preclinical models of cancer (213, 261). Other groups have demonstrated the potential efficacy of secreted tumor suppressors as therapeutic agents in pre clinical models (175, 262). With such studies in mind, we next asked if this putative tumor suppressor could affect tumor growth when given as a therapeutic agent.

Our findings that PTEN-Long could affect tumor growth and even cause tumor regression in several xenografted tumor models offers hope that PTEN-Long may be able to act as an anti-cancer agent if it were ever to be approved for use in the clinic. However, we do not know which patients might benefit from this therapy. One hypothesis is that PTEN-Long therapy will only be effective in tumors in which mutations to PTEN or the PI3K pathway are driving events in the evolution of the tumor. Through analysis of a larger cohort of tumors with known genetic alterations, it may be possible to more definitively elucidate tumor types, and thus which patient populations may benefit from PTEN-Long therapy.

The PDAC studies recapitulate many of the findings of our initial analysis regarding PTEN-Long in the specific setting of PDAC. This disease is almost uniformly lethal so learning more about it and finding effective therapies is critically important. In Chapter
4, we show that PTEN-Long is altered in human PDAC and we provide supporting evidence that the endogenous protein may play a tumor suppressive role in this disease. This idea is supported by several previous studies; one set demonstrates that PTEN loss accelerates PDAC tumor formation in several genetically engineered mouse models, while another set identified PTEN as cooperating with Kras mutations in unbiased screens\(^\text{138}, 250\). From our survival studies, we can conclude that the therapeutic effects of PTEN-Long in PDAC are dependent on being both an active phosphatase (as the PTEN-LongG302R combination failed to provide clinical benefits) and being able to enter cells (as the PTEN-Long\(\Delta R^6\) combination also failed to provide clinical benefits). Furthermore, we can also conclude that PI3K inhibition is not equivalent to restoration of PTEN activity via PTEN-Long in the setting of PDAC, as the GDC0941/gemcitabine combination also failed to cause tumor regressions or extend the median survival of the mice. These findings imply that the anti-tumor effect requires phosphatase activity, but that this activity alone may be insufficient to affect outcomes without the other functions of PTEN. The data leaves open the possibility that the regulation of an alternate PTEN-Long substrate such as an unidentified protein target could be responsible for the observed effects of PTEN-Long therapy. This could be tested by comparing the effects of lipid phosphatase dead/protein phosphatase active or lipid phosphatase active/protein phosphatase dead variants of PTEN-Long to test whether either of these variants is capable of inducing the effects seen by treatment with the wildtype protein\(^\text{263, 264}\). Based on the data of our preclinical studies, we believe that there may be therapeutic utility for PTEN-Long in PDAC. However, in order to optimize the potential impact of
PTEN-Long more studies are needed to understand the mechanisms by which it is affecting tumor growth and thereby how best to use it.
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