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lentivirus and oncolytic VSV virus**

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14. ABSTRACT

Prostate cancer is the most commonly diagnosed non-skin carcinoma and one of the leading causes of cancer-related mortality of men in western society. Approximately half of all men with clinically localized disease are not cured by surgery or radiotherapy. Presently there are no therapies available for advanced and metastatic prostate cancer, thus the emergence of new targeted therapies are urgently required. This project aims to provide a proof-of-principle that lentivirus can be engineered to infect cells in a tumor-specific fashion. Additionally, mutated form of Vesicular Stomatitis Virus (VSV), an oncolytic virus capable of replicating in interferon (IFN) response defective cells was used to selectively target prostate cancer cells *in vivo*.

Initially, we looked at the safety and efficacy of interferon-sensitive VSV (AV3 strain) treatment of prostate tumors that arise *in situ* in immunocompetent, transgenic prostate-specific PTEN-null (PTEN^{-/-}) mice. Prostates of PTEN^{-/-} and control mice were injected with 5×10^8 pfu/ml of VSV (AV3), which expresses luciferase, and monitored for luminescence over a 96 h time period. Although virus quickly dispersed throughout the bodies of mice after only 3 h, it persisted in the prostate tumors of PTEN^{-/-} mice up to 72 h; while viral distribution rapidly dissipated by 48 h in the control mice. This data was confirmed by plaque assay which showed a higher concentration of replicating virus in prostates of PTEN^{-/-} mice while sparing normal cells in control mice. Our results indicated that direct injection of VSV (AV3) intra-prostatically lead to selective infection, replication, and overall increase in apoptotic cell death in malignant tissue while sparing normal tissue due to a faulty IFN response. Furthermore, the observed cell death is not a by-product of neutrophil infiltration as previously reported in other cancer models. In summary, our data suggests that VSV (AV3) may be used as a potential oncolytic viral therapy for control and treatment of locally advanced prostate cancer.

As an alternate approach, we looked to enhance tumor specificity by constructing various lentiviruses containing different complex hairpin regions in the 5' untranslated region (5'UTR). This 5'UTR region requires the eukaryotic translation initiation factor eIF4E, an RNA capping factor, which is over-expressed in prostate cancer tissue biopsies. Four different lengths (149 bp, 274 bp, 533 bp, 619 bp) of 5'UTR from different species have been inserted into the lentiviral backbone. Various constructs have been transfected into both prostate epithelial and cancer cell lines and downstream GFP expression has been monitored. Correlation of GFP expression along with level of eIF4E protein has thus lead us to pick two (274bp and 533 bp) 5'UTR to further test in the PTEN^{-/-} mice to determine the best candidate for targeted tumor expression.

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Introduction:

Prostate cancer is the most prevalent malignancy and second leading cause of cancer related mortality in North American men, with over 186,000 new cases annually and approximately 28,000 deaths [1]. Almost half of all men with clinically localized disease are not cured by surgery or radiotherapy [2, 3]. Furthermore, survival prospects for patients with locally advanced or metastatic prostate cancer are low and current therapies have side effects that seriously compromise quality of life [2-6]. The high incidence of prostate cancer in men over 50 coupled with the rapidly aging North American male demographic, highlight the urgency for better management and treatment of this cancer [7].

There are a variety of transplantable human xenografts and transgenic mouse tumor models that can be used to test new strategies for the treatment of prostate cancer. While use of xenografts are limited by the necessity of using immunocompromised animals and need for surgical implantation at orthotopic or subcutaneous sites, transgenic mouse tumor models offer several key advantages for pre-clinical testing, including: the cancer arises *in situ* in the target tissue with the appropriate microenvironment, mice possess an intact immune system, cancers are frequently heterogeneous, and by arising *de novo*, undergo neoplastic progression similar to that seen in human cancers [8-10]. One such transgenic model for prostate cancer is the pbARR2x PTEN^{fl/fl} mouse or prostate-specific PTEN null mice (PTEN^{-/-}), which leads to *de novo* formation of prostate tumors. The disease progression of PTEN^{-/-} mice to prostate cancer is similar to that seen in humans [9, 10]. Deletion or mutation of the tumor suppressor PTEN gene has been implicated in many human cancers and has been seen in up to 30% of primary prostate cancers and over 64% of prostate metastases, making PTEN an important candidate gene for prostate cancer development and progression [9-12]. This study has utilized this PTEN^{-/-} mouse model to test different viral based strategies for prostate cancer therapy.

In recent years, oncolytic viruses have been tested in various clinical trials [13, 14] for their anti-tumor targeting capabilities, as they are often able to exploit tumor specific genetic defects [15-17]. Some of these viruses are fully replication-competent and rapidly spread through and kill cancerous cells. Vesicular Stomatitis Virus (VSV) is an oncolytic virus which can infect and kill cells which have defects in their cellular anti-viral immune response [18-20]. One of the key cellular antiviral immune reactions involves the interferon (IFN) response pathway [19]. Typically most cancer cells display a faulty interferon (IFN) response and therefore are susceptible to infection by VSV. Previous studies have demonstrated that selectivity of VSV for malignant cells in comparison to normal cells can be enhanced either by pre-treatment with IFNs or by using a mutated form of VSV-ΔM51 [20]; referred to as VSV(AV3), which better induces IFN production in infected cells. It has been

shown that deletion of methionine at position 51 in the matrix (M) protein of VSV renders it more susceptible to host innate immune response. This enhanced susceptibility is important since the virus is safely cleared from normal cells but destroys malignant cells, thereby making VSV (AV3) more cancer specific [20-23].

Thus one goal of this study was to determine whether VSV (AV3) can infect and kill the prostate tumors of PTEN null mouse. Our results indicate that this virus is able to selectively infect, replicate, and increase apoptosis in malignant tissue while sparing normal tissue. This occurs through a mechanism involving direct oncolysis due to a faulty IFN response in the PTEN^{-/-} mice, rather than neutrophil infiltration as has been previously reported in other cancer models [24].

Another strategy is to enhance the tumor specificity of the virus. As a result, eIF4E, an mRNA cap-binding protein, which has been shown to be over-expressed in a variety of tumors including prostate cancer, was chosen as a tumor specific target [25, 26]. Increase in eIF4E leads to increase in cell proliferation, suppression of apoptosis and other characteristics associated with malignant transformation. EIF4E has been implicated in the control of translation of a few select proteins involved in developmental processes such as growth factors, proto-oncogenes, and transcription factors [27]. Typically the 5' untranslated region (UTR) of these mRNAs acts to repress their translation. mRNAs which contain excessive secondary structure in 5'UTRs are normally discriminated against by the translational machinery and therefore require higher levels of eIF4E/eIF4F complex to unwind their 5'UTRs for efficient translation [28]. To this end the initial goal of this portion of the study is to determine which 5' UTRs should be constructed into the backbone of lentivirus that could lead to a tumor specific viral infection. Four different 5'UTRs were selected including two derived from fibroblast growth factor-2 (FGF-2) and two derived from Ornithine Decarboxylase (ODC). Our results have determined that two out of four 5'UTR tested showed potential for tumor specific targeting based on eIF4E and GFP expressions *in vitro*. Thus far the *in vitro* testing of these lentiviral constructs are completed, viruses from these constructs have been prepared. The next step is to test the expression level of these viruses in the PTEN^{-/-} mice and compare them to control animals. In general the goal of this portion of the study is to construct a lentivirus containing 5'UTR which is capable of selective gene expression in the prostates of the tumor bearing PTEN^{-/-} mice.

OVERALL HYPOTHESIS:

The overall hypothesis of this research project is to create a viral-based therapy that will target and destroy malignant prostate cells while sparing normal cells.

SPECIFIC AIMS:

- Aim 1.** Construct viral vectors that have exogenous cellular promoters and an eIF4E UTR recognition sequence. This will enable us to test differential tumor expression.
- Aim 2.** Test these vectors for prostate and tumor specific targeting *in vivo* utilizing prostate-restricted PTEN^{-/-} mice.
- Aim 3.** To test whether mutated form of VSV (AV3 strain) can selectively target and infect prostate cancer cells in the PTEN^{-/-} mouse model.

RESULTS:

SPECIFIC AIM 3: To test whether mutated form of VSV (AV3 strain) can selectively target and infect prostate cancer cells in the PTEN^{-/-} mouse model.

The following points were previously reported:

1. VSV (AV3) on cytotoxicity of prostate cancer cells.
2. Presence of live replicating virus in prostate tissues of PTEN^{-/-} mice.
3. VSV (AV3) causes preferential killing of prostate cancer cells *in vivo* while sparing normal prostate tissue.
4. *In vivo* cancer cell death is not associated with neutrophil infiltration in PTEN^{-/-} mice prostates.
5. VSV (AV3) infection leads to an increase in macrophage and B-lymphocyte response.
6. *In vivo* effects of VSV (AV3) on IFN receptor-1 alpha (IFNR-1 α).

The following points were addressed in order to better decipher the mechanism underlying enhanced viral infection in prostate tumors of PTEN^{-/-} mice. The data mentioned below, which completes aim 3 of our specific aims, has recently been published in Cancer Research (2010 Feb 15; 70 (4):1367-76) titled “Oncolysis of prostate cancers induced by vesicular stomatitis virus in PTEN knockout mice”.

⇒ To demonstrate that oncolytic effect of VSV (AV3) leads to apoptosis and not inhibition of cell growth the following study was performed.

VSV(AV3) causes preferential killing of prostate cancer cells *in vivo* while sparing normal prostate tissue.

The difference between viral levels and persistence in the prostates of PTEN^{-/-} and control mice led us to assess whether the virus was preferentially infecting and killing tumor

cells. To test this, VSV(AV3) infected prostate tissue from both PTEN^{-/-} and control mice were collected at various time points, embedded in paraffin and evaluated for apoptosis by TUNEL assay (Fig. 1 A). There was a substantial increase in apoptotic cell bodies observed in the prostates of VSV (AV3)-treated PTEN^{-/-} mice compared to virus treated controls, where virtually no change in apoptosis was observed (Fig. 1B).

To see the effect of VSV (AV3) infection on cell proliferation, prostate tissues were stained with Ki67 (proliferation marker) and positive cells were counted (Fig 1C and D). Although there was consistently higher proliferation seen in PTEN^{-/-} prostates compared to control, there was no significant difference in proliferation detected post VSV (AV3) infection. This increase in apoptosis and lack of difference in proliferation in the tumor-bearing mice correlated with the increased viral titer previously noted; indicating that active infection of tumor cells with VSV (AV3) is the cause of this increased apoptosis.

- ⇒ To expand on the mechanism of action of VSV(AV3) prostate cancer specific killing *in vivo* we have looked more closely at the status of interferon pathway and its components in the prostates of PTEN^{-/-} compared to control mice.

Effects of VSV (AV3) on IFN pathway *in vivo*.

Since VSV infection leads to activation of the IFN response pathway expression, prostates of control and PTEN^{-/-} mice challenged with VSV(AV3) infection were stained for various components of the IFN pathway such as interferon receptor-1 alpha (IFNR-1 α), ribonuclease L (RNase L), interferon regulatory transcription factors 3 (IRF-3) and 7 (IRF-7) (Fig. 2A). Immunohistochemical analysis of the prostates of control mice post VSV (AV3) injection demonstrates an increase in staining of all of the mentioned IFN pathway components, demonstrating that there is an intact initial IFN response towards the virus in the prostate tissues of control mice. However, in PTEN^{-/-} prostates, there was no change observed over time in any of the IFN pathway components as scored by a pathologist. This correlates with the increase propagation of virus as seen over time in prostate tumors of PTEN^{-/-} mice (previously shown).

Additionally, mRNA levels of IFN- α present in prostate tissues of VSV (AV3) infected PTEN^{-/-} and control mice were checked and compared by qPCR analysis. Figure 2B demonstrates that in control prostates treated with VSV (AV3), there is an approximately 500-

fold increase in IFN- α transcription by 48 h. However, in PTEN^{-/-} prostates treated with virus at the same time point there is only an approximately 40-fold increase in IFN- α mRNA levels. These data suggest that there is a partial IFN response to viral infection in PTEN^{-/-} mice that permits further viral infection and oncolysis of tumor cells (previously shown).

SPECIFIC AIM 1: Construct viral vectors that have exogenous cellular promoters and an eIF4E UTR recognition sequence. This will enable us to test differential tumor expression.

Expression of eIF4E in normal prostate epithelial cells compared to prostate cancer cell lines.

Western analysis comparing the relative expression of eIF4E from whole cell protein lysates of non-neoplastic human epithelial prostate cell lines (BPH-1 and PNT1B) were compared to tumorigenic prostate cancer cell lines (LNCaP, C4-2, DU145, PC-3 and MPPK-1 cell lines). Western blots were normalized by the house keeping protein β -actin. Figure 3 demonstrates an increase in eIF4E protein levels in tumorigenic cell lines when compared to control immortalized prostate epithelial cells. These results indicate that there is an elevated level of eIF4E protein in the majority of prostate cancer cell lines.

Lentiviral construction.

The 5' UTR constructs with various lengths were inserted into the backbone of a lentivirus vector called FUGW. FUGW vector was a gift from Dr. Christopher Ong (Department of Urologic Sciences at University of British Columbia). This lentiviral vector contains an enhanced green fluorescence protein (EGFP) which is downstream of the ubiquitin promoter. The ubiquitin promoter allows for expression of the plasmid in a non discriminatory fashion in both tumor and control cell lines. As previously shown, 5' UTR derived from rat FGF-2 cDNA was originally inserted in between the ubiquitin promoter and the EGFP expression gene at the XhoI-BamHI. This 5' UTR's length was 619 bp. Upon further sequencing analysis, we determined the presence of a signal enhancing sequence within the 619 bp. Thus we modified this plasmid to a truncated version where the signaling sequence was removed and the end product was a 533 bp 5'UTR which was again inserted upstream of EGFP after the ubiquitin promoter.

Another 5' UTR structure that we used for this study is derived from ornithine decarboxylase (ODC). The secondary 5' UTR structure of ODC, a rate determining enzyme in the polyamine synthesis, has been extensively studied. Due to complex secondary structures in its 5' UTR, the translation of the ODC enzyme is tightly controlled. The 5' UTR of ODC consists of stem and loop structure. Here we have made two plasmids using either the full length ODC 5' UTR at 274 bp or the loop portion of the 5' UTR at 149 bp. As before these different length 5' UTRs have been inserted upstream of EGFP expression gene and downstream of the ubiquitin promoter. All plasmids were confirmed by sequencing. Figure 4 is a schematic demonstration of the five different lentiviral plasmids used in this study.

Relative expression of each lentiviral plasmid with different lengths 5'UTR.

Prostate cancer cell lines and non-neoplastic cell lines were transfected with each of the plasmids containing different lengths of 5' UTR. To investigate which of these 5' UTR structures will most consistently be expressed at higher levels in the prostate cancer cells compared to non-neoplastic cells, protein expression of GFP were compared in each cell line both in presence of eIF4E or absence through siRNA knock down of eIF4E. Initially, the siRNA against eIF4E was tested in each tumorigenic cell line to demonstrate both that it is capable of lowering eIF4E levels and to determine optimal conditions for eIF4E knock down. Our results (Figure 5) demonstrated that the GFP levels produced by FU-533-GW and FU-274-GW were the most dependent on eIF4E protein levels. Thus, these two plasmids have been selected and virus has been propagated and purified in order to test this theory further *in vivo*.

Expression of eIF4E in prostates of control and PTEN^{-/-} transgenic mice.

Although increase expression levels of eIF4E have previously been demonstrated in the human prostate cancer tissue micro-arrays, here we need to demonstrate whether this holds true in our transgenic model. Thus, expression profile of eIF4E family of transcription factors were determined in prostate tissues of transgenic PTEN^{-/-} mice and compared to control mice. Figure 6 demonstrates a clear elevation of eIF4E protein levels when comparing the prostates of PTEN^{-/-} mice in to control mice. Similarly, levels of phosphorylated eIF4E binding protein-1 (4EBP-1), which is in its non-phosphorylated form sequesters eIF4E in the cell thus rendering it inactive, were markedly higher in the prostate tissues of PTEN^{-/-} mice when compared with control, hence, validating the use of this model for further testing the tumor specific expression

of the two different lengths of 5'UTRs lentivirus (FU-274-GW and FU-533-GW). These results were validated and scored by a pathologist.

REPORTABLE OUTCOME:

1. A summary of the above data has been presented in the following meetings in chronological order:

#	Conference Name	Date	Location	Type of Presentation
1	BC Genome centre & Student biotechnology network	April (2009)	Vancouver, BC (Canada)	Poster
2	American Association for Cancer Research (AACR)	April (2009)	Denver, CO (USA)	Poster
3	Department of Experimental Medicine student Research Day	June (2008)	Vancouver, British Columbia (Canada)	Oral
4	5th International meeting on tumor microenvironment	October (2009)	Versailles, (France)	Poster
5	Centre for Hip Health and Mobility Trainee poster Afternoon	April (2010)	Vancouver, British Columbia (Canada)	Poster
6	American Association for Cancer Research (AACR)	April (2010)	Washington DC (USA)	Poster

2. Manuscript in print: Oncolysis of prostate cancers induced by vesicular stomatitis virus in PTEN knockout mice. Moussavi M, Fazli L, Tearle H, Guo Y, Cox M, Bell J, Ong C, Jia W, Rennie PS. Cancer Research. 2010 Feb 15;70(4):1367-76. Epub 2010 Feb 9.
3. Manuscript in preparation: Titled "Utilizing 5'UTR to enhancing viral tumor specificity in prostates of PTEN^{-/-} mice" Moussavi M, Moshkabadi N, Fazli L, Tearle H, Jia W, Rennie PS. To be submitted by July to Cancer Gene Therapy.

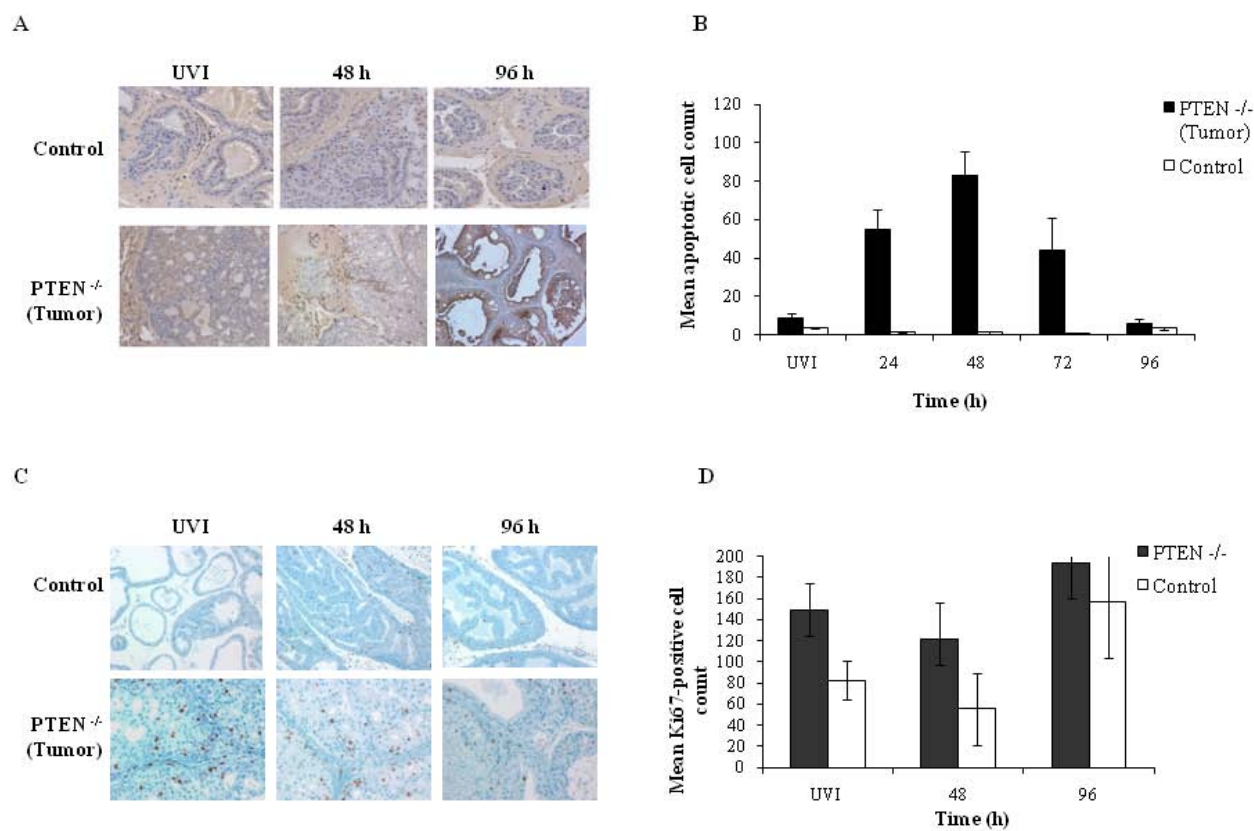
FIGURES:

Figure 1. Apoptosis in the prostates of PTEN^{-/-} and control mice. Each mouse was injected with 100 μ l of 5×10^8 pfu/ml of VSV(AV3). Mice were euthanized at 24, 48, 72 and 96 h post viral injection. Paraffin embedded prostates tissues were stained with TUNEL. (A) and Ki67 (C). Representative slides at 40x magnification show presence of apoptotic bodies (stained brown). (B) Apoptotic cells and cells stained with Ki67 (D) were counted in ten fields of view and presented as average number of apoptotic cells $\pm$ SD (n = 3).

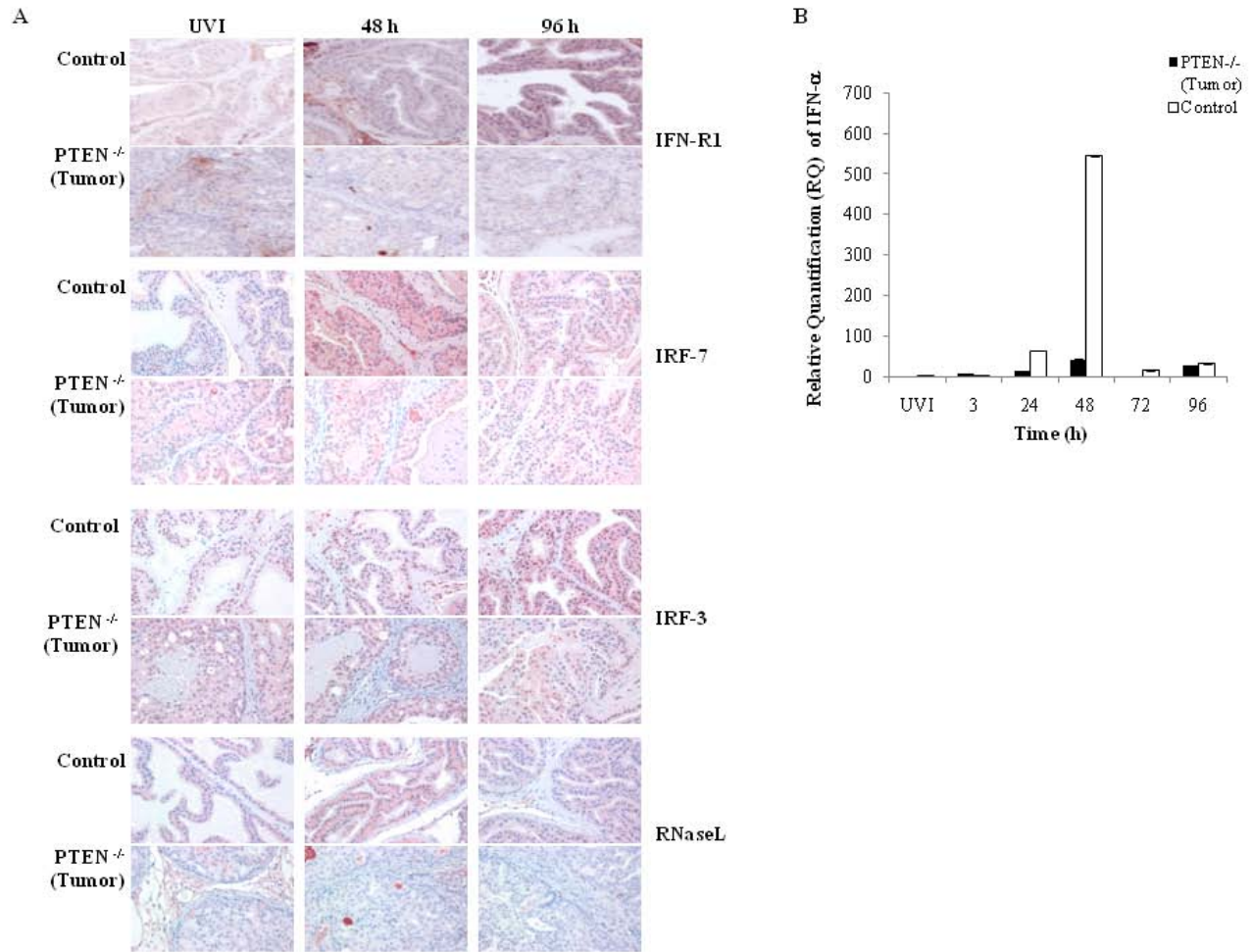


Figure 2. (A) Immunohistochemical staining of IFN- α/β receptor, IRF-3, IRF-7 and RNaseL. Paraffin embedded tissues were stained with IFN- α or IRF-7. Representative slides were prepared and visualized at 40x magnification. Results were scored by a pathologist (n = 3). (B) IFN- α mRNA levels in prostates of control and PTEN^{-/-} were compared post viral infection. IFN- α mRNA level was first normalized to ribosomal RNA level ($\Delta CT = CT_{IFN-\alpha} - CT_{rRNA}$) and then compared with the negative control group prostates treated with UVI virus ($\Delta\Delta CT = \Delta CT - \Delta CT_{UVI}$). The results were expressed as relative quantification (RQ) = $2^{-\Delta\Delta C}$.

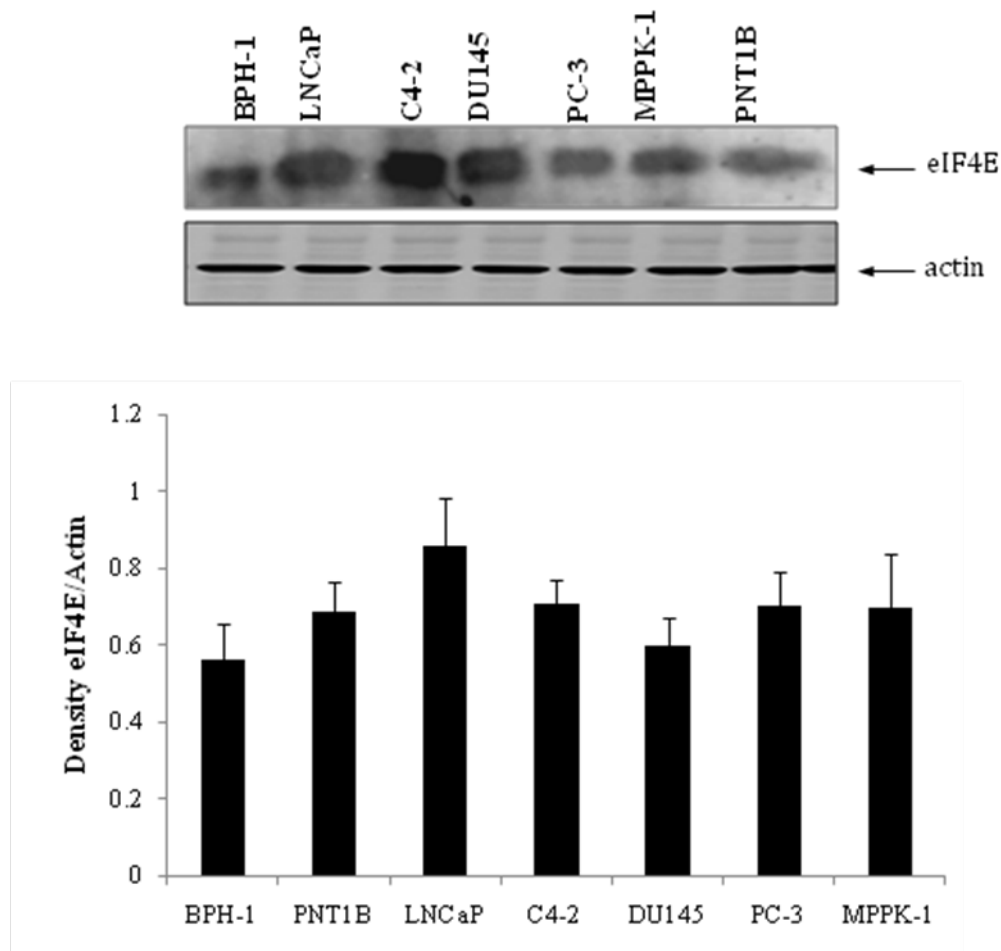


Figure 3. Western blot analysis of eIF4E protein levels in untreated neoplastic (LNCaP, C4-2, DU145, PC-3) and non-neoplastic (BPH-1, PNT1B) prostate cell lines.

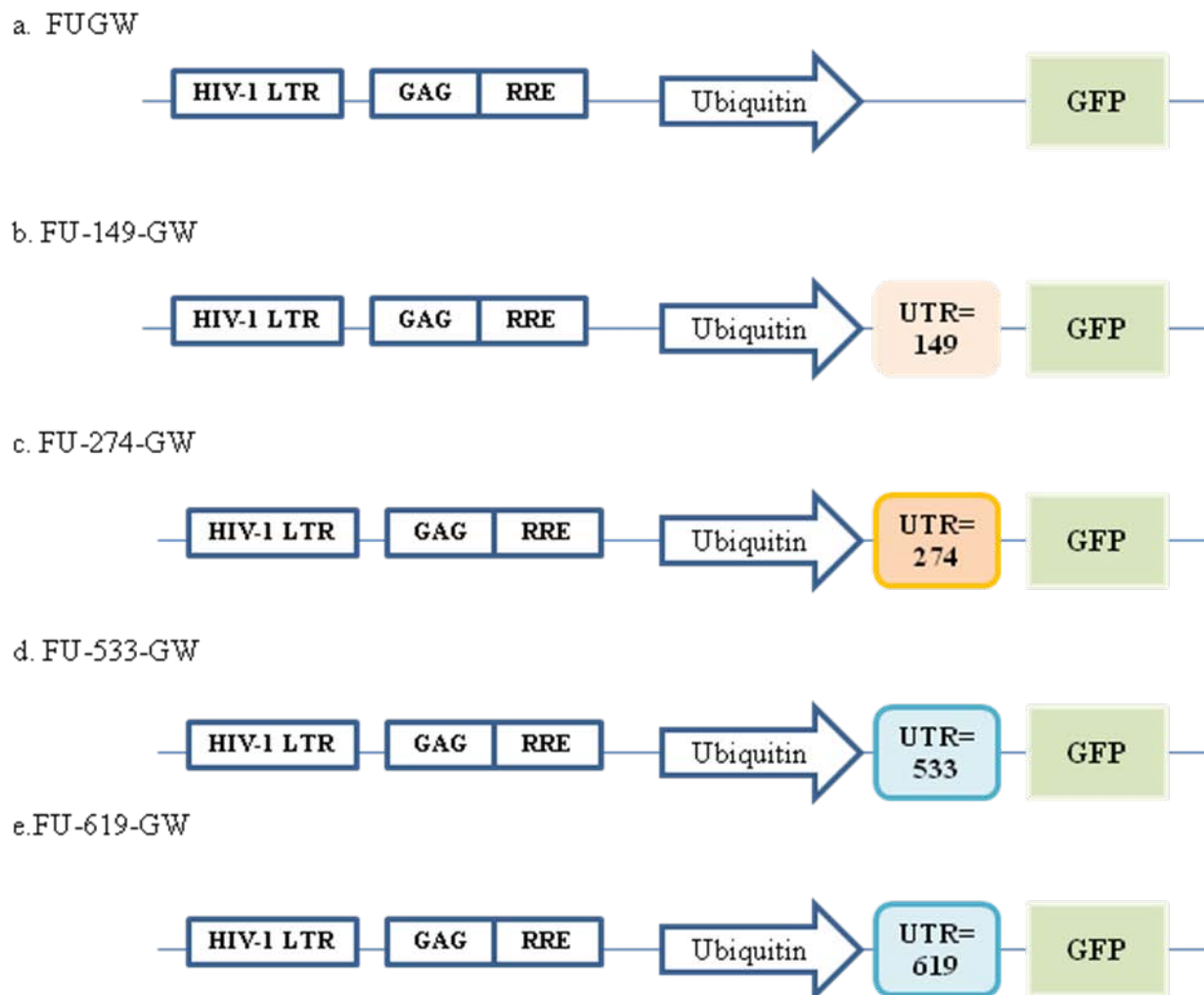


Figure 4. Schematic diagram of lentiviral transfer plasmids used in to make lentivirus in this study.

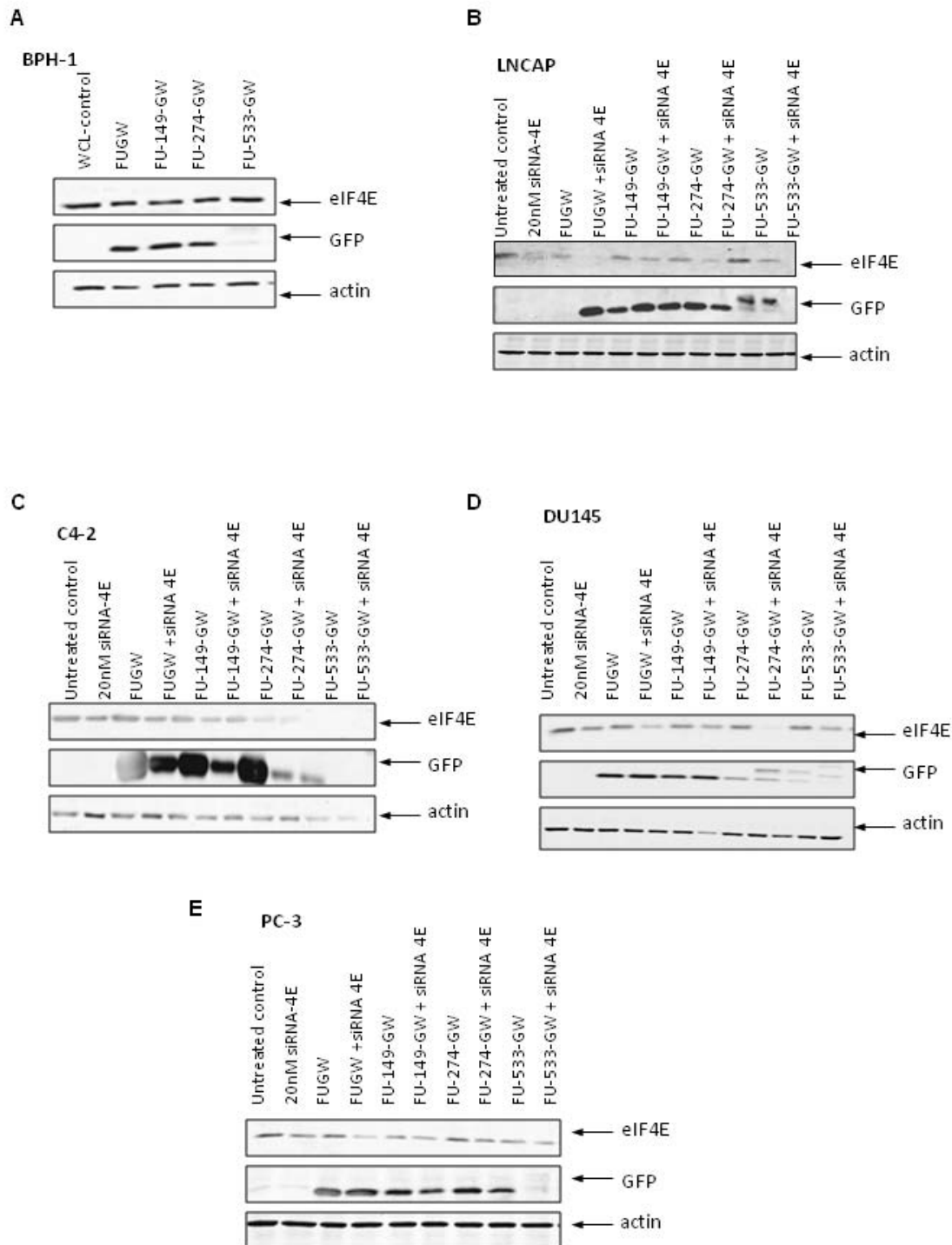


Figure 5. Western blot analysis of GFP levels after transfection with lentiviral plasmids containing different lengths of 5' UTR in presence or absence of siRNA against eIF4E. The above are representative blots of $n=3$ separate trials. (A) BPH-1 (B) LNCaP, (C) C4-2, (D) DU145 and (E) PC-3.

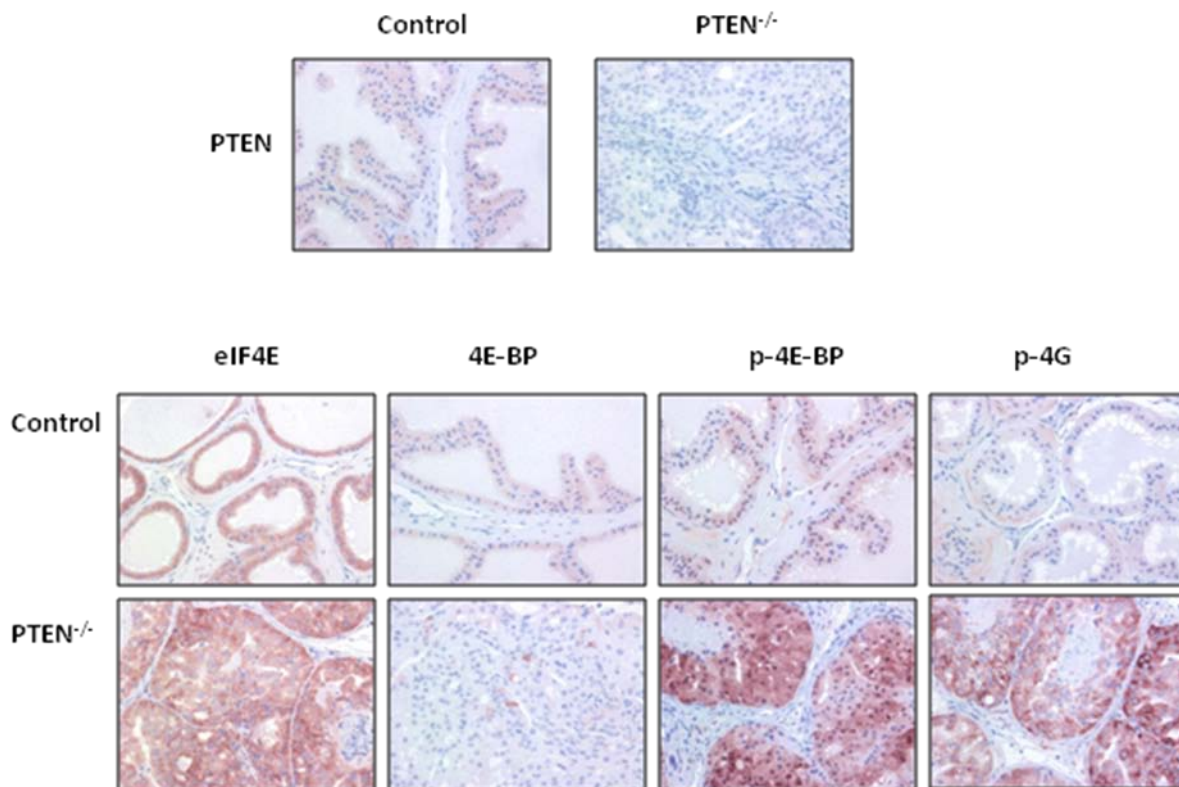


Figure 6. Prostate tissues of PTEN^{-/-} mice is compared with control PTEN^{+/+} mice. Tissue immunohistochemistry demonstrates distribution of eIF4E and its family of transcription regulatory proteins.

CONCLUSION:

1. There is a direct correlation between increased viral titer and decreased cell survival in PTEN^{-/-} derived cell lines.
2. Mutated M protein in VSV can restrict its infectivity to cancer cells with a malfunctioning interferon (IFN) response.
3. VSV (AV3) infection and replication in PTEN^{-/-} mice is higher than that of non tumor-bearing control mice, with an amplification of viral load detected between 24-48 h in PTEN^{-/-} mice.
4. VSV (AV3) infection leads to increase cell death in prostates of tumor bearing PTEN^{-/-} mice while little cell death is observed in prostates of control mice.
5. Cell death mediated by VSV (AV3) in PTEN^{-/-} mice is not a byproduct of neutrophil infiltration.
6. Cell death mediated by VSV(AV3) in PTEN^{-/-} mice is due to a faulty IFN response in these mice.
7. eIF4E transcription regulatory protein is over-expressed in cancer (both *in vitro* and *in vivo*).
8. Lentivirus with different length 5'UTR were constructed.
9. Lentivirus containing ODC 5'UTR (274) and FGF-2 (533) were shown to need eIF4E for expression. Both FU-274-GW and FU-533-GW were overall expressed in presence of eIF4E in various prostate cancer cell lines. However, their expression was markedly reduced in cells which had low or knocked down levels of eIF4E. .

Future Direction:

1. The limitation of prostate specific PTEN null mouse model is that it has very low levels of metastatic lesions reported. Therefore to see whether VSV (AV3) is capable of finding metastatic cells, we purpose to look at a more aggressive prostate cancer tumor model. The transgenic adenocarcinoma of the mouse prostate (TRAMP) has high frequency of metastatic lesions particularly to lungs and pelvic lymph nodes.
2. Further investigation *in vivo* of which 5' UTR is best suited for tumor specific expression will be tested in PTEN^{-/-} mice.

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