Changes in susceptibility to oncolytic vesicular stomatitis virus during progression of prostate cancer

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Abstract

A major challenge to oncolytic virus therapy is that individual cancers vary in their sensitivity to oncolytic viruses, even when these cancers arise from the same tissue type. Variability in response may arise due to differences in the initial genetic lesions leading to cancer development. Alternatively, susceptibility to viral oncolysis may change during cancer progression. These hypotheses were tested using cells from a transgenic mouse model of prostate cancer infected with vesicular stomatitis virus (VSV). Primary cultures from murine cancers derived from prostate-specific Pten deletion contained a mixture of cells that were susceptible and resistant to VSV. Castration-resistant cancers contained a higher percentage of susceptible cells than cancers from noncastrated mice. These results indicate both susceptible and resistant cells can evolve within the same tumor. The role of Pten deletion was further investigated using clonal populations of murine prostate epithelial (MPE) progenitor cells and tumor-derived Pten−/− cells. Deletion of Pten in MPE progenitor cells using a lentivirus vector resulted in cells that responded poorly to interferon and were susceptible to VSV infection. In contrast, tumor-derived Pten−/− cells expressed higher levels of the antiviral transcription factor STAT1, activated STAT1 in response to VSV, and were resistant to VSV infection. These results suggest that early in tumor development following Pten deletion, cells are primarily sensitive to VSV, but subsequent evolution in tumors leads to development of cells that are resistant to VSV infection. Further evolution in castration-resistant tumors leads to tumors in which cells are primarily sensitive to VSV.
There has been a great deal of progress in the development of replication-competent viruses that kill cancer cells (oncolytic viruses). However, a major problem is that individual cancers vary in their sensitivity to oncolytic viruses, even when these cancers arise from the same tissue type. The experiments presented here were to determine whether both sensitive and resistant cells are present in prostate cancers originating from a single genetic lesion in transgenic mice, prostate-specific deletion of the gene for the tumor suppressor Pten. The results indicate that murine prostate cancers are composed of both cells that are sensitive and cells that are resistant to oncolytic vesicular stomatitis virus (VSV). Furthermore, androgen deprivation led to castration-resistant prostate cancers that were composed primarily of cells that were sensitive to VSV. These results are encouraging for the use of VSV for the treatment of prostate cancers that are resistant to androgen deprivation therapy.
Introduction

There has been a great deal of progress in development of new replication-competent viruses that kill cancer cells (oncolytic viruses), in understanding their mechanisms of oncolysis, and in their advancement to clinical trials (1-3). The key biological underpinning of oncolytic virus therapy is that activation of proliferative signaling pathways in cancer cells often leads to down-regulation of antiviral pathways, making cancer cells more susceptible to virus infection than normal cells (4-7). Vesicular stomatitis virus (VSV) is a well-established example of a highly cytolytic virus with a tropism for cancers that have down-regulated their antiviral responses (5). Our laboratory and others have made a variety of genetic modifications to enhance the selectivity of VSV for cancers versus normal tissues (7-14). For example, viruses with mutations in the viral M protein, which is responsible for suppressing host antiviral responses, are defective in their ability to invade normal tissues (15, 16), but effectively infect cancers that are defective in their antiviral responses (3). Genetically engineered VSV is currently in a phase I clinical trial for localized treatment of hepatocellular carcinoma (2). However, one of the major challenges to oncolytic virus therapy is that individual cancer cell lines vary dramatically in their sensitivity to oncolytic viruses, even when these cancers arise from the same tissue type (7, 13, 17-22). The experiments presented here address the origin of these differences in susceptibility to oncolytic VSV among prostate cancers.

Human prostate cancer develops as normal prostate epithelium acquires a series of mutations and epigenetic changes that lead to invasive adenocarcinoma of the prostate (23). Further mutations lead to development of metastatic prostate cancer that
spreads to other organs. For patients with localized prostate cancer, radiation therapy and/or radical prostatectomy typically achieve > 90% disease-free survival within five years (24, 25). However, treatment for metastatic disease is less effective (26). Since androgenic effects are important for growth of normal and malignant prostatic cells, androgen deprivation therapy was developed to control prostate cancer growth. Nonetheless, nearly all men with metastatic prostate cancer eventually develop castration-resistant disease after treatment with androgen deprivation, such that the cancer cells continue to proliferate in the presence of low levels or absence of androgen (26). Patients who present with castration-resistant disease typically have poor prognosis, even with recent improvements in therapy for this disease (26). These patients would be candidates for oncolytic virus therapy.

Individual prostate cancer cell lines vary dramatically in their sensitivity to VSV. Some prostate cancer cell lines, such as LNCaP, have substantial defects in their antiviral responses and are highly susceptible to oncolysis by VSV (13, 17). Other cell lines, such as PC3, retain their ability to mount an antiviral response, and have constitutively high levels of expression of antiviral genes that render them more resistant to VSV than normal prostate epithelial cells (17, 27). The variability in response of prostate cancer cells to VSV infection may arise due to differences in the initial genetic lesions leading to prostate cancer development. Alternatively, susceptibility to VSV oncolysis may change during cancer progression. Human prostate cancers are heterogeneous in histologic architecture, immunophenotype and genetic diversity (28). This also raises the possibility that susceptible and resistant cells may coexist in the same tumor. These hypotheses were tested using primary prostate
epithelial cell cultures from transgenic Pb-Cre4 x \( Pten^{\text{loxP/loxP}} \) mice, in which prostate
tumors develop from a single genetic lesion, deletion of the \( Pten \) gene (29).

Results presented here show that primary cultures from murine cancers derived
from prostate-specific \( Pten \) deletion contained a mixture of cells that were susceptible
and resistant to VSV. Furthermore, castration-resistant cancers contained a higher
percentage of susceptible cells than cancers from noncastrated mice. These results
indicate that both susceptible and resistant cells can evolve within the same tumor due
to heterogeneity in tumor evolution. The role of \( Pten \) in sensitivity to VSV infection was
further investigated using clonal populations of murine prostate epithelial (MPE)
progenitor cells and tumor-derived \( Pten^{-/-} \) cells. Deletion of \( Pten \) in MPE progenitor
cells using a self-deleting lentivirus vector resulted in cells that were poorly responsive
to interferon (IFN) stimulation, and were susceptible to VSV infection. In comparison,
tumor-derived \( Pten^{-/-} \) cells constitutively expressed higher levels of the antiviral
transcription factor STAT1, activated STAT1 in response to VSV infection, and were
correspondingly resistant to VSV infection. These results suggest that in the early
stages of tumor development following \( Pten \) deletion, cells destined to become prostate
cancer are primarily sensitive to VSV, but subsequent evolution in tumors leads to
development of cells that are resistant to VSV infection. Further evolution in castration-
resistant tumors leads to tumors composed primarily of cells that are sensitive to VSV
oncolysis.
Materials and Methods

Mice. Prostate-specific Pten 
-/- mice were generated by crossing Pten 

Loxp/Loxp (Pten 

L/L) mice with mice of the ARR2Probasin-Cre transgenic line PB-cre4, in which expression of the Cre recombinase is under the control of a modified rat prostate-specific probasin promoter, as previously described (30, 31). Pten 

L/L mice backcrossed to C57BL/6 mice (32) were a generous gift from Dr. Yong Chen (Wake Forest School of Medicine). DNA was acquired via tail snipping for genotyping. Castration procedure was performed on prostate-specific Pten 

+/- mice at 3 months of age under isoflurane anesthesia. Recovery from the procedure was carefully monitored. All animals were maintained in an isolated environment. Animal care was conducted in compliance with the state and federal Animal Welfare Acts and the standards and policies from the U.S. Department of Health and Human Services. The protocol was approved by the Institutional Animal Care and Use Committee of Wake Forest School of Medicine.

Cells and Viruses. Primary cultures of epithelial cells were derived from prostates of normal Pten 

L/L, tumor-bearing Pten 

+/- and castration-resistant tumor-bearing Pten 

+/- mice at 3 and 6 months of age. The culture method was a modification of that described by Nandi et al. for primary cultures of mouse mammary epithelium (33) modified for prostate monolayer cultures (33, 34). Briefly, prostate lobes were microdissected and washed with HEPES-buffered saline (HBS) containing 5% penicillin/streptomycin. The tissues were minced using a sterile razor blade and digested for 2-3 hours using 225 U/mL of collagenase (type IV, Worthington). Digestion was considered complete when stroma was released from the organoids. The digested
tissue was layered onto a Percoll gradient, and the epithelial organoids were separated from the stromal cells and debris by gradient centrifugation (2000rpm, 20 mins). The fraction containing epithelial organoids was isolated and washed repeatedly. The organoids were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (1:1) containing 1% fetal bovine serum, 10% bovine serum albumin (Sigma), 10 ng/mL of cholera toxin (BD biosciences), 28 ug/mL of bovine pituitary extract (Hammond Cell Tech), 80 μg/mL of gentamycin, 8 μg/mL of insulin (Gibco), alpha tocopherol \((2.3 \times 10^{-6} \text{M})\), 5 μg/mL of transferrin (Sigma) and trace elements. The organoids were cultured in dishes coated with rat-tail collagen. Cell outgrowths from the organoids formed a confluent monolayer by day 6 of harvest and were used for experiments at passage 2 and passage 3.

Generation and culture of MPE progenitor cells from \(Pten^{L/L}\), acutely-deleted \(Pten^{-/-}\), and tumor-derived \(Pten^{-/-}\) were previously described (35) and maintained in the medium described above (34-36). Briefly, \(Pten^{L/L}\) cells were spontaneously immortalized from prostate epithelial cells of 8 week old \(Pten^{L/L}\) mice. Acutely-deleted \(Pten^{-/-}\) cells were derived by transducing \(Pten^{L/L}\) cells with a self-deleting lentivirus expressing cre recombinase to achieve \(Pten\) deletion \textit{in vitro} (37). Tumor-derived \(Pten^{-/-}\) cells were obtained by spontaneously immortalized prostate cells from 16 week old \(Pten^{-/-}\) mice.

The recombinant viruses, rwt and rM51R, as well as viruses encoding GFP and a palmitoylated form of dsRed were prepared in BHK cells as described (38).
Fluorescence microscopy and image analysis. Primary cells were seeded onto imaging plates coated with rat-tail collagen. Cells were infected with viruses that express GFP at a multiplicity of infection (MOI) of 10 for 16 hrs, were washed three times with phosphate-buffered saline (PBS), and were fixed with 4% paraformaldehyde at room temperature for 15 min. The cells were permeabilized with PBS containing 0.25% Triton X-100 (PBST) for 15 min. Cells were washed with PBS and blocked with 1% BSA in PBST for 30 mins. Cells were incubated with anti-pancytokeratin conjugated to phycoerythrin (Sigma) for 1 hr with constant agitation. Cells were then washed and incubated with 1 μg/mL of 4',6-diamidino-2-phenylindole (DAPI) to label cellular DNA.

High content images were acquired using a BD Pathway 855 instrument. Image analysis was performed using BD Attovision software. Briefly, a region of interest (ROI) was defined using the area occupied by DAPI fluorescence to denote single cells. For each ROI, fluorescence intensity in GFP and PE channels were determined. The number of cells that expressed both viral protein (GFP) and cytokeratin (PE) were expressed as a percentage of the total number of cells that expressed cytokeratin (PE).

Images shown are merged images of GFP, PE and DAPI channels.

Long term culture cells were seeded on 6 well tissue culture dishes. Cells were infected with VSV-GFP or VSV-dsRed at the multiplicity indicated in the figures. At 24 hrs postinfection, fluorescence and bright field images were captured using a Retiga EX 1350 digital camera (QImaging Corp) attached to a Nikon Eclipse TE 300 inverted microscope.
**Cell Viability Assay.** Cells were grown in 96 well tissue culture dishes until 70% confluent and were then infected with viruses at the MOI indicated in the figures. At various times postinfection, live cells were measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (cell proliferation kit 1, Roche Diagnostics).

**Metabolic Labeling.** Cells were grown in 6 well tissue culture dishes until 70-90% confluent and were then infected with viruses at MOI of 10. At the times postinfection indicated in the figures, cells were pulse-labeled for 20 mins using $^{35}$S-methionine at 100 $\mu$Ci/mL. Cells lysates were harvested in RIPA buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Radioactivity was analyzed by phosphorescence imaging (Typhoon FLA 9500, GE Healthcare Life Sciences).

**Immunoblotting Analysis.** Cells were seeded in 6 well tissue culture dishes until 70-90% confluent and were then mock or virus-infected at the indicated MOIs. At the indicated times postinfection, cell lysates were prepared in RIPA buffer containing 1 $\mu$M aprotinin, 1 $\mu$M leupeptin, 1 $\mu$M E-64 protease inhibitor, 500 $\mu$M AEBSF and 1X PhosSTOP (Roche Applied Sciences). Proteins were resolved by SDS-PAGE and were transferred on to 0.45 um polyvinyl difluoride (PVDF) membranes. The membranes were blocked for 1 hour in PBS containing 0.1% Tween 20 and 5% nonfat dried milk. Immunoblots were then probed with antibodies against the viral matrix (M) protein, phospho-STAT1 (tyrosine 701; Cell Signaling), or STAT1 (Cell Signaling). Phospho-
STAT1 was probed before total STAT1. Proteins were detected using enhanced chemiluminescence substrate (Thermo Scientific). The intensity of the bands was quantified using ImageJ software.

**IFN Responsiveness Assay.** Cells were pretreated with the concentrations of universal type I IFN (PBL interferon source) indicated in the figures. Cells were infected with virus at the indicated MOI. At 72 hours postinfection, cells were treated with MTT reagent and cell viability was determined as described above.

**Statistical Analysis.** Statistical analysis of two groups was performed using Student’s t-test. One way analysis of variance (ANOVA) was performed on data with two or more groups. Two way ANOVA was performed for all pairwise multiple comparisons. The Bonferroni post-test was performed for all multiple comparisons. Statistical analyses were performed with GraphPad Prism software.
Results

Infection of primary prostate tumor cells with VSV. The variability in response of prostate cancer cells to VSV infection may arise due to differences in the initial genetic lesions leading to prostate cancer development. Alternatively, susceptibility to VSV oncolysis may change during cancer progression. Furthermore, prostate cancers are heterogeneous in cellular phenotypes (28). This raises the possibility that susceptible and resistant cells may coexist in the same tumor. These hypotheses were tested using primary prostate epithelial cell cultures from transgenic Pb-Cre4 × \( Pten^{loxp/loxp} \) mice, in which prostate tumors develop from a single genetic lesion, deletion of the \( Pten \) gene (29).

Cells were cultured from mouse prostates according to the procedure described by Barclay et al., which promotes epithelial cell growth (34). Cells cultured from normal mouse prostates or prostate tumors were analyzed for cytokeratin expression to identify cells of epithelial origin (Fig. 1). Cells from normal \( Pten^{L/L} \) prostates, \( Pten^{-/-} \) prostate tumors, and castration resistant \( Pten^{-/-} \) prostate tumors were seeded at passage 2 or 3 onto imaging plates and labeled with DAPI as a marker for the nucleus, and immunolabeled using a pan-cytokeratin antibody as a marker of epithelial cells. Mouse embryo fibroblasts (MEFs) were used as a negative control for cytokeratin labeling. Fluorescence of individual cells was quantified using a high content cellular imaging system. 85-95% of the cultured prostate cells were positive for cytokeratin, indicating their epithelial origin. The fluorescence intensity among individual cells varied considerably, consistent with heterogeneity in cytokeratin composition among epithelial
cells and differential reactivity of the pan-cytokeratin antibody. In previously published data, these cells also express markers of both luminal and basal epithelium (34).

In order to determine whether cells derived from $Pten^{-/-}$ prostate tumors are susceptible to VSV infection, primary prostate cells from 3 month old prostate-specific $Pten^{-/-}$ mice and their littermate controls were infected with recombinant eGFP-expressing virus containing either a wt M protein (rwt-GFP virus) or an isogenic virus containing mutant M protein (rM51R-GFP virus) at MOI of 10 for 16 hrs. Cells were then fixed and stained with DAPI and anti-cytokeratin antibody. Fluorescence images of normal and tumor-derived cells infected with rwt-GFP are shown in Fig. 2A and B, respectively. The numbers of cells that express both GFP and cytokeratin were expressed as a percentage of total cytokeratin positive cells, and data from multiple experiments are shown in Fig. 2C.

Most of the normal $Pten^{+/+}$ prostate epithelial cells infected with rwt-GFP virus express GFP (approximately 75%). These results are similar to previous results indicating that normal human prostate epithelial cells are susceptible to VSV (13). In contrast, significantly fewer cells derived from $Pten^{-/-}$ prostate tumors (42%) expressed detectable GFP. The percentage of prostate cells that expressed detectable GFP from the M protein mutant virus (rM51R-GFP) was less than that of cells infected by rwt-GFP virus (Fig. 2D), likely due to the induction of antiviral responses in some of the cells by the M protein mutant virus. Nonetheless, the prostate epithelial cells infected with M protein mutant virus exhibited a similar trend in that fewer cells from $Pten^{-/-}$ tumors support GFP expression compared to normal $Pten^{+/+}$ prostate epithelial cells. These results indicate that even with a single defined initiating genetic lesion of $Pten$ deletion,
a heterogeneous response to VSV infection develops in prostate tumors during carcinogenesis.

In order to determine the susceptibility of castration resistant $Pten^{\Delta}$ prostate tumors to VSV infection, transgenic Pb-Cre4 x $Pten^{\text{loxp/loxp}}$ mice were castrated at 3 months of age, and cells were cultured from castration-resistant $Pten^{\Delta}$ prostate tumors at 6 months of age. Normal $Pten^{L/L}$ prostate cells and $Pten^{\Delta}$ prostate tumor cells from 6-month-old noncastrated mice served as controls. The cells were infected, stained, and imaged, and subjected to the same analysis as in Fig. 2. Shown in Fig. 3A and B are images of cells from castration-resistant $Pten^{\Delta}$ and 6 month old control $Pten^{\Delta}$ tumors following infection with rwt-GFP virus, and quantification of multiple experiments with rwt-GFP and rM51R-GFP viruses is shown in Fig. 3C and D, respectively. Most of the castration-resistant $Pten^{\Delta}$ prostate tumor cells expressed GFP similar to the results with normal $Pten^{L/L}$ cells. In contrast, the cells from control $Pten^{\Delta}$ mice at 6 months still consisted of a mixture of cells that were primarily resistant to VSV and support very little viral gene expression, similar to the results with 3 month old mice in Fig. 2. These results indicate that in the androgen-depleted environment of castrated mice, VSV-sensitive tumor cells are predominant over VSV-resistant tumor cells.

**Susceptibility to VSV in clonal populations of murine prostate epithelial progenitor cells and tumor-derived $Pten^{\Delta}$ cells.** The observation that $Pten^{\Delta}$ prostate tumors contain a mixture of VSV-sensitive and VSV-resistant cells suggests that resistance does not result from $Pten$ deletion alone but instead results from cellular changes during tumor progression. To further determine whether resistance to VSV develops from a single genetic lesion of $Pten$ deletion or from tumor progression, clonal
populations of mouse prostate epithelial cells in long-term culture were used. Murine prostate cells capable of long term growth are derived from primary cultures such as those in Figs. 1-3 after passage 20 or higher (34) and will be referred to here as murine prostate epithelial (MPE) progenitor cells. MPE progenitor cells bear markers of both luminal and basal epithelial cells, as well as stem cell markers, such as Sca-1 and CD49f, and have the key stem cell properties of self-renewal and multi-lineage differentiation (39). Deletion of the Pten gene in MPE progenitor cells was achieved by transducing Pten\textsuperscript{L/L} cells in vitro with lentivirus that expresses self-deleting Cre recombinase, and clonal populations of transduced and mock-transduced cells were derived as described (35). A clonal population of cells derived from tumors of 4 month old Pten\textsuperscript{-/-} mice was used to model tumor-derived Pten\textsuperscript{-/-} cells (40). Pten\textsuperscript{-/-} cells resulting from lentivirus transduction are referred to as acutely deleted to distinguish them from tumor-derived Pten\textsuperscript{-/-} cells.

Control Pten\textsuperscript{L/L}, acutely deleted and tumor-derived Pten\textsuperscript{-/-} cells were infected with rwt virus that encodes either GFP or a palmitoylated version of the fluorescent protein dsRed at increasing MOIs. Similar results were obtained with either virus. Shown in Fig. 4 are representative bright-field and fluorescence images of cells at 24 hours postinfection with VSV-dsRed. Most control Pten\textsuperscript{L/L} cells and acutely deleted Pten\textsuperscript{-/-} cells expressed dsRed at MOI of 1 and nearly all expressed dsRed at MOI of 10 or 50. However, tumor-derived Pten\textsuperscript{-/-} cells expressed only low levels of dsRed independently of MOI. This result supports the conclusion that resistance does not result from Pten deletion alone but instead results from cellular changes during tumor progression.
The difference in viral gene expression in tumor-derived Pten<sup>−/−</sup> cells versus acutely deleted or normal prostate epithelial cells was confirmed by immunoblot analysis of viral M protein and by radiolabeling infected cells with <sup>35</sup>S-methionine. Control Pten<sup>L/L</sup>, acutely deleted and tumor-derived Pten<sup>−/−</sup> cells were mock-infected or infected with rwt virus. At 6 and 12 hours postinfection, cell lysates were prepared and immunoblotted for VSV M protein (Fig. 5A). Individual band intensities were quantified and expressed as a percentage of band intensity of control Pten<sup>L/L</sup> cells at 12 hours postinfection, and results from multiple experiments are shown in Fig. 5B. There was no significant difference in M protein expression between control Pten<sup>L/L</sup> and acutely deleted Pten<sup>−/−</sup> cells, whereas M protein expression in tumor-derived Pten<sup>−/−</sup> cells was only detectable in longer exposures and was only about 10% of that of control cells.

The rates of viral protein synthesis were determined by pulse-labeling infected cells with <sup>35</sup>S-methionine at 6, 12, and 24 hours postinfection. Cell lysates were analyzed by SDS-PAGE and phosphorescence imaging. Shown in Fig. 5C are representative phosphoimages from vector control Pten<sup>L/L</sup> and acutely deleted Pten<sup>−/−</sup> cells, and in Fig. 5D, images from control Pten<sup>L/L</sup> and tumor-derived Pten<sup>−/−</sup> cells infected with rwt virus. Control Pten<sup>L/L</sup> and acutely deleted Pten<sup>−/−</sup> cells had a pattern of viral protein synthesis that is typical for permissive cell types, in which viral protein synthesis was detectable as early as 6 hours after infection, and continued from 12-24 hours after infection. However, synthesis of viral proteins in tumor-derived Pten<sup>−/−</sup> cells was barely detectable above the background of host protein synthesis.

Also apparent in Fig. 5C and D is the inhibition of host protein synthesis by VSV, in which labeling of host proteins visible in mock-infected cells declined during the
course of infection. The inhibition of host protein synthesis in tumor-derived Pten−/− cells was not notably different from that in control cells, suggesting that the low level of M protein expression in these cells was sufficient to inhibit host protein synthesis. For example, the amount of M protein required to inhibit host gene expression in permissive cells is 100- to 1000-fold less than that produced during virus infection (41).

**Susceptibility to VSV-induced cell death in MPE progenitor cells and tumor-derived Pten−/− cells.** VSV infection leads to cell death by multiple pathways, some of which are dependent on the ability of M protein to inhibit host gene expression, and others of which are independent of M protein’s inhibitory activity (42). These two classes of mechanisms can be distinguished by comparing cell death induced by virus with wt M protein (rwt virus) to that of M protein mutant virus (rM51R virus). Control PtenL/L, acutely deleted, and tumor-derived Pten−/− cells were infected with rwt or rM51R virus at MOI of 10. At various times after infection, cells were incubated with MTT reagent. Cell viability was expressed as a percentage of mock-infected cells at that time point (Fig. 6).

Most control PtenL/L and acutely deleted Pten−/− cells were killed by either virus by 36-48 hours postinfection (Fig. 6A, B). At 12 and 24 hours postinfection, the acutely deleted Pten−/− cells had significantly more viability than control PtenL/L cells. This may be due to more active survival signaling in Pten−/− cells. Tumor-derived Pten−/− cells were more resistant to virus-induced cell death than either PtenL/L or acutely deleted Pten−/− cells, with about 50% of tumor-derived Pten−/− cells remaining viable by 48 hours postinfection with rwt virus (Fig. 6C) and about 75% remaining viable after infection with rM51R virus (Fig. 6D). Some of the resistant cells continue to divide following infection with VSV (unpublished data), which may account for the transient increase in viability observed at
36 hours. Changes in the rate of MTT metabolism in individual cells could also contribute to this effect.

The difference in responses of tumor-derived Pten−/− cells was even more striking in multiple cycle infections (MOI of 0.1, Fig. 7). PtenL/L or acutely deleted Pten−/− cells were largely nonviable by 48 hours postinfection with either rwt or rM51R virus (Fig. 7A, B), indicating that virus had spread throughout the culture from the initially infected cells. In contrast, tumor-derived Pten−/− cells remained viable throughout the time course of infection with either virus.

Antiviral signaling through STAT1 in MPE progenitor cells and tumor-derived Pten−/− cells. One of the hallmarks of human prostate cancer cells that are relatively resistant to VSV, such as PC3 cells, is that they have constitutively high levels of expression of antiviral gene products, such as the IFN-responsive transcription factor STAT1, and that STAT1 is activated by phosphorylation in cells infected with wild-type VSV, which normally suppresses IFN production and STAT1 activation in most cell types (17). To determine whether this is also the case for murine tumor-derived cells, control PtenL/L, acutely deleted, and tumor-derived Pten−/− cells were analyzed for STAT1 expression and activation by phosphorylation by immunoblots. Cells were mock-infected or infected with rwt or rM51R virus at MOI of 10, and cell lysates were prepared at 6 and 12 hours postinfection. Lysates were analyzed by immunoblots using antibodies against phospho- or total STAT1 (Fig. 8). Mock-infected tumor-derived Pten−/− cells constitutively expressed much higher levels of STAT1 than PtenL/L or acutely deleted Pten−/− cells (Fig. 8A, C). Furthermore, STAT1 was phosphorylated in response to infection of tumor-derived cells with rwt virus (Fig. 8A, B), likely due to the production
of type I IFNs by these cells. In contrast, there was little if any phosphorylation of STAT1 in Pten\textsuperscript{L/L} or acutely deleted Pten\textsuperscript{-/-} cells infected with rwt virus (Fig. 8A, B), due to the inhibitory activity of the wt M protein, which prevents IFN production in most cell types.

All three cell types responded to infection with M protein mutant virus by phosphorylation of STAT1 (Fig. 8D, E), consistent with the induction of type I IFN by this virus (43). The levels of phospho-STAT1 at 6 hours postinfection differed among the three cell types in the order tumor-derived Pten\textsuperscript{-/-} cells > Pten\textsuperscript{L/L} cells > acutely deleted Pten\textsuperscript{-/-} cells. Also notable in Fig. 8D and F was the increase in total STAT1 expression following infection with rM51R virus, which is consistent with the positive feedback induction of STAT1 expression induced by type I IFNs. Again the three cell types differed in the magnitude of this effect in the order tumor-derived Pten\textsuperscript{-/-} cells > Pten\textsuperscript{L/L} cells > acutely deleted Pten\textsuperscript{-/-} cells.

**IFN responsiveness of Pten\textsuperscript{L/L} and Pten\textsuperscript{-/-} MPE progenitor cells.** The differential response of phospho-STAT1 in Pten\textsuperscript{L/L} versus acutely deleted Pten\textsuperscript{-/-} cells infected with rM51R virus in Fig. 8D and E suggested that the acutely deleted cells might be less responsive to type I IFN than control Pten\textsuperscript{L/L} cells. This hypothesis was tested in the experiments in Fig. 9 by pretreating the cells with the indicated doses of type I IFN for 16 hours. The cells were then infected with rwt virus at MOI of 10, and cell viability was determined at 72 hours. Control Pten\textsuperscript{L/L} cells responded to IFN in a dose-dependent manner and were protected from rwt virus-induced cell death at the high dose. In contrast, acutely deleted Pten\textsuperscript{-/-} cells were susceptible to rwt virus infection even at high doses of IFN, indicating that deletion of Pten inhibits the IFN responsiveness of these cells.
Discussion

VSV is an attractive candidate oncolytic virus. To date, the virus has been tested as an oncolytic agent against a variety of cancer types, including glioblastomas, head and neck cancers, pancreatic adenocarcinomas, hepatocellular carcinomas, colorectal cancers, and breast and prostate cancers, to name a few (17, 20, 44-46). Even more excitingly, a recombinant VSV has recently moved to a phase I clinical trial for treatment of patients with hepatocellular carcinoma (http://clinicaltrials.gov/ct2/show/NCT01628640?term=vesicular+stomatitis+virus&rank=1). However, the common trend observed throughout the literature is that some cancer cell lines are susceptible to VSV oncolysis, whereas others are resistant, despite originating from the same tissue type. The basis for this diversity in response among cancers could be due to two general classes of mechanisms. One possibility is that different initiating mutations cause cancers to evolve through different paths such that one type of initiating mutation leads to sensitivity whereas a different mutation leads to resistance. Alternatively, heterogeneity in the tumor microenvironment may lead to development of some cells that are sensitive to viral oncolysis and others that are resistant, even within the same tumor. The results presented here show that this second class of mechanism is responsible for the heterogeneity in responsiveness among prostate cancer cells originating from a single initiating mutation, deletion of the \textit{Pten} gene.

The data presented here together with earlier data support a model for the progression of susceptibility versus resistance in prostate cancers shown in Fig. 10. In primary cultures of murine prostate epithelial cells, most of the cells derived from normal
prostate epithelium were quite susceptible to VSV infection (Fig. 2, 3). Likewise, in murine clonal cell populations, normal MPE progenitor cells were sensitive to VSV infection (Figs. 4 – 7). VSV replication in normal murine prostates has been observed following intraprostatic inoculation in vivo (47, 48). However, the virus was quickly cleared in vivo, and there did not appear to be damage to the normal prostatic epithelium. This is likely due to the responsiveness of the normal epithelial cells to type I interferons (Fig. 9, 10a) and other antiviral cytokines produced by immune cells. These results are similar to results with human cells, in which normal human prostate epithelial cells are also susceptible to VSV, but mount antiviral responses in response to type I IFN that can protect them from VSV cytolysis (13).

Similar to normal MPE progenitor cells, acutely-deleted Pten -/- cells were susceptible to VSV infection. However, in contrast to normal MPE cells, acutely-deleted Pten -/- cells were poorly responsive to IFN stimulation (Figs. 4 - 7, 9). This suggests that in the early stages of tumor development, cells destined to become prostate cancer are primarily sensitive to VSV (Fig. 10b). Defects in the response to IFNs can also account for the observations of Moussavi et al. that virus replication in VSV-susceptible cells in Pten -/- tumors in vivo was greater than in normal prostates (47), suggesting that some of the cells in the tumors are defective in their antiviral responses. Together, these studies support the idea that loss of Pten can decrease the protective effect of IFN against VSV infection. This is likely due to crosstalk between the pathways activated by Pten deletion and the pathways activated by IFNs. For example, these pathways interact at the level of glycogen synthase kinase-3β (GSK-3β). GSK-3β is a serine/threonine kinase whose activity enhances STAT1 activation by inhibiting Src.
homology-2 domain-containing phosphatase 2 (SHP2) (49). GSK-3β is inhibited through phosphorylation by AKT following loss of Pten. Further, activation of AKT and inhibition of GSK-3β activity following Pten loss leads to downregulation of STAT1 (50). This could be one mechanism through which loss of Pten may serve to downregulate antiviral responses.

Despite the suppressive effects on the antiviral response of acute deletion of Pten, by the time prostate cancers were well established in Pten−/− mice at 3 months of age, most of the prostate cancer cells were more resistant to VSV than normal prostate epithelial cells (Figs. 2, 3, 10c). Similarly, a clonal population of tumor-derived Pten−/− cells was much more resistant to VSV infection than normal MPE progenitor cells (Figs. 4-7). These results are similar to results with human cells, in which normal human prostate epithelial cells are also susceptible to VSV, whereas some human prostate cancer cell lines such as PC3 are more resistant than normal cells (13). The resistance of PC3 cells is due to delays in several early steps in VSV replication that appear to be mediated by constitutive expression of antiviral genes, as well as robust responsiveness to type I interferons (17, 27). Similarly, murine tumor-derived Pten−/− cells expressed high levels of the antiviral transcription factor STAT1, which was activated upon virus infection, even with wt VSV, which normally suppresses interferon synthesis in most cell types (Fig. 8).

Loss of Pten does not account for the difference in susceptibility between VSV-sensitive LNCaP and VSV-resistant PC-3 human prostate cancer cells, as neither of these two cell lines have functional Pten protein, and both have constitutively active AKT pathway (51). Similarly, as shown here, a clonal population of tumor-derived Pten−/−
/- cells exhibited resistance to VSV infection (Figures 4-7), whereas another cell line derived from murine Pten^-/- tumors was shown to be susceptible to VSV (47). Together, these data are consistent with the idea that evolution within tumors can change the susceptibility to VSV infection in some of the cells by promoting antiviral gene expression.

What could be the cause of the changes that must have occurred during tumor evolution that enhance resistance to oncolytic VSV? Whereas activated AKT inhibits antiviral gene expression through inhibition of GSK-3β, activated AKT can also interact with the IFN response pathway at other levels to give a completely different phenotype, a phenotype that promotes IFN responsiveness and antiviral defense. For example, one downstream effector of AKT is protein kinase C-δ (PKC-δ). PKC-δ is activated by treatment with type I IFNs and associates with STAT1 to phosphorylate and activate STAT1 (reviewed in (52, 53)). Another downstream substrate of AKT1 is EMSY, a transcriptional repressor that in conjunction with other binding partners represses transcription of IFN-stimulated genes (54). AKT1 phosphorylates EMSY and removes this repressive activity from ISREs, which allows transcription of antiviral genes (54). Thus, activation of the AKT pathway can also have antiviral effects.

A key result presented here is that castration-resistant Pten^-/- tumor cells are primarily susceptible to virus infection (Figs. 3, 10d). This is important because men with castration-resistant disease would be the target population for oncolytic virus therapy. At least two possibilities that are not mutually exclusive could contribute to this effect. One possibility is that cells that are IFN-responsive and resistant to VSV-infection are more sensitive to effects of androgen withdrawal. As a result, the cells that remain and
undergo expansion are castration-resistant and susceptible to virus. Alternatively, androgen withdrawal induces death of tumor cells irrespective of their IFN-responsiveness and sensitivity to virus infection. However, the progression of surviving cells to castration resistance is accompanied by downregulation of antiviral genes. Both of these scenarios suggest a relationship between androgen signaling and antiviral response. Some castration-resistant tumors develop independence from androgen receptor (AR) signaling, such as appears to be the case of PC3 cells. However, in many castration-resistant prostate cancers, AR activation and signaling remains sustained through a variety of mechanisms (reviewed in (55)), such as amplification of AR gene copy number, gain of function mutations in the AR gene, and alternative splice isoforms that encode constitutively active AR variants. Activation of AR inhibits IFN-responsiveness and silencing AR stimulates IFN-induced gene expression (56), suggesting that androgen signaling and IFN signaling may be antagonistic. In a system in which AR signaling predominates, IFN responses may be attenuated whereas cancers that have little or no AR signaling may retain IFN responsiveness.

The observation that prostate cancers resulting from Pten deletion consist of a mixture of cells that are susceptible to VSV and cells that have developed resistance is an important result that can account for the wide variation in susceptibility among prostate cancer cell lines. The presence of cells in prostate cancers that are resistant to virus infection does not imply that virus will be ineffective. It is well established that the antitumoral effects of oncolytic viruses in vivo involve additional mechanisms besides direct viral oncolysis (reviewed in (2)). These mechanisms include disruption of the tumor vasculature and induction of antitumor immunity. Also, it may be possible to
enhance the susceptibility of cancer cells that are initially resistant to oncolytic viruses by treatment with pharmacologic agents that suppress antiviral gene expression (27, 57). That being said, cancers with a large percentage of resistant cells are not as likely to be responsive to oncolytic virus therapy as those with a large percentage of susceptible cells. In this respect, the observation that castration increases the percentage of VSV-susceptible cells in Pten−/− prostate cancers is encouraging for the use of oncolytic VSV for the treatment of prostate cancers for which androgen ablation therapy has failed.
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50. Tseng PC, Huang WC, Chen CL, Sheu BS, Shan YS, Tsai CC, Wang CY, Chen SO, Hsieh CY, Lin CF. 2012. Regulation of SHP2 by PTEN/AKT/GSK-


Figure 1. Primary cultures of mouse prostate epithelial cells express cytokeratins. Primary cultures of mouse prostate epithelial cells were cultured from normal Pten\textsuperscript{L/L} prostates, Pten\textsuperscript{-/-} prostate tumors, and castration resistant Pten\textsuperscript{-/-} prostate tumors. Cells were seeded into imaging plates and labeled with DAPI (blue) as a marker for cellular DNA, and immunolabeled for cytokeratin (red) as a marker for epithelial cells. Using the DAPI label to define the region of interest to denote single cells, the percentages of cells immunolabeled with cytokeratin were determined and shown in the graph. Murine embryonic fibroblasts (MEFs) were used as a negative control for cytokeratin immunolabeling.

Figure 2. Prostate tumors contain a mixture of resistant and susceptible cells. Primary cultures of prostate epithelial cells were established from Pten\textsuperscript{-/-} prostate tumors of 3 month old mice and their littermate normal Pten\textsuperscript{L/L} controls. Cells were seeded into imaging plates and were infected with rwt-GFP or rM51R-GFP viruses (green). Cells were fixed, permeabilized, and immunolabeled for cytokeratin (red) and stained with DAPI to label cellular DNA (blue), then analyzed with a high content imaging system. Representative fluorescence images are shown in A and B. The number of cells that were positive for GFP and cytokeratin were expressed as a percentage of total cytokeratin positive cells (C and D). Data shown are average of at least 3 experiments +/- SEM.
Figure 3. Castration resistant tumor cells are primarily susceptible to VSV infection. 
Pten⁻/⁻ mice were castrated at 3 months of age and by 6 months of age had developed castration resistant tumors. Primary cultures of prostate epithelial cells were established from Pten⁻/⁻ prostate tumors of 6 month old mice, castration resistant tumors, and their littermate normal Pten⁺/⁺ control prostates. Cells were seeded into imaging plates and were infected with rwt-GFP and rM51R-GFP viruses (green). Cells were fixed, permeabilized, and immunolabeled for cytokeratin (red) and stained with DAPI to label cellular DNA (blue), then analyzed with a high content imaging system. Representative fluorescence images are shown in A and B. The number of cells that were positive for GFP and cytokeratin were expressed as a percentage of total cytokeratin positive cells (C and D). Data shown are average of at least 3 experiments +/- SEM.

Figure 4. Tumor-derived Pten⁻/⁻ cells support very little viral gene expression even at high MOI. Vector control Pten⁺/⁺ and acutely-deleted Pten⁻/⁻ (A) or non-transduced control Pten⁺/⁺ and tumor-derived Pten⁻/⁻ cells (B) were infected with rwt-dsRed virus at MOIs of 1, 10 and 50. Bright field and fluorescence images were obtained at 24 hrs postinfection. Images shown are representative images from 2 - 3 independent experiments.

Figure 5. Viral protein expression in control Pten⁺/⁺ cells and acutely-deleted Pten⁻/⁻ cells versus tumor-derived Pten⁻/⁻ cells. (A) Control Pten⁺/⁺, acutely-deleted Pten⁻/⁻ and tumor-derived Pten⁻/⁻ cells were infected with rwt virus at MOI of 10. Cell lysates were
harvested at 6 and 12 hrs postinfection and probed for viral matrix protein (M) protein expression by immunoblots. Representative immunoblots of at least 3 experiments are shown. (B) Viral matrix (M) protein expression was quantified and expressed as a percentage of M protein expression in control Pten<sup>L/L</sup> cells at 12 hpi. * = p < 0.05. (C) Vector control Pten<sup>L/L</sup> and acutely-deleted Pten<sup>+/+</sup> cells were infected with rwt virus at MOI of 10 or mock-infected (M). At 6, 12 and 24 hrs postinfection, cells were pulse-labeled with <sup>35</sup>S-methionine. Cell lysates were harvested and resolved on SDS-PAGE gels. Representative phosphoiimages are shown. Viral proteins L, G, N, P and M are indicated on the right. (D) Non-transduced control Pten<sup>L/L</sup> and tumor-derived Pten<sup>-/-</sup> cells were infected with rwt virus at MOI of 10 or mock-infected (M). At 6, 12 and 24 hrs postinfection, cells were pulse-labeled with <sup>35</sup>S-methionine and analyzed as in (C).

**Figure 6.** In a single cycle infection, tumor-derived Pten<sup>-/-</sup> cells are more resistant to virus-induced cell death than control Pten<sup>L/L</sup> and acutely-deleted Pten<sup>+/+</sup> cells. Vector control Pten<sup>L/L</sup> and acutely-deleted (A and B) or non-transduced control Pten<sup>L/L</sup> and tumor-derived Pten<sup>+/+</sup> cells (C and D) were infected with rwt (A and C) or rM51R (B and D) viruses at MOI of 10. Cell viability at the indicated times was determined using an MTT assay and expressed as a percentage of mock-infected cells. Data shown are averages of at least 3 experiments +/- SEM. * = p < 0.05.

**Figure 7.** In a multiple cycle infection, tumor-derived Pten<sup>-/-</sup> cells are highly resistant to virus-induced cell death. Vector control Pten<sup>L/L</sup> and acutely-deleted (A and B) or non-transduced control Pten<sup>L/L</sup> and tumor-derived Pten<sup>+/+</sup> cells (C and D) were infected with
rwt (A and C) or rM51R (B and D) viruses at MOI of 0.1. Cell viability was determined using MTT assay and expressed as a percentage of mock-infected cells. Data shown are average of at least 3 experiments +/- SEM. * = p < 0.05.

**Figure 8.** Tumor-derived *Pten*−/− cells constitutively express high levels of STAT1, which is phosphorylated in response to infection with VSV with either wt or mutant M protein. Control *Pten*+/−, acutely-deleted and tumor-derived *Pten*−/− cells were infected with rwt (A) or rM51R (B) viruses at MOI of 10. Cell lysates were harvested at the indicated times and probed for phospho- and total STAT1 expression. Shown here are representative immunoblots of at least 3 experiments. Levels of phospho- and total STAT1 were quantified and expressed as a percentage of levels in tumor-derived *Pten*−/− cells at 6 hpi. Data shown are average of at least 3 experiments +/- SEM. * = p < 0.05.

**Figure 9.** Acutely-deleted *Pten*−/− cells respond poorly to IFN stimulation. Control *Pten*+/− and acutely-deleted *Pten*−/− cells were pretreated with the indicated concentrations of IFN, then infected with rwt virus at MOI of 10. Cell viability was determined using an MTT assay at 72 hrs postinfection and expressed as a percentage of mock-infected cells. * = p < 0.05.

**Figure 10.** Model for development of both VSV-susceptible and VSV-resistant cells in prostate cancers resulting from *Pten* deletion.
A. Normal Pten<sup>L/L</sup>

<table>
<thead>
<tr>
<th>rwt-GFP + DAPI</th>
<th>CK + DAPI</th>
<th>rwt-GFP + CK + DAPI</th>
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B. Tumor-derived Pten<sup>-/-</sup>

<table>
<thead>
<tr>
<th>rwt-GFP + DAPI</th>
<th>CK + DAPI</th>
<th>rwt-GFP + CK + DAPI</th>
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C. rwt-GFP

D. rM51R-GFP

\[ p < 0.05 \]
A. Castration-resistant Pten\(^{-/-}\) tumor

rwt-GFP + DAPI  CK + DAPI  rwt-GFP + CK + DAPI

B. Six months Pten\(^{-/-}\) tumor (noncastrated control)

rwt-GFP + DAPI  CK + DAPI  rwt-GFP + CK + DAPI

C. rwt-GFP

D. rM51R-GFP

\(\text{GFP}^+ \text{ cells} \) (% of CK\(^+\) cells)

\[
\begin{align*}
Pten^{L/L} & : 80 \pm 5 \quad \text{p < 0.05} \\
Pten^{-/-} & : 40 \pm 5 \\
Pten^{-/-} \text{ castrated} & : 20 \pm 5
\end{align*}
\]

\[
\begin{align*}
Pten^{L/L} & : 80 \pm 5 \quad \text{p < 0.05} \\
Pten^{-/-} & : 40 \pm 5 \\
Pten^{-/-} \text{ castrated} & : 20 \pm 5
\end{align*}
\]