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Selective Killing of Smad4-Negative Tumor Cells Via A Designed Repressor Strategy

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Running Title: Smad4 dependent selective toxicity

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Abbreviations: GCV, ganciclovir; HSV-TK, herpes simplex virus thymidine kinase; DPC-4, deleted in pancreatic cancer locus 4; BMP, bone morphogenetic protein; MCS, multiple cloning site; RIPA, radioimmunoprecipitation assay

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ABSTRACT

Smad4 is a key tumor suppressor that is frequently deleted or inactive in pancreatic and colon tumors. In this report we describe an approach for attaining selective killing of Smad4 deficient tumor cells. Using a vector system involving a designed repressor with zinc finger binding domains and the HSV-TK 'suicide gene', we demonstrate Smad4 responsive regulation of HSV-TK expression and consequent altered susceptibility to the pro-drug ganciclovir (GCV). In pancreatic tumor cell lines stably transfected with the vector system, a robust differential of HSV-TK expression and GCV toxicity was attained depending on the presence or absence of co-transfected Smad4. In matched colon tumor cell lines lacking Smad4 or expressing physiological levels of Smad4, an Adenoviral version of the vector system attained a significant degree of preferential killing of Smad4 negative tumor cells in response to GCV. These findings demonstrate the possibility of achieving selective killing of pancreatic and colon cells depending on their Smad4 status.

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INTRODUCTION

A major goal for cancer therapeutics is to be able to selectively compromise the growth or function of tumors by exploiting key differences in the molecular machinery of normal and malignant cells. One approach to this goal has been to maximize tumor cell expression of therapeutic proteins, such as 'suicide enzymes' capable of activating prodrugs (Dachs et al., 2005; Scanlon, 2004). A variety of enzyme/prodrug combinations have been utilized including herpes simplex virus thymidine kinase (HSV-TK) with ganciclovir (GCV), *E. coli* cytosine deaminase with 5-fluorocytosine, and bacterial nitroreductase with an alkylating agent (Ichikawa et al., 2000; Lipinski et al., 2001; Sethi and Palefsky, 2003). Suicide enzyme expression is often designed to be regulated by promoters thought to be especially active in particular types of tumors (Gommans et al., 2006; Rein et al., 2006; Sadeghi and Hitt, 2005; Sethi and Palefsky, 2003); however, the regulation provided by tissue selective or tumor selective promoters can be imperfect in terms of the magnitude or specificity of suicide enzyme expression.

Another strategy for tumor selective gene regulation is based on the fact that many types of cancers have lost the expression or function of key tumor suppressor proteins (Vogelstein and Kinzler, 2004). For example, functional P53 is absent in over 50% of human tumors (Lane and Lain, 2002; Vousden and Prives, 2005). This has been exploited in the development of oncolytic adenoviruses that can preferentially replicate in cells where the P53 pathway is inactive; this strategy has already reached advanced clinical trials (Chu et al., 2004; Dobbelstein, 2004). Since P53 is a transcription factor, its presence in normal cells and absence in tumor cells can also be used to selectively affect suicide gene

expression. This approach has previously been employed by us (Xu et al., 2003) and others (Andreu et al., 2001; Lipinski et al., 2001) to attain selective toxicity to P53 deficient tumor cells.

Smad4/DPC-4 is a tumor suppressor that is abrogated in approximately 50% of human pancreatic cancers and approximately 20% of colon cancers (Schutte et al., 1996; Takayama et al., 2006; Thiagalingam et al., 1996). Smad4 is part of the TGF-β/Activin/BMP signaling pathway (ten Dijke and Hill, 2004) and forms complexes with R-Smads that lead to altered transcription of genes involved in growth control and tumor progression. In the nucleus, activated Smads interact directly with genes containing the recognition element 5'-CAGAC-3'. However, binding to a single such site is relatively weak and Smads mainly act in cooperation with other transcription factors (Derynck and Zhang, 2003), including Forkhead family, Runx family, AP-1, Sp1, bHLH, Homeodomain and others; depending on the identity and status of the interacting partners. Smads then recruit co-activator or co-repressor complexes to control gene expression.

Malignancies such as colon, and especially pancreatic, cancer are aggressive and devastating diseases and there is an urgent need for additional novel therapies (Bhattacharyya and Lemoine, 2006; MacKenzie, 2004). We have exploited the absence of Smad4 in certain pancreatic and colon tumor cells to attain selective killing of these cells via a suicide enzyme approach. We have employed a designed transcription factor (Blancafort et al., 2004; Falke and Juliano, 2003; Juliano et al., 2005) termed K25F that is comprised of two KRAB-A repressor domains and five DNA-binding zinc fingers (Bartsevich and Juliano, 2000; Xu et al., 2002). Expression of this repressor is driven by a Smad4-responsive promoter, while the repressor in turn binds a site that is upstream of the minimal promoter and coding region of the HSV-TK suicide gene. Thus the system is designed so that K25F is expressed and TK is consequently repressed in cells with normal Smad4 function, but in cells lacking Smad4, K25F is not expressed and HSV-TK is more abundantly produced, thus increasing sensitivity to the

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prodrug ganciclovir. We have used this system to elicit selective toxicity in pancreatic and colon tumor cells depending on their Smad4 status.

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MATERIALS AND METHODS

Cell lines: HEK293, its variant HEK293AD and Panc-1 cells were obtained from Lineberger Cancer Center tissue culture facility, University of North Carolina. HCT116 colon cells expressing endogenous Smad4, or with the gene deleted, were kindly provided by Dr. B. Vogelstein (Zhou et al., 1998). Pancreatic CFPac-1 and AsPC-1 cells were purchased from the American Type Culture Collection (Manassa, VA). HEK293, HEK293 AD and Panc-1 were cultured in Dulbecco's minimum essential medium with L-glutamine and HCT116 cells in McCoy's 5A medium. CFPac-1 cells were grown in Isocove's modified Dulbecco's medium with supplemented 4mM L-glutamine and 1.5 g/L sodium bicarbonate. AsPC-1 cells were cultured in RPMI1640 medium with L-glutamine and 4.5g/L glucose supplemented with 10mM HEPES, 1mM sodium pyruvate and 1.5 g/L bicarbonate. All the growth media were obtained from Invitrogen, Carlsbad, CA and supplemented with 10% fetal bovine serum unless specified otherwise.

Plasmid and Viral Constructs: For the construction of the HSV-TK expressing plasmid, a multiple cloning site (MCS) created by annealing two oligonucleotides consisting of the forward sequence 5'GATCTCTCGAGATGCGGATCACTAGTTGCAACGTCATATGGCTCATCAGGTACCCAG and the reverse sequence 5' CTGGGTACCTGATGAGCCATATGACGTTGCAACTAGTGATCGCATCTCGAGA, was introduced at BglII and PvuII sites of pRL-TKp-TK (Xu et al., 2002). This resulted in the construct pRL-TKp-TK-MIN. Two copies of K25F binding sequence were PCR amplified from the plasmid pRL-2xMDR1-TKp-TK (Xu et al., 2002) and inserted at XhoI and SpeI sites in the MCS of pRL-TKp-TK-MIN. Two additional K25F binding sites were amplified from 2xMDR1-TKp-TK and inserted at

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SpeI and NdeI followed by two more additions at NdeI and KpnI sites. This resulted in the plasmid pRL-6xMDR1-TKp-TK-MIN.

All the luciferase reporter plasmids and the K25F expressing plasmids were constructed using the promoterless reporter plasmid pGL3-basic vector (Promega, Madison, WI). The promoter of human IgA1 gene was a kind gift from Dr. S. Paschalis, Athens, Greece (Lars and Paschalis, 1993). The TGF-β responsive region between -247 and +79 was PCR amplified and subcloned at XhoI and HindIII of pGL3-basic vector resulting in the plasmid pGL3-IgA1-Luc. Mutations in the IgA1 promoter were introduced by using the QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutant sequences are shown in **Figure 2b**. The resulting constructs pGL3-IgA1-Sp1mut-Luc, pGL3-IgA1-Sp1-CREBmut-Luc and pGL3-IgA1-Sp1-CREB-Eboxmut-Luc had Sp1, Sp1 plus CREB, and Sp1, CREB and E-box mutated respectively.

To construct the Smad4 driven K25F expressing plasmid, the IgA1 promoter with a mutant Sp1 site was PCR amplified from pGL3-IgA1-Sp1mut-Luc and ligated at SacI and SmaI of the pGL3-basic vector. This was followed by the addition of the entire coding region of the protein K25F from the plasmid pcK25F (Bartsevich and Juliano, 2000) in two steps. First, the kozak sequence, nuclear localization sequence and the two KRAB domains were amplified in a single PCR and ligated at SmaI and XhoI, and subsequently the remaining 5 zinc fingers along with myc and 6xHis tags were added at the XhoI and XbaI sites. This resulted in the construct pGL3-IgA1-Sp1mut-K25F. Smad4 expressing plasmid pRK5-Smad4 was a kind gift from Dr. R. Derynck (University of California, San Francisco, CA).

The 6xMDR1 sites, minimum TK promoter, coding region of HSV-TK and 6xHis tag were all amplified in a single PCR from the plasmid pRL-6xMDR1-TKp-TK-MIN and introduced at MfeI and XhoI site of cDNA3.1(+)/Hygro (Invitrogen, Carlsbad, CA) resulting in the plasmid pcDNA3.1-TK-

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Hygro. The IgA1-Sp1mut-K25F fragment from the construct pGL3-IgA1-Sp1mut-K25F was amplified and ligated at FspI and XbaI of pBudCE4.1 (Invitrogen, Carlsbad, CA). This resulted in the replacement of the original CMV promoter by the IgA1 promoter. The resulting plasmid was called pBudCE4.1-K25F-zeo. The Smad4 cassette was amplified from pRK5-smad4 and ligated at a BstBI site of pBudCE4.1-K25F-zeo resulting in the construct pBudCE4.1-K25F-Smad4-zeo.

AdenoQuick cloning system (O.D.260 Inc, Boise, ID) was used as per manufacturer's instructions to construct the dual cassette Adenoviruses Ad-TK and Ad-TK-K25F. Briefly, the HSV-TK expression cassette was PCR amplified from pRL-6xMDR1-TKp-TK-MIN and subcloned into the shuttle vector pE1.2 at BglII and SalI sites. The K25F expressing cassette was amplified from pGL3-IgA1-Sp1mut-K25F and subcloned at PstI and SpeI sites of pE3.1. The resulting plasmids were digested with PflMI or DraIII and ligated with SfiI-digested AdenoQuick13.1. The ligated DNA was transferred into *E. coli* preferentially via packaging into phage λ. The resulting cosmids were linearized with PacI and transfected into helper HEK293 AD cells to obtain the viruses Ad-TK and Ad-TK-K25F. Purified plaques were sent to O.D.260 Inc for the preparation of viral stocks. The control Ad-GFP virus was obtained from the Vector Core facility of the University of North Carolina. Standard plaque assays were performed to quantify the number of infectious particles in the viral stock suspensions. Briefly, confluent HEK293 AD cells were infected with various dilutions of the virus at 37^{o}C for 2 hours, followed by overlaying with 0.8% agar mixture. Plaques were counted 10-15 days after overlaying.

Transient transfections and adenoviral infections: Transient transfections with supercoiled plasmid DNA in HEK 293 cells were performed by using Lipofectamine2000 according to manufacturer's instructions. Panc-1, CFPac-1 and AsPC-1 cells were transiently transfected with Fugene (Roche, Indianapolis, IN) as per manufacturer's instructions. Amounts of plasmid DNA used and the times

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allowed for protein expression are indicated in the respective figure legends. HCT116 cells were infected at a 1:40 cell to adenovirus infectious particle ratio.

Stable cell line production: HEK 293 and CFPac-1 cells were plated in 6-well plates (4.0×10^5 cells/well) and first transfected with BglII linearized pcDNA3.1-TK-hygro or pcDNA3.1-hygro, which encoded for HSV-TK and empty cassettes respectively. Twenty four hours after transfection, cells were re-plated onto 10 cm tissue culture plates. Another 24 h later, 200 μg/ml hygromycin was added to the medium. Three weeks later, individual hygromycin resistant clones were picked and pooled. These cells were referred to as 293-TK-hygro, 293-empty-hygro, CFPac-1-TK-hygro or CFPac-1-empty-hygro. Next, each of the above four pooled cell types were transfected with FspI linearized pBud-Empty-zeo, pBud-K25F-zeo and pBud-K25F-Smad4-zeo and maintained under the dual selection of 100 μg/ml hygromycin and 100μg/ml zeocin. Cells resistant to hygromycin and zeocin were recovered. The resulting cell sublines were referred to as 293-TK-hygro-empty-zeo, 293-TK-hygro-K25F-zeo, 293-TK-hygro-K25F-Smad4-zeo, CFPac-1-TK-hygro-empty-zeo, CFPac-1-TK-hygro-K25F-zeo, CFPac-1-TK-hygro-K25F-Smad4-zeo and similarly their corresponding empty cassette controls introduced in 293-empty-hygro and CFPac-1-empty-hygro.

Luciferase reporter assays: Cells were usually harvested 48 hours after transfection and activity determined using Luciferase assay kit (Promega, Madison WI). Measurements were performed on a Monolight 2010 instrument (Analytical Luminescence laboratory, San Diego, CA).

Western blotting and Immunoprecipitation: Transfected cells were lysed in modified RIPA. Protein concentration was measured using the BCA kit (Pierce, Rockford, IL). Lysates (20ug) were resolved on

SDS-PAGE and detected by Western blotting as described (Xu et al., 2002). Expressed HSV-TK was detected by using either a mouse monoclonal anti-his antibody (Covance, Berkeley, CA) at 1:1000 dilution or anti-HSV-TK polyclonal antibody, kindly provided by Dr. M. E. Black (Washington State University, WA), at 1:5000 dilution. K25F protein was detected using 1:1000 dilution mouse anti-myc monoclonal antibody 9E10 (Covance, Berkeley, CA). Endogenous Smad4 was detected by rabbit polyclonal H-552 antibody (Santacruz Biotechnology, Santa Cruz, CA). Smad4 overexpression was detected using murine anti-flag M2 monoclonal antibody (Sigma-Aldrich Co., St Louis, MO). Actin and Tubulin were detected using a rabbit anti-α-actin and mouse anti-β-tubulin antibodies respectively (Sigma-Aldrich Co., St Louis, MO) at dilutions of 1: 5000. The secondary antibodies used were HRP-conjugated bovine anti mouse IgG antibody (Santacruz, Biotechnology, Santa Cruz, CA) and HRP-conjugated goat anti rabbit IgG antibody (Calbiochem, San Diego, CA) at a dilution of 1:5000. Signals were detected by enhanced chemiluminescence (ECL kit, Amersham, Arlington Heights, IL). Immunoprecipitation was carried out using the Catch and Release v2.0 Reversible Immunoprecipitation System (Upstate Biotech/Millipore, VA), as per manufacturer's instructions. Proteins were visualized by Western blotting as described above.

Pharmacologic experiments: Cells were plated in 6-well plates (4.0×10^5 cells/well) and 24 h later cells from each well were split into 6 wells of a 6-well plate. Another 24 h later GCV was added at the concentrations indicated. Cell number was counted 4 days post GCV treatment using an electronic particle counter and plotted as % surviving cells as compared to control cells.

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RESULTS

Strategy and vector constructs: An overview of the strategy is shown in **Figure 1a**. In order to achieve the selective killing of tumor cells that lack the tumor suppressor Smad4, we constructed two main vectors. The first, pGL3-Sp1mut-IgA1-K25F (**Fig. 1b**), contains a Smad4 responsive promoter taken from the IgA1 gene (Lars and Paschalis, 1993) upstream of the coding region of the K25F designed repressor protein (Bartsevich and Juliano, 2000). This vector will thus express K25F in the presence of functional Smad4. The second vector, pRL6xMDR1-TKp-TK-MIN (**Fig. 1c**), contains six copies of a 15-base sequence, taken from the MDR1 promoter, that specifically binds K25F. These were placed upstream from a minimum HSV-TK promoter, which in turn is upstream from the coding sequence of HSV-TK. This vector will constitutively express HSV-TK, but can be repressed by the expression of K25F. Thus when both the vectors are present in a cell, one would expect high levels of HSV-TK expression in the absence of Smad4 and lower levels in its presence.

The current study is designed primarily to test the principle of Smad4-regulated therapeutic gene expression. However, this strategy may ultimately be developed as a gene therapy approach; thus we sought to express K25F and HSV-TK from a typical gene delivery vector (**Fig. 1d**). We chose a dual cassette adenoviral vector because of its high transgene capacity, good tissue tropism, possible utility in future animal experiments, and because it's large genome separates the two expression cassettes, thereby reducing any concerns about inhibitory chromatin effects (Thiel et al., 2004) within the vector caused by the designed repressor. This dual cassette vector could thus express either the K25F or HSV-TK proteins (or both) from their respective regulated promoters within a single adenoviral vector.

We pursued our strategy in a step-wise manner. The first experiments dealt with optimization of vectors for K25F and HSV-TK. The second series of experiments involved transiently transfecting

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HEK293 cells with various combinations of vectors having cassettes capable of regulated expression of Smad4, K25F and HSV-TK, and monitoring TK expression. The next series of experiments involved using drug resistance markers to select stable sublines of HEK 293 and pancreatic cancer cells that contained combinations of regulated cassettes for the three proteins and then similarly testing for TK expression and cell killing by GCV. Finally, we developed an Adenoviral vector that contained either HSV-TK alone or both K25F and HSV-TK cassettes and tested these in a matched pair of colon carcinoma cell lines that either expressed Smad4 from its normal chromosomal site, or where both copies of the Smad4 gene were deleted.

Optimization of promoters: Prior to the construction of the final vectors described above, the various elements in the plasmids were optimized. Earlier our laboratory had described the use of pRL-2xMDR1-TKp-TK as a K25F responsive vector (Xu et al., 2002). We sought to increase the repression of HSV-TK by K25F by increasing the number of K25F binding sites on pRL-2xMDR1-TKp-TK, and since the intent was to express these proteins from a viral vector, we were also interested in minimizing the size of each cassette being used. The region between 109 and 52 nucleotides upstream of the HSV-TK structural gene efficiently promotes transcription of this gene (McKnight et al., 1984). Hence we reduced the HSV-TK promoter in the original pRL-2xMDR1-TKp-TK construct by 531 bases at the 5' end while still maintaining the transcriptional control region. This resulting construct, pRL-2xMDR1-TKp-TK-MIN, showed no loss in transcriptional activity (**Fig. 2a**, middle panel). Increasing the number of binding sites for K25F from 2 to 6 resulted in significantly greater repression of HSV-TK by K25F (**Fig. 2a**, lower panel). Thus these results show that in the final construct, pRL-6xMDR1-TKp-TK-MIN, the HSV-TK promoter was made more responsive to repression by K25F and at the same time was made more compact and thus suitable for transfer into viral vectors.

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The Smad4 responsive promoter was derived from the IgA1 gene. Previous reports had shown that TGF-β responsiveness is mediated by two regions on this promoter, the first between position -247 and –84, and the second between positions -84 and -20, with respect to the transcription start site (Lars and Paschalis, 1993). In order to ascertain the transcriptional activity of the Smad4 responsive region, the region between -247 to +79 was subcloned upstream of a luciferase reporter gene, resulting in the plasmid pGL3-IgA1-Luc. As shown in **Figure 2b**, the IgA1 promoter has binding sites for several transcription factors besides Smad4. Runx, belonging to the AML family of proteins, is known to synergistically confer TGF-β responsiveness to the IgA genes (Pardali et al., 2000). Our goal was to reduce as much as possible any transcriptional activity in the absence of Smad4 and to increase Smad4 inducibility. Hence the binding sites for transcription factors other than Smad and Runx were mutated using site directed mutagenesis. Consensus sequences for each of these sites, the actual sequence of the IgA1 promoter, and the mutations introduced at the various sites are shown in **Figure 2b**. The PU.1 site was not mutated since this transcription factor is expressed selectively in B-cells (Lloberas et al., 1999) and hence not relevant to studies in carcinomas. Smad4 inducibility of wild type (WT) and mutant IgA1 promoters were checked by transient co-transfection with a Smad4 expressing plasmid in pancreatic Panc-1 and AsPC-1 cells. As seen in **Figure 2c**, the WT IgA1 promoter showed higher basal activity in Panc-1 cells expressing endogenous Smad4 (Schutte et al., 1996; Subramanian et al., 2004), as compared to AsPC-1 cells lacking Smad4 (16). Upon co-transfection with Smad4 expressing plasmid, Panc-1 showed a 2.7 fold increase in transcriptional activity whereas AsPC-1 cells showed a 7-fold increase (**Figs. 2c and 2d**). The mutant Sp1 IgA1 promoter showed considerable reduction in background transcriptional activity in the absence of Smad4 while continuing to maintain 9-fold inducibility with Smad4. Other mutants lost Smad4 inducibility and hence were not considered for

further experiments. Thus the K25F region was cloned downstream of the IgA1-Sp1mut promoter to get pGL3IgA1-Sp1mut-K25F, a vector with a high degree of Smad4 inducibility.

Repression of HSV-TK by ectopically expressed Smad4: To initially assess the ability of Smad4-induced K25F to repress HSV-TK, we first used transient transfections in HEK293 cells. As seen from the Western blots of HSV-TK (**Fig. 3a**), co-transfection of Smad4 strongly enhanced the repression of HSV-TK by K25F, indicating that the vector system can function as designed. We next evaluated the pharmacological consequences of Smad4 driven K25F expression in stably transfected cells. HEK293 cells were stably co-transfected with various combinations of HSV-TK, K25F and Smad4 cassettes expressed from plasmids containing drug selectable markers; cells transfected with empty vector pcDNA3.1 and pBudCE4.1-zeo were used as controls. These stably transfected HEK293 lines were then used in pharmacologic experiments. After 3 days of treatment with GCV, surviving cells were counted. The repression of HSV-TK by K25F resulted in a significant right-shift (less sensitivity) of the dose-response profile in the cells co-transfected with Smad4 cassette as compared to those transfected only with the HSV-TK and K25F vectors; for example, at 40% cell survival this represented approximately a 2-log right shift (**Fig. 3b**).

Pharmacological response in stably transfected pancreatic cell lines: We wished to assess the effects of the vector system in pancreatic cancer cells. Thus we stably transfected the HSV-TK and K25F cassettes sequentially into pancreatic cancer cells using hygromycin and zeocin drug resistance markers. Due to the lack of Smad4-expressing and Smad4-deficient pancreatic cells derived from the same genetic background, in some cases Smad4 was stably introduced into Smad4-deficient (Subramanian et al., 2004) CFPac-1 cells. Pooled CFPac-1-TK-Hygro cells (which contain the K25F-

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responsive HSV-TK cassette) and control cells lacking HSV-TK were further stably transfected with linearized vectors pBudCE4.1 (a control vector), or pBud-K25F-zeo (a vector which contains a Smad4-responsive K25F expression cassette), or pBud-K25F-Smad4-zeo (a vector which contains both a Smad4-responsive K25F expression cassette and a cassette that constitutively expresses Smad4) (see Methods). A series of cell lines resistant to both hygromycin and zeocin were selected based on the above transfections. These lines were then used for pharmacological and biochemical experiments.

The various stably transfected sublines were tested for their response to GCV. CFPac-1 cells transfected with only an HSV-TK cassette showed profound killing in the presence of GCV while control cells transfected with an empty vector did not, thus demonstrating susceptibility to TK mediated toxicity in this cell type (data not shown). We then examined the effect of the presence of the K25F and Smad4 cassettes in the cells containing the HSV-TK expression cassette. In comparison to the cells expressing only K25F, cells containing both Smad4 and K25F showed a substantial 1.2 log right shift in the dose-response curve for GCV (**Fig. 4a**). In addition, western blot analysis showed a major repression of HSV-TK (**Fig. 4b**) and the presence of detectable K25F (**Fig. 4c**) only in the Smad4 containing cells. Thus our system was able to protect Smad4-containing cells against GCV toxicity via induction of the K25F repressor and subsequent reduction of HSV-TK expression.

Regulation of response to GCV by endogenous Smad4 using an adenoviral gene delivery vector: An important issue for this investigation was whether endogenous Smad4 would be able to provide significant regulation of the K25F/HSV-TK system. To address this we utilized a well-characterized matched pair of colon tumor cell lines that do or do not express physiological levels of Smad4 (Zhou et al., 1998). HCT116/smad4+ and HCT116/smad4- cells were infected with an adenoviral vector that contained both the K25F and HSV-TK expression cassettes, or with a vector containing only the TK

cassette. Cells were then treated with GCV, and cell viability was evaluated by counting cell number. As seen in **Figure 5a**, the GCV dose-response curve was right-shifted approximately 1.0 log units in the HCT116/smad4+ cells as compared to the HCT116/smad4- cells after both cell types had received the dual cassette adenoviral vector, while infection with an adenovirus expressing only HSV-TK did not result in a significant difference in GCV toxicity between the two cell lines. This correlates with levels of HSV-TK expressed in the two cases (**Fig. 5b**) and with the presence or absence of Smad4 (**Fig. 5c**). Thus the presence of physiological levels of Smad4 were sufficient to cause appreciable down regulation of HSV-TK and therefore partial protection of the Smad4+ cells against the toxic effects of GCV. This suggests that the strategy for selective toxicity developed here can be regulated by endogenous levels of Smad4 and thus may eventually be of value in the therapy of colon or pancreatic cancers.

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DISCUSSION

The basic concept underlying this study is that the presence of Smad4 in normal cells will sustain activity of promoters regulated by Smad-dependent signaling, while the lack of Smad4 in many pancreatic and colon cancers will result in reduced expression of Smad-regulated genes in those cells. Accordingly we have used the Smad-dependent IgA1 promoter to drive expression of a designed repressor protein K25F that in turn binds to and inhibits expression from a promoter driving the HSV-TK suicide gene. We have sought to optimize this system in two ways. First, we have made the promoter driving HSV-TK very susceptible to repression by K25F while maintaining relatively high basal levels of expression; this was done by reducing the extent of the minimal TK promoter and by including six copies of a site that binds K25F. This resulted in significant improvements in the degree of TK repression caused by expression of K25F. A second approach was to make the IgA1 promoter more selectively responsive to Smad4 by mutating some of the additional transcription factor binding sites in that promoter. This effort was only partially successful; thus mutating the Sp1 site resulted in a modest increase in the fold activation caused by Smad4 of an IgA1 promoter-reporter construct.

This information was used to produce vectors designed to confer Smad4-dependent drug sensitivity on cells. Initial tests in the HEK293 line showed that cells transiently transfected with a Smad4-dependent K25F-expressing vector and a K25F-dependent HSV-TK-expressing vector displayed substantially reduced amounts of TK protein upon co-transfection with pRK5-smad4, a Smad4 expressing vector. Further, in pharmacological experiments, HEK293 cells stably transfected with a K25F-responsive HSV-TK vector and a vector containing both a Smad4-responsive K25F cassette and a constitutive Smad4 expression cassette displayed a major right shift in the GCV dose-response curve as compared to several controls lacking the Smad4 cassette. After this validation of the

basic concept, we used the Smad4-deficient CFPac-1 pancreatic tumor cell line to prepare a series of stably transfected sublines that contained various combinations of the K25F and TK expression cassettes and, in some cases, a cassette constitutively expressing Smad4. In the absence of Smad4 there was little difference in the response to GCV between cells expressing only the TK vector and those expressing both TK and K25F vectors; however, the expression of Smad4 led to a substantial 1.2 log right shift in the GCV dose response curve and thus significant protection against GCV toxicity. Thus when high Smad4 levels are present, the dual vector system is efficient in shutting down HSV-TK production and preventing toxicity to the Smad4 positive cells.

However, a major issue is whether endogenous levels of Smad4 are sufficient to attain a differential toxic response. This was addressed by developing an Adenoviral system containing both the Smad-responsive K25F cassette and the K25F-responsive HSV-TK cassette in a single vector and using this to efficiently infect paired HCT116 colon tumor cell lines that were either wild type for Smad4 expression or had Smad4 expression deleted (Zhou et al., 1998). This resulted in appreciable differential toxicity, with the Smad4-positive HCT116 line displaying an approximately 1.0 log right-shift of the GCV dose-response curve (i.e. less sensitivity) as compared to the Smad4-negative line. Thus the overall conclusion of these studies is that it is indeed possible to attain selective expression of potentially therapeutic genes based on differential expression of physiological levels of the Smad4 tumor suppressor.

This work extends to Smad4, the concept developed in previous studies using P53 status to confer preferential toxicity to tumor cells. Earlier work from our laboratory used a P53 responsive expression plasmid to drive K25F expression and thus control TK expression (Xu et al., 2003). Another group used P53 to regulate expression of the lac repressor, which in turn controlled a nitroreductase gene cassette (Lipinski et al., 2001); additionally, another study used P53 regulated Cre recombinase to

excise a lox flanked TK cassette and thus block TK expression in P53 positive cells, but not in P53 deficient cells (Andreu et al., 2001). Regulation via P53 is complex since the protein is not very active normally but rather becomes activated subsequent to DNA damage or other stimuli (Vousden and Prives, 2005). Thus Smad4, although a relatively weak transactivator, may ultimately be more amenable for use in this type of targeted therapeutic approach than P53.

The present system for Smad4 dependent selective cell killing has attained a 1.0 log differential toxicity in response to physiological levels of Smad4. This demonstrates proof of concept but is probably not adequate for actual therapeutic utilization. Although we have taken a number of steps to improve the efficacy of our vector system for attaining selective toxicity in Smad4 deficient cells, additional potential improvements would seem to merit exploration in future studies. A number of approaches are possible; for example, the IgA1 promoter could be further modified by the inclusion of additional Smad4/Runx sites, or by changing the spacing between sites. Alternatively other Smad4 responsive natural promoters or completely designed promoters could be tested. With further improvements in the promoter, it seems likely that more robust enhancement of the Smad4–dependent selective toxicity can be attained.

A major issue with this study, as well as with all types of cancer-directed gene therapy, is the limited ability to deliver genes to tumors *in viv*o. The biological barriers to effective gene delivery to tumors via viral or non-viral vectors are now fairly well understood (Wang and Yuan, 2006) but nonetheless remain substantial. Offsetting this, there is currently a major effort involving many laboratories to improve the capabilities of gene delivery vehicles. This includes several strategies to alter the cell/tissue tropisms of viral vectors and to improve their ability to express therapeutic genes (Breckpot et al., 2007; Li et al., 2005; Rein et al., 2006; Schepelmann and Springer, 2006; Wu et al., 2006). To some degree the inability of viral vectors to deliver genes to all of the cells in a tumor can be

further countered by suicide enzyme-drug combinations that have significant 'bystander' effects thus allowing drug to reach cells other than those infected by the virus. The HSV-TK/ganciclovir combination has only limited bystander effects since cell to cell drug transfer is dependent on the presence of gap junctions between the cells whereas for other enzyme/prodrug combinations the products seem to diffuse freely (Dachs et al., 2005). Other suicide gene/drug combinations may thus offer more robust bystander effect and be advantageous to use in connection with further development of the Smad4 dependent gene regulation system described here. Additionally, the current strategy could be applied to regulation of other types of potentially therapeutic genes including anti-angiogenic factors or immunomodulatory factors that would not require expression in every cell in the tumor in order to be effective (Isayeva et al., 2004; Kong et al., 1998). However, the current study was designed to test the basic principle of Smad4-dependent regulation of gene expression rather than to develop a complete therapeutic approach.

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FIGURE LEGENDS

Figure 1: Strategy for selective killing of Smad4 deficient tumor cells and schematic representation of two essential constructs and an adenoviral dual cassette vector. (**a**) Construct pGL3-IgA1Sp1mut-K25F expresses the designed transcriptional repressor, K25F under the control of a Smad4 responsive promoter. Construct pRL-6xMDR1-TKpMIN-TK expresses the therapeutic gene HSV-TK under the control of a minimum TK promoter that contains six binding sites for K25F. In normal cells (upper panel), wild type Smad4 activates the expression of K25F, which binds to the TK vector and represses HSV-TK. In Smad4 negative tumor cells (lower panel), no K25F is made and hence HSV-TK is expressed, making cells susceptible to the prodrug ganciclovir (GCV) which is converted to the active drug ganciclovir-mono-phosphate (GCV-mP) by HSV-TK. (**b**) pGL3-IgA1-Sp1mut-K25F produces K25F and is regulated by Smad4. This vector contains the Smad4 responsive region from the IgA1 promoter. The Sp1 transcription factor binding site in close proximity is mutated for greater Smad4 specificity. (**c**) pRL-6xMDR1-TKp-TK-MIN produces HSV-TK and is regulated by K25F. This vector contains six copies of K25F binding sequence from the MDR1 promoter followed by the truncated HSV-TK promoter and the coding sequence of HSV-TK. (**d**) The adenoviral vectors have the HSV-TK expressing cassette on the right containing elements (a) through (f) from the plasmid pRL-6xMDR1-TKp-TK-MIN. On the left of the vector Ad-Tk-K25F, is the K25F expressing cassette containing elements (a) through (e) from the plasmid pGL3-IgA1-Sp1mut-K25F; an empty cassette from the AdenoQuick system is present at this site in the vector Ad-TK. Triangles and bars at the end of the linearized vector represent the viral ITRs and packaging signal.

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Figure 2: Optimization of HSV-TK and IgA1 promoters for enhanced efficiency and specificity. (**a**) Repression of HSV-TK by K25F in transiently transfected HEK293 cells. Cells were plated in 6-well plates (2.5 x 10^5 cells/well) and 48 h later were co-transfected with the indicated plasmids. Lanes 2 through 6 were transfected with 0.8 μg of the various HSV-TK expressing plasmids. CMV driven pcK25F is described in Methods and was co-transfected at concentrations 0.01, 0.06, 0.12 and 0.2 μg (lanes 3 through 6). pcDNA3.1 was used as an empty plasmid in order to transfect uniform amounts of DNA in each well. Whole cell lysates of cells were prepared 48 h after transfection and immunoblotted with monoclonal anti-6xhis antibody. (**b**) Schematic representation of the IgA1 promoter. The IgA1 promoter was cloned upstream of firefly luciferase resulting in the plasmid pGL3-IgA1-luc. Binding sites for transcription factors other than Smad4 and Runx were mutated using site directed mutagenesis. Mutated nucleotides are shown in italics. Top line, consensus sequence of the site, middle line, the sequence of site in the IgA1 promoter, and bottom line, mutated sequence. (**c**) Smad4 inducibility of mutant IgA1 promoters were checked in two pancreatic cell lines by measuring luciferase activities. Panc-1 (Smad4 expressing) and AsPc-1 (Smad4 negative) cells were plated in 12-well plates (1.0 x 10^5 cells/well) and 48 h later were transfected respectively with 0.5 μg of various mutant IgA1-luc plasmids alone (open bars) or co-transfected with 1.5 μg pRK5-smad4 plasmid (black bars). pcDNA3.1 was used as an empty vector to transfect uniform amounts of DNA. Luciferase activities were measured 48 h after transfection and normalized for protein concentration. (**d**) Fold increase in transcription activities of the various mutant promoters with pRK5-smad4 co-transfections. Left panel, Panc-1 cells, right panel, AsPC-1 cells.

Figure 3: Repression of HSV-TK by Smad4 induced K25F in HEK293 cells. (**a**) Cells were plated in 6-well plates (2.5 x 10^5 cells/well) and 48 h later were transiently co-transfected with the indicated

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plasmids. Smad4 expressing pRK5-smad4 was co-transfected at 0.5, 1.0 and 1.5 μg. pcDNA3.1 was used as the empty plasmid in order to transfect uniform amounts of DNA. Whole cell lysates were prepared 48 h after transfection and immunoblotted with monoclonal anti-6xhis and anti-flag antibody to detect HSV-TK and Smad4 respectively. (**b**) Regulation of the response to GCV by Smad4-induced K25F in stably transfected HEK 293 cells. Stable HEK 293 cells with various cassettes were prepared as described in the Methods. Cells were plated in 12-well plates (0.25×10^5 cells/well) and 24 h later GCV was added at concentrations indicated. Cell number was counted 3 days post GCV treatment and plotted as % surviving cells as compared to control cells not treated with GCV. 293-empty-hygro-empty-zeo (closed triangle), 293-empty-hygro-K25F-zeo (closed circle), 293-empty-hygro-K25F-Smad4-zeo (closed square), 293-TK-hygro (open diamonds), 293-TK-hygro-empty-zeo (open triangle), 293-TK-hygro-K25F-zeo (open circle), 293-TK-hygro-K25F-Smad4-zeo (open square).

Figure 4: Regulation of the response to GCV in pancreatic CFPac-1 cells. (**a**) Vectors containing various combinations of the HSV-TK, K25F and Smad4 cassettes were stably transfected into CFPac-1 cells as described in Methods. The respective stable cell lines were plated in 6-well plates (4.0×10^5 cells/well) and 24 h later cells from each well were distributed into a 6-well plate. Another 24 h later GCV was added at the concentrations indicated. Cell number was counted 4 days post GCV treatment and plotted as % surviving cells as compared to control cells. CFPac-1-empty-hygro-empty-zeo (closed triangles), CFPac-1-empty-hygro-K25F-zeo (closed circles), CFPac-1-TK-Hygro-Empty-Zeo (open triangles), CFPac1-TK-Hygro-K25F-Zeo (open circles), CFPac1-TK-Hygro-K25F-Smad4-Zeo (open squares). (**b**) Repression of HSV-TK in Smad4 containing stable cells. Whole cell lysates of the various stably transfected cell lines were prepared and immunoblotted with monoclonal anti-6xhis antibody to detect HSV-TK. Lane 1, untransfected CFPac-1; Lane 2, CFPac-1-TK-Hygro-Empty-Zeo; lane 3,

CFPac1-TK-Hygro-K25F-Zeo; lane 4, CFPac1-TK-Hygro-K25F-Smad4-Zeo. Blotting lysates with anti-actin is shown for normalization. (**c**) Detection of K25F in stably transfected cell lines. Expressed K25F was pulled down by immunoprecipitation with monoclonal anti-myc antibody and detected by Western blotting using anti-myc antibody. Lane 1, CFPac-1-TK-Hygro-Empty-Zeo; lane 2, CFPac1-TK-Hygro-K25F-Zeo; lane 3, CFPac1-TK-Hygro-K25F-Smad4-Zeo.

Figure 5: Regulation of the response to GCV by endogenous Smad4 in HCT116 colon cells. (**a**) HCT116/Smad4+ and HCT116/Smad4- cells were plated in 6-well plates (2.5 x 10^5 cells/well) and 24 h later cells were infected with either Ad-GFP, Ad-TK or Ad-TK-K25F at 10^7 infectious particles/well. Twenty four hours post infection, cells from each well were distributed into a 6-well plate. Another 24 h later GCV was added at the concentrations indicated. Cell number was counted 4 days post GCV treatment and plotted as % surviving cells as compare to control cells. Ad-GFP infected HCT116/Smad4+ (closed diamonds), Ad-GFP infected HCT116/Smad4- (closed squares), Ad-TK infected HCT116/Smad4+ (open circles), Ad-TK infected HCT116/Smad4- (open diamonds), Ad-TK-K25F infected HCT116/Smad4+ (open squares), Ad-TK-K25F infected HCT116/Smad4- (open triangles). This result is typical of several independent experiments. (**b**) Repression of HSV-TK in Smad4 positive HCT116 colon cells. HCT116/Smad4+ and HCT116/Smad4- cells were plated in 6-well plates (2.5 x 10^5 cells/well) and 24 h later cells were infected with either Ad-TK or Ad-TK-K25F with 10^7 infectious particles/well. HCT116/Smad4+ infected with Ad-GFP (lane 1), Ad-TK (lane 2) and Ad-TK-K25F (lane 3), HCT116/Smad4- infected with Ad-GFP (lane 4), Ad-TK (lane 5) and Ad-TK-K25F (lane 6). Forty eight hours post infection, whole cell lysates were prepared and immunoblotted with polyclonal anti-HSV-TK antibody. Note: there is a small amount of background staining at the site of the HSV-TK band in both cell lines. (**c**) Immunoblot to ascertain the Smad4 status

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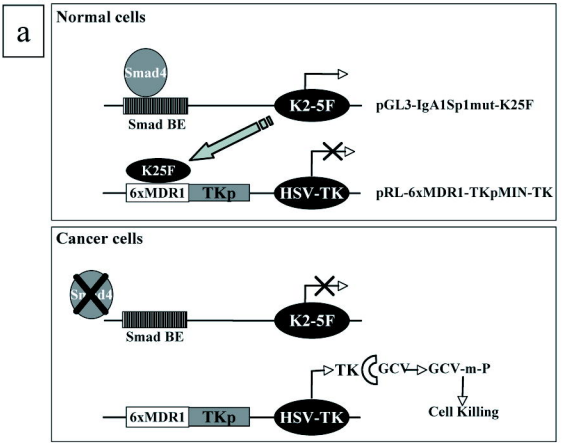
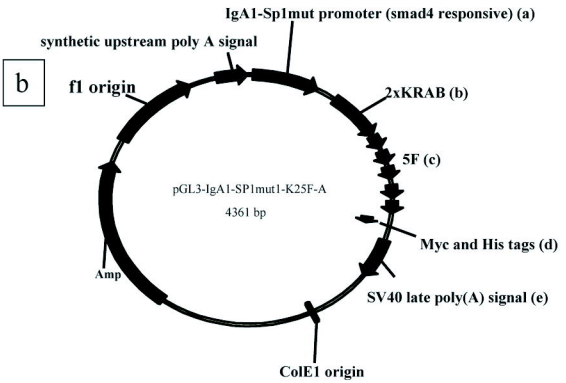
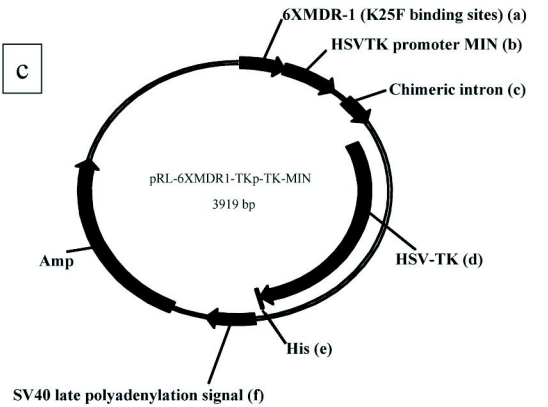
of HCT116/Smad4+ and HCT116/Smad4- cells. Whole cell lysates were prepared and immunoblotted for Smad4 as described in Methods.

Figure 1


a
Normal cells
Smad4
Smad BE
K2-5F
pGL3-IgA1Sp1mut-K25F
K25F
6xMDR1
TKp
HSV-TK
pRL-6xMDR1-TKpMIN-TK
Cancer cells
Smad4
Smad BE
K2-5F
TK
GCV
GCV-m-P
Cell Killing
6xMDR1
TKp
HSV-TK


b
IgA1-Sp1mut promoter (smad4 responsive) (a)
synthetic upstream poly A signal
f1 origin
2xKRAB (b)
5F (c)
pGL3-IgA1-SP1mut1-K25F-A
4361 bp
Myc and His tags (d)
Amp
SV40 late poly(A) signal (e)
ColE1 origin


c
6XMDR-1 (K25F binding sites) (a)
HSVTK promoter MIN (b)
Chimeric intron (c)
pRL-6XMDR1-TKp-TK-MIN
3919 bp
Amp
HSV-TK (d)
His (e)
SV40 late polyadenylation signal (f)

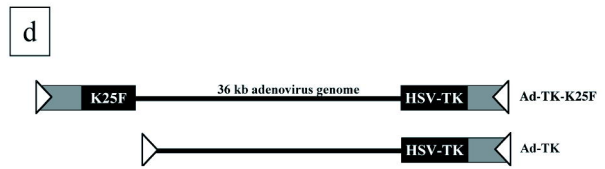
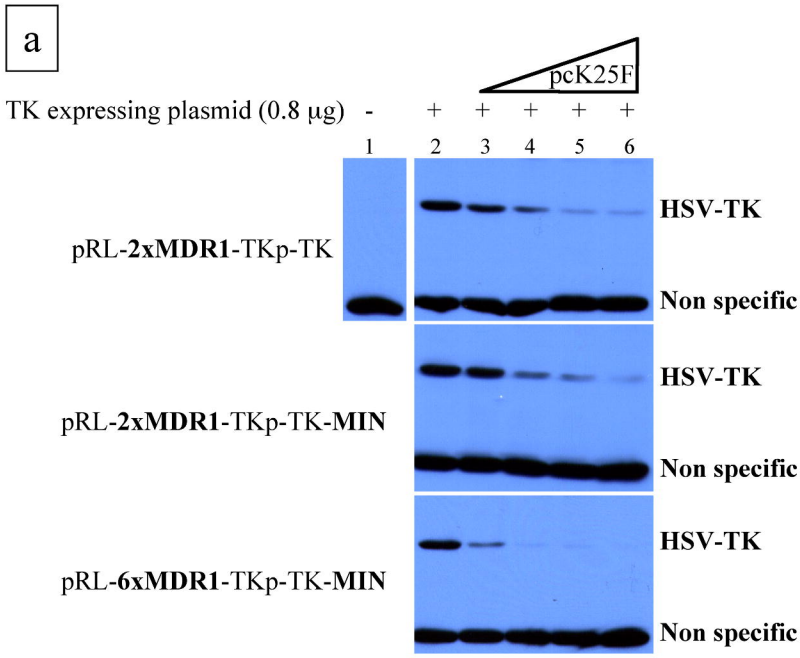
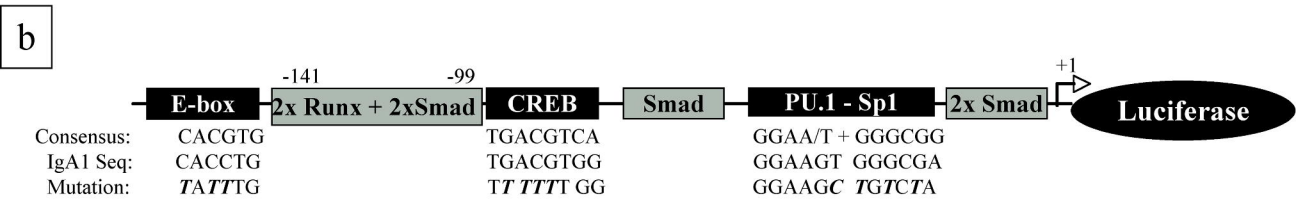
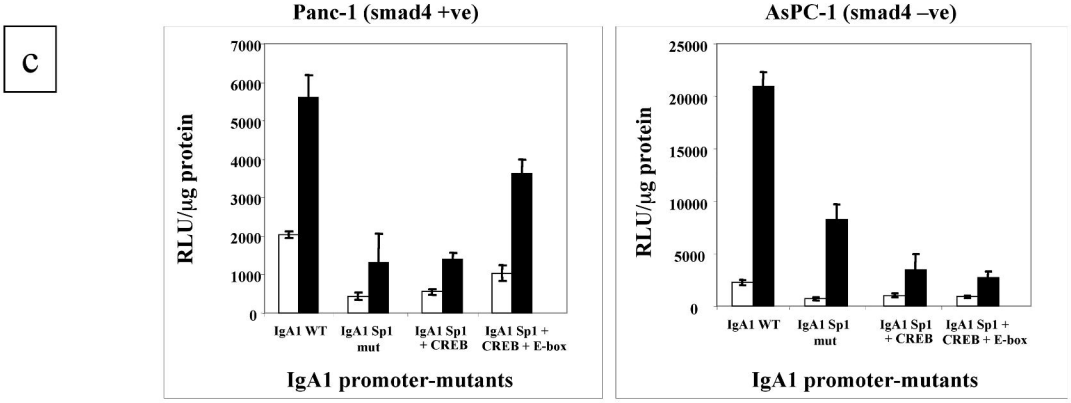

d
K25F
36 kb adenovirus genome
HSV-TK
Ad-TK-K25F
HSV-TK
Ad-TK

Figure 2


a
pcK25F
TK expressing plasmid (0.8 µg) - + + + + +
1 2 3 4 5 6
pRL-2xMDR1-TKp-TK
pRL-2xMDR1-TKp-TK-MIN
pRL-6xMDR1-TKp-TK-MIN
HSV-TK
Non specific
HSV-TK
Non specific
HSV-TK
Non specific


b
-141
-99
+1
E-box
2x Runx + 2xSmad
CREB
Smad
PU.1 - Sp1
2x Smad
Luciferase
Consensus: CACGTG TGACGTCA GGAA/T + GGGCGG
IgA1 Seq: CACCTG TGACGTGG GGAAGT GGGCGA
Mutation: TATTTG TT TTTT GG GGAAGC TGTCTA


c
Panc-1 (smad4 +ve)
AsPC-1 (smad4 –ve)
RLU/µg protein
IgA1 WT
IgA1 Sp1 mut
IgA1 Sp1 + CREB
IgA1 Sp1 + CREB + E-box
IgA1 promoter-mutants

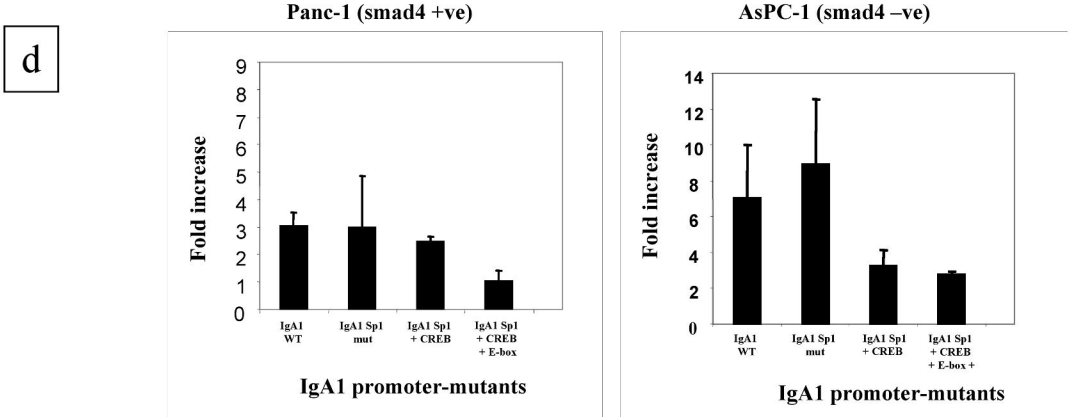
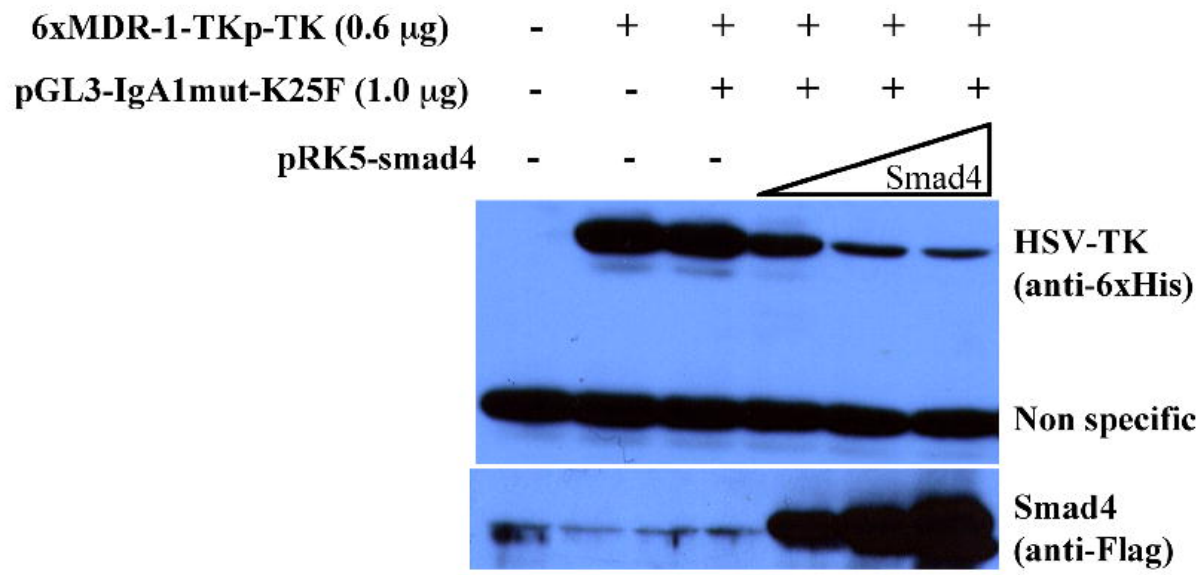

d
Panc-1 (smad4 +ve)
AsPC-1 (smad4 –ve)
Fold increase
IgA1 WT
IgA1 Sp1 mut
IgA1 Sp1 + CREB
IgA1 Sp1 + CREB + E-box
IgA1 promoter-mutants

Figure 3

a


6xMDR-1-TKp-TK (0.6 μg) - + + + + +
pGL3-IgA1mut-K25F (1.0 μg) - - + + + +
pRK5-smad4 - - -
Smad4
HSV-TK
(anti-6xHis)
Non specific
Smad4
(anti-Flag)

b

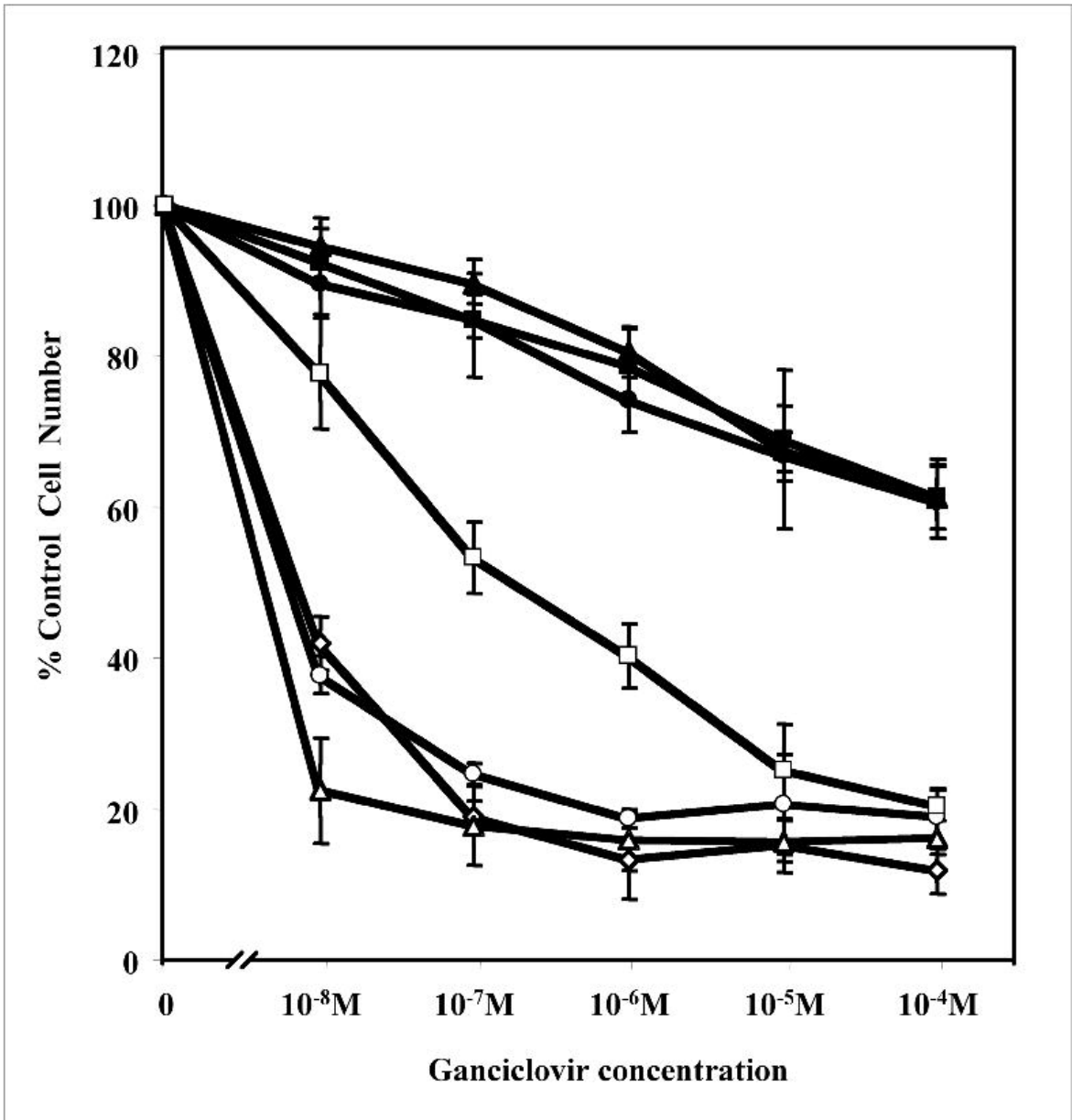
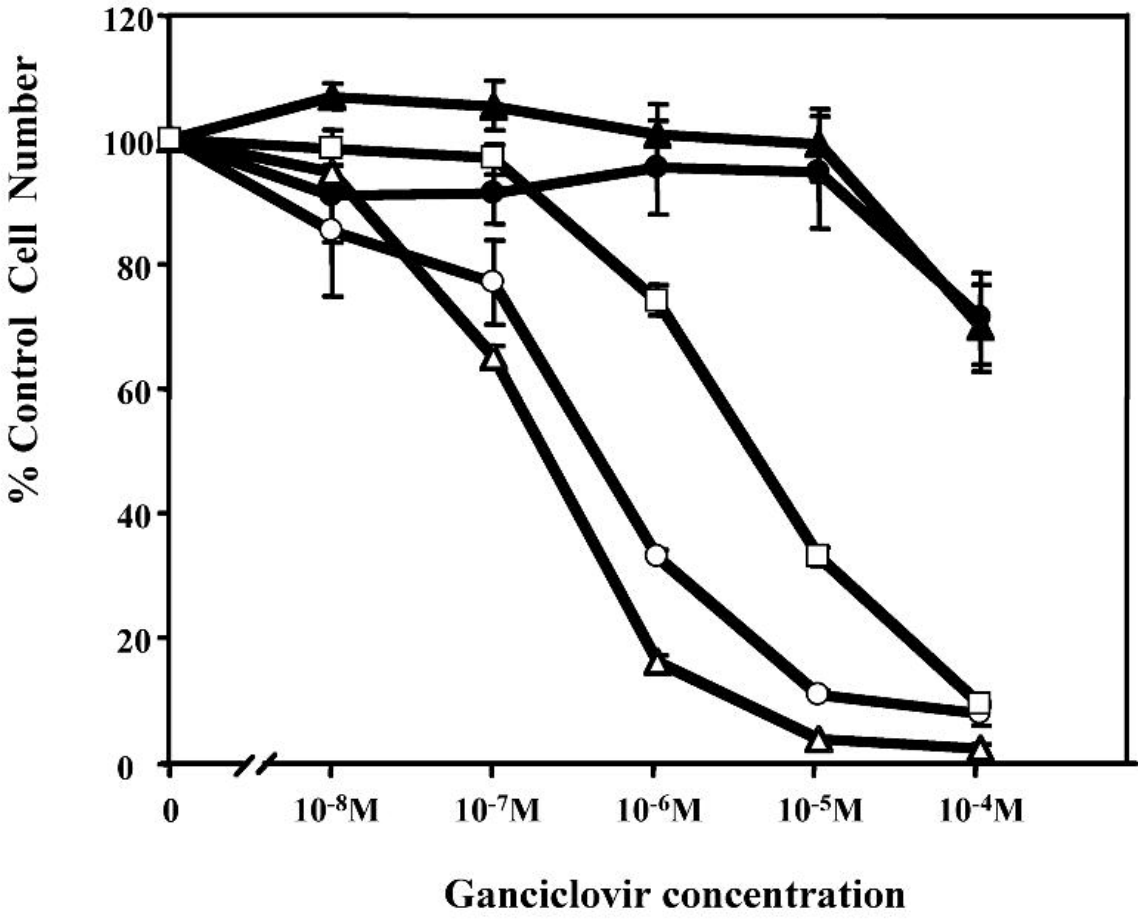
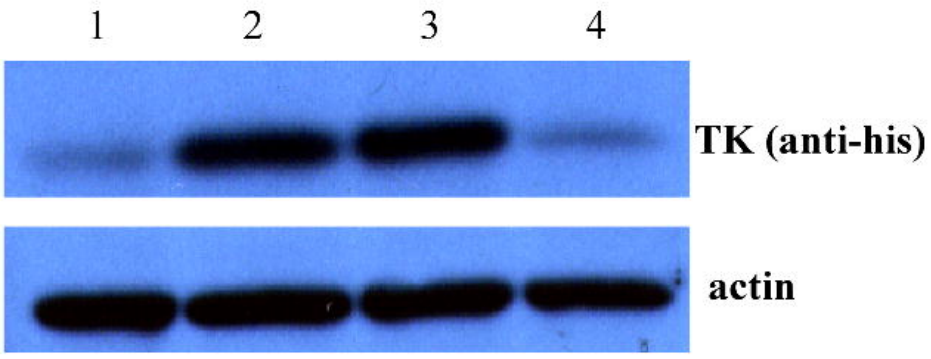

120
100
80
60
40
20
0
% Control Cell Number
0
10-8M
10-7M
10-6M
10-5M
10-4M
Ganciclovir concentration

Figure 4

a


120
100
80
60
40
20
0
% Control Cell Number
0
10-8M
10-7M
10-6M
10-5M
10-4M
Ganciclovir concentration

b


1
2
3
4
TK (anti-his)
actin

c

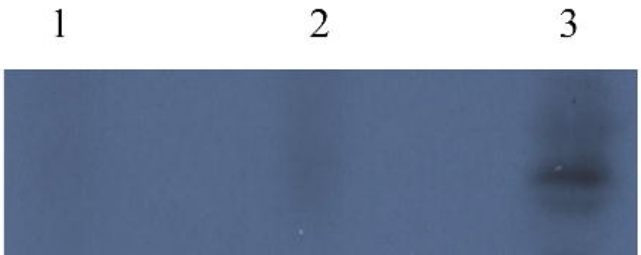
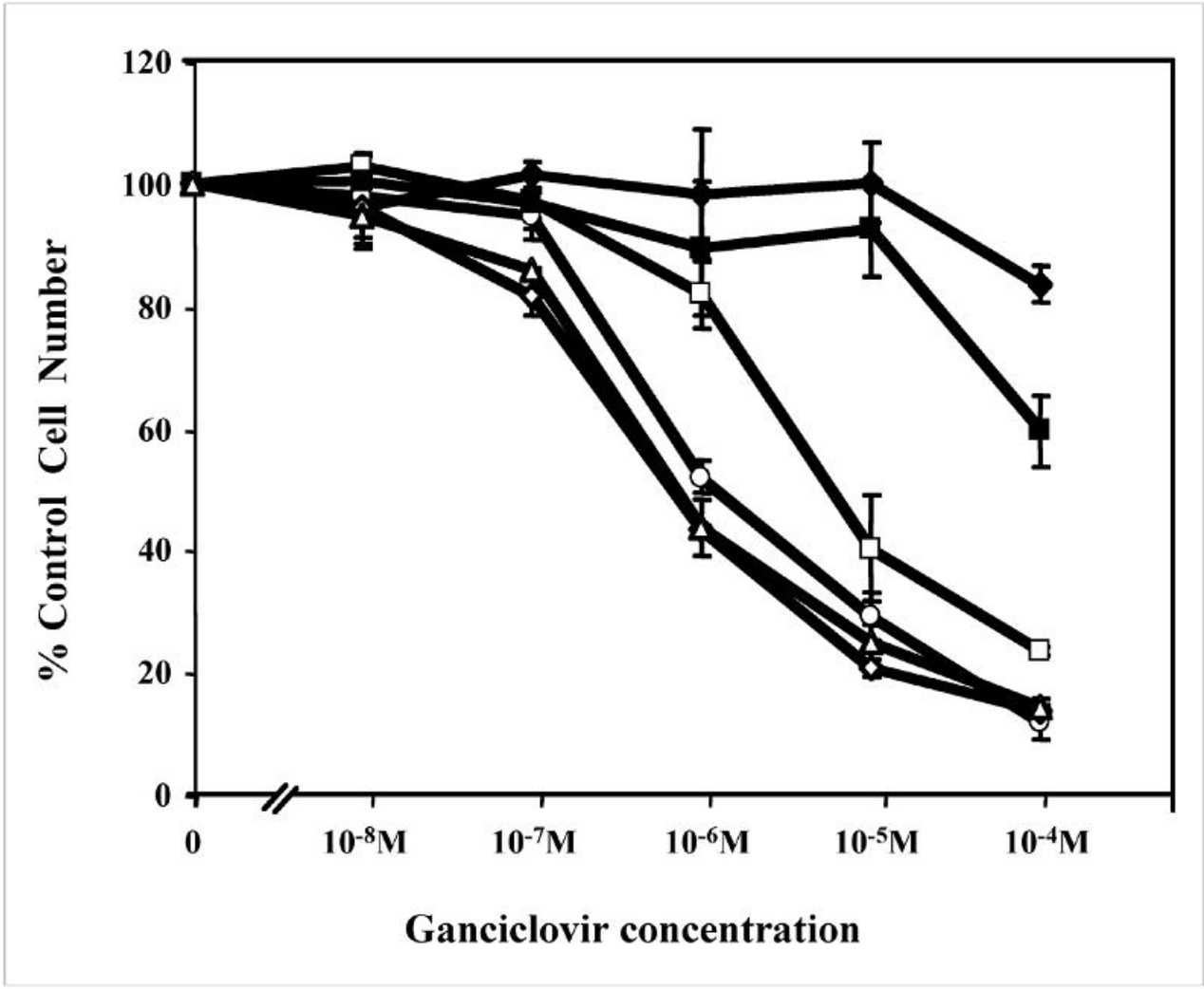
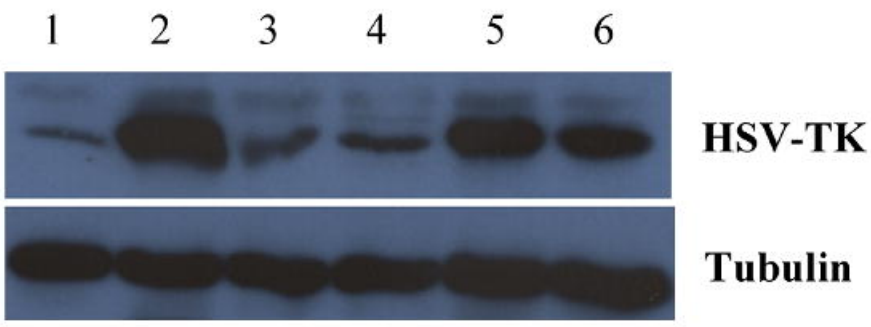

1
2
3
IP: K25F
(anti-myc)

Figure 5

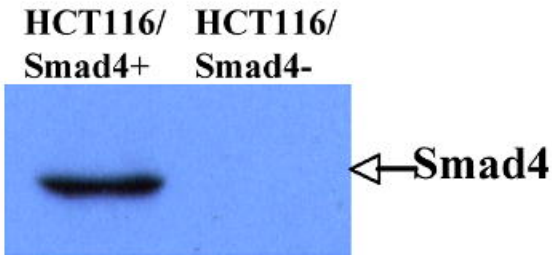
a


120
100
80
60
40
20
0
% Control Cell Number
0
10-8M
10-7M
10-6M
10-5M
10-4M
Ganciclovir concentration

b


1
2
3
4
5
6
HSV-TK
Tubulin

c


HCT116/ Smad4+
HCT116/ Smad4-
Smad4