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Selective Killing of Smad4-Negative Tumor Cells via a Designed Repressor Strategy

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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 herpes simplex virus thymidine kinase (HSV-TK) "suicide gene," we demonstrate Smad4-responsive regulation of HSV-TK expression and consequent altered susceptibility to the prodrug 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 cotransfected 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.

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 (Scanlon, 2004; Dachs et al., 2005). A variety of enzyme/prodrug combinations has been used, including herpes simplex virus thymidine kinase (HSV-TK) with ganciclovir (GCV), *Escherichia 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 believed to be especially active in particular types of tumors (Sethi and Palefsky, 2003; Sadeghi and Hitt, 2005; Gommans et al., 2006; Rein et al., 2006); 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 more than 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 in which the p53 pathway is inactive; this strategy has already reached advanced clinical trials (Chu et al., 2004; Dobbelstein, 2004). Because 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 been used previously by us (Xu et al., 2003) and others (Andreú et al., 2001; Lipinski et al., 2001) to attain selective toxicity to p53-deficient tumor cells.

Smad4/deleted in pancreatic cancer locus 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; Thiagalingam et al., 1996; Takayama et al., 2006). Smad4 is part of the TGF-β/activin/ bone morphogenetic protein signaling pathway (ten Dijke and Hill, 2004) and forms complexes with R-Smads that lead to the 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, activator protein-1, Sp1, basic helix-loop-helix protein, homeodomain, and others, depending on the identity and status of the interacting partners.

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ABBREVIATIONS: HSV-TK, herpes simplex virus thymidine kinase; GCV, ganciclovir; TGF-β, transforming growth factor-β; HEK, human embryonic kidney; CREB, cAMP response element-binding protein.

Smads then recruit coactivator or corepressor 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 (MacKenzie, 2004; Bhattacharyya and Lemoine, 2006). 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 used a designed transcription factor (Falke and Juliano, 2003; Blancafort et al., 2004; Juliano et al., 2005) termed K25F that is composed 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, whereas 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 prodrug ganciclovir. We have used this system to elicit selective toxicity in pancreatic and colon tumor cells depending on their Smad4 status.

Materials and Methods

Cell Lines. HEK293, its variant HEK293 AD, and Panc-1 cells were obtained from Lineberger Cancer Center tissue culture facility (University of North Carolina, Chapel Hill, NC). 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 (Manassas, 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 4 mM L-glutamine and 1.5 g/l sodium bicarbonate. AsPC-1 cells were cultured in RPMI 1640 medium with L-glutamine and 4.5g/l glucose supplemented with 10 mM HEPES, 1 mM sodium pyruvate, and 1.5 g/l bicarbonate. All of the growth media were obtained from Invitrogen (Carlsbad, CA) and supplemented with 10% fetal bovine serum unless otherwise specified.

Plasmid and Viral Constructs. For the construction of the HSV-TK-expressing plasmid, a multiple cloning site 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 multiple cloning site of pRL-TKp-TK-MIN. Two additional K25F binding sites were amplified from 2xMDR1-TKp-TK and inserted at SpeI and NdeI followed by two more additions at NdeI and KpnI sites. This resulted in the plasmid pRL-6xMDR1-TKp-TK-MIN.

All of 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 QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Mutant sequences are shown in Fig. 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; thereafter, the remaining five 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) resulting in the plasmid pcDNA3.1-TK-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). 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 according to the manufacturer's instructions to construct the dual-cassette adenoviruses Ad-TK and Ad-TK-K25F. In brief, 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 AdenoQuick 13.1. 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. In brief, confluent HEK293 AD cells were infected with various dilutions of the virus at 37°C for 2 h, followed by overlaying with 0.8% agar mixture. Plaques were counted 10 to 15 days after overlaying.

Transient Transfections and Adenoviral Infections. Transient transfections with supercoiled plasmid DNA in HEK293 cells were performed by using Lipofectamine 2000 according to manufacturer's instructions. Panc-1, CFPac-1, and AsPC-1 cells were transiently transfected with FuGENE (Roche, Indianapolis, IN) according to the manufacturer's instructions. Amounts of plasmid DNA used and the times 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. HEK293 and CFPac-1 cells were plated in six-well plates (4.0×10^5 cells/well) and were 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 replated 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 100 μg/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, and CFPac-1-TK-hygro-K25F-Smad4-zeo; likewise, their corresponding empty cassette controls introduced in 293-empty-hygro and CFPac-1-empty-hygro.

Luciferase Reporter Assays. Cells were usually harvested 48 h after transfection, and activity was determined using Luciferase assay kit (Promega). Measurements were performed on a Monolight 2010 instrument (Analytical Luminescence Laboratory, San Diego, CA).

Western Blotting and Immunoprecipitation. Transfected cells were lysed in modified radioimmunoprecipitation assay. Protein concentration was measured using the BCA kit (Pierce, Rockford, IL). Lysates (20 μg) were resolved on SDS-PAGE and detected by Western blotting as described previously (Xu et al., 2002). Expressed HSV-TK was detected by using either a mouse monoclonal anti-His antibody (Covance Reserch Products, Princeton, NJ) at 1:1000 dilution or anti-HSV-TK polyclonal antibody, kindly provided by Dr. M. E. Black (Washington State University, Pullman, WA), at 1:5000 dilution. K25F protein was detected using 1:1000 dilution mouse anti-myc monoclonal antibody 9E10 (Covance). Endogenous Smad4 was detected by rabbit *polyclonal H-552* antibody (Santa Cruz 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.) at dilutions of 1: 5000. The secondary antibodies used were horseradish peroxidase-conjugated bovine anti-mouse IgG antibody (Santa Cruz, Biotechnology) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Calbiochem, San Diego, CA) at a dilution of 1:5000. Signals were detected by enhanced chemiluminescence (ECL kit; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Immunoprecipitation was carried out using the Catch and Release version 2.0 Reversible Immunoprecipitation System (Upstate Biotechnology/Millipore, Billerica, MA) according to the manufacturer's instructions. Proteins were visualized by Western blotting as described above.

Pharmacological Experiments. Cells were plated in six-well plates (4.0×10^5 cells/well), and 24 h later, cells from each well were split into six wells of a six-well plate. Another 24 h later, GCV was added at the concentrations indicated. Cell number was counted 4 days after GCV treatment using an electronic particle counter and plotted as the percentage of surviving cells compared with control cells.

Results

Strategy and Vector Constructs. An overview of the strategy is shown in Fig. 1a. To achieve the selective killing

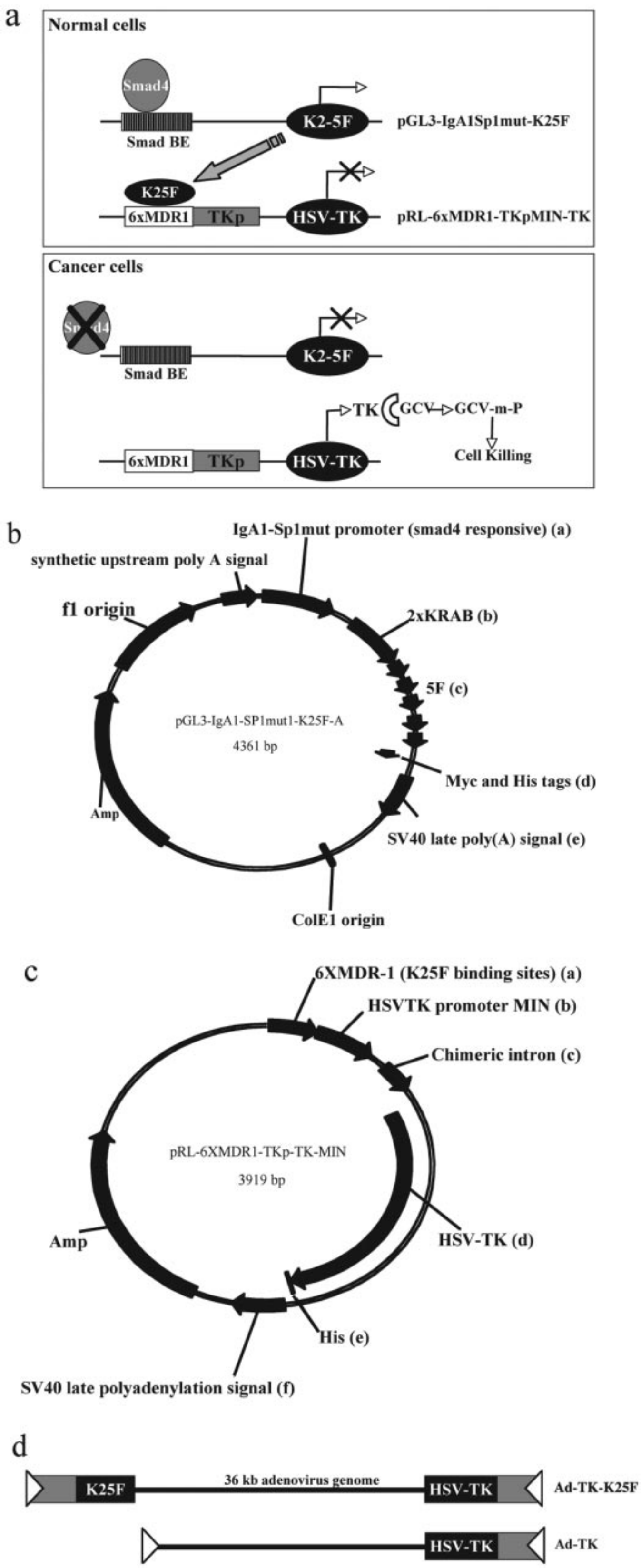

Fig. 1. Strategy for selective killing of Smad4-deficient tumor cells, and schematic representation of two essential constructs and an adenoviral dual-cassette vector. a, construct pGL3-IgA1Sp1mut-K25F expresses the designed transcriptional repressor, K25F, under the control of a Smad4-responsive promoter. 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 (top), wild-type Smad4 activates the expression of K25F, which binds to the TK vector and represses HSV-TK. In Smad4-negative tumor cells (bottom), no K25F is made, and hence, HSV-TK is expressed, making cells susceptible to the prodrug GCV, which is converted to the active drug ganciclovir-mono-phosphate 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.

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 of 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 was 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 stepwise manner. The first experiments dealt with optimization of vectors for K25F and HSV-TK. The second series of experiments involved transiently transfecting 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 HEK293 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. Before the construction of the final vectors described above, the various elements in the plasmids were optimized. Our laboratory had described the use of pRL-2xMDR1-TKp-TK as a K25F responsive vector previously (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 because 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). Increasing the number of binding sites for K25F from two to six resulted in significantly greater repression of HSV-TK by K25F (Fig. 2a, bottom). 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.

The Smad4-responsive promoter was derived from the IgA1 gene. Previous reports have shown that TGF-β responsiveness is mediated by two regions on this promoter, the first between positions -247 and -84, and the second between positions -84 and -20, with respect to the transcription start site (Lars and Paschalis, 1993). To ascertain the transcriptional activity of the Smad4-responsive region, the region between -247 and $+79$ was subcloned upstream of a luciferase reporter gene, resulting in the plasmid pGL3-IgA1-Luc. As shown in Fig. 2b, the IgA1 promoter has binding sites for several transcription factors other than 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 Fig. 2b. The PU.1 site was not mutated because this transcription factor is expressed selectively in B cells (Lloberas et al., 1999) and hence is not relevant to studies in carcinomas. Smad4 inducibility of wild-type and mutant IgA1 promoters were checked by transient cotransfection with a Smad4-expressing plasmid in pancreatic Panc-1 and AsPC-1 cells. As seen in Fig. 2c, the wild-type IgA1 promoter showed higher basal activity in Panc-1 cells expressing endogenous Smad4 (Schutte et al., 1996; Subramanian et al., 2004) compared with AsPC-1 cells lacking Smad4 (Takayama et al., 2006). Upon cotransfection with Smad4-expressing plasmid, Panc-1 showed a 2.7-fold increase in transcriptional activity, whereas AsPC-1 cells showed a 7-fold increase (Fig. 2, c and d). 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 pGL3-IgA1-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), cotransfection 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 cotransfected 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 pharmacological experiments. After 3 days of treatment with GCV, surviving cells were counted. The repression of HSV-TK by K25F resulted in a significant rightward shift (less sensitivity) of the dose-response profile in the cells cotransfected with Smad4 cassette compared with those transfected only with the HSV-TK and K25F vectors; for example, at 40% cell survival this represented approximately a 2-log rightward shift (Fig. 3b).

Pharmacological Response in Stably Transfected Pancreatic Cell Lines. We wanted 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. Because of 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-responsive HSV-TK cassette) and control cells lacking HSV-TK were further stably transfected with linearized vectors pBudCE4.1 (a control vector), pBud-K25F-zeo (a vector that contains a Smad4-responsive K25F expression cassette), or pBud-K25F-Smad4-zeo (a vector that contains both a Smad4-responsive K25F expression cassette and a cassette that constitutively expresses Smad4) (see *Materials and 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.

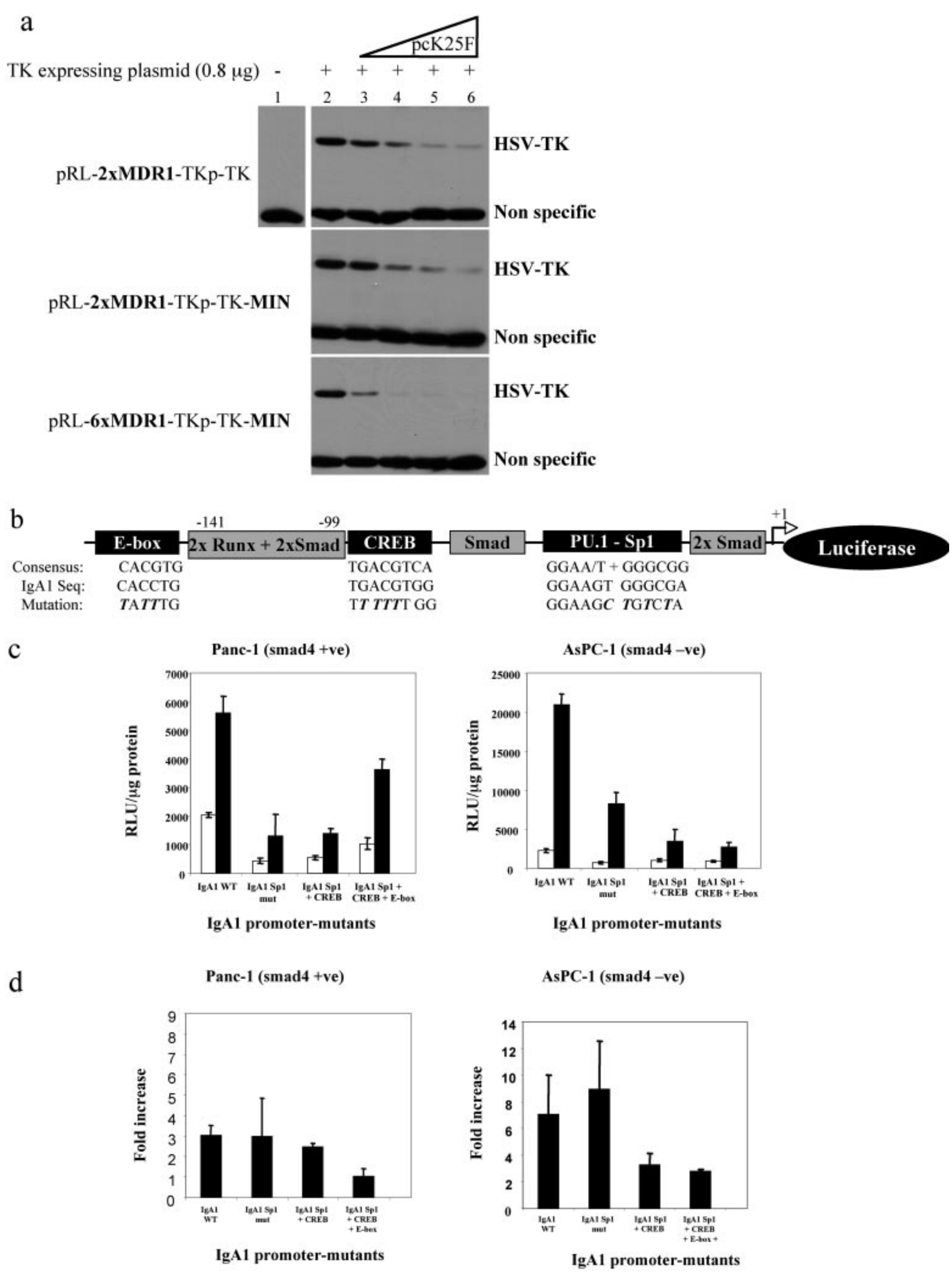

Fig. 2. Optimization of HSV-TK and IgA1 promoters for enhanced efficiency and specificity. a, repression of HSV-TK by K25F in transiently transfected HEK293 cells. Cells were plated in six-well plates (2.5×10^5 cells/well) and 48 h later were cotransfected with the indicated plasmids. Lanes 2 through 6 were transfected with 0.8 μg of the various HSV-TK expressing plasmids. CMV-driven pcK25F is described under *Materials and Methods* and was cotransfected at concentrations of 0.01, 0.06, 0.12, and 0.2 μg (lanes 3 through 6). pcDNA3.1 was used as an empty plasmid 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; 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 (1.0×10^5 cells/well) and 48 h later were transfected, respectively, with 0.5 μg of various mutant IgA1-luc plasmids alone (□) or cotransfected with 1.5 μg of pRK5-smad4 plasmid (■). 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 cotransfections. Left, Panc-1 cells; right, AsPC-1 cells.

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, whereas 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. Compared with the cells expressing only K25F, cells containing both Smad4 and K25F showed a substantial 1.2-log rightward 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

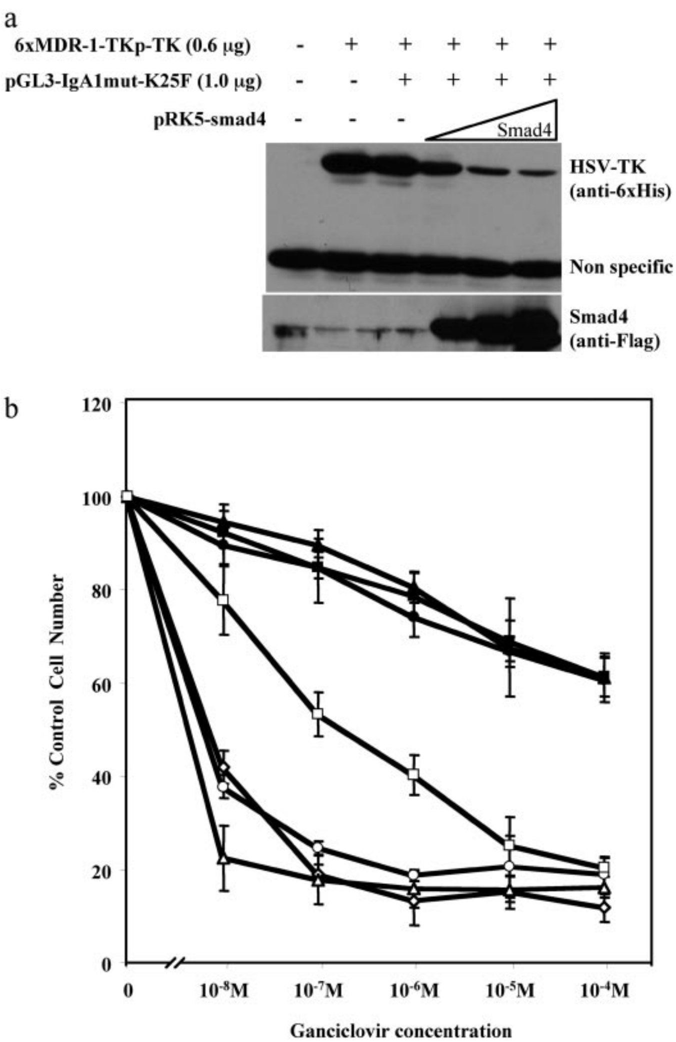

Fig. 3. Repression of HSV-TK by Smad4 induced K25F in HEK293 cells. a, cells were plated in six-well plates (2.5×10^5 cells/well) and 48 h later were transiently cotransfected with the indicated plasmids. Smad4-expressing pRK5-smad4 was cotransfected at 0.5, 1.0, and 1.5 μg. pcDNA3.1 was used as the empty plasmid 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 HEK293 cells. Stable HEK293 cells with various cassettes were prepared as described under *Materials and 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 after GCV treatment and plotted as the percentage of surviving cells compared with control cells not treated with GCV. ▲, 293-empty-hygro-empty-zeo; ●, 293-empty-hygro-K25F-zeo; ■, 293-empty-hygro-K25F-Smad4-zeo; ◇, 293-TK-hygro; △, 293-TK-hygro-empty-zeo; ○, 293-TK-hygro-K25F-zeo; □, 293-TK-hygro-K25F-Smad4-zeo.

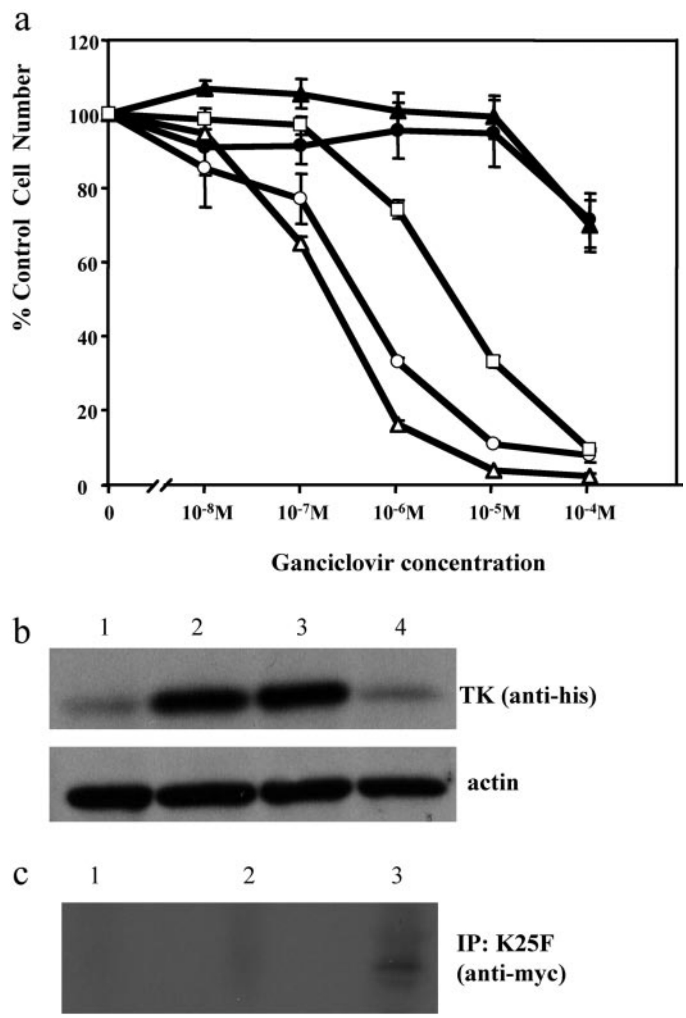

Fig. 4. Regulation of the response to GCV in pancreatic CFPac-1 cells. a, vectors containing various combinations of the HSV-TK, K25F, and Smad4 cassettes were stably transfected into CFPac-1 cells as described under *Materials and Methods*. The respective stable cell lines were plated in six-well plates (4.0×10^5 cells/well), and 24 h later, cells from each well were distributed into a six-well plate. Another 24 h later, GCV was added at the concentrations indicated. Cell number was counted 4 days after GCV treatment and plotted as the percentage of surviving cells compared with control cells. ▲, CFPac-1-empty-hygro-empty-zeo; ●, CFPac-1-empty-hygro-K25F-zeo; △, CFPac-1-TK-Hygro-Empty-Zeo; ○, CFPac1-TK-Hygro-K25F-Zeo; □, CFPac1-TK-Hygro-K25F-Smad4-Zeo. 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.

the K25F/HSV-TK system. To address this, we used 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 Fig. 5a, the GCV dose-response curve was rightward-shifted approximately 1.0 log units in the HCT116/smad4+ cells compared with the HCT116/smad4− cells after both cell types had received the dual-cassette adenoviral vector, whereas 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 treatment of colon or pancreatic cancers.

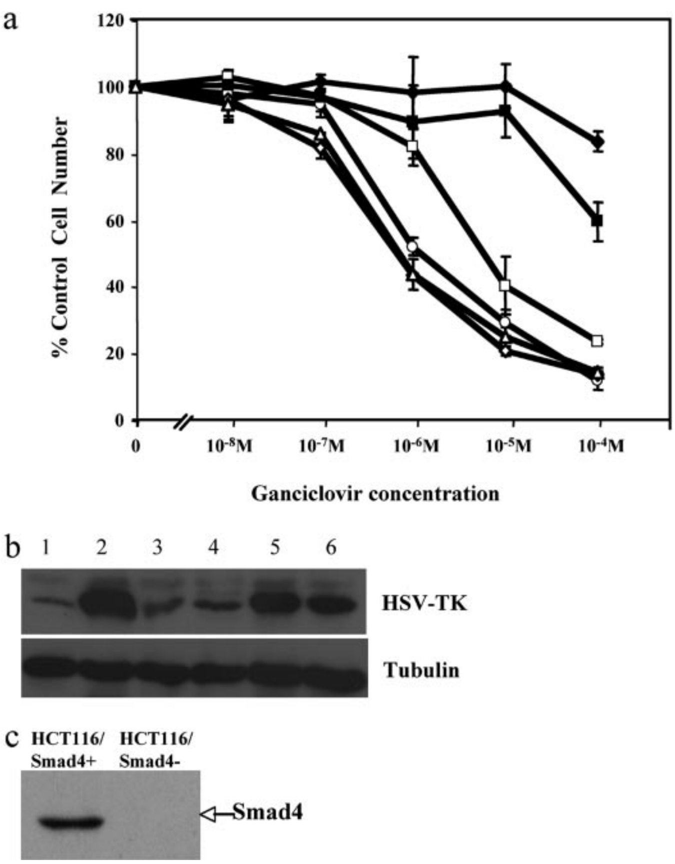

Fig. 5. Regulation of the response to GCV by endogenous Smad4 in HCT116 colon cells. a, HCT116/Smad4+ and HCT116/Smad4− cells were plated in six-well plates (2.5×10^5 cells/well), and 24 h later, cells were infected with either Ad-GFP, Ad-TK, or Ad-TK-K25F at 10^7 infectious particles/well. Twenty-four hours after infection, cells from each well were distributed into a six-well plate. Another 24 h later, GCV was added at the concentrations indicated. Cell number was counted 4 days after GCV treatment and plotted as the percentage of surviving cells compared with control cells. ♦, Ad-GFP-infected HCT116/Smad4+; ■, Ad-GFP-infected HCT116/Smad4−; ○, Ad-TK-infected HCT116/Smad4+; ◇, Ad-TK-infected HCT116/Smad4−; □, Ad-TK-K25F infected HCT116/Smad4+; △, Ad-TK-K25F-infected HCT116/Smad4−. 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 six-well plates (2.5×10^5 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 after 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 of HCT116/Smad4+ and HCT116/Smad4− cells. Whole cell lysates were prepared and immunoblotted for Smad4 as described under *Materials and Methods*.

Discussion

The basic concept underlying this study is that the presence of Smad4 in normal cells will sustain the activity of promoters regulated by Smad-dependent signaling, whereas the lack of Smad4 in many pancreatic and colon cancers will result in reduced expression of Smad-regulated genes in those cells. Therefore, we have used the Smad-dependent IgA1 promoter to drive the 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 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 the 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 cotransfection with pRK5-smad4, a Smad4 expressing vector. Furthermore, 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 rightward shift in the GCV dose-response curve compared with 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 rightward shift in the GCV dose-response curve and thus significant protection against GCV toxicity. Therefore, 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 rightward shift of the GCV dose-response curve (i.e., less sensitivity) compared with 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 (Andreú et al., 2001). Regulation via p53 is complex because the protein is not very active normally but rather becomes activated after 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 use. 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 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. On the other hand, 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 and with all types of cancer-directed gene therapy is the limited ability to deliver genes to tumors in vivo. The biological barriers to effective gene delivery to tumors via viral or nonviral 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 (Li et al., 2005; Rein et al., 2006; Schepelmann and Springer, 2006; Wu et al., 2006; Breckpot et al., 2007). 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 because 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. In addition, the current strategy could be applied to the regulation of other types of potentially therapeutic genes, including antiangiogenic factors or immunomodulatory factors that would not require expression in every cell in the tumor to be effective (Kong et al., 1998; Isayeva et al., 2004). 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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