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Scientific Article

INSM1 Promoter-Driven Adenoviral Herpes Simplex Virus Thymidine Kinase Cancer Gene Therapy for the Treatment of Primitive Neuroectodermal Tumors

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Abstract

The *INSM1* gene encodes a developmentally regulated zinc finger transcription factor. INSM1 expression is normally absent in adult tissues, but is reactivated in neuroendocrine tumor cells. In the present study, we analyzed the therapeutic potential of an adenoviral *INSM1* promoter-driven herpes simplex virus thymidine kinase (HSV-*tk*) construct in primitive neuroectodermal tumors (PNETs). We constructed an adenoviral *INSM1* promoter-driven HSV-*tk* gene for therapy in PNETs. The PNET-specific adeno-*INSM1* promoter HSV-*tk* construct was tested both *in vitro* and *in vivo* in a nude mouse tumor model. Northern blot analysis and transient transfection of an *INSM1* promoter-driven luciferase reporter gene indicated that the *INSM1* promoter was active in neuroblastoma (IMR-32), retinoblastoma (Y79), and medulloblastoma (D283 Med) cells, but not in glioblastoma (U-87 MG) cells. After Ad-INSM1p-HSV-tk infection, the levels of HSV-tk protein expression were consistent with *INSM1* promoter activities. Furthermore, *in vitro* multiplicity of infection and ganciclovir (GCV) sensitivity studies indicated that the *INSM1* promoter could mediate specific expression of the HSV-*tk* gene and selective killing of INSM1-positive PNETs. *In vivo* intratumoral adenoviral delivery demonstrated that the *INSM1* promoter could direct HSV-*tk* gene expression in a nude mouse tumor model and effectively repressed tumor growth in response to GCV treatment. Taken together, our data show that the *INSM1* promoter is specific and effective for targeted cancer gene therapy in PNETs.

Introduction

Cancer gene therapy has gained much interest as an alternative or combination treatment modality. The emergence of improved viral delivery vectors and the significant progress made in understanding the process of oncogenesis have allowed the field of cancer gene therapy to expand (Seth, 2005). The herpes simplex virus thymidine kinase/ganciclovir (HSV-*tk*/GCV) suicide gene system is amongst the oldest and most widely tested concepts for cancer gene therapy (Moolten *et al.*, 1990). Although initial studies of the HSV-*tk*/GCV system demonstrated excellent potential, the major roadblock to its usefulness is the significant liver toxicity (Chen *et al.*, 1995; O'Malley *et al.*, 1995; Brand *et al.*, 1997; van der Eb *et al.*, 1998). Ultimately, the goal of any cancer therapy is to efficiently eliminate the tumor cells with minimal deleterious side effects to the normal surrounding tissue. One strategy to ensure tumor-selective expression is through transcriptional targeting with a tissue-specific promoter. The current challenge is to identify potential regulatory elements that are highly active in tumor cells and have low to ideally no activity in normal cells, especially the liver.

Primitive neuroectodermal tumors (PNETs) are a heterogeneous group of tumors that generally occur in pediatric patients and include medulloblastoma, retinoblastoma, and neuroblastoma. Medulloblastoma is the most common pediatric central nervous system malignancy. Medulloblastoma is an aggressive, embryonal tumor derived from cerebellum. With combined surgery, irradiation, and chemotherapy, the 5-year recurrence-free survival rate has reached 60–65% (Packer *et al.*, 1999; Oyharcabal-Bourden *et al.*, 2005). However, nearly all patients exhibit debilitating side effects from radiation including cognitive impairment, psychiatric

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disorders, endocrine dysfunction, and skeletal growth retardation. Neuroblastoma is a solid tumor derived from cells of neural crest origin. Despite intensive therapy for high-risk neuroblastoma, the 5-year survival is ~33% (Berthold and Hero, 2000). Therefore, alternative treatment options for these forms of cancer are needed. Gene therapy approaches focused toward high-risk neuroblastoma and central nervous system tumors such as medulloblastoma have used common neuronal-specific promoters from the genes encoding midkine, tyrosine hydroxylase, neural crest homeobox (NCX), and Mash1 (Narita *et al.*, 2001; Adachi *et al.*, 2002; Steffens *et al.*, 2004; Arvidsson *et al.*, 2005). Despite promising results from these gene therapy strategies, one significant barrier is the continuous expression of these gene products, with the exception of *Mash1*, in normal adult neuronal cells. This aspect limits their usefulness for cancer gene therapy especially if delivered systemically. Therefore, new genes with tumor-selective expression need to be identified and tested.

INSM1 is a novel transcriptional repressor that was originally identified in an insulinoma subtractive hybridization screen (Goto *et al.*, 1992). INSM1 expression is both temporally and spatially restricted to the embryonic peripheral and central nervous system in cells of neuroendocrine origin including the pituitary, pancreas, thymus, thyroid, retina, olfactory bulb, brain, and spinal cord and is silenced during normal postnatal development (Zhu *et al.*, 2002; Xie *et al.*, 2002; Mellitzer *et al.*, 2006). Strikingly, *INSM1* is reactivated in tumors of neuroendocrine origin including insulinomas, pituitary tumors, pheochromocytomas, medullary thyroid carcinomas, small-cell lung carcinomas, medulloblastomas, neuroblastomas, and retinoblastomas (Goto *et al.*, 1992; Breslin *et al.*, 2003; DeSmaele *et al.*, 2008). These features make it an excellent candidate for use in promoter-regulated cancer gene therapy. One study using *INSM1* promoter-targeted HSV-*tk*/GCV gene therapy for small-cell lung cancer demonstrated effective killing of INSM1-expressing lung cancer cells *in vitro* (Pedersen *et al.*, 2006).

In the present study, we have defined a 1.7-kilobase (kb) region of the *INSM1* promoter that faithfully recapitulates endogenous *INSM1* gene expression in PNETs. We tested both adult and childhood tumors for response to the suicide gene therapy, including neuroblastoma (IMR-32), medulloblastoma (D283 Med), retinoblastoma (Y79), and glioblastoma (U-87 MG). Both Northern blot and luciferase reporter gene assays indicated that *INSM1* mRNA and promoter activities were specifically restricted in normal fetal brain and PNETs. Adenoviral INSM1p-HSV-*tk* constructs showed that the *INSM1* promoter could mediate selective expression of the HSV-*tk* gene and in the presence of GCV induced killing of INSM1-positive tumor cell lines IMR-32, Y79, and D283 Med. *In vivo* studies demonstrated that direct intratumoral injection or *ex vivo* transduction of Ad-INSM1p-HSV-tk significantly retarded tumor growth after treatment with GCV. Therefore, we propose the use of the *INSM1* promoter as an effective transcription-targeted viral gene therapy in PNETs.

Materials and Methods

Cell lines and transfection assay

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human neuroblastoma cell line IMR-32 was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (0.1 mg/ml) in a 5% CO_2 humidified atmosphere at 37°C. Retinoblastoma (Y79), medulloblastoma (D283 Med), and glioblastoma (U-87 MG) cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) in a 5% CO_2 humidified atmosphere at 37°C. Twenty-four hours before transfection, cells were seeded at a density of $0.25–0.3\times10^6$ cells per well in a 6-well culture dish. A ratio of 1 μg of INSM1p-pGL3-Basic vector DNA (Promega, Madison, WI) and 0.25 μg of pCMV-βGal vector to 2.5 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions. The DNA complex was added in the absence of serum for 5 hr at 37°C. The medium was removed and replaced with fresh fetal bovine serum containing medium for a total of 24 hr at 37°C. The cells were collected for luciferase and β-galactosidase (β-Gal) assays (Promega). All experiments were repeated at least three times, and averages with the SEM are shown.

Northern blot analyses

Human fetal tissues were obtained from the Central Laboratory for Human Embryology (University of Washington, Seattle, WA). Human adult brain was obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA). Total RNA was isolated from human adult and fetal tissues, and IMR-32, D283 Med, Y79, and U-87 MG cell lines, using TRIzol reagent according to the instructions of the manufacturer (Invitrogen). *INSM1* and β-actin cDNA probes were random-prime labeled with [^{32}P]dCTP (PerkinElmer Life Sciences, Waltham, MA). Labeled probe (1.0×10^6 cpm/ml) was added to the hybridization mixture as previously described (Breslin *et al.*, 2003). After overnight incubation at 55°C, the blot was washed in 0.1×SSPE (saline–sodium phosphate–EDTA)–0.1% SDS (sodium dodecyl sulfate) and exposed to autoradiography at −70°C for 2 days.

DNA construction and preparation of recombinant adenoviruses

The *INSM1* promoter (bp −1661 to +40) from pGL3-INSM1p (Breslin *et al.*, 2003) was digested with *Kpn*I, blunt-ended, and subsequently digested with *Hin*dIII. The pShuttle-RSV-HSV-tk vector (obtained from the Gene Therapy Core Facility, Louisiana State University Health Sciences Center, New Orleans, LA) was digested with *Hin*dIII to remove the Rous sarcoma virus (RSV)promoter and generate the pShuttle-HSV-tk construct. The pShuttle-HSV-tk plasmid was digested with *Xho*I, blunt-ended, and subsequently digested with *Hin*dIII. The 1.7-kb *INSM1* promoter was inserted into the pShuttle-HSV-tk vector, resulting in pShuttle-INSM1p-HSV-tk. The simian virus 40 (SV40) promoter was excised from the pGL3 control vector (Promega), using *Xho*I and *Hin*dIII, and inserted into the pShuttle-HSV-tk plasmid at the *Xho*I and *Hin*dIII sites. The pShuttle-INSM1p-HSV-tk, pShuttle-SV40p-HSV-tk, and pShuttle-RSV-HSV-tk vectors were electroporated into BJ5183-AD-1 cells. After selecting the correct recombinant clones, 10–20 μg of *Pac*I-digested recombinant adenoviral plasmid DNA was transfected into

AD-293 cells, using an MBS transfection kit (Stratagene, La Jolla, CA). The primary viral stock was amplified, titered, and verified for HSV-tk protein expression. High-titer recombinant adenovirus was purified from forty 150-cm^2 plates, using a two-step CsCl centrifugation method. Adenoviral stocks were titered, using an Adeno-X rapid titer kit (Clontech, Mountain View, CA). The viral titer is expressed as infectious units per milliliter (IFU/ml), as determined with the titer kit. Ad-CMV-LacZ virus was purchased from Vector BioLabs (Philadelphia, PA). Ad-RSV-ntLacZ virus was purchased from ViraQuest (North Liberty, IA). Both commercial adenovirus preparations were subsequently amplified in our laboratory in AD-293 cells, purified by CsCl centrifugation, and titered with the Adeno-X rapid titer kit.

Western blot analysis

After recombinant adenoviral transduction (multiplicity of infection [MOI] of 100), where MOI refers to the infectious units determined with the titer kit, the relative levels of HSV-tk protein expression in IMR-32, Y79, D283 Med, and U-87 MG cells were measured by Western blot analysis. Virally infected cells were collected 48 hr posttransduction, washed twice in 1×phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (25 m*M* Tris-HCl [pH 7.6], 150 m*M* NaCl, 1% Nonidet P-40 [NP-40], 1% sodium deoxycholate, 0.1% SDS with mini-complete protease inhibitor cocktail (Roche, Indianapolis, IN). To decrease whole cell lysate viscosity, samples were sonicated (two 10-sec bursts). The protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology/Thermo Fisher Scientific, Rockford, IL). Total cellular lysates, 100 μg/well, were separated on 10% SDS–polyacrylamide gels and transferred overnight to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline for 30 min and incubated with rabbit anti-HSV-tk antibody (1:1000 dilution; Dr. W. Summers, Yale University, New Haven, CT) for 2 hr. Membranes were washed three times with Tris-buffered saline with 0.5% Tween 20 (each time for 20 min) and then incubated with a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA) for 2 hr. Membranes were washed with Tris-buffered saline with 0.5% Tween 20 three times and developed with a SuperSignal West Pico chemiluminescent substrate kit (Pierce Biotechnology/Thermo Fisher Scientific).

3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

IMR-32 and Y79 cells (each, 2×10^4), D283 Med cells (3×10^4), and U-87 MG cells (1×10^4) were seeded into a 96-well plate. After a 12-hr incubation, the cells were infected with Ad-INSM1p-HSV-tk, Ad-SV40p-HSV-tk, Ad-CMV-LacZ, Ad-RSV-ntLacZ, and Ad-RSV-HSV-tk at various MOIs (0, 1, 10, 50, and 100 IFU). Twenty-four hours posttransduction the cells were treated with 100 μM GCV for an additional 5 days. Alternatively, the cells were infected with a fixed concentration of adenovirus (MOI of 100), and treated with various concentrations of GCV (0–100 μM). Five days later, cell viability was measured in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (ATCC) according to the manufacturer's instructions. The experiments were performed in triplicate on at least three separate occasions.

In vitro *and* in vivo *β-galactosidase staining of D283 Med cells*

D283 Med cells were analyzed both *in vitro* and *in vivo* to demonstrate the efficacy of the *INSM1* promoter-driven HSV-*tk* gene therapy approach. An evaluation of viral delivery was done to verify the viral efficiency of transduction to the tumor cells. D283 cells (2×10^5 per well) were seeded in a 12-well dish. Ad-RSV-ntLacZ virus (MOIs of 0, 1, 10, 50, and 100) was transduced into the cells. After a 48-hr incubation, the cells were fixed in β-Gal fixative (2% formaldehyde, 0.2% glutaraldehyde, 1×PBS [137 m*M* NaCl, 2.6 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4; pH 7.4]), washed twice in 1×PBS, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) stain [5 m*M* $K_3Fe(CN)_6$, 5 m*M* $K_3Fe(CN)_6$, 2 m*M* MgCl, 1×PBS, X-Gal (1 mg/ml)]. The cells were photographed with a×10 objective on an Olympus CX40 inverted microscope equipped with a Spot Insight QE digital camera. D283 subcutaneous tumors were injected with 1×10^9 IFU of Ad-RSV-ntLacZ virus. Forty-eight hours posttransduction, the tumors were removed, fixed, and stained as described previously. The tumors were processed and embedded in paraffin. Ten-micron-thick sections were cut to analyze the distribution of virus within the tumor mass. The sections were counterstained with nuclear fast red. The tumor sections were visualized with an Olympus CX40 inverted microscope equipped with a Spot Insight QE digital camera.

Nude mouse tumor model and Ad-INSM1p-HSV-tk/GCV treatment

Eight-week-old male *nu/nu* strain nude mice were purchased from Charles River Laboratories (Wilmington, MA). To generate the INSM1-positive tumor-bearing model, 5×10^7 D283 Med cells were injected subcutaneously into the hind flank of the nude mice. Four to 6 weeks after injection, the diameter of established tumor reached about 7–9 mm^3. Twenty-four hours before GCV administration, intratumoral injection of 1×10^9 IFU of Ad-INSM1p-HSV-tk ($n=5$), Ad-CMV-LacZ ($n=4$), Ad-RSV-ntLacZ ($n=3$), or Ad-RSV-HSV-tk ($n=4$) was performed. The mice received daily intraperitoneal injections of GCV (50 mg/kg body weight) for 18 days. Tumor volume was calculated on the basis of diameters measured at right angles, done every other day with calipers. Tumor volume was calculated according to the following formula: tumor volume (mm^3) $=4/3\times\pi r^3$ ($r=$ sum of the two diameters divided by 4). For measurement of liver toxicity, blood samples from the saphenous vein were collected on day 0 before adenoviral administration and on day 4 and day 18 of ganciclovir injections (end point) of the gene therapy protocol. Blood samples were collected in capillary tubes from the Ad-RSV-HSV-tk, Ad-INSM1p-HSV-tk, and Ad-RSV-ntLacZ treatment groups. The blood was allowed to clot overnight at 4°C. Blood samples were centrifuged at 15,000×*g* for 5 min and plasma was collected. Plasma samples were stored at −20°C until all samples were collected. Plasma was sent to the University of Missouri Research Animal Diagnostics Laboratory (RADIL, Columbia, MO) for measurement of alanine aminotransferase (ALT)

and aspartate aminotransferase (AST) liver enzymes. Additional liver toxicity studies were performed on three animals per group without tumors were injected intraperitoneally with 1×10^9 IFU of Ad-RSV-HSV-tk or Ad-INSM1p-HSV-tk. Twenty-four hours after virus administration, daily GCV injections were started. Blood samples were collected on days 0, 4, and 7 to assess for liver toxicity. Samples were collected and analyzed as described previously.

For the adenoviral *ex vivo* transduction experiments, 2×10^7 D283 Med cells were infected with 4×10^9 IFU of either Ad-INSM1p-HSV-tk or Ad-CMV-LacZ virus (MOI of 200). The cells were incubated for 24 hr in the presence of virus. The cells were collected by centrifugation, washed twice in sterile PBS, and injected into the left and right flanks of nude mice. Six mice from each group were used for the *ex vivo* study. Twenty-four hours after tumor cell inoculation, daily GCV injections (50 mg/kg body weight) were initiated and continued for 8 weeks. At the end of the 8 weeks, the animals were killed by CO_2 inhalation and any tumor growth was removed and weighed for comparison. At least four animals per group were tested. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Research Institute for Children, Children's Hospital (New Orleans, LA).

Results

Restricted expression of the INSM1 *gene in fetal brain and PNETs*

Northern blot analysis with normal human fetal and adult brain samples showed *INSM1* mRNA expression at the earliest prenatal brain time point, day 45, and absence from adult brain tissue (Fig. 1A). *INSM1* was highly expressed in the human medulloblastoma (D283 Med), retinoblastoma (Y79), and neuroblastoma (IMR-32) cell lines and was not present in the human glioblastoma (U-87 MG) cell line (Fig. 1B). Our previous studies indicated that the 1.7-kb 5′ upstream region of the human *INSM1* gene (bp −1661 to +40) is sufficient for strong, specific expression in the developing nervous system and neuroendocrine cell lines (Breslin *et al.*, 2003). To evaluate *INSM1* promoter specificity *in vitro*, we used the 1.7-kb *INSM1* promoter to drive expression of the luciferase reporter gene in various cell types. Transient transfection experiments demonstrated that *INSM1* promoter activity was highest in IMR-32 cells, had lower activity in D283 Med and Y79 cells, and was weakest in the *INSM1*-negative cell line U-87 MG (Fig. 1C). The promoter activities were consistent with the mRNA expression profiles: high expression in PNETs but low to no activity in adult glioblastoma (U-87 MG) cells. These observations indicate that the *INSM1* promoter would be a valuable tissue-specific promoter for targeted suicide gene therapy in PNETs.

Determination of multiplicity of infection in tumor cell lines

INSM1 promoter-luciferase reporter gene assays demonstrated selective activity of the *INSM1* promoter in INSM1-positive tumor cells, with IMR-32, D283 Med, and Y79 confirming the feasibility of our approach. To test the *in vitro* efficacy and specificity of the *INSM1* promoter, we constructed recombinant adenoviral vectors using the 1.7-kb

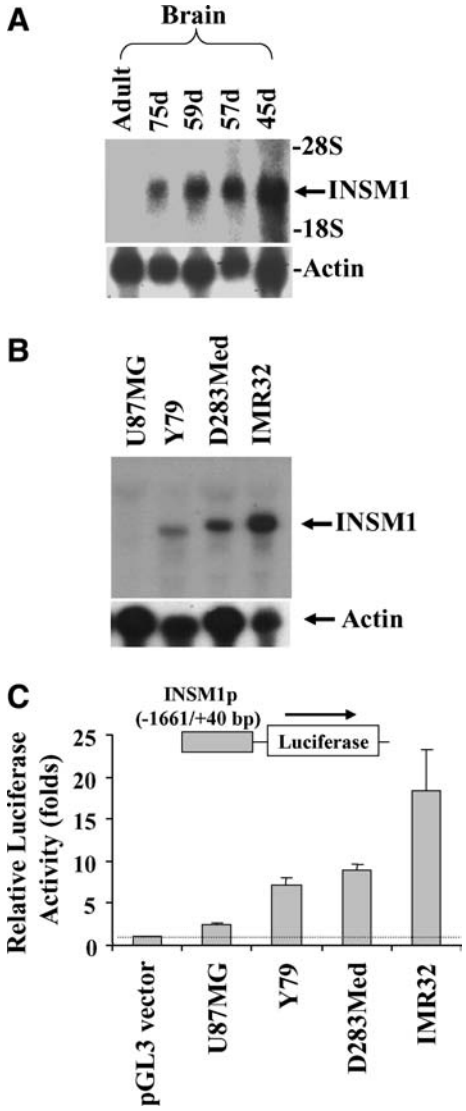

FIG. 1. Northern blot analysis of *INSM1* gene expression and *INSM1* promoter activity. (**A**) Total RNAs (20 μg) from human fetal and adult brain tissue were subjected to Northern blot analysis. Each blot was probed with a ^{32}P-labeled *INSM1* cDNA probe, stripped, and reprobed with actin (loading control). (**B**) Northern blot analysis of human brain tumor cell lines IMR-32 (neuroblastoma), Y79 (retinoblastoma), D283 Med (medulloblastoma), and U-87 MG (glioblastoma). (**C**) Transient transfections of *INSM1* promoter (bp −1661 to +40)-linked luciferase constructs into various tumor cell lines. Luciferase activities from various cell lines were compared with pGL3-Basic vector and presented as relative fold increase. All activities were normalized with β-galactosidase control and averaged from three different experiments. Data are presented as averages ± SEM.

INSM1 promoter, RSV promoter, or SV40 promoter to drive expression of the HSV-*tk* suicide gene. First, we verified the specificity of the *INSM1* promoter in the context of the adenoviral backbone. Ad-INSM1p-HSV-tk, Ad-SV40p-HSV-tk, Ad-CMV-LacZ, Ad-RSV-ntLacZ, or Ad-RSV-HSV-tk virus was transduced into the four tumor cell lines. Cell lysates were collected and subjected to Western blot analysis with a

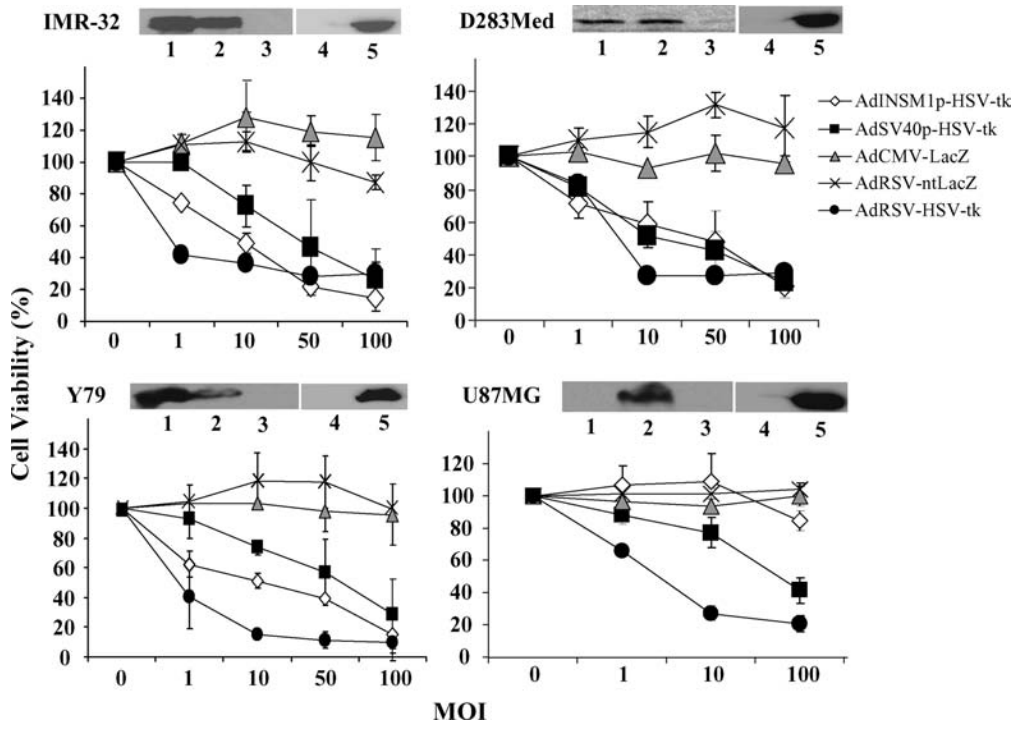

FIG. 2. *In vitro* efficacy of Ad-INSM1p-HSV-tk, Ad-RSV-HSV-tk, and Ad-SV40p-HSV-tk therapy. IMR-32, D283 Med, Y79, and U-87 MG cells were seeded at 2×10^4 cells per well in a 96-well dish. Cells were transduced with 0, 1, 10, 50, or 100 MOI of Ad-INSM1p-HSV-tk (open diamonds), Ad-SV40p-HSV-tk (solid squares), or Ad-RSV-HSV-tk (solid circles), or with control Ad-CMV-LacZ (shaded triangles) or Ad-RSV-ntLacZ (mults). Twenty-four hours after viral infection, the cells were treated with 100 μM GCV for 5 days. MTT proliferation assays were performed in triplicate. The percentage cell viability was calculated relative to no virus addition from three different experiments. *Top of each graph:* Each cell line was transduced with adenovirus (MOI of 100) for 24 hr and the cell lysates were harvested for Western blot analysis using anti-HSV-tk antibody to reveal the expression levels of thymidine kinase protein.

rabbit anti-HSV-tk antibody. As expected, there was strong expression of the HSV-tk protein from the 1.7-kb *INSM1* promoter in IMR-32, D283 Med, and Y79 cells and no expression in the U-87 MG INSM1-negative cell line, whereas expression of HSV-*tk* from the SV40 and RSV promoters was robust in all four cell lines tested (Fig. 2, compare lanes 1, 2, and 5). Next, we tested the *in vitro* efficacy of the HSV-*tk* adenoviral constructs. Various MOIs (0, 1, 10, 50, and 100) of Ad-INSM1p-HSV-tk, Ad-SV40p-HSV-tk, Ad-CMV-LacZ, Ad-RSV-ntLacZ, or Ad-RSV-HSV-tk were transduced into IMR-32, D283 Med, Y79, and U-87 MG cells, using a fixed ganciclovir (GCV) concentration (Fig. 2). Ad-SV40p-HSV-tk, Ad-RSV-HSV-tk, Ad-CMV-LacZ or Ad-RSV-LacZ viruses, was used as positive or negative controls, respectively. Twenty-four hours posttransduction, the cells were treated with 100 μM GCV and incubated for an additional 5 days. MTT cell proliferation assays showed a dose-dependent cytotoxic response with increasing MOI in Ad-INSM1p-HSV-tk-treated (Fig. 2, open diamonds) D283 Med, Y79, and IMR-32 cells. An approximately 80–90% kill rate was seen in IMR-32, Y79, and D283 Med cells at an MOI of 100 (Fig. 2). As expected, all the cell lines were susceptible to killing by the Ad-SV40p-HSV-tk and Ad-RSV-HSV-tk treatment (Fig. 2, solid squares and solid circles), with Ad-RSV-HSV-tk being the most effective in all cell lines even at low MOI. Ad-INSM1p-HSV-tk was more effective than Ad-SV40p-HSV-tk in IMR-32 and Y79 cells, but their results could almost be superimposed in D283 cells. Only Ad-RSV-HSV-tk and Ad-SV40p-HSV-tk exhibited cytotoxicity in U-87 MG cells. Despite the superior killing effect of Ad-RSV-HSV-tk at lower MOIs, at the highest MOI all three promoter-driven adenoviral constructs showed a similar maximal kill rate and no one viral construct was able to completely eliminate all the tumor cells *in vitro*. Even at the highest MOI, control Ad-CMV-LacZ and Ad-RSV-ntLacZ viruses had no significant effect on cell viability in any of the cell lines analyzed. Therefore, these results indicate that the *INSM1* promoter would mediate specific expression of HSV-*tk* gene and selective killing of INSM1-positive tumor cells even in the context of the adenoviral backbone.

In vitro *GCV sensitivity assay*

To compare the cellular toxicity of prodrug-mediated cell killing with Ad-INSM1p-HSV-tk, Ad-SV40p-HSV-tk, Ad-RSV-HSV-tk, Ad-RSV-ntLacZ, and Ad-CMV-LacZ, the D283 Med, Y79, IMR-32, and U-87 MG cell lines were infected at an MOI of 100 and exposed to various doses of GCV continuously for 5 days. Figure 3 shows the sensitivity curves of the four tumor cell lines. In a GCV dose-dependent manner, all promoter–HSV-*tk* viruses mediated efficient cell death. The Ad-INSM1p-HSV-tk virus was superior to both control HSV-*tk* viruses at all GCV concentrations in IMR-32 cells (Fig. 3, top left). In D283 Med cells, at the lower GCV concentration (0.1 and 1 μM) the HSV-*tk* viral constructs had a similar kill rate; however, at higher GCV concentrations

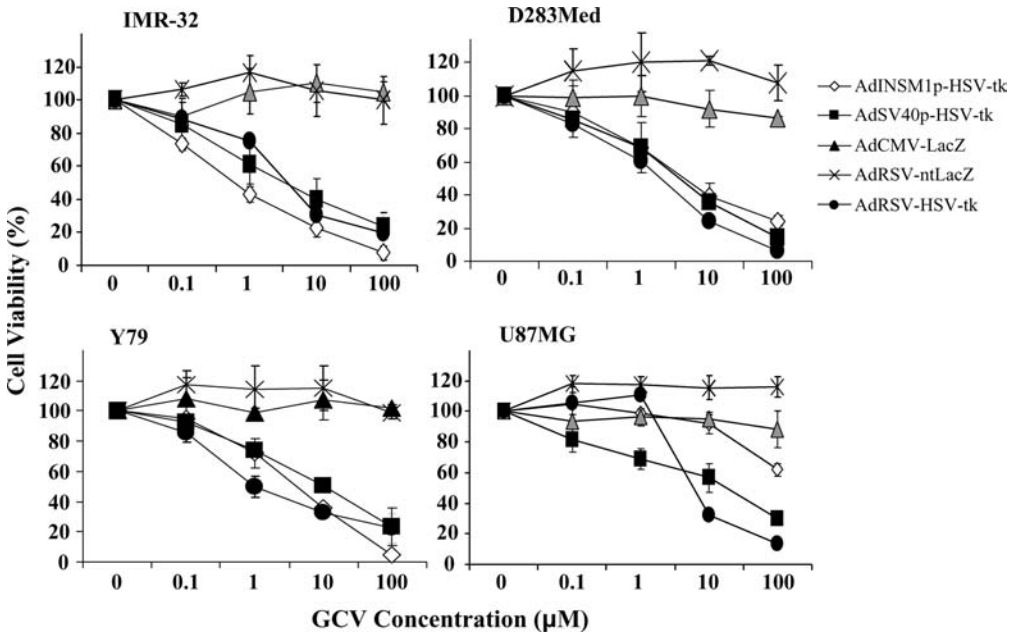

FIG. 3. Prodrug dose–response cell survival curves in various PNET cell lines. IMR-32, Y79, D283 Med, and U-87 MG cell lines were infected with Ad-INSM1p-HSV-tk (open diamonds), Ad-SV40p-HSV-tk (solid squares), Ad-RSV-HSV-tk (solid circles), Ad-RSV-ntLacZ (mults), or Ad-CMV-LacZ at an MOI of 100 and exposed to a range of concentrations (0–100 μM) of GCV. After 6 days, cell viability was measured in an MTT assay. Measurements were performed in triplicate and results are presented as the average of three experiments. The cell viability curve was calculated using zero GCV as 100%.

(10 and 100 μM) the RSV promoter was more effective than either the *INSM1* or SV40 promoter-driven HSV-*tk* viruses (Fig. 3, top right). In Y79 cells, Ad-RSV-HSV-tk showed greater sensitivity to lower doses of ganciclovir than either the Ad-INSM1p or Ad-SV40p-HSV-tk construct, but at the higher GCV doses *INSM1* is as effective if not better than the RSV construct (Fig. 3, bottom left). Again, in U-87 MG cells only at the highest dose of GCV did Ad-INSM1p-HSV-tk show a 30% reduction in cell viability whereas both the Ad-SV40p-HSV-tk and Ad-RSV-HSV-tk constructs showed a maximal kill rate of 70–80% (Fig. 3). Neither negative control (Ad-CMV-LacZ and Ad-RSV-ntLacZ) had any effect on cell viability in any of the cell lines tested, even at the highest dose of GCV (Fig. 3). *INSM1* promoter-driven adenoviral cancer gene therapy shows cell type-specific cellular toxicity consistent with the expression pattern and *in vitro* promoter reporter gene activity.

In vivo *suicide gene therapy*

The luciferase reporter gene assay and *in vitro* efficacy tests demonstrated the feasibility of our *INSM1* promoter-targeted suicide gene therapy. Therefore, we sought to evaluate the *in vivo* efficacy of Ad-INSM1p-HSV-tk therapy for the treatment of D283 Med medulloblastoma. Approximately 5×10^7 D283 Med cells were injected subcutaneously into athymic nude mice via the hind flank. Each tumor was allowed to grow to 0.7–1.0 cm in diameter (4 to 6 weeks) before gene therapy was started. A single intratumoral dose of 1×10^9 IFU of Ad-INSM1p-HSV-tk, Ad-RSV-HSV-tk, Ad-RSV-ntLacZ, or AdCMV-LacZ was injected into each nude mouse. Twenty-four hours after viral delivery, daily intraperitoneal GCV injections (50 mg/kg body weight) were started. Blood from each mouse was collected from the saphenous vein on day 0 and day 18. The tumor volume was measured with calipers every other day for the 18 days. Initial tumor volumes were arbitrarily set at 100% for comparison. Comparison of results from at least four individual mice showed that Ad-CMV-LacZ- and Ad-RSV-ntLacZ-injected tumors continued to grow more than 2-fold with respect to the initial tumor volume (Fig. 4, solid squares and solid circles). Although we were able to eliminate 80–90% of the tumor cells *in vitro*, our *in vivo* data for Ad-INSM1p-HSV-tk or control Ad-RSV-HSV-tk therapy was not as dramatic. In the Ad-INSM1p-HSV-tk-treated tumors only a 15–20% decrease in overall initial tumor volume was measured (Fig. 4, solid triangles). Similarly, treatment with the control Ad-RSV-HSV-tk virus showed a growth retardation curve strikingly similar to that of the Ad-INSM1p-HSV-tk-treated tumors (Fig. 4). Limited efficacy to eliminate the tumor mass could be due to inefficient delivery of the virus throughout the tumor because a single injection in one site was performed. To assess the viral distribution of the virus after direct intratumoral injection, nude mice bearing D283 tumors were injected with Ad-RSV-ntLacZ virus and the tumors were removed 48 hr later to assess viral distribution within the tumors. The tumors were fixed and stained for β-galactosidase activity. Not unexpectedly, the virus was localized primarily to cells in the periphery of the tumor, explaining at least in part the lack of a more impressive response (Fig. 5B). To evaluate whether Ad-INSM1p-HSV-tk was sufficient to suppress tumor growth in the nude mouse tumor model, we tested an alternative delivery route for the cancer gene therapy. *In vitro*, D283 cells are poorly transduced by adenovirus at lower MOIs of 1, 10, and 50; however, they reach a high level of infectivity at an MOI of 100

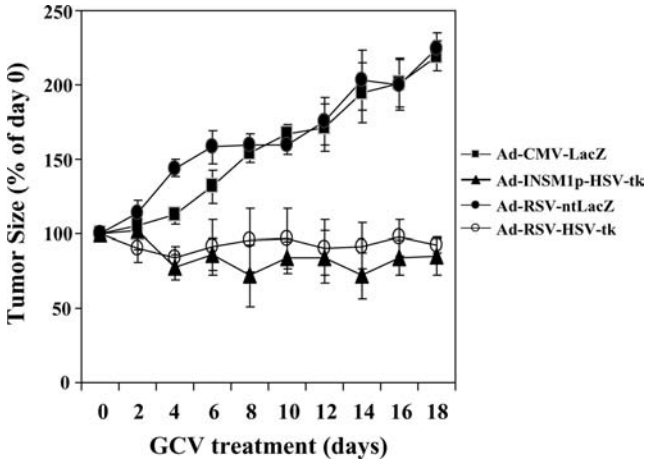

FIG. 4. *In vivo* subcutaneous xenograft nude mouse model. D283 Med cells (5×10^7) were injected subcutaneously into the flank of athymic nude mice. After tumor establishment, approximately 1×10^9 IFU of Ad-CMV-LacZ ($n=4$), Ad-INSM1p-HSV-tk ($n=5$), Ad-RSV-HSV-tk ($n=4$), or Ad-RSV-ntLacZ ($n=3$) was directly injected intratumorally. Twenty-four hours later, GCV (50 mg/kg body weight) was injected intraperitoneally daily. Tumor growth was measured with calipers every other day for 18 days. Tumor volume was calculated according to the following formula: tumor volume (mm^3) $=4/3\times\pi r^3$ ($r=$ sum of the two diameters divided by 4). Data are expressed as size relative to initial tumor volume on the day before initiation of GCV administration.

(Fig. 5A). We infected cells with Ad-INSM1p-HSV-tk virus or Ad-CMV-LacZ virus at an MOI of 200 *ex vivo*, 24 hr before transplantation, to ensure nearly 100% transduction efficiency. The virus-transduced cells were collected and injected subcutaneously into the hind flank of nude mice. Daily GCV injections were started and continued for 8 weeks. At the end of the study, the tumors were removed and weighed for comparison (Fig. 6). Five of six mice in the control group, Ad-CMV-LacZ, grew tumors and the average tumor weight was 0.4 g. In the Ad-INSM1p-HSV-tk group, only three of six mice grew tumors and the average tumor weight was 0.03 g (7% of the control). The three Ad-INSM1p-HSV-tk-treated mice that did not have visible tumors were monitored for an additional 4 weeks without any tumor growth. Using an approach to ensure that the majority of transplanted tumor cells were transduced by the adenovirus, we could effectively prevent up to 93% of tumor cell growth. This result is consistent with the *in vitro* efficacy data. Therefore, these data demonstrate the *in vivo* effectiveness of *INSM1* promoter-driven suicide gene therapy for PNETs.

In vivo *liver toxicity profile in* INSM1*-treated nude mice*

Because of highly selective regulation by the *INSM1* promoter, we anticipate that *in vivo* the promoter will limit expression of the therapeutic gene to the tumor and reduce the overall nontarget liver toxicity that is associated with strong constitutive viral promoters. To assess *in vivo* whether the *INSM1* promoter limits expression of the HSV-*tk* gene to the tumor and minimizes or prevents liver damage, blood samples were collected on day 0 before intratumoral delivery of adenovirus and on day 18 during ganciclovir treatment in nude mice treated with Ad-RSV-ntLacZ, Ad-RSV-HSV-tk, and Ad-INSM1p-HSV-tk. The serum samples were analyzed for levels of ALT and AST liver enzymes. Elevated levels of ALT and AST in the blood signify liver damage. Comparison of the animals treated with RSV-ntLacZ, RSV-HSV-tk, and INSM1p-HSV-tk showed no significant difference between the three treatment groups in terms of ALT and AST enzyme levels. In all three groups the ALT and AST levels remained within the normal range. Because of the limited utility of intratumoral delivery for highly metastatic forms of cancer

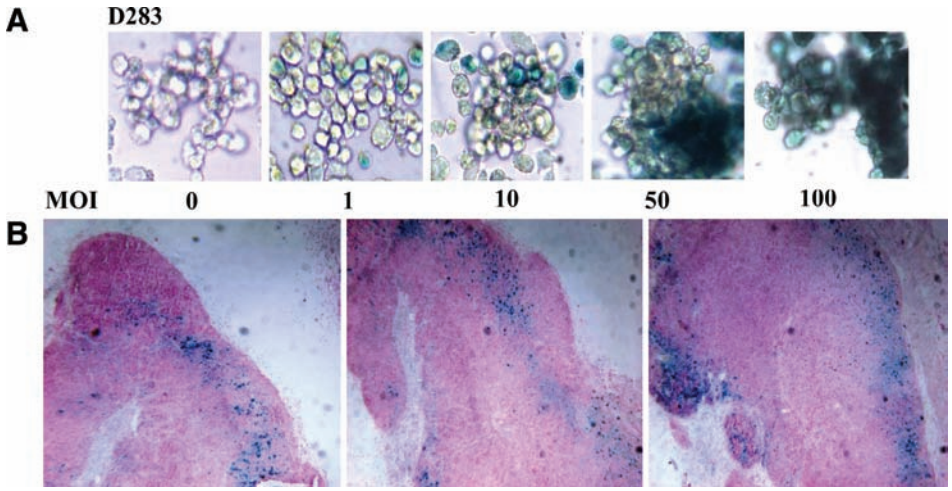

FIG. 5. *In vitro* and *in vivo* adenoviral transduction efficiency in D283 Med cells. (**A**) To determine the transduction efficiency of the adenovirus, 2×10^5 D283 Med cells were infected with Ad-RSV-ntLacZ virus at MOIs of 0, 1, 10, 50, and 100. Forty-eight hours postinfection, the cells were fixed and stained to detect β-galactosidase activity. (**B**) To assess the distribution of virus within D283 tumor, 1×10^9 IFU of Ad-RSV-ntLacZ virus was injected directly into subcutaneous D283 Med tumor. Forty-eight hours postinjection, the tumors were removed, fixed, and stained for β-galactosidase activity to localize the adenovirus within the tumor mass. Blue staining represents β-galactosidase activity. The tumor samples were counterstained with nuclear fast red to visualize the tumor. Color images available online at www.liebertonline.com/hum.

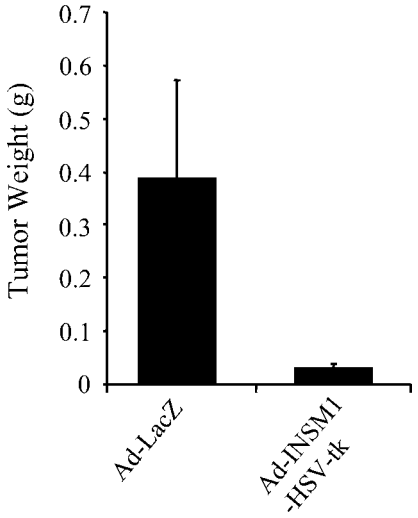

FIG. 6. *Ex vivo* viral transduction of D283 Med cells in nude mouse tumor model. D283 Med cells were transduced with Ad-INSM1p-HSV-tk virus or Ad-CMV-LacZ virus (MOI of 200) *ex vivo* 24 hr before transplantation. The virally transduced cells were collected and injected subcutaneously into the flanks of mice (six mice in each group). Daily GCV injections were continued for 8 weeks until tumor growth was detected. At the end of the study, the tumors were removed and weighed for comparison. Five of six mice in the control group grew tumors and the average tumor weight was 0.4 g. In the Ad-INSM1p-HSVtk group only three of six mice grew tumors and the average tumor weight was 0.03 g (7% of the control). The mice that did not have visible tumors were monitored for an additional 4 weeks without any tumor growth.

and lack of effect even with Ad-RSV-HSV-tk on liver enzymes, additional studies were performed on mice to verify enhanced liver protection when using the Ad-INSM1p-HSV-tk virus. Ad-INSM1p-HSV-tk or Ad-RSV-HSV-tk virus was injected intraperitoneally into male mice. Blood samples from the saphenous vein were collected on day 0 before virus administration and on days 4 and 7 during ganciclovir treatment to assess the safety of systemic Ad-INSM1p-driven suicide gene therapy. In the Ad-RSV-HSV-tk treatment group liver ALT was increased 1.5 to 5 times the normal range on day 7 and liver AST was increased 1.6- to 13-fold the normal range on day 7. All the Ad-INSM1p-HSV-tk mice showed both ALT and AST values to be below the normal range at all time points (days 0, 4, and 7). Our data demonstrate that *INSM1* promoter-driven suicide gene therapy exhibits similar antitumor effects *in vivo* as the strong viral RSV promoter but with no observable liver damage as assessed by serum ALT/AST enzyme levels. Taken together, all these results suggest the feasibility of Ad-INSM1p-HSV-tk as a cancer gene therapy approach even for metastatic disease.

Discussion

In the present study, we investigated whether the *INSM1* neuroendocrine-specific promoter could direct expression of the HSV-*tk* suicide gene in PNET cell lines. INSM1 is characterized as a transcription factor that functions during early nervous system and pancreatic development (Xie *et al.*, 2002; Zhu *et al.*, 2002; Breslin *et al.*, 2003; Gierl *et al.*, 2006; Mellitzer *et al.*, 2006). Our laboratory has shown that INSM1 is a novel transcriptional repressor of the NeuroD1 and insulin genes (Breslin *et al.*, 2002; Wang *et al.*, 2008) and both the upstream transcription factors, ngn3 and NeuroD1, activate the *INSM1* gene during pancreatic ductal epithelial cell differentiation (Mellitzer *et al.*, 2006; Breslin *et al.*, 2007). Other laboratories have demonstrated that INSM1 is critical in directing the proper differentiation of endocrine cells of the pancreas and intestine (Gierl *et al.*, 2006), the sympathoadrenal lineage (Wildner *et al.*, 2008), and for basal progenitor formation in the neocortex (Farkas *et al.*, 2008). *INSM1* expression peaks in early fetal development and is absent from normal adult tissues. Although *INSM1* is silenced in normal adult tissues, it is highly reactivated in tumors of neuroendocrine origin, which display an ideal expression pattern for use in transcriptionally targeted cancer gene therapy (Narita *et al.*, 2001; Adachi *et al.*, 2002; Steffens *et al.*, 2004; Arvidsson *et al.*, 2005). Using a 1.7-kb (bp -1661 to $+40$) *INSM1* promoter-driven *LacZ* transgenic mouse line, we convincingly demonstrated that the information required for tissue-specific expression of the *INSM1* gene is present in the 5′-upstream regulatory region (Breslin *et al.*, 2003). Another group used microarray analysis on multiple human small-cell lung cancer (SCLC) cell lines to identify prominent markers of SCLC (Pedersen *et al.*, 2003). INSM1 was identified as a major differential marker for SCLC along with Hash1 and gastrin-releasing peptide. Using the *INSM1* promoter, they demonstrated the *in vitro* efficacy and specificity of this promoter to drive expression of the HSV-*tk* gene in human SCLC cell lines (Pedersen *et al.*, 2006).

Our data establish *INSM1* gene expression in pediatric tumor cell lines IMR-32, D283 Med, and Y79. We confirmed that the 1.7-kb region of the *INSM1* promoter recapitulates both the tissue specificity and activity seen at the mRNA expression level. We detected the strongest to weakest mRNA signal in IMR-32, D283 Med, and Y79 cells, respectively, and no signal in U-87 MG cells. The same pattern of activity was seen in the *INSM1* promoter-luciferase reporter gene assays. Collectively, this result confirms the feasibility for use of the *INSM1* promoter to transcriptionally target suicide gene therapy to PNETs including medulloblastoma, retinoblastoma, and neuroblastoma.

The HSV-*tk* suicide gene under the control of a strong constitutive viral promoter such as CMV or RSV is an effective strategy for cancer treatment. However, its use is limited by severe liver toxicity (Chen *et al.*, 1995; O'Malley *et al.*, 1995; Brand *et al.*, 1997; van der Eb *et al.*, 1998). Because of the high level of liver toxicity associated with HSV-*tk*/GCV therapy in the context of a human adenoviral vector, combining a tissue-specific promoter with the HSV-*tk*/GCV system to transcriptionally limit the expression of the suicide gene to the tumor cell has become an attractive strategy. One of the major challenges to this approach is to identify a promoter that can limit expression in normal cells and have high expression in the tumor cells. We have characterized a 1.7-kb region of the *INSM1* promoter that directs proper spatial and temporal expression of the *INSM1* gene (Breslin *et al.*, 2003). In this study, we created recombinant adenoviral constructs containing the 1.7-kb *INSM1*, the control SV40 promoter, or the RSV promoter linked with the HSV-*tk*

suicide gene. *In vitro* studies using various MOIs of virus clearly demonstrated cell type-specific elimination of tumor cells in the presence of GCV. Dose-dependent cell killing was evident with the control Ad-SV40p-HSV-tk and Ad-RSV-HSV-tk constructs. However, in the Ad-INSM1p-HSV-tk-treated groups only IMR-32, D283 Med, and Y79 cells were sensitive to GCV-mediated cytotoxicity. As expected, INSM1-negative U-87 MG cells infected with Ad-INSM1p-HSV-tk remained resistant to GCV even at the highest MOI. The *in vitro* data show that the *INSM1* promoter maintains its tissue-specific fidelity in the adenoviral backbone and could efficiently eliminate 80–90% of the tumor cells. These results indicate the usefulness of the *INSM1* promoter to direct cancer gene therapy to PNETs including medulloblastoma, neuroblastoma, and retinoblastoma.

To demonstrate the *in vivo* efficacy of Ad-INSM1p-HSV-tk suicide gene therapy, immunodeficient nude mice were injected subcutaneously with human D283 Med cells to establish tumors. Once the tumors reached 0.7–1.0 mm^3, the mice were given a single intratumoral injection of Ad-INSM1p-HSV-tk, Ad-RSV-HSV-tk, Ad-RSV-ntLacZ, or Ad-CMV-LacZ virus. After adenoviral delivery, the animals were given daily intraperitoneal injections of GCV and the tumors were monitored with calipers. Ad-INSM1p-HSV-tk-treated tumors shrank by 20% relative to their initial size and did not grow for the duration of the GCV treatment. Similarly, Ad-RSV-HSV-tk-injected tumors shrank by a maximum of 15–20% relative to their initial size and all further growth was inhibited.Conversely, the Ad-CMV-LacZ and Ad-RSV-ntLacZ control animal tumors continued to grow 2 to 2.5 times the size of the initial tumors. Although we did not completely eradicate the tumors in our experiments, a partial therapeutic effect was evident and found to be as effective as for the Ad-RSV-HSV-tk treatment group. Our results are similar to those of Anderson and colleagues, who reported that breast tumors regressed dramatically but not completely after adenovirus-mediated tissue-targeted cancer gene therapy (Anderson *et al.*, 1999). There are several possible reasons for the partial regression of the tumors *in vivo*. One reason may be inefficient viral transduction into the tumor mass. The advantage of the HSV-tk/GCV system is the bystander effect, whereby the toxic drug formed is transferred to the nontransduced surrounding cells via gap junctions (Mesnil *et al.*, 1996). However, if the transduced cells are not in direct contact with the nontransduced cells the bystander effect will be limited. This possibility was explored by β-galactosidase staining of Ad-RSV-ntLacZ-injected tumor. Staining of xenograft D283 tumors showed that the virus was concentrated at the site of initial injection along the tumor periphery and not found throughout the tumor mass. In three separate sections, the virus was localized to the tumor borders. Therefore, future efforts need to be made to ensure more uniform delivery of the virus therapy within the tumor. Liver toxicity was measured to assess the safety of the viral gene therapy. When virus was delivered intratumorally, serum liver ALT or AST enzyme activity remained in the normal range even in the Ad-RSV-HSV-tk groups. This implies that the amount of virus that leaves the primary site of injection was limited. Thus, to assess any enhanced safety associated with Ad-INSM1p-HSV-tk therapy, intraperitoneal injections of Ad-RSV-HSV-tk and Ad-INSM1p-HSV-tk were directly compared after a short course of daily GCV injections. Clearly, the Ad-INSM1p-HSV-tk virus demonstrated enhanced safety because of the complete lack of change in liver enzymes in the plasma during the GCV treatment compared with a significant increase in both ALT and AST enzymes in the plasma of Ad-RSV-HSV-tk-treated mice. To ensure that the adenovirus efficiently transduced D283 Med tumor cells, we infected the cells *ex vivo* at an MOI of 200 before subcutaneous transplantation. After transplantation, GCV treatment was initiated and continued for approximately 8 weeks. Any visible tumors were removed and weighed. Just to prove the approach in principle, the outcome of this treatment showed a dramatic suppression of tumor growth similar to the *in vitro* study (Fig. 2), suggesting that ineffective viral delivery via the intratumoral injection could hamper eradication of the existing tumor. Staining of D283 cells infected with Ad-RSV-ntLacZ at various MOIs (0, 1, 10, 50, and 100) showed that close to 100% of the D283 cells are transduced at an MOI of 100. Other studies have clearly shown that the level of expression from various tissue-specific promoters is weak *in vivo* (Iyer *et al.*, 2001). However, this explanation is not as likely given that a similar tumor regression profile was observed with the Ad-RSV-HSV-tk and Ad-INSM1p-HSV-tk viruses (Fig. 4). One way to enhance the activity of the *INSM1* promoter is by incorporating an enhancer that increases promoter activity but maintains tissue selectivity. We incorporated a neuron-specific regulatory element with the *INSM1* promoter and showed *in vitro* that it can increase the activity of the promoter while still maintaining the specificity (M.B. Breslin and M.S. Lan, unpublished results). Another strategy to boost *in vivo* activity could be to link the *INSM1* promoter to a modified/mutated HSV-*tk* gene that demonstrates increased activity. One HSV-*tk* mutant, SR39, has been described and tested extensively in gene therapy studies (Black *et al.*, 2001; Wiewrodt *et al.*, 2003). Therefore, improving the delivery system, enhancing *INSM1* promoter activity, and linking a more active mutant HSV-*tk* gene could provide a more robust outcome for this cancer gene therapy strategy.

In summary, the *INSM1* promoter is a promising novel candidate tumor-specific promoter for use in PNETs such as neuroblastoma, medulloblastoma, and retinoblastoma. The key advantage of the *INSM1* promoter is that it has no or extremely low activity in normal adult tissues. This aspect makes the *INSM1* promoter particularly safe and suitable for systemic delivery of suicide gene therapy.

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Author Disclosure Statement

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References

Adachi, Y., Matsubara, S., Muramatsu, T., Curiel, D.T., and Reynolds, P.N. (2002) Midkine promoter-based adenoviral

suicide gene therapy to midkine-positive pediatric tumor. J. Pediatr. Surg. 37, 588–592.

Anderson, L.M., Swaminathan, S., Zackon, I., Tajuddin, A.K., Thimmapaya, B., and Weitzman, S.A. (1999) Adenovirus-mediated tissue-targeted expression of the HSVtk gene for the treatment of breast cancer. Gene Ther. 6, 854–864.

Arvidsson, Y., Sumantran, V., Watt, F., Uramoto, H., and Funa, K. (2005) Neuroblastoma-specific cytotoxicity mediated by the *Mash1*-promoter and *E. coli* purine nucleoside phosphorylase. Pediatr. Blood Cancer 44, 77–84.

Berthold, F., and Hero, B. (2000) Neuroblastoma: Current drug therapy recommendations as part of the total treatment approach. Drugs 59, 1261–1277.

Black, M.E., Kokoris, M.S., and Sabo, P. (2001) Herpes simplex virus-1 thymidine kinase mutants created by semi-random sequence mutagenesis improve prodrug-mediated tumor cell killing. Cancer Res. 61, 3022–3026.

Brand, K., Arnold, W., Bartels, T., Lieber, A., Kay, M.A., Strauss, M., and Dorken, B. (1997) Liver-associated toxicity of the HSV-tk/GCV approach and adenoviral vectors. Cancer Gene Ther. 4, 9–16.

Breslin, M.B., Zhu, M., Notkins, A.L., and Lan, M.S. (2002) Neuroendocrine differentiation factor, IA-1, is a transcriptional repressor and contains a specific DNA-binding domain: identification of consensus IA-1 binding sequence. Nucleic Acids Res. 30, 1038–1045.

Breslin, M.B., Zhu, M., and Lan, M.S. (2003) NeuroD1/E47 regulates the E-box element of a novel zinc-finger transcription factor, IA-1, in developing nervous system. J. Biol. Chem. 278, 38991–38997.

Breslin, M.B., Wang, H.W., Pierce, A., Aucoin, R., and Lan, M.S. (2007) Neurogenin 3 recruits CBP co-activator to facilitate histone H3/H4 acetylation in the target gene INSM1. FEBS Lett. 581, 949–954.

Chen, S.H., Chen, X.H., Wang, Y., Kosai, K., Finegold, M.J., Rich, S.S., and Woo, S.L. (1995) Combination gene therapy for liver metastasis of colon carcinoma *in vivo*. Proc. Natl. Acad. Sci. U.S.A. 92, 2577–2581.

DeSmaele, E., Fragomeli, C., Ferretti, E., Pelloni, M., Po, A., Canettieri, G., Coni, S., Dimarcotullio, L., Greco, A., Moretti, M., Dirocco, C., Pazzaglia, S., Maroder, M., Screpanti, I., Giannini, G., and Gulino, A. (2008) An integrated approach identifies Nhlh1 and Insm1 as Sonic Hedgehog-regulated genes in developing cerebellum and medulloblastoma. Neoplasia 10, 89–98.

Farkas, L.M., Haffner, C., Giger, T., Khaitovich, P., Nowick, K., Birchmeier, C., Paabo, S., and Huttner, W.B. (2008) Insulinoma-associated 1 has a panneurogenic role and promotes the generation and expansion of basal progenitors in the developing mouse neocortex. Neuron 60, 40–55.

Gierl, M.S., Karoulias, N., Wende, H., Strehle, M., and Birchmeier, C. (2006) The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. Genes Dev. 20, 2465–2478.

Goto, Y., Desilva, M.G., Toscani, A., Prabhakar, B.S., Notkins, A.L., and Lan, M.S. (1992) A novel human insulinoma-associated cDNA, IA-1, encodes a protein with zinc-finger DNA-binding motifs. J. Biol. Chem. 267, 15252–15257.

Iyer, M., Wu, L., Carey, M., Wang, Y., Smallwood, A., and Gambhir, S.S. (2001) Two-step transcriptional amplification as a method for imaging reporter gene expression using weak promoters. Proc. Natl. Acad. Sci. U.S.A. 98, 14595–14600.

Mellitzer, G., Bonne, S., Luco, R.F., Van De Casteele, M., Lenne, N., Collombat, P., Mansouri, A., Lee, J., Lan, M.S., Pipeleers, D., Nielsen, F.C., Ferrer, J., Gradwohl, G., and Heimberg, H. (2006) IA-1 is Ngn3-dependent and essential for differentiation of the endocrine pancreas. EMBO J. 25, 1344–1352.

Mesnil, M., Piccoli, C., Tiraby, G., Willecke, K., and Yamasaki, H. (1996) Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. Proc. Natl. Acad. Sci. U.S.A. 93, 1831–1835.

Moolten, F.I., Wells, J.M., Heyman, R.A., and Evans, R.M. (1990) Lymphoma regression induced by ganciclovir in mice bearing a herpes thymidine kinase transgene. Hum. Gene Ther. 1, 125–134.

Narita, M., Bahar, R., Hatano, M., Kang, M.M., Tokuhisa, T., Goto, S., Saisho, H., Sakiyama, S., and Tagawa, M. (2001) Tissue-specific expression of a suicide gene for selective killing of neuroblastoma cells using a promoter region of the NCX gene. Cancer Gene Ther. 8, 997–1002.

O'Malley, B.W., Chen, S.H., Schwartz, M.R., and Woo, S.L.C. (1995) Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. Cancer Res. 55, 1080–1085.

Oyharcabal-Bourden, V., Kalifa, C., Gentet, J.C., Frappaz, D., Edan, C., Chastagner, P., Sariban, E., Pagnier, A., Babin, A., Pichon, F., Neuenschwander, S., Vinchon, M., Bours, D., Mosseri, V., Le Gales, C., Ruchoux, M., Carrie, C., and Doz, F. (2005) Standard-risk medulloblastoma treated by adjuvant chemotherapy followed by reduced-dose craniospinal radiation therapy: A French Society of Pediatric Oncology Study. J. Clin. Oncol. 23, 4726–4734.

Packer, R.J., Goldwein, J., Nicholson, H.S., Vezina, L.G., Allen, J.C., Ris, M.D., Muraszko, K., Rorke, L.B., Wara, W.M., Cohen, B.H., and Boyett, J.M. (1999) Treatment of children with medulloblastomas with reduced-dose craniospinal radiation therapy and adjuvant chemotherapy: A Children's Cancer Group Study. J. Clin. Oncol. 17, 2127–2136.

Pedersen, N., Mortensen, S., Sorensen, S.B., Pedersen, M.W., Rieneck, K., Bovin, L.F., and Poulsen, H.S. (2003) Transcriptional gene expression profiling of small cell lung cancer cells. Cancer Res. 63, 1943–1953.

Pedersen, N., Pedersen, M.W., Lan, M.S., Breslin, M.B., and Poulsen, H.S. (2006) The insulinoma-associated 1: A novel promoter for targeted cancer gene therapy for small-cell lung cancer. Cancer Gene Ther. 13, 375–384.

Seth, P. (2005) Vector-mediated cancer gene therapy: An overview. Cancer Biol. Ther. 4, 512–517.

Steffens, S., Sandquist, A., Frank, S., Fischer, U., Lex, C., Rainov, N.G., and Kramm, C.M. (2004) A neuroblastoma-selective suicide gene therapy approach using the tyrosine hydroxylase promoter. Pediatr. Res. 56, 268–277.

van der Eb, M.M., Cramer, S.J., Vergouwe, Y., Schagen, F.H., van Krieken, J.H., van der Eb, A.J., Borel Rinkes, I.H., van de Velde, C.J., and Hoeben, R.C. (1998) Severe hepatic dysfunction after adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene and ganciclovir administration. Gene Ther. 5, 451–458.

Wang, H.W., Muguira, M., Liu, W.D., Zhang, T., Chen, C., Aucoin, R., Breslin, M.B., and Lan, M.S. (2008) Identification of an INSM1 binding site in the insulin promoter: Negative regulation of the insulin gene transcription. J. Endocrinol. 198, 29–39.

Wiewrodt, R., Amin, K., Kiefer, M., Jovanovic, V.P., Kapoor, V., Force, S., Chang, M., Lanuti, M., Black, M.E., Kaiser, L.R., and Albelda, S.M. (2003) Adenovirus-mediated gene transfer of enhanced herpes simplex virus thymidine kinase mutants

improves prodrug-mediated tumore cell killing. Cancer Gene Ther. 10, 353–364.
Wildner, H., Gierl, M.S., Strehle, M., Pla, P., and Birchmeier, C. (2008) Insm1 (IA-1) is a crucial component of the transcriptional network that controls differentiation of the sympathoadrenal lineage. Development 135, 473–481.
Xie, J.P., Cai, T., Zhang, H., Lan, M.S., and Notkins, A.L. (2002) The zinc-finger transcription factor INSM1 is expressed during embryo development and interacts with the Cbl-associated protein. Genomics 80, 54–61.
Zhu, M., Breslin, M.B., and Lan, M.S. (2002) Expression of a novel zinc-finger cDNA, IA-1, is associated with rat AR42J cells differentiation into insulin-positive cells. Pancreas 24, 139–145.

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