A combinatorial approach for selectively inducing programmed cell death in human pancreatic cancer cells

Zao-zhong Su*†, Irina V. Lebedeva*†, Rahul V. Gopalkrishnan*, Neil I. Goldstein‡, C. A. Stein§, John C. Reed¶, Paul Dent‖, and Paul B. Fisher*,††‡‡**

Departments of *Urology, **Pathology, §Medicine, and ††Neurosurgery, Herbert Irving Comprehensive Cancer Center, Columbia University, College of Physicians and Surgeons, New York, NY 10032; ‡DGI Biotechnologies, Edison, NJ 08818; ¶Burnham Institute, La Jolla, CA 92037; and ‖Department of Radiation Oncology, Medical College of Virginia, Richmond, VA 23298

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Pancreatic cancer is an extremely aggressive neoplasm whose incidence equals its death rate. Despite intensive analysis, the genetic changes that mediate pancreatic cancer development and effective therapies for diminishing the morbidity associated with this disease remain unresolved. Through subtraction hybridization, we have identified a gene associated with induction of irreversible growth arrest, cancer reversion, and terminal differentiation in human melanoma cells, melanoma differentiation associated gene-7 (*mda*-7). Ectopic expression of *mda*-7 when using a recombinant adenovirus, Ad.*mda*-7, results in growth suppression and apoptosis in a broad spectrum of human cancers with diverse genetic defects, without exerting deleterious effects in normal human epithelial or fibroblast cells. Despite the apparently ubiquitous antitumor effects of *mda*-7, pancreatic carcinoma cells are remarkably refractory to Ad.*mda*-7 induced growth suppression and apoptosis. In contrast, the combination of Ad.*mda*-7 with antisense phosphorothioate oligonucleotides, which target the K-ras oncogene (a gene that is mutated in 85 to 95% of pancreatic carcinomas), induces a dramatic suppression in growth and a decrease in cell viability by induction of apoptosis. In mutant K-*ras* pancreatic carcinoma cells, programmed cell death correlates with expression and an increase, respectively, in *MDA*-7 and *BAX* proteins and increases in the ratio of *BAX* to *BCL*-2 proteins. Moreover, transfection of mutant K-*ras* pancreatic carcinoma cells with an antisense K-*ras* expression vector and infection with Ad.*mda*-7 inhibits colony formation *in vitro* and tumorigenesis *in vivo* in nude mice. These intriguing observations demonstrate that a combinatorial approach, consisting of a cancer-specific apoptosis-inducing gene and an oncogene inactivation strategy, may provide the foundation for developing an effective therapy for pancreatic cancer.

Pancreatic cancer is the fourth leading cause of cancer deaths; it is estimated that 29,200 cases will be diagnosed in the US in 2001, and 28,900 of these patients will die (1). Moreover, long-term survival for patients with organ-confined disease is only 20%, and in the majority of cases, in which the disease when diagnosed has already spread past the pancreas, survival is only 4% (2–6). These findings underscore the need for developing improved therapies for this aggressive cancer. Although they are the subjects of intensive study, the defining molecular determinants of pancreatic cancer and effective therapies for this disease remain elusive (2, 6–8). Pancreatic cancer is a complex ailment in which multiple subsets of genes undergo genetic change, either activation or inactivation, during tumor development and progression (2, 7, 8). Frequent genetic modifications in pancreatic carcinomas include activation of the K-*ras* oncogene (85–95%) and inactivation of the p16/RB1 (>90%), p53 (75%), and DPC4 (55%) tumor suppressor genes (2, 7, 8). These findings highlight the complexity of this cancer and may provide a partial explanation for the aggressiveness and inherent resistance of this neoplasm to conventional therapies, including chemotherapy and radiation (3–6).

The effects of modifying the expression of the K-*ras* oncogene that is genetically altered in pancreatic carcinoma cells on growth and viability, both *in vitro* and *in vivo* in athymic nude mice, have been examined (9–11). Antisense (AS) targeting of K-*ras* using a plasmid (9, 10) or with mutation-specific phosphorothioate oligodeoxynucleotides (PS ODN) (11) inhibits the growth of pancreatic cancer cells containing K-*ras* mutations, but not pancreatic carcinoma cells containing a wild-type (wt) K-*ras* gene. Specificity of the antisense mutant (mut) K-*ras* PS ODN approach was indicated by the absence of a growth inhibitory effect when using mutation-mismatched (MM) PS ODN, a direct reduction in the levels of K-*ras* p21 protein in AS treated cells, and the absence of a growth-suppressive effect in wt K-*ras* pancreatic cancer cells (11). Additionally, liposome-mediated *in vivo* gene transfer of an AS K-*ras* expression plasmid in animals containing AsPC-1 tumor cells, which represents a peritoneal dissemination model of pancreatic cancer, significantly suppressed tumor development in the peritoneal cavity (9). An important role for K-*ras* in pancreatic cancer physiology is further suggested by the ability of the dominant negative H-*ras* mut, N116Y, to suppress pancreatic cancer cell growth *in vitro* and *in vivo*, including tumorigenesis and metastasis to the liver of nude mice (12, 13). Although promising, these studies demonstrate that a single approach of inhibiting K-*ras* is not sufficient to completely eradicate pancreatic carcinoma cells (9–13).

Treatment of human melanoma cells with a combination of fibroblast IFN and the protein kinase C-activating agent mezerein results in an irreversible suppression in cell growth, loss of tumorigenic potential, and induction of terminal cell differentiation (14). Using a modified subtraction hybridization protocol, genes displaying elevated expression resulting from changes in cancer cell physiology in combination-treated melanoma cells were isolated (15). This scheme identified melanoma differentiation-associated gene-7 (*mda*-7), which displays elevated expression in human melanoma cells induced to terminally differentiate (16). When transfected into a wide spectrum of human cancers, growth is suppressed (17, 18). In contrast, no significant growth inhibitory effect is apparent when this gene

Abbreviations: *mda*, melanoma differentiation-associated; PS ODN, phosphorothioate oligodeoxynucleotides; AS, antisense; MM, mismatched; SC, scrambled; wt, wild type; mut, mutant; Ad, adenovirus; MAPK, mitogen-activated protein kinase; PI3-kinase, phosphatidylinositol 3-kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

See commentary on page 10028.

†Z.-z.S. and I.V.L. contributed equally to this work.

‡‡To whom reprint requests should be addressed. E-mail: pbf1@columbia.edu.

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is transfected into normal human fibroblast or epithelial cells (17, 18). Similarly, when expressed from a replication-incompetent Ad, Ad.*mda*-7, induction of growth suppression and apoptosis is also apparent in diverse human cancers, whereas no harmful effect is evident in normal human cells (18–21). These studies indicate that *mda*-7 may prove useful for the gene-based therapy of diverse human tumors.

Pancreatic cancers are recognized as one of the most therapeutically refractory neoplasms, and they are inherently resistant to ectopic expression of *mda*-7, which in contrast causes significant growth suppression and apoptosis in most other human cancers (18–21). The extensive genetic changes that occur in this tumor may mediate both therapeutic resistance and lack of susceptibility to *mda*-7. Of the genetic changes that occur in pancreatic cancers, mutations in the K-*ras* gene, predominantly in codon 12, are the most frequent (2, 22, 23). This observation prompted us to investigate the putative role of mut K-*ras* in mediating resistance to *mda*-7. To achieve this aim, we evaluated the consequence of a combinatorial approach involving forced expression of *mda*-7 and targeted K-*ras* gene suppression on the growth and survival of pancreatic carcinoma cells. We presently demonstrate that a single treatment of mut and wt K-*ras* pancreatic carcinoma cell lines with AS PS ODN or infection with Ad.*mda*-7 variably modifies cell growth without significantly decreasing cell survival. In contrast, the combination treatment protocol results in a striking synergistic growth inhibitory and antisurvival effect, which is apparent strictly in mut K-*ras* pancreatic cancer cells. Moreover, when MIA PaCa-2 cells are infected with Ad.*mda*-7, transfected with an AS K-*ras* expression vector, and then injected into athymic nude mice, tumor formation is prevented. These intriguing observations suggest that a combinatorial approach consisting of a cancer-specific apoptosis-inducing gene and an oncogene inactivation strategy could provide the basis for a new and potentially effective therapy for pancreatic cancer.

Materials and Methods

Cell Lines, Culture Conditions, and Growth Assays. The AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1 human pancreatic carcinoma cell lines (American Type Culture Collection) were grown in RPMI medium 1640 containing 10% FBS at 37°C in a 95% air, 5% CO_2 humidified incubator. Cell growth and viable cell numbers were monitored by hemocytomer and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining as described (24).

Animal Studies. Tumorigenicity assays were performed as described (18, 19). Briefly, MIA PaCa-2 cells were untreated or infected with 100 plaque-forming units (pfu)/cell of Ad.*vec* or Ad.*mda*-7 and then untransfected or transfected with an expression vector containing a 346-nt K-*ras* gene fragment (nt 172 to nt 517) cloned in a sense or AS orientation, and 1×10^6 cells were mixed with Matrigel and injected 48 h later s.c. into athymic nude mice. Animals were monitored for tumor formation, and tumor volume was determined (18).

Viral Construction, Purification, and Infectivity Assays. The replication-defective Ad.*mda*-7 was created in two steps as described (17, 19). Ad.*mda*-7 and Ad.*vec* (control virus lacking the *mda*-7 gene) were grown in 293 cells, and recombinant structure, plaque purification, and titrations of virus were performed as described (25).

PS ODN. Eighteen-base PS ODN were synthesized and purified by HPLC (24). AS K-*ras* PS ODN, CACAAGTTTATATTCAGT, were synthesized that were complementary to wt K-*ras* nucleotides 196–213 (adjacent to the start codon). Based on previous studies (26), MM K-*ras* PS ODN, CACTTGTAAATATTCAGT, and scrambled (SC) K-*ras* PS ODN, ACTAGCTATACTAGCTAT, to the same region (nucleotides 196–213), were synthesized.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated by the guanidinium/phenol procedure, and Northern blots were performed as described (15, 16).

DNA Extraction, Fragmentation Assays, FACS Analysis, and Annexin V, PI, and 4′,6-Diamidino-2-phenylindole Staining. DNA was extracted, and fragmentation assays were performed as described (27) 3 days after a single or combination treatment protocol. FACS analysis and Annexin V and PI staining reactions were performed by using previously described methods (19, 24, 27, 28).

Western Blotting. Cell extracts in RIPA buffer were prepared, and equal concentrations of proteins were evaluated for *MDA*-7, *BCL*-2, *BAX*, and *EF-1α* protein levels by Western blotting as described (11, 24, 29). Radioautograms were scanned by densitometer.

Results and Discussion

The Combination of Ad.*mda*-7 and AS K-*ras* PS ODN Synergistically Suppresses Growth in mut K-*ras* Expressing Human Pancreatic Carcinoma Cells. *mda*-7 is a broad-spectrum cancer-specific growth-suppressing gene, which displays no apparent harmful effects in normal cells (16–21). Infection of a diverse group of human cancers with Ad.*mda*-7, including melanoma, glioblastoma multiforme, and osteosarcoma, and carcinomas of the breast, cervix, colon, endometrium, lung, and prostate, results in growth suppression and hypodiploidy, a cellular change frequently associated with apoptosis (17–21). In a detailed study with several breast carcinoma cell lines, the ability of Ad.*mda*-7 to induce growth suppression was found to be independent of *p53* status and to correlate with induction of apoptosis, as monitored by DNA nucleosomal laddering, the terminal deoxynucleotidyltransferase-mediated UTP end-labeling reaction, and Annexin V staining (18, 19, 21). In contrast, growth was minimally affected, and no induction of apoptosis was apparent in early passage normal mammary epithelial cells or the normal breast epithelial cell line, HBL-100, after infection with Ad.*mda*-7.

When evaluating the effect of *mda*-7 on diverse cancer subtypes, it was readily apparent that pancreatic carcinoma cells are inherently resistant to ectopic expression of *mda*-7. Infection of mut or wt K-*ras* expressing pancreatic carcinoma cells with 100 pfu/cell of Ad.*mda*-7 or Ad.*vec* (the Ad construct lacking the *mda*-7 gene insert) did not significantly effect growth, and no selective induction of apoptosis was evident (Fig. 1 and data not shown). A dose-dependent growth inhibitory effect was apparent when the different pancreatic carcinoma cells were treated with 0.1–5 μM AS K-*ras* PS ODN, with a maximum inhibition of ≈10–30% depending on the cell type when treated for 3 or 4 days with 5 μM AS K-*ras* PS ODN (Fig. 1). Growth of BxPC-3 was inhibited the least by the K-*ras* PS ODN. Selectivity of the AS K-*ras* PS ODN was suggested by the fact that treatment with either SC or MM PS ODN resulted in significantly less growth suppression than treatment with the AS K-*ras* PS ODN (Fig. 1 and data not shown). These studies document that a single application of Ad.*mda*-7 or AS K-*ras* PS ODN to mut or wt K-*ras* pancreatic carcinoma cell lines can induce variable degrees of growth suppression. However, in all cases, growth suppression was transient, and cells survived the single treatment and continued to proliferate, even when initially exposed to 5 μM PS ODN.

When mut K-*ras* pancreatic carcinoma cells were infected with Ad.*mda*-7 and then treated with 0.1–5.0 μM AS K-*ras* PS ODN, but not SC or MM PS ODN, a profound synergistic growth

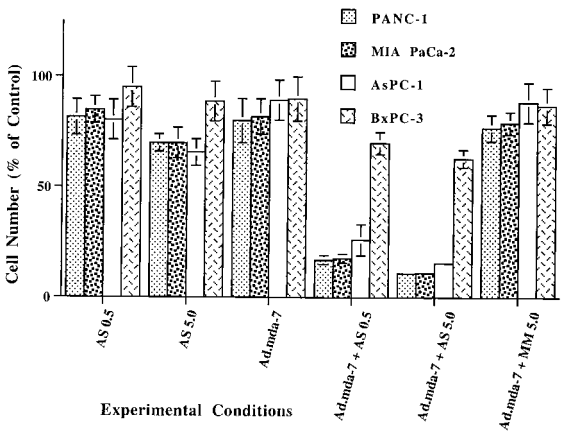

Fig. 1. Synergistic inhibition of growth in mut K-*ras* pancreatic carcinoma cells by the combination of Ad.*mda*-7 and AS K-*ras* PS ODN. Cells were treated with the indicated agents for 3 days, and viable cell counts were determined by hemocytometer. Qualitatively similar results were obtained by MTT staining. AS PS ODN, 0.5 or 5.0 μM; Ad.*mda*-7, 100 pfu per cell; MM PS ODN, 5.0 μM. Results are average of four plates $\pm$ SD from the mean. Qualitatively similar results were obtained in an additional experiment.

inhibitory effect and a decrease in cell survival were evident (Figs. 1 and 2). In contrast, no synergistic growth inhibition or decrease in cell viability was detected in wt K-*ras* BxPC-3 cells (Figs. 1 and 2). Additionally, no effect on growth or viability was apparent with any of the treatments in early passage normal human prostate epithelial cells or when pancreatic cancer cells were infected with an adenovirus expressing luciferase or β-galactosidase and then treated with AS K-*ras* PS ODN (data not shown). These results document an antisurvival effect of the combination of *mda*-7 and AS K-*ras* PS ODN in mut K-*ras* pancreatic carcinoma cells, but not in wt K-*ras* pancreatic cancer cells or normal epithelial cells.

A previous study has reported that AS K-*ras* PS ODN that target specific point mutations in K-*ras* codon 12 can reduce growth in mutant pancreatic carcinoma cell lines, but not in wt K-*ras* BxPC-3 cells (11). This effect was greater when using appropriate mutation-matched AS PS ODN versus mutation-mismatched PS ODN. Effects on growth, although less, were also

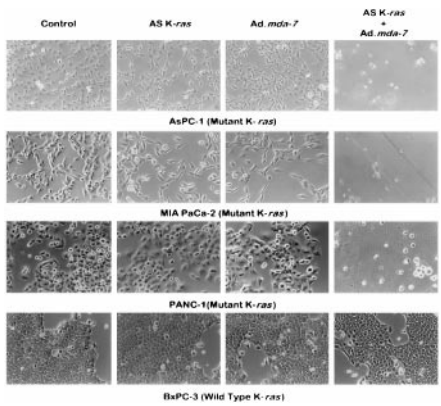

Fig. 2. The combination of Ad.*mda*-7 with AS K-*ras* PS ODN synergistically suppresses growth and decreases survival in mut K-*ras* pancreatic carcinoma cells. The different pancreatic carcinoma cell lines were treated as indicated, and representative microscopic fields were photographed 3 days later. Cells were untreated (control), treated with 0.5 μM AS K-*ras* PS ODN, infected with Ad.*mda*-7 (100 pfu per cell) or infected with Ad.*mda*-7 (100 pfu per cell) and then treated with the 0.5 μM AS K-*ras* PS ODN.

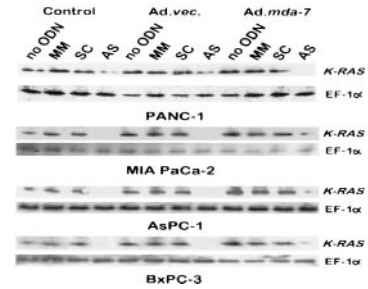

Fig. 3. AS K-*ras* PS ODN inhibits K-*RAS* protein synthesis in pancreatic carcinoma cells. Western blot analysis of K-*RAS* and EF-1α protein levels in cells treated with the various agents for 3 days. The concentration of MM, SC, and AS PS ODN was 0.5 μM, and the dose of virus was 100 pfu per cell.

apparent when using AS PS ODN that did not correspond precisely to the mutation in K-*ras* codon 12 of the particular pancreatic carcinoma analyzed. This observation supports numerous previous studies indicating that AS PS ODN can induce both specific and apparently nonspecific effects in target cells (30–32). In the present study, AS K-*ras* PS ODN were designed to interact with the AUG start codon of the K-*ras* gene. Treatment of both mut and wt K-*ras* expressing pancreatic carcinoma cells with AS K-*ras* PS ODN, but not MM or SC PS ODN, reduced K-*ras* p21 protein levels in both mut and wt K-*ras* cells by $> 80\%$ within 24 h (Fig. 3). This effect was observed with and without Ad.*vec* or Ad.*mda*-7 infection, which did not consistently cause a further alteration in K-*ras* levels (Fig. 3). Moreover, the growth inhibitory effect of the AS K-*ras* PS ODN was greater in the three mut K-*ras* pancreatic carcinoma cells than in the wt K-*ras* BxPC-3 cell line (Fig. 1). As observed in the study by Kita *et al.* (11), growth inhibition induced by AS K-*ras* PS ODN, either point or start codon specific (current study), exceeded that observed when using SC or MM PS ODN. In addition, no synergistic growth inhibitory effects or decreases in cell survival were apparent in Ad.*mda*-7 infected pancreatic carcinoma cells that were subsequently treated with SC or MM PS ODN. These results confirm a profound synergistic growth inhibitory effect specifically in mut K-*ras* pancreatic carcinoma cells after infection with Ad.*mda*-7 and treatment with AS K-*ras* PS ODN.

Plasma membrane-associated small molecular weight GTP-binding proteins are frequently used by cells in the process of signal transduction from the inner leaflet of the plasma membrane to the cytosol. The prototypical small molecular weight family of GTP-binding proteins is the *ras* gene family (33). K-*ras* is a member of the *ras* gene family, which consists of three members, K-*ras*, H-*ras*, and N-*ras* (34). When activated, the *RAS* proteins contain a bound GTP molecule, whereas the inactive form contains GDP (35). The process of *ras* activation involves an exchange of bound GDP with GTP. A common occurrence in pancreatic and other cancers involves point mutations of K-*ras*, which involve codon 12 (predominantly in pancreatic carcinoma) and codons 13 and 61 in other cancers (2, 22, 23). Moreover, based on the observation that K-*ras* mutations appear in atypical hyperplastic ducts that surround the ductal-like cancer cells (36), it is currently believed that K-*ras* mutations represent a very early event in pancreatic carcinogenesis. The resulting K-*ras* mutation induces a conformational change in the molecule and a concomitant maintenance of *ras* activation by decreasing hydrolysis of GTP to GDP (33, 34). When activated, K-*ras* can signal into the cytosol via multiple downstream signaling pathways such as the classical mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3-kinase) pathway, and the c-Jun NH_2-terminal kinase pathway to induce plethora cellular changes, including enhanced

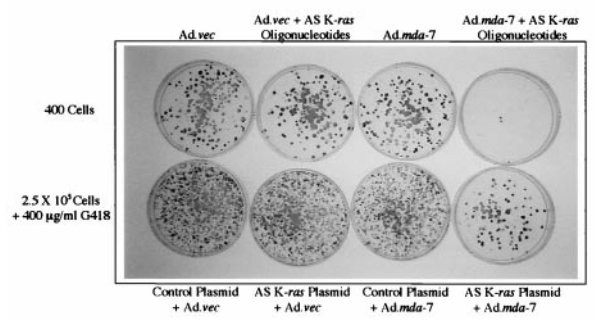

Fig. 4. The combination of Ad.*mda*-7 plus AS K-*ras* PS ODN or AS K-*ras* plasmids synergistically inhibits colony formation in mut K-*ras* MIA PaCa-2 pancreatic carcinoma cells. (*Upper*) Effect of Ad.*mda*-7 plus AS K-*ras* PS ODN on MIA PaCa-2 colony formation. Cells were infected with 100 pfu per cell of Ad.*vec* or Ad.*mda*-7, treated with 0.5 μM AS K-*ras* PS ODN plus 10 μl of Lipofectamine, reseeded at a density of 400 cells per plate, and fixed and stained with Giemsa after 3 weeks. (*Lower*) Effect of Ad.*mda*-7 plus AS K-*ras* plasmid transfection on MIA PaCa-2 G418-resistant colony formation. Cells were infected with 100 pfu per cell of Ad.*vec* or Ad.*mda*-7, transfected with 10 μg of plasmid (either control pcDNA3.1 lacking insert or the pcDNA3.1 vector containing a 346-nt AS K-*ras* fragment), reseeded at a density of 2.5×10^5 cells per plate, and selected in 400 μg/ml G418, and G418-resistant colonies were fixed and stained with Giemsa after 3 weeks.

proliferation (37–39). In these contexts, blocking K-*ras* expression may alter downstream pathway activities in mut K-*ras* pancreatic cancer cells, rendering these cells sensitive to *mda*-7 induction of growth suppression and effects on cell viability. This hypothesis is currently being tested.

Infection of mut K-*ras* Pancreatic Carcinoma Cells with Ad.*mda*-7 Followed by Transfection with an AS K-*ras* Expression Vector Inhibits Growth *in Vitro* and Tumorigenesis *in Vivo* in Nude Mice. As an additional approach to inhibiting K-*ras* expression, a K-*ras* gene fragment of 346 nt (consisting of nt 172–517), which was previously shown to inhibit pancreatic cancer cell growth *in vitro* and *in vivo* when used in an AS orientation (9, 10), was isolated by PCR from BxPC-3 cells and cloned into a pcDNA3.1 (neomycin resistance) expression vector. This expression vector was then tested for effects on pancreatic carcinoma cells, when used alone or in combination with Ad.*mda*-7. As can be seen in Fig. 4 (*Upper*), infection of MIA PaCa-2 cells with Ad.*vec* alone or in combination with AS K-*ras* PS ODN did not significantly alter colony formation. Similarly, infection of MIA PaCa-2 cells with Ad.*mda*-7 also did not modify cloning efficiency in monolayer culture. In contrast, the combination of Ad.*mda*-7 with AS K-*ras* PS ODN dramatically inhibited colony formation (confirming previous studies using cell counting by hemocytometer and MTT staining). To test the effect of the AS K-*ras* plasmid in combination with Ad.*mda*-7 on pancreatic carcinoma cell growth, MIA PaCa-2 cells were infected with Ad.*vec* or Ad.*mda*-7 and transfected with a control or the AS K-*ras* plasmid, and G418-resistant colony formation was determined. As can be seen in Fig. 4 (*Lower*), a dramatic suppression in growth was only observed in MIA PaCa-2 cells infected with Ad.*mda*-7 and transfected with the AS K-*ras* plasmid. Qualitatively similar growth inhibitory results were obtained when the same protocols were used with AsPC-1 or PANC-1 mut K-*ras* pancreatic carcinoma cells but not with wt K-*ras* BxPC-3 cells (data not shown). These results indicate that both AS PS ODN and AS K-*ras* expression by plasmid transfer can synergize with *mda*-7 to inhibit mut K-*ras* pancreatic carcinoma cell growth.

MIA PaCa-2 cells form tumors in athymic nude mice with a short latency time. Transfection with an AS K-*ras* plasmid or infection with Ad.*mda*-7 resulted in rapidly growing tumors in

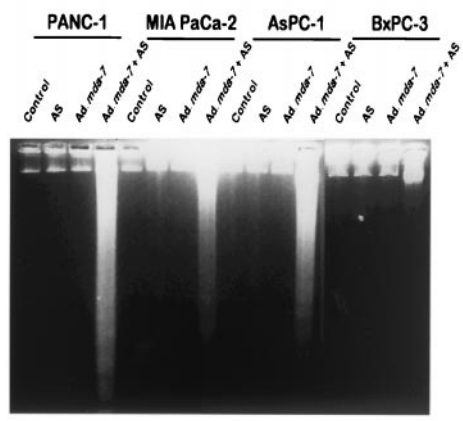

Fig. 5. Ad.*mda*-7 plus AS K-*ras* PS ODN induce nucleosomal DNA degradation in K-*ras* mutant human pancreatic cancer cells. The indicated cell types were treated as indicated for 3 days. AS, 0.5 μM AS K-*ras* PS ODN; Ad.*mda*-7, 100 pfu per cell; Ad.*mda*-7 infected (100 pfu per cell) + 0.5 μM AS K-*ras* PS ODN. Nucleosomal ladder formation was determined as described (33).

80% of animals (three independent experiments; $n = 26$). Similarly, infection with an Ad.*vec*, a plasmid lacking the gene inserts, or transfection with a plasmid construct containing a 346-nt K-*ras* gene fragment cloned in a sense orientation did not significantly inhibit tumor formation (76% tumors; $n = 17$; three independent experiments). In contrast, a remarkable complete suppression in tumor formation was apparent only when MIA PaCa-2 cells were infected with Ad.*mda*-7 and then transfected with the AS K-*ras* plasmid before injecting into athymic nude mice (no tumors formed in 13 animals; three independent studies). These findings document that in mut K-*ras* pancreatic carcinoma cells, infection with Ad.*mda*-7 combined with targeting the K-*ras* gene for inhibition in a small subset of cells by means of transfection with an AS K-*ras* expression plasmid eliminates *in vivo* tumor formation in nude mice. Because transfection is an inherently inefficient means of introducing genes into target cells, it is possible that cells receiving the combination treatment release factor(s) that sensitize adjacent tumor cells containing *mda*-7 to lose viability, thereby preventing tumor formation. Further studies are necessary to mechanistically explain this provocative finding.

The Combination of Ad.*mda*-7 and AS K-*ras* PS ODN Induces Apoptosis Selectively in mut K-*ras* Expressing Human Pancreatic Carcinoma Cells. The mechanism by which *mda*-7 selectively decreases colony formation and growth in human cancer cells involves induction of apoptosis (18–21). To determine whether the combination treatment of K-*ras* mut pancreatic cancer cells decreases cell survival by induction of apoptosis, we have performed a number of assays typically used to monitor programmed cell death. In many cell types, induction of apoptosis is associated with DNA degradation, which can be monitored by generation of nucleosomal DNA ladders (19, 40, 41). As can be seen in Fig. 5, treatment of mut K-*ras* expressing pancreatic carcinoma cells, but not wt K-*ras* expressing BxPC-3, with Ad.*mda*-7 plus AS K-*ras* PS ODN results in DNA fragmentation. The specificity of this effect is further documented by the lack of nucleosomal DNA ladders in pancreatic cancer cells infected with Ad.*mda*-7 or treated with 5.0 μM AS K-*ras* PS ODN alone or in cells treated with the combination of Ad.*mda*-7 with 5.0 μM MM K-*ras* PS ODN (data not shown). Confirmation of induction of apoptosis by combination treatment in the three mut K-*ras* pancreatic carcinoma cells was verified by 4′,6-diamidino-2-phenylindole and by propidium iodide staining, increases in hypodiploid cells,

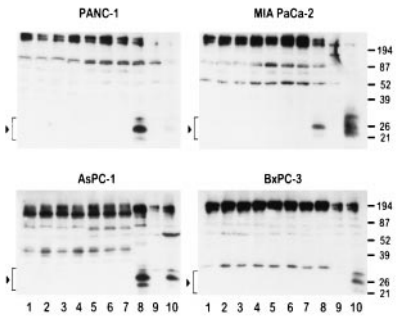

Fig. 6. MDA-7 protein is detected in mut K-*ras* pancreatic carcinoma cells infected with Ad.*mda*-7 and treated with AS K-*ras* PS ODN. The various cell lines were treated for 1 day as indicated: 1, control cells; 2, AS K-*ras* PS ODN; 3, Ad.*vec*; 4, Ad.*vec* + AS K-*ras* PS ODN; 5, Ad.*mda*-7; 6, Ad.*mda*-7 + MM PS ODN; 7, Ad.*mda*-7 + SC PS ODN; 8, Ad.*mda*-7 + AS K-*ras* PS ODN; 9, PC-3 human prostate carcinoma cells treated for 1 day with Ad.vec; and 10, PC-3 cells treated for 1 day with Ad.*mda*-7 (used as a positive control for MDA-7 expression). Lysates of treated cells were evaluated by Western blotting for MDA-7 and EF-1α protein as described (19, 24, 29). Arrowheads and brackets indicate MDA-7 proteins detected by Western blotting. The concentration of MM, SC, and AS PS ODN was 0.5 μM, and the dose of virus was 100 pfu per cell.

and Annexin V staining by FACS analysis (data not shown). These results confirm that the combination of Ad.*mda*-7 and AS K-*ras* PS ODN decreases viability in mut K-*ras* expressing pancreatic carcinoma cells by inducing apoptosis.

***MDA*-7 Protein Is Present in mut K-*ras*-Expressing Human Pancreatic Carcinoma Cells Following Infection with Ad.*mda*-7 and Treatment with AS K-*ras* PS ODN.** The reason pancreatic carcinoma cells are resistant to *mda*-7 and the mechanism by which the combination of Ad.*mda*-7 and AS K-*ras* PS ODN sensitizes specific pancreatic carcinoma cells to *mda*-7 induction of growth suppression and apoptosis are not known. One hypothesis is that the mut K-*ras* protein, or biochemical pathways modified by this protein, prevents synthesis, processing, and/or secretion of *MDA*-7 protein following infection with Ad.*mda*-7. To begin testing this possibility, we determined the effect of various treatment protocols on intracellular *MDA*-7 protein levels in the different pancreatic carcinoma cell lines (Fig. 6). No *MDA*-7 protein was detected in cell lysates from the four different pancreatic carcinomas 24 h after infection with Ad.*mda*-7 alone or in combination with MM or SC PS ODN. This occurred despite the production of *mda*-7 mRNA in all four pancreatic cancer cell lines following infection with Ad.*mda*-7 (Fig. 7). In contrast, *MDA*-7 protein was readily detected in the three K-*ras* mut pancreatic carcinoma cell lines after infection with Ad.*mda*-7 and treatment with AS K-*ras* PS ODN (Fig. 6). In the case of wt

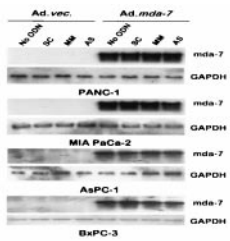

Fig. 7. Expression of *mda*-7 mRNA in Ad.*mda*-7 infected mut and wt K-*ras* pancreatic carcinoma cells. The indicated cell lines were treated for 3 days, and total mRNA was isolated and analyzed by Northern blotting for *mda*-7 and glyceraldehyde-3-phosphate dehydrogenase RNA. The concentration of SC, MM, and AS PS ODN was 0.5 μM, and the dose of virus was 100 pfu per cell.

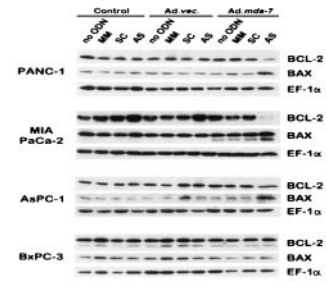

Fig. 8. Expression of BAX, BCL-2, and EF-1α proteins in pancreatic carcinoma cells after various treatment protocols. The different cell lines were treated for 3 days as indicated, and the levels of the respective proteins were determined by using 30 μg of total protein lysates by Western blotting using the respective antibodies as described (19, 24, 29). The concentration of MM, SC, and AS PS ODN was 0.5 μM, and the dose of virus was 100 pfu per cell.

K-*ras* expressing BxPC-3, *MDA*-7 protein was not detected (Fig. 6). These results suggest that mut K-*ras* may negatively effect *MDA*-7 protein processing in mut K-*ras* pancreatic carcinoma cells. The absence of *MDA*-7 protein, using similar protocols, in BxPC-3 cells suggests that other pathways may be operational that modify expression and/or retention of *MDA*-7 protein in these pancreatic carcinoma cells. Because apoptosis only occurs in the combinatorial treated mut K-*ras* pancreatic carcinoma cells, the present studies demonstrate a potential correlation between presence/retention of *MDA*-7 protein and induction of growth suppression and programmed cell death in pancreatic carcinoma cells.

The Combination of Ad.*mda*-7 and AS K-*ras* PS ODN Alters the Levels of Apoptosis-Associated Proteins. Previous studies indicate that infection of diverse cancer cells with Ad.*mda*-7 results in apoptosis, and in the majority of cases this process is associated with up-regulation of *BAX* protein and changes in the ratio of *BAX* to *BCL*-2 protein (18–21). However, the ability of Ad.*mda*-7 to induce apoptosis in specific cancer cells, such as DU-145 human prostate carcinoma cells that do not produce *BAX* protein (42), indicates that *mda*-7 can also mediate programmed cell death in certain cancer cells by a *Bax*-independent pathway (18, 21). Based on these considerations and the presence of *MDA*-7 protein specifically in combination-treated mut K-*ras* pancreatic carcinoma cells, experiments were performed to determine the levels of *BAX* and *BCL*-2 proteins in treated cells. When analyzed 3 days after combination treatment, in which the majority of K-*ras* mut cells were apoptotic, the levels of *BAX* protein were elevated in PANC-1, MIA PaCa-2, and AsPC-1 cells, ≈7.5-, ≈3-, and ≈10-fold, respectively, but not in BxPC-3 cells (Fig. 8). Moreover, the levels of *BCL*-2 protein were significantly reduced in PANC-1 (≈8-fold) and MIA PaCa-2 (≈13.5-fold) cells, marginally reduced in AsPC-1 cells (≈1.2-fold), and remained unchanged in BxPC-3 cells (Fig. 8). These results support a potential involvement of *BAX* protein and changes in the ratio of *BAX* to *BCL*-2 proteins in inducing apoptosis in combination-treated pancreatic carcinoma cells. Although further studies are necessary to define possible roles of additional apoptosis modulating molecules, the changes presently observed in *BAX* and the ratio of *BAX* to *BCL*-2 proteins are anticipated to induce downstream events, including induction of cytochrome *c* release from mitochondria and caspase activation (40, 41, 43), culminating in programmed cell death.

Summary and Future Perspectives. The present studies illustrate a fascinating phenomenon with potentially important clinical implications for the therapy of pancreatic cancer. A combinatorial

approach, forced expression of the cancer growth-suppressing and apoptosis-inducing gene *mda*-7 plus targeted inhibition of K-*ras*, results in growth suppression *in vitro* and *in vivo* in athymic nude mice and programmed cell death in mut K-*ras* expressing human pancreatic carcinoma cells. These observations suggest future avenues for investigation that offer potential for providing effective therapeutic approaches for this invariably fatal human cancer. These include targeting K-*ras* suppression by using a viral (or nonviral) delivery system to deliver an AS K-*ras* gene construct or AS PS ODN in combination with Ad.*mda*-7 and applying Ad.*mda*-7 together with agents that inhibit downstream mut K-*ras* activated genes and/or biochemical pathways.

Previous studies indicate that mutation-specific AS K-*ras* PS ODN, an AS wt K-*ras* plasmid, and a virus encoding a dominant negative H-*ras* mutant can inhibit pancreatic carcinoma cell growth *in vitro* (9–13). In addition, both an AS wt K-*ras* plasmid and an Ad expressing a dominant negative H-*ras* mutant can also inhibit human pancreatic carcinoma tumorigenesis and metastasis *in vivo* when tested in nude mouse human pancreatic cancer models (9, 10, 12, 13). These findings and the present AS K-*ras* transfection results suggest that either two viruses, one expressing Ad.*mda*-7 and one expressing AS wt K-*ras*, or a recombinant bipartite Ad expressing both *mda*-7 and AS wt K-*ras* could prove beneficial for inducing pancreatic carcinoma cell death. Because the AS K-*ras* plasmid sequence that shows suppressive effects in mutant pancreatic carcinoma cells does not show effects in wt K-*ras* pancreatic cancer cells, or nonspecific toxicity in animal models, this sequence incorporated into an Ad vector should provide a means of altering K-*ras* levels in mut K-*ras* pancreatic carcinoma cells. Moreover, the present studies indicate that infection with Ad.*mda*-7 and then transfection with Ad.K-*ras* AS induces a similar synergistic growth inhibitory effect as observed with Ad.*mda*-7 plus AS K-*ras* PS ODN. In this context, of even greater clinical potential as a therapeutic reagent, an Ad that expresses both *mda*-7 and AS K-*ras* should prove more efficacious with less potential toxicity than the use of two separate viruses. Studies are presently in progress to produce these viruses and test this hypothesis.

The ability of the combination of *mda*-7 and AS K-*ras* PS ODN to synergistically inhibit growth and promote apoptosis in mut K-*ras* pancreatic carcinoma cells probably occurs because K-*ras* expression is inhibited, thereby altering downstream signaling pathway functions, resulting in gene expression changes. Because primary targets of activated K-*ras* are likely to be the MAPK, PI3-kinase, and c-Jun NH_2-terminal kinase pathways, it may be possible, by directly altering the functions of these signaling cascades, to sensitize pancreatic carcinoma cells to *mda*-7-induced biological effects. Furthermore, because several studies have argued for cytoprotective signaling via MAPK and PI3-kinase (44–46), further investigations are planned to selectively target the MAPK and PI3-kinase pathways for inhibition, with appropriate pharmacological agents, and determine whether this renders pancreatic carcinoma cells responsive to induction of apoptosis by *mda*-7. If effective, this strategy would also provide a potentially powerful methodology for treating pancreatic cancer.

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