Reversing Translational Suppression and Induction of Toxicity in Pancreatic Cancer Cells Using a Chemoprevention Gene Therapy Approach [S]

Siddik Sarkar, Bridget A. Quinn, Xuening Shen, Paul Dent, Swadesh K. Das, Luni Emdad, Devanand Sarkar, and Paul B. Fisher

Department of Human and Molecular Genetics (S.S., B.A.Q., X.S., S.K.D., L.E., D.S., P.B.F.), Department of Biochemistry and Molecular Biology (P.D.), VCU Institute of Molecular Medicine (P.D., S.K.D., L.E., D.S., P.B.F.), and VCU Massey Cancer Center (P.D., L.E., D.S. P.B.F.), Virginia Commonwealth University, School of Medicine, Richmond, Virginia

ABSTRACT

Pancreatic cancer is an aggressive disease with limited therapeutic options. Melanoma differentiation–associated gene-7/interleukin-24 (mda-7/IL-24), a potent antitumor cytokine, shows cancer-specific toxicity in a vast array of human cancers, inducing endoplasmic reticulum stress and apoptosis, toxic autophagy, an antitumor immune response, an antiangiogenic effect, and a significant "bystander" anticancer effect that leads to enhanced production of this cytokine through autocrine and paracrine loops. Unfortunately, mda-7/IL-24 application in pancreatic cancer has been restricted because of a "translational block" occurring after Ad.5-mda-7 gene delivery. Our previous research focused on developing approaches to overcome this block and increase the translation of the MDA-7/IL-24 protein, thereby promoting its subsequent toxic effects in pancreatic cancer cells. We demonstrated that inducing reactive oxygen species (ROS) after adenoviral infection of mda-7/IL-24 leads to greater translation into MDA-7/IL-24 protein and results in toxicity in pancreatic cancer cells. In this study we demonstrate that a novel chimeric serotype adenovirus, Ad.5/3-mda-7, displays greater efficacy in delivering mda-7/IL-24 compared with Ad.5-mda-7, although overall translation of the protein still remains low. We additionally show that D-limonene, a dietary monoterpene known to induce ROS, is capable of overcoming the translational block when used in combination with adenoviral gene delivery. This novel combination results in increased polysome association of mda-7/IL-24 mRNA, activation of the preinitiation complex of the translational machinery in pancreatic cancer cells, and culminates in mda-7/IL-24–mediated toxicity.

Introduction

Pancreatic cancer is a deadly disease with a 5-year survival of less than 5%. The lack of early stage symptoms often leads to advanced disease at the time of diagnosis. Additionally, it exhibits intrinsic resistance to chemotherapy and radiation (Hezel et al., 2006). Consequently, there is a dire need for the development of novel therapies for this devastating disease.

Melanoma differentiation–associated gene-7/interleukin-24 (mda-7/IL-24) is an anticancer cytokine originally cloned in our laboratory during studies of terminal differentiation of human melanoma cells (Jiang and Fisher, 1993; Jiang et al., 1995, 1996). This gene is a member of the interleukin-10 family of cytokines and functions as a tumor suppressor (Dash et al., 2010a). Reintroduction of this cytokine into cancer cells leads to significant endoplasmic reticulum (ER) stress, apoptosis, and anticancer immune effects, but shows no toxicity in normal cells (Jiang et al., 1996; Su et al., 1998; Dash et al., 2010a). Additionally, mda-7/IL-24 exhibits a potent "bystander effect," wherein cells that express mda-7/IL-24 can also secrete it and further activate its expression through autocrine and paracrine loops in both normal and cancer cells (Dash et al., 2010a). Clinically, this suggests that mda-7/IL-24 has the capability of traveling from the original source of treatment to affect distant tumors and metastases (Sauane et al., 2003, 2008; Dash et al., 2010a).

Gene therapy studies using replication-incompetent adenovirus containing the full-length mda-7 gene (Ad.mda-7) have been shown to be extremely effective in multiple cancer types in both laboratory and clinical settings (Fisher, 2003; 2005, 2007; Tong et al., 2005; Cunningham et al., 2005; Eager et al., 2008). Although mda-7/IL-24 has ubiquitous activity in multiple cancer types, it is difficult to fully exploit these in pancreatic cancer owing to a block in mda-7/IL-24 translation after delivery into these cells. Infected cells accumulate mda-7/IL-24

mRNA, which exhibits a reduced ability to interact with polysomes and create MDA-7/IL-24 protein (Su et al., 2001; Lebedeva et al., 2005, 2006, 2007, 2008a,b; Sarkar et al., 2013).

Our previous work has demonstrated that the "translational block" seen after Ad.5-*mda*-7 treatment can be abrogated by two strategies: inhibition of oncogenic Kirsten-ras (K-*ras*) or in combination with a reactive oxygen species (ROS)–inducing agent (Su et al., 2001; Lebedeva et al., 2005, 2006, 2007, 2008a,b). We have shown that agents such as arsenic trioxide or perillyl alcohol (POH) are able to induce MDA-7/IL-24 protein translation and subsequent toxic effects in pancreatic cancer cells. The creation of a clinically useful drug that specifically targets K-*ras* has been a long-standing and complex problem that still remains unresolved. Additionally, targeting K-*ras* only enhances MDA-7/IL-24 expression in cells that express mutated forms of K-*ras*. Because of this, we have focused on identifying novel ROS-inducing agents that could translate easily into a clinical setting.

Furthermore, the use of adenoviruses (Ad) in pancreatic cancer cells is often hampered by poor infectivity rates. Serotype 5 Ad (Ad.5) viruses use Coxsackie and adenovirus receptors (CAR) to infect cells. Unfortunately, cancer cells, including pancreatic cancer cells, often downregulate these receptors, which leads to inefficient viral infectivity (Pearson et al., 1999). Using alternate viral serotypes to create chimeric viruses can help with this issue (Dash et al., 2010b, 2011; Hamed et al., 2010, 2013; Azab et al., 2012, 2014). The Ad.3 serotype relies on expression of CD46 and desmoglein for viral entry into cells (Wang et al., 2011). The creation of Ad.5/3 chimeric Ad combines both of these serotypes to create a virus that is capable of using CAR, CD46, and/or desmoglein for infection. We demonstrated that Ad.5/3-*mda*-7 is able to infect prostate, renal, glioblastoma multiforme, and colorectal cancer cells with greater efficiency compared with Ad.5-*mda*-7 and that this translates into greater toxicity (Dash et al., 2010b, 2011; Eulitt et al., 2010; Hamed et al., 2010; Azab et al., 2012).

In this study, we determined if Ad.5/3-*mda*-7 would improve the transduction efficiency in pancreatic cancer cells compared with Ad.5-*mda*-7. We also evaluated the ability of D-limonene, a dietary monoterpene, to increase the translation of *mda-7/IL-24* mRNA and lead to toxicity in combination with Ad.*mda*-7. Limonene is a major component of citrus oil, shows minimal toxicity in low doses, and, like POH, is also able to induce ROS (Rabi and Bishayee, 2009). We hypothesized that it would function just as well as would increasing *mda*-7/IL-24 mRNA translation following Ad.*mda*-7 infection.

Materials and Methods

Cell Lines and Generating Stable Clones. AsPC-1 and BxPC-3 cell lines were obtained from the American Type Culture Collection, maintained in RPMI-1640 media (Gibco; Invitrogen, Auckland, New Zealand) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). MIA PaCa-2 and PANC-1, also obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS. Immortalized normal pancreatic mesenchymal cell line LT2 was obtained from EMD Millipore (Billerica, MA) and maintained per Millipore instructions. All cell lines were cultured at 37°C in a 5% CO_2 and 95% air-humidified incubator. To obtain *mda-7/IL-24*–overexpressing clones, MIA PaCa-2 and PANC-1 cells were transfected with pc.DNA3.1-*mda*-7. Individual clones were selected after ~3–4 weeks of continuous maintenance in culture medium

containing hygromycin (Lebedeva et al., 2003), and were further characterized for the presence/expression of the inserted plasmid by real-time polymerase chain reaction (PCR).

Generation of Ad.5.mda-7 and Ad.5/3-mda-7. Recombinant serotype 5 and serotype 5/3 chimeric adenoviruses expressing *mda*-7/IL-24 (Ad.5-*mda*-7 and Ad.5/3-*mda*-7) and control empty adenovirus (Ad.5.*vec* and Ad.5/3.*vec*) were generated as described (Su et al., 1998; Dash et al., 2010b).

Adenoviral Infection. Pancreatic cancer cell lines were infected with Ad.*mda*-7 or Ad.5/3-*mda*-7 in medium lacking FBS for 3 hours followed by treatment with limonene (200 μM) in complete medium (10% FBS) for time points as described for particular assays.

Cell Proliferation Assays. Pancreatic cancer cell lines were infected with Ad.*mda*-7 or Ad.5/3-*mda*-7 in medium lacking FBS for 3 hours followed by treatment with D-limonene in complete medium (10% FBS) for 72 hours, and cell proliferation assays were performed at optical density 560 nm after adding 100 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye (1 mg/ml; Sigma-Aldrich). To further confirm cell proliferation, a chemiluminescent-based BrdU Cell Proliferation Assay kit (Cell Signaling Technology, Danvers, MA) was used.

Apoptotic Assays. Apoptotic assays were performed using an FITC Annexin V Apoptosis Detection Kit I (Pharmingen; BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Flow cytometry assays were performed immediately after staining using FACS Canto (BD Biosciences). Data were analyzed using FACSDIVA software (BD Biosciences).

ROS Measurements. To determine ROS production, cells were stained with 100 μl of 10 μM carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes; Life Technologies, Grand Island, NY) in phosphate-buffered saline for 30 minutes followed by treatment with limonene, and fluorescence was measured with a fluorometer using a green filter at the indicated time points.

Preparation of Whole-Cell Lysates and Western Blotting Analyses. Cells were treated for 48 hours, lysed using cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich) with protease inhibitor cocktail, phosphatase inhibitor (Roche, Indianapolis, IN). Whole-cell lysates were collected after centrifugation at 12,000 rpm at 4°C. For Western blotting analyses, the primary antibodies used were mouse monoclonal anti-MDA-7/IL-24 (1:2000; Gen Hunter Corporation, Nashville, TN), anti-EF1α (1:5000; EMD Millipore), mouse monoclonal anti-human K-Ras (1:1000; Bio-Rad Laboratories, Raleigh, NC), rabbit monoclonal anti–Bcl-xL, anti-PARP, anti-Mcl-1, anti–phospho-p70S6K (Thr-389), anti–phospho-eIF4E (Ser-209), anti–phospho-4EBP1 (Thr-37/46) (1:1000; Cell Signaling Technology), rabbit polyclonal anti–BiP/GRP-78 (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX). The secondary antibodies used were polyclonal goat anti-mouse IgG (1:1000; Dako, Carpinteria, CA) and polyclonal swine anti-rabbit IgG (1:3000; Dako).

Polysome Studies. Cells (2×10^6) were infected with Ad.*mda*-7 and 48 hours later harvested in 500 μl buffer A (200 nM Tris-HCl pH 8.5, 50 mM KCl, 25 mM $MgCl_2$, 2 mM EGTA, 100 mg/ml heparin, 100 mg/ml cycloheximide, 2% polyoxyethylene 10-tridecyl ether, and 1% sodium deoxycholate supplemented with cOmplete Mini protease inhibitor cocktail and RNase inhibitor (Roche). Cells were centrifuged at 12,000 rpm for 10 minutes at 4°C to clear cell debris. The supernatant was loaded on top of a 10–50% sucrose gradient prepared in buffer B (50 nM Tris-HCl, 25 nM KCl, and 10 mM $MgCl_2$) and centrifuged at 40,000 rpm for 1 hour at 4°C. Fractions of 500 μl were collected, the optical density at 260 nm was monitored, and polysome fractions were identified (typically fractions 10–20). The fractions were pooled into two separate tubes: fraction I (1–10 fraction number) and fraction II (polysome-enriched fraction; 11–20 fraction number). RNA was extracted by adding equal volume of 8 M guanidine hydrochloride, and precipitated by adding 2 volumes of ethanol. RNA was extracted from each fraction with a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was prepared and reverse transcription PCR was performed using a probe

for mda-7/IL-24 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for relative quantification of mRNA.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Student's *t* test or 1-way analysis of variance was used as indicated, to study the level of significance ($P < 0.05$).

Results

Enhanced Delivery of *mda-7/IL-24* Using Serotype Ad.5/3-*mda*-7 in Pancreatic Cancer Cells. Initial studies with *mda-7/IL-24* focused on gene delivery using the Ad.5 serotype, which utilizes CAR for infection. Pancreatic cancer cells express relatively low levels of CAR, making infection with Ad.5 inefficient. To circumvent this problem, a serotype chimeric recombinant adenovirus, Ad.5/3, was generated. Ad.5/3, while able to use CAR receptors, is also reliant on expression of CD46 and desmoglein for infection. Pancreatic cancer cell lines AsPC-1, PANC-1, MIA PaCa-2, and BxPC-3, as well as an immortal pancreas fibroblast cell line, LT2, all express CD46 protein at much higher levels compared with CAR (Fig. 1A). AsPC-1 and BxPC-3 cells also express high levels of desmoglein (Fig. 1B). These results were also confirmed by measuring mRNA transcript levels (Fig. 1C). To compare Ad.5/3 infection to that of Ad.5, we used viruses expressing a luciferase reporter gene. In all pancreatic cell lines, higher luciferase levels were found using the Ad.5/3 serotype compared with the Ad.5 serotype (Fig. 1D). Pretreatment of cells with blocking antibodies to CAR, CD46, desmoglein, or a combination of the three confirmed the importance of all three receptors in determining infectivity of pancreatic cancer cells, with infection being inhibited the most when antibodies to all three receptors were used in combination (Fig. 1E). These results support the use of the Ad.5/3 virus as a means of effectively delivering *mda-7/IL-24* to pancreatic cancer cells.

D-Limonene Inhibits Growth of Pancreatic Cancer Cells and Induces ROS. D-Limonene is able to inhibit the proliferation of multiple pancreatic cancer cells but does not affect growth of an immortalized pancreatic mesenchymal cell line, LT2 (Fig. 2A). D-Limonene is a monoterpene and capable of inhibiting the prenylation of GTPases, such as K-*ras* (Chen et al., 1999). However, the doses of D-limonene we used in our study did not affect the normal prenylation of K-*ras* (Fig. 2B). We next evaluated the ability of D-limonene to induce ROS and found that even at nontoxic or sublethal doses D-limonene promoted ROS induction, specifically peroxides, and this increase was abrogated with *N*-acetyl cysteine (NAC) pretreatment (Fig. 2C).

D-Limonene Enhances Translation of *mda-7/IL-24* mRNA and Promotes Toxicity of Ad.*mda*-7. Previous studies indicate that the translational block seen with *mda-7/IL-24* mRNA in pancreatic cancer cells can be overcome with ROS induction (Lebedeva et al., 2008a,b). Because D-limonene showed significant ROS induction, we infected cells with Ad.5-*mda*-7 or Ad.5/3-*mda*-7 in the presence or absence of D-limonene to determine if this compound could enhance *mda-7/IL-24* mRNA translation and promote toxicity. In LT2 cells, we found no effect on growth (Fig. 3A). However, in pancreatic cancer cell lines PANC-1 (Fig. 3B) and MIA PaCa-2 (Fig. 3C), the addition of D-limonene to Ad.*mda*-7-infected cells was able to significantly enhance growth inhibition. Additionally, in agreement with prior results comparing Ad.5 versus Ad.5/3, Ad.5/3-*mda*-7 alone was able to induce a greater growth inhibition compared with Ad.5-*mda*-7 (Dash
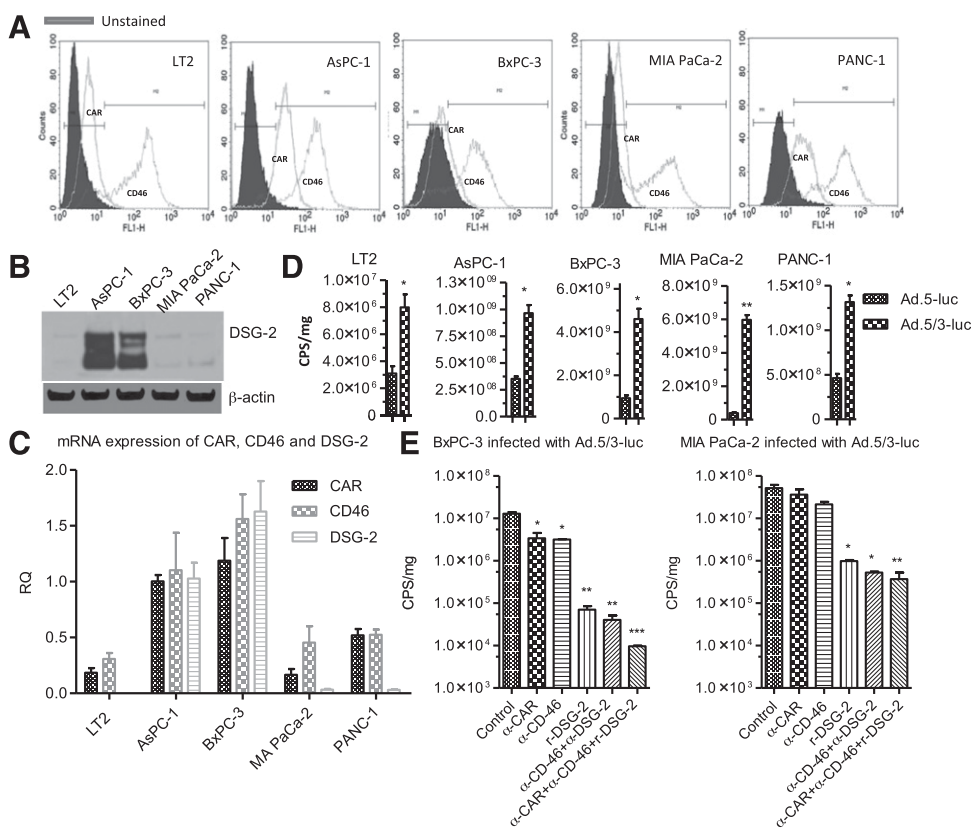

Fig. 1. Enhanced gene delivery using a serotype chimeric modified recombinant Ad.5/3. (A) Expression of CAR and CD46 in pancreatic cancer cells as measured by flow cytometry. Cells were stained with anti-CAR, anti-CD46, and unstained or isotype-specific primary antibody. (B) Western blotting of desmoglein-2 (DSG-2) expression. (C) mRNA levels of CAR, CD46, and DSG-2 determined by real-time PCR. RQ, relative quotient. (D) Pancreatic cell lines were infected with Ad.5-luc and Ad.5/3-luc and level of luciferase (luc) expression was measured 48 hours postinfection using a luminometer (CPS, counts per second). (E) Pancreatic cancer cell lines BxPC-3 and MIA PaCa-2 were pretreated with anti-CAR (α-CAR), anti-CD46 (α-CD46) and recombinant DSG-2 for 2 hours followed by Ad.5/3-luc infection, and luciferase expression was quantified using a luminometer (CPS, counts per second). Level of significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
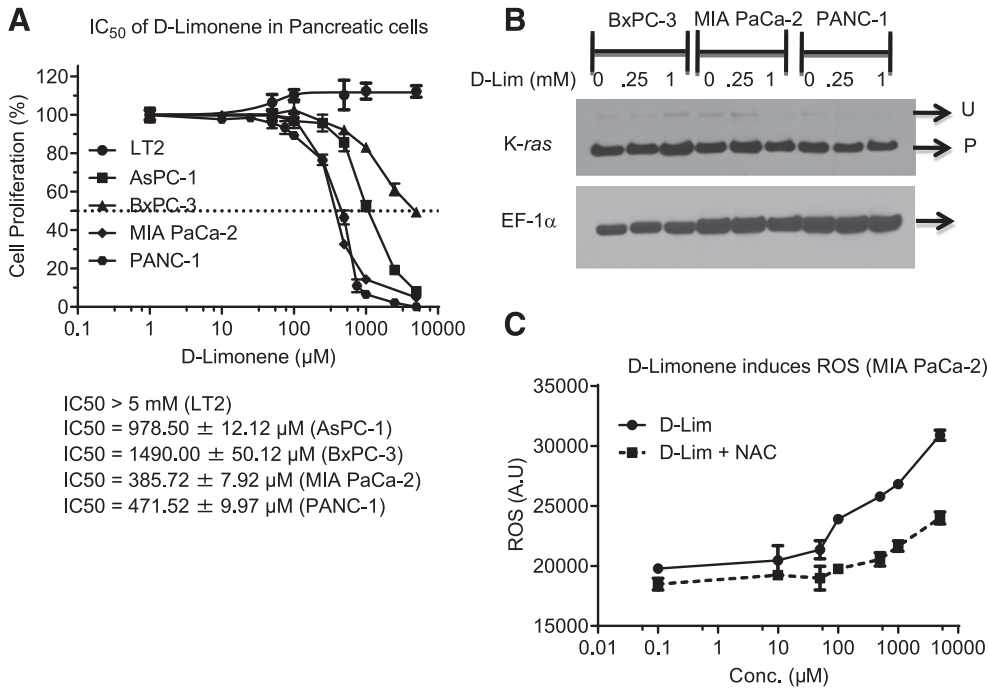
A
IC$_{50}$ of D-Limonene in Pancreatic cells

IC50 > 5 mM (LT2)
IC50 = 978.50 ± 12.12 µM (AsPC-1)
IC50 = 1490.00 ± 50.12 µM (BxPC-3)
IC50 = 385.72 ± 7.92 µM (MIA PaCa-2)
IC50 = 471.52 ± 9.97 µM (PANC-1)

B

C
D-Limonene induces ROS (MIA PaCa-2)

Fig. 2. D-Limonene (D-Lim) inhibits cellular proliferation by inducing ROS. (A) Pancreatic cells were treated with increasing doses of D-Lim, and MTT assays were performed after 72 hours to measure cell proliferation. (B) Western blotting analysis of K-*ras* prenylation status after treatment with D-Lim. (U, unprenylated, P, prenylated.) (C) MIA PaCa-2 cells were treated with D-Lim with or without NAC pretreatment and stained with dihydrodichlorofluorescein to detect ROS. ROS induction was quantified using fluorescence measurements. A.U., arbitrary units; Conc., concentration.

et al., 2010b; Azab et al., 2012, 2014). Western blotting showed similar results, with Ad.5/3-*mda*-7 inducing greater MDA-7/IL-24 protein expression, both with and without the addition of D-limonene compared with Ad.5-*mda*-7 (Fig. 3, D and E). This correlated with an increase in cell death, as indicated by increased poly(ADP-ribose) polymerase cleavage in these cells. This effect correlated with decreases in the expression of the Bcl-2 antiapoptotic proteins Mcl-1 and Bcl-xL.

Effects of D-Limonene in Inducing Cancer-Specific Cell Death Are Dependent on ROS Induction. To evaluate whether cell death induction and growth inhibition following treatment with D-limonene are related to its ability to induce
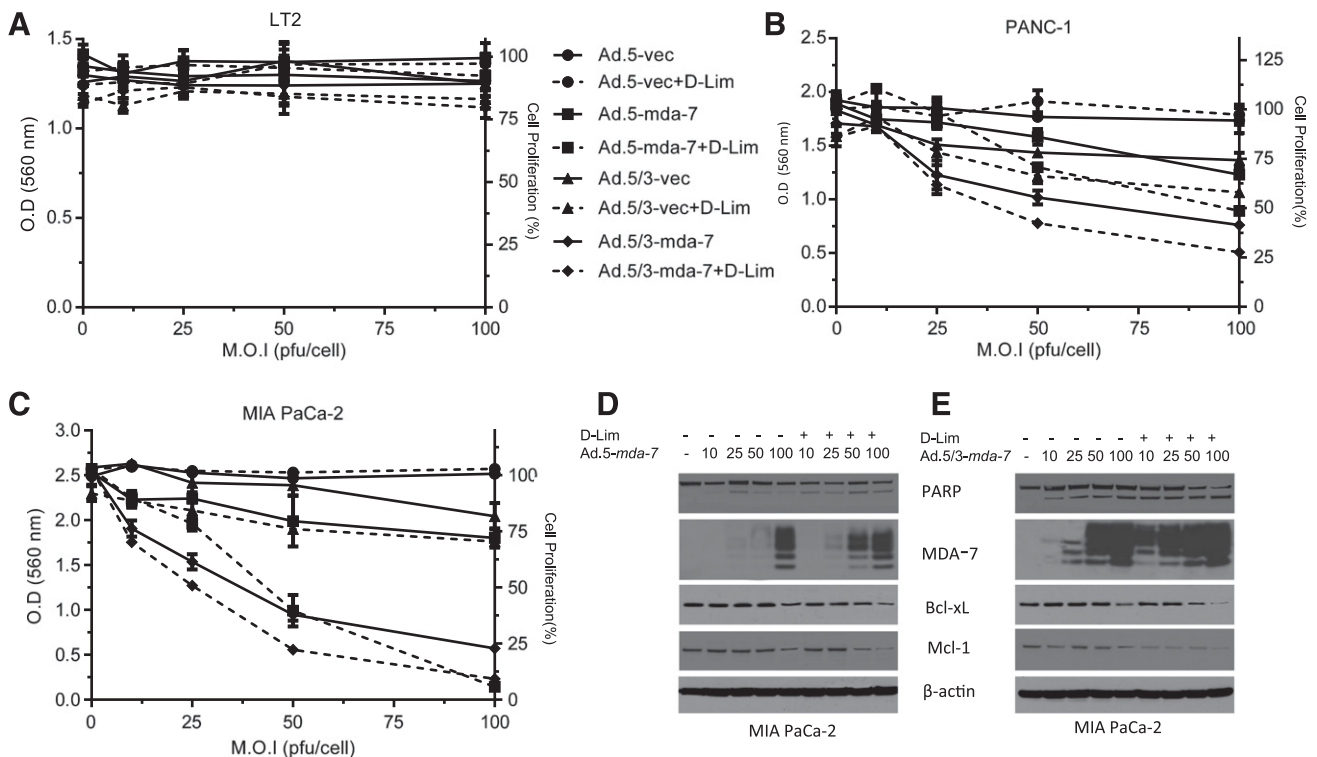
A
LT2

B
PANC-1

C
MIA PaCa-2

D

E

Fig. 3. D-Limonene (D-Lim) synergizes with *mda*-7/IL-24, in inducing cancer-specific apoptosis in pancreatic cancer cells. Pancreatic cells were infected with increasing m.o.i (multiplicity of infection; pfu/cell) of Ad.5-*mda*-7 and its recombinant chimeric Ad.5/3-*mda*-7, followed by 100 µM D-Lim treatment. MTT assays were performed in (A) LT2 cells, (B) PANC-1 and (C) MIA PaCa-2 cells 72 hours after treatment. (D and E) Western blotting of MIA PaCa-2 lysates for MDA-7 and markers of apoptosis. O.D., optical density.

ROS, we pretreated cells with NAC before Ad infection and D-limonene treatment. As expected, this addition did not show any change in proliferation in LT2 cells (Fig. 4A). However, in both mutant K-*ras*–bearing MIA PaCa-2 (Fig. 4B) and wild type K-*ras*–bearing BxPC-3 cells (Fig. 4C) pretreatment with NAC significantly rescued cells from the growth-inhibitory effects of Ad.5/3-*mda*-7 plus D-limonene. To further confirm that these changes were reflective of alterations in proliferation, bromo-deoxyuridine (5-bromo-2′-deoxyuridine) incorporation assays were done in both LT2 and MIA PaCa-2 cells. These studies confirmed the observations seen by MTT, showing very similar results (Supplemental Fig. 1). The observation of ROS levels (specifically peroxide) confirmed that NAC pretreatment prevented D-limonene–induced ROS generation (Fig. 4D). Pyruvate, another ROS scavenger, was also shown to inhibit this increase in ROS (Fig. 4D). Similar results were obtained when monitoring apoptosis induction, with LT2 cells showing no increase in apoptosis, but MIA PaCa-2 and BxPC-3 cells infected with Ad.5/3-*mda*-7 and treated with D-limonene showing increases in Annexin V staining, cleaved poly(ADP-ribose) polymerase, and cleaved caspase-3 (Fig. 5). Furthermore, these cells also showed increased *mda*-7/IL-24 protein expression as well as an increase in an ER stress marker, BiP/GRP-78, which is induced by *mda*-7/IL-24. These effects were also prevented by pretreatment with NAC (Fig. 5).

ROS Induction by D-Limonene Activates Translational Machinery in Cells. The effects of D-limonene when used in conjunction with Ad.*mda*-7 infection are similar to what we have observed previously with other ROS-inducing agents (Lebedeva, et al., 2005, 2008a,b). In an effort to enhance our understanding of the mechanism behind these observations, we evaluated the effects of D-limonene further. We found that D-limonene–induced ROS generation occurs in both a dose- and time-dependent manner (Fig. 6A). We know that without this compound, *mda*-7/*IL-24* mRNA is very weakly translated when introduced into pancreatic cancer cells via Ad infection and that the addition of D-limonene results in greater translation of *mda*-7/*IL-24* mRNA into protein. To further confirm that D-limonene is able to increase the translation of this mRNA into protein, we looked at the expression of the preinitiation complex proteins after D-limonene treatment. In accordance with our observed time-dependent ROS induction, we found that D-limonene is able to activate the transient translational machinery within a few hours of treatment. Increases in phosphorylated eIF4E were found to peak at around 3 hours post-treatment. Translational repressor 4EBP-1, which inhibits eIF4E in its unphosphorylated form, also demonstrated an increase in phosphorylation that peaked at 3 hours after D-limonene treatment. We also saw activation of upstream kinase P70S6K in this same time frame (Fig. 6, B and C). The dose of
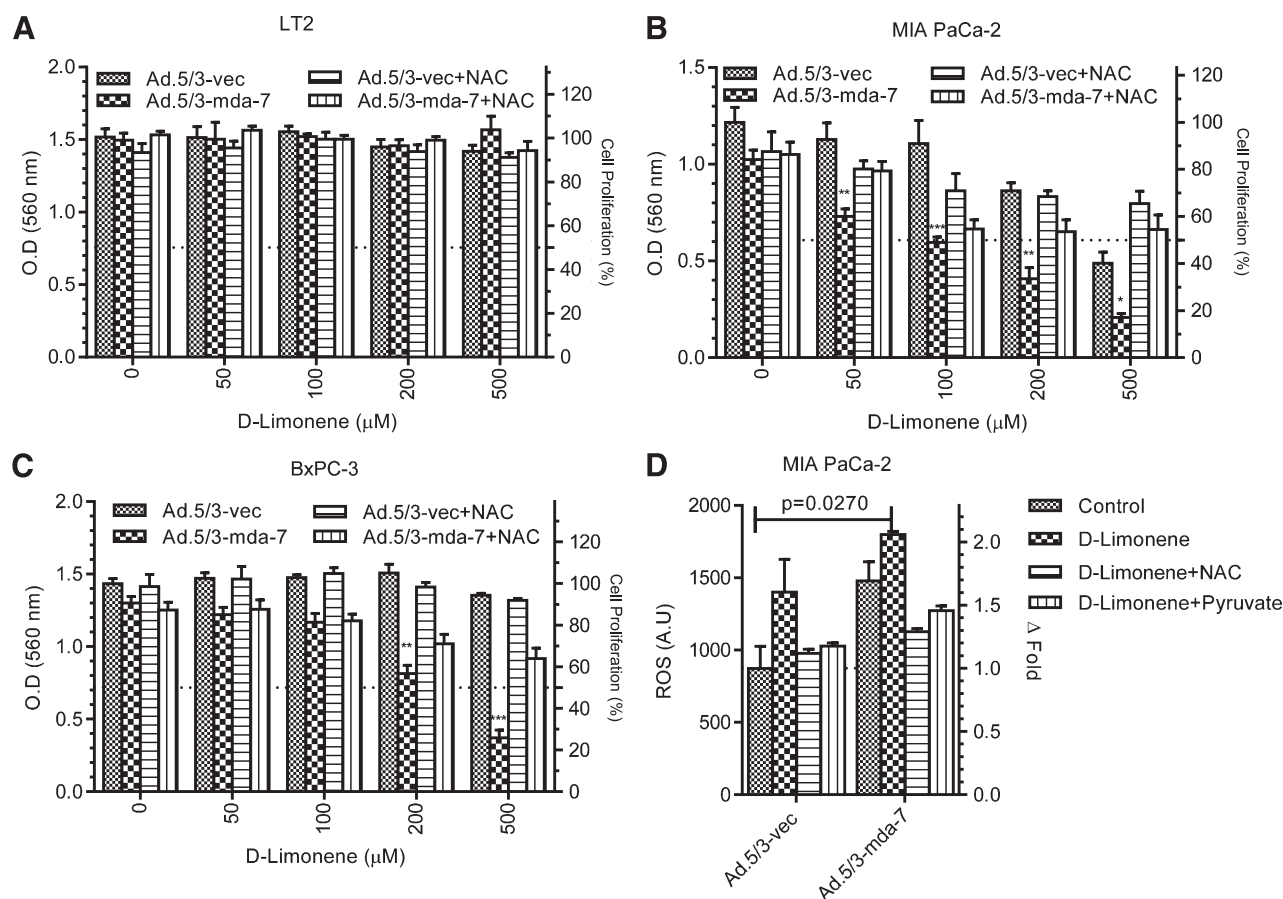

Fig. 4. D-Limonene enhances *mda*-7/IL-24–mediated inhibition of cell growth by inducing ROS (specifically peroxide). Pancreatic cells were pretreated with 10 mM NAC prior to treatment with Ad.5/3-*mda*-7 (25 pfu/cell) and D-Lim. MTT assays were performed on (A) LT2 cells, (B) MIA PaCa-2, and (C) and BxPC-3 cells 72 hours after treatment. (D) MIA-PaCa-2 cells were pretreated with either NAC or peroxide scavenger, pyruvate, and then stained with DCF to detect ROS induction (specifically peroxide). A.U., arbitrary units; O.D., optical density.
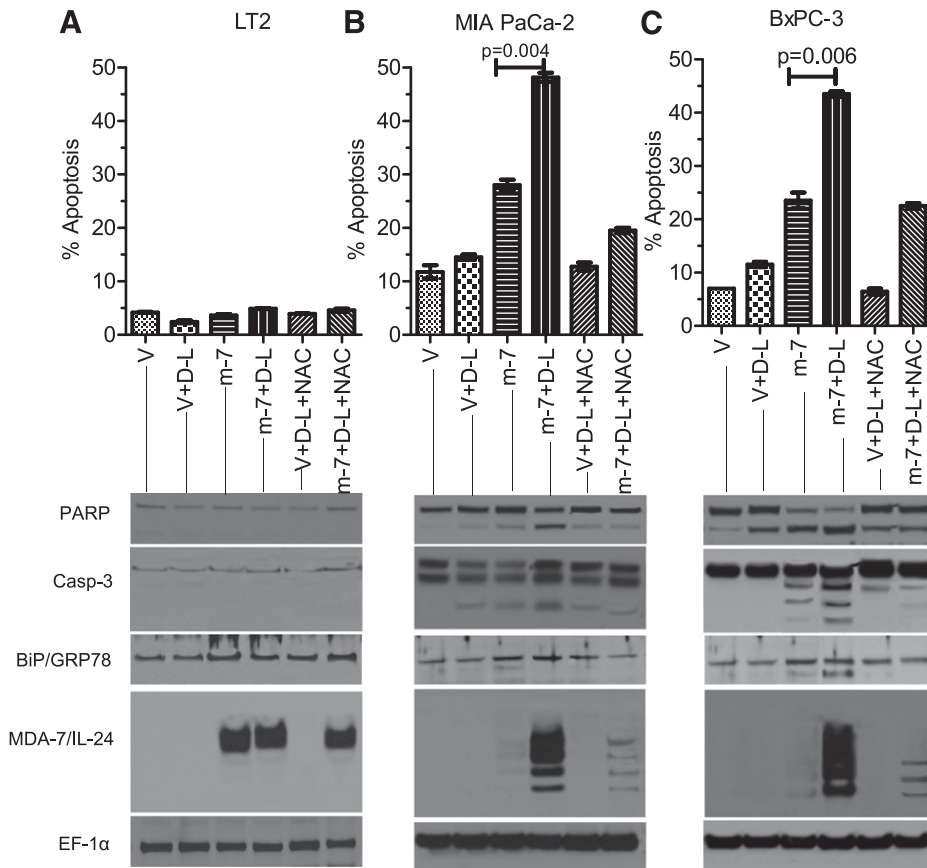
Fig. 5. D-Limonene enhances *mda-7/IL-24*–mediated apoptosis. (A) LT2, (B) MIA PaCa-2, and (C) BxPC-3 cells were treated with NAC prior to treatment with Ad.5/3-*mda-7* (m-7) (25 pfu/cell) and D-limonene (D-L) (100 μM). Apoptosis was measured after 48 hours by Annexin V/PI staining and flow-cytometer analysis. Western blotting was performed from the whole-cell lysates 48 hours post-treatment. V, Annexin V.

limonene used in these experiments showed potent induction of ROS at 3 hours after treatment (Fig. 6A), supporting a potential role of ROS in the activation of the translational machinery. To confirm that this activation of the preinitiation complex was related to the induction of ROS, we pretreated cells with NAC and also included the potent ROS-inducer arsenic trioxide as a positive control. D-Limonene was able to induce the phosphorylation of P70S6K, eIF4E, and 4EBP-1 to the same extent as arsenic trioxide. Importantly, these increases in phosphorylation were completely abrogated if cells were pretreated with NAC (Fig. 6D).

D-Limonene Increases *mda-7/IL-24* mRNA Polysome Association. These experiments provide a novel role for D-limonene, showing that this compound induces ROS that can subsequently activate the translational machinery in pancreatic cancer cells. We have also shown that the overall effect of this phenomenon is increased *mda-7/IL-24* translation, thereby resulting in cancer-specific toxicity. We finally wanted to determine if the activation of the translational machinery actually resulted in increased association of *mda-7/IL-24* mRNA with the polysomes. Although we have demonstrated that MDA-7/IL-24 protein levels increase, showing increased polysome association would confirm our hypothesis that the ROS generated by D-limonene not only activated the preinitiation complex of the translational machinery but also subsequently facilitated the association of *mda-7/IL-24* mRNA with polysomes. As a proof-of-principle, MIA PaCa-2 cells were infected with either Ad.5-*mda-7* or Ad.5/3-*mda-7*, with or without subsequent D-limonene treatment. Polysomal fractions from these cells were separated using sucrose gradient gels (Fig. 7A) and then assayed for the presence of *mda-7/IL-24*

mRNA using real-time PCR. We found that with both Ad serotypes, the addition of D-limonene resulted in a significantly increased polysome association of *mda-7/IL-24* mRNA as observed by a decreased Ct value (Fig. 7B). There was no apparent change in the association of the housekeeping gene GAPDH mRNA with polysomes (Fig. 7, B and C), indicating that D-limonene induces the translation of weakly translated mRNAs such as *mda-7/IL-24*. In order to see the fold-change in polysomal-associated *mda-7/IL-24* mRNA with respect to polysomal-associated GAPDH mRNA following treatment with Ad.*mda*-7/IL-24 and/or D-limonene, the relative quotient was calculated. It was found that D-limonene significantly increased the association of weakly translated *mda-7/IL-24* mRNA with polysomes when MIA PaCa-2 cells were treated with *mda*-7/IL-24 using either serotype of Ad (Fig. 7C). Furthermore, pretreating cells with NAC prevented increased polysome association, supporting our conclusion that it is indeed the induction of ROS that ultimately leads to increased *mda*-7/IL-24 translation. Western blots confirmed that this combination resulted in increased MDA-7/IL-24 protein expression following phosphorylation of translation machinery proteins P70S6K and eIF4E, which leads to the formation of the preinitiation complex. All of these changes were inhibited with NAC pretreatment (Fig. 7D).

D-Limonene Induces *mda-7/IL-24* Expression and Toxicity in Cell Lines That Stably Overexpress *mda-7/IL-24* mRNA. To ensure that the effects of D-limonene on *mda-7/IL-24* mRNA translation were not dependent on the adenoviral delivery of *mda-7/IL-24*, we evaluated this compound in pancreatic cancer cell lines that stably overexpress *mda-7/IL-24* mRNA, without detectable protein. PANC-1 (Fig. 8A) and MIA PaCa-2
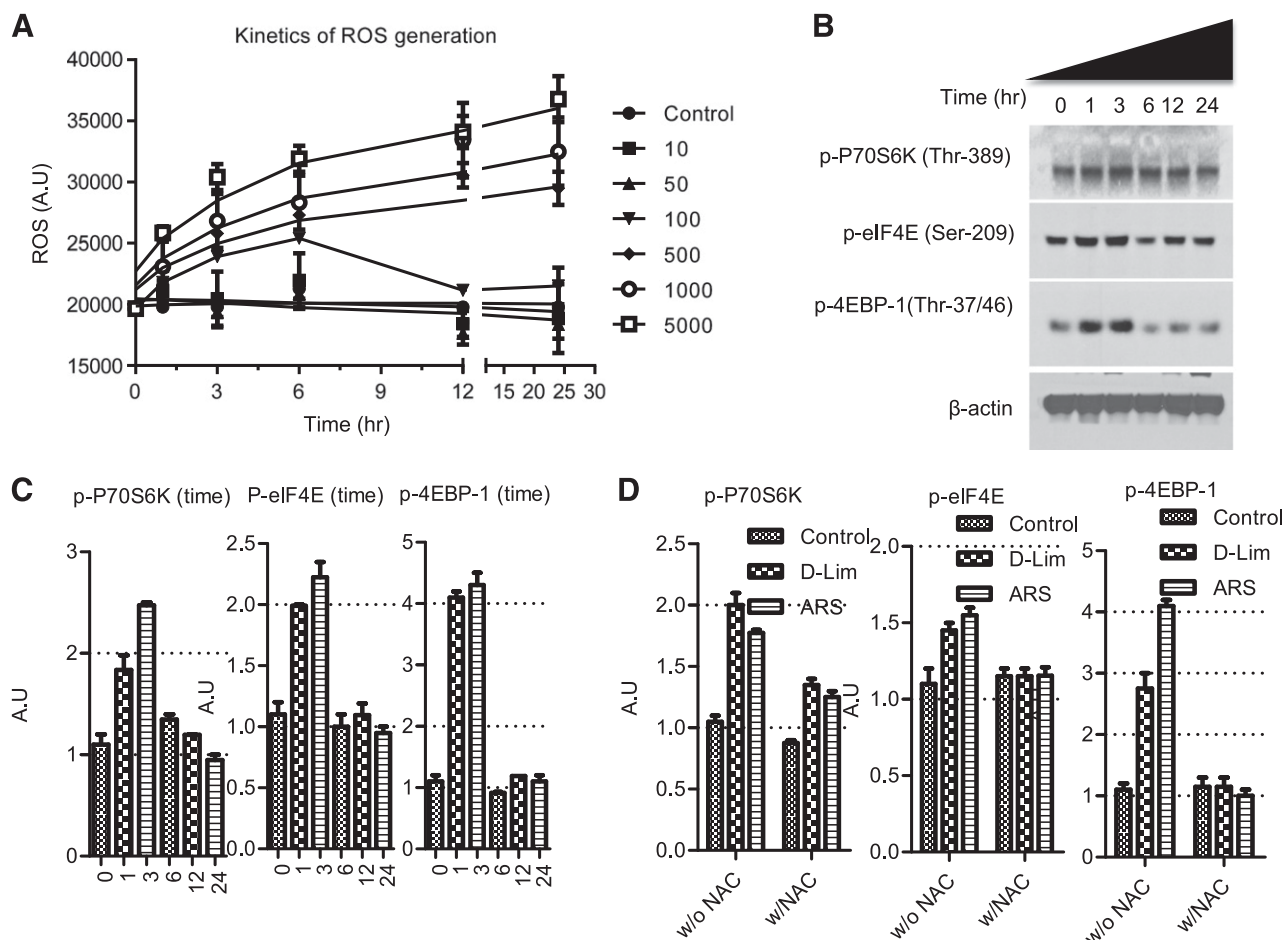
Fig. 6. D-Limonene (D-Lim) activates the translational machinery in the cell through the induction of ROS. (A) Dose and time kinetics of ROS generation by D-Lim. (B) Western blotting analysis of phosphorylated P70S6K, eIF4E, and 4EBP-1 after 100 μM limonene treatment. (C) Western blotting quantification of (B). (D) Quantification of Western blotting analysis of phosphorylated P70S6K, eIF4E, and 4EBP-1 after limonene (100 μM) or arsenic trioxide (ARS) (1 μM) treatment with or without NAC pretreatment. A.U., arbitrary units.

(Fig. 8B) stable *mda-7/IL-24* mRNA–expressing clones were treated with increasing doses of D-limonene for 3 days and evaluated for effects on proliferation by MTT assays. We found that compared with parental cell lines containing an empty vector, the *mda-7/IL-24*–overexpressing clones exhibited greater growth inhibition by D-limonene, with left-shifting dose curves and lower IC$_{50}$ values. The IC$_{50}$ values of PANC-1 and MIA PaCa-2 treated with D-limonene were 413.15 and 385.12 μM, respectively. The IC$_{50}$ values were decreased to 337.12 and 195.11 μM, respectively, for PANC-1 overexpressing *mda-7/IL-24* (PANC-1-m7) and MIA PaCa-2 overexpressing *mda-7/IL-24* (MIA PaCa-2-m7), respectively. Western blotting results confirmed that both D-limonene and POH were able to induce MDA-7/IL-24 protein expression in pancreatic cancer cells overexpressing *mda-7/IL-24*, and this expression resulted in cell death as demonstrated by poly(ADP-ribose) polymerase cleavage (Fig. 8C).

Discussion

Pancreatic cancer is an invariably fatal disease that currently lacks effective therapeutic options for patients (Hezel et al., 2006). The development of novel ways to combat this cancer is essential. *mda-7/IL-24* has shown great promise as a cancer therapeutic (Fisher et al., 2003, 2005; Cunningham et al., 2005;

Tong et al., 2005; Eager et al., 2008; Dash et al., 2010a). This tumor suppressor, when introduced back into cancer cells, is capable of inducing ER stress and apoptosis, initiating an autocrine and paracrine feedback-loop to increase *mda-7/IL-24* expression, and fostering an anticancer immune response (Fisher, 2005; Dash et al., 2010a). Furthermore, this cytokine shows a bystander effect as a result of its feedback loop, whereby MDA-7/IL-24 protein secretion from cells is able to bind to receptors on neighboring normal and cancer cells and induce *mda-7/IL-24* expression in those cells (Su et al., 2005; Sauane et al., 2008). This loop intensifies the overall anticancer response and contributes significantly to its efficacy as a cancer therapeutic.

Unfortunately, the use of *mda-7*/IL-24 in pancreatic cancer has been restricted, owing to decreased translation of its encoded mRNA into protein (Su et al., 2001; Lebedeva et al., 2008a,b; Dash et al., 2014). Adenoviral delivery of this cytokine results in robust *mda-7*/IL-24 mRNA expression with only low or limited levels of expressed MDA-7/IL-24 protein, and consequently minimal changes in cellular phenotype. We have studied strategies to overcome this block in protein translation of the *mda-7*/IL-24 mRNA (Su et al., 2001; Lebedeva et al., 2005, 2006, 2007, 2008a,b; Sarkar et al., 2013; Dash et al., 2014), and thereby increase the utility of *mda-7*/IL-24 for the therapy of pancreatic cancer. Our previous work has shown
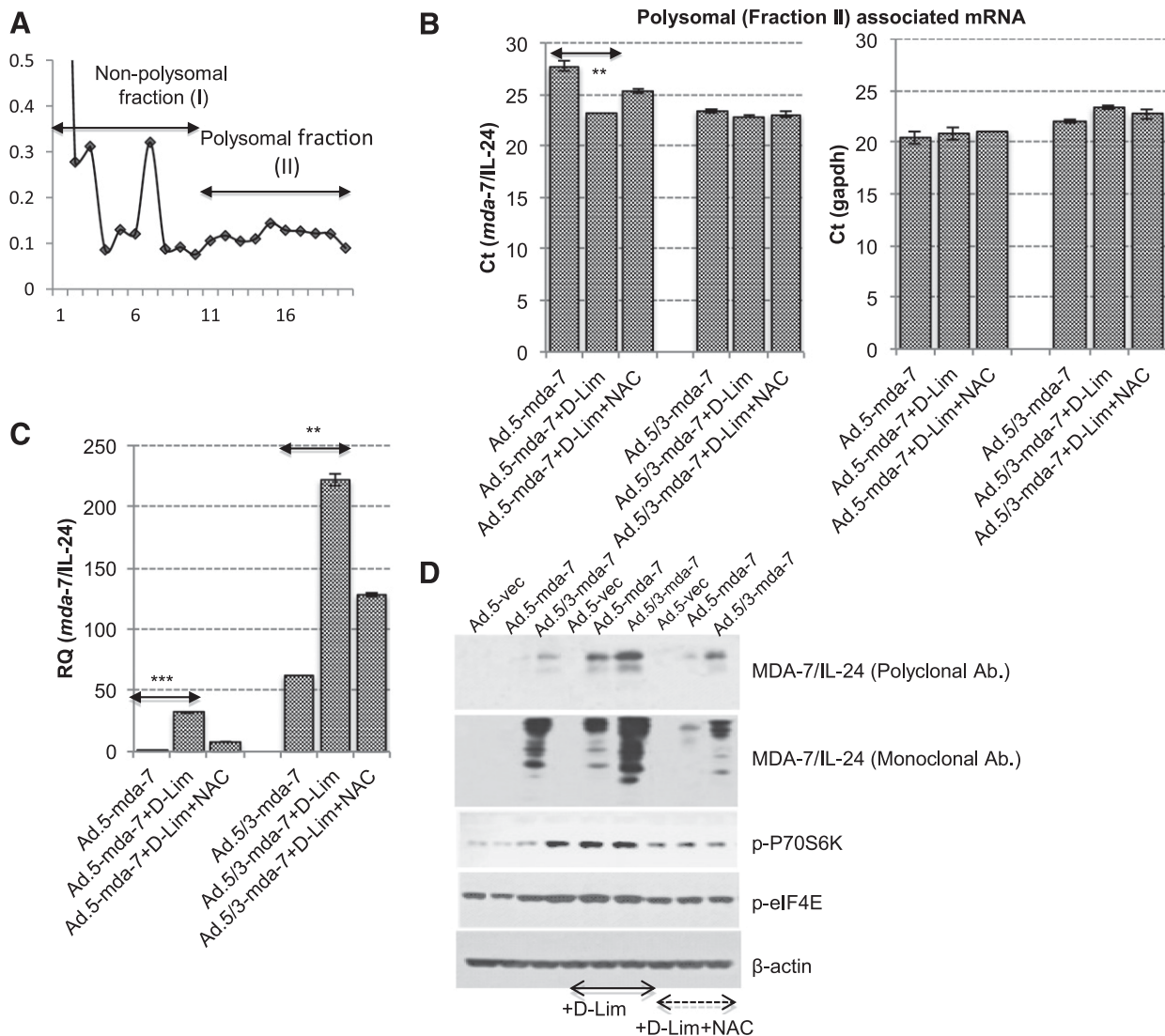
Fig. 7. D-Limonene (D-Lim) enhances the binding of weakly translated *mda-7/IL-24* mRNA with polysomes. (A) MIA PaCa-2 cells were infected with Ad.5-*mda-7* or Ad.5/3-*mda-7* (25 pfu/cell) and subsequently treated with 100 μM D-Lim. Polysomal fractions were separated using a 10–40% sucrose gradient as indicated by graph. Fractions were pooled into two sets, i.e., fraction I (1–10 fraction number) and fraction II (enriched with mRNA associated with polysomes). (B) mRNA was isolated and reverse transcription PCR was performed using a probe for *mda-7/IL-24* and GAPDH. (C) The relative quotient of *mda-7/IL-24* with respect to GAPDH. (D) Western blotting was performed on lysates from cells infected with Ad.5-*mda-7* or Ad.5/3-*mda-7* (25 pfu/cell), with or without D-Lim and/or NAC, using antibodies for preinitiation complex markers and MDA-7/IL-24. Level of significance: **$P < 0.01$; ***$P < 0.001$.

that inhibiting oncogenic K-*ras* is one mechanism of increasing the translation of *mda*-7/IL-24 mRNA. The mechanism through which K-*ras* influences MDA-7/IL-24 protein expression is currently unknown. K-*ras* signaling involves a multitude of downstream target proteins and it is possible that one of these K-*ras* effector proteins is involved in protein translation. While this is a useful strategy in vitro, it is difficult to translate this into the clinic, as inhibiting K-*ras* in patients is problematic because of the lack of success in the development of drugs specifically targeting this oncogene (Maurer et al., 2012; Sun et al., 2012; Hocker et al., 2013). We also found that the induction of ROS was able to increase expression of MDA-7/IL-24 protein and produce a subsequent therapeutic effect. Potent ROS inducers such as arsenic trioxide show great success when used in combination with *mda*-7/IL-24 in experimental settings (Lebedeva et al., 2005).

We then turned our studies to more natural compounds capable of inducing ROS. Drug toxicity is a major problem in the treatment of cancer and this only intensifies as patients

are treated with increasing numbers of drugs in combination. We evaluated POH, a dietary agent known to induce ROS at nontoxic doses. It was demonstrated that the combination of *mda*-7/IL-24 and POH also induced MDA-7/IL-24 expression and potent cell death in pancreatic cancer cells (Lebedeva et al., 2008b).

While we had made advances in increasing the utilization of *mda*-7/IL-24 as a therapy for pancreatic cancer, a few outstanding questions needed to be addressed. The goal of this study was to examine some of those questions. Could we improve our viral delivery of *mda*-7/IL-24 as a means of increasing therapeutic efficacy? Do dietary agents other than POH also function to increase *mda*-7/IL-24 mRNA translation and promote subsequent phenotypic effects?

We began this study by evaluating our delivery system of *mda*-7/IL-24. Adenoviral delivery mechanisms can be a successful means of gene therapy but are reliant on certain factors for success. Adenoviral serotypes use different cellular
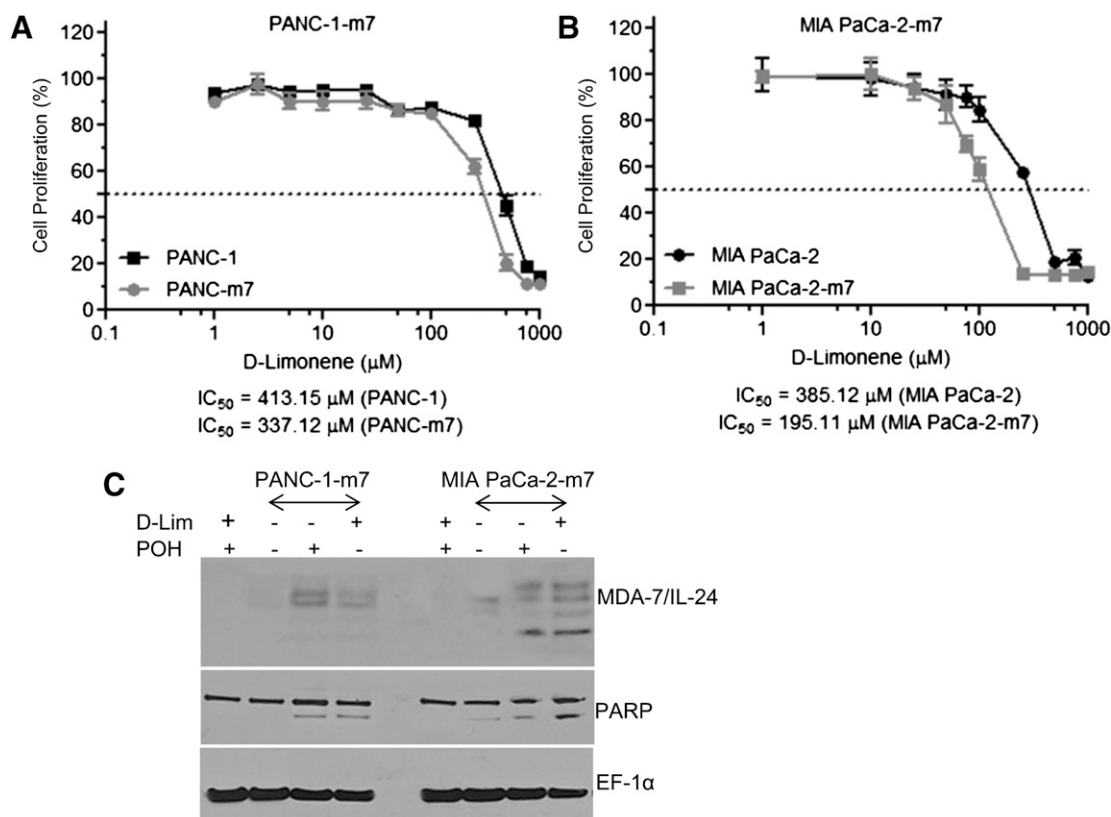
Fig. 8. D-Limonene (D-Lim) induces *mda*-7/IL-24 expression and toxicity in cell lines that stably overexpress *mda-7/IL-24* mRNA. (A) PANC-1 stably transfected with pcDNA3.1-*mda-7/IL-24* (PANC-1-m7) and (B) MIA PaCa-2 stably transfected with pcDNA3.1-*mda*-7 (MIA PaCa-2-m7) were treated with D-Lim (100 μM) and MTT assays were used to assess the effect on cellular proliferation 72 hours after treatment. (C) Western blotting of whole cell lysates of PANC-1-m7 and MIA PaCa-2-m7 with or without D-Lim (100 μM) or POH (100 μM).

receptors as a means of introducing their genetic material into target cells (Bergelson et al., 1998; Pearson et al., 1999; Dash et al., 2010b; Wang et al., 2011; Azab et al., 2012, 2014). Ad.5-*mda*-7, the traditional type 5 adenovirus serotype used in our prior work, is dependent on the expression of Coxsackie and adenovirus receptors (CAR) for efficacy. Our initial experiments showed that, as a whole, pancreatic cancer cells expressed relatively low levels of CAR, a factor that was most likely preventing us from successfully delivering optimum levels of *mda*-7/IL-24 to these cells. In contrast, the Ad.5/3 serotype uses desmoglein and CD46, as well as CAR, receptors for entry into cells. We show that pancreatic cancer cells express higher levels of these receptors, both on an mRNA and protein level and that expression of all three receptors yielded the highest rate of success in gene delivery (Fig. 1E).

However, despite being able to increase the infection efficacy of Ad, we still only observed minimal MDA-7/IL-24 protein expression. Our prior studies have shown that POH, a dietary monoterpene, is able to relieve this translational block when combined with Ad.*mda*-7 infection and enhance MDA-7/IL-24 protein production and subsequent expression through the induction of ROS (Lebedeva et al., 2008a,b). These observations supported the hypothesis that other monoterpenes that are capable of inducing ROS might also have similar effects on MDA-7/IL-24 protein production. In this study, we evaluated D-limonene, also a dietary monoterpene known to induce ROS. This compound has a low toxicity profile and has been shown to be safe when used in humans. Adverse effects reported include skin irritation when D-limonene was used in cosmetic products

(Kim et al., 2013). As a systemic therapy, this compound has been used in humans in clinical trials at up to 15 gm/day without major toxicities being observed. Some mild side effects, such as nausea or fatigue, were seen at higher doses (Sun, 2007). Given its safe drug profile, D-limonene is a viable candidate for inclusion as adjuvant treatment in patients.

We found that, similar to POH, nontoxic doses of D-limonene are able to enhance MDA-7/IL-24 protein production in cells infected with Ad.*mda*-7, which translates into *mda*-7/IL-24–induced toxicity. Our experiments demonstrated that this ROS induction was necessary for MDA-7/IL-24 protein production and that it enhanced translation through activation of the translation machinery and hence increased polysome association of *mda*-7/IL-24 mRNA and subsequent protein expression. These observations correlate with studies in the literature demonstrating that ROS generated via extracellular hydrogen peroxide is capable of inducing activation of p70S6K and its downstream target 4EBP-1 (Bae et al., 1999). We observed that ROS generated through D-limonene was also able to activate these members of the preinitiation complex and that led to subsequent activation of translation. Furthermore, we showed that this strategy was successful using both an adenoviral delivery strategy as well as stable transfection of *mda*-7/IL-24, resulting in mRNA expression with little protein production, in pancreatic cancer cells.

As a whole, our data supports the efficacy of *mda*-7/IL-24 gene therapy in pancreatic cancer. The use of *mda*-7/IL-24 as a cancer therapeutic is exciting and promising in many cancer types, but progress in pancreatic cancer has been slow. We

have made major strides in developing strategies to use this cancer-specific cytokine in this disease but continue to work on this issue. The presented data help to answer some of our outstanding questions and represent novel ways of increasing the efficacy and therapeutic use of *mda*-7/IL-24 in this devastating disease.

Authorship Contributions

Participated in research design: Fisher, D. Sarkar, Das, Emdad.
Conducted experiments: S. Sarkar, Quinn, Shen.
Contributed new reagents or analytic tools: Dent.
Performed data analysis: S. Sarkar, Quinn, Das, Emdad, Fisher.
Wrote or contributed to the writing of the manuscript: S. Sarkar, Quinn, Emdad, Das, D. Sarkar, Dent, Fisher.

References

Azab B, Dash R, Das SK, Bhutia SK, Shen XN, Quinn BA, Sarkar S, Wang XY, Hedvat M, and Dmitriev IP et al. (2012) Enhanced delivery of mda-7/IL-24 using a serotype chimeric adenovirus (Ad.5/3) in combination with the Apogossypol derivative BI-97C1 (Sabutoclax) improves therapeutic efficacy in low CAR colorectal cancer cells. *J Cell Physiol* 227:2145–2153.

Azab BM, Dash R, Das SK, Bhutia SK, Sarkar S, Shen XN, Quinn BA, Dent P, Dmitriev IP, and Wang XY et al. (2014) Enhanced prostate cancer gene transfer and therapy using a novel serotype chimera cancer terminator virus (Ad.5/3-CTV). *J Cell Physiol* 229:34–43.

Bae GU, Seo DW, Kwon HK, Lee HY, Hong S, Lee ZW, Ha KS, Lee HW, and Han JW (1999) Hydrogen peroxide activates p70(S6k) signaling pathway. *J Biol Chem* 274: 32596–32602.

Bergelson JM, Krithivas A, Celi L, Droguett G, Horwitz MS, Wickham T, Crowell RL, and Finberg RW (1998) The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J Virol* 72:415–419.

Chen X, Yano Y, Hasuma T, Yoshimata T, Yinna W, and Otani S (1999) Inhibition of farnesyl protein transferase and P21ras membrane association by d-limonene in human pancreas tumor cells in vitro. *Chinese Med Sci J* 14:138–144.

Cunningham CC, Chada S, Merritt JA, Tong A, Senzer N, Zhang Y, Mhashilkar A, Parker K, Vukelja S, Richards D, Hood J, Coffee K, and Nemunaitis J (2005) Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study. *Mol Ther* 11: 149–159.

Dash R, Bhutia SK, Azab B, Su ZZ, Quinn BA, Kegelmen TP, Das SK, Kim K, Lee SG, and Park MA et al. (2010a) mda-7/IL-24: a unique member of the IL-10 gene family promoting cancer-targeted toxicity. *Cytokine Growth Factor Rev* 21:381–391.

Dash R, Dmitriev I, Su ZZ, Bhutia SK, Azab B, Vozhilla N, Yacoub A, Dent P, Curiel DT, and Sarkar D et al. (2010b) Enhanced delivery of mda-7/IL-24 using a serotype chimeric adenovirus (Ad.5/3) improves therapeutic efficacy in low CAR prostate cancer cells. *Cancer Gene Ther* 17:447–456.

Dash R, Azab B, Quinn BA, Shen X, Wang XY, Das SK, Rahmani M, Wei J, Hedvat M, and Dent P et al. (2011) Apogossypol derivative BI-97C1 (Sabutoclax) targeting Mcl-1 sensitizes prostate cancer cells to mda-7/IL-24-mediated toxicity. *Proc Natl Acad Sci USA* 108:8785–8790.

Dash R, Bhoopathi P, Das SK, Sarkar S, Emdad L, Dasgupta S, Sarkar D, and Fisher PB (2014) Novel mechanism of MDA-7/IL-24 cancer-specific apoptosis through SARI induction. *Cancer Res* 74:563–574.

Eager R, Harle L, and Nemunaitis J (2008) Ad-MDA-7; INGN 241: a review of pre-clinical and clinical experience. *Expert Opin Biol Ther* 8:1633–1643.

Eulitt PJ, Park MA, Hossein H, Cruikshanks N, Yang C, Dmitriev IP, Yacoub A, Curiel DT, Fisher PB, and Dent P (2010) Enhancing mda-7/IL-24 therapy in renal carcinoma cells by inhibiting multiple protective signaling pathways using sorafenib and by Ad.5/3 gene delivery. *Cancer Biol Ther* 10:1290–1305.

Fisher PB (2005) Is mda-7/IL-24 a "magic bullet" for cancer? *Cancer Res* 65: 10128–10138.

Fisher PB, Gopalkrishnan RV, Chada S, Ramesh R, Grimm EA, Rosenfeld MR, Curiel DT, and Dent P (2003) mda-7/IL-24, a novel cancer selective apoptosis inducing cytokine gene: from the laboratory into the clinic. *Cancer Biol Ther* 2(4, Suppl 1)S23–S37.

Fisher PB, Sarkar D, Lebedeva IV, Emdad L, Gupta P, Sauane M, Su ZZ, Grant S, Dent P, and Curiel DT et al. (2007) Melanoma differentiation associated gene-7/ interleukin-24 (mda-7/IL-24): novel gene therapeutic for metastatic melanoma. *Toxicol Appl Pharmacol* 224:300–307.

Hamed HA, Yacoub A, Park MA, Eulitt PJ, Dash R, Sarkar D, Dmitriev IP, Lesniak MS, Shah K, and Grant S et al. (2010) Inhibition of multiple protective signaling pathways and Ad.5/3 delivery enhances mda-7/IL-24 therapy of malignant glioma. *Mol Ther* 18:1130–1142.

Hamed HA, Yacoub A, Park MA, Archer K, Das SK, Sarkar D, Grant S, Fisher PB, and Dent P (2013) Histone deacetylase inhibitors interact with melanoma differentiation

associated-7/interleukin-24 to kill primary human glioblastoma cells. *Mol Pharmacol* 84:171–181.

Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, and Depinho RA (2006) Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev* 20:1218–1249.

Hocker HJ, Cho KJ, Chen CY, Rambahal N, Sagineedu SR, Shaari K, Stanslas J, Hancock JF, and Gorfe AA (2013) Andrographolide derivatives inhibit guanine nucleotide exchange and abrogate oncogenic Ras function. *Proc Natl Acad Sci USA* 110:10201–10206.

Jiang H and Fisher PB (1993) Use of a sensitive and efficient subtraction hybridization protocol for the identification of genes differentially regulated during the induction of differentiation in human melanoma cells. *Mol Cell Differ* 1:285–299.

Jiang H, Lin JJ, Su ZZ, Goldstein NI, and Fisher PB (1995) Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene* 11:2477–2486.

Jiang H, Su ZZ, Lin JJ, Goldstein NI, Young CS, and Fisher PB (1996) The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. *Proc Natl Acad Sci USA* 93:9160–9165.

Kim YW, Kim MJ, Chung BY, Bang Y, Lim SK, Choi SM, Lim DS, Cho MC, Yoon K, and Kim HS et al. (2013) Safety evaluation and risk assessment of d-limonene. *J Toxicol Environ Health B Crit Rev* 16:17–38.

Lebedeva IV, Sarkar D, Su ZZ, Kitada S, Dent P, Stein CA, Reed JC, and Fisher PB (2003) Bcl-2 and Bcl-x(L) differentially protect human prostate cancer cells from induction of apoptosis by melanoma differentiation associated gene-7, mda-7/IL-24. *Oncogene* 22:8758–8773.

Lebedeva IV, Su ZZ, Sarkar D, Gopalkrishnan RV, Waxman S, Yacoub A, Dent P, and Fisher PB (2005) Induction of reactive oxygen species renders mutant and wild-type K-ras pancreatic carcinoma cells susceptible to Ad.mda-7-induced apoptosis. *Oncogene* 24:585–596.

Lebedeva IV, Sarkar D, Su ZZ, Gopalkrishnan RV, Athar M, Randolph A, Valerie K, Dent P, and Fisher PB (2006) Molecular target-based therapy of pancreatic cancer. *Cancer Res* 66:2403–2413.

Lebedeva IV, Washington I, Sarkar D, Clark JA, Fine RL, Dent P, Curiel DT, Turro NJ, and Fisher PB (2007) Strategy for reversing resistance to a single anticancer agent in human prostate and pancreatic carcinomas. *Proc Natl Acad Sci USA* 104:3484–3489.

Lebedeva IV, Su ZZ, Vozhilla N, Chatman L, Sarkar D, Dent P, Athar M, and Fisher PB (2008a) Chemoprevention by perillyl alcohol coupled with viral gene therapy reduces pancreatic cancer pathogenesis. *Mol Cancer Ther* 7:2042–2050.

Lebedeva IV, Su ZZ, Vozhilla N, Chatman L, Sarkar D, Dent P, Athar M, and Fisher PB (2008b) Mechanism of in vitro pancreatic cancer cell growth inhibition by melanoma differentiation-associated gene-7/interleukin-24 and perillyl alcohol. *Cancer Res* 68:7439–7447.

Maurer T, Garrenton LS, Oh A, Pitts K, Anderson DJ, Skelton NJ, Fauber BP, Pan B, Malek S, and Stokoe D et al. (2012) Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity. *Proc Natl Acad Sci USA* 109:5299–5304.

Pearson AS, Koch PE, Atkinson N, Xiong M, Finberg RW, Roth JA, and Fang B (1999) Factors limiting adenovirus-mediated gene transfer into human lung and pancreatic cancer cell lines. *Clin Cancer Res* 5:4208–4213.

Rabi T and Bishayee A (2009) d -limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. *J Carcinog* 8:9.

Sarkar S, Azab BM, Das SK, Quinn BA, Shen X, Dash R, Emdad L, Thomas S, Dasgupta S, and Su ZZ et al. (2013) Chemoprevention gene therapy (CGT): novel combinatorial approach for preventing and treating pancreatic cancer. *Curr Mol Med* 13:1140–1159.

Sauane M, Gopalkrishnan RV, Sarkar D, Su ZZ, Lebedeva IV, Dent P, Pestka S, and Fisher PB (2003) MDA-7/IL-24: novel cancer growth suppressing and apoptosis inducing cytokine. *Cytokine Growth Factor Rev* 14:35–51.

Sauane M, Su ZZ, Gupta P, Lebedeva IV, Dent P, Sarkar D, and Fisher PB (2008) Autocrine regulation of mda-7/IL-24 mediates cancer-specific apoptosis. *Proc Natl Acad Sci USA* 105:9763–9768.

Su Z, Lebedeva IV, Gopalkrishnan RV, Goldstein NI, Stein CA, Reed JC, Dent P, and Fisher PB (2001) A combinatorial approach for selectively inducing programmed cell death in human pancreatic cancer cells. *Proc Natl Acad Sci USA* 98: 10332–10337.

Su Z, Emdad L, Sauane M, Lebedeva IV, Sarkar D, Gupta P, James CD, Randolph A, Valerie K, and Walter MR et al. (2005) Unique aspects of mda-7/IL-24 antitumor bystander activity: establishing a role for secretion of MDA-7/IL-24 protein by normal cells. *Oncogene* 24:7552–7566.

Su ZZ, Madireddi MT, Lin JJ, Young CS, Kitada S, Reed JC, Goldstein NI, and Fisher PB (1998) The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice. *Proc Natl Acad Sci USA* 95:14400–14405.

Sun J (2007) D-limonene: safety and clinical applications. *Altern Med Rev* 12: 259–264.

Sun Q, Burke JP, Phan J, Burns MC, Olejniczak ET, Waterson AG, Lee T, Rossanese OW, and Fesik SW (2012) Discovery of small molecules that bind to K-Ras and inhibit Sos-mediated activation. *Angew Chem Int Ed Engl* 51:6140–6143.

Tong AW, Nemunaitis J, Su D, Zhang Y, Cunningham C, Senzer N, Netto G, Rich D, Mhashilkar A, Parker K, and et al. (2005) Intratumoral injection of INGN 241, a nonreplicating adenovector expressing the melanoma-differentiation associated gene-7 (mda-7/IL24): biologic outcome in advanced cancer patients. *Mol Ther* 11:160–172.

Wang H, Li ZY, Liu Y, Persson J, Beyer I, Möller T, Koyuncu D, Drescher MR, Strauss R, and Zhang XB et al. (2011) Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11 and 14. *Nat Med* 17:96–104.

Address correspondence to: Dr. Paul B. Fisher, VCU Institute of Molecular Medicine, Virginia Commonwealth University, School of Medicine, Richmond, VA 23298. E-mail: pbfisher@vcu.edu