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Reversing translational suppression and induction of toxicity in pancreatic cancer  
cells using a chemoprevention gene therapy (CGT) approach

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

Affiliations:

Department of Human and Molecular Genetics, Virginia Commonwealth  
University, School of Medicine, Richmond, Virginia, USA.  
S.S., B.A.Q., X.S., S.K.D., L.E., D.S., & P.B.F.

Department of Biochemistry and Molecular Biology, Virginia Commonwealth  
University, School of Medicine, Richmond, Virginia, USA.  
P.D.

VCU Institute of Molecular Medicine, Virginia Commonwealth University, School  
of Medicine, Richmond, Virginia, USA.  
P.D., S.K.D., L.E., D.S., & P.B.F.

VCU Massey Cancer Center, Virginia Commonwealth University, School of  
Medicine, Richmond, Virginia, USA  
P.D., L.E., D.S. & P.B.F.

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**Running Title:** CGT induces translation and toxicity in pancreatic cancer

**Corresponding author: Paul B. Fisher, VCU Institute of Molecular Medicine,  
Virginia Commonwealth University, School of Medicine, Richmond, VA  
23298; [pbfisher@vcu.edu](mailto:pbfisher@vcu.edu).**

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**Abbreviations:**

Ad, adenovirus; Ad.5, serotype 5 adenovirus; Ad.5/3, serotype 5 and serotype 3 chimeric adenovirus; Ad.*vec*, replication-incompetent adenovirus lacking a gene insert; Ad.*mda-7*, replication-incompetent adenovirus containing the full-length *mda-7* gene; ARS, Arsenic Trioxide; CAR, Coxsackie and Adenovirus receptors; CGT, chemoprevention gene therapy; D-Lim, D-Limonene; IL-10, Interleukin 10; K-*ras*, Kirsten ras; Melanoma differentiation associated gene-7/Interleukin-24 (*mda-7/IL-24*); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
POH, perillyl alcohol; ROS, reactive oxygen species

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## Abstract

Pancreatic cancer is an aggressive disease with limited therapeutic options. *mda-7/IL-24*, a potent anti-tumor cytokine, shows cancer-specific toxicity in a vast array of human cancers, inducing ER stress and apoptosis, toxic autophagy, an anti-tumor immune response, an anti-angiogenic effect and a significant 'bystander' anti-cancer effect that leads to enhanced production of this cytokine through autocrine and paracrine loops. Unfortunately, its applications in pancreatic cancer have been restricted due to 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* as compared to 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 pre-initiation complex of the translational machinery in pancreatic cancer cells, and culminates in *mda-7/IL-24*-mediated toxicity.

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## Introduction

Pancreatic cancer is a deadly disease with a five-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 anti-cancer cytokine originally cloned in our laboratory during studies of terminal differentiation of human melanoma cells (Jiang and Fisher, 1993; Jiang et al., 1995; Jiang et al., 1996). This gene is a member of the IL-10 family of cytokines and functions as a tumor suppressor (Dash et al., 2010a). Re-introduction of this cytokine into cancer cells leads to significant ER stress, apoptosis, and anti-cancer 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 effect distant tumors and metastases (Dash et al., 2010a; Sauane et al., 2003; Sauane et al., 2008).

Gene therapy studies using Ad.*mda-7* have been shown to be extremely effective in multiple cancer types in both lab and clinical settings (Cunningham et al., 2005; Eager et al., 2008; Fisher, 2005; Fisher et al., 2007; Fisher et al., 2003;

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Tong et al., 2005). Although *mda-7/IL-24* has ubiquitous activity in multiple cancer types, it is difficult to fully exploit these in pancreatic cancer due 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 (Lebedeva et al., 2006; Lebedeva et al., 2005; Lebedeva et al., 2008a; Lebedeva et al., 2008b; Lebedeva et al., 2007; Sarkar et al., 2013; Su et al., 2001).

Our previous work has demonstrated that this '*translational block*' seen after Ad.5-*mda-7* treatment can be abrogated by two strategies: inhibition of oncogenic *K-ras* or combination with a ROS-inducing agent (Lebedeva et al., 2006; Lebedeva et al., 2005; Lebedeva et al., 2008a; Lebedeva et al., 2008b; Lebedeva et al., 2007; Su et al., 2001). 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,

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which leads to inefficient viral infectivity (Pearson et al., 1999). Using alternate viral serotypes to create chimeric viruses can help with this issue (Azab et al., 2012; Azab et al., 2014; Dash et al., 2011; Dash et al., 2010b; Hamed et al., 2013; Hamed et al., 2010). 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 as compared to Ad.5-*mda-7* and that this translates into greater toxicity (Azab et al., 2012; Dash et al., 2011; Dash et al., 2010b; Eulitt et al., 2010; Hamed et al., 2010).

In this study, we determined if Ad.5/3-*mda-7* would improve the transduction efficiency in pancreatic cancer cells as compared to 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 similarly as well as increasing *mda-7/IL-24* mRNA translation post Ad.*mda-7* infection.

## **Materials and Methods**

### **Cell lines and generating stable clones**

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AsPC-1 and BxPC-3 cell lines were obtained from the ATCC, maintained in RPMI-1640 (GIBCO®, Invitrogen™, Auckland, NZ) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA). MIA PaCa-2 and PANC-1, also obtained from the ATCC, were maintained in DMEM (GIBCO®) supplemented with 10% FBS. Immortalized normal pancreatic mesenchymal cell line, LT2, was obtained from EMD Millipore (Billerica, MA, USA) and maintained per Millipore instructions. All cell lines were cultured at 37°C in a 5% CO<sub>2</sub> 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 qPCR.

### **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 h followed by treatment with Limonene (200 μM) in complete medium (10% FBS) for time points as described for particular assays.

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### **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 h followed by treatment with D-Limonene in complete medium (10% FBS) for 72 h, and cell proliferation assays were performed at O.D. 560 nm after adding 100  $\mu$ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich) (MTT) dye (1 mg/ml) (Sigma-Aldrich). To further confirm cell proliferation, a chemiluminescent-based BrdU Cell Proliferation Assay kit (Cell Signaling Technology) was used.

### **Apoptotic assays**

Apoptotic assays were performed using an FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™, San Diego, CA, USA), 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.

### **ROS measurements**

To determine ROS production, cells were stained with 100  $\mu$ l of 10  $\mu$ M carboxy-H<sub>2</sub>DCFDA (Molecular Probes™, Invitrogen) in PBS for 30 min 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**



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Cells were treated for 48 h, lysed using cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) supplemented with 1 mM PMSF (Sigma-Aldrich) with Protease cocktail inhibitor, Phosphatase inhibitor (Roche, Indianapolis, IN USA), and 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, USA), anti-EF1 $\alpha$  (1:5000; EMD Millipore), mouse monoclonal anti-human K-Ras (1:1000; Bio-Rad Laboratories, Raleigh, NC, USA), 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., Santa Cruz, CA, USA). The secondary antibodies used were polyclonal goat anti-mouse IgG (1:1000; Dako, Carpinteria, CA, USA) and polyclonal swine anti-rabbit IgG (1:3000; Dako).

### **Polysome Studies**

Cells ( $2 \times 10^6$ ) were infected with Ad.*mda-7* and 48 h later harvested in 500  $\mu$ l Buffer A (200 nM Tris-HCl pH 8.5, 50 mM KCl, 25 mM MgCl<sub>2</sub>, 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; Invitrogen Carlsbad, CA). Cells were centrifuged at 12,000 rpm for 10 min 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

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Tris-HCl, 25 nM KCl, and 10 mM MgCl<sub>2</sub>) and centrifuged at 40,000 rpm for 1 h at 4°C. Fractions of 500 µl were collected, the O.D. 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 further purified using 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 RT-PCR was performed using a probe for *mda-7/IL-24* and GAPDH for relative quantification of mRNA.

## STATISTICAL ANALYSES

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.). Student's t-test or 1-way ANOVA 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

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this problem, a serotype chimeric recombinant adenovirus, Ad.5/3, was generated. Ad.5/3, while able to use CAR receptors, are also reliant upon 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 as compared to CAR (Figure 1A). AsPC-1 and BxPC-3 cells also express high levels of Desmoglein (Figure 1B). These results were also confirmed by measuring mRNA transcript levels (Figure 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 as compared to the Ad.5 serotype (Figure 1D). Pre-treatment 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 (Figure 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 (Figure 2A). D-Limonene is a monoterpene and capable of inhibiting the prenylation of GTPases, such as *K-ras* (Chen et al., 1999). However, the

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doses of D-Limonene we used in our study do not affect the normal prenylation of K-*ras* (Figure 2B). We next evaluated the ability of D-Limonene to induce ROS and found that even at nontoxic or sub-lethal doses, D-Limonene promoted ROS, specifically peroxides, induction and this increase was abrogated with NAC pretreatment (Figure 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; Lebedeva et al., 2008b). 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 (Figure 3A). However, in pancreatic cancer cell lines PANC-1 (Figure 3B) and MIA PaCa-2 (Figure 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 as compared to Ad.5-*mda-7* (Azab et al., 2012; Azab et al., 2014; Dash et al., 2010b). 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 as compared to Ad.5-*mda-7* (Figure 3D-E). This correlated with an

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increase in cell death, as indicated by increased PARP cleavage in these cells. This effect correlated with decreases in the expression of the Bcl-2 anti-apoptotic proteins Mcl-1 and Bcl-xL.

### **The 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 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 (Figure 4A). However, in both mutant K-*ras* bearing MIA PaCa-2 (Figure 4B) and wild type K-*ras* bearing BxPC-3 (Figure 4C) cells 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, BrdU incorporation assays were done in both LT2 and MIA PaCa-2 cells. These studies confirmed the observations seen by MTT, showing very similar results (Supplementary Figure 1). The observation of ROS (specifically peroxide) levels confirmed that NAC pretreatment prevented D-Limonene-induced ROS generation (Figure 4D). Pyruvate, another ROS scavenger, was also shown to inhibit this increase in ROS (Figure 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,

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cleaved PARP, and cleaved caspase-3 (Figure 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 (Figure 5).

### **ROS induction by D-Limonene activates the 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; Lebedeva, et al., 2008a; Lebedeva, et al., 2008b). 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 (Figure 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 pre-initiation 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 h post-treatment. Translational repressor 4EBP-1, which inhibits eIF4E in its unphosphorylated form, also demonstrated an increase in phosphorylation that

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peaked at 3 h post D-Limonene treatment. We also saw activation of upstream kinase P70S6K in this same time frame (Figure 6B-C). The dose of Limonene used in these experiments showed potent induction of ROS at 3h post-treatment (Figure 6A), supporting a potential role of ROS in the activation of the translational machinery. To confirm that this activation of the pre-initiation complex was related to the induction of ROS, we pretreated cells with NAC and also included the potent ROS-inducer arsenic trioxide (ARS) as a positive control. D-Limonene was able to induce the phosphorylation of P70S6K, eIF4E, and 4EBP-1 to the same extent as ARS. Importantly, these increases in phosphorylation were completely abrogated if cells were pretreated with NAC (Figure 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 was actually resulting 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 pre-initiation complex of the translational machinery, but also

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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 (Figure 7A) and then assayed for the presence of *mda-7/IL-24* mRNA using qPCR. 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 (Figure 7B). There was no apparent change in the association of the housekeeping gene GAPDH mRNA with polysomes (Figure 7B 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 (RQ) 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 (Figure 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 pre-initiation complex. All of these changes were inhibited with NAC pretreatment (Figure 7D).



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## **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 (Figure 8A) and MIA PaCa-2 (Figure 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 to 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<sub>50</sub> values. The IC<sub>50</sub> values of PANC-1 and MIA PaCa-2 treated with D-Limonene were 413.15 and 385.12  $\mu$ M, respectively. The IC<sub>50</sub> values were decreased to 337.12  $\mu$ M and 195.11  $\mu$ M, 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 PARP cleavage (Figure 8C).

## **Discussion**

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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 (Cunningham et al., 2005; Dash et al., 2010a; Eager et al., 2008; Fisher, 2005; Fisher et al., 2003; Tong et al., 2005). 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 anti-cancer immune response (Dash et al., 2010a; Fisher, 2005). 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 (Sauane et al., 2008; Su et al., 2005). 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, due to decreased translation of its encoded mRNA into protein (Dash et al., 2014; Lebedeva et al., 2008a; Lebedeva et al., 2008b; Su et al., 2001). 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 (Dash et al., 2014; Lebedeva et al., 2006; Lebedeva et al., 2005; Lebedeva et al., 2008a; Lebedeva et al., 2008b; Lebedeva et al., 2007; Sarkar et al., 2013; Su et

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al., 2001), and thereby increase the utility of *mda-7/IL-24* for the therapy of pancreatic cancer. Our previous work has shown 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 due to the lack of success in the development of drugs specifically targeting this oncogene (Hocker et al., 2013; Maurer et al., 2012; Sun et al., 2012). 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 perillyl alcohol (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., 2008a; Lebedeva et al., 2008b).

While we have made advances in increasing the utilization of *mda-7/IL-24* as a therapy for pancreatic cancer, a few outstanding questions needed to be

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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 receptors as a means of introducing their genetic material into target cells (Azab et al., 2012; Azab et al., 2014; Bergelson et al., 1998; Dash et al., 2010b; Pearson et al., 1999; Wang et al., 2011). 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 (Figure 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

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production and subsequent expression through the induction of ROS (Lebedeva et al., 2008a; Lebedeva et al., 2008b). 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 included 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 observing major toxicities. 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, non-toxic 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 pre-initiation complex and that lead to

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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.

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## **Authorship Contributions**

Participated in research design: Fisher, D Sarkar, Das, Emdad

Conducted experiments: S Sarkar, Quinn, Shen

Contributed new reagents: Dent

Performed data analysis: S Sarkar, Quinn, Das, Emdad, Fisher

Wrote or contributed writing of the manuscript: S Sarkar, Quinn, Emdad, Das, D Sarkar, Dent, Fisher

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### **Footnotes.**

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Siddik Sarkar and Bridget A. Quinn Contributed equally to this paper.



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## Figure Legends

**Figure 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 (Des-2) expression. (C) mRNA levels of CAR, CD46, and DSG-2 as determined by qRT-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 h post-infection using a luminometer (cps = counts per second). (E) Pancreatic cancer cell lines BxPC-3 and MIA PaCa-2 were pretreated with anti-CAR ( $\alpha$ -CAR), anti-CD46 ( $\alpha$ -CD46) and recombinant DSG-2 for 2 h followed by Ad.5/3-luc infection, and luciferase expression was quantified using a luminometer (cps = counts per second). \*Indicates the level of significance ( $p < 0.05$ ); \*\*( $p < 0.01$ ); \*\*\*( $p < 0.001$ ).

**Figure 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 h 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 N-acetyl cysteine (NAC) pretreatment and stained with DCF to detect

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reactive oxygen species (ROS). ROS induction was quantified using fluorescence measurements (AU = arbitrary units).

**Figure 3: 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 (pfu/cell) of Ad.5-*mda-7* and its recombinant chimeric Ad.5/3-*mda-7*, followed by 100  $\mu$ M D-Lim treatment. MTT assays were performed in (A) LT2 cells, (B) PANC-1 and (C) MIA PaCa-2 cells 72 h after treatment. (D and E) Western blotting of MIA PaCa-2 lysates for MDA-7 and markers of apoptosis.

**Figure 4: D-Lim 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 h after treatment. (D) MIA-PaCa-2 cells were pretreated with either NAC or peroxide scavenger, pyruvate, and then stained with DCF to detect ROS (specifically peroxide) induction (AU = arbitrary units).

**Figure 5: D-Lim 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* (25 pfu/cell) and D-Lim (100  $\mu$ M). Apoptosis was measured after 48 h by Annexin V/PI staining and flow-cytometer analysis. Western blotting was performed from the whole cell lysates 48 h post-treatment.

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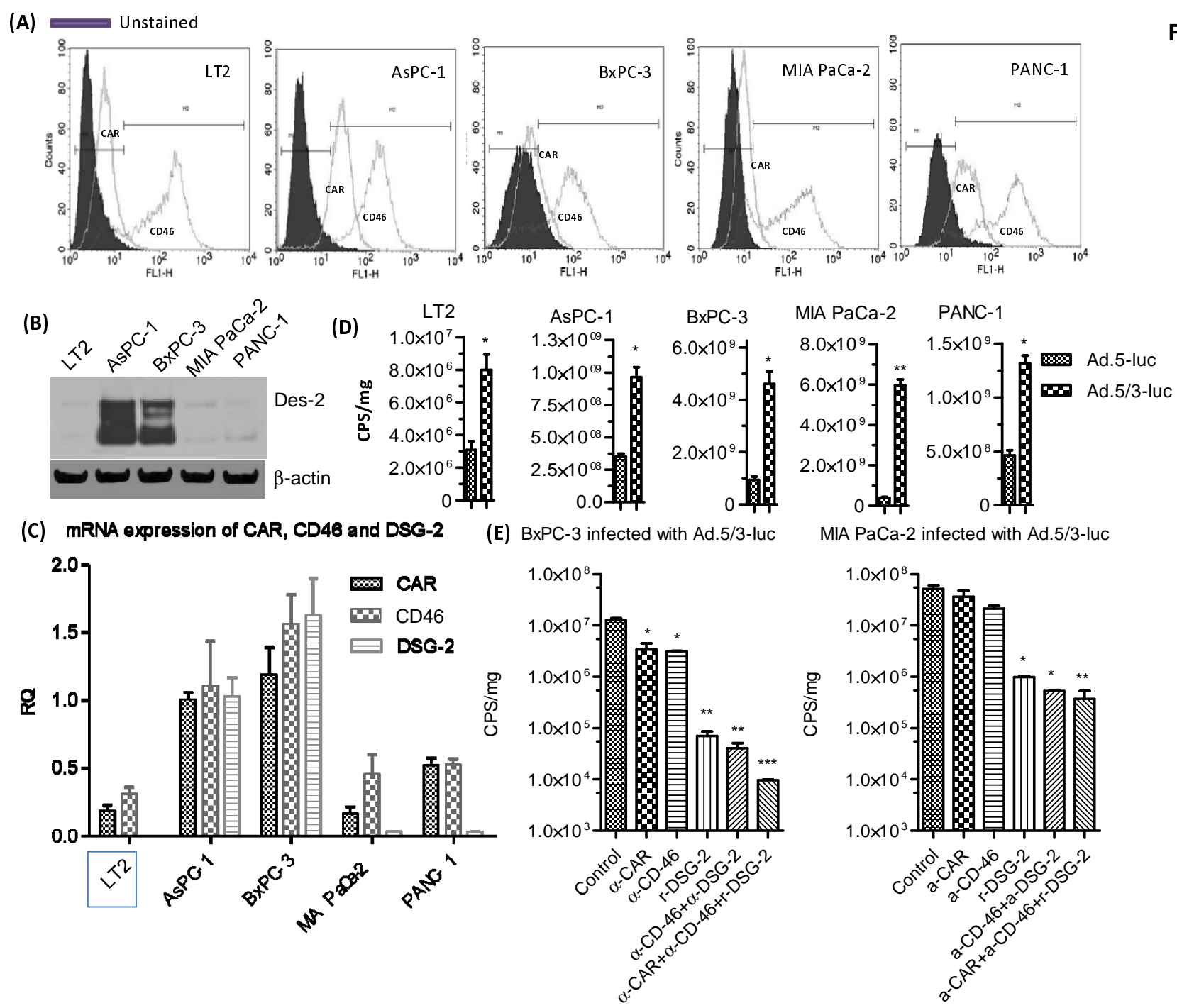
**Figure 6: D-Lim activates the translational machinery in the cell through the induction of ROS.** (A) Dose and time kinetics of ROS generation by D-Lim. AU = arbitrary units. (B) Western blotting analysis of phosphorylated P70S6K, eIF4E and 4EBP-1 after 100  $\mu$ M Limonene treatment. (C) Western blotting quantification of panel B. AU = arbitrary units. (D) Quantification of Western blotting analysis of phosphorylated P70S6K, eIF4E and 4EBP-1 after Limonene (100  $\mu$ M) or Arsenic trioxide (ARS)(1  $\mu$ M) treatment with or without NAC pretreatment. AU = arbitrary units.

**Figure 7: 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  $\mu$ M 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 no.) and Fraction II (enriched with mRNA associated with polysomes). (B) mRNA was isolated and RT-PCR was performed using a probe for *mda-7/IL-24* and GAPDH. (C) The relative quotient (RQ) 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 pre-initiation complex markers and MDA-7/IL-24. \*\*Indicates the level of significance ( $p < 0.01$ ); \*\*\*( $p < 0.001$ ).

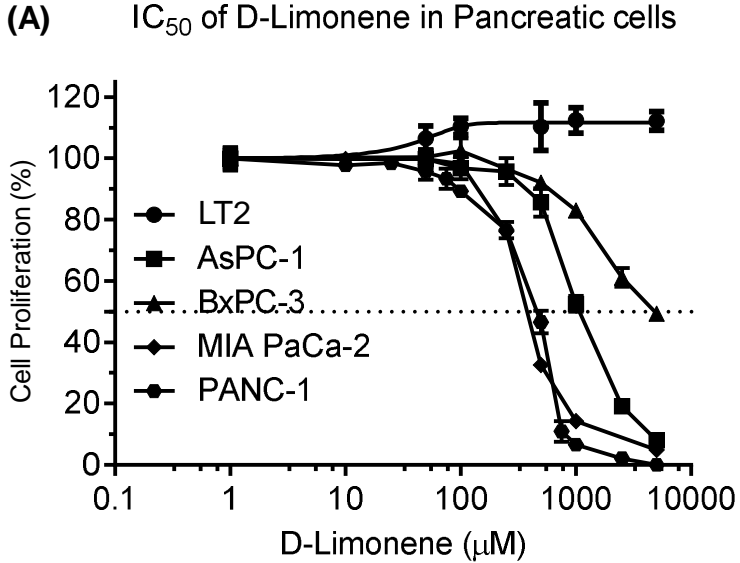
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**Figure 8: D-Lim induces *mda-7/IL-24* expression and toxicity in cell lines that stably overexpress *mda-7/IL-24* mRNA.** (A) PANC-1 stably transfected with pcDNA3.1-*mda-7/IL-24* (PANC-1-m7) and (B) MIA PaCa-2 stably transfected with pcDNA3.1-*mda-7* (MIA PaCa-2-m7) were treated with D-Lim (100  $\mu$ M) and MTT assays were used to assess the effect on cellular proliferation 72 h after treatment. (C) Western blotting of whole cell lysates of PANC-1-m7 and MIA PaCa-2-m7 with or without D-Lim (100  $\mu$ M) or POH (100  $\mu$ M).

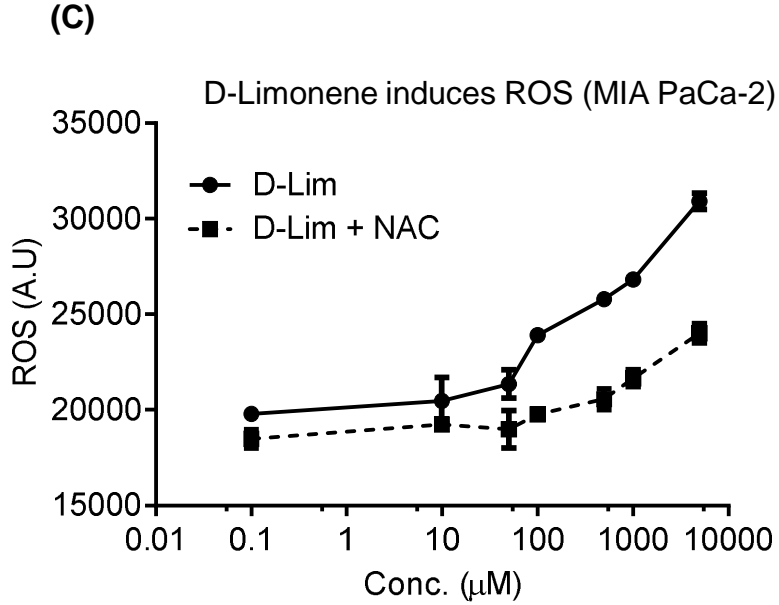
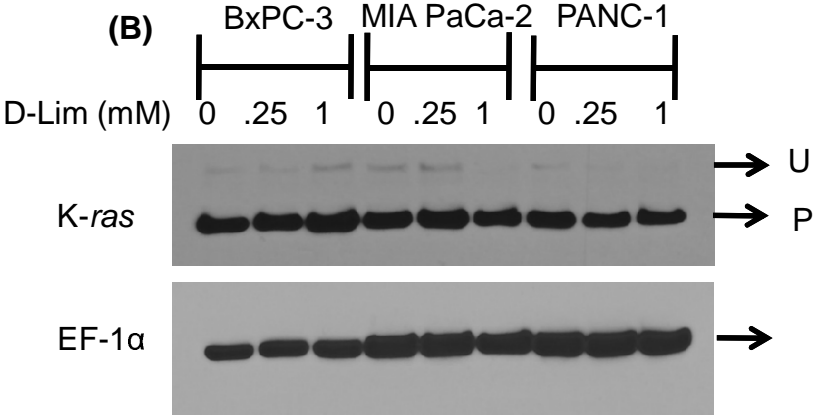
**Figure 1**



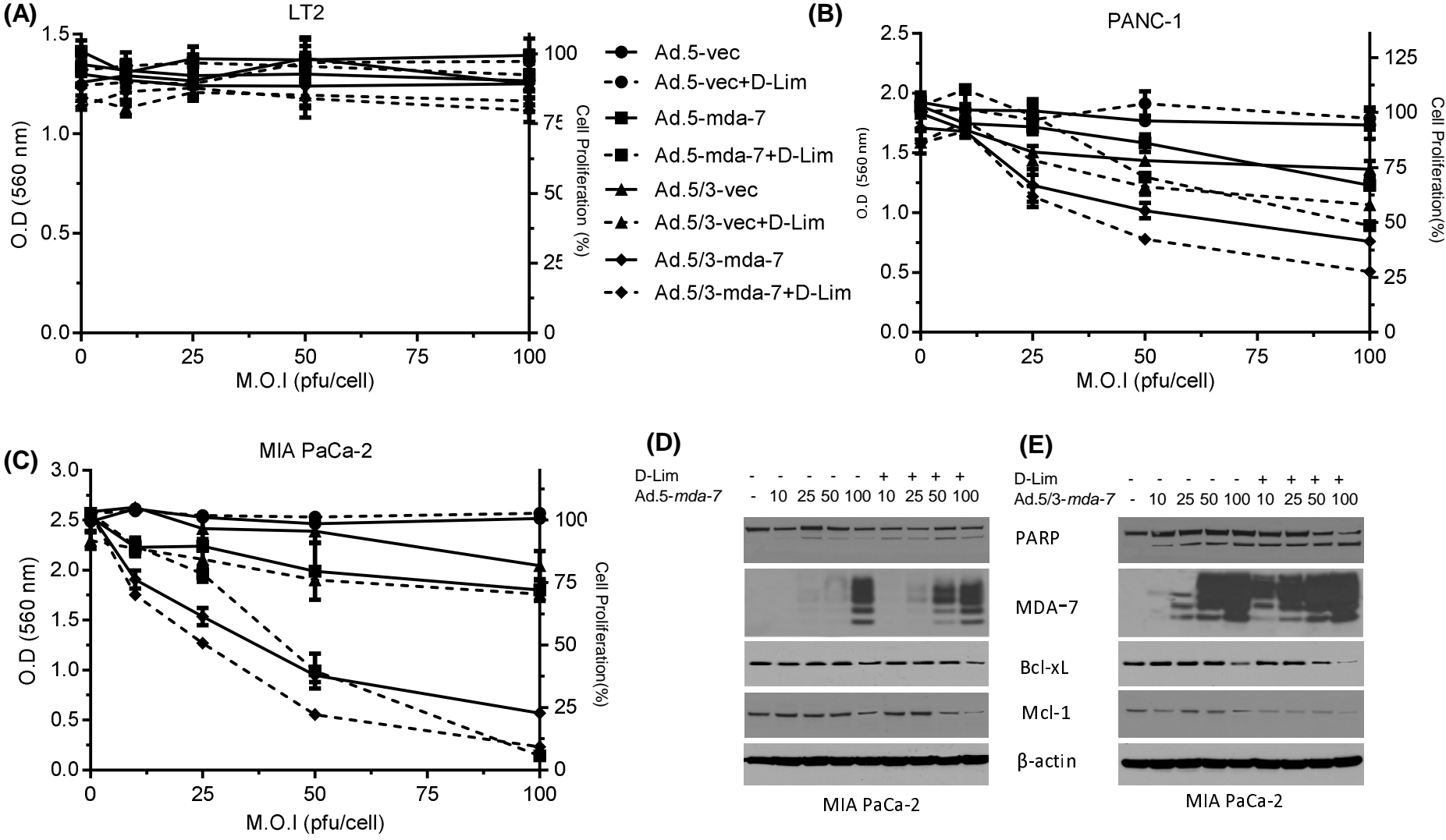
**Figure 2**



IC<sub>50</sub> > 5 mM (LT2)  
 IC<sub>50</sub> = 978.50 ± 12.12  $\mu\text{M}$  (AsPC-1)  
 IC<sub>50</sub> = 1490.00 ± 50.12  $\mu\text{M}$  (BxPC-3)  
 IC<sub>50</sub> = 385.72 ± 7.92  $\mu\text{M}$  (MIA PaCa-2)  
 IC<sub>50</sub> = 471.52 ± 9.97  $\mu\text{M}$  (PANC-1)

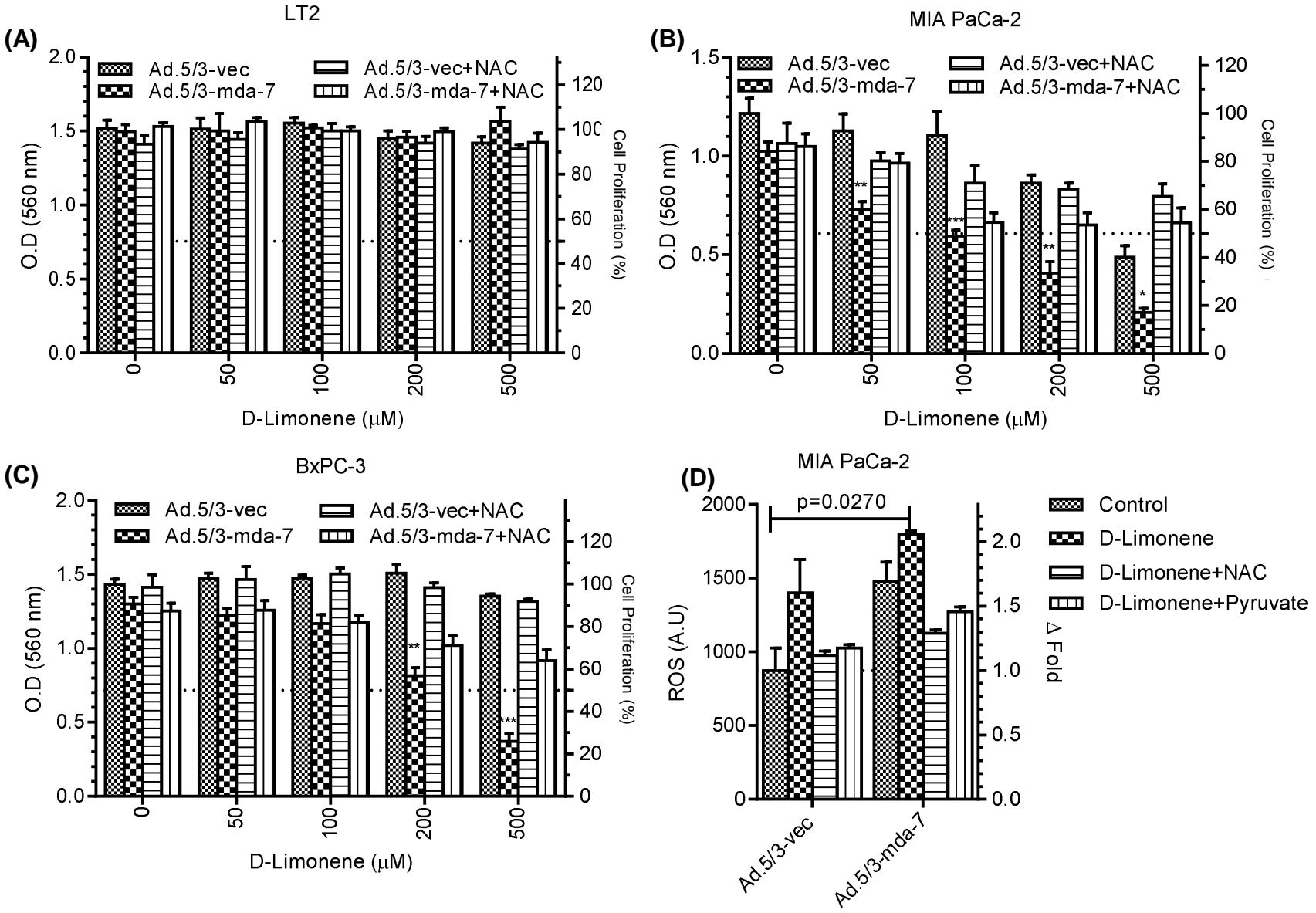


**Figure 3**



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Figure 4



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Figure 5

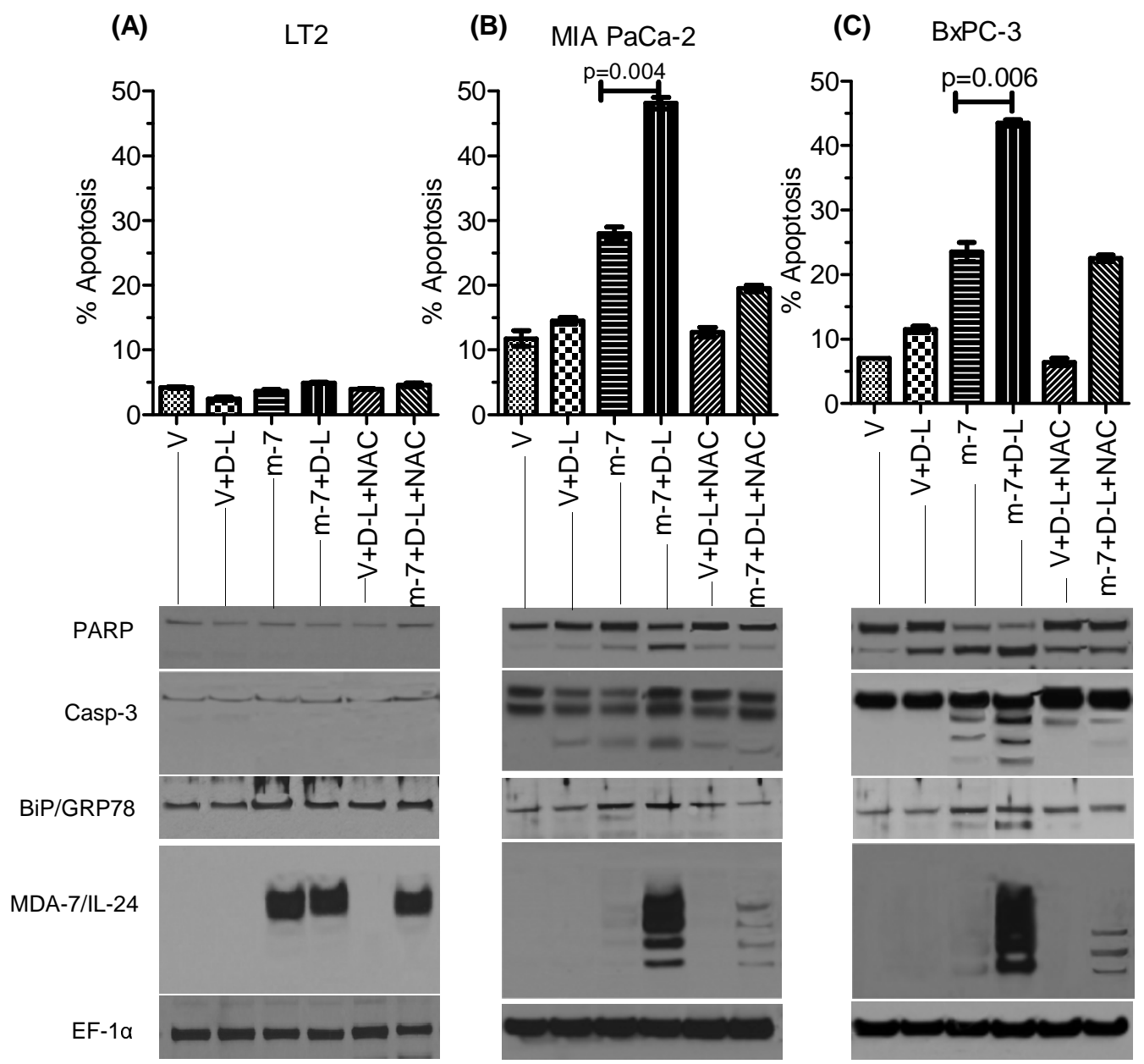
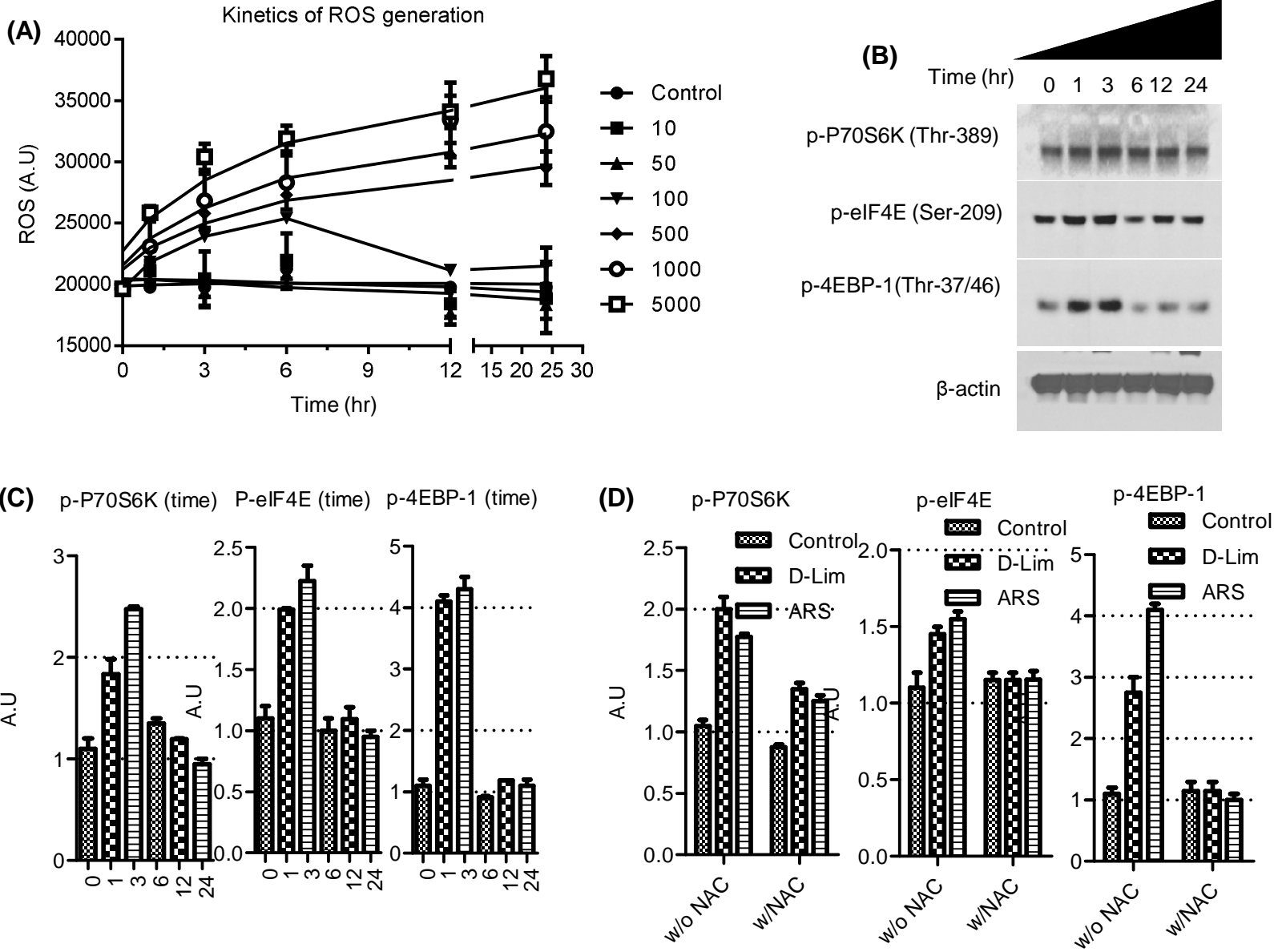


Figure 6



**Figure 7**

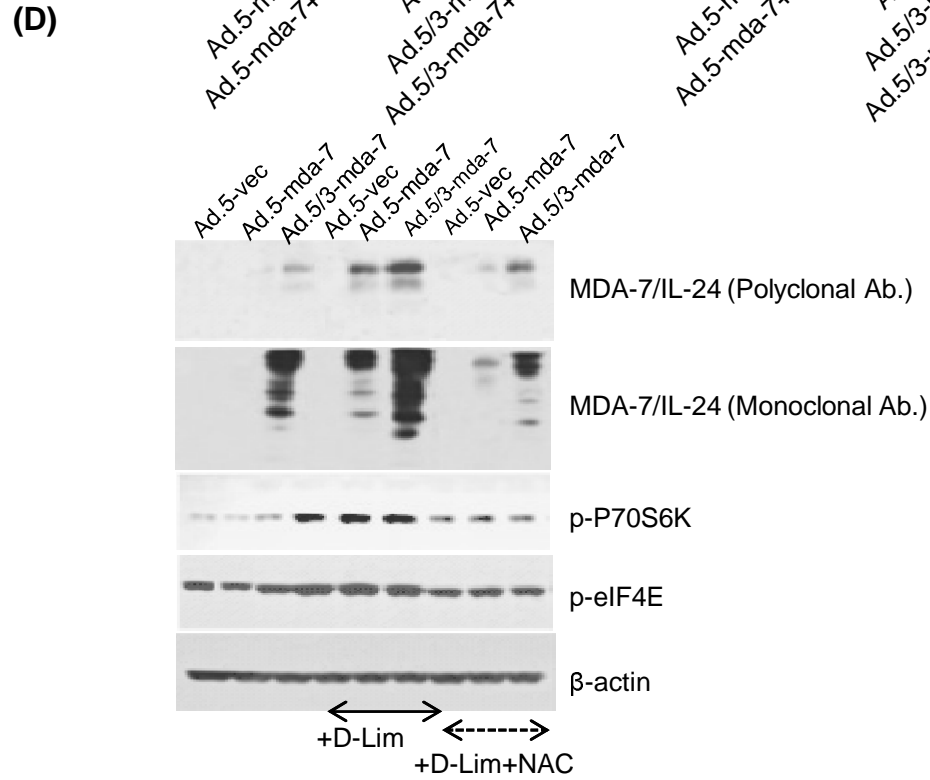
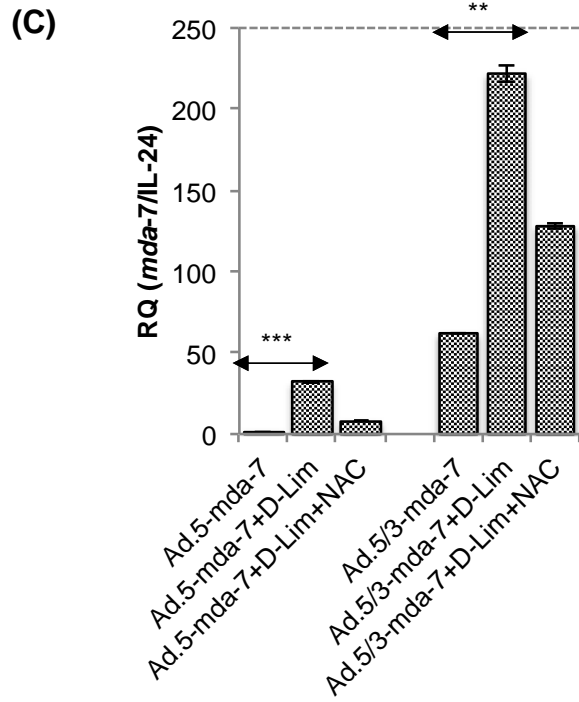
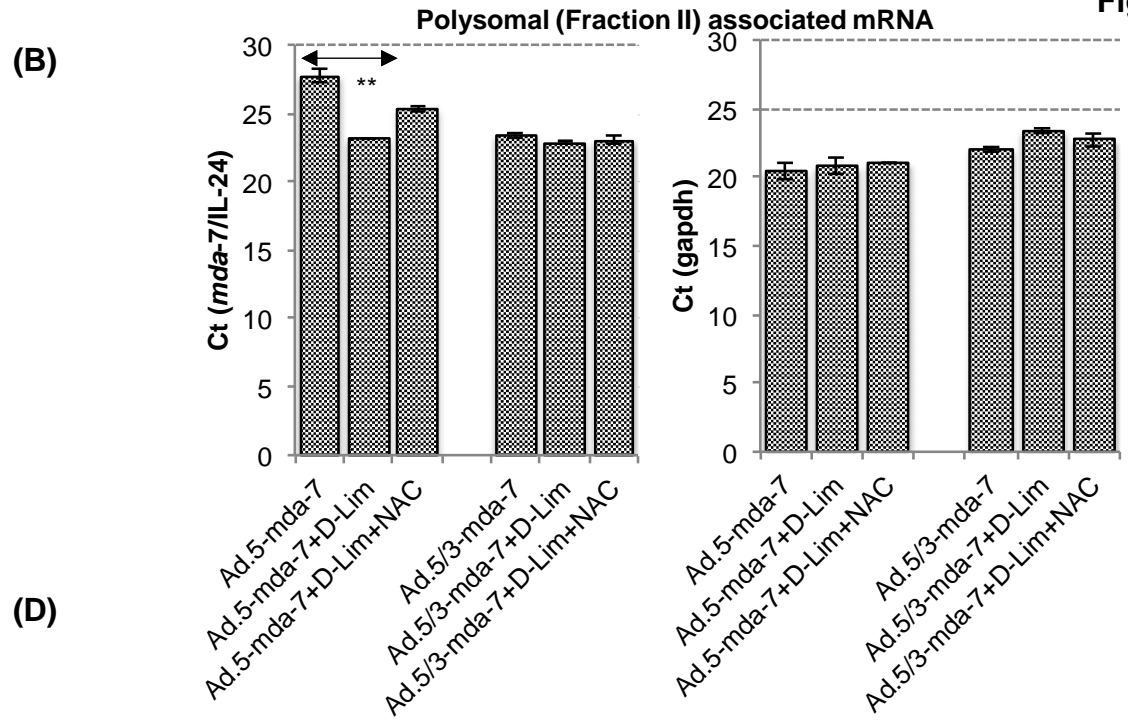
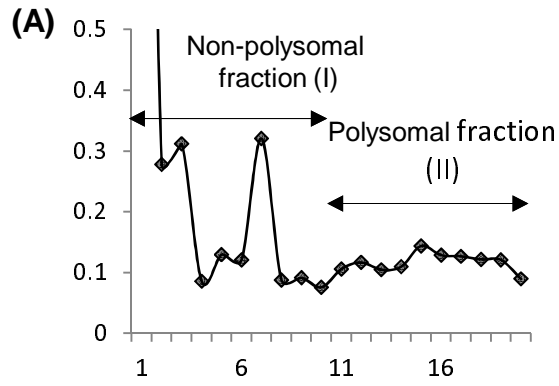
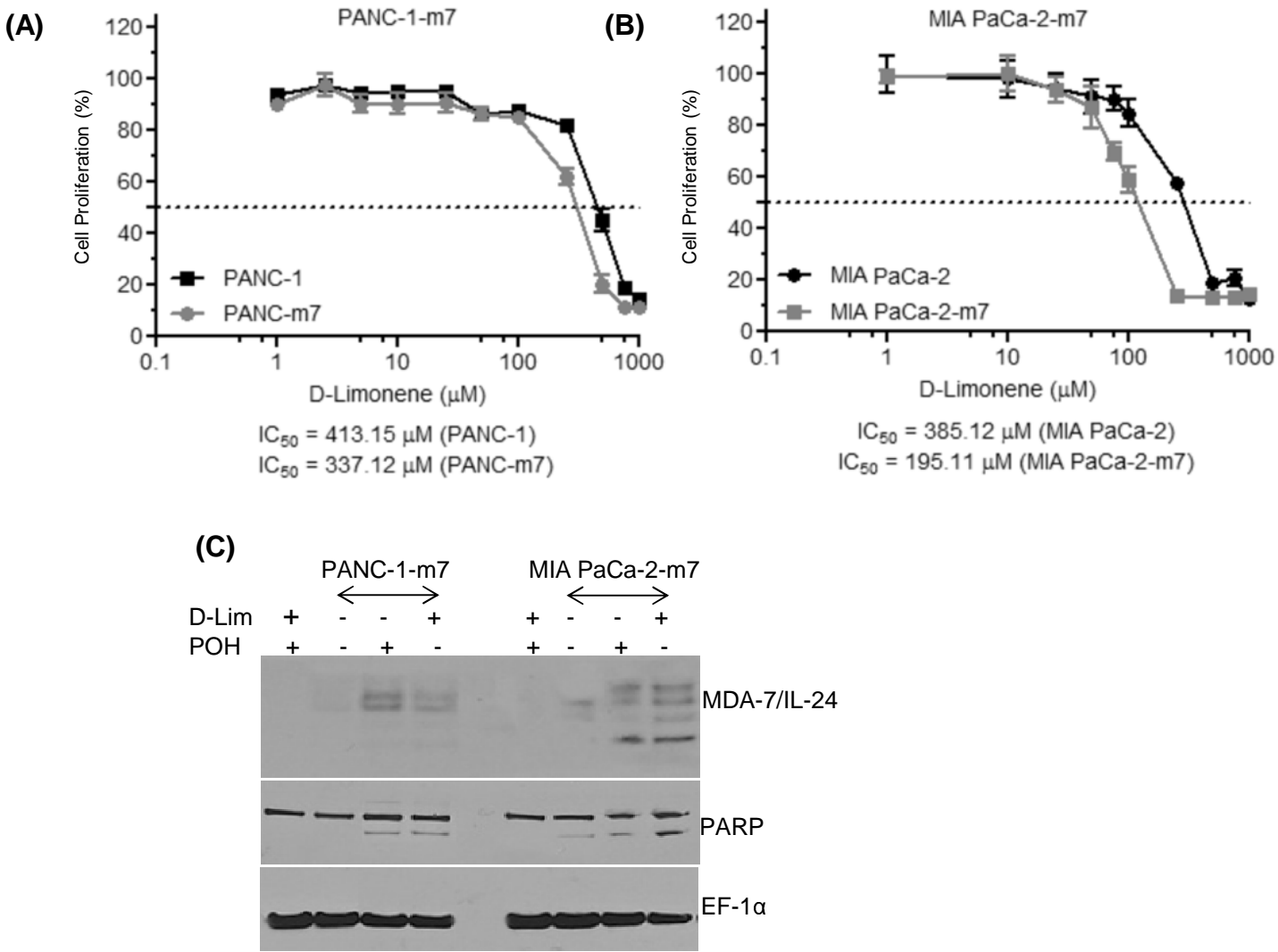
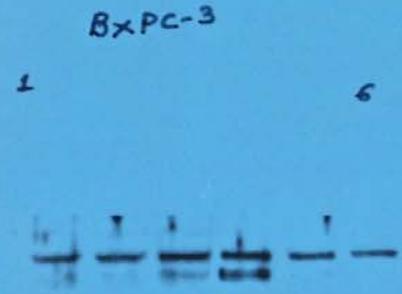
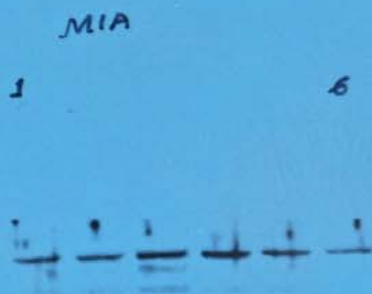
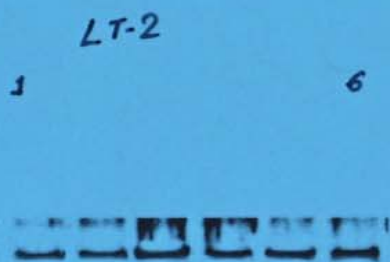


Figure 8



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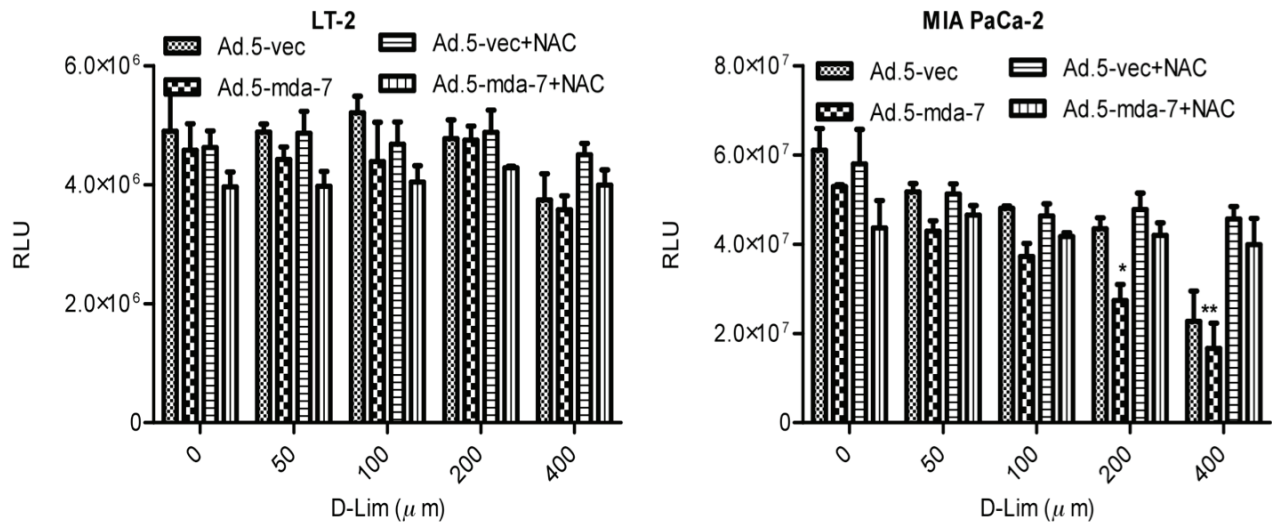


→Gp-78

Reversing translational suppression and induction of toxicity in pancreatic cancer cells using a chemoprevention gene therapy (CGT) approach

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

Supplementary Figure 1



**Figure 1: D-Lim enhances *mda-7*/IL-24-mediated inhibition of cell growth by inducing ROS.** Pancreatic cells were pretreated with 10 mM NAC prior to treatment with Ad.5-*mda-7* (25 pfu/cell) and D-Lim. BrdU incorporation assays were performed on (A) LT2 cells and (B) MIA PaCa-2 cells.