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Evidence for activation of mutated p53 by apigenin in human pancreatic cancer

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ABSTRACT

Pancreatic cancer is an exceedingly lethal disease with a five-year survival that ranks among the lowest of gastrointestinal malignancies. Part of its lethality is attributable to a generally poor response to existing chemotherapeutic regimens. New therapeutic approaches are urgently needed. We aimed to elucidate the anti-neoplastic mechanisms of apigenin—an abundant, naturally-occurring plant flavonoid—with a particular focus on p53 function. Pancreatic cancer cells (BxPC-3, MiaPaCa-2) experienced dose and time-dependent growth inhibition and increased apoptosis with apigenin treatment. p53 post-translational modification, nuclear translocation, DNA binding, and upregulation of p21 and PUMA were all enhanced by apigenin treatment despite mutated p53 in both cell lines. Transcription-dependent p53 activity was reversed by pifithrin-α, a specific DNA binding inhibitor of p53, but not growth inhibition or apoptosis suggesting transcription-independent p53 activity. This was supported by immunoprecipitation assays which demonstrated disassociation of p53/BclXL and PUMA/BclXL and formation of complexes with Bak followed by cytochrome c release. Treated animals grew smaller tumors with increased cellular apoptosis than those fed control diet. These results suggest that despite deactivating mutation, p53 retains some of its function which is augmented following treatment with apigenin. Cell cycle arrest and apoptosis induction may be mediated by transcription-independent p53 function via interactions with BclXL and PUMA. Further study of flavonoids as chemotherapeutics is warranted.

1. Introduction

Pancreatic cancer is a dismal diagnosis with a 5-year survival of only 5%. The lethality of pancreatic cancer is attributable to its exceedingly malignant biology with early metastatic spread to lymph nodes and distant organs such as the liver and lungs as well as to its aggressive local invasiveness. Furthermore, patients tend to present late in the disease progression at a time when extirpative surgery—currently the only potentially curative therapy—is not feasible. Chemotherapeutic regimens based on gemcitabine offer little potential for improved survival with randomized controlled studies demonstrating only a five-week survival benefit versus control patients [1]. New therapeutics capable of extending survival and down-staging otherwise inoperable tumors in the neoadjuvant setting are urgently needed in order to improve both survival and quality of life for pancreatic cancer patients.

In response to this need recent basic science and translational research has identified naturally-occurring plant polyphenols such as apigenin as attractive candidate chemotherapeutics. Apigenin is inexpensive to administer and is non-toxic at pharmacologic doses making it possible to administer concurrently with other chemotherapeutics while avoiding additive toxicity [2]. Apigenin has been shown to inhibit pancreatic cancer cell growth *in vitro* by induction of cell cycle arrest [3] and leads to synergistic effects when combined with gemcitabine by inhibiting pro-survival pathways involving pAKT which can contribute to gemcitabine resistance [4]. Breast and colon cancer cells respond similarly to apigenin treatment with cell cycle arrest and apoptosis induction which is linked to increased phosphorylation of p53 in both wild type and mutant p53-expressing cell lines [5,6]. Modulation of p53 activity appears to be central to the effectiveness of apigenin: mechanistic studies utilizing osteosarcoma cells identified phosphorylation of p53 at threonine 55 as a stimulus for nuclear export—and thus deactivation of wild type p53 which was specifically reversed by apigenin treatment [7].

However, the role of mutant p53 in cancer cells is multi-fold and exceedingly complex. In its native state, p53 is known to act as a transcription factor responsible for regulating the expression of cell-cycle regulatory genes such as p21/waf, and cell-death inducing proteins

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[8]. Classically, p53 induces cell-cycle arrest following DNA damage from UV exposure or chemical insult and initiates either DNA-repair or cell death machinery through its transcription-dependent regulation of gene transcription. More recently alternative roles of p53 have been discovered involving p53-Upregulated Modulator of Apoptosis (PUMA) and the mitochondrial pathway of programmed cell death [9]. Interactions between PUMA, p53, and anti-apoptotic Bcl-2-family proteins such as Bcl-X_L have been described in which p53 acts outside of the nucleus in a transcription-independent fashion. There is a poor understanding of the degree to which mutant p53 retains its transcription-dependent and -independent functions in pancreatic cancer cells [10]. Furthermore, in addition to loss of tumor suppressor functions, recent studies have identified gain of function properties of mutant p53 involving activation of proliferative and anti-apoptotic pathways and genes responsible for drug resistance [11].

Given the complexity of the roles p53 plays in tumorogenesis and aggressive tumor biology, the use of apigenin to re-establish or augment the growth-inhibitory and pro-apoptotic activity of mutant p53 could be a powerful strategy to check the growth of pancreatic cancer and improve the effectiveness of existing chemotherapeutics. We hypothesized that apigenin inhibits pancreatic cancer growth through reconstitution of mutated p53 function in pancreatic cancer cells, thereby inhibiting cancer cell proliferation and inducing apoptosis.

2. Methods

2.1. Cell culture and reagents

Human pancreatic cancer cell lines (MiaPaCa-2, BxPC-3) were obtained from American Type Culture Collection (ATCC) and maintained in DMEM (MiaPaCa-2) or RPMI culture medium (BxPC-3) (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin–streptamycin. Cells were cultured under standard culture conditions. All solvents and regents for HPLC were HPLC grade (Fisher Scientific, Fairlawn, NJ). Luteolin, quercetin and β-glucuronidase/sulfatase (type H-5 from Helix Pomatia) were purchased from Sigma-Aldrich (St. Louis, MO). Apigenin (Purity ≥98%; Cayman Chemical, Ann Arbor, MI) and Pifithrin-α (Purity ≥95%; Sigma-Aldrich, St. Louis, MO) were dissolved in DMSO as stock solutions and stored at −20 °C for in vitro experiments. Antibodies for total p53, phosphorylated p53 (ser-392), topoisomerase I, and Protein A-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies for acetylated p53 (lys-382), PUMA, and p21 were obtained from AbCam (Cambridge, MA); and antibodies for GAPDH, Cox IV, Bcl-X_L, Bax, and Bak were purchased from Cell Signaling Technology (Boston, MA).

2.2. Growth inhibition assay

Cells were seeded onto 96-well plates at 10^4 cells/well and allowed to attach overnight at 37 °C. Cells were serum starved using FBS-free DMEM or RPMI and allowed to grow for another 24 h at 37 °C after which varying concentrations of Apigenin ± pifithrin-α were incubated for 24 or 48 h. For growth inhibition, MTT (3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich, St. Louis, MO), was added for 3 h, cells solubilized in DMSO, and plates were read with an ELISA reader at 560 nm.

2.3. Apoptosis detection assay

Cells were plated to 6-well plates at 5×10^5 cells/well and allowed to attach overnight at 37 °C. Cells were serum starved as above and allowed to grow for 24 h at 37 °C followed by treatment as for growth inhibition assays. Ultraviolet (UV) irradiation was performed by exposing cells without medium with a standard UV lamp (Sylvania G30T8, 30 W; Danvers, MA) for a total dose of 50 mJ/cm^2. Medium was replaced following exposure and cells harvested as for apigenin and control-treated cells. A commercially available cell death detection ELISA kit (Roche Diagnostics, Mannheim, Germany) detecting nucleosomes in the cytoplasm was used according to instructions. Briefly, cells were harvested by trypsinization, counted, and 10^4 cells were subjected to lysis. Lysate was added to microplate wells coated with streptavidin along with anti-histone antibody conjugated with biotin and anti-DNA antibody conjugated with horseradish peroxidase (HRP) and incubated. Substrate was added and plates were read with an ELISA reader at 405 nm. Enrichment Factor (EF) was calculated as a ratio of absorbance of treated cells versus that of cells treated with vehicle alone (DMSO). EF = absorbance of sample/absorbance of vehicle control.

2.4. Whole cell lysate and western blotting

Cells were grown on 10 cm dishes in standard cell culture conditions to 80% confluence and were serum starved overnight. Cells were treated with apigenin ± pifithrin-α or UV irradiation and were incubated for 30 min to 48 h. Cells were washed twice with ice-cold PBS and then underwent lysis with ice cold RIPA buffer containing Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). Cells were disrupted by repeated aspiration through a 23 G needle prior to centrifugation at 14,000 × *g* for 10 min at 4 °C. Pellet was discarded and protein concentration determined by DC Protein Assay (BioRad, Hercules, CA). Proteins (20 μg/well) were separated on 10–15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk and probed with primary antibody overnight at 4 °C followed by secondary antibody. Bands were visualized by chemiluminescence substrate (Thermo Scientific, Rockford, IL) and autoradiography. Digital image analysis was performed with QuantityOne software (BioRad, Hercules, CA).

2.5. Nuclear extraction

Cells were treated as above for western blotting. Cells were washed twice with ice-cold PBS and then incubated with cold lysis buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40, 2 mM activated Na_3VO_4, 3 μg/mL Aprotinin, 25 μg/mL Leupeptin, 25 μg/mL Pepstatin, 25 μg/mL Chymostatin, 0.2 mM PMSF, and 5 mM NaF) for 10 min on ice. Cells were collected and solubilized by pipetting up and down followed by centrifugation at 16,000 × *g* for 5 min at 4 °C. Cytosolic supernatant was aspirated and stored at −80 °C. Nuclear pellet was solubilized in nuclear lysis buffer (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 25% glycerol, 2 mM activated Na_3VO_4, 3 μg/mL Aprotinin, 25 μg/mL Leupeptin, 25 μg/mL Pepstatin, 25 μg/mL Chymostatin, 0.2 mM PMSF, and 5 mM NaF) and incubated for 20 min on ice followed by centrifugation at 16,000 × *g* for 5 min at 4 °C. Protein concentration was determined and samples were stored at −80 °C prior to either western blotting or p53 binding activity assay.

2.6. Mitochondrial extraction

Cells were treated as above for western blotting. Cells were trypsinized, collected, and centrifuged at 370 × *g* for 5 min. Cells were washed with ice-cold NKM buffer (1 mM Tris–HCl [pH 7.4], 0.13 M NaCl, 5 mM KCl, 7.5 mM $MgCl_2$) and re-centrifuged at 370 × *g* for 5 min. Wash step was repeated once and then cell pellet was resuspended in homogenization buffer (10 mM Tris–HCl [pH 6.7], 10 mM KCl, 0.15 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT) and incubated for 10 min on ice. Cells were broken by 20 strokes of a Dounce homogenizer and incubated with 2 M sucrose for 10 min on ice followed by centrifugation at 1200 × *g* for 5 min at 4 °C. The pellet was discarded

and supernatant was combined with 2 M sucrose and samples spun at 7000 ×*g* for 5 min at 4 °C. Cytoplasmic supernatant was aspirated and stored at −80 °C. The pellet was re-suspended in mitochondrial suspension buffer (10 mM Tris–HCl [pH 6.7], 0.15 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, 1 mM DTT) and centrifuged at 9500 ×*g* for 5 min at 4 °C to pellet mitochondria. Pellet was resuspended in mitochondrial suspension buffer, protein quantified with DC protein assay, and stored at −80 °C.

2.7. p53 binding activity assay

Binding activity of nuclear extracts was assayed with Human Active p53 Activity Assay (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. Briefly, nuclear extracts (20 μg of total protein/well) were incubated with a biotin-labeled oligonucleotide corresponding to a consensus p53-binding sequence on ice. Samples were then added to microplate wells coated with a p53 capture antibody. Wells were treated with Streptavidin-HRP and the plate was read with an ELISA reader at 450 nm.

2.8. Co-immunoprecipitation

Cells were treated as above for western blotting. Cells were washed twice with ice-cold PBS and then incubated with cold lysis buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40, 2 mM activated Na_3VO_4, 3 μg/mL Aprotinin, 25 μg/mL Leupeptin, 25 μg/mL Pepstatin, 25 μg/mL Chymostatin, 0.2 mM PMSF, and 5 mM NaF) for 10 min on ice. Cells were collected and solubilized by pipetting up and down followed by centrifugation at 16,000 ×*g* for 5 min at 4 °C. Lysate was pre-cleared with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were pelleted by centrifugation at 1000 ×*g* for 5 min at 4 °C and protein quantified in the supernatant using DC protein assay. Lysate (250 μg total protein) was incubated with primary antibody for one hr on ice followed by addition of protein A-Agarose beads and incubation at 4 °C overnight on a rocker platform. Beads were pelleted by centrifugation at 1000 ×*g* for 5 min at 4 °C and pellet was washed with PBS + 1% Tween-20 four times, pelleting beads by centrifugation each time. Resulting pellet was resuspended in Laemmli buffer and boiled at 95 °C for 10 min prior to centrifugation at 1000 ×*g* for 10 min to pellet beads. Proteins were separated and visualized by SDS-PAGE as above for western blotting.

2.9. Cytochrome c immunofluorescence

Cells were treated as above for western blotting. Cells were trypsinized, collected, and centrifuged at 1000 ×*g* for 10 min. A commercially available kit was used to perform immunofluorescence for cytochrome c (EMD Chemicals, Darmstadt, Germany). Briefly, 10^6 cells were resuspended in permeabilization buffer and incubated for 10 min on ice. Cells were fixed with 8% paraformaldehyde for 5 min and pelleted by centrifugation at 1000 ×*g* for 5 min. Cells were washed ×3 with wash buffer, repeating centrifuge step each time, and then incubated with blocking buffer for 1 h. Samples were incubated with primary antibody followed by secondary antibody and then pelleted as above. Cells were resuspended in wash buffer and mounted on a microscope slide with Vectashield HardSet mounting medium + DAPI (Vector Labs, Burlingame, CA) and visualized with immunofluorescence microscopy.

2.10. Experimental animals

All animal experiments were performed according to protocols approved by the Chancellors Animal Research Committee at UCLA. Twelve four week-old male nude mice (Nu/Nu) were obtained from Charles River Laboratories (Wilmington, MA) and provided standard γ-irradiated rodent chow and autoclaved water *ad libitum* prior to orthotopic xenograft experiments.

2.11. Orthotopic xenograft model of pancreatic cancer

Human pancreatic cancer cells (MiaPaCa-2) were grown to 80% confluence, trypsinized, and counted. Cells were resuspended in serum free DMEM supplemented with 30% growth factor reduced Matrigel (BD Biosciences, San Jose, CA). Mice (n = 6 treatment; n = 6 control) were anesthetized with inhaled isoflurane (Phoenix Pharmaceutical, St. Joseph, MO), skin was prepared with Betadine (Purdue Products, Stamford, CT), and a one cm paramedian laparotomy in the left abdomen created. The spleen and tail of the pancreas was exteriorized and 5×10^5 cells injected into the tail of the pancreas. The viscera were reoriented within the abdomen and the wound closed in two layers with sutures.

2.12. Dietary supplementation with apigenin and tissue procurement

Apigenin (Purity ≥ 98%) was mixed with powdered AIN-76A rodent diet (Dyets; Bethlehem, PA) for in vivo feeding experiments. Animals were provided with either control diet (six animals; AIN-76A reconstituted with autoclaved water alone) or 0.2% apigenin-supplemented diet (six animals; AIN-76A + 0.2% apigenin [w/w] reconstituted with autoclaved water) *ad libitum* for 6 weeks following orthotopic xenograft tumor implantation. Apigenin dose was calculated based on pilot feeding experiments in which we assessed feeding tolerance and potential toxicity as well as apigenin levels in the serum and tissues. Feed supplemented with 0.2% apigenin was tolerated well by mice with no noted toxicity and tissue levels were detectable in the tens—of micromoles per liter—a level which showed cytotoxic effects in *in vitro* experiments (data not shown). Feed was replaced every-other day and intake of feed was measured daily. Mice were weighed weekly. At the end of the six week study period mice were sacrificed and tissues collected. Plasma was collected by exsanguinating cardiac puncture. Ten microliter heparin (1000 U/mL) was added to 1 mL of whole blood and centrifuged at 500 ×*g* for 10 min at 4 °C. Plasma was mixed with 10% (v/v) 0.58 M acetic acid and stored at −80 °C. Liver, pancreas, and tumor specimens were snap frozen in liquid nitrogen and stored at −80 °C. Additional liver, pancreas, and tumor specimens were fixed in 10% paraformaldehyde, paraffin embedded, and sectioned for immunohistochemical analysis.

2.13. Immunohistochemistry

Paraffin-embedded tissue sections (4 μm) were dewaxed and rehydrated in xylene and graded ethanol washes followed by deionized water and stained with the TUNEL method. Apoptosis was quantified by counting the number of TUNEL positive cells per 100 cells in 10 random microscopic fields (400×; 1000 cells counted/sample; control [n = 6] and 0.2% apigenin-fed [n = 6]).

2.14. HPLC for detection of apigenin in plasma and tumor samples

Plasma samples were acidified with 10% (v/v) 0.58 M acetic acid to limit flavonoid losses. Acidified plasma samples were further prepared with 0.2 M sodium acetate buffer (pH 5.0) with 1% ascorbic acid, β-glucuronidase, and sulfatase. The resulting mixture was vortexed and then incubated at 37 °C for 2 h. After incubation, ethyl acetate was added followed by 10 μL quercetin in MeOH as an internal standard. Apigenin and its metabolites were extracted with 800 μL ethyl acetate three times. In each extraction mixture was vortexed for 1 min and then centrifuged at 600 rpm for 5 min. Supernatant was transferred to a glass test tube and combined. Solvent was removed in a SpeedVac at room temp until completely dry. Residue was reconstituted in 100 μL

of methanol/acetone/water (3:3:4 by volume), vortexed and then sonicated for 1 min. An aliquot of 50 μL supernatant was injected into the HPLC column.

For tissue sample analysis, frozen tissue was weighed and homogenized in sodium acetate buffer with a tissue grinder and internal standard was added. The mixture was then hydrolyzed and extracted similar to plasma samples. HPLC analysis was performed with a RP-18 Luna column (150×4.6 mm, 3 μm, Phenomenex, Torrance, CA) on an Agilent 1100 HPLC system comprising of an autosampler, a quaternary pump and a photodiode array detector (Agilent Technologies, Palo Alto, CA). The mobile phase consisted of a binary gradient of 0.1% (v/v) ortho-phosphoric acid in water (eluent A) and methanol (eluent B), used with flow rate of 0.5 mL/min in the following conditions: 50–80% B (0–12 min); 80% B (12–17 min); and 80–50% B (17–20 min). Column temperature was held on 28 °C. The chromatograms were recorded at 337 nm and 255 nm for apigenin and its metabolites.

Data were analyzed with the Hewlett Packard Chemstation® software. Mouse plasma and tissue concentrations were determined by internal calibration. Concentration of the stock solutions was determined spectrophotometrically using Beer's Law. Calibration standards were prepared from the stock solutions by series dilution. The calibration curves generated from standard solutions of apigenin showed a linear relationship between peak area and concentration in the range of 22.07 ng/mL to 5.65 μg/mL. The limit of quantitation for apigenin was 11.0 ng/mL. Stability of apigenin in mouse diet was measured at 0, 1, 2, and 3 days following preparation by HPLC following excessive extraction with methanol.

2.15. Statistical analysis

Descriptive statistics such as mean and standard error (SE) were summarized for all variables using bar plots. P values were calculated with two-tailed *t*-test and statistical significance reached at the $p \leq 0.05$ level. For analysis of animal studies, logarithm transformation was applied to pancreas total apigenin and plasma free apigenin to improve normality. Two sample t-tests were performed to study the treatment effect of 0.2% apigenin relative to control for tumor weight. Scatter plots were used to illustrate patterns of tumor weight with respect to log-transformed pancreas total apigenin and log-transformed plasma free apigenin respectively, as well as the pattern of log-transformed pancreas total apigenin with respect to log-transformed plasma free apigenin. Pearson's correlation coefficients and p-values for testing the null hypothesis of no correlation were reported.

3. Results

3.1. Apigenin inhibits pancreatic cancer cell growth and induces apoptosis in vitro

MiaPaCa-2 and BxPC-3 cells treated with apigenin at concentrations of 1–100 μM resulted in dose-dependent decrease in cell proliferation as detected by MTT with an IC_{50} of ~25–50 μM for MiaPaCa-2 and ~1–10 μM for BxPC-3 at 24 h following treatment. Inhibition of proliferation was time-dependent as well (Fig. 1A and B). These data were confirmed by cell counts with trypan blue staining and 5-Bromo-2′-deoxy-uridine (BrdU) incorporation ELISA (Supplemental Fig. 1A and B). There was a 7.7 ± 2.9-fold increase in apoptosis for MiaPaCa-2 cells treated with 25 μM apigenin for 24 hours and 16.5 ± 5.0-fold increase for similarly treated BxPC-3 cells ($P<0.05$; Fig. 1C). This exceeded the amount of apoptosis for cells treated with UV irradiation (50 mJ/cm²).

3.2. Apigenin modulates post-translational modification, nuclear translocation, and DNA binding of p53

Apigenin treatment led to increased acetylation of lysine 382 (75.8 ± 12.5% at 8 h, $P<0.05$; Fig. 2A)—a signal associated with nuclear translocation and stabilization of the p53 tetramer. Increased acetylation followed a time course that showed a peak at 4 to 8 h for BxPC-3 and MiaPaCa-2 cells, respectively—though a measurable increase in acetylation was observed as early as 30 min following treatment with 25 μM apigenin (25.6 ± 9.3% increase). Accordingly, nuclear extracts showed increased levels of p53 protein relative to cytoplasmic extracts peaking at 2 h following apigenin treatment (52.4 ± 8.9% increase, $P<0.01$; Fig. 2B), indicating nuclear translocation of p53. Nuclear translocation was also accompanied by increased p53 binding to a consensus promoter sequence detected by ELISA despite the presence of mutated p53 in both cell lines tested (MiaPaCa-2 65.8 ± 0.6% increase and BxPC-3 48.9 ± 0.3% increase, $P<0.01$; Fig. 2C). These events were all in the context of a slight decrease in p53 expression observed in whole cell lysates (24.3 ± 3.5% decrease at 12 h, $P<0.01$; Fig. 2D). These data indicate that treatment with apigenin may increase the ability of p53 to bind to promoter regions of targeted genes and thus partially restore the function of the mutant p53 protein. Interestingly the magnitude of increased p53 DNA binding was similar for both cell lines indicating the possibility of additional mechanisms responsible for the anti-proliferative effects of apigenin.

3.3. Apigenin-induced apoptosis is not dependent on p53-mediated transcriptional regulation

In non-neoplastic cells, p53 activation has been shown to cause cell cycle arrest and cell death via activating expression of numerous cell cycle and/or apoptosis mediators such as p21 and PUMA (p53 Upregulated Mediator of Apoptosis). These proteins then mediate downstream events such as cyclin-dependent kinase inhibition (p21) and caspase activation (PUMA). Apigenin treatment (25 μM) induced the expression of p21 and PUMA, consistent with our data showing nuclear translocation and increased DNA binding of p53 (Fig. 3A). This upregulation was blocked by the addition of 25 μM pifithrin-α (PFT), a specific inhibitor of p53 DNA binding (Fig. 3B). However, the anti-proliferative effect of apigenin was not reversed by treatment with pifithrin-α nor was increased apoptosis (Fig. 3C,D) indicating that apigenin may activate p53 through a parallel, transcriptionally independent pathway of programmed cell death.

3.4. Apigenin induces mitochondrial translocation and modulates protein–protein interaction of p53 and PUMA with Bcl-X_L

Consistent with a model of transcriptionally-independent p53 activation we found there was increased translocation of p53 to the mitochondria in apigenin-treated cells (31% increase; Fig. 4A). Immunoprecipitation of the anti-apoptotic Bcl-2 protein Bcl-X_L showed there was dissolution of complexes between Bcl-X_L/p53 and Bcl-X_L/PUMA following apigenin treatment. Binding of p53 and PUMA with Bcl-X_L effectively sequestered these molecules in the unstressed cells (Fig. 4B). Following liberation from Bcl-X_L, p53 and PUMA both formed stable complexes with Bak leading to mitochondrial membrane permeabilization and cytochrome c release seen by immunofluorescence (Fig. 4C).

3.5. Dietary apigenin reduces the volume of orthotopic pancreatic cancer xenografts in nude mice

Using a well-established nude mouse model of human pancreatic cancer we tested the hypothesis that dietary apigenin supplementation would yield smaller pancreatic tumors compared to mice fed standard rodent diet. All mice fed and gained weight appropriately with no gross evidence of toxicity. Serum chemistries were performed at the conclusion of the feeding period and there were no significantly elevated indices among a panel testing for pancreatic, renal, and hepatic function. Finally, feed samples were tested for the

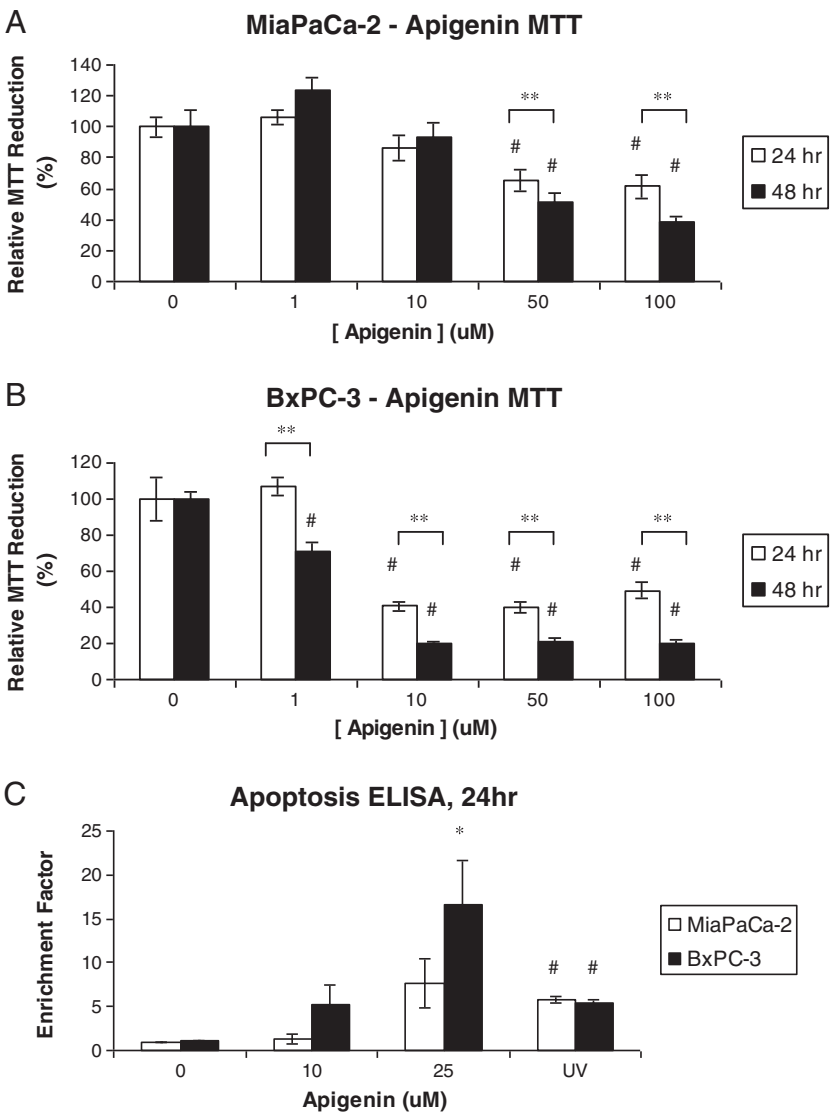

Fig. 1. Apigenin inhibited pancreatic cancer cell growth dose- and time-dependently for MiaPaCa (A) and BxPC-3 (B) cells. Apigenin dose-dependently induced apoptosis for MiaPaCa and BxPC-3 cells (C). $^{*}P \leq 0.05$; $^{**}P < 0.01$, $\#P < 0.01$ versus control.

stability of apigenin by HPLC and there was no significant degradation of apigenin over 72 h at ambient humidity and temperature (data not shown).

Apigenin-fed mice had significantly elevated levels of apigenin detected in both plasma (0.3 ± 0.04 vs. 0.2 ± 0.01 μg/mL, $P < 0.05$; Fig. 5A) and tumor (1.8 ± 0.7 vs 0.2 ± 0.1 mg/g tissue, $P < 0.05$; Fig. 5B). Tumors tended to be smaller in apigenin-fed mice (0.7 ± 0.1 vs. 0.9 ± 0.1 g, $P = 0.058$, Fig. 5C) and there was a strong correlation between tumor apigenin level and tumor size (Pearson correlation coefficient -0.65, $P < 0.05$; Fig. 5D) indicating mice that ingested the most apigenin had the smallest tumors. TUNEL staining performed on tumor sections demonstrated an increased proportion of apoptotic cells for the treated tumors (56.3 ± 6.2 vs. 33.2 ± 8.3 TUNEL positive cells/HPF; $P < 0.05$; Fig. 6).

4. Discussion

Our initial growth inhibition and apoptosis experiments indicated that apigenin exhibited potent anti-proliferative and pro-apoptotic effects on pancreatic adenocarcinoma cells *in vitro*. These results are consistent with prior reported work with pancreatic cancer cells treated with apigenin. The concentrations of apigenin required to inhibit growth and induce apoptosis were somewhat higher than those attained in *in vivo* murine experiments. Several factors may contribute to this phenomenon: cell culture experiments were performed in serum-free medium where the solubility of apigenin is limited, effectively decreasing its bioavailability *in vitro* [12]. Additionally, pharmacologic studies of apigenin have shown that apigenin sequesters in the lipid fraction of organs; the resulting high volume of distribution makes the serum concentration of apigenin a relatively small component of the total apigenin level *in vivo* [13]. These variables may increase the apparent IC_{50} *in vitro* by necessitating a large amount of apigenin dissolved in the medium to attain the same total apigenin concentration seen *in vivo*.

p53 has long been recognized to be a critical factor in cell cycle progression and apoptosis in untransformed cells following genotoxic insults such as UV or gamma irradiation [14]. Furthermore mutation of the Tp53 gene has been implicated as a transformative factor in numerous human malignancies, including pancreatic adenocarcinoma where it is mutated in up to 76% of resected tumors and 67% of established cell lines [15,16]. MiaPaCa-2 and BxPC-3 cells have Tp53 mutations in codons corresponding to the DNA-binding domain of the

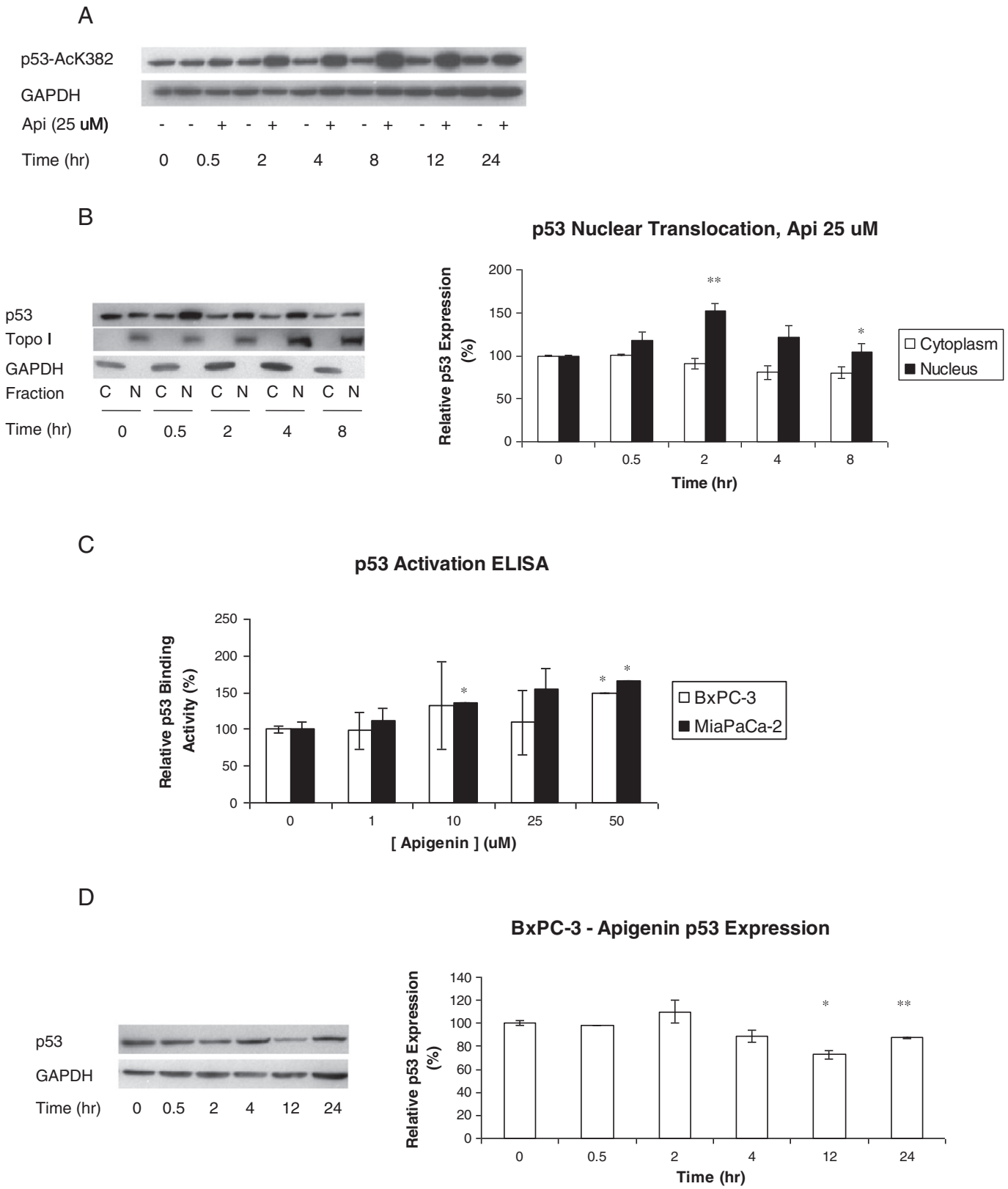

Fig. 2. Apigenin induced acetylation of p53 at lysine 382 (AcK382) time-dependently in pancreatic cancer cells (A), and was associated with nuclear translocation which peaked at 2 h (B), and increased DNA binding of p53 at 4 h (C) despite overall reduced expression of p53 (D). $^{*}P \leq 0.05$ versus control; $^{**}P < 0.01$ versus control.

p53 protein (codon 248 and 273 [M.P.] and 220 and 265 [Bx], [17]). Mutations in these codons are the most commonly seen alterations of the Tp53 gene for pancreatic cancers as well as many other types of human malignancy. There is evidence that these mutations may have characteristic functional consequences affecting protein half-life, conformation, and DNA sequence-specificity [18]. Tp53 mutation,

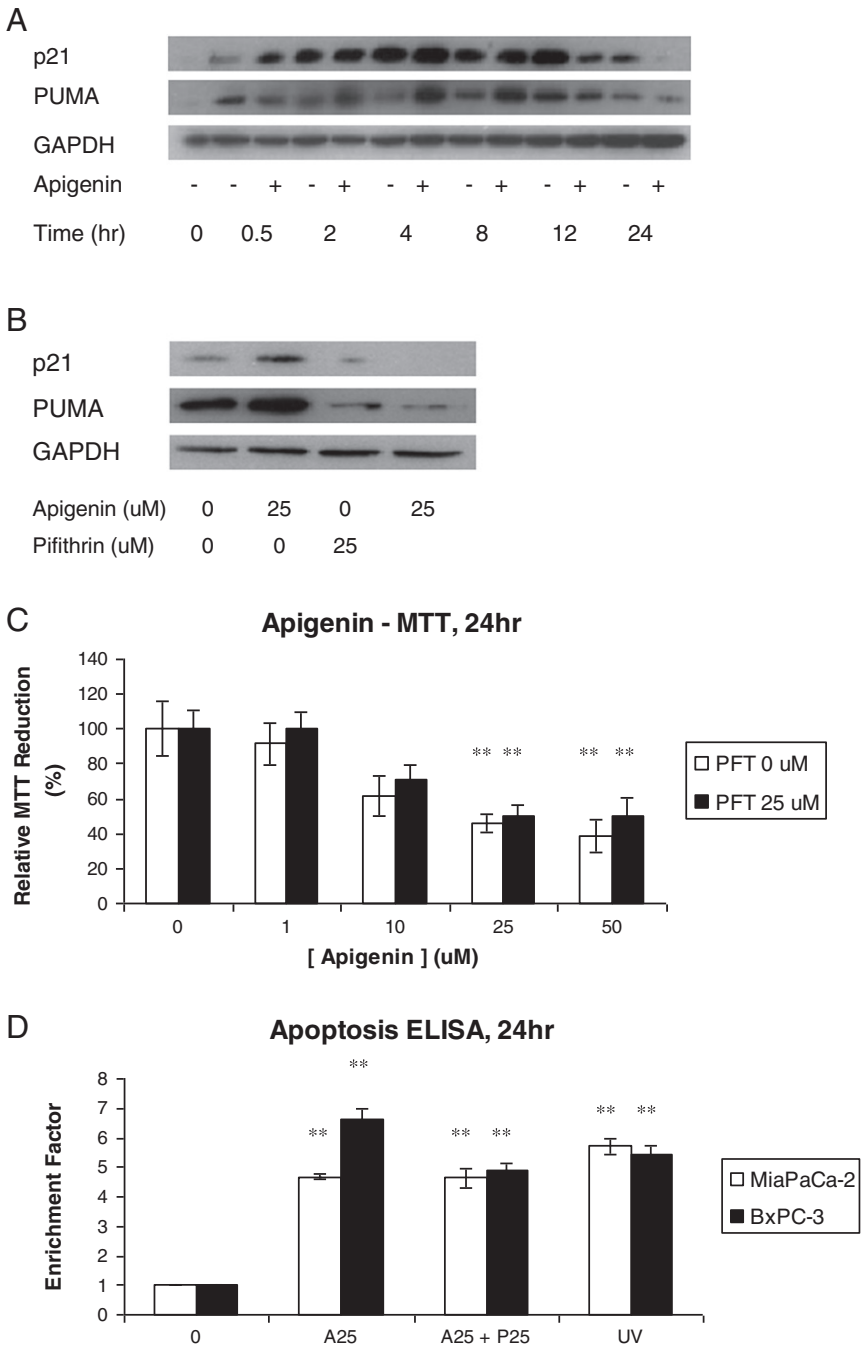

Fig. 3. Apigenin induced expression of p21 and PUMA (A) and co-treatment with pifithrin-α reversed p53-mediated expression of p21 and PUMA (B) but was not able to reverse growth inhibition (C) or apoptosis (D) following apigenin treatment. $^{**}P<0.01$ versus control.

along with activating mutation of Kras, is thought to be a key event in pancreatic carcinogenesis. Importantly, the MiaPaCa-2 cell line has mutated Kras while BxPC-3 is unusual in that it has a wild-type gene. Given both cell lines ultimately respond to apigenin treatment we surmised that apigenin may act through a mechanism independent of Kras. For this reason, our choice of both Kras mutant and wild-type cell lines was helpful in directing our investigation away from the RAS/RAF/MAPK pathway. We chose to study p53 because it is a key molecule uniquely situated in cellular machinery responsible for both cell cycle inhibition and apoptosis induction. Furthermore, we hypothesized that loss-of-function mutations in Tp53 seen in pancreatic cancer may be partially circumvented by treatment with apigenin leading to cell cycle arrest and apoptosis seen in *in vitro* growth assays.

In the current study, *in vitro* experiments demonstrated transcription-dependent functionality of p53 despite the presence of deactivating mutations in the Tp53 gene in both cell lines tested. This finding is in concordance with previous reports in the literature studying the effect of apigenin in widely disparate cancer cell lines, including carcinoma and sarcoma cell lines [5–7]. Apigenin treatment was accompanied by increases in acetylation of lysine-392. This modification is carried out by the acetyltransferases CBP/p300 and is known to affect p53 sequence-specific DNA binding and p53 half-life [19]. Consequently, binding of p53 to DNA was also promoted

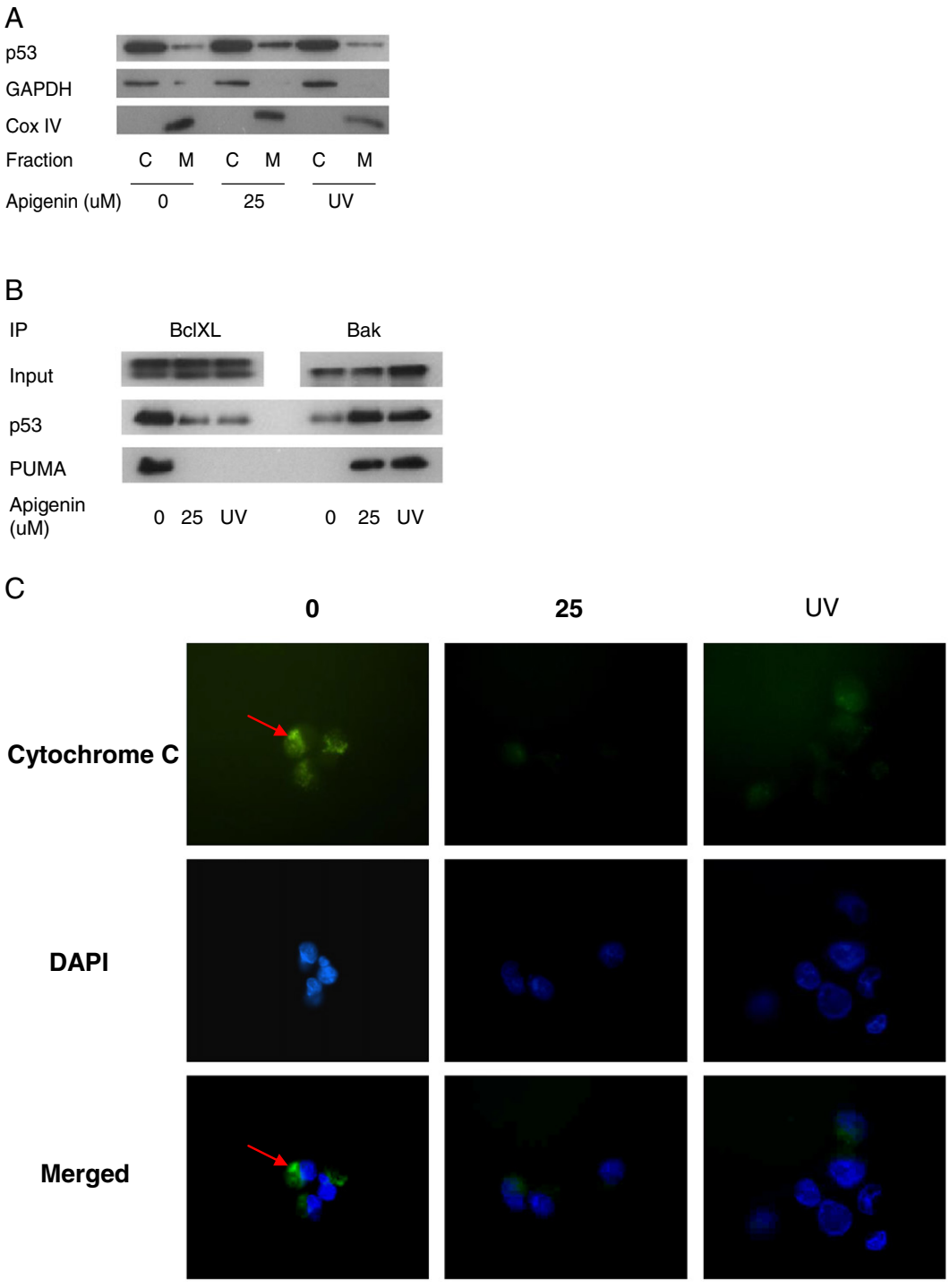

Fig. 4. Apigenin induced mitochondrial translocation of p53 (A) and influenced interaction of p53 and PUMA with the anti-apoptotic BH-3 domain protein Bcl XL and induced binding between p53 and Bak (B). Immunofluorescence of pancreatic cancer cells stained for cytochrome c. Release of cytochrome c is marked by dimunition of fluorescence signal from mitochondria (arrows) (C). In 4A, GAPDH loading controls for lanes 1, 3 and 5 represent protein loading for cytosolic fractions of the mitochondrial isolation experiment and are roughly equal. A GAPDH signal is missing in lanes 2, 4 and 6 and demonstrateS purity of the mitochondrial fraction (i.e.: minimal cytosolic contamination). Conclusions discussed in the Results and Discussion sections are made from the relative increase in p53 signal between lanes 2 and 4 where the loading control signal (Cox IV) is also roughly equal.

by apigenin treatment. p53 fulfilled its classically described role as an activator of transcription of the cell cycle regulatory gene p21 and the pro-apoptotic gene PUMA correlating to our findings of decreased cellular proliferation and increased apoptosis. Importantly, this transcriptional activation was promoted by apigenin treatment and completely abrogated by co-treatment with pifithrin-α—a specific inhibitor of p53/DNA binding—indicating p53 transcriptional activation was causally related to apigenin treatment.

Based upon these results apigenin appeared to restore the transcription-dependent activation of p53. However, we did not find a correlation between the relative sensitivity of the two cell lines to apigenin's anti-proliferative and pro-apoptotic effects to the increase in p53 activation (i.e.: BxPC-3 IC_{50} was two-to-five fold lower than MiaPaCa-2 while p53-DNA binding was similar). Furthermore, we found there was very little reversal of the anti-proliferative and pro-apoptotic effects of apigenin when transcription-dependent p53

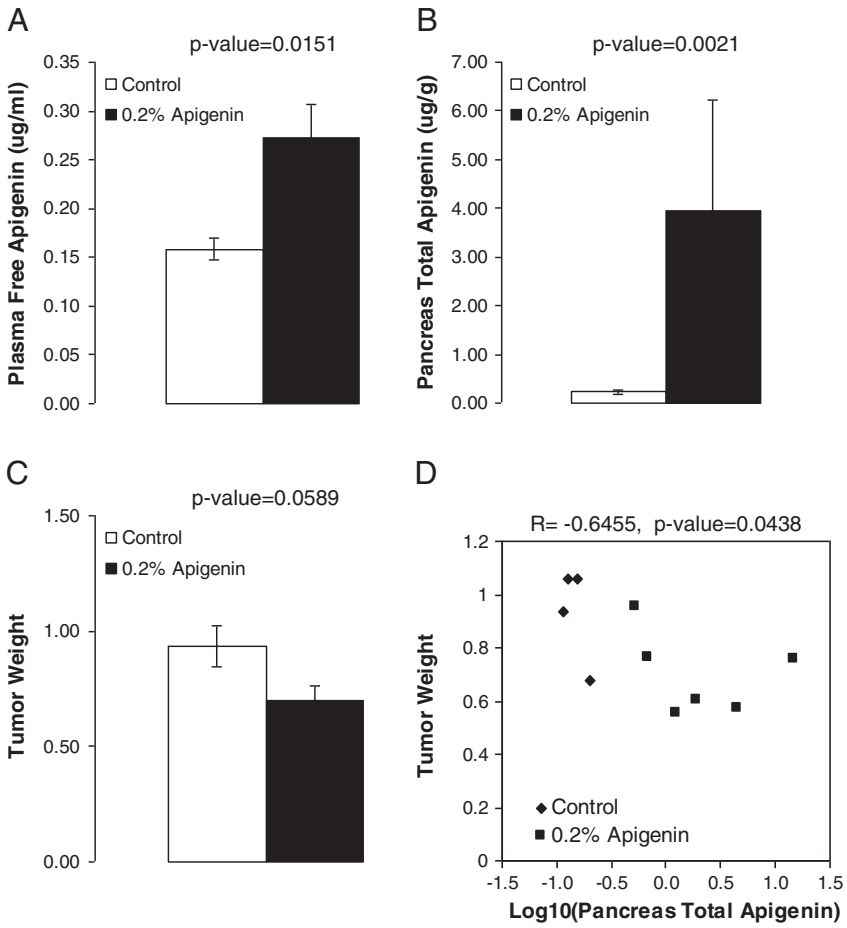

Fig. 5. Animals fed a diet supplemented with 0.2% apigenin (w/w) had higher levels of apigenin detected in their plasma (A) and pancreas (B) and trended towards smaller tumors compared to control animals (C). There was a correlation between tumor apigenin and tumor weight (Pearson's Correlation Coefficient = −0.65, $P = 0.04$; D); (n = 6 0.2% apigenin-fed; n = 6 control-fed).

activation was blocked with pifithrin-α. These date indicate the possibility of parallel pathway(s) involving the [co-] activation of PUMA as has been demonstrated in neoplastic and non-neoplastic cells with intact p53 function [20,21].

Current models of the p53/PUMA apoptotic pathway describe dissolution of the p53/Bcl-X_L complex accompanied by creation of a PUMA/Bcl-X_L pair which is required for 'freeing' p53 prior to its activation of the downstream BH123 proteins such as Bax or Bak [20]. These findings were reported by Chipuk et al. using HCT116 colorectal carcinoma cells and mouse embryonic fibroblasts which express wild-type p53. Both nuclear p53, which incites transcriptional activation of PUMA, as well as cytosolic p53, which binds to and activates Bax, are necessary for induction of apoptosis in this model as shown by the lack of PUMA-mediated apoptosis in p53 null cells. This model has been disputed by Callus et al who observed continued PUMA-mediated apoptosis in p53-null mouse embryonic fibroblasts and murine myeloid cell lines undergoing forced overexpression of PUMA (thus obviating the need for p53-mediated transcriptional upregulation of PUMA) and contended that the role of p53 in PUMA-mediated apoptosis was limited to transcriptional upregulation of PUMA [22].

Our results indicate that there may be a combination of these two models of transcription-independent p53 function in pancreatic cancer cell lines. Apigenin treatment resulted in increased expression of PUMA (via p53 transcriptional activation) and co-immunoprecipitation demonstrated dissociation of stable complexes between both p53/Bcl-X_L and PUMA/Bcl-X_L, indicating that p53 and PUMA may act in parallel to activate Bax following dissociation from Bcl-X_L. In the case of PUMA/Bcl-X_L, there was no detectable complex following treatment with apigenin as opposed p53/Bcl-X_L where there was a partial dissolution. This finding is supported by structure/function and affinity binding studies which have shown that p53—though it lacks a true BH3 domain—binds to Bcl-X_L through its DNA binding domain to the 'bottom side' of Bcl-X_L—at residues distinct from its BH3 domain [23]. The dissociation constant for the p53/Bcl-X_L complex was 164 ± 54 nM K_D versus 10 ± 4 nM K_D for PUMA/Bcl-X_L [20].

Once p53 and PUMA are freed from sequestration by Bcl-X_L both formed complexes with pro-apoptotic Bak, suggesting both proteins act as BH3-only molecules in this situation. These events appear to occur in parallel in contrast to the findings of Chipuk et al in which p53 and PUMA 'flip-flop' their association with Bcl-X_L so that p53 can then activate pro-apoptotic Bax [20].

Further support for this model comes from studies in which another polyphenolic flavonoid, rottlerin, was found to alter interactions of Bcl-X_L with the BH3-only proteins PUMA and Bim in a manner similar to a known Bcl-X_L inhibitor, BH3I-2′ which preferentially binds to the hydrophobic pocket of Bcl-X_L, preventing its binding with BH3-only proteins [24]. These experiments were performed in the MiaPaCa-2 and Panc-1 pancreatic cancer cell lines, making their findings directly applicable to this study. Based on these data we propose that apigenin induces dissociation of p53 and PUMA from sequestration by Bcl-X_L allowing activation of Bak by both p53 and PUMA at the mitochondrial membrane leading to cytochrome c release and initiation of the caspase-driven mitochondrial cell death cascade as has been shown by others [25]. Release of p53 from Bcl-X_L appears to have the added effect of nuclear translocation

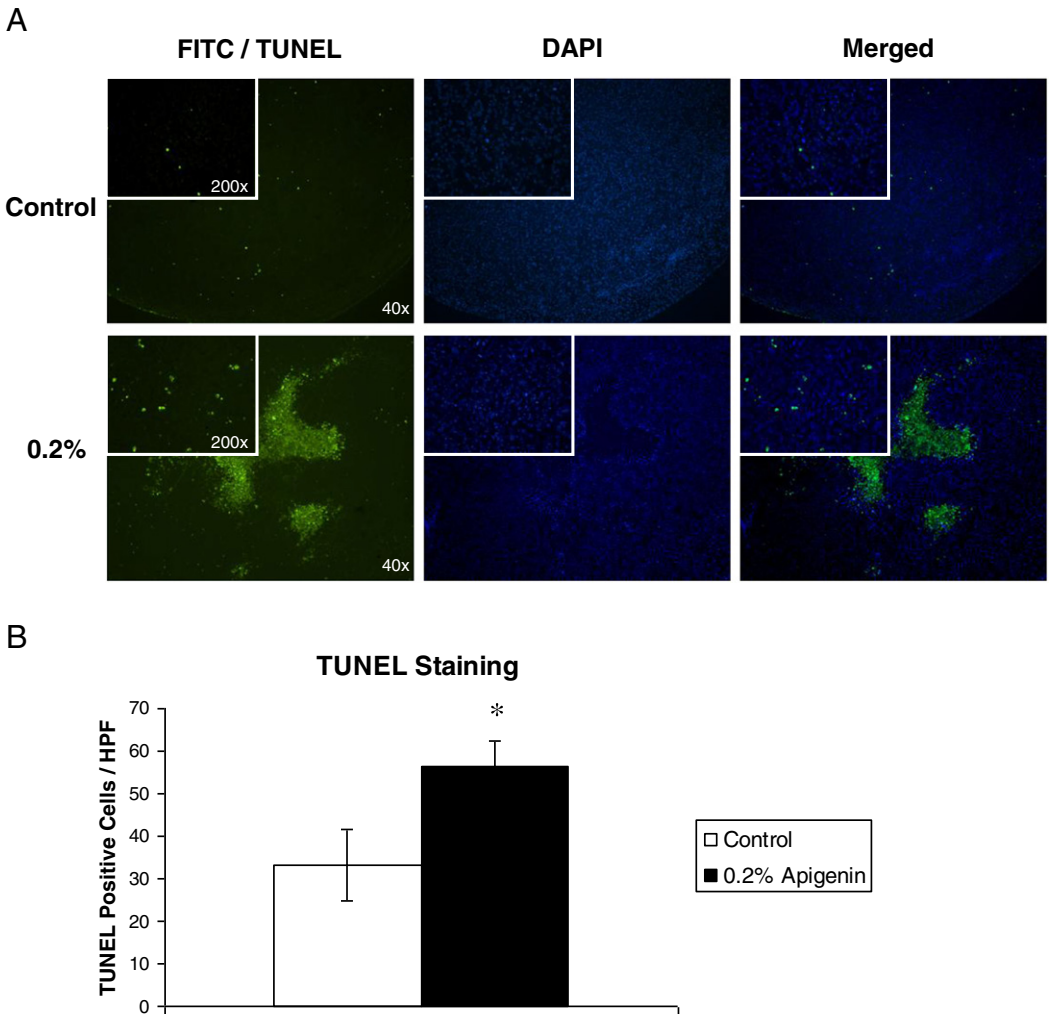

Fig. 6. TUNEL staining of tumor sections revealed more tumor cell apoptosis in apigenin-treated animals compared with control (A,B). *$P<0.05$ versus control (n = 6 0.2% apigenin-fed; n = 6 control-fed).

of p53 leading to increased transcription of p53 target genes, including PUMA. However, the transcriptionally-dependent function of p53 does not appear to be necessary for induction of apoptosis (Fig. 7). It is not clear whether apigenin's effect is direct through its binding to p53, PUMA, or Bcl-X_L or indirect via its known ability to inhibit the activity of numerous kinases including PKC, PI3-kinase, and the JAK/STAT pathway [26,27]. Additional experiments utilizing a p53 null cell line, or 'knock-down' of p53 may help to further elucidate the role of p53 in the mechanism of apigenin.

Our *in vivo* studies were designed to demonstrate the efficacy of apigenin as an orally administered chemotherapeutic. Our primary outcome measure was tumor size following 6 weeks of dietary supplementation of apigenin. While the tumors in treated animals were smaller than control diet-fed mice this difference was not statistically significant ($P = 0.058$). Correlation of apigenin levels with tumor size showed a significant reduction in tumor volume in mice with the highest tissue levels of apigenin. Because apigenin was administered as a dietary supplement *ad libitum* (as opposed to gavage feed), mice that ate more feed were exposed to greater doses of apigenin and may have achieved higher tissue levels. This along with individual differences in absorption and elimination of apigenin and the dose-dependency of apigenin's anti-neoplastic properties contributed to the inhomogeneity of the data, and the apparent lack of treatment effect when examining tumor volume alone. The small sample sizes (n = 6 treatment, n = 6 control mice) also may have contributed to the lack of significance through type II error (accepting null hypothesis when treatment effect truly exists due to insufficient sample size). While the administration method employed may be seen as a weakness in our study design it does allow us to conclude that apigenin supplementation at a level known to be non-toxic and well-tolerated (as opposed to mega-doses that may be intolerable or otherwise unachievable in human subjects) can have a positive effect on tumor size and lead to increased tumor apoptosis as seen with TUNEL staining.

Further study is warranted, particularly in the area of apigenin as a supplemental therapy in combination with standard chemotherapeutics such as gemcitabine. Studies have indicated a facultative role of apigenin in increasing the efficacy of gemcitabine's anti-tumor effects [4,28]. This polypharmacy strategy may also help to improve the tolerability of chemotherapeutic regimens by reducing the dose necessary to realize a treatment effect. It is not known whether apigenin would have a similar effect when combined with newer chemotherapy combinations. At least one study has indicated that apigenin may actually decrease the effectiveness of standard chemotherapeutics necessitating a deeper understanding of the exact mechanism of apigenin's effects and a more targeted approach to combination with existing drugs [27]. Finally, apigenin may have chemopreventative properties which would halt the progression of pre-neoplastic

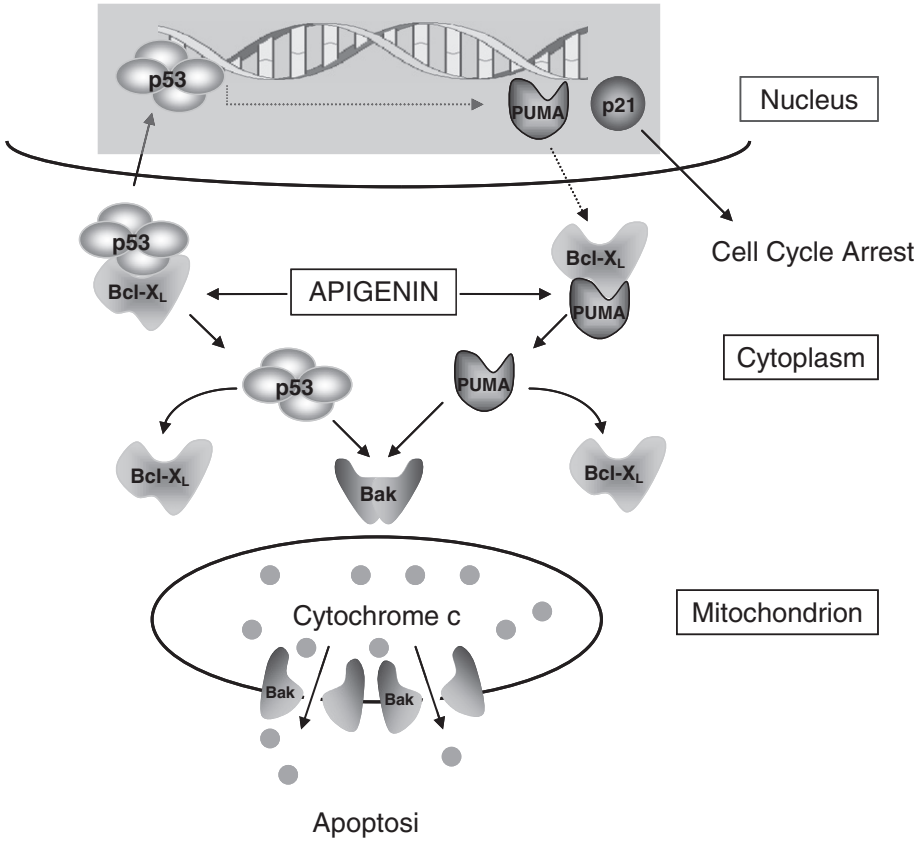

Fig. 7. Proposed mechanism of apigenin-induced apoptosis in pancreatic cancer cells. The shaded area indicates activity inhibited by pifithrin-α.

PanIN lesions to invasive carcinoma [29]. Apigenin's anti-neoplastic properties as they relate to the timing of p53 mutation, which is thought to be a late event in the dysplasia-carcinoma continuum, would be of particular interest in light of our findings here.

Although these data do not definitively establish a causal mechanism between Bcl-X_L, PUMA, and p53, they do indicate that the roles of p53 and PUMA may be parallel and facultative, not sequential and inexorably linked as has been previously proposed. Furthermore, our data show that even mutated p53 retains some of its cell cycle regulatory and pro-apoptotic function(s) in the presence of growth-inhibitory stimuli such as apigenin treatment. Most importantly from a translational perspective apigenin reduced the size of xenograft tumors in a dose-dependent fashion with greater cellular apoptosis, thus correlating *in vitro* observations with *in vivo* data. These findings contribute additional support to a growing body of evidence indicating apigenin may hold promise as a chemotherapeutic for the treatment of pancreatic cancer.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.12.008.

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