

Nuclear Factor- κ B p65/relA Silencing Induces Apoptosis and Increases Gemcitabine Effectiveness in a Subset of Pancreatic Cancer Cells

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Abstract Purpose: Nuclear factor κ B (NF κ B) activity may increase survival and protect cancer cells from chemotherapy. Therefore, NF κ B activity may be prognostic, and inhibition of NF κ B may be useful for pancreatic cancer therapy. To test these hypotheses, we examined NF κ B activity and the effects of inhibiting NF κ B in several pancreatic cancer cell lines with differing sensitivities to gemcitabine.

Experimental Design: The gemcitabine sensitivity of pancreatic cancer cell lines BxPC-3, L3.6pl, CFPAC-1, MPanc-96, PANC-1, and MIA PaCa-2 were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and fluorescence-activated cell sorting assays. NF κ B levels were determined by electrophoretic mobility shift assay and reporter assays. The effects of gemcitabine on NF κ B activity were determined *in vitro* and *in vivo*. NF κ B was inhibited by silencing of the p65/relA subunit using small interfering RNA *in vitro* and by neutral liposomal delivery of small interfering RNA *in vivo*, and the effects were evaluated on gemcitabine sensitivity.

Results: The cell lines L3.6pl, BxPC-3, and CFPAC-1 were sensitive, whereas MPanc-96, PANC-1, and MIA PaCa-2 were resistant to gemcitabine. No significant correlation was observed between basal NF κ B activity and gemcitabine sensitivity. Gemcitabine treatment did not activate NF κ B either *in vitro* or *in vivo*. Silencing of p65/relA induced apoptosis and increased gemcitabine killing of all gemcitabine-sensitive pancreatic cancer cells. No significant effects, however, were observed on gemcitabine-resistant pancreatic cancer cell lines either *in vitro* or *in vivo*.

Conclusions: NF κ B activity did not correlate with sensitivity to gemcitabine. Silencing of p65/relA was effective alone and in combination with gemcitabine in gemcitabine-sensitive but not gemcitabine-resistant pancreatic cancer cells. Thus, NF κ B may be a useful therapeutic target for a subset of pancreatic cancers.

Pancreatic cancer has the worst prognosis of all major cancers, with an overall 5-year survival rate of around 5% (1). The current clinical standard of care for advanced pancreatic cancer is gemcitabine, a cytotoxic nucleoside analogue. Gemcitabine results in a tumor response rate of 12% and offers a median survival time of 5 months (2). Unfortunately, this means that the best current treatment offers very modest benefits. Recent studies have indicated that targeted therapies in combination with gemcitabine can have statistically significant benefits (3). However, the results to date remain meager, and new approaches to improving the effectiveness of gemcitabine are

needed. One of the targets considered for combination therapy that has generated wide attention is the transcription factor NF κ B (4).

NF κ B is a ubiquitous transcription factor that is regulated by a vast array of stimuli, including growth factors, inflammatory mediators, cytotoxic agents like chemotherapeutic drugs, oxidative stress, UV light, and many others. NF κ B is a dimer composed of various combinations of the five mammalian Rel proteins, namely, p65/RelA, c-Rel, RelB, NF κ B1/p50, and NF κ B2/p52 (5). The most common form of NF κ B is a dimer of p65/relA and p50, and this dimer is often referred to simply

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Translational Relevance

Nuclear factor κ B (NF κ B) is widely considered a useful target for cancer therapy. Most studies in this regard, however, have used NF κ B inhibitors that are not entirely specific. Furthermore, most studies have investigated only a small number of cancer cell lines and have not taken into account the heterogeneity that exists among tumor cells. In the current study, the effects of the highly specific approach of silencing NF κ B using small interfering RNA were investigated in several pancreatic cancer cells with either high or low levels of resistance to gemcitabine. The data indicated that inhibition of NF κ B was highly effective in gemcitabine-sensitive pancreatic cancer cells but was without effect in gemcitabine-resistant cells. Thus, patients with gemcitabine-sensitive tumors would likely respond well to inhibition of NF κ B alone or in combination with gemcitabine. However, only a small percentage of patients have tumors that are sensitive to gemcitabine. Therefore, it seems unlikely that NF κ B inhibition will be widely useful in the treatment of pancreatic cancer. Clearly the development of biomarkers or diagnostic tests that could identify the subset of patients with gemcitabine-sensitive tumors would be valuable as this is the group that would be most likely to benefit from NF κ B inhibition.

as NF κ B. All Rel proteins share the NH₂-terminal Rel homology domain that mediates dimerization, DNA binding, and nuclear localization and interaction with the inhibitors of NF κ B, the inhibitory κ Bs (I κ B). The p65/RelA, c-Rel, and RelB proteins possess COOH-terminal transactivation domains that allow for transcriptional activation of target genes. The binding of Rel proteins with I κ Bs masks a nuclear localization signal and effectively sequesters them in the cytoplasm where they are inactive. The most common pathway for NF κ B activation (the canonical pathway) involves upstream activation of a complex of I κ B kinases leading to the phosphorylation and subsequent ubiquitination and degradation of I κ B α , which releases the Rel dimer that then enters the nucleus and activates gene transcription. Activity of NF κ B, however, is also regulated by a number of posttranslational modifications of Rel proteins and by interactions with other transcription factors and regulatory factors (5). Furthermore, there are several less common pathways to NF κ B activation, some of which are independent of I κ Bs. Which of these pathways is of primary importance in cancer therapy is currently unclear.

In cancer cells, NF κ B is thought to play an antiapoptotic role through its ability to induce the expression of several molecules, including inhibitors of apoptosis family members as well as Bcl-2 homologues (6). Thus, NF κ B is often regarded as being important for chemoresistance. This being the case, then the level of NF κ B may be an indicator of chemoresistance and NF κ B may be an excellent target for combined therapy with a cytotoxic such as gemcitabine. That NF κ B activity might be an indicator of chemoresistance is supported by the observation that in pancreatic cancer, which is highly chemoresistant, NF κ B is often constitutively activated (7–9). Furthermore, data suggesting that basal NF κ B activity is higher in highly resistant

cells compared with more sensitive cells have previously been reported (10). The concept of NF κ B as a therapeutic target in cancer is supported by the observation that inhibition of NF κ B seems to sensitize a wide variety of cancer cells to cytotoxics (11, 12) and this has also been reported for pancreatic cancer cells (10, 13–15).

Despite the abundance of data supporting the role of NF κ B in chemoresistance, there are also data that suggest this may be an oversimplification. Many studies have now shown that NF κ B can have either proapoptotic or antiapoptotic effects depending on the status of tumor suppressors and other mechanisms within a specific cell (16–18). The role of NF κ B is also determined by the specific cytotoxic being tested; for example, NF κ B activity is required for doxorubicin-induced cell death in N-type neuroblastoma cells (19). In addition, the effects of pharmacologic and molecular inhibition of NF κ B are often divergent (20). Unfortunately, most of the studies supporting NF κ B as a target for sensitization to cytotoxics have relied on pharmacologic inhibitors that are not specific for NF κ B. In summary, based on the complex pathways of NF κ B activity and function, the effects of NF κ B inhibition can be cell-type specific and may depend on the method of inhibition and on the specific cytotoxic stress being applied. Therefore, whether or not inhibiting NF κ B might improve gemcitabine therapy in patients with gemcitabine-resistant cancer cells remains a relevant issue.

The aim of the current study was to test the hypotheses that (a) levels of NF κ B activity could distinguish sensitive from resistant pancreatic cancer cells; and (b) that inhibition of NF κ B by small interfering RNA (siRNA)-directed gene silencing of the major DNA binding component p65/relA would increase the sensitivity of pancreatic cancer cell lines to gemcitabine-mediated apoptosis. The first hypothesis was not supported. The second hypothesis was partially supported, as we found that silencing p65/relA was effective alone and in combination with gemcitabine, but only for the subset of pancreatic cancer cells with native sensitivity to gemcitabine. Thus, our results suggest that the regulation and role of NF κ B in pancreatic cancer is more complex than previously recognized and do not support targeting NF κ B as a universal treatment for this disease.

Materials and Methods

Cell lines and culture conditions. Human pancreatic cancer cell lines BxPC-3, L3.6pl, CFPAC-1, MPanc-96, PANC-1 and MIA PaCa-2 were obtained from the American Type Culture Collection, and all except BxPC3 cells were routinely cultured in DMEM (BxPC-3 were in RPMI) supplemented with 10% fetal bovine serum in a 37°C incubator in a humidified atmosphere of 5% CO₂. Human pancreatic duct epithelial (HPDE) cells (21) kindly provided by Dr. Tsao (University of Toronto) were cultured in keratinocyte serum-free media.

Lentiviral constructs. To allow monitoring of NF κ B activity in living cells, a lentivirus NF κ B luciferase reporter gene was constructed. A NF κ B luciferase reporter gene consisting of repeats of a consensus κ B binding site coupled with a minimal promoter to drive firefly luciferase was excised from the pNF κ B-luc vector (Clontec) and cloned into the lentiviral vector FG9 (ref. 22; a gift from Dr. Xiao-Feng Qin, Department of Immunology, M. D. Anderson Cancer Center, Houston, TX), replacing UBIC promoters, to form p-Lenti-NF κ B-luc. As a control for infection efficiency, we used a modified version of the same FG9 lentiviral plasmid in which the ubiquitin promoter drives renilla luciferase gene expression (Lenti-Ubiquitin-renilla-luc), also from

Dr. Xiao-Feng Qin. To study pancreatic cancer growth *in vivo*, we used a control lentiviral luciferase construct in which the luciferase coding sequence was isolated from the pGL-3 vector (Promega) and cloned into the lentiviral vector FG9 behind the UBiC promoter to form the luciferase-expressing p-Lenti-luc. Lentiviral plasmid vectors were cotransfected with packaging vectors in 293T cells by the calcium transfection method, and Lenti-NF κ B-luc and Lenti-luc viruses were produced (22).

NF κ B activity assay. For analysis of basal NF κ B activity, cells were simultaneously infected with Lenti-NF κ B-luc and a Lenti-Ubiquitin-Renilla-Luc (25 μ L of each viral supernatant/mL of medium) mixed with polybrene (4 μ g/mL medium) to develop stable cell lines expressing the NF κ B reporter and the renilla luciferase control. Stable cells were cultured in a 96-well plate until cells were 70% confluent, and functional validation of the NF κ B reporter activity was conducted *in vitro* using tumor necrosis factor- α (TNF- α ; 10 ng/mL; Sigma) as a positive control. Cell lysates were prepared, and firefly and renilla luciferase activities were quantified according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System; Promega). Each luciferase signal was measured by using a luminometer (Lumistar). To control for different levels of infectivity and cell numbers, NF κ B promoter activities were calculated from firefly signals divided by renilla signals. To examine the response to gemcitabine, stable transfected NF κ B reporter cells (L3.6pl, BxPC-3, MPanc-96, and PANC-1, $\sim 1 \times 10^5$ cells) were cultured in a 96-well plate overnight and stimulated with various concentrations of gemcitabine for 24 h. For these experiments, stimulated levels of firefly luciferase were compared with control levels in the absence of gemcitabine.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from cells using the NE-PER nuclear and cytoplasmic extraction reagents kits according to the manufacturer's directions (Pierce Biotech). For NF κ B DNA binding, the 10,000 cpm of the 22-bp oligonucleotide 5'-AGTTGAGGGGACITTCAGGC-3' containing the NF κ B consensus sequence that had been labeled with [32 P]ATP (10 mCi/mmol) by T4 polynucleotide kinase was added to 15 μ g nuclear extract. The reaction was allowed to proceed for 30 min at room temperature. For cold competition experiments, unlabeled NF κ B oligonucleotide or OCT1 oligonucleotide as nonspecific competitor gels were dried and directly exposed to a B-1 phosphorimaging screen and visualized with a GS-250 Molecular Imaging System (Bio-Rad). TATA binding protein (TBP) was used as a nuclear loading control (Abcam Inc.).

SDS-PAGE and Western blot analysis. For analysis of protein expression, cells were lysed with 100 μ L Triton X-100 lysis buffer in 6-well plates by incubation for 1 h at 4 $^{\circ}$ C. Protein concentrations were measured by as per the manufacturer's Bio-Rad reagent protocol (Bio-Rad). Proteins (50 μ g) were separated by 10% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked for 1 h at room temperature in 5% milk solution. NF κ B p65/relA was detected by incubating the transferred membrane overnight at 4 $^{\circ}$ C with rabbit polyclonal antibody (Santa Cruz Biotech; cat #D2204) at 1:250 dilution in 5% milk solution, then washing and incubating with the secondary antibody (Goat antirabbit IRDye 680) for 1 h at room temperature and the signal was detected by Odyssey IR imaging system (Li-Cor Biosciences).

Transfection of small interfering RNA. Cells in 6-well plates or 96-well plates were grown to 50% confluence and transfected with double-stranded siRNA for relA/p65 (Dharmacon Res Inc.) target sequence (Sense 5' CCAUCAACUAUGAUGAGUU dTdT 3', Antisense 3' dGdTGGUAGUUGAUACUACUCAA 5') or with a siRNA nonspecific control (Ambion, Austin, TX cat# 4611) in serum-free medium without antibiotic supplements using Hiperfect Transfectin Reagent (Qiagen, Inc.). Cells were incubated under these conditions for 48 h and silencing was then confirmed by reporter assays as well as Western blotting.

Liposomal preparation. Neutral liposomes were used for siRNA delivery similarly to what has been previously described (23). Briefly, siRNA for *in vivo* delivery was incorporated into neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC). DOPC and siRNA were mixed in the presence of excess tertiary butanol at a ratio of 1:10

(w/w) siRNA/DOPC. Tween 20 was added to the mixture in a ratio of 1:19 Tween 20: siRNA/DOPC. The mixture was vortexed, frozen in an acetone/dry ice bath, and lyophilized. Before *in vivo* administration, this preparation was hydrated with normal 0.9% saline at a concentration of 50 μ g/mL to achieve the desired dose in 200 μ L per injection.

Cell growth studies. Analysis of cell growth was used to determine sensitivity of the cell lines to gemcitabine *in vitro*. Cells (10^4 cells/well) were seeded on 96-well plates in 100 μ L of complete culture medium, allowed to attach for 24 h, then transfected with siRNA mixtures. After 24 h, cells were treated with gemcitabine (2',2'-difluorodeoxycytidine; Eli Lilly) at indicated concentrations in a total volume of 150 μ L of culture medium. After 72 h, cell viability was assessed using MTS reagent (Promega) according to the manufacturer's directions. The percentage of viable cells was defined as therapy group divided by control group, multiplied by 100%. Fluorescence-activated cell sorting analysis and cell counting were done in parallel and gave similar results as the MTS assay.

Analysis of NF κ B *in vivo*. Male athymic nude mice (nu/nu) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center and housed in specific pathogen-free conditions. They were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the USPHS "Policy of Human Care and Use of Laboratory Animals," and all studies were approved and supervised by the University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee. Stable NF κ B reporter-bearing cells were grown to 80% confluence, harvested by trypsinization, washed twice in PBS, and 50 μ L of cell suspensions (1×10^6 cells) were used for intrapancreatic injection into 4-wk-old male nude mice to make an orthotopic pancreatic tumor model. For analysis of the effects of gemcitabine, after 3 wk the mice were injected with gemcitabine (50 mg/kg or 200 mg/kg i.p. injection) and bioluminescence imaging was done after 0, 6, 24, 48, and 72 h of gemcitabine treatment. Data were calculated as percentage of the NF κ B signals in each individual mouse before i.p. injection of gemcitabine, and experiments were done at least thrice. For *in vivo* analysis of the effectiveness of liposomal delivery of siRNA, after 1 wk the mice were injected with d-luciferin (150 mg/kg body weight, i.p. injection), and basal NF κ B activity was determined using the IVIS system (Xenogen Corp.). Subsequently, mice were injected with siRNAp65 or the control (siRNAC) coupled liposome complexes (10 μ g/kg body weight, i.p.), and NF κ B luciferase activity was reanalyzed after 48 h.

Analysis of tumor development. For monitoring of cancer cell growth *in vivo*, we used Lenti-luc-expressing cells and confirmed that there was

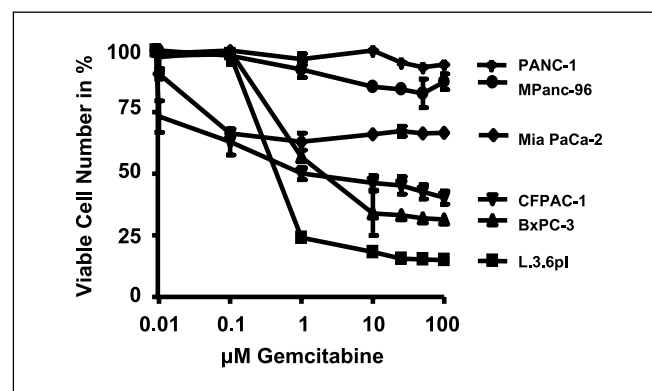


Fig. 1. Pancreatic cancer cells have differing levels of native resistance to gemcitabine. Human pancreatic cancer cell lines PANC-1, MPanc-96, MIA PaCa-2, CFPAC-1, BxPC-3, and L3.6pl were treated with increasing concentrations of gemcitabine (0–100 μ mol/L) for 72 h. The viabilities indicated on the y axis were determined by MTS assays and normalized to control. Data shown are means \pm SE for $n = 3$ independent experiments.

a linear relationship between cell numbers ($0-10 \times 10^5$ cells/well in a 24-well plate) and the light emission after adding luciferin (150 $\mu\text{g/mL}$) measured using the IVIS system. Cells carrying the Lenti-luc reporter gene were grown to 80% confluence, harvested by incubation with trypsin-EDTA, washed twice in PBS, and resuspended to a final concentration of 2.5×10^6 cells/mL (MPanc-96) or 5.0×10^6 cells/mL (BxPC-3) for 100 μL intrapancreatic injections ($n = 20$ mice/cell line) in 4-wk-old male nude mice. Tumors were allowed to become established for 1 wk, then bioluminescent imaging was done and the mice were grouped into four groups of five mice with equivalent mean tumor volumes between groups. The mice in group 1 were treated with liposomal relA siRNA (10 $\mu\text{g/mouse}$) and 24 h later the mice were treated with gemcitabine (100 mg/kg for MPanc96 orthotopic model, 50mg/kg for BxPC3 orthotopic model) via i.p. injection (2 times/wk).

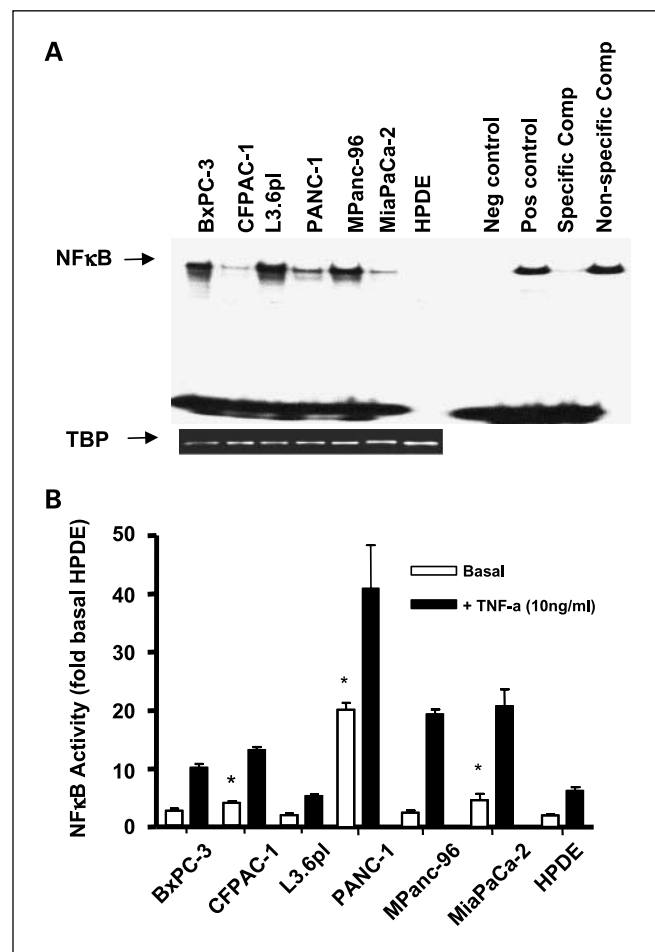


Fig. 2. Basal levels of NF κ B do not correlate with gemcitabine sensitivity. **A**, NF κ B nuclear binding was analyzed in human pancreatic cancer cell lines. Nuclear extracts from six pancreatic cancer cell lines and human pancreatic duct epithelial cells were prepared and EMSA using a labeled oligonucleotide (Oligo) containing a consensus κ B binding site was done. TBP was used as a loading control for quality and quantity of cell extracts. Negative control, ^{32}P -labeled NF κ B Oligo without HeLa nuclear extract; positive control, HeLa nuclear extract and ^{32}P -labeled NF κ B Oligo; specific competitor, HeLa nuclear extract with ^{32}P -labeled NF κ B Oligo plus unlabeled NF κ B Oligo; nonspecific competitor, HeLa nuclear extract with ^{32}P -labeled NF κ B Oligo plus unlabeled Oct1 Oligo. **B**, NF κ B transcriptional activity was estimated using an NF κ B reporter assay. Pancreatic cancer cells were coinfecting with an NF κ B reporter expressing firefly luciferase and a control lentivirus expressing renilla luciferase, and stable populations were developed. Basal NF κ B transcriptional activity determined as a ratio of firefly and Renilla luciferase signals and presented as a comparison with the level measured in human pancreatic duct epithelial cells. As a positive control, all cell lines were also challenged with TNF- α (10 ng/mL) for 6 h and the level of NF κ B activity was determined. Data shown are means \pm SE for three independent experiments.

The mice in group 2 were treated with liposomal relA siRNA and PBS (control) 24 h later via i.p. injection (2 times/wk). The mice in group 3 were treated with liposomal control siRNA (10 $\mu\text{g/mouse}$) and 24 h later the mice were treated with gemcitabine via i.p. injection (2 times/wk). The mice in group 4 were treated with liposomal control siRNA and PBS (control) 24 h later via i.p. injection (2 times/wk). At the end of the 5 wk the animals were sacrificed and the pancreas were removed and weighed then processed for histology.

In vivo bioluminescence imaging. Bioluminescence imaging was conducted using a cryogenically cooled IVIS 100 imaging system coupled to a data acquisition computer running Living Image Software. Before imaging, the mice were placed in an acrylic chamber, anesthetized with 1.5% isoflurane-air mixture, and injected i.p. with 15 mg/mL of luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. A digital gray scale image of each mouse was acquired, followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the mouse which was integrated for 5 min starting from 7 min after the injection of d-luciferin. Therapy was continued for 5 wk before the animals were sacrificed. The tissue was formalin-fixed, paraffin-embedded, and also frozen in -80°C in optimal cutting temperature compound.

Statistical analysis. All experiments were conducted in triplicate and carried out on three or more separate occasions. Data presented are means of the three or more independent experiments \pm SE. Statistically significant differences were determined by two-tailed unpaired Student's t test and were defined as $*P < 0.05$.

Results

Pancreatic cancer cell lines vary in resistance to gemcitabine. We examined the relative sensitivity of six commonly used pancreatic cell lines (PANC-1, MPanc-96, MIA PaCa-2, CFPAC-1, BxPC-3, and L3.6pl) to gemcitabine *in vitro*. Cells were treated with different concentrations of gemcitabine for 72 hours and the number of surviving cells was analyzed. Whereas the gemcitabine LD₅₀ was $>50 \mu\text{mol/L}$ for the PANC-1, MPanc-96, and MIA PaCa-2 cells, the LD₅₀ was around $1 \mu\text{mol/L}$ for BxPC-3, CFPAC-1, and L3.6pl (Fig. 1). The same sensitivities were obtained when the effects of gemcitabine were analyzed on apoptosis using fluorescence-activated cell sorting (data not shown). These data supported the classification of the PANC-1, MPanc96, and MIA PaCa-2 cell lines as gemcitabine-resistant and the BxPC3, CFPAC, and L3.6pl cell lines as gemcitabine-sensitive.

NF κ B levels in pancreatic cancer cell lines. To test the hypothesis that NF κ B basal levels could predict gemcitabine sensitivity, we initially evaluated NF κ B DNA binding by electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from the six pancreatic cancer cell lines and also from noncancer immortalized human pancreatic duct epithelial cells. Nuclear extracts from each of the cell lines cultured under basal conditions bound an NF κ B consensus oligonucleotide (Fig. 2A). Specificity was indicated by the observation that this binding was blocked by excess unlabeled oligonucleotide. Highest levels of binding were observed with MPanc-96, CFPAC-1, L3.6pl, and BxPC-3 cells, and lowest DNA binding was observed with PANC-1, CFPAC-1, MIA PaCa-2, and human pancreatic duct epithelial cells. Thus, pancreatic cancer cells exhibited elevated NF κ B, but there was no consistent pattern of NF κ B DNA binding in the resistant versus sensitive cell lines.

Because nuclear levels of NF κ B subunits do not directly correspond to NF κ B transcriptional activity, we examined NF κ B activity using a reporter assay. In preliminary studies conducted

using transient transfection assays we observed that the stress of transient transfection itself activated NF κ B and interfered with the analysis (data not shown). Therefore, to avoid transient transfection artifacts, we developed a lentiviral vector to stably express an NF κ B reporter. This reporter virus was coinfecting with a control lentivirus that expressed renilla-luciferase from an ubiquitin promoter as a control for infection efficiency. Using the ratio of firefly and renilla luciferase signals, we observed that basal levels of NF κ B transcriptional activity could be observed in all of the cell lines, including the control human pancreatic duct epithelial cells (Fig. 2B). Furthermore, all cells responded to TNF- α with increased NF κ B activity. NF κ B activity measured using the reporter assay did not perfectly match the level of nuclear NF κ B DNA binding observed in EMSA. Only three cancer cell lines, CFPAC-1, MIA PaCa-2, and PANC-1, had higher basal levels of NF κ B activity than the human pancreatic duct epithelial cells. Thus, no consistent difference was noted between NF κ B activities in gemcitabine-sensitive versus gemcitabine-resistant cells.

Gemcitabine treatment did not affect NF κ B activity. Although basal NF κ B activity did not reflect gemcitabine sensitivity, it remained possible that responses to stress may be different in sensitive versus resistant cells. Therefore, we wished to evaluate whether NF κ B activity in response to treatment with gemcitabine could predict sensitivity. Two sensitive cells (L3.6pl and BxPC-3) and two resistant cells (MPanc-96 and PANC-1) stably expressing the NF κ B reporter were stimulated with various concentrations (0.05–2 μ mol/L) of gemcitabine for 24 hours *in vitro* and NF κ B activities were measured using the reporter assay. Gemcitabine treatment (1 μ mol/L) for 24 hours had no significant effect on NF κ B activity in any of the cells analyzed (Fig. 3A). Because the results of *in vitro* treatments may not reflect the situation *in vivo*, we established mouse orthotopic pancreatic tumor models of the most sensitive (L3.6pl) and most resistant (PANC-1) cells stably expressing the NF κ B reporter gene. Animals were treated with either a lower dose (50 mg/kg) or higher dose (200 mg/kg) of gemcitabine and NF κ B activities were evaluated. Neither the more gemcitabine-sensitive tumor (L3.6pl) nor the more resistant tumor (PANC-1) showed any significant change in NF κ B activity from 0 to 72 hours after gemcitabine injection (Fig. 3B).

In vitro knockdown of p65/relA influenced sensitive but not resistant cells. To test the hypothesis that inhibition of NF κ B would increase the sensitivity of pancreatic cancer cell lines to gemcitabine-mediated apoptosis, we used small interfering RNA (siRNA) to knock down p65/relA. To validate the effectiveness of this approach, we examined basal and TNF- α -stimulated NF κ B-dependent luciferase activity in PANC-1 cells transfected with either control siRNA (siRNac) or p65/relA siRNA (siRNap65). After transfection with siRNap65, but not siRNac, the NF κ B-dependent signal was dramatically reduced from its basal level and the ability of TNF α to increase NF κ B transcriptional activity was completely blocked (Fig. 4A). As further confirmation of the silencing, Western blotting of the p65/relA subunit in the six cell lines transfected with siRNap65, but not siRNac, also showed complete silencing (Fig. 4B).

Next, we transiently expressed expression of p65/relA in each of the six pancreatic cell lines and examined the effects alone and with LD₅₀ concentrations of gemcitabine (Fig. 5). Silencing of p65/relA alone significantly reduced cell numbers *in vitro* of all gemcitabine-sensitive pancreatic cell lines (BxPC-3, L3.6pl,

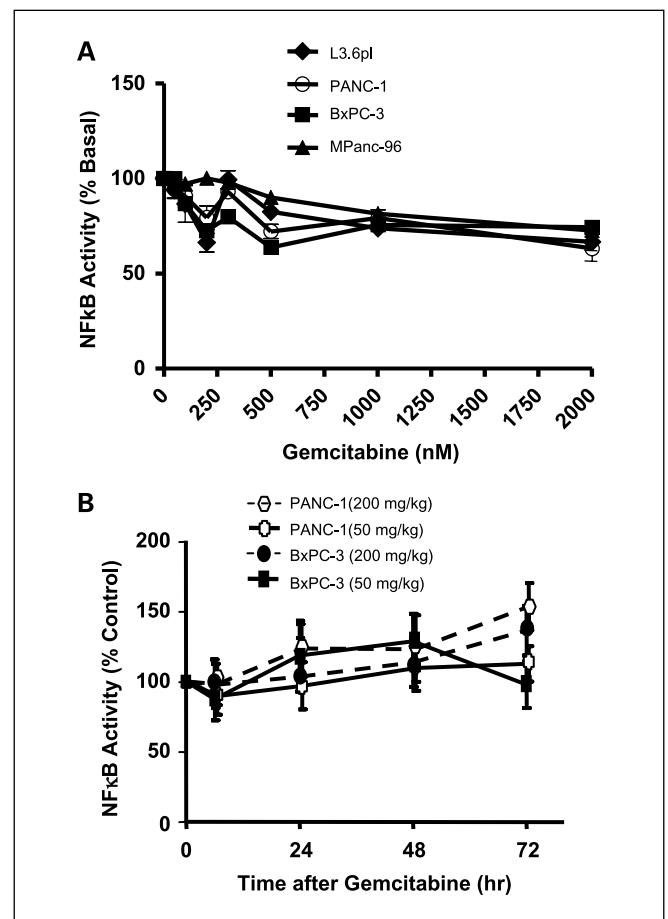


Fig. 3. Gemcitabine treatment does not influence NF κ B activity in pancreatic cancer cells *in vitro* or *in vivo*. **A**, two gemcitabine sensitive (L3.6pl and BxPC-3) and two gemcitabine resistant (MPanc-96 and PANC-1) cell lines were treated with indicated concentrations of gemcitabine for 24 h *in vitro* and activity of an NF κ B reporter was analyzed. **B**, the most gemcitabine-sensitive cell line (L3.6pl) and the most resistant (PANC-1) were implanted orthotopically in nude mice, allowed to form tumors, and then the level of NF κ B reporter activity was measured before and 24, 48, 72 h after treatment with either a low dose (50 mg/kg) or a high dose (200 mg/kg) of gemcitabine. All measurements are shown as a percentage of basal and are means \pm SE for three independent experiments.

CFPAC-1). Furthermore, in gemcitabine-sensitive cell lines p65/relA silencing also increased the effectiveness of gemcitabine treatment. In contrast, p65/relA silencing did not affect cell numbers of the gemcitabine-resistant cell lines (MPanc-96, PANC-1, MIAPaCa-2). Nor did silencing of p65/relA increase the effectiveness of gemcitabine killing of the gemcitabine-resistant cell lines.

Knockdown of p65/relA reduced in vivo tumor growth in sensitive but not resistant orthotopic tumors. To determine whether the results of the *in vitro* studies were relevant to the *in vivo* situation, we used neutral liposomes to deliver the siRNap65 to pancreatic orthotopic xenografts. To validate the approach, we first examined NF κ B activity of orthotopically transplanted MPanc-96 cells stably expressing an NF κ B luciferase reporter. After 1 week, NF κ B luciferase activity was determined before (0 time point) and 48 hours after a single injection of siRNap65 or siRNac liposomal complex (10 μ g/mouse by i.p. injection). Liposomal siRNap65 administration reduced NF κ B activity in the tumors by 90% versus the controls (Fig. 6A).

We then used this approach to analyze the effects of the liposomal delivery of siRNAs in orthotopic models of one gemcitabine resistant cell line (MPanc-96) and one gemcitabine sensitive cell line (BxPC-3). Mice with established orthotopic tumors were randomized ($n = 5$ mice/group) into four treatment groups, each with approximately equal mean tumor size. Groups of mice were treated with siRNAp65 liposomal complex with or without gemcitabine, or siRNAc liposomal complex with or without gemcitabine. Treatments were continued for 5 weeks and the tumor burden was analyzed by weekly bioluminescence imaging. In the gemcitabine-resistant MPanc-96 orthotopic model, neither siRNAp65 alone, gemcitabine alone, nor the combination influenced the time-course of tumor development (Fig. 6B). Likewise, none of the treatments had any significant effect on tumor weight at the end of the experiment (Fig. 6C). In contrast, in the gemcitabine-sensitive BxPC-3 orthotopic model, gemcitabine alone, even at a lower dose than used in the MPanc-96 model, had a significant inhibitory effect. Furthermore, treatment with siRNAp65 alone dramatically reduced tumor growth; in fact, no tumor could be found in those animals after 5 weeks. For this reason, it was not possible to determine whether siRNAp65 increased the effectiveness of gemcitabine in this model (Fig. 6B, C).

Discussion

Most pancreatic cancer patients receive little benefit from current adjuvant therapies largely because most pancreatic cancer cells are resistant to chemotherapeutic agents. NF κ B is thought to be important in cancer cell resistance to apoptosis and thus contribute to chemoresistance. More than 10 years ago it was noted that pancreatic cancer cells possess high levels of NF κ B (7) and the level of p65/relA observed in tissue sections has been reported to correlate with poor survival in patients who received resections (24). Furthermore, many studies have suggested that inhibition of NF κ B using a wide

variety of inhibitors sensitizes pancreatic cancer to cytotoxic treatments (25–27). Therefore, we hypothesized that NF κ B activity levels would predict chemoresistance. Furthermore, we predicted that inhibition of NF κ B activity using a siRNA directed at the most common transcriptionally active subunit, p65/rel, would reduce chemoresistance. However, our data did not confirm these hypotheses and indicated rather that the role and regulation of NF κ B in pancreatic cancer is more complex than previously recognized. Of particular significance, our results on the effects of NF κ B inhibition on sensitivity to gemcitabine indicated that natively sensitive but not resistant cells were affected. This suggests that clinical trials based on inhibition of NF κ B in pancreatic cancer may not be uniformly successful. This study differs in several specifics compared with previous studies including the technique of analysis of NF κ B activity, the means of NF κ B inhibition, the use of a large number of cell lines that were well defined in terms of native gemcitabine sensitivity, and the focus on gemcitabine as the cytotoxic.

Our initial goal was to determine whether NF κ B levels could predict chemosensitivity to gemcitabine. High basal levels of NF κ B in pancreatic cancer cells have previously been noted in a large number of studies by EMSA (8, 14, 25, 28–31). Some studies have suggested that the level of basal NF κ B is correlated with resistance to chemotherapies (10, 14). We also observed levels of nuclear NF κ B DNA binding that were higher in pancreatic cancer cells than in the nontumorigenic human pancreatic duct epithelial cell line. However, we found no correlation between the level of NF κ B observed by EMSA and the gemcitabine sensitivity of the cell lines. It is not possible to conclude from the small sampling of cell lines in this study that no statistical correlation exists between NF κ B activity and gemcitabine resistance. It is clear from these examples, however, that NF κ B activity of a specific cell line is not enough information to determine gemcitabine resistance. One important issue that has not been well investigated in pancreatic cancer is that EMSA does not directly relate to NF κ B transcriptional activity due to the large number of other regulatory mechanisms (5, 32). One previous study used an NF κ B reporter in transient reporter assays to help address this issue (8). However, we found that transient transfection of reporters gave highly variable results due to the dramatic effects of transfection procedures themselves on NF κ B activity. To avoid these complications, we used for the first time in pancreatic cancer studies lentiviral vectors to stably express the NF κ B reporter in the cells. The results indicated that all of the cell lines had basal NF κ B activity, even the nontumorigenic human pancreatic duct epithelial cells. This may reflect the basal level of cell stress involved in the cell culture procedures. There was no obvious relationship between DNA binding complexes observed in EMSA studies and NF κ B transcriptional activity evaluated using the reporter. Only three of the six cancer cell lines showed NF κ B activity levels significantly higher than that of the human pancreatic duct epithelial cells. There was no simple correlation between basal reporter activity and gemcitabine sensitivity, thus disproving our hypothesis.

We also investigated whether sensitive and resistant pancreatic cancer cells could be differentiated on the basis of the NF κ B response to gemcitabine treatment. Using a stably expressed NF κ B reporter, we did not observe any effect of gemcitabine on NF κ B activity *in vitro*. Furthermore, this was the first study to

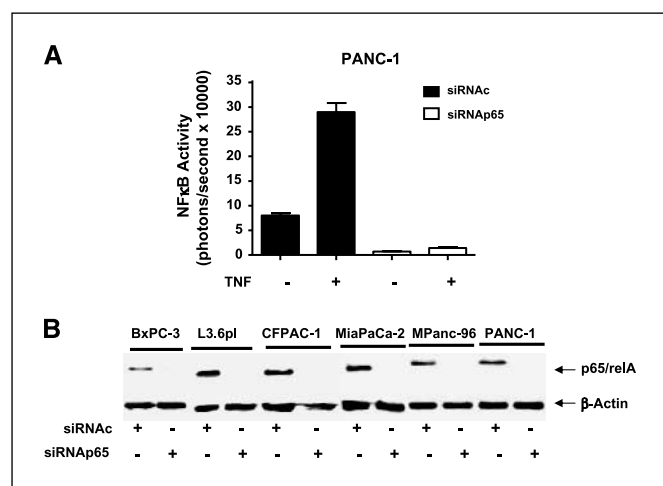
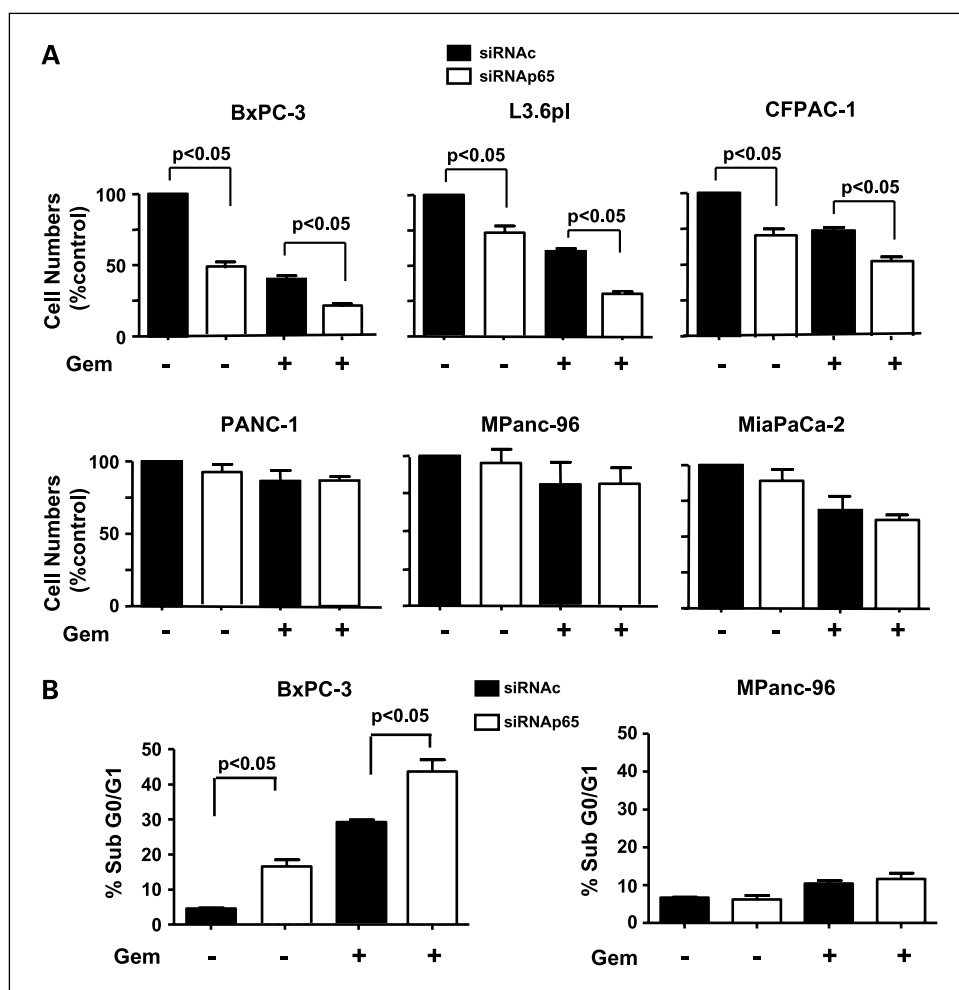


Fig. 4. siRNA silencing of p65/relA was highly effective *in vitro*. **A**, to examine the effectiveness of p65/relA silencing on NF κ B activity in the cancer cells, PANC-1 cells expressing NF κ B luciferase gene were transfected with control siRNA and relA siRNA. After 72 h, cells were treated with or without TNF- α (10 ng/mL) and bioluminescent imaging was used to estimate NF κ B reporter activity. **B**, Western blotting was conducted 72 h after treatment with control siRNA or siRNA against p65/relA in the six cell lines. Full-length gels are presented in Supplementary Fig. S1. Columns, mean for three experiments; bars, SE. *, $P < 0.05$, versus control.

Fig. 5. Silencing of p65/relA is effective against gemcitabine-sensitive but not resistant cells. **A**, effects of gemcitabine on cell viability of pancreatic cancer cell lines after silencing of relA/p65. Human pancreatic cancer cell lines BxPC-3, L3.6pl, CFPAC-1, MPanc-96, PANC-1, and MIA PaCa-2 were treated with different concentrations of gemcitabine for 48 h. The cell numbers indicated on the y axis were determined by MTS assays and normalized to control as described in Materials and Methods. **B**, cell death assay for measuring apoptosis induced by siRNAp65, gemcitabine, and combination. Cells were plated at an equal density and the sub-G₁ fraction was assessed by fluorescence-activated cell sorting analysis after propidium iodide staining in BxPC-3 and Mpanc-96 cells. Data are means \pm SE for $n = 3$ -5 experiments.



analyze the effects of gemcitabine on pancreatic cancer cell NF κ B activity *in vivo*, in which we also did not detect any effects. This is in contrast to a previous report that indicated that gemcitabine treatment stimulated NF κ B in pancreatic cancer cells *in vitro* as measured using EMSA (10). One important difference between the current study and the previous study is the use of the stable reporter, which measures transcriptional activity, rather than EMSA, which measures DNA binding. Another difference between the studies was the dosage of gemcitabine used. For the *in vitro* experiments Arlt et al. (10) used a concentration of 20 μ mol/L gemcitabine whereas our highest concentration was 2 μ mol/L. Perhaps the lower concentration is less effective. In the *in vivo* study, however, we used a maximal dose of gemcitabine at 200 mg/kg, which is higher than the level used in patients clinically. Therefore, it seems unlikely that the concentration of gemcitabine explains the lack of effects on NF κ B in the current study.

To evaluate the effects of inhibiting NF κ B, we used siRNA silencing of the p65/relA subunit. This subunit is responsible for the activation of gene expression on NF κ B activation by most known pathways and was identified as the important subunit in pancreatic cancer (7). However, the noncanonical pathway does not use p65/relA but rather relB, which would not be inhibited by p65/relA silencing. Silencing p65/relA would also not be expected to inhibit rel C-mediated gene expression. Thus, the pattern of inhibition expected after p65/

relA silencing would be similar, but not identical, to that of overexpressing an I κ B inhibitory subunit. It is important to be aware that either silencing or overexpression of NF κ B subunits may have effects on a variety of other molecules and signaling pathways. However, these approaches are still more specific than the use of pharmacologic agents and natural products. Several previous studies have used drugs such as genestein (25, 31), sulfasalazine (10, 26), or curcumin (27, 30) as NF κ B inhibitors. Each of these drugs is known to have many other targets besides NF κ B, such that interpretation of the actions of these drugs is quite difficult. We observed that the siRNA against the NF κ B p65/relA subunit very effectively reduced cellular levels of this subunit and blocked NF κ B transcriptional activity in all of the pancreatic cancer cells. Therefore, differences in the effectiveness of siRNA-mediated silencing do not explain the observation that p65/relA silencing inhibited gemcitabine-sensitive but not resistant cells.

Recently there has been a growing interest in the use of *in vivo* delivery of siRNAs as a potential novel approach to cancer treatment. Therefore, we tested whether *in vivo* delivery of the p65/relA siRNA using neutral liposomes in a manner previously described (23) would affect orthotopic pancreatic tumors. We found that delivery of the siRNA by i.p. injection reduced the basal level of NF κ B reporter activity. Furthermore, siRNA treatment had a dramatic inhibitory effect such that it completely eliminated tumors formed from the gemcitabine-sensitive

BxPC-3 cells. It was not possible to see an additional effect of gemcitabine in those mice, because the siRNA treatment was so highly effective. In contrast, the same siRNA treatment had no discernable effect on tumor development when using gemcitabine-resistant MPanc-96 cells. These observations strongly suggest that therapies directed toward NFκB may not be universally successful but could be advantageous in certain patients.

Our data indicate that the benefits of inhibiting NFκB are limited to cell lines that are natively gemcitabine-sensitive. Most previous studies have focused on one or two cell lines and have not determined the native gemcitabine resistance of the cells. One study that was somewhat similar to this one also characterized the cells being used for gemcitabine sensitivity, but in that study the BxPC-3 cells were considered gemcitabine-resistant. In contrast, we found BxPC-3 cells to be among the most gemcitabine-sensitive. The most likely explanation for this difference is that their analysis of gemcitabine effectiveness was conducted within 24 hours of treatment. Gemcitabine functions as a toxin by influencing DNA synthesis during S-phase. We did not observe large effects within 24 hours of gemcitabine (data not shown); therefore, in the current study the effects of gemcitabine were examined after 72 hours to allow time for cell killing.

The current data suggest that the resistant pancreatic cancer cells do not rely on NFκB as a major survival pathway under either control or gemcitabine-treated conditions. In contrast, sensitive pancreatic cancer cells rely on NFκB for survival under basal conditions and during treatment with gemcitabine. Thus, the sensitivity of pancreatic cancer cells to gemcitabine and to p65/relA silencing was correlated. The mechanisms responsible for gemcitabine resistance are not completely understood, but are largely thought to involve molecules involved in drug metabolism (33). Other molecules have also been suggested to be important in gemcitabine resistance, such as p8 (34), HSP27 (35), S100A4 (36), and Bcl-2 (37, 38). More recently it has been noted that resistant cells have more of a mesenchymal phenotype, suggesting epithelial-to-mesenchymal transition as an important resistance mechanism (39). Our data support the concept that NFκB-independent mechanisms determine chemoresistance in pancreatic cancer. Further investigations will be necessary to delineate these important mechanisms.

In summary, inhibition of NFκB seems to be a useful treatment in pancreatic cancer cell lines that are natively sensitive to gemcitabine. In such cells, silencing of p65/relA itself reduced cell numbers and improved the effectiveness of gemcitabine. In highly resistant cell lines, however, silencing of p65/relA had no effect alone or in combination with

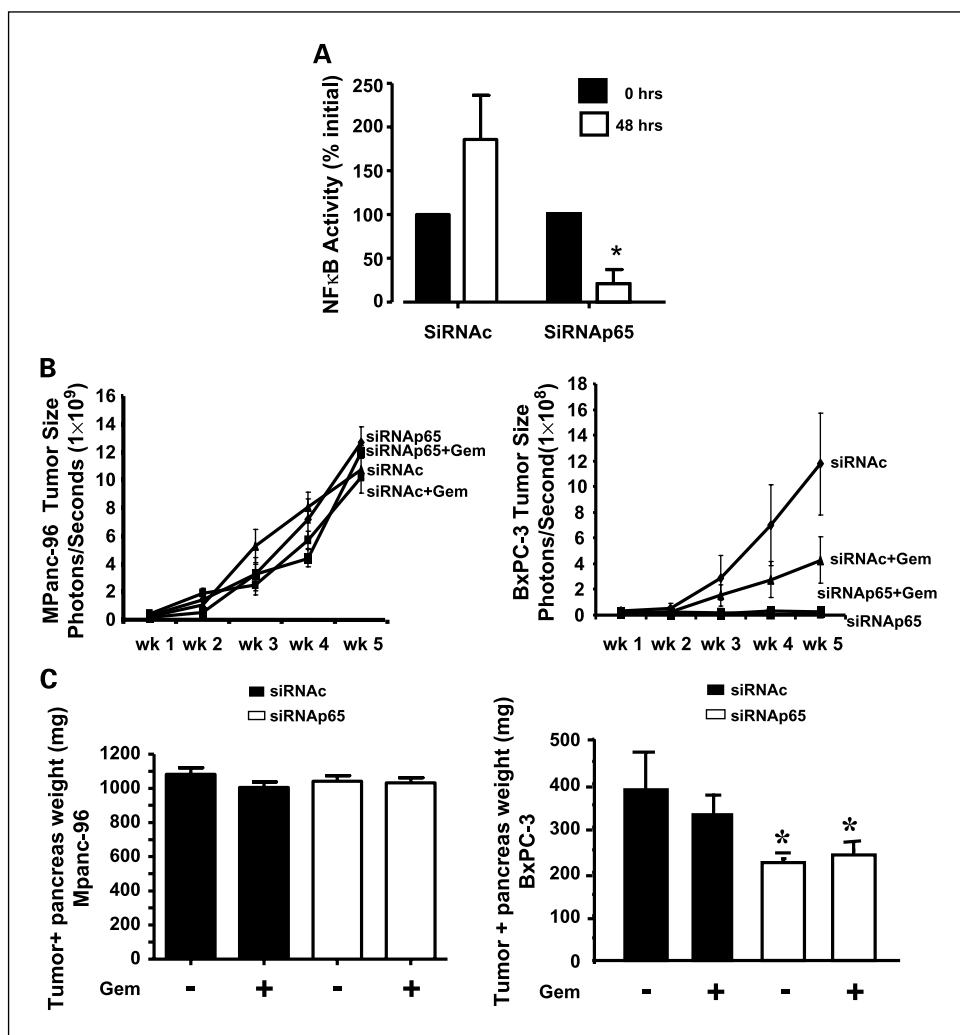


Fig. 6. Liposomal delivery of siRNA against p65/relA effectively inhibits NFκB activity and it also effectively inhibits growth of gemcitabine-sensitive pancreatic cancer cells but not gemcitabine-resistant pancreatic cancer cells *in vivo*. **A**, tumors were formed in nude mice with MPanc-96 cells stably expressing the NFκB reporter. The mice were treated with or without neutral liposomes bearing siRNAp65 and the effects on bioluminescence imaging indicated significant reduction. **B**, tumors were formed in nude mice with MPanc-96/BxPC-3 cells stably expressing a constitutively regulated luciferase for analysis of tumor formation. Bioluminescent imaging was done weekly to assess tumor growth. **C**, after 5 wk the weights of the pancreata including the primary tumors were quantified. Columns, mean for five animals; bars, SE. *, $P < 0.05$, versus control.

gemcitabine. Because clinical studies indicate that only about 12% of patients with advanced pancreatic adenocarcinoma have either a partial or complete response to gemcitabine treatment (33), it seems apparent that targeting NF- κ B will have limited utility in battling this disease. Nevertheless, our studies suggest that in a subset of patients in which gemcitabine has effectiveness, the combination of inhibition of NF- κ B could be very useful. Unfortunately, it is not possible at the current time

to predict which patients will respond to gemcitabine. Clearly future efforts should be directed to understanding the mechanisms of gemcitabine resistance and at developing biomarkers that can predict gemcitabine responsiveness.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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