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PEGylated Curcumin Conjugate Inhibits Pancreatic Cancer Cell Growth through Inactivation of Jab1

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Running Title: PEGylated curcumin inhibits pancreatic cancer cell growth

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ABBREVIATIONS: Jab1, Jun activation domain protein binding 1; CSN, COP9 signalosome complex; DPC4, deleted in pancreatic carcinoma locus 4; PEG, polyethylene glycol; BrdU, 5-Brini-2'-deoxyuridine; MTT, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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Abstract

Jab1 (Jun activation domain binding protein 1), integrated into COP9 signalosome complex (CSN), induces protein instability of many tumor suppressors and cell cycle regulators, and therefore is a novel target in cancer therapy. Curcumin, an inhibitor of Jab1/CSN-associated kinase(s), has been reported to suppress tumor growth; however, curcumin is highly hydrophobic and this feature prevents its usage as an anti-tumor drug. To increase the solubility and targeted delivery, we generated a water-soluble polyethylene glycol (PEG)-conjugated curcumin system, in which curcumin is covalently linked to PEG_{35kD}. PEGylated curcumin showed much greater reduction of cell growth than free curcumin in pancreatic cancer cells. Cells treated with PEGylated curcumin had increased arrest at the mitotic phase with the formation of abnormal multinucleated cells, indicating that this compound affects cell cycle progression which may contribute to cell growth inhibition. The stabilities of Jab1 target proteins were also examined. PEGylated curcumin increased protein stability of these proteins in pancreatic cancer cells and directly inhibited the activity of Jab1/CSN-associated kinases. Moreover, the inhibitory effect of PEGylated curcumin on cell proliferation was blunted in pancreatic cancer cells with Jab1 knockdown. The results suggest that PEGylated curcumin inhibits cell proliferation through suppression of Jab1/CSN activity. More importantly, the new compound sensitized pancreatic cancer cells to gemcitabine-induced apoptosis and cell proliferation inhibitory effects. Collectively, the PEGylated curcumin conjugate has much more potent effects than free curcumin on pancreatic cancer cell growth inhibition. The current study provides a biologic rationale to treat pancreatic adenocarcinoma patients with the nontoxic phytochemical conjugated to PEG for systemic delivery.

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Introduction

Pancreatic ductal adenocarcinoma represents greater than 80% of all pancreatic neoplasms with a death:incidence ratio of approximately 0.99 (Farrow et al., 2004; Brand et al., 2005). Although gemcitabine currently is the most commonly used drug for treatment of pancreatic cancer (Burris et al., 1997; Hui et al., 1997), only marginal improvements on survival and tumor response to this drug have been reported. One of the reasons for the low survival rate is the poor responses of pancreatic cancer cells to chemotherapy or radiotherapy. Therefore, the mechanisms responsible for the loss of growth regulation in pancreatic carcinoma cells should be defined, and new treatments based on a better understanding of the biology of pancreatic cancer must be established.

Recently, steps in the genetic basis of pancreatic ductal adenocarcinoma have been identified, with the identification of the activation of the oncogene Kras and inactivation of the tumor suppressor genes p16INK4a, p53 and DPC4 (Deleted in Pancreatic Carcinoma locus 4) as characteristic features of invasive pancreatic cancer (Hruban et al., 2001). The protein instability of DPC4, which is also known as Smad4, is a common phenomenon in pancreatic carcinoma cells (Wan et al., 2005) and is strongly correlated with pancreatic tumorigenesis and patient survival (Biankin et al., 2002; Hua et al., 2003). Our previous studies demonstrated that Jab1 (Jun activation domain binding protein 1) plays a key role in inducing protein degradation of DPC4/Smad4 (Wan et al., 2002). Jab1 is also known as CSN5 as it is the fifth component of the COP9 signalosome complex (CSN) (Seeger et al., 1999; Naumann et al., 1999). Jab1-integrated CSN also induces degradation of the tumor suppressor, p53 (Bech-Otschir et al., 2001), and the cell cycle inhibitor, $p27^{kip1}$ (Tomoda et al., 1999) and $p21^{cip1}$ (Peng et al., 2003), all of which are actively involved in suppressing the development of pancreatic cancer. Therefore, Jab1 in the CSN acts as a negative regulator of important pancreatic tumor suppressors by targeting them for degradation. Recent work

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suggests that the Jab1/CSN possesses kinase activity that phosphorylates proteins such as c-Jun and p53 with consequence for their protein stabilization (Naumann et al., 1999) or ubiquitin (Ub)-dependent degradation (Bech-Otschir et al., 2001; Uhle et al., 2003; Sun et al., 2002). Curcumin and emodin, two natural plant-derived compounds, have been identified as Jab1/CSN inhibitors as they potently inhibit the kinase activity of Jab1/CSN and have been shown to enhance the stability of the p53 protein (Bech-Otschir et al., 2001; Uhle et al., 2003; Sun et al., 2002). These results indicate that blocking of Jab1/CSN kinase activity causes stabilization of the tumor suppressors and cell cycle regulators and leads to the suppression of pancreatic tumor growth. Importantly, the level of Jab1 expression is significantly elevated in several human malignant cancers, providing additional evidence for the role of Jab1 in tumorigenesis (Sui et al., 2001; Korbonits, et al., 2002; Fukumoto et al., 2004).

Curcumin (difeuloymethane), a derivative of the spice turmeric (*curcuma longa*), is nontoxic to humans (Maheshwari et al., 2006) and has been widely studied for its anti-inflammatory, anti-angiogenic, anti-oxidant, and anti-cancer effects and its promotion of wound healing. This compound has antiproliferative and proapoptotic effects in many cancer cell lines and has tumor suppression effects in several tumor animal models. Multiple molecular mechanisms have emerged to elucidate its diverse biological effects. Curcumin has been shown to suppress NF-κB activation (Kunnumakkara et al., 2007), activate caspases and inhibit the expression of Bcl-2 and Bcl-xL and cellular inhibitor of apoptosis protein-1 (Sharma et al., 2005). Curcumin also affects cell proliferation, angiogenesis and metastasis by regulating the expression of a variety of related downstream genes (Sharma et al., 2005). The inhibitory effect of curcumin on Jab1/CSN kinase activity leads to stimulation of tumor suppressors, which may be an important pathway for its anti-tumor effects. Curcumin is highly hydrophobic and cannot be administered systemically. In addition, the bioavailability of oral curcumin is poor. Therefore, better strategies for the systemic delivery of this compound have to be developed. An approach that may solve the above problems of the usage of

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curcumin as an anti-tumor drug is the use of its conjugate with polyethylene glycol (PEG) (Greenwald et al., 2003; Greenwald et al., 1996). PEG is a water soluble amphiphilic polymer showing excellent biocompatibility and is frequently used in biomedical applications. A low molecular-weight drug conjugated to higher molecular weight PEG (M_w>20,000) results in high aqueous solubility, slower clearance, reduced systemic toxicity, and efficient accumulation in tumors through enhanced permeability and retention. Several small organic molecules such as camptothecin, doxorubicin and paclitaxel, have been conjugated to PEGs and these conjugates especially camptothecin are in clinical trials (Greenwald et al., 2003).

In the present study, we demonstrated that Jab1 overexpression increased pancreatic cancer cell growth and protein degradation of Jab1 target proteins, whereas Jab1 gene silencing by siRNA suppressed pancreatic cancer cell proliferation. PEGylated curcumin inhibited cell proliferation in pancreatic cancer cells and exhibited much greater effect on reduction of proliferation than free curcumin. This compound inhibited the activity of Jab1-associated kinases and changed protein stability of Jab1 target proteins, and the cell growth inhibitory effect of this compound was blunted in Jab1 knockdown cells. The results suggest that PEGylated curcumin suppresses pancreatic cancer growth at least partially through inactivation of Jab1. In addition, PEGylated curcumin sensitized pancreatic cancer cells to gemcitabine-induced apoptotic and cell growth inhibitory effects, therefore has the potential to be developed as an anti-pancreatic cancer drug.

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Materials and Methods

Cell Culture, Antibodies and Reagents. PANC-1, AsPC-1, MiaPaCa-2 and BxPC-3 human pancreatic cancer cells were obtained from the American Type Tissue Collection (ATCC, Rockville, MD). Cells were cultured in either DMEM or RPMI 1640 media with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100μg/ml streptomycin. All cells were maintained in 100-mm tissue culture dishes in a 37°C incubator equilibrated with 5% CO_2 in humidified air. Monoclonal antibodies recognizing human Smad4 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 5-Bromo-2′-deoxyuridine (BrdU) and monoclonal anti-p53 (Clone BP53) were from Calbiochem (San Diego, CA). Monoclonal anti-p27 (Clone 1B4) was from Vector Labs (Burlingame, CA). Monoclonal anti-BrdU (Clone BUZ0a) was from DakoCytomation (Carpinteria, CA). Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO).

Virus Infection. Generation and titration of retroviral supernatants were performed as described (Wan et al., 2005). PANC-1 was infected with retrovirus vector containing pMSCVneo-GFP, pMSCVneo/HA-Jab1, pMSCVneo/U6-GFP (siGFP) or pMSCVneo/U6-Jab1 (siJab1) as described above. For infection, the virus-containing supernatant in the presence of 4 μg/ml of polybrene (Sigma) was added to the culture medium. Six days post infection, the efficiency was assayed by immunoblotting.

Western Blot Analysis. Cell lysates in RIPA buffer (10 mM PBS, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 5mM ethylenediaminetetraacetic acid) were prepared and all the samples were measured for total protein content using a BCA assay (Pierce, Rockford, IL). Equal amounts of protein samples were loaded onto 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Bio-Rad). The blots were blocked in 5% skim milk in TBST buffer (10 mM Tris-HCl,

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150 mM NaCl, and 0.5% Tween-20) for 2 hours and then incubated overnight at 4°C with primary antibody. The blots were washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After washing, bands were detected on light sensitive film using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Clonogenic Assays. PANC-1 cells were seeded at 1.0×10^5 cells/well of a six-well plate and infected the next day with either GFP (vector control) or HA-Jab1. In separate experiments, cells were infected with either siGFP (siRNA control) or siJab1. Twenty four hours after infection, the cells were washed in PBS and trypsinized. Cells were seeded in 6cm plates at 1000 cells/well for the cells with GFP or Jab1 overexpression and 1500 cells/well for the cells with siGFP or siJab1, and the colonies were grown for 10 days. For compound treatment, cells were seeded in 6cm plates at 5000 cells/well and incubated with different concentration of PEGylated curcumin for 7 days. The colonies were stained with 2% crystal violet and counted. Only colonies containing >50 cells were counted. The survival fractions of HA-Jab1- or siJab1-transfected cells were normalized to GFP- or siGFP-transfected cells. For compound treatment, the survival fractions of PEGylated curcumin-treated cells were normalized to vehicle-treated control cells.

MTT Assays. Cellular proliferation was assayed by the MTT [3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO] method. Briefly, after transfection, 50 µl of MTT solution (5 mg/ml) was added to the culture medium. After 4 hours at 37°C the medium was removed and 50 µl of acidified isopropanol was added to each well. The color was allowed to develop for 5 minutes and optical density at 570 nm was determined with a microplate reader (Bio-Rad, Richmond, CA). The mean value and standard error for each treatment were determined, then converted to percent relative to control. The concentration at which cell growth was inhibited by 50% (IC_{50}) was determined by linear

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interpolation using the formula [(50%-low percentage) / (high percentage - low percentage)] × (high concentration - low concentration)+ low concentration.

Apoptosis Assays. Apoptotic cells were detected by dual staining with Annexin V-phycoerythrin (PE) and 7-amino-actinomycin (7-AAD) using a commercially available kit (PharMingen, San Diego, CA) following the manufacturer's protocol. Briefly, cells were washed in PBS and resuspended in binding buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$), and the pellet was resuspended in 5 µL Annexin V-PE, 5 µL 7-AAD and 100 µl binding buffer, followed by incubation for 15 min at room temperature in the dark. Measurement was performed using a Becton–Dickinson FACS II scanner (BD Biosciences, San Jose, CA). A predetermined count of 10,000 cells was set on forward light scatter. Annexin V-PE was measured in FL-2 and 7-AAD in FL-3. Only cells with an intact cell membrane, *i.e.* 7-AAD (–) and Annexin V-PE (+), were considered as apoptotic.

Immunocytochemistry Analysis. Cells were grown on glass coverslips in 6-well plates to 50% confluence, rinsed in PBS and fixed in neutral-buffered formalin (Fisher Scientific, Norcross, GA) overnight at 4°C. The cells were permeabilized with acetone for 15 seconds. Endogenous peroxidases were quenched using an aqueous solution of 3% H_2O_2. Goat serum (3%) was added to block nonspecific immunostaining. The coverslips were incubated with the appropriate primary monoclonal antibody, with p27 antibody at 1:10 and BrdU antibody at 1:60. The antibody-antigen complex was visualized using a 3, 3′-diaminobenzidine substrate kit (Biogenex, City, State). The coverslips were then counterstained lightly with hematoxylin for the evaluation of p27 and BrdU expression. The positively stained nuclei and negative nuclei on photographs from four random fields of view for each coverslip were counted. The percentage of stained nuclei was estimated by dividing positive nuclei by total nuclei.

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Design and Synthesis of PEG-curcumin Conjugate (Please provide detailed procedures). PEG_{35kD} diacid: The diacid was prepared by using a modification of a previously reported procedure (Greenwald et al., 1996). Specifically, PEG_{35kD} (3.5 g, 0.1 mmol) was azeotroped with freshly distillated toluene (2 x10 mL) and then dissolved in 20 mL of anhydrous toluene. To this solution, potassium *tert*-butoxide (1M in tert-butanol, 0.3 mL) was added. The mixture was stirred for 1h at room temperature followed by the addition of ethyl bromoacetate (122 μL, 1.1 mmol). The solution was heated to reflux for 1h and then cooled to room temperature. Toluene was removed under vacuum. The white precipitate was dissolved in water and extracted with methylene chloride (20 mL x 3). The organic layers were combined, washed with brine (30 mL) and concentrated to afford the PEG_{35kD} diethyl ester (3.5 g, 100%). Characteristic ^{1}H NMR peaks were evaluated: 4.21 (q, J = 7.1 Hz, 4 H), 4.15 (s, 4 H), and 1.29 (t, J = 7.1 Hz, 4 H). The purity of the PEG-Conjugate was assessed by proton NMR and known reactions. >90% of the carboxyl groups from the PEG-diacid were linked to curcumin, and the other of the COOH group reacted with the coupling reagent DCC (N,N'-dicyclohexylcarbodiimide) to form a urea type moiety. The PEGylated curcumine has a characteristic peak at 4.5 ppm (Ha labeled on NMR) while the correponding proton at the end bonded to DCC has a chemical shift at 4.2 ppm). The ratio was estimated on the basis of integration.

The PEG_{35kD} diester (3.5 g, 0.1 mmol) was dissolved in 15 mL of sodium hydroxide (1 N) and stirred at room temperature for 4 h. The solution was adjusted to pH 3-4 with HCl (2M) and extracted with methylene chloride (20 mL x 3). The combined organic solutions were washed with water, brine, and concentrated to 5 mL of residue, to which diethyl ether was added to crystallize the PEG_{35kD} diacid (3.1 g, 88%). Characteristic ^{1}H NMR peak: 4.16 (s, 4 H).

Curcumin-PEG_{35kD} Conjugate: The PEG_{35kD} diacid (3.2 g, 0.09 mmol) and curcumin (135 mg, 0.36 mmol) in a flame dried reaction flask were azeotroped with freshly distillated anhydrous toluene (10 mL x

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2). DMAP (1 mg) was then added, followed by 20 mL of freshly distillated methylene chloride. To the resulting yellow suspension, DCC (76 mg, 0.36 mmol) was added at room temperature and mixture was stirred for 2 h. The reaction mixture was concentrated to 5 mL of residue and recrystallized with methylene chloride/ethyl ether (three times) to provide the curcumin-PEG_{35kD} conjugate (2.9 g, >90%). Characteristic ^1H NMR peaks were evaluated: 7.62 (d, J = 15.6 Hz, 2 H), 7.61 (d, J = 15.6 Hz, 2 H), 6.93 (d, J = 8.2 Hz, 2 H), 6.56 (d, J = 15.8 Hz, 2 H), 6.50 (d, J = 15.8 Hz, 2 H), 5.84 (s, 2 H), 4.46 (s, 4 H), 3.96 (s, 6 H), and 3.88 (s, 6 H).

Note: For ^1H NMR, chemical shifts are expressed in parts per million (δ scale) downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent ($CHCl_3$: δ 7.26). Data were presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and /or multiple resonances), coupling constant in Hertz (Hz), integration.

Statistical Analysis. Results are presented as the mean ± SD. Data were analyzed by Student's *t*-test, and statistical significance was accepted at a P-value of less than 0.05. Each experiment was repeated independently at least three times.

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Results

Jab1 Is Both Sufficient and Necessary for Pancreatic Cancer Cell Growth. To determine the function of Jab1 in pancreatic cancer cells, we overexpressed Jab1 by infection of PANC-1 cells with a retrovirus containing pMSCVneo-HA-Jab1 and used pMSCVneo-GFP (control). Two stable cell lines (PANC-1-Jab1 and PANC-1-GFP) have been generated by infecting PANC-1 cells with these two viruses individually. The infection efficiency was determined to be approximately 90% (Fig. 1A). We examined whether the activity of Jab1 is enhanced in Jab1-overexpressed cells. Studies from others and our lab demonstrated that Jab1/CSN plays an important role in inducing the protein degradation of p27 (Tomoda et al., 1999) and Smad4 (Wan et al., 2002), but enhancing protein stabilization of c-Jun (Naumann et al., 1999). We then assessed the levels of Smad4, p27 and c-Jun in the cells with Jab1 overexpression. Overexpression of Jab1 resulted in a significant reduction in the levels of Smad4 and p27, whereas caused elevation of c-Jun level (Fig. 1B and 1C). As PANC-1 cells contain mutant allele of p53, the level of p53 was not affected by Jab1 overexpression (data not shown). We then assessed whether overexpression of Jab1 would affect cancer cell growth by clonogenic assay. Jab1 overexpression in PANC-1 cells resulted in a significant elevation of colony formation when compared with control (Fig. 1D and 1E).

We also developed retroviral siRNA delivery vector pMSCVneo/U6-GFP (siGFP, irrelevant siRNA control) and pMSCVneo/U6-Jab1 (siJab1) to determine the effects of a reduction in the levels of Jab1 in PANC-1 cells. siJab1 successfully reduced Jab1 expression, elevated the levels of Smad4 and p27, and decreased the level of c-Jun in PANC-1 cells (Fig. 1F and 1G), indicating that the activity of Jab1 was inhibited in Jab1 knockdown cells. Importantly, cell growth as represented by colony formation was suppressed (Fig. 1H and 1I). Taken together, these results support the concept that Jab1 activity in human pancreatic cancer cells is associated directly with cell growth.

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PEGylated Curcumin Inhibits Cell Growth in Pancreatic Cancer Cells. Curcumin was previously reported to inhibit cell proliferation in pancreatic cancer cells (Hidaka et al., 2002; Li et al., 2004; Li et al., 2005; Lev-Ari et al., 2005; Wang et al., 2006). This nature compound is a potent inhibitor of Jab1/CSN-associated kinase(s) (Bech-Otschir et al., 2001; Uhle et al., 2003; Sun et al., 2002). To increase its solubility, we generated a water-soluble PEG-conjugated curcumin system, in which curcumin (Fig. 2A) is covalently linked to PEG_{35kD} (Fig. 2B). Fig. 2C shows ^{1}H NMR spectrum of PEGylated curcumin. The solubility of PEGylated curcumin in water is much higher than that of curcumin as PEGylated curcumin is easily dissolved in PBS at 1mM, whereas curcumin can not be dissolved in PBS at much lower concentration. To determine whether PEGylated curcumin affects cell proliferation, we performed colony formation assays in PANC-1 cells. PEGylated curcumin inhibited cell proliferation in a dose-dependent manner (Figs. 2D). The inhibitory effects of PEGylated curcumin were not very great at concentrations of 1 μM, but there was a profound effect at concentrations of 5 μM and higher. Similar results were obtained using MTT assays in which PEGylated curcumin inhibited cell proliferation in a dose- and time-dependent manner (Figs. 2E and 2F). The growth inhibitory effects of the compound required a minimum of 24 hours treatment (Fig. 2F). The effect of free curcumin on cellular proliferation was also examined. 20 μM curcumin exhibited similar inhibitory effect as 5 μM PEGylated curcumin did on cell proliferation (Fig. 2G). Thus, PEGylated curcumin has greater inhibitory effects than that of free curcumin on cell proliferation.

PEGylated Curcumin Has Greater Effects than Free Curcumin on Inhibition of Cell Growth in pancreatic cancer cells. We then compared the effects of PEGylated curcumin with free curcumin on cellular proliferation in four human pancreatic cancer cell lines PANC-1, MiaPaCa-2, BxPC-3 and AsPC-1 treated with increasing doses of both compounds. MTT assays showed that dose dependent effect of free curmcumin on cell proliferation was detected and curcumin started to exhibit its inhibitory effects on cell

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proliferation at 10 μM. The antiproliferative effects of PEGylated curcumin were much stronger than free curcumin at equimolar concentrations in PANC-1 (approximately 2.64 fold at concentration of 10 μM, Fig. 3A), MiaPaCa-2 (approximately 2.28 fold at concentration of 10 μM, Fig. 3A) and AsPC-1 (approximately 2.38 fold at concentration of 10 μM, Fig. 3B). The effect of PEGylated curcumin on cell proliferation in BxPC-3 cells was greater than that of free curcurmin at lower concentration (5 μM), but was similar to that of free curcumin at higher concentrations (Fig. 3B). PEG alone as a control did not affect cell growth in these four cell lines tested (data not shown). These results suggest that the activity of PEGylated curcumin conjugates *in vitro* was much greater than that of free curcumin at equimolar concentrations.

To confirm the inhibitory effects of PEGylated curcumin on cell proliferation at the cellular level, we examined the changes in cellular proliferation by BrdU labeling and p27 expression by immunohistochemical analysis in pancreatic cancer cells treated with 5μM PEGylated curcumin, or PEG control. PEGylated curcumin elevated p27 protein levels in both PANC-1 cells (Figs. 4Aii and 4B) and MiaPaCa-2 cells (Figs. 4Fii and 4G) in comparison with controls (Figs. 4Ai and 4Fi). Consistently, BrdU staining was dramatically reduced after treatment of PEGylated curcumin in both cell lines (Fig. 4Aiv and 4C in PANC-1, and Fig. 4Fiv and 4H in MiaPaCa-2) in comparison with controls (Fig. 4Aiii and 4Fiii). Interestingly, most of the cells treated with PEGylated curcumin exhibited mitotic features in a prometaphase or metaphase-like state with condensed chromatin (Figs. 4Av, 4Avi and 4D in PANC-1 cells. and 4Fvi and 4I in MiaPaCa-2 cells). Another unique feature was that numerous large, multinucleated cells per field were visible in PEGylated curcumin treated cells (Figs. 4A*v*, 4E, 4Fiv and 4J). The aberrant mitosis and multinucleated cells were not seen in equimolor concentration of free curcumin-treated cells (data not shown). These results suggest that the inhibitory effects of PEGylated curcumin on cell proliferation may be through arresting cells at mitosis.

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PEGylated Curcumin Inhibits Cell Growth via Suppression of Jab1 Activity in Pancreatic Cancer Cells. We then investigated whether this PEGylated curcumin compound affects Jab1 activity. We first examined whether this compound affects the protein levels of Smad4, p27 and c-Jun, which are Jab1 target proteins, in pancreatic cancer cells. As shown in Figs. 5A-5D, treatment with 5 μM PEGylated curcumin for 24 hours resulted in a significant elevation of the protein levels of Smad4 and p27, and reduction of c-Jun in both PANC-1 and AsPC-1 cells. As a control, the level of Smad2, which is not a Jab1 target protein, was not affected by the treatment of this compound, indicating that the effect of PEGylated curcumin was Jab1-specific. To assess whether PEGylated curcumin directly inhibits Jab1/CSN-associated kinase activity, we performed an *in vitro* kinase assays in which CK2 and PKD, previously identified Jab1/CSN-associated kinases, were incubated with the protein target c-Jun. As we expected, PEGylated curcumin dramatically inhibited CK2- and PKD-induced phosphorylation of c-Jun (Fig. 5E). To further examine whether Jab1 is required in PEGylated curcumin-induced cell growth inhibition, we treated the cells with 20 μM free curcumin and 5 μM PEGylated curcumin in PANC-1 cells with Jab1 knockdown by siRNA introduction. The inhibitory effect of free curcumin and PEGylated curcumin on cell growth was blunted in Jab1 knockdown cells (Fig. 5F). The results suggest that the inhibitory effect of PEGylated curcumin on cell growth is, at least partially, through suppression of Jab1 activity.

Synergistic Effects of PEGylated Curcumin with Gemcitabine on Cell Growth Inhibition and Apoptosis in PANC-1 and AsPC-1 Cells. Gemcitabine (2′, 2′-difluorodeoxycytidine, Gemzar®), is the FDA-approved chemotherapeutic agent for the treatment of pancreatic adenocarcinoma (Burris et al., 1997; Hui et al., 1997). However, gemcitabine resistance occurs in most patients and limits its effectiveness. We examined whether PEGylated curcumin can act to sensitize pancreatic tumor cells to the effects of gemcitabine by analysis of growth inhibition and apoptosis. As shown in Fig. 6, gemcitabine

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inhibited cell proliferation of PANC-1 and AsPC-1 cells dose-dependently at concentrations lower than 100 μM by MTT assay, with the AsPC-1 cells being less sensitive (Fig. 6A and 6B). But when 1 μM PEGylated curcumin was added, cell growth inhibition was significantly induced in both cell types (Fig. 6A). 1 μM Curcumin, however, did not exert the same effect (Fig. 6B). Treatment of the cells with a combination of 10 μM gemcitabine and 1 μM PEGylated curcumin for 24 hours resulted in a markedly enhanced inhibition of proliferation (Figs. 6C and 6D) and increased apoptosis with Annexin V staining (Figs. 6E and 6F), suggesting that PEGylated curcumin sensitizes pancreatic cells to respond to gemcitabine.

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Discussion

The present study demonstrates that water soluble PEGylated curcumin conjugate that we generated increases the effects of curcumin on inhibition of cellular growth in pancreatic cancer cells. More importantly, the new compound sensitized pancreatic cancer cells to gemcitabine-induced cell apoptosis and growth inhibitory effects. These results provide evidence supporting the potential entry of this PEGylated curcumin into preclinical animal studies and clinical trials for treatment of patients with pancreatic cancer. Curcumin has been widely used as an anti-inflammatory and anti-cancer drug. This compound is nontoxic and virtually devoid of side effects in animals as well as in humans (Hsu et al., 2007), and therefore may be a potential therapeutic agent for cancer. However, even though free curcumin has antitumor effects against a variety of cancer cells including pancreatic cancer cells (Kunnumakkara et al., 2007; Hidaka et al., 2002; Li et al., 2004; Li et al., 2005; Lev-Ari et al., 2005; Wang et al., 2006), this agent is highly hydrophobic and cannot be administered systemically. In addition, the bioavailability and circulation half-life of oral curcumin are poor. Thus, the clinical application of curcumin has been limited. To overcome these problems, we developed a strategy that conjugates curcumin to a water soluble high molecular weight PEG_{35kD} and examined the effects of this conjugate on pancreatic cancer cell proliferation and apoptosis. PEG-curcumin inhibited cell proliferation and apoptosis in all four human pancreatic cancer cell lines, and its effects were much more potent than those of free curcumin. The reasons may be that the covalent conjugation of curcumin with PEG (PEGylation) increases its water solubility and its molecular size and steric hindrance may improve its cellular permeability and proteolytic stability, and therefore enhances its half-life in cells.

The current work demonstrated that PEGylated curcumin-induced cell growth inhibition is caused by, at least partially, inhibition of Jab1/CSN-associated kinase activity, providing a molecular mechanism for the

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anti-pancreatic cancer effect of this compound. Jab1, integrated into CSN complex, controls protein stability of several key mediators controlling cell proliferation, cell cycle progression and apoptosis such as the cyclin-dependent kinase inhibitors $p27^{kip1}$ (Tomoda et al., 1999), $p21^{cip1}$ (Peng et al., 2003), p53 (Bech-Otschir et al., 2001), Smad4 (Wan et al., 2002) and Smad7 (Kim et al., 2004). We also found that Jab1 is a critical factor governing pancreatic cancer cell proliferation by regulating the stability of these key regulatory proteins, consistent with previous results by others (Fukumot et al., 2004; Fukumoto et al., 2006; Kouvaraki et al., 2006). Importantly, PEGylated curcumin-treated cells exhibited similar effects as Jab1 knockdown on elevating protein levels of Jab1 target proteins as well as inhibiting pancreatic cancer cell proliferation. This effect was blunted in Jab1 knockdown cells. Thus, the effect of PEGylated curcumin is Jab1-dependent. We also found that most of the cells were arrested in mitosis and abnormally large multinucleated cells were observed after PEGylated curcumin treatment. Previous study by Fukumoto (Fukumoto et al., 2006) demonstrated that Jab1 knockdown also caused S/G2/M phase arrest, providing an indirect evidence that this effect of PEGylated curcumin may also due to Jab1 inactivation. Further identification of the mechanism of the effect of this conjugate on cellular mitosis is needed and is underway. Curcumin possesses a wide range of pharmacological properties including anti-inflammatory, anti-infectious, and anti-carcinogenic activities (Goel et al., 2008) and a number of different targets have been proposed to mediate these different effects which include transcription factors, enzymes, hormones, growth factors, apoptotic and mitochondrial signaling molecules, and their associated receptors, … (Goel et al., 2008). Therefore, PEGylated curcumin may also affect these signaling pathways in cancer cells. However, the fact that the effect of PEGylated curcumin on cell growth inhibition was antagonized by knockdown of Jab1 in cells indicates Jab1/CSN is one of the major targets of PEGylated curcumin in pancreatic cancer.

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Evaluation of the effects of PEGylated curcumin on tumor growth and survival in mouse pancreatic tumor models is, of course, the major subject for further studies. PEG has been used widely to increase the circulation half-life of rapidly eliminated drugs, to impart enhanced permeability and retention (EPR) (Maeda et al., 2001), and to increase passive targeting of anticancer drugs. Many studies have demonstrated that the residence time in the bloodstream of PEG-protein conjugates increases according to the molecular size (Caliceti et al., 2003). It is believed that size enlargement promotes the accumulation into tumor tissues by the passive EPR mechanism. In addition, optimal PEGylation of drugs could selectively improve their *in vivo* therapeutic potency and reduce side effects (Kamada et al., 2000; Tsutsumi et al., 2000). The *in vitro* results presented in the current study demonstrate that PEG-conjugated curcumin exhibited much higher ability to suppress cell proliferation and induce apoptosis of pancreatic carcinoma cells, which provides a basis for the further investigation of this drug in preclinical animal studies and clinical trials.

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Footnotes

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Figure Legends

Fig. 1. Jab1 overexpression increases, and Jab1 gene silencing by siRNA suppresses pancreatic cancer cell growth. A, GFP is efficiently overexpressed in PANC-1 cells. PANC-1 cells were infected with a retrovirus containing pMSCVneo-GFP. Green light (upper panel) representing GFP expression. Lower panel shows cell density. B and C, Jab1 overexpression decreases endogenous Smad4 and p27 expression and increases c-Jun level. PANC-1 cells were infected with virus containing GFP or HA-Jab1. Cells were harvested and expression levels of Jab1, Smad4, p27, c-Jun and β-actin were measured by Western blot analysis with antibodies against HA, Smad4, p27, c-Jun or β-actin (B). The intensity of the bands in (B) was quantified by phosphorimaging, and the ratio of protein level in Jab1 overexpressed cells to that in control cells was calculated (C). D and E, Jab1 overexpression increases colony numbers. PANC-1 cells were infected with virus containing GFP or HA-Jab1. Cells were reseeded in 6cm plates at 1000 cells/well and colony numbers were photographed (D) and counted (E). The survival fractions of HA-Jab1-transfected cells were normalized to GFP-transfected cells. The values represent means ± SD from three independent experiments. *: $p < 0.005$, compared with control. F and G, Suppression of Jab1 by retroviral delivery of siRNA increases endogenous Smad4 and p27 expression and decreases c-Jun level. PANC-1 cells were infected with virus containinig siGFP or siJab1. Cells were harvested and expression levels of Jab1, Smad4, p27, c-Jun and and β-actin were measured by Western blot analysis with antibodies against HA, Smad4, p27, c-Jun or β-actin (F). The intensity of the bands in (F) was quantified by phosphorimaging, and the ratio of protein level in cells with Jab1 knockdown to that in control cells was calculated (G). H and I, Jab1 knockdown reduces colony numbers. PANC-1 cells were infected with virus containing siGFP or siJab1. Cells were reseeded in 6cm plates at 1500 cells/well and colony numbers were photographed (H) and counted (I). The survival fractions of siJab1-transfected cells were normalized to

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siGFP-transfected cells. The values represent means ± SD from three independent experiments. *: p< 0.005, compared with control.

Fig. 2. PEGylated curcumin inhibits cell growth. A, General chemical structure of curcumin. B, Synthetic route of PEG-curcumin conjugation. C, ^{1}H NMR spectrum of a synthesized PEGylated curcumin. D, Dose-dependent inhibitory effect of PEGylated curcumin on colony formation in PANC-1 cells. Cells were seeded and incubated with different concentration of PEGylated curcumin or 20 μM of PEG (control) for 7 days. The survival fractions of PEGylated curcumin-transfected cells were normalized to PEG-treated cells. The values represent means ± SD from three independent experiments. *: p<0.001, compared with PEG-treated cells. E, Dose-dependent inhibitory effect of PEGylated curcumin on cell proliferation in PANC-1 cells. Cells were treated with different doses of PEGylated curcumin or 20 μM of PEG for 24 hours. MTT assays were performed. The cell survival rates were normalized to PEG-treated cells as percentage. The values represent means ± SD from three independent experiments. *: p<0.001, compared with PEG-treated cells. F, Time-dependent effects of PEGylated curcumin on cell proliferation. PANC-1 cells were treated with 5 μM PEGylated curcumin or 5 μM of PEG for different time periods. MTT assays were performed. The cell survival rates were normalized to PEG-treated cells as percentage. The values represent means ± SD from three independent experiments. *: p<0.001, compared with PEG-treated cells. G, Dose-dependent inhibitory effect of curcumin on cell proliferation in PANC-1 cells. Cells were treated with different doses of free curcumin or vehicle control (Con) for 24 hours. MTT assays were performed. The cell survival rates were normalized to vehicle control-treated cells as percentage. The values represent means ± SD from three independent experiments. *: p<0.05, **: p<0.001, compared with vehicle control-treated cells.

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Fig. 3. Comparison of the effects of curcumin and PEGylated curcumin on cell proliferation in different pancreatic cancer cell lines. A, PANC-1 or MiaPaCa-2 cells were treated with different doses of curcumin or PEG-curcumin for 24 hours. MTT assays were performed. The values represent means ± SD from three independent experiments. *: $p<0.01$, **: $p<0.001$, compared with vehicle control-treated cells. B, BxPC-3 or AsPC-1 cells were treated with different doses of curcumin or PEG-curcumin for 24 hours. MTT assays were performed. The values represent means ± SD from three independent experiments. *: $p<0.001$, compared with vehicle control-treated cells.

Fig. 4. Immunohistochemical analysis of the effects of PEGylated curcumin on cell proliferation. A-E, Responses of PANC-1 cells to PEGylated curcumin. Representative immunostaining image of PANC-1 cells to PEGylated curcumin (A). p27 was elevated in PEG-curcumin-treated PANC-1 cells (*ii*) compared with PEG control treated cells (*i*). BrdU incorporation was reduced in PEG-curcumin-treated PANC-1 cells (*iv*) compared with PEG control (*iii*). H&E staining of PEG-curcumin-treated PANC-1 cells (*v* and *vi*). The small square in *v* enlarged in *vi* indicate abnormal mitotic cells. Green arrows indicate abnormal multinuclear cells. p27-positive (B) and BrdU-positive (C) cells, and cells with condensed chromatin (D) and multinucli (E) were counted in three different fields in one slides. Three different slides for each treatment were counted. F-J, Responses of MiaPaCa-2 cells to PEG-curcumin. Representative immunostaining image of PANC-1 cells to PEGylated curcumin (F). p27 protein levels were elevated in PEG-curcumin-treated MiaPaCa-2 cells (*ii*) compared with PEG control (*i*). BrdU incorporation was reduced in PEG-curcumin-treated MiaPaCa-2 cells (*iv*) compared with PEG control (*iii*). Green arrows indicate positive BrdU-labeling in abnormal multinuclear cells. H&E staining of PEG control (*v*) and PEG-curcumin-treated PANC-1 cells (*vi*). Red arrows indicate abnormal mitotic cells. p27-positive (G) and BrdU-positive (H) cells, and cells with condensed chromatin (I) and multinucli (J) were counted in

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three different fields in one slides. Three different slides for each treatment were counted. *: $p<0.001$, compared with PEG-treated cells.

Fig. 5. PEGylated curcumin inhibits cell growth through inactivation of Jab1. A-D, PEGylated curcumin changes protein levels of Jab1 target proteins in pancreatic cancer cells. PANC-1 (A) and AsPC-1 (B) cells were treated with indicated compounds for 24 hours and lysed. Cell lysates were subjected to Western blotting using specific antibodies against Smad4, Smad2, p27, c-Jun and β-actin. The intensity of the bands in (A and B) was quantified by phosphorimaging, and the ratio of protein level in cells treated with curcumin or PEGylated curcumin to that in control cells was calculated (C and D). E, PEGylated curcumin inhibits the phosphorylation of c-Jun by Jab1/CSN associated kinases. Recombinant c-Jun was incubated with either recombinant CK2 or PKD with [γ-^{32}P]ATP in the presence of 5 μM PEG or PEGylated Curcumin. After 1 h at 37°C, the reaction mix was separated by SDS-PAGE and the dried gel was autoradiographed. F, Inhibitory effect of PEGylated curcumin on cell proliferation was blunted in Jab1 knockdown cells. PANC-1 cells were infected with virus containinig siGFP or siJab1 and treated with PEG (control) or PEGylated curcumin for 24 hours, and MTT assays was performed. The values represent means ± SD from three independent experiments. *: $p<0.001$, compared with PEG treatment of siGFP group; n.s.: no significance, compared with PEG treated siJab1 group.

Fig. 6. Synergistic effects of PEGylated curcumin with gemcitabine on cell growth. A, Enhancement of gemcitabine-induced cytotoxicity by PEG-Curcumin cotreatment in PANC-1 and AsPC-1 cells. Cells were exposed to 1 μM PEGylated curcumin with increasing concentrations (0.1-100 μM) of gemcitabine for 24 hours, after which cell survival was determined by MTT assays. *: $p<0.001$, compared with Gem alone treated group. B, Failure of free curcumin to increase gemcitabine-induced cytotoxicity in PANC-1 and AsPC-1 cells. Cells were exposed to 1 μM free curcumin with increasing concentrations (0.1-100 μM)

of gemcitabine for 24 hours, after which cell survival was determined by MTT assays. C and D, Enhancement of gemcitabine-induced cytotoxicity by PEG-Curcumin cotreatment in PANC-1 (C) and AsPC-1 (D) cells. Cells were treated with PEG-curcumin (1 μM) and/or gemcitabine (Gem, 10 μM) for 24 hours, after which cell survival was determined by MTT assays. The values represent means ± SD from three independent experiments.*: $p<0.001$, compared with both Gem alone and PEG-curcumin alone treatment group. E and F, Enhancement of gemcitabine-induced cell apoptosis by PEGylated curcumin cotreatment in PANC-1 (E) and AsPC-1 (F) cells. Cells were treated with PEGylated curcumin (1 μM) and/or gemcitabine (Gem, 10μM) for 24 hours, after which apoptosis cells were determined by flow cytometry using an Annexin-V-PE/7-AAD apoptosis detection kit. The values represent means ± SD from three independent experiments. *: $p<0.001$, compared with both Gem alone and PEG-curcumin alone treatment group.

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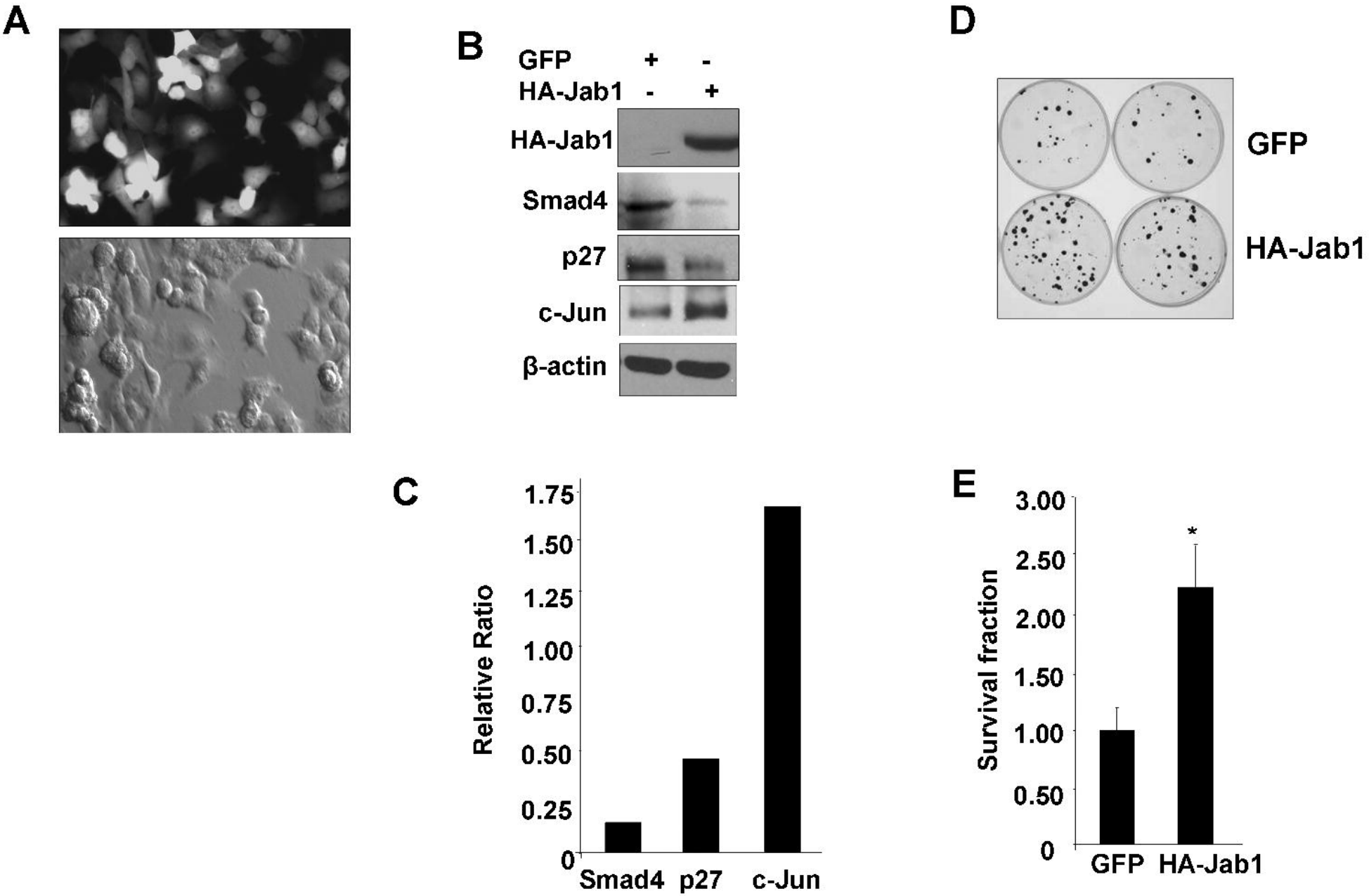
Table 1 Growth inhibitory effects of curcumin and PEGylated curcumin on a panel of human pancreatic cancer cell lines

	PANC-1	**MiaPaCa-2**	**BxPC-3**	**AsPC-1**
Curcumin	17μM	15μM	7μM	18μM
PEG-Curcumin	6μM	5μM	4μM	8μM

IC_{50} : concentration at which 50% growth inhibition occurred. Mean of triplicate experiments (MTT assay after 24 hours of exposure to curcumin or PEG-curcumin) was calculated.

Figure 1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HA-Jab1 - +
HA-Jab1
Smad4
p27
c-Jun
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D
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C
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1.50
1.25
1.00
0.75
0.50
0.25
0
Smad4
p27
c-Jun
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Survival fraction
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*
GFP
HA-Jab1

Figure 1 Cont'd

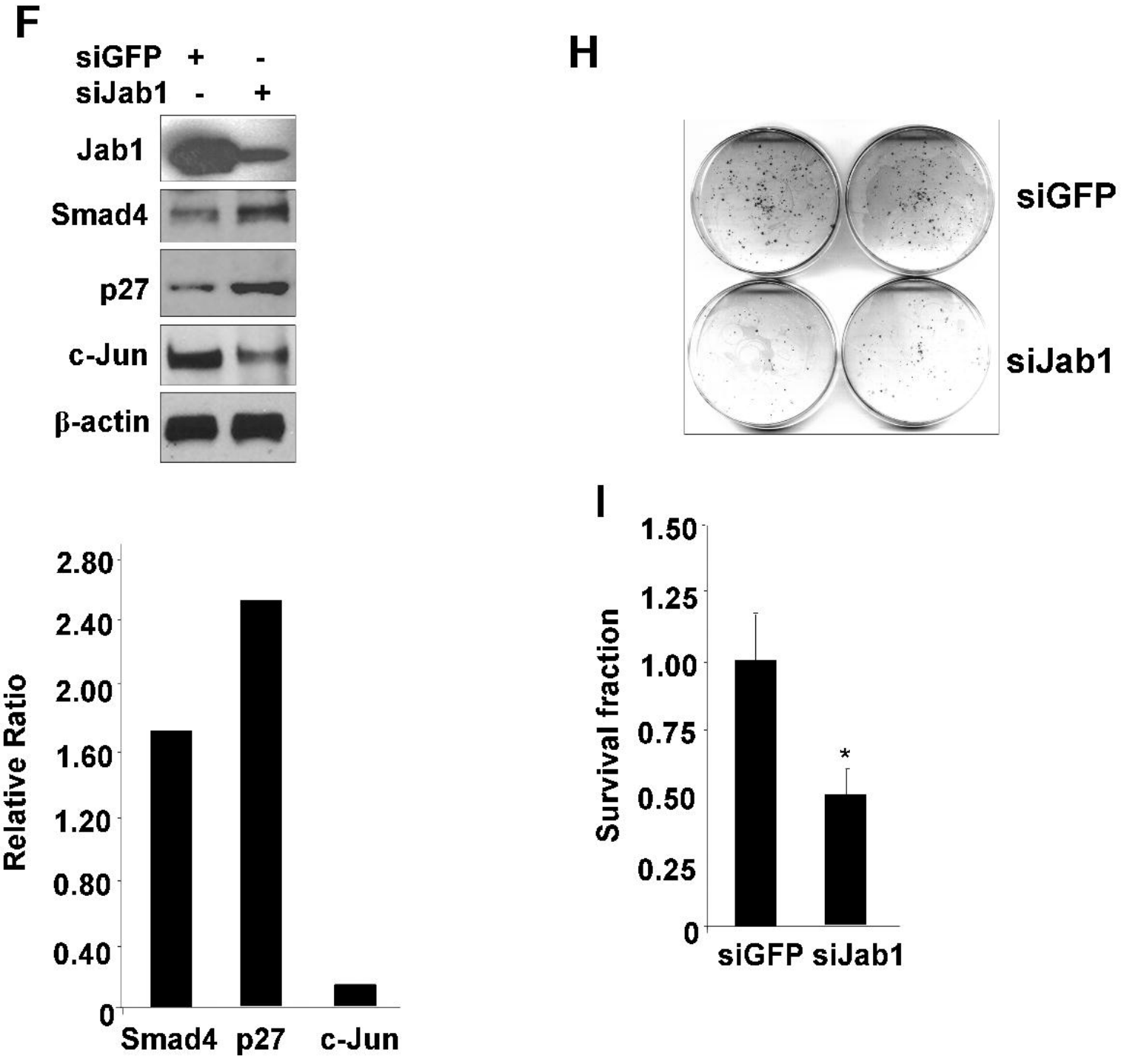
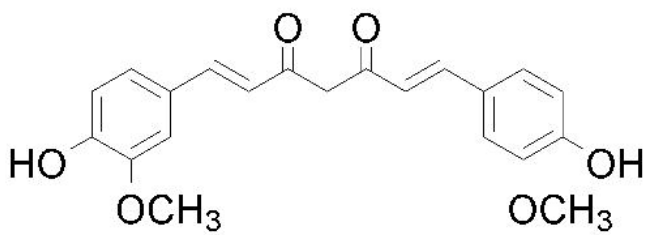


Figure 2

A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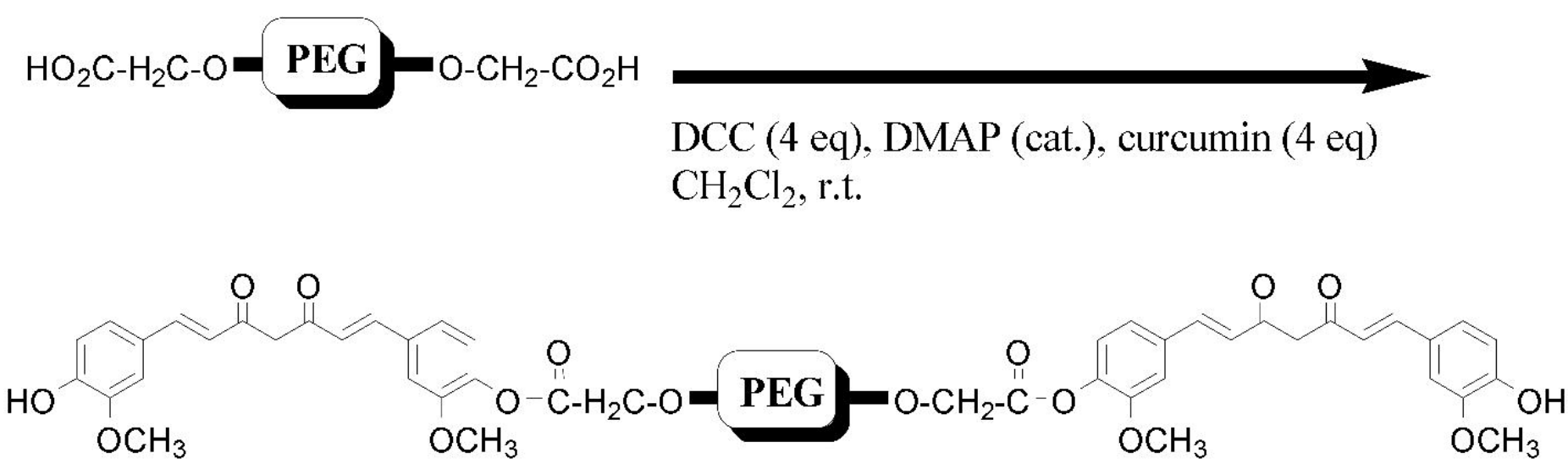
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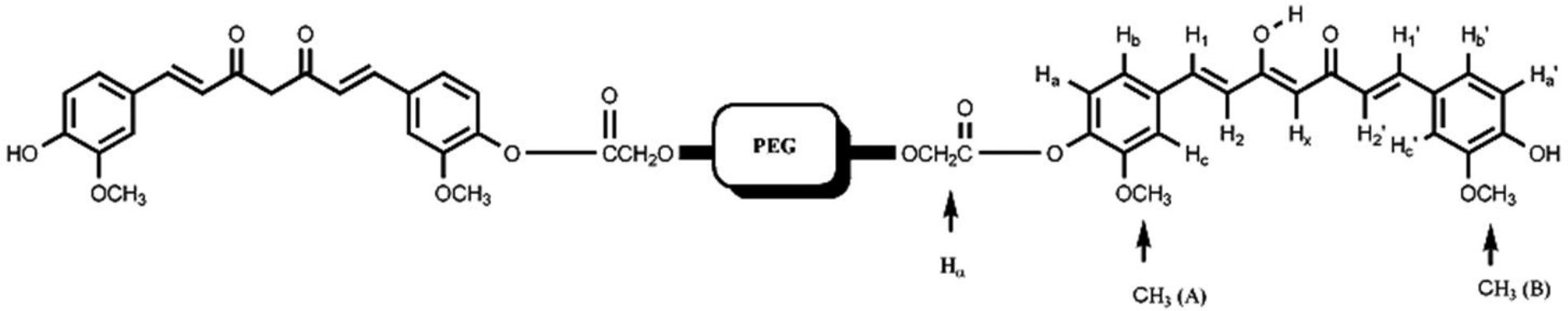
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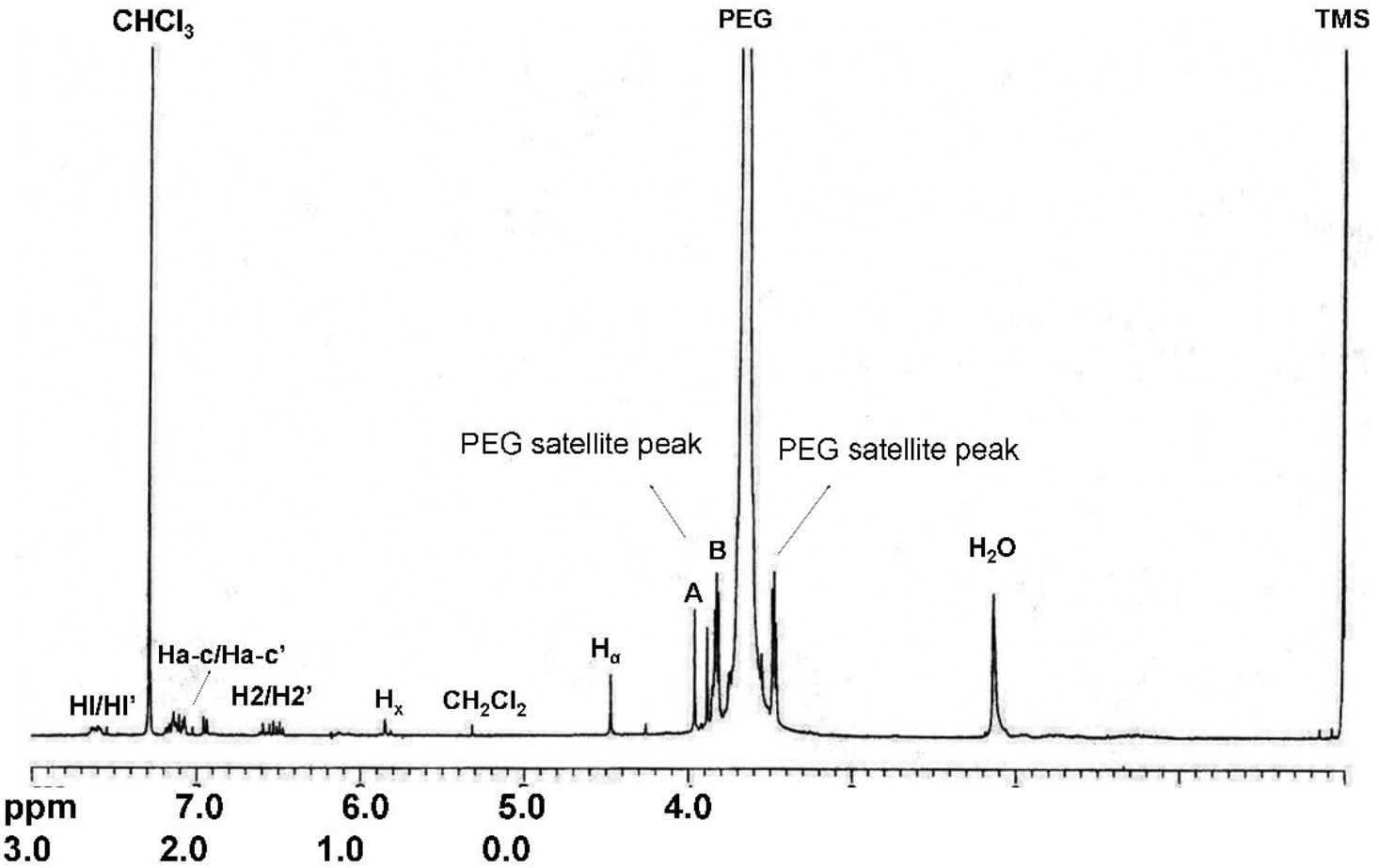
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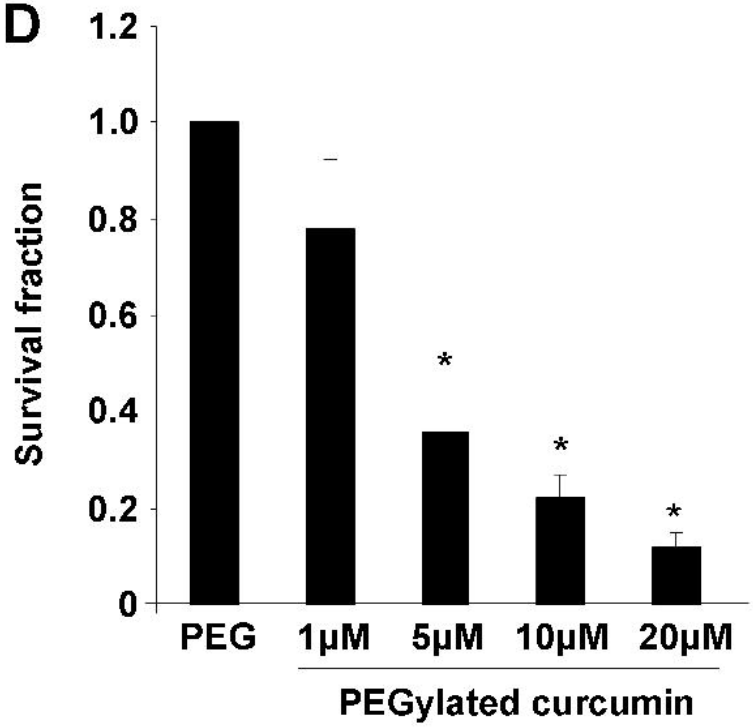
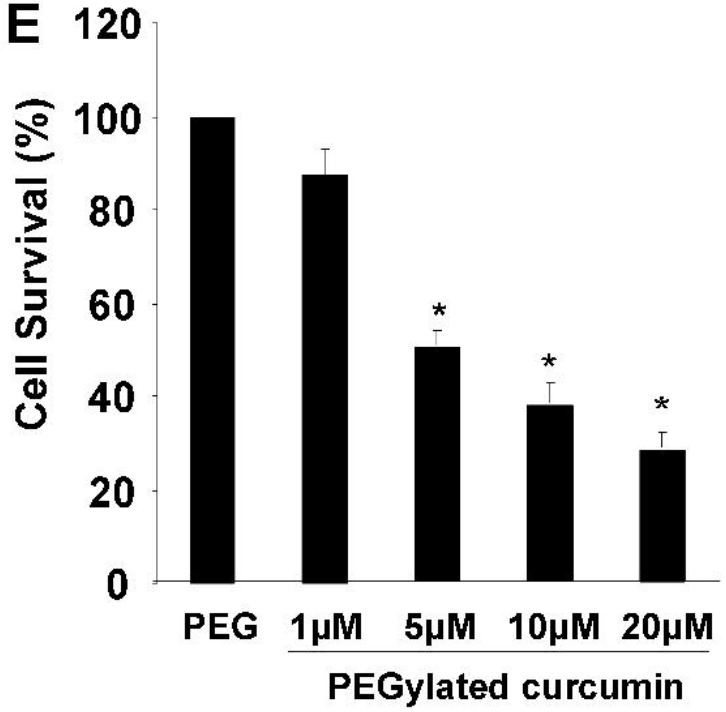
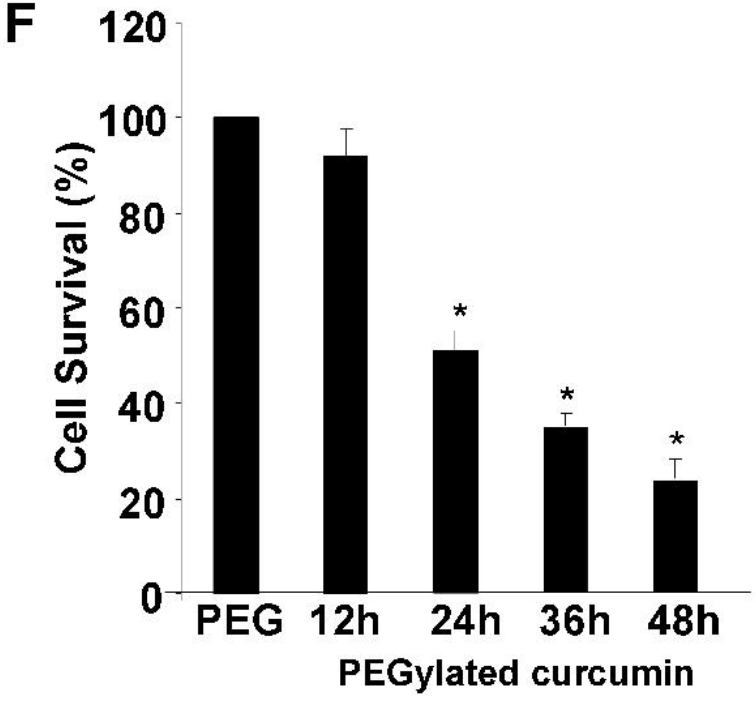
Figure 2 Cont'd







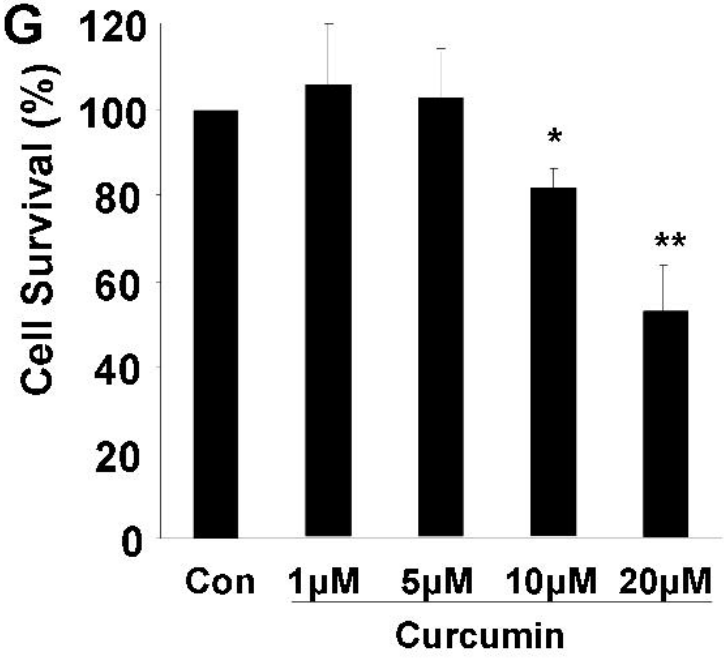
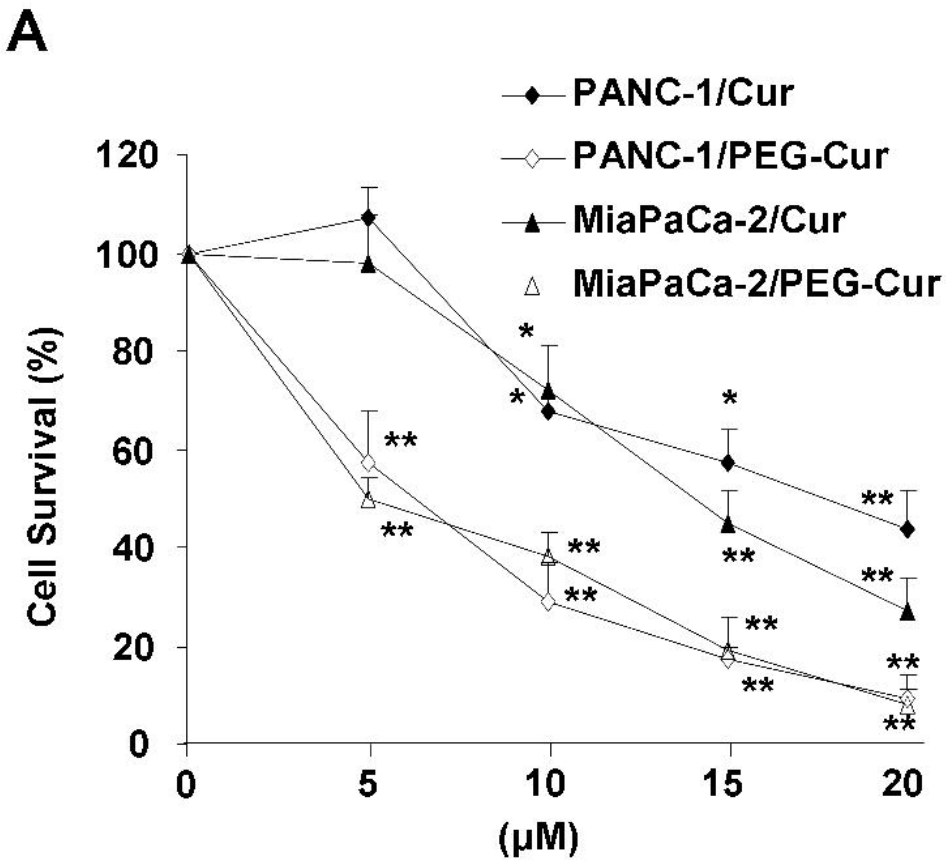


Figure 3


A
PANC-1/Cur
PANC-1/PEG-Cur
MiaPaCa-2/Cur
MiaPaCa-2/PEG-Cur
Cell Survival (%)
120
100
80
60
40
20
0
0
5
10
15
20
(μM)

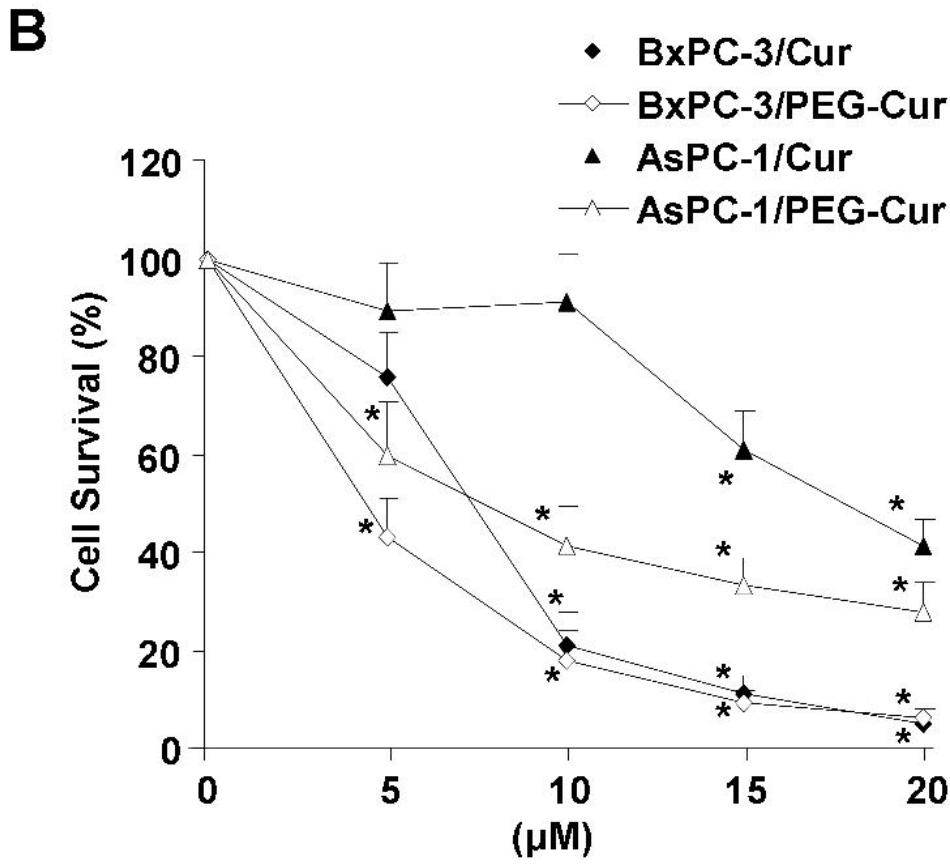
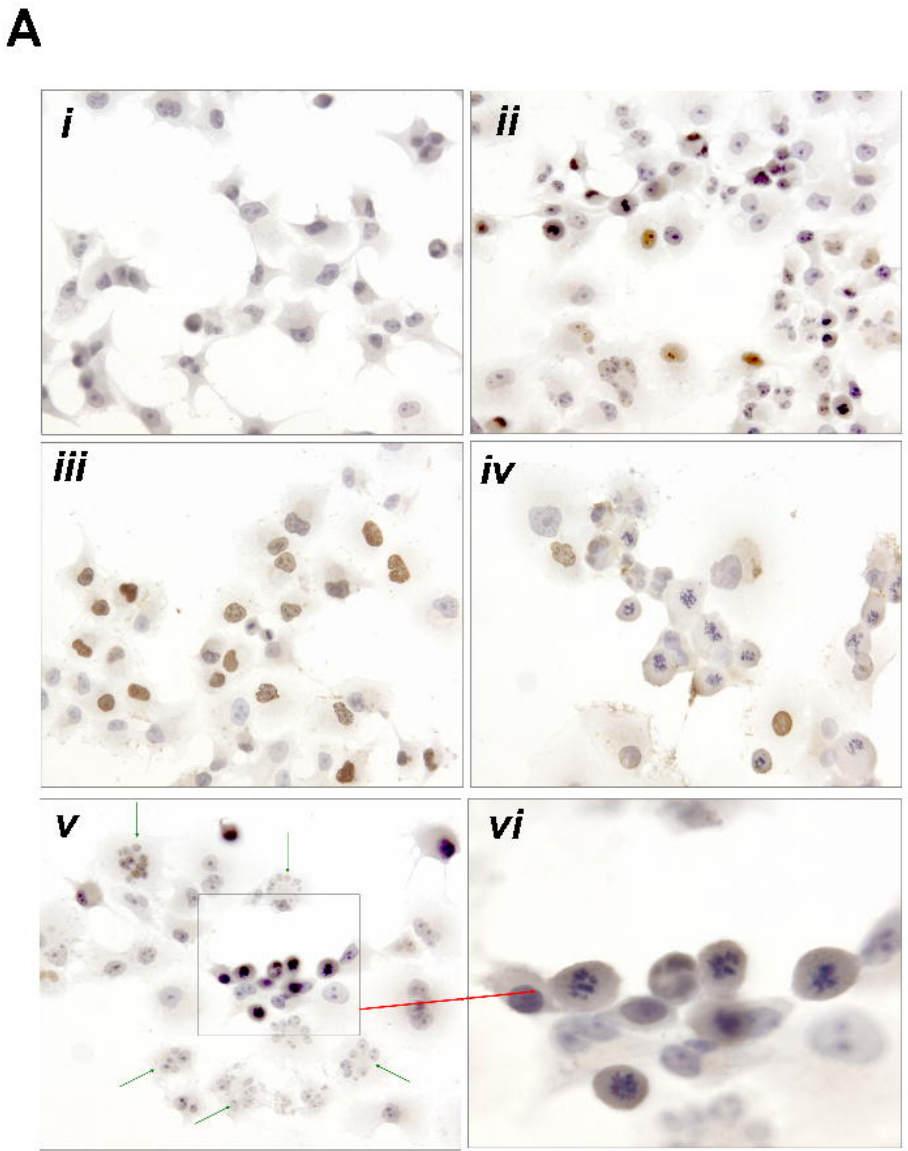
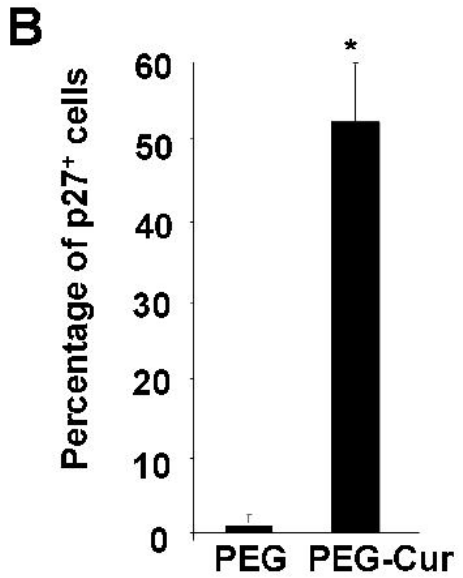
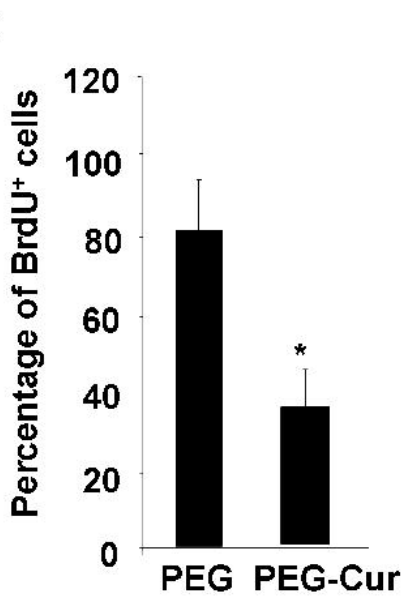
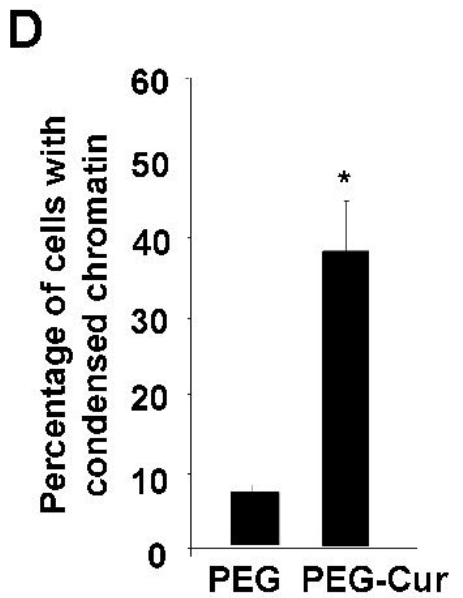

B
BxPC-3/Cur
BxPC-3/PEG-Cur
AsPC-1/Cur
AsPC-1/PEG-Cur
Cell Survival (%)
120
100
80
60
40
20
0
0
5
10
15
20
(μM)

Figure 4


A
i
ii
iii
iv
v
vi


B
Percentage of p27+ cells
60
50
40
30
20
10
0
*
PEG
PEG-Cur


C
Percentage of BrdU+ cells
120
100
80
60
40
20
0
*
PEG
PEG-Cur


D
Percentage of cells with condensed chromatin
60
50
40
30
20
10
0
*
PEG
PEG-Cur

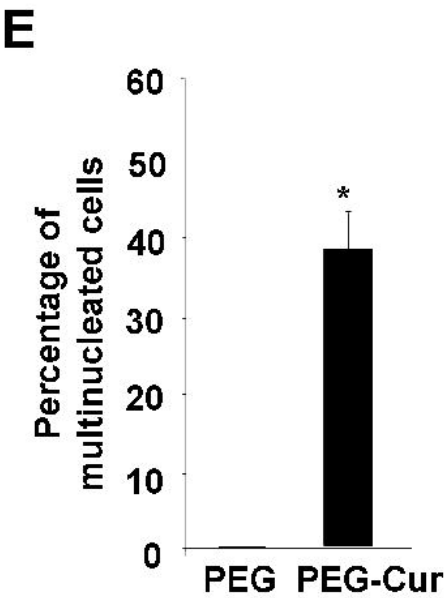

E
Percentage of multinucleated cells
60
50
40
30
20
10
0
*
PEG
PEG-Cur

Figure 4 Cont'd

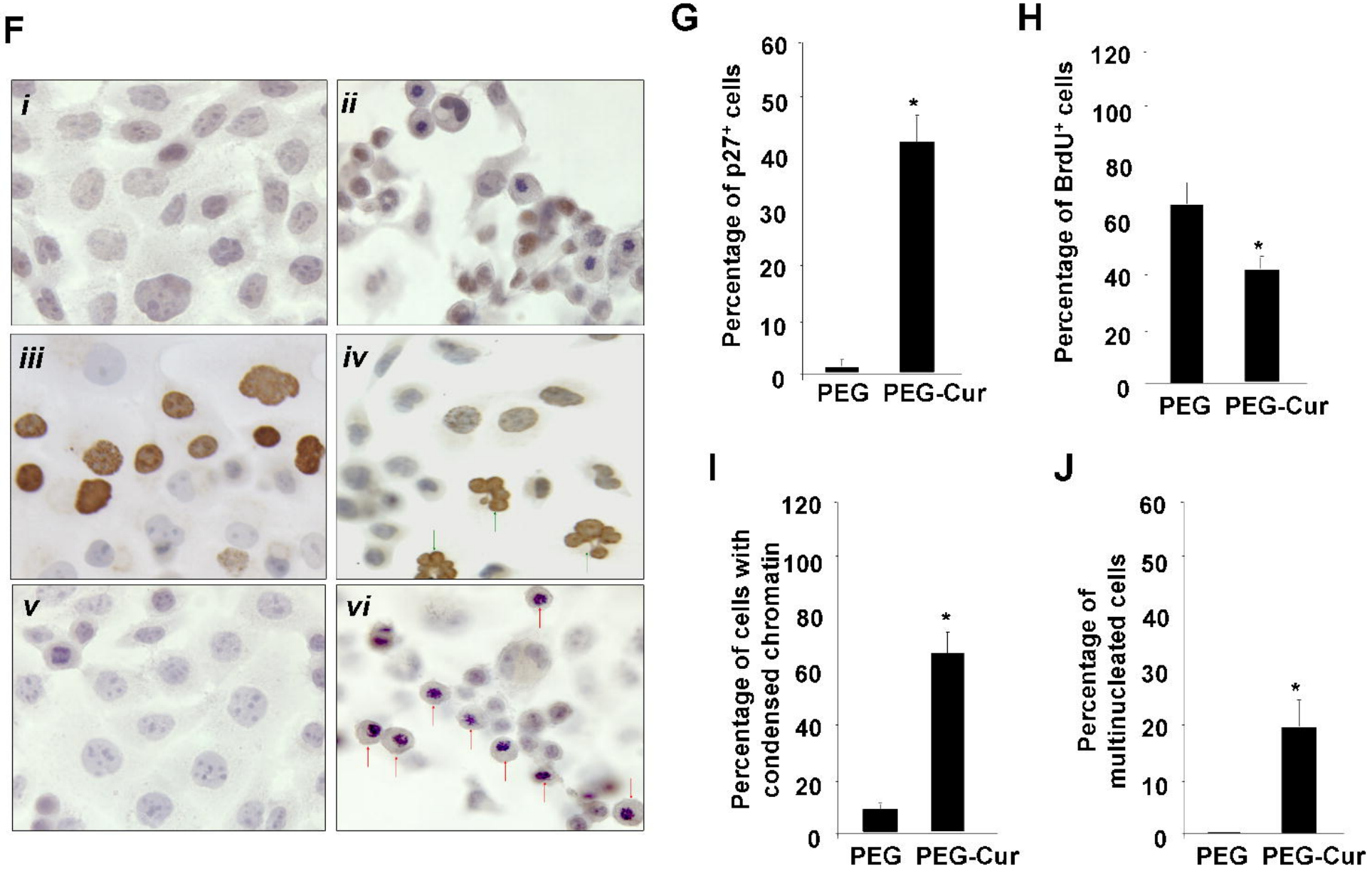


Figure 5

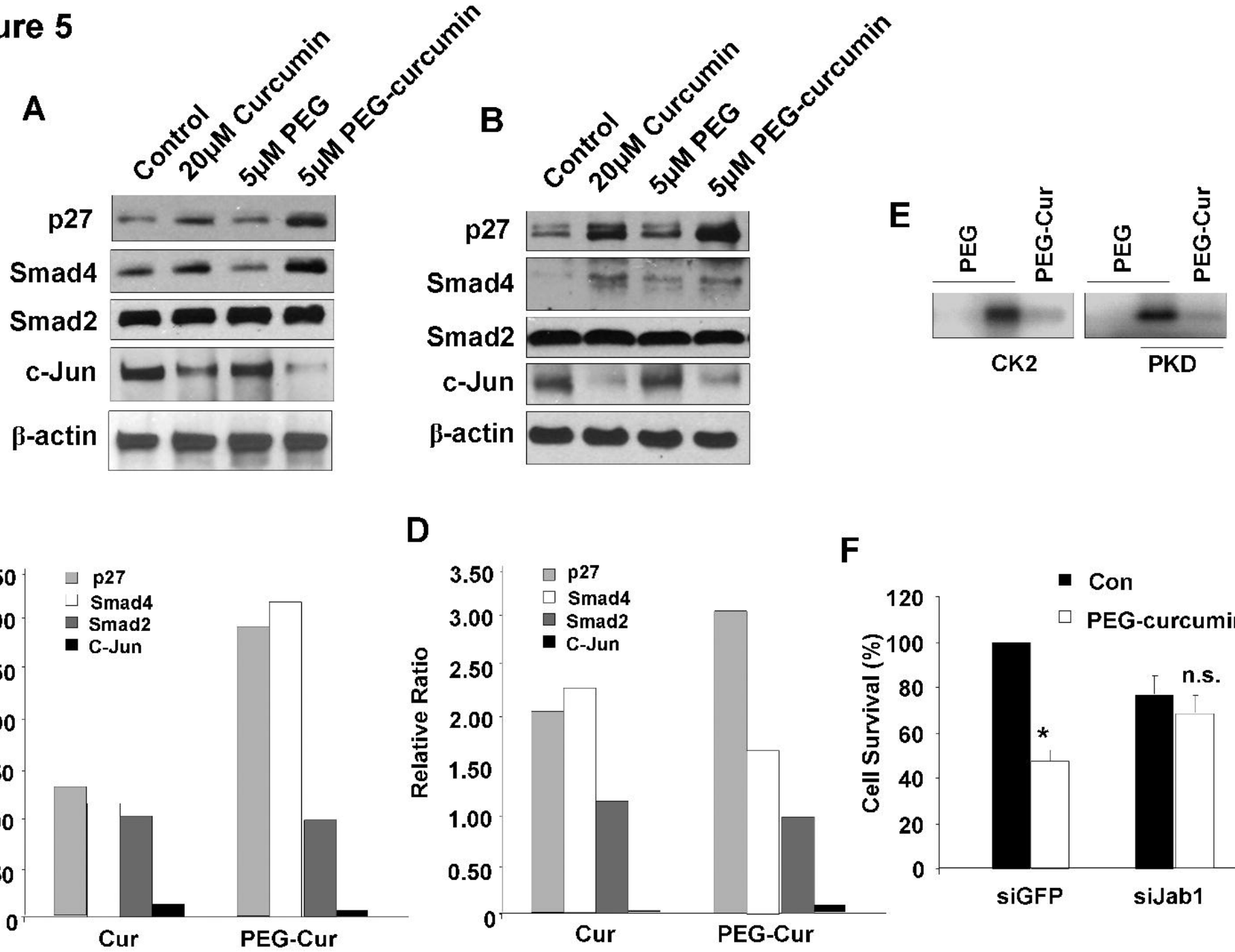
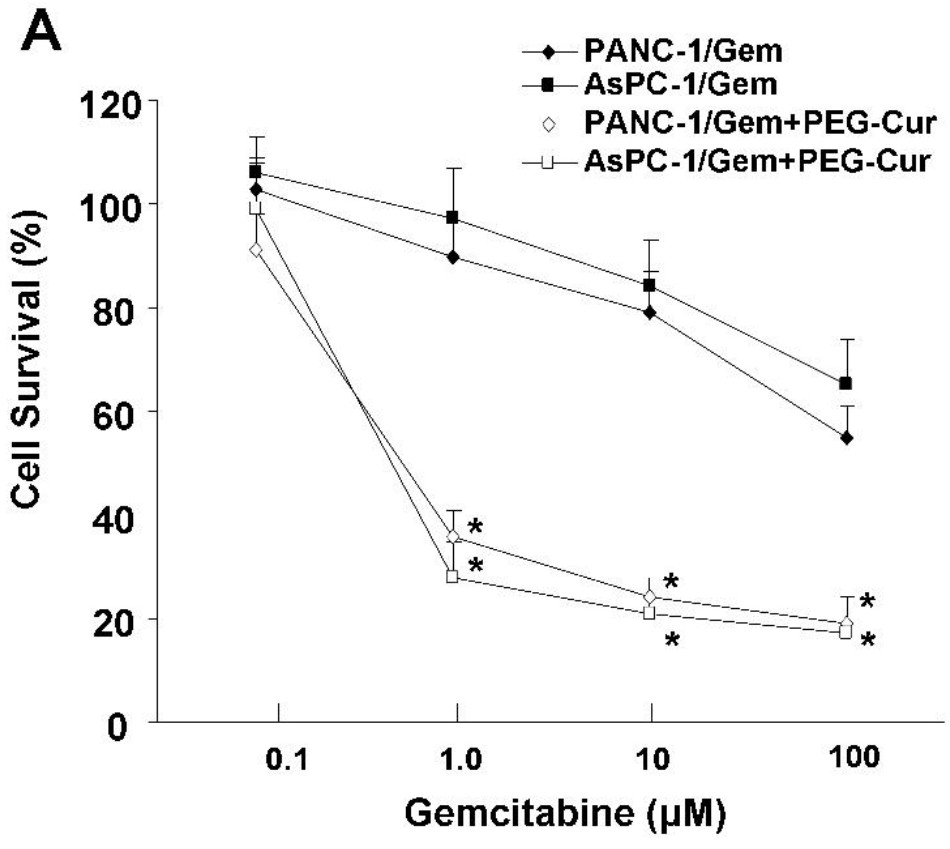

A
Control
20μM Curcumin
5μM PEG
5μM PEG-curcumin
p27
Smad4
Smad2
c-Jun
β-actin
B
Control
20μM Curcumin
5μM PEG
5μM PEG-curcumin
p27
Smad4
Smad2
c-Jun
β-actin
E
PEG
PEG-Cur
PEG
PEG-Cur
CK2
PKD
C
p27
Smad4
Smad2
C-Jun
Relative Ratio
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0
Cur
PEG-Cur
D
p27
Smad4
Smad2
C-Jun
Relative Ratio
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0
Cur
PEG-Cur
F
Con
PEG-curcumin
Cell Survival (%)
120
100
80
60
40
20
0
*
n.s.
siGFP
siJab1

Figure 6


A
PANC-1/Gem
AsPC-1/Gem
PANC-1/Gem+PEG-Cur
AsPC-1/Gem+PEG-Cur
Cell Survival (%)
120
100
80
60
40
20
0
0.1
1.0
10
100
Gemcitabine (μM)
*

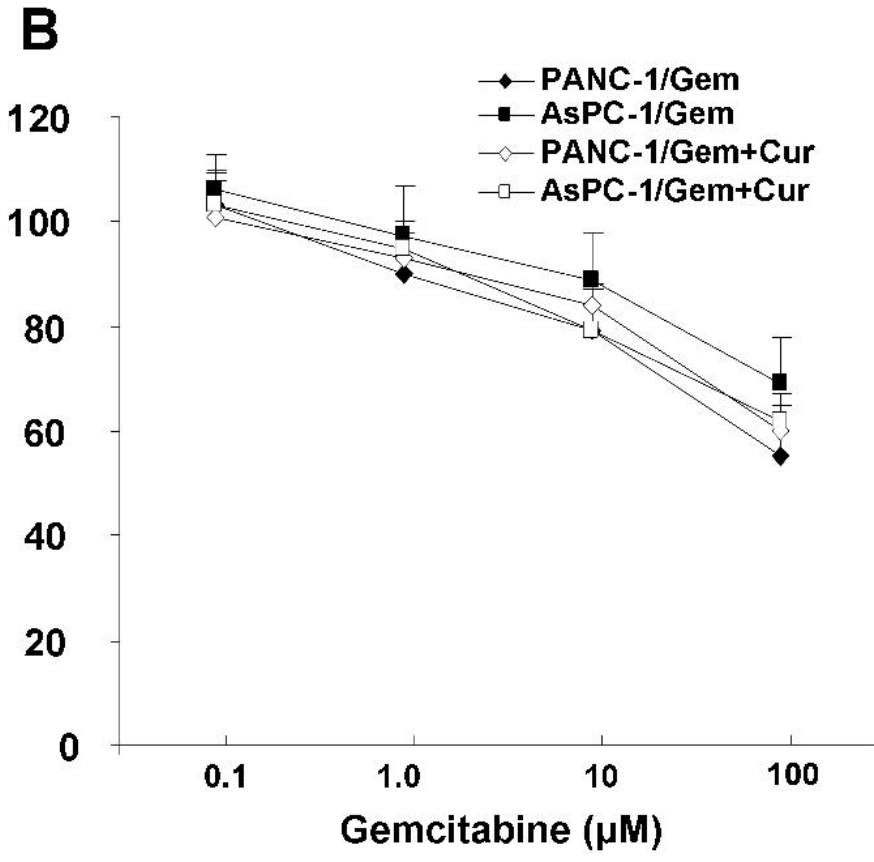

B
PANC-1/Gem
AsPC-1/Gem
PANC-1/Gem+Cur
AsPC-1/Gem+Cur
120
100
80
60
40
20
0
0.1
1.0
10
100
Gemcitabine (μM)

Figure 6 Cont'd