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EGR-1/Bax Pathway Plays a Role in Vitamin E δ -Tocotrienol-induced Apoptosis in Pancreatic Cancer Cells

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Abstract

The anticancer activity of δ -tocotrienol, a bioactive vitamin E present in whole grain cereals, annatto beans, and palm fruit, is strongly dependent on its effect on the induction of apoptosis. δ -Tocotrienol-induced apoptosis is associated with consistent induction in the expression of the pro-apoptotic protein Bax. The molecular mechanism by which δ -tocotrienol regulates Bax expression is unknown. We carried out a DNA microarray study that identified δ -tocotrienol induction of the zinc finger transcription factor, EGR-1, in pancreatic cancer cells. Here, we provide evidence linking δ -tocotrienol-induced apoptosis in pancreatic cancer cells to EGR-1 regulation of Bax expression. Forced expression of EGR-1 induces Bax expression and apoptosis in pancreatic cancer cells. In contrast, knockdown of δ -tocotrienol-induced EGR-1 by small interfering RNA attenuated δ -tocotrienol-induced Bax expression and reduced δ -tocotrienol-induced apoptosis. Further analyses showed that *de novo* protein synthesis was not required for δ -tocotrienol-induced EGR-1 expression, suggesting a direct effect of δ -tocotrienol on EGR-1 expression. Furthermore, a ChIP assay demonstrated that EGR-1 binds to the Bax gene promoter. Finally, δ -tocotrienol

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treatment induced Bax expression and activated EGR-1 in the pancreatic neoplastic cells of the PDX-Cre Kras genetically engineered model of pancreatic cancer. Our study provides the first evidence for EGR-1 as a direct target of vitamin E δ -tocotrienol, suggesting that EGR-1 may act as a pro-apoptotic factor in pancreatic cancer cells via induction of Bax.

Keywords

δ -tocotrienol; pancreatic cancer; early growth response protein 1; apoptosis

1. Introduction

Tocotrienols, the unsaturated forms of vitamin E, are some of the most bioactive anticancer constituents in whole grain food [1, 2]. Recently, tocotrienols have been the focus of preclinical studies and early-phase clinical trials due to their anticancer activity. Our group recently showed that vitamin E δ -tocotrienol is the most bioactive tocotrienol against pancreatic cancer [3]. In addition to augmenting the activity of gemcitabine in pancreatic cancer xenografts, we recently showed that δ -tocotrienol prolonged the survival and prevented the development of pancreatic cancer in the *LSL-Kras^{G12D/+};Pdx-1-Cre* and the *LSL-Kras^{G12D/+};Pdx-1-Cre; LSL-Trp53^{R172H/+}* genetically engineered models of pancreatic cancer [4, 5]. Treatment with δ -tocotrienol leads to inhibition of tumor growth and induction of tumor apoptosis [3–6]. Such effects have been reported to involve the interaction of δ -tocotrienol with apoptotic signaling pathways.

We have previously reported that the pro-apoptotic effect of δ -tocotrienol involves modulation of the B cell lymphoma-2 (Bcl-2) family proteins [3, 4]. The Bcl-2 family proteins, which include both anti- and pro-apoptotic proteins, are key regulators of the apoptotic signaling pathway [7–10]. The intrinsic apoptotic pathway can be activated by a range of stress stimuli, including ultraviolet radiation, heat, most DNA-damaging agents, growth factor deprivation, and some anticancer bioactive food components. These diverse stressors interact with multiple intracellular components that relay the signal to the mitochondria, resulting in mitochondrial outer membrane depolarization (MOMP). MOMP prompts apoptogenic proteins such as cytochrome c, which are normally confined to the mitochondrial intermembrane space, to diffuse into the cytosol. In the cytosol, the apoptogenic factors interact with various cellular components to initiate the death-inducing caspase cascade [10, 11]. MOMP is considered the critical event in the apoptotic signaling pathway that commits the cell to apoptosis. Therefore, the Bcl-2 family proteins serve as an “apoptotic switch” by turning MOMP on or off. In general, the relative ratio of pro- and anti-apoptotic Bcl-2 family proteins in a cell determines whether MOMP occurs and therefore whether the cell undergoes apoptosis [10]. Thus the regulation of the expression and function of Bcl-2 proteins is critical in mediating cell death. Disruption of the balance between pro- and anti-apoptotic Bcl-2 proteins can render cells resistant to apoptotic stimuli, thereby promoting cancer cell development and progression [12]. Both suppressed expression of pro-apoptotic members and overexpression of anti-apoptotic members have been reported in various cancers [3, 4, 9, 10]. Understanding how bioactive food components such as δ -tocotrienol modulate these proteins will give further insight into

developing strategies to target dysregulation of apoptosis in cancer using bioactive anticancer micronutrients.

In pancreatic cancer cells, δ -tocotrienol has been shown to both suppress the expression of anti-apoptotic members of the Bcl-2 family proteins, such as Bcl-2 and Bcl-2-related gene long isoform (Bcl-xl), and increase the expression of a prominent pro-apoptotic member of the Bcl-2 family protein, Bcl-2-associated X protein (Bax) [3–5]. Bax is ubiquitously expressed in all tissues; however, in many cancer cells including pancreatic cancer cells, Bax is down-regulated while Bcl-2 is up-regulated, leading to the pronounced imbalance between the pro-apoptotic and anti-apoptotic Bcl-2 members and to a dramatic increase in Bcl-2-to-Bax ratio and thus decreased sensitivity to apoptosis [4, 5, 9, 10]. Several studies have demonstrated a role for transcriptional regulation in Bax expression. The transcription factors p53, EGR-1, Id3, ELK-1, and Runx2 have been shown to activate the Bax gene [13–20]. To examine the role of these pathways in the regulation of δ -tocotrienol function, genes induced or repressed by δ -tocotrienol were determined in a pancreatic cancer cell model using cDNA microarray technology. In this report, we show that the immediate early gene, EGR-1, is constitutively expressed in pancreatic cancer cells and further induced in response to δ -tocotrienol. Furthermore, we show that EGR-1 induces Bax expression. δ -Tocotrienol-induced EGR-1 expression results in the induction of apoptosis, which is mediated in part by activation of the pro-apoptotic proteins Bax.

2. Materials and methods

2.1. Materials and animals

α -, β -, γ -, and δ -Tocotrienols and α -, β -, γ -, and δ -tocopherols were kindly provided as a gift by Davos Life Sciences (Helios, Singapore). EGR-1, β -actin, Bcl-2, Bax, XIAP, survivin, cIAP-1, and phospho-c-Jun antibodies were purchased from Cell Signaling Technology (Danvers, MA) or from Santa Cruz Biotechnology (San Diego, CA). PCMV6-EGR-1 plasmid and pCMV6-vector were purchased from OriGene Technologies (Rockville, MD). siRNA targeting EGR-1 and control siRNA were purchased from Sigma-Aldrich (St. Louis, MO). Other materials included the MAGnify Chip system (Invitrogen, Carlsbad, CA) and Bax promoter with luciferase reporter (Switch Gear Genomics, Menlo Park, CA). All chemicals were purchased from Sigma-Aldrich unless otherwise specified. LSL-Kras^{G12D} and PDX-1-Cre mice were obtained from the National Cancer Institute Mouse Models of Human Cancers Consortium (Frederick, MD). All animal studies were approved by our Institutional Animal Care and Use Committee, following the guidelines of the American Association for the Assessment and Accreditation of Laboratory Animal Care.

2.2. Cell culture and treatment

Our human pancreatic cancer cell line (MiaPaCa-2) was purchased from American Type Culture Collection (Manassas, VA). MiaPaCa-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 100 U/mL penicillin and 100 μ g/mL streptomycin. All cells were cultured with a humidified atmosphere containing 5% CO₂ at 37°C and collected using 0.5% trypsin EDTA following the completion of a specified incubation period before analyses.

2.3. Transfection of siRNAs and plasmids

Cells were seeded in six-well plates at a concentration of 1×10^5 and cultured in medium without antibiotics approximately 24 hours before transfection. Cells were transiently transfected with siRNA targeting EGR-1 or control (scramble) siRNA at 100 nM final concentration, pCMV6-EGR-1 plasmid, Bax promoter with Luciferase reporter, or pCMV6-vector at 2–4 $\mu\text{g}/\text{well}$. The siRNA, plasmid, and promoter transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.4. Cell viability assay

Cell viability was determined at 72 hours by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay. Cells (5×10^3 cells per well) were grown overnight in 96-well plates. Fresh medium (200 μL) with different doses of δ -tocotrienol or ethanol was added and incubated at 37°C at 5% CO_2 for 72 hours. After 72 hours of incubation, MiaPaCa-2 and Panc-1 cells were incubated for an additional 4 hours with 20 μL MTT (5 mg/mL). The supernatant was then removed, and 150 μL DMSO was added. Absorbance at 490 nm was measured with a microplate reader.

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

MiaPaCa-2 cells were cultured on four chamber coverslips (NUNC, Logan, UT) for 12 hours, with cell density of 1×10^5 per slide. Cells were treated with δ -tocotrienol (50 μM) or ethanol for 12 hours. To measure DNA fragmentation, the commercially available in situ death detection kit (Roche Diagnostics, Indianapolis, IN) was utilized. Nuclear apoptosis is accompanied by segmentation of the nucleus into dense nuclear parts that are further distributed into apoptotic bodies. These DNA breaks can be visualized by FITC-labeled staining. Slides were mounted in a mounting medium (Vectashield, Burlingame, CA) for fluorescence imaging. Images were captured with an immunofluorescence microscope at 63×1.4 oil magnification.

2.6. Annexin V-FITC/propidium iodide staining assay

The Annexin V-FITC/propidium iodide assay was performed using a commercial kit (BD Bioscience, San Jose, CA). Briefly, cells were washed twice with PBS and incubated in 500 μL binding buffer containing Annexin V-FITC and propidium iodide in the dark for 10 minutes at room temperature. The stained samples were then analyzed on a FACSsort flow cytometer as instructed by the manufacturer (BD Bioscience).

2.7. Microarray assay

We conducted global gene expression analyses with RNA prepared from MiaPaCa-2 cells exposed to 50 $\mu\text{mol}/\text{L}$ δ -tocotrienol or ethanol for 3 hours. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Gaithersburg, MD). Microarray analysis was performed using Affymetrix human U133 plus 2.0 GeneChips (Santa Clara, CA).

2.8. Real-time PCR assay

Total RNA was extracted with the RNeasy Mini Kit and reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) to form cDNA. To amplify transcripts, the cDNA was subjected to a SYBR green-based method for real-time polymerase chain reaction (PCR) relative quantification using QuantiTect Primer Assays systems (Qiagen). Real-time PCR was performed on an ABI PRISM 7900 analytical thermal cycler (Applied Biosystems, Carlsbad, CA) according to the manufacturer's recommendations. The mRNA expression level was calculated relative to the level of β -actin.

2.9. Chromatin immunoprecipitation assay

The ChIP assay was performed in MiaPaCa-2 cells using MAGnify Chromatin Immunoprecipitation System (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Briefly, the treated cells in dishes were cross linked using 37% formaldehyde (final concentration 1%) at room temperature for 10 minutes. The reaction was stopped with 1.25 M glycine for 10-minute incubation at room temperature. The cells were washed with cold PBS and harvested using a scraper into 1.5-mL tubes on ice. The cells were centrifuged, and the pellet was lysed using lysis buffer containing protease inhibitors. Chromatin was sheared using Branson Sonifier 450 (VWR Scientific, Arlington Heights, IL). Sheared chromatin was diluted with dilution buffer containing protease inhibitors. The EGR-1 antibody was coupled with magnetic Dynabeads, and beads were treated with sheared chromatin for binding for 2 hours followed by washing 3 times with washing buffer. The crosslinking was reversed using reverse crosslinking buffer and proteinase K. DNA was purified using DynaMag-PCR magnet and buffers. PCR was performed using primers for Bax promoter (5'-GCCTGGGCAACACAGTGAG-3' and 5'-GCAATCATCTCTGCAGCTCCAT-3') that yielded 500-bp products. For control PCR reaction, β -actin gene was used. The primers sequences were: 5'-GATGACCTGGCCGTCAGGCAGCTCATA-3' and 5'-ACGTAGCCATCCAGGCTGTGCTGTCCC-3'.

2.10. Luciferase reporter assay

MIAPaCa-2 cells were seeded in 6-well plates at 2×10^5 cells/well. Each well was transfected the following day with 2 μ g of Bax promoter with luciferase reporter (Switch Gear Genomics, Menlo Park, CA). Lipofectamine 2000 was used as the transfection reagent as per manufacturer's instructions. After 48-hour transfection, the cells were treated with δ -tocotrienol (50 μ M) for 6 hours and 5% ethanol as vehicle. Lysates were prepared using RIPA buffer and collected in tubes. Luciferase activity was measured by the luciferase assay system kit (Promega).

2.11. Western blot analysis

Cells in each dish, including dead cells floating in medium, were harvested and lysed in sampling buffer. Protein concentrations of the lysates were determined using the bicinchoninic acid protein assay kit (Pierce Biotech, Rockford, IL). An aliquot of the denatured supernatant containing 40 μ g of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride

membranes. Membranes were blocked with blocking buffer (TBST, containing 5% non-fat milk) for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with the primary antibodies (EGR-1, β -actin, Bcl-2, Bax, XIAP, survivin, cIAP-1, and phospho-c-Jun). After blots were washed, they were incubated with horseradish peroxidase-conjugated secondary antibody IgG (1:2,000) for 1 hour at room temperature. The washed blot was then treated with SuperSignal West Pico chemiluminescent substrate (Pierce Biotech) for positive antibody reaction. Membranes were exposed to X-ray film for visualization and densitometric quantization of protein bands using AlphaEaseFC software (Alpha Innotech, Santa Clara, CA).

2.12. DNA binding activity by ELISA assay

The DNA binding activity of EGR-1 was detected by enzyme-linked immunosorbent assay (ELISA) with the transcription factor ELISA kit (Panomics, Santa Clara, CA) according to the manufacturer's instructions. In brief, nuclear extracts were prepared and incubated in 96-well plates coated with immobilized oligonucleotide (5'-GCGGGGGCG-3') containing an EGR-1 binding site and quantified at 450 nm with a reference wavelength of 655 nm. Each sample was analyzed in duplicate, and results are reported as means $\pm$ SEM.

2.13. Cell death ELISA assay

Cell death as a result of apoptosis was quantified by measuring mono- and oligonucleosomes released using the Cell Death Detection ELISA kit (Roche, Indianapolis, IN) following manufacturer's instructions. Briefly, cells were seeded in 6-well plates and treated with δ -tocotrienol, siRNA target EGR-1, siRNA-control, pCMV6-EGR-1 plasmid, and pCMV6-vector or combined δ -tocotrienol with siRNA or pCMV6-EGR-1, respectively, for 12 hours at 37°C. Cells were lysed in buffer for 30 minutes at 25°C, and the supernatant was diluted 1:10 with incubation buffer and then transferred into the MP-modules. The conjugate solution and substrate solution were added following the protocol. Absorbance was read in a microplate reader at 405 nm. Experiments were performed in triplicate.

2.14. Conditional Kras^{G12D} mouse model of pancreatic cancer

LSL-Kras^{G12D} and PDX-1-Cre mice were maintained as heterozygous lines and crossed and bred in our institutional vivarium. Tail snips, harvested from offspring of LSL-Kras^{G12D} and PDX-1-Cre mice, were digested overnight, and genomic DNA was extracted and estimated using the DNAeasy kit (Qiagen, Gaithersburg, MD), with genotyping analysis performed with the following primers: PDX-forward = 5'-CTGGACTACATCTTGAGTTGC-3', PDX-reverse = 5'-GGTGTACGGTCAGTAAATTTG-3', Kras-forward = 5'-AGGTAGCCACCATGGCTTGAGTAAGTCTGCA-3', and Kras-reverse = 5'-CCTTTACAAGCGCACGCAGACTGTAGA-3'.

LSL-Kras^{G12D/+} x Pdx-1-Cre mice were randomized as follows: 1) no treatment control (n = 34), 2) vehicle (ethanol-extracted olive oil, 1.0 mL/kg twice a day by oral gavage) (n = 27), and 3) δ -tocotrienol (200 mg/kg twice per day by oral gavage) (n = 31), with treatment started at 10 weeks of age and continued for 12 months. After 12 months of treatment, animals were euthanized and pancreatic tissues were frozen in liquid nitrogen and fixed in buffered formalin for Western blot and immunohistochemistry, respectively.

2.15. Immunohistochemistry

Immunohistochemistry was performed using the Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) per manufacturer's protocol with proprietary reagents. Briefly, slides were deparaffinized on the automated system with EZ Prep solution. Sections were heated for antigen retrieval. For immunohistochemistry, tissue sections were incubated with EGR-1, Bax, and β -actin antibody. Detection was performed using the Ventana OmniMap kit.

2.16. Statistical analysis

Each experiment was repeated at least three times. Data are presented as means $\pm$ SD. Statistical comparisons were performed using Student's and unpaired t-tests or one-way analysis of variance (ANOVA) where appropriate. A *P* value of 0.05 was considered statistically significant.

3. Results

3.1. δ -Tocotrienol suppresses cell growth and increases apoptosis in pancreatic cancer cells by 12 hours

A number of studies indicate that δ -tocotrienol exhibits anti-tumorigenic activity in pancreatic cancer cells [3, 6, 21, 22]. To determine the dose and time of exposure to use in our δ -tocotrienol gene expression experiments, we evaluated the effects of δ -tocotrienol on exponentially growing pancreatic cancer cells. MiaPaCa-2 and Panc-1 human pancreatic cancer cells were treated in the presence of various concentrations (0–100 μ M) of δ -tocotrienol for 72 hours, and the cell viability rate was measured using MTT assay. δ -Tocotrienol significantly ($P < 0.001$) decreased the cell viability of MiaPaCa-2 cells in a dose-dependent manner (Figure 1A). The inhibitor concentrations required for 50% inhibition of cell proliferation (IC_{50}) values was about 50 μ M for MiaPaCa-2 cells. We subsequently used MiaPaCa-2 cells in the rest of the experiments using IC_{50} (50 μ M) of δ -tocotrienol. To determine whether the growth inhibition by δ -tocotrienol was associated with the induction of apoptosis, annexin V/propidium iodide flow cytometry and TUNEL analyses were performed with 50 μ M of δ -tocotrienol. As shown in Figure 1B and 1C, we confirmed the induction of apoptosis of MiaPaCa-2 cells by δ -tocotrienol. Results in Figure 1D indicate that induction of cell death was clearly observed at 12 hours but not by 6 hours or earlier of exposure.

3.2. Microarray analysis of δ -tocotrienol-inducible genes in MiaPaCa-2 cells

To identify genes that were induced by δ -tocotrienol in MiaPaCa-2 cells, we cultured MiaPaCa-2 cells with or without δ -tocotrienol (50 μ M) for 3 hours. This short exposure time point was selected because it is a time point prior to any visible signs of δ -tocotrienol-induced apoptosis (Figure 1D). Total RNA was extracted and subjected to microarray analysis using Affymetrix human U133 plus 2.0 GeneChips. By using the GeneChips analysis suite, we sorted the genes that were called "present" in control MiaPaCa-2 cells and were either up-regulated or down-regulated by greater than one-fold after treatment with δ -tocotrienol in three independent experiments (Table 1). Seven genes were up-regulated and 7 genes were down-regulated by δ -tocotrienol in all 3 experiments. The modulation of the

genes identified by the microarray analysis was confirmed by quantitative real-time reverse transcription PCR (RT-PCR; Table 1). In 10 of the 14 genes examined, the RT-PCR data were consistent with the GeneChips data, demonstrating that our microarray analysis was reliable.

3.3. δ -Tocotrienol induces EGR-1 in MiaPaCa-2 cells

The gene showing the highest fold induction following 3-hour treatment with δ -tocotrienol was EGR-1 (2.21-fold), which was further confirmed in MiaPaCa-2 cells by Western blot analysis. As shown in Figure 2A and 2B, although EGR-1 protein levels were negligible in untreated MiaPaCa-2 cells, δ -tocotrienol treatment resulted in markedly up-regulated EGR-1 expression in a dose- and time-dependent manner. Among four tocotrienols, only 3 tocotrienols (β , γ , δ) have inhibitory activity against pancreatic cancer cells. We tested all of the 8 vitamin E compounds (α -, β -, γ -, and δ -tocopherols and α -, β -, γ -, and δ -tocotrienols) for their ability to induce EGR-1 in MiaPaCa-2 cells. Only β -, γ - and δ -tocotrienols were able to induce EGR-1 protein (Figure 2C), suggesting that EGR-1 induction seems to correlate with vitamin E pro-apoptotic activity in pancreatic cancer cells. Consistent with our Western blot results, our DNA binding ELISA showed that δ -tocotrienol treatment enhanced the binding activity of EGR-1 in MiaPaCa-2 cells (Figure 2D). Experiments were done to determine whether the δ -tocotrienol-dependent increase in EGR-1 mRNA is a primary response or requires ongoing protein synthesis. MiaPaCa-2 cells were treated with cycloheximide (10 μ M) for 30 minutes followed by δ -tocotrienol (50 μ M) for 1, 3, 6, and 12 hours. Real-time RT-PCR showed that the expression profile of EGR-1 following δ -tocotrienol was not perturbed by pretreating the cells with cycloheximide, indicating that de novo protein synthesis was not required for δ -tocotrienol to exert its effect (Figure 2E) and suggesting a direct transcriptional regulation of EGR-1 by δ -tocotrienol.

3.4. δ -Tocotrienol interacts with EGR-1 to induce Bax expression and promote apoptosis in pancreatic cancer cells

To further explore the precise mechanism by which δ -tocotrienol-mediated EGR-1 expression induces pancreatic cancer cell apoptosis, we used Western blot analyses to examine the expression of potential EGR-1 target proteins, the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, and IAP family proteins (cIAP1, XIAP, and survivin). After a short (12-hour) exposure to δ -tocotrienol, EGR-1 and Bax were consistently and significantly ($P < 0.05$) up-regulated in MiaPaCa-2 cells (Figure 3A and 3B). In contrast, there were no significant consistent alterations of Bcl-2, survivin, XIAP, or cIAP1 in these cell lines after 12 hours of δ -tocotrienol exposure (Figure 3A and 3B). These data suggest that δ -tocotrienol-induced EGR-1 expression is temporarily associated with up-regulation of Bax. In addition, δ -tocotrienol treatment markedly induced Bax promoter activity and expression of Bax in control MiaPaCa-2 cells, but not in MiaPaCa-2 cells with EGR-1 knockdown using siRNA (Figure 3C). Furthermore, forced expression of EGR-1 in MiaPaCa-2 cells using EGR-1 cDNA demonstrated corresponding induction of Bax expression using Western blot (Figure 3D). Taken together, these data strongly suggest that δ -tocotrienol induction of EGR-1 expression results in increased expression of Bax with subsequent induction of apoptosis. δ -Tocotrienol increased Bax promoter enrichment after EGR-1 chromatin immunoprecipitation compared to vehicle in MiaPaCa-2 cells (Figure 3E),

indicating that EGR-1 binds to the Bax gene promoter leading to induction of Bax protein expression and thereby inducing apoptosis.

3.5. Suppression of EGR-1 reduces δ -tocotrienol-induced apoptosis and Bax promoter activity in MiaPaCa-2 cells

To evaluate the role of EGR-1 in δ -tocotrienol-mediated apoptosis induction, we applied siRNA to silence EGR-1 expression (Figure 4A). To test the effect of EGR-1 suppression on δ -tocotrienol-mediated cell death, MiaPaCa-2 cells were transfected with either control siRNA (scramble siRNA) or EGR-1 siRNA and grown either in the presence or absence of δ -tocotrienol (50 μ M) for 12 hours. Their survival rate was determined by cell death ELISA. As expected, δ -tocotrienol treatment resulted in a marked induction in cell apoptosis in the control-transfected cells (Figure 4B). On the other hand, the responsiveness to δ -tocotrienol, although not completely extinguished, was significantly reduced in MiaPaCa-2 cells transfected with the EGR-1 siRNA (Figure 4B). δ -Tocotrienol treatment resulted in a marked induction of Bax promoter activity in the control siRNA-transfected cells (Figure 4C). On the other hand, the responsiveness to δ -tocotrienol, although not completely extinguished, was significantly reduced in MiaPaCa-2 cells transfected with the EGR-1 siRNA (Figure 4C). These results indicate that EGR-1 plays a major role in δ -tocotrienol-mediated increased apoptosis through induction of Bax in MiaPaCa-2 cells.

3.6. Forced expression of EGR-1 inhibits the survival of MiaPaCa-2 cells

Further studies explored the biological function of EGR-1 in pancreatic cancer cells. We transiently transfected the EGR-1 expression vector into MiaPaCa-2 cells. Cell death ELISA showed that MiaPaCa-2 cells transfected with pCMV6-EGR-1 displayed increased apoptosis compared with the control MiaPaCa-2 cells transfected with the same amount of pCMV6 empty vector (Figure 5A). Western blot analysis showed that EGR-1 levels in the transiently transfected MiaPaCa-2 cells were comparable with levels in the δ -tocotrienol-treated MiaPaCa-2 cells (Figure 5B). Our results indicate that EGR-1 plays a major role in the induction of apoptosis in pancreatic cancer cells.

3.7. δ -Tocotrienol induces EGR-1 and Bax expression in the pancreatic neoplastic tissue of conditional *Kras*^{G12D} mouse model

Recently, we found that δ -tocotrienol prolongs the survival and prevents pancreatic cancer in *LSL-Kras*^{G12D/+};*Pdx-1-Cre* and *LSL-Kras*^{G12D/+};*Pdx-1-Cre*; *LSL-Trp53*^{R172H/+} mice [4, 5]. To determine whether δ -tocotrienol modulates EGR-1 and Bax *in vivo*, we examined the effects of δ -tocotrienol on EGR-1 and Bax expression in the pancreatic tumor tissue of *LSL-Kras*^{G12D/+};*Pdx-1-Cre* mice using Western blot (Figure 5C) and immunohistochemistry (Figure 5D). δ -Tocotrienol treatment significantly induced EGR-1 and Bax expression in pancreatic tumors compared with that shown in control vehicle or no treatment groups (Figure 5E and 5F). Furthermore, consistent with our *in vitro* data, δ -tocotrienol-induced EGR-1 expression in pancreatic tumors is associated with increased Bax expression as well as with induction of apoptosis. These data support the concept that δ -tocotrienol-induced apoptosis is directly related to induction of EGR-1 and Bax expression.

4. Discussion

Vitamin E δ -tocotrienol, a natural vitamin E available in whole grains and cereals, has gathered much attention as a potential chemopreventive and/or chemotherapeutic agent [5, 23–25]. Numerous studies, utilizing *in vitro* and *in vivo* model systems, have illustrated δ -tocotrienol's capacity to inhibit carcinogenesis and modulate a multitude of signaling pathways associated with cellular growth, apoptosis, angiogenesis, and metastases [3, 4, 6, 26, 27]. However, the underlying molecular mechanisms involved in the anti-tumorigenic activities of δ -tocotrienol, especially in pancreatic cancer, are complex and remain poorly defined. This study sought to investigate the effect of δ -tocotrienol on gene modulation in MiaPaCa-2 human pancreatic cancer cells in order to identify a novel target that may mediate the anticancer activities of this compound and to determine the mechanism involved in its regulation. Our study identified EGR-1 as being an early gene induced by δ -tocotrienol.

The family of EGR proteins encompasses four zinc finger transcription factors termed EGR-1, EGR-2, EGR-3, and EGR-4. All four EGR proteins exhibit a homologous domain structure, indicating that the functions of these proteins are similar. The EGR proteins contain an extended transcriptional activation domain on the N-terminus and a DNA binding domain, consisting of three zinc finger motifs toward the C-terminus. EGR proteins preferentially bind to the GC rich sequence 5'-GCGGGGCG-3', which is very similar to the DNA binding domain of the transcription factor Sp1 [28]. Although genes where competition for EGR-1 and Sp1 binding in composite GC rich DNA binding sites have been identified, there are also genuine EGR-1 and Sp1 controlled target genes showing no cross-regulation of EGR-1 or Sp1 through the same binding site [29]. Additional regulatory complexity of the EGR transcription factors is indicated by an inhibitory domain located between the activation and DNA binding domain of the EGR-1, EGR-2, and EGR-3 proteins that functions as a binding site for the transcriptional co-repressor proteins NGFI-A binding proteins 1 and 2 (NAB1 and NAB2) [30–32]. Both NAB1 and NAB2 block the biological activity of EGR-1. Thus, induction of transcription of the EGR-1 gene may have no biological effect when the transactivation function of EGR-1 is neutralized by NAB1 or NAB2. Many biological functions have been attributed to EGR proteins, including control of neuronal cell death [33], neuronal plasticity [34], proliferation [35], reproduction [36], T cell differentiation [37], macrophage maturation [38], myelination [39], inflammation [40], ossification [41, 42], and muscle spindle formation [43, 44]. EGR-1 has also been connected to the development of human cancers.

The functions of EGR-1 in oncogenesis have been contradictory with reports of both oncogenic and tumor-suppressive functions in tumor cells. On the one hand, high levels of EGR-1 expression have been observed in human prostate cancers and gastric cancers, with the increased EGR-1 expression correlated with advanced stages of malignancy and poor prognosis [45, 46]. Moreover, tumor progression in transgenic mouse models of prostate cancer was reported to be significantly impaired when EGR-1 was not expressed [47]. EGR-1 has been reported to inhibit apoptosis and enhance tumor growth in addition to inducing metastasis related factors *in vitro*, such as the VEGF receptor, *flt1*, and MMP, indicating that EGR-1 might act as a master protein in directing invasion and metastasis

during cancer progression [48, 49]. These observations would suggest that EGR-1 functions as an oncogene in cancer cells. On the other hand, many reports also indicate that EGR-1 acts as a tumor-suppressor gene. EGR-1 is down-regulated in several types of neoplasia and in an array of tumor cell lines. It induces cell growth arrest and apoptosis and is an important factor involved in neuronal apoptosis [50–53]. The contradictory functions of EGR-1 in cancer indicate that the consequences of EGR-1 over- or under-expression might be different, depending on cell context. The varied function of EGR-1 may be dependent on expression of other members of the EGR-1 family, Sp transcription factors, EGR-1 binding repressors, and other factors that are yet to be identified.

EGR-1 has been shown to have a dichotomous function in the human pancreas. Experimental data demonstrate both a proliferative (insulinoma cells and pancreatic β -cells) and proapoptotic (exocrine pancreatic cancer cells) role of EGR-1 in the pancreas. EGR-1 expression is induced in insulinoma cells and pancreatic β -cells following stimulation with either glucose or pregnenolone sulfate [43, 54, 55]. Recent studies have shown that EGR-1 controls insulin biosynthesis via binding to the regulatory region of the pancreatic duodenal homeobox-1 (PDX-1) gene, which encodes a major regulator of insulin gene transcription [56, 57]. RNA interference experiments performed with insulinoma cells suggest that EGR-1 regulates glucose-induced proliferation *in vitro* [58]. EGR-1 also regulates cyclin D1 expression in exendin-4-stimulated insulinoma cells, suggesting that EGR-1 is an essential regulator of the cell cycle in these cells [59]. In addition, Pdx-1 has been shown to stimulate cell replication in pancreatic β -cells, implying that EGR-1 may influence cell proliferation via activating Pdx-1 expression [60]. Transgenic mice expressing a dominant-negative mutant of EGR-1 had significantly smaller pancreatic islets and elevated caspase 3/7 activity, suggesting that reduced proliferation and enhanced cell death may be responsible for the reduction of the islet size in mice that have genetic inhibition of EGR-1 protein function [61]. These results demonstrate that EGR transcription factors are crucial regulators of pancreatic β -cells in controlling insulin biosynthesis, glucose homeostasis, and proliferation. The biological role of EGR-1 in promoting growth and proliferation has also been observed in many cell types in response to mitogens such as T cells [62], astrocytes [63], glioma cells [64], glomerular mesangial cells [65], and keratinocytes [66]. In contrast to the function of EGR-1 as a growth-promoting protein in pancreatic β -cells, there are several reports describing EGR-1 as a pro-apoptotic protein in pancreatic exocrine carcinoma cells. Genistein, a component of soybeans, was noted to inhibit the growth of human pancreatic cancer (Panc-1) cells and induce the expression of EGR-1 in these cells [67]. α -Bisabolol, a sesquiterpene alcohol found in essential oils derived from a variety of plants, was found to induce EGR-1 in human pancreatic cancer cell lines (KLM1, KP4, Panc-1, and MiaPaca-2) and to inhibit the growth and viability of these cells as well as induce apoptosis [68]. Moreover, the study showed that inhibition of the α -bisabolol-induced EGR-1 expression by EGR-1 siRNA in KLM1 cells rescued these cells from α -bisabolol-induced apoptosis. Methyl 2-cyano-3, 11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me), a synthetic triterpenoid derived from glycyrrhetic acid, a bioactive phytochemical in licorice, was shown to induce apoptosis in Panc-1 and Panc-28 human pancreatic cancer cells [69]. This induction of apoptosis was accompanied by induction of EGR-1. It is interesting that several natural products with anticancer activities have been

demonstrated to induce apoptosis in cancer cells via EGR-1 induction. For example, green tea catechin, epicatechin gallate [70], and resveratrol [71] have been shown to induce apoptosis and activate EGR-1 in colorectal cancer cells. The results of our study add vitamin E δ -tocotrienol to the list of natural compounds that induce EGR-1 in cancer cells with mechanistic data.

To elucidate the molecular mechanism by which δ -tocotrienol induces EGR-1 expression, the promoter region spanning -1704 to +21 bp was assessed by luciferase assay in response to δ -tocotrienol. We found that δ -tocotrienol transactivated the EGR-1 promoter and identified putative response elements located within the -875 to -163 bp region (Supplemental Figure S1A). Five SREs that bind serum response factor and ternary complex factors (TCFs) such as ELK-1 or SAP-1 have been identified in this region of the EGR-1 promoter [72, 73]. Since previous studies have found that JNK and c-Jun are required for the induction of EGR-1 gene and c-Jun was noted to be induced by δ -tocotrienol [74], we investigated the role of c-Jun in δ -tocotrienol-induced EGR-1 expression. We demonstrated in MiaPaca-2 human pancreatic cancer cells that δ -tocotrienol-induced EGR-1 expression was associated with phosphorylated c-Jun induction and that δ -tocotrienol EGR-1 expression was suppressed by the JNK inhibitor SP 600125 (Supplemental Figure S1B). These data indicate that induction of EGR-1 expression by δ -tocotrienol is at least in part under the control of the JNK-c-Jun pathway. In addition to the JNK-c-Jun pathway inducing EGR-1 expression, EGR-1 is also known to bind c-Jun directly to augment c-Jun proapoptotic transcriptional activity.

EGR-1 has also been shown to stimulate synthesis of apoptosis mediators via direct promoter activation [20, 75]. EGR-1 acts as a proapoptotic protein, by directly binding to p53, NAG-1, and PTEN promoters. For example, EGR-1 regulates radiation-induced cell apoptosis in solid tumors via p53, PTEN, Bcl-2, and Bax [76–79]. In our study, EGR-1 was up-regulated and apoptosis was induced in MiaPaCa-2 cells (no PTEN expression, mutated TP53) indicating that pancreatic cancer cell apoptosis induced by δ -tocotrienol-induced EGR-1 did not occur via PTEN or p53. As shown by Western blot analysis in Figure 3A and 3B, increased expression of EGR-1 by δ -tocotrienol is associated with induction of Bax expression, which was reduced by silencing of the δ -tocotrienol-induced EGR-1 expression by siRNA to EGR-1 following δ -tocotrienol treatment. Furthermore, consistent with our *in vitro* data, δ -tocotrienol-induced EGR-1 expression in pancreatic tumors of *LSL-Kras^{G12D/+};Pdx-1-Cre* mice is associated with increased Bax expression as well as with induction of apoptosis (Figure 5D). It is of interest that in untreated and control pancreas of this mouse model, EGR-1 has low expression in the cytoplasm and nucleus of acinar cells of the exocrine pancreas, with virtually minimal to no expression in the endocrine pancreas. Moreover, there appears to be no change in expression when acinar cells transformed into ductal cells in early pancreatic neoplasia, such as with pancreatic intraepithelial neoplasia. However, treatment with vitamin E δ -tocotrienol results in a significant increase in the expression of EGR-1 in the nucleus of acinar cells that are transforming into early pancreatic intraductal neoplasia. These cells also have increased Bax expression. Our *in vivo* data support the concept that δ -tocotrienol-induced apoptosis in transformed pancreatic ductal cells is directly related to induction of EGR-1 and Bax expression.

Recently, other investigators reported microarray analyses to identify target genes of tocotrienols in breast cancer cells [80, 81]. They used arrays carrying 1176 genes to analyze the effects of tocotrienol-rich fraction (TRF) from palm oil on MCF-7 and MDA-MB-231 human breast cancer cell lines and found only 3 significantly modulated genes that were common to both cell lines (the c-Myc binding protein MM-1, the 23-kDa highly basic protein, and the interferon-inducible protein 9–27). It is of interest that these investigators observed that EGR-1 was slightly induced by TRF in MCF-7 cells (+1.15) but not in MDA-MB-231 cells (–0.04). We note that, with the exception of EGR-1, the gene expression profiles generated by this study do not overlap with our study. It is likely that the dissimilarities are the result of different tocotrienols used (TRF vs. δ -tocotrienol), cancer cell lines from different tissues (breast vs. pancreas), or shorter exposure times used in our study (3 hours vs. 72 hours). Such results suggest a complex response of cancer cells to tocotrienols, which is dependent on cellular genetic background as well as the purity of the tocotrienol and conditions of exposure. In this study, we used microarray analyses to identify EGR-1 as a potential δ -tocotrienol target gene in conditions in which δ -tocotrienol subsequently caused apoptosis in human pancreatic cancer cells. Knockdown of EGR-1 expression by siRNA significantly reduced the ability of δ -tocotrienol to induce apoptosis in MiaPaCa-2 cells. Furthermore, forced expression of EGR-1 in MiaPaCa-2 cells induced apoptosis, suggesting that EGR-1 behaves as a tumor-suppressive molecule in these human pancreatic cancer cells. The other bioactive vitamin E tocotrienols, β - and γ -tocotrienols, were also shown to inhibit growth and induce apoptosis in pancreatic cancer cells [3]. In addition, β - and γ -tocotrienols also induced EGR-1 in MiaPaCa-2 cancer cells. These data suggest that the induction of EGR-1 mediated by β -, γ -, and δ -tocotrienols in MiaPaCa-2 cancer cells correlates with the anticancer activities of these bioactive vitamin E compounds in these cells.

In conclusion, we found the stress-inducible gene EGR-1 as a most highly induced early gene after δ -tocotrienol treatment in human pancreatic cancer cells. Here, we report for the first time the involvement of δ -tocotrienol in transcriptional regulation of EGR-1 and that this regulation is mediated by JNK-c-Jun signaling pathway. We demonstrate that EGR-1 induces the expression of the pro-apoptotic protein Bax in the presence of δ -tocotrienol which facilitates δ -tocotrienol induction of apoptosis in human pancreatic cancer cells *in vitro* and *in vivo*. These data provide a novel explanation for the anticancer properties of δ -tocotrienol in pancreatic cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Aggarwal B, Nesaretnam K. Vitamin E tocotrienols: life beyond tocopherols. *Genes Nutr.* 2012; 7:1. [PubMed: 21590364]
2. Aggarwal BB, Sundaram C, Prasad S, Kannappan R. Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases. *Biochem Pharmacol.* 2010; 80:1613–31. [PubMed: 20696139]
3. Husain K, Francois RA, Yamauchi T, Perez M, Sebti SM, Malafa MP. Vitamin E delta-tocotrienol augments the antitumor activity of gemcitabine and suppresses constitutive NF-kappaB activation in pancreatic cancer. *Mol Cancer Ther.* 2011; 10:2363–72. [PubMed: 21971120]
4. Husain K, Centeno BA, Chen DT, Fulp WJ, Perez M, Zhang Lee G, et al. Prolonged survival and delayed progression of pancreatic intraepithelial neoplasia in LSL-KrasG12D/+; Pdx-1-Cre mice by vitamin E delta-tocotrienol. *Carcinogenesis.* 2013
5. Husain K, Centeno BA, Chen DT, Hingorani SR, Sebti SM, Malafa MP. Vitamin E delta-tocotrienol prolongs survival in the LSL-KrasG12D/+; LSL-Trp53R172H/+; Pdx-1-Cre (KPC) transgenic mouse model of pancreatic cancer. *Cancer Prev Res (Phila).* 2013; 6:1074–83. [PubMed: 23963802]
6. Hodul PJ, Dong Y, Husain K, Pimiento JM, Chen J, Zhang A, et al. Vitamin E delta-tocotrienol induces p27(Kip1)-dependent cell-cycle arrest in pancreatic cancer cells via an E2F-1-dependent mechanism. *PloS one.* 2013; 8:e52526. [PubMed: 23393547]
7. Bai J, Sui J, Demirjian A, Vollmer CM Jr, Marasco W, Callery MP. Predominant Bcl-XL knockdown disables antiapoptotic mechanisms: tumor necrosis factor-related apoptosis-inducing ligand-based triple chemotherapy overcomes chemoresistance in pancreatic cancer cells in vitro. *Cancer Res.* 2005; 65:2344–52. [PubMed: 15781649]
8. Jones RL, Pepling ME. Role of the antiapoptotic proteins BCL2 and MCL1 in the neonatal mouse ovary. *Biol Reprod.* 2013; 88:46. [PubMed: 23269665]
9. Kontos CK, Christodoulou MI, Scorilas A. Apoptosis-related BCL2-family members: Key players in chemotherapy. *Anticancer Agents Med Chem.* 2014; 14:353–74. [PubMed: 23848200]
10. Sharawat SK, Bakhshi R, Vishnubhatla S, Gupta R, Bakhshi S. BAX/BCL2 RMFI ratio predicts better induction response in pediatric patients with acute myeloid leukemia. *Pediatr Blood Cancer.* 2013; 60:E63–6. [PubMed: 23553948]
11. Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene.* 2006; 25:4798–811. [PubMed: 16892092]
12. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011; 144:646–74. [PubMed: 21376230]
13. Boualleague A, Simo Cheyou ER, Anand-Srivastava MB, Srivastava AK. ET-1-induced growth promoting responses involving ERK1/2 and PKB signaling and Egr-1 expression are mediated by Ca²⁺/CaM-dependent protein kinase-II in vascular smooth muscle cells. *Cell Calcium.* 2013; 54:428–35. [PubMed: 24238620]
14. Cabodi S, Morello V, Masi A, Cicchi R, Broglio C, Distefano P, et al. Convergence of integrins and EGF receptor signaling via PI3K/Akt/FoxO pathway in early gene Egr-1 expression. *J Cell Physiol.* 2009; 218:294–303. [PubMed: 18844239]
15. Chu L, Wang T, Hu Y, Gu Y, Su Z, Jiang H. Activation of Egr-1 in human lung epithelial cells exposed to silica through MAPKs signaling pathways. *PLoS One.* 2013; 8:e68943. [PubMed: 23874821]
16. Eliseev RA, Dong YF, Sampson E, Zuscik MJ, Schwarz EM, O'Keefe RJ, et al. Runx2-mediated activation of the Bax gene increases osteosarcoma cell sensitivity to apoptosis. *Oncogene.* 2008; 27:3605–14. [PubMed: 18223689]
17. Hudson CD, Morris PJ, Latchman DS, Budhram-Mahadeo VS. Brn-3a transcription factor blocks p53-mediated activation of proapoptotic target genes Noxa and Bax in vitro and in vivo to determine cell fate. *J Biol Chem.* 2005; 280:11851–8. [PubMed: 15598651]
18. Shin SY, Kim CG, Lim Y, Lee YH. The ETS family transcription factor ELK-1 regulates induction of the cell cycle-regulatory gene p21(Waf1/Cip1) and the BAX gene in sodium arsenite-exposed human keratinocyte HaCaT cells. *J Biol Chem.* 2011; 286:26860–72. [PubMed: 21642427]

19. Trabosh VA, Daher A, Divito KA, Amin K, Simbulan-Rosenthal CM, Rosenthal DS. UVB upregulates the bax promoter in immortalized human keratinocytes via ROS induction of Id3. *Exp Dermatol*. 2009; 18:387–95. [PubMed: 19054058]
20. Zagurovskaya M, Shareef MM, Das A, Reeves A, Gupta S, Sudol M, et al. EGR-1 forms a complex with YAP-1 and upregulates Bax expression in irradiated prostate carcinoma cells. *Oncogene*. 2009; 28:1121–31. [PubMed: 19137013]
21. Hussein D, Mo H. d-delta-Tocotrienol-mediated suppression of the proliferation of human PANC-1, MIA PaCa-2, and BxPC-3 pancreatic carcinoma cells. *Pancreas*. 2009; 38:e124–36. [PubMed: 19346993]
22. Shin-Kang S, Ramsauer VP, Lightner J, Chakraborty K, Stone W, Campbell S, et al. Tocotrienols inhibit AKT and ERK activation and suppress pancreatic cancer cell proliferation by suppressing the ErbB2 pathway. *Free Radic Biol Med*. 2011; 51:1164–74. [PubMed: 21723941]
23. Ahsan H, Ahad A, Iqbal J, Siddiqui WA. Pharmacological potential of tocotrienols: a review. *Nutr Metab (Lond)*. 2014; 11:52. [PubMed: 25435896]
24. Ji X, Wang Z, Sarkar FH, Gupta SV. Delta-tocotrienol augments cisplatin-induced suppression of non-small cell lung cancer cells via inhibition of the Notch-1 pathway. *Anticancer Res*. 2012; 32:2647–55. [PubMed: 22753722]
25. Pierpaoli E, Viola V, Barucca A, Orlando F, Galli F, Provinciali M. Effect of annatto-tocotrienols supplementation on the development of mammary tumors in HER-2/neu transgenic mice. *Carcinogenesis*. 2013; 34:1352–60. [PubMed: 23430951]
26. Kannappan R, Gupta SC, Kim JH, Aggarwal BB. Tocotrienols fight cancer by targeting multiple cell signaling pathways. *Genes Nutr*. 2012; 7:43–52. [PubMed: 21484157]
27. Viola V, Cifollilli S, Legnaioli S, Piroddi M, Betti M, Mazzini F, et al. Mitochondrial-dependent anticancer activity of delta-tocotrienol and its synthetic derivatives in HER-2/neu overexpressing breast adenocarcinoma cells. *Biofactors*. 2013; 39:485–93. [PubMed: 23361894]
28. Christy B, Nathans D. DNA binding site of the growth factor-inducible protein Zif268. *Proc Natl Acad Sci U S A*. 1989; 86:8737–41. [PubMed: 2510170]
29. Al-Sarraj A, Day RM, Thiel G. Specificity of transcriptional regulation by the zinc finger transcription factors Sp1, Sp3, and Egr-1. *J Cell Biochem*. 2005; 94:153–67. [PubMed: 15523672]
30. Russo MW, Sevetson BR, Milbrandt J. Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription. *Proc Natl Acad Sci U S A*. 1995; 92:6873–7. [PubMed: 7624335]
31. Svaren J, Sevetson BR, Apel ED, Zimonjic DB, Popescu NC, Milbrandt J. NAB2, a corepressor of NGFI-A (Egr-1) and Krox20, is induced by proliferative and differentiative stimuli. *Mol Cell Biol*. 1996; 16:3545–53. [PubMed: 8668170]
32. Thiel G, Kaufmann K, Magin A, Lietz M, Bach K, Cramer M. The human transcriptional repressor protein NAB1: expression and biological activity. *Biochim Biophys Acta*. 2000; 1493:289–301. [PubMed: 11018254]
33. Xie B, Wang C, Zheng Z, Song B, Ma C, Thiel G, et al. Egr-1 transactivates Bim gene expression to promote neuronal apoptosis. *J Neurosci*. 2011; 31:5032–44. [PubMed: 21451041]
34. Wei F, Xu ZC, Qu Z, Milbrandt J, Zhuo M. Role of EGR1 in hippocampal synaptic enhancement induced by tetanic stimulation and amputation. *J Cell Biol*. 2000; 149:1325–34. [PubMed: 10871275]
35. Liao Y, Shikapwashya ON, Shteyer E, Dieckgraefe BK, Hruz PW, Rudnick DA. Delayed hepatocellular mitotic progression and impaired liver regeneration in early growth response-1-deficient mice. *J Biol Chem*. 2004; 279:43107–16. [PubMed: 15265859]
36. Lee SL, Sadovsky Y, Swirnoff AH, Polish JA, Goda P, Gavrilina G, et al. Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science*. 1996; 273:1219–21. [PubMed: 8703054]
37. Safford M, Collins S, Lutz MA, Allen A, Huang CT, Kowalski J, et al. Egr-2 and Egr-3 are negative regulators of T cell activation. *Nat Immunol*. 2005; 6:472–80. [PubMed: 15834410]
38. Gibbs JD, Liebermann DA, Hoffman B. Egr-1 abrogates the E2F-1 block in terminal myeloid differentiation and suppresses leukemia. *Oncogene*. 2008; 27:98–106. [PubMed: 17599039]

39. Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, et al. Krox-20 controls myelination in the peripheral nervous system. *Nature*. 1994; 371:796–9. [PubMed: 7935840]
40. Yan SF, Fujita T, Lu J, Okada K, Shan Zou Y, Mackman N, et al. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nat Med*. 2000; 6:1355–61. [PubMed: 11100120]
41. Levi G, Topilko P, Schneider-Maunoury S, Lasagna M, Mantero S, Cancedda R, et al. Defective bone formation in Krox-20 mutant mice. *Development*. 1996; 122:113–20. [PubMed: 8565822]
42. Ji B, Chen XQ, Misek DE, Kuick R, Hanash S, Ernst S, et al. Pancreatic gene expression during the initiation of acute pancreatitis: identification of EGR-1 as a key regulator. *Physiol Genomics*. 2003; 14:59–72. [PubMed: 12709512]
43. Tourtellotte WG, Milbrandt J. Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor Egr3. *Nat Genet*. 1998; 20:87–91. [PubMed: 9731539]
44. Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S, et al. A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci*. 2001; 4:289–96. [PubMed: 11224546]
45. Eid MA, Kumar MV, Iczkowski KA, Bostwick DG, Tindall DJ. Expression of early growth response genes in human prostate cancer. *Cancer Res*. 1998; 58:2461–8. [PubMed: 9622090]
46. Kobayashi D, Yamada M, Kamagata C, Kaneko R, Tsuji N, Nakamura M, et al. Overexpression of early growth response-1 as a metastasis-regulatory factor in gastric cancer. *Anticancer Res*. 2002; 22:3963–70. [PubMed: 12553019]
47. Abdulkadir SA, Qu Z, Garabedian E, Song SK, Peters TJ, Svaren J, et al. Impaired prostate tumorigenesis in Egr1-deficient mice. *Nat Med*. 2001; 7:101–7. [PubMed: 11135623]
48. Haas TL, Stitelman D, Davis SJ, Apte SS, Madri JA. Egr-1 mediates extracellular matrix-driven transcription of membrane type 1 matrix metalloproteinase in endothelium. *J Biol Chem*. 1999; 274:22679–85. [PubMed: 10428849]
49. Vidal F, Aragones J, Alfranca A, de Landazuri MO. Up-regulation of vascular endothelial growth factor receptor Flt-1 after endothelial denudation: role of transcription factor Egr-1. *Blood*. 2000; 95:3387–95. [PubMed: 10828020]
50. Fernandez-Busquets X, Burger MM. The main protein of the aggregation factor responsible for species-specific cell adhesion in the marine sponge *Microciona prolifera* is highly polymorphic. *J Biol Chem*. 1997; 272:27839–47. [PubMed: 9346930]
51. Liu C, Rangnekar VM, Adamson E, Mercola D. Suppression of growth and transformation and induction of apoptosis by EGR-1. *Cancer Gene Ther*. 1998; 5:3–28. [PubMed: 9476963]
52. Nair P, Muthukumar S, Sells SF, Han SS, Sukhatme VP, Rangnekar VM. Early growth response-1-dependent apoptosis is mediated by p53. *J Biol Chem*. 1997; 272:20131–8. [PubMed: 9242687]
53. Rao TK. Human immunodeficiency virus infection in end-stage renal disease patients. *Semin Dial*. 2003; 16:233–44. [PubMed: 12753686]
54. Thiel G, Cibelli G. Regulation of life and death by the zinc finger transcription factor Egr-1. *J Cell Physiol*. 2002; 193:287–92. [PubMed: 12384981]
55. Thiel G, Muller I, Rossler OG. Expression, signaling and function of Egr transcription factors in pancreatic beta-cells and insulin-responsive tissues. *Mol Cell Endocrinol*. 2014; 388:10–9. [PubMed: 24631481]
56. Eto K, Kaur V, Thomas MK. Regulation of insulin gene transcription by the immediate-early growth response gene Egr-1. *Endocrinology*. 2006; 147:2923–35. [PubMed: 16543365]
57. Eto K, Kaur V, Thomas MK. Regulation of pancreas duodenum homeobox-1 expression by early growth response-1. *J Biol Chem*. 2007; 282:5973–83. [PubMed: 17150967]
58. Garnett KE, Chapman P, Chambers JA, Waddell ID, Boam DS. Differential gene expression between Zucker Fatty rats and Zucker Diabetic Fatty rats: a potential role for the immediate-early gene Egr-1 in regulation of beta cell proliferation. *J Mol Endocrinol*. 2005; 35:13–25. [PubMed: 16087718]

59. Kang JH, Kim MJ, Ko SH, Jeong IK, Koh KH, Rhie DJ, et al. Upregulation of rat *Ccnd1* gene by exendin-4 in pancreatic beta cell line INS-1: interaction of early growth response-1 with cis-regulatory element. *Diabetologia*. 2006; 49:969–79. [PubMed: 16547599]
60. Hayes HL, Moss LG, Schisler JC, Haldeman JM, Zhang Z, Rosenberg PB, et al. Pdx-1 activates islet alpha- and beta-cell proliferation via a mechanism regulated by transient receptor potential cation channels 3 and 6 and extracellular signal-regulated kinases 1 and 2. *Mol Cell Biol*. 2013; 33:4017–29. [PubMed: 23938296]
61. Muller I, Rossler OG, Wittig C, Menger MD, Thiel G. Critical role of Egr transcription factors in regulating insulin biosynthesis, blood glucose homeostasis, and islet size. *Endocrinology*. 2012; 153:3040–53. [PubMed: 22597533]
62. Perez-Castillo A, Pipaon C, Garcia I, Alemany S. NGFI-A gene expression is necessary for T lymphocyte proliferation. *J Biol Chem*. 1993; 268:19445–50. [PubMed: 8366092]
63. Biesiada E, Razandi M, Levin ER. Egr-1 activates basic fibroblast growth factor transcription. Mechanistic implications for astrocyte proliferation. *J Biol Chem*. 1996; 271:18576–81. [PubMed: 8702507]
64. Kaufmann K, Thiel G. Epidermal growth factor and platelet-derived growth factor induce expression of Egr-1, a zinc finger transcription factor, in human malignant glioma cells. *J Neurol Sci*. 2001; 189:83–91. [PubMed: 11535237]
65. Hofer G, Grimmer C, Sukhatme VP, Sterzel RB, Rupperecht HD. Transcription factor Egr-1 regulates glomerular mesangial cell proliferation. *J Biol Chem*. 1996; 271:28306–10. [PubMed: 8910451]
66. Kaufmann K, Thiel G. Epidermal growth factor and thrombin induced proliferation of immortalized human keratinocytes is coupled to the synthesis of Egr-1, a zinc finger transcriptional regulator. *J Cell Biochem*. 2002; 85:381–91. [PubMed: 11948693]
67. Bai J, Sata N, Nagai H, Wada T, Yoshida K, Mano H, et al. Genistein-induced changes in gene expression in Panc 1 cells at physiological concentrations of genistein. *Pancreas*. 2004; 29:93–8. [PubMed: 15257099]
68. Seki T, Kokuryo T, Yokoyama Y, Suzuki H, Itatsu K, Nakagawa A, et al. Antitumor effects of alpha-bisabolol against pancreatic cancer. *Cancer Sci*. 2011; 102:2199–205. [PubMed: 21883695]
69. Jutooru I, Chadalapaka G, Chintharlapalli S, Papineni S, Safe S. Induction of apoptosis and nonsteroidal anti-inflammatory drug-activated gene 1 in pancreatic cancer cells by a glycyrrhetic acid derivative. *Mol Carcinog*. 2009; 48:692–702. [PubMed: 19125423]
70. Cho KN, Sukhtharankar M, Lee SH, Yoon JH, Baek SJ. Green tea catechin (–)-epicatechin gallate induces tumour suppressor protein ATF3 via EGR-1 activation. *Eur J Cancer*. 2007; 43:2404–12. [PubMed: 17764926]
71. Whitlock NC, Bahn JH, Lee SH, Eling TE, Baek SJ. Resveratrol-induced apoptosis is mediated by early growth response-1, Kruppel-like factor 4, and activating transcription factor 3. *Cancer Prev Res (Phila)*. 2011; 4:116–27. [PubMed: 21205742]
72. Sakamoto KM, Bardeleben C, Yates KE, Raines MA, Golde DW, Gasson JC. 5' upstream sequence and genomic structure of the human primary response gene, EGR-1/TIS8. *Oncogene*. 1991; 6:867–71. [PubMed: 2052361]
73. Whitmarsh AJ, Shore P, Sharrocks AD, Davis RJ. Integration of MAP kinase signal transduction pathways at the serum response element. *Science*. 1995; 269:403–7. [PubMed: 7618106]
74. Hoffmann E, Ashouri J, Wolter S, Doerrie A, Dittrich-Breiholz O, Schneider H, et al. Transcriptional regulation of EGR-1 by the interleukin-1-JNK-MKK7-c-Jun pathway. *J Biol Chem*. 2008; 283:12120–8. [PubMed: 18281687]
75. Woo SM, Min KJ, Kim S, Park JW, Kim DE, Chun KS, et al. Silibinin induces apoptosis of HT29 colon carcinoma cells through early growth response-1 (EGR-1)-mediated non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1) up-regulation. *Chem Biol Interact*. 2014; 211:36–43. [PubMed: 24440808]
76. Li YB, Guo CX, Wang ZC, Dong LH, Guan F, Liu Y, et al. Radiosensitization of breast cancer cells by TRAIL-endostatin-targeting gene therapy. *Neoplasma*. 2013; 60:613–9. [PubMed: 23906295]

77. Liu JL, Liu XL, Qian Q, Du Q, Guo SY, Li CJ, et al. Expression of the radio-inducible TK suicide gene controlled by Egr-1 promoter in pancreatic cancer cells: an in vitro experiment. *Zhonghua Yi Xue Za Zhi*. 2008; 88:1952–5. [PubMed: 19062733]
78. Wenke L, Peng L, Xing W, Yujun S, Qi Z, Haibo R, et al. Treatment of brain glioblastoma multiforme with pcDNA3.1-Egr. 1p-p16 combined with gamma knife radiation: an experimental study on nude mice. *Neurol India*. 2013; 61:491–6. [PubMed: 24262451]
79. Zwang Y, Oren M, Yarden Y. Consistency test of the cell cycle: roles for p53 and EGR1. *Cancer Res*. 2012; 72:1051–4. [PubMed: 22315347]
80. Nesaretnam K, Ambra R, Selvaduray KR, Radhakrishnan A, Reimann K, Razak G, et al. Tocotrienol-rich fraction from palm oil affects gene expression in tumors resulting from MCF-7 cell inoculation in athymic mice. *Lipids*. 2004; 39:459–67. [PubMed: 15506241]
81. Nesaretnam K, Meganathan P, Veerasenan SD, Selvaduray KR. Tocotrienols and breast cancer: the evidence to date. *Genes Nutr*. 2012; 7:3–9. [PubMed: 21516480]

Fig. 1.

(A) δ -Tocotrienol-inhibited pancreatic cancer cell proliferation was dose dependent (MTT assay). (B) δ -Tocotrienol (50 μ M, 24 hours) promoted apoptosis in MiaPaCa-2 cells (Annexin V/PI Assay) compared to control vehicle (* P <0.001). (C) δ -Tocotrienol (50 μ M, 12 hours) promoted apoptosis on MiaPaCa-2 cells (TUNEL assay) compared to vehicle control. (D) δ -Tocotrienol significantly promoted apoptosis in MiaPaCa-2 cells after 12 hours (cell death ELISA assay) compared to vehicle control (* P <0.001). Results are mean and SE (bars; n = 3–5). All statistical analyses were performed using ANOVA with Duncan.

Fig. 2.

(A) δ-Tocotrienol (6 hours) induced EGR-1 expression in MiaPaCa-2 cells in a dose-dependent manner using Western blot analysis. (B) δ-Tocotrienol (50 μM) induced EGR-1 expression in MiaPaCa-2 cells in a time-dependent manner using Western blot analysis. (C) β-, -γ, and δ-Tocotrienols (50 μM, 6 hours) were able to induce EGR-1 protein in MiaPaCa-2 cells. (D) δ-Tocotrienol significantly enhanced DNA binding activity of EGR-1 in MiaPaCa-2 cells in a time-dependent manner at 6 and 12 hours compared to at 0 hours (* $P < 0.05$). (E) Expression of EGR-1 induced by δ-tocotrienol was not changed by pretreating the cells with cycloheximide. Results are mean and SE (bars; $n = 3-5$). All statistical analyses were performed using ANOVA with Duncan.

Fig. 3. (A) Western blot of EGR1, Bax, Bcl-2, XIAP, cIAP-1, and survivin in MiaPaCa-2 cells treated with δ -tocotrienol at different time points. δ -Tocotrienol increased EGR-1 protein expression along with Bax protein expression after 3–12 hours. (B) Densitometry of Western blot showing significant ($aP < 0.05$) induction of EGR-1 and Bax expression in MiaPaCa-2 cells 12 hours after δ -tocotrienol treatment. (C) Expression of Bax in MiaPaCa-2 cells after transfection with siRNA-EGR1, δ -tocotrienol, or the combination. Knockdown of EGR1 significantly rescued δ -tocotrienol-induced Bax protein expression ($*P < 0.05$). (D) Expression of Bax in MiaPaCa-2 cells after transfection with pCMV6-EGR1 or pCMV6-vector. Forced expression of EGR-1 in MiaPaCa-2 cells correspondingly induced Bax protein expression. (E) δ -Tocotrienol increased Bax promoter enrichment after EGR-1 ChIP assay compared to vehicle in MiaPaCa-2 cells. NC, Negative control; IP: Immunoprecipitation. Results are mean and SE (bars; $n = 3-5$). All statistical analyses were performed using ANOVA with Duncan.

Fig. 4.

(A) Expression of EGR-1 in MiaPaCa-2 cells treated with siRNA-EGR1, δ-Tocotrienol, or the combination. (B) Cell death ELISA showed that knockdown of EGR-1 significantly rescued apoptosis induced by δ-tocotrienol in MiaPaCa-2 cells (* P <0.05). (C) Effect of δ-tocotrienol and EGR-1 knockdown on Bax promoter activity in MiaPaCa-2 cells. EGR-1 knockdown was rescued by δ-tocotrienol-induced Bax promoter activity. δ-Tocotrienol significantly increased Bax promoter activity compared to vehicle (** P <0.01), whereas EGR-1 knockdown significantly rescued the induced Bax promoter activity (* P <0.02). Results are mean and SE (bars; n = 3–5). All statistical analyses were performed using ANOVA with Duncan.

Fig. 5.

(A) Cell death ELISA showing that forced expression of EGR-1 significantly induced apoptosis in MiaPaCa-2 cells (* $P < 0.05$). (B) Expression of EGR-1 in MiaPaCa-2 cells after transfection with pCMV6-EGR1 or pCMV6-vector using Western blot. (C) Expression of EGR-1 and Bax in the pancreas of Kras^{G12D} mice per treatment group over 12 months. δ-Tocotrienol induced EGR-1 and Bax expression compared to control. N: no treatment; V: vehicle; δ-T3: δ-tocotrienol. (D) Immunohistochemical staining of EGR-1 and Bax in the pancreas of Kras^{G12D} mice per treatment group over 12 months. (E) δ-Tocotrienol significantly increased Bax expression ($P < 0.001$) in pancreatic tumor tissues of Kras^{G12D} mice compared to vehicle or no treatment groups. (F) δ-Tocotrienol significantly increased EGR-1 expression ($P < 0.001$) in pancreatic tumor tissues of Kras^{G12D} mice compared to vehicle or no treatment groups. Results are mean and SE (bars; $n = 3-5$). All statistical analyses were performed using ANOVA with Duncan.

Table 1

Genes either induced or repressed after exposure of MiaPaCa-2 cells to δ -tocotrienol

Gene name	Probe Set ID	Fold Change	
		Array	PCR
EGR-1	201693_s_at	2.51	2.21
SLC20A1	230494_at	1.95	1.86
SPRY4	1566968_at	1.71	1.91
ARRDC3	224797_at	1.40	1.69
JUN	201465_s_at	1.17	1.89
CFLAR	239629_at	1.11	1.12
CAPZA2	240146_at	1.04	1.68
RGS22	223691_at	-1.16	-1.36
SERPINA3	202376_at	-1.16	1.83
DOCK11	238356_at	-1.41	1.95
GJA7	228563_at	-1.55	1.65
GBP2	242907_at	-2.07	-2.41
SLCO2A1	204368_at	-4.04	-2.50
CCL14	205392_at	-6.27	3.49

Abbreviations: EGR-1, early growth response protein 1; SLC20A1, solute carrier family 20, member1; SPRY4, sprouty homolog 4; ARRDC3, arrestin domain containing 3; JUN, v-Jun sarcoma virus 17 oncogene homolog; CFLAR, casp8 and FADD-like apoptosis regulator; CAPZA2, capping protein muscle Z-line, alpha 2; RGS22, regulator of G-protein signaling 22; SERPINA3, serpin peptidase inhibitor, clade A; DOCK11, dedicator of cytokinesis 11; GJA7, gap junction protein, alpha7; GBP2, guanylate binding protein 2; SLCO2A1, solute carrier organic anion transporter family, member 2A1; CCL14, chemokine ligand 14.