**Tobacco Carcinogen-Induced Production of GM-CSF Activates CREB to Promote Pancreatic Cancer**

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**Supplementary Materials and Methods**

**Cell lines, drugs and antibodies**

Murine pancreatic intraepithelial neoplasia (PanIN) lesions were derived from the *LSL-KrasG12D/+; Pdx1Cre/+*(KC), and invasive PDAC (PDA) and liver metastasis (LMP) cell lines were derived from *LSL-KrasG12D/+;Trp53R172H/+; Pdx1Cre/+* (KPC) mouse models of PDAC respectively ([1](#_ENREF_1)) (kindly provided by Dr. Andrew Lowy, University of California). These were maintained as previously described ([2](#_ENREF_2)). The immortalized human pancreatic ductal cell line HPDE6-E6E7 (H6c7) was kindly provided by Dr. M.S. Tsao ([3](#_ENREF_3)) (mycoplasma-negative tested by a PCR detection method using the Sigma Venor-Gem Kit) and was maintained in keratinocyte growth media (Invitrogen) supplemented with human epidermal growth factor and bovine pituitary extract.

666-15, a CREB inhibitor, was purchased from Tocris (Ellisville, MO). Recombinant GM-CSF and GM-CSF blocking antibody was purchased from R&D Systems (Minneapolis, MN) and Life Technologies, Inc. (Gaithersburg, MD), respectively. MK2206, a Akt (1/2/3) inhibitor was purchased from Selleck Chemicals Co. Ltd (Houston, TX). Nitrosamine 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was purchased from Sigma Aldrich (St. Louis, MO).

**Western blotting and phosphokinase array**

Cell lysis and Western blotting was performed as previously described ([4](#_ENREF_4)). Briefly, cells were washed and lysed using RIPA buffer (0.1% SDS, 50 mM Tris·HCl, 150 mM NaCl, 1% NP-40, and 0.5% Na deoxycholate) with protease inhibitor cocktail (Sigma, St. Louis, MO) and PhosSTOP phosphatase inhibitor (Roche, Indianapolis, IN, USA). Lysates were sonicated and centrifuged at 10,000 g for 15 minutes at 40C to collect supernatant. The protein concentration of the lysate was determined by Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Per lane, 35 µg of whole-cell lysate was separated on NuPAGENovex 4-12% Bis-Tris Gels and transferred on iBlot transfer stack using iBlot dry blotting transfer system (Life Technologies). For immune-detection, membranes were incubated with antibodies listed in Supplementary Table 1. The membranes were subsequently incubated with secondary anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch). Finally, the immunoreactive bands were developed with Pierce ECL Western Blotting Substrate (Thermo Scientific) and recorded on blue basic autoradiography film (Bioexpress).

The human tyrosine kinase array was purchased from R&D Systems and used according to the manufacturer's recommended conditions. Both immunoblots and array intensity were then quantified using Image J image analysis software. Statistical analysis was performed using Prism software (Graphpad Software Inc., La Jolla, CA).

**Soft agar assays**

Cells at a density of 5x104 were suspended in media containing 0.33% Select Agar (Invitrogen, Carlsbad, CA) and plated on a bottom layer of media containing 0.5% Select Agar. The plates were incubated at 370C for 2-3 weeks prior to imaging. The colonies formed were photographed and analyzed, again using Image J and Prism software.

**Enzyme-linked immunosorbent assay (ELISA)**

Serum levels of GM-CSF were measured using mouse GM-CSF Quantikine ELISA Kit (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

**Immunofluorescence**

Formalin-fixed, paraffin-embedded tissue samples were cut at 6μm on microtome and dried overnight. Sections were re-hydrated with Histoclear and ethanol solutions of decreasing concentration. Tissue sections were permeabilized by soaking in a 10mM Tris buffer 7.4 with 0.1% Triton X-100. Antigen retrieval was performed by boiling slides in sodium citrate solution (10mM sodium citrate dihydrate pH 6.0, 0.5% Tween-20) for 10 minutes. Sections were blocked for 1 hour at room temperature and then stained with primary antibodies overnight at 4oC. The sections were washed three times and then labeled with secondary antibodies. Slides were again washed three times and then mounted using Vectashield hardset anti-fade medium with DAPI. Fluorescent images were acquired with a Leica DFC3000 camera mounted to a Leica DMi8 Microscope (Leica).

**RNA isolation, preparation and analysis**

Total RNA from cells was isolated using QIAGEN kits (Qiagen, Valencia, CA) and DNase-I treated, quantified by Nanodrop-1000 (Thermo Scientific, Rockford, IL) and assessed for quality using Agilent Bioanalyzer. RNA concentration was determined by UV spectrophotometry. cDNA synthesis was conducted using purified 300ng RNA and Superscript III reverse transcriptase (Roche, Indianapolis, IN) according to the manufacturer’s instructions. PCR amplification of cDNA was carried out using gene-specific primers (Universal Probe Library, Roche, Indianapolis, IN). The amplification protocol consisted of incubations at 95°C for 15s, 60°C for 1 min, and 72°C for 1 min for 45 cycles using the Light Cycler 480 (Roche, Indianapolis, IN).

DNA primers for real-time polymerase chain reaction **(**PCR) were synthesized by Invitrogen Life Technology after sequences were obtained from the Primer Bank software (<http://pga.mgh.harvard.edu/primerbank/>). All quantitative PCR reactions were done in quadruplicate. Statistical analysis of fluorescence was performed using Light Cycler 480 SW Version 1.5 (Roche, Indianapolis, IN). The gene specific primers are listed in Supplementary Table 2.

**Quantitative RT-PCR (qRT-PCR)**

Relative quantitation of CREB1, EGR1, FOS, CDH5, CSF2, PRKG1 and SOWAHC by real-time reverse-transcriptase PCR (RT-PCR) was done using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The cDNA prepared for microarray analysis was diluted to 1 ng/μl and 2 μl was used as a template for RT-PCR in a 25 μl reaction. Forward and reverse primer mix was added (3 μl, 1:1 mix, 0.3 μM each) in SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). RT-PCR cycles consisted of initial denaturing for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C/15s and annealing/extension at 60°C/1 min. Each reaction was performed in triplicate and ‘no-template’ controls were included in each experiment. Dissociation curves were run to eliminate non-specific amplification, including primer-dimers. The cycle threshold (CT) values were normalized to the house keeping gene β-actin and the fold change was calculated using 2T− vC method ([5](#_ENREF_5)).

**RNA sequencing (RNA-seq)**

Samples were processed for RNA-seq using standard methods on the Illumina HiSeq 2000 platform in the Vanderbilt Technologies for Advanced Genomics Next Generation Sequencing Core ([6](#_ENREF_6)). Sequencing was performed in two multiplexed lanes of 100-bp single-end sequencing, which resulted in 75 million mappable reads per lane. The Illumina pipeline was used for base calling and quality filtering of sequence reads ([6](#_ENREF_6)). Subsequent analysis was performed on the GALAXY open platform using the Tuxedo Tools analysis suite. Transcript assembly and abundance estimates of transcripts in fragments per kilobase of exon per million fragments mapped (FPKM) were performed by Cufflinks. Additional filtering removed any transcripts with <0.5 FPKM value. Significant differences in total gene and transcript expression, splice site, transcription start site (TSS) and promoter usage were determined using a false discovery rate (FDR)-adjusted *P*-value (*q*-value) in Cuffdiff, with *q* value) in Cuffdiff, with the Tuxedo Tools analysis suwas performed using PANTHER tools ([7](#_ENREF_7)). The CREB target genes were compiled based on the Chip data and as reported previously ([8](#_ENREF_8),[9](#_ENREF_9)).

**Flow Cytometry**

The PKT mouse pancreas was minced and digested with 1mg/ml Collagenase P (Roche, Indianapolis, IN) and 2mg/ml Collagenase V (Gibco, Grand Island, NY) for 30 minutes at 37ºC as detailed before ([10](#_ENREF_10)). Briefly, the dissociated tissue was filtered to remove the large chunks, washed with PEB, and stained with CD3e APC (Thermo Fisher), CD4 AF700 (eBioscience) and CD8 PerCPCy5.5 (TONBO). Samples were analyzed using BD LSRII flow cytometer and results were analyzed using FLOWJO.

**NNK delivery in *Ptf1aCreER; LSL-KrasG12D/+*mice**

The mice in the treatment groups received 10 ppm of the NNK in the drinking water, and the control mice were given just the drinking water as previously described ([11](#_ENREF_11)). Aqueous solutions of NNK was prepared weekly and stored at 40C. conditions under which they are known to be stable. These solutions were placed in amber plastic water bottles of the mice cages twice weekly and water consumption was recorded. The dose of 10 ppm in the drinking water was comparable to 470–1200 μg/kg/day, considering the daily water consumption and body weights.

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**Supplementary Figures**

**Supplementary Figure S1.** NNK activates CREB which is downstream of Akt signaling. **A.** H6c7 cells were treated with NNK (1 µmol/L) for up to 50 days, after which the cells were harvested and lysates were prepared using the buffer supplied by the (R&D systems). The lysates were incubated on human phospho-kinase array and the remainder of the procedure was followed according to the manufacturer’s instructions. NNK increases the pAkt and pCREB levels and modestly increases the Src family kinases. **B.** MiaPaCa2 cells treated with Akt inhibitor MK2206 inhibited pCREB levels in a dose-dependent manner, confirming pCREB is a downstream effector of Akt signaling.

**Supplementary Figure S2.** GM-CSF and nicotine acetylcholine receptor expression in PDAC cells. **A.** Expression of nicotinic acetylcholine receptors (nAChRs) expressing subunit α7 (α7nAchR) and GM-CSF receptor CSF2Rα in a panel of seven human pancreatic cell lines including PDAC cells. Cells were allowed to grow and reach up to 70-80% confluency. Cell lysates were collected and immunoblotted for CSF2Rα and α7nAChR expression. The blots were subsequently stripped and reprobed for β-actin. **B.** The normalized expression levels of nα7AchR and CSF2Rα in a panel of human pancreatic cells was quantified using Image J image analysis software.

**Supplementary Figure S3.** CREB inhibition using 666-15 drug in PDAC cells. **A.** BxPC3 cells were treated with CREB inhibitor (666-15) in a dose-dependent manner and analyzed for apoptotic Annexin + cells using flow cytometry. **B.** BxPC3 cells were treated with 666-15 in a dose-dependent manner and analyzed for proliferating EdU+ cells using flow cytometry. Mean of each condition assayed in triplicate. **C.** qPCR data of RNA collected from BxPC3 cells treated with 666-15 (250 nmol/L) or DMSO (control) and analyzed to validate the CREB targeted genes downregulated in RNA-seq data. (n=3). \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.

**Supplementary Figure S4. A.** Multiple CREB shRNA MiaPaCa2 and **B.** PANC1 clones were generated and Western blot confirmed attenuated expression of total CREB when compared with sh-Scrambled and parental controls. **C.** Fox1-nu/nu mice with subcutaneously established tumors from both Vector and shCREBPANC1 cells (3 × 106) were treated with or without NNK (n = 5) at 10 mg/100g or vehicle (n = 5) daily by intraperitoneal administration for 30 dafter tumors approached 200 mm3. Tumor volume was measured for a period of approximately 7 weeks using the formula: tumor volume [mm3] = (length [mm]) × (width [mm])2 × 0.52. At the end of the experiment, the tumors were harvested. Growth curves for the respective xenograft tumors are presented as mean ± S.D. of five tumors in each data point. **D.** Vector and shCREB MiaPaCa2 xenografts were treated as indicated and their respective weights were obtained twice a week. \*, P < 0.05; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001; ns, P > 0.05.

**Supplementary Figure S5.** Ingenuity Pathway Analysis (IPA) summary. **A.** Common top seven cellular functions identified by IPA functional analysis that were most significant to gene expression altered by the intake of NNK in PanIN cells. **B.** Overlap among the top ranked correlated gene pairs in two representative data sets (5d and 50d). Venn diagram showing the distribution of gene pairs that are unique or overlapping with other genes. **C.** The list of top analyses confirmed upstream and downstream molecules. **D**. The list of top five diseases obtained from IPA based on differentially expressed genes.

**Supplementary Figure S6.** CREBi reduces **r**ecombinant GM-CSF-dependent increase in number of colonies. HPNE-Kras (**A**) and MiaPaCa2 (**B**) cells were treated with recombinant GM-CSF (rGM-CSF) with or without CREB inhibitor (666-15) and analyzed for number of colonies. Pharmacological inhibition of CREB as single treatment modality significantly reduced rGM-CSF-induced colony formation. Colony size results are represented by eight photographs analyzed from triplicate wells. \*, P < 0.05; \*\*\*, P < 0.001; \*\*\*\*, P < 0.0001; ns, P > 0.05. **C.** Mouse weight assessment during therapeutic intervention.PKT mice were treated as indicated and their respective weights were obtained twice a week.