Am J Cancer Res 2021;11(7):3515-3536
www.ajcr.us /ISSN:2156-6976/ajcr0135128

Original Article

Doxycycline potentiates the anti-proliferation effects of gemcitabine in pancreatic cancer cells

Yong Weon Yi[1], Na Young Park[2], Joo-In Park[2,3], Yeon-Sun Seong[1,4,5], Young Bin Hong[2,3]

[1]Department of Nanobiomedical Science and BK21 PLUS Research Center for Regenerative Medicine, Dankook University, Cheonan, Korea; [2]Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea; [3]Department of Biochemistry, College of Medicine, Dong-A University, Busan 49201, Korea; [4]Department of Biochemistry, College of Medicine, Dankook University, Cheonan 31116, Korea; [5]Graduate School of Convergence Medical Science, Dankook University, Cheonan 31116, Korea

Received May 10, 2021; Accepted June 13, 2021; Epub July 15, 2021; Published July 30, 2021

Abstract: Gemcitabine is often recommended as a first-line treatment for patients with metastatic pancreatic cancer. However, gemcitabine resistance is a major challenge in the treatment of pancreatic ductal adenocarcinoma. Our group serendipitously identified the role of doxycycline as a potentiator of gemcitabine efficacy in pancreatic cancer cells. Doxycycline and gemcitabine co-treatment was significantly more cytotoxic to pancreatic cancer cells compared to gemcitabine alone. Interestingly, doxycycline only exerted synergistic effects when coupled with gemcitabine as opposed to other conventional chemotherapeutics including nucleoside analogs. The anti-clonogenic effects of gemcitabine on pancreatic cancer cells were also enhanced by doxycycline. According to cell cycle analyses, doxycycline prolonged gemcitabine-mediated S phase cell cycle arrest. Further, gene expression profiling analyses indicated that a small set of genes involved in cell cycle regulation were uniquely modulated by gemcitabine and doxycycline co-treatment compared to gemcitabine alone. Western blot analyses indicated that several cell cycle-related proteins, including cyclin D1, p21, and DNA damage inducible transcript 4 (DDIT4), were further modulated by doxycycline and gemcitabine co-treatment. Taken together, our findings indicate that doxycycline enhances the effects of gemcitabine on cell cycle progression, thus rendering pancreatic cancer cells more sensitive to gemcitabine. However, additional studies are required to assess the mechanisms of doxycycline and gemcitabine synergism, which might lead to novel treatment options for pancreatic cancer.

Keywords: Anticancer, doxycycline, gemcitabine, pancreatic cancer, drug resistance, synergism

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal diseases worldwide, with less than 5% five-year survival rates [1]. Current treatment options for PDAC largely encompass surgical and chemotherapy approaches. Surgical resection can improve the malignancy, however, only 10% of patients are eligible for this procedure [2, 3]. For most patients, chemotherapy agents such as gemcitabine alone or in combination with other compounds can be applied as palliative measures [4]. Particularly, gemcitabine (2'-deoxy-2',2'-difluorocytinide), a cytidine analog, has become a standard PDAC treatment [5, 6]. However, the benefits of gemcitabine monotherapy or in combination with other drugs are limited due to chemoresistance. Chemotherapy resistance can be attributed to intrinsic (primary) or acquired (secondary) factors, which are mediated by multiple mechanisms [6-10].

To circumvent gemcitabine resistance, many studies have assessed the efficacy of gemcitabine co-treatment with other chemotherapeutic agents [11]. However, only gefitinib [i.e., an epidermal growth factor receptor (EGFR) inhibitor] rendered meaningful clinical outcomes, as it increased overall survival [12]. Gemcitabine and nab-paclitaxel co-treatment is often used as neoadjuvant therapy, resulting in superior tumor objective response and disease control rates compared with gemcitabine alone [13, 14]. We previously demonstrated that several kinases including SRC family kinas-

es, phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), AKT, transforming growth factor beta receptor I (TβRI), and checkpoint kinase 2 (CHK2) can be targeted to prevent gemcitabine resistance in pancreatic cancer cells [15-21]. Aldehyde dehydrogenases (ALDHs), one of several cancer stem cell (CSC) markers, also reportedly confer drug resistance in pancreatic cancer cells [22, 23]. Nuclear factor (erythroid-derived 2)-like-2 (NRF2), a master regulator of detoxification genes [24, 25], also plays a role in regulating drug resistance in pancreatic cancers [26]. Interestingly, knockdown of *NRF2* suppressed the expression of *ALDH1A1* and *ALDH3A1* in pancreatic cancer cells, leading to sensitization of pancreatic cancer cells to 5-fluorouracil [23].

Our group has investigated the mechanisms of drug resistance in pancreatic and breast cancer cells for several years [15-32]. Recently, we identified a gene that strongly modulates gemcitabine resistance in pancreatic cancer cells (unpublished observation). While characterizing the function of this gene via Tet-On inducible system-based conditional knockdown, we serendipitously found that doxycycline, the antibiotic inducer of the Tet-On system, affects the viability of pancreatic cancer cells in the presence of gemcitabine. Therefore, the present study sought to evaluate the synergistic effects of doxycycline and gemcitabine co-treatment in pancreatic cancer cells, as well as to provide preliminary insights into the potential molecular mechanisms that mediate this synergism.

Materials and methods

Cell culture and reagents

Human pancreatic cancer cell lines (PANC1, Colo357, MiaPaCa2, and AsPC1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MiaPaCa2 and PANC1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2.5% horse serum and 10% fetal bovine serum (FBS) (for MiaPaCa2) or 5% FBS (for PANC1). AsPC1 and Colo357 were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with FBS (20% for AsPC1 and 10% for Colo357) and 1% sodium pyruvate. Gemcitabine-resistant cell lines (MiaPaCa2-GR and AsPC1-GR) were generated as described in a previous study [18, 22]. The cells were monitored via trypan blue dye exclusion-based cell counting [33].

Except for cisplatin, all compounds were dissolved according to the manufacturer's recommendations, after which small aliquots were stored at -20°C. Cisplatin was dissolved in a 0.9% NaCl solution and stored at 4°C [34].

Cell viability and drug combinations

To determine cell viability, the cells were plated onto 96 well plates. After treating the cells for 72 h with gemcitabine or doxycycline, cell viability was measured using the MTT (3-(4,5-dimethyl) thiazole) assay [19]. After a 2 h incubation with 20 ml of 5 mg/ml MTT solution per 100 ml of growth medium, the media were removed and MTT solvent (4 mM HCl and 0.1% Nonidet-40 in isopropanol or absolute DMSO) was added to dissolve the formazan. The absorbance of each well was measured using an ELx808 microplate reader (BioTek, Winooski, VT). Viable cells were calculated as a percentage of the control. The combination index at the half maximal effective concentration (CI_{50}) of each drug combination was determined using the CompuSyn software (ComboSyn Inc., Paramus, NJ) [16].

Clonogenic colony forming assay

Cells were seeded onto 6-well plates and treated with drugs as indicated above. The colonies were then stained using 0.5% crystal violet in DPBS containing 25% methanol as previously described [35].

Cell cycle analysis

MiaPaCa2 cells cultured with gemcitabine or doxycycline for 24 h were harvested and fixed with 70% ethanol. Afterward, the cells were treated with a DNA staining solution [3.4 mM Tris-Cl (pH 7.4), propidium iodide, 0.1% Triton X-100 buffer, and 100 μg/ml RNase A] and analyzed with a FACSort system (Becton Dickson, San Jose, CA). Cell cycle distributions were determined using the ModFit software (Verity Softwarehouse, Topsham, ME). At least 20,000 events were collected and analyzed for each measurement as previously described [36].

Western blot analysis

Standard western blot analyses were conducted to assess the effects of different treatments

and co-treatments on the protein expression levels of pancreatic cells. MiaPaCa2 cells treated with gemcitabine (2 μM) or doxycycline (2 μg/ml) were harvested, after which the total proteins were loaded into SDS-PAGE gels and transferred onto PVDF membranes. The primary antibodies used in this study included anti-phospho (p)-53-binding protein 1 (53BP1), anti-DNA damage inducible transcript 4 (DDIT4) (Cell Signaling Technology, Inc., Boston, MA), anti-budding uninhibited benzimidazole-related 1 (BuBR1), anti-Cyclin B, anti-Cyclin E, anti-Cyclin D1, anti-p21, anti-p-RB1, anti-heat shock protein 90 (HSP90), and anti-β actin antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Moreover, anti-mouse, anti-goat, and anti-rabbit IgG–peroxidase antibodies (Sigma, St. Louis, MO) were used as secondary antibodies and ECL solution (Santa Cruz Biotechnology, San Diego, CA) was used for detection.

Gene expression analyses

Microarray gene expression analyses were conducted using MiaPaCa2 cells treated with gemcitabine (2 μM) or doxycycline (2 μg/ml) for 48 h. Total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, USA), then purified using RNeasy columns (Qiagen, Hilden, Germany). To obtain biotinylated cRNA, the resulting total RNA was then amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX) according to the manufacturer's instructions. The labeled cRNA samples (750 ng) were then hybridized to human HT-12 expression v.4 bead arrays (Illumina, Inc., San Diego, CA) for 16-18 h at 58°C. Array signal detection was conducted using Amersham fluorolink streptavidin-Cy3 (GE Healthcare BioSciences, Little Chalfont, UK) and the arrays were scanned with an Illumina Bead Array Reader Confocal Scanner. Hybridization quality and overall chip performance were then assessed, after which raw data were extracted. Array data were filtered based on a detection p-value < 0.05 (similar to signal to noise). Afterward, the selected gene signal value was log-transformed and normalized via the quantile method. Gene ontology (GO) analysis for significant probe lists was performed using PANTHER (http://www.pantherdb.org/panther/ontologies.jsp) coupled with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The KEGG pathway map was kindly provided by Kanehisa Laboratories (Kyoto University, Kyoto, Japan).

Statistical analyses

The effects of single and combined treatments were assessed using two-tailed Student's t-tests.

Results

Synergistic effects of doxycycline and gemcitabine on the viability of pancreatic cancer cells

To evaluate the synergistic effects of doxycycline and gemcitabine co-treatment, we measured drug sensitivity in four pancreatic cancer cell lines including Colo357, PANC1, AsPC1, and MiaPaCa2. Doxycycline exerted little to no cytotoxic effects on the tested cell lines compared to gemcitabine (**Figure 1**). Further, the cytotoxicity of gemcitabine was more pronounced in the Colo357 and MiaPaCa2 cells than in the PANC1 and AsPC1 cells. However, the addition of doxycycline significantly increased the cytotoxicity of gemcitabine in all cell lines. To further evaluate the synergistic effects of doxycycline and gemcitabine, we assessed cell viability in two gemcitabine-resistant cell lines, AsPC1-GR and MiaPaCa2-GR [18, 22]. Doxycycline co-treatment also increased the sensitivity to gemcitabine in both gemcitabine-resistant cell lines. The combination index at half maximal effective concentration (CI_{50}) of gemcitabine and doxycycline co-treatment was substantially below 1 in four pancreatic cancer cell lines (Colo357, PANC1, MiaPaCa2, and MiaPaCa2-GR), thus confirming the synergism of the drug combination. In contrast, CI_{50} values could not be determined for either the AsPC1 or AsPC1-GR cell lines, as the individual gemcitabine or doxycycline treatments had no observable cytotoxic effects.

Gemcitabine-specific synergistic effects of doxycycline on pancreatic cancer cell viability

Given that doxycycline increases the sensitivity of pancreatic cancer cells to gemcitabine, we assessed whether doxycycline could also sensitize pancreatic cancer cells to other anti-tumor agents. Interestingly, co-treatment of doxycycline with conventional chemotherapeutics such as cisplatin, SN38 (7-ethyl-10-hydroxycamtothecin), and doxorubicin did not

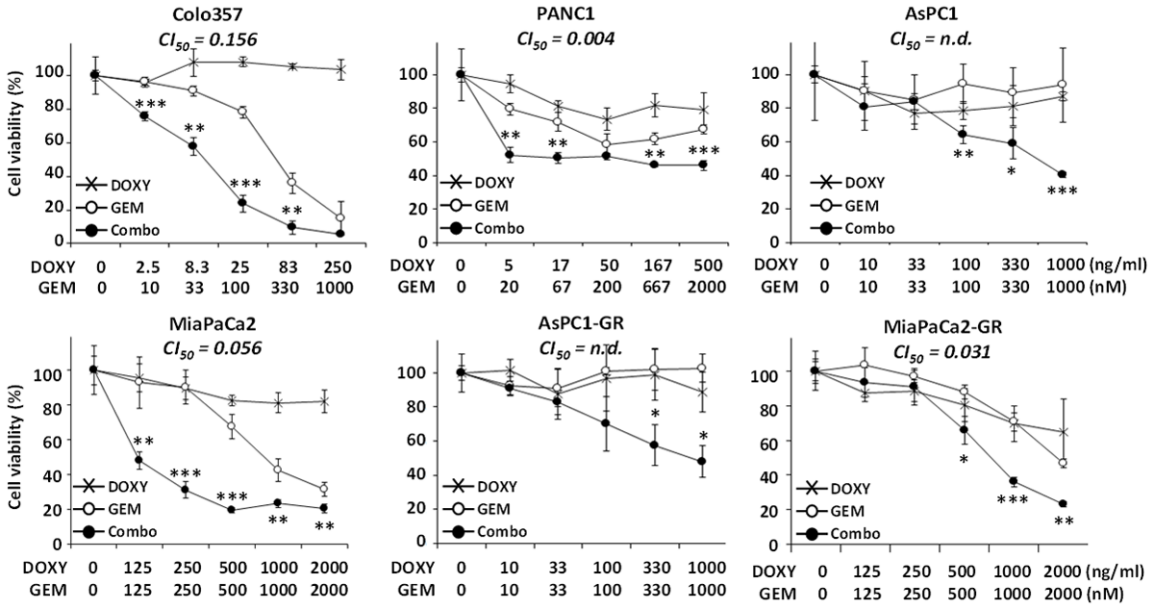

Figure 1. Synergistic effects of doxycycline and gemcitabine on pancreatic cancer cell viability. After exposure to doxycycline (DOXY), gemcitabine (GEM), or their combination for 72 h, pancreatic cancer cell viability was measured using the MTT assay. Combination index at half maximal effective concentration (CI_{50}) of gemcitabine and doxycycline was presented for each cell line. The effects of gemcitabine treatment alone and in combination with doxycycline were assessed using Student's t-test. **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

show synergism in MiaPaCa2 cells compared to the effects of each agent alone, although the CI_{50} index of doxorubicin co-treatment was < 1.0 (**Figure 2A**). Since these drugs have different cytotoxic mechanisms compared to gemcitabine, we next sought to assess whether doxycycline synergism was specifically elicited by nucleoside analogs. However, co-treatment of doxycycline with other nucleoside analogs [5-fluorouracil (5FU), 6-mercaptopurine (6MP), 6-thioguanine (6TG), fludarabine phosphate (FLU), and cladribine (CLA)] rendered no significant synergistic effects on the CI_{50} values of neither MiaPaCa2 nor MiaPaCa2-GR cells (**Figure 2B** and **2C**). Only azacytidine exhibited a synergistic interaction with doxycycline in MiaPaCa2 and MiaPaCa2-GR cells (CI_{50} = 0.005 and 0.650, respectively), however, this synergism was far less potent than that of gemcitabine and doxycycline co-treatment. Taken together, our findings suggest that the synergistic effects of doxycycline are gemcitabine-specific.

Synergistic effects of doxycycline and gemcitabine on MiaPaCa2 cell colony formation and cell proliferation

The combined effects of doxycycline and gemcitabine were further evaluated via long-term clonogenic assays. Incubation of 0.1 μg/ml doxycycline and 10 nM gemcitabine for 10 days almost completely blocked MiaPaCa2 cell colony formation compared to single treatment (**Figure 3A**). These data indicate that doxycycline decreases the survival of MiaPaCa2 cells in combination with gemcitabine. We also measured the effect of this combination on cell proliferation over time. Although doxycycline itself did not affect cell proliferation, gemcitabine-mediated anti-proliferation was significantly augmented by the addition of doxycycline (**Figure 3B**). Importantly, the proliferation of residual pancreatic cancer cells over time, which was monitored 72 h post-treatment, was effectively inhibited by doxycycline treatment. Taken together, our data suggest that doxycycline treatment decreased the survival rates of MiaPaCa2 cells by inhibiting the proliferation of gemcitabine-resistant cells.

Disturbance of cell cycle progression by doxycycline and gemcitabine co-treatment

Cell cycle analyses further demonstrated that doxycycline treatment prolonged gemcitabine-mediated S phase arrest (**Figure 4A**). Doxycycline alone did not affect cell cycle progression, however, the cells exhibited a longer S/G2 phase when treated with a high gemcitabine

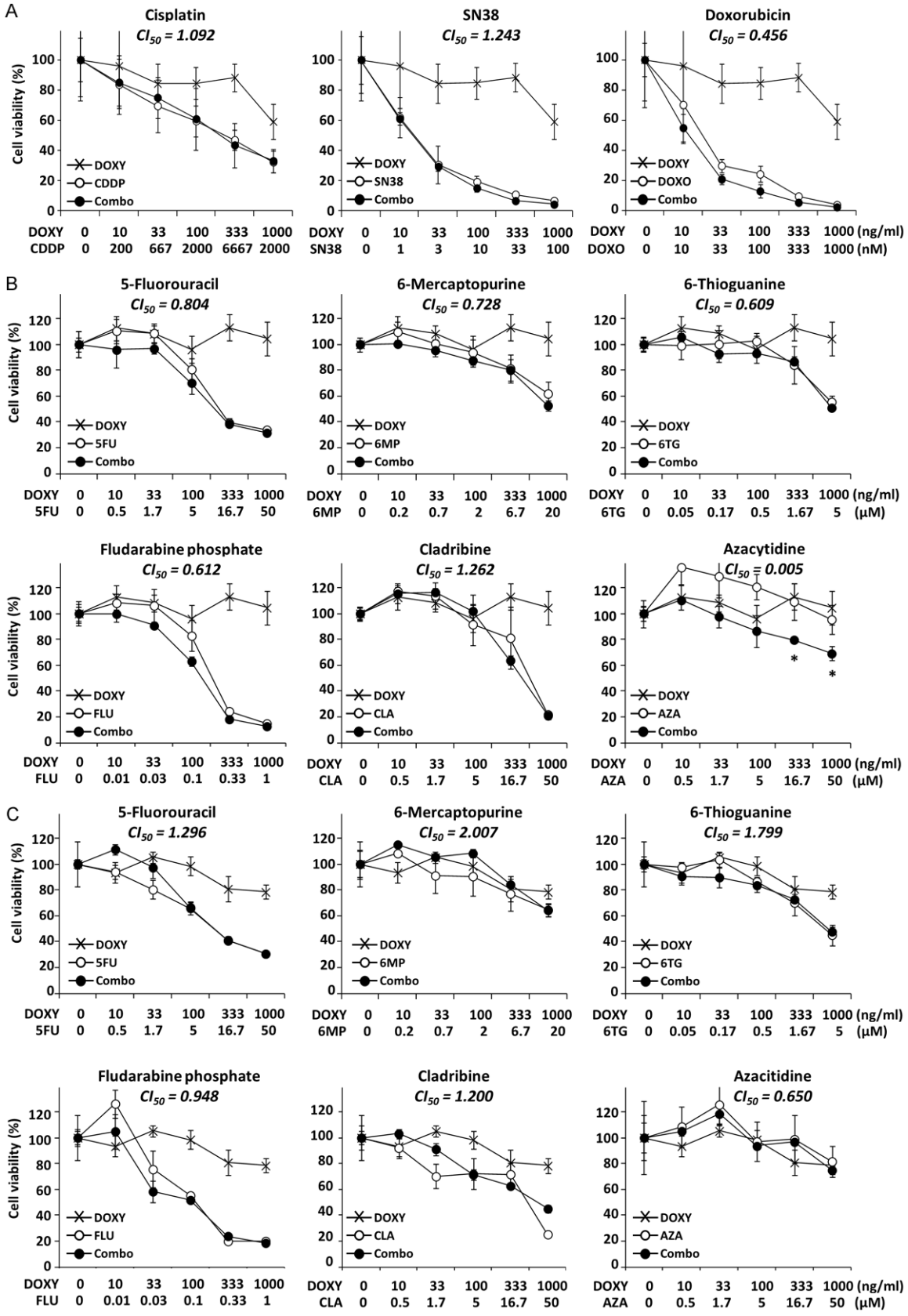

Figure 2. Combined effects of doxycycline and other conventional chemotherapeutics on the viability of MiaPaCa2 or MiaPaCa2-GR cells. A. MiaPaCa2 cells were treated with doxycycline (DOXY) and conventional anticancer drugs such as cisplatin (CDDP), SN38, and doxorubicin (DOXO). B. MiaPaCa2 cells were treated with doxycycline (DOXY)

and nucleoside analogs including 5-fluorouracil (5FU), 6-mercaptopurine (6MP), 6-thioguanine (6TG), fludarabine phosphate (FLU), cladribine (CLA), and azacytidine (AZA). After 72 h of incubation with the drugs, cell viability was measured using the MTT assay. CI_{50} between each chemotherapeutic agent and doxycycline was presented. The effects of gemcitabine treatment (alone and combined) were compared via Student's t-test. *$P < 0.05$. C. Combined effect of doxycycline with other nucleoside analogs in MiaPaCa2-GR cells. The cells were treated with doxycycline and other nucleoside analogs for 72 h, after which cell viability was measured using the standard MTT assay. CI_{50} of each chemotherapeutic agent and doxycycline at the half maximal effective concentration.

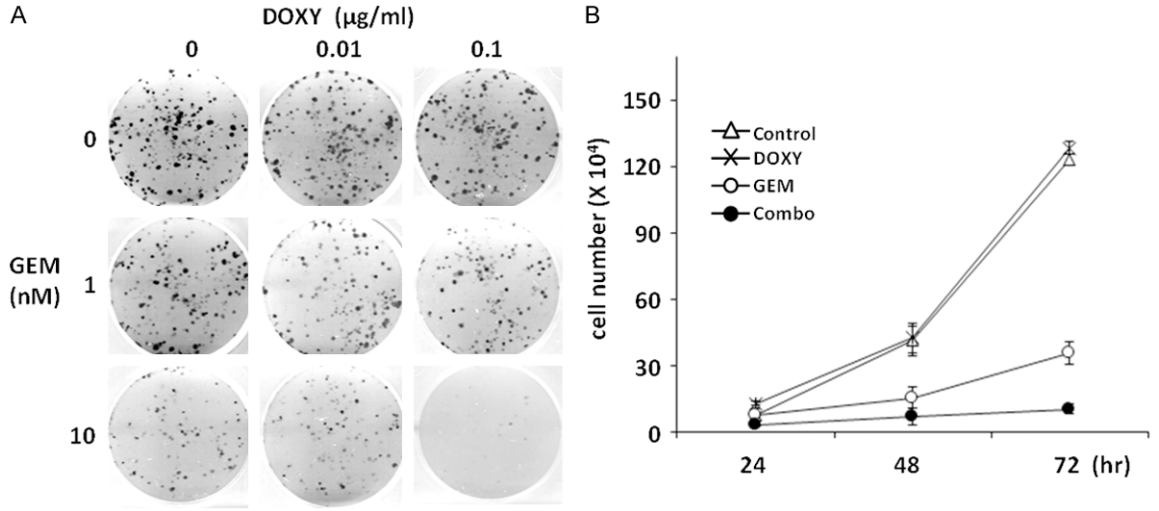

Figure 3. Synergistic effect of doxycycline (DOXY) and gemcitabine (GEM) on the colony-forming ability and proliferation of pancreatic cancer cells. A. Clonogenic assays were performed to demonstrate the long-term synergistic effect of doxycycline and gemcitabine. MiaPaCa2 cells (200 cells per well) were seeded onto 6-well plates and incubated for 24 h, then treated with the indicated drugs in triplicate for 10 days. The colonies were visualized by staining with 5% crystal violet, then washed with distilled water. B. Effect of doxycycline on cell proliferation. MiaPaCa2 cells (1×10^4 cells per well) were incubated with 0.5 μg/ml of doxycycline (DOXY) or 0.5 μM of gemcitabine (GEM). Cell proliferation was determined by direct cell counting over time.

dose (0.5 μM). Under these conditions, doxycycline addition further increased the S phase rather than the G2 phase (**Figure 4B**). Furthermore, apoptotic cell death analysis revealed that doxycycline addition did not increase apoptosis; however, doxycycline and gemcitabine co-treatment slightly increased apoptotic cell death following S phase arrest (**Figure 4C**). Moreover, doxycycline did not significantly increase gemcitabine-induced caspase activity (**Figure 4D**). These results suggest that doxycycline-mediated sensitization of cancer cells to gemcitabine might be due to cell cycle progression delays, which in turn increases apoptosis rates.

Effect of doxycycline and gemcitabine co-treatment on cell cycle-related protein expression

As indicated by our gene expression analyses, doxycycline and gemcitabine co-treatment induced changes in the expression of several genes involved in multiple biological processes such as signal transduction, developmental processes, immunity and defense, cell proliferation and differentiation, and electron transport (**Figure 5A**). However, doxycycline alone did not significantly affect the expression of genes in MiaPaCa2 cells, whereas gemcitabine upregulated genes involved in cell growth, detoxification, and cell cycle regulation (**Figure 5B**). Further, a small set of signaling genes were downregulated by gemcitabine treatment. Interestingly, the effect of gemcitabine on gene expression was dominant, whereas doxycycline exerted little to no effects (**Figure 5B**). Additionally, the gemcitabine-mediated downregulation of various cyclin genes (*CCNA2, CCNB1, CCNB2, CCNC,* and *CCND1*) was sustained in the presence of doxycycline (**Figure 5C**). Among the cyclin-dependent kinase inhibitors (CDKIs) assessed in our microarray analyses, only the *CDKN1A* gene (i.e., the gene that encodes the p21 protein) was upregulated by

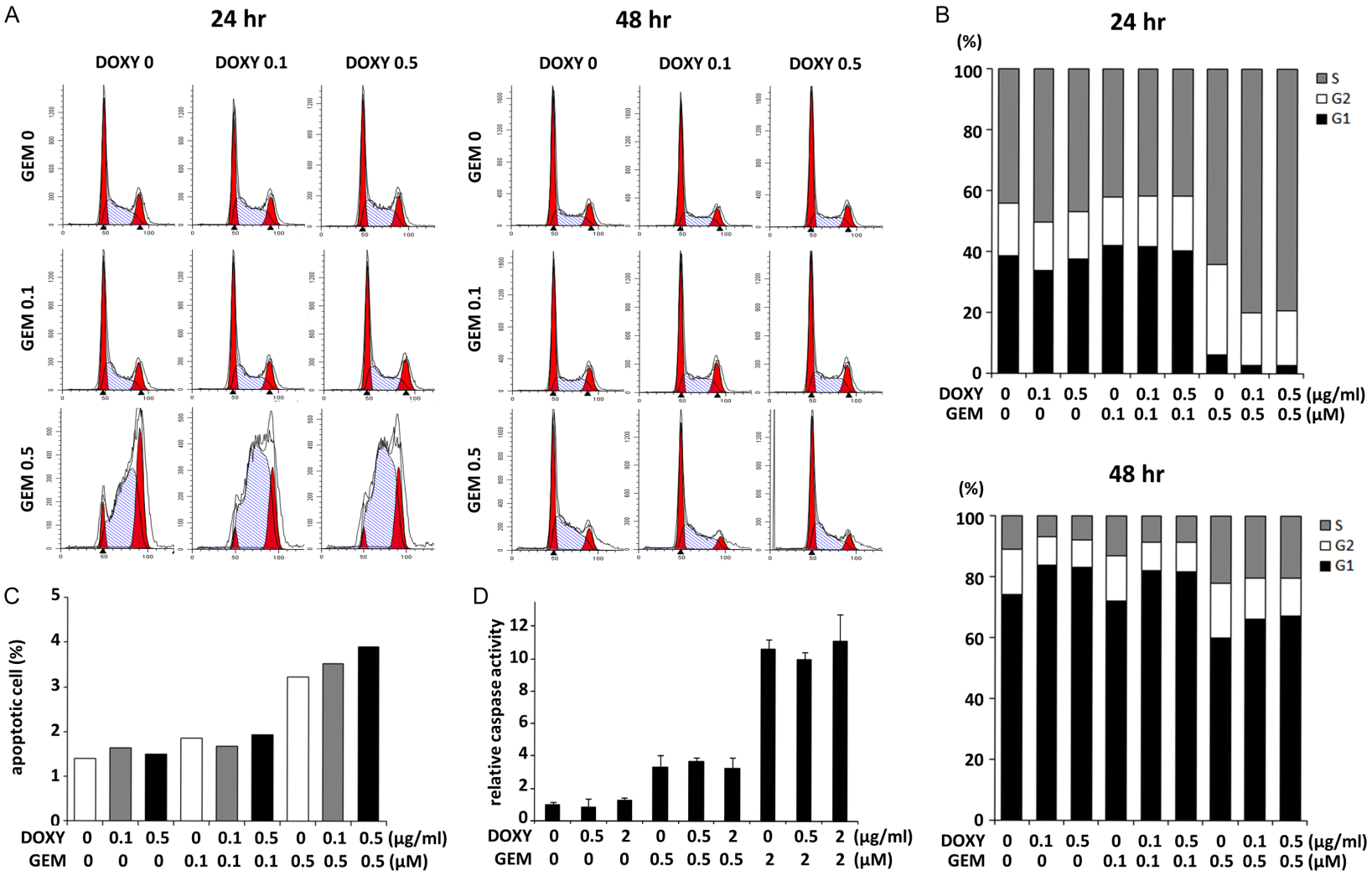

Figure 4. Effects of doxycycline and gemcitabine on cell cycle distribution. MiaPaCa2 cells were incubated with the indicated drugs for 24 or 48 h, after which cell cycle distribution was analyzed. (A) Representative results of flow cytometric analysis of cell cycle distribution. (B) Proportional analysis of cell cycle distribution. (C) Proportion of apoptotic cells obtained from (A). (D) Relative caspase activity in cells treated as described in (A).

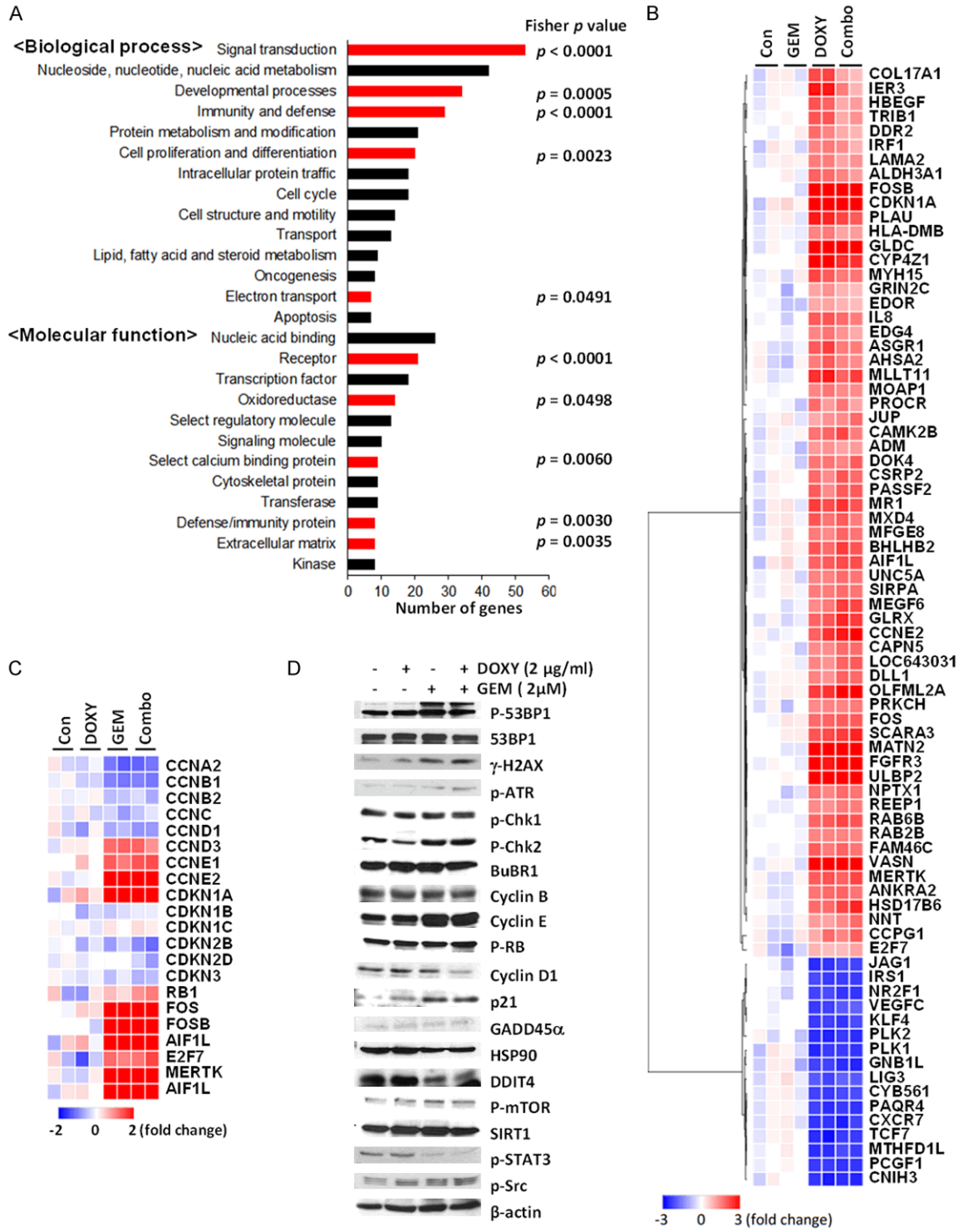

Figure 5. Effect of doxycycline and gemcitabine on gene and protein expression levels. A. MiaPaCa2 cells were incubated with doxycycline (2 μg/ml) or gemcitabine (2 μM) for 48 h, after which gene expression profiles were analyzed using microarrays. Gene ontology (GO) analysis was then conducted for the genes whose expression changed more than 1.5-fold upon gemcitabine treatment. B and C. Dysregulation of cell cycle-related genes (over 1.5-fold change) by gemcitabine compared to the control; the heatmap was generated using the MORPHEUS analysis software (https://software.broadinstitute.org/morpheus/). D. MiaPaCa2 cells were incubated with doxycycline (2 μg/ml) or gemcitabine (2 μM) for 24 h, after which protein expression levels were analyzed by western blot analysis using protein-specific antibodies.

both gemcitabine and doxycycline co-treatment and gemcitabine alone. However, no distinct synergistic effects of doxycycline and gemcitabine co-treatment on MiaPaCa2 cell gene expression were observed.

To further characterize the anti-proliferative effect of doxycycline and gemcitabine co-treatment, we monitored the levels of cell cycle-related proteins via western blot analysis. Doxycycline alone did not induce any changes in the expression levels of the tested proteins, whereas gemcitabine treatment increased the expression of DNA damage-related proteins including p-53BP1, p-ATR, γ-H2AX, p-Chk2, and p-Src (**Figure 5D**). Consistent with our gene expression analyses, the expression levels of the cyclin D1 and cyclin B proteins were downregulated by gemcitabine. In contrast, gemcitabine upregulated cyclin E and p21 protein expression. Further, gemcitabine reduced the expression of heat shock protein 90 (HSP90), DNA-damage induced transcript 4 (DDIT4), and phospho (p)-signal transducer and activator of transcription 3 (STAT3). On the other hand, the gemcitabine-induced upregulation of p-53BP1, p-ATR, γ-H2AX, p-Chk2, cyclin E, p21, and p-Src was preserved or enhanced after doxycycline treatment. More importantly, doxycycline accentuated the downregulation of cyclin D1, DDIT4, and p-STAT3.

Modulation of gemcitabine-mediated gene pathway modifications by doxycycline

To investigate the effects of doxycycline addition on the gemcitabine-induced modification of multiple gene pathways, pathway analysis was conducted using the gene expression analysis results. KEGG pathway maps were constructed as described in the Materials and Methods. The key pathways modified by the gemcitabine-only treatment were those associated with cancer, PI3K signaling, and necroptosis. Specifically, several components of the cancer pathway were modulated by gemcitabine treatment in MiaPaCa2 cells compared to the control. Doxycycline addition affected the gemcitabine-mediated modulation of several components of the aforementioned pathway. Specifically, doxycycline upregulated the expression of AML1, AML1-ETO, AML1-EV1, CBL, GMCSFR, integrin beta (*ITGB*), and TRAFs, whereas it downregulated Grb2, inhibitor of κB (*IκB*), and STAT2/4/6, compared to gemcitabine alone (**Figure 6**). In the PI3K pathway, PI3K, RTK, and TCL1 were upregulated by doxycycline co-administration, whereas ITGA and PKCs were downregulated compared to gemcitabine treatment alone (**Figure 7**). One component of the necroptosis pathway, PGAM5, was distinctly regulated by doxycycline (**Figure 8**). These results suggest that doxycycline fine-tunes the effects of gemcitabine on MiaPaCa2 cells at the gene expression level.

Discussion

In the present study, we serendipitously discovered that the anti-proliferative effects of gemcitabine on pancreatic cancer cells can be further enhanced by doxycycline co-treatment. Doxycycline alone did not affect the viability, long-term survival, cell cycle progression, and protein and gene expression of pancreatic cancer cells. However, doxycycline co-treatment enhanced the anti-proliferation effects of gemcitabine, as well as its cell cycle arrest-, and apoptosis-inducing effects. These two compounds also exhibited synergistic effects on the viability of gemcitabine-resistant pancreatic cancer cell lines. Intriguingly, doxycycline did not exhibit synergistic interactions with other conventional chemotherapeutic drugs such as SN38, cisplatin, and doxorubicin, as well as other nucleoside analogs. Therefore, our results suggest that doxycycline specifically augments the cytotoxicity of gemcitabine. In target cells, gemcitabine is known to be converted into various nucleotides that inhibit enzymes involved in nucleotide metabolism such as DNA polymerase (by difluorodeoxycytidine triphosphate; dFdCTP), ribonucleotide reductase (by difluorodeoxycytidine diphosphate; dFdCDP), and thymidylate synthase (by difluorodeoxyuridine monophosphate; dFdUMP) [37-41]. In contrast, doxycycline is not known to affect any of these enzymes.

Chemoresistance in cancer cells may have multiple mechanisms including the avoidance of intracellular accumulation of anticancer drugs, enhanced detoxification mechanisms, enhanced DNA repair capacity, and activation of survival pathways [42, 43]. Gemcitabine can disrupt DNA replication and activate the S phase checkpoint [44, 45]. However, alterations in certain pathways such as deoxycytidine kinase

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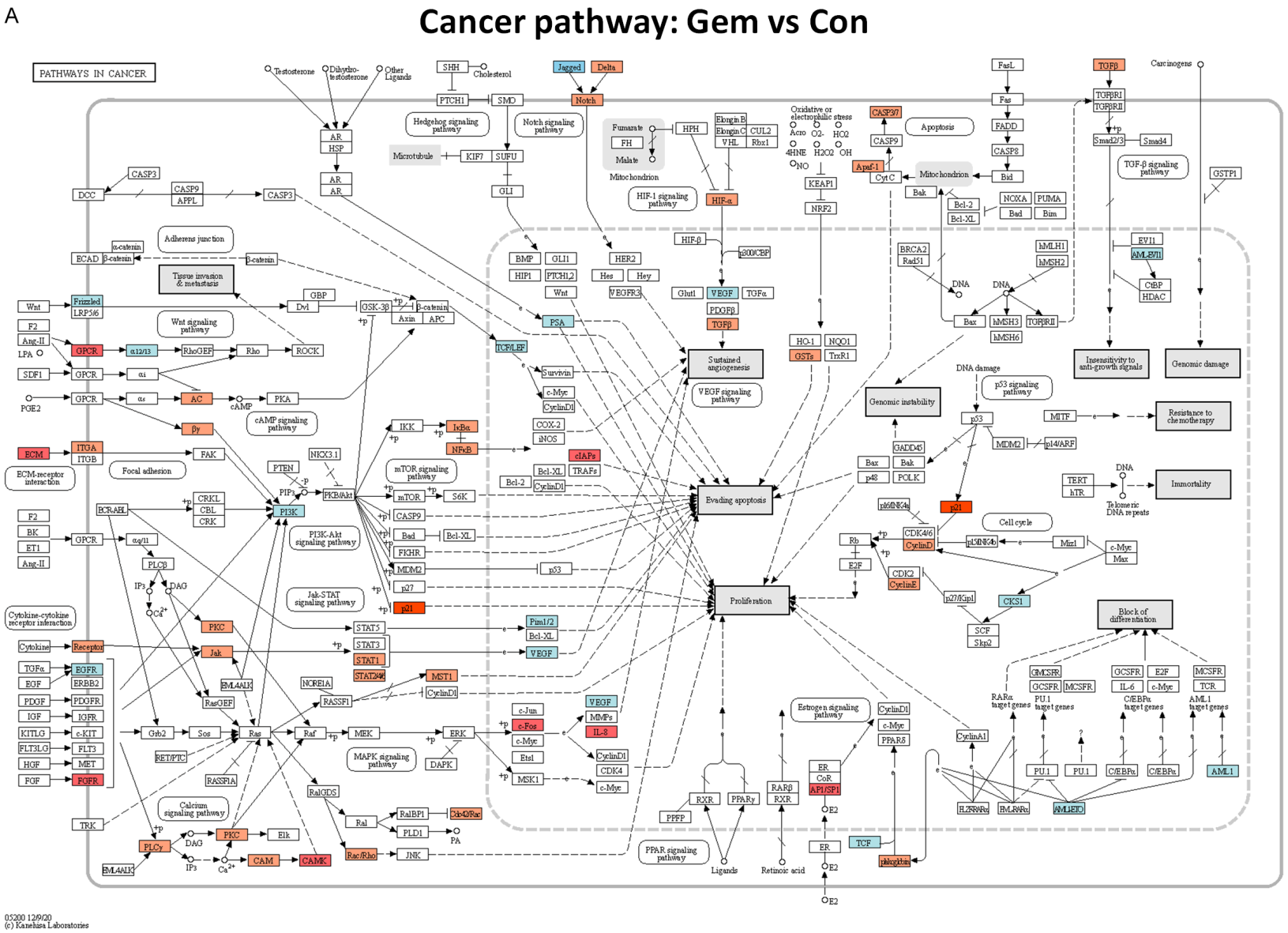
Cancer pathway: Gem vs Con
PATHWAYS IN CANCER
Testosterone
Dihydro-testosterone
Other Ligands
SHH
Cholesterol
Jagged
Delta
PTCH1
SMO
Notch
Hedgehog signaling pathway
Notch signaling pathway
Microtubule
KIF7
SUFU
GLI
AR
HSP
Fumarate
FH
Malate
Mitochondrion
HPH
Elongin B
Elongin C
CUL2
VHL
Rbx1
HIF-1 signaling pathway
HIF-α
HIF-β
p300/CBP
Oxidative or electrophilic stress
Acro
O2-
HCO
4HNE
H2O2
OH
NO
KEAP1
NRF2
CASP3/7
CASP9
Apaf-1
CytC
Apoptosis
FasL
Fas
FADD
CASP8
Bid
Mitochondrion
Bak
Bcl-2
Bcl-XL
NOXA
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Smad2/3
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GSTP1
CASP3
DCC
CASP9
APPL
CASP3
Adherens junction
α-catenin
ECAD
β-catenin
β-catenin
Tissue invasion & metastasis
BMP
GLI1
HIP1
PTCH1,2
HER2
Hes
Hey
Wnt
VEGFR3
Glut1
VEGF
TGFα
PDGFβ
TGFβ
Sustained angiogenesis
VEGF signaling pathway
BRCA2
Rad51
hMLH1
hMSH2
DNA
DNA
Bax
hMSH3
hMSH6
TGFβRII
EVI1
AML-EVI1
CtBP
HDAC
Insensitivity to anti-growth signals
Genomic damage
Wnt
Frizzled
LRP5/6
GBP
Dvl
GSK-3β
β-catenin
Axin
APC
Wnt signaling pathway
PSA
TCF/LEF
Survivin
c-Myc
CyclinD1
F2
Ang-II
LPA
SDF1
GPCR
α12/13
RhoGEF
Rho
ROCK
GPCR
αi
GPCR
αs
AC
cAMP
PKA
PGE2
cAMP signaling pathway
βγ
HO-1
NQO1
GSTs
TrxR1
Genomic instability
DNA damage
p53 signaling pathway
p53
MITF
Resistance to chemotherapy
ECM
ITGA
ITGB
ECM-receptor interaction
FAK
Focal adhesion
PTEN
NKX3.1
PIP3
PKB/Akt
IKK
IκBα
NFκB
COX-2
iNOS
cIAPs
Bcl-XL
TRAFs
Bcl-2
CyclinD1
mTOR signaling pathway
mTOR
S6K
Evading apoptosis
GADD45
Bax
Bak
p48
POLK
MDM2
p14/ARF
TERT
hTR
DNA
Telomeric DNA repeats
Immortality
p16INK4a
p21
Cell cycle
F2
BK
ET1
Ang-II
GPCR
αq/11
BCR-ABL
CRKL
CBL
CRK
PI3K
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CASP9
Bad
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MDM2
p53
p27
p21
CDK4/6
CyclinD
p15INK4b
Mxi1
c-Myc
Max
Rb
E2F
CDK2
CyclinE
p27/Kip1
CKS1
PLCβ
IP3
DAG
Ca2+
Jak-STAT signaling pathway
Proliferation
SCF
Skp2
Block of differentiation
Cytokine-cytokine receptor interaction
PKC
Cytokine
Receptor
Jak
STAT5
STAT3
STAT1
STAT2/p48
Pim1/2
Bcl-XL
VEGF
TGFα
EGF
PDGF
IGF
KITLG
FLT3LG
HGF
FGF
EGFR
ERBB2
PDGFR
IGFR
c-KIT
FLT3
MET
FGFR
EML4ALK
RasGEF
NORE1A
RASSF1
MST1
CyclinD1
Grb2
Sos
Ras
Raf
MEK
ERK
DAPK
MAPK signaling pathway
RET/PTC
RASSF1A
RalGDS
Ral
RalBP1
Cdc42/Rac
PLD1
PA
Rac/Rho
JNK
TRK
Calcium signaling pathway
PLCγ
DAG
IP3
Ca2+
PKC
Elk
CAM
CAMK
EML4ALK
c-Jun
c-Fos
c-Myc
Etsl
MSK1
VEGF
MMPs
IL-8
CyclinD1
CDK4
c-Myc
Estrogen signaling pathway
CyclinD1
c-Myc
PPARδ
CyclinA1
ER
CoR
AP1/SP1
E2
ER
E2
E2
TCF
pkcgbtm
RXR
PPARγ
PPRE
RARβ
RXR
PPAR signaling pathway
Ligands
Retinoic acid
RARα target genes
GMCSFR
GCSFR
MCSFR
PU.1 target genes
PU.1
PU.1
GCSFR
IL-6
c-Mye
C/EBPα target genes
C/EBPα
C/EBPα
MCSFR
TCR
AML1 target genes
AML1
FLZRARα
FMLRARα
AML1EID
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B

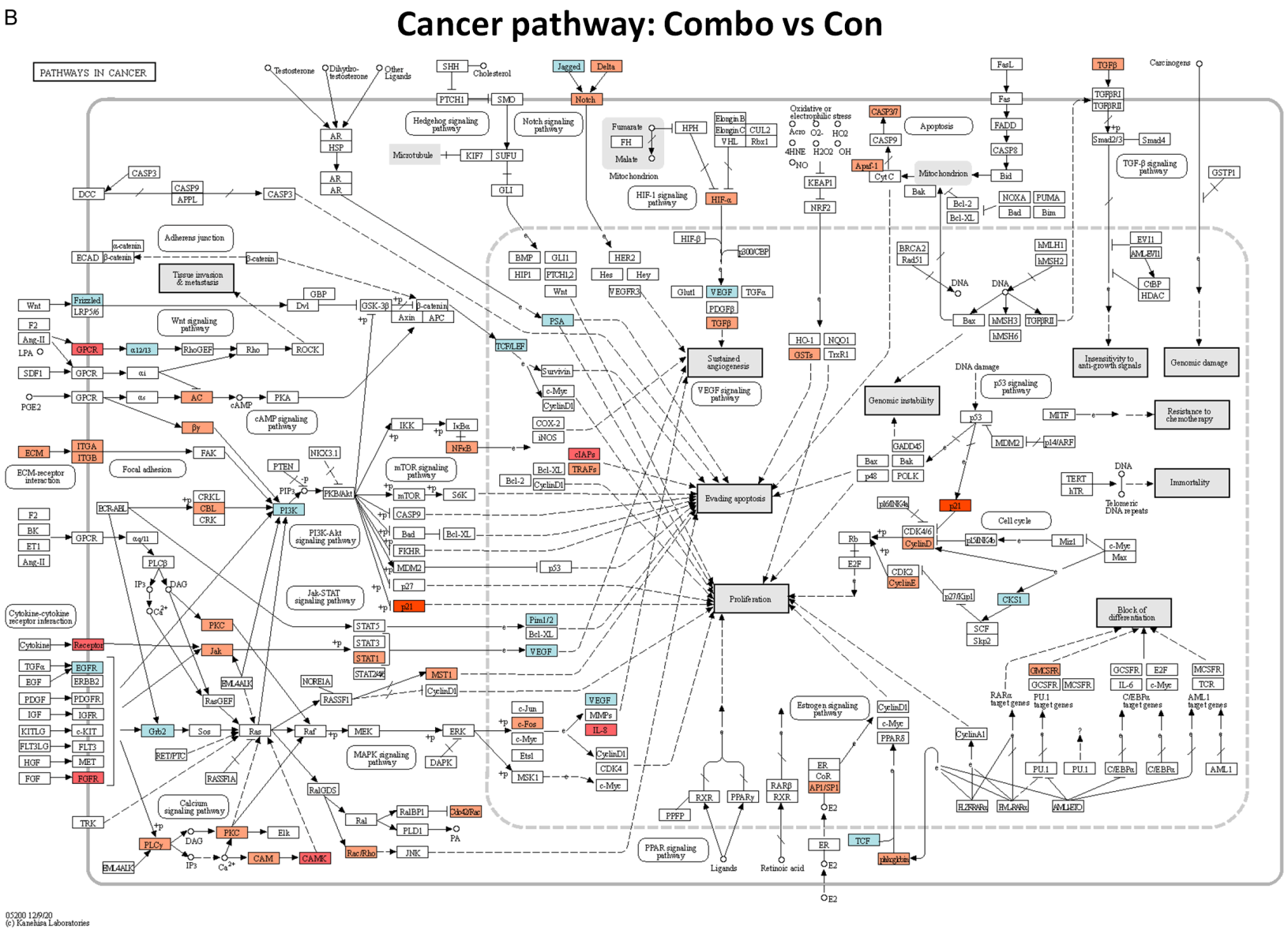
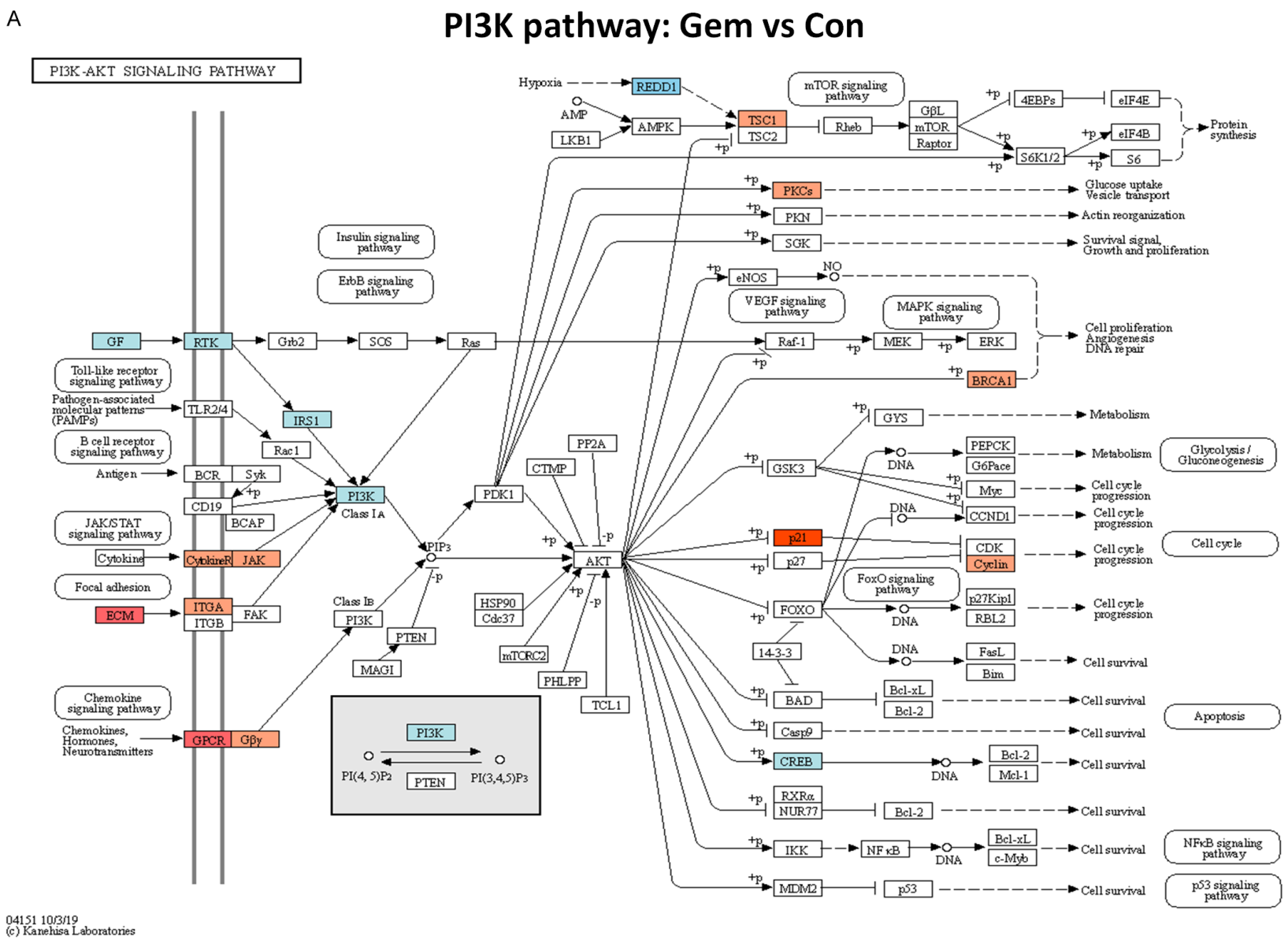


Figure 6. Changes in the cancer pathway analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps of relevant differentially-expressed genes. (A) Changes in the cancer pathway induced by gemcitabine alone and (B) in combination with doxycycline compared to the vehicle control.

A


PI3K pathway: Gem vs Con
PI3K-AKT SIGNALING PATHWAY
Hypoxia
REDD1
AMP
LKB1
AMPK
TSC1
TSC2
mTOR signaling pathway
Rheb
GβL
mTOR
Raptor
4EBPs
eIF4E
eIF4B
S6K1/2
S6
Protein synthesis
PKCs
Glucose uptake
Vesicle transport
PKN
Actin reorganization
SGK
Survival signal,
Growth and proliferation
Insulin signaling pathway
ErbB signaling pathway
eNOS
NO
VEGF signaling pathway
MAPK signaling pathway
GF
RTK
Grb2
SOS
Ras
Raf-1
MEK
ERK
Cell proliferation
Angiogenesis
DNA repair
BRCA1
Toll-like receptor signaling pathway
Pathogen-associated molecular patterns (PAMPs)
TLR2/4
IRS1
B cell receptor signaling pathway
Antigen
BCR
Syk
Rac1
PI3K
Class IA
CD19
BCAP
JAK/STAT signaling pathway
Cytokine
CytokineR
JAK
Focal adhesion
ECM
ITGA
ITGB
FAK
Chemokine signaling pathway
Chemokines, Hormones, Neurotransmitters
GPCR
Gβγ
PIP3
PDK1
CTMP
PP2A
AKT
HSP90
Cdc37
mTORC2
PHLPP
TCL1
Class IB
PI3K
PTEN
MAGI
PI3K
PI(4, 5)P2
PTEN
PI(3,4,5)P3
GYS
Metabolism
GSK3
DNA
PEPCK
G6Pace
Metabolism
Glycolysis / Gluconeogenesis
Myc
Cell cycle progression
CCND1
Cell cycle progression
p21
p27
CDK
Cyclin
Cell cycle progression
Cell cycle
FoxO signaling pathway
FOXO
p27Kip1
RBL2
Cell cycle progression
14-3-3
FasL
Bim
Cell survival
BAD
Bcl-xL
Bcl-2
Cell survival
Casp9
Cell survival
Apoptosis
CREB
Bcl-2
Mcl-1
Cell survival
RXRα
NUR77
Bcl-2
Cell survival
IKK
NFκB
Bcl-xL
c-Myb
Cell survival
NFκB signaling pathway
MDM2
p53
Cell survival
p53 signaling pathway
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B

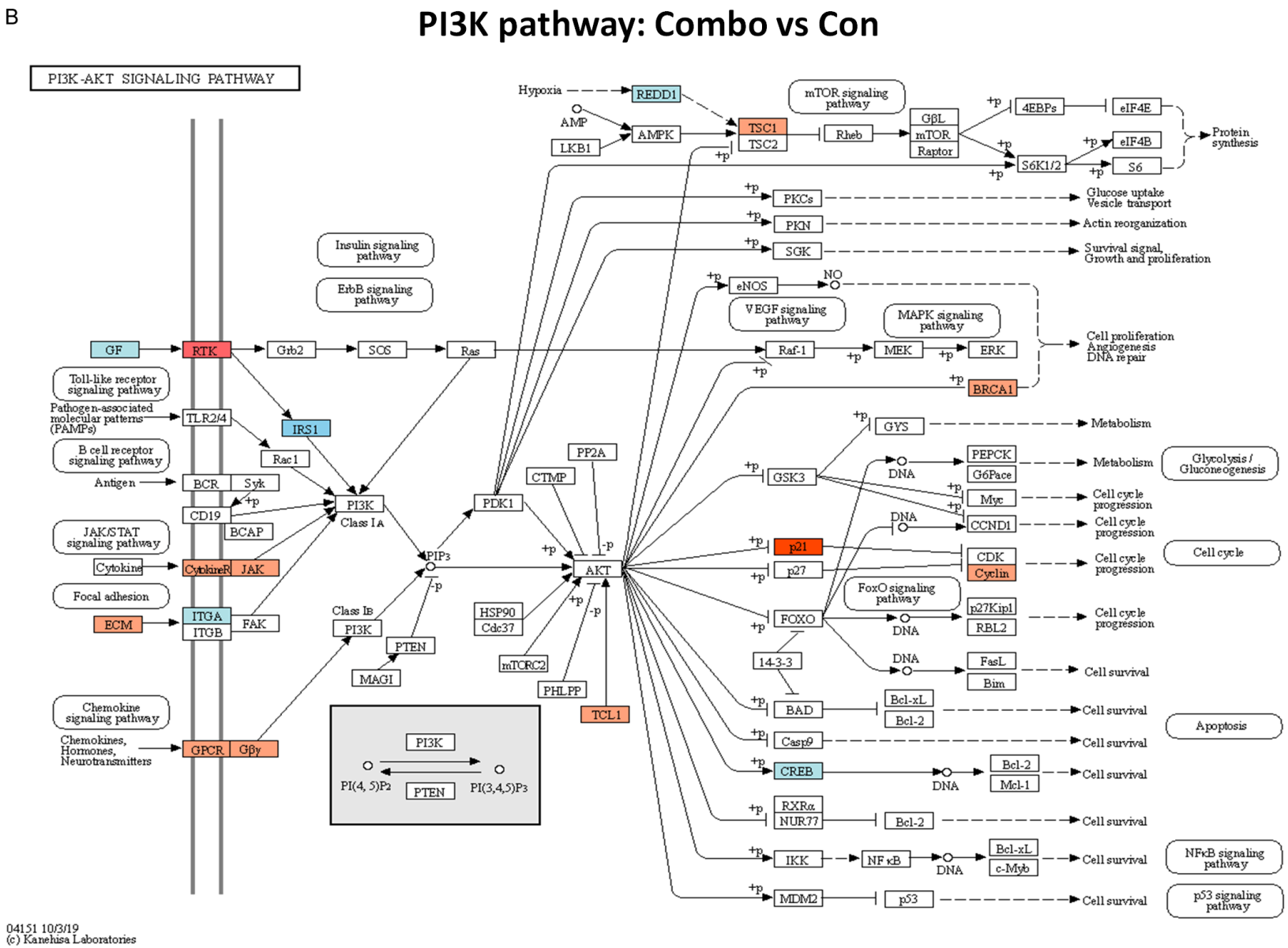
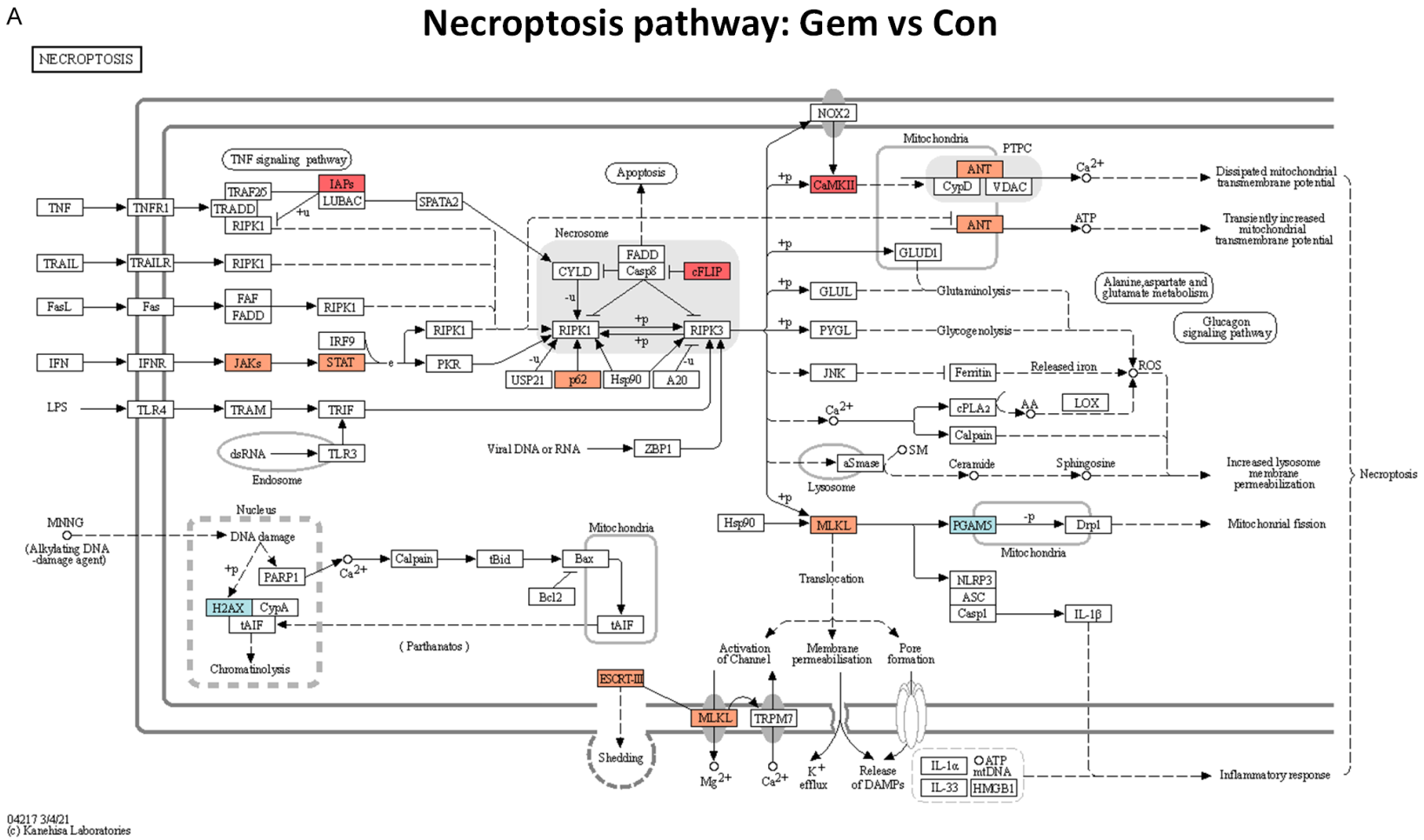


Figure 7. Changes in the PI3K pathway analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps of relevant differentially-expressed genes. (A) Changes in the PI3K pathway induced by gemcitabine alone and (B) in combination with doxycycline compared to the vehicle control.

A



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B

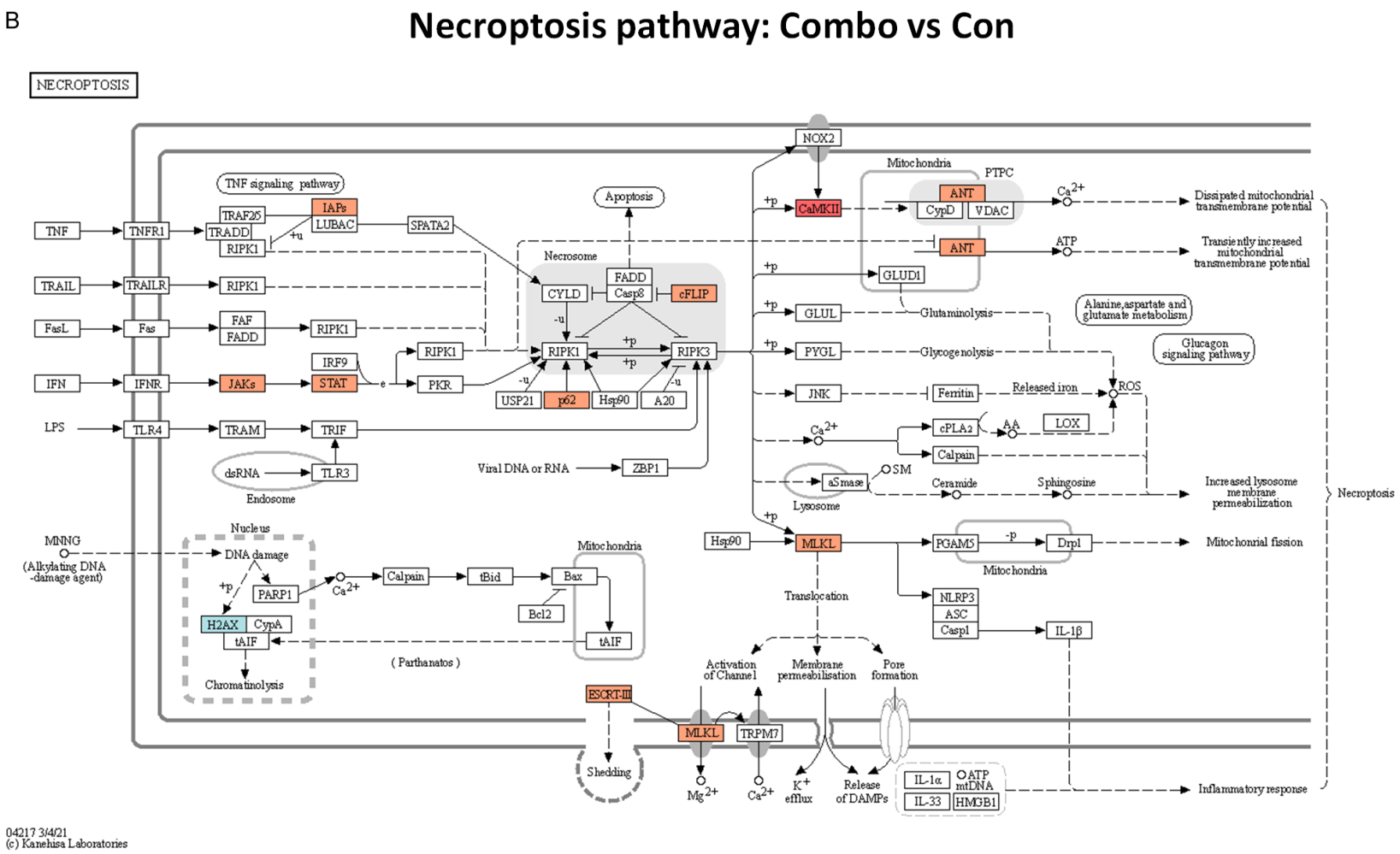

Figure 8. Changes in the Necroptosis pathway analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps of relevant differentially-expressed genes. (A) Changes in the necroptosis pathway induced by gemcitabine alone and (B) in combination with doxycycline compared to the vehicle control.

and ribonucleotide reductase M1 or M2 may confer gemcitabine resistance [46, 47]. Additionally, overexpression of ATP-binding cassette (ABC) transporters can lower the intracellular availability of gemcitabine in pancreatic cancer cells [48, 49]. Further, the induction of several crucial signaling proteins such as NF-κB and AKT can also confer gemcitabine resistance, thereby increasing cell viability or apoptosis in response to gemcitabine cytotoxicity [17, 18, 50, 51]. Recent studies have also linked gemcitabine resistance to the reactivation of several pathways such as Hedgehog, Wnt, and Notch [52].

Gene expression profiles were also analyzed to assess the molecular mechanisms that mediate doxycycline and gemcitabine synergism. Our findings indicated that gemcitabine treatment reduced cyclin D1 expression both at the protein and mRNA levels. Additionally, doxycycline treatment further reduced cyclin D1 levels. Cyclin D1 is involved in the G1-S progression and its overexpression is often observed in human cancer cells [53]. Therefore, cyclin D1 downregulation induces G1 cell cycle arrest [54]. Further, gemcitabine treatment increased p21 levels and this effect was further accentuated by doxycycline co-administration. p21 negatively controls cell-cycle progression into the S phase. The downregulation of cyclin D1 coupled with the upregulation of p21 observed herein might synergistically inhibit cell cycle progression. It is well known that gemcitabine treatment induces G2/S phase arrest [55, 56]. In the present study, we also observed that gemcitabine treatment for 24 h effectively prolonged the S phase in MiaPaCa2 cells. When combined with doxycycline, however, the duration of this gemcitabine-mediated S phase arrest was further increased. Therefore, doxycycline likely enhances pancreatic cancer cell sensitization to gemcitabine by downregulating cyclin D1 and upregulating p21.

Although our gene expression analyses failed to provide a concrete molecular basis for the synergistic interaction between doxycycline and gemcitabine, some of the affected genes may play important roles in the anticancer effects of gemcitabine in MiaPaCa2 cells. Junction plakoglobin (JUP; also known as γ-catenin) has been associated with tumor/metastasis suppressor activity. JUP activates the expression of the tumor suppressor 14-3-3δ by binding its promoter in association with p53 [57]. Additionally, it interacts and restores the tumor/metastasis suppressor activity of mutant p53R175H and promotes the proteasomal degradation of β-catenin [58]. JUP also inhibits the oncogenic activity of β-catenin by sequestering it from the cadherin-β-catenin-α-catenin complex or by inhibiting TCF-mediated β-catenin transactivation [59].

Interestingly, the expression of jagged-1 (JAG1) was downregulated by both gemcitabine alone and in combination with doxycycline (**Figure 5B**). The JAG1 protein is a ligand of the Notch receptor and has been reported to be highly expressed in pancreatic cancer cells at both the mRNA and protein levels [60]. Additionally, silencing JAG1 reportedly confers anticancer effects in vitro and in vivo and enhances the anticancer activity of gemcitabine in pancreatic cancer cells. Consistent with these observations, gemcitabine resistance has been linked to the activation of the Notch pathway [52].

In contrast to our gene expression analysis results, another study indicated that many proteins that are reportedly upregulated in MCF7 human breast cancer cells are downregulated by doxycycline treatment [61]. This discrepancy suggests that doxycycline mainly affects post-translational modifications (PTMs) rather than transcriptional regulation. In fact, both doxycycline and gemcitabine contribute PTM of proteins in pancreatic cancer cells: 1) doxycycline has been reported to induce the caspase-dependent apoptosis in pancreatic cancer cells with concordant increase of phosphorylation in p38, mitogen-activated protein kinase kinase 3/6 (MKK3/6), and MAP kinase-activated protein kinase 2 (MAPKAPK2) [62]; and 2) gemcitabine is also reported to induce PTM by ubiquitin and ubiquitin-like proteins through the canonical and noncanonical WNT and PI3K/AKT/mTORC1 pathways [63]. However, to the best of our knowledge there is no report on proteomic profiling in the gemcitabine and doxycycline co-treated pancreatic cancer cells. Proteomic analyses of specific gene-knockdown in pancreatic cancer cells are also of importance to understand the mechanism of this combination. These studies can provide novel knowledge on the mechanism of conven-

tional drug combination and alternative strategy for treating pancreatic cancer.

The upregulation of p-53BP1, p-ATR, and γ-H2AX was somewhat expected, as gemcitabine is known to induce DNA damage [64-67]. Interestingly, the addition of doxycycline marginally upregulated the gemcitabine-induced p-ATR and γ-H2AX (**Figure 5D**). Ataxia telangiectasia and Rad3-related protein (ATR) is a serine/threonine protein kinase that acts as a DNA damage sensor [68]. Given that the phosphorylation and subsequent activation of ATR are induced by DNA damage [69-71], the synergistic increase of p-ATR induced by both doxycycline and gemcitabine (**Figure 5D**) suggested that ATR kinase activity was further activated under these conditions. Notably, ATR inhibition is known to potentiate gemcitabine cytotoxicity in pancreatic cancer cells [72, 73].

γ-H2AX is a phosphorylated H2AX at S139 and a hallmark of DNA double-strand breaks [74]. Consistent with the prolongation of the S phase by gemcitabine treatment (both alone or in combination with doxycycline combination), γ-H2AX has been associated with the gemcitabine-induced inhibition of DNA synthesis, S phase prolongation, and checkpoint activation [64].

High doxycycline doses (50 μM) reportedly downregulate (i.e., >90% reduction) the expression of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [61]. A previous study using a chemical proteomics approach identified doxycycline as the first FDA-approved DNA-PKcs inhibitor, thus demonstrating the interaction between gemcitabine and DNA-PKcs [66]. Upon gemcitabine treatment, DNA-PKcs and p53 form a protein complex that interacts with the gemcitabine-containing DNA, thus inhibiting DNA synthesis. Therefore, the effect of doxycycline on the interaction between DNA-PKcs and gemcitabine would be an interesting research topic for future studies. Additionally, high doxycycline doses (50 μM) have been reported to reduce the NRF1/2 antioxidant response, as well as STAT1/3, sonic hedgehog (Shh), Notch, WNT, and TGF-β signaling [61]. In our study, doxycycline treatment alone did not affect p-STAT3 expression; however, doxycycline and gemcitabine co-administration accentuated the gemcitabine-mediated downregulation of p-STAT3. These results suggest that the low doses of doxycycline used in this study (2 μg/ml = ~4.5 μM) fine-tuned the gemcitabine-induced downregulation of p-STAT3.

DNA damage-inducible transcript 4 (DDIT4) is a negative regulator of the mammalian target of rapamycin complex 1 (mTORC1) [75], thus promoting tumorigenesis and conferring drug resistance [76-79]. DDIT4 is also known as RTP801 or regulated in development and DNA damage responses 1 (REDD1) [80, 81]. DDIT4, a p53 target gene, was previously found to be upregulated by gemcitabine in wild-type p53 breast cancer cells (MCF7) [82]. To the best of our knowledge, our study is the first to demonstrate the downregulation of DDIT4 at the protein level by gemcitabine in the MiaPaCa2 pancreatic cancer cell line. More importantly, DDIT4 protein expression was further downregulated by the synergistic interaction between gemcitabine and doxycycline. DDIT4 is a mitochondrial and tumor-related protein that plays an important role in drug resistance, as well as cancer cell proliferation and invasion [83]. Although the role of DDIT4 in tumorigenesis remains to be further confirmed, its expression has been reported to be induced by various stressors such as chemotherapy, hypoxia, reactive oxygen species (ROS), exercise, and DNA damage [80, 81, 84, 85]. Overexpression of DDIT4 has been associated with poor prognosis in pancreatic adenocarcinoma, acute myeloid leukemia, breast cancer, colon cancer, lung cancer, multiple glioblastoma, and skin cancer cases [83]. DDIT4 is also known as a convergence node of the hypoxia-inducible factor-1 (HIF-1) and phosphatidylinositol 3-kinase (PI3K), leading to invasive tumor growth in prostate cancer cells [86]. Further, mitochondria are known to accumulate DDIT4 to suppress tumorigenesis. In contrast, DDIT4 depletion increases mitochondrial ROS levels, which upregulates HIF-1 and its target gene *in vitro* and promotes tumorigenesis *in vivo* in a ROS-dependent manner [87]. A recent report demonstrated that doxycycline primes cancer cells for gemcitabine-induced apoptosis *via* inhibition of mitochondrial protein synthesis, which decreases mitochondrial ATP generation [86]. Moreover, doxycycline decreased the inner mitochondrial membrane potential resulting in oxidative stress. However, the role of DDIT4 in the synergistic interaction between

doxycycline and gemcitabine remains to be characterized.

The FDA approved doxycycline in 1967 as a once-a-day broad-spectrum antibiotic, and therefore the safety of this compound has been established for more than half a century [88]. However, additional studies are required to assess the interactions of doxycycline with anticancer agents *in vivo*, as this might lead to the development of novel pancreatic cancer therapies.

Acknowledgements

We would like to thank the late Dr. Insoo Bae, former Georgetown University professor, for his valuable support and assistance in this study. This work was partially supported by the NRF grants funded by the Korea Ministry of Science and ICT (NRF-2016R1A5A2007009, NRF-2019R1F1A1060313 and NRF-2021R1-A2C1007793).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yeon-Sun Seong, Department of Biochemistry, College of Medicine, Dankook University, Cheonan 31116, Korea. Tel: +82-41-550-3875; Fax: 82-41-550-1149; E-mail: seongys@dankook.ac.kr; Dr. Young Bin Hong, Department of Translational Biomedical Sciences, Graduate School of Dong-A University, Busan 49201, Korea. Tel: +82-51-240-2762; Fax: +82-51-240-2971; E-mail: ybhong@dau.ac.kr

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