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Indirubin 3′-Oxime Inhibits Migration, Invasion, and Metastasis *In Vivo* in Mice Bearing Spontaneously Occurring Pancreatic Cancer *via* Blocking the RAF/ERK, AKT, and SAPK/JNK Pathways

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Abstract

BACKGROUND: Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with high invasive and metastatic potential. We generated a spontaneous PDAC mouse model and examined the therapeutic potential of indirubin 3′-oxime (Indox) against PDAC bearing mouse *in vivo*. *METHODS:* Randomized 3-month-old *LSL-Kras$^{G12D/+}$;Trp53$^{flox/+}$;Pdx-1-cre* (*KPC^{flox}*) mice were intraperitoneally injected with 40 mg/kg Indox (n = 9) or a vehicle (n = 10) twice a week. At the end point, tumor status including proliferation, direct invasion, and distant metastasis was analyzed histopathologically. The inhibitory potentials of Indox for proliferation, migration/invasion, and the phosphorylation of target molecules were determined in *KPC^{flox}*-derived PDAC cells *in vitro*. *RESULTS:* Prolonged survival by Indox via intraperitoneal administration was observed in the *KPC^{flox}* mice. Indox inhibited tumor proliferation accompanied with low levels of nuclear phosphorylated cyclin-dependent kinase (p-CDK) and cyclin B1 *in vivo*. Furthermore, Indox inhibited the migration/invasive activities of PDAC via down-regulation of matrix metalloproteinase (MMP)-9 *in vitro* and *in vivo*. Antibody array and immunoblotting analysis revealed that Indox inhibited the phosphorylation of multiple molecules, including key upstream proteins of MMP-9 in RAF/extracellular signal-regulated kinase (ERK), AKT, and stress-activated protein kinase/c-Jun-N-terminal kinase (SAPK/JNK) pathways. *CONCLUSION:* Indox inhibited the proliferative, invasive, and metastatic potentials of PDAC *in vitro* and *in vivo*. Therefore, Indox could a therapeutic candidate for treating spontaneously occurring PDAC via blocking the RAF/ERK, AKT and SAPK/JNK pathways.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with a high mortality rate [1,2]. The 5-year survival rate of PDAC is approximately 8%, which is partially attributed to the difficulty of an early diagnosis, and the post-surgical 5-year survival rate is still around 20%. The poor prognosis in PDAC patients is due to its malignancy such as aggressive invasion and metastasis [3]. Patients with advanced and inoperable PDAC generally receive chemotherapy. A combination chemotherapy regimen using gemcitabine, S-1, and nab-paclitaxel has been demonstrated to prolong the overall survival of PDAC patients [4,5]. However, there are still issues of partially existing chemoresistant PDAC cells and clinical complications from the side effects of the chemotherapy. Therefore, novel anticancer drugs for the treatment of PDAC are necessary.

Indirubin is an active ingredient found in a traditional Chinese herbal medicine, *Danggui Longhui* Wan, which is used for the treatment of patients with chronic myelogenous leukemia [6]. Indirubin and its derivatives block the ATP-binding sites in cell cycle-related kinases such as cyclin-dependent kinases (CDKs) [7−10]. Several studies have shown that indirubin and its derivatives inhibit cell proliferation and partially induce apoptosis by inhibition of CDKs and induction of G2/M arrest in cancer cells [11−13]. We previously reported that indirubin 3′-oxime (Indox) inhibited the proliferation of PDAC cells by down-regulation of p-CDK1/cyclin B1 in PDAC cells *in vitro* and in a xenograft mouse model [14]. However, the inhibitory potentials of Indox against the progression stages, direct invasion, and distant metastasis in spontaneously occurring PDAC remain unclear.

Among the many kinds of mouse models generated for the investigation of PDAC [15], *LSL-Kras*$^{G12D/+}$*;LSL-Trp53*$^{R172H/+}$*; Pdx-1-Cre* (*KPC*) mouse is considered as an adequate model for human PDAC patients [16]. *KPC* mice show hypovascular tumors with abundant stromal reaction (desmoplasia), which is a characteristic phenotype of human PDAC and is considered as a factor in the chemoresistant mechanism in PDAC patients [17]. In the current report, we used *LSL-Kras*$^{G12D/+}$*;Trp53*$^{flox/+}$*;Pdx-1-Cre* (*KPC*^{flox}) mice, which are basically a similar phenotype to the *KPC* mouse, to determine the potential antitumor effects of Indox in spontaneously occurring PDAC.

Materials and Methods

Anticancer Drugs

The indirubin derivative, Indox, was prepared as described previously [14,18].

Genetically Engineered Mice and Animal Care

Three individual strains of *LSL-Kras*$^{G12D/+}$, *Trp53*$^{flox/+}$, and *Pdx-1-cre* mice were obtained from Jackson Laboratory (Sacramento). We crossed and generated the *LSL-Kras*$^{G12D/+}$*;Trp53*$^{flox/+}$*;Pdx-1-cre* (*KPC*^{flox}) mice in house. All animals were kept in specific pathogen-free housing with abundant food and water under guidelines approved by the Nihon University, School of Medical, Animal Care and Use Committee (AP15M001). At 3 months old, the mice were intraperitoneally injected with 40 mg/kg Indox or a vehicle (a mixture of DMSO/PEG400, 1:1 (v/v)) twice a week until the endpoint. The total pancreatic weight and tumor sizes were measured, and their volumes were calculated using the following formula: width x length x height. All samples were embedded in paraffin followed after fixing in 10% neutral buffered formalin.

Immunohistochemistry

Immunohistochemical staining was performed using antibodies against MMP-9 (1:100; Kyowa Pharma Chemical Co.), Ki-67 antigen, p-CDK1, cyclin B1, K-19 antigen, CD31, LYVE-1, and cleaved caspase-3. The number of positive cells in at least four microscopic fields (200×) in a representative specimen were determined using Image J software.

Mouse PDAC Cell Lines

Murine PDAC cell lines (#146, 147 and 244) were established from the primary site in *KPC*^{flox} mice (Supplementary Table 1). Therefore, all of these PDAC cells were genetically induced by *Kras*^{G12D} mutation. All cell lines were maintained at 37°C in 5% CO_2 in D-MEM (Wako Pure Chemical Industries, Ltd.) containing 10% fetal bovine serum (Equitech-Bio Inc.) and 1% penicillin/streptomycin.

Antibody Array Analysis

The mouse PDAC cell line (#146) was treated with 10 μM Indox for 24 h and then was subjected to protein analysis by the antibody arrays based on the instructions that accompanied the antibody array assay kit (Full Moon BioSystems, Inc.). The processed antibody arrays on slides were scanned by a Microarray Scanner System G2565CA and the data obtained were analyzed with Feature Extraction software (Agilent Technologies, Inc.).

Cell Cycle Analysis

Cell cycle analysis was performed using a Cell-Clock Assay Kit (Biocolor Ltd.) on a murine PDAC cell line (#146) treated with 3 or 10 μM Indox for 24 h.

Migration and Invasion Assays

Migration and invasion assays were performed by the method described previously [19]. Cells (2.5×10^4) were plated into either control or Matrigel-coated invasion chamber inserts (Becton Dickinson) and cultured with or without 10 μM Indox for 24 h.

Immunoblotting

Immunoblotting analysis was performed by the method described previously [19]. PDAC cell lines (#146, #147, and #244) were treated with Indox for 24 h. Antibodies to MMP-2, MMP-9 (1:100; Kyowa Pharma Chemical Co.), B-RAF (1:1000; Abcam); p-B-RAF (Ser446), p-ERK (Tyr204), p-AKT (Thr308), SAPK/JNK, p-SAPK/JNK (Tyr183), and p-c-Jun (Thr91) (1:1000; Cell Signaling Technology); Akt, c-Jun, GAPDH (1:1000; R&D systems); MMP-7 and ERK (1:1000; Santa Cruz) were used.

Statistical Analyses

Results are presented as average ± SD or percentage. Data were analyzed using one-way ANOVA with post-hoc Tukey tests. All statistical analyses were performed using SPSS software (version 25.0, IBM SPSS Statistics). P values of $<.05$ were classified to be significant.

Results

Indox Inhibits PDAC Proliferation and Prolongs KPC^{flox} *Mice Survival*

To investigate the antitumor effect of Indox on spontaneous a PDAC bearing mouse model, we generated *KPC*^{flox} mice and

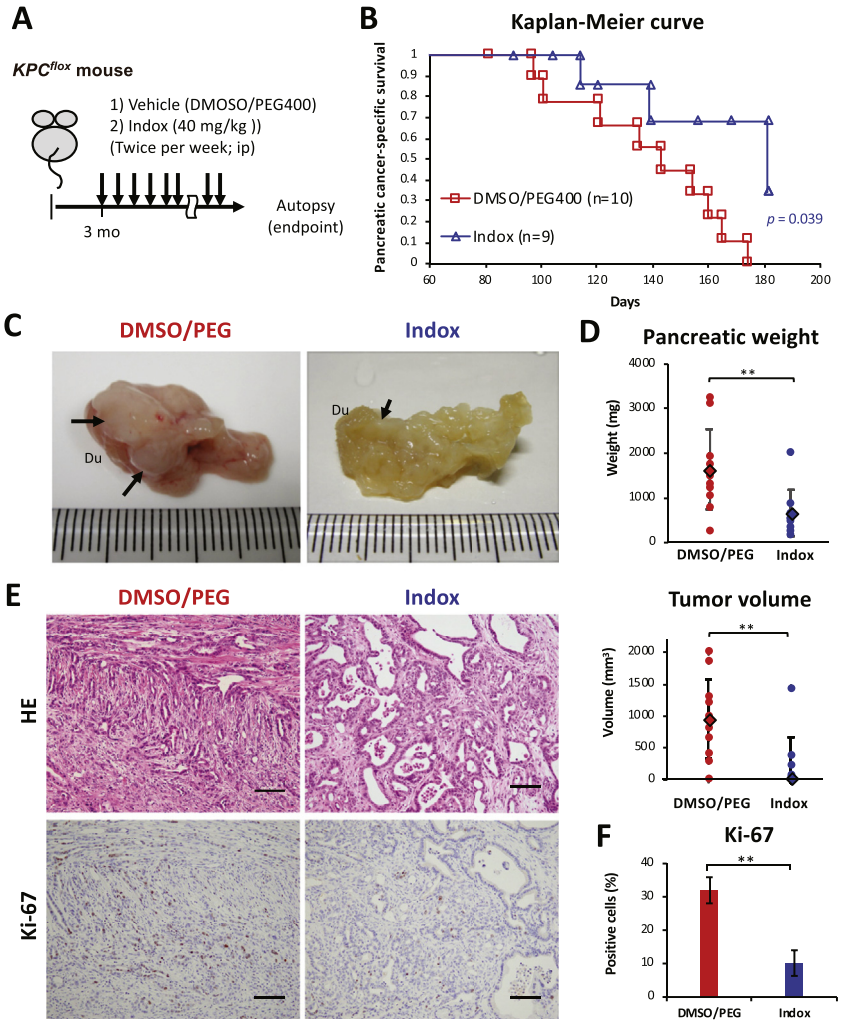

Figure 1. The effects of indirubin 3′-oxime (Indox) on pancreatic ductal adenocarcinoma (PDAC) occurring in a *KPC*^{flox} (*LSL-Kras*$^{G12D/+}$;*Trp53*$^{flox/+}$;*Pdx-1-cre*) mouse.(**A**) Schematic of the experimental treatment regimen. Three-month-old *KPC*^{flox} mice were intraperitoneally injected with 40 mg/kg Indox or vehicle control twice a week until the endpoint. (**B**) Kaplan—Meier survival analysis of the *KPC*^{flox} mice by log-rank test ($P = .039$). (**C**) Gross appearance of representative tumors (arrows) in the pancreas (6 months old). Du, duodenum. (**D**) Total pancreatic weights and pancreatic tumor volumes. Moderately to poorly differentiated PDAC by hematoxylin and eosin (HE) staining and Ki-67 proliferative marker in PDAC (**E**). Scale bars, 100 μm. (**F**) Quantification of data presented in E. *$P < .05$; **$P < .01$ vs. vehicle control by ANOVA Tukey's test.

intraperitoneally injected Indox (Figure 1*A*). Significant prolonged survival ($P = .039$) was observed by Indox administration (Figure 1*B*). The pancreatic tumors that occurred in the *KPC*^{flox} mice were whitish solid nodules with pancreatic atrophy (Figure 1*C*). Tumor volume and total pancreatic weight were reduced by Indox administration (Figure 1*D*). A summary of the phenotypes and pancreas analyses of the mice in the treatment groups is shown in Supplementary Table 1. In the *KPC*^{flox} mice without Indox administration, histopathological evidence of the PDAC differentiated between moderately and poorly with occasional sarcomatoid or anaplastic carcinoma component (Figure 1*E*, Supplementary Table 1). In contrast, well to moderately differentiated PDAC was frequently observed in the *KPC*^{flox} mice who received Indox (Supplementary Tables 1 and 2). Ki-67-positive cell content in the tumor portions were reduced by Indox-treatment (Figure 1, *E* and *F*).

Next, we determined the cell cycle-related molecules. Nuclear p-CDK1- and cyclin B1-positive PDAC cell percentages were immunohistochemically decreased in tumors in the *KPC*^{flox} mice that received Indox (Figure 2, *A* and *B*). Then the effects of Indox on CDKs and cyclins were examined in an established cell line from the PDAC in the *KPC*^{flox} mice. In this case, the PDAC cells were induced by *Kras*^{G12D} mutation. The decrease in the p-CDK1 level in the PDAC cells was supported by antibody array analysis (Figure 2*C*). The array analysis revealed that phosphorylated CDK1, CDK2, and CDK7 levels in the PDAC cells were significantly reduced by Indox treatment *in vitro* while changes of the non-phosphorylated CDKs levels were insignificant. The intensive suppression of phosphorylation on cyclins by Indox was observed on only cyclin D1 (Figure 2*D*). Furthermore, Indox reduced the levels of other cell cycle-associated molecules, including p-ATM, p-WEE1, p-cyclin-dependent kinase inhibitor (p-CKIs), and p-ChKs (Supplementary Figure 1). Since the effect of Indox on the cell cycle of PDAC cells is determined to be G2/M arrest by Cell-Clock Assay (Figure 2, *E* and *F*), we conclude that Indox inhibits PDAC proliferation by suppression of p-CDK1/

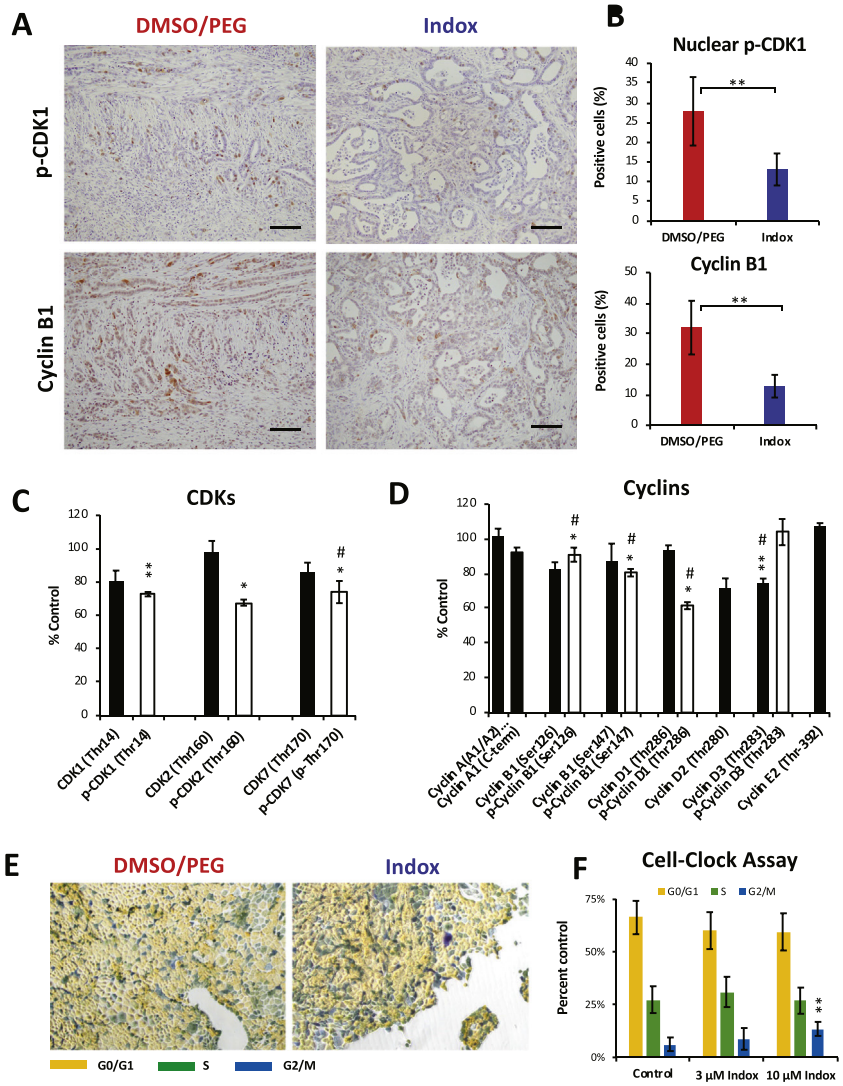

Figure 2. Effects of Indox administration on the proliferation of PDAC in the *KPC*^{flox} mice.(**A**) Cycle-related molecules p-CDK1 and cyclin B1 were markedly decreased in PDAC with the administration of Indox. (**B**) Quantification of the data presented in **A**. Levels of phosphorylated CDKs (**C**) and cyclins (**D**) in murine PDAC cells (#146) by antibody array (n = 6 each). Microscopic features of Cell-clock assay (**E**) and the quantification of data presented in E (**F**). *$P < .05$; **$P < .01$; ***$P < .001$ vs. vehicle control by ANOVA Tukey's test. #n = 5.

p-cyclin B1 levels, thereby inducing G2/M arrest (Figure 5 and Supplementary Figure 2).

Next, we evaluated the effect of Indox on apoptosis in PDAC in *KPC*^{flox} mice. At first we stained the PDAC tissues immunohistochemically using an anti-cleaved caspase-3 antibody and measured the positive areas microscopically. The cleaved caspase-3 positive area was deceased by Indox administration, but it was statistically insignificant (Supplementary Figure 3, *A* and *B*). On the other hand, anti-apoptotic p-BCL-xL and pro-apoptotic p-Bad and p-Bid levels determined by the antibody array analysis were decreased in the PDCA cells *in vitro* by Indox treatment (Supplementary Figure 4*A*). On the caspases 3, 6, 8, and 9 and cytochrome c (inter) in the PDAC cells, by Indox treatment *in vitro*, caspases 3 (phosphorylated/non-phosphorylated), 6 (non-phosphorylated), and 8 (non-phosphorylated) and cytochrome c (inter) levels were reduced significantly while caspases 9 (phosphorylated/non-phosphorylated) and 6 (phosphorylated) levels were not changed (Supplementary Figure 4*B*). This evidence leads us to conclude that the Indox induced cell death of PDAC in *KPC*^{flox} mice is non-apoptotic and is also not related to the modulation of mitochondrial BCL-2 family members and caspase signaling cascades (Supplementary Figure 5).

Indox Inhibits Migration and Invasion Activities of PDAC Cells via Down-Regulation of MMP-9 In Vitro and In Vivo

The pathological status of PDAC bearing *KPC*^{flox} mice is listed in Supplementary Table 2. The invasions of the vascular, lymph, and peripheral organs including the duodenum and common bile duct, metastasis to the lymph nodes, and distant metastasis and/or dissemination in the *KPC*^{flox} mice were remarkably suppressed by Indox administration *in vivo*. We thus determined the PDAC cell distribution in pancreatic tissue, microinvasion, in the *KPC*^{flox} mice by a K-19 antigen as a marker. Microinvasion of the K-19^{+} PDAC cells into the CD31^{+} tumor blood vessels and LYVE-1^{+} lymph vessels were observed in all of the control *KPC*^{flox} mice (Figure 3*A*). In

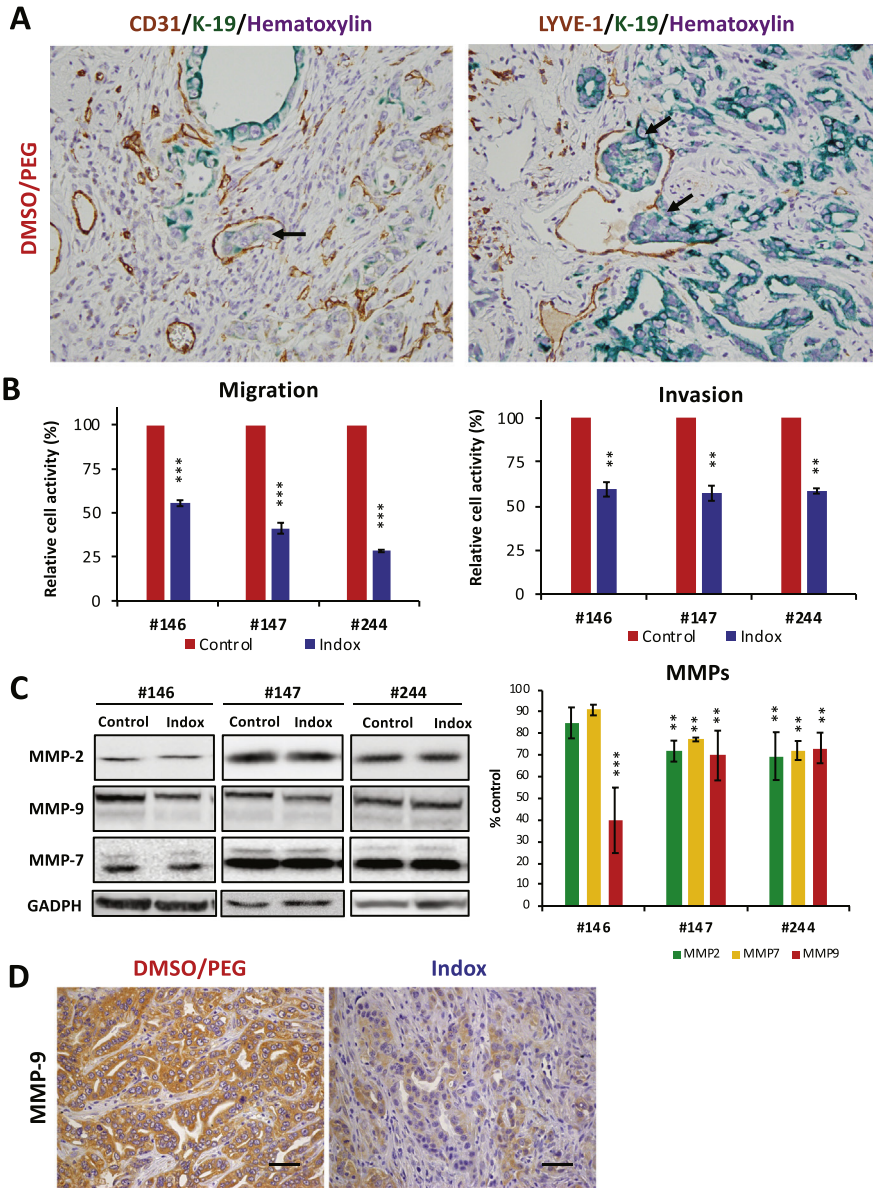

Figure 3. Effects of Indox on migration/invasion activities *in vivo* and *in vitro*.(**A**) Microscopic invasion of PDAC into CD31$^+$ veins and LYVE-1$^+$ lymph vessels were analyzed by staining with epithelial marker K-19 antigen (arrows). (**B**) Migration and invasion activities of mouse PDAC cells. (**C**) Expression levels of MMP-2, -7 and -9 in murine PDAC cell lines treated with 10 μM Indox for 24 h. (**D**) Expression of MMP-9 in PDAC cells was inhibited in the *KPC^{flox}* mice. Scale bars, 100 μm. *P < .05; **P < .01; ***P < .001 vs. vehicle control by ANOVA Tukey's test.

contrast, microinvasion was significantly suppressed by Indox administration (Data not shown).

Next, we performed *in vitro* migration and invasion assays using the mouse PDAC cell lines (Figure 3*B*). Consistent with the results in the *KPC^{flox}* mice, Indox blocked both the migration and invasion activities in the PDAC cells. Since matrix metalloproteinase (MMP) is the major invasion-associated molecule in various cancers, we next determined the expressions of MMPs in mouse PDAC cells by the western blotting method. The expression of MMP-9 in the PDAC cells was reduced by Indox treatment, especially the #146 cells (Figure 3*C*). Likewise, by Indox administration, the reduction of MMP-9 expression was observed in the PDAC in the *KPC^{flox}* mice *in vivo* (Figure 3*D*). The expressions of MMP-2 and -7 were also partially suppressed by Indox treatment in the PDAC cells with statistical significance. These results suggest there is an inhibitory effect of Indox on PDAC cell invasion via down-regulation of MMP-9.

To identify the molecules influenced by Indox treatment in up-stream of MMP-9 expression, we performed antibody array analyses again. The RAF/ERK, AKT, and SAPK/JNK signaling pathways have been reported to be the modulators for MMP-2 and -9 expression in cancer cells [20–22]. Interestingly, Indox suppressed the phosphorylation of A-RAF and B-RAF significantly in the PDAC cells (Figure 4*A*). Moreover, Indox slightly reduced the phosphorylated ERK1-p42/44 MAP kinase and p90RSK levels (Figure 4*A*, Supplementary Figure 4*D*). Phosphorylated AKT, AKT1/2 and p70S6K levels were reduced by Indox (Figure 5*B*, Supplementary Figure 4*C*). Additionally, phosphorylated MKK7/MAP2K7 levels

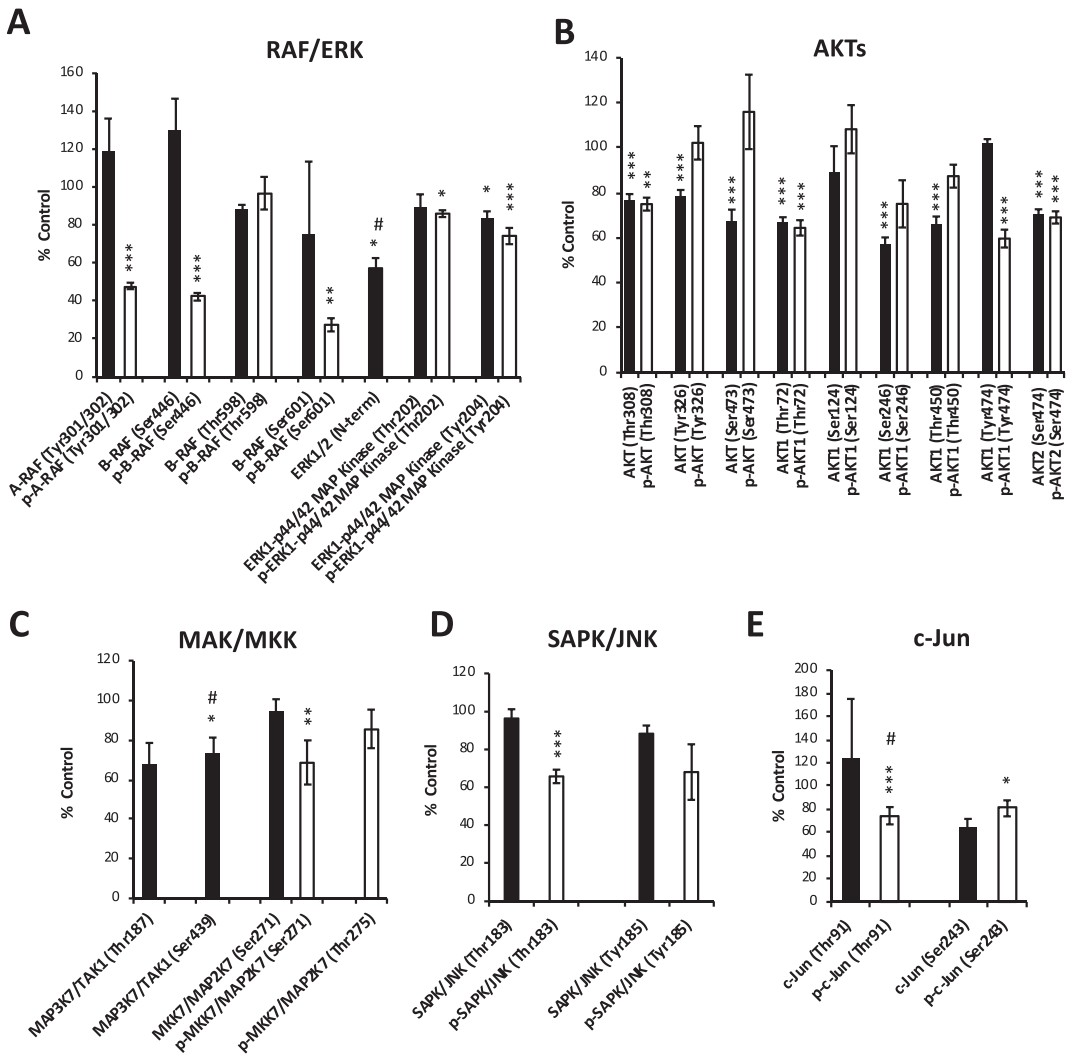

Figure 4. Effects of Indox on the levels of phosphorylated RAF/ERK, AKT, SAPK/JNK pathways.Antibody array revealed that phosphorylation of RAF/ERK (**A**), AKTs (**B**), MAP/MMK (**C**), SAPK/JNK (**D**), and c-Jun (**E**) in the murine cell line (#146) was inhibited by 10 μM Indox for 24 h (n = 6). **P* < .05; ***P* < .01; ****P* < .001 vs. vehicle control by ANOVA Tukey's test. #n = 5.

and their down-stream kinases, SAPK/JNK and c-Jun, were also suppressed by Indox (Figure 4, *C*—*E*). These results of the antibody array were basically supported by immunoblotting analysis in the mouse PDAC cell lines (Figure 5, *A* and *B*, Supplementary Figure 5, *A* and *B*). Therefore, these findings suggest that Indox inhibits RAF/ERK, AKT and SAPK/JNK signaling cascades, which most likely leads to the down-regulation of MMP-9 and inhibition of tumor migration and invasion (Figure 6*B*, Supplementary Figure 6).

Discussion

Previous reports have demonstrated the antiproliferation effects of indirubin derivatives against PDAC cell lines or cancer stem cells *in vitro* only [23—25]. We first show the antitumor effect of Indox to spontaneous PDAC model (*KPC*^{flox} mice) *in vivo* in addition to the mice-derived PDAC cell lines *in vitro* in this report. Most indirubin derivatives have been shown to inhibit CDKs by competitively binding to ATP-binding sites [7—9] and thereby inducing the G2/M arrest of cancer cells [11—13]. In this study, antibody array analysis showed that Indox reduced the levels of p-CDK1/2/7 and p-cyclin B1/D1 and other cell cycle-associated molecules such as p-ATM, p-WEE1, p-CKIs, p-CDCs, and p-ChKs. These molecules are involved in complex regulatory mechanisms in other phases of the cell cycle (G1 and S phases) and the checkpoints (G1/S and S/G2). Otherwise, Indox predominantly induced G2/M arrest indicating that the inhibition of p-CDK1/cyclin B1 is a key antiproliferative mechanism of Indox.

The current study first evaluated the anti-migration/invasion effects of Indox on a spontaneous PDAC mouse model and the underlying mechanisms using the mice-derived cell lines. Previous reports demonstrated that indirubin derivatives inhibited the migration and invasion activities in other cancer and endothelial cells [26—32]. Indox blocked the invasion of the human myeloid cell line KBM-5 by the down-regulation of MMP-9 [26]. In another study, 5-nitroindirubin 3′-oxime ($5NO_2$Indox) inhibited the migration and invasion of salivary gland ductal adenocarcinoma by suppression of beta1 integrin and MMP-2 and -9 [27]. Furthermore, 6-bromoindirubin 3′-oxime (6BrIndox) also blocked the migration and invasion potential of colorectal and invasive urinary bladder carcinomas, accompanied with down-regulation of MMP-2 and -9 [28,29]. These forms of Indox with different functional groups likely

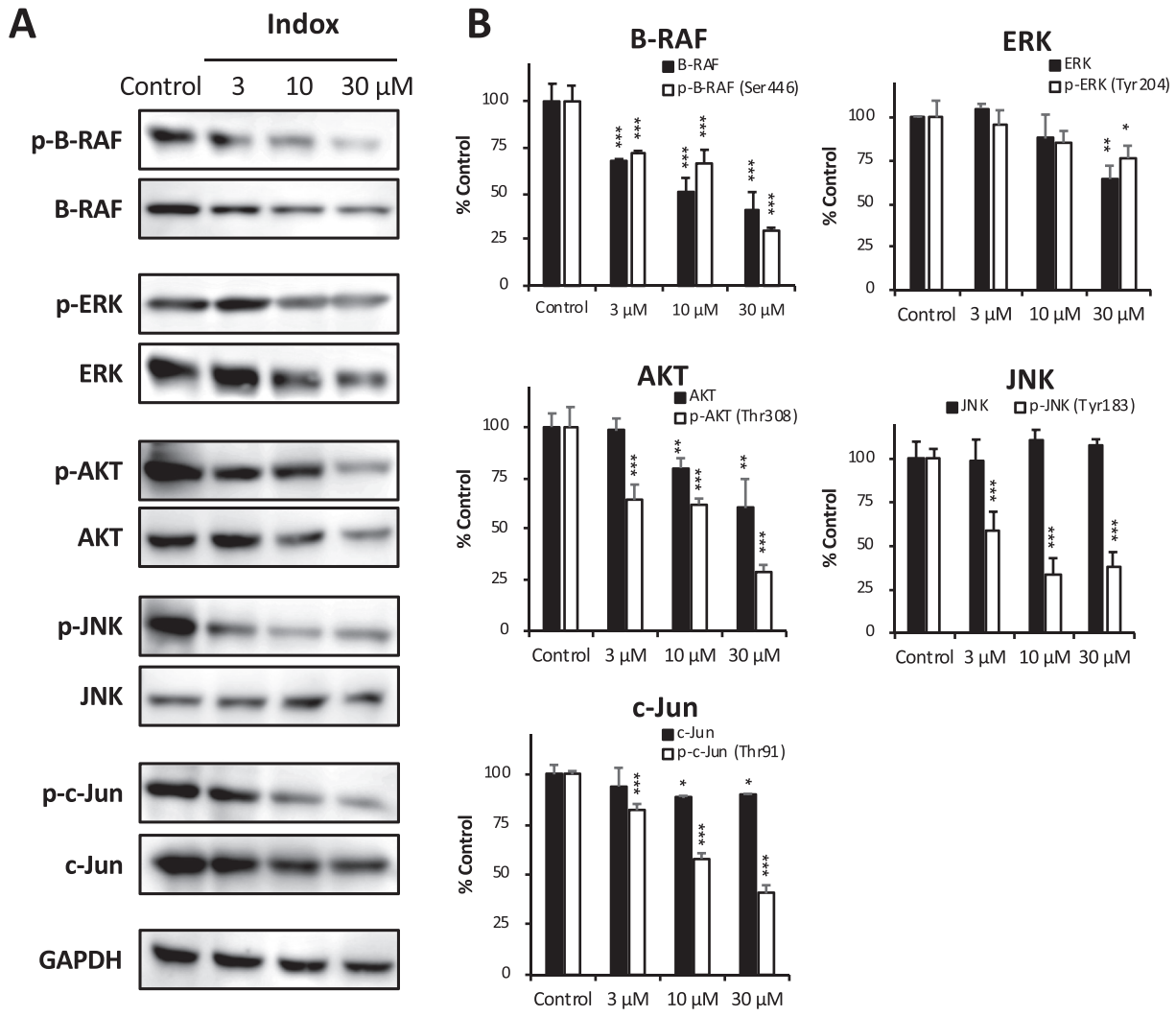

Figure 5. Inhibition of phosphorylated RAF/ERK, AKT, SAPK/JNK and c-Jun by Indox.(**A**) After treatment with indicated concentration of Indox for 24 h, low levels of p-B-RAF (Ser446), p-ERK (Tyr204), p-AKT (Thr308), p-SAPK/JNK (Tyr183) and p-c-Jun (Thr91) in the murine PDAC cell line (#146) were determined by immunoblotting. (**B**) Quantification of data presented in **A**. *$P < .05$; **$P < .01$; ***$P < .001$ vs. vehicle control by ANOVA Tukey's test.

showed similar biological effects but different inhibitory effects due to different affinities to the target molecules.

Indirubin and its derivatives are known to be multi-kinase inhibitors, but the target molecules might depend on cell type. In our study, Indox inhibited the B-RAF/ERK and SAPK/JNK signaling pathways as well as the AKT pathways in the PDAC cells *in vitro*. In mouse fibroblast NIH/3T3, Indox inhibited the fibroblast growth factor-induced phosphorylation of ERK and AKT, but did not inhibit the phosphorylation of JNK and p38 MAPK [33]. Meanwhile, Indox has been reported to directly inhibit the activity of JNK1 and JNK3 in neuronal cells [34]. Similar to our findings, $5NO_2$Indox showed inhibitory effects on the phosphorylation of RAF-1, ERK, JNK, and c-Jun, thereby blocking the neoplastic transformation of mouse epidermal cells [35]. Similarly, $5NO_2$Indox inhibited the metastatic ability of head and neck cancer cells by blocking the AKT pathway [36]. In carcinomas, MMP-2 and -9 expressions were modulated by the activation of the RAF/ERK, AKT, and SAPK/JNK signaling pathways [20—22]. Therefore, Indox might inhibit the migration/invasion and metastatic potentials of PDAC via down-regulation of MMP-9 modulated by the inhibition of the B-RAF/ERK, AKT and SAPK/JNK signaling pathways.

Recent clinical studies have shown that kinase inhibitors such as Vemurafenib and Dabrafenib were useful compounds for the treatment of malignant melanoma and thyroid papillary carcinoma with mutant B-RAF [37]. Interestingly, Indox strongly inhibited the phosphorylation of B-RAF (Ser446 and Ser601) in the *Kras-^{G12D}*-induced PDAC cells *in vitro*. Although future investigations are still needed to determine the effects on the B-RAF pathway, Indox may be an effective candidate for treating various cancers though the activation of the B-RAF signaling pathway.

Conclusions

Indox inhibited the proliferation, invasion and metastatic activities of PDAC cells *in vitro* and spontaneous PDAC model *KPC^{flox}* mice *in vivo*. Treatment with Indox could be a new therapeutic candidate for the treatment of advanced and aggressive PDAC.

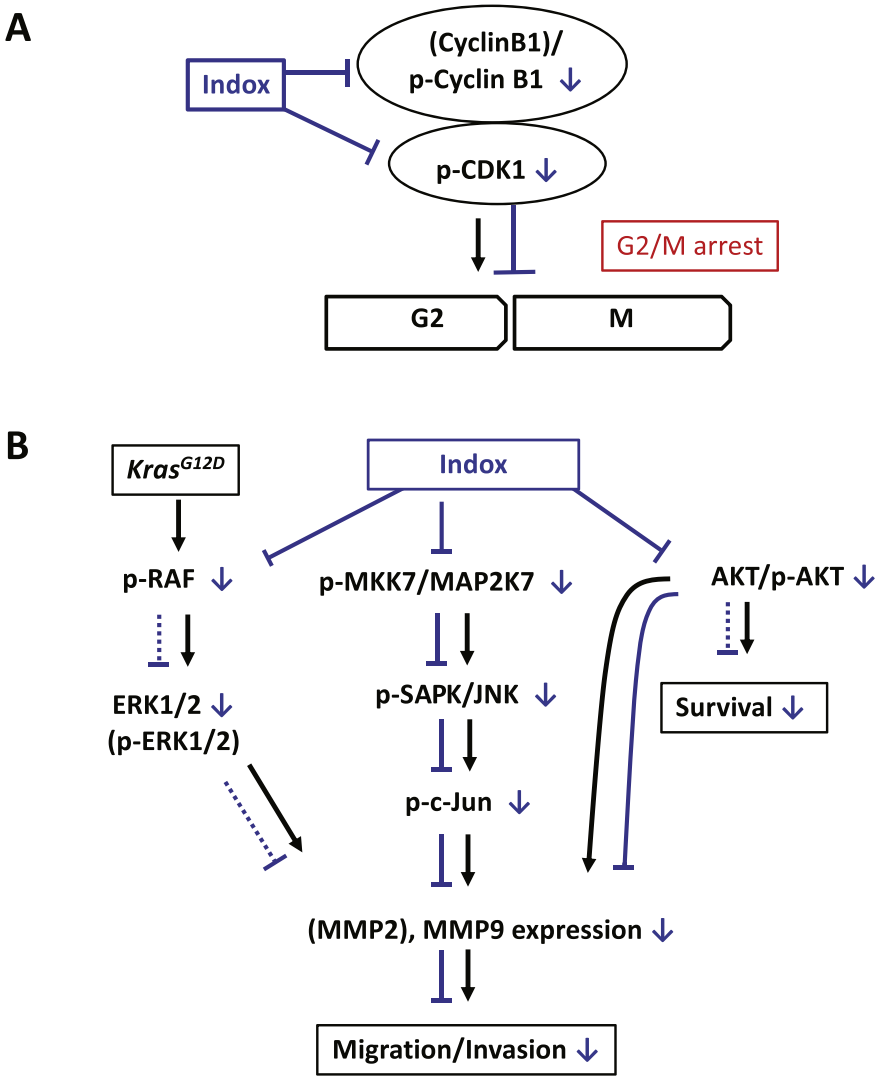

Figure 6. A schematic diagram of the effect of Indox on the *Kras*^{G12D}-induced PDAC cells.(**A**) Indox inhibits the phosphorylation of CDK1 and cyclin B level, leading to G2/M arrest in mouse PDAC cells. (**B**) Indox inhibits phosphorylation of B-Raf, and the MMK7/MAP2K7 and AKT pathways, which leads to down-regulation of MMP-9 and decreased migration and invasion activities.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.08.010.

Acknowledgments

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Conflict of Interest Statement

None declared.

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