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Bile acids-mediated overexpression of MUC4 via FAK-dependent c-Jun activation in pancreatic cancer

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ABSTRACT

The majority of pancreatic cancer (PC) patients are clinically presented with obstructive jaundice with elevated levels of circulatory bilirubin and alkaline phosphatases. In the current study, we examined the implications of bile acids (BA), an important component of bile, on the pathophysiology of PC and investigated their mechanistic association in tumor-promoting functions. Integration of results from PC patient samples and autochthonous mouse models showed an elevated levels of BA ($p < 0.05$) in serum samples compared to healthy controls. Similarly, an elevated BA levels was observed in pancreatic juice derived from PC patients ($p < 0.05$) than non-pancreatic non-healthy (NPNH) controls, further establishing the clinical association of BA with the pathogenesis of PC. The tumor-promoting functions of BA were established by observed transcriptional upregulation of oncogenic MUC4 expression. Luciferase reporter assay revealed distal MUC4 promoter as the primary responsive site to BA. *In silico* analysis recognized two c-Jun binding sites at MUC4 distal promoter, which was biochemically established using ChIP assay. Interestingly, BA treatment led to an increased transcription and activation of c-Jun in a FAK-dependent manner. Additionally, BA receptor, namely FXR, which is also up-regulated at transcriptional level in PC patient samples, was demonstrated as an upstream molecule in BA-mediated FAK activation, plausibly by regulating Src activation. Altogether,

Abbreviations: PC, pancreatic cancer; PDAC, pancreatic ductal adenocarcinoma; BA, bile acids; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; FXR, farnesoid-x-receptor; FAK, focal adhesion kinase; MAPK, mitogen activated protein kinase; JNK, c-Jun N-terminal kinase; PI3K, phosphoinositide 3-kinase; ChIP, chromatin immunoprecipitation.

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these results demonstrate that elevated levels of BA increase the tumorigenic potential of PC cells by inducing FXR/FAK/c-Jun axis to upregulate MUC4 expression, which is overexpressed in pancreatic tumors and is known to be associated with progression and metastasis of PC.

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1. Introduction

In 2014, about 45,000 new cases of pancreatic cancer (PC) were diagnosed in the United States, of which pancreatic ductal adenocarcinoma (PDAC) represents the major histological type (Siegel et al., 2014). The majority of tumors (about 75%) arise at the head of the pancreas (Wisinski et al., 2007). Anatomically, the pancreatic duct is placed close to the common bile duct, and unite at the point known as the ampulla of Vater, and secrete their contents into the duodenum, the proximal site of the intestine (Bardeesy and DePinho, 2002). Approximately, 70% of PC patients develop extrahepatic cholestasis due to blockage of the common bile duct by increasing tumor size and results in multiple organ failure and early death (Nakamura et al., 2002). Due to bile duct obstruction, extrahepatic cholestasis exhibits obstructive jaundice, hyperbilirubinemia and elevated circulatory levels of bile acids (BA).

BA are amphiphilic molecules and are the main component of bile along with cholesterol, phospholipids, and bilirubin (Baptissart et al., 2013). By utilizing a series of enzymatic modifications, BA are synthesized in the liver using cholesterol as a precursor. BA are further modified by bacterial species present in the colon to form secondary BA (Baptissart et al., 2013). Dietary fat is a stimulus for BA secretion into the intestine, which is required for the proper digestion of fatty foods (Baptissart et al., 2013). Though bile-reflux has been associated with esophageal and gastric cancers, its association with PC pathogenesis has not been investigated (Sifrim, 2013; Tsoukali and Sifrim, 2013). A recently performed meta-analysis has revealed an increased risk of PDAC in patients with the cholecystectomy history (Lin et al., 2012). It has been proposed that the increased levels of cholecystokinin, which is known to stimulate the growth of human PC cell lines, promote pancreatic carcinogenesis in hamsters (Howatson and Carter, 1985).

BA have been shown to participate in tumor progression using multiple mechanisms including, alteration in the expression of oncogenic mucins (Mariette et al., 2004; Piessen et al., 2007). Interestingly, PC is characterized by aberrant mucins expression (Kaur et al., 2013c; Joshi et al., 2014; Joshi et al., 2015). Among various mucins expressed in PC, MUC4 is one of the top-differentially expressed protein compared to normal pancreas (Andrianifahanana et al., 2001; Iacobuzio-Donahue et al., 2003). We and others have established the oncogenic role of MUC4 in PC, and inhibition of MUC4 expression led to reduced PC cell proliferation, migration, and chemoresistance (Moniaux et al., 2007; Chaturvedi et al., 2007; Lakshmanan et al., 2015). In the present study, we have evaluated the role of BA in the regulation of MUC4 expression in PC. The findings from the current

study, for the first time, have demonstrated that BA levels are significantly high in the serum and pancreatic juice samples obtained from PC patients. Using defined spontaneous mouse model of PC, we found that BA levels increased with the severity of PC, which we mechanistically linked with BA-mediated expression of oncogenic MUC4 through FAK-dependent activation of c-Jun. Further studies demonstrated the role of FXR as the upstream molecule in this FAK/c-Jun/MUC4 axis.

2. Materials and methods

2.1. Cell culture and reagents

All human PC cell lines were obtained from ATCC, except T3M4 and CD18/HPAF. CD18/HPAF is a metastatic clone derived from the HPAF cell line (Mullins et al., 1991), whereas T3M4 cell line is derived from lymph node metastasis of pancreatic adenocarcinoma (Okabe et al., 1983). Human ductal pancreatic epithelial (HDPE) cells were a generous gift of Dr. Thiru Arumugam (MD Anderson, Houston, Texas) and cultured in keratinocyte serum-free (KSF) medium supplemented with epidermal growth factor and bovine pituitary extract. All PC cell lines were cultured in DMEM (supplemented with 10% heat-inactivated FBS, penicillin, and streptomycin (100 µg/ml)) at 37 °C with 5% CO₂ and were tested mycoplasma-free before conducting the experiments. Deoxycholic (DCA) and chenodeoxycholic acid (CDCA) were dissolved in sterile ethanol. For inhibition studies, wortmannin (phosphoinositide 3-kinase (PI3K) inhibitor, 1 µM, Cell Signaling Technology), SP100625 (JNK inhibitor; 35 µM, Merck Millipore), FAK inhibitor 14 (FAK inhibitor, 15 µM, Cayman's chemical), U1026 (MAPK inhibitor, 10 µM, Promega) and actinomycin-D (2 µg/ml, Sigma–Aldrich) were given 1 h prior to BA treatment. To transiently knockdown FXR, commercially available FXR siRNA (Santa Cruz Biotechnologies (SCB); Dallas, TX, USA) were used. For transfection purposes, lipofectamine 2000 (Life Technologies; Carlsbad, CA, USA) was used, according to the manufacturer's protocol.

2.2. Procurement of human and murine PDAC samples

All human PDAC samples used in the present study were de-identified and a written informed consent was received from all recruited patients before enrollment at respective institutions. The collection of secreted pancreatic juice upon secretin induction from PC patients was approved by the Mayo Clinic Institutional Review Board (IRB#07-0000099) and the detailed information has been provided in our previous publication (Kaur et al., 2013a). Plasma samples were collected using an

approved protocol (IRB number PRO07030072) at University of Pittsburgh (Pittsburgh, PA) from PC patients (Kaur et al., 2013b). The samples from autochthonous murine model, KrasG12D/+; p53R172H/+; Pdx1-Cre (KPC), and their contemporary littermates, were collected at 5, 7, 10, 15, 20 and 25 weeks (wk) of age. The mouse model was housed at UNMC animals facility and generated by crossing LSL-KrasG12D with LSL-Trp53R172H/+ transgenic mice as described previously (Hingorani et al., 2005; Rachagani et al., 2012). The tissue specimen from Whipple procedure were obtained from UNMC tissue bank and used for immunohistochemistry analysis. For mRNA expression profiling, frozen PC tissues were obtained from cooperative human tumor network (CHTN) and UNMC.

2.3. Total BA estimation method

To analyze total BA concentration in pancreatic juice and plasma samples, we used a highly sensitive bile acid estimation assay kit (Diazyme, NBT, DZO92A-k). To increase the precision of the test, each sample was analyzed in triplicates. We used deoxycholic acid for making the reference plot using serial dilution from 1.25 $\mu\text{mol/L}$ to 150 $\mu\text{mol/L}$. After completing the BA estimation assay according to the manufacturer's protocol, ELISA plates were read at 405 nm and the collected data was analyzed using SOFTMAX PRO software (Molecular Devices Corp.; Sunnyvale, CA).

2.4. Immunoblotting

Briefly, cells were lysed with a radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl pH-7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), supplemented with protease inhibitor mixture, 2 mM Na_3VO_4 , 10 mM NaF and 1 mM PMSF, for 15 min at 4 °C. Cell lysates were quantified and proteins were resolved on 2% agarose gel electrophoresis for high molecular weight MUC4 protein, whereas proteins less than 250 kDa were resolved by 10% SDS-PAGE under reducing conditions and blotted onto a PVDF membrane (Millipore). Following electrophoresis, the membrane were blocked in 5% non-fat dry milk in PBS for 1 h and incubated overnight with primary antibodies at 4 °C. The primary antibodies used in the study are as follows: β -Actin (Sigma, 1:5000), c-Jun (SC-1694, Santa Cruz Biotechnologies (SCB), 1:500), p-c-Jun (9261, Cell Signaling, 1:1000), c-src (SC-18, SCB, 1:500), p-src (6943, Cell Signaling, 1:1000), FAK (SC-558, SCB, 1:500), p-FAK (8556, Cell Signaling, 1:1000), FXR (SC-134481, SCB, 1:500) and MUC4 (In house generated, 1:1000). Blots were washed and probed with respective secondary peroxidase-conjugated antibodies. Following, the protein bands were visualized using enhanced chemiluminescence (ECL) method (Thermoscientific) and quantified using image Studio Lite ver. 5.2 (LI-COR Biosciences).

2.5. Immunofluorescence microscopy

PC cells were grown on sterilized cover slips for 24 h, serum starved for 8 h and treated with appropriate vehicle control (media, ethanol or DMSO), DCA, CDCA, and inhibitors for indicated time-points. After the treatment, immunofluorescence staining with specific primary antibodies was performed as

described previously (Kumar et al., 2015b). For immunohistochemistry, we deparaffinized tissue sections with xylene, rehydrated with decreasing concentrations of ethanol and incubated tissues for 30 min with 3% H_2O_2 : methanol solution. Subsequently, antigen retrieval was performed and tissues sections were blocked in 2.5% horse serum for 2 h and incubated with primary antibodies. Following, tissues were processed as described previously (Kumar et al., 2015b). The primary antibodies used are c-Jun (SC-1694, SCB, 1:500), FXR (SC-558, SCB, 1:200) and MUC4 (In-house generated, 1:400). All the images were taken by using LSM 510 microscope, a laser scanning confocal microscope (Carl Zeiss GmbH; Thornwood, NY) in the respective channels.

2.6. RNA isolation and RT-PCR

Quantitative real-time PCR amplifications, using gene-specific primers (Table S1) were carried out using the standardized protocol established in our lab (Kumar et al., 2015b), using SYBR Green chemistry. The relative fold differences in gene expression were calculated using the $\Delta\Delta\text{Ct}$ method with β -actin as a normalization control (Livak and Schmittgen, 2001). For RT-PCR, amplification of both FXR and β -actin genes was done using the following steps: initial denaturation at 95 °C for 5 min, followed by 35 programmed cycles at 95 °C for 1 min, 58 °C for 1.5 min and 72 °C for 1 min, with a final incubation at 72 °C for 10 min. The amplified product was detected by electrophoresis on 2% agarose gels.

2.7. Chromatin immunoprecipitation (ChIP)

PC cells (CD18/HPAF) were serum starved for 8 h and treated with DCA or CDCA for 4 h. Afterward, the ChIP experiment was performed as described previously (Kumar et al., 2015a; Pai et al., 2016) and have been repeated more than three times. In brief, 1% formaldehyde was used to cross-linked chromatin, which was isolated and sheared into 500–1000 bp fragments by sonication (Bioruptor UCD-200, Diagenode; New York, NY, USA). As an input, 1% of the sonicated DNA was used. The remaining sonicated DNA fraction was used for the pull down using indicated antibodies overnight at 4 °C. Antibodies used are: 5 μg of anti-c-Jun (SCB#1694X) and IgG (negative control). MUC4 promoter targeted primers were used to identify enriched fragment through real-time qPCR. The details of the primers are given in Table S2.

2.8. Luciferase reporter assay

To perform this assay, previously designed and established pGL3-MUC4 deletion constructs were used (Andrianifahanana et al., 2005). PC cell lines were plated in six-wells in triplicates and repeated thrice. Transient transfection was performed with MUC4 deletion constructs using lipofectamine 2000 (Life Technologies; Carlsbad, CA, USA). Next day, the media was first changed to 10% FBS containing DMEM for 12 h (to alleviate cellular stress of transfected cells) and then to serum free media for additional 8 h. Subsequently, transfected cells were treated with BA for 4 h in serum free condition. Following treatment, cells were lysed using reporter lysis buffer (Promega; Madison, WI) and subsequently, the activity of luciferase and beta-

galactosidase activity was measured using Steady-Glo Luciferase assay system (Promega, E2510) and β -galactosidase assay kit (Promega, E2000). Fold activation of luciferase activity in BA-treated cells were calculated and compared with untreated cells. *In silico* analysis was performed on designated MUC4 promoter region using ALGGEN PROMO software (where similarity score of >0.85 was used to screen transcription factor binding sites) to predict the binding of putative transcription factors.

2.9. Cytoplasmic and nuclear fractionation

Briefly, after 4 h of 50 μ M of DCA or CDCA treatment, CD18/HPAF cells were washed with ice-cold PBS and incubated with a cytoplasmic extraction buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 0.2% NP-40, 0.1 mM EDTA, 10% glycerol, 1.5 mM $MgCl_2$, supplemented with protease inhibitor, 1 mM DTT, 1 mM PMSF, 5 mM Na_3VO_4 , 5 mM NaF) for 1 h at 4 °C. Cells were centrifuged at $800 \times g$ for 10 min and the supernatant was labeled as cytoplasmic extract, and the remaining pellet was washed with PBS and then incubated for 1 h with the nuclear extraction buffer (20 mM HEPES (pH 7.6), 420 mM NaCl, 1 mM EDTA, 20% glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 5 mM Na_3VO_4 , 5 mM NaF). Following incubation, the pellet was sonicated for 10 s at 60% amplitude, and then subjected to centrifugation at $13,000 \times g$ for 10 min. The obtained supernatant was collected as a nuclear extract. The purity of the fractions were checked by analyzing the expression of nuclear SP1 (#9389S, Cell Signaling, 1:1000) and cytoplasmic GAPDH (#5174S, Cell Signaling, 1:3000) proteins in collected lysates.

2.10. Statistical analysis

All results are representative of at least three independent experiments. The *in vitro* data are expressed as the mean \pm standard deviation (S.D.), whereas the *in vivo* data are represented as mean \pm standard error (S.E.). Statistical comparisons of the two groups were made using a student's t-test (two-tailed, unpaired) using Microsoft Excel 2010, where a *p* value of less than 0.05 was considered statistically significant. For correlation analysis, the coefficient of determination (R^2) was determined between two groups.

3. Results

3.1. BA levels are elevated in serum and pancreatic juice during PC

According to our hypothesis, BA play important roles in PC development by regulating the expression of oncogenic proteins, including MUC4. Therefore, we first analyzed the levels of BA in serum and pancreatic juice obtained from PC patients. There was significantly higher circulatory levels of BA ($p = 0.0014$) with a mean concentration of $68 \pm 4.39 \mu$ M as compared to $38 \pm 3.09 \mu$ M observed in the control group (Figure 1A). Similarly, we noticed a significant increase in mean value of circulatory BA levels in 10–15 wk ($19.96 \pm 4.15 \mu$ M, $p = 0.002$) and 20–25 wk-old ($27 \pm 6.31 \mu$ M, $p = 0.009$) KPC mice compared to their littermate controls

($1.17 \pm 0.97 \mu$ M) (Figure 1B), strengthening the association of BA with the pathobiology of PC. We included controls from different age group for BA estimation and did not observe any noticeable change in their serum BA levels, which is also evident from the demonstrated standard errors (Figure 1B). Additionally, earlier report by Uchida K et al. have demonstrated that circulatory BA levels when expressed in terms of units per rat did not ostensibly change, regardless of their age (Uchida et al., 1978). Consistently, pancreatic juice obtained from PC patients ($n = 18$) had significantly high BA levels ($65 \pm 12.26 \mu$ M, $p = 0.048$), compared to the non-pancreatic non-healthy (NPNH, patients with symptoms mimicking pancreatic disease but found to be free of pancreatic pathology) subjects ($n = 5$), where the mean concentration of BA was $13.63 \pm 12.55 \mu$ M (Figure 1C). Taken together, high BA levels in PC condition suggest their possible involvement in the pathobiology of PC.

3.2. BA up-regulate MUC4 expression in PC cells

BA modulates the expression levels of mucins such as MUC1, MUC2, MUC4 and MUC5AC in esophageal, gastric and colon cancers (Lee et al., 2010; Mariette et al., 2004, 2008; Song et al., 2011; Piessen et al., 2007; Martin et al., 2011). Interestingly, PC is characterized by altered mucins expression. We along with others have established that mucins play important role in the pathogenesis of PC (Kaur et al., 2013c; Joshi et al., 2014; Joshi et al., 2015). In order to analyze the effect of BA on MUC4 expression, we treated PC cell lines with different concentrations of DCA and CDCA in a dose and time dependent manner. We observed a significant increase in MUC4 expression in CD18/HPAF cells at all concentrations, ranging from 5 to 100 μ M with the maximal increase at 50 μ M concentration for both DCA and CDCA in 24 h (Figure 2A, Figure S1). BA-mediated increase in MUC4 expression was further confirmed in T3M4 (Figure 2B) and CAPAN1 cell lines (Figure 2C). Furthermore, immunofluorescence experiment revealed significant increase in MUC4 expression in DCA or CDCA treated CD18/HPAF cell line (Figure 2D). Altogether, the results suggest that BA may play important role in the pathogenesis of PC by upregulating MUC4 expression.

3.3. BA transcriptionally upregulates MUC4 expression in PC

In order to mechanistically evaluate BA-mediated upregulation of MUC4, PC cells were treated with DCA or CDCA in conjunction with actinomycin-D, which inhibits the process of transcription. There was 4.09- and 4.49-fold increase in MUC4 expression after DCA and CDCA treatments in CD18/HPAF cells, respectively, which was attenuated to 0.18- and 0.16-fold when treated in combination with actinomycin-D (Figure 3A). Similarly, in T3M4 cells, we observed a 2.40-fold increase in MUC4 expression upon DCA treatment was attenuated to 0.38-fold, when given in the presence of actinomycin-D, whereas a 2-fold MUC4 upregulation in CDCA treated T3M4 cells was reduced to 0.54-fold in the presence of CDCA and actinomycin-D treatment (Figure 3A).

To highlight the DCA and CDCA responsive regions on the MUC4 promoter, Luciferase reporter assay was performed

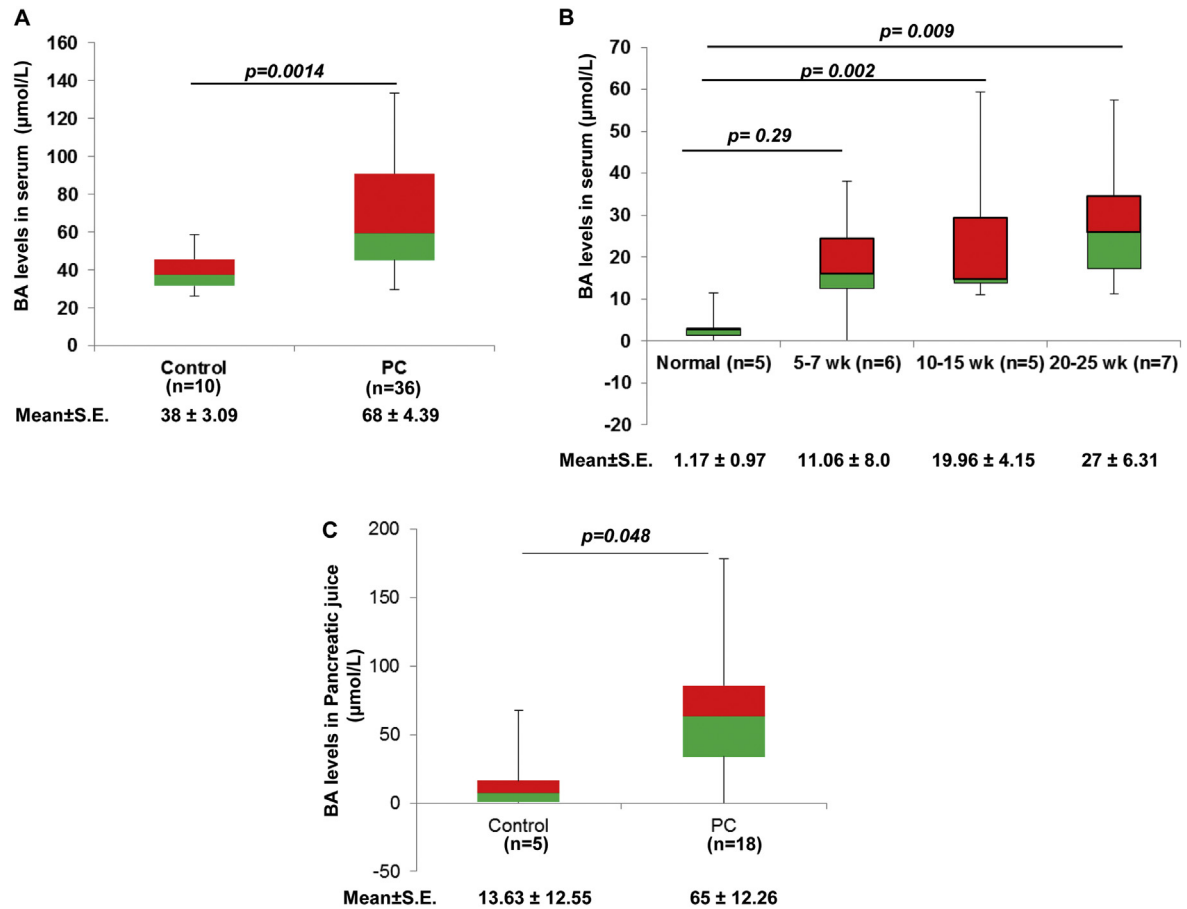


Figure 1 – Upregulation of BA in PC condition. **A.** The representative box-plot is showing levels of BA in the serum samples obtained from the PC patients ($n = 36$) and healthy ($n = 10$) individuals using a commercially available total BA estimation kit. The difference in BA levels between normal and PC patients were found to be statistically significant. **B.** To understand the association of BA with PC progression, we measured BA levels in established KPC mice model at early (5–7 wk), medium (10–15 wk) and advanced stages (20–25 wk). The BA levels were found to increase with the severity of the disease. **C.** Box-plot representing the levels of BA in the pancreatic juice obtained from PC patients. We observed significant increase in BA concentration in the pancreatic juice obtained from PC patients ($65 \mu\text{mol/L}$) compared to NPNH controls ($13 \mu\text{mol/L}$) (All values are mean \pm S.E, ns means non-significant).

(Andrianifahanana et al., 2005). Our results demonstrated that both distal (P-1641) and proximal (P-1809 and P-2150) constructs were responsive to BA in CD18/HPAF cells (Figure 3B). Of particular interest was the deletion construct P-1641, which evidenced a statistically significant 2.95- and 3.24-fold upregulation of the reporter gene in response to DCA and CDCA treatment, respectively (Figure 3B). A similarly enhanced transcriptional activity by 1.93-fold was also noticed in DCA and CDCA treated CD18/HPAF cells transfected with P-2150 construct, however, these changes were insignificant. P-1809 construct demonstrated increase in luciferase activity by 1.21- and 1.91- fold upon DCA and CDCA treatment, respectively (Figure 3B), nevertheless, these changes were significant only for CDCA treatment. Correspondingly, compared to untreated controls, T3M4 cells transfected with P-1641 fragments showed 3.04- and 2.55-fold increase ($p < 0.05$), in luciferase activity upon DCA and CDCA treatment, respectively (Figure 3C). P-1809 deletion construct demonstrated 1.53- and 1.78-fold increase in luciferase activity upon DCA and CDCA, respectively. Similarly, P-2150 construct exhibited

1.4- and 2-fold increase in luciferase activity in the presence of DCA and CDCA, respectively. However, the increase in luciferase activity at proximal promoter regions upon BA treatment were statistically insignificant in T3M4 cell lines. Taken together, our data suggests that the distal promoter region of MUC4 gene is mainly responsible for BA-mediated transcriptional upregulation of MUC4 in both CD18/HPAF and T3M4 cell lines.

3.4. BA increase the expression and nuclear localization of c-Jun

Due to an observed maximal increase in the region -2572 to -3135 (present in P-1641) to BA treatment in PC cell lines, we performed *in silico* analysis to delineate putative transcription factors binding sites for transcription factors on this promoter region (Figure S2). Two c-Jun binding sites were identified on MUC4 distal promoter (P-1641), which were absent on the proximal promoter fragment (P-1809 sequence) (Singh et al., 2007), and therefore, were suggestive of making distal

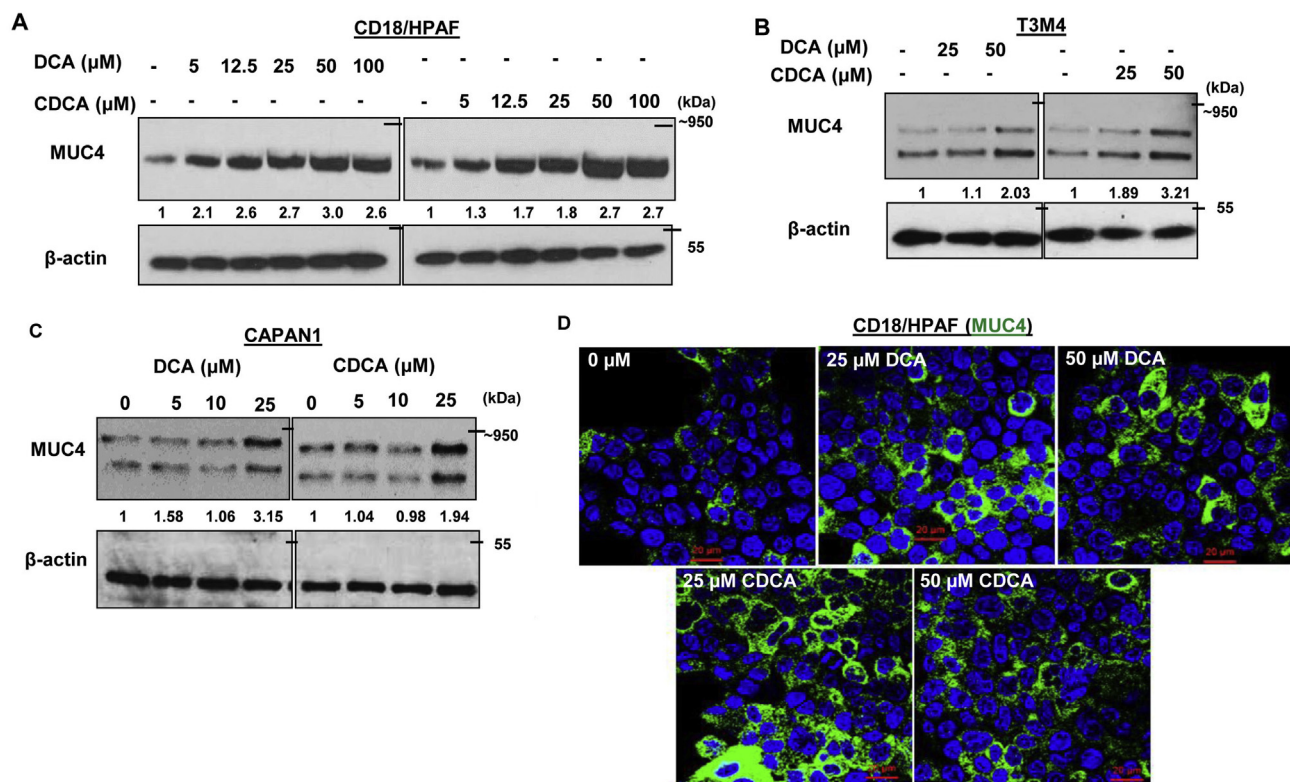


Figure 2 – BA-mediated positive regulation of MUC4 protein. **A**. CD18/HPAF cells were serum starved for 8 h prior to BA treatment. Following 24 h of BA treatment, cell lysates were collected, quantified and resolved using gel electrophoresis. Immunoblot showing increase in MUC4 expression upon DCA and CDCA treatment of CD18/HPAF cells at indicated concentrations. **B**. Immunoblot showing increase expression of MUC4 in DCA and CDCA treated T3M4 PC cells at indicated concentrations. **C**. Immunoblots confirming MUC4 upregulation by BA treatment in CAPAN1 cells. **D**. Representative confocal images showing the positive effect of BA on MUC4 expression in CD18/HPAF cells (scale bar = 20 μM).

promoter (P-1641) more responsive to BA treatment (Figure 4A). It incited us to propose that BA-facilitated upregulation in MUC4 expression in PC cell lines is c-Jun dependent. Firstly, we were interested to know whether BA itself has any effect on c-Jun expression levels. Intriguingly, in CD18/HPAF cells, we observed 1.95-, 2.9-, and 3.46-fold increase ($p < 0.05$) in c-Jun expression at 10, 50, and 100 μM of DCA treatment over untreated cells. On the other hand, 1.78-, 2.16-, and 3.87-fold increase ($p < 0.05$) in c-Jun expression was noticed at 10, 50 and 100 μM concentration of CDCA treatment, respectively (Figure 4B). The increased expression of c-Jun in response to both DCA and CDCA treatments was also confirmed by immunoblot analysis in PC cell lines (Figure 4C). Immunofluorescence experiments also revealed a significant increase in c-Jun expression and nuclear localization in both DCA- and CDCA-treated CD18/HPAF cells (Figure 4D). Further, nuclear and cytoplasm fractionation after BA treatment in CD18/HPAF cells, revealed significant increase in c-Jun expression in the nuclear extracts than untreated cells (Figure 4E).

To investigate the direct involvement of c-Jun in BA-induced MUC4 expression, we performed ChIP assay to analyze c-Jun binding on MUC4 distal promoter (Figure 4F). Using a primer set covering only one c-Jun binding site (or region-II), we observed 4.01- and 1.64-fold enrichment upon

DCA and CDCA treatment of CD18/HPAF cells, respectively. However, primers encompassing both c-Jun binding sites (region-I), showed a significant (<0.05) enrichment of 6.74- and 2.61-folds, compared to untreated cells after DCA and CDCA treatments in CD18/HPAF cells (Figure 4F), suggestive of the cumulative effects of both c-Jun binding sites in inducing the transcription of MUC4 gene. As a negative control, we synthesized primers against the non-c-Jun binding MUC4 promoter fragment and found no difference. Taken together, BA increase the expression and nuclear localization of c-Jun, which then occupy MUC4 promoter to increase its transcription.

3.5. BA mediated increase in FAK activation induced c-Jun expression

To elucidate the signaling pathways responsible for increased MUC4 transcription to BA treatment, CD18/HPAF cells were treated with a panel of inhibitors targeting different signaling pathways prior to BA treatment. Interestingly, pharmacological inhibition of both FAK and MAPK pathway showed attenuation of DCA- and CDCA-mediated MUC4 upregulation (Figure 5A). Inhibition of PI3K pathway did not have perceptible effect on MUC4 expression, whereas inhibition of JNK suppressed CDCA-mediated upregulation of MUC4

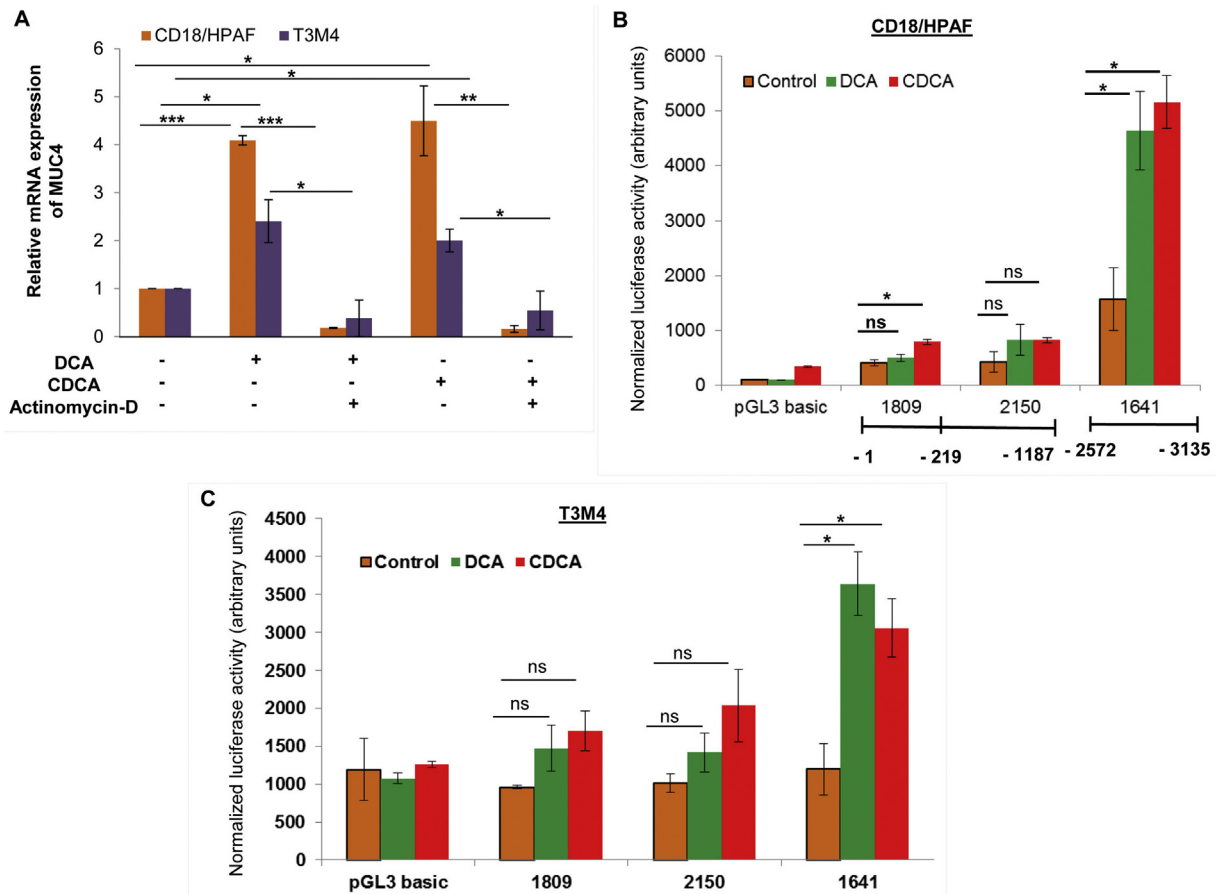


Figure 3 – BA-mediated transcriptional regulation of MUC4. **A**. After 8 h of serum starvation, both CD18/HPAF and T3M4 cell lines were treated for 12 h with 50 μ M of DCA, CDCA or vehicle control (ethanol) in the presence or absence of actinomycin-D (2 μ g/ml). Following treatment, cDNA was prepared from isolated RNA and used for real-time PCR to analyze the quantitative expression of MUC4 gene. The represented graph is demonstrating that inhibition of transcription attenuates DCA- and CDCA-mediated increase in MUC4 expression in both CD18/HPAF and T3M4 cell lines. **B**. Luciferase assay was performed in CD18/HPAF cell line transfected with MUC4 promoter-truncated constructs, followed by 4 h treatment of 50 μ M of DCA and CDCA. A significantly elevated luminescence was detected upon BA treatment, primarily at the distal promoter region. **C**. Similar to CD18/HPAF cells, T3M4 cells also showed significantly elevated luminescence at the distal promoter region upon BA (50 μ M) treatment (* p < 0.05, ** p < 0.01, *** p < 0.001, ns means non-significant).

(Figure 5A). The involvement of FAK pathway in BA-mediated upregulation of MUC4 was further confirmed using an immunofluorescence analysis (Figure 5B). Earlier, JNK and MAPK pathways have been associated with BA (Gupta et al., 2001; Qiao et al., 2001); however, effects of BA on FAK has not been studied so far, particularly, in terms of MUC4 regulation. Due to observed maximal effect of FAK pathway on BA-facilitated MUC4 expression, we decided to focus on FAK pathway and analyzed the activation status of FAK in BA-treated PC cells. As anticipated, we observed a high expression of activated FAK or pFAK (Y397) in BA-treated PC cells (Figure 5C), whereas expression of total FAK remains constant. Further, the selective pharmacological inhibition of FAK, led to significant decline in the expression levels of c-Jun and MUC4 in PC cell lines, both at transcript and protein levels, suggesting that c-JUN activation is mediated through FAK pathway (Figure 5D and E). To further substantiate our results, we performed ChIP experiment and observed significant reduction in enrichment for c-Jun binding on MUC4 promoter

when BA treatment was concomitantly given with FAK inhibitor, as compared to BA alone (Figure 5F), suggesting that FAK activation is a prerequisite for DCA- and CDCA-mediated MUC4 upregulation in PC cells due to its direct involvement in the induction of c-Jun expression.

3.6. FXR activation is an essential for BA-mediated MUC4 upregulation via src/FAK/c Jun axis

Farnesoid-X-receptor (FXR) is known to be activated by BA. Upon its activation, FXR gets translocated to the nucleus, where it alters the transcriptional expression of multiple genes (Figure 6A). Interestingly, the overall expression of FXR did not get influenced by BA treatment, as FXR levels were high in the cytoplasmic fraction of untreated cells than DCA and CDCA treated cells. The insignificant changes on FXR levels upon BA exposure were also noticed at mRNA level (Figure S3A). Expression profiling of FXR receptor in PC cell lines showed its significant overexpression in HPAF, CD18/

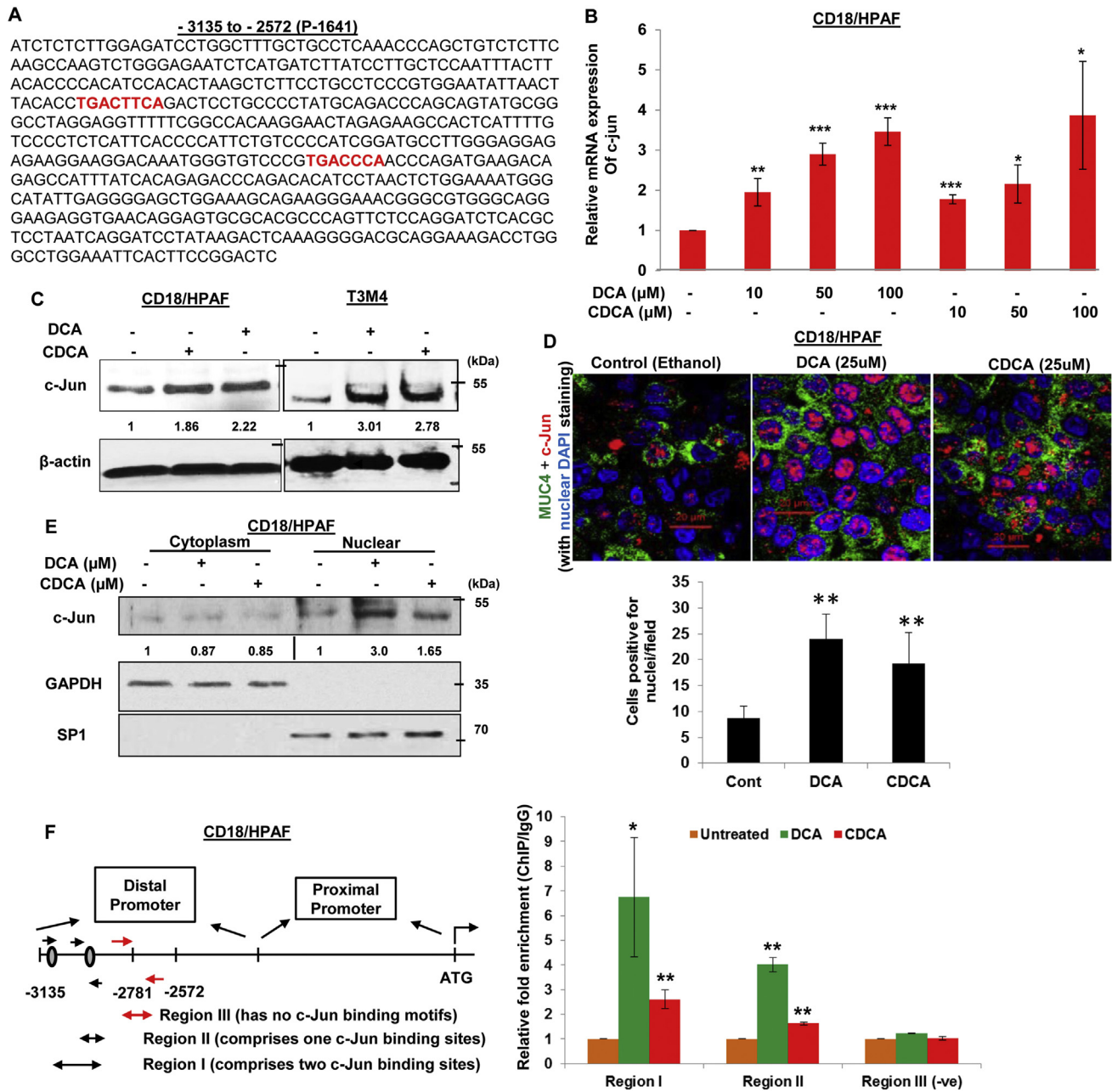


Figure 4 – The effects of BA on MUC4 expression *via* activation and nuclear translocation of c-Jun. **A**. Sequence of the MUC4 distal promoter (P-1641) which has two binding sites for c-Jun protein (marked red). **B**. Graph showing increase in c-Jun mRNA expression in a dose-dependent manner in CD18/HPAF cell line, treated for 2 h with DCA and CDCA. **C**. CD18/HPAF and T3M4 cell lines were treated with BA (50 μM) for 4 h and cell lysates were collected. Immunoblot was performed to observe change in c-Jun expression in DCA- and CDCA-treated CD18/HPAF and T3M4 cell lines, compared to their respective untreated controls. **D**. Confocal images showing significant increase in c-Jun and MUC4 protein expression in CD18/HPAF cells treated with DCA or CDCA. Graph showing the quantification of the c-Jun positive nuclei in DCA and CDCA treated CD18/HPAF cells. **E**. Immunoblot showing significant increase in the expression levels of c-Jun in the nuclear fraction obtained from BA (25 μM)-treated CD18/HPAF cells, whereas cytoplasmic fraction did not demonstrate any noticeable alteration in c-Jun expression. **F**. ChIP experiment was performed to observe the effect on enrichment for c-Jun binding on MUC4 distal promoter in the presence or absence of DCA (50 μM) and CDCA (50 μM). We observed a significant increase in fold-enrichment at both region-I (containing two c-Jun binding sites) and region-II (containing one c-Jun binding sites) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, scale bar = 20 μM).

HPAF, CAPAN1, Panc10.05 and Panc1 cell lines (Figure 6B, Figure S3B), compared to normal pancreatic cells (HDPE). Interestingly, significantly high FXR levels in CD18/HPAF cells explains drastic increase in MUC4 expression even at very low

concentration of BA treatment compared to T3M4 and CAPAN1 cell lines (Figure 2A–C). Due to observed downregulation of activated FAK expression levels along with c-Jun upon transient knockdown of FXR in CD18/HPAF and T3M4 PC cell

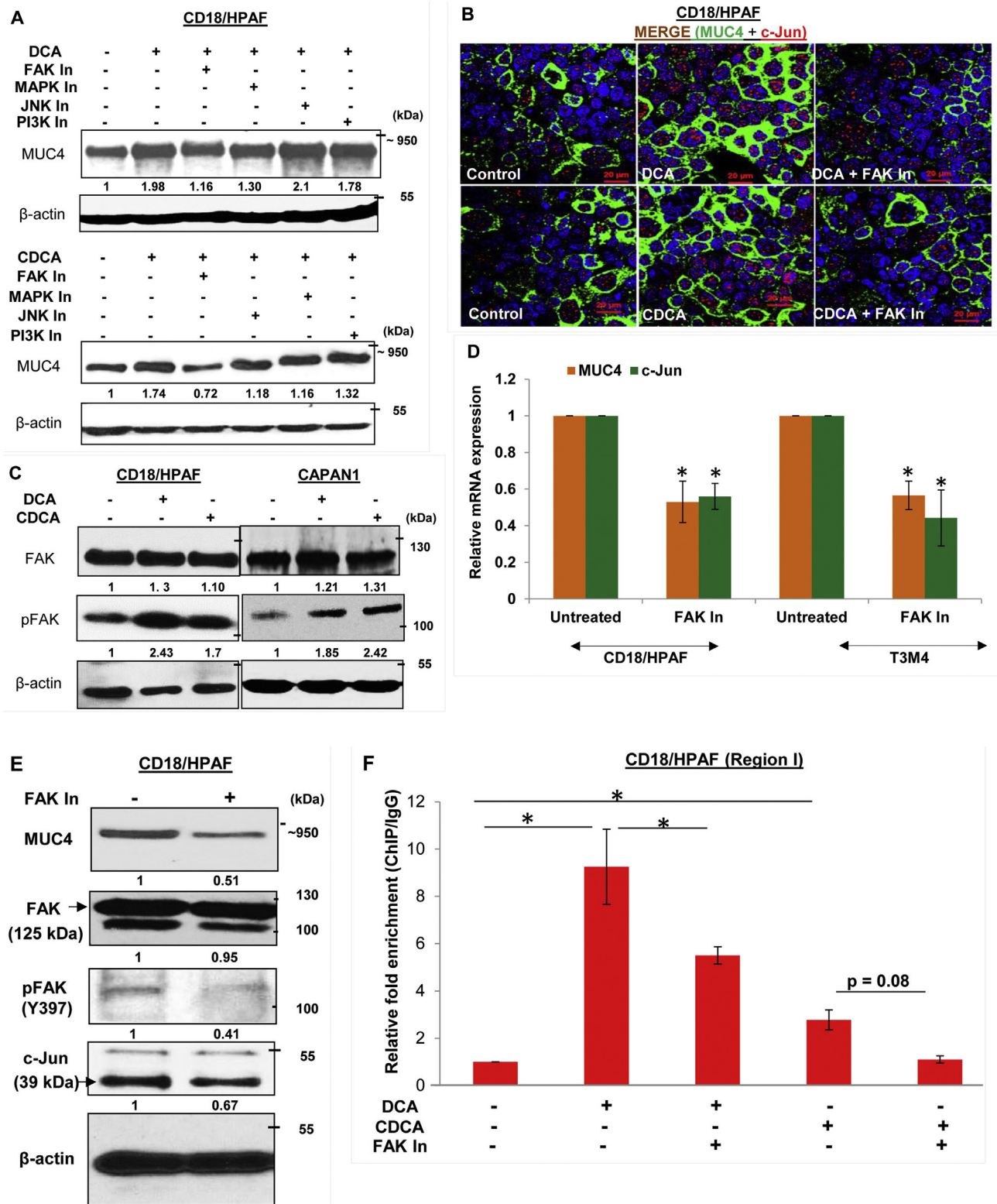


Figure 5 – BA-mediated upregulation of MUC4 is dependent on FAK activation. **A**. Concomitant treatment of 25 μ M of DCA or CDCA in the presence or absence of selective pharmacological signaling inhibitors for 12 h led us to know that the FAK pathway is mainly responsible for MUC4 upregulation upon BA exposure, as attenuation of this pathway maximally suppresses the BA-mediated upregulation of MUC4, compared to other signaling inhibitors. Besides FAK, inhibition of MAPK pathway also led to reduced MUC4 expression. **B**. Images obtained from immunofluorescence experiment showing MUC4 upregulation in DCA (25 μ M) and CDCA (25 μ M) treated CD18/HPAF cells, which is attenuated upon inhibiting FAK activity (or phosphorylation). **C**. Increase in FAK activity was confirmed by analyzing pFAK (Tyr397) expression upon BA (25 μ M) treatment of CD18/HPAF and CAPAN1 cell lines for 4 h. **D**. Graphical representation of relative mRNA expression for MUC4 and c-Jun gene altered upon inhibition of FAK pathway in both CD18/HPAF and T3M4 cell lines. **E**. Immunoblot showing that inhibition of FAK pathway, using 15 μ M of FAK Inhibitor 14, leads to downregulation of MUC4, pFAK and c-Jun in CD18/HPAF cells. **F**. Graph representing the relative fold enrichment for c-Jun on AP-1 sequence motifs present on MUC4 distal promoter when CD18/HPAF cells were concomitantly treated with DCA and CDCA in the presence and absence of FAK inhibitor for 4 h (* p < 0.05, scale bar = 20 μ m).

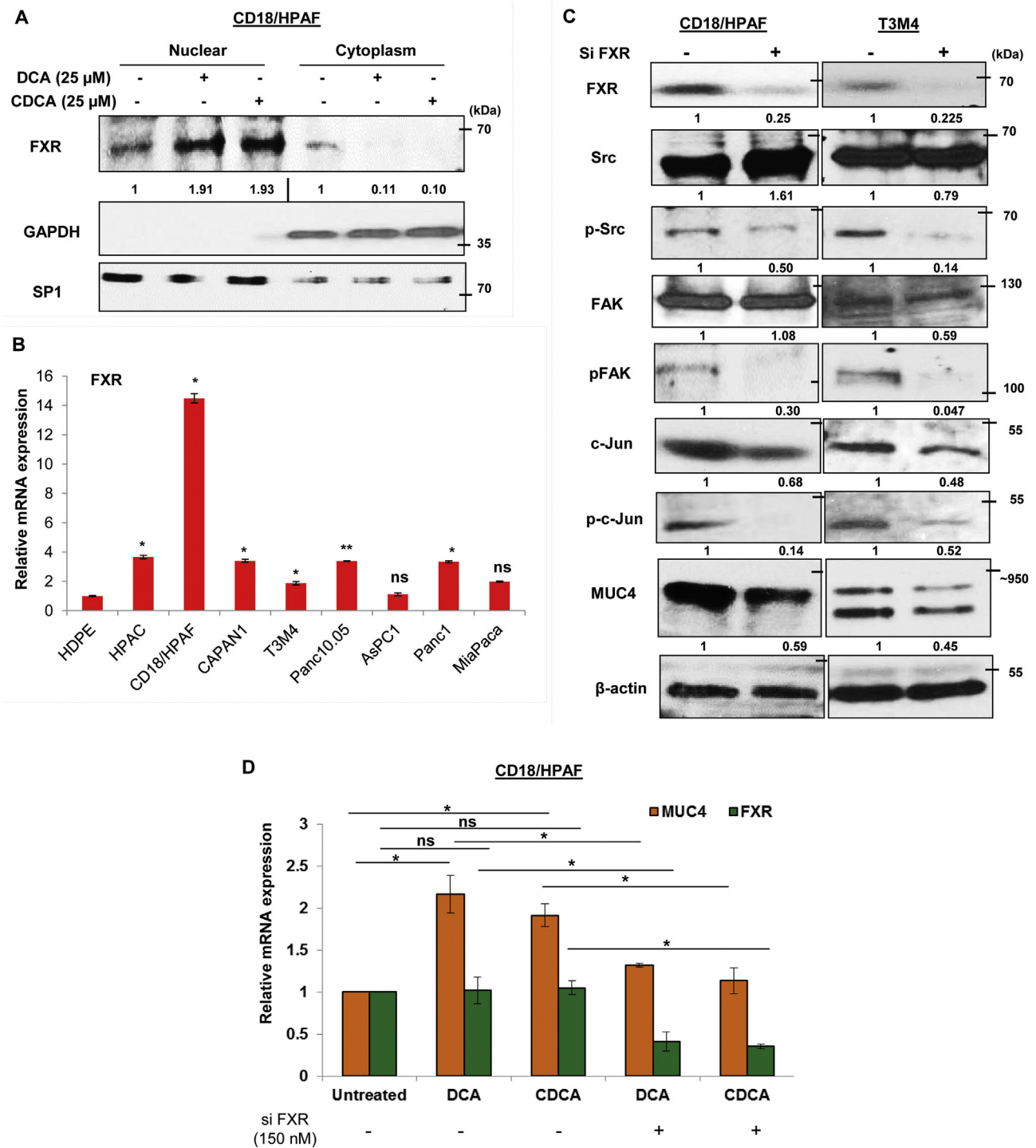


Figure 6 – Activation of FXR is required for BA-mediated MUC4 upregulation via Src/FAK/c-Jun axis. A. Nuclear fraction obtained from 50 μM of DCA and CDCA treated CD18/HPAF cells, demonstrated increased levels of FXR compared to the untreated control. On the other hand, FXR expression was more on the cytoplasmic fraction in untreated cells than DCA and CDCA treated cells. B. FXR expression was found to be significantly high in PC cell lines than normal pancreatic cells (HDPE). C. FXR was transiently knockdown in CD18/HPAF and T3M4 cell lines using 150 nM of siRNA oligos and confirmed using immunoblotting. Interestingly, FXR knockdown cells exhibited significant decline in FAK, pFAK (Tyr397), src, p-src (Tyr416), c-Jun, p-c-Jun (Ser63) and MUC4, suggestive of FXR involvement as the most upstream molecule in this BA-mediated FAK/c-Jun/MUC4 axis. D. The graphical representation of the result obtained from real-time PCR showing that knockdown of FXR in CD18/HPAF cell line leads to significant attenuation of both DCA (25 μM) or CDCA (25 μM)-mediated MUC4 upregulation (**p* < 0.05, ***p* < 0.01, ns means non-significant).

lines, it is likely that FXR is acting upstream in this FAK/c-Jun/MUC4 axis (Figure 6C). The key question which arises is that how FXR expression regulates the activity of FAK. It is well-known in the literature that src kinase is one of the critical regulator of FAK activity (Westhoff et al., 2004). As we have observed that BA treatment affects the phosphorylation of src (Figure S3C), we assumed that FXR-mediated phosphorylation of FAK is p-src-dependent, and FXR knocked down PC cells indeed showed significant reduction in p-src levels compared to si control (Figure 6C). To further substantiate our results, we gave BA treatment to FXR knockdown CD18/HPAF cells and found significant abrogation of BA-mediated MUC4 upregulation (Figure 6D). A 2.1-fold increase in MUC4 expression due to DCA treatment was reduced to 1.32-fold in FXR silenced CD18/HPAF cells (Figure 6D). Similarly, a 1.92-fold increase in MUC4 expression upon CDCA treatment was reduced to 1.13-fold when CDCA treatment was given to FXR knockdown cells (Figure 6D). Altogether, the results suggest that FXR activation due to BA exposure is responsible for the initiation of FAK/c-Jun/MUC4 axis in PC cells, by plausibly regulating the activity of src kinase.

3.7. Clinical association between MUC4 and BA receptor FXR

In PC patient samples, we clearly observed that similar to PC cell lines, mRNA expression for FXR was higher in 47% of PC tissues ($n = 15$), as compared to the tumor adjacent normal pancreatic tissues ($n = 4$) (Figure 7A). Though the upregulation of FXR in PC patients was not statistically significant ($p > 0.05$), but considering significant increase in the levels of BA, which are activators of FXR receptor, both in the circulation and pancreatic juice of PC patients, we can speculate that pancreatic tumors have increased activity of FXR receptor, which is sufficient to initiate FAK/c-Jun/MUC4 signaling cascade. In order to confirm an *in vivo* association between MUC4 and FXR, we measured the transcript levels of MUC4 in same clinical samples and performed regression analysis (Figure 7B). A fairly positive correlation ($R^2 = 0.60$) between MUC4 and FXR, further substantiated our *in vitro* findings. Moreover, using confocal microscopy, we observed co-expression of both FXR and MUC4 at the same PC tissue spots (Figure 7C).

4. Discussion

Anatomically, the common bile duct and the pancreatic duct are close in proximity, and unite at the ampulla of Vater. This led us to believe that BA can reflux to the pancreatic duct under pathological conditions. Growing pancreatic tumor often obstruct the bile ducts, preventing the flow of bile to the duodenum, leading to jaundice, a frequently occurring clinical manifestation in PC patients (Chen et al., 2015). Studies have established the tumor-promoting effects of BA in multiple cancers, including Barrett's metaplasia and colorectal, biliary, and hepatocellular cancers (Changbumrung et al., 1990; Degirolamo et al., 2011; Piessen et al., 2007; Souza et al., 2011). However, the role of BA in PC has not been clearly understood, which prompted us to investigate

its tumorigenic properties in PC. In order to establish our hypothesis, BA levels were measured in the serum and pancreatic juice obtained from PC patients and NPNH individuals. Encouragingly, we observed a significant increase in BA concentration in PC patients compared to controls. We also observed increased mRNA expression of BA receptor, FXR, in PC tumors compared to a normal pancreas. Due to increased BA levels, which act as FXR agonist, it can be speculated that not only its expression, activity of FXR also get increased under PC condition, which we have confirmed as well due to increased nuclear expression levels of FXR upon BA treatment. Similar to our observation, Lee et al. have also observed increased expression of FXR in the PC tissues and established its protumorigenic role in PC disease condition (Lee et al., 2011). Altogether, this is a first experimental evidence establishing that BA do enter the pancreatic duct and increases the tumorigenic potential of PC cells by altering the expression of oncogenic MUC4 mucin.

Our luciferase reporter assay established MUC4 distal promoter as the major BA responsive site. Further, *in silico* analysis demonstrated the presence of two activator protein 1 (AP-1) motifs in this region, which has also been reported in our earlier study (Singh et al., 2007). Consistent with the previous findings observed in gastric cells (Park et al., 2008), we noticed that BA treatment increase c-Jun expression, one of the members of the AP-1 family. Furthermore, ChIP experiments confirmed an increase in c-Jun binding onto MUC4 distal promoter when treated with BA. Interestingly, by utilizing the same c-Jun transcription factor, BA are known to increase the transcription of cyclooxygenase gene, by facilitating increased c-Jun binding on COX promoter in esophageal adenocarcinoma cells (Zhang et al., 1998). Importantly, c-Jun overexpression has already been associated with carcinogenesis and cancer progression in multiple cancers (Okutomi et al., 2003; Tessari et al., 1999). Although BA responsiveness was primarily observed at MUC4 distal promoter (P-1641), we also observed increased luciferase activity in proximal promoter region, P-1809 transfected CD18/HPAF cells, upon CDCA treatment, implying the involvement of other transcription factors in CDCA-mediated upregulation of CD18/HPAF cells, and requires further investigation (Singh et al., 2007). In spite of the presence of c-Jun bindings sites on MUC4 proximal promoter (P-2150), we observed an insignificant increase in luciferase activity upon BA treatment, suggesting that BA might be affecting the expression and binding of transcription factors having inhibitory effects on proximal promoter region, and therefore, neutralizing the positive effects of c-Jun.

Multiple forms of BA have been previously identified as potent inducers of MUC4 expression in esophageal carcinogenesis associated with bile reflux (Degirolamo et al., 2011; Mariette et al., 2004). Mechanistically, PI3K signaling, protein kinase C and hepatocyte nuclear factor-1 α were attributed to BA-facilitated increase in MUC4 expression (Mariette et al., 2004; Piessen et al., 2007). However, in the current study, we have established the role of FAK in MUC4 regulation in PC cells upon BA treatment. Moreover, downregulation of c-Jun expression upon FAK inhibition, suggests that c-Jun activation is a downstream event occurring after FAK activation. Nadruz et al. have also established the link between c-Jun

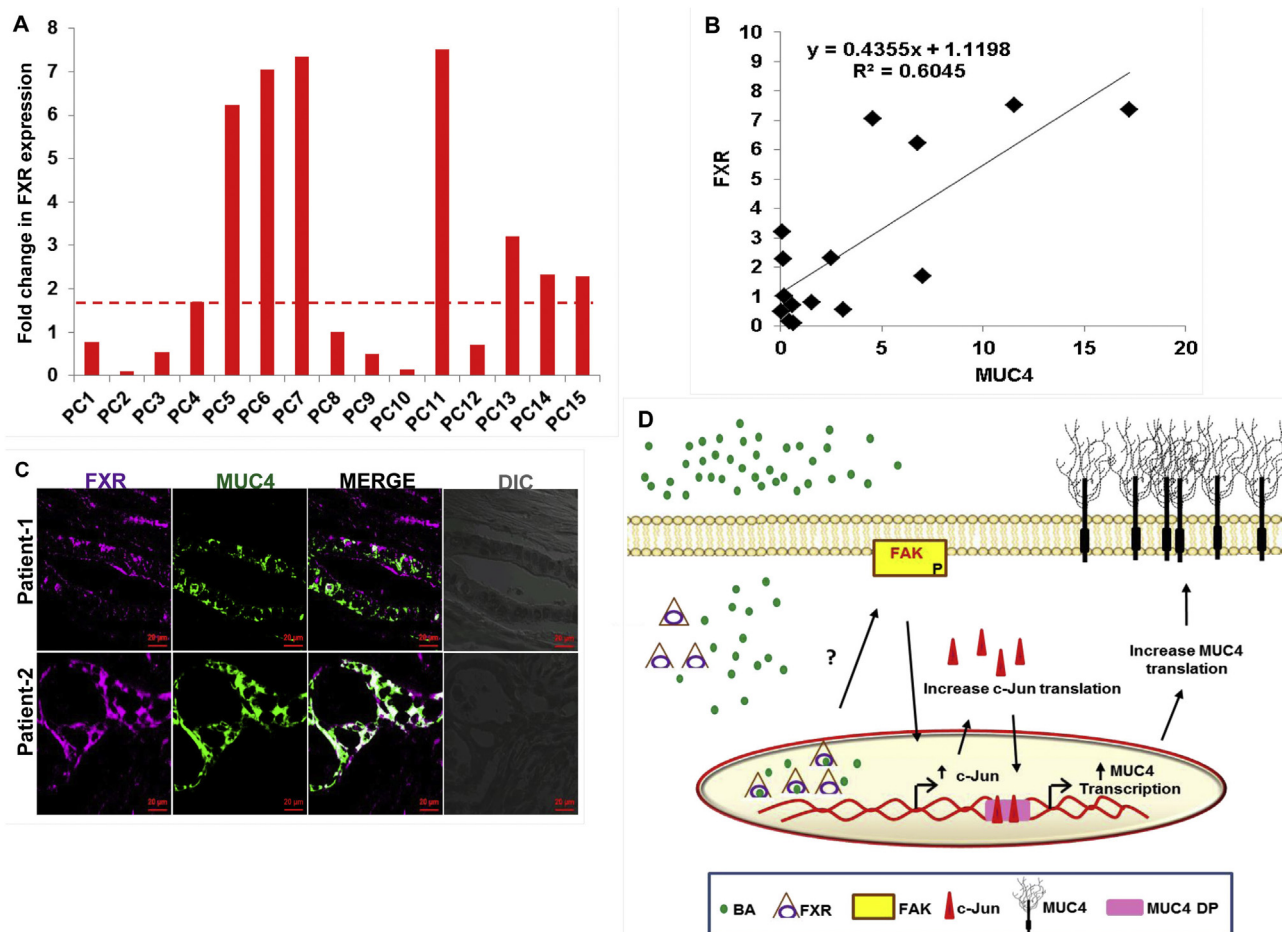


Figure 7 – Clinical association between MUC4 and FXR in PC tissues. **A.** Expression profiling of FXR was performed in cDNA samples prepared from pancreatic tumor ($n = 15$) and tumor adjacent normal tissues ($n = 4$). Similar to its agonists, the levels of FXR was upregulated in pancreatic tumors than adjacent normal tissues. **B.** Data showing regression analysis which was performed to correlate MUC4 and FXR in clinical samples at transcriptional levels. **C.** PC tissues (obtained from Whipple procedure) showed co-expression of both MUC4 and FXR, suggestive of their direct association (scale bar = 20 μ M). **D.** Diagrammatic representation of the overall summary of the paper: Treatment with BA leads to the activation of FXR receptor, which gets engage in the activation of FAK pathway, possibly by activating src kinase. Increase in FAK-mediated signaling leads to an increased transcription of c-Jun gene. Increased expression and activation of c-Jun is followed by its increased nuclear translocation, leading to increased MUC4 transcription, which plays an important role in the proliferation, survival, metastasis and chemoresistance of pancreatic tumors.

and FAK molecules in ventricular myocytes (Nadruz, Jr. et al., 2005). Unlike CDCA, the inhibition of the JNK pathway had no remarkable effect on DCA-induced MUC4 expression, suggesting that different BA transduce differential signaling to modulate the expression of the target molecules. Moreover, the data also implies differential mode of c-Jun activation in the presence of DCA and CDCA. Earlier studies have shown that c-Jun can get activated in a JNK-independent manner (Besirli and Johnson, Jr., 2003; Deng et al., 2012). For instance, in neuronal cells, DNA damage causing induction of neuronal c-Jun kinase has been shown to increase c-Jun phosphorylation (Besirli and Johnson, Jr., 2003). Upon injury, c-Jun is found to be activated in Schwann cells by MAP kinases, which is again occurring independent of JNK (Deng et al., 2012). In addition to FAK pathway, inhibition of MAPK pathway also led to attenuation of BA-mediated

MUC4 upregulation, which further strengthened our notion that MAPK pathway could be involved in c-Jun activation. Future studies will be focused to understand the in-depth involvement of different signaling pathways in MUC4 regulation after BA treatment.

BA are known to interact with nuclear receptors family including; FXR and pregnane X receptor (PXR) in order to modulate the transcription of their target genes. In the current study, for the first time, we have established the direct involvement of FXR protein in MUC4 regulation. In the clinical samples, we observed a positive correlation between FXR and MUC4 mRNA expression levels. Upon FXR knocked down, BA-mediated upregulation of MUC4 and activation of FAK and c-Jun were abrogated, placing FXR as an upstream molecule in this FAK/c-Jun/MUC4 axis in PC. Das A et al. have shown that FXR promotes the migration of endothelial cells by

regulating the expression of FAK and MMP9 (Das et al., 2009). However, the molecular mechanism of FXR-facilitated FAK activation is still unexplored. Due to observed increase in src kinase activity upon BA treatment, we assumed its role in this FXR-mediated increase in FAK activation, as FXR silencing led to reduced p-Src levels in PC cell lines. Interestingly, previous study in our lab has also shown that Guggulsterone, a selective pharmacological FXR inhibitor, also leads to a MUC4 downregulation at transcriptional level in PC cells by utilizing src/FAK pathway (Macha et al., 2013). In addition to FXR, other BA receptors could also be implicated in BA-facilitated MUC4 upregulation. Interestingly, TGR5 has found to be upregulated in 67% of PC patients (data not shown) and recent report has shown its tumorigenic role in gastrointestinal cancers, including PC (Nagathihalli et al., 2014). Further studies will be helpful and required to mechanistically delineate the association between TGR5 and PC disease condition.

Future studies will be directed to get the better insight of BA on the pathobiology of PC by bile duct ligation or cholecystectomy using autochthonous murine models, which will delineate the role of BA on pancreatic tumor growth and metastasis. Moreover, the significantly induced levels of BA indicates their possible usefulness for diagnostic purposes, and needs to be validated in more number of patient samples to assess and establish its clinical utility.

5. Conclusions

Altogether, the current study, for the first time, has established that BA levels rises both in the circulation and pancreatic juice in PC, and they exert their protumorigenic functions by upregulating oncogenic MUC4 expression. Mechanistically, we have demonstrated that BA facilitated activation of FXR leads to increased phosphorylation of FAK, followed by increased c-Jun expression and its nuclear translocation, which subsequently leads to increased transcription of the MUC4 gene (Figure 7D). The current study also supports emerging epidemiological data that, similar to colorectal cancer, fat-rich diet could be one of the risk factors for PC development and progression. Therefore, targeting BA receptors and administration of BA antagonists can significantly impact the outcome of PC patients.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.molonc.2016.04.007>.

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