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MUC1 Regulates PDGFA Expression During Pancreatic Cancer Progression

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Abstract

Pancreatic Ductal Adenocarcinoma (PDA) has one of the worst prognoses of all cancers. Mucin 1 (MUC1), a transmembrane mucin glycoprotein, is a key modulator of several signaling pathways that affect oncogenesis, motility, and metastasis. Its expression is known to be associated with poor prognosis in patients. However, the precise mechanism remains elusive. We report a novel association of MUC1 with Platelet-Derived Growth Factor-A (PDGFA). PDGFA is one of the many drivers of tumor growth, angiogenesis, and metastasis in PDA. Using mouse PDA models as well as human samples, we show clear evidence that MUC1 regulates the expression and secretion of PDGFA. This, in turn, influences proliferation and invasion of pancreatic cancer cells leading to higher tumor burden *in vivo*. In addition, we reveal that MUC1 over expressing cells are heavily dependent on PDGFA both for proliferation and invasion while MUC1-null cells are not. Moreover, PDGFA and MUC1 are critical for translocation of βcatenin to the nucleus for oncogenesis to ensue. Finally, we elucidate the underlying mechanism by which MUC1 regulates PDGFA expression and secretion in pancreatic cancer cells. We show that MUC1 associates with Hif1-α, a known transcription factor involved in controlling PDGFA expression. Furthermore, MUC1 facilitates Hif1-α translocation to the nucleus. In summary, we have demonstrated that MUC1-induced invasion and proliferation occurs via increased exogenous production of PDGFA. Thus, impeding MUC1 regulation of PDGFA signaling may be therapeutically beneficial for patients with PDA.

Keywords

Pancreatic cancer; MUC1-Mucin 1; PDGFA; β-catenin

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Introduction

Pancreatic Cancer is the 10th most commonly diagnosed cancer and the 4th leading cause of cancer-related deaths in the United States [1]. It is estimated in the year 2010, approximately 43,000 new cases will be diagnosed in the U.S. alone, with approximately 36,800 deaths [1]. Surgical resection remains the only potentially curative intervention for pancreatic cancer, but is contraindicated in most patients because their disease is either locally inoperable or metastatic at presentation [2]. Among the minority of patients who undergo surgical resection, the median survival is only 20 months, with a 5-year survival rate of 8–20% [3] due to high rate of recurrence. The median survival for patients with metastatic disease at presentation is less than 6 months [4]. Moreover, pancreatic cancer is largely resistant to radiation and chemotherapy. Early micro-metastasis to the distal organs such as lymph nodes, liver, and lung is one of the hallmarks of this disease [4]. Pancreatic cancer therefore remains a lethal diagnosis for the vast majority of patients due to high rate of recurrence and metastasis. Understanding the oncogenic role of proteins that are over expressed during pancreatic cancer progression and metastasis is essential for developing innovative therapies.

Mucin-1 (MUC1, CD227) is one such factor. MUC1 is a type-I transmembrane glycoprotein which is overexpressed and aberrantly glycosylated in many adenocarcinomas including PDA [5] [6]. MUC1 is detected in >60% of high-grade pancreatic intraepithelial neoplasia (PanIN) and its expression positively correlates with high metastasis and poor prognosis [7–8]. MUC1 has an N-terminal extracellular domain consisting of variable number tandem repeats (MUC1-TR) of 20 amino acids (aa) and a C-terminal domain which includes a 53aa extracellular region, a transmembrane domain (TM) and a 72aa cytoplasmic tail (MUC1CT) [9]. MUC1-CT plays a critical role in cell signaling during cancer progression (reviewed in [10–13]) and is known to associate with β-catenin and translocate to the nucleus. This association is known to influence the activities of several transcription factors [11, 14]. We have recently shown that translocation of MUC1 CT to the nucleus is essential to drive the transcription of genes responsible for Epithelial to Mesenchymal Transition (EMT) in PDA cells. The translocation of MUC1 CT directly contributes to the invasiveness and metastatic properties of these cells [14]. We have also shown that PDA mice that lack MUC1 have a profound defect in tumor growth and metastasis while PDA mice that express MUC1 have enhanced tumor progression and metastasis [15–16]. Taken together, these recent reports strengthen the important role of MUC1 in the development and progression of the disease [17–19].

Although there have been many recent advances in the role of MUC1 driven tumor progression, the underlying mechanism remains elusive. Given that MUC1 is known to affect epidermal growth factor (EGF) interaction with its receptor EGFR in breast cancer [20], we hypothesized that another growth factor may associate with MUC1. In the current study, we examined the association of MUC1 with Platelet-Derived Growth Factor-A (PDGFA), a factor known to be critical for PDA progression. In PDA, expression of PDGFA is associated with poor prognosis and high metastasis [21]. Interaction of PDGFA with its receptor leads to cellular responses such as proliferation and migration through MEK and PI3K signaling respectively [22]. *In vivo*, PDGF plays a significant role in

angiogenesis and metastasis (reviewed in [23] [24–25]). Indeed, inhibition of PDGFA signaling has been shown to reduce growth and metastasis of human pancreatic carcinoma [26–27]. Therefore, targeting PDGFA has been proposed as an adjuvant treatment in patients with PDA [28].

In this study, we demonstrate the first evidence for MUC1 regulation of PDGFA during progression of PDA. Analysis using both PDA mouse models and pancreatic cancer cell lines revealed a significant role for MUC1 in regulation of PDGFA expression. We report that MUC1 increases proliferation and invasion of pancreatic cancer cells via regulation of PDGFA expression. This may be responsible for higher tumor burden and metastasis in MUC1expressing PDA. Moreover, expression of MUC1 correlates with PDGFA expression during PDA. In this study, for the first time, the significance and mechanism by which MUC1 associates with PDGFA has been elucidated.

Results MUC1 expression was detected during all stages of PDA and is correlated with PDGFA expression

MUC1 is known to be over-expressed during human PDA and leads to an aggressive phenotype. To evaluate the mechanism underlying MUC1 regulation of PDA development, we subjected RNA samples from MiaPACA-2 cells treated with MUC1 siRNA to a Cancer Pathway RT-PCR array. This array profiles 84 genes representing 6 biological pathways involved in tumorgenesis. 15 genes were significantly altered in MiaPACA-2 cells treated with MUC1 siRNA (Figure 1A). Of these genes, PDGFA was most notably down-regulated with MUC1 knockdown. We next determined the levels of PDGFA expression in human PDA tissues by IHC. Our results show that although basal level expression of PDGFA is detectable in normal pancreas, there is over-expression of PDGFA in dysplastic ductal cells, in a stage dependent manner (Figure 1C). Representative images are shown from n=5 patients per stage. Representative H&E of the same area is also shown in Figure 1C. Although it is well established that human PDA expresses MUC1, we demonstrate here that MUC1 is clearly detected in early stage PanIN lesions using the MUC1 CT antibody and this expression increases in adenocarcinoma (Figure 1B). Although low level expression is detected in the adjacent normal pancreas tissue, the expression is localized to the apical surface. Further, in supplemental Figure 1A, we confirm MUC1 over expression in PDA tissues at different stages of the disease using a MUC1 TR antibody.

MUC1 regulates PDGFA expression in pancreatic cancer cell lines *in vitro*

To study whether MUC1 regulates PDGFA expression, several pancreatic cancer cell lines with various levels of MUC1 expression were utilized. We used human and mouse cell lines that were either positive or null for MUC1. BXPC3 ($MUC1^{low}$), SU86.86 ($MUC1^{low}$), MiaPACA-2($MUC1^{high}$), and CAPAN-1($MUC1^{high}$) as well as mouse cell lines KCM ($MUC1^{high}$) and KCKO ($MUC1^{null}$) were used. In addition, BXPC3 and SU86.86 cells that have low endogenous MUC1 were transfected with full length MUC1 or empty vector (Neo). Since MiaPACA-2 and CAPAN-1 cells have high endogenous MUC1, we utilized specific siRNA to knock-down MUC1 expression. First, we show the levels of MUC1 using both the MUC1-TR and MUC1-CT antibodies in all of the pancreatic cancer cell lines. As

predicted, in KCKO, BXPC3.Neo, and SU86.86.Neo cells, MUC1 was undetectable while in KCM, BXPC3.MUC1, and SU86.86.MUC1, high expression of MUC1 was detected (Figure 2A). Furthermore, MiaPACA-2 and CAPAN-1 cells expressed high MUC1 while MUC1 siRNA-treated MiaPACA-2 and CAPAN-1 cells expressed negligible levels of MUC1 at 72 hours post siRNA treatment (Figure 2A). Protein lysates from the same cells were then subjected to Western blot analysis for PDGFA expression. In KCKO cells, expression of PDGFA was undetectable while a robust expression of PDGFA was noted in KCM cells (Figure 2B). Similarly, BXPC3.MUC1 and SU86.86.MUC1 cells expressed high levels of PDGFA while BXPC3.Neo and SU86.86.Neo cells expressed lower levels. Moreover, in both of the siRNA models (MiaPACA-2 and CAPAN1), expression of PDGFA was significantly downregulated following MUC1 siRNA treatment (Figure 2B). Thus, data indicate that lack of MUC1 expression in pancreatic cancer cells leads to absence/downregulation of PDGFA expression while introduction of MUC1 to pancreatic cancer cells led to induction of PDGFA expression. Densitometric analysis was conducted for the western blots from n=3 experiments and the average density with the standard deviation is reported in Table 1.

To further test if MUC1-expressing cells release more PDGFA, supernatants from siRNA treated CAPAN-1, BXPC3.Neo and MUC1 and KCKO and KCM cells were analyzed for presence of PDGFA using a specific ELISA. Results correlated with the PDGFA expression data such that CAPAN-1, BXPC3.MUC1, and KCM cells secreted significantly higher levels of PDGFA compared to MUC1 siRNA treated CAPAN-1, BXPC3.Neo, and KCKO cells (Figure 2C). For the first time, we have shown that lack of MUC1 not only leads to downregulation of PDGFA expression in PDA cells but significantly reduces its secretion (Figure 2C).

MUC1 regulates PDGFA expression during tumor progression in a PDA mouse model as well as in human pancreatic tumors

We utilized our *in vivo* spontaneous model of PDA to assess if MUC1 regulates PDGFA expression during tumor progression. Pancreatic sections from PDA.Muc1$^{-/-}$ (no Muc1), PDA (with mouse Muc1), and PDA.MUC1 (with human MUC1) mice were stained for histological examination and PDGFA expression. At 6-weeks of age, the pancreatic architecture in all mice appeared similar to that of healthy C57BL/6 mice (Figure 2D). However, even at this early stage, PDGFA expression appeared to be the highest in PDA.MUC1 mice and lowest in PDA.Muc1$^{-/-}$ mice (Figure 2Di, ii). We have previously reported MUC1 expression in 6 week old PDA.MUC1 mice [16] (Supplemental Figure 1B). By 6-months of age, PanIN lesions and MUC1 expression are evident in both PDA and PDA.MUC1 mice [15–16, 29]. Furthermore, expression of PDGFA significantly increased in the pancreas of PDA and PDA.MUC1 mice as compared to PDA.Muc1$^{-/-}$ mice (Figure 2D). Most importantly, low PDGFA expression in the PDA.Muc1$^{-/-}$ (no Muc1) mice correlated with smaller numbers of low grade PanIN lesions compared to PDA and PDA.MUC1 mice. Similarly, strong expression of PDGFA in MUC1 expressing mice correlated with an increased number of higher grade PanIN lesions and disruption of pancreatic architecture as compared to PDA.Muc1$^{-/-}$ mice (Figure 2D iii). The same

phenomenon was recapitulated in 8-month old mice, where PDGFA expression levels correlated with higher numbers and grade of PanIN lesions and invasive adenocarcinoma. Thus, low PDGFA in PDA.Muc1$^{-/-}$ mice correlated with low numbers of late stage PanIN lesions. Whereas, PDA mice showed higher numbers of late stage PanIN lesions and higher PDGFA expression compared to PDA.Muc1$^{-/-}$ mice. The pancreas from PDA.MUC1 mice showed the highest numbers of late stage PanIN lesions and adenocarcinoma [15–16] which correlated with strongest expression of PDGFA (Figure 2D iv). Further, when we directly compared the 24 week PDA.MUC1 tumor with the 34week PDA tumor (when the levels of PanIN 3 lesions are similar between the two phenotypes [16]), we found that the PDGFA and MUC1 CT staining intensity and was similar (Supplemental Figure 3). The data confirms that at similar stage of tumor development, PDGFA and MUC1 CT staining are similar.

This was further confirmed using BXPC3.MUC1 and BXPC3.Neo tumors established *in vivo* in nude mice. Significantly higher levels of PDGFA were observed in the BXPC3.MUC1 tumors as compared to BXPC3.Neo tumors (Figure 2D v). Taken together, the data demonstrates a strong link between MUC1 and PDGFA expression during tumor progression *in vivo*.

Note: Since we have already published that MUC1 expression increases with tumor progression in PDA and PDA.MUC1 mice using MUC1 CT and MUC1 TR specific antibodies [16], we are providing that data as part of supplemental Figure 1B. There is no MUC1 expression in the PDA.Muc1$^{-/-}$ tumors. We have also compared the PDA.MUC1 tumors with PDA tumors based not only on age but stage of tumor development.

MUC1-expressing PDA cells have increased tumor burden *in vivo* and higher proliferative and invasive potential *in vitro* as compared to Muc1-null PDA cells

To determine if higher MUC1 and PDGFA expression translate to increased tumor growth *in vivo*, C57BL/6 mice were injected subcutaneously in the right flank with KCKO (derived from PDA.Muc1$^{-/-}$ tumors [15]) and KCM cells (derived from PDA.MUC1 tumors [15]). The tumor weight was monitored and assessed at sacrifice. Tumors resulting from KCM cells were significantly larger than those from KCKO cells (Figure 3Ai). Similarly, BXPC3.Neo and BxPC3.MUC1 cells were injected subcutaneously into nude mice and tumor weight was measured. Tumors resulting from BXPC3.MUC1 cells were significantly larger than that of BXPC3.Neo (Figure 3A ii) confirming the results of our previously published studies [14–15]. Thus, data provides strong evidence that tumor cells expressing MUC1 (high expression of PDGFA) have a significant growth advantage compared to tumors that are null for MUC1 (low expression of PDGFA). Indeed, using thymidine (^{3}H) uptake assay, we clearly demonstrate that cells expressing MUC1 have significantly higher proliferation when compared to cells lacking MUC1. This was observed in KCM versus KCKO; BXPC3.Neo versus BXPC3.MUC1; MiaPACA-2 versus siRNA-treated MiaPACA-2 and CAPAN-1 versus siRNA-treated CAPAN-1 cells (Figure 3Bi–iv).

Since PDGFA is also a known inducer of invasion, pancreatic cancer cell lines were subjected to the *in vitro* Boyden chamber invasion assay. Results demonstrate that invasion of KCKO and BXPC3.Neo cells through the growth factor reduced matrigel was significantly lower than that of KCM and BXPC3.MUC1cells respectively (Figure 3C i and ii). Similarly, both MiaPACA-2 and CAPAN-1 show significantly decreased invasion following 72 hours of MUC1 siRNA treatment (Figure 3C iii and iv). (*Note: all p values are provided in the figure legend*). Taken together, this data suggests strong evidence for the significant function of MUC1 in augmenting proliferation and the invasive properties of pancreatic cancer cells possibly via PDGFA. *In vivo*, we have recently reported that 1/10 PDA.Muc1$^{-/-}$ mice develop metastasis while 3/10 and 7/10 PDA.MUC1 mice develop metastasis to the lung and liver [14–15] and (Supplemental Figure 2A).

PDGFA expression in pancreatic cancer cell lines contributes to increased invasion and proliferation

Thus far, we have shown that high MUC1 levels in tumors correlate with high PDGFA levels leading to increased tumor burden. Therefore, to elucidate the role of PDGFA in the proliferation of MUC1-expressing cells, we neutralized PDGFA with a specific antibody prior to subjecting the tumor cells to a ^{3}H-thymidine proliferation assay. Proliferation of KCM cells was significantly reduced following PDGFA neutralization; however, proliferation of KCKO cells remained unchanged (Figure 4Ai). The same phenomenon was observed in BXPC3 cells where PDGFA neutralization significantly reduced BXPC3.MUC1 proliferation compared to untreated cells while BXPC3.Neo cells remained unaffected (Figure 4Bii).

Similarly, PDGFA neutralization significantly reduced the invasive index of MUC1-expressing tumor cells including KCM, BxPC3.MUC1, and MiaPACA-2 cells compared to cells that did not undergo PDGFA neutralization (Figure 4Bi–iii). As was expected, the MUC1-non expressing cells (KCKO, BXPC3.Neo, and MUC1 siRNA-treated MiaPACA-2) remained unaffected with PDGFA neutralization. Furthermore, there was no difference in the levels of PDGF-Rα (receptor responsible for PDGFA binding) in the MUC1-negative cells as compared to MUC1-positive cells (Supplementary Figure 2B). Taken together, our data suggests that cells lacking MUC1 not only produce less PDGFA but may not be dependent on PDGFA, a possible explanation for low growth and metastatic potential of MUC1-negative cancer cells.

MUC1 associates with Hif-1α and regulates its translocation to the nucleus

Since Hif-1α is a transcription factor known to regulate PDGFA production and is also associated with poor prognosis during PDA [21] [30], we hypothesized that MUC1 regulates translocation of this protein to the nucleus and thus its activation in PDA cell lines. Therefore, we first examined the interaction between Hif-1α and MUC1-CT in pancreatic cancer cell lines by co-immunoprecipitation assays. The results indicate that in MUC1 expressing PDA cell lines, Hif-1α associates with MUC1-CT (Figure 5A). This was validated using two different clones of the Hif-1α antibody (data not shown). *Note: We were unable to IP with MUC1 CT and IB with Hif-1α. This could be because MUC1 and HIF1-α*

interact at the CT portion of MUC1 (where the MUC1 CT antibody would bind poorly if at all during IP), thereby allowing this one-way IP/IB when using HIF1-α. Next, we examined the effects of this interaction on Hif1α nuclear translocation. Nuclear extracts of KCKO, KCM, BXPC3 and SU86.86 cells were subjected to Western blot analysis for presence of Hif-1α. We show that KCM cells have higher levels of Hif-1α translocation to the nucleus compared to KCKO cells (Figure 5Bi). This is exemplified in the BXPC3, SU86.86, and MiaPACA-2 cells where translocation of Hif-1α to the nucleus is evident only in the MUC1-expressing cells (Figure 5Bii and iii). To validate the role of Hif1-α in regulation of PDGFA, BXPC3.Neo and BXPC3.MUC1 cells were treated with a Hif1-α inhibitor for 24hours. Protein lysates were then subjected to Western blot analysis for presence of PDGFA. Our results show that following HIF1-α inhibition, expression of PDGFA is strongly downregulated in BXPC3.MUC1 cells (Figure 5C) with no significant change in the Neo cells. This suggests that regulation of PDGFA via MUC1 may require translocation of Hif1α to the nucleus. Densitometric analysis was conducted for the western blots from n=3 experiments and the average density with the standard deviation is reported in Table 2.

MUC1 associates with β-catenin and translocates to the nucleus

PDGFA and MUC1 have both been shown to induce translocation of β-catenin to the nucleus and promotion of tumorogenesis and EMT [14, 31–34]. Therefore, we investigated MUC1-CT association with β-catenin and its translocation to the nucleus. First, using several cell lines, we show that MUC1 and β-catenin associate with each other in all of the MUC1positive PDA cell lines (Figure 5D); second, we demonstrate that MUC1-CT translocates to the nucleus of all MUC1-positive PDA cell lines (Figure 5D). Likewise, although low level translocation of β-catenin was observed in KCKO, BXPC3, and SU86.86 cells, the levels were significantly higher in KCM, BXPC3.MUC1, and SU86.86.MUC1 cells (Figure 5E).

Studies have shown that PDGFA regulates β-catenin translocation to the nucleus of liver cancer cells [31], therefore, we hypothesized that translocation of β-catenin to the nucleus of pancreatic cancer cells is dependent on PDGFA secretion. In order to investigate this, we treated BXPC3.Neo and MUC1 cells with recombinant PDGFAA, or neutralizing PDGFA antibody and studied translocation of β-catenin to the nucleus of these cells. The results clearly demonstrate that treatment with exogenous PDGFAA increases translocation of β-catenin to the nucleus in the BXPC3.Neo cells, which normally do not express MUC1 and have very low levels of βcatenin in the nucleus (Figure 5F). In addition, neutralizing PDGFA in the supernatant of BXPC3.MUC1 cells resulted in decreased translocation of β-catenin to the nucleus (Figure 5F). Thus, we have shown that MUC1 regulation of β-catenin translocation to the nucleus is in part dependent on PDGFA. Densitometric analysis was conducted for the western blots from n=3 experiments and the average density with the standard deviation is reported in Table 2. Since these interactions are difficult to show *in vivo*, we have shown that MUC1 and β-catenin colocalize in the PDA.MUC1 tumors by confocal microscopy in supplemental Figure 2C. This could not be shown for HIF-1α and MUC1 because the antibody for HIF-1α does not work well on paraffin embedded tissue.

Discussion

To this date, PDA is one of the most challenging cancers. It is a highly metastatic tumor and greatly resistant to chemotherapy. While MUC1 has been proposed as a marker for pancreatic cancer detection [35], its significant role in PDA development has not been fully explored. We have shown that MUC1 enhances pancreatic tumor progression and invasion by directly regulating the levels of PDGFA expression and secretion. PDGFA expression during pancreatic cancer correlates with poor prognosis and is proposed to be a potential drug target for pancreatic cancer treatment [28]. Similarly, MUC1 has also been implicated as a marker for poor prognosis and a target for therapeutic intervention in pancreatic cancer [35]. This is the first study to show a direct relationship between these two pancreatic cancer promoting factors. The evidence for this association is overwhelming. We have shown that MUC1 over expression induces PDGFA in multiple human and mouse cell lines *in vitro* and *in vivo* as well as in models of PDA that either express MUC1 or are Muc1-null (Figure 1–2).

Since MUC1 antibodies against the tandem repeat are sensitive to the pattern of glycosylation, detection of MUC1 on early stage human PDA samples is challenging. In this study, using an antibody against the MUC1-CT we have shown that MUC1 is overexpressed in all stages of pancreatic cancer (Figure 1). We show that a lack of MUC1 in PDA cells, down regulates PDGFA expression (Figure 1A). In fact, we have shown a strong correlation between expression of these two proteins in human PDA samples at different stages (Figure 1B). We report that MUC1 expressing PDA cells and tumors express and secrete higher levels of PDGFA which may account for the increased proliferative and invasive index of these cells. In contrast, PDA cells and tumors that lack MUC1 and therefore also have lower expression of PDGFA show lower proliferation and invasion (Figure 3 and 4). Furthermore, PDA mice lacking Muc1 (PDA.Muc1$^{-/-}$ mice) have low levels of PDGFA expression correlating with low-grade PanIN lesions (Figure 2) [15]. In contrast, tumors from PDA.MUC1 mice (that have high PDGFA expression) progress relatively quickly to develop high-grade PanIN lesions and eventually develop into invasive adenocarcinoma (Figure 2D) [16].

PDGFA has long been associated with cancer progression [23] and can increase proliferation and invasion through the MEK and PI3K pathway respectively (as reviewed in [3637]). Thus, we propose for the first time that MUC1 modulates pancreatic cancer growth and progression via inducing expression and secretion of PDGFA. Our PDGFA neutralizing study demonstrates the significant role of PDGFA in induction of proliferation and invasion of pancreatic cancer cells (Figure 4). HIF1-α expression during PDA is correlated to PDGFA expression and poor prognosis [21]. Therefore we hypothesized that MUC1 association with Hif1-α leads to increased nuclear translocation of this transcription factor which is a known regulator of PDGFA [38]. Although Hif-1α expression is very low during normoxia, we were able to detect its association with MUC1-CT using two different antibodies against Hif-1α. In fact, we show that inhibition of Hif1-α leads to a significant decrease in the levels of PDGFA expression (Figure 5). These results provide us with indirect evidence that MUC1 up regulates PDGFA expression via up regulation of HIF-1α nuclear translocation. These findings supplement recent studies which have shown that

MUC1 translocation to the nucleus can alter expression of growth factors and therefore influence the tumor microenvironment [39].

Further, the association of MUC1-CT with β-catenin and translocation of the complex to the nucleus (Figure 5C–D) is critical for oncogenesis. It is of significance that translocation of βcatenin to the nucleus is strongly dependent on MUC1 levels. We have found that if cells do not have MUC1, very little β-catenin is found in the nucleus (Figure 5) although there is abundant βcatenin in the whole cell lysates. Previous data have shown the strong role of PDGFA in nuclear localization of β-catenin in liver cancer [31]. Other studies have elucidated the role of PDGFB in nuclear translocation of β-catenin in pancreatic cancer [33]. In this study, by neutralizing PDGFA, we have shown that translocation of β-catenin is decreased even if cells express high levels of MUC1. In addition, we have shown that by adding PDGFAA to MUC1-negative cells, β-catenin localizes to the nucleus (Figure 5F). Therefore, we report that MUC1 regulation of PDGFA has an additive effect on β-catenin translocation to the nucleus, which can consequently alter EMT, metastasis, and cancer stem cell production (as reviewed in [40] and schematically illustrated in Figure 6).

While MUC1-TR is proposed to be a targetable tumor antigen during PDA, growing evidence implicates the significant role of MUC1-CT in tumor progression. Moreover, clinical trials targeting PDGFA alone have shown limited success, therefore, targeting MUC1 signaling may be an alternative strategy that warrants further investigation. It is also plausible that MUC1 expression may be used as a surrogate biomarker for aggressive pancreatic tumors that over-express PDGFA.

Materials and Methods

Cell lines

BXPC3, SU86.86, MiaPACA-2, and CAPAN-1 (American Type Culture Collection (ATCC), Manassas, VA) were maintained in RPMI and DMEM (Invitrogen, Carlsbad, CA), 10%FCS, 1%penicillin/streptomycin and 1%glutamax (Invitrogen). KCM/KCKO, generated from tumors derived from PDA×MUC1.Tg mice and PDA×Muc1KO mice respectively [14–16] were maintained in DMEM. All cell types were maintained in 5% CO2 and 95% humidity.

RT-PCR Microarray

MiaPACA-2 cells were treated with MUC1 siRNA or scramble siRNA for 72 hours. RNA isolation was performed using RNAeasy Mini Kit (QIAGEN Sciences, Valencia, CA) according to the manufacturer's protocol. cDNA was constructed from RNA using TaqMan cDNA kit from Applied Bioscience (Foster City, CA) and subjected to real-time PCR Cancer PathwayFinder PCR Array (SABiosciences, MD). Arrays were performed independently at least three times for each treatment. Only genes showing consistent alteration with both controls were included in the results reported here. The reaction was performed using ABIPRISM7900HT thermocycler. Data were analyzed using SABiosciences software.

Human samples

Tissue sections of normal pancreas and PDA was acquired from the NIH/NCI tissue repository (http://seer.cancer.gov/biospecimen).

Immunohistochemistry

Tumor sections were formalin-fixed, paraffin-embedded, and sectioned. PDGFA expression was determined using the Santa Cruz Biotechnologies antibodies followed by the appropriate secondary antibody (DAKO, Carpenteria, CA). Sections were developed using 3,3”– Diaminobenzidine as the chromogen and hematoxylin was used as the counterstain. Pictures were taken at 40X magnification. Hematoxylin/Eosin (H&E) staining was performed using a standard protocol.

Cloning of MUC1 vectors

BXPC3 and SU86.86 are PDA cell lines that express low to no endogenous MUC1. For retroviral infection, GP2-293 packaging cells were co-transfected with the full-length MUC1 construct or empty vector expressing the neomycin resistance gene and VSV-G envelope protein as previously described [41]. Virus was subsequently pelleted, resuspended in medium containing 8 μg/mL polybrene and incubated overnight with PDA cells that had been pretreated for 2–3 hours with polybrene. Cells were selected with 0.5 mg/mL G418 for 48 hours post infection and were maintained as polyclonal populations until sorted for MUC1-positive and negative cells. Two independent infections of the constructs were carried out with similar results. Expression of the constructs was stable throughout the span of experiments. Cells infected with vector alone were used as control and designated as ‘Neo’. For MUC1 cells, MUC1+ve cells were sorted using the FACSAria. For Neo-infected cells, MUC1-ve cells were sorted. Level of MUC1 expression was validated using Western blot analysis (Figure 2A) and Flow cytometry (data not shown).

MUC1 knockdown

MiaPACA-2 and CAPAN-1 cells were seeded in 6 well plates and at 30% confluency were treated with 100nM siGENOME SMART pool siRNA targeting MUC1 or 100nM of scramble siRNA as control (DHARMACON, Thermo Fisher Sc.). siRNA was incubated with cells in presence of Lipofectamine (Invitrogen) for 5–6 hours in serum-free Opti-MEM (Invitrogen). Cells were then washed with PBS and FBS containing DMEM was added. Levels of MUC1 expression were checked 48, 72, and 96 hours post siRNA treatment.

PDGFA ELISA

Supernatants from PDA cells were collected. The levels of PDGFA secretion was analyzed using Human/Mouse PDGFAA Immunoassay (R and D systems, MN) according to the manufacturer’s protocol.

Mouse Model

PDA mice were generated in our laboratory congenic on the C57BL/6 background by mating the P48-Cre with the LSL-$KRAS^{G12D}$ mice [29] to produce PDA mice and further mated to the human MUC1.Tg mice to generate PDA.MUC1 mice [16, 42] or to the

Muc1$^{-/-}$ mice [43] to generate PDA.Muc1$^{-/-}$ mice. Primary tumors were dissociated using collagenase-IV and several lines of cells were generated in our laboratory. These cells are designated KCKO for PDA cells lacking Muc1 and KCM for PDA cells expressing human MUC1. Thus far, we have been unable to make cell lines from PDA mice.

PDA mice were sacrificed at 6 weeks (pre-neoplastic), 15 weeks (PanIN-1A), 26 weeks (PanIN1A), and 40 weeks (PanIN-1A,B, PanIN-2, and PanIN-3) of age [29]. The entire pancreas was removed and weighed including the duodenal lobe. Pancreas removal was consistent between all mice. Part of the tumor tissue was fixed in formalin for future IHC.

Proliferation

Cells were serum starved for 24 hours prior the assay. 5000 cells were seeded in 96 well plates and were incubated with 1μCu ^{3}H-thymidine (PerkinElmer, Waltham, MA). 24 hours later, ^{3}H-thymidine incorporation was measured using TopCount micro-scintillation counter (PerkinElmer).

In vivo tumor growth

For BXPC3 tumors, two-month old nude mice (Jackson Laboratory, Bar Harbor, ME) were subcutaneously (s.c.) injected with 5×10^{6} BXPC3.MUC1 or BXPC3.Neo cells into the flank of the mice. Tumors were allowed to grow for two months. For **KCKO/KCM** tumors, three-month old male C57BL/6 mice were injected with 1×10^{6} KCKO or KCM cells s.c. in the flank. Tumors were allowed to grow for 18 days. Upon sacrifice, tumor was dissected from under the skin and weighed and part of the tumor tissue was fixed in formalin for future IHC.

Invasion Assay

Serum starved cells (30,000 cells) were plated over trans-well inserts (BD Biosciences, Sparks, MD) pre-coated with growth-factor reduced matrigel (BD Biosciences) and were permitted to invade towards serum-contained in the bottom chamber for 48 hours. Non-invaded cells were swabbed from the tops of half of the inserts ('samples', containing only invaded cells), and retained in the others ('controls', all cells). Inserts were stained for 10 minutes with crystal-violet (0.5% in 20% methanol) and washed with water. Membranes were destained in 10% acetic acid and absorbance read at 570nm (Single Plate Reader, Molecular Devices, Sunnyvale, CA). Percent invasion was calculated as absorbance of samples/absorbance of controls×100.

PDGFA neutralization, Hif1-α inhibition, and PDGFA treatment

PDGF neutralization was performed using anti-PDGFA neutralizing antibody (Millipore, Billerica, MA) according to the company's instructions. Cells were cultured in 6 well plates and 24 hours post culture, 1 ml of supernatant was treated with 20μg of neutralizing antibody for 1 hour at 37°C. Cells were then subjected to either a proliferation or invasion assay. Hif1-α inhibition studies were done by treating cells with Hif1-α inhibitor (Santa Cruz Biotechnology). Cells were treated with 30 μM of Hif1-α inhibitor for 24 hours and then subjected to Western blot analysis. PDGFA treatment was performed by addition of 50ng/ml of rPDGFAA (Peprotech, Rocky Hill, NJ) for 4 hours.

Western Blots and Antibodies

Briefly, cells were lysed in HEPES buffer (20mmol/L HEPES, 150mmol/L NaCl, 1%Triton X100, 2mmol/L EDTA) containing protease (Complete inhibitor cocktail; Roche, Indianapolis, IN) and phosphatase inhibitors (Sigma). Equal quantities of lysate were loaded on SDS-PAGE gels. MUC1-CT antibody CT2, was made in Mayo Clinic Immunology Core [20]. MUC1-TR antibody (HMFG-2) was acquired as a kind gift from Prof. Joyce Taylor-Papadimitriou (King's College London School of Medicine, Guy's Hospital, London UK). All other antibodies (β-actin, PDGFA, β-catenin, Hif-1α (clones H26 and C19), Lamin-B, and IKK) (Santa Cruz Biotechnology) were used according to manufacturer's recommendations. Coimmunoprecipitation (co-IP) using CT2 and β-catenin were carried out as previously reported [44] using 1mg of protein lysate prepared in 1% Brij buffer (Sigma) followed by standard western blotting.

Sub-cellular fractionation

Cells were resuspended in buffer-A (10mM HEPES pH7.5, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA and 0.1%Nonidet-P40) on ice. Lysate was spun at 6000 rpm, 2min, 4°C and the resulting pellet was washed twice in 1× ice-cold PBS before sonication in buffer-B (20mM HEPES ph7.9, 25%glycerol, 400mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.5mM DTT) to obtain the nuclear fraction which was then spun at 13000 rpm for 10min, 4°C, for western blot analysis using MUC1-CT, β-catenin, and Hif-1α antibodies.

Statistical analysis

Statistical analysis was performed with GraphPad software. P-values were generated using the one way Anova and significance was confirmed using the Duncan and Student-Newman-Keul test. Values were considered significant if $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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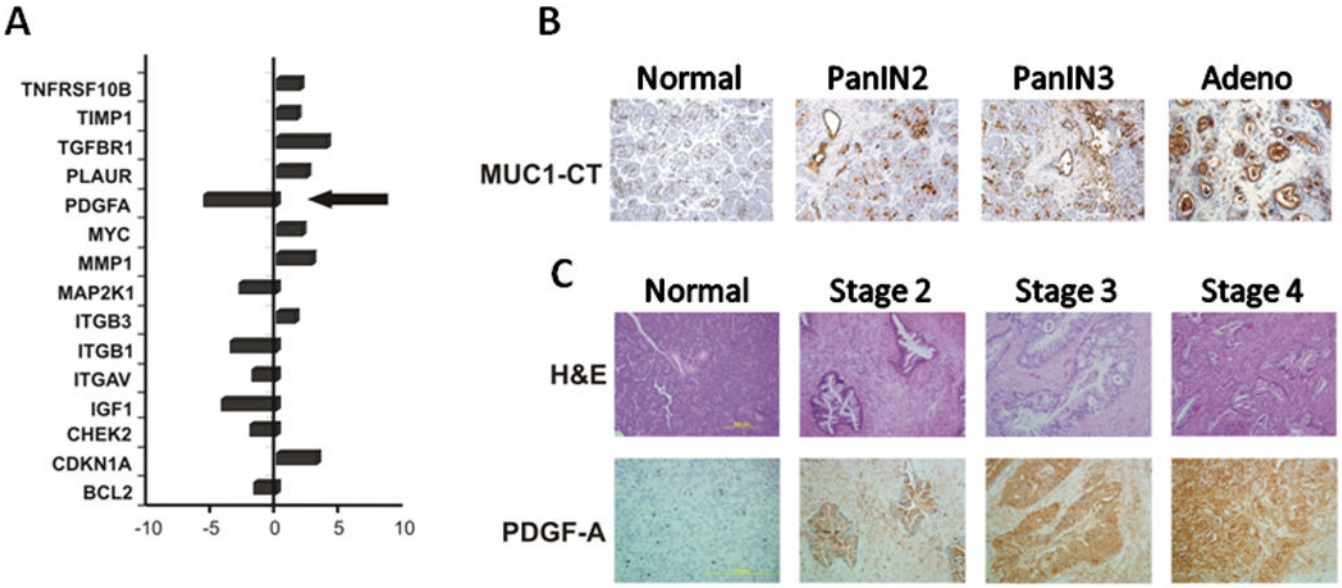

Figure 1. MUC1 overexpression is detected in all stages of PDA and is correlated to increased expression of PDGFA

A) RT-PCR data showing fold change in mRNA levels in WT versus MUC1siRNA-treated MiaPACA-2 cells. Differences over 2-fold are shown (n=3, $p<0.05$). B) MUC1 staining of primary human pancreatic tissue: Enhanced MUC1 expression in the early pre-neoplastic lesions and in late adenocarcinoma of the pancreas as compared to normal pancreas. C) H&E staining and IHC of PDGFA staining pattern showing over expression during all stages of human PDA. (Representative pictures are shown from n=5 patients for each stage with similar staining pattern)

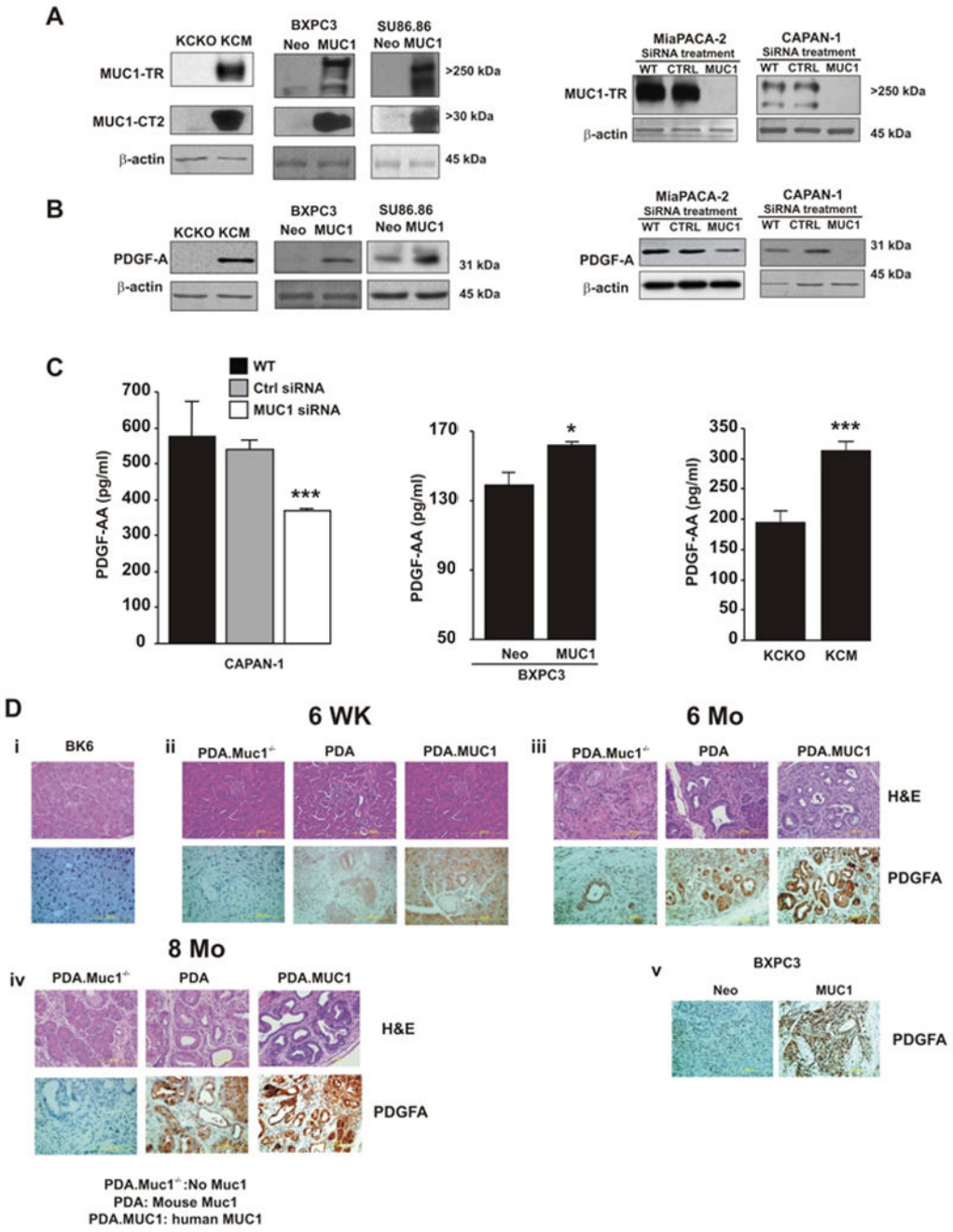

Figure 2. MUC1 regulates PDGFA expression during pancreatic cancer

A) Western blot analysis of MUC1 expression in human and mouse pancreatic cancer cell lines using both the MUC1 TR and MUC1 CT-specific antibodies. Additionally, downregulation of MUC1 expression in MiaPACA2 and CAPAN-1 cells 72 hours post siRNA treatment. B) Western blot analysis of PDGFA expression in various pancreatic cancer cell lines. β-actin served as the loading control. Representative blots from 3 separate experiments are shown. Average band intensity and standard deviation for 2B is reported in Table 1. C) Levels of PDGFA secreted in the supernatant of WT and MUC1 or control

siRNA-treated CAPAN-1, BXPC3.Neo/MUC1, and KCKO/KCM cells as measured by specific ELISA (n=4) (***P<0.0001, *P<0.05). D) i–iv: Histology and corresponding PDGFA expression in pancreas of PDA.Muc1$^{-/-}$, PDA, and PDA.MUC1 mice at 6-weeks (ii), 6-months (iii) and 8-months (iv) of age. One representative image from a normal 6-month old C57BL/6 pancreas is shown as control. v: PDGFA expression in BXPC3.neo and MUC1 tumors from nude mice. N=5 tumors/experimental group and 10 fields/section were examined with similar results. Images are captured at 40X magnification.

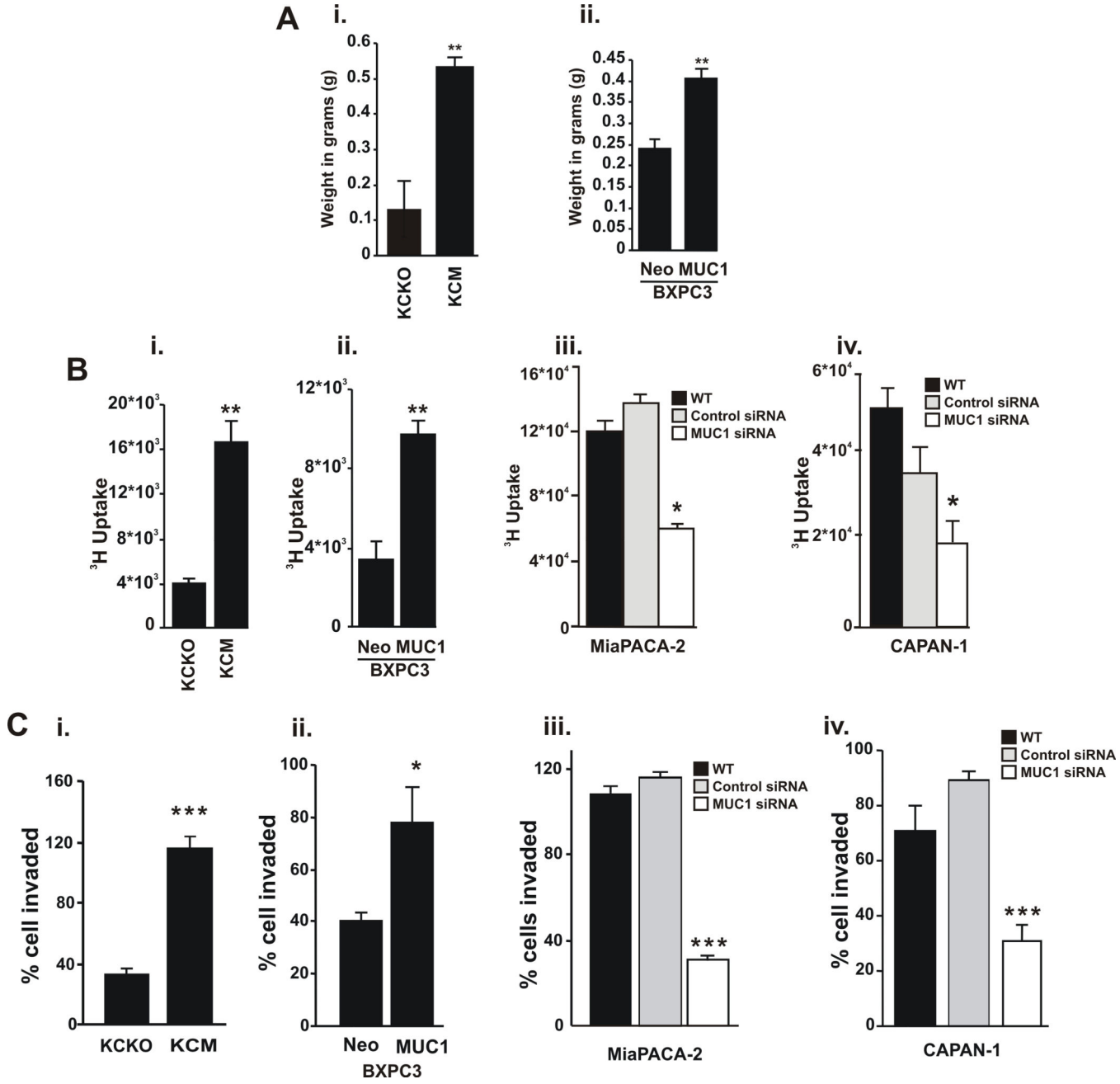

Figure 3. MUC1 regulates tumor burden, proliferation, and invasion of pancreatic cancer cell lines

A i and ii) Tumor wet weight of KCKO versus KCM (i) and BxPC3.Neo versus BxPC3.MUC1 tumors in vivo. 1×10^6 KCKO or KCM cells were injected in the flank of C57BL/6 mice and tumor wet weight measured 18-days post tumor cell inoculation (n=5) (**P<0.01) whereas 5×10^6 BXPC3.Neo or BXPC3.MUC1 cells were injected in the flank of nude mice and tumor wet weight taken 60-days post tumor cell inoculation (n=3) (**P<0.01). B) Proliferation of $MUC1^+$ and $MUC1^-$ pancreatic cancer cells as measured by ^{3}H-thymidine uptake (cpms) (n=4) (*P<0.05, **P<0.01). C) Percent cells that invaded

through growth factor-reduced matrigel in a Boyden chamber invasion assay (n=4) (***P=0.001, *P=0.05).

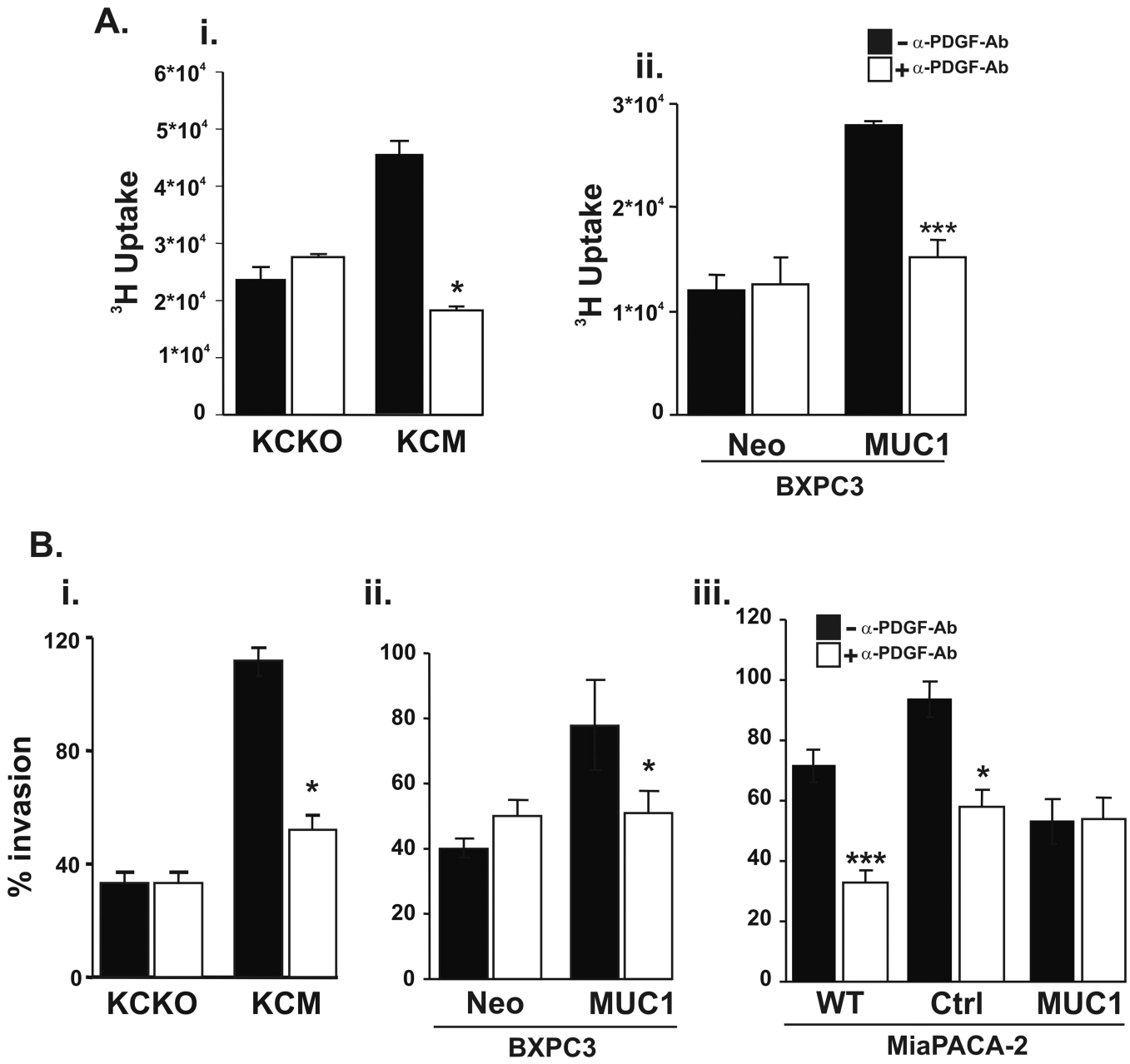

Figure 4. PDGF regulates invasion and proliferation of MUC1+ pancreatic cancer cells but has no effect on MUC1- cells

A) Proliferation of pancreatic cancer cells as measured by 3Hthymidine uptake (cpms) following PDGF neutralization (n=4) (*P<0.05, and ***P=0.001). B) Percent cells that invade through growth factor-reduced matrigel following PDGF neutralization. (n=3), (*P< 0.05 and ***P=0.001).

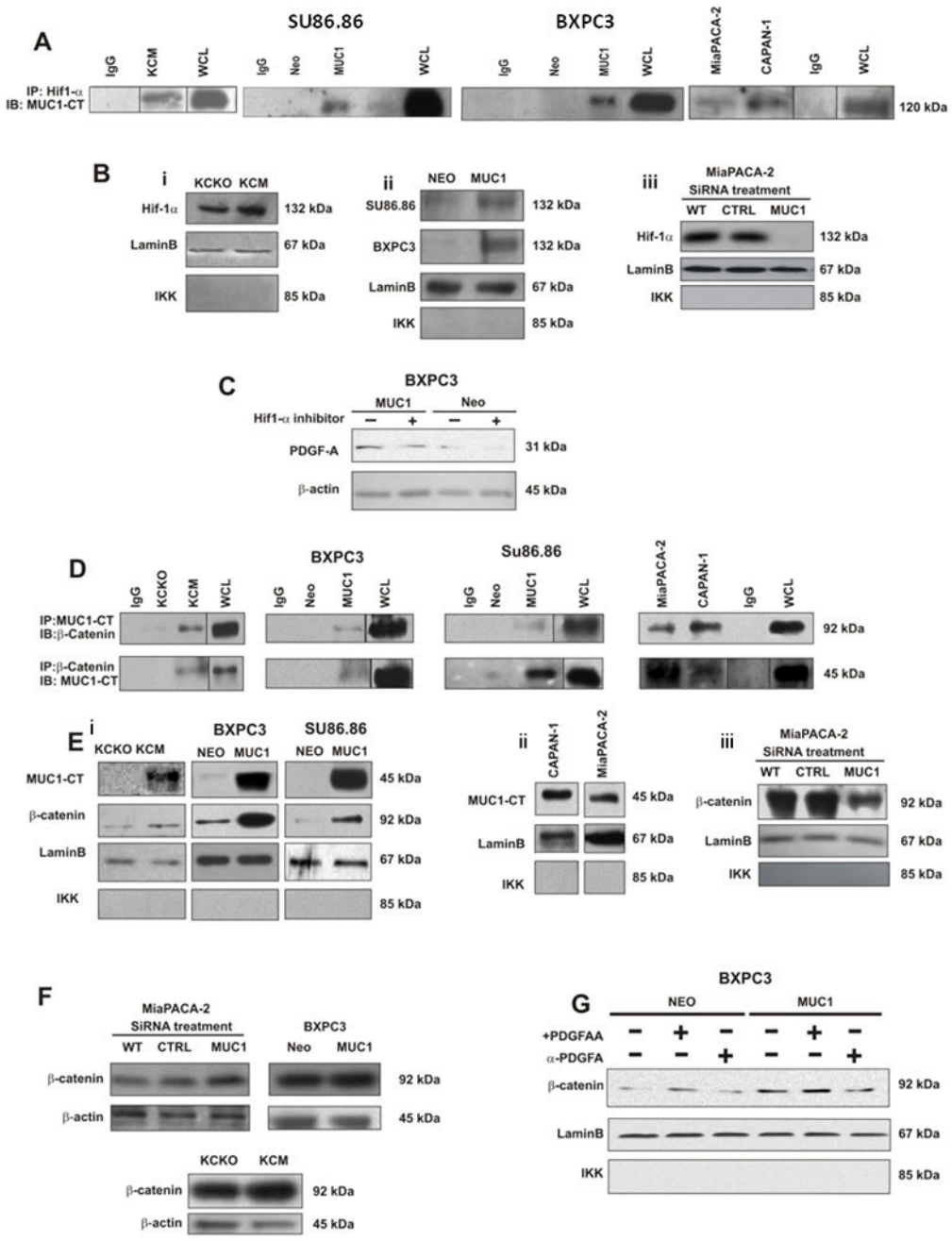

Figure 5. MUC1 interacts with HIF-1α and β-catenin. Nuclear translocation of MUC1, βcatenin and HIF-1α

A) Co-IP of MUC1-CT and HIF-1α in several cell lines demonstrating the interaction of MUC1-CT and HIF-1α. B) HIF-1α translocation to the nucleus of i) KCKO and KCM mouse PDA cells; and ii) SU86.86 and BxPC3 human cells. Lamin B and IKK serve as positive and negative markers for nuclear extracts. C) Western blot analysis for expression of PDGFA in BXPC3.Neo and MUC1 cells treated with 30μM Hif1-α inhibitor for 24hours. D) Co-IP of MUC1 CT and β-catenin in both directions in cell lines demonstrates the binding of these two proteins. E) i) Translocation of MUC1-CT and β-catenin to the nucleus

in KCKO, KCM, SU86.86, and BxPC3 cells indicating the activation of β-catenin as a transcription factor; ii) MUC1-CT in the nucleus of CAPAN-1, MiaPACA-2, and iii) β-catenin in the nucleus of MiaPACA-2 cells with and without siRNA treatment. F) Western blot analysis for presence of βcatenin in the whole cell lysate of MiaPACA-2 with and without siRNA treatment, BXPC3.MUC1, BXPC3.Neo, KCM and KCKO cells. G) Western blot analysis for presence of β-catenin in the nucleus in BXPC3.Neo and MUC1 cells treated with either 50ng/ml of rPDGFA, or 20μg of PDGFA neutralizing antibody for 24 hours. Lamin B and IKK serve as positive and negative markers for nuclear extracts while β-actin serves as loading control for whole cell lysate. For 5C, D, and G, representative blots from n=3 experiments is shown, and the average band intensity with standard deviation and significance is shown in Table 2.

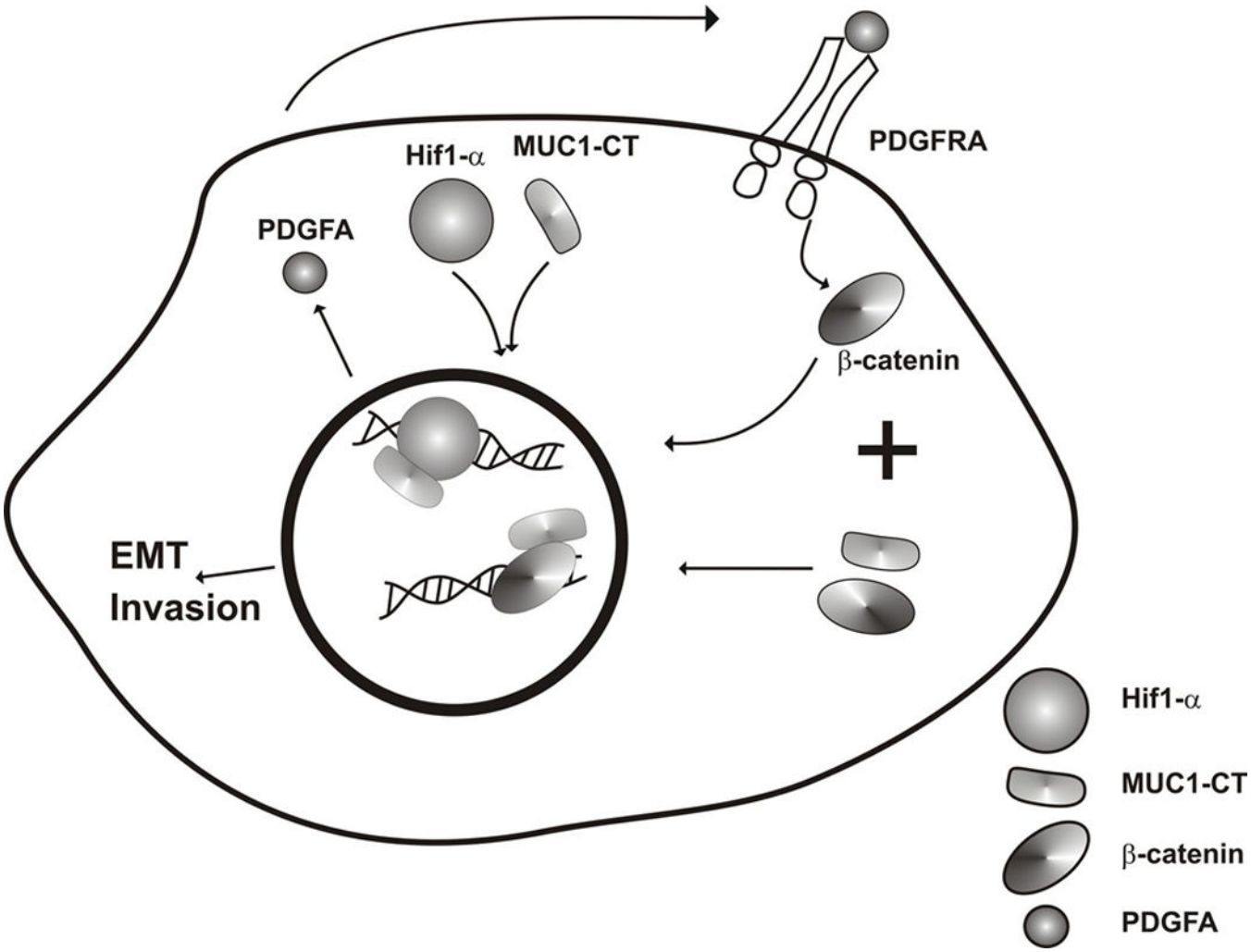

Figure 6. Schematic of the possible mechanism underlying MUC1 regulation of PDGFA

In MUC1 positive PDA cells, MUC1-CT associates with HIF1-α and facilitates its translocation to the nucleus. This results in expression of PDGFA which is then secreted and interacts with PDGFR-α. Signaling through PDGFR-α has an additive effect on β-catenin translocation and enhances proliferation and metastasis.

Table 1
Densitometric analysis of the blots from Figure 2B

Blots from n=3 experiments were analyzed and the average band intensity and standard deviation is tabulated. Values were normalized to control (β-actin). P values provided suggest significant difference between MUC1 high and MUC1 low cells: KCM versus KCKO; BXPC3.MUC1 and SU86.86.MUC1 versus Neo and between MUC1 siRNA treated versus control siRNA treated or WT MiaPACA-2 and CAPAN cells. Each experimental group is divided by the double-line border. Significance was calculated in comparison to the experimental group represented in the shaded cell. NS: not-significant.

Figure 2B	Density	P values
KCKO	0.9 ± 0.6	
KCM	32.6 ± 5.9	P=0.007
BXPC3.Neo	2.0 ± 1.2	
BXPC3. MUC1	14.8 ± 4.5	P=0.0015
SU86.86.Neo	32.6 ± 5.7	
SU86.86.MUC1	73.9 ± 14.3	P=0.003
MiaPACA2 WT	47.2 ± 10.2	NS
MiaPACA2 Ctrl	39.3 ± 12.2	
MiaPACA2 MUC1	9.5 ± 2.4	P=0.0006
CAPAN-1 WT	17.6 ± 3.4	NS
CAPAN-1 CTRL	12.2 ± 4.8	
CAPAN-1 MUC1	4.5 ± 3.4	P=0.006

Oncogene. Author manuscript; available in PMC 2013 May 22.

Table 2

Densitometric analysis of the blots from Figures 5 C, D, and G

Blots from n=3 experiments were analyzed and the average band intensity and standard deviation is tabulated. Values were normalized to control; Lamin B for nuclear proteins and β-actin for whole cell lysate. P values suggesting significant difference is provided. In 5C: significant difference is only noted when HIF1-α was inhibited in BXPC3.MUC1 cells. In 5D: Significant difference is noted in MUC1 and β-catenin co-IP between Neo and MUC1 cells and between IgG control and CAPAN or MiaPACA-2 cells. In 5G: Significant difference is noted in BXPC3.MUC1 cells when treated with PDGFA + α-PDGFA compared to untreated BXPC3.MUC1 or BXPC3.MUC1 treated with PDGFA. Each experimental group is divided by the double-line border. Significance was calculated in comparison to the experimental group represented in the shaded cell. NS: not-significant.

Figure 5C	**Density**	**P values**			
BXPC3.MUC1	19.6 ± 3.5	P=0.002			
BXPC3.MUC1+HIF1α INHIB	8.3 ± 2.3	NS			
BXPC3.Neo	3.5 ± 1.2				
BXPC3.Neo+HIF1α INHIB	2.1 ± 1.3	NS			
Figure 5D	**Density**	**P values**	**Figure 5D**	**Density**	**P values**
IP: MUC1 CT IB: β-catenin			**IP: β-catenin IB: MUC1 CT**		
IgG	0.8 ± 0.3		IgG	0.7 ± 0.3	
KCKO	1.1 ± 0,5	NS	KCKO	0.2 ± 0.1	NS
KCM	21.9 ± 13.1	P=0.0032	KCM	39.2 ± 5.6	P=0.0005
IgG	0.6 ± 0,1		IgG	0.6 ± 0.5	
BXPC3.Neo	0.1 ± 0,1	NS	BXPC3.Neo	0.6 ± 0.3	NS
BXPC3.MUC1	8.8 ± 2.2	P=0.0023	BXPC3.MUC1	41.8 ± 10.1	P=0.0003
IgG	0.1 ± 0.1		IgG	2.8 ± 1.2	
SU86.86.Neo	0.5 ± 0.2	NS	SU86.86. Neo	14.7 ± 4.3	P=0.001
SU86.86.MUC1	16.7 ± 3.2	P=0.0007	SU86.86. MUC1	87.6 ± 12.3	P=0.0001
IgG	1.5 ± 0,3		IgG	6.5 ± 3.2	
MiaPACA2	29.1 ± 4.3	P=0.0007	MiaPACA2	79.4 ± 9.8	P=0.0001
CAPAN-1	55.2 ± 6.7	P=0.0006	CAPAN1	45.7 ± 13.4	P=0.0004
Figure 5G	**Density**	**P values**			
BXPC3.Neo	5.7 ± 2.3				
BXPC3.Neo+PDGFA	9.5 ± 3.2	NS			

BXPC3.Neo+PDGFA+α-PDGFA	4.6 ± 1.6	NS			
BXPC3.MUC1	14.2 ± 2.0				
BXPC3.MUC1+PDGFA	16.9 ± 1.5	NS			
BXPC3.MUC1+PDGFA+α-PDGFA	8.7 ± 1.3	P=0.00086			