THE ROLE OF MUC1 IN REGULATION AND PROGRESSION OF CANCER

by

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ABSTRACT

MAHNAZ SAHRAEIMANOUCHEHRABADI. The role of MUC1 in regulation and progression of cancer. (Under the direction of Dr. PINKU MUKHERJEE)

MUC1 is a heavily glycosylated transmembrane glycoprotein which is overexpressed and aberrantly glycosylated in most human carcinomas. MUC1 contains a large tandem repeat, a short transmembrane, and a small cytoplasmic tail. Both tandem repeat and the cytoplasmic tail have been proposed to have significant contributions to cellular behavior. In the present study, we have investigated the role of MUC1 in cancer progression. First, we have studied the role of MUC1 in oncogenesis during pancreatic cancer. Our studies have shown that during pancreatic cancer, MUC1 regulates expression of PDGFA, an important growth factor which causes proliferation and invasion of cancer cells. Moreover, we have shown that MUC1 regulates epithelial to mesenchymal transition during pancreatic cancer and thereby contributes to metastasis. Second, we have investigated the role of MUC1 in regulation of myeloid derived suppressor cells. We have shown that under cancer progression MUC1 regulates expansion and suppressive function of myeloid derived suppressor cells. Genetic knockout of this protein causes mice to be more susceptible to cancer growth due to increased myeloid derived suppressor cells function and expansion. Taken together, MUC1 plays a dual role in oncogenesis and cancer immunology.

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On a personal note, I would like to thank my mother for her unconditional love which has given me the strength to go after what I want in life. I would also like to thank all of my friends who have been there for me through out the past years. This dissertation is dedicated to 3 unconventional and influential women in my life: my mother, my mentor, and my grandmother.

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CHAPTER 1: INTRODUCTION

1.1 MUC1

Mucin 1 (designated MUC1 for human and Muc1 for mouse) is an extensively O-glycosylated glycoprotein and the smallest member of the Mucin family. To this date, 11 members of this family have been discovered. The secreted Mucines: MUC2, MUC5AC, MUC5B, MUC6, and MUC7 and the membrane tethered Mucines: MUC1, MUC3, MUC4, MUC11, MUC12, and MUC13 (reviewed in (Gendler 2001)). Normally, MUC1 is expressed on the apical surfaces of all glandular epithelial organs including breast, pancreas, colon, stomach, lung, salivary glands, etc as a transmembrane protein (Gendler 2001). Recently, MUC1 has also been detected in low levels on hematopoietic cells as well (Gendler 2001). The extracellular portion of MUC1 can be enzymatically cleaved and secreted (Peat N 1992 ; Baeckström D 1993) and therefore can exist in a soluble form in the serum and bodily fluids. (Boshell M 1992). MUC1 consists of an extracellular domain of variable number of tandem repeats (VNTR) of 20 amino acids (aa) on the amino terminal, a transmembrane domain, and a 72 aa cytoplasmic tail (CT) domain at the carboxyl terminal (Figure 1) (Gendler SJ 1990). Due to differential mRNA splicing, several MUC1 variants have been identified. MUC1 can lack either the tandem repeat or the cytoplasmic tail (Zrihan-Licht S. 1994; Smorodinsky N. 1996; Oosterkamp H. M. 1997; Baruch A. 1999) or it can exist as a heterodimer containing both the tandem

repeat and the cytoplasmic tail. Presence of serine and threonine hydroxyl groups provides the sites for O-glycosylation on the tandem repeat domain. MUC1 is referred to as a Polymorphic Epithelial Mucin (PEM) and depending on the extent of glycosylation (which accounts for 50-90% of MUC1's weight), the molecular weight (MW) can range from 100 to >500 kDa. Because of extensive glycosylation and the size of the protein core, mature, full length MUC1 can extend from the cytoplasm up to 1500 nm and it resembles a bottle brush.

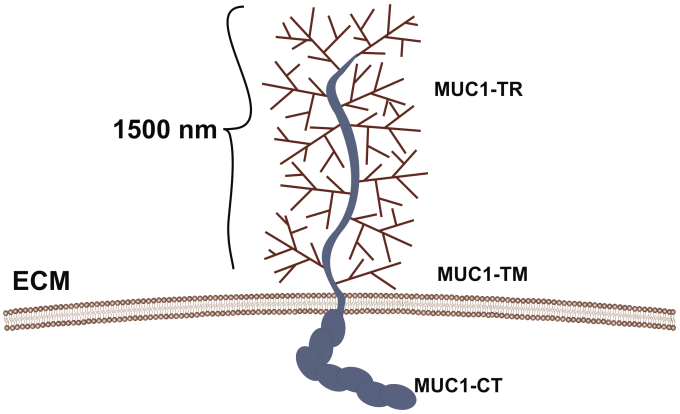

Figure 1. MUC1 structure. MUC1 contains a large extracellular tandem repeat domain, a short transmembrane domain and a small intracellular cytoplasmic tail.

In humans, *MUC1* is located on the chromosome 1q21 in a closely packed region of the DNA (Swallow DM 1987). MUC1 expression is developmentally regulated and can be detected in the pancreas, stomach, and lung on day 12 of the gestation period (as reviewed in (Gendler SJ 1995)). In fact, expression of MUC1 during embryogenesis is a marker of epithelial differentiation. *In-vivo,* hormonal modulation and soluble factors from the connective tissue of the colon can also stimulate and regulate expression of MUC1 (Irimura T 1990).

Following translation of MUC1 mRNA, the precursor of this protein is cleaved at the C-terminal in the Endoplasmic Reticulum (ER) into two fragments of the heterodimer (Ligtenberg MJ 1992). Depending on the tissue type and function of the MUC1 which determines the number of VNTRs, weight of MUC1 protein core ranges from 100-300 kDa. This weight can double after the final glycosylation. MUC1 glycosylation happens in the ER during translation and succeeding cleavage, MUC1 is exposed to further glycosylation in the Golgi apparatus (Rose MC 2006). Type and extend of glycosylation depends on the tissue type and the organ in which MUC1 is expressed. The N-terminal of MUC1 contains a sequence which directs its localization to the cell membrane.

MUC1, an epithelial cell marker is shown to be an anti-adhesive protein (Jentoft 1990) and can regulate epithelial morphogenesis by blocking cell-cell or cell-matrix interactions (Braga VM 1992 ; Hilkens J 1992). Expression of this protein on mucosal organs such as colon, can have protective function against pathogen entry, bacterial infection, and can regulate the host's reaction against pathogens (McAuley JL 2007 ; Ueno K 2008 ; Lindén SK 2009) in a matrix metalloproteinase-14 (MMP-14) dependant manner. In the lung, due to its vast carbohydrate chains, MUC1 can bind to an array of bacteria, viruses, and particles that have been inhaled. Mucins in general are important for hydration, lubrication, and protection from proteases. Due to its long extension from the cell membrane, MUC1 TR can act as an environmental sensor for the cell in addition to ligand binding and signal transduction. MUC1 can detect changes in the pH, chemical, and physical conditions outside the cells. If expressed on cancer cells however, MUC1's function and expression is drastically changed and its signaling mechanism is hijacked by the cancer cells to proliferate, metastasize, and evade death.

1.2 MUC1 and cancer

MUC1 is overexpressed and aberrantly (hypo)-glycosylated in all epithelial carcinomas including breast, ovarian, and pancreas as well as in some hematological malignancies. MUC1 overexpression on cancer cells causes increased shedding of MUC1 in the serum. In fact, in cancers such as ovarian and breast, detection of shed MUC1 via standardized methods has diagnostic and prognostic potential. MUC1 is known to significantly contribute to oncogenesis and metastasis. During carcinogenesis, MUC1 expression is no longer restricted to the apical surface of the cells and therefore cells lose their polarity. This allows MUC1 to interact with receptors which are physically unavailable to it under normal conditions (Figure 2). Therefore, overexpression of MUC1 in cancer patients is correlated to both poor prognosis and increased metastasis (Fung PY 1991 ; Kobayashi H 1992; Gendler 2001).

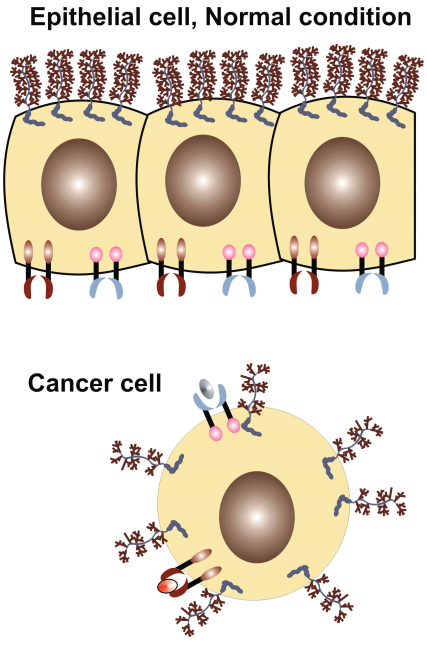

Figure 2. MUC1 expression in normal and transformed cells. MUC1 is expressed on the apical surface of normal epithelial cells. However, MUC1 is over expressed and aberrantly glycosylated in cancer cells. In addition, its expression is no longer restricted to the apical surface. Consequently, MUC1 can interact with proteins and receptors which are not available to it in normal condition.

MUC1 not only contributes to oncogenesis, but also inhibits body's natural attempt to eliminate cancer cells. Expression of MUC1 on cancer cells have been shown to limit the killing ability of Natural Killer (NK) and Cytolytic T-cells (CTLs) (Sherblom AP 1986; van de Wiel-van Kemenade E 1993) and therefore help immune evasion during cancer progression.

MUC1 has a dual role in regulation of cell adhesion to the extracellular matrix. During oncogenesis, this anti-adhesive functions of MUC1is utilized by cancer cells to evade binding to extracellular matrix in order to metastasize to the distal organs. MUC1 can interact with Intracellular Adhesion Molecule-1 (ICAM1) on endothelial cells to facilitate migration of cells (Rahn JJ 2005) or it can act as an adhesive protein by providing ligands for selectin-like molecules (Aruffo A 1992; Majuri ML 1992) and assist homing of metastatic cancer cell to the new organ.

Hypo- glycosylation of tumor associated MUC1 results in exposure of the immunogenic protein core to the immune system. This makes MUC1 a compelling tumor antigen (Agrawal B 1998; Brossart P 1999). Pulsing Dendritic Cells (DCs) with MUC1 peptide results in presentation of this peptide within Major Histocompatibility (MHC) molecules and generation of cytotoxic T-lymphocytes (CTLs) capable of lysing MUC1 expressing tumor cells (Brossart P 1999; Tinder 2008; Mukherjee 2009). Targeting MUC1 as a tumor antigen either through antibodies or vaccination in hopes of preventing recurrence is the focus of many laboratories as well as our laboratory. Tumor associated MUC1 is considered to be a very promising target for immune intervention and studies have shown that vaccination with MUC1 peptides can result in decreased tumor burden

in pancreas breast and colon cancer models (Mukherjee 2004; Mukherjee 2007; Akporiaye ET 2007 ; Mukherjee 2009).

Signaling through MUC1-CT in cancer: The cytoplasmic tail of MUC1 is highly conserved across species and plays a major role in oncogenic signal transduction (Figure 3).

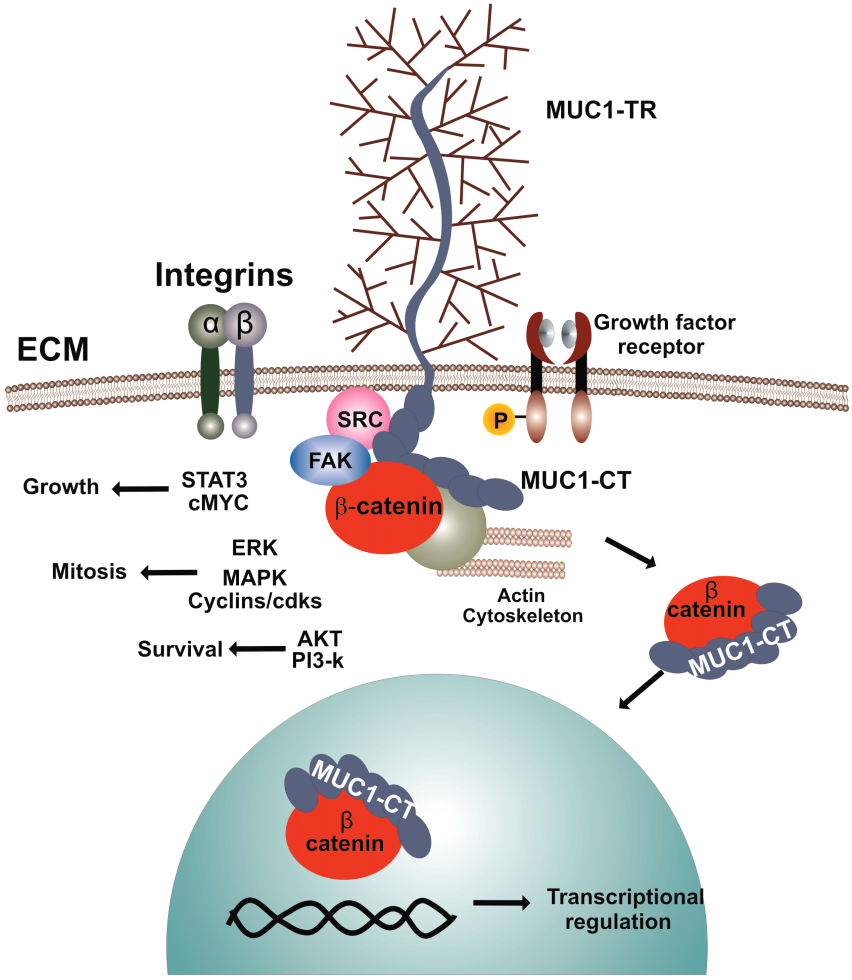

Figure 3. MUC1 regulates signaling pathways which contribute to oncogenesis. MUC1-CT can associate with SRC, FAK, and β-catenin to regulate growth, mitosis and survival of cancer cells. Moreover, MUC1 can associate with proteins such as β-catenin and translocate to the nucleus to alter transcriptional activity of transcription factors.

MUC1-CT contains 72 aa and resembles a cytokine receptor in its sequence with several tyrosines, serines, and threonines which can be subjected to phosphorylation, and proteolytic modification, thus affecting its interaction with other oncogenic proteins (Figure 4). MUC1-CT contains 7 tyrosines which when phosphorylated associates with several signaling proteins to modulate signal transduction events. Members of the Src family such as lyn (Li Y 2003), c-Src (Al Masri A 2005), and Lck (Mukherjee P 2005) can phosphorylate MUC1-CT and regulate its interaction with known transcription factors such as β-catenin. MUC1-CT association with the Armadillo repeats on β-catenin through its serine rich regions stabilizes β-catenin's translocation to the nucleus (Huang L 2005). Furthermore, MUC1 expression is known to increase cellular levels of β-catenin in cancer cells through blocking Glycogen Synthase Kinase-3β (GSK3β) mediated destruction of β-catenin (Huang L 2005). β-catenin in the nucleus regulates transcriptional activity of several proteins which are responsible for regulation of *c-myc* and *cyclinD.* These proteins are known to be crucial for cell cycle regulation in cancer. Moreover, MUC1-CT is also known to translocate to the nucleus of many MUC1+ cancer cells and modulate transcription (Wen Y 2003; Roy, Sahraei et al. 2011; Sahraei 2011). The exact mechanism behind MUC1-CT translocation to the nucleus is not yet known and to this date, no nuclear localization sequence has been found on MUC1-CT (Singh and Hollingsworth 2006). Therefore, MUC1-CT contributing to tumor progression is heavily dependent on its interactions with β-catenin and its transcriptional role.

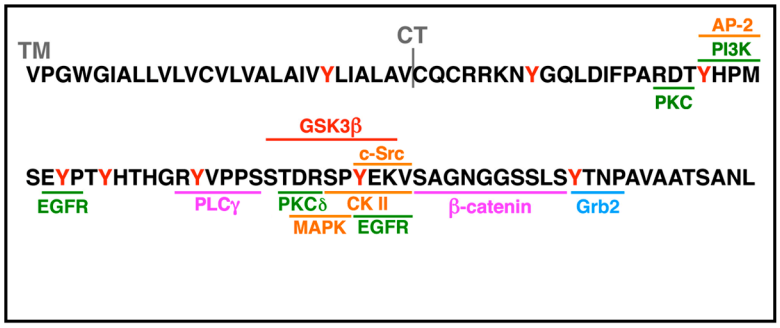

Figure 4. A graphic representation of the MUC1-CT. the tyrosines are shown in red. Known and potential interactions are indicated**.**

MUC1 itself is proposed to be an oncogene and its expression alone can increase chances of tumor formation. In fact, transfection of rat 3Y1 fibroblasts with full length MUC1 caused increased proliferation of these cells and injecting 3Y1-MUC1 cells to nude mice causes tumor formation, an event which does not happen with wild type 3Y1 cells (Li 2003).

One of the biggest contributions of MUC1 to increased survival is through regulation of NF-κB levels and activity (Ahmad R 2007). A thorough study by Ahmad et al has revealed that MUC1 regulates levels of NF-κB expression as well as its nuclear translocation via phosphorylation and degradation of IκB. Further, MUC1-CT directly binds to IKKα and IKKβ to activate their complex and augments NF-κB signaling in response to Tumor Necrosis factor-α (TNF-α) indicating that MUC1 contributes to cell survival, decreased apoptosis and increased transformation via activation of the NF-κB pathways (Ahmad R 2007).

Growth factors and their signaling play a crucial role in cancer progression and resistance to apoptosis. MUC1-CT can interact with and get phosphorylated by growth factor receptors such as Epidermal Growth Factor Receptor (EGF-R) both *in-vitro* and *in-vivo* (chroeder JA 2001; Li Y 2001; Schroeder JA 2001). Additionally, EGF-R can

regulate interaction of MUC1-CT with β-catenin and c-Src (Li Y 2001). This integrated signaling amongst MUC1-CT, EGF-R, c-Src, and GSK3β can modify c-Src/β-catenin or GSK3β signaling and induces further transformation of cancer cells.

Another growth factor receptor which interacts with MUC1 is Fibroblast Growth Factor Receptor-3 (FGFR3) (Ren J 2006). FGF stimulation can increase MUC1/FGFR-3 association and consequently MUC1 gets phosphorylated by FGFR3 to increase interaction of MUC1-CT /β-catenin. Consequently β-catenin is stabilized and translocated to the nucleus to alter Wnt signaling and induce further oncogenesis.

In addition to tumor progression and metastasis, MUC1 has long been associated with resistance to chemotherapy, stress, and apoptosis. MUC1 induces radiation resistance via contributing to DNA repair and indirect promotion of cell survival in response to DNA double strand breaks (Huang L 2010). Also, MUC1 blocks the genotoxic agent induced apoptosis, causing chemoresistance in cancer patients. (Raina D 2006). Taken together, there is mounting evidence pointing to MUC1's direct role in resistance to conventional cancer treatments.

MUC1's role in induction of proliferation and metastasis in some carcinomas is well established. Through interaction with growth factor receptors, signaling molecules and alteration of gene expression MUC1 plays a significant role in oncogenesis. Induction of proliferation, metastasis, chemo and radiation resistance, and stress resistance explains why MUC1 expression during cancer is associated with such bad prognosis. While it appears that MUC1 plays a similar role in cancers, recent findings point that MUC1's contribution to oncogenesis is dependent on the nature of the tumor. Our laboratory investigates the role of MUC1 in pancreatic cancer progression.

1.3 Pancreatic cancer

Pancreatic Ductal Adenocarcinoma (PDA) accounts for more than 85% of all pancreatic cancers and is the 4th leading cause of cancer related death in the United Stated. Every year, 270,000 patients are globally diagnosed with this disease and the 5 year survival rate it 5% as the patient's quality of life decreases dramatically (Li D 2004.). Despite significant improvements in the cancer treatment field, PDA remains to be one of the most challenging cancers to treat. To this date, surgical resection and early diagnosis are the only means by which this disease is less fatal. PDA is extremely resistant to chemotherapy and radiation. Early micrometastasis to distal organs is a hall mark of this cancer. Pancreatic cancer is not symptomatic in early stages and therefore it is rarely diagnosed early enough to be treated. Pancreatic cancer is highly invasive and by the time of diagnosis is already metastatic and therefore incurable.

Very little is known regarding the environmental risk factors or genetic conditions which cause PDA. Some conditions such as diabetes, obesity, genetic predisposition, and chronic pancreatitis have been linked to PDA development. (Everhart J. 1995; C.S 2001; Fryzek J.P. 2001; Virtamo J. 2005). Mutations in the tumor suppressor genes such as *BRCA2,BRCA1, INK4A, MLH1, PRSS1, and LKB1* have also been linked to development of PDA (Whitcomb D.C. 1996; Jaffee E.M. 2002; Thompson D. 2002). Changes in the pancreas environment such as pancreatitis or organ dysfunction can lead to production of ROS, growth factors, and inflammation which results in transformation of ductal cells and their unrestrained proliferation. Perhaps one of the most important mutations that lead to PDA development is mutations in the *K-RAS* oncogene. Activating mutations in the codon 12 of *K-RAS* (from G to D) replaces the asparate with glycine, or arginine, or

valine. These mutations are assumed to be the initiating mutations in the pancreas which lead to pancreatic cancer. The *K-RAS*^{G12D} mutation is detected in about 30% of early neoplasms of the PDA and 100% of advanced PDA (Almoguera C. 1988; Klimstra D.S. 1994; Rozenblum E. 1997). Dr. Tuveson's group has generated a mouse model which expresses the *K-RAS*^{G12D} in the ductal cells of the pancreas and has shown that this mutation alone can cause pancreatic cancer which progresses identical to that of humans (Hingorani 2003). This model is of great importance since it provides the opportunity to study pancreatic cancer in its natural microenvironment in immunocompetent mice with the proper hormonal setting.

In more than half of PDA specimens the tumor suppressor *P53* gene has deactivating mutations (Rozenblum E. 1997). Several pathways such as MAPK, Phosphatidilonositol-3 Kinase (PI3K), and Ral GDS become activated following the activating *K-RAS*^{G12D} mutation [as reviewed in (Campbell S.L. 1998)]. MAPK activity has been shown to play a significant role in proliferation of PDA cells (Hirano T. 2002; Gysin S. 2005). *K-RAS*^{G12D} mutations have been shown to regulate NF-κB transcriptional activity (Sclabas G.M. 2003) and NF-κB is found to be constitutively active in PDA cells but not normal pancreas cells (Wang W. 1999; Chandler N.M. 2004).

Other signaling pathways that can play a role in PDA progression are signaling from receptors of growth factors such as Vascular Endothelial Growth Factor (VEGF) (Itakura J. 1997; Seo Y. 2000), Fibroblast Growth Facrot (FGF) (Kornmann M. 1997; Ishiwata T. 1998), Insulin Growth Factor (IGF) (Nair P.N. 2001), EGF (Li J. 2004), and Hepatocyte Growth Factor (HGF) (Di Renzo M.F. 1995). Several other factors such as epigenetic events, telomerase shortening (Hruban R.H. 2000), Notch (Miyamoto Y.

2003) and Hedgehog signaling (Berman D.M. 2003; Thayer S.P. 2003) are also known to contribute to PDA development.

PDA precursor lesions are called Pancreatic Intraepithelial Neoplasia (PanIN lesions). Most of genetic alterations occur in these lesions. PanIN lesions have 3 grades: 1, 2, and 3 and represent various changes which occur in a transforming pancreas. PanIN stages associate with accumulation of mutations in the tumor site (Feldmann 2007). While early and late PanIN lesions contain the *K-RAS*^{G12D} mutation, later stage PanINs contain additional mutations such as *P53* and *SMAD4* mutations (Wilentz RE 2000; Maitra A 2003).

1.4 MUC1 expression during PDA

Healthy pancreas of both human (Adsay NV 2002) and mouse express MUC1 (Peat N 1992). More than 90% of human PDA specimen overexpress MUC1 (Plate 1999; Qu 2004). Most pancreatic cancer cell lines as well as pancreatic juice of PDA patients express high levels of MUC1 (Batra SK 1992 ; Ho JJ 1993; Hollingsworth 1994; borg 2004). In pancreatic carcinomas, expression of MUC1 has been associated with decreased survival (Sakamoto H 1997; Utsunomiya T 1998; Hinoda Y 2003) and increased metastasis (Masaki Y 1999). MUC1 expression is considered to be a valuable prognostic and diagnostic factor for patients with PDA (Gold, Karanjawala et al. 2007; Grote T 2007; Westgaard, Schjolberg et al. 2009) .

Previous studies have reported that MUC1 expression cannot be detected in early PanIN lesions (Adsay NV 2002; Maitra A 2003). MUC1 glycosylation is one of the factors that is known to pose a problem in detection of MUC1-TR since it is known to mask the protein core and limit antibody access (Ho JJ 1995). Using an antibody against

the cytoplasmic tail of MUC1 (CT-2), we have shown that MUC1 expression can be detected in early stages of PDA and low grade PanIN lesions as well suggesting MUC1 may play a role in early development of PDA as it can be a valuable marker for initiation of this disease (Sahraei 2011).

MUC1 signaling in PDA: More than a decade ago, Satoh et al have shown that transfection of PDA cells (S2-013) with full-length MUC1 results in increased proliferation of these cells *in-vitro* and once injected, the resulting tumor is significantly larger than that of mock transfected cells (Satoh S 2000). Moreover, high numbers of metastatic lesions in the lung of mice injected with MUC1+ cells indicate a considerable function for MUC1 in increasing metastasis during PDA progression. This was the first report which highlighted the role of MUC1 in PDA progression.

MUC1's regulation of cell adhesion plays a notable role in PDA metastasis. Human PDA cells are known to bind to E-selectin. MUC1 decreases the binding affinity of these cells to E-selectin and thereby, facilitate its detachment from extracellular matrix (McDermott KM 2001.). Meanwhile, PDA cells which are positive for MUC1-TR can aggregate with ICAM+ cells implicating that during PDA progression, MUC1-TR can help cancer cells detach from the basal membrane and metastasize to distal organs. Also, MUC1-TR can facilitate homing of PDA cell to the target organs (Kohlgraf KG 2003). Since early and micro metastasis is one of the major problems in PDA, MUC1's role in induction of such event is of great importance.

MUC1 expression and β-catenin signaling during PDA: It has been shown that in MUC1+ PDA cells, MUC1-CT translocates to the nucleus. Moreover, MUC1 appears to stabilize β–catenin levels and associate with it in order to increase its nuclear localization

(Wen Y 2003). β-catenin/wnt signaling in PDA cells has been linked to proliferation, migration, and invasion (Kobayashi T 2011; Cho IR 2011). MUC1 knock down in PDA cells lead to release of β-catenin from the nucleus and results in increased levels of cytoplasmic β-catenin (Yuan Z 2007). β-catenin then forms a complex with E-cadherin and promotes cell adhesion which decreases cell migration and possibly lowers metastasis. This implies that MUC1 regulation of β-catenin signaling and nuclear translocation can in fact regulate metastasis.

In PDA cells, PDGFR-β has been shown to phosphorylate MUC1-CT at the HGRYVPP sequence (Singh PK 2007). Phosphorylated MUC1-CT then associates with β-catenin and increases its translocation to the nucleus and ultimately results in increased invasion of pancreatic cancer cells. Overall, there is increasing amount of evidence showing one of the major ways by which MUC1 regulates proliferation and invasion of PDA cells is through regulation of β-catenin signaling.

Motility, invasiveness and metastasis of cancer cells is known to be associated with Epithelial to Mesenchymal Transition (EMT) [as reviewed in (Nieto 2002; Thiery 2002)]. During EMT, epithelial cells are shown to acquire fibroblastic phenotype and increase their motility as they loose their adherence properties. Functional loss of E-cadherin due to Snail expression is known to be associated with this event (Batlle, Sancho et al. 2000; Cano, Perez-Moreno et al. 2000; Guaita, Puig et al. 2002). In a thorough study by Roy LD et al, using several cell lines and *in-vivo* models it has been shown that MUC1 expression in PDA cells increases their invasiveness and that both *in-vitro* and *in-vivo*, MUC1 expression induces EMT (Roy LD 2010). Decreased metastasis and increased survival of spontaneous PDA mice which lack MUC1 was also associated

with decreased expression of mesenchymal markers such as vimentin and increased expression of epithelial markers such as E-cadherin in the pancreas of these mice. Cell lines extracted from PDA mice which express human MUC1 (PDA.MUC1) vs. cells that are isolated from PDA mice which totally lack expression of Muc1 (PDA.Muc1$^{-/-}$) express higher levels of snail, slug, N-cadherin, vimentin, and VEGF, indicating the role of MUC1 in regulation of the mesenchymal phenotype of PDA cells. Similar event was observed in human MUC1- PDA cells transfected with full length MUC1. Surprisingly, if MUC1-cells were transfected with a MUC1 plasmid which contained mutated tyrosines in the cytoplasmic tail, neither invasion nor mesenchymal markers were upregulated. Subcutaneous injection of these cells to nude mice resulted in increased tumor burden of MUC1+ cells as well as increased levels of circulating tumor cells (CTCs). Tumors resulted from MUC1- cells, or cells with mutated MUC1 showed both smaller tumors, and no CTCs were detected. Gene expression analysis revealed that MUC1 regulates such event at a transcriptional level. Also, MUC1 was shown to interact with β-catenin and translocate to the nucleus. Both their interaction and translocation was significantly lower in MUC1+cells which contained mutated cytoplasmic tail indicating that MUC1-CT's interaction with β-catenin is dependent on the tyrosines. Several studies have linked β-catenin transcriptional activity to EMT (von Gise, Zhou et al. 2011; Zhao, Luo et al. 2011). Therefore, it is tempting to conclude that MUC1 regulates the EMT process by regulation of β-catenin nuclear localization and transcriptional activity and consequently induce metastasis.

Both β-catenin (Takahashi K 1997) and MUC1 have been shown to associate with EGF-R and EGF-R has been shown to translocate to the nucleus (Wang SC 2009) and

induce radiation resistance (Liccardi 2011). It is possible that MUC1 is regulating nuclear localization of EGFR via β-catenin and by doing so modulates cell proliferation and sensitivity to genotoxic reagents and radiation (Wang SC 2006). Given the chemo and radiation resistant nature of PDA, it is appealing to blame this in part on MUC1.

Other MUC1 regulated PDA signaling events: Using Dr. Tuveson's PDA model, our laboratory has generated spontaneous PDA mice which either express normal mouse Muc1 (PDA.Muc1), or human MUC1 (PDA.MUC1), or lack expression of Muc1 (PDA.Muc1$^{-/-}$) (Tinder TL 2008; Roy LD 2010; Sahraei 2011; Besmer DM 2011). We have shown a significant survival advantage in PDA.Muc1$^{-/-}$ mice compared to Muc1/MUC1 expressing PDA mice (Tinder TL 2008). Increased survival was associated with decreased tumor burden as well as lower metastasis in these mice. Our studies have shown that pancreatic cancer in PDA.Muc1$^{-/-}$ mice never progressed as it did in PDA.Muc1 or PDA.MUC1 mice indicating a significant role for MUC1 in contributing to PDA progression. Decreased proliferations of MUC1-PDA cells have been speculated to be one of the reasons attributing to lower tumor burden. Increasing amount of support has been gathered which suggest MUC1 in fact regulates cell cycle arrest in PDA cells. MUC1+PDA cells have been shown to express higher levels of pP44/42 and Cdc-25c while expressing lower levels of P53 and P21 (Besmer DM 2011). This is in agreement with many other studies confirming that MUC1 induces proliferation of PDA cells. MUC1 siRNA treatment in PDA cells (Tsutsumida H 2006);(Yuan Z 2009; Sahraei 2011) decreases proliferation of these cells while transfection of MUC1- cells with MUC1 induced increased proliferation (Sahraei 2011).

Whereas many factors such as MAPK/ERK activity have been linked to proliferation, the upstream event which leads to their activity remains to be studied. It is known that many growth factors induce proliferation via MAPK/MEK. In fact, our studies have shown MUC1 regulates expression of Platelet-Derived Growth Factor-A (PDGFA), which induces proliferation in PDA cells via activation of MAPK (Sahraei 2011). In this study, neutralization of PDGF leads to reduction of both proliferation and invasion to the levels of MUC1-PDA cells. MUC1 regulation of PDGFA expression was confirmed in *in-vivo* studies of both spontaneous PDA as well as injectable models of tumor in nude mice. On the other hand, PDGFA secretion similar to PDGFB production was shown to regulate β-catenin translocation to the nucleus further signifying the role of MUC1 in regulation of oncogenic signaling in pancreatic cancer.

Invasion of pancreatic cancer cells to the peripheral nerves in PDA patients is particularly associated with poor prognosis. This event leads to morbidity in result of severe incurable pain. Myelin-associated glycoprotein (MAG) is known to be expressed on cells of the peripheral nervous system and interact with glycoproteins. It has been shown that MUC1 on PDA cells interacts with MAG and binds to them (Swanson, McDermott et al. 2007). This can in part explain the neurotropic nature of PDA cells and induction of pain and morbidity.

MUC1 expression during PDA influences tumor microenvironment: The tumor microenvironment has been shown to influence functionality of infiltrated immune cells. Dendritic Cells (DCs) are one of the most important cells in cancer immunology. DCs are known to uptake tumor antigens and present them to T-cells in order to induce immune response. Tumor microenvironment can influence DC maturation and differentiation. As

mentioned, MUC1 can be cleaved from tumor cells and enter the peripheral circulation via draining lymph nodes. It has been suggested that shed tumor associated MUC1 can in fact regulate immune responses (Agrawal 1998). Monti et al have shown that when MUC1+ PDA cells are co-cultured with human polymorphonuclear cells, differentiation and maturation of DCs is affected. (Monti P 2004). This study explains that shed MUC1, especially sialyl-Tn glycoform interacts with mannose receptor (MR) on DCs and increases IL-10 production and decreases IL-12 expression in these cells and thereby skews their phenotype towards regulatory DCs. IL-12^{low}IL-10^{high}DCs are known to induce T-cell anergy instead of Th1 response which is needed for anti-tumor immunity (Gao 1999; Jonuleit H 2001; Roncarolo MG 2001).

While several studies are focused on targeting MUC1 as a tumor antigen, many studies have shown that MUC1 expression leads to an immunosuppressive tumor microenvironment which causes an immense problem. Myeloid Derived Suppressor cells (MDSCs) and T-regulatory cells (T-regs) are known to suppress the antitumor immune response and allow tumor growth. We have shown that in the spontaneous model of PDA which expressed human MUC1, levels of both MDSCs and T-regulatory cells are higher within the pancreas and that this increase is associated with higher levels of Cox2/PGE-2 which apparently originates from MUC1+ PDA cells (Tinder 2008). Further, IDO activity was increased in PDA.MUC1 mice compared to PDA mice and resulted in decreased levels of tryptophan in the serum of these mice. This is of great importance, since efficient targeting of MUC1 via CTLs requires a less immunosuppressive microenvironment. Further studies are required to find the in-depth mechanism for such observation.

MUC1 and angiogenesis in PDA: MMP9 and MMP2, expression in the pancreatic tumor have been shown to be regulated by MUC1 (Roy LD 2010). MMP9 expression (Arnold, Mira et al. 2008; Zhang, Chen et al. 2011), as well as MMP2 expression (Binker 2010) have been shown to increase metastasis during PDA (Giannopoulos 2008). Furthermore, MUC1 has been shown to regulate transcription of Connective Tissue Growth Factor (CTGF) and induce its expression in pancreatic cancer cell lines. Behrens et al have shown that following phosphorylation of the MUC1-CT due to stimulation by EGF, PDGF-BB, and HGF, MUC1-CT translocates to the nucleus and localizes around the CTGF's promoter and induces β-catenin and P53 mediated expression of CTGF (Behrens ME 2010). CTGF then induces angiogenesis and therefore metastasis.

Other studies have shown that other metastatic factors such as IL-6, IL-6R, IGF, VEGF, SCF, EGF, and P-selectin are significantly higher in the tumor microenvironment of MUC1 expressing pancreatic cancers (Roy LD 2010). Spontaneous PDA.MUC1 mice have also shown significantly higher serum levels of VEGF in all stages of the disease (Besmer DM 2011). Interestingly, lack of intact MUC1-CT caused expression of these factors to be significantly reduced back to the levels of MUC1- PDA tumors signifying MUC1-CT signaling as a crucial player in induction of angiogenic tumor microenvironment and metastasis (Roy LD 2010).

Expression of PDGFA has long been associated with increased angiogenesis in cancer (Mantur M 2008). PDGFA is known to play a significant role in angiogenesis and metastasis during pancreatic cancer (Hwang RF 2003). A recent study from our lab has shown that MUC1 regulates PDGFA expression at the transcriptional level (Sahraei

2011). Moreover, we have shown that MUC1 associates with PDGFA's transcription factor Hypoxia Inducible Factor-α (HIF1-α) and induces its translocation the nucleus. Hif1-α is well recognized for its role in angiogenesis (Coma 2011) and immunosuppression (Corzo 2010).

Targeting MUC1 in PDA: While the protein core of MUC1 in normal cells is masked by glycosylation (Ho JJ 1995), during cancer progression aberrant glycosylation allows the immunogenic protein core to be exposed. Several types of MUC1 vaccines are under development: peptide based, fusion based, and DNA based vaccines. The ultimate goal of these vaccines includes DC uptake and CTL induction which leads to memory formation against a tumor antigen in hopes of inhibiting cancer recurrence. Tumor associated MUC1 can be recognized by non-MHC restricted CTLs (Barnd 1989; Magarian-Blander J 1993). It has been shown that healthy human's peripheral T-cells as well as bone marrow mononuclear T-cells can recognize unglycosylated MUC1 protein core (Noto 1997; Hiltbold EM 1998). In fact, mice with spontaneous MUC1+PDA are shown to have functional CTLs which recognize MUC1 and kill MUC1+ cancer cells. Adoptive transfer of these cells indeed resulted in rejection of MUC1+ tumor cells (Mukherjee 2000). This implies that shed MUC1 in the serum of PDA mice is in fact antigenic and can mobilize the low avidity T-cells to induce a CTL response. Further study revealed that vaccination with tumor cell fused DCs induced a strong anti-MUC1 CTL response which caused a survival benefit but not decreased tumor burden (McConnell 2002). In mouse models, vaccination with MUC1-TR cDNA induced in induction of MUC1 reactive CTL and anti-MUC1 antibody response which resulted in both decreased tumor burden and increased survival (Rong 2009). Transfection of DCs

with MUC1 cDNA also elicited an anti-MUC1 immune response in patients which did not inhibit disease progression (Pecher 2002).

One of the immune evasion tactics used by PDA tumors is activated cyclooxygenase-2 (Cox-2) (Juuti 2006). Cox-2 is known to induce Prostaglandin E-2 (PGE-2), a highly immunosuppressive agent. PGE-2 is known to induce and attract immunosuppressive cells such as MDSCs and T-regs (Ben-Baruch 2006; Muller 2006). Ultimately, PGE-2 downregulates CTL and T-helper lymphocyte function (Okuno 1995; Takayama 2002). We have shown that treatment of PDA mice with a Cox-2 inhibitor such as Celecoxib inhibits Cox-2 and IDO and allows peptide based MUC1 specific vaccine to be effective in inducing MUC1-specific CTLs and antibodies (Mukherjee 2009). Such change was associated with decreased T-regulatory and MDSC infiltration within the tumor microenvironment. Eventually, Celecoxib/vaccine treated mice did not develop invasive PDA and had reduced tumor burden.

A number of clinical trials using the MUC1-TR region as the antigen have been described (Qu 2004; North 2005; Ramanathan, Lee et al. 2005; Loveland 2006; North 2006; Wierecky 2006). These vaccines consist of non-glycosylated MUC1-TR peptides of different lengths (from 17-105 amino acids) with different carriers and adjuvants (diphtheria toxin, Bacillus Calmette-Guerin (BCG), mannan, keyhole limpet hemocyanin (KLH), DETOX, and QS-21. These clinical trials have either been ineffective or have reduced the progression of the disease, but did not cure/stop the PDA (Karanikas 1997; Adluri 1999; Gilewski 2000; Mukherjee 2000; Brossart 2001; Karanikas 2001; Salit 2002). While immunotherapy is a promising field in cancer treatment, several obstacles are in the way. Tumors are very efficient in evading the anti-tumor immune response and

therefore even an effective anti tumor immune response might not be functional within the tumor microenvironment. Moreover, most of the cancer immunotherapy clinical trials are performed on terminal patients, or patients who are recovering from radiation/chemotherapy. At this stage, the immune system is considerably defected and cannot mount the proper immune response (Pockaj 2004; I 2009). In order to target MUC1 as a tumor antigen in PDA patients, it is imperative to overcome the immunosuppression associated with this cancer. In addition, a better pool of patients with less advanced disease is needed to study MUC1 targeting.

1.5 Conclusion

Pancreatic cancer is a poorly understood cancer and a devastating disease with little promise in treatment. Radiation and chemotherapy even in combination have proven to be ineffective for patients. PDA is also a very painful disease due to the neurotropic nature of PDA cells. While expression of high levels of growth factors such as FGF, EGF, PDGF, IGF, VEGF and the highly metastatic nature of these cancer cells have been described, numerous studies have shown that MUC1 plays a significant role in regulation of many signaling pathways which cause PDA to be such an incurable disease. MUC1 induces increased tumor mass during PDA while allows cancer cells to survive and resist chemotherapy and radiation. It also induces invasion and metastasis of this cancer through many signaling pathways and in so doing, explains why its expression is associated with such bad prognosis. MUC1 also contributes to the notorious pain that is associated with PDA. Overall, there is mounting amount of evidence suggesting that targeting MUC1 signaling might be one of the best ways to increase survival of PDA patients.

In the cancer field, MUC1 is known as a promising tumor antigen which can induce a humoral response as well as being recognized by CTLs. While MUC1 can be targeted via antibodies and CTLs, its expression can also induce immunosuppression to the point that CTLs are almost ineffective within the tumor microenvironment. Studies on MUC1 signaling during PDA have shown that not only MUC1-TR but also MUC1-CT signaling must be targeted for chemotherapy/radiation and immunotherapy to be effective. Very few studies have been devoted to targeting MUC1-CT but due to all the studies on MUC1 signaling, we propose that in the future targeting MUC1-CT can be an effective therapeutic intervention not only for PDA, but also for other cancers which over express MUC1. Additionally, since MUC1 causes immune suppression, targeting MUC1-CT may make immunological targeting of MUC1-TR to be more effective.

CHAPETER 2: MUC1 AND PDGFA

MUC1 Regulates PDGFA Expression During Pancreatic Cancer Progression

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Running title: MUC1 regulates PDGFA expression

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2.1 Abstract

Pancreatic Ductal Adenocarcinoma (PDA) has one of the worst prognoses of all cancers. MUC1, a transmembrane mucin glycoprotein, is a key modulator of several signaling pathways that affect oncogenesis, motility, and metastasis and is positively associated with poor prognosis in patients. However, the precise mechanism by which this occurs remains elusive. We report a novel association of MUC1 with Platelet-Derived Growth Factor-A (PDGFA), one of the major drivers of tumor growth, angiogenesis, and metastasis in PDA. Using mouse and human PDA models, we show clear evidence that MUC1 regulates the expression and secretion of PDGFA and thus 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, and that PDGFA and MUC1 are critical for translocation of β-catenin 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 controlling PDGFA transcription and facilitates Hif1-α translocation to the nucleus. In summary, we have demonstrated that MUC1-induced invasion and proliferation occurs through the increased exogenous production of PDGFA. Thus, impeding MUC1 regulation of PDGFA signaling may be therapeutically beneficial for patients with PDA.

Key words: Pancreatic Cancer, MUC1, PDGFA, β-catenin

2.2 Introduction

Pancreatic Ductal Adenocarcinoma (PDA) is the fourth leading cause of cancer related deaths in the United States and has one of the worst prognoses of all cancers. Approximately, 95% of diagnosed patients die within the first 6 months of diagnosis and the 5 year survival rate is <5%. PDA is naturally resistant to radiation and chemotherapy. Early micrometastasis to the distal organs such as lymph nodes, liver, and lung is one of the hallmarks of this disease (Burris HA 3rd 1997). All these features have made pancreatic cancer a clinically terminal disease.

Mucin-1 (MUC1, CD227) is a type-I transmembrane glycoprotein which is overexpressed and atypically glycosylated during many adenocarcinomas including PDA (Lan MS 1990; Baldus SE 2004). MUC1 is associated with the metastatic phenotype of cancer cells and in PDA, MUC1 is commonly (~70%) detected in high-grade pancreatic intraepithelial neoplasia (PanIN) and its expression correlates with poor prognosis (Chhieng, Benson et al. 2003; Levi, Klimstra et al. 2004). 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 (MUC1-CT) (Gendler, Lancaster et al. 1990). MUC1-CT plays a critical role in cell signaling during cancer progression (reviewed in (Hollingsworth MA 2004 ; Singh PK 2006 ; DD 2008)). MUC1-CT has been shown to associate with β-catenin and translocate to the nucleus in several studies including ours and thereby influence activity of transcription factors (Hollingsworth MA 2004 ; Roy LD 2010). Recently, a direct contribution of MUC1 on invasive and metastatic properties of pancreatic cancer cell lines has been shown

(Kohlgraf, Gawron et al. 2003; Roy LD 2010) suggesting an important role in the development and progression of the disease (Adsay, Merati et al. 2002; Moniaux, Andrianifahanana et al. 2004; Adsay, Basturk et al. 2005). Using a spontaneous mouse model of PDA that expresses human MUC1, we have shown that *in-vivo,* MUC1 enhances pancreatic tumor progression and metastasis (Tinder, Subramani et al. 2008) and contributes to Epithelial to Mesenchymal Transition (EMT) in PDA cells (Roy LD 2010).

Expression of Platelet-Derived Growth Factor-A (PDGFA) and HIF1-α during PDA progression is associated with poor prognosis (Hoffmann AC 2008). In addition, many malignancies including PDA have been associated with altered PDGF signaling (reviewed in (A 2004)). Interaction of this growth factor with its receptor leads to cellular responses such as proliferation and migration through MEK and PI3K signaling respectively (A 2004). *In-vivo*, PDGF plays a significant role in angiogenesis and metastasis (reviewed in (A 2004; Mantur M 2008)). Indeed, inhibition of PDGFA signaling can reduce growth and metastasis of human pancreatic carcinoma (Hwang RF 2003). Therefore, targeting PDGFA has been proposed as a adjuvant treatment in PDA patients (Al-Batran SE 2007).

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 of MUC1 in regulation of PDGFA expression. We report that MUC1 increases proliferation and invasion of pancreatic cancer cells via regulation of PDGFA expression which is responsible for higher tumor burden and metastasis in MUC1-expressing PDA. Expression of MUC1 correlates with PDGFA

expression during PDA and for the first time we have shown the significance and mechanism associated with this correlation.

2.3 Results

MUC1 expression is detected during all stages of PDA and is correlated to PDGFA expression.

MUC1 is known to be over-expressed during 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 which 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. Next, using samples from different stages of PDA in human, we studied the correlation between PDA progression, MUC1 expression and PDGFA expression. Our results demonstrate that MUC1 expression is detected at early stages of the disease and increases as PanIN lesions evolve into adenocarcinoma (Figure 1B). In addition, we tested the same PDA specimens for expression of PDGFA by IHC. Our results show that although basal level expression of PDGFA is detectable in normal pancreas, there is a strong over-expression of PDGFA by dysplastic ductal cells of PDA patients in a stage dependent manner (Figure 1B).

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 that have low endogenous MUC1 were transfected with full length MUC1 or empty vector (Neo), while MiaPACA-2 and Capan-1 that have high endogenous MUC1 were knocked-down for its expression with a specific siRNA. 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). Next, protein lysates were 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 cells expressed high levels of PDGFA while BXPC3.Neo cells expressed negligible. Moreover, in both of the siRNA models (MiaPACA-2 and Capan-1), 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. In addition, introduction of MUC1 to pancreatic cancer cells which express very low endogenous MUC1 led to induction of PDGFA expression.

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 Capan1, BXPC3.MUC1, and KCM cells secreted significantly higher levels of PDGFA compared to MUC1 siRNA treated Capan1, BXPC3.Neo, and KCKO cells (Figure 2C). To this point, for the first time we have clearly shown that lack of MUC1 not only leads to downregulation of PDGFA expression in PDA cells but significantly hampers its secretion (Figure 2C).

MUC1 regulates PDGFA expression as tumors progress in a mouse model of PDA and in human pancreatic tumors grown *in-vivo*

Next, we utilized our *in-vivo* spontaneous model of PDA to study the role of MUC1 in PDGFA expression and its correlation with tumor development. Tumor sections were H&E stained for pathological assessment and analyzed for PDGFA expression. In 6-week old PDA mice, pancreas architecture in all mice appeared similar to that of healthy C57/B6 mice. However, PDGFA expression by ductal cells appeared to be the highest in PDA.MUC1 mice and lowest in PDA.Muc1$^{-/-}$ mice (Figure 2Di, ii). In 6-month old mice, at which time, PanIN lesions become evident (Hingorani 2003), expression of PDGFA was lowest in PDA.Muc1$^{-/-}$ mice and correlated with presence of small numbers of low grade PanIN lesions. However, pancreas from both PDA and PDA.MUC1 mice showed stronger expression of PDGFA with higher numbers of PanIN lesions and disruption of pancreas's architecture (Figure 2D iii). Finally, in 8- month old PDA.Muc1$^{-/-}$ mice, pancreas sections showed the presence of PanIN-1A lesions as well as low numbers of late stage PanIN lesions which correlated with low expression of PDGFA. However, at the same age, pancreas from PDA mice showed higher numbers of late stage PanIN lesions and higher PDGFA expression. Importantly, pancreas from

PDA.MUC1 mice showed the highest numbers of late stage PanIN lesions and adenocarcinoma which correlated with strongest expression of PDGFA (Figure 2D iv). Similarly, IHC of tumors derived from human pancreatic cancer cell lines, BXPC3.MUC1 established *in vivo* in nude mice showed high *in situ* levels of PDGFA while none was detectable in the BXPC3.Neo tumors (Figure 2D v). Taken together, data shows a strong link between MUC1 and PDGFA expression during tumor progression *in vivo.*

MUC1-expressing PDA cells have increased tumor growth *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*, two autochthonous models using mouse and human cell lines were used. C57/B6 mice were injected with KCKO and KCM cells and tumor weight was monitored and assessed at sacrifice. Tumors resulting from KCM cells were significantly larger than KCKO tumors (Figure 3Ai). Similarly, BXPC3.Neo and BxPC3.MUC1 cells were injected into nude mice and tumor weight measured. tumors resulting from BXPC3.MUC1 cells was significantly larger than that of BXPC3.Neo (Figure 3A ii) confirming the results of our previously published studies in the spontaneous model of PDA (Roy LD 2010). Thus, data provides strong evidence that tumor cells expressing MUC1 have a significant growth advantage compared to tumors that are null for MUC1 and this is most likely due to the role of MUC1 in regulating PDGFA which increases cell proliferation. 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 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, data presents strong evidence for the significant function of MUC1 in augmenting proliferation and the invasive properties of pancreatic cancer cells.

PDGFA expression by pancreatic cancer cell lines contributes to increased invasion and proliferation of these cells.

To this point, we have shown that high MUC1 levels in tumors correlate with high PDGFA levels leading to increased tumor burden. Therefore, to elucidate if the higher proliferation in MUC1-expressing cells is indeed due to regulation of PDGFA, we neutralized PDGFA with a specific antibody prior to subjecting the tumor cells to a ^{3}H-thymidine proliferation assay. We presume that neutralizing PDGFA will inhibit its interaction with the receptor. 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. Once PDGFA was neutralized in the supernatant of these cells, BXPC3.MUC1 showed

significantly less proliferation while proliferation of BXPC3.Neo cells remained unchanged (Figure 4Bii).

Similarly, PDGFA neutralization significantly reduced the invasive index of MUC1-expressing tumor cells including KCM, BxPC3.MUC1, and MiaPACA-2 cells but not so in KCKO, BXPC3.Neo, and MUC1 siRNA-treated MiaPACA-2 cells (Figure 4Bi-iii). Of note is that there was no difference in the levels of PDGF-Rα, which is the receptor responsible for PDGFA binding between the MUC1-negative and MUC1-positive cells (data not shown). Taken together, our data shows that cells lacking MUC1 not only produce less PDGFA but are also not 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.

To further elucidate the underlying mechanism, since HIF-1α is a transcription factor known to regulate PDGF-A production and is also associated with poor prognosis during PDA (Moon JO 2009) (Hoffmann AC 2008; Hoffmann AC 2008; Reiser-Erkan C 2008), we hypothesized that MUC1 regulates translocation of this protein to the nucleus and thus its activation in pancreatic cancer cell lines. Therefore, we first examined the interaction between HIF-1α and MUC1-CT in pancreatic cancer cell lines by co-immunoprecipitation assays. Results show that in all MUC1 expressing pancreatic cancer cell lines, Hif-1α associates with MUC1-CT (Figure 5A). This was validated using two different clones of Hif-1α antibody (data not shown). Next, we tested the effects of the interaction on Hif-1α nuclear translocation. Nuclear extracts of KCKO/KCM, BXPC3 and SU86.86 cells were subjected to Western blot 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). Further, BXPC3 and SU86.86 cells transfected with MUC1 plasmid show translocation of Hif-1α to their nucleus while BXPC3.Neo and SU86.86.Neo cells show no detectable levels of Hif-1α in their nucleus (Figure 5Bii). To validate the role of Hif1-α in regulation of PDGFA, BXPC3.Neo and MUC1 cells were treated with a Hif1-α inhibitor for 24hours. Protein lysates were then subjected to Western blot for presence of PDGFA. Our results show that following HIF1-α inhibition, expression of PDGFA is strongly downregulated in BXPC3.MUC1 cells (Figure 5C). This suggests that regulation of PDGFA via MUC1 may require translocation of Hif-1α to the nucleus which is known to affect PDGFA expression.

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 tumor genesis and EMT (Huang L 2003 ; Leng Y 2007; Fischer AN 2007 ; Hattrup CL 2008; Singh PK 2008; Roy LD 2010). Therefore, to study 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 MUC1-positive pancreatic cancer cell lines tested (Figure 5D); second, we demonstrate that MUC1-CT translocates to the nucleus of all MUC1-positive pancreatic cancer cell lines that were analyzed (Figure 5D). Similarly, 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 (Fischer AN 2007), therefore, we hypothesized that translocation of β-

catenin to the nucleus of pancreatic cancer cells is dependent on PDGFA secretion. Therefore, 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. Result clearly demonstrates 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 β-catenin 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). Taken all together, we have shown that MUC1 regulation of β-catenin translocation to the nucleus is in part dependent on PDGFA.

2.4 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 (Szajda SD 2011), its significant role in PDA development has not been fully explored. Data from this study shows that MUC1 enhances pancreatic tumor progression and invasiveness 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 (Al-Batran SE 2007). Similarly, MUC1 has also been implicated as a marker for poor prognosis and a target for therapeutic intervention in pancreatic cancer (Szajda SD 2011). This is the first study to show a direct relationship between these two pancreatic tumor promoting factors. The evidence for this association is overwhelming as 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 antibody against MUC1-CT we have shown that MUC1 is overexpressed in all stages of pancreatic cancer (Figure 1). The first evidence that lack of MUC1 in PDA cells indeed down regulates PDGFA expression came from the Human Cancer Pathway RT-PCR assay (Figure 1A) and 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-over expressing PDA cells and tumors express and secrete higher levels of PDGFA which accounts for the increased proliferative and invasive index of these cells as compared to PDA cells and tumors that lack MUC1 and therefore have lower levels of PDGFA (Figure 3). Of additional significance is that PDA mice lacking Muc1 (PDA.Muc1$^{-/-}$ mice) have low levels of PDGFA expression correlating with low-grade PanIN lesions that do not progress to invasive disease versus PDA.MUC1 mice that have high PDGFA expression with high-grade PanINs that do progress to invasive adenocarcinoma (Figure 2D).

PDGFA has long been associated with cancer progression (reviewed in (A 2004)) and can increase proliferation and invasion through the MEK and PI3K pathway respectively (as reviewed in (Heldin CH 1998 ; Castaneda CA 2010)) and 2). 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 (Hoffmann AC 2008; Hoffmann AC 2008), therefore we hypothesized that MUC1 association with Hif1-α leads to increased translocation of this transcription factor which is a known regulator of PDGFA induction (Moon JO 2009) and that is exactly what we report (Figure 5). Although Hif-1α expression is very low during normaxia, 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 significant decrease in the levels of PDGFA expression (Figure 5). These results provide us with in-direct evidence that MUC1 up regulates PDGFA expression via up regulation of HIF-1α translocation to the nucleus. MUC1 is a known regulator of transcription, and several studies including a recent study has shown that MUC1 translocation to the nucleus can alter expression of growth factors and therefore influence the tumor microenvironment (Behrens ME 2010).

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 β-catenin to the nucleus is strongly dependent on MUC1 levels such that if cells do not have MUC1, very little β-catenin is found in the nucleus (Figure 5) although there is abundant β-catenin in the whole cell lysates. Previous data have shown the strong role of PDGFA in nuclear localization of β-catenin during liver cancer (Fischer AN 2007). Also, other studies have pointed the role of PDGFB in nuclear translocation of β-catenin in pancreatic cancer (Singh PK 2007). In this study, through 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 via 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 (Singh A 2010) and schematically illustrated in Figure 6.

While MUC1-TR is proposed to be a targetable tumor antigen during PDA, growing evidence implicate 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.

2.5 Materials and methods

Cell lines

BXPC3 and 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 (Roy LD 2010), and 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 carried 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 (Thompson, Shanmugam et al. 2006). 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 PDGF-AA 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 (Hingorani 2003; Roy LD 2010) to produce PDA mice and further mated to the human MUC1.Tg mice to

generate PDA.MUC1 mice (Tinder, Subramani et al. 2008; Mukherjee, Basu et al. 2009) or to the $Muc1^{-/-}$ mice (Spicer, Rowse et al. 1995) 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 (PanIN-1A), and 40 weeks (PanIN-1A,B, PanIN-2, and PanIN-3) of age (Hingorani 2003). 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 uptake 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 $5x10^{6}$ 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 C57/B6 mice were injected with $1X10^{6}$ KCKO or KCM cells s.c. in the flank. Tumors were allowed to grow for 18 days. Upon sacrifice, tumor weight was measured 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 37ºC. 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 rPDGF-AA (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 X-100, 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 (chroeder JA 2001). 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. Co-immunoprecipitation (co-IP) using CT2 and β-catenin were carried out as previously reported (Al Masri and Gendler 2005) 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 1x 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 $p < 0.05$.

2.6 Figures

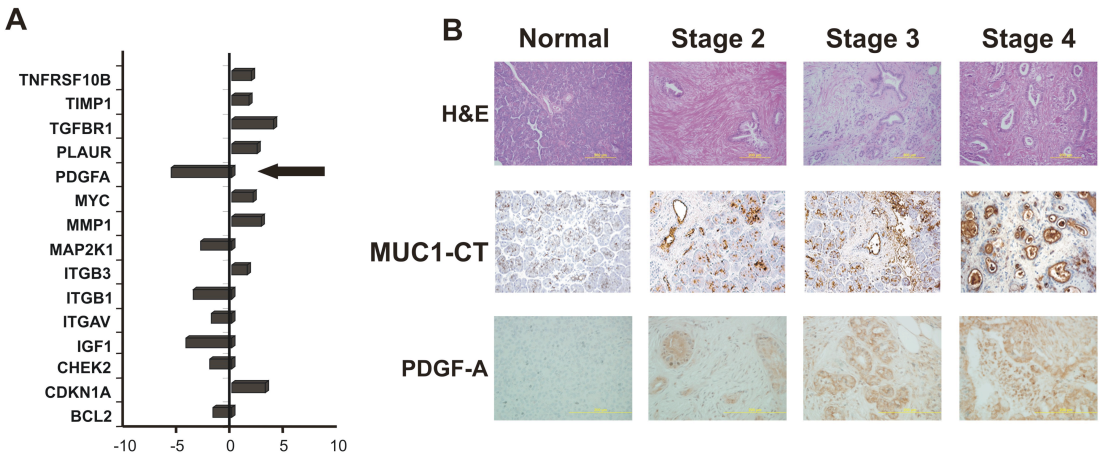

Figure 1. MUC1 overexpression is detected in all stages of PDA and is correlated to increased expression of PDGFA. A) Fold change in mRNA levels in WT versus MUC1siRNA-treated MiaPACA-2 cells. Differences over 2-fold are shown (n=3, $p<0.05$). B) H&E staining and detection of MUC1 and PDGFA over expression during all stages of human PDA. MUC1 is detected using antibody specific to the cytoplasmic tail.

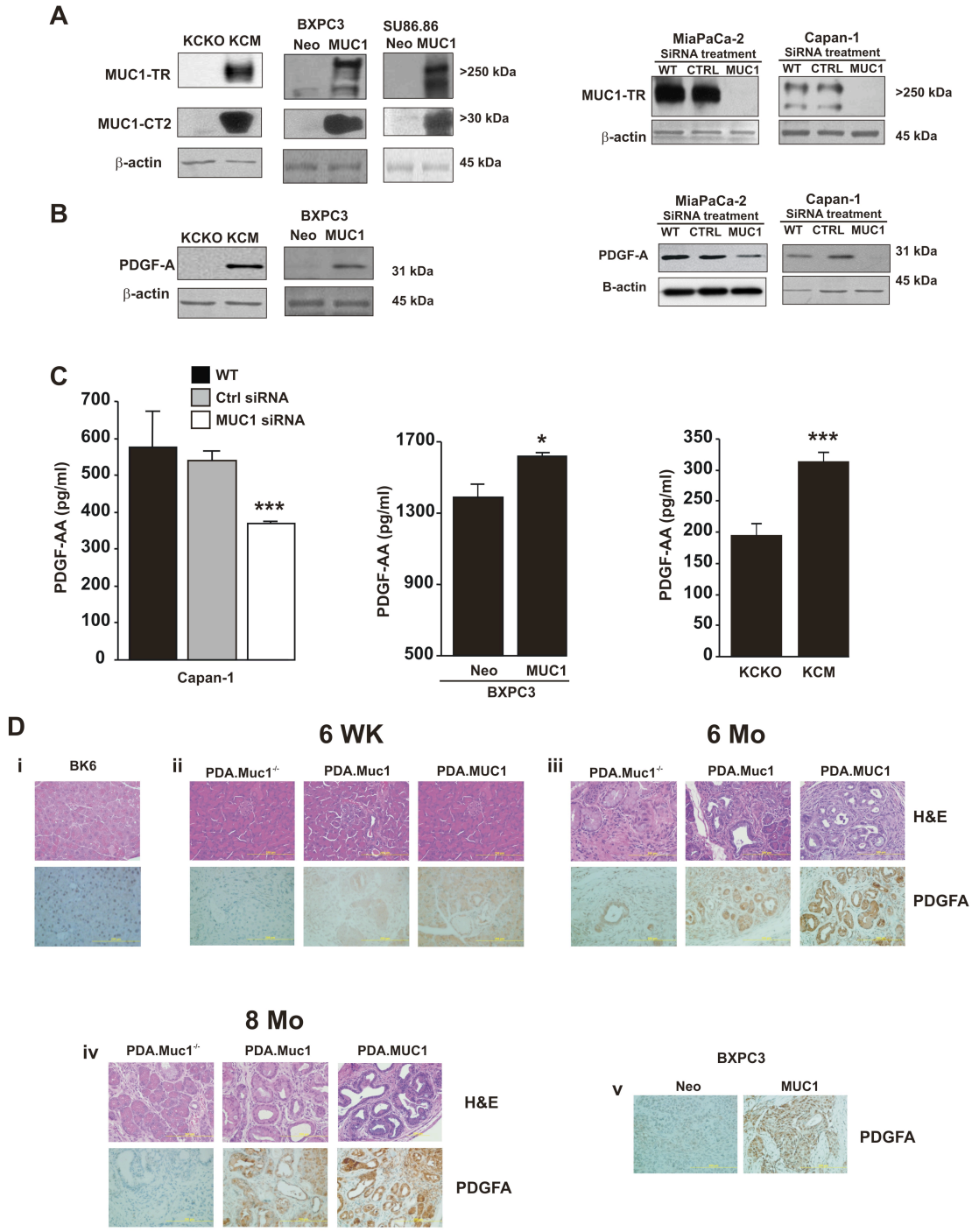

Figure 2. MUC1 regulates PDGFA expression during pancreatic cancer. A) Western blot analysis of MUC1 expression in human and mouse pancreatic cancer cell lines using both the MUC1 TR and MUC1 CT-specific antibodies. Additionally, downregulation of MUC1 expression in MiaPACA-2 and Capan-1 cells 72 hours post siRNA treatment. Representative blots from 3 separate experiments are shown. B) Western blotting analysis of PDGFA expression in various pancreatic cancer cell lines. C) Levels of PDGF-A

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 PDGF-A expression in pancreas of PDA.Muc1$^{-/-}$, PDA, and PDA.MUC1 mice at 6-weeks (ii), 6-months (iii) and 8-months (iv) of age (n=5). Representative images from normal 6-month old C57BL/6 pancreas is shown as control. v: PDGFA expression in BXPC3.neo and MUC1 tumors from nude mice. Images are captured at 40X magnification.

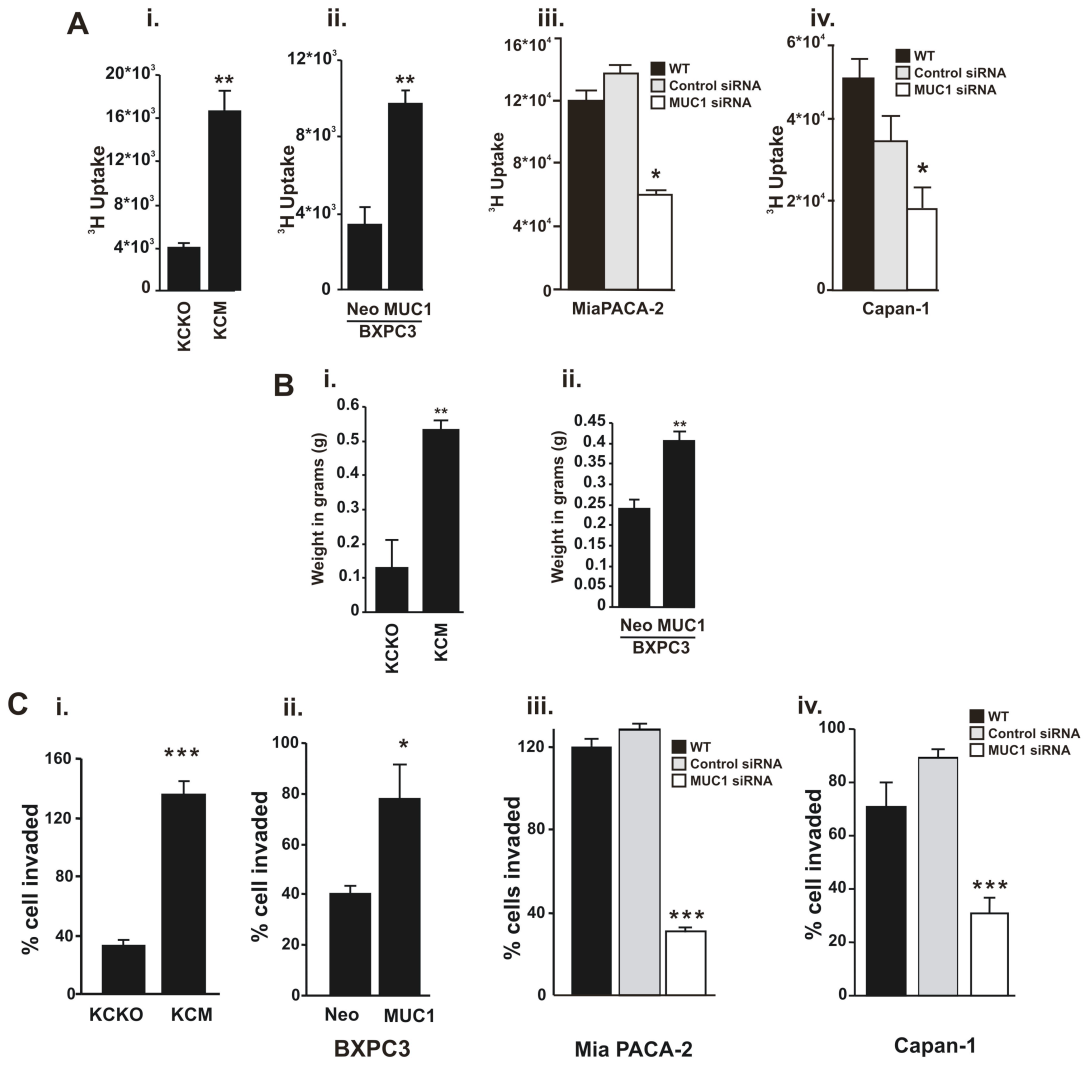

Figure 3. MUC1 regulates tumor burden, proliferation, and invasion of pancreatic cancer cell lines. B) Proliferation of $MUC1^+$ and $MUC1^-$ pancreatic cancer cells as measured by ^{3}H-thymidine uptake (cpms) (n=4) (*P<0.05, **P<0.01). A i and ii) Tumor wet weight of KCKO versus KCM (i) and BxPC3.Neo versus BxPC3.MUC1 tumors in vivo. $1x10^6$ 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 $5x10^6$ 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). C) Percent cells that invaded through growth factor-reduced matrigel in a Boyden chamber invasion assay (n=4) (***P=0.001, *P=0.05).

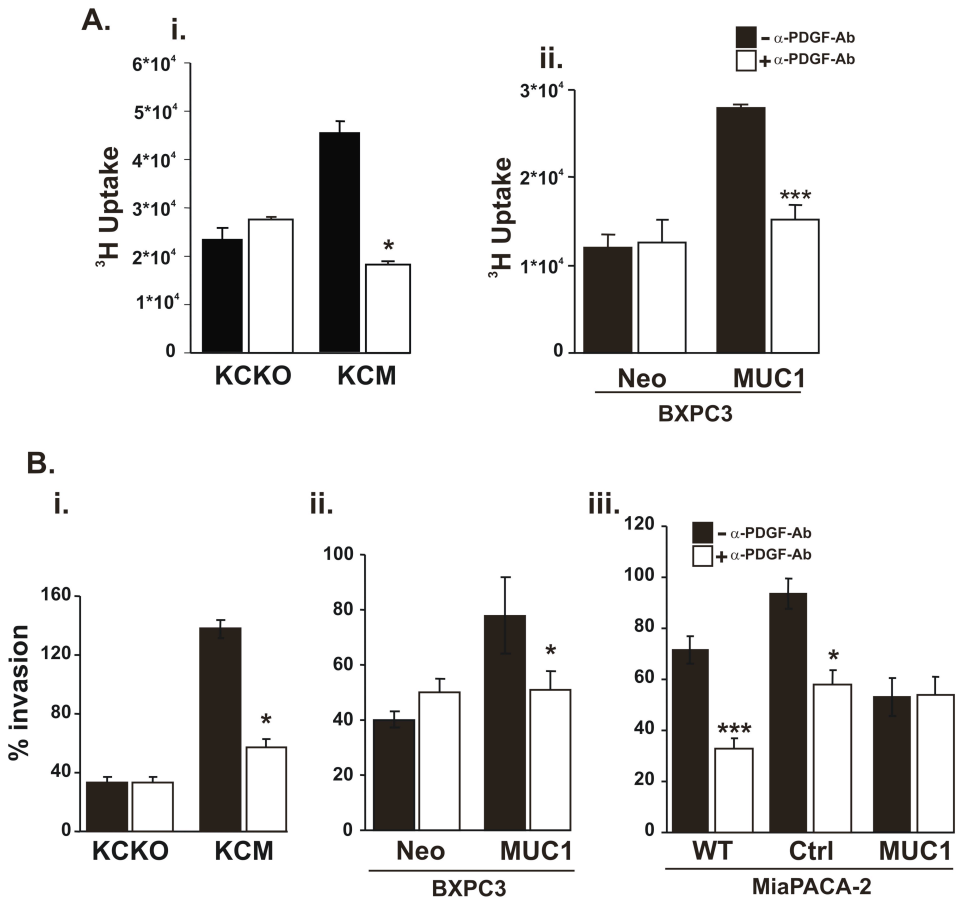

Figure 4. PDGF regulates invasion and proliferation of MUC1+ pancreatic cancer cells but has no effect on MUC1- cells A) Proliferation of pancreatic cancer cells as measured by 3H-thymidine uptake (cpms) following PDGF neutralization (n=4) (*P<0.05, and ***P=0.001). B) Percent cells that invade through growth factor-reduced matrigel following PDGF neutralization. (n=3), (*P< 0.05 and ***P=0.001).

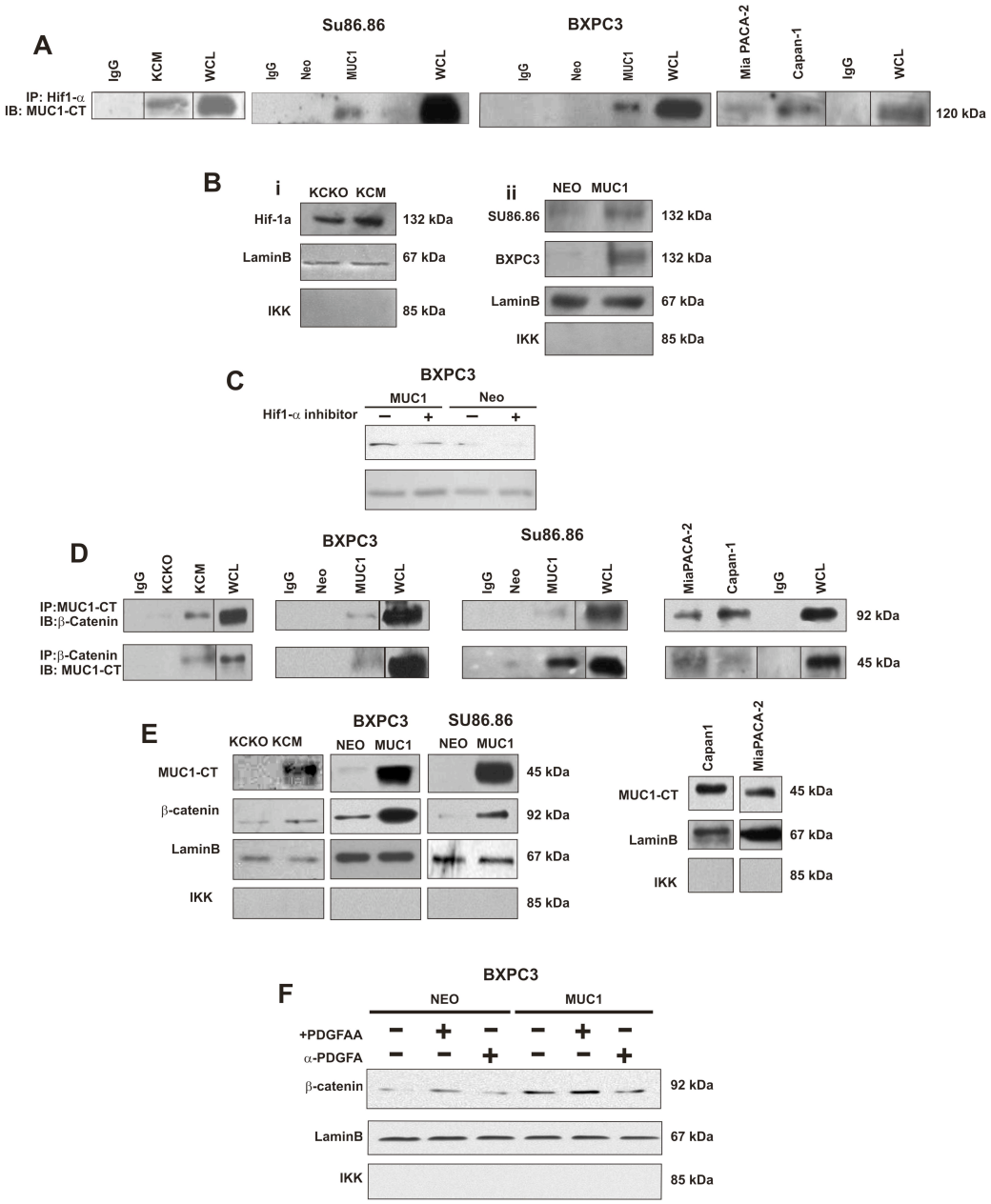

Figure 5. MUC1 interacts with HIF-1α and β-catenin. Nuclear translocation of MUC1, β-catenin and HIF-1α: A) Co-IP of MUC1-CT and HIF-1α in several cell lines demonstrating the interaction of MUC1-CT and HIF-1α. B) HIF-1α translocation to the nucleus of i) KCKO and KCM mouse PDA cells; and ii) Su86.86 and BxPC3 human cells. More HIF-1α in MUC1-expressing versus MUC1 null pancreatic cancer 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) Translocation of MUC1-CT and β-catenin to the nucleus i) KCKO, KCM, Su86.86, and BxPC3 cells

indicating the activation of β-catenin as a transcription factor; ii) MUC1-CT detected in high levels in the nucleus of Capan-1 and MiaPACA-2 cells. F) 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.

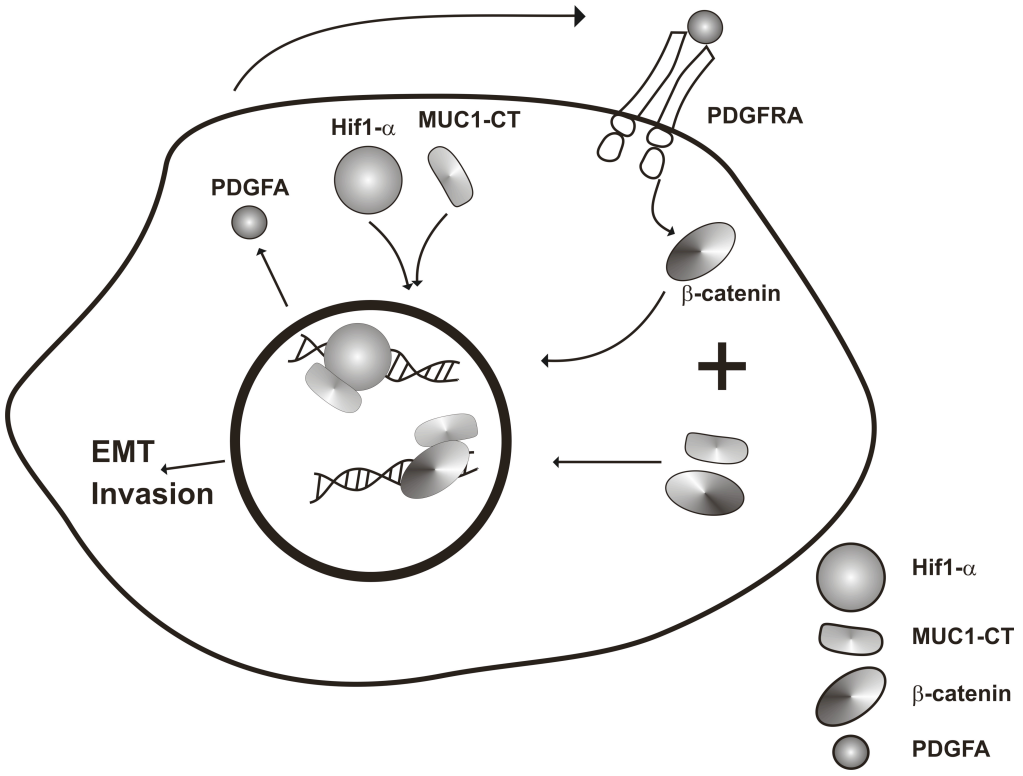

Figure 6. Schematic of the possible mechanism underlying MUC1 regulation of PDGFA. In MUC1 positive PDA cells, MUC1-CT associates with HIF1-α and facilitates its translocation to the nucleus. This results in expression of PDGFA which is then secreted and interacts with PDGFR-α. Signaling through PDGFR-α has an additive effect on β-catenin translocation and enhances proliferation and metastasis.

CHAPTER 3: MUC1 AND EMT

MUC1 enhances invasiveness of pancreatic cancer cells by inducing epithelial to mesenchymal transition

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3.1 Abstract

Increased motility and invasiveness of pancreatic cancer cells are associated with epithelial to mesenchymal transition (EMT). Snai1 and Slug are zinc-finger transcription factors that trigger this process by repressing E-cadherin and enhancing vimentin and N-Cadherin protein expression. However, the mechanisms that regulate this activation in pancreatic tumors remain elusive. MUC1, a transmembrane mucin glycoprotein, is associated with the most invasive forms of pancreatic adenocarcinomas (PDA). In this study, we show that over expression of MUC1 in pancreatic cancer cells trigger the molecular process of EMT which translates to increased invasiveness and metastasis. EMT was significantly reduced when Muc1 was genetically deleted in a mouse model of PDA or when all seven tyrosines in the cytoplasmic tail of MUC1 were mutated to phenylalanine (mutated MUC1 CT). Using proteomics, RT-PCR, and western blotting, we revealed a significant increase in vimentin, N-Cadherin, Slug and Snai1 expression with repression of E-Cadherin in MUC1-expressing cells compared to cells expressing

the mutated MUC1 CT. In the cells that carried the mutated MUC1 CT, MUC1 failed to co-immunoprecipitate with β-catenin and translocate to the nucleus thereby blocking transcription of the genes associated with EMT and metastasis. Thus, functional tyrosines are critical in stimulating the interactions between MUC1 and β-catenin and their nuclear translocation to initiate the process of EMT. This study signifies the oncogenic role of MUC1 CT and is the first to identify a direct role of the MUC1 in initiating EMT during pancreatic cancer. The data may have implications in future design of MUC1-targeted therapies for pancreatic cancer.

3.2 Introduction

Pancreatic Ductal Adenocarcinoma (PDA) is the fourth leading cause of cancer death in the United States (Saif 2006). This disease remains a major therapeutic challenge, as it is naturally resistant to current chemotherapy/radiation therapies. Poor prognosis has been attributed to delayed diagnosis and early vascular dissemination, spread to regional lymph nodes, and metastases to distant organs, especially the liver and peritoneum (Burris, Moore et al. 1997). One important insight came from the discovery that the increased motility and invasiveness of cancer cells are associated with EMT (Nieto 2002; Thiery 2002). In EMT, epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility. This process is associated with the transcriptional repression and functional loss of E-cadherin (Nieto 2002; Thiery 2002). Several transcription factors have been implicated in this repression, including the zinc-finger proteins of the Snai1/Slug family (Batlle, Sancho et al. 2000; Cano, Perez-Moreno et al. 2000; Guaita, Puig et al. 2002), δEF1/ZEB1, SIP1 (Comijn, Berx et al. 2001), and the basic helix-loop-helix E12/E47 factor (Bolos, Peinado et al. 2003). Snai1

was shown to repress the expression of E-cadherin and induce EMT in several cancer cells (Batlle, Sancho et al. 2000; Cano, Perez-Moreno et al. 2000; Guaita, Puig et al. 2002). Although several factors such as TGF-β and the estrogen receptor were shown to regulate Snail transcription (Fujita, Jaye et al. 2003; Peinado, Quintanilla et al. 2003), the mechanisms that modulate the function of Snail have remained largely elusive. Recently, dual regulation of Snai1 by GSK-3β-mediated phosphorylation has been implicated in EMT (Zhou, Deng et al. 2004).

MUC1 is a transmembrane mucin glycoprotein that is over-expressed and aberrantly glycosylated in 100% of metastatic PDA (Lan, Batra et al. 1990). Although elevated levels of MUC1 protein have been associated with higher metastasis and poor prognosis, its molecular role in metastasis remain unclear (Patton, Gendler et al. 1995; Spicer, Rowse et al. 1995; Schroeder, Adriance et al. 2003; Tinder, Subramani et al. 2008). Multiple reports have appeared over the past decade demonstrating an incredible range of intracellular signaling functions associated with the 72–amino acid residue of MUC1 CT which is reviewed in (Hollingsworth and Swanson 2004; Singh and Hollingsworth 2006; Carson 2008). MUC1 CT is a target for several kinases, including ζ chain–associated protein kinase of 70 kD (ZAP-70), the δ isoform of protein kinase C (PKCδ), glycogen synthase kinase-3β (GSK-3β), and the tyrosine kinases c-Src and Lck (Mukherjee, Tinder et al. 2005; Hattrup and Gendler 2008; Kufe 2008). Phosphorylation of Tyr within the TDRSPYEKV sequence by c-Src and Lck stimulates interactions between MUC1 and β-catenin whereas phosphorylation of Ser by GsK-3β inhibits this interaction (Ren, Li et al. 2002; Ren, Bharti et al. 2006). The same Tyr residue is critically important for nuclear localization of MUC1, apparently because of the

requirement for tyrosine phosphorylation to support the appropriate interactions necessary for intracellular trafficking. Binding to β- catenin appears to provide the signals required for movement of MUC1 to the nucleus in tumor cells (Carson 2008) thereby influencing transcription through TCF/LEF and/or other transcription factors (Hollingsworth and Swanson 2004).

We have generated mouse models of PDA in a congenic C57BL/6 background that either expresses human MUC1 (Tinder, Subramani et al. 2008) or genetically lacks Muc1. Cell lines have been developed from these mice to study the oncogenic role of MUC1 in pancreatic cancer progression. In this study, we show that EMT and secondary metastasis was significantly reduced in PDA mice that lack Muc1 compared to PDA mice that express MUC1. In contrast, over expression of MUC1 in both human and mouse pancreatic cancer cells initiated EMT by inducing the transcription factors, Snai1 and Slug and repressing E-Cadherin which lead to increased invasion and metastasis. Furthermore, mutating the tyrosines in MUC1 CT to phenylalanine completely blocked EMT and prevented metastasis. The data suggests direct oncogenic signaling through the tyrosines in MUC1 CT in the induction of EMT and metastasis during pancreatic cancer.

3.3 Materials and methods

Mouse Model

PDA mice was generated in our laboratory on the C57BL/6 background by mating the P48-Cre with the LSL-KRAS^{G12D} mice (Hingorani, Petricoin III et al. 2003) and further mated to the MUC1.Tg mice to generate PDA.MUC1 mice (Tinder, Subramani et al. 2008; Mukherjee, Basu et al. 2009) or to the Muc1KO mice (Spicer, Rowse et al. 1995) to generate PDA.Muc1KO mice (Figure 1A). Primary tumors were

dissociated using collagenase II and several lines of cells generated in our laboratory. These cells are designated KCKO for cells lacking Muc1 and KCM for cells expressing human MUC1.

Invasion Assays

Cells were serum-starved for 48hrs prior to plating for the invasion assay. Cells in serum free media (50,000 cells) were plated over transwell inserts (BD Biosciences) pre-coated with reduced growth factor matrigel or no matrix (control), 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 for 10 minutes in 10% acetic acid and absorbance read at 570 nm. Percent invasion was calculated as absorbance of samples/absorbance of controls × 100.

Western Blots and Antibodies

Briefly, cells were lysed in HEPES buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EDTA) containing protease (Complete inhibitor cocktail; Roche, Indianapolis, IN) and phosphatase inhibitors (10 mmol/L sodium fluoride, 2 mmol/L sodium vanadate, 50 μmol/L ammonium molybdate). Equal quantities of lysate were loaded on SDS-PAGE gels. MUC1 CT antibody CT2, was made in Mayo Clinic Immunology Core (Schroeder, Thompson et al. 2001). MUC1 TR (B27.29) antibody was acquired from Biomira Inc.,Edmonton, Alberta. All other antibodies (Snai1, Slug, N-Cadherin, Vimentin, Vegf, E-Cadherin, Twist, β-catenin, and β-actin) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA and were used according to

manufacturer's recommendations. Co-immunoprecipitation (co-IP) using CT2 (1:500 dilution) and β-catenin (1:100 dilution) were carried out as previously reported (Al Masri and Gendler 2005) using 1mg of protein lysate prepared in 1% Brij buffer (Sigma) followed by standard western blotting.

Cloning of MUC1 WT and MUC1 Y0 Vectors

MUC1 Y0 was created using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA) (Thompson, Shanmugam et al. 2006). Briefly, primers based on the MUC1 sequence were designed containing single-base alterations resulting in mutation of the tyrosine residues (Y) in MUC1 CT to phenylalanine (F) as shown schematically.

```
          1         10         20         30         40         50         60         70 72
          |         |          |          |          |          |          |          | |
WT MUC1 CQCRRKNYGQLDIFPARDTYHPMSEYPTYHTHGRYVPPSSTDRSPYEKVSAGNGGSSLSYTNPAVAATSANL
   Y0   -------F----------F-----F--F-----F----------F-------------F------------
```

Y=tyrosine; F=phenylalanine

Successful mutations were confirmed with DNA sequencing. MUC1 Y0 and MUC1 WT were cloned into the pLNCX.1 vector consisting of the neomycin resistance gene for retroviral infection.

Cell Culture and Retroviral Infection

BxPC3 cells and Su86.86 (American Type Culture Collection, Manassas, VA) are two human pancreatic cancer cell lines that express very little endogenous MUC1.The cells were maintained in complete RPMI (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 1% glutamax (Invitrogen), and 1% penicillin/streptomycin. For retroviral infection, GP2-293 packaging cells (stably expressing the *gag* and *pol* proteins) were co-transfected with the full-length MUC1 construct or the Y0 construct or empty vector expressing the VSV-G envelope protein as previously described (Thompson, Shanmugam et al. 2006). After 48 hours, virus was pelleted; resuspended in medium containing 8

μg/mL polybrene (hexadimethrine bromide) and incubated overnight with BxPC3 or Su86.86 cells that had been pretreated for 2 to 3 hours with polybrene. Cells were selected with 0.5 mg/mL G418, beginning 48 hours post infection, and cells were maintained as polyclonal populations until sorted. 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 Neo. For MUC1 and Y0-infected cells, MUC1+ve cells were sorted using the FACSAria. For Neo-infected cells, MUC1-ve cells were sorted.

Confocal Microscopy

Cells were plated on chamber slides and grown to the desired confluency. Cells were washed, fixed in –20°C ethanol, permeabilized with 0.5% Tween 20 and stained with the appropriate primary (Santa Cruz Biotechnologies) and secondary antibodies (Invitrogen, Carlsbad, CA). Nuclei were stained with To-pro-3 (Invitrogen). For tumor sections, similar protocol was followed. Pictures were taken at 400X using confocal microscopy (Carl Zeiss International, Thornwood, NY).

Real-time PCR in the Taqman low-density custom made array format

Total RNA was extracted according to standard protocol using the Qiagen RNeasy mini-kit protocol; (Invitrogen, Carson City, CA). cDNA was constructed using TaqMan® Reverse Transcription cDNA kit from Applied Bioscience (Foster City, CA). Each low-density custom array card was configured for 72 different genes in triplicates. The samples were distributed to the microwells by centrifugation for 1 min at 343 x *g*. The cards were sealed and placed in an ABIPRISM7900HT thermocycler for 40 cycles of 95°C to 60°C. The SDS2.2 software was used for qualitative analysis. Arrays were

performed independently at least three times for each cell line; values were obtained for the threshold cycle (Ct) for each gene and normalized using the average of four housekeeping genes on the same array (*HPRT1*, *RPL13A*, *GAPDH*, *ACTB*). Ct values for housekeeping genes and a dilution series of *ACTB* were monitored for consistency between arrays. Change (ΔCt) between BxPC3-neo, MUC1, and Y0 was found by: ΔCt = Ct (MUC1 or Y0) – Ct (neo) and fold change by: Fold change = 2(-ΔCt).Values are provided as fold change.

Isolation of Nuclear Protein

Cells were washed once with ice cold 1x PBS and then resuspended in buffer A (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 0.1% Nonidet-P40) on ice. Lysate was then spun at 6000 rpm, 1min, 4°C and the resulting pellet was washed twice in 1x ice cold PBS before sonication in buffer B (20mM HEPES ph 7.9, 25% glycerol, 400mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.5mM DTT) to obtain the nuclear fraction which was then spun at 13 000 rpm for 20min, 4°C, for western blot analysis using CT2 and β-catenin.

***In vivo* tumor growth**

Two-month old nude mice were injected with $5x10^6$ (in 100ul of phosphate buffer saline) BxPC3 MUC1, BxPC3 Neo or BxPC3 Y0 into the flank of the mice. Tumors were allowed to grow for two months. Mice were palpated every second day starting at 7 days post tumor injection until sacrifice. Tumor weight was calculated according to the following formula: grams=(length in centimeters x $(\text{width})^2$)/2 (Simpson-Herren and Lloyd 1970). Upon sacrifice, the tumors were prepared for lysates, and fixed for immunohistochemistry (IHC). Serum was collected.

Immunohistochemistry

Tumors sections were formalin fixed in 10% neutral-buffered formalin (pH 6.8-7.2) for a minimum of 24 hours. Paraffin embedded blocks were prepared by the Histology Core at The Mayo Clinic and 4-micron thick sections were cut for immuno-staining. VEGF expression was determined using the Santa Cruz Biotechnologies antibodies at 1:50 dilution followed by the appropriate secondary antibody (1:100 dilution, DAKO, Carpenteria, CA). Sections were developed using 3, 3"–Diaminobenzidine as the chromogen and hematoxylin was used as the counterstain. Slides were examined under light microscopy and pictures taken at 400X magnification.

Isolation of viable circulating human pancreatic cancer cells

Blood samples (0.5–1.0 ml) were obtained by cardiac puncture from individual tumor-bearing animals using heparin as the anticoagulant and processed immediately. Erythrocyte-free nucleated cell fractions were obtained using the RBC lysis by ammonium chloride solution according to the protocol supplied by the manufacturer (Stemcell Technologies, Inc., Vancouver, Canada). The erythrocyte-free nucleated cell fraction was immediately expanded in culture in DMEM complete media as previously described (Glinskii, Smith et al. 2003).

Protein Array Assay

Tumor lysates were analyzed using The RayBio® Custom human cytokines and growth factor antibody array kit (RayBiotech, Norcross, GA). Assay was conducted according to the manufacturer's instructions (Das Roy, Pathangey et al. 2009). Chemiluminescence was detected using a EpiChemi3® Darkroom imaging system and LabWorks® densitometry software (UVP Bioimaging, Upland, CA). Data was

corrected for background signal and normalized to positive controls using RayBio® Analysis Tool software. The protein analysis was done by the company.

Proteomics:

For the electrophoretic separation of each sample, 30 ug of protein was loaded on a 10 % Bis-Tris NuPAGE gel (Invitrogen, Carlsbad, CA). The gel slices were digested with trypsin and analyzed. A Nano Acuity UPLC system connected to an LTQ- Orbitrap hybrid MS system (Thermo Fisher, Pittsburg, PA) with Nanospray interface was used.. Data was acquired using Xcaliber (Thermo Fisher, Pittsburg, PA) and processed by using Bioworks software and mouse v.3.18 fasta database. SEQUEST parameters were used as follows: mass tolerance of 0.01 Da for precursor ions and 0.5 Da for fragment ions, variable modification on methionine of 16 Da, and maximum missed cleavage of 1. Search results were entered into Scaffold software (Proteome Software) for compilation, normalization, and comparison of spectral counts. Protein identifications were made at the peptide probability of 95 % and protein probability of 99 %. Experiments for Mass spectroscopy were duplicated for a power law global error model (PLGEM). Datasets were imported into R program for statistical computing.

Densitometry analysis

The densitometry analysis of the western blots was performed using NIH provided software. Results were presented as arbitrary densitometry units corrected for background intensity.

Statistical analysis

Statistical analysis was performed with SPSS 10 software. P-value was generated using the one way Anova and significance was confirmed using the Duncan and Student-Newman-Keul test. Values were considered significant if $p < 0.05$.

3.4 Results

PDA.MUC1 mice have significantly higher incidence of metastasis to the lungs, liver and peritoneum as compared to PDA.Muc1KO mice. This is characterized by increased Vimentin and decreased E-Cadherin in the primary pancreas tumors.

We have generated a model of spontaneous PDA that expresses human MUC1 as a self molecule (PDA.MUC1 mice) (Tinder, Subramani et al. 2008; Mukherjee, Basu et al. 2009). To further elucidate the role of MUC1 in PDA, we have also generated the PDA mice in a Muc1 null background (PDA.Muc1KO mice) (schematically represented in Figure 1A). Mice were sacrificed at ~36-40 weeks of age when all mice had primary pancreatic tumors. However, when organs were evaluated for macroscopic lesions, sixty percent (8/13 mice) of the PDA.MUC1 mice developed lung metastasis with ~40% (5/13) developing liver and ~20% (3/13) developing peritoneum metastasis (Figure 1B). This was in contrast to PDA.Muc1KO mice where only 1 out of 10 mice developed metastasis in all three organs. To investigate the mechanism by which MUC1 initiated higher metastasis, we evaluated if MUC1 plays a role in activating the EMT-associated proteins in the primary pancreatic tumors. Tumor lysates from n=5 PDA.MUC1 and PDA.Muc1KO tumors were analyzed by proteomics analysis. Several proteins were differentially expressed; however, the most notable difference was in the levels of Vimentin and E-Cadherin. In the PDA.MUC1 tumors, there was a significant increase

(5-fold) in Vimentin with concurrent decrease in E-Cadherin (Figure 1C). In contrast, the pattern was completely reversed in the PDA.Muc1KO tumors with significantly higher (4-fold) E-Cadherin levels and lower Vimentin levels (Figure 1D). The data was the first indication of possible induction of EMT in the MUC1-expressing tumors. Repression of E-Cadherin and induction of Vimentin in the PDA.MUC1 tumors directly correlated with increased metastasis in the PDA.MUC1 mice.

MUC1 promotes induction of mesenchymal markers and functionally enhances the invasive capacity of pancreatic cancer cells *in vitro.*

To further confirm the role of MUC1 in EMT, cells were isolated from PDA.Muc1KO mice (designated as KCKO cell line) and PDA.MUC1 mice (designated as KCM cell line). Expression of MUC1 was confirmed by western blotting (Figure 1E) using two monoclonal antibodies, one recognizing the extracellular tandem repeat domain (MUC1 TR) and the other recognizing MUC1 CT domain. As was expected, there was high expression of MUC1 in the KCM cells and no expression of MUC1 in the KCKO cells (Figure 1E). Both cell lines were subjected to an *in vitro* invasion assay, results of which showed significantly higher invasion index of the KCM cells compared to the KCKO cells ($p<0.01$, Figure1F), confirming MUC1 as the major contributor of increased motility and invasiveness. Furthermore, presence of MUC1 in KCM cells clearly repressed E-Cadherin expression (Figure 1D) and induced expression of transcription factors and proteins associated with the mesenchymal phenotype. These included Snai1, Slug, N-cadherin, and Vimentin (Figure 1G), which potentially contributed to the contact inhibition and high invasion *in vitro* and *in vivo* (Figure 1F and 1B). Lastly, KCM cells expressed higher levels of vascular endothelial growth factor

(VEGF) compared to KCKO cells (Figure 1G), another indicator of highly metastatic phenotype. Thus, we hypothesized that MUC1 initiates EMT and induces invasion in mouse pancreatic cancer cells.

Over expression of MUC1 in human pancreatic cancer cells promotes EMT and induces invasion: Role of tyrosines in the MUC1 CT.

To further test our hypothesis and delineate the potential mechanism, we stably infected human pancreatic cancer cell lines, BxPC3 and Su86.86 with full length MUC1 or mutated MUC1 CT. Cells expressing full –length MUC1 was designated BxPC3.MUC1 and Su86.86.MUC1 while the ones expressing mutant MUC1 CT was designated BxPC3.Y0 and Su86.86.Y0. Cells infected with an empty vector containing the neomycin resistance gene were used as controls and designated BxPC3.Neo and Su86.86.Neo. Expression of MUC1 was confirmed in both cell lines by western blotting (Figure 2A –D) using both the MUC1 TR (B27.29) and MUC1 CT (CT2) antibodies. B27.29 antibody detects the high molecular weight fragment (>200 kD) and CT2 detects a low molecular weight fragment (<45kD). The small shift in electrophoretic mobility noted in the Y0 cell lysate relative to MUC1 cells likely reflects a decrease in phosphorylation due to the mutations of the tyrosine residues in the Y0 cells (Figure 2B and D). Several bands appear in the blot for the MUC1, which may represent differences in glycosylation or other post-translational modifications of this subunit.

An *in vitro* invasion assay showed significantly higher invasive index for the BxPC3 and Su86.86 cells expressing full length MUC1 as compared to the cells expressing Y0 or Neo ($p<0.01$, Figure 2E and F). These results suggest that the increased invasiveness of the MUC1-expressing cells may be attributed to the tyrosines in the MUC1 CT. To

substantiate the role of the MUC1 CT tyrosines as an inducer of EMT, real-time PCR arrays were used to explore transcription of 72 genes implicated in EMT and metastasis. In these experiments, only the BxPC3 cells were ustilized. Genes whose transcription was altered by at least 2-fold compared to Neo-cells were considered significant. Several genes associated with tight junctions and epithelial phenotype was significantly reduced in BxPC3-MUC1 cells compared to the Neo cells. These included Tight Junction Proteins 1 and 3, Occludin, Laminin α5, β-and δ-catenin, Claudin, and E-cadherin (Figure 2G). In contrast, there was no such change in gene expression in the Y0 cells (Figure 2G). Conversely, genes associated with the mesenchymal phenotype and invasion/migration was significantly increased in the BxPC3 MUC1 cells compared to Neo cells. These included Vimentin, Twist, Slug, Snai1, and Goosecoid, (Figure 2H). Once again, the Y0 cells had lower expression of the mesenchymal-associated genes (Figure 2H). Both functional and molecular studies indicate that MUC1 initiates the process of EMT via signaling through the tyrosines in its CT and that no external factor is necessary to stimulate these changes. It is rather interesting that while most of the epithelial cell-associated genes remained unchanged between Neo and Y0, E-Cadherin and Claudin were significantly higher in the Y0 versus the Neo cells. Likewise, several of the mesenchymal associated gene transcription including Twist, Snai1, Goosecoid, and Fibronectin were significantly lower in the Y0 cells compared to Neo cells, implications of which are not yet known.

Loss of epithelial marker and gain of mesenchymal markers in BxPC3-MUC1 but not in Y0 cells directly corresponds to their *in vitro* invasive potential and metastatic gene expression.

At the protein level, gain of mesenchymal markers such as Slug, Snai1 and Vimentin and loss of E-cadherin expression was apparent in the BxPC3 MUC1 cells by western blot analysis even though the lysates were prepared from a heterogeneous population consisting of both the migrating and non-migrating cells (Figure 3A and B). Downregulation of E-cadherin was further confirmed by confocal microscopy (Figure3C). This was not the case with the BxPC3 Y0 cells implicating the importance of the tyrosines in MUC1 CT for effective oncogenic signaling and induction of EMT. It is well established that Snai1 represses the expression of E-cadherin and induces EMT in several cancer cells (Batlle, Sancho et al. 2000; Cano, Perez-Moreno et al. 2000; Guaita, Puig et al. 2002). Thus, taken together, the data clearly suggests a role for MUC1 in inducing EMT in human pancreatic cancer cell lines via signaling through its cytoplasmic tail tyrosines. Furthermore, genes associated with metastasis and angiogenesis such as VEGF, MMP-9, 3, & 2 and IL-6R were significantly increased in BxPC3-MUC1 but not in Y0 cells compared to Neo controls (Figure 3D). This confirms that EMT-linked phenotype such as repressed E-cadherin and increased Vimentin, Snai1, and Slug are associated with increased metastasis-associated genes. Thus, without the tyrosines, EMT and metastasis is blocked in human pancreatic cancer cells.

Circulating tumor cells (CTCs) detected *in vivo* with BxPC3-MUC1 but not with Neo or Y0 tumors.

To test tumor growth and metastasis *in vivo*, BxPC3-MUC1, Y0 and Neo cells were subcutaneously injected into nude mice. All cells formed tumors within 1 month of injection and the tumor growth was significantly higher in BxPC3 MUC1 as compared to BxPC3 Y0 and BxPC3 Neo cells (Figure 4A (*P<0.001)). Tumors were dissected two-month post challenge and evaluated for presence of MUC1 by western blotting. MUC1 TR (B27.29) and CT (CT2) antibodies were used and as was expected, BxPC3 MUC1 and Y0 tumors expressed high levels of MUC1 while Neo tumors expressed negligible amounts of MUC1 (Figure 4B). Since we were unable to detect gross metastatic lesions in the nude mice because BxPC3 cells do not metastasize from subcutaneous tumors, we evaluated if there were CTCs in the blood of these mice. Within 2-weeks of culture, we were able to detect colonies of tumor cells in the blood collected from BxPC3-MUC1 but not from Neo or Y0 tumor-bearing mice (Figure 4C). The data signifies the role of MUC1 CT tyrosines during pancreatic cancer growth and extravagation of the cells into the blood stream for future metastasis.

Detection of EMT *in vivo* in BxPC3-MUC1 tumors

Protein lysates from the tumors were analyzed for the epithelial and mesenchymal markers. Similar to the *in vitro* data in Figure 3, the BxPC3 MUC1 tumor lysate showed downregulation of E-Cadherin (Figure 4D) and upregulation of Slug, Snai1, and Vimentin (Figure 4E). The data clearly indicates the initiation of EMT *in vivo* in only the MUC1-expressing BxPC3 tumors and this process was completely abrogated

when the tyrosines were mutated even though high levels of MUC1 protein was being expressed in the Y0 tumors (Figure 4B).

Significantly higher levels of pro-metastatic and pro-angiogenic growth factors in the BXPC3 MUC1 tumor microenvironment compared to Y0 or Neo tumors.

To determine the effect of EMT on the tumor microenvironment itself, we tested the protein levels of some of the known mediators of metastasis and angiogenesis in the tumor lysates using the Ray Biotech protein array kit. Significant increase in levels of circulating VEGF (10-fold), insulin-like growth factor-1 (IGF-1, 2-fold), interleukin-6 (IL-6, 3-fold) and its receptor (IL-6R, 2.2-fold), stem cell factor (SCF, 2.5 fold), P-selectin (20-fold), and epidermal growth factor (EGF, 2.8-fold) were observed in the BxPC3-MUC1 tumors (Figure 4F) compared to the Neo and Y0 tumors. VEGF expression was also determined by IHC in the tumor sections with high levels detected in the BxPC3 MUC1 tumors versus Neo and Y0 tumors (Figure 4G).These results are novel and speak to the critical signaling role of the tyrosines within the MUC1 CT during oncogenesis. It is striking that simply abrogating the signal transduction events by mutating the seven tyrosines can completely reverse the process of EMT and significantly alter the tumor microenvironment, subsequently leading to a less aggressive, less metastatic, and less angiogenic phenotype. Lysates from n=3 mice were tested and average fold-increase is shown (Figure 4F). Two-fold or more increase was considered significant and is represented. Thus, BxPC3 MUC1 xenografted tumors exhibited an aggressive phenotype consistent with the human tumors that express high MUC1. This included loss of E-cadherin, induction of Vimentin and upregulation of metastatic and

angiogenic factors. The loss of tyrosine residues in MUC1 CT completely abrogated the entire process of EMT and metastasis.

MUC1 interacts with β-catenin and translocates to the nucleus in the BxPC3-MUC1 cells.

To delineate the underlying mechanism by which the Y0 tumors circumvent EMT, we tested the nuclear localization of MUC1 CT and β-catenin in the MUC1 and Y0 cells. It is known that tyrosine residues in MUC1 CT are critically important for nuclear localization of MUC1 CT. Previous reports have demonstrated that MUC1 CT tyrosine phosphorylation is required to support interaction of MUC1 with β-catenin which is necessary for intracellular trafficking (Hollingsworth and Swanson 2004) and possibly EMT. Thus, nuclear extracts from BxPC3 MUC1 and Y0 cells were analyzed for presence of MUC1 and β-catenin. Although β-catenin was present in the nuclear extracts in both cells, the levels were significantly higher in BxPC3 MUC1 cells compared to the Y0 cells (Figure 5A). Similar results was obtained with the KCM and KCKO cells where higher levels of β-catenin were detected in the nuclear extract of the KCM versus the KCKO cells (Figure 4B). More importantly, although both BxPC3 MUC1 and Y0 cells contained equal levels of MUC1, there was no detectable MUC1 CT in the nuclear extract of the Y0 cells while high levels was detected in the BxPC3 MUC1 cells (Figure 5A). The results were substantiated in Su86.86 MUC1 and Y0 cells (Figure 5C). The data clearly suggests that signaling through the tyrosines is critically important for efficient nuclear translocation of MUC1 CT and β-catenin. Purity of the nuclear extract was confirmed by the presence of lamin and the absence of IKK (Figure 5A-C).

Since tyrosine phosphorylation is also required for MUC1 to bind β-catenin and translocate to the nucleus, we examined if β-catenin and MUC1 co-IP in these cells. β-catenin pulled down MUC1 only in the BxPC3-MUC1 cells but not in the Y0 cells (Figure 5D). Similarly, MUC1 pulled down β-catenin more effectively in the BxPC3-MUC1 cells as compared to Y0 cells. Non-specific pull down was not detected using an IgG control antibody (data not shown). The co-IP of β-catenin and MUC1 was confirmed in the KCM and KCKO cells and in the Su86.86 cells (Figure 5E and F). Thus, we confirm that binding of MUC1 to β-catenin appears to provide the signal required for movement of MUC1 to the nucleus and that the tyrosines in MUC1 CT have to be functional for the two proteins to interact. *Note: Neo cells were not included in these experiments as they express minimal levels of endogenous MUC1.*

3.5 Discussion

Although it is known for decades that MUC1 is aberrantly over-expressed in greater than 65% of PDA and is associated with poor prognosis and metastasis, its precise role has remained obscure. We show for the first time in both mouse and human pancreatic tumors that 1) over-expression of MUC1 initiates the process of EMT and augments metastasis and 2) lack of tyrosines in the CT of MUC1 abrogates this process. The first evidence of EMT came from the proteomics data showing repression of E-Cadherin and induction of Vimentin expression in the PDA.MUC1 tumors when compared to the PDA.Muc1KO tumors. This correlated with significantly higher incidence of secondary metastasis in PDA.MUC1 mice as compared to the PDA.Muc1KO mice. The PDA mice are unique in that the pancreatic tumors arise spontaneously in an appropriate tissue background, within a suitable stromal and

hormonal milieu, and in the context of a viable immune system (Hingorani 2003; Hingorani, Petricoin III et al. 2003; Tinder, Subramani et al. 2008; Mukherjee, Basu et al. 2009). The tumor progression and the histopathology of the tumors in the PDA mice mimic the human disease with the initial development of pancreatic intra-epithelial neoplastic (PanIN) lesions progressing to carcinoma-in-situ (CIS), and invasive adenocarcinoma. The process of EMT and increased invasiveness in MUC1-expressing pancreatic tumors was confirmed in cell lines generated from the PDA mice and validated in two human pancreatic cell lines. The underlying molecular mechanism in all cell lines pointed to the induction of transcription factors, Snai1, and Slug which stimulated the expression of N-Cadherin and Vimentin and repressed E-Cadherin expression. E-Cadherin plays a key role in the establishment and maintenance of adherent junctions and loss of the same results in contact inhibition and cell motility. Interestingly, the molecular process of EMT was completely abrogated when all seven tyrosines in the CT of MUC1 were mutated to phenylalanine. Recent evidence shows that the phosphorylation of the tyrosines in the MUC1 CT are critical for the binding of β-catenin to MUC1 CT and that the MUC1 CT-β-catenin complex can be tranlocated to the nucleus to render its oncogenic signal (Wen, Caffrey et al. 2003). We show that lack of tyrosines in the MUC1 CT regulates the complexing of MUC1 CT with β-catenin and therefore its efficient translocation to the nucleus (Figure 5). It is also known that β-catenin binds to the SXXXXXSSL motif in the CT of MUC1 (Yamamoto, Bharti et al. 1997) and changes in tyrosine phosphorylation of MUC1 CT correlate with differences in cell adhesion (Yamamoto, Bharti et al. 1997; Quin and McGuckin 2000). Thus, we can speculate that the underlying mechanism for EMT might be the requirement of the tyrosines adjacent to

the β-catenin binding domain (possibly at the TDRSPYEKV site) to be phosphorylated. C-src and PKCδ have been shown to phosphorylate the tyrosines at that site and increase the interaction between β-catenin and MUC1 (Li and Kufe 2001; Ren, Li et al. 2002). We therefore propose that MUC1 may directly influence the transcriptional co-activator status of β-catenin, and up-regulate several genes that are associated with EMT such as Snai1, Slug, Twist, Vimentin and Goosecoid (Figure 2D) possibly through interactions with TCF/LEF1 and/or other transcription factors. Snai1 in turn represses E-cadherin message and protein levels in MUC1-expressing cells (Figures 1-4), perhaps through direct binding to the three E-boxes in the E-cadherin promoter (Guaita, Puig et al. 2002). E-cadherin plays a key role in the establishment and maintenance of adherent junctions, repression of which leads to down regulation of Tight Junction Proteins 1 and 3, Occludin, Laminin α5, and β-and δ-catenins (Figure 2C). Dissolution of pancreatic epithelial cell junctions is hence brought about by a concerted activation of mesenchymal proteins and repression of epithelial proteins *in vitro* and *in vivo* leading to EMT and metastasis exemplified by the presence of CTCs in BxPC3.MUC1 tumors (Figure 4C) and enhanced metastasis in the PDA.MUC1 mice (Figure 1B).

These molecular changes lead to an *in vivo* microenvironment in the MUC1 tumors favoring increased angiogenesis, and pro-inflammatory cytokines (Figure 4F and G), leading to increased metastasis. The glycoprotein P-selectin, an adhesion molecule involved in the initiation of the inflammatory process and induction of other pro-inflammatory cytokines such as IL-6 and its receptors (Kaikita, Ogawa et al. 1995; Dymicka-Piekarska, Matowicka-Karna et al. 2007) is increased by 20 fold in MUC1 tumors versus the Neo tumors (Figure 4F). In addition, link between IL-6R and

angiogenic cytokines such as VEGF has been shown in several cancers (Alexandrakis, Passam et al. 2003), with VEGF being a potent inducer of angiogenesis and promoter of tumor progression (Justinger, Schluter et al. 2008). VEGF is increased by 10-fold in the MUC1 tumors versus the Neo tumors (Figure 4F). Like P-selectin, SCF is also a member of a group of glycoprotein growth factors designated hematopoietic cytokines (HCs) known to affect the growth of pancreatic cancer (Mroczko, Szmitkowski et al. 2005). SCF is increased in the MUC1 tumors by 2.5 fold compared to Neo tumors (Figure 4F). Finally, IGF-I and EGF signaling induces growth and proliferation in pancreatic cancer cells (Bardeesy and DePinho 2002) (Wolpin, Michaud et al. 2007), and is increased by 2 and 3 fold respectively in MUC1 versus the Neo tumors (Figure 4F). Importantly, simultaneous targeting of both IGF-1 and EGF receptors has lead to enhanced antitumor activity (Lu, Zhang et al. 2005). Thus, it is indeed striking that the significant induction of all the above factors in the MUC1 tumors versus the Neo tumors was completely abrogated when the tyrosines in the MUC1 CT was deemed non-functional in the Y0 tumors (Figure 4F). This once again signifies the critical requirement of the MUC1 CT tyrosines in pancreatic cancer oncogenesis and metastasis.

The regulation of MUC1 CT phosphorylation and parameters that affect its association with β-catenin are not fully understood, although interaction of MUC1 with ErbB1 (Schroeder, Thompson et al. 2001) has been shown to enhance the binding between c-src, β-catenin and MUC1 (Hollingsworth and Swanson 2004) and increase ERK1/2 phosphorylation and NFκB activation (Schroeder, Thompson et al. 2001; Thompson, Shanmugam et al. 2006). Preliminary data from our laboratory shows that the active form of NFκB (p65 subunit) is significantly lower in Y0 cells compared to the

MUC1-expressing BxPC3 and Su86.86 cells (unpublished data). It has also been reported that phosphorylation of MUC1 CT by src, ErbBs, and PKCδ regulates its association with β-catenin and GSK-3β (Li, Kuwahara et al. 2001; Li, Ren et al. 2001; Ren, Li et al. 2002; Kohlgraf, Gawron et al. 2003). Another critical study demonstrates that met-mediated phosphorylation of MUC1 modulates signaling related to motility and invasion in pancreatic cancer (Singh, Behrens et al. 2008). Our study exemplifies the functional consequences (EMT and metastasis) of signaling through MUC1 CT, and for the first time delineates the role of MUC1 in a unique mouse model of PDA that lacks Muc1.

This study defines the role of MUC1 signaling (through its CT tyrosines) in the initiation of EMT perhaps by β-catenin-MUC1 interaction and translocation to the nucleus, leading to activation of the EMT-associated transcription factors Snai1 and Slug. This in turn enables the tumors to acquire a highly aggressive phenotype creating a pro-metastatic microenevironment *in vivo.* Future studies will focus on identifying the particular tyrosine/s within MUC1 CT and determining if inhibiting its phosphorylation using small molecule kinase inhibitors may lead to a novel treatment modality for pancreatic cancer.

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3.6 Figures

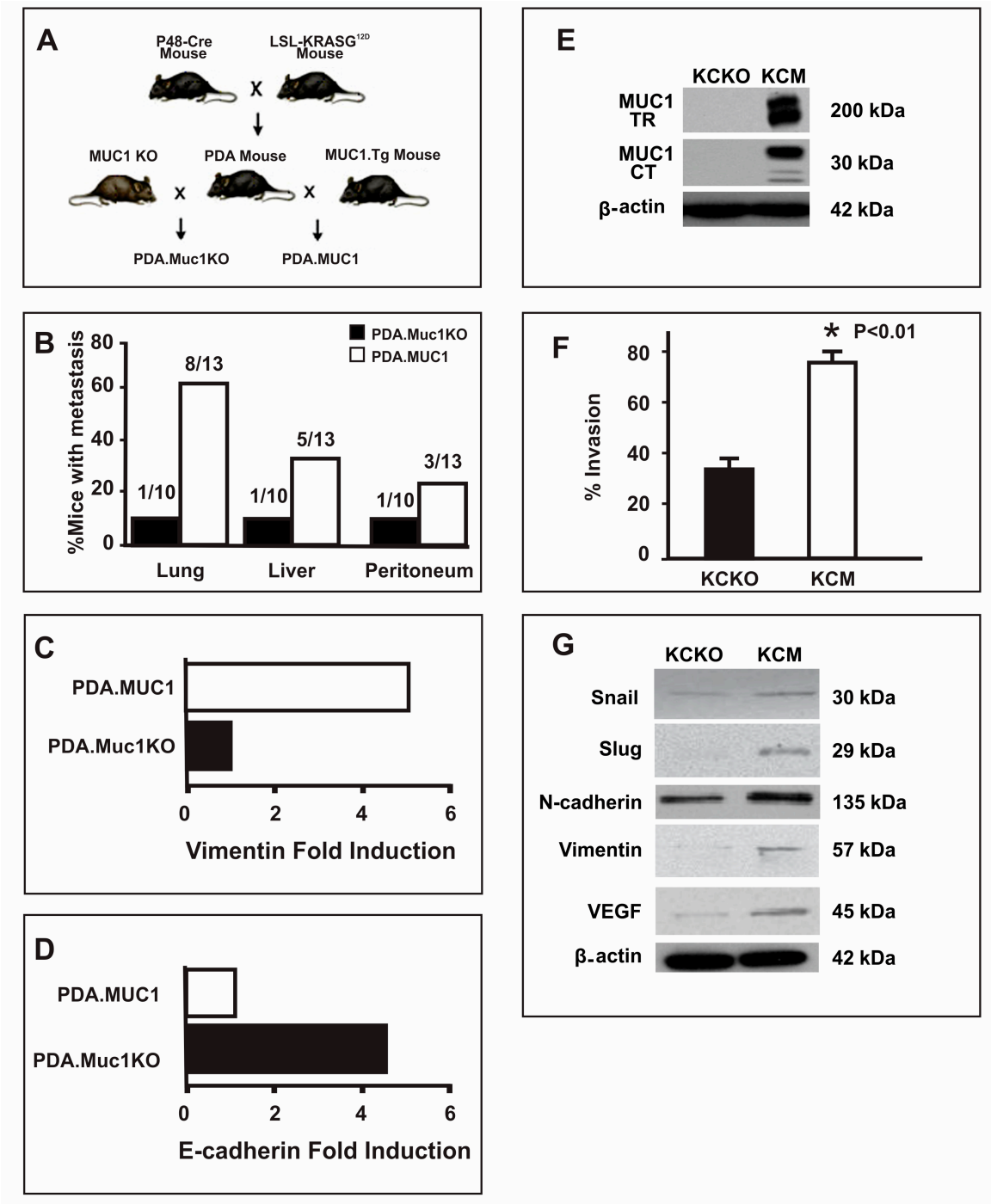


Figure 1: PDA mice lacking Muc1 has significantly lower incidence of secondary metastasis associated with decreased induction of EMT proteins in the tumors.

A. Schematic representation of PDA, PDA.MUC1 and PDA.Muc1KO mice. PDA mice were mated to the human MUC1.Tg and mouse Muc1 KO mice to get PDA.MUC1 mice and PDA.MUC1 KO mice respectively. **B.** Percent mice that developed metastasis in PDA.MUC1 versus PDA.MUC1 KO mice. **C and D.** Proteomics data on PDA.MUC1 and PDA.Muc1 KO tumor lysates showing E-cadherin repression and Vimentin upregulation. **E.** Western blot analysis of MUC1 expression for KCM and KCKO cells using MUC1 TR and MUC1 CT monoclonal antibodies. **F.** Transwell invasion assay showing significantly higher invasion index for KCM cells compared to KCKO cells (*P<0.001). **G.** Gain of mesenchymal proteins in KCM cells versus KCKO cells. β-actin serves as control for equal protein loading.

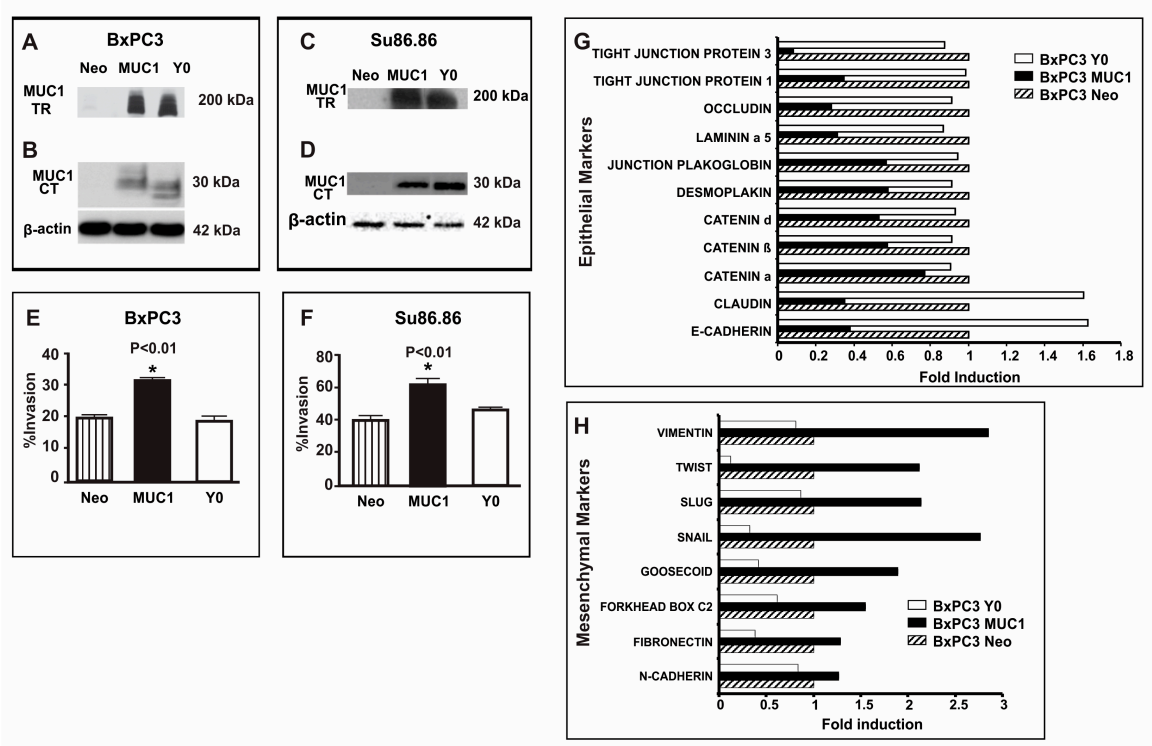


Figure 2: Significantly higher invasion and transcription of genes associated with a mesenchymal phenotype coupled with significantly lower transcription of genes associated with an epithelial phenotype in MUC1 over-expressing human pancreatic cancer cells as compared to control cells. Complete reversal in the MUC1.Y0 expressing cells.C1 expression by western blot analysis: **A.** MUC1 TR and **B.** MUC1 CT staining of BxPC3 Neo, MUC1, and Y0; **C.** MUC1 TR and **D.** MUC1 CT staining of Su86.86 Neo, MUC1, and Y0. >200kDa represents MUC1 TR domain and <45kD represents MUC1 CT domain. β-actin was used as loading control. **E - F.** *In vitro* trans-well invasion assay for BxPC3 and Su86.86 cells respectively; Compared to Neo and Y0 cells, MUC1-expressing cells have significantly higher invasion index (* p<0.001). **G - H.** RT-PCR analysis of BxPC3 Neo, MUC1, and Y0 cells: **G.** Transcription of genes generally associated with epithelial phenotype. **H.** Transcription of genes generally associated with mesenchymal phenotype. Genes whose transcription was altered by at least 2-fold or

more were considered significant. Average fold change is shown from three separate experiments. All experiments were repeated 3-5 times. Clones from three independent infections were analyzed with similar results.

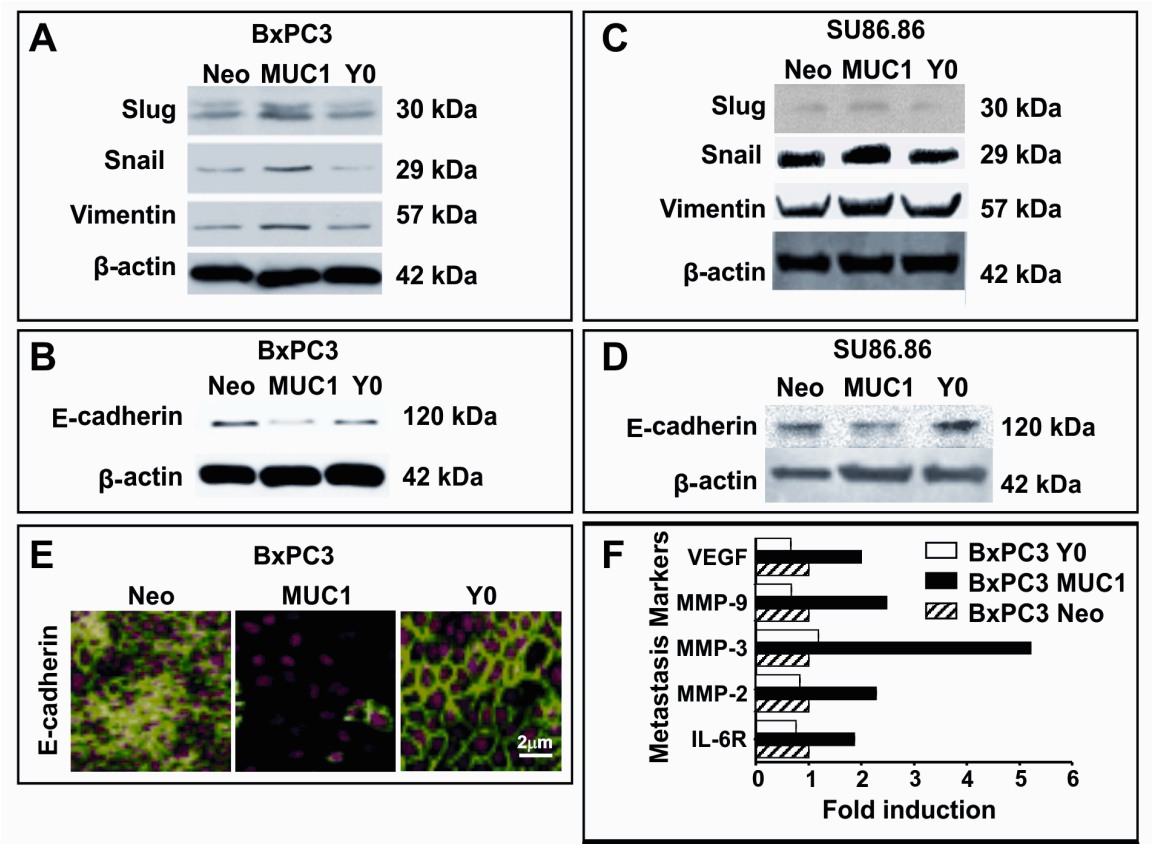


Figure 3: Repression of E-Cadherin and induction of Snai1, Slug, and Vimentin in BxPC3 MUC1 cells coupled with significantly increased transcription of genes associated with metastasis. Complete reversal in MUC1-Y0 expressing cells. **A and B.** Western blotting analysis of Slug, Snai1, Vimentin, and E-Cadherin expression in BxPC3 Neo, MUC1, and Y0 cells. **C.** Immunofluorescence staining and confocal microscopic image of E-Cadherin levels in BxPC3 Neo, MUC1 and Y0 cells. Images were taken at 400X magnification. The experiments were repeated 3 times with three separate clones with similar results. β-actin was used as loading control. **D.** RT-PCR analysis of genes generally associated with metastasis and angiogenesis. 2-fold or more difference was considered significant. Average fold change is shown from three separate experiments.

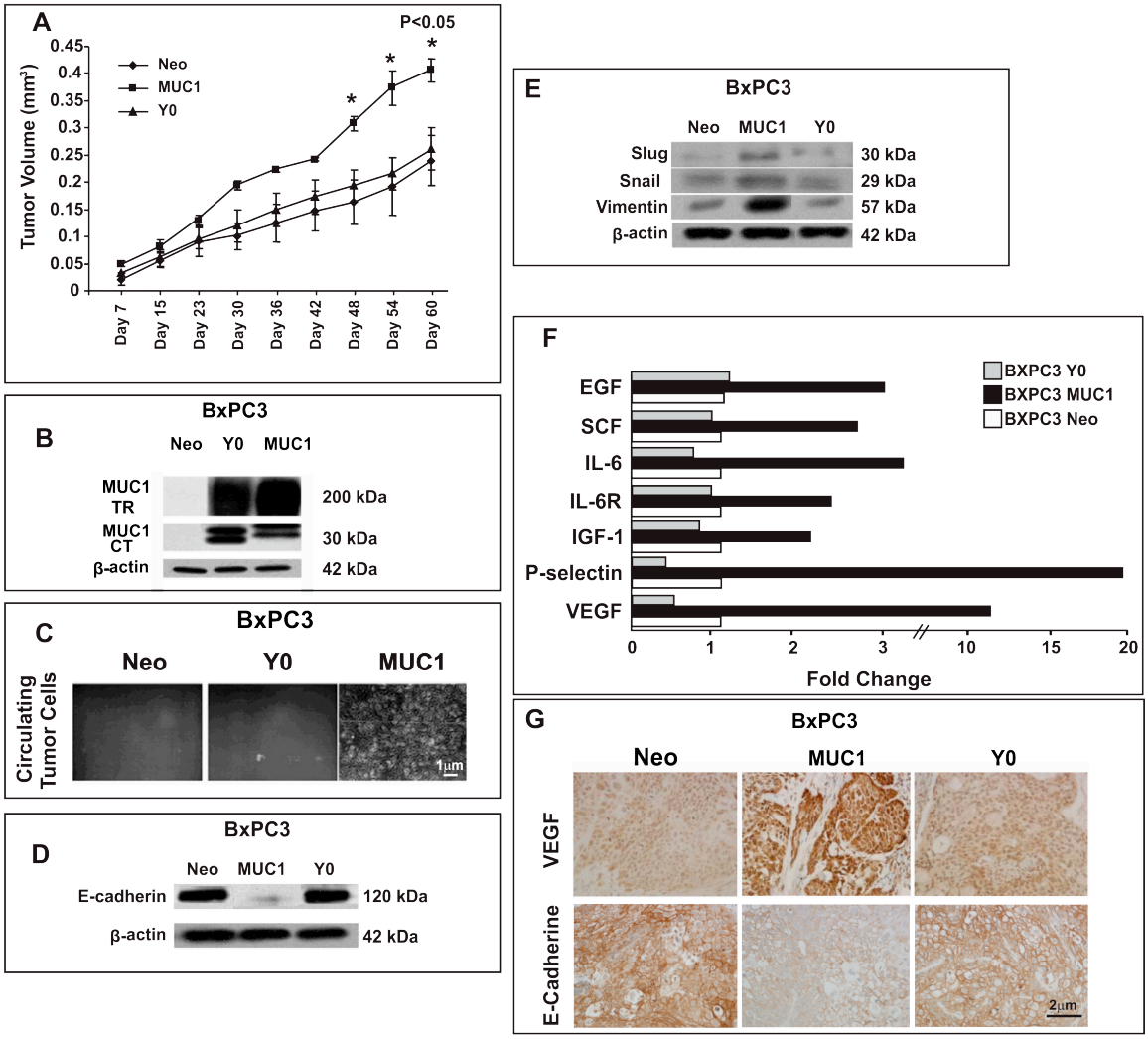


Figure 4: Higher tumor burden with loss of E-Cadherin and gain of mesenchymal and metastatic proteins in BxPC3 MUC1 versus Y0 and Neo tumors.
A. *In vivo* tumor growth in nude mice. Significantly higher tumor burden in BxPC3. MUC1 versus Neo and Y0 tumors (*p < 0.001). **B.** MUC1 expression by western blotting using the MUC1 TR and CT antibodies **C.** Tumor cells cultured from whole blood of tumor-bearing mice. Circulating tumor cells detected in the blood of mice bearing the BxPC3 MUC1 but not BxPC3 Y0 or Neo tumors. **D.** *E*xpression of E-Cadherin in primary tumors by western blotting. **E.** *E*xpression of Slug, Snai1, and Vimentin in primary tumors by western blotting. **F.** High levels of pro-metastatic and pro-angiogenic proteins detected in the BXPC3 MUC1 tumor lysate. Fold change in levels of various factors in BxPC3 MUC1 tumor lysate compared to Y0 and Neo**, G.** Expression of VEGF by IHC. Images were taken at 400X magnification. Similar results with n=3 mice were obtained.

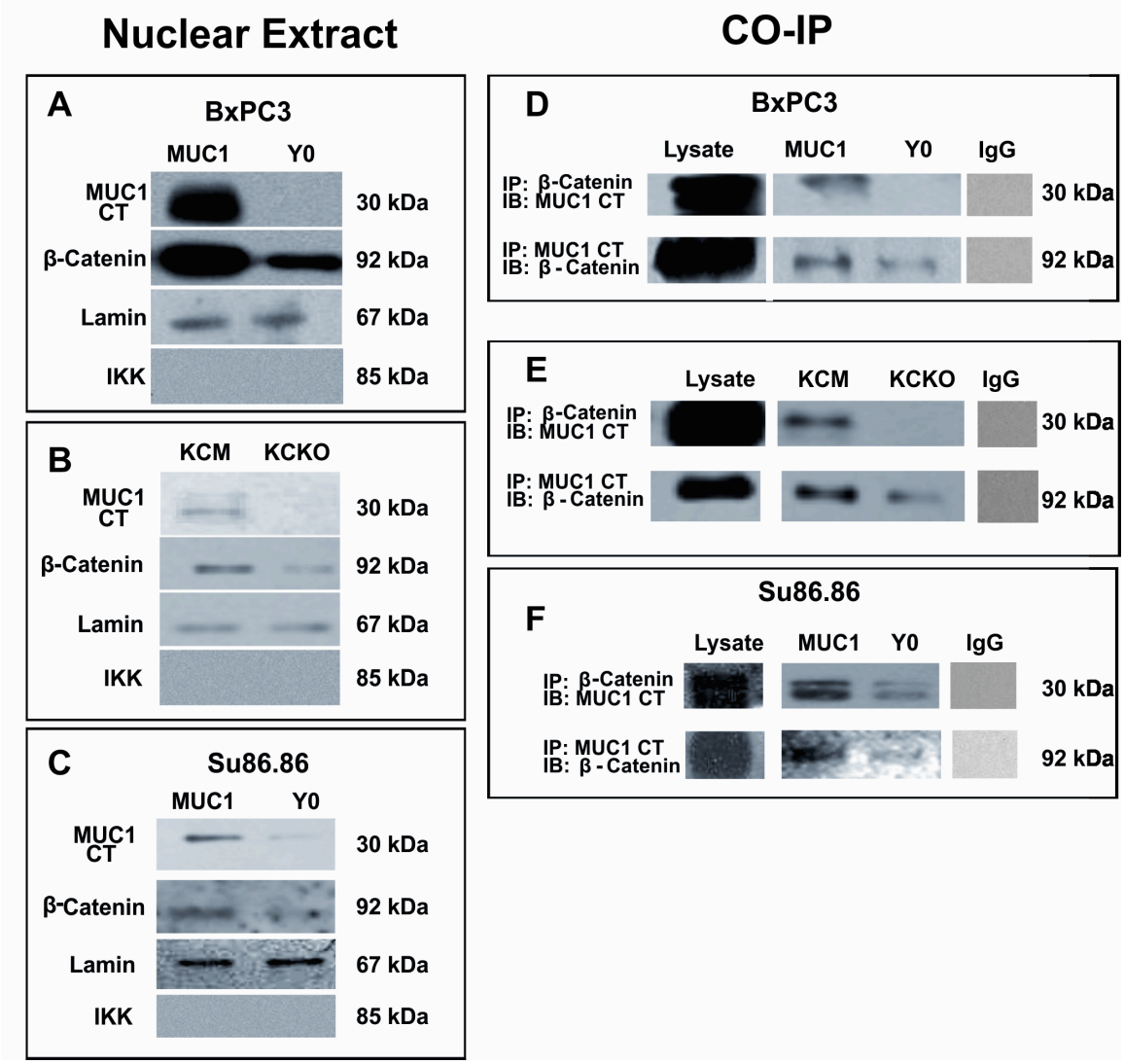


Figure 5: MUC1 interacts with β-catenin and translocates to the nucleus in the MUC1-expressing cells. Complete reversal in the Y0 cells.
Protein expression of MUC1 CT and β-catenin in the nuclear extracts of **A.** BxPC3 MUC1 and Y0**; B.** KCM and KCKO and **C.** Su86.86 MUC1 and Y0 cells. Lamin and IKK used as positive and negative control for nuclear extracts. Co-IP of MUC1 CT and β-catenin in both directions from **D.** BxPC3 MUC1 and Y0**; E.** KCM and KCKO and **F.** Su86.86 MUC1 and Y0 cells.

CHAPTER 4: MDSCS

4.1 Myeloid Derived Suppressor Cells (MDSC)

Tumor associated immune suppression: In recent years, new developments have been raised in the concept of cancer treatment in which the immune system is recruited in order to clear the transformed tumor cells. Studies have shown that cancer patients are capable of inducing expansion of tumor reactive $CD4^+$ and $CD8^+$ T-cells in their lymph nodes and peripheral blood in response to tumor associated antigens (Schmitz-Winnenthal FH 2005; Schmitz-Winnenthal FH 2006). Infiltration of lymphocytes to the site of tumor progression can be associated with a better prognosis in cancers such as breast, colon, esophageal, and ovarian cancer. (Yoshimoto M 1993; Naito Y 1998; Reichert TE 1998; Yasunaga M 2000; Strater J 2005; Sato E 2005).

However, presence of tumor infiltrating lymphocytes does not clear the tumor mass. In fact, the tumor microenvironment has been shown to be a site of ongoing inflammation and immune suppression. Presence of inflammatory cytokines and immune suppressive cells in the tumor microenvironment is well established (as reviewed in (Gordon S 2010)). It is believed that expression of some of the tumor derived soluble factors and inductions of regulatory cells are one of the major means by which tumor cells escape the anti-tumor immune response. Due to these factors, the elicited immune response whether through targeted immune therapy or through natural processes, is not

protective. Successful translation of immune therapy into a treatment option is dependent on reversal of the suppression of anti tumor immune response.

4.2 Functions of MDSCs

Myeloid Derived Suppressor Cells (MDSCs) were identified over 20 years ago when Young et al reported that bone marrow cells from tumor bearing mice can inhibit T-cell clonal activation (Young 1987). MDSCs are a heterogeneous population of cells which have originated from the myeloid cells in the bone marrow. Tumors can aggressively draft a network of regulatory cells such as MDSCs to weaken the anti tumor immune response in a local and systemic fashion. MDSCs are immature cells with a significant ability to repress T-cell responses. These cells expand under pathological conditions such as cancer progression, infection, sepsis, and trauma.

In the bone marrow of healthy individuals, common myeloid progenitor cells quickly differentiate into dendritic cells (DCs), Macrophages, or granulocytes. During cancer progression, cancer cells, activated T-cells and stromal cells express factors such as Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) (Obermueller E 2004), IL-6 (Trikha M 2003), Stem Cell Factor (SCF) (Melani C 2003), IL-1β, Prostaglandin E2 (PGE-2), and Vascular Endothelial Growth Factor (VEGF) (Bunt SK 2006; Tinder 2008). These factors and cytokines are known to influence hematopoiesis (Obermueller E 2004) and prevent maturation and differentiation of common myeloid progenitor cells into their destined cell type. This results in expansion of immature myeloid cells which under the influence of tumor derived factors gain suppressive ability and are eventually known as MDSCs (Figure 1).

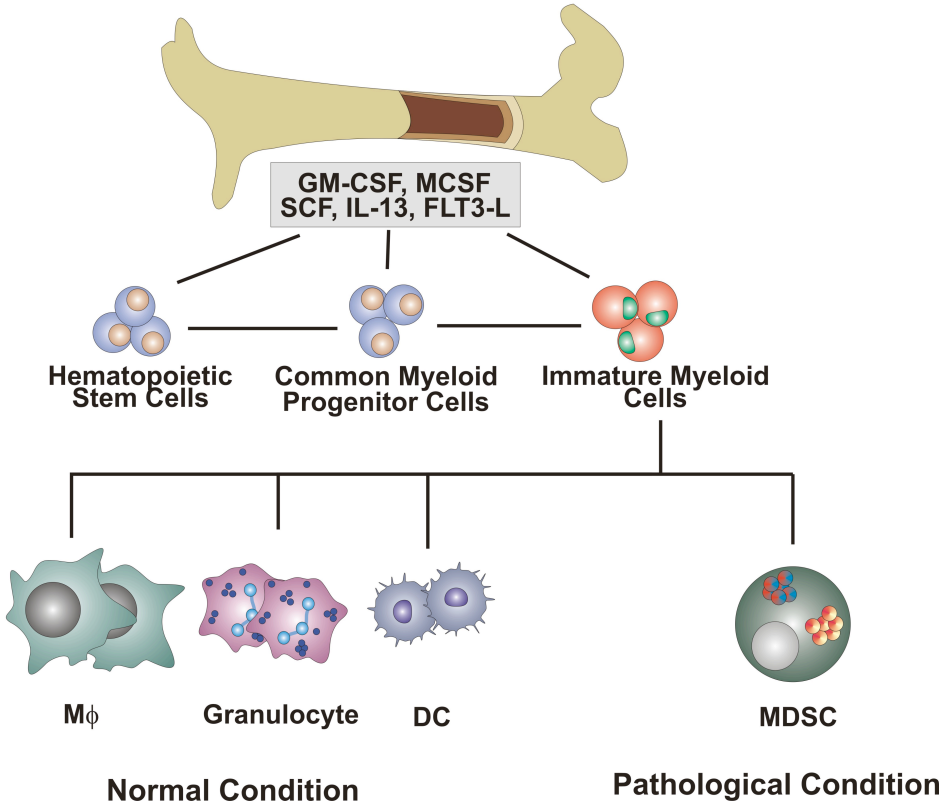


Figure1. MDSC expansion. Under pathological conditions, altered hematopoiesis results in decreased differentiation of myeloid cells and causes expansion of MDSCs.

Both in humans and mice accumulation of MDSCs has been observed in the bone marrow, spleen, blood, draining lymph nodes and tumor (Almand B 2001; Mirza N 2006; Ochoa AC 2007; Tinder 2008; Diaz-Montero CM 2009) . In addition, MDSCs are invariably associated with metastasis (Diaz-Montero CM 2009). Taken altogether, this has led scientists to link the presence of activated MDSCs to cancer-associated immune suppression and increased metastasis.

As mentioned, MDSCs are a heterogeneous population of cells and in mice are defined as cells which co-express Gr1 and CD11b (Gabrilovich DI 2007). The antibody which recognizes Gr1 can in fact recognize two different epitopes: Ly6C and Ly6G.

Phenotypically, MDSCs are divided into the granulocytic ($CD11b^{+}LY6G^{+}LY6C^{low}$) and the monocytic ($CD11b^{+}LY6G^{-}LY6C^{high}$) subsets (Hestdal K 1991; Youn 2008) which are proposed to have different functions during cancer and infection (Dietlin ta 2007; Zhu B 2007; Movahedi K 2008). A small portion of MDSCs are shown to form myeloid colonies and about 30% can differentiate into DCs and macrophages (Bronte 2000).

4.3 MDSC expansion

Many pathological conditions can induce MDSC expansion. Bacterial and parasitical infections as well as polymicrobial sepsis induce increased MDSC levels. Several studies have focused on identifying factors which can provoke MDSC expansion. It appears that activated immune system is one of the major factors which can cause these cells to expand. Such expansion is always associated with altered hematopoiesis. Type, tissue, and origin of the tumor can influence MDSC expansion. Below, we are going to introduce and briefly explain some of the prominent factors which are known to either alter hematopoiesis and cause MDSC expansion, or can act directly on MDSCs and activate them during tumor development. These factors can originate from the tumor cells, stromal cells, or activated T-cells.

- **GM-CSF**: Several groups have shown that high doses of Granulocyte-Macrophage Colony Stimulation Factor (GM-CSF) administration induce transient post-vaccine suppressive MDSCs and fail to induce anti tumor immunity in tumor bearing mice (Bronte 2000). GM-CSF dependent MDSC induction leads to both CD4 and CD8+ T-cell suppression which hampers post vaccine immune response (Fu 1990; Young 1991; Bronte V 1999).

- **G-CSF**: Tumor infiltrating MDSCs have both macrophage and neutrophilic phenotype. Lack of Chemokine Receptor-2 (CCR2) causes conversion of the macrophage phenotype to the neutrophile phenotype, indicating that excessive production of Granulocyte-Colony Stimulation Factor (G-CSF) causes induction of MDSCs with a neutrophilic characteristic (Sawanobori, Ueha et al. 2008).
- **M-CSF**: Macrophage Colony Stimulation Factor (M-CSF) expression by of tumor cells influences DC maturation and instead induces MDSC expansion (Cheng 2008).
- **Prostaglandins**: MDSCs express receptors for prostaglandins. EP2 deficient mice have been shown to have delayed tumor growth which is associated with decreased MDSC accumulation. Moreover, treatment of tumor bearing mice with a Cox-2 inhibitor resulted in reduced tumor burden associated with decreased MDSC levels indicating a significant role for Prostaglandins in MDSC expansion (Sinha 2007). Furthermore, Cox-2 mediated PGE-2 production induces T-cell suppressive activity in MDSCs, leading to a more immunosuppressive tumor microenvironment (Rodriguez 2005).
- **IFN-γ**: Interferon gamma (IFN-γ) expression during cancer induces suppressive activity of MDSCs as well as its expansion (Gallina G 2006). IFN-γ and IL-3 together can activate the metabolic pathways which lead to activation of MDSC suppressive function.
- **C5a:** Complement proteins are found on tumor tissue suggesting local complement activation. MDSCs are known to express Complement component -5a (C5a) receptors (Guo 2005) and accumulation and/or migration of MDSCs is

found to be regulated by complement components such as C5a (Markiewski 2008).

- **SCF:** One of the functions of MDSCs is induction of T-cell anergy. Blocking SCF function has been shown to reverse T-cell anergy. Further, blockage of SCF enhances tumor vaccine therapeutic function. Indeed, studies have revealed that SCF inhibition causes decreased MDSC function and suppressive outcome which can account for increased effectiveness of anti tumor immune response (Pan PY 2008).
- **S100A8 and S100A9**: Under pathological conditions, calcium binding proteins like S100A9 are shown to be required for MDSC generation (Cheng 2008). S100A9 regulates suppressive activity of MDSCs via ROS. MDSCs also synthesize S100A8/9 while express receptors for S100 proteins to which they chemotact (Sinha, Okoro et al. 2008).
- **TGF-β:** While transforming growth factor-beta (TGF-β) is known to downregulate antitumor immunesurveillance, CD1d restricted T-cells induce IL-13 production which in turn increases TGF-β expression and induce immunosuppression via MDSCs in tumor bearing mice (Terabe 2003).
- **IL-1β**: Tumor cells express substantial amounts of IL-1β. This inflammatory cytokine is known to both increase tumor burden and decrease survival time. This phenomena is associated with rapid induction of highly suppressive MDSCs (Bunt, Sinha et al. 2006). The effects of IL-1β on expansion of MDSCs are very strong and in fact, removal of the primary source of IL-1β (tumors) does not reverse levels of MDSCs.

- **IL-6**: According to epidemiologic studies, inflammation and especially chronic inflammation has long been associated with cancer initiation and progression. IL-6, a well known inflammatory factor which is an IL-1β downstream cytokine, can alter MDSC expansion and function and consequently tumor progression (Bunt, Yang et al. 2007). In lack of IL-1β, IL-6 can fully restore MDSC expansion and function under tumor progression.
- **IL-10**: IL-10, a known Th2 cytokine with strong immunosuppressive function has been shown to induce MDSCs. IL-10 production during cancer progression induces MDSC function which causes T-regulatory cell suppressive activity (Huang B 2006).
- **IL-13**: Perhaps one of the most important Factors which induces MDSC expansion and suppressive function is IL-13. While the source of IL-13 during cancer progression is a subject of debate, its role in MDSC induction is well established. MDSCs can respond to IL-13 in a both autocrine and paracrine manner and significantly suppress CTL generation and contribute to tumor progression (Gallina G 2006).

These factors can alter signaling pathways responsible for differentiation and cell survival during hematopoiesis. STAT-3 signaling pathway which causes cell proliferation and survival is one of the first pathways which gets activated in myeloid progenitor cells under the influence of TDSFs and causes MDSC expansion (Bromberg 2002). STAT3 activation causes increased levels of CyclinD1 and MYC expression to induce survival and proliferation and prevent maturation of myeloid progenitor cells (Nefedova 2004).

4.4 MDSC suppressive activities

Expansion of MDSCs is carefully regulated by the body. In fact, increased levels of MDSCs do not always mean immunosuppression. Induction of MDSC activity is another regulatory step taken by the immune system to ensure proper immune responses during infection, cancer, or trauma. Several studies have shown that along with MDSC expansion, TDSFs can induce MDSC activation to cause its suppressive function. Activated T-cells in patients suffering from infection or cancer produce high levels of IFN-γ which causes MDSC activation. Aside from inflammatory cytokines, MDSCs can sense stress and become suppressive.

TLRs are a family of proteins which are expressed on MDSCs and are known to regulate MDSC activity. TLR's role in innate immune response and pathogen perception is very well studied. During cancer, tumor cells shed exosomes or undergo apoptosis which cause Heat Shock Proteins (HSP) containing membrane bound cell parts to be shed to the bloodstream and reach MDSCs to stimulate TLR signaling (Delano 2007). TLR activity whether through interaction with microbial parts or its endogenous ligands such as heat shock proteins (HSPs) has been linked to MDSC suppressive activity and expansion in the spleen (Van Rompaey and Le Moine 2011; Zoglmeier, Bauer et al. 2011). Lack of MyD88, the adaptor for TLR, abrogates MDSC expansion indicating the regulatory role of TLR signaling in MDSC activity (Delano 2007). Furthermore, TLR activity in MDSCs induces IL-4, IL-13, or TGF-β expression. MyD88 signaling and cytokines induce signaling pathways which leads to NF-κB activity in MDSCs and ultimately causes immune suppression (Delano 2007).

MDSC suppressive mechanisms: MDSCs have been shown to exert their suppressive ability through both cell-cell contact dependent and independent manner. These cells use a variety of mechanisms to induce immune suppression via decreasing T-cell functionality. Various mechanisms have been described which include altering a) metabolism of amino acids necessary for T-cell function, b) T-cell receptor functionality, c) ROS production, and d) NK cell activity. These mechanisms are briefly explained below:

- **Arginase-1 expression**: Arginase-1 is an enzyme which metabolizes L-arginine and converts it to urea and L-ornithine. Arginine is a conditionally necessary amino acid for humans. T-cell functionality and T-cell proliferation depends on availability if this amino acid (Rodriguez 2005). Exposure to Th2 cytokines such as IL-13, IL-4 (Munder 1998; Munder 1999), TGF-β (Boutard 1995), or GM-CSF (Jost 2003) cause MDSCs to express high levels of this enzyme which catabolizes L-arginine and causes decreased Arginine availability in the tumor microenvironment or the lymph node. Lack of L-arginine causes decreased CD3ζ expression on T-cells (Rodriguez PC 2002). CD3ζ is the key signal transducer element of the TCR and its proper function is crucial for proper T-cell activation. Further, following TCR stimulation, T-cells undergo clonal expansion and without L-arginine in the microenvironment, CyclinD3 and cyclin-dependent kinase-4 are not expressed. Therefore, due to expression of high levels of Arginase-1 by MDSCs, T-cell proliferation is strongly reduced in the tumor microenvironment (as reviewed in (Gabrilovich DI 2009).

- **NO production**: Nitric Oxide Synthase (NOS) family of proteins uses L-arginine as a substrate for its enzymatic activity. MDSCs are known to express high levels of iNOS which causes T-cell suppression through reduced Arginine levels (Rodríguez PC 2008). Moreover, activation of JAK1 and 3 pathways as well as ERK in T-cells is blocked in presence of NO due to iNOS activity (Bingisser RM 1998). Furthermore, exposure of T-cells to NO causes T-cell apoptosis through Caspase independent pathway (Macphail 2003). Taken together, this means iNOS expression by MDSCs is one of the most potent ways to cause T-cell suppression.
- **Expression of peroxynitirite**: peroxynitirite is a very strong oxidant and one of the essential intermediary elements which mediate T-cell suppression. Following NO production, NO reacts with supreoxide anions to produce peroxynitirite. Peroxynitirite can then cause nitrosyalation and nitration of amino acids such as tryptophan, tyrosine, methionine, and cysteine. Through direct cell-cell contact between MDSCs and CD8+T-cells, peroxynitirite causes nitration of amino acids in the TCR and induces conformational changes in the TCR and alters its 3D structure and eventually leads to T-cell unresponsiveness in the tumor microenvironment (Vickers SM 1999; Bronte V 2005 ; Nagaraj 2007).
- **Induction of T-regs**: IFN-γ derived from tumor specific T-cells in combination with IL-10 induce expression of cytotoxic lymphocyte antigen-4 in MDSCs which causes *de-novo* FoxP3 expression and T-reg cell induction. (Yang R 2006). Such event is linked to antigen specific T-cell suppression via MDSCs (Serafini, Mgebroff et al. 2008).

- **ROS expression**: In human and mice tumors, MDSCs have increased ROS production (Ribechini, Greifenberg et al. 2010; Zhang and Meadows 2010). Factors such as IL-6, IL-13, TGF-b, IL-10, PDGF, and GM-CSF can induce ROS expression by MDSCs and cause T-cell unresponsiveness (Sauer 2001)
- **NK cell inhibition**: Natural Killer cells (NK cells) are very effective cells in clearing tumor cells and are an important member of the innate immunity which exert their cytotoxic effect on tumor cells and cause their death. Using several mouse models, MDSCs have been shown to decrease NK cell cytolytic functionality (Hoechst 2009; Li, Han et al. 2009; Elkabets 2010).

In addition to these mechanisms, different subsets of MDSC (monocytic and granulocytic) use different mechanisms to induce immune suppression. In most tumor models, it is the granulocytic subset that expands more (70-80%) and often uses ROS production as a suppressive mechanism (Youn 2008) and can modify the T-cell repertoire to induce antigen specific T-cell unresponsiveness. The monocytic subset expands to a smaller degree and induces suppression through NO expression (Youn 2008) and within the tumor microenvironment can differentiate into $F4/80^{+}Gr1^{-}$ Tumor Associated Macrophages (TAMs) (Gabrilovich 2005; Kusmartsev S 2005 ; Sica A 2007). TAMs are alternatively activated macrophages which skew the immune response towards a TH2 type of response and are considered one of the biggest contributors to the immunosuppressive tumor microenvironment. MDSCs and TAMs can interact with each other and skew the immune response towards a Th2 type of response which results in

increased IL-10 production by MDSCs and decreased IL-12 expression by macrophages (P. Sinha 2007) which ultimately results in promotion of tumor growth.

MDSCs serve as a serious obstacle in the effectiveness of immunotherapy as a treatment option for cancer patients. Moreover, MDSCs can promote tumor growth, cancer progression, and metastasis. Since MDSCs induce immunosuppression through various means, it is tempting to assume that targeting them can eliminate various immunosuppression mechanisms and allow immunotherapy to be an effective way to target tumor cells. However, targeting an entire cell population which happens to be heterogeneous is not an easy task. It is more practical to study key regulators of MDSC suppressive function and target them.

While MUC1 is known to be an epithelial cell marker, its expression and function on hematopoietic cells has been described (Agrawal B 1998; Treon 1999; Li 2004; Mukherjee P 2005). We have previously shown that in Muc1 null mice, altered DC differentiation leads to increase MDSC expansion in response to GM-CSF and IL-4 *in-vitro* (Poh, Bradley et al. 2009). Our study has revealed that Muc1 expression on hematopoietic cells acts as a signaling molecule to control MDSC expansion via stabilization of β-catenin and preventing aberrant expansion of MDSCs. Further, adoptive transfer of MDSCs from Muc1KO mice to WT mice challenged with allogeneic tumor resulted in tolerance to that tumor indicating that MDSCs from KO mice are more suppressive than that of WT mice. The mechanism and reason behind such observation was not studied.

In our current study, we investigated MDSC expansion, differentiation, and suppressive function in WT and Muc1 null mice under cancer conditions. Using two models of

epithelial originated cancers (PDA and Breast Cancer), we have shown that not only Muc1KO mice have higher levels of MDSC expansion in the spleen, but that these MDSCs are more suppressive compared to that of WT mice. The fact that MUC1 can act as a regulatory mechanism to prevent increased MDSC expansion is of great importance since systemic targeting of MUC1 in many carcinomas is being actively explored. From our studies, it becomes clear that therapeutic targeting of MUC1 must be localized to the epithelial tumors because down-regulating MUC1 expression in hematopoietic cells may be harmful and lead to increased MDSCs.

4.5 Results

Hematopoietic Stem Cells (HSCs) and MDSCs express Muc1.

Previously, MUC1 expression on hematopoietic cells has been described (Agrawal B 1998; Treon 1999; Li 2004; Mukherjee P 2005). We wanted to know if MUC1 is expressed on HSCs. Through flow cytometry, we found that 100% of HSC cells from C57/B6 mice express Muc1 indicating that Muc1 may play a role in hematopoiesis (Figure 2A). Further, since functionality of MDSCs from Muc1 null mice is altered (Poh TW 2009), we hypothesized that immature myeloid cells (IMCs) express Muc1. In-fact, we have shown that a high percentage of IMCs express Muc1-CT indicating that Muc1 possibly directly regulate MDSC function (Figure 2B). More interestingly, our data shows a significant decrease in Muc1 expression on MDSCs as cancer progresses indicating that increased suppressive function of MDSCs may be associated with decreased Muc1 expression by these cells.

MDSC expansion under cancer progression in the BM of Muc1 null mice is not different from that of WT mice.

To study whether Muc1 expression on HSCs leads to differential expansion of MDSCs in the bone marrow of cancer bearing mice, freshly isolated BM cells were analyzed for co-expression of Gr1 and CD11b. We found that Muc1KO mice have 15% higher levels of MDSCs in the BM indicating that Muc1 may regulate ontogeny of these cells. Further, while we detected a significant induction of BM-MDSCs in mice injected with a pancreatic cancer tumor cell line (KCKO), we did not find any difference in the levels of MDSCs between tumor-bearing WT and Muc1KO mice (Figure 3A). In contrast, the breast tumor cell line, C57MG, completely failed to induce MDSC expansion in the BM.

To further characterize the BM-MDSCs from WT and Muc1KO mice, levels of Arginase and iNOS expression were studied via flow cytometry. Our results indicate that higher percent of MDSCs from healthy non-tumor-bearing Muc1KO mice express iNOS (Figure 3B) than its WT counterpart. Moreover, MDSCs from KCKO tumor-bearing Muc1KO mice express higher levels of iNOS than WT mice suggesting a more suppressive phenotype in Muc1KO MDSCs compared to WT MDSCs (Figure 3B). With regards to Arginase-1 expression on MDSCs, although tumor induced higher levels of Arginase-1 on MDSCs, there was no difference between WT and Muc1KO MDSCs (Figure 3C). Data on Arginase-1 expression is presented as MFI since all MDSCs appeared positive for Arginase expression.

BM-MDSCs from Muc1KO mice have a less differentiated phenotype compared to WT-MDSCs.

To check whether MDSCs from WT and Muc1KO mice differ in their differentiation/maturation state, we checked for Stem Cell Antigen-1 (Sca1), a stem cell marker known to be expressed on early MDSCs which are not close to terminal differentiation (Zoglmeier, Bauer et al. 2011). Our data demonstrates that under cancer condition not only a higher percentage of BM-MDSCs from Muc1KO mice are positive for Sca1 (Figure 3D), but also the levels of Sca1 expression was higher in BM-MDSCs from these mice signifying that these MDSCs are in a less differentiated stage compared to WT BM-MDSCs (Figure 3D). We then checked levels of c-MYC expression on MDSCs and found that under cancer condition, BM-MDSCs from Muc1KO mice express significantly higher levels of c-MYC than the WT MDSCs. C-Myc protein is responsible for MDSC survival (as reviewed in (Nagaraj and Gabrilovich 2007)). Our data suggests that BM-MDSCs in the absence of Muc1 are more likely to survive and proliferate and probably have a longer life span (Figure 3F).

MDSC expansion and migration to the spleen of cancer bearing Muc1KO mice is higher than that of WT mice.

Spleen is the first major lymphatic organ to which MDSCs migrate to exert suppressive function prior to infiltrating tumors. To study whether Muc1 regulates MDSC expansion and migration to the spleen of tumor bearing mice, splenocytes from these mice were analyzed for Gr1+CD11b+ cells. According to our results, KCKO cells induced significant expansion/migration of MDSCs in the spleen and this expansion was significantly higher in the Muc1KO versus the WT mice (Figure 4A). In the breast cancer

model, C57MG cell line failed to induce MDSC expansion in the spleen of WT mice; however, in the Muc1KO mice, higher levels of Gr1+CD11b+ cells were detected compared to non-tumor bearing Muc1KO and tumor-bearing WT mice (Figure 4B). Our results indicated that increased migration and expansion of MDSCs in the spleen of Muc1KO versus WT mice.

MDSCs from the spleen of Muc1KO tumor bearing mice are more suppressive compared to WT MDSCs.

We hypothesized that differential expression of suppressive enzymes in BM-MDSCs might cause spleen MDSCs to be more effective in T-cell suppression. Using a Mixed Lymphocyte Reaction (MLR), we co-cultured MDSCs from WT and Muc1KO mice (bearing C57MG tumors) with naïve syngeneic T cells stimulated with α-CD3/CD28 antibody and collected the supernatants to test for IL-2 production. Our results show that MDSCs from the spleen of cancer bearing Muc1KO mice were more effective in inducing T-cell suppression indicated by lower IL-2 production (Figure 4C). Thus, Muc1 regulates the suppressive function of MDSCs under cancer conditions.

To further study the mechanism behind increased MDSC suppressive activity in Muc1KO mice, we studied whether levels of suppressive enzymes were any different in MDSCs from Muc1KO versus WT mice. Our results show increased Arginase-1 expression in the MDSC of Muc1KO and WT mice bearing the KCKO tumor compared to non-tumor bearing mice. However, there was no difference between levels of Arginase-1 expression between WT and Muc1KO mice (Figure 4D, E). No difference was detected in the levels of iNOS expression as well (data not shown).

We then hypothesized that even though Arginase-1 expression in KO and WT MDSCs is not different; it is possible that its activity is different. Since Arginase-1 converts L-arginine to urea, we tested the levels of urea production by Gr1+CD11b+ cells sorted from the spleen. For the KCKO model, male mice were used and for the C57 model, female mice were injected with cancer cells. To our surprise, we found that Arginase-1 in MDSCs from healthy male Muc1KO mice are significantly more active in metabolizing Arginine indicating a more suppressed phenotype in Muc1KO mice. In healthy female Muc1KO mice, we saw a similar trend which was not statistically significant (Figure 4F, G). However, we found that under cancer progression in both models, the levels of urea in MDSCs from tumor-bearing Muc1KO mice were significantly higher than its WT counterpart (Figure 4F, G). In agreement with our hypothesis, our data indicate that Muc1 regulates MDSC suppressive functions. Increased Arginase1 activity in Muc1KO MDSCs explain the reason behind decreased T-cell IL-2 production observed in Figure 3C. Next, since TGF-β expression by MDSCs is known to be associated with induction of MDSC suppression activity, we measured levels of TGF-β production by sorted MDSCs from WT and Muc1KO mice. Amazingly, our results show that MDSCs from healthy Muc1KO mice express significantly higher levels of TGF-β, indicating again a more suppressive phenotype even in Muc1KO MDSCs (Figure 4H). Breast cancer progression caused increased production of TGF-β in both WT and Muc1KO mice and while there is a trend of increased TGF-β production by Muc1KO MDSCs, the difference was not statistically significant. We further analyzed cytokine secretion from Splenic MDSCs and found that IL-6 secretion in both tumor bearing and

healthy Muc1KO MDSCs is significantly higher than that of WT mice signifying the increased inflammatory function of these cells (Figure 4H).

Overall, our results have provided evidence supporting the hypothesis that Muc1 not only regulates MDSC expansion, but also regulates MDSC suppressive function in both healthy condition and during cancer progression.

Muc1KO mice have higher levels of TGF-β but not IL-6 in their serum.

Due to higher levels and suppressive function of MDSCs in Muc1KO mice, and the inflammatory nature of these cells we wanted to know whether these mice express higher levels of TGF-β or IL-6. Serum from tumor-bearing and healthy mice was analyzed for certain cytokines known to induce MDSC expansion and are secreted by MDSCs. Our results show that in both cancer models, levels of TGF-β are significantly higher in Muc1KO mice (Figure 5A, B). We did not detect any differences in IL-6 levels in the serum of these mice (Figure 5C, D). Overall, our results show that during cancer progression, Muc1KO mice have higher levels of the immunosuppressive cytokine, TGF-β circulating in the serum. Although the source of increased production of this cytokine is not known to use, its increase is known to influence MDSC expansion and function.

Muc1KO mice are more susceptible to tumor growth.

Increased MDSCs cause susceptibility to tumor growth. In our study, a comparison of tumor growth and final wet weight between WT and Muc1KO mice indicate that in the absence of Muc1, mice are more susceptible to tumor growth with both KCKO and C57mg cell lines. In fact, not only tumors grow at a significantly faster rate in Muc1KO mice (Figure 6A, C), but tumors from WT mice never match the weight of that of Muc1KO mice (Figure 6B, D).

Muc1KO mice have decreased NK cell cytotoxicity.

As mentioned before, one of the suppressive functions of MDSCs is reducing NK cell cytotoxic activity. Therefore, we hypothesized that due to increased MDSC expansion and function, NK cells from Muc1KO cancer bearing mice are not as effective in inducing cell death. NK cells from the spleen of WT and KO mice with KCKO were sorted and incubated with Yac1 cells. Indeed, our results show that in the absence of Muc1, NK cells have decreased cytotoxic activity.

4.6 Conclusion

MDSCs are a heterogeneous population of cells which expand under pathological conditions such as cancer and have an extraordinary ability to reduce antitumor immune response. Furthermore, MDSCs pose a significant problem to effectiveness of cancer immunotherapy. The underlying mechanism in regulation of MDSC expansion and function is not yet clear.

In this study, for the first time we have shown that Muc1 affects hematopoietic differentiation. Both increased levels of IMCs in the BM of Muc1KO mice, as well as increased levels of c-MYC expression by IMCs show that Muc1 regulates terminal differentiation of hematopoietic cells. Most interestingly, our findings show that MDSCs express Muc1 and this expression is significantly reduced as the tumor develops. Decreased Muc1 expression by MDSCs was associated with increased expansion and suppressive functions of these cells. Consistent with our data, Muc1 null mice have MDSCs which exhibit both a less differentiated phenotype (Figure 3D), and express higher levels of suppressive enzymes (Figure 3B). Injecting Muc1Ko mice with tumor cells resulted in increased tumor burden (Figure 6A-D) which is partially explained by

higher suppressive functions of their MDSCs (Figure 4C). Increased expansion of MDSCs from Muc1KO mice was also associated with decreased NK cell functionality (Figure 5E). NK cells play an important role in induction of innate immune response against cancer cells and their decreased cytotoxic function can in part explain the reason for increased susceptibility of Muc1KO mice to tumor growth.

Although, the mechanisms by which Muc1 regulates hematopoiesis are not fully elucidated, its role in regulation of STAT3 is well studied. STAT3 signaling is one the most important signaling pathways which induces MDSC expansion and suppression. It is tempting to speculate that in the absence of Muc1, STAT3 signaling is not carefully regulated and therefore these mice have such immunosuppressed phenotype. In fact our preliminary data suggests that in the absence of Muc1, MDSCs in the spleen have higher levels of p-STAT3. Also, we have previously shown that BM progenitor cells from Muc1Ko mice differentiate into Gr1+CD11b+ cells under GM-CSF/IL-4 (Poh TW 2009). We showed that in lack of Muc1, β-catenin stability in myeloid progenitor cells is reduced and therefore, altered Wnt signaling contributes to aberrant expansion of Muc1.

Our data show that in future, targeting MUC1 as a tumor antigen must be carefully reviewed. It is possible that blindly targeting MUC1 in cancer patients may also target MUC1 on MDSCs and induce its expansion and suppressive function. Most likely, novel tolls must be designed to only target tumor associated MUC1 on epithelial cells and not MUC1 on hematopoietic cells.

4.7 Materials and methods

Cell line

PDA cell line called KCKO was generated by our lab. Pancreas was dissociated using collagenase and protease. Single cell suspension was plated in DMEM containing 10% FBS. Several passages were passed and Pancytokeratin levels were checked to ensure purity of the cells. C57MG breast cancer cells were a kind gift from Dr. Gendler, Mayo clinic. All cell lines were maintained in DMEM (Invitrogen, Carlsbad, CA), 10%FCS, 1%penicillin/streptomycin and 1%glutamax (Invitrogen). All cell types were maintained in 5% CO2 and 95% humidity.

Mice

For this study, 3 month old C57/B6 and Muc1KO mice were used. For the PDA model, 106 KCKO cells were injected in the right flank of the mouse. For the breast cancer model, 106 C57MG cells were injected in the right low mammary fat pad. Tumors were allowed to grow and their volume was measured using a caliber every two days until mice were euthanized.

Flow cytometry

$5*10^5$ cells were collected and labeled with fluorescent dye conjugated antibodies. Acquisition was performed by BD FACS Calibur and BD Fortessa. Data was analyzed using FlowJO (Ashland, OR).

MDSC Suppression Assay

CD3+T-cells were isolated from healthy C57/B6 mice using a Miltenyi kit (Miltenyi Biotec, Auburn, CA). Round bottom plates were coated with 10μg of functional α-CD3 antibody (eBiosciences, San Diego, CA) and 5 μg α-CD28 antibody

overnight. 10^5 CD3+T-cells were plated in RPIMI containing 10% heat inactivated FCS, 1%penicillin/streptomycin and 1%glutamax (Invitrogen). MDSCs were sorted first by enriching the CD11b+ cells from the spleen and then sorted for Gr1+cells using a Miltenyi kit. 10^5 MDSCs were incubated with CD3+T-cells for 72 hours. Supernatants were collected at 72hrs and were then subjected to IL-2 ELISA assay (eBiosciences).

Arginase assay

$2*10^5$ MDSCs were subjected to Arginase activity assay. Briefly, cells were lysed with 1% Trition-X in 37ºC. 25mM Tris-HCl and 10mM MnCl2 was added to samples. Lysates were then heated in 56º to activate Arginase-1. 0.5M L-arginine was added to the lysates and the reaction was stopped using phosphoric and suphoric acid. 40 ul of a-isonitrosopropiophenone was added and samples were heated at 95ºC for 30 minutes. Samples were read at 450 nm.

ELISA

Levels of cytokines were analyzed using sandwich ELISA. IL-6 in the serum was analyzed using purified and biotynilated Ab from BD (Franklin Lakes, NJ). TGF-β in the serum of from MDSC sups were analyzed using a kit from eBioscience.

Antibodies

Fluorescent antibodies against Gr1 (Miltenyi), CD11b (BD), iNOS (BD), Arginase-1, c-MYC (SantaCruz biotech, Santa Cruz, CA), Sca1 (Stem cell technologies, Vancouver, Canada) were used at a concentration of 1μg/sample.

NK cytotoxicity assay

Untouched NK cells were isolated form splenocytes using the NK cell isolation kit (Miltenyi, Cat# 130-090-864) and assessed for NK cell cytotoxic activity against the

NK cell-sensitive target cell line, YAC-1. 1 x 107 YAC-1 cells were incubated in RPMI with 500μCi 51Cr (Perkin-Elmer Analytical Sciences) for 1 hour at 37°C and then washed with PBS. Target cells were adjusted to 10^5 cells/mL and 100μL (10^4 cells per well) were plated in a 96-well V-bottom plate. NK cells were incubated with YAC-1 target cells at decreasing effector:target ratios for 4 hours at 37°C in a total volume of 200μL. The plate was again centrifuged at 1400 rpm for 8 minutes and then 30μL of supernatant was collected and counted on a gamma counter. Spontaneous release from labeled target cells was determined incubated in medium alone, while maximal release was obtained from labeled target cells lysed with 10% TritonX. The following formula was used to calculate the percentage of YAC-1 cells lysed: Percent specific cytotoxicity = [(experimental release - spontaneous release) / (maximal release - spontaneous release)] × 100.

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 $p < 0.05$.

4.8 Figures

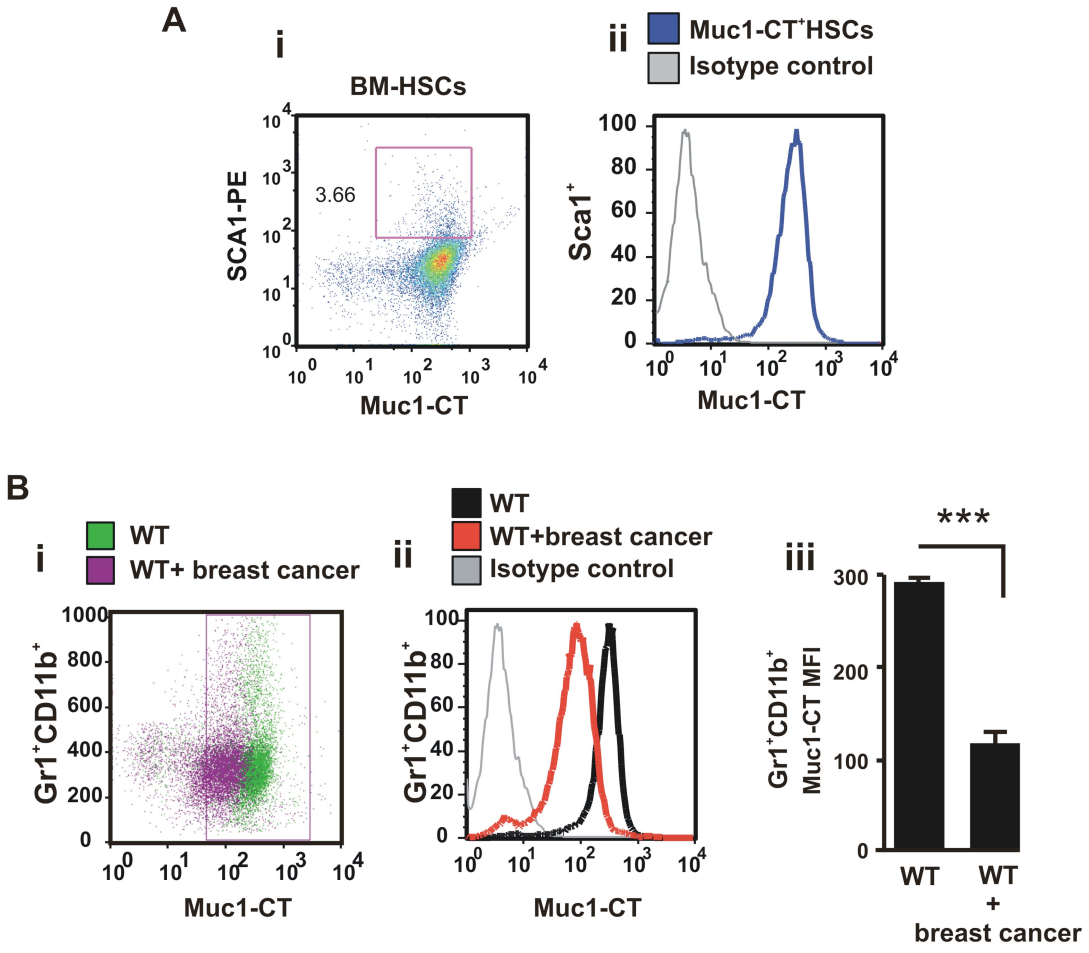
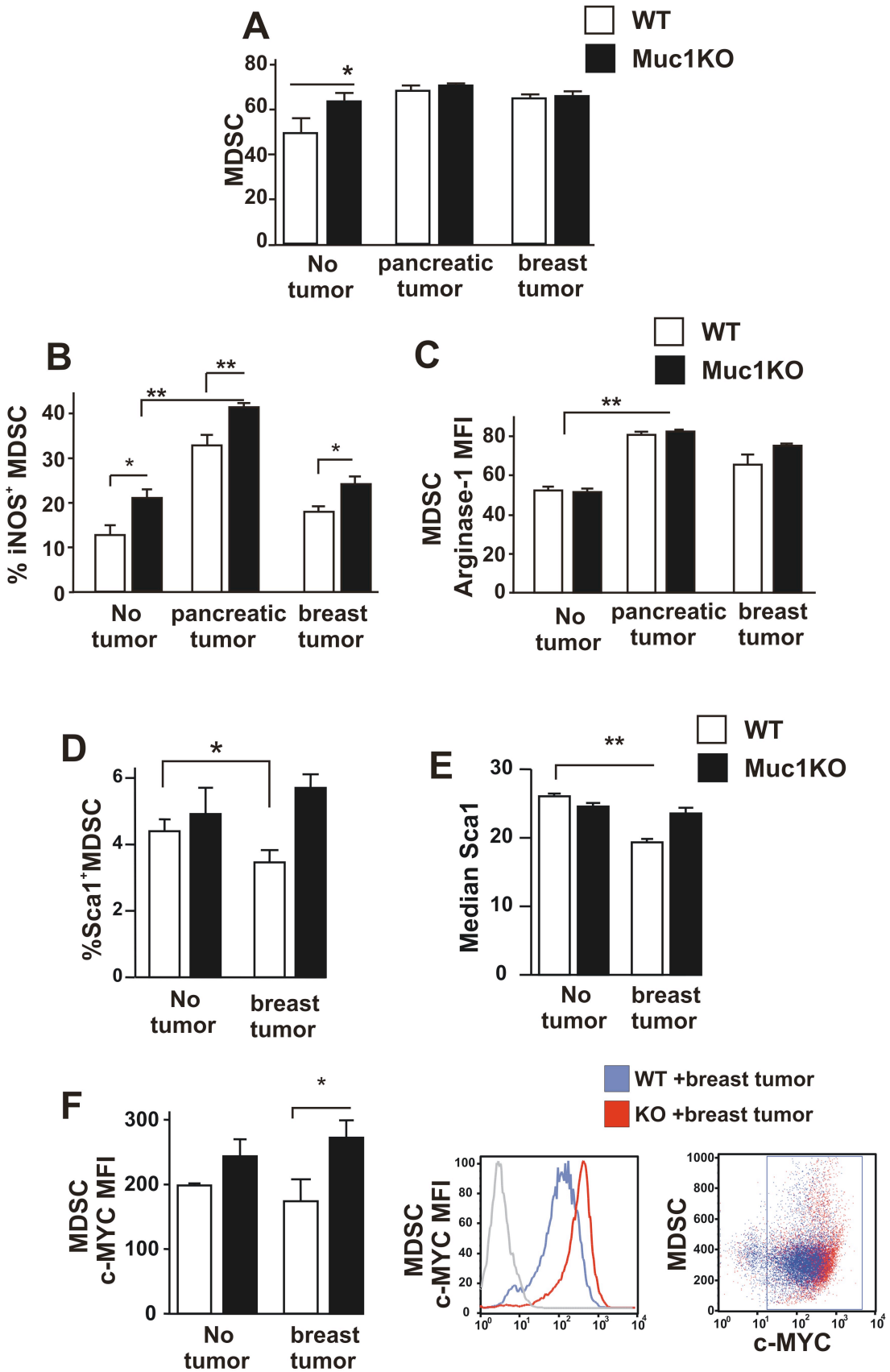


Figure 2. Both HSCs and MDSCs express Muc1. A) Fresh BM cells from healthy mice were isolated and labeled with fluorescent Abs against Sca1 and Muc1-CT. B) BM cells from WT mice and WT mice with C57MG tumor were labeled with fluorescent antibodies against Gr1, CD11b, and Muc1-CT. samples were analyzed via flow cytometry. First, cells were gated on Gr1-CD11b and then analyzed for Muc1-CT expression. Representative i) dot-plot, ii) histogram and iii) bar graph is shown (***, $P<0.0001$)


A
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10^2
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c-MYC

Figure 3: MDSC phenotype in the BM of WT and Muc1KO mice. A) BM cells from WT and Muc1Ko mice were isolated and labeled with Abs against Gr1 and CD11b. samples were analyzed via flow cytometry. (*, $P<0.05$) B) BM cells were gated on Gr1+CD11b+ cells and then analyzed for %cells which express iNOS. (*, $P<0.05$; **, $P<0.01$) C) BM cells were analyzed via flow cytometry for the level of expression of Arginase-1. (**, $P<0.001$). D, E) BM cells were gated on Gr1+CD11b+ and then were analyzed for expression of Sca1. Data is presented as both % cells which express Sca1 (D) and the levels of Sca1 expression (E) (* $P<0.05$; ** $P<0.001$). F) BM cells were gated on $Gr1^{+}CD11b^{+}$ markers and were then analyzed for the levels of c-MYC expression. Data is shown in bar graph, histogram, and dot-plot (* $P<0.05$)

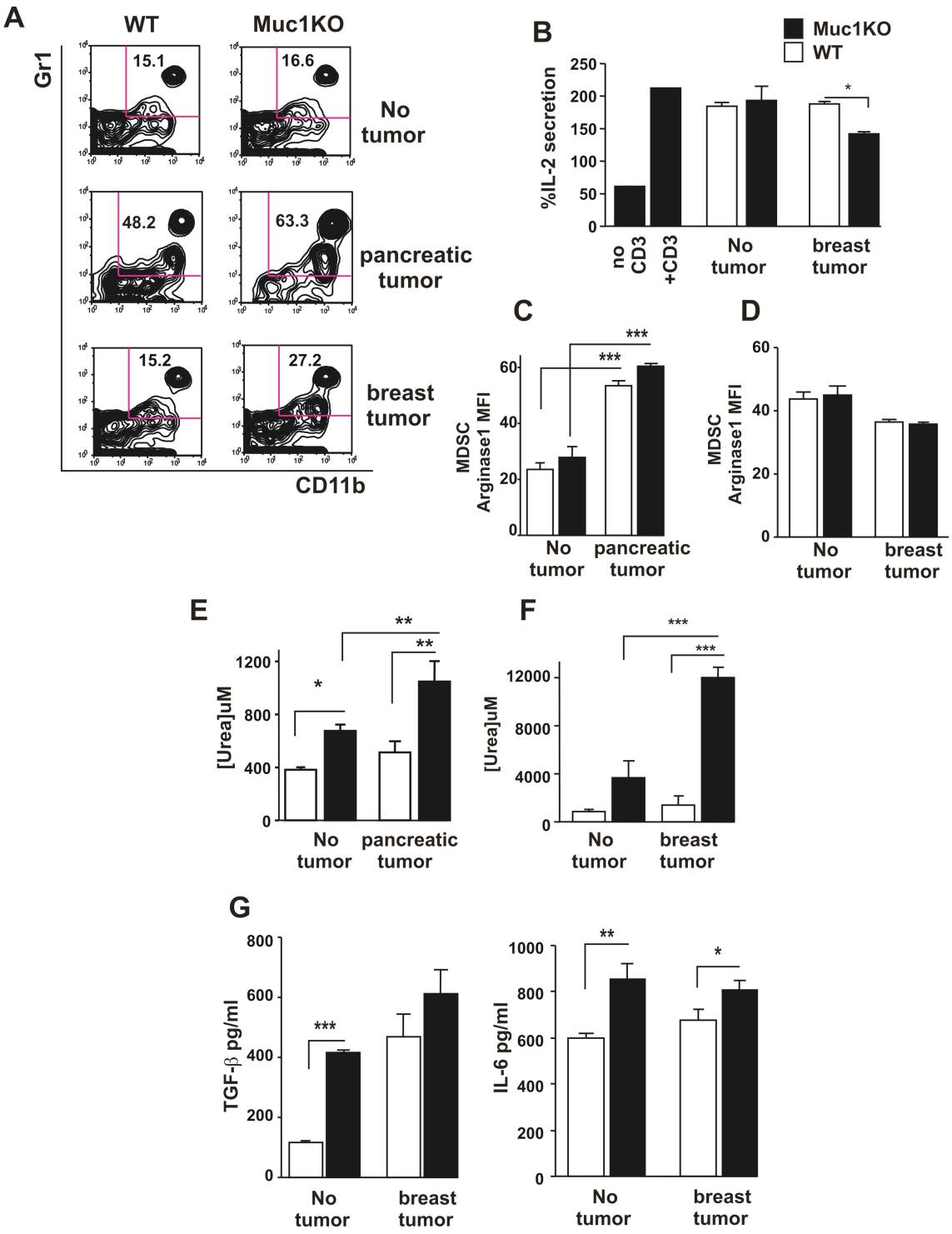

Figure 4. MDSC phenotype and function in the spleen of WT and Muc1KO mice. A) Splenocytes were isolated from WT and Muc1KO mice and immediately labeled with fluorescent antibodies against Gr1 and CD11b. representative contour plot is shown. B) Gr1+CD11b+ cells were isolated from the spleen on C57MG tumor bearing mice and incubated with naïve CD3+T-cells which were stimulated by α-CD3/28 antibody. $1*10^5$

MDSCs and $1*10^5$ CD3+T-cells were co-cultured for 72 hours. Supernatant was analyzed via ELISA for production of IL-2. (*, P<0.01) C,D) Splenocytes were gated on Gr1+CD11b+ cells and analyzed for expression of Arginase-1. (*** P<0.0001). E.F) Sorted MDSCs were subjected to an Arginase-1 activity assay. Cell lysates were exposed to Arginine and urea production was measured (*, P<0.01; **, P<0.01; *** P<0.0001). G) TGF-β and IL-6 production by MDSCs from WT and Muc1KO mice injected with C57MG cells was analyzed through ELSIA.

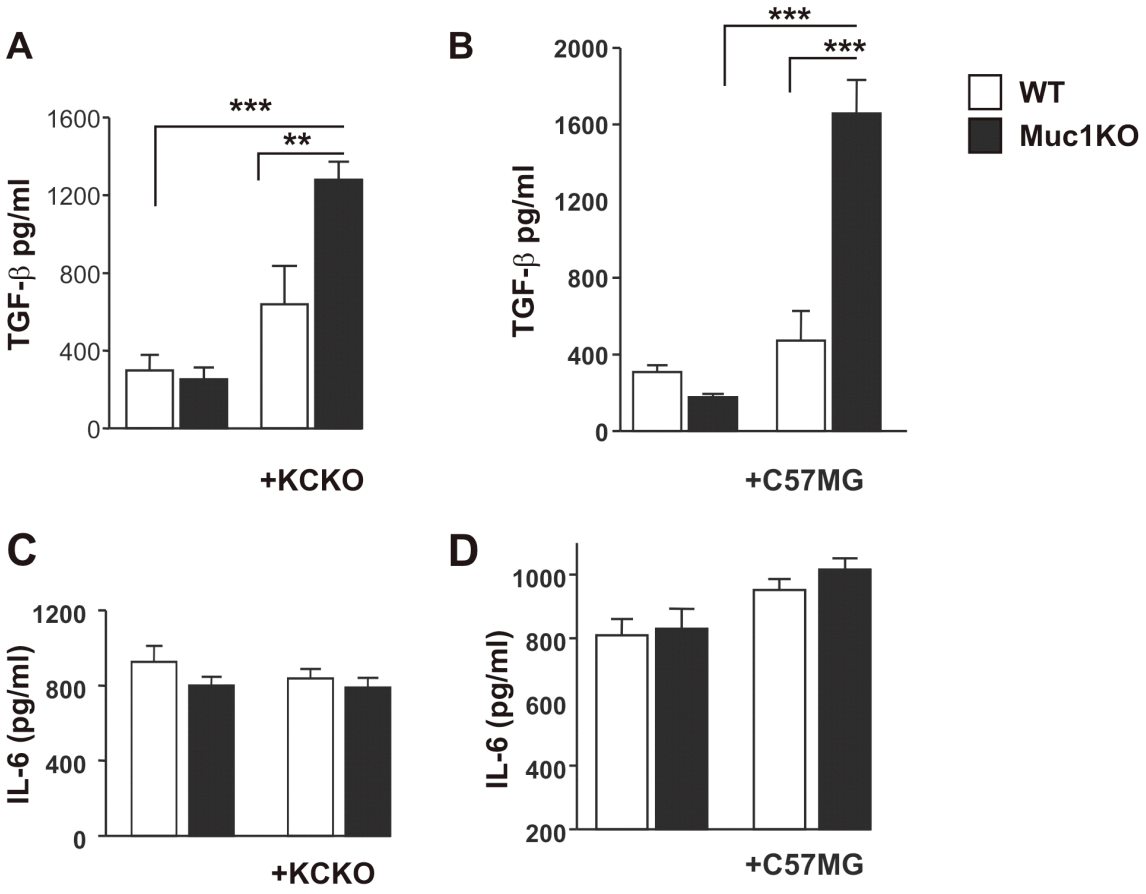

Figure 5. Inflammatory cytokine production by WT and Muc1Ko mice with cancer. A,B) Serum of WT and KO healthy and cancer bearing mice were subjected to ELISA to measure TGF-β and C,D) IL-6 production. (**, P<0.001; ***, P<0.0001).

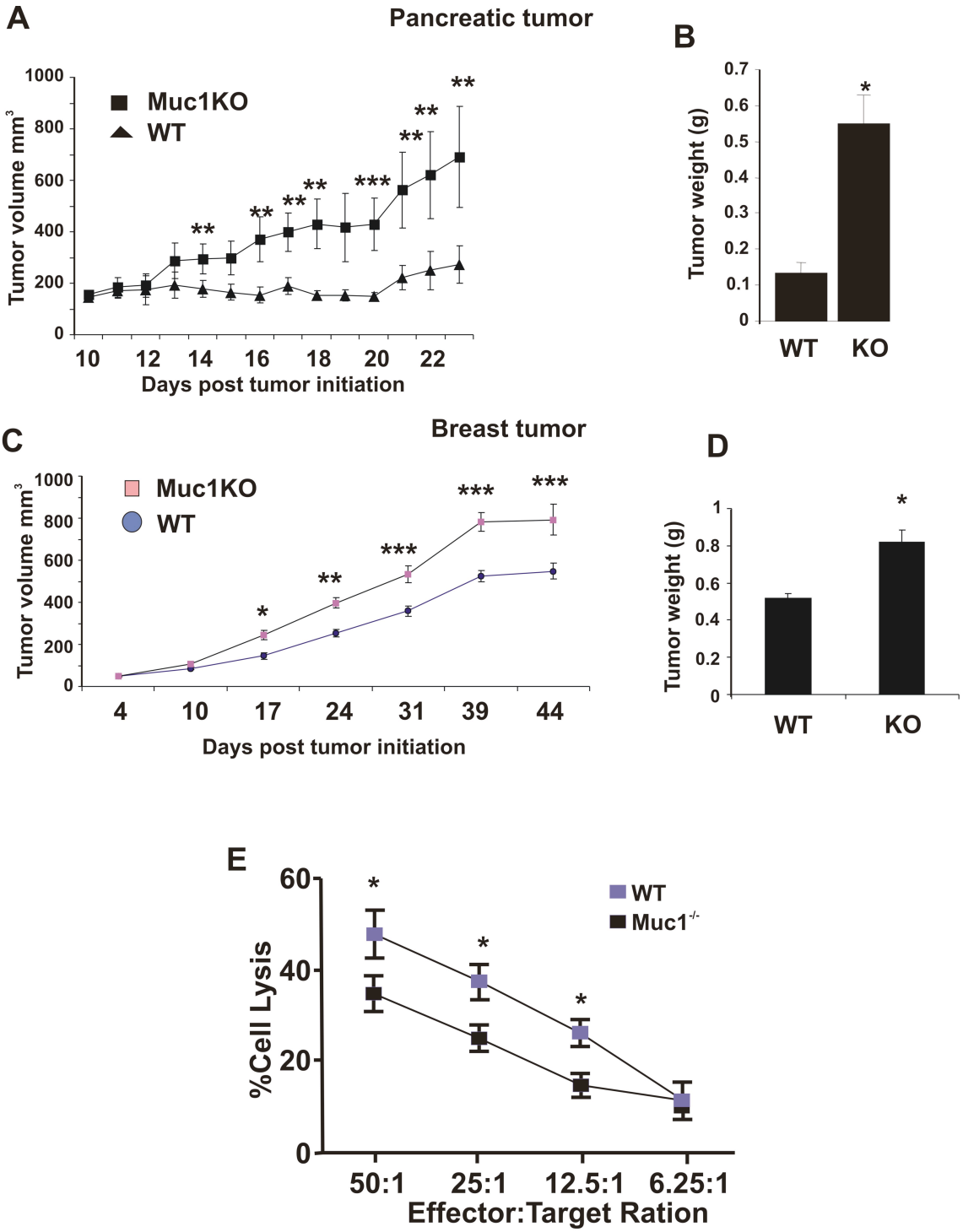

Figure 6: Muc1KO mice are more susceptible to tumor growth compared to WT mice. A) 3 month old male BK6 and Muc1KO mice were injected subQ with 10^6 KCKO cells (PDA cells). Tumor was measured every two days. B) Final tumor wet weight of WT and Muc1Ko mice with KCKO on day 23. B) 3 month old female WT and Muc1KO mice were injected in the mammary fat par with C57MG mice. Tumor volume was measured every 2 days. C) Final tumor wet weight of WT and KO +C57MG. D) NK cells were

isolated from the spleen of WT and Muc1KO KCKO bearing mice. NK cells were incubated with Yac1 cells and their cytotoxic ability was measured by a chromium release assay.

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