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Regulation of the transmembrane mucin MUC4 by Wnt/β-catenin in gastrointestinal cancers

By

PRIYA PAI

A DISSERTATION

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Regulation of the transmembrane mucin MUC4 by Wnt/β-catenin in gastrointestinal cancers

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University of Nebraska Medical Center, 2015

Supervisor: Surinder K. Batra, PhD.

The transmembrane mucin MUC4 is a high molecular weight glycoprotein that is expressed *de novo* in pancreatic ductal adenocarcinoma (PDAC). MUC4 has been shown to play a tumor-promoting role in malignancies such as PDAC, ovarian cancer and breast cancer. Unlike the normal pancreas, MUC4 is ordinarily expressed by goblet and absorptive cells in the normal colonic epithelium. However, its expression/role in colorectal cancer (CRC) is not well studied.

In this dissertation, the goal was to identify factor(s) that may differentially regulate MUC4 in these two disparate malignancies. Furthermore, in light of its pro-tumorigenic role in other malignancies, we analyzed the functional implications of MUC4 expression in CRC. A *MUC4* promoter analysis showed the presence of three putative TCF/LEF sites located in the proximal and distal promoters. Importantly, multiple TCF/LEF sites are typically present in the promoters of Wnt/β-catenin pathway target genes. In order to ascertain whether MUC4 was a Wnt/β-catenin target gene, we performed β-catenin knock down (KD) studies, treatment with Wnt3a ligand, as well as *MUC4* promoter luciferase studies in the first section of this dissertation. In PDAC, it was observed that *MUC4* transcript and protein were decreased upon β-catenin KD, WNT3a conditioned medium treatment increased MUC4 and *MUC4* promoter luciferase activity was increased upon transfection with stabilized β-catenin. Furthermore, immunohistochemistry (IHC) with rapid autopsy PDAC tissues showed a positive correlation between MUC4 and β-catenin expression. Mutation of each of the three putative TCF/LEF sites showed that the sites closest to and furthest from the ATG site were critical for *MUC4* promoter luciferase activity in

the presence of stabilized β-catenin. A Chromatin immunoprecipitation assay (ChIP) confirmed that β-catenin associates with the *MUC4* promoter at these two sites in PDAC. Functional studies with the β-catenin KD cells showed that migratory properties were decreased significantly upon KD, concomitant with altered levels of epithelial to mesenchymal transition (EMT) markers. We thus concluded that β-catenin up-regulates MUC4 in PDAC, and that the β-catenin-MUC4 axis likely contributes to the metastatic properties of PDAC cells.

The second part of this dissertation deals with the regulation of *MUC4* by β-catenin in CRC. Here, we observed that the KD of β-catenin induced an increase in *MUC4* transcript and protein. This was corroborated by transient overexpression of stabilized β-catenin, which resulted in decreased MUC4. *MUC4* promoter luciferase studies showed that KD of β-catenin resulted in increased promoter luciferase activity. The mutation of each of the three TCF/LEF sites and subsequent promoter luciferase assays showed that the second and third sites appeared to be repressive and that mutation of all three sites in combination caused an increase in *MUC4*. It was also observed that the Wnt/β-catenin pathway can indirectly repress MUC4 by suppression of Hath1, a Notch pathway target gene. Functional studies with MUC4 KD showed that MUC4 confers proliferative but not migratory properties to cells.

The third part of this dissertation describes the generation of a mouse model aimed at delineating the role of Muc4 in CRC progression. Mice with colon-preferential heterozygous loss of Apc and mutant Kras, i.e. CDX2 P-NLS -Cre $kras^{G12D/+}Apc^{loxP/+}$ were crossed with the $Muc4^{-/-}$ mice generated in our lab. Preliminary results indicate the expression pattern of Muc4 is altered during the progression of CRC.

Taken together, studies in this dissertation demonstrate the differential regulation of MUC4 by the Wnt/β-catenin pathway in PDAC and CRC, and that MUC4 expression may be pro-tumorigenic in CRC.

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ABBREVIATIONS

AP activator protein

AOM azoxymethane

ACF aberrant crypt foci

APC Adenomatous polyposis coli

ASGP Ascites sialoglycoprotein

Min multiple intestinal neoplasia

ATCC American Type Culture Collection

bHLH Basic helix loop helix

BRAF v-raf murine sarcoma viral oncogene homolog B1

CDX caudal type homeobox

ChIP Chromatin immunoprecipitation

CIMP CpG island methylator phenotype

CFTR Cystic fibrosis transmembrane conductance regulator

CS Composite score

CRC colorectal cancer

CREB cAMP response element-binding protein

DMEM Dulbecco's modified eagle's medium

DNA Deoxyribonucleic acid

DSS dextran sodium sulfate

ECM Extracellular matrix

EGFR Epithelial growth factor receptor

EMT Epithelial to Mesenchymal transition

ERBB2 erythroblastic leukemia viral oncogene homolog 2

ERK Extracellular signal-regulated protein kinases

FAP familial adenomatous polyposis

FUT fucosyltransferase

FAK focal adhesion kinase

FNA fine-needle aspirate

FOXA1 forkhead Box A1

GATA globin transcription factor

GTP guanosine triphosphate

HDAC histone deacetylase

HNF hepatocyte nuclear factor

HNPCC hereditary nonpolyposis syndrome

IBD inflammatory bowel disease

IHC Immunohistochemistry

IFN interferon

JAK janus kinase

KLF4 Kruppel-like factor 4

KRAS Kirsten rat sarcoma viral oncogene homolog

KPC PDX-1-Cre, LSL-$Kras^{G12D}$, LSL-$Trp53^{R172H/-}$

LEF lymphoid enhancer-binding factor

mRNA Messenger RNA

miRNA microRNA

μg Microgram

μL Microliter

μM Micromolar

MDF mucin depleted foci

MLH1 MutL Homolog 1

MSH2 MutS Homolog 2

MSI microsatellite instability

NLS nuclear localization signal

NCOA3 Nuclear receptor coactivator3

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

NOD-SCID nonobese diabetic/severe combined immunodeficient

PanIN Pancreatic intraepithelial neoplasia

PBS Phosphate-buffered saline

PCR Polymerase chain action

PDAC pancreatic ductal adenocarcinoma

PI3K phosphoinositide 3-kinase

PKC protein kinase C

qRT-PCR quantitative Reverse Transcription PCR

RAL ras related protein

RAR retinoic acid receptor

RNA Ribonucleic acid

RT-PCR Reverse transcription PCR

RXR retinoid X receptor

SDS Sodium dodecyl sulfate

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SMC sialomucin complex

STAT signal transducer and transcription factor

TCF T-cell factor

TNF tumor necrosis factor

TMA tissue microarray

TSA trichostatin A

TGF transforming growth factor

UTR untranslated region

VNTR variable number of tandem repeats

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CHAPTER 1 A

Introduction

The material covered in this chapter is the subject of 1 review article

1. Pai P., Rachagani S., Dhawan P. and Batra S.K., *Mucins and β-catenin in Gastrointestinal Cancers: A Pernicious Partnership;* **Carcinogenesis, 2015** (*under review*)

Mucins and β-catenin in Gastrointestinal Cancers

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1. Synopsis

The Wnt/β-catenin signaling pathway is indispensable for embryonic development, maintenance of adult tissue homeostasis, and repair of epithelial injury. Not surprisingly, aberrations in this pathway occur frequently in many cancers and often result in increased nuclear β-catenin. While mutations in key pathway members, such as β-catenin and adenomatous polyposis coli, are early and frequent occurrences in most colorectal cancers (CRC), mutations in canonical pathway members are rare in pancreatic ductal adenocarcinoma (PDAC). Instead, in the majority of PDACs, indirect mechanisms, including promoter methylation, increased ligand secretion, and decreased pathway inhibitor secretion, work in concert to promote aberrant cytosolic/nuclear localization of β-catenin. Concomitant with alterations in β-catenin localization, changes in mucin expression and localization have been documented in multiple malignancies. Indeed, numerous studies over the years suggest an intricate and mutually regulatory relationship between mucins and β-catenin. In this chapter, I summarize several studies that describe the relationship between mucins and β-catenin in gastrointestinal malignancies, with particular emphasis upon colorectal and pancreatic cancer.

2. Introduction to the Wnt/ β-catenin pathway

The Wnt signaling pathway is an important developmental regulatory pathway and plays critical roles in embryogenesis, including roles in regulating delineation of the body axis and in the formation of the germ layer [1]. The binding of Wnt ligands, a group of secreted lipid-modified proteins, activate both the canonical and non-canonical Wnt-signaling pathways [2]. The canonical Wnt pathway in particular hinges upon the activity of β-catenin, a molecule important for both cell adhesion and signaling, both functions being indispensable for normal cellular processes.

There are two separate pools of β-catenin – cytosolic and membrane-localized [2-5]. The membrane-localized fraction participates in cell adhesion, where it forms part of the adherens junction. Here, membrane-localized β-catenin links E-cadherin to the cytoskeleton via α-catenin. On the other hand, the cytosolic fraction is typically degraded through phosphorylation at the N-terminus by a *destruction complex*. This complex consists of glycogen synthase kinase β (GSKβ), Axin1, and casein kinase 1 (CK1) [5]. In the presence of a Wnt ligand, which binds to the Frizzled seven-pass transmembrane receptor and a co-receptor, the Low density lipoprotein Receptor-related protein (LRP), this complex is destroyed via a cascade of reactions triggered by the recruitment of dishevelled segment polarity protein 1 (DVL-1) to the receptor complex. Here, DVL-1 recruits Axin and glycogen synthase kinase 3 (GSK3) to form part of the Wnt signalosome, thus destabilizing the destruction complex [2-5]. Next, β-catenin is released from the destruction complex and enters the nucleus through direct contact with the nuclear pore complex [6]. Nuclear β-catenin upregulates a host of tissue-specific target genes, typically partnering with the TCF/LEF family of transcription factors, which usually function as transcriptional repressors in the absence of nuclear β-catenin [2]. Wnt ligands can also activate the non-canonical pathway, which is independent of β-catenin and comprises the planar cell polarity and the Wnt/Ca(2+) pathways [2].

The β-catenin molecule is remarkably well conserved, as evidenced by the presence of a β-catenin-like molecule in all metazoans. The *Drosophila* analogue of β-catenin, *armadillo*, was crucial in the discovery of the signaling function of β-catenin in a screen for mutations that affect segmentation of the embryo [7]. Remarkably, an amoebozoan, *Dictyostelium discoideum*, expresses a β-catenin analogue Aardvark, which maintains cell-cell junctional polarity in multicellular aggregates that comprise the fruiting bodies of the normally single-celled organism [8]. In the developing embryo, β-catenin is required for mesoderm formation, where the signaling function of the molecule plays a crucial role. The β-catenin molecule is also required for formation of the neuroepithelial structures and the endoderm. However, here the structural, junction-forming function of β-catenin takes precedence over the signaling function [9]. While Wnt/β-catenin signaling is not as active in adult tissue as the embryo, the Wnt/β-catenin pathway is required for the maintenance of tissue homeostasis and cell renewal, in addition to maintenance of the cell-cell junctions [2].

Given the multifarious nature of β-catenin and far-ranging effects of the perturbations in this critical pathway, Wnt/β-catenin signaling plays an important role in both normal tissue homeostasis and tumorigenesis. This chapter summarizes the significance of the Wnt/β-catenin signaling pathway in gastrointestinal malignancies, with an emphasis upon PDAC and colorectal cancer (CRC). Also, this chapter describes the relationship between the Wnt/β-catenin pathway and mucins, which are glycoproteins that play important roles in various malignancies.

3. The role of Wnt/β-catenin signaling in cancer

The Wnt/β-catenin signaling plays important role in development as well as homeostasis of adult tissue. As expected, mutations in this pathway occur frequently in cancer, most commonly in CRC, where around 80% of the patient population possesses either inactivating mutations in adenomatous polyposis coli (APC) or activating mutations in β-catenin [5]. However, aberrant activation of this pathway also occurs in pancreatic cancer, breast cancer,

multiple myelomas, melanoma, hepatocellular carcinoma, and other malignancies [4, 10-12]. Both mutations in Axin 1/2 [13] and activating mutations in β-catenin [3] occur in hepatocellular carcinoma. Mutations that prevent the phosphorylation-mediated degradation of β-catenin also occur in medulloblastoma [3] and the pediatric renal cancer Wilm's tumor [14]. Activation of Wnt/β-catenin signaling may also be wrought by epigenetic mechanisms, as observed in colorectal and pancreatic cancers, as well as medulloblastoma, where the promoters of Wnt inhibitors were found to be hypermethylated [15, 16].

Activation of the Wnt/β-catenin pathway can be precipitated either by overt mutations in pathway components or indirect mechanisms, such as increased secretion of ligands or decreased secretion of inhibitors. These two mechanisms of Wnt/β-catenin activation are exemplified by CRC and pancreatic cancer (PC), both gastrointestinal malignancies where the Wnt/β-catenin pathway plays a significant role in disease progression, albeit through distinct mechanisms.

4. Wnt/β-catenin in CRC

Aberrations in the Wnt/β-catenin pathway frequently occur in CRC. While mutations in several Wnt/β-catenin pathway have been recorded, an overwhelming majority of CRCs (70-80%) possess truncating mutations in APC [17]. Individuals with familial adenomatous polyposis (FAP) possess truncating mutations in APC, rendering them liable to the formation of hundreds of polyps in their colon, ultimately leading to CRC [1]. The increase in cytosolic/nuclear β-catenin could also be due to mutations in the exon 3 of β-catenin, which render it resistant to degradation, seen in less than 5% of CRCs [17]. Mutations in Transcription factor 7-like 2 (TCF7L2 or TCF4), the nuclear partner of β-catenin, have also been observed in roughly 5% of CRCs [17]. It must be noted, however, that distinct molecular subtypes of CRC exist, and that while mutations in the Wnt/ β-catenin pathway are very frequent, not all CRCs are driven by aberrant Wnt/ β-catenin signaling.

The majority of CRCs follow what is often referred to as the ‘suppressor’ pathway [18]. Here, both precursor lesions; ‘traditional’ adenomas, as well as full-blown tumors are characterized by aberrantly localized β-catenin, typically a consequence of truncated APC mutations [1]. Further, truncating mutations in APC are present in the earliest lesions, aberrant crypt foci, suggestive of a driving role for the Wnt/ β-catenin pathway [1]. In addition, around 50% of these tumors have a Kras mutation [19], which has been shown to aid in nuclear localization of β-catenin [20]. The levels of nuclear β-catenin steadily increase during the progression of CRC, starting from adenomas to full-blown carcinomas [1]. A subset of CRCs are characterized by frequent aberrations in the DNA mismatch repair machinery, often called the ‘mutator’ pathway [18]. These tumors possess microsatellite instability (MSI) and are less likely to possess Wnt/ β-catenin driver mutations [21, 22]. Yet another subtype, mucinous CRC, comprising roughly 10% of all CRC [23] has also been observed. These tumors are characterized excessive mucin production (chiefly MUC2) are also less likely to have aberrations in the Wnt/ β-catenin as driving mutations since they also frequently possess MSI-high (MSI-H) status[24]. Each of these CRC subtypes are further stratified by varying frequencies of BRAF, Kras mutations as well as CIMP (CpG island methylator phenotype) [25]. A detailed analysis of the various subtypes of CRC is, however, beyond the purview of this chapter.

5. Wnt/β-catenin in pancreatic cancer (PC)

Unlike CRC, where mutations in the Wnt pathway are important driver mutations, PC does not usually display such mutations. However, around 65% of PCs show aberrant nuclear/cytosolic localization of β-catenin and active Wnt signaling [26]. A significant fraction of PC patients also show elevated Axin2 expression, widely regarded as a universal marker of active Wnt/β-catenin signaling [27]. Further, the Wnt pathway was found to be one of the 12 core signaling pathways most frequently dysregulated in PDAC [28].

The Kras mutation is considered to be the driving mutation in PDAC, the most prevalent type of pancreatic neoplasm present in around 90% of patients [29]. Mice that express the $Kras^{G12D}$ mutation, driven by the expression of a pancreas-specific Cre (Pdx or p48), develop precursor lesions (mPanINs) that eventually form PDACs reminiscent of the majority of human lesions [30]. In contrast, mice that express mutant, stabilized β-catenin (exon 3-deleted), driven by a pancreas-specific p48-Cre, develop solid pseudopapillary neoplasms, an extremely uncommon form of the disease [31]. Mice that express both stabilized β-catenin and mutant Kras, driven by p48-Cre (p48-Cre; $Cttnb1^{exon3/+}$; $Kras^{G12D}$), develop tumors that resemble intraductal tubular neoplasms, yet another extremely rare disease [31]. Despite these findings, activation of the canonical Wnt pathway is necessary for the formation of pancreatic intra-epithelial neoplasia (PanINs) and full-blown PDAC in the Ptf1a-Cre; $Kras^{G12D}$ (KC) mouse model [32], albeit at levels substantially lower than observed in CRC, as demonstrated by Zhang *et al*, who generated a Ptf1a-Cre; $Kras^{G12D}$; $\beta\text{-catenin}^{f/f}$ mouse model. It was observed that the loss of β-catenin prevented the formation of mPanINs (mouse precursor lesions). Notably, β-catenin-depleted cells expressed lower levels of mucins in these mice, as determined by Periodic acid–Schiff (PAS) staining.

The proposed causes for the increase in Wnt/β-catenin signaling in PC include epigenetic regulation of the Wnt pathway components, increased ligand secretion, and decreased expression of pathway inhibitors. For example, the promoter of the Wnt-inhibitor SFRP1 was found to be hypermethylated in PDAC [16]. Also, the canonical Wnt ligand, Wnt 7b, was found to be over-expressed in PC [33]. Further, Wnt 7b independently confers a poorer prognosis in patients who over-express this protein. On the other hand, oncogenic Kras has been shown to induce expression of the ataxia telangiectasia group D complementing gene (ATDC) [34], which activates β-catenin signaling via stabilization of Dishevelled-2 (Dv2), thereby abolishing the *destruction complex*. Further, in a mouse model that expressed both transgenic ATDC and mutant

Kras driven by a pancreas-specific promoter p48-Cre, ATDC was found to induce the epithelial-to-mesenchymal transition (EMT) and metastasis via β-catenin [29, 34]. It has also been reported that Wnt ligand agonists Sulfatase 1 (SULF-1) and SULF-2 are overexpressed in PDAC [35]. Other developmental pathways, such as the Notch and Hedgehog pathways, have also been shown to cause increased nuclear/cytosolic β-catenin in PDAC [36]. Thus, while β-catenin alone is unable to initiate pancreatic tumorigenesis, canonical Wnt signaling is active in PDAC and contributes to the EMT and metastasis. The temporal regulation of activation of the Wnt pathway appears critical considering that β-catenin has been reported to actively suppresses Kras-mediated tumorigenesis by acinar cell regeneration in a mouse model of pancreatitis [37]. **Figure 1** pictorially summarizes the mechanism by which the Wnt/β-catenin pathway is dysregulated in both PDAC and CRC.

6. Mucins and their roles in cancer

Mucins are heavily O-glycosylated proteins that are normally expressed in the epithelial lining of the lungs, and gastrointestinal and reproductive tracts [38]. Mucins can be broadly categorized as follows: (i) membrane-bound/trans-membrane mucins, which include MUC1, MUC3A/MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC21, (ii) secreted (gel-forming) mucins, which include MUC2, MUC5AC, MUC5B, MUC6, and MUC19, and (iii) soluble (non-gel-forming) mucins, which include MUC7, MUC8, MUC9, and MUC20 [38]. The normal functions of mucins involve protection of the epithelial surfaces via entrapment of pathogens, which is primarily a function of secreted mucins [39]. The transmembrane mucins can also be involved in cell signaling [39]. Thus, mucins are critical in maintaining cellular functions, particularly those of epithelial surfaces.

While critical in maintaining and protecting the normal epithelium, decades of research have revealed that a number of mucins are aberrantly expressed in cancer. For instance, the transmembrane mucin MUC4, which is normally absent in the pancreas, is aberrantly

overexpressed in PDAC [40]. Here, MUC4 has been shown to act as a binding partner for HER2 and thereby play a role in promoting metastasis, cell proliferation, and invasion [41-43]. Furthermore, MUC4 can interact with secreted endothelial proteins such as Galectin [44], thus aiding in the invasion and metastasis of PDAC cells. Likewise, mucins MUC1, MUC16, and MUC5AC are also overexpressed in PDAC [39].

Interestingly, while some mucins are aberrantly overexpressed in cancer, expression of other mucins decreases in certain malignancies. For instance, expression of the secreted-mucin MUC2, which comprises most of the secreted mucus layer in the colon, is markedly reduced in most CRCs, with certain notable exceptions, such as mucinous CRCs [45, 46]. The role played by MUC4 in CRC is, however, controversial [47, 48]. For example, while some reports suggest that MUC4 is lost as CRC progresses [47], another report indicates that MUC4 expression is enhanced in a subset of patients where it confers a poorer prognosis [48]. No correlation between high MUC4 expression and MSI or mucinous status of CRC has been observed [23]. MUC1 is expressed in both normal and cancerous colons, but its expression increases in CRC and strongly confers with disease progression [49]. As with MUC4, MUC1 expression does not correlate with MSI status [23]. Other mucins, such as MUC5AC, which is not normally expressed in the colon, are also aberrantly overexpressed in CRC [50]. The *de novo* expression of MUC5AC is more frequently observed in mucinous and MSI-high tumors [51, 52]. Thus, despite varying expression levels in disparate malignancies, the importance of mucins in disease progression is evident.

Given their role in promoting cancer progression, mucins have been proposed to be important diagnostic and prognostic markers. Consequently, the mechanisms by which these molecules promote and/or suppress the progression of cancer have been subject of intense investigation. In this regard, a number of studies have focused on the interaction of β-catenin and mucins. Most prominently, the cytoplasmic tail of the transmembrane mucin MUC1 has been shown to interact with β-catenin in various malignancies and aid in the nuclear localization of the

molecule [53]. Other mucins, like MUC16 and MUC4, have also been shown to influence the localization and/or stabilization of β-catenin [54, 55].

While many studies have shown the regulation of β-catenin by mucins, the Wnt/ β-catenin has also been shown to regulate mucin expression. In CRC, where the β-catenin pathway is a driving force, β-catenin has been shown to suppress mucin expression. A siRNA targeting β-catenin resulted in the loss of mucin expression, as measured by alcian blue staining, in the CRC cell line LS174T [56]. Activation of β-catenin has been shown to result in the loss of colonocyte differentiation, resulting in a crypt progenitor phenotype in CRC [57]. The loss of mucin expression is among the myriad of changes associated with this de-differentiated phenotype. Mucin-depleted foci, first identified in rats treated with the carcinogen azoxymethane (AOM), are pre-cancerous lesions in CRC characterized by both aberrant β-catenin signaling and loss of mucins, [58]. Mucin-depleted foci have also been described in human colon tissue samples [59]. Thus, a number of studies have implied that the loss of certain mucins are a consequence of Wnt/ β-catenin pathway activation in CRC. The following sections of the chapter summarize the diverse, and occasionally contradictory, roles played by mucins and β-catenin in cancer.

7. The relationship between β-catenin and membrane-bound mucins in cancer, MUC1, MUC4 and MUC16

(a) MUC1

The relationship between MUC1 and β-catenin has been extensively studied in various malignancies. The MUC1 cytoplasmic domain (CD) has been shown to possess a serine-rich motif (SXXXXXSSL) required for the binding of β-catenin [60], which is used to bind the Armadillo repeat domain of β-catenin, thus preventing the phosphorylation induced degradation of β-catenin by GSK3β. Cleavage of the MUC1 CD has been demonstrated to occur through γ-secretase, thus untethering the cytoplasmic tail from the membrane [61]. In addition, the MUC1 CD possesses a binding site for GSK3β, at the STDRSPYEKV site [62]. Phosphorylation by

GSK3β of the serine residue next to the proline at this site inhibits the MUC1-β-catenin interaction and stimulates the formation of the β-catenin-E-cadherin complex at the membrane. The MUC1 CD also possesses a phosphorylation site for the epidermal growth factor receptor (EGFR), which phosphorylates the tyrosine residue in the YEKV motif on the MUC1 CD, thus priming the tail for binding by the c-Src tyrosine kinase, which leads to increased β-catenin-MUC1 CD interaction [63, 64]. Protein kinase-C δ phosphorylates the tail at the TDR site, also leading to increased β-catenin binding to the MUC1 CD [63-65]. The MUC1CD-β-catenin complex can enter the nucleus, where it partners with TCF4 to up-regulate β-catenin target genes, such as cyclin D1 [66, 67]. Not only does MUC1 stabilize β-catenin, it also binds the nuclear co-factor TCF4, preventing binding of the repressive C-terminal binding proteins to TCF4 and recruiting transcriptional co-activators such as p300 on the cyclin D1 promoter [68]. The expression of MUC1 has been linked to the Wnt target gene Cyclin D1 in a number of cancers, such as breast cancer [68], *H. pylori*-induced gastric cancer [66], and PDAC [67, 69].

The expression of MUC1 and aberrant β-catenin at the invasive front in gastric cancer and CRC has been shown to be independent predictors of poorer prognoses [70, 71]. The MUC1-β-catenin interaction is implicated in inducing invasion and EMT in breast, renal, gastric, and pancreatic cancers [68, 72, 73]. In pancreatic cancer, the seven tyrosine residues present in the MUC1 cytoplasmic tail were found to be critical for its interaction with β-catenin and mediation of EMT [73]. In mouse NIH3T3 fibroblast cells, the interaction between Galectin-3 and the N-terminal domain of MUC1 was found to trigger recruitment of β-catenin to the C-terminus of MUC1 [74]. In renal carcinoma, the MUC1-β-catenin complex has been found to directly bind the Zinc finger protein SNAI1 (SNAIL) promoter, thus triggering EMT and invasion [72, 75]. Additionally, the KL6 variant of MUC1 has been found to exacerbate metastasis of PDAC through interactions with β-catenin [76]. Further, the MUC1 cytoplasmic tail has been shown to interact with APC in some breast cancer cell lines and in human metastatic breast cancer tissue

[77]. Moreover, MUC1 was found to aid in the nuclear localization of β-catenin in CRC [78]. Thus, multiple lines of evidence show a direct relationship between MUC1 and EMT, metastasis and progression of various cancers.

In contrast to the aforementioned findings, in HEK293 cells, MUC1 has been implicated in suppressing the proliferation of cancer by preventing nuclear localization of β-catenin [79], thus contradicting a number of studies. The MUC1- β-catenin interaction may also promote cancer progression without necessitating the nuclear localization of β-catenin. For example, in breast cancer, the deletion of MUC1 in MMTV-Wnt-1 transgenic mice prolonged the time required for tumor formation [80]. However, the MUC1-β-catenin complex was observed in the membrane and cytosol of wild-type mice, as opposed to the nucleus. Further, the MUC1-β-catenin complex was present at the invading edge of the cell membrane connecting to the collagenous matrix; this complex co-localized with the focal adhesion proteins fascin and vinculin, thus presumably aiding in invasion and metastasis despite preventing the nuclear localization of β-catenin. Accordingly, MUC1-β-catenin interactions were found to be greatly enriched in metastatic tumors [80].

In summary, MUC1-β-catenin interactions may either (a) promote nuclear localization of β-catenin, thereby upregulating numerous EMT-, metastasis-, and proliferation-related genes or (b) prevent nuclear localization of β-catenin by sequestering it at the membrane/cytoskeleton. It has been suggested that the relative abundance of these two proteins may determine which path is followed [79]. A recent study of PDAC observed that the MUC1-β-catenin regulation of cyclin D1 requires the presence of p120 catenin, which sequesters the transcriptional repressor Kaiso. This observation suggests that the relative abundance of various p120 isoforms determines the ability of MUC1-β-catenin to activate gene transcription [67]. Thus, while the MUC1- β-catenin interaction possesses the ability to promote EMT and metastasis, several variables such as the

relative abundance of MUC1/ β-catenin and the presence of requisite isoforms of as p120 catenin influence the MUC1- β-catenin dynamic.

(b) MUC4

While the relationship between MUC1 and β-catenin has been extensively studied, the potential relationship between β-catenin and other membrane-bound mucins, such as MUC4, is less known. In PDAC, MUC4 induces the dissociation of β-catenin from E-cadherin, by triggering lysosomal degradation of E-cadherin via HER2/Src/FAK signaling, and thereby causes nuclear localization of β-catenin [54]. Gao *et al.* proposed that MUC4 can also inhibit nuclear localization of β-catenin in lung cancer, where MUC4 plays a protective role [81]. MUC4 may also be governed by β-catenin. For example, a recent study, using genetically ablated β-catenin by zinc finger nucleases in the PDAC cell line BXPC3, applied a subsequent microarray to demonstrate that MUC4 was one of the most significantly down-regulated genes upon the depletion of β-catenin (Supplementary Table 3 of paper by Olson *et al.*) [82]. Moreover, whole-exome sequencing of a case of osteosarcoma showed that the Wnt/β-catenin pathway is an important disease driver and that MUC4 was up-regulated, hinting at a regulatory relationship between β-catenin and MUC4 [83].

A number of other studies also support the existence of a β-catenin- MUC4 regulatory relationship. A study by Hashimoto *et al.*, which examined the role of β-catenin in developing lungs, used a lung-specific rCCSP-Cre recombinase in mice and found that when a constitutively active (exon 3-deleted) β-catenin was overexpressed, MUC4 transcript levels were significantly increased in the bronchial epithelium [84]. Our *MUC4*-promoter analysis using the MatInspector (Genomatix) software showed presence of 3’ putative TCF/LEF binding sites (one in the proximal promoter and two in the distal promoter) in the *MUC4* promoter. Additional studies conducted in our laboratory further suggest that, in PDAC, MUC4 is a direct transcriptional target of β-catenin [85].

Overall, most studies thus far indicate that the Wnt/β-catenin pathway likely regulates the expression of MUC4. Furthermore, MUC4 may regulate nuclear localization of β-catenin through its interactions with HER2, which then triggers a cascade of signaling events that culminate in the Src-mediated phosphorylation of E-cadherin and result in the release of β-catenin from E-cadherin. However, MUC4 has also been shown to prevent the nuclear localization of β-catenin in lung cancer through a mechanism that has not yet been delineated [81], thus contributing to a certain degree of uncertainty in the field.

(c) MUC16

MUC16 is a membrane-bound mucin that is upregulated in various cancers, including ovarian, pancreatic, and breast cancers [86]. The first report suggesting an interaction between MUC16 and β-catenin was published by Comamala *et al.* in 2011; here, MUC16 was shown to interact with β-catenin in the ovarian carcinoma cell line OVCAR3 [87]. Comamala *et al.* proposed that MUC16 interacts with E-cadherin and β-catenin, thus ensuring their membrane localization and preventing EMT. This co-related with the fact that expression of MUC16 is lost in late-stage, metastatic ovarian cancer. A different study by Akita *et al.* focused on the interaction between the cytoplasmic tail of MUC16 and β-catenin in a colon cancer cell line, HCT116. It was determined that overexpression of the MUC16 cytoplasmic tail resulted in reduced expression of membranous E-cadherin and β-catenin. This reduced expression was attributed to the increased recruitment of Src family kinases to the membrane, which in turn caused degradation of E-cadherin and dissociation of β-catenin from E-cadherin [88].

In 2014, Giannakouros *et al.* proposed that the MUC16- β-catenin complex promotes the formation of multicellular aggregates. These aggregates precede dissemination of ovarian cancer cells and the MUC16-β-catenin interaction tethers β-catenin at the membrane, preventing phosphorylation-mediated degradation of β-catenin by GSK-3β [55]. Thus, while most studies indicate that the MUC16 cytoplasmic tail does indeed interact with β-catenin, the role played by

the MUC16-β-catenin complex appears to be context-dependent, and either causes increased cytosolic/nuclear localization of β-catenin or prevents the nuclear localization and enhances the E-cadherin binding propensities of β-catenin.

8. The relationship between β-catenin in cancer and secreted-mucins MUC2, MUC5AC, and MUC6

MUC2 is the most abundantly secreted mucin in the intestines, produced primarily by goblet cells [47]. Notably, unlike most colorectal carcinomas in humans, *Muc2*$^{-/-}$ mice develop colorectal carcinomas in the absence of any other mutations [89, 90] and without aberrantly localized β-catenin. However, when *Muc2*$^{-/-}$ mice were crossed with *Apc*$^{1638N/+}$ or *Apc*$^{Min/+}$ mice in a study by Yang *et al.*, increased tumor formation occurred in the distal colon [91]. This is unlike mice for which the *Apc* gene alone has been mutated or lost, and tumor lesions are located primarily in the small intestine [91]. Importantly, these tumors in the distal colon showed an increase in aberrant Wnt/β-catenin signaling, which suggests that the loss of MUC2 acts in concert with Wnt/ β-catenin signaling to cause CRC [91]. Because these mice also showed signs of an inflammatory response in their tumors, it was suggested that loss of MUC2 results in tumors through an inflammatory mechanism that complements activation of the Wnt pathway [91]. Interestingly, in colon carcinomas, β-catenin has also been shown to negatively regulate MUC2 expression [92]. This negative regulation of MUC2 was found to be driven by Sox9, which in turn is upregulated by β-catenin [93]. Another mechanism of down-regulation of MUC2 by β-catenin involves Hath-1, a transcription factor that is proteasomally degraded by active Wnt/β-catenin signaling [94]. Hath-1 up-regulates MUC2 expression [92] and is repressed by the Wnt/β-catenin pathway [94]. Other mechanisms such as MUC2 promoter methylation have also been shown to contribute to the MUC2 mucin loss in CRC [95]. In conclusion, the loss of MUC2 plays an important role in CRC progression and the Wnt/β-catenin pathway aids in precipitating

the loss of MUC2 expression. The loss of MUC2 may promote inflammatory responses which exacerbate the severity of the disease.

The gastric, gel-forming, secreted-mucin MUC5AC is aberrantly overexpressed in colorectal carcinoma [47], PDAC [39] and in certain infections, including the infection caused by the bacterial pathogen *Shigella dysenteriae* [96]. Some studies suggest that β-catenin can regulate MUC5AC. It has been shown that *S. dysenteriae* stimulates secretion of interleukin-1β, which in turn causes Trefoil factor 3 to stimulate the Akt pathway by phosphorylating the EGF receptor [96]. The Akt pathway then potentiates the nuclear localization of β-catenin, which in turn upregulates MUC5AC [96]. In rats treated with 1, 2-dimethylhydrazine, a carcinogen, it was seen that crypts that contained aberrant localization of β-catenin showed progressively increasing levels of MUC5AC (33% immunopositivity at eight weeks and 90% immunopositivity at 36 weeks) concurrent with progressively decreasing levels of MUC2 [97]. A study by Mucenski *et al.* used transgenic mice that constitutively overexpressed transcriptionally active, exon 3-deleted β-catenin, which was achieved using doxycycline-regulated Cre recombinase regulated by the lung-specific rat Clara cell secretory protein (rCCSP) promoter. Here, it was observed that mice displayed goblet cell dysplasia and increased MUC5AC expression [98]. All the aforementioned studies suggest that MUC5AC expression is likely governed by β-catenin, although the precise manner in which β-catenin regulates MUC5AC has not yet been studied.

Concurrent with findings that suggest a regulatory relationship between β-catenin and MUC5AC, it has also been found that MUC5AC can increase nuclear accumulation of β-catenin. Specifically, a study by Inaguma *et al.,* which investigated the effect of the Hedgehog pathway effector GLI-1 in PDAC, determined that MUC5AC can prevent membranous accumulation of E-cadherin, and therefore untether β-catenin from the adherens junction complex and stimulate nuclear accumulation of β-catenin [99]. Thus, MUC5AC expression has been reported to be governed by Wnt/β-catenin signaling in *S. dysenteriae* infections in the colon, and possibly also

in colon cancer and in the developing lungs. On the other hand, MU5AC has also been shown to regulate β-catenin localization in pancreatic cancer.

The secreted-mucin MUC6, in conjunction with MUC1 and MUC2, is frequently associated with the presence of nuclear β-catenin in gastrointestinal type gastric cancer and has been proposed for a prognostic marker by Aihara *et al.* [100]. A separate study by Silva *et al.* found that patients with gastric cancer who were younger (less than or equal to 40 years old) were more likely to express MUC6, MUC5AC, and MUC2, as well as β-catenin, compared to older patients (above 40 years old) [101]. However, the prognostic value of these observations remains unclear.

9. Conclusions and future directions

Despite the proven importance of the Wnt/β-catenin pathway and mucins in regulating neoplastic transformation and malignant growth, a number of questions remain unanswered. While the MUC1-β-catenin relationship is the most well delineated of all mucin-β-catenin relationships, the determinants or context that governs whether the MUC1-β-catenin complex enters the nucleus or remains bound to members of the cytoskeleton have not yet been identified. Notably, another transmembrane mucin, MUC16, has been shown to interact with β-catenin. While the cleavage and nuclear localization of the MUC16 cytoplasmic tail has been demonstrated [102], it is not known whether the β-catenin-bound MUC16 cytoplasmic tail can also enter the nucleus. Also, the role played by the MUC16-CT-β-catenin complex in ovarian cancer, where it prevents EMT by sequestering β-catenin at the membrane, and in colon and breast cancers, where it enhances EMT by enabling cytosolic/nuclear localization of β-catenin, is lacking sufficient explanation. A similar pattern has been observed for MUC4, where MUC4 prevents β-catenin nuclear localization in lung cancer but enhances nuclear localization in pancreatic cancer.

Another stream of research focuses on the regulation of mucin expression by β-catenin. This field is replete with findings reflective of the disparate roles played by the collusion of mucins and β-catenin in various malignancies. For example, the depletion of β-catenin in the Ptf1a-Cre; $Kras^{G12D}$; $\beta\text{-catenin}^{f/f}$, as well as inhibition of β-catenin in the Ptf1a-Cre; LSL-$Kras^{G12D}$; Rosa26rtTa/+; TetO-Dkk1 mouse models of PDAC, resulted in reduced overall mucin staining compared to KC (Ptf1a-Cre;$Kras^{G12D}$) mice, hinting at a regulatory β-catenin–mucin relationship [32]. While this is not conclusive proof of a direct β-catenin-mucin relationship, a study that performed a microarray on β-catenin-null PDAC cells found that MUC4 was one of the top significantly down-regulated transcripts, implying that MUC4 is regulated by β-catenin [82]. Studies from our laboratory support direct β-catenin-mediated up-regulation of the MUC4 transcript in pancreatic cancer [85]. In CRC, however, β-catenin has been shown to repress mucin expression. The β-catenin-mediated repression of MUC2 in CRC occurs via Sox9 up-regulation. Additional studies from our laboratory (unpublished data) indicate that β-catenin can repress the MUC4 transcript in CRC. The reasons for this seemingly contradictory relationship could stem from differing statuses of MUC4 promoter methylation in these two cancers. Studies by Yamada *et al.* showed using the CaCo2 CRC cell line negative for *MUC4* mRNA, that while the MUC4 promoter is methylated at key positions (CpG sites 108-112 in the proximal promoter), these sites are un-methylated in PDAC cell lines expressing *MUC4* [103]. A repressive histone code including deacetylated Histone 3 and trimethylated K27H3 in the *MUC4* 5' UTR is also present in MUC4-negative cell lines [104]. One possibility is that, given the wide disparity in the degree of Wnt/β-catenin pathway activity in PDAC and CRC (50 - 80% nuclear β-catenin in CRC versus 4 - 11% nuclear β-catenin in PDAC [5]), a different set of target genes are activated. A study by Hlubek *et al.* supports this possiblity. Here, it was observed that even within CRC tumors, varying degrees of nuclear β-catenin activate different target genes in the tumor center as opposed to the invasive front [105]. Another factor to consider is that while aberrant β-catenin is the driving force in CRC progression, dysregulation of β-catenin occurs at a later stage in the natural

history of PDAC. Thus, a number of other mutations are at play, possibly influencing methylation of the mucin promoter and other variables that affect the β-catenin-mucin relationship. Furthermore, β-catenin regulates a number of micro-RNAs (mi-RNAs) in CRC [106]. Interestingly, MUC4 has also been shown to be regulated by mi-RNAs such as miR-200c, miR-219-1-3p, and m-iR-150 [107-109], suggesting the possibility that β-catenin may indirectly regulate MUC4 via mi-RNAs. Further studies to address the potentially disparate roles played by β-catenin vis-à-vis MUC4 in these two cancers can delineate the precise relationship between β-catenin and MUC4.

The apparent discordance between mucin- β-catenin relationships in PDAC and CRC could be due to the distinct functions of secretory and membrane bound mucins in normal and cancerous conditions. The normal adult gastrointestinal system is characterized by specific levels of secretory and transmembrane mucins in every tract/organ. For instance, MUC2 is the predominant secretory mucin in the intestine, while MUC5AC is highly preponderant in the stomach [110]. The outer, loose layer of MUC2 harbors the intestinal microflora, while the dense inner MUC2 layer protects the colonic epithelium from microbial assault [110]. Transmembrane mucins such as MUC1, MUC3, MUC17, MUC13 and MUC4 form the protective glycocalyx while also participating in cell signaling events, although their functions in normal conditions are not as well studied [110]. The variable lengths of the extracellular portions of each mucin form a multi-tiered barrier to pathogen invasion [111]. This delicate balance between the levels and functions of transmembrane and secreted mucins is disrupted in cancer. In conjunction with aberrant β-catenin, it is likely that each transmembrane and secretory mucin plays a highly context-dependent role in cancer, based on its expression and role in the normal tissue, thus likely accounting for the disparate roles played by mucins and β-catenin in various malignancies.

Figure 2 and **Table 1** summarize the relationship between the Wnt/β-catenin pathway and mucins. Notably, the cytoplasmic tails of MUC1 and MUC16 have been shown to interact

with β-catenin. In both instances, the mucin-β-catenin complexes can either promote or repress tumor progression. The MUC1 cytoplasmic tail-β-catenin complex, in particular, plays an important role in various cancers. Likewise, in PDAC and lung cancer, MUC4 has been shown to affect β-catenin localization. In colorectal and pancreatic cancer, the Wnt/β-catenin pathway, on the other hand, can also regulate expression of MUC5AC, MUC2, and MUC4. Thus, mucins and the Wnt/β-catenin pathway have an intricate, mutually regulatory relationship that often culminates in cancer progression. As mentioned earlier, further studies are needed to determine the factors governing β-catenin-MUC1 mucin nuclear localization as well as the individual roles played by β-catenin-mucin complexes in different cancers. While it is apparent that β-catenin can govern mucin expression, both indirectly (*i.e.*, MUC2 in CRC) and directly (*i.e.*, MUC4 in pancreatic cancer- unpublished study), future studies are needed to explain the seemingly contradictory nature of the β-catenin-mucin relationship in various malignancies.

Table 1: The relationship between β-catenin and mucins in cancer				
Mucin	**Type**	**Cancer**	**Relationship with β-catenin**	**References**
MUC1	Transmembrane	PDAC	The cytoplasmic tail binds β-catenin, enters the nucleus, and up-regulates Cyclin D1, which induces EMT.	[67, 69]
		CRC	The cytoplasmic tail binds β-catenin, enters nucleus, and co-localizes with β-catenin at invasive front.	[71, 78]
		Breast cancer	The cytoplasmic tail binds β-catenin, enters nucleus, co-localizes with β-catenin at the invasive front.	[68]
MUC16	Transmembrane	Ovarian cancer	The cytoplasmic tail binds β-catenin, suppresses EMT by preventing cytosolic localization, and helps form multicellular aggregates that precede metastasis by tethering β-catenin to membrane.	[55, 87]
		CRC	The cytoplasmic tail binds β-catenin and recruits Src family kinases, thus triggering degradation of E-cadherin and enhancing cytosolic β-catenin	[88]
MUC4	Transmembrane	PDAC	MUC4 causes dissociation	[54]

		Lung cancer	of β-catenin from E-cadherin by HER2/Src/FAK signaling, enhances nuclear β-catenin, and is transcriptionally up-regulated by β-catenin. MUC4 prevents nuclear localization of β-catenin.	[81]
MUC5AC	Secreted, gel forming	PDAC CRC	Enhances nuclear β-catenin by disrupting E-cadherin at the membrane. β-catenin up-regulates MUC5AC in HT29 CRC cell line; nuclear β-catenin is associated with enhanced MUC5AC expression.	[99] [96, 97]
MUC2	Secreted, gel forming	CRC	β-catenin represses MUC2; loss of MUC2 co-operates with β-catenin signaling to aid in cancer progression.	[91-94]
MUC6	Secreted, gel forming	Gastric cancer	Expression frequently associated with nuclear β-catenin; co-expression is prognostic marker.	[100, 101]

Fig.1. Mechanisms of Wnt/β-catenin up-regulation in CRC and PDAC (A.) In CRC, mutations in Adenomatous polyposis coli (APC; most common), β-catenin, and Axin1 contribute to increased nuclear β-catenin. (B.) In pancreatic ductal adenocarcinoma (PDAC), no mutations in canonical pathway members are typically found. However, an increase in the WNT 7B ligand, SULF1, and SULF2, which enhance Wnt ligand binding and an increased expression of the ataxia telangiectasia group D complementing gene (ATDC), inhibit formation of the destruction complex and work together to precipitate cytosolic/nuclear β-catenin. In addition, other developmental pathways, such as Notch and Hedgehog, can contribute to the pool of nuclear β-catenin.

Figure 1

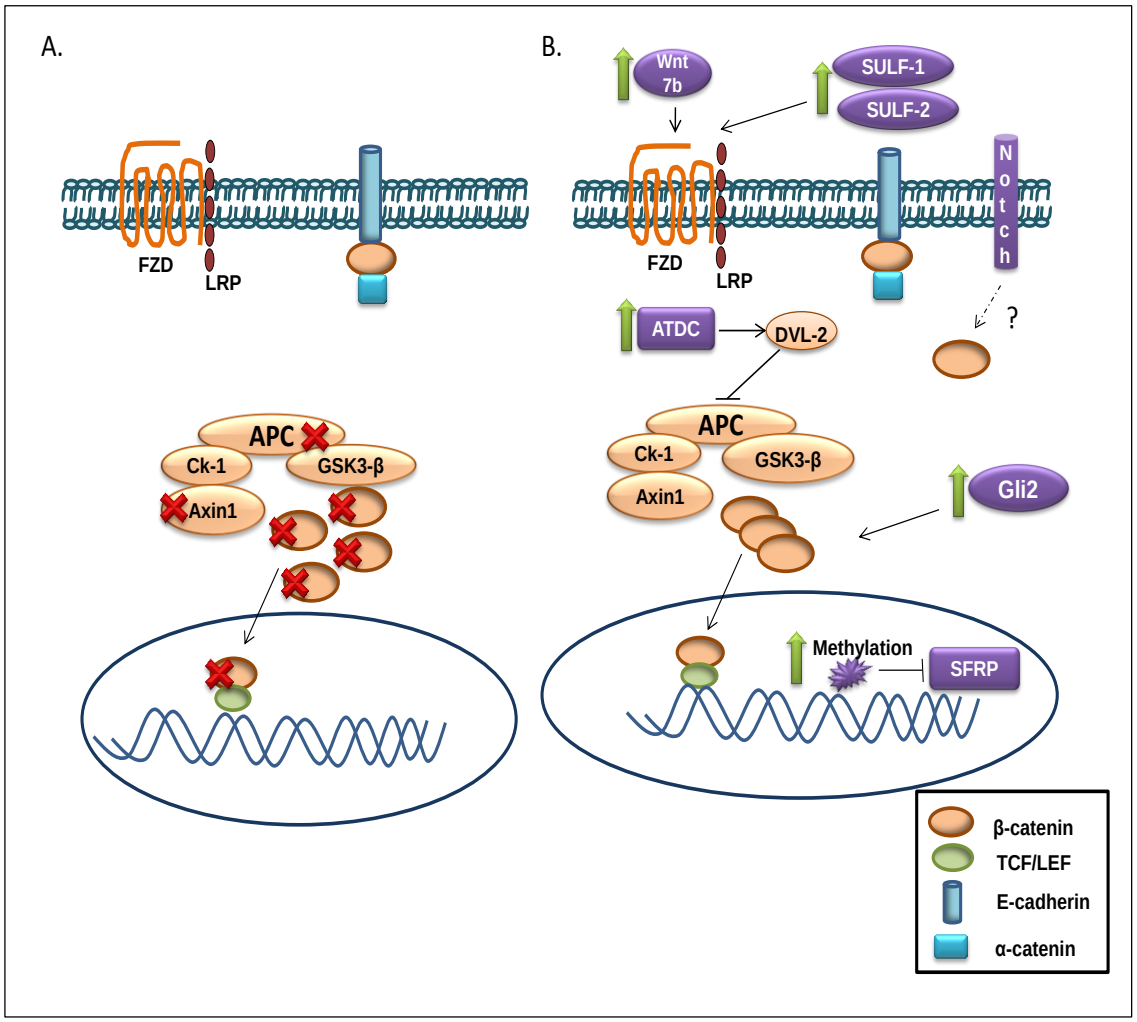
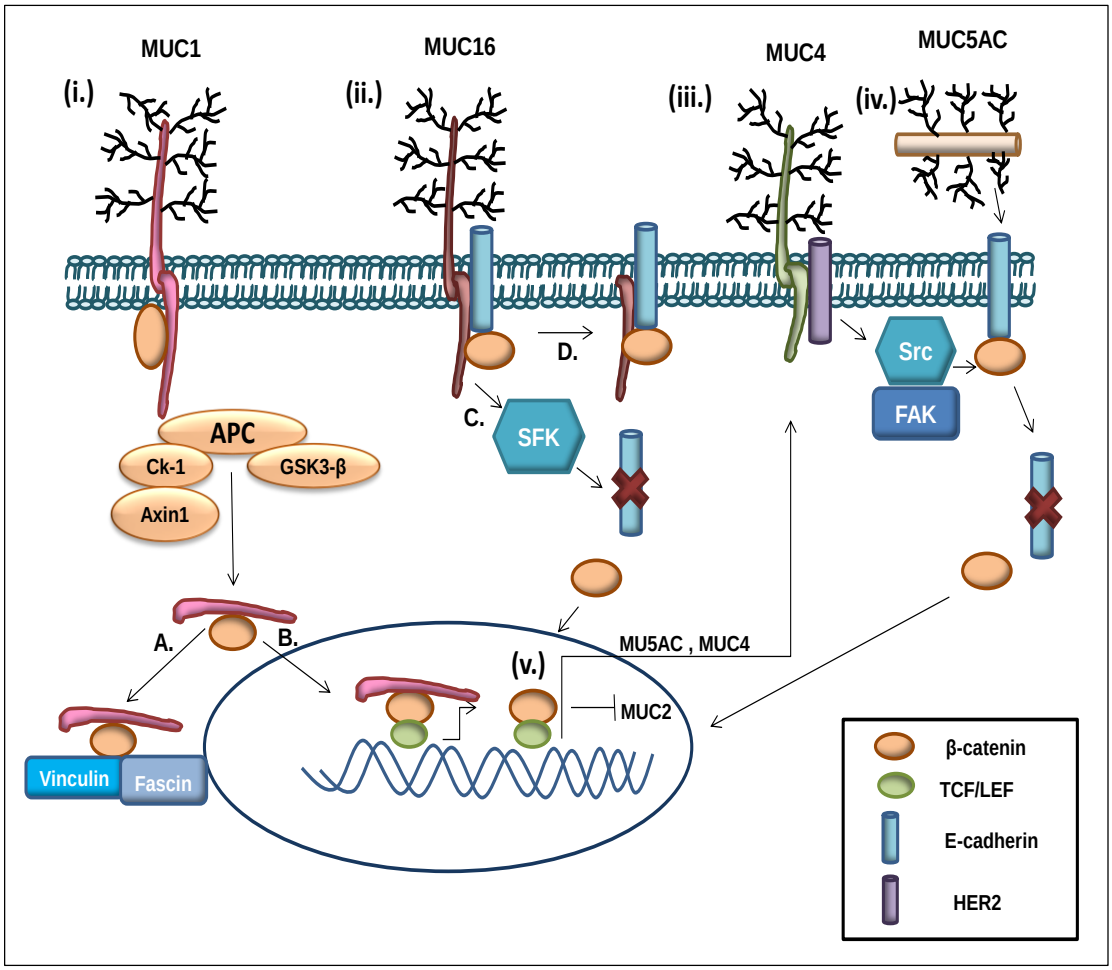

A.
FZD
LRP
APC
Ck-1
GSK3-β
Axin1
B.
Wnt 7b
SULF-1
SULF-2
FZD
LRP
Notch
?
ATDC
DVL-2
APC
Ck-1
GSK3-β
Axin1
Gli2
Methylation
SFRP
β-catenin
TCF/LEF
E-cadherin
α-catenin

Fig.2. Schematic representation of β-catenin-mucin dynamics (i.) MUC1: The cytoplasmic tail of MUC1 binds to β-catenin at the serine-rich domain. Formation of the MUC1- β-catenin complex stabilizes β-catenin by preventing its phosphorylation-mediated degradation. Following cleavage of the cytoplasmic tail, the MUC1- β-catenin complex can either (A) localize adjacent to the membrane and bind cytoskeleton members Fascin and Vinculin, or (B) enter the nucleus, where MUC1 can also bind Transcription factor 7-like 2 (TCF7L2/TCF4) and aid in transcription of β-catenin target genes such as Cyclin D1. (ii.) The MUC16 cytoplasmic tail can bind both β-catenin and E-cadherin. This may either cause C., the recruitment of Src family kinases (SFKs) resulting in the degradation of E- cadherin and release of β-catenin in the cytosol or D., the stabilization of the β-catenin-E-cadherin complex. (iii.) MUC4 can act as a binding partner for HER2, triggering the activation of Src and FAK, which cause the lysosomal degradation of E-cadherin and release of β-catenin into the cytosol. (iv.) MUC5AC in the extracellular space can hinder the membranous localization of E-cadherin and stimulate cytosolic and nuclear β-catenin. (v.) β-catenin has been shown to transcriptionally upregulate MUC5AC and MUC4, and transcriptionally repress MUC2.

Figure 2


MUC1
MUC16
MUC4
MUC5AC
(i.)
(ii.)
(iii.)
(iv.)
D.
C.
SFK
Src
FAK
APC
Ck-1
GSK3-β
Axin1
A.
B.
(v.)
MU5AC , MUC4
MUC2
Vinculin
Fascin
β-catenin
TCF/LEF
E-cadherin
HER2

References

[1] Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55-67.

[2] Valenta T, Hausmann G, Basler K. The many faces and functions of beta-catenin. EMBO J 2012;31:2714-36.

[3] Polakis P. The many ways of Wnt in cancer. Curr Opin Genet Dev 2007;17:45-51.

[4] Polakis P. Wnt signaling in cancer. Cold Spring Harb Perspect Biol 2012;4:a008052.

[5] White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. Gastroenterology 2012;142:219-32.

[6] Jamieson C, Sharma M, Henderson BR. Targeting the beta-catenin nuclear transport pathway in cancer. Semin Cancer Biol 2014;27:20-9.

[7] Nusslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. Nature 1980;287:795-801.

[8] Coates JC, Grimson MJ, Williams RS, Bergman W, Blanton RL, Harwood AJ. Loss of the beta-catenin homologue aardvark causes ectopic stalk formation in Dictyostelium. Mech Dev 2002;116:117-27.

[9] Lyashenko N, Winter M, Migliorini D, Biechele T, Moon RT, Hartmann C. Differential requirement for the dual functions of beta-catenin in embryonic stem cell self-renewal and germ layer formation. Nat Cell Biol 2011; 13:753-61.

[10] Schmeel LC, Schmeel FC, Kim Y, Endo T, Lu D, Schmidt-Wolf IG. Targeting the Wnt/beta-catenin pathway in multiple myeloma. Anticancer Res 2013;33:4719-26.

[11] Geyer FC, Lacroix-Triki M, Savage K, et al. beta-Catenin pathway activation in breast cancer is associated with triple-negative phenotype but not with CTNNB1 mutation. Mod Pathol 2011;24:209-31.

[12] Damsky WE, Curley DP, Santhanakrishnan M, et al. beta-catenin signaling controls metastasis in Braf-activated Pten-deficient melanomas. Cancer Cell 2011;20:741-54.

[13] Salahshor S, Woodgett JR. The links between axin and carcinogenesis. J Clin Pathol 2005;58:225-36.

[14] Su MC, Huang WC, Lien HC. Beta-catenin expression and mutation in adult and pediatric Wilms' tumors. APMIS 2008;116:771-8.

[15] Kongkham PN, Northcott PA, Croul SE, Smith CA, Taylor MD, Rutka JT. The SFRP family of WNT inhibitors function as novel tumor suppressor genes epigenetically silenced in medulloblastoma. Oncogene 2010; 29:3017-24.

[16] Vincent A, Omura N, Hong SM, Jaffe A, Eshleman J, Goggins M. Genome-wide analysis of promoter methylation associated with gene expression profile in pancreatic adenocarcinoma. Clin Cancer Res 2011;17:4341-54.

[17] Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011;6:479-507.

[18] Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. Gastroenterology 2010;138:2059-72.

[19] Wilson PM, Labonte MJ, Lenz HJ. Molecular markers in the treatment of metastatic colorectal cancer. Cancer J 2010;16:262-72.

[20] Janssen KP, Alberici P, Fsihi H, et al. APC and oncogenic KRAS are synergistic in enhancing Wnt signaling in intestinal tumor formation and progression. Gastroenterology 2006;131:1096-109.

[21] Jass JR, Barker M, Fraser L, et al. APC mutation and tumour budding in colorectal cancer. J Clin Pathol 2003;56:69-73.

[22] Lovig T, Meling GI, Diep CB, et al. APC and CTNNB1 mutations in a large series of sporadic colorectal carcinomas stratified by the microsatellite instability status. Scand J Gastroenterol 2002;37:1184-93.

[23] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Mucin core protein expression in colorectal cancers with high levels of microsatellite instability indicates a novel pathway of morphogenesis. Clin Cancer Res 2000;6:1909-16.

[24] Debunne H, Ceelen W. Mucinous differentiation in colorectal cancer: molecular, histological and clinical aspects. Acta Chir Belg 2013;113:385-90.

[25] Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77-87.

[26] Zeng G, Germinaro M, Micsenyi A, et al. Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma. Neoplasia 2006;8:279-89.

[27] Lowe AW, Olsen M, Hao Y, et al. Gene expression patterns in pancreatic tumors, cells and tissues. PLoS One 2007;2:e323.

[28] Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 2008;321:1801-6.

[29] Collins MA, Pasca di MM. Kras as a key oncogene and therapeutic target in pancreatic cancer. Front Physiol 2014;4:407.

[30] Hingorani SR, Petricoin EF, Maitra A, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003;4:437-50.

[31] Heiser PW, Cano DA, Landsman L, et al. Stabilization of beta-catenin induces pancreas tumor formation. Gastroenterology 2008;135:1288-300.

[32] Zhang Y, Morris JP, Yan W, et al. Canonical wnt signaling is required for pancreatic carcinogenesis. Cancer Res 2013;73:4909-22.

[33] Arensman MD, Kovochich AN, Kulikauskas RM, et al. WNT7B mediates autocrine Wnt/beta-catenin signaling and anchorage-independent growth in pancreatic adenocarcinoma. Oncogene 2014;33:899-908.

[34] Wang L, Yang H, Abel EV, et al. ATDC induces an invasive switch in KRAS-induced pancreatic tumorigenesis. Genes Dev 2015;29:171-83.

[35] Nawroth R, van ZA, Cervantes S, McManus M, Hebrok M, Rosen SD. Extracellular sulfatases, elements of the Wnt signaling pathway, positively regulate growth and tumorigenicity of human pancreatic cancer cells. PLoS One 2007;2:e392.

[36] Morris JP, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. Nat Rev Cancer 2010;10:683-95.

[37] Morris JP, Cano DA, Sekine S, Wang SC, Hebrok M. Beta-catenin blocks Kras-dependent reprogramming of acini into pancreatic cancer precursor lesions in mice. J Clin Invest 2010;120:508-20.

[38] Andrianifahanana M, Moniaux N, Batra SK. Regulation of mucin expression: mechanistic aspects and implications for cancer and inflammatory diseases. Biochim Biophys Acta 2006;1765:189-222.

[39] Kaur S, Kumar S, Momi N, Sasson AR, Batra SK. Mucins in pancreatic cancer and its microenvironment. Nat Rev Gastroenterol Hepatol 2013;10:607-20.

[40] Andrianifahanana M, Moniaux N, Schmied BM, et al. Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res 2001;7:4033-40.

[41] Rachagani S, Macha MA, Ponnusamy MP, et al. MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1. Carcinogenesis 2012;33:1953-64.

[42] Senapati S, Gnanapragassam VS, Moniaux N, Momi N, Batra SK. Role of MUC4-NIDO domain in the MUC4-mediated metastasis of pancreatic cancer cells. Oncogene 2012;31:3346-56.

[43] Singh AP, Moniaux N, Chauhan SC, Meza JL, Batra SK. Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis. Cancer Res 2004;64:622-30.

[44] Senapati S, Chaturvedi P, Chaney WG, et al. Novel INTeraction of MUC4 and galectin: potential pathobiological implications for metastasis in lethal pancreatic cancer. Clin Cancer Res 2011;17:267-74.

[45] Imai Y, Yamagishi H, Fukuda K, Ono Y, Inoue T, Ueda Y. Differential mucin phenotypes and their significance in a variation of colorectal carcinoma. World J Gastroenterol 2013;19:3957-68.

[46] Ogata S, Uehara H, Chen A, Itzkowitz SH. Mucin gene expression in colonic tissues and cell lines. Cancer Res 1992;52:5971-8.

[47] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Immunohistochemical staining patterns of MUC1, MUC2, MUC4, and MUC5AC mucins in hyperplastic polyps, serrated adenomas, and traditional adenomas of the colorectum. J Histochem Cytochem 1999;47:1039-48.

[48] Shanmugam C, Jhala NC, Katkoori VR, et al. Prognostic value of mucin 4 expression in colorectal adenocarcinomas. Cancer 2010;116:3577-86.

[49] Kesari MV, Gaopande VL, Joshi AR, Babanagare SV, Gogate BP, Khadilkar AV. Immunohistochemical study of MUC1, MUC2 and MUC5AC in colorectal carcinoma and review of literature. Indian J Gastroenterol 2015;34:63-7.

[50] Kocer B, Soran A, Erdogan S, et al. Expression of MUC5AC in colorectal carcinoma and relationship with prognosis. Pathol Int 2002;52:470-7.

[51] Renaud F, Mariette C, Vincent A, et al. The serrated neoplasia pathway of colorectal tumors: Identification of MUC5AC hypomethylation as an early marker of polyps with malignant potential. Int J Cancer 2015; *in press*.

[52] Renaud F, Vincent A, Mariette C, et al. MUC5AC hypomethylation is a predictor of microsatellite instability independently of clinical factors associated with colorectal cancer. Int J Cancer 2015;136:2811-21.

[53] Kufe DW. MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. Oncogene 2013;32:1073-81.

[54] Zhi X, Tao J, Xie K, et al. MUC4-induced nuclear translocation of beta-catenin: a novel mechanism for growth, metastasis and angiogenesis in pancreatic cancer. Cancer Lett 2014;346:104-13.

[55] Giannakouros P, Comamala M, Matte I, Rancourt C, Piche A. MUC16 mucin (CA125) regulates the formation of multicellular aggregates by altering beta-catenin signaling. Am J Cancer Res 2014;5:219-30.

[56] van de Wetering M, Oving I, Muncan V, et al. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep 2003;4:609-15.

[57] van de Wetering M, Sancho E, Verweij C, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241-50.

[58] Caderni G, Femia AP, Giannini A, et al. Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. Cancer Res 2003;63:2388-92.

[59] Sakai E, Morioka T, Yamada E, et al. Identification of preneoplastic lesions as mucin-depleted foci in patients with sporadic colorectal cancer. Cancer Sci 2012;103:144-9.

[60] Yamamoto M, Bharti A, Li Y, Kufe D. Interaction of the DF3/MUC1 breast carcinoma-associated antigen and beta-catenin in cell adhesion. J Biol Chem 1997;272:12492-4.

[61] Julian J, Dharmaraj N, Carson DD. MUC1 is a substrate for gamma-secretase. J Cell Biochem 2009;108:802-15.

[62] Li Y, Bharti A, Chen D, Gong J, Kufe D. Interaction of glycogen synthase kinase 3beta with the DF3/MUC1 carcinoma-associated antigen and beta-catenin. Mol Cell Biol 1998;18:7216-24.

[63] Li Y, Kuwahara H, Ren J, Wen G, Kufe D. The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3 beta and beta-catenin. J Biol Chem 2001;276:6061-4.

[64] Li Y, Ren J, Yu W, et al. The epidermal growth factor receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and beta-catenin. J Biol Chem 2001;276:35239-42.

[65] Ren J, Li Y, Kufe D. Protein kinase C delta regulates function of the DF3/MUC1 carcinoma antigen in beta-catenin signaling. J Biol Chem 2002;277:17616-22.

[66] Udhayakumar G, Jayanthi V, Devaraj N, Devaraj H. Interaction of MUC1 with beta-catenin modulates the Wnt target gene cyclinD1 in H. pylori-induced gastric cancer. Mol Carcinog 2007;46:807-17.

[67] Liu X, Caffrey TC, Steele MM, et al. MUC1 regulates cyclin D1 gene expression through p120 catenin and beta-catenin. Oncogenesis 2014;3:e107.

[68] Rajabi H, Ahmad R, Jin C, et al. MUC1-C oncoprotein induces TCF7L2 transcription factor activation and promotes cyclin D1 expression in human breast cancer cells. J Biol Chem 2012;287:10703-13.

[69] Wen Y, Caffrey TC, Wheelock MJ, Johnson KR, Hollingsworth MA. Nuclear association of the cytoplasmic tail of MUC1 and beta-catenin. J Biol Chem 2003;278:38029-39.

[70] Retterspitz MF, Monig SP, Schreckenberg S, et al. Expression of beta-catenin, MUC1 and c-met in diffuse-type gastric carcinomas: correlations with tumour progression and prognosis. Anticancer Res 2010;30:4635-41.

[71] Baldus SE, Monig SP, Huxel S, et al. MUC1 and nuclear beta-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis. Clin Cancer Res 2004;10:2790-6.

[72] Gnemmi V, Bouillez A, Gaudelot K, et al. MUC1 drives epithelial-mesenchymal transition in renal carcinoma through Wnt/beta-catenin pathway and interaction with SNAIL promoter. Cancer Lett 2014;346:225-36.

[73] Roy LD, Sahraei M, Subramani DB, et al. MUC1 enhances invasiveness of pancreatic cancer cells by inducing epithelial to mesenchymal transition. Oncogene 2011;30:1449-59.

[74] Tanida S, Mori Y, Ishida A, Akita K, Nakada H. Galectin-3 binds to MUC1-N-terminal domain and triggers recruitment of beta-catenin in MUC1-expressing mouse 3T3 cells. Biochim Biophys Acta 2014;1840:1790-7.

[75] Bouillez A, Gnemmi V, Gaudelot K, et al. MUC1-C nuclear localization drives invasiveness of renal cancer cells through a sheddase/gamma secretase dependent pathway. Oncotarget 2014;5:754-63.

[76] Xu H, Inagaki Y, Seyama Y, et al. Expression of KL-6/MUC1 in pancreatic cancer tissues and its potential involvement in tumor metastasis. Oncol Rep 2011;26:371-6.

[77] Hattrup CL, Fernandez-Rodriguez J, Schroeder JA, Hansson GC, Gendler SJ. MUC1 can interact with adenomatous polyposis coli in breast cancer. Biochem Biophys Res Commun 2004;316:364-9.

[78] Wang Z, Sun J, Hu X, Huang S. Interference of mucin 1 inhibits progression of colon carcinoma by repression of Wnt/beta-catenin signaling. DNA Cell Biol 2014;33:162-70.

[79] Lillehoj EP, Lu W, Kiser T, Goldblum SE, Kim KC. MUC1 inhibits cell proliferation by a beta-catenin-dependent mechanism. Biochim Biophys Acta 2007;1773:1028-38.

[80] Schroeder JA, Adriance MC, Thompson MC, Camenisch TD, Gendler SJ. MUC1 alters beta-catenin-dependent tumor formation and promotes cellular invasion. Oncogene 2003;22:1324-32.

[81] Gao L, Liu J, Zhang B, et al. Functional MUC4 suppress epithelial-mesenchymal transition in lung adenocarcinoma metastasis. Tumour Biol 2014;35:1335-41.

[82] Olsen PA, Solberg NT, Lund K, et al. Implications of targeted genomic disruption of beta-catenin in BxPC-3 pancreatic adenocarcinoma cells. PLoS One 2014;9:e115496.

[83] Reimann E, Koks S, Ho XD, Maasalu K, Martson A. Whole exome sequencing of a single osteosarcoma case--integrative analysis with whole transcriptome RNA-seq data. Hum Genomics 2014;8:20.

[84] Hashimoto S, Chen H, Que J, et al. beta-Catenin-SOX2 signaling regulates the fate of developing airway epithelium. J Cell Sci 2012;125:932-42.

[85] Pai P, Rachagani S, Lakshmanan I, et al. The canonical Wnt pathway regulates the metastasis-promoting mucin MUC4 in pancreatic ductal adenocarcinoma. Mol Oncol 2015; *in press*.

[86] Haridas D, Ponnusamy MP, Chugh S, Lakshmanan I, Seshacharyulu P, Batra SK. MUC16: molecular analysis and its functional implications in benign and malignant conditions. FASEB J 2014;28:4183-99.

[87] Comamala M, Pinard M, Theriault C, et al. Downregulation of cell surface CA125/MUC16 induces epithelial-to-mesenchymal transition and restores EGFR signalling in NIH:OVCAR3 ovarian carcinoma cells. Br J Cancer 2011;104:989-99.

[88] Akita K, Tanaka M, Tanida S, Mori Y, Toda M, Nakada H. CA125/MUC16 interacts with Src family kinases, and over-expression of its C-terminal fragment in human epithelial cancer cells reduces cell-cell adhesion. Eur J Cell Biol 2013;92:257-63.

[89] Velcich A, Yang W, Heyer J, et al. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science 2002;295:1726-9.

[90] Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;131:117-29.

[91] Yang K, Popova NV, Yang WC, et al. Interaction of Muc2 and Apc on Wnt signaling and in intestinal tumorigenesis: potential role of chronic inflammation. Cancer Res 2008;68:7313-22.

[92] Leow CC, Romero MS, Ross S, Polakis P, Gao WQ. Hath1, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells. Cancer Res 2004;64:6050-7.

[93] Blache P, van de Wetering M, Duluc I, et al. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. J Cell Biol 2004;166:37-47.

[94] Tsuchiya K, Nakamura T, Okamoto R, Kanai T, Watanabe M. Reciprocal targeting of Hath1 and beta-catenin by Wnt glycogen synthase kinase 3beta in human colon cancer. Gastroenterology 2007;132:208-20.

[95] Vincent A, Perrais M, Desseyn JL, Aubert JP, Pigny P, Van S, I. Epigenetic regulation (DNA methylation, histone modifications) of the 11p15 mucin genes (MUC2, MUC5AC, MUC5B, MUC6) in epithelial cancer cells. Oncogene 2007;26:6566-76.

[96] Raja SB, Murali MR, Devaraj H, Devaraj SN. Differential expression of gastric MUC5AC in colonic epithelial cells: TFF3-wired IL1 beta/Akt crosstalk-induced mucosal immune response against Shigella dysenteriae infection. J Cell Sci 2012;125:703-13.

[97] Zoghbi S, Drouin E, Claustre J, Bara J, Scoazec JY, Plaisancie P. Intestinal MUC2 and gastric M1/MUC5AC in preneoplastic lesions induced by 1,2-dimethylhydrazine in rat: a sequential analysis. Int J Oncol 2007;30:489-97.

[98] Mucenski ML, Nation JM, Thitoff AR, et al. Beta-catenin regulates differentiation of respiratory epithelial cells in vivo. Am J Physiol Lung Cell Mol Physiol 2005;289:L971-L979.

[99] Inaguma S, Kasai K, Ikeda H. GLI1 facilitates the migration and invasion of pancreatic cancer cells through MUC5AC-mediated attenuation of E-cadherin. Oncogene 2011;30:714-23.

[100] Aihara R, Mochiki E, Nakabayashi T, Akazawa K, Asao T, Kuwano H. Clinical significance of mucin phenotype, beta-catenin and matrix metalloproteinase 7 in early undifferentiated gastric carcinoma. Br J Surg 2005;92:454-62.

[101] Silva EM, Begnami MD, Fregnani JH, et al. Cadherin-catenin adhesion system and mucin expression: a comparison between young and older patients with gastric carcinoma. Gastric Cancer 2008;11:149-59.

[102] Das S, Majhi PD, Al-Mugotir MH, Rachagani S, Sorgen P, Batra SK. Membrane proximal ectodomain cleavage of MUC16 occurs in the acidifying Golgi/post-Golgi compartments. Sci Rep 2015;5:9759.

[103] Yamada N, Nishida Y, Tsutsumida H, et al. Promoter CpG methylation in cancer cells contributes to the regulation of MUC4. Br J Cancer 2009;100:344-51.

[104] Vincent A, Ducourouble MP, Van S, I. Epigenetic regulation of the human mucin gene MUC4 in epithelial cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases. FASEB J 2008;22:3035-45.

[105] Hlubek F, Brabletz T, Budczies J, Pfeiffer S, Jung A, Kirchner T. Heterogeneous expression of Wnt/beta-catenin target genes within colorectal cancer. Int J Cancer 2007;121:1941-8.

[106] Schepeler T, Holm A, Halvey P, et al. Attenuation of the beta-catenin/TCF4 complex in colorectal cancer cells induces several growth-suppressive microRNAs that target cancer promoting genes. Oncogene 2012;31:2750-60.

[107] Lahdaoui F, Delpu Y, Vincent A, et al. miR-219-1-3p is a negative regulator of the mucin MUC4 expression and is a tumor suppressor in pancreatic cancer. Oncogene 2015;34:780-8.

[108] Radhakrishnan P, Mohr AM, Grandgenett PM, Steele MM, Batra SK, Hollingsworth MA. MicroRNA-200c modulates the expression of MUC4 and MUC16 by directly targeting their coding sequences in human pancreatic cancer. PLoS One 2013;8:e73356.

[109] Srivastava SK, Bhardwaj A, Singh S, et al. MicroRNA-150 directly targets MUC4 and suppresses growth and malignant behavior of pancreatic cancer cells. Carcinogenesis 2011;32:1832-9.

[110] Johansson ME, Sjovall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol 2013;10:352-61.

[111] Pelaseyed T, Bergstrom JH, Gustafsson JK, et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. Immunol Rev 2014;260:8-20.

CHAPTER 1 B

MUC4 regulation and expression patterns in pancreatic ductal adenocarcinomas (PDAC) and Colorectal Cancer (CRC)

1. Synopsis

Numerous studies have focused on factors regulating MUC4 expression. In particular, pancreatic cancer has been of interest to researchers due to the *de novo* expression of MUC4 in this malignancy. MUC4 has been shown to be regulated by various factors including epigenetic factors, transcriptional and post-transcriptional, as well as post-translational mechanisms. While most studies have used pancreatic cancer as their model system, other ailments such as breast, esophageal, colorectal as well as ulcerative colitis and Crohn's disease have also been studied. Here, I attempt to summarize studies on the epigenetic, transcriptional, post-transcriptional, and translational regulation of MUC4. Secondly, while studies on MUC4 in PDAC have been unequivocal in asserting that MUC4 is incrementally increased during PDAC progression in a *de novo* fashion, the MUC4 expression pattern in CRC is less clear, confounded by the fact that MUC4 is expressed by the normal healthy colon. In addition, the presence of a multitude of molecular subtypes in CRC likely leads to an additional level of complexity. Therefore a survey of literature examining the MUC4 expression patterns in colorectal cancer was performed and their findings have been summarized.

2. The regulation of *MUC4* expression

The *MUC4* promoter was first characterized by Perrais *et al* who established that the promoter is 3.7 kb long, with a long 5' UTR of 2.7 kb [1]. The first TATA box was found to be located at -2672/-2668, upstream of the ATG site. There were 2 highly transcriptionally active regions, located at -219/-1 and -2781/-2572 and binding sites for a number of transcription factors were found in the 5'UTR and proximal promoter. Four alternative transcription start sites have been identified, at positions −2603, −2604, −2605, and −199, lending variability to the length of the 5' UTR. Owing to the *de novo* expression of MUC4 in PDAC, many studies have focused on factors regulation *MUC4* in this disease. It has been observed that extensive promoter methylation in combination with a repressive histone code suppresses *MUC4* in non-expressing cells [1-3]. Furthermore, numerous spatio-temporally regulated transcription factors and inflammatory cytokines work in concert to regulate *MUC4* in development and disease [1]. Below, I discuss the epigenetic, transcriptional, post-transcriptional and translational factors regulating *MUC4* expression.

A. The epigenetic regulation of *MUC4* expression

The 5'UTR and the proximal promoter (3' of the TATA box) are both GC rich and thus show potential for methylation, while the distal promoter is not GC rich [1, 2]. In a study that examined the effect of histone acetylation and promoter methylation in pancreatic cancer cells, it was observed that methylation at five CpG sites in the 5' UTR, located at -81, -93, -102, -113 and -121 were critical for the transcriptional regulation of *MUC4* [2]. They used three cell lines that served as a model for different degrees of MUC4 expression; the pancreatic cancer cell line Panc1 (MUC4 negative), the gastric cancer cell line KATO-III (low MUC4 levels) and the pancreatic cancer cell line Capan1 (high MUC4 levels). It was observed that the vast majority of potential methylation sites in the proximal promoter and 5'UTR were highly methylated regardless of *MUC4* expression, with the exception of the aforementioned 5 sites [2].

Histone acetylation was also found to regulate *MUC4* expression; however, it was observed that when treated with the histone deacetylase inhibitor Trichostatin A (TSA), there was an increase in MUC4 only in MUC4 negative and low-expressing cell lines, while suppressing MUC4 high cell lines [2]. The hypothesis put forward to explain this discrepancy was that the promoter occupancy of the Sp1 transcription factor, which contributes to constitutive *MUC4* transcription, was reduced by TSA. In addition, DNMT1, 3A and 3B were found to be responsible for promoter methylation [2]. It was hypothesized that in non-expressing cells, promoter hypermethylation and histone deacetylation keep MUC4 suppressed, while in early stage cancer hypomethylation but no histone acetylation occurs, allowing for low levels of *MUC4*. Finally, in advanced stage cancer, both promoter hypomethylation and permissive chromatin allow for full *MUC4* transcription [2].

Another study by Yamada *et al* compared the methylation of the MUC4 promoter in pancreatic, breast, lung and colon cancer cell lines with varying levels of MUC4 [3]. Interestingly, it was found that regardless of cellular origin, methylation of five residues, from -170 to -102 in the 5' UTR was requisite for *MUC4* expression, with the exception of LS174T, where the promoter was hypomethylated at these sites despite very low *MUC4* expression [3]. However, two of these five residues differed from the ones identified by Vincent *et al*. This was ascribed to technical differences in the two studies. The rest of the potential methylation sites in the *MUC4* promoter were mostly methylated in all the cell lines studied.

While the aforementioned studies used cell lines to show methylation of the MUC4 promoter, a study by Zhu *et al* used a set of 57 pancreatic cancer tissues and performed methylation specific PCR on microdissected tissue to show that promoter hypomethylation incrementally increases from low grade PanINs (25% hypomethylation) to high grade PanINs and PDAC (80% hypomethylation) [4]. Recently, a study using breast cancer tissues showed that late stage, invasive breast cancer showed reduced MUC4 which corresponded to increased promoter hypomethylation [5]. Recently, a study from our lab has shown that NCOA3, a histone

acetyltransferase, binds the *MUC4* promoter, thus creating a favorable chromatin environment for *MUC4* inducing factors such as retinoic acid to increase *MUC4* transcription [6]. NCOA3 was also found to induce an increase in MUC4 stability by altering glycosylation related genes such as FUT8.

In conclusion, the epigenetic regulation of *MUC4* has been found to chiefly involve a combination promoter methylation of key residues in the 5' UTR and histone acetylation. A combination of both these factors is likely required for full *MUC4* transcription to occur.

B. Transcriptional regulation of *MUC4*

In the study where the *MUC4* promoter was first characterized [1], it was noted that the *MUC4* promoter (proximal and distal) contains numerous putative transcription factor binding sites for factors such as AP-1 and 2, Sp1 and 3, the GATA family of transcription factors as well as the STATs. It was observed that Sp1 could promote transcription, while Sp3 repressed MUC4 transcription, although the extent of activation/repression by these factors varied between cell lines. Furthermore, it was found that EGF and TGF-α were able to induce MUC4 transcription via intracellular tyrosine kinases. A synergistic up-regulation of *MUC4* transcription by IFN-γ and TNF-α as well as with TGF-α was found to occur in the Capan-2 cell line but not Capan-1. This was proposed to occur via the binding of STATs and/or NF-κB on the *MUC4* promoter. Protein Kinase C (PKC) was also found to up-regulate *MUC4.* Pancreatic cancer cell lines were used by this study.

More recently, the same group used a panel of lung, colon and pancreatic cancer cell lines to show that a number of developmentally important transcription factors such as hepatocyte nuclear factors (HNFs), GATAs, FOXAs and CDXs regulate *MUC4* [7], suggesting that MUC4 may play an important role in cytodifferentiation in the lung and intestine. This was supported by IHC staining of MUC4 in the developing mouse small intestine, lung, colon and stomach that showed MUC4 was expressed prior to and after cellular differentiation. It was observed that the

transcription factors CDX-1, CDX-2, FOXA1 and FOXA2 induced *MUC4* more strongly in colon cancer cell lines, while HNF-1α and HNF-1β induced *MUC4* in all cell lines. The factors HNF-4α, HNF-4β, FOXA2 and GATA-5 were proposed to induce *MUC4* in an indirect fashion. The binding sites for all these transcription factors were in the distal promoter and it was proposed that the spatio-temporal expression of transcription factors as well as indirect mechanisms involving other co-factors contribute to the differential expression of *MUC4* both developmentally and in various cancers.

Since the very first study where the *MUC4* promoter was characterized, most research has focused on the regulation of *MUC4* in pancreatic cancer. For instance, the TGF-β pathway has been shown to regulate *MUC4* transcription in PDAC [8], both via Smad2 and Smad4, which bind to Smad binding sites in the proximal and distal promoter, as well as through the PKC, PKA, PI3K and MAPK pathways in Smad4 negative PDAC cell lines. Another study used the PDAC cell line BXPC3 to show that MUC4 can also be regulated by CREB, Ets-1 and Elk-1[9].

As mentioned previously, pro-inflammatory cytokines such as IFN-γ can regulate *MUC4*. This regulation has been shown to be mediated by the JAK/STAT pathway as follows: IFN-γ binds the IFN-γ receptor (INGR), which in turn activates the JAK receptor, ultimately resulting in the phosphorylation of STAT-1 at Ser^{727}, which binds the *MUC4* promoter causing increased transcription [10]. Another secreted factor, retinoic acid, has also been shown to increase *MUC4* transcription by activating the TGFβ-2 pathway [11]. Interestingly, when PDAC cells were treated with both IFN-γ and retinoic acid, there was a synergistic effect on *MUC4* [12]. However, an alternate mechanism was found to be involved in this synergistic up-regulation [12]. It was found that retinoic acid partially represses STAT-1 induction while IFN-γ can inhibit TGFβ-2. It was proposed that STAT1 and RAR/RXR may act as transcriptional co-factors to up-regulate *MUC4*. A recent study has shown that nicotine can collaborate with IFN-γ and retinoic acid to induce *MUC4* and that all three of these entities converged on the E2F1 and STAT1, which

mediates *MUC4* up-regulation [13]. It was observed that nicotine required the presence of α7-nicotinic acetylcholine receptor subunit to mediate its effect on STAT1 [13]. These findings were corroborated by a study from our lab, which showed that nicotine could also activate *MUC4* via the JAK/STAT3 and MEK/ERK1/2 pathways [14]. All of the aforementioned studies on IFN-γ, retinoic acid and nicotine were conducted in PDAC cells. A very recent study in PDAC has demonstrated that Kras, the most commonly mutated gene in pancreatic cancer, up-regulates MUC4 via the MAPK/AP-1 and NF-κB pathways [15]. AP-1 and NF-κB were found to bind the *MUC4* proximal promoter as a consequence of the $Kras^{G12V}$ mutation [15].

While all studies thus far have focused on the transcriptional up-regulation of *MUC4* in PDAC, only one study has demonstrated the potential suppression of *MUC4* by a transcription factor in PDAC. Fauquette *et al* showed that the AP2-α transcription factor, which is a tumor suppressor in PDAC, binds the proximal promoter at sites in the -475/-238 region, thereby suppressing *MUC4* [16].

Some studies have also studied *MUC4* regulation in other malignancies such as esophageal cancer. Here, it has been demonstrated that bile acids and their various conjugates can up-regulate *MUC4* by stimulating the PI3K pathway [17]. Bile acids and their conjugates are present in the gastro-esophageal reflux that leads to Barrett's esophagus, a precursor to esophageal cancer [17]. It has also been demonstrated that the effect of bile acids on MUC4 is also mediated by HNF1-α, which binds the distal *MUC4* promoter [18]. In colon cancer, a recent study has shown that the epigenetic regulation of HNF4-α regulates MUC4 expression [19]. It was shown that the use of HDAC inhibitors and HDAC siRNAs caused a reduction in *HNF4-α*, thus indirectly reducing *MUC4*.

In conclusion, *MUC4* is governed by both spatio-temporally regulated transcription factors, as well as inflammation and disease related cytokines such as IFN-γ, nicotine and TNFα. In diseases such as PDAC, It is likely that a permissive chromatin environment in combination with

transcription factors that are possibly activated by factors such as IFN-γ, nicotine and TNFα, are required for *MUC4* transcription.

C. Post-transcriptional regulation of *MUC4*

The burgeoning field of microRNA (miRNA) research has shown that *MUC4*, like numerous other transcripts, is also regulated by miRNAs. Here also, most studies have focused on pancreatic cancer. Typically, miRNAs bind to a sequence in the 3'UTR of a transcript, targeting it for degradation or inhibiting translation [20]. The tumor suppressor miRNA-150 binds to a highly conserved sequence in the 3' UTR of *MUC4*, thereby suppressing translation [20]. This was associated with decreased pHER2, pFAK, pERK1/2, migration, and proliferation of PDAC cells. The miRNA-150 was found to be reduced in PDAC tissues [20].

Another miRNA, miR-200c, was shown to bind base pairs 820-842 in exon 1 of the *MUC4* transcript , causing reduced mRNA and protein levels [21]. The PDAC cell lines S2.028 and T3M4 were used in this study. Recently, another tumor suppressor miRNA in PDAC, miR-219-1-3p, has been shown to bind two sequences in the 3'UTR, causing a reduction in MUC4 protein [22].

D. Post-translational regulation of MUC4

The post-translational regulation of the rat homolog of MUC4, the Muc4/Sialomucin complex (SMC) has been extensively studied. The Muc4/SMC is composed of two non-covalently bound subunits, the O-glycosylated ASGP-1 and the transmembrane N-glycosylated ASGP-2 [23]. Studies in the lactating rat mammary gland showed that while the expression is tightly regulated at basal levels in the virgin and non-pregnant animal, there is a dramatic increase mid-pregnancy and during lactation [23]. The low basal levels of SMC were found to be due to a post-translational regulation of SMC by the TGF-β pathway via the intermediates Smad2/Smad4 by an indirect mechanism, wherein there was a transcriptional modulation of a gene required for the

post translational processing of SMC [23]. This gene was speculated to be a serine protease required for the cleavage of the two subunits. Subsequently, it was shown that TGF-β exerted its effects by the inhibition of the processing of the two subunits, ASGP-1 and ASGP-2, ultimately causing a proteasomal degradation of the protein precursor. This mechanism was followed in both cancer cells [24] as well as the rat corneal epithelium [25].

In a study which used a human pancreatic cancer cell line deficient in CFTR and a sub clone expressing functional CFTR, it was observed that functional CFTR suppressed MUC4 post translationally by reducing protein half-life in a cell-confluency dependent manner [26]. A transcriptional suppression was also observed and the *MUC4* promoter regions -1187/-488 and -3135/-2782 were found to be critical for this regulation [26]. A recent study has shown that Kras can increase MUC4 protein levels via the RalB GTPase [15]. However, the mechanism of the RalB mediated MUC4 up-regulation was not examined.

D. Summary and conclusions

In conclusion, numerous epigenetic, transcriptional, post-transcriptional and post-translational factors have been shown to regulate MUC4. Firstly, a permissive chromatin milieu and promoter hypomethylation are prerequisites for *MUC4* transcription. Secondly, any of several spatio-temporally regulated transcription factors may enhance *MUC4* transcription. Furthermore, inflammatory cytokines such as TGF-β, TNF-α and IFN-γ may also influence *MUC4*. Third, post-transcriptional regulatory factors such as miRNAs and cross-talk with other signaling pathways such as the TGF-β pathway, Kras/MAPK pathways may affect MUC4 protein stability.

Currently, most studies on MUC4 regulation have been conducted in PDAC cell lines. Future studies could address MUC4 expression regulation in other malignancies, such as colorectal, breast and ovarian cancers. Also, studies could also address *MUC4* regulatory mechanisms in non-pathological conditions such as the normal human colon, where *MUC4* is first expressed in embryonic week 6.5 [27].

3. MUC4 expression patterns in PDAC and CRC

A. MUC4 in pancreatic cancer

In the normal pancreas, MUC4 is not expressed at the RNA level, while appearing *de novo* at the RNA and protein level in pancreatic cancer [28]. This aspect of MUC4 expression sets it apart from all the other mucins and can possibly be exploited for early diagnosis [28]. When a panel of seven well characterized mucins was examined, only MUC4 was found to be absent in the normal pancreas and cases of chronic pancreatitis while being present in both PDAC cell lines and tissue [28]. Furthermore, MUC4 expression incrementally increases during PDAC progression, first appearing at PanIN stage I with expressing culminating in full-blown PDAC [29].

Over the years, studies have shown that MUC4 can interact with HER2, acting as a ligand, thus triggering an intracellular cascade of signaling events involving the MAPK and PI3K pathways [30, 31]. Recently, it has been reported that MUC4 can also interact with other EGFR family members such as HER3 and HER4 [32]. Furthermore, MUC4 on circulating tumor cells can potentiate the cell surface localization of endothelial Galectin-3, and the MUC4-Galectin interaction can result in the temporary docking of tumor cells on the endothelium, leading to a permanent attachment of the tumor cell to the endothelium [33]. MUC4 is indiscriminately expressed on all surfaces of the tumor cell and can therefore interact with extracellular matrix proteins (ECM) such as Nidogen, present in the basement membrane, thus disrupting normal ECM protein-protein interactions [34]. As a consequence of all the aforementioned features of MUC4 function, MUC4 can modulate phenomena as diverse as epithelial to mesenchymal transition (EMT) [35], metastasis [35], drug resistance [36], stem cell-like properties [37], tumor cell proliferation [30], invasion, and thus PDAC patients that express MUC4 have a poorer prognosis[38].

Given the multifarious and tumor promoting nature of MUC4, a number of studies have focused on harnessing its tumor specific expression for therapy and using serum levels as a diagnostic/prognostic marker [39-41]. MUC4 has also been proposed as a useful diagnostic tool for PDAC in fine-needle aspirates (FNA) [40, 41]. In conclusion, MUC4 has been unequivocally established as a tumor specific molecule that can promote tumor progression and metastasis in PDAC, as well as serve as a useful diagnostic marker.

B. MUC4 in colorectal cancer

While a multitude of studies in PDAC have established the importance of MUC4 expression, its status in CRC is less well studied. MUC4 is ordinarily expressed in the colon, with expression being concentrated in the lower 2/3rd of the crypt [42]. MUC4 is expressed by both goblet cells and absorptive cells [42]. Furthermore, *MUC4* is one of the earliest mucins expressed in the embryonic gut, appearing at embryonic week 6.5, even before secreted mucins such as *MUC2* [27], suggestive of a role in epithelial cell differentiation.

Unlike PDAC, a number of molecular subtypes that originate via distinct mutational pathways have been identified in CRC. Also, there are distinct precursor lesions associated with each subtype. Briefly, CRCs can arise either through somatic mutations or through germ line mutations that predispose individuals to CRC. The ‘traditional’ pathway typically requires truncations of the APC tumor suppressor gene and is also sometimes referred to as the ‘tumor suppressor’ pathway [43]. This could either be due to sporadic somatic mutations or due to germ line mutations, as seen in Familial Adenomatous Polyposis (FAP) [44]. Another pathway typically involving the activation of oncogenes such as BRAF and KRAS also exists and is usually associated with a high microsatellite instability phenotype (MSI-H) implying the loss of mismatch-repair genes [45]. This is sometimes referred to as the ‘mutator pathway’. In terms of precursor lesions, most tumors usually arise from adenomatous polyps that progress into adenomas, ultimately forming carcinomas [44]. Hyperplastic polyps and serrated adenomas are less likely to from CRCs, but

when they do, they are typically associated with the ‘mutator pathway’[44]. Furthermore, while the majority of CRCs are associated with a loss of mucin expression, a minority (roughly 10%) are associated with abundant mucin secretion [46]. These tumors are called mucinous CRCs and are more frequently associated with the ‘mutator pathway’[47].

Ogata *et al* first examined the MUC4 expression levels in 8 CRC tumors; 4 tumors had diminished MUC4, the rest had either an increased expression, or levels comparable to normal tissue [48]. When CRC precursor lesions were stained for MUC4 by IHC, MUC4 was reduced in serrated adenomas and 50% of hyperplastic polyps, while being present at roughly normal levels in traditional adenomas [42]. Another study examined the association between MUC4 expression and lesion type. The majority of non-mucinous tumors (66%) had low-moderate expression of MUC4 while 34% of the CRC examined had high MUC4 [46]. Of the subset of patients that expressed high MUC4, an overwhelming majority (79%) were grade I lesions. Also, 90% of the lesions that expressed MUC4 were well/ moderately differentiated. Furthermore MUC4 was not associated with either the MSI-H or the mucinous phenotype [46].

Very few studies have examined the prognostic significance of MUC4 in CRC. In 2010, Shanmugam *et al* performed an analysis of a large cohort of CRC samples [49]. It was observed that while 75% of tumors displayed a loss of MUC4, 25% showed an increase in MUC4 expression. However, MUC4 expression conferred a significantly worse prognosis to stage I and Stage II patients [49]. Another recent study reiterated these findings, when they noted that while only a minority (33%) of tumors showed high MUC4, MUC4 was associated with a poorer prognosis [50]. Thus, MUC4, when present in high levels in early stages of CRC, appears to confer a worse prognosis.

In conclusion, despite the paucity of studies that examine the role of MUC4 in CRC, most studies indicate that MUC4 is lost in late stage CRC, but when present in high levels, especially in early stages, confers a worse prognosis. It has been proposed that cancer associated truncated glycan

epitopes may cause the seemingly altered expression of MUC4 due to altered affinity of antibodies to MUC4[46]. Nevertheless, a recent study performed by our lab [51]appears to support the pro-tumorigenic role of MUC4. When $Muc4^{-/-}$ mice were subjected to AOM/DSS treatment, it was observed that the presence of Muc4 led to a significant increase in colitis and colorectal cancer.

C. MUC4 in inflammatory bowel disease: ulcerative colitis and Crohn's disease

Inflammatory bowel disease (IBDs) refers to two distinct diseases: Crohn's disease and ulcerative colitis. Both these diseases are characterized by a deranged, inappropriate immune response against commensal microflora in the lumen of the gut [52]. Importantly, these diseases confer a strong predisposition towards CRC [53-55]. This predisposition is stronger in Crohn's disease where a 20 fold higher-than-normal risk of CRC was observed [53], in comparison to ulcerative colitis, where the increase in risk is not as significant [54]. In addition, individuals with Crohn's disease tend to develop mucinous CRCs with a significantly poor prognosis [53]. These diseases also differ in other respects; Crohn's disease tends to be transmural (involving the entire gut wall) while ulcerative colitis only involves the mucosa and submucosa [52].

Since the primary function of mucins in the gut is to act as a barrier between intestinal microflora and the surface epithelium, one would expect an alteration in mucin expression in IBD. Studies have shown that there is a more significant depletion of mucins in ulcerative colitis in than in Crohn's disease [56]. The most abundant mucin in the gut; MUC2, as well as MUC4 are both depleted in ulcerative colitis [56]. On the contrary, MUC4 is up-regulated in Crohn's disease [56, 57]. This has been shown to be a consequence of increased cytokines such as TNFα and TGFβ, as well as intestinal differentiation factors such as Hath1 and KLF4 [57]. Thus, MUC4 appears to be increased by inflammatory cues in Crohn's disease but not ulcerative colitis.

In light of the aforementioned facts, it appears likely that the AOM/DSS $Muc4^{-/-}$ model developed in our lab may mimic the inflammation-induced CRC formation as seen in IBD. Since

IBD patients tend to develop mucinous carcinomas, a distinct entity differing in many respects from the conventional APC driven CRC, it is likely that this the AOM/DSS Muc4$^{-/-}$ model may not accurately reflect the role played by MUC4 in the majority of human CRCs.

D. Summary and conclusions

In conclusion, while MUC4 is a cancer-specific tumor promoting molecule in PDAC, its expression/role in CRC is less clear. MUC4 is highly expressed in a subset of non-mucinous early stage CRC tumors, likely conferring a worse prognosis. Further analysis of the mutational status of this subset is required to ascertain the possible diagnostic/prognostic significance of MUC4. Thus far, only a single study has suggested that MUC4 is not associated with the MSI-H and mucinous sub-type of CRC [46]. However, given the inherent overlap between mutations in the canonical CRC subtypes and the possibly heterogenous expression of MUC4 within CRC tumors, further studies are needed to establish MUC4 expression patterns in CRC.

References

[1] Perrais M, Pigny P, Ducourouble MP, et al. Characterization of human mucin gene MUC4 promoter: importance of growth factors and proinflammatory cytokines for its regulation in pancreatic cancer cells. J Biol Chem 2001;276:30923-33.

[2] Vincent A, Ducourouble MP, Van S, I. Epigenetic regulation of the human mucin gene MUC4 in epithelial cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases. FASEB J 2008;22:3035-45.

[3] Yamada N, Nishida Y, Tsutsumida H, et al. Promoter CpG methylation in cancer cells contributes to the regulation of MUC4. Br J Cancer 2009;100:344-51.

[4] Zhu Y, Zhang JJ, Zhu R, et al. The increase in the expression and hypomethylation of MUC4 gene with the progression of pancreatic ductal adenocarcinoma. Med Oncol 2011;28 Suppl 1:S175-84.

[5] Cho JS, Park MH, Lee JS, Yoon JH. Reduced MUC4 expression is a late event in breast carcinogenesis and is correlated with increased infiltration of immune cells as well as promoter hypermethylation in invasive breast carcinoma. Appl Immunohistochem Mol Morphol 2015;23:44-53.

[6] Kumar S, Das S, Rachagani S, et al. NCOA3-mediated upregulation of mucin expression via transcriptional and post-translational changes during the development of pancreatic cancer. Oncogene 2014; 34(37):4879-89.

[7] Jonckheere N, Vincent A, Perrais M, et al. The human mucin MUC4 is transcriptionally regulated by caudal-related homeobox, hepatocyte nuclear factors, forkhead box A, and GATA endodermal transcription factors in epithelial cancer cells. J Biol Chem 2007;282:22638-50.

[8] Jonckheere N, Perrais M, Mariette C, et al. A role for human MUC4 mucin gene, the ErbB2 ligand, as a target of TGF-beta in pancreatic carcinogenesis. Oncogene 2004;23:5729-38.

[9] Zhang J, Zhang X, Zhu Y, Chen Z, Xu Z, Miao Y. Transcriptional regulation of human mucin gene MUC4 in pancreatic cancer cells. Mol Biol Rep 2010;37:2797-802.

[10] Andrianifahanana M, Singh AP, Nemos C, et al. IFN-gamma-induced expression of MUC4 in pancreatic cancer cells is mediated by STAT-1 upregulation: a novel mechanism for IFN-gamma response. Oncogene 2007;26:7251-61.

[11] Choudhury A, Singh RK, Moniaux N, El-Metwally TH, Aubert JP, Batra SK. Retinoic acid-dependent transforming growth factor-beta 2-mediated induction of MUC4 mucin expression in human pancreatic tumor cells follows retinoic acid receptor-alpha signaling pathway. J Biol Chem 2000;275:33929-36.

[12] Andrianifahanana M, Agrawal A, Singh AP, et al. Synergistic induction of the MUC4 mucin gene by interferon-gamma and retinoic acid in human pancreatic tumour cells involves a reprogramming of signalling pathways. Oncogene 2005;24:6143-54.

[13] Kunigal S, Ponnusamy MP, Momi N, Batra SK, Chellappan SP. Nicotine, IFN-gamma and retinoic acid mediated induction of MUC4 in pancreatic cancer requires E2F1 and STAT-1 transcription factors and utilize different signaling cascades. Mol Cancer 2012;11:24.

[14] Momi N, Ponnusamy MP, Kaur S, et al. Nicotine/cigarette smoke promotes metastasis of pancreatic cancer through alpha7nAChR-mediated MUC4 upregulation. Oncogene 2013;32:1384-95.

[15] Vasseur R, Skrypek N, Duchene B, et al. The mucin MUC4 is a transcriptional and post-transcriptional target of K-ras oncogene in pancreatic cancer. Implication of MAPK/AP-1, NF-kappaB and RalB signaling pathways. Biochim Biophys Acta 2015, *in press*.

[16] Fauquette V, Aubert S, Groux-Degroote S, et al. Transcription factor AP-2alpha represses both the mucin MUC4 expression and pancreatic cancer cell proliferation. Carcinogenesis 2007;28:2305-12.

[17] Mariette C, Perrais M, Leteurtre E, et al. Transcriptional regulation of human mucin MUC4 by bile acids in oesophageal cancer cells is promoter-dependent and involves activation of the phosphatidylinositol 3-kinase signalling pathway. Biochem J 2004;377:701-8.

[18] Piessen G, Jonckheere N, Vincent A, et al. Regulation of the human mucin MUC4 by taurodeoxycholic and taurochenodeoxycholic bile acids in oesophageal cancer cells is mediated by hepatocyte nuclear factor 1alpha. Biochem J 2007;402:81-91.

[19] Algamas-Dimantov A, Yehuda-Shnaidman E, Peri I, Schwartz B. Epigenetic control of HNF-4alpha in colon carcinoma cells affects MUC4 expression and malignancy. Cell Oncol (Dordr) 2013;36:155-67.

[20] Srivastava SK, Bhardwaj A, Singh S, et al. MicroRNA-150 directly targets MUC4 and suppresses growth and malignant behavior of pancreatic cancer cells. Carcinogenesis 2011;32:1832-9.

[21] Radhakrishnan P, Mohr AM, Grandgenett PM, Steele MM, Batra SK, Hollingsworth MA. MicroRNA-200c modulates the expression of MUC4 and MUC16 by directly targeting their coding sequences in human pancreatic cancer. PLoS One 2013;8:e73356.

[22] Lahdaoui F, Delpu Y, Vincent A, et al. miR-219-1-3p is a negative regulator of the mucin MUC4 expression and is a tumor suppressor in pancreatic cancer. Oncogene 2015;34:780-8.

[23] Soto P, Price-Schiavi SA, Carraway KL. SMAD2 and SMAD7 involvement in the post-translational regulation of Muc4 via the transforming growth factor-beta and interferon-gamma pathways in rat mammary epithelial cells. J Biol Chem 2003;278:20338-44.

[24] Lomako WM, Lomako J, Soto P, Carraway CA, Carraway KL. TGFbeta regulation of membrane mucin Muc4 via proteosome degradation. J Cell Biochem 2009;107:797-802.

[25] Lomako J, Lomako WM, Carothers Carraway CA, Carraway KL. Regulation of the membrane mucin Muc4 in corneal epithelial cells by proteosomal degradation and TGF-beta. J Cell Physiol 2010;223:209-14.

[26] Singh AP, Chauhan SC, Andrianifahanana M, et al. MUC4 expression is regulated by cystic fibrosis transmembrane conductance regulator in pancreatic adenocarcinoma cells via transcriptional and post-translational mechanisms. Oncogene 2007;26:30-41.

[27] Buisine MP, Devisme L, Savidge TC, et al. Mucin gene expression in human embryonic and fetal intestine. Gut 1998;43:519-24.

[28] Andrianifahanana M, Moniaux N, Schmied BM, et al. Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res 2001;7:4033-40.

[29] Swartz MJ, Batra SK, Varshney GC, et al. MUC4 expression increases progressively in pancreatic intraepithelial neoplasia. Am J Clin Pathol 2002;117:791-6.

[30] Chaturvedi P, Singh AP, Moniaux N, et al. MUC4 mucin potentiates pancreatic tumor cell proliferation, survival, and invasive properties and interferes with its interaction to extracellular matrix proteins. Mol Cancer Res 2007;5:309-20.

[31] Chaturvedi P, Singh AP, Chakraborty S, et al. MUC4 mucin interacts with and stabilizes the HER2 oncoprotein in human pancreatic cancer cells. Cancer Res 2008;68:2065-70.

[32] Lakshmanan I, Seshacharyulu P, Haridas D, et al. Novel HER3/MUC4 oncogenic signaling aggravates the tumorigenic phenotypes of pancreatic cancer cells. Oncotarget 2015;6:21085-99.

[33] Senapati S, Chaturvedi P, Chaney WG, et al. Novel INTeraction of MUC4 and galectin: potential pathobiological implications for metastasis in lethal pancreatic cancer. Clin Cancer Res 2011;17:267-74.

[34] Senapati S, Gnanapragassam VS, Moniaux N, Momi N, Batra SK. Role of MUC4-NIDO domain in the MUC4-mediated metastasis of pancreatic cancer cells. Oncogene 2012;31:3346-56.

[35] Rachagani S, Macha MA, Ponnusamy MP, et al. MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1. Carcinogenesis 2012;33:1953-64.

[36] Bafna S, Kaur S, Momi N, Batra SK. Pancreatic cancer cells resistance to gemcitabine: the role of MUC4 mucin. Br J Cancer 2009;101:1155-61.

[37] Mimeault M, Johansson SL, Senapati S, Momi N, Chakraborty S, Batra SK. MUC4 down-regulation reverses chemoresistance of pancreatic cancer stem/progenitor cells and their progenies. Cancer Lett 2010;295:69-84.

[38] Huang X, Wang X, Lu SM, et al. Clinicopathological and prognostic significance of MUC4 expression in cancers: evidence from meta-analysis. Int J Clin Exp Med 2015;8:10274-83.

[39] Krasnoslobodtsev AV, Torres MP, Kaur S, et al. Nano-immunoassay with improved performance for detection of cancer biomarkers. Nanomedicine 2015;11:167-73.

[40] Jhala N, Jhala D, Vickers SM, et al. Biomarkers in Diagnosis of pancreatic carcinoma in fine-needle aspirates. Am J Clin Pathol 2006;126:572-9.

[41] Horn A, Chakraborty S, Dey P, et al. Immunocytochemistry for MUC4 and MUC16 is a useful adjunct in the diagnosis of pancreatic adenocarcinoma on fine-needle aspiration cytology. Arch Pathol Lab Med 2013;137:546-51.

[42] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Immunohistochemical staining patterns of MUC1, MUC2, MUC4, and MUC5AC mucins in hyperplastic polyps, serrated adenomas, and traditional adenomas of the colorectum. J Histochem Cytochem 1999;47:1039-48.

[43] Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55-67.

[44] Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011;6:479-507.

[45] Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77-87.

[46] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Mucin core protein expression in colorectal cancers with high levels of microsatellite instability indicates a novel pathway of morphogenesis. Clin Cancer Res 2000;6:1909-16.

[47] Tanaka H, Deng G, Matsuzaki K, et al. BRAF mutation, CpG island methylator phenotype and microsatellite instability occur more frequently and concordantly in mucinous than non-mucinous colorectal cancer. Int J Cancer 2006;118:2765-71.

[48] Ogata S, Uehara H, Chen A, Itzkowitz SH. Mucin gene expression in colonic tissues and cell lines. Cancer Res 1992;52:5971-8.

[49] Shanmugam C, Jhala NC, Katkoori VR, et al. Prognostic value of mucin 4 expression in colorectal adenocarcinomas. Cancer 2010;116:3577-86.

[50] Khanh dT, Mekata E, Mukaisho K, et al. Transmembrane mucin MUC1 overexpression and its association with CD10(+) myeloid cells, transforming growth factor-beta1 expression, and tumor budding grade in colorectal cancer. Cancer Sci 2013;104:958-64.

[51] Das S, Rachagani S, Sheinin Y, et al. Mice deficient in Muc4 are resistant to experimental colitis and colitis-associated colorectal cancer. Oncogene 2015; 1-10.

[52] Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol 2008;8:458-66.

[53] Freeman HJ. Colorectal cancer risk in Crohn's disease. World J Gastroenterol 2008;14:1810-1.

[54] Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 2001;48:526-35.

[55] Choi PM, Zelig MP. Similarity of colorectal cancer in Crohn's disease and ulcerative colitis: implications for carcinogenesis and prevention. Gut 1994;35:950-4.

[56] Dorofeyev AE, Vasilenko IV, Rassokhina OA, Kondratiuk RB. Mucosal barrier in ulcerative colitis and Crohn's disease. Gastroenterol Res Pract 2013;2013:431231.

[57] Gersemann M, Becker S, Kubler I, et al. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. Differentiation 2009;77:84-94.

Chapter 1C

Dissertation General Hypothesis and Objectives

1. Background and rationale

The aberrant expression of mucins and the inappropriate activation of Wnt/β-catenin are inextricably linked with the neoplastic process in many malignancies. Moreover, mucins and β-catenin have a mutually regulatory relationship that aid cancer progression, as has been described in Chapter1A.

In PDAC, several studies have hinted at a regulatory relationship between β-catenin and MUC4. Firstly, when β-catenin was inhibited in the well-established KPC (PDX-1-Cre, LSL-$Kras^{G12D}$, LSL-$Trp53^{R172H/-}$) mouse model, a depletion of mucins as measured by alcian blue was observed [1]. Second, a microarray performed upon the depletion of β-catenin in the BXPC3 PDAC cell line showed that *MUC4* was one of the most significantly reduced transcripts [2]. Third, both aberrant β-catenin and MUC4 have been documented to appear at roughly the same stage of progression in in early PanIN precursor lesions [3, 4]. We performed a *MUC4* promoter analysis using the Genomatix MatInspector program, which showed the presence of 3 putative TCF/LEF sites, required for β-catenin mediated transcriptional upregulation (**Figure 1**).

In CRC, where aberrant β-catenin is the predominant, cancer-driving anomaly [5], a siRNA targeting β-catenin showed resulted in reduced mucins as measured by alcian blue staining in the LS174 cell line [6]. The loss of mucins such as MUC2 during CRC progression is a generally accepted phenomenon [7], and many studies suggest that MUC4 expression is also lost in CRC [8-10]. The Apc^{Min} mouse model for CRC, where truncations in Apc are the only genetic abnormality, shows a reduced number of goblet cells and alcian blue staining in lesions [11, 12].

While a number of studies suggest that MUC4 expression is reduced in CRC, paradoxically, it has also been claimed that its expression confers a significantly worse prognosis to the subset of patients that express MUC4 [13, 14]. This notion has been substantiated to a

degree by a recent study in our lab that showed that Muc4 loss was protective towards DSS induced colitis and CRC in mice [15].

2. Hypothesis

Based on previous studies suggesting that β-catenin can modulate mucin/MUC4 expression and a *MUC4* promoter analysis performed by our lab showed the presence of three putative TCF/LEF sites, we **hypothesized** that β-catenin can modulate *MUC4* expression in PDAC and CRC.

3. Objectives

Aim 1: To determine whether β-catenin can modulate MUC4 expression in PDAC and determine the mechanism of this regulation as well as the functional implications of the β-catenin-MUC4 axis in PDAC progression and metastasis.

Aim 2: To determine whether β-catenin can regulate MUC4 expression in CRC and establish the mechanism of the possible differential modulation of MUC4 expression by β-catenin in PDAC and CRC.

Aim 3: To establish the functional role played by MUC4 in CRC.

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Figure1 Sequence of the MUC4 promoter and 5' UTR showing the three TCF/LEF sites(red text) and the Hath1 site (green text) unearthed by the MatiInspector promoter analysis. The ATG site is in bold type

Figure 1


taaaagcaaggcccaccctgcaggggccagcagctgggagctgtccactaaccactatccttgcagctggacagcgaggcccctccaaaaggC
CGTCTCCACCTGCCACCGGGAAAGGACCCGGAGCGAAGGATGTCGTGGGCGGTCAGCCCAGGTGA
TTGCCCTTCTTTTgctttggagccaggcagaacagagctcaaattccagctttgGGACAGTGTGTGGCCCAGAAGGCTGT
AACTGCGGAGTTAACACTCTCCAGTTTCCCTAGATGGCCCGACCTGGCCGGTGACTCGGGGATGAT
GAAGCTCCTGGAGCCTCAGCGTGCTCACTTGAGAGGTAGGGCAGCGCTTTGTACTTCACAGGGCC
ATGGGGACAGGCTCAGGGCCTGGCACTCTAGTGACCGGCTTCATGCTGGGTCACTGACTAGGGAAT
CCACACACCTTTTTAGTCCAGACTGAGTAGGTAAATCAGACTTAATAAACATCCAGCTTCCTGCTCGA
GATCAGCCTAGTTGTCCTAAGTCTGACAAGATTGAACTTTATTCACAGCACGAGGCATAAATGATAT
AAAGGTAAGCAATTTTAGGGGACCCAAAAAACTAGGGACTCCTACTTGGGTAGCTTAGAAGGGGAAT
CTCTCTTGGAGATCCTGGCTTTGCTGCCTCAAACCCAGCTGTCTCTTCAAGCCAAGTCTGGGAGAAT
CTCATGATCTTATCCTTGCTCCAATTTACTTACACCCCACATCCACACTAAGCTCTTCCTGCCTCCCG
TGGAATATTAACTTACACCTGACTTCAGACTCCTGCCCCTATGCAGACCCAGCAGTATGCGGGCCTA
GGAGGTTTTTCGGCCACAAGGAACTAGAGAAGCCACTCATTTTGTCCCCTCTCATTCACCCCATTCT
GTCCCCATCGGATGCCTTGGGAGGAGAGAAGGAAGGACAAATGGGTGTCCCGTGACCCAACCCAG
ATGAAGACAGAGCCATTTATCACAGAGACCCAGACACATCCTAACTCTGGAAAATGGGCATATTGAG
GGGAGCTGGAAAGCAGAAGGGAAACGGGCGTGGGCAGGGAAGAGGTGAACAGGAGTGCGCACGC
CCAGTTCTCCAGGATCTCACGCTCCTAATCAGGATCCTATAAGACTCAAAGGGGACGCAGGAAAGA
CCTGGGCCTGGAAATTCACTTCCGGACTCCTCCTGTATCCTGGCTGGCCCTCGTTTGTGTCTCCCTC
CAGGGATGATGCCCGGATGGAAGGAGGAGCCACGTTCCTCCACAGGTGTCCTCCACAACAGGTGT
CCTCACAACAGGTGTCGTAACAACAGGTGTCCTCACAACAGGTGTCCTAATACTCCCGCagcccctcagc
atctacaggctcactcccaccctacaacctttcagataggtgttatccccatttttacaggtgaacgaggaaaacagctcgaagaggctgtgactttgtg
gtgacgcggtggcagagctgggatcaagcccaggtttgTAACGCCCTCCTGGAGTCTCGGCCGTCACAGCAGCTTCC
GGAGAGCGCTGTGTGCCTCTGGCCTTCCTGGCATGGGAAAGAGGTCAGCTACCTGTGTTCGGTCAA
CAAGATGTCCAGCGCTTAGTTTGGTCTCACCTGGATGCTCCTTTCTCCTCTAGAGATGGAAAACTGT
TGTCCTGAAAGCGTCACCAAACAAAACCTGTGGAAAGGTAATGGGGGGACCCACGAGCAAGAGCTG
ACAGAAAAATGACCACGggccgggtgcagtggctcacgcctgtaatcccagcactttgggaggccaaggcgggcggatcatctgagttc
aggggttcgagaccaacctggccaacatggtgaaacctcgtcctactaaaaatacaaaaaaaattagccgggcgtggtggcaggtgcctgtagt
cccagctactagggaggctgaggcaggggaatggcgtgaacccgggaggcggagcttgcagtgagctgagatggtgccactgcactccagcctg
ggcgacagagcaagactccatctcaaaaaaaaaaaaaaGTGCAAAATGATCACGAAGGCCAAGCTGGGCCTTGGTG
TCTGCGAATTCCCTGCAGTAGGTCCCTCCTGCTGCCCCGTCCAGGCGCCCACAGGATGCTCCACAT
GACTCTTTACCTTTATGATTTTCTTACCTCCAGGAAAGAACAGGGTCAGCTCGTCCACCCAGAGCTG
GAGGGACTGTTGGGGGACCACGCCACTTCCCCACGGTATCCGCATTATTATGGCGTGGCCCAGGCT
TTGGCCTGGGGCCATGAACAGACTTTCAGCTTCTAGAAGCAAGTGGTCACAGGGCCTCCGTGACCC
TCGGAGACAGCGCCACAGCGTCGGTTGCCTACTCCTTGCTGGACAAGGCTGGCTCTGCTTGGGAAT
GCAGAAACCCTGAGTCCTTGTCCTGGGCCCCACAGTGTCTGAGCATCGAAGGTCCCTGCTCTGGGA
CGAGTCACCCCACAGACACCCCACATTCGGGAATCAAGTCTTGAGGAATCAGAAGCAGAGATTCAC
ACTGTGGCAAAGAGGGGAGCGGAGGTATCCCCGTTTTCCTTTGCAGAGAGCTACACCAACTGCCCT
CTTCGTCTTTACAACCCTACTTGGGGTCCCTTTCCCTCCTCGCTTGTGCCTGTGAGTTCCAAGAGGT
GGAACTGGGGTGAGCAGAGCCCCCAGGTCTCCAGGAGCAGCTCCACCTCCTCACAGGTTACAGTC
GCTTCCTCCTACCGAGGTTCTGCCTTCAACCCACTGCCCATGCCAGCTCCCTCAGGCCAGTCTCCC
AGACTGTCCCTGGCTCCCTGCACCCTGGCTCCAGCTCCCTGCCCTGGAAAGAAGAAGTCCACCATC
TGGTGAGGGTCTGCCACGCATCTGGGGTCTGCCATGCGTCTGCACTGAGGTTCTGCCACGAGTCTG
CACTGAGGTTCTGCCATGCGTCTGGAGTCTGCCACGCGTCTGGGGTCTGCCACGCGTCTGGAGTCT
GCCATGGGTCTGGGGTCTGCCATGTGTCTGGGATCTGCCGTGCGTCTGGGGTCTGCCACGCATCT
GCACTGAGGTTCTGCCACGCGTCTGGGGTCTGCCACGCGTCTGGGGTCTGCCACGCGTCTGGGGT
CTGCCACGCGCCTGCACTGAGCAGATATTCCAAGCACACCACCCCTTTCCAAAAGACGGCATACAG
TGCATTTCTGTTCCTGCCCCTCACCCACATGGTTCCCCGCTTCCCAATTCCTTGGGGTCTGCTTAAG
TCTCACTCTCTTTCCCCCATTCATACAGCCCCAAGGTCGCTCCCTCTGGGGCCCTTTCTTCCCCATT
CTTCCCAGCAGCCCAAAGCTCTGGTGGGACAGGGGCAGCCCCTGGGGAGGGAGGAGAGGACCCA
GGAACCCGGCTAGGAGGGTGGCCCACCCATTTCCAGTGTGACCTGTTCCCATTCCCCCATGTCTCC
TCCCATCCCTCCCGCCACTCAGCTCAGGCTGATGAGAAGCAGAGCAACGGGTGTATCGGTGTTTTC
TTTCCTGGTGGGGTAGTGGGGTGGGGCTGAGGAGAGAAAAGGGTGATTAGCGTGGGGCCCCGCCC
TCTTTTGTCCTCTTCCCAGGTTCCCTGGCCCCTTCGGAGAAACGCACTTGGTTCGGGCCAGCCGCC
TGAGGGGACGGGCTCACGTCTGCTCCTCACACTGCAGCTGCTGGGCCGTGGAGCTTCCCCAGGGA
GCCAGGGGGACTTTTGCCGCAGCCATG

References

[1] Zhang Y, Morris JP, Yan W, et al. Canonical wnt signaling is required for pancreatic carcinogenesis. Cancer Res 2013;73:4909-22.

[2] Olsen PA, Solberg NT, Lund K, et al. Implications of targeted genomic disruption of beta-catenin in BxPC-3 pancreatic adenocarcinoma cells. PLoS One 2014;9:e115496.

[3] Al-Aynati MM, Radulovich N, Riddell RH, Tsao MS. Epithelial-cadherin and beta-catenin expression changes in pancreatic intraepithelial neoplasia. Clin Cancer Res 2004;10:1235-40.

[4] Andrianifahanana M, Moniaux N, Schmied BM, et al. Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res 2001;7:4033-40.

[5] Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55-67.

[6] van de Wetering M, Oving I, Muncan V, et al. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep 2003;4:609-15.

[7] Velcich A, Yang W, Heyer J, et al. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science 2002;295:1726-9.

[8] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Immunohistochemical staining patterns of MUC1, MUC2, MUC4, and MUC5AC mucins in hyperplastic polyps, serrated adenomas, and traditional adenomas of the colorectum. J Histochem Cytochem 1999;47:1039-48.

[9] Khanh dT, Mekata E, Mukaisho K, et al. Transmembrane mucin MUC1 overexpression and its association with CD10(+) myeloid cells, transforming growth factor-beta1 expression, and tumor budding grade in colorectal cancer. Cancer Sci 2013;104:958-64.

[10] Ogata S, Uehara H, Chen A, Itzkowitz SH. Mucin gene expression in colonic tissues and cell lines. Cancer Res 1992;52:5971-8.

[11] Hulit J, Wang C, Li Z, et al. Cyclin D1 genetic heterozygosity regulates colonic epithelial cell differentiation and tumor number in ApcMin mice. Mol Cell Biol 2004;24:7598-611.

[12] Liu HP, Gao ZH, Cui SX, et al. Chemoprevention of intestinal adenomatous polyposis by acetyl-11-keto-beta-boswellic acid in APC(Min/+) mice. Int J Cancer 2013;132:2667-81.

[13] Huang X, Wang X, Lu SM, et al. Clinicopathological and prognostic significance of MUC4 expression in cancers: evidence from meta-analysis. Int J Clin Exp Med 2015;8:10274-83.

[14] Shanmugam C, Jhala NC, Katkoori VR, et al. Prognostic value of mucin 4 expression in colorectal adenocarcinomas. Cancer 2010;116:3577-86.

[15] Das S, Rachagani S, Sheinin Y, et al. Mice deficient in Muc4 are resistant to experimental colitis and colitis-associated colorectal cancer. Oncogene 2015; *in press*.

CHAPTER 2

Materials and Methods

1. Cell Culture

CD18/HPAF was a metastatic clone derived from the HPAF PDAC cell line [1]. T3M4 was derived from lymph node metastasis of a tumor in the exocrine pancreas [2]. Other PDAC cell lines, Capan1, BXPC3, Panc1, AsPc1, and MiaPaCa, were cultured in DMEM containing 10% fetal bovine serum supplemented with 100 µg/ml penicillin and streptomycin. Colorectal cancer cell lines HCT-8, LS180, HCT15, SW480, CaCo2 and HCT116 were maintained in 10% fetal bovine serum containing α-MEM supplemented with 100 µg/ml penicillin and streptomycin. Cells transfected with lentiviral constructs were maintained in 5 µg/ml Puromycin as a selection agent. The L Wnt-3A cells were a gift from Dr. Jing (aka. Jenny) Wang at the University of Nebraska Medical Center (UNMC). Wnt-3A conditioned medium was collected as stipulated by American Type Culture Collection (ATCC). All cells were maintained at 37ºC with 5% CO_2 in a humidified atmosphere.

2. Lentiviral and Retroviral Transfection

The lentiviral plasmids used included the following: pLKO.1.sh.beta-catenin.2279 (Addgene plasmid #19762), pLKO.1.sh.beta-catenin.1248 (Addgene plasmid #19761), pLKO.1 shSCR (Addgene plasmid #17920), and packaging plasmid pCMV-dR8.2 dvpr and envelope plasmid pCMV-VSVG, which were a kind gift from Dr. Yuzuru Shiio (University of Tennessee Health Science Center). The Lenti-X-293T cell line #632180 (Clontech; Mountain View, CA, USA) was used for transfection, and the Lenti-X™ Concentrators #631231 and #631232 from Clontech were used to concentrate the lentiviral supernatant. Briefly, 2 X 10^6 cells were plated in 10 cm plates, and transfection using a calcium phosphate precipitate method with 20 µg transfer vector, 15 µg packaging plasmid, and 6 µg envelope plasmid was performed the next day. Concentration of the supernatant collected after 48 hours was done per manufacturer's instructions (Clontech). Following concentration, lentiviral supernatant was used to transfect 2X

10^5 cells plated per well of a 12-well plate. Puromycin (5 µg/ml) was used to select for positive clones.

For generation of the HCT-8 Scr and shMUC4 cell lines, a MUC4 targeting oligonucleotide was cloned into the pSUPER.retro.puro vector using the HindIII and BglII restriction sites. Phoenix cells were transfected with Scr control and MUC4 targeting oligonucleotide, and the supernatant was collected 48 and 72 hours after transfection. This supernatant was used to infect the target, i.e., HCT-8 cells. Following selection with Puromycin, stably selected pooled populations were maintained in 5µg/ml Puromycin.

3. Tissues specimens and Immunohistochemistry

The UNMC Rapid Autopsy Program was used to obtain tissue microarrays (**TMA**) containing primary PDAC tissue specimens (IRB-091-01) and normal pancreas, kidney, and colon tissue spots as controls (n = 30, 25 PC spots). TMAs containing metastatic spots (n = 26; 25 liver metastatic spots, 1 lung metastatic spot) with normal pancreas, colon, and kidney tissues as controls were also used (IRB-091-01). Prior consent was obtained from all participants in the Rapid Autopsy Program, and the UNMC Institutional Review Board (**IRB**) approved this study. The following antibodies were used: anti-β-catenin #610154, (BD Biosciences; San Jose, CA, USA) anti-MUC4 monoclonal antibody clone 8G7 (developed in our lab). The protocols used for immunohistochemical (**IHC**) detection of β-catenin and MUC4 have been described previously [3, 4]. The tissue microarrays (**TMAs**) were evaluated by a UNMC pathologist and were given a composite score ranging from 0 – 12, which was a product of the intensity of staining (range 1 – 3) and number of cells stained (range 1 – 4; 0 - 25% area stained = score of 1, 26 - 50% = score 2, 51 - 75% = score 3, and 76 - 100% = score 4).

Tissues from *Apc*^{Min} mice treated with dextran sodium sulfate (DSS) tissues and mice treated with DSS alone were a kind gift from Dr. Punita Dhawan, UNMC. Tissues were evaluated by a UNMC pathologist and composite score for immunohistochemical staining in mouse tissue

was calculated by estimating the number of positively stained cells per hundred cells and multiplying this number by the intensity of staining, which was given a range from 1-3. Pictures were taken using a Nikon Eclipse E400 light microscope (Kawasaki, Japan). For staining of mouse tissue, the IHC protocol was modified as follows: 5% hydrogen peroxide in methanol instead of 3% for human tissue was used to block endogenous peroxide activity and the duration of this treatment was extended to 1.5 hours instead of 1 hour.

4. Transient Transfection and Luciferase Assays

All transient transfections were performed with Lipofectamine 2000 (Life Technologies; Carlsbad, CA, USA). Lysates/RNA/luciferase readings were taken 48 hours post-transfection. The pGL4.17 vector was a gift from Dr. Robert Bennett at UNMC. The dominant-negative TCF4 plasmid pPGS dnTcf-4(deltaN41) was a gift from Eric Fearon (Addgene plasmid #19284) [5]. M50 Super 8x TOPFlash and M51 Super 8x FOPFlash (TOPFlash mutant) were gifts from Randall Moon (Addgene plasmids # 12456 and # 12457) [6]. The TOPflash vector contains seven TCF/LEF-binding sites upstream of a firefly luciferase gene, while the FOPflash vector contains seven mutant TCF/LEF sites and was used as a negative control. The pRenilla-CMV luciferase vector #E2261 (Promega; Madison, WI, USA) was used as an internal transfection control in all luciferase assays, which were performed in triplicate and repeated twice. Briefly, 2 X 10^5 cells were seeded per well in 12 well plates and were transfected the following day. Luciferase readings were taken 48 hours later, using the Dual-Glo luciferase assay kit (Promega) as per the manufacturer's instructions.

5. RNA Isolation and Real-Time PCR Analysis

RNA was isolated and purified using the QIAGEN RNeasy mini kit (Qiagen; Valenica, CA, USA); the RNA concentration was measured using a NanoDrop ND 1000 Spectrophotometer. The Oligo(dT)12-18 Primer #18418-012 (Life Technologies) and Super Script II RNase reverse transcriptase (Invitrogen, Life Technologies) were used to obtain cDNA

from 1.5 µg of RNA per cell line. The PCR primers used are enumerated in **Table B.1**. Real-time PCR analysis was performed using the 480 Real-Time PCR System (Roche; Indianapolis, IN, USA). A master mix comprised of 2 X Sybr green mix (Life Technologies) with primers and nuclease-free water was used to constitute a 10 µl reaction mixture consisting of 1 µl cDNA and 9 µl of the master mix.

6. Immunofluorescence

For IHC with CD18/HPAF, 2 X 10^5, cells were seeded on coverslips in a 12-well plate and processed 48 hours later using a previously described protocol described [7]. The antibodies and dilutions used are listed in **Supplementary Table A**. Images were taken using an LSM 710 Zeiss Confocal Microscope located at UNMC Advanced Microscopy Core Facility.

7. Migration and Invasion Assay

For migration assays, 1.5 X 10^6 cells were plated in 1.5 ml serum-free DMEM on an 8 µm pore polyethylene cell culture insert in a 6-well plate (Falcon/VWR #353093; Radnor, PA, USA) and Matrigel-coated membrane inserts (BD Biosciences, Bedford, MA) for invasion assay. The lower chamber contained 2 ml DMEM supplemented with 10% FBS. The inserts were removed 36 hours after seeding. Next, the cells at the top of the chamber were scraped off, and the cells that had traversed the membrane were stained with a Diff-Quick cell staining kit (Dade Behring Inc.; Westwood, MA, USA). Images of inserts were taken using QCapture (Surrey, BC, Canada) software version 2.0.12 at a 10X magnification.

8. Cell Proliferation and Colony Formation Assays

The cell proliferation reagent WST-1 (Roche Life Science, #05015944001; Penzberg, Upper Bavaria, Germany) was used to measure the proliferative rate of 1000 cells/well in a 96-well plate over three days in DMEM that contained 1% fetal bovine serum. The readings were taken as per the manufacturer's instructions. For the colony formation assay, 1,000 cells were

seeded per well in a 6-well plate in triplicate. The cells were maintained in 10% DMEM and allowed to form colonies for 21 days. Cells were then fixed with 100% methanol, stained with 0.4% crystal violet in methanol, and colonies were manually counted.

9. Scratch assay

For the scratch assay, 1 X 10^6 cells were seeded in triplicate in a 6 well plate such that they were 100% confluent and 24 hours subsequent to seeding; two scratches perpendicular to each other were made with a sterile 200 µl pipette tip. Photographs of the scratch were taken after washing twice with PBS 24 and 48 hours after seeding.

10. Western Blot Analysis

Western blot analysis was performed as previously described [7]. Briefly, 1.5-2 X 10^6 cells were seeded in a 10 cm plate, and lysates/RNAs were extracted 48 hours later, such that cells were 70 - 80% confluent. After a freeze-thaw cycle, lysates were thawed and syringe-passed through a $21^{5/8}$ gauge needle. Next, cells were quantified using Bio-Rad protein assay kit (Hercules, CA, USA). A 10% SDS-PAGE gel was used to resolve 20 - 40 µg of whole cell lysates for all proteins described, except MUC4, which was resolved on a 2%-agarose gel owing to its high molecular weight. The proteins were transferred onto a polyvinylidene difluoride membrane (Millipore; Billerica, MA, USA) and probed with primary antibodies overnight at 4ºC. The antibodies used and their respective dilutions are described in **Supplementary Table A**.

11. Quantitative ChIP assay

A total of 2 X 10^7 cells per cell line were used for the chromatin immunoprecipitation (**IP**), which was performed as described previously [8]. The 1% formaldehyde cross-linked chromatin was isolated and sheared into 500-1000 bp fragments by sonication (Bioruptor UCD-200, Diagenode; New York, NY, USA). Prior to IP, 1% of the sonicated DNA was taken as input. The concentrations of antibodies used for overnight incubation at 4ºC were as follows: 2.5 µg of

anti-β-catenin #610154 (BD Biosciences), -, 2 µg of IgG (negative control). Primers for TCF/LEF included Site #1, #2, and #3 on the *MUC4* promoter; the TCF/LEF site on the c-myc promoter (positive control) and primers for the promoter of an unrelated gene (negative control) were also used (Supplementary Table B.2.). Immunoprecipitated qPCR Ct (cycle threshold) values were normalized to input Ct values, and all data are represented as a percentage of input.

12. Generation of Constructs

For the 4ACAT construct, the β-catenin transcript was amplified using the appropriate primers (**Table B.3**) from the cDNA of a PDAC cell line, T3M4 that expressed the wild-type transcript. The amplicon was cloned into a p3XFLAG-CMV10 vector (Sigma-Aldrich; St. Louis, MO) digested with the Not1 enzyme (New England Biolabs; Ipswich, MA). Point mutations were introduced at Ser33, Ser37, Thr41, Ser45, which were mutated to alanine using appropriate primers **(Table B.3)**. The MUC4-promoter fragment was generated from genomic DNA (CD18/HPAF cell line) using primers that incorporated the HindIII and KpnI restriction sites (**Table B.3**). The amplicon was cloned into a pGL4.17 vector digested with HindIII and KpnI (New England Biolabs). The p3778 construct encompasses the entire *MUC4* promoter (proximal and distal promoter); the p3000 construct encompasses two TCF/LEF sites (proximal promoter and part of distal promoter), and the p2700 construct encompasses one TCF/LEF site (primarily proximal promoter). Primers used to generate these constructs are enumerated in **Table B.3**. Mutations at the TCF/LEF sites were introduced using primers enumerated in **Table B.2**. The instructions from the QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies; Santa Clara, CA) were used for primer design. Platinum® Taq DNA Polymerase High Fidelity Assay (Life Technologies) was used for all site-directed mutagenesis PCR reactions. Luciferase experiments were performed in triplicate and repeated a minimum of three times. Figure 4 B, C represent the average of a minimum of three attempts.

13. Promoter Analysis

Promoter analysis was performed using the MatInspector program (Genomatix GmbH; Munich, Bavaria, Germany). A matrix similarity score of > 0.85 was used to screen transcription factor binding sites.

14. Tumorigenicity Assay

For the tumorigenicity assay, sub-confluent cultures of CD18/HPAF Scr/sh-β-catenin cells were trypsinized and then counted using the Countess™ Automated Cell Counter (Life Technologies) after their viability had been ascertained (>95%). Next, 0.25 X 10^6 cells/50 µl PBS were orthotopically implanted in the head of the pancreas of 14 female athymic nude mice obtained from Harlan Sprague Dawley in Indianapolis, IN (7 per group). Mice were observed for five weeks, and were subsequently sacrificed and weighed. The animals were treated in accordance with guidelines from the UNMC Institutional Animal Care and Use Committee (**IACUC**). The primary tumors were excised and weighed. Tumor metastases sites were counted and dissected after a thorough physical examination. Both primary tumors and metastases were kept in 10% formalin for 48 hours, after which they were embedded in paraffin blocks that were sectioned into 0.5 micron-thick sections.

15. Gamma Secretase Inhibitor treatment

Cells were seeded at 50-60% confluency, i.e., 4X 10^5 cells per well in a 6 well plate 24 hours prior to treatment. The gamma secretase inhibitor dibenzazepine (DBZ) was purchased from EMD Milipore (CAS 209984-56-5 , catalog # 565789). DBZ was diluted in DMSO and treatment concentration was 500nM. Control cells were treated with an equal volume of DMSO.

16. Statistical Analysis

All data were analyzed using two-tailed T test with unequal variance.

Table A

Antibody	Supplier	Catalog no.	WB dilution
β-catenin	Sigma-Aldrich	C2206	1:4000
β-catenin	BD Biosciences	610154	1:1000
MUC4(8G7)	Generated in our lab	Clone 8G7	1:1000
MUC4(2214)	Generated in our lab	Clone 2214	
pSer9GSK3β	Cell Signaling	9336S	1:2000
GSK3β	Santa Cruz	9166	1:2000
N-cadherin	Dr. Keith R. Johnson, UNMC	-	1:500
E-cadherin	Dr. Keith R. Johnson, UNMC	-	1:500
Vimentin	Abcam	ab8978	1:1000
pY1248HER2	Cell Signaling	2247	1:1000
HER2	Cell Signaling	29D8	1:1000
pERK1/2	Cell Signaling	9101	1:1000
Total ERK1/2	Cell Signaling	9102	1:1000
Zo-1	Cell Signaling	5406	1:1000
β-actin	Sigma Aldrich	A5316	1:10,000
CD44 (8E2)	Cell Signaling	5640	1:1000
TCF4	EMD Millipore	17-10109	1:1000
c-Myc	Abcam	ab32072	1:2000
Cyclin D1	Santa Cruz	sc-753	1:500
FLAG	Cell Signaling	8146	1:1000
Hes1	Santa Cruz. Gift; Dr. Punita Dhawan, UNMC	sc-25392	1:800
Snail	Cell Signaling	3879	1:1000

Table B.1. Real-time PCR/RTPCR primers

Primer	Forward primer (5'-3')	Reverse primer (5'-3')
β-catenin	CCTGGTGAAAATGCTTGGTTCAC	GAAGGCAGTCTGTCGTAATAGCC
MUC4	GACTTGGAGCTCTTTGAGAATGG	TGCAATGGCAGACCACAGTCC
β-actin	TGGACATCCGCAAAGACCTG	CCGATCCACACGGAGTACTT
N-cadherin	CCTGATATATGCCCAAGACAAAGAGA	CACTGTGCTTACTGAATTGTCTTGGGA
Vimentin	GCAGCTCAAGGGCCAAGGCA	CCTGCAATTTCTCCCGGAGGCG
TCF1	CATCAGCCAGAAGCAAGTTCACAGGC	CAGAACCTAGCATCAAGGATGGGTG
TCF4	GGAAGAAGCGGCCAAGAGGCAAGATG	GGACTGAAAATGGAGGGTTCG
LEF1	CCATCCCGAGAACATCAAATAAAGTGC	GGACATGCCTTGTTTGGAGTTGACATC
TCF3	GAGAATGAACCAGCCGCAGA	CGGTCCTCAAGACCTGAACC
Hath1	CGAGAGAGCATCCCGTCTAC	TCCGGGGAATGTAGCAAATA

Table B.2. Quantitative PCR primers for ChIP assay

Primer	Forward primer (5'-3')	Reverse Primer (5'-3')
TCF site 1	GAGGGGAGCTGGAAAGCAGAAGGGAAAC	GAATTTCCAGGCCCAGGTCTTTC
TCF site 2	CAGACTTAATAAACATCCAGCTTCCTG	CATTTATGCCTCGTGCTGTGAAT
TCF site 3	TGGAGCCTCAGCGTGCTCACTTGA	CAGCATGAAGCCGGTCACTAGAGT
TCF-c-Myc	CCCAAAAAAAGGCACGGAA	TATTGGAAATGCGGTCATGC
Negative control	TGAACTGTGGTGGAGAGTGC	AGGAAGGGCTAGGACGAGAG

Table B.3. Cloning primers

Primer	Forward primer (5'-3')	Reverse primer (5'-3')
CMV10-bCat-NOT1	GTTATAGCGGCCGCGGCTACTCAAGCTGATTTGATGGAGTTG	GTTATGCGGCCGCTTACAGGTCAGTATCAAACCAGGCCAG
S45A	CACTACCACAGCTCCTGCTCTGAGTGGTAAAGG	CCTTTACCACTCAGAGCAGGAGCTGTGGTAGTG
S33A	AACAGTCTTACCTGGACGCTGGAATCCATTCTGGT	ACCAGAATGGATTCCAGCGTCCAGGTAAGACTGTT
S37A	CTGGACGCTGGAATCCATGCTGGTGCCAC	GTGGCACCAGCATGGATTCCAGCGTCCAG-
T41A	GGAATCCATGCTGGTGCCACTGCCACAGCTCCT	AGGAGCTGTGGCAGTGGCACCAGCATGGATTCC
MUC4_3788_KpnI FP	GTTATAGGTACCGACTGCCTGTTGGCGGAGTCTTGTGGGGTGGAAATGG	
MUC4_3788_HindIII_RP		GTTATAAGCTTGAACCAAGTGCGTTTCTCCGAAGGGGCCAGGGAACCTG
MUC4_2743_KpnI_FP	GTTATATGGTACCGAGGTGAACAGGAGTGCGCACGCCCAGTTCTCCAGG	
pGL4.17_del3087_FP	CTCAATATGCCCATTTTGGTACCGGCCAGTTAGG	CTAACTGGCCGGTACCCTGGGTCACTGACTAG
TCFMUT1	TCTCACGCTCCTAATCAGGATCCTATAAGACGTGCGAGGGACGCAGGAAAGACCTGGGCCTGGAAAT	ATTTCCAGGCCCAGGTCTTTCCTGCGTCCCTCGCACGTCTTATAGGATCCTGATTAGGAGCGTGAGA
TCFMUT2	GAGATCAGCCTAGTTGTCCTAAGTCTGACAAGGCCACGCTTTATTCACAGCACGAGGCATAAATGATATA	TATATCATTTATGCCTCGTGCTGTGAATAAAGCGTGGCCTTGTCAGACTTAGGACAACTAGGCTGATCTC
TCFMUT3	GCTCACTTGAGAGGTAGGGCAGCGACCCAGGCTTCACAGGGCCATGGGGACAGGC	GCCTGTCCCCATGGCCCTGTGAAGCCTGGGTCGCTGCCCTACCTCTCAAGTGAGC

Table B.4. Genotyping primers		
Primer	**Forward primer (5'-3')**	**Reverse Primer (5'-3')**
APC$^{flox/flox}$	gag aaa ccc tgt ctc gaa aaa a	agt gct gtt tct atg agt caa c
Cdx2-Cre	gcg gtc tgg cag taa aaa cta tc	gtg aaa cag cat tgc tgt cac tt
Kras^{G12D}	gca ggt cga ggg acc taa ta	ctg cat agt acg cta tac cct gt
Muc4 KO	atcagtaaattggtgtgtacttgtgtgcag	ctgtcagaagatgttgatgaggtcgatgct
Muc4 WT allele	atcagtaaattggtgtgtacttgtgtgcag	gtttccttgaaggactccaatagggtaccc

References

[1] Mullins TD, Kern HF, Metzgar RS. Ultrastructural differentiation of sodium butyrate-treated human pancreatic adenocarcinoma cell lines. Pancreas 1991;6:578-87.

[2] Okabe T, Yamaguchi N, Ohsawa N. Establishment and characterization of a carcinoembryonic antigen (CEA)-producing cell line from a human carcinoma of the exocrine pancreas. Cancer 1983;51:662-8.

[3] Barker N, van den Born M. Detection of beta-catenin localization by immunohistochemistry. Methods Mol Biol 2008;468:91-8.

[4] Kaur S, Momi N, Chakraborty S, et al. Altered expression of transmembrane mucins, MUC1 and MUC4, in bladder cancer: pathological implications in diagnosis. PLoS One 2014;9:e92742.

[5] Kolligs FT, Hu G, Dang CV, Fearon ER. Neoplastic transformation of RK3E by mutant beta-catenin requires deregulation of Tcf/Lef transcription but not activation of c-myc expression. Mol Cell Biol 1999;19:5696-706.

[6] Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr Biol 2003;13:680-5.

[7] Seshacharyulu P, Ponnusamy MP, Rachagani S, et al. Targeting EGF-receptor(s) - STAT1 axis attenuates tumor growth and metastasis through downregulation of MUC4 mucin in human pancreatic cancer. Oncotarget 2015;6:5164-81.

[8] Kumar S, Das S, Rachagani S, et al. NCOA3-mediated upregulation of mucin expression via transcriptional and post-translational changes during the development of pancreatic cancer. Oncogene 2014;10.

CHAPTER 3

The β-catenin/TCF-mediated regulation of MUC4 in pancreatic ductal adenocarcinoma

The material of this chapter is the subject of 1 research article.

1. Pai P, Rachagani S, Lakshmanan I, Macha MA, Sheinin Y, Smith LM, Ponnusamy MP, Batra SK *The Canonical Wnt Pathway Regulates the Metastasis-Promoting Mucin MUC4 in Pancreatic Ductal Adenocarcinoma*, **Molecular Oncology** (*In press*)

The Canonical Wnt Pathway Regulates the Metastasis-Promoting Mucin MUC4 in Pancreatic Ductal Adenocarcinoma

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1. Synopsis

Aberrant Wnt signaling frequently occurs in pancreatic cancer (PC) and contributes to disease progression/metastases. Likewise, the transmembrane-mucin MUC4 is expressed *de novo* in early pancreatic intraepithelial neoplasia (PanINs) and incrementally increases with PC progression, contributing to metastasis. To determine the mechanism of MUC4 upregulation in PC, we examined factors deregulated in early PC progression, such as Wnt/β-catenin signaling. *MUC4* promoter analysis revealed the presence of three putative TCF/LEF-binding sites, leading us to hypothesize that *MUC4* can be regulated by β-catenin. Immunohistochemical (IHC) analysis of rapid autopsy PC tissues showed a correlation between MUC4 and cytosolic/nuclear β-catenin expression. Knock down (KD) of β-catenin in CD18/HPAF and T3M4 cell lines resulted in decreased *MUC4* transcript and protein. Three *MUC4* promoter luciferase constructs, p3778, p3000, and p2700, were generated. The construct p3778, encompassing the entire *MUC4* promoter, elicited increased luciferase activity in the presence of stabilized β-catenin. Mutation of the TCF/LEF site closest to the transcription start site (i.e., -2629/-2612) and furthest from the start site (i.e., -3425/-3408) reduced *MUC4* promoter luciferase activity. Transfection with dominant negative TCF4 decreased MUC4 transcript and protein levels. Chromatin immunoprecipitation confirmed enrichment of β-catenin on -2629/-2612 and -3425/-3408 of the *MUC4* promoter in CD18/HPAF. Functionally, CD18/HPAF and T3M4 β-catenin KD cells showed decreased migration and decreased Vimentin, N-cadherin, and pERK1/2 expression. Tumorigenicity studies in athymic nude mice showed CD18/HPAF β-catenin KD cells significantly reduced primary tumor sizes and metastases compared to scrambled control cells. We show for the first time that β-catenin directly governs MUC4 in PC.

2. Background and rationale

Pancreatic cancer (PC) is a highly lethal disease with a rather dismal prognosis, showing a five-year survival rate of only 7.2% (SEER Stat Fact Sheet, 2005 - 2011). While numerous studies have focused on the genetic abnormalities that underpin this disease, much remains unknown regarding this complex, intractable malignancy that unfortunately often only manifests symptoms in patients at advanced, metastatic stages [1, 2]. Most prominent among the mutations driving PC is the *Kras* oncogene, which is mutated into a constitutively active form ($Kras^{G12D}$) in around 90% of PC patients [3]. In addition to mutations in Kras, the Wnt signaling pathway has been described as one of the 12 pathways most commonly deregulated in pancreatic ductal adenocarcinoma (PDAC), which is the most prevalent type of pancreatic neoplasms [4].

A central mediator of the canonical Wnt pathway is β-catenin, a molecule that plays an important role in both cell adhesion and signaling. There are two distinct pools of β-catenin - one cytosolic and the other membranous [5]. The membranous fraction participates in cell adhesion through interactions with E-cadherin, while the cytosolic fraction is ordinarily degraded by a destruction complex, which is comprised of Adenomatous polyposis coli (APC), Glycogen Synthase Kinase β, Axin1, and Casein Kinase1 [5]. In the presence of a Wnt ligand, which binds the Frizzled/LRP receptor, this complex is abolished and β-catenin is released, whereupon β-catenin enters the nucleus and upregulates a host of tissue-specific target genes, typically partnering with the TCF/LEF family of transcription factors [5]. Wnt ligands can also activate the non-canonical pathway, which is independent of β-catenin [5]. While mutations in this pathway are rare in PDAC, a spate of recent studies demonstrates the importance of both the canonical and non–canonical pathways in PDAC [6-10]. Specifically, the gene expression signature of the Wnt/β-catenin pathway as well as aberrant β-catenin localization are implicated in conferring a poorer prognosis in patients [11], as well as promoting PC metastases [9].

Aberrant cytosolic and nuclear localization of β-catenin occurs early on in PDAC and steadily increases with disease progression, starting from the earliest stage of pancreatic intraepithelial neoplasia 1 (PanIN-1) [7]. Sustained low-grade activation of the canonical Wnt pathway is essential for PDAC progression, subsequent to the Kras mutation, in a mouse model of PDAC [12]. Further, the Wnt/ β-catenin pathway is active in most PDAC cell lines and confers increased proliferative and anti-apoptotic properties to PDAC cells [8].

MUC4 is a transmembrane mucin that is absent in the normal pancreas but incrementally increases as PDAC advances, with expression commencing at the PanIN-1 stage [13-15]. Importantly, our lab has shown the importance of MUC4 in the invasion and metastases of PDAC [16-19]. A 2008 study by Chaturvedi *et al.* proposed that the epidermal growth factor (EGF) domains of MUC4 act as ligands for HER2, thereby triggering an intracellular cascade of signaling events involving the MAPK and AKT pathways [20]. Other studies have shown that knock down (KD) of MUC4 is sufficient to induce a decrease in mesenchymal markers, such as Vimentin, and increase in epithelial markers, such as E-cadherin, in PDAC cell lines [21, 22]. These alterations in epithelial and mesenchymal markers suggest that MUC4 expression alone induces epithelial-mesenchymal transition (EMT) in PDAC. Notably, MUC4 expression is also a marker for poor prognosis in PDAC [23].

The *de novo* expression of MUC4 in PDAC has been attributed to factors such as nicotine, retinoic acid, interferon-γ, CFTR, TGF-β, and several miRNAs including miR-200c, miR-219-1-3p, m-iR-150 [24-31]. The *de novo* expression of MUC4 has been further attributed to the transcription factor NCOA3 [32] as well as epigenetic mechanisms like hypomethylation and histone acetylation [33].

The *MUC4* promoter is well characterized; it is around 3.7 kb long [34], and its TATA box is located at -2672/-2668 [34]. The MUC4 proximal promoter contains two highly transcriptionally active regions; -219/-1 and -2781/-2572 [34]. Interestingly, MUC4 has been

hypothesized to aid in the nuclear localization of β-catenin in PDAC by inducing dissociation of β-catenin from E-cadherin via HER2/Src signaling [21].

The objective of this study was to examine the nature and functional implications of the β-catenin-MUC4 relationship and the effect of this relationship on PDAC metastasis. For this purpose, the study herein involved *MUC4* promoter analysis, which showed the presence of three putative TCF/LEF sites. This finding implies that *MUC4* is a putative transcriptional target of the Wnt/β-catenin pathway in PDAC. Another piece of evidence suggesting a β-catenin-MUC4 relationship was the observation that when β-catenin was depleted using a pancreas-specific Cre in the KPC (PDX-1-Cre, LSL-$Kras^{G12D}$, LSL-$Trp53^{R172H/-}$) mouse model, β-catenin-negative cells showed significantly reduced mucin expression, as measured by alcian blue staining [12]. Another recent study by Olsen *et al.* showed genetically ablated β-catenin by zinc finger nucleases in the PDAC cell line BXPC3 [35]. A subsequent microarray showed that MUC4 was one of the most significantly downregulated genes in PDAC [35], giving further impetus to our hypothesis that Wnt/β-catenin pathway colludes with transcription factors including retinoic acid, interferon-γ, and NCOA3 to precipitate MUC4 expression in PDAC.

3. Results

A. Nuclear/cytosolic β-catenin was associated with MUC4 expression in PDAC

Western blot analysis was used to screen panels of PDAC cell lines for expressions of MUC4 and β-catenin (**Figure 1A**). MUC4 was shown to be expressed in all β-catenin-expressing cell lines (*i.e.*, BXPC3, Capan1, CD18/HPAF, T3M4) but was absent in β-catenin-non-expressing cell lines (*i.e.*, MiaPaCa, Panc1), with the exception of AsPc1. Next, IHC was performed on a PDAC TMA, and it was found that both MUC4 and cytosolic/nuclear β-catenin, which is a hallmark of active Wnt/β-catenin signaling [36], were present in 80% of the PDAC tissue spots examined (n = 25, mean composite score for MUC4= 3.52 [21/25] and for β-catenin = 7.92

[24/25]) (**Figure 1B**). Further, tissue immunofluorescence of human PDAC showed that MUC4 was expressed in cells that expressed nuclear/cytosolic β-catenin (**Figure 1C**).

B. Wnt/β-catenin regulated MUC4 expression in PDAC

Two lentiviral shRNAs that targeted β-catenin were used to stably KD expression of β-catenin in PDAC cell lines T3M4 and CD18/HPAF. It was found that upon the KD of β-catenin, expressions of MUC4 transcripts and proteins were reduced in comparison to the scrambled control-transfected cells in both cell lines (**Figure 2A, Supplementary Figure 1B**). Confocal microscopy was used to confirm the decrease in MUC4 expression in CD18/HPAF and T3M4 cell lines (**Figure 2B**, **Supplementary Figure 1C**). For further confirmation, Lithium Chloride (LiCl) was used; LiCl inhibits the GSK3-β enzyme [37] and potentiates increased nuclear β-catenin due to the prevention of its N-terminal phosphorylation-mediated degradation [37]. Next, 20 mM and 50 mM LiCl treatment was performed for 48 hours in CD18/HPAF, resulting in increased MUC4 expression in a dose-dependent manner (**Figure 2C**). These findings are concurrent with the increase in the inhibitory expression of pSer9GSK3-β. In order to employ a more specific inducer of Wnt/β-catenin signaling, CD18/HPAF cells were treated with Wnt-3A conditioned medium. Compared to untreated control cells, increasing doses of Wnt-3A conditioned medium resulted in increased MUC4 protein expression (**Figure 2D**). The level of c-Myc, which is a Wnt/β-catenin target gene in PDAC [38], was used as a positive control.

C. β-catenin directly regulated MUC4 transcript expression

In light of observations for a correlation between aberrant β-catenin and MUC4 expression in PDAC, and the decrease in MUC4 protein and RNA levels upon β-catenin KD, it was next determined whether β-catenin can directly regulate *MUC4* transcription. *MUC4*-promoter analysis by the Matinspector program (genomatix.de) revealed the presence of three putative TCF/LEF sites at the following positions: -3408 (Site #3), -3226 (Site #2), and -2612 (Site #1), with matrix similarity scores of 0.91, 0.84, and 0.907, respectively (**Figure 3, Table 1**).

Next, the following three *MUC4* promoter constructs were generated: (i) p3778, which encompasses the entire *MUC4* promoter and has three TCF/LEF sites, (ii) p3000, which encompasses the proximal promoter as well as part of the distal promoter and has two TCF/LEF sites, and (iii) p2700, which encompasses the proximal promoter as well as TATA box and has one TCF/LEF site (**Figure 3**). Following the generation of promoter constructs, a stabilized β-catenin construct, 4ACAT, was generated with four mutations (S33A, S37A, T41A, and S45A) that prevent degradation; the TOPflash/FOPflash assay was used to test the efficacy of this construct. Notably, CD18/HPAF that was transiently transfected with either 4ACAT or the empty vector showed a significantly elevated TOPflash/FOPflash luciferase activity in the 4ACAT cells (**Figure 4A**), indicating that 4ACAT elicits an increased β-catenin/TCF-mediated transcription activity. Next, the luciferase activities were compared for all three promoter constructs in the presence of 4ACAT and the empty vector. Compared to the other two constructs p3000 and p2700, the promoter construct p3778 elicited significantly increased luciferase activity compared to the empty vector (**Figure 4B**). On the other hand, the p2700 construct displayed a higher basal level of luciferase activity, but no significant increase in luciferase activity was seen in the presence of 4ACAT.

Following these findings from the luciferase assays for 4ACAT cells, luciferase assays were then performed for T3M4 Scr and KD cells. Subsequently, p3778-driven luciferase activity was shown to be reduced in the KD cells but not in the T3M4 Scr cells (**Supplementary Figure 2A**). Each TCF/LEF site on the p3778 construct was mutated individually as well as in combination; the following five different constructs were then generated: MUT1, MUT2, MUT3, MUT2,3, and MUT1,2,3, which are enumerated in **Table 1**. Interestingly, after CD18/HPAF cells were transfected with p3778, MUT1, MUT2, and MUT3 in the presence of 4ACAT, it was seen that MUT1 elicited significantly reduced luciferase activity in comparison to the un-mutated promoter (**Figure 4C**. Surprisingly, in comparison to p3778, MUT2 elicited an increased

luciferase activity, while MUT3 elicited a decreased luciferase activity. Additionally, when all three sites were mutated, luciferase activity was diminished compared to p3778, but was increased compared to MUT1 and MUT3 (**Figure 4C**). A similar pattern was observed for T3M4 (**Supplementary Figure 2B**).

Because Site #1 appeared to be critical for *MUC4* transcription, another construct was generated, MUT2,3, for which Sites #2 and #3 were mutated, but Site #1 was retained. The levels of luciferase activity for MUT2,3 were decreased compared to that of p3778, suggesting that Sites #2 and/or #3 also play a role in 4ACAT-mediated transcription of *MUC4*. To confirm if a β-catenin/TCF complex physically occupies the *MUC4* promoter at one or more TCF/LEF sites, quantitative Chromatin immunoprecipitation (ChIP) assays were performed in CD18/HPAF Scr and CD18/HPAF β-catenin KD cells (**Figure 4D**). A pull down was performed with a β-catenin antibody and IgG as a negative control. Real-time PCR was performed using primers that amplified regions containing the TCF/LEF Sites #1, #2, and #3 on the *MUC4* promoter. As a positive control, the TCF/LEF site on the *c-Myc* promoter was used, which is a β-catenin target gene in PDAC; a non-specific primer pair was used as a negative control. In CD18/HPAF Scr, but not CD18/HPAF sh-β-catenin, significant enrichment of β-catenin was seen for TCF Site #1, but not for TCF Site #2; further, roughly two-fold enrichment was seen for Site #3 compared to the negative control.

D. β-catenin partnered with TCF4 to regulate MUC4 expression

Cell lines used in this study (*i.e.,* CD18/HPAF and T3M4) were profiled for the expression of TCF/LEF factors. Notably, TCF1, 3, and 4 were found to be abundantly expressed in both cell lines (**Supplementary Figure 3A**.). Because TCF4 is the most significantly over-expressed TCF/LEF factor in PDAC [39], CD18/HPAF cells were transfected with dominant-negative TCF4 (*i.e.*, dnTCF4), which lacks an N-terminal β-catenin-binding domain. Decreases in MUC4 protein levels and RNA expression were observed upon transfection with dnTCF4

(**Supplementary Figure 3B**), suggesting that TCF4 partners with β-catenin on the *MUC4* promoter. Further, confocal microscopy for HPAF/CD18 cells showed that TCF4 primarily co-localized with β-catenin in the nucleus (**Supplementary Figure 3C**).

E. β-catenin contributed to migratory and mesenchymal properties of PDAC cell lines

In order to ascertain the functional implications of the β-catenin-MUC4 axis, migration, proliferation, and colony formation assays were performed with CD18/HPAF Scr and KD cells. Because MUC4 can mediate migration, metastasis, and EMT via HER2/RAF/MEK/ERK signaling [21, 22, 40], it was hypothesized for the present study that β-catenin causes EMT partly via MUC4 upregulation. It was seen that, while migration was significantly reduced upon β-catenin KD in both CD18/HPAF and T3M4 cells (**Figure 5a, Supplementary Figure 4C**), there was a non-significant decline in proliferation and colony formation (**Supplementary Figure 4B**). It was further observed that the CD18/HPAF KD cells also assumed a more cobblestone-like morphology compared to the dispersed, spindle-shaped Scr cells (**Figure 5B**).

Given the change in morphology and the decreased migration upon β-catenin KD, the markers of EMT were then analyzed. Notably, in T3M4 KD cells, marked decreases at the protein levels were observed for the mesenchymal markers N-cadherin, CD44, and Vimentin, and increases were observed at the protein level for epithelial markers E-cadherin and Zo-1. On the other hand, CD18/HPAF KD cells showed a decrease in the EMT-regulator Snail and an increase in E-cadherin, as well as an increase in the molecular weight of Zo-1 (**Figure 5C, Supplementary Figure 5A**). Further, a significant reduction in phospho-ERK1/2 occurred in the KD cells for both cell lines (**Figure 5C, Supplementary Figure 5A)**; phospho-ERK1/2 is a downstream effector of MUC4-HER2 signaling [20]. In contrast, the total ERK levels did not significantly decrease in the KD cells for both cell lines. Next, considering that CD18/HPAF cells express very low protein levels of N-cadherin and Vimentin, the RNA levels of these mesenchymal makers were examined. Interestingly, RNA levels were decreased in the

CD18/HPAF KD cells (**Figure 5D**). In addition, the T3M4 KD cells showed a decrease in phospho-HER2.

Given that the relationship between β–catenin and other transmembrane mucins such as MUC1 and MUC16 is well studied, we sought to determine whether β–catenin KD affects other mucins in addition to MUC4. In the CD18/HPAF KD cells, a significant decrease was seen in MUC16 levels (**Supplementary Figure 5B**), while no significant difference was seen in T3M4 KD cells (data not shown). Interestingly, an increase in MUC1 levels occurred in both KD cell lines (**Supplementary Figure 5B**). Overall, these observations suggest that β-catenin contributes to an increase in the migratory and mesenchymal properties of PDAC cells as well as alterations in mucin levels.

F. Orthotopic implantation of β-catenin KD cells affected metastases

In order to examine tumorigenesis and metastases of PDAC, equal amounts of HPAF/CD18 Scr and β-catenin KD cells (0.25 x 106 cells / 50µl) were orthotopically implanted at the head of the pancreases of seven athymic nude mice, who were monitored for the next five weeks. While the Scr group showed high cachexia, mortality, elevated tumor burden, and metastases, the β-catenin KD group showed significantly lower tumor burden and reduced metastases (**Figure 6A and 6C, Supplementary Figure 6A**). Sites of metastases were most significantly reduced for the diaphragm and peritoneal cavity (**Figure 6C**). IHC and Western blot analysis of tumor lysates from primary tumors confirmed that β-catenin KD was maintained *in vivo*. Further, MUC4, pHER2, and tHER2 levels were also reduced in the KD tumor lysates compared to the control cells (**Figure 6B, Supplementary Figure 6B**).

In order to ascertain the effect of the β-catenin-MUC4 axis on metastasis in human tissue, two metastatic TMAs were analyzed for β-catenin and MUC4 expression (25 spots, liver metastasis, UNMC Rapid Autopsy Program). Both aberrant β-catenin and MUC4 were elevated in spots corresponding to the same patient (**Figure 7**). Of the liver metastatic spots examined,

40% (10/25) expressed both MUC4 and β-catenin, while MUC4 was expressed in 40% (10/25) of the spots (mcs = 5.7). Notably, aberrant β-catenin was universally expressed in all metastatic spots.

4. Discussion

Both aberrant β-catenin signaling and MUC4 overexpression have been shown to contribute to PDAC progression [6, 8, 12, 14, 17]. Mutations in the key components of Wnt signaling, β-catenin, and APC are uncommon in PDAC [6]. Despite this, pronounced aberrant β-catenin signaling has been observed in over 65% of patients with PDAC [6]. Importantly, aberrant β-catenin signaling has been attributed to factors such as overexpression of ataxia-telangiectasia group D complementing gene (ATDC) [41, 42], upregulation of Wnt-7b [9], the c-met receptor tyrosine kinase [43], and the chemokine receptor CXCR4 [44]. While β-catenin signaling does not appear to drive the formation of PDAC, it was shown to be a strong driver of metastases and tumor cell invasion in mice with a Kras-mutant background [12]. Further, oncogenic Kras has been shown to induce the expression of the *ATDC* gene [41], which activates β-catenin signaling via stabilization of Dishevelled-2, abolishing the *destruction complex*. Likewise, the *ATDC* gene was found to induce EMT and metastases via β-catenin in a mouse model that expressed both transgenic *ATDC* and mutant Kras, which was driven by a pancreas-specific promoter p48-Cre [41].

While MUC4 expression incrementally increases from PanIN-1A to PDAC [13], aberrant (cytosolic/nuclear) β-catenin first occurs in the PanIN-1 stage, with increasing aberrant localization occurring in advanced PanIN lesions and PDAC [7]. Our expression analysis of primary PDAC tissues suggests that aberrant β-catenin localization and MUC4 occur in most (80%) cases of PDAC. In a 2014 study, Olsen *et al.* performed a microarray analysis of the human PDAC cell line BXPC3, which was completely depleted of β-catenin using zinc-finger nucleases. Their analysis showed that β-catenin potentially regulates a wide array of cell adhesion

molecules, including integrins, laminins, tight junction proteins, and other members of the adherens junction [35]. Interestingly, *MUC4 was* included in their list of the most significantly downregulated transcripts upon depletion of β-catenin, which is a molecule shown to contribute to EMT by its ability to act as a ligand for HER2. These observations, as well as the fact that PDAC cell lines that express the β-catenin protein also express MUC4, spurred further analyses of the *MUC4* promoter, which was found to contain three putative TCF/LEF sites.

The *MUC4* promoter has been shown to contain binding sites for numerous transcription factors, such as STATs, GATA, Sp1, and GR [34]. Further, the first TATA box is located at −2672/−2668 [34]. Our analysis showed that the first putative TCF/LEF site was located just proximal to the TATA box, at -2629/-2612, while the other two putative sites were located distal to the TATA box, at -3226 and -3408. The TCF/LEF binding site is a conserved sequence in the minor groove of DNA, with the consensus sequence being (A/T)(A/T)CAA(A/T)G [45]. Having confirmed that β-catenin can regulate MUC4 protein and RNA levels, we generated a construct that encompasses the entire *MUC4* promoter (p3778), which contains all three TCF/LEF sites. We further generated two other constructs (p3000 and p2700) that contain two and one TCF/LEF sites, respectively. Promoter luciferase studies with 4ACAT showed that, despite the presence of TCF/LEF sites in p2700 and p3000, there was significantly increased *MUC4* promoter luciferase activity only in the presence of all three sites (*i.e.*, p3778; -3408 [Site #3], -3226 [Site #2], and -2612 [Site #1]). Our promoter luciferase studies indicated that (i) the *MUC4* transcript is directly regulated by β-catenin when all three TCF/LEF sites are present, and (ii) the TCF/LEF site proximal to the TATA box is critical for β-catenin mediated *MUC4* regulation. The latter result was surprising given that p2700 construct, which containing only one TCF/LEF site, was unable to elicit significantly increased *MUC4* promoter luciferase activity in the presence of 4ACAT. However, we also observed that MUT3 reduces *MUC4* promoter luciferase activity, and that some β-catenin/TCF binding also takes place at Site #3, suggesting that Site #3 also regulates

MUC4 transcription. Likewise, MUT2 increased *MUC4* promoter luciferase activity, suggesting that Site #2 ordinarily represses *MUC4* transcription. However, we did not observe any discernible β-catenin/TCF-binding at Site #2 via ChIP analysis. Thus, it appears that while TCF/LEF Site #1 is the most critical for *MUC4* transcription, Site #3 is also required for β-catenin-mediated *MUC4* regulation. Further, there appears to be a combinatorial enhancement of transcription via Sites #1 and #3. Another factor to be considered is that TCF/LEF Site #1 is in extreme proximity to, but does not overlap with, the TATA box. As such, mutating this site may also affect numerous other factors that bind in this very active promoter region [34]. However, our ChIP results suggest that β-catenin does indeed bind this site. Given that *MUC4* transcription has been shown to be governed by several other factors, such as STAT1 [16], NCOA3 [32], IFNγ, and retinoic acid [26], it is likely that multiple disease-stage specific factors govern *MUC4* expression, and may collude to increase MUC4 expression.

Functionally, several studies have shown that aberrant β-catenin signaling contributes to the migratory and metastatic properties of PDAC cells [8, 41]. Further, it has been suggested that β-catenin signaling is epistatic to the MAPK/ERK pathway in PDAC [12], although the precise mechanism has not been delineated. Notably, the ERK pathway has been implicated in PDAC metastases and invasion [46]. Incidentally, KD of MUC4 in PDAC has been shown to decrease pERK1/2 levels [22]. The mesenchymal marker Vimentin has been shown to be a direct target of β-catenin/TCF-LEF in breast cancer [47]. The reduction in Vimentin protein and RNA in our KD cells suggests that Vimentin may also be a target in PDAC. Furthermore, Zo-1 is considered an epithelial marker, but some studies indicate that Zo-1 is overexpressed in PDAC and contributes to metastasis [48]. While we saw an increase in Zo-1 levels in T3M4 KD cells, we observed a shift to a slightly higher molecular weight Zo-1 in CD18/HPAF KD cells. The occurrence of two distinct isoforms of Zo-1 is documented for different cell types [49]. Although we did not determine whether there was indeed a shift to a different isoform in the KD cells, in light of the

changes in morphology observed in the KD cells, we surmise that this could be a possibility. What we did observe CD18/HPAF KD cells was a reduction in the EMT regulator Snail, which been shown to repress E-cadherin and MUC1 expression, as well as promote transcription of Vimentin [50]. This could be an explanation for the increased levels of MUC1 observed in both our KD cell lines. Alternatively, the increase in MUC1 could represent a compensatory mechanism for the loss of other transmembrane mucins such as MUC16 (in CD18/HPAF) and MUC4. Given that the MUC1-β-catenin interaction is well studied; these interesting observations with regard to MUC1 and β–catenin warrant further study. Notably, MUC16 has also been shown to interact with β-catenin [51]. However, while we observed a significant reduction in MUC16 protein expression in the CD18/HPAF KD cells, no significant change was seen in T3M4 KD cells. These observations also warrant further studies.

KD of β-catenin in T3M4 cells resulted in decreased levels of MUC4, accompanied by reduced pHER2, suggesting that MUC4/HER2-driven oncogenic signaling was reduced in this cell line. While we did not observe a significant difference in pHER2 in the CD18/HPAF KD cells *in vitro*, we observed reduced pHER2 in the tumor lysates from the KD cells that were orthotopically implanted. Our earlier studies [52] showed that MUC4 is promiscuous and can also partner with other Erbb family members such as HER3 and HER4, whose expression increases as a compensatory mechanism when HER2 is knocked down. However, given that the MUC4 levels were significantly reduced in both our KD cell lines, the likelihood of any MUC4-HER3/HER4 coupling is low.

All the aforementioned studies indicate that β-catenin acts an EMT/metastasis driver in the context of a Kras mutation [12, 41]. In addition to up-regulating conventional EMT-related genes, such as CD44 [41], it is likely that Vimentin, which has been shown to be a direct target of β-catenin signaling in breast cancer [47], and MUC4 represent other genes that are up-regulated by β-catenin as part of a larger EMT program. Our tumorigenicity studies showed that both β-

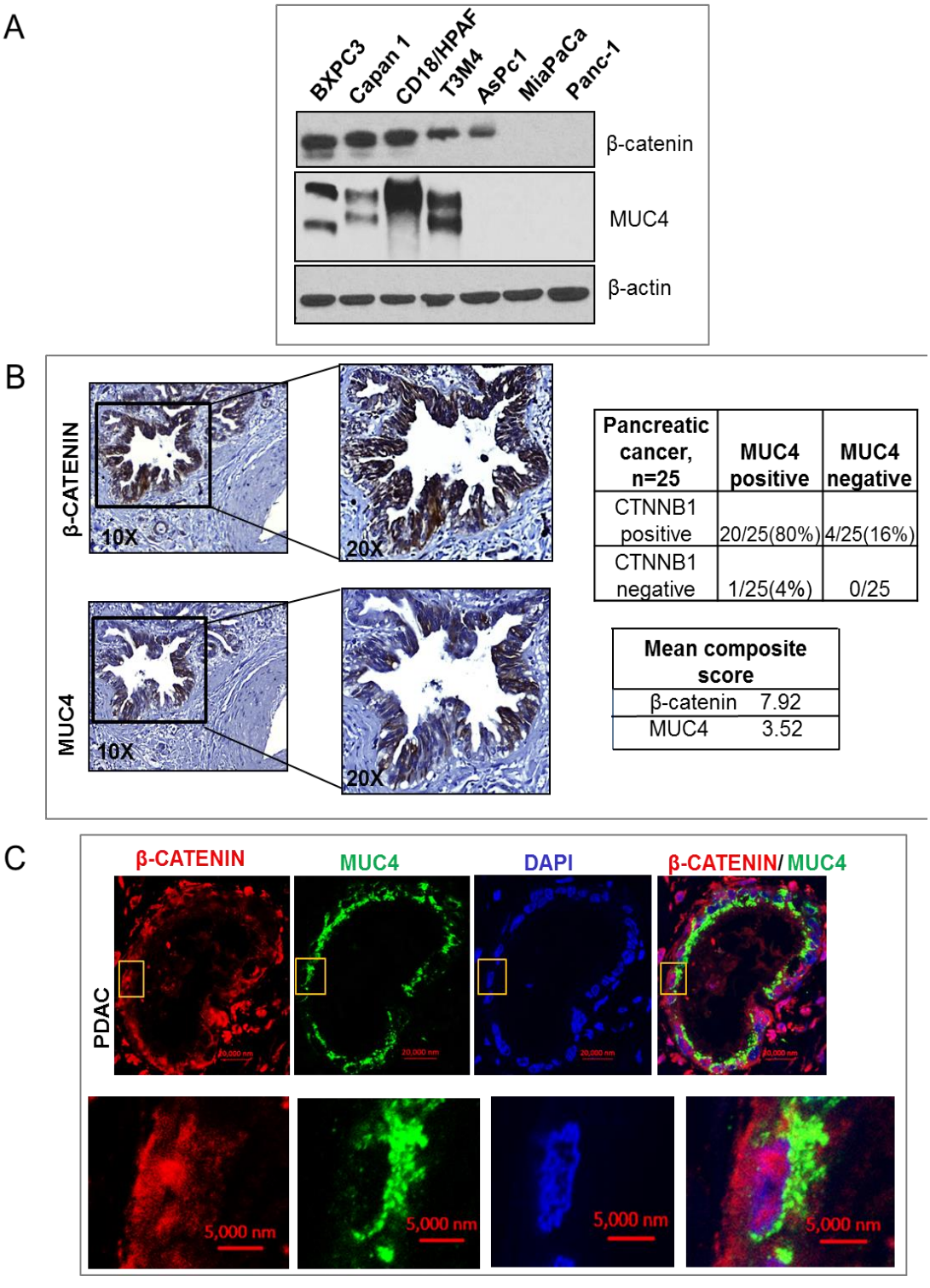
catenin and MUC4 expression contribute to enhanced tumorigenicity. Specifically, IHC analysis of metastatic human tissues showed that β-catenin and MUC4 were expressed in 40% of the liver metastatic tissues examined.

MUC4 is theorized to play an important role in EMT in PC [22] as well as in ovarian [40] and breast cancers [53]. For example, MUC4 stabilizes fibroblast growth factor receptor 1 (FGFR), thereby stabilizing N-cadherin [22]. The N-cadherin and FGFR complex then potentiates activation of the AKT and ERK pathways and stabilizes NF-KB and AP-1 transcription factors [22]. Interestingly, it has also been posited that MUC4 induces nuclear localization of β-catenin in PDAC [21], indicating the existence of a possible feed-forward loop.

In conclusion, our study demonstrates, for the first time, that β-catenin directly regulates MUC4 transcription in PC and that MUC4 may exacerbate the β-catenin-induced invasive and metastatic phenotypes of PDAC cells by contributing to the upregulation of several EMT markers. The findings of this study are summarized in **Figure 8.**

Figure 1. The expression pattern of MUC4 and β-catenin in PC tissue and cell lines. (A) Western blot analysis of protein lysates from a panel of PDAC cell lines showed that β-catenin (upper panel) was expressed in cell lines that expressed MUC4 (except the AsPc1 cell line). β-actin was used as a loading control. **(B)** Immunohistochemistry (**IHC**) of a tissue microarray (**TMA**) obtained from the UNMC Rapid Autopsy Program (n = 25) showed that both β-catenin (average composite score 7.92) and MUC4 (average composite score 3.52) were co-expressed in 80% of the PDAC spots examined. **(C)** Tissue immunofluorescence in pancreatic ductal adenocarcinoma (**PDAC**) tissue showed that MUC4 (green) was expressed in cells that also expressed nuclear/cytosolic β-catenin (red).

Figure 1



Pancreatic cancer, n=25	MUC4 positive	MUC4 negative
CTNNB1 positive	20/25(80%)	4/25(16%)
CTNNB1 negative	1/25(4%)	0/25

Mean composite score	
β-catenin	7.92
MUC4	3.52

Figure 2. MUC4 protein and RNA expression are governed by β-catenin. (A) CD18/HPAF and T3M4 pancreatic ductal adenocarcinoma (**PDAC**) cell lines were transfected with two lentiviral shRNAs that targeted β-catenin (shRNA-cat1, shRNA-cat2) or a scrambled sequence in the PLKO.1 vector. Further, knock down (**KD**) of β-catenin resulted in reduced MUC4 protein expression. **(B)** Confocal microscopy analysis was used to analyze MUC4 (green) and β-catenin (red) levels in CD18/HPAF Scr and CD18/HPAF shRNA-β-catenin (shRNA-cat) cells. **(C)** Treatment with lithium chloride (LiCl, a GSK3-β inhibitor) at 20 mM and 50 mM concentrations was used to induce nuclear β-catenin. Western blot analysis showed a dose-dependent effect on MUC4 levels and an increase in phosphorylation of the inhibitory Ser9 residue of GSK3-β, while total GSK3-β levels were unaffected. **(D)** CD18/HPAF cells were treated with increasing amounts of Wnt-3A-conditioned medium; levels of c-Myc were used as a positive control.

Figure 2

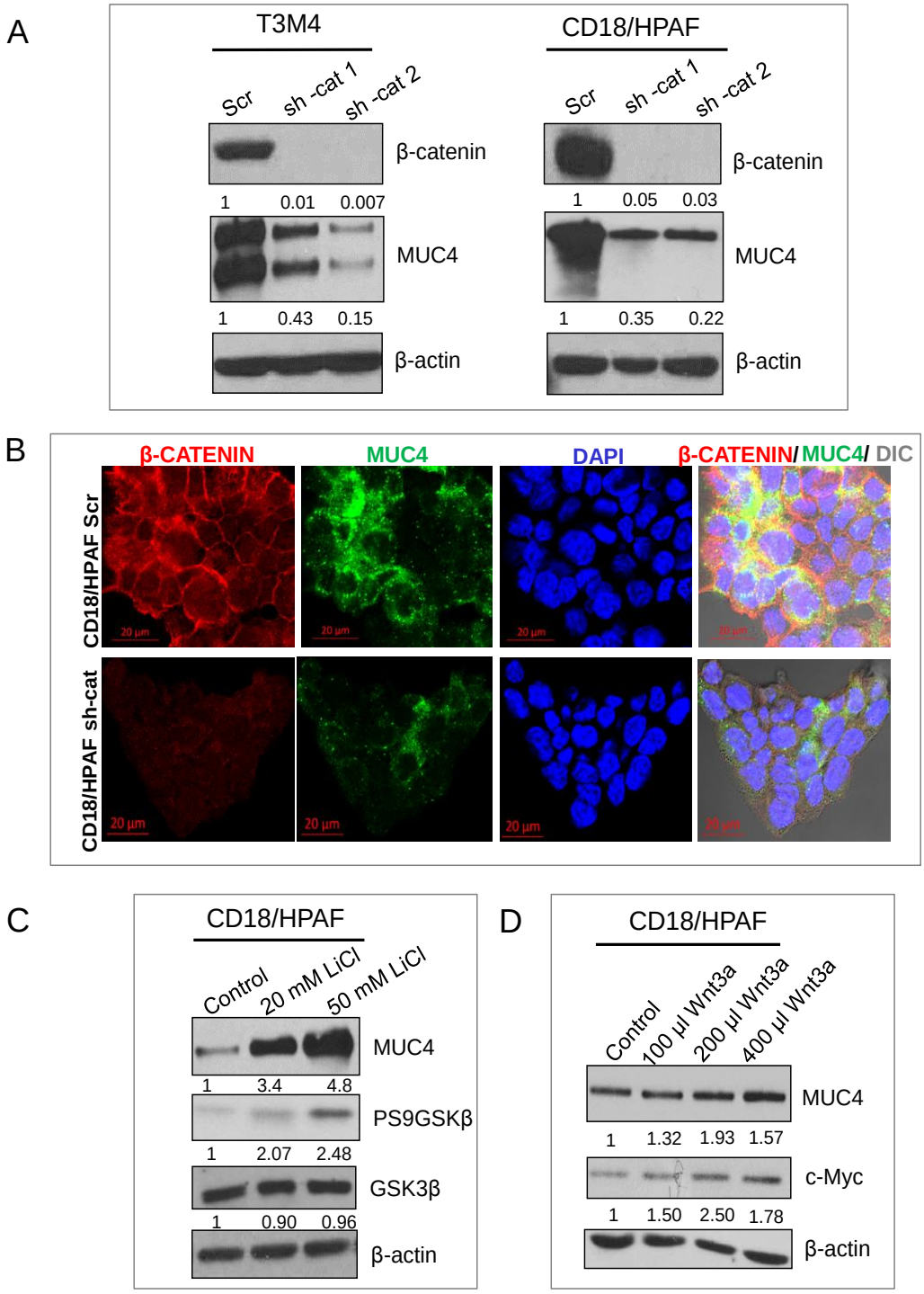

A
T3M4
CD18/HPAF
Scr
sh -cat 1
sh -cat 2
β-catenin
1
0.01
0.007
MUC4
1
0.43
0.15
β-actin
1
0.05
0.03
1
0.35
0.22
B
β-CATENIN
MUC4
DAPI
β-CATENIN/ MUC4/ DIC
CD18/HPAF Scr
CD18/HPAF sh-cat
20 µm
C
CD18/HPAF
Control
20 mM LiCl
50 mM LiCl
MUC4
1
3.4
4.8
PS9GSKβ
1
2.07
2.48
GSK3β
1
0.90
0.96
β-actin
D
CD18/HPAF
Control
100 µl Wnt3a
200 µl Wnt3a
400 µl Wnt3a
MUC4
1
1.32
1.93
1.57
c-Myc
1
1.50
2.50
1.78
β-actin

Figure 3. Schematic representation of the *MUC4* promoter constructs generated. Three MUC4 promoter constructs were generated and cloned into pGL4.7; p3778 encompasses the full promoter and incorporates all three putative TCF/LEF sites, p3000 encompasses the proximal promoter and part of the distal promoter and two putative TCF/LEF sites, while p2700 incorporates mainly the proximal promoter and one putative TCF/LEF site.

Figure 3

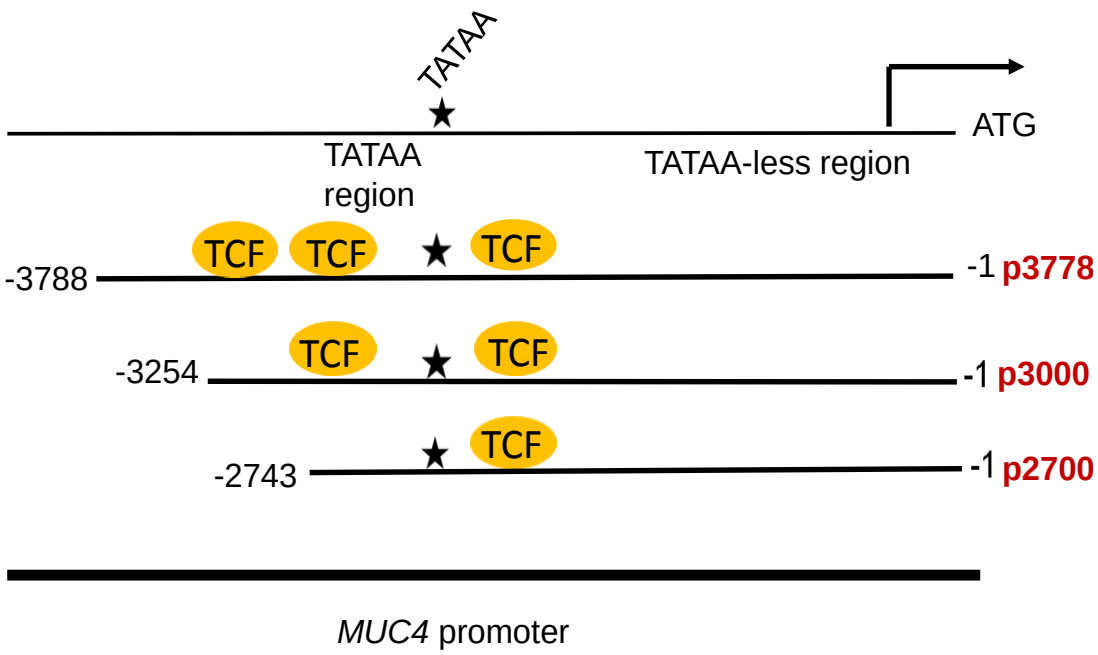

TATAA
TATAA
region
TATAA-less region
ATG
TCF
TCF
TCF
-3788
-1 p3778
TCF
TCF
-3254
-1 p3000
TCF
-2743
-1 p2700
MUC4 promoter

Figure 4. β-catenin directly regulates MUC4 transcription. (A) A stabilized β-catenin construct (4ACAT; S33A, S37A, T41A, T45A) elicited increased TOP/FOP luciferase activity in comparison to the empty vector control in CD18/HPAF cells. **(B)** Luciferase activity for *MUC4* promoter fragments p3778, p3000, and p2700 in the presence of 4ACAT compared to the empty vector control. Luciferase readings are the average of three or more separate experiments performed in triplicate; the standard deviation shown is for three or more separate experiments. **(C)** The p3778 promoter construct with each of the three putative TCF/LEF sites mutated (*i.e.,* -2612:MUT1, -3226:MUT2, -3408: MUT3) was transfected into CD18/HPAF cells in the presence of 4ACAT. The pCMV9-Renilla vector was used as an internal transfection control; all luciferase experiments were performed in triplicate and repeated a minimum of three times. Images represent the average of at least three experiments, each performed in triplicate. **(D)** Quantitative ChIP assay using β-catenin antibody to pull down sheared chromatin isolated from CD18/HPAF Scr and CD18/HPAF sh-cat. Primer pairs specific to the TCF/LEF Sites #1, #2, and #3 on the *MUC4* promoter were used. Primers amplifying the TCF/LEF site on the c-Myc promoter were used as a positive control; an unrelated primer pair was used as a negative control. All real-time values were normalized to the 1% input control.

Figure 4

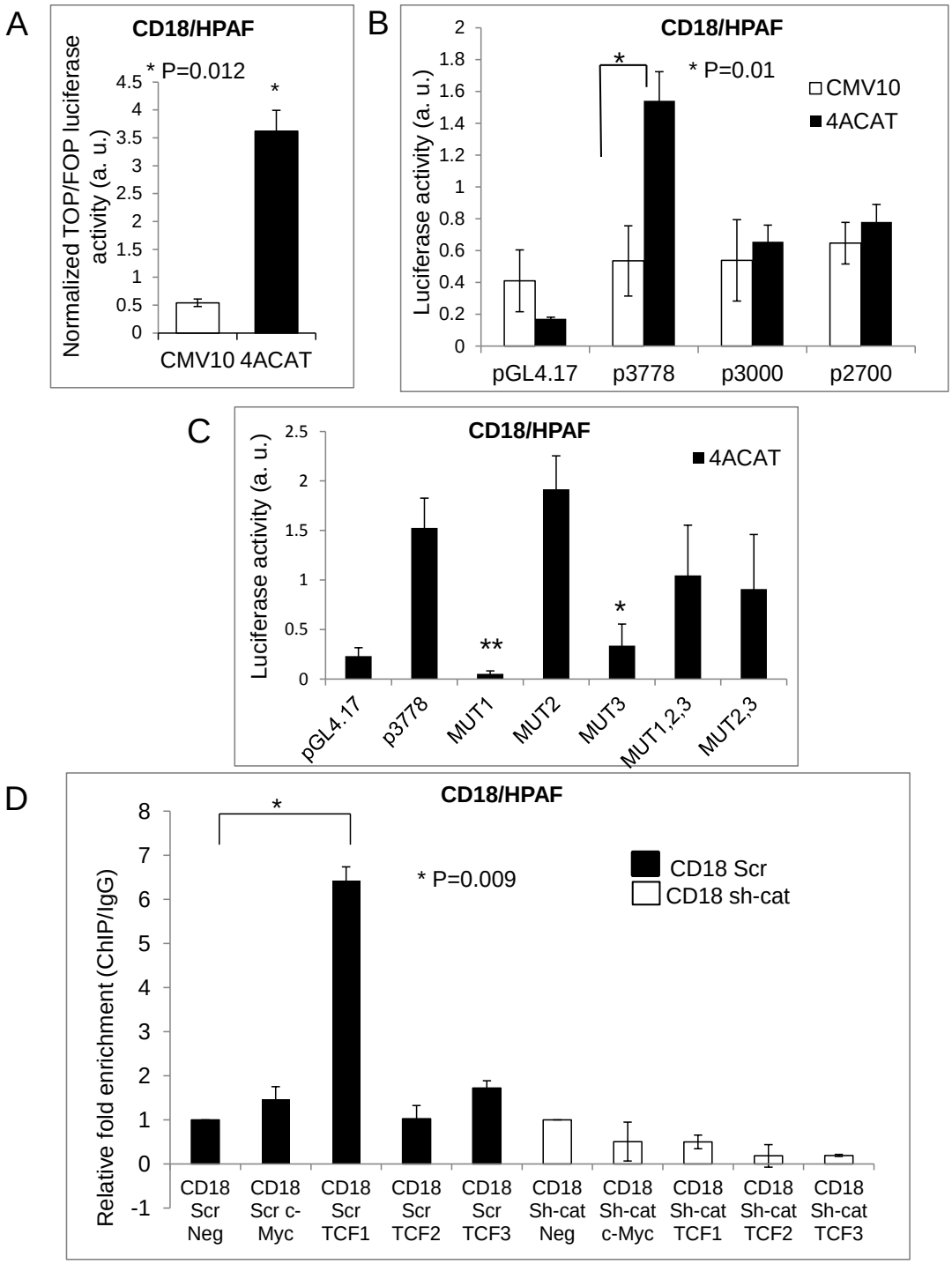

A
CD18/HPAF
* P=0.012
Normalized TOP/FOP luciferase activity (a. u.)
CMV10 4ACAT
B
CD18/HPAF
* P=0.01
CMV10
4ACAT
Luciferase activity (a. u.)
pGL4.17
p3778
p3000
p2700
C
CD18/HPAF
4ACAT
Luciferase activity (a. u.)
pGL4.17
p3778
MUT1
MUT2
MUT3
MUT1,2,3
MUT2,3
D
CD18/HPAF
* P=0.009
CD18 Scr
CD18 sh-cat
Relative fold enrichment (ChIP/IgG)
CD18 Scr Neg
CD18 Scr c-Myc
CD18 Scr TCF1
CD18 Scr TCF2
CD18 Scr TCF3
CD18 Sh-cat Neg
CD18 Sh-cat c-Myc
CD18 Sh-cat TCF1
CD18 Sh-cat TCF2
CD18 Sh-cat TCF3

Figure 5. Effect of β-catenin on migratory properties/EMT. (A) Knockdown (KD) of β-catenin in the CD18/HPAF cell line significantly reduced the migration of cells, as measured by a trans-well migration assay using 1.5 X 10^6 cells seeded in uncoated Boyden's chambers (8 μm pore size) in triplicate. Cells were seeded in serum-free medium in the chamber, and 10% fetal bovine serum containing DMEM was used as a chemoattractant below the chamber in a 6-well plate. Cells were allowed to traverse the membrane for 36 hours, following which the chamber was removed, non-migrant cells were scraped off, and cells that traversed the membrane were stained. The image on the left is representative of 10 random fields that were analyzed as depicted in the image on the right, which quantifies the number of cells per field in CD18/HPAF Scr and CD18/HPAF sh-cat. **(B)** Morphological changes observed in CD18/HPAF sh-cat cells in comparison to the Scr vector control transfected cells. **(C)** Western blot analysis showing that KD of β-catenin in T3M4 cells resulted in reduced N-cadherin, E-cadherin, Vimentin, pERK1/2, and p HER2 (1248Y), while E-cadherin levels marginally increased; Zo-1 levels also increased. **(D)** Real-time PCR in CD18/HPAF Scr and sh-cat cells showed decreased N-cadherin and Vimentin (*$p < 0.05$).

Figure 5

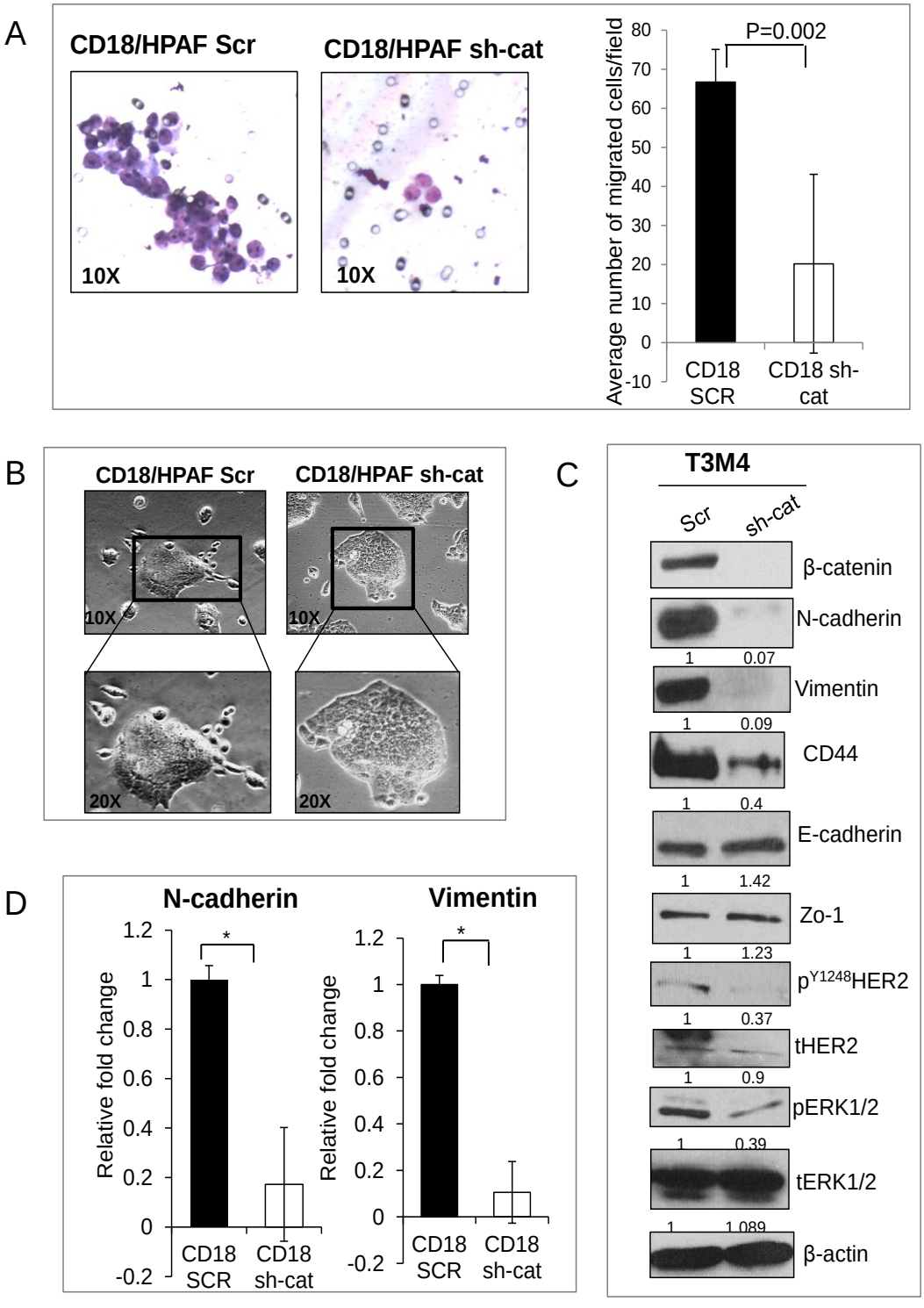

A
CD18/HPAF Scr
CD18/HPAF sh-cat
10X
10X
P=0.002
Average number of migrated cells/field
80
70
60
50
40
30
20
10
0
-10
CD18 SCR
CD18 sh-cat
B
CD18/HPAF Scr
CD18/HPAF sh-cat
10X
10X
20X
20X
C
T3M4
Scr
sh-cat
β-catenin
N-cadherin
1
0.07
Vimentin
1
0.09
CD44
1
0.4
E-cadherin
1
1.42
Zo-1
1
1.23
pY1248HER2
1
0.37
tHER2
1
0.9
pERK1/2
1
0.39
tERK1/2
1
1.089
β-actin
D
N-cadherin
Vimentin
Relative fold change
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
*
*
CD18 SCR
CD18 sh-cat
CD18 SCR
CD18 sh-cat

Figure 6. β-catenin KD reduces tumorigenicity/metastasis. (A) Significantly reduced tumor weight and increased body weight were observed for β-catenin KD tumors. (**B**) IHC for β-catenin, MUC4, and Haematoxylin and Eosin staining for the CD18/HPAF Scr and sh-cat tumors. Images were taken at a 20X magnification. (**C**) Analysis of metastases to various organs (*p = 0.03, **p = 0.02, ***p = 0.008, ****p = 0.0009). No metastases were detected in organs where the white bars are absent in the CD18 sh-cat xenografted mice. The panel on the right shows Haematoxylin and Eosin staining for representative metastatic tumors taken from the Scr cohort. Dotted line indicates border between tumor and normal tissue. T = tumor, N = normal. Images were taken at a 20X magnification.

Figure 6

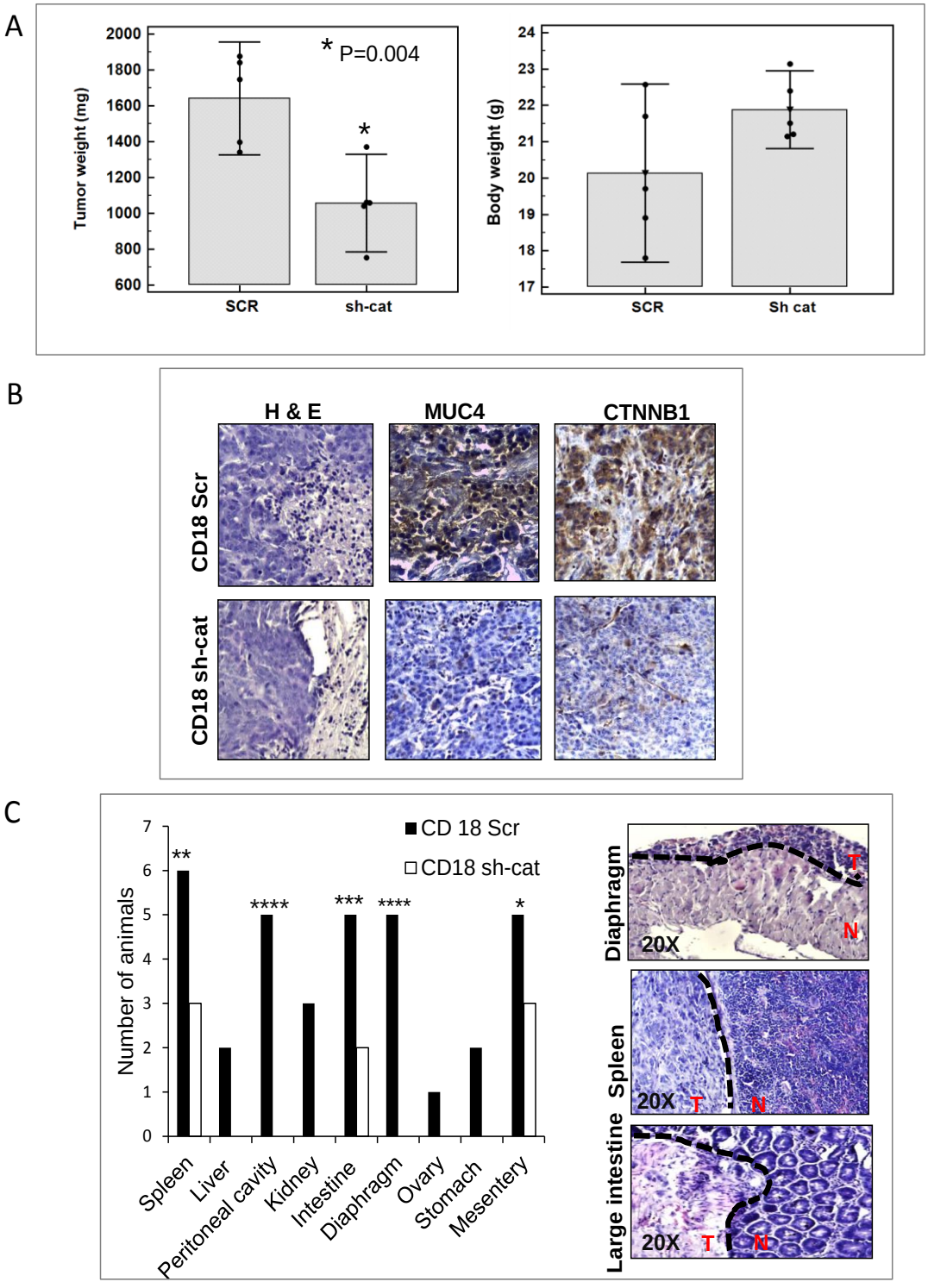
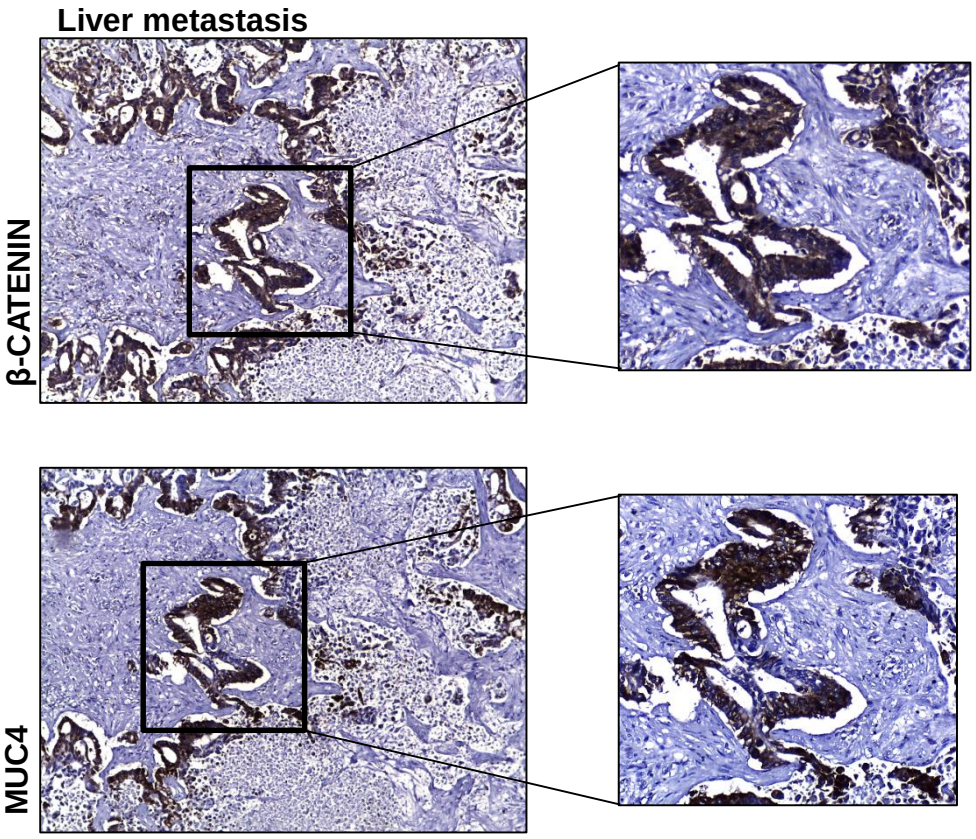

A
Tumor weight (mg)
2000
1800
1600
1400
1200
1000
800
600
* P=0.004
*
SCR
sh-cat
Body weight (g)
24
23
22
21
20
19
18
17
SCR
Sh cat
B
H & E
MUC4
CTNNB1
CD18 Scr
CD18 sh-cat
C
CD 18 Scr
CD18 sh-cat
Number of animals
7
6
5
4
3
2
1
0
**
****
***
****
*
Spleen
Liver
Peritoneal cavity
Kidney
Intestine
Diaphragm
Ovary
Stomach
Mesentery
Diaphragm
20X
T
N
Spleen
20X
T
N
Large intestine
20X
T
N

Figure 7. MUC4 and β-catenin are co-expressed in a subset of human metastatic lesions Immunohistochemical (**IHC**) staining for β-catenin (upper panel) and MUC4 (lower panel) in serial sections of a liver metastasis taken from the UNMC Rapid Autopsy Program's tissue microarray (**TMA**) containing metastatic lesions.

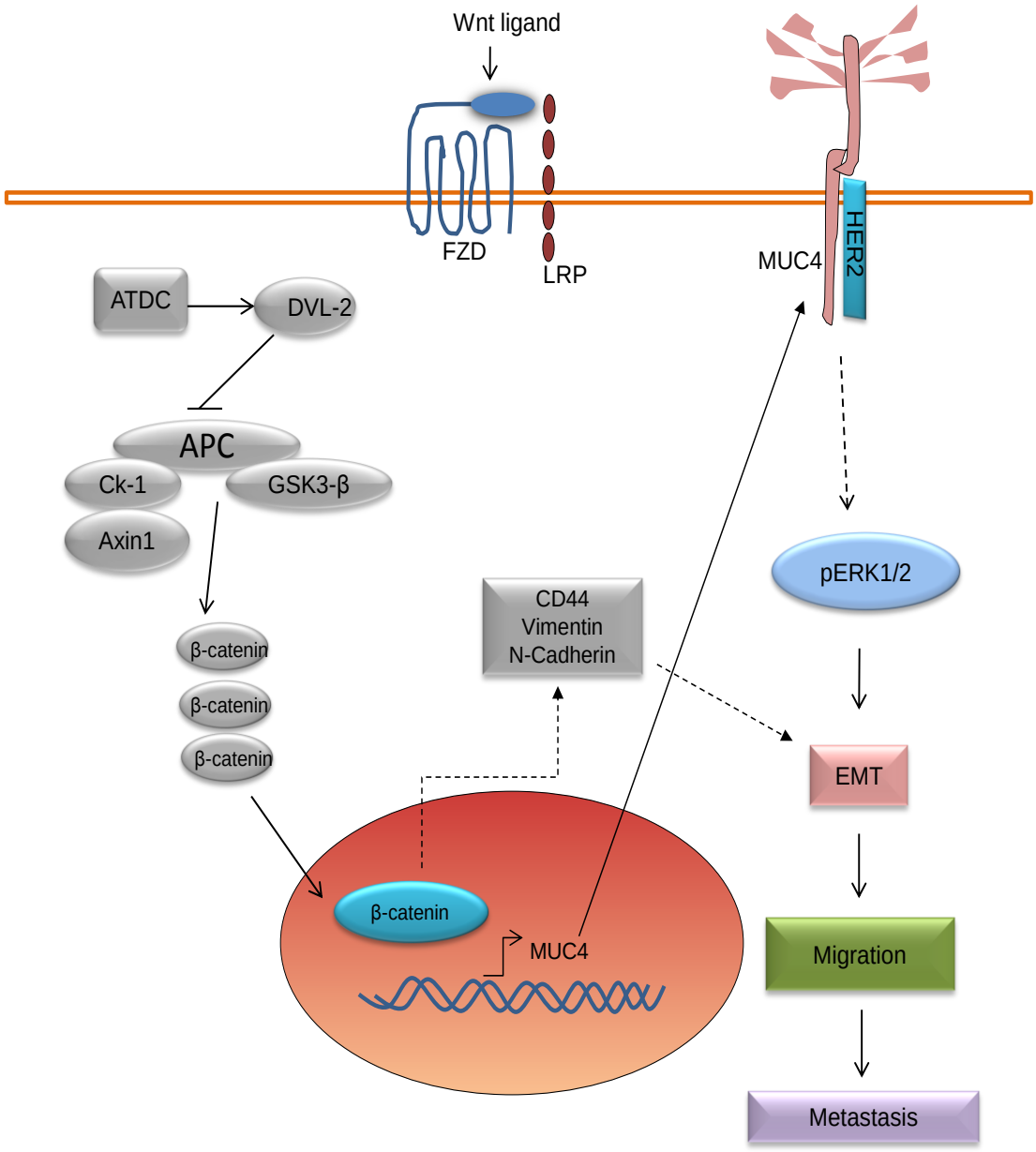
Figure 7



Liver metastasis, n=25	MUC4 positive	MUC4 negative
CTNNB1 positive	10/25(40%)	15/25(60%)
CTNNB1 negative	0/25	0/25

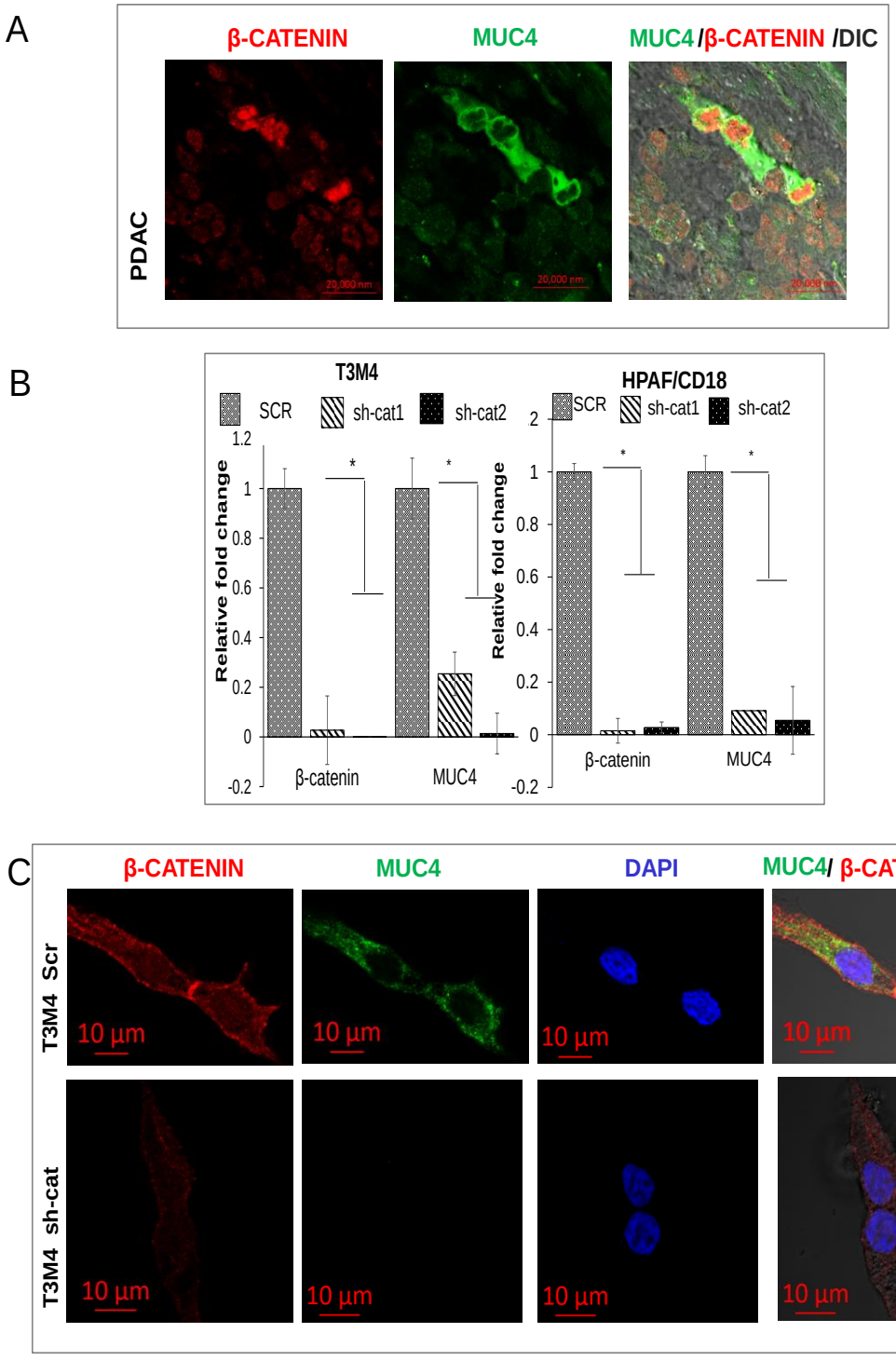
Figure 8: Schematic representation of the role of β-catenin regulated MUC4 ATDC stabilizes Dishevelled 2, thus inhibiting the 'destruction complex' comprising GSK3β, Axin2 and APC. The β-catenin sequestered in this complex is released, whereupon it enters the nucleus. Our findings show that β-catenin/TCF4 up-regulates *MUC4* transcription. MUC4 can then partner with HER2, triggering an intracellular cascade that culminates in activation of the ERK pathway and up-regulation of mesenchymal markers. Also, β-catenin has been shown to independently up-regulate mesenchymal markers such as Vimentin and CD44, contributing to EMT and metastasis.

Figure 8


Wnt ligand
FZD
LRP
MUC4
HER2
ATDC
DVL-2
APC
Ck-1
GSK3-β
Axin1
β-catenin
β-catenin
β-catenin
CD44
Vimentin
N-Cadherin
pERK1/2
EMT
β-catenin
MUC4
Migration
Metastasis

Supplemental Figure 1. (A) Tissue confocal microscopy image of PDAC tissue showing co-localization of nuclear β-catenin (red) and MUC4 (green). (**B**) The qPCR analysis of β-catenin and MUC4 RNA levels in CDA8/HPAF and T3M4; *$p < 0.0004$. (**C**) Confocal microscopy analysis was used to analyze MUC4 (green) and β-catenin (red) levels in T3M4 Scr and KD cells.

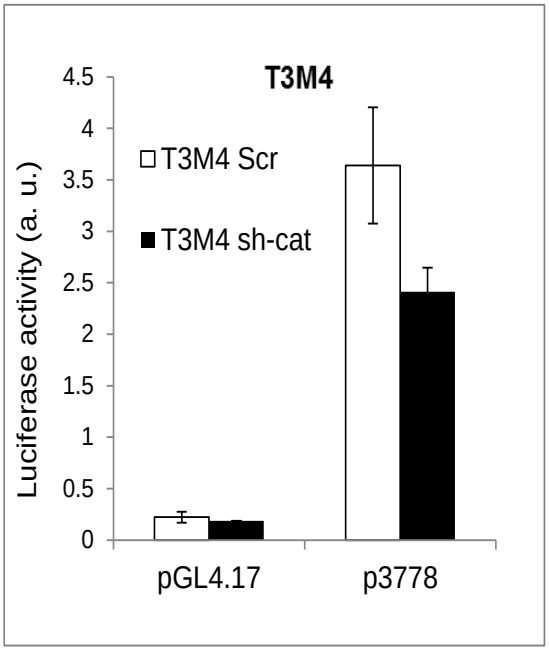
Supplementary Figure 1



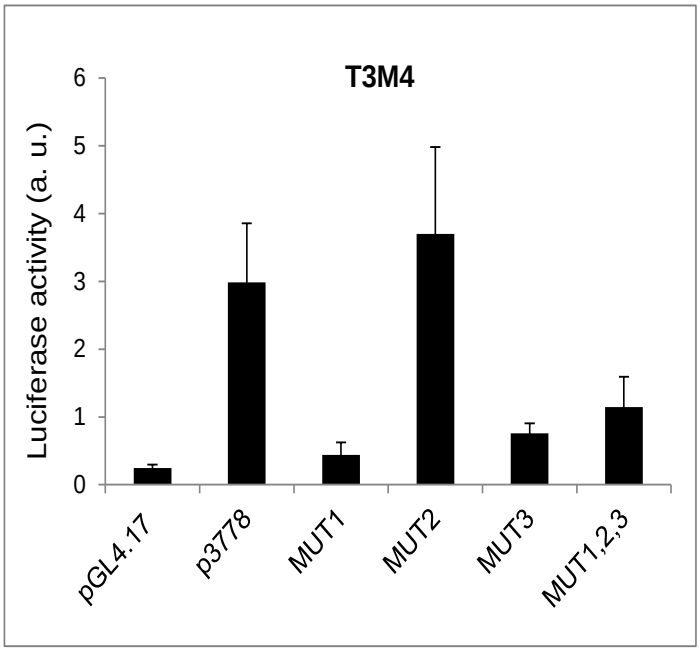
Supplemental Figure 2 (**A**) *MUC4* promoter luciferase studies with p3778 in T3M4 Scr and KD cells. (**B**) The p3778 promoter construct with each of the three putative TCF/LEF sites mutated (*i.e.*, -2612:MUT1, -3226:MUT2, -3408: MUT3) was transfected into T3M4 cells in the presence of 4ACAT. The pCMV9-Renilla vector was used as an internal transfection control.

Supplementary Figure 2

A



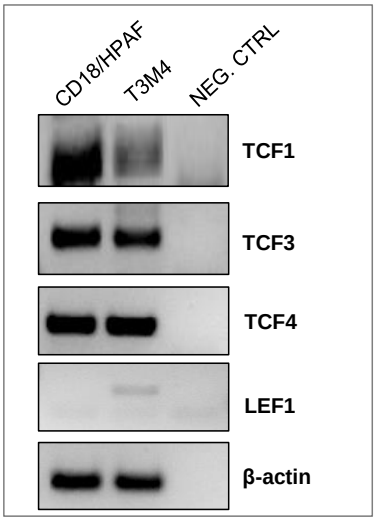
B



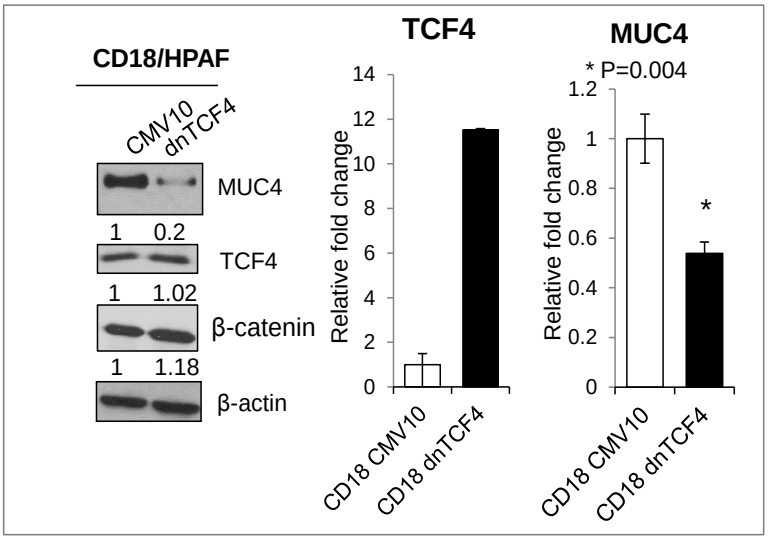
Supplemental Figure 3. (**A**) CD18/HPAF and T3M4 cells were profiled for the expression of the TCF/LEF group of transcription factors. Figure shows Real-time PCR results. (**B**) Transient transfection with dominant-negative TCF4 (dnTCF4) in CD18/HPAF cells resulted in decreased MUC4 protein and RNA levels compared to the vector control. (**C**) Confocal microscopy analysis was used to analyze TCF4 (green) and β-catenin (red) levels in CD18/HPAF cells.

Supplementary Figure 3

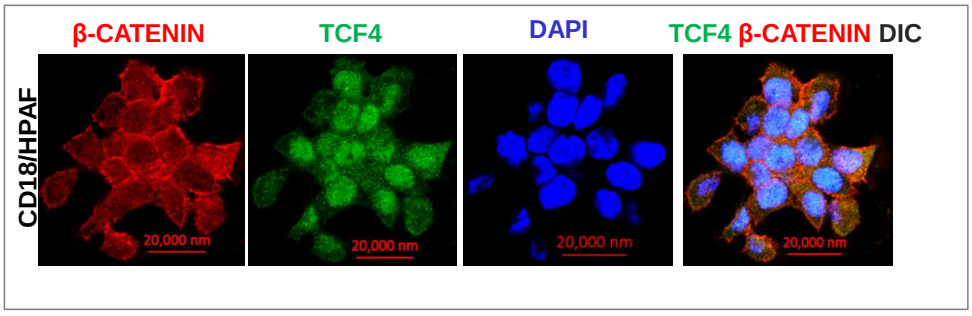
A



B

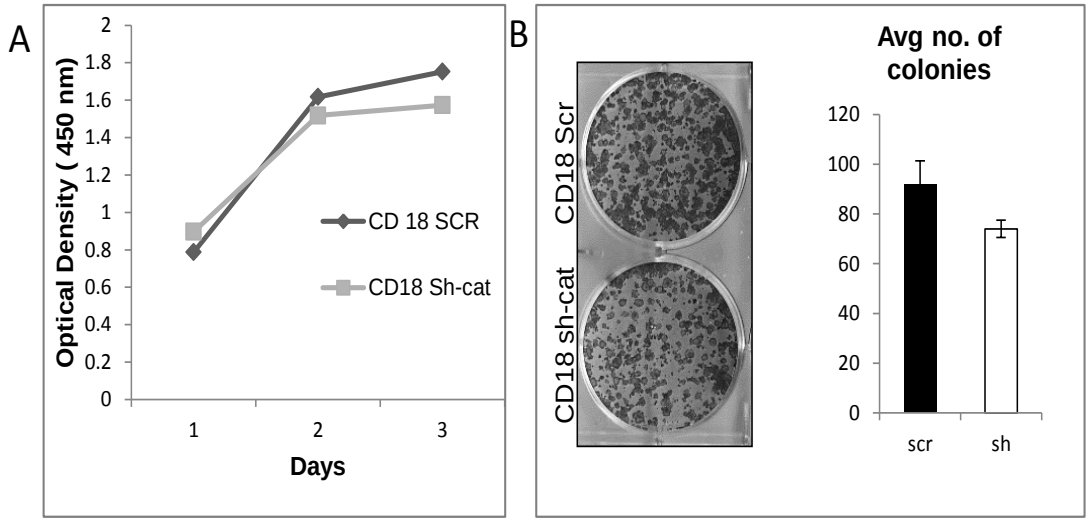


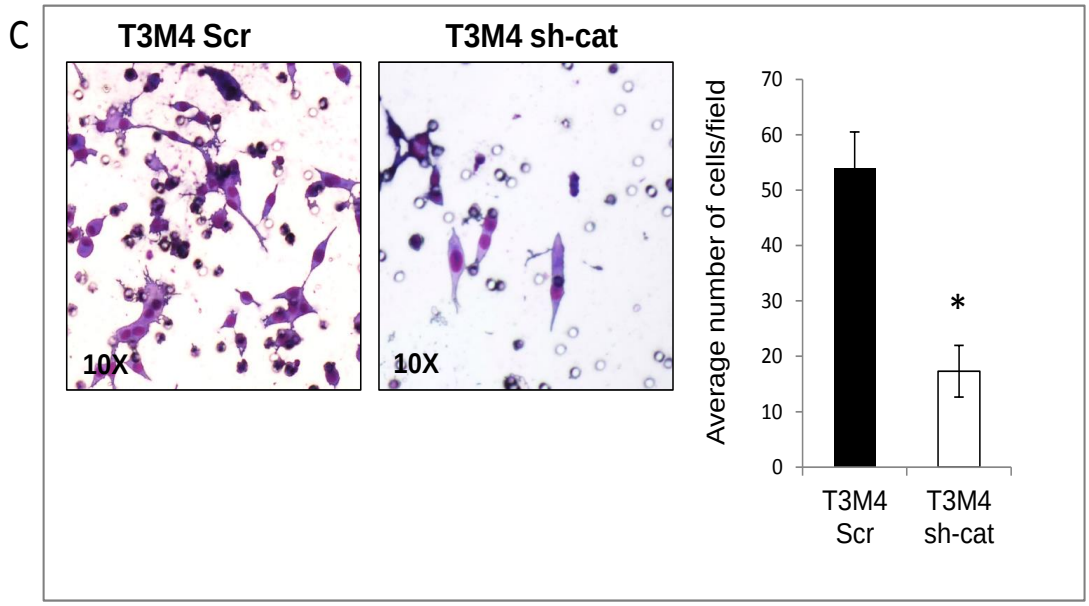
C



Supplemental Figure 4. (A) The cell proliferation assay (WST-1) showed that CD18/HPAF shRNA-cat cells proliferated at a slower rate than Scr control cells. **(B)** The colony formation assays performed for CD18/HPAF Scr and CD18/HPAF shRNA-cat cells showed that the CD18/HPAF shRNA-cat cells formed fewer colonies than CD18/HPAF Scr cells. (**C**) Transwell migration assay with T3M4 Scr and KD cells.

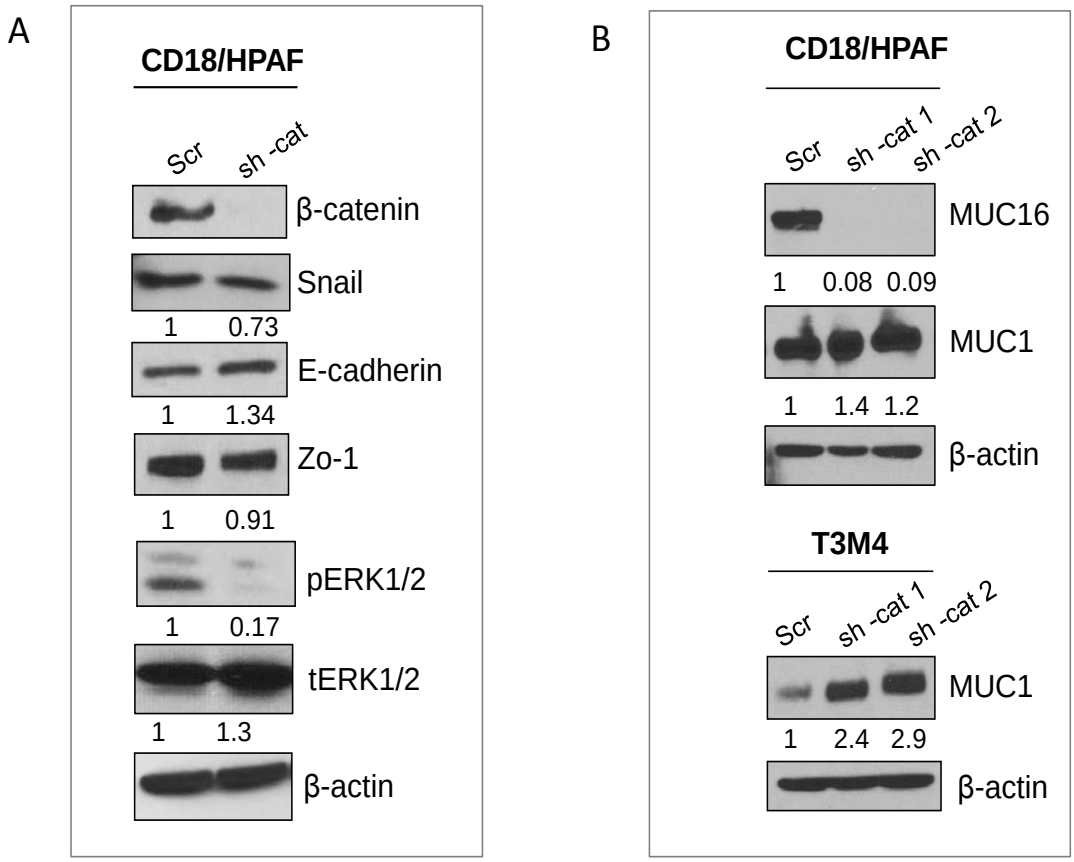
Supplementary Figure 4





Supplemental Figure 5. (A) Western blot analysis of CD18/HPAF Scr and CD18/HPAF shRNA-cat cells showing levels of β-catenin, Snail, E-cadherin, Zo-1, pERK1/2, total ERK, and β-actin (loading control). (**B**) Protein expression levels of MUC1 and MUC16 in CD18/HPAF, T3M4 Scr, and KD cells.

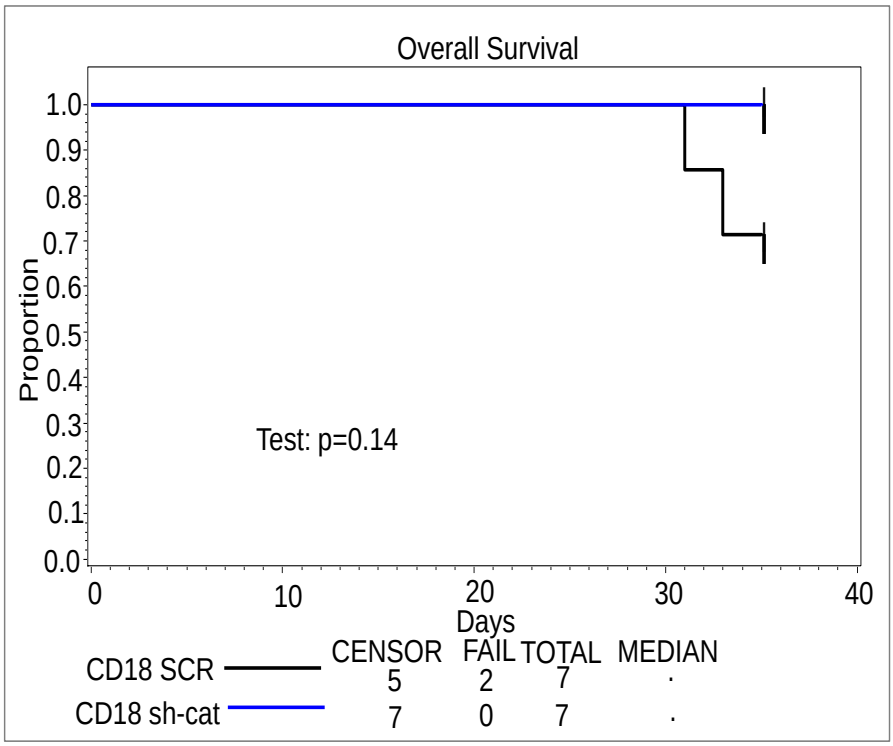
Supplementary Figure 5



Supplemental Figure 6. (A) Kaplan-Meier curve showing reduced survival of mice injected with CD18/HPAF Scr control cells compared to KD cells. (**B**) Tumor lysates from mice orthotopically implanted with CD18/HPAF Scr and KD cells were probed with antibodies for β-catenin, MUC4, pHER2, and tHER2.

Supplementary Figure 6

A



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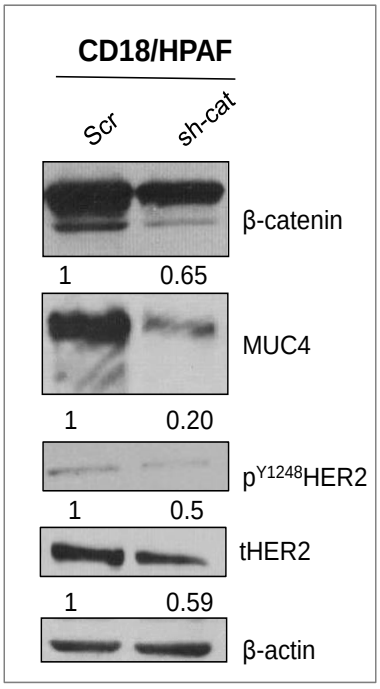


References

[1] Rhim AD, Mirek ET, Aiello NM, et al. EMT and dissemination precede pancreatic tumor formation. Cell 2012;148:349-61.

[2] Morris JP, Wang SC, Hebrok M. KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. Nat Rev Cancer 2010;10:683-95.

[3] Collins MA, Pasca di MM. Kras as a key oncogene and therapeutic target in pancreatic cancer. Front Physiol 2014;4:407.

[4] Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 2008;321:1801-6.

[5] Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55-67.

[6] Zeng G, Germinaro M, Micsenyi A, et al. Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma. Neoplasia 2006;8:279-89.

[7] Al-Aynati MM, Radulovich N, Riddell RH, Tsao MS. Epithelial-cadherin and beta-catenin expression changes in pancreatic intraepithelial neoplasia. Clin Cancer Res 2004;10:1235-40.

[8] Pasca di MM, Biankin AV, Heiser PW, et al. Common activation of canonical Wnt signaling in pancreatic adenocarcinoma. PLoS One 2007;2:e1155.

[9] Arensman MD, Kovochich AN, Kulikauskas RM, et al. WNT7B mediates autocrine Wnt/beta-catenin signaling and anchorage-independent growth in pancreatic adenocarcinoma. Oncogene 2014;33:899-908.

[10] Jiang H, Li Q, He C, et al. Activation of the Wnt pathway through Wnt2 promotes metastasis in pancreatic cancer. Am J Cancer Res 2014;4:537-44.

[11] Qiao Q, Ramadani M, Gansauge S, Gansauge F, Leder G, Beger HG. Reduced membranous and ectopic cytoplasmic expression of beta -catenin correlate with cyclin D1 overexpression and poor prognosis in pancreatic cancer. Int J Cancer 2001;95:194-7.

[12] Zhang Y, Morris JP, Yan W, et al. Canonical wnt signaling is required for pancreatic carcinogenesis. Cancer Res 2013;73:4909-22.

[13] Andrianifahanana M, Moniaux N, Schmied BM, et al. Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res 2001;7:4033-40.

[14] Ansari D, Urey C, Gundewar C, Bauden MP, Andersson R. Comparison of MUC4 expression in primary pancreatic cancer and paired lymph node metastases. Scand J Gastroenterol 2013;48:1183-7.

[15] Swartz MJ, Batra SK, Varshney GC, et al. MUC4 expression increases progressively in pancreatic intraepithelial neoplasia. Am J Clin Pathol 2002;117:791-6.

[16] Seshacharyulu P, Ponnusamy MP, Rachagani S, et al. Targeting EGF-receptor(s) - STAT1 axis attenuates tumor growth and metastasis through downregulation of MUC4 mucin in human pancreatic cancer. Oncotarget 2015;6:5164-81.

[17] Chaturvedi P, Singh AP, Moniaux N, et al. MUC4 mucin potentiates pancreatic tumor cell proliferation, survival, and invasive properties and interferes with its interaction to extracellular matrix proteins. Mol Cancer Res 2007;5:309-20.

[18] Senapati S, Gnanapragassam VS, Moniaux N, Momi N, Batra SK. Role of MUC4-NIDO domain in the MUC4-mediated metastasis of pancreatic cancer cells. Oncogene 2012;31:3346-56.

[19] Singh AP, Moniaux N, Chauhan SC, Meza JL, Batra SK. Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis. Cancer Res 2004;64:622-30.

[20] Chaturvedi P, Singh AP, Chakraborty S, et al. MUC4 mucin interacts with and stabilizes the HER2 oncoprotein in human pancreatic cancer cells. Cancer Res 2008;68:2065-70.

[21] Zhi X, Tao J, Xie K, et al. MUC4-induced nuclear translocation of beta-catenin: a novel mechanism for growth, metastasis and angiogenesis in pancreatic cancer. Cancer Lett 2014;346:104-13.

[22] Rachagani S, Macha MA, Ponnusamy MP, et al. MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1. Carcinogenesis 2012;33:1953-64.

[23] Saitou M, Goto M, Horinouchi M, et al. MUC4 expression is a novel prognostic factor in patients with invasive ductal carcinoma of the pancreas. J Clin Pathol 2005;58:845-52.

[24] Andrianifahanana M, Agrawal A, Singh AP, et al. Synergistic induction of the MUC4 mucin gene by interferon-gamma and retinoic acid in human pancreatic tumour cells involves a reprogramming of signalling pathways. Oncogene 2005;24:6143-54.

[25] Andrianifahanana M, Singh AP, Nemos C, et al. IFN-gamma-induced expression of MUC4 in pancreatic cancer cells is mediated by STAT-1 upregulation: a novel mechanism for IFN-gamma response. Oncogene 2007;26:7251-61.

[26] Kunigal S, Ponnusamy MP, Momi N, Batra SK, Chellappan SP. Nicotine, IFN-gamma and retinoic acid mediated induction of MUC4 in pancreatic cancer requires E2F1 and STAT-1 transcription factors and utilize different signaling cascades. Mol Cancer 2012;11:24.

[27] Singh AP, Chauhan SC, Andrianifahanana M, et al. MUC4 expression is regulated by cystic fibrosis transmembrane conductance regulator in pancreatic adenocarcinoma cells via transcriptional and post-translational mechanisms. Oncogene 2007;26:30-41.

[28] Jonckheere N, Perrais M, Mariette C, et al. A role for human MUC4 mucin gene, the ErbB2 ligand, as a target of TGF-beta in pancreatic carcinogenesis. Oncogene 2004;23:5729-38.

[29] Lahdaoui F, Delpu Y, Vincent A, et al. miR-219-1-3p is a negative regulator of the mucin MUC4 expression and is a tumor suppressor in pancreatic cancer. Oncogene 2015;34:780-8.

[30] Srivastava SK, Bhardwaj A, Singh S, et al. MicroRNA-150 directly targets MUC4 and suppresses growth and malignant behavior of pancreatic cancer cells. Carcinogenesis 2011;32:1832-9.

[31] Radhakrishnan P, Mohr AM, Grandgenett PM, Steele MM, Batra SK, Hollingsworth MA. MicroRNA-200c modulates the expression of MUC4 and MUC16 by directly targeting their coding sequences in human pancreatic cancer. PLoS One 2013;8:e73356.

[32] Kumar S, Das S, Rachagani S, et al. NCOA3-mediated upregulation of mucin expression via transcriptional and post-translational changes during the development of pancreatic cancer. Oncogene 2014; 34(37):4879-89.

[33] Vincent A, Ducourouble MP, Van S, I. Epigenetic regulation of the human mucin gene MUC4 in epithelial cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases. FASEB J 2008;22:3035-45.

[34] Perrais M, Pigny P, Ducourouble MP, et al. Characterization of human mucin gene MUC4 promoter: importance of growth factors and proinflammatory cytokines for its regulation in pancreatic cancer cells. J Biol Chem 2001;276:30923-33.

[35] Olsen PA, Solberg NT, Lund K, et al. Implications of targeted genomic disruption of beta-catenin in BxPC-3 pancreatic adenocarcinoma cells. PLoS One 2014;9:e115496.

[36] Barker N, van den Born M. Detection of beta-catenin localization by immunohistochemistry. Methods Mol Biol 2008;468:91-8.

[37] Cai G, Wang J, Xin X, Ke Z, Luo J. Phosphorylation of glycogen synthase kinase-3 beta at serine 9 confers cisplatin resistance in ovarian cancer cells. Int J Oncol 2007;31:657-62.

[38] Li YJ, Wei ZM, Meng YX, Ji XR. Beta-catenin up-regulates the expression of cyclinD1, c-myc and MMP-7 in human pancreatic cancer: relationships with carcinogenesis and metastasis. World J Gastroenterol 2005;11:2117-23.

[39] Uhlen M, Fagerberg L, Hallstrom BM, et al. Proteomics. Tissue-based map of the human proteome. Science 2015;347:1260419.

[40] Ponnusamy MP, Lakshmanan I, Jain M, et al. MUC4 mucin-induced epithelial to mesenchymal transition: a novel mechanism for metastasis of human ovarian cancer cells. Oncogene 2010;29:5741-54.

[41] Wang L, Yang H, Abel EV, et al. ATDC induces an invasive switch in KRAS-induced pancreatic tumorigenesis. Genes Dev 2015;29:171-83.

[42] Wang L, Heidt DG, Lee CJ, et al. Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization. Cancer Cell 2009;15:207-19.

[43] Danilkovitch-Miagkova A, Miagkov A, Skeel A, Nakaigawa N, Zbar B, Leonard EJ. Oncogenic mutants of RON and MET receptor tyrosine kinases cause activation of the beta-catenin pathway. Mol Cell Biol 2001;21:5857-68.

[44] Wang Z, Ma Q, Liu Q, et al. Blockade of SDF-1/CXCR4 signalling inhibits pancreatic cancer progression in vitro via inactivation of canonical Wnt pathway. Br J Cancer 2008;99:1695-703.

[45] Gustavson MD, Crawford HC, Fingleton B, Matrisian LM. Tcf binding sequence and position determines beta-catenin and Lef-1 responsiveness of MMP-7 promoters. Mol Carcinog 2004;41:125-39.

[46] He X, Zheng Z, Li J, et al. DJ-1 promotes invasion and metastasis of pancreatic cancer cells by activating SRC/ERK/uPA. Carcinogenesis 2012;33:555-62.

[47] Gilles C, Polette M, Mestdagt M, et al. Transactivation of vimentin by beta-catenin in human breast cancer cells. Cancer Res 2003;63:2658-64.

[48] Kleeff J, Shi X, Bode HP, et al. Altered expression and localization of the tight junction protein ZO-1 in primary and metastatic pancreatic cancer. Pancreas 2001;23:259-65.

[49] Balda MS, Anderson JM. Two classes of tight junctions are revealed by ZO-1 isoforms. Am J Physiol 1993;264:C918-C924.

[50] Nieto MA. The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol 2002;3:155-66.

[51] Giannakouros P, Comamala M, Matte I, Rancourt C, Piche A. MUC16 mucin (CA125) regulates the formation of multicellular aggregates by altering beta-catenin signaling. Am J Cancer Res 2014;5:219-30.

[52] Lakshmanan I, Seshacharyulu P, Haridas D, et al. Novel HER3/MUC4 oncogenic signaling aggravates the tumorigenic phenotypes of pancreatic cancer cells. Oncotarget 2015;6:21085-99.

[53] Mukhopadhyay P, Lakshmanan I, Ponnusamy MP, et al. MUC4 overexpression augments cell migration and metastasis through EGFR family proteins in triple negative breast cancer cells. PLoS One 2013;8:e54455.

CHAPTER 4

The β-catenin/TCF-mediated regulation of MUC4 in colorectal cancer

1. Synopsis

MUC4 is a transmembrane mucin normally lining the epithelial surface of the colon. While the aberrant over-expression of MUC4 in malignancies such as pancreatic, ovarian and breast [1-3] cancer has been shown to confer proliferative and metastatic properties to tumor cells, the functional role played by MUC4 in colorectal cancer (CRC) has not been extensively studied. Moreover, while some studies have reported the loss of MUC4 expression in the majority of CRCs [4-6], it is apparent that a subset of CRCs and precursor lesions retain express high MUC4, and somewhat paradoxically, MUC4 appears to confer a worse prognosis to these patients [5, 6]. A *MUC4* promoter analysis performed in our lab showed the presence of three putative TCF/LEF sites, implying a possible regulation by the Wnt/β-catenin pathway, a well-established driver of CRC progression [6, 7]. Thus, the objective of our study was two pronged: (a) establish the functional role of MUC4 in CRC (b) examine the possible regulation of MUC4 in CRC by β-catenin. To this end, we first transiently and stably knocked down (KD) β-catenin in three CRC cell lines; LS180, HCT-8 and HCT116. This resulted in increased *MUC4* transcript and protein. Overexpression of stabilized β-catenin resulted in a decrease in MUC4 expression. Luciferase assays with the *MUC4* promoter construct p3778, which encompasses the entire *MUC4* promoter, showed decreased *MUC4* promoter luciferase activity in the presence of β-catenin KD. Mutation of all three putative TCF/LEF sites showed that *MUC4* promoter luciferase activity is increased when all 3 sites are mutated. Further, we show that β-catenin KD can also regulate MUC4 indirectly via the up-regulation of *Hath1*, a tumor suppressor in CRC [8]. Functional studies with MUC4 KD cells showed significantly reduced proliferation and colony formation but not migration and invasion. Taken together, we show that MUC4 expression is repressed by β-catenin in CRC. Also, MUC4, when present in either precursor lesions or full blown CRCs may confer a proliferative advantage to cells.

2. Background and rationale

Colorectal cancer (CRC) is the third leading cause of cancer deaths in the United States, accounting for 49,700 estimated total deaths in the year 2015 alone. Broadly, CRCs can be characterized as having chromosomal instability typified by the loss of heterozygosity of tumor suppressor genes, loss of DNA mismatch-repair genes and methylation of a large cohort of genes, referred to as CIMP (CpG island methylator phenotype) [9, 10]. This genomic instability can be acquired (somatic) or inherited (germline) such as in cases of hereditary nonpolyposis syndrome (HNPCC), where individuals possess defects in DNA mismatch repair genes like MLH1 and MSH2 [9]. Additionally, CRC is characterized by the mutational inactivation of tumor suppressor genes and activation of oncogenes [11]. Most frequently, tumors possess mutations in the Adenomatous polyposis coli gene (APC), a tumor suppressor gene that results in the activation of the canonical Wnt pathway. An overwhelming majority of CRCs (70-80%) possess APC mutations [11]. Here also, certain individuals may possess germline mutations in APC, as seen in familial adenomatous polyposis, where virtually all those afflicted develop CRC by age 40 [12]. A small subset of patients possess activating mutations in β-catenin, also resulting in the activation of the Wnt/β-catenin pathway [11]. Other tumor suppressor genes that are commonly lost/mutated include p53 and components of the TGF-β pathway [11].

Oncogenes that are mutated in CRC include KRAS and BRAF [11]. Notably, the CIMP phenotype, KRAS and BRAF mutations have been used to distinguish individuals who develop precursor lesions called ‘traditional adenomas’ and those that develop serrated adenomas and hyperplastic polyps [10].

As mentioned previously, precursor lesions typically follow a polyp-adenoma-carcinoma sequence. The degree of nuclear β-catenin progressively increases during CRC progression, as a consequence of mutations in APC/ β-catenin [7]. Another feature associated with early CRC progression is the presence of dysplastic crypts or aberrant crypt foci (ACF) [13]. These lesions

precede the formation of adenomas and are also sometimes associated with mucin depleted foci (MDF) [13, 14]. MDFs are characterized by the absence of mucins and were originally identified in the colon of rats treated with Azoxymethane and dextran sodium sulfate (carcinogens) [13, 14].

Mucins are high molecular weight glycoproteins and usually line the epithelial surfaces of the digestive and reproductive tracts [15]. Mucins can be broadly categorized as follows: (i) membrane-bound/trans-membrane mucins, which include MUC1, MUC3A/MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC21, (ii) secreted (gel-forming) mucins, which include MUC2, MUC5AC, MUC5B, MUC6, and MUC19, and (iii) soluble (non-gel-forming) mucins, which include MUC7, MUC8, MUC9, and MUC20 [15]. MUC4 ordinarily lines the goblet cells and epithelial cells of the normal human small and large intestine. A number of studies have suggested that MUC4 expression is generally lost in CRC [4, 16]. However, certain other studies suggest that while the majority (around 75%) of CRC tumors have reduced or zero MUC4 expression relative to normal tissue, the subset (around 25%) that have high MUC4 expression have a worse prognosis, specifically in the early stages (stage I and II) of the disease [5, 6]. Thus, the precise role played by MUC4 in CRC progression is unclear.

A number of studies have probed the effect of perturbations in the Wnt pathway on mucins in CRC. When a siRNA targeting β-catenin was used in the CRC cell line LS174, the mucin staining as measured by alcian blue was decreased [17]. The most abundantly expressed mucin in the normal colon, MUC2, is repressed by β-catenin via an indirect mechanism involving Sox9 in CRC [18]. The Wnt/ β-catenin pathway also indirectly regulates the level of mucins in CRC, notably via regulation of the Notch pathway target, Hath1 [8]. Hath1 (also called Atoh1) is suppressed by active Notch signaling [19]. Both the Notch and Wnt pathways have been shown to collaborate in CRC progression, in part, by the suppression of Hath1 [19]. Hath1 is a tumor suppressor in CRC [8] and has been shown to be reduced in the majority of CRCs. The Wnt/ β-catenin pathway has been shown to directly reduce Hath1 at the protein as well as the RNA level

in CRC [8, 20]. Hath1, in turn, has been shown to regulate MUC2 in CRC [8] and MUC5AC and MUC6 in gastric cancer [21]. Importantly, our MUC4 promoter analysis showed the presence of a putative Hath1 binding site at -3102/-3089. Thus, a number of factors collude to alter the expression of mucins in CRC.

Given the importance of both β-catenin and MUC4 in the normal colon as well as in light of the fact that the role of MUC4 in CRC is still unclear, we decided to examine the relationship between β-catenin and MUC4 in CRC. A *MUC4* promoter analysis showed the presence of three TCF/LEF sites, suggesting that *MUC4* can be regulated by β-catenin in CRC. Based on our *MUC4* promoter analysis, as well as the generally reduced MUC4 expression in CRC, we hypothesized that β-catenin can regulate MUC4 expression in CRC. Furthermore, a retrospective study showing that high MUC4 expression in stage I and II conferred a worse prognosis to CRCs [5] spurred us to examine the functional aspects of MUC4 function.

3. Results

A. MUC4 expression is lost during the progression of CRC concomitant with aberrant β-catenin localization.

We examined the expression of MUC4 and β-catenin in tissue sections from the normal colon as well as from a polyp. It was observed that the expression of MUC4 was lower in the polyp sections in comparison to the normal colon (**Figure 1A**). Concurrent with the loss of MUC4 expression, we observed an increased degree of aberrant (cytoplasmic/nuclear) staining of β-catenin. Furthermore, we analyzed the levels of MUC4 and β-catenin in a panel of CRC cell lines (**Figure 1B**). While all cell lines examined expressed β-catenin, only two of the 7 cell lines examined expressed MUC4 abundantly; LS180 and HCT-8. The cell line HCT116 expressed very low levels of MUC4.

B. Knock down of β-catenin induces the expression of MUC4 in CRC

In order to delineate the precise relationship between MUC4 and β-catenin in CRC, we knocked down β-catenin using lentiviral shRNA as well as siRNA in three CRC cell lines: HCT-8, HCT116 and LS180. Upon the knock-down (KD) of β-catenin, there was an increase in MUC4 in all three cell lines (**Figure 2 A**). Since the 8G7 antibody used to detect MUC4 protein targets the variable number of tandem repeats (VNTR) domain and may therefore be affected by variations in the glycosylation state of the protein, we used the 2214 antibody (targeting the MUC4-α-N-Ter [22]) to confirm the increase in MUC4 upon KD of β-catenin (**Figure 2A**).

In order to determine whether the β-catenin KD induced MUC4 up-regulation occurred at the transcript level, we examined the *MUC4* levels in all three cell lines and we confirmed that the *MUC4* RNA was also increased upon β-catenin KD (**Figure 2B**).

C. Overexpression of β-catenin results in down-regulation of MUC4

Transient overexpression of β-catenin using the 4ACAT stabilized β-catenin construct resulted in a decreased MUC4 protein expression in the LS180 and HCT-8 cell lines (**Figure 3**).

D. *MUC4* transcript stability is not affected by β-catenin

In light of the observation that *MUC4* was increased upon β-catenin KD, we decided to ascertain whether β-catenin can affect *MUC4* RNA stability. For this purpose, we treated our HCT116 Scr and sh-cat cells with Actinomycin D (10µg/ml) for 6 hours. It was observed that there was a reduction in *MUC4* (the half-life of *MUC4* mRNA is 5 hours [24]), concurrent with the reduction in *β-catenin* in both the Scr and sh-cat cells (**Figure 4**), thus indicating that β-catenin KD does not increase the mRNA stability of *MUC4*.

E. Luciferase studies with *MUC4* promoter construct show that *MUC4* can be governed by β-catenin

LS180 cells were transfected with the TOPflash plasmid and its negative control FOPflash, which are a measure of the β-catenin/TCF signaling. As expected, there was a decrease in the TOP/FOPflash luciferase activity in LS180 β-catenin siRNA transfected cells in comparison to the control (Si-ctrl) cells (**Figure 5A**). We then transfected the LS180 Si-cat and Sictrl cells with the *MUC4* promoter luciferase construct p3778. There was an increased *MUC4* promoter driven luciferase activity in the Si-cat cells in comparison to the Si-ctrl transfected cells. However, this difference was not statistically significant.

Since our promoter analysis indicated the presence of three putative TCF/LEF sites in the *MUC4* promoter: at positions -2612, -3226 and -3408, i.e., site #1, site #2 and site #3 respectively (**Table 1, Chapter 3**), we went on to mutate the TCF/LEF sites both individually as well as in combination (MUT1, MUT2, MUT3, and MUT123) in the p3778 construct. All luciferase assays were performed in triplicate and repeated a minimum of three times. Results represent the mean of three separate experiments. It was observed that while transfection with MUT1 caused a reduction in the luciferase activity (**Figure 5B**), MUT2, MUT3 and MUT123 caused an increase in luciferase activity presence of 4ACAT, suggesting that the mutation of all 3 TCF/LEF sites reduces *MUC4*. Since MUT1 decreased luciferase activity and therefore appeared to promote *MUC4* transcription, we generated another construct MUT23, which possessed an intact site #1 but mutant site #2 and #3. This luciferase reading was higher than that of p3778 but lower than MUT123. Although mutation of all three TCF/LEF sites resulted in an increase in *MUC4* promoter driven luciferase activity, overall, this difference was not statistically significant.

F. Tubular adenomas and hyperplastic polyps in DSS treated *Apc*^{Min} mice show reduced MUC4 expression

In order to determine whether the expression of MUC4 is governed by β-catenin *in vivo*, we looked at levels of mouse MUC4 and β-catenin in lesions from *Apc*^{Min} mice that were treated with the colitis and CRC inducer DSS [25] or mice that were treated with DSS alone. The *Apc*^{Min} mice treated with DSS possessed adenomatous polyps and displayed an increased cytosolic/nuclear β-catenin in the lesion in comparison to the adjacent normal regions. These lesions also displayed a virtual absence of MUC4, while adjacent normal regions showed intense MUC4 staining, particularly in goblet cells (**Figure 6**). The goblet cells in normal areas showed strong globular cytoplasmic staining, while lesions showed occasional faint apical staining. Similar results were obtained with the DSS treated mice, which possessed hyperplastic polyps (**Figure 6**).

G. MUC4 expression is also regulated by Hath1

Although luciferase studies with the *MUC4* promoter constructs indicated that the *MUC4* transcript can be governed by β-catenin, the results were not statistically significant. Therefore, it was decided to explore the possibility that β-catenin governs MUC4 expression via indirect mechanisms. We examined the level of Hath1 in CRC cell lines. It was seen that the *Hath1* was higher in MUC4 expressing cell lines, HCT116, HCT8, and LS180 in comparison to MUC4 non-expressing cell lines HCT15, CaCo2 and HT29 (**Figure 7 A**). Microarray data from Oncomine indicated that *Hath1* mRNA expression decreases in CRC in comparison to normal tissue (**Figure 7 B**). Furthermore, we observed that there was a significant increase in *Hath1* mRNA expression upon β-catenin KD (**Figure 8A**). In order to modulate the levels of Hath1 in CRC cell lines, we treated LS180 with 500nM DBZ, a γ-secretase inhibitor, which has been proven to increase Hath1 [26] for 72 hours. There was an increase in MUC4 (**Figure 8A**) in the DBZ treated cells in comparison to the DMSO treated control cells, concurrent with an increase in *Hath1*.

H. MUC4 confers increased proliferative and colony forming properties to CRC cells

Having established that MUC4 is governed by β-catenin in CRC, our objective was to delineate the role MUC4 in CRC. Of all the CRC cell lines examined, two cell lines: HCT-8 and LS180 expressed abundant MUC4 protein, while HCT116 expressed very low levels of MUC4 protein (**Figure 1B**).Given that the role played by MUC4 in CRC is not well defined, we knocked down MUC4 in the HCT-8 cell line and performed functional studies in this cell line. Proliferation assays in HCT-8 Scr and HCT-8 Sh-MUC4 showed that the KD of MUC4 caused a significant reduction in proliferation (**Figure 9A**). Further, colony formation assay showed that MUC4 KD significantly reduced colony formation (**Figure 9B**).

I. MUC4 KD does not affect the invasive and migratory properties of cells.

In order to gauge the effect of MUC4 KD on the invasive and migratory properties of CRC cells, we performed a transwell invasion and migration assay. MUC4 KD did not have a significant effect on the invasion and migration of the KD cells (**Figure 10A, B**). Furthermore, we performed a scratch assay, which also did not show any significant difference in the scratch area between Scr and KD cells (**Figure 11**). We thus concluded that MUC4 does not affect the invasive and migratory properties of HCT-8 CRC cells.

4. Discussion

Aberrations in the Wnt/ β-catenin pathway are well established initiating events in CRC [7, 9, 11]. Most frequently, truncating mutations in APC, present in 85-90% of all tumors, prevents the phosphorylation mediated degradation of β-catenin and drives the molecule into the nucleus [7, 11]. Less frequently, activating mutations in β-catenin that prevent its degradation and mutations in Axin2/1 can also cause activation of the Wnt/ β-catenin pathway [7, 11]. The fact that aberrant Wnt/ β-catenin pathway activation is an initiating event is underscored by the fact that aberrant activation of the Wnt/ β-catenin pathway is the only abnormality seen in early CRC

precursor lesions such as aberrant crypt foci and adenomas [27]. The Wnt/ β-catenin pathway activates the transcription of a host of tissue specific genes. Overall, β-catenin causes the loss of differentiation of CRC cells and pushes the cells into a crypt progenitor phenotype [27]. This includes the loss of mucin expression, chiefly MUC2, and the gain of several genes commonly active in the proliferative base of the normal colonic crypt such as CD44 [27].

As hinted at previously, the loss of mucin expression is one of the defining characteristics of precursor lesions such as aberrant crypt foci and mucin depleted foci. The most extensively studied mucin in CRC is MUC2, which is the main secreted mucin in the colon [28]. MUC2 expression is usually lost during CRC progression and this loss has been shown to be mediated by β-catenin, albeit via an indirect mechanism involving Sox9 [18]. Other factors, such as the loss of Hath1, a transcription factor that ordinarily governs colonocyte differentiation, contribute to the loss of MUC2 expression [8]. Hath1 has also been shown to be repressed by β-catenin [20].

The expression pattern of MUC4, a transmembrane mucin typically expressed in goblet cells and in the lower two-thirds of the normal crypt [29], in CRC progression has been the subject of some controversy. While most studies concur that the majority of CRCs display a loss/reduced MUC4 expression, somewhat conflictingly, it has been proposed that MUC4 expression, when present, confers a worse prognosis to patients with early stage (grade I and II) CRCs [5, 6]. A meta-analysis of all patient data involving MUC4 showed that MUC4 expression was associated with a poorer prognosis in CRC [30]. Also, a recent study from our lab showed that Muc4 expression in mice led to increased susceptibility to AOM/DSS induced colitis and CRC [31]. In one study, serrated adenomas displayed a complete loss of MUC4 expression while 50% of hyperplastic polyps showed reduced MUC4 expression and traditional adenomas showed no change in MUC4 expression compared to normal [29]. Thus, while MUC4 expression appears to be lost in full blown CRCs, it is likely that some precursor lesions and early stage carcinomas retain MUC4 expression and that this expression may have a pro-tumorigenic role.

The current study aimed to delineate the role of MUC4 in tumorigenesis in CRC and determine whether MUC4 expression is governed by β-catenin, since the *MUC4* promoter was found to contain 3 TCF/LEF sites. To this end, we first analyzed the expression pattern of MUC4 and β-catenin in seven commonly used CRC cell lines. It was observed that only two cell lines expressed MUC4 abundantly, LS180 and HCT8. These cell lines are moderate/well differentiated and secrete Carcinoembryonic antigen (CEA) which is associated with a more differentiated and less tumorigenic state [32]. HCT116 expressed very low levels of MUC4. All three MUC4 expressing cell lines have mutations in APC /β-catenin, Kras and a wild type p53. Most of the MUC4 non-expressing cell lines (HCT15, HT29, and CaCo2) possess a mutant p53. Interestingly, p53 loss typically occurs at a later stage of CRC progression [11] and therefore the MUC4 expressing cell lines may represent an earlier stage in CRC progression. Our confocal microscopy analysis showed that MUC4 is lost in polyps, concurrent with increased β-catenin in the nucleus. This was consistent with earlier reports that report a loss of MUC4 expression in polyps [29].

The KD of β-catenin in the three cell lines that express MUC4 showed that there was a significant increase in MUC4 protein expression upon KD of β-catenin. This was consistent at the the RNA level, where *MUC4* levels were found to be significantly higher in the KD cells. These results imply that β-catenin ordinarily represses *MUC4*; seemingly contradicting our earlier findings in pancreatic cancer, where we showed that *MUC4* is increased by β-catenin. However, one must note that these two diseases are completely different entities with distinct mutational profiles and β-catenin typically has different tissue specific target genes. Moreover, nuclear β-catenin is typically 5-20 times higher in CRC than in PDAC [33], thus possibly altering levels of a different set of target genes, which, in turn, could affect factors such as *MUC4* promoter methylation and histone acetylation. One study has shown that the *MUC4* promoter is methylated at certain key residues in the proximal promoter in the cell line Caco2 and that treatment with the histone deacetylase inhibitor Trichostatin A and DNA methylation inhibitor 5-aza-2'-

deoxycytidine caused increased *MUC4* mRNA [34]. For further confirmation, we transiently transfected LS180 and HCT-8 with 4ACAT, the stabilized β-catenin construct, which caused decreased MUC4 protein levels. Staining of mouse tissue showed that Muc4 staining intensity was significantly reduced in both tubular adenomas and hyperplastic polyps, and that this reduction corresponded with increased β-catenin staining in the same areas, thus confirming our findings *in vivo*.

The Wnt/ β-catenin pathway has been shown to perturb the levels of numerous miRNAs in CRC [35], likely affecting mRNA levels of many genes. Moreover, *MUC4* has been shown to be targeted by several miRNAs [36, 37]. In light of these facts, we asked whether β-catenin can alter the *MUC4* mRNA. To this end, we treated the HCT116 Scr and sh-cat cells with 10μg/ml Actinomycin D, and it was seen that following 6 hours of treatment, *MUC4* mRNA levels in the KD cells were not significantly enriched compared to the Scr control cells, implying that β-catenin does not increase *MUC4* mRNA stability.

Having confirmed that the β-catenin KD induces increased *MUC4* mRNA and protein levels, we decided to determine whether this β-catenin induced MUC4 repression occurs via a direct or an indirect mechanism. To this end, we used a *MUC4* promoter luciferase construct, p3778 that encompasses all three of the putative TCF/LEF sites. It was observed that when LS180 cells were transfected with p3778 in the presence of β-catenin siRNA, there was an increase in the *MUC4* promoter driven luciferase activity. However, this increase was not significant. Furthermore, when we mutated each of the three TCF/LEF sites both individually and in combination, there was an increase in *MUC4* promoter driven luciferase activity when sites #2, #3, sites #2 and #3 in combination, as well as when all three sites were mutated together in comparison to p3778. However, when only site #1 was mutated, there was a decrease in *MUC4* promoter driven luciferase activity. This appeared to conflict our other results where mutation of either site #2 or #3 or all three sites in combination caused the increase in luciferase activity.

However it is possible that binding of β-catenin does not occur at site #1 due to factors such as differential promoter accessibility. Despite the observed differences in *MUC4* promoter luciferase activity due to mutations in TCF/LEF sites, none of the differences were statistically significant.

Owing to the fact that we were unable to obtain a statistically significant result with our promoter luciferase assays, we went on to examine alternate pathways downstream of β-catenin that may regulate *MUC4*. We focused on Hath1 because this well-established tumor suppressor gene in CRC has been shown to be repressed by β-catenin and regulate MUC2 in CRC [8]. Hath1 is a basic helix-loop-helix transcription factor that is repressed by the Notch pathway and determines cell fate determination of intestinal cells into secretory and goblet cells [38]. Interestingly, our promoter analysis showed that the *MUC4* promoter contains a Hath1 binding site. Our results suggest that Hath1 may regulate *MUC4* and that the loss of Hath1 may bolster the repressive effect of β-catenin on MUC4 expression.

The second part of our study focused on the functional aspects of MUC4 expression in CRC. As mentioned previously, MUC4 expression is generally lost in CRC but is retained/over-expressed in some early stage lesions and CRCs. Our proliferation assay showed that MUC4 expression confers increased proliferative properties and colony formation abilities to cells. This is in agreement with most studies in other malignancies such as pancreatic cancer [2, 3, 39, 39].

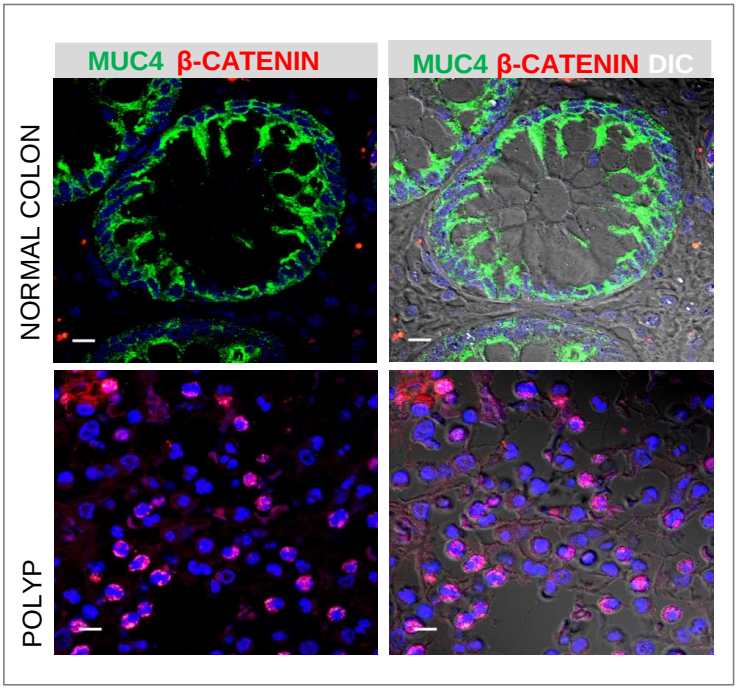
Our migration, scratch and invasion studies did not show any significant difference with MUC4 KD, suggesting that while the presence of MUC4 does confer proliferative properties to cells, it may not confer migratory and metastatic properties. This is in keeping with current literature, which suggests that MUC4, when present in CRC, confers a worse prognosis only in the early, non-metastatic stages of CRC. As mentioned earlier, both high MUC4 expressing cell lines are well/moderately differentiated and possess wild type p53, thus likely resembling the early precursor lesions that are non-metastatic.

In conclusion, this study shows for the first time that β-catenin can repress MUC4 expression in CRC, and that other factors deregulated in CRC such as Hath1 also contribute to MUC4 loss in CRC. However, the presence of MUC4 confers proliferative but not migratory properties to CRC cells, suggesting that MUC4, while aiding in tumor cell proliferation, does not aid in tumor cell migration. However, some unanswered questions remain. For example, if the Wnt/ β-catenin pathway suppresses MUC4, why does a subset of CRCs express high MUC4? This could be answered by a detailed mutational analysis of tumors/cell lines expressing MUC4 by techniques such as RNA-seq. It is possible that MUC4 is expressed by tumors having an un-mutated Wnt/ β-catenin pathway, such as MSI-H (microsatellite instability – high) tumors. MSI-H tumors show a lower frequency of APC/ β-catenin mutations [40, 41]. Interestingly, all three of the MUC4 expressing cell lines; HCT-8, HCT116, LS180 are MSI-H [42].Only one study thus far has examined the possible association between MSI-H status and MUC4 expression, and did not find a significant correlation [16]. However, a larger scale study may yield different results. Alternatively, MUC4 expression may be disease-stage specific, and is perhaps only expressed in early stage CRCs, when nuclear β-catenin levels are low. However, all these hypotheses are purely speculative, and need to be addressed by more comprehensive studies in the future.

Figure 1 MUC4 and β-catenin expression in colorectal carcinoma (CRC) tissues and cell lines (A.) Confocal microscopy showed that MUC4 (green) was reduced in a polyp in comparison to the normal colon, concurrent with an increase in aberrant (cytosolic/nuclear) β-catenin (red). (**B.)** A panel of CRC cell lines was profiled for the expression of MUC4 and β-catenin. β-actin was used as a loading control.

Figure 1

A


MUC4 β-CATENIN
MUC4 β-CATENIN DIC
NORMAL COLON
POLYP

B

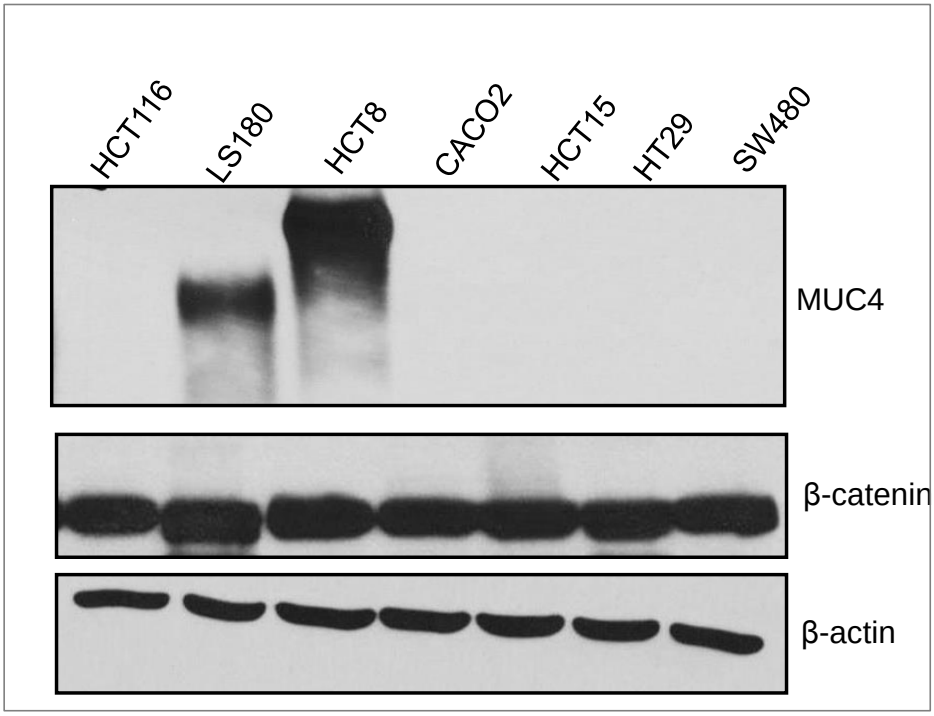

HCT116
LS180
HCT8
CACO2
HCT15
HT29
SW480
MUC4
β-catenin
β-actin

Figure 2: Knockdown (KD) of β-catenin induces MUC4 expression. (A) Lentiviral shRNA and siRNA were used to KD β-catenin in HCT-8, HCT116, and LS180. The levels of MUC4 protein were increased in the KD cells when probed with the 8G7 and 2214 antibodies. (**B.**) Real time PCR was used to assess *MUC4* mRNA levels upon β-catenin KD.

Figure 2

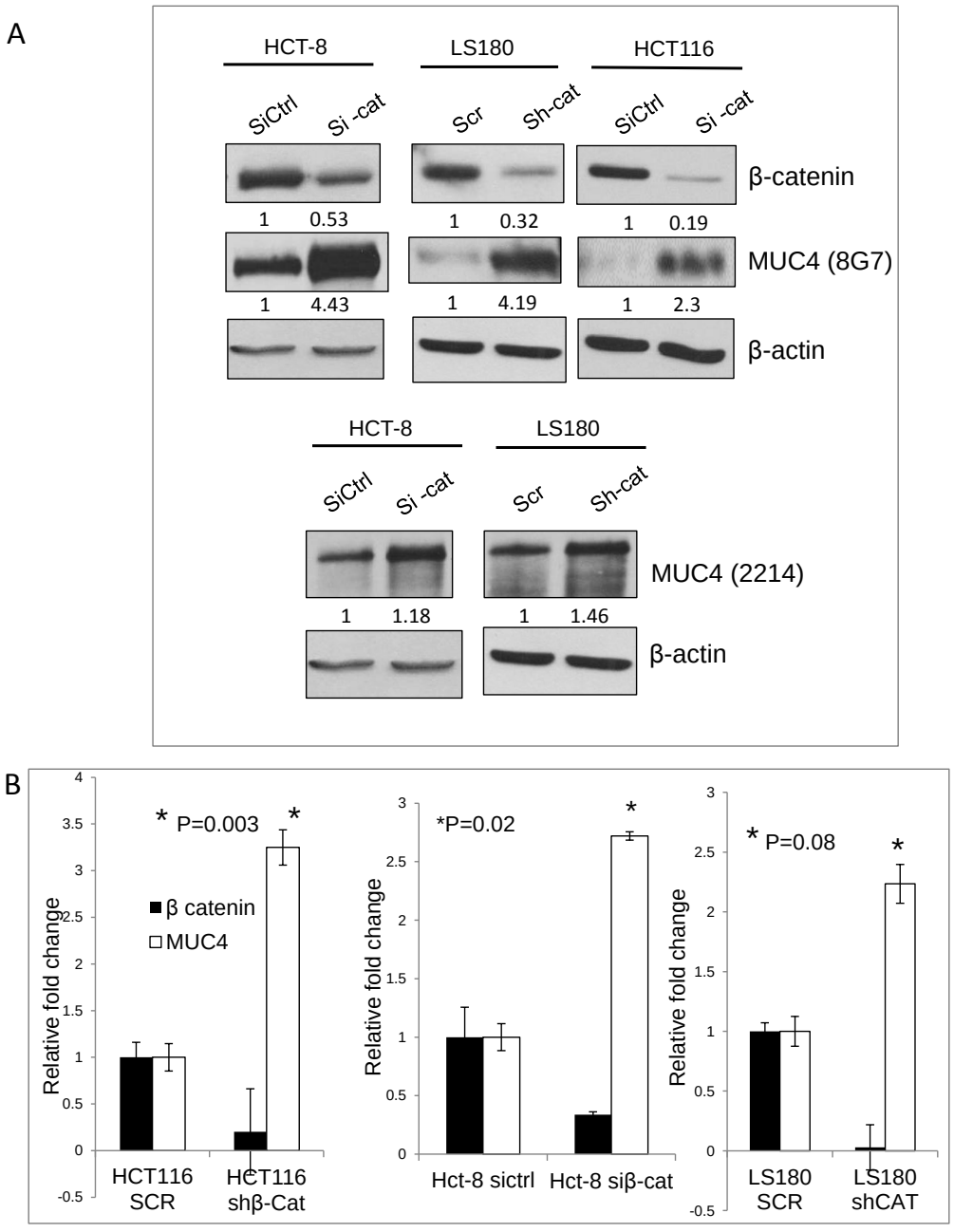

A
HCT-8
LS180
HCT116
SiCtrl
Si -cat
Scr
Sh-cat
SiCtrl
Si -cat
β-catenin
1 0.53
1 0.32
1 0.19
MUC4 (8G7)
1 4.43
1 4.19
1 2.3
β-actin
HCT-8
LS180
SiCtrl
Si -cat
Scr
Sh-cat
MUC4 (2214)
1 1.18
1 1.46
β-actin
B
* P=0.003
*
β catenin
MUC4
Relative fold change
HCT116 SCR
HCT116 shβ-Cat
*P=0.02
*
Relative fold change
Hct-8 sictrl
Hct-8 siβ-cat
* P=0.08
*
Relative fold change
LS180 SCR
LS180 shCAT

Figure 3: Transient over-expression of β-catenin induces MUC4 expression. A FLAG tagged stabilized β-catenin construct (4ACAT) was transiently transfected in LS180 and HCT-8. The levels of MUC4 protein were reduced.

Figure 3

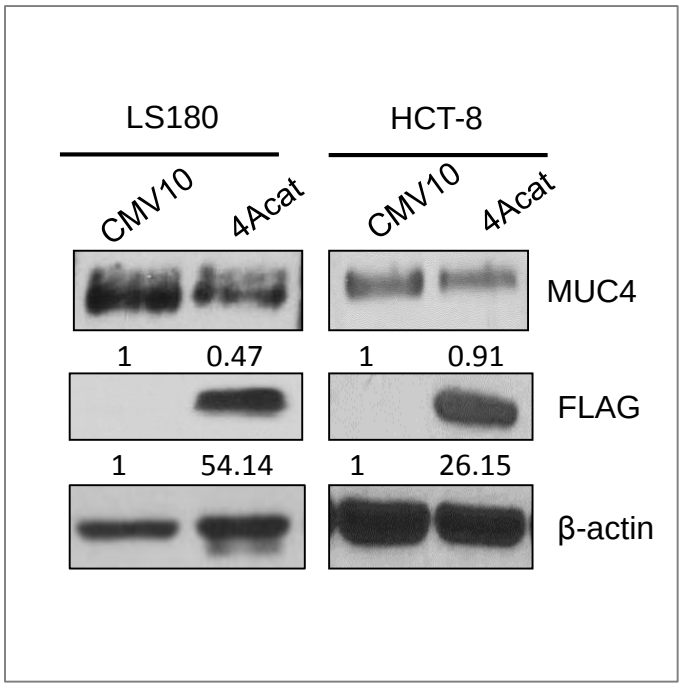

LS180
HCT-8
CMV10
4Acat
CMV10
4Acat
MUC4
1
0.47
1
0.91
FLAG
1
54.14
1
26.15
β-actin

Figure 4: β-catenin does not increase *MUC4* transcript stability. HCT116 SCR and HCT116 sh-cat were treated with 10μg/ml Actinomycin D. After 6 hours of treatment, the levels of MUC4 mRNA were not enriched in the sh-cat cells in comparison to the SCR Actinomycin D treated cells.

Figure 4

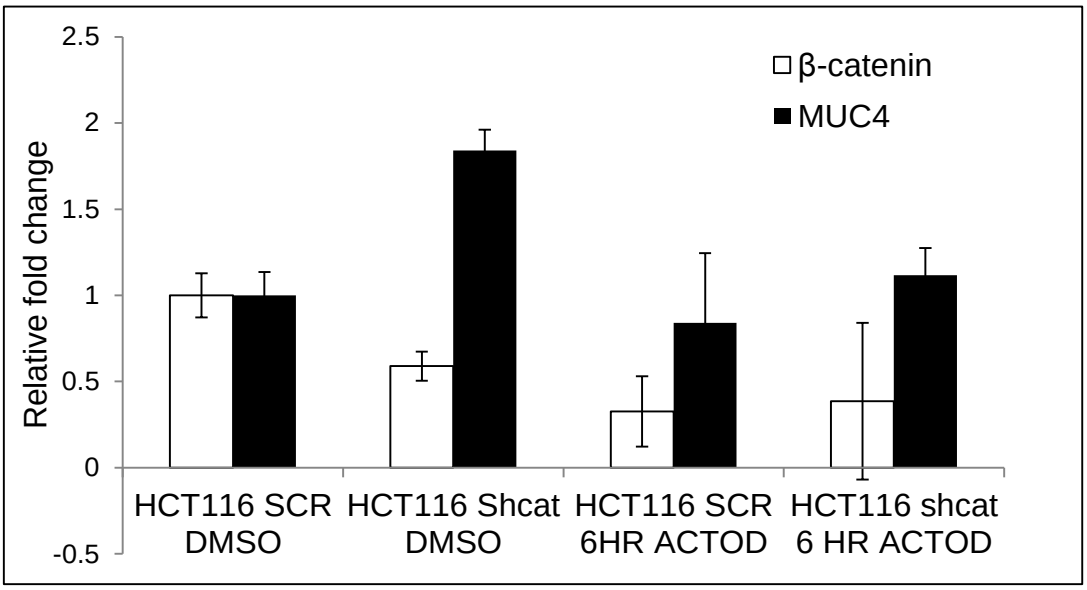
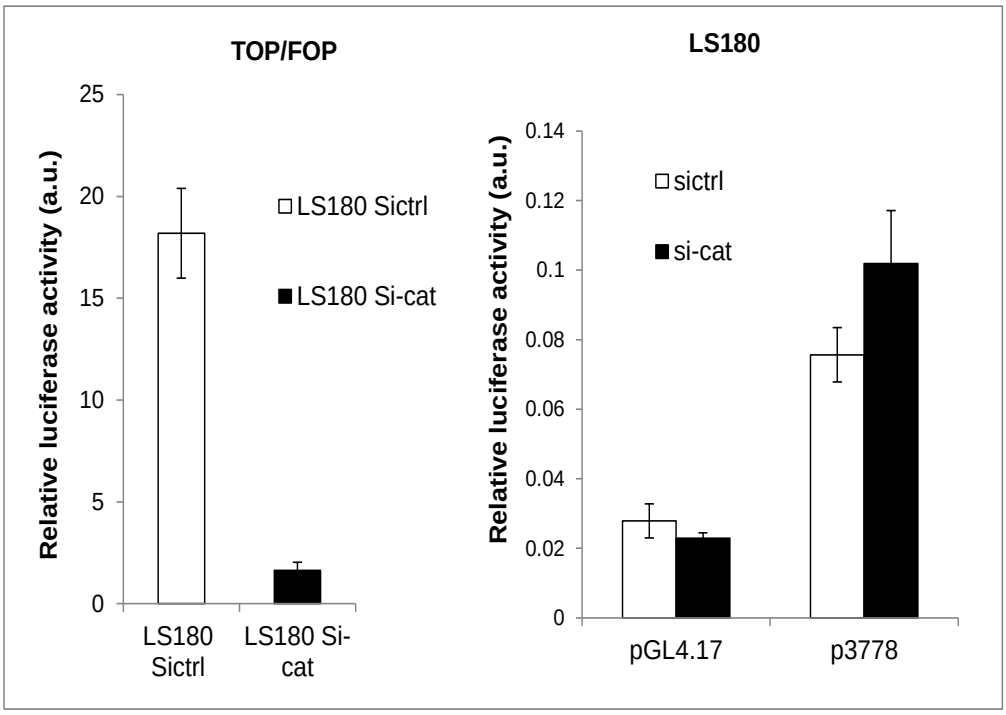

2.5
2
1.5
1
0.5
0
-0.5
Relative fold change
β-catenin
MUC4
HCT116 SCR DMSO
HCT116 Shcat DMSO
HCT116 SCR 6HR ACTOD
HCT116 shcat 6 HR ACTOD

Figure 5: Luciferase studies with the *MUC4* promoter luciferase construct. LS180 cells were transiently transfected with si-RNA targeting β-catenin. (**A.**)TOP/FOPflash studies showed that there was reduced β-catenin mediated transcription in the cells transfected with siRNA. When siRNA transfected cells were also transfected with the p3778 MUC4 promoter luciferase construct, there was an increase in *MUC4* promoter driven luciferase activity. (**B.**) The p3778 promoter construct with each of the three putative TCF/LEF sites mutated (*i.e.,* -2612:MUT1, -3226:MUT2, -3408: MUT3) was transfected into LS180 cells in the presence of 4ACAT. The pCMV9-Renilla vector was used as an internal transfection control; all luciferase experiments were performed in triplicate and repeated a minimum of three times. Images represent the average of at least three experiments, each performed in triplicate.

Figure 5

A


TOP/FOP
Relative luciferase activity (a.u.)
25
20
15
10
5
0
LS180 Sictrl
LS180 Si-cat
LS180 Sictrl
LS180 Si-cat
LS180
Relative luciferase activity (a.u.)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
sictrl
si-cat
pGL4.17
p3778

B

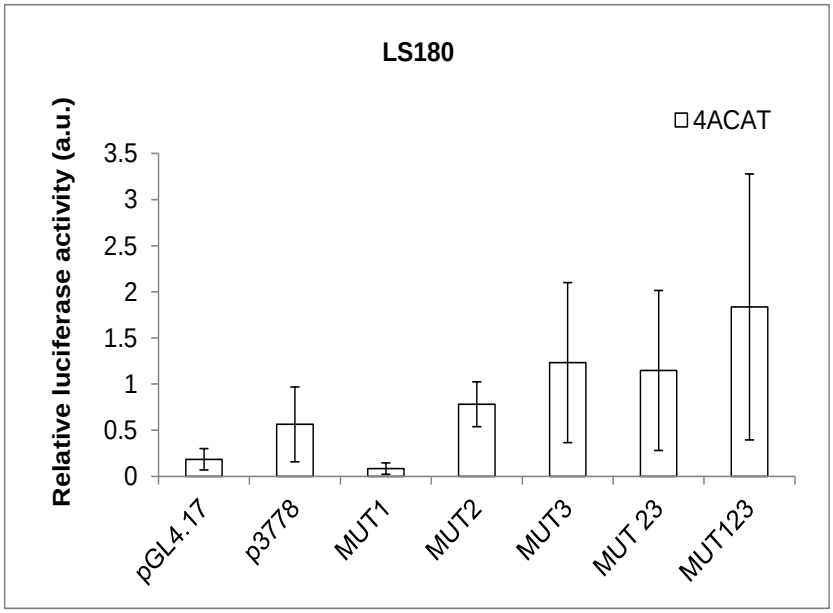
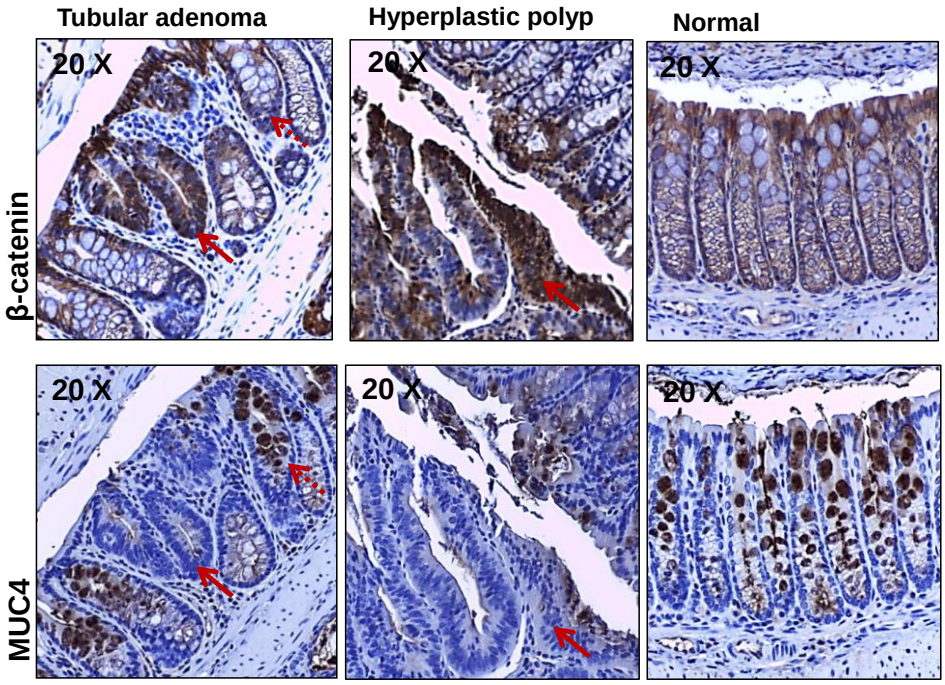

LS180
Relative luciferase activity (a.u.)
4ACAT
3.5
3
2.5
2
1.5
1
0.5
0
pGL4.17
p3778
MUT1
MUT2
MUT3
MUT 23
MUT123

Figure 6 Immunohistochemical staining for mouse Muc4 and β-catenin in colon sections from *Apc*^{Min} mice treated with DSS. (A.) Staining for β-catenin (upper panel) and Muc4 (lower panel) showed intense cytosolic/nuclear staining for β-catenin and depletion of Muc4 in lesions (solid arrow), while surrounding normal areas showed reduced β-catenin and intense goblet cell staining for Muc4 (dotted arrow).**(B.)** Table showing type, number of lesions in in mice either treated with DSS alone or *Apc*^{Min} mice treated with DSS.

Figure 6

A



B

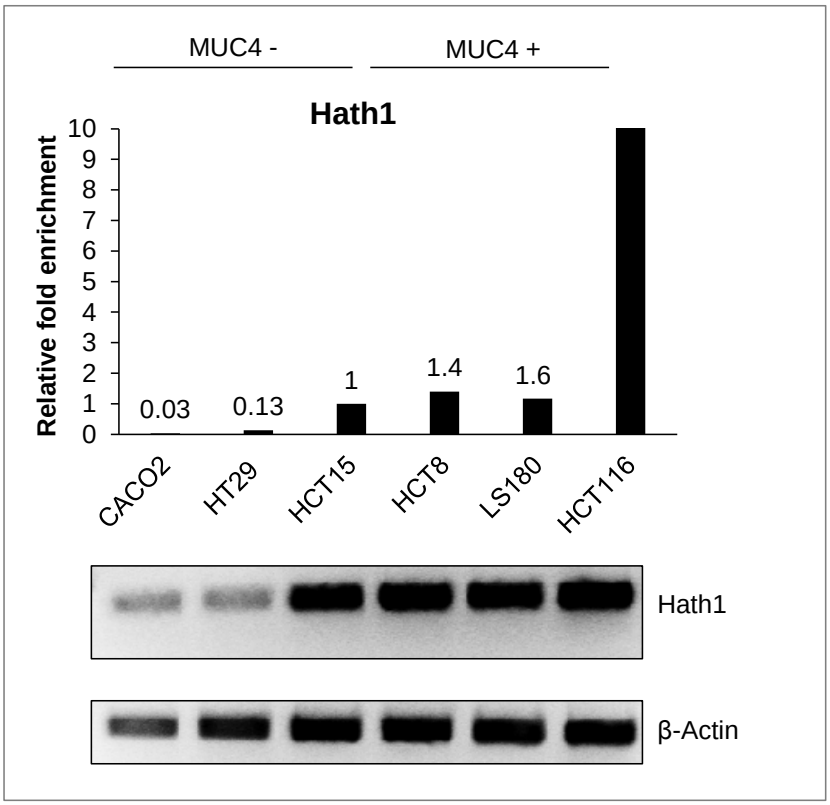
Type of tissue	Number of lesions	Type of lesion	Composite score: lesion	Composite score: normal mucosa
Apc^{Min}/DSS	9	Tubular adenoma	MUC4 = 0 CTNNB = 12	MUC4 = 3 CTNNB = 3
DSS	1	Hyperplastic polyp	MUC4 = 0 CTNNB = 12	MUC4 = 3 CTNNB = 3

Note: Composite score= percentage of cells stained/100 cells x intensity

Figure 7 Hath1 expression levels in CRC (A.) Real time PCR for Hath1 was performed in a panel of cell lines. The PCR products were then run on a 2% agarose gel. β-actin was used as a reference gene.(**B.**) Data extracted from the Oncomine database shows that Hath1 levels are reduced in CRC compared to normal colon tissue.

Figure 7

A


MUC4 -
MUC4 +
Hath1
Relative fold enrichment
10
9
8
7
6
5
4
3
2
1
0
0.03
0.13
1
1.4
1.6
CACO2
HT29
HCT15
HCT8
LS180
HCT116
Hath1
β-Actin

B

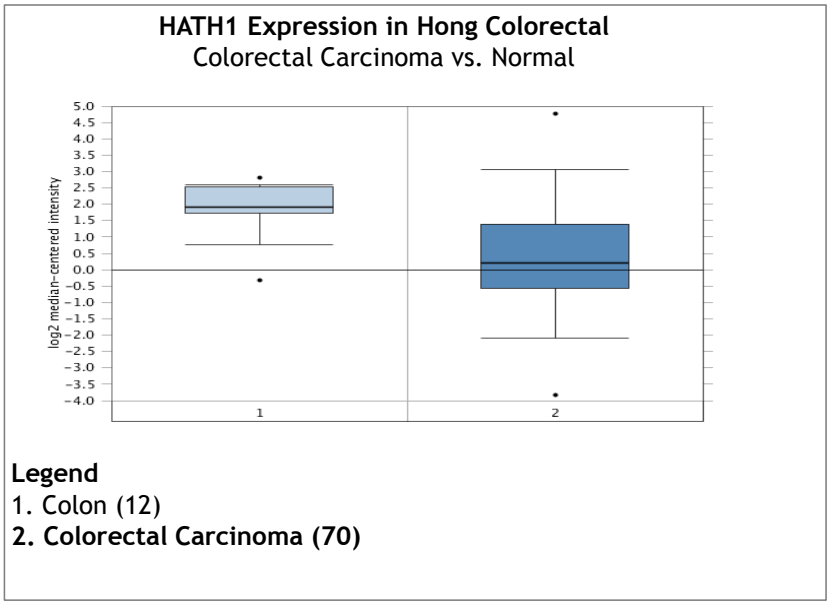
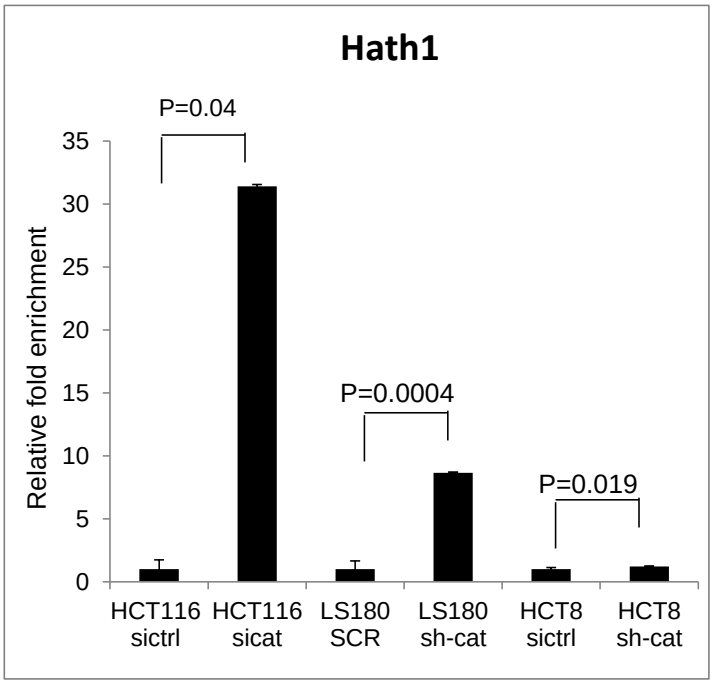

HATH1 Expression in Hong Colorectal
Colorectal Carcinoma vs. Normal
log2 median-centered intensity
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
−3.0
−3.5
−4.0
1
2
Legend
1. Colon (12)
2. Colorectal Carcinoma (70)

Figure 8 β-catenin regulated MUC4 via Hath1 (A.) Real time PCR for Hath1 in CRC cell lines where β-catenin was knocked down showed a significant increase in *Hath1* levels (B.) LS180 cells were treated with the gamma secretase inhibitor DBZ (500nm), which resulted in an increase in MUC4 protein. Hes1, a Notch pathway target gene, was used as a verification of treatment efficacy.

Figure 8

A


Hath1
P=0.04
P=0.0004
P=0.019
Relative fold enrichment
35
30
25
20
15
10
5
0
HCT116 sictrl
HCT116 sicat
LS180 SCR
LS180 sh-cat
HCT8 sictrl
HCT8 sh-cat

B

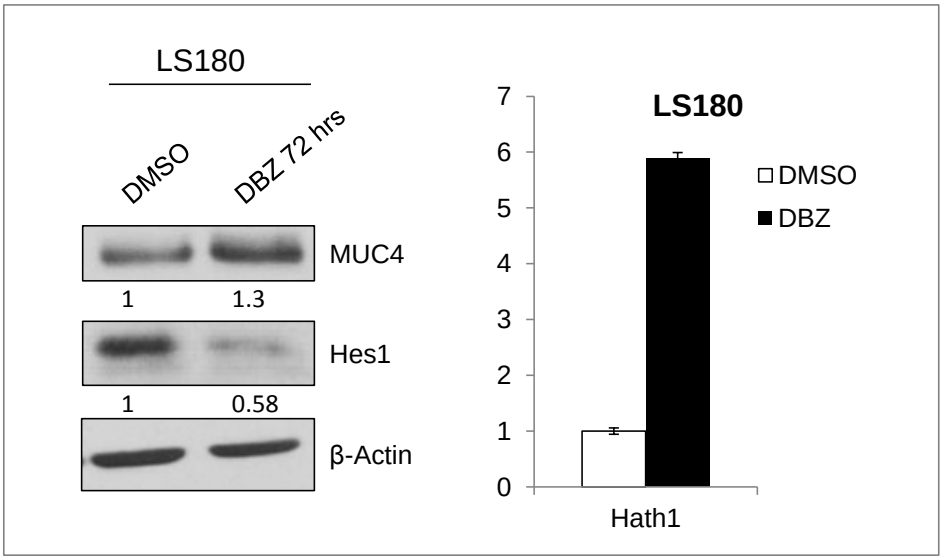
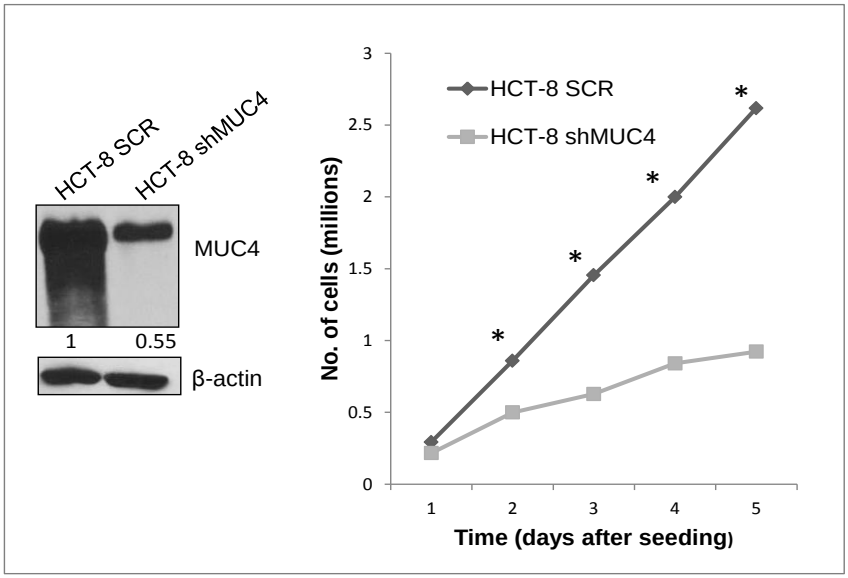

LS180
DMSO
DBZ 72 hrs
MUC4
1
1.3
Hes1
1
0.58
β-Actin
LS180
DMSO
DBZ
7
6
5
4
3
2
1
0
Hath1

Figure 9 Functional studies with MUC4 KD in CRC. (**A**) MUC4 was knocked down in the HCT-8 cell line. It was observed that there was a significantly reduced proliferation in the KD cells in comparison to the SCR control cells. * $p < 0.005$. (**B**) Colony formation assay with HCT-8 SCR and sh-MUC4 showed significantly reduced colony formation with the KD cells.* $p<0.05$. The assay was performed in triplicate in a 6 well plate. Image represents one replicate.

Figure 9

A


HCT-8 SCR
HCT-8 shMUC4
MUC4
1
0.55
β-actin
HCT-8 SCR
HCT-8 shMUC4
No. of cells (millions)
Time (days after seeding)
0
0.5
1
1.5
2
2.5
3
1
2
3
4
5
*
*
*
*

B

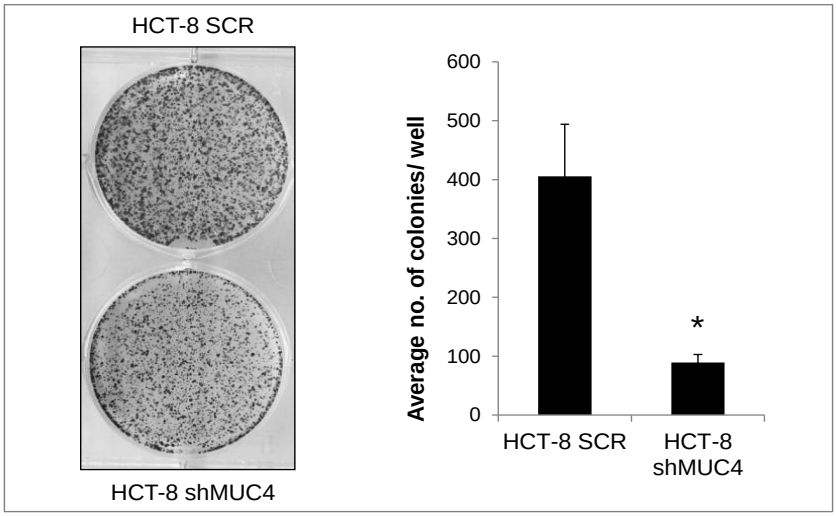

HCT-8 SCR
HCT-8 shMUC4
Average no. of colonies/ well
0
100
200
300
400
500
600
*
HCT-8 SCR
HCT-8 shMUC4

Figure 10 Functional studies with MUC4 KD in CRC. (**A**) Invasion assay performed with HCT-8 SCR and KD showed no significant alteration in the number of cells invading the matrigel coated insert. Experiment was performed in triplicate. Image represents a single field representative of data obtained. Images of 10 arbitrary fields were taken and the numbers of cells in each were manually counted. (**B.**) Migration assay performed with HCT-8 SCR and KD showed no significant difference between the numbers of cells that traversed the transwell insert.

Figure 10

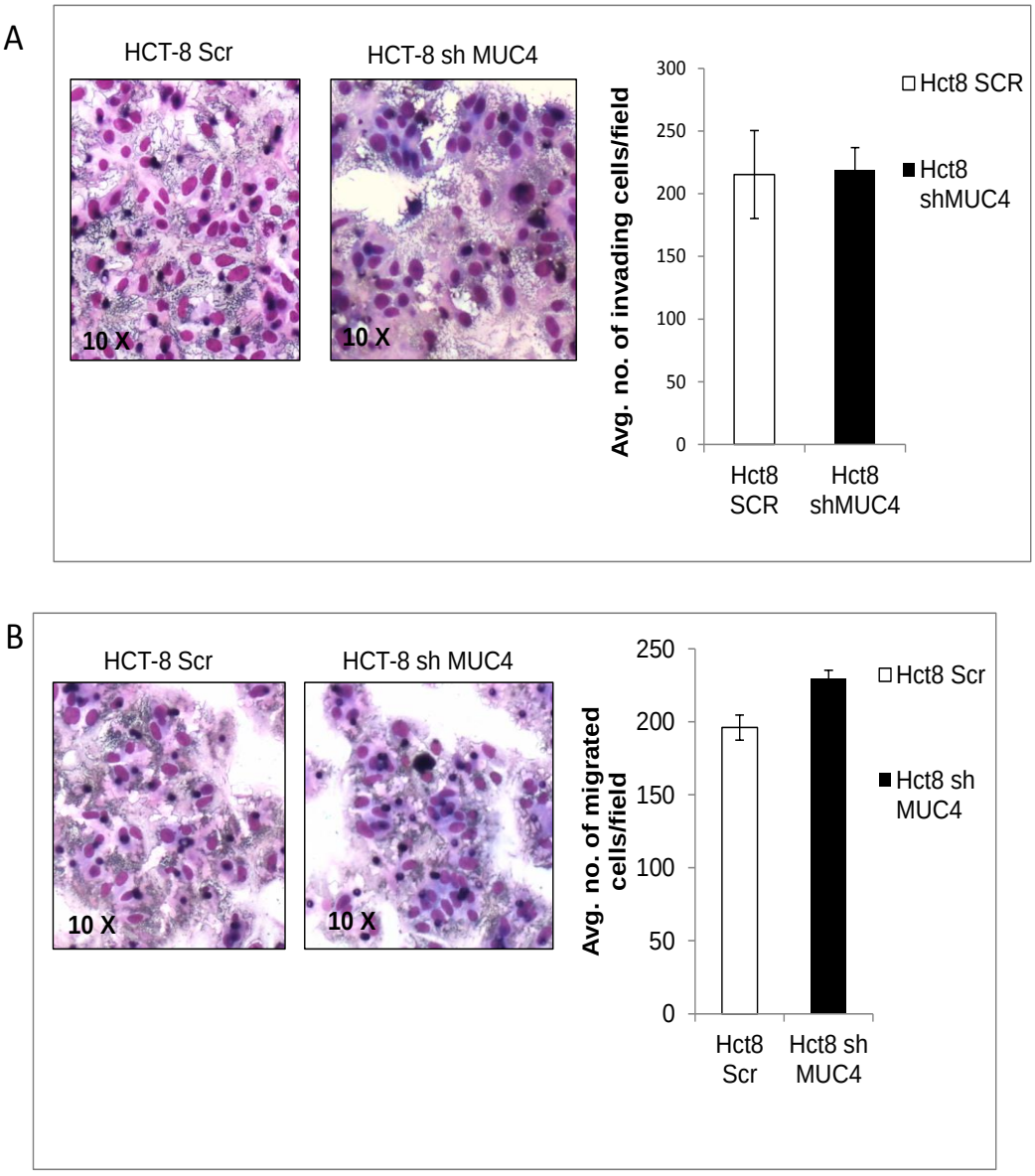
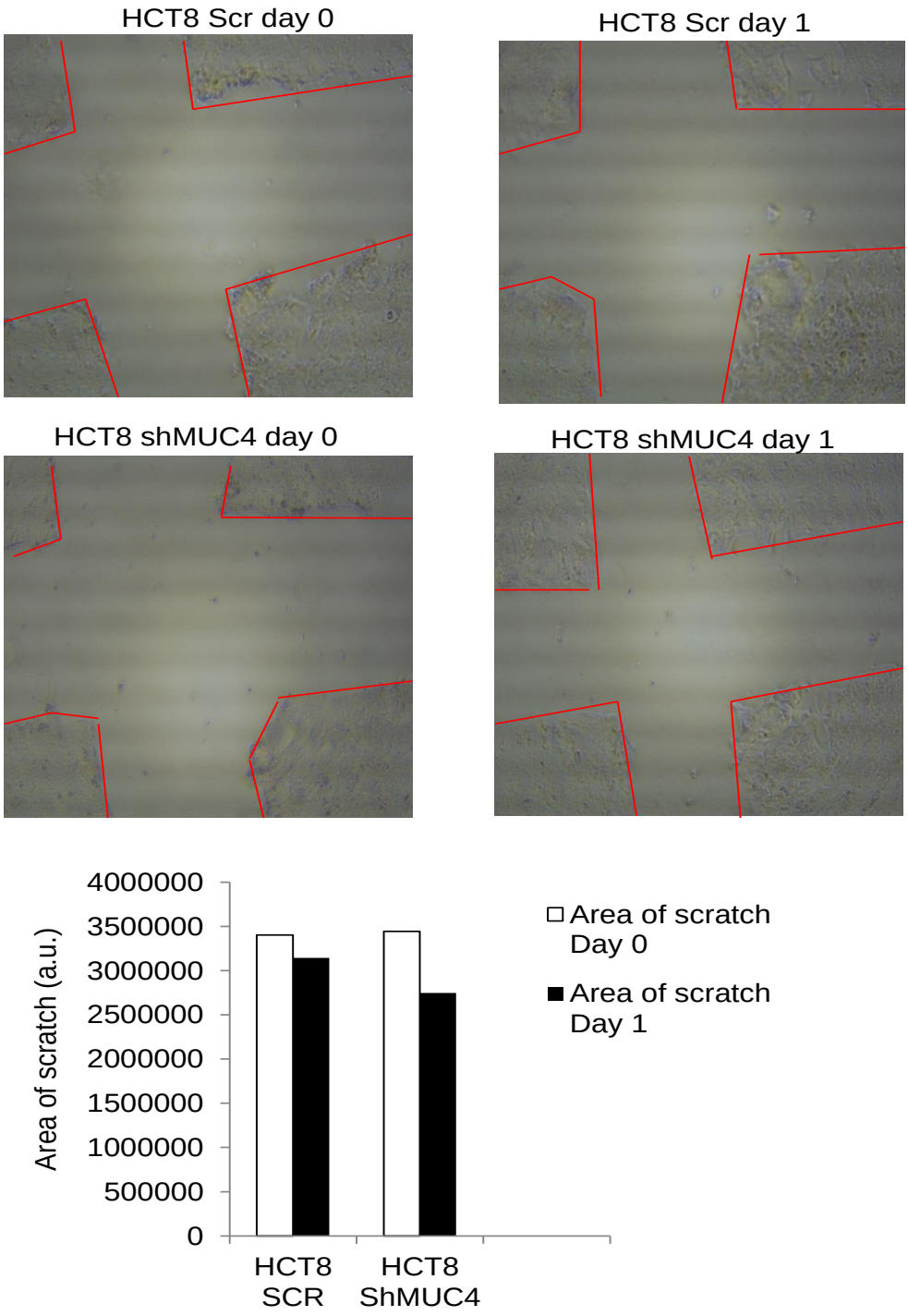

A
HCT-8 Scr
HCT-8 sh MUC4
10 X
10 X
Avg. no. of invading cells/field
300
250
200
150
100
50
0
Hct8 SCR
Hct8 shMUC4
Hct8 SCR
Hct8 shMUC4
B
HCT-8 Scr
HCT-8 sh MUC4
10 X
10 X
Avg. no. of migrated cells/field
250
200
150
100
50
0
Hct8 Scr
Hct8 sh MUC4
Hct8 Scr
Hct8 sh MUC4

Figure 11 Functional studies with MUC4 KD in CRC. A wound healing (scratch) assay was performed with HCT-8 SCR and KD cells. Breifly, 1 X 10^6 cells were plated in a 6 well plate and a wound (scratch) was made 24 hours later. Images of the scratch were taken at 0 hours and 24 hours after the scratch was made. The area of the scratch was measured using ImageJ software (arbitrary units). No significant difference between HCT-8 SCR and KD was observed.

Figure 11


HCT8 Scr day 0
HCT8 Scr day 1
HCT8 shMUC4 day 0
HCT8 shMUC4 day 1
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Area of scratch (a.u.)
HCT8 SCR
HCT8 ShMUC4
Area of scratch Day 0
Area of scratch Day 1

References

[1] Andrianifahanana M, Moniaux N, Schmied BM, et al. Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res 2001;7:4033-40.

[2] Mukhopadhyay P, Lakshmanan I, Ponnusamy MP, et al. MUC4 overexpression augments cell migration and metastasis through EGFR family proteins in triple negative breast cancer cells. PLoS One 2013;8:e54455.

[3] Ponnusamy MP, Lakshmanan I, Jain M, et al. MUC4 mucin-induced epithelial to mesenchymal transition: a novel mechanism for metastasis of human ovarian cancer cells. Oncogene 2010;29:5741-54.

[4] Ogata S, Uehara H, Chen A, Itzkowitz SH. Mucin gene expression in colonic tissues and cell lines. Cancer Res 1992;52:5971-8.

[5] Shanmugam C, Jhala NC, Katkoori VR, et al. Prognostic value of mucin 4 expression in colorectal adenocarcinomas. Cancer 2010;116:3577-86.

[6] Khanh dT, Mekata E, Mukaisho K, et al. Transmembrane mucin MUC1 overexpression and its association with CD10(+) myeloid cells, transforming growth factor-beta1 expression, and tumor budding grade in colorectal cancer. Cancer Sci 2013;104:958-64.

[7] Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55-67.

[8] Leow CC, Romero MS, Ross S, Polakis P, Gao WQ. Hath1, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells. Cancer Res 2004;64:6050-7.

[9] Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med 2009;361:2449-60.

[10] Phipps AI, Limburg PJ, Baron JA, et al. Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77-87.

[11] Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011;6:479-507.

[12] Lynch HT, Lynch JF, Lynch PM, Attard T. Hereditary colorectal cancer syndromes: molecular genetics, genetic counseling, diagnosis and management. Fam Cancer 2008;7:27-39.

[13] Sakai E, Morioka T, Yamada E, et al. Identification of preneoplastic lesions as mucin-depleted foci in patients with sporadic colorectal cancer. Cancer Sci 2012;103:144-9.

[14] Caderni G, Femia AP, Giannini A, et al. Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. Cancer Res 2003;63:2388-92.

[15] Andrianifahanana M, Moniaux N, Batra SK. Regulation of mucin expression: mechanistic aspects and implications for cancer and inflammatory diseases. Biochim Biophys Acta 2006;1765:189-222.

[16] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Mucin core protein expression in colorectal cancers with high levels of microsatellite instability indicates a novel pathway of morphogenesis. Clin Cancer Res 2000;6:1909-16.

[17] van de Wetering M, Oving I, Muncan V, et al. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep 2003;4:609-15.

[18] Blache P, van de Wetering M, Duluc I, et al. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. J Cell Biol 2004;166:37-47.

[19] Peignon G, Durand A, Cacheux W, et al. Complex interplay between beta-catenin signalling and Notch effectors in intestinal tumorigenesis. Gut 2011;60:166-76.

[20] Tsuchiya K, Nakamura T, Okamoto R, Kanai T, Watanabe M. Reciprocal targeting of Hath1 and beta-catenin by Wnt glycogen synthase kinase 3beta in human colon cancer. Gastroenterology 2007;132:208-20.

[21] Sekine A, Akiyama Y, Yanagihara K, Yuasa Y. Hath1 up-regulates gastric mucin gene expression in gastric cells. Biochem Biophys Res Commun 2006;344:1166-71.

[22] Jain M, Venkatraman G, Moniaux N, et al. Monoclonal antibodies recognizing the non-tandem repeat regions of the human mucin MUC4 in pancreatic cancer. PLoS One 2011;6:e23344.

[23] Li YJ, Wei ZM, Meng YX, Ji XR. Beta-catenin up-regulates the expression of cyclinD1, c-myc and MMP-7 in human pancreatic cancer: relationships with carcinogenesis and metastasis. World J Gastroenterol 2005;11:2117-23.

[24] Fischer BM, Cuellar JG, Diehl ML, et al. Neutrophil elastase increases MUC4 expression in normal human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 2003;284:L671-L679.

[25] Tanaka T, Kohno H, Suzuki R, et al. Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc(Min/+) mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. Int J Cancer 2006;118:25-34.

[26] Souaze F, Bou-Hanna C, Kandel C, et al. Differential roles of Hath1, MUC2 and P27Kip1 in relation with gamma-secretase inhibition in human colonic carcinomas: a translational study. PLoS One 2013;8:e55904.

[27] van de Wetering M, Sancho E, Verweij C, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241-50.

[28] Johansson ME, Sjovall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol 2013;10:352-61.

[29] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Immunohistochemical staining patterns of MUC1, MUC2, MUC4, and MUC5AC mucins in hyperplastic polyps, serrated adenomas, and traditional adenomas of the colorectum. J Histochem Cytochem 1999;47:1039-48.

[30] Huang X, Wang X, Lu SM, et al. Clinicopathological and prognostic significance of MUC4 expression in cancers: evidence from meta-analysis. Int J Clin Exp Med 2015;8:10274-83.

[31] Das S, Rachagani S, Sheinin Y, et al. Mice deficient in Muc4 are resistant to experimental colitis and colitis-associated colorectal cancer. Oncogene 2015;10.

[32] Wagner HE, Toth CA, Steele GD, Jr., Thomas P. Metastatic potential of human colon cancer cell lines: relationship to cellular differentiation and carcinoembryonic antigen production. Clin Exp Metastasis 1992;10:25-31.

[33] White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. Gastroenterology 2012;142:219-32.

[34] Yamada N, Nishida Y, Tsutsumida H, et al. Promoter CpG methylation in cancer cells contributes to the regulation of MUC4. Br J Cancer 2009;100:344-51.

[35] Schepeler T, Holm A, Halvey P, et al. Attenuation of the beta-catenin/TCF4 complex in colorectal cancer cells induces several growth-suppressive microRNAs that target cancer promoting genes. Oncogene 2012;31:2750-60.

[36] Lahdaoui F, Delpu Y, Vincent A, et al. miR-219-1-3p is a negative regulator of the mucin MUC4 expression and is a tumor suppressor in pancreatic cancer. Oncogene 2015;34:780-8.

[37] Radhakrishnan P, Mohr AM, Grandgenett PM, Steele MM, Batra SK, Hollingsworth MA. MicroRNA-200c modulates the expression of MUC4 and MUC16 by directly targeting their coding sequences in human pancreatic cancer. PLoS One 2013;8:e73356.

[38] Kazanjian A, Shroyer NF. NOTCH Signaling and ATOH1 in Colorectal Cancers. Curr Colorectal Cancer Rep 2011;7:121-7.

[39] Chaturvedi P, Singh AP, Moniaux N, et al. MUC4 mucin potentiates pancreatic tumor cell proliferation, survival, and invasive properties and interferes with its interaction to extracellular matrix proteins. Mol Cancer Res 2007;5:309-20.

[40] Lovig T, Meling GI, Diep CB, et al. APC and CTNNB1 mutations in a large series of sporadic colorectal carcinomas stratified by the microsatellite instability status. Scand J Gastroenterol 2002;37:1184-93.

[41] Jass JR, Barker M, Fraser L, et al. APC mutation and tumour budding in colorectal cancer. J Clin Pathol 2003;56:69-73.

[42] Li HR, Shagisultanova EI, Yamashita K, Piao Z, Perucho M, Malkhosyan SR. Hypersensitivity of tumor cell lines with microsatellite instability to DNA double strand break producing chemotherapeutic agent bleomycin. Cancer Res 2004;64:4760-7.

CHAPTER 5

Understanding the role of MUC4 in CRC: Generation of *Muc4$^{-/-}$;CDX2 P-NLS -Cre kras$^{G12D/+}$Apc$^{loxP/+}$* mice

1. Synopsis

The role played by MUC4 in CRC progression has been the subject of much speculation. While numerous studies have noted that MUC4 is lost in some CRCs [1-3], other studies indicate that MUC4 expression in unchanged/elevated and when present, is significantly correlated with a worse prognosis, particularly in the early stages of the disease [4, 5]. Furthermore, functional characterization of a MUC4 KD CRC cell line in our lab (unpublished studies) showed that the presence of MUC4 conferred proliferative properties to cells. Thus, the exact nature of the role played by MUC4 in CRC progression remains largely unclear. We sought to address this question by generating $Muc4^{-/-}$;CDX2P-NLS-Cre;$Kras^{G12D/+}$;$Apc^{loxP/+}$ ($Muc4^{-/-}$; CKA) mice, i.e., mice that lacked Muc4 expression and had $Kras^{G12D/+}$ activation and Apc loss induced via a colon preferential Cre recombinase. Our preliminary studies show that Muc4 expression is lost in $Apc^{-/-}$;Cdx2P NSLCre lesions, concomitant with aberrant β-catenin expression. However, we anticipate that the comparison of $Muc4^{-/-}$; CKA mice with CKA mice will lead to a better understanding of the role of Muc4 in CRC initiation and progression.

3. Background and Rationale

The vast majority of CRCs possess truncations in APC or activating mutations in β-catenin. Chief among the myriad changes associated with aberrantly activated Wnt/ β-catenin signaling in CRC is the loss of differentiation, or crypt progenitor-like phenotype [6]. The normal colonic epithelium is lined by villi that possess several types of differentiated cells, however; this is lost during the process of neoplastic transformation [6]. This loss of differentiation is associated with a loss of mucin expression, mainly comprising MUC2 as well as other mucins such as MUC4 [1, 7]. Other mucins ordinarily absent from the normal colon, such as MUC5AC are expressed *de novo* in CRC, while mucins such as MUC1 are increased in expression [7]. These changes in mucin expression are also accompanied by altered glycosylation that aids in cancer metastasis [8]. However, the manner in which these changes in mucin expression levels affect CRC progression is not entirely clear.

The loss of MUC2 has been demonstrated to aid in CRC formation, as demonstrated by $Muc2^{-/-}$ mouse models, where it was seen that the loss of Muc2 caused increased susceptibility to colitis and that the loss of Muc2 alone was sufficient for CRC formation [9, 10]. These tumors were atypical in the sense that they did not possess cytosolic/nuclear β-catenin. However, when these mice were crossed with Apc^{Min} mice, tumors that were concentrated toward the distal part of the large intestine were observed [11]. Apc^{Min} mice do not ordinarily develop full blown tumors and typically develop lesions that are concentrated in the upper gastrointestinal tract [12]. This suggests that the loss of Muc2 colludes with alterations in β-catenin. Moreover, the fact that these tumors possessed elevated transcripts of genes typically up-regulated during inflammation suggested that loss of Muc2 leaves the colon susceptible to inflammation aided tumorigenesis. $Muc1^{-/-}$ mice showed increased susceptibility to infection by *Campylobacter jejuni* [13]. Our lab has recently generated a whole body $Muc4^{-/-}$ mouse model which was treated with the chemical carcinogens Azoxymethate (AOM) and Dextran sodium sulfate (DSS) [14]. It was observed that

the loss of Muc4 conferred reduced susceptibility to DSS induced colitis and colitis induced CRC, suggesting that Muc4 aids in CRC mediated tumorigenesis and therefore apparently contradicting the results obtained from knock-out models of other mucin genes. Tumors in wild type and $Muc4^{-/-}$ mice exhibited increased nuclear β-catenin. Interestingly, wild-type mice showed increased Ki67 staining, indicative of increased proliferation. This ties in with the *in vitro* data presented in this thesis that shows that MUC4 confers increased proliferative properties to CRC cells. These results may present an explanation of the presence of MUC4 expression in a subset of human early CRCs [5].

Despite the utility of the aforementioned studies in shedding light on the role played by MUC4 in CRC, no study thus far has studied the manner in which the loss of MUC4 expression ties in with mutations in APC and/or Kras, genetic aberrations that are present in a large proportion of human CRC tumors [15]. Therefore, the goal of this study was to analyze the role played by MUC4 in the conventional genetic mutation induced CRC progression model. Thus, in this study, we have sought to generate $Muc4^{-/-}$; CDX2P-NLS-Cre; $Kras^{G12D/+}$; $Apc^{loxP/+}$ ($Muc4^{-/-}$; CKA) mice. The CDX2P-NLS-Cre confers increased lesion formation in the colon compared to other intestine-specific Cre based models [16]. Our breeding strategy is outlined in **Figure1**.

3. Results/Materials and Methods

A. Procurement of animals

The CDX2P-NLS-Cre; $Apc^{loxP/+}$ mice was characterized and generated previously [16]. We obtained the B6.Cg-Tg(CDX2-cre)101Erf/J mice from the Jackson Laboratory (Stock No: 009350). These mice express a nuclear localized Cre recombinase regulated by a CDX2 promoter and are on a C57BL/6J genetic background. This promoter is expressed in the ileum, caecum and colon. The depositing laboratory noted that when $Apc^{loxP/+}$ mice were crossed with CDX2P-NLS-Cre mice; they developed lesions mainly in the colon. The B6.Cg-Apc^{tm2Rak}/Nci (strain number: 01XAA) mice were obtained from the National Cancer Institute (NCI) mouse repository. These

mice have the exon 14 of the Apc gene flanked by loxP sites. When crossed with mice expressing a tissue specific Cre-recombinase, the loxP sites are excised by Cre- recombinase resulting in truncated Apc protein, which is 605 amino acids long, of which only the first 580 are present in the normal protein. These mice also had a C57BL/6J genetic background. The B6.129S4-Kras^{tm4Tyj}/J (LSL-K-ras G12D) strain was obtained from the NCI. Here, the Kras gene contains a point mutation: G12D and is followed by Lox-Stop-Lox codon. In the presence of Cre, the stop codon is excised and the mutant protein is expressed. These mice also possessed a C57BL/6J genetic background. The whole body Muc4$^{-/-}$ mice were generated in our lab and have been described in a recent paper [14]. These mice have a mixed genetic background

B. DNA isolation, genotyping and maintenance of animals

Animals were maintained in accordance with guidelines and protocols approved by the by the Institutional Animal Care and Use Committees (IACUC) of the University of Nebraska Medical Center. The animals were exposed to a 12 hour light/dark cycle and were allowed access to food and water *ad libitum*. The tails of mice were clipped at the age of 8 days and the DNA was isolated using the Maxwell 16 mouse tail DNA purification kit, Promega, Madison, WI, USA. Following DNA isolation, genotyping was performed using primers listed in Table B.4. in the materials and methods section (Chapter 2). Mice were observed daily for rectal bleeding and sacrificed once observed, and anal prolapse or any other signs of distress were carefully recorded. A representative genotyping gel picture is shown in **Figure 2.**

C. **Preliminary analysis of animals sacrificed**

We observed that CDX2P-NLS-Cre; Apc$^{loxP/loxP}$ mice developed severe rectal bleeding within 14 weeks of age and had large lesions primarily in the colon. It has been reported by an earlier study that homozygosity for Apc^{loxP} allele along with CRX2P-NLS-Cre was embryonically lethal [16]. However, it was observed that our mice had very faint bands for CRX2P-NLS-Cre when genotyped, suggesting that they were likely heterozygous for Cre, thus had a very low dose

of Cre, likely accounting for the non-lethality of this genotype. A single CDX2P-NLS-Cre; $Apc^{loxP/loxP}$ mouse that was sacrificed due to signs of distress and rectal bleeding showed extensive presence of macroscopic lesions concentrated in the colon (**Figure 3**) Further, we also euthanized and collected tissues from 5-6 months of age $Apc^{loxP/+}$;$Muc4^{+/-}$;CDX2P-NLS-Cre. These mice displayed splenomegaly; as well as numerous polyps throughout the intestine with an increased concentration in the colon. Currently, we are generating composite mice with $Muc4^{-/-}$;CKA and their contemporary littermate controls (CKA) mice, in future we will comparing $Muc4^{-/-}$;CKA mice with $Muc4^{+/+}$;CKA as well as $Muc4^{-/-}$;Cdx2P-NLS-Cre; $Apc^{loxP/+}$ ($Muc4^{-/-}$;CA) and $Muc4^{-/-}$; Cdx2P-NLS-Cre; $kras^{G12D/+}$ ($Muc4^{-/-}$;CK) mice.

C. **Analysis of tissue sections from CDX2P-NLS-Cre; $Apc^{loxP/loxP}$**

The part of the colon containing lesions was excised and kept in formalin for 48 hours, after which it was transferred to 70% ethanol. Tissue was embedded in paraffin and serial sections were made by the Tissue Sciences Core Facility at UNMC. Histological analysis of the the colon showed the presence of 1 ductal adenocarcinoma *in situ* and one adenoma showing low grade dyplasia. Further, immunohistochemical analysis showed that the lesions expressed dramatically increased cytosolic and occasionally nuclear β-catenin, in comparison to adjacent normal tissue, while intense Muc4 staining was present in the goblet cells in normal tissue and was dramatically reduced in the lesions, where faint cytoplasmic Muc4 staining was seen uniformly in all cells (**Figures 3-5**). The intensity of β-catenin staining in the lesions was variable, and this was possibly due to technical issues with the IHC process. Nevertheless, there was a clear intensification of β-catenin staining in all dysplastic/neoplastic areas, which is consistent with literature in CRC. Of note, while the overall reduction in Muc4 staining in lesions was quite obvious, the shift from staining in goblet cells alone (normal tissue) to being uniformly present in all cells, albeit at very low levels in the lesion(s) was striking.

4. Discussion

Our preliminary data confirms earlier studies performed in our lab where lesions from *Apc*^{Min} tissues were stained, showing that the loss of Muc4 is an inherent characteristic of colonic lesions with APC loss. However, our ultimate goal is to establish whether (a) the loss of MUC4 is a consequence of the mutations driving the disease and is merely a byproduct of the overall loss of differentiation with no significant functional role, or, (b) Muc4 has a significant role to play in CRC progression, by either imparting proliferative properties to CRC cells, a condition which will be simulated by the Muc4$^{+/+}$; CKA mice or aiding CRC progression by ablating the presumably protective role (i.e., against factors such as inflammation) via its loss of expression, a condition simulated by Muc4$^{-/-}$; CKA mice. We will compare the number and location of lesions and overall survival in these two groups of mice.

Interestingly, two other mice that developed lesions and were sacrificed were heterozygous for Muc4 while having a heterozygous loss of Apc. Could this point toward the fact that Muc4 is protective in the colon, much like Muc2? However, one must be cautious before coming to a conclusion from the very limited sample size and also given the fact that these mice express Muc4. We have as yet not obtained any Muc4$^{-/-}$; CKC mice, which will likely provide a more conclusive answer.

Figure legends

Figure 1 Breeding strategy Muc4$^{-/-}$ mice were generated in our lab in an earlier study. These mice were crossed with Apc$^{flox/flox,}$ CDX2-Cre and kras$^{G12D/+}$ to generate intermediate crosses, which were inter-crossed in order to generate the Muc4$^{-/-}$;CDX2-Cre kras$^{G12D/+}$Apc$^{flox/+}$ final genotype.

Figure 1

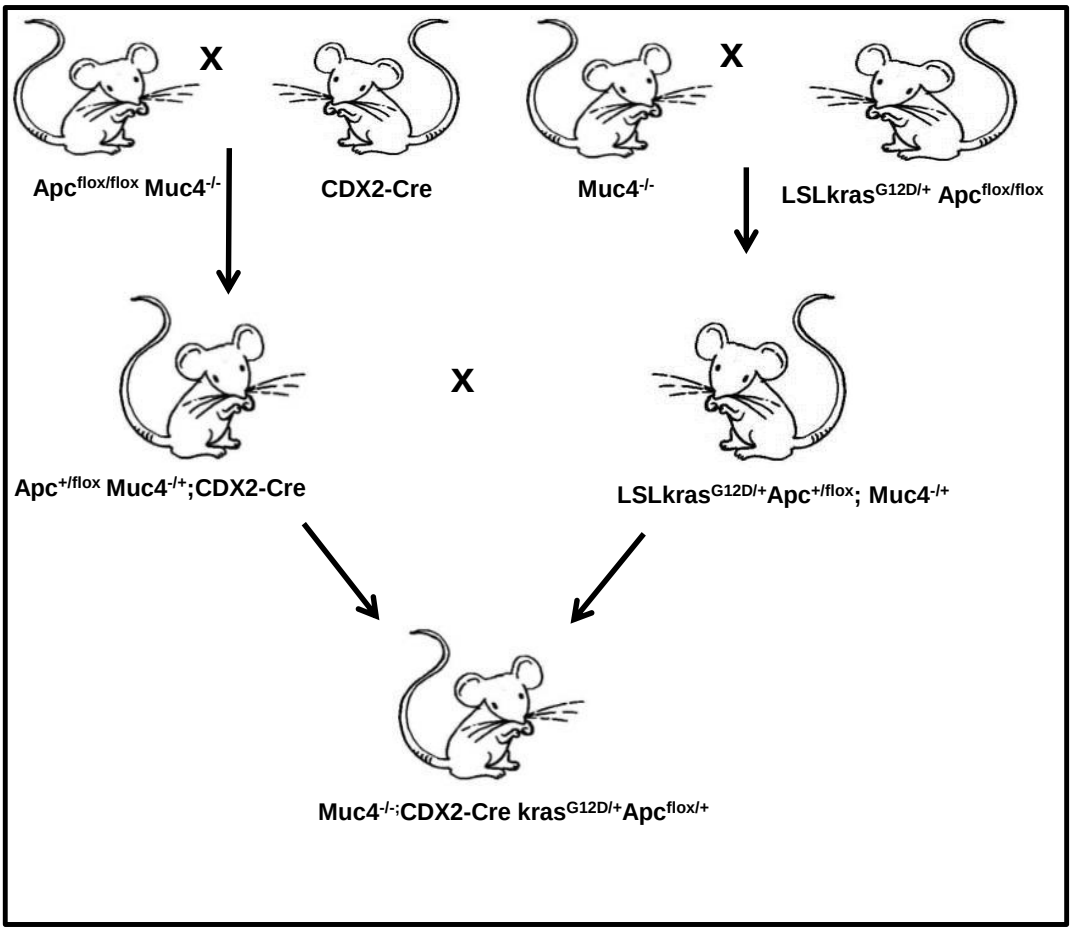

X
X
Apc^flox/flox Muc4^-/-
CDX2-Cre
Muc4^-/-
LSLkras^G12D/+ Apc^flox/flox
X
Apc^+/flox Muc4^-/+;CDX2-Cre
LSLkras^G12D/+Apc^+/flox; Muc4^-/+
Muc4^-/-;CDX2-Cre kras^G12D/+Apc^flox/+

Figure 2 A representative genotyping gel. Mouse tails were clipped and after DNA extraction, PCR using appropriate primers was performed and the products were run on 2% agarose gels. Encircled in red boxes are the bands for a mouse positive for Apc (2 bands indicative of heterozygosity for the floxed allele), Cdx2-Cre, $Kras^{G12D}$ and heterozygous for Muc4.

Figure 2

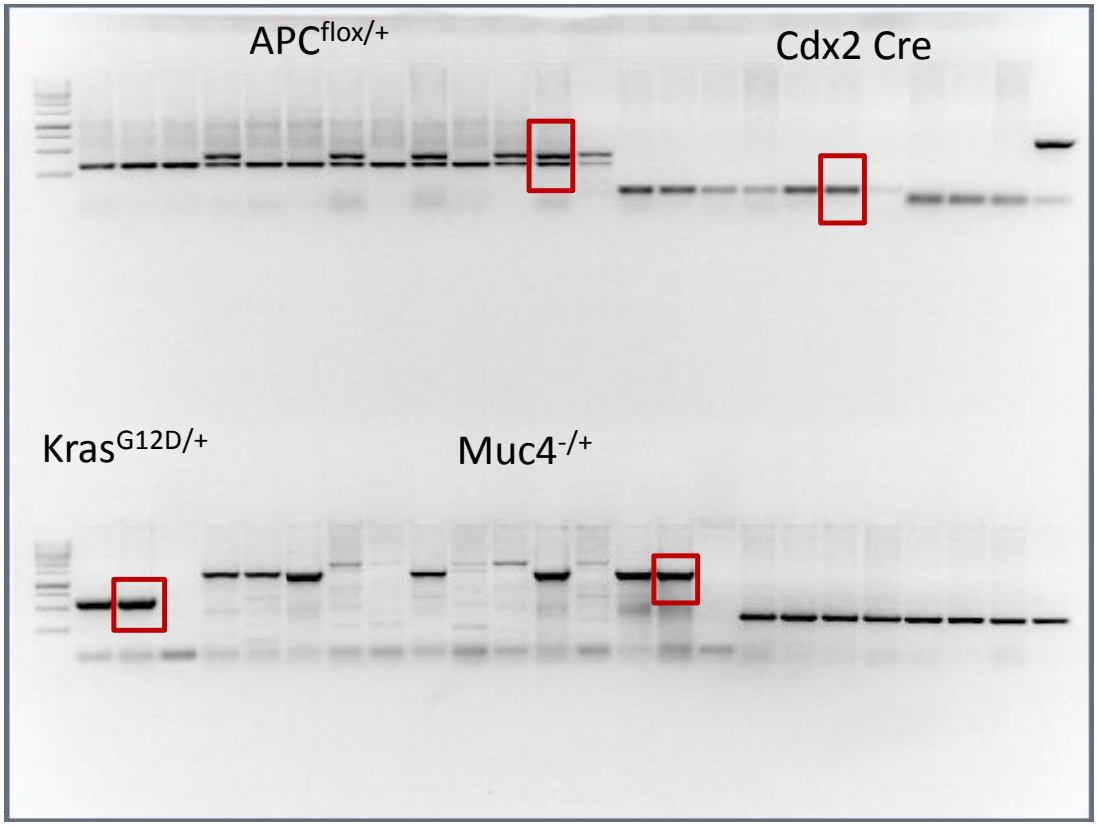


Figure 3 Gross appearance of lesions in mice sacrificed. Lesions were concentrated in the colon and large intestine.

Figure 3

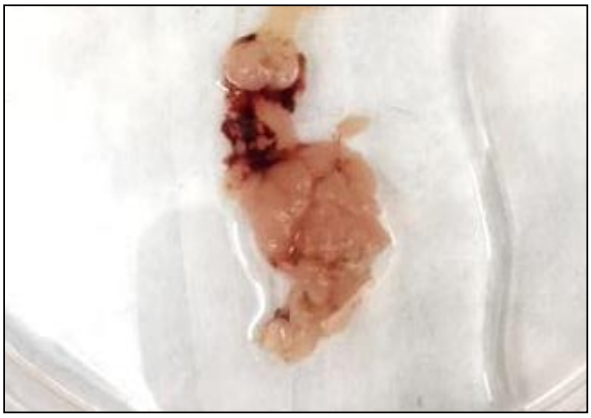


Figure 4 Immunohistochemical staining of mouse adenoma. (A.) Staining for β-catenin showed intense, widespread (80-90%) cytoplasmic/nuclear staining (solid arrow) in the lesion while adjacent normal tissue showed low to moderate staining (dotted arrow). Note that goblet cells in normal mucosa appear to be negative for β-catenin. (**B**.) Staining for Muc4 showed intense staining concentrated in the goblet cells (dotted arrow) in the normal mucosa, while showing weak cytoplasmic staining in the adenoma cells (solid arrow). Dotted lines demarcate lesion.

Figure 4

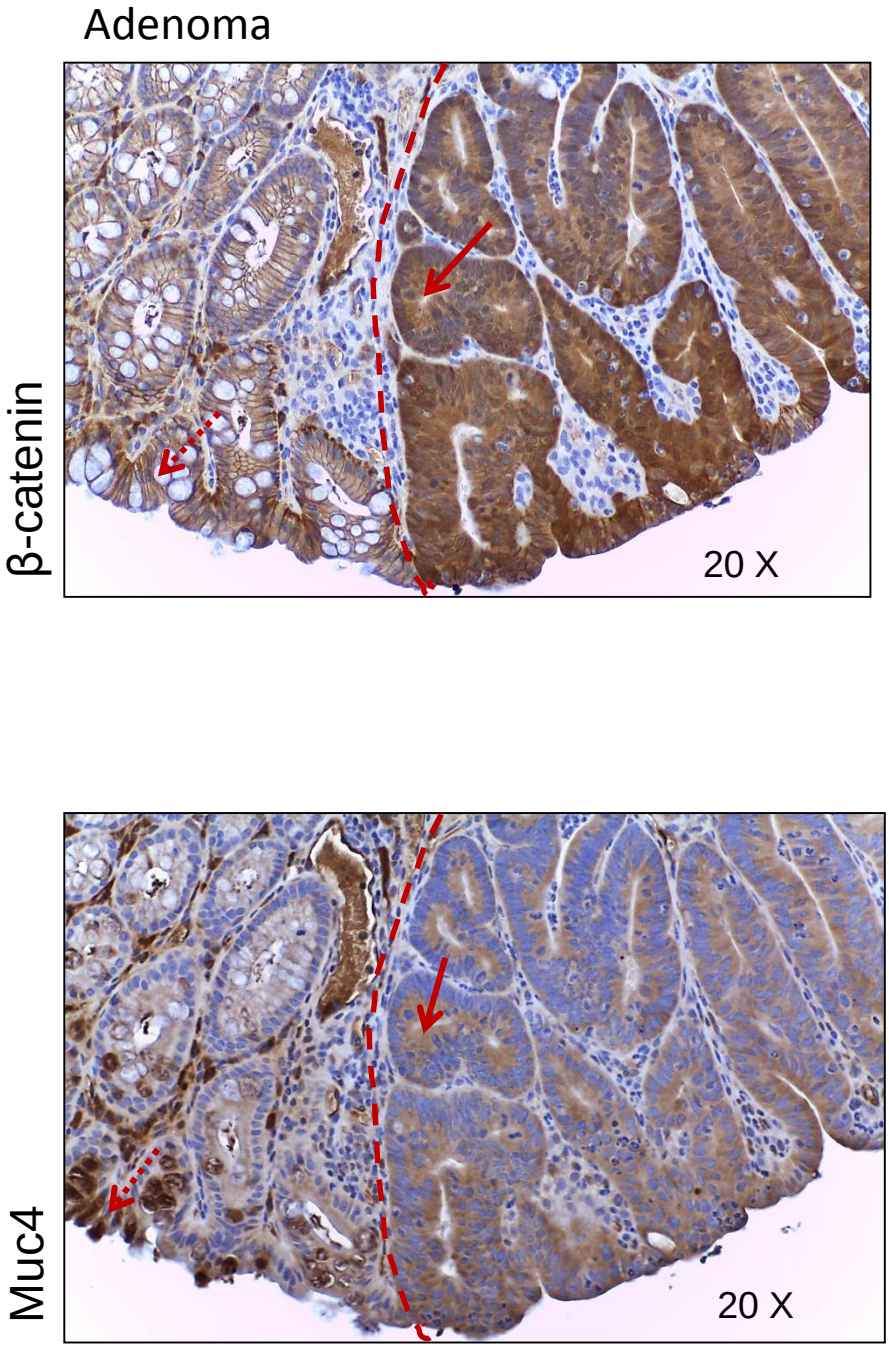


Figure 5 Immunohistochemical staining of mouse adenocarcinoma *in situ* (A.) Intense cytosolic/nuclear staining for β-catenin in the lesion (solid arrow) and weak staining in the adjacent normal areas (dotted arrow) was observed. (**B**.) Intense staining for Muc4 in goblet cells (dotted arrow) and weak, cytoplasmic staining in tumor cells (solid arrow) was observed. Dotted lines demarcate lesion.

Figure 5

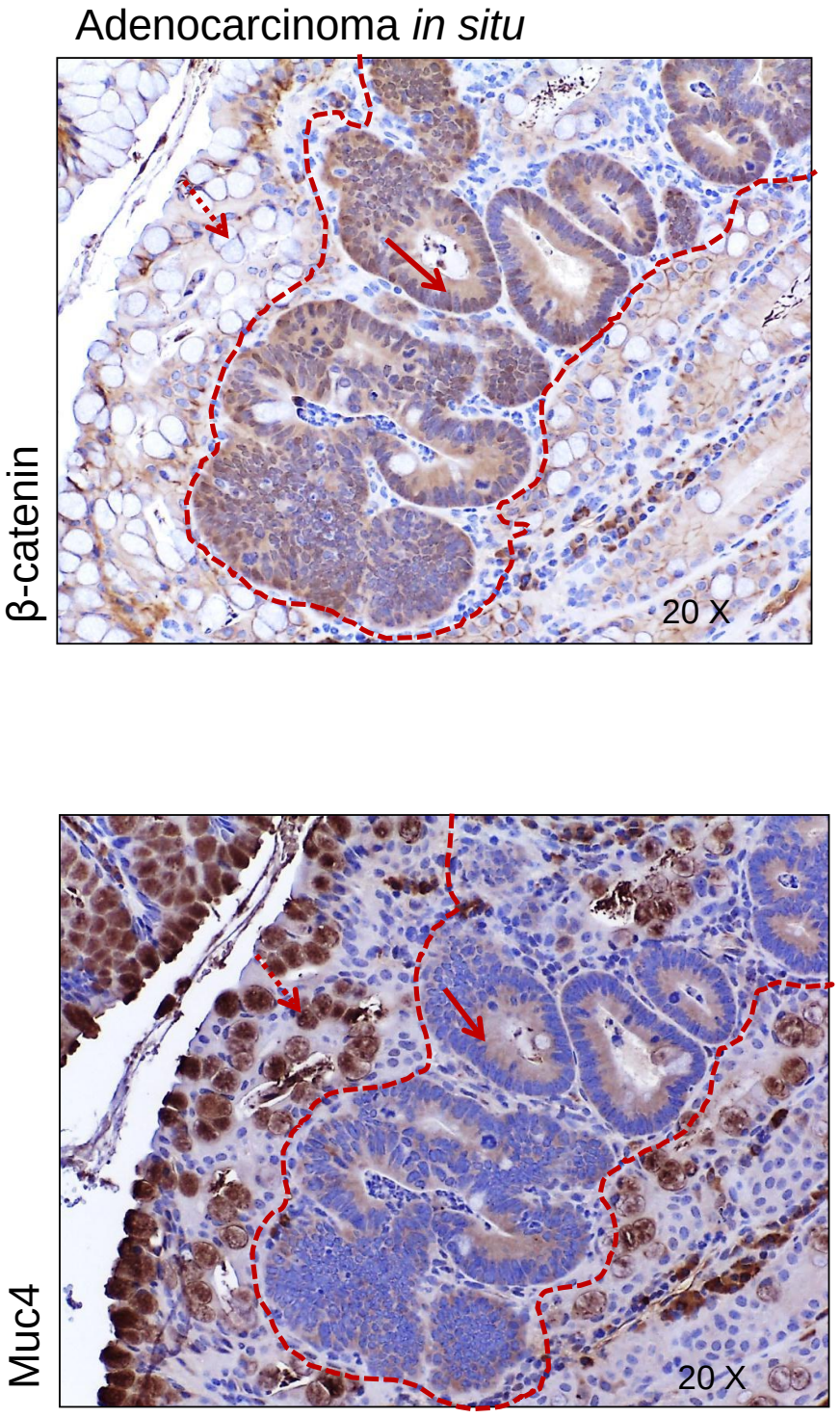
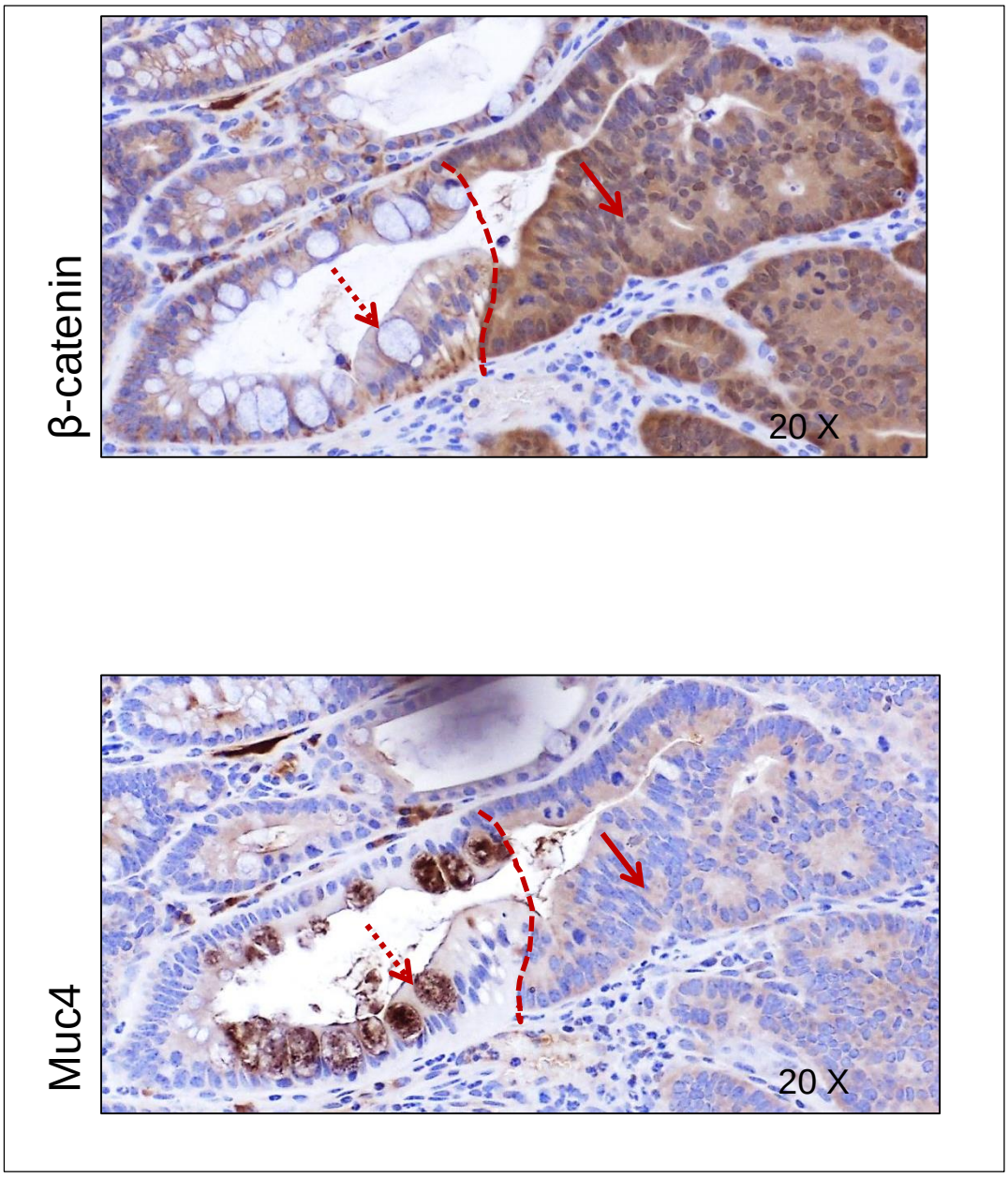


Figure 6: A single crypt showing neoplastic transformation in the upper half while having an untransformed basal crypt. Note sharp demarcation in the staining pattern, normal lower crypt shows goblet cell Muc4 staining and weak β-catenin staining (dotted arrows) while upper transformed half shows intense β-catenin staining and weak Muc4 staining (solid arrows).

Figure 6



References

[1] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Immunohistochemical staining patterns of MUC1, MUC2, MUC4, and MUC5AC mucins in hyperplastic polyps, serrated adenomas, and traditional adenomas of the colorectum. J Histochem Cytochem 1999;47:1039-48.

[2] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Mucin core protein expression in colorectal cancers with high levels of microsatellite instability indicates a novel pathway of morphogenesis. Clin Cancer Res 2000;6:1909-16.

[3] Ogata S, Uehara H, Chen A, Itzkowitz SH. Mucin gene expression in colonic tissues and cell lines. Cancer Res 1992;52:5971-8.

[4] Khanh dT, Mekata E, Mukaisho K, et al. Transmembrane mucin MUC1 overexpression and its association with CD10(+) myeloid cells, transforming growth factor-beta1 expression, and tumor budding grade in colorectal cancer. Cancer Sci 2013;104:958-64.

[5] Shanmugam C, Jhala NC, Katkoori VR, et al. Prognostic value of mucin 4 expression in colorectal adenocarcinomas. Cancer 2010;116:3577-86.

[6] van de Wetering M, Sancho E, Verweij C, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241-50.

[7] Kesari MV, Gaopande VL, Joshi AR, Babanagare SV, Gogate BP, Khadilkar AV. Immunohistochemical study of MUC1, MUC2 and MUC5AC in colorectal carcinoma and review of literature. Indian J Gastroenterol 2015;34:63-7.

[8] Byrd JC, Bresalier RS. Mucins and mucin binding proteins in colorectal cancer. Cancer Metastasis Rev 2004;23:77-99.

[9] Van der Sluis M, De Koning BA, De Bruijn AC, et al. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;131:117-29.

[10] Velcich A, Yang W, Heyer J, et al. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science 2002;295:1726-9.

[11] Yang K, Popova NV, Yang WC, et al. Interaction of Muc2 and Apc on Wnt signaling and in intestinal tumorigenesis: potential role of chronic inflammation. Cancer Res 2008;68:7313-22.

[12] Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55-67.

[13] McAuley JL, Linden SK, Png CW, et al. MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. J Clin Invest 2007;117:2313-24.

[14] Das S, Rachagani S, Sheinin Y, et al. Mice deficient in Muc4 are resistant to experimental colitis and colitis-associated colorectal cancer. Oncogene 2015;10.

[15] Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011;6:479-507.

[16] Hinoi T, Akyol A, Theisen BK, et al. Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. Cancer Res 2007;67:9721-30.

CHAPTER 6

Summary, Conclusions and Future directions

1. Summary

Over the past several years, our lab has studied various aspects of MUC4 function and regulation in various malignancies [1-5]. However, a number of questions remain unanswered. For instance, why does MUC4 confer metastatic and proliferative properties to cells in pancreatic and ovarian cancer [3, 4] while suppressing proliferation and migration in other malignancies such as lung cancer [6]? Also, while many factors such as miRNAs, retinoic acid, inflammatory cytokines, transcription factors such as GATAs, FOXOs etc. [1, 2, 7]; regulate *MUC4*, might other factors also cause MUC4 up-regulation/suppression? The studies presented in this thesis attempt to address parts of these questions.

The overarching goal of the studies presented in this thesis has been to examine the regulation of the transmembrane mucin MUC4 by the Wnt/β-catenin pathway in pancreatic ductal adenocarcinoma (PDAC) as well as in colorectal cancer (CRC). Briefly, we examined the following aspects of MUC4 regulation and function: 1) the regulation of MUC4 by Wnt/ β-catenin in pancreatic cancer and colorectal cancer, 2) the significance and functional implications of Wnt/β-catenin up-regulation in pancreatic cancer and 3) the functional implications of MUC4 expression in colorectal cancer as well as its contribution to disease progression.

In a nutshell, our major findings were: 1) MUC4 is up-regulated by β-catenin in pancreatic cancer while it is repressed by β-catenin in colorectal cancer. However, factors such as promoter accessibility and cross-talk with other signaling pathways may also affect this dynamic. 2) The Wnt/ β-catenin pathway contributes to the migratory properties of pancreatic cancer cells, by up-regulating mesenchymal markers. These results, when viewed in conjunction with the up-regulation of MUC4 by β-catenin, suggest that the Wnt/ β-catenin pathway contributes to metastasis of pancreatic cancer, in part, through MUC4 up-regulation 3) It was observed that MUC4 expression contributes to the proliferative and colony forming properties of colorectal cancer cells, in an apparent contradiction of the fact that the loss of MUC4 expression is observed

in the majority of colorectal cancers. We generated a mouse model to determine the effect of Muc4 loss on colorectal cancer progression. Our results with the mouse model thus far indicate that Muc4 expression appears to be lost during the course of CRC progression in mice. However, these mice need to be characterized further in order to determine the impact of Muc4 loss on Apc/Kras driven CRC in mice. Below, I summarize the findings of each project and implications thereof.

A. The Wnt/β-catenin pathway regulates *MUC4* in pancreatic ductal adenocarcinoma (PDAC)

The rationale for this study was based on a *MUC4* promoter analysis showing the presence of 3 TCF/LEF sites as well several other recent studies, including one that reported *MUC4* as one of the most significantly down-regulated transcripts in a microarray performed upon the depletion of β-catenin in the BXPC3 PDAC cell line [8]. First, we examined the expression of MUC4 and β-catenin in panel of PDAC cell lines and PDAC autopsy tissues. We observed a β-catenin-MUC4 co-expression in all cell lines, with the exception of AsPc1. Furthermore, IHC and confocal analysis of PDAC tissue showed that 80% of PDACs expressed MUC4 and cytosolic/nuclear β-catenin. Also, cells expressing MUC4 had strong cytosolic/nuclear β-catenin staining.

Having established an apparent correlation between MUC4 and β-catenin expression in human cell lines and tissues, we went on to knock-down (KD) β-catenin using two lentiviral β-catenin shRNA constructs in the cell lines CD18/HPAF and T3M4. We observed a significant reduction in *MUC4* RNA and protein. For further confirmation, lithium chloride (LiCl) treatment (which enhances nuclear β-catenin by inhibiting GSK3β) and Wnt3a conditioned medium treatment were used, resulting in increased MUC4.

The next step was to confirm whether *MUC4* up-regulation occurred at the transcript level, and to this end we generated three *MUC4* promoter luciferase constructs, each containing

one (p2700), two (p3000) or three (p3778) TCF/LEF sites. In the presence of 4ACAT, the stabilized β-catenin construct, only p3778 (all three sites) showed a significant increase in luciferase activity compared to the empty vector. This suggested that all three sites are required for the β-catenin mediated *MUC4* up-regulation. Next, we mutated each site individually in order to determine which site(s) was most critical for the β-catenin mediated *MUC4* up-regulation. In the presence of 4ACAT, MUT1 (mutation in site #1, closest to the ATG site) showed a dramatically reduced luciferase activity compared to p3778 while MUT2 (mutant site #2), quite surprisingly, showed an increased luciferase activity compared to p3778, implying that this was ordinarily a repressive site. MUT3 (mutant site #3, furthest from the ATG site) showed a significantly decreased luciferase activity in comparison to p3778, although this was not as dramatic as the reduction seen with MUT1. Due to the dramatic reduction in luciferase activity seen with MUT1, we expected that p2700 (promoter luciferase fragment containing only site #1) would show an increased luciferase activity in the presence of 4ACAT, however, this fragment did not show any significant increase in luciferase activity. This led us to conclude that the full promoter containing all three TCF/LEF sites is required for 4ACAT mediated *MUC4* up-regulation.

Given that our promoter luciferase studies indicated that site #1 and #3 were *MUC4* transcription promoting, while site #2 was repressive, it was deemed necessary to determine which sites were actually bound to the β-catenin/TCF complex. A Chromatin immunoprecipitation (ChIP) was performed, which showed that while significantly elevated β-catenin binding occurred at site #1compared to the negative control, no binding was observed at site #2 and some binding occurred at site #3. Thus, our overall conclusions were that TCF/LEF site #1 and #3 are required for β-catenin mediated *MUC4* up-regulation. This is in line with literature, which suggests that most β-catenin target genes usually have more than one TCF site

and that the β-catenin enhanceosome complex typically recruits two or more distant TCF sites in order to create a locally conducive chromatin environment [9].

The second part of this project dealt with the functional properties imparted by β-catenin to PDAC cells. It was observed that β-catenin KD reduced the migratory properties of PDAC cells but did not significantly affect proliferation and colony formation. In line with these findings, we observed a significant reduction in mesenchymal markers such as Vimentin, N-cadherin, CD44 and increased E-cadherin and Zo-1. This was confirmed by tumorigenicity studies in mice. Our conclusions from this part of the study were that aberrantly localized β-catenin appears to act a driver of metastasis in PDAC, concurring with earlier studies with β-catenin in pancreatic cancer [10, 11].

B. The Wnt/β-catenin pathway regulates *MUC4* in colorectal cancer (CRC)

Given the proven significance of altered Wnt/ β-catenin signaling in CRC [12, 13], and the fact that several studies indicate a loss of MUC4 expression in CRC progression [14, 15]; it was decided to examine whether *MUC4* is also a β-catenin target gene in CRC. Furthermore, no study thus far has examined the functional significance of MUC4 in CRC. Numerous studies indicate that MUC4 expression is generally lost in CRC [14, 16]. However, others claim that while MUC4 is indeed lost in a large cohort of non-mucinous CRCs, MUC4 confers a worse prognosis to the subset of patients that retain high MUC4 expression, specifically in the early stages of the disease (stage I and II) [15, 17]. Most recently, a study from our lab observed that $Muc4^{-/-}$ mice are less susceptible to dextran sodium sulfate (DSS) induced colitis and CRC [18]. Thus, the goal of this part of the study was to examine the functional implications of MUC4 loss in human CRC cell line(s).

As part of this project, a panel of seven CRC cell lines was profiled for MUC4 and β-catenin expression. As expected, all cell lines expressed β-catenin abundantly while MUC4 was expressed in only three cell lines. Tissue immunofluorescence showed a decrease in MUC4 and

increase in cytosolic/nuclear β-catenin in colonic polyps compared to the normal colon. KD of β-catenin using lentiviral shRNA and siRNA resulted in increased *MUC4* RNA and protein expression. Transient over-expression of 4ACAT and treatment with Wnt3a conditioned medium caused a reduction in MUC4. Treatment with Actinomycin D showed that β-catenin KD does not prolong the *MUC4* mRNA stability. Luciferase studies using β-catenin siRNA showed that there was an increase in p3778 driven luciferase activity in the presence of the siRNA. However, this difference was not statistically significant. Luciferase studies with MUT1, MUT2 and MUT3 showed that mutation of site 2 and 3 caused an increased luciferase activity in comparison to p3778. When all three sites were mutated, there was increased luciferase activity. These results were also not statistically significant.

Since our promoter luciferase studies did not yield conclusive results, we looked at other factors that may bolster the effect of Wnt/β-catenin on MUC4 in CRC. Hath1 is a basic helix-loop-helix transcription factor that regulates the differentiation of colonocytes into secretory cells in the colonic epithelium [19]. It is ordinarily repressed by the Notch pathway and is a tumor suppressor in CRC [19]. MUC2, the major mucin in the intestine, has been shown to be governed by Hath1 [19, 20]. Our promoter analysis showed that the *MUC4* promoter contains a Hath1 binding site at position -3102/-3089 upstream from the ATG site. We observed that MUC4 expressing CRC cell lines expressed higher levels of *Hath1* than the non-expressing cells. KD of β-catenin resulted in an increase in *Hath1*and when the LS180 cell line was treated with a gamma secretase inhibitor, which has been shown to stimulate Hath1 [20], an increase in MUC4 expression was observed, concurrent with increased *Hath1*.

The second part of this project focused on a functional characterization of MUC4 in CRC. It was seen that MUC4 increased the proliferation and colony formation in CRC cells, but not migration or invasion. Thus, in conclusion, we found that *MUC4* is likely regulated by both β-catenin and Hath1 in CRC, and that MUC4 confers proliferative properties to CRC cells.

C. Generation of *Muc4$^{-/-}$; CDX2 P-NLS -Cre kras$^{G12D/+}$Apc$^{loxP/+}$* mice

As mentioned previously, while *in vitro* studies with human cell lines indicate MUC4 is likely tumor-promoting in CRC, it is apparent that MUC4 is lost during the course of CRC progression and yet, confers a worse prognosis to early -stage patients that retain MUC4 expression. Therefore there is considerable confusion regarding the precise role played by Muc4 in CRC. Our lab has recently generated a Muc4$^{-/-}$ mouse model [18]. No study thus far has analyzed the effect of Muc4 loss in conjunction with a conventional genetically driven mouse model of CRC. The goal of this project was to generate a *CDX2 P-NLS –Cre* driven colorectal cancer mouse model that will be crossed with the Muc4$^{-/-}$ mouse model generated in our lab. Thus far, we have generated *CDX2 P-NLS -Cre Apc$^{loxP/loxP}$* mice, which developed tumors at 14 weeks of age. The tumors in these mice show altered Muc4 expression pattern in comparison to adjacent normal tissue. We expect that upon obtaining mice with the full *Muc4$^{-/-}$; CDX2 P-NLS -Cre kras$^{G12D/+}$Apc$^{loxP/+}$* genotype, we will be able to definitively answer questions regarding whether Muc4 loss aids or hinders CRC progression.

2. Future directions

A. The Wnt/β-catenin pathway regulates MUC4 expression in pancreatic ductal adenocarcinoma (PDAC)

(i) Could Wnt/ β-catenin contribute to *MUC4* promoter hypomethylation and histone acetylation?

While our studies in PDAC proffer definitive evidence of a Wnt/ β-catenin mediated up-regulation in pancreatic cancer, these studies raise a number of questions. For instance, numerous studies [21-23] have stated that *MUC4* promoter hypomethylation and histone acetylation occur early on in PDAC progression, yet what factor(s) might cause these epigenetic changes? Coincidentally, the β-catenin has been shown to induce widespread chromatin modifications,

including histone acetylation and methylation [9, 24]. Given that both aberrant β-catenin and increased MUC4 appear at roughly the same time during PanIN progression [25, 26]; might β-catenin contribute to the permissive chromatin milieu? However, it is also likely that a number of factors including β-catenin collude to cause *MUC4* promoter accessibility. These tantalizing questions could be addressed by future studies.

(ii) Could Wnt/ β-catenin affect other mucins?

During the course of our analysis of the β-catenin KD cells, we observed that the levels of other mucins were also affected by the KD of β-catenin. In both CD18/HPAF and T3M4, MUC1 levels were increased upon the KD of β-catenin i.e, the presence of β-catenin co-related with decreased MUC1 protein while RNA levels were unaltered. Given that the MUC1 cytoplasmic tail has been shown to interact with, stabilize and potentiate nuclear β-catenin [27], this result appears rather counter-intuitive. One possible explanation is that β-catenin, which has been shown to target glycosylation related genes [28], alters the glycosylation of MUC1. The HMFG2 antibody for MUC1 recognizes sparsely glycosylated MUC1 [29, 30] and thus the altered glycosylation of MUC1 may account for the apparent change in protein levels. However, these ideas are purely speculative and need to be verified. Alternatively, as mentioned in Chapter 3, a decrease in other EMT related molecules such as Snail, which has been shown to repress MUC1 expression [31, 32], could be responsible for this increase. In the CD18/HPAF cell line there was a significant decrease in MUC16 protein levels while RNA levels were unchanged in the KD cells. This also suggests that β-catenin might alter *MUC16* RNA/protein stability and could be addressed by future studies.

B. The Wnt/β-catenin pathway regulates MUC4 expression in colorectal cancer (CRC)

(i) What could possibly cause the differential regulation of the same transcript, *MUC4*, by β-catenin in these two malignancies?

There are several plausible explanations for this apparent contradiction, all of which could be addressed by future studies and that are discussed below.

Firstly, the starkest difference between PDAC and CRC is the disparity in the levels of aberrant β-catenin and the stage at which the Wnt/ β-catenin pathway is activated during disease progression. The levels of β-catenin transcriptional activity are 5-20 fold higher in CRC compared to PDAC [33] as well as appearing much earlier on in disease progression. A study has shown that even within CRCs, the invasive front of the tumor expresses much higher nuclear β-catenin than the tumor center, thus activating a different cohort of target genes, leading to heterogenous target gene expression [34]. It has been observed that the tissue specific regulation of target genes by β-catenin is due to both differential promoter/histone modifications and the relative abundance of different TCF/LEF factors [35]. Interestingly, when CD18/HPAF was treated with incrementally increasing doses of Wnt3a, low to moderate levels of Wnt3a caused an increase in MUC4 and c-Myc (held to be a β-catenin target gene in PDAC [36]), higher levels of Wnt3a did not induce MUC4 while the c-Myc levels were actually reduced in comparison to the control (**Figure 1**). Thus, it is tempting to conclude from this experiment that nuclear β-catenin represses MUC4 at higher levels while promoting MUC4 expression at lower levels. This hypothesis dovetails neatly with studies in CRC showing moderate/increased MUC4 in early grade lesions [37](moderate nuclear β-catenin), while advanced grade CRCs (high nuclear β-catenin) mostly lacked MUC4 [15]. However, this experiment needs to be validated in other cell lines and with more specific inducers of nuclear β-catenin (for example, the 4ACAT stabilized β-catenin construct) since Wnt3a has been shown to also have β-catenin independent functions [38, 39]. Another experiment that could validate this hypothesis would be an IHC analysis of large

tumors to see whether MUC4 is differentially expressed at the invasive front vis-à-vis the tumor center, concurrent with differential nuclear β-catenin expression.

The second plausible explanation for the observed discrepancy between PDAC and CRC could be differential TCF/LEF factor expression. While there are primarily four major TCF/LEF factors in humans: TCF4, TCF3, TCF1 and LEF1, these factors have numerous splice variants, each of which can either promote/repress target gene transcription [40]. For instance, there are 14 alternatively spliced TCF4 isoforms in hepatocellular carcinoma alone [41]. Furthermore, the presence of alternative promoters means that truncated 'dominant negative' isoforms also exist [40]. Adding to this already mind-boggling complexity is the fact that β-catenin has been shown to occasionally partner with unconventional nuclear binding parkers, such as HIF1α [42], SOX family proteins [43] and FOXO proteins [44]. Any or several of these proteins are likely differentially expressed in PDAC and CRC, which could possibly lead to the differential regulation of the same gene. When a panel of CRC cell lines and the PDAC cell line T3M4 were profiled for the expression levels of TCF/LEF factors (**Figure 2**), we observed that while LEF1, TCF1 and TCF4 were expressed in all cell lines, TCF3 was expressed in T3M4 alone while being completely absent in CRC. TCF3 is generally held to be a repressive TCF factor [40]. Having stated that, one cannot rule out the presence of alternate splice forms/truncated isoforms that may potentially promote transcription. T3M4 also showed an overall lower level of TCF1, TCF4 and LEF1, which is perhaps expected given the lower level of Wnt/ β-catenin signaling in PDAC. Given the paucity of studies on the TCF/LEF factors and their functions in PDAC, future studies could address the function/isoforms of these factors in pancreatic cancer, more specifically, the function/isoforms of TCF3 in light of the differential expression we observed.

A third possible cause, which was touched upon in Chapter 4, is cross-talk with other pathways. In Chapter 4, evidence pointing towards the notion that β-catenin can influence MUC4 levels by suppressing Hath1, which ordinarily increases MUC4 expression was presented. Hath1

is also suppressed by the Notch pathway, shown to be active in CRC [45]. The role of Notch in PDAC, however, is controversial [46], while interplay between Notch and Wnt, leading to disease progression has been reported in CRC [45]. Future studies could address the precise mechanism by which multiple pathways such as Notch and Wnt converge upon MUC4 regulation in CRC. Specifically, Hath1 could be over-expressed and knocked down in CRC cells and the MUC4 levels could be subsequently observed, the Hath1 binding site in the *MUC4* promoter could be mutated and we could perform *MUC4* promoter luciferase assays to ascertain the regulation of *MUC4* by Hath1. In **Figure 3**, I summarize the presumptive mechanism for the differential regulation of *MUC4* in CRC and PDAC.

C. Generation of *Muc4$^{-/-}$; CDX2 P-NLS -Cre kras$^{G12D/+}$Apc$^{loxP/+}$* mice

(i) Does Muc4 aid tumor progression despite being diminished in the majority of CRCs?

While we haven't yet generated the Muc4$^{-/-}$; CKA mice, which will likely provide a definitive answer to this question, a recent study from our lab [18] gives us reason to believe that the presence of Muc4 may aid tumor progression. In the *CDX2 P-NLS -Cre Apc$^{-/-}$* mice that were examined, lesions showed dramatically reduced Muc4 compared to surrounding normal areas. However, while normal crypts showed focally intense staining in goblet cells, tumor cells showed weak cytoplasmic Muc4 staining that was uniformly distributed throughout the lesion. When viewed in conjunction with *in vitro* human cell line data that show increased proliferation in the presence of Muc4, one could ask whether the presence of even low amounts of Muc4, such as that seen in mouse tumors, contribute to tumor proliferation.

In a recent study from our lab where a Muc4$^{-/-}$ mouse was generated, it was observed that a compensatory increase in Muc2 occurred upon depletion of Muc4, which was ascribed to increased inflammatory cues such as TNF-α and IL-1β [18]. In the proposed mouse model, the mice will not be subjected to any inflammation inducing agents such as DSS, and therefore we may not see any increase in Muc2 expression. This observation could also have implications for

our functional studies with MUC4 KD in human cell lines. Das *et al* showed that MUC2 is also increased as a compensatory mechanism in HCT-8 MUC4 KD cells [18]. Therefore, in order to ascertain that MUC2 up-regulation, which has been shown to suppress proliferation [47] is not confounding our results, we could over-express MUC4 using the miniMUC4 vector developed in our lab in MUC4 negative cell lines. Furthermore, we could over-express MUC4 in MUC2 low/negative cell lines such as CaCo2 [48].

Another factor to be considered is whether the mouse model will necessarily be an accurate reflection of the human disease. For instance, while in the normal human colon MUC4 has been reported to be present in columnar and goblet cells in the lower 2/3rds of the crypt [37], in the mouse we observed Muc4 staining virtually exclusively in the goblet cells throughout the crypt, concentrated towards the outer 2/3rds of the crypt. These issues related to whether studies in mice are applicable to human cancer could be addressed by more extensive studies with human cell lines to bolster our findings in mice, such as corroborating our findings in HCT-8 with another MUC4 expressing CRC cell line, LS180 and orthotopic implantation of human MUC4 expressing/depleted cell lines in athymic nude mice.

Figure 1 Treatment of CD18/HPAF with Wnt3a conditioned medium. CD18/HPAF cells were treated with increasing volumes of Wnt3a conditioned medium. It was observed that at higher doses of Wnt3A, there was no induction of MUC4 expression. As a positive control, the levels of c-Myc, an established β-catenin target in PDAC was used. It was observed that there was an increase in c-Myc only at lower volumes of Wnt3A, while reduced levels of c-Myc were seen with higher levels of Wnt3A. β-actin was used as a loading control.

Figure 1

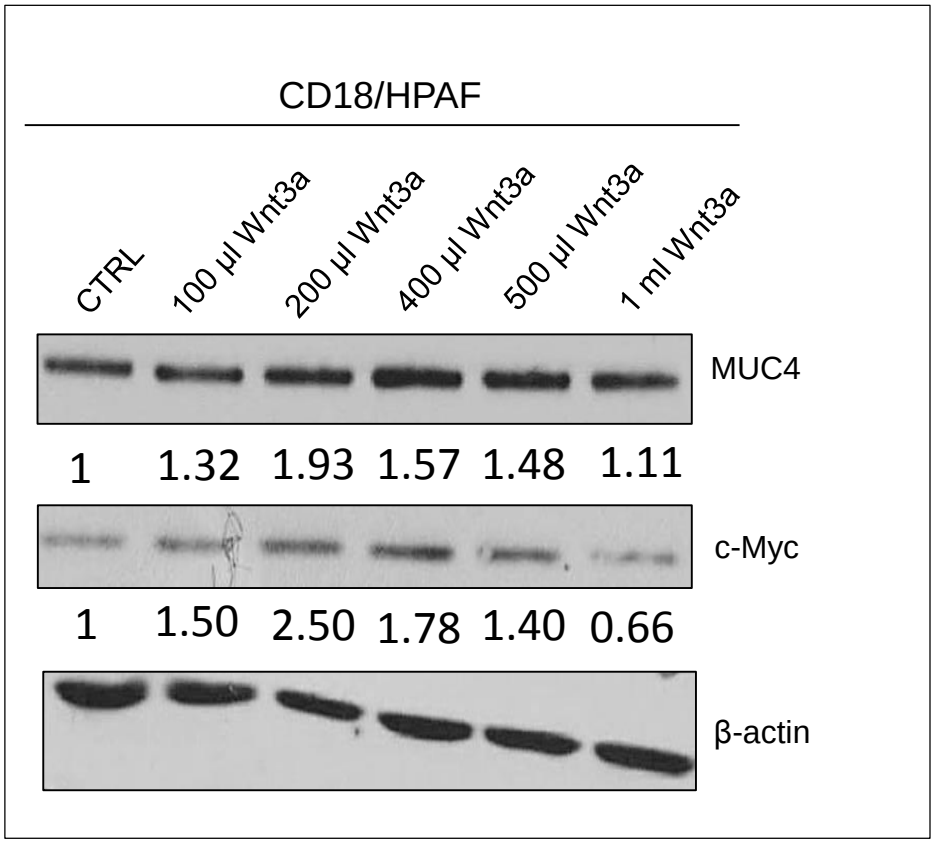

CD18/HPAF
CTRL
100 µl Wnt3a
200 µl Wnt3a
400 µl Wnt3a
500 µl Wnt3a
1 ml Wnt3a
MUC4
1 1.32 1.93 1.57 1.48 1.11
c-Myc
1 1.50 2.50 1.78 1.40 0.66
β-actin

Figure 2 Expression profiles of TCF/LEF factors in CRC and PDAC. Real time PCR for the TCF and LEF factors in a panel of CRC and the PDAC cell line T3M4 was performed.

Figure 2

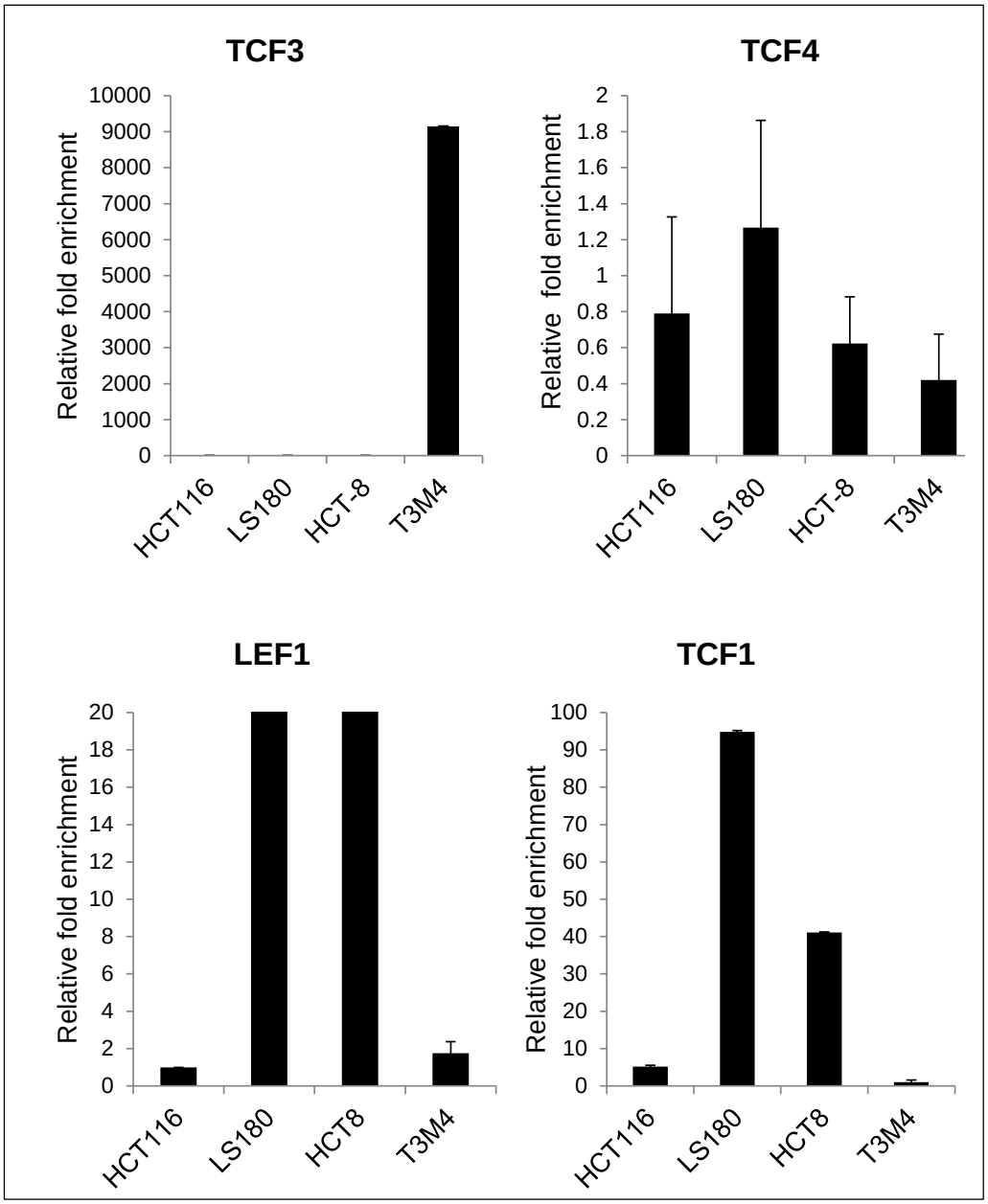
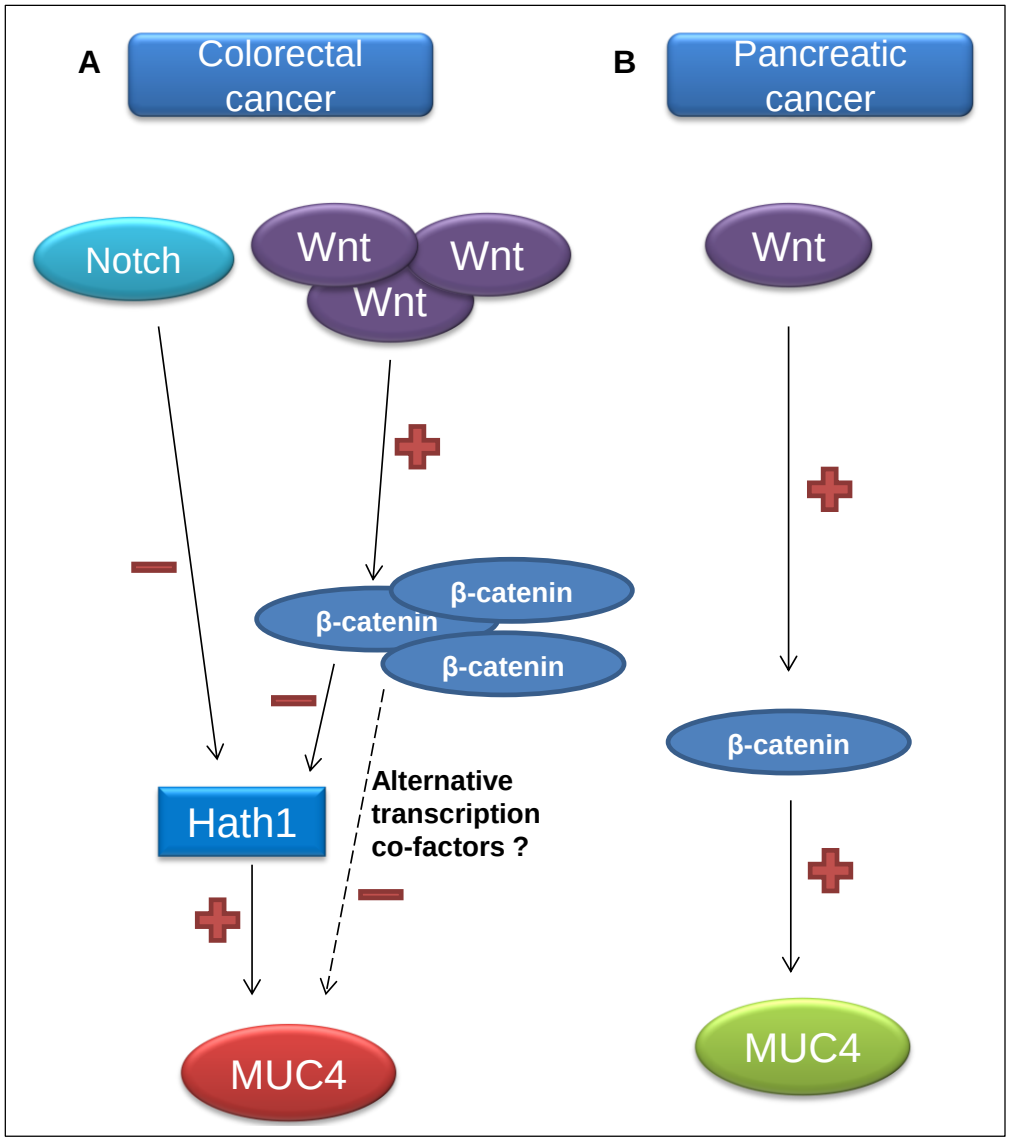

TCF3
Relative fold enrichment
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
HCT116
LS180
HCT-8
T3M4
TCF4
Relative fold enrichment
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HCT116
LS180
HCT-8
T3M4
LEF1
Relative fold enrichment
20
18
16
14
12
10
8
6
4
2
0
HCT116
LS180
HCT8
T3M4
TCF1
Relative fold enrichment
100
90
80
70
60
50
40
30
20
10
0
HCT116
LS180
HCT8
T3M4

Figure 3 Proposed mechanisms for the regulation of MUC4 expression in colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC). A. in CRC, high levels of nuclear β-catenin alters *MUC4* levels by directly binding the *MUC4* promoter and forming a repressive complex. Also, the Notch and Wnt/ β-catenin pathways may converge upon the repression of Hath1, which ordinarily up-regulates *MUC4*, thus attenuating *MUC4* transcription. **B.** in PDAC, relatively low levels of nuclear β-catenin/TCF4 form a transcription activating complex on the *MUC4* promoter. Note that Wnt/β-catenin transcriptional activity is more active and occurs earlier on in disease progression in CRC in comparison to PDAC.

Figure 3


A
Colorectal cancer
B
Pancreatic cancer
Notch
Wnt
Wnt
Wnt
Wnt
β-catenin
β-catenin
β-catenin
β-catenin
Hath1
Alternative transcription co-factors ?
MUC4
MUC4

References

[1] Andrianifahanana M, Moniaux N, Batra SK. Regulation of mucin expression: mechanistic aspects and implications for cancer and inflammatory diseases. Biochim Biophys Acta 2006;1765:189-222.

[2] Andrianifahanana M, Singh AP, Nemos C, et al. IFN-gamma-induced expression of MUC4 in pancreatic cancer cells is mediated by STAT-1 upregulation: a novel mechanism for IFN-gamma response. Oncogene 2007;26:7251-61.

[3] Ponnusamy MP, Lakshmanan I, Jain M, et al. MUC4 mucin-induced epithelial to mesenchymal transition: a novel mechanism for metastasis of human ovarian cancer cells. Oncogene 2010;29:5741-54.

[4] Rachagani S, Macha MA, Ponnusamy MP, et al. MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1. Carcinogenesis 2012;33:1953-64.

[5] Seshacharyulu P, Ponnusamy MP, Rachagani S, et al. Targeting EGF-receptor(s) - STAT1 axis attenuates tumor growth and metastasis through downregulation of MUC4 mucin in human pancreatic cancer. Oncotarget 2015;6:5164-81.

[6] Majhi PD, Lakshmanan I, Ponnusamy MP, et al. Pathobiological implications of MUC4 in non-small-cell lung cancer. J Thorac Oncol 2013;8:398-407.

[7] Radhakrishnan P, Mohr AM, Grandgenett PM, Steele MM, Batra SK, Hollingsworth MA. MicroRNA-200c modulates the expression of MUC4 and MUC16 by directly targeting their coding sequences in human pancreatic cancer. PLoS One 2013;8:e73356.

[8] Olsen PA, Solberg NT, Lund K, et al. Implications of targeted genomic disruption of beta-catenin in BxPC-3 pancreatic adenocarcinoma cells. PLoS One 2014;9:e115496.

[9] Mosimann C, Hausmann G, Basler K. Beta-catenin hits chromatin: regulation of Wnt target gene activation. Nat Rev Mol Cell Biol 2009;10:276-86.

[10] Wang L, Heidt DG, Lee CJ, et al. Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization. Cancer Cell 2009;15:207-19.

[11] Wang L, Yang H, Abel EV, et al. ATDC induces an invasive switch in KRAS-induced pancreatic tumorigenesis. Genes Dev 2015;29:171-83.

[12] Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011;6:479-507.

[13] Fodde R, Smits R, Clevers H. APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55-67.

[14] Khanh dT, Mekata E, Mukaisho K, et al. Transmembrane mucin MUC1 overexpression and its association with CD10(+) myeloid cells, transforming growth factor-beta1 expression, and tumor budding grade in colorectal cancer. Cancer Sci 2013;104:958-64.

[15] Shanmugam C, Jhala NC, Katkoori VR, et al. Prognostic value of mucin 4 expression in colorectal adenocarcinomas. Cancer 2010;116:3577-86.

[16] Ogata S, Uehara H, Chen A, Itzkowitz SH. Mucin gene expression in colonic tissues and cell lines. Cancer Res 1992;52:5971-8.

[17] Huang X, Wang X, Lu SM, et al. Clinicopathological and prognostic significance of MUC4 expression in cancers: evidence from meta-analysis. Int J Clin Exp Med 2015;8:10274-83.

[18] Das S, Rachagani S, Sheinin Y, et al. Mice deficient in Muc4 are resistant to experimental colitis and colitis-associated colorectal cancer. Oncogene 2015, *in press*.

[19] Leow CC, Romero MS, Ross S, Polakis P, Gao WQ. Hath1, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells. Cancer Res 2004;64:6050-7.

[20] Sekine A, Akiyama Y, Yanagihara K, Yuasa Y. Hath1 up-regulates gastric mucin gene expression in gastric cells. Biochem Biophys Res Commun 2006;344:1166-71.

[21] Vincent A, Ducourouble MP, Van S, I. Epigenetic regulation of the human mucin gene MUC4 in epithelial cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases. FASEB J 2008;22:3035-45.

[22] Yamada N, Nishida Y, Tsutsumida H, et al. Promoter CpG methylation in cancer cells contributes to the regulation of MUC4. Br J Cancer 2009;100:344-51.

[23] Zhu Y, Zhang JJ, Zhu R, et al. The increase in the expression and hypomethylation of MUC4 gene with the progression of pancreatic ductal adenocarcinoma. Med Oncol 2011;28 Suppl 1:S175-84.

[24] Cadigan KM. TCFs and Wnt/beta-catenin signaling: more than one way to throw the switch. Curr Top Dev Biol 2012;98:1-34.

[25] Al-Aynati MM, Radulovich N, Riddell RH, Tsao MS. Epithelial-cadherin and beta-catenin expression changes in pancreatic intraepithelial neoplasia. Clin Cancer Res 2004;10:1235-40.

[26] Andrianifahanana M, Moniaux N, Schmied BM, et al. Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res 2001;7:4033-40.

[27] Kufe DW. MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. Oncogene 2013;32:1073-81.

[28] Sengupta PK, Bouchie MP, Kukuruzinska MA. N-glycosylation gene DPAGT1 is a target of the Wnt/beta-catenin signaling pathway. J Biol Chem 2010;285:31164-73.

[29] Burchell J, Durbin H, Taylor-Papadimitriou J. Complexity of expression of antigenic determinants, recognized by monoclonal antibodies HMFG-1 and HMFG-2, in normal and malignant human mammary epithelial cells. J Immunol 1983;131:508-13.

[30] Sakurai J, Hattori N, Nakajima M, et al. Differential expression of the glycosylated forms of MUC1 during lung development. Eur J Histochem 2007;51:95-102.

[31] Nieto MA. The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol 2002;3:155-66.

[32] Guaita S, Puig I, Franci C, et al. Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. J Biol Chem 2002;277:39209-16.

[33] White BD, Chien AJ, Dawson DW. Dysregulation of Wnt/beta-catenin signaling in gastrointestinal cancers. Gastroenterology 2012;142:219-32.

[34] Hlubek F, Brabletz T, Budczies J, Pfeiffer S, Jung A, Kirchner T. Heterogeneous expression of Wnt/beta-catenin target genes within colorectal cancer. Int J Cancer 2007;121:1941-8.

[35] Wohrle S, Wallmen B, Hecht A. Differential control of Wnt target genes involves epigenetic mechanisms and selective promoter occupancy by T-cell factors. Mol Cell Biol 2007;27:8164-77.

[36] Li YJ, Wei ZM, Meng YX, Ji XR. Beta-catenin up-regulates the expression of cyclinD1, c-myc and MMP-7 in human pancreatic cancer: relationships with carcinogenesis and metastasis. World J Gastroenterol 2005;11:2117-23.

[37] Biemer-Huttmann AE, Walsh MD, McGuckin MA, et al. Immunohistochemical staining patterns of MUC1, MUC2, MUC4, and MUC5AC mucins in hyperplastic polyps, serrated adenomas, and traditional adenomas of the colorectum. J Histochem Cytochem 1999;47:1039-48.

[38] Yun MS, Kim SE, Jeon SH, Lee JS, Choi KY. Both ERK and Wnt/beta-catenin pathways are involved in Wnt3a-induced proliferation. J Cell Sci 2005;118:313-22.

[39] Kim SE, Lee WJ, Choi KY. The PI3 kinase-Akt pathway mediates Wnt3a-induced proliferation. Cell Signal 2007;19:511-8.

[40] Cadigan KM, Waterman ML. TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harb Perspect Biol 2012;4:a007906.

[41] Tsedensodnom O, Koga H, Rosenberg SA, et al. Identification of T-cell factor-4 isoforms that contribute to the malignant phenotype of hepatocellular carcinoma cells. Exp Cell Res 2011;317:920-31.

[42] Kaidi A, Williams AC, Paraskeva C. Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. Nat Cell Biol 2007;9:210-7.

[43] Kormish JD, Sinner D, Zorn AM. Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease. Dev Dyn 2010;239:56-68.

[44] Almeida M, Han L, Martin-Millan M, O'Brien CA, Manolagas SC. Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. J Biol Chem 2007;282:27298-305.

[45] Peignon G, Durand A, Cacheux W, et al. Complex interplay between beta-catenin signalling and Notch effectors in intestinal tumorigenesis. Gut 2011;60:166-76.

[46] Avila JL, Kissil JL. Notch signaling in pancreatic cancer: oncogene or tumor suppressor? Trends Mol Med 2013;19:320-7.

[47] Bu X, Li L, Li N, Tian X, Huang P. Suppression of mucin 2 enhances the proliferation and invasion of LS174T human colorectal cancer cells. Cell Biol Int 2011;35:1121-9.

[48] Bu XD, Li N, Tian XQ, Huang PL. Caco-2 and LS174T cell lines provide different models for studying mucin expression in colon cancer. Tissue Cell 2011;43:201-6.

Bibliography of Priya Pai

1. Pai P., Rachagani S., Dhawan P. and Batra S.K., *Mucins and β-catenin in Gastrointestinal Cancers: A Pernicious Partnership;* (2015), **Carcinogenesis** (*under review*)

2. Pai P, Rachagani S, Lakshmanan I, Macha MA, Sheinin Y, Smith LM, Ponnusamy MP, Batra SK *The Canonical Wnt Pathway Regulates the Metastasis-Promoting Mucin MUC4 in Pancreatic Ductal Adenocarcinoma*, (2015) **Molecular Oncology** (*In press*)

3. Rachagani S, Macha MA, Menning MS, Dey P, **Pai P**, Smith LM, Mo YY, Batra SK. *Changes in microRNA (miRNA) expression during pancreatic cancer development and progression in a genetically engineered KrasG12D;Pdx1-Cre mouse (KC) model.* (2015) **Oncotarget**. (*In press*).

4. Macha M, Rachagani S, **Pai P**, Gupta S, Lydiatt W, Smith R, Johansson S, Lele S, Kakar S, Ibrahim F, Lee J, Jain M, Batra SK. *MUC4 Regulates Cellular Senescence in Head and Neck Squamous Cell Carcinoma (HNSCC) throughp16/Rb Pathway*. (2014) **Oncogene**. 26; 34(13):1698-708.

5. Macha MA, Seshacharyulu P, Krishn SR, **Pai P**, Rachagani S, Jain M, Batra SK. *MicroRNAs (miRNA) as Biomarker(s) for Prognosis and Diagnosis of Gastrointestinal (GI) Cancers.* (2014) **Curr Pharm Des** 20(33):5287-97.

6. Macha MA, Rachagani S, Gupta S, **Pai P**, Ponnusamy MP, Jain M. , Batra SK. *Guggulsterone decreases proliferation and metastatic behavior of pancreatic cancer cells by modulating JAK/STAT and Src/FAK signaling.*(2013) **Cancer Lett.**; 341(2):166-77.

7. Pai P, Rachagani S, Are C, Batra SK. *Prospects of miRNA-based therapy for pancreatic cancer.* (2013) **Curr Drug Targets;** 14(10):1101-9.

8. Mukhopadhyay P, Lakshmanan I, Ponnusamy MP, Chakraborty S, Jain M, **Pai P**, Smith LM, Lele SM, Batra SK. *MUC4 overexpression augments cell migration and metastasis through EGFR family proteins in triple negative breast cancer cells* (2013) **PLoS One.**; 8(2):e54455.