University of Nebraska Medical Center
BREAKTHROUGHS FOR LIFE.

University of Nebraska Medical Center
DigitalCommons@UNMC

Theses & Dissertations
Graduate Studies

Spring 5-7-2016

Novel Regulatory Mechanisms and Functions of MUC4 in Pancreatic Cancer

Suhasini Joshi
University of Nebraska Medical Center

Follow this and additional works at: https://digitalcommons.unmc.edu/etd

Part of the Life Sciences Commons

Recommended Citation
Joshi, Suhasini, "Novel Regulatory Mechanisms and Functions of MUC4 in Pancreatic Cancer" (2016). *Theses & Dissertations*. 112.
https://digitalcommons.unmc.edu/etd/112

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@UNMC. It has been accepted for inclusion in Theses & Dissertations by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.

NOVEL REGULATORY MECHANISMS AND FUNCTIONS OF MUC4 IN PANCREATIC CANCER

By

Suhasini Joshi

A DISSERTATION

Presented to the Faculty of

The Graduate College in the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Department of Biochemistry and Molecular Biology

Under the Supervision of Dr. Surinder K. Batra

University of Nebraska Medical Center

Omaha, Nebraska

April, 2016

Novel regulatory mechanisms and functions of MUC4 in Pancreatic cancer

Suhasini Joshi, Ph.D.

University of Nebraska, 2016

Advisor: Surinder K Batra, Ph.D.

Mucins are high molecular weight glycoproteins and have critical functions in protecting epithelial cells from a myriad of cellular stress. However, mucins are expressed aberrantly under cancer conditions that allow tumors to progress and metastasize. Among many mucins, Mucin 4 (MUC4) serves as one of the top-differentially expressed proteins in pancreatic cancer (PC), however, the precise mechanism responsible for its aberrant expression is still not clear. The evolving view of cancer as an energetic and growing ecosystem underlines an intricate interplay between cancer and its microenvironment. In spite of being recognized as one of the most critical oncogenic proteins in PC, MUC4 regulation in terms of micro-environmental stress has not been determined. In my dissertation research, I have investigated the role of PC microenvironment in the regulation of MUC4. From my studies, I have demonstrated that MUC4 stability is significantly reduced due to hypoxia-mediated induction of reactive oxygen species (ROS), which promotes autophagy by inhibiting pAkt/mTORC1 pathway. Hypoxia-mediated degradation of MUC4 provides necessary metabolites to ensure the survival of highly stressed PC cells.

The longstanding model of cancer development involves that presence of cytokines can trigger chronic inflammation and impact tumor development, including PC. In addition to cytokines, bile acids (BA) facilitated chronic inflammation has shown to induce intestinal metaplasia, but their role in PC is still elusive. Elevated levels of BA ($p<0.05$) and its receptor were observed in pre-clinical and clinical serum samples from human and mouse models. Further, their significantly higher levels were also observed

in pancreatic juice obtained from PC patients in comparison to controls, establishing the direct involvement of BA in PC pathobiology. It prompted us to hypothesize that BA have tumor promoting functions in PC. Mechanistically, the tumorigenic functions of BA were explained by BA-mediated upregulation of mRNA expression of MUC4, which, in turn, is primarily dependent on FXR-mediated activation of FAK. Activation of FXR further leads to an increase in the expression of c-Jun that binds to AP-1 motifs present on MUC4 distal promoter region resulting in transcriptional upregulation of MUC4.

In addition to the regulation, I have pinpointed the novel functional roles of MUC4 in determining the fate of receptor tyrosine kinases (RTKs) in PC. Multiple studies have associated MUC4 overexpression with increased stability of RTKs for sustained proliferation; however, no studies have so far highlighted the implicated mechanism. I have demonstrated that the presence of MUC4 leads to increased internalization and recycling of EGFR and HER2 to the plasma membrane compared to MUC4 silenced PC cells. Mechanistically, the impact of MUC4 on RTKs trafficking is associated with its ability to regulate the activity of RAB5A, which is known to catalyze the rate-limiting step in receptor internalization. Lastly, I have detected the presence of MUC4 in pancreatic cancer associated stellate cells (PaSC). This was an unexpected finding given that MUC4 is normally expressed in the epithelial cells. These results indicate towards the involvement of MUC4 expression in determining the activation status of PaSC and provide us an additional strong rationale to therapeutically target MUC4.

Altogether, in my dissertation research, I have elucidated the novel regulatory mechanisms and functions of MUC4 in PC condition.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACKNOWLEDGEMENTS

CHAPTER I: INTRODUCTION 1

CHAPTER IA: Regulation of Mucins by the microenvironmental stress of pancreatic cancer microenvironment 2

1A.1 An outline of mucins 2

1A.2 Types of mucins 6

1A.3 MUC4 and its genomic structure 13

1A.4 PC microenvironment 14

1A.5 Hypoxia in PC 16

1A.6 Hypoxia inducible factors (HIFs) in PC 17

1A.7 Oxidative stress association with PC progression 19

1A.8 Autophagy status in PC condition 20

1A.9 Role of hypoxia in the regulation of autophagy 24

1A.10 Role of oxidative stress in autophagy regulation in PC 25

1A.11 Regulation of mucins by hypoxia in cancer condition 27

1A.12 References 30

CHAPTER IB: Mucins Regulation by Bile acids (BA) in PC condition 42

1B.1 An outline of BA 43

1B.2 BA under cancer condition 44

1B.3 BA receptors and their role in cancer 47

1B.4 Association between BA and mucins regulation 50
1B.5 References 54
CHAPTER IC: Novel mechanism implicated in MUC4-mediated increase in the stability of EGFR-family members in PC 58
1C.1 General introduction of altered mucins expression and localization under disease condition 59
1C.2 Altered localization of MUC and its association with cancer condition 63
1C.3 EGFR family receptors 66
1C.4 General mechanism involved in the regulation of EGFR trafficking 67
1C.5 Role of EGFR family members in PC development and progression 68
1C.6 Modulation of sub-cellular protein trafficking of EGFR family members by mucins 70
1C.7 Summary and Conclusions 74
1C.8 References 74

General Hypothesis and Objectives 80

CHAPTER II: Materials and methods 82
II.1. Cell culture 83
II.2. Procurement of human and murine PDAC samples 83
II.3. Treatment of cells with hypoxia and pharmacological reagents 84
II.4. Knocked down and overexpression experiments 85
II.5. Immunoblotting 86
II.6. Isolation of cytoplasmic and nuclear fractions 86
II.7. Confocal Immunofluorescence microscopy 87
II.8. Immunohistochemical staining 88

II.9. Measurement of florescence to analyze ROS levels and autophagic vacuoles 88
II.10.RT- PCR 89
II.11. Quantitative chromatin immunoprecipitation (ChIP) 89
II.12. Total BA estimation method 90
II.13. Growth inhibition and growth kinetics assay 90
II.14. Anchorage-dependent colony formation assay 91
II.15. Luciferase promoter assay 91
II.16. Plasmids and cloning strategy 91
II.17. Internalization experiment via different approaches 92
II.18 Cell motility assay 93
II.19 GTP-loaded Rab5 pull-down assay 93
II.20. Statistical analysis 94
II.21 References 102

CHAPTER III: Hypoxia-induced oxidative stress promotes MUC4 degradation via autophagy to enhance pancreatic cancer cells survival 104
III.1 Synopsis 106
III.2 Background and rationale 107
III.3 Results
A. MUC4 expression is down-regulated in PC cell lines in response to hypoxia 108
B. Decrease in MUC4 expression under hypoxia is HIF-1α independent 109
C. Decrease in MUC4 expression under hypoxia is ROS-dependent 110
D. Hypoxia-mediated autophagy induction leads to reduced MUC4 stability 111
E. Hypoxia inhibits Akt/mTORC1 pathway to induce autophagy 112
F. MUC4 gives survival advantage to hypoxia-stressed PC cells 114

G. Clinical validation of MUC4 association with oxidative stress and degradation *via* lysosomal pathway 115

III.4 Discussion 116

III.5 References 146

CHAPTER IV: Bile acids-mediated overexpression of MUC4 via FAK-dependent c-Jun activation in pancreatic cancer 151

IV.1 Synopsis 153

IV.2 Background and rationale 154

IV.3 Results

A. BA levels are elevated in serum and pancreatic juice during pancreatic cancer 155

B. BA up-regulate MUC4 expression in PC cells 156

C. BA transcriptionally upregulates MUC4 expression in PC 157

D. BA increase the expression and nuclear localization of c-Jun 159

E. BA mediated increase in FAK activation induced c-Jun expression 160

F. FXR activation is a prerequisite for BA-mediated MUC4 upregulation via src/FAK/c Jun axis 161

G. Clinical association between MUC4 and BA receptor FXR 162

IV.4 Discussion 163

IV.5 References 191

CHAPTER V: Multifaceted role of MUC4 in regulating the trafficking of RTKs in Pancreatic Cancer 195

V.1 Synopsis 196

V.2 Background and rationale 198

V.3 Results 199

A. MUC4 increased the stability of RTKs in PC cells 199

B. MUC4 influences the stability of RTKs by altering their rate of internalization 200

C. MUC4 impacts receptor internalization by altering the expression of Rab5A 201

D. MUC4-mediated transcriptional regulation of Rab5A is CREB dependent 202

E. MUC4-mediated regulation of the expression of EGFR family ligands also determine the fate of receptors 203

F. MUC4 increases the sensitivity of EGF-mediated migration and proliferation by increasing oncogenic signaling 204

V.4 Discussion 205

V.5 References 234

CHAPTER VI: Identification of MUC4 in Pancreatic stellate cells (PaSC) and putative MUC4 alternate promoter 238

VI.1 Synopsis 239

VI.2 Background and rationale 241

VI.3 Results 242

A. Presence of MUC4 mucin on activated cancer associated fibroblast cells. 242

B. Presence of MUC4 determines the activation status of PaSC. 243

C. Suppression of MUC4 reduces the proliferation and migration of PaSC. 243

D. Identification of novel MUC4 promoter in PC cell lines and patients. 244

E. Differential localization of MUC4 in PaSC and PC cells. 245

VI.4 Discussion 246

VI.5 References 265

CHAPTER VII: General Conclusions and Future Directions 267

Other contributions 281

LIST OF FIGURES

Chapter I

Figure 1A.1 Illustration of the various physiological outcomes of aberrant mucin expression under normal and pathological conditions. 3

Figure 1A.2 Representation of the prototype structure of mucins along with the characterized and putative roles of their functional domains. 10

Figure 1C.1 Diagrammatic representation of the intracellular transport of glycoproteins along endocytic and exocytic pathways. 61

Figure 1C.2 Mechanisms of intracellular transport and sorting of MUC1. 64

Chapter II

Figure 2A Strategy to design deletion constructs for MUC4 cytoplasmic domain and their confirmation at protein level. 95

Chapter III

Figure 3.1 MUC4 is negatively regulated by hypoxia in PC cell lines. 120

Figure 3.2 HIF-1α independent mechanisms play predominant role in hypoxia-mediated suppression of MUC4. 122

Figure 3.3 MUC4 expression is negatively regulated by hypoxia induced ROS. 125

Figure 3.4 Hypoxia-mediated ROS production induces autophagy, which leads to reduced MUC4 stability. 129

Figure 3.5 Hypoxia-mediated oxidative stress promotes autophagy by inhibiting pAkt/mTORC1 axis and reduces cell viability. 134

Figure 3.6 Hypoxia-mediated reduction in cell viability is rescued by MUC4 overexpression. 139

Figure 3.7 *In vivo* validation of MUC4 association with oxidative stress and degradation *via* lysosomal pathway. 141

Figure 3.8 Schematic presentation of the summary of the paper. 144

Chapter IV

Figure 4.1 BA are significantly upregulated in PC condition. 168

Figure 4.2 BA are positive regulators of MUC4 expression. 170

Figure 4.3 BA-mediated positive regulation of MUC4 is at transcriptional level. 173

Figure 4.4 BA affects the expression, activation and nuclear translocation of c-Jun, which led to enhanced c-Jun binding on MUC4 distal promoter. 176

Figure 4.5 BA-mediated upregulation of MUC4 is dependent on FAK activation. 180

Figure 4.6 Activation of FXR is required for BA-mediated MUC4 upregulation v*ia* Src/FAK/c-Jun axis. 184

Figure 4.7 Clinical association between MUC4 and FXR in PC tissues. 188

Chapter V

Figure 5.1 *In vitro* and *In vivo* data shows an association between MUC4 and EGFR in PC cells. 210

Figure 5.2 Internalization and recycling experiments revealed that MUC4 significantly impact EGFR trafficking. 214

Figure 5.3 MUC4 regulates the expression and activity of RAB5A. 218

Figure 5.4 MUC4-mediated regulation of CREB activity is leading to RAB5A regulation. 223

Figure 5.5 MUC4 regulates the expression of EGFR ligands. 226

Figure 5.6 MUC4 potentiates EGF-mediated proliferative and migratory potential. 229

Figure 5.7 Schematic diagrams showing that presence of MUC4 determines the fate of EGFR. 232

Chapter VI

Figure 6.1. Identification of MUC4 expression in activated pancreatic stellate cells. 249

Figure 6.2 MUC4 kd has significant impact on the activation status of PaSC. 253

Figure 6.3. MUC4 kd PaSC exhibits negative effects on their proliferative and migratory potential. 256

Figure 6.4. Identification of MUC4 promoter in PaSC cells, PC cell lines and PC tissues. 260

Figure 6.5. Altered MUC4 localization in PaSC and PC cells. 263

LIST OF TABLES

Chapter I

Table 1A.1. Specific expression patterns of different mucins in the human body. 5

Table 1A.2. Different human mucin homologues of mice and their genomic localization. 12

Chapter II

Table 2A. Primary antibodies used in the study for Immunoblotting (IB) and Immunofluorescence (IF) and ChIP purposes. 97

Table 2B. Human primers sequences used for quantitative PCR. 99

Table 2C. CHIP primer sequence for MUC4 distal promoter. 101

ABBREVIATIONS

AP: Alternate promoter

a-SMA: Alpha-smooth muscle actin

α-TS: alpha-tocopherol succinate

BA: Bile Acids

BE: Barrett's oesophageal

CA125: Cancer antigen 125

CA: Cholic acid

CDCA: Chenodeoxycholic acid

cDNA: Complimentary DNA

CE: Cytoplasmic extract

ChIP: Chromatin immunoprecipitation

CHX: cycloheximide

$CoCl_2$: Cobalt chloride

CP: Classical/conventional promoter

CRE: cAMP response elements

CREB: cAMP response element-binding protein

CT: Cytoplasmic tail

CTD: Cytoplasmic tail domain

DCA: Deoxycholic acid

DCFDA: dihydrodichlorofluorescein diacetate

DMEM: Dulbecco’s modified Eagle’s medium

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EGF: Epidermal growth factor-like domain

ERK: Extracellular signal-regulated kinases

FAK: Focal adhesion kinase

FBS: Fetal bovine serum

FXR: Farnesoid-x-receptor

GFAP: Glial fibrillary acidic protein

GI: Gastrointestinal

HIF-1α: Hypoxia inducible factor-1 alpha

H_2O_2: Hydrogen peroxidase

HRE: Hypoxia response element

8-OHG: 8 Hydroxyguanosine

IB: Immunoblotting

IF: Immunoflorescence

IHC: Immunohistochemistry

JNK: c-Jun N-terminal kinase

Kd: Knocked down

KDa: Kilo Dalton

LC3: Microtubule-associated protein 1A/1B-light chain 3

LCA: Lithocholic acid

LOH: Loss of heterozygosity

MAPK: Mitogen activated protein kinase

MDC: Monodansylcadaverine

MFI: Mean florescence intensity

MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal

mRNA: Messenger RNA

MTT: 3-[4,5-dimethylthiozol-2-yl]-2,5diphenyltetrazoliumbromide

MUC: Mucins

MUC4-CD: MUC4 cytoplasmic domain

NAC: N-acetyl-cysteine

NCI: National cancer institute

NE: Nuclear extract

NLS: Nuclear localization signal

ns: non-significant

PaSC: Pancreatic stellate cells

PC: Pancreatic cancer

Pdx1: Pancreatic and duodenal homeobox 1

PDAC: Pancreatic ductal adenocarcinoma

PTEN: Phosphatase and tensin homolog

PTS: Proline threonine serine

q-RT-PCR: Quantitative real-time Polymerase chain reaction

RA Retinoic acid

RAP: Rapamycin

ROS: Reactive oxygen species

RTK: Receptor tyrosine kinases

SCB: Santa Cruz Biotech

SEA: Sea urchin sperm, Enterokinase and Agrin

siRNA: Small interfering RNA

TCA: Taurocholic acid

TGF-α: Transforming growth factor-alpha

TM: Transmembrane

TR: Tandem repeat

VB: Vinblastine

VNTR: Variable number of tandem repeats

vWD: Von Willebrand factor D-like domain

WB: Western blot

WT: Wild-type

ACKNOWLEDGEMENTS

I would like to thank my Ph.D advisor Dr. Surinder K. Batra for his guidance and teaching during my graduate studies. I greatly appreciate him for being so encouraging and persevering with me. His constant encouragement has nurtured my independent thinking as a graduate student. Also, I would like to thank my supervisory committee members Dr. Michael A. Hollingsworth, Dr. Parmender P. Mehta, Dr. Michele Ouellette, and Dr. Kaustubh Datta for their suggestions, criticisms and encouragement throughout my graduate program.

I would like to thank both the past and present members of the Batra Laboratory: Dr. Sushil Kumar, Dr. Moorthy P. Ponnusamy, Dr. Maneesh Jain, Dr. Wade Junker, Dr. Murielle Mimeault, Dr. Imay Lakshmanan, Dr. Satyanarayana Rachagani, Dr. Sukhwinder Kaur, Dr. Parthasarathy, Dr. Maria P. Torres- Gonzalez, Dr. Parama Dey, Dr. Naveet Momi, Dr. Michael Baine, Dr. Joshua Souchek, Dr. Dhanya Haridas, Dr. Muzafar Macha, Dr. Shaktivel, Dr. Prakash Kshirsagar, Dr. Parthasarathy, Dr. Priya Pai, Shivram Ktishn, Dr. Arokia Priyanka Vaz, Dr. Ashish Patel, Dr. Abhijit Aithal, Dr. Shailendra Gautam, Dr. Asif Qazi, Dr. Mohd. Wasim Nasser, Eric Cruz, , Seema Chugh, Kasturi Banerjee, Rahat Jahan, Garima Sharma, Saswati Karmakar, Rama Nimmakayala, Suprit Gupta, Erik Moore and Kavita Mallya. I would like to thank all for their support in scientific discussions, technical assistance, sharing resources and friendship.

I would like to thank Dr. Jerred Garrison (UNMC) for letting us his hypoxia chamber, Dr, Shilpa Buch (UNMC) for allowing us to her instruments, Dr. Prakash Radhakrishnan for providing us antibodies, Dr. Pankaj K. Singh (UNMC) for providing us HIF-1α shRNA construct, and Dr. Michael Hollingsworth for gifting us MUC1 (HMGF2) antibody. I would like to thank UNMC Confocal core, UNMC flow cytometry and the UNMC DNA Sequencing Core Facilities.

I would like to thank everyone at the UNMC, especially the Department of Biochemistry and Molecular Biology for their support and friendly environment to pursue my graduate studies. I would specifically like to thank everyone in the BMB office Amy Dodson, Jennifer Pace, Susan Klima, Jeanette Gardner and Karen Hankins, Coleen and April Busch. I would also like to thank Graduate Studies at UNMC for funding me throughout my graduate program.

Finally, I would like to thank my spiritual mentor for instilling me with lot of positivity and happiness. I would specifically like to thank my wonderful parents for their unconditional love and support throughout my life. I would like to thank my brother Devashish and sisters; Medha and Sangeeta, for being there for me and for always making me happy. I thank my Nephew Mrinal and my brother-in-law for all the laughs that we have shared. I thank my childhood friends; Harpreet, Anika, Thejus and Yagya for their love and support all throughout my life. I would like to thank my roommate, Naaz Al-Shawi for all the immense love and care she has given me in last two years. I thank all my teachers starting from my primary school to date for their constant encouragement and support to make me what I am today. Thank you all so very much.

Dedicated to

my Spiritual mentor &

Ever-supporting Parents

CHAPTER I

INTRODUCTION

CHAPTER IA: Regulation of Mucins by the microenvironmental stress of pancreatic cancer microenvironment

CHAPTER IB: Regulation of MUC4 by the Bile acids (BA) in PC condition

CHAPTER IC: Novel mechanism implicated in MUC4-mediated increase in the stability of EGFR-family members in PC

Parts of this chapter are driven from:

1. **Joshi S**, Kumar S, Bafna S, Rachagani S, Jain M, Wagner KU *et al*. Genetically-Engineered Mucins Mouse Models for Inflammation and Cancer. **Cancer Metastasis Rev.** 2015; 34(4): 593-609.

2. **Joshi S**, Kumar S, Choudhary A, Ponnusamy MP, Batra SK. Altered Mucins (MUC) Trafficking in Benign and Malignant Conditions. **Oncotarget.** 2014; 5(17): 7272-84.

CHAPTER IA: Regulation of Mucins by the microenvironmental stress of pancreatic cancer microenvironment

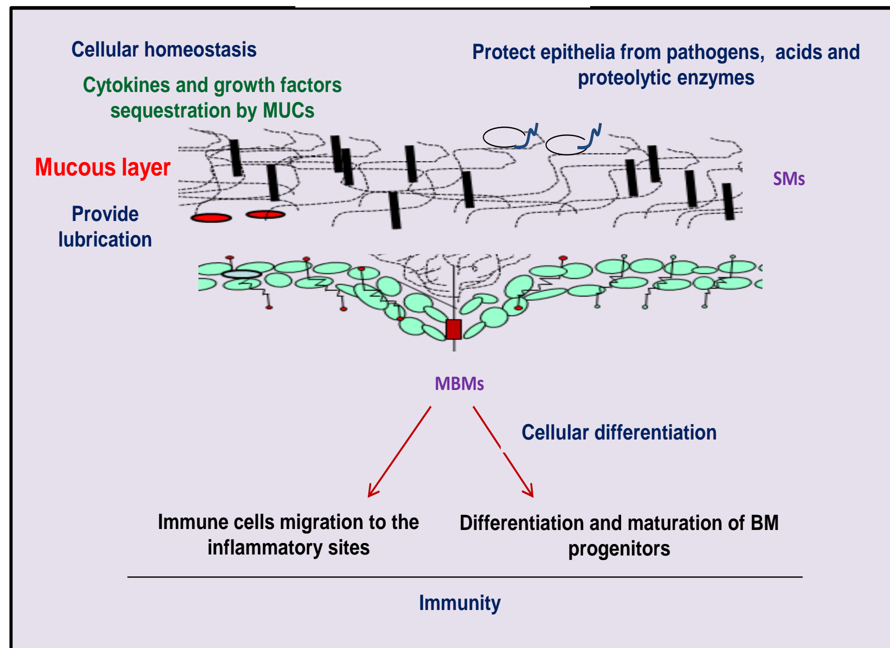
1A.1 An outline of mucins

Mucins comprise a complex family of high molecular weight, membrane-bound or secreted O-glycoproteins which are produced by glandular and ductal epithelial cells. Mucins play critical roles in lubrication and protection of mucosa, renewal and differentiation of the epithelia, cell adhesion, and cellular signaling (**Figure 1.A.1**) (3-5). So far twenty one mucins have been recognized in human; out of them twelve are attached to the cell membrane, whereas the others are secreted by the cells [1]. Multiple studies have shown the diverse and tissue-specific expression profile of mucins. Nonetheless, a single tissue can express number of different mucins (**Table 1A.1)**. Qualitative and quantitative alterations in mucins have been correlated with the inflammatory, pre-neoplastic and neoplastic conditions (6-12). Studies have shown that some of the membrane-spanning mucins could serve as cell-surface receptors and facilitate signal transduction in response to external stimuli that lead to cell proliferation, differentiation, apoptosis, migration, and invasion of cancer cells (**Figure 1A.1**) (3, 13-18). Despite ongoing research efforts, the structure and function of various mucins and mucin-mediated molecular mechanisms under normal and pathological conditions remain poorly understood. Moreover, the awry molecular and cellular mechanisms which lead to the aberrant expression and upregulation of various mucins under different disease conditions have not been completely comprehended (19).

Figures and Figure Legends

Figure 1A.1. Illustration of the various physiological outcomes of aberrant mucin expression under normal and pathological conditions: A) Under normal physiological condition, mucins provide lubrication and protection to the epithelial surface by providing a physical barrier from a hostile environment. Mucins shield the epithelium against the action of various pathogens ( ), enzymes, gastric and bile acids.(20). Mucins are involved in the cellular differentiation of epithelial and immune cells. The expression of mucins in BM progenitors and mature immune cells are involved in hematopoiesis. **B)** Under pathological conditions, mucins are aberrantly expressed and undergo differential post-translational modifications. The mucous layer sequesters many molecules involved in inflammation, cellular migration and healing processes [1]. Mucins help transformed cells to avoid immune surveillance by masking epitopes of tumor antigens on the cell surface. Loss of apical-basolateral polarity allows interaction between membrane bound mucins (MBMs) and growth factor receptors such as receptor tyrosine kinases ( ), leading to sustained proliferative signaling cascades. Furthermore, the overexpression of mucins promotes cells motility, invasiveness and induces resistance to chemotherapeutic agents. Interestingly, the aberrant expression of secretory mucins (SMs) occasionally facilitates pathogenic infection, though the exact mechanism is still not understood.

Figure 1A.1



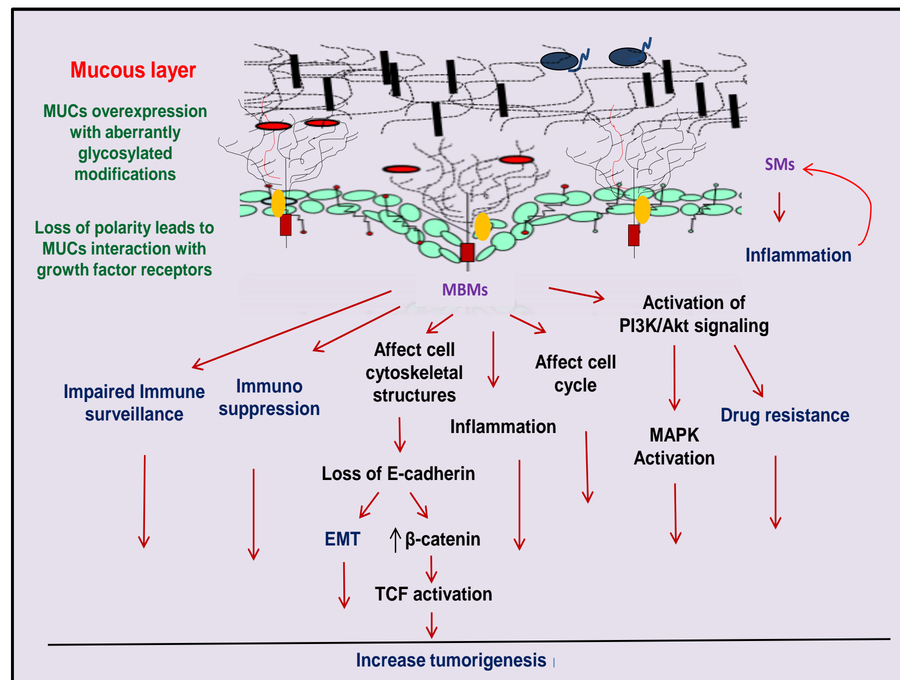


Table 1A.1 Specific expression patterns of different mucins in the human body

Mucin	**Normal Expression Pattern** (3, 5, 12, 21)
MUC1	Expressed in the epithelial surfaces of the respiratory, female reproductive and gastrointestinal tracts as well as in the middle ear, salivary and mammary glands.
MUC2	Expressed in the intestinal and colonic goblet cells.
MUC3	MUC3 is the product of two genes, MUC3A and MUC3B that are both present in the gastrointestinal epithelium.
MUC4	Mainly expressed by the epithelial surfaces of the eye, oral cavity, middle ear, lachrymal glands, salivary glands, mammary gland, prostate gland, stomach, colon, lung, trachea, and female reproductive tract.
MUC5	MUC5 is the product of two genes, MUC5AC and MUC5B. MUC5AC is primarily expressed in the tracheobronchial goblet cells and in the gastric epithelial cells, whereas MUC5B is present in the salivary, tracheobronchial and esophageal mucous glands as well as in the pancreatobiliary and endocervical epithelial cells.
MUC6	Detected in the gastric and duodenal mucous glands, pancreatobiliary and endocervical epithelial cells.
MUC7	Expressed in the oral cavity epithelial cells, minor salivary gland, and possibly in the respiratory tract. Its expression is also detected in the pancreas and bladder.
MUC8	Expressed in the airway and middle ear epithelial cells and male and female reproductive tracts.
MUC10	The expression pattern of MUC10 has not been determined to date.
MUC11	The MUC11 sequence is part of the very large VNTR domain of MUC12 and may represent a differential splice variant, which is normally expressed in the colon and stomach. Its expression has also been shown in the middle ear and lung epithelium.
MUC12	Normally expressed by the stomach and colon.
MUC13	Highly expressed in the epithelium of the gastrointestinal and respiratory tracts.
MUC15	Expressed in the lung, mammary gland, hematopoietic tissues, gonads, and gastrointestinal tract.
MUC16	Expressed in the ocular surface, respiratory tract, and female reproductive tract epithelia.
MUC17	Expressed in the gastrointestinal tract with the highest expression in the duodenum and conjunctival epithelium.
MUC19	Mainly expressed in the mucosal cells of major salivary glands and the epithelial cells from corneal, conjunctival, lacrimal gland, middle ear and trachea.
MUC20	Highly expressed in the kidneys and moderately in the placenta, colon, lung, prostate, and liver.
MUC21	It is a novel transmembrane mucin and normally expressed in the lung, large intestine, thymus, and testis.

1A.2 Types of mucins

Mucins consist of multiple domains (**Figure 1A.2**): Sperm protein enterokinase, and the agrin (SEA) domain involved in protein interactions; epidermal growth factor (EGF)-like domain that can act as a ligand; cysteine-rich dimerization or D domain (including D1, D2, D', D3 similar to vWD domains) for oligomerization; variable number of tandem repeats (VNTR or TRs) rich in serine (Ser), threonine (Thr), proline (Pro) (collectively known as S/T/P) for O-linked glycosylation; the hydrophobic transmembrane (TM) domain for cell surface localization and cytoplasmic tail (CT) to facilitate signal transduction (22, 23).

1A.2.1 Membrane-bound mucins

Muc1 was the first murine mucin gene identified and characterized (24). The human *MUC1* gene and its murine ortholog is 87% identical in the non-TR domains and 74% in the promoter regions. The VNTR region of human MUC1 consists of 20 amino acid repeats, while that of mouse Muc1 has 20 to 21 amino acids each (25). The maximum similarity between Muc1 and MUC1 exist in their TM and cytoplasmic domains. The tissue-specific expression pattern of the mouse Muc1 is also very similar to that of its human counterpart **(Table 1A.2).** Similarities in the sequence and expression pattern of human *MUC1* and murine *Muc1* are indicative of their similarities in function(s), interacting partners, mode of internalization, sub-cellular localization and routing to the plasma membrane during their recycling or after their synthesis.

Muc4, like its human ortholog, is encoded by 25 exons (26). It consists of at least 20 TRs of 124-126 amino acids each, whereas human MUC4 has 146-500 repeats of 16 amino acid residues. The Ser/Thr region located upstream of TRs in murine Muc4 is significantly different and much smaller in size (63 amino acids) as compared to human sequence (951 amino acids) (26). Interestingly, 12 potential N-glycosylation sites, which

are downstream of TR region, are perfectly conserved in *Muc4* and *MUC4* and both orthologs exhibit similar expression patterns (26).

Two large exons of mouse *Muc16* at the N terminal region have sequence homology to exons 1 and 3 of human *MUC16*. Murine Muc16 possesses only one SEA domain in its extracellular (EC) region, whereas the number of SEA domains in the EC region of human MUC16 goes upto 60. Muc16 also shares the similar characteristic repeat structure of human MUC16 along with 66% homology in their C-terminus (27). The overall expression pattern of Muc16 and MUC16 is similar (**Table 1A.2**). Both of them are expressed by the ovarian surface epithelial cells, though their cellular localization is different. Human MUC16 is present on the cell surface and soluble fraction due to its shedding from the cell membrane, whereas murine Muc16 has shown to be secreted by MOVCAR ovarian cells (28).

Other membrane-bound murine mucins including Muc13, Muc15, Muc3, Muc20, and Muc21 are either partially characterized or have not been characterized yet. The C-terminus of Muc13 shows 52% identity to the human MUC13 ortholog. However, the N-terminus of the Muc13 mucins domain shows a significant divergence from the human MUC13, as the murine form has a nearly perfect repeat structure in contrast to the human form which retains many degenerate repeats (29, 30). The carboxyl terminal of MUC17 was found to be 59.6% similar to murine Muc3, while there is only 46.4% amino acid sequence similarity between murine and human MUC3. MUC17 has 52% similarity with the first EGF domain and 63.5% similarity with the second EGF domain of Muc3. Altogether, there is greater similarity between Muc3 and MUC17 compared to Muc3 and MUC3, suggesting that MUC17 is the ortholog of Muc3 (31). Comparison of the amino acid sequences of human and mouse *Muc20* showed 48% overall similarity (32). Both

mucins comprise several hydrophobic domains and three mucin-like repeats of 18 amino acid residues in their N-terminal regions, and are expressed predominantly in kidney.

1A.2.2 Gel-forming mucins

Gel-forming mucins are the main components of mucus and consist of multiple "cysteine-rich" vWF C and vWF D domains in the flanking region of the mucin-like Thr/Ser-rich repeats and C-terminal cystine knot-like domain (CTCK) (33), which allow them to oligomerize by forming intermolecular disulfide bonds. Currently, five gel-forming murine mucins have been recognized; Muc2, Muc5ac, Muc5b, Muc6 and Muc19. Interestingly, four of these genes (*Muc2, Muc5ac, Muc5b* and *Muc6)* are clustered on chromosome 7F5 (34), a region that exhibits synteny with the human chromosome 11p15 (35). The order of clustering of secretory mucin genes is Muc6–Muc2–Muc5ac–Muc5b, which is conserved in both human and mouse (35).

Muc2 forms the basic framework for the formation of an intra-luminal mucus gel of various gastrointestinal (GI) organs (36). Apart from their 75% homology at the N-terminus, mouse and human *MUC2* promoter regions also exhibit a strong sequence similarity which might subject them to similar transcriptional regulation (37). Muc2, like its human counterpart, is predominantly expressed in the colon, to a lesser extent in the small intestine and undetectable in the stomach (38).

The TR of Muc5ac contains a 16 amino acid sequence, whereas the human MUC5AC has only 8 amino acid residues per repeat (25, 39). The TR domain of Muc5ac is followed by a 133 amino acid cysteine-rich non-repetitive region (CRRI), a 63-residue non-repetitive Ser/Thr-rich domain and a second cysteine rich region (CRRII) which share around 81% and 76% similarity, respectively (40). Despite the lack of sequence

similarity between the TR units of the murine and human MUC5AC, their non-repetitive regions are nearly identical.

Alignment of the *Muc5b* gene with its human orthologue indicated few common features. Overall, there is 43% identity between the murine Muc5b and human MUC5B which is predominantly contributed by N- and C-terminal regions which are 64% and 62% similar, respectively (41). However, the expression pattern of *Muc5b* does not match the human *MUC5B* as the murine form is principally expressed in the laryngeal mucous glands and at a low level in the stomach and duodenum, whereas the *MUC5B* gene is expressed in many tissues including the airway, gall bladder, and tongue (41).

The mouse *Muc19* gene is located on chromosome 15, which is homologous to human *MUC19* on chromosome 12 (33). Like other gel-forming mucins, Muc19 also has vWD, vWC and CTCK domains (33). Paired analysis of mouse Muc19 and human MUC19 has shown 27% homology (42), mainly at the C-terminus and the putative N-terminus of the peptide sequences, whereas the central repetitive regions did not show any homology (33). Similar to the human MUC19, Muc19 is predominantly expressed in the salivary glands.

The murine *Muc6* is composed of 33 exons and comparative analysis suggested that the human and mouse Muc6 lack both the cysteine-rich domains and the cysteine-rich subdomains which are frequently found in the S/T/P-rich regions of other human and mouse secretory mucins (MUC2, 5AC, and 5B). The absence of these cysteine-rich domains and sub-domains possibly make them resistant to proteolytic degradation (43) and could be the reason for their high expression in the stomach in both humans and mice. In addition to the stomach, murine Muc6 also exhibits high expression in the duodenum, whereas it is expressed at low levels in the salivary glands (34).

Figure 1A.2. Representation of the prototype structure of mucins along with the characterized and putative roles of their functional domains: MBMs and SMs have a TR domain with variable numbers and lengths of the repeats. They are predominantly get O-glycosylated and separated by unique sequences. They also have few N-glycosylation sites with varying localization with different mucins. Most of the MBMs possess SEA domains with a potential cleavage site (G/SVVV), except MUC4 where GDPH (also present in MUC2 and MUC5ac secretory mucins) is considered to be a putative site for cleavage. Mucins have varying lengths of cytoplasmic tails, (MUC4 CT is shortest with 22 amino acids) which are believed to facilitate signal transduction due to the presence of potential phosphorylation sites such as Ser, Thr and Tyr residues. Other domains present in mucins include EGF-like motifs, nidogen and adhesion-associated NIDO and AMOP, and vWD domains. SMs are rich in cys-rich domains (D1, D2, D3 and D4), which are similar to the D domains of the vWD factor and flank the TR region. These domains are important for disulfide cross-linking to allow oligomerization between the mucin molecules required for gel-forming network.

Figure 1A.2

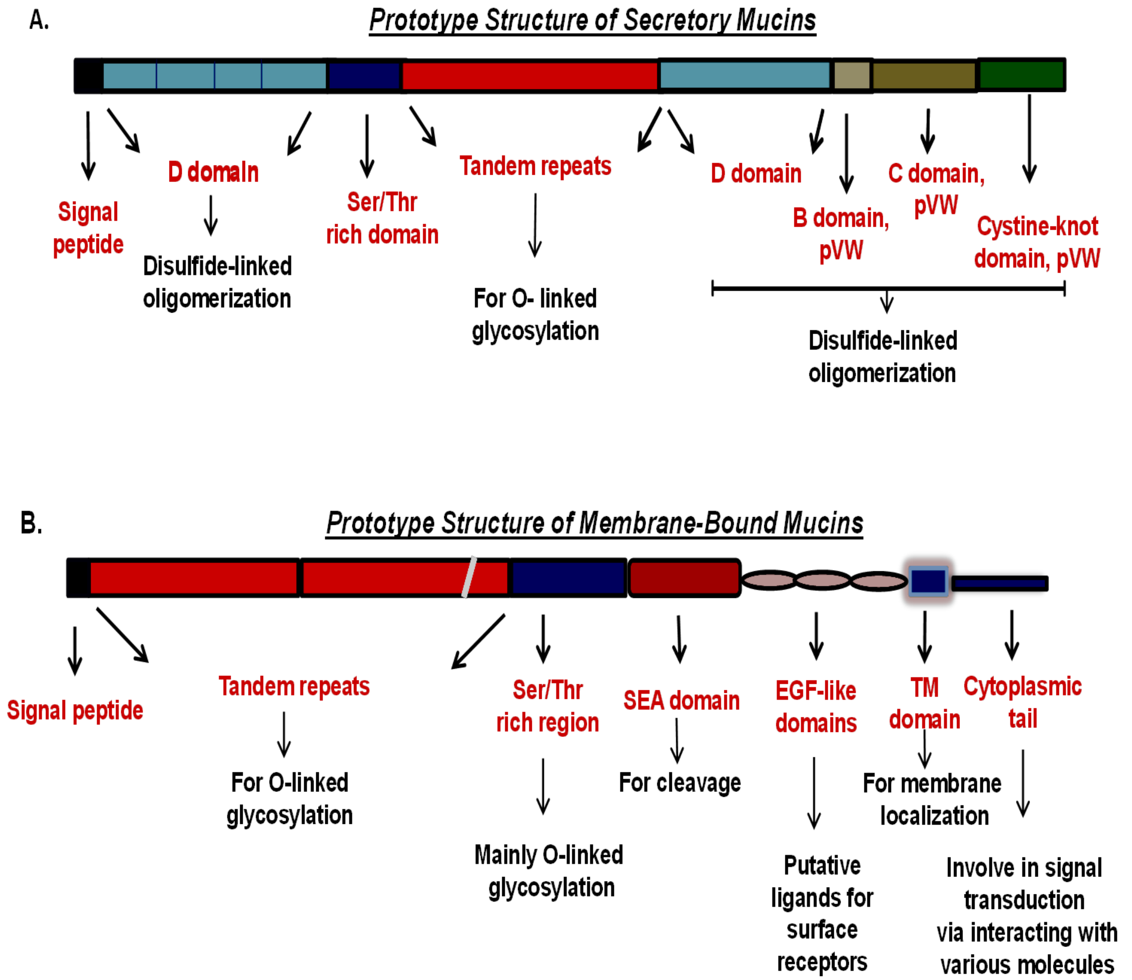


Table 1A.2. Different human mucin homologues of mice and their genomic localization

Human Mucins	Type	Chromosomal Location in Human	Mouse Homologue of Human	Chromosomal Location in Mice
MUC1	TM	1q21	Muc1	3F1
MUC2	Gel	11p15	Muc2	7F5
MUC3A	TM	7q22	Muc3*	5G2
MUC3B	TM	7q22		
MUC4	TM	3q29	Muc4	16B3
MUC5AC	Gel	11p15	Muc5ac	7F5
MUC5B	Gel	11p15	Muc5b	7F5
MUC6	Gel	11p15.5 - p15.4	Muc6	7F5
MUC7	Soluble	4q13–q21	Muc7	NP
MUC8	Gel	12q24.3	Muc8	NP
MUC10	NP	NP	Muc10	5qE1+
MUC11	TM	7q22	Muc11	NP
MUC12	TM	7q22	Muc12	NP
MUC13	TM	3q13.3	Muc13	16B3
MUC15	TM	11p14.3	Muc15	2E3
MUC16	TM	19p13.2	Muc16	9A3
MUC17	TM	7q22	Muc3*	5G2
MUC19	Gel	12q12	Muc19	15E3
MUC20	TM	3q29	Muc20	16B3
MUC21	TM	6p21	Muc21	17B1

Abbreviations: TM, transmembrane; NP, Not present

(* Muc3 is 46.4% and 59.6% similar to human MUC3 and MUC17, respectively [36, 37]. Therefore, murine Muc3 is considered as true ortholog of human MUC17)

1A.3 MUC4 and its genomic structure

In our laboratory, we have extensively studied human *MUC4.* The first partial cDNA of MUC4 was obtained from a human tracheobronchial library (44), where it was found to be localized on chromosome 3q29 (45). The 5′-region of the MUC4 gene is characterized by an extremely lengthy exon-2 which is mainly comprised of 48-bp minimal unit repeated in tandem and encodes for a large Ser/Thr-rich domain (46). The tandem repeat could vary from 7 to 19 kb and gives rise to variable number of tandem repeat polymorphism (46). On the other hand, the 3′-end region of MUC4 is primarily made up of two EGF-like domains, a transmembrane domain, and a short cytoplasmic tail (47). Due to the presence of 26 exons having number of repetitive sequences, MUC4 extensively generates many splice variants which give rise to a family of putative secreted and membrane-associated MUC4 isoforms.

In situ hybridization studies have detected MUC4 expression on various normal tissues such as trachea, lung, stomach, colon, uterus, and prostate, whereas normal pancreas, gall bladder, biliary epithelial cells, liver, or intrahepatic bile ducts were negative for MUC4 expression (48-50). MUC4 expression appears very early during the development of the primitive gut (6.5 weeks of gestation) (51). Expression of MUC4 has shown to be developmentally regulated in the pulmonary and GI segments, and was associated with cell and tissue differentiation. Remarkably, aberrant expression of MUC4 has been noticed in multiple human epithelial cancers such as lung and pancreatic carcinomas (5, 52). Silencing of MUC4 expression led to noticeable decrease in the proliferation, migration and chemo-resistance of PC cells, points out an important role for MUC4 in human tumor biology (18). Therefore, understanding of the underlying molecular mechanisms responsible for the dysregulation of MUC4 is necessary to understand its precise role and contribution during carcinogenesis. Studies have highlighted the importance of soluble and insoluble factors in the regulation of MUC4

expression. For instance, Gollub *et al.* have shown that MUC4 expression is induced at the transcriptional level upon estrogen and dexamethasone treatment in the endometrial Ishikawa epithelial cell line. RA and all-trans-RA have also showed to induce MUC4 at the transcriptional level in PC cell lines (53). However, the exact reason that leads to an aberrant expression of MUC4 in pancreatic ductal adenocarcinoma (PDAC) is still unknown.

Earlier studies have revealed that rat Muc4 has a TATA less promoter with 2.4 kb of size (54, 55). On the other hand, functional studies of the 5′-flanking region of MUC4 in human PC cells have demonstrated that MUC4 transcription is regulated by two regulatory regions (−219/−145 and −2781/−2572) (55). This led to the characterization of a classical TATA box flanked by an extremely long 5′-UTR which is generally referred as a distal promoter, and the 3′-end of the 5′-UTR is characterized by a GC-rich region that serves as a second transcription unit and generally called as proximal promoter (56). Due to the presence of two promoters with numerous binding sites for transcription factors which gets activated in response to growth factor stimulation, MUC4 regulation is somewhat complicated.

1A.4 PC microenvironment

The tumor microenvironment is the environment at the cellular and acellular level in which cancer cells either interact with each other (homotypic interactions), different cell types (heterotypic interactions) and with the extracellular matrix (ECM) (57). This interaction is highly critical for the sustained tumor development and growth. Under normal and healthy conditions, extracellular signals play a critical role by tightly regulating the growth and differentiation programs of epithelial cells. However, defects in such signaling pathways may circumvent the normal pathway of epithelial differentiation and drives the cells towards malignant transformation (57, 58). As repeatedly mentioned in multiple scientific reports, the PC microenvironment is extremely complex and

consists of components of the ECM, connective tissues, stromal cells, and polypeptide growth factors. The ECM itself is composed of complex components of fibronectins, laminins, collagens, glycoaminoglycans and proteoglycans (59). In this microenvironment, epithelial cancer cells do not only interact with each other, but also interact with mesenchymal cells (which includes, cancer associated fibroblasts and stellate cells) and the ECM. These interactions are quite specific. Cell-cell interactions are mediated by specific cell-cell adhesion molecules, while cell-matrix interactions are mediated by specific integrin receptors for each of the major components of the ECM. It has long been recognized that changes in the microenvironment accompany the transformation process. This is often indicated by increased activation of cancer associated fibroblasts (CAFs) and pancreatic stellate cells (PaSCs), which in turn is accompanied by increased proliferation, aberrant expression or overexpression of proteins, increased migration and extensive ECM remodeling, particularly in areas where cancer cells are found. The tumor stroma, in many aspects, resembles the processes of wound healing and inflammatory response.

Despite of the presence of tremendous amount of literature regarding cellular and acellular component of PC tumor microenvironment, there are limited studies which has extensively concentrated to link microenviromental stress, such as hypoxia, serum starvation and oxidative stress, with PC aggressiveness. Increasing evidence strongly emphasizes that hypoxia exerts profound impact on the development and advancement of the tumor microenvironment which in turn controls the differentiation of tumor and stromal cells (60). In detail, tumor cells and their microenvironment reciprocally regulate each other. In the following chapters and section, I am going to present the background information in pertinent to the following chapters where I have elucidated the novel regulatory mechanisms which are involved in the aberrant overexpression of MUC4 mucin.

1A.5 Hypoxia in PC

Under cancerous condition, imbalance between cellular supply and consumption of oxygen leads to depletion of oxygen (O_2), a condition known as hypoxia (61). Ambient air is approximately 21% O_2 (or 150 mmHg), however, most human organs are exposed to 2% to 9% of O_2 (average, 40 mmHg) (62) . The levels of oxygen varies among organs, however, most experimental studies consider ≤ 2% O_2 as hypoxia (62). Hypoxia has been acknowledged as one of the distinctive and common feature observed for locally advanced solid cancers (63). In multiple cancer models, hypoxia has been associated with poor clinical outcomes; including, cancer cell invasion and metastasis. In order to adjust with the hypoxic microenvironment, tumor cells have to alter the expression of multiple genes, which encodes for metabolic enzymes, vasoactive, and proangiogenic molecules and so on. Tumor hypoxia activates multiple signal transduction pathways, which produce significant impact on the tumor biology by promoting metastasis, angiogenesis and tumor progression (64, 65).

Koong and colleagues were the first one who demonstrated that PC are notoriously hypoxic in nature, where they directly measured intratumoral O_2 levels in patients undergoing a Whipple procedure (n=7) (66). It was revealed that areas of pancreatic carcinoma had a median pO_2 level of 0–5.3 mmHg. Contrarily, tumor adjacent areas consisting normal pancreatic tissue had median tissue pO_2 levels of 24–92.7 mmHg (66). Such momentous reduced tissue oxygenation has also been noticed in chronic pancreatitis, a condition that usually co-exists in PC patients (67). This is already known that pancreatic tumors are highly resistant to common therapies (68, 69). It could be attributed to the presence of low oxygenation and the extraordinary ability of PC cells to withstand and grow aggressively in highly stressed microenvironment (5, 70). These reasons are further supported by the studies which have directly associated hypoxic

tumor regions with the resistance against chemo- and radiation therapy, both of which are clinical hallmarks of human PC (71, 72). Besides therapy resistance, recent studies have implicated tumor hypoxia with a variety of growth-modulating effects including tumor metastasis. Altogether, hypoxia, a condition when cancer cells are deprived of oxygen, has profound effect on its overall growth, development and therapy resistance.

1A.6 Hypoxia inducible factors (HIFs) in PC

A crucial component required for the induction of hypoxia-regulated genes is the hypoxia inducible factor-1 (HIF-1) complex, which is composed of HIF-1α and HIF-1β subunits (73, 74). The HIF-1β subunit (also known as aryl hydrocarbon nuclear translocator, ARNT) is constitutively expressed, whereas the HIF-1α subunit is accumulated only under hypoxic exposure by evading proteasome-mediated degradation (74). Under hypoxic conditions, the active HIF-1α/β heterodimer is translocated to the nucleus and binds to a specific cis-acting regulatory sequence referred to as the hypoxia response element (HRE) in target genes, which leads to transcriptional activation of their target genes. HIF-1α serves as a master regulator of several hypoxia-inducible genes, including vascular endothelial growth factor (VEGF), glucose transporter-1 (Glut-1) and survivin (75). VEGF plays a central role in the tumor neo-angiogenesis which is a crucial step in tumor growth and progression. Glut-1 mediates cellular glucose uptake, and thus facilitates anaerobic glycolysis, a prerequisite for cancer cell proliferation under hypoxic microenvironment. Survivin, a member of the apoptosis inhibitor protein family, is uniquely expressed in various kinds of human malignances but not in normal adult cells and its over-expression in cancerous condition has been associated with reduced cell death (75). In PDAC condition, immunohistochemistry performed in 58 PDACs and 20 normal pancreatic tissue samples by Sun HC *et al*, have clearly demonstrated expression of HIF-1α, VEGF, Glut-1 and survivin in 70.7%, 77.6%, 67.2% and 84.5% of

the patients with PDACs, respectively, which is substantially higher than in the normal controls (75).

Under chronic hypoxia, stabilized HIF-1 complex binds to HRE2 in 5'flanking region of miR-191 in PC cells, which is followed by increased transcription of miR-191 (76). Expression of miR-191 was significantly higher in pancreatic tumor tissues, compared to normal pancreas (76). The overexpression of miR-191 was associated with increased tumor size, pTNM stage, lymph node metastasis, perineural invasion and poor prognosis of the disease (76). Similar to miR-191, increased HIF-1α expression also indicates increased lymph node metastasis and a tendency of larger tumor size as well as advanced TNM stage, irrespective of its expression pattern (75). Multiple studies have shown that 50-70% of the investigated PDA samples have positive staining for HIF-1α molecule. HIF-1α expression has shown primarily two patterns depending upon the underlying source. For example, lack of oxygen leads to necrosis where HIF-1α exhibits prominent focal positive staining, whereas in an oxygen-independent pathway, HIF-1α has strong diffused nuclear and/or cytoplasm staining in the tumor cells (75).

Due to the implicated role of HIF-1 in increasing the expression of genes required for increased tumor growth and metastasis, disruption of the HIF-1 pathway could be an effective strategy to treat PC. In a study by Chen *et al*, it has been demonstrated that expression of dominant-negative HIF-1α in PC cells leads to reduction in tumorigenicity due to interruption of glucose metabolism, which made cancer cells sensitive to apoptosis and growth inhibition upon hypoxic condition (77). Taken together, it can be concluded that PC patients may noticeably receive clinical advantage from treatments targeting HIF-1α, and a routine assessment of these proteins by IHC may expand our understanding into improved treatment after surgery.

1A.7 Oxidative stress association with PC progression

Around 90% of PC patients have K-ras mutations, implying their critical role in the molecular pathogenesis (78), which has been associated with ROS homeostasis under cancer condition. Vaquero *et al.* have recently shown that ROS have pro-survival and anti-apoptotic functions in PC (79). They showed that ROS generation by the activation of nonmitochondrial NAD(P)H oxidase due to the stimulation of growth factors facilitates survival of PC cells, whereas suppression of ROS production leads to PC cell death. Therefore, the pro-survival effect of ROS may be an important mechanism which is utilized by PC cells to evade therapy response. Further, Santillo *et al.* have shown that in K-ras–transformed mouse fibroblasts, there is elevated ROS levels which leads to the activation of signal transduction pathways (80). Not only k-ras, transfection of viral H-ras oncogene in mouse fibroblasts also led to increased synthesis of superoxide (O_2^-), where they execute their cell proliferating functions by plausibly acting as a second messenger molecule. In addition to fibroblasts, ras-transformed keratinocytes also demonstrated increased generation of O_2^-, and this augmented production was effectively paused when an adenovirus construct containing the cDNA of the antioxidant protein superoxide dismutase was expressed (81). Contrarily, most of the studies have encouraged the usage and clinical testing of ROS-inducing small molecules for the treatment of PC. For instance, in NAD(P)H:quinone oxidoreductase (NQO1)-overexpressing pancreatic tumors, administration of ROS inducer β-lapachone leads to significant cytotoxicity by tempering PARP, NAD+/ATP levels. It is subsequently followed by increased single-stranded DNA breaks, and results in necrosis (82, 83). Another small molecule pro-oxidant, imexon, which is an aziridine-derived iminopyrrolidone, has shown to induce apoptosis in multiple PC cell lines by increasing ROS levels and facilitating cell cycle arrest at G2 phase (84). Considering its effective anti-cancer

properties, this drug has been used for phase I and II clinical studies for PC patients (85).

In a recent study, Dhillon and colleagues have investigated the *in vitro* and *in vivo* effects of the ROS-inducer piperlongumine (PPLGM) on PC cell death (86). PPLGM is an bioactive alkaloid found in the fruits of long pepper plants and have potent growth-inhibitory properties in a variety of cancer cell lines and animal models (86). Though it exhibits cytotoxic effects on tumor cells, PPLGM is not at all toxic to its normal counterparts. In this study, authors have evaluated the therapeutic potential of PPLGM for PC treatment. Similar to other cancer types, PPLGM exhibited growth inhibitory effects on PC cells by inducing ROS levels and generating DNA damage (86). The effect of PPLGM was found to be ROS-dependent because its effect on cytotoxicity and DNA damage was reversed, when PC cells were concomitantly treated with antioxidant. These anticancer effects of PPLGM were confirmed in a xenograft mouse model of human PC, where they observed significant reduction in tumor size. IHC analysis further revealed that PPLGM-treated animals had reduced Ki-67 and increased 8-OHdG expression. Taken together, it can be proposed that further studies evaluating the anti-tumor effects of ROS-inducer on normal and K-ras mutant pancreatic tumors in combination with chemotherapy should be encouraged and executed.

1A.8 Autophagy status in PC condition

Autophagy is a highly regulated destructive cellular mechanism in which autophagosomes are fused with lysosomes that lyse or recycle the contents (87). Therefore, this catabolic process provides building blocks for use within the cell and dynamically control and maintain cell function. Particularly, stress conditions, such as starvation, are known to induce autophagy which leads to energy redistribution to

sustain cell survival; however, if the attempts to sustain viability are failed, autophagy facilitates cell death. The dual role of autophagy is also applied to tumorigenesis because it can serves both as a guardian for cancer initiation and as a stimulator for cancer growth, by providing energy for advanced malignancies (88). For instance, loss of Atg5 predisposes mice to develop benign liver adenomas; however, these lesions do not headway to form malignant tumors (89). Supportively, upon lung specific concomitant loss of Atg5 or Atg7 in conjunction with oncogenic Kras, mice developed increased benign lesions (adenomas) which were again failed to progress to malignancy (90, 91).

In PC, the role of autophagy has been examined by different research groups and the results are quite interesting. Mukubou and colleagues have investigated the *in vivo* and *in vitro* effects of gemcitabine and ionizing radiation (IR) upon modulation of autophagy (92). Treatment with gemcitabine and/or IR had significant inhibitory effects on cellular viability and tumor growth, but addition of autophagy inhibitors ostensibly increased the dose requirement of gemcitabine in order to suppress cell viability, suggesting that autophagy sensitize PC cells for gemcitabine-mediated cell death (92). However, most of the available literature has associated autophagy with increased survival, tumorigenicity and aggressiveness of PDAC. Yang and colleagues, for instance, have suggested presence of constitutively instigated autophagic pathway in PC neoplasm (93). They assessed the autophagic status using both static and flux measurements in different cells. Upon comparison, it was revealed that all PDAC cell lines showed a significant increase in autophagic activity as well as flux compared to non-transformed pancreatic cells, breast and lung cancer cell lines (93). To directly relate the induction of autophagy with the survival of PC cells, ATG5 was silenced using targeted small interfering RNA (siRNA), which led to 50% reduction in the growth of PDAC cell lines compared to the other cancer cell lines. Altogether, their results

provided evidence that activation of autophagy is required for the maintenance of PDAC (93). Further, clinicopathological study in a cohort of 71 resected PDAC patients revealed an association between activated autophagy (high LC3 protein expression) and poor outcome of PDAC patients (94).

Increased basal levels of autophagy has been observed in human cancer cell lines bearing activating mutations in H-ras or K-ras, even in a nutrient abundant microenvironment, suggesting that autophagy maintains tumor cell survival. Therefore, by subduing the expression of proteins required for autophagy, the growth of cancer cells was significantly suppressed, indicating that Ras-driven cancers are addicted to autophagy, and blocking autophagy in such tumors could be an effective treatment approach (95). Interestingly, xenografts obtained from PC patients who contain Kras mutation were extraordinarily susceptible for the treatment of anti-autophagy based therapies including, chloroquinone derivatives (96). In a recent report, Perera *et al.* have gauged both autophagosomes and lysosomes using IHC and found them significantly larger in PDAC cell lines than in controls (97, 98). Additionally, high resolution transmission electron microscopy showed that not only the size, numbers of lysosomes are also higher in PDAC samples than in normal pancreatic tissues. Mechanistically, the authors have associated this increase with an elevated expression of genes involved in lysosomal biogenesis (98). It is already known in the literature that MiT/TFE family of transcription factors play important role in increased lysosome and autophagosome (99). After melanoma and kidney cancer, PDAC had the third highest expression levels for MiT/TFE factors; particularly TFE3, TFEB and MITF. Suppression of these MiT/TFE factors in PDAC cells led to significant change in lysosomal morphology and functionality. Particularly, metabolite profiling in TFE3 kd PC cells revealed a substantial reduction in the cellular levels of amino acids and their breakdown products due to impaired uptake of extracellular factors through macro-pinocytosis (98). In a parallel

experiment, PDAC cells overexpressing TFE3 or MITF exhibited increased clonogenic growth compared with controls when cultured in low amino acid, indicating that induction of autophagy make PC cells to survive better under nutrient deprived conditions. Moreover, MITF overexpression considerably increased the tumorigenicity of mouse pancreatic epithelial cells expressing KRAS-G12D when orthotopically injected into recipient mice.

The pro-survival role of autophagy in PDAC progression has been further substantiated by using highly defined genetically engineered mouse models (GEMMs) (100). For example, pancreas-specific depletion of Atg5 or Atg7 in the presence of constitutively active Kras, significantly abrogated the progression of pre-malignant lesions to invasive cancer; however, due to the embryonic homozygous p53 deletion in the pancreas, these observation might not be physiologically relevant (100). Because p53 alterations are mainly loss of heterozygous (LOH) in human PC condition, these conclusions derived from p53 homozygous model might not be representative of human tumors. Interestingly, Atg5 deletion impairs the progression of pre-malignant PanIN to invasive PDAC in the setting of heterozygous deletion of p53 (101). Altogether, it led us to conclude that in the physiological setting of p53 loss during tumor progression, autophagy seems to be required for optimal development of PC. The intricate and highly complexed relationship between autophagy and p53 is of great importance and required further studies. To increase the clinical relevance of the study, authors did acute inhibition of autophagy by chloroquinone (CQ)-treatment or RNAi approaches, and observed significant growth inhibition of murine PDAC cell lines with various p53 alterations, which is quite consistent with the prior data using p53 harboring human PDAC cell lines. Upon treating of patient derived PDAC xenografts that harbor p53 mutations with hydrochloroquinone (HCQ), significant attenuation in the growth was observed (101). Taken together, majority of the evidence indicates that autophagy has

oncopromoting functions in PDAC, which makes this process to look appealing as a potential target in PC therapy and has prompted multiple anti-autophagy based ongoing preclinical and clinical trials.

1A.9 Role of hypoxia in the regulation of autophagy

Previous reports have confirmed that hypoxia induces autophagy in a HIF-1α dependent manner in both normal and cancer cell lines to promote cell survival, implying that HIF-1α plays an important role in maintaining and regulating cell autophagy. However, the underlying mechanism of hypoxia-induced autophagy has started to get unearthed by recent studies. The activation of HIF-1α-dependent autophagy occurs *via* the induction of Bcl-2 (B-cell lymphoma 2)/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), which has two HRE (HRE-1 and 2) sites on its promoter region. HIF-1 directly binds to HRE2 site on BNIP3 promoter and leads to the transcriptional upregulation of BNIP3 (102). Due to established role of BNIP3 in disrupting the autophagy inhibitory Bcl-2: Beclin 1 complex, BNIP3 overexpression under hypoxia led to the release of BECN1, which is now free to initiate autophagy by assembling a pre-autophagosomal structure (103).

In addition to HIF-1 dependent pathways, there are HIF-1 independent pathways as well, known to regulate autophagy under hypoxic condition. One of such pathway is the regulation of the mammalian target of rapamycin (mTOR), which consists of two specific mTOR complexes; mTORC1 and mTORC2. Their downstream effectors orchestrate several biological processes, including autophagy. mTORC1 has been associated with autophagy suppression by inhibiting ATG1 ser/thr protein kinase, which is involved in the formation of autophagosomes (104). Under hypoxia, mTORC1 pathway is suppressed by cancer cells by utilizing multiple pathways; one of them is mediated through hypoxic activation of the tuberous sclerosis protein (TSC) complex

(104). It is a heterodimeric complex formed by TSC1 and TSC2. Under nutrient deprived conditions, there is an increase in AMP/ATP ratio, which is sensed by the heterodimeric 5′ AMP-activated protein kinase (AMPK) complex, which phosphorylates many downstream targets, including TSC2 on serine residues positioned at 1270 and 1388 (104). Phosphorylation of these sites has inhibitory effect on the activity of the TSC1/TSC2 complex. Due to the inactivation of the TSC1/TSC2 complex, Ras homolog enriched in brain (RHEB)-dependent activation of mTOR is prevented. Activation of the unfolded protein response (UPR), a program which leads to major transcriptional and translational alteration due to endoplasmic reticulum (ER) stress with a goal to clear misfolded proteins, is an additional HIF-1-independent pathway that activates autophagy (105). There are mainly three ER stress sensors which facilitate UPR program: PKR-like ER kinase (PERK), ER to nucleus signaling 1 (ERN1), and activating transcription factor (ATF). Under hypoxic conditions, autophagy seems to be facilitated by PERK. Phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α) in a PERK-dependent manner leads to the activation of ATF4 transcription factor which subsequently induce the expression of MAP1LC3B and ATG5 at mRNA level (105).

1A.10 Role of oxidative stress in autophagy regulation in PC

Different stress signals or genetic alterations prompt ROS production leading to the stimulation of autophagy, which regulates cell death or cell survival pathways. In a classical paper by Scherz-Shouval and colleagues, it has been clearly demonstrated that during amino acid starvation, PtdIns3K class III-dependent generation of H_2O_2 by the mitochondria, serves as a local signaling molecule to modulate the activity of the cysteine protease Atg4 (106). In their *in vitro* studies, they have demonstrated that a critical cysteine residue near the catalytic site of Atg4 undergo oxidative alteration, which inhibits the activity of Atg4, and thus ensures the conjugation of Atg8 to the

autophagosomal membrane (106). This could lead to a ROS-dependent accumulation of LC3-PE on the autophagosomal membranes in close vicinity, thereby facilitating the first steps in autophagosome formation.

Furthermore, autophagy regulation by intracellular ROS levels during starvation has shown to involve p53-inducible protein TP53-induced glycolysis and apoptosis regulator (TIGAR). TIGAR functions as a fructose- 2,6-bisphosphatase, and diverts glycolytic metabolic intermediates to the oxidative branch of the hexose monophosphate pathway, which led to increased cellular production of NADPH, and thereby, lowers intracellular ROS levels and the sensitivity of cells to oxidative stress associated apoptosis (107). Therefore, suppression of TIGAR expression increases the production of ROS and autophagy in hepatocellular cancer cells, independent of mTOR- and p53, However, induction of autophagy due to loss of TIGAR is considered to be cytoprotective and reduces the apoptotic response by limiting oxidative stress (plausibly by the degradation of ROS producing mitochondria) (108).

The redox-regulation by Akt-mTOR and AMPK signaling systems are other critical mechanisms responsible for ROS-mediated induction of autophagy. ROS production following growth factor-stimulation results in the inactivation of phosphatase and tensin homologue (PTEN) through the formation of a disulfide bridge between a cysteine in the catalytic site with a proximal cysteine residue. This PTEN inactivating process leads to an increment in the levels of PtdIns(3,4,5)P3, which promotes the activation of Akt, a well-known proto-oncogene (109). Furthermore, the selective degradation of the H_2O_2 converting enzyme, catalase, by autophagy can provide an auto-stimulatory feedback loop, while the autophagic degradation of mitochondria or other ROS-producing organelles alleviates oxidative stress progression and acts as a negative feedback loop (110). These results are also consistent with a model in which

ROS act as signaling molecules upstream of autophagy, whereas the stimulation of autophagy further limits ROS levels by removing ROS-generating mitochondrial therapies.

1A.11 Regulation of mucins by hypoxia in cancer condition

Accumulating evidence suggests a number of physiological roles and regulatory mechanisms for mucins; however, there are limited studies which have demonstrated the effects of local microenvironment on mucins regulation. Studies have identified and appreciated the involvement of HIF-1α regulated pathway in mucins regulation. The first study came back in 1990s, where a molecular link between hypoxia and mucins regulation was observed in intestinal epithelial cells. The hypoxia-dependent induction of MUC3 signified a novel innate mechanism that may protect the immunologic components of the lamina propria from exposure to various insults including, pathogenic luminal bacteria, antigens, and toxins under oxygen deficient conditions (111). Of note, HRE was not precisely mapped on MUC3 promoter, which could be due to the complexity of the flanking region around the HIF-1 consensus sequence (111). Following HIF-1 consensus site, the immediate region contains potential binding sites for the transcription factors such as, c-rel, NFκB, glucocorticoid receptor, the estrogen receptor, and CREB, where some of these transcription factors have been implicated in either gene induction or repression under hypoxia. Therefore, MUC3 gene at this site is quite complicated and might involve interplay between positive and negative regulatory signals. Efforts to better understand MUC3 signaling pathways and to identify other hypoxia-elicited protective elements could provide future focus for development of novel treatments.

Polosukhin *et al.* demonstrated that hypoxia induces metaplasia in goblet cells which is followed by increased gel-forming MUC5AC mucin expression in primary human bronchial epithelial cell lines (112). Zhou *et al* validated the presence of HIF-1α

transcription factor–binding sites (HREs) on the MUC5AC promoter region (113). The expression and secretion of MUC5AC in human bronchial epithelial cells was found to be significantly reduced when HIF-1α expression was inhibited using HIF-1α inhibitor (YC-1) and HIF-1α small interfering RNA (siRNA).

Another transmembrane mucin, MUC17, has been reported to be overexpressed in pancreatic ductal adenocarcinomas (PDAC) compared with its lack of expression in normal pancreas or pancreatitis. Additionally, Hirono *et al.* also reported that MUC17 is an independent prognostic factor associated with lymph node metastasis in PDAC (114). MUC17 expression has also been found to be enhanced under hypoxic condition in a HIF-1α dependent manner (115). Further investigations revealed that DNA methylation of HRE is a key determinant of the hypoxic inducibility of MUC17 in PC (115). Clinically, hypomethylation of HRE within the MUC17 promoter is a frequently occurring event in the pancreatic tissues of patients with PDAC. In the future, the significance of these findings in PC pathogenesis needs to be reconnoitered.

MUC1 is the most studied mucin in terms of hypoxia under cancerous condition. The first study which showed the connection between MUC1 and hypoxia came in 2007, where Yin *et al.* first related MUC1 expression with reduced ROS production which had inhibitory effects on the activity of HIF-1α expression (116). According to the report, MUC1 overexpression blocks hypoxia-induced apoptosis and necrosis by suppressing accumulation of ROS, and therefore, MUC1 expression leads to better survival response in response to hypoxic stress (116). The subsequent studies have related HIF-1 signaling mechanism in MUC1 upregulation, which is the main pathway involved in renal carcinogenesis. Hypoxia-derived conditioned media (HCM) from MUC1 kd AsPC1 cells demonstrated profound inhibitory effect on the migration and proliferation ability of endothelial cells compared to hypoxia-treated control cells, suggesting the potential involvement of MUC1 in the process of hypoxia-driven angiogenesis (117). Not only wt-

MUC1, this study has also shown that hypoxia strongly induced the translocation of MUC1-CT to the nucleus as well as HIF1α in AsPC1 cells, but not under normoxic culture conditions. In another study, hypoxia-mediated induction of MUC1 has been linked with increased glucose uptake and glycolysis by nutrient-deficient PC cells (118). The physical interaction of MUC1 with HIF-1α and p300 in a hypoxia-dependent manner is ensued by enhanced promoter occupancy of the HIF- 1α and p300 on glycolytic gene promoters, which regulates the expression of multiple metabolic genes. This is further supported by the observed positive correlation of MUC1 expression with the expression of glucose metabolic enzymes such as GLUT1 and LDHA (118). Presence of MUC1 has also been associated with enhanced stability by diminishing the levels of 2-oxoglutarate in PC cells. Altogether, the interrelationship between MUC1–HIF-1α oncogenic signaling networks serves to facilitate tumor growth and metastasis and could present a potential therapeutic target for the treatment of malignant diseases that rely upon MUC1 and HIF-1α.

In addition to PC and CRC, the association between HIF-1α and MUC1 is also established in invasive ductal breast carcinoma (n=243). MUC1 overexpression was observed in 37.0% of patients and it correlated positively with estrogen receptor ($p = 0.0001$), progesterone receptor ($p = 0.0001$), HIF-1α ($p = 0.006$), VEGF ($p = 0.024$), and p53 ($p = 0.025$) (119). Here, authors have also demonstrated that MUC1 overexpression leads to the increased degradation of inhibitor of NF-κB (IκBα). It subsequently promotes the nuclear translocation of NF-κB which blocks apoptosis and promotes cell survival (119). Moreover, it has been reported that MUC1 has the ability to promote autophagy which provides a survival advantage in a low glucose-stressed microenvironment by suppressing excessively generated ROS levels in colon cancer condition.

1A.12 Reference List

(1) Joshi S, Kumar S, Bafna S, Rachagani S, Wagner KU, Jain M, Batra SK. Genetically engineered mucin mouse models for inflammation and cancer. Cancer Metastasis Rev 2015;34:593-609.

(2) Joshi S, Kumar S, Choudhury A, Ponnusamy MP, Batra SK. Altered Mucins (MUC) trafficking in benign and malignant conditions. Oncotarget 2014;5:7272-84.

(3) Kufe DW. Mucins in cancer: function, prognosis and therapy. Nat Rev Cancer 2009;9:874-85.

(4) Rachagani S, Torres MP, Moniaux N, Batra SK. Current status of mucins in the diagnosis and therapy of cancer. Biofactors 2009;35:509-27.

(5) 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.

(6) Buisine MP, Desreumaux P, Leteurtre E, Copin MC, Colombel JF, Porchet N, Aubert JP. Mucin gene expression in intestinal epithelial cells in Crohn's disease. Gut 2001;49:544-51.

(7) Corfield AP, Myerscough N, Longman R, Sylvester P, Arul S, Pignatelli M. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. Gut 2000;47:589-94.

(8) Singh AP, Chauhan SC, Bafna S, Johansson SL, Smith LM, Moniaux N, Lin MF, Batra SK. Aberrant expression of transmembrane mucins, MUC1 and MUC4, in human prostate carcinomas. Prostate 2006;66:421-9.

(9) 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. PLoS One 2013;8:e54455.

(10) Senapati S, Chaturvedi P, Sharma P, Venkatraman G, Meza JL, El-Rifai W, Roy HK, Batra SK. Deregulation of MUC4 in gastric adenocarcinoma: potential pathobiological implication in poorly differentiated non-signet ring cell type gastric cancer. Br J Cancer 2008;99:949-56.

(11) Chauhan SC, Singh AP, Ruiz F, Johansson SL, Jain M, Smith LM, Moniaux N, Batra SK. Aberrant expression of MUC4 in ovarian carcinoma: diagnostic significance alone and in combination with MUC1 and MUC16 (CA125). Mod Pathol 2006;19:1386-94.

(12) 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.

(13) Chaturvedi P, Singh AP, Chakraborty S, Chauhan SC, Bafna S, Meza JL, Singh PK, Hollingsworth MA, Mehta PP, Batra SK. MUC4 mucin interacts with and stabilizes the HER2 oncoprotein in human pancreatic cancer cells. Cancer Res 2008;68:2065-70.

(14) Ponnusamy MP, Singh AP, Jain M, Chakraborty S, Moniaux N, Batra SK. MUC4 activates HER2 signalling and enhances the motility of human ovarian cancer cells. Br J Cancer 2008;99:520-6.

(15) Singh PK, Hollingsworth MA. Cell surface-associated mucins in signal transduction. Trends Cell Biol 2006;16:467-76.

(16) Ahmad R, Raina D, Trivedi V, Ren J, Rajabi H, Kharbanda S, Kufe D. MUC1 oncoprotein activates the IkappaB kinase beta complex and constitutive NF-kappaB signalling. Nat Cell Biol 2007;9:1419-27.

(17) Rachagani S, Macha MA, Ponnusamy MP, Haridas D, Kaur S, Jain M, Batra SK. MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1. Carcinogenesis 2012;33:1953-64.

(18) Chaturvedi P, Singh AP, Moniaux N, Senapati S, Chakraborty S, Meza JL, Batra SK. 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.

(19) Kumar S, Das S, Rachagani S, Kaur S, Joshi S, Johansson SL, Ponnusamy MP, Jain M, Batra SK. NCOA3-mediated upregulation of mucin expression via transcriptional and post-translational changes during the development of pancreatic cancer. Oncogene 2014.

(20) Montagne L, Piel C, Lalles JP. Effect of diet on mucin kinetics and composition: nutrition and health implications. Nutr Rev 2004;62:105-14.

(21) Itoh Y, Kamata-Sakurai M, Denda-Nagai K, Nagai S, Tsuiji M, Ishii-Schrade K, Okada K, Goto A, Fukayama M, Irimura T. Identification and expression of human epiglycanin/MUC21: a novel transmembrane mucin. Glycobiology 2008;18:74-83.

(22) Senapati S, Das S, Batra SK. Mucin-interacting proteins: from function to therapeutics. Trends Biochem Sci 2010;35:236-45.

(23) Joshi S, Kumar S, Choudhury A, Ponnusamy MP, Batra SK. Altered Mucins (MUC) trafficking in benign and malignant conditions. Oncotarget 2014;5:7272-84.

(24) Spicer AP, Parry G, Patton S, Gendler SJ. Molecular cloning and analysis of the mouse homologue of the tumor-associated mucin, MUC1, reveals conservation of potential O-glycosylation sites, transmembrane, and cytoplasmic domains and a loss of minisatellite-like polymorphism. J Biol Chem 1991;266:15099-109.

(25) Shekels LL, Lyftogt C, Kieliszewski M, Filie JD, Kozak CA, Ho SB. Mouse gastric mucin: cloning and chromosomal localization. Biochem J 1995;311 (Pt 3):775-85.

(26) Desseyn JL, Clavereau I, Laine A. Cloning, chromosomal localization and characterization of the murine mucin gene orthologous to human MUC4. Eur J Biochem 2002;269:3150-9.

(27) Maeda T, Inoue M, Koshiba S, Yabuki T, Aoki M, Nunokawa E, Seki E, Matsuda T, Motoda Y, Kobayashi A, Hiroyasu F, Shirouzu M, Terada T, Hayami N, Ishizuka Y, et al. Solution structure of the SEA domain from the murine homologue of ovarian cancer antigen CA125 (MUC16). J Biol Chem 2004;279:13174-82.

(28) Goodell CA, Belisle JA, Gubbels JA, Migneault M, Rancourt C, Connor J, Kunnimalaiyaan M, Kravitz R, Tucker W, Zwick M, Patankar MS. Characterization of the tumor marker muc16 (ca125) expressed by murine ovarian tumor cell lines and identification of a panel of cross-reactive monoclonal antibodies. J Ovarian Res 2009;2:8.

(29) Dougherty GJ, Kay RJ, Humphries RK. Molecular cloning of 114/A10, a cell surface antigen containing highly conserved repeated elements, which is expressed by murine hemopoietic progenitor cells and interleukin-3-dependent cell lines. J Biol Chem 1989;264:6509-14.

(30) Williams SJ, Wreschner DH, Tran M, Eyre HJ, Sutherland GR, McGuckin MA. Muc13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. J Biol Chem 2001;276:18327-36.

(31) Gum JR, Jr., Crawley SC, Hicks JW, Szymkowski DE, Kim YS. MUC17, a novel membrane-tethered mucin. Biochem Biophys Res Commun 2002;291:466-75.

(32) Higuchi T, Orita T, Nakanishi S, Katsuya K, Watanabe H, Yamasaki Y, Waga I, Nanayama T, Yamamoto Y, Munger W, Sun HW, Falk RJ, Jennette JC, Alcorta DA, Li H, et al. Molecular cloning, genomic structure, and expression analysis

of MUC20, a novel mucin protein, up-regulated in injured kidney. J Biol Chem 2004;279:1968-79.

(33) Chen Y, Zhao YH, Kalaslavadi TB, Hamati E, Nehrke K, Le AD, Ann DK, Wu R. Genome-wide search and identification of a novel gel-forming mucin MUC19/Muc19 in glandular tissues. Am J Respir Cell Mol Biol 2004;30:155-65.

(34) Desseyn JL, Laine A. Characterization of mouse muc6 and evidence of conservation of the gel-forming mucin gene cluster between human and mouse. Genomics 2003;81:433-6.

(35) Pigny P, Guyonnet-Duperat V, Hill AS, Pratt WS, Galiegue-Zouitina S, d'Hooge MC, Laine A, Van-Seuningen I, Degand P, Gum JR, Kim YS, Swallow DM, Aubert JP, Porchet N. Human mucin genes assigned to 11p15.5: identification and organization of a cluster of genes. Genomics 1996;38:340-52.

(36) Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4659-65.

(37) Aslam F, Palumbo L, Augenlicht LH, Velcich A. The Sp family of transcription factors in the regulation of the human and mouse MUC2 gene promoters. Cancer Res 2001;61:570-6.

(38) van Klinken BJ, Einerhand AW, Duits LA, Makkink MK, Tytgat KM, Renes IB, Verburg M, Buller HA, Dekker J. Gastrointestinal expression and partial cDNA cloning of murine Muc2. Am J Physiol 1999;276:G115-G124.

(39) Inatomi T, Tisdale AS, Zhan Q, Spurr-Michaud S, Gipson IK. Cloning of rat Muc5AC mucin gene: comparison of its structure and tissue distribution to that of human and mouse homologues. Biochem Biophys Res Commun 1997;236:789-97.

(40) Shekels LL, Lyftogt C, Kieliszewski M, Filie JD, Kozak CA, Ho SB. Mouse gastric mucin: cloning and chromosomal localization. Biochem J 1995;311 (Pt 3):775-85.

(41) Escande F, Porchet N, Aubert JP, Buisine MP. The mouse Muc5b mucin gene: cDNA and genomic structures, chromosomal localization and expression. Biochem J 2002;363:589-98.

(42) Culp DJ, Latchney LR, Fallon MA, Denny PA, Denny PC, Couwenhoven RI, Chuang S. The gene encoding mouse Muc19: cDNA, genomic organization and relationship to Smgc. Physiol Genomics 2004;19:303-18.

(43) Toribara NW, Ho SB, Gum E, Gum JR, Jr., Lau P, Kim YS. The carboxyl-terminal sequence of the human secretory mucin, MUC6. Analysis Of the primary amino acid sequence. J Biol Chem 1997;272:16398-403.

(44) Porchet N, Nguyen VC, Dufosse J, Audie JP, Guyonnet-Duperat V, Gross MS, Denis C, Degand P, Bernheim A, Aubert JP. Molecular cloning and chromosomal localization of a novel human tracheo-bronchial mucin cDNA containing tandemly repeated sequences of 48 base pairs. Biochem Biophys Res Commun 1991;175:414-22.

(45) Gross MS, Guyonnet-Duperat V, Porchet N, Bernheim A, Aubert JP, Nguyen VC. Mucin 4 (MUC4) gene: regional assignment (3q29) and RFLP analysis. Ann Genet 1992;35:21-6.

(46) Nollet S, Moniaux N, Maury J, Petitprez D, Degand P, Laine A, Porchet N, Aubert JP. Human mucin gene MUC4: organization of its 5'-region and polymorphism of its central tandem repeat array. Biochem J 1998;332 (Pt 3):739-48.

(47) Joshi S, Kumar S, Bafna S, Rachagani S, Wagner KU, Jain M, Batra SK. Genetically engineered mucin mouse models for inflammation and cancer. Cancer Metastasis Rev 2015.

(48) Audie JP, Janin A, Porchet N, Copin MC, Gosselin B, Aubert JP. Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by in situ hybridization. J Histochem Cytochem 1993;41:1479-85.

(49) Audie JP, Tetaert D, Pigny P, Buisine MP, Janin A, Aubert JP, Porchet N, Boersma A. Mucin gene expression in the human endocervix. Hum Reprod 1995;10:98-102.

(50) Vandenhaute B, Buisine MP, Debailleul V, Clement B, Moniaux N, Dieu MC, Degand P, Porchet N, Aubert JP. Mucin gene expression in biliary epithelial cells. J Hepatol 1997;27:1057-66.

(51) Buisine MP, Devisme L, Savidge TC, Gespach C, Gosselin B, Porchet N, Aubert JP. Mucin gene expression in human embryonic and fetal intestine. Gut 1998;43:519-24.

(52) Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth MA, Buchler MW, Aubert JP, Batra SK. 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.

(53) Andrianifahanana M, Agrawal A, Singh AP, Moniaux N, Van S, I, Aubert JP, Meza J, Batra SK. 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.

(54) Carraway KL, Price-Schiavi SA, Komatsu M, Idris N, Perez A, Li P, Jepson S, Zhu X, Carvajal ME, Carraway CA. Multiple facets of sialomucin complex/MUC4, a membrane mucin and erbb2 ligand, in tumors and tissues (Y2K update). Front Biosci 2000;5:D95-D107.

(55) Price-Schiavi SA, Perez A, Barco R, Carraway KL. Cloning and characterization of the 5' flanking region of the sialomucin complex/rat Muc4 gene: promoter activity in cultured cells. Biochem J 2000;349:641-9.

(56) Perrais M, Pigny P, Ducourouble MP, Petitprez D, Porchet N, Aubert JP, Van S, I. 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.

(57) Li H, Fan X, Houghton J. Tumor microenvironment: the role of the tumor stroma in cancer. J Cell Biochem 2007;101:805-15.

(58) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.

(59) Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol 2012;196:395-406.

(60) Kim Y, Lin Q, Glazer PM, Yun Z. Hypoxic tumor microenvironment and cancer cell differentiation. Curr Mol Med 2009;9:425-34.

(61) Michiels C. Physiological and pathological responses to hypoxia. Am J Pathol 2004;164:1875-82.

(62) Bertout JA, Patel SA, Simon MC. The impact of O2 availability on human cancer. Nat Rev Cancer 2008;8:967-75.

(63) Lopez-Lazaro M. Role of oxygen in cancer: looking beyond hypoxia. Anticancer Agents Med Chem 2009;9:517-25.

(64) Kunz M, Moeller S, Koczan D, Lorenz P, Wenger RH, Glocker MO, Thiesen HJ, Gross G, Ibrahim SM. Mechanisms of hypoxic gene regulation of angiogenesis factor Cyr61 in melanoma cells. J Biol Chem 2003;278:45651-60.

(65) Kunz M, Ibrahim SM. Molecular responses to hypoxia in tumor cells. Mol Cancer 2003;2:23.

(66) Koong AC, Mehta VK, Le QT, Fisher GA, Terris DJ, Brown JM, Bastidas AJ, Vierra M. Pancreatic tumors show high levels of hypoxia. Int J Radiat Oncol Biol Phys 2000;48:919-22.

(67) Patel AG, Toyama MT, Alvarez C, Nguyen TN, Reber PU, Ashley SW, Reber HA. Pancreatic interstitial pH in human and feline chronic pancreatitis. Gastroenterology 1995;109:1639-45.

(68) Long J, Zhang Y, Yu X, Yang J, LeBrun DG, Chen C, Yao Q, Li M. Overcoming drug resistance in pancreatic cancer. Expert Opin Ther Targets 2011;15:817-28.

(69) Oberstein PE, Olive KP. Pancreatic cancer: why is it so hard to treat? Therap Adv Gastroenterol 2013;6:321-37.

(70) Sullivan R, Pare GC, Frederiksen LJ, Semenza GL, Graham CH. Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity. Mol Cancer Ther 2008;7:1961-73.

(71) Durand RE, Aquino-Parsons C. The fate of hypoxic (pimonidazole-labelled) cells in human cervix tumours undergoing chemo-radiotherapy. Radiother Oncol 2006;80:138-42.

(72) Rockwell S, Dobrucki IT, Kim EY, Marrison ST, Vu VT. Hypoxia and radiation therapy: past history, ongoing research, and future promise. Curr Mol Med 2009;9:442-58.

(73) Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510-4.

(74) Ziello JE, Jovin IS, Huang Y. Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. Yale J Biol Med 2007;80:51-60.

(75) Sun HC, Qiu ZJ, Liu J, Sun J, Jiang T, Huang KJ, Yao M, Huang C. Expression of hypoxia-inducible factor-1 alpha and associated proteins in pancreatic ductal adenocarcinoma and their impact on prognosis. Int J Oncol 2007;30:1359-67.

(76) Song Z, Ren H, Gao S, Zhao X, Zhang H, Hao J. The clinical significance and regulation mechanism of hypoxia-inducible factor-1 and miR-191 expression in pancreatic cancer. Tumour Biol 2014;35:11319-28.

(77) Chen J, Zhao S, Nakada K, Kuge Y, Tamaki N, Okada F, Wang J, Shindo M, Higashino F, Takeda K, Asaka M, Katoh H, Sugiyama T, Hosokawa M, Kobayashi M. Dominant-negative hypoxia-inducible factor-1 alpha reduces

tumorigenicity of pancreatic cancer cells through the suppression of glucose metabolism. Am J Pathol 2003;162:1283-91.

(78) Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell 1988;53:549-54.

(79) Vaquero EC, Edderkaoui M, Pandol SJ, Gukovsky I, Gukovskaya AS. Reactive oxygen species produced by NAD(P)H oxidase inhibit apoptosis in pancreatic cancer cells. J Biol Chem 2004;279:34643-54.

(80) Santillo M, Mondola P, Seru R, Annella T, Cassano S, Ciullo I, Tecce MF, Iacomino G, Damiano S, Cuda G, Paterno R, Martignetti V, Mele E, Feliciello A, Avvedimento EV. Opposing functions of Ki- and Ha-Ras genes in the regulation of redox signals. Curr Biol 2001;11:614-9.

(81) Yang JQ, Li S, Domann FE, Buettner GR, Oberley LW. Superoxide generation in v-Ha-ras-transduced human keratinocyte HaCaT cells. Mol Carcinog 1999;26:180-8.

(82) Lewis AM, Ough M, Hinkhouse MM, Tsao MS, Oberley LW, Cullen JJ. Targeting NAD(P)H:quinone oxidoreductase (NQO1) in pancreatic cancer. Mol Carcinog 2005;43:215-24.

(83) Ough M, Lewis A, Bey EA, Gao J, Ritchie JM, Bornmann W, Boothman DA, Oberley LW, Cullen JJ. Efficacy of beta-lapachone in pancreatic cancer treatment: exploiting the novel, therapeutic target NQO1. Cancer Biol Ther 2005;4:95-102.

(84) Dorr RT, Raymond MA, Landowski TH, Roman NO, Fukushima S. Induction of apoptosis and cell cycle arrest by imexon in human pancreatic cancer cell lines. Int J Gastrointest Cancer 2005;36:15-28.

(85) Cohen SJ, Zalupski MM, Modiano MR, Conkling P, Patt YZ, Davis P, Dorr RT, Boytim ML, Hersh EM. A phase I study of imexon plus gemcitabine as first-line therapy for advanced pancreatic cancer. Cancer Chemother Pharmacol 2010;66:287-94.

(86) Dhillon H, Chikara S, Reindl KM. Piperlongumine induces pancreatic cancer cell death by enhancing reactive oxygen species and DNA damage. Toxicol Rep 2014;1:309-18.

(87) Kim J, Klionsky DJ. Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. Annu Rev Biochem 2000;69:303-42.

(88) Kimmelman AC. The dynamic nature of autophagy in cancer. Genes Dev 2011;25:1999-2010.

(89) Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K, Mizushima N. Autophagy-deficient mice develop multiple liver tumors. Genes Dev 2011;25:795-800.

(90) Guo JY, Karsli-Uzunbas G, Mathew R, Aisner SC, Kamphorst JJ, Strohecker AM, Chen G, Price S, Lu W, Teng X, Snyder E, Santanam U, Dipaola RS, Jacks T, Rabinowitz JD, et al. Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. Genes Dev 2013;27:1447-61.

(91) Rao S, Tortola L, Perlot T, Wirnsberger G, Novatchkova M, Nitsch R, Sykacek P, Frank L, Schramek D, Komnenovic V, Sigl V, Aumayr K, Schmauss G, Fellner N, Handschuh S, et al. A dual role for autophagy in a murine model of lung cancer. Nat Commun 2014;5:3056.

(92) Mukubou H, Tsujimura T, Sasaki R, Ku Y. The role of autophagy in the treatment of pancreatic cancer with gemcitabine and ionizing radiation. Int J Oncol 2010;37:821-8.

(93) Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, Bause A, Li Y, Stommel JM, Dell'antonio G, Mautner J, Tonon G, Haigis M, Shirihai OS, Doglioni C, et al. Pancreatic cancers require autophagy for tumor growth. Genes Dev 2011;25:717-29.

(94) Fujii S, Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M, Kinoshita T, Ueno T, Esumi H, Ochiai A. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. Cancer Sci 2008;99:1813-9.

(95) Amaravadi RK. Transcriptional regulation of autophagy in RAS-driven cancers. J Clin Invest 2015;125:1393-5.

(96) Morgan MJ, Gamez G, Menke C, Hernandez A, Thorburn J, Gidan F, Staskiewicz L, Morgan S, Cummings C, Maycotte P, Thorburn A. Regulation of autophagy and chloroquine sensitivity by oncogenic RAS in vitro is context-dependent. Autophagy 2014;10:1814-26.

(97) Perera RM, Bardeesy N. Pancreatic Cancer Metabolism: Breaking It Down to Build It Back Up. Cancer Discov 2015;5:1247-61.

(98) Perera RM, Stoykova S, Nicolay BN, Ross KN, Fitamant J, Boukhali M, Lengrand J, Deshpande V, Selig MK, Ferrone CR, Settleman J, Stephanopoulos G, Dyson NJ, Zoncu R, Ramaswamy S, et al. Transcriptional control of autophagy-lysosome function drives pancreatic cancer metabolism. Nature 2015;524:361-5.

(99) Sardiello M, Palmieri M, di RA, Medina DL, Valenza M, Gennarino VA, Di MC, Donaudy F, Embrione V, Polishchuk RS, Banfi S, Parenti G, Cattaneo E, Ballabio A. A gene network regulating lysosomal biogenesis and function. Science 2009;325:473-7.

(100) Rosenfeldt MT, O'Prey J, Morton JP, Nixon C, MacKay G, Mrowinska A, Au A, Rai TS, Zheng L, Ridgway R, Adams PD, Anderson KI, Gottlieb E, Sansom OJ, Ryan KM. p53 status determines the role of autophagy in pancreatic tumour development. Nature 2013;504:296-300.

(101) Yang A, Rajeshkumar NV, Wang X, Yabuuchi S, Alexander BM, Chu GC, Von Hoff DD, Maitra A, Kimmelman AC. Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations. Cancer Discov 2014;4:905-13.

(102) Kothari S, Cizeau J, McMillan-Ward E, Israels SJ, Bailes M, Ens K, Kirshenbaum LA, Gibson SB. BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene 2003;22:4734-44.

(103) Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, Mazure NM. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol 2009;29:2570-81.

(104) Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. FEBS Lett 2010;584:1287-95.

(105) Rouschop KM, van den Beucken T, Dubois L, Niessen H, Bussink J, Savelkouls K, Keulers T, Mujcic H, Landuyt W, Voncken JW, Lambin P, van der Kogel AJ, Koritzinsky M, Wouters BG. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. J Clin Invest 2010;120:127-41.

(106) Scherz-Shouval R, Sagiv Y, Shorer H, Elazar Z. The COOH terminus of GATE-16, an intra-Golgi transport modulator, is cleaved by the human cysteine protease HsApg4A. J Biol Chem 2003;278:14053-8.

(107) Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 2006;126:107-20.

(108) Ye L, Zhao X, Lu J, Qian G, Zheng JC, Ge S. Knockdown of TIGAR by RNA interference induces apoptosis and autophagy in HepG2 hepatocellular carcinoma cells. Biochem Biophys Res Commun 2013;437:300-6.

(109) Leslie NR. The redox regulation of PI 3-kinase-dependent signaling. Antioxid Redox Signal 2006;8:1765-74.

(110) Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, Baehrecke EH, Lenardo M. Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci U S A 2006;103:4952-7.

(111) Louis NA, Hamilton KE, Canny G, Shekels LL, Ho SB, Colgan SP. Selective induction of mucin-3 by hypoxia in intestinal epithelia. J Cell Biochem 2006;99:1616-27.

(112) Polosukhin VV, Cates JM, Lawson WE, Milstone AP, Matafonov AG, Massion PP, Lee JW, Randell SH, Blackwell TS. Hypoxia-inducible factor-1 signalling promotes goblet cell hyperplasia in airway epithelium. J Pathol 2011;224:203-11.

(113) Zhou X, Tu J, Li Q, Kolosov VP, Perelman JM. Hypoxia induces mucin expression and secretion in human bronchial epithelial cells. Transl Res 2012;160:419-27.

(114) Hirono S, Yamaue H, Hoshikawa Y, Ina S, Tani M, Kawai M, Ushijima M, Matsuura M, Saiki Y, Saiura A, Yamamoto J, Miki Y, Noda T. Molecular markers associated with lymph node metastasis in pancreatic ductal adenocarcinoma by genome-wide expression profiling. Cancer Sci 2010;101:259-66.

(115) Kitamoto S, Yokoyama S, Higashi M, Yamada N, Matsubara S, Takao S, Batra SK, Yonezawa S. Expression of MUC17 is regulated by HIF1alpha-mediated hypoxic responses and requires a methylation-free hypoxia responsible element in pancreatic cancer. PLoS One 2012;7:e44108.

(116) Yin L, Kharbanda S, Kufe D. Mucin 1 oncoprotein blocks hypoxia-inducible factor 1alpha activation in a survival response to hypoxia. J Biol Chem 2007;282:257-66.

(117) Kitamoto S, Yokoyama S, Higashi M, Yamada N, Takao S, Yonezawa S. MUC1 enhances hypoxia-driven angiogenesis through the regulation of multiple proangiogenic factors. Oncogene 2013;32:4614-21.

(118) Chaika NV, Gebregiworgis T, Lewallen ME, Purohit V, Radhakrishnan P, Liu X, Zhang B, Mehla K, Brown RB, Caffrey T, Yu F, Johnson KR, Powers R, Hollingsworth MA, Singh PK. MUC1 mucin stabilizes and activates hypoxia-inducible factor 1 alpha to regulate metabolism in pancreatic cancer. Proc Natl Acad Sci U S A 2012;109:13787-92.

(119) Zanetti JS, Soave DF, Oliveira-Costa JP, da Silveira GG, Ramalho LN, Garcia SB, Zucoloto S, Ribeiro-Silva A. The role of tumor hypoxia in MUC1-positive breast carcinomas. Virchows Arch 2011;459:367-75.

CHAPTER IB

Mucins Regulation by Bile acids (BA) in PC condition

1B.1 An outline of Bile acids

Bile acids (BA) are amphiphilic molecules and are the main component of bile along with cholesterol, phospholipids, and bilirubin (1). BA are derived from cholesterol and synthesized primarily in the liver. They are initially synthesized as primary bile acids, namely cholic acid (CA) and chenodeoxycholic acid (CDCA), but get conjugated with glycine and taurine (2). Subsequently, these conjugated BA are excreted from the liver into the gall bladder, at a concentration of approximately 100 mM, and remains stored, until gall bladder receives stimulus (2). Dietary fat is a stimulus for BA secretion into the intestine, and the primary function of BA is to facilitate digestion absorption of fats and liposoluble vitamins in the intestine throughout the enterocyte barrier (1). It is the same region, where approximately 95% of the BA are re-absorbed into ileal columnar epithelium cells, by an active apical sodium-dependent BAs transporter ASBT (Apical Sodium-Dependent Bile Acid transporter) (3). After uptake into enterocytes of the ileum, bile salts are transported to the basolateral domains of the cells for efflux into the portal circulation, and mined from portal blood plasma by liver cells. Liver does it job efficiently and eliminate BA from the circulation; therefore, the circulatory BA levels in healthy individuals are generally maintained at around 0.003 mM. The remaining unabsorbed 5% of BA pool enters to the colon, where it gets further metabolized by the anaerobic bacterial species using two major and a number of minor reactions (4). The first major reaction is the process of deconjugation which is followed by the release of free BA, whereas the second major reaction is 7-α dihydroxylation, which converts CA and CDCA to DCA and lithocholic acid (LCA), respectively (4). Although partially, DCA gets reabsorbed in the colon and enters into enterohepatic circulation, where it undergoes conjugation in the liver and secreted in the bile. On the other hand, very little amount of LCA is reabsorbed in the colon which can be attributed to its fairly insoluble nature. As a

result, the circulating BA pool (after undergoing both conjugation and deconjugation process) contains about 30–40% each of CA and CDCA, and about 20–30% of DCA, with less than 5% of LCA (5). Besides primary and secondary BA, bacterial degradation in the colon and alterations in the liver produce tertiary BA, such as ursodeoxycholic acid, which is also present in the circulating BA pool (6). It has been noticed that people on a high fat diet have substantially elevated levels of both DCA and LCA, in the fecal water. Before delivery, BA levels are maximally present in the gallbladder, but after their delivery towards small intestine, their maximum levels are present near the Ampulla of Vater than any other region of the body.

1B.2 BA under cancer condition

The first evidence demonstrating the involvement of BA in cancer development came from Cook *et al.* in 1940, where authors have clearly shown the induction of malignant tumors when DCA was injected into the right flank of mice. In another model, administration of CDCA to APC (Min/+) mice model has shown to increase number of duodenal tumors (7). Administration of BA alone did not lead to the formation of cancer in both mice and rat, but their treatment along with carcinogens has shown to significantly promote colon carcinogenesis (8).

Using multiple experimental models of rodents, particularly between 1974 and 1993, different types of BA have shown to exert their tumor-promoting functions. Administration of BA alone was failed to induce colon tumors. However, administration of LCA, taurine conjugated and unconjugated DCA had a significant promoting impact on colon carcinogenesis in rat after intrarectal instillation of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), which is a well-known mutagen (8). Similarly, CA also had a promoting effect on colon tumor formation in rats after intrarectal instillation of N-methyl-

N-nitrosourea (MNU) or sub-cutaneous injection of azoxymethane (9). In addition to colon, BA shown enhanced hyperplastic and neoplastic lesions in stomach mucosa, when administered in the presence of MNNG (10). Therefore, BA is considered as tumor-promoters. However, in spite of all these experimental models and related literature, considerable indirect evidence and logical argument supports the view that BA are carcinogens in humans. The GI tract of rodents and humans are exposed to BA for different duration of time. Most of the discussed experiments establishing the tumor promoting roles of BA were conducted for one year or less. However, most of the GI cancers in humans are primarily developed at around 60 years of age. Additionally, colonic epithelium renews themselves around 365 times during the lifespan of mice and rats and 5110 times in human. Due to DNA damaging or mutation inducing ability of BA, sustained exposure of BA to human colonic epithelium could have tumor initiating actions.

In addition to colon cancer, significant amount of literature has made it apparent that BA is also important in other gastrointestinal (GI) cancers (11). High or abnormal BA exposure is associated with increased incidence of cancer in the laryngopharyngeal tract, esophagus, stomach, pancreas, the small intestine (near the Ampulla of Vater) and the colon. As the local microenvironment (both cellular and acellular) varies from one GI organ to another, it can be speculated that both the extent of effect and the underlying mechanism involved upon BA exposure, could be different at different organs.

The involvement of BA in the progression from Barrett's esophagus (BE) to esophageal cancers, which is the seventh leading cause of cancer-related death worldwide, is well established. Patients with BE disease have an estimated 30–125 fold increased risk of developing esophageal cancers (12). BE disease occurs during healing

of esophageal mucosal injury typically triggered by gastro-esophageal reflux diseases (GERD), in which the exposure of esophagus is significantly increased to acidity from stomach and to BA from duodenum. Using surgical models, several studies have shown that GERD development is indeed result in esophageal adenocarcinoma (EA) development without exogenous carcinogen. Furthermore, feeding mice with zinc deficient diet containing DCA led to increased production of ROS with the visible appearance of BE-like lesions (13). Moreover, BA induces expression of inflammatory mediators (e.g. Interleukin-8 (IL-8), cyclo-oxygenase (COX-2), oxidative stress and DNA damage that could be linked to mutational events over a longer period (14), to development of resistant apoptotic cells, and ultimately cancer. However, the exact molecular pathways involved remain unclear. Few studies have investigated the implication of BA receptors in the development of BE disease and adenocarcinoma.

Several lines of evidence have implicated BA in liver tumorigenesis. Rodent models showed prominent appearance of preneoplastic lesions of hepatocellular carcinoma upon exogenous administration of DCA. Diet enriched with 0.2% CA has shown to intensely enhance *N*-nitrosodiethylamine-initiated liver carcinogenesis in WT-mice (15). In addition, children with progressive familial intrahepatic cholestasis type 2 (PFIC type 2), which is genetic deficiency of the canalicular bile salt export pump BSEP or ABCB11, are known to be predisposed to hepatocellular carcinoma (HCC). Similar to colon cancer, *in vitro* studies have indicated that BAs may directly affect hepatocytes by inducing ROS production which is followed by DNA damage and apoptosis. For example, hydrophobic BAs like DCA, glucuro-CDCA or tauro-CDCA have been reported to produce ROS in rat hepatocytes, human hepatoma cell line or primary human hepatocytes. Treatment of human hepatocarcinoma cells with DCA leads to the transcription of genes that participates in oxidative stress (NF-κB, c-fos) or DNA damage

(gadd153, c-fos). Moreover, several studies have also shown that BA induce apoptosis in liver cells, which can be reduced by an anti-oxidant treatment like α-tocopherol or β-carotene, suggesting that increased ROS production due to BA exposure is responsible for this cell death event. Even though we know that BA has carcinogenic potential in the pathogenesis of liver cancer, the defined mechanisms by which they act is not known and involvement of their receptors FXRα and TGR5 are poorly understood.

1B.3 BA receptors and their role in cancer

In addition to their mechanical role, BAs have been described as signaling molecules binding receptors. So far, four receptors, namely nuclear receptor farnesyl-X-receptor (FXRα, NR1H4), vitamin D receptor (VDR), G-protein-coupled receptor TGR5 (GPBAR1, G-protein-coupled bile acid receptor) and pregnane X receptor (PXR)/steroid xenobiotic receptor (SXR), have been recognized to bind to BA and perform tumorigenic functions.

FXR is present in high levels in liver, intestine, or kidney and it belongs to nuclear receptor superfamily. It acts as a mandatory heterodimer with retinoid X receptor (RXR) and binds to specific IR1 (inverted repeat-1) sequences on target gene promoters to regulate gene transcription. Using Fxrα gene (Fxrα−/−) mouse model, its involvement in regulating BAs biosynthesis and entero-hepatic cycle was highlighted. Fxrα−/− mice revealed high BA plasma levels due to abnormal hepatic biosynthesis, as FXRα represses the gene expression of cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1), key enzymes involved in BA biosynthesis (16). This is how FXRα limits the deleterious effects of accumulated BA. In hepatocytes, FXRα decreases BA uptake via repression of Na+-taurocholate co-transporting polypeptide (NTCP), organic anion-transporting polypeptide (OATP)-1 and OATP-4 expressions. It also promotes BA excretion in bile ducts through transcriptional induction of the specific BA

transporter BSEP (Bile salt export pump) in hepatocytes. Due to the involvement of FXR on BA homeostasis, significant upregulation was observed for the genes implicated in inflammation and cell cycle in aging FXR−/− mice, whereas WT control mice did not exhibit such effects. FXR-/- mice showed liver tumor formation between 13 and 15 months of age, whereas WT mice did not have any tumor formation (15). Interestingly, feeding mice with 2% cholestyramine, a bile acid–sequestering resin, to lower BA pool in FXR−/− mice had significant negative impact on the formation of malignant lesions, establishing the causal relationship between BA and liver tumors (15).

The involvement of FXRα in BE disease and adenocarcinoma development has been elucidated by several studies. In patients with BE disease, FXRα overexpressed in both esophageal squamous epithelium and specialized intestinal BE cells, while almost no FXRα was found in healthy squamous epithelium, whereas advanced EA patients exhibited loss of FXRα expression (17, 18). FXR expression has shown to be reduced remarkably in intestinal tumors developed both in human and mice models. Using two mouse models of intestinal tumorigenesis results, it has been demonstrated that loss of FXR is related with early death and increased size of small intestine adenocarcinomas, indicating that loss of FXR and not merely elevated BA concentrations increases susceptibility to tumorigenesis (19). In the absence of FXR, enhanced Wnt signaling is observed which is attributed to increased infiltrating immune cells (neutrophils) and cytokines (TNFα) production. It is also accompanied with an increased basal proliferative compartment both in the ileal portion of small intestine and colon along with simultaneous decrease in the apically localized differentiated compartment. This scenario leads to increased progression of colon tumors along with early mortality in utilized mice model (20). On the other hand, when FXR is activated in the differentiated normal enterocytes and in colon cancer cells, there is an induction of apoptosis and

removal of genetically altered cells, which may otherwise progress to complete transformation (20). Thus, from a therapeutic standpoint, strategies aimed at reactivating FXR expression in colon tumors might be useful in the treatment of colon cancer.

The first established G-protein coupled receptor specific for BA binding is TGR5 (21). TGR5 expresses ubiquitously both in humans and animals, and it is known to activate multiple intracellular signaling cascades upon binding to BA (22). TRG5 activation has well established functions in the maintenance of metabolic homeostasis and energy expenditure. IHC study has shown that TGR5 protein expression is present throughout the GI tract (22). Unlike FXRα, significantly high levels of TGR5 was observed both at mRNA and protein levels in human EA compared to normal esophageal mucosa or Barrett's mucosa (23), suggesting that it might play a central role in adenocarcinoma development. Additionally, TGR5 was established as a mediator of ROS generation, which leads to increased proliferation of esophageal cancer cells upon exposure to BA. Thus, TGR5 activity could be involved in the evolution of Barrett's syndrome to adenocarcinoma (23). Additionally, prominent staining for TGR5 has been observed in 12% of human intestinal metaplasia, without any detectable expression in normal gastric epithelium controls (24). In a recent study, TGR5 overexpression in gastric adenocarcinoma has been associated with poor survival (25).

Vitamin D deficiency has been considered as one of the major risk factors for the development of GI malignancies, such as pancreatic and colon cancers (26). Vitamin D binding to VDR, activates the receptor which makes it an active nuclear transcription factor and leads to the transcriptional induction of the expression of various genes (27). This is how vitamin D execute its profound antimitogenic and prodifferentiating effects on normal and malignant cells (28). Therefore, inadequacy in the levels of Vitamin D is plausibly causing abrupt regulation of cellular functions and growth. Besides these

functions, VDR has also been established as a receptor for secondary BAs such as LCA, and has a key role in the initiation of pathways which leads to the detoxification of LCA (29). Similar to VDR, activated human xenobiotic receptor (SXR) and its mouse homolog (PXR) have been associated with the detoxification of BA by activating enzymes, involved in BA metabolism (30, 31). Under cancer condition, PXR/SXR receptor is considered to be protective against oxidant induced apoptosis. Upon progression from normal epithelia to dysplastic lesions, the enteric NR transcriptome has been found to be reduced in both mouse and human models of colorectal cancer (20), suggesting that these transcription factors could have therapeutic and/or diagnostic potential that could be exploited to treat colorectal cancer.

1B.4 Association between BA and mucins expression

The first evidence that BA is important in the regulation in mucins secretion was provided by Klinkspoor JH *et al.* in 1995 (32). Hypersecretion of gallbladder mucin has been proposed as a pathogenic factor in gallstone formation. Therefore, the primary objective of the study was to understand the effect of biliary constituents on mucin secretion using normal, well-differentiated dog gallbladder epithelial cells. Interestingly, it was observed that alteration in the concentration of cholesterol or phospholipid had no effect on the extent of mucin secretion (32). However, TCA showed a dose-dependent increase in mucin secretion, signifying that bile salts, one of the major functional components of bile, are responsible for these stimulatory effects. Compared to hydrophilic BA, hydrophobic bile salts; TCDCA and TCA, showed more increased mucin secretion at 0.5 mmol/L ($p < 0.01$). A shift in the bile salt composition of bile towards more hydrophobic bile salts may cause mucin hypersecretion, and thereby participate in the initiation of cholesterol gallstone formation (32). Following, another study by Dray-Charier N and colleagues have implicated the predominant involvement of calcium-

dependent signaling pathways, particularly Ca2+/CaM-kinase II and protein kinase C (PKC) in BA-mediated hypersecretion by human gallbladder epithelial cells (33). Similarly, human colonic epithelial cells demonstrated increased mucin secretion upon BA stimulation. Taken together, these reports suggest that bile salts-mediated regulation of mucins secretion might be a common mechanism and one of the plausible reasons of increased secretion is to protect epithelia against the deleterious detergent action of bile salts as an adaptive response throughout the gastrointestinal tract. Later studies have apparently shown that BA play crucial role in the increased synthesis of both transmembrane and secretory mucins (34, 35).

Clinically, atypical gastro-oesophageal reflux and BA have been associated with the occurrence of BE premalignant lesions, which are linked with an increase in mucin-producing goblet cells and overexpression of mucins (36, 37). Multiple studies have postulated that a pattern of mucin staining in BE patients might be indicative of their increased tendency to progress from BE to adenocarcinoma (38). In normal esophagus, which has stratified squamous epithelium, there is positive expression for membrane bound MUC1 and MUC4 mucins. In the preneoplastic stage or BE, secretory mucins, such as MUC2, MUC5AC, MUC5B, MUC6, are expressed with MUC1 and MUC4. In high-grade dysplasia and adenocarcinoma of BE, downregulation in secreted mucins have been observed, whereas MUC1 and MUC4 mucin expression remain persistently high. It is therefore evident that there is a definite order in the appearance and subsequent decrease of various mucins in the Barrett's-adenocarcinoma sequence.

MUC5AC mucin is strongly expressed in almost 100% of BE and in 61.5% of tissues obtained from EA (39). MUC5AC mucin has been found to express at high levels in BE tissues stimulated by duodenoesophageal in rat reflux model (39). Conjugated BA had more impact in the induction of MUC5AC gene expression at the transcriptional

level compared to unconjugated BA, by utilizing PI3K/AKT/AP-1 signaling pathway (39). Similar to MUC5AC, another secretory mucin, MUC2 also showed transcriptional induction in esophageal carcinoma cell lines (40). PKC-dependent activation of nuclear factor-κB (NF-κB) was implicated in BA-mediated transcriptional upregulation of MUC2 in esophageal cancer (40). In addition to esophageal cancer, induction of MUC2 mRNA has also been noticed in DCA treated gastric cancer cell lines (41). DCA showed inhibitory effects on the invasion and migration of SNU-216 and MKN45 cell lines (41). Supportively, increased expression in E-cadherin along with reduced expression of snail and MMP-9 was observed upon DCA treatment. Multiple forms of BA have been previously identified as potent inducers of MUC4 expression in esophageal carcinogenesis associated with bile reflux (34, 42). Mechanistically, PI3K signaling, PKC and hepatocyte nuclear factor-1α were attributed to BA-facilitated increase in MUC4 expression (34, 35). BA also leads to MUC1 upregulation via a PI3K–mediated molecular transcriptional mechanism in human esophageal adenocarcinomatous cell lines. The biologic consequences of the induction of mucins expression by BA in cancer cells are still unknown, but considering the association of both BA and mucins overexpression in cancer condition, all this data favors a role of this mode of mucins regulation by tumor cells for their progression and metastasis. The development of a mouse or rat model of carcinogenesis in which epithelial mucosa is exposed to bile or acid reflux or both will be very informative regarding the following: (1) deciphering the precise molecular mechanisms activated by BA to induce mucins expression, (2) evaluating the consequences of mucins overexpression during the carcinogenetic sequence on tumor cell behavior and biologic properties, and (3) demonstrating the pivotal role of BA as the main inducer of the expression of both membrane and secreted mucins.

However, it is still unclear whether this is the primary cause for the persistence of the BE in-spite of successful anti-reflux surgery, or if it reflects the genetic instability associated with this preneoplastic condition. Further studies in patients undergoing surveillance for Barrett's and dysplasia will help answer whether mucin gene expression has a diagnostic role in predicting those at risk and does not merely represent an artifact of progression. An open question is whether therapeutic manipulation of MUC gene expression will decrease the risk of malignancy for patients with BE and dysplasia.

1B.5 Reference List

(1) Baptissart M, Vega A, Maqdasy S, Caira F, Baron S, Lobaccaro JM, Volle DH. Bile acids: from digestion to cancers. Biochimie 2013;95:504-17.

(2) Staels B, Fonseca VA. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. Diabetes Care 2009;32 Suppl 2:S237-S245.

(3) Alrefai WA, Gill RK. Bile acid transporters: structure, function, regulation and pathophysiological implications. Pharm Res 2007;24:1803-23.

(4) Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev 2009;89:147-91.

(5) Lafleur B, Lee W, Billhiemer D, Lockhart C, Liu J, Merchant N. Statistical methods for assays with limits of detection: Serum bile acid as a differentiator between patients with normal colons, adenomas, and colorectal cancer. J Carcinog 2011;10:12.

(6) Sagar NM, Cree IA, Covington JA, Arasaradnam RP. The interplay of the gut microbiome, bile acids, and volatile organic compounds. Gastroenterol Res Pract 2015;2015:398585.

(7) Mahmoud NN, Dannenberg AJ, Bilinski RT, Mestre JR, Chadburn A, Churchill M, Martucci C, Bertagnolli MM. Administration of an unconjugated bile acid increases duodenal tumors in a murine model of familial adenomatous polyposis. Carcinogenesis 1999;20:299-303.

(8) Narisawa T, Magadia NE, Weisburger JH, Wynder EL. Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of N-methyl-N'-nitro-N-nitrosoguanidine in rats. J Natl Cancer Inst 1974;53:1093-7.

(9) Cohen BI, Raicht RF, Deschner EE, Takahashi M, Sarwal AN, Fazzini E. Effect of cholic acid feeding on N-methyl-N-nitrosourea-induced colon tumors and cell kinetics in rats. J Natl Cancer Inst 1980;64:573-8.

(10) Kobori O, Shimizu T, Maeda M, Atomi Y, Watanabe J, Shoji M, Morioka Y. Enhancing effect of bile and bile acid on stomach tumorigenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine in Wistar rats. J Natl Cancer Inst 1984;73:853-61.

(11) Bernstein H, Bernstein C, Payne CM, Dvorak K. Bile acids as endogenous etiologic agents in gastrointestinal cancer. World J Gastroenterol 2009;15:3329-40.

(12) Hage M, Siersema PD, van DH, Steyerberg EW, Dees J, Kuipers EJ. Oesophageal cancer incidence and mortality in patients with long-segment Barrett's oesophagus after a mean follow-up of 12.7 years. Scand J Gastroenterol 2004;39:1175-9.

(13) Guy NC, Garewal H, Holubec H, Bernstein H, Payne CM, Bernstein C, Bhattacharyya AK, Dvorak K. A novel dietary-related model of esophagitis and Barrett's esophagus, a premalignant lesion. Nutr Cancer 2007;59:217-27.

(14) Payne CM, Bernstein C, Dvorak K, Bernstein H. Hydrophobic bile acids, genomic instability, Darwinian selection, and colon carcinogenesis. Clin Exp Gastroenterol 2008;1:19-47.

(15) Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res 2007;67:863-7.

(16) Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, Shan B, Schwarz M, Kuipers F. Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. J Biol Chem 2003;278:41930-7.

(17) van de Winkel A, Menke V, Capello A, Moons LM, Pot RG, van DH, Siersema PD, Kusters JG, van der Laan LJ, Kuipers EJ. Expression, localization and polymorphisms of the nuclear receptor PXR in Barrett's esophagus and esophageal adenocarcinoma. BMC Gastroenterol 2011;11:108.

(18) van de Winkel A, van Zoest KP, van DH, Moons LM, Kuipers EJ, van der Laan LJ. Differential expression of the nuclear receptors farnesoid X receptor (FXR) and pregnane X receptor (PXR) for grading dysplasia in patients with Barrett's oesophagus. Histopathology 2011;58:246-53.

(19) Maran RR, Thomas A, Roth M, Sheng Z, Esterly N, Pinson D, Gao X, Zhang Y, Ganapathy V, Gonzalez FJ, Guo GL. Farnesoid X receptor deficiency in mice leads to increased intestinal epithelial cell proliferation and tumor development. J Pharmacol Exp Ther 2009;328:469-77.

(20) Modica S, Murzilli S, Salvatore L, Schmidt DR, Moschetta A. Nuclear bile acid receptor FXR protects against intestinal tumorigenesis. Cancer Res 2008;68:9589-94.

(21) Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K. Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun 2002;298:714-9.

(22) Duboc H, Tache Y, Hofmann AF. The bile acid TGR5 membrane receptor: from basic research to clinical application. Dig Liver Dis 2014;46:302-12.

(23) Hong J, Behar J, Wands J, Resnick M, Wang LJ, DeLellis RA, Lambeth D, Souza RF, Spechler SJ, Cao W. Role of a novel bile acid receptor TGR5 in the development of oesophageal adenocarcinoma. Gut 2010;59:170-80.

(24) Tatsugami M, Ito M, Tanaka S, Yoshihara M, Matsui H, Haruma K, Chayama K. Bile acid promotes intestinal metaplasia and gastric carcinogenesis. Cancer Epidemiol Biomarkers Prev 2012;21:2101-7.

(25) Cao W, Tian W, Hong J, Li D, Tavares R, Noble L, Moss SF, Resnick MB. Expression of bile acid receptor TGR5 in gastric adenocarcinoma. Am J Physiol Gastrointest Liver Physiol 2013;304:G322-G327.

(26) Garland CF, Garland FC. Do sunlight and vitamin D reduce the likelihood of colon cancer? Int J Epidemiol 1980;9:227-31.

(27) Pike JW, Meyer MB. The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3). Endocrinol Metab Clin North Am 2010;39:255-69, table.

(28) Newmark HL, Yang K, Kurihara N, Fan K, Augenlicht LH, Lipkin M. Western-style diet-induced colonic tumors and their modulation by calcium and vitamin D in C57Bl/6 mice: a preclinical model for human sporadic colon cancer. Carcinogenesis 2009;30:88-92.

(29) Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ. Vitamin D receptor as an intestinal bile acid sensor. Science 2002;296:1313-6.

(30) Zhang B, Xie W, Krasowski MD. PXR: a xenobiotic receptor of diverse function implicated in pharmacogenetics. Pharmacogenomics 2008;9:1695-709.

(31) Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, Kliewer SA. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc Natl Acad Sci U S A 2001;98:3369-74.

(32) Klinkspoor JH, Kuver R, Savard CE, Oda D, Azzouz H, Tytgat GN, Groen AK, Lee SP. Model bile and bile salts accelerate mucin secretion by cultured dog gallbladder epithelial cells. Gastroenterology 1995;109:264-74.

(33) Dray-Charier N, Paul A, Combettes L, Bouin M, Mergey M, Balladur P, Capeau J, Housset C. Regulation of mucin secretion in human gallbladder epithelial cells: predominant role of calcium and protein kinase C. Gastroenterology 1997;112:978-90.

(34) Mariette C, Perrais M, Leteurtre E, Jonckheere N, Hemon B, Pigny P, Batra S, Aubert JP, Triboulet JP, Van S, I. 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.

(35) Piessen G, Jonckheere N, Vincent A, Hemon B, Ducourouble MP, Copin MC, Mariette C, Van S, I. 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.

(36) Debruyne PR, Bruyneel EA, Li X, Zimber A, Gespach C, Mareel MM. The role of bile acids in carcinogenesis. Mutat Res 2001;480-481:359-69.

(37) Jankowski J. Gene expression in Barrett's mucosa: acute and chronic adaptive responses in the oesophagus. Gut 1993;34:1649-50.

(38) Szachnowicz S, Cecconello I, Ribeiro U, Iriya K, El IR, Takeda FR, Corbett CE, Vaz Safatle-Ribeiro A. Mucin pattern reflects the origin of the adenocarcinoma in Barrett's esophagus: a retrospective clinical and laboratorial study. World J Surg Oncol 2009;7:27.

(39) Song S, Byrd JC, Guha S, Liu KF, Koul D, Bresalier RS. Induction of MUC5AC mucin by conjugated bile acids in the esophagus involves the phosphatidylinositol 3-kinase/protein kinase C/activator protein-1 pathway. Cancer 2011;117:2386-97.

(40) Wu J, Gong J, Geng J, Song Y. Deoxycholic acid induces the overexpression of intestinal mucin, MUC2, via NF-kB signaling pathway in human esophageal adenocarcinoma cells. BMC Cancer 2008;8:333.

(41) Pyo JS, Ko YS, Kang G, Kim DH, Kim WH, Lee BL, Sohn JH. Bile acid induces MUC2 expression and inhibits tumor invasion in gastric carcinomas. J Cancer Res Clin Oncol 2015;141:1181-8.

(42) Degirolamo C, Modica S, Palasciano G, Moschetta A. Bile acids and colon cancer: Solving the puzzle with nuclear receptors. Trends Mol Med 2011;17:564-72.

CHAPTER IC

Novel mechanisms implicated in MUC4-mediated increase in the stability of EGFR-family members in PC

1C.1 General introduction of altered mucins expression and localization under disease condition

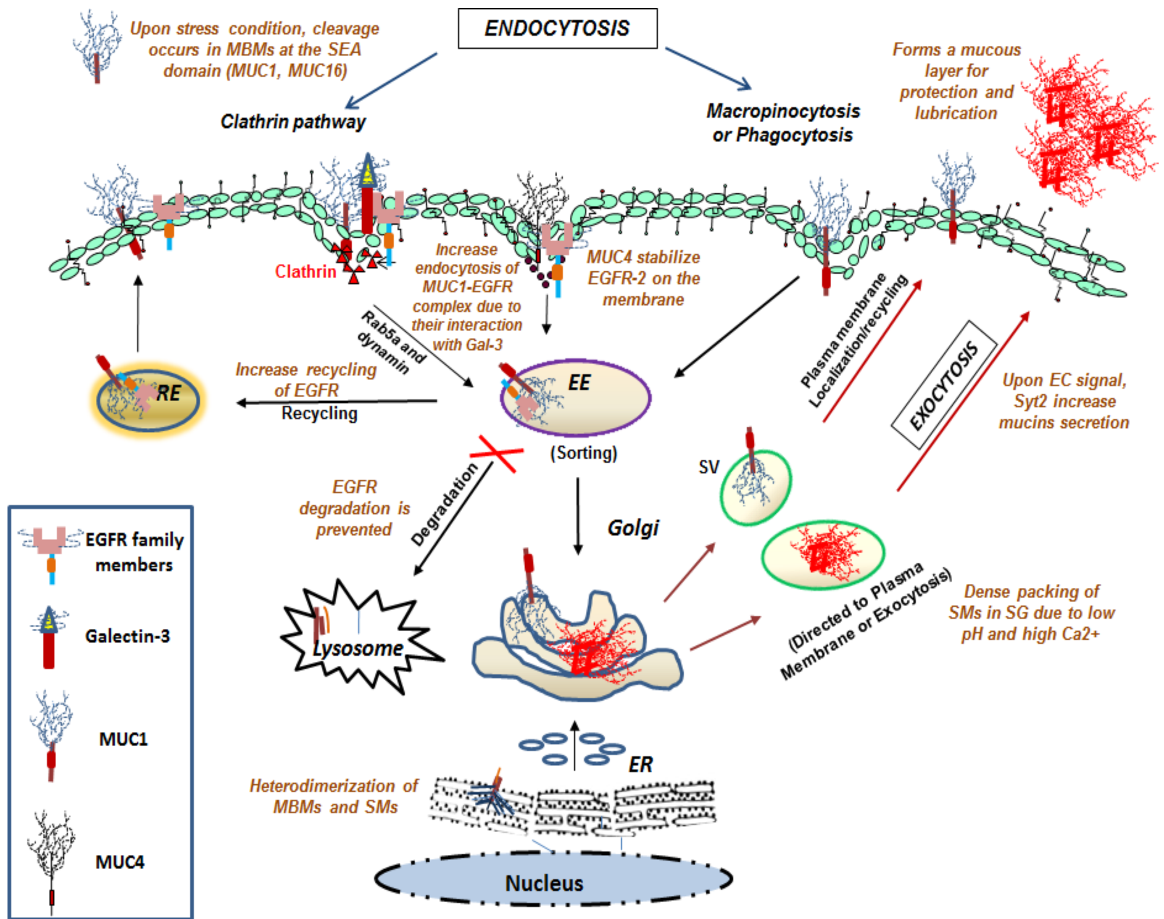
Mucins (MUC) are high molecular weight O-glycoproteins, predominantly expressed at the apical surface of the epithelial cells, and are classified into membrane bound MUC and secreted MUC (1-4). Tissue specific expressions of MUC have essential functions to provide protection, lubrication to epithelial cells, maintenance of epithelial characteristics, cellular adhesion, differentiation, and immunity (1-5). The expression of MUC is significantly altered during tumorigenesis and other pathological conditions. For example, MUC4 is not expressed in the normal pancreas, but the early pancreatic intraepithelial neoplasia (PanINs) precursor lesions have been shown to express MUC4, which further increases as the disease progresses (4-6). In addition, MUC4 is also overexpressed in breast cancer, gastric cancer and ovarian cancer (7-9), and its overexpression has also been associated with the poor prognosis of pancreatic cancer and cholangiocarcinoma (10, 11). However, MUC4 expression is down-regulated during prostate carcinomas (12) and urothelial cancer (13), suggesting the complicated context-dependent role of mucins. Another example, MUC1 is overexpressed in various malignancies and inflammatory conditions (1, 14-16). Besides the aberrant expression of mucins, emerging evidence suggests that anomalies in their subcellular localization and resultant changes in their endocytic trafficking play critical roles under pathological conditions (17).

In a cell, the majority of proteins are not pre-set to any single location and are in a steady-state distribution due to opposing egress (exocytosis) and entrance (endocytosis) pathways (18). These two pathways are extremely dynamic and are regulated by highly sensitive cross talks between different subcellular compartments. Endocytic pathways have always been considered as enduring mechanisms for

recycling molecules from the plasma membrane to different intracellular compartments, and reduce receptor density at the cell surface resulting in signal attenuation. Proteins could be endocytosed by utilizing; clathrin-mediated pathway, caveolae-mediated pathway, macropinocytosis, and phagocytosis (19). The MUC1 utilizes these pathways for endocytosis and cell surface localization (20-22) (**Fig. 1C.1**). Like other glycoproteins, MUC are also sorted after their internalization in the early or sorting endosome, where their fates are decided including, their recycling, transportation to the Golgi (retrograde), and proteosomal or lysosomal degradation. This is not only responsible for efficient and regulated cellular metabolism and signal transduction, but is also required for coordinating the functions of each intracellular compartment by maintaining their specific compositions. Intriguingly, the trafficking of MUC and other glycoproteins is mainly regulated by post-translational modifications, including phosphorylation, glycosylation, palmitoylation and ubiquitylation. In this chapter, we provide a perspective on MUC trafficking and its pertinence to pathological conditions.

Figure 1C.1 Diagrammatical representation of the intracellular transport of glycoproteins along endocytic and exocytic pathways. Internalization of cell surface glycoproteins occurs by clathrin-mediated, caveolin-mediated, or clathrin- & caveolin-independent pathways, followed by the fusion of internalized vesicles with early endosomes (EE) where the cargo is sorted and targeted for either recycling (from trans-Golgi, late endosome and recycling endosome) or for degradation (in lysosomes). The other exocytic route are representative of the secretory pathways, where glycoproteins are first synthesized and processed in the rough ER followed by their entry into the Golgi, where they are further modified, packaged and either targeted to the plasma membrane or secreted by the exocytic machinery.

Figure 1C.1

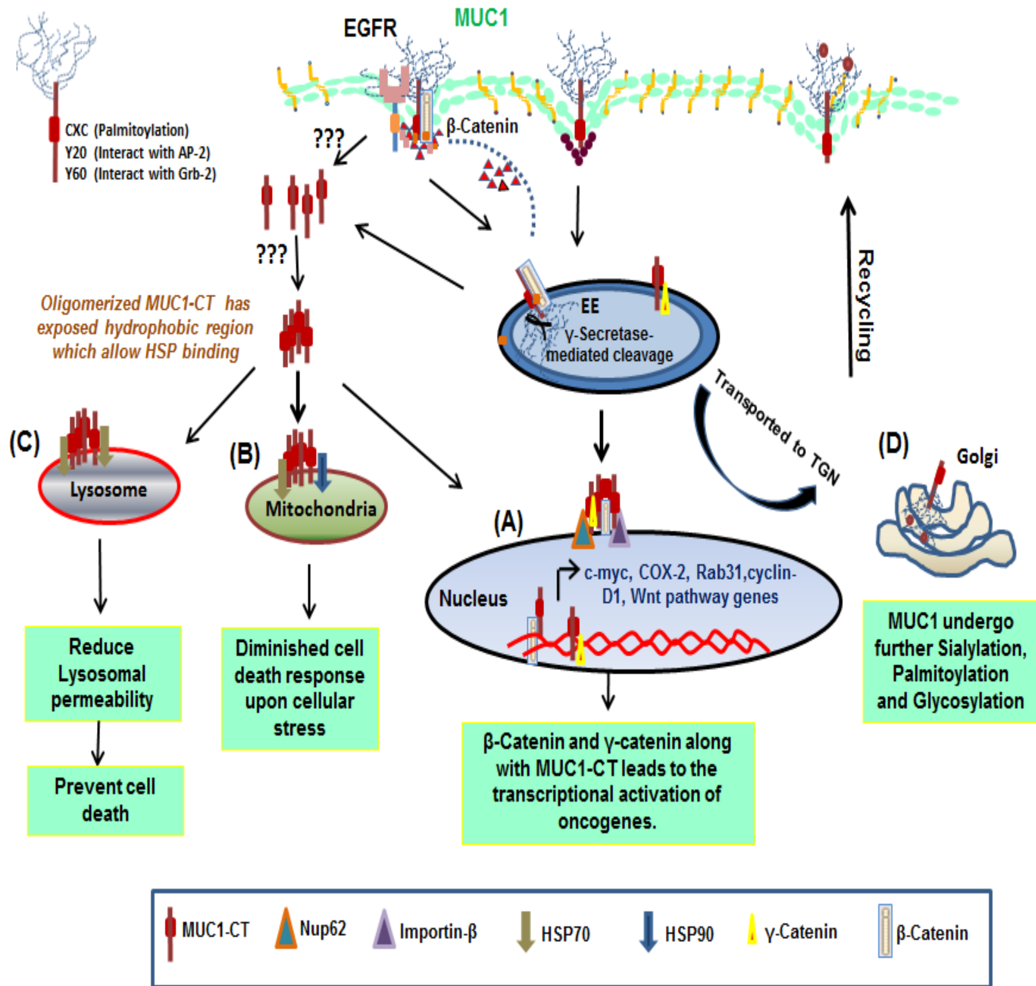


1C.2 Altered localization of MUC and its association with cancer condition

In 1992, Ceriani and colleagues conducted immunohistochemistry (IHC) analysis of MUC1 cytoplasmic and membranous expression/localization on 227 breast cancer patients. They found that low cytoplasmic intensity and high cell surface localization of MUC1 correlated with better prognosis of breast cancer patients and survival (23). This observation was further validated by Rahn *et al.*, who found that increased cell surface MUC1 expression in lower grade and estrogen receptor (ER)-positive tumors have better prognosis, whereas aberrant MUC1 cytoplasmic localization in tumors correlated with worse prognosis (24). Aberrant cytoplasmic MUC1 localization has also been correlated with high-risk papillary thyroid carcinoma (25). In breast ductal adenocarcinomas, MUC2 and MUC5AC are localized in cytoplasm with granular staining pattern (14, 26, 27), whereas distribution of MUC5B expression changes from apical localization in non-malignant breast cells to cytoplasmic and non-apical localization in malignant ductal breast carcinoma (28). Similarly, MUC3 cell surface expression has been correlated with poor prognosis, higher grade and negative ER expression in breast carcinoma (29). These studies clearly demonstrate that, aberrant localization of MUC is associated with cancer pathology (14, 23, 25, 26), and therefore, it is essential to investigate the mechanisms that alter trafficking of MUC among different subcellular compartments. So far, no definite mechanism has been established to understand the elevated intracellular presence of MUC in cancer, but different postulations, specifically for MUC1, have been put forth including; its impaired recycling, altered glycosylation, altered endocytosis and other presumed changes in MUC dynamics (**Fig. 1C.2**), which will be discussed in detail in the next sections.

Figure 1C.2: Mechanisms of intracellular transport and sorting of MUC1. MUC1 has demonstrated to be internalization by using clathrin and caveolin-mediated pathway, which is dependent on Rab5a, an early endosome marker. MUC1 has many interacting partners including EGFR family proteins, AP-2, Grb2 and β-catenin. MUC1 possess a γ-secretase cleavage site and cleaved by the same enzyme in early endosome. Cleaved MUC1-C, which is still in contact with β-catenin travels to nucleus to increase the transcription of various genes that are regulated by the TCF promoter. MUC1, like other glycoproteins, undergoes multiple rounds of sialylation and glycosylation while continuing on the itinerary to the Golgi. MUC1 also has a CQCRRK sequence motif, which undergoes palmitoylation. These post-translational modifications and interacting protein partners play important roles in deciding the fate of MUC1.

Figure 1C.2



1C.3 EGFR family receptors

Sustained proliferative signaling is the fundamental trait of cancer hallmarks (30) Normal cells regulate their growth by modulating the expression of growth factors and their receptors at an optimal time and concentration. On the other hand, cancer cells abrupt this crucial regulation in order to grow unrestrictedly (30). Role of aberrant glycoprotein trafficking in this sustained proliferative signaling can be exemplified by the understanding of Epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs) which constitute four receptors; epidermal growth factor receptor (EGFR), erbB2/HER2, erbB3/HER3 and erbB4/HER4 (31). Structurally, these receptors have a glycosylated extracellular N-terminal ligand-binding domain, a transmembrane region, and a C-terminal intracellular domain, which includes the kinase domain and multiple phosphorylation sites. EGFR and erbB4 are considered to be fully functional receptors due to their ability to bind ligands and autophosphorylate C-terminal tails through functional intracellular tyrosine kinase domains (32, 33). On the other hand, erbB2 is unique in that it does not have ligand binding domain, therefore, it has to heterodimerize with other EGFR family members in order to execute active signal transductions. Similar to erbB2, erbB3 needs to heterodimerize, but due to different reason, which is the lack of intrinsic tyrosine kinase activity (32). Interestingly, erbB4 is known for its established ability to transduce direct nuclear signaling by releasing its intracellular fragment that is cleaved by ligand-dependent dual protease (34).

Upon ligand binding (EGF, transforming growth factor-alpha (TGF-α) or amphiregulin), receptors undergo homo- or hetero-dimerization, which is subsequently followed by the autophosphorylation of the receptor in specific tyrosine kinase residues within the cytoplasmic tail (35). The activated receptor recruits signaling complexes and activates the Ras/mitogen-activated protein kinase (MAPK), extracellular signal-

regulated kinase, phosphatidylinositol 3-kinase (PI3K)/Akt, signal transducers and activators of transcription, and phospholipase C gamma pathways (31, 36). These pathways are potent oncogenic regulators of tumor cell growth, invasion, transformation, and survival. Moreover, EGFR-mediated signaling has also been implicated in angiogenesis, promotes invasiveness through matrix metalloproteinases, and stimulates tumor-cell motility that furthers metastasis (37, 38).

1C.4 General mechanism involved in the regulation of EGFR trafficking and signaling

Multiple studies have led us to know that fate of EGFR is dependent on various parameters comprising; the phosphorylation and ubiquitination status of the receptor and pH sensitivity of the ligand bound receptor (39). All these regulatory mechanisms are crucial for EGFR signaling. It is well established that binding of EGF to EGFR is followed by receptor internalization and multi-ubiquitylation by Cbl E3 ligase inside the endosomes while it is concomitantly participating in active cellular signal transductions (40). This multi-ubiquitination facilitates the sorting of EGFR towards the degradative route to lysosomes. Unlike EGF ligand, binding of EGFR to transforming growth factor-alpha (TGF-α) ligand leads to different outcome by avoiding prolonged ubiquitination, and thus hypo-ubiquitylated form would be prevented to take entry into the degradative lysosomal pathway, and prefer its routing directly to the recycling route back to the cell surface which helps cancer cells to accomplish sustain growth and proliferation (41). Interestingly, ubiquitination of the receptor is reliant on pH tolerance of ligand bound receptor. For example; EGF-EGFR binary complex is comparatively stable at low pH of endosomes, and can undergo polyubiquitination by Cbl ubiquitin ligases, followed by their targeting to the lysosomes for degradation (41). On the contrary, increased pH sensitivity of TGF-α, makes it to disassociate rapidly from the receptor and

prevents its polyubiquitination, and finally, facilitates its recycling back to the plasma membrane (41).

Besides ligands, Cbl ubiquitin ligases are also among the primary regulators of RTKs (42). As they act as a negative regulator of RTKs, cancer cells have to come up with different strategies to inhibit their function. For instance, by facilitating its degradation or by the presence of inactivating mutations in the RING domain of Cbl. Approximately, 5% of human myeloid neoplasms have missense mutations, frameshift mutations or deletion in the E3 activity in the RING domain of Cbl resulting in the loss of its activity (43). Overexpression and mutational activation of Src along with overexpressed EGFR, found very frequently in cancers, which constitute one of the mechanisms that lead to Cbl degradation. Src is a non-receptor tyrosine kinase which is known to be activated by various stimuli including, EGFR. Bao and colleagues have reported the involvement of active Src in the obliteration of Cbl functions by inducing its tyrosine phosphorylation and polyubiquitination, with subsequent proteasomal degradation of Cbl (44). This decrease in Cbl protein expression leads to increased EGFR localization on the cell surface, which further facilitates Src activation, and therefore, promotes sustained cell proliferation. Receptor recycling and disintegration of the degradative mechanisms make cells to undergo repeated rounds of EGFR activation. Similar mechanisms have further been explored in other RTKs as well under tumorigenic condition.

1C.5 Role of EGFR family members in PC development and progression

The phosphorylation of protein kinases leads to the activation of multiple signal-transduction pathways, which have a critical role in a many cellular processes, including cell growth, differentiation, and death. In PC, many tyrosine kinases were found to be

degregulated and overexpressed, such as EGFR (ErbB1), HER2/neu (ErbB2), VEGFR2, platelet-derived growth factor-α, FGF-1, CSF- 1 receptor, steroid receptor co-activator, and others. Interestingly, studies have shown that >70% of the known oncogenes and proto-oncogenes in cancer are tyrosine kinases (45). Therefore, blocking tyrosine kinase activity signifies a highly rationale and potential approach to treat cancer.

Among multiple tyrosine kinases which are overexpressed in PC, EGFR axis is known to play the most important role in PC development and progression. EGFR overexpression has been observed in human PDAC and PDAC spontaneous mouse models (46). Primarily, its functions have been associated with increased proliferation and invasiveness of PC. The expression of activated EGFR was undetectable in WT-mice, however, in the KrasLSL-G12D/+; Ptf1aCre/+ mouse model, at ~8 weeks of age, when these mice exhibit metaplasia and mPanIN formation, had detectable expression of activated EGFR (46). In addition to EGFR, levels of transcripts for both EGFR and TGF-α, an EGFR ligand, were significantly upregulated by ~2-fold. Amphiregulin (AREG), another EGFR ligand, was also upregulated relative to WT controls (46). Due to the observed highly prominent immunofluorescence staining of EGFR in larger acinar clusters of KrasG12D pancreata, especially near areas of metaplasia and mPanIN, it seems that activation of EGFR pathway is plausibly an early event in pancreatic tumorigenesis. Additionally, EGFR is found to be critical for acinar cell proliferation and its stimulation of MEK is necessary for trans-differentiation and transformation of acinar cells to a progenitor cell-like, metaplastic ductal epithelial cells. Thereby, EGFR controls the differentiation of neoplastic precursors and participates in pancreatic tumorigenesis (46).

Chronic activation of EGFR alone is sufficient to cause acinar to ducal metaplasia (ADM) both *in vitro* and *in vivo*. Importantly, blocking EGFR activity effectively eradicates KRAS-initiated pancreatic tumorigenesis, with or without pancreatitis induction, due to its critical role in amplifying ERK activation within the pancreas. Although subgroups of patients with defined molecular subtypes did respond to EGFR targeting, overall, the effect of anti-EGFR therapy on the survival of metastatic PDA patients was quite modest (47). Interestingly, $Kras^{G12D}$; p53KO mice exhibited no added survival benefit in response to erlotinib treatment, suggesting towards the involvement of EGFR-independent mechanisms on the development and progression to fully developed PDAC in this spontaneous mouse model. Interestingly, increased MET activity has been observed in minority of EGFR negative tumors, suggesting that MET activation is plausibly required to circumvent EGFR deficiency. Altogether, these studies encourage us to define the essential molecular signals and the exact role of p53 inactivation in order to improve the efficiency and efficacy of EGFR-targeted therapies for the better clinical outcome of PC patients.

1C.6 Modulation of sub-cellular protein trafficking of EGFR family members by mucins

Due to the loss of polarity in cancer, MUC1 and MUC4 localize all over the cell surface, instead of restricted confinement at the apical surface. This allows them to interact with cell surface proteins such as the EGFR family members, which normally exist at the basolateral sides of polarized cells (48, 49). MUC4 has shown to interact with HER2/ErbB2 in ovarian and pancreatic cancers (48, 50). MUC4-ErbB2 complex lead to the activation of various signaling pathways leading to cell proliferation and survival through stimulation of p38 MAPK phosphorylation (51). In the absence of the soluble ligand, the MUC4-ErbB2 complex leads to ErbB2 phosphorylation, which in turn, leads to

the phosphorylation of the ErbB2-ErbB3 heterodimer in the presence of neuregulin (52). MUC4 did not demonstrate interaction with ErbB3 in polarized cells, but loss of polarity also leads to MUC4-ErbB3 interaction.

The tradeoff between phosphorylation and glycosylation (O-GlcNAc) is known to regulate intracellular trafficking of EGFR (53). MUC1 is known to interact with EGFR at plasma membranes of non-polarized breast epithelia which resulted in increased EGFR internalization, reduced lysosomal degradation and increased EGFR recycling back to the plasma membrane (49). Likewise, MUC4 has also shown to interact with the other EGFR family member, HER2, via its EGF-like motifs located at the juxtamembrane domains (54). The EGF-like motif is also present in other MUC like MUC17, and has been implicated in the pathogenesis of colonic inflammation and cancer, can presumably initiates EGFR mediated oncogenic signaling. Interestingly, activated EGFR phosphorylates YEKV motif in MUC1-CT to induce MUC1 interaction with c-Src and β-catenin. MUC1-CT also has a γ-secretase cleavage motif and the cleavage by γ-secretase results in the release of intracellular MUC1-CT to regulate MUC1 mediated cellular proliferation (55). The MUC1-CT and E-cadherin both compete for β-catenin binding due to the loss of the cellular polarity (56, 57). In breast cancer, silencing of galectin-3 strongly enhanced the interaction between MUC1 and EGFR in response to EGF stimulation and the reduced rate of their endocytosis, which leads to the noticeable cell surface localization of MUC1 and EGFR (58). Therefore, possibly galectin-3 overexpression in cancer may be related to the frequently observed intracellular retention of MUC1.

Despite of the absence of a classical mitochondrial localization signal, MUC1-C gets localized to the outer membrane of mitochondria by its interaction with cytosolic chaperones such as HSP70 and HSP90 (59). MUC1-C mitochondrial

localization has been correlated with the diminished cell death response to the DNA damage and other cellular stress by inhibiting the release of cell-death causing factors. Cytosolic sequestration of MUC1 exposes its hydrophobic TM domains that facilitate their binding with chaperones, and thus targeting to the mitochondria. Interestingly, Heregulin (HRG), a ligand of EGFR family receptor family, enhances the association between MUC1-C and HSP90 due to autophosphorylation and activation of c-Src in HCT116/MUC1 cells (60) and facilitates the translocation of MUC1-C to the mitochondria. In breast cancer cells, FGF1 plays similar role in the mitochondrial localization of MUC1 using similar molecular mechanism (56).

Like EGFR family members, β-catenin also resides at the lateral side of the cell. The loss of polarity allows β-catenin to interact with the SAGNGGSSL motif present in MUC1-C and the loss of E-cadherin-mediated cell-to-cell interaction at MUC1 positive sites. Under normal conditions, β-catenin interacts with the similar S*XXXXX*SSL motif of E-cadherin, which is required for the maintenance of the adherent junction. This interaction between β-catenin and MUC1 is regulated by EGFR mediated phosphorylation of the crucial tyrosine residues present on MUC1-CT (61). Additionally, phosphorylation of the serine residue in SPYEKV sequence by glycogen synthase kinase 3β (GSK3β), a site adjacent to the β-catenin binding motif inhibits the interaction between MUC1 and β-catenin (62); whereas c-Src mediated phosphorylation of the tyrosine residue in that same SPYEKV site enhances this interaction (63). MUC1 shows binding affinity to the Armadillo repeats and the non-repetitive COOH-terminal region of β-catenin (64). MUC1 and β-catenin, once in complex, mitigates GSK-3β phosphorylation of β-catenin and translocate to the nucleus to transcriptional activation of various genes implicated in increased carcinogenic potential and metastasis (65). In breast and colon cancers, HRG stimulation facilitates the binding between MUC1-CT

and γ-catenin, allowing MUC1 to function as a vehicle for γ-catenin nuclear translocation (66). These findings indicate that MUC1-CD has crucial functions in integrating signals from the EGFR and Wnt signaling pathways. Unlike in MUC1 and MUC4, the RTK binding motif is not present in MUC16-CT (67). MUC16 secretion is influenced by EGF stimulation through phosphorylation of MUC16-CT (68). MUC16 knockdown in ovarian cancer cell lines caused increased cytoplasmic localization of β-catenin and E-cadherin, and was linked with greater cellular motility and invasiveness (67). In agreement, reduction of MUC16 expression has been related with advanced ovarian cancer (69). Taken together, these studies pointed towards the possibility that the interactions between MUC16, E-cadherin and/or β-catenin permit MUC16 to modulate various signaling pathways.

Bitler *et al.* found evidence that MUC1 has regulatory functions in the trafficking and nuclear activity of EGFR (61). Presence of MUC1 showed enhance interaction between EGFR and phosphorylated RNA polymerase II, which implies that MUC1 can impact the association of EGFR with transcriptional machinery at the promoter region as the loss of MUC1 reduces the occupancy of EGFR at the cyclin D promoter region (61). Besides controlling such inter-molecular interactions, MUC1-C also regulates Rab31 expression, which is an early endosome protein belonging to the subfamily of small GTPase Rab5 (70). MUC1-C and estrogen receptor form a complex at the Rab31 promoter and are responsible for the transcriptional activation of Rab31. According to this study, patients who express MUC1-C and Rab31 are resistant to tamoxifen treatment indicating the possible involvement of these two molecules in determining the efficacy of tamoxifen therapy (70).

1C.7 Summary and Conclusions

MUC are the chief macromolecular components of epithelial mucus and have been incriminated in the pathogenesis of various diseases. Their mislocalization has been well associated with the pathobiology of several cancers such as, breast and colorectal cancer. Under normal condition, MUC are localized predominantly on the apical surface, but loss of polarity allows them to extend all over the cell. It favors multiple MUC-protein interactions that suppose not to occur in a polarized cell. Several unique domains present in MUC play crucial important role in determining these interactions. Mislocalization of MUC also facilitates MUC interactions with other novel proteins, which further help MUC as well as other proteins (Y-catenin) to translocate to the different subcellular compartments. Though many postulations have been kept forth to attribute the altered localization of MUC including altered glycosylation, sialylation, and differential protein-protein interactions, still the exact mechanism behind it has not been explored and need immediate attention to make better therapeutic interventions.

1C.8 Reference List

(1) Kufe DW. Mucins in cancer: function, prognosis and therapy. Nat Rev Cancer 2009;9:874-85.

(2) Senapati S, Das S, Batra SK. Mucin-interacting proteins: from function to therapeutics. Trends Biochem Sci 2010;35:236-45.

(3) Rachagani S, Torres MP, Moniaux N, Batra SK. Current status of mucins in the diagnosis and therapy of cancer. Biofactors 2009;35:509-27.

(4) 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.

(5) Chaturvedi P, Singh AP, Batra SK. Structure, evolution, and biology of the MUC4 mucin. FASEB J 2008;22:966-81.

(6) Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth M, Buchler MW, Aubert JP, Batra SK. Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitits: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res 2001;7:4033-40.

(7) 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. PLoS One 2013;8:e54455.

(8) Senapati S, Chaturvedi P, Sharma P, Venkatraman G, Meza JL, El-Rifai W, Roy HK, Batra SK. Deregulation of MUC4 in gastric adenocarcinoma: potential pathobiological implication in poorly differentiated non-signet ring cell type gastric cancer. Br J Cancer 2008;99:949-56.

(9) Chauhan SC, Singh AP, Ruiz F, Johansson SL, Jain M, Smith LM, Moniaux N, Batra SK. Aberrant expression of MUC4 in ovarian carcinoma: diagnostic significance alone and in combination with MUC1 and MUC16 (CA125). Mod Pathol 2006;19:1386-94.

(10) Saitou M, Goto M, Horinouchi M, Tamada S, Nagata K, Hamada T, Osako M, Takao S, Batra SK, Aikou T, Imai K, Yonezawa S. MUC4 expression is a novel prognostic factor in patients with invasive ductal carcinoma of the pancreas. J Clin Pathol 2005;58:845-52.

(11) Shibahara H, Tamada S, Higashi M, Goto M, Batra SK, Hollingsworth MA, Imai K, Yonezawa S. MUC4 is a novel prognostic factor of intrahepatic cholangiocarcinoma-mass forming type. Hepatology 2004;39:220-9.

(12) Singh AP, Chauhan SC, Bafna S, Johansson SL, Smith LM, Moniaux N, Lin MF, Batra SK. Aberrant expression of transmembrane mucins, MUC1 and MUC4, in human prostate carcinomas. Prostate 2006;66:421-9.

(13) Kaur S, Momi N, Chakraborty S, Wagner DG, Horn AJ, Lele SM, Theodorescu D, Batra SK. Altered expression of transmembrane mucins, MUC1 and MUC4, in bladder cancer: pathological implications in diagnosis. PLoS One 2014;9:e92742.

(14) Diaz LK, Wiley EL, Morrow M. Expression of epithelial mucins Muc1, Muc2, and Muc3 in ductal carcinoma in situ of the breast. Breast J 2001;7:40-5.

(15) Schroeder JA, Masri AA, Adriance MC, Tessier JC, Kotlarczyk KL, Thompson MC, Gendler SJ. MUC1 overexpression results in mammary gland tumorigenesis and prolonged alveolar differentiation. Oncogene 2004;23:5739-47.

(16) Tsutsumida H, Swanson BJ, Singh PK, Caffrey TC, Kitajima S, Goto M, Yonezawa S, Hollingsworth MA. RNA interference suppression of MUC1 reduces the growth rate and metastatic phenotype of human pancreatic cancer cells. Clin Cancer Res 2006;12:2976-87.

(17) Kinlough CL, McMahan RJ, Poland PA, Bruns JB, Harkleroad KL, Stremple RJ, Kashlan OB, Weixel KM, Weisz OA, Hughey RP. Recycling of MUC1 is dependent on its palmitoylation. J Biol Chem 2006;281:12112-22.

(18) McDermott AE. Structural and dynamic studies of proteins by solid-state NMR spectroscopy: rapid movement forward. Curr Opin Struct Biol 2004;14:554-61.

(19) Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. Nat Rev Mol Cell Biol 2007;8:603-12.

(20) Altschuler Y, Kinlough CL, Poland PA, Bruns JB, Apodaca G, Weisz OA, Hughey RP. Clathrin-mediated endocytosis of MUC1 is modulated by its glycosylation state. Mol Biol Cell 2000;11:819-31.

(21) Lloyd KO, Burchell J, Kudryashov V, Yin BW, Taylor-Papadimitriou J. Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. Demonstration of simpler and fewer glycan chains in tumor cells. J Biol Chem 1996;271:33325-34.

(22) Liu X, Yuan Z, Chung M. MUC1 intra-cellular trafficking is clathrin, dynamin, and rab5 dependent. Biochem Biophys Res Commun 2008;376:688-93.

(23) Ceriani RL, Chan CM, Baratta FS, Ozzello L, DeRosa CM, Habif DV. Levels of expression of breast epithelial mucin detected by monoclonal antibody BrE-3 in breast-cancer prognosis. Int J Cancer 1992;51:343-54.

(24) Rahn JJ, Dabbagh L, Pasdar M, Hugh JC. The importance of MUC1 cellular localization in patients with breast carcinoma: an immunohistologic study of 71 patients and review of the literature. Cancer 2001;91:1973-82.

(25) Bieche I, Ruffet E, Zweibaum A, Vilde F, Lidereau R, Franc B. MUC1 mucin gene, transcripts, and protein in adenomas and papillary carcinomas of the thyroid. Thyroid 1997;7:725-31.

(26) Pereira MB, Dias AJ, Reis CA, Schmitt FC. Immunohistochemical study of the expression of MUC5AC and MUC6 in breast carcinomas and adjacent breast tissues. J Clin Pathol 2001;54:210-3.

(27) Lau SK, Weiss LM, Chu PG. Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study. Am J Clin Pathol 2004;122:61-9.

(28) Sonora C, Mazal D, Berois N, Buisine MP, Ubillos L, Varangot M, Barrios E, Carzoglio J, Aubert JP, Osinaga E. Immunohistochemical analysis of MUC5B apomucin expression in breast cancer and non-malignant breast tissues. J Histochem Cytochem 2006;54:289-99.

(29) Rakha EA, Boyce RW, Abd El-Rehim D, Kurien T, Green AR, Paish EC, Robertson JF, Ellis IO. Expression of mucins (MUC1, MUC2, MUC3, MUC4, MUC5AC and MUC6) and their prognostic significance in human breast cancer. Mod Pathol 2005;18:1295-304.

(30) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.

(31) Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. Cell Mol Life Sci 2008;65:1566-84.

(32) Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. Exp Cell Res 2003;284:54-65.

(33) Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 1997;16:1647-55.

(34) Schlessinger J, Lemmon MA. Nuclear signaling by receptor tyrosine kinases: the first robin of spring. Cell 2006;127:45-8.

(35) Linggi B, Carpenter G. ErbB receptors: new insights on mechanisms and biology. Trends Cell Biol 2006;16:649-56.

(36) Folgiero V, Bachelder RE, Bon G, Sacchi A, Falcioni R, Mercurio AM. The alpha6beta4 integrin can regulate ErbB-3 expression: implications for alpha6beta4 signaling and function. Cancer Res 2007;67:1645-52.

(37) Hudson LG, Zeineldin R, Silberberg M, Stack MS. Activated epidermal growth factor receptor in ovarian cancer. Cancer Treat Res 2009;149:203-26.

(38) Hudson LG, Moss NM, Stack MS. EGF-receptor regulation of matrix metalloproteinases in epithelial ovarian carcinoma. Future Oncol 2009;5:323-38.

(39) Tomas A, Futter CE, Eden ER. EGF receptor trafficking: consequences for signaling and cancer. Trends Cell Biol 2014;24:26-34.

(40) Goh LK, Sorkin A. Endocytosis of receptor tyrosine kinases. Cold Spring Harb Perspect Biol 2013;5:a017459.

(41) Macdonald-Obermann JL, Pike LJ. Different epidermal growth factor (EGF) receptor ligands show distinct kinetics and biased or partial agonism for homodimer and heterodimer formation. J Biol Chem 2014;289:26178-88.

(42) Thien CB, Langdon WY. c-Cbl and Cbl-b ubiquitin ligases: substrate diversity and the negative regulation of signalling responses. Biochem J 2005;391:153-66.

(43) Kales SC, Ryan PE, Nau MM, Lipkowitz S. Cbl and human myeloid neoplasms: the Cbl oncogene comes of age. Cancer Res 2010;70:4789-94.

(44) Bao J, Gur G, Yarden Y. Src promotes destruction of c-Cbl: implications for oncogenic synergy between Src and growth factor receptors. Proc Natl Acad Sci U S A 2003;100:2438-43.

(45) Hahne JC, Kummer S, Heukamp LC, Fuchs T, Gun M, Langer B, Von RA, Wernert N. Regulation of protein tyrosine kinases in tumour cells by the transcription factor Ets-1. Int J Oncol 2009;35:989-96.

(46) Ardito CM, Gruner BM, Takeuchi KK, Lubeseder-Martellato C, Teichmann N, Mazur PK, Delgiorno KE, Carpenter ES, Halbrook CJ, Hall JC, Pal D, Briel T, Herner A,

Trajkovic-Arsic M, Sipos B, et al. EGF receptor is required for KRAS-induced pancreatic tumorigenesis. Cancer Cell 2012;22:304-17.

(47) Collisson EA, Sadanandam A, Olson P, Gibb WJ, Truitt M, Gu S, Cooc J, Weinkle J, Kim GE, Jakkula L, Feiler HS, Ko AH, Olshen AB, Danenberg KL, Tempero MA, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. Nat Med 2011;17:500-3.

(48) Chaturvedi P, Singh AP, Chakraborty S, Chauhan SC, Bafna S, Meza JL, Singh PK, Hollingsworth MA, Mehta PP, Batra SK. MUC4 mucin interacts with and stabilizes the HER2 oncoprotein in human pancreatic cancer cells. Cancer Res 2008;68:2065-70.

(49) Pochampalli MR, el Bejjani RM, Schroeder JA. MUC1 is a novel regulator of ErbB1 receptor trafficking. Oncogene 2007;26:1693-701.

(50) Ponnusamy MP, Singh AP, Jain M, Chakraborty S, Moniaux N, Batra SK. MUC4 activates HER2 signalling and enhances the motility of human ovarian cancer cells. Br J Cancer 2008;99:520-6.

(51) Funes M, Miller JK, Lai C, Carraway KL, III, Sweeney C. The mucin Muc4 potentiates neuregulin signaling by increasing the cell-surface populations of ErbB2 and ErbB3. J Biol Chem 2006;281:19310-9.

(52) Ramsauer VP, Pino V, Farooq A, Carothers Carraway CA, Salas PJ, Carraway KL. Muc4-ErbB2 complex formation and signaling in polarized CACO-2 epithelial cells indicate that Muc4 acts as an unorthodox ligand for ErbB2. Mol Biol Cell 2006;17:2931-41.

(53) Kaleem A, Ahmad I, Hoessli DC, Walker-Nasir E, Saleem M, Shakoori AR, Nasir UD. Epidermal growth factor receptors: function modulation by phosphorylation and glycosylation interplay. Mol Biol Rep 2009;36:631-9.

(54) Jonckheere N, Skrypek N, Merlin J, Dessein AF, Dumont P, Leteurtre E, Harris A, Desseyn JL, Susini C, Frenois F, Van S, I. The mucin MUC4 and its membrane partner ErbB2 regulate biological properties of human CAPAN-2 pancreatic cancer cells via different signalling pathways. PLoS One 2012;7:e32232.

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

(56) Ren J, Raina D, Chen W, Li G, Huang L, Kufe D. MUC1 oncoprotein functions in activation of fibroblast growth factor receptor signaling. Mol Cancer Res 2006;4:873-83.

(57) 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.

(58) Argueso P, Guzman-Aranguez A, Mantelli F, Cao Z, Ricciuto J, Panjwani N. Association of cell surface mucins with galectin-3 contributes to the ocular surface epithelial barrier. J Biol Chem 2009;284:23037-45.

(59) Ren J, Agata N, Chen D, Li Y, Yu WH, Huang L, Raina D, Chen W, Kharbanda S, Kufe D. Human MUC1 carcinoma-associated protein confers resistance to genotoxic anticancer agents. Cancer Cell 2004;5:163-75.

(60) Ren J, Bharti A, Raina D, Chen W, Ahmad R, Kufe D. MUC1 oncoprotein is targeted to mitochondria by heregulin-induced activation of c-Src and the molecular chaperone HSP90. Oncogene 2006;25:20-31.

(61) Bitler BG, Goverdhan A, Schroeder JA. MUC1 regulates nuclear localization and function of the epidermal growth factor receptor. J Cell Sci 2010;123:1716-23.

(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) Huang L, Chen D, Liu D, Yin L, Kharbanda S, Kufe D. MUC1 oncoprotein blocks glycogen synthase kinase 3beta-mediated phosphorylation and degradation of beta-catenin. Cancer Res 2005;65:10413-22.

(65) Huang L, Ren J, Chen D, Li Y, Kharbanda S, Kufe D. MUC1 cytoplasmic domain coactivates Wnt target gene transcription and confers transformation. Cancer Biol Ther 2003;2:702-6.

(66) Li Y, Yu WH, Ren J, Chen W, Huang L, Kharbanda S, Loda M, Kufe D. Heregulin targets gamma-catenin to the nucleolus by a mechanism dependent on the DF3/MUC1 oncoprotein. Mol Cancer Res 2003;1:765-75.

(67) Comamala M, Pinard M, Theriault C, Matte I, Albert A, Boivin M, Beaudin J, Piche A, Rancourt C. 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.

(68) Konishi I, Fendrick JL, Parmley TH, Quirk JG, Jr., O'Brien TJ. Epidermal growth factor enhances secretion of the ovarian tumor-associated cancer antigen CA125 from the human amnion WISH cell line. J Soc Gynecol Investig 1994;1:89-96.

(69) Hogdall EV, Christensen L, Kjaer SK, Blaakaer J, Kjaerbye-Thygesen A, Gayther S, Jacobs IJ, Hogdall CK. CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients. From The Danish "MALOVA" Ovarian Cancer Study. Gynecol Oncol 2007;104:508-15.

(70) Jin C, Rajabi H, Pitroda S, Li A, Kharbanda A, Weichselbaum R, Kufe D. Cooperative interaction between the MUC1-C oncoprotein and the Rab31 GTPase in estrogen receptor-positive breast cancer cells. PLoS One 2012;7:e39432.

General Hypothesis and Objectives

Over the past few decades, multiple studies have established key roles of mucins in malignant diseases. The expression of mucins is significantly altered during tumorigenesis and other pathological conditions. In this dissertation, I have primarily focused on MUC4, which is one of the most differentially expressed proteins in PC and has strongly been implicated in the progression, metastasis and chemoresistance of PC. MUC4 is not expressed in the normal pancreas, but the early pancreatic intraepithelial neoplasia (PanINs) precursor lesions have been shown to express MUC4, which further increases as the disease progresses. The ability of NIH3T3 fibroblasts cells to form tumors in nude mice upon ectopic expression of MUC4 was the first evidence which has experimentally proved its oncogenic function. Considering the significant role of MUC4 in tumor biology, additional studies are required to highlight its novel functions and regulatory mechanisms. Although studies have associated extrinsic (cytokines) and intrinsic factors (NCOA3) with the regulation of MUC4, there is no study which has addressed the role of PC microenvironmental stress (hypoxia and oxidative stress) on MUC4 expression. Both Hypoxia and MUC4 has been associated with PC aggressiveness and chemoresistance. Moreover, hypoxia has been shown to regulate mucins expression in solid tumors. All these studied led me to hypothesize that hypoxia has a significant impact on MUC4 expression in PC, which aggravate the PC conditions. Besides PC microenvironment, the critical anatomical position of pancreas can influence the growth of pancreatic tumors. However, these mechanisms are unexplored. The majority of tumors (about 75%) arise at the head of the pancreas. Most of the PC patients develop extrahepatic cholestasis due to common bile duct obstruction by increasing tumor size which results in hyperbilirubinemia and elevated circulatory levels of bile acids (BA). Multiple studies have implicated BA as tumor promoter for various

cancers. A recently performed meta-analysis has shown that patients with the history of cholecystectomy have significantly higher risk to develop PDAC. These studies incited me to hypothesize that BA play important role in PC tumorigenesis by regulating the expression MUC4 oncogene.

In addition to regulation, I have also focused to elucidate the novel functional properties of MUC4 in PC. MUC4 is known to regulate the fate of EGFR family proteins in several cancers including PC. However the precise mechanism involved is still ascertain. Emerging reports have shown altered expression of RAB proteins in various cancers. Additionally, a recent study has shown that mucins can also regulate the expression of RABs to influence the trafficking of oncogenic proteins in cancer. It brought me to my next hypothesis that MUC4 determines the fate of EGFR family members by modulating the expression and activity of RAB GTPases in PC. In addition to PC cells, MUC4 expression has recently been detected in activated PaSC. Interestingly, our preliminary studies have shown reduction of MUC4 expression upon treatment with RA, which is known to change the status of activated PaSC to quiescent, suggesting a plausible link between MUC4 expression and activation status of PaSC. It led me to hypothesize that MUC4 regulates the activation status of PaSC and thereby, promotes desmoplastic reactions in PC microenvironment, which is known to exacerbate PC condition.

Broadly, the aims for my dissertation research were as follows:

1. To elucidate the role of microenvironment stress (hypoxia and oxidative stress) on MUC4 regulation in PC.

2. To investigate the impact of bile acids (BA) on MUC4 expression in PC.

3. To identify the novel functions of MUC4 in epithelial (ductal tumor) and nonepithelial (PaSC) cells under PC condition.

CHAPTER II

Materials and methods

II.1 Cell culture: All PC (CAPAN1, Colo357, HPAC, AsPC1, MIA PaCa-2, and Panc1) and LS180 colon cancer cell lines were obtained from American Type Culture Collection [ATCC] (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin (100 μg/ml)) at 37°C with 5% CO_2 and were tested mycoplasma-free before conducting the experiments. CD18/HPAF is a metastatic clone derived from the HPAF cell line (1), whereas T3M4 cell line is derived from lymph node metastasis of pancreatic exocrine adenocarcinoma (2). T3M4 PC cell line was a gift from Dr. RS Metzgar (Duke University, Durham, NC) and cultured in 10% DMEM media. UMSCC1 and UMSCC10B (head & neck cancer) cell lines were a kind gift from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI, USA) and cultured in 10% minimal essential medium (Invitrogen, Carlsbad, CA). Human ductal pancreatic epithelial (HDPE) cells were a kind gift of Dr. Thiru Arumugam (MD Anderson, Houston, Texas) and cultured in keratinocyte serum-free (KSF) medium supplemented with epidermal growth factor and bovine pituitary extract. The method of generation and maintenance of stable clones of MiniMUC4 expressing MIA PaCa-2 and MUC4 kd CAPAN1 cells have been described previously (3, 4). Pancreatic stellate cells (PaSC) derived from PC patient and immortalized with E6/E7 were obtained from Dr. Pankaj K. Singh (UNMC) and were cultured in 10% DMEM.

II.2 Procurement of human and murine PDAC samples: Formalin-fixed and paraffin-embedded PC Whipple tissue specimen and Rapid Autopsy (IRB-091-01) tissue array (consisting of 3 normal pancreas, 25 primary PC with 1 colon and 1 kidney as controls) were obtained from University of Nebraska Medical Center and used for immunohistoflorescence analysis. For mRNA expression profiling, frozen PC tissues were obtained from cooperative human tumor network (CHTN) and UNMC.

The detailed information for pancreatic juice collection from PC patients has been provided in our previous publication (5). Plasma samples were collected using an Institutional Review Board (IRB number PRO07030072) approved protocol at University of Pittsburgh (Pittsburgh, PA) from PC patients. Further details of the study design (including exclusion and inclusion criteria) have been mentioned earlier (6). A written informed consent was obtained from all patients and controls prior to entrance into the study. From triple-transgenic animals, $Kras^{G12D/+}$; $p53^{R172H/+}$; Pdx1-Cre (KPC), and their contemporary littermates, mice were sacrificed and blood collection was done at 5, 7, 10, 15, 20 and 25 weeks (wk) of age. The mouse model was maintained at UNMC by crossing LSL-KrasG12D with LSL-Trp53R172H/+ transgenic mice as described previously (7, 8). Whipple tissue specimens were obtained from UNMC and used for immunohistoflorescence analysis. For mRNA expression profiling, frozen PC tissues were obtained from cooperative human tumor network (CHTN) and UNMC (IRB- 491-97). For tissues obtained through UNMC, a written informed consent was obtained for all non-archival tissue before their collection.

II.3 Treatment of cells with hypoxia and pharmacological reagents

Chapter 3

Hypoxic exposure was carried out at 37°C in a humidified incubator (CoyLab, MI, USA) with 94% N_2, 5% CO_2, and 1% O_2. For inhibition studies, before hypoxia or H_2O_2 exposure, cells were treated with pharmacologic inhibitors: MG132 (Sigma Aldrich, St. Louis, MO), CHX (Sigma), YC-1 (Sigma), NAC (Sigma), α-TS (Sigma), VB (Sigma) and RAP (Sigma).

Chapter 4

Deoxycholic (DCA) and chenodeoxycholic acid (CDCA) were dissolved in sterile ethanol. For inhibition studies, wortamannin (phosphoinositide 3-kinase (PI3K) inhibitor, 1 μM, Cell Signaling Technology), SP100625 (JNK inhibitor; 35 μM, Merck Millipore), FAK

inhibitor 14 (FAK inhibitor, 15 μM, Cayman's chemical), U1026 (MAPK inhibitor, 10 μM, Promega) and actinomycin-D (2 μg/ml, Sigma) were given 1h prior to BA treatment.

Chapter 5

CBP: CREB interaction was prevented by using 20 μM of selective pharmacological inhibitor (CAS 92-78-4-Calbiochem) to block CREB-mediated upregulation of Rab5A gene. CAPAN1 and CD18/HPAF cells were cultured in serum free media 12h prior to treatment. Following, cells were treated with 200 nM of insulin (CAS Number 11061-68-0, Sigma) for 4h and RNA isolation was performed.

Chapter 6

PaSC cells were serum starved for 8h and then overnight RA (Sigma) treatment was given at indicated concentrations.

II.4 Knocked down and overexpression experiments: Transient knocked down of HIF-1α and ATG7 in PC cell lines was done by established targeted ShRNA sequence and commercially available siRNA oligonucleotides (Cell Signaling Technology, Catalogue no. 6604 S), respectively, by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To transiently knockdown FXR, commercially available FXR siRNA (Santa Cruz biotechnologies (SCB), Dallas, TX, USA) were used. For transient overexpression of RAB5A in PC cells, we used commercially available mRFP-Rab5A construct (addgene). For transfection purposes, lipofectamine 2000 (Life Technologies; Carlsbad, CA, USA) was used, according to the manufacturer's protocol.

MUC4 was stably knockdown in PaSC using targeted sh-RNA constructs: Shmuc4-1(referred as Sh-1 MUC4):5'AGCTTAAAAAGGAGATGGCTATTTCGAAATCTC TTGAATTTCGAAATAGCCATCTCCGGG-3'and shmuc4-31 (referred as Sh-3 MUC4 on chapter 6) 5'AGCTTAAAAAGCATGAAACTCGACGCGTTTCTCTTGAAAACGCGTCGA GTTTCATGCGGG-3' construct (pSUPER-Retro-sh-MUC4) in PaSC. Scramble control (sh-control) and pSUPER-Retro-sh-MUC4 has been transfected into packaging cell

Phoenix using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48h post transfection, viral particles were collected and used to infect PaSC cells. Pooled population of MUC4 kd cells were obtained using antibiotic selection (Puromycin 4 μg/ml), and were further expanded to confluent levels to obtain stably transfected cells.

II.5 Immunoblotting: Immunoblot analysis was performed as described previously (9). Briefly, cells were lysed with radioimmunoprecipitation buffer (50 mM Tris-HCl pH-7.5, 150 mM NaCl, 1% Nonidet P- 40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with complete protease inhibitor mixture (Roche), 2mM Na_3VO_4, 10mM NaF and 1mM PMSF on ice. Cell lysates were cleared by centrifugation and quantified using the bicinchoninic acid method. Due to high-molecular weights of MUC4 (~ 950 kDa) and MUC1 (~ 250 kDa), electrophoresis was performed on 2% SDS-agarose gel, whereas molecules less than 250 kDa were resolved by SDS–PAGE under reducing conditions and blotted onto a polyvinylidene difluoride membrane membrane (Millipore). Membranes were incubated overnight at 4^0C with primary antibodies (**Table 2A**). Blots were washed and probed with peroxidase-conjugated secondary antibodies, and the bands were visualized using enhanced chemiluminescence (ECL) method (Thermoscientific).

II.6 Isolation of cytoplasmic and nuclear fractions: Briefly, after 4h of 50 μM of DCA or CDCA treatment, CD18/HPAF cells were washed with ice-cold PBS and incubated with a cytoplasmic extraction buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 0.2% NP-40, 0.1 mM EDTA, 10% glycerol, 1.5 mM $MgCl_2$, supplemented with protease inhibitor, 1 mM DTT, 1 mM PMSF, 5 mM Na_3VO_4, 5 mM NaF) for 1h at 4^0C. Cells were centrifuged at 800 × g and the supernatant was labeled as cytoplasmic extract, and the remaining pellet was washed with PBS and then incubated for 1h with the nuclear extraction buffer (20 mM HEPES (pH 7.6), 420 mM NaCl, 1 mM EDTA, 20% glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 5 mM Na_3VO_4, 5 mM NaF). Following incubation, the pellet was

sonicated for 10 s at 60% amplitude, and then subjected to centrifugation at 13,000 xg for 10 min. The obtained supernatant was collected as a nuclear extract. The purity of the fractions were checked by analyzing the expression of SP1 (nuclear protein) and GAPDH (cytoplasmic protein) in collected lysates (**Table 2.A**).

II.7 Confocal Immunofluorescence microscopy: PC cells ($1X10^5$) were grown on sterilized cover slips for 24h and treated with appropriate vehicle control (media or DMSO), $CoCl_2$ (150 μM), H_2O_2, hypoxia and VB, and further incubated for 24h. Following treatment, cells were fixed with 4% formaldehyde, permeabilized (0.2% saponin), blocked (with normal goat serum) and incubated with the primary antibodies. For immunohistofluorescence, we deparaffinized tissue sections with xylene, rehydrated with decreasing concentrations of ethanol and incubated tissues for 30 min. with 3% H_2O_2: methanol solution. Tissues sections were blocked in 2.5% horse serum for 2h and incubated with primary antibodies (**Table 2A**). Following primary antibody incubation, cells were washed and incubated with FITC and Texas red conjugated secondary antibodies.

To label autophagic vacuoles, hypoxia and H_2O_2-treated PC cells were incubated with 50 μM of MDC (Sigma) at 37°C for 10 minutes (10). After incubation, cells are washed four times with PBS; coverslips were mounted and immediately analyzed. All the images were taken by using LSM 510 microscope, a laser scanning confocal microscope (Carl Zeiss GmbH, Thornwood, NY) in the respective channels. The intensity colocalization plot was made by using the Zen lite 2012 software. Image J software was used to determine the Pearson correlation coefficient and MFI values for both 8-OHG and MUC4. For box plot, the fluorescence intensities (FI) of 8-OHG were sorted, according to the median FI of MUC4. Values more than median FI of MUC4 is considered as MUC4 high or $MUC4^H$ and values lower than median FI was taken as MUC4 low or $MUC4^L$.

II.8 Immunohistochemical staining: Paraffin embedded mouse and human tissues were deparaffinised in xylene for 4 X 10 min washes, followed by rehydration through graded ethanol. To block endogenous peroxidase, tissue slides were kept in 3% hydrogen peroxide/methanol for 30 min. 0.01 M preheated citrate buffer (pH −6.0, 90°C) was used for antigen retrieval for 15 min, and the slides were cooled and then washed with PBS. Following, slides were blocked using horse serum (ImmPRESS kit; Vector Labs) for 2 h and sections were incubated overnight at 4°C with primary antibodies (**Table 2A**) diluted in PBS. Slides were washed with PBS, incubated with the secondary antibody (peroxidase-labeled universal anti-mouse/anti-rabbit IgG, ImmPRESS kit; Vector Labs) for 1h at RT and then washed four times in PBS and were developed using DAB as substrate (DAB substrate kit; Vector Labs). The sections were counterstained with haematoxylin (Vectors Lab) and washed in tap water, dehydrated in increasing grades of alcohol (20–100%), washed with xylene for 5 min and dried overnight at RT. Lastly, slides were mounted using paramount mounting medium (Fisher Scientific) and images were obtained by using Nikon Eclipse E400 light microscope (Kawasaki, Japan).

II.9 Measurement of florescence to analyze ROS levels and autophagic vacuoles: To analyze ROS production, PC cells were incubated with 1μM 2′-7′-Dichlorofluorescein diacetate (DCFDA) (Sigma) for 15 min (11). After three washes with PBS, cells were collected in 500 μl of PBS and analyzed (at 488 nm) using flow cytometry.

For the detection of autophagic vacuoles (positive for MDC) (Suppl. Fig 4A), cell lines were incubated with 50 μM of MDC at 37°C for 10 minutes. Following, cells were washed thrice with PBS and florescence was measured immediately at 300 nm using florescence reader (Biotek, SMATBLD). To quantify number of apoptotic and necrotic cells, PC cells were serum starved for 12h, followed by 48h treatment of CD18/HPAF cells with 1% hypoxia, either alone or in the presence of NAC (2.5 mM) or CQ (50 uM).

After the completion of treatment, Annexin-V-cy5 and propidium iodide (PI) (BD biosciences) staining were performed and analyzed by flow cytometry.

II.10. RT- PCR: RNA was isolated using QIAGEN RNeasy mini kit (Qiagen, Valenica, CA, U.S.A.) and cDNA was synthesized using 2 μg RNA, random hexamer primers, and Super Script II RNase reverse transcriptase (Invitrogen) and quantitative real-time PCR (qRT-PCR) was performed using gene specific primers (**Table 2B**) using the standardized protocol established in our lab, using SYBR Green chemistry. β-actin was used as an internal control. The relative fold differences in gene expression were calculated using the ΔΔCt method with β-actin as a normalization control (12). For reverse transcriptase-PCR (RT-PCR), RNA was isolated and cDNA was prepared as mentioned earlier for qRT-PCR, using the following steps: initial denaturation at 95°C for 5 min., followed by 35 programmed cycles at 95°C for 1 min., 58°C for 1.5 min. and 72°C for 1 min., with a final incubation at 72°C for 10 min. The amplified product was detected by electrophoresis on 2% agarose gels.

II.11 Quantitative chromatin immunoprecipitation (ChIP): PC cells (CD18/HPAF) were starved of serum for 8h and treated with DCA or CDCA for 4h. Afterward, the ChIP experiment was performed as described previously (13) and has been repeated more than three times. In detail, 1% formaldehyde was used to cross-linked chromatin, which was isolated and sheared into 500–1000 bp fragments by sonication (Bioruptor UCD-200, Diagenode; New York, NY, USA). As an input, 1% of the sonicated DNA was used. The remaining sonicated DNA fraction was used for the pull down experiment. The concentrations of antibodies used for overnight incubation at 4°C were as follows: 5 μg of anti-c-Jun (SCB#1694X) and IgG (negative control). MUC4 promoter targeted primers were used to amplify and study the enrichment of the fragmented DNA using real-time qPCR. The details of the primers are given in **Table 2C**. Immunoprecipitated qPCR Ct (cycle threshold) values were normalized to input Ct values.

II.12 Total BA estimation method: To analyze total BA concentration in pancreatic juice and plasma samples, we used a highly sensitive bile acid estimation assay kit (Diazyme, NBT, DZO92A-k). To increase the precision of the test, each sample was analyzed in triplicates. We used deoxycholic acid for making the reference plot and were serially diluted from 1.25 μ mol/L to 150 μ mol/L. After completing the BA estimation assay according to the manufacturer's protocol, ELISA plates were read at 405 nm and the collected data was analyzed using SOFTMAX PRO software (Molecular Devices Corp., Sunnyvale, CA).

II.13 Growth inhibition and growth kinetics assay

Chapter 3

For the growth inhibition assay, 5 X 10^3 PC cells were plated onto flat-bottomed 96-well plates (Costar, Corning, NY). After 24h, cells were treated with 1% hypoxia and indicated concentrations of H_2O_2, NAC and VB for an additional 24h. Subsequently, MTT assay was performed as per the standard procedure.

For growth kinetics assay, 50 X 10^3 PC cells were plated in triplicates into six-well plates in triplicates and cultured in serum-free DMEM media for 12h. Following, cells were first pre-treated with NAC (2.5 mM) for 30 mins and then incubated with 1% hypoxia or oxidative stress condition (H_2O_2). Cells were counted at indicated time-points by using automated cell counter (Invitrogen, Countess™).

Chapter 5

20 x 10^3 scr and MUC4 kd CAPAN1 cells were plated in triplicates into six-well and cultured in serum-free DMEM media for 12h. Following, cells were treated with EGF (20ng/ml) containing DMEM media supplemented with 2% FBS. Cells were counted at indicated time-points by using automated cell counter (Invitrogen, Countess™).

Chapter 6

For growth kinetics assay, 50 X 10^3 PaSC cells were plated in triplicates into six-well plates in triplicates and cultured in serum-free DMEM media for 12h. Following, cells were cultured in 2% serum containing DMEM media and counted at indicated time-points by using automated cell counter (Invitrogen, Countess^{TM}).

II.14 Anchorage-dependent colony formation assay: Briefly, PaSC cells were plated at densities of 2000 cells per well in 6-well plates and cultured in regular DMEM medium. After overnight incubation, unattached cells were removed and attached cells were fed with fresh regular medium every three days for 2 weeks. At the end of the experiment, the colonies were stained with 0.2% crystal violet solution (Sigma-Aldrich) containing 50% methanol. At least two independent experiments were performed in triplicate.

II.15 Luciferase promoter assay: To perform this assay, previously designed and established pGL3-MUC4 deletion constructs were used (14). PC cell lines were plated in six-wells in triplicates and repeated thrice. Transient transfection was performed with MUC4 deletion constructs using lipofectamine 2000 (Life Technologies; Carlsbad, CA, USA). Next day, the media was first changed to 10% FBS containing DMEM for 12h (to alleviate cellular stress of transfected cells) and then to serum free media for additional 8h. Subsequently, transfected cells were treated with BA for 4h in serum free condition. Following treatment, cells were lysed using reporter lysis buffer (Promega; Madison, WI) and subsequently, the activity of luciferase and beta-galactosidase activity was measured using Steady-Glo Luciferase assay system (Promega, E2510) and β-galactosidase assay kit (Promega, E2000). Fold activation of luciferase activity in BA-treated cells were calculated and compared with untreated cells. Putative transcription binding sites on MUC4 distal promoter were determined by ALGGEN PROMO software (where similarity score of >0.85 was used to screen transcription factor binding sites).

II.16 Plasmids and cloning strategy: Standard PCR and molecular cloning techniqueswere used to make constructs. For expression in the mammalian system,

p3X-FLAGCMV9 (vector contain N-terminal plasma membrane targeted sequence, Sigma) plasmids were used to make various constructs. DNA fragments encoding the carboxyl-terminal region of MUC4 (150 amino acids) with deletion of first EGF-L, second and third EGF-L, all three EGF-L motifs, cytoplasmic tail (of 21 amino acids) and truncated constructs (expressing only MUC4-EGF and CT in different combinations) as depicted in **Fig. 2A** were amplified using specific primers from synthesized cDNA. In frame GFP was placed upstream of MUC4 fragment for the live cell imaging (as our EGF ligand is tagged with Rhodamine). The protein expressions of the designed 3XFLAG.GFP.MUC4 truncated constructs were confirmed in the AsPC1 PC cell line by performing transfection, using lipofectamine 2000 according to manufacturer's protocol (**Fig. 2B**). All the constructs were verified by sequencing.

II.17 Internalization experiment *via* different approaches

Live Imaging by Time-Lapse Fluorescence Microscopy: Cells seeded on a Lab-Tek chambered coverglass system (Nunc, Rochester, NY). Live images of the cells were obtained a Zeiss LSM 5 Pascal confocal microscope (Zeiss). Temperature was maintained at 37°C with a water heated stage and lens warmer (Zeiss). The laser excitation setting was maintained at 543 nm for Rhodamine-tagged EGF (Invitrogen), with emission detected by appropriate filter sets as supplied by the manufacturer. For live pulse chase, cells were starved for 8h followed by a 15 min. binding of the EGF ligand at 4^0C. Cells were then washed three times with PBS followed by incubation in complete media, and then the movement of EGF bound EGFR was monitored for the indicated time-points.

Flow cytometry: PC cells were seeded in triplicates on six-well plate. After cells confluency reach to 60-70%, cells were serum starved for 8h followed by EGF stimulation at 37^0C for indicated time points which leads to EGFR internalization. Following internalization, we replaced the media to 10% serum containing media for

recycling purpose for 30 to 60 mins. Subsequently, cells will be washed 3 times with cold PBS and fixed with 4% paraformaldehyde. The mean florescence intensity will be measured by using flow cytometry approach at 568 nm as the ligand is labeled with Rhodamine.

Confocal microscopy: PC cells were seeded on coverslips. After cells confluency reach to 60-70%, cells were serum starved for 8h followed by EGF stimulation (unlabeled) at 37^0C for indicated time points which leads to EGFR internalization. Following internalization, we replaced the media to 10% serum containing media for recycling purpose for indicated time-points. Subsequently, cells will be washed 3 times with cold PBS and fixed with 4% paraformaldehyde. To pinpoint EGFR localization, colocalization experiment of EGFR with Rab5A (early endosome marker) and Rab7 (late endosome marker) was performed using the similar above mentioned protocol (**section II.7**)

II.18 Cell motility assay: A six-well chamber insert containing polyethylene teraphthalate membranes with a pore size of 8 μM (Becton Dickinson, Franklin Lakes, NJ, USA) was used for motility assay. The PC (CD18/HPAF) and PaSC cells were seeded at 1×10^6 and 0.5×10^6 in serum free media. After 48 h, cells reached to the lower chamber (serum containing media) were stained with Quick-Diff kit staining solution, while cells still present on the upper chamber were removed. Stained migrated cells were counted in 8 different random fields and the average number of motile cells per representative field was calculated.

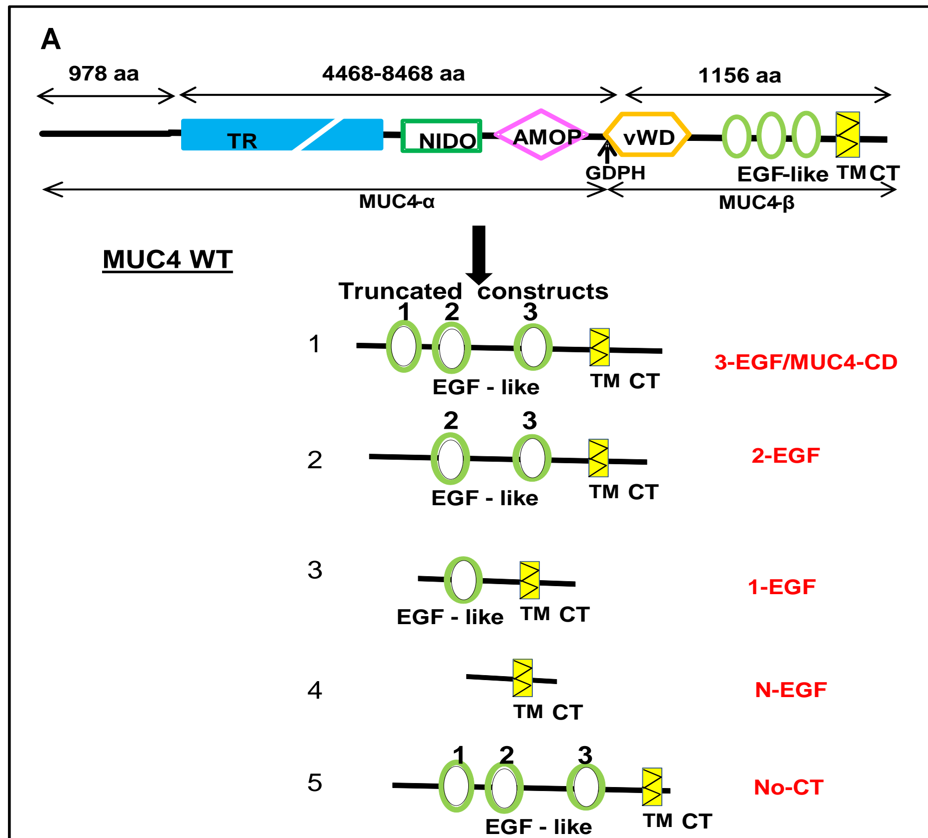
II.19 GTP-loaded Rab5 pull-down assay: Protein A+ G-Sepharose beads (Sigma-Aldrich Corp., St Louis, MO, USA) were coated with anti-Rab5-GTP mouse monoclonal antibody (Cat No. 26911, NewEast Biosciences). Beads were then incubated with protein lysates obtained from scr and MUC4 kd PC cells for 2h at 4^oC. Precipitated

active Rab5 was detected by immunoblotting using anti-Rab5A polyclonal antibody (SC-309, Santa Cruz Biotech (SCB), Dallas, Texas, USA).

II.20 Statistical analysis: All results are representative of at least three independent experiments. The *in vitro* data are expressed as the mean±standard deviation (S.D.), whereas the *in vivo* data are represented as mean±standard error (S.E.). Statistical comparisons of the two groups were made using a student's t-test (two-tailed, unpaired) using Microsoft Excel 2010, where a p value of less than 0.05 was considered statistically significant. For correlation analysis, the Pearson and regression coefficients were determined between two groups.

Figure 2. Strategy to design deletion constructs for MUC4 cytoplasmic domain and their confirmation at protein level. A. Pictorial representation of the proposed construct designs for MUC4 cytoplasmic domain which consists of 3-EGF-L domains, transmembrane domain and cytoplasmic tail. The overall MUC4 structure consist of large tandem repeat (TR) domain at N-terminal end and NIDO, AMOP, vWD, EGF-like and Trans-membrane (TM) domain towards 3'extermities. MUC4 also consist of short (22aa) cytoplasmic tail (CT) and one putative cleavage site (GDPH) supposed to generate mucin like MUC4-α and growth factor like MUC4-β subunits. DNA fragments encoding the carboxyl-terminal region of MUC4 (150 amino acids) with deletion of first EGF-L, second and third EGF-L, all three EGF-L motifs, cytoplasmic tail (of 21 amino acids) and truncated constructs (expressing only MUC4-EGF and CT in different combinations) were amplified using specific primers from synthesized cDNA. In frame GFP was placed upstream of MUC4 fragment for the live cell imaging (as our EGF ligand is tagged with Rhodamine). **B.** IB confirming the protein expression of synthesized MUC4 deletional constructs in transfected AsPC1 cell line. Here, NO-CT represents MUC4-CD without CT; N-EGF represents MUC4-CD without any EGF domain; 1-EGF represents MUC4-CD without 2nd and 3rd EGF; 2-EGF represents MUC4-CD without 1st EGF and 3-EGF is the construct which has the full intact MUC4-CD.

Figure 2A



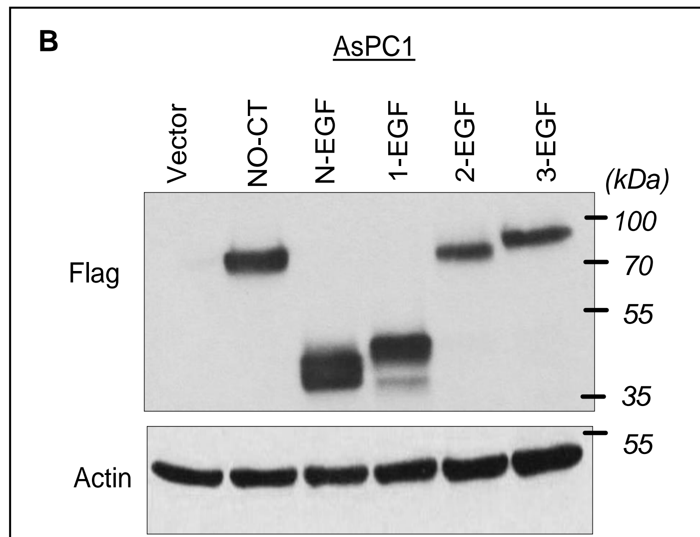


Table 2A Primary antibodies used in the study for Immunoblotting (IB) and Immunofluorescence (IF) and ChIP purposes

Serial no.	Antibody	Company	Dilution	Analysis performed
1	β-Actin	Sigma	1:5000	IB
2.	HIF-1α	NB100-479, Novus Biologicals	1:500	IB
3.	EGFR	SC-03, SCB	1:1500	IB
4.	phospho-EGFR (Ser1046)	SC-101665	1:500	IB
5.	LC3	2775, Cell Signaling	1:3000	IB
6.	LC3	AP1802a, Abgent	1:200	IF
7.	p62	PAB1750, Abnova	1:3000	IB
8.	p70 S6Kinase	2708, Cell Signaling	1:1000	IB
9.	phospho-p70 S6Kinase (Thr389)	9234, Cell Signaling	1:1000	IB
10.	p53	SC-126, SCB	1:1000	IB
11.	p21	SC-6246, SCB	1:1000	IB
12.	MDM2	SC-965, SCB	1:500	IB
13.	Akt	4691. Cell signaling	1:1000	IB
14.	phospho-Akt (Ser473)	4060, Cell signaling	1:1000	IB
15.	LAMP1	ab24170, Abcam	1:200	IF
16.	ATG7	2054-1, Epitomics	1:5000	WB
17.	8-OHG	ab10802, Abcam	1:150	IF
18.	MUC4 (8G7)	In-house generated	1:1000 1:400 1:800	IB IF IHC
19.	MUC4 (2175)	In-house generated	1:1000 1:500	IB IF
20.	Muc4 (4A-rabbit polyclonal against mice)	Designed by us and developed by GenScript (Piscataway, NJ, USA)	1:400	IHC
21.	MUC1 (HMFG2)	In-house generated (Gifted by Dr. Hollingsworth)	1:5 1:5	IB IF

Serial no.	Antibody	Company	Dilution	Analysis performed
22.	c-Jun	SC-1694,SCB	1:500 1:500 5 µg	IB IF ChIP
23.	p-c-Jun	9261, Cell Signaling	1:1000	IB
24.	FAK	SC-558, SCB	1:500	IB
25.	FXR	SC-134481, SCB	1:500 1:200	IB IF
26.	src	SC-18, SCB	1:500	IB
27.	p-src	6943, Cell Signaling	(1:1000	IB
28.	SP1	9389S, Cell Signaling	1:1000	IB
29.	GAPDH	5174S, Cell Signaling	1:3000	IB
30.	RAB5A	SC-309, SCB	1:500 1:50	IB IF
31.	RAB11	SC-6565, SCB	1:500	IB
32.	EEA1	SC-6415, SCB	1:700 1:50	IB IF
33.	RAB7	2094, Cell Signaling	1:1000 1:200	IB IF
34.	RIN1	bs-6094R, Bioss antibodies	1:1000	IB
35.	Alpha-SMA	ab7817, abcam	1:500 1:200	IB IF
36.	GFAP	SC-9065,SCB	1:1000 1:500	IB IF
37.	MUC16	Clone M11, Dako	1:1000	IB
38.	Alpha-tubulin	236-10501, Thermofisher scientific	1:400	IF
39.	β-catenin	C2206, Sigma	1:300	IF
40.	Calnexin	C5C9,Cell Signaling	1:300	IF
41.	Giantin	ab37266, abcam	1:1000	IF

Table 2B Human primers sequences used for PCR

(qRT:quantitative real-time)

Gene	Primers sequence
FXR_ qRT_F	5′-GCGCGTCAGCAGGGAGGATC-3′
FXR_ qRT_R	5′-CACACAGTTGCCCCCGTTTTTAC-3’
MUC4_ qRT_F	5’-GCAGAGAGCCAGTGTTTGTACAATCAG-3’
MUC4_ qRT_R	5’-AGGCCTCGCAGCCCTTCCCAGGAA-3’
c-Jun_ qRT_F	5’- TCCACGGCCAACATGCT -3’
c-Jun_ qRT_R	5′- CCACTGTTAACGTGGTTCATGAC-3’
β-actin_ qRT_F	5′- GACCTGTACGCCAACACAGT -3
β-actin_ qRT_R	5′- AGTACTTGCGCTCAGGAGGA -3′
EGFR_ qRT_F	5’-AGGCAC AAGTAA CAGGCTCAC-3’
EGFR_ qRT_R	5’-AAGGTCGTAATTCCTTTGCAC-3’
TGF-α_ qRT_F	5'- AGATAGACAGCAGCCAACCCTGA-3'
TGF-α_ qRT_R	5'- CTAGGGCCATTCTGCCCATC-3’
EGF_ qRT_F	5'-CCTGCCTAGTCTGCGTCTTT-3'
EGF_ qRT_R	5'-CACAATACCCAGAGCGAACA-3'
Cyclin D1_ qRT_F	5’-CCTCTGTGCCACAGATG-3’
Cyclin D1_ qRT_R	5’-GGGTCACACTTGATCACTC-3’
Rab5A_ qRT_F	5’-ACTTCTGGGAGAGTCCGCTGTT-3’
Rab5A_ qRT_R	5’- GTGTCATCAAGACATACAGTTTGG-3’
Twist_ qRT_F	5’ CGGGTCATGGCTAACGTG -3’
Twist_ qRT_R	5’-CAGCTTGCCATCTTGGAGTC -3’
Vimentin_ qRT_F	5’-GACAATGCGTCTCTGGCACGTCTT-3’
Vimentin_ qRT_R	5’-TCCTCCGCCTCCTGCAGGTTCTT-3’

Gene	Primers sequence
MUC4_Intron1_F	5'-GTCTATGTCCTGAATGGTATTGCCTA-3'
MUC4_Ex-2_R	5’-GAGGAGCTGTCTCCATCACATTGT-3’
MUC4-5’UTR_F	5’- CTTCGGAGAAACGCACTTGGTTCG-3’
MUC4_Ex-1_F	5’- CTGGAGGAGGGTCCCCTGGGTG-3’
MUC4_Ex-2_R	5'-GTCACACAACCCAGTCAACAACCGA-3'
MUC4_Ex-3_F	5’-GACAACACCGTCACTGAAGACAGACG-3’
MUC4_Ex-7_R	5’- GAGAAGCCCATGAGCACCGGGTTG-3’
MUC4_Ex-8_F	5’- GATGGCTATTTCGAAAACAGCCCACTG-3’
MUC4_Ex-12_R	5’- TGGAGCGGTACTGAGCCGCAAA-3’
MUC4_Ex-16_F	5’- CTGCGCAACGCAAGCATCGGACT-3’
MUC4_Ex-22_F	5’- TTGCTGTGGACACCCAAGTCGC-3’
MUC4_Ex-23_F	5’- CCAACACTGGATGGTCATCTCGGAG-3’
MUC4_Ex-26_R	5’- CAGCTGAGTTCAGGAAATAGGAGAACCTG-3’
MUC4_qRT_Int-1_F	5’-ATTCTATTTGTAGCAATTGTGA-3’
MUC4_qRT_Ex-2_R	5’-TTGAAGAAGCTGCAGTTGATTGTC-3’
MUC4_qRT_5’UTRF	5’-CTCTTTTGTCCTCTTCCCAGGTTCCCT-3’
MUC4_qRT_EX-1R	5’-ACATGCGGAAGGAGGCAGAGACACA-3’

Table 2C CHIP primer sequence for MUC4 distal promoter

Gene	Primers sequence
hmuc4Promo_c-jun2s ***(Comprising two c-Jun sites)***	5'-TCCCGTGGAATATTAACTTACA-3'
hmuc4Promo_c-jun1s ***(Comprising one c-Jun site)***	5'-ATCGGATGCCTTGGGAGGAGAGAA -3'
hmuc4Promo_c-jun_R ***(Common reverse primer)***	5'- AAATGGCTCTGTCTTCATCTGGGT -3'
hmuc4Promo_c-jun0s_F ***(No c-Jun binding sites)***	5'-ACTCTGGAAAATGGGCATATTGA-3'
hmuc4Promo_c-jun0s_R ***(No c-Jun binding sites)***	5'-CGTGCGCACTCCTGTTCACCTCTT-3'

II.21 Reference List

(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) Moniaux N, Chaturvedi P, Varshney GC, Meza JL, Rodriguez-Sierra JF, Aubert JP, Batra SK. Human MUC4 mucin induces ultra-structural changes and tumorigenicity in pancreatic cancer cells. Br J Cancer 2007;97:345-57.

(4) Rachagani S, Macha MA, Ponnusamy MP, Haridas D, Kaur S, Jain M, Batra SK. MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1. Carcinogenesis 2012;33:1953-64.

(5) Kaur S, Baine MJ, Guha S, Ochi N, Chakraborty S, Mallya K, Thomas C, Crook J, Wallace MB, Woodward TA, Jain M, Singh S, Sasson AR, Skinner V, Raimondo M, et al. Neutrophil gelatinase-associated lipocalin, macrophage inhibitory cytokine 1, and carbohydrate antigen 19-9 in pancreatic juice: pathobiologic implications in diagnosing benign and malignant disease of the pancreas. Pancreas 2013;42:494-501.

(6) Baine MJ, Menning M, Smith LM, Mallya K, Kaur S, Rachagani S, Chakraborty S, Sasson AR, Brand RE, Batra SK. Differential gene expression analysis of peripheral blood mononuclear cells reveals novel test for early detection of pancreatic cancer. Cancer Biomark 2011;11:1-14.

(7) Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, Tuveson DA. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. Cancer Cell 2005;7:469-83.

(8) Rachagani S, Torres MP, Kumar S, Haridas D, Baine M, Macha MA, Kaur S, Ponnusamy MP, Dey P, Seshacharyulu P, Johansson SL, Jain M, Wagner KU, Batra SK. Mucin (Muc) expression during pancreatic cancer progression in spontaneous mouse model: potential implications for diagnosis and therapy. J Hematol Oncol 2012;5:68.

(9) Kumar S, Das S, Rachagani S, Kaur S, Joshi S, Johansson SL, Ponnusamy MP, Jain M, Batra SK. NCOA3-mediated upregulation of mucin expression via transcriptional and post-translational changes during the development of pancreatic cancer. Oncogene 2014.

(10) Biederbick A, Kern HF, Elsasser HP. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol 1995;66:3-14.

(11) Torres MP, Rachagani S, Purohit V, Pandey P, Joshi S, Moore ED, Johansson SL, Singh PK, Ganti AK, Batra SK. Graviola: a novel promising natural-derived drug that inhibits tumorigenicity and metastasis of pancreatic cancer cells in vitro and in vivo through altering cell metabolism. Cancer Lett 2012;323:29-40.

(12) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.

(13) Kumar S, Das S, Rachagani S, Kaur S, Joshi S, Johansson SL, Ponnusamy MP, Jain M, Batra SK. NCOA3-mediated upregulation of mucin expression via transcriptional and post-translational changes during the development of pancreatic cancer. Oncogene 2015;34:4879-89.

(14) Andrianifahanana M, Agrawal A, Singh AP, Moniaux N, van S, I, Aubert JP, Meza J, Batra SK. 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.

CHAPTER III

Hypoxia-induced oxidative stress promotes MUC4 degradation via autophagy to enhance pancreatic cancer cells survival

This chapter has been adapted from:

Joshi S, Kumar S, Ponnusamy MP, Batra SK. Hypoxia-induced oxidative stress promotes MUC4 degradation via autophagy to enhance pancreatic cancer cells survival. Oncogene. April 25, 2016. [Epud ahead of print]

Hypoxia-induced oxidative stress promotes MUC4 degradation via autophagy to enhance pancreatic cancer cells survival

Suhasini Joshi[1], **Sushil Kumar***'[1], **Moorthy P. Ponnusamy**[1]

and Surinder K. Batra*[,1,2]

[1]Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA, [2]Buffett Cancer Center, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA.

Running title: Hypoxia-mediated negative regulation of MUC4

Keywords: Hypoxia, MUC4, Pancreatic cancer, Oxidative stress, Autophagy

Conflict of interest: The authors declare no conflict of interest

*For correspondence:

Surinder K. Batra, Ph.D. and Sushil Kumar, Ph.D.

Department of Biochemistry and Molecular Biology,

University of Nebraska Medical Center,

Omaha, Nebraska, 68198-5870, U.S.A.

Phone: 402-559-5455,

Fax: 402-559-6650,

Email: sbatra@unmc.edu; skumar@unmc.edu

III.1 Synopsis

Pancreatic cancer (PC) and associated pre-neoplastic lesions have been reported to be hypoxic, primarily due to hypovascular nature of PC. Though presence of hypoxia under cancerous condition has been associated with the overexpression of oncogenic proteins (MUC1), multiple emerging reports have also indicated the growth inhibitory effects of hypoxia. In spite of being recognized as the top-most differentially expressed and established oncogenic protein in PC, MUC4 regulation in terms of micro environmental stress has not been determined. Herein, for the first time, we are reporting that MUC4 protein stability is drastically affected in PC, under hypoxic condition in a HIF-1α independent manner. Mechanistically, we have demonstrated that hypoxia-mediated induction of reactive oxygen species (ROS) promotes autophagy by inhibiting pAkt/mTORC1 pathway, one of the central regulators of autophagy. Clinically, immunohistoflorescence analyses revealed significant negative correlation (p value = 0.017) between 8-hydroxy Guanosine (8-OHG) and MUC4 in primary tumors (n=25). Moreover, we found pronounced colocalization between MUC4 and LAMP1/LC3 in PC tissues and also observed their negative relationship in their expression pattern, suggesting that areas with high autophagy rate had less MUC4 expression. We also found that hypoxia and resultantly arise ROS have negative impact on overall cell growth and viability, which was partially, though significantly ($p<0.05$), rescued in the presence of MUC4. Altogether, hypoxia-mediated oxidative stress induces autophagy in PC, leading to the MUC4 degradation to enhance survival, possibly by offering required metabolites to stressed cells (1).

III. 2 Background and rationale

Pancreatic cancer (PC) is the fourth leading cause of cancer related mortalities in United States with an overall survival rate of only 6% (2). Currently gemcitabine is used as a standard therapy for advanced PC; however, its clinical outcome is quite modest due to development of acquired and inherent chemo-resistance. One of the prominent features of PC which contributes to this chemoresistance and cancer progression is the presence of extreme hypoxia. Unlike other solid tumors, PC is hypovascular and characterized by enormous desmoplastic reactions (3, 4). Tumor hypoxia is a condition when cancer cells are deprived of oxygen and is primarily found in regions that are distant from the tumor blood vessels, particularly, center of the tumor. Therefore, these microenvironments suffer from low nutrient availability and production of waste products (acidosis). Ultimately, it results in the development of a stressful environment which adversely affects tumor cell proliferation and survival, and leads to the clonogenic selections of only those cells who can withstand hostile environment (5). In order to survive and remain viable, cancer cells induce both HIF-1α dependent and independent mechanisms.

PC is characterized by aberrant mucins expressions, such as MUC1, MUC4 and MUC5AC (6-9). Under normal condition, the expressions of these mucins are low or undetectable, but under disease conditions, their expression increases. Studies have established that MUC1, a transmembrane protein, is positively regulated by hypoxia and has been linked with increase survival, angiogenesis and altered metabolomics in PC (10-12). Similar to MUC1, MUC4 is also a transmembrane protein, but it does not express in normal pancreas (13). MUC4 appears quite early in preneoplastic stage (PanIN-I) and its expression increases with the severity of the disease (8). We have

previously established that aberrant overexpression of MUC4 leads to increased tumor growth, survival, metastasis and therapy-resistance in PC (14-16). So far, various intrinsic and extrinsic factors have been associated with its aberrant expression during PC progression (17). However, how environmental stimuli such as hypoxia can regulate MUC4 expression is still not clear.

Therefore, in the present study, we investigated the regulation of MUC4 expression by hypoxia, and examined the clinical significance of this association in PC. Our findings indicate that hypoxia negatively regulates MUC4 expression in PC, and also provided evidence for a novel regulatory mechanism which leads to MUC4 degradation due to hypoxia-induced oxidative stress.

III.3 Results

A. MUC4 expression is down-regulated in PC cell lines in response to hypoxia

To understand the effect of hypoxia in MUC4 expression, we treated MUC4 expressing PC cell lines, CAPAN1, CD18/HPAF and T3M4, with 1% of hypoxia for 24 hours (h). There was significant downregulation of MUC4 at the protein level in all three PC cell lines (**Fig.3.1A**), with concomitant increase in HIF-1α levels. Substantially, we observed similar decrease in MUC4 levels in hypoxia treated Colo357 cells (**Fig.3.1B**). Immunofluorescence analysis also validated reduction in MUC4 expression, whereas MUC1, an established HIF-1α target, was significantly increased in CD18/HPAF cells (**Fig.3.1C**). To further substantiate our findings, we gave prolong (or chronic) hypoxia to CD18/HPAF cells for 72h and 96h. Consistently, we observed significant downregulation of MUC4, whereas MUC1 expression remains persistently high (**Fig.3.1D**). The qRT-PCR analysis showed insignificant reduction in MUC4 expression at transcript levels in all tested PC cell lines (**Fig.3.1E**), suggesting that hypoxia may affect the stability of

MUC4 protein. Altogether, the results indicate that MUC4 expression reduces under hypoxic condition due to modulation in MUC4 protein stability.

B. Decrease in MUC4 expression under hypoxia is HIF-1α independent

Previous studies have linked hypoxia-mediated alterations in mucins expression with induced HIF-1α expression (10-12, 18, 19), which led us to ask whether hypoxia-mediated downregulation of MUC4 expression is HIF-1α dependent. To ascertain the role of HIF-1α transcription factor in MUC4 reduction, we silenced HIF-1α expression by utilizing ShRNA approach and by pharmacological inhibitor, YC-1. Under both normoxic and hypoxia conditions, HIF-1α knocked down (kd) led to MUC4 downregulation in CAPAN1, as compared to its respective control (**Fig. 3.2A**). Furthermore, treatment of both CD18/HPAF and CAPAN1 cells with YC-1 inhibited the expression of MUC4 in a dose-dependent manner (**Fig.3.2B**), suggesting the role of HIF-1α in the upregulation of MUC4 expression. Additionally, inhibition of HIF-1α degradation upon MG132 (ubiquitin-proteasome inhibitor) treatment of CD18/HPAF cells did not rescue MUC4 degradation, in fact further downregulation in MUC4 expression was observed (**Fig.3.2C**), possibly due to MG132-mediated induction of autophagy (20-22). This data further strengthened the fact that reduced MUC4 protein expression under hypoxia is HIF-1α independent, and it is the stability of MUC4 which is primarily affected under hypoxia. To further prove our conjecture, we treated CD18/HPAF cells with cycloheximide (CHX, protein translation inhibitor) for indicated time-points and observed significant decrease in MUC4 expression under hypoxic condition as compared to normoxia (**Fig.3.2D**), establishing that MUC4 protein stability is reduced under hypoxic conditions. Immunofluorescence analysis in PC tissues (n=25) also revealed 56% (14/25) and 68% (17/25) expression of MUC4 and HIF-1α, respectively. MUC4 and HIF-1α were co-expressed in 44% (11/25)

of patients, however, were simultaneously absent in 20% (5/25) of PC patients (**Fig.3.2E-F**). Altogether, the results indicate that MUC4 expression is positively associated with HIF-1α; therefore, hypoxia-mediated downregulation of MUC4 is HIF-1α independent.

C. Decrease in MUC4 expression under hypoxia is ROS-dependent

Because hypoxia-mediated reduction in MUC4 is HIF-1α independent, therefore, our next question was to explore the mechanism responsible for significant downregulation of MUC4 expression under hypoxia. It is already known that hypoxia has various HIF-1α dependent and independent functions (23). Recent studies have shown that mucins expression is regulated by reactive oxygen species (ROS) (24), and induction of ROS under hypoxia, is an established feature. It prompted us to ask whether hypoxia-mediated ROS induction is responsible for MUC4 reduction. To address this question, we treated CD18/HPAF cells with 5 mM of ROS scavenger; N-acetyl cysteine (NAC), for 24h in the presence and absence of hypoxia. Interestingly, we observed attenuation of MUC4 reduction under hypoxic condition in NAC treated cells (**Fig.3.3A**). Notably, NAC treatment alone was sufficient for MUC4 upregulation (**Fig.3.3B**), by attenuating basal levels of ROS already present in cancer cell lines (**Fig 3C**). The measurement of 2′,7′-Dichlorofluorescin diacetate (DCFDA) florescence showed 41% and 63% reduction in ROS levels upon NAC treatment under both normoxic and hypoxic condition, respectively, further strengthening that NAC-mediated neutralization of ROS is responsible for MUC4 upregulation (**Fig. 3D**). Treatment of both CD18/HPAF and CAPAN1 PC cells with another antioxidant, α-tocopherol succinate (α-TS), also showed similar increase in MUC4 expression (**Fig. 3E**). Additionally, treatment of CAPAN1 with exogenous hydrogen peroxide (H_2O_2), a form of non-ionic ROS, resulted in concomitant

reduction in MUC4 expression in a dose-dependent manner (**Fig.3F),** which was further confirmed in CD18/HPAF cells (**Fig. 3G**). Immunofluorescence experiment also exhibited that the negative impact of hypoxia and ROS on MUC4 expression was abolished in the presence of NAC (**Fig. 3H**). Altogether, these data suggests that ROS is playing a key role in hypoxia-mediated negative regulation of MUC4 in PC.

D. Hypoxia-mediated autophagy induction leads to reduced MUC4 stability

As demonstrated earlier, inhibition of ubiquitin-proteasome pathway was failed to rescue MUC4 suppression under hypoxic condition (**Fig.3.2C**). Multiple studies have established that autophagy and ubiquitin proteasome systems (UPS) are functionally coupled, and inhibition of UPS system by MG132 induces autophagy (20-22). Furthermore, the link between ROS and autophagy is also well established (25, 26). Altogether, these studies incited us to propose that HIF-1α independent hypoxia-mediated induction of oxidative stress promotes autophagy which reduces the protein stability of MUC4. Therefore, we first evaluated the status of autophagy in PC cells, under hypoxic and oxidative stress conditions. Interestingly, the levels of LC3-I and II were significantly increased in hypoxia treated CAPAN1 and CD18/HPAF cells compared to normoxic controls (**Fig.3.4A**). Further, treatment of CAPAN1 cells with H_2O_2 showed increased LC3-I and LC3-II expression levels in a dose-dependent manner (**Fig.3.4B**). The results were verified in CD18/HPAF cells where increase in LC3 was accompanied with the concomitant reduction in p62 expression (**Fig.3.4C**), further emphasizing autophagy induction under oxidative stress conditions. Increased autophagosome formation in oxidative stress and hypoxic condition was also confirmed by monodansylcadaverine (MDC) staining in CAPAN1 cells (**Fig.3.4D**). Further, treatment of CAPAN1 cells with increasing doses of rapamycin (RAP), an autophagy

inducer by inhibiting mTORC1 complex, resulted in reduction in MUC4 expression in a dose-dependent manner, with concomitant increase in LC3-I and II levels (**Fig.3.4E**). These results were substantiated by treatment of CAPAN1 cells with autophagy inhibitor, vinblastine (VB) (27, 28) in the presence and absence of ROS. Consistent to our premise, the suppression of MUC4 expression by ROS was significantly abolished by VB treatment, as compared to H_2O_2 treated CAPAN1 cells (**Fig.3.4F**). VB inhibits the fusion of LC3 carrying autophagosome vesicles with lysosomes, and thus, prevents the degradation of proteins, causing accumulation of LC3. Immunofluorescence experiment further confirmed increase in MUC4 expression and colocalization with accumulated LC3 positive vesicles in VB-treated CD18/HPAF and CAPAN1 cells (**Fig.3.4G and H**). To further substantiate our findings, we did immunofluorescence staining for MUC4 and LAMP1+ lysosomal vesicles in CAPAN1 and observed their colocalization (**Fig.3.4I**). Moreover, significant increase in MUC4 expression upon ATG7 kd in CD18/HPAF cell line, establish the involvement of autophagy in MUC4 degradation (**Fig.3.4J**). Altogether, we have demonstrated that hypoxia-mediated ROS stimulation causes induction of autophagy process, which leads to MUC4 degradation and reduced stability.

E. Hypoxia inhibits Akt/mTORC1 pathway to induce autophagy

Recent report by Wang *et al* has demonstrated the involvement of Akt activation in mTORC1 regulated autophagy process (29). Additionally, chronic hypoxia has also shown to suppress Akt activation in hypoxia treated PC cells (30). Similarly, we also observed that levels of phosphorylated Akt and mTORC1 effector, pS6 kinase, were consistently reduced in hypoxia-treated PC cells, whereas expression of EGFR, pEGFR, Akt and S6 kinase remained unchanged (**Fig.3.5A**). We observed significant reduction in p53 expression in hypoxia-treated cells lines, suggesting the possible accumulation of

genomic and cellular defects in stressed PC cells. We also observed increase in p21 expression in hypoxia-treated T3M4 and CD18/HPAF cells, suggestive of growth arrest of PC cells (**Fig.3.5B**), which was corroborated by our growth kinetics analysis in hypoxia-treated and untreated CD18/HPAF cells (**Fig.3.5C**) and by a recent study where hypoxia has shown to cause growth inhibition in PC cell lines (31). To assess the role of ROS on pAkt reduction, we analyzed its expression in the presence and absence of NAC. Interestingly, hypoxia-mediated downregulation of pAkt in CD18/HPAF cells was abolished upon NAC administration, further emphasizing that the reduction in pAkt levels under hypoxia is ROS-dependent (**Fig.3.5D**). From this data, we were also able to reasoned that p53 downregulation under hypoxia is occurring due to induced expression of MDM2 (ubiquitin ligase), though we did not see any change in their levels after NAC treatment, implying the involvement of ROS-independent mechanisms in these alterations. Further, treatment with NAC attenuates the growth inhibitory effects of hypoxia (**Fig.3.5E**) and H_2O_2 (ROS stress) on PC cells (**Fig.3.5F**). These results were further supported by performed MTT assay as significant loss in cell viability ($p<0.05$) was noticed in H_2O_2 treated PC cells (**Fig.3.5G**). In order to know the effect of hypoxia on cell viability and death, MTT assay was performed. CD18/HPAF cells exhibited significant loss of viability under hypoxia, which was partially rescued in the presence of ROS scavenger (NAC) and further augmented upon autophagy inhibitor chloroquinone treatment (CQ) (**Fig.3.5H**). Similar to cell growth results, under normoxia, NAC did not demonstrate any change in cell viability, whereas, CQ significantly reduces the cell viability. Interestingly, PC cell lines demonstrate high autophagy rate even at basal levels (**Fig. 3.5I**), affirming protumorigenic role of autophagy in PC (32). Consistent to cell viability results, we observed increased cellular apoptosis and necrosis upon hypoxia treatment ($p<0.05$), which was significantly ($p<0.05$) suppressed by NAC and

augmented by CQ treatment (**Fig. 3.5I**), suggesting that reduction in PC cell viability and death under hypoxic condition is oxidative stress-dependent, and induction of autophagy is a survival mechanism.

F. MUC4 gives survival advantage to hypoxia-stressed PC cells

To determine the role of MUC4 in the survival of PC cells under hypoxia and oxidative stress, we gave 1% hypoxia treatment to MUC4 kd and scrambled (Scr) CAPAN1 cells (**Fig.3.6A**). Noticeably, under hypoxia, MUC4 scr CAPAN1 cells exhibited 6%, 21% and 53% reduction in cell viability on day 1, 3 and 5, respectively, compared to normoxic cells. On the other hand, MUC4 kd cells exhibited 10%, 27% and 64% loss in cell viability on day 1, 3 and 5, respectively, compared to normoxic kd cells, suggesting the role of MUC4 in maintaining the viability of PC cells under stressed condition (**Fig. 3.6B**). Similar results were obtained when MiniMUC4 overexpressing MIA PaCa-2 cell model was used (33). In this model, MUC4 non-expressing MIA PaCa-2 cell lines ectopically express MiniMUC4, which consists only 10% of the total VNTR of wild-type MUC4 (**Fig. 3.6C**). Growth kinetics was performed in these cells for 24 and 48h after H_20_2 treatment in the presence and absence of NAC. Intriguingly, we observed 85% and 63% reduction in cell viability in H_2O_2 treated vector and MiniMUC4 expressing MIA PaCa-2 cells, upon 24h of H_2O_2 treatment (**Fig. 3.6D**). At 48h, we observed 71% and 55% reduction in cell numbers in H_2O_2 treated vector and MiniMUC4 expressing MIA PaCa-2 cells, respectively. Administration of NAC was able to rescue H_2O_2-mediated decrease in cells numbers in vector and MiniMUC4 expressing MIA PaCa-2 (**Fig. 3.6D**). These results indicate that presence of MUC4 alone cannot completely abolish oxidative stress-facilitated PC death. However, presence of MUC4 does offer better survival and viability

advantage to PC cells under hypoxic and oxidative stress conditions than MUC4 kd or null cells.

G. Clinical validation of MUC4 association with oxidative stress and degradation *via* lysosomal pathway

To validate the link between MUC4 and hypoxia-induced autophagy, we performed immunofluorescence analysis for MUC4 and LAMP1 in PC tissues, and observed significant co-localization between them (**Fig.3.7A**). One of the consistent and intriguing finding was the inverse relationship between LAMP1 and MUC4 expression. Ducts having high MUC4 expression exhibited low expression of LAMP1 and *vice versa,* as demonstrated in the intensity plot diagram (**Fig.3.7A**). Due to the established association of increased expression of LAMPs with increased lysosomal function and autophagy involvement (34), their inverse expression pattern may indicate that MUC4 does enter to the lysosomes, and may undergo degradation. Additionally, presence of MUC4 in LC3-positive vesicles in PC tissues, confirmed the association between MUC4 with autophagy (**Fig.3.7B**).

To know the clinical association between MUC4 and oxidative stress, we performed immunofluorescence analysis by staining PC tissues for MUC4 and 8-hydroxy guanosine (8-OHG, commonly used marker for oxidative stress) (35, 36). We observed 8-OHG and MUC4 expression in 64% and 56% of PC patients, respectively. Validating our *in vitro* data, MUC4 and 8-OHG exhibited significantly inverse expression status under *in vivo* condition, as shown in representative images (**Fig.3.7C**). It was further established by quantifying the mean florescence intensities (MFI) of 8-OHG in MUC4 low ($MUC4^L$) and MUC4 high ($MUC4^H$) regions, and the difference was found to be statistically significant ($p=0.017$) (**Fig.3.7C**). In our analysis, we also observed that

oxidative stress does not always correlate with increase HIF-1α expression (**Fig.3.7D**). The statistical analysis of MFI of different spots/fields (n=40) of RAPID autopsy tissue array (having 25 PC patients tissues) revealed their Pearson correlation of 0.56 with an R^2 value of 0.31 (**Fig.3.7E**). Altogether, we can conclude that MUC4 expression is differentially regulated by HIF-1α and oxidative stress, which is possible in varied PC microenvironment.

III. 4 Discussion

By far, PC has one of the most complicated microenvironment among other solid cancers due to its myriad of unique properties (4). Unlike most of the solid tumors, PC is characterized by hypo-vascularization due to the deposition of extracellular matrix, which causes extreme hypoxia and oxidative stress (4). Chronic and severe hypoxia has been shown to inhibit tumor cell proliferation, which ultimately led to cell death (37). Nevertheless, tumor hypoxia is also the predict marker for the worse clinical outcome. To resolve these two opposite observations, hypoxia has been projected to create a selection pressure which causes the survival of only those clones which are highly aggressive and resistant towards fluctuating microenvironmental stress (38).

Alike, aberrant overexpression of mucins has been implicated in PC survival, aggressiveness, drug resistance and maintenance of stem cell phenotype (3, 13-15). Most of these attributes are frequently assign to their interaction with receptor tyrosine kinases, cell surface proteins and components of extracellular matrix (39, 40). Present study provides an additional oncogenic mechanism by which MUC4 contributes to the survival of PC cells under hypoxic conditions through its degradation *via* autophagy. Among cancers, such as renal and pancreatic cancer, the hypoxia-mediated induction of MUC1 has been associated with HIF-1α (10-12). Nevertheless, we observed significant reduction in MUC4 expression under hypoxia in multiple PC cell lines. Intriguingly, we

observed that similar to MUC1, MUC4 is also positively regulated by HIF-1α, however, in spite of increased HIF-1α stability (by inhibiting its proteasomal degradation); MUC4 was degraded persistently under hypoxic condition. Therefore, downregulation of MUC4 expression under hypoxia, even in the presence of induced HIF-1α expression, signifies the presence of other predominant pathways independent of HIF-1α.

Studies have demonstrated that ROS induction is one of the most common HIF-1α independent mechanism activated under hypoxic conditions (41). Moreover, established role of ROS in autophagy induction (42) and emerging data linking mucins regulation by ROS (24), prompted us to postulate that ROS induced autophagy plays crucial role in MUC4 downregulation. Consistent to our proposition, we did observe MUC4 downregulation under hypoxic, oxygen-deficient or chemically-induced (rapamycin) autophagy, which was attenuated upon inhibition of ROS and autophagy. So far, studies have not demonstrated the involvement of autophagy in mucins degradation. The apparent presence of MUC4 in LAMP1 and LC3-positive vesicles in PC tissue, imply that MUC4 does enter to autophagy/lysosomal pathway under *in vivo*, and provided the first evidence of mucins degradation by autophagy pathway. Despite of both cancer promoting and suppressing role of autophagy, majority of the available data hints toward its role in promoting survival and proliferation of PC cells (26, 43). Our study also suggests that MUC4 degradation *via* ROS-mediated autophagy might be a survival mechanism in PC, as MUC4 kd CAPAN1 and MUC4-null MIA PaCa-2 cell lines were less viable under microenvironmental stress conditions compared to CAPAN1/Scr and MiniMUC4 expressing MIA PaCa-2 cells, respectively. Recent studies have clearly established that pancreatic tumors are nutrient deprived and heavily-dependent on macropinocytosis, leading to uptake of small extracellular proteins by cancer cells (44, 45). These internalized proteins then undergo autophagy process and provides

necessary metabolites to ensure the survival of highly stressed PC cells. Due to reportedly reduced levels of extracellular proteins concentration under clinical settings (46, 47), we anticipate that requirement or dependency to internalize and degrade overexpressed membrane proteins (such as MUC4) by hypoxic/oxidatively stressed/nutrient deprived PC cells is conceivably more than extracellular proteins and needs further investigations.

Mechanistically, we observed significant downregulation of phospho-Akt in hypoxia treated PC cells. Attenuation of ROS level by NAC treatment, suppresses the hypoxia facilitated Akt activation, which was further related with the resumption of cell proliferation. These data were further supported by a recent report by Sayin VI *et al.* where *in vivo* administration of NAC and vitamin-E have demonstrated to increase the tumorigenicity of lung cancer by downregulating the levels of ROS, DNA damage, and p53 (48). We also observed downregulation of p53 under hypoxia, which further reduces upon ROS attenuation, and therefore, questioned the utility of antioxidant-based therapies in PC. Looking into earlier clinical trials on dietary antioxidants in cancer condition, we have not received encouraging results (49, 50). Moreover, NAC treatment leads to the attenuation of apoptotic functions of ROS-inducers, further emphasizing towards the optimization of antioxidant therapies against PC (51). However, due to observed overexpression of HIF-1α even under normoxic condition, current study encourages HIF-1α targeting, which will led to the downregulation of multiple oncogenic proteins, including mucins. It will definitely be our future interest to observe how HIF-1α inhibition leads to MUC4 downregulation. Our *in silico* analysis has clearly shown that MUC4 promoter does not contain HIF-1α bindings sites, indicating the involvement of other protein mediators in HIF-1α facilitated MUC4 regulation. One of the possible mechanisms could be EGFR downregulation upon HIF-1α inhibition, as recent study

from our lab has shown that inhibition of EGFR leads to MUC4 downregulation in PC cells, and need to be investigated (52).

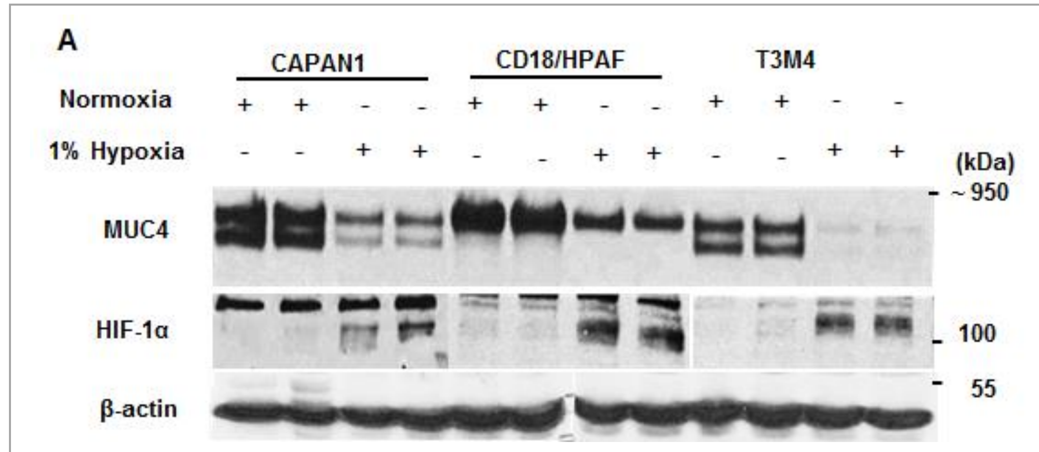
In conclusion, the present study provides evidence that hypoxia negatively regulates MUC4 expression in PC by affecting its stability. Moreover, we found that hypoxia-mediated reduction of MUC4 is HIF-1α independent, and further investigation directed us to know the involvement of ROS induced autophagy in MUC4 degradation (**Fig.3.8**). Similar to cytokines, we observe functional redundancy in mucins, implying that induction in MUC1 expression under hypoxia may be sufficient to compensate for MUC4 downregulation, and need to be addressed. Lastly, due to the diverse effect of hypoxia and highly complicated PC microenvironment, we can speculate that MUC4 expression could be differentially regulated by HIF-1α and oxidative stress, which leads to differential expression and regulation of MUC4 within the same tumor due to the different local microenvironment.

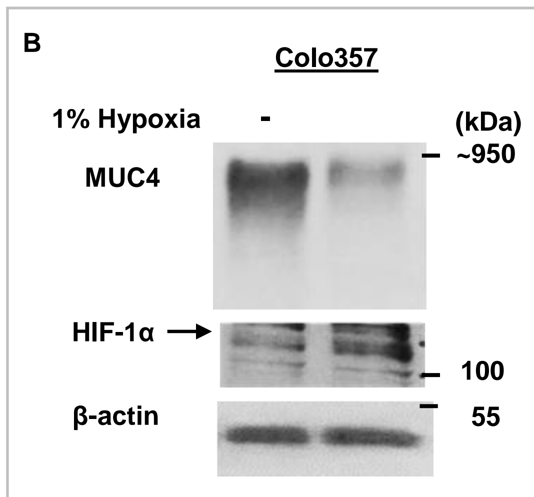
Figures and Figure legends

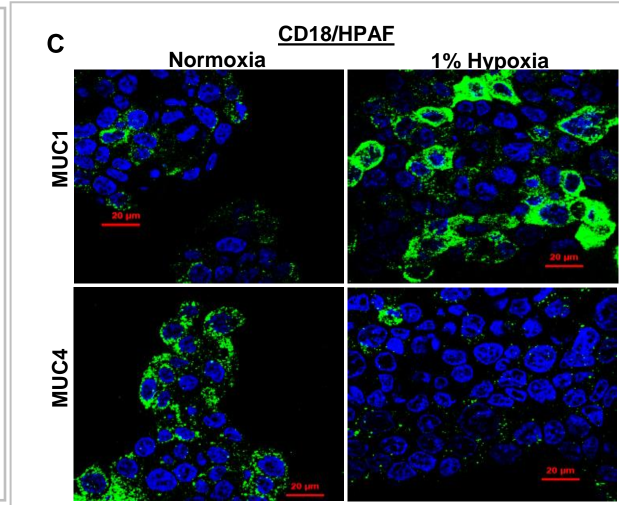
Figure 3.1. MUC4 is negatively regulated by hypoxia in PC cell lines. **A.** CAPAN1, CD18/HPAF and T3M4 cells were cultured under normoxia or hypoxic (1% O_2) conditions for 24 h. Following treatment, lysates were collected and western blots were performed. Protein expression of MUC4 and HIF-1α was analyzed by 2% agarose and 10% polyacrylamide gel-based electrophoresis, respectively. **B.** MUC4 expressing PC cell line, Colo357, was exposed to 1% hypoxia for 24 h. As anticipated, upon hypoxia treatment, HIF-1a expression was significantly induced, whereas MUC4 protein showed significant reduction, as compared to untreated controls. **C.** CD18/HPAF cells were grown on coverslips followed by 24 h incubation under normoxia or hypoxia. After the completion of treatment, cells were fixed, permeabilized and then subjected to immunofluorescence experiment to observe changes in the expression of MUC1 and MUC4. **D.** Prolong hypoxia treatment was given to CD18/HPAF cells for 72 h and 96 h and the expression of MUC4 and MUC1 was analyzed. **E.** qRT-PCR experiment was performed to detect changes in the mRNA expression levels of MUC4 in hypoxia treated and untreated CD18/HPAF, T3M4 and CAPAN1 PC cell lines.

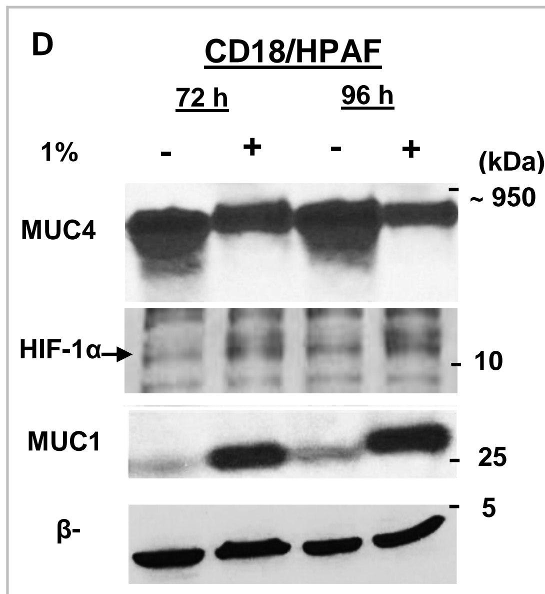
(ns stands for no significant difference, Scale bar = 20 μM).

Figure 3.1









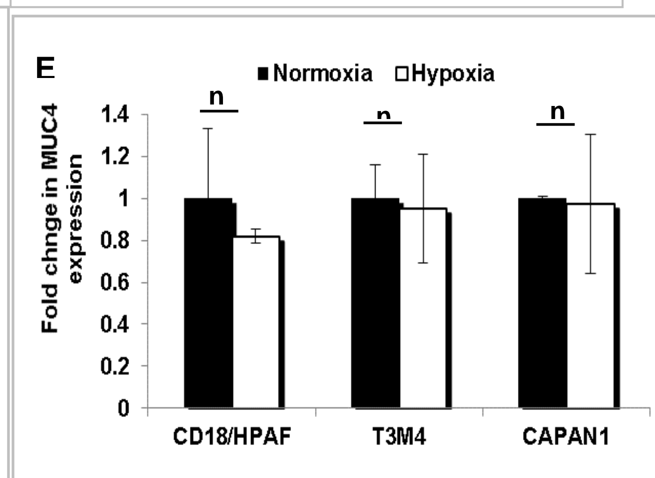
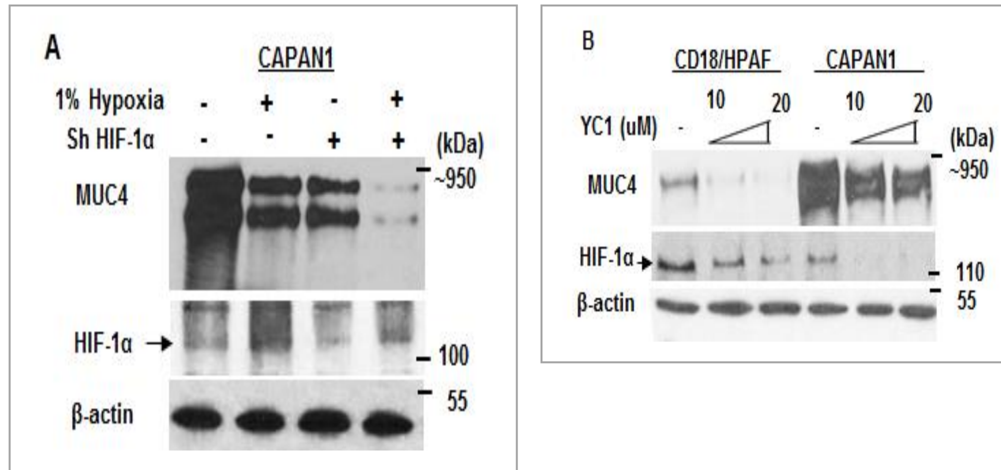
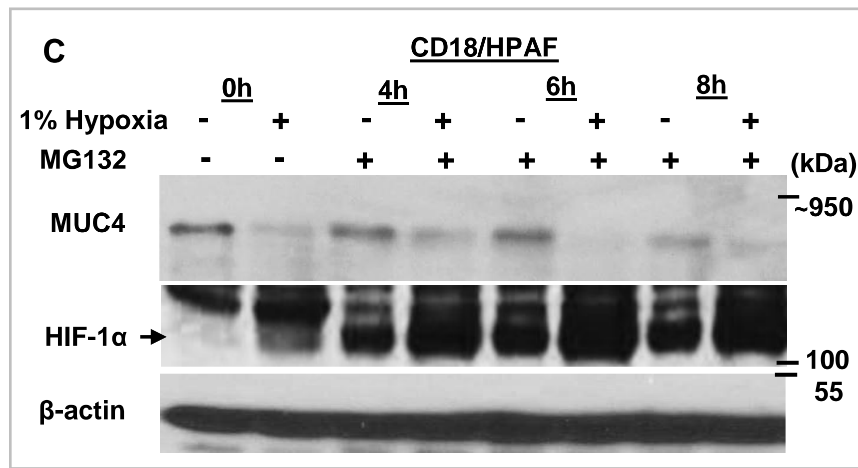


Figure 3.2. HIF-1α independent mechanisms play predominant role in hypoxia-mediated suppression of MUC4. A. After transiently knocking down HIF-1α, CAPAN1 cells were incubated under 1% hypoxic conditions for 24 h. Following treatment, total protein was isolated and western blot was performed to observe the effect of HIF-1α silencing on MUC4 expression under both hypoxic and normoxic conditions. **B.** CD18/HPAF and CAPAN1 cells were exposed to different concentration of YC-1 (10 or 20 μM), an inhibitor of HIF-1α, for 16 h. Immunoblotting was performed to detect changes in MUC4 and HIF-1α expression. **C.** CD18/HPAF cells were first pre-treated with MG132 (10 μM) for 30 mins. Following pre-treatment, cells were incubated under 1% hypoxic conditions for 4, 6 and 8 h in the presence of MG132. Even after inhibition of ubiquitin-proteasome pathway, MUC4 degradation did not prevent, whereas, HIF-1α protein which is known to be degraded solely by proteasome pathway was stabilized upon MG132 treatment under both normoxic and hypoxic condition. **D.** Similar to MG132, CD18/HPAF cells were pre-treated with CHX (50 μg/ml) for 30 mins followed by 1% hypoxia treatment for 2, 4 and 6 h in the presence and absence of CHX. 2% agarose gel electrophoresis was performed to see the effect of these inhibitor treatments on MUC4 expression in the presence or absence of hypoxia. We observed that CHX-treatment significantly reduces the levels of MUC4 under hypoxic condition, compared to CHX treated cells alone, confirming the negative effect of hypoxia on MUC4 protein stability. **E.** Representative images obtained from normal colon and PC tissues (from three different patients) showing MUC4 and HIF-1α co-expression at same tissue spots. **F.** The bar graph showing the percentage positive and negative expression for MUC4 and HIF-1α in stained PC tissue arrays. (Scale bar = 20 μM).

Figure 3.2





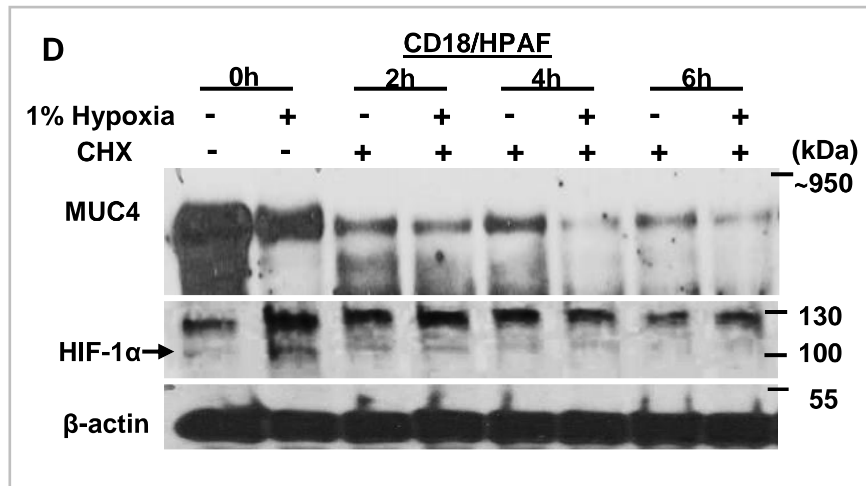
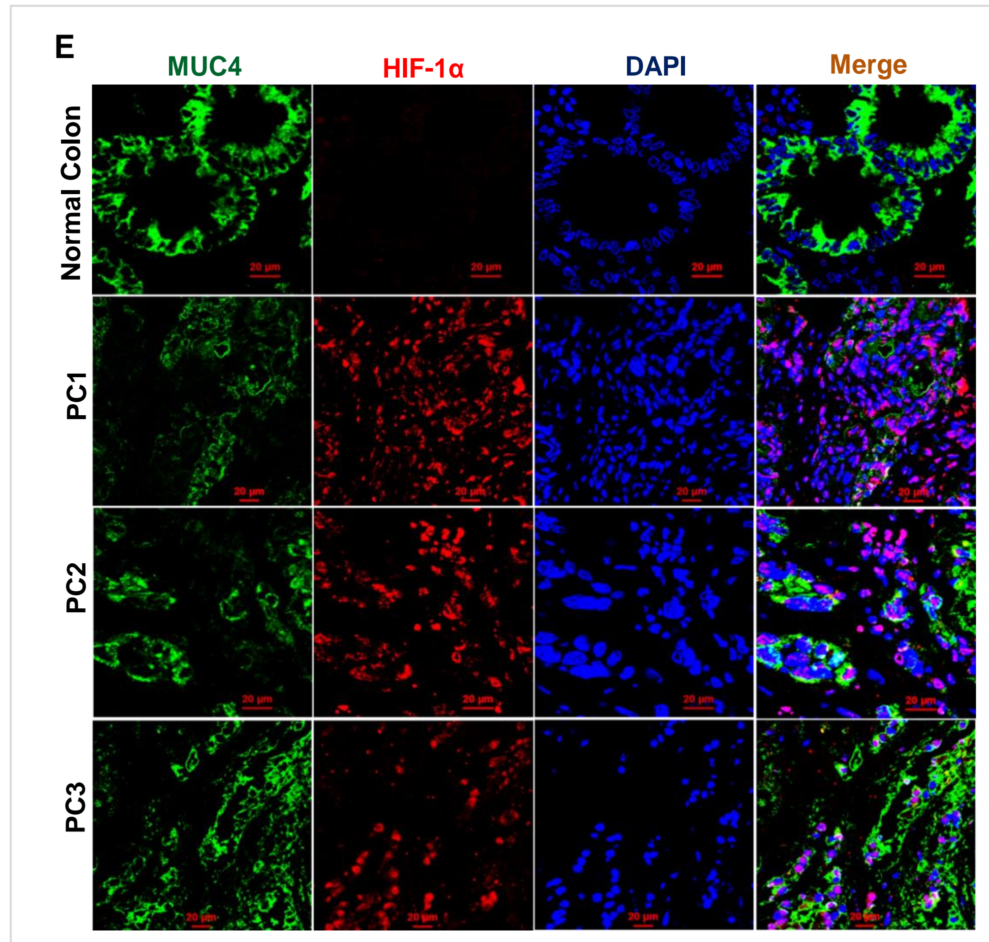


Figure 3.2



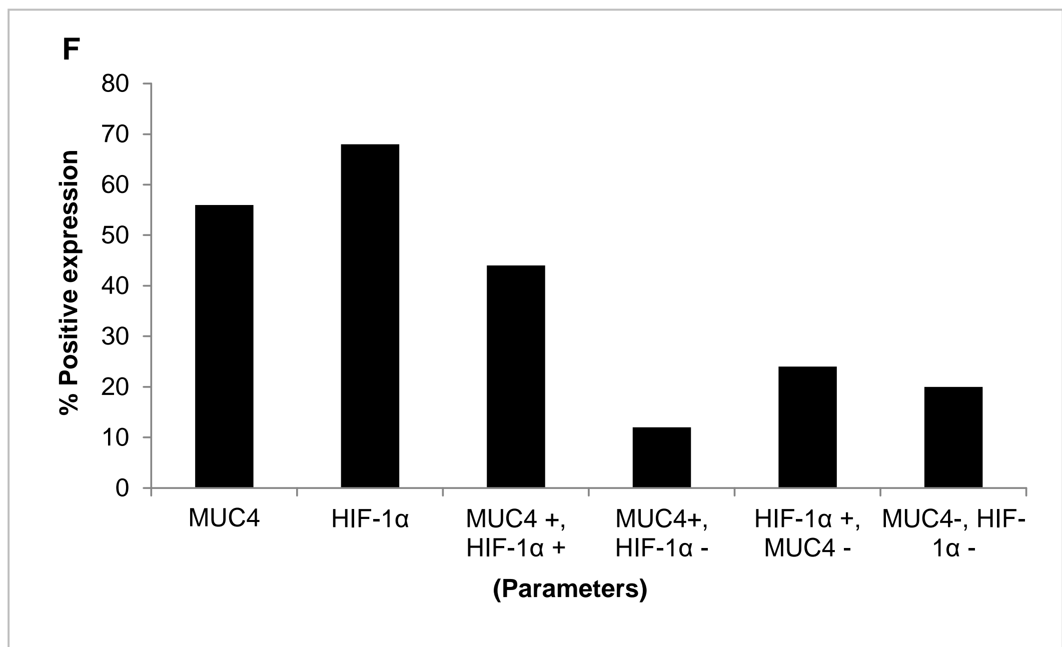
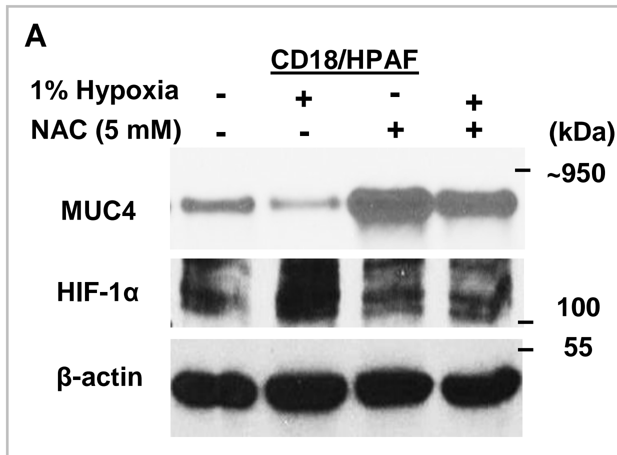
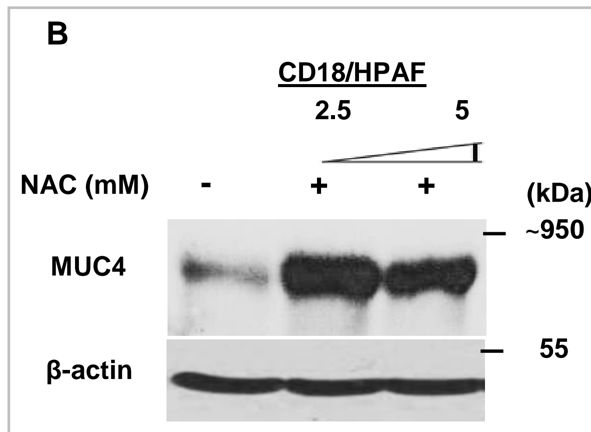


Figure 3.3. MUC4 expression is negatively regulated by hypoxia induced ROS. A. CD18/HPAF cells were treated with NAC in the presence and absence of hypoxia for 24 h. Western blot was performed to analyze alteration in the expression of MUC4 and HIF-1α. **B.** MUC4 expression was analyzed in lysates obtained from CD18/HPAF cell line treated with different concentrations of NAC for 24 h. **C.** Flow cytometry was performed to measure DCFDA florescence in order to detect changes in ROS levels in CD18/HPAF cells upon NAC treatment in the presence and absence of hypoxia. **D.** The bar graph showing mean florescence intensity (MFI) measured for DCFDA dye in indicated immortalized normal pancreatic and cancer cell lines. (**E**). After 12 h of serum starvation, CD18/HPAF and CAPAN1 cells were treated with α-tocopherol succinate (α-TS) for 24 h at indicated concentrations. Following treatment, MUC4 expression was analyzed by 2% agarose gel electrophoresis. **F** and **G.** CAPAN1 cells and CD18/HPAF cells were treated with H_2O_2 followed by MUC4 expression analysis. **H**. Immunofluorescence experiment was performed to further confirm the effect of hypoxia and exogenous ROS on MUC4 at protein level in the presence and absence of ROS neutralizer, NAC.

(Scale bar = 20 μM).

Figure 3.3





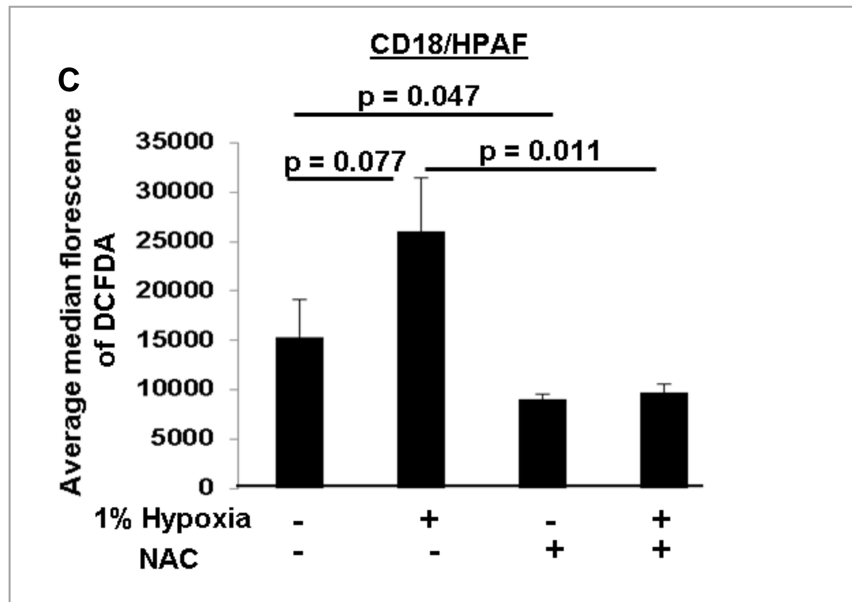
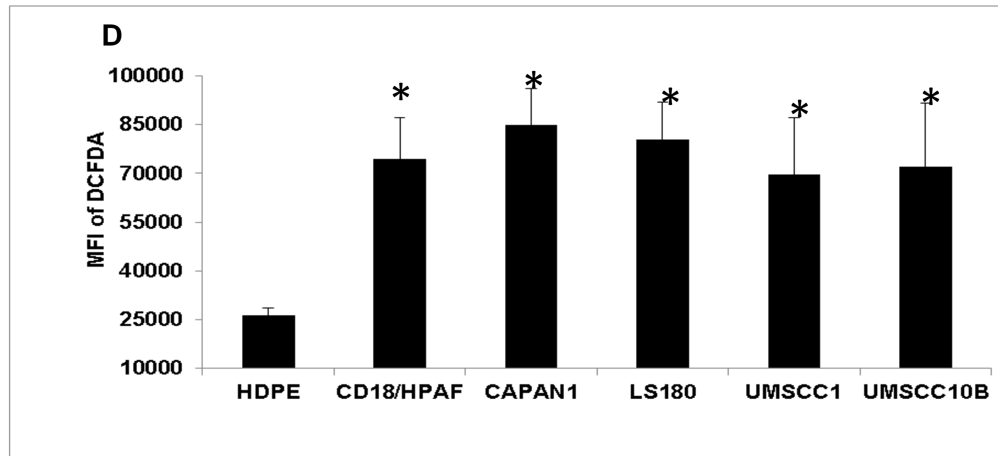
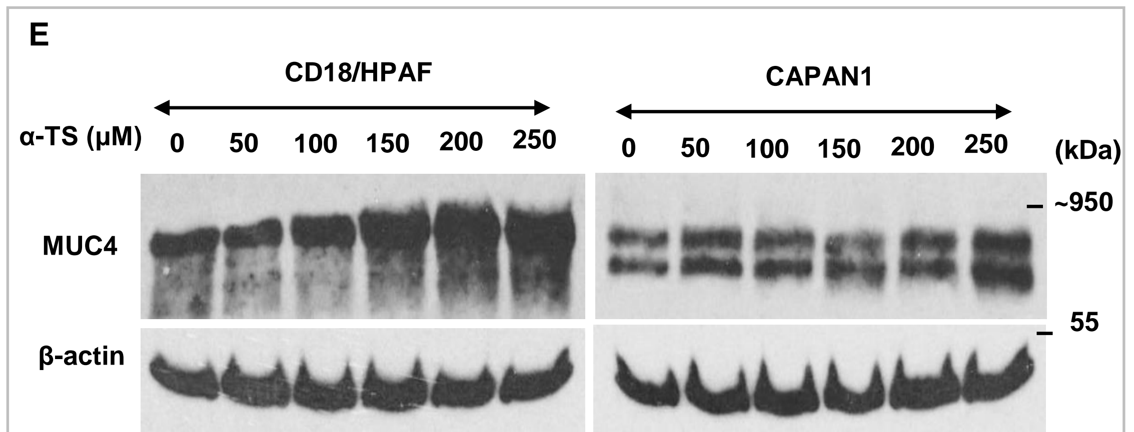
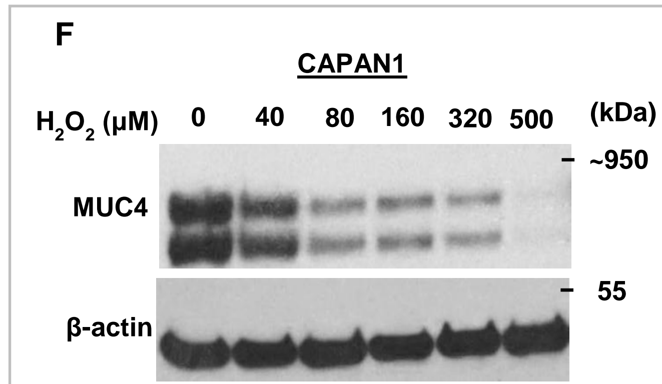


Figure 3.3







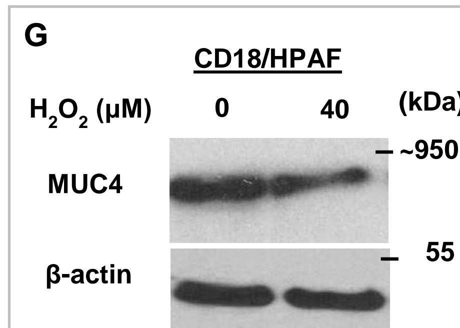


Figure 3.3

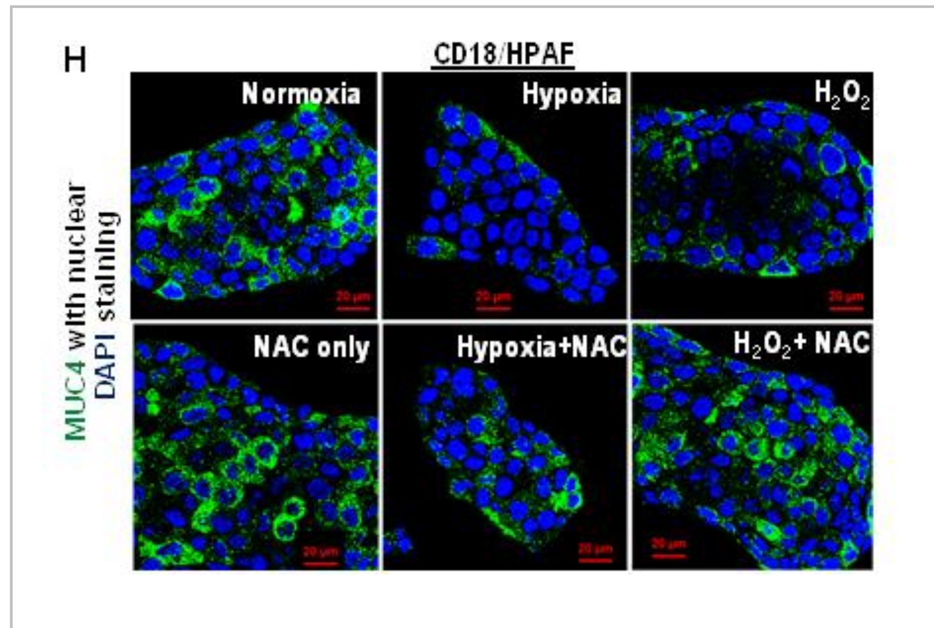
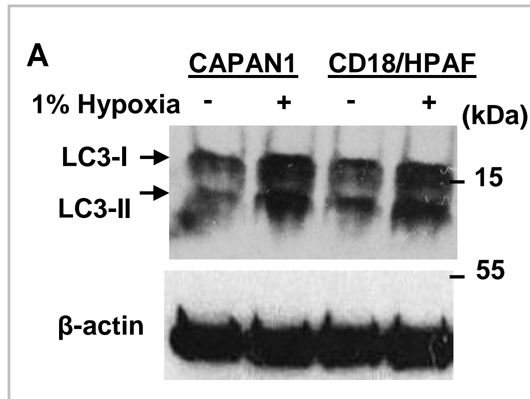
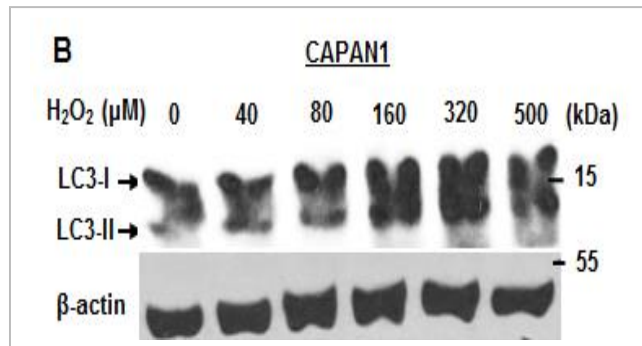


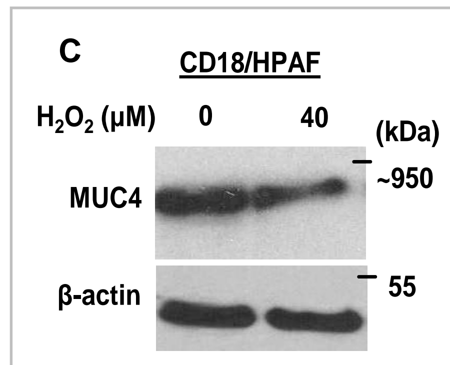
Figure 3.4. Hypoxia-mediated ROS production induces autophagy, which leads to reduced MUC4 stability. A. Cell lysates of CD18/HPAF and CAPAN1 were collected after 24 h incubation with or without 1% hypoxia to analyze the expression of LC3-I and II by western blot. **B.** CAPAN1 cells were treated with increasing concentrations of H_2O_2 to observe the effect of oxidative stress on autophagy. **C**. To further substantiate that presence of oxidative stress induces autophagy, CD18/HPAF cell line was treated with 40 and 80 μM of H_2O_2 followed by the analysis of LC3 and p62 levels, using immunoblotting. **D.** Representative image showing increased autophagosome formation in H_2O_2 and $CoCl_2$ (hypoxia mimetic) treated CAPAN1 cells. For the detection of autophagy vacuoles, MDC staining was performed (Scale bar = 10 μM). **E.** CAPAN1 cells were treated with 10, 20 and 50 nM of rapamycin (RAP), an autophagy inducer, for 24 h. Cell lysates were prepared to analyze the expression of MUC4 and LC3. **F.** CD18/HPAF cells were treated with VB (10 μg/ml) for 24 h under hypoxic conditions to observe the effect of autophagy inhibition on MUC4 expression. **G.** Additionally, confocal microscopy revealed that inhibition of autophagy due to VB treatment leads to increased expression and retention of MUC4 in LC3-positive vesicles. The bar graph is showing the person correlation coefficient between MUC4 and LC3 colocalization in VB-treated and untreated CD18/HPAF cells. (Scale bar = 20 μM). **H.** To confirm an association between MUC4 and autophagy, autophagy was blocked in CAPAN1 cells (plated on the coverslips) by treating them with VB (10 μM) for 8 h. Cells were fixed and immunofluorescence staining was performed to look for the colocalization between MUC4 and LC3 (Scale bar = 20 μM). VB treated PC cells exhibited increased expression and retention of MUC4 in accumulated LC3-positive vesicles. **I.** Confocal image demonstrating significant co-localization between MUC4 and LAMP1 in CAPAN1 cell line. **J.** To specifically pinpoint the role of autophagy in MUC4 degradation, we used

targeted siRNA oligonucleotides to transiently knock down ATG7 in CD18/HPAF PC cells to inhibit autophagy. Consistently, we observed significant increase in MUC4 expression upon ATG7 silencing. (**$p<0.01$: statistically highly significant, Scale bar = 20 μM).

Figure 3.4







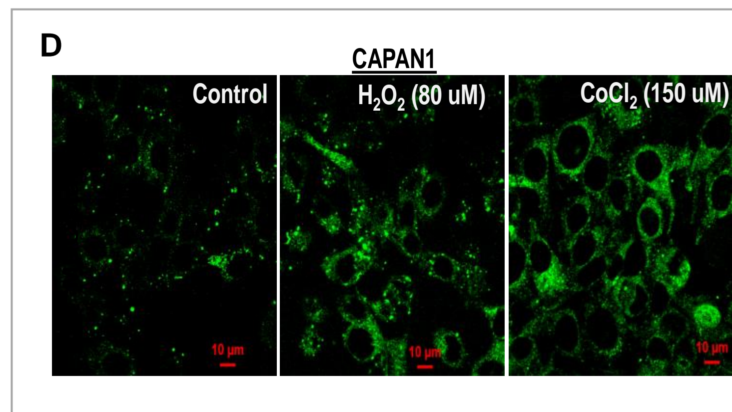


Figure 3.4

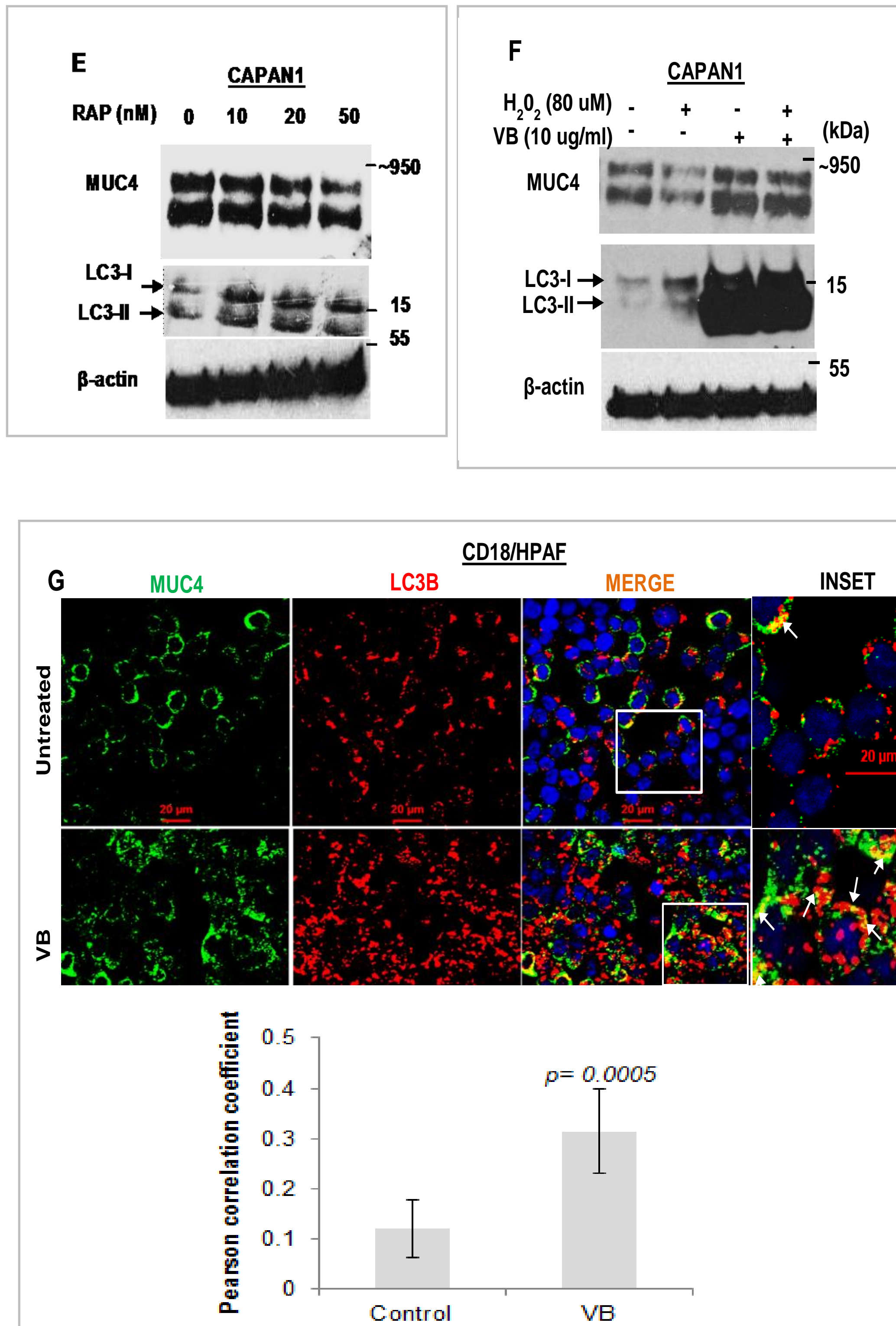
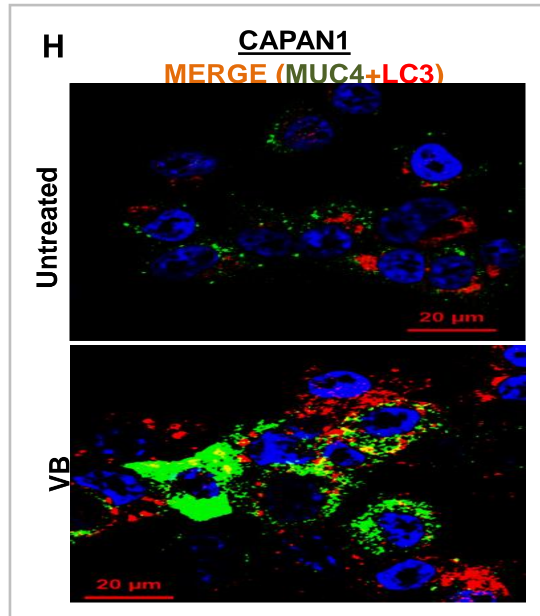
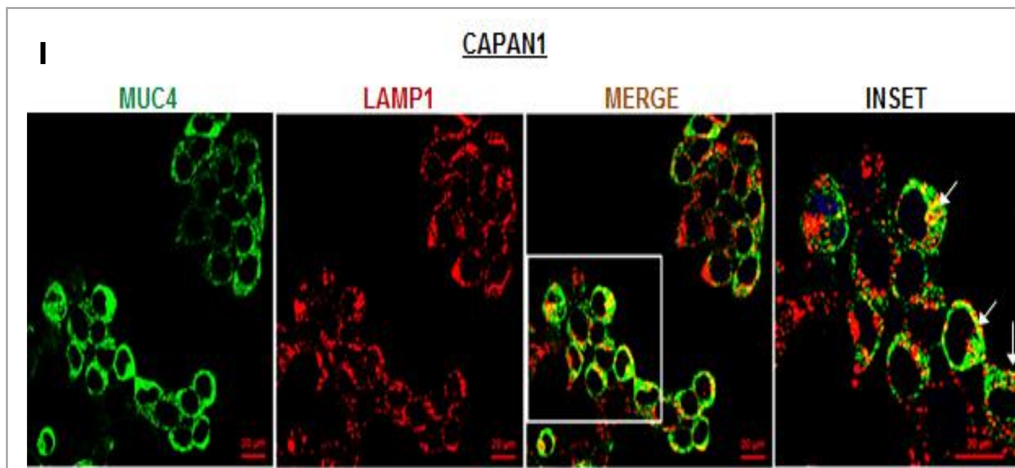


Figure 3.4





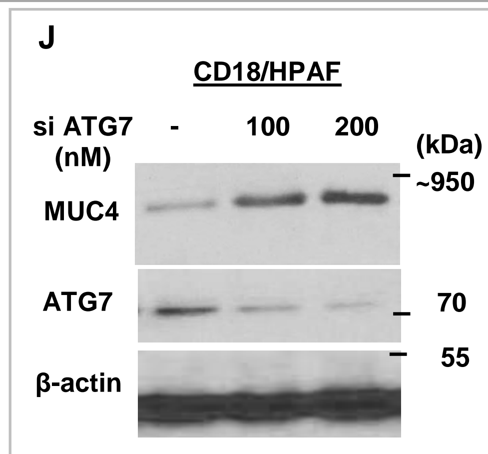


Figure 3.5. Hypoxia-mediated oxidative stress promotes autophagy by inhibiting pAkt/mTORC1 axis and reduces cell viability. (A) T3M4, CD18/HPAF and CAPAN1 cells were incubated under 1% hypoxic conditions for 24 h. Following treatment, cell lysates were collected and used for western blotting to observe changes in the proteins expression of HIF-1α, EGFR, pEGFR (Ser1046), Akt, pAkt (Ser473), S6kinase, pS6kinase (Thr389) and p53. **B.** Immunoblots showing changes in the expression of p21 in hypoxia (1% O_2) treated T3M4 and CD18/HPAF cell lines. **C.** Growth kinetics was performed for CD18/HPAF for 24 and 48 hrs in the presence and absence of 1% hypoxia. **D.** To know whether hypoxia-mediated suppression of pAkt and p53 is ROS-dependent, CD18/HPAF cells were first pre-treated with NAC (5 mM) for 30 mins. Following pre-treatment, cells were incubated under 1% hypoxia. Cell lysates were subsequently collected and immunoblot experiment was performed for Akt, pAkt (Ser473), p53, and MDM2 expression levels. **E.** The graphical representation to demonstrate the effect of hypoxia and neutralization of consequently produced ROS (by concomitant treatment with 2.5 mM of NAC) on the proliferation of CD18/HPAF and CAPAN1 cell lines. **F.** Cell numbers were quantified after 24 h of H_2O_2, NAC and NAC+H_2O_2 treatment of CD18/HPAF and CAPAN1 PC cells. **G.** CD18/HPAF and CAPAN1 cell lines were treated with different concentrations of exogenous H_2O_2. Following treatment, MTT assay was performed to analyze the effect of treatment on cellular viability. **H**. To explore the role of hypoxia-induced oxidative stress and autophagy on cell death and viability, MTT assay was performed. CD18/HPAF cells were exposed to 1% hypoxia in the presence and absence of NAC (2.5 mM) and CQ (50 μM) for 24 h. Post-treatment, MTT assay was performed and optical density was measured at 570 nm. **I.** The graphical representation of Annexin (indicator of early-apoptosis) and propidium iodide (PI, indicator of late apoptosis and necrotic cells)

staining performed on CD18/HPAF cells treated for 24 h with hypoxia alone, hypoxia followed by NAC (2.5 mM) or CQ (50 μM) treatment for further 12 h.

Figure 3.5

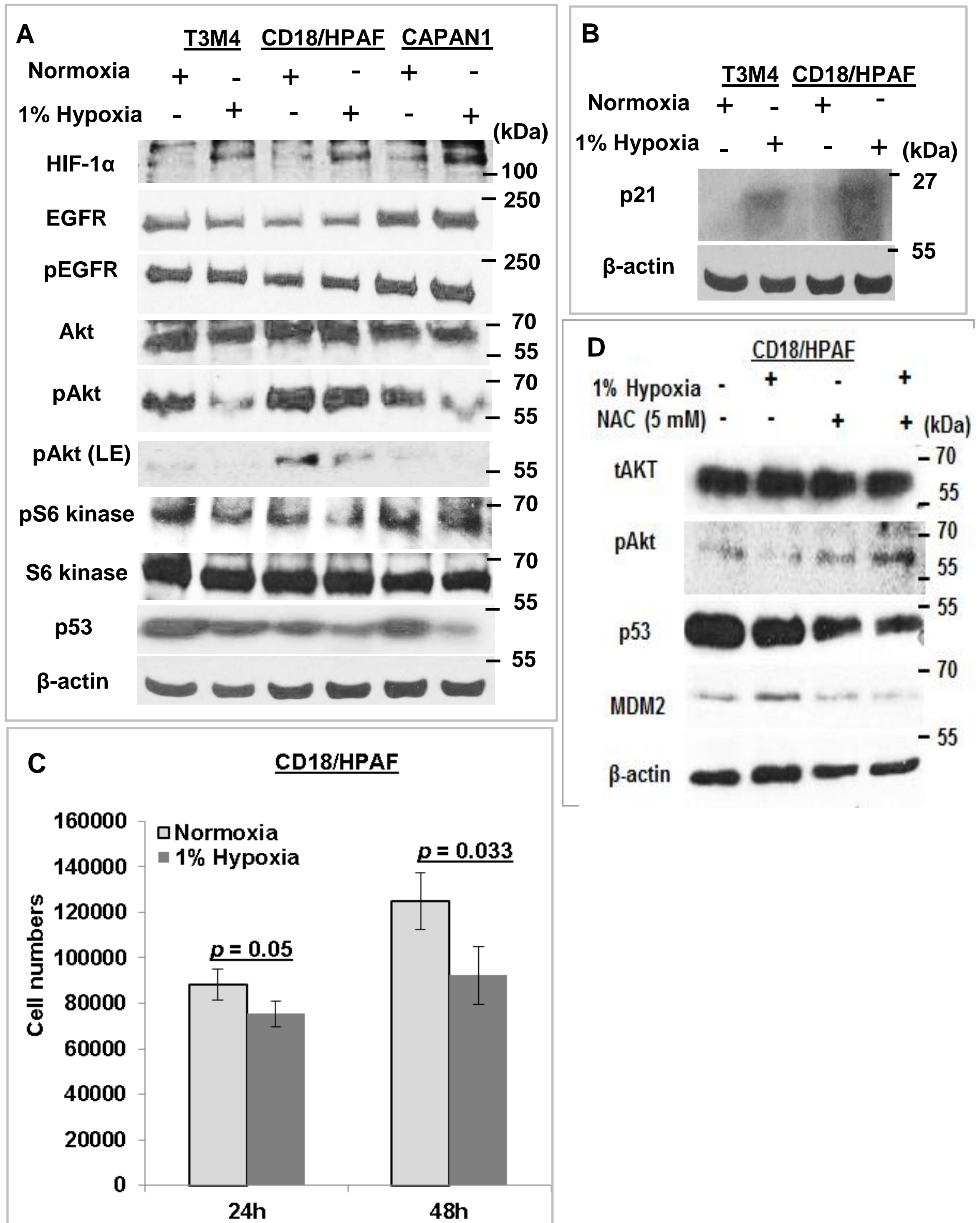
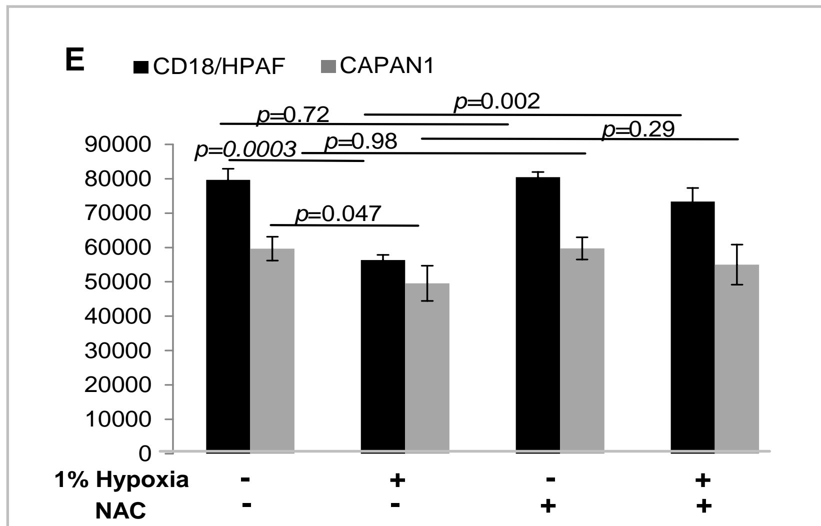
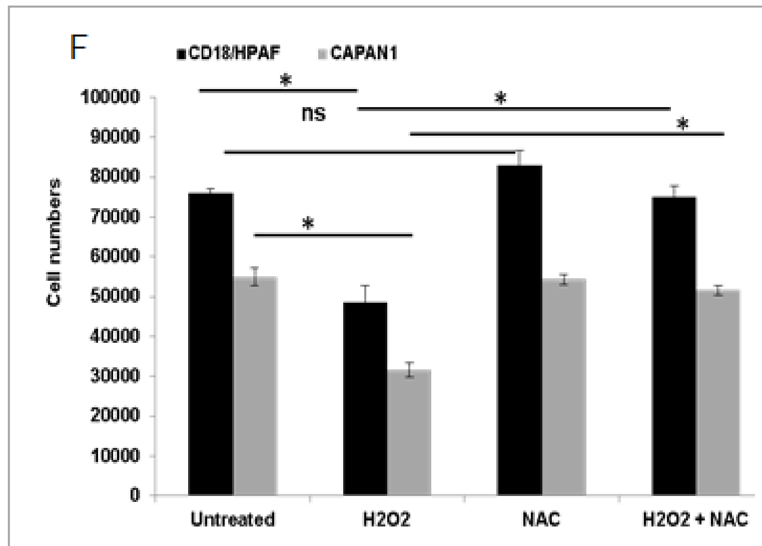


Figure 3.5





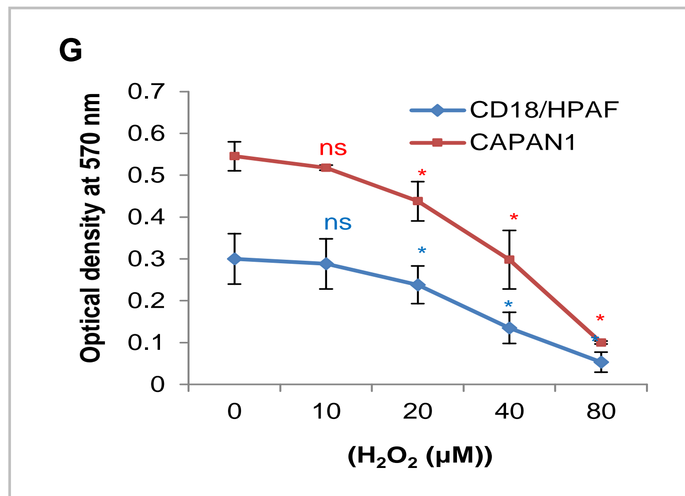
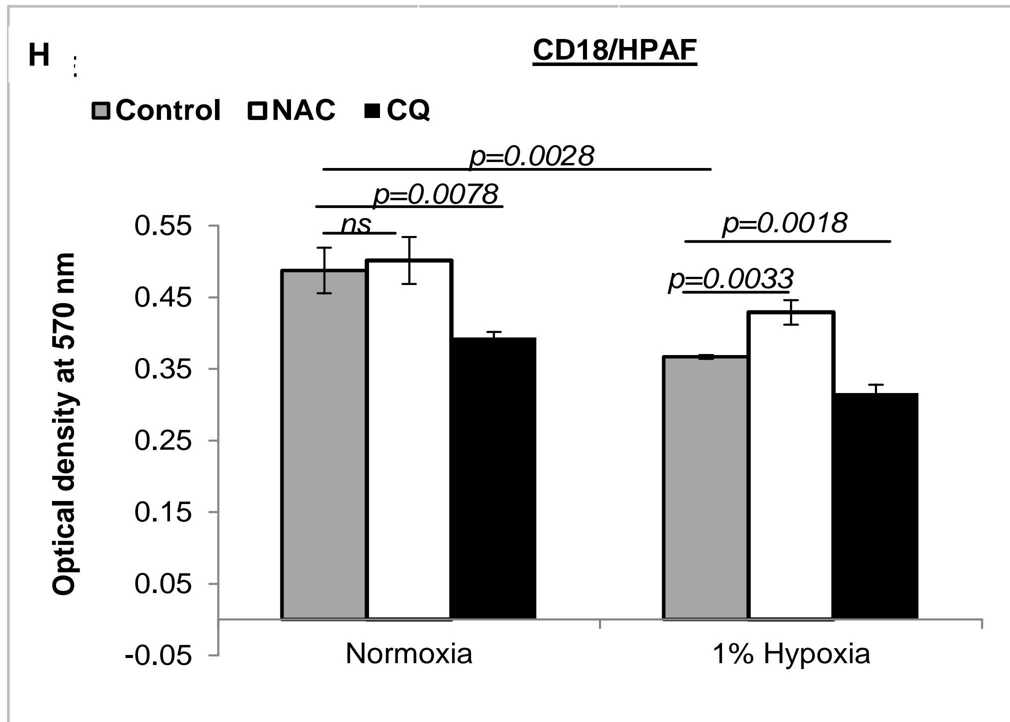


Figure 3.5



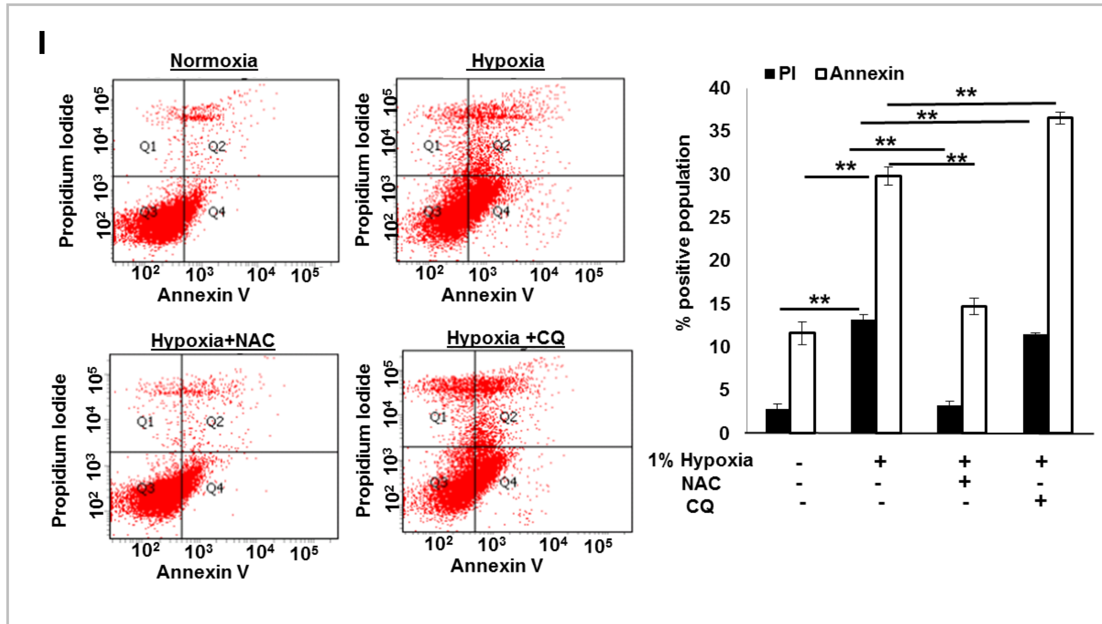


Figure 3.6. Hypoxia-mediated reduction in cell viability is rescued by MUC4 overexpression. A. Immunoblot confirming MUC4 knocked down in CAPAN1 cells. **B.** The graphical representation to demonstrate the effect of 1, 3 and 5 days of hypoxia treatment on the proliferation of MUC4 kd and scr CAPAN1 cells. (LE: Low exposure; $p<0.05$: statistically significant; **$p<0.01$: statistically highly significant; ns: no significant difference). **C.** Immunoblot representing the ectopic expression of MiniMUC4 in MUC4 non-expressing MIA PaCa-2 cell line. **D.** The graphical representation to demonstrate the effect of 24 h and 48 h of combinatorial or individual treatment of H_2O_2 and NAC on the proliferation of MIA PaCa-2/psectag and MIA PaCa-2/MiniMUC4 expressing cell lines. (*$p<0.05$ signifies statistically significant results; ns means insignificant changes; Scale bar =20 μM)

Figure 3.6

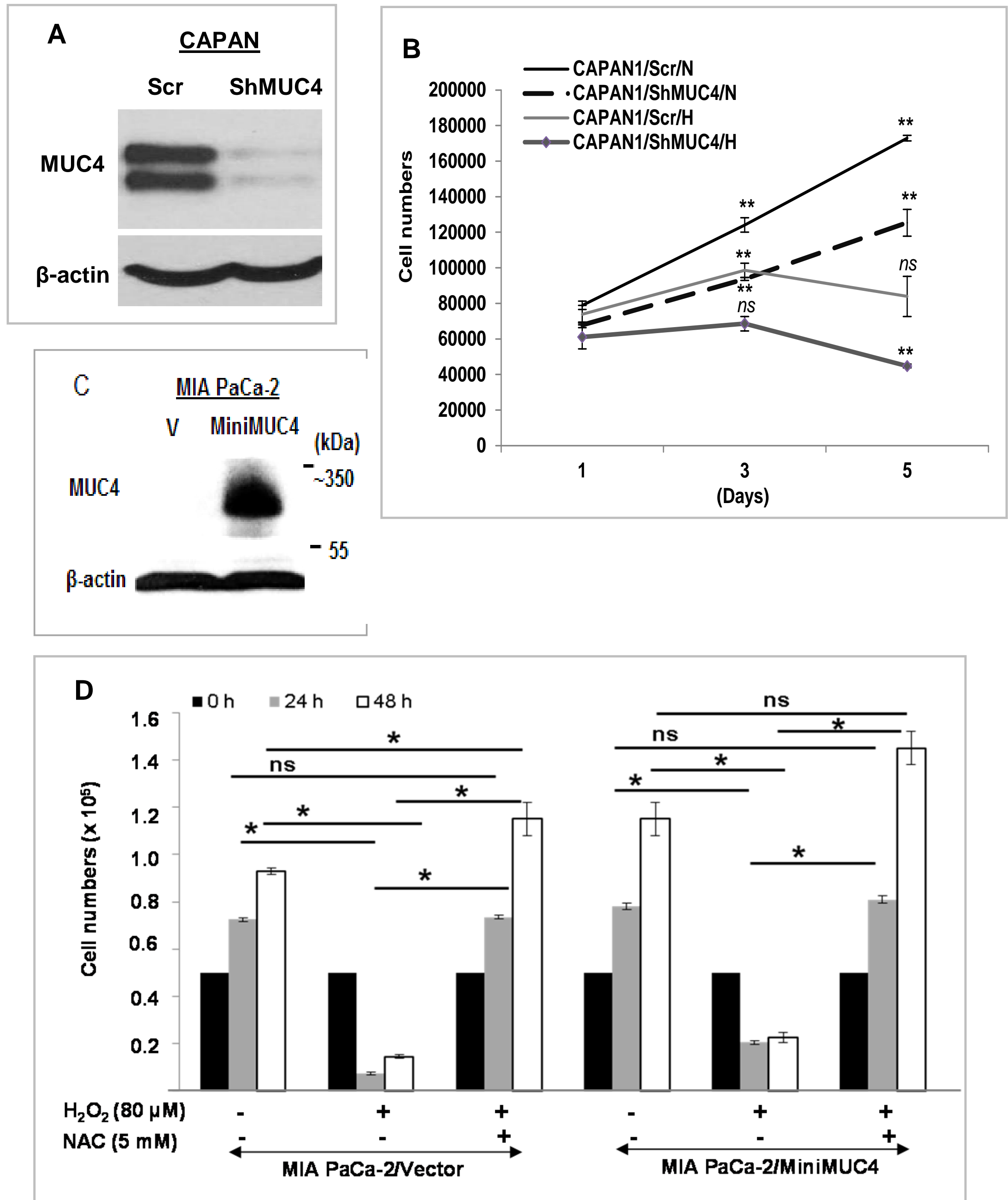
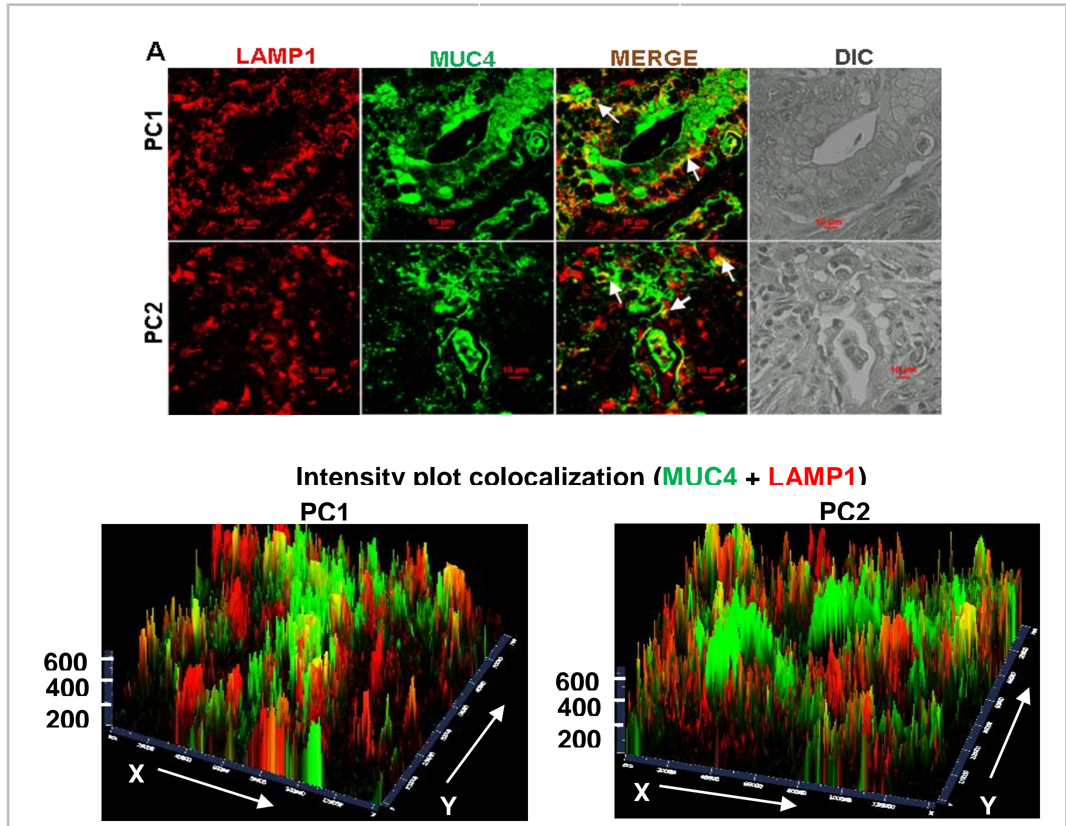


Figure 3.7. *In vivo* validation of MUC4 association with oxidative stress and degradation *via* lysosomal pathway. A. Confocal images showing colocalization between MUC4 and lysosomal marker (LAMP1), and thus clearly indicate that MUC4 does enter to lysosomal compartment. In spite of significant colocalization between MUC4 and LAMP1, similar to CAPAN1 cell line, MUC4 and LAMP1 expression pattern was inversely associated under *in vivo* settings. Tumor cells having more LAMP1 expression exhibited reduce MUC4 expression in stained Whipple tissue samples. Histogram representation of the intensities plots for MUC4 and LAMP1 is further confirming our observation. **B.** Immunofluorescence staining was performed in PC tissue section to observe the colocalization between MUC4 and LC3 molecules by confocal microscopy. **C.** Representative images of PC tissues stained with MUC4 and oxidative stress marker (or high ROS indicator; 8-OHG). Box-plot showing the significant difference between the MFI observed for 8-OHG in $MUC4^{L}$ (n=20) and MUC^{H} (n=16) fields. **D.** Representative images obtained from confocal microscopy showing that presence of oxidative stress does not always correlate with HIF-1α expression, as PC tissue spots demonstrating high 8-OHG expression had less HIF-1α expression and *vice versa*. **E.** Scatter graph showing relationship between the MFI levels of HIF-1α and 8-OHG in clinical samples.

(Scale bar = 10 μM).

Figure 3.7



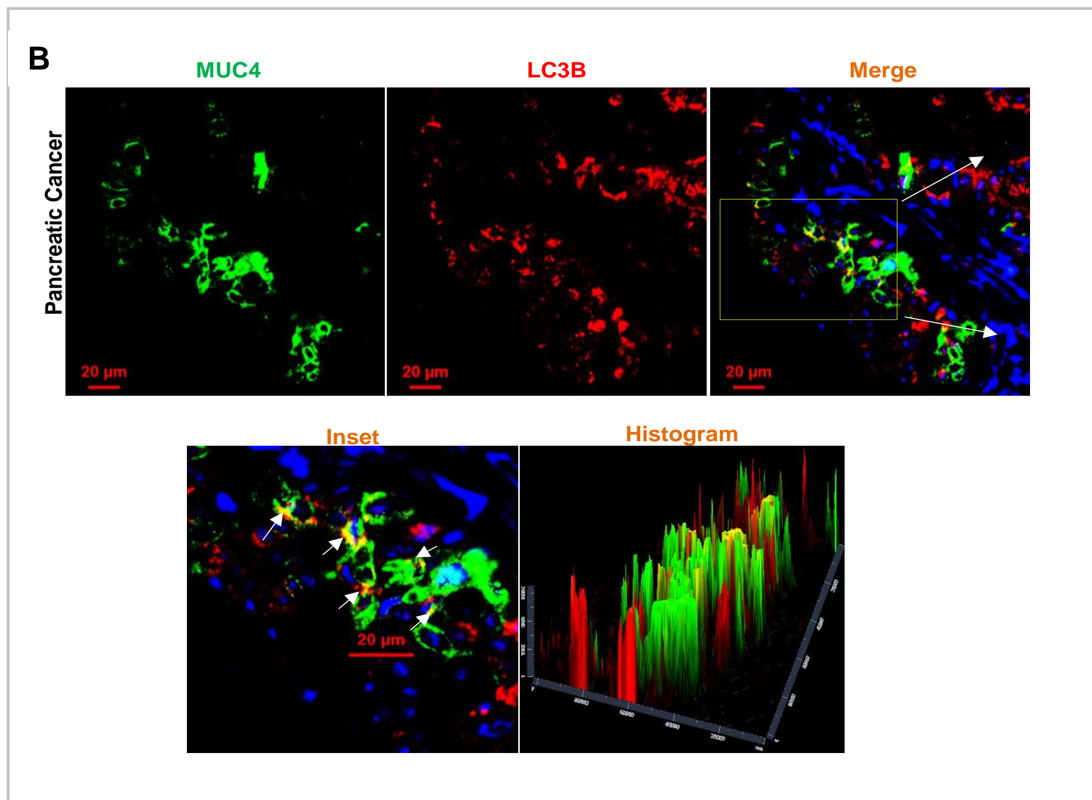
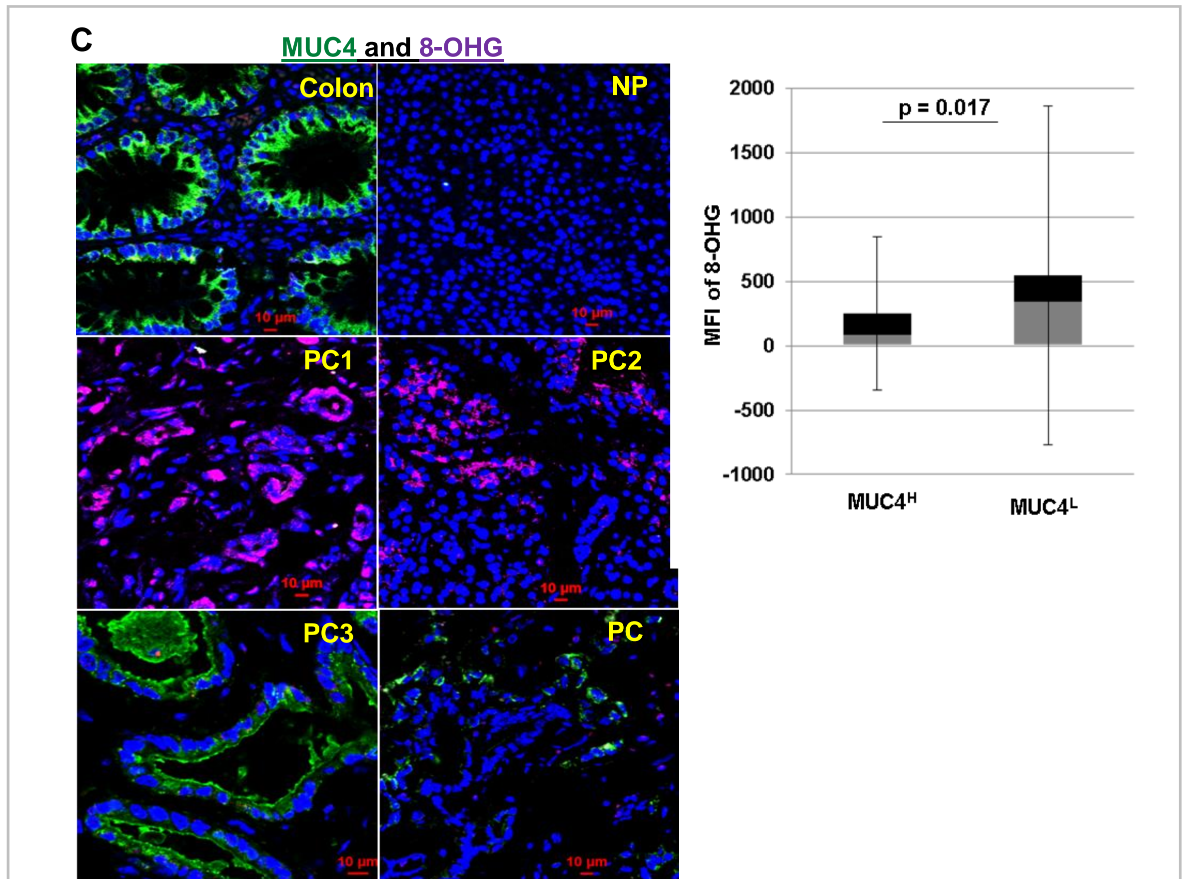


Figure 3.7



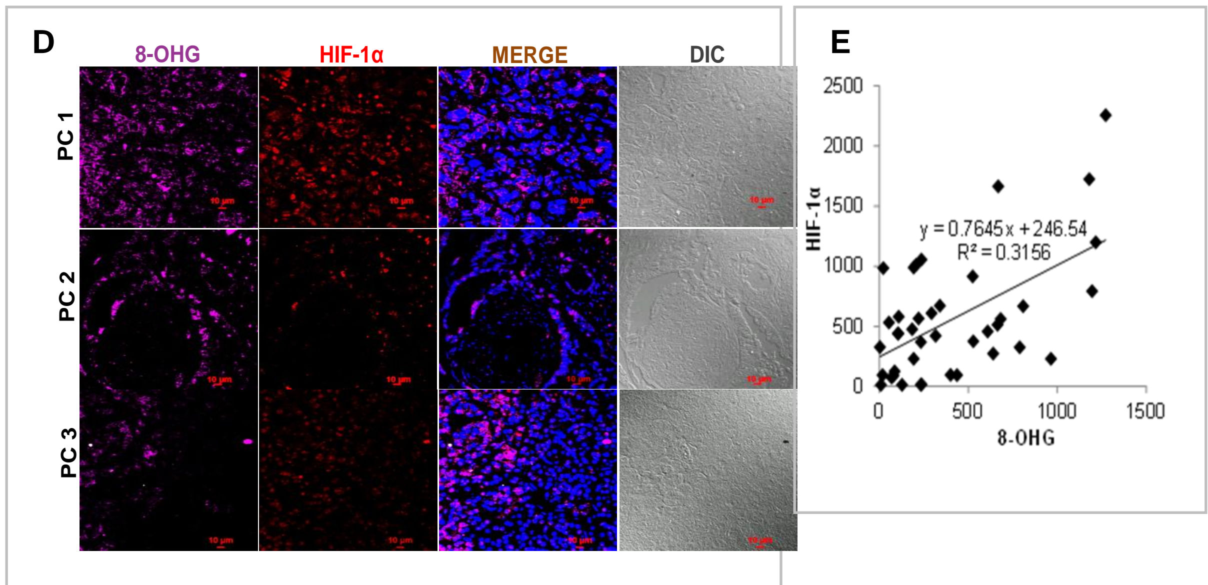
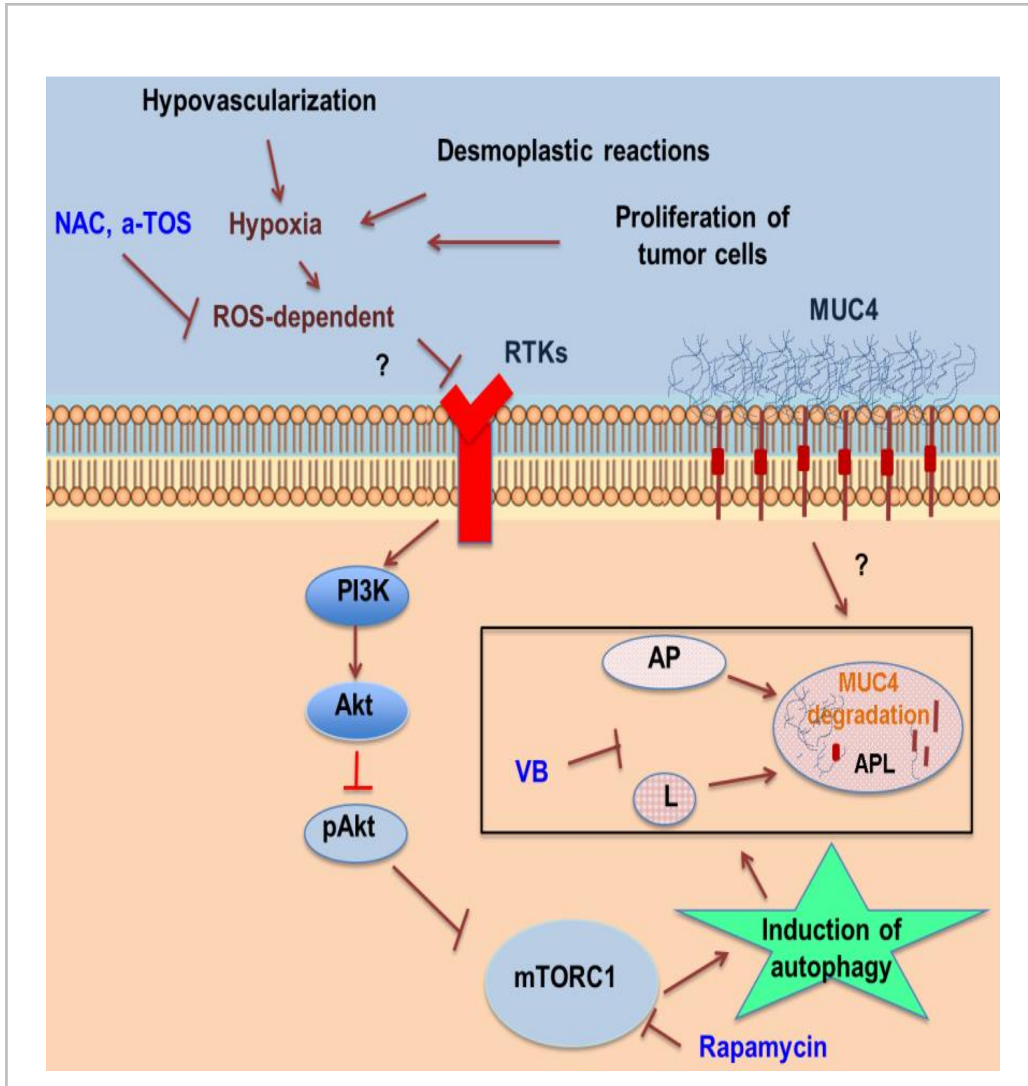


Figure 3.8. Schematic presentation of the summary of the paper. Hypoxia is induced collaboratively by hypovascularization, desmoplastic reactions and continuous proliferation of tumor cells, which further leads to increase ROS production and generate oxidative stress condition. Produced ROS inhibits the activation of Akt which further leads to mTORC1 inhibition and induction of autophagy. Induce autophagy facilitates MUC4 degradation. The inhibitors used in this study suppress the activity of different proteins. For example, NAC and α-TOS act as ROS scavenger, rapamycin inhibits mTORC1 and VB inhibits the fusion of autophagosomes (AP) with lysosomes (L) and thus, prevent the formation of autophagolysosomes (APL) which causes MUC4 accumulation.

Figure 3.8



III. 5 References

(1) Joshi S, Kumar S, Ponnusamy MP, Batra SK. Hypoxia-induced oxidative stress promotes MUC4 degradation via autophagy to enhance pancreatic cancer cells survival. Oncogene 2016.

(2) Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9-29.

(3) Sullivan R, Pare GC, Frederiksen LJ, Semenza GL, Graham CH. Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity. Mol Cancer Ther 2008;7:1961-73.

(4) 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.

(5) Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996;379:88-91.

(6) Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth MA, Buchler MW, Aubert JP, Batra SK. 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.

(7) Hinoda Y, Ikematsu Y, Horinochi M, Sato S, Yamamoto K, Nakano T, Fukui M, Suehiro Y, Hamanaka Y, Nishikawa Y, Kida H, Waki S, Oka M, Imai K, Yonezawa S. Increased expression of MUC1 in advanced pancreatic cancer. J Gastroenterol 2003;38:1162-6.

(8) Rachagani S, Torres MP, Kumar S, Haridas D, Baine M, Macha MA, Kaur S, Ponnusamy MP, Dey P, Seshacharyulu P, Johansson SL, Jain M, Wagner KU, Batra SK. Mucin (Muc) expression during pancreatic cancer progression in spontaneous mouse model: potential implications for diagnosis and therapy. J Hematol Oncol 2012;5:68.

(9) Joshi S, Kumar S, Bafna S, Rachagani S, Wagner KU, Jain M, Batra SK. Genetically engineered mucin mouse models for inflammation and cancer. Cancer Metastasis Rev 2015.

(10) Aubert S, Fauquette V, Hemon B, Lepoivre R, Briez N, Bernard D, Van S, I, Leroy X, Perrais M. MUC1, a new hypoxia inducible factor target gene, is an actor in clear renal cell carcinoma tumor progression. Cancer Res 2009;69:5707-15.

(11) Chaika NV, Gebregiworgis T, Lewallen ME, Purohit V, Radhakrishnan P, Liu X, Zhang B, Mehla K, Brown RB, Caffrey T, Yu F, Johnson KR, Powers R, Hollingsworth MA, Singh PK. MUC1 mucin stabilizes and activates hypoxia-

inducible factor 1 alpha to regulate metabolism in pancreatic cancer. Proc Natl Acad Sci U S A 2012;109:13787-92.

(12) Kitamoto S, Yokoyama S, Higashi M, Yamada N, Takao S, Yonezawa S. MUC1 enhances hypoxia-driven angiogenesis through the regulation of multiple proangiogenic factors. Oncogene 2013;32:4614-21.

(13) Joshi S, Kumar S, Choudhury A, Ponnusamy MP, Batra SK. Altered Mucins (MUC) trafficking in benign and malignant conditions. Oncotarget 2014;5:7272-84.

(14) Senapati S, Chaturvedi P, Chaney WG, Chakraborty S, Gnanapragassam VS, Sasson AR, Batra SK. Novel INTeraction of MUC4 and galectin: potential pathobiological implications for metastasis in lethal pancreatic cancer. Clin Cancer Res 2011;17:267-74.

(15) 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.

(16) 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.

(17) Kumar S, Das S, Rachagani S, Kaur S, Joshi S, Johansson SL, Ponnusamy MP, Jain M, Batra SK. NCOA3-mediated upregulation of mucin expression via transcriptional and post-translational changes during the development of pancreatic cancer. Oncogene 2014.

(18) Kitamoto S, Yokoyama S, Higashi M, Yamada N, Matsubara S, Takao S, Batra SK, Yonezawa S. Expression of MUC17 is regulated by HIF1alpha-mediated hypoxic responses and requires a methylation-free hypoxia responsible element in pancreatic cancer. PLoS One 2012;7:e44108.

(19) Kim YJ, Cho HJ, Shin WC, Song HA, Yoon JH, Kim CH. Hypoxia-mediated mechanism of MUC5AC production in human nasal epithelia and its implication in rhinosinusitis. PLoS One 2014;9:e98136.

(20) Ding WX, Ni HM, Gao W, Yoshimori T, Stolz DB, Ron D, Yin XM. Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. Am J Pathol 2007;171:513-24.

(21) Ge PF, Zhang JZ, Wang XF, Meng FK, Li WC, Luan YX, Ling F, Luo YN. Inhibition of autophagy induced by proteasome inhibition increases cell death in human SHG-44 glioma cells. Acta Pharmacol Sin 2009;30:1046-52.

(22) Liu C, Yan X, Wang HQ, Gao YY, Liu J, Hu Z, Liu D, Gao J, Lin B. Autophagy-independent enhancing effects of Beclin 1 on cytotoxicity of ovarian cancer cells mediated by proteasome inhibitors. BMC Cancer 2012;12:622.

(23) Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 2007;26:225-39.

(24) Shao MX, Nadel JA. Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-alpha-converting enzyme. J Immunol 2005;175:4009-16.

(25) Liu Z, Lenardo MJ. Reactive oxygen species regulate autophagy through redox-sensitive proteases. Dev Cell 2007;12:484-5.

(26) Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, Bause A, Li Y, Stommel JM, Dell'antonio G, Mautner J, Tonon G, Haigis M, Shirihai OS, Doglioni C, et al. Pancreatic cancers require autophagy for tumor growth. Genes Dev 2011;25:717-29.

(27) Munafo DB, Colombo MI. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 2001;114:3619-29.

(28) Zhou J, Tan SH, Nicolas V, Bauvy C, Yang ND, Zhang J, Xue Y, Codogno P, Shen HM. Activation of lysosomal function in the course of autophagy via mTORC1 suppression and autophagosome-lysosome fusion. Cell Res 2013;23:508-23.

(29) Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, White M, Reichelt J, Levine B. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. Science 2012;338:956-9.

(30) Endo H, Okuyama H, Ohue M, Inoue M. Dormancy of cancer cells with suppression of AKT activity contributes to survival in chronic hypoxia. PLoS One 2014;9:e98858.

(31) Blanco FF, Jimbo M, Wulfkuhle J, Gallagher I, Deng J, Enyenihi L, Meisner-Kober N, Londin E, Rigoutsos I, Sawicki JA, Risbud MV, Witkiewicz AK, McCue PA, Jiang W, Rui H, et al. The mRNA-binding protein HuR promotes hypoxia-induced chemoresistance through posttranscriptional regulation of the proto-oncogene PIM1 in pancreatic cancer cells. Oncogene 2015.

(32) Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, Bause A, Li Y, Stommel JM, Dell'antonio G, Mautner J, Tonon G, Haigis M, Shirihai OS, Doglioni C, et al. Pancreatic cancers require autophagy for tumor growth. Genes Dev 2011;25:717-29.

(33) Moniaux N, Chaturvedi P, Varshney GC, Meza JL, Rodriguez-Sierra JF, Aubert JP, Batra SK. Human MUC4 mucin induces ultra-structural changes and tumorigenicity in pancreatic cancer cells. Br J Cancer 2007;97:345-57.

(34) Yu L, McPhee CK, Zheng L, Mardones GA, Rong Y, Peng J, Mi N, Zhao Y, Liu Z, Wan F, Hailey DW, Oorschot V, Klumperman J, Baehrecke EH, Lenardo MJ.

Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 2010;465:942-6.

(35) Zhang X, Wu RS, Fu W, Xu L, Lam PK. Production of reactive oxygen species and 8-hydroxy-2'deoxyguanosine in KB cells co-exposed to benzo[a]pyrene and UV-A radiation. Chemosphere 2004;55:1303-8.

(36) Kasai H. Chemistry-based studies on oxidative DNA damage: formation, repair, and mutagenesis. Free Radic Biol Med 2002;33:450-6.

(37) Papandreou I, Krishna C, Kaper F, Cai D, Giaccia AJ, Denko NC. Anoxia is necessary for tumor cell toxicity caused by a low-oxygen environment. Cancer Res 2005;65:3171-8.

(38) Vasseur S, Tomasini R, Tournaire R, Iovanna JL. Hypoxia induced tumor metabolic switch contributes to pancreatic cancer aggressiveness. Cancers (Basel) 2010;2:2138-52.

(39) Chaturvedi P, Singh AP, Chakraborty S, Chauhan SC, Bafna S, Meza JL, Singh PK, Hollingsworth MA, Mehta PP, Batra SK. MUC4 mucin interacts with and stabilizes the HER2 oncoprotein in human pancreatic cancer cells. Cancer Res 2008;68:2065-70.

(40) Zhi X, Tao J, Xie K, Zhu Y, Li Z, Tang J, Wang W, Xu H, Zhang J, Xu Z. MUC4-induced nuclear translocation of beta-catenin: a novel mechanism for growth, metastasis and angiogenesis in pancreatic cancer. Cancer Lett 2014;346:104-13.

(41) Sendoel A, Hengartner MO. Apoptotic cell death under hypoxia. Physiology (Bethesda) 2014;29:168-76.

(42) Filomeni G, De ZD, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. Cell Death Differ 2015;22:377-88.

(43) Hashimoto D, Blauer M, Hirota M, Ikonen NH, Sand J, Laukkarinen J. Autophagy is needed for the growth of pancreatic adenocarcinoma and has a cytoprotective effect against anticancer drugs. Eur J Cancer 2014;50:1382-90.

(44) Commisso C, Davidson SM, Soydaner-Azeloglu RG, Parker SJ, Kamphorst JJ, Hackett S, Grabocka E, Nofal M, Drebin JA, Thompson CB, Rabinowitz JD, Metallo CM, Vander Heiden MG, Bar-Sagi D. Macropinocytosis of protein is an amino acid supply route in Ras-transformed cells. Nature 2013;497:633-7.

(45) Kamphorst JJ, Nofal M, Commisso C, Hackett SR, Lu W, Grabocka E, Vander Heiden MG, Miller G, Drebin JA, Bar-Sagi D, Thompson CB, Rabinowitz JD. Human pancreatic cancer tumors are nutrient poor and tumor cells actively scavenge extracellular protein. Cancer Res 2015;75:544-53.

(46) Siddiqui A, Heinzerling J, Livingston EH, Huerta S. Predictors of early mortality in veteran patients with pancreatic cancer. Am J Surg 2007;194:362-6.

(47) Gupta D, Lis CG. Pretreatment serum albumin as a predictor of cancer survival: a systematic review of the epidemiological literature. Nutr J 2010;9:69.

(48) Sayin VI, Ibrahim MX, Larsson E, Nilsson JA, Lindahl P, Bergo MO. Antioxidants accelerate lung cancer progression in mice. Sci Transl Med 2014;6:221ra15.

(49) Chandel NS, Tuveson DA. The promise and perils of antioxidants for cancer patients. N Engl J Med 2014;371:177-8.

(50) Greco E, Basso D, Fadi E, Padoan A, Fogar P, Zambon CF, Navaglia F, Bozzato D, Moz S, Pedrazzoli S, Plebani M. Analogs of vitamin E epitomized by alpha-tocopheryl succinate for pancreatic cancer treatment: in vitro results induce caution for in vivo applications. Pancreas 2010;39:662-8.

(51) Dhillon H, Chikara S, Reindl KM. Piperlongumine induces pancreatic cancer cell death by enhancing reactive oxygen species and DNA damage. Toxicol Rep 2014;1:309-18.

(52) Seshacharyulu P, Ponnusamy MP, Rachagani S, Lakshmanan I, Haridas D, Yan Y, Ganti AK, Batra SK. 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.

CHAPTER IV

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

Part of this chapter are driven from:

Joshi S, Cruz E, Rachagani S, Guha S, Brand RE, Ponnusamy MP *et al*. Bile acids-mediated overexpression of MUC4 via FAK-dependent c-Jun activation promotes the aggressiveness of pancreatic cancer. Accepted in **Mol. Oncol.** (**#** MOLONC-D-16-00080)

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

Suhasini Joshi[1], Eric Cruz[1], Satyanarayana Rachagani[1], Sushovan Guha[2], Randall E. Brand[3], Moorthy P. Ponnusamy[1,4,5], Sushil Kumar[1,*], and Surinder K. Batra[1,4,5,6,*]

Author Affiliations

[1]Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center; [2]Division of Gastroenterology, Hepatology and Nutrition, UT Health-UT Health Science Center and Medical School at Houston, Houston, Texas, [3]Division of Gastroenterology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, [4]Buffett Cancer Center, [5]Eppley Institute for Research in Cancer and Allied Diseases; [6]Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198, United States of America

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

*To whom correspondence should be addressed:
Drs. Surinder K. Batra and Sushil Kumar, Ph.D. Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5870, U.S.A.
Phone: 402-559-5455; Fax: 402-559-6650, E-mail: sbatra@unmc.edu, skumar@unmc.edu

IV. 1 Synopsis

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

IV.2 Background and rationale

In 2014, about 45,000 new cases of pancreatic cancer (PC) were diagnosed in the United States, of which pancreatic ductal adenocarcinoma represents the major histological type (1). The majority of tumors (about 75%) arise at the head of the pancreas (2). Anatomically, the pancreatic duct is placed close to the common bile duct, which both unite at the point known as the ampulla of Vater, and secrete their contents into the duodenum, which is the proximal site of the intestine (3). Approximately, 70% of PC patients develop extrahepatic cholestasis due to blockage of the common bile duct by increasing tumor size and results in multiple organ failure and early death (4). Due to this bile duct obstruction, extrahepatic cholestasis exhibits obstructive jaundice, and indication of both hyperbilirubinemia and the increased circulatory levels of BA.

BA are amphiphilic molecules and are the main component of bile along with cholesterol, phospholipids, and bilirubin (5). By utilizing a series of enzymatic modifications, BA are synthesized in the liver using cholesterol as a precursor. Even after their synthesis, they are further modified by bacterial species present in the colon and form secondary BA (5). Dietary fat is a stimulus for BA secretion into the intestine, which is required for the proper digestion of fatty foods (5). Though bile-reflux has been associated with esophageal and gastric cancers, BA association with PC pathogenesis has not been investigated (6, 7). A recently performed meta-analysis has revealed increased risk of PDAC with patients having the history of cholecystectomy (8). It has been proposed that the mechanism attributed to this is the increased levels of cholecystokinin, which is known to stimulate the growth of human PC cell lines and promote pancreatic carcinogenesis in hamsters (9).

BA have been shown to participate in the progression of tumors using multiple mechanisms including, alteration in the expression of oncogenic mucins (10, 11). Interestingly, PC is characterized by aberrant mucins expression (12-14). Among multiple mucins expressed in PC, MUC4 is one of the top-differentially expressed protein (15). We and others have established the oncogenic functions of MUC4 in PC, and inhibition of MUC4 expression has been associated with reduced PC cell proliferation, migration, and chemoresistance (16-18). MUC4 is one of the most differentially expressed proteins in PC; therefore, comprehending the mode of its regulation will give us an opportunity to develop novel therapeutic strategies. In the present study, we have evaluated the role of BA in the regulation of MUC4 expression in PC. The findings from the current study, for the first time, have demonstrated that BA levels are significantly high in the serum and pancreatic juice samples obtained from PC patients. Using highly defined spontaneous mouse model of PC, we found that BA levels increase with the severity of PC disease condition, which led us to propose its tumor-promoting functions, which we have mechanistically explained by BA-mediated induced expression of oncogenic MUC4 mucin. Mechanistically, BA-mediated upregulation of MUC4 was found to be primarily dependent on FAK-dependent c-Jun activation. Further studies led us to establish the role of FXR as the upstream molecule in this FAK/c-Jun/MUC4 axis.

IV.3 Results

A. BA levels are elevated in serum and pancreatic juice during pancreatic cancer

According to our hypothesis, BA play important roles in PC development by regulating the expression of oncogenic proteins, including MUC4. Therefore, we

first analyzed the *in vivo* levels of BA under PC disease condition. We observed that PC patients had significantly ($p<0.05$) higher circulatory bile acid levels as compared to the control group (**Fig.4.1A**). Additionally, we observed a significant increase in circulatory BA levels in 10-15-wk- and 20-25-wk-old (fully developed PC tumor) of KPC mice compared to their littermate controls (**Fig.4.1B),** strengthening the association of BA with the pathobiology of PC disease. We included controls from different age group for BA estimation and did not observe any noticeable change in their serum BA levels, which is also evident from the demonstrated standard errors (**Fig.4.1B**). Additionally, earlier report by Uchida K *et al*. have demonstrated that circulatory BA levels when expressed in terms of units per rat did not ostensibly change, regardless of their age (19). Consistently, pancreatic juice obtained from PC patients (n=18) had significantly high BA levels ($p = 0.048$) of 65 μM, compared to the non-pancreatic non-healthy (NPNH, patients with symptoms mimicking pancreatic disease but found to be free of pancreatic pathology) subjects (n=5), where the mean concentration of BA was 13.65 μM (**Fig.4.1C**). Taken together, high BA levels in PC condition suggest their possible involvement in the pathobiology of PC.

B. BA up-regulate MUC4 expression in PC cells

BA are known to execute their oncogenic functions by altering the expression levels of mucins such as MUC1, MUC2, MUC4 and MUC5AC in oesophageal, gastric and colon cancers (10, 11, 20-23). Interestingly, PC is characterized by altered mucins expression. We along with others have clearly established that mucins play important role in the pathogenesis of PC (12-14). In order to analyze the effect of BA on MUC4 expression, we treated PC cell lines with different concentrations of DCA and CDCA for 24 h. We observed a significant increase in

MUC4 expression in CD18/HPAF cells at all concentrations, ranging from 5-100 μM with the maximal increase at 50 μM concentration for both DCA and CDCA (**Fig.4.2A**). Corrobortively, our time course experiment in DCA and CDCA treated CD18/HPAF cells revealed increase in MUC4 expression starting from 6h treatment with maximum increase at 24h (**Fig. 4.2B**). BA-mediated increase in MUC4 expression was further confirmed in T3M4 (**Fig.4.2C**) and CAPAN1 cell lines (**Fig.4.2D**). Unlike CD18/HPAF, presence of two bands for MUC4 protein in T3M4 and CAPAN1 cells indicates the presence of allelic VNTR polymorphism in MUC4 genes in these cell lines (24). Furthermore, immunofluorescence experiment revealed significant increase in MUC4 expression in DCA or CDCA treated CD18/HPAF cell line (**Fig.4.2E**). Altogether, the results suggest that BA may play important role in the pathogenesis of PC by positively regulating MUC4 expression.

C. BA transcriptionally upregulates MUC4 expression in PC

In order to know whether BA-mediated upregulation of MUC4 is at transcriptional level, PC cells were treated with DCA or CDCA in conjunction with actinomycin-D, which inhibits the process of transcription. Intriguingly, we observed a significant increase in MUC4 expression by 4.09- and 4.49-fold in DCA and CDCA treated CD18/HPAF cells, respectively, which was attenuated to 0.18- and 0.16-fold in DCA and CDCA treated CD18/HPAF cells when treated in combination with actinomycin-D (**Fig.4.3A**). Similarly, in T3M4 cells, we observed a 2.40-fold increase in MUC4 upon DCA treatment, was attenuated to 0.38-fold, when given in the presence of actinomycin-D, whereas a 2-fold MUC4 upregulation in CDCA treated T3M4 cells was reduced to 0.54-fold in the presence of CDCA and actinomycin-D treatment (**Fig.4.3A**).

To highlight the DCA and CDCA responsive regions on the MUC4 promoter, Luciferase reporter assay was performed (Andrianifahanana et al., 2005). Our results demonstrated that both distal (P-1641) and proximal (P-1809 and P-2150) constructs were responsive to BA in CD18/HPAF cells (**Fig. 4.3B**). Of particular interest was the deletion construct P-1641, which evidenced a statistically significant 2.95- and 3.24-fold upregulation of the reporter gene in response to DCA and CDCA treatment, respectively (**Fig. 4.3B**). A similarly enhanced transcriptional activity by 1.93-fold was also noticed in DCA and CDCA treated CD18/HPAF cells transfected with P-2150 construct, however, these changes were insignificant. P-1809 construct demonstrated increase in luciferase activity by 1.21- and 1.91- fold upon DCA and CDCA treatment, respectively (Fig. 3B), nevertheless, these changes were significant only for CDCA treatment. Correspondingly, compared to untreated controls, T3M4 cells transfected with P-1641 fragments showed 3.04- and 2.55-fold increase ($p<0.05$), in luciferase activity upon DCA and CDCA treatment, respectively (**Fig. 4.3C**). P-1809 deletion construct demonstrated 1.53- and 1.78-fold increase in luciferase activity upon DCA and CDCA, respectively. Similarly, P-2150 construct exhibited 1.4- and 2-fold increase in luciferase activity in the presence of DCA and CDCA, respecitively. However, the increase in luciferase activity at proximal promoter regions upon BA treament were statistically insignificant in T3M4 cell lines. Taken together, our data suggests that the distal promoter region of MUC4 gene is mainly responsible for BA-mediated transcriptional upregulation of MUC4 in both CD18/HPAF and T3M4 cell lines.

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

Due to an observed maximal increase in the region -2572 to -3135 (present in P-1641) to BA treatment in PC cell lines, we performed *in silico* analysis to delineate putative transcription factors binding sites for transcription factors on this promoter region (**Fig. 4.4A**). Two c-Jun binding sites were identified on MUC4 distal promoter (P-1641), which were absent on the proximal promoter fragment (P-1809 sequence) (Singh *et al.*, 2007), and therefore, were suggestive of making distal promoter (P-1641) more responsive to BA treatment (**Fig. 4.4B**). It incited us to propose that BA-facilitated upregulation in MUC4 expression in PC cell lines is c-Jun dependent. Firstly, we were interested to know whether BA itself has any effect on c-Jun expression levels. Intriguingly, in CD18/HPAF cells, we observed 1.95-, 2.9-, and 3.46-fold increase ($p<0.05$) in c-Jun expression at 10, 50, and 100 μM of DCA treatment over untreated cells. On the other hand, 1.78-, 2.16-, and 3.87-fold increase ($p<0.05$) in c-Jun expression was noticed at 10, 50 and 100 μM concentration of CDCA treatment, respectively (**Fig.4.4C**). The increased expression of c-Jun in response to both DCA and CDCA treatments was also confirmed by immunoblot analysis in PC cell lines (**Fig.4.4D**). Immunofluorescence experiments also revealed a significant increase in c-Jun expression and nuclear localization in both DCA- and CDCA-treated CD18/HPAF cells (**Fig.4.4E)**. Further, nuclear and cytoplasm fractionation after BA treatment in CD18/HPAF cells, revealed significant increase in c-Jun expression in the nuclear extracts than untreated cells (**Fig.4.4F**).

To investigate the direct involvement of c-Jun in BA-induced MUC4 expression, we performed ChIP assay to analyze c-Jun binding on MUC4 distal promoter (**Fig.4.4G**). Using a primer set covering only one c-Jun binding site (or region-II),

we observed 4.01- and 1.64-fold enrichment upon DCA and CDCA treatment of CD18/HPAF cells, respectively. However, primers encompassing both c-Jun binding sites (region-I), showed a significant (<0.05) enrichment of 6.74- and 2.61-folds, compared to untreated cells after DCA and CDCA treatments in CD18/HPAF cells (**Fig.4.4G**), suggestive of the cumulative effects of both c-Jun binding sites in inducing the transcription of MUC4 gene. As a negative control, we synthesized primers against the non-c-Jun binding MUC4 promoter fragment and found no difference. Taken together, BA increase the expression and nuclear localization of c-Jun, which then occupy MUC4 promoter to increase its transcription.

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

To elucidate the signaling pathways responsible for increased MUC4 transcription to BA treatment, CD18/HPAF cells were treated with a panel of inhibitors targeting different signaling pathways prior to BA treatment. Interestingly, pharmacological inhibition of both FAK and MAPK pathway showed attenuation of DCA- and CDCA-mediated MUC4 upregulation (**Fig.4.5A**). Inhibition of PI3K pathway did not have perceptible effect on MUC4 expression, whereas, inhibition of JNK did suppress MUC4 upregulation but only in the presence of CDCA (**Fig.4. 5A**). The attenuation of BA-mediated upregulation of MUC4 upon FAK inhibition was further confirmed using an immunofluorescence experiment (**Fig.4.5B**). Earlier JNK and MAPK pathways have been associated with BA, however, effects of BA on FAK has not been studied so far, particularly, in terms of MUC4 regulation. Moreover, due to observed maximal effect of FAK pathway on BA-facilitated MUC4 expression, we decided to focus on FAK pathway and analyzed the activation status of FAK in BA-treated PC cells. As

anticipated, we observed a high expression of activated FAK or pFAK (Y397) in DCA and CDCA-treated CAPAN1 and CD18/HPAF cells (**Fig.4.5C**), whereas expression of total FAK remains constant. As earlier experiments have linked c-Jun expression with BA-mediated upregulation of MUC4, our next question was to explore whether alteration in FAK has any impact on c-Jun expression. Interestingly, we observed that selective pharmacological inhibiton of FAK, led to significant decline in the expression levels of c-Jun and MUC4 in PC cell lines, both at transcript and protein levels (**Fig.4.5D and E**). To further substantiate our results, we performed ChIP experiment and observed significant reduction in enrichment for c-Jun binding on MUC4 promoter when BA treatment was concomitantly given with FAK inhibitor, as compared to BA alone (**Fig.4.5F**), suggesting that FAK activation is a prerequisite for DCA- and CDCA-mediated MUC4 upregulation in PC cells due to its direct involvement in the induction of c-Jun expression.

F. FXR activation is a prerequisite for BA-mediated MUC4 upregulation via src/FAK/c Jun axis

Farenosoid-X-receptor (FXR), a well established nuclear receptor, is known to be activated by BA. Upon its activation, FXR gets translocated to the nucleus, where it alters the trancriptional expression of multiple genes (**Fig.4.6A)**. Interestingly, the overall expression of FXR did not get influence by BA treatment, as FXR levels were high in the cytoplasmic fraction of untreated cells than DCA and CDCA treated cells. Expression profiling of FXR receptor in PC cell lines showed its significant overexpression in HPAC, CD18/HPAF; CAPAN1, Panc10.05 and Panc1 cell lines (**Fig.4.6B**-**C**), compared to immortalized normal pancreatic cells (HDPE). Interestingly, significantly high FXR levels in CD18/HPAF cells explains

drastic increase in MUC4 expression even at very low concentration of BA treatment, compared to T3M4 and CAPAN1 cell lines (**Fig.4.1B,C and D**). Due to observed downregulation of activated FAK expression levels along with c-Jun levels upon transient knockdown of FXR in CD18/HPAF and T3M4 PC cell lines, it is likely that FXR is acting upstream in this FAK/c-Jun/MUC4 axis (**Fig.4.6D**).The key question which arises is that how FXR expression regulates the activity of FAK. It is well-known in the literature that src kinase is one of the critical regulator of FAK activity (26). As we have observed thar BA treatment do affect the phosphorylation of src (**Fig.4.6E**), we assumed that FXR-mediated phosphorylation of FAK is p-src-dependent, and FXR knocked down PC cells indeed showed significant reduction in p-src levels compared to si control (**Fig.4.6E**). To further substantiate our results, we gave BA treatment to FXR knockdown CD18/HPAF cells and found significant abrogation of BA-mediated MUC4 upregulation (**Fig.4.6F**). A 2.1-fold increase in MUC4 expression due to DCA treatment was reduced to 1.32-fold in FXR silenced CD18/HPAF cells (**Fig.4.6F**). Similarly, a 1.92-fold increase in MUC4 expression upon CDCA treatment was reduced to 1.13-fold when CDCA treatment was given to FXR knockdown cells (**Fig.4.6F**). Altogether, the results suggest that FXR activation due to BA exposure is responsible for the initiation of FAK/c-Jun/MUC4 axis in PC cells, by plausibly regulating the activity of src kinase.

G. Clinical association between MUC4 and BA receptor FXR

In clinical samples, we clearly observed that similar to PC cell lines, mRNA expression for FXR was higher in 47% of PC tissues (n=15), as compared to the tumor adjacent normal pancreatic tissues (n=4) (**Fig.4.7A**). Though the upregulation of FXR in PC patients was not statistically significant ($p>0.05$), but

considering significant increase in the levels of BA, which are activators of FXR receptor, both in the circulation and pancreatic juice of PC patients, we can speculate that pancreatic tumors have increased activity of FXR receptor, which is sufficient to initiate FAK/c-Jun/MUC4 signaling cascade. In order to confirm an *in vivo* association between MUC4 and FXR, we measured the transcript levels of MUC4 in same clinical samples and performed regression analysis (**Fig.4.7B**). A fairly positive correlation (R^2=0.60) between MUC4 and FXR, further substantiated our *in vitro* findings. Moreover, using confocal microscopy, we observed co-expression of both FXR and MUC4 at the same PC tissue spots (**Fig.4.7C**).

IV.4 Discussion

Anatomically, the common bile duct and the pancreatic duct are close in proximity, and reunite at the ampulla of Vater. This led us to believe that BA can reflux to the pancreatic duct under pathological conditions. Growing pancreatic tumor often obstruct the bile ducts, preventing the flow of bile to the duodenum, leading to jaundice, a frequently occuring clinical manifestation in PC patients (27). Multiple studies have established BA as tumor-promoting agents in multiple cancers, including Barrett's metaplasia and colorectal, biliary, and hepatocellular cancers (11, 28-30). However, the role of BA in PC has not been clearly understood, which prompted us to study its influence on the tumorigenic properties of PC. In order to establish our hypothesis, BA levels were measured in the serum and pancreatic juice obtained from PC patients and NPNH individuals. Encouragingly, we observed a significant increase in BA concentration in those PC patients compared to controls. We also observed increased mRNA expression of BA receptor, FXR, in PC tumors compared to a

normal pancreas. Due to increased BA levels, which act as FXR agonist, it can be speculated that not only its expression, activity of FXR also get increased under PC condition, which we have confirmed as well due to increased nuclear expression levels of FXR upon BA treatment. Similar to our observation, Lee *et al.* have also observed increased expression of FXR in the PC tissues and established its protumorigenic role in PC disease condition (31). Altogether, this is a first experimental evidence establishing that BA do enter the pancreatic duct and increases the tumorigenic potential of PC cells by altering the expression of oncogenic MUC4 mucin.

Our luciferase promoter assay revealed MUC4 distal promoter as the major BA responsive site. Further, *in silico* analysis demonstrated the presence of two activator protein 1 (AP-1) motifs on this region, which has also been reported in our earlier publication (32). Consistent with the previous findings observed in gastric cells (33), we noticed that BA treatment increase c-Jun expression, one of the members of the AP-1 family. Furthermore, ChIP experiments confirmed an increase in c-Jun binding on MUC4 distal promoter when exposed to BA treatment. Interestingly, by utilizing the same c-Jun transcription factor, BA are known to increase the transcription of cyclooxygenase gene, by facilitating increased c-Jun binding on COX promoter in esophageal adenocarcinoma cells (34). Importantly, c-Jun overexpression has already been associated with carcinogenesis and cancer progression in multiple cancers (35, 36). Although BA responsiveness was maximally observed at distal promoter (P-1641), we also observed increased luciferase activity in proximal promoter region, P-1809 transfected CD18/HPAF cells, upon CDCA treatment, implying the involvement of other transcription factors in CDCA-mediated

upregulation of CD18/HPAF cells due to the absence of c-Jun binding sites on this region and requires further investigation (32). Inspite of the presence of c-Jun bindings sites on MUC4 proximal promoter (P-2150), we observed an insignificant increase in luciferase activity upon BA treatment, suggesting that BA might be affecting the expression and binding of transcription factors having inhibitory effects on proximal promoter region, and therefore, neutralizing the positive effects of c-Jun.

Multiple forms of BA have been previously identified as potent inducers of MUC4 expression in esophageal carcinogenesis associated with bile reflux (10, 29). Mechanistically, PI3K signaling, protein kinase C and hepatocyte nuclear factor-1α were attributed to BA-facilitated increase in MUC4 expression (10, 11). However, in the current study, we have established the role of FAK in MUC4 regulation in PC cells upon BA treatment. Selective pharmacological inhibition of FAK led to the attenuation in BA-mediated MUC4 upregulation. Moreover, we observed downregulation of c-Jun expression upon FAK inhibition, suggesting that c-Jun activation is a downstream event occurring after FAK activation. Nadruz *et al.* have also established the link between c-Jun and FAK molecules in ventricular myocytes (37). Unlike CDCA, the inhibition of the JNK pathway had no remarkable effect on DCA-induced MUC4 expression, suggesting that different BA transduce differential signaling, and therefore, affects the expression of molecules, which is MUC4 in our case, to different extent. Moreover, the data also implies differential mode of c-Jun activation in the presence of DCA and CDCA. Earlier studies have shown that c-Jun can get activated in JNK-independent manner (38, 39). For instance, in neuronal cells, DNA damage causing induction of neuronal c-Jun kinase has been shown to

increase c-Jun phosphorylation (Besirli and Johnson, Jr., 2003). Upon injury, c-Jun is found to be activated in Schwann cells by MAP kinases, which is again occurring independent of JNK (Deng *et al.*, 2012). In addition to FAK pathway, inhibition of MAPK pathway also led to attenuation of BA-mediated MUC4 upregulation, which further strengthened our notion that MAPK pathway could be involved in c-Jun activation. Future studies will be focused to understand the in-depth involvement of different signaling pathways in MUC4 regulation after BA treatment.

BA are known to interact with nuclear family receptors including;FXR and pregnane X receptor (PXR) in order to influence the transcription of their target genes. In the current study, for the first time, we have established the direct involvement of FXR protein in MUC4 regulation. In the clinical samples, we observed a fair positive correlation between FXR and MUC4 mRNA expression profiles. Upon FXR kd, BA-mediated upregulation of MUC4, FAK and c-Jun was abrogated, placing FXR as an upstream molecule in this FAK/c-Jun/MUC4 axis in PC. Das A *et al* have shown that FXR promotes the migration of endothelial cells by regulating the expression of FAK and MMP9 (40). However, the molecular mechanism of FXR-facilitated FAK activation is still unexplored. Due to observed increase in src kinase activity upon BA treatment, we assumed its role in this FXR-mediated increased FAK activation and found to be plausible as FXR silencing led to reduced p-Src levels in PC cell lines, and needs to be further validated. Interestingly, previous study in our lab has also shown that Guggulsterone, a selective pharmacological FXR inhibitor, also leads to a MUC4 downregulation at transcriptional level in PC cells by utilizing src/FAK pathway (41). In addition to FXR, other BA receptors could also be implication in BA-

facilitated MUC4 upregulation. Interestingly, TGR5 has found to be upregulated in 67% of PC patients (data not shown) and recent report has shown its tumorigenic role in gastrointestinal cancers, including PC (42). Further studies will be helpful and required to mechanistically delineate the association between TGR5 and PC disease condition.

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

Altogether, the current study, for the first time, has established that BA levels rises both in the circulation and pancreatic juice in PC, and they exert their protumorigenic functions by upregulating oncogenic MUC4 expression. Mechanistically, we have demonstrated that BA binding to FXR receptor leads to FAK activation, followed by increased c-Jun expression and its nuclear translocation, which *in turn* causes increased transcription of the MUC4 gene (**Fig. 4.7D**). The current study also supports emerging epidemiological data that, similar to colorectal cancer, fat-rich diet could be one of the risk factors for PC development and progression. Therefore, targeting BA receptors an administration of BA antagonists can significantly impact the outcome of PC patients.

Figures and Figure legends

Figure 4.1. BA are significantly upregulated in PC condition. **A.** Representation of BA levels in the serum samples obtained from the PC patients (n=36) and healthy (n=10) individuals using a commercially available total BA estimation kit. The difference in BA levels between normal and PC patients were found to be statistically significant. **B.** To understand the association of BA with PC progression, we measured BA levels in established KPC mice model at early (5-7 wk), medium (10-15 wk) and advanced stages (20-25 wk). The BA levels were found to increase with the severity of the disease. **C.** Box-plot representing the levels of BA in the pancreatic juice obtained from PC patients. We observed significant increase in BA concentration in the pancreatic juice obtained from PC patients (65 μmol/L) compared to NPNH controls (13 μmol/L). (All values are mean ±S.E, ns means non-significant)

Figure 4.1

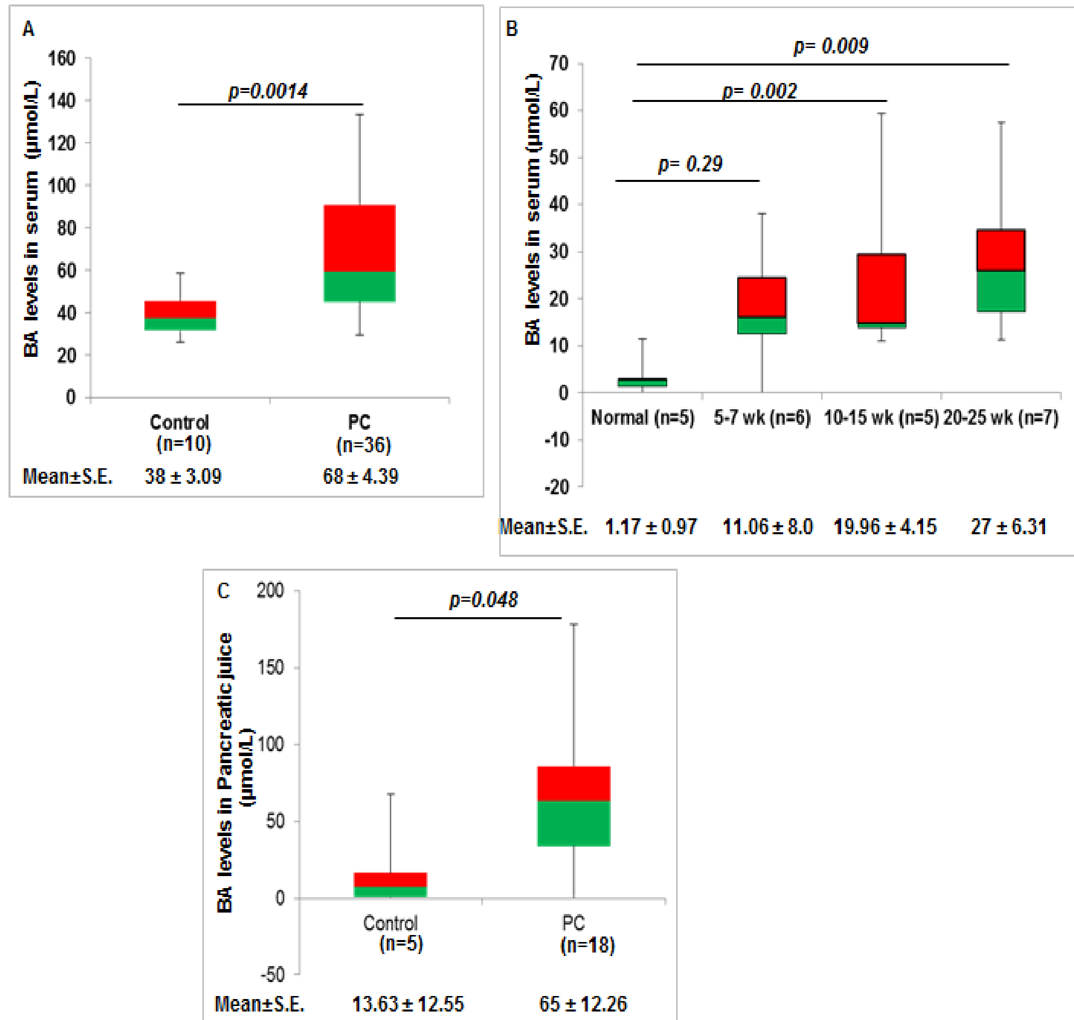
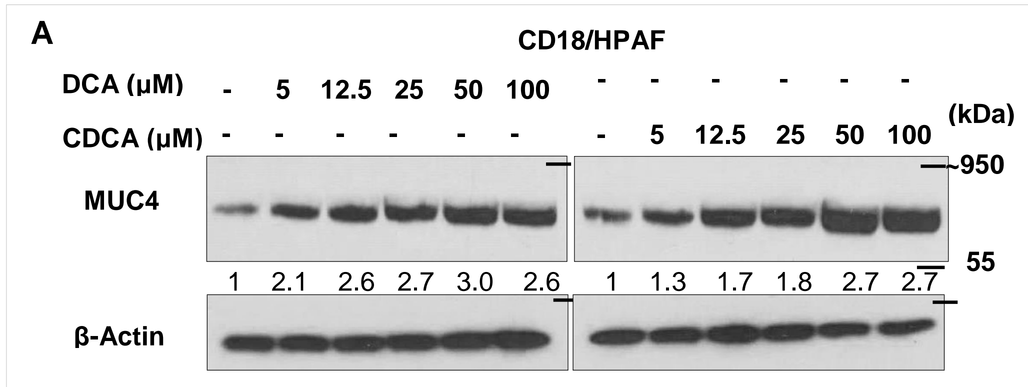
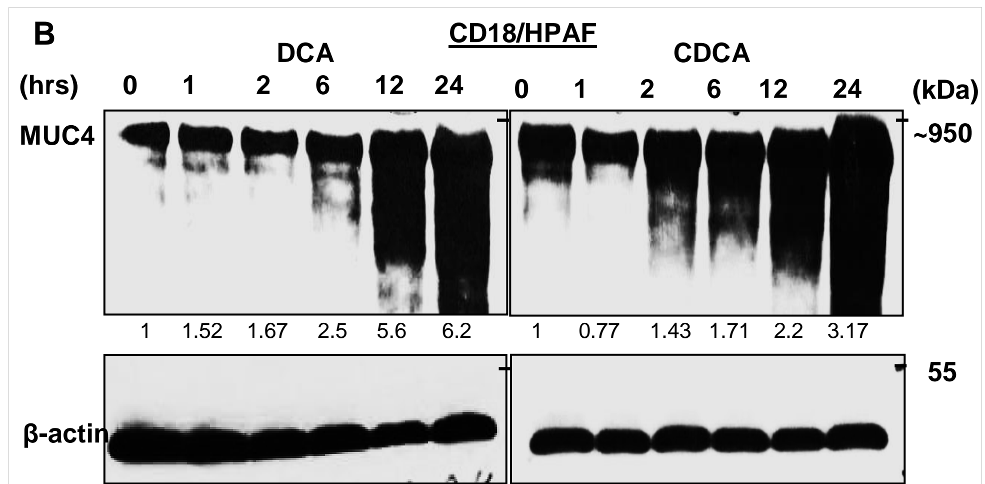


Figure 4.2. BA are positive regulators of MUC4 expression. A. CD18/HPAF cells were serum starved for 8h prior to BA treatment. Following 24h of BA treatment, cell lysates were collected, quantified and resolved using gel electrophoresis. Immunoblot showing increase in MUC4 expression upon DCA and CDCA treatment of CD18/HPAF cells at indicated concentrations. **B.** CD18/HPAF cells were treated with 50 μM of DCA or CDCA for indicated time points. MUC4 protein expression starts increasing at 6 h and maximal increase was noticed at 24 h. **C.** Immunoblot showing increase expression of MUC4 in DCA and CDCA treated T3M4 PC cells cells at indicated concentrations. **D.** Immunoblots confirming MUC4 upregulation by BA treatment in CAPAN1 cells. **E.** Representative confocal images showing the positive effect of BA on MUC4 expression in CD18/HPAF cells. (LE: Low exposure, scale bar = 20 μM)

Figure 4.2





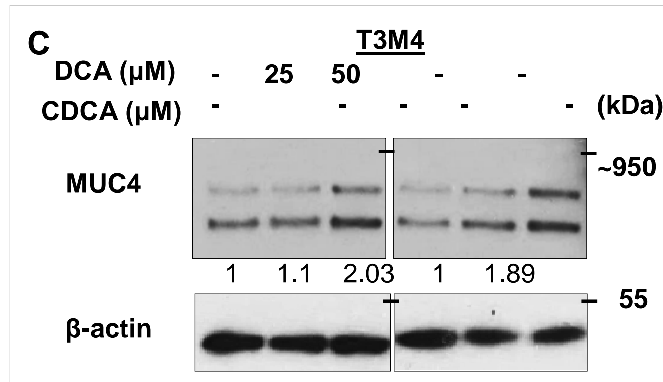
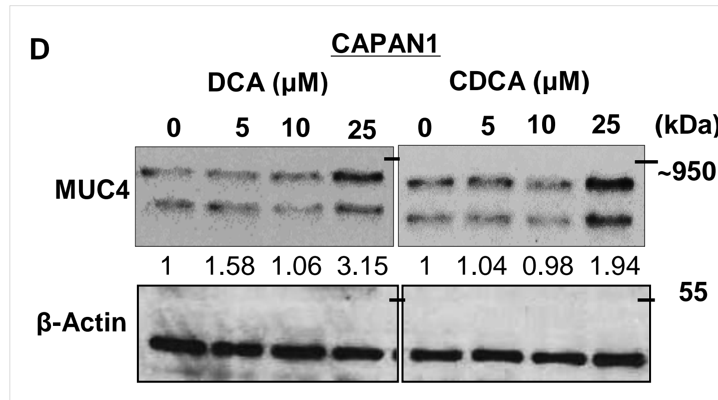


Figure 4.2



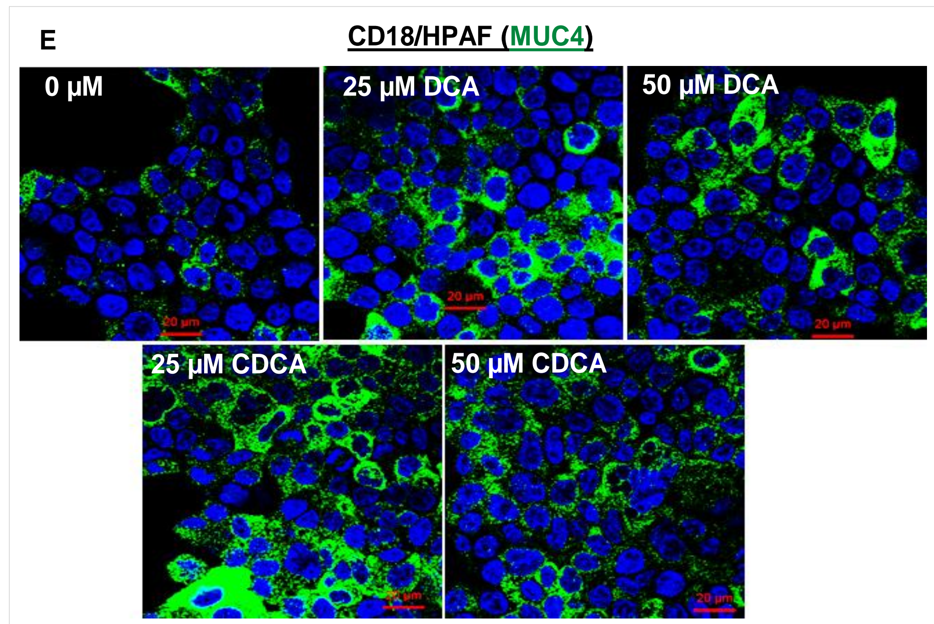
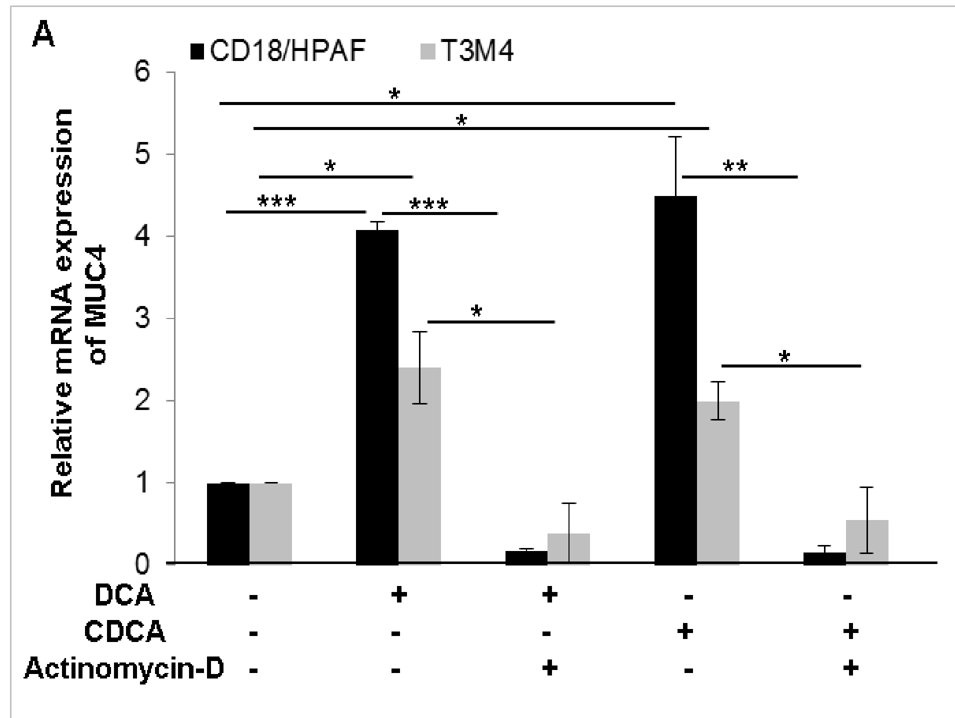


Figure 4.3. BA-mediated positive regulation of MUC4 is at transcriptional level. A. After 8h of serum starvation, both CD18/HPAF and T3M4 cell lines were treated for 12h with 50 µM of DCA, CDCA or vehicle control (ethanol) in the presence or absence of actinomycin-D (2 µg/ml). Following treatment, cDNA was prepared from isolated RNA and used for real-time PCR to analyze the quantitative expression of MUC4 gene. The represented graph is demonstrating that inhibition of transcription attenuates DCA- and CDCA-mediated increase in MUC4 expression in both CD18/HPAF and T3M4 cell lines. **B.** Luciferase assay was performed in CD18/HPAF cell line transfected with MUC4 promoter-truncated constructs, followed by 4h treatment of 50 µM of DCA and CDCA. A significantly elevated luminescence was detected upon BA treatment, primarily at the distal promoter region. **C.** Similar to CD18/HPAF cells, T3M4 cells also showed significantly elevated luminescence at the distal promoter region upon BA (50 µM) treatment. (*$p<0.05$, ** $p<0.01$, *** $p<0.001$, ns means non-significant)

Figure 4.3



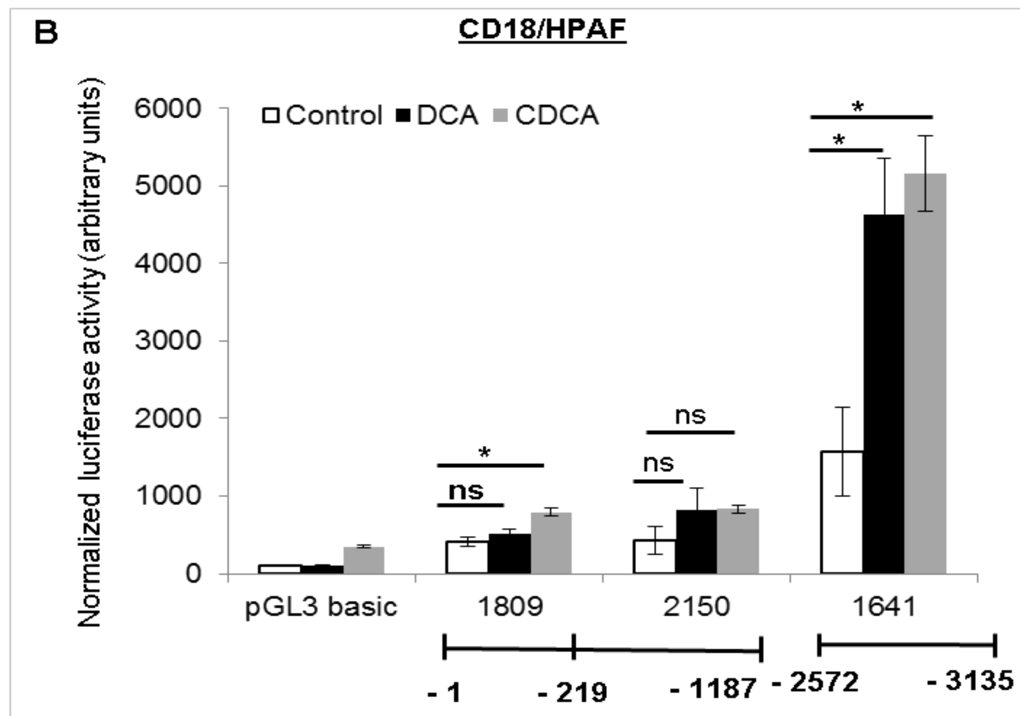


Figure 4.3

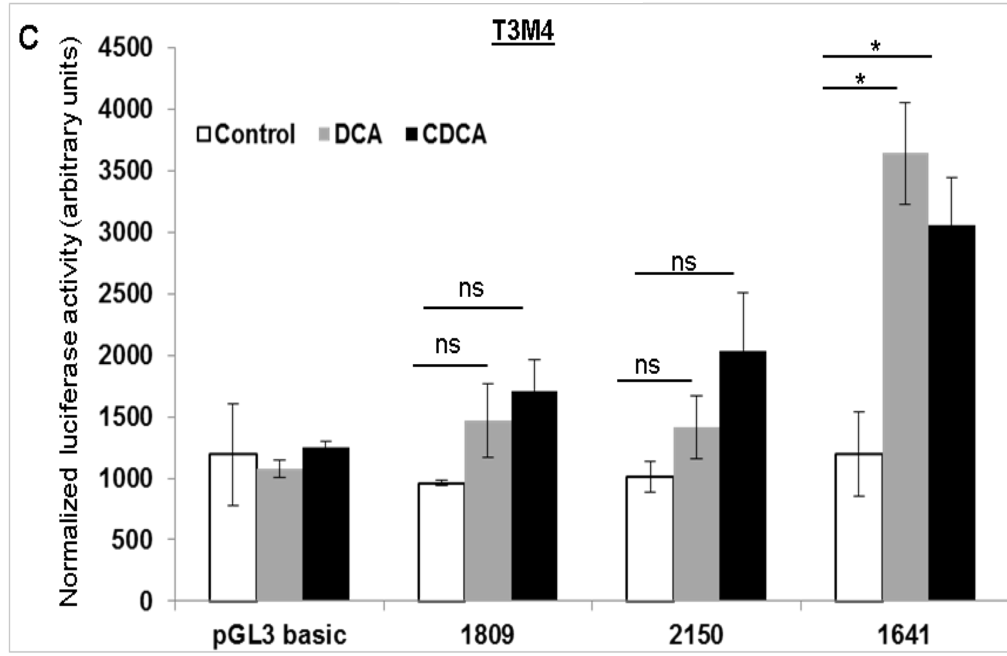


Figure 4.4. BA affect the expression, activation and nuclear translocation of c-Jun, which led to enhanced c-Jun binding on MUC4 distal promoter. A. By utilizing PROMO software, we obtained differential transcription factors binding sites on the highly responsive region for BA on MUC4 promoter region. **B.** Sequence of the MUC4 distal promoter (P-1641) which has two binding sites for c-Jun protein (marked red). **C.** Graph showing increase in c-Jun mRNA expression in a dose-dependent manner in CD18/HPAF cell line, treated for 2 h with DCA and CDCA. C. CD18/HPAF and T3M4 cell lines were treated with BA (50 μM) for 4 h and cell lysates were collected. **D.** Immunoblot was performed to observe change in c-Jun expression in DCA- and CDCA-treated CD18/HPAF and T3M4 cell lines, compared to their respective untreated controls. **E**. Confocal images showing significant increase in c-Jun and MUC4 protein expression in CD18/HPAF cells treated with DCA or CDCA. Graph showing the quantification of the c-Jun positive nuclei in DCA and CDCA treated CD18/HPAF cells. **F**. Immunoblot showing significant increase in the expression levels of c-Jun in the nuclear fraction obtained from BA (25 μM)-treated CD18/HPAF cells, whereas cytoplasmic fraction did not demonstrate any noticeable alteration in c-Jun expression. **G.** ChIP experiment was performed to observe the effect on enrichment for c-Jun binding on MUC4 distal promoter in the presence or absence of DCA (50 μM) and CDCA (50 μM). We observed a significant increase in fold-enrichment at both region-I (containing two c-Jun binding sites) and region-II (containing one c-Jun binding sites). (*$p<0.05$, ** $p<0.01$, *** $p<0.001$, scale bar = 20 μM)

Figure 4.4

A

Promoter region	Transcription factors
1641	c-Jun, GATA-1, RXRα, NF-kB, NF-1, Myo-D

B

- 3135 to - 2572 (P-1641)

ATCTCTCTTGGAGATCCTGGCTTTGCTGCCTCAAACCCAGCTGTCTCTTCAAGC
CAAGTCTGGGAGAATCTCATGATCTTATCCTTGCTCCAATTTACTTACACCCCAC
ATCCACACTAAGCTCTTCCTGCCTCCCGTGGAATATTAACTTACACC**TGACTTCA**
GACTCCTGCCCCTATGCAGACCCAGCAGTATGCGGGCCTAGGAGGTTTTTCGG
CCACAAGGAACTAGAGAAGCCACTCATTTTGTCCCCTCTCATTCACCCCATTCT
GTCCCCATCGGATGCCTTGGGAGGAGAGAAGGAAGGACAAATGGGTGTCCCG**T**
GACCCAACCCAGATGAAGACAGAGCCATTTATCACAGAGACCCAGACACATCC
TAACTCTGGAAAATGGGCATATTGAGGGGAGCTGGAAAGCAGAAGGGAAACGG
GCGTGGGCAGGGAAGAGGTGAACAGGAGTGCGCACGCCCAGTTCTCCAGGAT
CTCACGCTCCTAATCAGGATCCTATAAGACTCAAAGGGGACGCAGGAAAGACCT
GGGCCTGGAAATTCACTTCCGGACTC

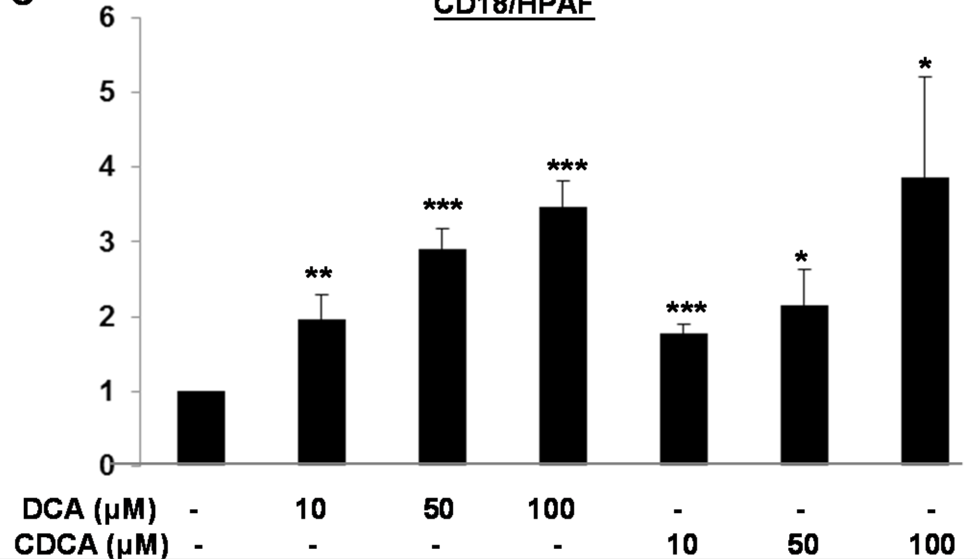
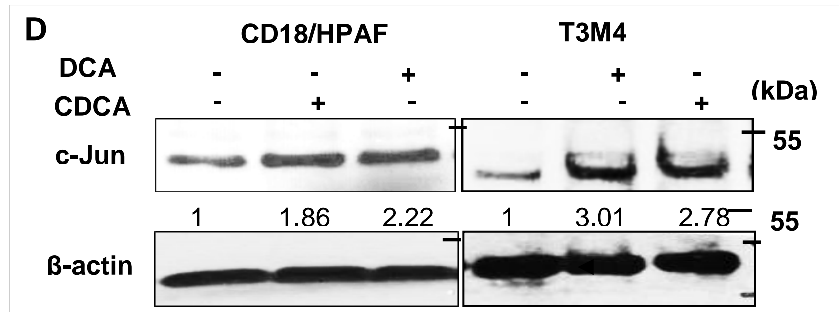
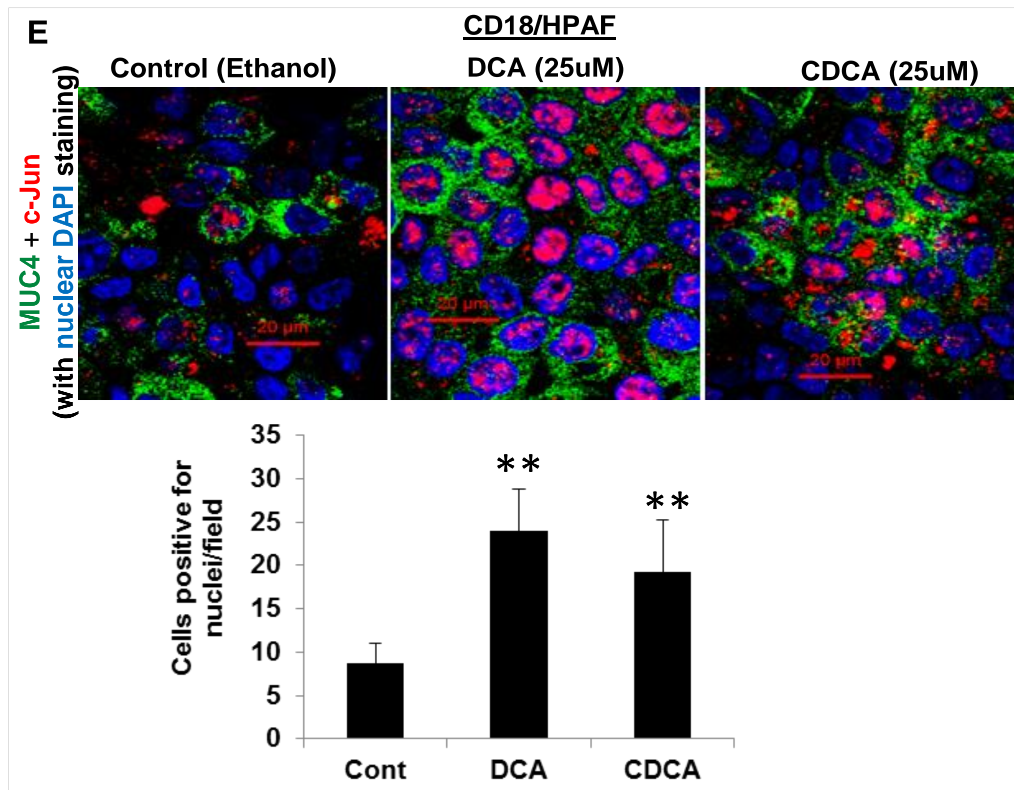


Figure 4.4





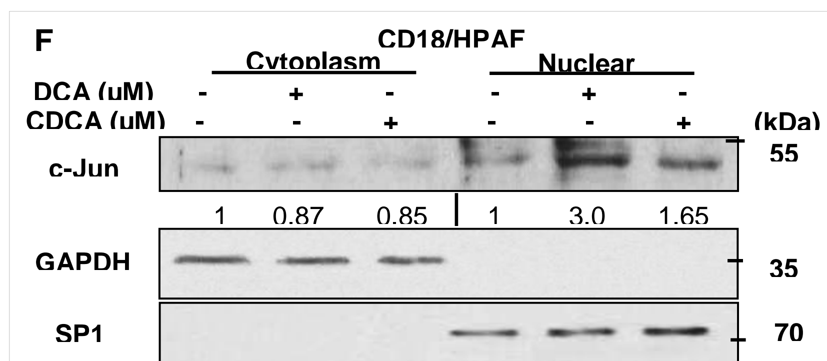


Figure 4.4

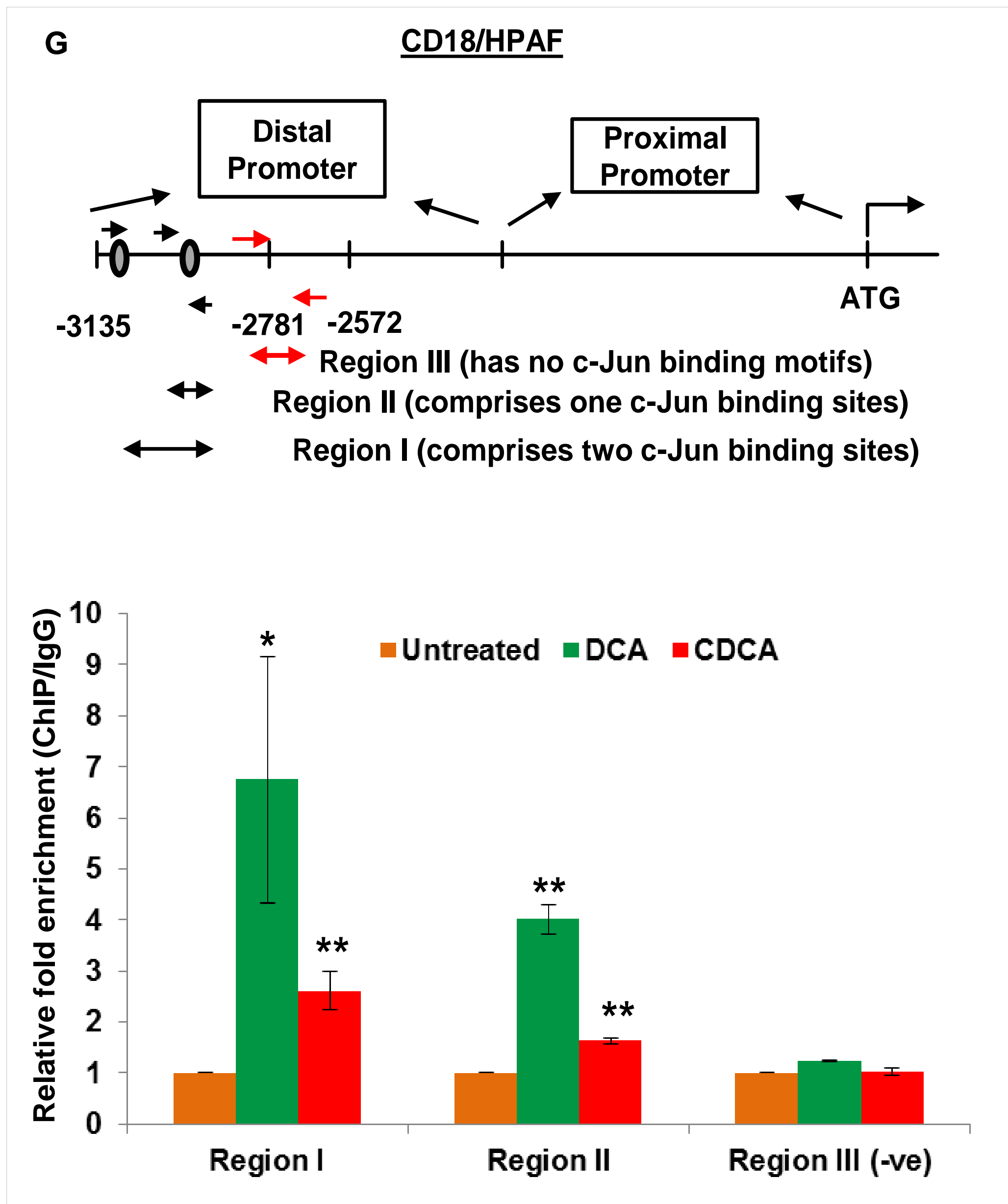
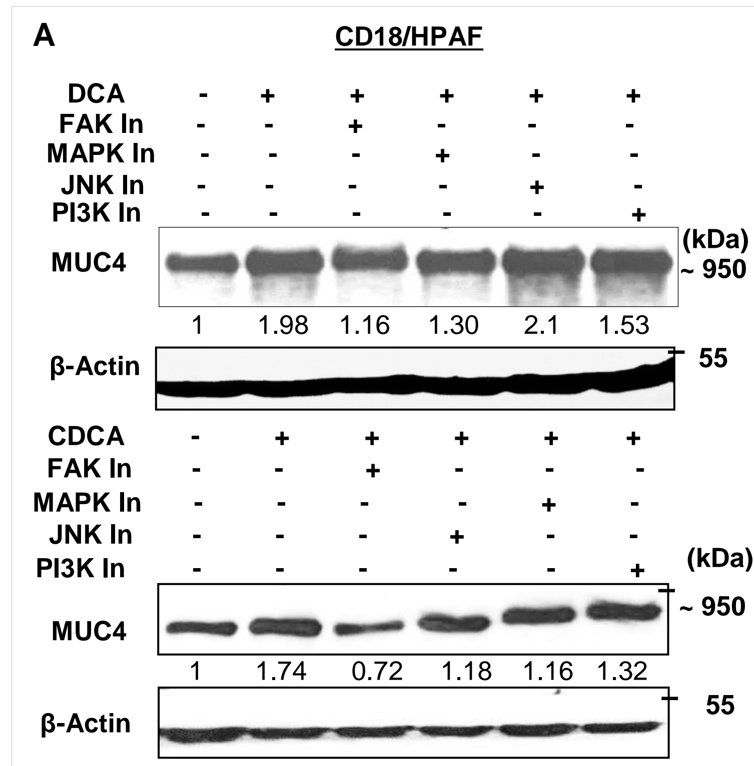
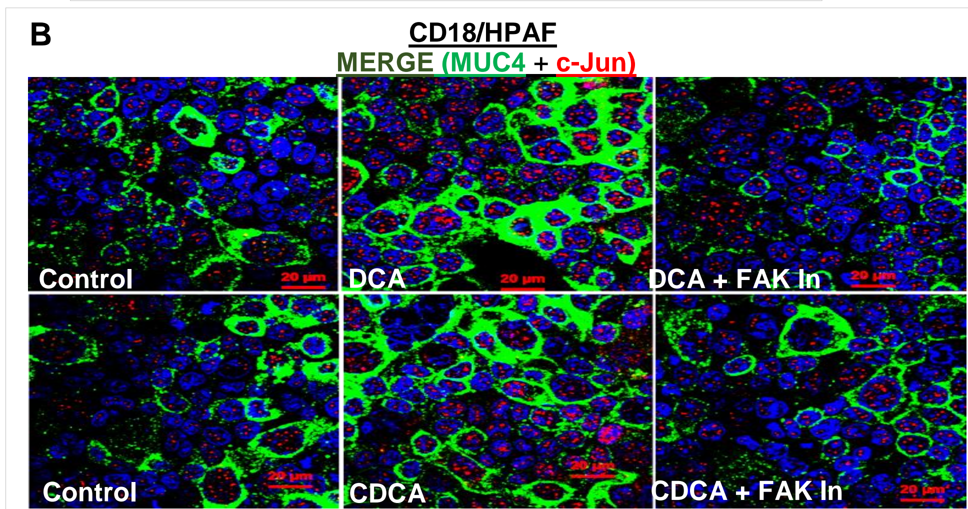


Figure 4.5. BA-mediated upregulation of MUC4 is dependent on FAK activation. A. Concomitant treatment of 25 µM of DCA or CDCA in the presence or absence of selective pharmacological signaling inhibitors for 12h led us to know that the FAK pathway is mainly responsible for MUC4 upregulation upon BA exposure, as attenuation of this pathway maximally suppresses the BA-mediated upregulation of MUC4, compared to the other signaling inhibitors. Besides FAK, inhibiton of MAPK pathway also led to reduced MUC4 expression. **B.** Images obtained from immunofluorescence experiment showing MUC4 upregulation in DCA (25 µM) and CDCA (25 µM) treated CD18/HPAF cells, which is attenuated upon inhibiting FAK activity (or phosphorylation). **C.** Increase in FAK activity was confirmed by analyzing pFAK (Tyr397) expression upon BA (25 µM) treatment of CD18/HPAF and CAPAN1 cell lines for 4h. **D.** Graphical representation of relative mRNA expression for MUC4 and c-Jun gene altered upon inhibition of FAK pathway in both CD18/HPAF and T3M4 cell lines. **E.** Immunoblot showng that inhibition of FAK pathway, using 15 µM of FAK Inhibitor 14, leads to downregulation of MUC4, pFAK and c-Jun in CD18/HPAF cells. **F.** Graph representing the relative fold enrichment for c-Jun on AP-1 sequence motifs present on MUC4 distal promoter when CD18/HPAF cells were concomitantly treated with DCA and CDCA in the presence and absence of FAK inhibitor for 4 hours. (*$p<0.05$, scale bar = 20 µM)

Figure 4.5





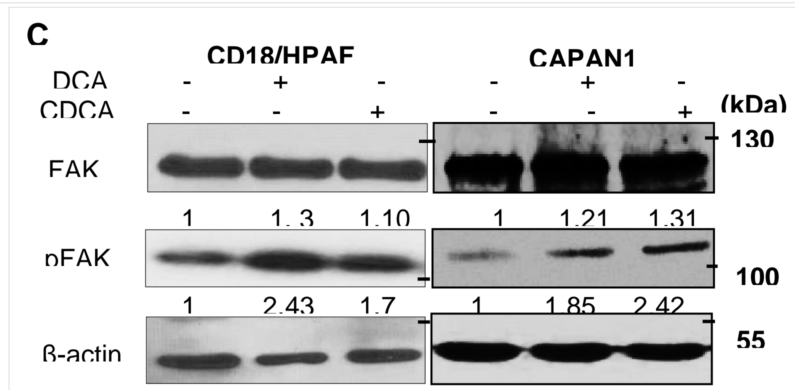
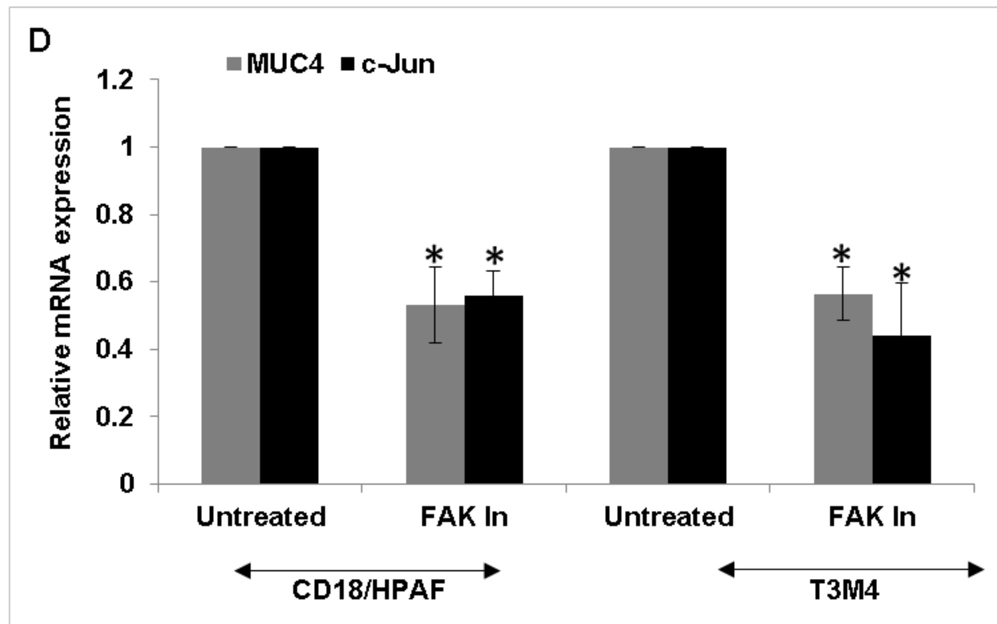


Figure 4.5



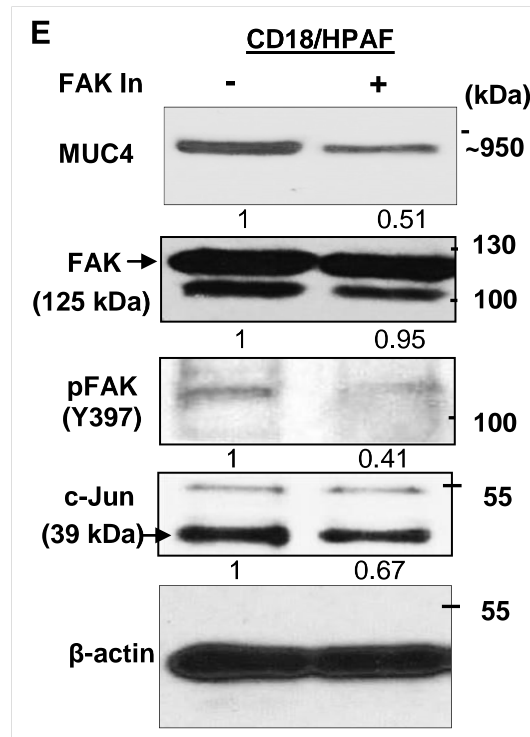


Figure 4.5

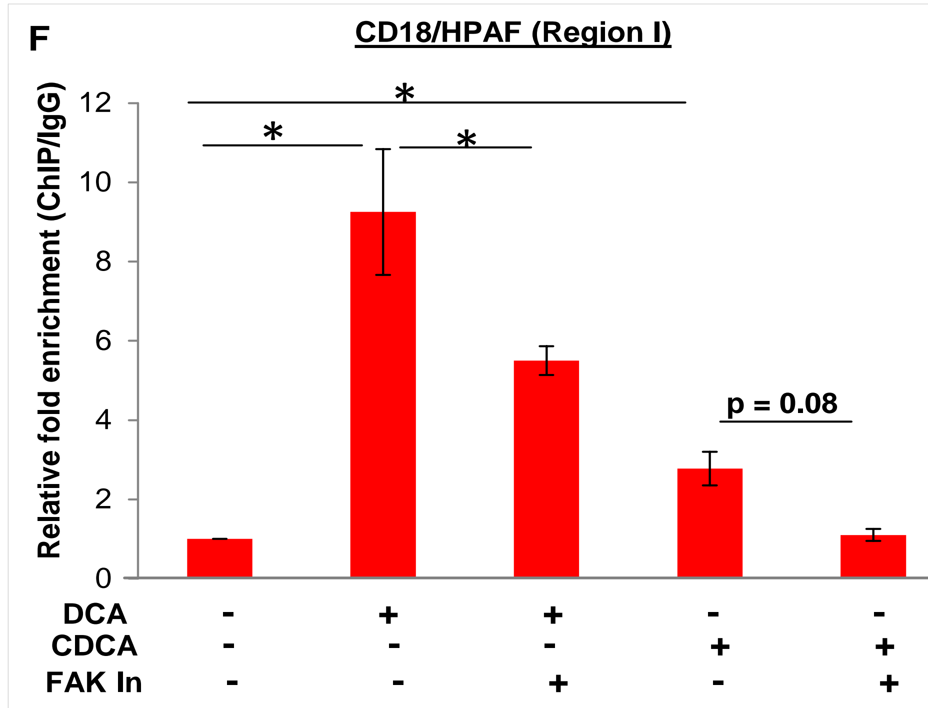
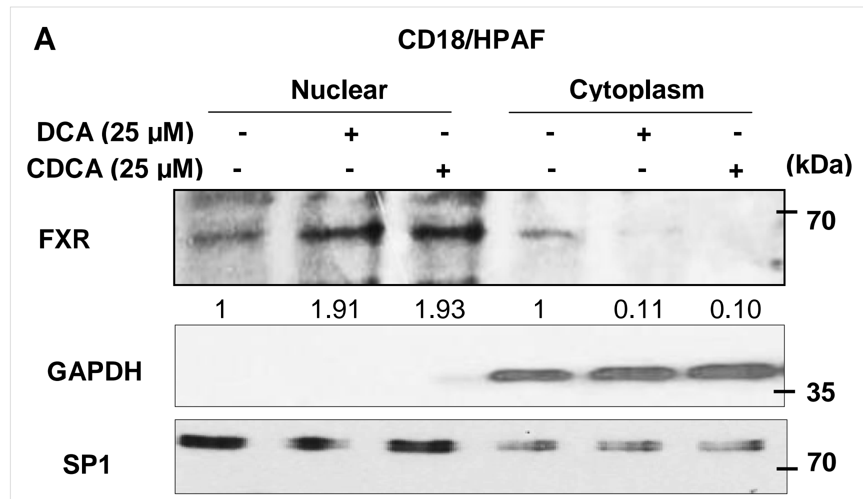
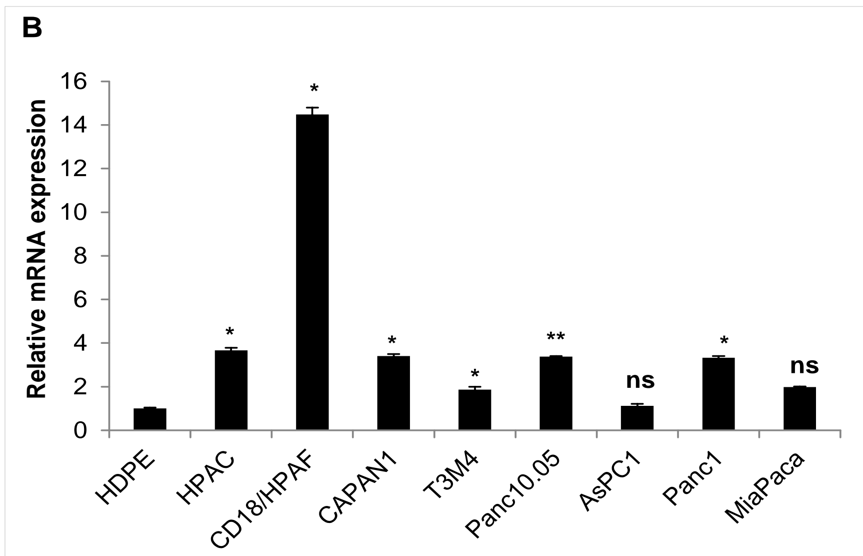


Figure 4.6. Activation of FXR is required for BA-mediated MUC4 upregulation v*ia* Src/FAK/c-Jun axis. A. Nuclear fraction obtained from 50 μM of DCA and CDCA treated CD18/HPAF cells, demonstrated increased levels of FXR compared to the untreated control. On the other hand, FXR expression was more on the cytoplasmic fraction in untreated cells than DCA and CDCA treated cells. **B.** FXR expression was found to be significantly high in PC cell lines than normal pancreatic cells (HDPE). **C.** Reverse-transcriptase PCR was performed to analyze FXR expression in PC cell lines panel. Following PCR, 2% agarose gel was run to detect the bands using ethidium bromide dye. **D.** FXR was transiently knockdown in CD18/HPAF and T3M4 cell lines using 150 nM of siRNA oligos and confirmed using immunoblotting. Interestingly, FXR knockdown cells exhibited significant decline in FAK, pFAK (Tyr397), src, p-src (Tyr416), c-Jun, p-c-Jun (Ser63) and MUC4, suggestive of FXR involvement as the most upstream molecule in this BA-mediated FAK/c-Jun/MUC4 axis. **E.** CD18/HPAF cells were treated for 2h with 25 μM of DCA and 50 μM of CDCA. Following treatment, the expression of src and p-src (Tyr 416) were analyzed using 10% PAGE. (*$p<0.05$, **$p<0.01$, ns means non-significant). **F.** The graphical representation of the result obtained from real-time PCR showing that knockdown of FXR in CD18/HPAF cell line leads to significant attenuation of both DCA (25 μM) or CDCA (25 μM)-mediated MUC4 upregulation.

Figure 4.6





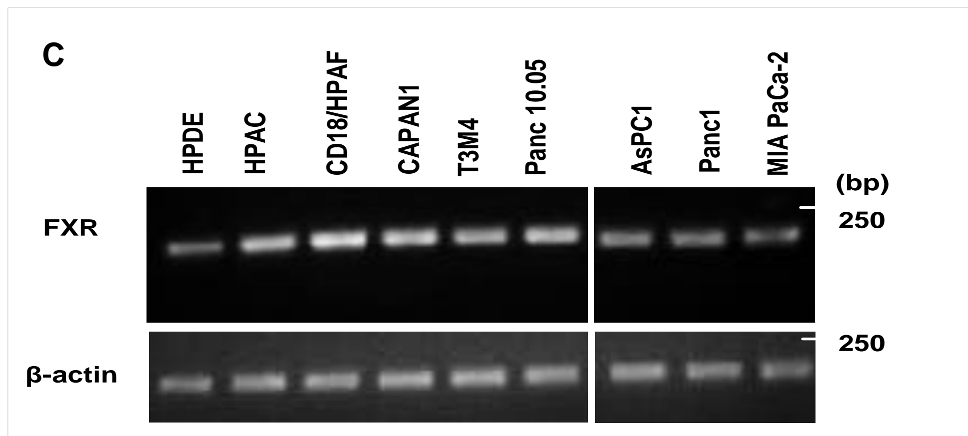
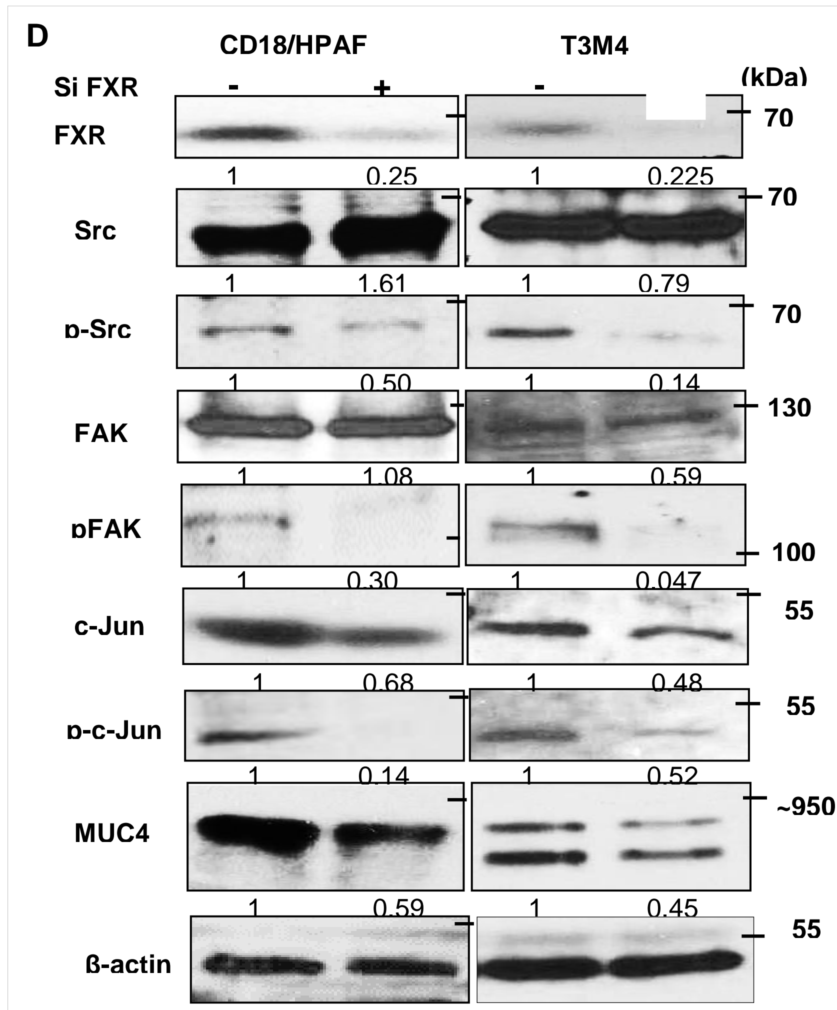


Figure 4.6



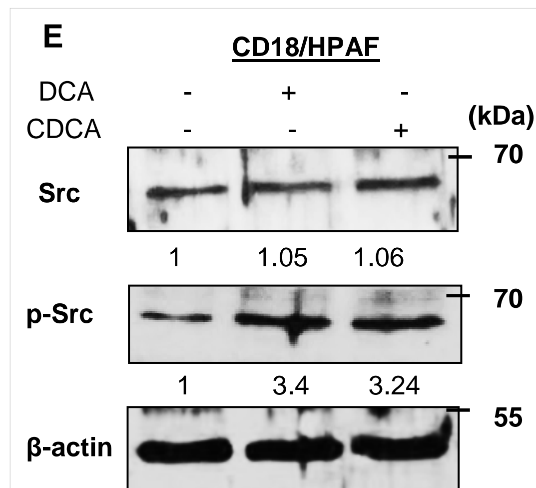


Figure 4.6

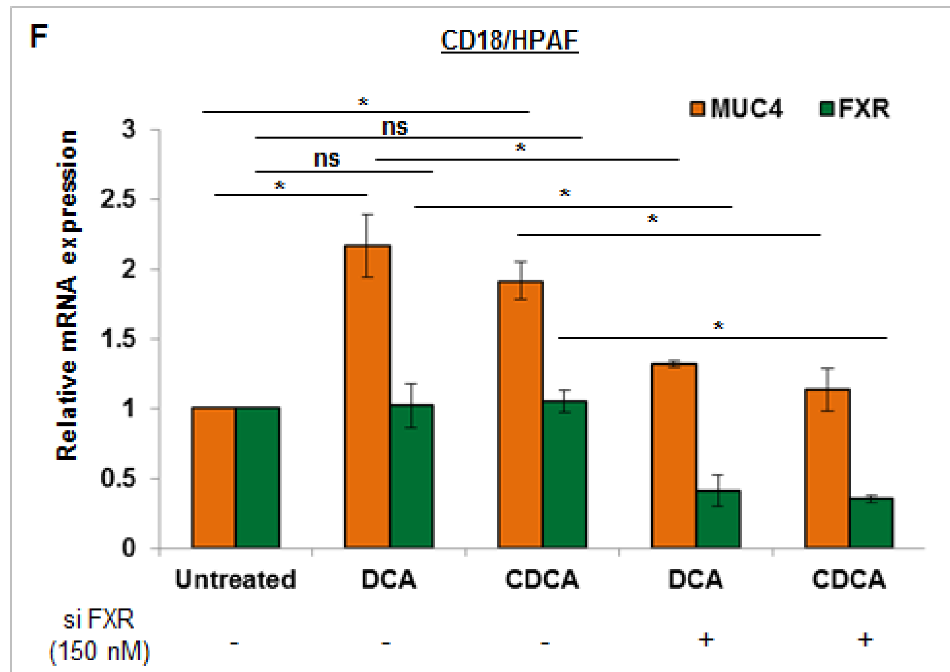
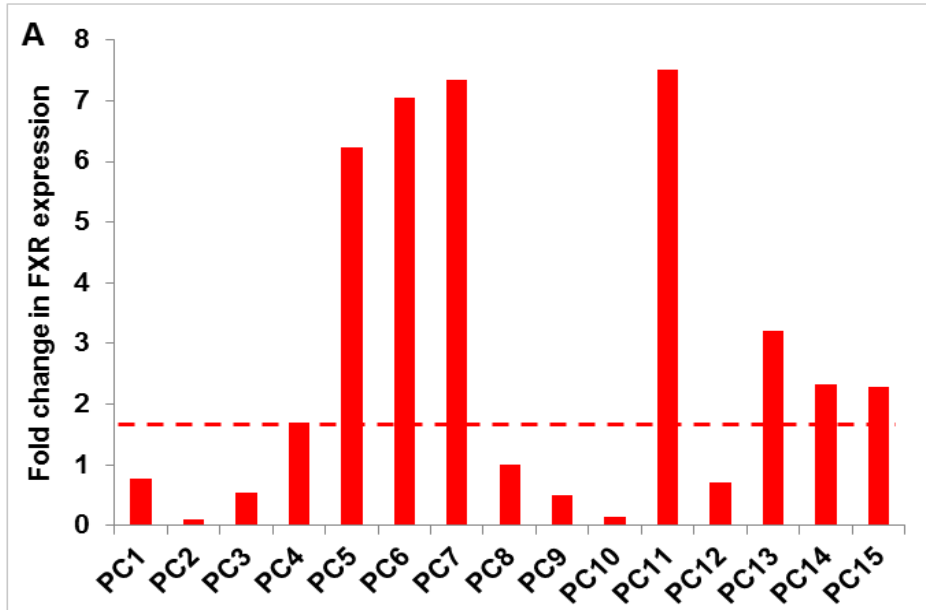
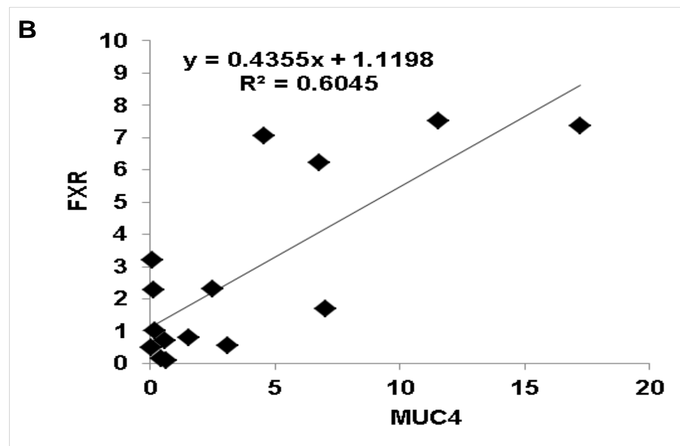


Figure 4.7. Clinical association between MUC4 and FXR in PC tissues. A. Expression profiling of FXR was performed in cDNA samples prepared from pancreatic tumor tissues (n=15) and tumor adjacent normal (n=4). Similar to its agonists, the levels of FXR was upregulated in tissues obtained from PC patients than tumor adjacent tissues. **B.** Data showing regression analysis which was performed to correlate MUC4 and FXR in clinical samples at transcriptional levels. **C.** PC tissues (obtained from Whipple procedure) showed the co-expression of both MUC4 and FXR at same tissue spots, suggestive of their direct association. (scale bar = 20 μM). **D.** Schematic representation of the overall summary of the paper: Treatment with BA leads to the activation of FXR receptor, which gets engage in the activation of FAK pathway, possibly by activating src kinase. Increase in FAK-mediated signaling leads to an increased transcription of c-Jun gene. Increased expression and activation of c-Jun is followed by its increased nuclear translocation, leading to increased MUC4 transcription, which plays an important role in the proliferation, survival, metastasis and chemoresistance of pancreatic tumors.

Figure 4.7





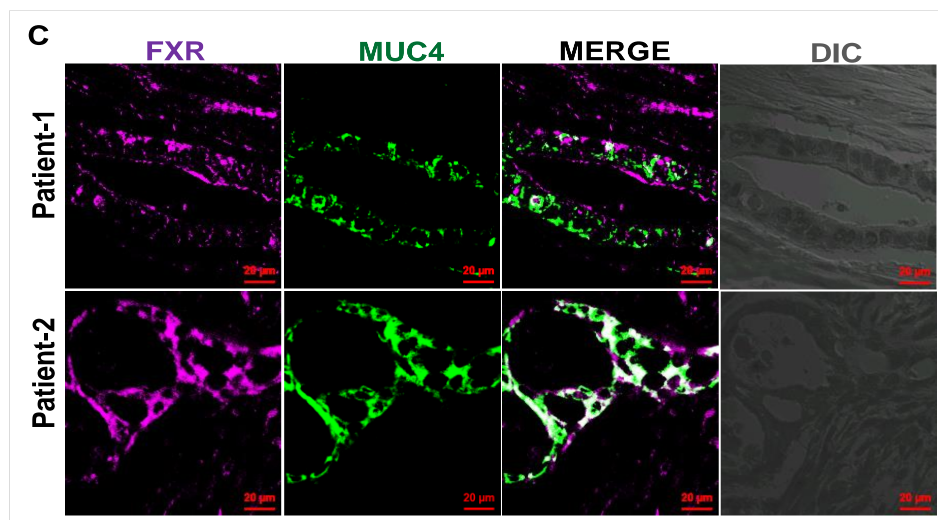
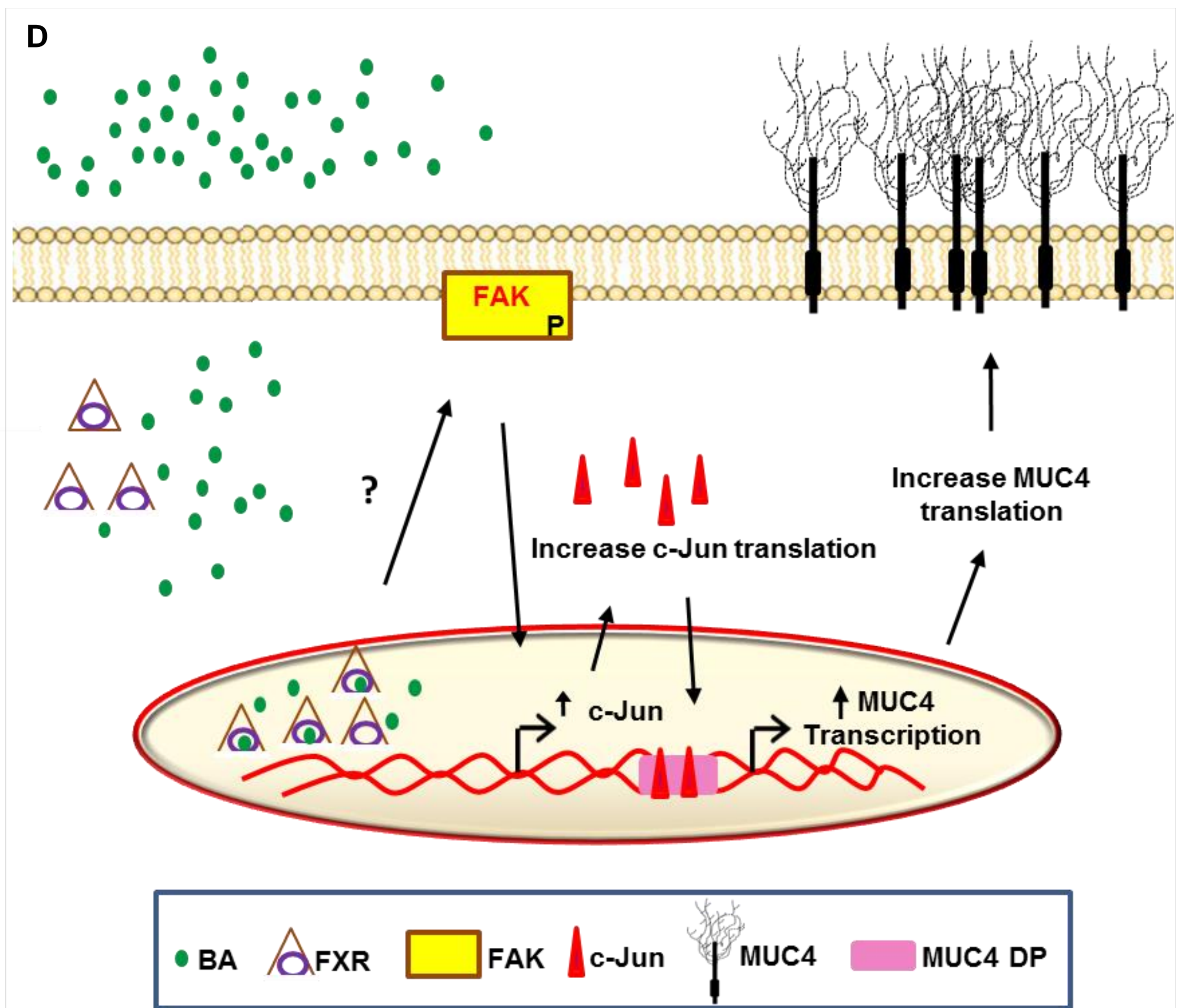


Figure 4.7



IV. 5 Reference List

(1) Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9-29.

(2) Wisinski KB, Wahl AO, Small W, Jr., Benson AB, III. Inoperable pancreatic cancer: standard of care. Oncology (Williston Park) 2007;21:1558-64.

(3) Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat Rev Cancer 2002;2:897-909.

(4) Nakamura T, Hirai R, Kitagawa M, Takehira Y, Yamada M, Tamakoshi K, Kobayashi Y, Nakamura H, Kanamori M. Treatment of common bile duct obstruction by pancreatic cancer using various stents: single-center experience. Cardiovasc Intervent Radiol 2002;25:373-80.

(5) Baptissart M, Vega A, Maqdasy S, Caira F, Baron S, Lobaccaro JM, Volle DH. Bile acids: from digestion to cancers. Biochimie 2013;95:504-17.

(6) Sifrim D. Management of bile reflux. Gastroenterol Hepatol (N Y) 2013;9:179-80.

(7) Tsoukali E, Sifrim D. Investigation of extraesophageal gastroesophageal reflux disease. Ann Gastroenterol 2013;26:290-5.

(8) Lin G, Zeng Z, Wang X, Wu Z, Wang J, Wang C, Sun Q, Chen Y, Quan H. Cholecystectomy and risk of pancreatic cancer: a meta-analysis of observational studies. Cancer Causes Control 2012;23:59-67.

(9) Howatson AG, Carter DC. Pancreatic carcinogenesis-enhancement by cholecystokinin in the hamster-nitrosamine model. Br J Cancer 1985;51:107-14.

(10) Mariette C, Perrais M, Leteurtre E, Jonckheere N, Hemon B, Pigny P, Batra S, Aubert JP, Triboulet JP, Van S, I. 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.

(11) Piessen G, Jonckheere N, Vincent A, Hemon B, Ducourouble MP, Copin MC, Mariette C, van S, I. 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.

(12) 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.

(13) Joshi S, Kumar S, Choudhury A, Ponnusamy MP, Batra SK. Altered Mucins (MUC) trafficking in benign and malignant conditions. Oncotarget 2014;5:7272-84.

(14) Joshi S, Kumar S, Bafna S, Rachagani S, Wagner KU, Jain M, Batra SK. Genetically engineered mucin mouse models for inflammation and cancer. Cancer Metastasis Rev 2015.

(15) Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth MA, Buchler MW, Aubert JP, Batra SK. 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.

(16) Moniaux N, Chaturvedi P, Varshney GC, Meza JL, Rodriguez-Sierra JF, Aubert JP, Batra SK. Human MUC4 mucin induces ultra-structural changes and tumorigenicity in pancreatic cancer cells. Br J Cancer 2007;97:345-57.

(17) Chaturvedi P, Singh AP, Moniaux N, Senapati S, Chakraborty S, Meza JL, Batra SK. 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) Lakshmanan I, Seshacharyulu P, Haridas D, Rachagani S, Gupta S, Joshi S, Guda C, Yan Y, Jain M, Ganti AK, Ponnusamy MP, Batra SK. Novel HER3/MUC4 oncogenic signaling aggravates the tumorigenic phenotypes of pancreatic cancer cells. Oncotarget 2015.

(19) Uchida K, Nomura Y, Kadowaki M, Takase H, Takano K, Takeuchi N. Age-related changes in cholesterol and bile acid metabolism in rats. J Lipid Res 1978;19:544-52.

(20) Lee HY, Crawley S, Hokari R, Kwon S, Kim YS. Bile acid regulates MUC2 transcription in colon cancer cells via positive EGFR/PKC/Ras/ERK/CREB, PI3K/Akt/IkappaB/NF-kappaB and p38/MSK1/CREB pathways and negative JNK/c-Jun/AP-1 pathway. Int J Oncol 2010;36:941-53.

(21) Mariette C, Piessen G, Leteurtre E, Hemon B, Triboulet JP, Van S, I. Activation of MUC1 mucin expression by bile acids in human esophageal adenocarcinomatous cells and tissues is mediated by the phosphatidylinositol 3-kinase. Surgery 2008;143:58-71.

(22) Song S, Byrd JC, Guha S, Liu KF, Koul D, Bresalier RS. Induction of MUC5AC mucin by conjugated bile acids in the esophagus involves the phosphatidylinositol 3-kinase/protein kinase C/activator protein-1 pathway. Cancer 2011;117:2386-97.

(23) Martin NA, Mount Patrick SK, Estrada TE, Frisk HA, Rogan DT, Dvorak B, Halpern MD. Active transport of bile acids decreases mucin 2 in neonatal ileum: implications for development of necrotizing enterocolitis. PLoS One 2011;6:e27191.

(24) Choudhury A, Moniaux N, Winpenny JP, Hollingsworth MA, Aubert JP, Batra SK. Human MUC4 mucin cDNA and its variants in pancreatic carcinoma. J Biochem 2000;128:233-43.

(25) Andrianifahanana M, Agrawal A, Singh AP, Moniaux N, van S, I, Aubert JP, Meza J, Batra SK. 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.

(26) Westhoff MA, Serrels B, Fincham VJ, Frame MC, Carragher NO. SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. Mol Cell Biol 2004;24:8113-33.

(27) Chen YG, Pan HH, Dai MS, Lin C, Lu CS, Su SL, Chang PY, Huang TC, Chen JH, Wu YY, Chen YC, Ho CL. Impact of Comorbidity and Age on Determinants Therapeutic Strategies in Advanced Pancreatic Head Cancer Patients With Obstructive Jaundices. Medicine (Baltimore) 2015;94:e1298.

(28) Changbumrung S, Tungtrongchitr R, Migasena P, Chamroenngan S. Serum unconjugated primary and secondary bile acids in patients with cholangiocarcinoma and hepatocellular carcinoma. J Med Assoc Thai 1990;73:81-90.

(29) Degirolamo C, Modica S, Palasciano G, Moschetta A. Bile acids and colon cancer: Solving the puzzle with nuclear receptors. Trends Mol Med 2011;17:564-72.

(30) Souza RF, Freschi G, Taddei A, Ringressi MN, Bechi P, Castiglione F, Rossi DD, Triadafilopoulos G, Wang JS, Chang AC, Barr H, Bajpai M, Das KM, Schneider PM, Krishnadath KK, et al. Barrett's esophagus: genetic and cell changes. Ann N Y Acad Sci 2011;1232:18-35.

(31) Lee JY, Lee KT, Lee JK, Lee KH, Jang KT, Heo JS, Choi SH, Kim Y, Rhee JC. Farnesoid X receptor, overexpressed in pancreatic cancer with lymph node metastasis promotes cell migration and invasion. Br J Cancer 2011;104:1027-37.

(32) Singh AP, Chauhan SC, Andrianifahanana M, Moniaux N, Meza JL, Copin MC, Van S, I, Hollingsworth MA, Aubert JP, Batra SK. 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.

(33) Park WI, Park MJ, An JK, Choi YH, Kim HY, Cheong J, Yang US. Bile acid regulates c-Jun expression through the orphan nuclear receptor SHP induction in gastric cells. Biochem Biophys Res Commun 2008;369:437-43.

(34) Zhang F, Subbaramaiah K, Altorki N, Dannenberg AJ. Dihydroxy bile acids activate the transcription of cyclooxygenase-2. J Biol Chem 1998;273:2424-8.

(35) Okutomi Y, Shino Y, Komoda F, Hirano T, Ishihara T, Yamaguchi T, Saisho H, Shirasawa H. Survival regulation in pancreatic cancer cells by c-Jun. Int J Oncol 2003;23:1127-34.

(36) Tessari G, Ferrara C, Poletti A, Dubrovich A, Corsini A, Del FG, Naccarato R. The expression of proto-oncogene c-jun in human pancreatic cancer. Anticancer Res 1999;19:863-7.

(37) Nadruz W, Jr., Corat MA, Marin TM, Guimaraes Pereira GA, Franchini KG. Focal adhesion kinase mediates MEF2 and c-Jun activation by stretch: role in the activation of the cardiac hypertrophic genetic program. Cardiovasc Res 2005;68:87-97.

(38) Besirli CG, Johnson EM, Jr. JNK-independent activation of c-Jun during neuronal apoptosis induced by multiple DNA-damaging agents. J Biol Chem 2003;278:22357-66.

(39) Deng Z, Sui G, Rosa PM, Zhao W. Radiation-induced c-Jun activation depends on MEK1-ERK1/2 signaling pathway in microglial cells. PLoS One 2012;7:e36739.

(40) Das A, Yaqoob U, Mehta D, Shah VH. FXR promotes endothelial cell motility through coordinated regulation of FAK and MMP-9. Arterioscler Thromb Vasc Biol 2009;29:562-70.

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

(42) Nagathihalli NS, Beesetty Y, Lee W, Washington MK, Chen X, Lockhart AC, Merchant NB. Novel mechanistic insights into ectodomain shedding of EGFR Ligands Amphiregulin and TGF-alpha: impact on gastrointestinal cancers driven by secondary bile acids. Cancer Res 2014;74:2062-72.

CHAPTER V

Multifaceted role of MUC4 in regulating the trafficking of RTKs in Pancreatic Cancer

V.1 Synopsis

The importance of EGFR signaling in PC has been acknowledged recently by multiple groups. However, the most interesting came in 2013, where Ardito and colleagues have clearly shown that EGFR activation is indispensable for the progression and development of PC. Interestingly, aberrant expression of MUC has been associated with the protein trafficking of EGFR family proteins. For instance, MUC4 expression has been linked with the increased protein stability of HER2 in PC cell lines, which was further attributed to MUC4-mediated reduced internalization of HER2. In breast cancer, modulation of MUC4 expression had significant impact on the expression of EGFR family members including, EGFR/HER1 and HER2. In breast cancer, MUC4 silencing led to decrease in the expression of Sprouty 2, an intracellular protein with established functions in stabilizing EGFR receptor. Besides breast cancer, MUC4 has shown to affect EGFR protein expression in glioblastoma. However, the precise mechanism involved in MUC4-facilitated impact on EGFR family members in cancer condition is still unexplored. In this chapter, I have disseminated the novel mechanisms involved in the regulation of the trafficking of EGFR and HER2 proteins by MUC4 mucin in PC. Using time-lapse live-cell imaging and confocal microscopy experiments, I have demonstrated that presence of MUC4 is increasing both the internalization and recycling rate of RTKs in PC cell lines upon ligand stimulation. It has been further associated with MUC4-mediated regulation of RAB5A, a GTPase which regulates the rate-limiting step in protein endocytosis, at the transcriptional as well as protein level. At mRNA level, MUC4 is inducing the activity of CREB via ERK activation, which is causing increased transcriptional activation of RAB5A by binding to the cyclic-AMP response element (CRE) present on RAB5A promoter. Moreover, I have observed that MUC4 regulates the expression of EGFR ligands, particularly TGF-α, and thereby regulating the receptor

recycling. Altogether, in this current chapter, I am presenting the multi-faceted roles of MUC4 in regulating the fate and trafficking of EGFR family members.

V.2 Background and rationale

PC progression is accompanied by multiple genetic mutations such as, K-ras, p53, SMAD4, and so on [1]. Mutations along with inflammation can turned on various genes which does not express in normal conditions. MUC4 is one of such aberrantly overexpressed protein (~60%-70% of PC patients) in pancreatic cancer condition [2, 3]. MUC4 promotes tumorigenicity and has been directly associated with the growth, survival and chemoresistance of the PC cells, and its inhibition suppresses pancreatic tumor cell growth and metastasis [4-7]. Due to loss of cellular polarity, which is one of the hall-marks of cancer, MUC4 spread over the entire cell surface and start interacting with various cell-surface RTKs, including EGFR family members [8, 9]. Overexpression as well as functional importance of EGFR family members is quite evident in PC [10, 11]. Based on numerous experimental approaches, it has been suggested that loss of EGFR signaling could decline K-ras activity by 50%, and thus block the process of PC tumorigenesis [12]. Though multiple studies have associated MUC4 overexpression with the increased stability of RTKs over the cell surface, so far no studies have highlighted any mechanism which contributes to this process. Being a well-established model system to study receptor trafficking, it is known that EGFR fate is determined and decided at multiple steps by myriad of trafficking proteins. Several line of evidence has also established that proteins, which are known to participate in prolonging the RTKs-initiated active signal transductions, overexpress in cancerous condition. One of such proteins is Rab GTPases subfamily which has been implicated in the regulation of intracellular vesicle transport, such as receptor-mediated endocytosis, exocytosis, degradation and recycling [13, 14]. Studies have demonstrated their aberrant expression and activity in multiple cancers; for instance, Rab5A has been implicated in the progression of multiple cancers, such as, lung, hepatocellular, cervical and ovarian

cancers [15-18]. A study by Fukui K *et al* has clearly shown the involvement of Rab5A in potentiating EGFR-mediated signal transductions in hepatocellular carcinoma [19]. Moreover, Rab5A is involved in the rate-limiting step of EGFR endocytosis, and therefore, acts as a critical link between signal transduction and protein trafficking [20]. Considering the importance of MUC4 and Rab5A on the expression of EGFR family members, we hypothesized that MUC4 regulates the endocytosis of EGFR by regulating Rab5A expression and activity. Therefore, in the current study, for the first time, we have addressed the novel role of oncogenic MUC4 protein in determining the fate of RTKs endocytosis by Rab5A regulation in PC condition. To address this, we have performed biochemical, time-lapse microscopy and qRT-PCR approaches. Our biochemical data upon inhibiting protein synthesis and degradation has evidently revealed the involvement of MUC4 in increasing the half-life of EGFR family members. Considering the importance of RTKs in PC, deeper understanding of its prolonged presence as well as activation onto the cell membrane due to MUC4 overexpression, will give us better opportunity to therapeutically target PC.

V.3 Results

A. MUC4 increased the stability of RTKs in PC cells

Studies have evidently shown that MUC4 increase the stability of EGFR family members in various cancers [8, 9], which we have also confirmed as significant reduction in the EGFR and HER2 levels were noticed in MUC4 knocked down (kd) CD18/HPAF and CAPAN1 cells (**Fig. 5.1A**). Digital merging of confocal microscopic images of MUC4 (green) and EGFR (red) exhibited a noticeable colocalization (yellow) of these proteins at both membrane (non-permeabilized) and cytoplasmic regions (permeabilized) in PC cell lines (**Fig. 5.1B**). Corroboratively, our clinical data exhibited substantial MUC4 and EGFR co-expression and co-localization in PC tissues (n=13) than normal pancreas (n=3) and colon (n=1) (**Fig. 5.1C**), and further establishing their

association both under *in vivo* and *in vitro* settings. To directly implicate the role of MUC4 in EGFR stability, MUC4 scr and kd CD18/HPAF cells were treated with cycloheximide, which is an inhibitor of protein translation, for indicated time points. As anticipated, we observed significant depletion of EGFR in MUC4 silenced CD18/HPAF cells at all time-points compared to their respective scr controls (**Fig. 5.1D**). Altogether, these results indicate that in addition to HER2, MUC4 increases the stability of EGFR in PC cells.

B. MUC4 influences the stability of RTKs by altering their rate of internalization

Because it is reported earlier that MUC4 modulates the rate of HER2 internalization and consequently its turnover [8], we hypothesized that MUC4 utilizes the same mechanism to regulate EGFR expression. The kinase domain of HER3 shares 60% and 62% similarity with EGFR and HER2, respectively. However, both EGFR and HER2 share 83% identity in their amino acid sequence encoding for kinase domains [21]. It suggests that both EGFR and HER2 are more closely related to each other than they are to HER3 [21], which further support our hypothesis. To address that, we utilized time-lapse live-cell microscopy to monitor the fate of EGFR using rhodamine-tagged EGF ligand in MUC4 kd and control CAPAN1 cells. It is well-known in the literature that unlike TGF-α, EGF remains bound to EGFR and this EGF-EGFR complex undergoes dissociation and degradation in the lysosomes, therefore, movement of labeled vesicles from the cell membrane to endosomes actually indicates the levels and status of EGFR [22]. At 50 ng/ml of EGF, a concentration which is known to induce receptor degradation [23], significantly faster internalization was observed in MUC4 scr compared to MUC4 kd cells. As soon as chamber was kept at 37^0C, scr cells exhibited pronounced punctate formation, whereas in MUC4 kd cells, EGF vesicles remain persistently on the membrane (**Fig. 5.2A**). Despite of increased internalization, control cells had insignificant loss of EGF vesicles at 60 mins, whereas in MUC4 kd cells, there was

significant loss of vesicles, which is highly apparent in the marked (by white box) cells (**Fig. 5.2A**). Similar findings were observed in MUC4 kd CD18/HPAF cells, where control cells had significantly higher internalization (after 10 and 30 mins. of pulse) followed by insignificant depletion of internalized vesicles when compared to MUC4 kd cells (**Fig. 5.2B**).

To know EGFR localization, internalization experiment followed by colocalization experiment was performed. After 15 min. of pulse and 30 min. of chase, most of the EGFR vesicles are being recycled back to the plasma membrane in MUC4 scr CAPAN1 cells, whereas most of the internalized vesicles are depleted in MUC4 silenced cells and accumulated in Rab5A and Rab7 positive compartments (**Fig. 5.2C**). Increased EGFR staining on the Rab11-positive compartments at 30 and 60 mins of internalization in MUC4 scr CD18/HPAF cells compared to kd cells, further confirms increased receptor recycling, whereas more pronounced colocalization between LAMP1 and EGFR in MUC4 kd CD18/HPAF cells compared to scr cells, suggests increased degradation of EGFR receptor (**Fig. 5.2D**).

Earlier studies have implicated MUC4 cytoplasmic domain (MUC4-CD) as an interacting partner of HER2. Moreover, CD of mucins have shown to regulate EGFR trafficking [24], therefore, our next objective was to see whether overexpression of MUC4-CD has any effect on EGFR internalization and stability. Intriguingly, we observed increase in EGFR and p-EGFR expression in MUC4-CD overexpressing PC cell lines, however, changes in EGFR expression was insubstantial in MUC4-CD overexpressing MIA PaCa-2 cell line (**Fig. 5.2E**). Altogether, the results suggest that cytoplasmic domain of MUC4 has stabilizing effect on EGFR expression in PC cells.

C. MUC4 impacts receptor internalization by altering the expression of Rab5A

As mentioned earlier, Rab5A catalyzes the rate-limiting step in the internalization of RTKs and alterations in its expression have been noticed in various cancers [15-18].

Therefore, we next wanted to analyze the expression status of Rab5A in PC cell lines. Intriguingly, it was noticed that MUC4-expressing PC cell lines exhibited high Rab5A transcript levels compared to non-expressing PC cells (**Fig. 5.3A).** Furthermore, downregulation in Rab5A mRNA expression in MUC4 kd CAPAN1 cells compared to scr cells and upregulation of Rab5A expression in MUC4 expressing MIA PaCa-2 compared to MUC4 null MIA PaCa-2 cells (**Fig. 5.3B**), were enough to directly relate MUC4 with Rab5A expression. Similar results were obtained at protein level **Fig. 3C**). In addition to that, active-Rab5A (GTP bound) pull down assay revealed reduced Rab5A activity in MUC4 kd CAPAN1 cells compared to control cells (**Fig. 5.3D**). Further, IF analysis revealed co-localization between MUC4 and Rab5A in CD18/HPAF cell line and confirmed their concomitant expression and localization in PC cells (**Fig. 5.3E**). Increased Rab5A expression in MUC4-CD overexpressing AsPC1 cells further associated the involvement of Rab5A with MUC4-mediated increased RTKs internalization (**Fig. 5.3F**). To solely implicate the role of Rab5A in MUC4-mediated regulation of RTKs stability, Rab5A was overexpressed in MUC4 kd and scr CAPAN1 cells (**Fig. 5.3G**). Interestingly, we observed that loss of both EGFR and HER2 receptors upon MUC4 silencing were attenuated when RAB5A was overexpressed. We also validated the positive association between MUC4 and Rab5A expression levels in PC tissues using confocal microscopy (**Fig.5.3H**). Increased RIN1 mRNA expression in PC tissues compared to their adjacent controls (**Fig. 5.3I**); further established increase activity of Rab5A in PC patients. Altogether, MUC4 regulates the expression and activity of Rab5A and plausibly affects the internalization rate of RTKs in PC.

D. MUC4-mediated transcriptional regulation of Rab5A is CREB dependent

Earlier studies have clearly shown that promoter of Rab5A gene has cAMP-responsive element or CRE [25], which instigated us to propose that MUC4-mediated transcriptional regulation is CREB-dependent. To validate the importance of CREB in

Rab5A upregulation, we treated PC cell lines with CREB inhibitor to suppress CREB-mediated transcription by inhibiting its interaction with CBP, and observed downregulation in Rab5A expression compared to untreated cells (**Fig. 5.4A**). Treatment of CD18/HPAF cells with CREB activator, insulin, led to increased Rab5A expression at mRNA level (**Fig. 5.4B**), further establishing the involvement of CREB activity on the transcription of Rab5A gene.

Due to observed involvement of MUC4 in the regulation of Rab5A expression, our next objective was to determine whether MUC4 expression impacts the expression and activation status of CREB molecule. As anticipated, significant downregulation in p-CREB expression was observed in MUC4 kd PC cell lines compared to the control cells, without alteration in the expression of total CREB protein (**Fig. 5.4C**). ERK signaling has established association with the phosphorylation of CREB [26, 27], which was also found to be downregulated in MUC4 kd cells [28], and thus, provided the plausible link between MUC4 and CREB activation. Expression profiling revealed significant overexpression of both CREB and p-CREB in a panel of PC cell lines compared to HDPE (immortalized normal pancreatic cells). Moreover, this increase was more prominent in MUC4-expressing PC cell lines than MUC4 non-expressing PC cells, supporting the role of MUC4 in CREB activation (**Fig. 5.4D**). Co-expression of MUC4 and p-CREB in PC tissues further signifies their positive association and validated our *in vitro* results (**Fig. 5.4E**). Interestingly, p-CREB staining was highly nuclear and expressed both in the ductal as well as in the stromal compartments. Taken together, the data provides the mechanistic link which leads to MUC4-mediated increase in Rab5A transcription *via* CREB regulation in PC condition.

E. MUC4-mediated regulation of the expression of EGFR family ligands also determine the fate of receptors

Although in our study, we have mentioned the role of MUC4 in determining the stability of EGFR and HER2, the basal level of EGFR remains unchanged in MUC4-expressing and non-expressing PC cell lines, suggesting that PC cell lines do possess some unique mechanisms to stabilize the expression of EGFR receptor (**Fig.5.5A**). Interestingly, addition of EGF ligand in these cell lines impacts the fate of EGFR quite differently in MUC4 expressing *vs.* MUC4 non-expressing cell lines. As demonstrated, MUC4 non-expressing; Panc1 and MIA PaCa-2 cell lines, had significant depletion of EGFR receptor at both 60 and 120 mins, compared to MUC4 expressing CD18/HPAF and CAPAN1 cell lines (**Fig.5.5B**). Due to these unique observations and the established involvement of EGFR ligands on determining their fate, we decided to analyze the expression levels of EGFR ligands in a panel of PC cell lines. Compared to MUC4 non-expressing cell lines (HPNE, Panc1, AsPC1 and MIA PaCa-2), MUC4-expressing PC cell lines exhibited significantly elevated levels of EGF and TGF-α ligands (**Fig.5.5C**). TGF-α has established role in facilitating the recycling of EGFR [22], which can be attributed to the observed increased recycling of EGFR receptor in the presence of MUC4 and needs to be validated. Altogether, these results suggest that in the absence of MUC4, PC cell lines attempts to stabilize EGFR expression by limiting the concentration of ligands.

G. MUC4 increases the sensitivity of EGF-mediated migration and proliferation by increasing oncogenic signaling

To understand the functional impact of prolonged cell surface localization of EGFR in the presence of MUC4, cell-growth kinetics and Boyden chamber migration assays were performed in the presence and absence of EGF ligand. CD18/HPAF MUC4 Scr cells showed significant increase by 39% ($p<0.05$) in cell growth in ligand treated as compared to untreated cells (**Fig. 5.6A**). Silencing of MUC4 exhibited less impact on cell growth as only 18% increase after EGF treatment. CD18/HPAF MUC4 Scr cells showed

significant increase in cell migration by 39% ($p<0.05$) in ligand treated as compared to untreated cells. Silencing of MUC4 exhibited less impact on cell migration as only 18% increase in cell migration was noticed after EGF treatment in MUC4 kd CD18/HPAF cells (**Fig. 5.6B**). Similar changes were also noticed in CAPAN1 cells, where presence of MUC4 increases the responsiveness of EGF-mediated migration by 40%, whereas loss of MUC4 attenuate such effects as only 25% increase in cell migration has been noticed. Taken together, we can conclude from these results that MUC4 role in providing stability to EGFR by preventing its internalization is definitely making cancer cells more sensitive for EGF-mediated proliferative and migratory potential.

V.4 Discussion

Normal cells regulate their growth by regulating the secretion of growth factors and expression of their respective cell surface receptors at the optimal time and concentration to maintain their function [30]. On the other hand, cancer cells abrupt this crucial regulation in order to grow unrestrictedly [30]. In this study, we have addressed the implicated mechanisms by which EGFR and HER2 receptors provide prolong proliferative advantage to PC cells. Normal epithelial cells with a well-defined morphology with apical, basal, and lateral organization restrict the interaction of proteins from one region to another. However, under cancer condition, cells lose its polarity which subsequently leads to the disruption of this organization and facilitates novel protein-protein interactions [31, 32]. For instance, MUC4 which expresses in apical regions in normal polarized epithelial cells can interact with basolateral proteins, including EGFR/Erbb family receptors in non-polarized cancerous cells, which has been evidently shown in multiple cancers such as, pancreatic and ovarian cancers [8, 9]. As a consequence, these interactions lead to the induction of multiple downstream signaling events such as PI3K and MAPK-mediated signaling [33]. Although earlier studies have indicated that presence of MUC4 regulates the stability of RTKs, the exact mechanism

which can be attributed to this phenomenon has not been pinpointed. In the current study, for the first time, we have indicated that MUC4 regulates the expression of Rab5A, a key protein involved in EGFR/HER2 trafficking. Being a critical molecule involve in protein endocytosis, alteration in the expression of Rab5A can significantly impact the protein trafficking. Nevertheless, we have also shown that MUC4-mediated regulation of Rab5A is responsible for the regulation of both HER2 and EGFR. MUC4 has been demonstrated to increase EGFR expression in triple-negative breast cancer and glioblastoma [34, 35], but in the current study, we have clearly shown the involvement of MUC4 in EGFR regulation, using both *in vivo* and *in vitro* studies. Previous studies have shown that MUC1, a well-established mucin, imparts oncogenicity in cancerous condition, by influencing EGFR internalization and nuclear translocation [24]. Altogether, THE current study along with other studies have suggested that upregulation of mucin, one of the characteristics of PC, plays important role in stabilizing RTKs to support tumor growth.

We have demonstrated that MUC4 interaction with EGFR occurs in the membranous and cytoplasmic regions under *in vitro* and *in vivo* settings [8]. Attenuation of EGFR reduction upon MUC4 silencing in CD18/HPAF cells when treated with lysosomal inhibitor, chloroquinone (CQ), indicates that MUC4 does regulate EGFR and HER2 fate. Under physiological condition as well, MUC4-mediated regulation of EGFR plays more significant role than MUC4-mediated HER2 regulation because of significantly higher incidence of EGFR overexpression (~70%) than HER2 (`18%) in PC condition. Nevertheless, EGFR is expressed primarily on the ductal epithelial cells, unlike HER2, which expressed predominantly on stromal cells than pancreatic ducts [36]. Though MUC4 interactions with HER2 has been attributed to HER2 increased stability on the pancreatic cancer cell surface by our previous study [8], but the general mechanism which actually leads to this phenomenon has not been addressed. In the

current study, we have established that MUC4-mediated regulation of EGFR family members is dependent on Rab5A.

Rabs family members play critical role in controlling protein trafficking. Change in their expression has been observed in multiple cancers [37-39]. Due to established role of Rab5A in cancer and EGFR internalization, we focused ourselves on this molecule and received intriguing observations. Due to increased expression of Rab5A in PC cell lines as compared to normal pancreatic cells (HDPE), we proposed that Rab5A negatively regulates EGFR internalization. Indeed, similar to MUC4, overexpression of Rab5A leads to an increased rate of internalization and recycling and *vice versa*. Moreover, overexpression of Rab5A in MUC4 kd CAPAN1 cells attenuates the downregulation of both EGFR and HER2 compared to their respective controls. Interestingly, MUC4 is a critical regulator of transcription of Rab5A gene. Previous study has shown that Rab5A has multiple CRE sites [25], suggesting the plausible role of CREB in Rab5A transcriptional regulation, which we have established using inhibitors approach. Inhibition of CREB causes reduction in Rab5A expression, whereas CREB activation by insulin is responsible for Rab5A upregulation at both mRNA and protein levels. The connection between ERK and MUC4 signaling is well understood in the literature [40]. Similarly, ERK-mediated regulation of CREB phosphorylation is also known, which led us to comprehend that MUC4-mediated CREB activation is *via* ERK signaling cascade. Besides regulating the expression of Rab5A, we believe that MUC4 also regulates its activity by regulating RIN1 expression [41, 42]. Interestingly, frequent overexpression of RIN1 at mRNA level was observed in PC patients. Further studies are still required to experimentally establish this link.

As mentioned in the introductory chapter-1c, there is significant increase in the levels of EGFR ligands in both PDA spontaneous mouse models and human PC. Interestingly, we observed significant influence of MUC4 expression on EGFR ligands,

including, EGF and TGF-α. Of particular interest was TGF-α ligand due to its established role in facilitating increased recycling of EGFR, which can possibly be attributed to the MUC4-mediated increased recycling of both EGFR and HER2 [22]. However, it is still a critical question how does MUC4 mechanistically regulate the expression of EGFR ligands at transcriptional level? Altogether, from this data, it seems that MUC4 regulates the fate of EGFR family members at multiple steps. Our functional studies have shown that presence of MUC4 makes cancer cells more responsive to EGF-mediated oncogenic effects. We observed significant increase in cell growth and migration upon the addition of EGF in MUC4 Scr than the MUC4 kd PC cells. There are myriad of evidence suggesting the overexpression of EGF under cancerous condition, including PC [12, 43]. EGF overexpression has been linked with increased cancer cell migration and proliferation due to its ability to activate multiple downstream signaling elements *via* EGFR receptor.

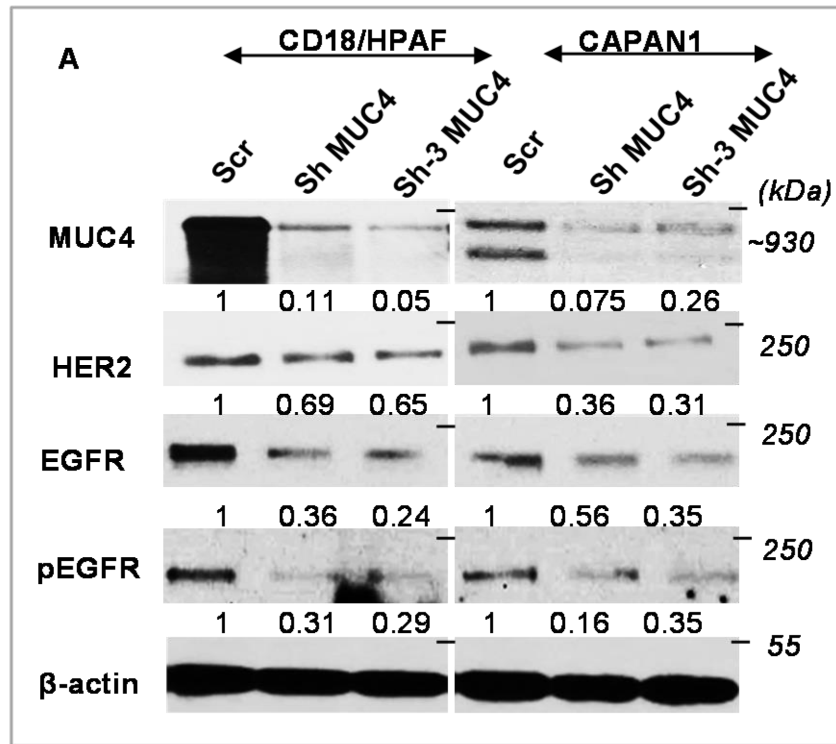
Besides regulating the oncogenicity of pancreatic tumor cells, MUC4 mediated regulation of EGFR internalization could also affect the therapeutic response, as one of the widely accepted mechanisms of action of EGFR based mAbs is to induce receptor internalization followed by degradation. We have observed significant attenuation of EGFR specific monoclonal antibody (mAb) mediated EGFR degradation in the presence of MUC4, suggesting that MUC4-EGFR interaction is not only stimulating the proliferation of the cells but also makes cells resistant towards EGFR-based therapies. In fact, presence of MUC4 has been associated with herceptin resistance in breast cancer [44]. Altogether, these observations led us to think that in spite of having such enormous importance of EGFR in PC; EGFR mAbs such as cetuximab, did not deliver anticipated clinical benefits to the patients. Physical interaction between MUC4-EGFR interactions, which is inhibiting mAbs-mediated internalization and receptor degradation to attenuate signaling, could be an important contributing factor in drugs failure and

resistance. Therefore, by modulating their interaction, we can possibly achieve increase efficacy of EGFR-mediated therapeutic response. Further studies are required to understand the relevance of MUC4 domains in terms of EGFR-directed monoclonal antibodies. Conclusively, this study has highlighted a novel function of MUC4 in regulating the trafficking of EGFR to potentiate EGF-mediated effects.

Figure and Figure legends

Figure 5.1 *In vitro* and *In vivo* data shows an association between MUC4 and EGFR in PC condition. A. CD18/HPAF and CAPAN1 cells were serum-deprived for 12 hours, and then cell lysates were collected. IB results showing significant reduction in HER2, EGFR and pEGFR protein levels in MUC4 kd CD18/HPAF and CAPAN1 PC cell lines, compared to scr control cells. **B.** IF images showing significant colocalization and interaction between MUC4 and EGFR in CD18/HPAF and CAPAN1 cell lines under both permeabilized and non-permeabilized conditions. A 0.2% saponin was used with antibody solution for permeabilized condition, whereas antibody solution without detergent was used for non-permeabilized condition. **C**. Confocal images demonstrating co-expression of MUC4 and EGFR in stained PC tissues. Pearson correlation was calculated using Image J software for each field from tissue spots of normal colon (NC), normal pancreas (NP) and PC. The graph is clearly showing that colocalization between MUC4 and EGFR was significantly high in PC P. **D**. IB showing significant depletion of EGFR protein levels upon cycloheximide (50 µg/ml) treatment in MUC4 kd CD18/HPAF cells compared to scr cells, suggesting that MUC4 has significant role in stabilizing EGFR expression in PC cancer condition.

Figure 5.1



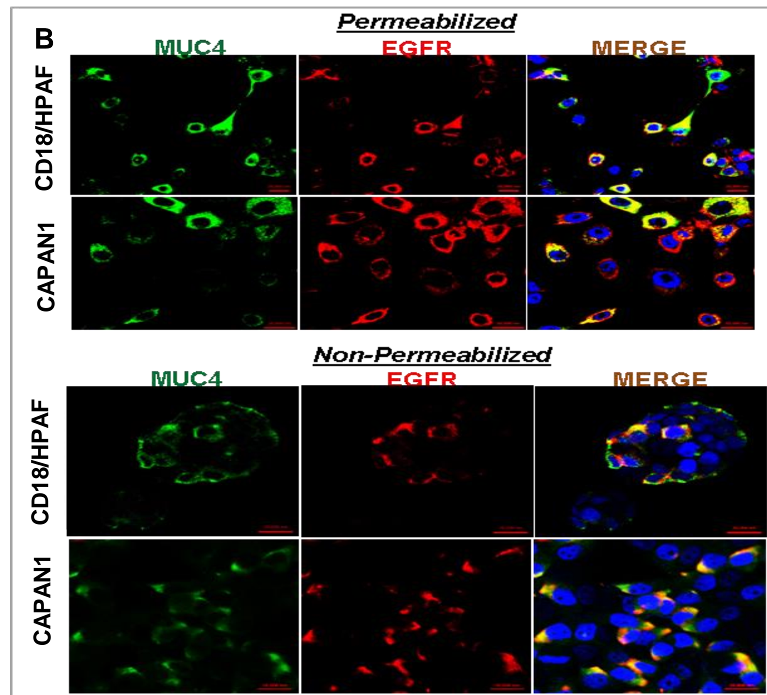


Figure 5.1

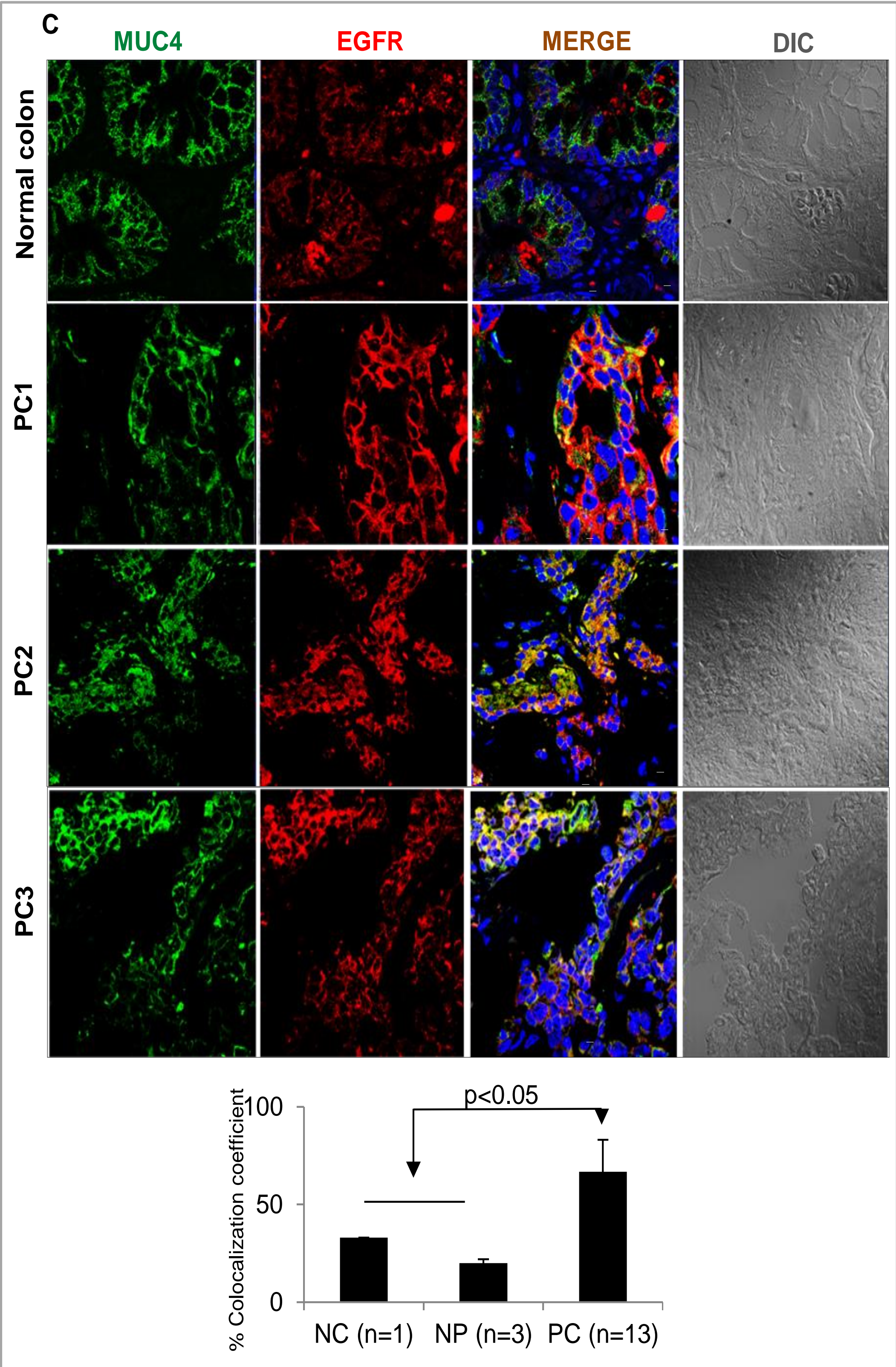


Figure 5.1

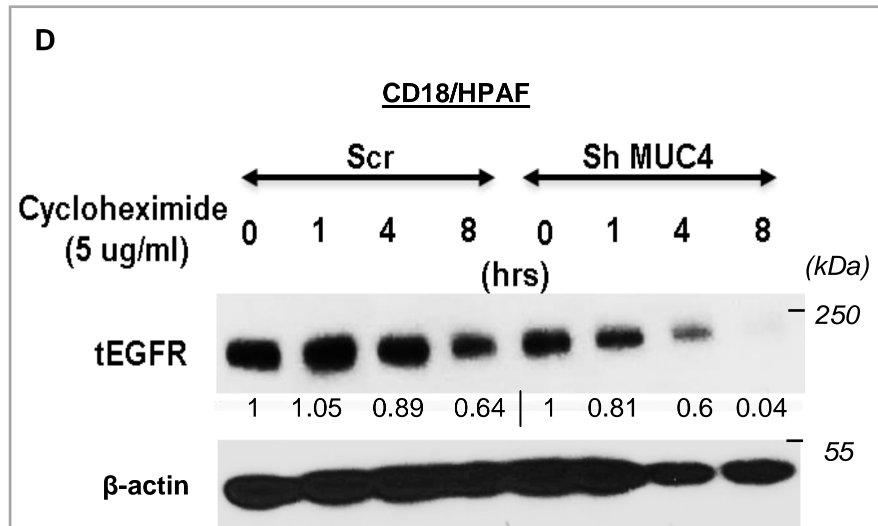


Figure 5.2 MUC4 significantly impact EGFR trafficking. A. Images obtained from live cell imaging experiment have revealed that EGF ligand tagged with Rhodamine (but represented as yellow color for more clarity) is internalizing at very faster rate in MUC4 control cells compared to MUC4 scr cells. However, CAPAN1/scr cells also had significant recycling of EGF-vesicles at 45 and 60 mins, compared to MUC4 kd PC cells. **B.** Flow cytometry data revealed that MUC4 scr cells had significantly higher level of uptake or internalization of EGF bound EGFR at 30 mins compared to MUC4 scr cells, however, the bound receptor is getting depleted in MUC4 kd CD18/HPAF cells than scr control cells. **C.** Confocal images showing the presence of EGFR and Rab5A in scr and MUC4 kd CAPAN1 cells. Here, cells were stimulated with unlabeled EGF for 15 min. and then EGF bound EGFR was chased after 30 mins at 37^0C. **D.** Confocal images showing significant colocalization between EGFR and LAMP1 at 60 mins in MUC4 kd CAPAN1 cells compared to scr cells, suggesting increased degradation of receptor in scr cells. On the other hand, increased colocalization between EGFR and RAB11 receptor in MUC4 scr cells compared to MUC4 kd cells, suggestive of increased recycling. **E.** Ectopic expression of MUC4 cytoplasmic domain (MUC4-CD) in MIA PaCa-2 and AsPC1 cell lines led to increased expression of both EGFR and p-EGFR compared to their respective vector controls.

Figure 5.2

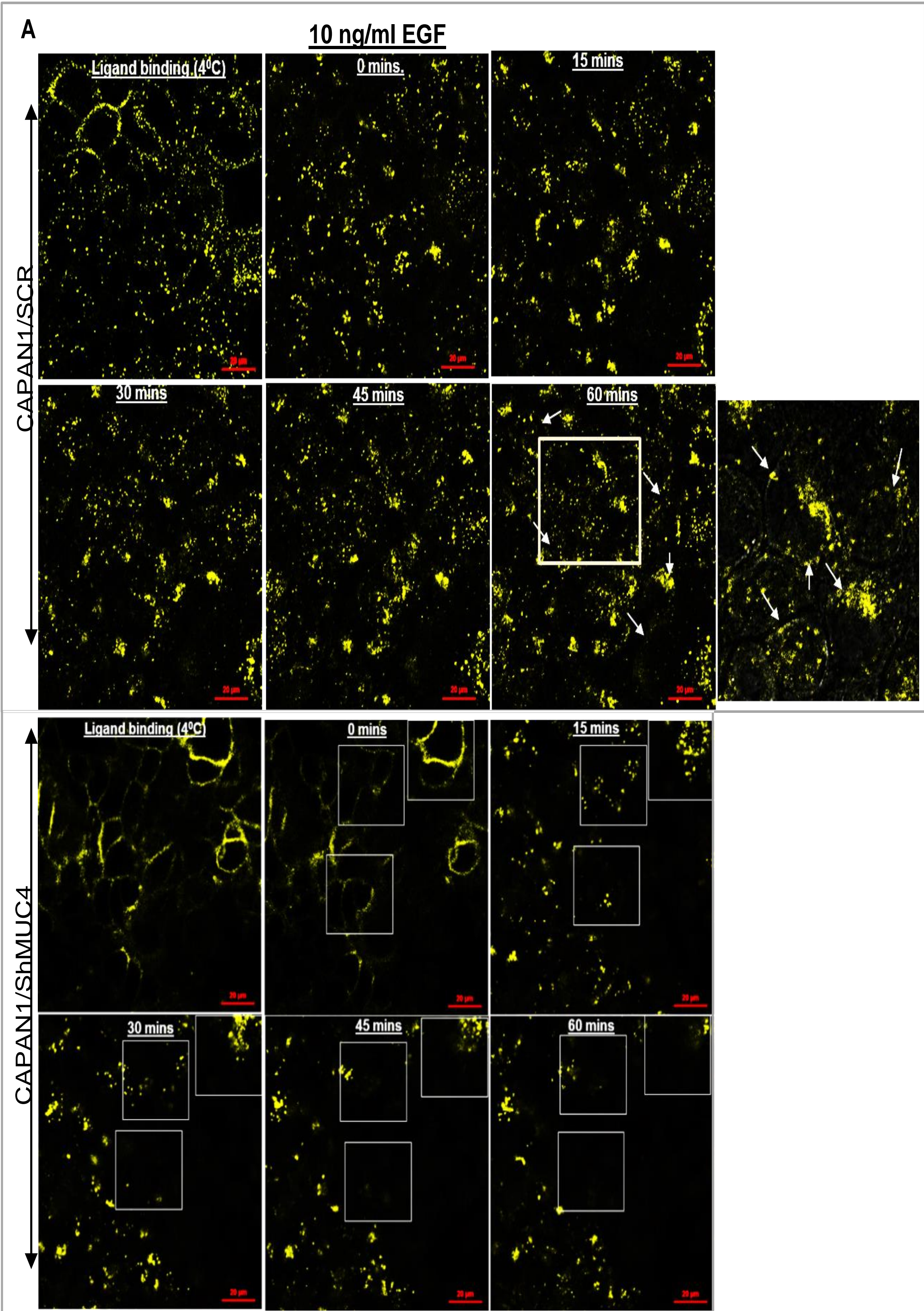
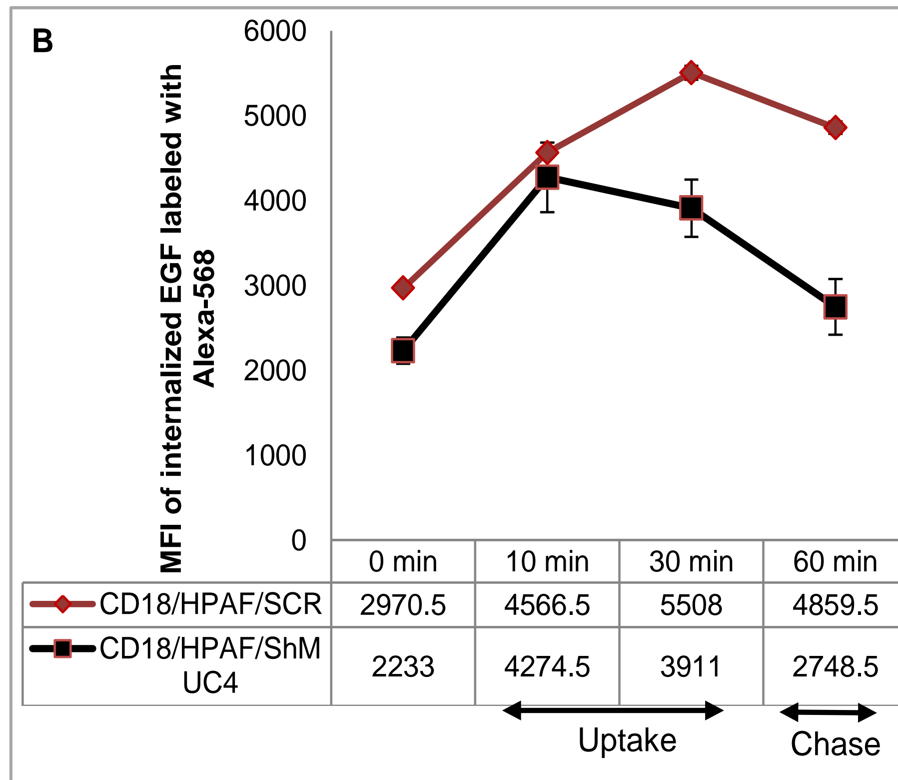


Figure 5.2



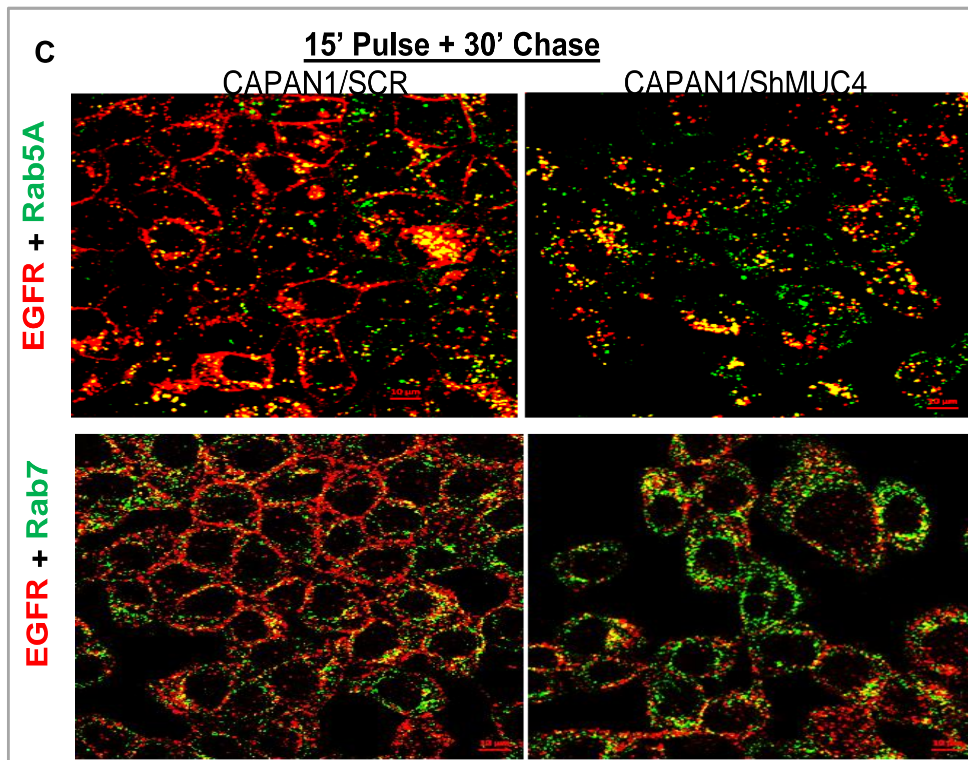
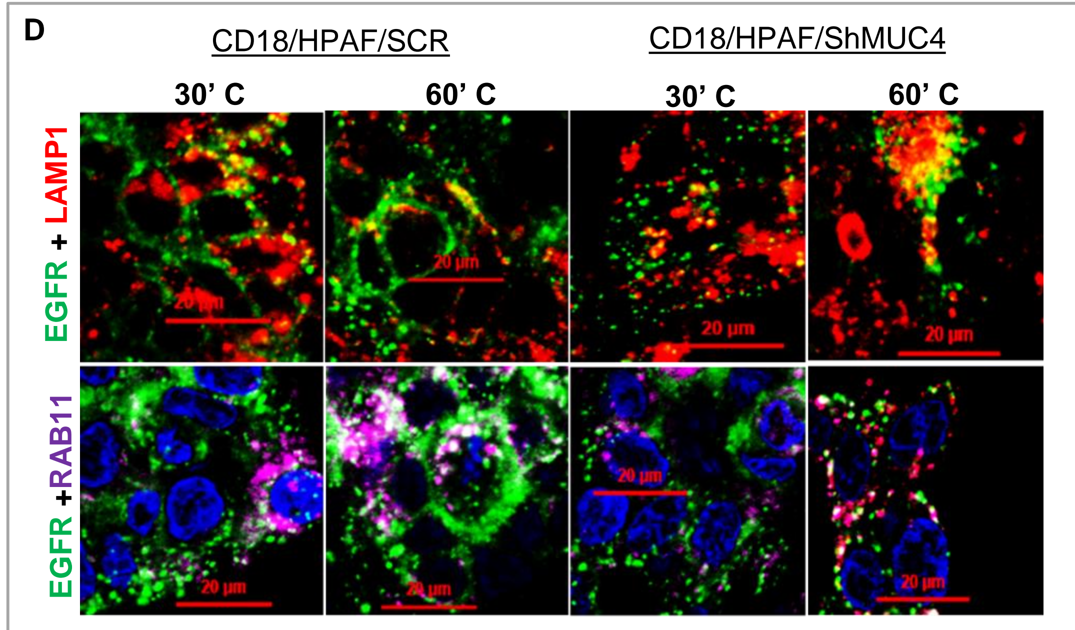


Figure 5.2



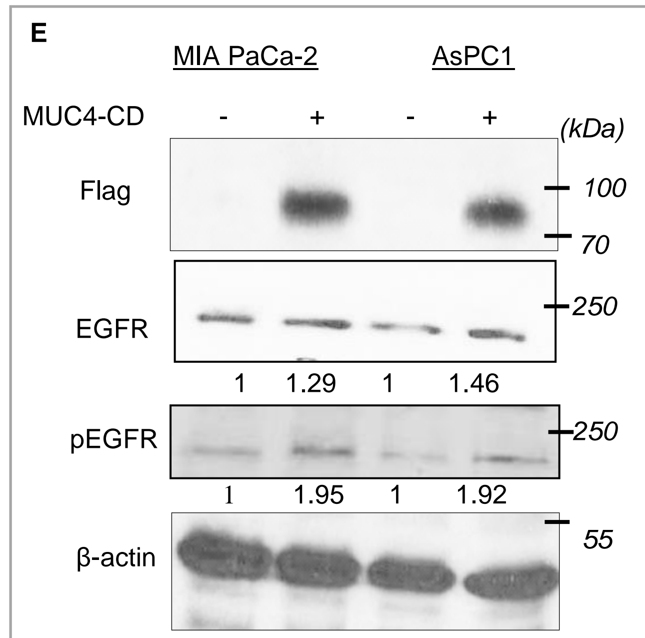
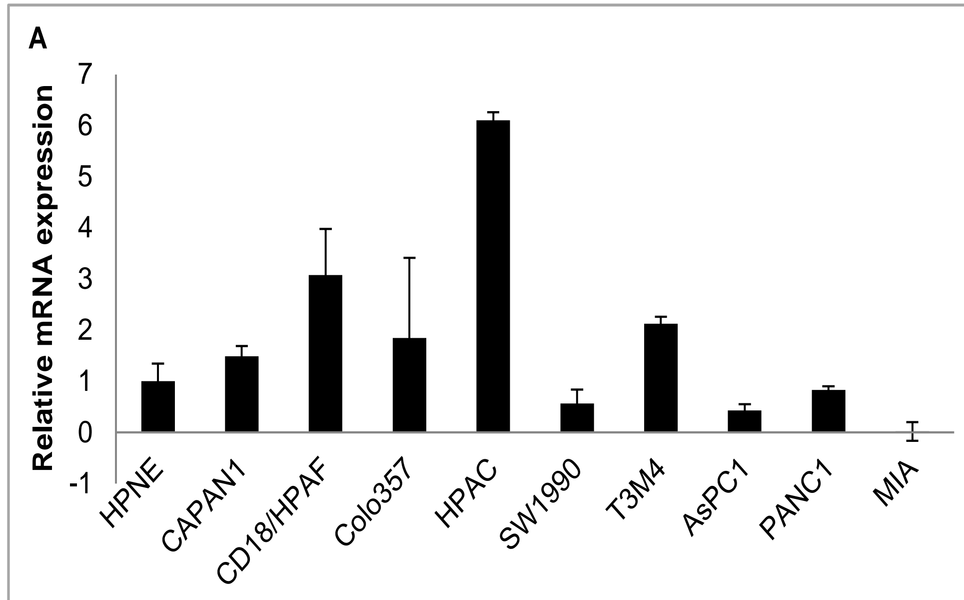


Figure 5.3 MUC4 regulates the expression and activity of Rab5A. A. Quantitative real-time PCR data showing increased expression of Rab5A in MUC4 expressing (CAPAN1, CD18/HPAF, Colo357, HPAC, SW1990 and T3M4) PC cell lines, compared to MUC4 non-expressing (HPNE, AsPC1, Panc1,MIA PaCa-2) cells. **B**. MUC4 kd CAPAN1 and MUC4 ectopic expression in MIA PaCa-2 cell lines exhibited reduced and high expression of Rab5A at mRNA levels, compared to their respective controls. **C**. These results were also confirmed at protein levels. **D.** Active Rab5A was pulled down in MUC4 scr and kd CAPAN1 cells. IB results are clearly showing that Rab5A pull down was significantly more in MUC4 kd CAPAN1 cells compared to scr cells. **E.** Confocal images demonstrating Rab5A expression in MUC4 scr and kd CD18/HPAF cells. **F**. IB showing increased expression of Rab5A upon ectopic expression of MUC4-CD in MIA PaCa-2 and AsPC1 cell lines. **G.** IB showing the expression levels of EGFR and HER2 in Rab5A overexpressing MUC4 kd and scr CAPAN1 cells. H**.** MUC4 and Rab5A colocalization (or co-expression) was determined using immunofluorescence experiment in RAPID autopsy tissue samples. I**.** Increased mRNA expression of RIN1 in PC patients compared to tumor adjacent tissues, further confirm increased activity of Rab5A in PC tissues than control.

Figure 5.3



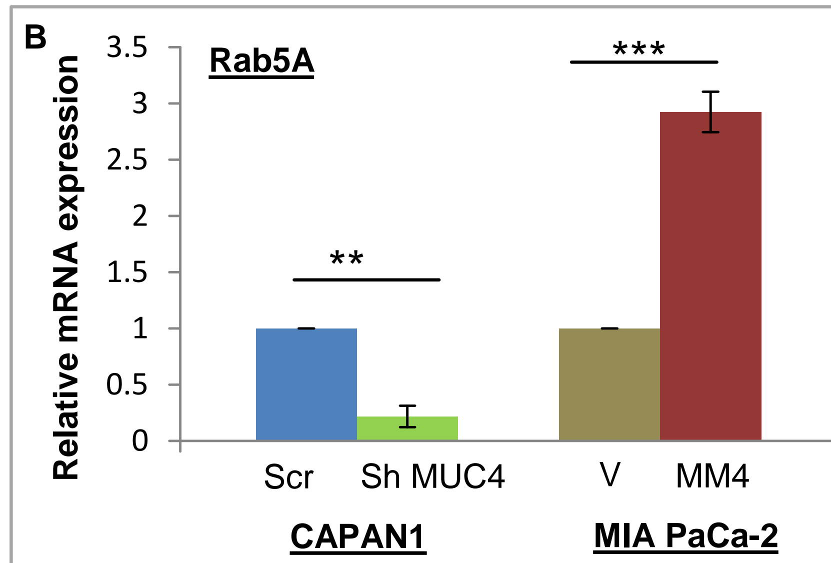
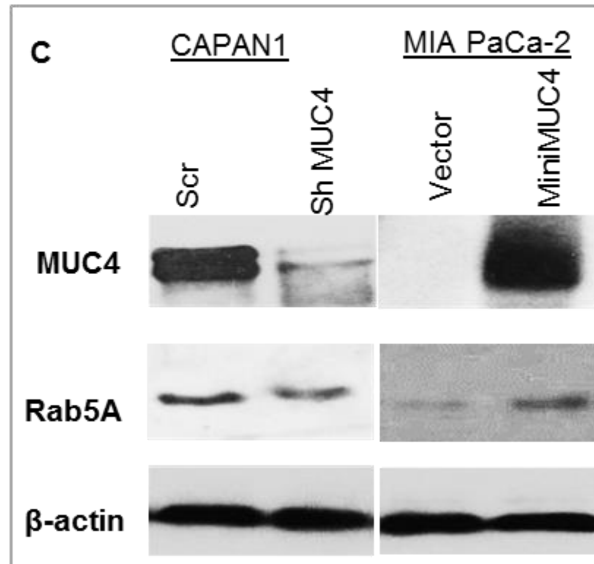
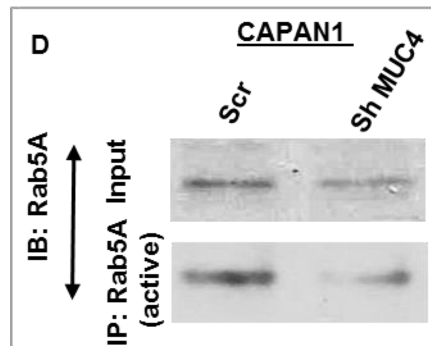


Figure 5.3





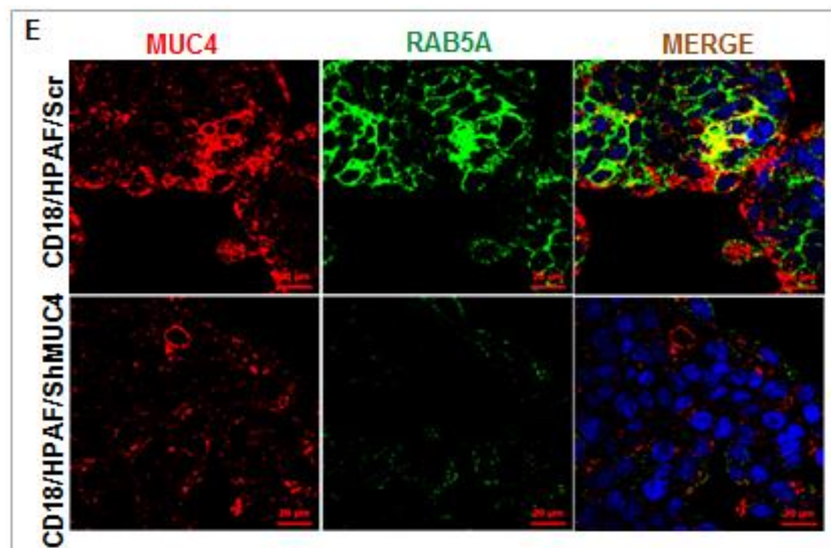
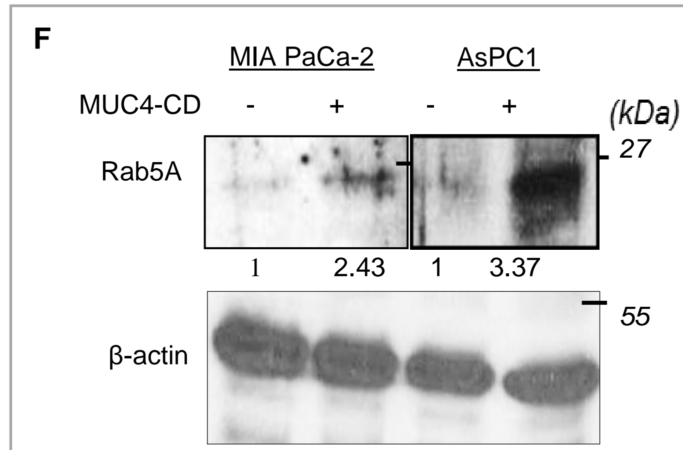


Figure 5.3



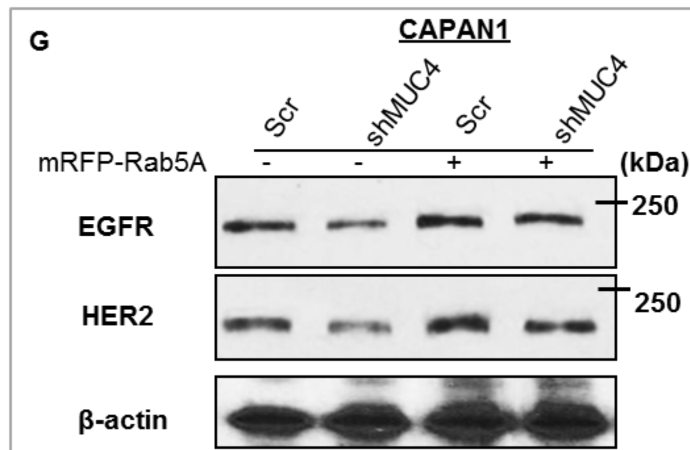
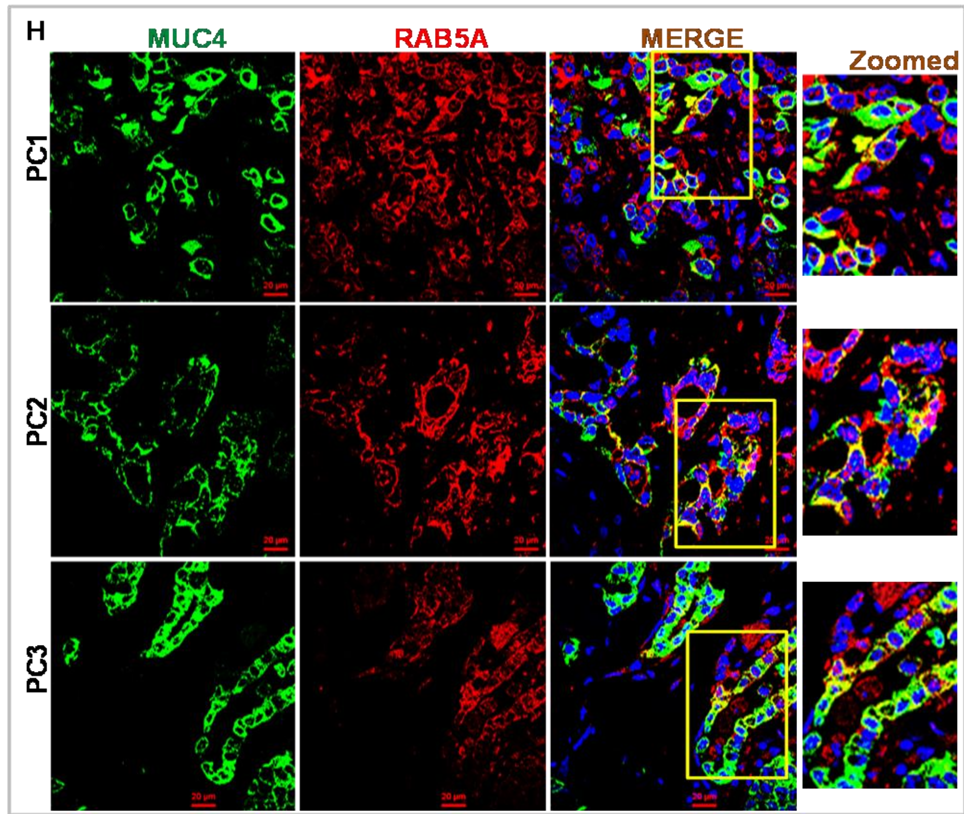


Figure 5.3



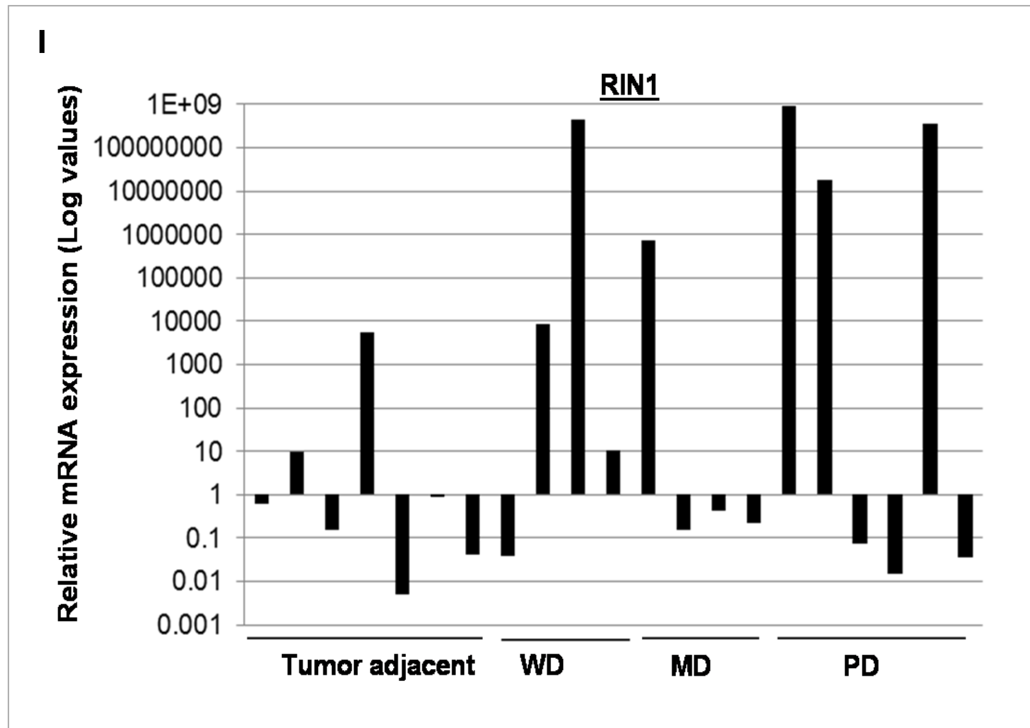


Figure 5.4 MUC4-mediated regulation of CREB activity is leading to Rab5A regulation. **A.** Inhibition of CBP:CREB interaction by using pharmacological inhibitor significantly reduces Rab5A expression in CAPAN1 and CD18/HPAF cells. **B**. CAPAN1 and CD18/HPAF cells were cultured in serum free media 12h prior to treatment. Following, cells were treated with insulin (200 nM) for 4h and RNA isolation was performed. The graph is showing upregulation of Rab5A mRNA expression upon insulin treatment in both the tested cell lines, confirming the positive involvement of activated CREB on the transcriptional induction of Rab5A gene **C**. IB showing reduced expression of p-CREB and p-ERK in MUC4 kd PC cells, while total CREB remains constant. **D.** A panel of PC cell lines was screened for p-CREB and CREB molecules using IB analysis. Both CREB and p-CREB levels were relatively high in MUC4 expressing than MUC4 non-expressing PC cell lines, confirming the link between MUC4 and CREB activation. **E.** IF images were taken for p-CREB and MUC4 in stained PC tissues. The significant coexpression of both of these molecules on PC tissue spots was validating our *in vitro* results under clinical settings.

Figure 5.4

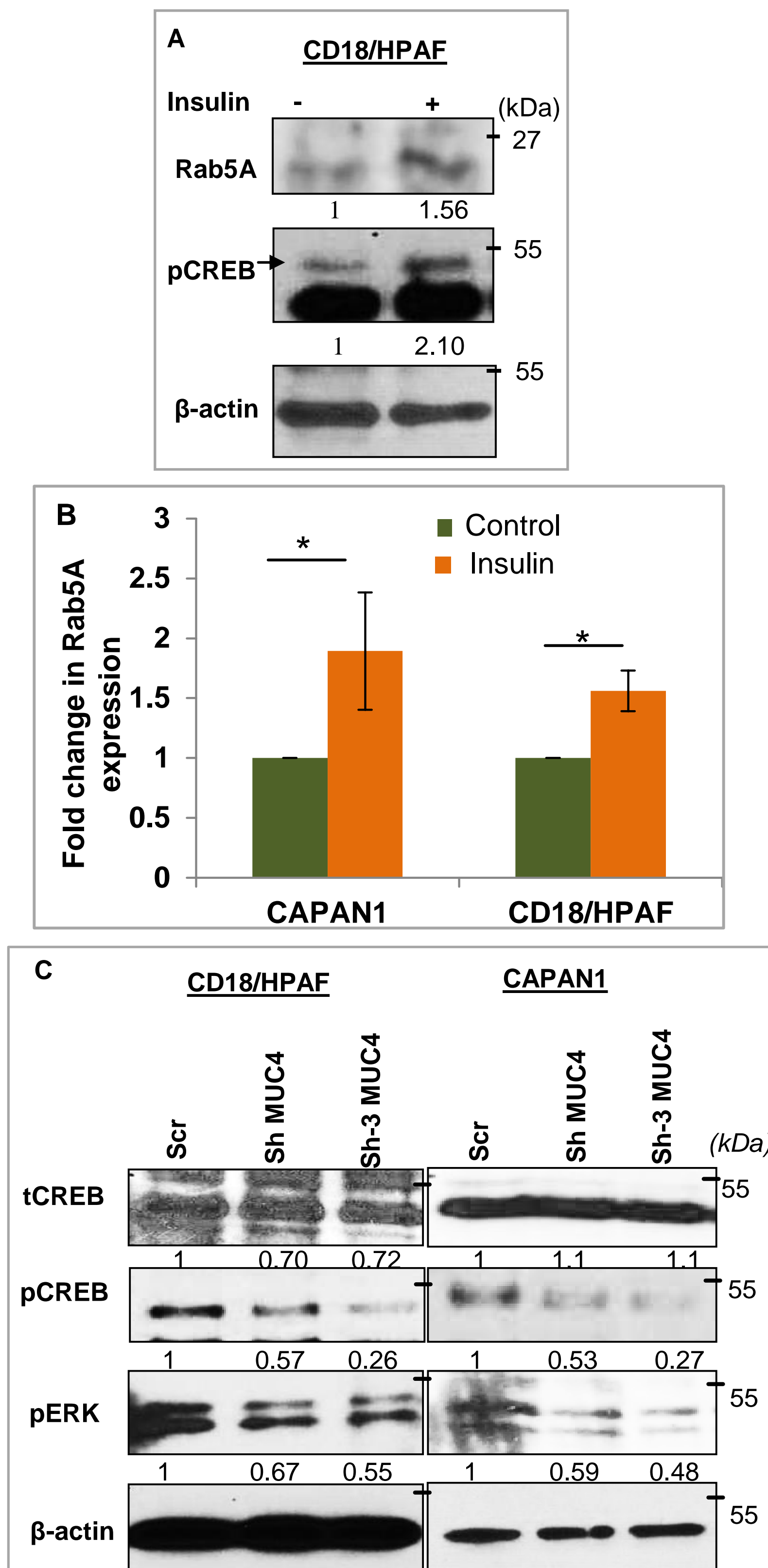
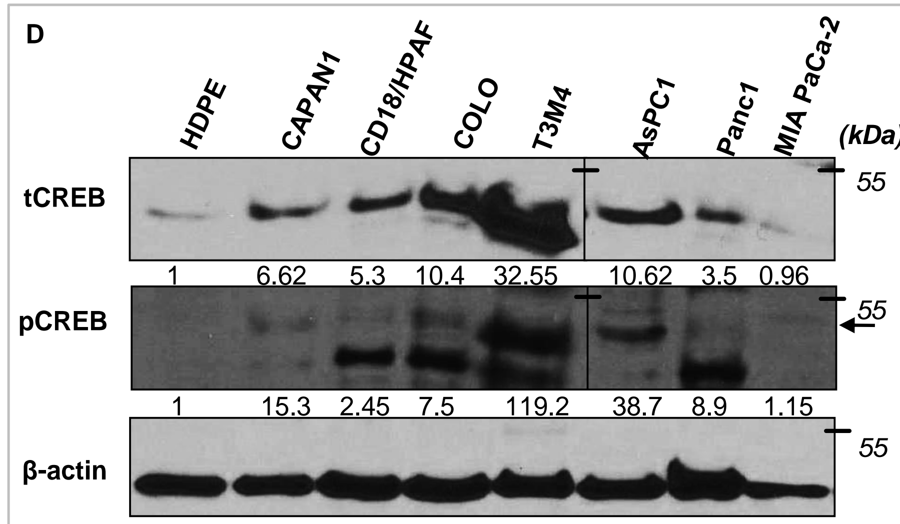


Figure 5.4



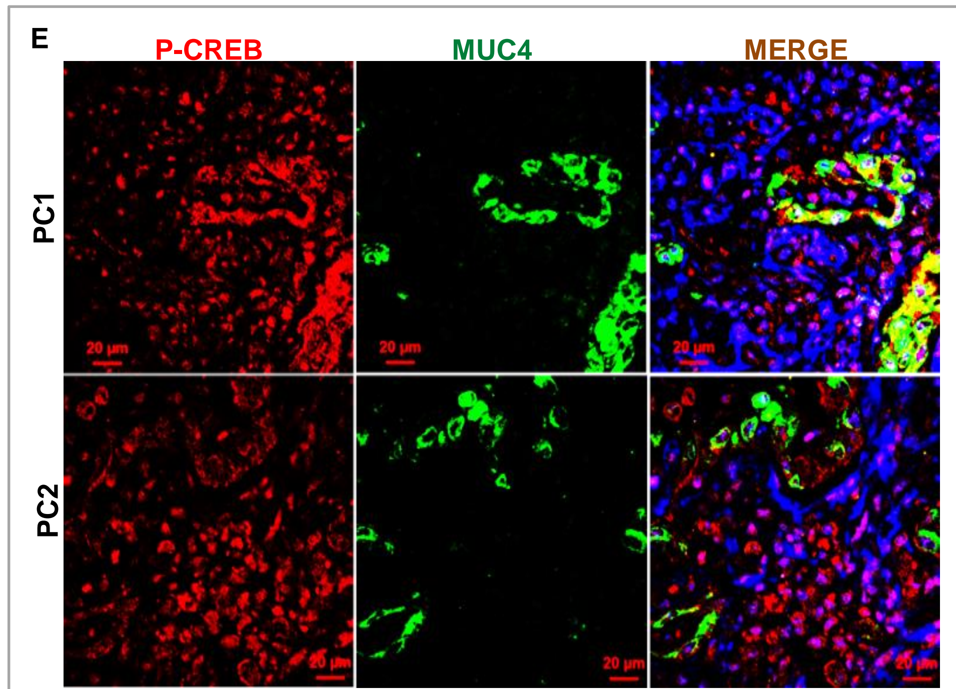
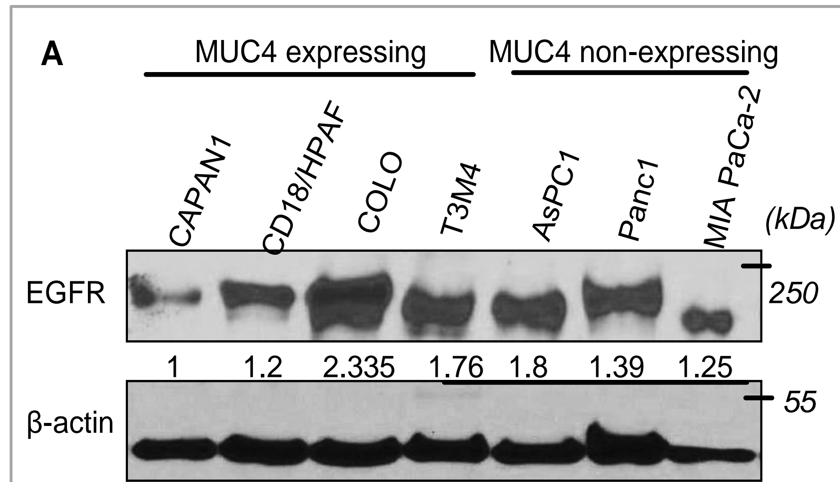


Figure 5.5 MUC4 regulates the expression of EGFR ligands. A. A panel of PC cell lines was screened for EGFR expression using IB. **B**. PC cell lines were stimulated with unlabeled EGF ligand (20ng/ml) for 60 and 120 min. Following stimulation, cells were stained with EGFR. Significant depletion of EGFR was noticed in MUC4 non-expressing MIA PaCa-2 and Panc1 cell lines, whereas CAPAN1 and CD18/HPAF cells also exhibited depletion of the receptor; however it was significantly less than MUC4 non-expressing cell lines. **C**. A panel of PC cell lines was screened for EGFR ligands; EGF and TGF-α. The data showing the relative mRNA change value normalized with β-actin.

Figure 5.5



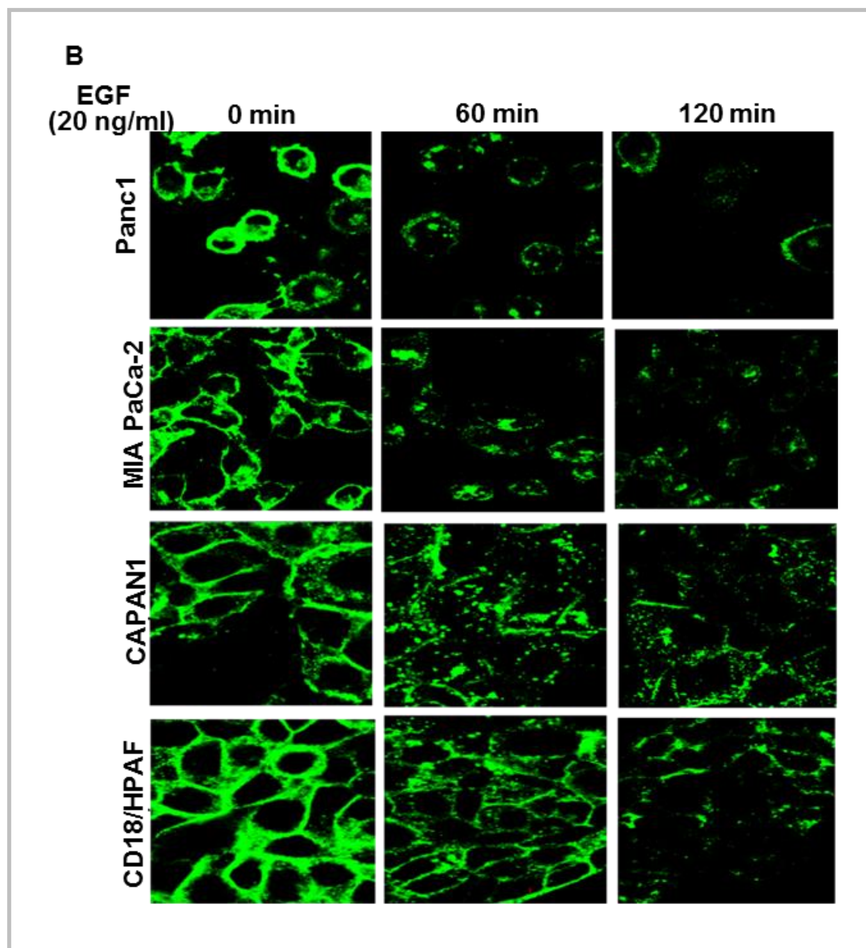


Figure 5.5

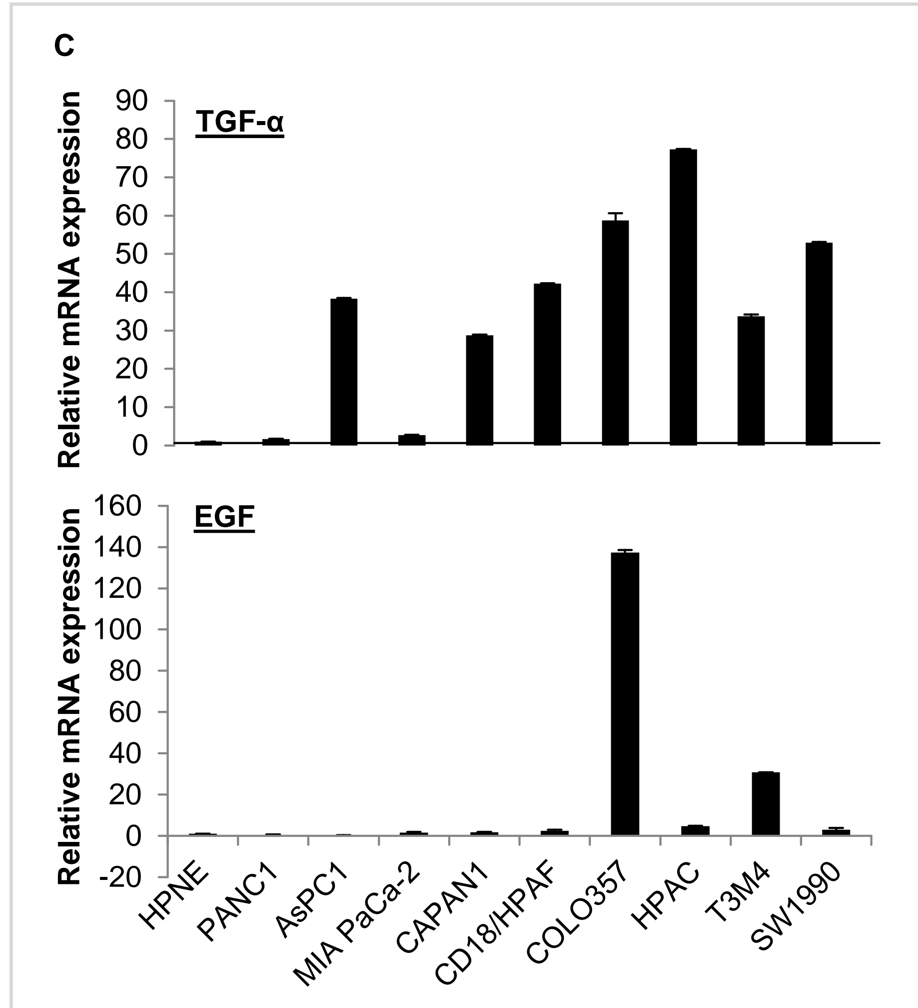


Figure 5.6 MUC4 potentiates EGF-mediated proliferative and migratory potential. A. 0.5X10^6 of scr and MUC4 kd CD18/HPAF cells were grown on the surface of Boyden Chamber assay in serum free media. After 12h, EGF ligand (20ng/ml) was added on the bottom chamber and cells movement was traced. Following 48h of treatment, cells reached to the lower chamber were stained, while cells still present on the upper chamber were removed. Stained cells were quantified by the use of microscope. Images obtained are showing the effect of EGF ligand on the migration of MUC4 scr and kd CD18/HPAF cells, and suggesting that presence of MUC4 does make PC cells more responsive for EGF-mediated effects on cellular migration. **B.** Similar to migration, scr cells also exhibited higher levels of proliferation upon EGF treatment, compared to MUC4 kd cells, as depicted by growth kinetics experiment.

Figure 5.6

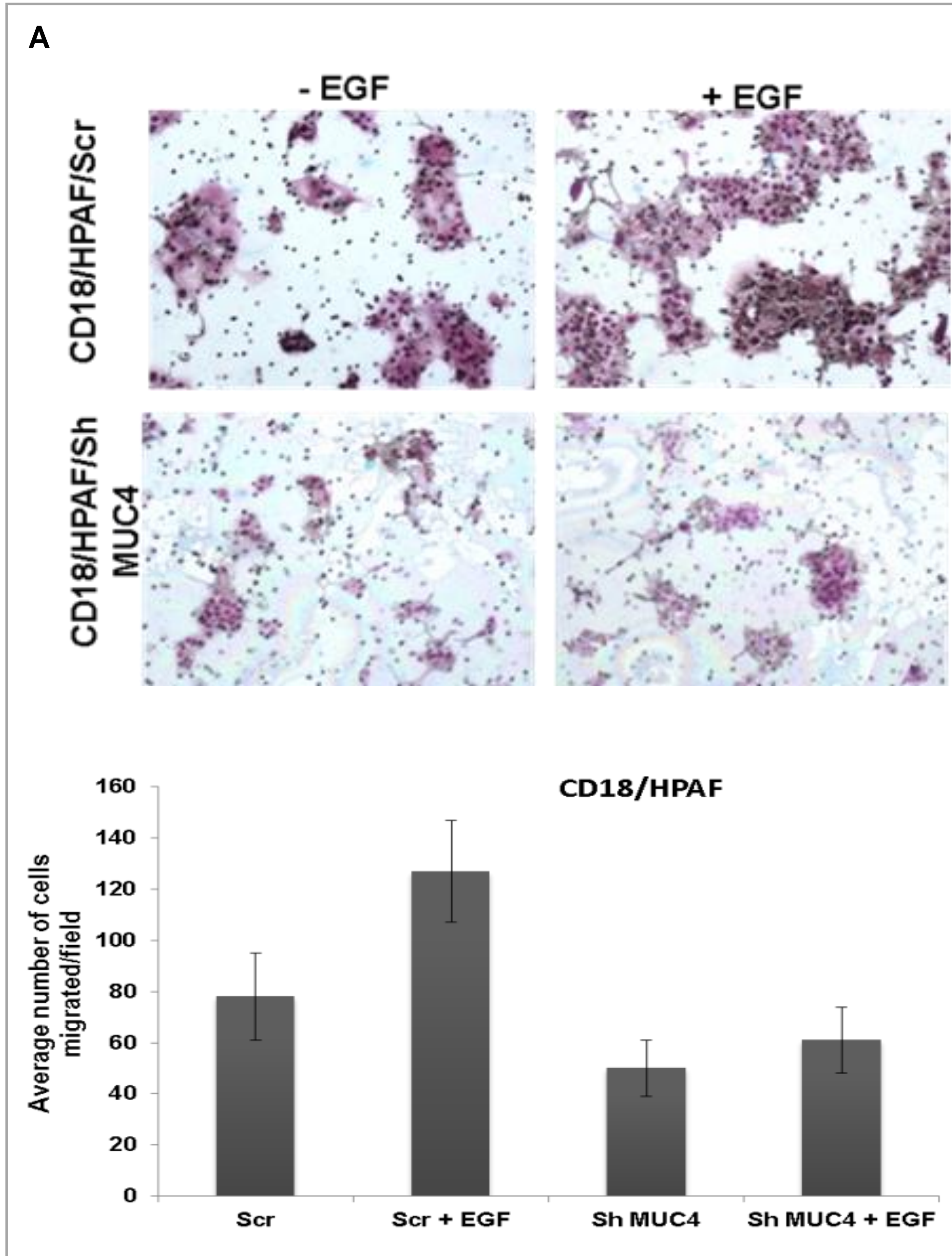


Figure 5.6

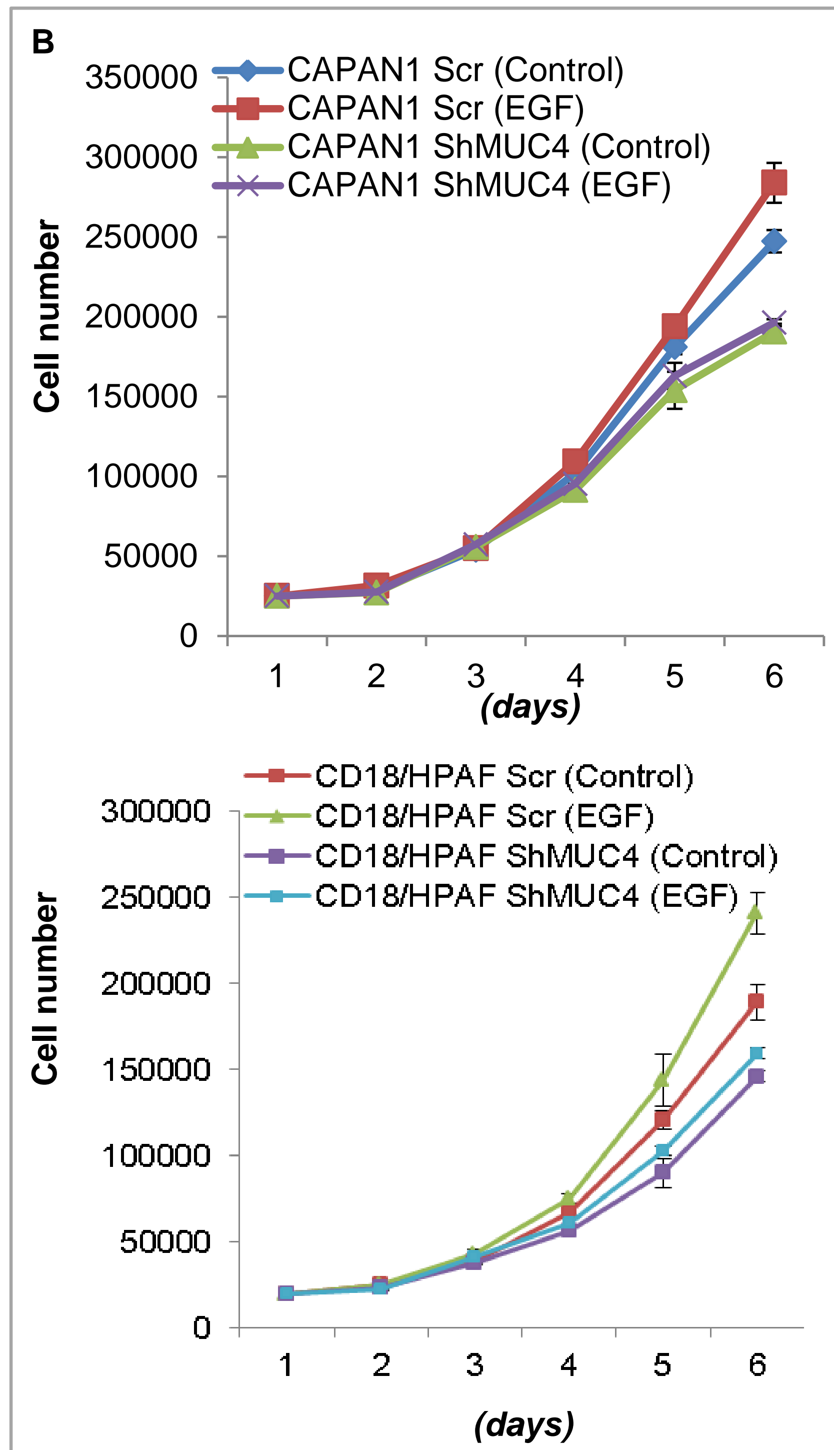
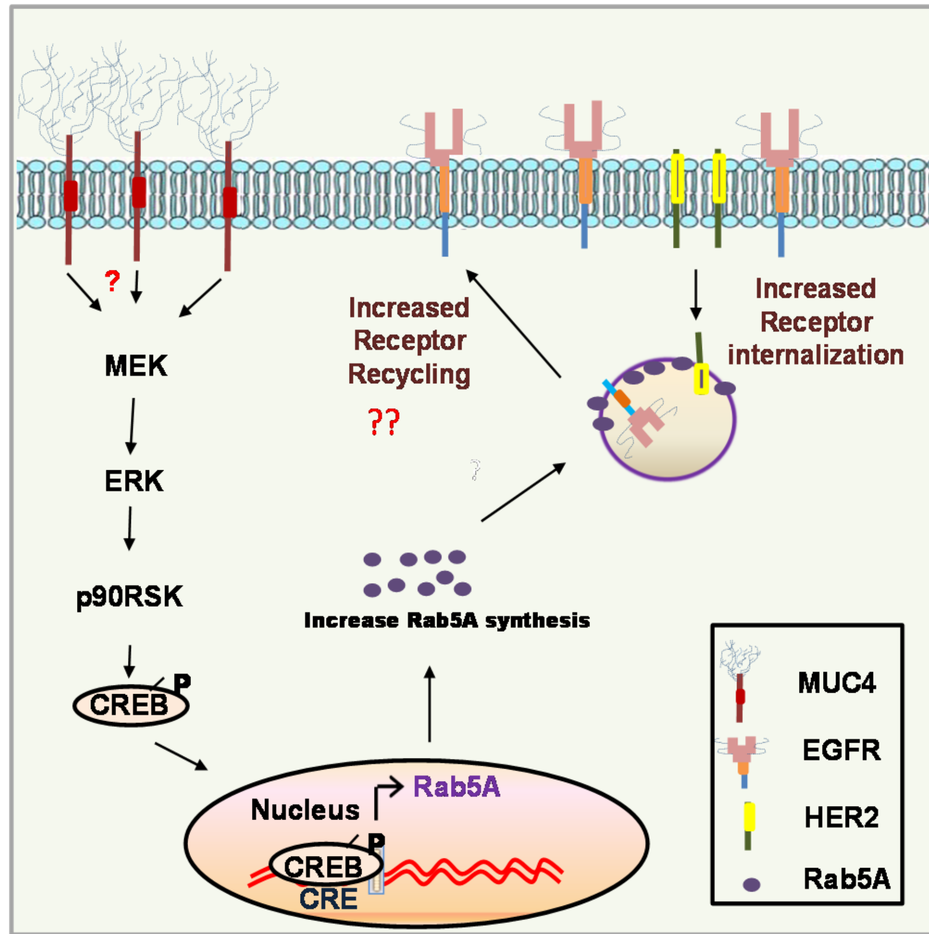


Figure 5.7 Schematic diagrams showing that presence of MUC4 determines the fate of EGFR. In the presence of MUC4, through the activation of MAPK pathway, CREB is getting phosphorylated to p-CREB, which allows it to enter to the nucleus. Inside the nucleus, p-CREB binds to the CRE element present on RAB5A promoter, which increases the transcription of RAB5A gene. It is followed by increased synthesis of RAB5A. Increased RAB5A expression will then leads to increased internalization of EGFR receptor. However, internalized EGFR will be redirected to the recycling pathway to the plasma membrane, rather than lysosomal degradation pathway. This preference of EGFR for recycling route in the presence of MUC4 could be attributed to induce TGF-α expression. This is one of the mechanism by which aberrant expression or overexpression of MUC4 helps cancer cells to proliferate in an unrestricted manner.

Figure 5.7



V. 5 Reference List

[1] Ghiorzo P. Genetic predisposition to pancreatic cancer. World J Gastroenterol 2014;20:10778-89.

[2] 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.

[3] 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.

[4] 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.

[5] Bafna S, Kaur S, Batra SK. Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. Oncogene 2010;29:2893-904.

[6] 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.

[7] Moniaux N, Chaturvedi P, Varshney GC, et al. Human MUC4 mucin induces ultra-structural changes and tumorigenicity in pancreatic cancer cells. Br J Cancer 2007;97:345-57.

[8] 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.

[9] Ponnusamy MP, Singh AP, Jain M, Chakraborty S, Moniaux N, Batra SK. MUC4 activates HER2 signalling and enhances the motility of human ovarian cancer cells. Br J Cancer 2008;99:520-6.

[10] Ioannou N, Seddon AM, Dalgleish A, Mackintosh D, Modjtahedi H. Expression pattern and targeting of HER family members and IGF-IR in pancreatic cancer. Front Biosci (Landmark Ed) 2012;17:2698-724.

[11] 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.

[12] Ardito CM, Gruner BM, Takeuchi KK, et al. EGF receptor is required for KRAS-induced pancreatic tumorigenesis. Cancer Cell 2012;22:304-17.

[13] Stenmark H. Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 2009;10:513-25.

[14] Hutagalung AH, Novick PJ. Role of Rab GTPases in membrane traffic and cell physiology. Physiol Rev 2011;91:119-49.

[15] Liu SS, Chen XM, Zheng HX, Shi SL, Li Y. Knockdown of Rab5a expression decreases cancer cell motility and invasion through integrin-mediated signaling pathway. J Biomed Sci 2011;18:58.

[16] Li Y, Meng X, Feng H, Zhang G, Liu C, Li P. Over-expression of the RAB5 gene in human lung adenocarcinoma cells with high metastatic potential. Chin Med Sci J 1999;14:96-101.

[17] Geng D, Zhao W, Feng Y, Liu J. Overexpression of Rab5a promotes hepatocellular carcinoma cell proliferation and invasion via FAK signaling pathway. Tumour Biol 2015.

[18] Zhao Z, Liu XF, Wu HC, et al. Rab5a overexpression promoting ovarian cancer cell proliferation may be associated with APPL1-related epidermal growth factor signaling pathway. Cancer Sci 2010;101:1454-62.

[19] Fukui K, Tamura S, Wada A, et al. Expression of Rab5a in hepatocellular carcinoma: Possible involvement in epidermal growth factor signaling. Hepatol Res 2007;37:957-65.

[20] Chen PI, Kong C, Su X, Stahl PD. Rab5 isoforms differentially regulate the trafficking and degradation of epidermal growth factor receptors. J Biol Chem 2009;284:30328-38.

[21] Plowman GD, Whitney GS, Neubauer MG, et al. Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proc Natl Acad Sci U S A 1990;87:4905-9.

[22] Roepstorff K, Grandal MV, Henriksen L, et al. Differential effects of EGFR ligands on endocytic sorting of the receptor. Traffic 2009;10:1115-27.

[23] Sigismund S, Argenzio E, Tosoni D, Cavallaro E, Polo S, Di Fiore PP. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev Cell 2008;15:209-19.

[24] Bitler BG, Goverdhan A, Schroeder JA. MUC1 regulates nuclear localization and function of the epidermal growth factor receptor. J Cell Sci 2010;123:1716-23.

[25] Croizet-Berger K, Daumerie C, Couvreur M, Courtoy PJ, van den Hove MF. The endocytic catalysts, Rab5a and Rab7, are tandem regulators of thyroid hormone production. Proc Natl Acad Sci U S A 2002;99:8277-82.

[26] Chang F, Steelman LS, Lee JT, et al. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. Leukemia 2003;17:1263-93.

[27] Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 1996;273:959-63.

[28] Pino V, Ramsauer VP, Salas P, Carothers Carraway CA, Carraway KL. Membrane mucin Muc4 induces density-dependent changes in ERK activation in mammary epithelial and tumor cells: role in reversal of contact inhibition. J Biol Chem 2006;281:29411-20.

[29] Hu F, Deng X, Yang X, et al. Hypoxia upregulates Rab11-family interacting protein 4 through HIF-1alpha to promote the metastasis of hepatocellular carcinoma. Oncogene 2015;34:6007-17.

[30] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.

[31] Lee M, Vasioukhin V. Cell polarity and cancer--cell and tissue polarity as a non-canonical tumor suppressor. J Cell Sci 2008;121:1141-50.

[32] Royer C, Lu X. Epithelial cell polarity: a major gatekeeper against cancer? Cell Death Differ 2011;18:1470-7.

[33] Kaur S, Sharma N, Krishn SR, et al. MUC4-mediated regulation of acute phase protein lipocalin 2 through HER2/AKT/NF-kappaB signaling in pancreatic cancer. Clin Cancer Res 2014;20:688-700.

[34] Li W, Wu C, Yao Y, et al. MUC4 modulates human glioblastoma cell proliferation and invasion by upregulating EGFR expression. Neurosci Lett 2014;566:82-7.

[35] 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.

[36] Lakshmanan I, Seshacharyulu P, Haridas D, et al. Novel HER3/MUC4 oncogenic signaling aggravates the tumorigenic phenotypes of pancreatic cancer cells. Oncotarget 2015.

[37] Dong WW, Mou Q, Chen J, Cui JT, Li WM, Xiao WH. Differential expression of Rab27A/B correlates with clinical outcome in hepatocellular carcinoma. World J Gastroenterol 2012;18:1806-13.

[38] Wang JS, Wang FB, Zhang QG, Shen ZZ, Shao ZM. Enhanced expression of Rab27A gene by breast cancer cells promoting invasiveness and the metastasis potential by secretion of insulin-like growth factor-II. Mol Cancer Res 2008;6:372-82.

[39] Shi C, Yang X, Ni Y, et al. High Rab27A expression indicates favorable prognosis in CRC. Diagn Pathol 2015;10:68.

[40] Jonckheere N, Skrypek N, Merlin J, et al. The mucin MUC4 and its membrane partner ErbB2 regulate biological properties of human CAPAN-2 pancreatic cancer cells via different signalling pathways. PLoS One 2012;7:e32232.

[41] Tall GG, Barbieri MA, Stahl PD, Horazdovsky BF. Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. Dev Cell 2001;1:73-82.

[42] Woller B, Luiskandl S, Popovic M, et al. Rin-like, a novel regulator of endocytosis, acts as guanine nucleotide exchange factor for Rab5a and Rab22. Biochim Biophys Acta 2011;1813:1198-210.

[43] Kumar S, Torres MP, Kaur S, et al. Smoking accelerates pancreatic cancer progression by promoting differentiation of MDSCs and inducing HB-EGF expression in macrophages. Oncogene 2015;34:2052-60.

[44] Nagy P, Friedlander E, Tanner M, et al. Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. Cancer Res 2005;65:473-82.

CHAPTER VI

Identification of MUC4 in Pancreatic stellate cells (PSCs) and putative MUC4 alternate promoter

VI.1 Synopsis

Pancreatic ductal adenocarcinoma (PDAC) is an extremely malignant disease with an equally poor prognosis. Till date, no significant improvement has been made to improve clinical outcomes of the PDAC patients, primarily due to the limited number of patients eligible for surgical resections and the frequently occurring problem of radiation and chemotherapy resistance of these tumors. Due to consistent failure of both conventional and novel therapies, researchers are forced to re-scrutinize the involvement of tumor environment in PDAC. The pancreatic tumor microenvironment is comprised of abundant amount of stroma containing many cells types, but the majority of them are pancreatic stellate cells (PaSC). It is well established that PaSC are responsible for enormous desmoplastic reactions which is apparent in almost 90% of the PC patients. These desmoplastic reaction leads to fibrosis which is observed in two major diseases of the pancreas—chronic pancreatitis and pancreatic cancer. Considering the importance of PaSC in the pathobiology of PC disease, there has been an exponential upsurge in research in this field in last few years, with numerous research groups channelizing their energies to elucidate the biology and function of these cells. However, the major problem that we are currently facing is the lack of consensus among PaSC markers. Still, alpha smooth muscle actin (a-SMA) is widely used to detect activate stellate cells in PC.

While studying the role of different cell types on MUC4 expression, I came to this very intriguing observation that PaSC does have MUC4 mucin expression, whereas other transmembrane mucins such as MUC1 and MUC16 were completely absent. This is the first report which has shown MUC4 expression in cells of non-epithelial origin. Interestingly, inactivation of PaSC by using retinoic acids (RA) leads to drastic reduction in MUC4 expression. Further, confocal microcopy has shown significant expression

levels of MUC4 in glial fibrillary acidic protein (GFAP, marker of myofibroblasts) positive PaSC. Encouragingly, PC tissue samples have also shown presence of MUC4 in few α-SMA-positive cells, a marker of activated PaSC. Moreover, MUC4 knocked down (kd) in PSC, using two targeted ShRNA constructs, has shown significant reduction ($p>0.05$) in a-SMA expression. We also evidenced decline in the proliferative and migratory properties of MUC4 kd PaSC, as compared to the Scr control cells, emphasizing that MUC4 plays an important role in determining the activation status of PaSC. This study gives us an additional reason to target MUC4, which not only leads to the killing of PC cells, but will also inhibits the activation of PaSC.

VI.2 Introduction

Pancreatic cancer (PC) is the fourth leading cause of cancer mortality in United States with 5-year survival of only 6% (1). Due to lack of diagnostic markers, early metastasis (invasion to local and distant organs), frequently developed chemo-resistance and lack of reliable therapies, PC is a very lethal disease. Moreover, PC has expected to become the second leading cause of cancer-related deaths in next 15 years (2). Among many unique hallmarks, PC is characterized by extreme desmoplastic reactions, which has shown to accelerate PC growth and metastasis. One of the major contributors of pancreatic desmoplasia is pancreas residing fibroblasts cell population, also known as pancreatic stellate cells (PaSC) or myofibroblasts. In normal pancreas, PaSC are quiescent in nature, but inflammation, hypoxia and pancreatic injury leads to their activation, which is accompanied by the absence of lipid droplets, and their increased migratory and proliferative potential (3, 4). Activated PaSC synthesizes and secretes excessive extracellular matrix proteins, which reduces the accessibility of chemotherapy agents to the cancer site. In addition, activated PaSC are also known to contribute in PC metastasis.

Considering the highly important role of PaSC in PC progression, metastasis and chemo-resistance, recent studies have been directed to inhibit their activation (3, 4). Our research group from past two decades has established MUC4, a transmembrane protein, as one of the critical oncogenic protein because it facilitates survival, growth and metastasis of PC (5-7). MUC4 is one of the top differentially expressed proteins in PC (8). Earlier, expression of mucins was thought to be confined to epithelial cells. However, emerging reports have demonstrated presence of mucins on non-epithelial cells. For instance, MUC3 expression has been noticed in synovial lining cells, macrophages and fibroblasts derived from rheumatoid arthritis (RA), osteoarthritic (OA) and normal human

synovial tissues (ST) (9). Similarly, these cell types also exhibited expression for MUC5AC, though at extremely low levels. In addition, MUC1 expression was observed in activated human T-cells (10, 11). However, there is no report so far which has shown the presence of MUC4 in non-epithelial cells. While studying the role of PC tumor microenvironment in MUC4 regulation, we obtained one of the most intriguing findings, which was the presence of MUC4 in PaSC. In addition to its expression, we have also demonstrated the role of MUC4 protein on the activation status and functional properties of PaSC.

VI.3 Results

A. Presence of MUC4 mucin on activated cancer associated fibroblast cells

Normal and healthy pancreas contains less number of fibroblast cells, and they are generally inactive until or unless there is any stimulatory signal present. When cultured in the presence of tumor (KCT961)-derived conditioned media, inactivated fibroblasts cells (ImPaSC, obtained from WT mice) become activated, marked by significant increase in the expression of both α-SMA and Ki67 staining (F**ig. 6.1A**). Real-time PCR experiment led us to know significant increase in MUC4 expression by ~20-fold in activated ImPaSC, compared to inactivated ImPaSC (**Fig. 6.1B)**. To further establish these findings, we used another PaSC cell lines (immortalized with E6/E7 antigen) derived from human PC patients. Similar to CD18/HPAF PC cells, human PaSC cells, which are already activated, showed positive expression for MUC4 promoter, though at significantly less molecular weight (**Fig. 6.1C)**. However, other transmembrane mucins such as MUC1 and MUC16, were absent in PaSC cells (**Fig. 6.1C)**. We further verified the presence of MUC4 in PaSC using different anti-MUC4 antibody (2175) (**Fig. 6.1D**), however, difference in its molecular weight in PaSC and CD18/HPAF cells was relatively less than our previous observation (**Fig. 6.1C**). Immunofluorescence staining showed

significant colocalization between MUC4 and GFAP (marker of PaSC) which further supported our findings (**Fig. 6.1E**). Confocal microscopy performed for MUC4 and α-SMA in PC tissue array further validated our *in vitro* findings (**Fig. 6.1F**). We observed that small fractions of PaSC cells were positive for MUC4 expression in PC tissue spots. IHC performed for MUC4 in human PC tissues and KPC mice model also demonstrated the presence of MUC4 in cells other than epithelial cells, with morphology similar to myofibroblast cells (**Fig. 6.1G-H**).

B. Presence of MUC4 determines the activation status of PaSC

Due to observed MUC4 expression in activated PaSC, we were prompted to hypothesize that MUC4 expression is required for the functionality and activation of PaSC. To address our hypothesis, we made stable MUC4 kd PaSC using two different MUC4 targeting constructs. As depicted in **Fig. 6.2A**, MUC4 was significantly downregulated in PaSC cells at protein levels, which was further confirmed with the help of IF and qRT-PCR techniques (**Fig. 6.2B-C**). In MUC4 kd PaSC cells, we observed significant decrease in α-SMA expression, which is a marker of activated cancer associated fibroblasts or PaSC, at both mRNA and protein levels (**Fig. 6.2D-E**), suggesting that MUC4 does involve in the maintenance of the activation status of PaSC. Alternatively, upon inducing quiescence in PaSC by using RA (12, 13), significant decline in MUC4 expression was noticed in RA-treated PaSC at both protein (**Fig. 6.2F**) and mRNA level (**Fig. 6.2G**). Altogether, our results demonstrate that MUC4 expression and activation of PaSC cell are directly associated with each other.

C. Suppression of MUC4 reduces the proliferation and migration of PaSC

It is well-established in the literature that activation of PaSC is accompanied by increase in their migration and proliferation (3). Therefore, analysis of the migratory and proliferative potential upon MUC4 silencing would be another way to prove that MUC4

plays important role in the activation status of PaSC cells. Similar to PC cell lines, suppression of MUC4 expression led to significant decrease in cell proliferation ($p<0.05$), particularly on day 5 and 6, compared to scr control cells (**Fig. 6.3A**). Further, we observed decrease in number of colonies in our anchorage dependent assay upon MUC4 kd PaSC cells compared to control (**Fig. 6.3B**). At molecular level, we observed decrease in the EGFR and cyclin D1 protein expression, without any noticeable effect on Akt and ERK activation (**Fig. 6.3C**). To relate MUC4 kd with the migration of PaSC, we did Boyden chamber motility assay and observed that kd of MUC4 led to significant reduction in migration of PaSC cells to the lower chamber compared to scr control cells (**Fig. 6.3D**). We observed reduction in the expression and arrangement of the tubulin proteins in MUC4 kd PaSC than scr cells (**Fig. 6.3E**). Further, significant decrease in the expression of vimentin protein in MUC4 kd cells than control cells, validate the role of MUC4 in the motility of PaSC, however, N-cadherin expression did not change with MUC4 silencing (**Fig. 6.3F**). Similar to their protein data, we observed decline in the mRNA expression of twist, vimentin, cyclin d1 and EGFR in MUC4 kd PaSC compared to control cells (**Fig. 6.3G**).

D. Identification of novel MUC4 promoter

From earlier results, it is quite apparent that molecular weight of MUC4 is less in PaSC cells compared to PC cells; which led us to postulate that the observed difference is possibly due to exon deletion, which is quite common among mucins. Screening of MUC4 cDNA from exon-1 to exon-26 using RT-PCR revealed that MUC4 in PaSC cells does not utilize classical promoters (CP) and has exon-1 deletion (**Fig. 6.4A**). Using promoter prediction V2 software, two highly likely predicted promoter sites on intron-1 (primarily at the end) were recognized, which we have referred as alternate promoter or AP (**Fig. 6.4B**). Certainly, we did observe amplified expected PCR product in PaSC

using forward primer against intron-1 and reverse primer against exon-2, and sequencing was done to confirm that the amplified band is MUC4 only. Further, we found that this identified AP is present only in MUC4-expressing PC cells and is completely absent in MUC4 non-expressing PC cell lines, excluding the possibility of DNA contamination in utilized cDNAs obtained from these PC cell lines (**Fig. 6.4C**). These results were further verified using quantitative RT-PCR. We observed that AP is maximally expressed by HPAC and Panc10.05 cell lines, whereas CP expression is maximal in CAPAN1, Panc10.05, Colo357 and QGP-1 cell lines (**Fig. 6.4D**). To validate the presence of AP in clinical samples, we did RT-PCR in cDNA obtained from PC tissues. We observed the presence of MUC4 at transcriptional level in tumor adjacent and tumor tissues, whereas AP was found to be utilized by tumor tissues only, associating the presence of AP with the aggressiveness of pancreatic tumors (data not shown). Using *in silico* analysis, a putative AUG translation site in-frame codon was detected at the beginning of exon 2 of MUC4 gene. It suggested that the protein which is being synthesized by utilizing MUC4 AP will have most likely all the MUC4 domains present, except leader sequence due to exon-1 deletion.

E. Differential localization of MUC4 in PaSC and PC cell lines

As mentioned earlier that MUC4 in PaSC has an exon-1 deletion, which led us to assume that MUC4 localized differentially in these two different cell types. For that, we utilized confocal microscopy approach. As anticipated, we observed that MUC4 in PaSC cells have more surface localization, whereas its localization in early endosomes (signified by EEA-1 staining) and Golgi (marked by giantin staining) was significantly less compared to PC cell line (**Fig. 6.5**). In spite of the absence of leader sequence, MUC4 does enter into endoplasmic reticulum (ER), which is possible and has been observed for other proteins that do not possess leader sequence (**Fig. 6.5**). We observed that

MUC4 enters to lysosomes in both PaSC and CD18/HPAF PC cells. Altogether, these results suggest that MUC4 localization is different between PaSC and PC cell lines.

VI.4 Discussion

In this study, we have analyzed in detail the expression and functions of MUC4 in activated PaSC cells. The current study is the first evidence where presence of MUC4 has been observed in cell type other than epithelial cells. Upon exploring its function, we realized that MUC4 increase the activation status of PaSC under both *in vitro* and *in vivo* settings. Metastasis is one of the main reasons of PC related deaths, in which activated PaSC has shown to play important role. By orthotopic transplantation of a suspension of human PC cells (MiaPaCa-2, AsPC-1, BxPC-3), alone or in combination with primary human PaSCs, directly onto the mouse pancreas, two separate studies by Vonlaufen *et al.* and Hwang *et al.* have shown that the combination of human PaSCs and PC cells has significantly high degree of desmoplasia, increased primary tumor growth and increased regional/distal metastasis compared to PC cells alone (4, 14). Therefore, increased activation of PaSC and the implicated role of MUC4 further strengthened the rationale to target MUC4, which not only leads to the killing of PC cells, but will also inhibits the activation of PSC.

Moreover, we have also seen that this alternate form of MUC4 is present on aggressive PC tissues (n=6), whereas it was completely absent in tumor adjacent tumors (data not shown), suggesting that during the course of evolution, PC cells undergo many changes in which utilization of MUC4 AP is one of the them. Although in this study, we have highlighted the expression and functions of MUC4, it is still not clear when and how AP of MUC4 is being regulated and utilized by PC cells, which we would like to delineate in future.

Importantly, we have identified the usage of AP in MUC4-expressing PC cell lines and human PC tissues using RT-PCR approach. The AP of MUC4 was identified on intron-1. The concept that intronic sequences can also act as alternate promoters is not new. For instance, in a study by Scohy *et al.*, authors have demonstrated the presence of a novel, tissue-specific α-fetoprotein mRNA isoform in the yolk sac and fetal liver, with a transcription site present on the first intron of the α-fetoprotein gene (15). This isoform is synthesized by the usage of an AP located ∼100 bp downstream of the enhancer element (15). However, the direct evidence validating the presence of MUC4 AP is still lacking and needs to be established. Currently, these results are being validated using 5'Rapid Amplification cDNA ends (RACE) experiment. With the help of *in silico* analysis, we predicted that MUC4 isoform driven by alternate promoter is using the translation site present on exon-2. Further studies also prompted us to assume that identified isoform of MUC4 has altered localization in PaSC *vs.* PC cell lines due to the absence of exon-1 which encodes for MUC4 leader sequence. Along with aberrant expression, accumulating evidence has associated altered subcellular localization of mucins with the poor prognosis and survival of cancer patients (5). Loss of leader sequence encoding exon-1 in MUC4 due to alternate promoter usage will definitely impact MUC4 subcellular localization. This study has paved the way to initiate investigations which will provide us in-depth understanding of MUC4 both at the cellular and molecular levels.

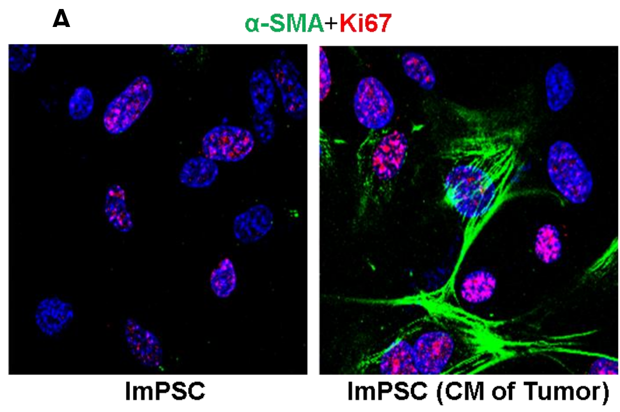
Taken together, this study for the first time has shown the expression of MUC4 in non-epithelial cells. Further functional studies established that presence of MUC4 does alter the migratory and proliferative potential of PaSC cells and is directly related with the activation status of PaSC. Further, studies with more number of activated pancreatic

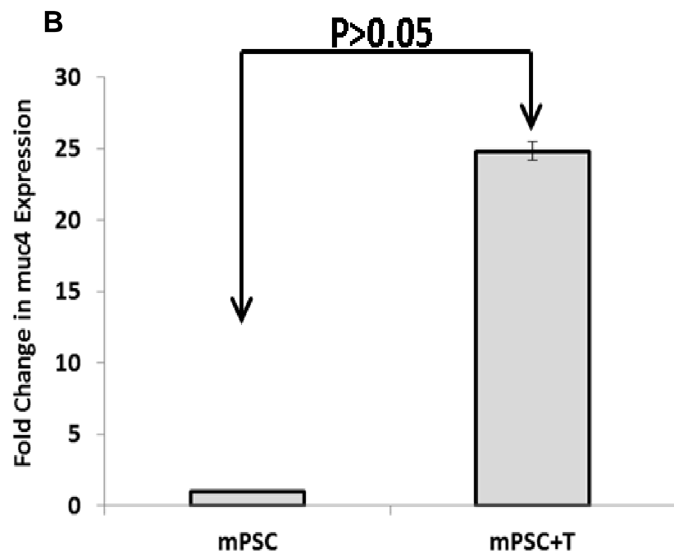
cancer fibroblast or stellate cells will strengthen our observations and is a part of our future investigations.

Figure and Figure legends

Figure 6.1. Identification of MUC4 expression in activated pancreatic stellate cells. **A.** IF experiment revealed that ImPaSC cells obtained from WT mice become activated when cultured in tumor-derived (KCT961) conditioned media, as marked by induced expression of α-SMA and ki-67, compared to untreated control cells. **B**. The bar graph is showing Muc4 expression at mRNA level in mice PaSC co-cultured with or without tumor cells (KCT961). **C.** The transmembrane mucins; MUC1, MUC4 and MUC16 were profiled in CD18/HPAF and PaSC cells by immunoblotting, which did reveal the presence of MUC4, whereas other two membranous mucins were absent. **D.** Immunoblotting by using another antibody against MUC4 (2175) is confirming the presence of MUC4 in PaSC and CD18/HPAF cells. **E.** IF analysis revealed noticeable co-localization between MUC4 and PaSC cells. GFAP is a marker of stellate/fibroblast cells, whereas 8G7 and 2175 antibodies were used to detect MUC4 expression. **F.** Confocal microscopy was utilized to detect MUC4 expression in activated stellate cells (marked by positive α-SMA staining) in pancreatic tissues obtained from RAPID autopsy program. **G.** IHC images obtained from stained human PC tissues are showing the presence of MUC4 in cells other than ductal epithelial cells. **H.** In 25-wk age of KPC mice, when PC is fully developed, MUC4 was stained using IHC protocol. Correspondingly, Muc4 was observed in non-ductal cells, which share a significant similarity in the morphology with myofibroblast cells.

Figure 6.1





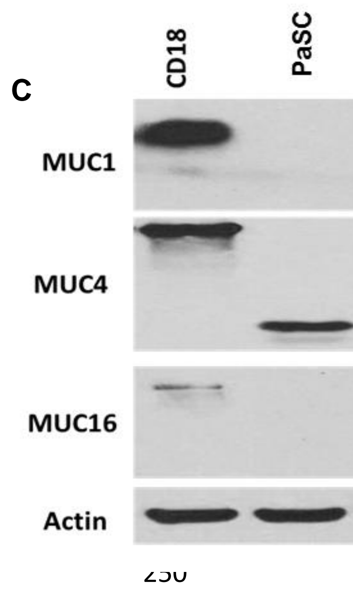
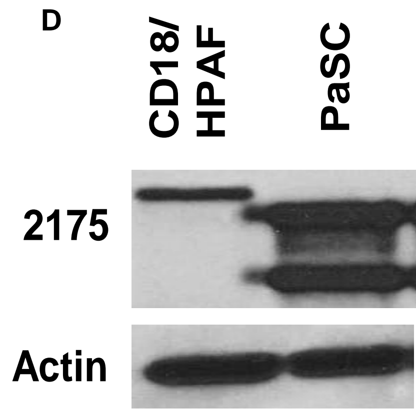


Figure 6.1



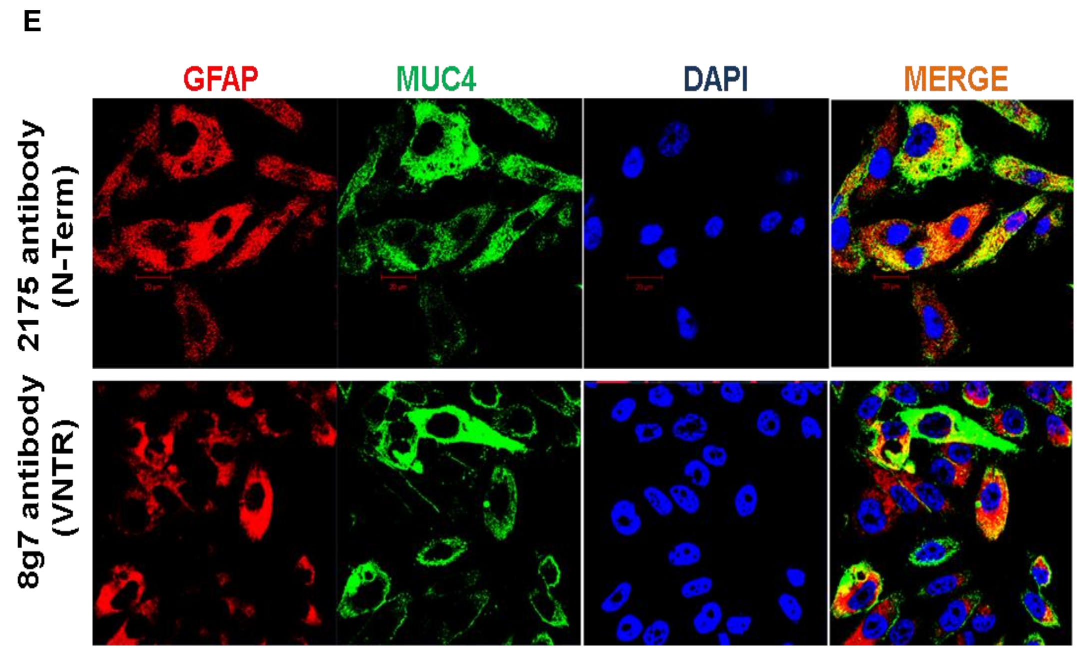
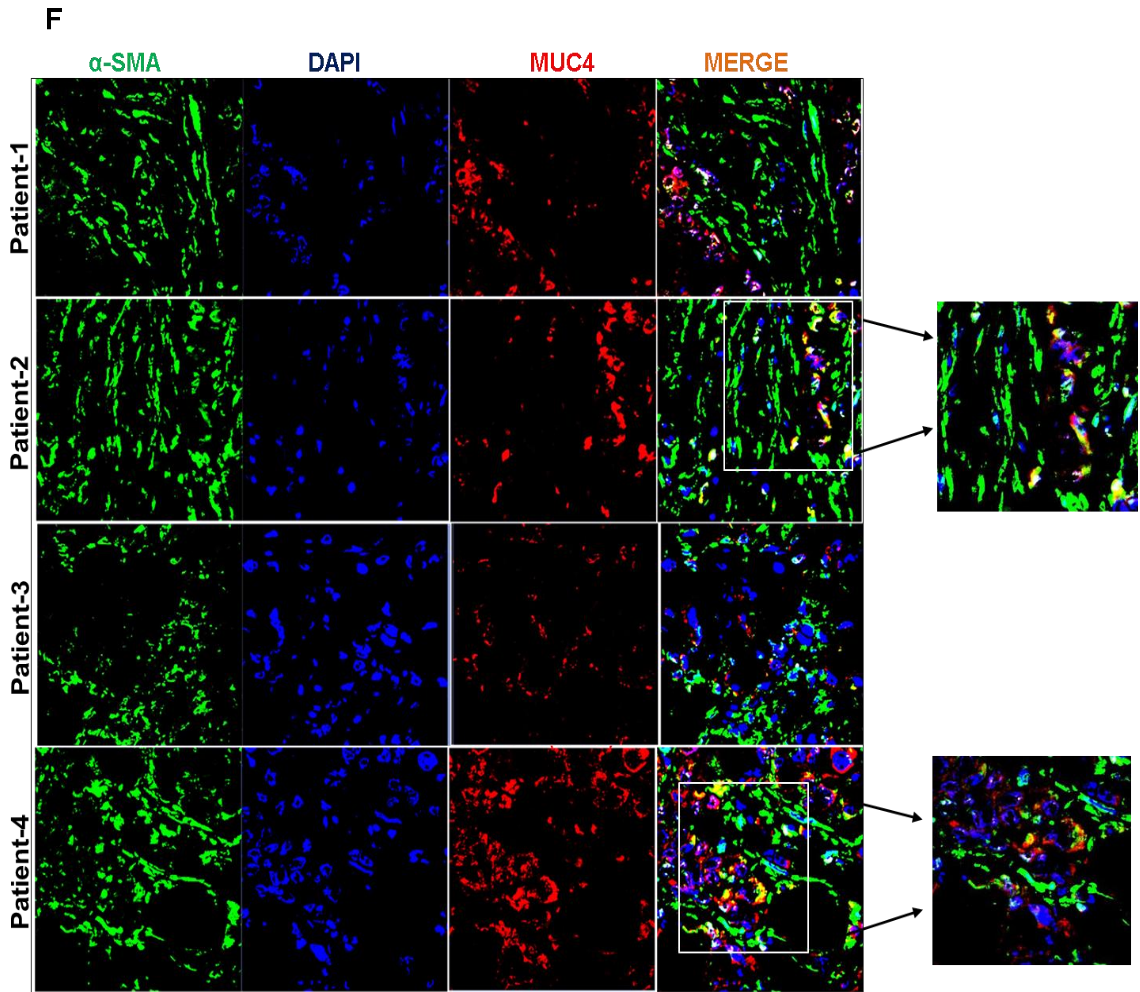
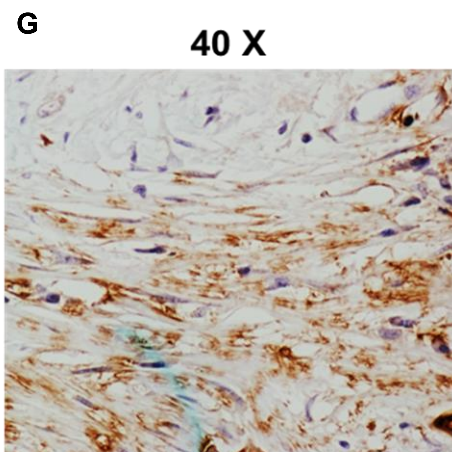


Figure 6.1

F



G



H

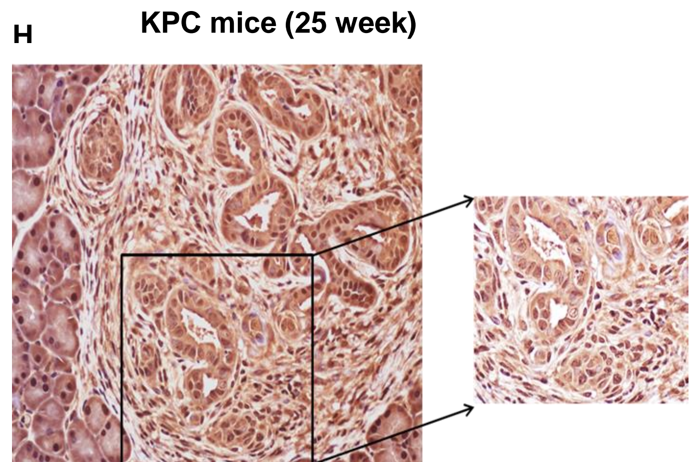


Figure 6.2. MUC4 kd has significant impact on the activation status of PaSC. A. Stable kd for MUC4 was performed in PaSC cells and immunoblot technique was used to confirm MUC4 silencing in PaSC. **B.** Images obtained from IF experiment further confirmed significant kd of MUC4 protein expression in PaSC. **C.** The graph is representing the data obtained from qRT-PCR and showing fold change in MUC4 expression in scr and MUC4 kd PaSC cells. **D**. Scr and MUC4 kd PaSC were cultured alone or in the presence of CD18/HPAF derived conditioned media (tumor conditioned media or TCM). Here, bar graph is showing the effect of MUC4 silencing on the expression of α-SMA at transcriptional level. Supportively, MUC4 kd led to significant decline in α-SMA mRNA expression in both TCM treated and untreated PaSC cells, compared to respective scr control cells. **E.** IB showing the effect of MUC4 silencing on the protein expression for α-SMA and GFAP in PaSC cells. **F.** To induce quiescence, PaSC were treated with RA (500 nM) which led to significant reduction in MUC4 expression as shown by immunofluorescence staining. **G.** Inhibition of MUC4 expression was further noticed at transcriptional level in RA treated and untreated PaSC cells.

Figure 6.2

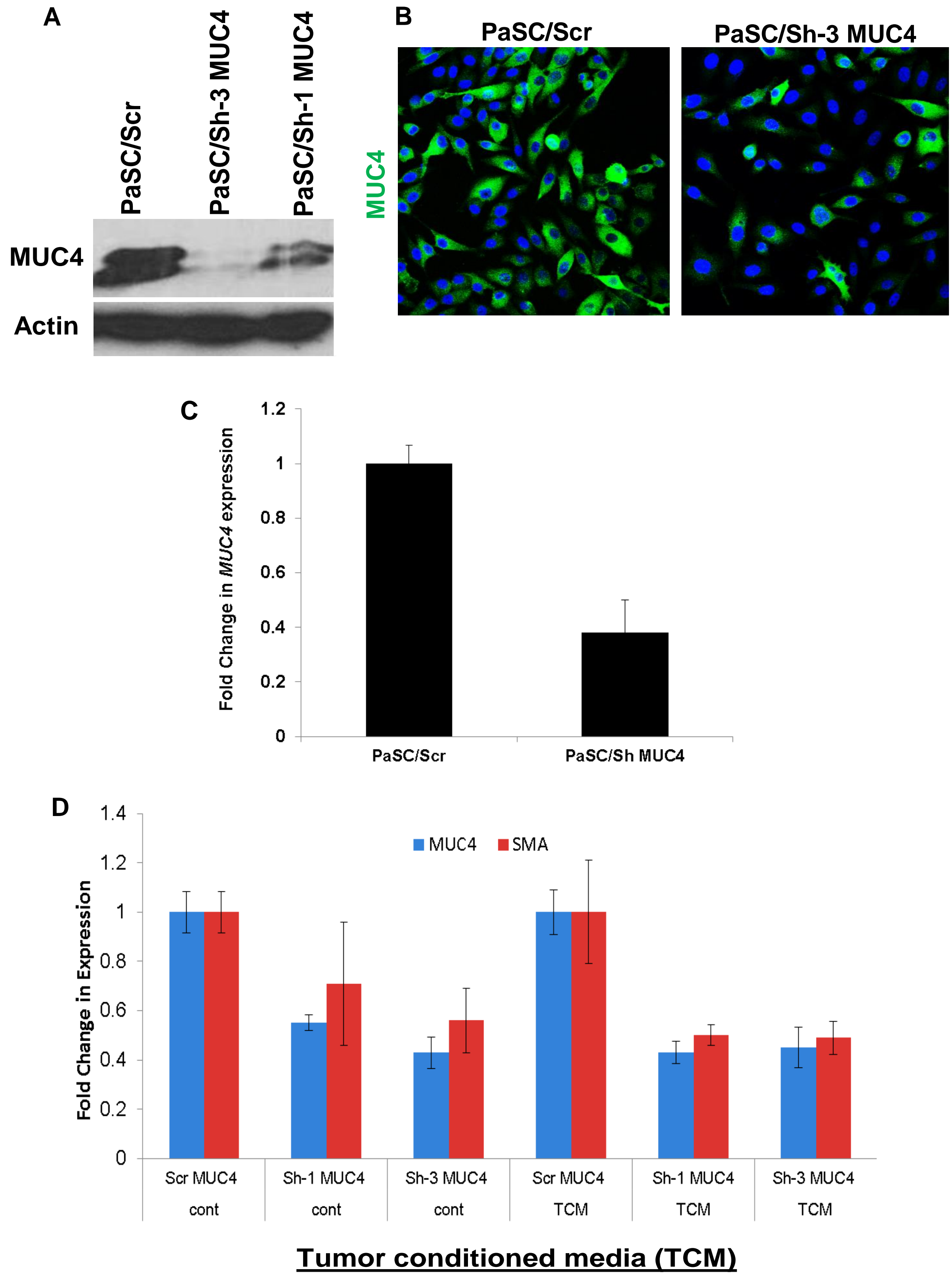
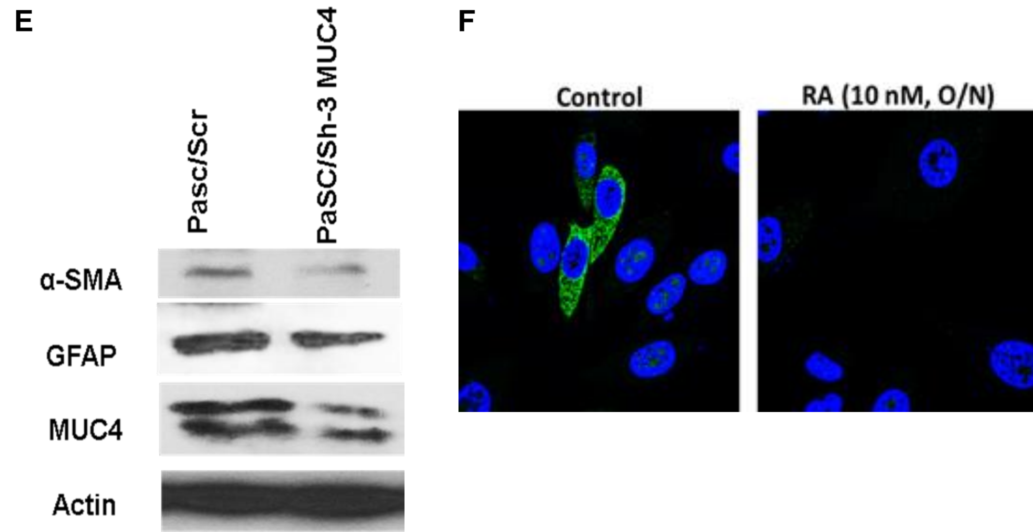


Figure 6.2



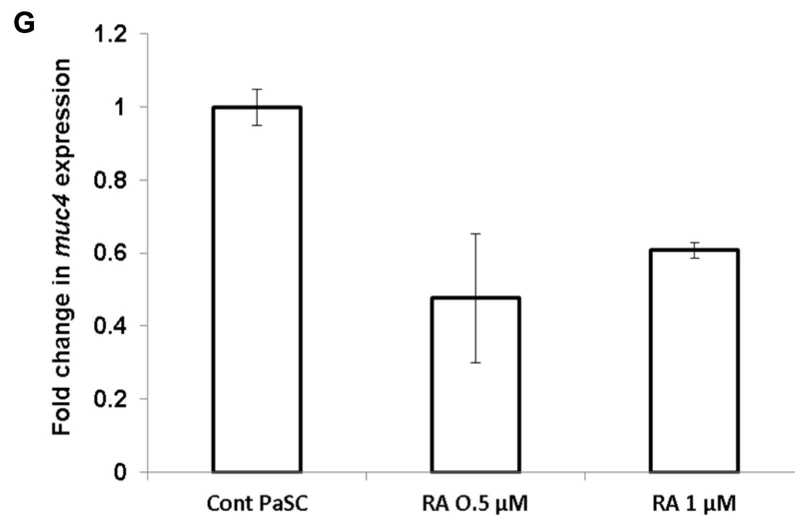


Figure 6.3. MUC4 kd PaSC exhibits negative effects on their proliferative and migratory potential. **A.** Growth kinetic experiment was performed for indicated time-points in MUC4 scr and kd PaSC cells, which showed significant reduced cell number at day 6 in both MUC4 kd PaSC than scr cells. **B.** The bar graph is showing the results obtained from anchorage dependent assay which was performed on MUC4 kd and scr PaSC cells. **C.** IB results showing the effect of MUC4 suppression in PaSC on the protein expression of EGFR, pEGFR, Akt, pAkt, ERK, pERK and cyclin D1. Reduced EGFR and cyclinD1 expression in MUC4 kd PaSC than control cells, signifying that aberrant expression of MUC4 does influence the proliferation of PaSC cells. **D**. Images showing the results obtained from Boyden chamber assay, which was performed to analyze the effect of MUC4 kd on the motility of PaSC cells. **E.** IF images showing the impact of MUC4 silencing on the intensity and arrangement of α-Tubulin of PaSC cells. **F.** IB showing the impact of MUC4 kd on the protein expression of N-cadherin and vimentin molecules. **G.** The bar graph is showing the influence of MUC4 suppression on the mRNA expression of twist, vimentin, cyclinD1, EGFR and MUC4.

Figure 6.3

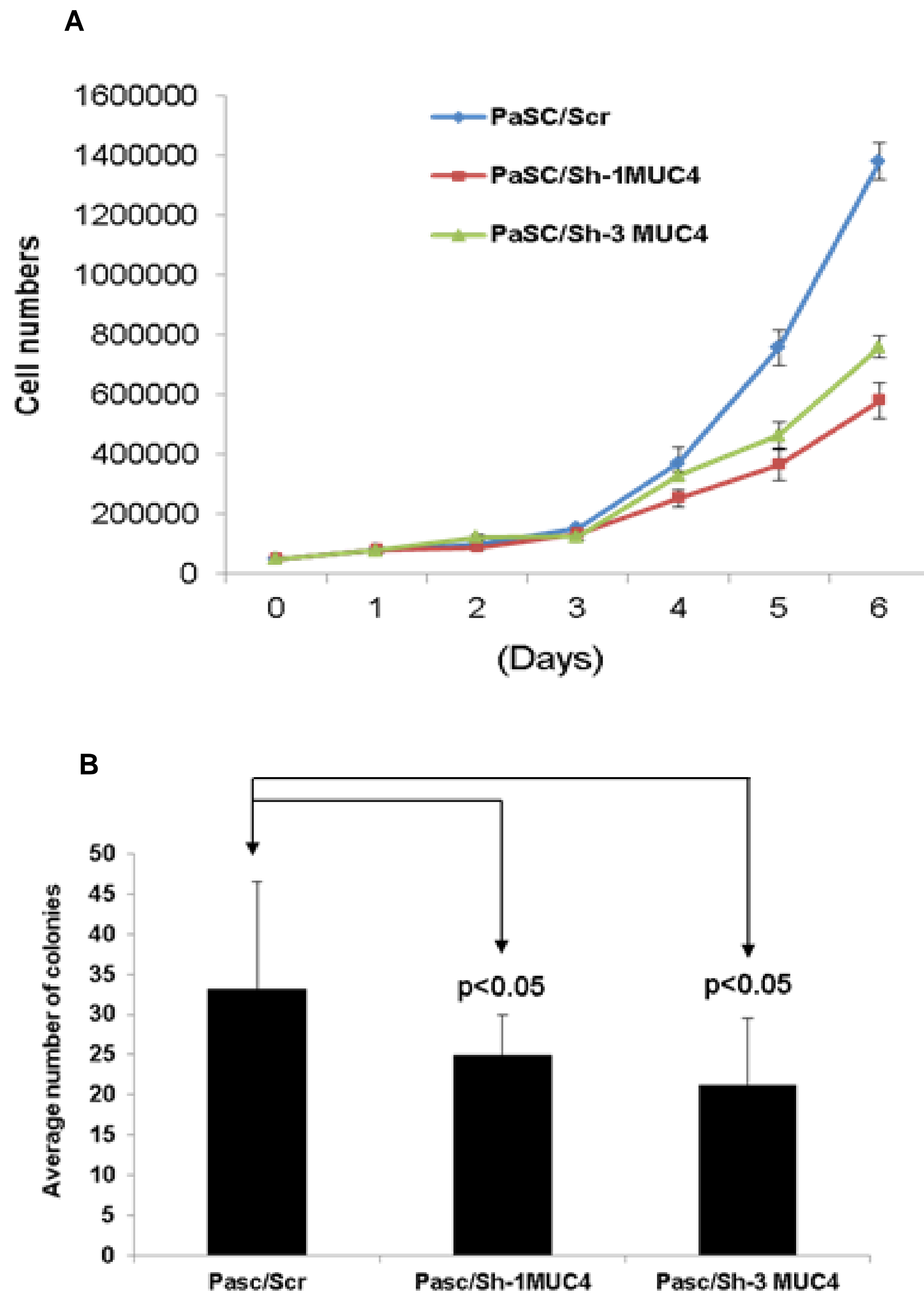
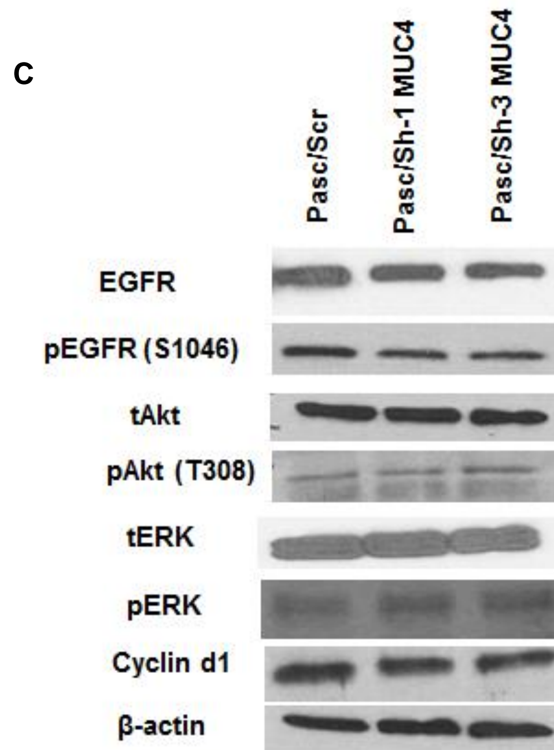


Figure 6.3



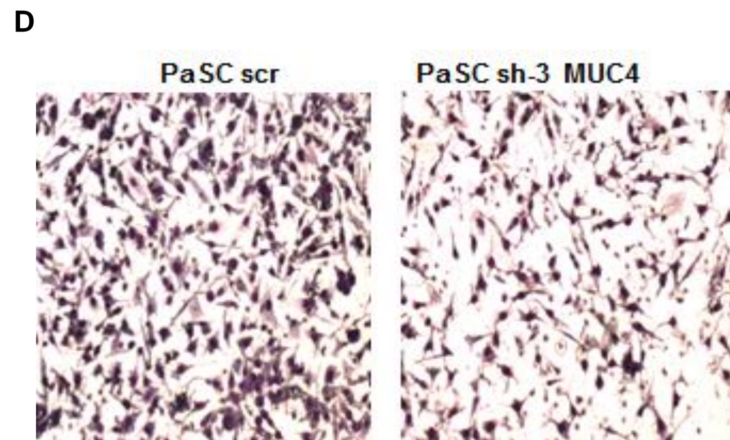


Figure 6.3

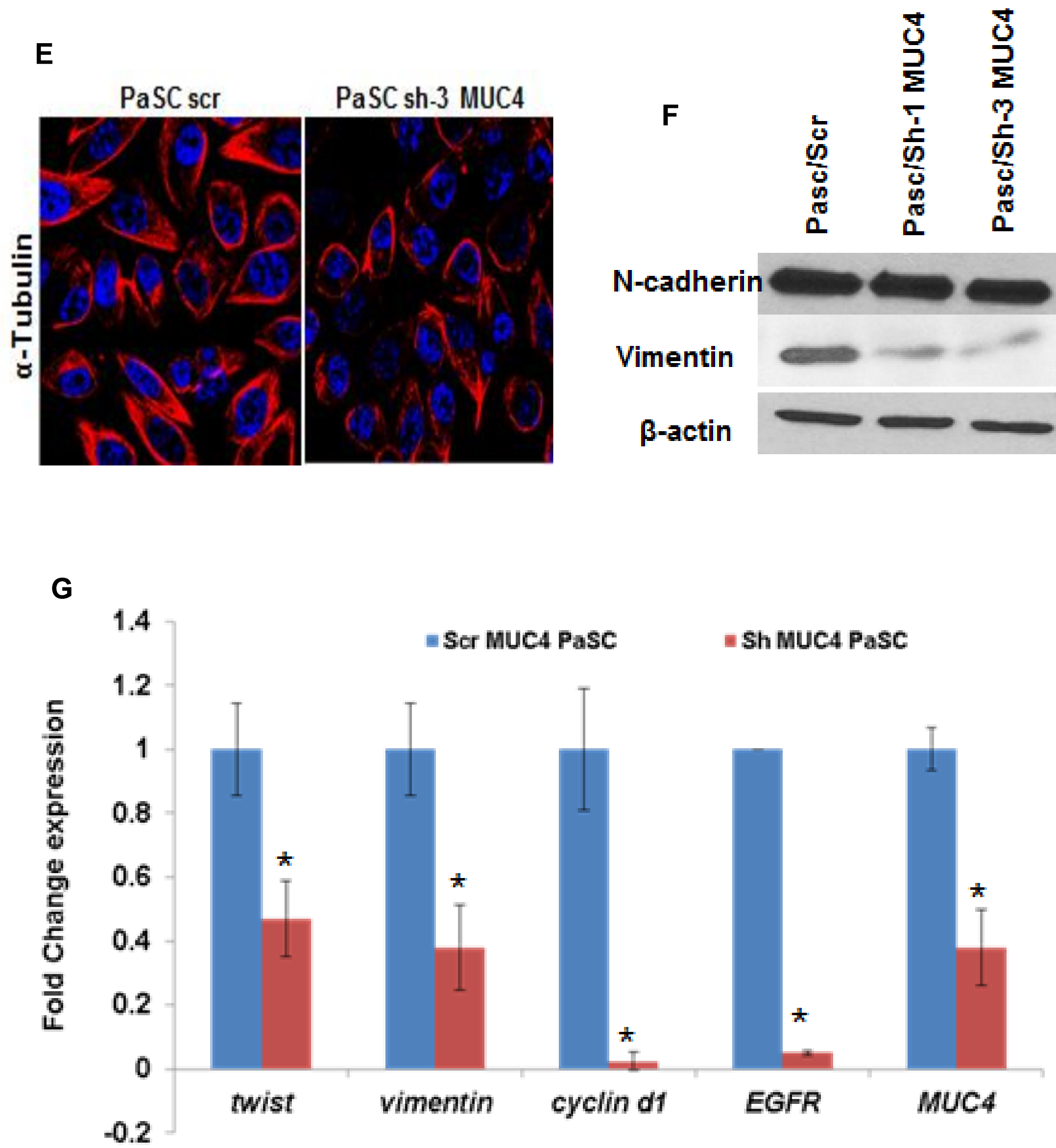


Figure 6.4. Identification of MUC4 promoter in PaSC cells, PC cell lines and PC tissues. A. 2% Agarose gel was ran to detect the PCR product amplified using RT-PCR technique, which was performed to screen PaSC from exon-1 to exon-26 of MUC4 gene. **B.** Promoter V2 software was used to identify the presence of highly likely prediction sites for MUC4 promoter on intron-1. **C.** Agarose gel showing the expression of classical promoter (detected by using 5'UTR+Ex-2) and AP (Intron-1+ex-2) in MUC4 expressing and non-expressing PC cell lines. **D**. Quantitative RT-PCR was done to measure the levels of both ALP and CLP in PC cell lines.

Figure 6.4

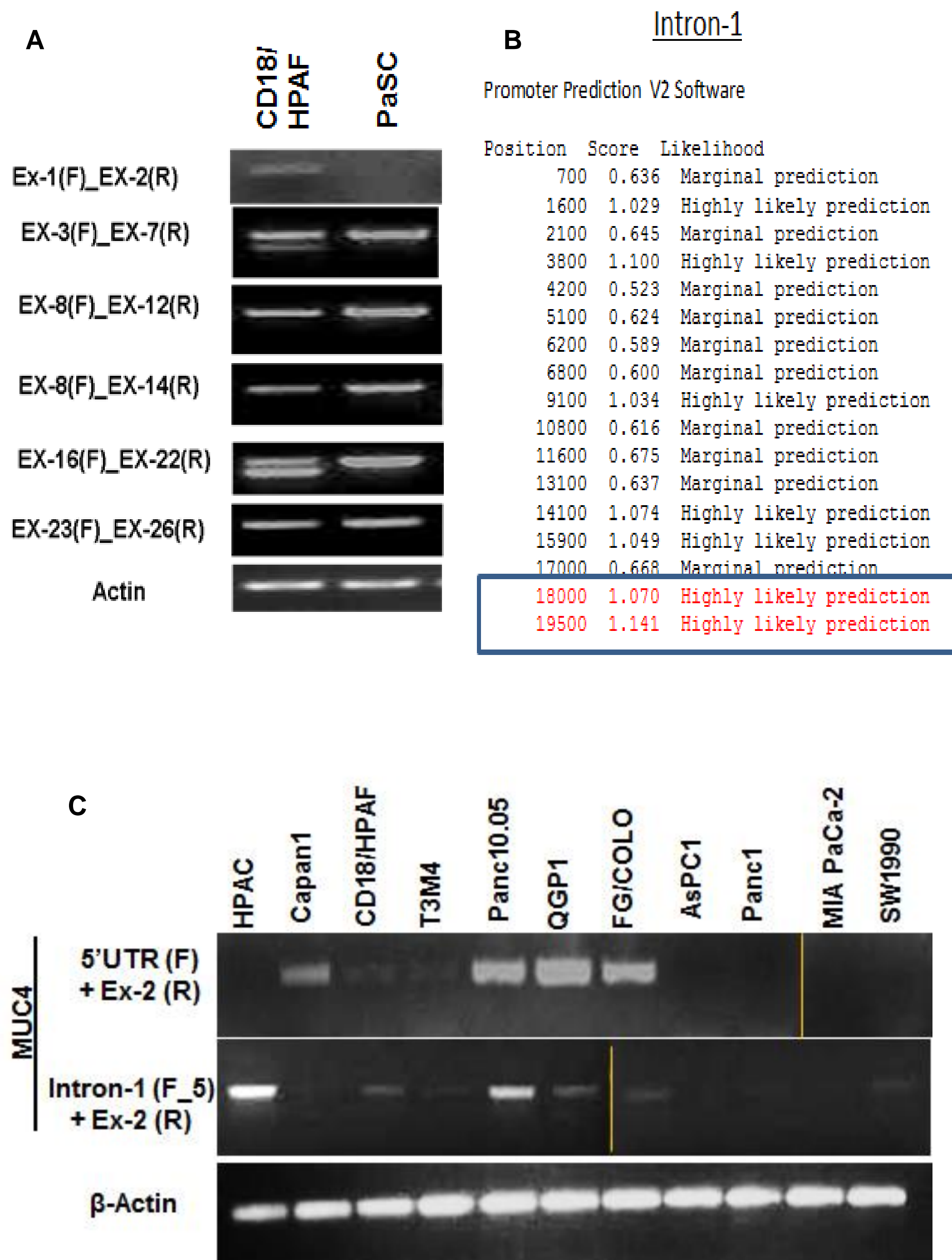


Figure 6.4

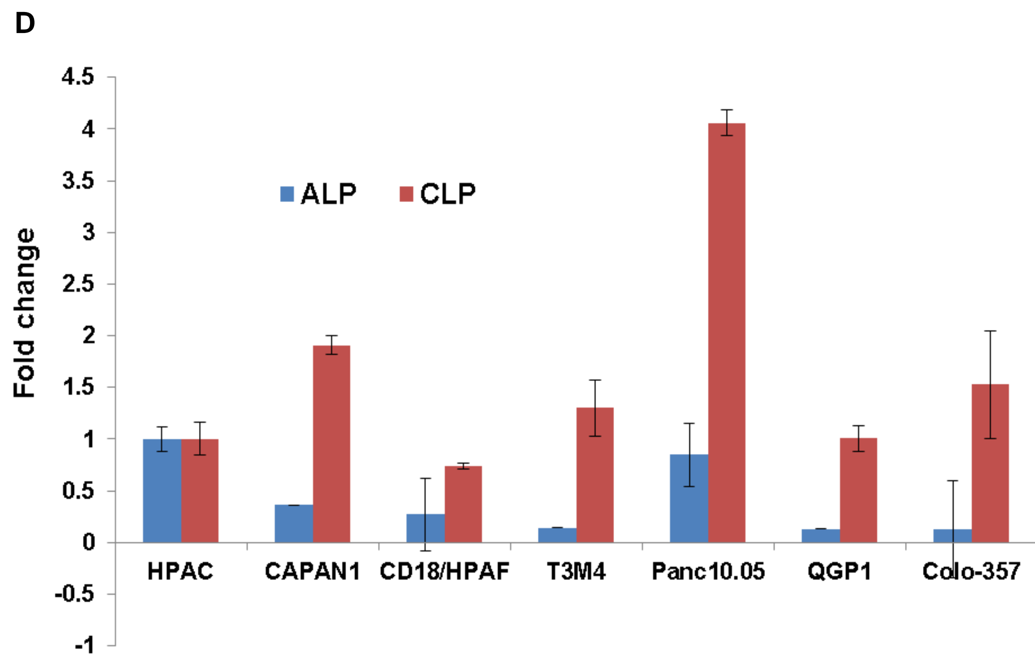
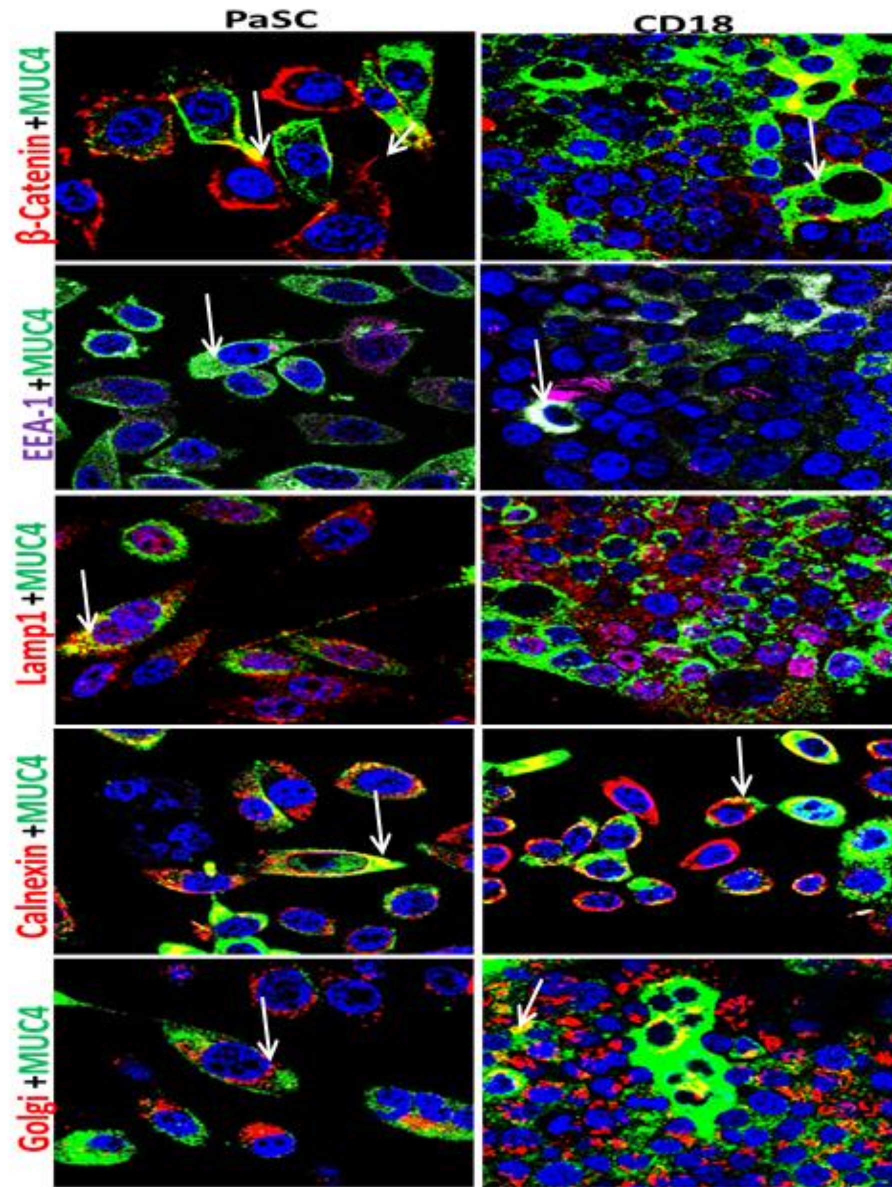


Figure 6.5. Altered MUC4 localization in PaSC and PC cells. Confocal images obtained the difference in the localization of MUC4 protein in PaSC and CD18/HPAF cells. Here, β-catenin is used as a plasma membrane marker, EEA-1 is used as a marker of early endosomes, Lamp1 is used as a marker for lysosomes, calnexin is an ER marker and Giantin is a Golgi marker.

Figure 6.5



VI.5 Reference List

(1) Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014;64:9-29.

(2) Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res 2014;74:2913-21.

(3) Omary MB, Lugea A, Lowe AW, Pandol SJ. The pancreatic stellate cell: a star on the rise in pancreatic diseases. J Clin Invest 2007;117:50-9.

(4) Vonlaufen A, Phillips PA, Xu Z, Goldstein D, Pirola RC, Wilson JS, Apte MV. Pancreatic stellate cells and pancreatic cancer cells: an unholy alliance. Cancer Res 2008;68:7707-10.

(5) Joshi S, Kumar S, Choudhury A, Ponnusamy MP, Batra SK. Altered Mucins (MUC) trafficking in benign and malignant conditions. Oncotarget 2014;5:7272-84.

(6) Joshi S, Kumar S, Bafna S, Rachagani S, Wagner KU, Jain M, Batra SK. Genetically engineered mucin mouse models for inflammation and cancer. Cancer Metastasis Rev 2015.

(7) 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.

(8) Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth MA, Buchler MW, Aubert JP, Batra SK. 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.

(9) Volin MV, Shahrara S, Haines GK, III, Woods JM, Koch AE. Expression of mucin 3 and mucin 5AC in arthritic synovial tissue. Arthritis Rheum 2008;58:46-52.

(10) Agrawal B, Krantz MJ, Parker J, Longenecker BM. Expression of MUC1 mucin on activated human T cells: implications for a role of MUC1 in normal immune regulation. Cancer Res 1998;58:4079-81.

(11) Correa I, Plunkett T, Vlad A, Mungul A, Candelora-Kettel J, Burchell JM, Taylor-Papadimitriou J, Finn OJ. Form and pattern of MUC1 expression on T cells activated in vivo or in vitro suggests a function in T-cell migration. Immunology 2003;108:32-41.

(12) McCarroll JA, Phillips PA, Santucci N, Pirola RC, Wilson JS, Apte MV. Vitamin A inhibits pancreatic stellate cell activation: implications for treatment of pancreatic fibrosis. Gut 2006;55:79-89.

(13) Xiao W, Jiang W, Shen J, Yin G, Fan Y, Wu D, Qiu L, Yu G, Xing M, Hu G, Wang X, Wan R. Retinoic Acid Ameliorates Pancreatic Fibrosis and Inhibits the Activation of Pancreatic Stellate Cells in Mice with Experimental Chronic Pancreatitis via Suppressing the Wnt/beta-Catenin Signaling Pathway. PLoS One 2015;10:e0141462.

(14) Hwang RF, Moore TT, Hattersley MM, Scarpitti M, Yang B, Devereaux E, Ramachandran V, Arumugam T, Ji B, Logsdon CD, Brown JL, Godin R. Inhibition of the hedgehog pathway targets the tumor-associated stroma in pancreatic cancer. Mol Cancer Res 2012;10:1147-57.

(15) Scohy S, Gabant P, Szpirer C, Szpirer J. Identification of an enhancer and an alternative promoter in the first intron of the alpha-fetoprotein gene. Nucleic Acids Res 2000;28:3743-51.

Chapter VII

General Conclusions and future directions

A.Summary/Conclusions

B. Future directions

A. Summary/Conclusions

Aberrant overexpression of various members of the mucin family of proteins (i.e., MUC1, MUC4, MUC16, and MUC5AC) is one of the hallmarks of PC. It starts appearing in precursor lesions (i.e., pancreatic intraepithelial neoplasia [PanIN]) and increases with severity of disease. There are multiple studies which has associated the functional significance of mucins in PC pathobiology, and recognized their usefulness as diagnostic and therapeutic targets. In terms of their regulation, mucin expression is known to be controlled by inflammatory cytokines, including TGF-β, interferon-γ (IFN-γ), interleukins, and tumor necrosis factor-α (TNF-α) (1-3). MUC4 is one of the most differentially overexpressed mucins associated with oncogenic transformation in PC (4-8). It functionally contributes to enhanced motility, invasiveness, metastasis, and drug resistance of PC cells (9, 10). Earlier, our studies have demonstrated that IFN-γ, an inflammatory cytokine, and all-trans retinoic acid (RA) synergistically upregulate MUC4 in PC cells (11). The cellular complexity of the PC stoma leads to hypoxia due to huge desmoplastic reactions (12, 13). Moreover, as the size of tumor grows, metabolic activities expedite which cause increased generation of ROS, which prompted us to speculate that microenvironmental stress also has role in MUC4 regulation in PC. In this dissertation, I have examined the role of microenvironmental stress and bile acids on MUC4 expression. Moreover, I have also highlighted the novel mechanism by which MUC4 regulates the expression of EGFR family members. Further, I have discussed one of the most intriguing finding that MUC4 is expressed by PaSC, where its transcription is facilitated by an alternate promoter, which is locate on intron-1 of MUC4 gene. The detailed summary of all these observations is as follows

a. The role of PC micro-environmental stress on MUC4 regulation: Pancreatic cancer (PC) has been reported to be most hypoxic among solid tumors, primarily due to its hypovascular nature and extreme desmoplastic reactions (14). Emerging evidence demonstrates that HIF1α regulates MUC1 expression under hypoxia in PC. It enhances hypoxia driven angiogenesis and tumor cells survival by regulating the metabolic programming of PC cells. Our group has demonstrated that MUC4, which remains undetectable in the normal pancreas, is aberrantly overexpressed in the precursor lesions and progressively increase with the severity of PC. In this study, for the first time, I have demonstrated that under hypoxia, the stability of MUC4 protein is significantly affected in PC, in a HIF-1α independent manner. Further, I have demonstrated that hypoxia-mediated induction of reactive oxygen species (ROS), promotes autophagy by inhibiting pAkt/mTORC1 pathway, one of the central regulators of autophagy, leading to MUC4 degradation. Due to established functional redundancy in mucins, hypoxia-mediated induction of MUC1 may be sufficient to functionally compensate for autophagic degradation of MUC4. Altogether, hypoxia-mediated degradation of MUC4 provides necessary metabolites to ensure the survival of highly stressed PC cells.

b. The pathobiological implications of BA in PC: The pancreatic duct is placed in close proximity to the common bile duct, and they both unite at the point called as the ampulla of Vater. Approximately, 70% of PC patients develop extrahepatic cholestasis due to obstruction of common bile duct by gradually increasing tumor size, and exhibits obstructive jaundice which is characterized by increased circulatory BA levels. Moreover, most of the pancreatic tumors (about 75%) occur at the head of the pancreas. The tumor-promoting functions of BA

are established for multiple cancers such as esophageal, gastric and colorectal cancers, which made me inquisitive to comprehend whether BA play tumorigenic role in PC development and progression. Interestingly, I observed high circulatory BA levels ($p<0.05$) and its receptor expression, namely FXR, in PC patients compared to controls. Moreover, significantly high levels of BA in pancreatic juice obtained from PC patients than non-pancreatic non-healthy (NPNH) controls, suggest that there is a direct involvement of BA in the pathobiology of PC disease. Using defined spontaneous mouse model of PC (KPC), BA levels has shown to progressively increase with the severity of PC, which supported our postulation that BA do have tumor-promoting functions. Mechanistically, I have demonstrated that BA exposure led to induced mRNA expression of MUC4, which is primarily dependent on FAK-mediated induced expression of c-Jun. Using quantitative ChIP assay, I have shown that c-Jun binds to the AP-1 motifs present on MUC4 distal promoter region, a maximal responsive region for BA. This is the first study which has shown the direct involvement of c-Jun in MUC4 regulation. Further studies established FXR as the upstream molecule in this FAK/c-Jun/MUC4 axis. Therefore, targeting BA receptors or administration of BA antagonists can significantly impact the disease outcome.

c. MUC4 potentiates EGF-mediated signaling by regulating receptor trafficking in PC: Multiple studies have linked aberrant overexpression of MUC4 in cancer condition with the increased stability of HER2 over the cell surface, which facilitates sustained proliferation (15-17), one of the hallmarks of cancers; however, no studies have so far highlighted the implicated mechanism. In this study, I have highlighted the novel role of oncogenic MUC4 protein in

determining the fate of RTKs internalization, particularly EGFR. In PC tissues, calculated Pearson colocalization coefficient led me to know that ~60% of patients have both MUC4 and EGFR expression. By utilizing time-lapse live-cell imaging and confocal microscopy, it became apparent that presence of MUC4 increases the rate of internalization and recycling of EGFR and HER2 receptors to the plasma membrane compared to MUC4 kd PC cells. Mechanistically, the altered rate of EGFR and HER2 receptors in MUC4 kd cells was associated with the role of MUC4 in regulating the activity of Rab5A, one of the member of Rab GTPase family, which is known to catalyze the rate-limiting step of receptor internalization. Overexpression of Rab5A in MUC4 kd PC cells was able to attenuate the loss of EGFR and HER2 receptors, suggesting that MUC4 utilizes the common mechanism to regulate their fate. Besides Rab5A, we have also demonstrated that MUC4 regulates the expression of EGFR ligands such as, TGF-α and EGF. Altogether, we have found that MUC4 has multifaceted role in the regulation of the EGFR family receptors in PC. Considering the importance of RTKs in PC, deeper understanding of its prolonged presence as well as activation onto the cell membrane due to MUC4 overexpression, will give us better opportunity to therapeutically target PC.

d. Discovery of MUC4 mucin in activated pancreatic stellate cells (PaSC): While studying the role of PC tumor microenvironment in the regulation of MUC4, I obtained one of the most intriguing findings, which was the presence of MUC4 in PaSC cells (immortalized with E6/E7), whereas other mucins (MUC1 and MUC16) were absent. This was an unexpected finding given that MUC4 is normally expressed in the epithelial cells. Using confocal microscopy, these results have further confirmed the presence of MUC4 expression in α-SMA

(representing active PaSC) positive cells in PC tissue sections. Unlike PC cell lines, treatment with RA, which has well-established role in inducing PaSC quiescence, leads to reduced transcriptional expression of MUC4 in PaSC. It directed us to postulate that MUC4 expression determines the activation status of PaSC, which we have addressed by silencing MUC4 expression in PaSC by using targeted ShRNA constructs. Suppression of MUC4 expression leads to significant reduction ($p>0.05$) in α-SMA, vimentin and EGFR expression. Functionally, significant decline in the proliferative and migratory potential was observed in MUC4 kd PaSC cells, compared to the control cells. Altogether, these results indicate towards the involvement of MUC4 expression in determining the activation status of PaSC. This study has given us an additional strong rationale to therapeutically target MUC4, as it will not only kill PC cells, but will also inhibit the activation of PaSC cells, which has prominent role in PC desmoplasia.

e. Identification of Alternate promoter for MUC4 gene: MUC4 gene contains a GC-rich and TATA-less proximal regulatory region and a distal regulatory region flanked by a TATA box (18, 19). In addition to these promoters, I have identified a putative alternate promoter (AP), which is located on the intron-1 region of MUC4 gene. The size of MUC4 protein in PaSC was significantly smaller than PC cell lines, which was indicating a plausible deletion of exon. Screening of MUC4 cDNA from exon-1 to exon-26 using RT-PCR revealed that MUC4 in PaSC cells does not utilize CP and has deletion of exon-1 which encodes for the leader sequence of MUC4. Using promoter prediction V2 software, two highly likely predicted promoter sites on intron-1 (primarily at the end) were recognized. Certainly, I observed amplified expected PCR product in PaSC using forward

primer against intron-1 and reverse primer against exon-2, and further confirmed using sequencing. Further, I found that this identified AP is being used by MUC4-expressing PC cells as well. Supportively, *in silico* analysis has revealed that there is a presence of open reading frame at the beginning of exon-2, from where active translation could take place. Along with aberrant expression, accumulating evidence has associated altered subcellular localization of mucins with the poor prognosis and survival of cancer patients. Loss of leader sequence in MUC4 due to AP usage leads to significant change in MUC4 subcellular localization in PaSC compared to CD18/HPAF PC cell line (which primarily utilize MUC4 CP). This study has paved the way to initiate investigations which will provide us in-depth understanding of MUC4 both at the cellular and molecular levels.

B. Future directions

a. The role of PC micro-environmental stress on MUC4 regulation

Similar to cytokines, functional redundancy also exists among mucins, implying that hypoxia-mediated induction of MUC1 expression may be sufficient to compensate for MUC4 downregulation. We would like to experimentally authenticate our assumption by using MUC1 kd CD18/HPAF and CAPAN1 cell lines. Being the least molecular weight, it seems logical that nutrient-deprived, hypoxic and oxidatively stressed PC cells are inducing MUC1 (~250 kDa) expression, while simultaneously facilitating the degradation of high molecular weight MUC4 protein to save cellular energy expenditure. It would be interesting to study the impact of hypoxia on the expression and stability of other high molecular weight mucins, such as MUC16 and MUC5AC in PC condition. As shown in chapter 3, presence of MUC4 provides survival benefits to cancers cells residing in highly stress conditions and led us to hypothesize that MUC4

degradation in hypoxia may be critical for the viability of PC cells by reducing energy consumption, which is an urgent need for the survival of hypoxic cells. To directly relate MUC4 with the metabolism of stressed PC cells, we would like to do the quantitative measurement of metabolites in MUC4 kd and control PC cells with or without hypoxia treatment. Moreover, we have seen that HIF-1α and MUC4 has direct relationship both *in vitro* and *in vivo* system. One of the possible mechanisms could be EGFR downregulation upon HIF-1α inhibition, as recent study from our lab has shown that inhibition of EGFR leads to MUC4 downregulation in PC cells, and need to be investigated.

b. The pathobiological implications of BA in PC

In this project, we have associated BA-induced MUC4 expression with FXR expression, which is found to be overexpressed in 47% of PC patients. However, another BA receptor, TGR5 was upregulated in 67% of PC patients and emerging studies have shown its tumorigenic role in GI cancers, including PC. Further studies will be helpful and required to mechanistically delineate the association between TGR5 and PC disease condition. We would also like to study whether TGR5 is involved in mucins regulation. As mentioned earlier, PC also has aberrant expression for MUC1, MUC5AC and MUC16; we would like to see the impact of BA exposure on these mucins. BA levels have been found to be elevated in chronic pancreatitis condition, one of the known risk factor for PC development. As mentioned earlier, mucins overexpression appears early and increases with the progression of PC. Although in my dissertation research, I have primarily focused on the role of BA at the late stages of PC when most of the patients exhibit obstructive jaundice, it will be important to study the implications of the BA at the initial stages of PC. To get the better insight of BA

on the pathobiology of PC, bile duct ligation or cholecystectomy using autochthonous murine models could be extremely important to reveal its importance at the initial and later stages of pancreatic tumor. Moreover, the significantly induced levels of BA indicates their possible usefulness for diagnostic purposes, and needs to be validated in more number of patient samples to assess and establish its clinical utility. In addition to FAK pathway, inhibition of MAPK pathway also led to attenuation of BA-mediated MUC4 upregulation. Future studies will be focused to understand the in-depth involvement of different signaling pathways in MUC4 regulation after BA treatment.

c. MUC4 potentiates EGF-mediated signaling by regulating receptor trafficking in PC

In this project (chapter 5), we have shown that MUC4 is plausibly regulating the expression of TGF-α at transcriptional level. TGF-α has known function to direct EGFR towards recycling route. Therefore, by delineating the mechanism involved in MUC4-mediated regulation of TGF-α ligand, we would be able to explain the observed involvement of MUC4 in increased EGFR receptor recycling to the plasma membrane. Because we did not see an apparent interaction of MUC4-CD either with EGFR or HER2, we believe that the observed interaction between these receptors and MUC4 is primarily occurring at the MUC4 N-terminus. We would like to study this in-depth using MUC4 N-ter. We already have MiniMUC4 construct which consists 10% of VNTR of WT-MUC4, from which MUC4 C-ter can be detached using restriction enzymes. The remaining MUC4 N-ter could be used to do pull down experiments to confirm that this site is mainly interacting with EGFR and HER2 proteins. Although recent studies have shown the success

of erlotinib therapy in PC, treatment of PC patients with this drug did not lead to anticipated improvement on the clinical outcome of PC patients. Interestingly, in breast cancer, MUC4 overexpression has been considered as one of the reasons for Herceptin failure, which further provide more strength to our rationale to address this question. Therefore, our next objective would be to analyze whether presence of MUC4 influences the sensitivity and efficacy of EGFR-targeting therapies.

d. Discovery of MUC4 mucin in activated pancreatic stellate cells (PaSC)

In this research project (chapter-6), I have clearly shown that presence of MUC4 in PaSC affects its activation status, migration and proliferation. However, how does control and MUC4 kd PaSC affect the migration and proliferation of PC cells is still need to be explored. For that, we would take the help of 3D co-culture technique. Moreover, PaSC are considered to be partner-in-crime with PC cells and increase the incidence of both regional and distant metastasis of PC cells. Therefore, we would like to address whether expression of MUC4 in PaSC is responsible for increasing the metastatic potential of PC cells? Orthotopic implantation of the combination of PC cells with MUC4 scr or kd PaSC cells would be extremely helpful to delineate the functional importance of MUC4 expression on PaSC in PC development and progression.

Because we have observed that MUC4 cDNA in PaSC have all the exons present, except extremely short exon-1. Therefore, the observed difference in the molecular weight of MUC4 protein between PaSC and PC cell lines is plausibly due to other reasons. One of the most likely reasons could be the presence of allelic VNTR polymorphism in MUC4 gene in these cell lines, which also cause large variations in the size of MUC4 protein among PC cell lines (20).

Importantly, most of the conducted experiments involve only one PaSC, which is one of the biggest limitations of this study. Therefore, we would like to confirm MUC4 expression with additional PaSC cell lines, where UNMC rapid autopsy program could be of great help. We will obtain PaSC and PC cells from the same PC patients and analyze MUC4 expression both at genomic and protein levels.

e. Identification of Alternate promoter for MUC4 gene

The identification of alternate promoter of MUC4 gene in PC cell lines, PaSC and human PC tissues is primarily done by using RT-PCR technique followed by sequencing. Although we have considered all the precautions required to negate the possibility of DNA contamination, we will further evidence the presence of AP using more confirmatory techniques, such as 5’ rapid amplification of cDNA ends (RACE). Moreover, as mentioned earlier, we detected the presence of AP only on cDNA derived from PC tissues, whereas tumor adjacent tissues did not demonstrate the presence of AP. We correlated the usage of MUC4 AP with the aggressiveness of PC cancer, which needs to be studied in details. Additional studies focused to delineate the regulation of MUC4 AP would be advantageous to understand when and why MUC4 AP usage is preferred over MUC4 CP. For that, we would like to perform i*n silico* analysis on MUC4 AP which will highlight the putative sites for the binding of transcription factors. Construction of luciferase promoter constructs against AP segment will further help us to confirm the influence of revealed transcription factors.

Reference List

(1) Borchers MT, Carty MP, Leikauf GD. Regulation of human airway mucins by acrolein and inflammatory mediators. Am J Physiol 1999;276:L549-L555.

(2) Koo JS, Kim YD, Jetten AM, Belloni P, Nettesheim P. Overexpression of mucin genes induced by interleukin-1 beta, tumor necrosis factor-alpha, lipopolysaccharide, and neutrophil elastase is inhibited by a retinoic acid receptor alpha antagonist. Exp Lung Res 2002;28:315-32.

(3) Li X, Wang L, Nunes DP, Troxler RF, Offner GD. Pro-inflammatory cytokines up-regulate MUC1 gene expression in oral epithelial cells. J Dent Res 2003;82:883-7.

(4) Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth MA, Buchler MW, Aubert JP, Batra SK. 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) Iacobuzio-Donahue CA, Ashfaq R, Maitra A, Adsay NV, Shen-Ong GL, Berg K, Hollingsworth MA, Cameron JL, Yeo CJ, Kern SE, Goggins M, Hruban RH. Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. Cancer Res 2003;63:8614-22.

(6) Joshi S, Kumar S, Choudhury A, Ponnusamy MP, Batra SK. Altered Mucins (MUC) trafficking in benign and malignant conditions. Oncotarget 2014;5:7272-84.

(7) Joshi S, Kumar S, Bafna S, Rachagani S, Wagner KU, Jain M, Batra SK. Genetically engineered mucin mouse models for inflammation and cancer. Cancer Metastasis Rev 2015.

(8) 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.

(9) Chaturvedi P, Singh AP, Moniaux N, Senapati S, Chakraborty S, Meza JL, Batra SK. 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.

(10) Chaturvedi P, Singh AP, Batra SK. Structure, evolution, and biology of the MUC4 mucin. FASEB J 2008;22:966-81.

(11) Andrianifahanana M, Agrawal A, Singh AP, Moniaux N, Van S, I, Aubert JP, Meza J, Batra SK. 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.

(12) Koong AC, Mehta VK, Le QT, Fisher GA, Terris DJ, Brown JM, Bastidas AJ, Vierra M. Pancreatic tumors show high levels of hypoxia. Int J Radiat Oncol Biol Phys 2000;48:919-22.

(13) Omary MB, Lugea A, Lowe AW, Pandol SJ. The pancreatic stellate cell: a star on the rise in pancreatic diseases. J Clin Invest 2007;117:50-9.

(14) Olive KP, Jacobetz MA, Davidson CJ, Gopinathan A, McIntyre D, Honess D, Madhu B, Goldgraben MA, Caldwell ME, Allard D, Frese KK, Denicola G, Feig C, Combs C, Winter SP, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 2009;324:1457-61.

(15) Chaturvedi P, Singh AP, Chakraborty S, Chauhan SC, Bafna S, Meza JL, Singh PK, Hollingsworth MA, Mehta PP, Batra SK. MUC4 mucin interacts with and

stabilizes the HER2 oncoprotein in human pancreatic cancer cells. Cancer Res 2008;68:2065-70.

(16) Pochampalli MR, el Bejjani RM, Schroeder JA. MUC1 is a novel regulator of ErbB1 receptor trafficking. Oncogene 2007;26:1693-701.

(17) Ponnusamy MP, Singh AP, Jain M, Chakraborty S, Moniaux N, Batra SK. MUC4 activates HER2 signalling and enhances the motility of human ovarian cancer cells. Br J Cancer 2008;99:520-6.

(18) Van S, I, Pigny P, Perrais M, Porchet N, Aubert JP. Transcriptional regulation of the 11p15 mucin genes. Towards new biological tools in human therapy, in inflammatory diseases and cancer? Front Biosci 2001;6:D1216-D1234.

(19) Perrais M, Pigny P, Ducourouble MP, Petitprez D, Porchet N, Aubert JP, Van S, I. 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.

(20) Choudhury A, Moniaux N, Winpenny JP, Hollingsworth MA, Aubert JP, Batra SK. Human MUC4 mucin cDNA and its variants in pancreatic carcinoma. J Biochem 2000;128:233-43.

General Hypothesis and Objectives

Over the past few decades, multiple studies have established key roles of mucins in malignant diseases. The expression of mucins is significantly altered during tumorigenesis and other pathological conditions. In this dissertation, I have primarily focused on MUC4, which is one of the most differentially expressed proteins in PC and has strongly been implicated in the progression, metastasis and chemoresistance of PC. MUC4 is not expressed in the normal pancreas, but the early pancreatic intraepithelial neoplasia (PanINs) precursor lesions have been shown to express MUC4, which further increases as the disease progresses. The ability of NIH3T3 fibroblasts cells to form tumors in nude mice upon ectopic expression of MUC4 was the first evidence which has experimentally proved its oncogenic function. Considering the significant role of MUC4 in tumor biology, additional studies are required to highlight its novel functions and regulatory mechanisms. Although studies have associated extrinsic (cytokines) and intrinsic factors (NCOA3) with the regulation of MUC4, there is no study which has addressed the role of PC microenvironmental stress (hypoxia and oxidative stress) on MUC4 expression. Both Hypoxia and MUC4 has been associated with PC aggressiveness and chemoresistance. Moreover, hypoxia has been shown to regulate mucins expression in solid tumors. All these studied led me to hypothesize that hypoxia has a significant impact on MUC4 expression in PC, which aggravate the PC conditions. Besides PC microenvironment, the critical anatomical position of pancreas can influence the growth of pancreatic tumors. However, these mechanisms are unexplored. The majority of tumors (about 75%) arise at the head of the pancreas. Most of the PC patients develop extrahepatic cholestasis due to common bile duct obstruction by increasing tumor size which results in hyperbilirubinemia and elevated circulatory levels of bile acids (BA). Multiple studies have implicated BA as tumor promoter for various

cancers. A recently performed meta-analysis has shown that patients with the history of cholecystectomy have significantly higher risk to develop PDAC. These studies incited me to hypothesize that BA play important role in PC tumorigenesis by regulating the expression MUC4 oncogene.

In addition to regulation, I have also focused to elucidate the novel functional properties of MUC4 in PC. MUC4 is known to regulate the fate of EGFR family proteins in several cancers including PC. However the precise mechanism involved is still ascertain. Emerging reports have shown altered expression of RAB proteins in various cancers. Additionally, a recent study has shown that mucins can also regulate the expression of RABs to influence the trafficking of oncogenic proteins in cancer. It brought me to my next hypothesis that MUC4 determines the fate of EGFR family members by modulating the expression and activity of RAB GTPases in PC. In addition to PC cells, MUC4 expression has recently been detected in activated PaSC. Interestingly, our preliminary studies have shown reduction of MUC4 expression upon treatment with RA, which is known to change the status of activated PaSC to quiescent, suggesting a plausible link between MUC4 expression and activation status of PaSC. It led me to hypothesize that MUC4 regulates the activation status of PaSC and thereby, promotes desmoplastic reactions in PC microenvironment, which is known to exacerbate PC condition.

Broadly, the aims for my dissertation research were as follows:

1. To elucidate the role of microenvironment stress (hypoxia and oxidative stress) on MUC4 regulation in PC.

2. To investigate the impact of bile acids (BA) on MUC4 expression in PC.

3. To identify the novel functions of MUC4 in epithelial (ductal tumor) and nonepithelial (PaSC) cells under PC condition.