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Role of Endothelin Axis in Pancreatic Tumor Microenvironment

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Role of Endothelin Axis in Pancreatic Tumor Microenvironment

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Role of Endothelin Axis in Pancreatic Tumor Microenvironment (TME)

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Endothelins (ETs) are a family of three 21 amino-acid vasoactive peptides ET-1, ET-2 and ET-3 that mediate their effects via two G-protein couple receptors ET_AR and ET_BR which are expressed on various cell types. Apart from their physiological role in vasoconstriction, there is emerging evidence supporting the role of endothelin axis (ET-axis) in cancer. Due to the expression of ET receptors on various cell-types, ET-axis can exert pleotropic effects and contribute to various aspects of cancer pathobiology. Several studies have provided a fragmented picture of the diverse roles or ET-axis in various tumors. However, the comprehensive picture of the pathobiological role of this axis in any given cancer is poorly understood.

Given that PC epitomizes the complexity of tumor microenvironment (TME), which is an active player in disease progression and therapy resistance, the overarching goal of this dissertation was to define the role of ET-axis in this lethal malignancy. Specifically, the dissertation was aimed at defining the expression pattern of ET axis in PC TME and elucidating the pathobiological significance of ET axis in PC. Immunohistochemistry (IHC) analysis of surgically resected tumor tissues from PC patients indicated expression of ECE-1, ET-1, ET_AR and ET_BR expression in both primary and metastatic lesions. In addition to tumor cells, ET_AR and ET_BR expression was observed on blood vessels (BV), stromal cells including stellate cells and infiltrating immune cells. The expression of ET_AR and ET_BR in various cellular compartments was also analyzed using marker for tumor cell (CK19), blood vessel (CD31), stellate cell (alpha SMA) and macrophages (CD68 and F4/80). Importantly, analysis of survival data showed ET_BR positivity on BV is correlated with poor prognosis of the PC patients.

Bioinformatics analysis of TCGA database revealed high positive correlation of the pro-fibrotic gene signatures with both ET_AR and ET_BR particularly Collagen I (Col1A2, Col3A1, Col5A2, Col6A3), Platelet derived growth factor receptor beta (PDGFRβ), Fibroblast activation protein (FAP), suggesting a pro-fibrotic role of ET axis in PC.

In the second part of the dissertation, we studied the impact of ET-axis inhibition in autochthonous tumors that develop in genetically engineered mouse model (KPC). Treatment with dual ET receptor antagonist Bosentan induced cell death in the autochthonous tumors, decreased IHC signal for extracellular matrix proteins (α-SMA, Collagen I, Fibronectin and CTGF). Transcriptomic analysis using fibrosis gene array indicated anti-fibrogenic effects of Bosentan in KPC tumors. Further, treatment of murine pancreatic stellate cells (PSCs) and human cancer associated fibroblasts (CAFs) with recombinant ET-1 *in vitro* induced the expression of pro-fibrotic genes was abrogated by selective inhibition of ET_AR (BQ123) and ET_BR (BQ788) signaling with synergistic effects observed with dual receptor inhibition. Further, ET-1 stimulation induced a significant increase in the p-ERK and p-AKT in a time dependent manner and dual receptor antagonist Bosentan significantly attenuated the ET-1 mediated induction. Our study also demonstrates that targeting ET_AR with a specific inhibitor BQ123 enhances perfusion selectively in the tumor and reduces hypoxia in xenograft PC tumors.

The third part of the dissertation describes a possible involvement of ET axis in inflammation associated pancreatic tumor progression in presence if mutated $Kras^{G12D}$. The expression of ET axis components initially is restricted to pancreatic acinar and islet cell compartment in physiological conditions. However, during inflammation or injury the acinar expression is abrogated and is seen in early pre-cancerous lesions and neoplastic cells. The reprogramming of acinar phenotype into early pre-neoplastic lesions indicates an essential role of ET axis in pancreatic acinar to ductal metaplasia. This trans-differentiation is followed by excessive accumulation of ECM proteins and

inflammatory reaction in the pancreas, indicating further involvement of ET axis in influencing micro-environmental factors in initiation and progression of pancreatic cancer.

The fourth part of the dissertation describes the generation of the mouse model aimed at delineating the role of ET-1 in PC progression. Genetically engineered mouse model of PC (K-ras^{G12D}; *Trp53*$^{R172H/+}$; Pdx-1-Cre) that harbors a Kras and p53 mutation in the pancreas were crossed with the ET-1 $^{flox/flox}$ mice.

Taken together, studies in this dissertation demonstrate that ET axis plays a pleotropic role in the TME, and targeting ET axis can modulate the obstructive and immunosuppressive TME and make it potentially more amenable for chemotherapy and immunotherapy.

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ABBREVIATIONS

A

α-SMA alpha smooth muscle actin

AP-1 Activator protein

AT-II Angiotensin II

ADM Acinar to ductal metaplasia

AKT Protein kinase B

AP Acute pancreatitis

ANP acute necrotizing pancreatitis

B

BAL Bronchoalveolar lavage

BUILD Bosentan Use in Interstitial Lung Disease

BV Blood vessel

BMP Bone morphogenetic protein

C

CS Composite Score

CTGF Connective tissue growth factor

Col I Collagen I

CHO Chinese Hamster Ovary

COPD Chronic obstructive pulmonary disease

CAFs Cancer associated fibroblasts

CTL Cytotoxic T cells

COX Cyclooxygenase

CCK Cholecystokinin

CP Chronic pancreatitis

CCr Chemokine receptor 5

D

DMEM Dulbecco’s modified eagle’s medium

DNA Deoxyribonucleic acid

DC Dendritic cells

DBTC Dibutyltin dichloride

E

ET-1 Endothelin-1

ET-2 Endothelin-3

ET-3 Endothelin-3

ECE Endothelin converting enzyme

ET_AR Endothelin A receptor

ET_BR Endothelin B receptor

ET_CR Endothelin C receptor

ECM Extracellular matrix

EMT Epithelial to Mesenchymal transition

ERK Extracellular signal regulated protein kinases

EGFR Epidermal growth factor receptor

F

FN1 Fibronectin

FAP Fibroblast activation protein

FAK Focal adhesion kinase

G

GATA Globin transcription factor

GPA Granulomatosis polyangiitis

H

HIF-1 Hypoxia inducible factor 1

HH Hedhehog

HELLP Hemolysis-elevated liver enzymes-low platelet

I

IHC Immunohistochemistry

IFN- γ Interferon-γ

IGF-1 Insulin like growth factor

IBD Inflammatory bowel disease

IPF Idiopathic pulmonary fibrosis

ICAM-1 Intercellular adhesion molecule-1

IDMM Insulin dependent diabetes mellitus

IL Interleukin

IC Immune cells

Itga Integrin alpha subunit

J

JNK JUN terminal kinase

K

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

KC PDX-1-Cre, LSL-$Kras^{G12D}$

L

LUM Lumican

Lox Lysyl Oxidase

Ltbp Latent transforming growth factor beta binding protein

M

µg Microgram

µL Microliter

µM Micromolar

mRNA messenger RNA

MAPK Mitogen activated protein kinase

MMP Matrix metalloproteinase

MVD Micro vessel density

N

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

NFAT Nuclear factor of activated T cells

NO Nitric oxide

NMLA N-monomethyl-L-arginine

NIDMM Non-insulin dependent diabetes mellitus

NID Nidogen

O

OCTT Orocecal transit time

P

PLC Protein lipase C

PC Pancreatic Cancer

PanIN Pancreatic intraepithelial neoplasia

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PI3K Phosphoinositide-3-Kinase

PSC Pancreatic stellate cells

PHD Prolyl hydroxylase domain

PKC Protein kinase C

PLA Phospholipase A

PAF Platelet receptor activating receptor

PDTC Pyrrolidine dithiocarbonate

PDGF Platelet derived growth factor

Q

qRT-PCR Quantitative Reverse Transcription PCR

R

RNA Ribonucleic acid

RT-PCR Reverse Transcription PCR

ROS Reactive oxygen species

RBC Red blood cells

S

SDS Sodium dodecyl sulphate

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SPARC Secreted protein acidic and cysteine rich

T

TME Tumor Microenvironment

TMA Tissue Microarray

TGF-β Transforming growth factor-beta

TNF-α Tumor Necrosis factor

TAM Tumor associated macrophages

TBS Tris buffer saline

Thbs Thrombospondin

Timp Tissue inhibitor of metalloproteinase

U

UTR Untranslated region

V

VEGF Vascular endothelial growth factor

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CHAPTER 1 A

Introduction

1. Synopsis

Pancreatic cancer (PC) represents one of the deadliest malignancies with high incidence worldwide and poor survival. Despite recent advances in the treatment, modest benefits have been achieved. The complex tumor microenvironment of PC including desmoplasia, poor and heterogeneous blood flow, and a hypoxic environment limits the delivery and efficacy of therapeutic drugs. Endothelin (ET) isoforms, endothelin converting enzymes (ECEs) and receptors (endothelin A receptor, ET_AR and endothelin B receptor, ET_BR) referred to as the ET axis, plays a vital role in vascular homeostasis and tumor progression. Recently, role of ET axis is regulating various aspects of the tumor microenvironment has gain considerable interest such as a potential target for clinical opportunities and improving the clinical cancer management. The presence of well-characterized antagonist(s) for each of the molecular components of the axis holds promise for the treatment of cancer, however, the application of ET antagonists in PC therapy warrants further investigation. This review outlines and discusses the multiple roles of the ET axis in cancer progression, with major emphasis on pancreatic inflammation, cancer and cancer microenvironment.

2. Introduction to Endothelin(s)

PC is among the most aggressive and intractable human malignancies and is a leading cause of cancer related deaths worldwide. Despite intensive clinical research efforts the last decade, we have witnessed the failure of many clinical trials with only marginal increase in survival benefit [1] [2]. A striking histological feature of PC is a pronounced desmoplastic tumor stroma comprised of cancer-associated fibroblasts, pancreatic stellate cells, infiltrating immune cells, macrophages and perturbed vascular network. The interactions between these cell types makes a dynamic reactive microenvironment around the pancreatic ductal cells which serves as physical barrier for drug delivery [3] [4]. In PC, the microenvironment under goes constant change in its composition during the course of tumor progression and the stromal compartment out numbers the tumor cells [5]. The autocrine and paracrine secretions of these cellular components with the tumor cells facilitate tumor progression. In the micro ecology of tumor progression, growth factors exchange between these participating players stimulate proliferation, migration, extracellular matrix synthesis, invasiveness, angiogenesis and metastasis of cancer cells [6] [7] [8]. One such mediator that is secreted by the cancer cells and tumor microenvironment components, is endothelins (ET) family members, first identified by Yanagisawa *et al* in 1988 form the porcine endothelial cells [9]. The ETs comprise three, 21 amino acids, endogenous, vasoactive isoforms ET-1, ET-2 and ET-3. ETs are characterized by a single alpha helix and two intra-molecular disulfide bridges produced by endothelial and epithelial cells and are widely distributed in various tissues. The pleiotropic effects attributed to this family of endothelin ligands are mediated by two G protein couple receptors, ETA receptor (ET_AR) and ET B receptor (ET_BR) [10] [11]. Collectively, endothelins, their receptors and the endothelin converting enzymes are referred as “endothelin (ET) axis” which participates

in physiological functions such as vaconstriction vasoconstriction, vasodilation, cell growth, differentiation and hormone production. Pathophysiological implication of the axis is seen in aberrant expression of the ET-1 and the receptors in congestive heart failure, pulmonary hypertension, diabetes, and renal failure [12] [13]. Recently, the ET axis was implicated in signaling and formation of signaling complexes by coupling to other G family of proteins as well as scaffold proteins like β-arrestin [14]. In addition to its potent vasoconstrictive ability, aberrant expression of ET axis is also reported in many solid tumors such as breast, ovarian, prostate, bladder and lung cancers [15]. The autocrine and paracrine feedback loops between tumor cells and stromal cells facilitated by ET axis promotes tumor growth by activation of diverse array of signaling networks such as proliferation, apoptosis, formation of new vessels, immune modulation, invasion and metastatic dissemination [16]. As a result, the cellular behavior of ET axis depends on environmental cues and insults from the microenvironment. This review outlines the role of ET axis in physiological and pathological conditions of the pancreas with potential implications for future therapy

3. The Endothelin System

ET isoforms are synthesized in a three-step process. The primary translation product is a 212 amino acid pre-proendothelin and is cleaved by furin proteases to yield a biologically inactive 38 amino acid (pro-peptide) endothelin-1. The pro-peptide form is then processed by endothelin converting enzymes (ECEs) to yield the biologically active 21 amino acid cyclic peptide **(Figure 1)**. Of the three isoforms, ET-1 is the most well characterized which is synthesized and released continuously from endothelial cells. In humans ET-1 gene maps to chromosome 6, contains five exons, four introns and 5' and 3' flanking regions that spans approximately 6.8 kb of DNA. The mature ET-1 peptide has two disulfide bridges. Its carboxy terminus is for receptor binding and the amino terminus is required for determining the receptor binding affinity, which makes it unusual compared to other bioactive peptides. The expression of the pre-proET-1 is determined by transcription activation of EDN1 gene and is under the control of TATA box containing promoter [17]. Regulation of the EDN1 gene occurs at the level of transcription and is induced by different stimuli including hormones (insulin, cortisol, aldosterone, leptin, and adrenaline), growth mediators (angiotensin II, transforming growth factor beta, and inflammatory mediators), shear stress and hypoxia. Various transcription factors are known to stimulate the transcriptional activation of EDN1 gene such as functional activator protein-1 (AP-1), proto-oncogenes (c-fos, c-jun), hypoxia inducible [17, 18] factor-1 (HIF-1), GATA protein binding-2, transforming growth factor-β (TGF-β) at the proximal promoter and nuclear factor-κB (NF- κB), interferon-γ (IFN- γ), tumor necrosis factor-α (TNF- α), E box and nuclear factor of activated T cells (NFAT) at the distal promoter. Once activated, ET-1 activates several signaling pathways such as protein kinase C (PKC), c-JUN terminal kinase, insulin like growth factor, mitogen activated protein kinase (MAPK) and epidermal growth factor receptor (EGFR). In addition, the

tissue specific regulation of ET-1 is accomplished by epigenetic modifications. For example, in renal duct cells and fibroblasts, EDN1 is hypo methylated and hyper methylated respectively to influence transcriptional activity [19]. Also, in embryonic stem cells using a stable reporter gene system, methylation increased the level of EDN1 in endothelial cells [20]. In renal epithelial cells Stow *et al* showed that aldosterone stimulated ET1 expression by methylation at H3 lysine 4 residues [21]. In breast cancer cells, Matteucci *et al* demonstrated an impairment of ET-1 expression and decreased mRNA level due to epigenetic disturbances [22].The presence of AUUUA motifs at the 3'-untranslated region of EDN1 mRNA is responsible for regulating mRNA stability [23] [24]. The ET-1 mRNA has a short half-life and half-life of secreted ET-1 peptide in circulation is 1 min [15]. In endothelial cells, dual pathways synthesize ET-1. In regulated pathway, ET-1 is stored in the secretory granules known as Weilbel-Palade bodies, which are responsible for maintaining the vascular tone. Once activated in response to external stimulus, these bodies de-granulate and fuse with the plasma membrane, releasing its contents causing further vasoconstriction [25]. ET-1 is also released from a constitutive secretory pathway that appears to be the prime secretory mechanism operating at the level of transcription in most cell types [26]. Additionally, the clearance of ET-1 is a dynamic process and is governed by two pathways. Using Chinese Hamster Ovary (CHO) and COS cells, Bremnes and colleagues demonstrated that both ET receptors follow different routes of intracellular trafficking following a clathrin dependent endocytosis [27]. After co-localization in early Rab5 endosome upon ET-1 stimulation, ET_AR recycling progressed through a pericentriolar dependent pathway and appeared on the plasma membrane whereas ET_BR is subject to degradation through the lysosomal pathway and is responsible for clearance of ET-1. Abassi *et al* studied the metabolism *in vivo* and suggested the catabolism of ET-1 is by neural endopeptidase EC.3.4.24.11 [28]

ECEs belong to the M13 group of proteins and has three isoforms: ECE-1, ECE-2 and ECE-3. These enzymes share 59% homology with each other and their function is inhibited by phosphoramidon [29]. ECE-1 and ECE-2 are most prominent ECEs, belong to the type II membrane bound metalloprotease family and show preference for cleavage of ET-1 over ET-2 or ET-3 [30]. While ECE-1 is predominantly expressed on the cell surface and has broader tissue distribution, ECE-2 exhibits primarily neuroendocrine distribution [31] [32]. In addition, Nakano *et al* reported that conversion of pro-ET-1 by chymase cleavage of the Tyr31-Gly32 bond to generate a novel 31 amino acid ET peptide, that causes constriction of smooth muscle cells [33] [34]. ECE-1 is comprised of a short cytoplasmic N terminal tail, is localized to chromosome 1 and has four isoforms (ECE-1a-d) derived from single gene from differential gene splicing [35] [36] [37]. The ECE-1a isoform is mainly responsible for generating the functional secreted ET-1 after cleavage of pro-ET-1 [35]. All these isoforms differ in their N-terminal amino acid sequences and reveal different subcellular localization [38]. ECE-1c is the predominant isoform expressed on the cell surface, with ECE-1a and ECE-1d, whereas ECE-1b is found intercellular [36]. In addition, Kuruppu and co-workers reported that the expression and localization of the ECE1 is the rate-limiting step in the generation of ET-1 [39]. Further insights in the biology of ECE-1 come from the study of Muller *et al* who demonstrated different subcellular localizations of ECE-1 isoforms [40]. In neuroendocrine AtT-20 cells, ECE-1b and ECE-1d are present in late endosomes and early recycling compartments respectively. Additionally, in human endothelial cells, both ECE-1a and ECE1c isoforms are present, however ECE-1a shows nuclear immunoreactivity and co-localization with nucleolin [41]. Interestingly, difference in the sub-cellular localization of these isoforms has opposing effects on cellular behavior. ECE-1a and ECE-1c promote and suppress invasion respectively, whereas overexpression of ECE-1a abolishing the activity of ECE-1c in prostate cancer cells [42].

The other two isoforms ET-2 (EDN2) and ET-3 (EDN3) are present on chromosome 1 and chromosome 2 respectively [43]. The kidney and the intestine primarily express ET-2, ET-3 is found in brain, intestine, lung and kidney [12]. ET-2 differs from ET-1 by two amino acids and both isoforms has equal affinity for both ET_AR and ET_BR. In contrast, ET-3 differs from ET-1 by six amino acids and is selective for ET_BR compared to ET_AR [10]. Interactions of all three isopeptides with receptor controls several fundamental processes of cell growth, tissue differentiation and repair. The gene for ET_AR and ET_BR is located on chromosome 4 and 13 respectively and shows a high degree of conservation between human and animal species. Both receptors share 63% sequence homology with each other over a 420-residue match length. The EDNRA gene contains 427 amino acids and has eight exons and seven introns while EDNRB gene contains 442 amino acids and has 7 exons and 6 introns [10] [44]. The differences in the C-terminal sequences of two ET receptors allow G-protein coupling and results in divergent effects following ligand activation [45]. Both receptors are present in variety of cell types including vascular smooth muscle cells, endocrine cells and reproductive cells whereas the ET_BR is also abundantly expressed in endothelial cells [46] [47] [48]. The vasoconstriction response is through ET-1/ET_AR interaction in the vascular smooth muscle cells. In the absence of smooth muscle, binding of ET-1 to ET_BR causes vasodilation by the release of vasodilator such as nitric oxide (NO) [49]. In addition, Karne *et al* reported the presence of a third ET receptor subtype (ETCR) specific for ET-3, in Xenopus laevis. ET_CR is comprised of a 424 amino acids and shares 47% and 52% sequence similarity to ET_AR and ET_BR respectively [50]. Receptor activation is complex exhibiting diverse cellular outcomes by interaction with different G-protein coupled cells surface receptors. Extracellular binding of ET-1 to ET_AR induces activation and coupling to $G\alpha_{q/11}$ and stimulation of phospholipase C. This in turn leads to increase inositol triphosphate and diacylglycerol levels and enhance protein kinase C expression with

rapid elevation in Ca2+ levels which are responsible for vasoconstriction. On the contrary; engagement of ET_BR with ET-1 stimulates vasodilation by $G\alpha_{q/11}$ to induce phosphoinositide hydrolysis [35]. In the context of cancer, ET-1 and its receptors are de-regulated in many solid tumors and melanoma which contribute to tumor growth and progression. Studies have also indicated that autocrine signaling across the ET-1 axis plays a vital role in tumor cell proliferation. The interaction of the ET-1 ligand to its cognate receptor in turn activates a sequence of intracellular events that act in a synergistic fashion to foster cell proliferation [15]. Interestingly, ET-1 also promotes cell proliferation and act as a protective factor by inhibiting apoptosis in different cell types. In ovarian and prostate carcinoma cells, ET-1 modulates phosphatidyinositol 3-kinase activation and protects cells against paclitaxel-induced apoptosis by significantly inhibiting the levels of pro-apoptotic proteins such as Bad and Bax [51] [52]. In addition to contributing in tumorigenic potential, ET-1 also regulates the tumor microenvironment interactions that are important in maintenance and metastatic spread of the disease. Through autocrine and paracrine signaling across the axis and amplification of the cross talk between different components of microenvironment, ET-1 facilitates tumor-stroma interactions, angiogenesis and lymphangiogenesis, epithelial to mesenchymal transition, stemness and therapy resistance [10]. In contrast, the role of other two isoforms in human cancer is not much explored and few studies have attempted to define their possible role in cancer biology. In basal cell carcinoma aberrant activation of Hedhehog (HH) signaling is associated with ET-2 over-expression and the presence of Gli-1 binding sites at the 3’ promoter of EDN2 gene indicate that the expression is under the influence of HH signaling pathway [53]. Moreover, in human breast cancer pathogenesis, EDN2 is overexpressed and co-localizes with the hypoxic areas within the tumors [54]. Both ET-1 and ET-2 enhance the invasive and metastatic potential of tumor cells when co-cultured in presence of macrophages, suggesting ET-2 would mimic the

actions of ET-1 [55]. In addition, ET-2 is a potent chemo-attractant for macrophages and leads to increased activation dependent on ET_BR signaling [56]. Interestingly, in breast and cervical cancers, epigenetic inactivation of the EDN3 gene is associated with reduced expression and gene silencing [57] [58] [59]. Also, hyper-methylation of both ET-2 and ET-3 is reported in human colon cancer suggesting that silencing of these genes is essential step and pre-requisite for cancer development to evade competition with ET-1 for both receptors [60] [61]. Recent evidence indicates that a reciprocal relationship exists in expression of ET-1 and ET-3 in glioblastoma stem cells and glioblastoma tumors [62] inferring different isoforms are expressed in distinct cell populations. Evidence indicates that ET-3 (expressed by the metastatic melanoma cells) promotes survival, proliferation and invasion via ET_BR signaling and interaction with HIF-1α dependent machinery [63] [64].

4. Endothelin in Inflammation

Rudolf Virchow first observed the presence of leukocytes within tumors and indicated a possible association between inflammation and cancer [65]. To date, several lines of evidence support this notion that inflammatory insults are a trigger and are pre-requisite for cancer initiation In physiological conditions, in response to tissue damage or attack by pathogens, neutrophils first infiltrate the inflamed site, followed by macrophages and mast cells via activation of diverse array of signaling pathways and various soluble and insoluble mediators. The orchestrated actions of inflammatory cells strengthen the host defense, participate in tissue repair and pathogen elimination to decrease inflammation and regain normal tissue homeostasis. However, if the inflammation continues or it is not decreased, the cellular response changes to chronic inflammation, which leads to dysplasia and metaplastic changes thereby promoting the

risk of cancer initiation and progression. Epidemiological and clinical studies suggest that 15-20% of all cancer deaths are correlated with chronic inflammation and infection [66]. In the gastrointestinal tract, prolonged infection by *Helicobacter pylori* is coupled with gastric cancer and mucosa-associated lymphoid tissue with lymphoma. In the liver, chronic infection caused by Hepatitis C and B viruses increases the risk of hepatocellular carcinoma. Infection with *Schistosoma haematobium* and human herpes virus type 8 induces chronic inflammation and increases the risk in urinary bladder and Kaposi's sarcoma respectively [67] [68]. Also, cholangiocarcinoma and nasopharyngeal carcinoma are preceded by chronic inflammatory infiltrate stimulated by *Clonorchis sinensis* and Epstein Barr virus infection respectively [69]. In addition to microbial infection, some chemical irritant or immune deregulation and autoimmunity can also contribute to chronic inflammation and contribute significantly to tumor development. For example, in inflammatory bowel disease (IBD) the pro-tumorigenic niche created by effector CD4+ T cells and IL-6 increases the risk of colorectal cancer [70]. Similarly, inflammatory diseases such as chronic pancreatitis, barrett's metaplasia, chronic cholecystitis, chronic asthma and endometriosis may increase the risk of pancreatic cancer, esophageal cancer, gall bladder cancer, bronchial cancer and endometrial carcinoma respectively [69] [71] signifying the critical role of inflammation in cancer growth. In contrast, a chronic cutaneous inflammatory condition, psoriasis depicts an inflammatory state that is rarely associated with cancer risk [72]. On the other hand, exposure to environmental cues can also potentiate chronic inflammation. Long-term exposure to cigarette smoke can cause chronic obstructive pulmonary disease (COPD), a potential risk factor for lung cancer by activating IKK-β/NF-κB and JNK-1 dependent inflammation [73] [74]. Dostert *et al* demonstrated that the Nalp3 inflammasome present in asbestos/silica particulate matter contribute to hepatic fibrosis and lung cancer by release of pro-inflammatory cytokine IL-1β [75]. Also, obesity can play a role in

development of hepatocellular carcinoma by release of inflammatory mediators like IL-6 and TNF-α [76].

Several studies showed the pro-inflammatory role of ET-1 in the pathogenesis of many infectious diseases like bacterial meningitis, cerebral malaria, HIV encephalitis, sepsis and chagas disease in addition to its function as a potent vasoconstrictor [77]. ET-1 is ubiquitously expressed by the endothelium throughout the body in normal physiological conditions, however, in response to external stimuli such as hypoxia, cytokines, reactive oxygen species, angiotensin II or shear stress exerted on the endothelium facilitate increase secretion of ET-1 [78]. Elevated levels of ET-1 is found in macrophages [79], fibroblasts [80], mesenchymal stem cells [81] neutrophils and leukocytes [82]. Interactions of these cell types with each other and with the tumor cells is believed to drive inflammation associated cancers. The cellular cascade of events mediated by ET-1 within the tumor microenvironment releases various cytokines and chemokines, promotes trafficking of immune cells and play a crucial role in tumor growth and progression.

4.1. ET in fibroblasts

The strength and elasticity provided by the extracellular matrix components play a major role in maintenance of cellular architecture and tissue homeostasis. In normal wound healing, inflammatory cells are recruited to the site of injury to remove necrotic and apoptotic cells followed by limited deposition of ECM. In pathological conditions, due to excessive scarring or repeated injury, the conditions persist and inflammation fails to decrease and eventually contribute to development of fibrosis, which is commonly observed in idiopathic pulmonary fibrosis, hepatic fibrosis and systemic sclerosis [83] [84]. An increasing body of evidence indicate the induction to various cell types such as resident fibroblast, stellate cells, vascular pericytes, circulating bone marrow derived

monocyte to a myofibroblast like phenotype upon ET-1 stimulation [85]. The role of ET-1 as a pro-fibrogenic factor in the pathologies of various organs has gained considerable attention and interest as witnessed during the last decade. Mechanistic and experimental evidences implicate the regulation of ET-1 at the transcriptional level. Several factors such as shear stress, hypoxia, TGF-β, IFN-γ, angiotensin II and thrombin up-regulate; whereas, nitric oxide down regulate the expression of ET-1 mRNA [86] [87]. The involvement of ET-1 in diverse biological processes can be attributed to its interaction with various transcription factors such as GATA, Smad, TGF-β and activator protein-1 (AP-1) [23] [88].

The pro-fibrotic role of ET-1 in lung pathology is well studied. Elevated levels of ET-1 peptide and ET receptors are associated with scleroderma associated lung disease. In addition, increased concentrations of ET-1 in the plasma and bronchoalveolar lavage (BAL) fluids of patients with scleroderma and idiopathic pulmonary fibrosis is reported [89] [90]. Fibroblasts isolated from the lungs of systemic sclerosis patients showed elevated levels of ET-1; and ET-1 applied to lung fibroblasts induced a contractile phenotype with enhanced expression of alpha smooth muscle actin (α-SMA) that could be reversed by antagonizing ET-1 signaling [91]. In a lung fibroblast cell line, ET-1 via ETA receptor showed a dose and time-dependent increase in the CTGF and α-SMA by a JNK-AP1 dependent pathway [92] or by acting downstream of TGF-β signaling pathway [93]. *In vivo* evidence suggests increased ET-1 immunoreactivity in the epithelium surface and inflammatory cells of bleomycin-induced lung fibrosis has a significant effect on collagen deposition upon treatment with dual ET receptor antagonist, Bosentan [94] [95], suggesting the ET-1/ET receptor axis an attractive target for treatment of lung fibrosis. The first large, multinational, double blind clinical study to test the efficacy of Bosentan in IPF patients was BUILD-1 (Bosentan Use in Interstitial Lung Disease). Pharmacologial inhibition of the axis in patients with

IPF did not show superiority over placebo [96]. Compare to BUILD-1, a second clinical trial BUILD-3 failed to provide any improvement in the length of life using the primary end point analysis in IPF patients [97]. In context of liver fibrosis, activation of hepatic stellate cells is a central event, which subsequently progresses to cirrhosis from prolonged injury, results in excessive scarring. ET-1 is constantly produced by liver parenchymal cells and stellate cells in response to injury and induces a pro-fibrogenic response in an autocrine and paracrine manner [98]. ET-1/ET receptors signaling in stellate cells promotes proliferation, enhanced contraction, increase hepatic resistance and blood flow, which is abrogated by the mixed ET_A/ET_B antagonist Bosentan and ROCK inhibitor [99] [100] [101] [102]. In fact, ET-1 increases TGF-β expression and collagen synthesis by eliciting Ca2+ release, however selective blockade of ET_AR inhibits collagen deposition [103] [104] [105]. Further, pre-clinical studies also indicate the anti-fibrotic effects of ET_AR antagonism by ambrisentan and darusentan by inhibiting the activation of stellate cells, extracellular matrix synthesis and ischemia respectively in animal models [106] [107]. The pro-fibrotic actions of ET-1 and its ability to promote major phenotypic transformation in hepatic fibrosis are regulated by many factors. Tumor necrosis factor-α (TNF- α) has shown to increase ET-1 levels in rat hepatic stellate cells through an IKK-JNK dependent signaling [108]. In human stellate cells, treatment with angiotensin II and TGF-β stimulate ET-1 release by stabilization of ET-1 mRNA by a PI3K/AKT dependent pathway which was abolished by ET_AR (BQ123) and Ang-II (losartan) blockers [109] [110]. In addition, studies by Zhan and co-workers demonstrated the effects of ECM protein fibronectin in enhanced ET-1 release from activated stellate cells in an integrin and ERK dependent fashion [111]. The beneficial effects of ET axis antagonism encourage the use of testing of antagonists in clinical settings; however liver toxicity observed in patients with pulmonary hypertension limits it's use [112]. In a double-blind randomized study, treatment with BQ123 and BQ788 on

hemodynamics in liver cirrhosis patients fails to provide significant benefit over palcebo control; suggesting the use in clinical settings warrants further investigation [113].

All three ET isoforms and both receptors are present in the kidney and the expression of ET-1 and ET_AR progressively increase with nephritis [114]. The podocytes cells present in the Bowman's capsule, and mesangial cells express increased levels ET-1 and receptors in response to cytoskeletal remodeling and glomerular permeability [115] [116] [117] [118]. In renal physiology and pathophysiology, ET-1 plays a pleiotropic role in cell proliferation, podocyte dysfunction, inflammation, fibrosis and pathobiology of the chronic kidney disease [119]. Enhanced expression of ET-1 and the ET_BR is reported in glomerular endothelial cells in patients with renal disease, with no difference in ETA receptor expression [120] [121]. From the perspective of fibrosis, over expression of ET-1 and ET-2 in transgenic mice and rats respectively is associated with kidney pathophysiology, pronounced renal fibrosis, collagen accumulation and glomerulosclerosis [122] [123]. Increased production of ET-1 in renal vessels is correlated with increase resistance and collagen I formation in hypertensive rats [124]. Treatment with recombinant TGF-β2 increase tissue ET-1 levels and fibrosis, however, systemic treatment with TGF-β2 neutralizing antibody, CAT-152 ameliorates the disease [125] [126]. Inhibition of ET signaling has pronounced effects in various renal injury models; with attenuation of fibrosis, hypo cellularity, a decrease in the ECM matrix components, and regression of vascular fibrosis. [127] [128] [129]. Compare to hepatic fibrosis, the pro-fibrogenic outcomes of ET-1 are mediated by Ang II where increased accumulation of collagen I is observed, and treatment with either ET receptor or Ang II inhibitors reduces the renal fibrogenic response by blocking TGF-β signaling [130] [131]. Despite of their encouraging results in pre-clinical settings, ET axis antagonists achieved moderate success in clinical trials. In diabetic nephropathy patient treatment with ET_AR inhibitor Avosentan was associated with congestive heart failure, although a reduction in

proteinuria was observed [132]. In a randomized double blind study, a protective effect of avosentan in combination with sitaxsentan (ET_AR selective) was observed in kidney and hearts in patients [133]. In a separate study, another ET_AR antagonist, Atrasentan in combination with a renin-angiotensin system blocker reduces albuminuria and provided better renal outcome than Atrasentan alone [134] [135].

4.2. ET in Immune System

The biological role of ET-1 in immune response and regulation of the immune microenvironment is well documented. The classic antigen presenting cells, dendritic cells (DC), which are responsible for anti-tumor immunity, also produce ET-1 ligand and express both functional ET receptors, at significantly increased levels during DC maturation. However, in the context of DC maturation, both receptors display reciprocal effects, posing a unique target for immunotherapy [136]. Guruli *et al.* showed that selective targeting of ET_AR using Atrasentan promotes DC apoptosis, decrease expression of DC marker CD83 and cytokine IL-12, and abrogates the ability of DCs to stimulate T cells. However, ET_BR blockade by A192621 counteracted the effects of ET_AR antagonism and promoted DC survival, implicating the involvement of autocrine and paracrine loops in DC function and maturation. Additionally, stimulation of human monocyte derived DCs with TLR2 and TLR4 agonists enhanced ET-1 production in a dose and time dependent manner, which could subsequently be inhibited by a HIF-1α inhibitor [137]. In addition to DCs, both ET receptors are present in B-lymphocytes, a subset of T cells (CD4 and CD8), monocytes and neutrophils isolated from normal and systemic sclerotic patients [138] [139]. Compared to normal B cells, over expression of ET-1 and ET_AR is reported in circulating chronic lymphocytic leukemia B cells. Moreover, ET-1 mediated survival; proliferation and drug resistance was abolished by ET_A receptor antagonism [140]. In addition, B cell depletion with rituximab blunted ET-1 mRNA levels in response to placental ischemia [141]. In human ovarian tumors, transcriptional

profiling of endothelial cells suggests over expression of ET_BR and is correlated with poor prognosis and absence of T infiltrating lymphocytes (TILs). Moreover, blockade of ET_BR by BQ788 leads to an increase in intercellular adhesion molecule-1 (ICAM-1) expression, increased T cell homing and adhesion to tumors [142]. In contrast, selective ET_AR inhibition prevents the stimulation of ICAM-1 and recruitment of anti-tumor immune cells [142]. Altogether, these studies suggests the potential of ET axis in cancer immunotherapy and underscore an inverse association of ET_AR and ET_BR where dual ET receptors antagonism target tumor cells and enhance T cell infiltration by ET_AR and ET_BR respectively. Recently, study in malignant gliomas demonstrated an increased expression of ET_BR with tumor grade correlated with fewer infiltrations of cytotoxic T cells (CTLs), signifying the critical role of ET_BR signaling in the homing of T cell population to tumors [143] [144]. In contrast, ET_BR expression correlated with micro-vessel density and tumor angiogenesis in oesophageal squamous cell carcinoma and not with TILs [145]. In hemolysis-elevated liver enzymes-low platelet (HELLP) syndrome, ET_AR antagonism decreased ET-1 and IL-17 levels and was accompanied by paucity of CD4+ and CD8+ T lymphocytes [146]. Additionally, in multiple sclerosis signaling through ET_AR induced IL-17 secretion from CD+T cells and was significantly inhibited by BQ123 [147]. Also, in granulomatosis polyangiitis (GPA) patients, ET_BR expression in the lesions T lymphocytes predominates the ET_AR expression [148]. Further, characterization of ET-1 promoter identified two binding sites for nuclear factors of activated T cells (NFAT) [149], which is a key regulator of T cell development and function [150]. In both pulmonary arteries and osteoblasts, ET-1 activates calcineurin and promotes nuclear transport of NFAT, which indicates a potential interaction within the immune microenvironment [151] [152]. In transgenic mice, over expression of ET-1 significantly increased the density of CD4+ T cells compared to age matched controls [153].

Several observations have also linked the orchestrated actions of ETs in neutrophils, monocytes and macrophages within the complex host microenvironment. Previous studies have indicated contradictory roles of ETs as chemoattractant for monocytes and neutrophils. ET-1 ligand has been earlier shown to be strong chemo attractant [154] or non-chemo attractant for blood monocytes [155]. Interestingly, a 31 amino acid bioactive peptide exhibited significant chemotactic activity towards monocytes and neutrophils compared to 21 amino acid ET-1 peptide and big ET-1. The enhanced chemo taxis is complemented by increase in intracellular calcium levels and was inhibited by selective A receptor antagonism (BQ123), and not by selective B receptor inhibition (BQ788) [156]. Also, ET-1 induced a chemo kinetic migration of rabbit peritoneal monocytes dependent on ETA receptor [157]. In addition to ET-1, interaction of ET-2 and ET-3 with both ET_AR and ET_BR stimulated migration of neutrophils in a concentration dependent manner [158] [159]. Grimshaw *et al.* reported that hypoxia induced increase in ET-2 production act as a chemo attractant for macrophages and monocytes dependent on ET_BR, indicating a possible role of ET-2 in facilitating the recruitment of inflammatory cells [56] [160]. In human monocytes, ET-1 has been shown to stimulate pro-inflammatory NF-κB pathway and drive the expression of CD40 but not IL-6 in an ET_AR dependent manner [161] [162]. Moreover, infection of the U937 monocytic cell line with Chlamydia pneumonia increased expression of ET-1, further indicating its possible involvement in acute and chronic inflammation [163] [164]. Also, Trypanosoma cruzi infection in cardiac myocytes induces pro-inflammatory mediators and ET-1 release and subsequent activation of NFAT signaling pathway [165]. More recently, in apolipoprotein E knockout transgenic mice, ET-1 over expression in the endothelium increases oxidative stress, inflammation and monocyte/macrophage accumulation [166]. However, reduction in macrophage dependent inflammation in Csf1 mutant mice with over expressing ET-1 diminishes vascular injury and the inflammatory

cell population [167]. Within the tumor microenvironment, autocrine and paracrine signaling between the cancer cells and macrophages accelerate the infiltration towards the inflamed sites. In bladder cancer, ET-1/ET_AR interaction increases migration of both tumor cells and tumor-associated macrophages (TAMs) with enhance production of IL-6, MMPs and CCL2, accompanied by increase in metastatic colonization in the lung via ruptured vascular integrity. Interestingly, dissemination of tumor cells and diminution of TAMs is blocked by ET_AR antagonist, zibotentan [168].In an experimental breast cancer model, ET axis mediates lung metastasis and trans-endothelial migration of breast cancer cells stimulated by macrophages [169]. Additionally in a rat model, stromal deficiency of the ET_BR was correlated with reduced metastatic spread, infiltration of TAMs and production of TNF-α, suggesting a pivotal role of the ET_BR in tumor progression [170].

4.3. ET in endothelial cells

In addition to its potent mitogenic effect on fibroblasts, smooth muscles and tumor cells, ETs also control the proliferation of blood and lymphatic endothelial cells. Interaction of ET-1 and ET-3 with ET_BR stimulate the proliferation and migration of endothelial cells in a dose dependent manner [171] [172] [173]. In endothelial cells, ET-1 modulates the angiogenesis process and exhibits a potent effect in combination with VEGF. ET-1 stimulates release of MMP-2, which allows sprouting and migration of endothelial cells and formation of vascular cord-like structures indicating that ET-1/ET_BR interaction favors neovascularization in concert with VEGF [174] [175]. Previous observations demonstrated that both VEGF and ET-1 stimulate each other's expression. In bovine aortic endothelial cells, VEGF induces ET-1 mRNA expression and ET-1 secretion while in vascular smooth muscle cells, ET-1 acts on ET_AR to stimulate VEGF mRNA and VEGF secretion [176], showing a coordinated role of VEGF and ET-1 in a physiological conditions. In pathological conditions, including angiogenesis and lymph

angiogenesis, the levels of VEGF and ET-1 are influenced by various environmental cues, including hypoxia. Some of the earliest evidence about the role hypoxia in influencing endothelin expression comes from the studies by Kourembanas *et al.* who demonstrated that oxygen tension increases ET-1 transcription and secretion in endothelial cells [177] by induction of the AP-1 transcription factor [178]. Under normal oxygen conditions, HIF-1α is subjected to proteasomal degradation by E3 ubiquitin ligase complex, catalyzed by the prolyl hydroxylase domain (PHD), which acts as an oxygen sensor [179]. However, during oxygen tension conditions, HIF-1α is stabilized and binds to hypoxia responsive elements (HREs) to induce transcription of angiogenic genes such as ET-1, VEGF and erythropoietin [180] [181] [182]. In cancer cells, ET-1 stabilizes HIF-1α in normoxic and hypoxic conditions by inhibiting PHD2 expression and promoter activity by acting on the ET_AR and ET_BR, indicating that a reciprocal relationship exists between ET-1 and HIF-1α [183] [184]. In chondrosarcoma cells as well, ET-1/ ET_AR interaction promotes tumor growth and angiogenesis by inducing HIF-1α and VEGF levels [185]. Also, in human lymphatic endothelial cells, ET-1 control the stability of both HIF-1α and HIF-2α by promoting its nuclear accumulation and by down regulating PHD2 hydroxylation [186]. Once stabilized, the ET axis promotes and interacts with the HIF-1α dependent machinery to drive the expression of VEGF family of proteins VEGFA, VEGFB, VEGFC and VEGFR3 receptor [64] [175]. Indeed a reciprocal interplay exists within the hypoxic microenvironment mediated by HIF-1α/HIF-2α in melanoma and endothelial cells linking ET-1/ET_BR to VEGF A/C accelerating neovascularization and tumor growth, signifying the presence of autocrine/paracrine circuit [187]. Within the tumor microenvironment, β-arrestin controls ET-1 and VEGF activity epigenetically by interacting with HIF-1α, and ET-1 itself to modify the microenvironment by driving the expression of chemokine receptors dependent on ET_AR and hypoxic machinery [188] [189]. In many solid malignancies, increased ET-1 and ET

receptors expression is coupled with increase VEGF expression. In ovarian carcinoma, increase expression of ET-1 and both GPCRs is significantly correlated with VEGF expression and micro vessel density implying a mutual relationship between ET-1 and VEGF during neovascularization, as this angiogenic response can be altered by ET receptor blockade [190]. In addition to ET-1, HIF-1α also modulates and stabilizes ET-2 and acts as a survival factor in breast tumor cells via ET_BR signaling [54]. In breast carcinoma, a significant relationship exists between HIF-1α and the pro-angiogenic genes, ET-1 and VEGF-C, where enhanced invasion of MCF-7 mediated by ET-1 is counteracted by the ET_AR antagonist atrasentan [191] [192]. Expression of ET-1, ET_AR and ET_BR has been correlated with VEGF expression, intratumoral vascularization, increased lymphatic dissemination, and the elevated levels of ET_AR in primary breast cancer are associated with decreased overall diseased free survival [193]. Furthermore, studies have shown that ET-1 induces lymphatic vessels to grow and invade. ET_BR and ET-1 were significantly upregulated in lymphatic endothelial cells derived from metastatic lymph nodes, suggesting the role of ET-1/ET_BR signaling in angiogenesis and lymph angiogenesis [175] [194] [195]. Interestingly, mathematical modeling and global transcript analysis of melanoma cell lines identified ET_BR as one of the genes linked with aggressive phenotype and the ability to form tubular networks in the absence of endothelial cells thus mimicking a true vascular endothelium [196]. The *de novo* generation of capillary like channels is known as vasculogenic mimicry [197]. Very recently research in melanoma cells, showed ET-1 induced phosphorylation of VEGFR3, MAPK and AKT formed tubular capillary network via ET_BR signaling. Moreover, in combination with VEGF-C, a synergistic effect was observed in fostering vasculogenic mimicry [198].

In addition, in ovarian carcinoma ET-1/ET_AR interaction stimulated downstream activation of cyclooxygenase (COX) 1/2 and production of prostaglandin E2. COX

inhibitors inhibit the angiogenic effect mediated by ET-1 to stimulate VEGF levels, specifying its role neovascularization [199]. Also, elevated levels of COX1 is reported in ovarian cancer and enhanced expression is associated with tumor regions undergoing extensive angiogenesis that have pro-angiogenic VEGF and HIF-1α levels [200]. Exposure of endothelial cells to hypoxic conditions induces expression of COX1 and COX2, however treatment with the non-selective COX inhibitor, indomethacin reversed the hypoxic effects and ameliorate endothelial cell injury [201]. ET-1 significantly increases COX-1 and COX-2 by increasing their promoter activity; and pharmacological inhibition of COX-2 using NS-398 significantly impaired the levels of ET-1, PEG2 and VEGF [202]. Selective ET_AR inhibition by atrasentan and ZD-4054 drastically reduced the expression of angiogenic and invasive mediators such as COX 1/2, VEGF and MMPs, thus implicating that the VEGF and COX genes acts downstream of the ET-1 gene and aid in neoangiogeneis [203] [202].

5. Endothelin in Normal Pancreas

5.1. Role in pancreatic islet cells

The effect of endothelium derived vasoactive peptide ET-1 on the islet cells of the pancreas has been well studied and been shown to support the pancreatic beta cell function **(Figure 2)**. Co-immunostaining studies performed in human and rat tissues using islet specific hormones suggest the presence of the ET-1 and endothelin receptors [204]. In both human and rat islet beta cells, positive staining for the components of endothelin axis was observed. In addition, alpha cells of the human islets show colocalization only with ET-1; however, rat islet shows weak immunoreactivity for receptor A and positive staining for receptor B and ET-1. As opposed to beta and islet cells, somatostatin-expressing delta cells show no expression with ET-1 and ET receptors in human pancreas. The presence of ET-1 and its receptors were also

confirmed in rat beta cell lines INS-1 and RINm5f by radiolabel binding studies with ET-1 using selective inhibitors for ET_AR and ET_BR respectively. Kakugawa *et al.* also reported the presence of ET-1 like immunoreactivity in the normal pancreatic islets and it was shown to co-exist with insulin and glucagon but not with somatostatin [205], indicating an insulinotropic role for ET-1. The ET-1 stimulated glucose induced insulin release by direct action on mice islets was also observed by Gregerson *et al.* [206] and involved calcium ions influx indicating direct involvement in glucose homeostasis. The insulinotropic effect of ET-1 is mediated by the endothelin A receptor involving PKC activation, as the specific inhibitor BQ123 attenuated the secretion while specific ET_BR antagonist BQ3020 did not alter the glucose stimulated release [207]. In contrast, Brock *et al.* suggested that the mechanism of insulin release is an indirect effect due to paracrine activation from the alpha cells of pancreatic islets. Using purified beta cells from normal rat islet and three beta cell lines (INS-1, MIN6 and βTC3), exogenous administration of ET-1 failed to elicit insulin secretion. In addition, competitive receptor binding studies on βTC3 cells displayed no specific binding, with only ET_AR expression in rat islet and alpha cell line αTC1.9. Alternatively, ET-1 potentiated the release of insulin when both purified beta and non-beta cells were mixed in presence of glucose [208]. In a rat experimental model, Zimmerman and Maymind described a decrease in the blood glucose levels and enhanced insulin release within 15 minutes after infusion of ET-1 in anesthetized rats. This increase in the insulin level was associated with a decrease in the glucagon hormone levels suggesting a possible impact on the endocrine pancreas [209]. Another possibility is that nitric oxide (NO) release, due to ET-1 stimulation contributes to insulin secretion, as the nitric oxide inhibitor N-monomethyl-L-arginine (NMLA) abrogated insulin release; signifying the involvement of endothelium in the pancreatic islet contributes to ET-1 induced insulin secretion [210]. In healthy human subjects, pharmacological infusion of ET-1 intravenously reduced the splanchnic blood

flow. Interestingly, this decrease is shadowed by a significant reduction in glucose concentration, splanchnic glucose production, and a transient decrease in insulin and glucagon levels [211] [212]. The inconsistency observed in both human and rat experimental model system can be accredited to species specific differences, structural differences and distribution of the receptors [204].

In addition to ET-1, Carlo and his colleagues also evaluated the insulinotropic role of ET-3 in isolated rat islets. Both ET isoforms stimulated insulin release in rat islets when cultured in presence in medium containing a physiological concentration of glucose and calcium, indicating a direct interaction with the islets. Alternatively, incubation in glucose and calcium deprived medium abrogated the release. The difference in insulin secretion mediated by ET-1 and ET-3 can be explained on the basis of the structural disparities and their binding affinities towards the ET receptors [213]. Additionally, evidence for insulin stimulated ET-1 release is also reported in *in vitro* studies. In bovine endothelial cells insulin stimulates the secretion and production of ET-1 in a dose related fashion through a PKC dependent mechanism [214] [215] [216]. This insulin-mediated release of ET-1 was also reported in human endothelial cells when cultured in presence of glucose [217]. In contrast, exposure of porcine endothelial cells to hyperglycemic conditions resulted in reduction of immunoreactive ET-1 secretion [218]. Interestingly, studies have demonstrated elevated levels of ET-1 are positively associated with both Type I (insulin dependent) and Type II (non-insulin dependent) diabetes mellitus. Haak *et al.* observed increase plasma endothelin levels in 69 patients and 83 patients of Type II and I respectively as compared to 152 controls patients [219]. Additionally, in patients with non-insulin dependent diabetes mellitus (NIDMM) and insulin dependent diabetes mellitus (IDMM) circulating levels of ET-1 are higher as compared to controls [220]. Using radioimmunoassay analysis Takahaski and his colleagues demonstrated significant increase in the levels of ET-1 as compared to

healthy controls [221] with no change in the ET-2 and ET-3, signifying the crucial role of ET-1 peptide on the endocrine pancreas. Examination of the circulating peptide concentrations in diabetic patients and obese hypertensive patients indicate that hypersinsulinemia is a potent inducer of ET-1 in humans [222] [223]. On the contrary, Bertello *et al.* did not observe any changes in the plasma ET-1 levels in patients with NIDMM compared to healthy human subjects [224]. Despite its role in enhanced insulin production, studies also indicate that the ET-1 peptide modulates glucose metabolism by inducing insulin resistance by inhibiting beta cell function. In a rat model, intraperitoneal administration of ET-1 increased plasma glucose level in dose dependent fashion by lowering the insulin sensitivity [225]. Additionally, in healthy human subjects exogenously administered ET-1 reduces the glucose uptake in skeletal muscle causing insulin resistance with no effect on its vasoactive role [226]. In a randomized study involving healthy men, pharmacological elevation of ET-1 following intravenous administration abolished insulin release with simultaneous reduction in C-peptide secretion but did not alter insulin sensitivity [227]. Further evidence of a role for ET-1 in reducing the insulin sensitivity, comes from hyperinsulinemic NIDMM patients, where a negative correlation exists with total uptake of glucose and circulating plasma ET-1 peptide levels [228]. Measurement of insulin sensitivity in response to intravenous glucose using minimal modeling technique indicates that the association between insulin release and beta cell is linked via close loop feedback system involving mutual alterations in insulin release and sensitivity [229] [230]. Kahn *et al* further strengthen this close coordinated relationship in healthy human subjects and suggested the relationship between beta cells and insulin sensitive tissues is dictated in a hyperbolic manner and alterations in insulin release have a minuscule effects on insulin sensitivity [231].

5.2. Role in pancreatic acinar cells

Similar to islet cells, pancreatic acinar cells also express endothelin receptors **(Figure 2)**. Using radiolabeled binding studies in rat model; high specific affinity receptors for all three forms of endothelins were identified, with ET_A subtype present in higher numbers compared to ET_BR. Additionally, further studies from the same group also indicate that ET isoform 1 and 2 bind to the ET_A subtype whereas ET-3 binds only to the ET_BR. Indirect interaction of ET receptors with phospholipase C pancreatic secretagogues favors internalization of the receptors and reduces the binding of ET peptides by activation of protein kinase C without altering the intracellular calcium levels [232]. In contrast, Yule *et al.* demonstrated that exposure of acini cultured in calcium free medium to ET-1 induces the release of intracellular calcium levels similar to cholecystokinin (CCK) although with less potency. Interestingly, as opposed to CCK or other PI-PLC like agonists, ET-1 failed to stimulate amylase secretion [233]. Similar to previous reports, the presence of receptor B was also seen in the rat acinar cells using anti-serum against the specific amino residues of the ET_BR [234].

5.3. Role in pancreatic microcirculation

The impact of potent vasoconstrictor, ET-1, on pancreatic microcirculation is well studied in many species due to its significance in regulating blood flow under physiological conditions. Takaori and his colleagues utilized anesthetized mongrel dogs and evaluated the effect of all three ET isoforms on pancreatic blood flow. Both ET-1 and ET-3 decrease the pancreatic blood flow in a dose dependent fashion, with more potency than the effects observed with ET-2, without significantly alerting systemic blood pressure. Interestingly, no effect in the liver was reported indicating the specific effect of ETs on pancreas vasculature [235]. Similarly, in an experimental rat system, topical superfusion of 100 pmol ET-1 in exteriozed pancreas significantly reduced the blood flow velocity accompanied by stasis of red blood cells (RBCs) as compared to control animals. In addition, administration of ET-2 and ET-3 also diminished blood flow velocity

in a dose dependent manner although with less prominent effects as compared to ET-1. This decrease in pancreatic microcirculation is paralleled by reduced functional capillary density, with ET-1 being more potent followed by ET-3 and ET-2 [236] [237]. Using a microsphere technique Lai *et al.* characterized the effects of exogenous ET-1 on the vasculature of pancreatic islets [238]. A few minutes after administration, 5nmol/kg dose of ET-1 significantly decrease perfusion in pancreas islets with minuscule effects on the blood pressure. Interestingly, islets were more responsive to the vasoconstrictive effects caused by ET-1 as compared to exocrine pancreas blood vessels. However, this decrease was not attenuated either in presence of selective A receptor antagonist (BQ123) or selective B receptor antagonist (BQ788) when used alone or in combination. The inability of selective A and B receptor antagonists to reverse the ET-1 mediated effects indicate the involvement of a third ET receptor subtype. Cloning and characterization of ETC receptor form Xenopus laevis dermal melanophores suggest high affinity for ET-3 peptide and a 47% and 52% sequence similarity with ET_AR and ET_BR respectively that could possibly explain persistent vasoconstriction. However, the mammalian complement is yet to be recognized [50]. Analogous to the effects observed with exogenous ET-1, the vasoconstriction caused by endogenous ET-1 in rat pancreas was also not hindered by BQ123, however surprisingly BQ788 diminished the total pancreatic and islet blood flow. Selective antagonism of B receptor impedes the release of nitric oxide (NO) responsible for vasodilation, which explains the reduced blood flow. Another possibility is that, in addition to its role as a vasodilator, ET_B receptors also act as “clearance” receptors for endothelins [239]. Fukuroda *et al.* demonstrated that intravenous infusion of BQ788 but not BQ123 reduced the uptake of ^{125}I-ET-1 in the lungs and significantly inhibited the clearance of ET-1 from circulation [240]. Also, in transgenic ET_BR deficient mice, increased circulating levels of ET-1 is reported provides further support for the role of ET_BR in clearance [241]. Additionally, *ex vivo,* perfusion

caused by ET-1 in isolated mice islets is reversed by ET_AR and ET_BR antagonism; indicating the presence of both receptors on the islets.

6. Endothelins in Pancreas Pathophysiology

6.1. Role in Pancreatitis and Inflammation

The deregulation of pancreatic microcirculation and capillary leakage plays a major role in the pathogenesis and is hallmark of severe pancreatitis. A growing body of literature suggests that these microcirculatory disorders facilitate necrosis of acinar cells, release of proteases, activation of pro-inflammatory cytokines, the infiltration of neutrophils, and are believed to contribute to organ dysfunction [242] [243] [244]. Alterations in the pancreatic blood flow may include activation of endothelial cells and release of endothelin peptides, which are important mediators in the determining the pathophysiology of pancreatitis [245] [246]. Plasma endothelin-1 levels correlate with pancreatitis severity and are a useful indicator for diagnosis. Using enzymo-immunological analysis Milnerowicsz *et al.* demonstrated that the plasma ET-1 concentration in patients with severe acute pancreatitis with necrosis was 6.37± 1.9 pg/ml as compared to control 1.37 pg/ml. Additionally, patients with severe acute pancreatitis without necrosis, mild acute pancreatitis and chronic pancreatitis had increased levels of ET-1 [247]. Additionally, immunohistochemical localization of ET-1 shows a significant correlation of ET-1 immunostaining and the degree of severity in patients with chronic pancreatitis [205]. Further, a marked increase in the plasma ET-1 levels and strong immunohistochemical reaction for ET-1 is reported in individuals who smoke and in patients with chronic pancreatitis compared to non-smoking healthy subjects [248]. Additionally, Borissova *et al.* and Goerre *et al.* also demonstrated a significant increase in plasma ET-1 levels after exposure to cigarette smoke [249] [250] suggesting a direct effect of tobacco smoke on the peptide release. The elevated levels

of ET-1 exhibit positive correlation with orocecal transit time (OCTT) indicating a close association with gastrointestinal dysmotility in patients with acute pancreatitis [251]. Moreover, elevated serum concentration of ET-1 in circulation is associated with leukocytosis and correlates with AP and multi-organ dysfunction syndrome [252].

Though well studied in context of acute pancreatitis (AP) and in experimental disease systems, doses, time of application, and the effects of endothelin-1 and its antagonists are controversial, as ET-1 has been shown to play dual role in both protection and disease aggravation. Gene expression profiling of dibutyltin dichloride (DBTC) induced pancreatitis in Lewis rats revealed upregulation of ET-1 as the candidate gene associated with pancreatic inflammation. In addition, treatment with selective ET_AR (BQ123) and ET_BR (BQ788) inhibitors reverses the inflammatory response [253]. Zhang *et al*. observed in a severe acute pancreatitis model that therapy with the anti-inflammatory dexamethasone drug significantly reduced levels of ET-1 and NO compared to sham operated animals [254] [255]. Zhang *et al.* also reported that retrograde infusion of sodium taurocholate in a multiple injury model of pancreatitis showed a remarkable increase in serum ET-1 levels [256]. Assessment of early pathological abnormalities included evident edema and necrosis in the pancreas and multiple organ injury accompanied by prominent increase in levels of ET-1, NO, IL-6, TNF-α and PLA2 in severe AP induced by sodium taurocholate [257].

Similar to sodium taurocholate induced pancreatitis, histological examination after topical ET-1 administration induces alterations in tissue integrity, profound edema, focal acinar cell necrosis and a marginal increase in serum amylase levels compared to control group [237]. Liu *et al.* observed that in presence of ET-1 cerulean-stimulated acute pancreatitis exhibit remarkable changes in pancreas morphology. This was followed by hemorrhage and an increase in serum amylase and elastase levels. Interestingly, intra-aortal infusion of ET-1 alone failed to alter the pancreas integrity

[258]. Foitzik *et al.* also observed similar results where ET-1 superimposed on mild acute necrotizing pancreatitis (ANP) favored acinar and peripancreatic fat necrosis with prophylactic treatment of ET_AR antagonist LU-135252 ameliorating the disease. Also, a synergistic effect is witnessed in capillary blood flow reduction in rats infused with alcohol and ET-1 and was subsequently reversed by ET_AR antagonist LU-135252 [259]. In addition, transgenic mice with ET-2 over-expression develop severe pancreatitis and significant increase in acinar cell necrosis with mild increase in trypsinogen activated peptides (TAP) levels compared with non-transgenic littermates controls [260]. Further, studies from the same group also suggest a significant decrease in mortality in ANP induced rats exposed to pharmacological ET_AR inhibitor LU-135252 compared to the untreated group. Pharmacological inhibition using LU-135252 is paralleled by improved capillary blood flow, stabilized capillary permeability, reduced extravascular fluid sequestration and intravascular fluid loss, and a decrease in hematocrit concentration without mitigating signature of disease severity such as acinar cell necrosis [261] [262] [263]. Activation of trypsinogen and its presence in the plasma is an accurate indicator of AP and correlates with necrosis, disease severity and hemorrhage [264] [265]. Ultra-structural examination of Wistar rats with AP induced by intra-peritoneal injection of sodium taurocholate treated with either selective ET_AR (LU-302146) or dual ET_AR and ET_BR (LU-302872) antagonists reveal a 2-3 fold reduction of trypsinogen activation index in pancreas tissue, mainly by blockade of ET_AR. In addition, pharmacological inhibition of both the ET_A and ET_B receptors showed necrosis in a few acinar cells, and enhanced auto phagocytosis with insignificant effects in recruitment of inflammatory cells [266]. Additionally, Dlugosz *et al.* identified similar degree of trypsinogen attenuation and reduced score of necrosis using either mixed ET_A/ET_B or ET_A inhibitors in AP [267]. Contrasting results were reported by the same group in cerulean induced rat AP treated with either selective ET_AR (LU-302146) or non-selective ET_A/ ET_B receptor (LU-302872)

antagonists [268]. Electron microscopy visualization of cellular organelles reveals a slight increase in vacuolization of acinar cells, with disorganized endoplasmic reticulum, and total cytoplasm degradation with no alteration in pancreatic edema and necrotic score upon therapy. Surprisingly, treatment displayed no effects on the trypsinogen activation index as compared to untreated AP, although infiltration of inflammatory cells was decreased. The authors rationalize that the difference in the outcomes of ET receptor antagonism in different models of AP can be explained by discrepancies in the pancreatic microcirculation. Also, the divergences of potency, dosing of ET inhibitors and the pancreatitis experimental model system used can explain the contradiction in both the studies. The decrease in the inflammatory cell population is in harmony with previous findings as well. Plusczyk *et al.* demonstrated that pre-treatment with low dose (4mg/kg body weight) and not high dose (10mg/kg body weight) of ET antagonist Tenzostan (Ro-61-0612) is effective in abrogation of pancreatitis induced parenchymal tissue injury, microcirculatory disorders, improve perfusion and infiltration of leukocytes in the tissue [269]. An increasing body of evidence indicates that coupling of ET-1 to the ET_AR is responsible for chemotaxis, migration and adhesion of leukocytes and selective targeting of ET_A and not ET_B receptor diminish the migration [156] [270] [271] [272]. The high concentration of the inhibitor used in this particular study mediates simultaneous inhibition of both receptors while at low dose inhibits only ET_AR. The involvement of the ET_B receptor in clearance of ET-1 isoform from circulation and release of vasodilator nitric oxide (NO) to counteract the release of ET-1 and mediate vasoconstriction is well studied [240] [273] [274]. Thus, synchronized blockade of both receptors using high dose of antagonist abolishes the clearance mechanism thereby maintaining high levels of ET-1, which masks the favorable outcomes of ET_AR inhibition indicating that the coupling of ET peptide with ET_AR is associated with pathogenesis of pancreatitis. These findings were confirmed and extended in a bile salt induced rat pancreatitis model where

mixed therapy using dual ET_A and ET_B antagonist Bosentan failed to provide survival benefit [275].

Contrary to the previous findings, Todd *et al.* investigated first the consequences of ET receptors blockade in a murine model of acute hemorrhagic pancreatitis. Treatment with mixed ET_A/ET_B antagonist PD145065 decreased the severity of pancreatitis with no effects in the mortality. Additionally, reduction in serum amylase levels, markers of systemic inflammation (IL-6 and IL-10) and diminished myeloperoxidase activity was also noted upon treatment [276]. In addition, further studies by Eibl and co-workers also support the favorable outcomes of ET_AR antagonism over ET_BR using LU-135252 which blocks receptor A and B at low (50mg/kg body weight) and high doses (100mg/kg body weight) respectively. After induction of AP by retrograde infusion of glycodeoxycholic acid, blockade of ET_AR and not ET_BR was effective in stabilizing the capillary permeability; decrease fluid loss and leukocyte rolling [277]. In addition, an increase in capillary permeability after exogenous administration of ET-1 in AP is reversed by ET_AR antagonism thereby improving the capillary blood flow and reduced leukocyte rolling. However, simultaneous inhibition of both receptors displays comparable effects than that ET_AR inhibition alone [263] [278]. In a separate approach to evaluate the efficacy of different therapeutic regimens, Eibl and his co-workers utilized ET_AR, platelet receptor activating receptor (PAF) antagonists, and intercellular cell adhesion molecule-1 antibody (ICAM-1) either alone or in combination in rats after 6 hours of administration of bile salt and cerulein to induced pancreatitis. In harmony with previous reports, ET_AR antagonist LU-135252 was found to be more effective than PAF antagonist and ICAM-1 antibody in improving or reducing capillary leakage. Also, combination therapy of ET_AR antagonist either with PAF or ICAM-1 was found to be more beneficial than the combination of PAF antagonist and ICAM-1 antibody in stabilizing capillary permeability [279]. Similar to previous observation, ET_AR

antagonist LU-135252 was found be more advantageous in enhancing capillary blood flow and reducing leukocyte rolling than ICAM-1 antibody in a delayed model of AP after 12 hours infusion of glycodeoxycholic acid and cerulein [280].

Despite its role in promoting damage to pancreatic parenchyma and deterioration of acinar cells, some conflicting reports also suggests the protective role of ET-1 in context of AP. Kogire and colleagues witnessed decrease in pancreatic edema, acinar cell vacuolization and inflammatory cells infiltration in pancreas tissue after intravenous infusion of ET-1 plus cerulein compared to cerulein alone in a rat model system. Surprisingly, this ET-1 mediated amelioration of experimental pancreatitis was neutralized by selective ET_AR antagonist BQ123 [281]. Martignoni and coworkers examined the influence of selective ET_AR (LU135252 and BSF208075) and non-selective, ET_A/ET_B (BSF420627) antagonists in three severity groups of AP (4%, 5% and 6% sodium taurocholate). Prophylactic application of both selective and non-selective antagonists failed to provide survival benefit and restoration of pancreatic damage [282]. Besides its involvement in maintaining the vascular tone, ET-1 also participates in the synthesis of prostaglandins [283] [284] and beneficial results seen could be dependent on secondary production of prostaglandins. The cytoprotection offered by prostaglandins (type A, E or F) in response to noxious agents and anti-inflammatory compounds is reported in gastric mucosa [285] [286], small intestine [287], kidney [288] and liver [289]. An increasing body of evidence also suggests the protective role of prostaglandins and its analogs in cerulein and sodium taurocholate induced AP by hindering the activation of pancreatic digestive enzymes [290] [291] [292] [293]. On a similar note, Dlugosz and co-workers demonstrated the favorable role of all three ET isoforms on the histology and ultrastructure of the pancreas. Exogenous application is followed by diminution of trypsinogen activation and reduced polymorphonuclear cells migration in the pancreas; however aggravating results was seen with ET_AR antagonist [294] [295]. In addition,

using NF-kB inhibitor (pyrrolidine dithiocarbonate, PDTC) in combination with ET-1 and ET-3 a synergistic effect was observed in attenuation of trypsinogen activation in cerulein-induced pancreatitis [296].

Hypovascularity of the pancreas makes it vulnerable to ischemia/re-perfusion injury and is a major complication associated with graft pancreatitis. Given the involvement of ET-1 in microcirculatory disturbances of the ischemia/re-perfusion, recent studies have indicated a beneficial role of ET inhibitors in attenuating disorders in the liver [297] [298] [299], kidney [300] and small intestine [301]. Recently the blockade of ET receptors and its impact on the pancreatic transplantation is being addressed in few studies. In a pig model, selective antagonism of ET_AR using BSF 208075 reduces ischemia/re-perfusion induced injury accompanied by significant reduction in injury to pancreatic parenchyma and tissue edema. The prophylactic treatment is also paralleled by decrease in inflammatory cell infiltration and pro-inflammatory cytokine IL-6 and ET-1 levels [302] [303] [304]. Also in a rat experimental model, systemic oxygen perfusion using nitric oxide gas followed by orthotropic liver transplant reduced ET-1 levels, neutrophil infiltration and production of prostacyclin [305] [306]. Findings by Marada *et al.* suggested the promising effects of cold storage conditions in a pancreas transplantation model and showed significant reduction in TGF-β and ET-1 levels [307]. **Figure 2** describes the sequence of ET-1 mediated events in inflammation.

6.2. Role in Pancreatic Stellate cells and Pancreatic Cancer

Pancreatic stellate cells (PSCs) are the resident cells of the pancreas located in the peri-acinar region and are characterized by the presence of vitamin A droplets in the cytoplasm in quiescent state [308]. Once activated in response to external stimuli, inflammation or injury, PSCs acquire a myofibroblasts like phenotype and secrete increase amount of extracellular matrix (ECM) proteins rich in collagen. The excess deposition of this fibrous tissue rich in collagen is termed as fibrosis or desmoplasia,

regarded as a hallmark for pancreatitis and pancreatic cancer [309] [310] [311]. Literature reports have suggested several extracellular mediators and intracellular pathways in PSCs activation. The external stimuli include soluble mediators, cytokines, oxidative stress, hypoxia, TNF-α; platelet derived growth factor (PDGF), ethanol and its metabolites and TGF-β. Accumulating evidence reveal PDGF as mitogen for PSCs and TGF-β1 as a main stimulator to induce collagen and α-SMA (smooth muscle actin) to maintain the pro-fibrotic phenotype of PSCs [312] [313] [314]. In the context of acute pancreatitis in response to necro-inflammation where the injury is limited, PSC proliferation occurs at an early stage and is believed to play a role in repair or regeneration of the pancreas tissue and maintenance of the tissue architecture by maintaining the ECM turnover. In contrast, in chronic pancreatitis the PSCs are activated due to repeated inflammatory insults resulting in fibrosis and aggravation of disease [315]. Additionally, this aggravation results in sustained increase expression of α-SMA and confers augmented contractile potential. Masumune *et al.* observed that this contraction is caused by ET-1 via the ET_BR as selective inhibition abolished the contractility. Additionally, both receptors are involved in enhanced migration of PSCs isolated from male Wistar rats, revealing the varied role of receptors in response to ET-1 peptide [316]. Further, studies by Stumpe and coworkers implied that excess secretion of ET-1 from activated but not quiescent PSCs possibly impacts the pathogenesis of pancreatitis through an autocrine and paracrine loop [317]. To gain mechanistic insights in the induction and maintenance PSCs activation in pancreatitis, Jonitz and coworkers observed that treatment of PSCs with TGF-β1 and TNF-α stimulated the secretion of ET-1 peptide by increased binding to smad3 and NF-kB respectively. Also, treatment with ET-1 induced the phosphorylation of ERK1/2 and p38, a marker of PSCs activation (α-SMA) and pro-inflammatory cytokines (IL-1β and IL-6) [318]. Nevertheless, dual ET_A/ET_B antagonist Bosentan displays an anti-tumor effect in PC cells and anti-fibrotic effects in

PSCs by marked reduction in the expression of CTGF (connective tissue growth factor) and α-SMA [319] indicating a pro-fibrogenic role of ET-1 in PC. Previous investigations have suggested the inhibitory role of interferons in the activation of PSCs and extracellular matrix deposition in pancreatic and hepatic fibrosis [320] [321] [322]. Using gene expression analysis in IFN-γ treated PSCs, Fitzner and his group showed down regulation of the ET-1 isoform and CTGF in a STAT-1 dependent manner [323]. Further, proteomic analysis of the conditionally generated immortalized human non-tumor (NPSC) and tumor derived (TPSC) pancreatic stellate cells showed differential expression of ET-1 with a significant p value in TPSC compared to NPSC cells [324].

Soon after the discovery of ET-1 in 1988 from porcine endothelial cells earlier reports have also revealed the secretion of all three ET isoforms from pancreatic cancer cell lines. With specific ET-1 radioimmunoassay, production and concentration of immuno-reactive ET-1 was detected in PC cell lines, more commonly than CA19-9, CEA and DUPAN-2 tumor markers. In contrast, ET-2 and ET-3 were detected in only one cell line tested [325] [326]. Further *in vitro* and *in vivo* evidence of ET-1/ET receptor system in PC comes from the study by Bhargava and his colleagues. Using selective ET_A antagonist LU-302146, the proliferations of three PC cell lines was prominently decreased. In addition, LU-302146-treated orthotropic tumors displayed reduced tumor burden and metastatic spread of the disease, reduction in micro-vessel density and increase in overall survival [327]. In an experimental model of PC, treatment with the angiotensin inhibitor losartan target pro-fibrotic gene signatures ET-1 and CTGF thereby enhancing drug delivery and potentiating chemotherapy [328]. Recently, Fukuda *et al.* reported the diagnostic method to detect ET_BR expression in cell lines and tissues using an ET_BR transfected cells. Immunostaining and immunoblot analysis suggested no biologically specific staining in PC cells, xenograft tumors and human pancreatic cancer tissues; however, mild staining in acinar and islet cells was reported [329]. Contrary to

previous reports, a recent study stated the expression of ET-1 axis in human PC cases. Cook and his co-workers determined the immuno-histochemical expression of ET-1 and ET_BR in surgically resected human PC specimens and found no expression of ET_AR Additionally, this increase in expression of ET-1/ET_BR axis was found to be correlative to micro-vessel density and VEGF expression [330].

7. Conclusions and Future perspective

The endothelin axis is a critical regulator of various physiological and pathophysiological processes extending from maintaining vasomotor tone to cancer progression. The central involvement of this axis in promoting cellular cross talk and signaling within the human microenvironment holds promise for the future clinical trials. Given the extensive fibrosis and hypo-vascular nature of pancreatic tumors and its involvement in fostering the stromal-tumor cell interaction this axis represents a new and yet unexplored target for pancreatic cancer targeted therapies. Pre-clinical and experimental studies in different cancer settings with either selective or dual ET receptor antagonists used as monotherapy or in combination with cytotoxic drugs can provide considerable therapeutic benefit. Additionally, the favorable effects of ET axis antagonism observed in fibrosis and vascular disorders further represents a potential target for cancer treatment. However, systematic targeting of the axis with ET antagonists in combination with conventional chemotherapy or radiotherapy in PC need to be evaluated.

Figure 1: Endothelin axis: molecular components and physiological roles. Schematic representation of the synthesis of the three isoforms of endothelins. The primary translational product is a pre-proendothelin form, which is 203 amino acid polypeptide. This peptide is cleaved by furin like proteases to yield biologically active big ET-1. Big ET-1 is further cleaved by endothelin converting enzyme (ECEs) to produce mature ET-1. Amino acids that differ between ET-1 and ET-2 and ET-3 are shown in yellow**.** ECEs have four isoforms, ECE-1, ECE-2, ECE-3, and ECE-4 and have different cellular localization. Once formed the mature peptides can bind to either endothelin A receptor (ET_AR) or endothelin B receptor (ET_BR). ET-1 has an equal binding affinity for both the ET_AR and ET_BR receptors; however, there is difference in binding affinity for other two ET isoforms. Both ET receptors belong to G-protein–coupled receptors and are differentially expressed according to cell type. ET_AR is expressed predominantly on smooth muscle cells whereas ET_BR is expressed on endothelial cells. Both receptors are expressed in various other cell types and regulate diverse functions.

Figure 1

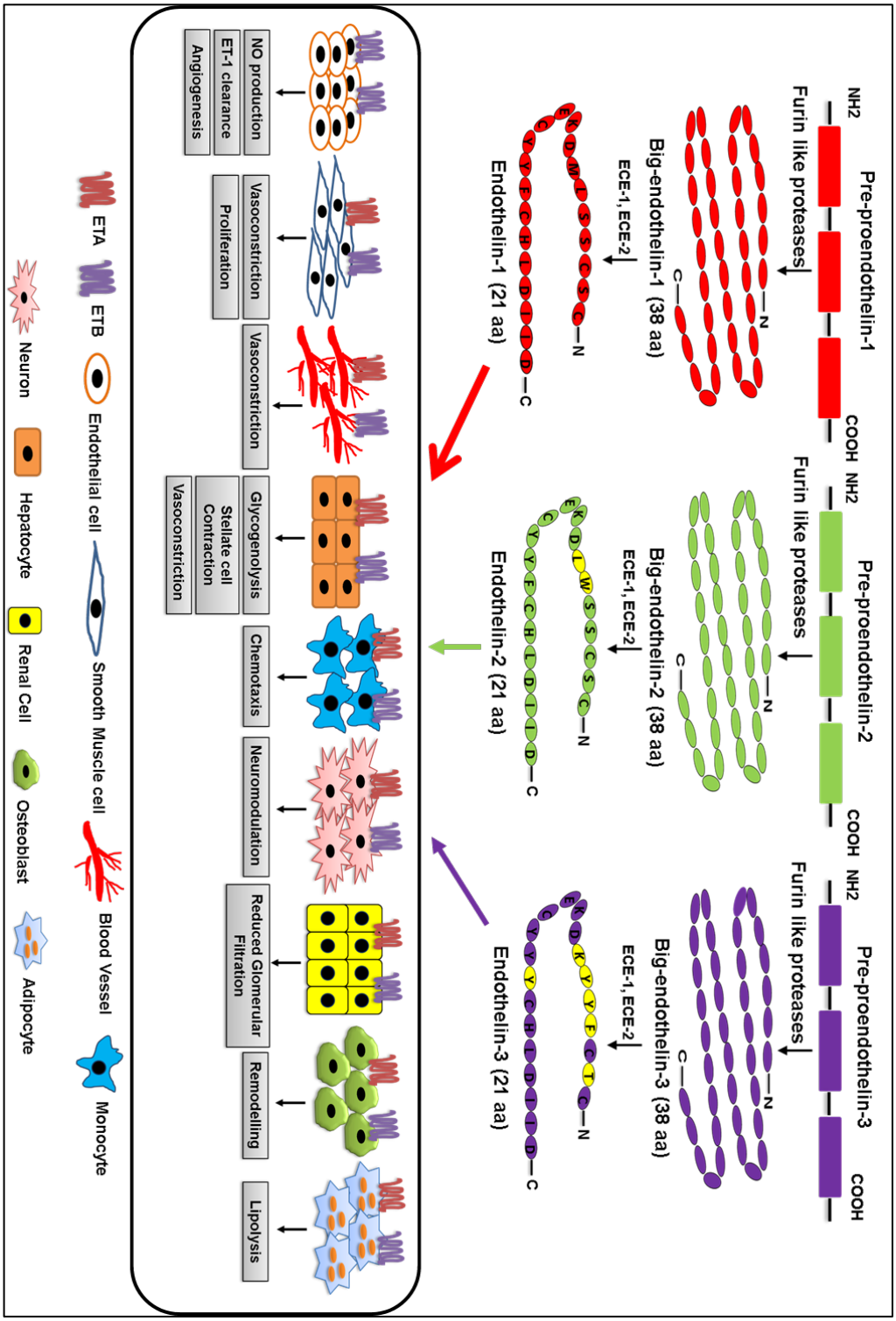
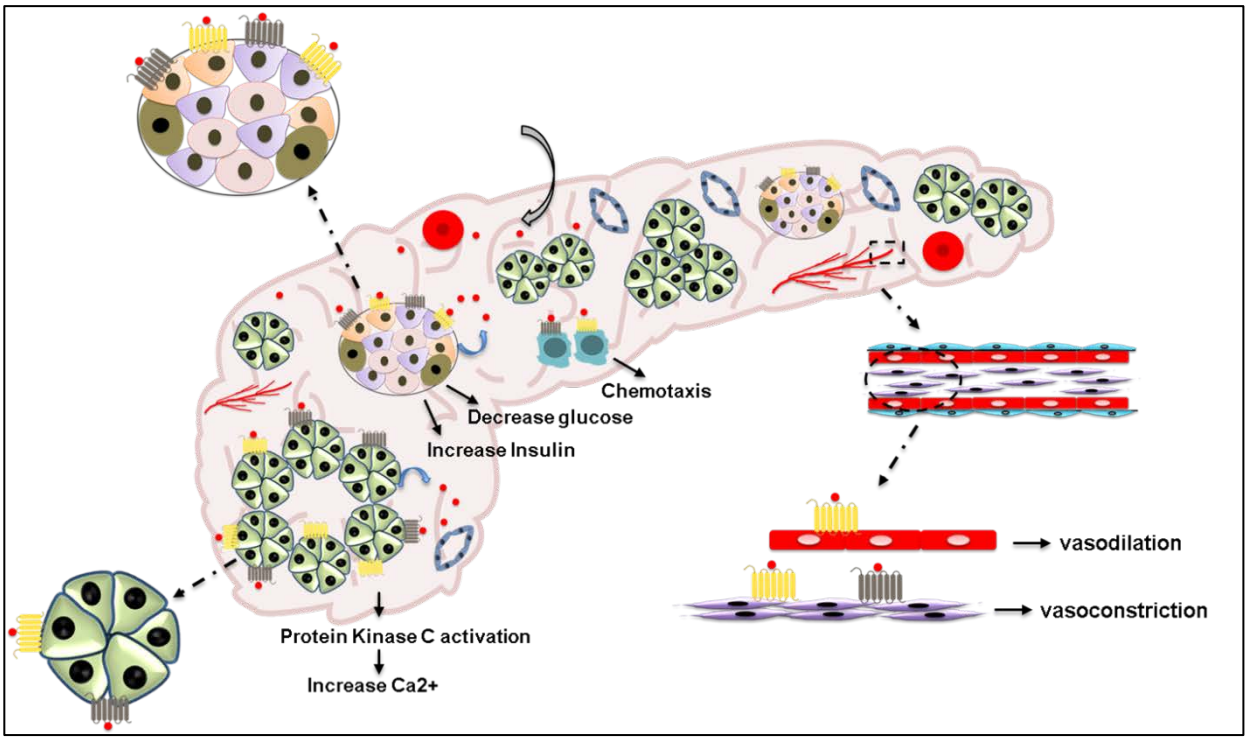

Pre-proendothelin-1
NH2
COOH
Furin like proteases
Big-endothelin-1 (38 aa)
ECE-1, ECE-2
Endothelin-1 (21 aa)
Pre-proendothelin-2
Big-endothelin-2 (38 aa)
Endothelin-2 (21 aa)
Pre-proendothelin-3
Big-endothelin-3 (38 aa)
Endothelin-3 (21 aa)
NO production
ET-1 clearance
Angiogenesis
Vasoconstriction
Proliferation
Vasoconstriction
Glycogenolysis
Stellate cell Contraction
Vasoconstriction
Chemotaxis
Neuromodulation
Reduced Glomerular Filtration
Remodelling
Lipolysis
ETA
ETB
Endothelial cell
Smooth Muscle cell
Blood Vessel
Monocyte
Neuron
Hepatocyte
Renal Cell
Osteoblast
Adipocyte

Figure 2: Role of Endothelin(s) in pancreas physiology. In normal pancreas, infusion of endothelin-1 (ET-1) stimulates insulin secretion and decreases the blood glucose levels by acting on the islet cells expressing endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR). The insulinotropic action mediated by the ET-1 indicates a direct involvement in regulating the glucose homeostasis by glucagon release involving influx of Ca2+ influx and protein kinase C activation. Additionally, in pancreatic acini, interaction of ET-1 with both ET_AR and ET_BR induces internalization involving increase in intracellular calcium with no effects on amylase secretion. Vascular smooth muscle cells express both ET receptors whereas endothelial cells express only ET_BR. In the pancreas microcirculation, ET-1 acting on the blood vessels induces vasoconstriction, however, nitric oxide release by ET-1/ET_BR interactions favors vasodilation. Administration of ET-1 decreases pancreatic blood flow and perfusion with minuscule effects on blood pressure.

Figure 2


Chemotaxis
Decrease glucose
Increase Insulin
vasodilation
vasoconstriction
Protein Kinase C activation
Increase Ca2+

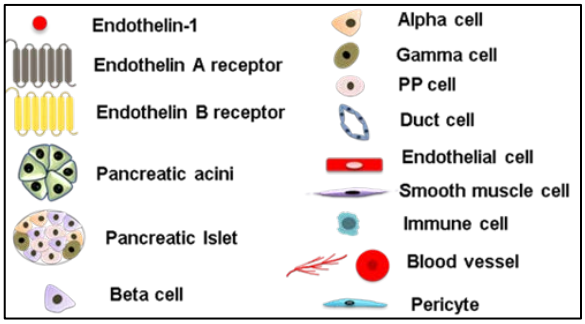
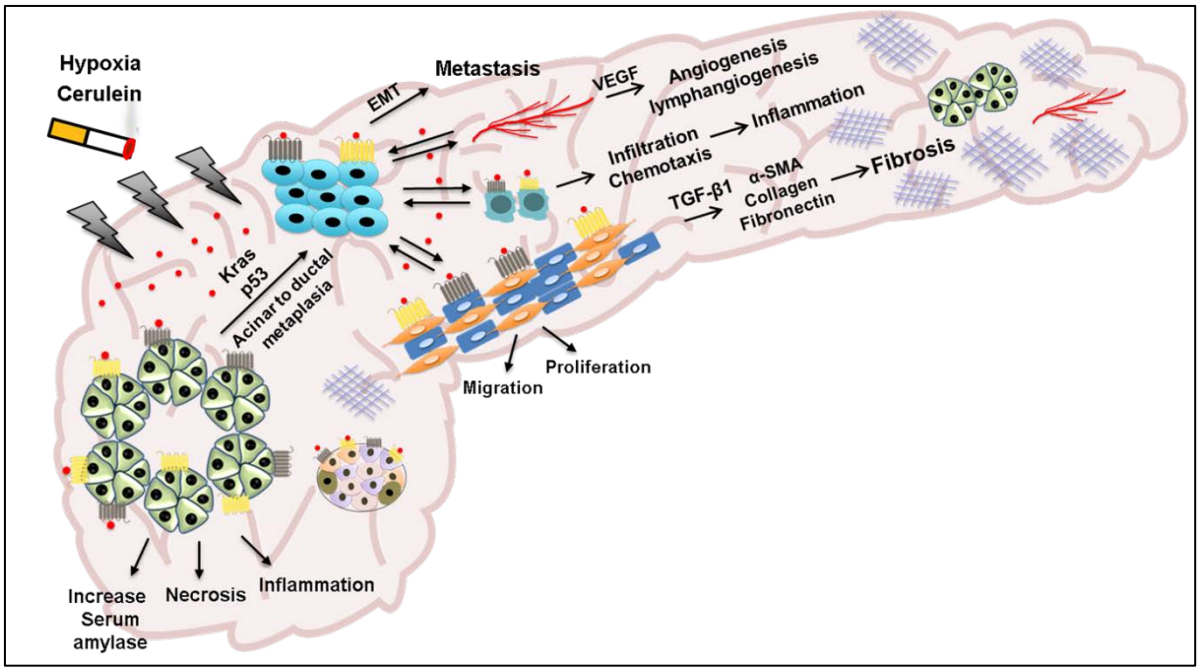
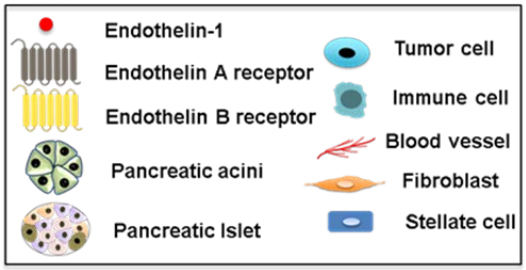

Endothelin-1
Endothelin A receptor
Endothelin B receptor
Pancreatic acini
Pancreatic Islet
Beta cell
Alpha cell
Gamma cell
PP cell
Duct cell
Endothelial cell
Smooth muscle cell
Immune cell
Blood vessel
Pericyte

Figure 3: Role of endothelin(s) in pancreas pathophysiology. In response to stress due to hypoxia, acute, or chronic insults elevated production of ET-1 induces alterations in pancreas integrity, promotes acinar cell necrosis, increases in serum amylase and elastase levels and promotes inflammation. However, the prolonged inflammatory insult and damage to pancreatic parenchyma and gain of mutations such as Kras can lead to trans-differentiation of acinar cells to ductal phenotype. The tumor cells expressing ET_AR and ET_BR also secrete ET-1, which can interact with different cellular components within the microenvironment, and promote tumor progression and metastasis. Interaction of ET-1 with receptors present on the cancer associated fibroblasts and stellate cells promote proliferation and modulate tumor stroma by increase expression of extracellular matrix proteins (α-SMA, collagen, fibronectin) thereby promoting fibrosis. In response to ET-1/ET_BR interaction, endothelial cells promote angiogenesis and neo-vascularization in a VEGF dependent manner. Within in the immune microenvironment, ET-1 signaling can facilitate chemotactic migration of monocytes and macrophages at the inflamed sites and homing of T cell population within the tumors.

Figure 3


Hypoxia
Cerulein
Metastasis
EMT
VEGF
Angiogenesis
lymphangiogenesis
Infiltration
Chemotaxis
Inflammation
TGF-β1
α-SMA
Collagen
Fibronectin
Fibrosis
Kras
p53
Acinar to ductal metaplasia
Proliferation
Migration
Increase Serum amylase
Necrosis
Inflammation


Endothelin-1
Endothelin A receptor
Endothelin B receptor
Pancreatic acini
Pancreatic Islet
Tumor cell
Immune cell
Blood vessel
Fibroblast
Stellate cell

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CHAPTER 1 B

Dissertation General Hypothesis and Objectives

1. Background and Rationale

Endothelins (ETs) are family of three 21 amino acid vasoactive peptides; ET-1, ET-2 and ET-3 that exerts their effects via two G-protein couple receptors ET_AR and ET_BR expressed on various cell types. Endothelin-1 (ET-1) is an important signal messenger in various pathological malignancies including cancer [1] [2]. The peptide regulates and control different aspects in the cancer progression such as proliferation, angiogenesis, epithelial to mesenchymal transition (EMT), immune modulation and metastasis [3] indicating its potential impact on multitude processes in the tumor microenvironment (TME) of cancer.

Several studies have hinted the involvement of ET-1, ET_AR and ET_BR in pancreatic inflammations and are important mediators in the determining the pathophysiology of pancreatitis [4] [5] [6]. The autocrine and paracrine effects mediated by endothelin (ET) axis induce the pro-inflammatory cytokines, exacerbating the inflammatory process [7]. Recent evidence indicates the role of endothelin(s) in TME in few scattered studies, however the comprehensive representation of the overall axis in any given cancer type is unknown and expression pattern in human PC is not well studied.

PC is characterized by extensive desmoplasia and heterogeneous blood flow, which is a major cause for the poor sensitivity of PC to chemo-and radiation therapy. Once activated in response to stimuli or injury, pancreatic stellate cells (PSCs) acquire a myofibroblasts like phenotype and secrete increase amount of extracellular matrix (ECM) proteins rich in collagen [8] [9]. Treatment with ET-1 induces enhance contraction and migration of PSCs in ERK and MAPK dependent manner. Additionally, it also stimulates marker of PSCs activation, αSMA and CTGF and enhance secretion of pro-inflammatory cytokines, IL-1 and IL-6 [10] [11] [7]

While a number of studies have suggested that ET-1 levels are enhanced in pancreatic inflammations, it has also been claimed that the levels are correlated with the disease severity and inflammation [12] [13]. This notion has been substantiated to a degree by recent studies that demonstrated that marked increase in the plasma ET-1 levels in patients with severe acute pancreatitis and chronic pancreatitis [14] [15] [16].

2. Hypothesis

Autocrine and paracrine signaling along the ET axis promotes pancreatic cancer initiation and progression and contributes to pathophysiological characteristics and aggressiveness of PC

3. Objectives

Aim 1: To determine the expression pattern of ET axis in human PC and mice model with disease progression as well as in various components of tumor microenvironment.

Aim 2: To determine the impact of targeting the ET axis in in vivo (autochthonous tumors in KPC model of PC) and *in vitro* (murine pancreatic stellate cells ad human cancer associated fibroblasts) and investigate the molecular mechanisms responsible for ET-1 mediated induction of pro-fibrotic genes.

Aim 3: To analyze the expression pattern of ET axis in the pancreatic inflammations (acute and chronic) in presence of oncogenic Kras and in pre-neoplastic lesions.

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CHAPTER 2

Materials and Methods

1. Cell lines and culture conditions

The expression of ET-1, ET_AR and ET_BR was analyzed in panel of 13 human PC cell lines (Capan1, MiaPaca, HCG25, Suit2, QGP1, SW1990, Panc1, AsPC1, CD18/HPAF, BxPC3, Colo357, T3M4 and HPAC), human pancreatic duct epithelial cells (HPDE), murine PC cell line (UN-KPC-961) and mouse pancreatic stellate cells (ImPSC.c2). These human PC cell lines were obtained from ATCC and grown in 10% DMEM supplemented with fetal calf serum (FCS) and 100ug/ml of antibiotics (Penicillin and Streptomycin). Human promonocytic cell line, U937 was cultured in 10% RPMI supplemented with FCS, 10mM HEPES buffer, 1mM pyruvate and 100ug/ml of antibiotics. All cell lines were maintained at 37^0C under 5% CO_2. Human fibroblasts cell lines derived from normal, chronic pancreatitis and cancer patients were cultured in 10 % RPMI supplemented with fetal calf serum (FCS) 100ug/ml of antibiotics (Penicillin and Streptomycin) and maintained in either Puromycin (5µg/ml) or Blasticidin (5µg/ml) as a selection agent. Normal fibroblasts, chronic pancreatitis fibroblasts (Patient 1 and Patient 2) and cancer associated fibroblasts (CAFs) (10-32, 10-15, 10-03, E6.E7) were cultured in puromycin while the 10-11 CAFs were maintained in blasticidin. Murine ImPSC.c2 and 10-03 human CAFs were serum starved for 24 hours and stimulated with 100nm of ET-1 for 30 mins, 1hr, 4hr, 8hr and 16hr in either DMEM or RPMI containing 1 % FCS. The antagonists BQ123 (100µM) (Peptides International, PED-3512-PI), BQ788 (20µM) (American peptide, 88-255), Bosentan (25µm) (Key organics, KS1231) were dissolved according to the manufacturer's instructions and added 45 min prior to the addition to the recombinant ET-1.

2. Tissue specimens

Formalin fixed and paraffin embedded pancreatic tissue samples from 38 Whipple resected PC patients were procured from University of Nebraska Medical Centre and

were analyzed for expression of ECE-1, ET-1, ET_AR and ET_BR. These samples were obtained from the UNMC tissue bank following approval from the Institutional regulatory board (IRB # 186-14). Additionally, tissue samples from PC patients from University of Nebraska Medical Centre's Rapid Autopsy Program (RAP) (IRB approved) comprising of normal pancreas, primary tumor, lung metastasis, liver metastasis, lymph node metastasis, omentum/diaphragm metastasis were also evaluated for expression of ET-1, ET_AR and ET_BR for PC metastasis. In this Rapid Autopsy Program, after three hours of death, tissues are harvested from donor patients and are put in liquid nitrogen or formalin for fixation. Microarrays made from these embedded tissues were analyzed for expression after mounting on charged slides. Within the tissue microarray (TMA), control specimen from normal kidney, colon in addition to tumor tissues is also present.

3. Immunohistochemistry and Immunofluorescence

Slides were baked overnight at 56^0C and deparaffinised with xylene followed by rehydration with increasing concentration of ethanol. The slides were then treated for 1 hour with 3% H_2O_2 in methanol to quench the peroxidase activity. 0.01M preheated citrate buffer (pH 6, 95^0C) in microwave for 15 mins was used for antigen retrieval. After cooling at room temperature (RT), the non-specific reaction was blocked using 2.5% horse serum (ImmPress Universal antibody kit; Vector Laboratories, Burlingame, California, USA) for 2h. The sections were then incubated overnight at 4^0C with respective primary antibodies diluted in PBS (anti- ET_AR, 1:1000, ab117521; anti-ET_BR, 1:2000, ab117529; anti-ET-1,1:2000, ab117521; anti ECE-1,1:750, ab189843 anti-F4/80, 1:80, e-Biosciences, 14-4801-82; anti-α-SMA,1:100, ab7817; anti-CTGF, 1:500, ab ab6992; anti-fibronectin, 1:400, ab 2413; anti-Collagen I, 1:500, ab34710; anti cleaved caspase-3, 1:300, cell signaling 9661,). The slides were washed with PBST (4 washes for 10 mins each) followed by anti-rabbit secondary antibody (ImmPress

Universal antibody kit; Vector Laboratories, Burlingame, California, USA) incubation for 30 min at RT. The slides were washed with PBST (4 washes for 10 mins each) and color was developed using 3-3' diaminobenzidine solution (DAB substrate kit, Vector Laboratories). After washing with distilled water, slides were counterstained with Gill's hematoxylin for 5 mins (Vector Laboratories) and dehydrated with graded ethanol and then mounted with Permount solution (Fischer Scientific, Pittsburg, Pennsylvania, USA). All stained slides were scored by a pathologist at UNMC using a Nikon light Microscope and images of the particular area were taken. The staining intensity for ECE-1, ET-1 ET_AR, and ET_BR were graded on a scale of 0-3, 0 being negative and 3 strongly positive. The proportion positive of cell for each of the molecule in a given specimen were scored between 1-4 indicating 0-25% for intensity 1, 26-50% for intensity 2, 51-75% for intensity 3 and 76-100% for intensity 4. Composite score was then calculated by multiplying the staining intensity and proportion positivity in a range between 0-12. Quantification of F4/80 and CD206 positive macrophages and cleaved caspase 3 positive cells were performed by counting cells in 15-20 high-powered fields from each mouse.

For immunofluorescence, the tissues were blocked with 10% goat serum at room temperature for 2h followed by overnight incubation at 4^0C with respective primary antibodies diluted in PBS (anti- ET_AR, 1:300, AER-001; anti-ET_BR, 1:300, AER-002; anti CK19, 1:200, TROMA III, anti- CD31, 1:300, ab32457, anti CD68, 1:100, 14-0688-82, anti-F4/80, 1:80, e-Biosciences, 14-4801-82; anti-α-SMA,1:200, ab7817, anti FSP1,1:300, ab27957, anti-FAP, 1:300, ab53066, anti CD206, 1:300, bs4727R, anti CD4, 1:100, ebioscience, 14-9766-80, anti-CD8, 1:100, ebioscience, 14-0808-82, Anti-FOXP3, 1:100, ab20034, anti CD4, 1:100, ab133616). After overnight incubation the slides were washed with 1X PBS thrice for 10 mins and incubated for 30 mins at room temperature with secondary antibody (FITC conjugated anti-rat, Texas Red conjugated anti-rabbit and FITC conjugated anti-mouse). The slides were washed with 1X PBS

thrice for 10 mins, mounted by DAPI containing Vectashield mounting solution and analyzed using Zeiss (Carl Zeiss Microimaging, Thornwood, NY) confocal laser-scanning microscope, and representative images were captured digitally using the 710 LSM software.

4. RNA isolation, reverse transcription and RT- PCR analysis

QIAGEN RNeasy kit (QIAGEN, Valencia, CA, USA) was used to isolate RNA and cell lines according to manufacturer's protocol. The isolated RNA was converted to cDNA after hybridization with oligodT using Superscript II Reverse Transcriptase (Invitrogen). The cDNA was amplified by PCR using Taq Polymerase in reaction volume of 50ul. For human endothelin -1 and receptors following primer pairs were used: ET-1 (GTC AAC ACT CCC GAC GAC GTT/ CTG GTT TGT CTT AGG TGT TCC TC), ET_AR (CAC TGG TTG GAT GTG TAA TC/ GGA GAT CAA TGA CCA CAT AG), ET_BR (TCA ACA CGG TTG TGT CCT GC/ ACT GAA TAG CCA CCA ATC TT,). For Mouse ECE-1, ET-1 and receptors following primers were used: ECE-1 (GTG GCA TTG GTG TCG TAG TG/ GGA AGA AGA GCT GGT TGC TG), ET-1 (CTT CCC AAT AAG GCC ACA GAC CAG/ AGC CAC ACA GAT GGT CTT GCT AAG), ET_AR (ACC GCC ATT GAA ATC GTC TCC ATC/ TTA GCA AGA AGC TGA GCA GTT), ET_BR (TGA CGC CAC CCA CTA AGA CCT CC/ GCC TTC TGT ATG AAG GGC ACC AG). Obtained PCR products were run on 2% Agarose gel containing ethidium bromide. Human β actin and Mouse GAPDH was co-amplified using β actin and GAPDH primers respectively to ensure the quality of cDNA for PCR in every case.

5. *In vitro* assays of cell migration

To assess migration of ImPSC.c2 in presence of ET inhibitors, ImPSC.c2 ($1x10^6$) were seeded at in a 6 well plate in DMEM supplemented with 10% FBS and incubated overnight. After obtaining approximately 90% confluency, the cells were serum starved

for another 24 hours and an artificial wound is made using 200µl pipette tip. The detach cells were washed with PBS and remaining cells were treated with either ET-1 or antagonists for 45 mins prior to addition of ET-1 in DMEM containing 1% FBS while the control cells were left untreated. Representative phase contrast images were taken at 0 and 24 hours at 10X magnification using Accu-scope microscope attached with Moticam 580 digital camera. The wound closure was measured by calculating the distance between the two edges and ten independent areas in the wound per image was counted using a straight line tool in the Image J software. The mean distance of the ten independent areas at 0 and 24 hours of control and treatment sets was calculated and the percentage of wound closure was measured as mean area at 24 hour/mean area at 0 hour multiplied by 100. The migratory potential of murine macrophages (RAW 264.7) and human monocytes (U937) towards UN-KPC-961 and Panc1 cells respectively was assessed using boyden chamber consisting of a 0.8 µm transwell membrane. Briefly, $1x10^6$ UN-KPC-961 and Panc1 cells were seeded in six well plates in DMEM containing 10% FBS. After overnight incubation the cells were treated with ET antagonists and the macrophage/monocyte cell suspension ($0.5x10^6$ cells) in serum free medium was seeded in the upper chamber of the insert. The migration of these cells was allowed for 24 hours in a humidified atmosphere at 37°C with 5% CO_2. The migratory cells on the lower chamber that invaded through the pores were fixed and stained with Diff-quick staining kit (Dade Behring, Inc). For each membrane the number of cells migrated was counted in 10 random fields at 10X magnification.

6. *In vitro* 2D co-culture using tumor and stellate cells

In cell-cell non-contacted coculture system using boyden chamber the murine stellate cells (ImPSC.c2) were separated from murine tumor cells (UN-KPC-961) derived from KPC mice tumors using a 0.8 µm transwell membrane. $0.5x10^6$ ImPSC.c2 were

seeded in a 6 well plate in DMEM supplemented with 10% FBS. After 24 hours the cells were washed with PBS and treated with either specific ET_AR inhibitor (BQ123), specific ET_BR inhibitor (BQ788), or dual inhibitor (Bosentan) and cultured in presence and absence of same numbers of UN-KPC-961cells cultured in serum free DMEM on the inner chamber of transwell membrane and allowed to interact with ImPSC.c2 for another 24 hours. After the end of the treatment period, ImPSC.c2 were harvested and processed for western blot analysis.

7. *In vitro* assays of cell proliferation

Cell proliferation was measured by the WST-1 assay according to the manufacturer's instructions (Roche). Briefly, $3x10^3$cells were seeded into 96 well plates in triplicates for each cell type. Following 24hr of seeding, the cells were changed to 1% DMEM and treated with ET-1 (1nm, 10nm, 100nm an 1000nm) for 24hr, 48 hr and 72 hr. The absorbance was measured at 440 nm using a SpectraMax 190 (Molecular Devices) microplate reader.

8. Cell cycle analysis

Briefly, $1x10^6$cells were seeded in 60 mm petri dishes in 10% DMEM and allowed to grow for 24 hrs. Cells were serum starved for 48 hr and treated with BQ123 (100μM), BQ788 (20μM) and Bosentan (50μM) for 24 hr. Following treatment cells were trypsinized and fixed in 70% ice-cold methanol for 60 mins, washed in PBS and incubated for 30 min at 4^0C in Telford reagent (90 mM EDTA, 0.1% Triton-X-100, 50μg/ml propidium iodide, 25μg/ml RNase-A in PBS). The total DNA content was measured by fluorescence activated cell sorting method.

9. Annexin V staining and flow cytometry

A total of $1x10^6$cells were seeded in 60 mm petri dishes in 10% DMEM and allowed to grow for 48 hrs. Following treatment with BQ123 (100μM), BQ788 (20μM)

and Bosentan (50μM) for 24 hr, the early and late apoptotic cells were detected using annexin V-FLUOS staining kit (Roche) according to the manufacturer's instructions. Cells were collected and resuspended in the HEPES buffer containing annexin-V – fluorescein and propidium iodide in the dark for 15 mins, and the data were acquired by the flow cytometry

10. Cytotoxicity assay using MTT

Briefly, cells were seeded in 96 well plates in triplicates in DMEM supplemented with 10% FCS and after 24 hours the cells were treated with indicated concentrations of BQ123, BQ788 and Bosentan and incubated at 37^0C for 24, 48 and 72 hours. At the end of each time point 100 μl 5mg/ ml MTT (3-4,5 dimethylthiazol-2yl)-2,5 diphenyltetrazolium bromide was added and the plates were incubated for an additional 4hours at 37^0C. Post incubation, the solution was removed and 100μl of DMSO was added and absorbance was measured at 490nm using microplate reader.

11. Western blotting

Cell lines were processed for protein extraction followed by Immunoblot analysis. Cells were washed with PBS twice and then lysed using Radioimmunoprecipation assay buffer (RIPA) containing 50mM Tris, 5mM EDTA, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate,1mM Na_3VO_4. 200mM sodium fluoride and Protease Inhibitor (Roche diagnostics) and kept at 4^0C for 20 min under mild shaking. Cell lysates were incubated in -70^0C for at least 1hr and subjected to freeze thaw and pass through syringe to disrupt the cell membrane. Lysates were then centrifuged at 14000 rpm for 30 min at 4^0C and the supernatant were collected and protein quantification was done using Bio-Rad protein estimation kit. Immunoprecipitates were then resolve on 12% Polyacrylamide gel under reducing conditions, transferred to polyvinylidene fluoride membrane (PVDF) and blocked with 5% nonfat dry milk in PBS for 2h at RT. The

membranes were then incubated overnight at 4^0C with respective primary antibodies diluted in PBS (anti- ET_AR, 1:5000, ab117521; anti-ET_BR, 1:5000, ab117529; anti-ET-1,1:5000, ab117521; anti-α-SMA,1:200, ab7817; anti-CTGF, 1:2000, ab ab6992; anti-fibronectin, 1:3000, ab 2413; anti-Collagen I, 1:5000, ab34710; anti-desmin,1;10000, ab32632; anti FSP1,1:3000, ab27957, anti-CK19,1:3000, TROMA III, anti-pERK,1;1000, cell signaling 9101, anti-t-ERK, 1:1000, cell signaling 9102; anti-p-AKT, 1:1000, cell signaling, 4060S; anti-t-AKT, 1:1000, cell signaling 4691S; anti-β actin, 1:5000, Sigma A1978). Membranes were washed with Tris buffer saline (TBS) containing 0.1% Tween followed by incubation at RT in HRP (Horse Radish Peroxidase) conjugated anti rabbit, anti-mouse or anti-goat secondary antibodies in 3% nonfat dry milk in PBS at 1:5000 dilutions. The signal was detected using ECL western blotting detection reagents (Amersham, Biosciences, and Buckinghamshire, UK).

For treatment of monocytes, U937 cells were differentiated into macrophages in six well plate containing 10nM/ml phorbol myristate acetate (PMA) for 72 hours. The supernatant was collected and the attached cells were washed with PBS and lysed using RIPA buffer for immunoblot analysis. For IL-4 or IL-13 or IL-10 treatments, the macrophages differentiated using PMA were washed with PBS and incubated with 10ng/ml of each of IL-4, IL-13 and IL-10 for 24 hours. The supernatant was collected and the attached cells are lysed using RIPA buffer. Lysates collected were further analyzed for the expression of ET-1, ET_AR and ET_BR using specific antibodies through Western blot.

12. *In vivo* endothelin axis antagonism using Bosentan

20 weeks old KPC mice (n=4 control group and n=5 mice in the Bosentan group) were housed under specific pathogen-free conditions. All mouse experiments were performed in compliance with protocols approved by the Institutional Animal Care and

Use Committees of the University of Nebraska Medical Center. The dual ET receptor antagonist, Bosentan (Key Organics) was administered daily for continuous 10 days by intraperitoneal injection at a dose of 1 mg/kg whereas the control group received saline. Throughout the course study, mice were monitored for weight loss and others signs of abnormalities. Mice were sacrificed at the end of the treatment period and the weight of pancreas was recorded. Tissues were then fixed in 10% formalin and embedded in paraffin for histopathological analysis and RNA analysis.

13. RNA extraction from mouse tissues, reverse transcription and real time PCR analysis

RNA was extracted from the tumors removed from KPC (Pdx1-Cre, p53 $^{(R172H)}$ Kras^{G12D}) mice (saline or Bosentan treated), KC (Pdx1-Cre, Kras^{G12D}) mice (LSL-K-ras^{G12D} and K-ras^{G12D}; Pdx-1cre referred as unfloxed and floxed, respectively exposed to cigarette smoke and KC (Pdx1-Cre, Kras^{G12D}) mice exposed to cerulein day 0, 2, 7 and 21 post treatment using mirVANA isolation kit (Ambion, Foster city, CA, USA) by a standard protocol. All animal experiments were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC). The animals were exposed to cigarette smoke or cerulein treatment as previously described [1] [2]. Genomic DNA contamination was removed by Dnase treatment using RNeasy mini kit (Qiagen) and RNA integrity was measured spectrophotometrically. The isolated RNA was converted to cDNA after hybridization using random hexamers using Superscript II Reverse Transcriptase (Invitrogen). Following primer pairs were used: ECE-1 (TCA CGC TTT CGA TGA TCA AG/GTA TTG CTG CAC CAT GCA CT), ET-1 (GCT GGT GGA AGG AAG GAA AC/TTG TGC GTC AAC TTC TGG TC), ET-2 (AGA CTG GCA AGA TGT GGA CT/ TTC TTG TCA CCT CTG GCT GT), ET-3 (TGC GTT GTA CTT GTA TGG GG/ AGT CTC CCG CAT CTC TTC TG), ET_AR (CGG CAT TAA

CCT GGC AAC/ATG AGG CTT TTG GAC TGG TG), ET_BR (TCG CTC TGT ATT TGG TGA GC/ TTC AGG CAG GAC TGC TTC TC). Real time PCR was performed using Light Cycler 480 SYBR green I master mix (Roche Diagnostics, Indianapolis, IN, USA) in the Light Cycler 480II (Roche Diagnostics). The relative amount of expression was calculated using $2^{\Delta\Delta}C_T$ method. Statistical comparisons were made using student's *t*-test and $P<0.05$ was considered statistically significant. The average of the three independent analysis for each gene was calculated and normalized to Gapdh.

14. Microarray analysis

Mouse fibrosis RT^2 Profiler PCR Array (PAMM-120ZF, Qiagen) was used to evaluate the differential gene expression. The array evaluated the expression of 84 genes involved in facets of fibrosis, like extracellular matrix and cell adhesion, growth factor production and signal transduction. Real time PCR detection was carried out according to manufacturer's instructions. The PCR components cocktail was prepared by adding 1350µl real time PCR SYBR green master mix and 1350µl of nuclease free water to 20µl of cDNA. For the real time PCR detection 25 µl of the PCR cocktail was added to each well of 96 well RT^2 Profiler PCR Array. The array was then cycled on a Roche Light Cycler 480. The thermocycler parameters were 95^0C for 10 min, followed by 45 cycles of 95^0C for 15 s and 60^0C for 1 min. The experiment was performed in triplicates. For data analysis, the delta-delta CT method was used and the values obtained were exported to template excel sheet containing the algorithms provided by the manufacturer. The fold changes were calculated as the difference in gene expression between the treated and the control tumors. A positive value indicates gene upregulation whereas a negative value indicate downregulation.

15. Perfusion analysis using BQ123

Mice bearing T3M4 (tumor1) and Colo 357 (tumor 2) xenografts were administered with interaperitoneal injection of BQ123 (2 mg/kg) [3]. Perfusion mapping was done by flow sensitive alternating inversion recovery (FAIR) with a rapid acquisition by refocused echo (RARE) readout (RARE factor = 16). Perfusion was measured prior to administration of BQ123 (0 min) to establish baseline and subsequently imaged for 120 min post-administration to monitor the change in perfusion. Anatomic MRI scan was used to indicate the position of tumors and the regions of interest (ROI) in the tumors and muscle used for perfusion analysis. Perfusion maps were captured before and 120 min after injection of BQ123 and were windowed between 0 to 500 ml/(100g tissue per min). Blood flow values were determined from the serial perfusion maps between 0-120 min and plotted as a function of time. Tumor bearing animals were treated with saline (left) or BQ123 (right). 90 min after treatment animals were injected with pimonidazole HCl (Hypoxyprobe), which forms stable adducts with proteins in hypoxic cells. Animals were euthanized 45 mins thereafter and tumors were harvested and processed for immunohistochemistry. Hypoxy-probe-Plus kit was used to detect hypoxia using manufacturer's instructions.

16. Procurement of Animals

The ET-$1^{flox/flox}$ mice were obtained as a kind gift from Dr. Donald Kohan at University of Utah Health Sciences Center [4]. These mice have the exon 2 of the ET-1 gene flanked by loxP sites. The ET-1 mice were crossed with the KPC mice (Pdx1-Cre, p53 $^{(R172H)}$ $Kras^{G12D}$). These KPC mice express localized Cre recombinase regulated by Pdx1 promoter and are on C57BL/6 background. This promoter is expressed in pancreas. Here the Kras gene contains a point mutation G12D and is followed by Lox-Stop-Lox codon. In the presence of Cre, the stop codon is excised and the mutant protein is expressed. The ET-$1^{flox/flox}$ mice were crossed with KPC mice to generate the

intermediate crosses, which were intercrossed in order to generate the ET-$1^{-/-}$ Pdx1-Cre, p53 $^{(R172H)}$ $Kras^{G12D}$final genotype.

17. DNA isolation, genotyping and maintenance of animals

Animals were maintained in accordance with guidelines and protocols approved by the Institutional Animal care and Use Committees (IACUC) of the University of Nebraska Medical Center. The animals were exposed to 12-hour light/dark cycle and were allowed access to food and water *ad libitum*. The tails of mice were clipped at the age of 8 days and the DNA isolated using Maxwell 16 mouse-tail DNA purification kit, Promega, Madison, WI, USA. Following DNA isolation, genotyping was performed using following primers: Wild-type ET-1 (gct gcc caa ttc tga att ctg / gat gat gtc cag gtg gca gaa g), Flox ET-1 (ccc aaa gat tct gaa ttg ata act tcg/ gat gat gtc cag gtg gca gaa g), Kras (cct tta caa gcg cac gca gac tgt aga/ agc tag cca cca tgg ctt gag taa gtc tgc a), p53 (ctt gga gac ata gcc aca ctg/ agc tag cca cca tgg ctt gag taa gtc tgc a), Pdx-Cre (ctg gac tac atc ttg agt tgc/ ggt gta cgg tca gta aat ttg). The reaction for ET-1 primers is cycled 35 times (1 minute at 94°C, 2 minutes at 60°C, and3 minutes at 72°C), which amplifies an approximately 900-bp fragment for both the wild type and flox ET-1 alleles. Mice were observed daily and anal prolapse or any other signs of distress were carefully recorded.

18. Statistical Analysis

Student t test was used to determine the statistical significance between control and treatment group in all the experiments and p value less than 0.05 was considered to be statistically significant. Error bars were given on the basis of calculated standard error values. To measure colocalization using confocal microscopy the images were assessed using ImageJ (National Institute of Health, Bethesda, MD) with Manders colocalization using JaCoP plug-in.

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Chapter 3

Expression of endothelin converting enzyme (ECE-1), endothelin-1 (ET-1), endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR) in pancreatic cancer and its microenvironment

1. Synopsis

Overexpression of Endothelin-1 (ET-1) and its receptors (Endothelin A receptor, ET_AR and Endothelin B receptor, ET_BR) is observed in many solid cancers and is associated with poor prognosis. However, their expression pattern in pancreatic cancer (PC) and its tumor microenvironment (TME) have not been well studied. We have analyzed the expression pattern of Endothelin- converting enzyme (ECE-1), ET-1, ET_AR and ET_BR in both Whipple resected human PC patients and patient tissue microarray, which have both the primary and metastatic sites harvested. Immunohistochemical analysis indicated that expression of the ET axis is restricted to the acinar compartment and islet cells of the normal pancreas whereas a predominant expression of all four molecules is seen the duct cells of the PC. In addition to the primary site, expression was also seen in various metastatic sites. Careful observation suggests that in addition to tumor cells, the expression is observed in tumor blood vessels and immune cells and the expression on tumor blood vessels is correlated with the poor prognosis of the PC patients. Additionally, expression of the axis was also determined in a genetically engineered mouse model of PC (K-ras^{G12D}; *Trp53*$^{R172H/+}$; Pdx-1-Cre). RT-PCR and immunohistochemical analysis suggested that expression increases gradually with disease progression in both pancreatic ducts and the stromal compartment of PC. The expression on various cellular components was analyzed in both murine and human PC tissues using dual confocal microscopy with markers for blood vessels (CD31), tumor cells (CK19), stellate cells (α-SMA) and macrophages (F4/80 & CD68). Further, bioinformatics analysis with the TCGA database revealed positive correlation of ET axis expression with advance tumor grade and stage; and a significant association of fibrotic associated genes and the pathways regulating fibrosis seen upregulated in tumors exhibiting over expression of the ET axis. We show for the first time components of the

ET axis are over-expressed not only in PC cells but also in various cellular compartments of the surrounding microenvironment.

2. Background and Rationale

Pancreatic cancer (PC) is the 10th most commonly diagnosed cancer and the fourth leading cause of cancer-related deaths in the United States [1]. Due to the lack of early diagnostic and therapeutic modalities, PC has an extremely poor prognosis with a dismal five years survival rate of only 2-5% [2]. At the time of diagnosis, the disease has usually metastasized locally to the lymph nodes and to distant organs. While numerous studies have focused on the genetic abnormalities that underpin this disease, much remains unknown regarding this complex, intractable malignancy that unfortunately often only manifest symptoms in patients at advanced, metastatic stages [3]. Most prominent among the mutations driving PC is the Kras oncogene, which is mutated into a constitutively active form (Kras^{G12D}) in around 70% of PC patients [4]. Morphologically PC is characterized by a highly complex stromal compartment consisting of cellular components that include pancreatic stellate cells, endothelial cells, immune cells, neuronal cells, endocrine cells and extracellular matrix [5, 6]. These cellular components of tumor microenvironment (TME), have complex interactions with each other and with the cancer cells. The intricate autocrine and paracrine signaling between these cellular components is believed to orchestrate the initiation, progression and metastasis of PC and contribute to the pathophysiological hallmarks like hypoxia, desmoplasia, perineural invasion and resistance to therapy that define this lethal malignancy.

Endothelins (ETs) are a family of three 21 amino-acid vasoactive peptides ET-1, ET-2 and ET-3 that mediate their effects via two G-protein couple receptors, ET_AR and ET_BR that are expressed on various cell types. While under normal physiological conditions, the ET system is involved primarily in the regulation of basal vascular tone [7], there is emerging evidence demonstrating its expression and role in tumor progression of

several malignancies including melanoma [8], glioblastoma [9], prostate, ovarian, hepatocellular, breast and colorectal carcinomas thus making it a potential therapeutic target [10-14]. Elevated ET-1 levels are observed in many tumors where it is produced both by tumor and stromal cells [15]. Further, overexpression of the two endothelin receptors is observed on tumor cells, endothelial cells, infiltrating immune cells and tumor associated fibroblasts in various malignancies. Activation of the ET_AR in tumor cells promotes proliferation, migration, invasion and cell survival (anti-apoptotic signals) by activating different signaling pathways including MAPK, PKC, EGFR and Akt [15]. The ET axis also plays a critical role in tumor neovascularization by regulating HIF-1α, VEGF, COX2 and prostaglandin E2 and induces proliferative signaling in endothelial cells, pericytes and vascular smooth muscle cells. ET-1 potentiates hypoxia signaling via regulation of hypoxic inducible factor-1α (HIF-1α). Indeed, a reciprocal relationship has been proposed in which ET-1 stabilizes HIF-1α resulting in the activation of HIF-1α-regulated angiogenic genes, including HIF-1α- mediated transcription of ET-1 itself. Thus, ET expression can be influenced by the tumor microenvironment, and ETs then modify that environment through the actions of HIF-1α [16, 17]. These interactions are generally amplified under conditions of hypoxia as compared with normoxic conditions. Due to the high expression of ET_AR on osteoblasts, the ET-1/ET_AR axis promotes osteoblast proliferation and facilitates bone metastasis in prostate and breast cancer. ET_AR antagonism reduces bone metastasis in experimental models and several clinical trials have evaluated the role of ET_AR antagonists in patients with advanced bone metastatic breast and prostate cancer [18-22]. While most of the studies center on the ET_AR-mediated pro-tumorigenic effects, emerging evidence also suggests the critical involvement of ET_BR. In ovarian cancer, high expression of ET_BR on tumor blood vessels is associated with poor infiltration of anti-tumor T-lymphocytes and the ET_BR antagonist, BQ788, enhanced the efficacy of immunotherapy [23]. In contrast, ET_AR activation was

associated with enhanced T-cell infiltration in ovarian tumors [24]. In breast cancer ET-2 is involved in the recruitment of macrophages via ET_BR expressed on macrophages [25].

Very recently, the role of endothelins in the TME has come up in a few scattered studies, however the comprehensive picture in any given cancer type still remains unknown [26]. Tumor cells secreting ET-1 peptide also expresses both ET_AR and ET_BR thereby activating autocrine and paracrine interactions with the tumor stroma [27]. Further, the remodeling of tumor stroma can occur due to ET-1 mediated interactions with ET_AR and ET_BR expressed on stellate cells, and cancer associated fibroblasts . The signals or cues coming from stromal cells facilitate reprogramming of the tumor cells and favor epithelial-to-mesenchymal phenotypic transition or acquisition of stem cell like phenotype [28, 29]. Alternatively, blood and lymphatic endothelial cells increase angiogenesis and lymphangiogenesis in response to ET-1-ET_BR activation. In parallel, the tumor secretes vascular endothelial growth factor (VEGF) in an ET1-dependent manner, inducing sprouting and branching of new vessels from existing vessels [30, 31].

Recently, Cook et al. demonstrated overexpression of ET-1 and ET_BR in human PC [32], however the expression pattern of the overall axis (ECE-1, ET-1, ET_AR and ET_BR) in PC and its complex microenvironment is still under explored. The study here in provides for the first time the expression pattern of the entire ET axis in PC, and in various cellular compartments using both a genetically engineered mouse model and human PC cases. Importantly, TCGA database analysis indicated a positive correlation of pro-fibrogenic genes with the ET axis over expression giving further impetus to our hypothesis that the axis possible plays a pleotropic role in the tumor microenvironment of PC.

3. Results

A. Endothelin-1 and its receptors are expressed in human pancreatic cancer cells

The expression pattern of enodthelin-1 and its receptors was determined in 13 human pancreatic cancer cells and ductal epithelial cells (HPDE) using immunoblot and RT-PCR analysis **(Figure 1A and 1B).** The colon cancer cell line, SW480 and human melanoma cell line SKMEL28 were taken as positive controls for ET-1/ET_AR and ET_BR respectively [33, 34]. Interestingly our results indicate the ubiquitous expression of ET-1 expression in the majority of cell lines tested. The ductal epithelial cell line,HPDE, showed expression of ET-1 and ET_AR with no expression of ET_BR. In majority of cell lines, expression of endothelin receptors (ET_AR and ET_BR) showed an inverse relationship, with either one or the other expressing in a cell line one at a time. In SW1990, a low level of endothelin A receptor was observed however no expression of endothelin B receptor was seen. High levels of both ET_AR and ET_BR were present in poorly differentiated (AsPC1), moderately differentiated (BxPC3) and well differentiated (Colo357, HPAF II and Capan-1) PC cell lines as indicated by RT-PCR and immunblot analysis -indicating the differential expression pattern of ET_AR and ET_BR in these cells, suggesting a potential association between receptors expression and differentiation of PC cells. Actin was used as an internal control to normalize transcript levels.

B. ET axis components are overexpressed in PC tissues

To further delineate the expression and investigate the clinical significance of ECE-1, ET-1, ET_AR and ET_BR in human PC we first performed immunohistochemistry on formalin fixed paraffin embedded tissue samples from 38 pancreatic cancer patients who underwent Whipple procedure and on five normal pancreas samples. Immunohistochemistry (IHC) analysis revealed that within the axis the expression in tumors was found to be higher for ECE-1 and was expressed in 34 cases (89%). The

expression of ET-1 was observed in 33 cases (86%), whereas the expression of ET_AR and ET_BR were seen in 29 (75%) and 24 (68%) cases respectively **(Figure 2A).** In normal human pancreas, low immunoreactivity of ECE-1, ET-1, ET_AR and ET_BR was seen in the pancreatic acini, however islet cell were found to be strongly positive for all four molecules. **Figure 2B and 2C** shows the quantification of composite immunohistochemistry score and a heat map representation respectively of the expression pattern on case-to-case basis. Interestingly, in the normal pancreatic ducts weak immunostaining can be seen for ECE-1 compared to ET-1, ET_AR and ET_BR. To evaluate the clinical significance of ET axis expression in PC, the expression was correlated with the clinicopathological characteristics. The expression was correlated with the age, gender, tumor stage, tumor grade, lymph node metastasis and distant metastasis (**Table I)**. The incidence (positivity) of ET-1, ET_AR and ET_BR was correlated with patient characteristics using the chi-square test for the categorical variables and the t-test for age. For both ET_AR and ET_BR no patient characteristics were associated with marker positivity. Interestingly, ET-1 positivity was associated with a higher proportion of moderate grade patients and was negatively associated with a higher proportion of poor and well differentiated patients with a significant p value ($p=0.04$).

To study the expression of ET-1 and its receptors during PC metastasis immunohistochemical staining was performed using tissue microarrays (TMA) from UNMCs unique rapid autopsy program (RAP), which included patient samples from both primary and metastatic sites. We examined pancreatic cancer tissue samples from the primary and metastatic sites to measure the incidence of ET axis proteins and the average composite scores. Generalized estimating equations (GEE) was used to calculate the proportion with the positive staining results for each of the molecules examined for each tissue type as well as the mean composite score for each. The expression was compared between non-neoplastic ducts and pancreatic

adenocarcinoma having normal pancreas and tissues from primary PC. **Figure 3** represents the immunohistochemical analysis and heat map representation of ET-1 and both the receptors respectively in primary and metastatic tissues. We next determine the change ET axis expression with progression of PC on the basis of tumor grade and differentiation. **Table II** shows the expression of ET-1, ET_AR and ET_BR in primary tissues of 44 patients on the basis of loss of tumor differentiation. A progressive increase in the expression of ET-1 and receptors was observed from poorly differentiated to well differentiated carcinoma. Further, within the axis ET_AR was found to be highly expressed in all three histologic grades and was higher than ET-1 and ET_BR. In addition, patients who express all three of the molecules in primary sites also expressed the same in various metastatic sites suggesting the role of ET-1 and the receptors in metastatic spread of disease. **Table III** shows the distribution of TMA utilized in this study and the number of tissue specimens analyzed for ET-1, ET_AR and ET_BR. The expression of ET-1, ET_AR and ET_BR was seen in 37.1% (13/35), 74.3% (29/39), 31.5% (12/30) and 73% (27/37) of the primary cases. We then determined the incidence rate (proportion positive) for each of the molecules in each of the tissue, using GEE methods to take into account multiple tissue samples per patient. As shown in table IV, compared to metastatic tissue the incidence rate in primary tumor was found to be significantly higher for ET-1 ($p=0.0020$), ET_AR ($p=0.022$) and ET_BR ($p=0.020$). In addition, the incidence for ET_AR on blood vessels was found to be significant in metastatic tissues, however no significant difference for ET_BR was observed in these tissues.

C. Elevated expression of ET_BR on tumor blood vessels is correlated with patient survival

Very interestingly, in addition to tumor cells where the expression of ET axis components was identified in the cytoplasm, a strong immunostaining was also seen in

the stromal compartment of these patients particularly infiltrating immune cells and blood vessels, suggesting a potential involvement in the pancreas microenvironment **(Figure 4A).** To further confirm the expression in the stromal compartment, the expression was scored by a pathologist in a double blinded condition in Whipple resected PC tissues. Of all the cases analyzed the expression in blood vessels was seen in 60.5 % (23/38) and 31.5% (12/38) for ET_AR and ET_BR respectively. Additionally, expression in the immune cells indicate the expression of ET-1 in 31.5% (12/38), ET_AR in 34.1% (13/38) and ET_BR in 39.4 (15/38) of the cases **(Figure 4B).** Importantly, analysis of survival data from 33 patients showed that elevated ET_BR positivity on blood vessels is correlated with poor prognosis of the PC patients. Representative immunohistochemistry images of the low ET_BR expression on normal pancreas and elevated expression on tumor blood vessels in PC patients are shown in **(Figure 6).** The median survival of the patients with low ET_BR expression on blood vessels was 14.7 months as compared to 10.3 months in patients with high ET_BR blood vessel positivity **(Figure 5E).** However, no significant correlation of ET-1, ET_AR and ET_BR expression on tumor cells was seen with survival of these patients. Even in the small sample size this was a significant finding suggesting that analyzing the expression in the context of microenvironment in addition to tumor cells is equally important to determine the pathobiological significance of the pathway.

D. Expression of ET axis in mouse progression model of PC

Genetically engineered mouse (GEM) models are potential alternatives to xenograft subcutaneous models to study the tumor microenvironment and evaluate therapeutic strategies *in vivo*. We have procured the most widely used mouse model in pancreatic cancer, which expresses constitutively activated $Kras^{G12D}$ in the pancreas. This double transgenic line was developed by Dr. David Tuveson by crossing LSL-$Kras^{G12D}$ with Pdx-1-Cre. The resultant double transgenic animals ($Kras^{G12D}$; Pdx-1-Cre)

develop PanIN lesions at age of 9 weeks, advanced PanINs at 30 weeks and few animals developed PC with metastasis at 50 weeks of age [35]. However, this model does do not develop aggressive nor metastatic tumors. We have utilized widely used KPC model which consists of a triple transgenic animal (K-ras^{G12D}; *Trp53*$^{R172H/+}$; Pdx-1-Cre). The KPC model involves targeted expression of an endogenous *Kras*^{G12D} allele and *Trp53*$^{R172H/+}$ in murine pancreatic progenitor cells and closely recapitulates many of the genetic alterations, histopathology and metastatic features of human pancreatic cancer. The KPC mice develop pre-neoplastic lesions at 4-6 weeks after birth and invasive PDAC at 22 weeks of age with a median survival of 5 months and 100% mortality by 12 months of age. We studied the expression of ECE-1, ET-1, ET_AR and ET_BR in the autochthonous tumors of KPC mice. The expression was analyzed at the mRNA levels form tissues obtained from 5wk, 7 wk, 10wk, 15wk, 20 wk and 25wk animals. Low expression of ECE-1, ET-1 and both receptors was seen in animals of 5 wk age and a progressive increase was observed to 25 wk KPC animals **(Figure 7A).** In contrast, low expression of ET axis components was seen in the control animals, which have WT Kras in the pancreas. The expression was also evaluated by tissue IHC where the onset of expression of ECE-1, ET-1, ET_AR and ET_BR was observed in early PanIN lesions in 5 wk animals **(Figure 7B).** In the advanced lesions of 25 week animals, significant expression was seen both in cancer cells and tumor microenvironment components similar to human PC. In the 25 wk control animals having WT Kras, low expression was seen in the acinar compartment whereas the strong immunoreactivity was seen in the islet cells, which is similar to the normal human pancreas. In addition, in the normal pancreatic duct similar to normal human pancreas prominent immunostaininig of ECE-1 was seen. The progressive increase in the expression of ET-1 axis components with increase tumor grade and stage similar to human PC suggests the therapeutic potential of targeting this axis and study thereof using the KPC model.

E. Expression of ET axis in tumor microenvironment of human PC and mice KPC tissues

PC represents one of the most complex tumor microenvironments (TME) and consists of pancreatic stellate cells, endothelial cells, immune cells, neuronal cells, endocrine cells, and highly obstructive extracellular matrix. These cellular components of the TME have complex interactions with each other and with the cancer cells. To confirm the expression on components of PC microenvironment the expression was determined using dual confocal microscopy utilizing markers for blood vessels, tumor cells and stellate cells and macrophages. In both human PC and mouse KPC tissues, we observed that epithelial marker CK19 positivity is associated with both ET_AR and ET_BR **(Figure 8A & 8B).** Additionally, we found that both ET_AR and ET_BR are associated with CD31 **(Figure 9A & 9B).** In contrast, α-SMA positive stellate cells display low expression of ET_AR and high expression of ET_BR **(Figure 10A & 10B).** As macrophages are an integral part of TME and play a key role in its establishment, we analyzed the expression on tumor infiltrating macrophages. Using CD68 specific antibody we found that expression of ET_BR is mainly associated with CD68 positive macrophages, whereas low expression of ET_AR was seen **(Figure 11A).** In addition, dual immunofluorescence analysis on the F4/80 positive murine infiltrating macrophages in the stromal compartment of KPC mouse tissue suggests low expression of ET_AR and predominant expression of ET_BR **(Figure 11B).** We have analyzed the expression of both ET receptors in cancer stem cells (CSCs) in KPC mice tissues. Expression of both ET receptors was observed in CD133 positive CSCs **(Figure 12A & 12B).** In addition to the ET receptors, the expression of ECE-1 and ligand ET-1 was also studied in these tissues using triple co-immunofluorescence analysis. Examination revealed expression of ECE-1 and ET-1 on tumor cells and stellate cells that displayed co-expression with

the CK19 **(Figure 13A & 13B)** and α-SMA **(Figure 14A & 14B)** respectively in both PC and KPC tissues. Furthermore, co-immunostaining using ECE-1 and ET-1 specific antibodies showed extensive co-expression with CD68 and F4/80 positive macrophage populations in human PC and mice KPC tissues **(Figure 15A & 15B).** An overall summary of the ET axis expression profile on various cellular compartments of microenvironment is given in **Table V.**

F. ET-1 and receptors correlate with tumor grade and stage in human TCGA database

With the limitation in sample size in these two data sets (Whipple resected and RAP cases), we conducted a TCGA analysis on 162 PC patients **(Table VI i).** The total number of patients in stage 1 is 20 whereas stage 2 has the information on 126 patients. In addition, the sample size of the patients in stage 3 and 4 has information on 3 patients each. We correlated the expression of ET-1 and its receptors on the basis of tumor grade and stage **(Table VI ii).** The expression of ET-1 and both receptors correlated with advanced tumor grade and stage. Higher expression of the ET-1 was observed in stage 3 & 4 patients (p=0.05) and stage 2 patients (p=0.05) compared to stage 1 patients. Similarly, expression of ET_AR (p=0.01) and ET_BR (p=0.03) showed higher expression in stage 2 compared to stage 1 with increase in tumor stage, suggesting a positive correlation with advance tumor stage. To further determine the functional implication and pathobiological significance of this overexpression in PC tissues, we looked for genes in the TCGA database which has high correlation coefficients with overexpression of both ET_AR and ET_BR. To further determine the consequences of the overexpression of the axis in these PC patients we utilized bioinformatics approach to screen for the genes which correlate with the tumors with overexpression of ET-1 and its receptors. Using Pearson correlation coefficient ‘r’ the strength of the linear relationship between two

variables is determined. An 'r' value of 0 indicates no association between the two variables whereas 'r' value of +1 and -1 indicates the perfect positive and negative association respectively between the variables. Bioinformatics analysis of the TCGA database cases revealed high positive correlation of the genes associated with both ET_AR and ET_BR. Interestingly, significant association of pro-fibrotic genes were found to be correlated with ET_AR expression particularly Collagen I (Col1A2, Col3A1, Col5A2, Col6A3), Platelet derived growth factor receptor-beta (PDGFRβ), Fibroblast activation protein (FAP), Lumican (LUM) and Fibrillin 1 (FBN1), Sulfatase 1 (SULF1), Fibrillin 1 (FBN1), Follistatin like 1 (FSTL1), Secreted protein acidic and cysteine rich (SPARC), Chondroitin sulfate synthase (CHSY3). **Table VII** lists the top 30 genes that were found to correlative on patient-to-patient basis with both ET_AR and ET_BR. **Figure 16** shows the association of PDGFRβ with ET_AR expression (r=0.93), indicating a stronger association of the two variables. Additionally, pathways regulating hepatic fibrosis, SHH signaling, and tumor growth and metastasis, were also upregulated in tumors exhibiting overexpression of the ET axis **(Figure 17).** These findings indicate that over expression of ET-1 axis particularly ET_AR correlates with a pro-fibrotic gene signatures suggesting an active involvement of the ET-1 axis in the stromal compartment of PC.

Discussion

The ET system is comprised of a strong vasoactive peptide and receptors that play a crucial role in physiological events. In addition, a link has been suggested between the axis components and various pathological malignancies including cancer. Although a few studies have also reported a potential role of ET-1 in human pancreatic cancer, the data are very limited. Kushuhara *et al* first witnessed and reported evidence for the presence of ET-1 peptide in the pancreatic cancer cell lines using radioimmunoassay. However, no ET-1 receptors were detected in a panel of PC cell

lines tested [36]. Oikawa *et al* further confirmed these findings and analyzed the expression of all three ET isoforms in human PC cell lines postulating that the expression pattern of all three isoforms is similar to endothelial cells [37]. Inconsistent with the previous finding, Bhargava *et al* reported the presence of ET receptors in pancreatic cancer cell lines and suggested that mRNA expression of ET_AR is limited to some cell lines (MiaCaPa-2 and AsPC-1, not Panc-1) with no detectable expression of ET_BR [38]. In this study, we delineate the expression of ET-1, ET_AR and ET_BR in a panel of 13 PC cell lines using SW480 cells and SKMel 28 as positive controls for ET-1/ET_AR and ET_BR respectively. Similar to previous observations, ET-1 was detected in all PC cell lines, with the receptors showing inverse association with the exception of AsPC1, BxPC3, Colo357 and Capan-1. Inconsistent with the previous findings we observed expression of ET_AR and ET_BR in Panc1 and MiaPaCa respectively, whereas detectable expression of both receptors is observed in AsPC-1 cells.

Using islet specific antibodies, previous studies have demonstrated the presence of ET-1 and receptors in human and rat pancreas tissues thus suggesting the involvement of ET axis in supporting the beta cell function [39]. Using radiolabeled binding experiments; the presence of ET-1 and its receptors was also demonstrated in rat beta cell lines INS-1 and RINm5f. Similar to pancreatic islets, radiolabeled binding studies also indicate the presence the higher of ET_AR than ET_BR in the pancreatic acini [40]. Cook *et al* recently reported the higher expression of ET-1 and ET_BR in 45 pancreatic cancer samples compared to normal pancreas, however no positive staining for ET_AR was detected in the epithelium of tissues studied [41]. Our study to investigate the expression pattern of ET axis components, ECE-1, ET-1 and both receptors in tumor samples from Whipple resected PC cases and microarray from UNMC rapid autopsy program suggests over expression of the axis as compared to normal pancreas. We

have demonstrated that in normal pancreas, the expression is limited to acinar region and low immunoreactivity for all four molecules is seen with prominent expression is seen in pancreatic islets. In contrast, minimal immuno-staining is seen in the normal pancreatic ducts. We observed that in Whipple resected PC cases, within the axis ECE-1 has highest incidence and expressed in 89% cases (34/38). Analysis of the PC specimens in the tumor samples further indicates that ET-1, ET_AR and ET_BR are expressed in 86% (33/38), 76% (29/38) and 63% (24/38) of cases. The discrepancy in the ET_AR staining can be explained on the basis of antibody use in the study. Our immunohistochemical analysis in human PC samples used a polyclonal antibody (used by Cook *et al*) and demonstrated low staining intensity in the ductal cells of the pancreas. We further confirmed our findings by immunostaining the serial sections to compare the reactivity and intensity of the both the antibodies. **Figure 18** shows clear differences in the staining intensities in the three PC cases used; suggesting the low ET_AR incidence observed can be attributed to the selection of the antibody in the study. We also investigated the expression pattern in different tumor samples (obtained from the UNMC rapid autopsy program, RAP that included those with both the primary and metastatic sites harvested). Similar to our previous analysis, overexpression of axis components is seen compared to normal pancreas. The incidence of ET-1, ET_AR and ET_BR is seen in 37.1% (13/35), 74.3% (29/39) and 31.5% (27/37) of the cases respectively. The higher expression of ET_AR specific antibody in both primary and metastatic lesions of RAP tumor samples further strengthen our findings obtained in Whipple resected tumor samples.

The tumor microenvironment of the PC is of particular interest, as the primary tumor possesses an extensive and obstructive stromal compartment. This microenvironment is comprised of endothelial cells, fibroblasts, immune cells and vascular smooth muscle cells. ET-1 acts as a modulator of stromal response and

facilitates tumor progression either in autocrine signaling on tumor cells or by paracrine effect on nearby stromal cells [42]. Studies have shown that an elevated level of the ET axis is correlated with tumor progression, intra-tumoral vascularization and tumor angiogenesis [43] [44]. In breast cancer, increased ET-1, ET_AR and ET_BR expression is associated with tumor progression, disease free survival and overall survival [45]. Also, in bladder cancer overexpression of the axis is correlated with tumor grade and longer disease free survival [46] [47]. In endothelial cells, ET-1 modulates the angiogenesis process and exhibits a potent effect in combination with VEGF. ET-1 stimulates the release of MMP-2, which allows sprouting and migration of endothelial cells and formation of vascular cord-like structures indicating that ET-1/ET_BR interaction favors neovascularization in concert with VEGF [31] [30]. Previous observations have demonstrated that both VEGF and ET-1 stimulate each other's expression. In bovine aortic endothelial cells, VEGF induces ET-1 mRNA expression and ET-1 secretion while in vascular smooth muscle cells, ET-1 acts on ET_AR to stimulate VEGF mRNA and VEGF secretion showing a coordinated role of VEGF and ET-1 [48]. According to our data, in addition to the expression on tumor cells, expression of ET axis is also observed in the stroma of PC, particularly on immune cells and tumor blood vessels. We also observed that an association between the elevated levels of ET_BR on blood vessels with poor overall survival. Our findings indicate that overexpression of ET axis proteins on tumor cells is not correlated with patients overall survival. Importantly, our main finding is that investigation of the tumor microenvironment components is necessary to establish prognostic significance of the pathway and ET_BR expression on blood vessels is an independent prognostic factor for patient survival. To our knowledge, this is the first report to demonstrate that ET_BR is an independent prognostic marker for human pancreatic cancer. It may follow from our data that even in a small data set, this is a significant finding and further characterization of the different microenvironment

components can add significant prognostic information

To further investigate the pathobiological significance of the pathway, we also analyzed the expression of ET axis proteins in the TCGA database and their correlation with tumor grade. Next, we investigated the implications of overexpression of ET axis proteins in PC. In this study, we found significant positive correlation of ET_AR and ET_BR with pro-fibrotic genes (Collagen I/III/VI, PDGFRβ, FAP) and pathways using a bioinformatics approach. Pancreatic stellate cells (PSCs) are the resident cells of the pancreas and are the principal source of fibrosis in the stroma that interact closely with the surrounding tumor cells and stimulate tumor growth and metastasis [49]. Once these cells are activated in response to external stimuli, inflammation (pancreatitis) or cancer, PSCs acquire a myofibroblast like phenotype and secrete increase amount of extracellular matrix (ECM) [50]. Tumor cells secreting ET-1 participate in the recruitment and activation of PSCs. Alternatively, ET-1 is released from PSCs in an autocrine manner and can also perpetuate their activation [51]. In the context of PC, Jonitz and coworkers observed that treatment of PSCs with TGF-β1 and TNF-α stimulated the secretion of ET-1 peptide by increased binding to smad3 and NF-kB respectively. Also, treatment with ET-1 induced the phosphorylation of ERK1/2 and p38, a marker of PSCs activation (α-SMA) and pro-inflammatory cytokines (IL-1β and IL-6) [52]. This ET-1 induced effect was abrogated by the dual ET_AR and ET_BR inhibitor, Bosentan and displays anti-fibrotic effects in PSCs by marked reduction in the expression of CTGF (connective tissue growth factor) and α-SMA [53] indicating a pro-fibrogenic role of ET-1 in PC. Our present study suggests that ET receptors are expressed in PC and in the TME exhibit a significant correlation with fibrosis associated genes. This knowledge, may provide a rationale for developing a novel cancer therapy for targeting the ET axis to combat this fatal malignant disease. However, more experimental and clinical

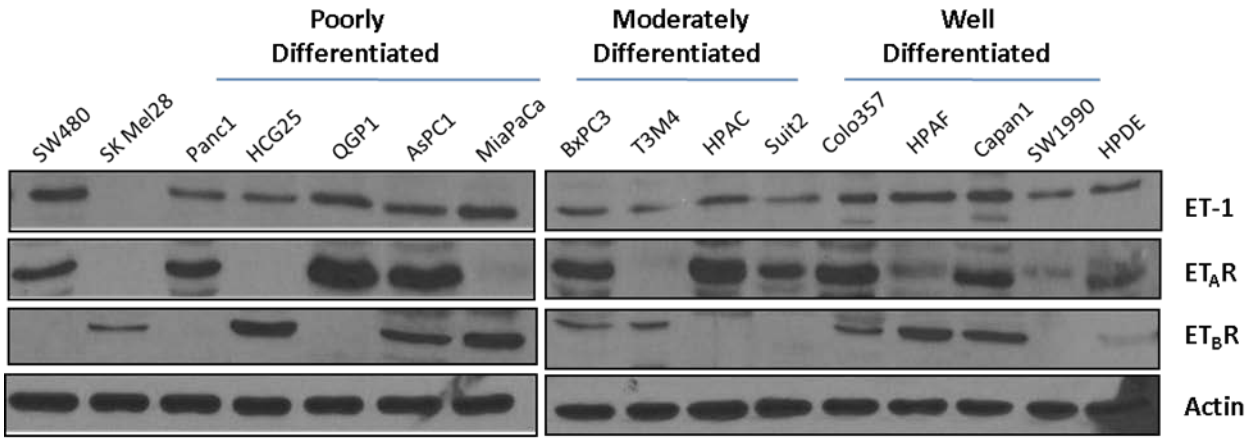
evidence are needed to prove the significance of this axis a therapeutic target. To determine the clinical significance of targeting this axis *in vivo*, we have utilized the widely used genetically engineered KPC mouse model (K-ras^{G12D}; *Trp53*$^{R172H/+}$*;* Pdx-1-Cre) that harbors a Kras and p53 mutation in the pancreas.[54] [55]. We observed the expression of ECE-1, ET-1; and both receptors increase gradually with the disease progression in these mice. Our data also infers that in addition to tumor cells, a prominent expression is seen in the stromal region (an observation similar to what we witnessed in human PC cases indicating the potential of targeting this axis). Because the main goal of the present study was to define and characterize the expression pattern in various cell types in the microenvironment of PC, we utilized the -markers specific for fibroblasts (α-SMA), endothelial cells (CD31), tumor-associated macrophages (CD68 & F4/80), tumor cells (CK19) and stem cells (CD133). In this study, we found that CD31^{+} blood vessels and CD133^{+} stem cells show co-expression with both ET_AR and ET_BR. In contrast, α-SMA+ fibroblasts, CD68^{+} and F4/80^{+} macrophages show predominant expression of ECE-1, ET-1 and ET_BR, however, low expression of ET_AR was observed in both KPC mouse and human PC tissues.

In conclusion, we have shown that, ET axis components are over-expressed in pancreatic cancer cells, human PC tissues and in the KPC mouse progression model of PC. Further, the proteins associated with this axis plays a pleotropic role in the TME and are expressed in various cellular compartments. This study establishes the prognostic significance of the ET axis in lethal PC indicating that TME components can also be of prognostic significant. Importantly, positive correlation of extracellular matrix associated genes further sheds light on the therapeutic potential of the axis and can be exploited for improving the delivery and efficacy of existing drugs.

Figure 1: **Expression of ET-1, ET_AR and ET_BR in pancreatic cancer cell lines. A.** Protein lysates from a panel of PC cell lines and normal pancreatic ductal epithelial cells, HPDE were resolved on 10% SDS-PAGE gel using western blot analysis. The expression of ET-1, ET_AR and ET_BR was observed in these cell lines when incubated with anti- ET-1, anti- ET_AR and anti- ET_BR antibody. Colon cancer (SW480) and melanoma cell line (SKMel28) were used as positive control for ET-1/ET_AR and ET_BR respectively. β-actin was used as an internal control. **B.** RT-PCR using ET-1, ET_AR and ET_BR gene specific primers in PC cell lines. β-actin was used as an internal control.

Figure 1

A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Moderately Differentiated
Well Differentiated
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SK Mel28
Panc1
HCG25
QGP1
AsPC1
MiaPaCa
BxPC3
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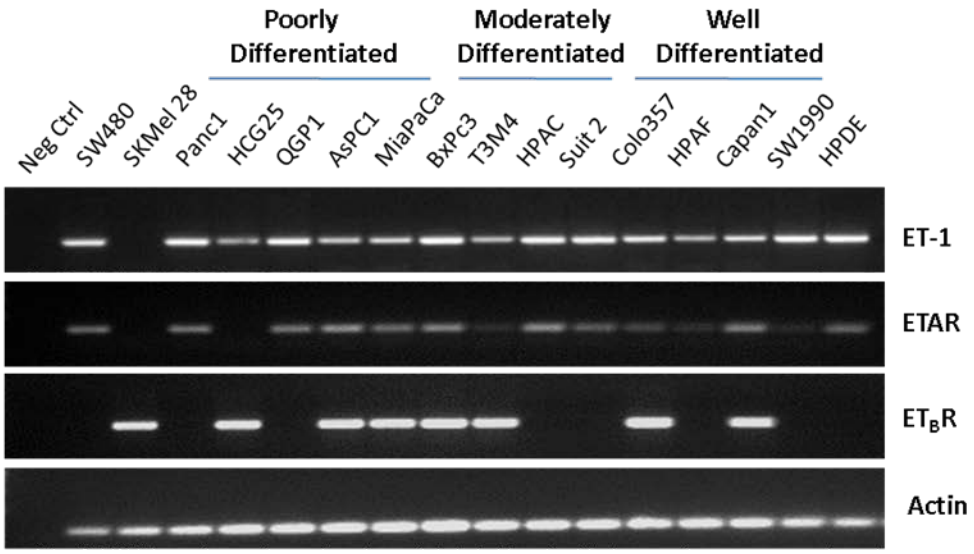
HPAC
Suit2
Colo357
HPAF
Capan1
SW1990
HPDE
ET-1
ETAR
ETBR
Actin

B


Poorly Differentiated
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Neg Ctrl
SW480
SKMel 28
Panc1
HCG25
QGP1
AsPC1
MiaPaCa
BxPc3
T3M4
HPAC
Suit 2
Colo357
HPAF
Capan1
SW1990
HPDE
ET-1
ETAR
ETBR
Actin

Figure 2: **Expression of ECE-1, ET-1, ET_AR and ET_BR in pancreatic cancer tissues.** **A.** Immunohistochemical analysis of ECE-1, ET-1, ET_AR and ET_BR in the pancreatic acini and ducts of normal pancreas (magnified image) with strong staining observed in islet cells for all four molecules (black arrow). There is a significant increase in expression of ECE-1, ET-1, ET_AR and ET_BR in the PC tissues when compared to normal pancreas. The magnified image shows over-expression in the pancreatic ducts. **B.** The staining score and intensity score were multiplied to obtain the composite scores. Composite scores of ECE-1, ET-1, ET_AR and ET_BR in normal pancreas ducts and PC tissues are shown as box plots. **C.** Heat map representation of ET axis expression in normal pancreas and PC patients on case to case basis.

Figure 2

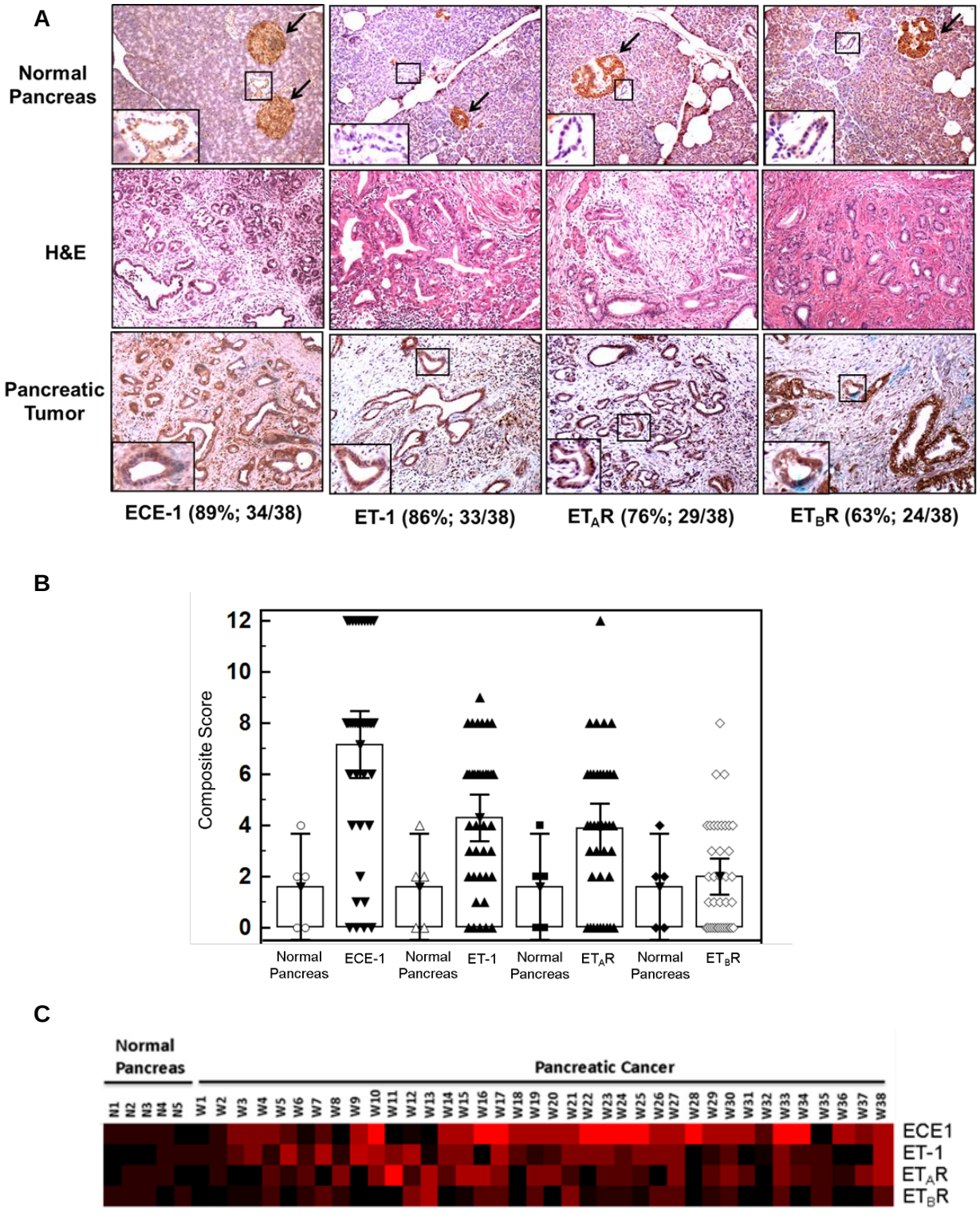

A
Normal Pancreas
H&E
Pancreatic Tumor
ECE-1 (89%; 34/38)
ET-1 (86%; 33/38)
ETAR (76%; 29/38)
ETBR (63%; 24/38)
B
Composite Score
0
2
4
6
8
10
12
Normal Pancreas
ECE-1
Normal Pancreas
ET-1
Normal Pancreas
ETAR
Normal Pancreas
ETBR
C
Normal Pancreas
Pancreatic Cancer
N1 N2 N3 N4 N5 W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12 W13 W14 W15 W16 W17 W18 W19 W20 W21 W22 W23 W24 W25 W26 W27 W28 W29 W30 W31 W32 W33 W34 W35 W36 W37 W38
ECE1
ET-1
ETAR
ETBR

Table I: Clinicopathological characteristics of Whipple resected PC patients. ET-1 positivity was significantly associated with a higher proportion of moderate grade patients (p=0.04) and was negatively associated with a higher proportion of poor and well grade patients.

Table I

		All patients (n=38)	ET-1 negative (n=5)	ET-1 positive (n=33)	p-value	ET_AR negative (n=9)	ET_AR positive (n=29)	p-value	ET_BR negative (n=14)	ET_BR positive (n=24)	p-value
Age	Mean (SD)	67.2 (12.3)	65.7 (19.3)	67.5 (11.4)	0.85	69.6 (9.6)	66.5 (13.1)	0.51	67.8 (13.3)	66.9 (12.0)	0.84
Gender	F M	16 (42%) 22 (58%)	1 (20%) 4 (80%)	15 (45%) 18 (55%)	0.37	3 (33%) 6 (67%)	13 (45%) 16 (55%)	0.71	4 (29%) 10 (71%)	12 (50%) 12 (50%)	0.20
Stage	pT1/pT2 pT3/pT4	9 (24%) 29 (76%)	2 (40%) 3 (60%)	7 (21%) 26 (79%)	0.57	2 (22%) 7 (78%)	7 (24%) 22 (76%)	1.0	5 (36%) 9 (64%)	4 (17%) 20 (83%)	0.25
Nodes	N0 N1 unknown	8 (22%) 28 (78%) 2	1 (20%) 4 (80%) -	7 (23%) 24 (77%) 2	1.0	3 (33%) 6 (67%)-	5 (19%) 22 (81%) 2	0.38	5 (36%) 9 (64%) -	3 (14%) 19 (86%) 2	0.22
Metastasis	M1 MX	7 (18%) 31 (82%)	0 (0%) 5 (100%)	7 (21%) 26 (79%)	0.56	2 (22%) 7 (78%)	5 (17%) 24 (83%)	1.0	2 (14%) 12 (86%)	5 (21%) 19 (79%)	1.0
Grade	Moderate/Well Poor	29 (76%) 9 (24%)	2 (40%) 3 (60%)	27 (82%) 6 (18%)	0.04	7 (78%) 2 (22%)	22 (76%) 7 (24%)	1.0	11 (79%) 3 (21%)	18 (75%) 6 (25%)	1.0

Primary Tumor (T): T1, T2, T3, T4: Size and/or extent of the primary tumor

Distant Metastasis: MX: Distant metastasis cannot be evaluated

M0: No distant metastasis

M1: Distant metastasis is present

Figure 3: Expression of ET-1, ET_AR and ET_BR in pancreatic cancer tissues from Rapid Autopsy Program (RAP). The staining score and intensity score were multiplied to obtain composite scores and are represented in the heat map with a scale from 0 to12 from low expression to high expression respectively. Heat map representation of immunohistochemical analysis of ET-1, ET_AR and ET_BR in metastatic sites in the primary tumor **(A)**, and metastatic sites of liver **(B)**, lung **(C)**, lymph node **(D)**, omentum/ diaphragm **(E)** from tissue microarray obtained from rapid autopsy program. **F.** Immunohistochemical representation of ET-1, ET_AR and ET_BR expression and incidence in metastatic sites.

Figure 3

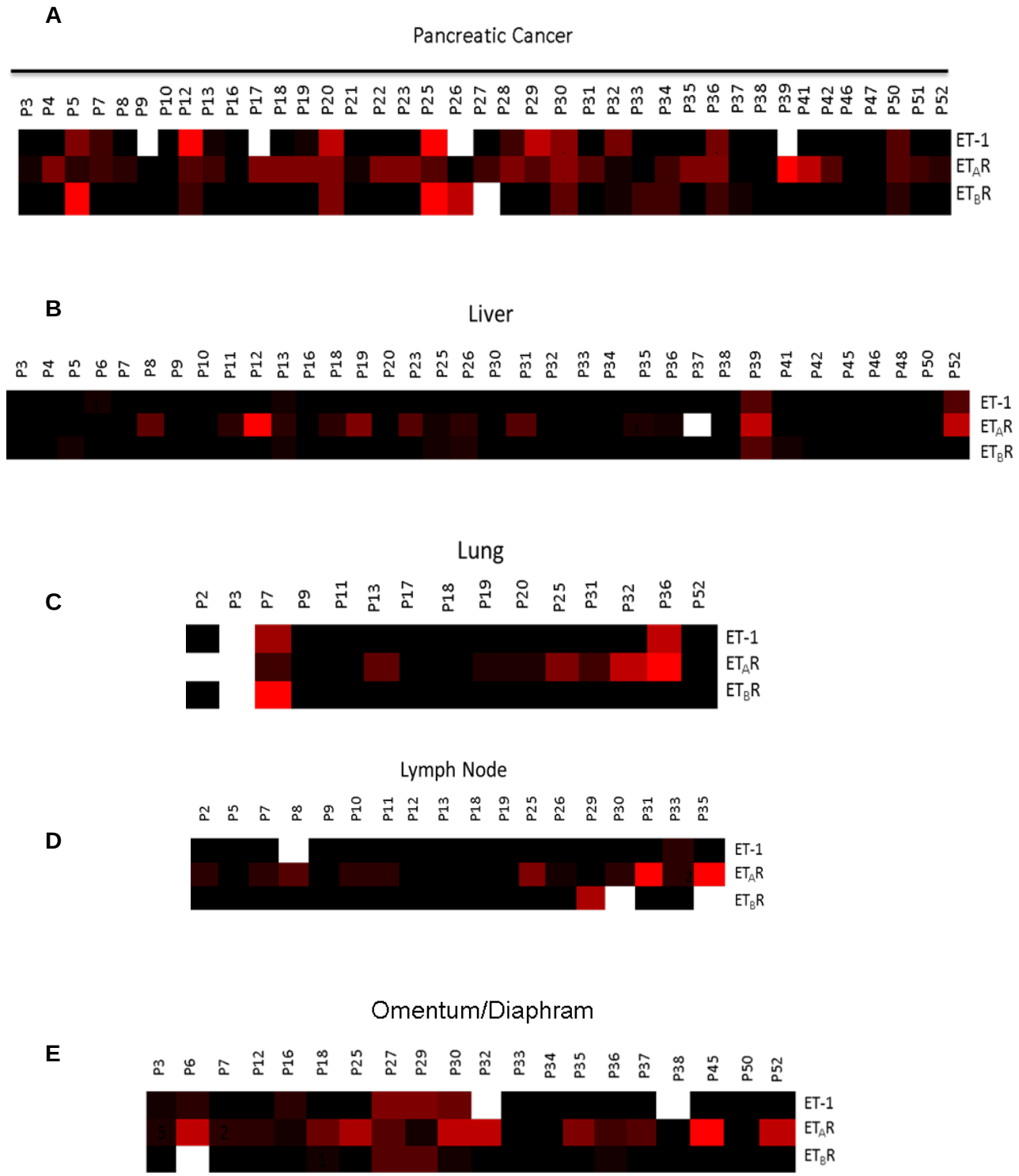

A
Pancreatic Cancer
P3 P4 P5 P7 P8 P9 P10 P12 P13 P16 P17 P18 P19 P20 P21 P22 P23 P25 P26 P27 P28 P29 P30 P31 P32 P33 P34 P35 P36 P37 P38 P39 P41 P42 P46 P47 P50 P51 P52
ET-1
ETAR
ETBR
B
Liver
P3 P4 P5 P6 P7 P8 P9 P10 P11 P12 P13 P16 P18 P19 P20 P23 P25 P26 P30 P31 P32 P33 P34 P35 P36 P37 P38 P39 P41 P42 P45 P46 P48 P50 P52
ET-1
ETAR
ETBR
C
Lung
P2 P3 P7 P9 P11 P13 P17 P18 P19 P20 P25 P31 P32 P36 P52
ET-1
ETAR
ETBR
D
Lymph Node
P2 P5 P7 P8 P9 P10 P11 P12 P13 P18 P19 P25 P26 P29 P30 P31 P33 P35
ET-1
ETAR
ETBR
E
Omentum/Diaphram
P3 P6 P7 P12 P16 P18 P25 P27 P29 P30 P32 P33 P34 P35 P36 P37 P38 P45 P50 P52
ET-1
ETAR
ETBR

Figure 3

F

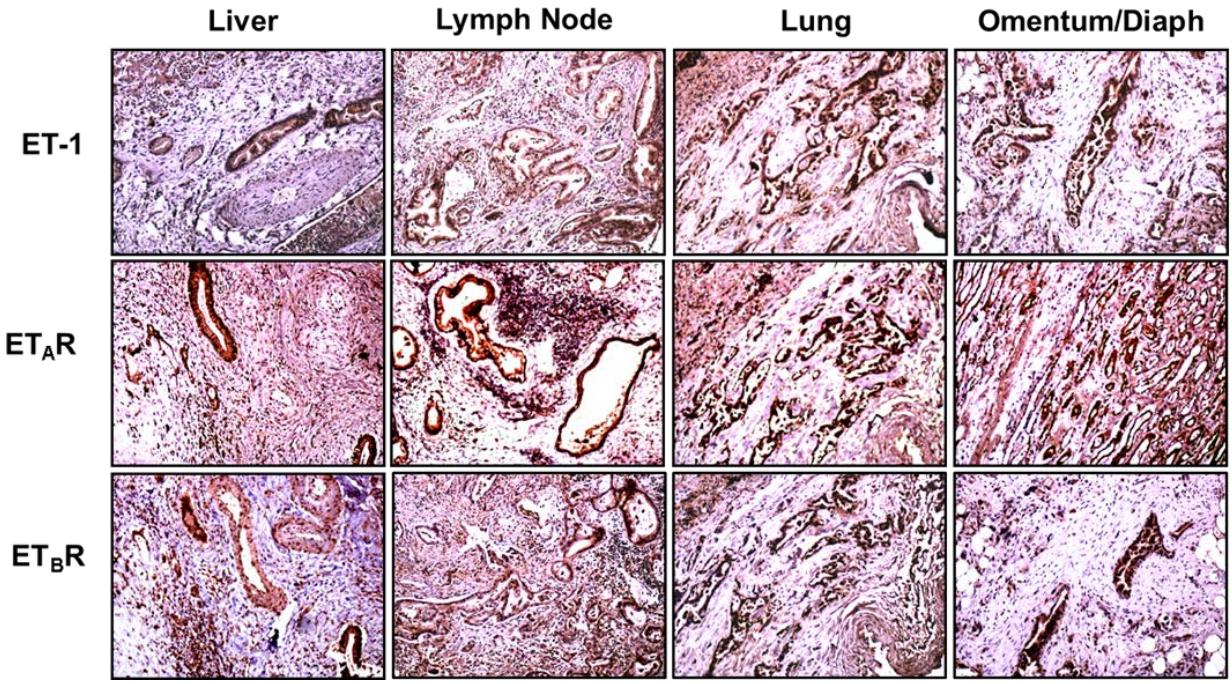


Table II: Expression of ET-1, ET_AR and ET_BR with tumor grade in tissue microarray (TMA)

Table II

Tumor Grade	ET_AR expression	ET_BR expression	ET-1 expression
Poorly differentiated	64% (9/14)	28% (4/14)	28% (4/14)
Moderately differentiated	83% (10/12)	41.6% (5/12)	41.6% (5/12)
Well differentiated	76% (10/13)	30.7% (4/13)	50% (6/12)

Table III: Distribution of tissue microarray (TMA) indicating number of cases analyzed for primary and metastatic sites for ET-1, ET_AR and ET_BR expression.

Table III

Tissue	No. of patients	No. of spots	ET-1 positivity
Omen/Dia	18	27	33.3% (6/18)
Liver	33	54	12.1% (4/33)
Lung	14	24	21.4% (3/14)
Lymph node	18	19	11.1% (2/18)
Pancreas	35	46	37.1%(13/35)

Tissue	No. of patients	No. of spots	ET_AR positivity
Omen/Dia	20	30	90% (18/20)
Liver	32	53	37.5% (12/32)
Lung	13	25	61.5% (8/13)
Lymph node	18	19	61.1% (11/18)
Pancreas	39	58	74.3%(29/39)

Tissue	No. of patients	No. of spots	ET_BR positivity
Omen/Dia	19	28	55.5% (5/19)
Liver	33	56	21.2% (7/33)
Lung	14	25	7.1% (1/14)
Lymph node	16	20	6.25% (1/16)
Pancreas	38	52	31.5%(12/38)

Table IV: Incidence of ET-1, ET_AR and ET_BR in TMA. The incidence rate (the proportion positive) for each of the molecules for each of the tissues, using GEE methods to take into account multiple tissue samples per patient with significant p value.

Table IV

	ET_AR Tumor			ET_AR BV			ET-1		
Tissue	proportion	Lower 95% CI	Upper 95% CI	proportion	Lower 95% CI	Upper 95% CI	proportion	Lower 95% CI	Upper 95% CI
Omem/Diap	0.570	0.424	0.768	0.773	0.615	0.971	0.191	0.098	0.373
liver	0.357	0.246	0.517	0.224	0.146	0.344	0.086	0.038	0.192
lung	0.437	0.294	0.648	0.882	0.704	1.106	0.151	0.043	0.539
lymph	0.523	0.333	0.819	0.588	0.383	0.904	0.103	0.029	0.368
metastatic	0.446	0.359	0.553	0.488	0.414	0.574	0.121	0.078	0.190
pancreas	0.590	0.483	0.721	0.309	0.216	0.442	0.319	0.216	0.473

p= 0.022 **p=0.024** **p=0.0020**

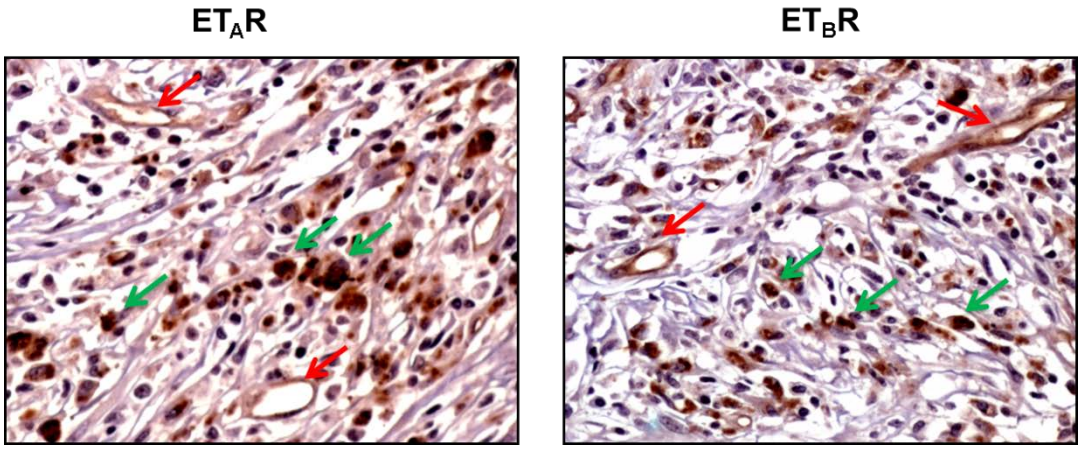
	ET_BR Tumor			ET_BR BV		
Tissue	proportion	Lower 95% CI	Upper 95% CI	proportion	Lower 95% CI	Upper 95% CI
Omem/Diaph	0.167	0.086	0.325	0.635	0.477	0.845
liver	0.129	0.065	0.258	0.380	0.268	0.540
lung	0.052	0.009	0.298	0.298	0.147	0.604
lymph	0.053	0.008	0.355	0.224	0.098	0.515
metastatic	0.115	0.073	0.182	0.404	0.311	0.523
pancreas	0.241	0.152	0.383	0.370	0.261	0.524

0.020 **p= 0.67**

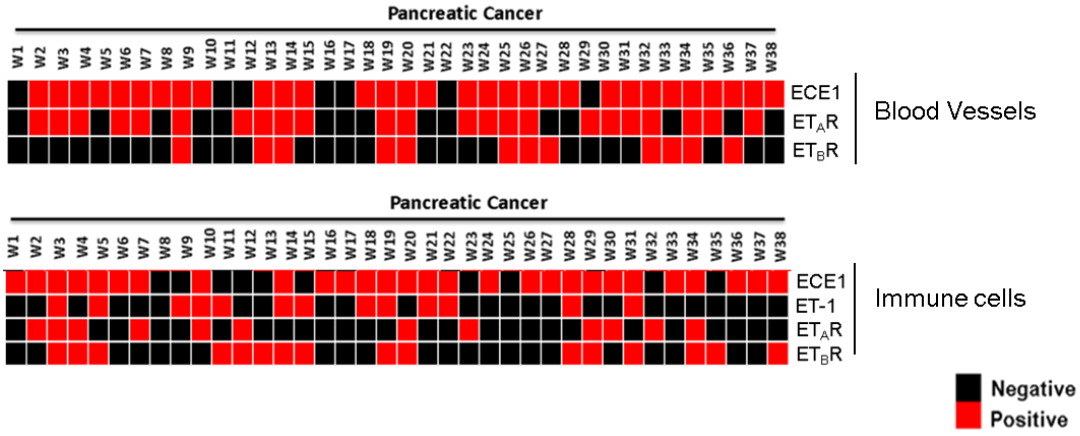
Figure 4: **Expression of ET_AR and ET_BR in the stromal compartment of human PC.** **A.** Immunohistochemical analysis of ET_AR and ET_BR in tumor blood vessels (red arrows) and immune cells (green arrows). **B.** Grid map representing immunohistochemical analysis of ECE-1, ET-1, ET_AR and ET_BR in immune cells and tumor blood vessels along with the overall incidence in whipple resected PC patients. Red and black squares represents the positive and negative cases respectively. **C.** Table represents the incidence of ET_AR and ET_BR on blood vessels and immune cells

Figure 4

A



B



C

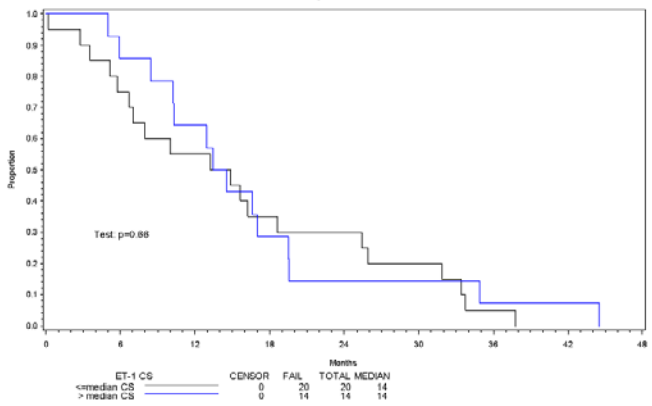
Marker	Positive cases (Higher than normal)	B.V. Incidence
ECE-1	31	81.5%
ET_AR	23	60.5%
ET_BR	12	31.5%

Marker	Positive Cases	Immune cell Incidence
ECE1	28	73.6%
ET-1	12	31.5%
ET_AR	13	34.1%
ET_BR	15	39.4%

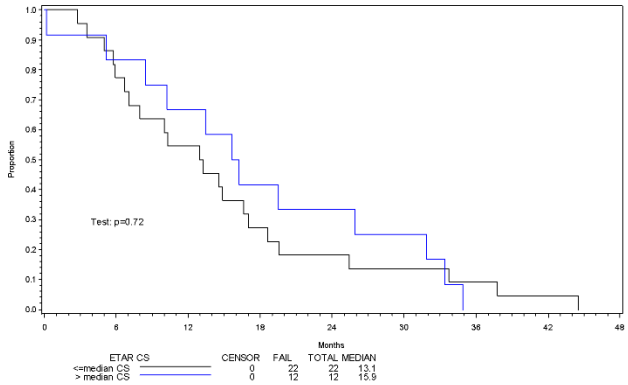
Figure 5: **Comparison of survival curves associated with ET axis expression in PC.** Kaplan Meier curves showing the overall survival in 33 whipple resected PC patients. Tumor positivity for ET-1 **(A)**, ET_AR **(B)** and ET_BR **(C)** are not significant independent predictors of overall survival. Kaplan Meier curves showing ET_AR **(D)** and ET_BR **(E)** expression on tumor blood vessels (BV). Survival curve indicate that the poor prognosis of PC patients is associated with elevated expression of ET_BR expression on BV (p=0.024). Negative and Positive indicates low and high expression respectively.

Figure 5

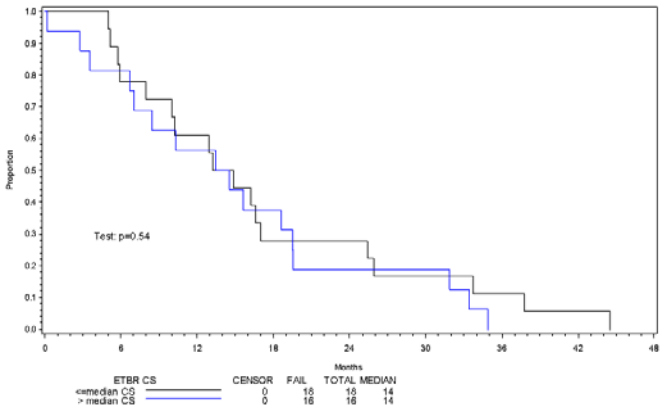
A



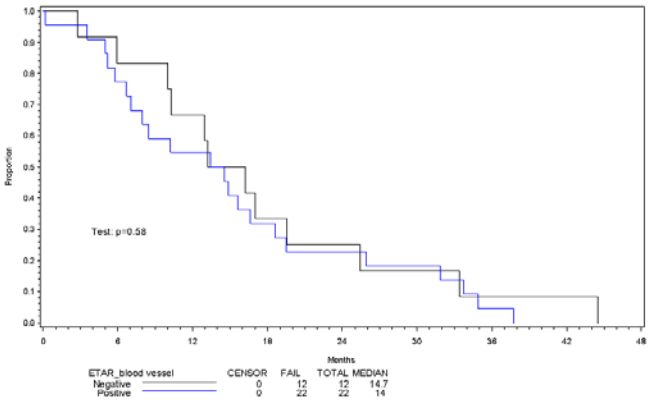
B



C



D



E

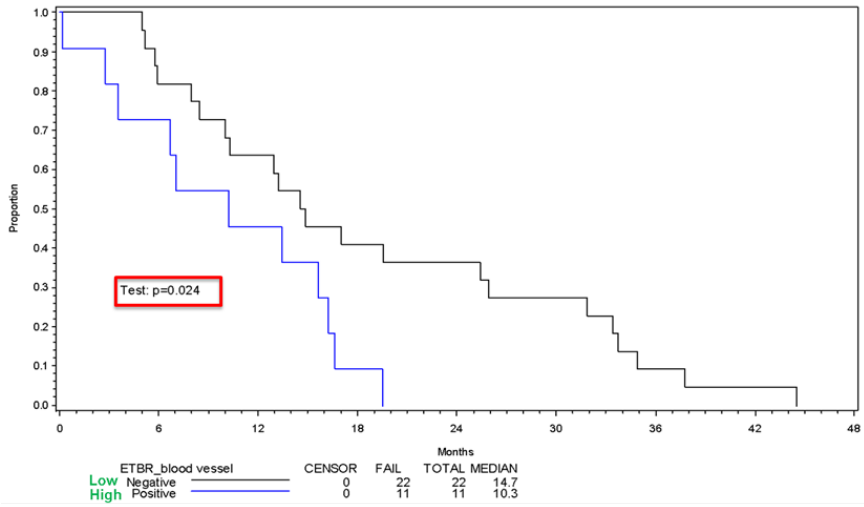


Figure 6: Comparison of ET_BR expression on blood vessels. Immunohistochemical analysis of ET_BR in blood vessels (BV) in normal pancreas and in PC patients. In the normal pancreas, ET_BR expression can be seen in blood vessels with enlarged lumen and restored architecture. Similar, in the PC (patient 3 and patient 4) comparable expression can be seen in tumor blood vessels, however heterogeneous vascular structure is seen. In contrast, in patient 5 and patient 6, elevated expression of ET_BR on BV can be seen and the expression is correlated with poor prognosis of the patients.

Figure 6

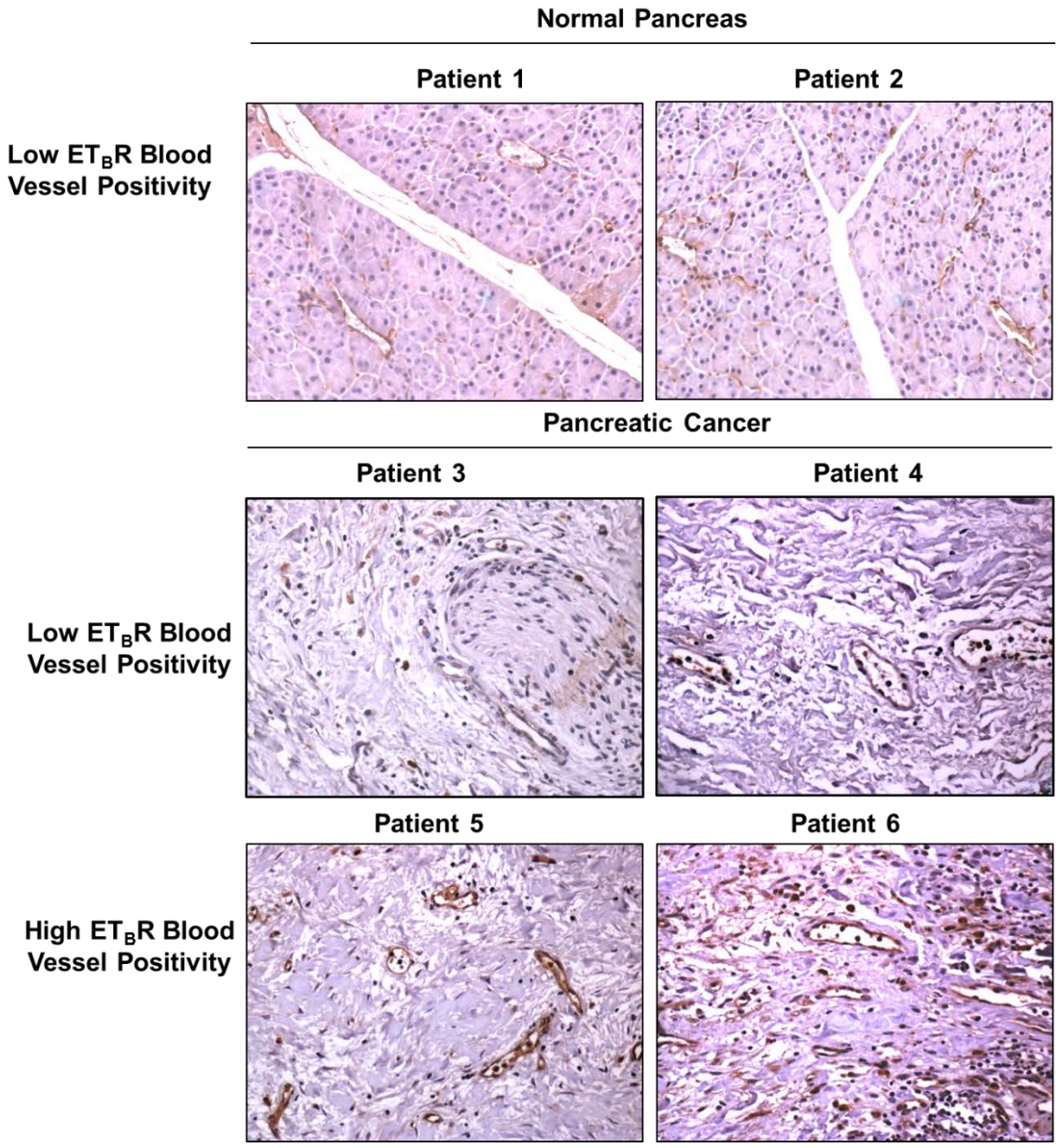

Normal Pancreas
Patient 1
Patient 2
Low ET_BR Blood Vessel Positivity
Pancreatic Cancer
Patient 3
Patient 4
Low ET_BR Blood Vessel Positivity
Patient 5
Patient 6
High ET_BR Blood Vessel Positivity

Figure 7: Expression of ECE-1, ET-1, ET_AR and ET_BR in triple transgenic mouse model of PC. A. RT-PCR analysis of ECE-1, ET-1, ET_AR and ET_BR mRNA expression in various ages of mice from $Kras^{G12D}$ P53; Pdx-1-Cre (KPC) mice compared to corresponding age-matched normal mice. A progressive increase in the expression of ECE-1, ET-1 and both receptors is observed in KPC mice compared to healthy age matched controls. GAPDH was used as a loading control. **B.** Immunohistochemical analysis of ECE-1, ET-1, ET_AR and ET_BR in pancreatic tissues from normal wild type 25 week animals versus KPC mice at ages 5, 10 and 25 weeks. Control mice showed low expression in the acinar compartment with predominant expression in the islet cells for ECE-1, ET-1, ET_AR and ET_BR as indicated by black arrowheads. On the other hand, increased expression was observed in the ductal compartment with advanced age in KPC mice.

Figure 7

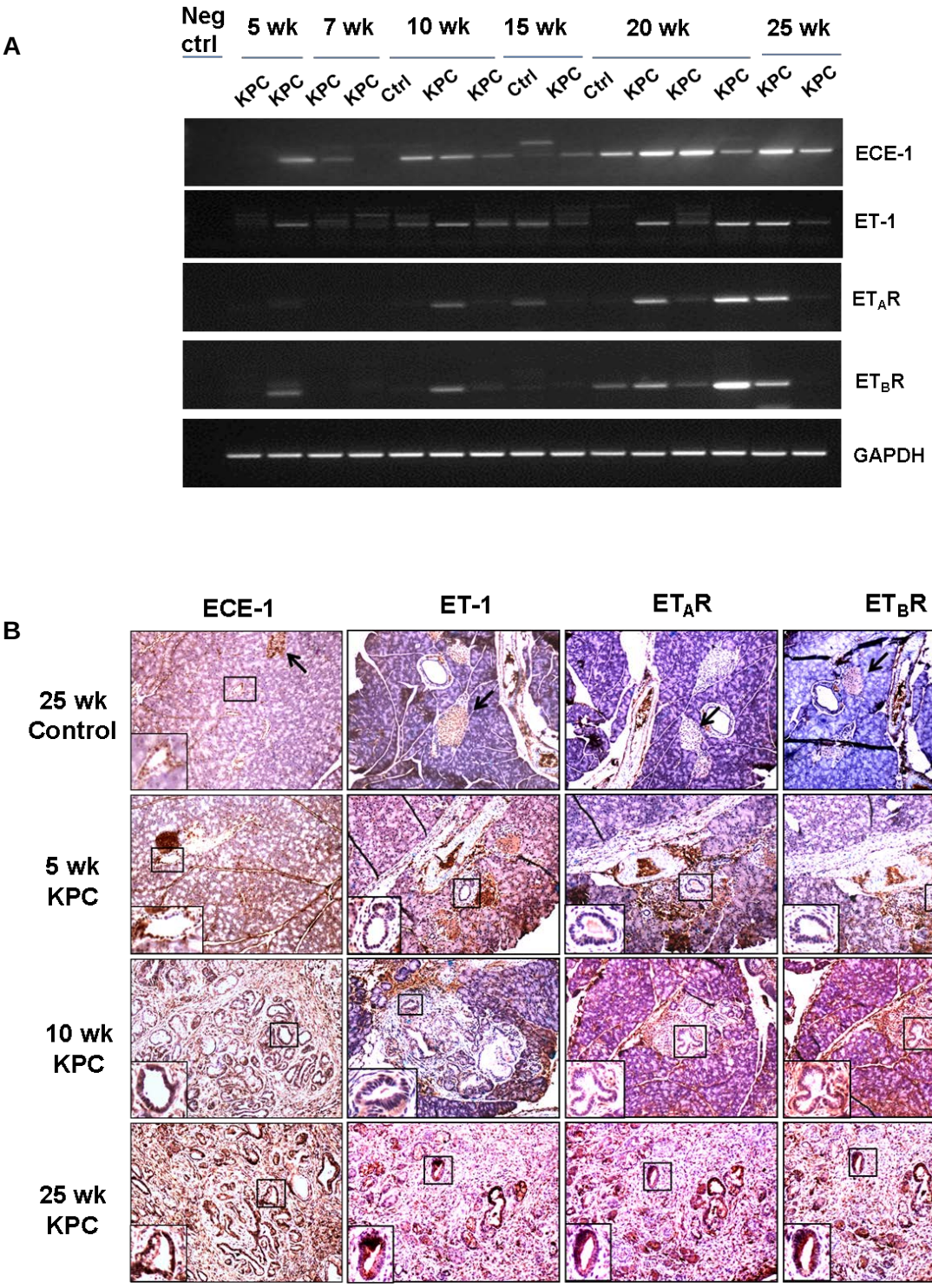

A
Neg ctrl
5 wk
7 wk
10 wk
15 wk
20 wk
25 wk
KPC
Ctrl
ECE-1
ET-1
ETAR
ETBR
GAPDH
B
ECE-1
ET-1
ETAR
ETBR
25 wk Control
5 wk KPC
10 wk KPC
25 wk KPC

Figure 8: Expression of ET_AR and ET_BR in tumor cells in human and mouse PC tissues. Dual confocal microscopy images show co-expression of ET_AR (red) and ET_BR (red) with epithelial marker CK19 (green) in human PC **(A)** and in 25 week KPC mouse **(B)** tissue. Scale bar = 50µm.

Figure 8

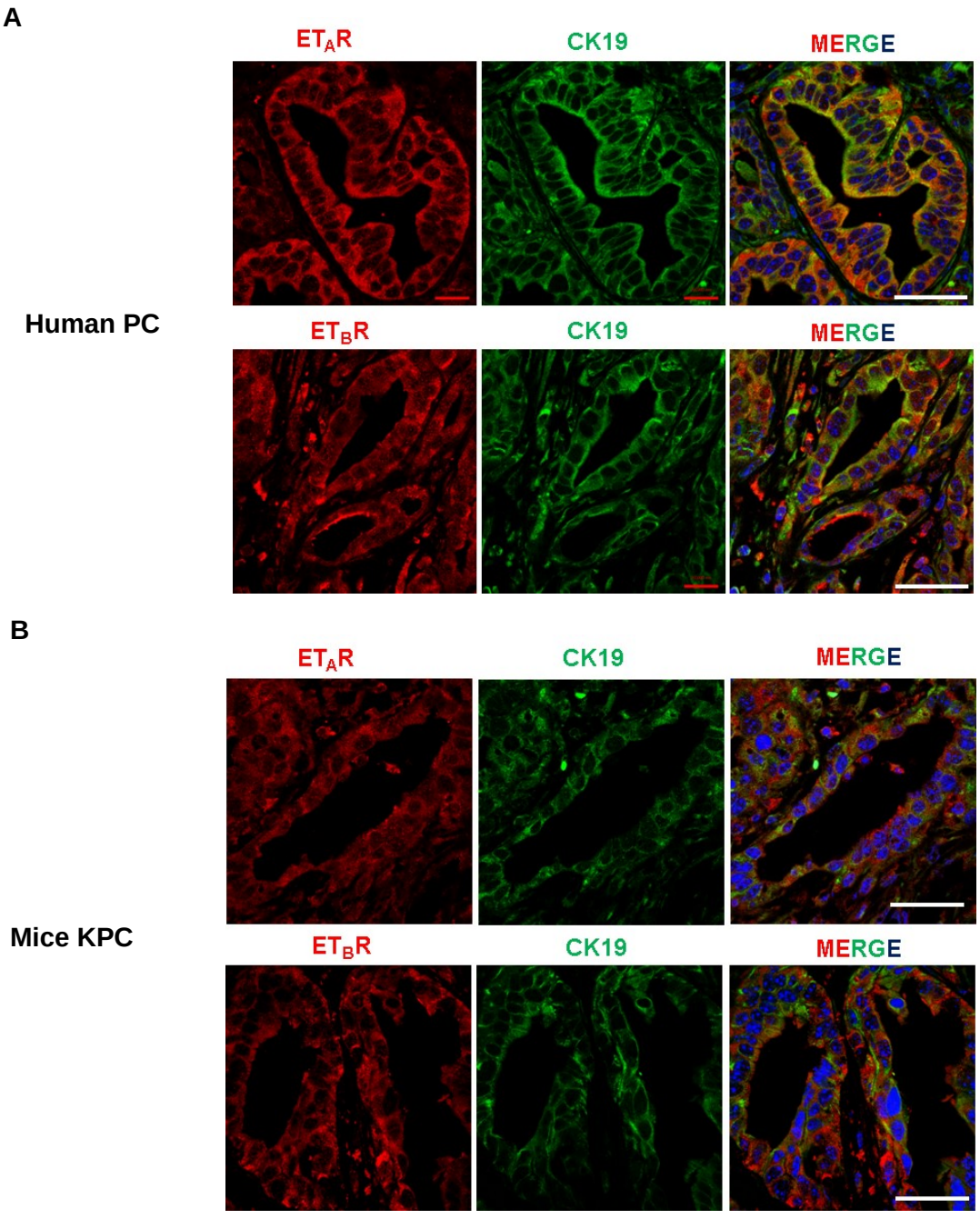

A
ETAR
CK19
MERGE
Human PC
ETBR
CK19
MERGE
B
ETAR
CK19
MERGE
Mice KPC
ETBR
CK19
MERGE

Figure 9: Expression of ET_AR and ET_BR in blood vessels in human and mouse PC tissues. Dual confocal microscopy images show co-expression of ET_AR (green) and ET_BR (green) with endothelial marker CD31 (red) in human PC **(A)** and in 25 week KPC mouse **(B)** tissue. Scale bar = 50µm.

Figure 9

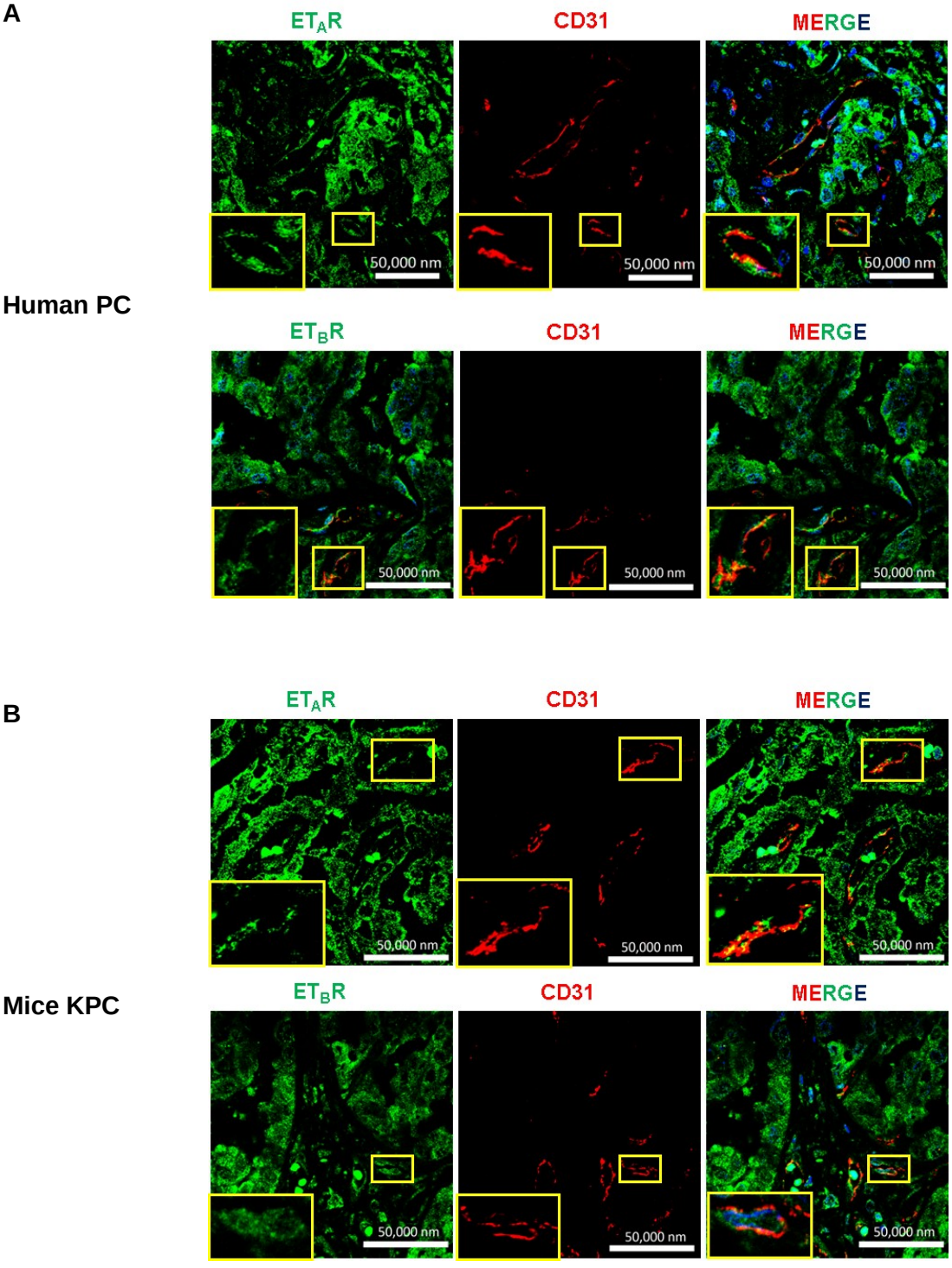
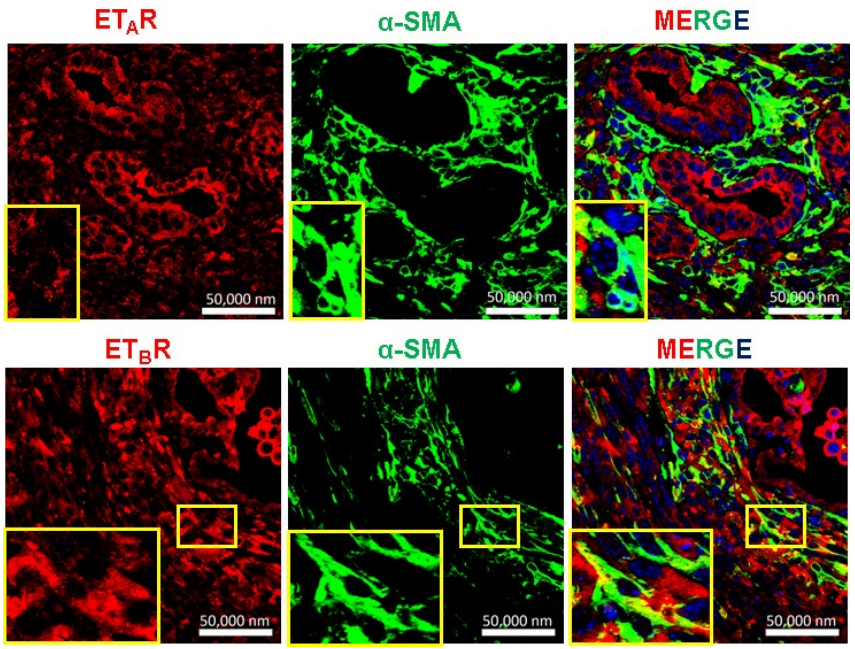

A
Human PC
ETAR
CD31
MERGE
50,000 nm
ETBR
CD31
MERGE
50,000 nm
B
Mice KPC
ETAR
CD31
MERGE
50,000 nm
ETBR
CD31
MERGE
50,000 nm

Figure 10: Expression of ET_AR and ET_BR in pancreatic stellate cells in human and mouse PC tissues. Dual confocal microscopy images show co-expression of ET_AR (red) and ET_BR (red) with stellate cell marker α-SMA (green) in human PC **(A)** and in 25 week KPC mouse **(B)** tissue. As compared to ET_BR, low expression of ET_AR is seen in α-SMA positive cells. Scale bar = 50μm.

Figure 10

A

Human PC



B

Mice KPC

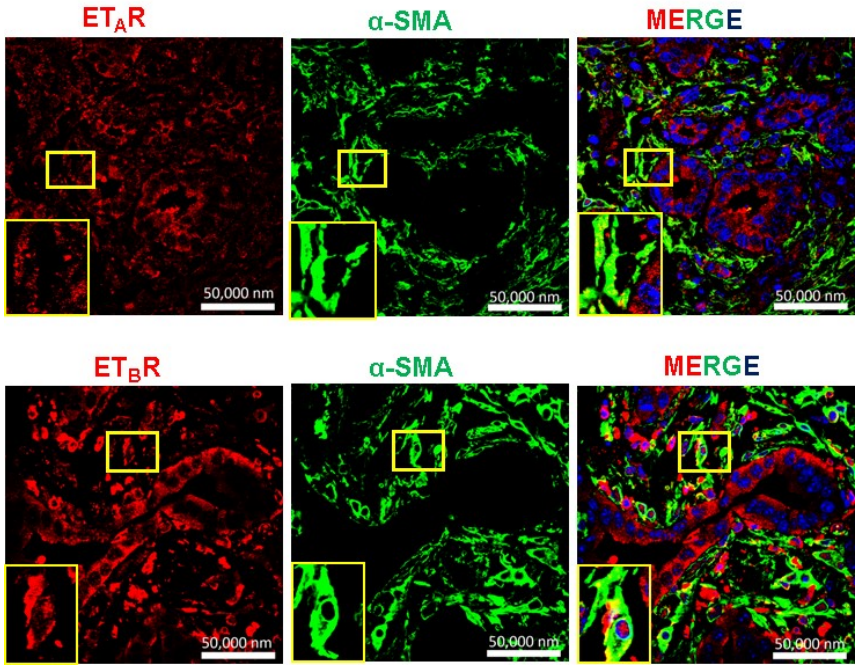


Figure 11: Expression of ET_AR and ET_BR in macrophages in human and mouse PC tissues. Dual confocal microscopy images show co-expression of ET_AR (red) and ET_BR (red) with macrophage marker, CD68 and F4/80 (green) in human PC **(A)** and in 25 week KPC mouse **(B)** tissue. As compared to ET_BR, low expression of ET_AR is seen in CD68 and F4/80 positive macrophages. Scale bar = 50µm.

Figure 11

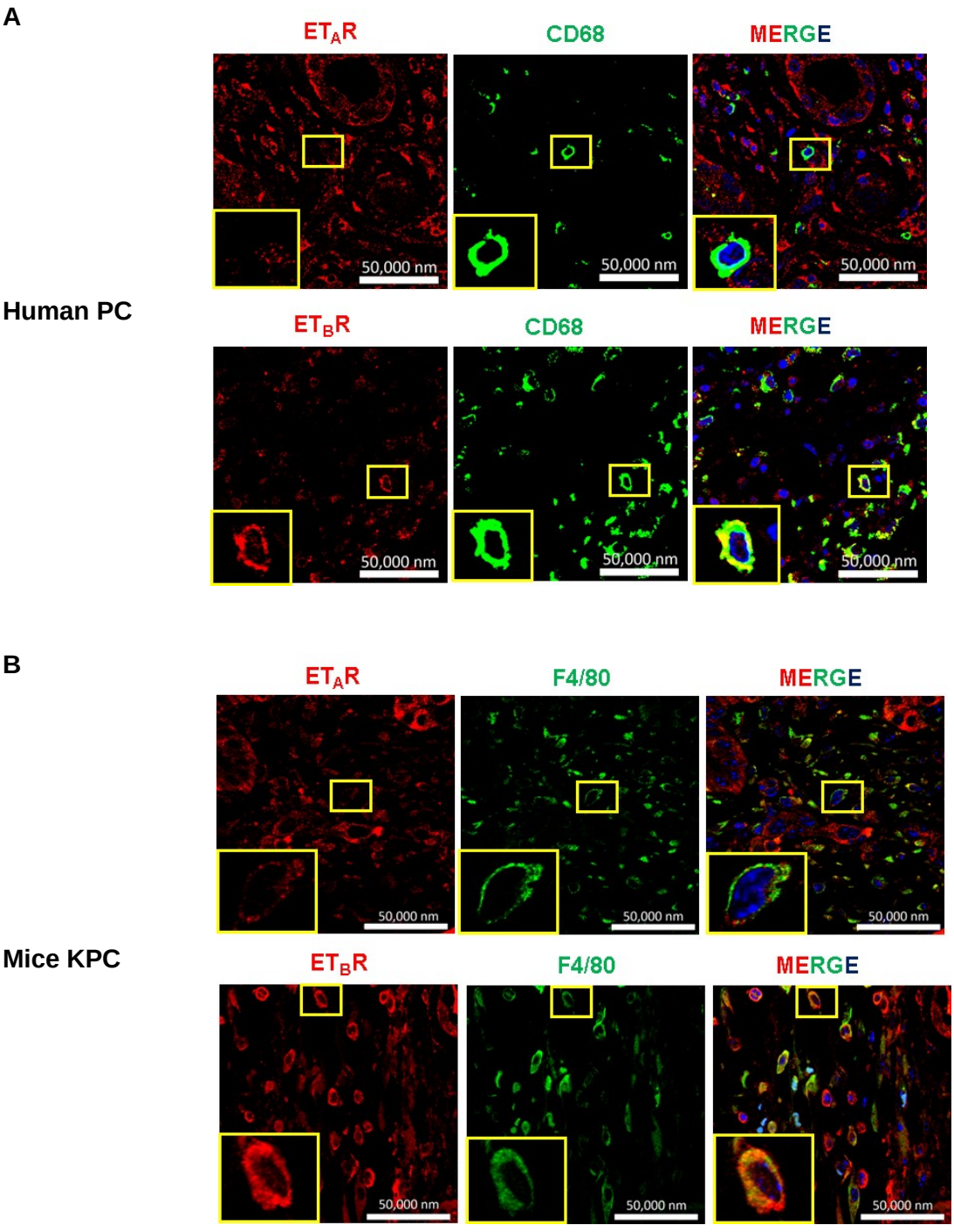

A
ETAR
CD68
MERGE
50,000 nm
Human PC
ETBR
CD68
MERGE
50,000 nm
B
ETAR
F4/80
MERGE
50,000 nm
Mice KPC
ETBR
F4/80
MERGE
50,000 nm

Figure 12: Expression of ET_AR and ET_BR in stem cells in mouse KPC tissues. Dual confocal microscopy images show co-expression of ET_AR (red) **(A)** and ET_BR (red) **(B)** with stem cell marker CD133 (green) in 25 week KPC mouse tissue and control. As compared to age-matched control, increase co-expression is seen for both ET_AR and ET_BR is seen in CD133 positive stem cells. Scale bar=50μm.

Figure 12

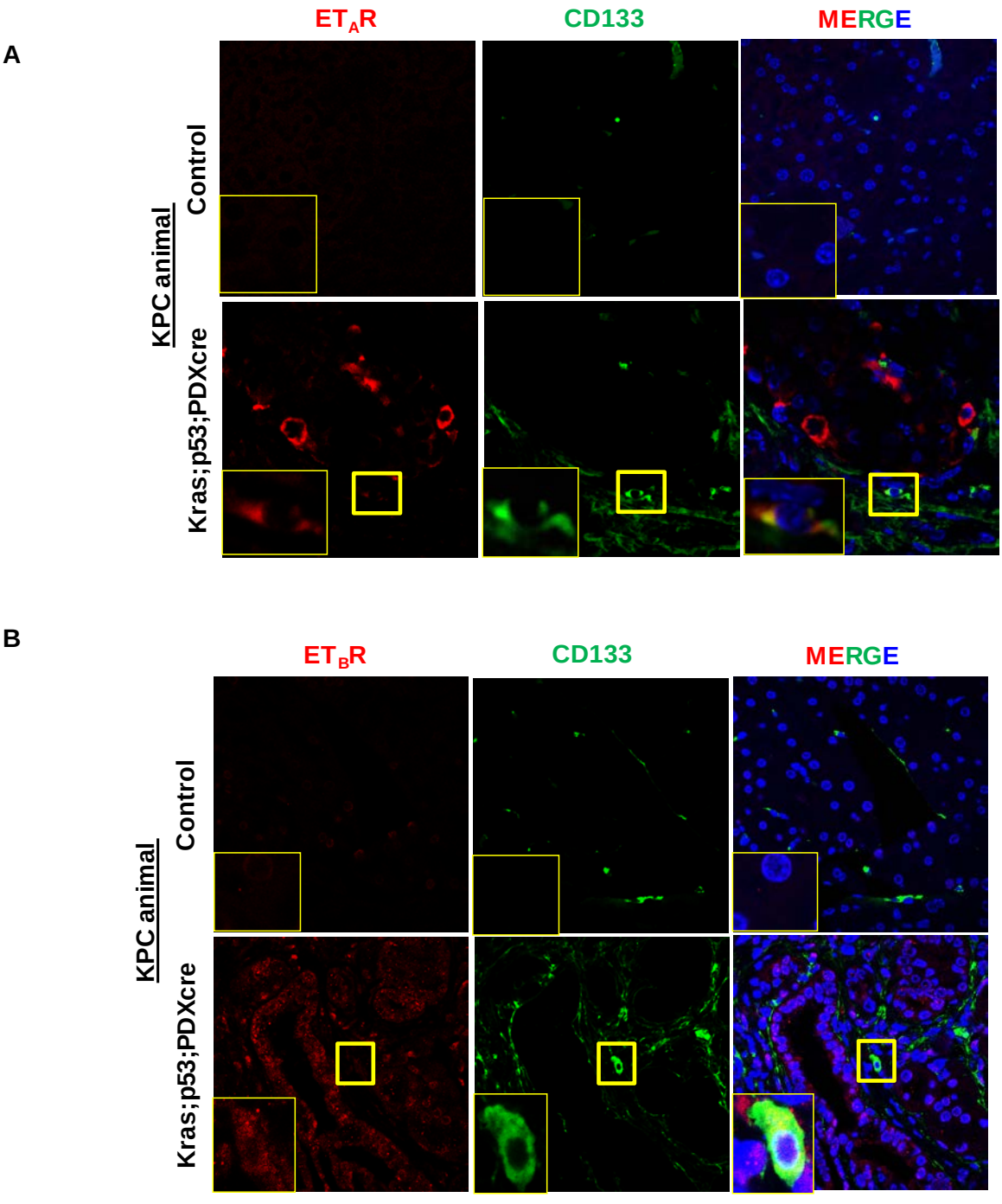
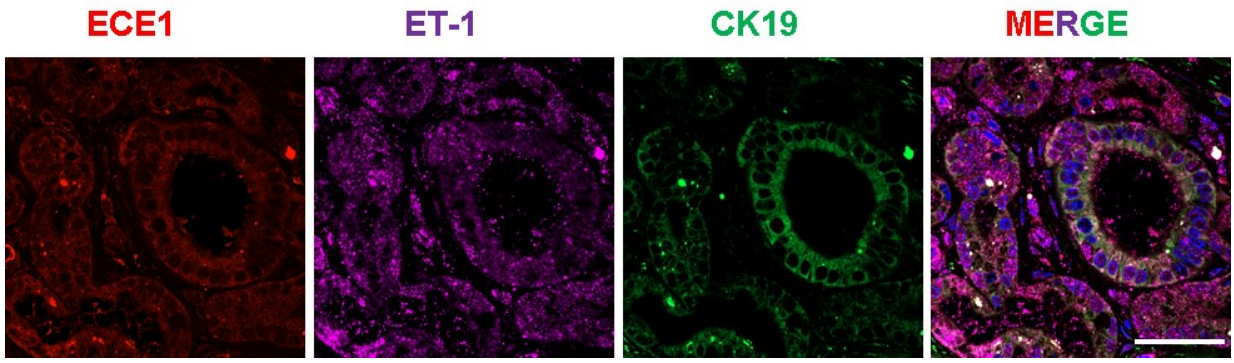

A
ETAR
CD133
MERGE
KPC animal
Control
Kras;p53;PDXcre
B
ETBR
CD133
MERGE
KPC animal
Control
Kras;p53;PDXcre

Figure 13: Expression of ECE-1, ET-1 in tumor cells in human and mouse PC tissues. Three color immunofluorescence analysis show co-expression of ECE-1 (red), ET-1 (purple) with epithelial marker CK19 (green) in human PC **(A)** and in 25 week KPC mouse **(B)** tissue. Both ECE-1 and ET-1 showed predominant co-expression with CK19 positive tumor cells. Scale bar= 20µm.

Figure 13

A

Human PC



B

Mice KPC

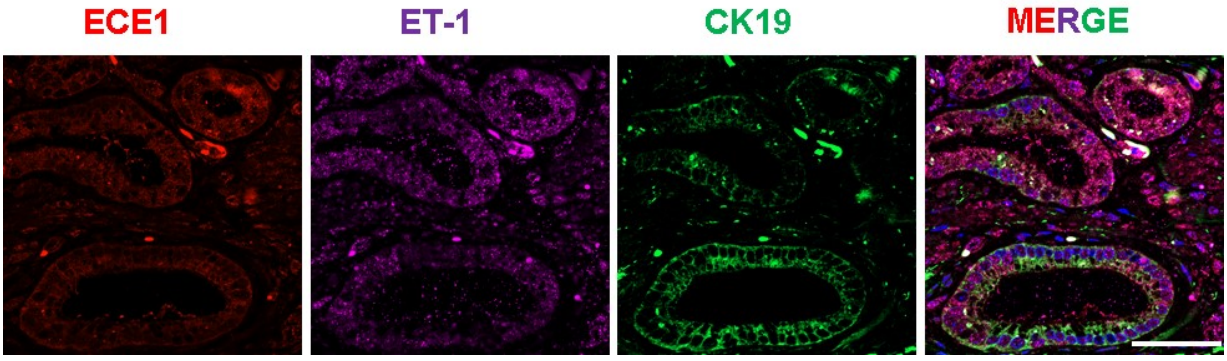
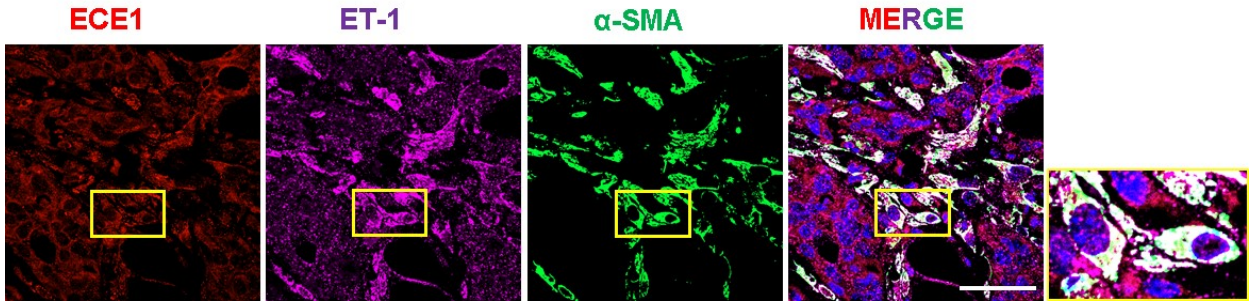


Figure 14: Expression of ECE-1, ET-1 in pancreatic stellate cells in human and mouse PC tissues. Three color immunofluorescence analysis show co-expression of ECE-1 (red), ET-1 (purple) with stellate cell marker α-SMA (green) in human PC **(A)** and in 25 week KPC mouse **(B)** tissue. Both ECE-1 and ET-1 showed predominant co-expression with α-SMA positive stellate cells. Scale bar= 20µm.

Figure 14

A

Human PC



B

Mice KPC

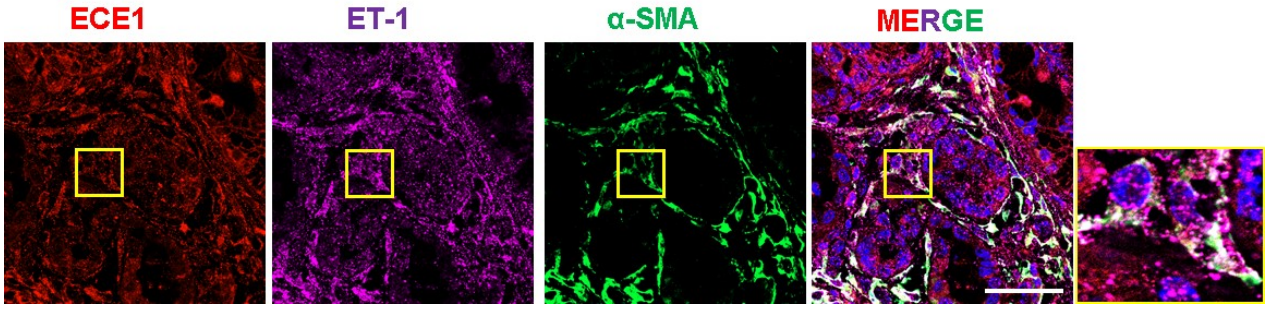
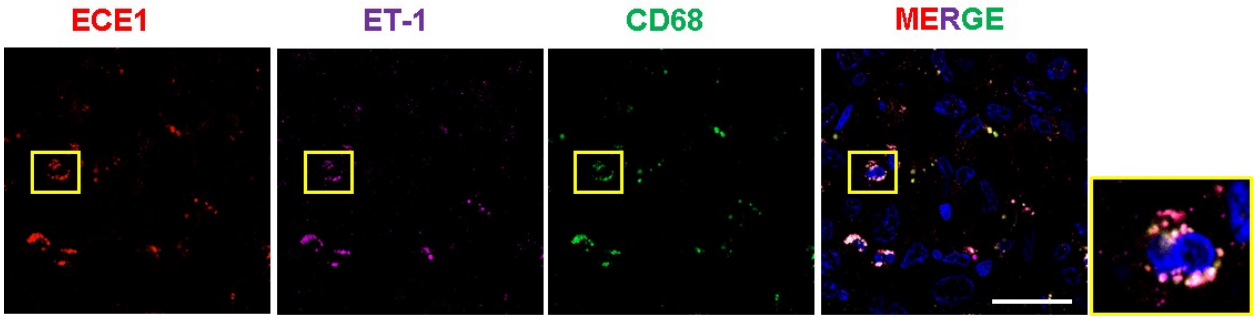


Figure 15: Expression of ECE-1, ET-1 in macrophages in human and mouse PC tissues. Three color immunofluorescence analysis showing co-expression of ECE-1 (red), ET-1 (purple) with macrophage marker CK19 (green) in human PC **(A)** and in 25 week KPC mouse **(B)** tissue. Both ECE-1 and ET-1 showed predominant co-expression with CD68 and F4/80 positive macrophages. Scale bar= 20μm.

Figure 15

A

Human PC



B

Mice KPC

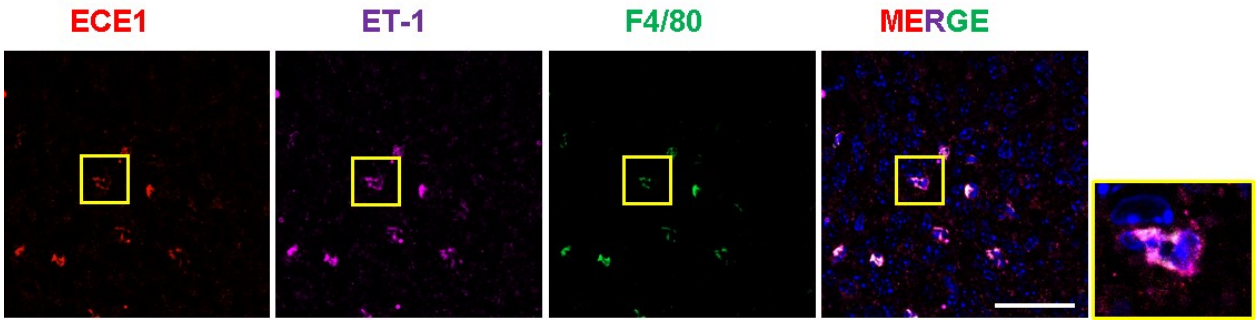


Table V: Expression profile of ET axis in TME of PC. Summary of the ET axis components (ECE-1, ET-1, ET_AR and ET_BR) expression in tumor cells, stellate cells, blood vessels, macrophages and stem cells. (NA= not analyzed).

Table V

Cellular Component	ECE-1	ET-1	ET_AR	ET_BR
Tumor cells	High	High	High	High
Stellate cells	High	High	Low	High
Tumor associated Macrophages	High	High	Low	High
Blood Vessels	NA	NA	High	High
Stem cells	NA	NA	High	High

Table VI: Expression of ET 1, ET_AR and ET_BR in TCGA databse. i. Representation of the number of TCGA cases on the basis of tumor stage. **ii.** Correlation of ET-1 (EDN1), ET_AR (EDNRA) and ET_BR (EDNRB) with disease stage in the TCGA sample set showed higher expression is significantly correlated with disease stage.

Table VI

i

Stage	# sample
1	20
2	136
3	3
4	3

ii

Comparison	Gene	p-value	Expression difference
Stage 1 vs. Stage 2	EDN1	0.05	Higher expression in Stage2
Stage 1 vs. Stage 3&4	EDN1	0.05	Higher expression in Stage3&4
Stage 1 vs. Stage 3&4	EDNRA	0.01	Higher expression in Stage3&4
Stage 1 vs. Stage 2	EDNRA	0.05	Higher expression in Stage2
Stage 1 vs. Stage 2	EDNRB	0.03	Higher expression in Stage2

Table VII: Bioinformatics analysis of TCGA database identified pro-fibrotic gene signatures associated with ET_AR and ET_BR over expression. The top 30 genes exhibiting strongest positive correlation with ET-axis included signaling receptor (PDGFRβ), extracellular matrix (ECM) proteins [collagens (COL1A2, COL6A3, COL5A2; fibro-nectin (FBN1), SPARC, lumican (LUM)], ECM modifying enzymes [sulfatase 1 (SULF1); chondritin sulfate synthase 3 (CHSY3)], basement membrane proteins [Nido-gen 2 (NID2)] and marker of fibroblast activation (FAP).

Table VII

Gene	Correlation Coefficient
EDNRA	1
PDGFRB	0.93229006
COL6A3	0.923354065
LUM	0.919764034
COL3A1	0.912931722
SULF1	0.908283067
COL5A2	0.904241078
FBN1	0.900111908
FSTL1	0.89695128
COL1A2	0.892809221
ANTXR1	0.888729065
FAP	0.887355808
RAB31	0.887014518
PRRX1	0.883714363
CTSK	0.882868859
VCAN	0.881828175
CDH11	0.878554108
SPARC	0.875505791
CHSY3	0.874950415
NID2	0.874049822
ST6GAL2	0.872265697
COL5A1	0.872014059
ZFPM2	0.871704495
ADAMTS12	0.869356289
OLFML1	0.867684557
COL8A1	0.86762915
NTM	0.867365174
THBS2	0.86728247
COL8A2	0.864088832
BNC2	0.863216

Gene	Correlation Coefficient
EDNRB	1
S1PR1	0.829567668
ERG	0.800204304
GIMAP8	0.796626128
CD93	0.79634361
SHE	0.792942146
GIMAP6	0.790079358
ABCA8	0.788097886
MYCT1	0.781620871
APOLD1	0.779309109
FZD4	0.778145346
CALCRL	0.773982364
DLG2	0.76891117
CYYR1	0.767162723
SDPR	0.76689
PREX1	0.76614093
BCL2	0.762736852
ABCC9	0.76161131
COL14A1	0.759946556
GPR116	0.759456347
SASH1	0.756571606
FBLN5	0.756516114
IGF1	0.755473139
TEK	0.754873115
PLCL1	0.7547968
SPARCL1	0.752258133
SLIT3	0.751699078
ELTD1	0.751505666
TMEM150C	0.748035643
PPAB2B	0.746605

Figure 16: Correlation of pro-fibrotic gene PDGFR-β with ET_AR over expression. Example of one gene showing positive correlation with ET_AR over-expression. Pearson correlation coefficient 'r' indicates the strength of the linear relationship between two variables. An 'r' value of 0 indicates no association between the two variables whereas an 'r' of +1 and -1 indicates the perfect positive and negative association respectively between the variables. Every dot indicates one patient. The association of PDGFR-β with ET_AR expression (r=0.93), indicates a stronger association of the two variables.

Figure 16

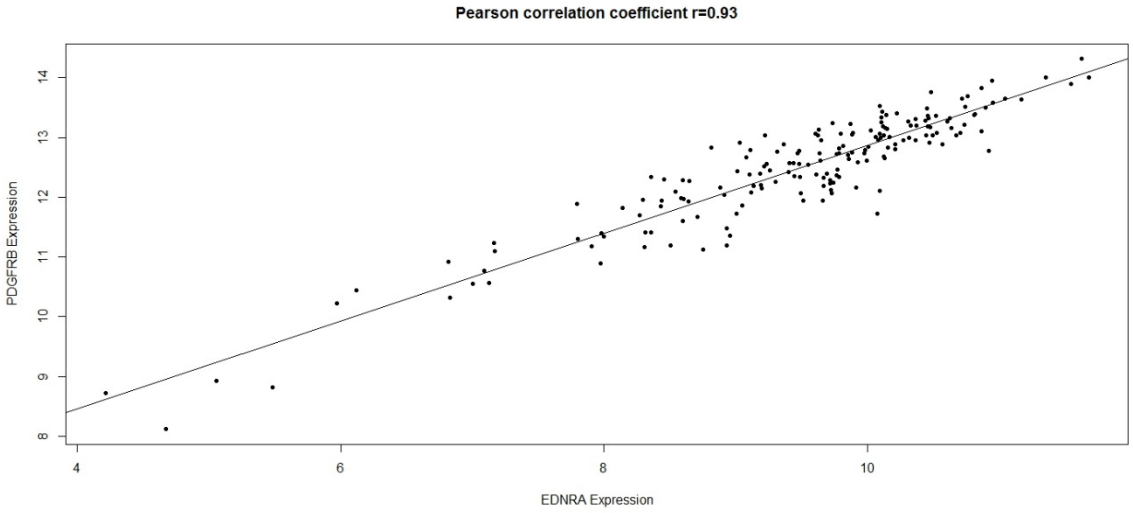

Pearson correlation coefficient r=0.93
PDGFRB Expression
EDNRA Expression
8
9
10
11
12
13
14
4
6
8
10

Figure 17: Correlation of pathways associated with ET axis over expression. Significant pathways were identified by Ingenuity pathway analysis that are upregulated and positively correlated with ET axis over expression.

Figure 17

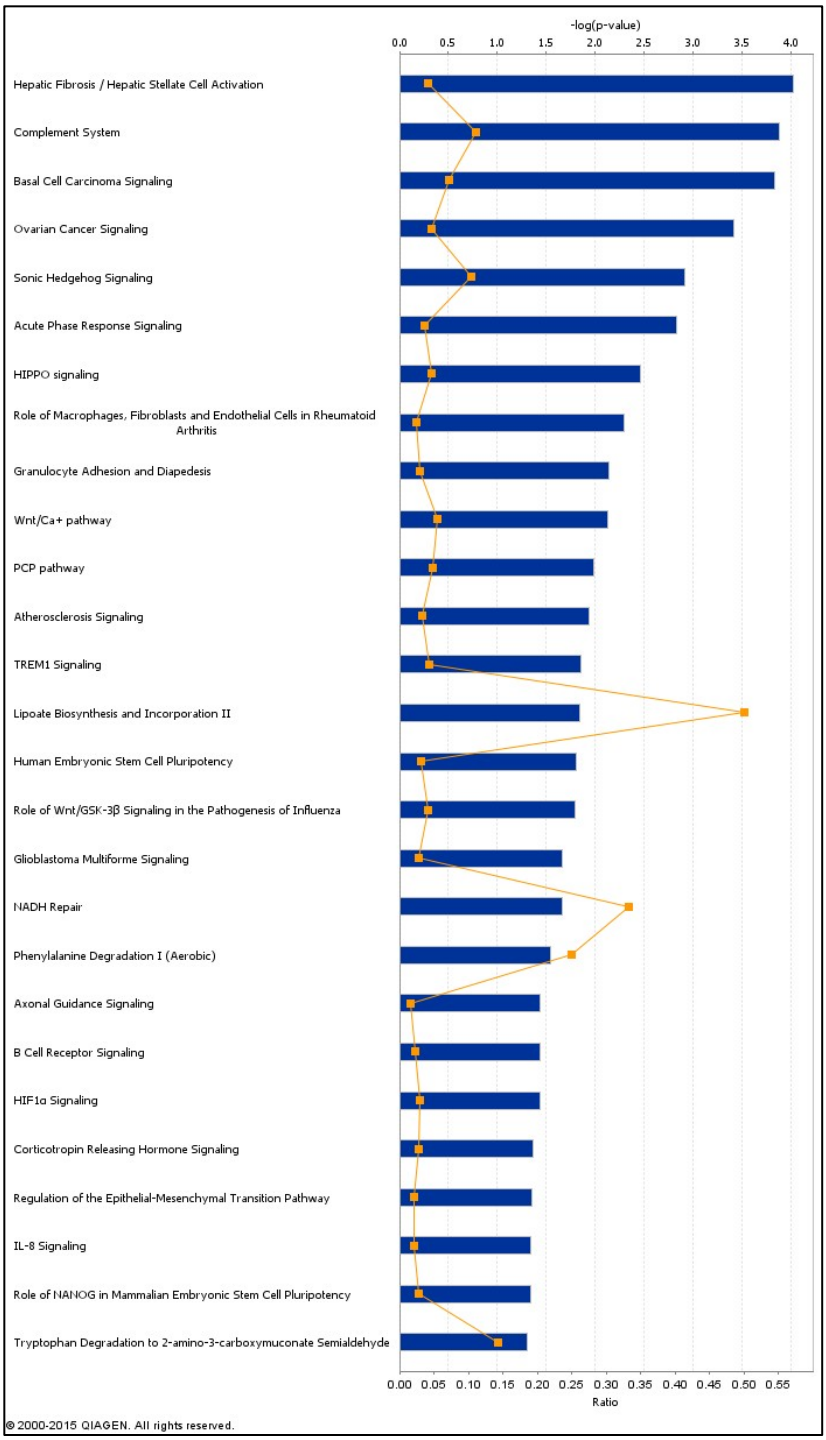

-log(p-value)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
Hepatic Fibrosis / Hepatic Stellate Cell Activation
Complement System
Basal Cell Carcinoma Signaling
Ovarian Cancer Signaling
Sonic Hedgehog Signaling
Acute Phase Response Signaling
HIPPO signaling
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis
Granulocyte Adhesion and Diapedesis
Wnt/Ca+ pathway
PCP pathway
Atherosclerosis Signaling
TREM1 Signaling
Lipoate Biosynthesis and Incorporation II
Human Embryonic Stem Cell Pluripotency
Role of Wnt/GSK-3β Signaling in the Pathogenesis of Influenza
Glioblastoma Multiforme Signaling
NADH Repair
Phenylalanine Degradation I (Aerobic)
Axonal Guidance Signaling
B Cell Receptor Signaling
HIF1α Signaling
Corticotropin Releasing Hormone Signaling
Regulation of the Epithelial-Mesenchymal Transition Pathway
IL-8 Signaling
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency
Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55
Ratio
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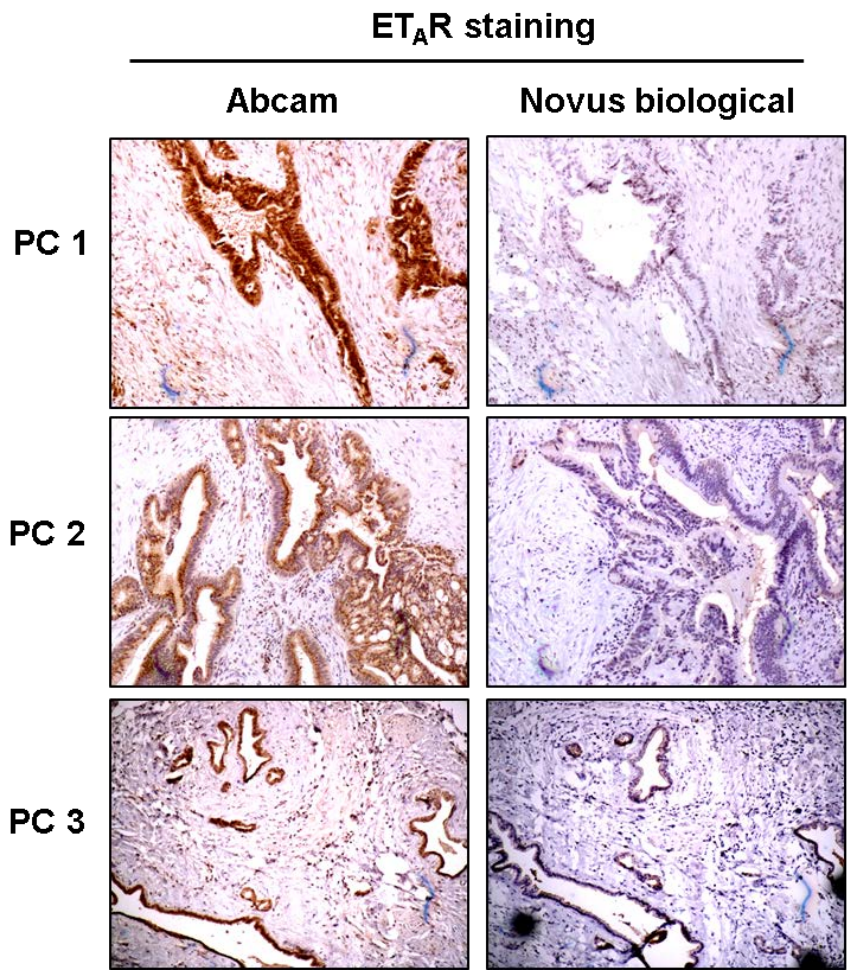
Figure 18: **Comparison of ET_AR staining in human PC tissues.** Immmunohistochemical analysis of ET_AR in serial sections of three human PC cases was compared to assess the immuno-reactivity of two different antibodies. A prominent increase in the ET_AR expression was seen in pancreatic ducts with Abcam antibody as compared to the Novus biologicals antibody.

Figure 18



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Chapter 4

Targeting endothelin axis in pancreatic cancer using selective and dual receptor inhibitors under *in vivo* (KPC, K-ras^{G12D}; *Trp53*$^{R172H/+}$; Pdx-1-Cre) and *in vitro* (pancreatic stellate cells and cancer associated fibroblasts) system

Synopsis

One of the hallmarks of pancreatic cancer (PC) is extensive and dense fibrous stroma that makes up 60-70% of the total tumor volume. Interaction of stromal cells with the cancer cells further modulates the malignant properties and favor metastasis of cancer cells. The cross talks between the stromal cells with each other and also with the tumor cells contribute to tumor growth and aggressiveness. The role of Endothelin(s) in the context of PC is not well studied and provides indirect evidence in fibrosis. In this study, we have evaluated the impact of targeting this axis using dual endothelin receptor antagonist, Bosentan using *in vitro* (murine stellate cells and human cancer associated fibroblasts) and *in vivo* mouse ($Kras^{G12D}$, $Trp53R172H^{+/-}$, Pdx-1-Cre) model. Bosentan treatment resulted in decrease in desmoplasia accompanied by decrease expression of extracellular matrix proteins. Further, the treatment induced direct killing of tumor cells as indicated by increase in cleaved caspase-3 positive cells. *In vitro,* pre-treatment of murine pancreatic stellate cells and human cancer associated fibroblasts with ETs inhibitors abrogated ET-1 mediated up regulation of fibrotic genes and suggested that ET-1 primarily induces pro-fibrotic gene signatures by both ET receptors. Further, ET-1 stimulation in PSCs induced a significant increase in the p-ERK and p-AKT in a time dependent manner. This ET-1 induced increase was inhibited by selective ET_BR antagonist BQ788 and to a lesser extent by selective ET_AR antagonist BQ123, however dual receptor antagonist Bosentan significantly attenuated the ET-1 mediated induction. Our studies suggest that signaling through ET-1 axis regulates pro-fibrogenic genes through ERK and AKT dependent pathway in PSCs. In addition, pharmacological inhibition of the ET axis reduces the infiltration of tumor-associated macrophages and increases infiltration of cytotoxic T cells in the PC stroma. Altogether, our studies also demonstrate that targeting ET axis can modulate the obstructive and

immunosuppressive TME and make it potentially more amenable for chemotherapy and immunotherapy.

Background and Rationale

PC is the 4th leading cause of cancer-related deaths in the United States accounting for nearly 37,390 deaths annually [1]. Due to the lack of early diagnostic and therapeutic modalities, PC has extremely poor prognosis with a dismal five year survival rate of <8%. Despite notable advances in surgical technique and postoperative care, and the use of adjuvant chemical and radiotherapies, virtually all early-stage patients who undergo resection also eventually succumb to recurrence and/or metastasis [2] that account for lethality of the disease. One contributing factor to the failure of systemic therapies may be the abundant tumor stromal contents forming the physical barrier and thereby impeding the drug delivery [3]. The stromal microenvironment is a complex structure composed of an extracellular matrix (ECM), cancer associated fibroblasts (CAFs), inflammatory cells and blood and lymphatic vessels that distort the normal architecture of pancreatic tissue. A major source of CAFs in PC is pancreatic stellate cells (PSCs), which are resident cells of the pancreas that store lipid droplets and express fibroblast activation protein-α (FAP α) [4]. Once activated in response to external stimuli, inflammation or injury, PSCs express the myofibroblast protein α-smooth muscle actin (α-SMA) and secrete factors that stimulate tumor growth and metastasis.

Recently, activation of sonic hedgehog (SHh) pathway has been identified to promote stromal desmoplasia [5, 6]. Inhibition of SHh by neutralizing antibody or small molecule inhibitor has been shown to decrease desmoplasia [7] and improve small molecule drug uptake in pancreatic tumors in KPC mice. However, most of the tumors in KPC mice resumed growth after a transient response [8]. Another microenvironment targeting strategy, utilizes CD40 agonist to drive antitumor T cell response-facilitating depletion of tumor stroma [9], making PC stroma as an attractive therapeutic target for

drug development. However, controversy exists and recent evidence has questioned the role of stroma in PC progression. Transgenic mice with αSMA(+) myofibroblasts depletion in the tumor stroma induces immune suppression and accelerates cancer progression [10]. Also, genetic ablation or prolonged inhibition of sonic hedgehog ligand led to non-invasive and poorly differentiated tumors and decreased survival in mice [11]. These findings demonstrate the need to use caution when targeting stroma in PC and highlight the importance of selective modulation of stroma is prerequisite to achieve considerable therapeutic benefit and improve the effectiveness of stroma- targeting drugs.

Endothelins (ETs) are a family of three peptides, ET-1, ET-2 and ET-3, which mediate pleotropic effects via two G-protein coupled receptors ET_AR and ET_BR and have key physiological functions in normal tissue, and act as modulator of vasomotor tone [12] Accumulating evidences indicate the pro-fibrogenic role of ET-1 in the various pathological malignancies including cancer and have gain considerable interest and attention[13] Mechanistic and experimental evidences implicate the regulation of ET-1 at the transcriptional level. Several factors such as shear stress, hypoxia, TGF-β, IFN-γ, angiotensin II and thrombin up-regulate; whereas, nitric oxide down regulate the expression of ET-1 mRNA [14] [15]. The involvement of ET-1 in diverse biological processes can be attributed to its interaction with various transcription factors such as GATA, Smad, TGF-β and activator protein-1 (AP-1) [12]. The role of ETs in the context of pathogenesis of PC and fibrosis is not well studied and provides incomplete evidence. The effect of targeting ET axis and its impact on fibrotic genes was evaluated using dual ET receptor inhibitor, Bosentan in rat model. Bosentan treatment was found to inhibit the proliferation of PSCs and the genes involved in PSC activation like α-SMA and CTGF [16]. In addition, ET-1 induced phosphorylation of ERK and MLC promotes migration and

contraction of stellate cells. This ET-1 induced migration and contraction was inhibited by selective ET_A and ET_B antagonist[17]. In addition to contraction and migration caused by ET-1, stimulation led to the activation of PKCs, MAPK and ERK pathway. Further, it also enhances the production of pro inflammatory cytokines like IL-1 and IL-6 and expression of α-SMA and CTGF (connective tissue growth factor) suggesting an autocrine and paracrine loop mediated by ET-1 which acts on activated stellate cells[18].

In this study, we have evaluated the impact of ET axis inhibition using autochthonous tumors that develop in genetically engineered mouse model (KPC) of PC. The study here in provides for the first time the anti-tumor and anti-fibrotic effects of ET axis antagonism in lethal PC *in vivo*. Importantly, we also examine the effect of selective and dual receptor antagonists on the proliferation of murine PSCs and human CAFs and investigated the molecular mechanisms responsible for ET-1 mediated induction of the pro-fibrotic gene signatures. Our results also suggest that pharmacological targeting of the ET axis in KPC mice reduces the infiltration of tumor-associated macrophages and increases recruitment of cytotoxic T lymphocytes (CTLs).

Results

A. Inhibition of ET axis *in vivo* using Bosentan increases apoptosis and reduces fibrosis in KPC autochthonous tumors

We evaluated the impact of ET-axis inhibition in autochthonous tumors that develop in genetically engineered mouse model (KPC) of PC that is driven by pancreas-specific expression of mutant K-ras and p53. Dual endothelin receptor antagonist, Bosentan (1mg/kg body weight) was administered for a period of 10 days in 20 wk KPC mice (n=5) having high stromal content whereas the control animals (n=5) received saline. At the end of the treatment period the primary tumors were harvested and analyzed for various histopathological features such as fibrosis and apoptosis. No difference in the weight and volume of pancreas was observed. Bosentan treatment resulted in a significant increase in the number of apoptotic cells (p=0.01) compared to saline treated control group as indicated by immunohistochemical staining against cleaved-caspase 3 and TUNEL staining **(Figure 1A).** Using Trichrome-Masson staining we observed that Bosentan treatment reduces stromal growth in KPC derived tumors compared to control group **(Figure 1B).** The Trichrome-Masson staining for fibrosis/desmoplasia was evaluated in mouse pancreatic tissue sections in a semi-quantitative fashion. Moderate to strong staining was seen in areas with control mice with 51-75 and 76-100 percent positivity respectively. In treated group, the staining shows variability with areas having poor to moderate staining with 0-25 and 26-50 percent positivity. The composite score is obtained by multiplying the numerical value and staining intensity for both the groups.

To further determine the impact on fibrosis following Bosentan treatment we sought to investigate the effect on matrix associated genes using RT^2 profiler fibrosis PCR array. Following 10-day treatment the tumors were harvested and used in the array as described in the materials and methods section. Data were normalized to six house

keeping genes in the array. Heat map represents the expression of 84 genes involved in the PCR array **(Table I)**. The array contains the genes encoding ECM remodeling enzymes, TGFβ signaling molecules and inflammatory cytokines, as well as additional genes important for fibrosis. The volcano plot in **Figure 2A** was used to identify genes that are 2-fold differential expressed upon treatment with ET antagonist as compared to control. We observed that Bosentan treatment has a significant impact and down regulated the pro-fibrotic gene signatures such as CTGF (5.22 fold), α-SMA (1.51 fold). In addition, we also found that a reduction in the extracellular matrix proteins such as Col3a1 (5.12 fold), Col1a2 (4.54 fold) with significant p value **(Figure 2B).** Pharmacological inhibition of the ET axis using Bosentan also decreased the levels of matrix remodeling enzymes such as Lysyl oxidase (Lox) (2,87 fold), Mmp2 (3.57 fold), Mmp14 (3.58 fold) and cellular adhesion proteins such as Integrin alpha V (Itgav) (2.55 fold), Integrin subunit beta 1 (Itgb1) (2.31 fold) and Integrin subunit beta 1 (Itgb5) (2.30 fold) with significant p value **(Table I)**. Further we also observed that Bosentan treated KPC mice displayed reduce levels of members of TGFβ superfamily such as Tgfb3 (2.45 fold), Tgfbr1 (1.64 fold), Smad 3 (1.75 fold), serine/threonine kinase AKT 1 (1.93 fold) and growth factors such as Pdgfa (1.39 fold) and Pdgfb (2.25 fold). **Table II** represents the overall summary of the gene expression profile. The pro-fibrotic associated gene signatures identified through TCGA database displayed significant association with ET axis overexpression and the gene signature targeted by Bosentan in fibrosis array is in concordant with ET axis association as predicted by TCGA analysis. These findings indicate that Inhibition of ET-axis by dual-specificity inhibitor Bosentan resulted in significant decrease in desmoplasia and increase in apoptosis in autochthonous tumors in KPC mice, suggesting a possible a possible impact on fibrosis on targeting ET axis. The decrease in the expression of these genes was confirmed by western blot analysis using tumor lysates and there was significant reduction in the

expression of CTGF, αSMA, fibronectin and FSP1 **(Figure 3A).** These findings were further confirmed through immunohistochemistry analysis as well. Bosentan treatment reduces the extracellular matrix proteins mainly collagen and αSMA which is associated with stellate cells activation and also fibronectin and CTGF, suggesting a possible impact on fibrosis on targeting this axis.

B. Bosentan treatment decreases the FSP1 positive but not FAP positive fibroblasts in PC tumor stroma

To further determine the impact on various populations of stromal fibroblasts following Bosentan treatment triple immunofluorescence analysis was done using CK19, αSMA and FSP1 specific antibodies **(Figure 4A)**. Bosentan treatment reduces both FSP 1 positive and αSMA positive fibroblasts in the PC tumor stroma with significant p value. Interestingly, α-SMA expressing fibroblasts surround the CK19 positive ducts whereas the FSP1 staining is present in different sub population and exhibits no overlap with α-SMA **(Figure 4B)**. In addition, Bosentan treatment has no significant effect on FAP positive fibroblasts cells however co-localization with α-SMA was seen **(Figure 4C)**.

C. ET-1 promotes proliferation and migration of pancreatic stellate cells (ImPSCc2)

As pancreatic stellate cells are predominantly responsible for desmoplastic reaction in PC, the effect on growth on immortalized pancreatic stellate cell line (ImPSC.c2) was evaluated by MTT assay. Treatment of ImPSCc2 cells with recombinant ET-1 increased their proliferation in a time and dose dependent manner, with significant effects at 100nm **(Figure 5A).** ET_AR specific antagonist (BQ123) has negligible effect on growth of these ImPSC.c2 **(Figure 5B)** however; both ET_BR specific (BQ788) **(Figure**

5C) and dual receptor antagonists (Bosentan) **(Figure 5D)** reduced the growth in time and dose dependent manner. To further assess the mechanism of underlying cause of the growth inhibition of ImPSCc2 following treatment with endothelin antagonists we performed cell cycle analysis and Annexin V staining of the control and treated cells by fluorescence activated cell sorting analysis. Increase apoptosis in stellate cells compared to control was seen following treatment with receptor B antagonist BQ788 and dual receptor antagonist Bosentan **(Figure 6A & 6B)**. Also, treatment with BQ788 induced G1/S cell cycle arrest. The percentage of cells increased from 48.2% to 54.5% and 32.9% to 35.7% in G1 and S phase respectively. Treatment with endothelin A/B receptor antagonist arrest cells in S phase of the cycle. The percentage of cells increased from 32.9 % to 48.2 % and decreased from 48.2 % to 37.8% in the S phase G1 phases respectively **(Figure 6C)**. We then investigated the effect of ET-1 and inhibitors on the migratory potential of ImPSCc2 using wound healing assay **(Figure 7).** The cells were seeded in a 6 well plate and after the cells reached approximately 90% confluency an artificial wound was made using 200µl pipette. The cells were then washed with PBS and were then incubated for 24 hr in presence of endothelin antagonists after pretreatment for 45 mins before addition of 100nm of ET-1. Phase contrast images were obtained at 0h, and 24hr time points. As compared to the control group, treatment with recombinant ET-1 displayed a higher migration rate of ImPSCc2 in a time dependent manner with significant effects observed at 24 hr ($p<0.01$). In contrast, selective A receptor ($p<0.01$) and B receptor ($p<0.01$) inhibitor treated cells resulted in significant decrease in the cellular migration compared to rET-1 group. This observation of decreased cellular migration was further strengthened using dual ET axis antagonism as it also demonstrated a significant decrease in migration of PSCs ($p<0.01$).

D. ET-1 stimulates expression of pro-fibrotic genes predominantly through ET_BR and to lesser extent by ET_AR in murine pancreatic stellate cells *in vitro*

We also evaluated the anti-fibrotic effects of ET axis antagonism *in vitro* and investigated the expression of ET axis in immortalized mouse pancreatic stellate cells (ImPSCc2) and tumor cell line derived from KPC model of PC. We have successfully derived and characterized aggressive cell line UN-KPC-961, from the primary tumors that developed in KPC mice [19]. To study the cellular cross talk between tumor and stellate cells along ET axis in vitro, 2D co-culture using Boyden chamber was utilized where immortalized mouse stellate cells are cultured in presence of tumor cells alone or in the presence of selective ET_AR (BQ123), selective ET_BR (BQ788) and dual (Bosentan) ET receptor antagonists. Our results indicated that tumor cells express receptor A whereas stellate cells predominantly express receptor B and low levels of receptor A **(Figure 8A).** The co-culture of ImPSCc2 with UN-KPC-961 cells did not alter the matrix-associated markers. Co-culture of ImPSCc2 treated with BQ123 did not influence expression of stromal genes. ET axis antagonism resulted in inhibition of genes associated with PSC activation (α-SMA) predominantly through ET-1/ET_BR signaling however, dual receptor inhibition using Bosentan significantly inhibit the expression of α-SMA, CTGF fibronectin and Collagen-1 in these cells **(Figure 8b).** Additionally, serum starved stellate cells were pre-treated with endothelin inhibitors, which is not toxic to cells for 45 mins prior to addition of recombinant ET-1. Exogenous administration of ET-1 caused timely dependent increase in expression of αSMA, CTGF, fibronectin and collagen I in stellate cells **(Figure 8c)**.This ET-1 induced expression was inhibited by selective ET_BR antagonist BQ788 and not by selective ET_AR antagonist, BQ123. Moreover, ET-1 induced expression was significantly abrogated by dual receptor antagonist Bosentan, suggesting that ET-1 stimulates

expression of matrix-associated genes mainly through ET_BR and to less extent by ET_AR.

E. Expression of ET-1 and receptors in patients derived fibroblasts

We further extended our studies in fibroblasts derived from the pancreas of healthy individuals, chronic pancreatitis and cancer associated fibroblasts. These cell lines were developed and immortalized in our lab. We first characterized the purity of these cell lines using immunoblot analysis against specific myo-fibroblast marker α-SMA and epithelial marker CK19. The HEK 293 and Panc1 cells were used as positive controls for α-SMA and CK19 respectively **(Figure 9A).** As compared to normal fibroblasts, a robust expression of α-SMA was observed in these cell lines whereas no expression of CK19 was evident. In addition to α-SMA, the expression of other pro-fibrotic genes like collagen I, fibronectin, CTGF was analyzed. Desmin, which is supposed to be a marker for quiescent fibroblasts, is expressed at very low levels in these cells **(Figure 9B).** The expression pattern of ET-1 and its receptors in fibroblasts isolated from chronic pancreatitis and cancer patients were also studied by RT-PCR analysis using PC3 as a positive control. Expression of ET-1 and ET_AR was seen in all cell lines tested except 10-32 fibroblasts. However, the expression of ET_BR is mainly observed in cell lines derived from cancer-associated fibroblasts **(Figure 9C).**

F. Inhibition of ET-1 axis inhibits fibrosis in human CAFs *in vitro*

The effect of selective ET_AR antagonist, BQ123, selective ET_BR antagonist, BQ788 and dual receptor antagonist, Bosentan on proliferation of 10-03 CAFs (express both ET_AR and ET_BR) was also determined by MTT assay. Similar to the effects observed on ImPSCc2 cells, BQ123 has negligible effect on growth of these cells **(Figure 10A),** however both BQ788 **(Figure 10B)** and Bosentan **(Figure 10C)** reduce

the growth in a dose and time dependent manner. We also evaluated the anti-fibrotic effects of ET axis antagonism in vitro using 10-03 CAFs. These serum CAFs were pre-treated with endothelin inhibitors, which is not toxic to cells for 45 mins prior to addition of recombinant ET-1. The ET-1 treatment caused time dependent increase in the expression of α-SMA, fibronectin and collagen I **(Figure 11A & 11B),** an effect similar as observed in ImPSCc2 cells. Both selective ET_AR and ET_AR inhibitors abrogated the ET-1 induced expression in pro-fibrotic gene signatures after 4 hr of treatment. However, a synergistic effect was observed with dual receptor antagonism, Bosentan **(Figure 11A & 11B).**

G. ET-1 promotes the expression of pro-fibrotic genes through a p42/44 MAPK and AKT dependent pathway

To probe the signaling mechanism through which ET-1 induces the expression of pro-fibrotic genes we perform the western blot analysis. We found that treatment with recombinant ET-1 in murine ImPSCc2 induces a robust expression in the p-ERK and p-AKT in a time dependent manner with a peak observed at 1hr of treatment **(Figure 12A & 12B).** Pretreatment of selective A and B inhibitors decrease the ET-1 mediated increase in the p-ERK levels, however no significant effect was observed in p-AKT levels. In addition, a 45-min pre-incubation with the dual specificity ET_AR and ET_BR antagonist Bosentan blocked the ability of ET-1 to induce phosphorylation of p-AKT and p-ERK levels, suggesting that ET-1 induces the expression of pro-fibrotic genes through ERK and AKT dependent mechanism **(Figure 12A & 12B)**. Similar to murine stellate cells, we found that treatment with recombinant ET-1 in 10-03 CAFs induces a robust expression in the p-AKT in a time dependent manner and persistent activationwas seen up to 16 hrs **(Figure 13A & 13B).** Pretreatment with both selective ET_A and ET_B receptors inhibitors decrease the ET-1 mediated increase in the p-AKT levels after 4 hrs

of treatment. In contrast, Bosentan abrogated the ET-1 mediated increase after 30 mins of treatment. Additionally, ET-1 also induces the expression of p-ERK with a peak observed after 1 hr of treatment and sustained increase in the p-ERK levels was seen up to 16 hrs. Both BQ123 and BQ788 reduces the phosphorylation of ERK at 16 hrs of treatment, however, dual receptor inhibition abrogated the ET-1 induces increase after 8 hrs of treatment **(Figure 13A & 13B),** suggesting that both p-AKT and p-ERK signaling are inhibited at different time points.

To summarize the overall findings,endothelin-1 release from the tumor cells act on the nearby pancreatic stellate cells in a paracrine manner expressing endothelin receptors and activate the downstream signaling which in turn induces the expression of pro-fibrotic genes through ERK/AKT dependent manner. However, in presence of Bosentan the binding of ligand to the receptor is inhibited resulting in the decrease expression of pro-fibrotic genes **(Figure 14).**

H. ET axis antagonism reduces infiltration of macrophages and increases cytotoxic T cell populations

We further determine the impact on recruitment of macrophages and T cells population in PC stroma following Bosentan treatment in autochthonous tumors (in KPC mouse model of PC). Dual specificity ET axis antagonist Bosentan reduces the number of tumor associated macrophages (TAMs) in KPC mice compared to age matched controls with significant p value ($p<0.008$) **(Figure 15A).** In addition, we also observed that pharmacological inhibition of ET axis reduces M2 marker (CD206) in KPC tumors with significant p value **(Figure 15B).** Interestingly, Bosentan treatment enhance recruitment of CD3 positive T cells in the stromal compartment of KPC tumors **(Figure 16).** Importantly, our immunofluorescence analysis also suggests increased infiltration

of cytotoxic T cells (CTLs) in the treated KPC tumors. Quantitative examination of the mice tumor tissues infers infiltration of $CD8^+$ T cells with significant p value (p=0.007) **(Figure 17C &17D)** compared to age matched controls, whereas no significant difference in $CD4^+$ T cells was observed **(Figure 17A & 17B).**

Expression of ET receptors in human macrophage-like U937 cells was also determined using Immunoblot analysis using Colo357 and T3M4 PC cells as postive controls for ET_AR and ET_BR. The U937 cells predominantly express ET_BR, which was not impacted by the differentiation status of the macrophages following treatments with PMA and cytokines **(Figure 18A).** The effect of ET antagonism using seletive and dual inhibitor on migration of human monocytes U937 **(Figure 18B)** and murine macrophages RAW264.7 **(Figure 18C)** towards tumor cells was analyzed using Boyden chamber assay. Monocytes and macrophage were cultured in serum free media and their migration towards Panc 1 and UN-KPC-961 cells in presence and absence of inhibitors was evaluated. In both U937 and RAW cells increased migration was seen towards tumor cells. Importantly, both specific and dual inhibitors significantly reduce the tumor cell induced migration of U937 cells. In addition, ET_BR-specific inhibitor, BQ788 and dual ET receptor antagonist, Bosentan significantly inhibited tumor cell induced migration of murine macrophage-like RAW 264.7 line while minimal effects with ET_AR-specific inhibitor, BQ123 was observed.

I. Selective ET_AR antagonism increases tumor perfusion and decreases tumor hypoxia in xenografts tumors

Selective ET_AR antagonism using BQ123 has been demonstrated to specifically induce dilation of tumor vessels and improve perfusion in low-perfused tumor areas [20] We evaluated the effect of ET_AR antagonist BQ123 in xenograft murine model derived from pancreatic cancer cell lines. The change in perfusion was studied by MRI using

flow sensitive alternating inversion recovery (FAIR) **(Figure 19A).** Perfusion was measured prior to adminsitrationof BQ123 to establish base line and subsequently imaged for 120 mins post adminsitration. Left panel shows the anatomic MRI scan to indicate the postion of the tumor and the region of interest in the tumor and muscle for perfusion analysis. Changes in perfusion in response to BQ123 were more pronounced in tumors than in the muscle. Further, we observed that the kinetics of perfusion changes were notably different in the T3M4 and Colo 357 tumors**(Figure 19B).** We also found that enhanced perfusion induced by BQ123 resulted in decreased hypoxia in T3M4 tumors **(Figure 19C).**

Discussion

An intense stromal desmoplastic reaction surrounding the tumor cells is the typical histological hallmark feature in PC and pancreatic stellate cells (PSCs), one of the major components of the TME are most important cellular source of CAFs in PC [21]. Evidences indicate that ET-1 mediate recruitment of various cell types such as resident fibroblast, stellate cells, vascular pericytes, circulating bone marrow derived monocyte responsible for pro-fibrotic potential of ET-1 [22]. Studied have indicated that ET-1 mediate the contraction, migration and expression of matrix associated genes in PSCs are abolished by dual receptor ET_AR and ET_BR inhibitor, Bosentan [23], however the data is very limited and provide inconclusive role of ET axis in generating the stromal reaction in PC. Our preliminary investigation suggests the direct correlation of stromal associated genes with ET axis in lethal PC. Bioinformatics analysis of the TCGA database infers that over expression of ET-1, ET_AR and ET_BR in PC has significant correlation with the fibrosis associated genes and pathways demonstrating the therapeutic potential of the axis. Our more recent data from the animal experiments confirm the pro-fibrogenic effect of ET-1 *in vivo*. We evaluated the impact of ET axis

antagonism using spontaneous KPC mice model treated with Bosentan. Bosentan is currently used in clinics to treat pulmonary hypertension and the formation of new digital ulcers in scleroderma patients [24] [25]. Our data show that Bosentan treatment of 20-week KPC mice, which has abundant tumor stroma, has global impact on the pro-fibrotic gene signatures compared to control mice. Further visualization of treated tumor tissues indicates decrease in the IHC and Immunoblotting signals for Collagen I, fibronectin, α-SMA and CTGF. Additionally, inhibition of the ET axis using FDA approved drug, Bosentan induces significant increase in the TUNEL positive cells and increased positivity of cleaved caspase 3. To our knowledge this is the first study to demonstrate the anti-fibrotic and anti-tumor effect of ET axis inhibition using genetically engineered mouse model in PC. Further comparative gene expression analysis on fibrosis gene array implies down- regulation of transcription factors such as Smad and TGF-β. Moreover, studies have shown that the pleotropic action mediated by ET-1 can be attributed to its interaction with various factors such as GATA, Smad, TGF-β and AP-1 [26, 27]

We speculate that anti-fibrotic effects of Bosentan can be attributed to its reduce interactions with the transcription factors. Further observation that ET-1 is a downstream mediator of TGFβ is elucidated by the observation that Bosentan blocks the ability of TGFβ to induce the expression of the fibrotic gene signatures such as α-SMA in fibroblasts isolated from scars of scleroderma patients [28]. Also, signaling pathways induced by ET-1 and TGFβ signaling led to increase ECM synthesis and contraction converge on a similar signaling pathway indicating synergistic association between the two [29] [30]. Evidence also suggests that, ET-1 increases TGF-β expression and collagen synthesis by eliciting Ca2+ release in an ET_AR dependent manner [31, 32]. Also, in human stellate cells, TGF-β stimulated ET-1 release by stabilization of ET-1 mRNA through PI3K/AKT dependent pathway was abolished by ET_AR antagonist,

BQ123 [33]. One of the important finding of our study is that the stroma associated genes identified through human TCGA database analysis displayed positive and significant correlation with ET axis overexpression and gene expression analysis in fibrosis array indicates significant down-regulation of the gene signature targeted by Bosentan.

Accumulating evidence indicates the presence of heterogeneous population of cancer-associated fibroblasts within the tumor microenvironment and controversy exists whether it can promote or inhibit tumor growth. Alpha-smooth muscle actin (α-SMA)-positive myofibroblasts have long been recognized as a prominent component of the activated fibroblasts. In addition to α-SMA, various other markers such as FAP, FSP1, vimentin can also detect stromal fibroblasts in tumors. Interestingly, FSP1 identifies a unique population of fibroblasts, which is, distinct from alpha SMA positive fibroblasts and it is also expressed on immune cells [34, 35]. Lineage tracing experiments also implies that FSP-1 is not a marker for precursors of myofibroblasts but co-expressed on cells with F4/80 and other markers of the myeloid-monocytic lineage [36]. We have demonstrated that in addition to α-SMA, Bosentan treatment decreases the FSP1 positive cells in the PC stroma, however no colocalization was observed with α-SMA positive myofibroblasts, an observation similar to earlier findings. One the contrary, our immunofluorescence analysis using fibroblast activation protein (FAP) antibody indicates no significant difference in the treated mice KPC tumor tissues. Elevated macrophage infiltration in tumor tissues is associated with metastasis of many solid tumors [37]. Several observations have also linked the orchestrated actions of ETs in neutrophils, monocytes and macrophages within the complex host microenvironment. ET-1 ligand has been earlier shown to be strong chemo attractant for blood monocytes [38]. Interestingly, a 31 amino acid bioactive peptide exhibited significant chemotactic activity towards monocytes and neutrophils compared to 21 amino acid ET-1 peptide and big

ET-1. Grimshaw *et al.* reported that hypoxia induced increase in ET-2 production acted as a chemo attractant for macrophages and monocytes, which are dependent on ET_BR, indicating a possible role of ET-2 in facilitating the recruitment of inflammatory cells [39, 40] Within the tumor microenvironment, autocrine and paracrine signaling between the tumor and macrophages accelerate the infiltration towards the inflamed sites. In bladder cancer, ET-1/ET_AR interaction increase migration of both tumor cells and tumor associated macrophages (TAMs) with enhance production of IL-6, MMPs and CCL2, accompanied by increase in metastatic colonization in the lung via ruptured vascular integrity [41].Very recently, in an experimental breast cancer model, it was reported that ET axis mediates lung metastasis and transendothelial migration of breast cancer cells stimulated by macrophages [42]. Additionally, in a rat model stromal deficiency of the ET_BR was correlated with reduced metastatic spread, infiltration of TAMs and production of TNF-α, suggesting a pivotal role of the ET_BR in tumor progression [43]. Our data revealed that pharmacological inhibition of ET receptors significantly reduced infiltration of F4/80 positive macrophages in the spontaneous mouse model. Our *in vitro* findings also demonstrate that selective ET_AR (BQ123) and ET_BR (BQ788) antagonists inhibited the increase migration of both murine macrophages (RAW 264.7) and human monocytic cells (U937) towards UN-KPC-961 and Panc 1 cells respectively. These findings indicate that ET-1 mediate chemo taxis of macrophages in PC stroma is dependent on both ET receptors, however further studies are required to delineate the molecular mechanisms of ET-1 mediated chemotactic interaction between tumor cell and TAMs.

We also evaluated the association of ET axis with tumor immunity following Bosentan treatment in KPC tumor model. Previous studies have shown that ET-1 axis also has a unique role to regulate immune response in tumor environment. In human ovarian tumors selective blockade of ET_BR by BQ788 leads to an increase in intercellular adhesion molecule-1 (ICAM-1) expression and increased T cell homing and

adhesion to tumors [44]. Also, in malignant gliomas, increased expression of ET_BR with tumor grade correlated with fewer infiltrations of cytotoxic T cells (CTLs), signifying the critical role of ET_BR signaling in the recruitment of T cell population to the tumors [45]. Therefore we hypothesized that ET axis antagonism can possibly regulate the T cell homing. Thus, our data suggests that Bosentan treatment increases the CD3 positive T cells compared to control. Furthermore, our immunofluorescence study indicates the increase infiltration of cytotoxic T cell population, signifying the critical role of ET axis modulating the immune response. However, further studies are required to decipher the immunological potential of ET axis in PC. Experiments with selective and dual ET inhibitors show that ET-1 treatment promotes migration of murine PSCs congruent with the previous findings [46] [47]. The stimulation of migratory phenotype is dependent on both ET_AR and ET_BR, which is in harmony with previous findings [48]. In addition, anti-proliferative effects on both murine ImPSC.c2 and human CAFs were observed using BQ788 and Bosentan in dose and time dependent manner with antagonism of ET_AR only having a minor effect. However, the mechanism by which ET-1 stimulates phenotypic changes warrants further investigation.

In this study we have used murine ImPSC.c2 and human CAFs as an *in vitro* model system with which we probe the role of ET-1 in the production of matrix associated proteins. In this study we demonstrated the involvement of ET receptor subtypes and the molecular mechanisms that govern the process. ET-1 induces CTGF (connective tissue growth factor), a member of extracellular matrix proteins involved in various biological process including migration, proliferation, wound healing and fibrosis [49] [50]. Report suggests that the expression of this protein is induced by TGF-β in Smad dependent manner [51] [52]. As previously stated, both ET-1 and TGF-β work in a synergistic manner to induce expression of fibrotic genes, and it is speculated by CTGF facilitates the downstream action mediated by TGF-β. Our study indicates that ET-1

stimulates the synthesis of pro-fibrogenic genes in both murine ImPSC.c2 and human CAFs. In this study we investigated the ability of selective and dual receptor inhibitors to prevent the overexpression of pro-fibrotic genes. Enhanced production of ET-1 during tissue repair [53] and patients with fibrotic disease implies that in addition to wound healing process it also plays a key role in pathogenesis of fibrosis [54] [55] [56]. Evidence also indicates that endogenous ET-1 signaling appears to play a role in the persistent fibrotic phenotype in lung fibroblasts [57]. However, we observed that endogenous ET-1 signaling was unable to induce the expression of ECM proteins in murine ImPSC.c2 when cultured in presence of UN-KPC-961 cells, however selective ET_BR and dual receptor antagonism reduced the expression of ECM proteins in these cells, implicating that the fibrotic phenotype is independent of endogenous ET-1 signaling. Interestingly, exogenous treatment of ET-1 caused the induction of α-SMA, collagen I, CTGF and fibronectin in ImPSC.c2. Perhaps more intriguingly, BQ788 and Bosentan appeared to reverse the ET-1 mediated induction with antagonism of ET_AR having only a minimal effect. These results indicate that ET-1/ET_BR signaling predominantly stimulates the expression of pro-fibrotic gene signatures in murine ImPSC.c2 cells. We also extended our studies in fibroblasts isolated from normal, chronic pancreatitis and cancer patients. Using western blot , we first confirmed the purity of our CAFs by examining the expression of ECM genes using appropriate controls. We found that elevated expression of α-SMA, Collagen I, CTGF and fibronectin in chronic pancreatitis and cancer associated fibroblasts compared to control. Using RT-PCR analysis we also investigate the expression pattern of ET axis. Expression of ET-1 and ET_AR was observed in all the fibroblasts tested, whereas ET_BR is restricted to cancer-associated fibroblasts and predominant expression is seen. Similar to murine ImPSC.c2, our results also demonstrated increase expression of ECM proteins in time dependent manner in human 10-03 CAFs, expressing both ET_AR and ET_BR. Contrary to

our previous observations, our data revealed that both BQ123 and BQ788 abrogated the ET-1 mediated effects in 10-03 CAFs. Our current data also indicate that both ET_A/ET_B receptor antagonism synergistically appeared to reverse the phenotype of activated fibroblasts. The results clearly indicate that ET-1 stimulates the expression of matrix-associated genes through both ET_AR and ET_BR. The inability of ET_AR antagonism to attenuate ET-1 effects and involvement of ET-1/ET_BR signaling to promote the ECM genes production in murine ImPSC.c2 can be explained on the low expression and distribution of ET_AR on ImPSC.c2, suggesting ET_BR, but not ET_AR is involved.

Indeed, our mechanistic data also implies ET-1 is able to aggravate the production of matrix associated proteins via ERK/AKT dependent mechanism requiring either the ET_A or the ET_B receptors. Our results are consistent with the previous reports that have linked the ERK/AKT signaling cascade and fibrosis. For examples, in lung fibroblasts ET-1 induces expression of matrix-associated genes through MEK/ERK kinase pathway [58]. Also, in lung fibroblasts ET-1 mediated myofibroblast induction is inhibited by blockade of PI3K/AKT pathway [59]. Altogether, our results indicate that collective antagonism of ERK/AKT and ET axis may be of benefit as a part of combinatorial drug strategy to combat fibrosis.

PC stroma is associated with structurally and functionally abnormal blood vessels, which are tortuous, poorly connected, and irregularly shaped with areas of dilation and constriction, thus resulting in turbulent and inefficient blood flow to the tumors [60] [61]. Additionally, the tumor blood vessels have discontinuous endothelial lining, abnormal pericytes and basement membrane, and are hence hyper permeable [62] [63]. The leaky nature of the tumor vessels coupled with the absence of efficient lymphatic system results in elevated interstitial fluid pressure (IFP) which in turn results in poor uptake and heterogeneous distribution of both micro and macromolecules in the

tumor tissue [64] [65]. Moreover, poor blood supply to the tumor results in regions of hypoxia, which contributes to radioresistance and chemoresistance of tumor cells. Therefore, selective enhancement in tumor blood flow can improve the drug delivery and efficacy. Due to the increased production of ET1 and overexpression of ET_AR in several epithelial tumors, this vasomodulatory activity is believed to be an important contributor to the tumor blood flow heterogeneity [66].Thus, ET_A antagonists, in addition to their direct antitumor effects also have an adjuvant effect that enhances tumor perfusion and increased drug uptake [66]. Several ET_AR antagonists including ZD4054, ABT-627 (atrasentan), are being investigated in clinical and preclinical studies for the treatment of various cancers [67] [68]. These inhibitors specifically bind to ET_AR and inhibit downstream signaling pathways in tumor and stromal cells that are implicated in tumor growth and metastasis. ET_AR antagonists, ZD4054 and ABT-627 have been demonstrated to inhibit tumor growth and metastasis and shown synergistic effects with cytotoxic drugs in prostate and ovarian cancers [69] [70]. Although, ET_AR antagonists have been shown to improve tumor perfusion and decrease tumor blood flow heterogeneity and improve uptake of cytotoxic drugs in the tumor [66], their utility to improve the tumor perfusion in PC is yet to be demonstrated. Here, in this study we show for the first time selective ET_AR antagonism using subcutaneous xenograft tumors derived from PC cell lines can improve perfusion in response to BQ123 indicating tumor selective modulation. Additionally, our analysis also suggested that enhanced perfusion induced by BQ123 resulted in decrease hypoxia in T3M4 tumors.

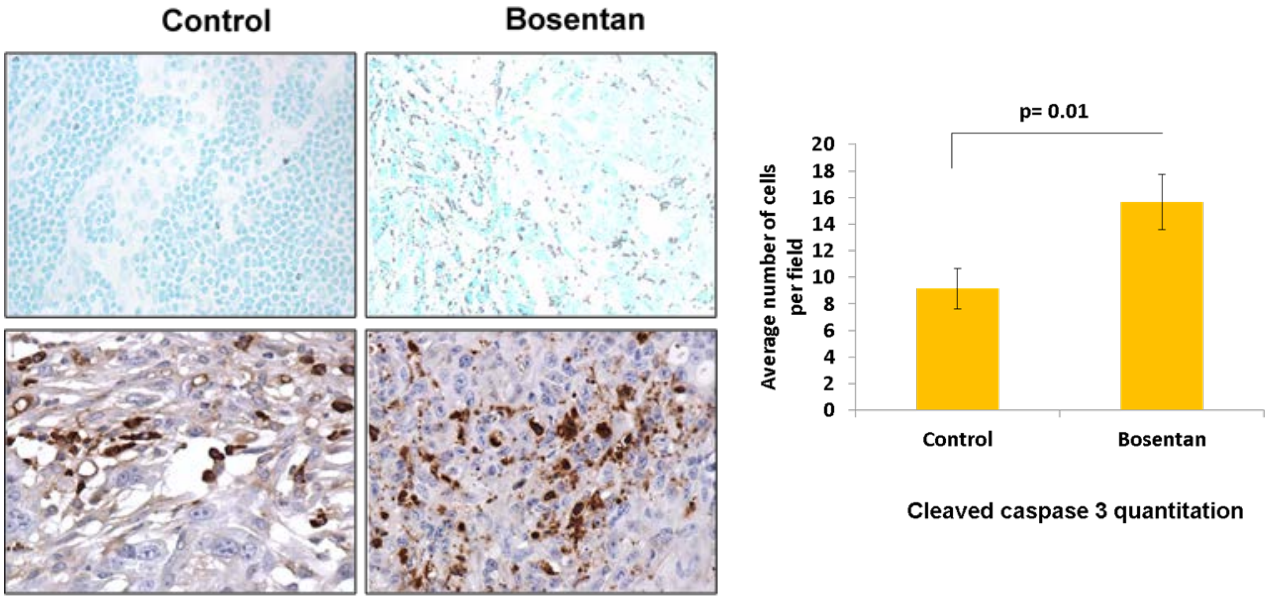
In conclusion , we have provided evidence that ET-1 signaling appears to play a central role in the persistent of pro-fibrotic phenotype in PC. Our data provide an experimental based rationale for the use of dual ET_AR and ET_BR antagonist, Bosentan to ameliorate pathological scarring observed in pancreatic fibrosis. Our results further

suggest that there may be a potential therapeutic advantage in selective ET_AR inhibitor, BQ123 to enhance tumor perfusion and increase drug uptake.

Figure 1: ET axis antagonism increases apoptosis and decreases fibrosis in KPC mice. A. Immunohistochemical analysis of cleaved caspase 3 and TUNEL staining in tumor sections obtained from control and Bosentan treated mice showed increase in the apoptotic cells. Quantitation of cleaved caspase -3 positive cells shows significant increase in the number of apoptotic nuclei ($p=0.01$) after Bosentan treatment (average count of cleaved caspase positive cells in 20 independent fields/section). **B.** Representative Masson Trichrome staining of the 20 weeks KPC mice tissue after challenged with either saline (control) or Bosentan (treated) for a period of 10 days. The zoomed magnified image indicates reduction in the desmoplasia. Augmented fibrosis is further represented in the semi-quantitative fashion where the staining score and intensity score were multiplied to obtain the composite score and subsequently represented as bar diagram.

Figure 1

A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Bosentan
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Average number of cells per field
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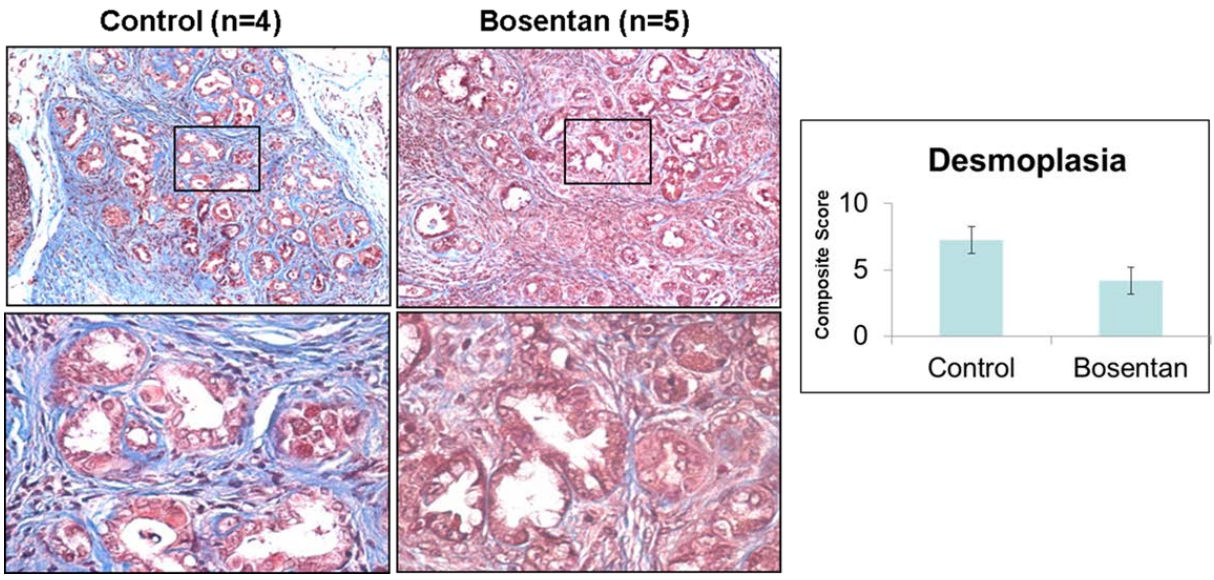
18
16
14
12
10
8
6
4
2
0
Control
Bosentan
Cleaved caspase 3 quantitation

B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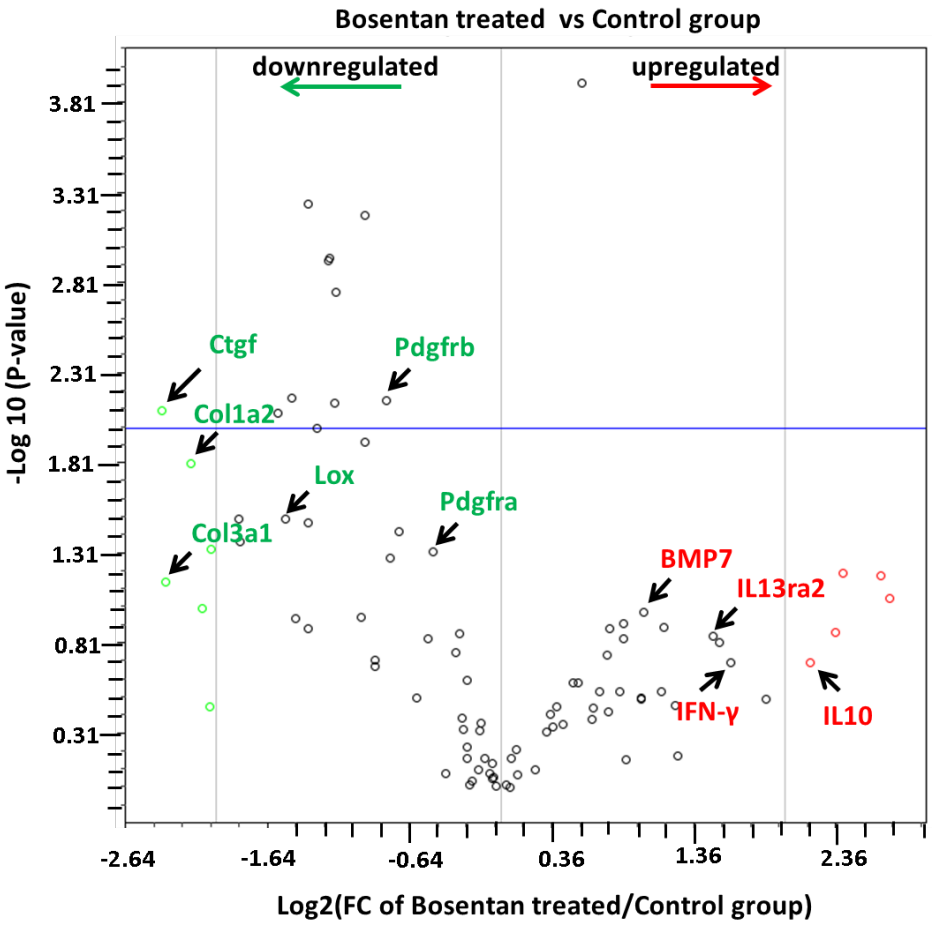
Bosentan (n=5)
Desmoplasia
Composite Score
10
5
0
Control
Bosentan

Figure 2. Bosentan treatment reduces the pro-fibrotic gene signatures in KPC mice. Volcano plot of the PCR array analysis in KPC mice treated with Bosentan or control saline showing the distribution of gene expression (represented by white circles). The genes in the green and red indicate the down regulated and upregulated genes respectively on the plot. The table in the bottom depicts the absolute fold changes of genes with p values. The pro-fibrotic and anti-fibrotic genes are indicated in green and red respectively.

Figure 2

A



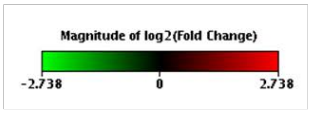
B

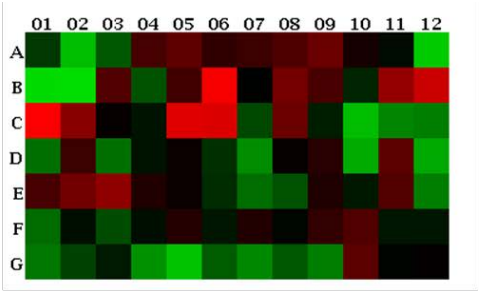
Symbol	p-value	Fold regulation
Ctgf	0.007925	-5.22
Col1a2	0.015632	-4.54
Col3a1	0.070879	-5.12
Pdgfra	0.048787	-1.39
Pdgfrb	0.007202	-2.25
Lox	0.031883	-2.87
Il10	0.200	4.53
Il13ra2	0.1436	2.81
Bmp7	0.105	2.01
IFN-γ	0.201	3.07

Table I. Representation of the genes in the microarray. Heat map representation of the expression of 84 genes in the PCR array. The array includes: **Pro-Fibrotic genes** Acta2 (α-SMA), Agt, Ccl11 (Eotaxin), Ccl12, Ccl3(MIP-1a),Ctgf,Grem1,Il13,Il13ra2,Snai1(Snail). **Anti-Fibrotic:** Bmp7, Hgf, Ifng, Il10, Il13ra2**. ECM Components:**Col1A2, Col3A1. **Remodeling Enzymes:** Lox, Mmp1a (Collagenase 1), Mmp13, Mmp14, Mmp2 (Gelatinase A), Mmp3, Mmp8, Mmp9 (Gelatinase B), Plat (tPA), Plau (uPA), Plg, Serpina1a (a1-antitrypsin), Serpine1 (PAI-1), Serpinh1, Timp1, Timp2, Timp3, Timp4. **Cellular Adhesion:** Itga1, Itga2, Itga3, Itgav, Itgb1, Itgb3, Itgb5, Itgb6, Itgb8. **Inflammatory Cytokines & Chemokines:** Ccl11 (Eotaxin), Ccl12, Ccl3 (MIP-1a), Ccr2, Cxcr4, Ifng, Il10, Il13, Il13ra2, Il1a, Il1b, Tnf. **Growth Factors:** Agt, Ctgf, Edn1, Egf, Hgf, Pdgfa, Pdgfb, Vegfa. **Signal Transduction:** TGFß Superfamily: Bmp7, Cav1, Dcn, Eng (EVI-1), Grem1, Inhbe, Ltbp1, Smad2, Smad3, Smad4, Smad6, Smad7, Tgfb1, Tgfb2, Tgfb3, Tgfbr1 (ALK5), Tgfbr2, Tgif1, Thbs1, Thbs2. Transcription Factors: Cebpb, Jun, Myc, Nfkb1, Sp1, Stat1, Stat6. **Epithelial-to-Mesenchymal Transition**: Akt1, Bmp7, Col1a2, Col3a1, Itgav, Itgb1, Mmp2 (GelatinaseA), Mmp3, Mmp9, Serpine1 (PAI-1), Smad2, Snai1 (Snail), Tgfb1, Tgfb2, Tgfb3, Timp1.The map indicates up regulation (red) and down regulation (green). The fold change of each individual gene is shown in the table.

Table I

Visualization of log2(Fold Change)





Green: Downregulated genes
Red: Upregulated genes

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	Acta2 -1.51	Agt -4.13	Akt1 -1.93	Bcl2 1.68	Bmp7 2.01	Cav1 1.43	Ccl11 1.56	Ccl12 1.78	Ccl3 2.21	Ccr2 1.18	Cebpb -1.08	Col1a2 -4.54
B	Col3a1 -5.12	Ctgf -5.22	Cxcr4 1.82	Dcn -1.84	Edn1 1.61	Egf 6.37	Eng -1.02	Fasl 2.38	Grem1 1.70	Hgf -1.31	Ifng 3.07	Il10 4.53
C	Il13 6.67	Il13ra2 2.81	Il1a 1.04	Il1b -1.16	Il4 5.31	Il5 5.13	Ilk -1.71	Inhbe 2.20	Itga1 -1.25	Itga2 -4.11	Itga3 -2.72	Itgav -2.55
D	Itgb1 -2.31	Itgb3 1.57	Itgb5 -2.30	Itgb6 -1.15	Itgb8 1.09	Jun -1.43	Lox -2.87	Ltbp1 1.05	Mmp13 1.35	Mmp14 -3.58	Mmp1a 1.98	Mmp2 -3.57
E	Mmp3 1.70	Mmp8 2.34	MMp9 2.91	Myc 1.27	Nfkb1 1.08	Pdgfa -1.39	Pdgfb -2.25	Plat -1.85	Plau 1.25	Plg -1.20	Serpin1a 1.85	Serpine1 -2.56
F	Serpinh1 -2.23	Smad2 -1.12	Smad3 -1.75	Smad4 -1.11	Smad6 1.31	Smad7 -1.18	Snai1 1.29	Sp1 -1.04	Stat1 1.46	Stat6 1.82	Tgfb1 -1.18	Tgfb2 -1.18
G	Tgfb3 -2.45	Tgfbr1 -1.64	Tgfbr2 -1.21	Tgif1 -2.97	Thbs1 -4.28	Thbs2 -1.97	Timp1 -2.77	Timp2 -1.93	Timp3 -2.55	Timp4 1.98	Tnf -1.04	Vegfa 1.03

Table II: Overall summary of gene expression profile identified by TCGA database and targeted by Bosentan in fibrosis array. The pro-fibrotic genes have significant correlation with overexpression of ET axis as predicted by human TCGA analysis and the gene signatures targeted by Bosentan in KPC mice indicates decrease in the absolute fold change with significant p value.

Table II

	Human TCGA analysis	Mouse fibrosis array		
Gene Name	**Gene Symbol**	**Correlation Coefficient**	**Fold change after treatment**	**p value**
Platelet-derived growth factor receptor, beta	PDGFR-β	0.93229	-2.25	0.007202
Collagen type III	Col3A1	0.912932	-5.12	0.0070879
Collagen type I	Col1A2	0.892809	-4.54	0.015632
Connective tissue growth factor	CTGF	0.66999	-5.22	0.007925
Lysyl Oxidase	LOX	0.846525	-2.87	0.031883

Figure 3: *In vivo* evidence for effect of dual ET receptors antagonism in fibrosis. A. The reduction in exaggerated fibrogenic response in Bosentan treated KPC mice is further represented by western blot in tumor lysates against CTGF, α-SMA, Fibronectin and FSP-1. **B.** Immunohistochemical staining against Collagen I, α-SMA, fibronectin, FSP1 and CTGF in treated mice tissues compared to control shows decrease in fibrotic growth.

Figure 3

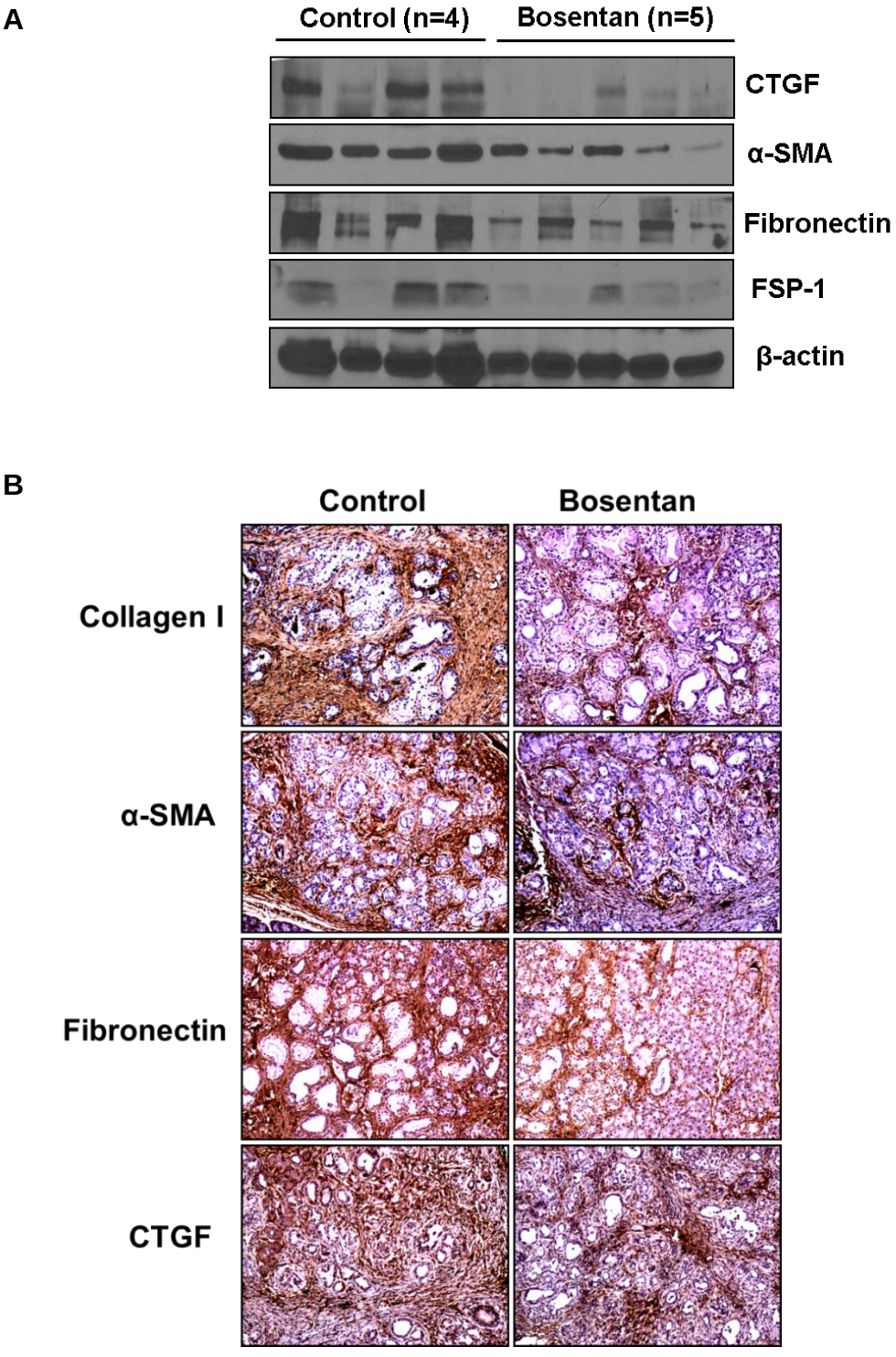

A
Control (n=4)
Bosentan (n=5)
CTGF
α-SMA
Fibronectin
FSP-1
β-actin
B
Control
Bosentan
Collagen I
α-SMA
Fibronectin
CTGF

Figure 4: Bosentan treatment reduces the FSP1 but not FAP positive cancer associated fibroblasts (CAFs). Three-color immunofluorescence showing the expression of epithelial marker, CK19 (green) and cancer associated fibroblasts, FSP1 (red) and α-SMA (purple) in KPC mice. Both the FSP1 and α-SMA positive fibroblasts populations surround the CK19 positive ducts in the microenvironment and exhibit minimal or no overlap with each other (Scale bar 20 μm). **B.** Dual confocal microscopy indicates decrease in the α-SMA positive fibroblasts (red) and FSP1 positive fibroblasts (green) following Bosentan treatment with significant p value. **C.** Immunofluorescence analysis indicates co-localization of both α-SMA and FAP positive fibroblasts (green) and no significant difference in the FAP positive fibroblasts population post Bosentan treatment. (Scale bar 20 μm).

Figure 4

A

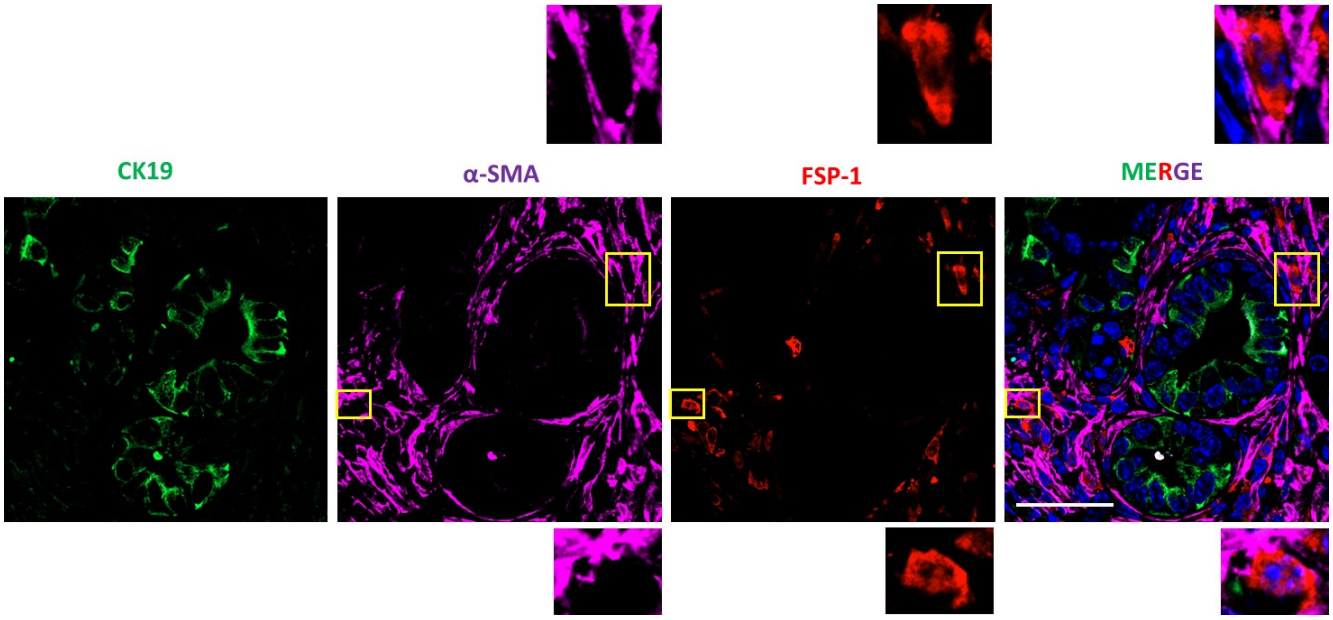
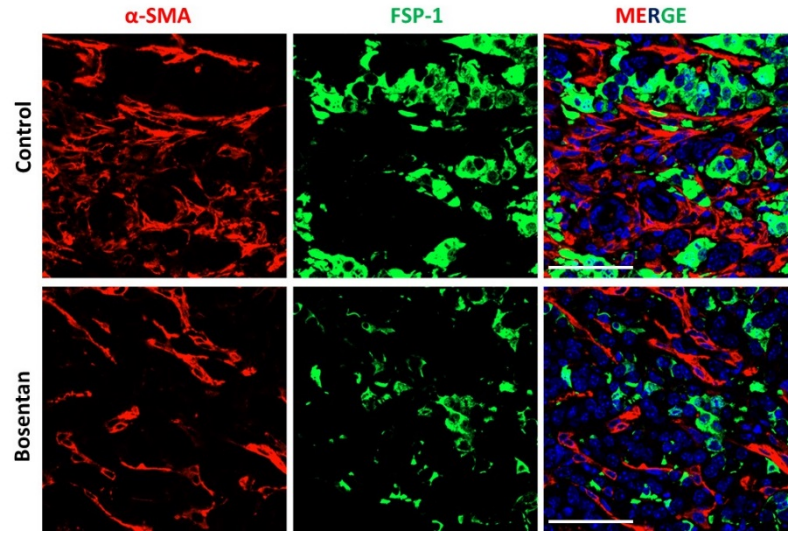
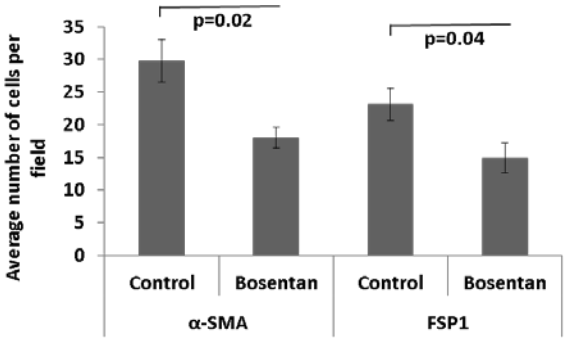


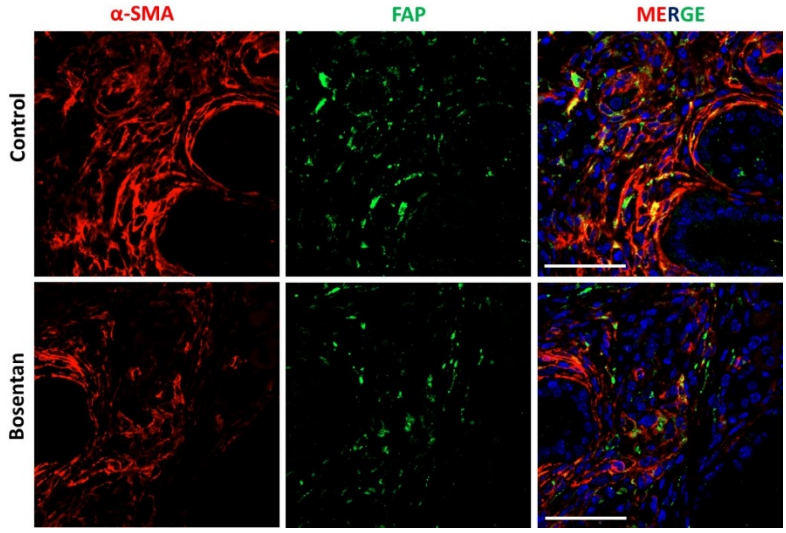
Figure 4

B


α-SMA
FSP-1
MERGE
Control
Bosentan


Average number of cells per field
35
30
25
20
15
10
5
0
p=0.02
p=0.04
Control
Bosentan
Control
Bosentan
α-SMA
FSP1

C


α-SMA
FAP
MERGE
Control
Bosentan

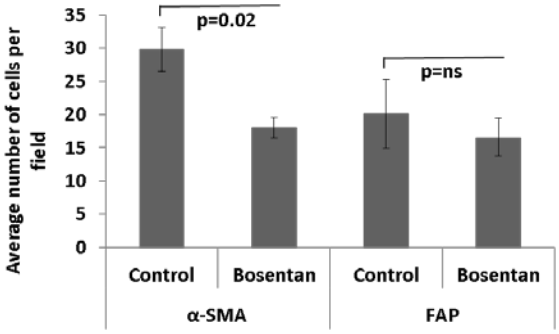
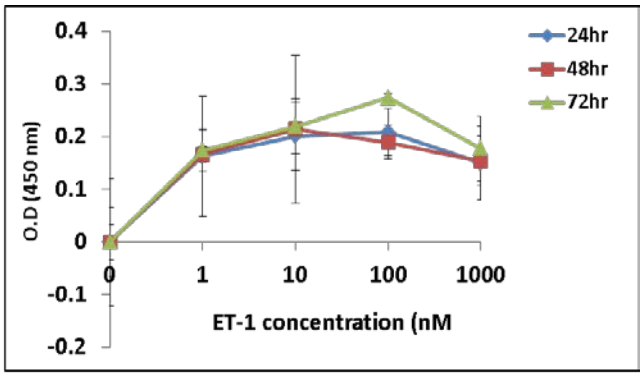

Average number of cells per field
35
30
25
20
15
10
5
0
p=0.02
p=ns
Control
Bosentan
Control
Bosentan
α-SMA
FAP

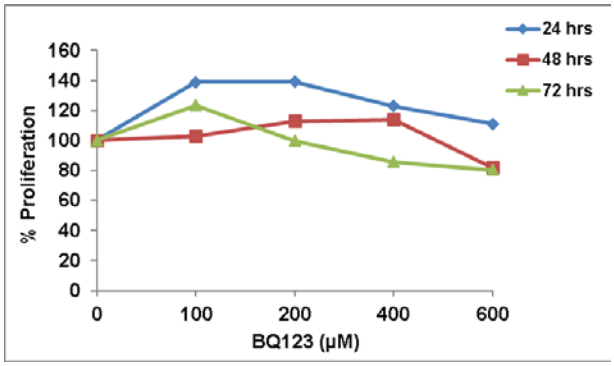
Figure 5: ET receptors antagonism inhibits proliferation of murine pancreatic stellate cells. (A) ET-1 promotes proliferation of ImPSC.c2 in concentration dependent manner. Cells were plated in 96 well format (3000 cells/ 100µl/well) in DMEM containing 10% FBS for 24 hours and then stimulated with indicated concentrations of ET-1 in DMEM containing 1% FBS for 24, 48 and 72 hours. Cell growth was measured by MTT assay. Selective ET_AR inhibitor, BQ123 **(B)**, have minimal effects on the growth of ImPSC.c2 cells whereas both selective ET_BR, BQ788 **(C)** and dual ET receptor inhibitor Bosentan **(D)** inhibits the proliferation of ImPSC.c2 as indicated by MTT assay in a dose and time dependent manner.

Figure 5

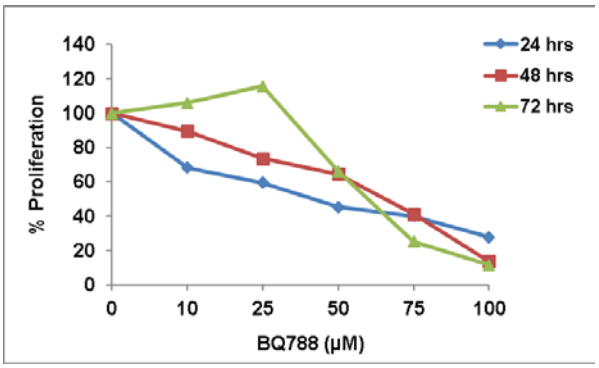
A



B



C



D

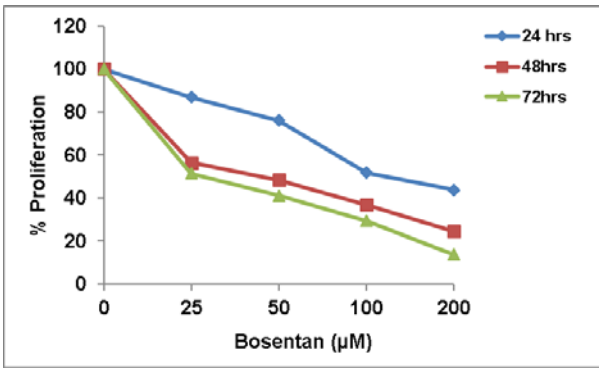


Figure 6: ET receptor antagonists induce apoptosis and cell cycle arrest in ImPSC.c2 cells. A. The percentage of apoptotic cells was measured by Annexin-V and propidium iodide (PI) staining using flow cytometric analysis following treatment with ET inhibitors. Annexin-V+ and PI- cells are considered to be early apoptotic while Annexin V+ and PI+ cells are considered to be late apoptotic. Treatment with both BQ788 (ET_BR inhibitor) and Bosentan (dual inhibitor) increases the apoptotic cells compared to control cells whereas minimal effect with BQ123 (ET_AR inhibitor) was observed. **B.** Flow cytometry based cell cycle analysis was carried out in ImPSC.c2 cells after treatment with inhibitors and the percentage of cells in G1, S, and G2/M phases of cell cycle are represented. As compared to untreated cells, BQ788 and Bosentan treatment induces cell cycle arrest in G1/S phase.

Figure 6

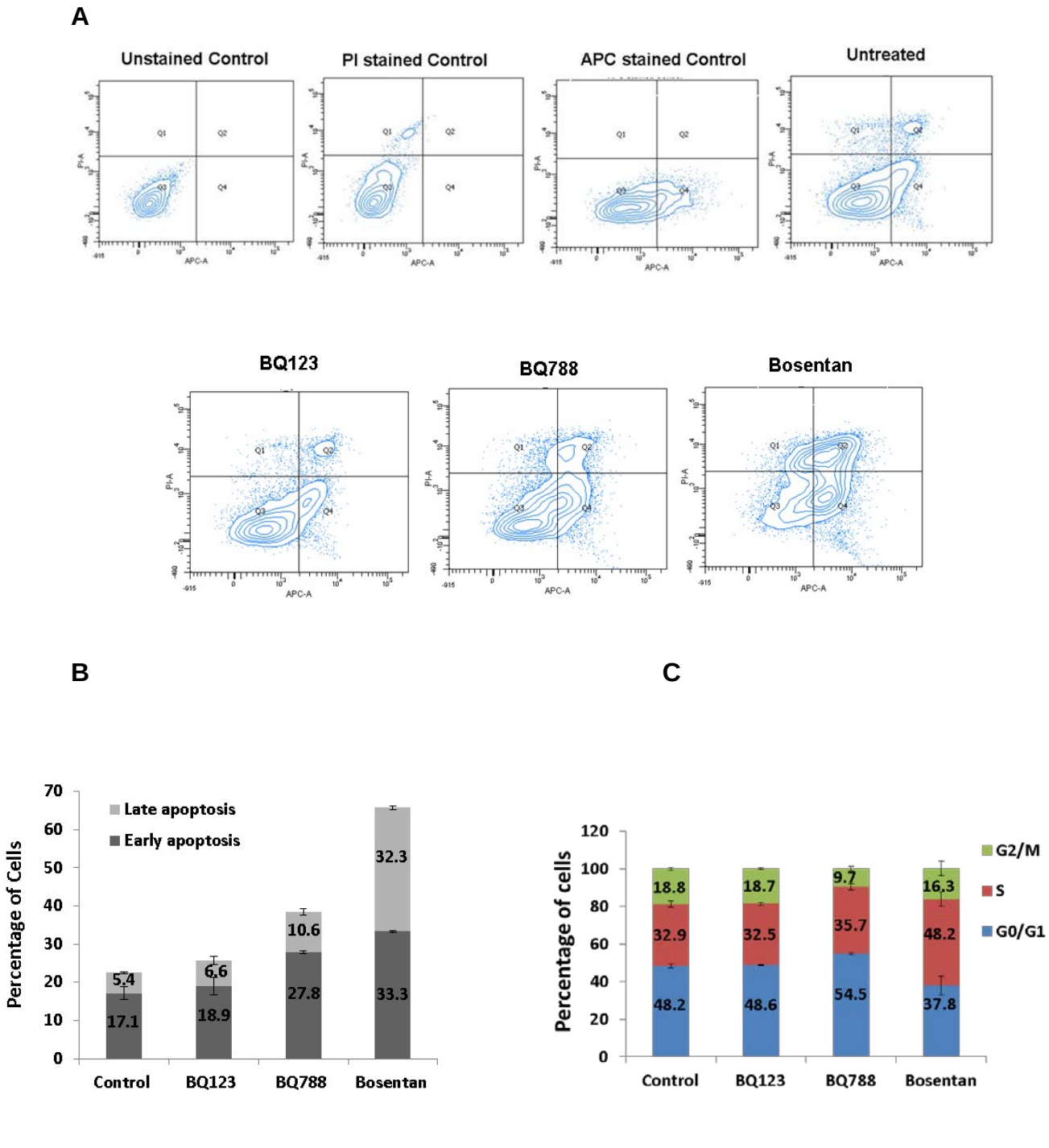

A
Unstained Control
PI stained Control
APC stained Control
Untreated
BQ123
BQ788
Bosentan
B
Percentage of Cells
Late apoptosis
Early apoptosis
5.4
17.1
6.6
18.9
10.6
27.8
32.3
33.3
Control
BQ123
BQ788
Bosentan
C
Percentage of cells
G2/M
S
G0/G1
18.8
32.9
48.2
18.7
32.5
48.6
9.7
35.7
54.5
16.3
48.2
37.8

Figure 7: ET receptors antagonism inhibits migration of murine pancreatic stellate cells. Endothelin receptors antagonists inhibit the recombinant ET-1 induced migration of ImPSC.c2. Cells were seeded at a density of 2×10^6 cells in 6 well plates and kept in 10% DMEM overnight. To determine the effect of BQ123, BQ788 and Bosentan upon wound closure artificial wounds were created in 90% confluent cells. The cells were then treated with ET-1 (100nm) or BQ123 (100µM), BQ788 (20µM) and Bosentan (25µM) in complete medium. Representative images of the wounds at 0 and 24 h in the presence of recombinant ET-1, antagonists or their combination. Histogram illustrates the relative wound width at 0 and 24 h. ($^{*}p<0.01$ vs. the Untreated group, $^{\#\#}p<0.01$ vs. the rh ET-1 group).

Figure 7

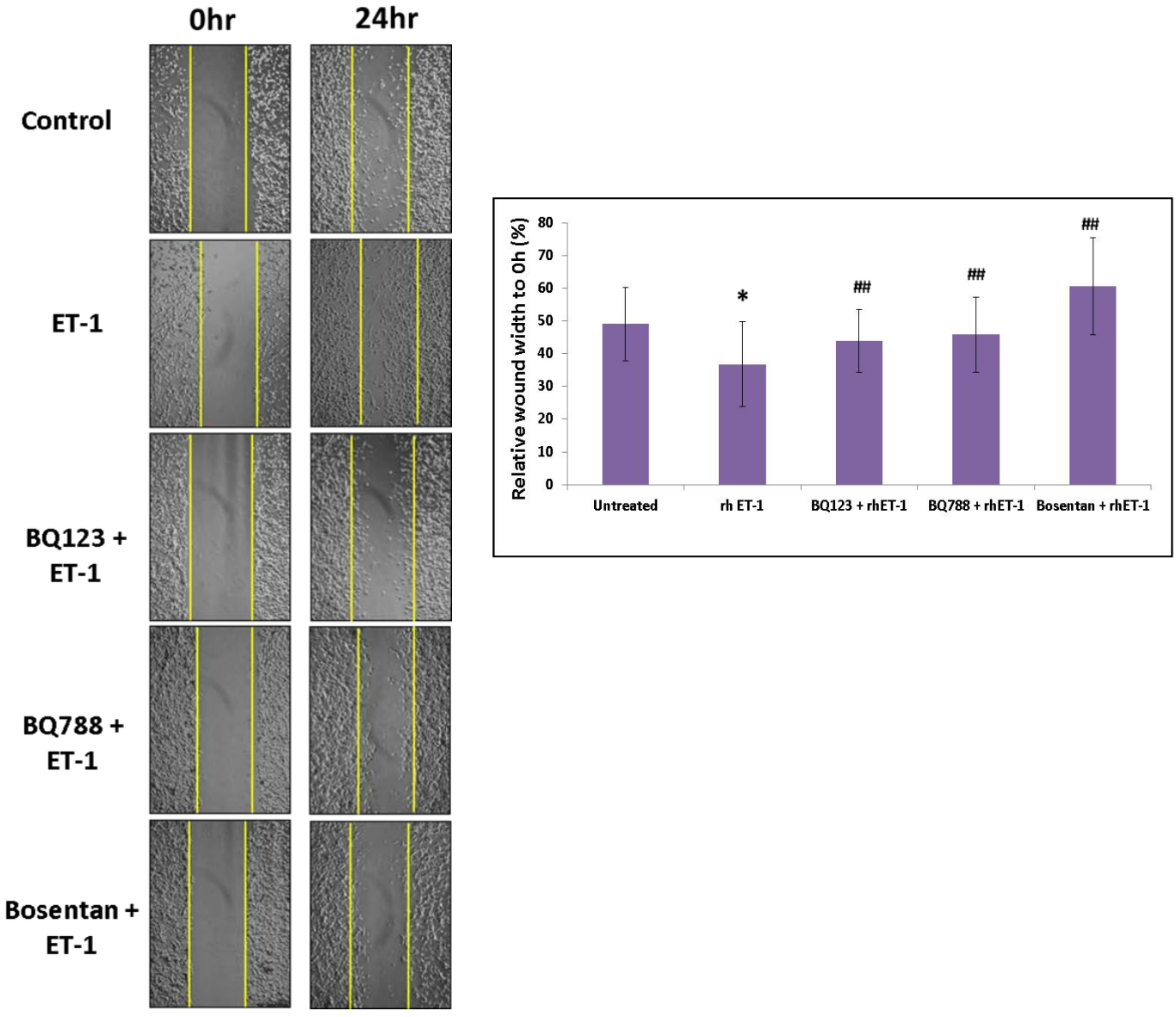

0hr
24hr
Control
ET-1
BQ123 + ET-1
BQ788 + ET-1
Bosentan + ET-1
Relative wound width to 0h (%)
80
70
60
50
40
30
20
10
0
*
##
##
##
Untreated
rh ET-1
BQ123 + rhET-1
BQ788 + rhET-1
Bosentan + rhET-1

Figure 8: Anti-fibrotic effects of ET axis antagonism *in vitro* in ImPSC.c2 cells. A. Expression of ET-1, ET_AR and ET_BR in UN-KPC-961 cells and ImPSC.c2 cells. **B.** After 24 hours coculture either in presence of UN-KPC-961 alone or ET inhibitors total protein of ImPSC.c2 was extracted. Western blot analysis was performed using an antibody against Collagen I, α-SMA, fibronectin and CTGF. **C.** Cells were plated in six well format ($0.8x\ 10^6$/ 100µl/well) in DMEM containing 10% FBS for 24 hours. After 24 hours, cells were serum starved for 24 hours and treated with ET-1 (100nm) or pre-treated with ET inhibitors, BQ123 (100µM), BQ788 (20 µM) and Bosentan (25 µM) for 45 mins prior to addition of ET-1. Protein lysates made was subjected to western blot analysis against Collagen I, α-SMA, fibronectin and CTGF. β-actin was used as a loading control.

Figure 8

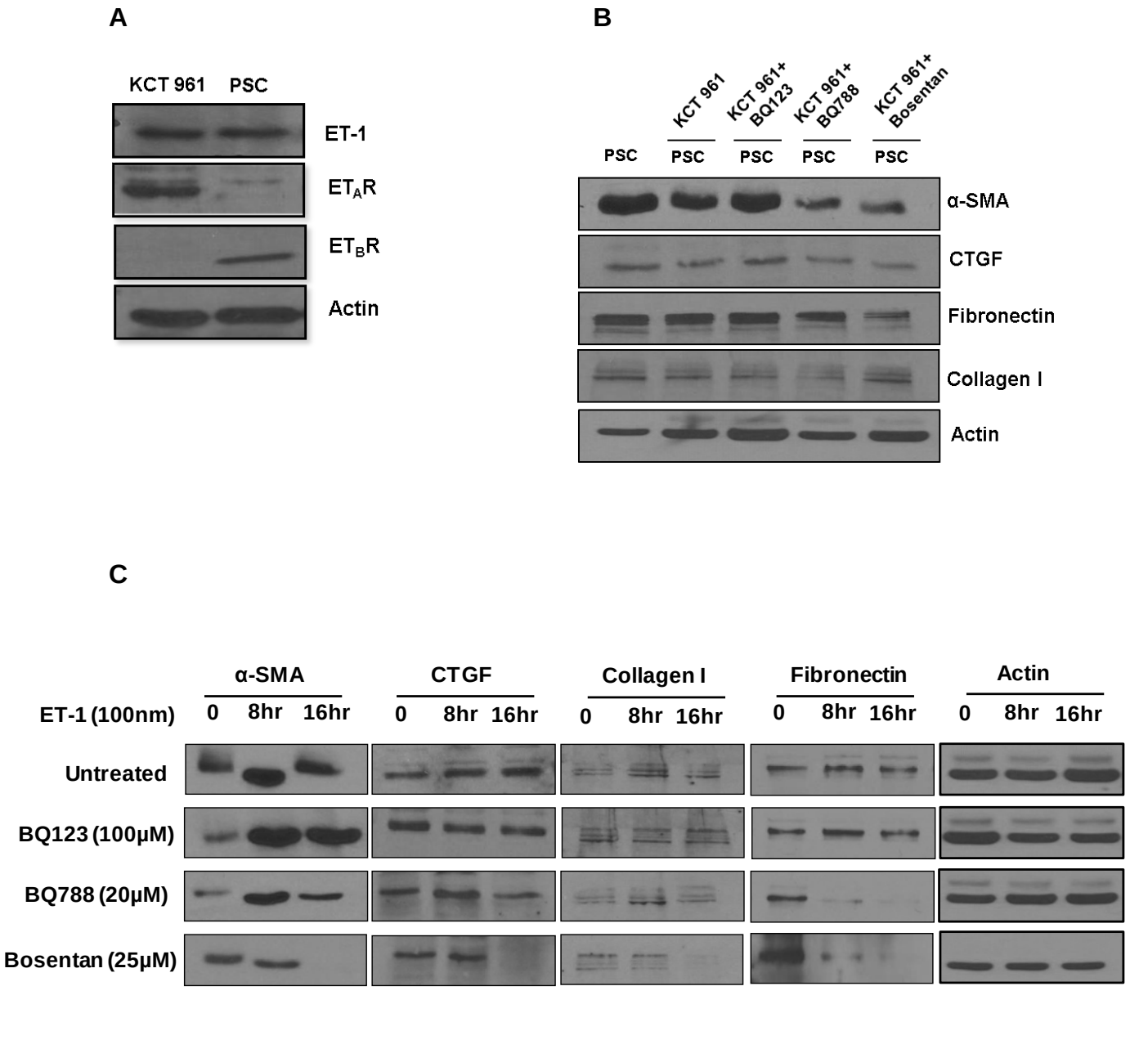

A
KCT 961
PSC
ET-1
ETAR
ETBR
Actin
B
KCT 961
KCT 961+ BQ123
KCT 961+ BQ788
KCT 961+ Bosentan
PSC
PSC
PSC
PSC
PSC
α-SMA
CTGF
Fibronectin
Collagen I
Actin
C
α-SMA
CTGF
Collagen I
Fibronectin
Actin
ET-1 (100nm)
0
8hr
16hr
Untreated
BQ123 (100μM)
BQ788 (20μM)
Bosentan (25μM)

Figure 9: Characterization of patient derived fibroblasts. Expression was determined in fibroblasts derived from normal pancreas, chronic pancreatitis and cancer patients. **A.** Western blot analysis was done against α-SMA (fibroblast marker) and CK-19 (epithelial marker) to demonstrate no contamination from other cellular components. HEK-293 and Panc1 cell lines was used as positive control for α-SMA and CK19 respectively. **B.** To probe for other stromal associated genes protein lysates made were subjected to western blot analysis against Collagen I, Fibronectin, CTGF and desmin. β-actin as used as a loading control. RT-PCR was done using ET-1, ET_AR and ET_BR gene specific primers in RNA isolated from patient derived fibroblasts. PC3 cell line was used as positive control. β-actin as used as a loading control.

Figure 9

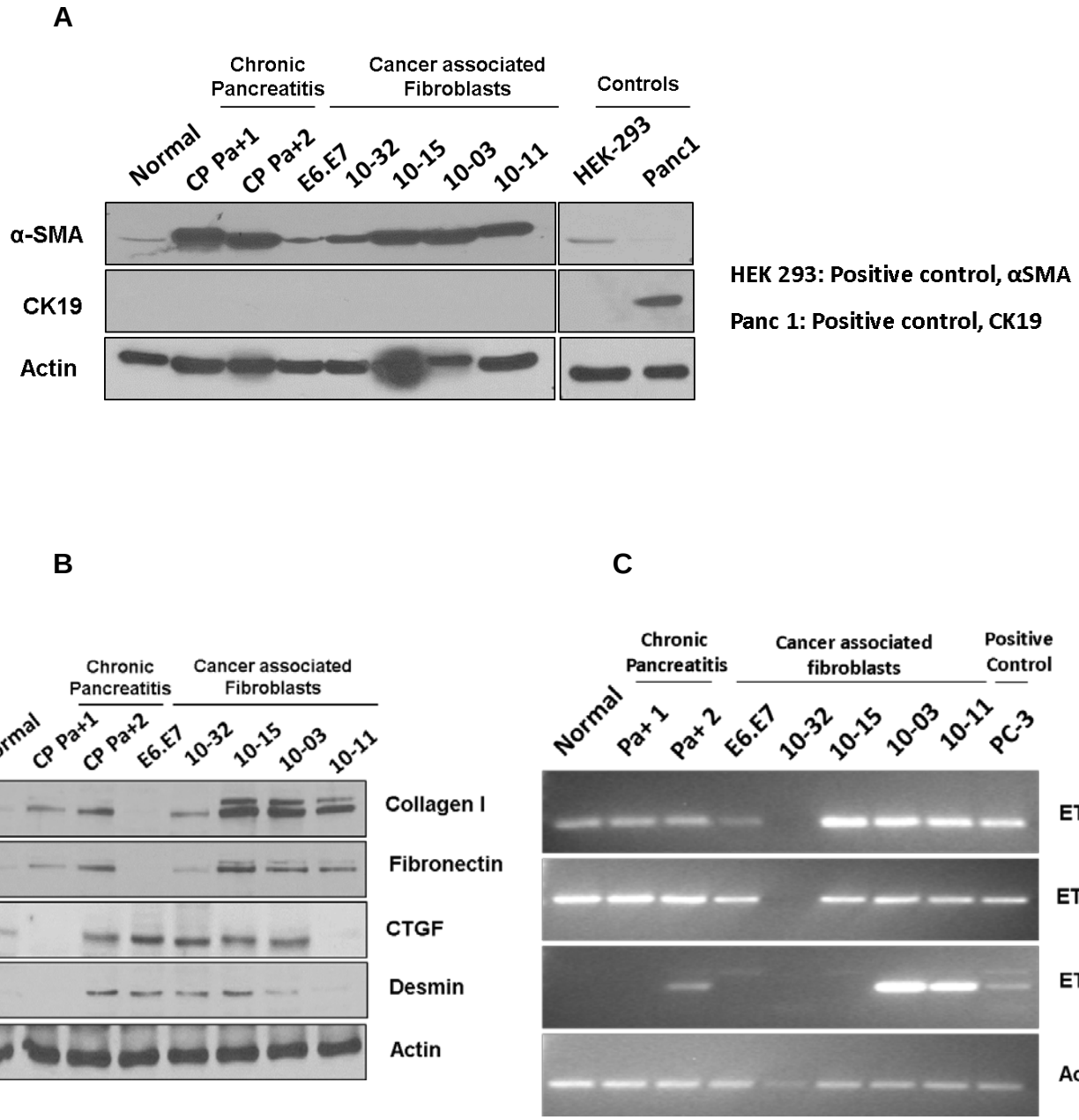
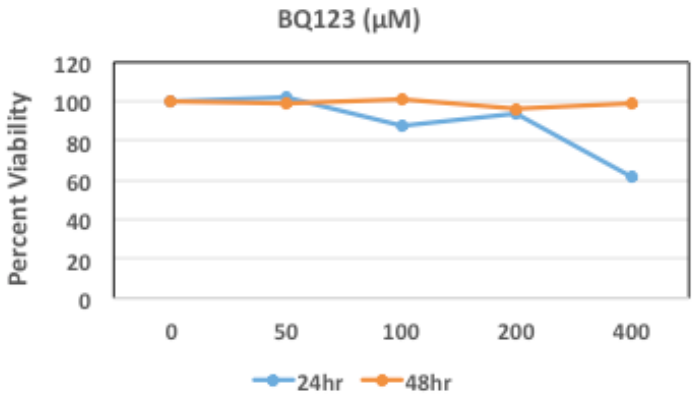

A
Chronic Pancreatitis
Cancer associated Fibroblasts
Controls
Normal
CP Pa+1
CP Pa+2
E6.E7
10-32
10-15
10-03
10-11
HEK-293
Panc1
α-SMA
CK19
Actin
HEK 293: Positive control, αSMA
Panc 1: Positive control, CK19
B
Chronic Pancreatitis
Cancer associated Fibroblasts
Normal
CP Pa+1
CP Pa+2
E6.E7
10-32
10-15
10-03
10-11
Collagen I
Fibronectin
CTGF
Desmin
Actin
C
Chronic Pancreatitis
Cancer associated fibroblasts
Positive Control
Normal
Pa+ 1
Pa+ 2
E6.E7
10-32
10-15
10-03
10-11
PC-3
ET-1
ETAR
ETBR
Actin

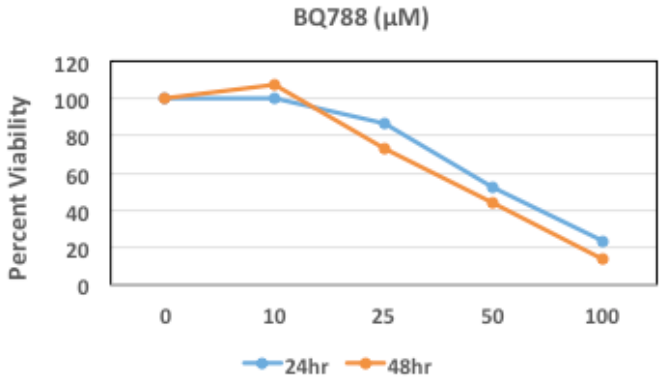
Figure 10: Effect of endothelin receptor antagonists on growth of 10-03 cancer fibroblasts. Cells were grown for 24hr, 48hr and 72hr in presence of increasing concentration ET_A receptor antagonist, BQ123 **(A)**, ET_B receptor antagonist, BQ788 **(B)** and dual ET_A and ET_B receptor antagonist, Bosentan **(C)**. The effect on cell growth after ET antagonists treatment was measured by MTT assay and read as absorbance at 490nm.

Figure 10

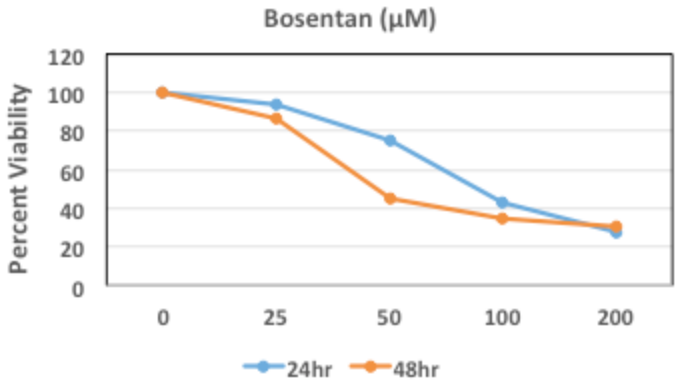
A



B



C



Figures 11: Anti fibrotic effect of ET axis antagonism *in vitro* in human CAFs. 10-03 fibroblasts were grown in grown in complete DMEM for 24 hr. Cells were serum starved for 24 hrs and treated with 100 nm of ET-1 or pre-treated with BQ123 (100 μM), BQ788 (20 μM) and Bosentan (25 μM) 45 mins prior to addition of ET-1. Whole cells protein extracts were collected at different time points and equal amounts of protein were subjected to immunoblotting analysis with anti- α-SMA, anti-fibronectin **(A)** anti-collagen I **(B)**, β-actin as used as a loading control.

Figure 11

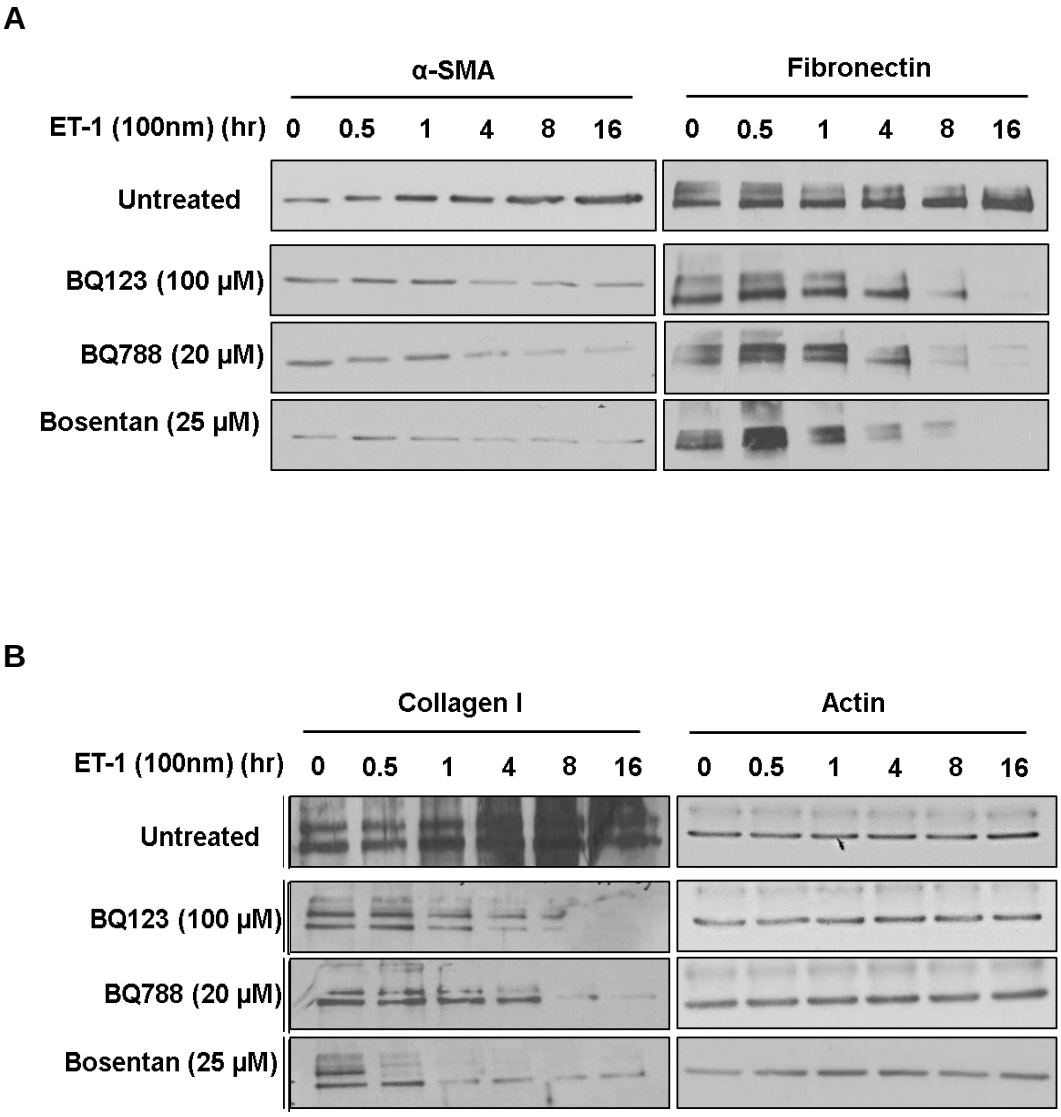
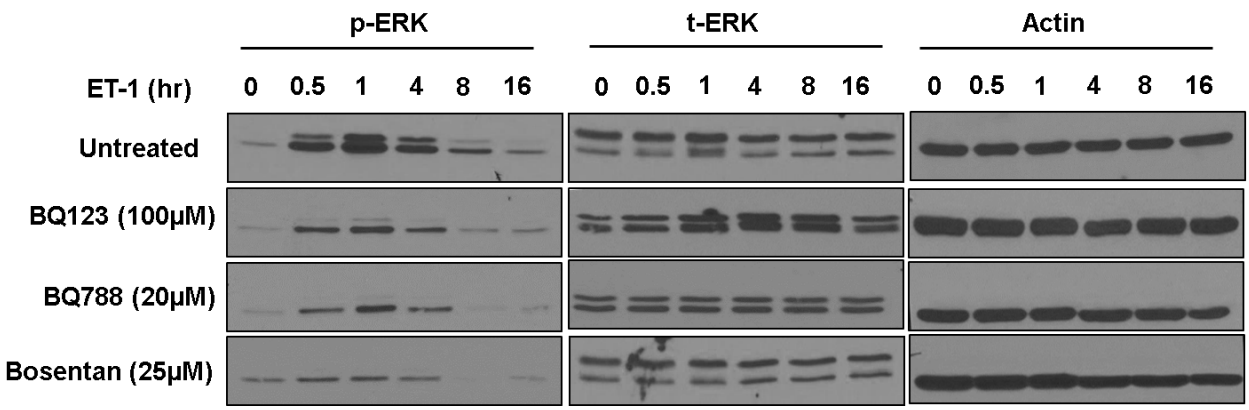

A
α-SMA
Fibronectin
ET-1 (100nm) (hr)
0 0.5 1 4 8 16
0 0.5 1 4 8 16
Untreated
BQ123 (100 μM)
BQ788 (20 μM)
Bosentan (25 μM)
B
Collagen I
Actin
ET-1 (100nm) (hr)
0 0.5 1 4 8 16
0 0.5 1 4 8 16
Untreated
BQ123 (100 μM)
BQ788 (20 μM)
Bosentan (25 μM)

Figure 12: ET-1 induces p42/44 MAPK and AKT phosphorylation in ImPSC.c2 stellate cells. Cells were cultured in complete DMEM medium for 24 hr, serum starved for 24 hr and treated with 100nm of ET-1 or pre-treated with BQ123 (100 μM), BQ788 (20 μM) and Bosentan (25 μM) 45 mins prior to addition of ET-1. Whole cells protein extracts were collected at different time points and equal amounts of protein were subjected to immunoblotting analysis with anti-ERK, anti-phospho-ERK **(A)**, anti-AKT and anti-phospho-AKT **(B)** antibodies. β-actin as used as a loading control.

Figure 12

A


p-ERK
t-ERK
Actin
ET-1 (hr)
0 0.5 1 4 8 16
Untreated
BQ123 (100μM)
BQ788 (20μM)
Bosentan (25μM)

B

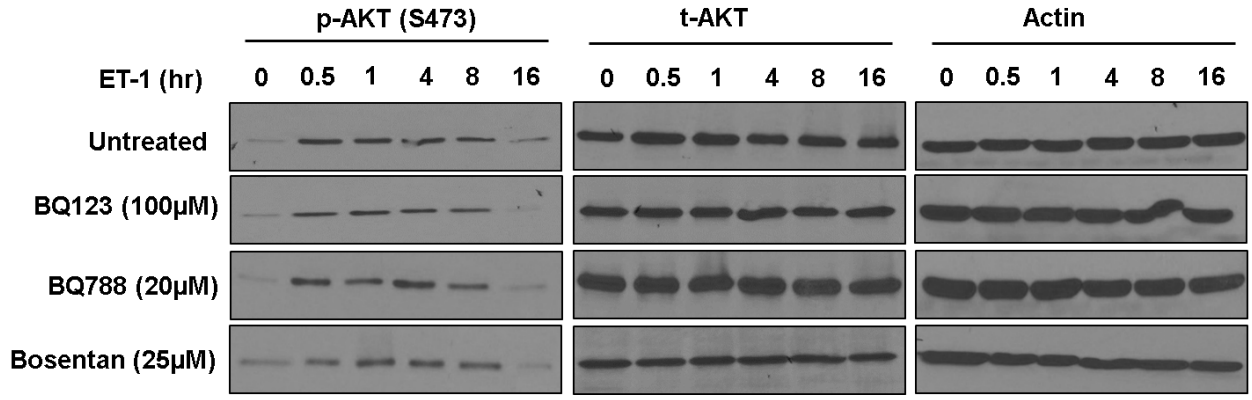
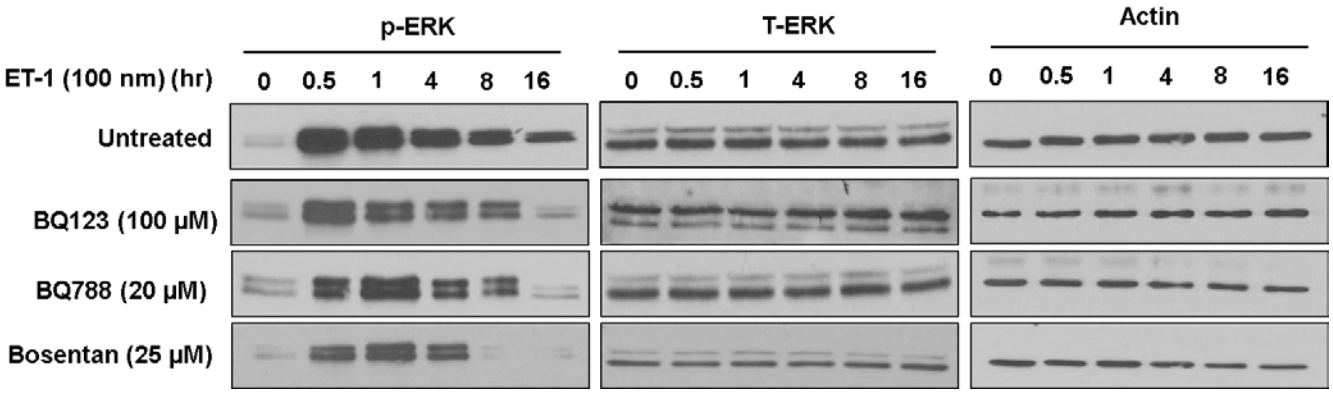

p-AKT (S473)
t-AKT
Actin
ET-1 (hr)
0 0.5 1 4 8 16
Untreated
BQ123 (100μM)
BQ788 (20μM)
Bosentan (25μM)

Figure 13: ET-1 induces p42/44 MAPK and AKT phosphorylation in 10-03 fibroblasts. Cells were cultured in complete DMEM medium for 24 hr, serum starved for 24 hr and treated with 100nm of ET-1 or pre-treated with BQ123 (100 μM), BQ788 (20 μM) and Bosentan (25 μM) 45 mins prior to addition of ET-1. Whole cells protein extracts were collected at different time points and equal amounts of protein were subjected to immunoblotting analysis with anti-ERK, anti-phospho-ERK **(A)**, anti-AKT and anti-phospho-AKT **(B)** antibodies. β-actin as used as a loading control

Figure 13

A


p-ERK
T-ERK
Actin
ET-1 (100 nm) (hr)
0 0.5 1 4 8 16
Untreated
BQ123 (100 μM)
BQ788 (20 μM)
Bosentan (25 μM)

B

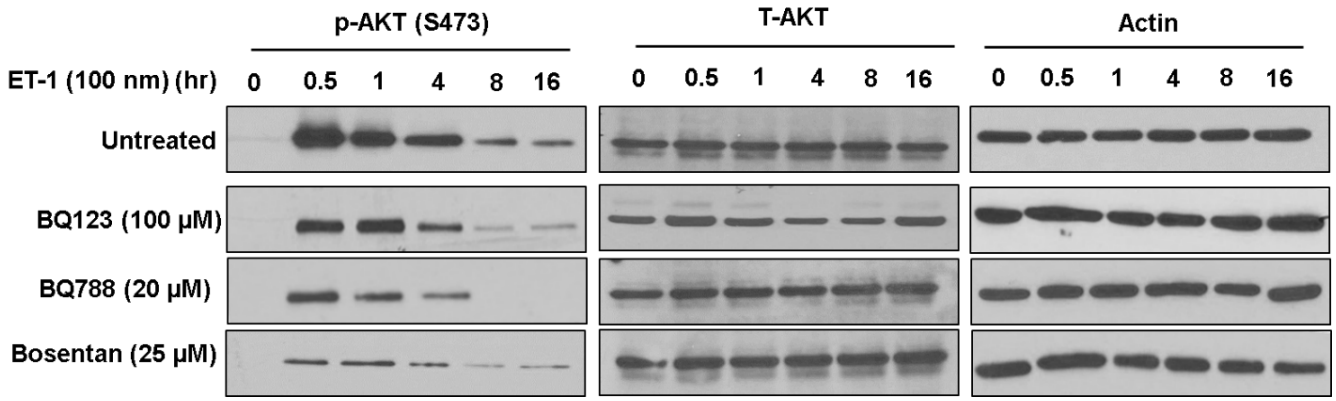

p-AKT (S473)
T-AKT
Actin
ET-1 (100 nm) (hr)
0 0.5 1 4 8 16
Untreated
BQ123 (100 μM)
BQ788 (20 μM)
Bosentan (25 μM)

Figure 14: Proposed Model. Pancreatic cancer cells expressing the endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR) also secret high levels of endothelin-1 (ET-1). The ET-1 released from the tumor cells act on the nearby pancreatic stellate cells and cancer associated fibroblasts in a paracrine manner expressing endothelin receptors and activate the downstream signaling which in turn induces the expression of pro-fibrotic gene signatures through ERK/AKT dependent manner. However, in presence of dual receptor Bosentan the binding of ligand to the receptor is inhibited resulting in the decrease expression of pro-fibrotic genes in an ERK/AKT dependent fashion.

Figure 14

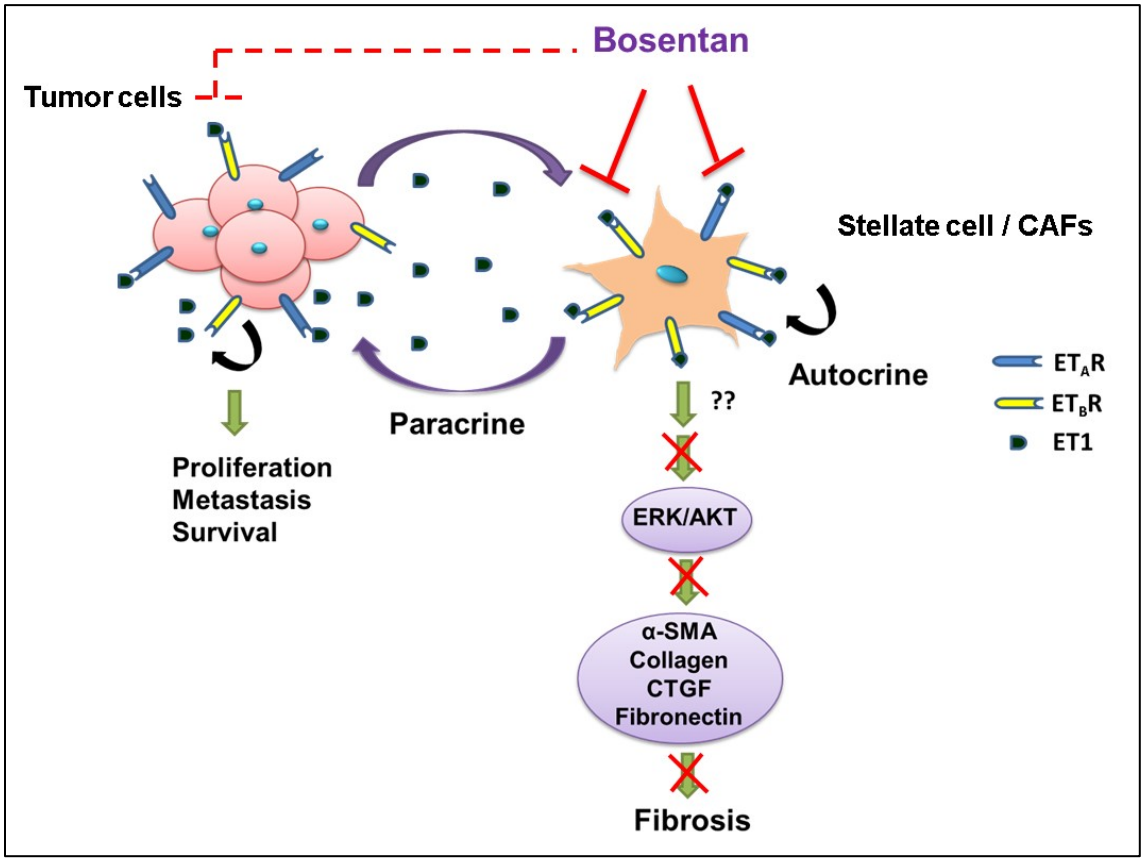
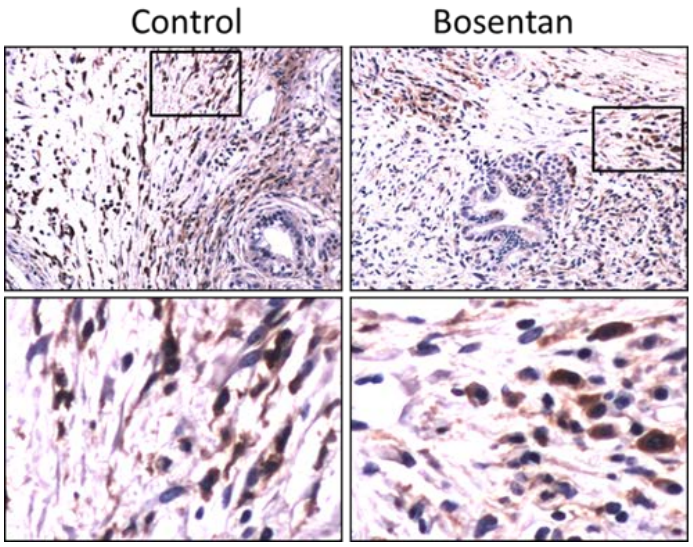
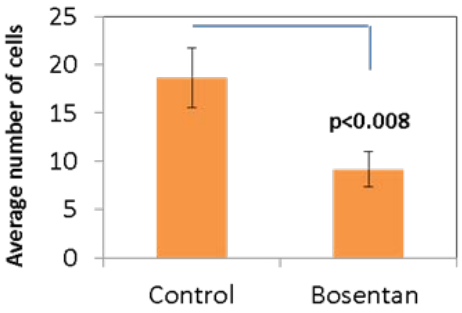

Bosentan
Tumor cells
Stellate cell / CAFs
Autocrine
Paracrine
??
Proliferation
Metastasis
Survival
ERK/AKT
α-SMA
Collagen
CTGF
Fibronectin
Fibrosis
ET$_A$R
ET$_B$R
ET1

Figure 15: ET axis antagonism inhibits infiltration of tumor-associated macrophages. A. Dual specificity ET axis antagonist b osentan reduces the number of tumor associated macrophages in KPC mice. Macrophages were visualized by immunostaining for F4/80 staining. Representative IHC images under low power (top) and high power (bottom- boxed area represented). The number of F4/80 positive cells per high power field (HPF) was counted in both control and Bosentan treated KPC tumors. 26 HPFs in Bosentan treated mice (n=6) and 19 HPFs in control mice (n=4) were counted. **B.** Immunofluorescence expression of CD206 was evaluated in KPC mice sections following Bosentan treatment. The number of CD206 positive cells per high power field (HPF) was counted in both control and Bosentan treated KPC tumors. Reduced infiltration of CD206 positive macrophages is observed in the stromal compartment in treated mice compared to control with significant p value.

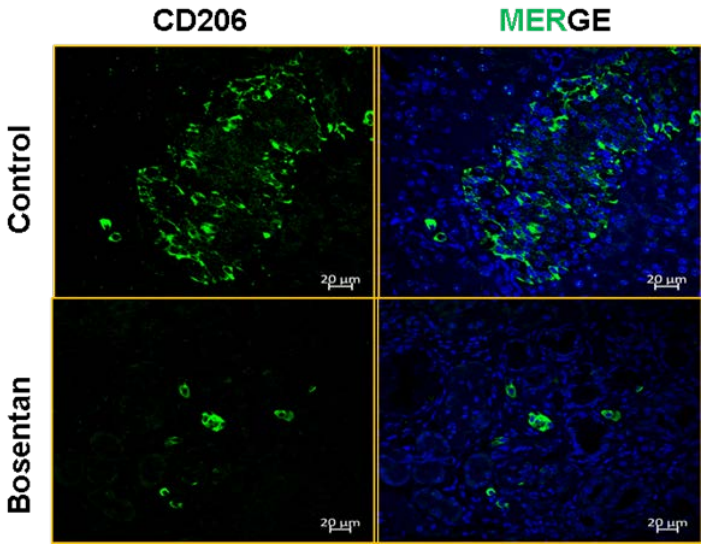
Figure 15

A


Control
Bosentan


Average number of cells
25
20
15
10
5
0
p<0.008
Control
Bosentan

B


CD206
MERGE
Control
Bosentan
20 µm

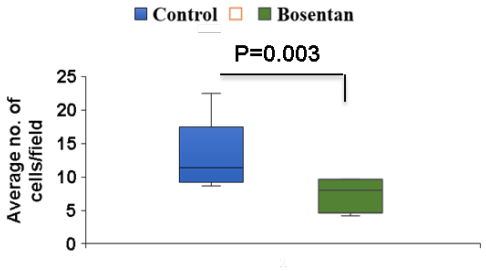

Control
Bosentan
P=0.003
Average no. of cells/field
25
20
15
10
5
0

Figure 16: Bosentan treatment increases the CD3 T cell infiltration in KPC tumors: Immunohistochemical analysis of CD3 was evaluated in KPC mice sections following Bosentan treatment. Increase in the recruitment of CD3 positive T cells is observed in the stromal compartment and tumor associated lymph node in treated mice compared to control.

Figure 16

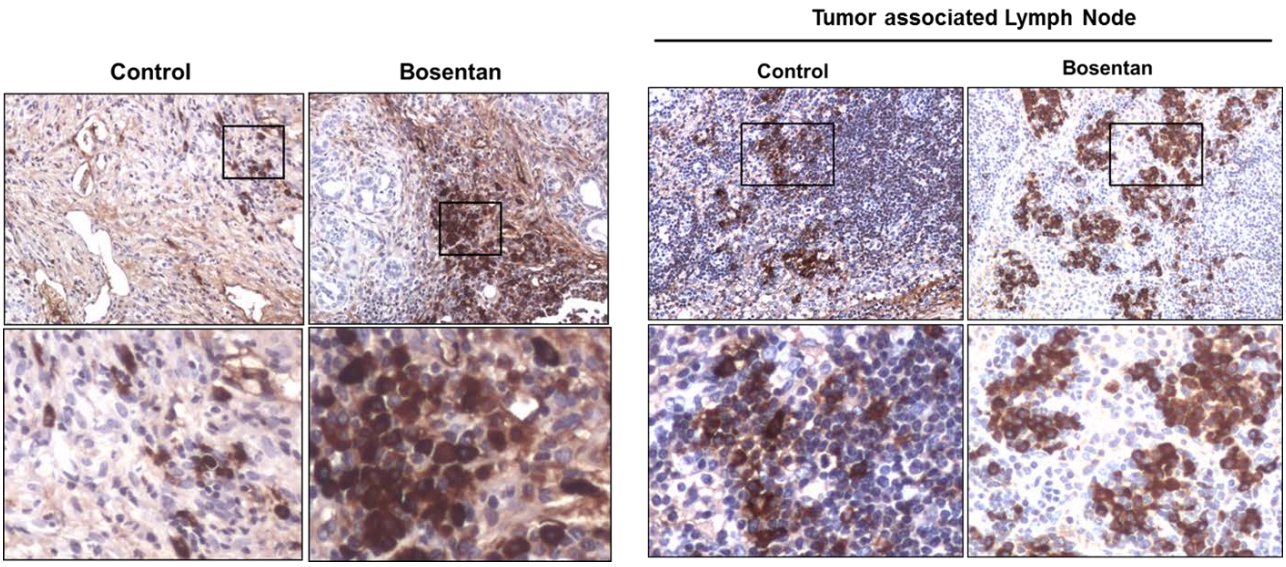

Tumor associated Lymph Node
Control
Bosentan
Control
Bosentan

Figure 17: Bosentan treatment increases the CD8 T cell infiltration in KPC tumors: Immunofluorescence analysis showing the distribution of CD4 **(A)** and CD8 **(C)** positive T cells in the microenvironment using mouse spleen as a positive control. Compared to control, Bosentan administration in KPC mice increases CD8 T cell infiltration **(D)** with significant p value whereas no difference in CD4 T cell is seen **(B)** (Scale bar 20 μm).

Figure 17

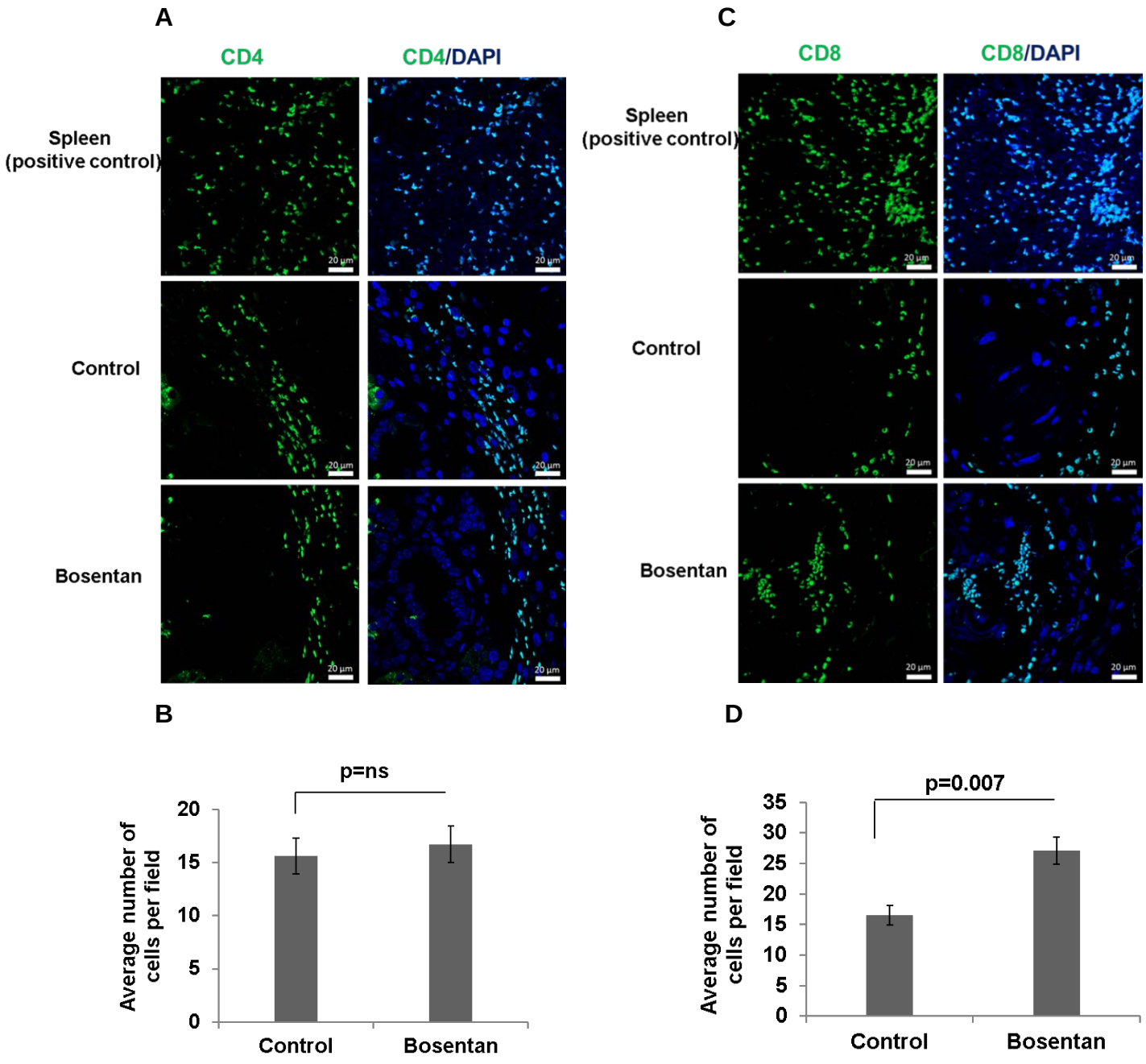
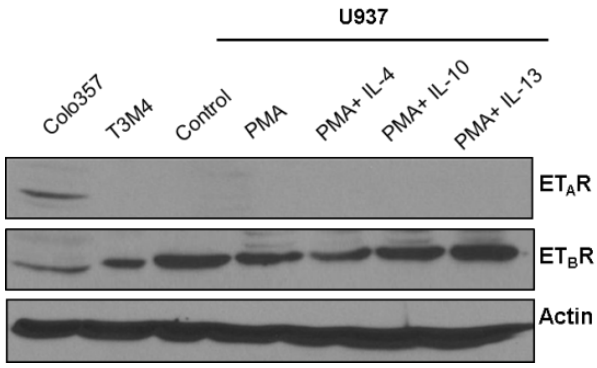

A
CD4
CD4/DAPI
Spleen
(positive control)
Control
Bosentan
20 µm
C
CD8
CD8/DAPI
Spleen
(positive control)
Control
Bosentan
20 µm
B
p=ns
Average number of cells per field
0
5
10
15
20
Control
Bosentan
D
p=0.007
Average number of cells per field
0
5
10
15
20
25
30
35
Control
Bosentan

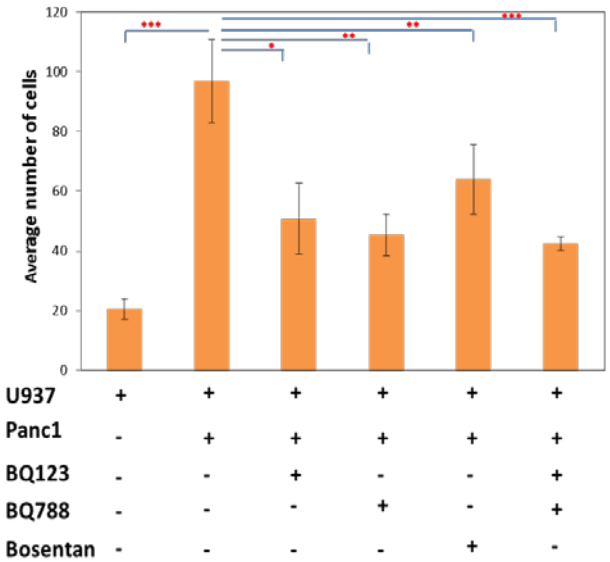
Figure 18: ET axis antagonism inhibits tumor induced migration of human monocytes and murine macrophages. A. Expression of ET receptors in human macrophage-like U937 cells. Colo357 and T3M4 PC cells were used as controls for ET_AR and ET_BR. U937 cells predominantly express ET_BR and this expression does not appear to be impacted by the differentiation status of the macrophages following treatments. Actin was used as a loading control. ET-axis antagonists inhibit tumor cell-induced migration of human **(B)** and murine macrophage-line **(C)**. Human (U937) and Mouse (RAW264.7) macrophage-like cells were either cultured alone or co-cultured with human PC cell line Panc 1 or KPC mouse tumor derived cell line (UN- KPC-961) with or without selective or dual receptor specific antagonists of ET axis. The tumor induced migration was inhibited by selective ET_AR (BQ123), ET_BR (BQ788) and dual ET_AR/ET_BR (Bosentan) antagonists in U937 and RAW264.7 cells. (* $p<0.05$, ** $p<0.005$, ***$p<0.0005$)

Figure 18

A



B



C

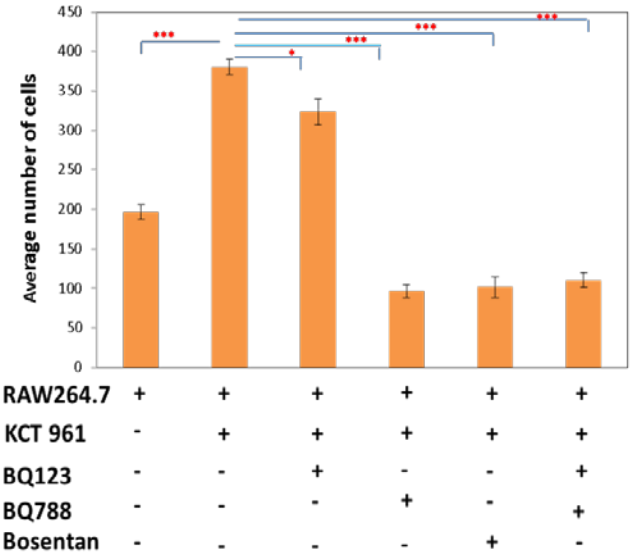
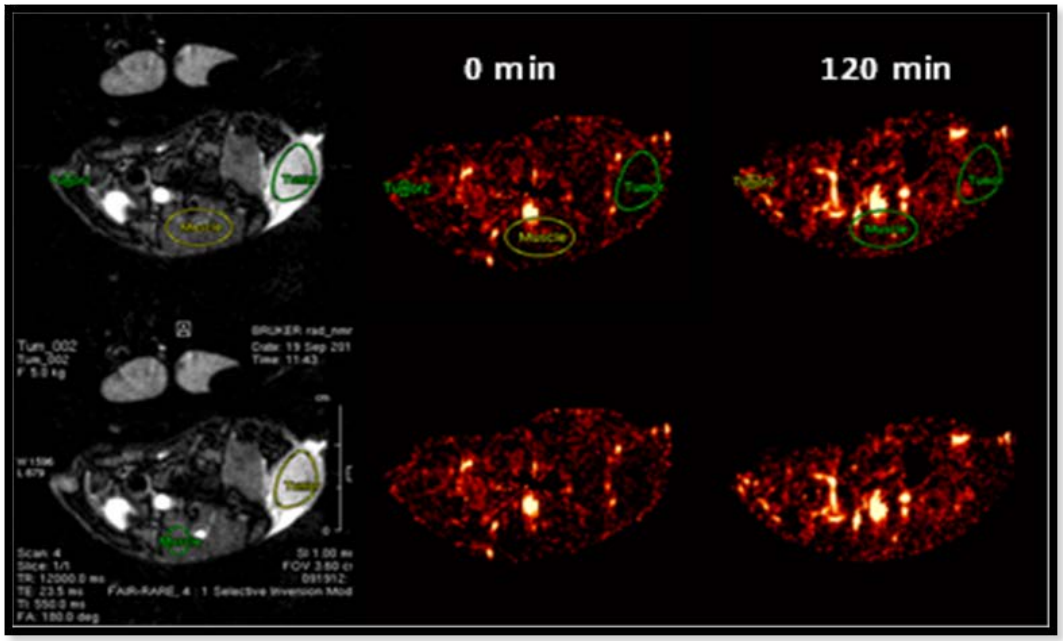


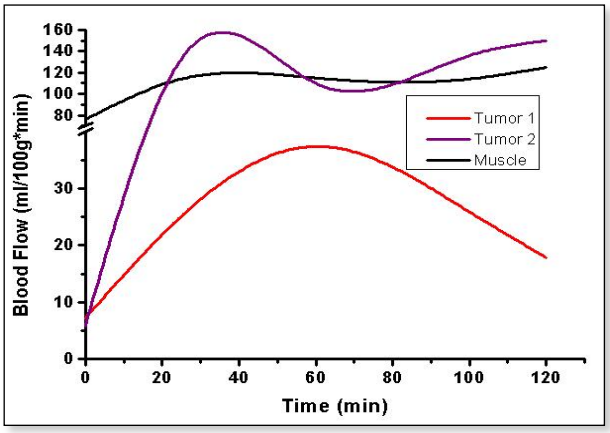
Figure 19: ET_AR antagonism increases tumor perfusion and decreases hypoxia in xenografts tumors. BQ123 improves perfusion and reduces hypoxia in xenograft PC tumors. **A.** Perfusion mapping of BQ123 administered tumor. Mice bearing T3M4 (tumor1) and Colo 357 (tumor 2) xenografts were administered with BQ123 (2 mg/kg). Perfusion mapping was done by flow sensitive alternating inversion recovery (FAIR) with a rapid acquisition by refocused echo (RARE) readout (RARE factor = 16). Perfusion was measured prior to administration of BQ123 (0 min) to establish baseline and subsequently imaged for 120 min post-administration to monitor the change in perfusion. Left panel shows the anatomic MRI scan to indicate the position of tumors and the regions of interest (ROI) in the tumors and muscle used for perfusion analysis. The right two panels show perfusion maps before and 120 min after injection of BQ123. Perfusion maps were windowed between 0 to 500 ml/(100g tissue * min). In the lower right two panels the ROI marker is removed to allow appreciation of the increased perfusion in regions of interest. It is notable that the phantoms at the top of the image (left) are completely absent in the perfusion maps, indicating proper balance of magnetization transfer effects. **B.** Kinetics of changes in perfusion in response to ET_AR antagonist BQ123 in xenograft PC tumors. Blood flow values were determined from the serial perfusion maps obtained by FAIR MRI. At zero time point, it can be appreciated that the tumors were considerably less perfused than the muscle tissue. Changes in perfusion in response to BQ123 were more pronounced in tumors than in the muscle. **C.** BQ123 treatment reduces hypoxia in T3M4 tumors. Tumor bearing animals were treated with saline (left) or BQ123 (right). 90 min after treatment animals were injected with pimonidazole HCl (Hypoxyprobe), which forms stable adducts with proteins in hypoxic cells. Animals were euthanized 45 mins thereafter; tumors were harvested and processed for immunohistochemistry for detecting Hypoxyprobe adducts (brown staining).

Figure 19

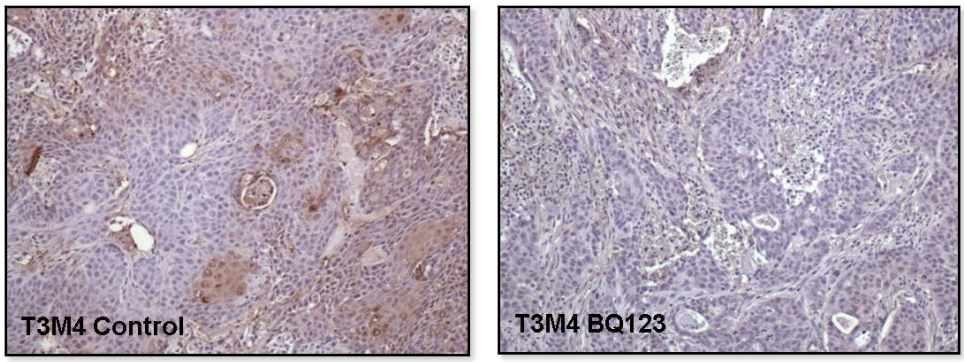
A



B



C



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Chapter 5

Irreversible and sustained upregulation of endothelin axis during K-Ras-oncogene associated pancreatic inflammation and cancer

1. Synopsis

Endothelin-1 (ET-1) and its receptors, endothelin A (ET_AR) and endothelin B (ET_BR) are overexpressed and deregulated in pancreatic cancer and pancreatitis. Here, we report that the expression of endothelin converting enzyme-1 (ECE-1), ET-1, ET_AR and ET_BR is predominantly associated with acinar and islet cells of the pancreas and progressive increase in ductal cells and stromal compartment is seen in the KC model (Pdx-1 Cre; KrasG12D) of pancreatic cancer with increasing age. Upon cerulein challenge, ET_AR and ET_BR expression is seen in amylase and CK19 double positive ducts during pancreatic acinar to ductal metaplasia (ADM) in mouse pancreas harboring KrasG12D mutation. In normal mice with wild type Kras, upon cerulein insult after an initial increase in expression of ET-1 and ET_AR and decrease expression of ET_BR, recovery in pancreatic parenchyma was observed. However, the kinetics of ET-1, ET_AR and ET_BR alterations was distinct in in presence of KrasG12D mutation. Also, expression of ECE-1 ET-1, ET_AR and ET_BR in spontaneous KrasG12D model subjected to cigarette smoke also suggests increase expression in the pre-cancerous lesions compared to sham control animals with WT kras. In addition to expression in early pancreatic cancer lesions (smoking) and metaplastic ducts (cerulein), ET_AR and ET_BR expression in also seen in infiltrating F4/80 positive macrophages and α-SMA positive fibroblasts and high colocalization was seen in presence of oncogenic Kras. In conclusion, presence of mutant KrasG12D in pancreas results the sustained activation of ET axis components with increasing dysplasia in early lesions and stromal region of pancreatic tissues following cerulein and smoke mediated inflammatory insult suggests a possible role in tumor initiation and progression.

2. Background and Rationale

Cellular plasticity is critical for tissue repair and regeneration. Cellular reprogramming in the exocrine pancreas under benign conditions involves a reversible trans-differentiation sequence of acinar cells to ductal- phenotype during repair and resolution of inflammation [1]. The presence of constitutively active K-ras mutations however, disrupt this sequence and initiate ductal reprogramming leading to the development of pre-neoplastic lesions termed as pancreatic intraepithelial neoplasia (PanIN), which are the precursor lesions the malignant pancreatic ductal adenocarcinoma (PC). These PanIN lesions undergo a series of histological and morphological changes during the course of cancer progression. The first genetically engineered mouse model generated by knock-in of mutant K-ras allele (G12D) exhibits acinar to ductal metaplasia (ADM) and develop a complete spectrum of PanIN lesions leading to PC. This process is characterized by the loss of acinar cell compartment (markers like amylase) and gain of the ductal phenotype, characterized by CK19 expression [2]. [3] utilizing transgenic mice overexpressing TGF-α provide earliest evidence of ADM. These animals develop fibrosis and dysplastic lesions accompanied by loss of zymogen granules and acquisition of ductal features [4], implying that maintenance of acinar cell organization is critical to prevent trans-differentiation into ductal architecture [5]. Activation of the mutant Kras in the cells of the acinar lineage under the control of elastase or Mist1 promoter lead to spontaneous low-grade lesion formation in the mouse pancreas. However, high-grade PanIN formation is observed following chronic inflammation [6]. Epidemiological studies also suggest that patients with chronic pancreatitis exhibit an elevated risk of developing PC. In chronic pancreatitis (CP) patients as well, constitutive activation of K-ras gene is associated with malignant transformation of the pancreas [7]. Furthermore, cerulean-induced chronic

pancreatitis in the mice expressing oncogenic Kras (KrasG12V) results in the development of aggressive PC along with increased infiltration of inflammatory cells [8]. Similarly, repeated episodes of cerulein mediated insult in the presence of K-ras mutation result in high-grade lesions, atrophy of pancreatic parenchyma, development of metaplastic cells, and enhanced inflammatory response [9]. Carriere *et al* demonstrated that episodes of cerulein induced pancreatitis favors rapid cancer progression and initiate cascade of events in mice expressing mutated Kras in the nestin cell lineage [10] [11]. Interestingly, in acute pancreatitis model, mutant Kras favors reprogramming and metaplastic conversion of acinar cells into pre-cancerous lesions in a beta catenin dependent manner [1].

Components of endothelin axis, which include endothelin-1 (ET-1), endothelin-2 (ET-2), endothelin-3 (ET-3), endothelin converting enzymes (ECEs) along with high affinity G-protein couple receptors, endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR), are deregulated in inflammation and cancer. Accumulating evidence suggest that ET-1 plays a significant role in the in pathophysiology of pancreatic inflammation. In patients with severe acute pancreatitis, an elevated level of ET-1 is correlated with disease severity and inflammation [12]. In addition, significantly elevated circulating levels of ET-1 in the plasma and its strong expression in the pancreas of the patients of chronic pancreatitis with a history of smoking was observed [13]. In experimental pancreatitis model, the role of ET-1 and its antagonists has been well studied. A remarkable increase in the serum ET-1 level and damage to pancreatic parenchyma is observed upon cerulein or sodium taurocholate induced pancreatic inflammation [14-16]. However, a synergistic effect is observed and a remarkable change in the pancreas morphology is seen in presence ET-1 plus cerulein compared to cerulein alone [17]. Administration of ET-1 favors acinar cell necrosis, edema, increase in serum amylase and elastase levels and inflammatory response indicating the role of

ET-1 in disease aggravation. Studies demonstrating the ability of several pancreatic cancer cell lines to produce high levels of ET-1 suggested the possible role in ET-axis in PC. [18, 19]. .A recent study has demonstrated the overexpression of ET-1 and ET_BR in human PC tissues [20] while we have observed the upregulation of ET-1, ET_AR and ET_BR in tumor cells and various components of tumor microenvironment.

Given the functional involvement of ET-1 in pancreatic inflammation, expression of ET-axis components in PC, the role of K-ras associated inflammation in driving pancreatic neoplastic transformation, and ability of ET-axis to exert pleotropic effects to promote tumorigenesis, it is possible that ET-axis plays a key role inflammation-driven pancreatic tumorigenesis in the presence of K-ras oncogene. However, the expression of ET axis proteins in pre-malignant lesions in acute and chronic pancreatic inflammation associated with oncogenic Kras remains explored. We therefore examined the expression pattern of ET-axis components in the murine models of chronic and acute inflammation in the presence and absence of oncogenic K-ras. The findings from the current study, demonstrated that cerulein induced inflammatory insult, a model of acute inflammation, initially up-regulates the expression of ET-1 and ET_AR, which was subsequently restored to basal levels in normal mice. However, in the KC mice the expression increases with progressive neoplasia. Smoke exposure, a model of chronic inflammation in KC mice results in increase expression of ET axis in the pre-cancerous lesions and also in pancreatic stroma indicating that mutated Kras results in sustained activation of ET axis in the pancreatic tissues, suggesting its possible role in pancreatic inflammation, repair and development of cancer.

3. Results

A. Expression of ECE-1, ET-1, ET_AR and ET_BR in murine pancreas in the presence of activated K-ras.

We first analyzed the expression of ET-axis components in the presence of activated $Kras^{G12D}$ mutation at the transcript level in comparison to their levels in the pancreas of age matched controls **(Figure 1A).** The levels of ECE-1, ET-1, ET_AR and ET_BR transcripts were comparable in the pancreas of 20 week old WT and KrasG12D mice. However, there was a progressive increase in the expression of ET axis components in the pancreas of 30, 40 and 50 weeks old KrasG12D mice as compared to that of WT animals. We next profiled their expression using immunohistochemistry on the pancreatic tissues harvested from 20-50 week old mice. In the control pancreas from 50-week-old WT mice, a robust expression of ECE-1, ET-1, ET_AR and ET_BR was seen in the islets and a relatively weak reactivity was detected in the acinar compartment **(Figure 1B)**. Importantly, the pancreatic ducts in the WT animals exhibited undetectable expression of ET-1, ET_AR and ET_BR. In the presence of oncogenic K-ras, the expression ECE-1, ET-1, ET_AR and ET_BR progressively increased in the ductal cells from pre-neoplastic lesions at 30 week of age to dysplastic lesions observed at 50 week of age **(Figure 1B)**. Interestingly, the expression was particularly localized to the ductal structures exhibiting transition from acinar to ductal phenotype, a characteristic observed during ADM. Further analysis revealed that the expression was not only restricted to the ductal lesions but was present in the surrounding stromal compartment.

B. Expression of ET axis during Cerulein induced pancreatic injury.

Cerulein is a cholecystokinin analogue that aggravates secretion of pancreatic enzymes, promotes inflammation, and induces ADM. The injury to pancreatic

parenchyma in the presence of oncogene results in in the development of s pre-neoplastic lesions and transformation of acinar cells to the ductal phenotype [23]. To analyze the changes in the ET-axis components in response to acute pancreatic inflammation in the presence and absence of oncogenic K-ras, we examined pancreatic tissues from 6 week old KC and WT mice treated with cerulein. On day 0 after treatment, both the WT and KC mice show acinar specific expression for ET_AR and ET_BR, as evident by their co-localization with amylase **(Figure 2A & 2B)**. On day 2-post cerulein treatment, in KC mice metaplastic ducts undergoing ADM observed, characterized by simultaneous expression of both amylase and CK19, and elevated ET_AR and ET_BR expression. The expression of ET receptors were more pronounced in CK19 positive ducts with increased dysplasia at day 7 and day 21-post cerulein administration. In contrast, the WT mice showed recovery of pancreatic parenchyma and restoration of pancreatic architecture **(Figure 3)**. Further, comparison of the WT and KC mice at the transcript levels after cerulein treatment showed difference in the kinetics and magnitude of ET axis upregulation. While ET-1 and ET_AR expression between WT and KC mice were unaltered and comparable on day 2, there was significant increase in both ET-1 and ET_AR levels in Kras mutant animals at day 7 and day 21-post cerulein administration **(Figure 4A & 4B)**. In contrast, the levels of ET_BR showed increase at day 2 and day 7-post cerulein injection and decreased at day 21 **(Figure 4C)**. Inflammatory insult in WT animals increased the expression of ET-1, ET_AR at day 2 while the levels of ET_BR decreased gradually. However, during recovery phase the expression restored to basal level at day 7 and 21 **(Figure 5A)**. In KC mice harboring $Kras^{G12D}$ mutation in the pancreas, after initial increase during inflammation the levels of ET_BR returned to normal whereas the ET_AR levels remained high with increasing dysplasia **(Figure 5B)**. Quantitative qRT- PCR showed no difference in the levels of ET-2 and ET-3 in $Kras^{G12D}$ mice compared to WT mice after cerulein treatment **(Figure 6A &6B).**

C. Expression of ET axis during Smoke induced pancreatic injury.

The effect of cigarette smoke induced alterations in the ET axis in presence of K-ras mutation showed on statistically significant difference in ECE-1 transcript levels in WT and K-ras animals however, there was slight increase in ECE-1 transcript levels. **(Figure 7A)**. Interestingly, qRT-PCR analysis revealed 7 fold increase in the ET-1 transcript levels in the K-ras mutant mice compared to sham control (p=0.04) **(Figure 7B)**. Additionally, the ET-3 mRNA levels in the WT and K-ras mice showed no significant difference with and without exposure to cigarette smoke **(Figure 8).** Comparison of the ET_AR and ET_BR mRNA levels in KrasG12D mice reveals significantly increase in both ET receptors **(Figure 7C & Figure 7D)**. Smoke-induced inflammation in the mutant K-ras mice showed increase of 4.5 fold and 5 fold in ET_AR (p=0.03) and ET_BR (p=0.01) mRNA levels respectively compared to sham controls. Further, histological analysis of the mice pancreas also showed increase in the expression of the ET axis components in response to smoke mediated inflammatory insult. Similar to our previous observation in spontaneous KC model, in the unfloxed mice pancreas with and without exposure to cigarette smoke, the expression of ECE-1, ET-1 and both ET receptors is associated with acinar compartment of the pancreas and prominent staining in islet cells is seen **(Figure 7E & Figure 7F)**. Interestingly, smoke exposure in the K-ras mice resulted in the increase in ECE-1 expression in the pancreatic duct while no difference was observed in the expression of ET-1, ET_AR and ET_BR in pancreatic ductal cells. In addition, comparison of the sham control mice reveals significant expression of ECE-1, ET-1 and the receptors in the pre-cancerous lesions and acinar compartment of the pancreas. Further analysis of the mice pancreas also demonstrated expression of ET axis proteins in the stromal region, and infiltrating immune cells. As compared to sham control, smoke

exposure in K-ras mice pancreas showed significant expression of ECE-1, ET-1, ET_AR and ET_BR in the pre-neoplastic lesions. Importantly, the increased expression in the early pre-cancerous lesions is accompanied by enhanced expression ET axis in the pancreatic stroma and immune cells. The co-expression of epithelial marker CK19 and ET-1, ET_AR and ET_BR in the pancreatic ductal cells further validated our findings **(Figure 9).**

D. Increase infiltration of F4/80 positive macrophages is associated with ET_AR and ET_BR expression in Cerulein and Smoke induced pancreatitis.

One of the key events that determines the onset of inflammation and severity of acute pancreatitis is the activation of macrophages [24] [25]. Similar to acute pancreatitis, increased infiltration of macrophages is reported in inflamed sites in both human and mouse chronic pancreatitis and are considered as master regulators of inflammation and disease progression [26]. Previous studies has reported increased accumulation of F4/80 positive macrophages in response to smoking in spontaneous KC mice model [27] [22] . In response to cerulein induced inflammation the median number of F4/80 positive macrophage population was not significantly different at day 2 of treatment, however a substantial increase was seen at day 7 (average number of cells/ field =13) and day 21 (average number of cells/ field =17) of cerulein treatment in mutant K-ras mice as compared to WT **(Figure 10A).** Immunohistochemical analysis in both KC mice model and smoking induced inflammation demonstrated increased expression of both ET_AR and ET_BR in ductal cells as well as stromal compartment, particularly on the infiltrating immune cells. We further confirmed our findings using immunofluorescence analysis in both cerulein and smoking model of pancreatitis. Validating our earlier findings, in at day 0 both ET_AR and ET_BR show expression in the acinar region and pancreas show fewer number of macrophages **(Figure 11A & Figure 11C).** At day 2-

post cerulein in the KC mutant mice the infiltrating F4/80 positive macrophages were found to be co-localized with ET_AR, although the co-expression was not statistically significant. On the other hand, as demonstrated, the expression of both ET_AR and F4/80 appeared in the double positive macrophages and significant co-localization was seen at day 7 and day 21 of treatment in the KC mice. Quantitative analysis of the F4/80 positive macrophages with ET_AR showed significant increase in the overlapping fractions at day 7 ($p=0.0035$) and day 21 ($p=0.0029$) of treatment **(Figure 11B).** Unlike ET_AR, analysis of the mice pancreas for the dual expression of ET_BR and F4/80 in the macrophages suggests higher degree of co-association at day 2 of treatment in mutant mice compared to control with significant p value ($p=0.0001$). The greater degree of co-localization is also witnessed in pancreas at day 7 ($p=0.0005$) and day 21 ($p=0.0008$) post cerulein trauma in presence of oncogenic Kras **(Figure 11D).** We also extended our studies in the smoking induced pancreatitis using three-color immunofluorescence. In WT animals with and without exposure to cigarette smoke the expression of both ET receptors is seen in pancreatic acini and no significant association was observed **(Figure 12A)**. In sham control mice the increase infiltration of F4/80 positive macrophages in the pancreatic stroma is accompanied by co-expression of both ET_AR and ET_BR with the F4/80 macrophages with significant p value ($p=0.003$ for ET_AR and $p=0.005$ for ET_BR) as compared to unfloxed mice subjected to smoke exposure. Smoke exposed animals in presence of Kras oncogene driven progression of PC demonstrate high co-expression of both ET_BR and F4/80 in double positive macrophages. Quantitative analysis using ImageJ further indicates that the F4/80 positive fractions overlapping with ET_BR show significant p value ($p=0.05$) as observed by Mander's correlation coefficient **(Figure 12C).** Unlike ET_BR, the increase infiltration of macrophages in the mice pancreas reveal limited co-expression with ET_AR and the overlapping fractions did not show statistical significance ($p=0.06$) **(Figure 12B).**

E. Increase accumulation of α-SMA fibroblasts in Cerulein and Smoke induced pancreatitis is associated with associated ET_AR and ET_BR.

Accumulating evidence indicate that activation of pancreatic stellate cells (PSCs) is pivotal in development of pancreatitis and pancreatic cancer by excessive deposition of extracellular matrix proteins [28] [29]. Studies have demonstrated that smoking leads to activation of PSCs and express myofibroblasts marker α-SMA in KrasG12D genetically engineered mouse model of PC [22]. Histological examination of the mice tissues from KrasG12D progression model and smoke exposed KrasG12D animals reveals enhanced expression of the ET axis components, particularly ET receptors in the pancreatic stroma. We further corroborated our findings using dual confocal microscopy utilizing the well-established myofibroblasts marker α-SMA in both acute and chronic models of pancreatitis. The degree of PSCs activation following cerulein administration was analyzed in both normal and KC mutant mice. In KrasG12D mice significant increase in the number of myo-fibroblasts is seen at day 2 post cerulein induced inflammatory insult (p=0.04), reflecting increased activation of PSCs **(Figure 10B).** Additionally, in KC mice harboring KrasG12D mutation in the pancreas substantial increase in the number of α-SMA positive myofibroblasts is seen at day 7 (average number of cells/field= 27) and day 21 (average number of cells/field= 32) with significant p value (p=0.02 for both ET_AR and ET_BR) compared to mice with WT Kras. Further, dual color immunofluorescence analysis reveals limited colocalization of both ET_AR and ET_BR with α-SMA positive myofibroblasts as demonstrated by no overlapping of the two fractions in the pancreas stroma at day 0 and day 2 of cerulein-mediated insult in normal and mutant mice **(Figure 13A & 13C).** Compared to wild type, in KC mice the increase activation of PSCs is followed by enhanced expression of ET_AR in the fibroblasts fractions at day 7-post cerulein administration with significant p value (p=0.001). Similar

to day 7-post trauma, the fraction of α-SMA fibroblasts shows high degree of co-expression with ET_AR in KC mutant mice as compared to WT mice with significant p value (p=0.003) **(Figure 13B).** Similar to ET_AR, co-association with activated PSCs after day 2 treated KC mice reveal limited colocalization with ET_BR compared to control mice **(Figure 13C).** Similar to ET_AR, co-association with activated PSCs after day 2 treated KC mice reveal limited colocalization with ET_BR compared to control mice (Figure 7c). In addition to ductal expression in the pancreas at day 7 and day 21 post cerulein treatment in KC mice, the fibroblasts surrounding the duct cells also show positivity for ET_BR expression and demonstrate extensive colocalization at day 7 (p=0.0006) and day 21 (p=0.0002) post cerulein trauma **(Figure 13D).** The activation status of PSCs in smoke treated unfloxed and floxed KC mice and its possible association with ET receptor expression was evaluated using immunofluorescence in tissues. Analysis of the unfloxed and floxed mice tissues shows prominent expression of both ET_AR and ET_BR in the pancreatic islet cells and acinar compartment with and without smoke exposure **(Figure 14A & 14C).** In sham control KC mice, in addition to the prominent expression in the early cancerous lesions, activated PSCs displays high colocalization with ET_AR (p=0.0001) compared to smoked unfloxed mice. Importantly, quantitative analysis of the smoke exposed KC mice show increase co-expression in the α-SMA and ET_AR double positive fibroblasts (p=0.04) (**Figure 14B).** Analysis of the histological features in the sham control mice compared to smoked unfloxed mice illustrate prominent increase and association of ET_BR with the stromal fibroblasts (p=0.0002). Contrary to ET_AR, α-SMA positive fibroblasts exhibit minimal and non-significant overlap with ET_BR compared to sham control **(Figure 14D).**

Discussion

Acinar to ductal metaplasia is the earliest recognizable morphological event in the pancreas in response to inflammation or oncogene activation and is regarded as a key event during the development of PC. It is characterized by the trans-differentiation of amylase-expressing acinar cell into CK19 positive ductal phenotype. The trigger for such intense morphological and histological change in the pancreas can either be due to accumulation of genetic changes or an inflammatory insult leading to increasing degree of atypia and ultimately to cancer. It has been firmly established that formation of precursor lesions (PanINs) precedes the formation of Kras driven PC initiation in experimental mouse model [8] [9]. These precursor lesions are derived from acinar cells of the pancreas undergoing a trans-differentiation from acinar to ductal phenotype, an event usually induced by pancreatitis [6] [30]. Further support for this view comes from various mechanistic studies implicating various transcription factors and signaling pathways in driving this switch from acinar to ductal phenotype [5] [31] [32] [33].

Endothelin axis plays significant role in tissue repair and inflammation of various tissues and has been implicated in the pathophysiology of pancreatic inflammation. Previous studies have shown aberrant expression of ET axis components in surgically resected pancreatic cancer patients and its association with tumorigenesis [20]. ET-1, most predominant and well-characterized ligand of the family is detected abundantly in pancreatic cancer cell lines as compared to the other two isoforms, ET-2 and ET-3 [18] [19]. In addition to its pathophysiological role in pancreas evidence also suggests its crucial role in normal pancreas physiology and also pancreatitis. Studies have demonstrated the presence of ET_AR and ET_BR in the rat pancreas and their differential binding affinities towards the three endothelin ligands [34]. The present study revealed the expression of ET axis components, ECE-1, ET-1, ET_AR and ET_BR in pancreatic acinar cells and islet cells and their minimal or low levels in the pancreatic ducts of WT

mice. In murine progression model of pancreatic cancer, a progressive increase in the expression was observed in the trans-differentiated or neoplastic ductal cells. The acinar specific expression of the ET axis in the normal pancreas implies a possible role of this axis in maintenance of acinar cell differentiated state. Metaplasia is regarded as the conversion or transformation of one cell type into another by an abnormal stimulus and are associated with early phase of tumor development. In the context of acinar to ductal metaplasia, metaplastic ducts are transitional structures and are characterized by presence of both acinar cell markers such as amylase and duct cell marker CK19. Our analysis reveals elevated expression of both ET_AR and ET_BR in amylase and CK19 double positive metaplastic ducts in the KrasG12D mutant mice pancreas following cerulean-induced injury.

Pancreatitis is an established risk factor for pancreatic cancer and is characterized by acinar cell necrosis, infiltration of inflammatory cell populations, stromal fibrosis and release of insoluble and soluble mediators [35] [36]. Previous study from our lab has revealed that ceruelin induced inflammatory damage to the pancreas is followed by decrease in the amylase expression in the mutant KrasG12D mice compared to control [21]. We observed that post cerulein induced inflammation metaplastic ducts can be seen in the mice pancreas and appearance of both ET_AR and ET_BR can be witnessed. In the KrasG12D mutant mice at day 7 and 21 post trauma, cerulein induced acinar to ductal metaplasia in pancreas display greater dysplasia and higher co-localization with CK19 positive ducts and progressed more rapidly to tumor development. In contrast, in normal mice with WT kras a recovery in the pancreatic parenchyma is observed after day 2-cerulein treatments, suggesting that oncogenic Kras results in enhanced and sustained activation of ET axis following inflammatory insults. In mice with WT Kras, cerulein treatment resulted in a notable increase in the expression of ET-1 and ET_AR mRNA levels at day 2, while the levels of ET_BR increased marginally;

however a recovery to basal level was observed for all three molecules by day 7. In contrast, significant increase in ET-1, ET_AR and ET_BR transcripts was observed following cerulein treatment and these levels continued to remain high even at 21 days post-trauma in KC mice. The expression of ET receptors in the normal mice is constrained in the pancreatic acini suggests its possible role in maintain the differentiating state of the acinar cells. Our data indicate that in mice harboring KrasG12D mutation in the pancreas the persistent activation of the ET axis components following cerulein treatment favors reprogramming of acinar cells into ductal morphology and favors neoplastic transformation. Therefore, we may speculate that the elevated levels of ET-1 in presence of KrasG12D oncogene favors acinar to ductal metaplasia changes to promote formation of pre-neoplastic lesions.

In the context of acute pancreatitis, the role of ET-1 has been widely studied and is known to promote disease aggravation. In experimental rat model of pancreatitis, ET-1 was identified as one of the candidate gene associated with pancreatic inflammation [37]. Additionally, in both sodium taurocholate and cerulein induced pancreatitis, exogenous administration of ET-1 damages the pancreatic parenchyma, promote acinar cell necrosis and increases amylase and elastase levels [38] [17]. ET-1 is considered to be significant risk factor for acute pancreatitis and elevated levels correlates with disease severity [12]. Similar to acute pancreatitis, elevated levels of ET-1 and significant correlation was observed in smoking individuals with chronic pancreatitis when compared to normal pancreatic tissues and non-smoking chronic pancreatitis patients [13]. In addition, studies also demonstrate a significant increase in the plasma ET-1 levels under the influence of tobacco smoking suggesting direct effect on endothelium facilitating the peptide release [39] [40]. Cigarette smoke is an established risk factor for PC and smoke induced inflammation accelerates the cancer progression in presence of constitutively active Kras mutation. Our recent study revealed that smoking

accelerates tumor progression by aggravating lesion formation in the pancreas and influences tumor microenvironment of the pancreas by activating pancreatic stellate cells and increase accumulation of macrophages [22]. We observed that cigarette smoke up regulates the transcript levels of ET-1, ET_AR and ET_BR in KrasG12D mice after smoke exposure as compared to mice with WT Kras. Interestingly mouse genotype determined the response to smoke induced inflammation. Further observation also suggests the acinar specific expression of ET axis components in WT mice with and without exposure to smoke further signifying its possible role in maintenance of differentiated state of acinar cells. Our results further indicate that in presence of KrasG12D oncogene smoke exposure significantly increase the expression in the pancreatic ductal cells.

In vivo observations suggest that the pancreatic inflammation elicits macrophage infiltration and secreted cytokines are considered as potent inducers of pancreatitis initiated acinar cell transformation to ductal progenitor phenotype [41]. These infiltrated macrophages are considered as drivers of ADM formation and in presence of oncogenic signaling favors tumor development [42]. A recent study demonstrated that acinar cells harboring mutant Kras up regulates ICAM-1 expression, which serves as chemo attractant for macrophages to drive tumor initiation [43] [44]. Several observations have also shown to modulate the migration of monocytes and macrophages in complex tumor microenvironment in endothelin dependent manner. Very recent study demonstrated that ET-1 through its interaction with receptors induced polarization of human macrophages [45] and favors cross talk between breast cancer cells and endothelial cells in an integrin dependent manner [46]. Also, in bladder cancer, ET-1/ET_AR interaction favors stromal cross talk and enhances metastatic colonization in the lung by increase migration and infiltration of tumor cells and tumor associated macrophages respectively [47]. In addition to promote tumor- stromal interactions, endothelin(s) also serves as potent chemoattractant for monocytes and macrophages. ET-1 has been shown to stimulate

chemotaxis of blood monocytes [48] and induced chemo-kinetic migration of peritoneal macrophages in an ET_AR dependent manner [49]. In addition to ET-1, ET-2/ET_BR signaling promotes chemotaxis and modulates the distribution of macrophages in tumor via MAPK pathway [50]. Therefore we may speculate that the elevated levels of ET-1 in pancreatitis associated inflammation in presence of $Kras^{G12D}$ oncogene drives acinar to ductal conversion in pancreas by increase infiltration of macrophages in an ET-1 dependent manner. We found that in response to inflammation (cerulein or smoking) the recruitment of activated macrophages in the pancreas stroma is dependent on mouse genotype and degree of inflammation. Also, the expression on the ET receptors on the infiltrating macrophage population is dependent on the mouse genome. In KC mice upon cerulein inflammation, significant co-localization of ET_BR and not ET_AR can be observed with F4/80 positive macrophages. However, F4/80 macrophages show high degree of co-localization with both the ET receptors at day 7 and day 21-post trauma. Our careful observation in the smoke treated mice tissues in presence of mutant $Kras^{G12D}$ also indicate higher fractions of F4/80 positive macrophages overlapping with ET receptors. Smoke treated mice carrying the KrasG12D oncogene show higher co-localization of F4/80 positive macrophages with ET_AR and ET_BR in the stromal compartment. It appears that the elevation in the ET-1 levels in inflammation associated pancreatitis serves as a potential chemo attractant for infiltrating macrophages expressing the receptors and favors neoplastic transformation by allowing acinar cells to be reprogrammed into ductal phenotype. However, to fully understand and delineate the molecular mechanisms of ET- axis mediated acinar cell reprogramming by recruiting macrophages in mutant $Kras^{G12D}$ mice, further studies involving macrophage targeted conditional knock-out of ET_AR and ET_BR need to be undertaken.

In addition to active infiltration of immune cell populations in response to pancreatic injury, activation of PSCs is recognized as central event in development of

pancreatitis and PC. These PSCs are activated by a variety of soluble and insoluble mediators such as cytokines, growth factors, oxidative stress, ethanol and its metabolites and pancreatitis. Once activated, they trans-differentiate into myo-fibroblasts like cells and the phenotypic transformation results in fibrosis and extensive deposition of extracellular matrix proteins [51]. The pro-fibrotic role of ET-1 in various pathologies is well documented and is regulated at transcriptional levels by interaction with various transcription factors such as Smad, TGF-β and activator protein-1 (AP-1) [52] [53]. ET-1 induces a pro-fibrogenic response in lung fibroblasts by increase expression of α-SMA and CTGF by JNK-AP1 and TGF-β pathway [54] [55]. Accumulating evidence imply that activated PSCs play a pivotal role in development of pancreatic fibrosis and inflammation. Activated PSCs express ET-1, ET_AR and ET_BR and are ET-1 responsive suggesting an autocrine and paracrine loop to stimulate contraction and migration of PSCs by inducing phosphorylation of ERK and MLC but not AKT [56]. Further, studies by Stumpe and co-workers indicate that ET-1/ET_AR interactions increase cytosolic calcium concentrations and possibly act as an autocrine and paracrine factor for activated PSCs [57]. In addition to stimulate myofibroblasts differentiation, ET-1 has also been shown to promote inflammatory reaction in the pancreas by release of pro-inflammatory mediators such as IL-6 and IL-1β [58]. Moreover, Fitzner *et al* demonstrated that the pro-fibrogenic effect of ET axis is attenuated by dual ET_AR and ET_BR antagonist Bosentan in experimental chronic pancreatitis model [59]. Our results suggest that acute and chronic inflammation induced by cerulein and smoking respectively accelerates the desmoplastic reaction in the $Kras^{G12D}$ mice and the concomitant expression of endothelin receptors on activated PSCs can possibly promote stromal cross talk by autocrine and paracrine interactions and aid in tumor progression. Our current study revealed that endothelin receptors are expressed in metaplastic ducts signifying its possible role in trans-differentiation. In addition, increase accumulation of

fibrotic stroma in mutant Kras mice after post cerulein administration suggests in addition to metaplastic ducts, the expression of ET receptors is also observed in microenvironment of the pancreas. Importantly, significant co-localization of α-SMA positive fibroblasts is observed with both ET_AR and ET_BR dependent on mouse genome and degree of inflammation. Our real time PCR analysis infers significant increase in the ET-1 transcript levels in pre-cancerous lesions in the $Kras^{G12D}$ mice further strengthen the involvement of possible interaction with the activated stellate cells. In addition to acute inflammation model, in smoke induced chronic pancreatitis elevated levels of α-SMA in presence of oncogenic signaling is followed by prominent expression of ET_BR and limited co-association of ET_AR. Quantitative analysis further indicates that fractions of α-SMA fibroblasts exhibit significant overlapping fractions with ET_BR positive fibroblasts. We speculate that persistent increase and activation of ET_AR and ET_BR with increasing dysplasia in the activated myo-fibroblasts in presence oncogenic Kras signaling plays an essential role in maintenance of PSC activation through ET-1 autocrine loops. However, the molecular mechanisms underlying this phenomenon are not completely understood and warrant further investigation.

To summarize, our study profiles the expression of endothelin axis during acute and chronic inflammation associated pancreatic tumor progression in presence of mutated KrasG12D. Under physiological conditions, the expression of ET axis components is restricted to pancreatic acinar and islet cell compartments **(Figure 15).** However, during inflammation or injury the acinar expression is abrogated and elevated expression is seen in early pre-cancerous lesions and neoplastic cells. The sustained upregulation of ET-axis components in the presence of oncogenic K-ras and overexpression in advanced lesions, suggest that signaling along ET-axis possibly contributes to reprogramming of acinar cells into metaplastic ductal cells and drives their transformation into neoplastic lesions. The increased expression in pre-neoplstic lesions

is followed by excessive accumulation of ECM proteins and inflammation in the pancreas, indicating further involvement of ET axis in influencing microenvironmental factors during the initiation and progression of pancreatic cancer.

Figure 1: Expression pattern of ECE-1, ET-1, ET_AR and ET_BR in Pdx1-Cre; $KrasG^{12D}$ (KC) murine pancreatic cancer model. A. RT-PCR analysis showing age wise expression of ECE-1 ET-1, ET_AR and ET_BR on pancreas of control and KC mutant mice. The expression for all four molecules can be observed at all weeks analyzed and the levels maintained throughout. In contrast, a robust expression in the expression is seen in 20, 30,40 and 50 wk KC mice compared to littermates controls. Actin was used as loading control. **B.** Immunohistochemistry analysis of ET-1, ET_AR and ET_BR after staining with respective antibodies (1b). Normal pancreas (unfloxed mice) isolated from 50 week old mice shows predominant expression for ECE-1, ET-1 and receptors in the islet cells (black arrow heads) while low immunoreactivity is seen in the pancreatic acini (zoom). In the control mice (unfloxed) pancreas the low expression of ECE-1 can be seen in the pancreatic duct (inset) whereas no expression was detected for ET-1 and receptors. In the early PanIN lesions of pancreas of Pdx1-Cre;$Kras^{G12D}$ mice starting from 30 weeks of age, the elevated expression can be seen and progressive increased expression is observed both in the tumor cells and the stromal compartment (inset) with the mutant Kras expression.

Figure 1

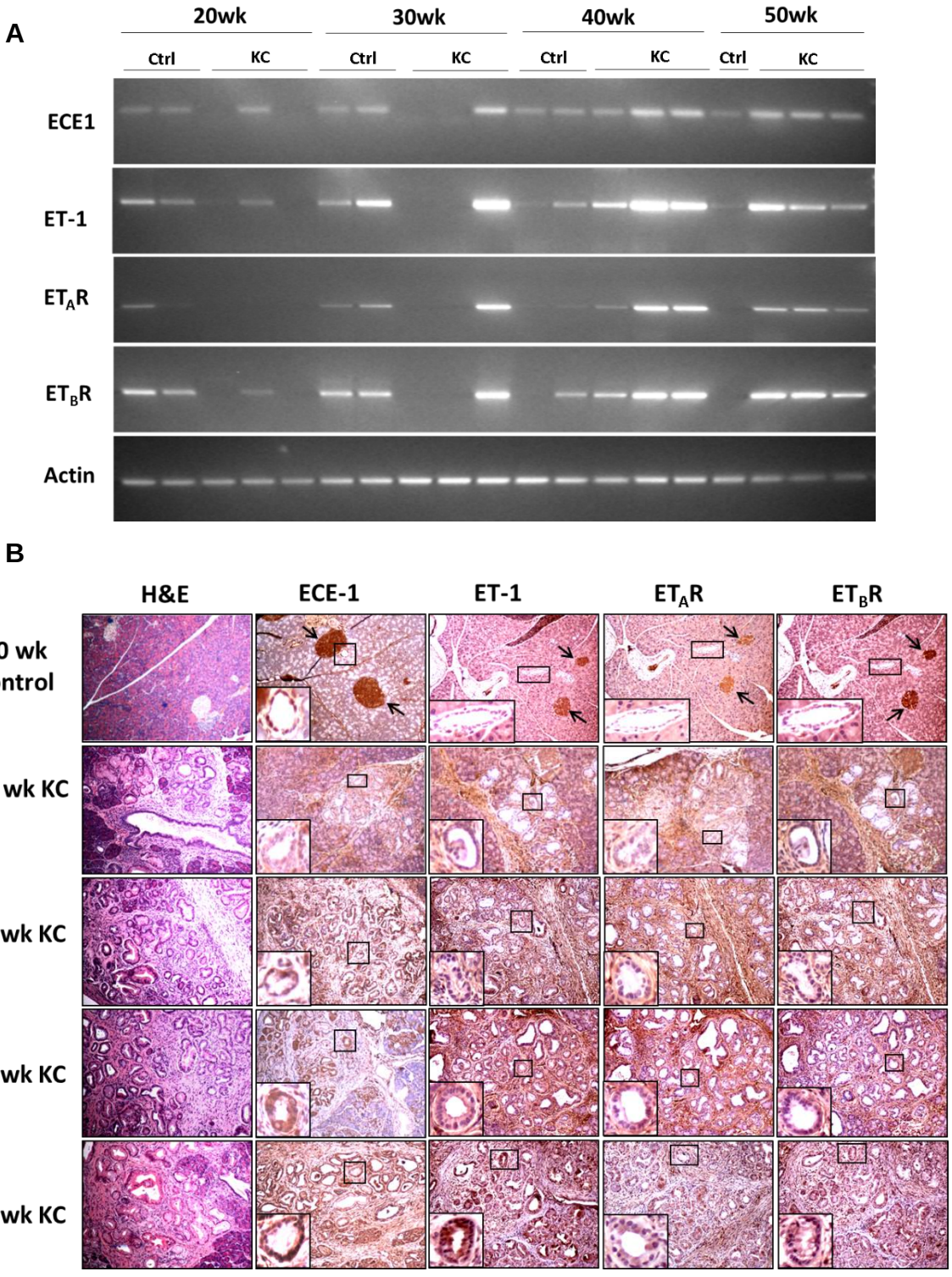

A
20wk
30wk
40wk
50wk
Ctrl
KC
Ctrl
KC
Ctrl
KC
Ctrl
KC
ECE1
ET-1
ETAR
ETBR
Actin
B
H&E
ECE-1
ET-1
ETAR
ETBR
50 wk control
20 wk KC
30 wk KC
40 wk KC
50 wk KC

Figure 2: ET_AR and ET_BR expression during pancreatic injury in normal and KC mice. Three color Immunofluorescence images of ET_AR **(A)** and ET_BR **(B)** expression (purple) of with acinar cell marker amylase (red) and ductal cell marker CK19 (green) in cerulein and saline treated mice at days 2, 7 and 21 post cerulein injection. Expression of both ET_AR and ET_BR is seen in the pancreatic acini in normal and KC mutant mice under control conditions. At day 2 post trauma, ET_AR and ET_BR expression appears in amylase and CK19 double positive metaplastic ducts in KC mice under cerulein treated metaplastic conversion. In control mice, normal pancreatic architecture is restored and the acinar cells displaying increases expression of amylase and colocalization with ET_AR and ET_BR. In the KC mice at day 7 and 21 post inflammatory insult, the pancreas show dysplastic changes and appearance of metaplastic ducts and gradual increase expression of both ET receptors can be seen in the CK19 positive ducts.

Figure 2

A

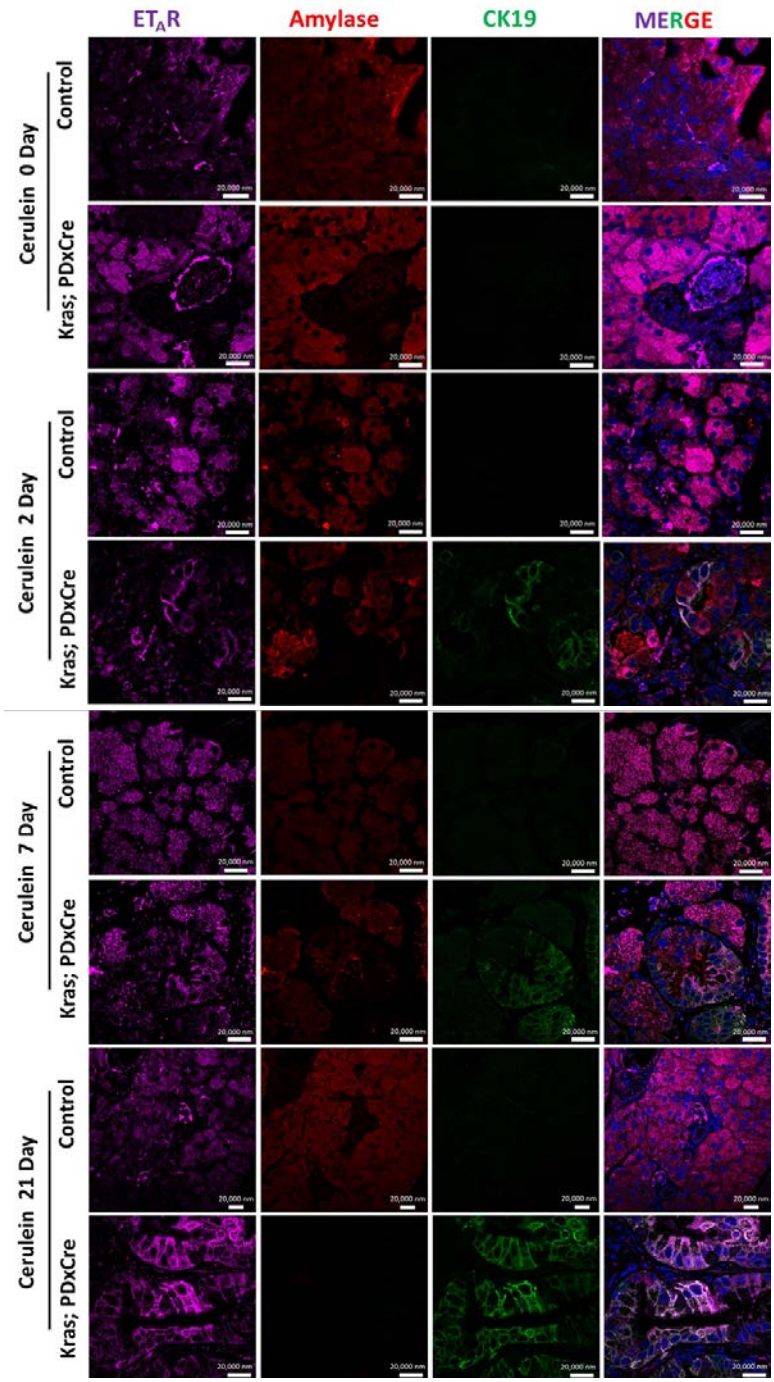

ETAR
Amylase
CK19
MERGE
Cerulein 0 Day
Control
Kras; PDxCre
Cerulein 2 Day
Control
Kras; PDxCre
Cerulein 7 Day
Control
Kras; PDxCre
Cerulein 21 Day
Control
Kras; PDxCre

Figure 2

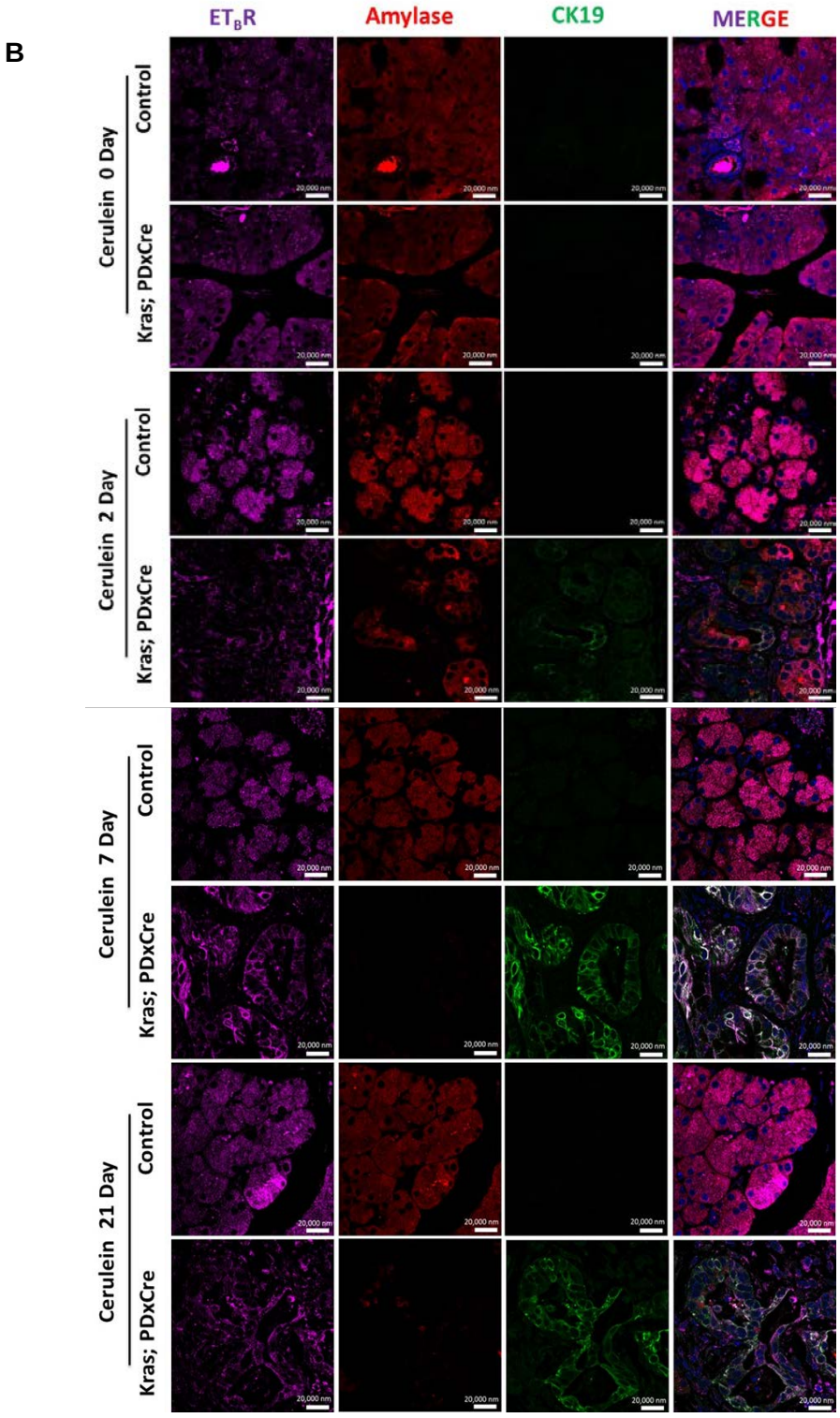

B
ET$_B$R
Amylase
CK19
MERGE
Cerulein 0 Day
Control
Kras; PDxCre
Cerulein 2 Day
Control
Kras; PDxCre
Cerulein 7 Day
Control
Kras; PDxCre
Cerulein 21 Day
Control
Kras; PDxCre
20,000 nm

Figure 3: Histological analysis of ET_AR and ET_BR expression in normal mice with and without cerulein treatment. Immunohistochemical analysis of ET_AR and ET_BR in the mice pancreas after cerulein treatment. Analysis reveal low expression of both receptors in the pancreatic acini whereas a predominant expression in the islet cells is seen (black arrowheads) at all day 0, 2, 7 and 21 of treatment. Interestingly, no expression in the normal pancreatic duct was seen (inset) and recovery in the pancreatic parenchyma is observed at day 7 and day 21 of treatment

Figure 3

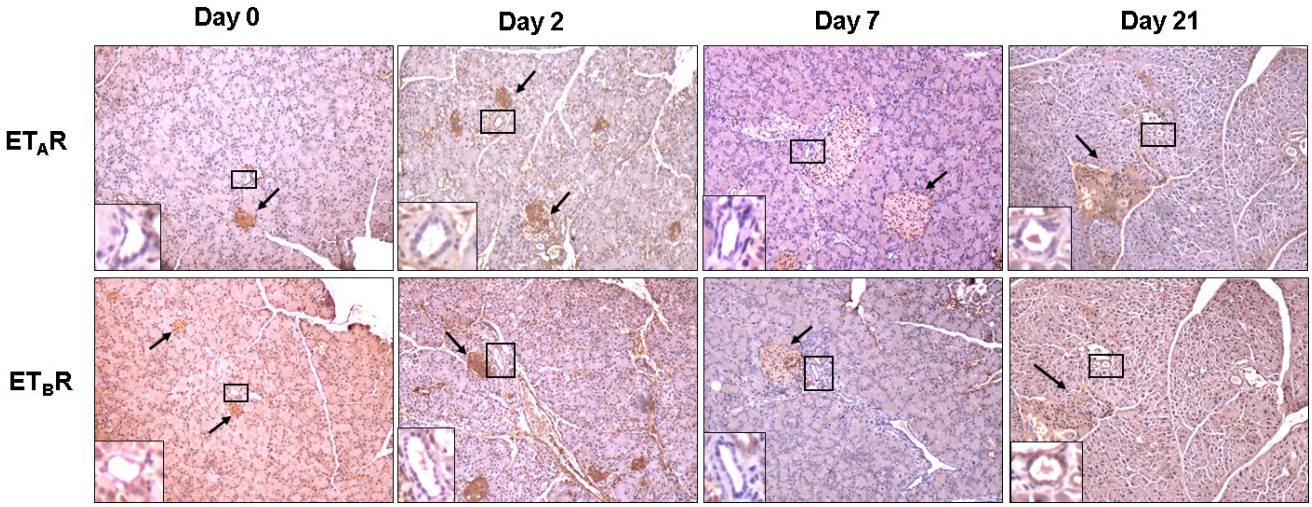


Figure 4: ET-1, ET_AR and ET_BR expression during pancreatic injury in normal and KC mice. Real time PCR analysis of ET-1 mRNA levels between normal and KC mice under control and cerulein treated conditions showed significant increase in the ET-1 mRNA levels in the $Kras^{G12D}$ mutant mice at day 7 and day 21 after cerulein administration **(A)**. Comparison of the of ET_AR transcript levels showed elevated expression in the Kras mutant mice at day 7 and day 21 of treatment with significant p value **(B)**. A predominant and significant increase in the ET_BR mRNA was seen at day 7 of treatment with a marginal increase being observed at day 21-post trauma **(C)**.

***** $p<0.05$, ****** $p<0.005$, ns=not significant.

Figure 4

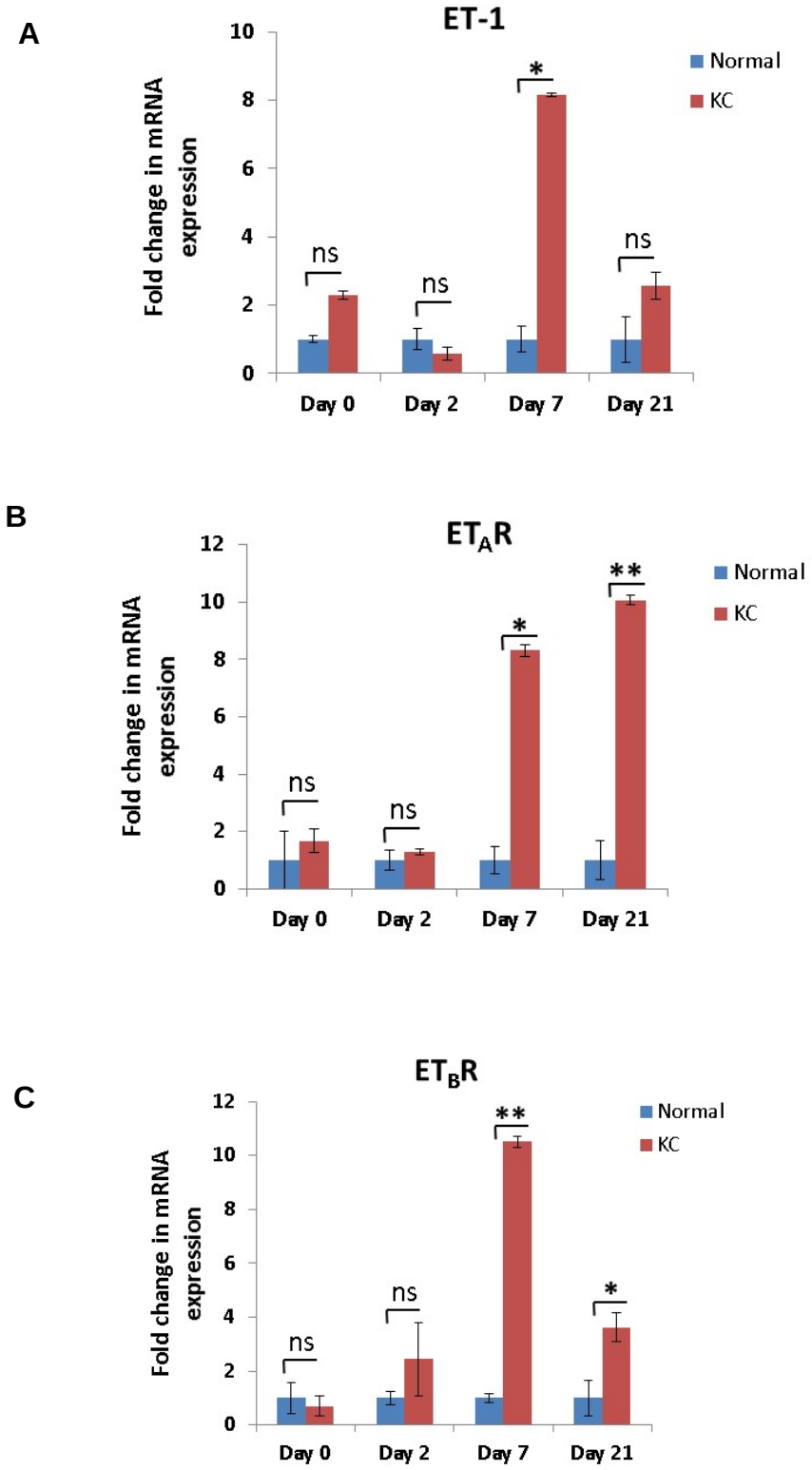
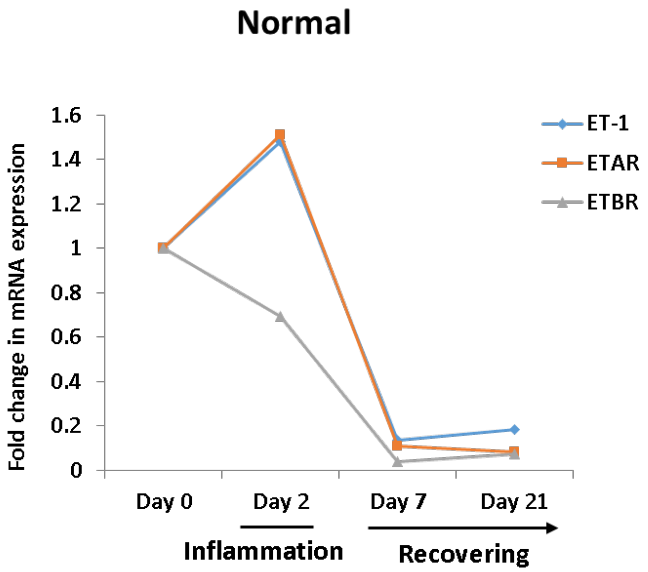

A
ET-1
Fold change in mRNA expression
Normal
KC
ns
ns
*
ns
Day 0
Day 2
Day 7
Day 21
B
ETAR
Fold change in mRNA expression
Normal
KC
ns
ns
*
**
Day 0
Day 2
Day 7
Day 21
C
ETBR
Fold change in mRNA expression
Normal
KC
ns
ns
**
*
Day 0
Day 2
Day 7
Day 21

Figure 5: ET-1, ET_AR and ET_BR expression during pancreatic injury in normal and KC mice. Real time PCR analysis of ET-1, ET_AR and ET_BR expression alterations in the normal **(A)** and KC **(B)** mice under control and cerulein treated conditions at various time points. In the normal mice, increase expression of ET-1 and ET_AR and decrease expression of ET_BR was seen due to cerulein induced inflammatory insult at day 2 and recovery for all three molecules being observed for all three molecules by day 21. In KC mice, harboring Kras mutation in the pancreas ET_AR mRNA levels gradually increased with progressive acinar to ducal phenotype switch following cerulein treatment. ET_BR mRNA levels on the other hand, increases due to metaplastic changes in the mice pancreas at day 7 but falls backs to basal levels subsequently.

* $p<0.05$, ** $p<0.005$, ns= not significant

Figure 5

A


Normal
Fold change in mRNA expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day 0
Day 2
Day 7
Day 21
Inflammation
Recovering
ET-1
ETAR
ETBR

B

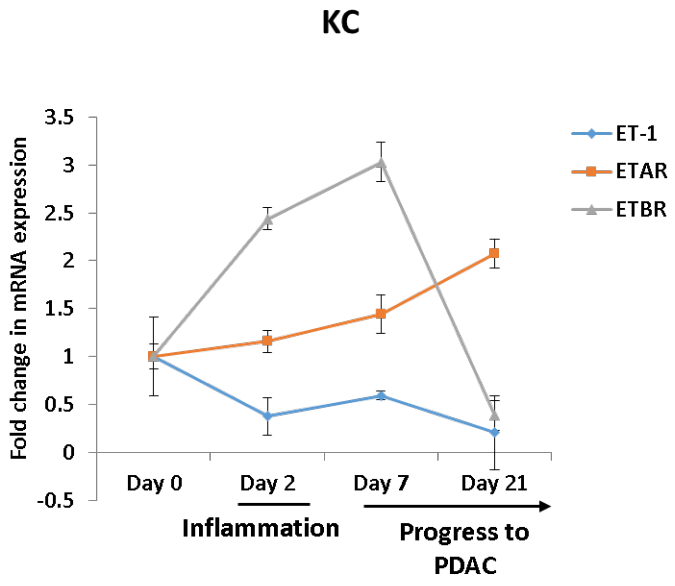
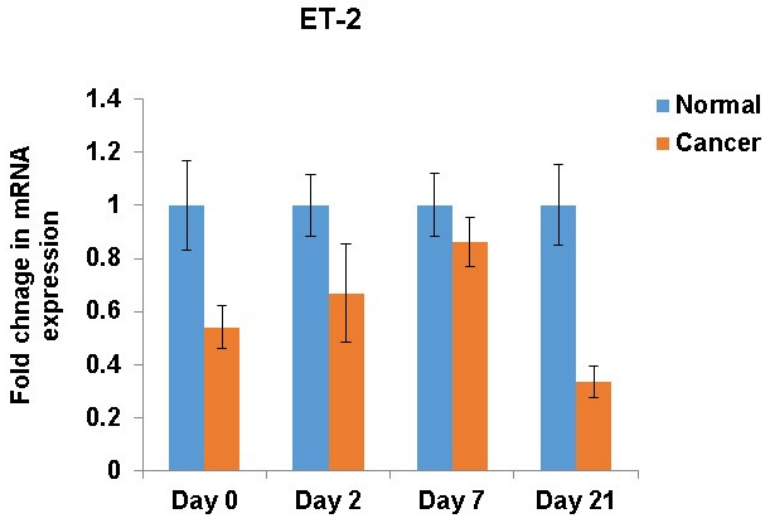

KC
Fold change in mRNA expression
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
Day 0
Day 2
Day 7
Day 21
Inflammation
Progress to PDAC
ET-1
ETAR
ETBR

Figure 6: Expression of ET-2 and ET-3 in normal and KC mice with or without cerulein treatment. Real time PCR analysis show no significant difference in the expression of ET-2 **(A)** and ET-3 **(B)** in KC mice compared to control over the treatment period. ***** $p<0.05$, ****** $p<0.005$, ns=not significant.

Figure 6

A


ET-2
Fold chnage in mRNA expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day 0
Day 2
Day 7
Day 21
Normal
Cancer

B

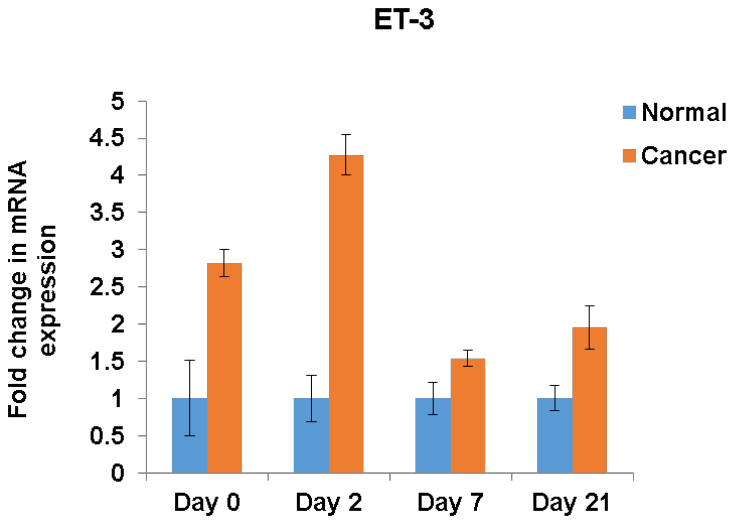

ET-3
Fold change in mRNA expression
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Day 0
Day 2
Day 7
Day 21
Normal
Cancer

Figure 7: Expression of ET-1, ET_AR and ET_BR in spontaneous KC mice model subjected to cigarette smoke. Quantitative real time PCR analysis show no significant difference in the expression of ECE-1 in the RNA isolated from the pancreas of sham and smoke exposed mice **(A)**. Comparison of ET-1**(B)**, ET_AR **(C)** and ET_BR **(D)** transcript levels show significant difference in mRNA levels in Kras mutant mice exposed to cigarette smoke as compared to sham controls. **E.** Immunohistochemical analysis of the unfloxed animals with and without exposure to cigarette smoke induced inflammatory insult show difference in the expression of ET axis components and low immunostaining in acinar cells and predominant staining in islet cells is observed (inset). Smoke exposure to unfloxed mice reveal increase expression of ECE-1 in normal pancreatic duct while the expression of other markers remain unchanged (inset). The expression of ECE-1, ET-1 and receptors can be seen in pre-cancerous lesions in the Sham floxed animals and stromal region. In presence of oncogenic Kras overexpression of ECE-1, ET-1 and receptors can be observed in both tumor lesions and stromal compartment. The box plot represents the quantitative expression of ECE-1 **(F)**, ET-1 **(G)**, ET_AR **(H)** and ET_BR **(I)**.

Figure 7

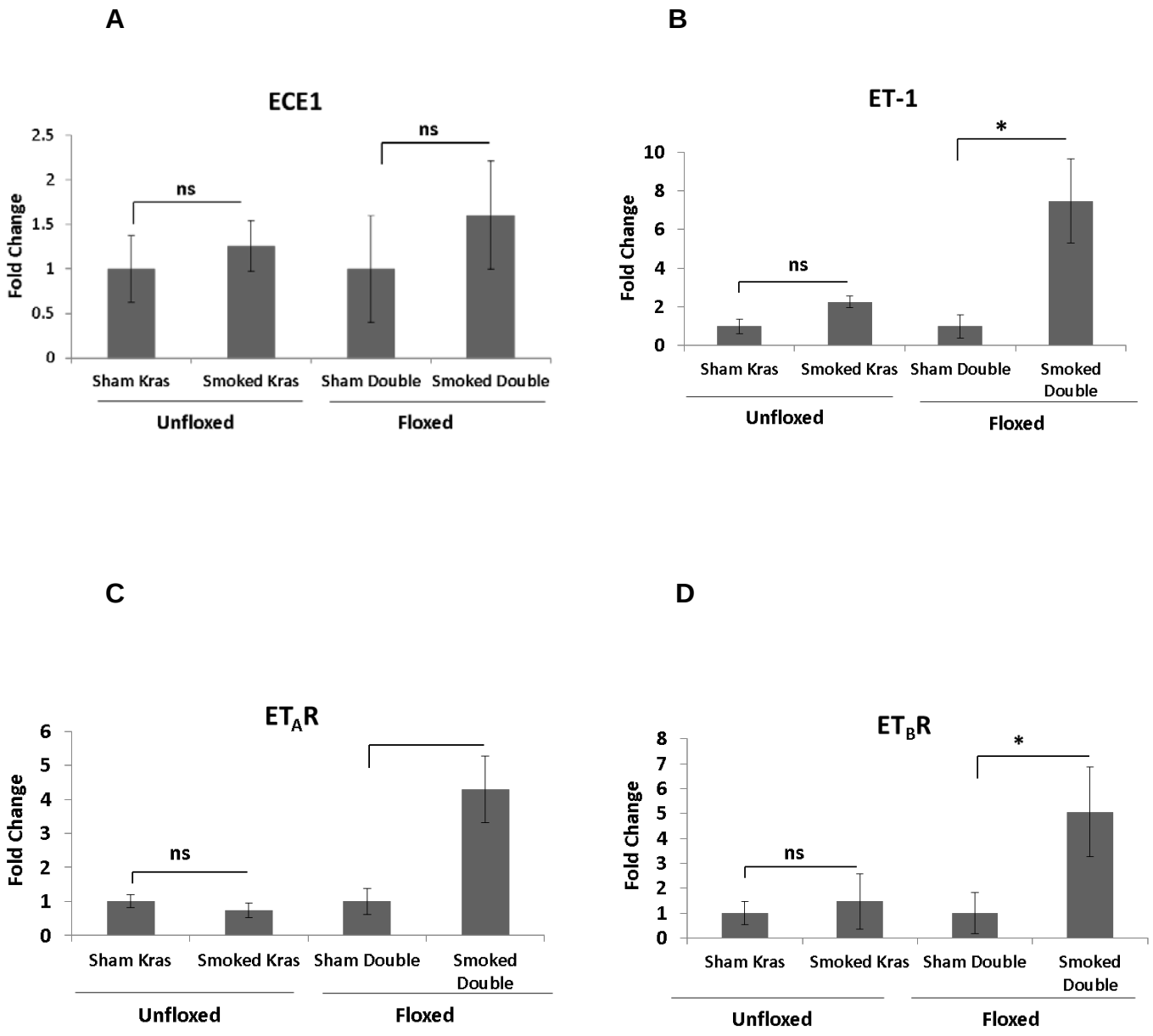
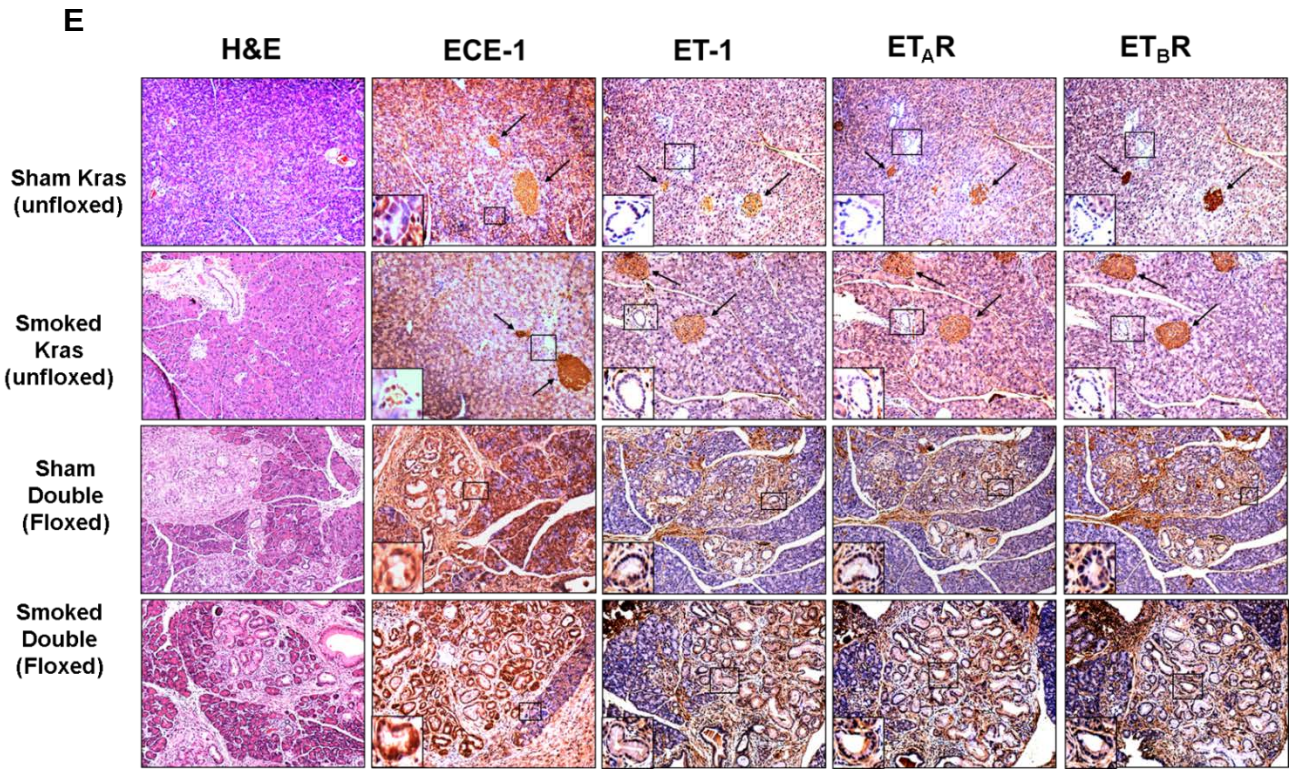
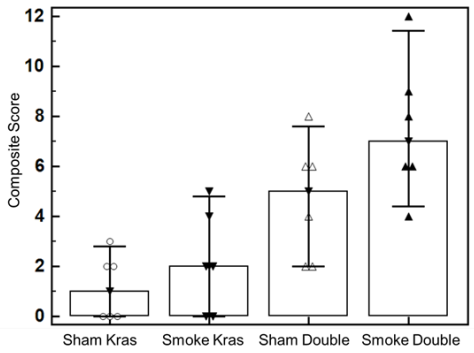
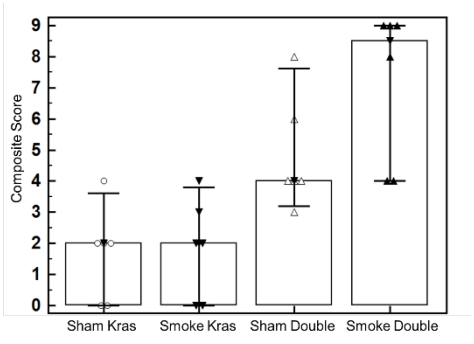
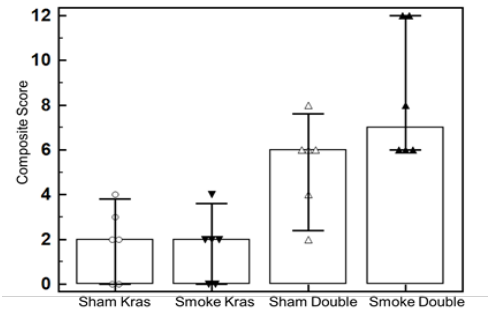

A
ECE1
Fold Change
2.5
2
1.5
1
0.5
0
ns
ns
Sham Kras
Smoked Kras
Sham Double
Smoked Double
Unfloxed
Floxed
B
ET-1
Fold Change
10
8
6
4
2
0
ns
*
Sham Kras
Smoked Kras
Sham Double
Smoked Double
Unfloxed
Floxed
C
ETAR
Fold Change
6
5
4
3
2
1
0
ns
Sham Kras
Smoked Kras
Sham Double
Smoked Double
Unfloxed
Floxed
D
ETBR
Fold Change
8
7
6
5
4
3
2
1
0
ns
*
Sham Kras
Smoked Kras
Sham Double
Smoked Double
Unfloxed
Floxed

Figure 7


E
H&E
ECE-1
ET-1
ET$_A$R
ET$_B$R
Sham Kras (unfloxed)
Smoked Kras (unfloxed)
Sham Double (Floxed)
Smoked Double (Floxed)


F
Composite Score
Sham Kras
Smoke Kras
Sham Double
Smoke Double


G
Composite Score
Sham Kras
Smoke Kras
Sham Double
Smoke Double


H
Composite Score
Sham Kras
Smoke Kras
Sham Double
Smoke Double

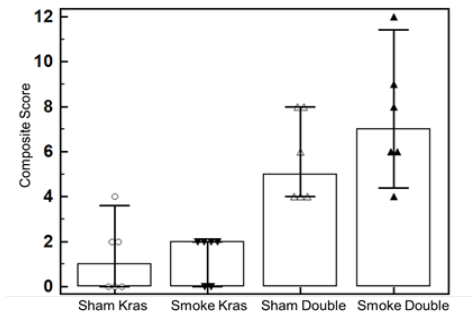

I
Composite Score
Sham Kras
Smoke Kras
Sham Double
Smoke Double

Figure 8. Expression of ET-3 in unfloxed and floxed KC mice with and without cigarette smoke exposure. Real time PCR analysis show no significant difference in the expression of ET-3 in the RNA isolated from the pancreas of sham and smoke exposed mice. ***** $p<0.05$, ****** $p<0.005$, ns=not significant.

Figure 8

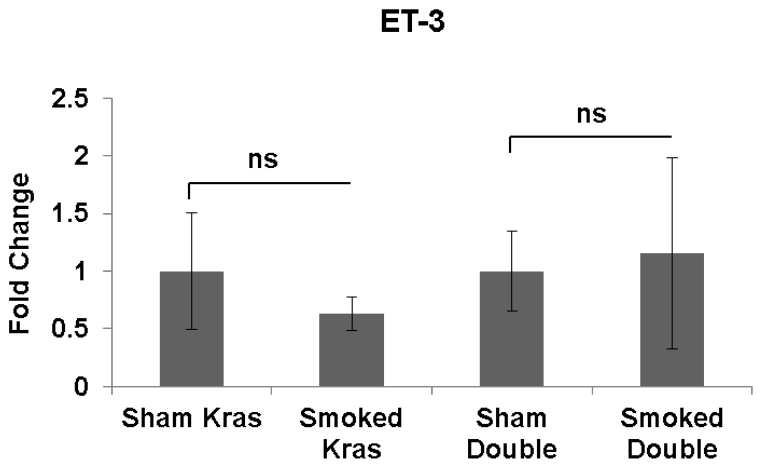

ET-3
Fold Change
2.5
2
1.5
1
0.5
0
ns
ns
Sham Kras
Smoked Kras
Sham Double
Smoked Double

Figure 9: Expression of ET-1, ET_AR and ET_BR in spontaneous KC mice model with and without cigarette smoke exposure. Dual immunofluorescence analysis showing the expression of ET-1, ET_AR and ET_BR in sham controls and smoke exposed floxed mice. Smoking in the Kras mutant mice accelerated the PanIN formation and increase expression in the CK19 positive ducts.

Figure 9

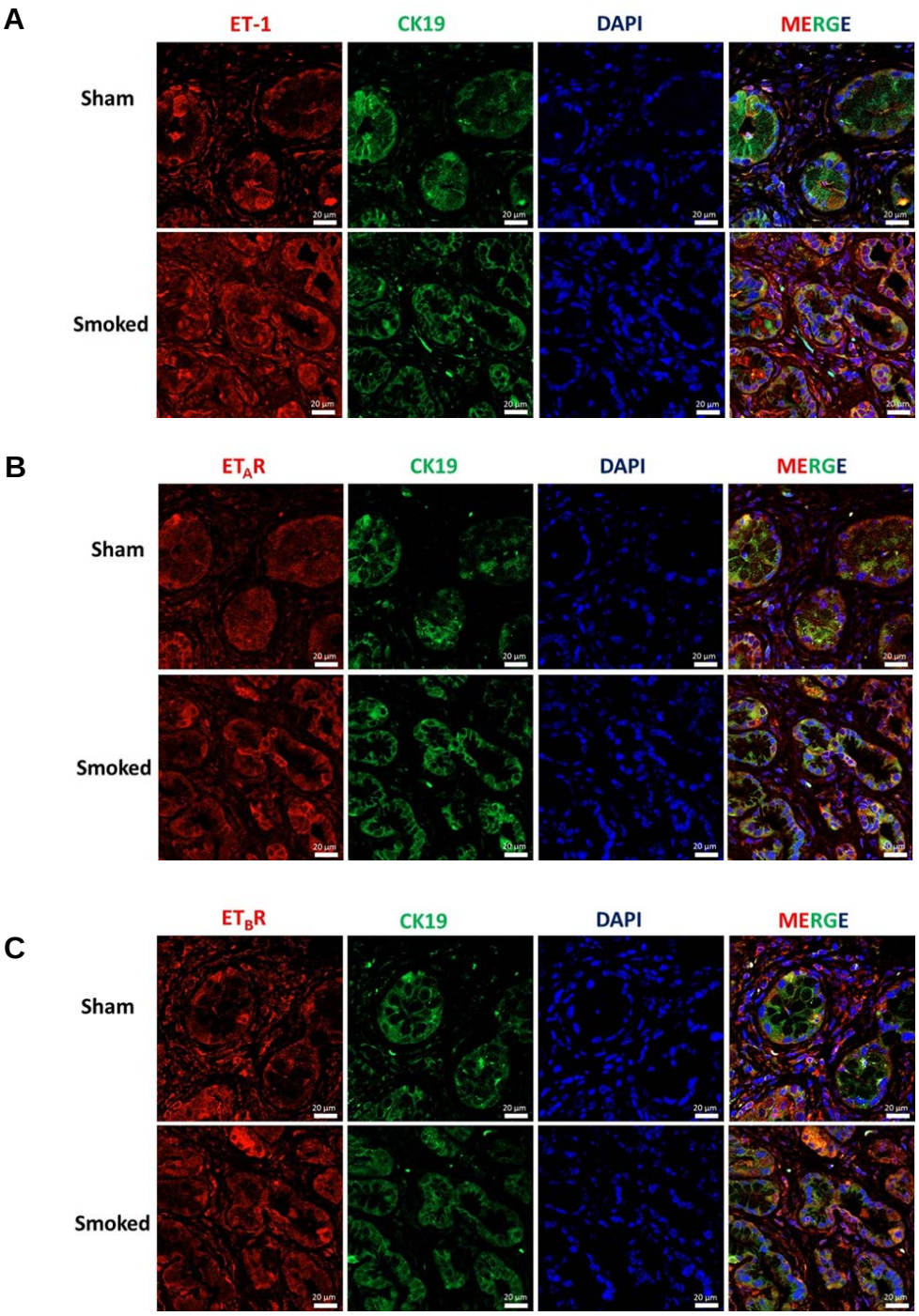
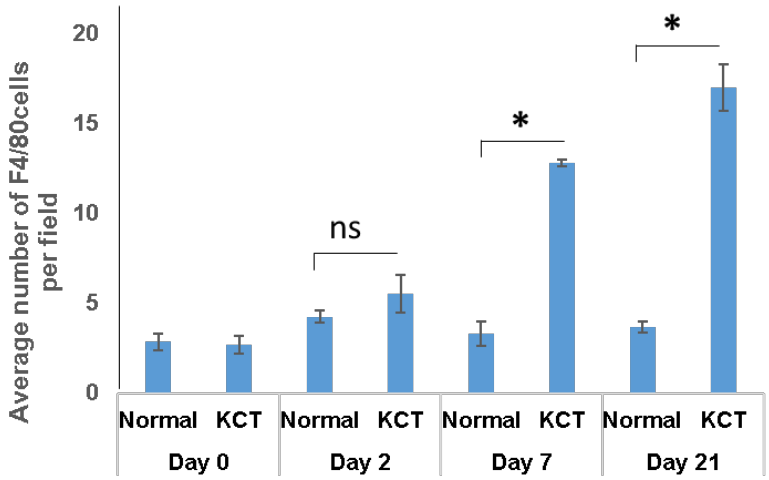

A
ET-1
CK19
DAPI
MERGE
Sham
Smoked
B
ET_AR
CK19
DAPI
MERGE
Sham
Smoked
C
ET_BR
CK19
DAPI
MERGE
Sham
Smoked

Figure 10: Increase accumulation of F4/80 positive macrophages and α-SMA fibroblasts following cerulein treatment. Bar diagram representation Immunofluorescence analysis of the pancreas of saline and cerulein treated mice show significant increase in the F4/80 cells after cerulein induced injury at day 7 and day 21 treatment (average count of F4/80-positive cells in five independent fields/tissue section). Quantitative analysis of the α-SMA fibroblasts indicate significant increase starting at day 2 of treatment compared to control (average count of α-SMA positive cells in five independent fields/tissue section). ***** $p<0.05$, ****** $p<0.005$, ns=not significant.

Figure 10

A


Average number of F4/80cells per field
20
15
10
5
0
ns
*
*
Normal KCT Normal KCT Normal KCT Normal KCT
Day 0
Day 2
Day 7
Day 21

B

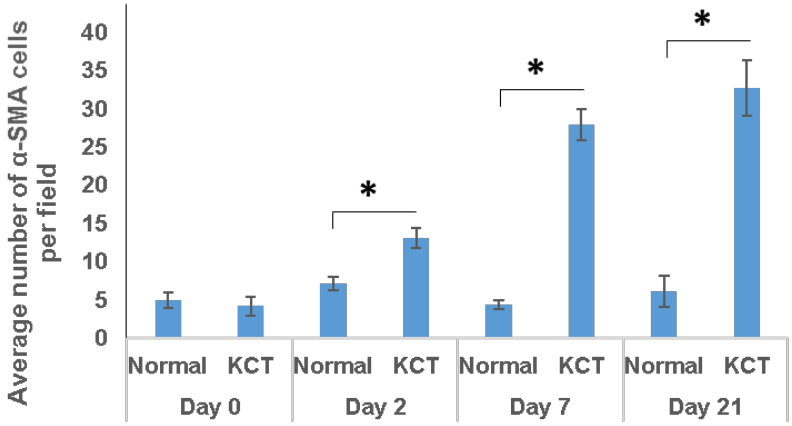

Average number of α-SMA cells per field
40
35
30
25
20
15
10
5
0
*
*
*
Normal KCT Normal KCT Normal KCT Normal KCT
Day 0
Day 2
Day 7
Day 21

Figure 11: Increase infiltration of F4/80 positive macrophages is associated with both ET_AR and ET_BR expression in cerulein induced inflammation: Dual color immunofluorescence images of the ET_AR and ET_BR expression (red) with macrophage marker F4/80 (green) in cerulein and saline treated mice at days 2, 7 and 21 post cerulein administration. **A.** In the control and KC mutant mice under control conditions, expression of ET_AR is restricted to acinar compartment and no overlap is seen with F4/80 positive macrophages. At day 2 post cerulein treatment in KC mice, limited localization of ET_AR is seen (inset) with F4/80 while no difference is seen in normal mice. In KC mice at day 7 and 21 post inflammatory insult, significant overlap of ET_AR is observed with the infiltrating F4/80 populations in the stromal region (inset), while minimal or no overlap is seen in control mice. **B.** The degree of overlap between F4/80 positive macrophages and ET_AR was measured using ImageJ using Manders overlap coefficients. **C.** Similar to ET_AR, acinar specific expression of ET_BR shows no colocalization with F4/80 in normal and KC mice under control conditions. Brief episode of cerulein treatment at day 2 in KC mice shows substantial colocalization with F4/80 populations (inset) with significant p value compared to control mice. In KC mice at day 7 and 21 of cerulein treatment, the ET_BR expression in the metaplastic ducts is accompanied by significant overlap with F4/80 positive macrophages in the stroma, while limited colocalization is seen in the normal mice. **D.** The degree of overlap between F4/80 positive macrophages and ET_BR was measured using ImageJ using Manders overlap coefficients * $p<0.05$, ** $p<0.005$, ns= not significant (Scale bar = 20µm; zoom scale bar =10µm).

Figure 11

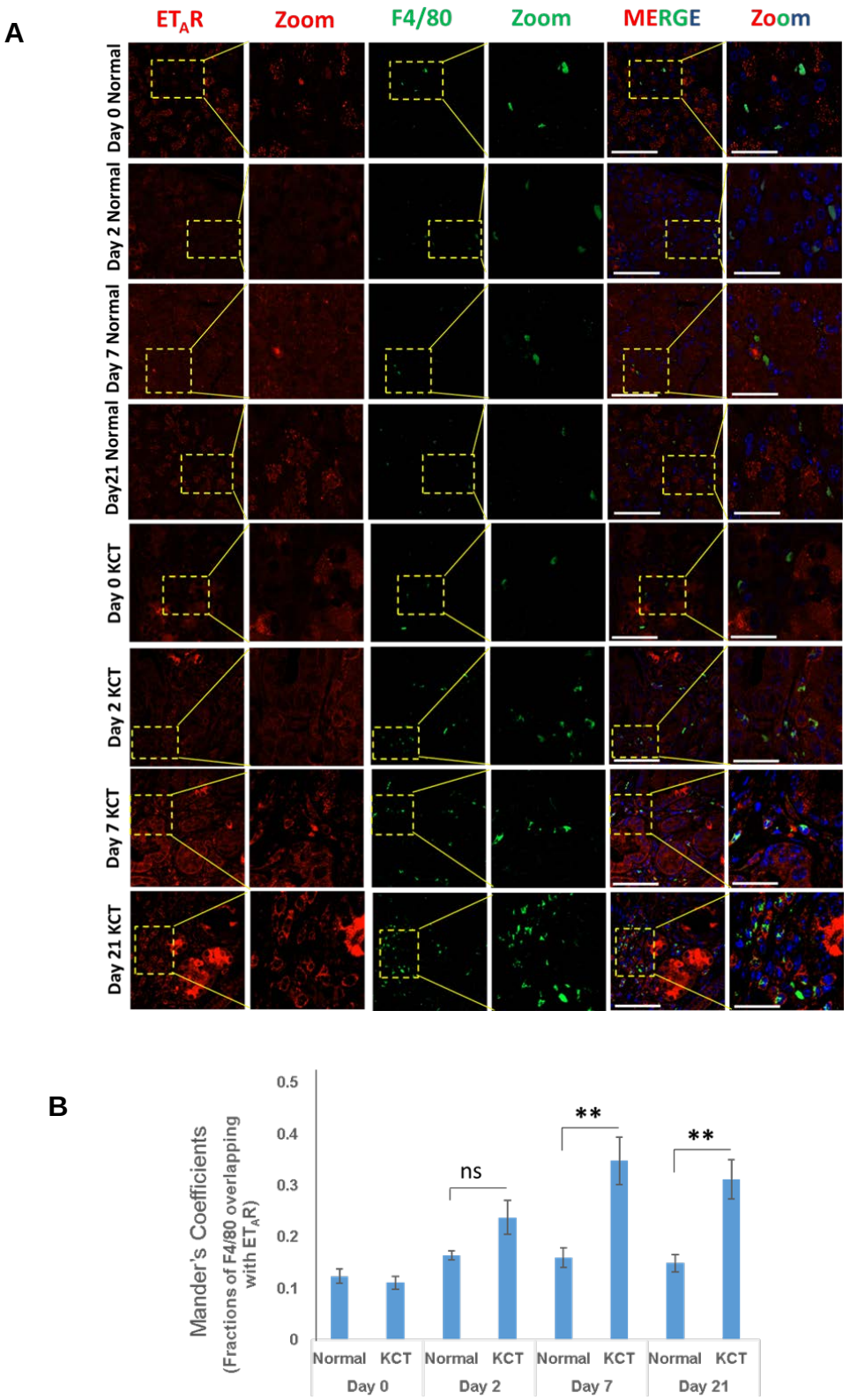

A
ETAR
Zoom
F4/80
Zoom
MERGE
Zoom
Day 0 Normal
Day 2 Normal
Day 7 Normal
Day21 Normal
Day 0 KCT
Day 2 KCT
Day 7 KCT
Day 21 KCT
B
Mander's Coefficients
(Fractions of F4/80 overlapping with ETAR)
0.5
0.4
0.3
0.2
0.1
0
ns
**
**
Normal
KCT
Day 0
Day 2
Day 7
Day 21

Figure 11

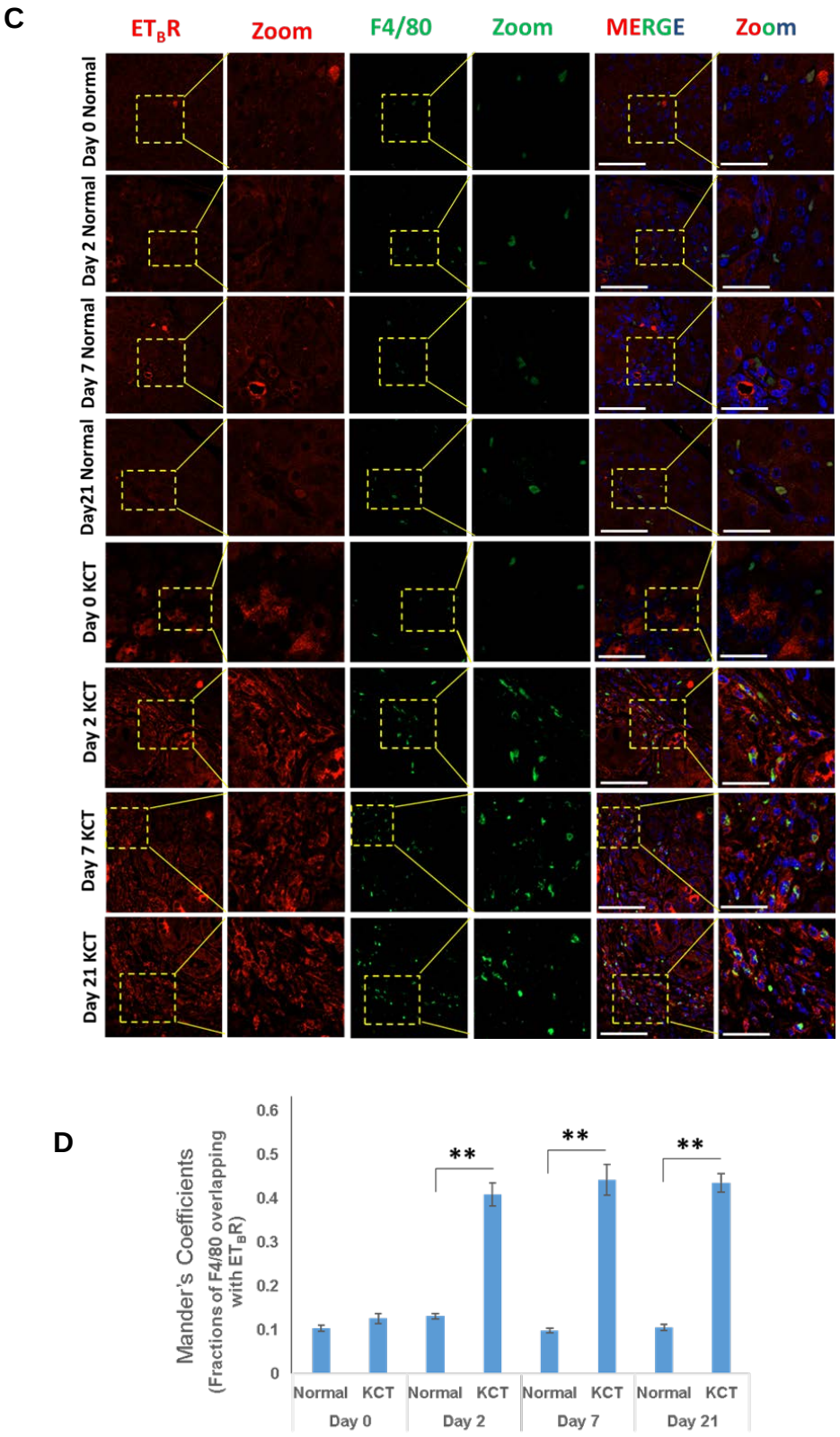
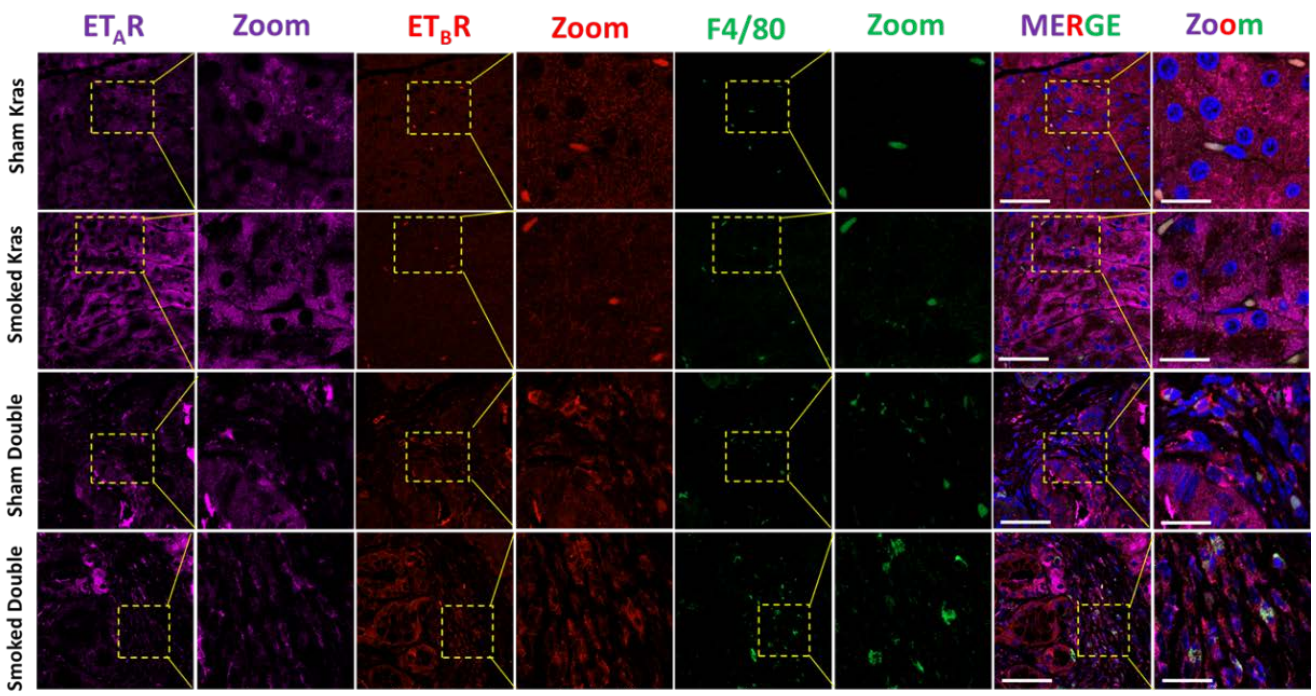

C
ETBR
Zoom
F4/80
Zoom
MERGE
Zoom
Day 0 Normal
Day 2 Normal
Day 7 Normal
Day21 Normal
Day 0 KCT
Day 2 KCT
Day 7 KCT
Day 21 KCT
D
Mander's Coefficients
(Fractions of F4/80 overlapping with ETBR)
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
**
Normal
KCT
Normal
KCT
Normal
KCT
Normal
KCT
Day 0
Day 2
Day 7
Day 21

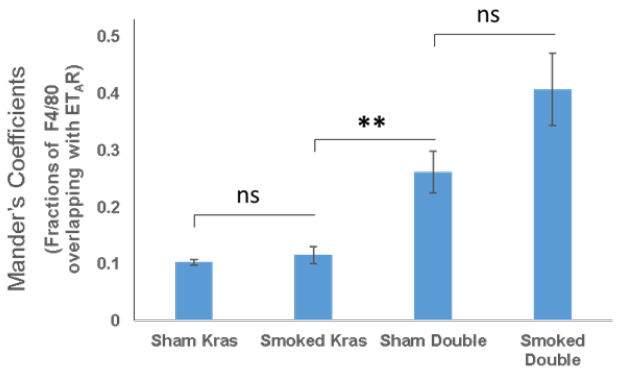
Figure 12: Increase infiltration of F4/80 positive macrophages is associated with ET_BR expression in smoke induced inflammation. Three color immunofluorescence images of the ET_AR (purple), ET_BR (red) and F4/80 (green) expression in the spontaneous KC model with and without exposure to cigarette smoke. **A.** In the unfloxed mice with and without exposure to cigarette smoke exposure expression of both ET receptors is seen in acinar region of pancreas . The pancreas displays fewer infiltration of F4/80 macrophages and exhibits minimal overlap with both ET_AR and ET_BR (zoom). Smoke exposed KC mutant mice exhibits significant colocalization of both ET_BR and F4/80 (zoom) as compared to sham control. **C.** The degree of overlap between ET_AR and F4/80 (zoom) in smoke exposed animals reveals no significant fractions of F4/80 overlapping fractions with ET_AR **(B)** as measured using ImageJ with Manders overlap coefficient * $p<0.05$, ** $p<0.005$, ns= not significant (Scale bar = 20µm; zoom scale bar =10µm).

Figure 12

A



B



C

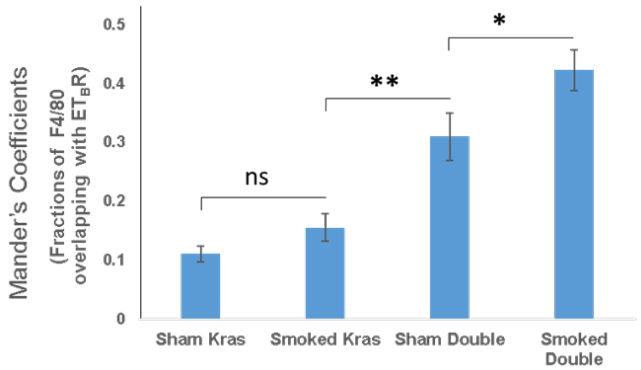
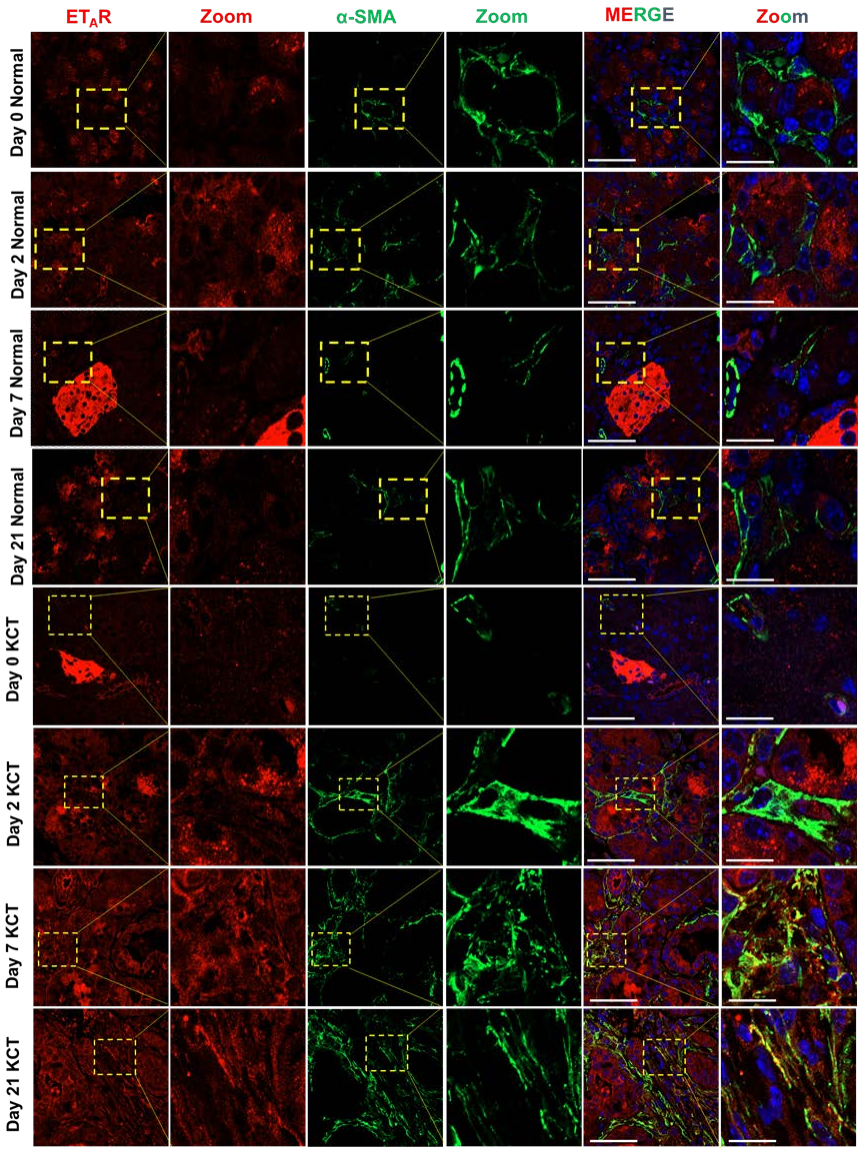


Figure 13: Increase expression of α-SMA positive fibroblasts is associated with both ET_AR and ET_BR expression in cerulein induced inflammation. Dual color immunofluorescence images of the ET_AR and ET_BR expression (red) with α-SMA (myofibroblast marker) (green) in cerulein and saline treated mice at days 2, 7 and 21 post cerulein administration**. A.** In the control and KC mutant mice under control conditions, expression of α- SMA is detected in the mice pancreas and shows limited colocalization with ET_AR. At day 2 post cerulein treatment in KC mice, increase expression of α- SMA is seen in the KC mutant mice as compared to control, however limited colocalization with ET_AR is detected in mice pancreas (zoom). In KC mice with constitutive active Kras mutation, higher expression of α-SMA in the pancreas is associated with significant overlap with ET_AR in the mice pancreas at day 7 and 21 cerulein insult with significant p value. **B.** The degree of overlap between α-SMA positive fibroblasts and ET_AR was measured using ImageJ using Manders overlap coefficients. **C.** Parallel to ET_AR, expression of α-SMA fibroblasts reveals limited co-association with ET_BR in the control and KC mutant mice under control conditions. At day 2 post injection, under cerulein induced metaplastic conversion, the increase number of α-SMA fibroblasts is marginally associated with ET_BR expression. With increase in dysplasia at days 7 and 21 of treatment increase activation of stellate cells and higher expression of α-SMA fibroblasts in pancreas is shows extensive colocalization with ET_BR with significant p value. **D.** The degree of overlap between α-SMA positive fibroblasts and ET_BR was measured using ImageJ using Manders overlap coefficients. ***** $p<0.05$, ****** $p<0.005$, ns= not significant (Scale bar = 20μm; zoom scale bar =10μm).

Figure 13

A


ETAR
Zoom
α-SMA
Zoom
MERGE
Zoom
Day 0 Normal
Day 2 Normal
Day 7 Normal
Day 21 Normal
Day 0 KCT
Day 2 KCT
Day 7 KCT
Day 21 KCT

B

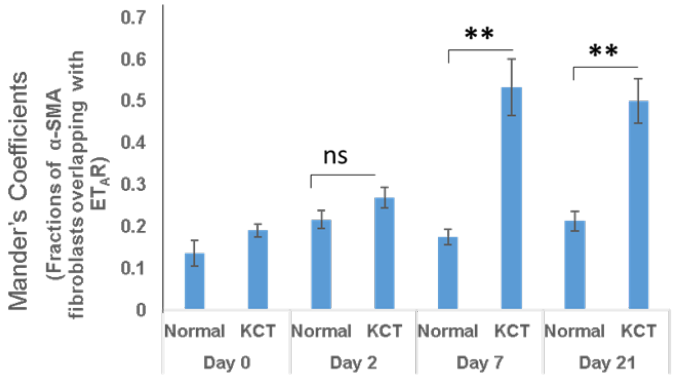
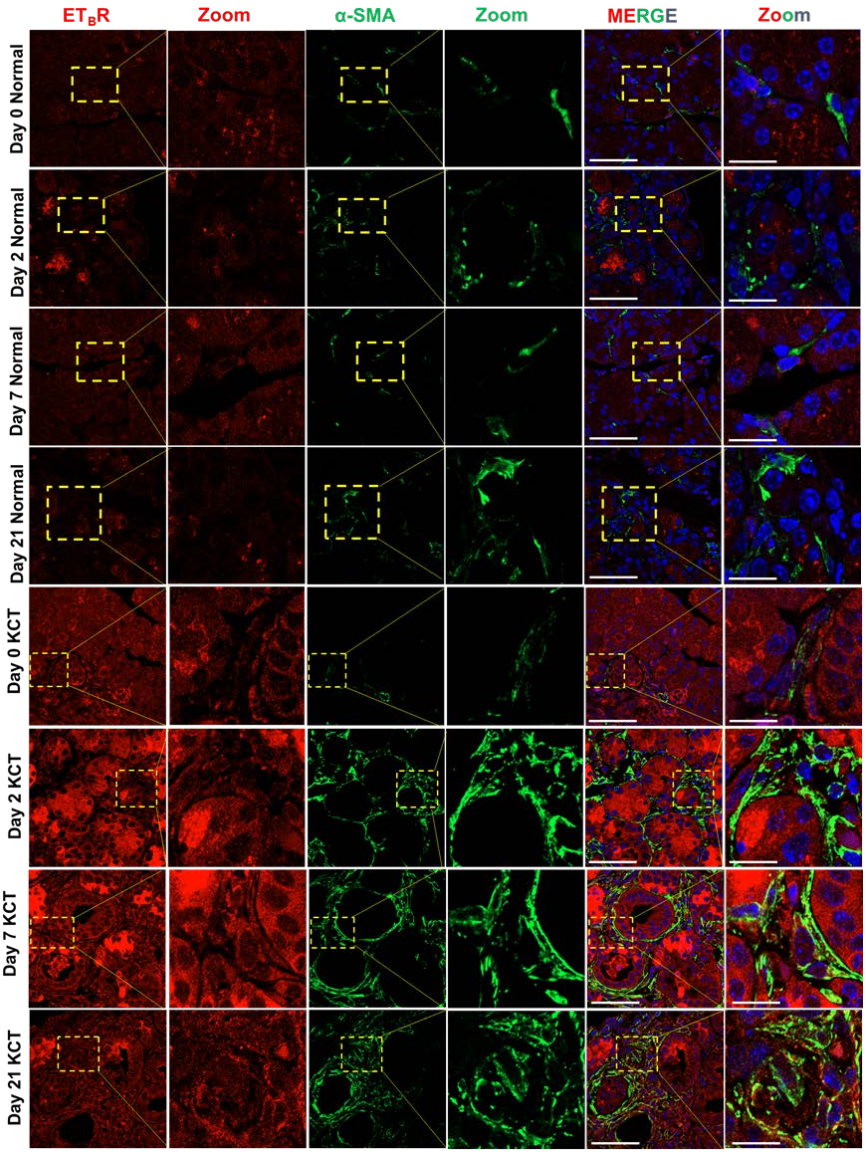

Mander's Coefficients
(Fractions of α-SMA fibroblasts overlapping with ETAR)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ns
**
**
Normal
KCT
Normal
KCT
Normal
KCT
Normal
KCT
Day 0
Day 2
Day 7
Day 21

Figure 13

C


ET$_B$R
Zoom
α-SMA
Zoom
MERGE
Zoom
Day 0 Normal
Day 2 Normal
Day 7 Normal
Day 21 Normal
Day 0 KCT
Day 2 KCT
Day 7 KCT
Day 21 KCT

D

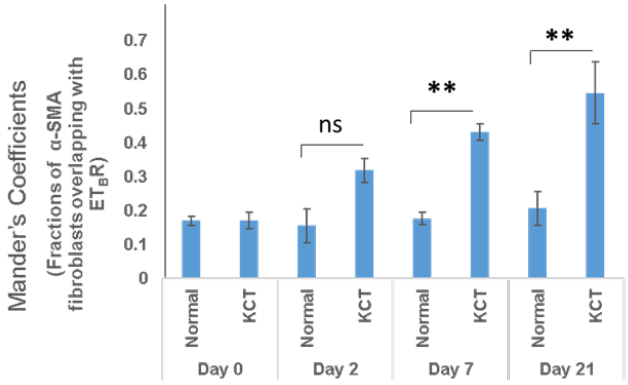

Mander's Coefficients
(Fractions of α-SMA fibroblasts overlapping with ET$_B$R)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
ns
**
**
Normal
KCT
Day 0
Day 2
Day 7
Day 21

Figure 14: Increase expression of α-SMA positive fibroblasts is associated with ET_AR epression in smoke induced inflammation. Dual color immunofluorescence images of the ET_AR and ET_BR staining (red) with α-SMA (myofibroblast marker) staining (green) in smoke exposed and sham control floxed mice. **A.** Immunofluorescence images of the ET_AR and α-SMA colocalization in the pancreas of sham and smoke exposed unfloxed mice reveals no significant difference in the fractions of α-SMA myofibroblasts overlapping with ET_AR. In kras mutant mice exposed to cigarette smoke higher expression of α-SMA in the pancreas of smoke-exposed animals is accompanied by significant colocalization was observed (zoom). **B.** The degree of overlap between α-SMA myofibroblasts and ET_AR was measured using ImageJ using Manders overlap coefficients. **C.** Dual confocal microscopy analysis of ET_BR and α-SMA overlap in the pancreas of sham and smoke exposed unfloxed mice displays no significant overlap between two fractions. Contrary to ET_AR, smoke exposure in KC mutant mice shows no significant co-association between the two populations in the pancreas stromal region as compared to control (zoom). **D.** The degree of overlap between α-SMA myofibroblasts and ET_AR was measured using ImageJ using Manders overlap coefficients ***** $p<0.05$, ****** $p<0.005$, ns= not significant (Scale bar = 20μm; zoom scale bar =10μm).

Figure 14

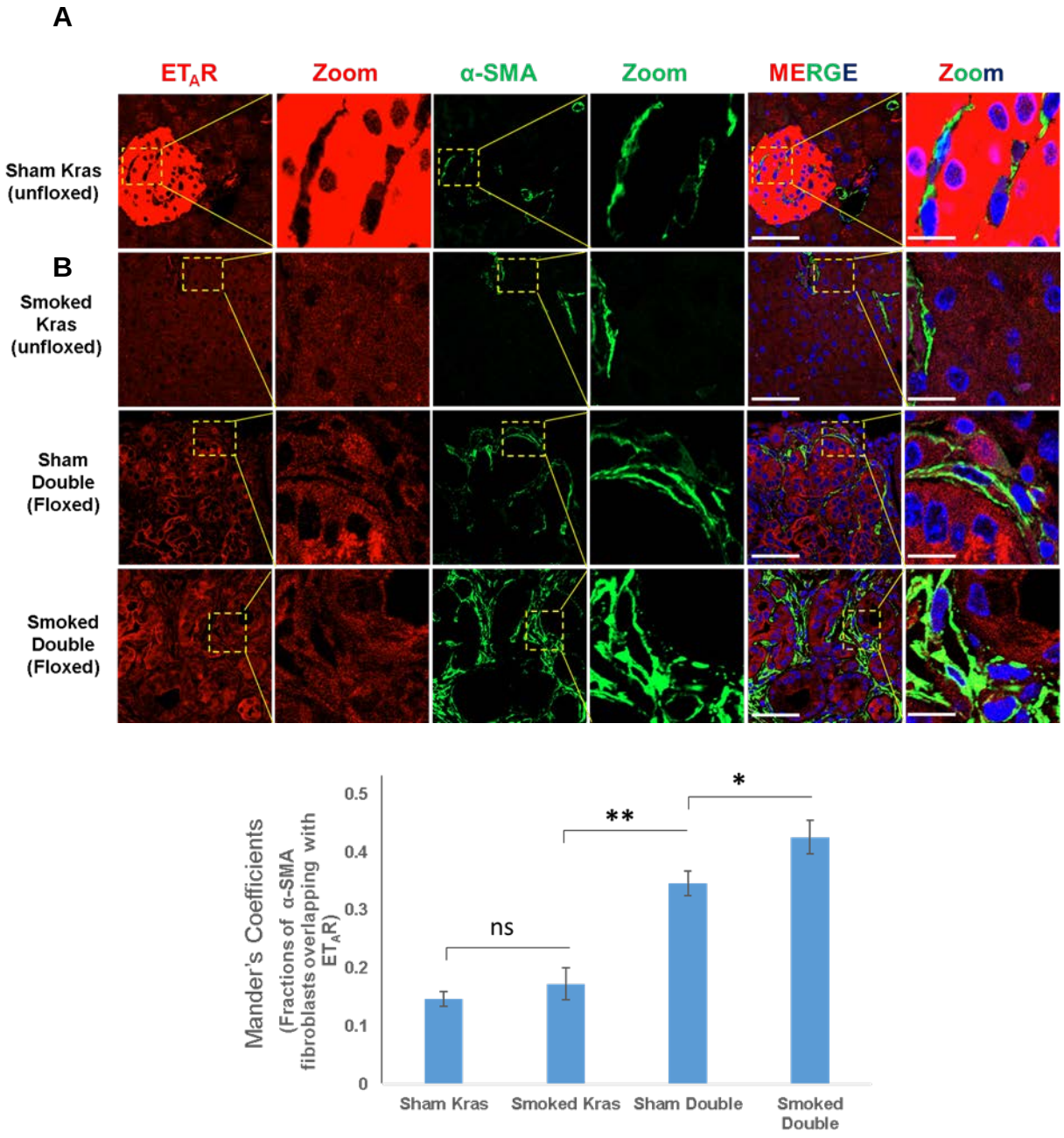

A
ETAR
Zoom
α-SMA
Zoom
MERGE
Zoom
Sham Kras (unfloxed)
B
Smoked Kras (unfloxed)
Sham Double (Floxed)
Smoked Double (Floxed)
Mander's Coefficients (Fractions of α-SMA fibroblasts overlapping with ETAR)
0.5
0.4
0.3
0.2
0.1
0
ns
**
*
Sham Kras
Smoked Kras
Sham Double
Smoked Double

Figure 14

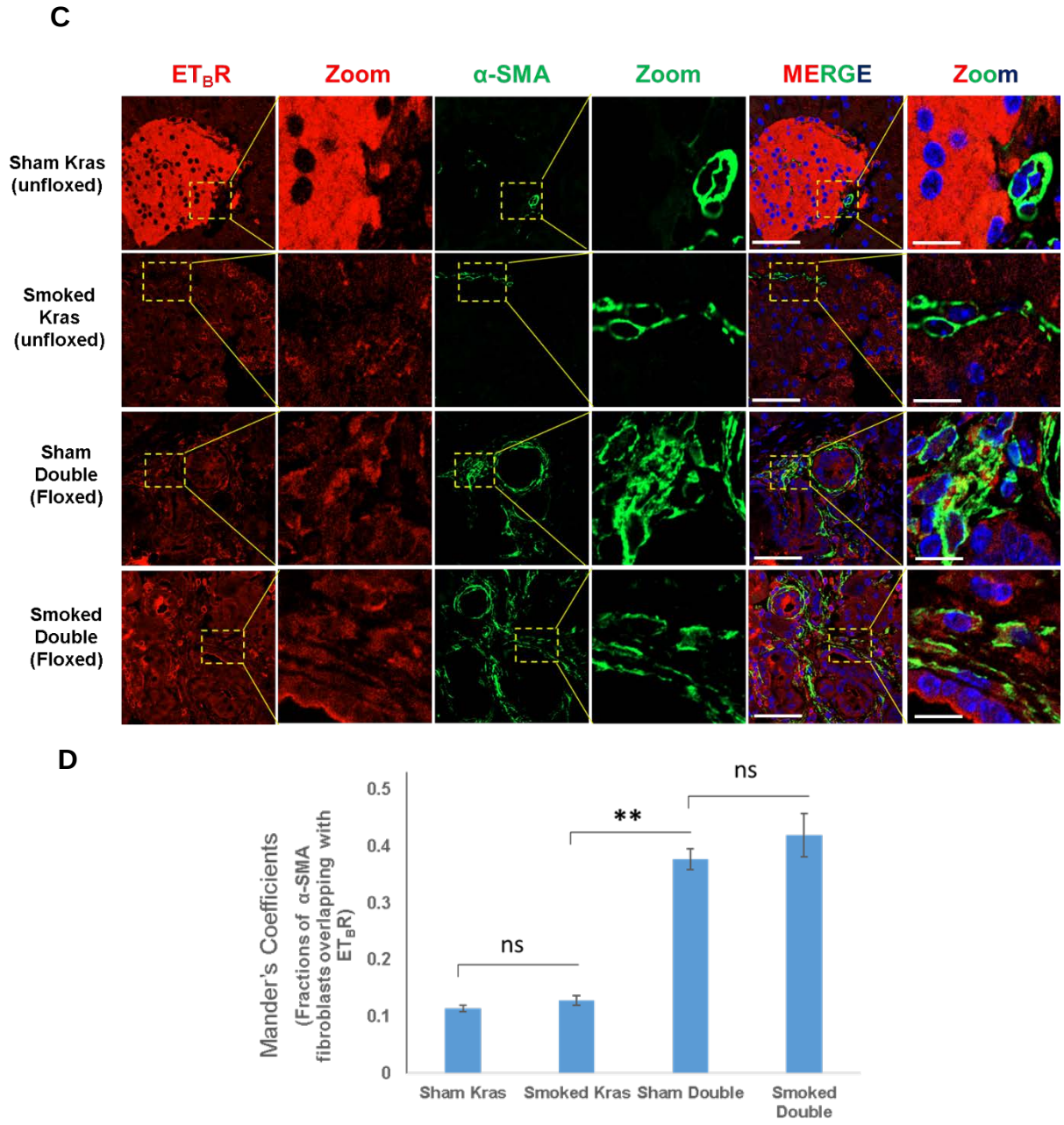
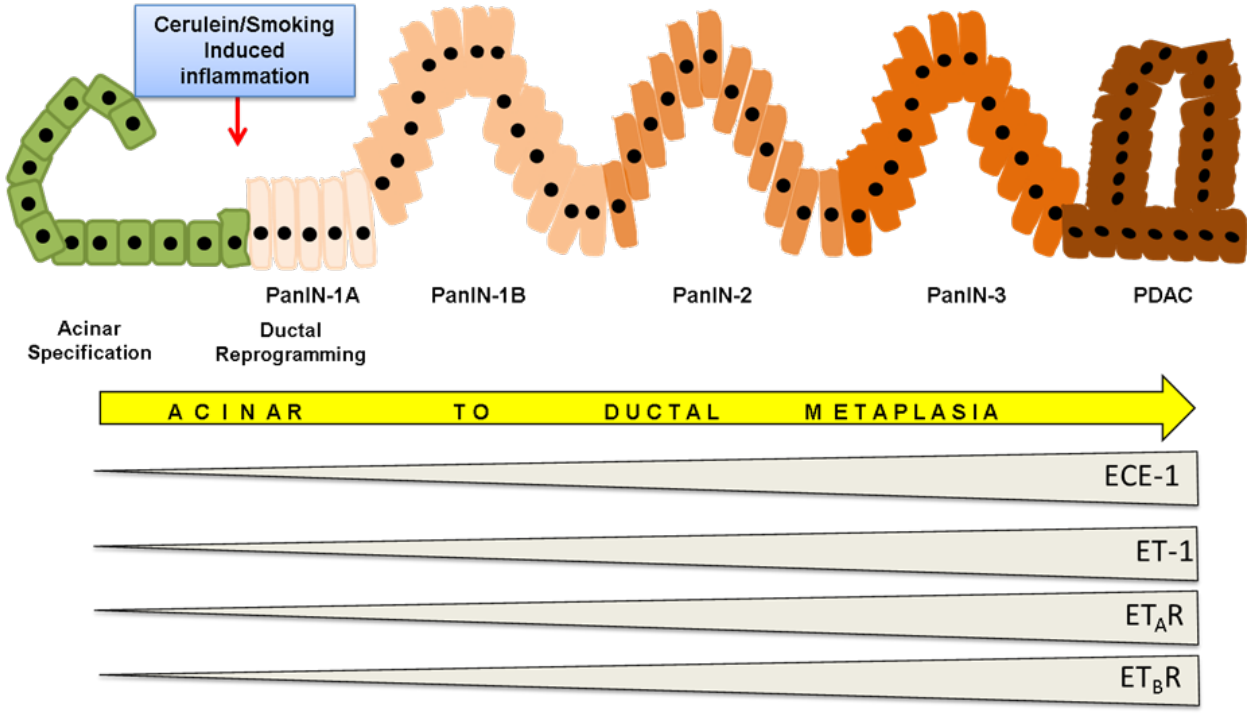

C
ETBR
Zoom
α-SMA
Zoom
MERGE
Zoom
Sham Kras (unfloxed)
Smoked Kras (unfloxed)
Sham Double (Floxed)
Smoked Double (Floxed)
D
Mander's Coefficients (Fractions of α-SMA fibroblasts overlapping with ETBR)
0.5
0.4
0.3
0.2
0.1
0
ns
**
ns
Sham Kras
Smoked Kras
Sham Double
Smoked Double

Figure 15: Proposed Model. In summary, in the normal pancreas, expression of both endothelin receptors (ET_AR and ET_BR) is restricted to pancreatic acinar and islet cells. In presence of mutated Kras (G12D), inflammatory insult (cerulein or smoking) results in transdifferentiation of acinar phenotype into ductal phenotype and favors metaplastic conversion accompanied by downregulation acinar cell marker genes (Amylase) with simultaneous upregulation of ductal markers (CK19). In the Kras mutant mice, the expression of the endothelin axis components is seen in early pre-neoplastic lesions and in tumor microenvironment of PC and increase gradually with increase in neoplasia. The sustained activation of the ET axis in presence of oncogenic Kras both in the tumor cells and in stromal compartment of PC, suggest its possible role in tumor initiation and progression.

Figure 15


Cerulein/Smoking Induced inflammation
PanIN-1A
PanIN-1B
PanIN-2
PanIN-3
PDAC
Acinar Specification
Ductal Reprogramming
ACINAR TO DUCTAL METAPLASIA
ECE-1
ET-1
ETAR
ETBR

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Chapter 6

Summary, Conclusions and Future directions

1. Summary

Over the past several years various studies have attempted to understand the role of endothelin axis in solid cancers and melanomas[1]. In the context of pancreatic cancer a recent study has shown the overexpression of ET-1ligand and endothelin B receptor (ET_BR) but not endothelin A receptor (ET_AR) [2]. However, a number of questions remain unanswered. For instance, what is the expression of overall ET axis as such in the in PC and in early pancreatic lesions? Also, what is the expression status of the axis in the context of complex TME of PC? What is the pathobiological significance of the ET axis proteins? Importantly, what is the impact of targeting the axis in PC microenvironment?

The overarching goal of the studies presented in this thesis was to examine the pleotropic actions of the ET axis in pancreatic tumor microenvironment. Briefly, we examined the following aspects of ET axis expression and function: 1) Expression in tumor cells and stromal compartment, 2) Pathobiological significance of ET axis upregulation in PC 3) Impact of ET-axis antagonists in the murine model of autochthonous tumorigenesis 4) Involvement of ET-axis in K-ras oncogene associated chronic and acute pancreatic inflammation

Overall, our major findings were: 1) Compared to normal pancreas, components of ET axis, ECE-1, ET-1, ET_AR and ET_BR are overexpressed in primary tumor and metastatic sites. 2) ET_AR and ET_BR are expressed in immune cells and tumor blood vessels. The expression of ET_BR on blood vessels is associated with poor prognosis of PC patients 3) It was observed that overexpression of the ET receptors is associated with pro-fibrotic gene signatures, suggesting that signaling along the ET promotes fibrosis in PC 4) Pharmacological inhibition of the axis using dual ET_AR and ET_BR antagonist Bosentan reduces the pro-fibrotic genes in autochthonous mice model. These results suggest that targeting this axis can possibly modulate the complex TME of PC. 5)

In stellate cells and cancer-associated fibroblasts, exogenous administration of ET-1 induces expression of stromal associated genes in an ERK/AKT dependent manner. We are generating mouse model to determine the effect of ET-1 loss on pancreatic cancer progression. The mice will be analyzed further to determine the loss of ET-1 in Kras/p53 driven PC in mice. Below, I summarize the findings of each project and implications thereof.

A. Expression of endothelin converting enzyme (ECE-1), endothelin-1 (ET-1), endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR) in PC and its microenvironment

The rationale for this study was based on earlier scattered evidence about the expression pattern of ET axis in PC, including a very recent study, which reported the ET-1 and ET_BR expression in PC patients [2] . First, we examined the expression of ECE-1, ET-1 and both ET receptors in PC cell lines and PC tissues. We observed that ET-1 expression is seen in majority of cell lines tested, however both ET_AR and ET_BR showed an inverse association, either of one expressing at a time with few exceptions. Immunohistochemical analysis in PC tissues isolated from Whipple resected patients (RAP) and tissue microarray from rapid autopsy program suggests overexpression both in the primary tumor as well metastatic sites. The incidence of ECE-1, ET-1, ET_AR and ET_BR was 88%, 86%, 75% and 68% cases respectively in the primary tumors. Our tissue analysis also revealed expression of the ET-1 and its receptors in the stromal region of the tissues studied. The expression was predominantly seen in the infiltrating immune cells and the blood vessels. Further scoring of the slides by a pathologist implied elevated expression of ET_AR and ET_BR on tumor vessels in 60.5% and 31.5% cases respectively. In the immune cells, ET- 1 expression was seen in31.5% of cases while the incidence of ET_AR and ET_BR expression was 34.1% and 39.4% ,respectively. Very importantly, survival analysis from surgically resected primary tumors suggested

that elevated expression of ET_BR on blood vessels and not tumor cells was associated with poor prognosis in these patients. The median survival of the patients with elevated expression for ET_BR was 14.7 months as compared to 10.3 months with low ET_BR positivity, indicating that looking at the expression in the tumor microenvironment is also essential to delineate the pathological significance of the axis. Due to limitation in the sample size of the Whipple and RAP cases we screened the TCGA database and expression of ET-1 and receptors was correlated with tumor grade and stage. To further demonstrate the pathobiological implication of axis we did bioinformatics study to look for the genes that correlated with ET axis overexpression. Interestingly, significant correlations of the fibrotic associated genes, predominantly collagen isoforms, FAP, PDGRFβ, SPARC were seen. In addition, regulatory pathways involved in fibrosis, tumor growth and metastasis were also upregulated, suggesting that overexpression of ET axis correlates with the extracellular matrix associated gene signatures.

One of our long-term quests with this study was to develop approaches to selectively modulate the TME to improve the therapeutic efficacy of the chemotherapeutic regimens. So to explore the potential of targeting this ET axis we also analyzed the expression in KPC model of PC.

Our RT-PCR and immunohistochemical result indicated progressive increase in the expression of ET axis components in advanced lesions at 25 weeks of age. Similar to human PC tissues, the expression of axis components is also seen in tumor stroma. The expression in various compartments of TME was confirmed using dual immunofluorescence studies using markers for stellate cells (α-SMA), blood vessels (CD31) and tumor associated macrophages (CD68 and F4/80). These observations indicate that mouse pancreas recapitulates the expression pattern in human PC.

Taken all together, the study for the first time established the prognostic potential of ET axis in PC. The expression on TME compartments and correlation with pro-fibrotic

genes further establishes the potential of axis as a “druggable” target that can be exploited in PC therapy.

B. Targeting endothelin axis in pancreatic cancer using selective and dual receptor inhibitors under *in vivo* (KPC, K-ras^{G12D}; Trp53$^{R172H/+}$; Pdx-1-Cre) and *in vitro* (pancreatic stellate cells and cancer associated fibroblasts) system

Our aforementioned expression and bioinformatics analysis data suggested that ET receptors are expressed in TME and exhibit significant correlation with the pro-fibrotic gene signatures. Due to the expression in of ET receptors on various cell types in TME, the ET axis possibly exerts pleotropic effects to modulate the pathophysiological hallmarks of PC such as fibrosis and hypo-vascularity [3]. Given the attraction of this pathway as a “druggable” target and the availability of both selective and dual inhibitors for ET receptors, an overarching goal was to unravel the pathobiological significance of ET-axis PC in the context of TME and define the manner in which the ET axis antagonism can be exploited in PC therapy.

Having established an apparent correlation between fibrotic gens and ET axis we investigated the consequences of targeting ET axis *in vivo* with Bosentan, a dual ET receptor antagonist. Bosentan is a FDA approved drug and is firmly established in the clinics for the treatment of pulmonary arterial hypertension (PAH) and chronic heart diseases [4, 5]. We utilized the 20-week KPC animal (widely used model for PC) where the tumor is fully formed and abundant stroma outnumbers the cancer cells. Bosentan given as a monotherapy increases apoptosis and decreases desmoplasia in KPC tumors evident by IHC analysis against cleaved caspase-3 and Masson’s trichrome staining respectively. Further using mouse fibrosis array, we observed Bosentan treatment induced significant reduction in the profibrogenic gene signatures which were concordant with the genes found to associated with ET-axis upregulation in TCGA database analysis. This led us to conclude that ET axis promotes desmoplasia in PC

and ET axis antagonist's exhibit anti-fibrotic effects. In addition to pro-fibrotic genes, dual ET axis antagonism also resulted in marginal increase in in anti-fibrotic genes such as IL-10, Bmp 7 and IFN-γ. The next step was to identify the specific population(s) of cancer-associated fibroblasts that are affected by ET axis antagonism. Immunofluorescence analysis confirmed that Bosentan treatment reduces the FSP1 and α-SMA positive fibroblasts but not FAP positive fibroblasts.

The next step was to validate ET axis mediated fibrogenic effects *in vitro*. It was observed that treatment of murine pancreatic stellate cells (ImPSC.c2) and human cancer associated fibroblasts (10-03) with ET-1, resulted in increased expression of pro-fibrotic genes (α-SMA, Collagen I, CTGF, Fibronectin) in time dependent manner. To identify the ET receptor exerting this effect, we used selective inhibitor for ET_AR (BQ123) and ET_BR (BQ788). ET-1 mediated induction of matrix associated genes was attenuated by BQ788 whereas minimal effects were observed with BQ123. A synergistic effect was seen using dual ET_AR and ET_BR inhibition using Bosentan. In contrast, in human CAFs, both BQ123 and BQ788 abrogated ET-1 induced expression. One speculation could be the distribution of ET_AR on murine stellate cells. The ImPSC.c2 expresses high level of ET_BR and low level of ET_AR, while 10-03 CAFs expresses both.

We next investigate the molecular mechanism(s) underneath the ET-1 stimulated fibrosis. Immunoblotting analysis confirmed that ET-1 treatment provokes a rapid phosphorylation of the p-ERK and p-AKT in both ImPSC.c2 and 10-03 CAFs. This is in line with the literature, which suggests that in rat PSCs and lung fibroblasts ET-1 stimulate phosphorylation of ERK/MEK pathway [6] [7]. In conclusion, our *in vivo* and *in vitro* findings demonstrates that ET-1 release from tumor cells act on acts on stellate cells and CAFs and through and ERK/AKT dependent manner induces pro-fibrotic phenotype and promotes extensive desmoplastic reaction in PC. The second part of the study dealt with the functional effects of ET inhibitors on these cells. It was observed that

both BQ788 and Bosentan displayed anti-proliferative effects on the growth of ImPSC.c2 and 10-03 CAFs, with minimal effects were observed with BQ123. Further, flow cytometry studies indicate that both BQ788 and Bosentan stimulate apoptosis and G1/S arrest in ImPSC.c2 cells. Additionally, we observed that ET-1 promotes migration of ImPSC.c2 cells which was inhibited by selective and dual ET antagonists.

Given the significance of infiltration of immune cells in PC progression [8] [9], we examined the consequence to ET axis inhibition on the infiltration of immune cells. Tissue immunohistochemistry and immunofluorescence analysis showed reduction in F4/80 positive macrophages and increase in the recruitment of cytotoxic T cells in the KPC mice stroma upon Bosentan treatment. In addition, our *in vitro* studies also demonstrated that the migration of RAW264.7 macrophages and U937 monocytic cells in response to murine and human pancreatic cancer cell lines respectively, was abrogated by Bosentan and ET_BR antagonist. .

In addition to fibrosis or desmoplasia, tumor hypovascularity and heterogenous tumor blood flow is also one of the hallmarks of PC. Due to increased production of ET-1 and overexpression of ET_AR, the vasomodulatory activity of ET axis is believed to contribute to tumor blood flow heterogeneity by selectively modulating the tone of vessels. Thus, ET_AR antagonists, in addition to their direct anti-tumor effects also exhibit an adjuvant effect that enhances tumor perfusion and increases drug uptake [10]. We studied the effect of selective ET_AR antagonist BQ123 on subcutaneous xenograft tumors derived from human pancreatic cancer cell lines. The change in perfusion was studied by MRI using flow sensitive alternating inversion recovery (FAIR). Baseline perfusion was higher in muscles as compared to tumors, however, the chnages in perfusion in response to BQ123 were more pronounced in tumors than in the muscle where only marginal increase in perfusion was observed. We also found that enhanced perfusion induced by BQ123 reduces tumor hypoxia in xenograft tumors.

Overall, these results demonstrate for the first time, the unequivoal role of ET axis in establishing desmoplastic, poorly perfused and possibly immunosuppresive micrenvironement in PC. These studies also suggest that it is possible to selectively modulate the determinants of therapy resistance (tumor stroma and vasculature) that contribute to poor drug delivery by targeting ET axis. suggests.

C. Irreversible and sustained upregulation of endothelin axis during K-ras-oncogene associated pancreatic inflammation and cancer.

Inflammation is believed to promote tumorigenesis and chronic pancreatitis and smoking (causes chronic pancreatic inflammation) are well recognized risk factors for PC. Given the potential involvement of ET-1 ligand in promoting pancreatic inflammation [11] and the fact that several studies indicating elevated ET-1 levels during acute and chronic pancreatitis [12-14], we examined the role of ET axis in the pancreatic inflammations (acute and chronic) in presence and absence of oncogenic Kras and in pre-neoplastic lesions. No study thus far has examined the expression of ET axis proteins in acute and chronic pancreatic inflammation in the presence of K ras oncogene. Thus the goal of this part of the study the role of ET axis in inflammation associated pancreatic cancer initiation and progression in presence of mutated $Kras^{G12D}$.

As a part of this project we have utilized murine models of acute (cerulean-induced) and chronic (smoking-induced) pancreatic inflammation described previously by our lab [15] [16]. We first analyzed the expression of ET axis components in the KC model (Pdx-1 Cre; KrasG12D) of the PC. Similar to our observations in KPC model, there was a progressive increase in the expression of ET axis components with the advancement of preneoplastic lesions. Further, careful analysis of the tissues revealed that the expression was not only restricted to be lesions but can also be detected in the surrounding stromal compartment and inflammatory cells. In cerulein induced pancreatic injury in $Kras^{G12D}$ mutant mice, metaplastic ducts can be seen suggesting acinar-to-

ductal transdifferentiation. Both ET_AR and ET_BR were found to be expressed in such metaplastic ducts that stained positively for amylase (acinar marker) and CK19 (ductal marker); however, no change in normal mice was observed. At day 7 and day 21-post-cerulein administration, expression of both ET receptors was more pronounced in CK19 positive ducts with increase in dysplasia accompanied by loss in amylase staining. In addition, real time PCR analysis of ET-1 and ET_AR mRNA levels in KC mice showed significant increase in transcript levels at day 7 and day 21-post trauma. In contrast, ET_BR mRNA levels showed increase at day 2 and day 7-post cerulein injection; however; at day 21 a fall in expression was seen. In contrast to KC mice, the wild-type mice exhibited restoration of the pancreatic acinar architecture and pancreatic parenchyma and recovery of transcripts of ET-axis components to basal level at day 7 and 21.

Cigarette smoke induced alterations in the ET axis in the pancreas of KC mice harboring $Kras^{G12D}$ mutation. There was significant increase in the transcripts of ET-1, ET_AR and ET_BR compared to sham control. Similar to our observations in spontaneous KC model, in the mice pancreas with and without exposure to cigarette smoke, the expression of ECE-1, ET-1 and both ET receptors was associated with acinar compartment of the pancreas and prominent staining in islet cells was noticable for all four molecules. In sham control mice prominent expression of ECE-1, ligand ET-1 and the receptors was observed in the pre-cancerous lesions and acinar region of the pancreas.

In addition to expression in early pancreatic cancer lesions (smoking) and metaplastic ducts (cerulein), ET_AR and ET_BR expression was also seen in infiltrating F4/80 positive macrophages and α-SMA positive fibroblasts. Tissue immunofluorescence analysis demonstrated that both ET_AR and ET_BR are expressed in F4/80 positive macrophages in KC mutant mice in response to smoke and cerulein induced injury. Quantitative analysis

using ImageJ software further indicated significantly higher number of F4/80 positive cells expressing ET_AR and ET_BR as determined by Mander's correlation coefficient. Dual color immunofluorescence analysis revealed enhanced expression of both ET_AR and ET_BR in the fibroblasts at day 7 and day 21-post cerulein administration. Importantly, quantitative analysis of the smoke exposed double transgenic KC mice showed increased co-expression of ET_AR and not ET_BR in α-SMA positive fibroblasts.

In conclusion, in the presence of oncogenic Kras, acute and chronic inflammation resulted in irreversible reprogramming of acinar phenotype into metaplastic ducts and accelerated progression of pre-neoplastic lesions respectively, along with increased and persistent elevation of ET axis components. These observations suggest a possible role of ET-axis in the neoplastic transformation and early stages of pancreatic cancer.

2. Future directions

A. Expression of endothelin converting enzyme (ECE-1), endothelin-1 (ET-1), endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR) in pancreatic cancer (PC) and its microenvironment

(i) Could loss of ET-2 and ET-3 in cancer development contribute to ET-1 overexpression and circumvent competition with ET-1 for its receptors?

While out studies in PC provide evidence supporting the involvement of ET-1 and ET receptors in pancreatic cancer, these studies raise number of questions. For instance, numerous studies have stated silencing of ET-2 and ET-2 in early cancer development. For example in colon cancer, ET-2 and ET-3 are potential targets for epigenetic inactivation [17]. In addition, Espinosa *et al* and Sun *et al* revealed that expression of ET-3 was down regulated in cervical cancer [18] [19] and levels are decreased in cervical cancer cells as compared to normal epithelial cells, respectively [20]. In breast cancer as well, frequent inactivation of ET-3 is observed due to promoter

methylation in cell lines and carcinoma tissues [21]. Earlier studies in PC also revealed that ET-1 is frequently produced by PC cell lines but not ET-2 and ET-3 [22]. Given that epigenetic modification of EDN2 and EDN3 favor the overexpression of EDN1 gene potentially causing the aberrant activation of ET axis, it will be of interest to study the interrelationship of the three ET ligands in the context of pathophysiology of PC and pancreatitis. A critical question that remains unanswered is: what mechanism(s) and factors contrinbute to deregulated expression of ET-axis components? Also, whether the loss of EDN2 and EDN3 is associated with adverse patient outcome in human PC? These tantalizing questions could be addressed by future studies.

(ii) Do ET-2 and ET-3 play a role in PC?

Our analyses on the expression pattern of ET axis components revealed their presence in various cellular compartments of tumor microenvironment. , We observed the expression of ECE-1 enzyme, ligand ET-1 and both ET receptors on pancreatic stellate cells, endothelial cell and immune cells. Given that ET-2, like ET-1, enhances the invasive and metastatic potential of tumor cells when co-cultured in presence of macrophages [23] it will be of interest to examine the effect of ET-2 on cancer and stromal cells. Also, ET-2 is a potent chemo-attractant for macrophages and leads to their increased activation via ET_BR signaling [24], suggesting a reciprocal cross talk between the tumor cells and the macrophages along ET-2-ET_BR axis. One possible explanation is that autocrine and paracrine signaling across the ET-2/ET_AR or ET-2/ET_BR signaling between tumor cells and tumor microenvironment components might contribute to tumor aggressiveness and metastasis. However, these ideas are purely speculative and need to be verified. As already mentioned in chapter 3, significant expression of ET-1 is seen in TME of pancreatic cancer, it is likely that ET-2 and ET-3 are also expressed on these cell types and further contribute to permissive tumor milieu. These aspects can be addressed by future studies.

B. Targeting endothelin axis in pancreatic cancer using selective and dual receptor inhibitors under *in vivo* (KPC, K-ras^{G12D}; Trp53$^{R172H/+}$; Pdx-1-Cre) and *in vitro* (pancreatic stellate cells and cancer associated fibroblasts) system

(i) Will ET_AR and ET_BR inhibition improve the delivery and efficacy of chemotherapeutic agents ?

Earlier studies have shown that targeting stromal components like hyaluronan and collagen using collagenase and hyaluronidase [25-27] decrease both interstitial fluid pressue (IFP) and microvascular pressure (MVP). resulting in enahancement in delivery and efficacy of Gemcitabine (Gem) in KPC mouse models. However, both collagenase and hyaluronidase lack specificity and cannot distinguish their targets in normal and cancer tissues and would lead to harmful side effects if administered in patients [28] thus, limiting their use in clinical settings**.** Similarly, inhibition of SHh signalling has been shown to increase uptake and efficacy of Gem in KPC mouse tumors [29]. Yet targeting TME in PC has recently become controversial due to the failure of clinical trials and preclinical studies demonstrating that genetic ablation of stroma results in more agrresive tumors [30] [31]. Our studies using dual ET_AR and ET_BR antagonist, Bosentan represents more tunable pharmacological approach that selectively modulates desmoplsia (not altogether abrogates) in the KPC mice model.

In addition our studies on xenografts tumors also demonstrate that ET-1/ET_AR axis contributes to heterogenous blood flow and selective ET_AR antagonist BQ123 enhances perfusion in xenograft tumors and reduces tumor hypoxia. Hence selective modulation of TME particularly blood vessels and extracellular matrix can not only improve the delivery of macromolecule (Abraxane) or small molecule (Gemctabine) into tumors but also improve the the sensitivity of tumor cells to cytotoxic effects of Gemcitabine. In future we will be interested in determining the effect of targeting ET_AR alone (BQ123) and both ET_AR and ET_BR (Bosentan) on the delivery and distribution of

chemotherapeutic agents in spontaneous tumor models of PC. However, these ideas are purely hypothetical and the effect of ET antagonists on the uptake and distribution of Gemcitabine *in vivo* can be addressed by future studies.

(ii) Will ET_AR and ET_BR inhibition enhance the therapeutic efficacy of chemotherapeutic agents?

Our expression analysis of ET-axis indicated that in addition to the stromal compartment, tumor cells also express ET_AR and $E_{TB}R$. Further, inhibition of ET-axis in autochthonous tumors (in the absence of any chemotherapeutic agent) resulted in the apoptosis of tumor cells. Studies have demonstrated that activation of ET_AR in tumor cells promotes proliferation, and cell survival (anti-apoptotic signals) by activating different signaling pathways including MAPK, PKC, EGFR and Akt [32]. Thus, it is likely that ET axis antagonism can have direct anti-tumor effects and synergistically enhance the efficacy of other chemotherapeutic drugs. Studies have demonstrated that pancreatic stellate cells promote therapy resistance in cancer cells and assays involving co-culture of both cell types provide a more accurate prediction of therapeutic response in vivo [33].

In future, we will be interested in investigating the molecular mechanisms by which ET-axis contribute to PSC-mediated chemoresistance in PC? Does ET axis activate pathways of cancer stem cells (CSCs) enrichment and maintenance to drive chemo resistance? These questions can be addressed by using knockdown, overexpression and inhibitor based studies in using 3D co-culture and organoid models.

(iii) Does the cellular cross talk between tumor and stellate cell along ET-axis contribute to desmoplastic tumor microenvironment in PC?

Our preliminary studies indicated that inhibition of ET- axis by Bosentan reduces desmoplasia. To elucidate the underlying mechanism, it is important to understand the cellular cross talk between cancer and stellate cells. In vitro system allows for the

analysis of cell-cell interaction in a controlled environment without the interference from other components. Our results indicated that ET_AR is expressed on tumor cells while pancreatic stellate cells predominantly expressed ET_BR and low levels of ET_AR. In our studies we performed limited studies *in vitro* studies using immortalized pancreatic stellate cells to elucidate the signaling network via which ET-axis regulates profibrotic genes. However, these studies were perfumed using monoculture approaches and do provide intricacies of cellular cross-talk between cancer cells and pancreatic stellate cells. Elucidating the mechanisms and significance of the cellular cross talk along ET-axis would entail co-culture studies in the presence of specific ET-receptor antagonist to define the predominant receptor involved in the cellular cross talk. It will be more informative if such in vitro studies are undertaken using approaches that closely recapitulate in vivo environment. For example, 2D- cultures in the presence of extracellular matrix proteins, 3D culture in scaffolds or mixed organoid cultures involving cancer cells and immortalized pancreatic stellate cells can be used. It will be of interest to determine: a) which signaling pathways are specifically regulated by ET_AR and ET_BR? b) Is there a functional overlap between the two ET-receptors? c) What specific genes are regulated by ET axis and what is the pathobiological significance of these genes? d) How does this cellular cross talk contribute to the survival, proliferation, migration and aggressiveness of tumor cells?

(iv) What are the molecular mechanisms that govern the infiltration of macrophages via ET axis?

Our *in vitro* studies indicated that U937 cells express high levels of ET_BR at all stages of differentiation and ET_BR signaling possibly contributes to monocyte/macrophage migration and recruitment in TME. Previous reports indicated that ET_BR on macrophage surface has been shown to promote the recruitment of

macrophages in breast cancer [24] while ET_AR was shown to be involved in the tumor cells and macrophage recruitment during metastatic colonization of lungs in bladder cancer [34]. While we observed that the tumor cell-induced migration of RAW 264.7 and U937 cells was abrogated by Bosentan and BQ123, our studies did not provide information about the underlying mechanisms. It will be of interest to define the cellular mechanisms by which ET-receptors contribute to macrophage migration and define the downstream signaling along ET axis. It will be equally important to determine the impact of ET-axis in regulating the chemotactic molecules (chemokines and cytokines) in both cancer cells and macrophages. Another important question to address would be to define what role ET-axis plays in TAM differentiation and polarization. Such studies can be undertaken using bone marrow derived macrophages isolated from conditional knockout mice for ET-receptors and studying their differentiation, polarization and migration in the presence of tumor cells.

(v) Does endothelin axis play a role in Immunomodulation in PC?

In addition to high desmoplasia and heterogeneous tumor vasculature in PC extreme immunosuppressive microenvironment, also pose a challenge in successful immunotherapy. Our data suggests that pharmacological inhibition of the ET axis using Bosentan not only reduces the stromal growth in KPC mice tumors but also affects the infiltration of immune cells in PC stroma. ET axis antagonism resulted in a significant decrease F4/80 positive and CD206 positive tumor associated macrophages (TAMs). Like macrophages, solid tumors are spontaneously infiltrated by T cells and trafficking through lymphoid organs is tightly regulated by endothelium. In human ovarian tumors, it has been shown that endothelial permeability to cytotoxic T lymphocytes cells is negatively associated with ET_BR overexpression on endothelial cells, and correlates with

poor prognosis [46]. In response to Bosentan treatment in KPC mice, we observed an increased accumulation of CD8 T cells, while the number of CD4 T cells remained unchanged, suggesting a similar role of ET-axis in T-cell recruitment in PC. These observations further raise the possibility of the involvement of ET-axis in immunomodulation of pancreatic TME. To understand the significance of ET-axis upregulation in the context of tumor immune environment, we performed bioinformatics analysis on TCGA database using Immuno Quant tool developed at Harvard Medical School (by Dr, Manoj Bhasin). This tool utilizes gene signature patterns associated with the differentiation, trafficking, and polarization of immune cells thereby, allowing virtual immunophenotyping of the tumor. These analyses indicated that tumors with high expression of ET-1, ET_AR and ET_BR were enriched in gene signatures associated with elevated levels of immunosuppressive myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg), regulatory B cells (Bregs) and TAMs **(Figure 1)**. The data suggests that ET-axis upregulation possibly contributes to establishing an immunosuppressive microenvironment in PDAC. Our experimental observations indicating reduced TAMs and increased CTLs following Bosentan treatment in KPC mice, align well this predicted role of ET axis. As I mentioned earlier **(Chapter 3)**, we observed that elevated ET_BR positivity on blood vessels is correlated with poor prognosis of the PC patients. Also, a recent report in patients with malignant gliomas also suggested that the increase expression of ET_BR on blood vessels interfere with homing of cytotoxic T lymphocytes and exhibit more infiltration of Tregs around the tumor [47] suggesting a potential role of ET_BR in maintaining the immunosuppressive microenvironment. We performed a preliminary analysis of the abundance of T-regs using dual immunofluorescence staining for CD4 and FoxP3 (master regulator of Tregs) in samples from patients with high and low levels of ET_BR expression on tumor blood vessels. Our data indicated that the tumors of patients, with elevated expression of ET_BR

in blood vessels have higher proportion of Tregs as compared to those with basal level of blood vessel ET_BR **(Figure 2)**. These preliminary observations open an exciting avenue to investigate the role of ET-axis in establishing an immunosuppressive TME in PC. An immediate first step would be to expand these observations in a larger cohort and study the correlation of ET-axis with tumor immunophenotype. Why a subset of patients fail to respond to checkpoint blockade agents remains an open question and resistance has been attributed in part, to the poor infiltration of CTLs. It is likely that ET-axis contributes to CTL exclusion in TME and pharmacological modulation may tilt the balance towards improved outcome on immunotherapy with vaccines and checkpoint blockade agents. Thus, it will be of interest to evaluate the impact of ET axis anatgonism in combination with anti-PD1 antibody on anti-tumor immunity in autochthonous models.

C. Irreversible and sustained upregulation of endothelin axis during K-ras-oncogene associated pancreatic inflammation and cancer.

(i) Does Endothelin axis play a role in acinar to ductal metaplasia?

As mentioned previously in chapter 5, our *in vivo* studies in cerulein treated KC mutant mice suggested that ET_AR and ET_BR are expressed in the metaplastic ducts (intermediate between acinar and ductal cells) at day 2 post trauma.; With the increase in dysplasia at day 7 and day 21 elevated expression of both ET_AR and ET_BR is maintained in CK19 positive ducts. These observations suggest the possible involvement of ET_AR and ET_BR in acinar to ductal metaplasia (ADM). A previous study from our own lab has demonstrated that cerulein treated KC mice progress towards neoplastic transformation with increased CK19 and decreased amylase expression [15] . Further, in order to elucidate the role of ET_AR and ET_BR in ADM *in vitro,* cerulein treated mouse pancreatic acinar cells (266-6) will be analyzed for ET receptors expression. In

order to explore whether ET axis directly participates in the process of ADM, we will knock down of ET_AR and ET_BR using shRNA in 266-6 cells. Another experiment that could validate this hypothesis would be analyzing the acinar cell markers such as elastase, amylase and ductal markers CK19, CA II (carbonic anhydrase). These experiments can suggests that variation in acinar and ductal lineage markers due to knockdown of ET_AR and ET_BR can favor acinar cell plasticity and trans-differentiation into ductal phenotype.

(ii) What are the molecular mechanism(s) through which cigarette smoke upregulates the expression of ET axis?

As previously discussed in chapter 5, our IHC and real time PCR analyses suggested an increase expression of ET axis in KC mice tissues subjected to cigarette smoke. Our results also demonstrated that, in addition to increase expression on the pre-cancerous lesions, expression of ET receptors was also enhanced in α-SMA positive fibroblasts and F4/80 positive macrophages. Given the aberrant expression of ET-1 and receptors in response to cigarette smoke extract will be of interest to elucidate the mechanisms by which such deregulation of ET-axis components occur. Accumulating evidence indicate that smoke extract increases ET-1 and receptors expression in endothelial cells, pulmonary artery and bronchi by ERK and PKC dependent pathway [35] [36] [37]. However, this effect was abolished by dual ET receptors antagonism using Bosentan [35]. Given the paucity of studies in the context of cancer, future studies could address the function and mechanism of ET axis in PC in presence of oncogenic Kras. More specifically studies should be focused on cell lines developed in our lab derived from KC (UN-KC-6141) and KPC (UN-KPC-961) mice models [38] or organoid cultures from conditional ET-receptor mice. The role of ET-axis in cigarette smoke induced accelerated progression can be examined in vivo using selective inhibitors or conditional

ET receptor KO mice. These studies will entail examination of alterations in the Et-axis components in response to cigarette smoke, impact of ET-axis antagonists on the progression of smoke-induced injury to pancreatic cells and impact of ET- receptor KO in tumor progression in response to smoking. Focus of such studies should be to delineate the cellular mechanisms, identify of signaling networks and discern the role of immune cells in exacerbating the effects of smoking via ET axis

D. Generation of ET-1-/-, Kras^{G12D}, Trp53$^{R172H/+,}$ Pdx1-Cre mice model

(i) Does ET-1 aid in pro-fibrotic phenotype in pancreatic cancer?

Previous studies using knockout mice models have indicated that ET-1 is necessary for normal mouse development and play a key role in normal homeostasis. For example, mice homozygous for ET-1 gene deletion were neonathal and displays craniofacial and cardiac abnormalities [39]. In contrast mice with heterozygous deletion of ET-1 gene (ET-1$^{-/+}$) appeared normal but had elevated mean arterial blood pressure [39]. Furthermore, mice with ET-1 deletion from the endothelium using Tie2-Cre have reduced cardiac hypertrophy and lower levels of TGFβ, collagen I and III, suggesting that endothelium derived ET-1 is a crucial mediator of fibrosis [40] [41]. In addition, increased oxidative stress and inflammation resulting due to ischemia/reperfusion mediated injury is attenuated in ET-1 KO mice [42]. Likewise, ET-1 overexpressing mice is presented with increase chronic inflammation, renal cyst formation and renal interstitial fibrosis [43] [44] [45].

Despite the utility of the aforementioned studies in shedding light on the role played by ET-1 in normal physiological and pathological conditions, no study thus far has studied whether the loss of ET-1 in the pancreas has any significant effect in promoting the pro-fibrogenic phenotype in pancreatic cancer. With a goal to analyze the role played by ET-1 ligand, we initiated efforts to generateET-1$^{-/-}$KPC mice. For this, we crossed the ET-

$1^{flox/flox}$ mice with K-ras^{G12D}; $Trp53^{R172H/+;}$ Pdx-1-Cre (KPC) mice to generate the intermediate crosses, which were intercrossed in order to generate the final ET-$1^{-/-}$ K-ras^{G12D}; $Trp53^{R172H/+;}$ Pdx-1-Cre ($ET^{-/-}$ KPC) genotype. We euthanized and collected tissues from the 5 week, 15 week and 25 week of age from the ET-$1^{-/-}$KPC mice and KPC mice. Our preliminary analysis of the animals scarified indicated that loss of ET-1 has minuscule effect on the stromal score compared to mice with wild type ET-1. In addition, H&E analysis of the ET-1 KO mice also reveals small effect on the inflammatory score **(Figure 3)**. We further analyzed the tissues with IHC and observed slight reduction of in the expression of pro-fibrotic genes such as α-SMA **(Figure 4A and 4C)** and Collagen I **(Figure 4B and 4C).** However, due to the limitation in the sample size one must be cautious before coming to a conclusion from a very limited sample size. However, a marginal effect is not surprising given the fact that ET-1 and other ligands can also be produced by other cell types (fibroblasts, immune cells and endothelial cells). Given the mosaic expression patterns of the ET-receptors in the tumor microenvironment, more definitive answers will come from models with ET-receptor knockout in various cellular compartments (tumor cells, stellate cells, immune cells and tumor vasculature) to fully comprehend the complex role of this axis in the evolution of complex tumor microenvironment.

Figure 1. ET axis overexpression is correlated with Immuno-suppressive phenotype in PC. Immune Quant analysis of the TCGA database for ET-1, ET_AR and ET_BR demonstrating high tumor expression is significantly correlated with elevated pathways involved in MDSCs, Tregs, Bregs and TAMs recruitment/differentiation.

Figure 1

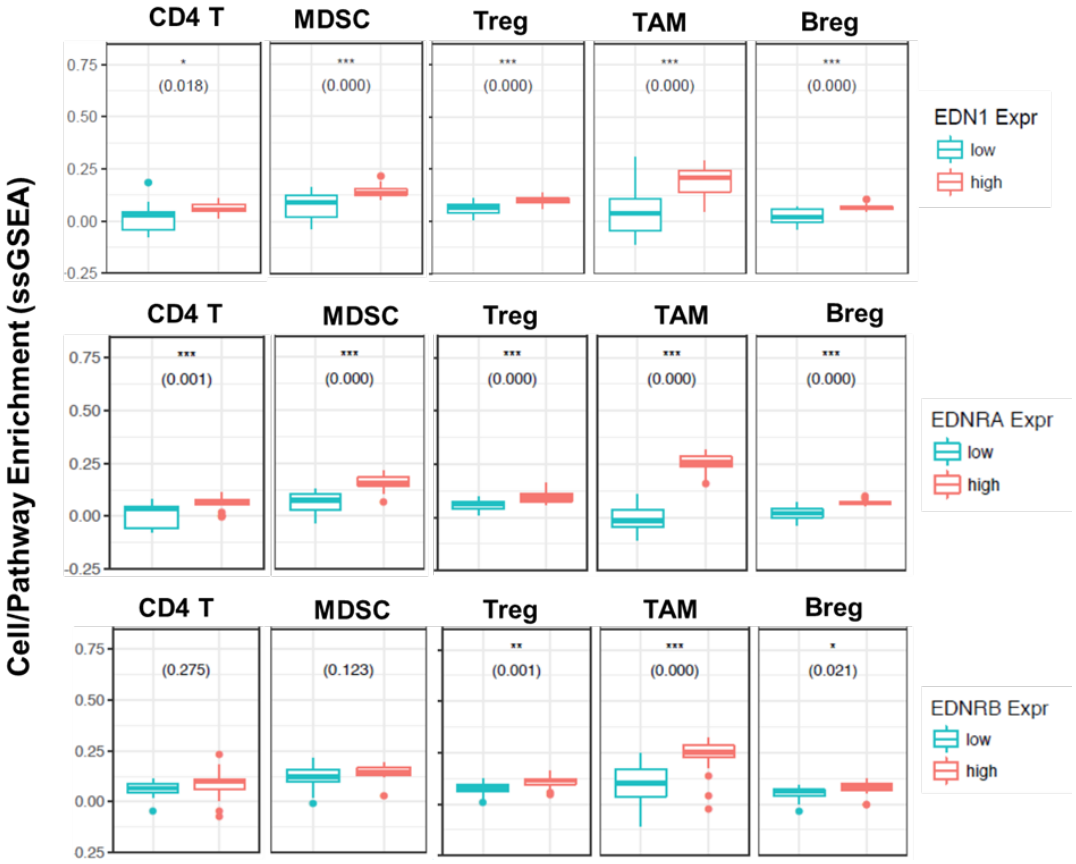
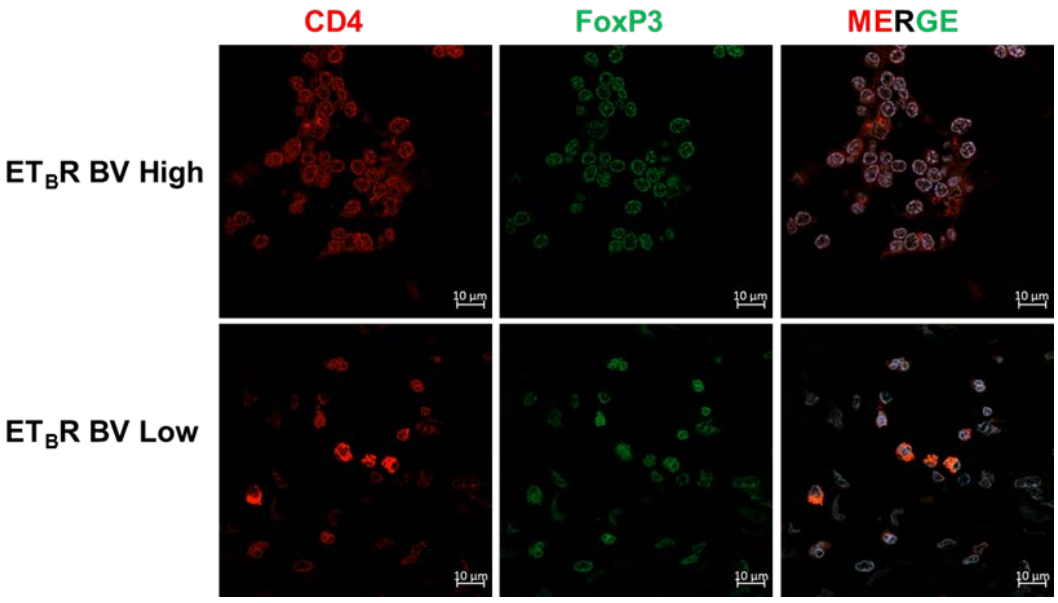

Cell/Pathway Enrichment (ssGSEA)
CD4 T
MDSC
Treg
TAM
Breg
(0.018)
(0.000)
(0.000)
(0.000)
(0.000)
EDN1 Expr
low
high
(0.001)
(0.000)
(0.000)
(0.000)
(0.000)
EDNRA Expr
low
high
(0.275)
(0.123)
(0.001)
(0.000)
(0.021)
EDNRB Expr
low
high
0.75
0.50
0.25
0.00
-0.25

Figure 2. Immunofluorescence analysis of CD4 T cells and FoxP3 in human PC A. Dual immunofluorescence staining for CD4 and FoxP3 in human PC samples with low and elevated ET_BR expression on blood vessels (BV). **B.** Quantitation of proportion of Tregs in the total T cells population suggest higher infiltration of Tregs in PC tissue with elevated ET_BR BV positivity.

Figure 2

A


CD4
FoxP3
MERGE
ETBR BV High
ETBR BV Low
10 µm

B

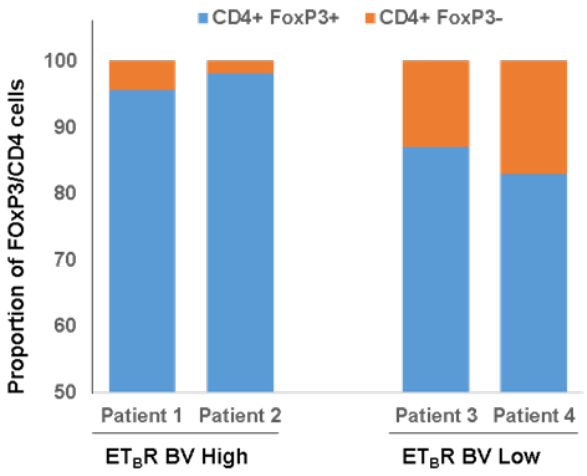

CD4+ FoxP3+
CD4+ FoxP3-
Proportion of FOxP3/CD4 cells
100
90
80
70
60
50
Patient 1
Patient 2
Patient 3
Patient 4
ETBR BV High
ETBR BV Low

Figure 3. Representation of the stromal and inflammatory score in the mice pancreas. Bar graph representation of the stromal score and inflammatory score in ET-$1^{-/-}$ KPC mice and ET-$1^{+/+}$KPC based on the H&E staining. The intensity was calculated on a scale of 0-4, with '0' being the lowest and '4' being the highest. Compared to ET-$1^{+/+}$KPC mice, pancreas with homozygous deletion of ET-1 showed minimal difference in the stromal and inflammatory score.

Figure 3

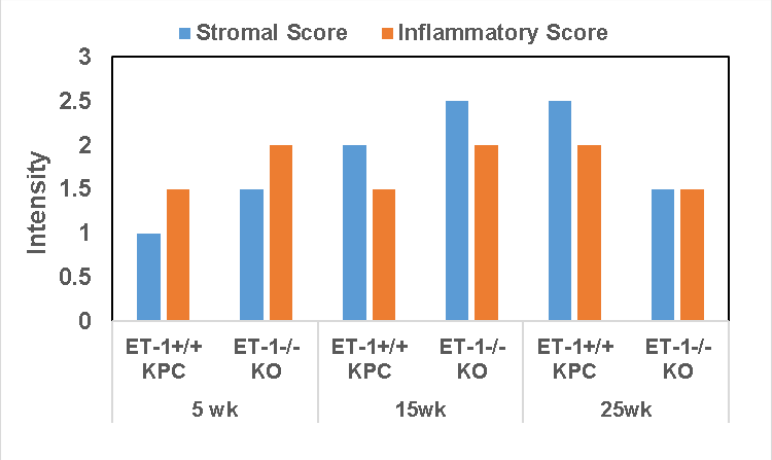

Stromal Score
Inflammatory Score
Intensity
3
2.5
2
1.5
1
0.5
0
ET-1+/+ KPC
ET-1-/- KO
ET-1+/+ KPC
ET-1-/- KO
ET-1+/+ KPC
ET-1-/- KO
5 wk
15wk
25wk

Figure 4. Immunohistochemical analysis of α-SMA and Collagen I in mice pancreas. A. Staining of α-SMA in pancreas of mice isolated from 5 week, 15 week and 25 week of age. The staining shows gradual increase in the α-SMA positive fibroblasts surrounding the pancreatic duct with increase in age of mice. Staining intensity and the overall composite score reveals slight reduction in 25week ET-$1^{-/-}$ KPC mice compared to ET-$1^{+/+}$KPC mice. **B.** Immunostaining for Collagen I in ET-$1^{-/-}$ KPC mice pancreas suggest minor decrease in the composite score as compared to ET-$1^{+/+}$KPC mice. Note the prominent collagen I staining in the tumor stroma surrounding the ducts. **C.** Quantitation of the composite score for SMA and collagen I in ET-$1^{-/-}$ KPC mice and ET-$1^{+/+}$KPC.

Figure 4

A

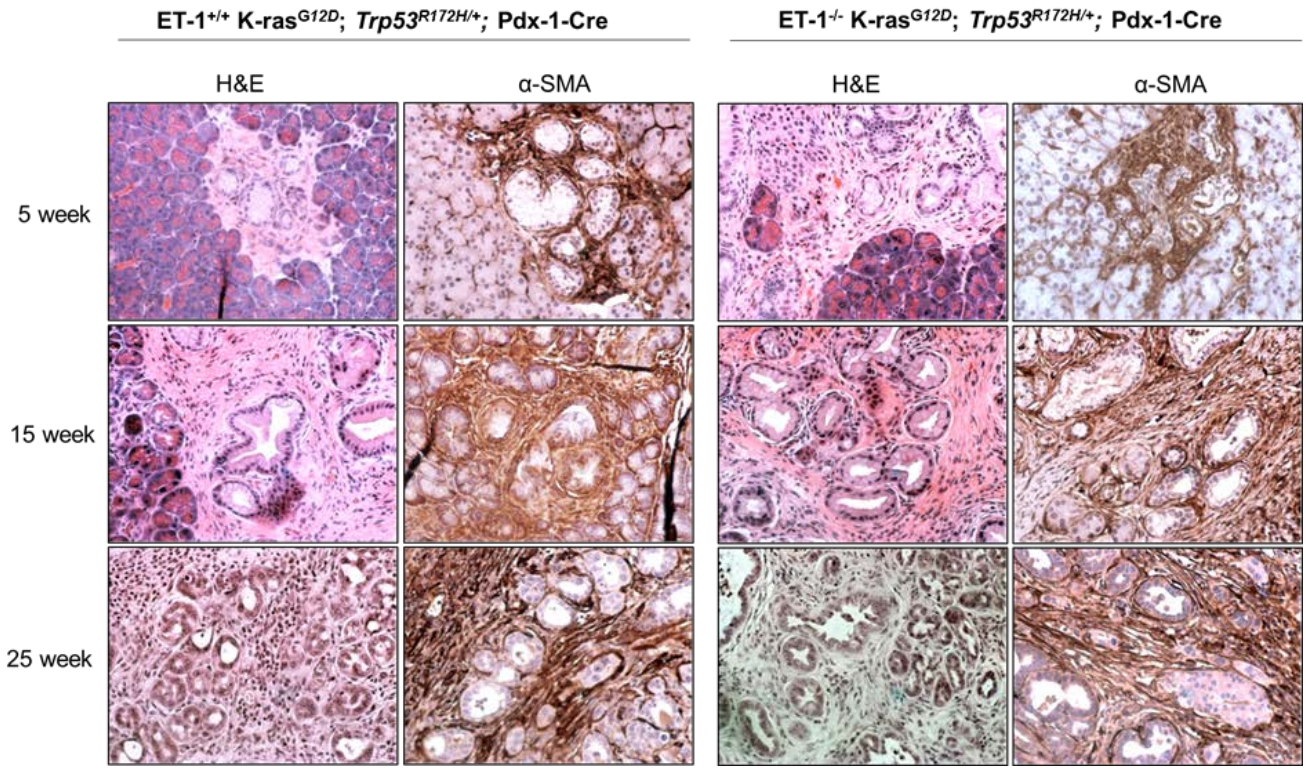
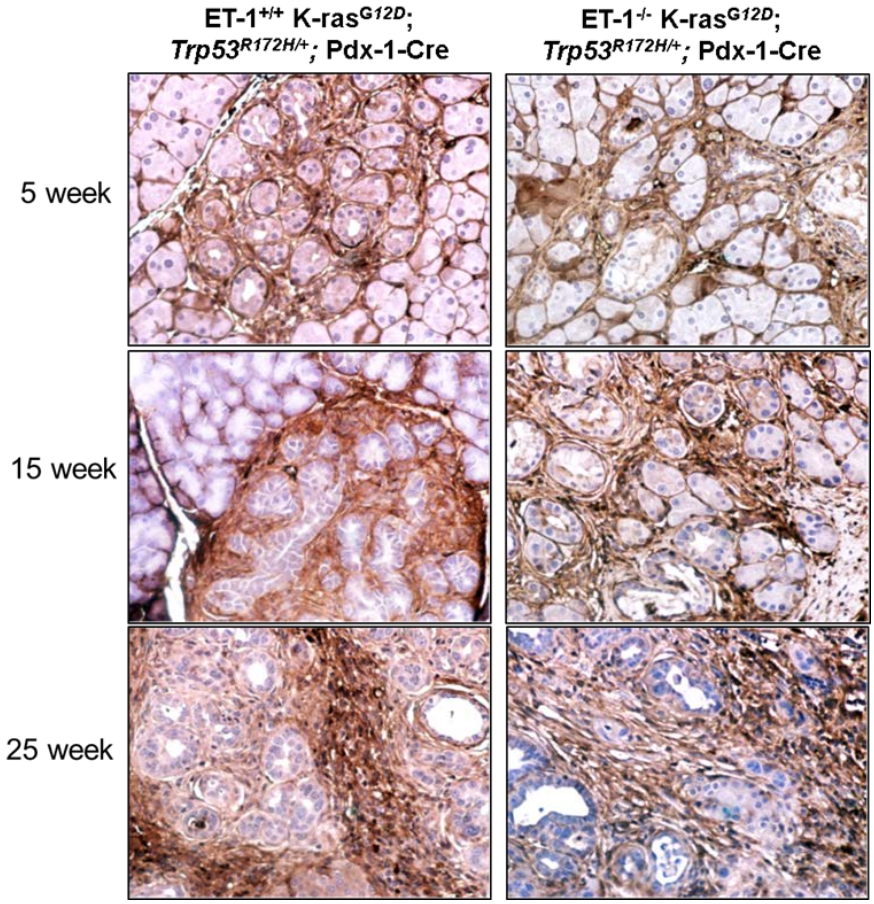
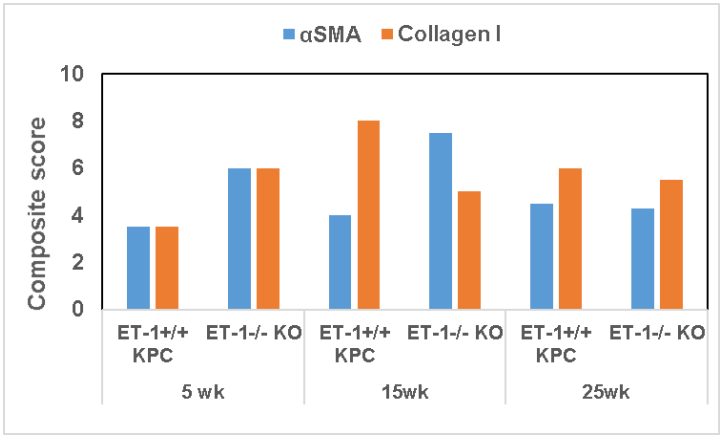

ET-1+/+ K-rasG12D; Trp53R172H/+; Pdx-1-Cre
ET-1-/- K-rasG12D; Trp53R172H/+; Pdx-1-Cre
H&E
α-SMA
H&E
α-SMA
5 week
15 week
25 week

Figure 4

B


ET-1+/+ K-rasG12D; Trp53R172H/+; Pdx-1-Cre
ET-1-/- K-rasG12D; Trp53R172H/+; Pdx-1-Cre
5 week
15 week
25 week

C


αSMA
Collagen I
Composite score
10
8
6
4
2
0
ET-1+/+ KPC
ET-1-/- KO
ET-1+/+ KPC
ET-1-/- KO
ET-1+/+ KPC
ET-1-/- KO
5 wk
15wk
25wk

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