Characterising the tumour microenvironment in pancreatic cancer and the changes elicited by targeted therapies

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SUMMARY

Pancreatic cancer represents less than 3% of cancers diagnosed each year in the United Kingdom yet despite this low number, it is the fifth highest cause of death by cancer. This situation has changed little in the past few decades with median survival barely altering between 1971 and 2007. The availability of well characterised *in vivo* models that histologically recapitulate pancreatic ductal adenocarcinoma (PDAC) have revolutionised the field of PDAC research. These models not only recapitulate the central epithelial component of human pancreatic cancer but also the incredibly complex microenvironment, a feature for which PDAC is well known. Due to the failure of therapies targeting the neoplastic epithelial cells within PDAC, increasing interest has been given to targeting the tumour microenvironment. The tumour microenvironment is extremely complex and consists of both cellular and non-cellular components and in PDAC exhibits a number of characteristic features including the presence of pancreatic stellate cells. Work in our lab has also highlighted stromal constituents such as lysyl-oxidase and tenascin C which are vital for PDAC viability and/or metastasis. In recent years increasing numbers of stromal targets have been evaluated in mouse models of PDAC with varying success. To date work characterising the stromal changes elicited by targeted therapies has utilised methods which we believe lack the required fastidiousness required to obtain reliable and meaningful results. In this work we have established reliable methods for stromal characterisation, we have established methods to characterise the expression of tenascin C on formalin fixed specimens and we have applied these methods to determine the changes elicited by stromal targeting therapies.

OBJECTIVES

In this study we aimed to establish methods to reliably characterise the composition of the tumour microenvironment. We aimed to evaluate the expression of tenascin C in our murine models of PDAC and determine its importance in both these models and in human disease. Finally we aimed to utilise these methods to determine the effects of lysyl-oxidase inhibition and CXCR2 inhibition on the stroma in murine models of PDAC.

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AUTHOR'S DECLARATION

I declare that I am the sole author of this thesis and the work presented here has been done by myself in collaboration with other members of the lab.

The results from the work undertaken determining the effects of CXCR2 inhibition discussed here will form part of a thesis to be submitted by Colin Steele for PhD to the Faculty of Medicine, University of Glasgow in 2014.

The results of the work undertaken determining the effects of CXCR2 inhibition and lysyl-oxidase inhibition on the survival of mice developing pancreatic cancer have been included in this thesis in order to present the thesis in the relevant wider context.

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ABBREVIATIONS

alphaSMA	alpha smooth muscle actin
BMP-1	Bone morphogenetic protein 1
Brca2	Breast cancer 2, early onset
CD3	Cluster of differentiation protein 3
CD31	Cluster of differentiation protein 31
CTGF	Connective tissue growth factor
CXCL	C-X-C motif ligand
CXCR1	C-X-C chemokine receptor type 1
CXCR2	C-X-C chemokine receptor type 2
DAB	3,3'-Diaminobenzanthracene
DKK1	Dickkopf-related protein 1
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediainetetraacetic acid
EGF	Epidemal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
Erk	Extracellular-signal-related kinases
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
FOV	Field of view
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
H&E	Haematoxylin and eosin
HIF-1alpha	Hypoxia inducible factor 1alpha

HGF	Hepatocyte growth factor
H2AX	H2A histone family member X
IGF	Insulin-like growth factor
IL-1beta	Interleukin 1beta
IL-8	Interleukin 8
IL-10	Interleukin 10
Ki67	Antigen Ki-67
KO	Knock out
KPC	Pdx-Cre, LSL-Kras^{G12D}, LSL-Trp53^{R172H}mouse
KPflC	Pdx-Cre, LSL-Kras^{G12D}, LSL-Trp53^{loxP} mouse
KRas	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LOX	Lysyl-oxidase
LOX-Ab	Lysyl-oxidase neutralising antibody
LSL	LoxP-STOP-LoxP
MET (HGFR)	Hepatocyte growth factor receptor
MMP	Matrix metalloproteinase
MOM	Mouse on mouse
MPO	Myeloperoxidase
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NGS	Normal goat serum
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
Pdx1	Pancreatic and duodenal homeobox 1
Pepducin	½i-pal peptide (Genscript) CXCR2 inhibiting peptide
PI3K	Phosphatidylinositol 3-kinase

qPCR	quantitative polymerase chain reaction
RBCs	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
Shh	Sonic hedgehog
shRNA	Short hairpin RNA
TBS	Tris-buffered Saline
TBST	Tris-buffered saline ad Tween
TGFβ	Transforming Growth Factor β
TLR4	Toll-like receptor 4
TMA	Tissue microarray
TNC	Tenascin C
VEGF	Vascular endothelial growth factor
WT	Wild type

Chapter 1
Introduction

1.1 Biology of the pancreas

The pancreas is both an endocrine and an exocrine gland, the endocrine pancreas being composed of the islets of Langerhans and the exocrine pancreas being composed of ducts and acini. These acini connect to the gastrointestinal tract through a system of ducts which converge eventually joining the common bile duct which enters the duodenum. The ducts are lined by a simple cuboidal epithelium surrounding a central lumen (see Fig. 1).

1.2 Pancreatic cancer

Pancreatic cancer represents less than 3% of cancers diagnosed each year in the United Kingdom (http://www.cancerresearchuk.org/cancer-info/cancerstats/). Despite this low number, it is the fifth highest cause of death by cancer with a median survival post-diagnosis of only 6 months and a five year survival rate of less than 5%. This situation has changed little in the past few decades with median survival barely altering between 1971 and 2007. Patients are often diagnosed late with aggressive and metastatic cancer making the disease particularly difficult to treat. For this reason, surgical resection is possible for only 20% of pancreatic cancer patients. Even in patients with surgically resectable tumours who receive adjuvant chemotherapy the five year survival rate only increases from 5% to 15-20%.

Over the same time period during which the prognosis for pancreatic cancer has remained unchanged other tumour types such as melanoma have seen dramatic increases in survival as a result of the development of therapies that target specific molecular alterations within the cancer cells themselves (Jang and Atkins 2013; Yauch and Settleman 2012). This approach is yet to yield similar results in pancreatic cancer with the current standard-of-care chemotherapeutic gemcitabine producing only a minimal survival increase in addition to its palliative effects.

The most common cancer affecting the pancreas is Pancreatic Ductal Adenocarcinoma (PDAC), with the majority of cases being sporadic. A number of risk factors have been associated with PDAC including; *breast cancer type 2*

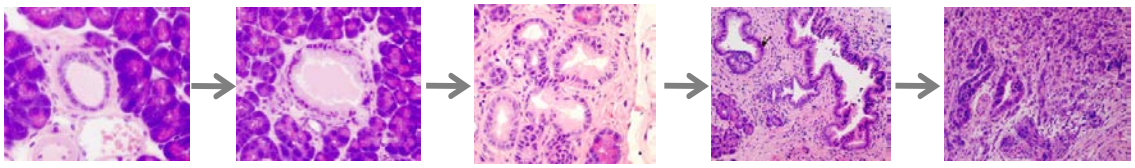
(brca2) and *liver kinase B1* (lkb1) germline mutations (Jaffee, Hruban et al. 2002). These risk factors however only account for around 5% of PDAC cases.

In common with a number of other cancers, pancreatic cancer is characterised by a series of early preneoplastic lesions. These lesions are called pancreatic intraepithelial neoplasia (PanIN) and occur in three stages, PanIN 1, 2 and 3 (see Fig. 1). The PanIN1 stage however is divided into PanIN1A and PanIN1B, defined by specific histological characterisics (Hruban, Goggins et al. 2000; Hruban, Adsay et al. 2001; Kern, Hruban et al. 2001). PanIN1A lesions are characterised by flat epithelial lesions composed of tall columnar cells which have basally situated nuclei and abundant supranuclear mucin. PanIN1B lesions although similar to PanIN1A lesions demonstrate a papillary, micropapillary or basally pseudostratified architecture. PanIN2 lesions may be flat but are generally papillary and must exhibit nuclear abnormalities with loss of polarity, hyperchromatism, nuclear crowding and/or anisokaryosis. PanIN3 lesions show greater nuclear abnormalities and disorganisation than PanIN2 lesions with duct epithelial cells appearing to “bud off” into the lumen of the duct, loss of cell polarity, abundant mucin production and increased/abnormal mitoses. Finally carcinoma occurs when neoplastic cells invade through the basement membrane. Carcinomas may further progress with loss of differentiation, generation of a marked stromal reaction and ultimately disseminated metastatic disease.

With KRas being mutated in over 90% of cases and genes such as $p16^{INK4A}$ and p53 being mutated in over 50% of cases a genetic progression model has been described for PDAC (Hruban, Wilentz et al. 2000; Almoguera, Shibata et al. 1988; Smit, Boot et al. 1988; Hruban, van Mansfield et al. 1993; van Es, Polak et al. 1995). *KRas* mutation alone in mouse models of pancreatic cancer is capable of reproducing the stepwise progression to PDAC seen in human patients (Hingorani, Perticoin et al. 2003) however this model has a long latency suggesting a requirement for further mutations, such as loss or mutation of p53, in order to allow progression to PDAC.

Figure 1: Overview of the evolution of PDAC

Initially KRas mutation alone is sufficient to allow PanIN 1 formation. Gradual evolution subsequently occurs with the accumulation of further mutations allowing progression through PanIN stages to adenoma and eventually adenocarcinoma. (Images by Jennifer Morton).



1.3 KRas and MAPK signalling

KRas is a small GTP-binding protein and an important component of the MAPK signalling pathway. Ras when released from its association with GDP is able to bind GTP and subsequently activate downstream signalling predominantly through Raf, MEK and ERK. Importantly oncogenic forms of Ras are constitutively active and have been shown to trigger transformation, invasion and angiogenesis (Ellis and Clark 2000).

1.4 *p53*

Encoded by the gene *TP53*, p53 has a vital role in regulating the cell cycle and conserving the stability and integrity of the genome (Strachan and Read, 1999). Under situations of stress, such as DNA damage or hypoxia, activation of p53 permits its translocation to the nucleus where it modulates the transcription of a wide variety of gene targets. This altered transcription results in quiescence, senescence or apoptosis (Shaw, Bovey et al. 1992; Diller, Kassel et al. 1990). TP53 mutation occurs in 50-70% of human PDAC following an initiating KRas mutation. Mutation of p53 does not necessarily result in the loss of p53 protein expression, in fact commonly it results in the expression of a stable gain-of-function p53 protein that when present specifically promotes metastasis (Morton, Timpson et al. 2010).

1.5 Genetically engineered mouse (GEM) models of pancreatic cancer

The availability of well characterised *in vivo* models that histologically recapitulate pancreatic ductal adenocarcinoma have revolutionised the field of PDAC research. These models not only recapitulate the central epithelial component of human

pancreatic cancer but also the incredibly complex microenvironment, the importance of which will be discussed in detail in this thesis. As such they provide a unique opportunity, unavailable *in vitro*, to develop a deeper understanding of the processes governing the composition of the microenvironment as well as the ability to trial targetted therapies, often combinatorial, and determine their therapeutic potential.

The models used in this work have been previously described and include the Pdx-Cre, LSL-Kras^{G12D}, LSL-Trp53^{R172H}, LSL-Trp53^{loxP}, CXCR2-/- and Tenascin C-/- mouse strains (Hingorani, Perticoin et al. 2003; Olive, Tuveson 2004; Jackson, Willis et al. 2001; Jamieson, Clarke et al. 2012). The main mouse model used in this work is the KPC model previously described by Hingorani et al. 2003. This model utilises Pdx driven Cre recombinase in conjunction with LSL-Kras^{G12D} and LSL-Trp53^{R172H}. The resulting expression of the Cre recombinase enzyme under the control of the Pancreatic and duodenal homeobox 1 promoter results in recombination and expression of Kras^{G12D} and LSL-Trp53^{R172H} within pancreatic tissues.

1.6 Tumour microenvironment

Due to the failure of therapies targeting the neoplastic epithelial cells within PDAC, increasing interest has been given to targeting the tumour microenvironment, a characteristic for which PDAC is well known (Feig, Gopinathan et al. 2012). Many epithelial cancers are able to induce a desmoplastic reaction with the accumulation of stromal cells and their products around the tumour epithelium. Pancreatic cancer, particularly PDAC, shows a particularly prominent desmoplastic reaction which can account for up to 90% of the tumour volume.

The tumour microenvironment is an extremely complex “ecosystem” consisting of both cellular and non-cellular components (Feig, Gopinathan et al. 2012). The non-cellular fraction is made up of an extracellular matrix (ECM) including structural proteins such as collagens and fibronectin as well as soluble factors and enzymes such as cytokines, growth factors and proteinases. The cellular components of the tumour microenvironment include; myofibroblasts (activated

fibroblasts), inflammatory/immune cells, blood vessels and pancreatic stellate cells (Feig, Gopinathan et al. 2012).
In addition to the high percentage of the tumour that is made up by stroma; PDAC is also unique in the composition of that stroma. The pancreas, similar to the liver, contains a population of stellate cells which, upon activation, are considered vital in the production of the characteristic desmoplastic reaction seen in PDAC (Apte, Park et al. 2004).

Pancreatic stellate cells in the normal pancreas are located surrounding the base of pancreatic acinar cells and are characterised by a central cell body and long cytoplasmic processes (Apte, Haber et al. 1998). Quiescent pancreatic stellate cells contain large numbers of cytoplasmic lipid droplets that are high in vitamin A; these are lost once a stellate cell becomes activated (Apte, Haber et al. 1998). Pancreatic stellate cells are also characterised by the presence of Glial Fibrillary Acidic Protein (GFAP), desmin, vimentin, nestin and synemin (Wehr, Furth et al. 2011; Apte, Park et al. 2004). Upon activation pancreatic stellate cells take on a myofibroblast-like phenotype expressing alphaSMA (Apte, Park et al. 2004). They are highly motile and contractile and have a high mitotic index. They are thought to be responsible for the production of a wide variety of ECM proteins (e.g. collagen I, III, XI, fibronectin, periostin, tenascin C), matrix metalloproteases (MMPs), neurotrophic fators (e.g. NGF, Ach), growth factors and cytokines (e.g. PDGF, FGF, TGFβ, CTGF, IL-1beta, IL-8, VEGF) and are therefore seen to be centrally placed in orchestrating the desmoplastic reaction characteristic of PDAC (Apte, Park et al. 2004). Additional roles for stellate cell derived myofibroblasts in immune suppression have also been highlighted by Fearon et al. in which ablation of fibroblast activation protein alpha (FAP) positive cells, a population of alphaSMA positive myofibroblasts, permitted immunological control of tumour growth (Kraman, Bambrough et al. 2010).

One further feature that characterises the PDAC microenvironment is the low density, poorly functional vascular supply within the tumour (Provenzano, Cuevas et al. 2012). It is this poor vascular supply in conjunction with the desmoplastic reaction that is thought to play a major role in limiting the efficacy of current

cytotoxics, such as gemcitabine, through limited drug delivery and hypoxia induced drug resistance (Provenzano, Cuevas et al. 2012).

As well as being incredibly complex in its composition, the tumour microenvironment is also in constant flux. This flux is controlled and coordinated by continuous crosstalk between the neoplastic cells and the environment, involving a wide variety of autocrine and paracrine signalling pathways including TGFβ, Shh, CXCLs, HGF/Met, FGFs, IGF-1 and EGF among others (Neesse, Michl et al. 2011). Additionally there are also direct interactions between structural ECM molecules and neoplastic cells through cell surface receptors such as integrins which signal via focal adhesion complexes and in turn via the actin cytoskeleton (Chung, Tan et al. 2012). This cross-talk results in the production of enzymes such as lysyl-oxidase and MMPs, alterations in the quantity and quality of structural ECM molecules such as collagen and tenascin C, alterations in the immune cell infiltrate and alterations in the levels of chemokines and cytokines. This constant cross-talk is vital in many areas of tumourigenesis, tumour progression, invasion and metastasis.

Increasingly therapies targeting stroma in PDAC are being explored in mouse models of PDAC and in human disease. Clinical trials with marimastat an MMP inhibitor showed no improvement compared to gemcitabine alone whereas recent work in mice has highlighted new potential targets with inhibition of hedgehog signalling and enzymatic depletion of hyaluronan separately enhancing delivery of chemotherapy agents to pancreatic tumours through suggested “stromal softening” and increased vascular delivery of chemotherapy agents (Bramhall, Rosemurgy et al. 2001; Moore, Goldstein et al. 2007; Provenzano, Cuevas et al. 2012; Olive, Jacobetz et al. 2009; Jacobetz, Chan et al. 2013). Unfortunately these studies although showing altered vasculature and increased gemcitabine delivery did not extensively characterise stromal changes in response to hedgehog signalling inhibition or hyaluronan depletion.

1.7 Available methods for tumour microenvironment characterisation

With the increasing interest in stromal targeting in cancer therapy, particularly in PDAC, there have been increasing numbers of publications evaluating the effects

of different stromal targeting therapies (Olive, Jacobetz et al. 2009; Provenzano, Cuevas et al. 2012; Jacobetz, Chan et al. 2013; Ijichi, Chytil et al. 2011). Unfortunately the methods used in these publications vary greatly in their fastidiousness. Some groups have used FACS analysis to quantify cellular constituents of tumours, others have used Western blot and qPCR analyses to determine levels of non-cellular stromal constituents and others have used histological assessment to determine either cellular or non-cellular constituents. FACS analysis alone provides robust counts of cellular constituents of tissues however it provides no information regarding tissue organisation and architecture and there are also a multitude of issues surrounding tissue selection and preparation for analysis. Similar difficulties arise with Western-blot or qPCR analysis for non-cellular constituents as without histological assessment there is no confirmation of the piece of tissue actually being analysed or its organisation and architecture. Broadly speaking the histological methods used to date involve subjectively selecting areas with highest staining and then selecting small numbers of representative fields on which to score the stromal constituent of interest. From these selected fields an average is taken which is assumed to represent the tumour as a whole.

1.8 Important stromal targets established within our lab

Work by Jen Morton and Bryan Miller using *in vitro* screening techniques in combination with microarray data from murine pancreatic cancer models (both metastatic and non-metastatic) and human pancreatic cancer data has previously highlighted potential stromal targets within PDAC including, tenascin C, lysyl-oxidase and CXCR2 signalling. As such we have applied a number of stromal targeting therapies to the KPC model of PDAC and have shown significant effects on survival in the KPC model.

1.9 Tenascin C

The tenascin family has four members: tenascin C, tenascin R, tenascin X and tenascin W. These members all share a characteristic modular structure with an oligomerization domain followed by EGF-like repeats, fibronectin (FN) type III repeats and a fibrinogen globe (Chiquet-Ehrismann 2004). In the case of tenascin

C and R alternative splicing can lead to the generation of multiple isoforms that contain additional FN type III repeats.

Tenascin C is produced in response to a wide variety of cellular signals such as in states of hypoxia under the control of the transcription factor HIF-1α or in response to mechanical strain provided by the stiff stroma present within tumours (Jones and Jones 2000).

Tenascin C has a number of receptors and mechanisms through which it signals to cells. These include signalling via integrins, EGFR, c-MET, TLR4, annexin II and through mechanotransduction by direct cytoskeletal rearrangements. It has also been shown to modulate TGFβ, Notch and WNT signalling pathways. One mechanism by which tenascin C modulates the WNT signalling pathway is via downregulation of Dkk1 a known inhibitor of the WNT pathway (Ramos, Chen et al. 1997; Sriramarao, Mendler et al. 1993; Yokosaki, Palmer et al. 1994; Varnum-Finney, Venstrom et al. 1995; Yokosaki, Monis et al. 1996; Iyer, Tran et al. 2008; Midwood, Sacre et al. 2009; Brosig, Ferralli et al. 2010; Thomasset, Lochter et al. 1998; Taraseviciute, Vincent et al. 2010; De Wever, Nguyen et al. 2004).

Tenascin C has been shown to be expressed in the haematopoietic stem cell environment and the hair follicle bulb (Klein, Beck et al. 1993; Kloepper, Tiede et al. 2008) and is therefore considered to be an important component within the stem cell niche. Tenascin C has also been shown to be overexpressed in tumour associated stroma in a variety of cancers including pancreatic cancer (Juuti, Nordling et al. 2004; Bourdon, Wikstrand et al. 1983; Chiquet-Ehrismann, Mackie et al. 1986). Tenascin C is involved in many of the steps of tumorigenesis from early tumour development through to metastatic spread and colonisation.

Work by Bryan Miller in our lab has shown the importance of tenascin C to pancreatic cancer cell lines growing *in vitro*. By using sh-RNA to block production of tenascin C by the tumour cells he has shown a significant decrease in cell viability. Tenascin C production by tumour cells has also been shown to be vital to breast cancer cells upon initial metastatic colonisation of the lungs. It has been shown that inhibiting tenascin C production by metastatic tumour cells at any time point up to 21 days post tail vein inoculation results in apoptosis of tumour cells and regression of any establishing micrometastases. After 21 days, inhibition of

tenascin C production by tumour cells had no effect on metastasis growth. It has been suggested that this is because the production of tenascin C which is initially undertaken by the colonising tumour cells themselves switches over to the mesenchymal cells in response to stimulation by the now established metastatic cells (Oskarsson, Acharyya et al. 2011). In contrast to these findings which highlight the importance of epithelial tenascin C production, it is generally considered that the source of tenascin C in epithelial tumours is the mesenchymal cells themselves. Indeed, it has been shown that fibroblasts produce tenascin C when co-cultured with neoplastic epithelial cells (Chiquet-Ehrismann, Kalla et al. 1989).

A major function of tenascin C is modulation of the adhesion status of cells. In turn, the state of cell adhesion is thought to modulate pathways controlling genomic stability. It is therefore possible that tenascin C through modulation of the adhesion status of cells may indirectly have an effect on genomic stability (Tlsty 1998; Chiquet-Ehrismann and Tucker 2011). Indeed it has been shown that molecules with known functions in controlling genome stability such as H2AX and Brad1 are down-regulated in the presence of tenascin C in glioblastoma cells (Ruiz, Huang et al. 2004) and that tenascin C rich environments favour the development of tumours (Thomasset, Lochter et al. 1998).

In addition to promoting genomic instability, the altered state of adhesion mediated by tenascin C also aids tumour cell migration. Tenascin C was initially shown to be an anti-adhesive molecule inhibiting *in vitro* tumour cell adherence to fibronectin. In fact it is increasingly apparent that tenascin C is able to specifically modulate adhesion status which results in an intermediate adhesion state in tumour cells thus allowing migration as opposed to firm attachment or total loss of adhesion. (Chiquet-Ehrismann, Kalla et al. 1988; Wenk, Midwoos et al. 2000; Murphy-Ullrich 2001).

Epithelial-mesenchymal transition (EMT) is a process utilised by epithelial cells developmentally to aid tissue formation and remodelling. EMT is characterised by loss of cell adhesion and increased cell mobility with additional loss of epithelial markers such as E-cadherin. Tenascin C appears to have a significant role in the induction of EMT as it has been consistently linked with cancer cells undergoing EMT and is vital in injury induced EMT in the lens epithelium of the eye (Dandachi,

Hauser-Kronberger et al 2001; Tanaka, Sumioka et al. 2010). EMT is normally under tight control as it is vital to tissue remodelling and development however it is also a process utilised by tumour cells allowing migration and invasive behaviours.

Tenascin C has also been associated with increased tumour cell proliferation. It has been shown that melanoma sphere growth is severely diminished in the absence of tenascin C. High levels have also been demonstrated at the invasive edges of breast cancer where there is also a significantly higher proliferation rate (Fukunaga-Kalabis, Martinez et al. 2010; Jahkola, Toivonen et al. 1998).

In addition to its functions triggering growth and migration, tenascin C is also pro-angiogenic and able to stimulate endothelial cells to acquire a sprouting phenotype and become migratory (Canfield, Schor et al 1995; Chung, Murphy-Ullrich et al. 1996). In any tissue, cells must reside within 100μm of a capillary blood vessel and this limitation inhibits tumour growth unless the tumour can induce the formation of new blood vessels (Bouck, Stellmach et al. 1996). Tenascin C has been shown to play an important role in stimulating angiogenesis in a number of models. Importantly, *in vivo*, it has been shown that xenograft tumours grown in tenascin C deficient mice had significantly reduced vasculature compared to xenografts grown in control animals. This reduced vasculature was due to the altered regulation of VEGF expression by tenascin C (Tanaka, Hiraiwa et al. 2004)

Finally tenascin C has also been linked to chemotherapy resistance in a number of cancers including; melanoma, breast cancer and pancreatic cancer. Tenascin C has been shown to induce gemcitabine resistance in pancreatic cancer through annexin II signalling and activation of PI3K/Akt and ultimately activation of NF-kB (Gong, Lv et al. 2010; Fukunaga-Kalabis, Martinez et al. 2010; Wang, Liu et al. 2010; Helleman, Jansen et al. 2008).

Due to the wide range of roles that tenascin C plays in the microenvironment of tumours and the importance of tenascin C to tumour cells in culture and at metastatic sites we aim to further characterise tenascin C expression patterns in our models of pancreatic cancer and the changes in expression elicited by different microenvironment targeting strategies. We aim to determine the importance of tenascin C in human disease and determine the similarities in tenascin C expression between human disease and the KPC model.

1.10 Lysyl-oxidase

ECM remodelling is a constant but highly regulated process in normal tissue development (Baker, Cox et al. 2011). Malignant cell growth is ordinarily suppressed by the normal microenvironment through the provision of appropriate forces that govern normal tissue organisation, cell growth, adhesion and migration. The absence of these normal forces in tumour ECM supports malignant cell proliferation, motility and adhesion (Weaver, Petersen et al. 1997; Paszek, Zahir et al. 2005). Enzymes that control ECM remodelling, which include members of the LOX family, are frequently either upregulated or downregulated in both tumour and stromal cells in various types of cancer (Payne, Hendrix et al. 2007; Zitka, Kukacka et al. 2010). Lysyl-oxidase (LOX) is a member of a multigene family with five members; LOX, LOXL1, LOXL2, LOXL3 and LOXL4. LOX is a secreted copper-dependent amine oxidase which functions to catalyse the cross-linking of collagens and elastins in the ECM resulting in increased tissue stiffness and tensile strength (Kagan and Trackman 1991). It is secreted as a proenzyme and subsequently activated by BMP-1. LOX family members have paradoxical roles as both tumour suppressors and metastasis promoters although many of the tumour suppressive roles of LOX have been attributed to the LOX-pro-peptide rather than LOX itself (Kagan and Li 2003). LOX has been shown to be elevated in invasive and metastatic breast cancer, has been validated as a prognostic marker in head and neck cancer and through inhibition with beta-aminoproprionitrile it has been shown to be vital to the invasion of melanoma cell lines *in vitro* (Kirschmann, Seftor et al. 2002; Erler, Bennewith et al. 2006; Le, Harris et al. 2009). LOX has been found to be highly expressed in stromal cells surrounding mammary ductal carcinoma *in situ* and increased tissue stiffness has been shown to promote progression to malignancy in *in vivo* models of breast cancer (Decitre, Gleyzal et al. 1998). It has also been suggested that LOX dependent collagen cross-linking is required for the provision of a microenvironment capable of supporting metastatic cell colonisation at distant sites (Erler, Bennewith et al. 2006; Erler, Bennewith et al. 2009). The progression to malignancy driven by LOX induced tissue stiffness is mediated primarily by altered focal adhesions, growth factor receptor signalling and altered cytoskeletal-dependent cell contractility (Butcher, Alliston et al. 2009; Discher, Janmey et al. 2005; Levental, Yu et al. 2009; Yeung, Georges et al. 2005). LOX also promotes the secretion of VEGF both *in vitro* and *in vivo* and

inhibition in an *in vivo* breast cancer model resulted in decreased intra-tumoral blood vessel numbers (Baker, Bird et al. 2013). In breast cancer and head and neck cancer upregulation of LOX closely correlates with hypoxia which is itself well known for its ability to enhance both metastasis and resistance to both chemotherapy and radiotherapy (Erler, Bennewith et al. 2006). LOX acts in conjunction with a wider repertoire of ECM molecules and has been shown to interact with fibronectin which in turn increases the catalytic activity of LOX. Finally, secreted LOX has been shown to recruit inflammatory cells to distant sites thereby helping to establish suitable niches for metastatic cells (Erler, Bennewith et al. 2009). Work in our lab has shown that lysyl-oxidase inhibition in the KPC model of PDAC leads to a significant increase in survival. As such we aim to characterise the stromal changes elicited by this treatment both alone and in conjunction with gemcitabine therapy.

1.11 CXCR2 signalling

The link between inflammation and tumorigenesis, tumour maintenance and tumour progression is well established. Patients with a history of chronic pancreatitis have a seven fold increased risk of developing PDAC (Duell, Casella et al. 2006) and patients with ulcerative colitis have a twenty fold increased risk of colorectal cancer which is reduced 50% by NSAID use (Xie and Itzkowitz 2008).

CXCR2 ligands are not produced in normal pancreatic tissue but have been shown to be highly expressed in cases of pancreatitis. Additionally CXCR2 expression has been noted in up to 65% of surgically resected human pancreatic tumours and expression is associated with poor survival (Kuwada, Sasaki et al. 2003; Li, King et al. 2011; Wente, Keane et al. 2006; Baggiolini, Dewald et al. 1994; Takamori, Oades et al. 2000).

CXCR2 is a G-protein-coupled cell surface receptor which binds to a number of low molecular weight chemotactic chemokines. Chemokines play important roles in the development of tumours and metastases by modifying the tumour immune response through co-ordinating leukocyte infiltration, angiogenesis and acting as growth factors (Murphy 2001; Raman, Baugher et al. 2007; Singh, Sadanandam et al. 2007).

CXCR2 is commonly found on neutrophils although it is also expressed on monocytes, dendritic cells, macrophages, endothelial cells, bone marrow derived endothelial progenitor cells, myeloid derived suppressor cells, mast cells, fibroblasts and can also be induced in tumour cells by activated oncogenes (Heidemann, Ogawa et al. 2003; Strieter, Burdick et al. 2006; Li, Cheng et al. 2011; Soehnlein, Drechsler et al. 2013; Hallgren and Gurish 2011; Ijichi, Chytil et al. 2011; Sharma, Nawandar et al. 2013; Feijoo, Alfaro et al. 2005; Marotte, Ruth et al. 2010).

CXCR2 predominantly interacts with ELR+ (glutamic acid-leucine-arginine) chemokines, including CXCL1, 2, 3, 4, 5, 6, 7 and 8. These chemokines are all proangiogenic and stimulate neutrophil chemotaxis (Li, Cheng et al. 2011; Rainczuk, Rao et al 2012; Ahuja and Murphy 1996).

Multiple studies to date have shown that CXCR2 participates in chronic inflammation, sepsis, lung pathology, atherosclerosis, neuroinflammation and has critical roles in angiogenesis, tumourigenesis and metastasis of colorectal cancer, melanoma, lung cancer, prostate cancer, pancreatic cancer and head and neck cancers (Stadtmann and Zarbock 2012; Veenstra and Ransohoff 2012; Gabellini, Trisciuoglio et al. 2009; Baier, Wolff-Vorbeck et al. 2005; Singh, Singh et al. 2010; Ohri, Shikotra et al. 2010; Reiland, Furcht et al. 1999; Liu, Yang et al. 2011; Mestas, Burdick et al. 2005; Wang, Hendricks et al. 2006; Matsuo, Ochi et al. 2009; Yang, Rosen et al. 2010; Han, Jiang et al. 2012; Li, Cheng et al. 2011; Li, King et al. 2011; Wente, Keane et al. 2006).

The roles of inflammatory cells within the tumour microenvironment are varied with cells capable of being either tumorigenic or tumour suppressive. Macrophages and neutrophils for example in their pro-tumourigenic M2 or N2 phenotype may promote tumour angiogenesis, matrix breakdown and tumour cell mobility as well as producing oxidative bursts with the release of reactive oxygen species (ROS) that have many roles in tumourigenesis and maintenance but importantly are also mutagenic (DeNicola, Karreth et al. 2011; Sica and Mantovani 2012; Mentzel, Brown et al. 2001; Benelli, Morini et al. 2002; Van Coillie, Van Aelst et al. 2001; Nozawa, Chiu et al. 2006).

As well as through the indirect actions of inflammatory cells, CXCR2 has also been shown to directly promote angiogenesis with CXCR2 signalling vital to both endothelial cell and endothelial progenitor cell mobilisation and chemotaxis (Li, Cheng et al. 2011).

Due to a central role in the co-ordination of the PDAC microenvironment the CXCR2 signalling pathway provides a clear target requiring evaluation for the treatment of PDAC. Work in our lab has shown a significant increase in survival in KPC mice treated with CXCR2 inhibitors. We therefore aim to determine the significant effects this inhibition is having on the tumour microenvironment.

1.12 Project aims

Given the importance of the tumour microenvironment and the increasing use of stromal targeting therapies we aim to establish rigorous methods for the quantification of stromal constituents. Given the importance of tenascin C we also aim to investigate the role of tenascin C in PDAC. Finally we will utilise these methods and staining procedures to evaluate the stroma of the models of PDAC used in our lab and the changes elicited by targeted stromal therapies.

Chapter 2

MATERIALS AND METHODS

2.1 *In vivo* experiments

All experiments were performed in accordance with UK Home Office guidelines and the EU directive 2010 with local ethical approval. Mice were maintained under non-barrier conditions and given a standard diet [CRM (E) expanded diet from Special Diets Services; Cat nº 801730] and water *ad libitum*.

Mice were examined three times per week for the development of pancreatic tumours. These signs include; hunched back, central abdominal distension, weight loss and/or palpable tumour. Once an endpoint was reached mice were euthanized and necropsied and tissues collected. Some mice developed skin papillomas and in these cases, the papilloma was measured three times weekly and once it reached 1.5cm in diameter the mouse was sacrificed. These rules were all clearly defined in the project licence as approved by the Home Office.

2.2 Mouse genotyping

Mice were genotyped externally using the service provided by Transnetyx (http://www.transnetyx.com/). The company uses a proprietary method based on real time PCR and DNA hybridisation to determine which alleles are present in the mice.

2.3 Drug treatments

Mice were selected according to genotype and Pdx1-Cre, $Kras^{G12D/+}$; $Trp53^{R172h/+}$ mice were randomised to treatment or control groups. Mice were aged to 70 days at which time they were commenced on the randomised treatment. Gemcitabine (LC labs) made up in phosphate buffered saline (PBS) was given at 100mg/Kg twice weekly via intraperitoneal injection (IP). Scrambled 14 amino acid sequence, 'scrambled pepducin' (Genscript) made up in saline was given 200µl subcutaneously (SC), ½i-pal peptide (Genscript), a CXCR2 inhibiting peptide, 'pepducin' was given 200µl subcutaneously daily. ½i-pal peptide is a cell-penetrating lipopeptide directed against the third intracellular loop of the CXCR2 G-protein coupled receptor and results in selective inhibition (Kaneider, Agarwal et al. 2005). Lox blocking antibody (Lox-Ab) and isotype control (kind donation by

Janine Erler) made up in saline were given 100 µl IP daily. Drug doses utilised were consistent with those previously published (Jamieson, Clarke et al. 2012; Erler, Bennewith et al. 2006; Morton, Timpson et al. 2010).

2.4 Tissue sampling

Pancreas, lungs, liver, spleen and other tissues where appropriate were collected and fixed as necessary. Subsequent to fixation all samples were embedded in paraffin, sectioned at 5-10µm and stained with haematoxylin and eosin prior to microscopic analysis.

2.5 Fixation protocols

2.5.1 Long Fixation

The tissues were incubated in 4% formalin [Leica; Cat nº 3800600E] for 24 hours before processing and paraffin embedding.

2.5.2 Methacarn Fixation

A solution of methanol [Sigma; Cat nº 32213], chloroform [Fisher Scientific; Cat nº C4960/PB17] and acetic acid [Sigma; Cat nº 695092] was made fresh at a ratio of 4:2:1 respectively and used to fix tumour tissue samples for no more than 24 hours. Tissues were then placed in formalin overnight before being processed and paraffin embedded.

Methacarn fixation was undertaken for tumours requiring MPO staining.

2.6 Histology

Mouse tissues were embedded and cut by the Beatson Institute histology services. Routine histochemistry and immunohistochemistry (IHC) was performed by the Beatson Institute histology services except IHC for CD31, GFAP (mouse and human) and Tenascin C (mouse and human). Routine IHC was performed for CD3 (Abcam, ab16669 rabbit monoclonal), alphaSMA (Abcam, ab15734 rabbit polyclonal), F4/80 (Abcam, ab16911 rat monoclonal), MPO (Abcam, ab9535 rabbit polyclonal), cleaved caspase 3 (Abcam, ab4052 rabbit polyclonal).

2.7 Immunohistochemistry on paraffin sections

Slides were de-waxed for a minimum of 7 minutes in xylene and rehydrated for 2 minutes each in decreasing concentrations of ethanol (100% - 95% - 70%) before finally being washed in dH_2O. After immunohistochemistry, counterstaining was performed. Following which slides were dehydrated by immersion in increasing concentrations of ethanol (70%-95%-100%) before a final immersion in xylene for 5 minutes. After dehydration slides were then mounted with a coverslip.

2.7.1 CD31

Sections were de-waxed as described previously. Antigen retrieval was performed by placing slides in pre-heated Citrate buffer (Thermo scientific) in a pressure cooker for 20 minutes. Following heating, pressure was released and slides were allowed to cool in the solution for 60 minutes at room temperature (RT). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide (Fluka) in de-ionized water. Slides were washed in Tris-Buffered Saline and Tween 20 (TBST). Non-specific binding was then blocked by incubation with 5% normal goat serum (NGS) for 30 minutes at RT. Primary anti-CD31 antibody (Abcam, ab28364 rabbit polyclonal) was applied overnight at 4^oC at a 1/100 dilution in 5% NGS. After washing in TBST, secondary antibody (Vector ABC Kit) was applied for 30 minutes at a dilution of 1/200 in 5% NGS. After washing, signal amplification was performed using the ABC Complex (Vector ABC Kit), applied for 30 minutes. After washing, positivity was then visualised with DAB, slides were washed in dH_2O and counterstained and mounted as described previously.

2.7.2 Tenascin C (anti-human)

Sections were de-waxed as described above and antigen retrieval was performed using Proteinase K antigen retrieval for 7mins. Endogenous peroxidase activity was blocked as described above and non-specific binding was blocked with 5% NGS for 30 minutes at RT. Primary anti-Tenascin C antibody (Abcam antiTNC clone BC-24 ab6393) was applied and slides incubated overnight at 4^oC at a dilution of 1/2000 in 5% NGS. After washing in TBST, secondary antibody (Vector ABC Kit) was applied for 30 minutes at a dilution of 1/200 in 5% NGS. After washing, signal amplification was performed using the ABC Complex (Vector ABC

Kit), applied for 30 minutes. After washing, positivity was then visualised with DAB, slides were washed in dH_2O and counterstained and mounted as described previously.

2.7.3 Tenascin C (anti-mouse)

Sections were de-waxed as described above and antigen retrieval was performed boiling slides in 1L of 1mM EDTA pH 8 for 20 minutes . Slides were allowed to cool for 20 minutes before endogenous peroxidase activity was blocked as described above. Slides had non-specific binding blocked with 5% NGS for 30 minutes at RT before primary anti-Tenascin C antibody (Sigma, anti-TNC T3413 rabbit polyclonal) was applied overnight at 4^oC at a dilution of 1/2000 in 5% NGS. Slides were washed in TBST and secondary antibody (Vector ABC Kit) was applied for 30 minutes at a dilution of 1/200 in 5% NGS. After washing, signal amplification, DAB positivity visualisation and mounting of the slides were performed as described previously.

2.7.4 Glial acidic fibrillary protein (GFAP)

Sections were de-waxed as described above and antigen retrieval was performed by placing slides in pre-heated Citrate buffer in a pressure cooker for 20 minutes. Following heating, pressure was released and slides were allowed to cool in the solution for 60 minutes at room temperature (RT). Endogenous peroxidase activity was blocked as described above and then washed in Tris-Buffered Saline (TBS). Non-specific binding was blocked with incubation in MOM mouse Ig blocking reagent for 1hour. Next slides were incubated in MOM diluent for 5 minutes before incubation in anti-GFAP (mouse monoclonal GA5 cell signalling) 1:50 in MOM diluent for 30 minutes at RT. Slides were washed in TBS before incubation in MOM biotinylated anti-mouse IgG reagent (Vector labs) for 10 minutes at RT. After washing; signal amplification, DAB positivity visualisation and mounting of the slides were performed as described previously.

2.8 Primary cell culture

2.8.1 Medium for cell culture

DMEM [Gibco; Cat nº 21969] with 10% Fetal Bovine Serum [PAA; Cat nº A15-101], 1% Penicillin-Streptomycin [Gibco; Cat nº 15070], and 2mM L-Glutamine [Gibco; Cat nº 25030].

2.8.2 PDAC, primary cell line establishment and culture

Pancreatic tumour tissue was collected in PBS at necropsy. The tissue was then homogenised with a scalpel blade and placed in 5mL of medium and shaken for 30 seconds. The supernatant containing PDAC cells was collected, placed in a 75cm^2 flask [NUNC; Cat nº 153732] and a further 10mL of DMEM was added. The cells were cultured at 37ºC in a humidified 5% CO_2 atmosphere until reaching 90% confluence at which time they were split using trypsin dissociation .

2.9 Subcutaneous tumour growth in mice

Primary cell lines derived from C57Bl/6 PDAC tumours were suspended in PBS and injected subcutaneously into the flank of control C57Bl/6 or Tenascin C-/- mice on a C57Bl/6 background. 1,000,000 cells were injected per mouse. Mice were monitored three times a week and tumours were measured from 6mm size. Mice were culled when tumours ulcerated or exceeded 15mm in size. The tumour was removed along with the overlying skin and underlying abdominal muscle and fixed in 10 % neutral buffered formalin pinned on wax discs. Organs (pancreas, liver, spleen and lungs) were also collected and evaluated for evidence of metastasis.

2.10 Orthotopic models of pancreatic cancer

Primary cell lines derived from C57Bl/6 PDAC tumours were suspended in matrigel (BD biosciences) and injected orthotopically into the tail of the pancreas of C57Bl/6 mice and Tenascin C-/- mice by Dr J Morton. 100 cells were injected per mouse.

Mice were examined three times per week for the development of pancreatic tumours. These signs include; hunched back, central abdominal distension, weight

loss and/or palpable tumour. Once an endpoint was reached mice were euthanized and necropsied and tissues collected.

2.11 Scoring:

All scoring was performed blinded, counting 30 non-consecutive x20 magnification field of view (FOV) or 60 x40 magnification FOV always avoiding areas of necrosis except where necrosis itself was being scored. For cell counts numbers of positive staining cells per FOV were counted, for other immunostaining quantification either pixel counting or semiquantitative methods were used as described below.

2.11.1 Necrosis scoring:

Whole representative slides of tumours were scored at X20 magnification and average percentage necrosis was determined for each tumour. Necrosis was defined by set histological characteristics on haemotoxylin and eosin staining.
These included; nuclear changes (pyknosis, karyorrhexis, karyolysis, and nuclear absence), cytoplasmic changes (early increased eosinophilia advancing to pale pink ghost like appearance), cell rupture (with release of dark basophilic nuclear remnants and eosinophilic protein). Necrotic areas may also contain RBCs in areas of haemorrhagic necrosis.

2.11.2 Immunohistochemistry pixel quantification:

30 x20 magnification images were taken at random from a representative slide from each tumour. The microscope (Olympus BX51) was optimised before taking complete sets of images therefore all images were taken with identical light levels and condenser setup. A manual exposure of 720µs and ISO 1600 were set as fixed parameters and all images were saved as TIFF files to standardise image quality. Finally all images were white balanced.

Once acquired immunostaining was quantified using Adobe Photoshop (version 5; Adobe Systems, San Jose, CA) using a method that has been previously described (Lehr, van der Loos et al. 1999). Briefly, in photoshop with the picture open, click window option in the task bar and open the histogram window. Once this window is open click on the expand icon in the top right corner of the histogram window and select expanded view this will then display the total number

of pixels in the image and once a selection is made will show the number of selected pixels. Next click on the select option on the top bar of the photoshop window and click on colour range. Use the colour dropper within the colour range window to select the colour of interest and then alter the tolerance “fuzziness” setting to achieve a selection setting where the highlighted stained tissue is appropriate. This adjustment is made possible as the selected area is automatically highlighted on the image and therefore allows close control of the process. Once optimised this setting may then be saved as an .AXT file extension and reused for every image to be scored in a group. For each image selection the total number of stained pixels can then be recorded and the total staining represented as a percentage of the entire image in μm^2 or cm^2.

2.12 Statistics:

2.12.1 Scoring experiments:

Three to eight mice per group were used in order to comply with guidelines recommended by “the three Rs”. These guidelines summarised as Refine, Reduce and Replace promote the ethical use of animals in research. Due to low numbers data cannot be assumed to be normally distributed and therefore non-parametric statistical tests are most appropriate. Here a Mann-Whitney test which allows the comparison of small groups of mice was used to determine the presence of statistically significant differences between groups.

2.12.2 Tissue microarray analysis

A human pancreatico-biliary tissue microarray was created within the West of Scotland Pancreatic Unit, University Department of Surgery, Glasgow Royal Infirmary. All patients gave written, informed consent for the collection of tissue samples, and the local Research Ethics Committee approved collection. All cases had undergone a standardized pancreaticoduodenectomy. A total of 1500 cores from a total of 224 cases with pancreatico-biliary cancer (including 119 pancreatic ductal adenocarcinomas) with a full spectrum of clinical and pathological features were arrayed in slides. At least 6 tissue cores (0.6mm diatmeter) from tumour and 2 from adjacent normal tissue were sampled. Complete clinical follow up data was available for all cases within the TMA.

2.12.3 TMA histoscoring

Tenascin C levels were scored based on staining intensity and area of positive staining stroma using a weighted histoscore (Morton, Timpson et al. 2010). The histoscore was calculated based on the sum of (1x% weak staining)+(2x% moderate staining)+(3x% strong staining), therefore providing a semi-quantitative classification of tenascin C levels in TMA cores. Tenascin C expression was defined as either high or low either being above or below the median histoscore value.

2.12.4 TMA survival analysis

Kaplan-Meier survival analysis was used to analyse the overall survival from the time of surgery and a log-rank test was performed to compare length of survival between the resulting two curves.

2.12.5 TMA correlation

Correlation between parameters within the TMA data set was determined using Spearman's Rank-Order Correlation Coefficient.

2.13 RNA microarray analysis

Microarray analysis was performed on request by Nigel Jamieson at the Cancer Research Microarray Facility, Paterson Institute for Cancer Research, University of Manchester. 40ng of RNA was amplified using WT-Ovation Pico RNA amplification system (NuGEN, San Carlos, CA) with subsequent labelling and hybridisation to HG_U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) using the FL-Ovation cDNA Biotin Module v2. Full datasets have been made public in MIAME VICE http://bioinformatics.picr.man.ac.uk/vice/Welcome.vice. Accession code is GE_PA(4).

Chapter 3

Results

3.1 Establishing a method for quantification of stromal constituents in the KPC model

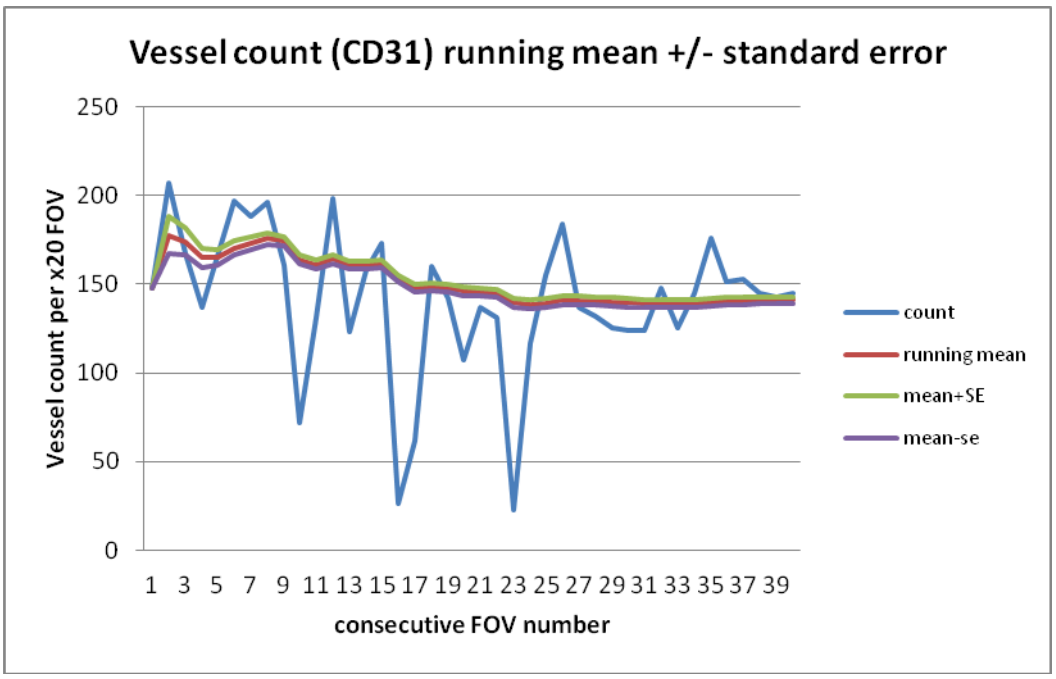
Given the interest in targeting stroma it was first important to carefully characterise and develop reliable methods to quantify the stromal constituents within our models of PDAC. Previous work characterising the stromal constituents of various models including the KPC model of PDAC is limited and methods of quantification vary in their fastidiousness. Previous work characterising stromal characteristics such as intratumoural microvessel density have subjectively identified three to five representative fields of view and counted the stromal constituent of interest in these fields (Ijichi, Chytil et al. 2011; Olive, Jacobetz et al. 2009). The count from these three to five fields is then used to represent the entire tumour. Due to the inherent subjectivity of selecting representative fields of view our first aim was to establish methods that provided more objective quantification of stromal constituents for the tumours as a whole.

To establish a reliable method I began by counting all non-consecutive fields of view in a single tumour at either X20 or X40 magnification depending on the ability to reliably identify stromal constituents at each magnification. During these counting procedures I calculated a running mean and the standard error and graphically it was possible to see when a suitable estimate of the mean had been reached for each constituent such that a truly representative value had been determined. I found that, for any stromal constituent, counting 30 fields of view was sufficient to generate a representative count (See Fig. 2). The procedure of counting 30 fields of view at x20 magnification generally sampled the majority of any given tumour and even in larger tumours 30 fields of view never accounted for less than half of a tumour. When counting at x40 magnification 30 fields of view was still adequate at generating a stable and reliable mean. However given the smaller field size I decided to count 60 fields of view such that the area of tumour sampled still accounted for over half of the tumour.

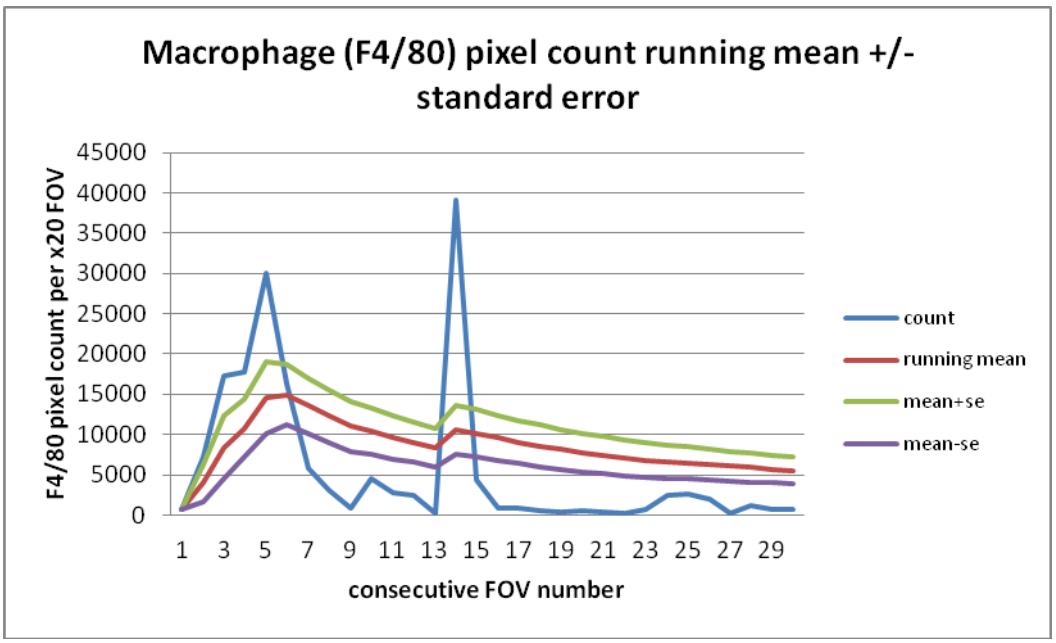
Figure 2: Counting 30 non-consecutive fields of view reliably quantifies any stromal constituent within a tumour.

Representative graphs for single tumours showing consecutive FOV counts (blue line), running mean (red line) and running mean +/- standard error (green and purple lines), a) CD31, b) F4/80, c) MPO quantification and d) Sirius red quantification. CD31, F4/80 and Sirius red quantification were performed at x20 magnification and MPO at x40 magnification.

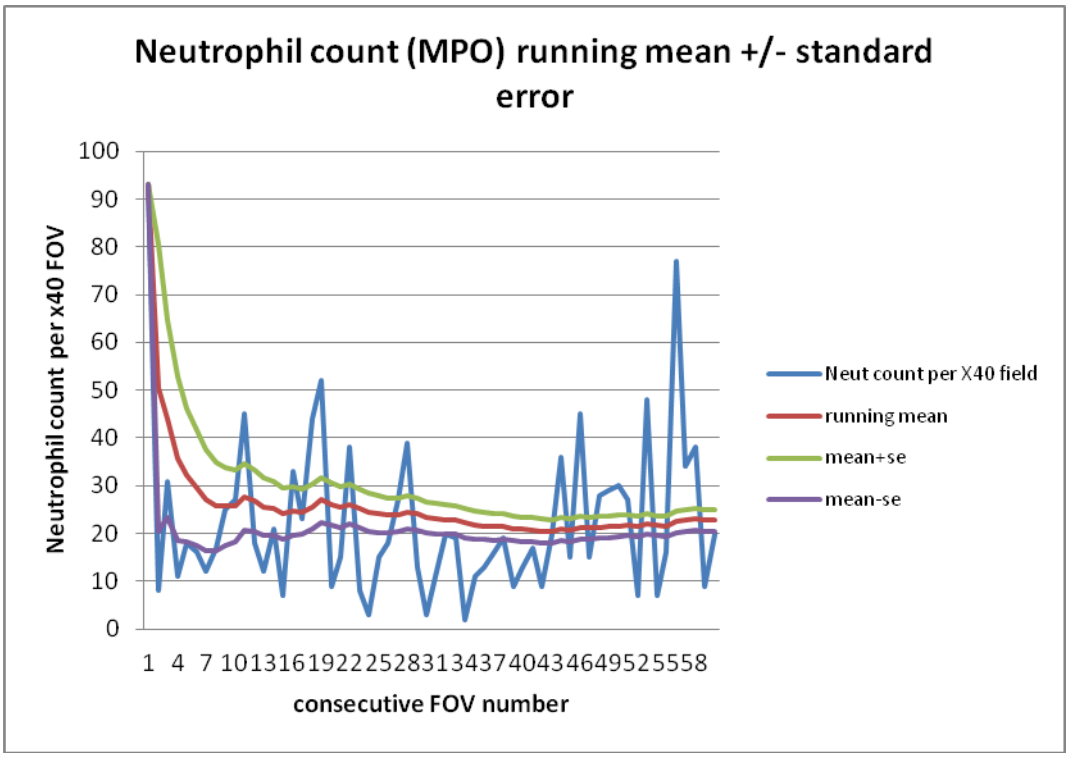
a)



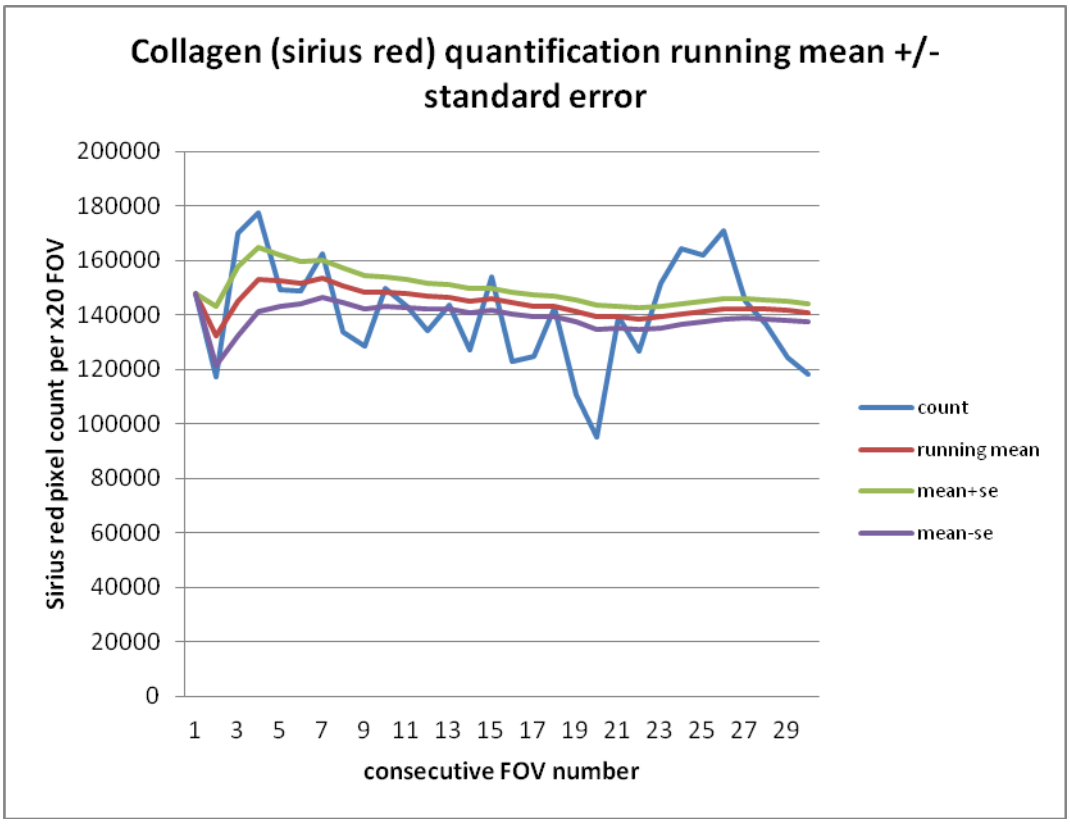
b)



c)


Neutrophil count (MPO) running mean +/- standard error
Neutrophil count per x40 FOV
100
90
80
70
60
50
40
30
20
10
0
1 4 7 10131619222528313437404346495255 58
consecutive FOV number
Neut count per X40 field
running mean
mean+se
mean-se

d)


Collagen (sirius red) quantification running mean +/- standard error
Sirius red pixel count per x20 FOV
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
consecutive FOV number
count
running mean
mean+se
mean-se

3.2 Characterising the stroma of normal pancreata and KPC PDACs

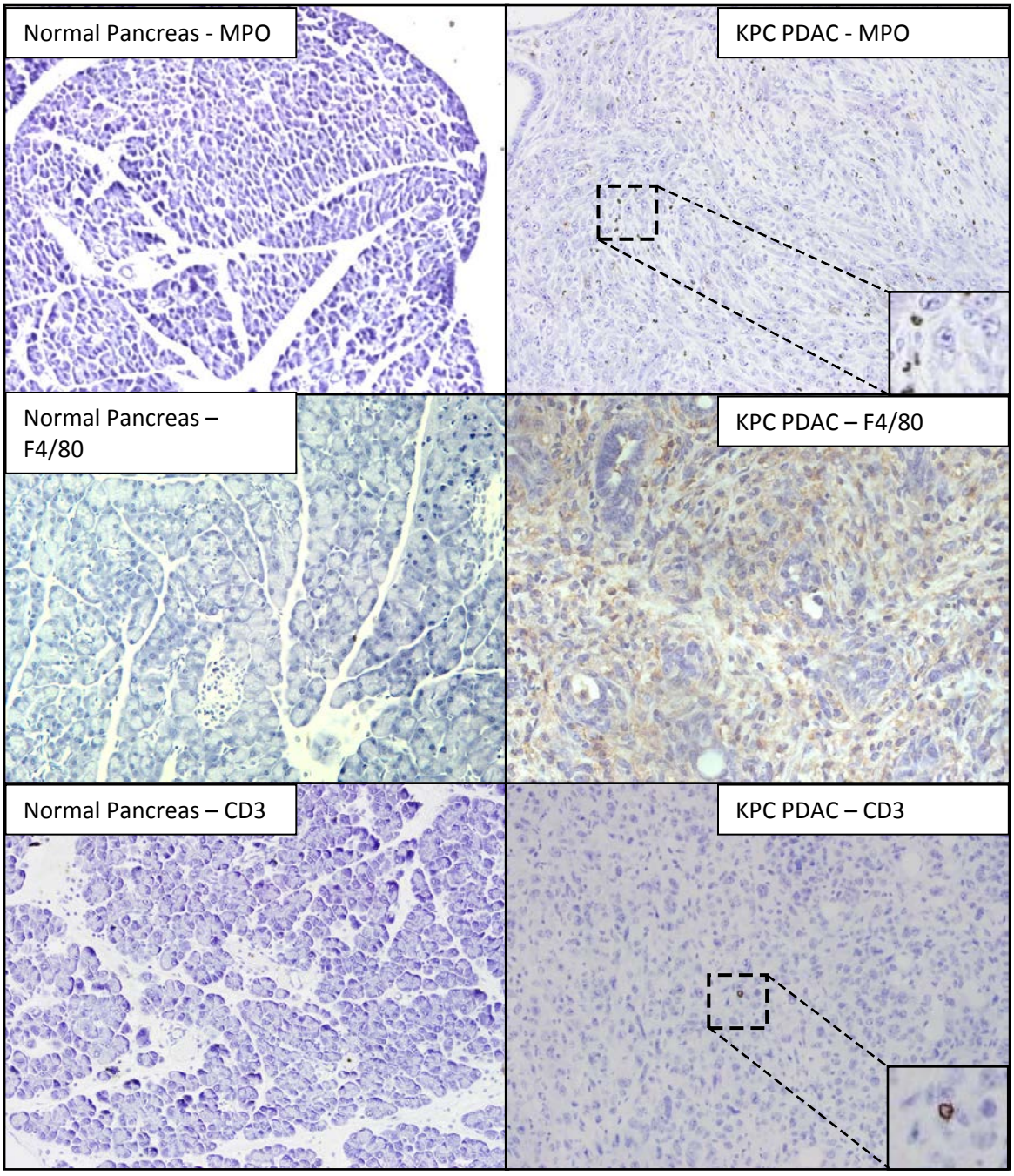
I first performed initial stromal characterisation of normal pancreata and KPC PDAC tissue and in doing so validated staining techniques and highlighted significant constituents of the KPC tumour stroma.

3.2.1 Establishing the normal presence of immune cells within normal pancreata and KPC PDAC stroma

Utilising characteristic cellular and nuclear morphology and MPO immunopositivity I have shown that, as expected, neutrophils are not a normal stromal constituent of the pancreas whereas they are present in the stroma of KPC tumours. I have also shown that in KPC PDACs as opposed to normal pancreata there are large numbers of F4/80 positive macrophages but only small numbers of CD3 positive T cells (see Fig. 3).

Figure 3: Establishing the normal presence of neutrophils (MPO), macrophages (F4/80) and T cells (CD3) within normal pancreata and KPC PDAC stroma

Representative images of MPO, F4/80 and CD3 immunostaining in normal murine pancreata and in KPC PDAC tissue. Note the lack of immune cell infiltrate in normal pancreata (n=5-8 for each stain).

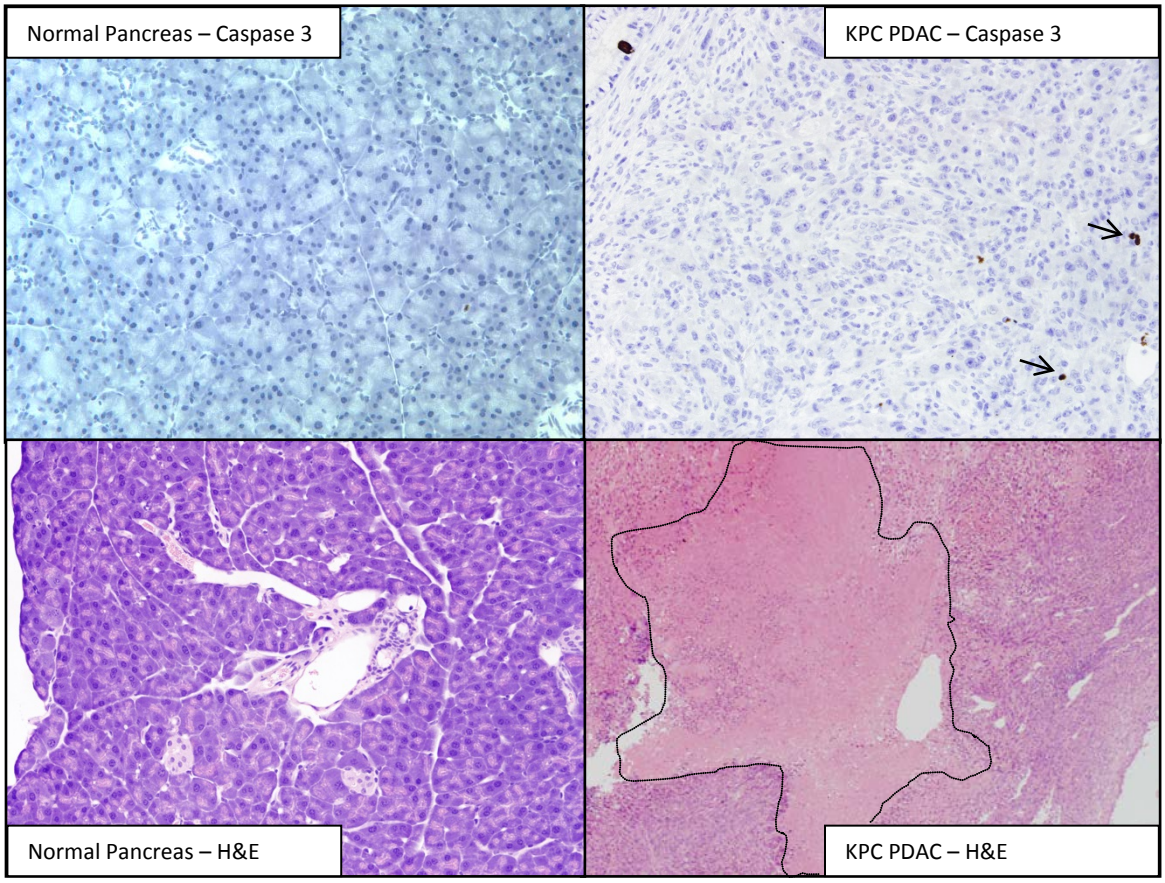


3.2.2 Determining the presence of apoptosis and necrosis in normal pancreata and KPC PDACs

Utillising the characteristic appearance of necrotic tissue and positivity of cells for cleaved caspase 3 it is apparent that apoptosis and necrosis are absent in the normal pancreas compared with KPC PDACs (see Fig. 4).

Figure 4: Determining the presence of apoptosis and necrosis in normal pancreata and KPC PDAC tissue

Representative images of cleaved-caspase 3 immunostaining (arrows) and H&E characteristics of necrosis (dashed black line) in normal pancreata and KPC PDAC tissue. Note the lack of apoptosis and necrosis in normal pancreata (n=5).



3.2.3 Characterisation of myofibroblasts/stellate cells in KPC models of PDAC

Utilising alphaSMA immunohistochemistry I have confirmed that in the normal pancreas there are small numbers of alphaSMA positive cells. These cells are predominantly perivascular smooth muscle cells as would be expected. I have also shown that there are large numbers of alphaSMA positive activated myofibroblasts in KPC PDACs (see Fig. 5). As previously discussed pancreatic stellate cells are considered to be the primary source of alphaSMA positive cells in both human PDAC and mouse models of PDAC. Unfortunately alphaSMA is not a specific marker for activated stellate cells as other cell types will also stain for this marker. These include; pericytes, smooth muscle cells and any other activated myofibroblast not originating from a pancreatic stellate cell. Due to the lack of specificity of alphaSMA as a marker of stellate cell origin I looked to use GFAP expression which is generally considered to be a more specific marker of pancreatic stellate cells. Using cell morphology, tissue location and GFAP staining of normal murine pancreata and both human and KPC PDAC tumour tissue I have shown that GFAP specifically stains both Schwann cells and pancreatic stellate cells within the normal murine pancreas (as expected for cells of neural crest origin) (see Fig. 6). I have also shown that there are GFAP positive cells present in both KPC and human PDAC tissue (see Figs 7 and 8 respectively). Using GFAP and alphaSMA staining on serial sections of KPC PDAC tissue it is also clear that there are significantly more alphaSMA positive cells than GFAP positive cells (see Fig. 9).

Figure 5: Characterisation of alphaSMA positive cells within normal pancreata and KPC PDAC tissue

Shows representative images of alphaSMA staining within normal pancreata and KPC PDAC tissue. Notice the immunopositivity of cells surrounding vessels within the normal pancreas consistent with perivascular smooth muscle cells (see arrows) (n=5).

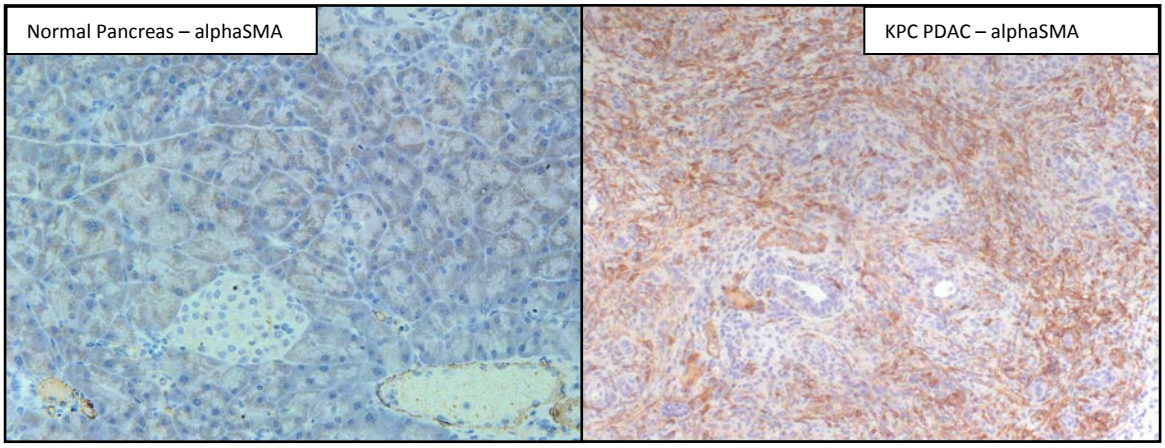


Figure 6: Stellate cells within normal murine pancreas

Shows a representative X20 magnification image of GFAP stained murine pancreatic tissue. The Inset image represents the highlighted red box showing a periacinar, GFAP positive pancreatic stellate cell (n=3). Inset shows magnified image of GFAP positive “stellate” cell in periacinar location.

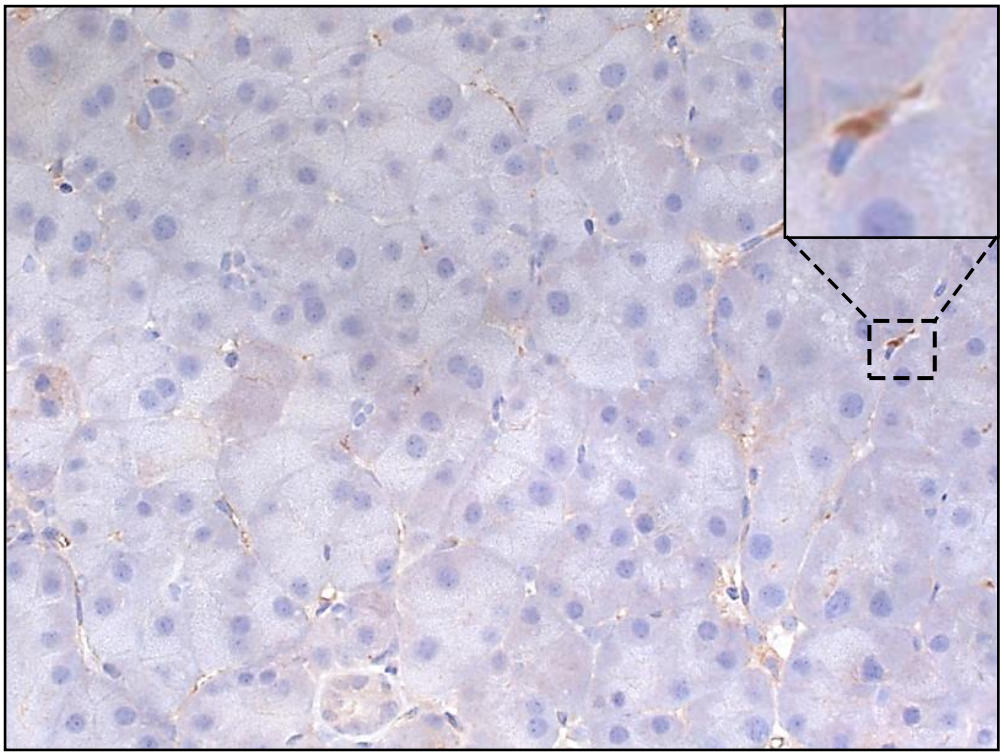


Figure 7: Stellate cells in pancreatic tumour tissue in KPC mice.

Shows representative X40 magnification images of GFAP stained tumour tissue (n=5).

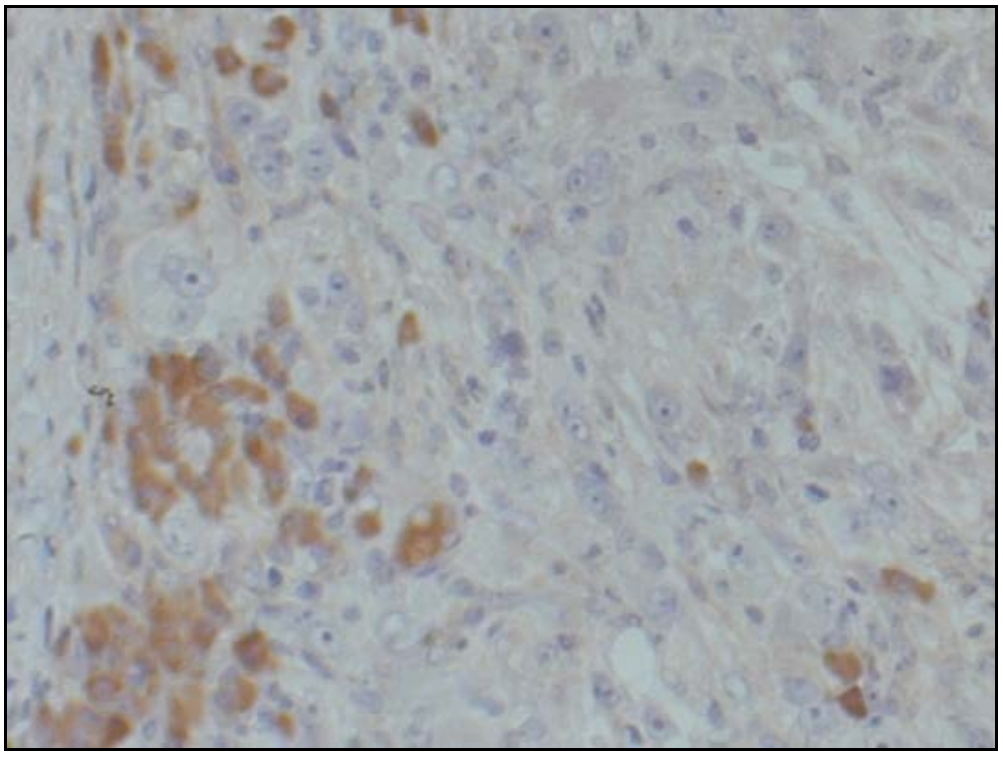


Figure 8: Stellate cells in pancreatic tumour tissue in human PDAC.
Shows representative X40 magnification images of GFAP stained human PDAC tissue (n=2). Arrow points to GFAP positive spindle shaped “stellate” cell.

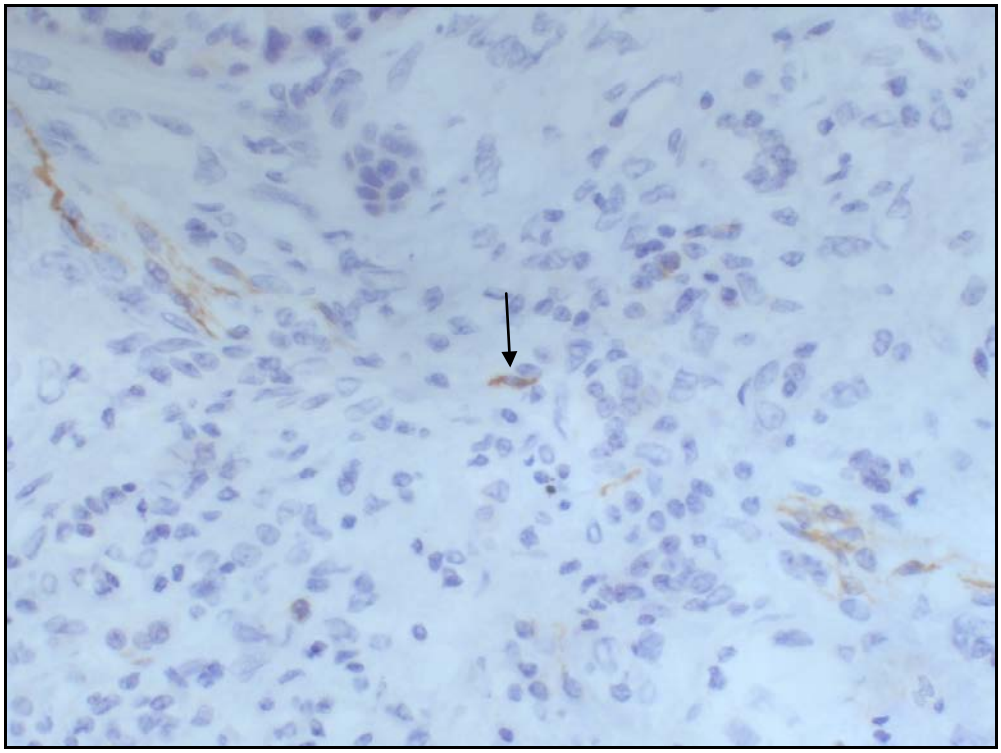
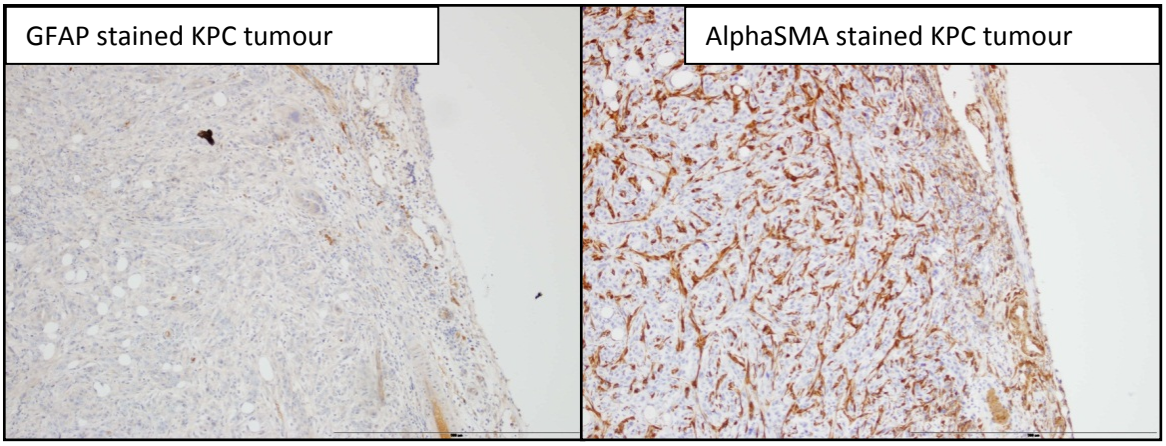


Figure 9: Stellate cells in pancreatic tumour tissue in KPC mice.
Shows representative X10 magnification images of GFAP and alphaSMA staining on consecutive tumour tissue sections (n=3).

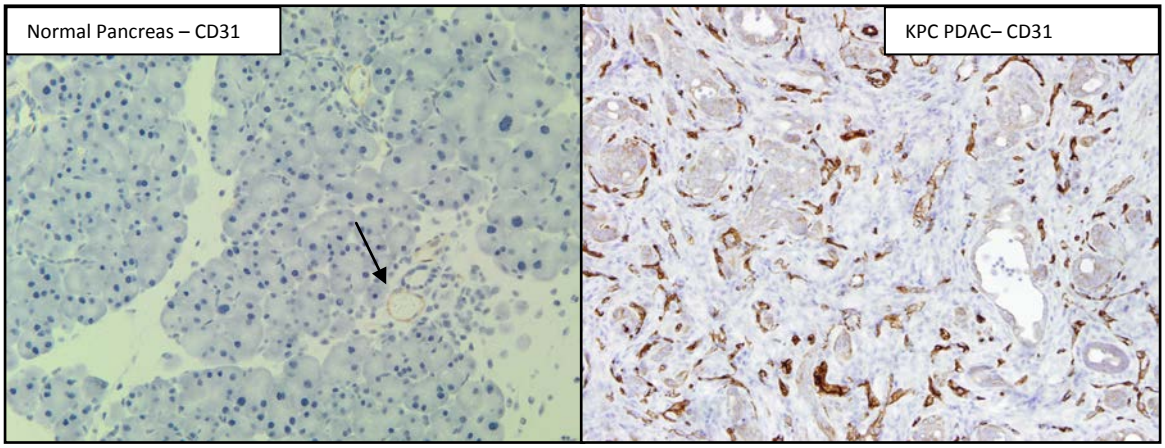


3.2.4 Establishing the presence of blood vessels in the stroma of normal pancreata and KPC PDACs

In the normal pancreas there are small to moderate numbers of well-formed blood vessels present between pancreatic acini, lobules and ducts. Within the stroma of KPC PDACs there are moderate numbers of poorly formed CD31 positive blood vessels (see Fig. 10).

Figure 10: Microvessels within normal pancreata and KPC PDAC tissue

Representative images of CD31 staining of endothelial cells of vessels within normal pancreata and KPC PDAC tissue (see arrows) (n=5-8). Arrow points to CD31 positive interlobular vessel.

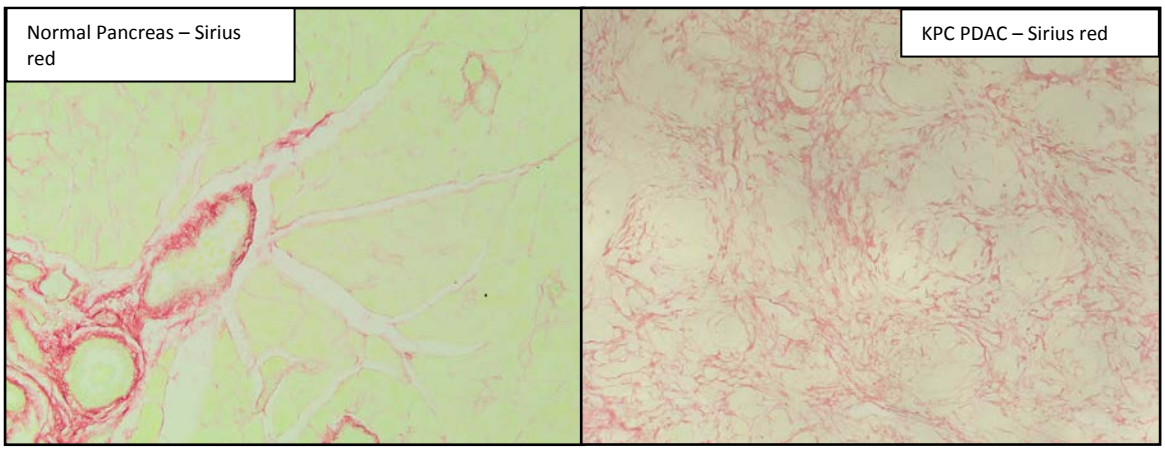


3.2.5 Establishing the presence of collagen within the stroma of normal pancreata and KPC PDACs

Sirius red staining highlights, as expected, that there are moderate amounts of collagen present within the interlobular and periductal regions of the normal pancreas. It is also clear that there are large amounts of collagen present within the stroma of KPC PDACs surrounding the neoplastic epithelium (see Fig. 11).

Figure 11: Stromal collagen in normal pancreata and KPC PDAC tissue

Representative images of Sirius red staining (collagen and elastin) of normal pancreata and KPC PDAC tissue (n=5-8)

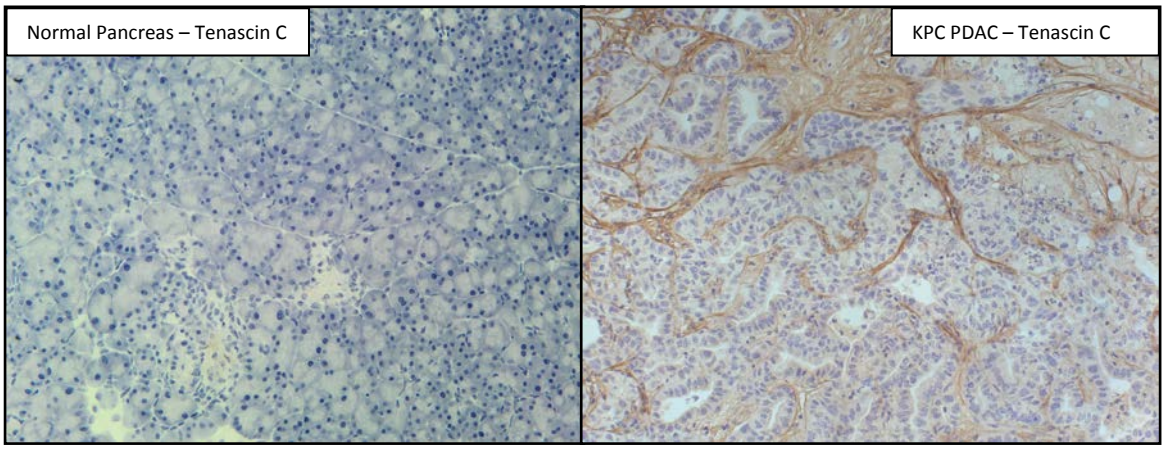


3.2.6 Establishing the presence of tenascin C within the stroma of normal pancreata and KPC PDACs

Due to the wide range of roles that tenascin C plays in the microenvironment of tumours and the importance, as shown by our lab, of tenascin C to tumour cell viability *in vitro* I next characterised the presence of tenascin C in the normal pancreas and in KPC PDACs. Through establishing and optimising an immunohistochemistry protocol for staining tenascin C in formalin fixed, paraffin embedded tissue I have shown that there is significant production of tenascin C in the stroma of KPC PDACs whereas there is limited production of tenascin C in the normal pancreas (see Fig. 12).

Figure 12: Tenascin C presence within normal pancreata and KPC PDAC tissue

Representative images showing tenascin C immunostaining in normal pancreata and KPC PDAC tissue. Notice the increased expression in the stroma of the PDAC tissue (n=5-8).



3.3 Tenascin C in Pancreatic ductal adenocarcinoma

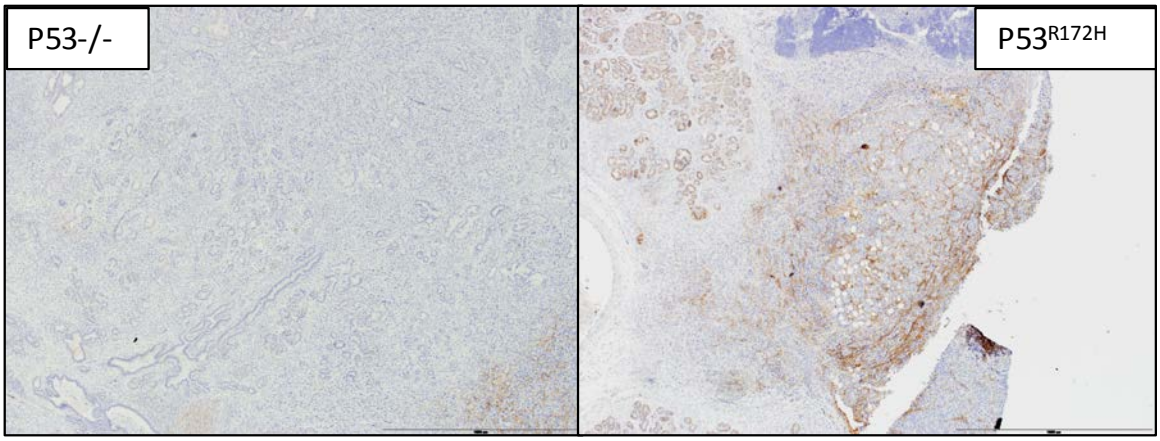
In response to our earlier findings and the wide ranging roles tenascin C plays from tumourigenesis through to metastatic spread I next looked to further characterise tenascin C expression in the murine models of PDAC available in our lab and to determine the importance of tenascin C in human PDAC

3.3.1 Mutant p53 causes an increase in intra-tumoural tenascin C expression compared with loss of p53

It has previously been shown by Jen Morton that PDAC expressing mutant p53 as opposed to loss of p53 is capable of metastatic spread (Morton, Timpson et al. 2010). Most work regarding the effects of p53 loss versus p53 mutation in PDAC has focussed on the changes to the tumour cells themselves. Due to the lack of metastasis in KPflC mice and the important role tenascin C plays in supporting

metastasis in other epithelial cancers we looked to characterise the expression of tenascin C in both the KPC and the KPflC models. We have shown that loss of p53 does not lead to production of tenascin C whereas the presence of a gain-of-function mutant p53 does (see Fig. 13).

Figure 13: Presence of gain-of-function mutant p53 as opposed to loss of p53 causes an increase in the production of tenascin-C in tumours in mice.
Shows representative X4 magnification images of tenascin-C stained p53-/- (KPflC) and $p53^{R172H}$ (KPC) tumours. There is markedly reduced tenascin-C expression in p53-/- tumours compared with $p53^{R172H}$ tumours (n=5-8).



3.3.2 Tenascin C is produced by the tumour stroma and not the tumour epithelium

Having shown high expression of tenascin C within the stroma of KPC PDAC tumours (see Figs. 12 and 13) I next looked to further characterise the production of tenascin C utilising other models of PDAC. As discussed previously, work in our lab by Bryan Miller has shown that tenascin C production by the tumour epithelium itself is vital to cell viability *in vitro*. This finding is interesting given the stromal staining pattern of tenascin C that I have demonstrated in KPC PDAC tumours (see Fig. 12) and the generally accepted view that in epithelial cancers it is stromal cells that are responsible for the production of tenascin C. Due to these contradictory findings I next used orthotopic and subcutaneous human PDAC cell line xenograft models in nude mice to confirm the source of tenascin C in PDAC. Tenascin C immunohistochemistry is species specific (see Fig. 14), therefore by utilising this species specific staining it is clear that tenascin C is not directly produced by the tumour epithelium but is produced by stromal cells (see Fig. 15). I further confirmed the finding that the stroma is responsible for the production of

tenascin C in epithelial cancers using human colorectal carcinoma HCT 116 tumour cell line subcutaneous xenografts (see Fig. 15).

Figure 14: Tenascin-C antibodies provide species specific immunohistochemical staining of human and murine tissues.
Shows representative X10 magnification images of tenascin-C stained mouse and human primary PDAC tissue with anti-mouse and anti-human tenascin-C antibodies. Note that there is no inter-species cross-reactivity.

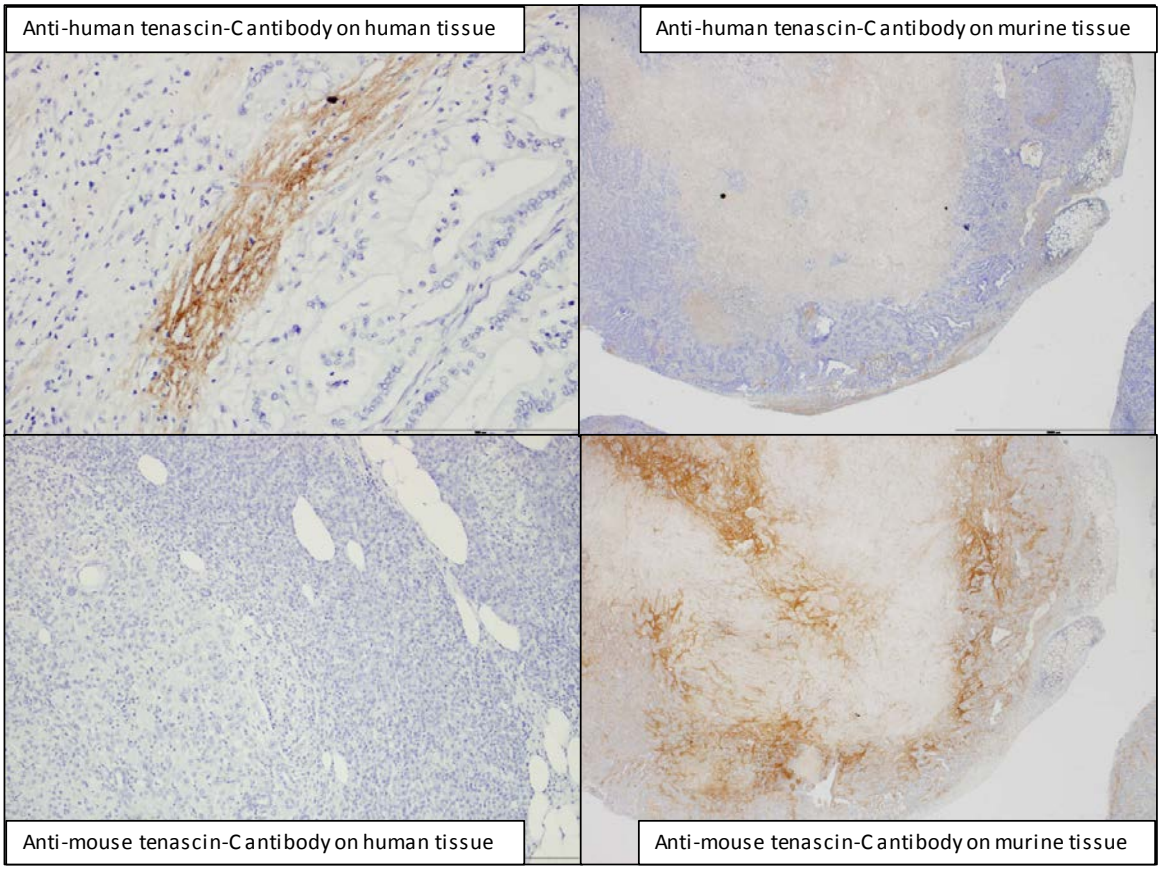
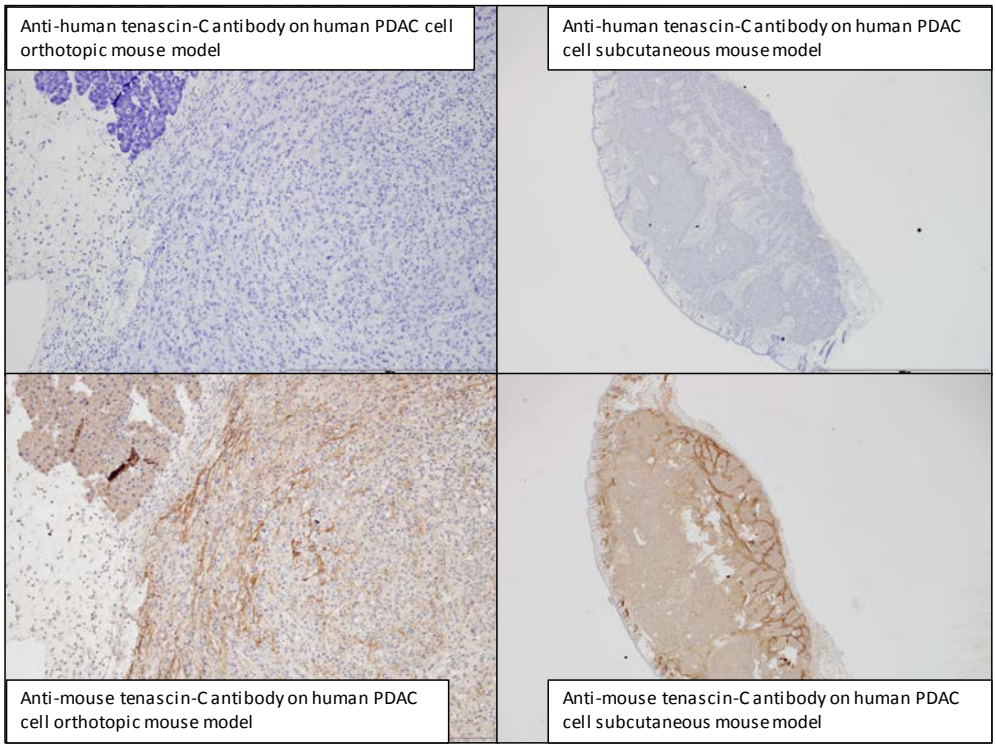


Figure 15: Species specific Tenascin-C immunohistochemistry provides evidence for the stromal production of tenascin-C.

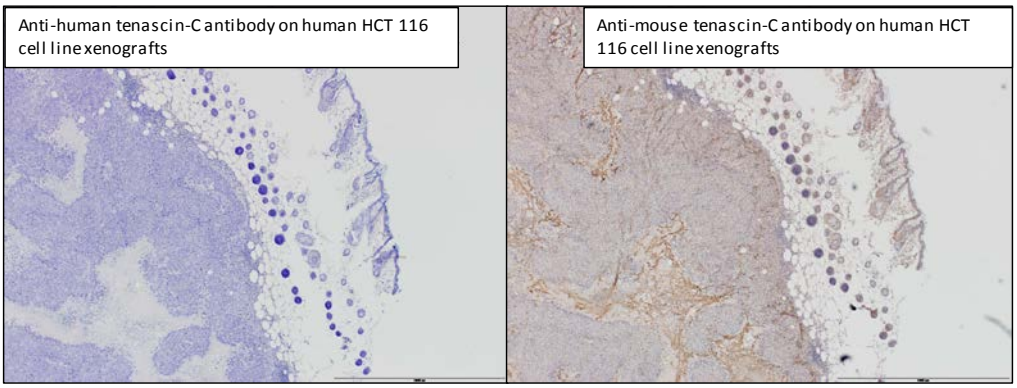
a) Shows representative X10 magnification images of anti-human and anti-mouse anti-tenascin-C stained human PDAC cell xenograft nude mouse models. Staining is only seen using the anti-mouse anti-tenascin-C staining therefore confirming tenascin-C production by the microenvironment and not the tumour cells themselves.

b) Shows representative X10 magnification images of anti-human and anti-mouse anti-tenascin-C staining in human colorectal carcinoma HCT 116 tumour cell line subcutaneous xenografts.

a)



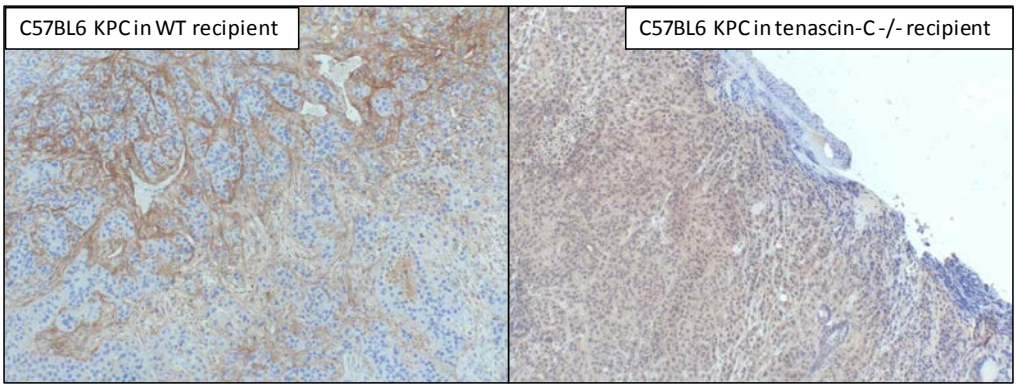
b)



3.3.3 Tenascin C is produced by the tumour epithelium in the absence of stromal production

Due to previous results in our lab and work by others showing that tenascin C production by the tumour epithelium is vital in establishment and survival of early breast cancer metastases (Oskarsson, Acharyya et al. 2011) I next looked to determine whether tenascin C could be produced by the tumour epithelium *in vivo*. I used subcutaneous allograft models of C57Bl6 KPC cell lines in syngeneic wild type (WT) and tenascin C knockout C57Bl6 mice to determine the importance of stromal tenascin C production. In this model allografted tumour cells are able to produce tenascin C but in the tenascin C knockout mice the stroma cannot. I have shown that in a WT recipient the stroma produces the tenascin C and the tumour epithelium does not stain, whereas in the tenascin C knockout recipient the stroma is unable to produce tenascin C and we see production of tenascin C by the tumour epithelium itself (see Fig. 16)

Figure 16: Tenascin-C expression is predominantly stromal however it may also be expressed within the tumour epithelium.
Shows representative X10 magnification images of tenascin-C stained C57BL/6 KPC cell line subcutaneous allografts in WT and tenascin-C -/- mice. Note the stromal expression in the wild type recipients and the tumoural expression in the tenascin-C -/- recipient.



3.4 Tenascin C and its importance in human PDAC

Having shown the importance of tenascin C production in murine models of PDAC I next looked to confirm the production of tenascin C and determine its importance in human PDAC.

3.4.1 Tenascin C is produced by the stroma of human PDAC and high levels of expression correlate significantly with survival

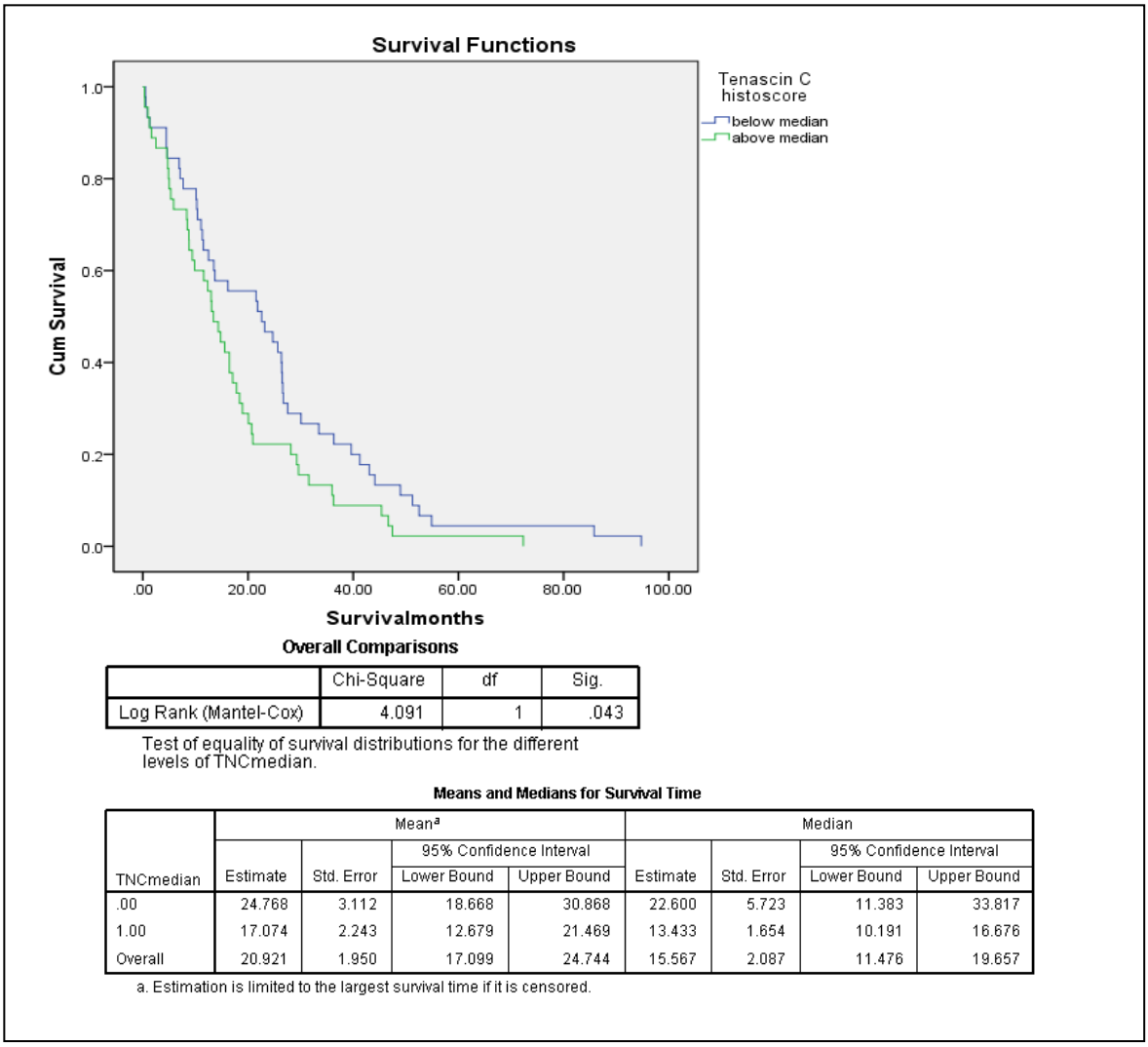
Previous studies have found increased expression of tenascin C in acute and chronic pancreatitis, early PanIN lesions and PDAC in humans. Tenascin C expression has also been shown to correlate with differentiation of PDAC (Juuti, Nordling et al. 2004; Esposito, Penzel et al. 2006). Utilising a human PDAC tissue microarray I have shown that tenascin C is a significant stromal constituent and that expression significantly correlates with survival (Log-Rank p=0.043). Also patients expressing high levels of stromal tenascin C (n=59) had a median survival of 13.4 months whereas patients expressing low levels of tenascin C (n=59) had nearly a 50% increase in survival with a median survival of 22.6 months (see Fig. 17). Immunohistochemical staining of this TMA also confirms that tenascin C production in these tumours is exclusively stromal and highly variable (see Fig. 17).

Figure 17: Tenascin-C expression is significantly associated with survival in human PDAC.

a) Kaplan-Meier survival analysis. There was a significant difference in survival between the survival of patients with tenascin-C expression above or below the median as determined by TMA histoscore (Log-Rank p=<0.043, n=118).

b) Representative images of tenascin C immunohistochemistry in human PDAC TMA samples highlighting high and low stromal staining.

a)



Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	4.091	1	.043

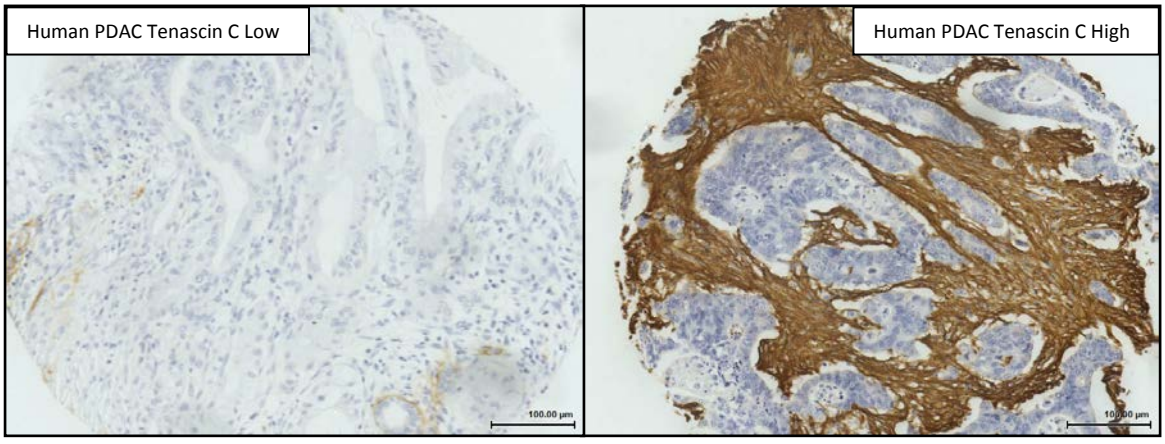
Test of equality of survival distributions for the different levels of TNCmedian.

Means and Medians for Survival Time

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
TNCmedian	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
.00	24.768	3.112	18.668	30.868	22.600	5.723	11.383	33.817
1.00	17.074	2.243	12.679	21.469	13.433	1.654	10.191	16.676
Overall	20.921	1.950	17.099	24.744	15.567	2.087	11.476	19.657

a. Estimation is limited to the largest survival time if it is censored.

b)



3.4.2 Tenascin C expression strongly correlates with hypoxia in human PDAC

Previous studies have found increased production of tenascin C in states of hypoxia (Jones and Jones 2000). In my analysis of the human PDAC TMA there is a strong positive correlation between the transcription factor Hypoxia-inducible factor 1-alpha (Hif1α) and tenascin C levels in human disease (p=0.018, see Fig. 18).

3.4.3 Tenascin C expression strongly correlates with proliferation in human PDAC

Tenascin C has been shown to promote both glioblastoma and breast carcinoma cell proliferation *in vitro* and *in vivo* (Chiquet-Ehrismann, Mackie et al. 1986; Huang, Chiquet-Ehrismann et al. 2001; Fukunaga-Kalabis, Martinez et al. 2010). In my analysis of the human PDAC TMA I have shown a significant correlation between tenascin C expression and proliferation in human PDAC (p=0.045 see Fig. 18).

3.4.4 Tenascin C expression strongly correlates with integrin signalling in human PDAC

Tenascin C is known to signal through integrins a large family of cell surface receptors. Tenascin C has been shown to signal through α2β1, αvβ3, α7β1, α8β1, α9β1, α5β3 and α5β6 integrins (Sriramarao, Mendler et al. 1993; Yokosaki, Palmer et al. 1994; Varnum-Finney, Venstrom et al. 1995; Yokosaki, Monis et al. 1996). Interestingly in my analysis there is a strong correlation between tenascin C expression and αvβ6 integrin (see Fig. 18).

3.4.5 Tenascin C expression is significantly associated with both tumour grade and vascular invasion in human PDAC

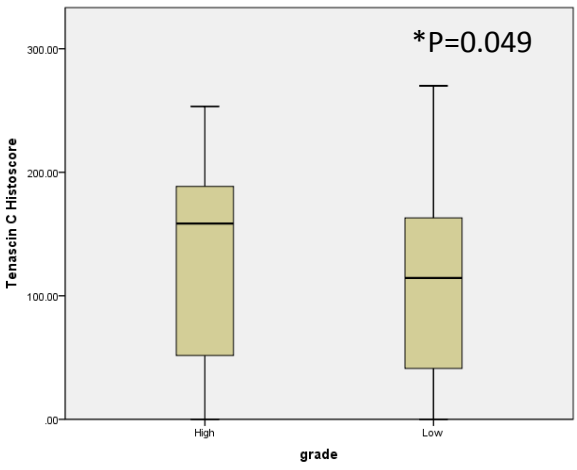
Tenascin C is considered to have important roles in both epithelial to mesenchymal transition (EMT) and tumour cell migration (Deryugina, Bourdon et al. 1996; Nishio, Kawaguchi et al. 2005; De Wever, Nguyen et al. 2004; Maschler, Grunert et al. 2004; Dandachi, Hauser-Kronberger et al. 2001; Tanaka, Sumioka et al. 2010). I have shown that high expression of tenascin C significantly

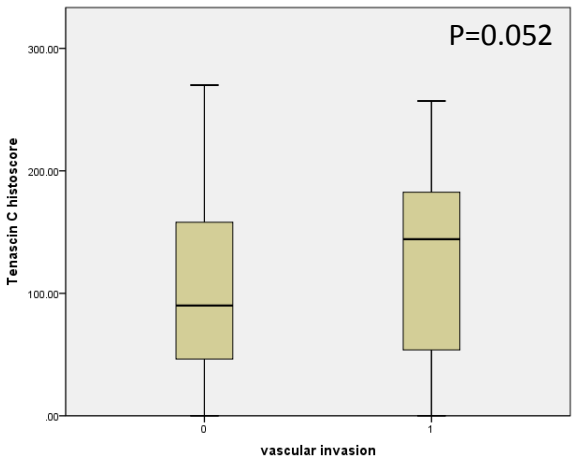
correlates with tumour grade (p=0.049) and there is also a trend towards increased vascular invasion (p=0.052) (see Fig. 19).

Figure 18: Tenascin C expression significantly correlates with proliferation, hypoxia and integrin αvβ6 in human PDAC TMA analysis.
There is a strong positive correlation with Ki67 a proliferation marker, Hif1alpha which is induced in states of hypoxia and integrin αvβ6 (n=118).

(n = 118)		
Correlates with:	**Spearman's ρ coefficient**	**Sig. (2-tailed)**
Tenascin	1	.
Ki67	0.185	0.045
nuclear Smad4	− 0.187	0.043
αvβ6 integrin	0.338	0.000
Hif1α	0.225	0.018
nucleophosmin	0.233	0.011

Figure 19: Tenascin-C expression significantly correlates with tumour grade
There is a positive correlation between tumour grade and tenascin-C expression with higher grade tumours producing more tenascin-C. There is also a trend towards tumours with higher tenascin-C expression exhibiting vascular invasion (n=118). Vascular invasion 1= invasion, 0= no invasion.

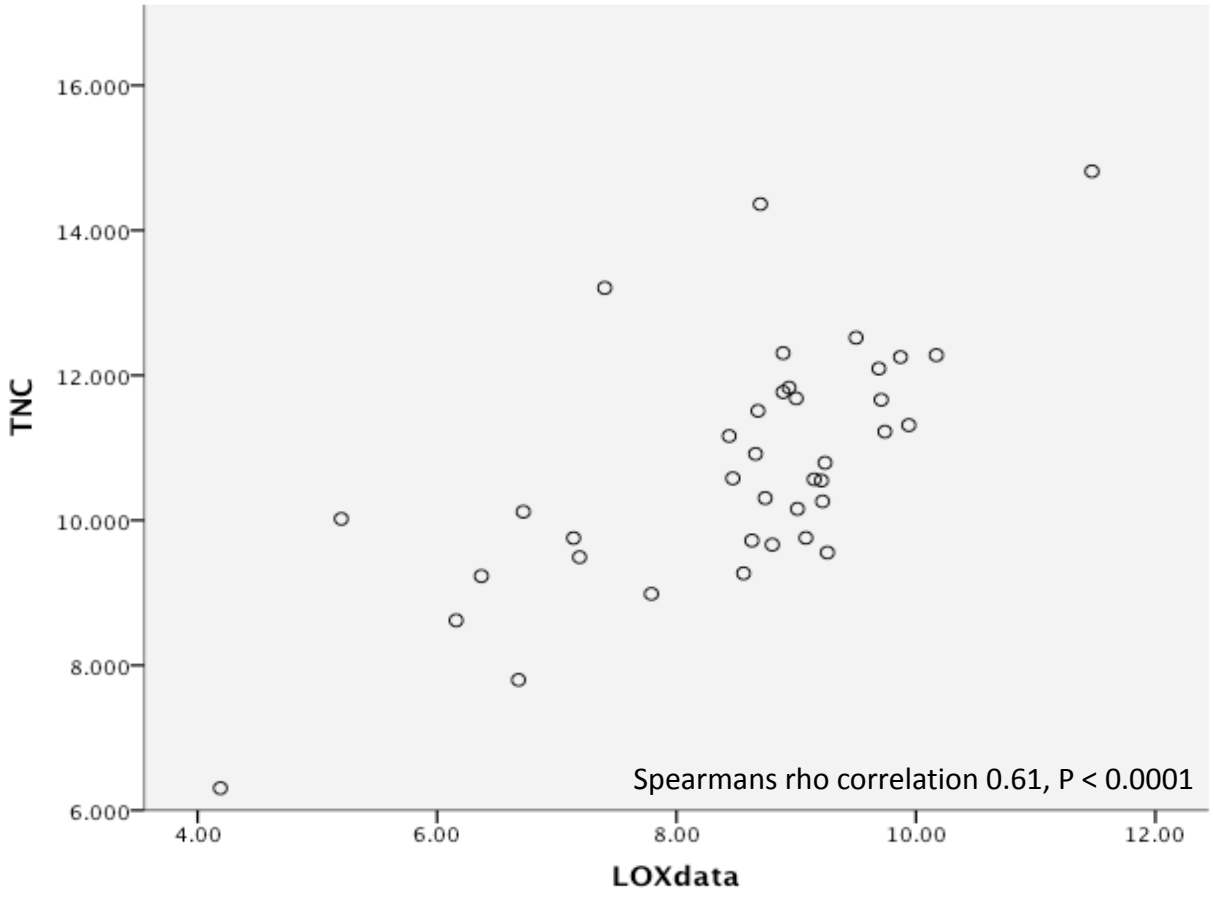




3.4.6 Tenascin C expression is significantly associated with lysyl-oxidase expression in human PDAC

Finally we have shown in a human PDAC RNA microarray that there is a significant positive correlation between lysyl-oxidase expression and tenascin C expression ($p<0.0001$ see Fig. 20).

Figure 20: Tenascin-C expression significantly correlates with lysyl-oxidase expression in human PDAC RNA microarray analysis.
There is a significant positive correlation between lox expression and tenascin-C expression in human PDAC (spearmans rho correlation 0.61, p=<0.0001).



3.5 Evaluating stromal changes elicited by stromal targeting treatments in the KPC model of PDAC

Having established reliable methods for quantifying cellular and non-cellular constituents of tumour stroma and establishing the presence and possible importance of tenascin C in PDAC we next looked to use these methods and staining protocols to evaluate stromal changes elicited in the KPC model by lysyl-oxidase inhibition and CXCR2 inhibition.

3.6 Lysyl-oxidase inhibition in the KPC model

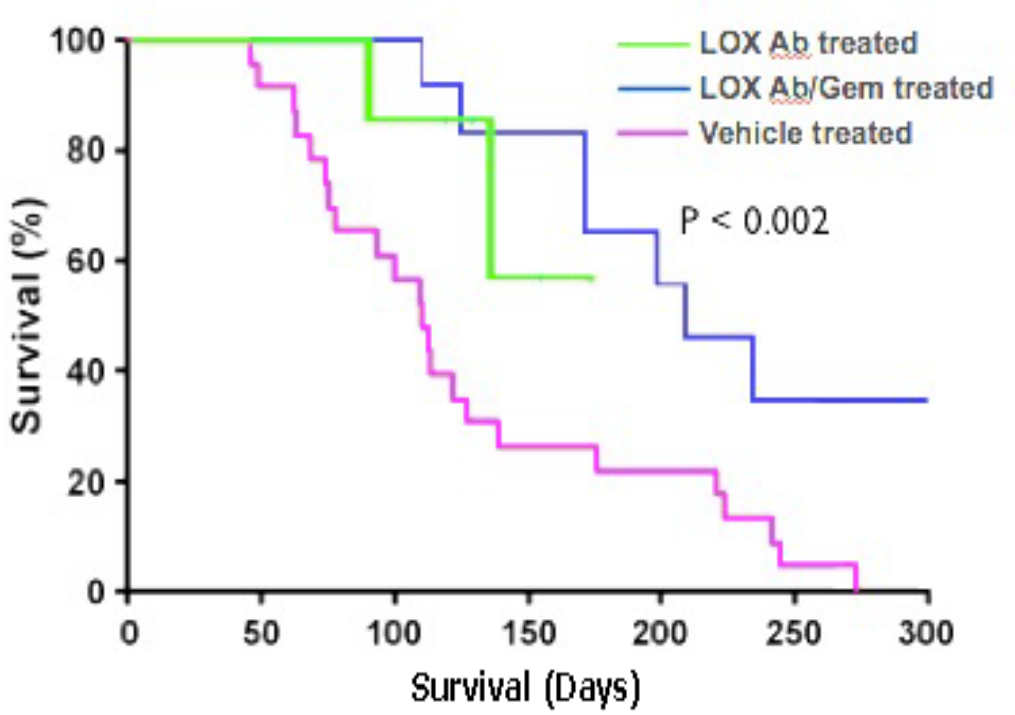
The role of lysyl-oxidase in tumour metastasis is well established, and it has been shown that inhibition of lysyl-oxidase reduces metastatic spread rather than reducing tumour initiation (Erler, Bennewith et al. 2006). In light of recent work showing that tumour stroma inhibits penetration of gemcitabine into PDAC (Olive, Jacobetz et al. 2009), and given the role that lysyl-oxidase plays in cross-linking collagen, we next determined the effect of lysyl-oxidase inhibition alone and in combination with gemcitabine on both metastatic spread and on the primary tumour itself.

3.6.1 Lysyl-oxidase inhibition delays tumorigenesis and stops metastasis

Work by Jen Morten and Bryan Miller in our lab has shown that KPC mice treated with LOX-Ab show a significant increase in survival, furthermore mice treated with the combination of gemcitabine and LOX-Ab had a further increase in survival, with a median survival of 226 days (Log-Rank $p<0.002$) (see Fig. 21). Importantly in the groups of mice treated with LOX-Ab alone or LOX-Ab/gemcitabine combination there were reduced instances of metastasis (0/8 and 2/13 respectively, compared with 9/11 treated with gemcitabine alone).

Figure 21: LOX inhibition significantly delays tumorigenesis and stops metastasis in KPC mice.

Kaplan-Meier survival analysis. There was a significant difference in survival between the LoxAB treated and the LoxAB/Gemcitabine treated cohorts (Log-Rank p=<0.002). There was also reduced incidence of metastasis noted in the Lox-Ab and Lox-Ab/Gemcitabine treatment cohorts.
(Results and figure courtesy of Jen Morton)



3.6.2 Characterising the effects of Lox-Ab and gemcitabine treatment on the KPC tumour epithelium and microenvironment

Having shown the significant effect of lysyl-oxidase inhibition on survival, alone and in combination with gemcitabine, it was next important to characterise the effects treatment was having on both the tumour epithelium and the tumour microenvironment

3.6.3 Lysyl-oxidase inhibition significantly reduces the tumoural expression of tenascin C in KPC mice

Considering the previously highlighted significant correlation between lysyl-oxidase and tenascin C expression in human PDAC we next looked to determine the effects of lysyl-oxidase inhibition on the expression of tenascin C in the KPC model of PDAC. Additionally tenascin C is produced in response to a wide variety of cellular signals such as hypoxia and in response to mechanical strain (Jones

and Jones 2000) therefore we looked to quantify tumoural tenascin C expression in Lox-Ab treated mice. In the context of lysyl-oxidase inhibition elicited by Lox-Ab treatment there is markedly decreased tenascin C expression in tumours. We have also shown that gemcitabine treatment induces increased stromal tenascin C expression when compared with vehicle treated mice. It is also clear that Lox-Ab treatment is able to inhibit the increased stromal expression of tenascin C in response to gemcitabine (see Fig. 22). Interestingly in the treatment KPC tumours treated with the combination of gemcitabine and lysyl-oxidase inhibition we also begin to see increased expression by the tumour epithelium itself as opposed to the increased stromal expression noted in vehicle or gemcitabine alone treated KPC mice (see Fig. 23).

Figure 22: Lysyl-oxidase inhibition significantly reduces the expression of tenascin-C in KPC tumours.

Representative x4 magnification images of tenascin-C staining of tumour tissues.
Lox inhibition markedly decreases the expression of tenascin-C in Lox treated KPC tumours.
Gemcitabine is also noted to markedly increase expression of tenascin-C, a change that is not noted when used in conjunction with Lox inhibition.

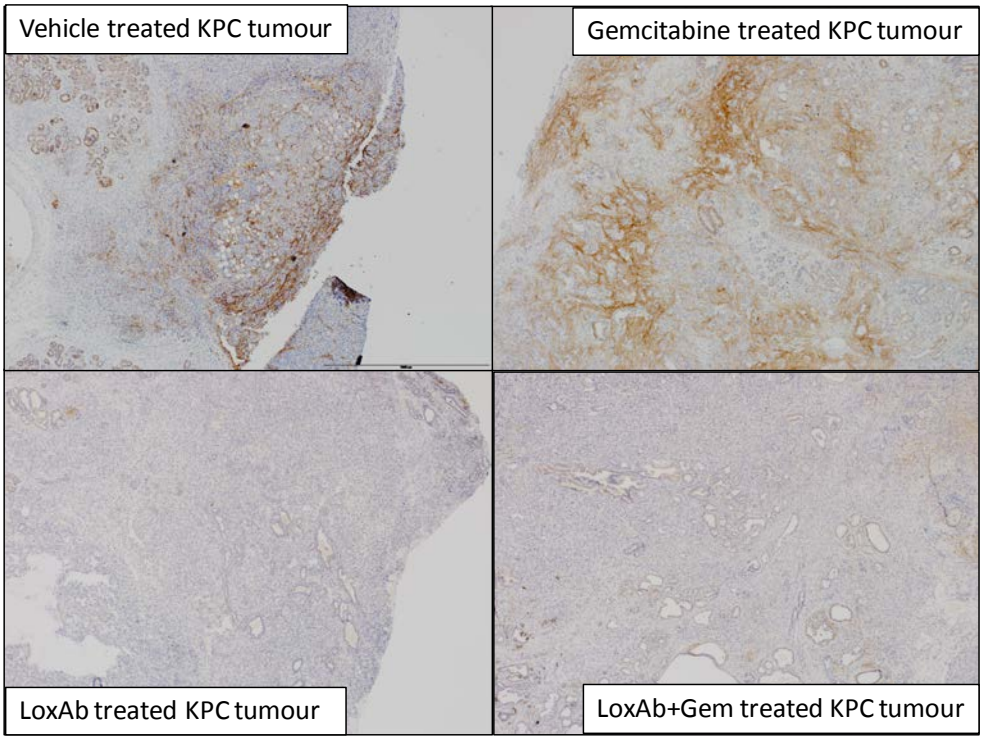
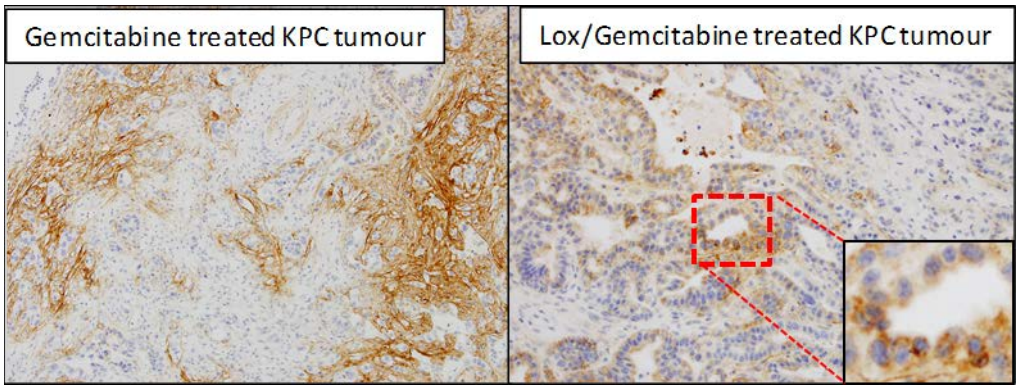


Figure 23: Tenascin-C expression is predominantly stromal however it may also be expressed within the tumour epithelium.
Shows representative X20 magnification images of tenascin-C stained gemcitabine treated and lox/gemcitabine treated KPC tumours. Note the stromal staining pattern in the gemcitabine treated tumour compared to the mild cytoplasmic staining of tumour cells in the Lox/gemcitabine treated tumour.



3.6.4 Lysyl-oxidase inhibition significantly increases intra-tumoural microvessel density

Given the decrease in the stromal tenascin C expression caused by Lox-Ab treatment we looked to determine whether there was an effect on the tumour microvasculature. Amy Au in our lab has shown that there is a significant increase in intra-tumoural microvasculature in Lox-Ab and Lox-AB/gemcitabine combination treatment groups compared to vehicle treated and gemcitabine alone (data not shown).

3.6.5 Lysyl-oxidase inhibition in conjunction with gemcitabine treatment significantly increases intra-tumoural necrosis but has no significant effect on apoptosis

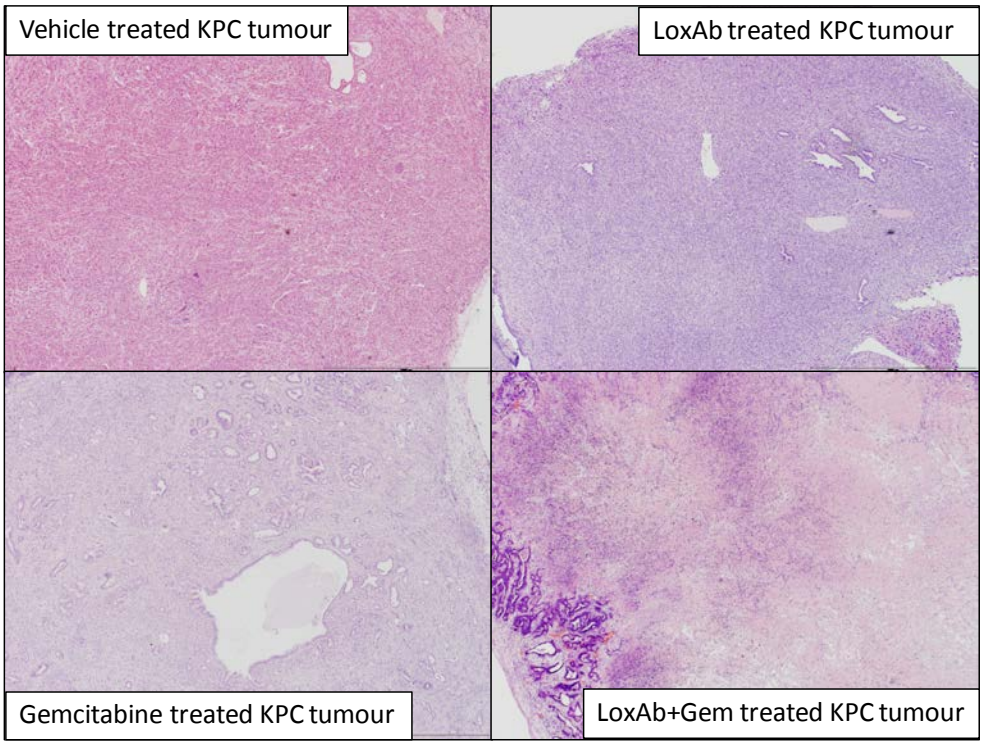
Work done in the lab by Jen Morten in KPC mice had previously shown that there was no significant change in tumour epithelium proliferation rate as assessed by Ki67 immunostaining and quantification. Given that there was no change in tumour proliferation I next characterised the changes in tumour cell survival by quantification of apoptosis and necrosis. Through quantification of intra-tumoural necrosis I have shown a significant increase in intra-tumoural necrosis in the Lox-Ab/gemcitabine combination treatment group compared with gemcitabine alone and vehicle treated mice ($p=0.034$ and 0.022 respectively, see Fig. 24). There is also a trend towards increased necrosis in the Lox-AB/gemcitabine combination treatment group compared to lysyl-oxidase inhibition alone ($p=0.101$ see Fig. 24).

Through quantification of apoptosis (positive immunostaining for cleaved caspase-3) I have shown that there is no significant alteration in apoptosis in the tumour epithelium (see Fig. 25). These findings are interesting given the increased intra-tumoural microvessel density previously noted in the Lox-Ab treatment groups.

Figure 24: Lysyl-oxidase inhibition in combination with gemcitabine treatment significantly increases intra-tumoural necrosis in KPC mice.

a) Shows representative X4 magnification images of PDAC necrosis in Lox treatment cohorts
b) Quantification of necrosis in Lox treatment cohorts. There was a significant difference in necrosis between the LoxAB/Gemcitabine treatment cohort and vehicle treated and gemcitabine alone treated cohorts (Mann-Whitney U test, p=0.022 and 0.034 respectively, group size n≥3) and a trend towards increased necrosis over Lox treatment alone.

a)



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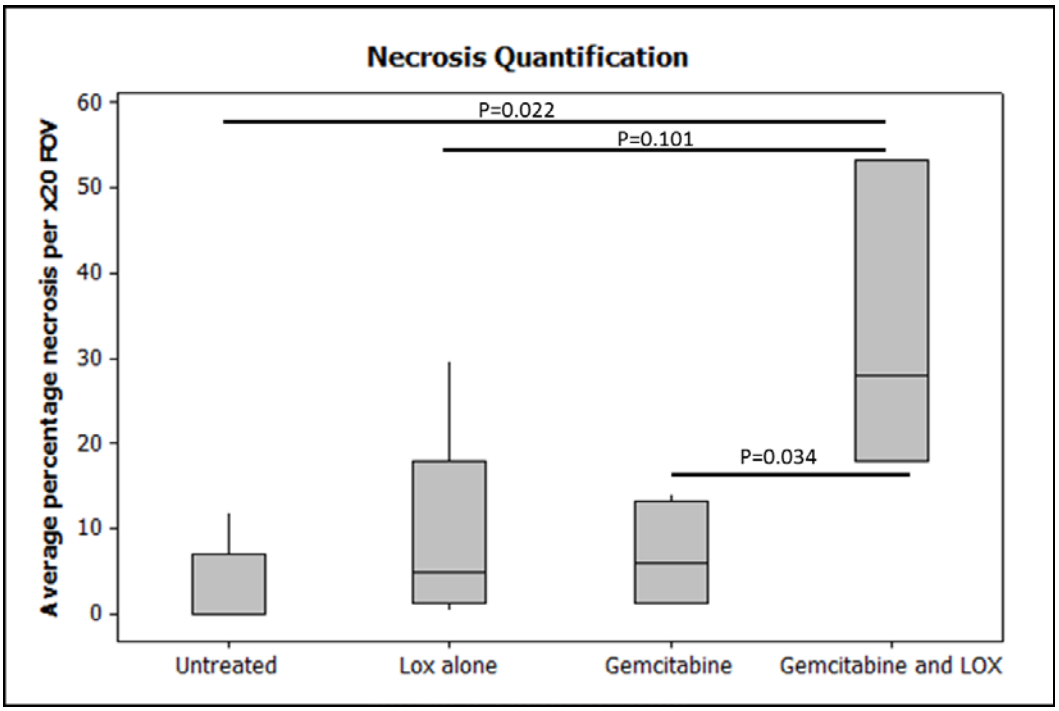
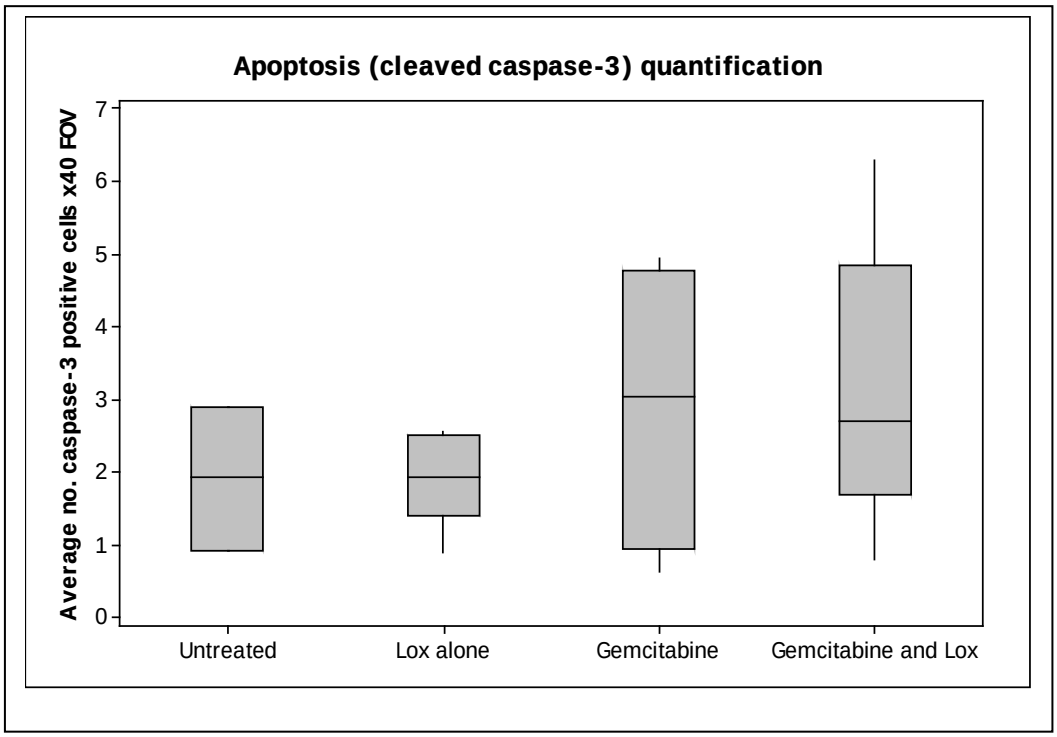


Figure 25: Lysyl-oxidase inhibition has no significant effect on apoptosis in PDAC in KPC mice

Quantification of apoptosis (cleaved Caspase-3 positive cells) in Lox treatment cohorts. There was no significant difference in apoptosis between treatments (Mann-Whitney U test, group size n≥3).



3.6.6 Lysyl-oxidase inhibition in combination with gemcitabine significantly increases intra-tumoural leukocyte infiltration

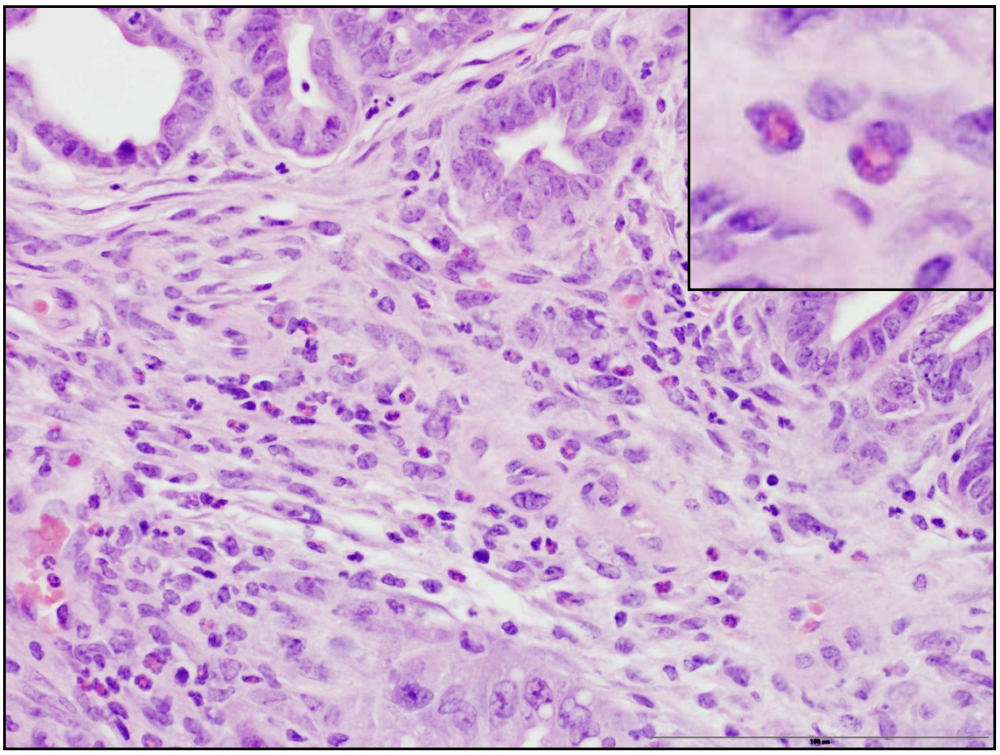
Utilising characteristic morphology and F4/80 staining I have quantified the intra-tumoural infiltrate of neutrophils and macrophages respectively. I have shown a significant increase in intra-tumoural neutrophils in the Lox-Ab/gemcitabine combination treatment group compared with gemcitabine alone and vehicle treated mice and a trend toward increased intratumoural neutrophils compared to lysyl-oxidase inhibition alone (p=0.027, 0.027 and 0.117 respectively) (see Fig. 26). There is also a significant increase in intra-tumoural infiltration of macrophages in the Lox-Ab/gemcitabine combination treatment group compared to the vehicle treated, Lox-AB alone and gemcitabine alone treatment groups (p=0.014, 0.004, 0.023 respectively) (see Fig. 27).

Figure 26: Lysyl-oxidase inhibition in combination with gemcitabine treatment significantly increases intra-tumoural neutrophil infiltrates in KPC mice.

a) Shows a representative image X40 magnification of neutrophil appearance on standard H&E stained tumour section. Inset note the characteristic nuclear shape and eosinophilic cytoplasm.

b) Quantification of intra-tumoural neutrophil numbers in Lox treatment cohorts. There was a significant difference in intra-tumoural neutrophil numbers between the LoxAB/Gemcitabine treatment cohort and vehicle treated and gemcitabine alone treated cohorts (Mann-Whitney U test, p=0.027 and 0.027 respectively, group size n≥3) and a trend to increased intra-tumoural neutrophil infiltrate over Lox treatment alone.

a)



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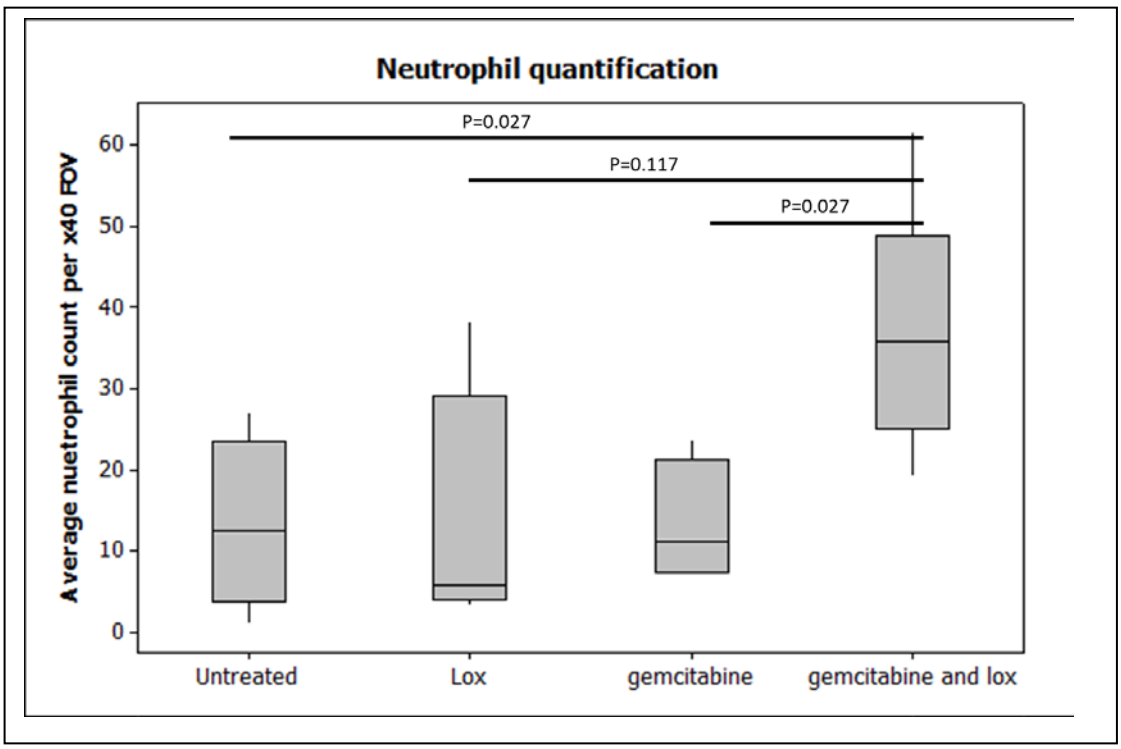
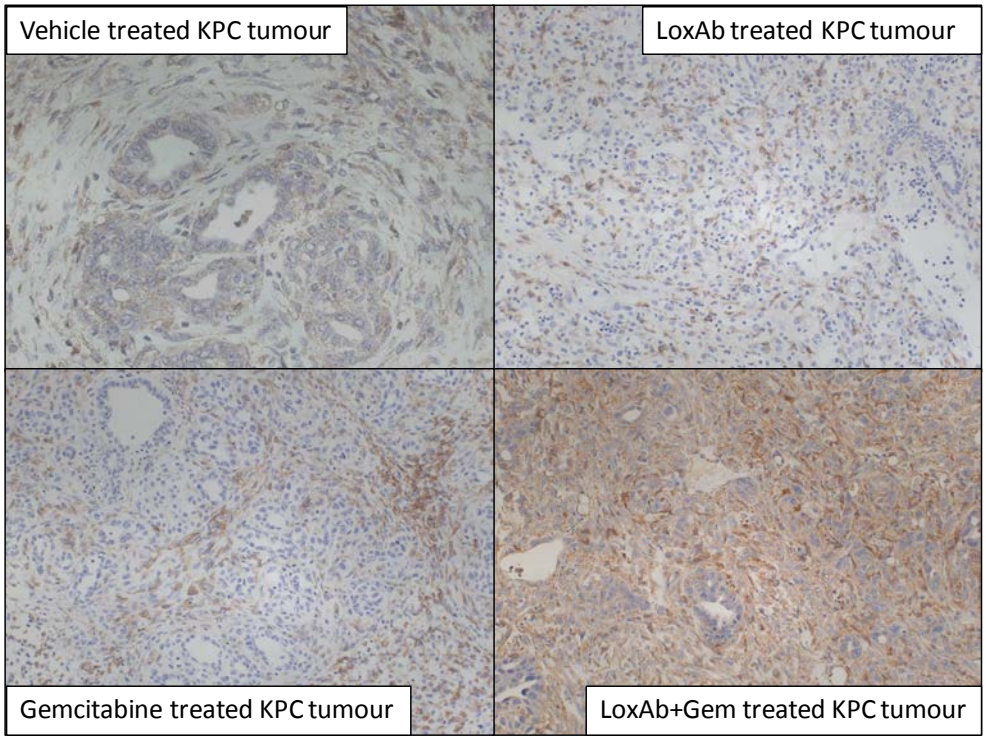


Figure 27: Lysyl-oxidase inhibition in combination with gemcitabine treatment significantly increases intra-tumoural macrophage infiltration in KPC mice.

a) Shows representative X40 magnification F4/80 stained images in Lox treatment cohorts.
b) Quantification of intra-tumoural macrophage infiltration in Lox treatment cohorts by pixel count analysis of F4/80 stained tissues. There was a significant difference in intra-tumoural macrophage infiltration between the LoxAB/Gemcitabine treatment cohort and vehicle treated, LoxAB alone and gemcitabine alone treated cohorts (Mann-Whitney U test, p=0.014, p=0.004 and 0.023 respectively, group size n≥3).

a)



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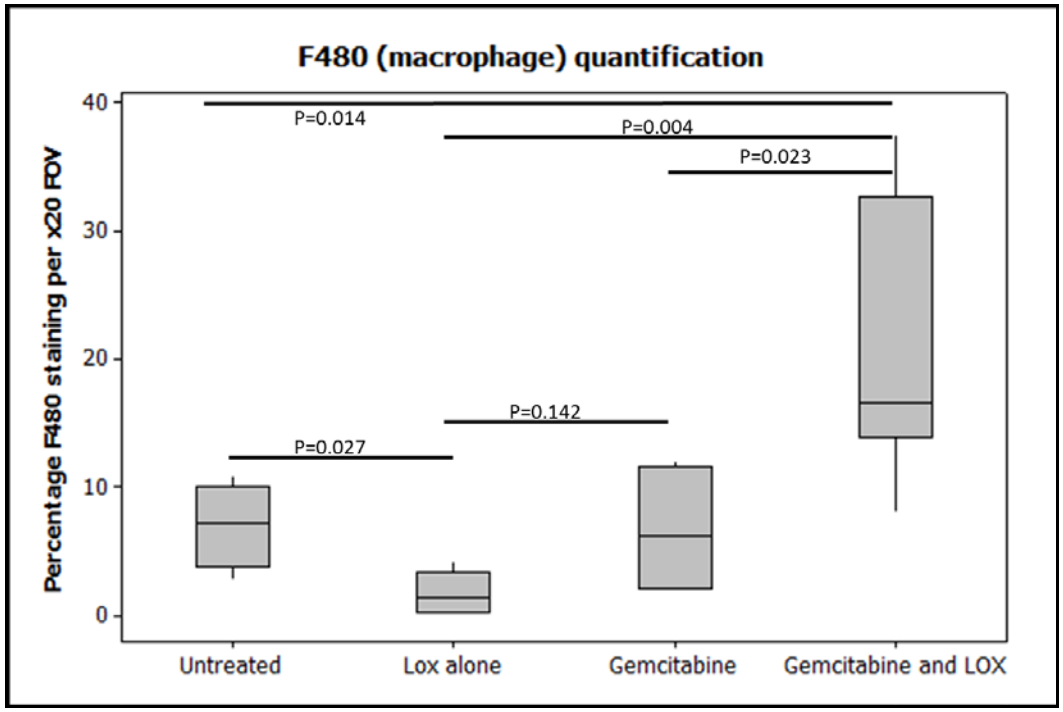


3.7 CXCR2 inhibition in the KPC model

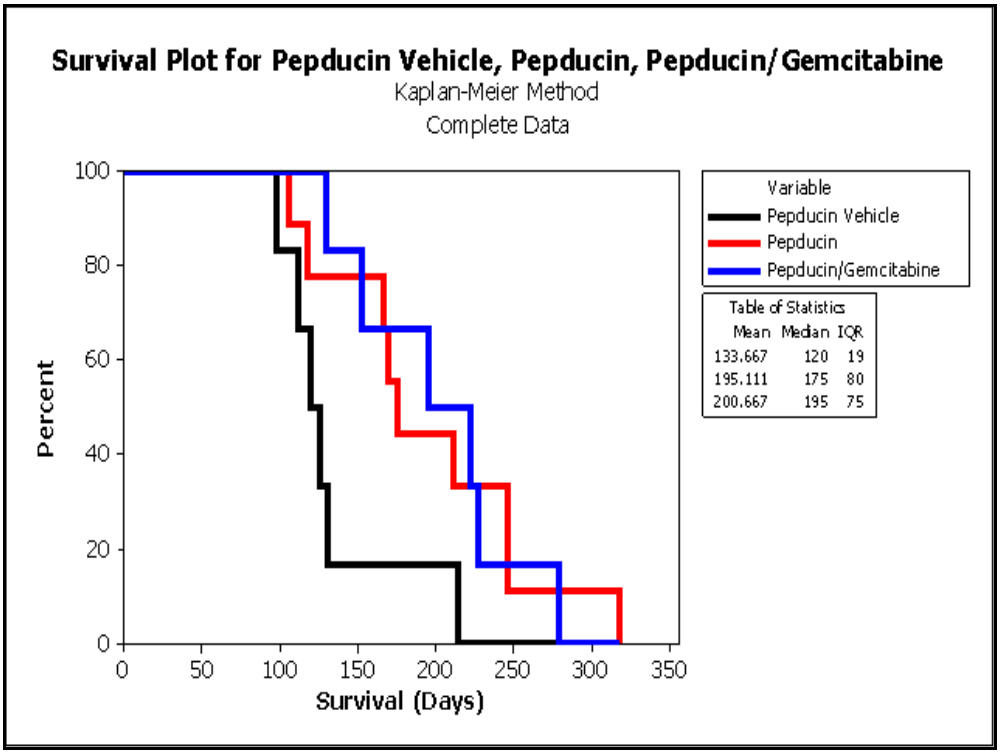
CXCR2 signalling has increasingly been implicated in cancer and inflammatory conditions (Matsuo, Ochi et al. 2009; Li, King et al. 2011; Gabellini, Trisciuoglio et al. 2009) and it is well known that inflammation may in part lead to the development of PDAC (Duell, Casella et al. 2006). CXCR2 is expressed on a wide variety of cell types including inflammatory cells, tumour epithelial cells and other stromal cells such as endothelial cells. Therefore we looked to determine the effects of CXCR2 inhibition alone and in combination with gemcitabine treatment on the both the tumour epithelium and the tumour microenvironment.

3.7.1 CXCR2 inhibition significantly increases survival in KPC mice

Work undertaken by Colin Steele in our lab has shown that pepducin treatment (inhibition of CXCR2) significantly increases survival in KPC mice (Log-Rank p=<0.002) (see Fig. 28). Furthermore it has been shown that combination of pepducin treatment with gemcitabine treatment also reduces incidence of metastasis.

Figure 28: CXCR2 inhibition significantly increases survival in KPC mice.

Kaplan-Meier survival analysis. There was a significant difference in survival between the pepducin treated and the vehicle treated cohorts (Log-Rank p=<0.002, group size n=20). There was also reduced incidence of metastasis noted in the pepducin/gemcitabine treatment cohort. (Results and figure courtesy of Colin Steele).



3.7.2 CXCR2 inhibition significantly increases intra-tumoural necrosis but has no effect on apoptosis

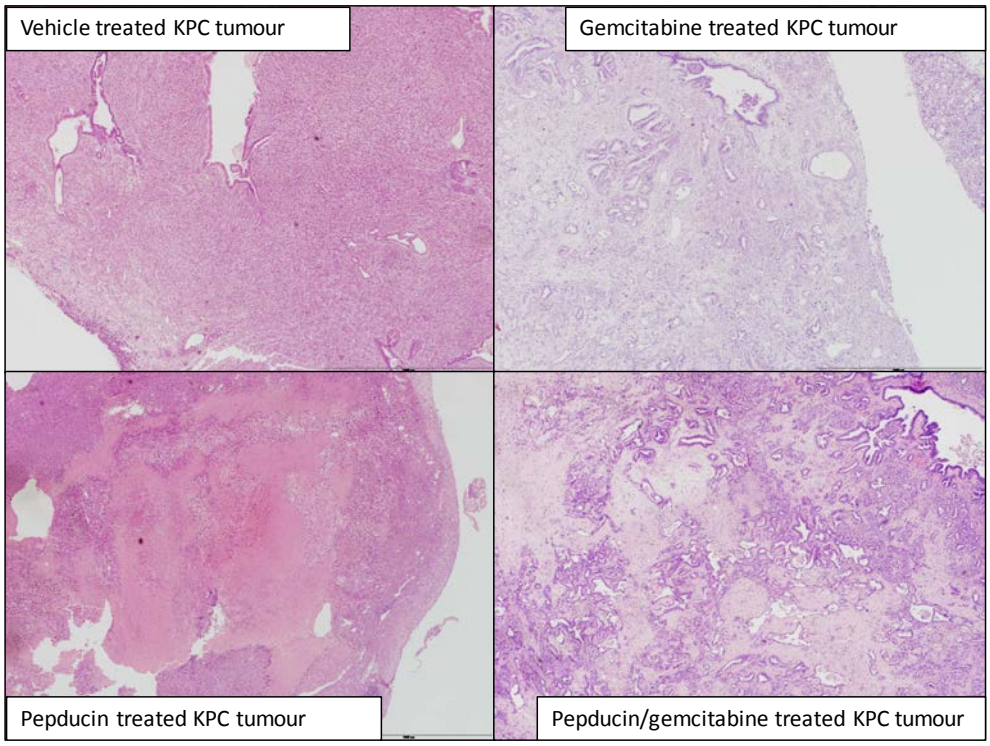
Due to the increase in survival we first characterised the effects of CXCR2 inhibition on the tumour epithelium itself. Work done in the lab by Colin Steele had previously highlighted that there was no significant change in cell proliferation rate as assessed by Ki67 immunostaining and quantification. Due to the lack of effect on proliferation I next characterised the changes in tumour cell viability by quantifying levels of apoptosis and necrosis. Quantification of intra-tumoural necrosis shows that pepducin treatment significantly increases intra-tumoural necrosis when compared to vehicle treatment or gemcitabine treatment alone ($p=0.007$ and 0.034 respectively). This increase in necrosis compared to vehicle was not seen in the gemcitabine alone treatment group and combination treatment with gemcitabine does not further increase intra-tumoural necrosis (see Fig. 29). Quantification of apoptotic tumour epithelium showed no significant difference between treatment groups (see Fig. 30).

Figure 29: CXCR2 inhibition significantly increases necrosis in KPC tumours.

a) Shows representative X4 magnification images of PDAC necrosis in pepducin treatment cohorts

b) Quantification of necrosis in pepducin treatment cohorts. There was a significant difference in necrosis between pepducin and pepducin/gemcitabine treatment compared with vehicle treatment (Mann-Whitney U test, p=0.007 and p=0.009 respectively, group size n≥3). There was a significant increase in necrosis in pepducin treatment alone compared to gemcitabine treatment alone (Mann-Whitney U test, p=0.034, group size n≥3) and there was a trend towards increased necrosis in the pepducin/gemcitabine treatment compared to the gemcitabine alone.

a)



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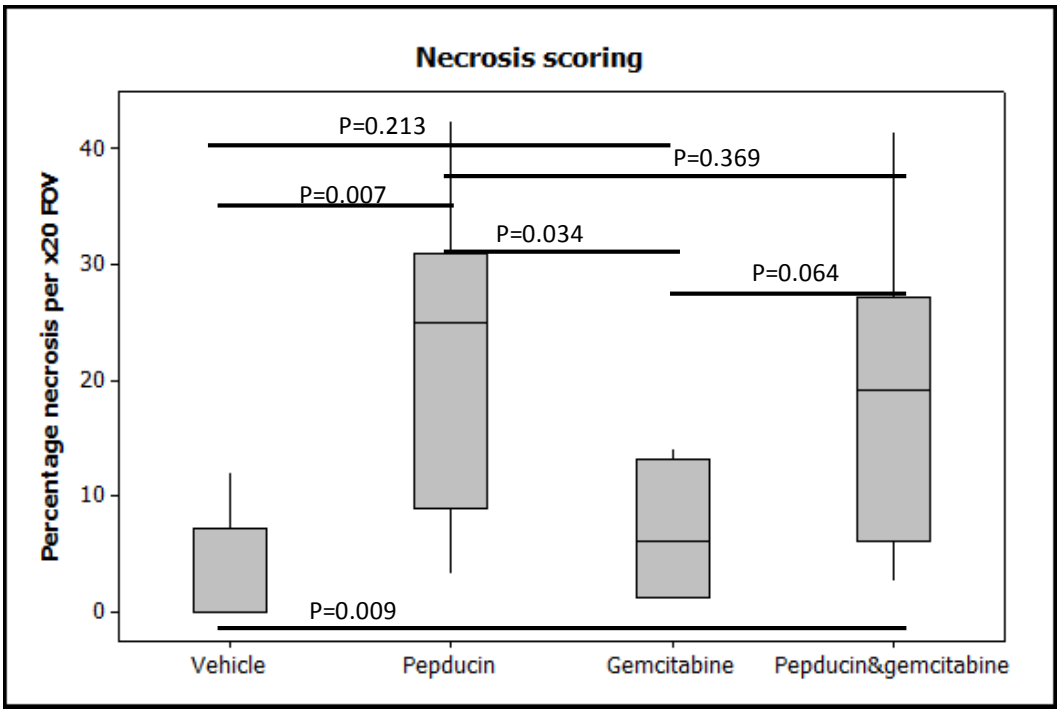
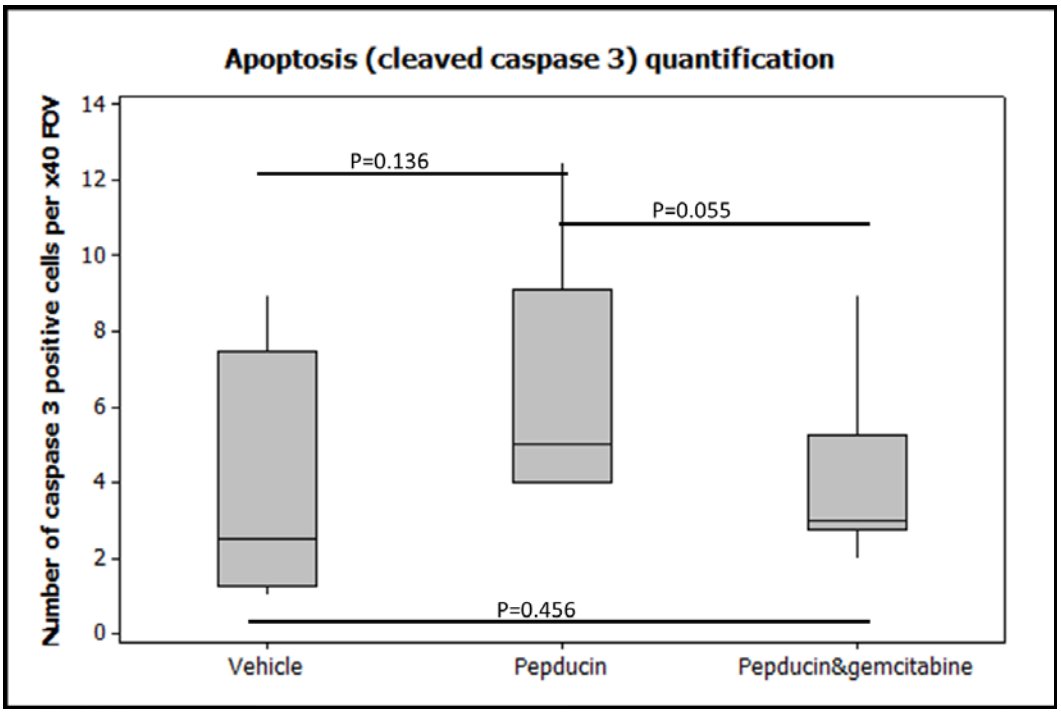


Figure 30: CXCR2 inhibition has no effect on apoptosis in KPC tumours.
Quantification of apoptosis (cleaved caspase-3 positive cells) in pepducin treatment cohorts.



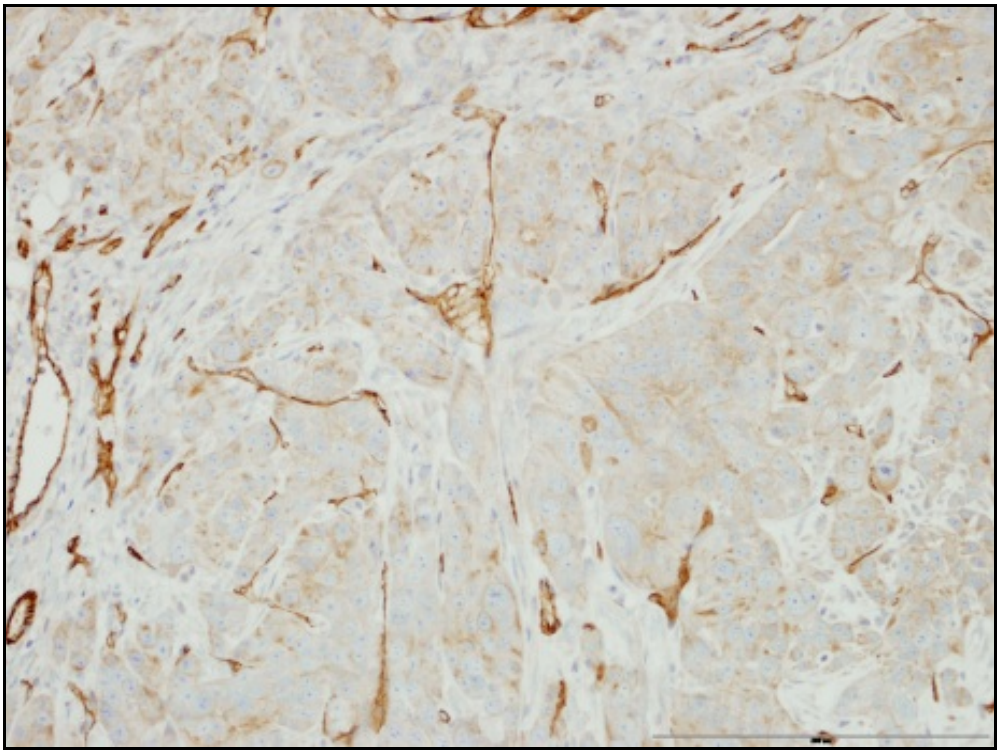
3.7.3 CXCR2 inhibition significantly reduces intra-tumoural microvessel density

Given the increase in intra-tumoural necrosis produced by pepducin alone, the inability of gemcitabine to further increase necrosis when given in combination with pepducin and the importance of CXCR2 signalling in angiogenesis (Li, Cheng et al. 2011) I next quantified intra-tumoural microvessel density. Pepducin treatment (CXCR2 inhibition) significantly decreases intra-tumoural microvessel density when compared with vehicle treatment (p=0.025) (see Fig. 31). This change was consistent and unaffected by combination of pepducin with gemcitabine treatment (p=0.025) (see Fig. 31).

Figure 31: CXCR2 inhibition significantly decreases vessel count in KPC tumours.

a) Shows a representative X20 magnification image of PDAC CD31 immunostaining in tumour tissue.
b) Quantification of vessel counts in pepducin treatment cohorts. There was a significant difference in vessel counts between pepducin and pepducin/gemcitabine treatment compared with vehicle treatment (Mann-Whitney U test, p=0.025 and p=0.025 respectively, group size n≥3) and gemcitabine alone (Mann-Whitney U test, p=0.027 and p=0.050 respectively group size n≥3).

a)



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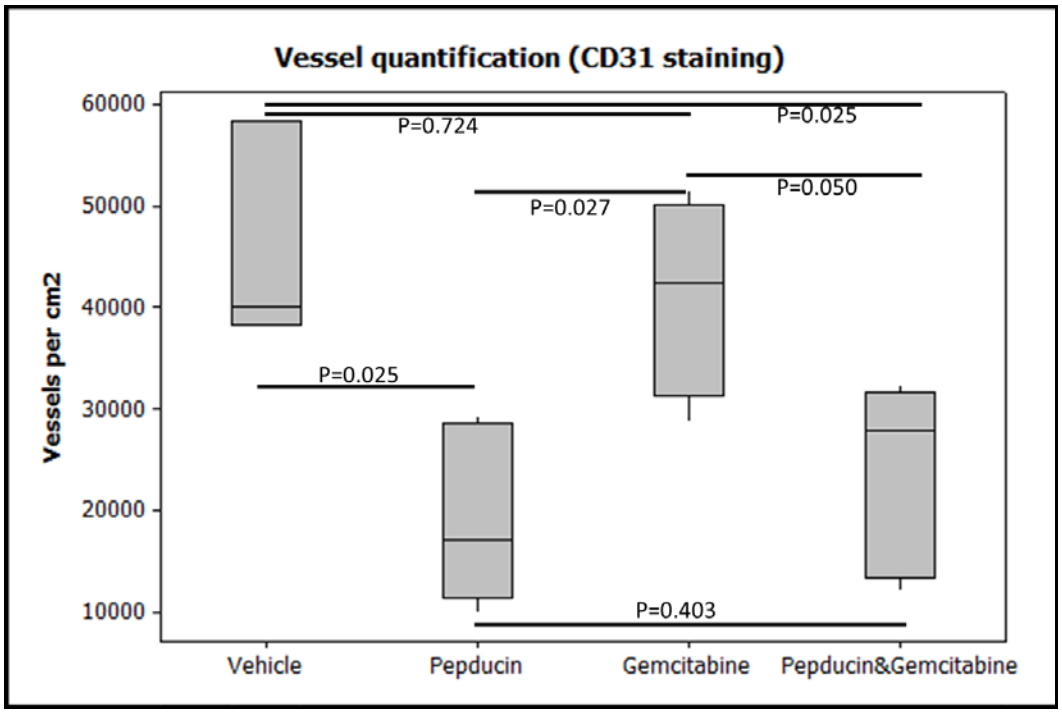
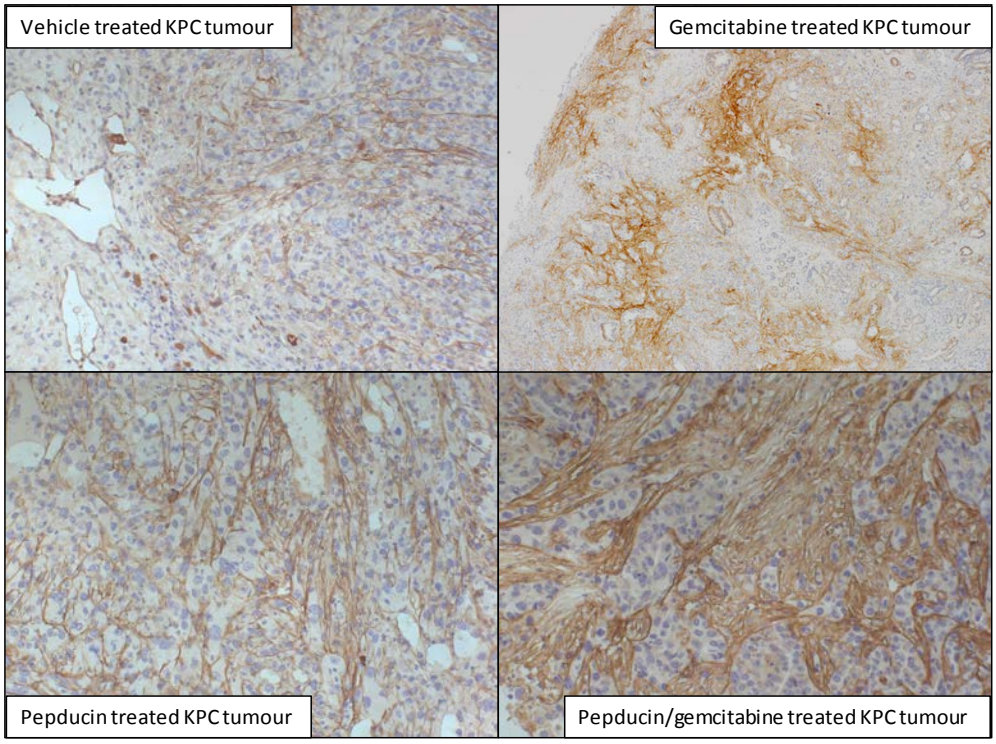


3.7.4 CXCR2 inhibition increases intra-tumoural expression of tenascin C in KPC mice

As tenascin C is expressed in situations of hypoxia, I next characterised the change elicited in tenacin C expression in response to CXCR2 inhibition. I stained for tenascin C which shows that CXCR2 inhibition leads to increased tenascin C production within the stroma of KPC tumours (see Fig. 32).

Figure 32: CXCR2 inhibition increases the expression of tenascin-C in KPC tumours.

Representative x10 magnification images of tenascin-C staining of pepducin treatment cohorts. CXCR2 inhibition markedly increases the expression of tenascin-C in pepducin treated KPC tumours. Gemcitabine treatment is also noted to markedly increase expression of tenascin-C. Pepducin and gemcitabine when given in combination have an additive effect on the increase in tenascin-C expression (group size n=5).



3.7.5 CXCR2 inhibition reduces intra-tumoural leukocyte infiltration in KPC mice

Previous studies have shown a significant reduction in neutrophil and macrophage numbers in response to CXCR2 inhibition (Ijichi, Chytil et al. 2011). In agreement with this finding I have shown that in pepducin treated KPC mice there is a non-significant trend towards decreased numbers of intra-tumoural neutrophils and macrophages (see Figs. 33, 34 and 35).

Figure 33: CXCR2 inhibition decreases neutrophil tumour infiltration in KPC mice (Haematoxylin and Eosin stain).
Quantification of neutrophils (haematoxylin and eosin stain) in pepducin treatment cohort. There was a non-significant trend towards decreased neutrophil numbers in pepducin treated tumours. This reduction was not seen when combination treatment with gemcitabine was given (Mann-Whitney U test, group size n≥3).

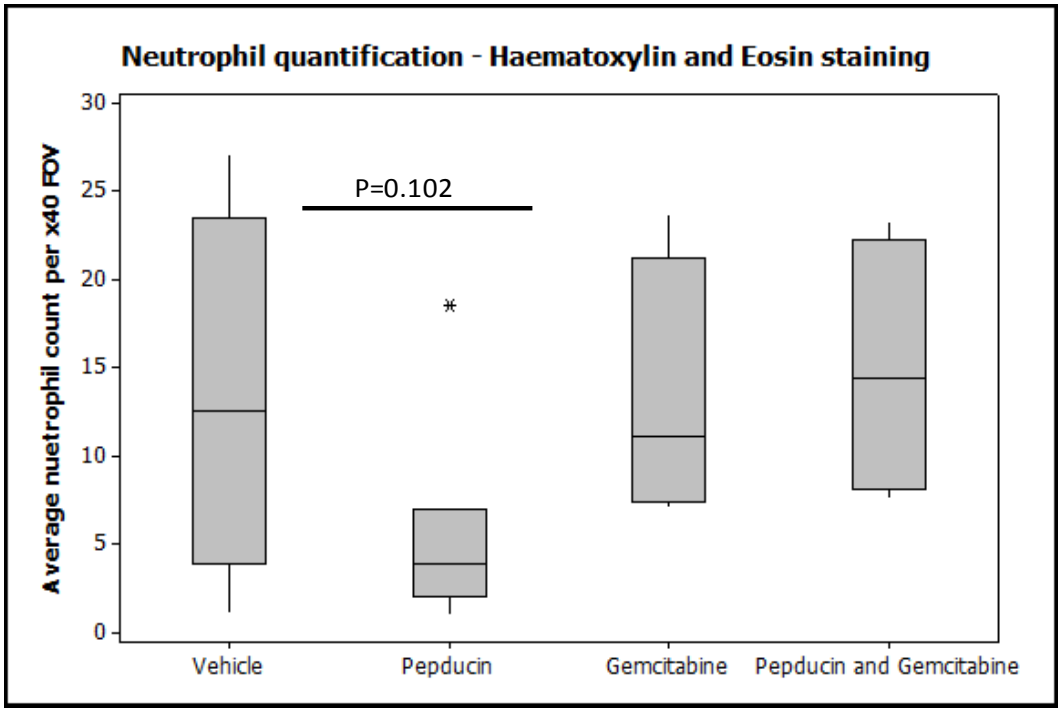
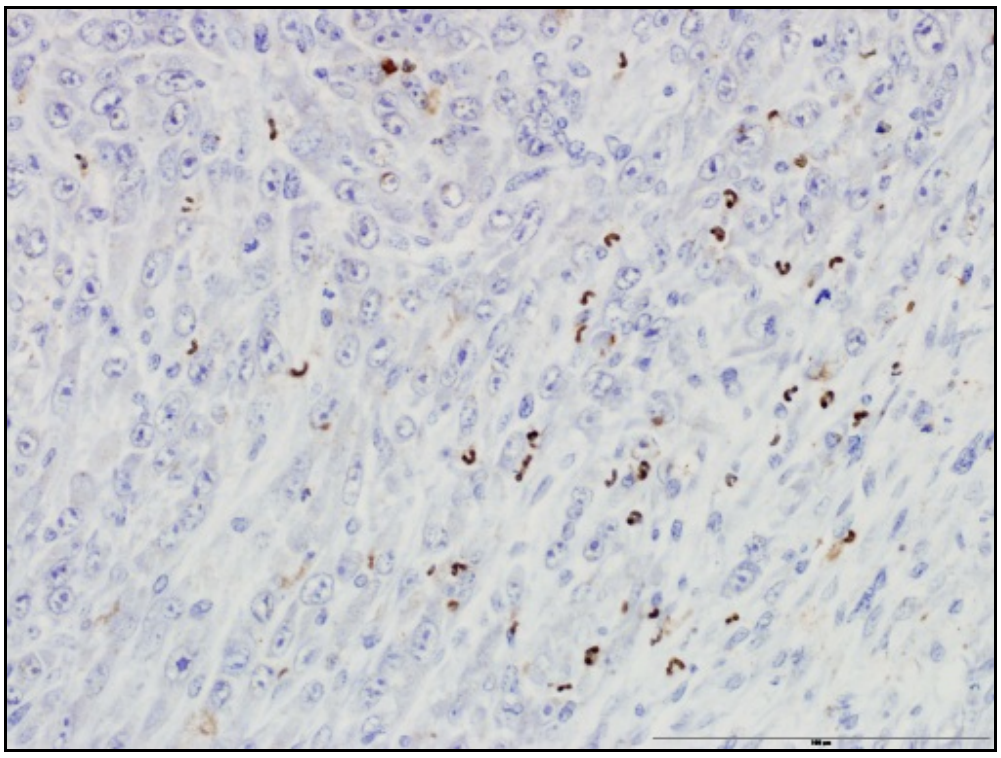


Figure 34: CXCR2 inhibition decreases neutrophil tumour infiltration in KPC mice (MPO immunostaining).

a) Shows a representative X40 magnification image of PDAC myeloperoxidase (MPO) immunostaining in tumour tissue.
b) Quantification of neutrophils (MPO positive cells) in pepducin treatment cohorts. There was a non-significant trend towards decreased neutrophil numbers in pepducin treated tumours. This reduction was not seen when combination treatment with gemcitabine was given (Mann-Whitney U test, group size n≥3).

a)



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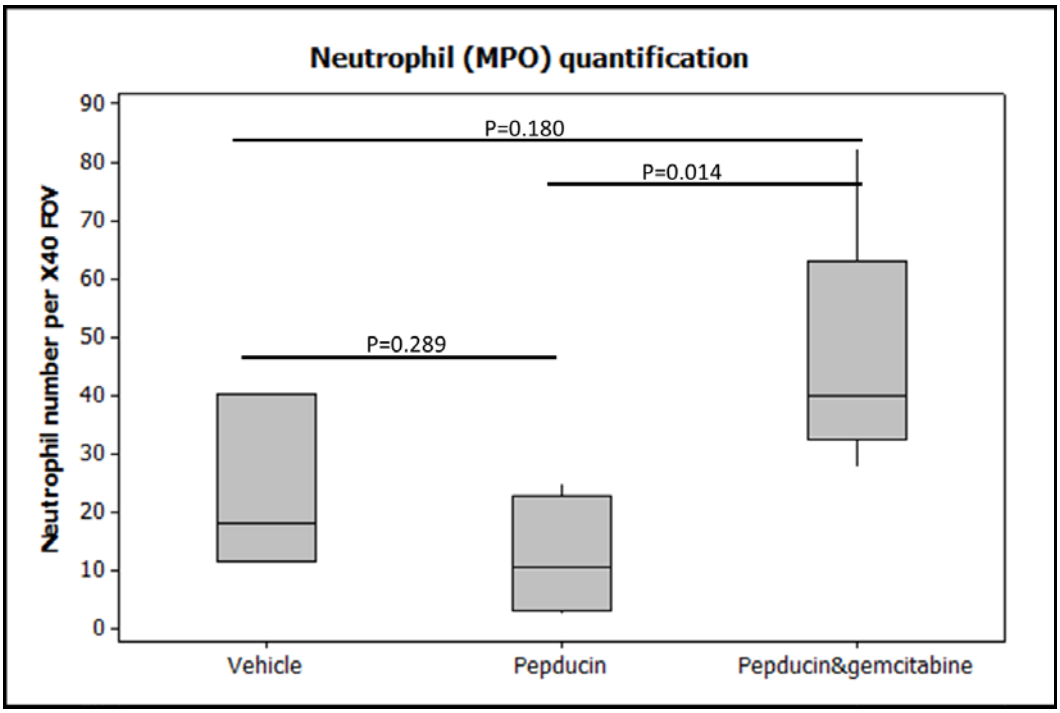
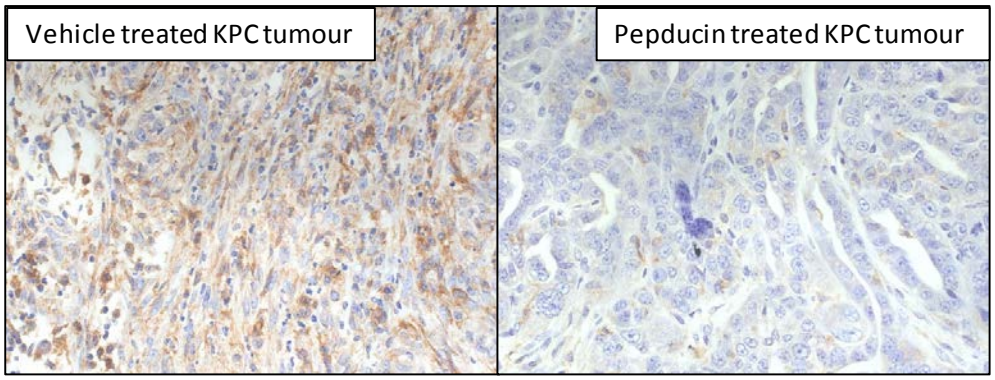


Figure 35: CXCR2 inhibition decreases macrophage tumour infiltration in KPC mice.

a) Shows representative X40 magnification images of F4/80 stained vehicle treated and pepducin treated tumour tissue.

b) Quantification of macrophages (F4/80 staining by pixel count analysis) in pepducin treatment cohorts. There was a non-significant trend towards decreased macrophage numbers in pepducin treated tumours. This reduction was not seen when combination treatment with gemcitabine was given (Mann-Whitney U test, group size n≥3).

a)



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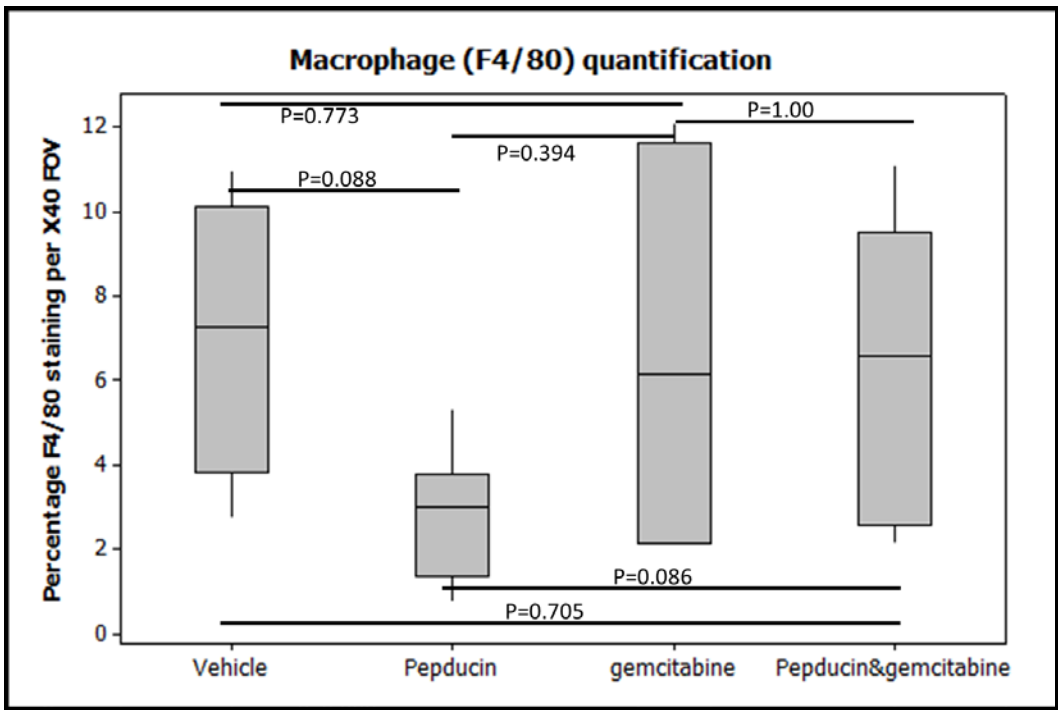


3.7.6 CXCR2 inhibition reduces intra-tumoural collagen and elastin and intra-tumoural myofibroblast number in KPC mice

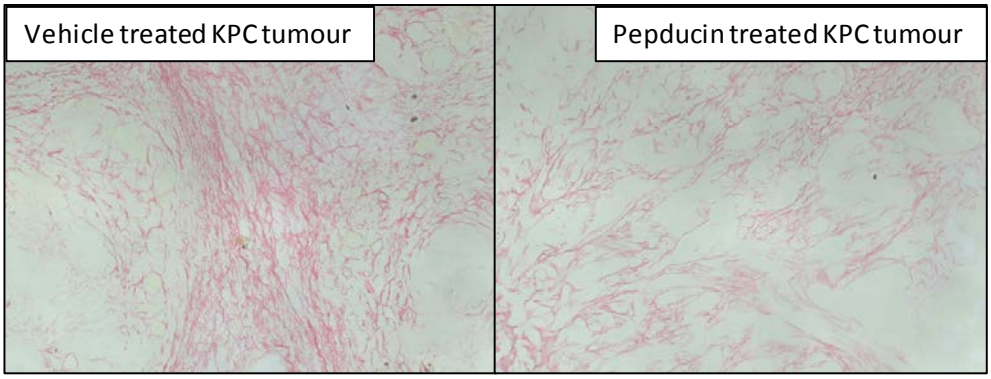
CXCR2 expression by neutrophils is widely appreciated, however other cell types including fibroblasts have also been shown to express CXCR2 (Ijichi, Chytil et al. 2011; Marotte, Ruth et al. 2010). I quantified the levels of collagen and elastin in tumours utilising Sirius red staining and through immunohistochemical staining for alpha-SMA evaluated the numbers of myofibroblasts in treatment groups. CXCR2 inhibition caused a significant decrease in the amounts of intra-tumoural collagen and elastin ($p=0.021$) (see Fig. 36) and a decrease in the number of alpha-SMA positive cells (see Fig. 37).

Figure 36: CXCR2 inhibition decreases collagen and elastin in KPC tumours.

a) Shows representative X20 magnification images of Sirius Red stained vehicle treated and pepducin treated tumour tissue.

b) Quantification of collagen and elastin (Sirius red staining by pixel count analysis) in pepducin treatment cohorts. There was a significant decrease in collagen and elastin in pepducin treated tumours (Mann-Whitney U test, p=0.021, group size n≥3). This reduction was not seen when combination treatment with gemcitabine was given.

a)



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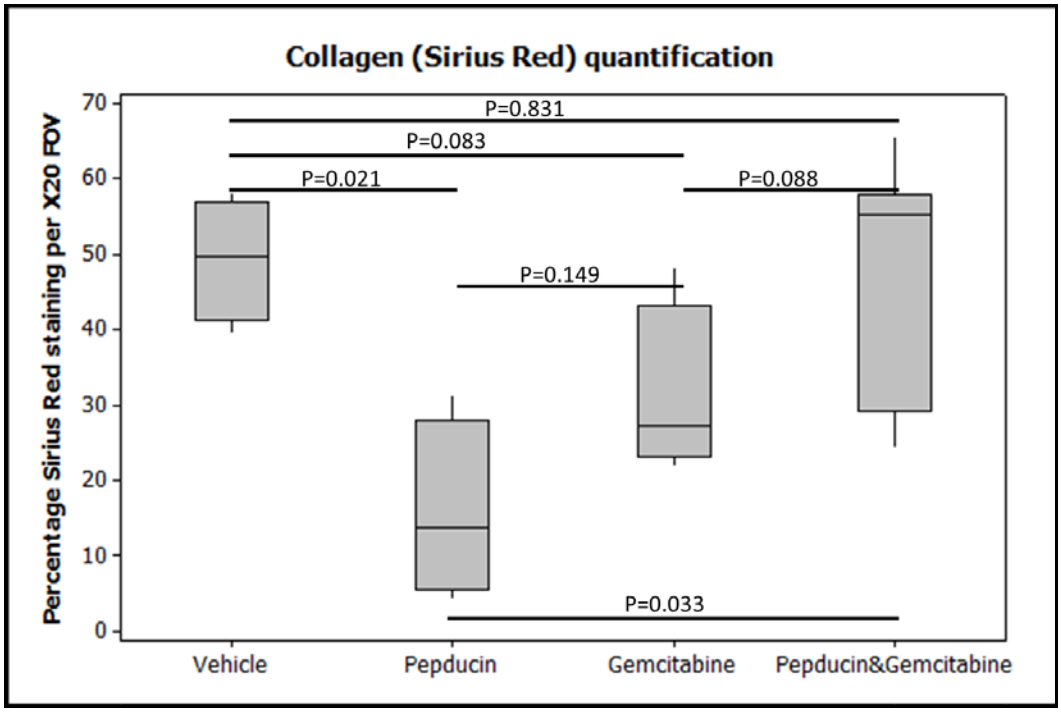
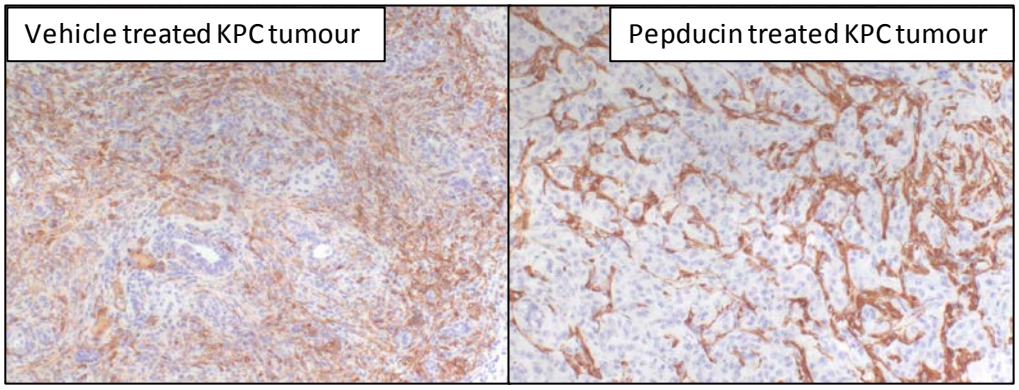


Figure 37: CXCR2 inhibition decreases myofibroblast numbers in KPC tuours.
Shows representative X20 magnification images of alphaSMA stained vehicle treated and pepducin treated tumour tissue.



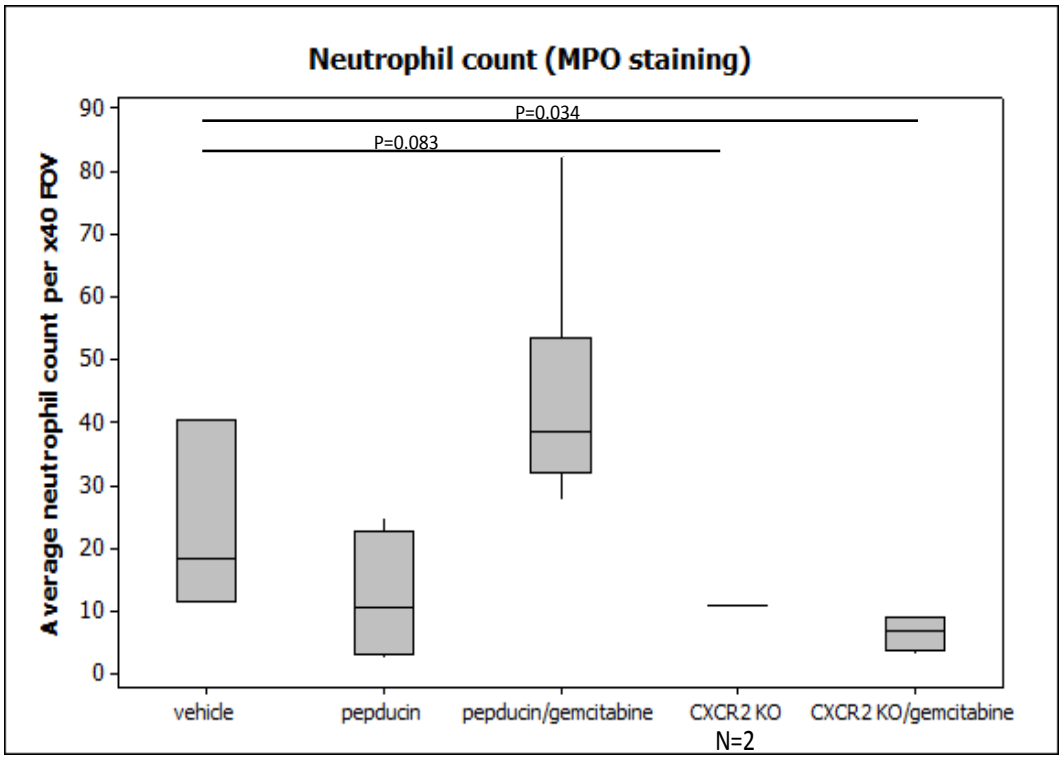
3.8 Constitutive CXCR2 knock out in the KPC model

Our lab has shown that inhibition of CXCR2 by pepducin has significant effects on overall survival in the KPC model of PDAC (pharmacological deletion) as well as having profound effects on the tumour epithelium and the tumour microenvironment. Work by Colin Steele in the lab is on-going to characterise the effect of constitutive knock out of the CXCR2 gene in the KPC model using a genetic model and the following are early results highlighting the changes seen in the microenvironment.

3.8.1 Constitutive CXCR2 knock out reduces intra-tumoural neutrophil infiltration in KPC mice

CXCR2 signalling is vital for neutrophil chemotaxis to sites of inflammation. I have shown that constitutive CXCR2 knock out significantly reduces intratumoural neutrophil infiltration in KPC mice treated with gemcitabine and a there is a trend towards decreased neutrophil numbers in CXCR2 knock out KPC mice for which we currently have small numbers ($p=0.034$, $p=0.083$ respectively) (see Fig. 38). This trend is also seen with pepducin inhibition of CXCR2 signalling but is found to be a significant finding in the context of a constitutive CXCR2 knock out.

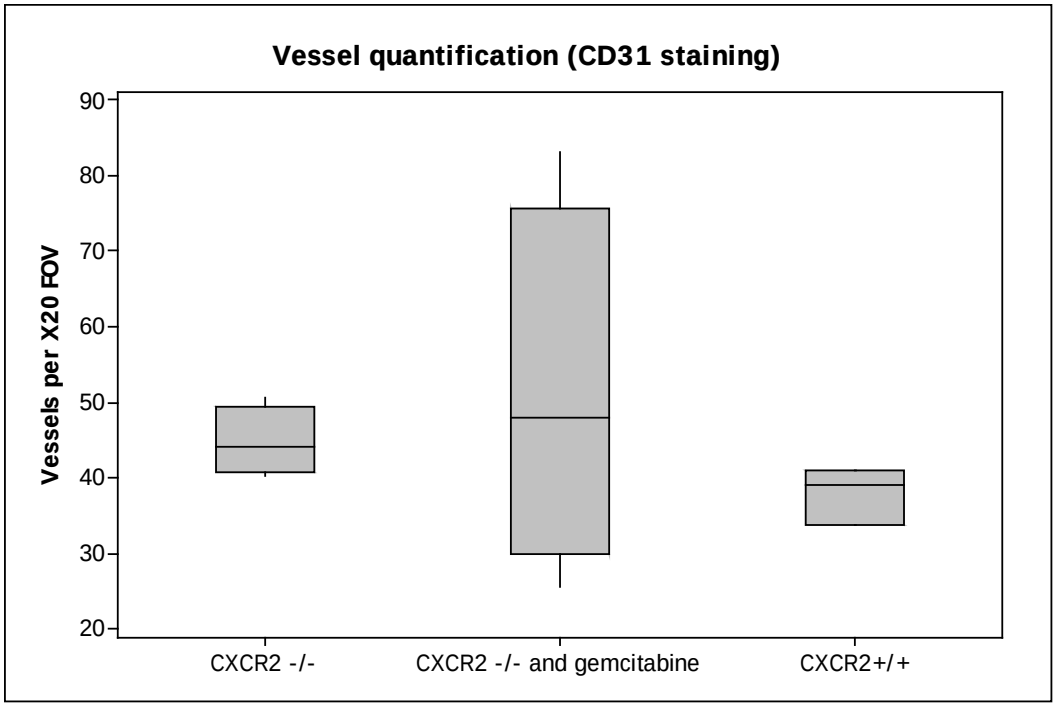
Figure 38: CXCR2 KO decreases neutrophil tumour infiltration in KPC mice.
Neutrophil quantification (MPO positive cells) in tumours in CXCR2 KO mice and mice expressing functional CXCR2 (group size n=2, ongoing cohort).



3.8.2 Constitutive CXCR2 knock out does not alter intra-tumoural microvessel density in KPC mice

I have previously shown that inhibition of CXCR2 signalling with pepducin treatment significantly reduces intra-tumoural microvessel density in KPC mice. I therefore looked to characterise changes to the tumour vasculature in the context of constitutive knock out of CXCR2. In contrast to pepducin mediated CXCR2 inhibition, constitutive knock out of CXCR2 does not have a significant effect on the intra-tumoural microvessel density (see Fig. 39).

Figure 39: CXCR2 KO has no effect on vessel count in KPC tumours.
There is no change in vessel count between tumours in CXCR2 knockout mice and mice expressing CXCR2.

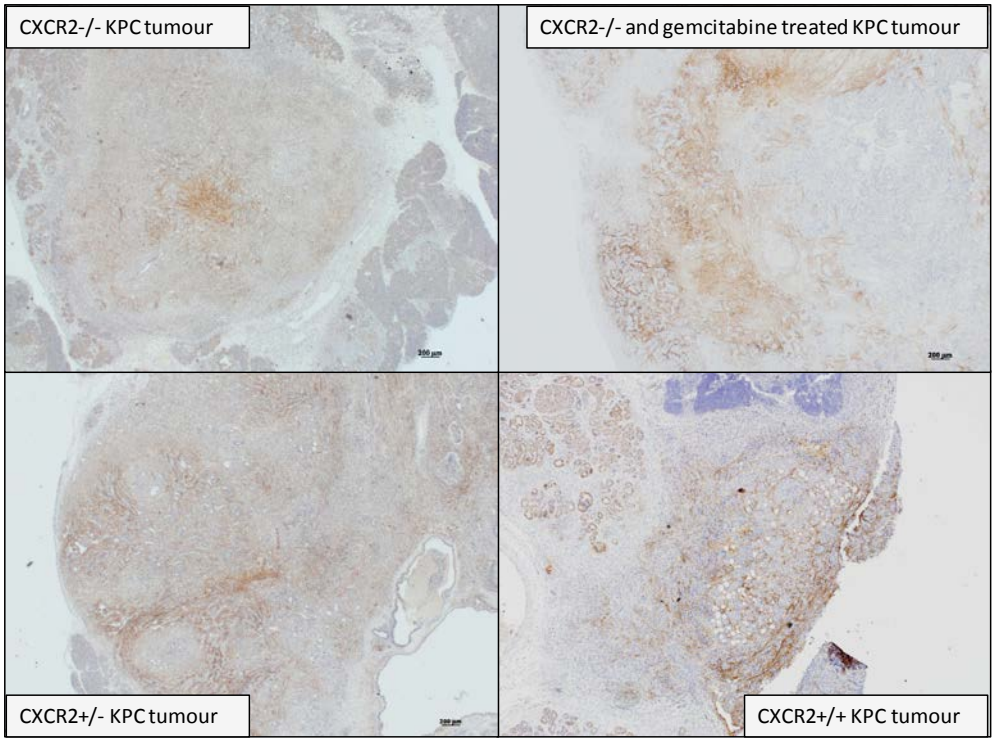


3.8.3 Constitutive CXCR2 knock out does not alter intra-tumoural expression of tenascin-C in KPC mice

Having shown that constitutive knockout of CXCR2 has no effect on intra-tumoural microvessel density I next demonstrated that constitutive knock out of CXCR2 also has no effect on levels of intra-tumoural tenascin C (see Fig. 40).

Figure 40: CXCR2 KO has no effect on tenascin-C expression in KPC tumours.

There is no change in tenascin-C production between CXCR2-/- tumours and CXCR2 expressing tumours (group size n≥2).



Chapter 4

Discussion

4.1 Pancreatic ductal carcinoma and the importance of its microenvironment

In this thesis I have established robust methods of stromal characterisation and novel immunohistochemistry protocols. I have subsequently applied these methods and characterised the stroma of the KPC model of PDAC and compared this with the normal pancreas as well as determining the stromal changes caused by specific targeted therapies. In the UK pancreatic cancer is the fifth highest cause of death by cancer with a median survival post-diagnosis of only 6 months. This situation has changed little in the past few decades with median survival barely altering between 1971 and 2007. To date therapies that target specific molecular alterations within the cancer cells themselves, which have been successful in other tumour types, have not been successful in treating PDAC (Jang and Atkins 2013; Yauch and Settleman 2012). In response to the lack of success in directly targeting the tumour epithelial cells, increasing interest has been given to targeting the tumour microenvironment, a characteristic for which PDAC is well known (Feig, Gopinathan et al. 2012). In our lab a number of approaches have been taken to target the tumour microenvironment and these have greatly increased survival in mouse models of PDAC. In order to begin to further understand the mechanisms by which these approaches affect the mouse tumours it was first vital to establish robust methods for stromal characterisation.

4.2 Establishing robust methods for stromal characterisation

With the increasing interest in stromal targeting in cancer therapy, particularly for pancreatic cancer, there have been increasing numbers of publications evaluating the effects of different stromal targeting therapies (Bramhall, Rosemurgy et al. 2001; Provenzano, Cuevas et al. 2012; Olive, Jacobetz et al. 2009; Jacobetz, Chan et al. 2013). Unfortunately the methods used in these publications vary greatly in their fastidiousness. We argued that it should be possible to develop a methodological approach that is less open to variation due to the subjectivity that affects these other scoring methods. Through evaluating a broad range of cellular and non-cellular stromal constituents and scoring all non-overlapping tumour FOV whilst simultaneously evaluating the running mean and standard error in each example I have established a more robust method for stromal characterisation. I

have shown that reliable estimations of stromal constituents may be obtained by scoring a minimum of 30 FOV at X20 magnification or 60 FOV at X40 magnification. This method, although more time consuming, is achievable and provides a minimum number of fields that must be evaluated in order to produce a representative average for any stromal constituent. I aimed to establish improved methods for histological analysis of stromal constituents and in so doing have increased the reliability of this method in stromal evaluation. Importantly however this approach will benefit from being run in tandem where possible with flow cytometry for cellular constituents and Western-blot and/or qPCR analysis where appropriate for other non-cellular components. Alone, and importantly in conjunction with these other methods of evaluation, the more robust method for histological assessment and quantification I have developed and used for all my analyses will lead to greater accuracy and more meaningful results when characterising stromal changes in models of cancer.

4.3 Stromal characterisation of the KPC model of PDAC

Through our initial analyses we have shown that the stromal constituents of PDAC differ greatly in composition and proportion from the stroma of the normal pancreas. We have shown that there is a prominent increase in the amount of stroma with a dramatic increase in the amount of collagen and the number of activated myofibroblasts. We have shown that there is an increase in the number of inflammatory cells within the stroma of PDAC compared to the normal pancreas. We have also shown that there are significant changes in the tumour epithelium with moderate to marked amounts of necrosis compared with the parenchyma of the normal pancreas.

4.3.1 Characterisation of myofibroblasts and pancreatic stellate cell numbers in PDAC

Existing literature regarding PDAC refers extensively to the significant contribution that pancreatic stellate cells make to the population of activated myofibroblasts in the stroma (Apte, Park et al. 2004). In this literature cellular alphaSMA positivity has been used to label and quantify pancreatic stellate cells. As discussed previously alphaSMA is not a specific marker for pancreatic stellate cells and any activated fibroblast from any origin as well as other cells such as pericytes will

stain for alphaSMA. The lack of specificity of alphaSMA as a marker for pancreatic stellate cells led us to investigate the utility of GFAP as a more specific marker for pancreatic stellate cells in PDAC. Pancreatic stellate cells are considered to be of neural crest origin and as such produce the intermediate filament GFAP. Unfortunately the characterisation of pancreatic stellate cells has been limited by the ability to isolate and study these cells in culture (Apte, Haber et al. 1998; Apte, Park et al. 2004; Wehr, Furth et al. 2011). One limitation is the fact that pancreatic stellate cells growing in an *in vitro* setting immediately become activated. Additionally the availability of specific markers which may be used to specifically isolate pancreatic stellate cells is also limited. GFAP is generally considered to be produced by quiescent pancreatic stellate cells however the literature surrounding activated pancreatic stellate cells is unfortunately less clear. I have shown that GFAP immunohistochemistry stains a small population of cells within the normal pancreas which are situated in the periacinar region as would be expected for pancreatic stellate cells. I have also shown that there is a small population of GFAP positive cells present in both human and KPC PDACs. Importantly however, the number of GFAP positive cells does not match the number of alphaSMA positive myofibroblasts in KPC tumours and this discrepancy is large in scale with the number of alphaSMA positive cells vastly exceeding the small number of GFAP positive cells present (see Fig. 9). This discrepancy may be explained by the fact that there are potentially multiple sources capable of contributing to the myofibroblast population in the KPC tumours. This is certainly a possibility with pericytes, fibrocytes, endothelial cells (through the process of endothelial to mesenchymal transition) and even epithelial cells (through epithelial to mesechymal transition) all capable of contributing to the cell population with a spindloid morphology and alphaSMA positivity (Kalluri and Neilson. 2003; van Meeteren and ten Dijke. 2012). It is also possible that although GFAP is a useful marker of quiescent stellate cells it may be unreliable as a marker once the cells have undergone activation and the associated cellular changes undertaken when becoming myofibroblasts. Indeed the morphology of the few GFAP positive cells within our KPC tumours is that of quiescent stellate cells (round and plump) and it is also possible that GFAP expression upon activation is dramatically reduced. Further evaluation of the origin of the myofibroblast population in pancreatic cancer will be required, utilising better and more specific markers, in order to

elucidate the true role played by pancreatic stellate cells in PDAC. The possibility of performing lineage tracing experiments utilising GFAP driven expression of Cre recombinase and LSL-GFP under the alphaSMA promoter is under investigation in our lab and may provide useful and vital insight into this population of cells. If confirmed, the finding that the proportion of pancreatic stellate cell derived myofibroblasts is small does not rule out the possibility that these cells are central to guiding the formation and ongoing remodelling of the tumour stroma. However it would seem prudent, until further characterised, to designate these cells generically as activated myofibroblasts rather than implying they are derived from pancreatic stellate cells.

4.3.2 Stromal characterisation highlights the increased levels of tenascin C in KPC stroma

Having characterised the constituents of the stroma within the KPC model of PDAC I next looked to determine levels and location of expression of tenascin C. Tenascin C has been previously shown to be produced in human PDAC however tenascin C production has not been previously reported in the KPC model of PDAC (Juuti, Nordling et al. 2004). I have developed and optimised an immunohistochemistry protocol for staining formalin fixed, paraffin embedded tissues for tenascin C which has shown that tenascin C is not produced in the normal pancreas but is present in the stroma of KPC-driven PDAC.

4.4 Tenascin C in PDAC

Tenascin C expression has been detected with increased frequency in the progression from early PanIN lesions through to PDAC (Esposito, Penzel et al. 2006). As a component of the extracellular matrix with significant effects on tumour cell behaviour and viability I looked to further characterise the expression of tenascin C in human PDAC and in our murine models of PDAC. I also looked to characterise changes in tenascin C expression in response to stromal targeted therapies.

Having established and optimised an immunohistochemistry protocol for staining tenascin C in formalin fixed, paraffin embedded tissue I was able to show that there is significant stromal production of tenascin C in the KPC model of PDAC.

This showed, as we suspected, that the KPC model of PDAC mirrors the stromal production of tenascin C as seen in the human disease. This finding further highlights the usefulness of the KPC model for studying the complex stroma found within these tumours.

Utilising the immunohistochemistry protocol for tenascin C I was next able to show that tenascin C expression is higher in tumours with mutated p53 (KPC) compared to those which have lost p53 (KPflC). It has previously been shown by Jennifer Morton in our lab that the presence of mutant p53 as opposed to loss of p53 results in the capacity for tumour cells to metastasise. Interestingly the majority of the subsequent work focussing on the effects of mutant gain-of-function p53 has focussed on the changes resulting in the tumour epithelium yet here we have shown a clear difference between the stromal constituents of these tumours. This interesting finding perhaps provides a mechanism by which gain-of-function p53 mutations may alter the stromal microenvironment and, given the established role of tenascin C in metastasis, increase their metastatic ability in a non-tumour cell autonomous way.

Using human PDAC cell line xenografts and species specific tenascin C staining, I have shown that tenascin C production in PDAC is undertaken by stromal cells as opposed to the tumour cells themselves. Importantly however, using KPC cell line allografts in tenascin C knockout mice I have shown that the tumour epithelium itself will produce tenascin C in the absence of stromal production, therefore potentially highlighting a significant requirement for tenascin C expression within PDAC. This finding is consistent with the findings of Oskarsen et al. in which they found that production of tenascin C by metastatic mammary epithelial cells was vital for tumour cell survival until stromal production in the metastatic niche had reached adequate levels at 21 days (Oskarsson, Acharyya et al. 2011). Interestingly where the combination of Lox-Ab and gemcitabine is given eliciting dramatically reduced stromal tenascin the tumour epithelium itself appears to upregulate production of tenascin C. These results all confirm the importance of tenascin C to PDAC epithelial cells which was initially highlighted by the work of Bryan Miller in our lab showing that the production of tenascin C by PDAC cell lines *in vitro* is vital to cell viability. In this context it would seem that tenascin C is upregulated *in vitro* by cells growing in the absence of stromal cells and

associated ECM as a “stress response” due to a lack of supportive stromal signalling including altered mechanotransduction and other cytokine pathways. Taken together these findings all point to the vital role tenascin C plays in both the primary tumour and the metastatic niche and as such when the stroma is not capable of producing tenascin C the epithelium will produce tenascin C itself.

In the KPC mouse model of PDAC I have shown that treatment with gemcitabine triggers a significant increase in the expression of tenascin C. It is interesting to speculate that this may be a specific response leading to resistance to gemcitabine therapy as has been shown previously (Gong, Lv et al. 2010) or a general tumour stress response.

I have shown that lysyl-oxidase inhibition impairs the ability of the tumours to express stromal tenascin C. This impaired tenascin C expression may in part explain the loss of tumour viability through lack of supportive tumour-ECM signalling or it is tempting to speculate that the lack of protective annexin II signalling triggered by tenascin C possibly leads to a specific increase in susceptibility to gemcitabine therapy as demonstrated by Gong et al. (Gong, Lv et al. 2010). It is equally possible that the lack of tenascin C production is simply a result of reduced tissue hypoxia, due to the increase in vascular supply that we have demonstrated in response to lysyl-oxidase inhibition. If this is the case then it is possible that the tumoural responses noted are due to increased gemcitabine delivery and perhaps increased chemosensitivity due to increased vascular supply which in turn will reduce hypoxia.

Interestingly in contrast to the changes elicited by lysyl-oxidase inhibition I have also shown that tenascin C is upregulated in KPC PDAC tumours in response to CXCR2 inhibition with pepducin. This increase is likely a stress response which may be a direct result of increased tissue hypoxia due to decreased intra-tumoural microvessel density. It is interesting to speculate that this increase in tenascin C is due to increased tissue hypoxia especially given the reduction in tenascin C production noted in the context of lysyl-oxidase inhibition which increases vascular supply. Importantly lysyl-oxidase inhibition leads to increased intratumoural microvessel density, increased necrosis, a reduction in tenascin C expression and an increase in infiltrating leukocytes whereas CXCR2 inhibition leads to decreased

intratumoural microvessel density, increased necrosis, increased tenascin C expression but no increase in infiltrating leukocyte numbers. Both however lead to increased survival and neither result in increased incidence of metastasis which is important especially in the context of CXCR2 inhibition given previous findings that hypoxia appears to be associated with increased metastasis in pancreatic ductal adenocarcinoma (Matsuo, Ding et al. 2013). The reduced incidence of metastasis in this example of increased hypoxia is in agreement with other findings in our lab where VEGF inhibition had no effect on metastasis in the KPC model. Importantly in the context of both lysl-oxidase inhibition and CXCR2 inhibition the presence or absence of hypoxia will need to be confirmed utilising methods such as pimonidazole or GLUT-1 staining. Tenascin C production however is also triggered by alterations in tissue stiffness (Jones and Jones 2000) so it is also possible that the increased production of tenascin C noted upon CXCR2 inhibition could be the result of altered ECM composition and architecture as highlighted by the reduction in collagen and elastin and also the reduction in myofibroblast numbers in treated tumours. Given preliminary findings in the setting of PDAC in a genetic model of CXCR2 deletion (CXCR2 -/-) where there is no decrease in tumour vasculature and concurrently there is also no increase in tenascin C production it seems plausible that the increased tenascin C production in response to pepducin treatment is likely due to hypoxia. This finding is consistent with my finding that the production of tenascin C in the human PDAC TMA strongly correlates with levels of HIF-1alpha.

In agreement with others I have shown that tenascin C significantly correlates with differentiation in human PDAC (Juuti, Nordling et al. 2004). Additionally our data, to the best of our knowledge, show for the first time a significant correlation between high tenascin C expression and decreased survival in human PDAC patients (see Fig. 17). In my analysis of the tenascin C levels in the human TMA I have also shown that there is a significant positive correlation between tenascin C levels and tumour cell proliferation, tumour grade and there is also a trend towards a positive correlation between tenascin C expression and vascular invasion. As stated previously this analysis of the human PDAC TMA has also confirmed the positive correlation between hypoxia and tenascin C expression. These findings, which would benefit from confirmation in whole tumour sections in addition to this TMA, may in part explain the reasons for decreased survival in patients with high

levels of tenascin-C expression and again highlight the important role tenascin C plays in both the primary PDAC tumours and in the metastatic process.

Interestingly in agreement with others, analysis of tenascin C production in the human PDAC TMA highlights a significant positive correlation between integrin signalling and tenascin C (Sriramarao, Mendler et al. 1993; Yokosaki, Palmer et al. 1994; Varnum-Finney, Venstrom et al. 1995; Yokosaki, Monis et al. 1996). Analysis has also shown a significant correlation with tenascin C and the integrin αvβ6 which to our knowledge has not been shown previously.

Finally data from a human PDAC RNA tissue microarray has confirmed that there is a significant positive correlation between lysyl-oxidase and tenascin-C expression in human PDAC. This finding supports the finding that lysyl-oxidase inhibition in the KPC model significantly decreases tenascin C expression.

4.5 Lysyl-oxidase in PDAC

The characteristic desmoplastic stroma found in PDAC has been implicated in promoting tumour growth, progression, invasion and metastasis. Recent studies have also suggested that the stroma functions to prevent drug delivery to the tumour epithelium (Provenzano, Cuevas et al. 2012). Lysyl-oxidase, an enzyme which catalyses the crosslinking of collagen and elastins, plays a central role in the generation and maintenance of this “stiff” desmoplastic stroma (Baker, Cox et al. 2011).

To date, lysyl-oxidase has been associated predominantly with the metastatic spread of cancers however through specific inhibition of lysyl-oxidase we have shown that it also plays a vital and ongoing role in an established primary tumour.

Work in our lab has shown that inhibition of lysyl-oxidase alone increases survival in KPC mice and that combination with gemcitabine therapy further increases survival to a median of 226 days.

Jen Morton and Bryan Miller in our lab have shown that expression of lysyl-oxidase is high in all our mouse models of PDAC when compared with primary pancreatic ductal epithelial cells. Work by Jen Morton has shown that lysyl-oxidase expression is most significantly increased in those tumours that carry gain-of-

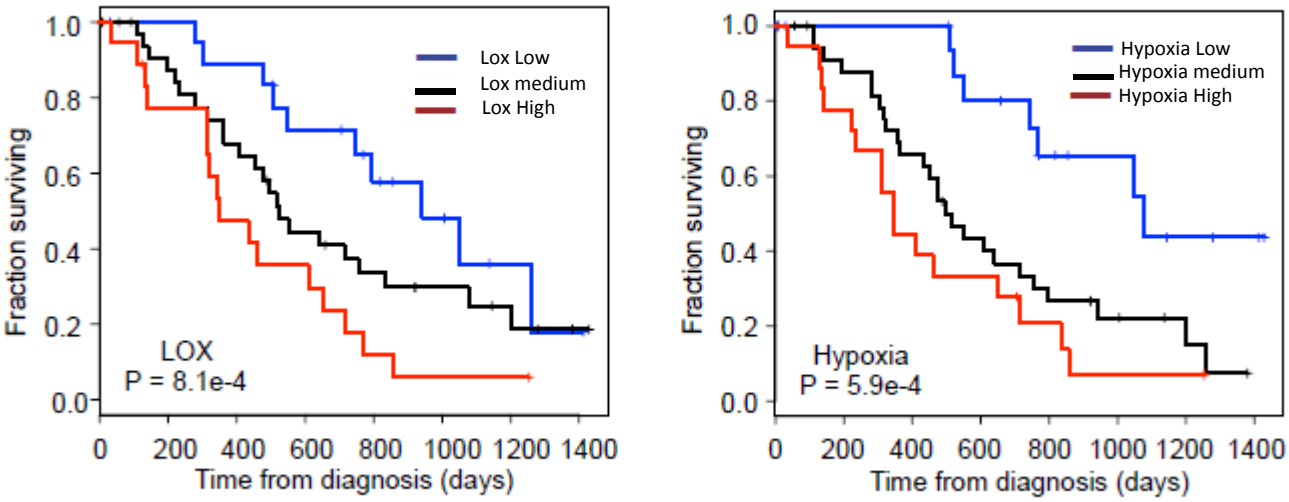
function mutant p53. Interestingly this finding that lysyl-oxidase expression is highest in these tumours correlates with my work showing increased tenascin C expression in the same model. I have also shown that inhibition of lysyl-oxidase inhibits expression of tenascin C which again correlates with other work in the human PDAC RNA microarray that has shown that there is a strong positive correlation between lysyl-oxidase expression and tenascin C expression. These findings taken together are highly suggestive that lysyl-oxidase and tenascin C may play an important role in the mechanism through which gain-of-function mutant p53 (KPC) tumours are able to successfully metastasize to distant sites whereas tumours with only loss of p53 (KPflC) cannot.

Work by Ewan McGhee, Paul Timpson and Jen Morton confirmed a significant decrease in collagen-crosslinking in Lox-Ab treated tumours through evaluation of the second harmonic resonance signal using multiphoton microscopy. This finding confirms the efficacy of the Lox-Ab being used in this study and the resultant “stromal softening”.

Work undertaken in our lab has shown that in resectable human PDAC, lysyl-oxidase expression correlates with expression of Hypoxia-inducible factor 1-alpha. The same work showed that high expression of both lysyl-oxidase and Hypoxia-inducible factor 1-alpha signifies a bad prognosis in resectable human pancreatic ductal adenocarcinoma (See Fig. 41). This finding raises the interesting possibility that ultrasound elastography may provide a clinically useful biomarker which would mirror the lysyl-oxidase/HIF-1a axis and could be assessed utilising endoscopic ultrasound scanning in the clinic.

Figure 41: LOX expression and Hypoxia are significantly associated with survival in human PDAC.

Kaplan-Meier survival analysis. There was a significant difference in survival between the Lox High and the Lox Low cohorts (Log-Rank p=8.1e-4) and patients with high levels of hypoxia also had significantly reduced survival (Log-Rank p=5.9e-4). (Figure and results courtesy of Jen Morton).



It is well established that PDAC tumours have a poor vascular supply which leads to a state of hypoxia within tumours and has also been shown to limit drug delivery to tumour cells (Provenzano, Cuevas et al. 2012). Amy Au in our lab has shown that inhibition of lysyl-oxidase results in an increase in the intra-tumoural vasculature. This finding contradicts work that has shown that inhibition of lysyl-oxidase leads to a reduction in the intra-tumoural vasculature (Osawa, Ohga et al. 2013). It is possible that the reduction in the "stiffness" of the stroma due to lysyl-oxidase inhibition permits increased migration of endothelial cells into the tumour thereby increasing blood supply to the tumour. It is also possible that the resultant stromal softening allows further opening of already existing vasculature, a mechanism highlighted in the context of hedgehog signalling inhibition (Olive, Jacobetz et al. 2009).

The ability to stimulate angiogenesis is a process considered to be one of the major hallmarks of cancer (Hanahan and Weinberg 2000). We might therefore find it concerning that a treatment aiming to treat cancer, through increasing vascular supply, is actually augmenting one of the processes considered fundamental to tumourigenesis. Contrary to this view we have shown that treatment with lysyl-oxidase alone significantly increases survival and more

importantly in combination with gemcitabine provides an additional survival advantage.

This benefit may be explained by the selective increase in intra-tumoural necrosis I have shown in the Lox-Ab/gemcitabine combination treatment group. This increase in necrosis with the addition of gemcitabine suggests that lysyl-oxidase may be increasing delivery of gemcitabine to the tumour cells thereby triggering tumour cell death by necrosis. It is also well established that hypoxia itself can decrease the susceptibility of tumour cells to chemotherapeutic agents; therefore it is also possible that lysyl-oxidase by increasing tumour blood supply also decreases the tissue hypoxia and in so doing increases the sensitivity of the tumour to gemcitabine.

In addition to the increased necrosis seen in the Lox-Ab/gemcitabine treatment group I have also shown an increase in the infiltration of intra-tumoural neutrophils and macrophages. It has been suggested that the poor vascular supply in PDAC may limit leukocyte infiltration in vehicle treated tumours however as this infiltration is not seen in KPC tumours treated with Lox-Ab alone it is likely that this infiltration is occurring in response to the significant intra-tumoural necrosis in the Lox-Ab/Gemcitabine combination treatment group with leukocyte infiltration being aided by the increased tumour vasculature.

As mentioned previously I have also shown that inhibition of lysyl-oxidase leads to dramatically reduced stromal tenascin C expression. It is possible that inhibition of lysyl-oxidase by increasing tumour vasculature in turn reduces tissue hypoxia thereby decreasing the drive for production of tenascin C. It is also possible that altered tissue “stiffness” reduces the expression of tenascin C by stromal cells. Additionally, tenascin C also has a large number of binding domains for extracellular matrix molecules such as fibronectin (Chiquet-Ehrismann 2004). As such it is possible that inhibition of lysyl-oxidase resulting in reduced crosslinking of collagen and elastin results in a significantly altered extracellular matrix architecture in which tenascin C is unable bind and interact in a normal manner.

Finally as discussed previously I have shown that there is an increase in stromal tenascin C in response to gemcitabine treatment. Interestingly tenascin C has been shown to induce gemcitabine resistance in pancreatic cancer through

annexin II signalling in a PI3K/Akt/NF-kB dependent manner (Gong, Lv et al. 2010). It is therefore possible that one mechanism by which lysyl-oxidase inhibition sensitizes PDAC to gemcitabine treatment in addition to increasing drug delivery is through the prevention of the protective upregulation of tenascin C.

4.6 CXCR2 signalling in PDAC

The role of CXCR2 signalling in epithelial cancers is well established and CXCR2 and its ligands have been shown to be upregulated in both human disease and mouse models of PDAC (Takamori, Oades et al. 2000; Kuwada, Sasaki et al. 2003; Hill, Gaziova et al. 2012; Ijichi, Chytil et al. 2011). Expression of CXCR2 is not limited to the PDAC tumour cells as a wide variety of cells including neutrophils, macrophages, endothelial cells and fibroblasts have been shown to express the receptor (Heidemann, Ogawa et al. 2003; Strieter, Burdick et al. 2006; Li, Cheng et al. 2011; Soehnlein, Drechsler et al. 2013; Hallgren and Gurish 2011; Ijichi, Chytil et al. 2011; Sharma, Nawandar et al. 2013; Feijoo, Alfaro et al. 2005; Marotte, Ruth et al. 2010). Given the high levels of expression of CXCR2 ligands and the widespread expression of the receptor; CXCR2 signalling has increasingly gained attention as a possible therapeutic target in pancreatic cancer.

The expression of CXCR2 by a variety of cell types including the tumour cells themselves means that the effects of CXCR2 inhibition are potentially wide ranging within the tumour and its stroma.

Inhibition of CXCR2 signalling in the KPC model of PDAC significantly increases survival (Colin Steele, personal communication). Furthermore combination therapy with gemcitabine provides a further but only moderate increase in survival. There is also no significant effect on tumour cell proliferation in response to CXCR2 inhibition and I have shown that CXCR2 inhibition causes a dramatic increase in intra-tumoural necrosis with no alteration of tumour cell apoptosis.

The expression of the CXCR2 receptor on endothelial cells and bone marrow derived endothelial progenitor cells is well characterised (Li, Cheng et al. 2011). The importance of CXCR2 signalling to the stimulation of angiogenesis in tumours is therefore of great importance. I have shown that inhibition of CXCR2 signalling with pepducin causes a significant reduction in the tumour vasculature. This reduction in vascular supply to the tumour may well be one of the important factors

triggering the dramatic increase in intra-tumoural necrosis. In contrast to this, Lox-Ab treatment increases vascular supply resulting in increased drug delivery/ sensitivity and therefore increased necrosis. It is likely that the tumours which are characteristically hypovascular and hypoxic are on a knife-edge and through pepducin induced CXCR2 inhibition the decrease in tumour vasculature tips the tumour into a state of hypoxia in which tumour cells are no longer viable. Although results are preliminary it is interesting to note that constitutive knock out of the CXCR2 receptor in the KPC model of PDAC does not cause any significant alteration in tumour vasculature. The lack of suppression of tumour angiogenesis in this model may be due to the fact that, from early tumourigenesis tumours have developed in the absence of CXCR2 signalling and as such will have circumvented the requirement for CXCR2 signalling. Whereas pepducin induced CXCR2 inhibition was initiated after tumours had developed. Similarly, previous literature highlighting the importance of CXCR2 signalling in tumour induced angiogenesis has not evaluated tumour tissue that has developed in a CXCR2 -/- context and as such although they have shown that CXCR2 is vital to angiogenesis they do not prove that other mechanisms are not capable of fulfilling this role if required (Ijichi, Chytil et al. 2011). Utilising a pepducin molecule which inhibits CXCR2 signalling in the KPC model I have shown that CXCR2 signalling, if present during tumour development, is vital for triggering angiogenesis.
Importantly work by others and results obtained in our pepducin treated mice have not shown that CXCR2 inhibition leads to a complete absence of vasculature in the tissues being investigated; therefore there must be other mechanisms capable of triggering angiogenesis in this context (Ijichi, Chytil et al. 2011). It is possible that although CXCR2 is the dominant receptor for proangiogenic ELR+ CXC chemokines other receptors such CXCR1 may still be able to play a significant role. Indeed CXCR1, although not the primary proangiogenic ELR+ chemokine receptor, is a receptor for a number of these chemokines (Strieter, Burdick et al. 2006). Interestingly this raises the possibility that the CXCR2 inhibiting pepducin molecule used in this study may have additional minor inhibitory actions such as inhibition of CXCR1 signalling in addition to inhibiting CXCR2 which together results in greater inhibition of angiogenesis. In light of this it will be prudent to determine whether the pepducin used in this work has any effect on angiogenesis, as well as other parameters, in the KPC model on a CXCR2-/- background. In

addition to the CXCR2 and CXCR1 receptors there are also a variety of other ligands and receptors that have been shown to be important in triggering angiogenesis. For example CXCR4 and its ligand CXCL12 and nitric oxide synthase have been shown to have important roles in angiogenesis in xenotransplantation models of adenoid cystic carcinomas of the oral floor in mice (Takoaka, Hidaka et al. 2013). With further work the intricacies of the mechanisms of induction of angiogenesis utilised by tumours will be further clarified. It is likely that there are multiple pathways that tumours may utilise to trigger angiogenesis however given a greater understanding of these pathways the possibility of determining which signalling pathways are most important for angiogenesis in any given tumour may allow for patient and tumour specific targeted therapies.

Interestingly given the massive increase in necrosis triggered by pepducin induced inhibition of CXCR2 signalling there is a trend towards decreased intra-tumoural leukocyte infiltration. Preliminary results indicate that this change is mirrored in KPC mice with constitutive knock out of CXCR2. The usual response to necrosis *in vivo* is the initial influx of neutrophils which are subsequently followed into the tissue by macrophages, a response that is conspicuously lacking in response to CXCR2 inhibition. We have shown that CXCR2 inhibition decreases the intra-tumoural infiltration by leukocytes most likely by preventing leukocyte chemotaxis although it is possible that the reduced tumour vasculature also reduces the available window of entry for leukocytes into the tumour. This reduction of leukocytes is important but needs further investigation as the role of intra-tumoural leukocytes is highly context dependent and they may be either anti or pro-tumourigenic. It has been shown that neutrophils and macrophages may exhibit either an N1/M1 anti-tumour phenotype or an N2/M2 pro-tumourigenic phenotype in response to polarizing cytokine signalling with macrophages for example in response to IL-10 and TGFβ signalling switching from an M1 to M2 phenotype (Sica and Mantovani 2012). It will therefore be interesting to determine whether an alteration in leukocyte phenotype occurs in response to CXCR2 inhibition.

Macrophages and neutrophils have also both been shown to play important roles in angiogenesis (Sica and Mantovani 2012; Tazzyman, Lewis et al. 2009) and the reduction in tumour vasculature by CXCR2 inhibition may in part be due to the reduction in intra-tumoural leukocytes.

Macrophages and neutrophils have additionally been shown to promote tumour cell migration and invasion through release of enzymes such as MMPs and through opening holes in vessel basement membranes on extravasation (Smith and Kang 2013). Therefore reduction in intra-tumoural leukocyte numbers may additionally reduce the invasive and metastatic potential of a tumour which in addition to the reduced vascular window due to decreased vessel numbers may in part explain the reduced metastatic spread noted with CXCR2 inhibition in our model (Tazzyman, Lewis et al. 2009; Bohrer, Schwertfeger et al. 2012).

In addition to reduced vasculature and leukocyte infiltration, CXCR2 inhibition caused a reduction in the number of myofibroblasts and amounts of collagen and elastin within treated tumours. It has been shown by others that CXCR2 signalling between tumour epithelium and fibroblasts leads to connective tissue growth factor (CTGF) expression which is important to tumour progression (Ijichi, Chytil et al. 2011). The importance of the desmoplastic stroma to PDAC is well established and therefore any reduction in the stroma likely has a significant effect on the viability of the tumour.

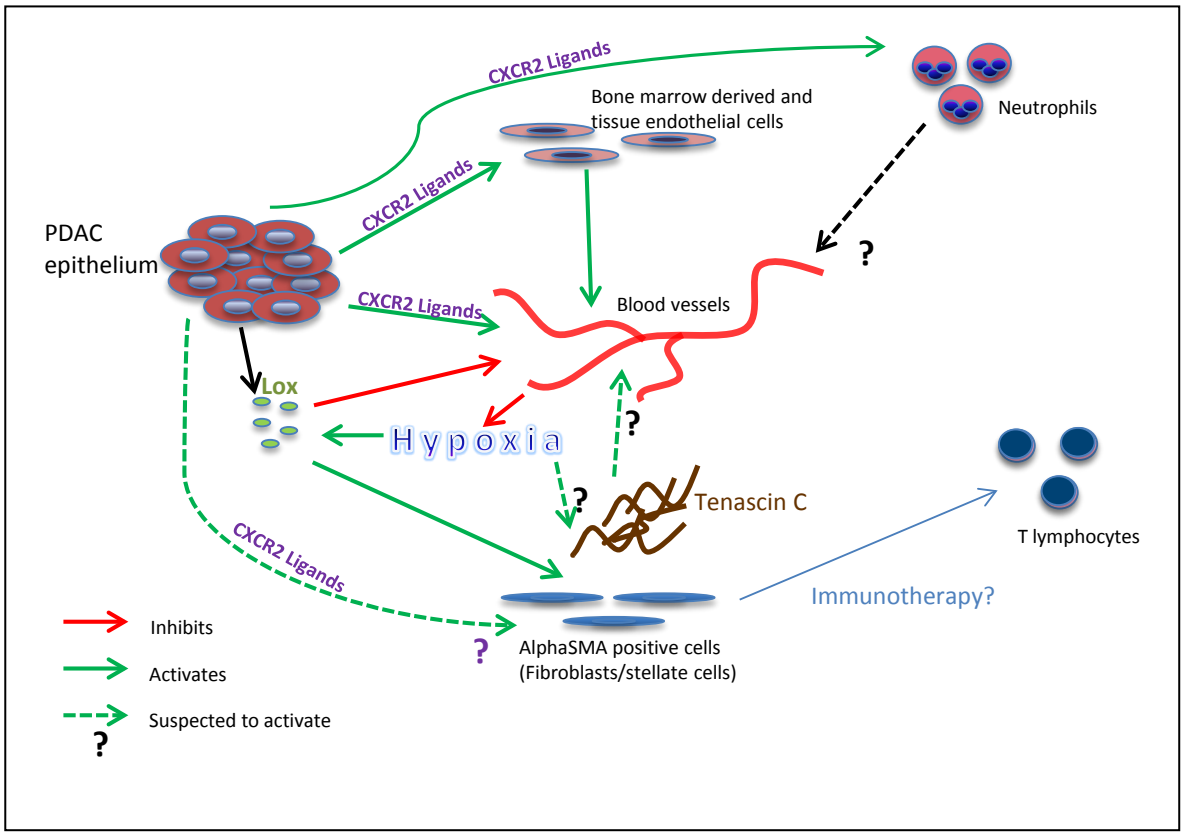
Interestingly, given the reduced stromal cell population in response to CXCR2 inhibition, expression of tenascin C is markedly increased. Tenascin C is involved in tumour progression and maintenance, angiogenesis, metastasis migration and invasion. It is produced in a variety of situations including mechanical stress and hypoxia (Jones and Jones 2000). The increase in tenasin C expression in this instance is most likely a stress response as a result of decreased tumour vasculature resulting in tissue hypoxia which is driving production. The increased tenascin C is likely providing tumour epithelium with support for example through attempts at stimulating angiogenesis and through augmentation of WNT and Notch signalling. Finally we have shown that combination with gemcitabine again causes a further increase in tenascin C production as noted in our work characterising stromal changes elicited by lysyl-oxidase inhibition. As discussed previously this may be additional evidence of a tenascin C induced mechanism of gemcitabine resistance.

4.7 The interlinked roles of lysyl-oxidase, CXCR2 and tenascin C in PDAC

The tumour microenvironment is an extremely complex “ecosystem” in a constant state of flux (Feig, Gopinathan et al. 2012). Within the microenvironment there are many factors seemingly essential for the maintenance of tumour cell viability however there is also extreme plasticity within the microenvironment allowing for continuous tumour adaptation and survival. The cellular and protein constituents of the microenvironment and the pathways that control them are complex and interlinked but crucial to this plasticity. CXCR2, lysyl-oxidase, tenascin C and hypoxia appear to be central components with significant roles within the tumour microenvironment (see Fig. 42) which is itself considered to promote tumorigenesis, maintenance of tumour cell viability, drug resistance and metastasis.

Figure 42: The roles of Lysyl-oxidase, CXCR2 and Tenascin C in PDAC

Summary of the roles of lysyl oxidase, CXCR2 and tenascin C as highlighted in this work.



Future Work

Through the work undertaken in this thesis I have developed more robust methods for histological characterisation of stromal constituents in tumour tissues. I have shown that tenascin C is important in human PDAC having a significant effect on survival in patients and significantly correlating with hypoxia, lysyl-oxidase expression, differentiation and possibly invasive behaviour. I have also shown that it is produced and potentially has important roles in the KPC model of PDAC that mirror those found in human disease. In agreement with others we have shown that the stroma in PDAC is a useful and effective therapeutic target. We have shown that both CXCR2 inhibition and lysl-oxidase inhibition have significant effects on survival in KPC models of PDAC. I have also highlighted significant effects on tenascin C expression in the context of CXCR2 inhibition and lysyl-oxidase inhibition.

In the lab we are currently generating a cohort of tenascin-C knock out KPC mice which will allow us to assess the survival benefit in PDAC. From these mice it will be possible to generate tenascin C knock out KPC PDAC cell lines with which, in conjunction with tenascin C-/- and tenascin C +/+ allograft recipients, we will be able to further characterise the importance of tumour derived tenascin C as opposed to that produced by the stroma. The observation that tenascin C is upregulated in response to gemcitabine treatment also requires further assessment, specifically in reference to the mechanism by which lysyl-oxidase inhibition in combination with gemcitabine treatment triggers PDAC tumour cell necrosis. Tenascin C may also be a potential target in combination with other treatments. For example in pepducin treated KPC mice where there is an upregulation of tenascin C in response to CXCR2 inhibition as a result of hypoxia, tenascin C is likely playing a tumour supportive role. It will therefore be vital to determine the effects of CXCR2 inhibition in tenascin C knockout KPC models of PDAC or in combination with lysyl-oxidase inhibitors. The role of tenascin C and lysyl-oxidase in mutant p53 mediated metastasis is also potentially of great importance. Given that in the non-metastatic p53 null (KPflC) mouse model of PDAC there is minimal production of tenascin C and lysyl-oxidase evaluating the effect of overexpression of tenascin C and/or lysyl-oxidase in p53 null (KPflC) PDAC cell line xenografts and allografts will be of great interest.

I have also shown that CXCR2 inhibition significantly decreases the number of myofibroblasts in KPC PDAC models. Unfortunately I was unable to fully characterise the source of these myofibroblasts. It is generally accepted that pancreatic stellate cells are the source of myofibroblasts in PDAC (Apte, Park et al. 2004). This is a finding that we were unable to confirm. Work to attempt to lineage trace these cells is necessary and is ongoing in the lab. This work will allow clarification of the specific contribution pancreatic stellate cells make to the generation of the characteristic desmoplastic stroma in PDAC and allow characterisation of the changes that occur in this population of cells by stroma targeted therapies. This will be particularly important given the early work being undertaken by Fearon et al. which is highlighting the potential significance of a FAP alpha positive “stellate cell” population, the ablation of which results in T cell mediated immune control of the growth of pancreatic ductal adenocarcinoma (Kraman, Bambrough et al. 2010).

Finally the failure of the human trial of IPI-926, a SHH inhibitor, likely highlights the need to fully and carefully evaluate stromal changes in response to targeted therapies in order to highlight robust alterations which are likely to represent significant changes relevant to human disease (Infinity pharmaceuticals. http://phx.corporate-ir.net/pheonix.zhtml?c=12194&p=irol-newsArticle&ID=1653550&highlight= (accessed 09.08.13)) This we believe is achievable utilising the thorough methods that we have established.

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