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MMP-7 modulates the transition of normal pancreatic acinar cells to metaplastic ducts associated with the neoplastic epithelium of pancreatic ductal adenocarcinoma

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Abstract of the Dissertation

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Pancreatic ductal adenocarcinoma (PDA) is the most common among pancreatic tumors and one of the most fatal human cancers, killing approximately 30,000 people each year in the United States. Pathological evidence strongly suggests that PDA arises from premalignant lesions known as pancreatic intraepithelial neoplasia (PanIN). While there is evidence that PanINs arise from a preexisting stem cell population, a second possible preneoplastic lesion that is always associated with PDA, as well as patients at risk for PDA, such as those with chronic pancreatitis (CP), is known as the metaplastic duct lesion (MDL). MDLs appear to arise from acinar-to-ductal metaplasia (ADM), a process in which healthy acinar cells are progressively replaced by duct-like epithelia that show progenitor-cell properties.

The matrix metalloproteinases (MMPs) are a family of extracellular, zinc dependent proteinases frequently expressed in cancer. Initially, MMPs were studied only as enzymes that degrade the extracellular matrix, lending credence to a role in tumor invasion and metastasis. However, evidence derived from the advent of mouse molecular genetics shows that MMPs have functions throughout tumor progression, including tumorigenesis.

MMP-7 is a member of the MMP family that is expressed in the tumor cells of many adenomas and adenocarcinomas and associated metaplasia, including those of the colon, breast, stomach and the vast majority of PDAs, at all PanIN stages as well as in 100% of tumor-associated MDLs. Animal tumor models have strongly supported a role for MMP-7 in tumorigenesis and early tumor growth. For instance, in the multiple intestinal neoplasia (MIN) mice, MMP-7 deficiency inhibits intestinal tumor formation compared to controls. Conversely, transgenic mice overexpressing MMP-7 in the mammary gland show epithelial hyperplasia and accelerated tumor formation. Though its role in PDA has not been directly addressed, in a mouse model of CP, all aspects of disease progression are strongly inhibited in the MMP-7 deficient mouse, including formation of MDLs.

Together, these data suggest a role for MMP-7 early in adenoma progression, and perhaps in tumorigenesis. Unfortunately, in the whole animal context, it is impossible to know if MMP-7 expression is induced by something intimately associated with the metaplastic process or if the metaplastic epithelium is responding to interaction with other tissue components, such as the fibrotic stroma or inflammatory cells that are inherent in this system. In order to address these questions, I used an *in vitro* model that simulates acinar-to-ductal metaplasia with out the context of fibrosis or inflammation in

order to test the direct contribution of MMP-7 to acinar-to-ductal metaplasia. I have determined that MMP-7 is expressed in this model in response to transforming growth factor-alpha (TGF-α) and that primary acinar cells derived form MMP-7 null pancreata were incapable of converting to cytokeratin-19 positive duct-like cells. Furthermore, experiments to determine the hierarchy of MMP-7 and other molecules associated with acinar transdifferentiation have implicated a Nestin-positive intermediate event that is dependent on Notch pathway signaling.

Dedication

I would like to dedicate my accomplishment of this dissertation to my wife, Joyce White.

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Acknowledgements

This dissertation would not have been possible without the support of many people. Many thanks to my advisor, Howard Crawford who guided me and supported me for the past 3 years. I would also like to thank my committee members, Thomas White, Roger Johnson, Nicolas Nassar, and David Lee Gorden who have remained supportive of both my graduate school experience as well as well as direction of my research project.

Although it would be futile to try and list them here, many people at Stony Brook have been fundamental to my research and well being. I would like to formally thank the Physiology and Biophysics Department for accepting me as a student and providing me with an opportunity to study at Stony Brook University. Though I am a student in the Physiology and Biophysics department, for the past 3 years I have worked in the Pharmacology Department. As I have become familiar with the staff and faculty of the Pharmacology Department I would certainly be remised if I did not acknowledge their continual support of their students, of whom I was also considered.

Chapter 1

Introduction

Pancreatic ductal adenocarcinoma

General Characteristics

Pancreatic ductal adenocarcinoma (PDA) is the most common among pancreatic tumors and one of the most fatal human cancers, killing approximately 30,000 people each year in the United States (Cancer Statistics, 2005). PDA is a disease that is associated with advancing age and is rarely detected before the age of 40 (Anderson, K.E., et al., 1996). Upon diagnosis PDA is almost unvaryingly fatal, with a five year survival rate of less than 5% (Warshaw, A.L. and Fernando de-Catillo, 1992). The median survival rate for patients with PDA is 4-6 months, partly because by the time of detection the pancreatic tumors are at an advanced stage and have often metastasized. Consequently, the precursor event leading to stepwise pancreatic tumor progression remains hidden from detection. Another factor conspiring to obscure diagnosis of the early stages of PDA is the lack of specific symptoms. The inflammatory response to the diseased pancreas results in extensive fibrosis, causing the diseased pancreas to impose on the stomach or the spine, mimicking stomach or back pain. Also, because the common bile duct enters at the head of the pancreas, joining the main pancreatic duct (Figure 1), often the common bile duct is obstructed due to pancreatic inflammation. As a result, bilirubin is found in excessive amounts in the blood stream, causing yellowing of the eyes and skin (jaundice). So while stomach pain, back pain and jaundice are all general symptoms of pancreatic cancer, due to the lack of specific symptoms and limitations in diagnostic methods, the disease often eludes detection during its formative stages.

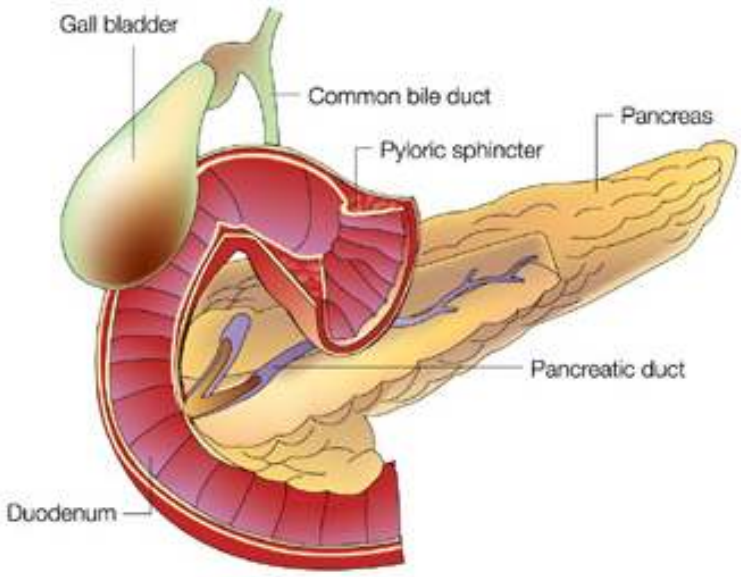

Figure 1. Gross anatomy of the pancreas. Diagram of the normal human pancreas; demonstrating the joining of the common bile duct with the main pancreatic duct at the head of the pancreas (adapted from Bardeesy, N. and DePinho, R., 2002).

Molecular genetics of pancreatic ductal adenocarcinoma

It is generally believed that PDA arises from pancreatic duct cells (Hruban, R.H., et al., 2001). As defined in Cubilla and Fitzgerald's classic study, the increased appearance in abnormal duct structures in patients with PDA and the similar spatial distribution of such lesions to malignant tumors, support the hypothesis that such lesions represent early PDA (Cubilla, A.L, and Fitzgerald, P.J., 1976). These lesions were recently codified under the collective term pancreatic intraepithelial neoplasia (PanIN) and are grouped into three histologic stages based on increasing degrees of architectural and nuclear atypia (Figure 2) (Kern, S., et al., 2001). PanIN-1 is often characterized by the elongation of cells surrounding an apparent lumen (Figure 2). The loss of polarity and the crowding of cells into the lumen defines PanIN-2, while PanIN-3 is characterized by severe nuclear atypia and cell budding into the lumen (Figure 2).

As to what induces the appearance of these pre-neoplastic lesions remains poorly defined. However there are a number of risk factors that have been identified that predispose patients for PDA. Environmental insults, such as ionizing radiation, smoking and diet, may modulate PDA by activating oncogenes or inactivating tumor suppressor genes (Anderson, K.E., et al., 1996). Furthermore, numerous studies have documented an increased risk in relatives of PDA patients, and it is estimated that 10% of pancreatic cancers are due to inherited predisposition (Lynch, H.T. et al., 1996). However, unlike familial cancer syndromes for breast, colon and melanoma, PDA that is linked to familial settings has a lower penetrance (~10%) and maintains a comparable age of onset to the sporadic cases in the general population (Lynch, H.T. et al., 1996).

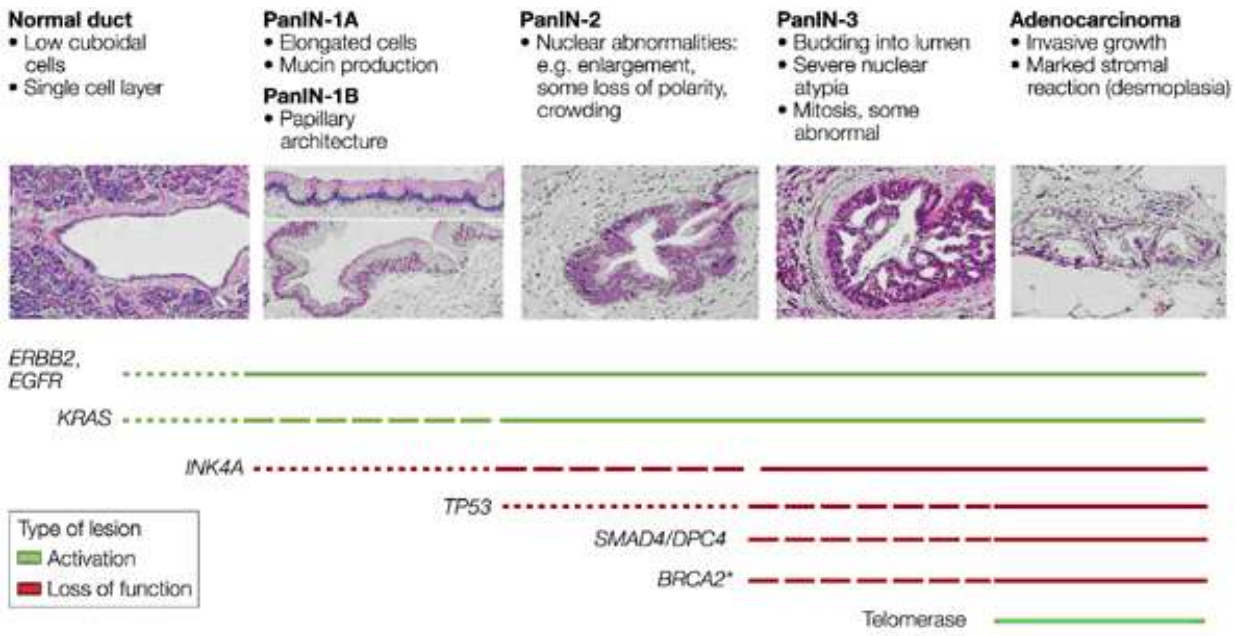

Figure 2. Genetic progression model of pancreatic adenocarcinoma. Pancreatic intraepithelial neoplasias (PanINs) seem to represent progressive stages of neoplastic growth that are precursors to pancreatic adenocarcinomas. The genetic alterations documented in adenocarcinomas also occur in sequence in PanINs. The stage of onset of these lesions is depicted (adapted from Bardeesy, N. and DePinho, R., 2002).

Further molecular and pathological analysis of PanINs and PDA has also revealed a characteristic pattern of genetic lesions that involve mutations of KRAS (derived from Kirsten rat sarcoma), cyclin dependent kinase-2A (CDKN2A) (Moskaluk, C.A et al., 1997), tumor protein-53 (TP53) (Luttges, J. et al., 2001), breast cancer type 2 (BRCA2) (Heipmoller, E. et al., 2000), and mothers against decapentaplegic homolog/deleted in pancreatic cancer locus 4 (SMAD4/DPC4) (Luttges, J. et al., 2001). Because these studies have identified genetic mutations that are common in both PanIN lesions and PDA, this data provides supportive evidence of the relationship between PanINs and the pathogenesis of PDA. Interestingly, the mutational events mentioned above seem to take place in series relative to PanIN progression to PDA (Figure 2). Activating KRAS mutations are the most common genetic changes that are detected throughout the PanIN series (Klimstra, D.S and Longnecker, D.S. et al., 1994), followed by CDKN2A (which encodes the tumor suppressor INK4A, an inhibitor of cyclin-dependent kinase CDK4) (Moskaluk, C.A et al., 1997), TP53 (Luttges, J. et al., 2001), SMAD4/DPC4 (Luttges, J. et al., 2001) and BRCA2 (Heipmoller, E. et al., 2000).

Kirsten rat sarcoma (KRAS) Mutations

Mutations in the RAS family of protoocogenes (comprising of KRAS, NRAS and HRAS) are common in 20-30% of all human tumors. On average, 30% of PanIN-1A and B lesions showed mutated KRAS (Luttges, J. et al., 2003). In PanIN-2 and PanIN-3 lesions, the rate of mutated KRAS increases to 45% and 56%, respectively (Luttges, J. et al., 2003). Furthermore KRAS mutations and are found in nearly 100% of PDA (Rozenblum, E. et al., 1997). Taken together these data demonstrate that KRAS mutations increase in frequency with disease progression. Although the roles of specific

KRAS effector pathways in pancreatic cancer pathogenesis have not been completely elucidated, there is evidence that autocrine signaling is a contributor to the oncogenic effects of KRAS. To gain insight into the molecular mechanism of epidermal growth factor receptor (EGFR)-dependent transformation and to examine whether autocrine-EGFR signaling was a prerequisite for oncogenic transformation by components of the RAS signaling pathway in other cell types, immortalized 3T3 fibroblasts isolated from EGFR−/− fetuses were used (Sibilia, M. et al., 2000). Both EGFR−/− and +/+ immortalized fibroblasts infected with a control virus were not tumorigenic, whereas wild-type cells expressing constitutively activate RAS efficiently formed tumors in nude mice (Sibilia, M. et al., 2000). In contrast, fibroblasts lacking the EGFR failed to be transformed by constitutively activate RAS, indicating that EGFR signaling is required for oncogenic transformation by constitutively activate RAS (Sibilia, M. et al., 2000). Consistent with the requirement of EGFR for the oncogenic transformation by constitutively activate RAS, pancreatic ductal adenocarcinomas overexpress EGFR ligands, like transforming growth factor-alpha (TGF-α), as well as EGFR (Korc, M. et al., 1992). EGFR induction occurs in the early stages of PanINs, indicating that autocrine EGF-family signaling might be operative at the onset of pancreatic tumorigenesis (Day, J.D. et al., 1996). The functional significance of this pathway is illustrated by the inhibited growth of PDA cell lines in vitro following attenuation of EGFR signaling by blocking antibodies (Watanabe, M. et al., 1996). Therefore a deeper understanding of this oncogenic pathway will be vital for the development of new treatment approaches towards this disease.

Cyclin dependent kinase-2A (CDKN2A) mutations

Loss of CDKN2A function, usually brought about by mutation, deletion or hypermethylation (the addition of a methyl group to cytosine and adenine residues in DNA that leads to the modification of DNA and the reduction of gene expression and protein production), also occurs in 80-95 % of sporadic PDA (Rozenblum, E. et al., 1997). CDKN2A loss is generally seen in moderately advanced lesions that show features of dysplasia, further implicating CDKN2A as a tumor suppressor. A closer look at how CDKN2A contributes to tumor suppression resulted in the interesting discovery of two tumor suppressors encoded at the CDKN2A locus 9q21 (Sher, C.J. et al., 2001). Given the physical location and frequent homozygous deletion of 9p21, many pancreatic cancers sustain the loss of the inhibitor of cyclin-dependent kinase CDK4 (INK4A) and the alternate reading frame-p14 (p14ARF) (Moskaluk, C.A et al., 1997). INK4A inhibits cyclin dependent kinase 4 (CDK4) mediated phosphorylation of retinoblastoma (RB), thereby blocking entry into the S (DNA synthesis) phase of cell cycle. p14ARF stabilizes p53 (a protein involved in tumor suppression) by inhibiting its proteolysis, resulting in the inactivation of CDK complexes, which would otherwise promote transcription of genes that would carry the cell through the cell cycle. Loss of p14ARF by a homozygous mutation in the CDKN2A (INK4A) gene will lead to loss of p53 function and cell cycle control. Nonetheless, INK4A seems to be the more prominent pancreatic cancer suppressor at the 9p21 locus because both germline and sporadic mutation have been identified that target INK4A, but spare p14ARF (Rozenblum, E. et al., 1997).

Tumor protein-53 (TP53) mutations

TP53 is a tumor suppressor gene, which means that it regulates the cycle of cell division by keeping cells from growing and dividing too fast or uncontrollably (Rosenblum, E. et al., 1997). TP53 mutations arise in later-stage PanINs that have acquired significant features of dysplasia, reflecting the function of TP53 in preventing malignant progression (Rosenblum, E. et al., 1997). A study of the structure of chromosome material (cytogenetic study) demonstrated that these lesions have profound aneuploidy (change in the number of chromosomes that can lead to a chromosomal disorder) (Gorunova, L. et al., 1998). Further cytogenetic studies have provided evidence that telomere dynamics might contribute to the genomic instability in lesions induced by TP53 mutations. A telomere is a region of highly repetitive DNA at the end of a linear chromosome that functions to maintain vital genetic information, which is needed to sustain a cell's activities (Joeng K.S., Song, E.J., Lee, K.J., Lee, J. 2004). In humans, cancerous tumors acquire indefinite replicative capacity by over-expressing telomerase, an enzyme involved in synthesis of telomeres (Joeng K.S., Song, E.J., Lee, K.J., Lee, J. 2004). However, although reactivation of telomerase is crucial to the emergence of immortal cancer cells, a preceeding period of telomere shortening and dysfunction might also contribute to carcinogenesis by leading to the formation of chromosomal rearrangements during DNA replication (Maser, R.S. and DePhino, R.A., 2002). The survival of cells with significantly shortened telomeres is enhanced by inactivation of p53, allowing the acquisition of oncogenic chromosomal alterations (Gisselsson, D. et al., 2001). Studies in the telomere knock out mice support this model, as telomere dysfunction and p53 loss cooperate to promote the development of carcinomas in

multiple tissues (Artandi, S.E. et al., 2002). An analysis of a large series of human pancreatic cancer cell lines revealed that telomeres were frequently lost from chromosome ends. As these observations were made in both early and late stage tumors, telomere dysfunction is believed to be an early event in the pathogenetic pathway (Gisselsson, D. et al., 2001).

Mothers against decapentaplegic homolog/deleted in pancreatic cancer locus 4 (SMAD4/DPC4) mutations

SMAD4/DPC4 encodes a transcriptional regulator that is a key component in the transforming growth factor-beta (TGF-β)-family signaling cascade (Inman, G.J., 2005). The activation of this pathway can result in a number cell functions, such as differentiation, apoptosis, embryonic development and the cell cycle (Inman, G.J., 2005). The SMAD4/DPC4 gene maps to a region on the chromosome (18q21) that sustains deletion in approximately 30% of pancreatic cancers (Hahn, S.A. et al., 1996). The mechanism by which SMAD4/DPC4 loss contributes to tumorigenesis is likely to involve its role in TFG-β mediated inhibition (Massague, J., Blaun, S.W. and Lo, R.S., 2002). TFG-β inhibits the growth of most normal epithelial cell either by blocking cell cycle transition or by promoting apoptosis (Massague, J., Blaun, S.W. and Lo, R.S., 2002). Cellular responses to TFG-β are at least partially SMAD4/DPC4 dependent (Sirard, C. et al., 2002). However, PDAC cell lines also show variable sensitivity to TGF-β-induced cytotoxicity in a manner that appears independent of *SMAD4* status (Dai, J.L. et al., 1999; Subramanian, G. et al., 2004). These contrasting observations have complicated the interpretation of the role of SMAD4 in human PDA progression.

To date, genetically engineered mice have provided tractable genetic models to dissect the complexities of a number of cancers in the physiological context. In the mouse

pancreas, expression of an activated KRAS knock-in allele induces PanIN lesions that can gradually progress to PDAC over a period of l year (Aguirre,A.J. et al., 2003; Hingorani, S.R. et al,. 2003). Inactivation of the Ink4a/Arf tumor suppressor locus, or of p53, does not induce pancreatic neoplasia; however, simultaneous KRAS expression and inactivation of Ink4a/Arf or p53 drives the development of advanced PDAC with short latency (Aguirre A.J. et al., 2003; Hingorani' S.R. et al., 2005; Bardeesy, N. et al., 2006). Although SMAD4 is inactivated in the majority of PDAs with simultaneous mutational inactivation of the INK4A/ARF tumor suppressor locus and activation of the KRAS oncogene, until recently the role of SMAD4 had not yet been investigated in the context of these models. Therefore, the impact of SMAD4 deficiency on the development of the pancreas and on the initiation and/or progression of PDA alone, or in combination with PDA relevant mutations, was analyzed using genetically engineered mice (Bardeesy, N. et al., 2006).

Selective SMAD4 deletion in the pancreatic epithelium had no discernable impact on pancreatic development or physiology (Bardeesy, N. et al., 2006). However, when combined with the activated KRAS allele, SMAD4 deficiency enabled rapid progression of KRAS initiated neoplasms (Bardeesy, N. et al., 2006). While KRAS alone induced PanINs that progressed slowly to carcinoma, the combination of KRAS and SMAD4 deficiency resulted in an alternative histological pathway to advanced PDAC, one that is characterized by intraductal papillary mucinous neoplasia (IPMN), a precursor to PDA in humans (Bardeesy, N. et al., 2006). SMAD4 deficiency also accelerated PDA development of KRAS INK4A/ARF heterozygous mice and induced IPMN (Bardeesy, N. et al., 2006). In addition, in vitro studies with a PDA cell line revealed that while

SMAD4 deficiency was associated with attenuated TGF-β responsiveness, SMAD-4 expressing lines showed increased proliferation and migration of undifferentiated $SMAD4^{+/+}$ lines and cell death in well-differentiated lines. These results provide genetic confirmation that SMAD4 is a PDA tumor suppressor, functioning to block the progression of KRAS initiated neoplasms, whereas in a subset of advanced tumors, intact SMAD4 facilitates TGF- β-dependent growth.

Breast cancer type 2 (BRCA2) mutations

BRCA2 mutations are typically associated with familial breast and ovarian cancer syndrome, but also carry a significant risk for the development of pancreatic cancer (Goggins, M., Hruban, R.H. and Kern, S.E., 2001). Approximately 17% of pancreatic cancers that occur in familial settings harbor mutations in this gene (Goggins, M., Hruban, R.H. and Kern, S.E., 2001). Similar to those individuals with germline CDKN2 mutations, the penetrance of pancreatic cancer in BRCA2 mutation carries is relatively low (7%), yet the age of onset is similar to that of patients with the sporadic form of the disease (Goggins, M., Hruban, R.H. and Kern, S.E., 2001).

Although the structures of the BRCA1 and BRCA2 genes are very different, their functions are thought to be very similar (Yoshida, K. and Miki, Y., 2004). However, it is important to note that the familial breast cancer alleles other than BRCA2 (like BRCA1) do not predispose patients to pancreatic cancer (Yoshida, K. and Miki, Y., 2004). Loss of wildtype BRCA2 seems to be a late event in those individuals who inherit germline heterozygous mutations in BRCA2 (Goggins, M., Hruban, R.H. and Kern, S.E., 2001). As a result of these mutations, the protein product of the BRCA2 gene is abnormally short and does not function properly (Goggins, M., Hruban, R.H. and Kern, S.E., 2001).

Researchers believe that the defective BRCA2 protein is unable to help fix mutations that occur in other genes (Yoshida, K. and Miki, Y., 2004). As a result, mutations build up and can cause cells to divide uncontrollably. Loss of wild type BRCA2 is restricted to late stage PanINs and adenocarcinomas (Goggins, M., Hruban, R.H. and Kern, S.E., 2001). These data support the possibility that BRCA2 loss promotes the malignant progression of existing lesions in pancreatic tumorigenesis.

Genes involved in familial pancreatic ductal adenocarcinoma (PDA)

As with most cancers, important insights have emerged from the study of rare family members that demonstrate an increased incidence of pancreatic adenocarcinoma. It is estimated that at least 10% of pancreatic cancers are due to an inherited predisposition (Jaffe, E.M. et al., 2002). Among the genetic lesions that are linked to familial pancreatic carcinoma are CDKN2, BRCA2 (which have already been described), serine/threonine kinase-11 (STK11) (also called LKB1) and micronuclear linker histone-1 (MLH1).

LKB1 is a member of the serine/threonine kinase family that provides instructions for making the STK11. In addition to regulating cell division, STK11 helps cells correctly orient themselves within tissues and assists in determining the amount of energy a cell uses via adenosine monophosphate kinase (AMPK) pathways. AMPK is a protein kinase cascade that plays an important role in regulating energy homeostasis. In vitro, STK11 has been observed to directly phosphorylate AMPK, an event that is correlated with activation of this kinase. STK11 regulates p53-dependent apoptosis pathways, and has also been found to play a role in cell cycle arrest. Restoring STK11 activity into cancer cell lines defective for its expression results in a G1 cell cycle arrest (Carretero, J. et al., 2007). Through a combination of these mechanisms, STK11 aids in the prevention of

tumors, especially in the gastrointestinal tract. Mutations in STK11 gene are associated with Peutz-Jeghers syndrome (PJS), an autosomal dominant disorder characterized by the growth of polyps in the gastrointestinal tract, pigmented macules (dermatological lesions) on the skin and mouth, and other neoplasms (Hemminki, A. et al., 1998). PJS patients are normally afflicted with benign intestinal polyposis at a young age (Cooper, H. S. et al., 1998), and not only does advancing age carry an increased risk of developing gastrointestinal malignancies, but there is also a 40-fold increase risk of developing pancreatic cancer (Giardiello, F. M. et al., 2000).

Microsatellite instability is a genomic instability that is characterized by very high mutation rates at small DNA repeat sequences (Boland, C. et al., 1998). An alteration of microsatellite repeats is the result of strand misalignment during DNA replication (Boland, C. et al., 1998). This phenotype is caused by mutations in DNA-mismatch repair genes such as MLH1 (Aaltonen, L. A. et al., 1998). The repair of mismatch DNA is essential to maintaining the integrity of genetic information over time. These defects in DNA repair pathways have been related to many human carcinogenesis. The importance of mismatch repair genes became apparent with the identification of the genetic basis for hereditary nonpolyposis colon cancer (HNPCC). There seems to be an elevated risk of pancreatic cancer in HNPCC families (Aarnio, M. et al., 1995). Although HNPCC is most commonly associated with an increased risk of colorectal cancer, it accounts for less than 5% of hereditary pancreatic cancer cases (Aaltonen, L. A. et al., 1998). The pancreatic adenocarcinomas in HNPCC patients show distinct molecular genetic profiles such as KRAS and TP53 mutation, as well as characteristic histopathology (Goggins, M. et al., 1998).

In summary, molecular pathology and cancer genetic studies have provided an outline of celluar disruptions that are associated with pancreatic adenocarcinoma. However, the specific biochemical and cellular events resulting from these mutations are not known. Therefore, a more direct mechanistic view of how lesions influence the biology of pancreatic cancer is necessary in order to answer key questions that have been a part of the ongoing study of pancreatic cancer. What is the cell of origin of pancreatic cancer? Do other signaling pathways regulate tumorigenesis? How do the cells of origin influence the developmental stage of the genetic lesions? In this dissertation, I have referred to these questions in order to stimulate my investigation into the early events of pancreatic cancer and the agents that are necessary to influence the early onset of the disease. While I have cited significant evidence of a common mutation patterns that link PanINs with PDA, in order to attempt to address the key questions mentioned above, the knowledge of pancreatic tumor biology is of extreme relevance.

Biology of pancreatic adenocarcinoma

As mentioned previously pancreatic ductal adenocarcinoma (PDA) is believed to arise from pancreatic duct cells (Hruban, R.H., et al., 2001), as pancreatic adenocarcinomas cells show phenotypic resemblance to normal duct cells, displaying cuboidal shape (Figure 2), ductal antigen expression and growth into tubular structures (Solcia, E. Capella, C. and Kloppel, G., 1995). Moreover, as I have referenced the molecular genetics of pancreatic adenocarcinoma, the genes involved in the early progression of pancreatic adenocarcinoma provides strong support for a ductal origin of this malignancy. However, parallel observations in rat and hamster carcinogen models

implicate acinar cells as the cells origin for pancreatic adenocarcinoma (Jimenez, R.E. et al., 1999).

Acinar cells are implicated as cells of origin for pancreatic adenocarcinoma

Implantation of the carcinogen, dimethylbenzanthracene (DMBA) into the head of the pancreas induces pancreatic adenocarcinomas in rats 9 months after carcinogen exposure. Precursor duct-like lesions (resembling PanINs) develop 1 month after initiation of treatment (Jimenez, R.E. et al., 1999). Lesions studied included 10 early precursor lesions (DMBA for 2 weeks), 8 precursor lesions after 1 month of DMBA treatment, and 10 adenocarcinomas after 9 months of DMBA treatment. Normal rat pancreas served as a control. For comparison, 5 human ductal adenocarcinomas were also evaluated. Immunohistochemistry with ductal (keratin, cytokeratin 19, cytokeratin 20), and acinar (chymotrypsin), cell markers was performed to analyze the tissues. Rat precursor lesions and adenocarcinomas revealed strong expression of keratin, cytokeratin 19, and cytokeratin 20 in the cytoplasm of all neoplastic cells, and absence of chymotrypsin (Jimenez, R.E. et al., 1999). Human adenocarcinomas showed strong expression of keratin and cytokeratin 19 in all neoplastic cells, expression of cytokeratin 20 in 5–20% of cells, and absence of chymotrypsin (Jimenez, R.E. et al., 1999). These data demonstrate that pancreatic adenocarcinomas induced by DMBA in rats express markers consistent with a ductal phenotype, as observed in human adenocarcinomas. Therefore it is possible, that ductal cell markers are acquired by acinar cells during the early stages of tumor development. These data suggest that the loss of acinar cells and the reciprical accumulation of duct-like precursor lesions is the direct result of DMBA treatment in

rats. Perhaps even more significant is the fact that after 9 months of treatment these rats develop pancreatic adenocarcinomas.

Recent studies demonstrate that expression of oncogenic KRAS transgenes in ductal lineages fail to induce PanINs or PDA (Brembeck, F.H. et al., 2003). These observations suggest that PDA originates either from early precursors before they are committed to ductal lineages or by transdifferentiation of other pancreatic cell types. The latter hypothesis has been explored by expressing KRAS oncogenes in cells of acinar lineage (Grippo, P.J.et al., 2003). Although some of these mice occasionally display low-grade PanINs, they do not develop high-grade lesions or PDA (Grippo, F.H. et al., 2003). More recently, knockin of a KRAS oncogene in the locus encoding Mist1, a transcription factor required for proper acinar organization, induces invasive and metastatic pancreatic tumors (Tuveson, D.A. et al., 2006). However, these tumors display mixed histological characteristics that do not recapitulate the basic properties of human PDA.

More recent studies report that expression of the endogenous KRAS oncogene in acinar/centroacinar cells results in a full range of PanINs and PDA histologically identical to those of human patients (Hruban, R.H. et al., 2006). Interestingly, using a mouse model that allows temporal control of KRAS oncogene expression, in adult acinar cells KRAS expression failed to induce PanINs and PDA (Guerra, C. et al., 2007). However, if these adult mice are subjected to tissue damage, they develop the full spectrum of PanINs and invasive PDA. These observations suggest that not only does PDA arise from the exocrine component of the pancreas, but also raises the possibility that human PDA may require, in addition to activation of KRAS oncogenes, nongenetic events involving tissue damage.

An introduction to metaplasia

As these mouse models indicate, the cells of origin of pancreatic cancer are represented by either the duct cells or acinar cells of the exocrine pancreas. Nonetheless these models do not explain whether progenitor cells specific to exocrine pancreas can give rise to duct-like cells associated with pancreatic adenocarcinoma. In addition, these models do not establish whether there is a selective expansion of differentiated cell types ordinarily present in low abundance. Nonetheless, what is possibly the more significant aspect of these rivaling compenents of the pancreas for the title of "cell of origin," is a distinct similarity in their ability to transform from one cell type into another. Whether it is the conversion of a normal duct or islet cells into pancreatic adenocarcinoma, tissue specific progenitors cells developing into pancreatic adenocarcinoma, or the loss of acinar cells and the accumulation of precursor lesions associated with pancreatic adenocarcinoma, these mechanism are indicative of process known as transdifferentiation (Bardeesy, N. and DePhino, R., 2002).

Transdifferentiation is defined by the conversion of one differentiated cell type into another, an event also regarded as metaplastic conversion. In the case of the acinar cells of the exocrine pancreas, the loss of acinar cells and subsequent accumulation of duct-like precursor lesions represents a transdifferentiation event known as acinar-to-ductal metaplasia (ADM) (DeLisle and Logsdon, 1990). During this process, the characteristic pyramidal morphology of healthy acinar cells containing basal nuclei and apical zymogen granules are transformed into duct-like cells characterized by lumen containing structures, lined by low cuboidal cells with simple squamous epithelia (Figure 3b) (Means, A.L. et al., 2005). The conversion of islet, duct or undifferentiated progenitor

(or stem) cells of different tissues can also be regarded as a metaplasic event (Bardeesy, N. and DePhino, R., 2002). Therefore metaplasia is believed to represent an early event in the accumulation of duct cell lesions (metaplastic ducts) associated with pancreatic adenocarcinoma.

Metaplastic ducts arise from pancreatic acinar cells

Until recently, in adult mammalian tissues the specific mechanisms underlying metaplastic events were not completely defined. Elucidating this mechanism would not only result in understanding the cellular basis of metaplasia, but it could also help determine the cellular origin of pancreatic adenocarcinoma. To further define the role of acinar cells during transdifferentiation, acinar cells, labeled in a cell type specific manner (genetic lineage label with lacZ), were cultured for 5 days in the presence of transforming growth factor alpha (TGF-α). The TGF-α ligand, mediates the autophosphorylation of the epidermal growth factor receptor (EGFR). As a result, down stream signaling of pathways involved in cell survival and proliferation are activated. The idea that TGF-α is involved in transdifferentiation comes from the observation that many human epithelial cancers exhibit over-expression of TGF-α /EGFR complexes. Furthermore TGF-α has been shown to induce pancreatic ductal metaplasia in vitro and in vivo (DeLisle and Logsdon, 1990; Wagner, M. et al., 1998).

As confirmed by acinar morphology and by immunohistochemical labeling with an acinar cell marker (amylase), the genetic lineage label was initially expressed exclusively by fully differentiated acinar cells (Figure 3A) (Means, A. et al., 2005). Although most cells at the start of culture were amylase positive, there was no specific amylase immunoreativity by day 5 (Figure 3D). Rather, the majority of the cells had adapted

classic cuboidal or simple squamous ductal morphology and were found to express duct cell markers (Figure 3F) (cytokeratin-19). The majority of these cytokeratin-19 positive duct cells were also positive for the genetic lineage label (Figure 3 D), indicating that the genetic lineage labeled duct cells arose by transdifferentiation of acinar cells into duct cells. Although the mechanism for generation of metaplastic ductal epithelium may certainly differ from the mechanisms employed for renewal of normal ductal epithelium, the results suggest that fully differentiated acinar cells retain a latent precursor potential. This model is consistent with the view that precursor activity may not necessarily be limited to a discrete population of undifferentiated stem cells within a given tissue, but rather might considered an inducible biologic function of fully differentiated cells (Blau et al., 2001; Shen et al., 2000). These data support an ability for differentiated acinar cells to assume a precursor function in the exocrine pancreas.

Mouse models demonstrate that metaplastic ducts arise from pancreatic acinar cells

More evidence established previously by mouse models of acinar-to-ducatal metaplasia supports the emergence of duct cells from acinar cells (Wagner, M. et al., 2001). Because of the availability of promoters that are capable of directing transgene expression to the pancreas, transgenic mice over-expressing TGF-α in the pancreas were developed in order to dissect the complexities of pancreatic tumorigenesis in a physiological context (Wagner, M. et al., 2001). Within months of exposure to TGF-α, ductal metaplasia is induced in the acinar cells of the exocrine pancreas (Figure 3b). After 1 year pancreatic adenocarcinoma is discernable in these mice, thereby confirming the cellular lineage of the disease (Figure 3c-d) (Wagner, M. et al., 2001). Not only is the metaplastic phenotype found in the pancreas a result of TGF-α exposure, but the

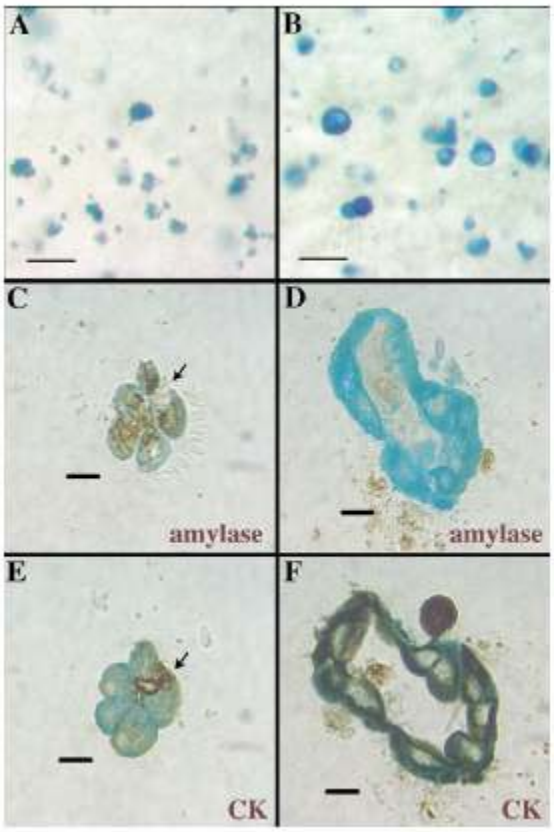

Figure 3. Lineage tracing reveals that acinar cells transdifferentiate into ductal cells. Pancreatic epithelium from mice with genetically labeled acinar cells was mice was isolated, cultured and fixed immediately after plating (A,C,E) or after culture in the presence of TGF-α for 5 days (B,D,F). On day 0 most cells (A) contained β-gal activity (blue) and were amylase positive (C) and cytokeratin negative (E), confirming that β-gal expression was confined to acinar cells. Following 5 days of TGF-α treatment, most cells were still β-gal positive (B). No amylase positive cells were observed (D) and β-gal activity was present in cells expressing ductal cytokeratins (F), indictatin that the β-gal expressing acinar cells had transdifferentiated into ductal cells (adapted from Means, A. et al., 2003).

deposition of collagen (fibrosis) and the infiltration of leukocytes (inflammation) is also apparent (Wagner, M. et al., 2001). Taken together, metaplasia, fibrosis, and inflammation represent yet another predisposing factor common to pancreatic adenocarcinoma.

Another predisposing factor links metaplastic ducts, PanINs and pancreatic adenocarcinoma.

Among the predisposing factors I mentioned previously were environmental insults as well as inherited genetic mutations that regulate oncogenes or tumor suppressor genes. Another predisposing factor common to pancreatic adenocarcinoma is chronic pancreatitis or inflammation of the pancreas. As a common response to tissue damage, chronic inflammation has been identified as part of an initiating event for many cancers (Schwartz, L. et al., 2002). For example, as a response to chronic inflammation, inducible nitric oxide synthase (iNOS) is expressed and can, in turn, lead to genetic mutations responsible for the induction neoplastic transformation of epithelial cells (Jaiswal, M. et al., 2001). Inflammation can also induce cycloxygenase-2 (COX-2) expression which can initiate a proliferative response and induce angiogenesis (the formation of new blood vessels) (O'Byrne, K.J. et al., 2001). While the promotion of oncogenic mutation, proliferation and angiogenesis provides an obvious link between inflammation and tumorigenesis, another event associated with inflammation (as well as fibrosis) also provides a link between chronic pancreatitis and tumorigenesis. Epithelial metaplasia has further been identified as a major hallmark of chronic pancreatitis (Warshaw, A.L. et al., 1998). In chronic inflammation-associated metaplasia, many epithelia undergo

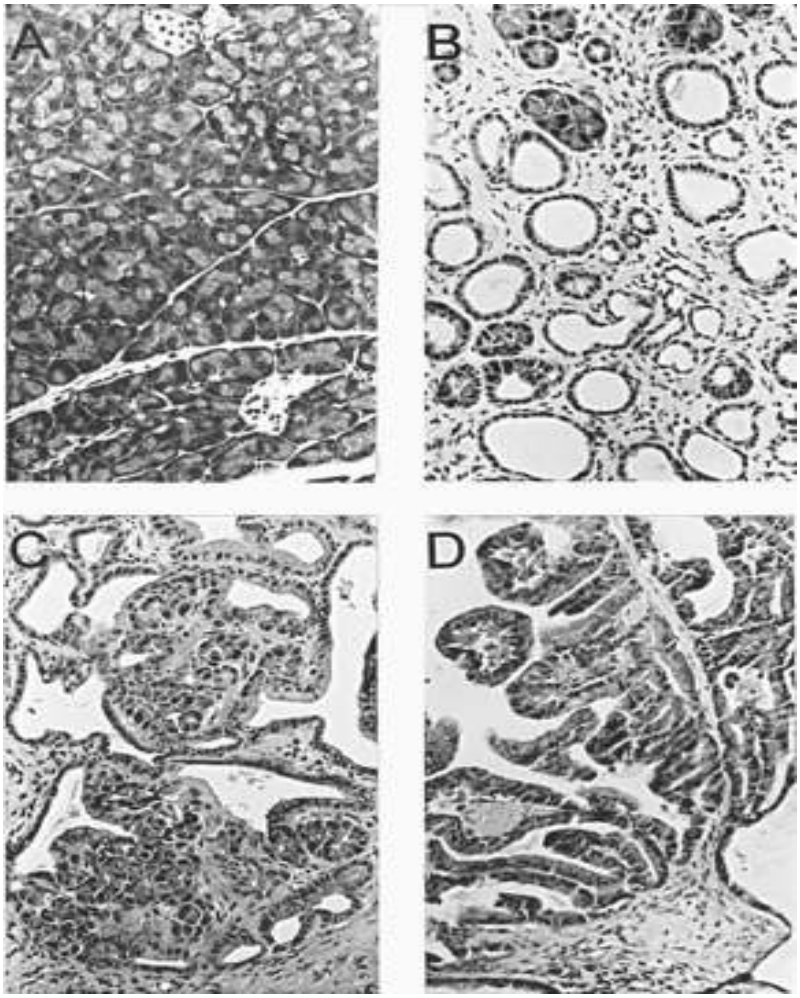

Figure 4. Acinar to ductal Carcinoma sequence in TGF-α transgenic mice. (A) Wild type littermate controls show regular acinar morphology. (B) whereas TGF-α transgenic mice develop acinar to ductal metaplasia within 3-4 months. (C) In older animals dysplastic changes occur within the metaplastic ducts, (D) which can also transform into tumors (adapted from Schmid, R.M., 1999).

morphological changes that resemble the transition of normal cells to tumors (Schwartz, L. et al., 2002). This is clearly true for gastrointestinal tissues where epithelial metaplasia is associated with sites of chronic inflammation, as well as tumor formation in the esophagus (DeMeester, T.R. et al., 2001), stomach (Kawachi, T. et al., 1976), colon (Jass, J.R. et al., 1994), and pancreas (Kloppel, G. et al., 1999). Therefore, it is possible that metaplasia could itself be a predisposing factor for cancer.

In fact, as I mentioned previously, in the pancreas, pancreatic ductal adenocarcinoma (PDA) is thought to arise from premalignant lesions known as pancreatic intraepithelial neoplasia (PanIN) (Kloppel, G. et al., 1999; Hruban, R.H. et al., 2001). Moreover, a preneoplastic lesion that is always associated with PDA as well as patients at risk for PDA, such as those with chronic pancreatitis (CP), is known as the metaplastic duct lesion (MDL) (Kloppel, G. et al., 1999). MDLs arise from the process previously described as acinar-to-ductal metaplasia (ADM). The association with full blown PDA and patients at elevated risk for PDA has led to the hypothesis that MDLs act as PanIN precursors associated with pancreatic ductal adenocarcinoma (Kloppel, G. et al., 1999).

Matrix Metalloproteinases

The matrix metalloproteinase (MMP) family of extracellular proteinases regulates development and physiologic events and are frequently expressed with cancer (Powell, W.C. et al., 1996). Genetic analyses using transgenic mice that have gain and loss of function of MMPs or of their endogenous inhibitors, the TIMPs, and pharmacogenetic studies with chemical inhibitors have begun to elucidate the roles that they play (Vu, T.H., Werb, Z., 2000). It is now clear that these enzymes are important for cell migration, invasion (Sternlicht, M. et al., 2000), proliferation (Johnson, S. and Knox, A., 1999), and

apoptosis (Crawford, H. et al., 2002). They regulate many developmental processes, including branching morphogenesis (Brown, L. et al., 1997), angiogenesis (OByrne, K.J. et al., 2001) wound healing (Lund, L. et al., 1999), and extracellular matrix degradation (Gross, J. and Lapiere, C.M., 1962)

The matrix metalloproteinases (MMPs) are a family of proteases capable of degrading the extracellular matrix (ECM). They share common functional domains and activation mechanisms (Figure 5) (Sternlicht et al. 2000 and Vu, T.H. et al., 2000). These are Ca^{2+}- and Zn^{2+}-dependent endopeptidases that are active at neutral pH (Vu, T.H., Werb, Z., 2000). They are secreted or transmembrane enzymes that remain inactive (proenzymes) until processed to an active form by the removal of an amino-terminal propeptide. MMP family members can be identified by their distinctive domain structure and several highly-conserved sequences, like the HEXGH zinc-binding site in the catalytic domain and the PRCGVPD cysteine-switch region in the pro-domain, responsible for maintaining enzyme latency (Figure 5) (Nagase, H. et al., 1999). The propeptide functions to keep the enzyme in latent form by the interaction of a cysteine residue in this peptide with the zinc moiety in the enzyme active site (Nagase, H. et al., 1999). Disruption of this interaction triggers the cysteine switch mechanism and results in activation of the enzyme (Nagase, H. et al., 1999). MMPs can be activated by chaotropic agents (which disrupt noncovalent bonds in molecular structures) or by cleavage of the propeptide by members of the MMP family or by other proteases. They are inhibited by a family of tissue inhibitors of metalloproteinases, the TIMPs (Brew, K. Dinakarpandian, D. Nagase, H., 2000). As a family, MMPs degrade most components of the ECM (Vu, T.H., Werb, Z., 2000). Currently, 25 members of the MMP family have been identified in humans (Sternlicht et

al. 2000 and Vu, T.H. et al., 2000). They are separated by subgroups based on preferential substrates or similar structural domains: Collagenases that are active against fibrillar collagen, gelatinases that have high activity against denatured collagens, stromelysins that degrade noncollagen components of the ECM, membrane-type MMPs (MT-MMPs) that are transmembrane molecules, and other less characterized members (Vu, T.H., Werb, Z., 2000) (Figure5).

Since the discovery of the first MMP by Gross and Lapiere in 1962, it was believed that MMPs primarily function to degrade the protein components of the extracellular matrix (ECM) (Gross, J. and Lapiere, C.M., 1962). This discovery was based on the observation that during amphibian metamorphosis, collagenolytic activity is required to digest the collagens in tadpole tails (Gross, J. and Lapiere, C.M. 1962). However, the activity of MMPs during embryonic development extends to more than the removal of ECM molecules. Not only can MMPs degrade the ECM, it is now evident that they are required during development and normal physiology in several ways: (1) to degrade ECM molecules and allow cell migration (Sternlicht, M. et al., 2000); (2) to alter the ECM micro-environment and result in alteration in cellular behavior (Johnson, S. and Knox, A., 1999); (3) or modulate the activity of biologically active molecules by direct cleavage (O'Reilly, M.S. et al., 1994).

Extracellular matrix degradation and cell migration by MMPs

Studies using assays of cell migration through extracellular matrix (ECM) barriers in culture have implicated MMPs in the migration of a variety of epithelial, mesenchymal (supportive frame work of epithelial cells consisting mainly of collagen and fibroblast),

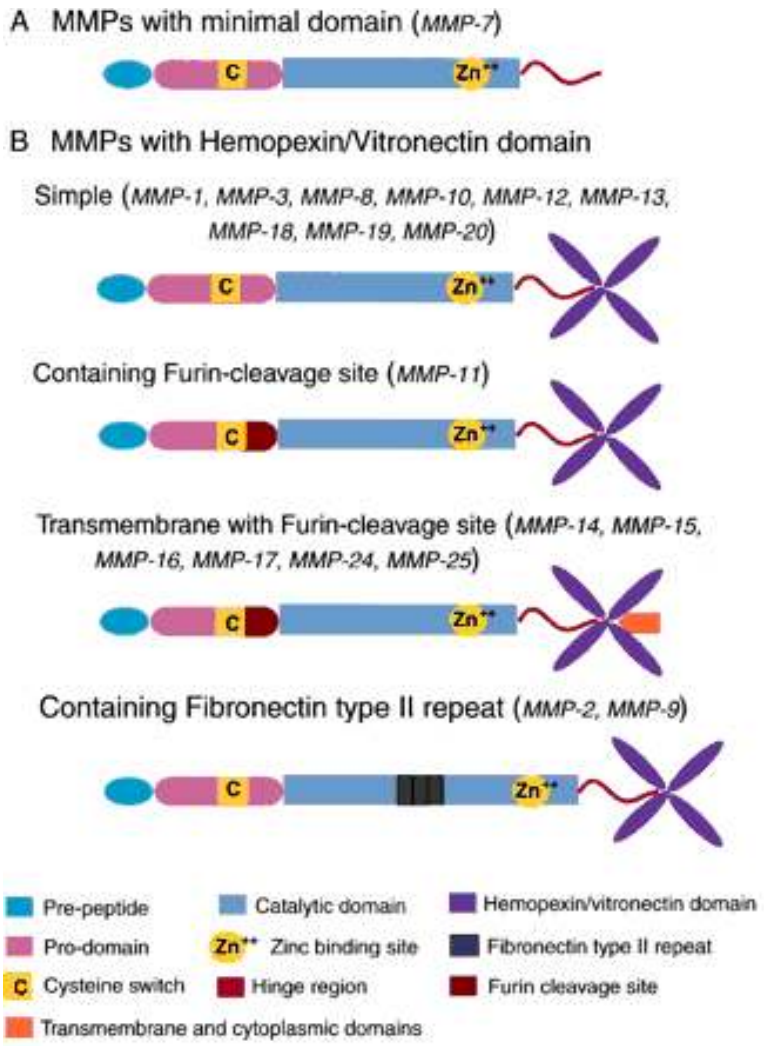

Figure 5. Structural domains of the matrix metalloproteinase. (A)MMPs with minimal domain structure and (B) MMPs with vitronectin/hemopexin domains (less characterized members) (adapted from Vu, T. and Werb, Z., 2000).

and neuronal cells either through the ECM or on specific ECM substrates. For example, in mammals trophoblasts mediate the implantation of the fetus into the lining of the uterus (endometrium), by invading the uterine lining of the female placenta at pregnancy. These cells express high levels of MMP-9 (Vu, T.H., Werb, Z., 2000). In culture, a neutralizing antibody against MMP-9 inhibits trophoblast from invading and degrading ECM (Behrendtsen, O. and Werb, Z. 1997). During angiogenesis biological signals known as angiogenic growth factors activate receptors present on endothelial cells that line the blood vessels. As a result, the activated endothelial cells release proteases that degrade the basement membrane, allowing endothelial cells to migrate from the original vessel walls in order to form new vessels (Burri, P.H. 2004). In culture, the migration of endothelial cells through collagen gels is impaired by MMP inhibitors (Fisher, C et al., 1994; Hiraoka, N. et al., 1998). More evidence demonstrating MMPs ability to aid in migration is found during bone remodeling. In this process the secretion of collagenases and other enzymes by osteoclasts (bone cells) aid in the breakdown of bone (bone resorption) allowing release of minerals into the blood (Schoppet, M. Preissner, K. and Hofbauer, L., 2002). Initially osteoclast are recruited to the bone surfaces, and their migration is believed to be MMP dependent. In culture, MMP inhibitors curb the migration of purified osteoclasts through collagen (Sato, T. et al., 1998).

MMPs and cell behavior

The ECM can serve many functions, such as providing support and anchorage for cells, providing a way of separating the tissues, and regulating intercellular communication. The ECM regulates a cell's behavior through its interactions between

cell surface molecules (Junqueira, L.C.U., Bignolas, G. and Brentani, R.R., 1979). In a changing environment, the ability of cells to proliferate, survive, or differentiate changes as well. Although studies on the roles of MMPs on cell behavior in culture are few they have shown effects on cell proliferation, survival, apoptosis, differentiation, and organization (Vu, T.H., Werb, Z., 2000). Inhibition of MMPs either by a chemical inhibitor or a neutralizing antibody to MMP-2 reduces the proliferative response of cultured vascular smooth muscle cells to growth factors (Uzui, H. et al. 2000). Proliferation of cultured airway smooth muscle cells is also reduced by MMP inhibitors (Johnson, S. and Knox, A., 1999). MMPs have also been shown to cause cell death. The cleavage of the transmembrane protein Fas ligand (FasL) by MMP-7 is one example (Fingleton, B. et al., 2001). Its ability to release the proapoptotic molecule FasL from the cell surface has been shown to enhance apoptoisis in susceptible cells (Fingle, B. et al., 2001). MMP inhibitors also suppress smooth muscle cell proliferation and induce cell apoptosis in hypertrophied (is the increase in size of an organ) rat pulmonary arteries in organ culture (Cowan, K.N., et al. 2000). Thus, MMPs affect cell behavior by regulating survival signals as well as proliferation. These differences in the effects of MMPs may reflect the differences in MMP substrates involved in these processes.

MMP cleavage can result in biologically active molecules

It is now well known that the precise activity of any proteinase is determined by the function of its available substrates. However, a frequent pattern in biologically active molecules is the generation of fragments with new activities by proteolytic cleavage. MMP cleavage of the protein plasmin demonstrates its ability to generate fragments with new activities. Plasmin is an enzyme present in blood that degrades many blood plasma

proteins, most notably fibrin clots (Cesarman-Maus, G., 2005). A number of MMPs can cleave plasmin, and one of the cleavage products, agiostatatin, acts as an inhibitor of angiogenesis (O'Reilly, M.S. et al., 1997). Other angiogenic inhibitors generated by proteolysis have also been reported. Endostatin is a fragment of collagen (type XVIII) and also has antiangiogenic activity (O'Reilly, M.S. et al. 1997). MMPs may also control the function of biologically active molecules by other mechanisms. For example, by releasing them from bound protein or ECM stores they can regulate a molecules' bioavailability (Whitelock, J.M. et al., 1996). Furthermore, a molecules' activity or inactivity can be regulated by MMPs via proteolytic processing (Rajah, R. et al., 1995). Thus, MMP activity can lead to both gain and loss of function of biologically active molecules. Examples for each of these mechanisms have been reported (Vu, T.H., Werb, Z., 2000). However, it is important to note that the experiments described here derive there conclusions based on the use of broad spectrum inhibitors not necessarily specific to MMPs. Furthermore, these in vitro experiments do not take into account the physiological response to MMP inhibition inherent in whole animal model. Therefore, analysis of transgenic mice that have gain and loss of function of MMPs or their endogenous inhibitors will give better insight into the roles that MMPs play in these processes.

MMPs are involved in tumor formation

While the effects of MMPs on ECM degradation, cell migration, cellular behavior and cleavage demonstrate physiological roles of MMPs, evidence derived from the advent of mouse molecular genetics shows that MMPs also have functions throughout tumor progression, including tumor formation (Chambers, A.F. et al., 1997). As I mentioned

previously, since the discovery of the first MMP by Gross and Lapiere in 1962, it was believed that MMPs primarily function to degrade the protein components of the extracellular matrix (ECM), lending credence to a role in tumor invasion and metastasis (Powell, W.C. et al., 1996). Although the involvement of MMPs in early tumor formation is in stark contrast to the majority of MMP literature, which is dedicated to the function of MMPs as promoters of tumor cell invasion, recent studies on many MMPs have shown that their activities are more complex than simple ECM degradation to promote tumor invasion.

Tumor progression is a multistep process. To complete this process, the tumor must not only grow at the primary site, but also avoid detection by the immune system, establish blood supply and invade the surrounding tissue. It is now established that MMPs play critical roles in all these processes, while their expression in normal tissue is highly restricted (Chambers, A.F. and Matrisian, L. M 1997). In addition to allowing for invasion, the discovery that MMPs can cleave non-ECM components has expanded the proposed role of MMPs to include functions in primary tumor growth as well as angiogenesis (Egeblad, M. and Werb, Z., 2002). For example, cell migration and invasion can be induced by MMP-3 and MMP-7 mediated cleavage of E-cadherin (a cell-cell adhesion molecule) (Lochter, A. et al., 1997; Noe, V. et al., 2001). Growth factors like fibroblast growth factor (FGF), and interleukin-8 (IL-8) can be cleaved and thus activated by MMPs, resulting in cell proliferation (Schonbeck, U. et al., 1998; Yu, W. et al., 2000).

MMPs as oncogene induced genes

Accumulated data has shown that the likely reason for this strong association of MMP expression and cancer is the array of oncogenic signaling molecules and transcription

factors that turn on MMP gene expression (Crawford, H.C. et al., 1996). For example, in the majority of MMPs, binding sites for the family of nuclear protoocogenes, AP-1 (Fos/Jun) are found within the first 75 base pairs of the promoter region (Benbow, U. and Brinckerhoff, C.E., 1997). Similarly, most MMP promoters have binding sites for the family of Ets nuclear protoocogenes. Regulation of Ets and AP-1 transcriptional activity occurs primarily through Mitogen-activated protein (MAP) kinases. MAP-kinases are involved in cell response to growth factors, cytokines, as well as cell-cell interaction (Robinson, M.J. and Cobb, M.H. 1997). Abnormal activation MAP-kinase pathways is associated with transformation process (Sivaraman, V.S et al., 1997). Of particular significance is the involvement of RAS in mediating activation of MAP-kinase. Inappropriate activation of the gene has been shown to play a key role in signal transduction, proliferation and malignant transformation. Mutations in the Ras family of proto-oncogenes (comprising H-Ras, N-Ras and K-Ras) are very common, being found in 20% to 30% of all human tumours (Reuter, C. et al., 2000). In summary, although other regulatory sites exist for specific MMP promoters, the Ets and AP-1 binding elements are the key sites of regulation for MMP promoters and are key elements responsible for abnormal regulation of MMPs in cancer.

MMP-7 and pancreatic tumor progression

MMP-7 is a member of the MMP family that is expressed by tumor cells in adenocarcinomas of many tissues including 98% of PDA samples at all stages of tumor progression, including 100% of MDLs (Crawford, H.C. et al., 2002). MMP-7 appears to play a role in the earliest stages of tumor progression, based on several pieces of

evidence, primarily gained from models of colon and breast cancers (Wilson, C. et al., 1997; Rudolph –Owen, L.A., et al., 1998)

The potential function of MMP-7 in colorectal cancer was originally tested by manipulating its expression levels in colon tumor cell lines and injecting the cells into the cecum (a pouch connected to the ascending colon of the large intestine) of nude mice (Witty, J.P. et al., 1994). For these experiments, SW480, a colon tumor cell line that makes very little endogenous MMP-7, was engineered to overexpress MMP-7. Conversely, SW620, a genetic relative of SW480 that expresses endogenous MMP-7, had MMP-7 levels reduced by stable expression of antisense MMP-7 RNA. When injected into the mouse cecum, the SW480 cells expressing MMP-7 were more tumorigenic than control SW480 cells and SW620 cells with reduced MMP-7 were less tumorigenic when compared to control SW620 cells. These data are consistent with MMP-7 playing an important role in tumor formation.

More definitively, the Multiple Intestinal Neoplasia (MIN) mouse, which normally forms multiple benign tumors in its small intestine, was mated into the MMP-7 null (MMP-$7^{-/-}$) genetic background (Wilson, C.L. et al., 1997). Compared to control MIN mice, the MIN/MMP-$7^{-/-}$ mice formed 60% fewer tumors, strongly suggesting an important role for MMP-7 in tumor formation or early tumor growth. In a complementary set of experiments, MMP-7 expression was transgenically targeted to the mouse mammary gland using the Murine Mammary Tumor Virus (MMTV) promoter (Rudolph-Owen, L.A et al., 1998). The resultant MMTV-MMP-7 mice were mated to the MMTV-Neu model of mammary cancer. Compared to control MMTV-Neu mice, MMTV-Neu/MMP-7 mice formed tumors at a significantly faster rate.

These data provide evidence that expression of MMP-7 in 100% of MDLs and 80% of PDA samples examined may be indicative of an early role for MMP-7 during tumor formation. Its preferential expression in glandular epithelium as a result of initial insult, and its lack of expression in normal epithelia, has generated interest about the pathological role of MMP-7 during tumor formation (Dunsmore, S.E. et al., 1998).

Observations demonstrating MMP-7 mRNA expression in 80% of colorectal adenocarcimonas (Newell, K.J. et al., 1994) and in more than 70% in benign polyps (Newell, K.J. et al., 1994; Takeuchi, N. et al., 1997) suggest that it is unlikely that the primary role of MMP-7 is only to enhance the metastatic phenotype. Thus, MMP-7 appears to play a role in the early stages of tumor formation, which is consistent with a growing body of literature establishing MMPs as important regulators of tumor formation (Noel, A.C. et al, 1996; Strernlicht, M.D. et al., 1999; Coussens, L.M. et al., 2000). More evidence of MMP-7's role in tumor formation comes from its ability to cleave cell surface molecules that are relevant in the process.

MMP-7 substrates relevant to tumor formation

Currently, a number of potentially relevant cell surface molecules associated with tumor formation have been identified as MMP-7 substrates (Figure 6). For instance, MMP-7 can release the proapoptotic molecule Fas ligand (FasL) from the cell surface, enhancing apoptosis in susceptible cells (Fingleton, B. et al., 2001). MMP-7 has also been shown to activate cellular proliferation by releasing growth factors like heparin-binding epidermal growth factor (HB-EGF) from the cell surface (Yu, W.H. et al., 2002). As a result, tyrosine phosphorylation of the EGF receptor family member ERβ4 is enhanced (Yu, W.H. et al., 2002). Release of HB-EGF has been shown to enhance

cellular proliferation and to contribute to apoptotic resistance via the phosphoinositol 3-kinase/Akt signal transduction pathway (Danielsen, A.J. et al., 2002).

Finally, MMP-7 can contribute to cellular invasion by release of cell surface adhesion molecules and ECM components. MMP-7 has been shown to cleave the homotypic cell-cell adhesion molecule E-cadherin, releasing it from the cell surface (Noe, V. et al., 2001). The newly soluble E-cadherin ectodomain is capable of interacting with intact E-cadherin molecules, breaking apart cell-cell junctions and inducing invasion through fibrillar collagen. Similarly, β4 integrin, which promotes interaction with laminin-containing ECM, is a substrate for MMP-7 (Von Bredow, D.C. et al., 1997). Cleavage of β4 integrin can disrupt cell-matrix interactions, thus promoting migration and invasion. MMP-7 can also cleave the serum/ECM molecule osteopontin (Agnihotri, R. et al., 2001) creating a bioactive epitope that can disrupt cellular interactions with fibronectin potentially having a similar effect as cleavage of β4 integrin. Thus, MMP-7 can

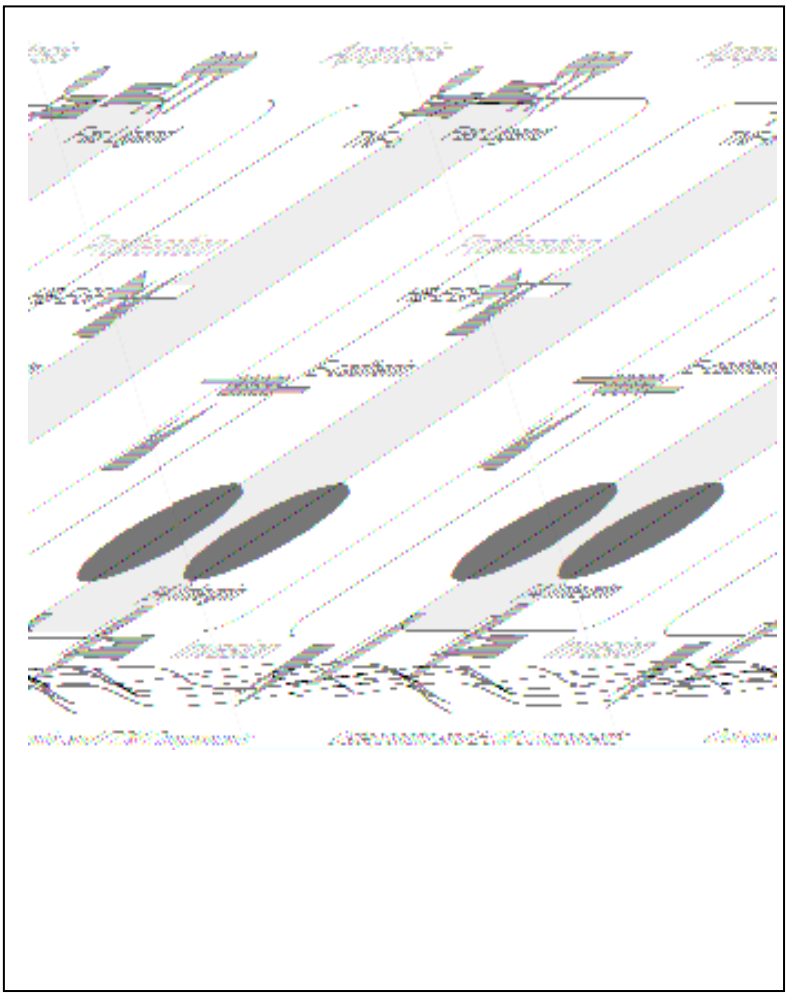

Figure 6. MMP-7 substrates with implications toward tumor progression. MMP-7 has been shown to release factors that influence apoptosis (Fas ligand, TNF-α), proliferation (HB-EGF) and invasion (E-cadherin, β4 integrin, osteopontin and other ECM components) (created by Dr. Howard Crawford).

contribute to migration and invasion through its cleavage of a variety of substrates, not just components of the ECM.

CP is inhibited in all regards in MMP-7 null mouse model of CP

Although MMP-7 has been shown to contribute to both the formation and invasion of a number of adenocarcinomas, the frequency of MMP-7 expression in pancreatic tumors exceeds expression of MMP-7 in other tumors (Crawford, H.C. et al., 2002). Consistent with the role of MMP-7 in the earliest stages of pancreatic tumor formation, all MDLs in tumor samples of patients with PDAC express MMP-7 (Crawford, H.C. et al., 2002). And as mentioned previously, MDLs have been proposed as PanIN precursors associated with PDA as well as patients at risk for PDA, such as those with CP (Kloppel, G. et al., 1999). Furthermore, because MMP-7 has been shown to be important for tumor formation in multiple tissues (Wilson, C.L. et al., 1997; Rudolph-Owen, L.A. et al., 1998) and because its expression correlates strongly with the formation of PanIN lesions, it was previously hypothesized that MMP-7 activity is important for the formation of pancreatic MDLs in vivo (Crawford, H.C. et al., 2002).

In order to determine if the induction of ADM was associated with MMP-7 expression in vivo, a mouse model was used that accurately simulates human CP (Crawford, H.C. et al., 2002). Obstruction of the main pancreatic duct by surgical ligation (PDL) can induce all the hallmarks of CP, including an inflammatory response, extensive fibrosis, and ADM, within days (Satake, K. et al., 1998; Scoggins, C.R. et al., 2000). PDL was performed on wild type (control) mouse pancreas as well as MMP-$7^{-/-}$ mouse pancreas.

After PDL, wild type mouse pancreatic tissue proximal to the point of the main pancreatic duct obstruction, exhibited CP by day 5 (Figure 7A-C) (Crawford, H.C. et al.,

2002). Conversely, the multiple pathological states of CP are inhibited in all regards in MMP-7$^{-/-}$ mouse pancreas (Figure 7D-F) (Crawford, H.C. et al., 2002). Furthermore, 5 days after PDL, pancreata isolated from both wild type and MMP-7$^{-/-}$ mice showed no MMP-7 immunoreactivity was in the acinar cells, fibrotic stroma, or inflammatory cells in the pancreas of these mice (Crawford, H.C. et al., 2002). However, MMP-7 expression was found exclusively in the MDLs of wild type mouse pancreas. Taken together, these data suggest that the expression of MMP-7 in the complex environment of the intact pancreas is clearly associated with metaplastic ductal epithelial cells specifically, not with pancreatic epithelial cells in general.

Acinar-to-ductal metaplasia (ADM) can be simulated in vitro

Unfortunately, in the whole animal context, it is impossible to know if MMP-7 is inducing an event intimately associated with the metaplastic process or if the metaplastic epithelium is responding to interaction with other tissue components, such as the fibrotic stroma or inflammatory cells that are inherent in this system. Therefore determining a role for MMP-7 with out the context of these inherent complications is of paramount importance. The identification of pathway by which MMP-7 mediates its affects is equally significant. To resolve these issues I have referred specifically to previous in vitro studies demonstrating the ability of recombinant human (rh) TGF-α to induce ADM (Figure 8A-C) via a Notch-dependent pathway (De Lisle and Logsdon, 1990; Githens et al., 1994; Roman et al., 2000; Miyamoto, Y.M. et al., 2003; Sphyris, et al., 2005; Means, A.L. et al., 2005).

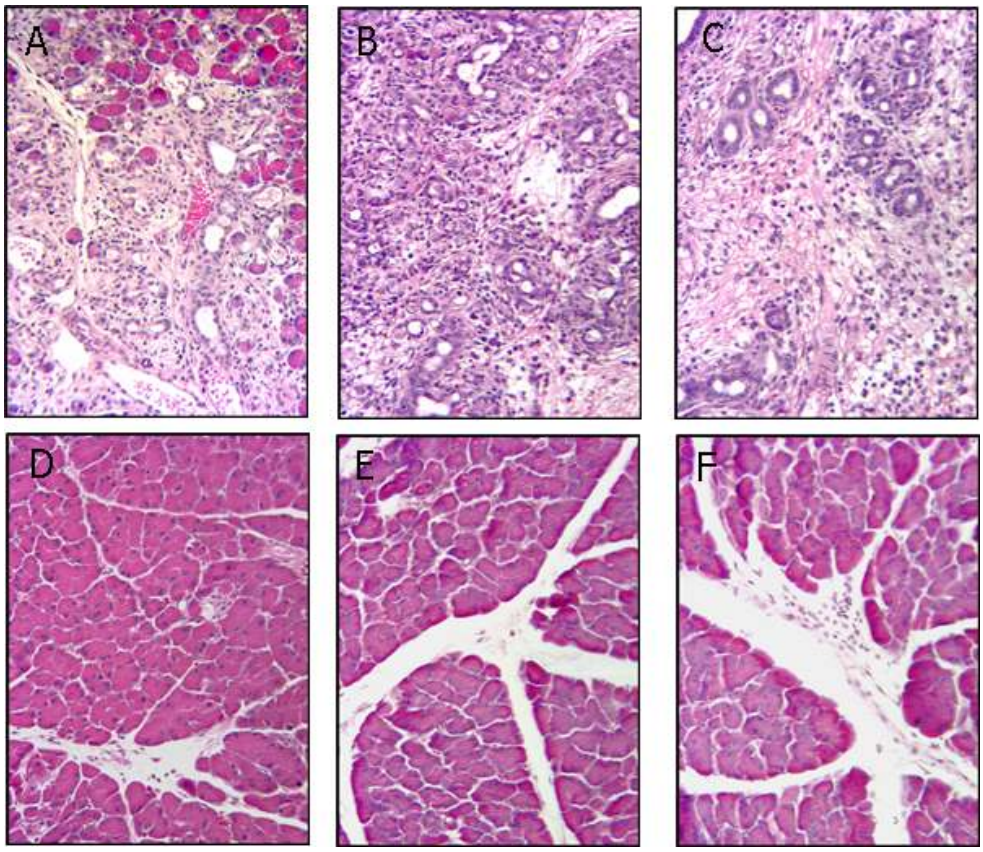

Figure 7. Inhibition of pancreatic ductal metaplasia following duct ligation in MMP-7-/- pancreas. (A-C) Pancreatic tails of wild type mice (left to right), 5,7 and 10 days after ductal ligation. Pancreatic tails of MMP-7-/- mice (left to right), 5,7 and 10 days after ductal ligation (adapted from Crawford, H. C. et al., 2002).

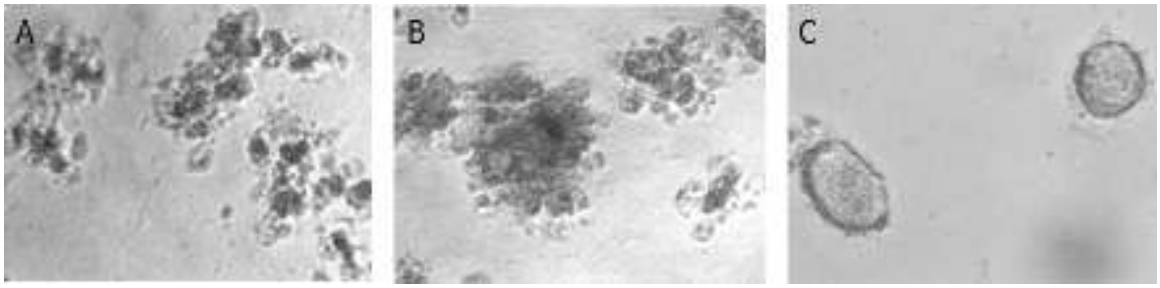

Figure 8. Exposure of the acinar cells to TGF-α induces an ductal metaplasia over a period of 5 days. (A, and B) show acinar cells cultured at day 0, and 3 respectively. (C) Conversion of acinar cells to duct cells is apparent by day 5.

The Notch signaling pathway

On both a histologic and molecular level, metaplastic duct epithelium arising under the influence of TGF-α has been characterized as a precursor for tumor formation (Song, Y. et al., 1999; Wagner, M. et al., 2002). The resemblance of undifferentiated cell populations in metaplastic epithelia to the embryonic pancreas during development implicated a role for the highly conserved, Notch pathway (Song, Y. et al., 1999), which is a known regulater of cell fate decisions in embryonic cells (Artavanis-Tsakonas, S. et al., 1999; Mumm and Kopan, 2000). It was later shown that the Notch pathway activation mediated TGF-α induced metaplastic conversion between differentiated cell types (Miyamoto, Y. et al., 2003). Therefore, as pathway for TGF-α dependent metaplasia has recently been defined by Notch expression (Miyamoto, Y. et al., 2003), I used the Notch pathway as a mechanistic reference to elucidate a pathway for MMP-7.

Notch/Delta signaling pathway has been conserved through evolution from nematodes to human. This pathway is involved in local cell-cell inductive processes that specify embryonic cell fates. Typically, Notch receptors (Notch 1-4) are activated upon interaction with one of their cognate ligands (Jagged or Delta-like) on an adjacent cell (Figure 9). Upon binding of the ligand to the Notch receptor, Notch undergoes proteolytic events during maturation and signal transduction. Ligand binding triggers cleavage of the extracellular region of Notch by metalloproteinase activity. Proteolytic cleavage within the transmembrane domain by γ-secretase releases the intracellular domain (ICD) so that it can translocate to the nucleus and activate transcription of target genes (Figure 9) (Struhl and Adachi, 2000; Struhl and Greenwald, 2001).

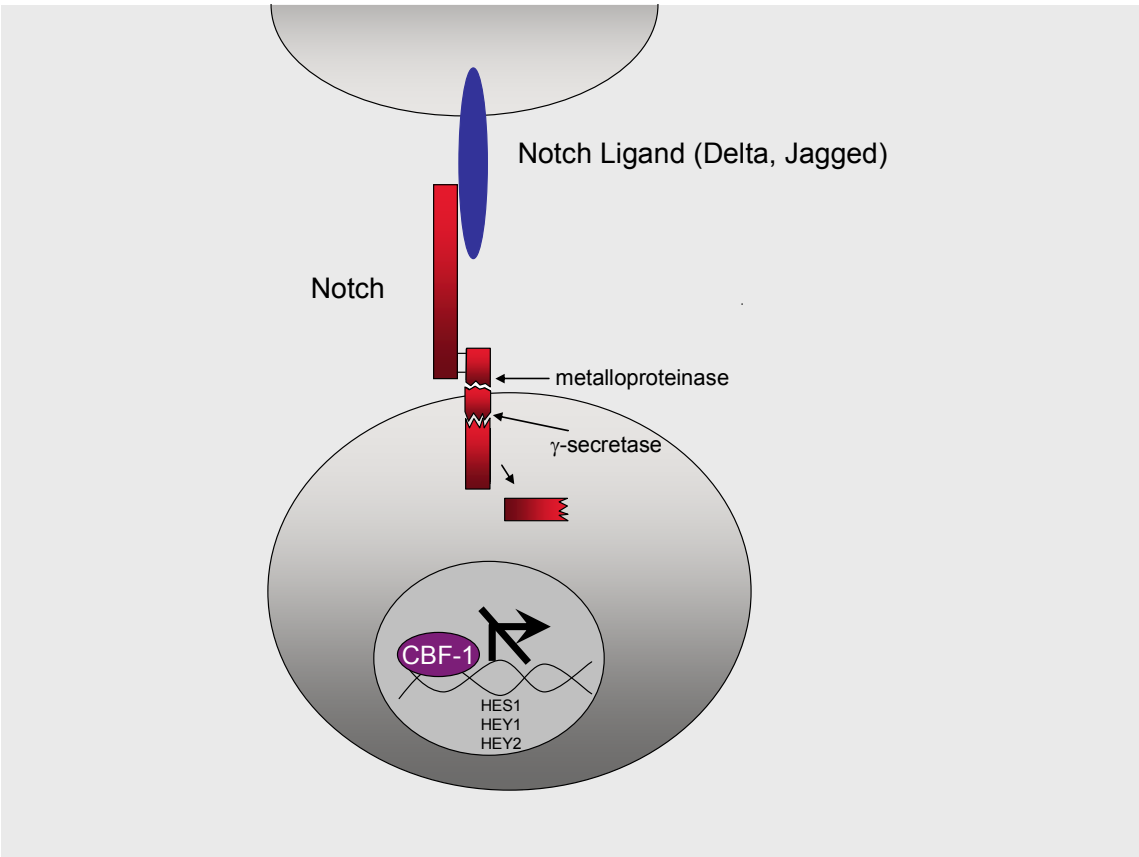

Figure 9. Notch receptors are activated upon interaction with one of their cognate ligands. Upon binding of the ligand to the Notch receptor, Notch undergoes proteolytic events during maturation and signal transduction. Ligand binding triggers cleavage of the extracellular region of Notch by metalloproteinase activity. Proteolytic cleavage within the transmembrane domain by γ-secretase releases the intracellular domain (ICD) so that it can translocate to the nucleus and activate transcription of target genes (image created by Dr. Howard Crawford).

The intracellular domain of Notch contains ankyrin repeats, which behave as transcriptional activators upon interaction with the DNA-binding protein CSL (CBF-1, Su(H), Lag-1) which is normally found associated with the transcriptional repressor, retinoblastoma protein (RBP-Jk). RBP-Jk represses the activity of several target genes, among them the genes of the Hairy Enhancer of Split-1 (HES-1) complex (Jennings et al., 1994). Translocation to the nucleus of the intracellular domain of Notch displaces RBP-Jk and transforms the repressor to an activator, thereby promoting expression of the HES-1 complex genes. As a result of HES-1 activation, pro-differentiation factors required to commit cells to a specific differentiation programs are down regulated. In the context of development, Notch appears to prevent cellular differentiation and maintain a population of undifferentiated precursor cells (Mayimoto, Y. et al., 2003). These precursor cells are believed to be a part of an intermediate event that is characterized by the expression of Nestin (Means, A.L. et al., 2005).

An intermediate event involved in acinar-to-ductal metaplasia

Nestin refers to a member of the family of intermediate filaments and comes from the fact that this protein is expressed mainly in neuroepithelial stem cells (Ehrmann, J. et al., 2005). Nestin is not expressed in mature cells and terminal cell differentiation is associated with loss of immunoreactivity to this protein. (Ehrmann, J. et al., 2005). Therefore, immunohistochemical assessment of Nestin expression has proven to be useful when evaluating the difference between mature and immature cells.

Although the use of Nestin as a label for undifferentiated pancreatic epithelial progenitors remains controversial, recent studies have confirmed that Nestin-expressing epithelial cells are indeed present during early pancreatic development, and that these

cells represent the cell of origin for differentiated exocrine cells (Delacour et al., 2004; Esni etal., 2004). Based on the role of Nestin as a marker of precursor cells in the developing exocrine pancreas, the expression of Nestin has been evaluated at various stages of TGF-α induced ADM. Nestin immunoreactivity was detected in a majority of cells following 2 days in culture with TGF-α. (Means, A.L. et al., 2005). The ability of TGF-α to reactivate Nestin expression in mature amylase positive acinar cells may therefore represent an adult recapitulation of the embryonic events mentioned previously (Means, A.L. et al., 2005).

Hypothesis

Evidence for a direct role for MMP-7 during tumor formation has been demonstrated in multiple tissues in vivo. As mentioned previously, MDLs expressing MMP-7 were found in a mouse model of CP (Crawford, H.C. et al., 2002); and because the multiple pathological states of CP were inhibited in all regards in MMP-$7^{-/-}$ mice pancreas, this has peaked our interest about the functional role of MMP-7 during ADM.

Furthermore, the in vivo data suggest that in the complex environment of the intact pancreas MMP-7 is clearly associated with metaplastic ductal epithelial cells specifically, not with pancreatic epithelial cells in general. Thus, in the context of the whole animal, it is impossible to know if MMP-7 is inducing an event intimately associated with the metaplastic process or if the metaplastic epithelium is responding to interaction with other tissue components inherent in this system. To gain understanding about the molecular role of MMP-7 during the formation of MDLs, I formulated an overall objective for my dissertation based on the hypothesis that MMP-7 modulates the transition of acinar cells to duct cells (independent of inflammatory or fibrotic

responses). Therefore my objective has been to determine specific the molecular role of MMP-7 in ADM that is associated with pancreatic tumor formation.

Using a simplified in vitro model of ADM to test the direct contribution of MMP-7 to ADM, I determined that in primary acinar cells derived pancreata of wild type mice, MMP-7 expression was discernable by day 2 of this model. However, in the pancreata of MMP-$7^{-/-}$, MMP-7 expression is not detected in this model in response to TGF-α. Furthermore, MMP-$7^{-/-}$ pancreatic acinar cells were incapable of converting to duct like cells that were cytokeratin-19 positive. By day 5, the majority of acinar cells were replaced by metaplastic ducts, demonstrated by the expression of a duct cell marker, cytokeratin-19. These results initiated a series of experiments to determine if MMP-7 is necessary to induce ADM. In addition, experiments to determine the hierarchy of MMP-7 and other molecules associated with acinar cell transdifferentiation (for example, Nestin) have demonstrated a requirement for Notch pathway activation in ADM.

Chapter 2

Materials and Methods

Mice

The Matrix metalloproteinase-7 null mice in a C57Bl/6J background were obtained from Dr. Lynn Matrisian (Vanderbilt University). Wild type C57Bl/6J animals were purchased from The Jackson Laboratory (Bar Harbor, Maine). Animals were bred and maintained in a maximum isolation facility in the Department of Laboratory Animal Resources at Stony Brook University. All protocols herein were approved by the Institutional Animal Care and Use Committee at Stony Brook University.

Acinar cell preparation

Primary acinar cell cultures were prepared by modifying previously published protocols (De Lisle and Logsdon, 1990; Githens et al., 1994; Wagner et a., 2002). Pancreata were removed from 4 week old male wild type or MMP-7$^{-/-}$ mice and minced into 1-5 mm pieces using sterile scissors. After centrifugation (2000 rpm for 2 minutes), the supernatant was removed and the acinar cells were digested in 0.2 mg/ml collagenase-P (Roche) at 37°C for 10 minutes.

Following multiple washes in Hanks balanced salt solution (HBSS) supplemented with 5% fetal bovine serum (FBS), collagenase digested pancreatic tissue was filtered through 500µm and 105µm polypropelene mesh (Spectrum Laboratories, Laguna, CA), in succession. The filtrate was centrifuged through HBSS supplemented with 30% FBS at 1000 rpm for 2 minutes. The pellet was re-suspended in 3 mls of 1X Waymouth MB 752/1 media (Sigma-Aldrich) supplemented with Gentamicin (100 µg/ml), 1% heat-

inactivated FBS, 0.1 mg/ml soybean trypsin inhibitor (USB Corporation, Cleveland, OH) and 1 μg/ml dexamethasone (Sigma).

An equal volume of rat tail collagen type 1 (RTC) (BD Biosciences) was prepared and added to the cellular suspension. The acinar cell/RTC mixture was pipetted (500μl) into 12 wells of a 24-well plate. The 24-well plate was placed in a 37°C and 5% CO_2 incubator for 30 minutes to allow the collagen to solidify. Following solidification, 1X Waymouth MB 752/1 media (prepared as previously described) supplemented with appropriate growth factors or inhibitors was added.

Transdifferentiation events were induced by the addition of 50 ng/ml of recombinant human transforming growth factor-alpha (rhTGF-α) (Chemicon International) or 200 ng/ml of active recombinant human matrix metalloproteinase-7 (rhMMP-7) (Calbiochem) to 1X Waymouth MB 752/1 media (prepared as previously described). Where appropriate, acinar cells were additionally treated with the peptidomimetic inhibitor of γ-secretase, WPE-III-31C (20 μM) (Calbiochem). Cultures were maintained in 37°C and 5% CO_2 incubator for 5 days with media supplemented with the appropriate rhTGF-α and/or inhibitors was replaced on days 1 and 3.

Immunostaining

The following antibodies were used for immunoflourescence analysis: purified mouse monoclonal anti-rat nestin (BD Pharmingen), rabbit anti-α-amylase (Sigma), mouse monoclonal anti-cytokeratin-19 (Novocastra Laboratories Ltd), and rabbit anti-MMP-7 (Sigma). For immunoflourescent labeling of cells suspended in collagen, collagen disks containing the cells were removed from the 24 well plate and fixed in methanol and DMSO (4:1) over night at 4°C. The disks were stored in 100% methanol at -20°C. Upon

immunostaining with appropriate antibodies as described above, the disks were re-hydrated, and washed with PBS. Cells were blocked with PBSMT (PBS+ 0.5% Triton-X-100 + 2% milk) for 2 hours at room temperature, then incubated sequentially with the primary and the secondary antibodies diluted in PBSMT overnight at 4°C. After the addition of the antibodies, disks were washed extensively in PBST and counterstained with a nuclear dye, DAPI (4',6-diamidino-2-phenylindole). Images were captured on a Zeiss LSM-510 Meta confocal microscope.

Ectopic Notch pathway activation in pancreatic acinar cells

Pancreatic acinar cell cultures from WT or MMP-7 null mice were prepared as previously described. Prior to re-suspending the acinar cells into collagen, 1.5 mls of the acinar cell suspension was pipetted into 35 X 10 mm disposable petri dishes. Adenoviral vectors encoding GFP alone or GFP with the Notch2-intracellular domain (ICD) were added to the acinar cell suspension at this time. All infections were performed at an (multiplicity of infection) MOI of 20:1. After the addition of the adenovirus, cells were gently rocked every 15 minutes for 1 hour at 37°C, to avoid aggregation. Acinar cells remained in suspension with the adenovirus over night. Confirmation of successful viral transduction was accomplished by examination of high-efficiency GFP expression. Acinar cell suspensions were plated in collagen as described above. After collagen solidification, 1X Waymouth MB 752/1 media was added without growth factors.

MT-TGF-α mice on $ZnSO_4$ -H_20

Mice harboring a TGF-α transgene under regulation of a metallothionein promoter (MT-TGF-α) maintained on a C57Bl/6 background were a gift of Dr. Eric Sandgren (University of Wisconsin). These mice were crossed onto a MMP-7 null background

using MMP-7 null mice also maintained on the C57Bl/6 genetic background. At 5 weeks old, expression of the transgene was induced in both C57Bl/6 MT-TGF-α (control) and C57Bl/6 MT-TGF-α/MMP-7 null mice by administration of drinking water containing 25 mM of $ZnSO_4$ for 1- 4 months.

Five control mice and five C57Bl/6 MT-TGF-α/MMP-7 null mice were sacrificed at the end of various points of time (1-4 months) and the pancreas was harvested. Wet weight was recorded. The pancreas was cut in half, length wise from head to tail. One half was formalin fixed and paraffin embedded, while the other half was split again, length wise and used to make tissue lysates. The paraffin embedded tissue was analyzed by hematoxylin and eosin (H&E) staining as well as Masson's trichrome stain to determine the extent of metaplasia and fibrosis, respectively. Tissue lysates were used to quantitate fibrosis via hydroxyproline measurements, normalized to total protein content as measured by BCA assay (Reddy, et al., 1996).

MT-TGF-α mice on $ZnSO_4$ -H_20 for 4 months and distilled H_20 for 4 months

In a separate experiment, control mice (as previously defined) and C57Bl/6 MT-TGF-α/MMP-7 null mice were placed on $ZnSO_4$-H_20 for 4 months. At the end of 4 months the $ZnSO_4$-H_20 was removed and replaced with distilled H_20 for 4 months, after which time the mouse pancreas was harvested and analyzed as mentioned previously.

Chapter 3

Results

MMP-7 is necessary for in vitro acinar-to-ductal transdifferentiation

Previously it was demonstrated that MMP-7 is necessary for most aspects of the progression of CP in vivo, including the formation of metaplastic ducts. However, the complexity of the in vivo model makes it difficult to distinguish between MMP-7 playing a direct role in MDL formation versus an indirect role via inhibition of other aspects of disease progression that, in turn, induce the MDL response. In order to test the hypothesis that MMP-7 directly affects ductal metaplasia, I employed a simplified in vitro system in which primary acinar cells were imbedded in a three-dimensional fibrillar collagen matrix and treated with TGF-α. The ensuing transition, which has been determined to be transdifferentiation, passes through a Nestin positive intermediate, ultimately to a ductal phenotype, usually within 5 days in culture.

To test the appropriateness of this system for assessing the function of MMP-7 in acinar-to-ductal transdifferentiation, I first determined whether MMP-7 was expressed under these culture conditions. Acinar cells were isolated from the pancreata of wild type or MMP-7$^{-/-}$ mice, embedded in collagen and treated with TGF-α under low serum conditions to minimize the effects of other growth factors. Duplicate cultures were harvested and fixed on consecutive days and MMP-7 expression was determined by immunofluorescence. MMP-7$^{-/-}$ cultures were used as a negative control for the specificity of the antibody. MMP-7 expression was never detected in the MMP-7$^{-/-}$ cultures during any time point. Only background signal, localized to the matrix, was

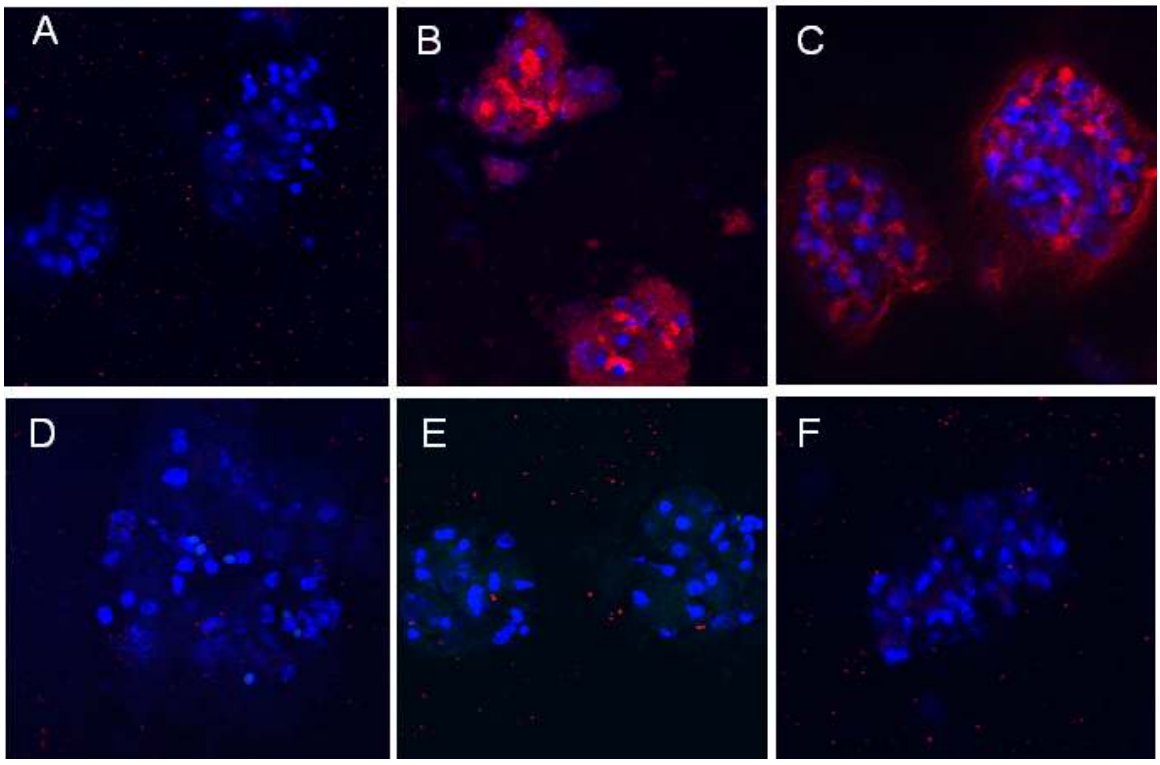

Figure 10. MMP-7 expression detected at day 2 in TGF-α induced ADM. (A-C) Immunofluorescence on wild type pancreatic acinar cells on days 1,2, and 3 (respectively) (X40). (B) MMP-7 is detected at day 2 in wild type acinar cells (X40). (D**,** E, and F) No immunoreactivity was observed in MMP-7-/- acinar cells at days 1, 2, and 3 (X40).

detected in the MMP-7$^{-/-}$ cultures (Figure 10A-C). However, by the second day of culture, cell-associated immunofluorescence was detected specifically in the wild type cultures (Figure 10E-F).

Next, using identical culture conditions, I tested whether MMP-7 was necessary for ADM. Wild type and MMP-7$^{-/-}$ cultures were harvested on days 0, 3, and 5, and were costained for the acinar and ductal markers, amylase and cytokeratin-19 (CK-19), respectively, and were examined by immunofluorescence. By day 5, acinar cells from wild type mice developed a ductal phenotype, evidenced by cell clusters losing their refractory acinar nature and becoming more clearly spherical and transparent (Figure 11C), as has been described previously. Expression of molecular markers confirmed transdifferentiation in that amylase had disappeared and CK-19 had appeared by day 5 (Figure 12A-C & J). In contrast, pancreatic acinar cells from MMP-7$^{-/-}$ mice maintained a predominantly acinar appearance by phase contrast microscopy (Figure 11F), confirmed by the persistence of amylase and the absence of CK-19 expression throughout the culture period **(**Figure 12D-F & J).

To confirm that the block in transdifferentiation in the MMP-7$^{-/-}$ cultures was in fact due to a lack of MMP-7, I tested whether recombinant MMP-7 (rMMP-7) could restore a wild type phenotype. I found that inclusion of rMMP-7 in the culture medium allowed MMP-7$^{-/-}$ acinar cells to convert to CK-19 positive duct cells (Figure 12G-I). Thus, I conclude that MMP-7 is required for in vitro acinar-to-ductal transdifferentiation, suggesting that it plays a direct role in the formation of ductal metaplasia in vivo. To test the complementary hypothesis that MMP-7 is sufficient to induce ADM, I replaced

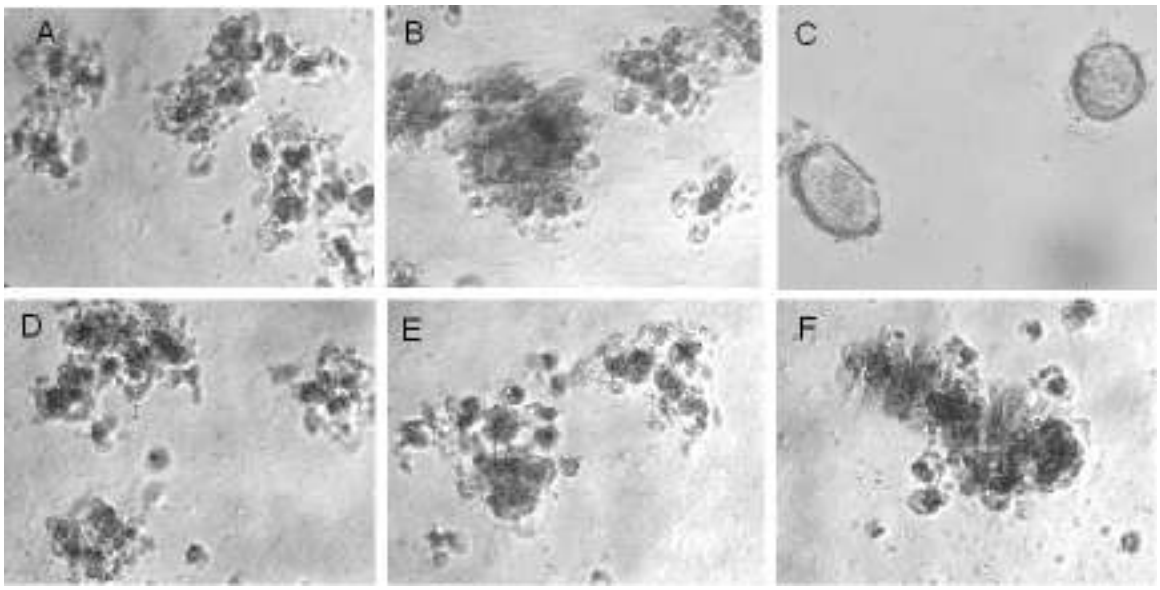

Figure 11. Bright field image of primary acinar cell cultures, derived from MMP-7$^{-/-}$ mice embedded in fibrillar collagen, and treated with TGF-α show no conversion of acinar cells to duct cells. (A) Wild type primary acinar cell cultures embedded in fibrillar collagen and treated with TGF-α day 0 (X20), (B) and Day 3 (X20). (C) At day 5 wild type acinar cell culture treated with TGF-α shows characteristics of acinar-to-ductal metaplasia on (X20). (D) MMP-7$^{-/-}$ acinar cell culture treated with TGF-α shows no characteristics of acinar-to-ductal metaplasia on day 0 (X20). **(**E) MMP-7$^{-/-}$ acinar cell culture treated with TGF-α Day 3 (20X). (F) MMP-7$^{-/-}$ acinar cells show no apparent duct formation at day 5 when treated with TGF-α (X20).

TGF-α in the culture media with rMMP-7. As a control, TGF-α was added to a separate culture as previously described and acinar-to-ductal transdifferentiation occurred within 5 days (Figure 13B). In the cultures treated with rMMP-7, I found that rMMP-7 induced acinar cell transdifferentiation with kinetics similar to that of TGF-α induced acinar cell transdifferentiation (Figure 13C), clearly bypassing the need for exogenous EGF-R ligand. These data suggest that MMP-7 is not only necessary to induce acinar-to-ductal transdifferentiation, but that rMMP-7 is also sufficient to induce the metaplastic event in vitro.

MMP-7 and Notch are required for the transition to the Nestin positive intermediate

The Notch pathway has been shown to be responsible for maintaining stem and progenitor cell populations in the pancreas and other tissues (Means, A. et al 2005). In in vitro acinar-to-ductal transdifferentiation, transition to the final ductal phenotype has been shown to require the activation of the Notch pathway. In order gain insight as to how the activities of Notch and MMP-7 may relate to one another during acinar transdifferentiation, I set out to define precisely where both act in the process. Initially, I examined the effects of γ-secretase inhibition, which blocks Notch processing, on transition to the Nestin-positive intermediate. In wild type acinar cells treated with DMSO as a vehicle control, Nestin expression was discernable by day 3 (Figure 14A), in agreement with previous studies (Means, A. et al., 2005). In contrast, in the presence of the γ-secretase inhibitor WPE III-31C, Nestin expression was not detected throughout the time course (Figure 14B). In parallel studies, I found that MMP-7$^{-/-}$ acinar cells also failed to initiate Nestin expression (Figure 14C). These experiments suggest that the

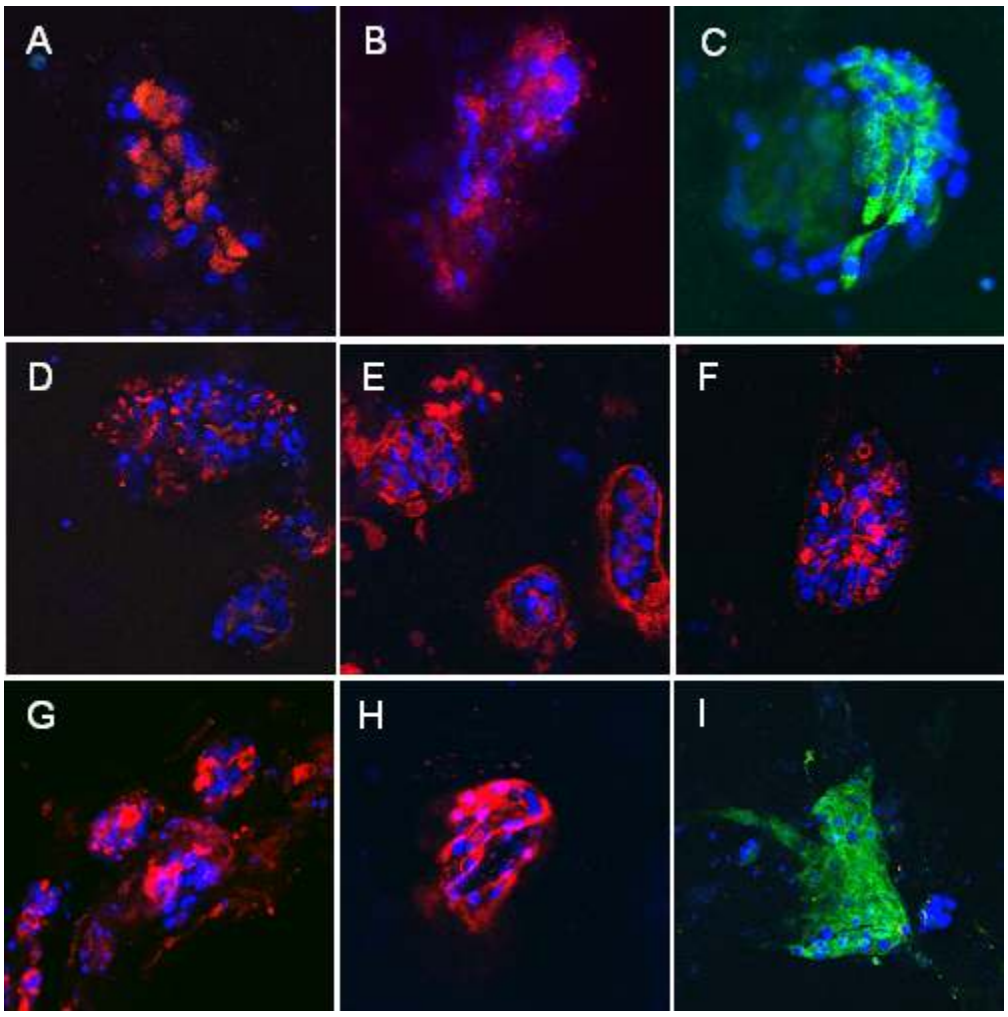

Figure 12. MMP-7 is required for the induction of acinar to ductal metaplasia. Coimmunoflourescence for amylase (red) and CK-19 (green) in wild type and MMP-7-/- cultured acinar cells. (A, B, and C) TGF-α treatment of wild type acinar cells on day 0, 3, and 5 (respectively); ADM occurs by day 5 (X40). (D, E, and F) TGF-α treatment of MMP-$7^{-/-}$ acinar cells on day 0, 3, and 5; at day 5 ADM is inhibited (respectively)(X40). (G, H, and I) Addition of 200ng/ml of recombinant active MMP-7 to MMP-$7^{-/-}$ acinar cells at day 0, 3, and 5 (respectively); recombinant active MMP-7 rescues ADM inhibition by day 5(X40).

activity of both Notch and MMP-7 act prior to, and are required for, the acquisition of the Nestin-positive intermediate phenotype.

In light of my observation that the activity of both Notch and MMP-7 are required for the acquisition of an intermediate phenotype, I tested whether the Notch pathway was necessary for MMP-7 to induce transdifferentiation. Based on the ability of the γ-secretase inhibitor to inhibit TGF-α induced ADM in vitro (Means, A.L. et al., 2005), I induced ADM using rMMP-7 in the presence of DMSO (vehicle control) or the γ-secretase inhibitor. Unlike acinar cells treated with MMP-7 alone (Figure 15C), by day 5 there was no appearance of ductal phenotype in the cultures treated with γ-secretase inhibitor. Expression of amylase confirmed the inhibition of ADM in the presence of the γ-secretase inhibitor (Figure 15D). These observations strongly suggests that like TGF-α, MMP-7 requires Notch activation to induce ADM.

MMP-7 is required for Notch activation in acinar to ductal transdifferentiation.

To further support the hypothesis that MMP-7 acts upstream of Notch, I set out to test whether Notch signaling affected MMP-7 expression. To this end, I assessed MMP-7 expression in wild type acinar cultures treated with WPE III-31C. Under these conditions, cell-associated immunofluorescence for MMP-7 expression was easily discernable by day 2 (Figure 16B) and showed no apparent difference from control cultures. Thus, MMP-7 does not appear to be a Notch target gene in this system.

Given that Notch and MMP-7 appear to be working in the same part of the transdifferentiation pathway, I next wanted to test whether they acted serially in the same pathway or in parallel in independent, but equally critical pathways. To distinguish these possibilities, I utilized an adenovirus encoding the dominant active intracellular domain

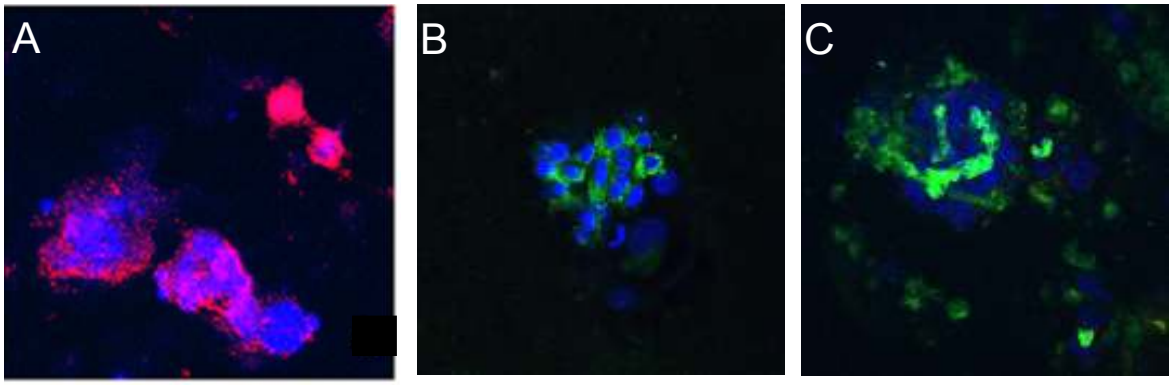

Figure 13. Addition of exogenous MMP-7 in the absence of TGF-α induces acinar-to-ductal trandifferentiation. (A) Co-immunofluorescence for amylase and CK-19 on acinar cells derived from wild type mice on day 5 without exogenous TGF-α (X20). (B) Day 5 with TGF-α (X20). (C) Day 5 with 200ng/ml of purified active recombinant MMP-7 (X20).

of Notch 2 (N2-ICD) that had been used previously to show that Notch activation is sufficient to induce transdifferentiation, bypassing the requirement for TGF-α (Miyamoto, Y. et al., 2003). Wild type and MMP-7$^{-/-}$ acinar cells, were infected with either GFP-encoding control adenovirus or N2-ICD adenovirus, using GFP signal to monitor infection efficiency (Figure 17). Five days post infection, cells were fixed and analyzed for CK-19 expression to test for ductal character. GFP controls showed no spontaneous conversion to the ductal phenotype (Figure 17C), whereas N2-ICD infected cultures showed conversion of a majority of cells, roughly equivalent to the infection efficiency in both wild type (Figure 17F) and MMP-7$^{-/-}$ cultures (Figure 17L). Thus, Notch induced transdifferentiation does not require MMP-7 activity, suggesting that they do not act in parallel necessary pathways. Having eliminated this possibility, these data also require that in the alternative model, where these proteins are part of the same pathway, that Notch acts downstream of MMP-7. Although our in vitro data are supportive of MMP-7 mediating the interaction between EGF-R and Notch, a mechanism has not been completely defined. Therefore, not only have my experiments determined in part the hierarchy of MMP-7 and other molecules associated with acinar transdifferentiation in vitro, these experiments also support a role for the direct cleavage of Notch by MMP-7, in order to induce Notch pathway activation. Evidence of this is provided by in vitro studies using antibodies against full length Notch-1 as well as antibodies specific for γ-secretase cleaved Notch-1.

Using primary acinar cell cultures from wild type and MMP-7$^{-/-}$ mice, Eric Sawey, a fellow graduate student in the Crawford lab, demonstrated that full length Notch-1 is expressed in both wild type and MMP-7$^{-/-}$ acinar cells. In additional studies, Eric Sawey

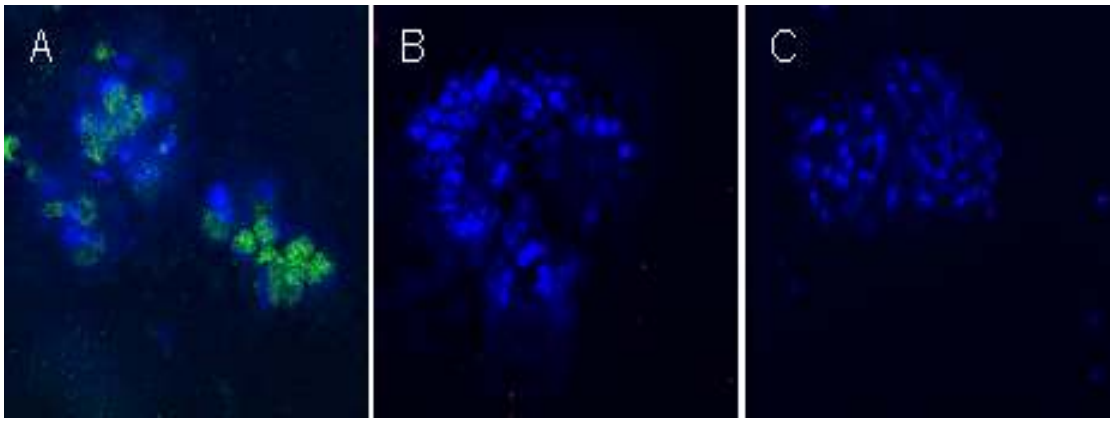

Figure 14. Nestin expression is not detected in MMP-7-/- acinar cells. (A) Control cells (without WPE III-31C) demonstrate nestin positive immunoreactivity at day 3 (X20). (B) Nestin positive cells are not detected in the presence of WPE III-31C at day 3 (X20). (C) TGF-α induced acinar-to-ductal transdifferentiation in MMP-7-/- acinar cells demonstrating no nestin positive immunoreactivity (X20).

demonstrated that γ-secretase cleaved Notch-1 expression is discernable in wild type acinar cells by day 3 when treated with TGF-α (control) (Figure 18A). In contrast, pancreatic acinar cells from MMP-7$^{-/-}$ show no cleaved Notch-1 expression during any time of the experiment (Figure 18B). To confirm that the lack of cleaved Notch-1 detection was a result of a lack of MMP-7, he tested if rMMP-7 could restore the cleavage of Notch-1 in the MMP-7$^{-/-}$ acinar cells. He found that inclusion of rMMP-7 in the culture medium restored cleavage of Notch-1 with kinetics similar to the control experiment (Figure 18C). These data suggest that MMP-7 can mediate cleavage of Notch-1 in vitro.

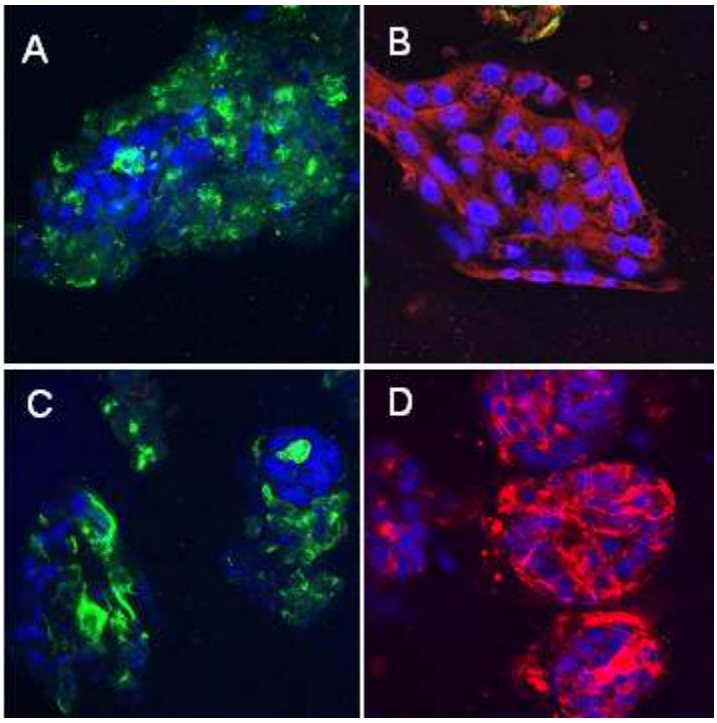

Figure 15. Inhibition of Notch signaling blocks MMP-7 induced acinar-to-ductal transdifferentiation. Co-immunofluorescence of day 5 cultures treated with (A) TGF-α +DMSO (X20), (B) TGF-α + WP III-31C (X20), (C) MMP-7+ DMSO (X20), (D) MMP-7 + WP III-31C (X20).

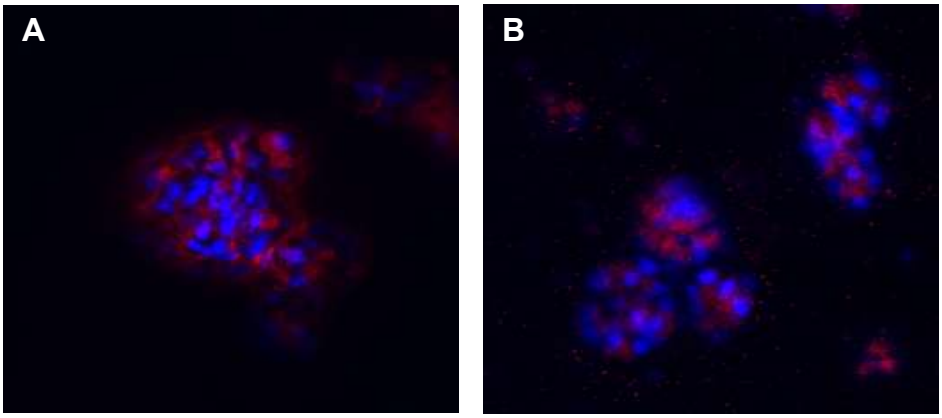

Figure 16. Notch signaling is not required for MMP-7 expression. (A) Immunoflouresence demonstrating MMP-7 expression at day 2 in TGF-α induced ADM (X20). (B) Immunoflouresence demonstrating MMP-7 expression at day 2 with WPE III-31C (X20).

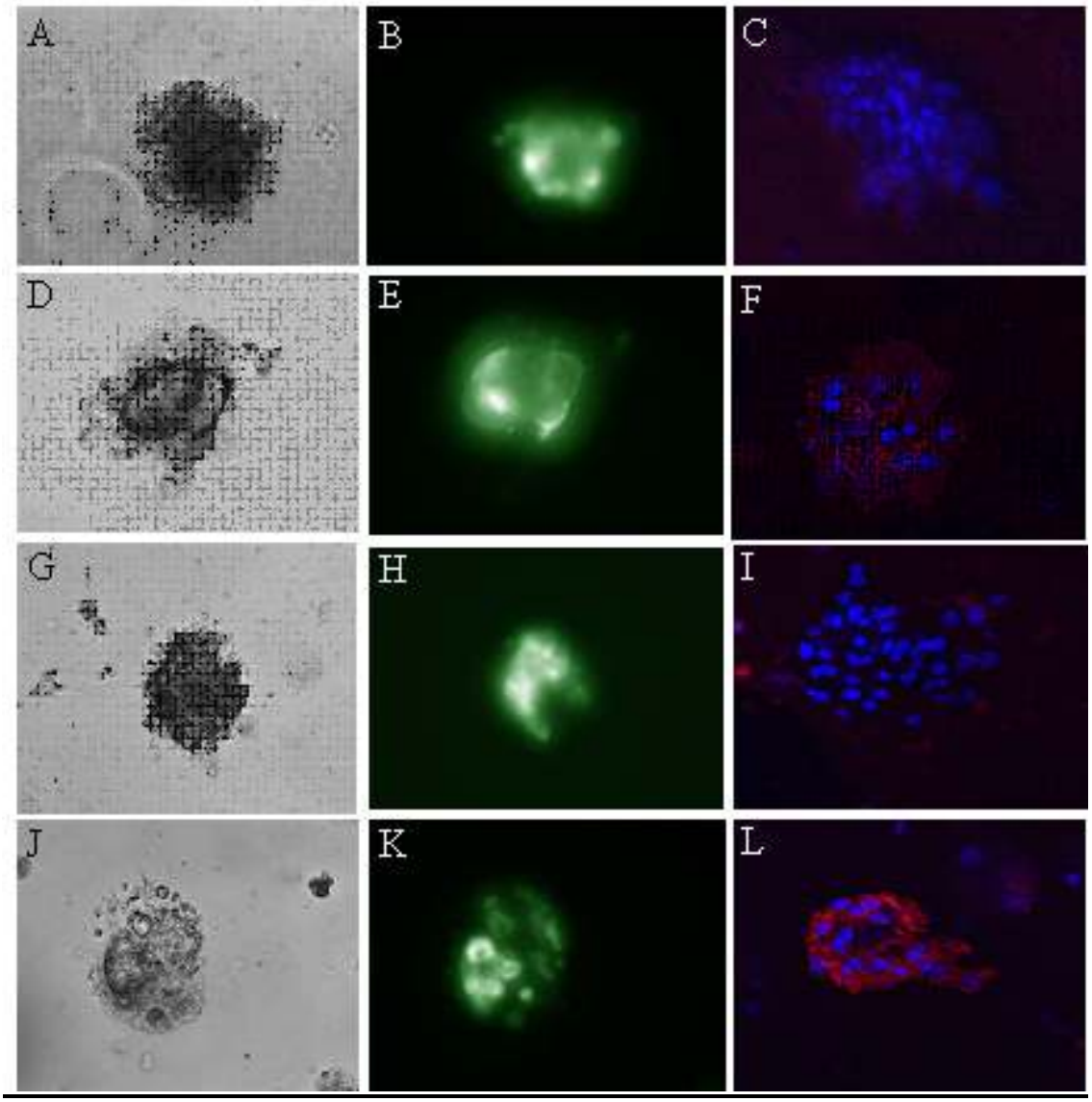

Figure 17. Notch activation induces acinar to ductal metaplasia in MMP-7$^{-/-}$ pancreas by day 3. Wild type (A-F) or MMP-7$^{-/-}$(G-L) primary acinar cells were infected either with an adenovirus encoding GFP (A-C, G-I) or the Notch-2 intracellular domain with GFP (D-F, J-L) and embedded in collagen. Three days after infection, phase contrast (A, D, G, J) and green fluorescence (B, E, H, K) were examined. Cultures were also analyzed for expression of CK-19 (C, F, I, L).

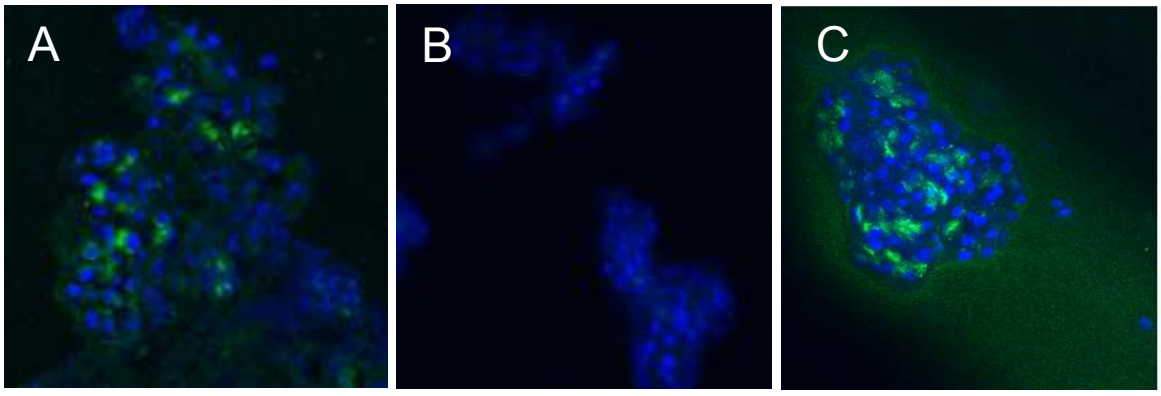

Figure 18. Addition of exogenous MMP-7 rescues the cleavage of Notch-1 in MMP-7$^{-/-}$ acinar cells. (A) Immunoflourescence for cleaved Notch-1 in wild type acinar cells treated with TGF-α, day 3 (X20). (B) Immunoflourescence for cleaved Notch-1 in MMP-7$^{-/-}$ acinar cells treated with TGF-α, day 3 (X20). (C) Addition of 200ng/ml recombinant active MMP-7 to MMP-7$^{-/-}$ acinar cells rescues the cleavage of Notch-1, day 3 (X20). Picture provided by Eric Sawey.

Chapter 4

Conclusions

A number of studies have provided evidence that implicate a role for MMP-7 during tumor formation. In addition, because MMP-7 expression correlates strongly with the metaplastic duct lesions (MDLs) found in chronic pancreatitis (CP) patients samples associated with PDA, Crawford et al., tested the affects of MMP-7 in a mouse model of human CP (Crawford, H.C. et al., 2002). They demonstrate that the pathological hallmarks of CP are inhibited in MMP-$7^{-/-}$ mice pancreas after PDL, compared to wild type (control). However, the complexity of the in vivo model makes it difficult to distinguish between MMP-7 playing a direct role in MDL formation versus an indirect role via inhibition of other aspects of disease progression, that in turn, induce the MDL response.

The data presented in this dissertation have made important contributions to the study of MMP-7 and its direct role in the formation of MDLs associated with PDA. I tested the hypothesis that MMP-7 directly affects the formation of MDLs by using an in vitro system involving primary acinar cells. Initially, I determined that MMP-7 was expressed in this system using wild type mouse pancreatic acinar cells. Additionally, I found that MMP-7 expression was not detected in the acinar cells of MMP-$7^{-/-}$ mice. These data suggest that MMP-7 expression is an early event in ADM.

Having shown that MMP-7 is necessary for the metaplastic duct formation in CP *in vivo* (Crawford et al., 2002), I tested whether MMP-7 activity is necessary for in vitro acinar transdifferentiation. I found that acinar cells isolated from MMP-$7^{-/-}$ mice do not undergo metaplastic conversion in response to TGF-α as do their wild type counterparts.

This inhibition was rescued by inclusion of rMMP-7 in the culture medium. These results show that MMP-7 is, in fact, necessary for *in vitro* transdifferentiation and acts downstream of TGF-α/EGF-R signaling. I tested the complementary hypothesis that MMP-7 is sufficient to induce acinar-to-ductal transdifferentiation by replacing TGF-α in the culture media with rMMP-7. In the cultures treated with rMMP-7, I found that rMMP-7 induced acinar cell transdifferentiation with kinetics similar to that of TGF-α induced controls, clearly bypassing the need for exogenous EGF-R ligand. These data suggest that MMP-7 is not only necessary to induce acinar-to-ductal transdifferentiation, but that rMMP-7 is also sufficient to induce the metaplastic event.

Since TGF-α has been previously shown to mediate ADM through the EGF-R/Notch pathway (Miyamoto, Y. eat al., 2003), I investigated how MMP-7 acts with known signaling pathways necessary for ADM, namely Notch. While the inhibition of the intracellular cleavage of Notch (via γ-secretase inhibition) abrogates the effect of TGF-α mediated ADM, using immunoflourescence I demonstrated that MMP-7 expression is still discernable by day 2 of the ACP. By contrast, Nestin immunoreactivity was never detected during any of the days following the ACP with the γ-secretase inhibitor. These data suggest that the MMP-7 induced metaplastic event is Notch dependent and that MMP-7 expression is not induced by the Notch pathway. Futhermore, the lack of Nestin expression in these cells, compared to Nestin positive immunoreactivity at day 3 of the control ACP (without γ-secretase inhibitor), suggests that Notch is necessary to induce an intermediate event during ADM.

As mentioned, TGF-α is not capable of inducing ADM in MMP-7$^{-/-}$ cells, suggesting that MMP-7 also acts downstream of EGF-R or in parallel with it, controlling a

previously unidentified pathway critical for acinar transdifferentiation. One possibility for a downstream role for MMP-7 is that MMP-7 can induce the extracellular cleavage of Notch proteins, an activity critical for Notch signaling (Miyamoto et al., 2003). To test whether MMP-7 acts upstream of Notch, I used an adenovirus encoding a constitutively active Notch-2 intercellular domain (Notch-2ICD) (gift of Steven Leach, Johns Hopkins University), to infect MMP-$7^{-/-}$ primary acinar cells. I observed that Notch similarly bypassed the need for MMP-7 to induce ADM, which suggests that MMP-7 does not control a critical parallel pathway and more likely mediates the interaction between EGF-R and Notch.

Although our in vitro data is supportive of MMP-7 mediating the interaction between EGF-R and Notch, a mechanism has not yet been completely defined. Therefore, not only have my experiments determined in part, the hierarchy of MMP-7 and other molecules associated with acinar transdifferentiation in vitro, these experiments also support a role for the direct cleavage of Notch by MMP-7, in order to induce Notch pathway activation.

Future Directions

Evidence that MMP-7 is expressed in 98% of human PDA has been previously been demonstrated (Crawford, H.C. et al., 2002), suggesting its potential as a marker for the disease. Its function in chronic pancreatitis supports the usefulness of matrix metalloproteinase inhibitors (MMPIs) as a preventative therapy for patients with chronic pancreatitis and early stage PDA. In vitro, I have also acquired data that supports the usefulness of MMPIs in the early stages of tumor formation. Using a broad spectrum MMPI (GM6001) I demonstrate that ADM is inhibited with varying concentrations of GM6001 (Figure 19). These data emphasize a potential benefit of MMPI treatment to patients with earlier stage cancer.

As I have demonstrated in my dissertation, that the lack of MMP-7 blocks ADM in both a complex in vivo environment induced by ductal ligation (Crawford, H.C. et al., 2002) as well as a simplified in vitro model induced by TGF-α. The latter observations lead me to ask the question if TGF-α induced metaplasia in vivo is similarly dependent on MMP-7 activity. Under the control of the metallothionein (MT) promoter, TGF-α can be chronically induced in the mouse pancreas by heavy metals in the drinking water, resulting in extensive ADM and fibrosis, without a significant induction of inflammation (Sandgren, E.P. et al., 1990). In support of the in vitro model of metaplasia, I tested if ablation of MMP-7 in an in vivo mouse model of metaplasia would reduce the metaplastic phenotype. MT-TGF-α mice maintained on a C57Bl/6 back ground were crossed onto a MMP-7 null background using MMP-7null mice also maintained on the C57Bl/6 genetic background. At 5 weeks old, expression of the transgene was induced in

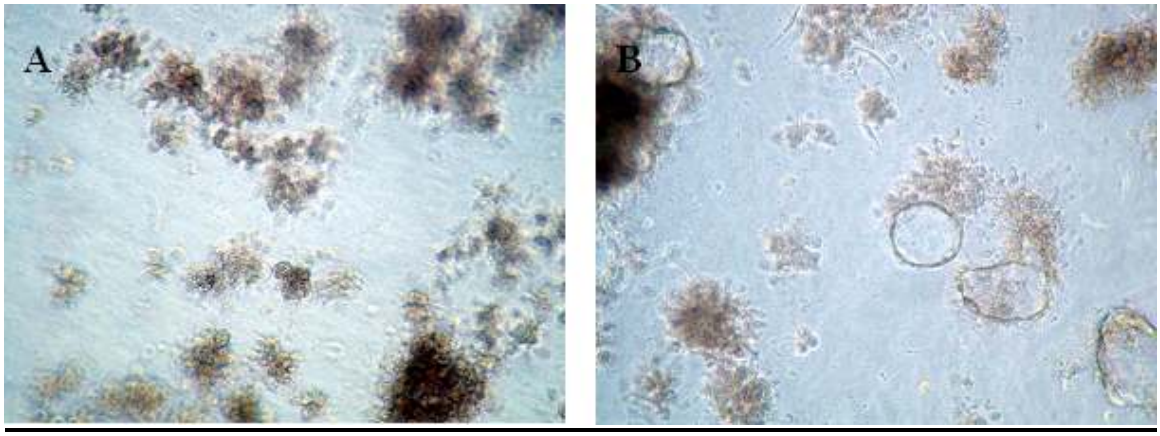

Figure 19. ADM is inhibited by MMPI by day 5, in vitro. (A) ADM induced by TGF-α in wild type acinar cells treated with DMSO (X20). **(B)** ADM induced by TGF-α in wild type acinar cells treated with broad spectrum MMPI (X20).

both C57Bl/6 MT-TGF-α (control) and C57Bl/6 MT-TGF-α/MMP-7 null mice by administration of drinking water containing 25 mM of $ZnSO_4$ for 4 months. Control mouse pancreas harvested after 4 months on drinking water containing 25 mM of $ZnSO_4$ exhibited metaplastic ducts when stained with hematoxylin and eosin (H&E) (Figure 20). To my surprise, the C57Bl/6 MT-TGF-α/MMP-7 null mice pancreas showed no difference in phenotype when stained with H&E. As a result of this, I hypothesized that the compensatory mechanisms inherent in the context of the whole animal model are bypassing MMP-7 to induce metaplasia. Since MMP-7 has been shown to directly affect MDL formation in the CP model as well as the in vitro model of metaplasia, I thought that if I remove the 25 mM of $ZnSO_4$-H_20 after 4 months, and allow the mice to recover by drinking distilled water for 4 months, the endogenous expression of MMP-7 in the control mouse would inhibit the ability of the MDLs to revert back to healthy acinar cells. Conversely I expected C57Bl/6 MT-TGF-α/MMP-7 null mice to exhibit a decrease in MDL formation as well as fibrosis as a result of recovery. After 4 months on the 25 mM of $ZnSO_4$- H_20 and subsequently replacing 25 mM of $ZnSO_4$-H_20 with distilled water for 4 months, I found that the control mice exhibited continued metaplastic duct formation, as well as fibrosis shown by Masson's trichrome stain (blue) (Figure 21A). However, the C57Bl/6 MT-TGF-α/MMP-7 null mice exhibited little to no metaplasia or fibrosis (Figure 21B). These results suggest that MMP-7 plays a direct role in the maintainence of MDLs in vivo, and that during recovery the lack of MMP-7 expression promotes the repopulation of healthy acinar cells. Furthermore, these data emphasize a potential benefit of MMP inhibitor treatment to patients with early stage pancreatic cancer. Unfortunately because of the amount of time it takes to generate and analyze the MT-

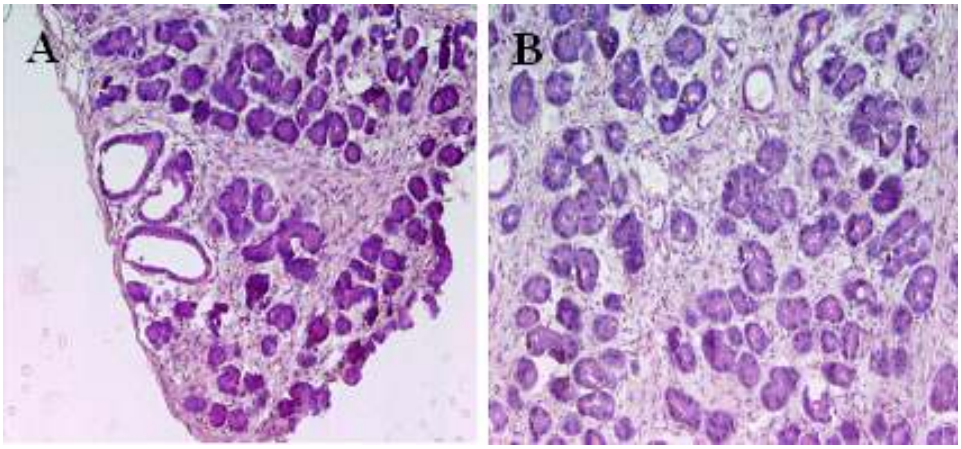

Figure 20. Hematoxylin and eosin (H&E) stain on mouse pancreas after administration of 25mM $ZnSO_4$-H_20 treatment. (A) C57Bl/6 MT-TGF-α (control) after 4 months of 25mM $ZnSO_4$-H_20 treatment (20X). (B) C57Bl/6 MT-TGF-α/MMP-7 null mice after 4 months of 25mM $ZnSO_4$-H_20 treatment (20X).

TGF-α mice, I was unable to analyze an adequate number of mice for my data to be significant. Nonetheless, as these mice are being generated and placed on 25 mM of $ZnSO_4$-H_20, further analysis will be accomplished in the months to come.

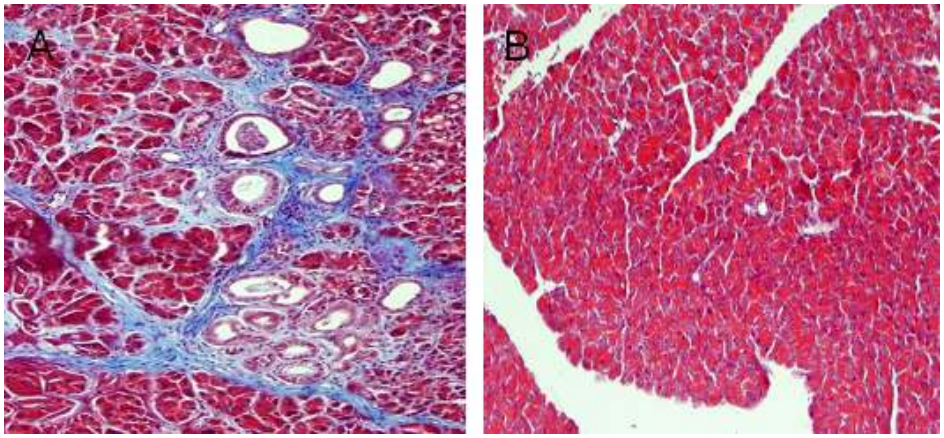

Figure 21. Masson's Trichrome stain (blue) on mice pancreas after 25mM $ZnSO_4$-H_20 treatment for 4 months and replaced by distilled drinking water for 4 months. (A) C57Bl/6 MT-TGF-α (control) after 4 months on 25mM $ZnSO_4$-H_20 and 4 months of distilled water (20X). (B) C57Bl/6 MT-TGF-α/MMP-7 null mice after 4 months on 25mM $ZnSO_4$-H_20 and 4 months on distilled water (20X).

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