University of Southampton

Faculty of Medicine, Health and Biological Sciences

Pancreatic Research Group

An investigation into soluble growth factors of TIMP-1, IGF-1 and Insulin on Pancreatic stellate cell survival

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DM Thesis

February 2014

University of Southampton

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Faculty of Medicine, Health and Biological Sciences

During pancreatic injury, the pancreatic stellate cell(PSC) become activated to a myofibroblast-like phenotype, proliferate and are known to be the major source of matrix which characterise pancreatic fibrosis in chronic pancreatitis and pancreatic cancer. Activated PSC also express matrix degrading metalloproteinases(MMPs) and their tissue inhibitors(TIMPs).

Previous work has demonstrated that during spontaneous recovery from experimental liver fibrosis after 4 weeks of carbon tetrachloride injections, there is a fall in the expression of TIMP-1, a loss of the hepatic stellate cells (HSCs) by apoptosis, and an increase in liver collagenolytic activity with the return of the liver to a near normal histology. This correlation between PSC survival and expression of TIMP-1 in vivo highlighted potential roles for TIMP-1, IGF-1 and insulin in PSC survival and apoptosis which was the subject of this study.

The initial aim this thesis was to establish and characterise human PSC in culture. This was achieved and cells were studied between passage 1 and 4 in order to determine the effect of TIMP-1, IGF-1 and insulin on PSC survival.

These data suggest that TIMP-1, showed a dose dependent anti apoptotic effect in activated rat and human PSCs, with no effect on proliferation. Additionally, successful silencing of TIMP-1 at the protein and mRNA level using electroporation was achieved, without significantly effecting PSC cytotoxicity. Furthermore, electroporation with TIMP-1 significantly increased the level of apoptosis compared with control, and this effect was attenuated by the addition of recombinant TIMP-1.

IGF-1 and insulin receptors were expressed in vitro, in activated rat PSCs. Immunohistochemistry demonstrated weak IGF-1R positive staining within areas of fibrosis in pancreatic resection specimens, which co-localised to cells which also stained positive for α-SMA a marker of activated PSCs. IGF-1 induced an increase in PSC numbers by promoting survival as well as increasing proliferation. On serum withdrawal IGF-1 is secreted by activated PSC. Insulin promotes survival but has no significant effect on proliferation.

In conclusion these data have identified TIMP-1, IGF-1 and insulin as mediators of stellate cell survival via inhibition of apoptosis. Furthermore, a successful technique has been identified to knockdown siRNA in PSC to allow further mechanistic studies to be performed.

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Academic Thesis: Declaration Of Authorship

I, MANISH PATEL

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

An investigation into soluble growth factors of TIMP-1, IGF-1 and Insulin on Pancreatic stellate cell survival

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Either none of this work has been published before submission, or parts of this work have been published as: [please list references below]:

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Acknowledgments

I would like to thank my supervisors Dr David Fine, Dr RC Benyon and Dr Jane Collins for their guidance, support and encouragement throughout my research, particularly through the most difficult times.

I would also like to thank Mr Colin Johnson and Mr Mo Abu-Hilal of Southampton University Surgical Unit, for their help in the provision of pancreatic resection specimens. I would like to thank Dr Andrew Fowell for his advice on the TIMP-1 experiments.

I thank my parents, for their unwavering support and love.

Above all, I would like to thank my wife Shruti for her patience throughout. None of this would have been possible without her.

Finally, I would also like to thank the Wellcome Trust for their generous sponsorship.

1 Chapter 1

1.1 Normal Pancreatic anatomy

1.1.1 Macroscopic anatomy

The pancreas is a retroperitoneal organ which consists of a head, body and tail and extends along the posterior wall of the abdomen from the duodenum to the spleen (Figure 1.1). Anteriorly its surface is covered by peritoneum. It is surrounded by connective tissue, blood vessels, nerves, and lymphatic vessels and lymph nodes. It lies anterior to the aorta at a level just below the entry of the aorta into the abdominal cavity through the diaphragm. The head is located within the curve of the duodenum. An extension from the head, the uncinate process projects upwards behind the superior mesenteric artery and vein. The tail of the pancreas narrows as it extends toward the hilus of the spleen.

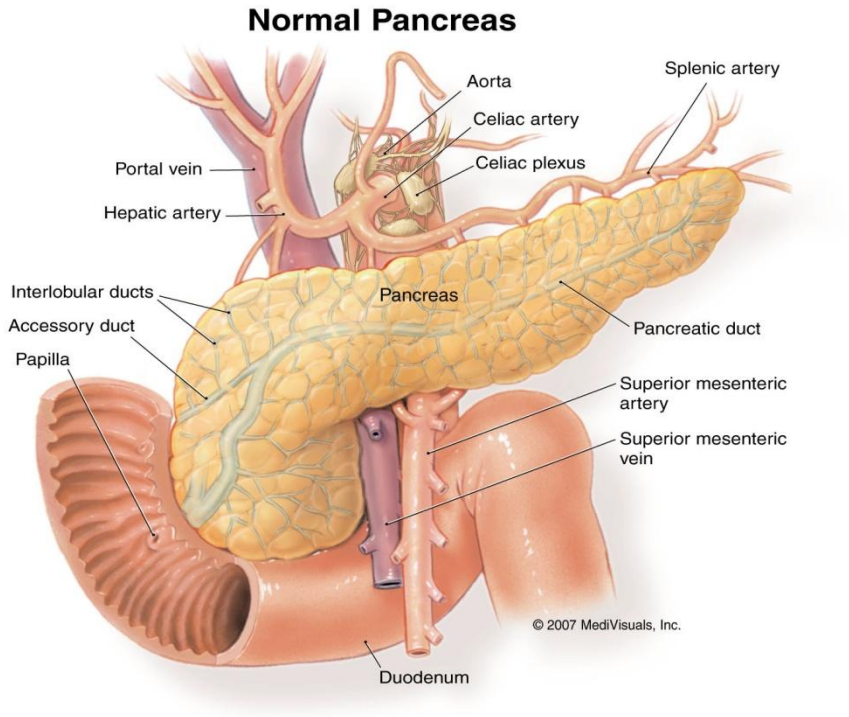


Figure 1-1 Normal Pancreas (©MediVisuals, Inc)

The body lies between head and tail. The arterial supply to the pancreas and related structures is derived from two main branches of the abdominal aorta namely the celiac trunk and the superior mesenteric artery. Macroscopically the pancreas appears as a lobulated gland and in its normal state its body is approximately 2 cm thick and 5 cm from superior to inferior border. Its dimensions are greater in the head and tapers towards the tail. However, the dimensions of any region are can be variable. The main pancreatic duct begins in the tail of the pancreas, and traverses from left to right ending by emptying into the duodenum at the major papilla (Figure 1.2). It takes a relatively constant course through the tail and body receiving tributaries throughout. In the head it comes into close association with the common bile duct. The main pancreatic duct and the common bile duct empty through a common channel, the ampulla of Vater, or very close to each other. The accessory duct when present anastomoses with the main pancreatic duct, and when patent enters the duodenum at the minor papilla. The major and minor papillae are located in the second part of the duodenum on the medial wall.

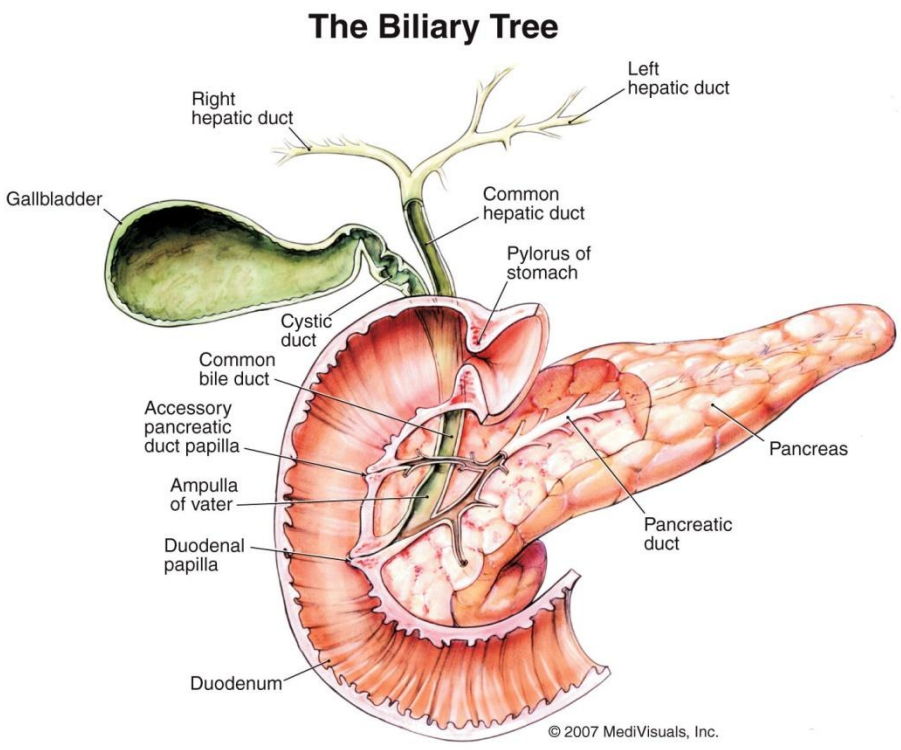


Figure 1-2 Biliary Tree (©MediVisuals, Inc)

The nervous system within the pancreas contains a mixture of motor nerve fibres from both the sympathetic and parasympathetic divisions with associated nerve fibres. There are numerous interactions with hormonal mechanisms to provide a well-coordinated response to stimuli associated with eating and digestion. The rich innervations of the pancreas also serve as the route by which pathological changes are brought to awareness in the form of pain.

Under normal physiological conditions the lymphatic vessels of the pancreas drain fluid from the extracellular matrix (ECM). They serve as an overflow system for fluid in the ECM of the pancreas. Lymphatic capillaries unite to form larger lymphatic vessels that pass through the connective tissue that separates lobules. Lymph vessels are closely associated with blood vessels and nerves. With the development of pancreatic disease, the lymphatic system may become more prominent. Pancreatic cancer frequently metastasizes through lymphatic vessels and proliferates within lymph nodes. This often occurs early in the disease.

1.1.2 Microscopic anatomy

The primary functional units of the pancreas can be divided into endocrine cells and exocrine cells, both of which arise from a primordial outgrowth of the primitive gut. Both neural and hormonal factors in the form of gastrointestinal peptides control the secretions of the exocrine pancreas.

The main exocrine function of the pancreas is to produce, store and release digestive enzymes such as trypsin, chymotrypsin, aminopeptidases and phospholipases. Acinar exocrine cells are essentially of one type, although each one contains a number of different substances. The secretions of these cells are carried away through the ductal system, which is composed of mostly plain-appearing epithelial cells, some of which display mucous granules. Ductal cells, however, have important secretory functions.

Normal function of the pancreas is possible due in part to compartmentalisation. Secretory products stored in acinar and endocrine cells are sequestered in membrane-bound packets (Figure 1.3). The interior of epithelial cells is separated from the ECM by a plasma membrane, so signalling for secretion is controlled through receptors and channels. Basal laminae provide a micromolecular sieve along the base of epithelial cells. The lumina of the exocrine pancreas are separated from the ECM by tight junctions between acinar cells and ductal cells. Secretion

products are thereby modified within the lumen and maintained without entering or altering the ECM.

An acinar unit (acinus) is a cluster of acinar cells (Figure 1.3c). Zymogen granules containing the proenzyme form of the digestive enzymes, occupy the apical region of each acinar cell and thus occupy the central zone of the acinar unit. The basal region of each acinar cell is filled with layers of rough endoplasmic reticulum.

The endocrine cells are arranged in clusters, as the islets of Langerhans (Figure 1.3d). They are specialised in that different peptides (glucagon, insulin, somatostatin, pancreatic polypeptide) are located in different cells (α, β, δ, and PP), whose secretions are delivered to other parts of the body via the bloodstream. A review of all of the known actions of these hormones is outside the scope of this thesis.

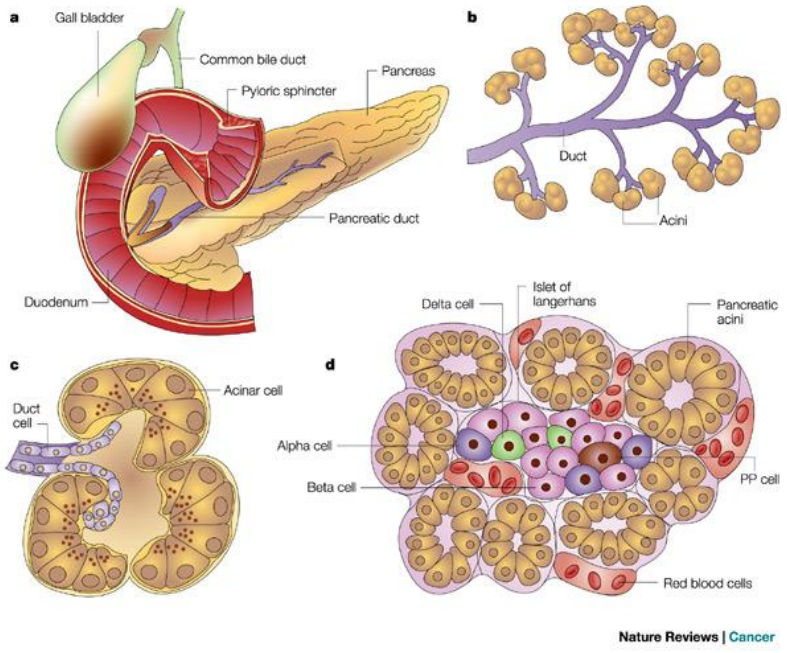


Figure 1-3 Microanatomy of the Pancreas(taken from Nabeel, Bardeesy & Ronald A. DePinho Nature reviews 2002)

The enzymes characteristic of the pancreas are synthesised by ribosomes associated with the membranes and inserted into the lumen of the rough endoplasmic reticulum. The nuclei of

acinar cells display a prominent nucleolus. In acinar cells with a large number of zymogen granules, the nucleus is usually located in the basal part of the cell. In the supranuclear region, the Golgi complex receives the products of the rough endoplasmic reticulum and processes them further. Carbohydrate moieties may be added to the protein enzymes. The Golgi apparatus also sorts the hydrolytic enzymes destined to produce lysosomes from the exocrine enzymes that will be stored and then released into the acinar lumen as part of the pancreatic juice. Zymogen granules store the enzymes until secretion is initiated. Each zymogen granule contains multiple digestive enzymes surrounded by a membrane. Secretion is initiated when membrane receptors are stimulated by acetylcholine or cholecystokinin. Secretion is mediated in part by $Ca^{2+,}$ causing fusion of the zymogen granule membrane with the apical plasma membrane of the acinar cell. The contents of the zymogen granules are then secreted into the acinar lumen.

1.1.3 Fine structure of ducts

Large ducts, like the main and accessory ducts, are lined by a single layer of tall columnar cells, producing a smooth inner surface. Many of the cells have mucin granules within their cytoplasm, with their secretions helping to form a protective layer along the luminal surface. Progression from large ducts to intralobular ductules is marked by the progressive reduction in the height of the epithelial cells and reduction in their mucin content. Connective tissue is arranged to support the epithelial cells, and forms the walls of ducts, and surrounds and penetrates the lobules. It serves as the environment for transmission of vessels and nerves. Two chronic diseases affecting the pancreas; pancreatic cancer and chronic pancreatitis share the distinguishing feature of extensive fibrosis which occurs due to a disruption to the usual composition of the ECM. The basal lamina marks the interface of the epithelial cell and connective tissue along the ductules and acini. The composition of the basal lamina includes type IV collagen, laminin, and fibronectin. In pancreatic disease, the basal lamina may become disrupted. Pancreatic cancer penetrates the basal lamina, spreading into and through the connective tissue. The cells comprising the islets of Langerhans lie in clusters and cords among numerous large fenestrated capillaries that supply oxygen and nutrients, and which carry away the secreted hormones.

1.1.4 Insulo-acinar portal system

The pancreatic islets contain four major endocrine cells that synthesise and secrete glucagon (α cells), insulin (β cells), somatostatin (δ cells), and pancreatic polypeptide (PP cells). The β cells account for approximately 80% of these cells; β and δ cells are present in all islets, whereas α cells are almost exclusively present in the tail, body, and superior part of the head of the pancreas. PP cells are observed in the middle and inferior portions of the head of the pancreas. There is no significant capsule or basement membrane surrounding the islets and they are therefore in close contact with acinar cells. The novel concept of an insulin–acinar axis based on morphologic and hemodynamic studies of the islet– acinar portal system and physiologic regulation of acinar cell function by islet peptides was first proposed by Williams and Goldfine (Williams & Goldfine, 1985).

The exocrine pancreas receives a significant part of its blood supply via the islets, and islet hormones reach the acinar cells in high concentrations through this insulo-acinar portal system. It has been observed that intralobular islets receive one or more arterioles and are drained by insulo-acinar portal vessels, which continue into the lobular capillaries(Murakami, Hitomi, Ohtsuka, Taguchi, & Fujita, 1997). Acinar cells located around the islets are called peri-insular acini, and are recognised by their larger cells containing larger nuclei and more abundant zymogen granules than the remote acini, which are called tele-insular acini. Insulin has a trophic effect on pancreatic acinar tissue, and locally high insulin levels are responsible for the morphologic characteristics of the peri-insular acini. Prominent peri-insular acini are seen in hyperinsulinaemia and disappear after β-cell destruction (Hellman, Wallgren and Petersson, 1962; von & Muller, 1994) .

These findings suggest that the islets exert control over the functions of the exocrine acini of the pancreas. It has been speculated that the parenchyma of the pancreas is exposed to concentrations of insulin 20-50 times higher than that in the circulation. Exocrine function has been found to be reduced in human diabetics (Cey, Shay, & Shuman, 1963; Vacca, Henke, & Knight, Jr., 1964), and experimental animal models with spontaneous or drug-induced diabetes. However, the cellular mechanisms underlying the impairment in pancreatic acinar cell function have yet to be elucidated.

1.2 Chronic pancreatitis

The inaccessibility of the pancreas has led to poor anatomical and physiological functional knowledge of this organ. Pathological conditions of this organ were therefore difficult to study despite the observation of chronic pancreatitis first reported in 1870 by Edwin Klebs.

Chronic pancreatitis can be defined as a continuing or iterative inflammatory disease of the pancreas with irreversible morphological changes in the ductal system and parenchyma. These changes persist even if the primary cause has been removed and the result is functional exocrine and endocrine insufficiency.

Chronic pancreatitis has a prevalence of 10–30 per 100 000 population with an annual incidence of 3–4 per 100,000 population (Etemad & Whitcomb, 2001; Otsuki, 2003). Clinically, patients suffer recurrent attacks of abdominal or back pain, and approximately 30% will develop pancreatic stones. In the advanced stages the exocrine and endocrine failure results in maldigestion manifesting with steatorrhoea and diabetes mellitus respectively. This disorder contrasts with acute pancreatitis, which is an acute inflammatory response to pancreatic injury and is usually non progressive, although the two conditions may overlap. Chronic pancreatitis may be asymptomatic over long periods of time and subsequently present with a fibrotic mass, or there may be symptoms of pancreatic insufficiency without pain. Acute pancreatitis is almost always painful. The serum amylase and lipase concentrations tend to be normal in patients with chronic pancreatitis, and they are almost always elevated with acute disease. Recurrent episodes of acute pancreatitis may lead to chronic pancreatitis over time.

Patients are usually affected by the disease in their early twenties but the clinical manifestations do not occur until the fourth or fifth decades. Males are three to four times more likely to be affected than females. Patients with chronic pancreatitis represent a considerable burden to the healthcare system with an average hospital admission of 10 days for acute exacerbation with most of the patients becoming dependent on opiates for pain relief. By the time the diagnosis is made, the disease is usually well established with chronic pancreatic inflammation and fibrosis, and on the basis of current evidence, this process is considered irreversible. Consequently the prognosis at this stage tends to be very poor.

Until recently, most research and treatment focused on managing end-stage CP and its complications. However, new research is focusing on identifying environmental, genetic, and other risk factors, as well as on the inflammatory and cellular mechanisms driving the process. Furthermore, efforts are underway to improve the diagnosis of CP at the earliest stages when the organ might be saved and returned toward normal.

Research has been hampered by the lack of disease markers, and classifications of the disease which focus on the destructive processes rather that the aetiology of the disease. Poor stratification of the disease has also impeded research in this field. Therefore, there is an urgent need to identify key mediators in the process of early disease, both at the molecular and genetic levels. The recent discovery of the genes responsible for cystic fibrosis and hereditary pancreatitis may herald a breakthrough in elucidating the underlying mechanisms of this poorly understood disease.

1.2.1 Aetiology of chronic pancreatitis

It is important to define the cause of chronic pancreatitis because this may have implications for ongoing management and prognosis. With the discovery of pancreatitis associated gene mutations and the recognition that alcohol only occasionally causes pancreatitis, a new classification system has been proposed, the TIGAR- O classification (Etemad & Whitcomb, 2001). This system is based on the mechanism of injury, and simultaneously addresses the risk, aetiology, and other complexities that are associated with chronic pancreatitis.

1.2.2 Toxic and metabolic factors.

Comfort characterised the connection between alcohol abuse and chronic pancreatitis(Comfort, 1946). He described the recurrent abdominal pain and the disturbance of acinar function and endocrine function. There appears to be a relationship between the amount of alcohol consumed and the risk of developing chronic pancreatitis, however there is no lower threshold value below which the disease does not occur. The duration of consumption of alcohol may also be important (Lankisch et al, 2005). Alcohol was previously reported to account for 70 to 90 percent of cases of chronic pancreatitis, but the association between alcohol and chronic pancreatitis is complex and these high percentages may be lower in some countries. However, the absolute risk of pancreatitis from alcohol consumption is much lower

than that for chronic liver disease or cirrhosis and ranges from 2% to 5% among patients who consume large amounts of alcohol (Lankish et al, 2005; Yadav et al, 2007 & Kristiansen et al 2008). This has suggested a role for other important cofactors, including smoking, deficiency in antioxidants and trace elements, and diets high in fat and protein.

Smoking has been confirmed as an independent risk factor for chronic pancreatitis (Maisonneuve et al., 2005). The exact mechanism remains unknown. Therefore, patients with early signs of CP should be persuaded to stop smoking for this and other health reasons.

1.2.3 Idiopathic chronic pancreatitis.

This is reported to account for 10-30% of all cases of chronic pancreatitis(Keller & Layer 2008). The majority of cases of chronic pancreatitis that are not related to alcohol abuse are classified as idiopathic. As new environmental, metabolic and genetic factors are identified, and as patients who are misdiagnosed are studied and reclassified, the number of patients in this category is likely to diminish. Hypersensitivity to small amounts of alcohol, unreported pancreatic trauma may be contributing factors in this group of patients.

1.2.4 Genetic predisposition.

A genetic background for CP was first described in a pedigree with an obviously autosomal dominant inheritance pattern in 1952(Comfort & Steinberg, 1952).

The three major genetic mutations leading to CP include mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR), cationic trypsinogen gene (PRSS1), and the pancreatic secretory trypsin inhibitor (serine protease inhibitor, Kazal-type, 1; SPINK1;). Cystic fibrosis is due to mutations in the CFTR gene. Most patients with cystic fibrosis develop progressive pancreatic damage, secondary to defective acinar and ductular secretions. Mutations in CFTR have been identified in some adults with idiopathic chronic pancreatitis who have no other evidence of cystic fibrosis (Sharer et al., 1998; Cohn et al, 1998). These mutations may also contribute to the development of chronic pancreatitis in patients who have additional risk factors for chronic pancreatitis. It has been demonstrated that CFTR variants increase the risk of developing chronic pancreatitis by up to 2.7-fold (Rosendahl et al., 2012).

Hereditary pancreatitis (HP) accounts for a small subset of all cases of chronic pancreatitis. It is transmitted as an autosomal dominant trait with a penetrance rate of 80 per cent (Whitcomb, 1999). Most individuals develop symptoms before the age of 20, but frequently before the age of 5. It is associated with a significant increased risk of pancreatic adenocarcinoma. Although the exact consequences of these mutations on trypsin activity remain unclear, they are known to interfere with inactivation of prematurely activated trypsin, permitting autodigestion of the pancreas. Mutations in the PRSS1 gene are responsible for approximately 60% of all cases of HP (Gorry et al., 1996; Whitcomb et al., 1997). Trypsinogen is the proenzyme that becomes trypsin, a pancreatic digestive enzyme that has a key role in pancreatic physiology. Mutations in the trypsinogen gene predispose to AP and CP. These observations suggest that enhanced activation or reduced inhibition of trypsin is a key factor in increasing the risk for pancreatitis in affected individuals.

Pancreatic secretory trypsin inhibitor (PSTI) also known as SPINK1, plays a major protective role in the pancreas by inhibiting prematurely activated trypsinogen. SPINK1 normally inhibits trypsin by directly blocking the active catalytic site, however it only accounts for inactivation of only about 20% of all activated trypsin. It is therefore unlikely that the SPINK1 gene mutation alone will cause pancreatitis, but it might be a disease modifier that lowers the threshold for pancreatitis (Pfützer et al., 2000; Witt et al., 2001).

1.2.5 Autoimmune pancreatitis

This is a distinct clinical entity characterised by the presence of auto antibodies. Clinical characteristics include symptomatic or asymptomatic diffuse enlargement of the pancreas, diffuse and irregular narrowing of the main pancreatic duct, increased circulating levels of gamma globulin, and the presence of autoantibodies (Dmitry et al., 2006). There are unique histopathological features with lymphocytic infiltration seen on pathology. The disorder is associated with elevated immunoglobulin IgG 4 concentrations (Hamano et al, 2001; Hirano et al., 2006).

AIP can occur as a primary pancreatic disorder or in association with other autoimmune diseases such as Sjogren's syndrome, autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis (Huang and Lichtenstein 2002; Ichimura et al., 2002; Kulling et al.,

2003). There is increasing recognition that AIP is actually an IgG4 systemic disease (Kamisawa, Egawa & Nakjima, 2003; Stone, Zen & Deshpande, 2006).

1.2.6 Recurrent and severe pancreatitis

The association between recurrent acute and chronic pancreatitis has been established by careful clinicopathological studies(Ammann & Muellhaupt 1994; Ammann, Heitz &Kloppel, 1996a) and some animal models of chronic pancreatitis (Freiburghaus, Redha &Ammann, 1995). Furthermore, clinical studies have demonstrated that recovery from acute pancreatitis may not always be complete, requiring the aetiology of some cases of chronic pancreatitis to be classified as recurrent and severe acute pancreatitis (Somogyi et al., 2001)

1.2.7 Obstructive chronic pancreatitis

This refers to a distinct entity that usually refers to pancreatitis caused by a single dominant narrowing of the pancreatic duct, usually the main duct. It is characterised by dilatation of the ducts above the stricture, and is characterised by diffuse fibrosis and acinar cell atrophy (Suda et al., 1990). Sphincter of Oddi dysfunction has also been associated with chronic pancreatitis in a small subset of patients (Tarnasky et al., 1997); whether this is a causative factor remains unknown.

1.2.8 Diagnosis of Chronic Pancreatitis

The diagnosis of chronic pancreatitis is usually suggested by the clinical features such as abdominal pain, steatorrhoea or diabetes.

The gold standard for diagnosing chronic pancreatitis is histology. Morphologically, chronic pancreatitis is characterised by the Marseilles classification as irregular sclerosis with destruction and/or loss of the exocrine parenchyma which may be focal, segmental or diffuse(Sarles et al., 1989; Singer, Gyr, & Sarles, 1985). These findings are found regardless of aetiology. Representative specimens demonstrate the presence of chronic inflammatory cells, acinar atrophy, and fibrosis. Other pathological features, include duct proliferation and nerve inflammation. The Islets of Langerhans are often relatively well preserved in comparison with

the degree of acinar atrophy. The ducts are often distorted, and stricturing is commonly seen often containing eosinophilic protein plugs and intraductal calcification. Pseudocysts are commonly found, and communicate with the ductal system.

Despite this classification system and characteristic findings, pancreatic tissue specimens are rarely available. Routine biopsy specimens are only of clinical importance in cases of pancreatic cancer. Additionally, specimens may show histopathological changes which are non specific for acute or chronic inflammation. Non representative sampling of the pancreas may not reflect focal or diffuse changes within the gland. The changes seen in aging may also be difficult to distinguish from chronic pancreatitis.

Confirmation of the diagnosis of chronic pancreatitis often relies on imaging studies. Computed tomography findings show atrophy of the gland, irregular contour of the pancreas, dilatation or irregularity of the duct and pancreatic calculi. Although major findings on imaging studies correlate with histology in moderate-to-severe CP, early diagnosis of CP remains challenging. Although, newer imaging techniques such as endoscopic ultrasound appear promising, clear and consistent criteria are yet to be developed and validated.

Pancreatic function tests may be helpful, such as faecal elastase. However, the overall sensitivity of the test is approximately 90% in advanced disease but drops to 50-60% in early disease(Ammann et al., 1996b). Pancreatic exocrine insufficiency determined by a tubed secretin test appears to be one of the most sensitive and earliest signs of CP (Clain &Pearson, 1999). However, these tests are rarely available and are only occasionally performed in expert centres.

Currently, an ideal diagnostic test which is accurate in early and advanced disease, and which is both safe and inexpensive does not exist. Thus, there remains room for improvement in the methods for diagnosing CP.

1.3 Complications

In addition to the loss of function effects on the exocrine and endocrine components of the pancreas, a variety of other complications may also be experienced. The most common are pseudocyst formation and mechanical obstruction of the duodenum and common bile duct; less

frequent complications include pancreatic ascites or pleural effusion, splenic vein thrombosis with portal hypertension, and pseudoaneurysm formation, particularly of the splenic artery. In numerous studies CP has also been shown to be a risk factor for pancreatic cancer (Karlson et al., 1997; Bansal & Sonnenberg, 1995; Lowenfels et al., 1993; Mack, Yu, Hanisch, & Henderson, 1986).

1.4 Treatment of chronic pancreatitis

CP can be a severe and disabling disease. Treatment options for chronic pancreatitis remain relatively limited and are aimed at symptom relief, as well as addressing the inevitable problems of pancreatic exocrine and endocrine insufficiency.

Pain is the symptom that most often requires treatment. The evaluation of pain should focus on identification of complications, for which therapy exists. Current therapies are aimed at limiting oxidative stress on the pancreas, reducing excessive pancreatic stimulation through smaller meals and pancreatic enzyme supplements, and eliminating known risk factors, such as alcohol consumption and smoking. Management of pain is often unsatisfactory, frequently requiring strong opioid analgesics or nerve plexus blocks. Often surgical procedures are required. Steatorrhoea is managed by pancreatic enzyme replacement therapy. Appropriate use of these enzymes often leads to resolution of diarrhoea and weight loss. Diabetes can be difficult to control, and is an independent predictor of mortality in patients with CP. Some patients will respond to oral hypoglycaemics, however many require insulin. Treatment induced hypoglycaemia is frequently seen, due to the fact that these patients will have also lost their pancreatic reserve of glucagon.

Ideally, individuals progressing toward CP could be identified early, the underlying risk factors and aetiologies identified, and effective preventative strategies adopted. Evidence-based guidelines have been difficult to develop, partly because disease progression is often slow and patients are heterogeneous. In addition, the number of patients in one centre is relatively small.

1.5 Pancreatic fibrosis and the role of pancreatic stellate cells

Extensive fibrosis is a hallmark of chronic pancreatitis irrespective of the aetiology, and is also a characteristic feature of the desmoplastic reaction seen in pancreatic cancer. Pancreatic stellate

cells were first identified by Bachem and Apte in 1998(Apte et al., 1998; Bachem et al., 1998). These cells in culture not only display a stellate-like morphology, but also undergo phenotypic changes that resemble the process of HSC activation. Pancreatic fibrosis is a dynamic process that is potentially reversible, particularly in its early stages. Considerable progress has been made in recent years with respect to our understanding of the pathogenesis of pancreatic fibrosis. In fact, it is now well established that the cell playing a key role in the fibrogenic process is the pancreatic stellate cell (PSC).

1.5.1 Quiescent Pancreatic Stellate cells

In the normal pancreas, the vitamin-A storing phenotype of PSCs constitute approximately 4% of the pancreatic cell population (Apte et al., 1998). They are found in the periacinar space, in the so-called "quiescent" state, which is characterised by the presence of vitamin A-containing fat droplets within the cytoplasm and absent expression of α-SMA. The precise function of quiescent pancreatic stellate cells remains unknown.

Following pancreatic injury or in pancreatic carcinoma, PSCs change their phenotype from the fat-storing phenotype to a highly active myofibroblastic phenotype. These activated PSCs are characterised by loss of retinoid, expression of α-smooth muscle actin (α-SMA) and secretion of ECM components, such as collagen I, III and fibronectin(Bachem et al., 1998; Apte et al., 1998), as well as matrix metalloproteinases (MMPs) and their inhibitors, TIMPs(Shek et al., 2002; Phillips et al., 2003a). They also become capable of migration(Phillips et al;., 2003 & McCarroll et al., 2004) and phagocytosis(Shimizu et al.,2005 & Rickmann et al., 2007). Activation of PSCs is regulated by a complex interaction of cytokines, growth factors, oxidant stress, alcohol and its metabolite acetaldehyde. Development of pancreatic fibrosis is associated with a net deposition and accumulation of ECM. It appears that PSC activation is part of a wound healing response after pancreatic injury. However, the perpetuation of the activated phenotype in chronic pancreatitis and pancreatic cancer are responsible for the pathological process of fibrosis and scarring.

1.5.2 Similarities between HSC and PSC

PSCs are similar to HSCs, which have been known for many years to play a key role in liver fibrosis. There are many morphological similarities between HSC and PSC, and they are both

found in areas of organ specific fibrosis. Much of the work carried out previously, investigating the role of HSC in hepatic fibrosis, is now being re-examined in the context of PSCs role in pancreatic fibrosis. Gene expression profiling using microarray technology demonstrates many similarities between PSCs and HSCs, clearly differentiating them from skin myofibroblasts(Buchholz et al., 2005). Despite this high degree of similarity, distinct differences in gene expression patterns have also been observed between HSCs and PSCs (Buchholz et al., 2005). Much of the work that has been performed in liver fibrosis and hepatic stellate cells has been recapitulated in pancreatic stellate cells.

It is now an emerging concept that both PSC and HSCs appear to share many anatomic and phenotypic similarities with pericytes in other organs, which are likely to represent the pan organ precursor of the fibrogenic cell type (Lee et al., 2007; Hutchinson, Fligny & Duffield, 2013).

1.5.3 Isolation of pancreatic stellate cells and in vitro activation

The lower density of quiescent cells due to their vitamin A content, is used to isolate PSCs with high purity by using density gradient centrifugation after collagenase digestion of normal pancreas (Apte et al., 1998; Bachem et al., 1998). On untreated plastic, cultured PSCs are autoactivated changing their morphological and functional features. This technique typically yields 1-2 million cells.

Due to the limited number of cells obtained with this technique, other methods to obtain activated stellate cells have been used. This includes an injection of a supraphysiological dose of the cholecystokinin analogue, cerulein, into a rats inducing acute pancreatitis . Subsequent harvest of tissue blocks and cell culture of this injured pancreas, allows “outgrowth” of activated stellate cells (Bachem et al., 1998). Human activated pancreatic stellate cells are also cultured from surgically resected human fibrotic pancreas or pancreatic cancer specimens using this technique (Bachem et al., 1998).

To overcome low availability of PSCs, immortalised human PSC lines using SV40 large T-antigen transfer have been generated (Sparmann et al., 2004; Satoh et al., 2002). Other groups have described a spontaneously immortalised rat PSC line (Masamune, Satoh, Kikuta, Suzuki, &

Shimosegawa, 2003). Whether these immortalised pancreatic stellate cell lines really represent activated PSCs and react like natural PSCs remains unclear.

1.5.4 Mechanisms of PSC activation

Many of our insights into the factors that cause activation of pancreatic stellate cells comes from studies of PSCs in tissue culture. Studies of human and rodent primary PSCs in tissue culture have identified numerous growth factors, cytokines, hormones, intracellular signalling molecules, transcription factors, ethanol, acetaldehyde and oxidant stress as regulators of PSC activation. These fibrogenic mediators promote fibrogenesis by stimulating PSC motility, proliferation, or matrix synthesis or by reducing matrix degradation.

It is not currently understood how transient activation of PSC in acute pancreatitis becomes perpetuated in chronic pancreatitis and pancreatic cancer. In particular few studies have been performed specifically looking at the resolution of pancreatic fibrosis. We know from animal models that unless an injury is sustained, resolution of fibrosis can and does occur (Elsasser, Haake, Grimmig, Adler, & Kern, 1992). The mechanisms that are involved in this process have not been fully investigated.

Potential activators of PSCs in vivo include paracrine factors growth factors; PDGF and TGF-β_1, cytokines; IL-1, IL-6, IL-8, and TNF-α, angiotensin II, and reactive oxygen species released by damaged neighboring cells as well as leucocytes recruited in response to pancreatic injury.

PDGF represents the most effective mitogen in both human and rat PSCs (Schneider et al., 2001; Luttenberger et al., 2000; Bachem et al., 1998; Bachem et al., 2005; Schmid-Kotsas et al., 1999). PDGF also stimulates PSC motility and chemotaxis (Luttenberger et al., 2000; McCarroll et al., 2004; Phillips et al., 2003c).PSC motility and proliferation are also stimulated by endothelin-1 (Masamune, Satoh, Kikuta, Suzuki, & Shimosegawa, 2005; Klonowski-Stumpe et al., 2003) and angiotensin II (Hama et al., 2006; Reinehr, Zoller, Klonowski-Stumpe, Kordes, & Haussinger, 2004).

TGF-β1 appears to a major stimulus of fibrogenesis (Apte et al., 1999; Shek et al., 2002), not only by increasing collagen production but also by inhibiting MMPs in the pathway of collagen degradation (Shek et al., 2002). Additionally, TGF-β1 enhanced the production of connective

tissue growth factor (CTGF) in cultured rat PSCs (Gao & Brigstock, 2005). CTGF is a mediator of the fibrogenic actions of TGF-β and stimulates PSC proliferation, integrin-dependent adhesion, migration, and collagen synthesis (Gao & Brigstock, 2006; Gao & Brigstock, 2005).

TNF-α also increases PSC proliferation and collagen synthesis(Mews et al., 2002) . PSC have also been shown to secrete IL-6, IL-8, monocyte chemotactic protein (MCP)-1, and RANTES in response to IL-1β and TNF-α. It has been suggested that activated PSCs may therefore mediate infiltration and accumulation of inflammatory cells in the pancreas(Andoh et al., 2000). The same group also demonstrated that IL-1β and TNF-α stimulate MMP-1 secretion of culture-activated human PSCs in a dose and time dependent manner(Tasaki et al., 2003) . TNF-α also increases the expression of inter-cellular adhesion molecule (ICAM)-1(Masamune et al., 2002).

Oxidative stress and ethanol metabolites have also been shown to activate PSCs. Ethanol can be metabolised by pancreatic acinar cells and PSCs, resulting in toxic metabolites and oxidative stress that can induce pancreatic damage (Apte et al., 2000; Apte & Wilson, 2003; Haber et al., 1998). In vitro studies indicate that ethanol and its metabolite acetaldehyde promote the activation of rat PSCs and cause lipid peroxidation in these cells (Apte et al., 2000).PSC activation caused by ethanol or its metabolite acetaldehyde can be prevented by the antioxidant vitamin E, implicating oxidative stress in the mediation of the pro-fibrogenic action of ethanol(Apte et al., 2000).

1.5.5 Autocrine loops and intracellular signal mediation in PSC

While in the initial phase of PSC activation extracellular mediators such as cytokines, free radicals and ethanol metabolites play a key role, it is probable that autocrine loops contribute to the perpetuation of PSC activation at later stages. PSC also have the capacity to synthesise growth factors, and various polypeptides, as well as being cytokine responsive themselves. It has also been demonstrated that some of these growth factors and cytokines may act in an autocrine fashion and contribute to the maintenance of PSC activation in the diseased pancreas. Activated PSC produce autocrine factors such as PDGF,TGF-β, and CTGF (Shek et al., 2002; Kruse, Hildebrand, Timke, Folsch, & Schmidt, 2000; Gao & Brigstock, 2005) which perpetuate the activated phenotype. Additionally, activin-A, a member of the TGF-β family of soluble factors, also functions in an autocrine manner increasing collagen secretion and augmenting

TGF-β expression and secretion (Ohnishi et al., 2003). PSC also produce proinflammatory molecules such as COX-2 (Yoshida et al., 2005), that can perpetuate the activated phenotype. PSC motility and proliferation is also stimulated by endothelin-1(ET-1)(Masamune, Satoh, Kikuta, Suzuki, & Shimosegawa, 2005; Klonowski-Stumpe et al., 2003). Elevation in the local expression of ET-1 has previously been suggested to be associated with the morphological and haemodynamic changes of chronic pancreatitis.

Development of autocrine loops in the course of PSC activation may provide a further molecular explanation of the clinical observation that fibrosis in chronic pancreatitis patients often persists even though the original injury such as alcohol, has been removed.

It is clear that multiple factors are involved in PSC activation, and cells are exposed to a variety of cytokines and growth factors that are secreted at increased levels during tissue injury, repair, and inflammatory processes.

1.5.6 Signalling Pathways

Several research groups have focused on the signalling pathways involved in PSC activation, proliferation, matrix synthesis, and migration. The PI3-kinase pathway appears to play a role in PSC migration(McCarroll et al., 2004). PDGF has been shown to induce rapid activation of Raf-1, ERK, and AP-1 proteins(Jaster, Sparmann, Emmrich, & Liebe, 2002). Ethanol and acetaldehyde both induce activation of all three subfamilies of the MAPK pathways (ERK 1/2, JNK, p38 MAPK in PSCs (Sparmann et al., 2005; Aoki et al., 2006). However, activation by ethanol and acetaldehyde is only inhibited by a p38 MAPK inhibitor, suggesting that only the p38 MAPK pathway is responsible for this alcohol effect (McCarroll et al., 2003). In addition, ethanol and acetaldehyde activate activator protein-1 (AP-1) but not nuclear factor κβ(NFκ-β) (Masamune, Kikuta, Satoh, Satoh, & Shimosegawa, 2002). The antioxidant *N*-acetylcysteine blocks ethanol and acetaldehyde induced activation of AP-1 and MAPK, suggesting a further role for oxidative stress (Masamune et al., 2002).

These signalling pathways represent potential therapeutic targets for the modulation of PSC function.

1.5.7 High Glucose and pancreatic stellate cells

Although glucose is a major energy source in mammalian cells, high glucose levels can have toxic effects and aggravate diabetic complications in various organs such as the liver (Angulo, Keach, Batts, & Lindor, 1999; Muller et al., 1992) kidney (Ayo et al., 1991; Lam et al., 2003) and vascular system (Natarajan, Gonzales, Xu, & Nadler, 1992; Williams, Tsai, & Schrier, 1992). High glucose concentration also induces fibrosis in non-alcoholic steatohepatitis (Paradis et al., 2001) in which HSCs are involved.

There have been several recent reports exploring the relationship of high glucose and insulin levels and the activation and proliferation of pancreatic stellate cells. The Otsuka Long Evans Tokushima Fatty (OLETF) rat is an animal model of type II diabetes with obesity. It shows a clinically relevant phenotype of type II diabetes including hyperinsulinaemia, hyperglycaemia, insulin resistance, hypertriglycaemia and mild obesity (Kawano, Hirashima, Mori, & Natori, 1994). Treatment of OLETF rats with the angiotensin- converting enzyme (ACE) inhibitor, ramipril, improved glucose tolerance by a significant reduction of islet destruction by fibrosis (Ko et al., 2004). Therefore, protection against islet fibrosis could be a new target for prevention of the development and progression of Type 2 diabetes mellitus. A subsequent study also confirmed that high glucose treatment produced a fourfold increase of ECM protein synthesis in PSCs, suggesting that hyperglycaemia may have an important role in the pathogenesis of pancreatic fibrosis (Ko et al., 2006). Insulin and insulin-like growth factor (IGF)-1 are mitogenic for fibroblasts and smooth muscle cells, and there is evidence that HSCs are sensitive to these molecules, resulting in mitogenesis and collagen synthesis (Svegliati-Baroni et al., 1999). Because insulin is a well-known growth factor for various cells in the body, PSCs in the islets might be predisposed to activate and proliferate by hyperinsulinaemia. The same group have also gone on to demonstrate that, the islet-specific environment represented by hyperinsulinaemia and hyperglycaemia had additive effects on the activation and proliferation of cultured rat PSCs, and this may further contribute to progressive pancreatic islet fibrosis observed in Type 2 diabetes(Hong et al., 2007). Both stimuli promoted PSC proliferation and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation independently, but there was also an additive effect. Glucose and insulin-induced ERK 1/2 phosphorylation also stimulated CTGF expression.

The WBN/Kob rat, represents another animal model of chronic pancreatitis (Ohashi et al, 1990). They become spontaneously diabetic at 60 to 90 weeks of age with the development of pancreatic fibrosis. Histology demonstrates α-sma expression by PSCs located in interlobular areas. The advancement of fibrosis is associated with progressive hyperglycaemia and reduction of β-cell mass(Mori et al., 1990). The mechanisms are not currently fully understood, but there appears to be enhanced apoptosis of acinar cells preceding the infiltration of inflammatory cell. Mitochondrial swelling after ischaemia and stasis of pancreatic juice were further reported to be responsible for early pancreatic changes (Hashimoto et al., 2000).

In another recent study , it has been demonstrated that high glucose increases PSC proliferation and collagen production through the protein kinase C (PKC) signalling pathway(Nomiyama et al., 2007). Protein kinase C is an important mediator of signal transduction in response to several cellular signals and is involved in tumour promotion, gene expression, cell proliferation and cell differentiation. These animal models of chronic pancreatitis demonstrate a potential role of hyperglycaemia in perpetuating the active myofibroblastic phenotype of PSCs.

1.6 Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases

Following parenchymal injury in most organs, the healing process involves the regeneration of both cellular and the ECM components. This wound healing process follows a conserved pattern of events which includes the initiation of the coagulation cascade, an inflammatory response which involves the granulation and re-epithelialisation of the affected area and finally a fibroproliferative response by the mesenchymal cells and the laying down of ECM.

In normal wound healing there is a network of negative regulatory mechanisms which terminates the proliferative and fibrotic process allowing the tissue to be repaired completely. In cases where these mechanisms fail or where there is a perpetuation of the injury, there is continuous ECM secretion and subsequent development of fibrosis.

Although various experimental models have improved our understanding of the fibrotic process, the mechanisms underlying pancreatic fibrogenesis remain unclear. A generally accepted theory is that fibrosis may result as a consequence of repeated injury, and that a

failure in this repair process contributes to sustained organ damage (Bataller & Brenner, 2005). This process can occur independently of the original injury, with organ recovery requiring extensive ECM remodelling.

Adequate remodelling of this provisional matrix allows cell proliferation, migration and assembly of new parenchymal cells. These processes are coordinated via production of ECM proteins, growth factors, adhesion proteins and extracellular-degrading proteases, and complex cell-matrix interactions. Alterations in this coordinated process leads to an imbalance between biosynthesis and proteolytic clearance of matrix components, impaired regeneration and ultimately to fibrosis.

Matrix metalloproteinases (MMPs) are a family of structurally related proteolytic enzymes which degrade components of the ECM. They all contain a zinc ion bound at the active site and are calcium-dependent endopeptidases. With the exception of membrane type MMPs (MT-MMPs) which are membrane bound, all MMPs are secreted in an inactive form as proenzymes and require activation to degrade the ECM. Conversion to the active form occurs by the proteolytic removal of the propeptides. The enzymatic activity occurs at neutral pH and has a combined ability to degrade a variety of substrates present in the ECM. There are at least 25 members of the MMP family divided into 5 groups according to their major substrates . MMPs are inhibited by specific natural tissue inhibitors called tissue inhibitors of metalloproteinases (TIMPs). Four members of the TIMP family have been characterised to date, and are called TIMPs 1, 2, 3 and 4 (Herron, Werb, Dwyer, & Banda, 1986; Stetler-Stevenson, Krutzsch, & Liotta, 1989; Pavloff, Staskus, Kishnani, & Hawkes, 1992; Leco et al., 1997). The relative molecular mass ranges from 22 to 30 kDa with a 40–50% sequence identity. TIMPs bind to MMPs with low selectivity forming tight non covalent 1:1 complexes. It has been postulated that any small change in the levels of either leads to a significant alteration in overall enzyme activity.

1.6.1 MMPs and TIMPs in pancreatic disease

The overall increase in ECM in fibrotic diseases, consists of collagen, proteoglycans, and glycoproteins such as fibronectin and laminin, although the exact composition of the fibrotic neomatrix differs from one organ to another. In the normal pancreas, type IV collagen and laminin are found deposited in a linear fashion along the basement membrane of epithelial cells

and blood vessels. In pancreatic fibrosis there is a shift from the normal low density type IV and VI collagens to fibril-rich collagens such as types I and III (Kennedy et al., 1987). The accumulation of ECM reflects an alteration of the balance of the matrix degradation pathways and the cellular drive to secrete ECM.

In liver fibrosis with increasing duration of culture and the enhanced level of activation of HSC, MMPs become down-regulated in conjunction with up-regulation of TIMP-1 and TIMP-2 (Iredale, Goddard, Murphy, Benyon, & Arthur, 1995). MMP-2 partly degrades the ECM produced by activated HSC and promotes HSC proliferation (Benyon et al., 1999). The switch to the production of molecules that inhibit MMP activation coupled with the increase production of ECM tips the balance towards a profibrotic state.

MMP-2 and MMP-9 may be particularly important in regulating fibrogenesis and scar degradation as they are known to degrade collagens I and III and basement membrane collagen. The activity of MMPs is regulated at the levels of transcription and pro-enzyme activation, and by the inhibition of activated enzyme by TIMPS.

Immunohistochemical distribution of MMP-2 and MMP-9 in normal pancreas is confined to the vessels and not seen in the ductal epithelia whereas in chronic pancreatitis, there is significant staining of MMP-2 in the epithelia and loss of MMP-9 staining (Ishihara, Hayasaka, Yamaguchi, Kondo, & Saisho, 1998).

PSC have the capacity to synthesise a number of matrix metalloproteinases, including MMP2, MMP9, and MMP13 (Phillips et al., 2003b). In this study, MMP2 has been shown to be the dominant MMP secreted by PSCs. However, the amount of MMP2 produced by quiescent PSCs was significantly lower than that produced by the activated PSC. The mechanisms by which increased MMP-2 may be profibrogenic is not fully understood, but may be due to increased degradation of normal basement membrane, type IV collagen. This may subsequently facilitate the deposition of pathological fibrillar collagen. Alternatively, MMP2 may exert a proliferative effect on PSCs leading to an increase in the number of activated PSCs at sites of injury, which has been shown to occur in HSC with an overall result of increase in collagen synthesis (Benyon et al., 1999).

TIMPs 1 and TIMP-2 have also been shown to be expressed by pancreatic stellate cells in tissue culture, and they are likely to have a critical role in pancreatic fibrosis (Phillips et al., 2003b; Shek et al., 2002b). The clear localisation of TIMP-1 to areas of fibrosis in chronic pancreatitis specimens (Shek et al., 2002b) suggests that these proteins are being expressed by activated PSCs in vivo. Similar to liver fibrosis, it is probable that the proportion between MMPs and TIMPs changes in pancreatic fibrosis toward a higher TIMP expression.

The activated stroma of pancreatic cancer is an important feature of pancreatic ductal adenocarcinoma (PDAC). Activated PSCs have also been recently identified in fibrotic areas of pancreatic cancer by staining for glial fibrillary acidic protein (GFAP), desmin, and α-SMA (Apte et al., 2004; Bachem et al., 2005). It is probable that the interactions between tumour cells and stromal cells play an important role in the pathobiology of pancreatic cancer. Pancreatic cancer cells have been shown to stimulate PSC proliferation and TIMP-1 production (Armstrong et al., 2004).

The presence of MMPs and TIMPS in pancreatic fibrosis suggests a role for PSC in controlling matrix degradation and deposition. Under normal physiological conditions MMPs are tightly regulated by growth factors and cytokines. These findings are important because it suggests that in addition to their well documented role in ECM synthesis, PSCs may also play a role in ECM degradation. TIMP-1 production by PSCs might therefore promote fibrogenesis by preventing the degradation of collagen, which may contribute to fibrosis in chronic pancreatitis. A further role for TIMP-1 in preventing stellate cell apoptosis is discussed below.

1.7 The effects of lipopolysaccharide on stellate cells

The first report of paracrine stimulation of pancreatic stellate cells via soluble mediators, was performed with lipopolysaccharide(LPS) stimulated macrophages(Schmid-Kotsas et al., 1999). Supernatants of these cultured mononuclear cells stimulated the synthesis and secretion of collagen type I and fibronectin in cultured human and rat PSC. This was shown to be a result of TGF-β released by macrophages in response to LPS.

Alcoholic pancreatitis demonstrates histological features of both acute necroinflammatory changes, as well as chronic fibrosis and acinar atrophy. It is generally is accepted that chronic pancreatitis due to alcohol results from repeated episodes of acute injury, which eventually

leads to irreversible fibrosis and atrophy of the pancreas. It has been observed that patients with increased frequency of attacks of alcoholic pancreatitis, develop chronic disease more rapidly (Ammann & Muellhaupt, 1994). This observation that not all alcoholics develop chronic pancreatitis, has led to the suggestion that additional trigger factors may be required to sustain pancreatic injury (Steinberg & Tenner, 1994).

PSCs can be activated directly by ethanol and its metabolite acetaldehyde (Apte et al., 2000). In vivo studies of the pathogenesis of alcoholic pancreatitis often have been hampered by the lack of a suitable animal model. It has been demonstrated that ad libitum administration of alcohol alone to rodents fails to cause overt pancreatic injury (Singh, 1987). Consequently, additional toxic agents or manipulations have been administered in an attempt to produce a model of alcoholic pancreatitis. These include; injecting high doses of cerulein (Pandol et al., 1999; Perides, Tao, West, Sharma, & Steer, 2005) , chemical toxins such as trinitrobenzene sulfonic acid (Puig-Divi et al., 1996) or pancreatic duct ligation (Tanaka et al., 1998).

Studies to date have failed to unequivocally identify an initiating factor for this condition. It is known that gut permeability is increased significantly in alcoholics, allowing translocation of gram-negative bacteria across the mucosal barrier and thus enabling bacterial endotoxins such as LPS to enter the circulation (Parlesak, Schafer, Schutz, Bode, & Bode, 2000). A recent study has demonstrated a possible role for LPS as a candidate trigger factor/cofactor for alcoholic pancreatitis (Vonlaufen et al., 2007). This rodent model of alcoholic pancreatitis utilises administration of alcohol and LPS together, to induce chronic pancreatitis. This study also demonstrated that PSCs can be directly activated by endotoxin both in vivo and in vitro, and that they express the LPS receptors TLR4 and CD14 (Vonlaufen et al., 2007).

Sepsis induces a catabolic state with negative nitrogen balance, which leads to reduced lean body mass and cachexia. LPS triggers the initiation of host responses to sepsis by gram-negative bacterial infection. Endotoxin administration in rats decreases circulating growth hormone (GH) and IGF-I (Fan, Molina, Gelato, & Lang, 1994). A decrease in serum concentrations of IGF-I in septic patients and in different inflammatory diseases has also been observed (Dahn, Lange, & Jacobs, 1988; Katsanos et al., 2001) The decrease in circulating IGF-I is secondary to a decrease in IGF-I gene expression in the liver and other peripheral tissues such as muscle (Lang, Frost, Jefferson, Kimball, & Vary, 2000). The inhibitory effect of LPS on the IGF-I axis is also exerted on

insulin-like growth factor-binding protein-3 (IGFBP-3) when measured in the serum and liver. The functional significance of this impact on IGFBPs is not fully understood. The decrease in this binding protein may increase IGF-I turnover and contribute to the catabolic state in sepsis, or, on the contrary, it may increase the IGF-I bioavailability and counteract the catabolic response. To my knowledge there is no available data on the effects of LPS on IGF axis components on the pancreas, although it is known that LPS can directly activate PSC (Vonlaufen et al., 2007). The mechanism by which endotoxin inhibits liver IGF-I and IGFBP-3 is not fully understood.

1.8 Apoptosis in the resolution of fibrosis

Apoptosis or programmed cell death is an essential physiological process that is required for the normal development and maintenance of tissue homeostasis. In liver fibrosis there is evidence of a constant background of apoptosis in activated hepatic stellate cells, and it is likely that this is a major mechanism regulating overall stellate cell numbers. Historically, both liver fibrosis and cirrhosis were considered as irreversible. However, serial liver biopsies have shown that there is remodelling of the liver with resolution of fibrosis, following removal of injurious agents. Studies using animal models have supported this concept of reversibility (Issa et al., 2004; Issa et al., 2001).

In common with other cells, hepatic stellate cells demonstrate susceptibility to apoptosis induced via two basic intracellular pathways. The first pathway is the so-called extrinsic pathway initiated by the binding of an extracellular death ligand, such as FasL, to its cell-surface death receptor. This initiates an intracellular cascade of proteolytic enzymes known as caspases, which results in apoptosis. The other major pathway involves the stability and integrity of the mitochondrial membrane. Mitochondrial membrane permeabilisation is regulated by a balance of pro and anti-apoptotic proteins of the Bcl-2 family. When pro-apoptotic proteins predominate and are allowed to homo-dimerise, cytochrome C is released from the mitochondrion which results in activation of specific caspases and apoptosis of the cell. The balance of mitochondrial membrane proteins is regulated in part via specific signals received by the cell, including those derived from soluble factors, cell-cell and cell-matrix interactions. A recent review highlights the current known mechanisms by which apoptosis is regulated in HSC (Elsharkawy, Oakley, & Mann, 2005).

It has been proposed that cells are imminently prone to undergo apoptosis as the default position (Raff et al., 1993; Raff et al., 1994), but are prevented by the presence of survival signals. Studies suggest HSC, fit nicely into this model (Murphy et al., 2002; Issa et al., 2001). These signals may be derived from the cellular environment in the form of soluble growth factors and cytokines, or in the example of hepatic stellate cell from the matrix. Following cessation of injury, apoptosis occurs when these survival factors fall below a critical level and the balance of pro and anti-apoptotic factors in the cells shift. Several cytokines and growth factors released during liver cell injury may impact on stellate cell apoptosis.

These include IGF-1 released in an autocrine manner by damaged hepatocytes and by stellate cells. IGF-1 is a powerful survival factor for stellate cells and may act together with other soluble factors to promote stellate cell survival (Issa et al., 2001).

Models of liver fibrosis in animals have provided key experimental data which identify the events determining resolution of fibrosis. Prominent amongst these is loss of the activated hepatic stellate cells through apoptosis. This has the effect of removing the major source of fibrillar collagen. Increasing evidence indicates that stellate cell apoptosis is determined by the balance of various survival factors and pro-apoptotic stimuli. HSCs are known to express a number of cell surface death receptors including Fas(Saile, Knittel, Matthes, Schott, & Ramadori, 1997) and low affinity nerve growth factor receptor (p75)(Trim et al., 2000)

Exposure of rodents to chronic carbon tetrachloride intoxication results in the development of fibrosis and ultimately cirrhosis. Spontaneous resolution occurs with a return to normal liver architecture and loss of the activated stellate cells. This process of resolution is accompanied by a decrease in the hepatic expression of TIMP-1 and an increase in overall hepatic collagenase activity (Iredale et al., 1998). A similar change can also be demonstrated following bile duct ligation induced fibrosis, and subsequent biliary decompression (Iredale et al., 1996). In each of these models, the loss of α-SMA positive activated stellate cells is mediated by apoptosis.

Thus, resolution is characterised not only by changes in the pattern of matrix degradation but by apoptosis of activated hepatic stellate cells. Attention has been directed at the identification of therapeutic agents that could promote activated stellate cell apoptosis. Gliotoxin, proteasome inhibitors and sulfasalazine have been shown to induce hepatic stellate cell apoptosis. Inhibition of NF-κB activity may be achieved by exposure to the fungal metabolite

gliotoxin. This has been shown to promote apoptosis in rat and human hepatic stellate cells and reduces fibrosis in experimental models of liver fibrosis (Wright et al., 2001). It unfortunately also promotes apoptosis in other cells that require NF-κB for survival, such as cells in the thymus, thus compromising the immune system.

Apoptosis may play a key role in the elimination of activated pancreatic stellate cells. There are currently few studies that have considered the role of pancreatic stellate cell apoptosis in the resolution of pancreatic fibrosis. Stellate cell activation is initiated via soluble factors secreted by components of the inflammatory response in addition to products released by damaged acinar cells. Once activated, the stellate cells may persist through a number of autocrine and paracrine mechanisms, several of which perpetuate the activation state.

Expression of collagen type 1 is significantly up regulated whilst concurrently its degradation is inhibited by expression of TIMPs -1 and -2(Shek et al., 2002). However stellate cells also express MMPs, including those with collagenalyitc activity. Analogous, to the liver the balance between these factors, will determine whether fibrillar matrix is continually laid down or resolution of fibrosis occurs. In human and experimental chronic pancreatitis there is an association with persistently activated PSCs in areas of fibrosis(Apte et al., 1998; Yamada et al., 2003; Haber et al., 1999; Vaquero, Molero, Tian, Salas, & Malagelada, 1999) In reversible models of pancreatic fibrosis, in which multiple acute episodes of acute pancreatitis are induced clearance of activated PSCs parallels both spontaneous(Yokota et al., 2002)and pharmacological (Yamada et al., 2003; van Westerloo et al., 2005; Kuno et al., 2003; Gomez et al., 2004) regression of pancreatic fibrosis.

It seems reasonable to speculate that apoptosis of activated pancreatic stellate cells may terminate ongoing fibrosis within the pancreas. Any therapeutic strategy would ideally target activated PSCs while sparing quiescent PSCs or other cells such as acinar, ductal, or islet cells.

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and CD95L are ligands to receptors belonging to the TNF receptor family of death receptors. The receptors are characterised by an intracellular death domain that recruits various adapter proteins and cysteine proteases such as caspase-8. Activation of caspase-8 then leads to apoptosis. Although the TRAIL receptors 1 and 2 are widely expressed in several tissues (Ashkenazi & Dixit, 1998), most cell types are not sensitive to TRAIL-mediated cell killing (Wiley et al., 1995).

Activated PSC have been shown to undergo apoptosis after incubation with the two death receptor ligands CD95L and TRAIL (Klonowski-Stumpe, Fischer, Reinehr, Luthen, & Haussinger, 2002). In this study cells it was demonstrated that there was a time-dependent sensitisation, that was accompanied by an increased expression of CD95 receptor and the TRAIL receptor. In this same study they showed that pancreatic acinar cells were not susceptible to TRAIL or CD95 mediated cell death. This was presumed to be due to expression of the antagonistic receptors TRAIL 3 and 4, and the lack of expression of CD95 on normal acinar cells.

Further investigation determined the effect of apoptosis induction by PK-11195(Klonowski-Stumpe et al., 2002), a specific ligand of the peripheral-type benzodiazepine receptor. HSCs demonstrated a transient sensitivity to PK-11195-induced apoptosis, which is maximal at about the 7th day of culture and disappears within 4 wk (Fischer, Schmitt, Bode, & Haussinger, 2001), whereas PSC appear to become increasingly susceptible to PK-11195 induced apoptosis over the same time period. The mechanisms underlying the culture-dependent sensitisation of PSC toward CD95 ligand and TRAIL induced apoptosis is unclear but may be due to increased expression of CD95 and TRAIL receptors. Targeting death receptors and inducing ligand-mediated apoptosis may be an efficient way to eliminate transformed PSC in chronic and acute pancreatitis.

Another study has shown that PSCs in culture can either die by apoptosis or acquire a fibroblastic differentiated state that is more resistant to apoptosis(Manapov, Muller, & Rychly, 2005). Whether these observations relate to PSC activation in vivo is unclear, which highlights the need for more information about the phenotypic states of PSCs during disease progression, and the mechanisms underlying the conversion between these states. These authors found that in an experimental model of acute pancreatitis , activated stellate cells were susceptible to apoptosis with subsequent return of the pancreas to normal after removal of the injury. On the other hand, cells with a “fibroblastic” phenotype persisted in an experimental model of progressive pancreatic fibrosis. The authors demonstrated that in the activated stellate cell susceptible to apoptosis, a cell cycle inhibitory protein $p21^{Cip1/WAF1}$ is present in the nucleus. With conversion of the stellate cell to the “fibroblastic” state, $p21^{Cip1/WAF1}$ translocates to the cytoplasm. This then binds to and inhibits activities of Rho kinase 2 and apoptosis signal regulating kinase 1(ASK1), resulting in decreased proliferation signals and apoptosis resistance.

In both the liver and pancreas, so-called “quiescent” SC have a low rate of proliferation and produce negligible ECM. However, during chronic inflammation, growth factors and cytokines released by resident damaged cells and infiltrating inflammatory cells stimulate SC to proliferate and adopt the myofibroblastic phenotype. These activated SCs express α-SMA and effect tissue repair by secreting a wide variety of ECM proteins and protect ECM from degradation by MMPs by producing potent TIMPs. If tissue injury is acute and fibrosis resolves, then activated SCs become lost from the tissues. In rats, cerulein induced pancreatitis results in a 20-30 fold increase in fibroblastic cells after 36 hours, but even after repeated episodes, fibrosis resolves in 6 weeks(Elsasser et al., 1992). During resolution of moderate liver fibrosis in rodents, regeneration of normal liver involves degradation of the scar and clearance of activated HSC by apoptosis (Iredale et al., 1998). Products of activated SCs such as TIMP-1, α–SMA and collagen I decrease as fibrosis resolves.

In most studies in which PSCs are activated after damage to the pancreas, the inflammatory process resolves and activated PSCs progressively disappear after removal of the injurious agent. However, repeated pancreatic damage and failure of the mechanisms regulating tissue repair can both lead to chronic inflammation with persistent activation and proliferation of PSCs. In experiments of repeated episodes of acute experimental pancreatitis, there are changes that resemble those found in chronic pancreatitis (Neuschwander-Tetri, Bridle, Wells, Marcu, & Ramm, 2000; Perides et al., 2005). If the matrix-forming activity of PSCs fails to cease after pancreatic repair has been completed, fibrogenesis will continue until the pancreatic endocrine and exocrine is replaced with fibrotic tissue, resulting in loss of function. The chronic injury results in perpetuation of the activated PSC phenotype. Furthermore, chronic pancreatitis is associated with reduced production of MMPs by PSCs, which probably helps promote and sustain the fibrotic phenotype (Shek et al., 2002).

Studies of liver fibrosis have shown that extensive ECM degradation is accompanied by apoptosis of HSCs, as a result of either increased proapoptotic signalling or reduced survival signals from the ECM (Issa et al., 2004), but this is yet to be determined in the pancreas.

More recently, impaired extracellular proteolysis of the ECM in mice lacking plasminogen has been associated with persistent PSC activation and accumulation of collagens during the recovery phase of mouse cerulein-induced acute pancreatitis (Lugea et al., 2006). Levels of

pancreatic TGF-β1, plasminogen activator inhibitor-1, as well as TIMP-1, also remained elevated persistently in plasminogen- deficient mice.

In contrast, in models of more severe liver injury akin to clinical disease, there was delayed and incomplete resolution and HSC and TIMP expression persisted for months. Further studies have demonstrated TIMP-1 added into stellate cell cultures protected them from apoptotic stimuli (Murphy et al., 2002). Complementary studies of liver fibrosis in normal and mutant mice which have non-degradable type I collagen showed that HSC apoptosis following cessation of liver injury required degradation of type I collagen, suggesting TIMP-1 might prevent HSC apoptosis in vivo by inhibiting breakdown of this protein.

During pancreatic fibrosis and cell culture activation in vitro, activated PSC express TIMP-1(Phillips et al., 2003b). Previous studies by our group using HSC cultures, rodent liver fibrosis models and clinical specimens, all suggest that TIMP-1 is a major driver of fibrosis (Murphy et al., 2002; Iredale et al., 1995). These studies suggest TIMP-1 promotes liver fibrosis by blocking apoptosis of activated HSC, essentially acting as a survival factor. Soluble growth factors may also support survival of PSC. In the pancreas these cells exist in a uniquely high-insulin environment. Insulin supports growth and survival of several cell types but its effects on PSC apoptosis have not been assessed.

If successful anti-fibrotic therapies are to be developed, it will be important to understand the mechanisms that lead to termination of PSC activation during regeneration of the pancreas. Several studies have demonstrated that it is possible to revert to the quiescent phenotype, on exposure to retinoic acid (McCarroll et al., 2006). However this has yet to be demonstrated in vivo. Retinoic acid is stored in pancreatic stellate cells, and is known to keep mesenchymal stem cells in their undifferentiated state, and it has been suggested that this might be also the case in stellate cells.

These factors suggest a potentially wide role of quiescent PSC and formation of fibrotic tissue reflect a process of dysregulated tissue repair and cell differentiation under the pathological conditions of CP and PC.

Taken together, in vitro and in vivo data suggest that pancreatic fibrosis is the result of increased synthesis and reduced degradation of fibrillar ECM, although it has been reported

that activated PSCs colocalise with MMP-2, MT1- MMP, and tissue inhibitor of metalloproteinases TIMP-1 and TIMP-2 within areas of abnormal periacinar and intraacinar fibrosis. It is probable that the proportion between MMPs and TIMPs changes in pancreatic fibrosis toward a higher TIMP expression.

1.9 Potential therapeutic approaches for pancreatic fibrosis

At present, no treatment has been shown to be effective in halting the fibrogenic process Previous studies have demonstrated that α-tocopherol inhibits PSCs activation in vitro (Apte et al., 2000) and also reduces pancreatic fibrosis in a rat model of chronic pancreatitis (Gomez et al., 2004b). A recent paper highlights the possibility of potential antifibrogenic value of natural tocotrienols(Rickmann, et al., 2007). This group showed tocotrienols induce PSC death in vitro PSC via apoptosis and autophagy, by targeting the mitochondrial permeability transition pore. These data demonstrate that tocotrienols exert selective cytotoxicity toward activated PSCs, suggesting that, in vivo administration may ameliorate the fibrogenesis associated with chronic pancreatitis.

1.10 Hypothesis and Aims

In chronic pancreatitis, pancreatic stellate cells (PSCs) become activated to a myofibroblastic phenotype secreting abundant fibrillar collagen leading to pancreatic fibrosis. Data from the injured liver shows that resolution of fibrosis requires the apoptosis of activated hepatic stellate cells(HSC). Therefore, the resolution of pancreatic fibrosis may also necessitate apoptosis and factors which inhibit PSC apoptosis might promote pancreatic fibrosis.

Regulation of PSC apoptosis is poorly understood. Two key polypeptide hormones, produced in the pancreas, are insulin-like growth factor-1 (IGF-1) and insulin, with PSCs being exposed to high levels of insulin in the insulo-acinar portal system. Insulin and IGF-1 prevent apoptosis of various cell types, including HSCs and therefore may inhibit PSCs from undergoing apoptosis. Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) was also shown to inhibit HSC apoptosis, thus contributing to the persistence of fibrosis. Acknowledging the effects IGF-1 , insulin and TIMP-1 on HSC survival, the following hypothesis is proposed:

1.10.1 Hypothesis

Insulin, IGF-1 and TIMP-1 inhibit apoptosis occurring in response to serum withdrawal thus promoting PSC survival.

1.10.2 Aims of the study:

- To establish whether insulin, IGF-1 and TIMP-1 protect PSC from apoptosis.
- To determine if TIMP-1, IGF-1 and insulin act in autocrine or proxicrine fashion to prevent pancreatic stellate cell apoptosis.
- Determine if IGF-1 and insulin promote survival of activated PSC.
- Identify siRNA which effectively silence TIMP-1

2 Chapter 2

2.1 Methods

2.1.1 Cell Culture

2.1.1.1 Isolation of rat PSCs

Rat PSCs were extracted from normal rat pancreas by pronase and collagenase digestion and purified by density centrifugation as previously described. In brief, normal Sprague-Dawley rats (200-250grms) were culled using carbon dioxide and cervical dislocation. The pancreas was then harvested and finely minced using a pair of sterile scissors. Next enzymatic digestion of the pancreas was undertaken with 10mls of 0.02% pronase (20mg/10ml HBSS with Ca^{2+}, Roche Diagnostics) and 10 mls of 0.05% collagenase (50mg/10ml HBSS with Ca^{2+}, Roche Diagnostics) solution at 37°C for approximately 20 minutes with gentle agitation. Next the pancreas was transferred to the tissue culture hood and the pancreas digest filtered through a Nybolt filter. DNase solution 0.1% (100mg/10mls HBSS with Ca2+, Roche Diagnostics) was used to help wash the pancreatic extract through the Newbolt mesh. The resulting pancreatic digest was transferred to 2 x 50 ml falcons and centrifuged at 1500 rpm for 5 minutes. The pellets were resuspended in HBBS with Ca^{2+}, and a further wash was performed at 1500 rpm.

A 12% density gradient was made from 35.2ml HBSS with Ca^{2+} and 4.8ml of Optiprep (Axis-Shield, Oslo, Norway, Density 1.320 g/l). The resulting pellet was then resuspended in this gradient and a 3ml buffer layer of HBSS with Ca^{2+} was carefully pipetted on to the top of this. The PSCs are then separated from other cells on the basis of their lower density due to high vitamin A content. The cells are spun in a centrifuge at 2000 rpm for 20 minutes with the brake off. Once the centrifuge has stopped the PSC appear as a “fuzzy band” visible immediately beneath the cushion of HBSS. This layer was carefully removed and resuspended in 40mls of HBSS with Ca^{2+}, and centrifuged at 1500 rpm. The pellet was once again resuspended and this wash step repeated. A typical rat preparation from one pancreas would yield approximately 1-2million PSCs with 95% viability by trypan blue staining. Cell numbers were determined with the use of a counting chamber (Neuebauer haemocytometer).

2.1.2 Isolation of human PSCs

Quiescent Human PSCs were extracted from the normal margins of human pancreas resected for pancreatic cancer or chronic pancreatitis. Local ethics committee approval was obtained from Southampton and South West Hampshire Research Ethics Committee B, and prior consent was taken from the patient. A letter was also sent to the patients General Practitioner.

Briefly, the pancreatic specimen was collected from the operating theatres. The operating surgeon would cut a piece of macroscopically normal pancreas from the resected pancreatic specimen. The pancreas specimen was taken to the tissue culture hood and minced into fine pieces with scissors and decanted into a sterile glass bottle. A mixture of collagenase (50mg/10ml HBSS with calcium), pronase (20mg/10ml HBSS with calcium) and 10ml of DNase solution (100mg/10ml HBSS with calcium) was added to the pancreas. This was then incubated at 37°C with shaking. After 20 minutes the pancreas digest was filtered through a Nybolt filter. This crude extract is processed in the same manner as the rat stellate cell preparation described above. The results using this technique are highly variable.

Activated human pancreatic stellate cells can be isolated using the "outgrowth" technique (Bachem et al., 1998). Resection specimens from patients undergoing pancreatic resection for chronic pancreatitis will have a highly fibrotic pancreas. In these cases the pancreas is cut up into small tissue blocks using a scalpel. Blocks measuring approximately 0.5–1 mm^3 are placed onto 10-cm^2 uncoated tissue culture plates. Complete media containing 16 %FCS were then placed into these plates taking care not to disturb the tissue blocks. Tissue blocks were cultured at 37°C in a 5% CO_2-air humidified atmosphere. 18 hours after plating, the culture medium was changed, and subsequently 24 hours later. At this stage the tissue blocks were transferred to new culture plates. Typically cells grow out of the tissue blocks between 1-3 days later.

2.1.3 Culture of pancreatic stellate cells

Quiescent PSCs were identified by their characteristic autofluorescence due to the presence of vitamin A droplets within the cells. Cell preparations were typically 90-95% pure. Extracted PSCs were cultured on plastic until they became activated to pancreatic stellate cells after 7-10 days.

Human and rat PSC were used for experiments after activation in primary culture or after passage (<3). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, UK) in the presence of 16% foetal calf serum and antibiotics including penicillin and streptomycin (Gibco, UK).

2.1.4 Trypsinisation of pancreatic stellate cell

Cells were passaged after reaching approximately 70-80% confluence. Complete media were removed from the flask and the cell monolayer was washed twice with HBSS without Ca^{2+}. Next the cells were detached by exposing them to 5ml of 1x trypsin for 5 minutes at 37^{o}C. 5ml of complete media containing 16% FCS were added to neutralise the trypsin. The cell suspension was then centrifuged in a 14ml centrifuge tube at 1500rpm for 5 minutes. The pellet obtained was then resuspended in 1ml of complete medium, and the cells split into new plasticware. For the purpose of experiments cell numbers were determined by counting with a haemocytometer, and plated at the required density. The cell pellet was then re-suspended in complete media and split between new T75cm^2 tissue culture flasks. Primary PSC were used experimentally between passages 1-3.

2.2 Stimulation and quantification of apoptosis

Various methods to measure apoptosis have been described , each with their own limitations. The process of apoptosis is defined by a series of morphological changes. The characteristic features can be seen best by electron microscopy but can be observed under the light microscope using nucleic acid binding dyes, such as haematoxylin, acridine orange, or propidium iodide. The biochemistry of apoptosis is now known in some detail but can vary depending on the apoptogen studied. Many methods use one specific part of the apoptotic pathway as a surrogate marker of apoptosis. Annexin V expression on the cell surface is thought to be an early event in apoptotic cell. Caspase-3 activation is considered an intermediate step in apoptosis. DNA fragmentation is thought to be a late manifestation of apoptosis. A generic problem with apoptosis measurement is that for a given population of cultured cells exposed to an apoptotic stimulus at the same time, the lead in or lag time before morphologic features of apoptosis occur is variable. Once apoptosis has started, it occurs in an efficient manner and some reports claim that in as little as 1-2hour a cell can undergo apoptosis and the apoptotic

cell debris be removed by neighbouring cell by phagocytosis. To attempt to measure apoptosis by a single method is therefore subject to caveats. Measurement of apoptosis usually involves static measurement and so it is incorrect to discuss “apoptotic rates”. For this reason, morphological techniques e.g. acridine orange staining are normally expressed as an apoptotic index or percentage.

Apoptosis of PSCs was induced exposing the cells to 24 hours of serum deprivation followed by cycloheximide treatment, as previously described. PSCs were cultured in 24-well tissue culture plates. PSCs were then exposed to pro-apoptotic stimuli with the following experimental conditions described below.

2.2.1 Detection of apoptosis using acridine orange staining to look at nuclear morphology.

Each experimental condition was left to incubate for a defined time following 24 hours of serum deprivation. Then nuclear morphology was assessed by adding 1 μL of 1mg/ml acridine orange (Sigma, UK) to each well (final concentration 1ug/ml) and observing the cells under blue fluorescence with a Zeiss inverted fluorescence microscope. The total number of apoptotic bodies were counted and any apoptotic bodies floating in the supernatant included by racking up the objective lens. The total number of cells per field was counted and an apoptotic index calculated. Each condition was performed in duplicate and a random low power field was counted (typically 800 cells).

2.2.2 Caspase-3 Activity assay

To verify data obtained with acridine orange staining assays, caspase-3 activity assays were performed. This was quantified by a colorimetric assay for Caspase-3 activity (Promega, Southampton, UK), according to the manufacturer's instructions. This kit utilises a colorimetric substrate to active Caspase-3 that yields p-nitroanaline (pNA) which is yellow in colour. The difference in absorption at wavelength 405nm is proportional to the caspase-3 activity within the protein extracts. Cells were cultured in T75 tissue culture flasks and then serum deprived overnight. They were then exposed to 50 μM cycloheximide for 6 or 24 hours with bovine

insulin (Sigma), recombinant IGF-1(R and D Systems), and recombinant rat or human TIMP-1(R and D Systems). An apoptotic index was calculated for each experiment. Cells were collected by cell scraping into 1ml of ice cold sterile PBS, pelleted and resuspended in a cell lysis buffer supplied with the kit (50mM HEPES,1mM DTT, 0.1mM EDTA, 0.1% CHAPS, pH 7.4). Cells were lysed at 4^{o}C for 5 minutes then centrifuged at 10,000rpm for 10 minutes at 4^{o}C. Protein concentration was determined by BCA assay described below. Typically 30μg of protein extract was used in each reaction. A positive and negative control was included by way of recombinant active Caspase-3 and z-VAD-fmk (broad spectrum caspase inhibitor) respectively.

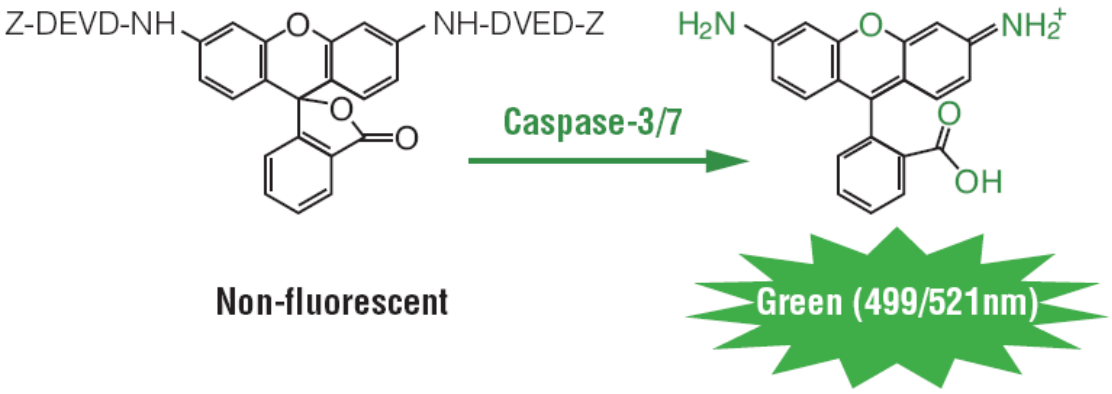
2.2.3 Quantification of apoptosis using Apo-ONE Homogenous Caspase 3/7 Assay in siRNA treated PSC

For the measurement of apotosis in siRNA treated cells, a commercially avaialable kit was used, Apo-ONE homogeneous caspase-3/7 assay (Promega, UK).

This assay uses a profluorescent substrate (rhodamine 110, bis-(N-CBZL-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110) and an optimised bifunctional cell lysis/activity buffer for caspase-3 and -7 (DEVDase). The buffer rapidly and efficiently lyses/permeabilises cultured cells and allows optimal caspase-3/7 enzymatic activity.

To perform the assay, the buffer and profluorescent substrate are mixed and added to the cells in culture. Cleavage of the non-fluorescent caspase substrate Z-DEVD-R110 by caspase-3/7 creates the fluorescent rhodamine 110. With excitation at 499nm,the rhodamine 110 leaving group becomes intensely fluorescent(Figure). The emission maximum is 521nm.

Figure 2-1 Cleavage of the non-fluorescent caspase substrate Z-DEVD-R110 (Promega Technical Bullletin)



PSC treated with siRNA by electroporation were cultured at a density of 10,000 cells well for 24 hours in 96-well black opaque plastic tissue culture plate. These were then washed with serum-free medium and incubated in the presence of serum-free medium or with the addtion TIMP-1 200ng/mL, for six and 24 hours at 37°C.

Caspase-3/7 activity corrected for cell number was determined as described above with caspase-3/7 related fluorescence measured 1 hour after addition of the assay reagent (excitation wavelength 485nm, emission wavelength 530nm).

Blank controls are used as a measure of background fluorescence associated with the culture system and Caspase-3/7 reagent and are subtracted from experimental values.

2.3 Quantification of DNA synthesis

The effects of insulin, IGF-1 and TIMP-1 on DNA synthesis of activated rat PSC was measured using [3H]-thymidine incorporation. Experiments were conducted in duplicate wells for each condition in 24-well plates. Activated PSCs, between passage 1 and 3, were trypsinised and plated into 24-well plates at a density of 20,000 cells/well and grown in media containing 16% FCS for 16 hours.

The cells were then washed twice with serum free medium and this was followed by complete serum deprivation for 24 hours, to synchronise the cells in the G_0 phase of the cell cycle. Cells

were then cultured for an additional 24 hours in serum-free media in the presence of recombinant IGF-1, insulin or recombinant TIMP-1 and 16 %FCS as a (positive control) prior to the addition of 1 μCi of [methyl-3H]-thymidine (Amersham Biosciences). Cells were incubated for a further 24 hours before washing with HBSS, and fixing in ice-cold methanol in the freezer for 20 minutes at minus 20°C. The fixed cells were then solubilised in 0.25 mol/L sodium hydroxide/0.2% (w/v) SDS (Sigma) and neutralised with 5M hydrochloric acid. The lysates were added to OptiPhase HiSafe 3 scintillation fluid (Wallac, Turku, Finland), and scintillation was counted using a Scintillation Counter (Elkay).

2.4 Protein methods

2.4.1 Protein extraction

In order to obtain the protein for western blotting and caspase assays, the PSC lysates were prepared. Each sample was also analysed for protein concentration. An appropriate volume of ice cold 1x RIPA buffer (Millipore, UK) was prepared. Cultured PSC were washed three times in ice cold PBS prior to removal with a cell-scraper. The cell suspension was then centrifuged to obtain a pellet. The cell pellets were then re-suspended in an equal volume of 1 x RIPA lysis buffer containing protease inhibitor cocktail at 1:100 (Sigma, UK). The sample was then centrifuged for 20 minutes at 4°C at 10000rpm and the supernate containing protein was retained. Samples were then assayed to establish protein concentration and aliquoted for storage at -20°C to keep freeze-thawing to a minimum.

2.4.2 Measurement of protein concentration in cell extracts

Protein concentration of cell extracts was determined using the bicinchoninic acid (BCA) assay (Sigma, UK). Proteins reduce alkaline Cu (II) to Cu (I) in a concentration dependent manner (Lowry, OH. 1951). Bicinchoninic acid (BCA) is a highly specific chromogenic reagent for Cu (I) forming a purple complex with an maximum absorbance at 562 nm. Briefly , a protein standard curve was set up using bovine serum albumin 0-2000 g/ml in distilled water. Cell lysates required a 1 in 10 dilution to achieve concentration ranges detectable by the bicinchoninic acid assay kit. The protein samples were diluted with distilled water to a final volume of 25μl. The standards and working samples were set up in a 96-well plate in duplicate. The BCA Working

Reagent is prepared by mixing 50 parts of Reagent A(Bicinchoninic Acid Solution) with 1 part of Reagent B(Copper(II) Sulphate Pentahydrate 4% Solution). 200 µl of BCA working reagent was added to each well and the plate was then incubated at 37°C for 30 minutes. Absorbance was measured at 540 nm. A standard curve was plotted and the concentrations of the protein in the samples were then determined from the standard curve equation which was obtained using linear regression in Microsoft Excel.

2.4.3 Western blotting technique

2.4.4 SDS-PAGE Gel Electrophoresis

All reagents were provided by Invitrogen, UK. 10 µg of protein was loaded for each sample. To ensure equal quantities of protein were used for each sample, cell lysates were assayed for protein concentration as described. For each protein sample, NuPage LDS sample buffer was used and made up as fo llows:

Reagent	Reduced sample	Non-reduced sample
Sample(10ug of protein)	X µL	X µL
NuPage sample buffer(4X)	2.5 µL	2.5 µL
Nu Page reducing agent(10x)	1.0 µL	--
Deionised water	Up to 6.5µL	Up to 7.5 µL
Total volume	10 µL	10 µL

Samples were heated at 70^{o}C for 10 minutes and then centrifuged briefly at 10,000 rpm. Samples were then loaded into wells of a precast Bis-tris 4-12% gel(Novex Nupage, Invitrogen) and run with pre-prepared 1X MES/SDS running buffer (+ anti-oxidant for reduced samples) on

an Invitrogen XCell SureLock Mini-Cell system with 5μL of MultiMark (Invitrogen) and/or 2.5μL of MagicMarker XP Western protein standard (Invitrogen). Electrophoresis was performed at 200 V constant for 35 minutes.

2.4.5 Western transfer

Samples were transferred onto PVDF using the Invitrogen XCell II Blot Module. 1xtransfer buffer plus methanol was prepared according to the manufacturer's instructions and anti-oxidant added for reduced samples. 2 pieces of high-grade filter paper and cassette sponges (Invitrogen) were soaked in transfer buffer. The PVDF membrane (Hybond) was cut to size and was soaked for 15 seconds in methanol. A "sandwich" was then assembled as follows: pre-soaked filter paper was placed on top of 2 sponges, followed by the PVDF membrane, then by the gel and lastly 1 more pre-soaked filter paper and 2 further sponges. The transfer step was then performed at 30V constant for 1 hour. The membrane was the carefully removed. A successful transfer was verified by observation of the coloured ladder on the membrane.

2.4.6 Blocking and detection

After successful transfer, the membrane was placed in plastic box with the protein facing upward and blocked in 10ml of ECL advance Blocking agent (ECL, Amersham, UK) for 1 hour at room temperature or 4^{o}C overnight. Blocking solution was then decanted. 10ml of primary antibody (appropriately diluted with blocking solution and 0.05% Tween 20) was added and the tube placed onto a shaker and incubated for 1 hour at room temperature (IGF-1R; rabbit polyclonal Ab (Santa Cruz sc-), 1:200, Ins -R; rabbit polyclonal Ab (Santa Cruz sc-) 1:200. The solution was again decanted. The membrane was briefly washed twice with wash buffer(1x TBS/0.05% Tween 20), and then 15ml of wash buffer was added to the membrane and placed on the shaker at room temperature for 15 minutes. Three further washes with wash buffer were repeated for 5 minutes each. The secondary HRP-linked antibody, goat anti-rabbit secondary,(GE Healthcare, UK) 1:2000 dilution) was diluted appropriately with blocking solution with 1xTBS/0.05% Tween 20 and incubated with the PVDF membrane for 1 hour at room temperature on a shaker. This solution was discarded and an identical series of washes was performed to that after the primary antibody step. The advanced ECL detection kit (Amersham) was removed from the fridge and allowed to equilibrate to room temperature. The

chemiluminescent substrate was then applied to the membrane and left in the dark for 10 minutes. Reactive bands were identified using the Versadoc imager (BioRad).

2.4.7 Immunohistochemical techniques

Archival tissue blocks of human chronic pancreatitis, pancreatic cancer and normal pancreas were used. All tissue samples were fixed in formalin and embedded in paraffin. Paraffin blocks were cut into 5μm sections, and consecutive sections were stained serially for Sirius red, α-SMA, IGF-1 receptor or insulin receptor.

2.4.8 Sirius red staining

The following solutions were made :

Solution A: Sirius red F3B (C.I. 35782) 0.5g dissolved in saturated aqueous picric acid solution 500ml(with a little solid picric acid to ensure saturation)

Solution B- acidified water: Add 5ml acetic acid (glacial) to 1 litre of water (distilled)

Sections are deparaffinised and hydrated, and then stained in solution A for 1 hour. Sections are washed in 2 changes of solution B, and the slides are then dried by shaking prior to dehydration, clearing in xylene and then mounting with DPX.

2.4.9 Antigen Retrieval

Tissue sections were microwaved on full power (850W) for 10 minutes in 0.01 M tri-sodium citrate buffer, pH 6.

2.4.10 General immunohistochemical techniques

Antigen retrieval was followed by 3 x 5 minute wash steps in phosphate-buffered saline (PBS). 0.5% H_2O_2/ methanol was then applied for 30 minutes to block endogenous peroxidase activity. Sections were then washed again 3 x 5 minutes in PBS. Sections were blocked with 2.5% normal horse blocking serum for 30 minutes. The primary antibody was added 1:200 concentration and

incubated overnight at 4^{o}C. Non immune rabbit immunoglobulin was used in place of the primary antibody of interest, to control for non-specific staining. Care must be taken to prevent the slides from drying out. The slides were then rinsed with PBS 3 x 5 minutes. Then a secondary biotinylated antibody was applied for 2 hours at room temperature. The slides were then rinsed 3 x 5 minutes with PBS. Then the streptavidin-biotin solution was then applied for 30 minutes at room temperature. Slides were then rinsed twice with PBS (5 minutes each rinse). DAB chromagen substrate was incubated for 5-10 minutes until a colour change was observed. Stained slides were rinsed in tap water. A brief counterstain was performed using Meyers Haematoxylin (Gurr). Then the slides were rinsed with tap water for 5 minutes. The slides were dehydrated using graded alcohols; Ethanol 80% (1 minute), then Ethanol 95% twice (1 minute each) and finally Ethanol 100% twice (10 minutes each). Finally 3 washes with xylene (3 minutes each) as a clearing agent were applied, the excess xylene was wiped off and the slides were mounted using DPX mounting solution and a cover slip applied.

2.4.11 Enzyme linked immunosorbent assay (ELISA)

ELISA utilises the ability of antibodies or antigens to be absorbed onto solid surfaces such as plastic. ELISAs for TIMP-1 and IGF-1 were performed using a commercially available kit, DUOset(R and D Sytems, Abingdon, UK). The principal use a sandwich ELISA to measure natural and recombinant Tissue Inhibitor of Metalloproteinases 1 (TIMP-1) or IGF-1. Cell culture supernates were used in all experiments. In brief, the ELISA plate was prepared as follows. The capture antibody was diluted to the working concentration and 100μl was immediately coated onto a 96-well ELISA plate. The plates were then sealed and incubated overnight at room temperature. Each well was washed with 400 μl wash buffer, repeating the process two times for a total of three washes. Wash steps were performed by filling each well with wash buffer. Complete removal of liquid at each step is essential for good performance. After the final wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. Plates were then blocked by adding 300 μL of blocking agent specific to the kit used. This was then incubated at room temperature for a minimum of 1 hour. The wash steps described above were repeated.

Assay

100 μL of sample or standards in reagent diluents were added in duplicate to each well. The plate was then covered with an adhesive strip and incubated for 2 hours at room temperature. The aspiration and washing described above was repeated 3 times. Next 100 μL of the reconstituted detection antibody, diluted in reagent diluent was added to each well. The plates were then incubated for a further 2 hours at room temperature. Again the aspiration and washing steps were repeated 3 times. Then 100 μL of the working dilution of streptavidin-HRP was added to each well. The plates are then covered and kept at room temperature for 20 minutes. The plates were protected from light by wrapping in foil. The aspiration and wash steps were repeated 3 times. Finally 100 μL of substrate solution was added to each well and incubated for 20 minutes at room temperature. 50 μL stop solution was added to each well. The optical density of each well was determined immediately, using a microplate reader set to 450 nm with wavelength correction set to 540nm.

2.5 RNA methods

2.5.1 General Methods

Due to the susceptibility of RNA to degradation appropriate precautions were taken. All procedures were performed with gloves. All plasticware was DNase/RNase-free. All buffer solutions were made using DEPC water. DNase/RNase-free filter tips (Greiner) were used for all procedures.

2.5.2 RNA extraction with RNeasy Mini and Micro Kit (Qiagen)

First, 1μL of β-mercaptoethanol (β-ME) was added per 1ml of buffer RLT. Next, 4 volumes of 96-100% ethanol were added to buffer RPE for a working solution. Adherent PSC were then washed twice with ice cold PBS. 350 μl of buffer RLT containing the β-ME was added to the tissue culture plate. Further homogenisation was performed by adding the lysed cells in RLT buffer to a Qiashredder column (Qiagen) and centrifuging at room temperature at 10000 rpm for 2 minutes. Supernatants were then transferred to new microcentrifuge tubes by pipette. 350μl of 70% ethanol was added to the cleared lysates and mixed immediately by pipetting. Up to 700μl of sample, including any precipitate formed, was then transferred to an RNeasy mini

column placed in a 2ml collection tube. Samples were centrifuged for 15 seconds at 10000 rpm. The follow-through was discarded and collection tubes reused. If the volume exceeded 700µl, the excess was reloaded into the mini column and spun again as above. 700µl of buffer RW1 was added to each RNeasy column. Samples were centrifuged for 15 seconds at 10000 rpm. Follow-through and collection tube were discarded. RNeasy columns were transferred into a new 2ml collection tube. 500µl buffer RPE (containing ethanol) was pipetted into each RNeasy column. Samples were centrifuged for 15 seconds at 10000 rpm to wash the columns. Follow-through was discarded and the collection tubes reused. Another 500µl of buffer RPE was added to each RNeasy column. Samples were centrifuged for 2 minutes at 10000 rpm to dry the RNeasy silica-gel membranes of ethanol.

RNeasy columns were transferred to new 1.5ml collection tubes. 10-20µl of RNase free water was pipetted directly onto silica membranes. Samples were finally centrifuged for 1 minute at 10,000 rpm to elute RNA.

2.5.3 Assessment of RNA quality

The quantity and quality of extracted RNA was assessed using the Nanodrop ND-1000 Spectrophotometer. 1µl of sample was taken and a reading comparing 260/280 ratio was taken. Absorbance at 260nm was measured to quantify RNA. Purity of the RNA was assessed by the 260nm/280nm ratio (RNA / Protein ratio). A ratio of greater than 1.8 was considered to be satisfactory in purity for the RNA to be used in further analysis. Lower values indicate the presence of proteins or residual organic contamination.

2.5.4 Preparation of cDNA from total RNA - Reverse Transcription

The first strand cDNA synthesis was undertaken using Precision Reverse Transcription Kit (Primer Design, Southampton, UK). Briefly, template RNA was thawed on ice, along with other reagents, then vortexed and centrifuged.

Annealing Step

For each RNA sample the following reagents were added to a thin walled 500 µL tube;

RNA template (recommended 50ng-2ug)	X µl
RT primer	1.0 µl
dNTP mix 10mM	1.0 µl
RNAse/DNAse free water	X µl
Total	10 µl

Each sample was then heated for 5 minutes at 65^{o}C using a thermostatically controlled “hot block”. The samples were then removed and immediately placed onto ice.

Extension step

For each sample the following reaction was made up.

MMLV 5Xbuffer	4.0 µl
RNA/DNAase-free water	5.2 µl
MMLV enzyme	0.8 µl
Final volume	10 µl

10 µL was added to each sample, and the tubes were mixed by brief vortexing. This was followed by a further incubation at 42°C for 60 minutes. cDNA samples were then stored at -20ºC until required.

2.5.5 Determination of messenger RNA using TaqMan real time quantitative PCR

2.5.5.1 General principles of Taqman PCR technique

The Taqman technique uses primers and probes that are designed to detect a specific target region in the cDNA of interest. The Taqman probe is labelled with a quenching molecule and a fluorescent molecule. Cleavage of the annealed probe by Taq polymerase results in a loss of quenching, and an increase in fluorescence signal. Normal PCR products are formed leading to an accumulation of cleaved probe during each cycle (see figure). After each thermocycle the fluorescence signal increases log linearly and reaches a threshold (ΔRn) that is set to be the same for the gene of interest and the reference gene. The threshold cycle (C_T) is the number of PCR cycles after which there is a detectable fluorescent signal from the reaction tube and this is directly related to the starting quantity of cDNA.

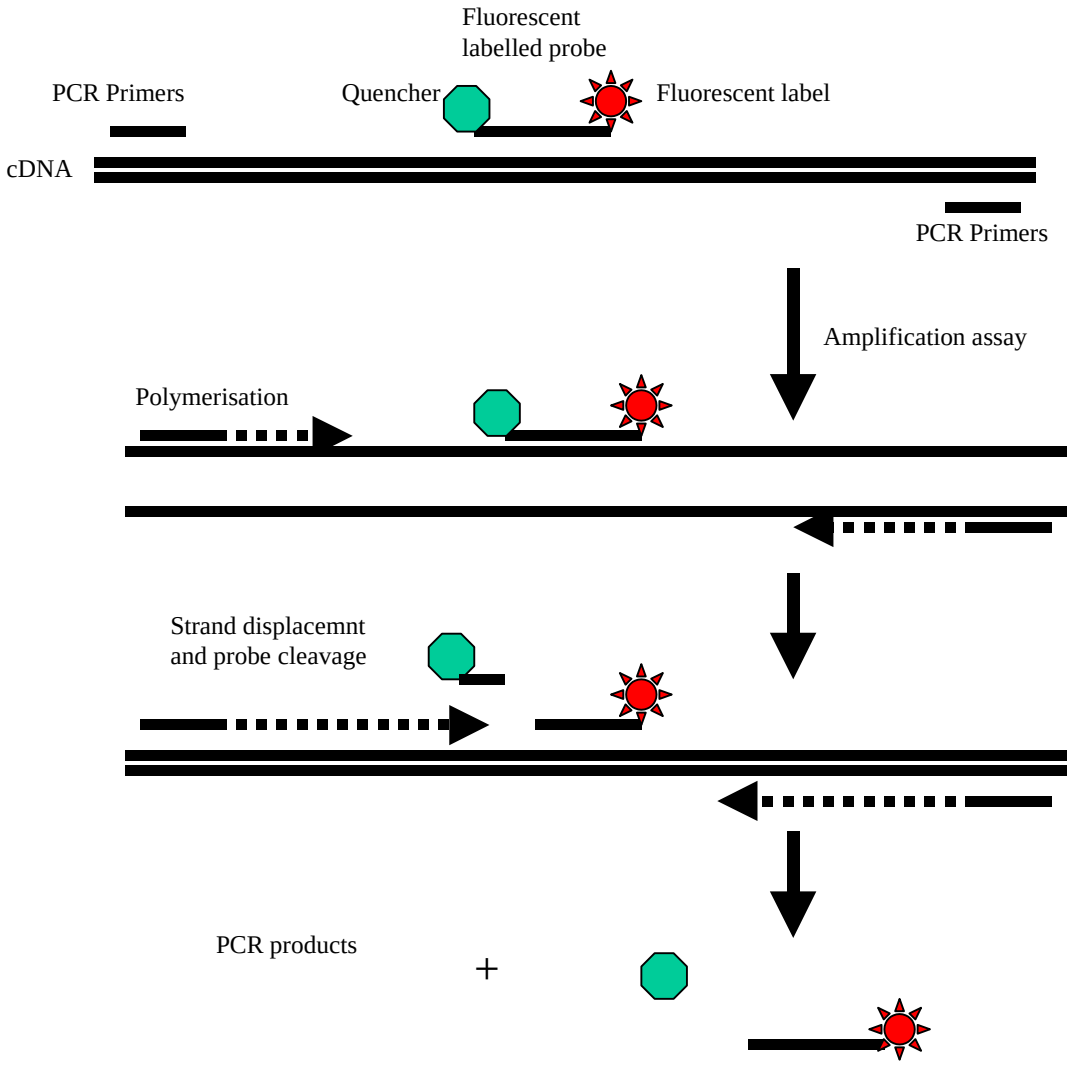


Figure 2-2 Principles of Taqman PCR

To quantitate the amount of cDNA in a sample from data generated by real-time PCR a number of methods are available. However in many cases the key information is the change in gene expression, hence cDNA copy number, in cells or tissue after an experimental manipulation. In this situation expression of a target gene after an experimental treatment is compared with expression of the same gene under control conditions, i.e.:

$^{\Delta C}{}_{T}$value for gene of interest= Ct(gene of interest) -Ct(reference gene).

An ideal reference gene should be expressed at the same level in all cells and its expression should not be influenced by the experimental manipulation being examined.

2.5.5.2 Arithmetic formula for relative RNA quantification

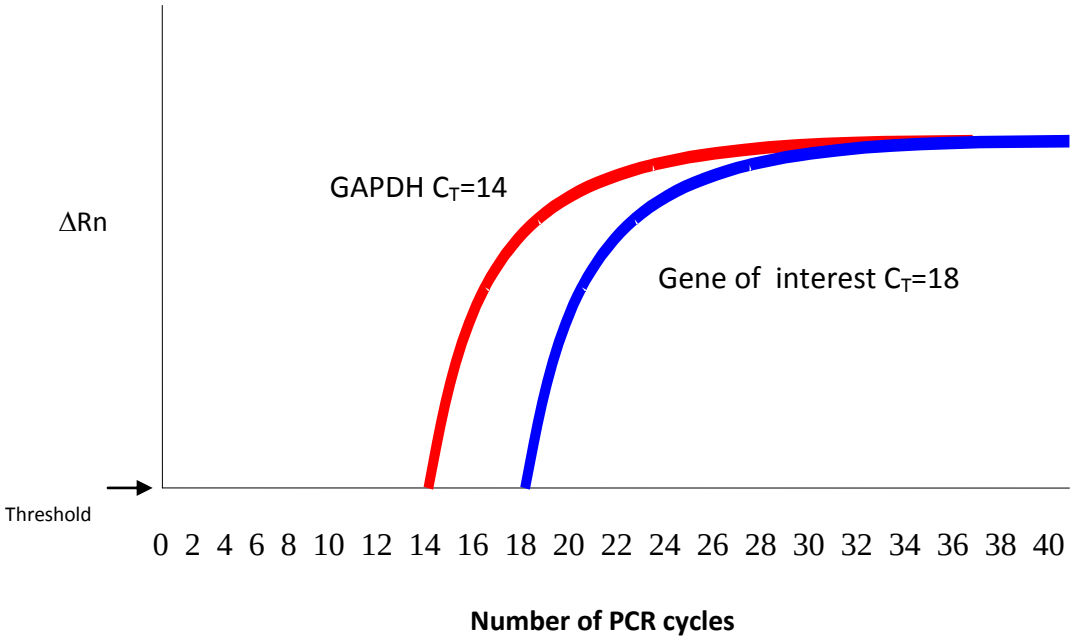


Figure 2-3 arithmetic formula for RNA quantification

The example above will be used to explain relative RNA quantification by Taqman PCR, by the technique known as Comparative C_T method(Applied Biosystems User Bulletin No 2 P/N 4303859, 1997, Livaka & Schmittgen 2001). The threshold cycle (C_T) for the reaction with the primers designed to recognise GAPDH is 14, the threshold cycle for the reaction with the primers designed to recognise the gene of interest is 18. Assuming the PCR reaction for both amplicon occurs with efficiency close to 100%, the amount of target RNA, normalised to the endogenous reference is given by:

$2^{-\Delta\Delta C_T}$

Where:

$^{\Delta\Delta C}{}_T = {}^{\Delta C}{}_T$ (gene of interest under experimental condition)-$^{\Delta C}{}_T$(gene of interest under control conditions)

For example if C_T GAPDH =14 and C_T Gene of interest =18

$^{\Delta\Delta C}{}_T$ = 14 - 18 = -4

Amount of target relative to GAPDH = $2^{-(-4)}$ = 16

In the above example there is 16 fold more GAPDH signal compared to the gene of interest. For parallel samples, for example at different time points, the relative differences in the gene of interest from two different RNA samples may be compared by comparing their amount of target relative to GAPDH.

2.5.6 Primer sequences

For determination of the expression of IGF-1 receptor, IGF-1 and insulin receptor in rat samples, primers and probes were purchased from Primer Design with a FAM labelled probe. Each was designed and manufactured by Primer Design, based on accession numbers from public databases (See Appendix). The housekeeping genes GAPDH and B Actin were supplied by PrimerDesign.

2.5.7 TaqMan real-time PCR protocol

DNA amplification was performed using Taq DNA polymerase for semi-quantitative PCR comparing the levels of particular gene mRNA in different samples. Taqman style double-dye primer and probes (PrimerDesign, Southampton, UK) were used for all reactions. Briefly, the PCR reactions were set up in the following manner. Lyophilised primer and probe mix was resuspended in RNAse/DNAse free water. A mastermix for each reaction was made up as follows.

Resuspended primer/probe mix	5 µl
Primer Design 2X PrecisionTM Mastermix	5 µl
RNAse/DNAse free water	5 µl

Final volume	15 µl

15µl of this mastermix was used for each real-time PCR reaction. 5 ?L of 1:10 diluted cDNA was also added to each reaction. The final volume in each well should be 20 µL. The following negative control wells were also performed to exclude contamination. cDNA was replaced with RNAse/DNAse free water. Any amplification in this sample is indicative of cDNA cross contamination between wells, or contamination of one or more reagents. Wells where the equivalent concentration of RNA is added, minus the reverse transcription step are also analysed. Amplification of these wells may indicate genomic DNA contamination of the RNA sample

Amplification conditions using Primer Design 2X PrecisionTM MasterMix.

Standard Taqman® cycling conditions

	Step	Time	Temp
	Enzyme activation	10min	95ºC
Cycling X50	Denaturation	15s	95ºC
	Data collection*	60s	60ºC

*Flurogenic data was collect during this step through the FAM channel. Determination of the expression of the house keeping genes, GAPDH and B Actin were employed and all reactions were undertaken in triplicate. After detection of the threshold cycle for each mRNA in each sample, relative concentrations were calculated and normalised to the average CT value of GAPDH and β Actin.

Agarose gel electrophoresis

PCR reactions were loaded onto a 1 or 2% agarose gel containing ethidium bromide and separated for 45-60 minutes by electrophoresis at 80V, along with a 100 base pair ladder (Promega) to allow estimation of the fragment size. DNA was visualised under UV light and a photographic record taken.

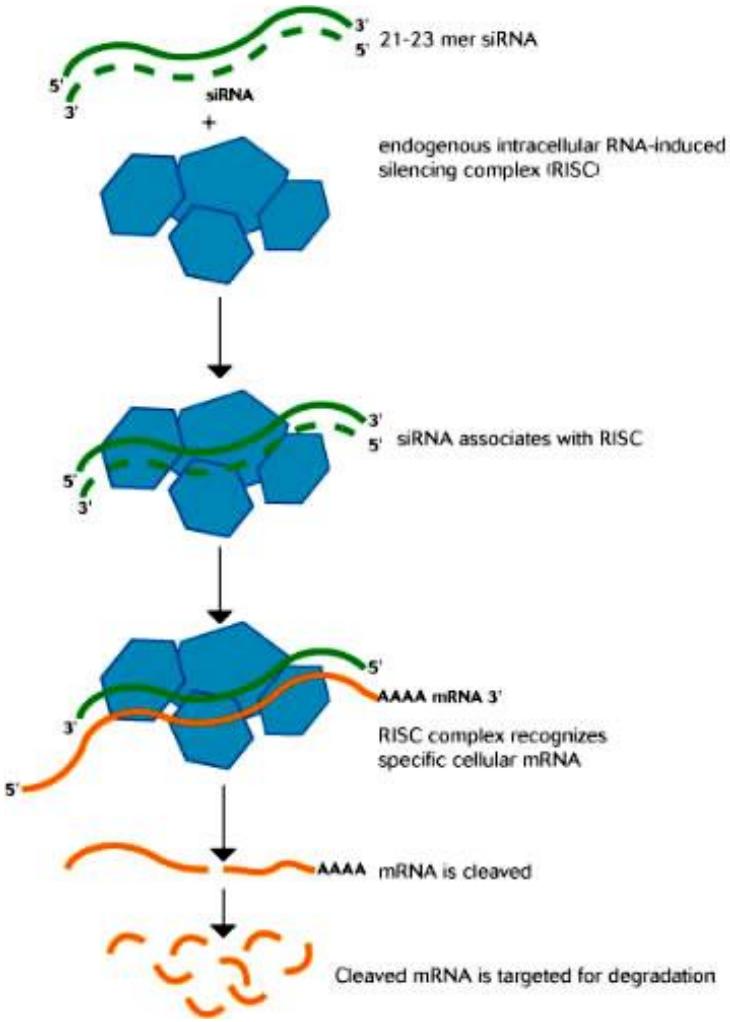
2.6 Silencing by small interference RNA (siRNA)

RNA interference (RNAi) has been proven to be a powerful method of knocking down gene activity by the introduction of a long double-stranded RNA (dsRNA) molecule in invertebrates such as Drosophila and C. elegans. Double stranded RNA is introduced either by chemical transfection using a detergent to temporarily alter the phospholipid cell membrane, or by electroporation using electric currents across the cell membrane which momentarily expands the cell membrane allowing RNA through. However, in most mammalian cells the introduction of long dsRNA either by chemical transfection or electroporation initiates a cellular interferon response that ultimately causes cell shutdown and leads to apoptosis.

This technique exploits a highly conserved evolutionary mechanism designed to protect the mammal from viral infection. In mammalian cells the introduction of short sequence-specific RNA duplexes that are 21-23 nucleotides long may be able to initiate post-transcriptional gene knockdown and avoid triggering this non-specific effect.

The RNAi process occurs in eukaryotes by the cleavage of long double-stranded RNA (dsRNA) into 21-23 nucleotide short (or small) interfering RNA (siRNA) duplexes, facilitated by an enzyme called Dicer. The siRNA associates with an intracellular multi-protein RNA induced silencing complex (RISC). This complex recognises and cleaves mRNA using the sense siRNA strand as a guide to target, cleave and degrade complementary cellular mRNA with the same sequence, ultimately leading to knock down of post-transcriptional gene expression in the cell.

Figure:2-4 An overview of siRNA mechanism



(Taken from Invitrogen catalogue)

Transfecting Primary pancreatic stellate cells with TIMP-1 siRNA using electroporation

Previous experience in our laboratory of using electroporation to introduce DNA into HSC resulted in low success rates. HSC are relatively fragile cells and the large voltages involved have previously reduced cell viability. Electroporation protocols with the use of siRNA have been optimised with hepatic stellate cells by Dr Aqeel Jamil and Dr Andrew Fowell. Voltages and currents required for RNA introduction were found to be lower when using the Ambion siPORT

electroporation buffer. Also rates of transfection were greater than with chemical transfection techniques.

Electroporation works by expanding the cell membrane for a few milliseconds. If the cells are suspended in PBS this would normally result in cytoplasmic solutes escaping out of the cell down a concentration gradient into the PBS. However, the buffer supplied by Ambion closely resembles cell cytoplasm in its constitution and therefore maintains cells viability by avoiding large shifts in solutes down a concentration gradient.

PSCs were selected before third passage and electroporated following a protocol optimised by Dr. Andrew Fowell. PSCs were cultured in complete media containing 16% FCS for 24 hours. Media were removed from the adherent PSC monolayer and washed in HBSS without Ca^{2+}, subsequently cells were trypsinised as previously described. Cells were then pelleted before being resuspended in HBSS. The resuspended cells were counted and 200,000 cell aliquots were added to microfuge tubes. These were then centrifuged and the supernatant discarded. The pellets were resuspended in 200µL of electroporation buffer (Ambion cat no 16704) and transferred to 4mm electroporation cuvettes (Biorad cat 165-2081). Cells were then treated with either 1.25µM rat TIMP-1 siRNA (Ambion), or 1.25µM negative control siRNA, reported to have no silencing effect on any constitutive gene. Controls included electroporation in the absence of siRNA, and an untreated group. The cells were electroporated using the BTX ModelL:ECM 830, using a single square wave pulse at an optimised voltage of 800V and a pulse length of 300 ms. Cells were then incubated in 16% FCS DMEM but without antibiotics which can stress cells following transfection.

Cells were plated in duplicate in a 24 well plate, at density of 20,000cells/well. Cells were cultured in 500µl of 16% FCS complete media. Media were collected every 24 hours for TIMP-1 ELISA.

2.7 Assessing the cytotoxic effects of TIMP-1 Electroporation.

The cytotoxic effect of electroporation on pancreatic stellate cells with TIMP-1 siRNA and relevant control groups were quantified using a colorimetric assay of lactate dehydrogenase(LDH) released during cell lysis. (CytoTox 96, Promega USA). This assay is a colorimetric technique, used to determine the number of viable cells present. It uses the

bioreduction of an tetrazolium salt into a red formazan product. This is achieved by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The soluble product formed is measured by recording absorbance at 490nm with a 96-well plate reader.

Following electroporation cell supernatants were collected at 24 hours. Media were immediately replaced and cells were lysed by undergoing two freeze thaw cycles. Cells were incubated at -80°C for 30 minutes, and then at 37°C for thirty minutes. The cell lysate was then collected. All lysates and supernatants were then centrifuged at 10000 rpm at 4°C to remove particulate matter. This procedure was repeated at 48 hours.

50µl of each lysate and supernatant were placed in separate wells of a 96 well plate in triplicate. 50µl of 16% FCS MEM was added as a background control, and 50µl of positive control that was included in the assay kit (1µl of bovine heart LDH diluted in 5ml PBS and 1% BSA). 50µl of reconstituted substrate mix was added to each well. The plate was incubated at room temperature away from light for 30 minutes. After this time the reaction was stopped on addition of 50µl 1M acetic acid and the optical density was read immediately at 492nm. Readings were corrected for background media absorbance. Percentage cytotoxicity was calculated as the percentage of LDH as a result of experimental cell death in the supernatant divided by the total amount of LDH contained within the PSC from the same well.

$$\% \text{ Cytotoxicity} = \frac{\text{LDH in supernate}}{(\text{LDH in supernate} + \text{LDH in Lysate})} \times 100$$

2.8 Statistical Analysis

Results are presented as the mean +/- standard error of the mean (SEM). The means were compared using a paired Student T test, and a p value <0.05 was considered as statistically significant. All means obtained for experiments were obtained on different cell preparations.

3 Chapter 3

3.1 Pancreatic stellate cell and Characterisation

3.2 Introduction

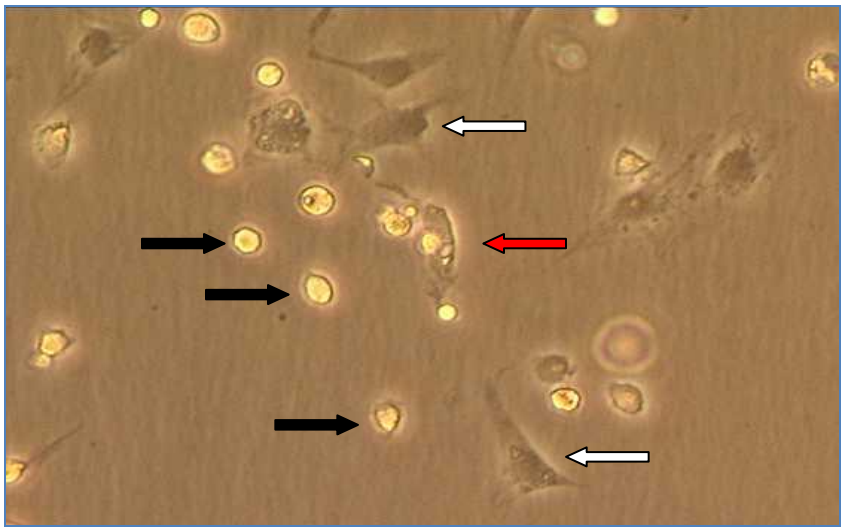
Pancreatic fibrosis is one of the main histological features of chronic pancreatitis, regardless of the underlying aetiology of the disease. It represents the final common pathological outcome despite the mode of injury(Kloppel, Detlefsen, & Feyerabend, 2004). Current evidence indicates that the central mediator of pancreatic fibrosis is the PSC. Following injury, these retinoid rich periacinar cells proliferate and undergo a phenotypic transformation to myofibroblast-like cells, a process termed activation (Apte et al., 1998; Bachem et al., 1998).

Quiescent pancreatic stellate cells have been isolated from both rodent and normal human pancreata. This technique is based on density centrifugation and the knowledge that quiescent PSCs contain abundant lipid droplets in the form of Vitamin A(Apte et al., 1998; Bachem et al., 1998). This property allows the cells to be readily separated from other pancreatic cells. Once placed in culture medium and grown on plastic tissue culture dishes these cells undergo a process of activation where they lose their retinoids and start to change morphologically into contractile myofibroblasts(Bachem et al., 1998; Apte et al., 1998).

In our laboratory the technique used for isolation of pancreatic stellate cells was first described by Dr David Fine and Dr Fiona Walker. This technique was further optimised by Dr Fanny Shek in 2002. The critical stages identified for the isolation of stellate cells include digestion, purification and dispersal. This technique gives a regular yield of between 1-2 million cells, and is based primarily on the technique used to isolate hepatic stellate cells in the laboratory(Issa et al., 2001). The stellate cell preparation would be placed into a T75 flask with cells routinely reaching 70-80% confluence in 7-10 days.

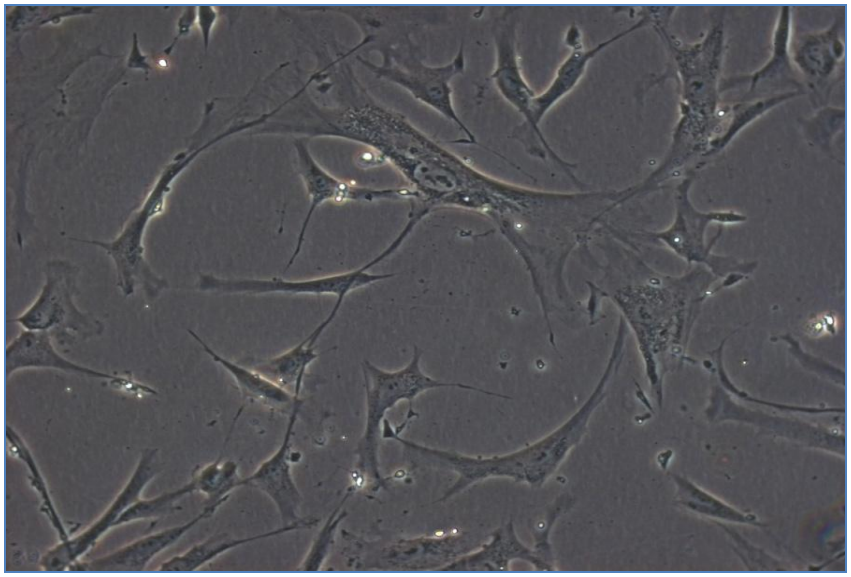
The yields obtained using this protocol are slightly lower than other groups who use an alternative protocol, obtaining approximately 3-4 million cells per preparation(Apte, 2011). The principal of this method still relies on density centrifugation.

Figure 3-1 Activated Rat Pancreatic stellate cells after 48 hrs plating on tissue culture plastic



Phase contrast microscopy demonstrating quiescent rat pancreatic stellate cell containing cytoplasmic vitamin A(black arrows) after 48 hrs. Subsequent loss of cytoplasmic vitamin A and transformation to a myofibroblastic phenotype (white arrows) with a flattened and angular appearance. The red arrow indicates a cell in the process of activation (x20 objective lens).

Figure 3-2 Human Pancreatic stellate cells 48 hrs plating on tissue culture plastic

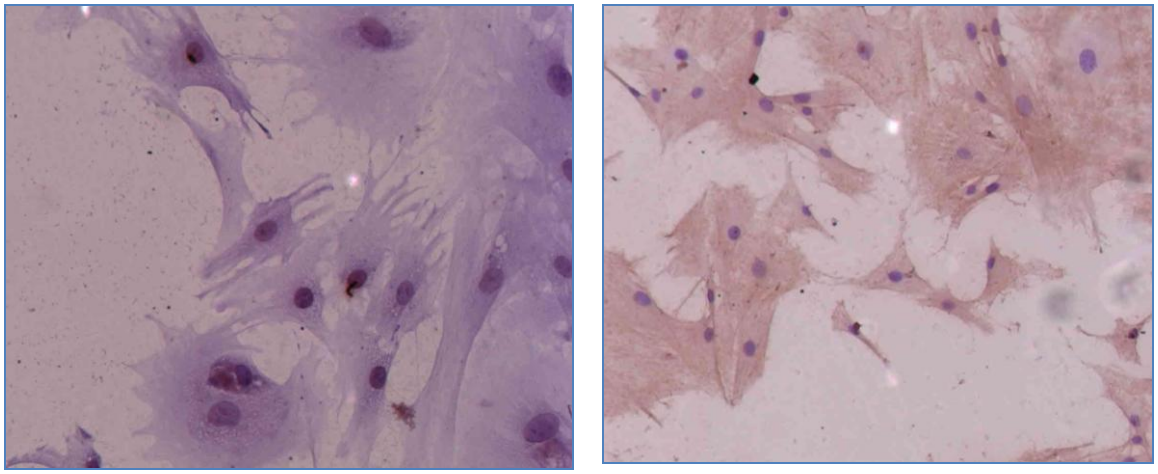


Phase contrast microscopy activated human pancreatic stellate cells after 48 hrs(x20 objective lens)

The normal transformation of quiescent vitamin A containing cells, to that of the activated phenotype was observed in all cells used.

3.3 Activated pancreatic stellate cells characteristically express α-smooth muscle actin (α-SMA)

Figure 3-3 Rat PSC stained for α-SMA

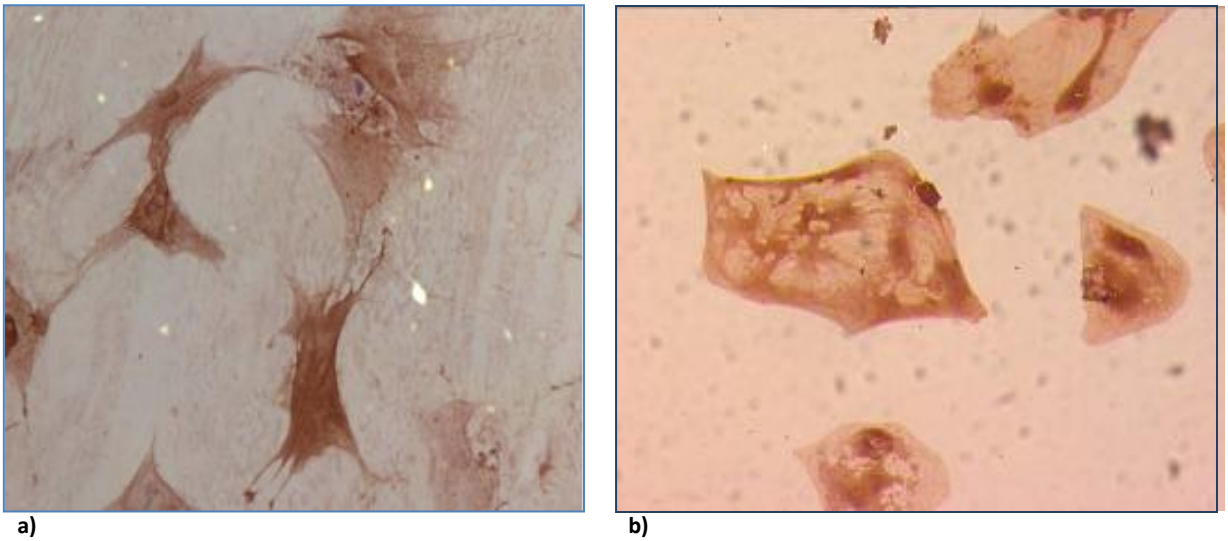


a) **b)**

Activated cultured rat PSC immunostained and photographed with x100 magnification a)negative control b)α-sma as a marker of activation

Activated pancreatic stellate cells expressed α-sma the characteristic marker of myofibroblasts. Cells isolated with density centrifugation were cultured and passaged between 1-3 times for use in these experiments.

Figure 3-4 Activated PSC undergoing change of phenotype with repeat passage



a) **b)**

PSCs and photographed x 100 magnification. Activated pancreatic stellate cells express α-SMA a) Usual PSC morphology p3 b) PSC morphology following serial passage, p5 cells. After multiple passages cells adopt atypical morphology, but continue to express α-SMA.

After a number of passages, isolated cells adopted atypical morphologies when examined under the microscope. These were stained for α-SMA to check for cell population contamination. The figure demonstrates that there is a significant change in the morphology of stellate cells on repeated passage. For this reason the cells were discarded beyond passage 4.

Observations of cells suggested that cells of higher passage number greater than 4 undergo a process consistent with senescence, and appear to resist apoptosis. These observed changes in morphology were not formally studied but is consistent with the past experience of Dr Shek and Dr Fine and others(Masamune et al., 2003; Jesnowski et al., 2005; Satoh et al., 2002)

3.4 Discussion

It is known that in chronic pancreatitis and pancreatic cancer, persistent activation of pancreatic stellate cells converts the wound healing response into a pathological process resulting in organ fibrosis (Kloppel et al., 2004).

In vitro research using pancreatic stellate cells remains inconsistent, with recent studies being performed on various pancreatic stellate cell lines. At the time this work was being undertaken, these were not readily available. Additionally, using immortalised cell lines to study apoptosis would be counterintuitive and potentially yield misleading data as those cells have been modified to resist or disable apoptotic mechanisms. It was felt that it was important to study cells that were isolated as quiescent cells, representing cells *in vivo* as closely as possible. This would enable more reliable assessment of their phenotypic responses to stimuli. The aim was also to perform these experiments with freshly isolated stellate cells from normal pancreas to ensure consistency of experimental data.

A considerable amount of time was spent isolating pancreatic stellate cells, which was a crucial element of this project. The technique for isolating quiescent human pancreatic stellate cells in these experiments was adapted from experience using rat pancreas, and is identical to the techniques described by Dr Shek. However, the results of human stellate cell isolation using density centrifugation was highly variable. Whilst pancreatic cancer resection specimens and chronic pancreatitis specimens were readily available, the tissue was not readily amenable to enzyme digestion, because of the intense fibrotic reaction within the specimens. This limited their use significantly. Yields were extremely variable and bacterial infection was also commonly encountered, as these specimens are not handled in a sterile manor prior to arrival in the laboratory. In view of these technical difficulties, most of the experiments were performed using rat pancreatic stellate cells, and limited confirmatory experiments were performed with human cells.

Other techniques such as “outgrowth”, using blocks of pancreatic tissue removed from the edge of resection specimens (Bachem et al., 1998), did not yield reliable results and again it was felt that cells isolated in this fashion may have undergone a high degree of differentiation and studies on apoptosis may not have been consistent. Observations also suggested a highly variable cell population of cells cultured in this manner. There has been minimal data published to date on the use of normal human pancreatic stellate cells, and this is likely a reflection of the scarcity of such specimens (Apte, 2011). Also, the techniques used to isolate these cells, have varied between groups, and it is likely that those groups which have the strongest track record of isolating these cells from normal rats will have the highest yields from normal human pancreatic specimens.

In fact it is only recently that methods have been published on the isolation of normal human pancreatic resection specimen, excluding chronic pancreatitis and pancreatic cancer (Apte, 2011; Vonlaufen et al., 2010). The Sydney group, who are leaders in this area, speculate that there are a few key areas that are likely to contribute to increased stellate cell yield from the normal rat pancreas(Apte, 2011). These include decapitation of rodents to ensure adequate exsanguination, thereby reducing congestion of the pancreas with subsequent injection of enzyme solution into the pancreas to aid tissue digestion by proteases. These expert insights are most likely to contribute to the more consistent yields that are to be obtained whilst harvesting pancreatic stellate cells from normal human pancreas. Useful pointers identified for isolating human PSCs, included trimming pancreatic specimens of excess adipose and connective tissue and adjusting the enzyme concentration to the weight of the piece of tissue. This was to ensure that the tissue is not over digested. Injection with enzyme solution was also routinely performed in this step. Despite these techniques, experience remains variable when isolating human pancreatic stellate cells, in contrast to the regular yields obtained from isolation of rat PSCs. Reasons for this may include; the way in which tissue was harvested during surgery, and the processing time of specimens following ligation from the blood supply. Additionally, pancreata were obtained from a diverse group of individuals with different underlying pathology and co-morbidities. This contrasts to rat pancreata, which come from a more homogenous population of Sprague Dawley rats.

It was observed that unlike rat PSCs, which attach to culture flasks within 8-12 hours, it could take between 8 hours and 4 days. It is also noted from the methodology (Apte, 2011; Vonlaufen et al., 2010) that FBS was used 20% to maintain human stellate cells in culture, which is higher than that used in my experiments.

It appears that pancreatic stellate cells undergo a process of senescence, and stop proliferating with multiple passaging. Recently there has been interest in this area of senescence in pancreatic stellate cells (Fitzner et al., 2012). This group have analysed senescence as a novel mechanism which may be involved in the termination of PSC activation and tissue repair. Long term cultures were established *in vitro* by applying chemical triggers such as doxorubicin, H_2O_2 and staurosporine. Further, co-culture experiments identified that senescence increased the susceptibility of PSC to cytolysis by lymphocytes. It was concluded that inflammation, PSC activation and senescence are coupled processes which occur in the microenvironment of the

inflamed pancreas. It was speculated lymphocytes may have dual role in pancreatic fibrogenesis, triggering both the initiation of wound healing by activating PSC, and its completion by killing senescent stellate cells.

A broad range of surrogate markers has been established to characterise senescent cells. Two commonly used phenotypic markers are telomere shortening and expression of senescence-associated β-galactosidase (SA β-gal) (Krizhanovsky et al., 2008). Senescent hepatic stellate cells, display a typical flat polygonal morphology and overexpress genes encoding secreted proteins that can alter the tissue microenvironment; a feature termed senescence-associated secretory phenotype (Krizhanovsky et al., 2008). Other typical characteristics of senescent cells are associated with the process of chromatin remodelling and include the exhibition of senescence-associated heterochromatic foci, increased methylation of histone H3 lysine K9, and increased phosphorylation of histone H2AX on serine 139 (Krizhanovsky et al., 2008).

Although the process of cellular senescence utilises evolutionary conserved pathways, there are likely to be type-specific peculiarities. Additionally, not all markers are equally suitable to monitor cell ageing in each individual tissue. The ideal senescent markers in pancreatic stellate cells has yet to be determined and is currently an active field of research (Müller, Fitzner & Jaster, 2013). Therefore, it is essential to test multiple parameters to fully establish senescence in different types of cells.

It was felt that for the experiments that needed to be performed for this thesis, it was essential to continue to use pancreatic stellate cells that did not exhibit these features of senescence. Due to the precious nature of these human stellate cell preparations, they were routinely used in experiments between passage 1-4.

4 Chapter 4

4.1 Studies of TIMP-1 pancreatic stellate cell survival

4.2 Introduction

Previous work has demonstrated activated PSC express TIMPs- 1 and -2(Phillips et al., 2003b; Shek et al., 2002). This has led to the hypothesis in liver fibrosis that matrix degradation is inhibited during progressive fibrosis . This hypothesis is supported by findings that over expression of TIMP-1 enhances experimental fibrosis(Iredale et al., 1996; Benyon, Iredale, Goddard, Winwood, & Arthur, 1996; Arthur, Mann, & Iredale, 1998; Murphy et al., 2002 and that spontaneous recovery from liver fibrosis is associated with reduction in TIMP expression and an increase in collagenase activity with consequent matrix degradation (Iredale et al., 1998). Mechanistic studies using transgenic mice overexpressing human TIMP-1, have demonstrated failure of matrix degradation and spontaneous recovery in a CCl_4 model of liver fibrosis (Yoshiji et al., 2000 & 2002). Overexpression of TIMP-1 in mice was not associated with fibrosis in the absence of injury, demonstrating that TIMP-1 does not by itself result in liver fibrosis, but strongly promotes liver fibrosis development.

Additionally, it has been found that apoptosis of HSC occurred during recovery from liver fibrosis (Gong et al., 1998; Issa et al., 2001b; Iredale et al., 1998; Saile et al., 1997) . This has highlighted the control of stellate cell apoptosis as a key process regulating fibrosis in the liver, which may have particular relevance for pancreatic fibrosis following injury

TIMP-1 was originally identified as a growth factor for myeloid elements and has also been shown to promote fibroblast proliferation (Kikuchi, Kadono, Furue, & Tamaki, 1997; Docherty et al., 1985)

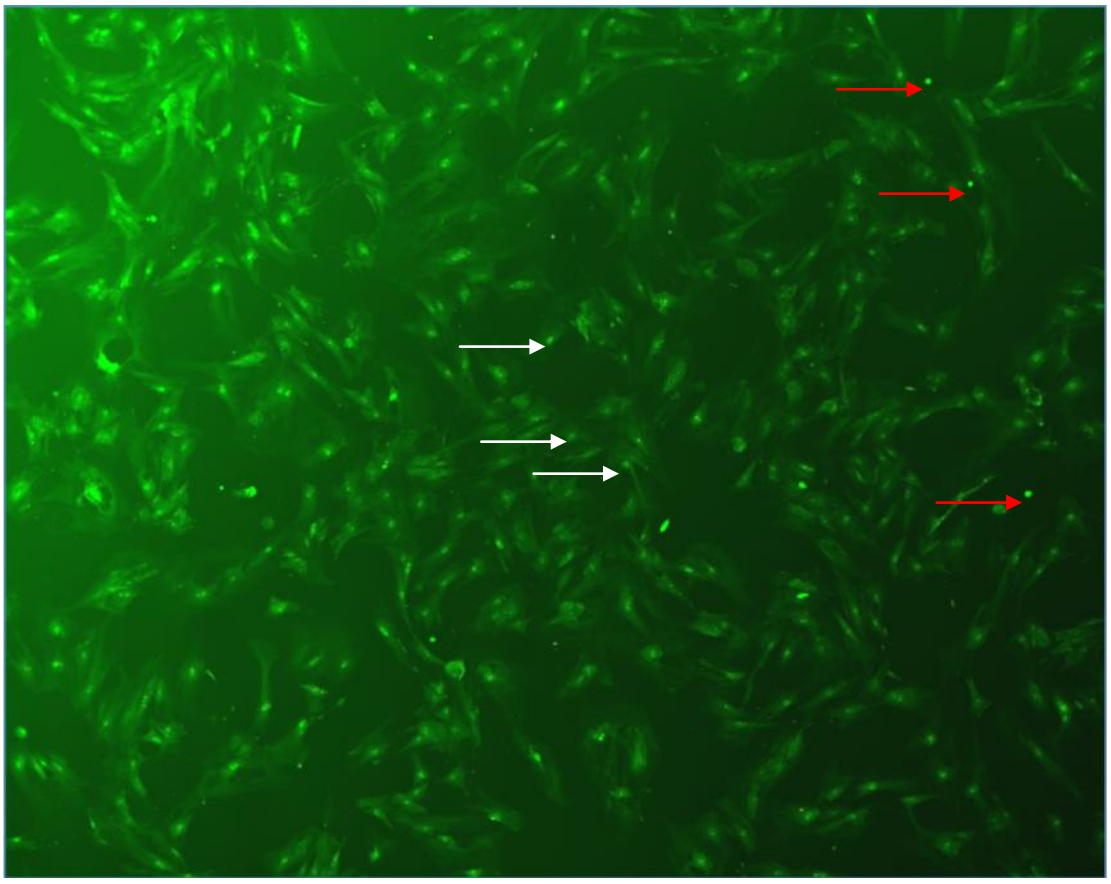
The TIMPs appear to have divergent effects on proliferation and apoptosis in different cell types. In HSC there is no apparent effect of TIMP-1 on HSC proliferation (Murphy et al., 2002b). Further interest in the mechanism of action of TIMP-1 in preventing resolution of fibrosis comes from the observation that in vitro, TIMP-1 was able to suppress apoptosis of human breast epithelial cells (Li, Fridman, & Kim, 1999) and Burkitt's lymphoma cell lines (Guedez et al., 1998).

The anti apoptotic effect of TIMP-1 was independent of its ability to inhibit MMP activity. These effects have subsequently been confirmed in models of liver fibrosis on HSC (Murphy et al., 2002; Murphy et al., 2004). These data suggest that TIMPs may be important regulators of cell growth and apoptosis.

4.3 In vitro studies of pancreatic stellate cell apoptosis

4.3.1 Acridine Orange staining for apoptotic index calculation

Figure 4-1: Acridine orange stained cells for counting

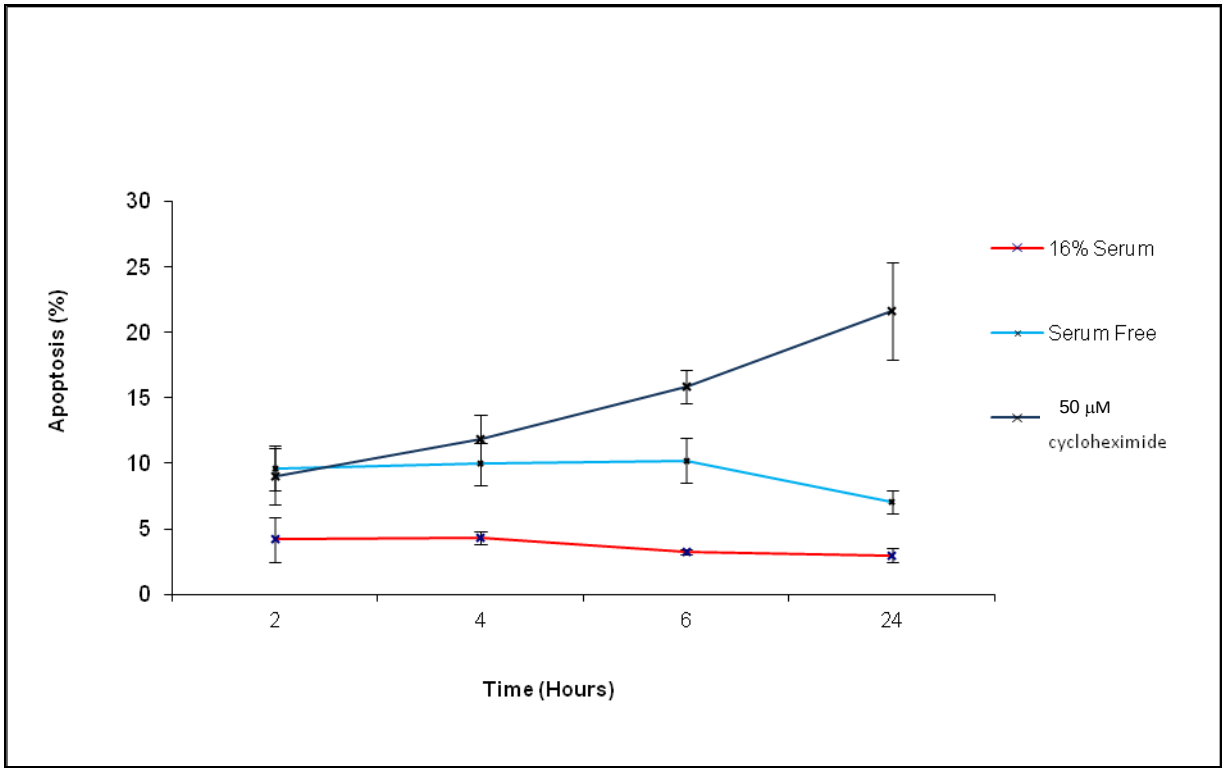


PSCs viewed under UV light (FITC filter). An example of a low power field(x 50) for calculating apoptotic index of activated PSC (50X). Red arrows indicate dense chromatin staining in apoptotic PSC. White arrows indicate normal nuclei of activated pancreatic stellate cells.

When cells undergo apoptosis certain morphological changes take place that are distinguishable from senescence or necrosis. Morphologically cell shrinkage, chromatin condensation, nuclear fragmentation and DNA degradation can be seen.

4.3.2 Determining the time course for studies of pancreatic stellate cell apoptosis following serum deprivation and induction of apoptosis with cycloheximide 50μM

Figure 4-2 Time course experiment for apoptosis experiments



A time course study was undertaken to determine the earliest time activated PSC showed apoptotic morphology after a pro apoptotic stimulus. Cells were exposed to 50μM cycloheximide after 24hrs of serum deprivation. After 4-6 hours, cycloheximide began to induce rat PSCs apoptosis. This effect peaked at 24 hours. At 6 and 24 hours cycloheximide induces apoptosis at levels higher than achieved using serum deprivation alone

Results show a background apoptosis of approximately 4% in cells cultured in serum across all four time points. Cycloheximide 50 μM induced the highest proportion of apoptosis, with the most pronounced effect seen at six and twenty four hours. From these results it was determined that cycloheximide would be used to induce a level of background apoptosis from which to assess any TIMP-1 effect , and that apoptosis would be assessed at 6 and 24 hrs.

All experiments were therefore performed after exposure to cycloheximide 50μM. This was to ensure that there was sufficient degree of apoptosis to be able to detect small changes in the apoptotic index on exposure to various growth factors and TIMPs.

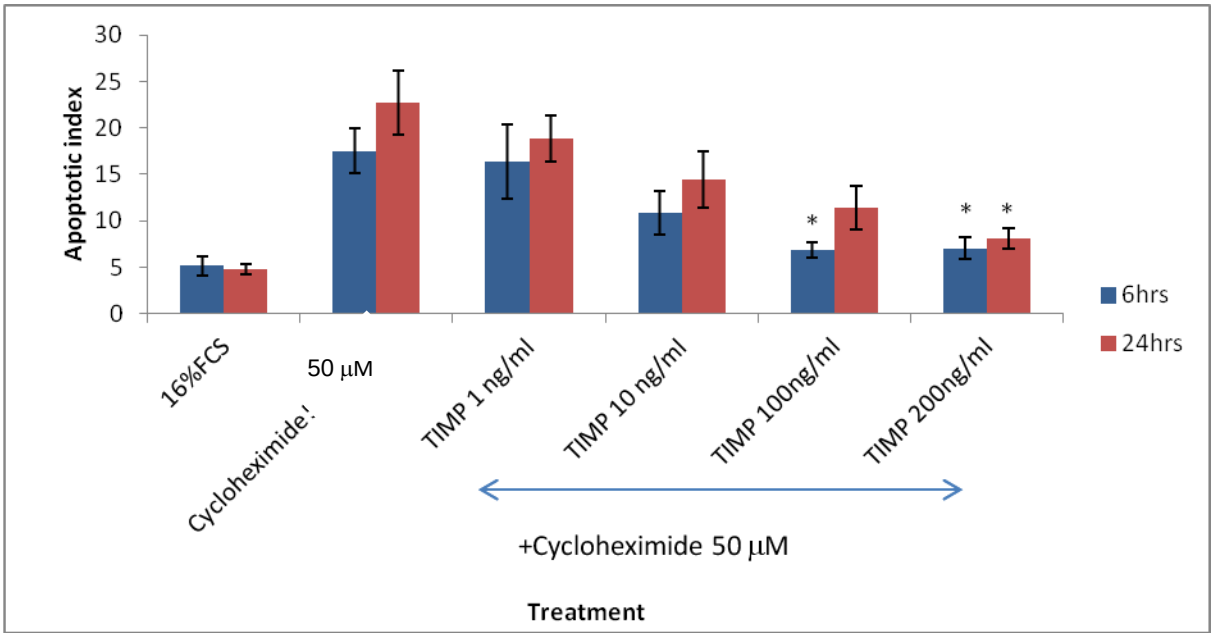
The effect of serum withdrawal suggested that growth factors are important regulators of PSC apoptosis in culture. To explore this concept further I undertook a series of experiments to examine the effect of the growth factors IGF-1, insulin, and TIMP-1 on PSC apoptosis.

4.4 In vitro studies of TIMP-1

To determine the effects of recombinant TIMP-1 on apoptosis, activated stellate cells were incubated with TIMP-1 (1-200ng/mg), following 24 hours of serum deprivation. They were subsequently exposed to cycloheximide 50 μM, including 0.1% bovine serum albumin which is used to as a carrier protein for TIMP-1.

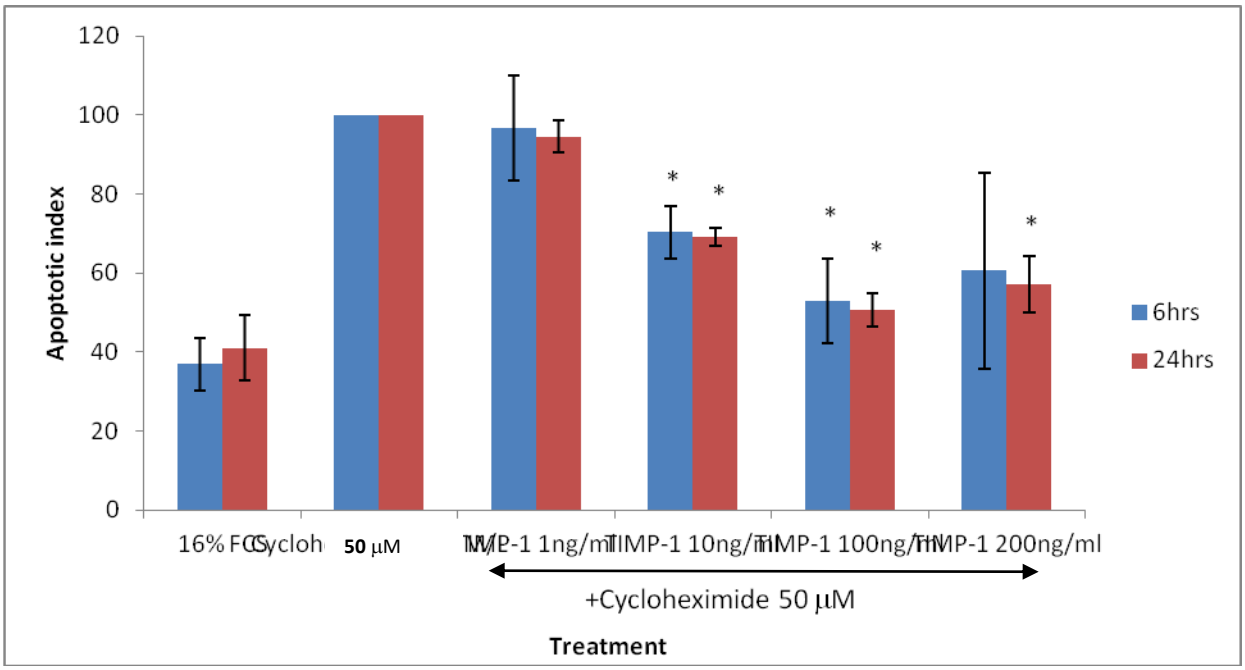
4.4.1 TIMP-1 inhibits apoptosis induced by cycloheximide

Figure 4-3:TIMP-1 significantly reduced apoptosis of activated rat pancreatic stellate cells



TIMP-1 significantly reduced apoptosis of activated rat pancreatic stellate induced by 6 and 24 hours cycloheximide exposure in a dose dependent manner over the concentration range 1-200ng/ml. Apoptosis was determined by acridine orange staining and counting. (Data presented are mean +/-SEM * p<0.05) for cycloheximide versus cycloheximide with 1- 200ng/ml TIMP-1 by Student's t-test, n=3)

Figure4-4 TIMP-1 significantly reduced apoptosis of activated human pancreatic stellate cells



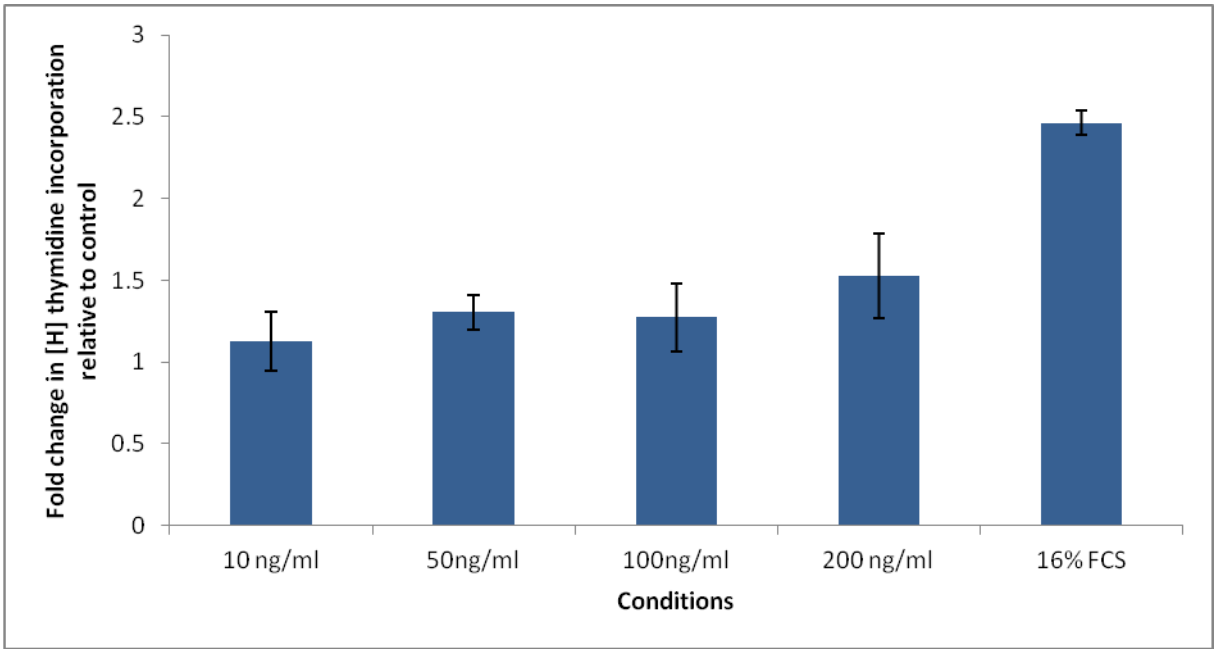
TIMP-1 significantly reduced apoptosis of activated human pancreatic stellate induced by 6 and 24 hours cycloheximide exposure in a dose dependent manner over the concentration range 1-200ng/ml. Apoptosis was determined by acridine orange staining and counting. (Data presented are mean +/-SEM * p<0.05) for cycloheximide versus cycloheximide with 1- 200ng/ml TIMP-1 by Student's t-test, n=3)

It was found that cells exposed to recombinant TIMP-1 had reduced levels of apoptosis at 6 and 24 hours compared with cycloheximide exposure alone. This was a consistent finding and occurred in a dose dependant manner. This occurred in both rat and human pancreatic stellate cells. The background apoptosis in human pancreatic stellate cells was significantly higher than that observed in rat PSC.

4.4.2 The effects of TIMP-1 on stellate cell proliferation

Other studies in different cell types have demonstrated a possible pro proliferative effect for TIMP-1 .This was therefore analysed in activated rat PSC cultured on plastic.

Figure 4-5:TIMP-1 has no effect on proliferation of activated rat pancreatic stellate cells



Rat activated PSC cultured on plastic were used to determine if TIMP-1 could change the rate of cellular proliferation. Proliferation was determined by the tritiated thymidine technique as described in the methods. TIMP-1 did not significantly increase the rate of proliferation in the concentration range 10-200ng/ml. (Data are expressed as mean+/- SEM, as normalised to control (serum free conditions), each condition was performed in duplicate, n=3, meaning three separate experiments using cells extracted from different animals)

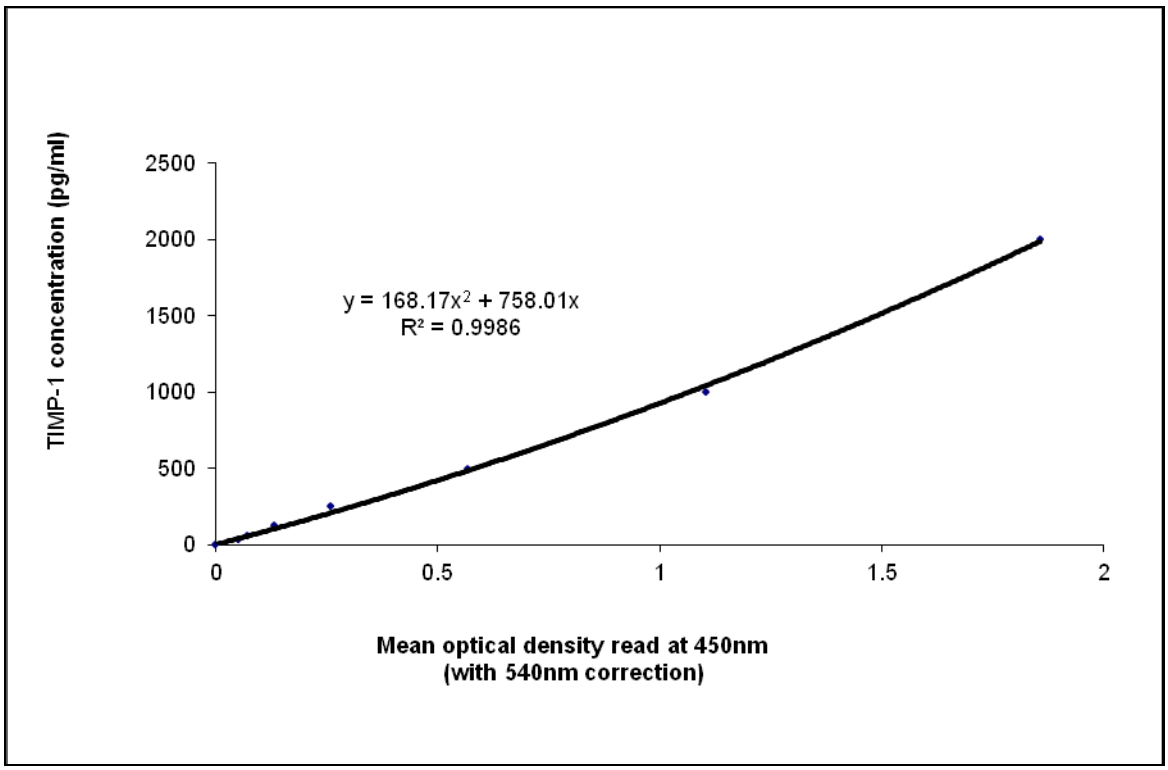
There was no evidence that TIMP-1 at concentrations of 10-200ng/ml had any effect on proliferation of rat PSC over a 24 hr incubation period. Serum containing 16% FCS was used as a positive control. Proliferation was quantified by the tritiated thymidine technique as described in the methods.

4.5 TIMP-1 ELISA

Pancreatic stellate cell are known to produce TIMP-1(Shek et al., 2002; Phillips et al., 2003a). R&D rat TIMP-1 DuoSet immunoassay was used to quantify TIMP-1 expression.

Determination of TIMP-1 concentration by ELISA using a standard curve.

Figure 4-6: Standard Curve produced by TIMP-1 ELISA (R&D DuoSet).



The standard curve was plotted using Microsoft Excel using polynomial regression.

A standard curve was produced by serial dilution of a known concentration of TIMP-1. Polynomial regression using Microsoft Excel was used to generate the standard curve. This equation was used to calculate the unknown TIMP-1 concentrations of the sample supernatants.

For subsequent experiments with RNA interference supernatants were collected every 24 hours, with replacement of complete media on each occasion. Samples were collected up to 96 hrs. Subsequently samples were examined by TIMP-1 ELISA.

4.6 Inhibition of TIMP-1 protein expression by electroporation with TIMP-1 siRNA

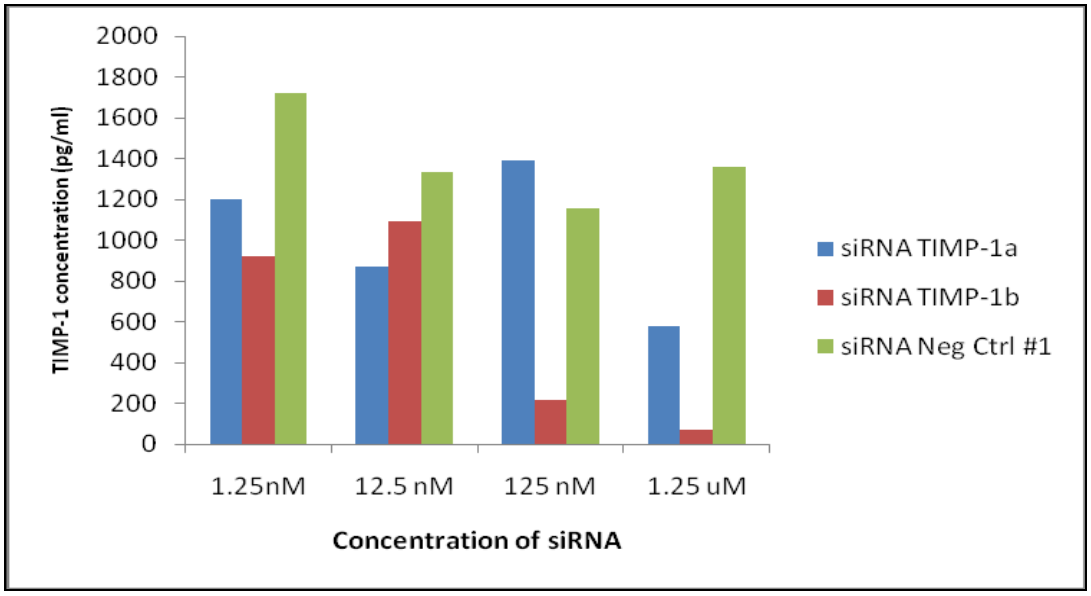
RNA interference (RNAi) involves the process of mRNA degradation, induced by double stranded RNA (dsRNA) in a sequence specific manner, and is a highly conserved mechanism of gene suppression found in plant, yeast and mammalian cells. It is thought to be an ancient mechanism for protecting the host and its genome against viruses and rogue genetic elements that use double stranded DNA (dsDNA) in their life cycles . In RNA silencing dsRNA is cleaved by the RNase III enzyme DICER into 21-24 nucleotide duplexes of siRNA . The antisense strand of siRNA is incorporated into the RNA-induced silencing complex (RISC) which then targets complementary mRNA sequences in the cytoplasm. mRNA is then cleaved by RNase at a site bound by siRNA, therefore preventing translation.

Extensive experience within the Liver and Pancreatic Research group was drawn upon to identify electroporation as a means to transfect cells with siRNA. It has been found by our group that traditional methods of siRNA transfection can prove problematic for transfection of hepatic stellate cells. Electroporation is the recommended technique for difficult to transfect primary cells. Due to the limitations of pancreatic stellate cell numbers, the techniques used were based on those optimised by Dr Andrew Fowell on hepatic stellate cells. Pancreatic stellate cells and hepatic stellate cells demonstrate a very similar morphology and this would appear to be a reasonable approach. Various electroporation parameters had already been optimised for hepatic stellate cells, including pulse number, pulse length, pulse voltage and concentration of siRNA. Identical parameters were applied for the electroporation of pancreatic stellate cells. Electroporation was performed as previously described in the methods.

Culture supernatants were harvested from pancreatic stellate cells that had undergone electroporation with TIMP-1 siRNA or negative control siRNA. The culture medium was collected every 24 hrs and replaced with fresh medium. The concentration of TIMP-1 was determined with the use of TIMP-1 ELISA kit.

4.6.1 Determination of the optimal concentration of siRNA for maximum silencing of TIMP-1 protein

Figure 4-7 Dose dependent inhibition of rat TIMP-1 protein

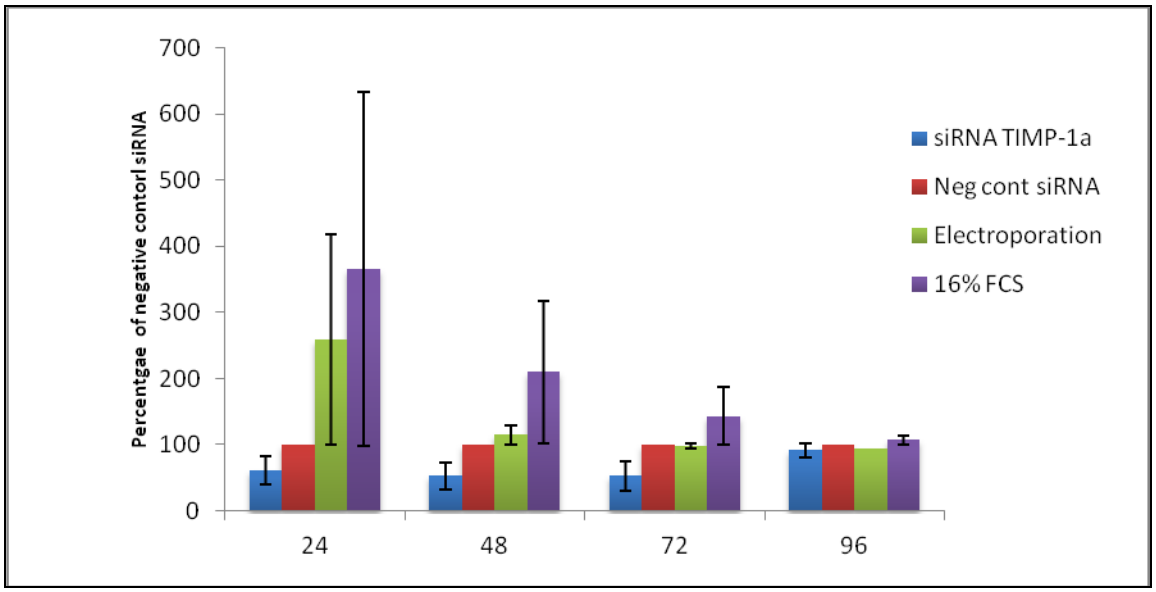


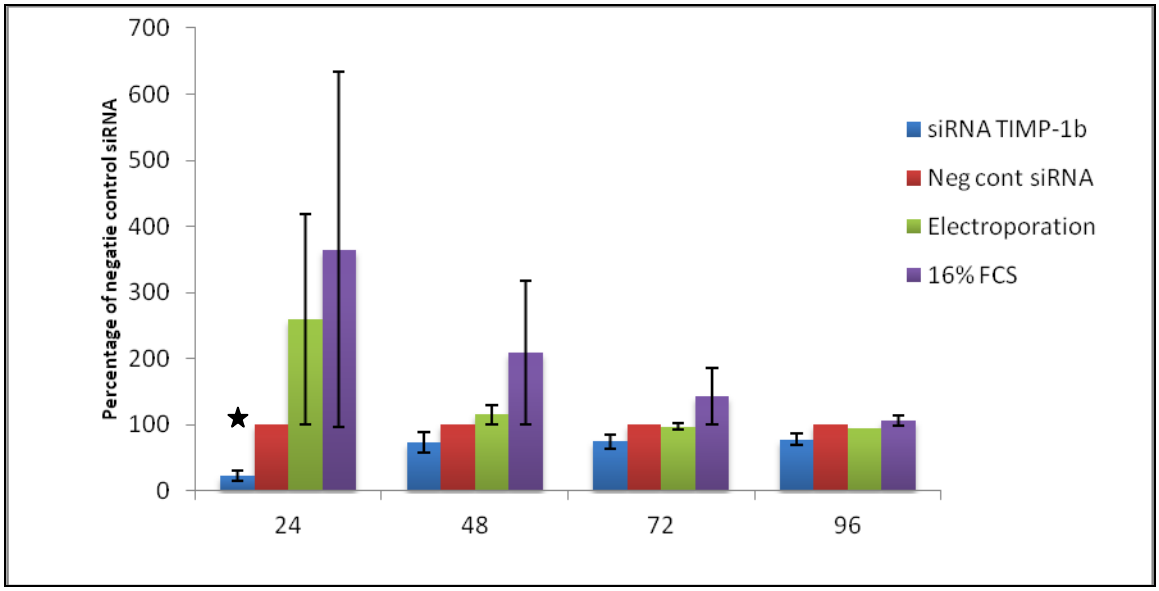
Dose dependent inhibition of rat TIMP-1 protein using different siRNAs targeting TIMP-1. These data represent the first 24 hours following electroporation. The concentration of TIMP-1 was assayed by rat TIMP-1 ELISA. There was a 95% reduction for the siRNA labelled as TIMP-1b compared with negative control siRNA, at 1.25 uM, with only a 58 % reduction with TIMP-1a.

The concentration for optimal silencing of TIMP-1 was determined to be 1.25µM. Therefore all further experiments were performed with rat 1.25µM TIMP-1 siRNA (Ambion), or 1.25µM negative control siRNA . Experiments were simultaneously performed using both preparations of siRNA, due to the fact that they both appeared to be capable of silencing TIMP-1 protein. The concentration of siRNA used in these experiments represents a final concentration of 25nM, ,2.5 nM, 025.nM, 0.025nM in tissue culture.

4.6.2 Silencing of TIMP-1 protein expression in primary activated rat pancreatic stellate cells

Figure4-8a,b Silencing of TIMP-1 protein expression in primary activated rat PSC

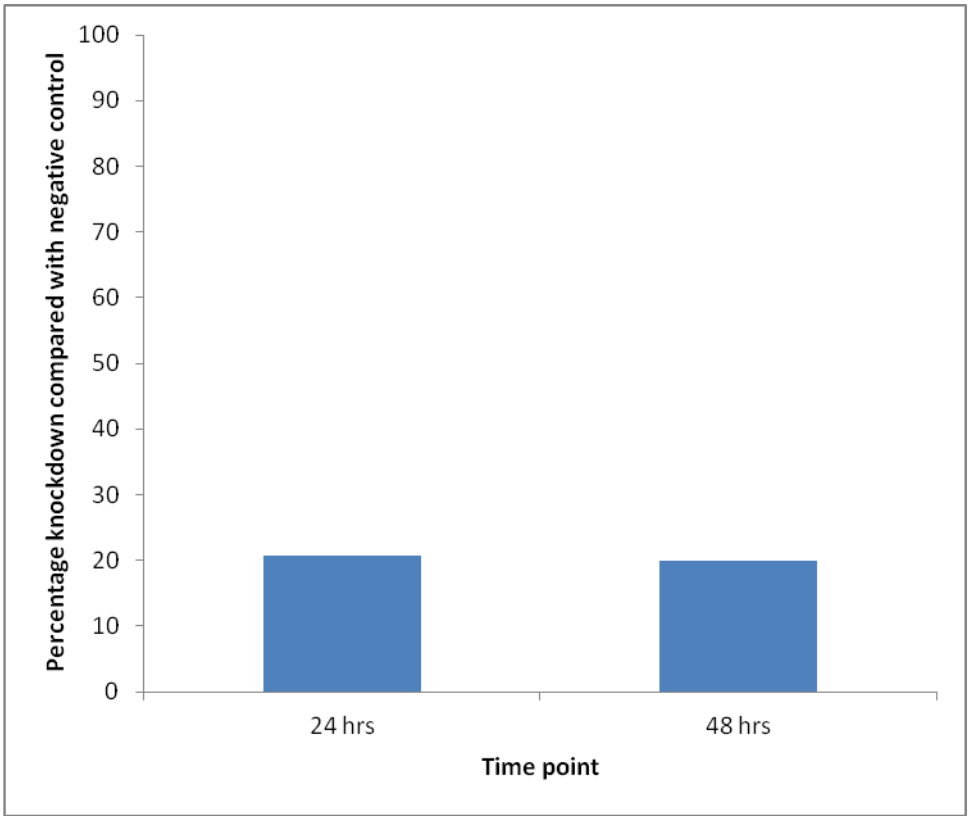




Electroporation with 2 different TIMP-1 siRNAs(a,b). Culture supernatants were harvested from pancreatic stellate cells that had undergone electroporation with TIMP-1 siRNA or control. The culture medium was collected every 24 hrs and replaced with fresh medium. The concentration of TIMP-1 was determined with the use of a TIMP-1 ELISA kit. a) There was 38% silencing of TIMP-1 protein expression achieved at 24 hrs, however this did not reach significance(p=0.22) b) There was an 86% inhibition of TIMP-1 protein expression achieved at 24 hrs, however this effect did not persist at a significant level beyond 24 hrs. ★p=0.012

(Data are expressed as mean+/- SEM, as normalised to control (negative control siRNA), n=3, meaning three separate experiments using cells extracted from different animals)

Figure 4-9 qPCR result of TIMP-1 knockdown at 24 hrs and 48 hrs



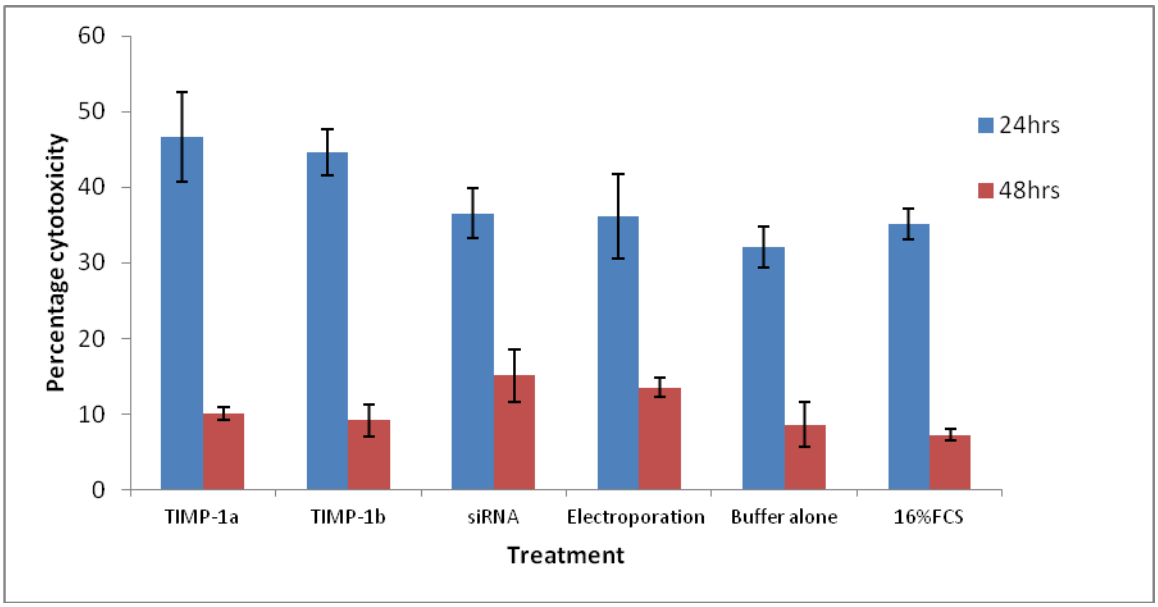
TaqMan® Quantitative PCR of TIMP-1 following electroporation with TIMP-1 siRNA. There was 79.2% silencing of TIMP-1 mRNA at 24 hrs, and 80% % inhibition of TIMP-1 protein expression achieved at 48 hrs.(n=1)

Despite using the same siRNA and protocols as in hepatic stellate cells, initial experiments failed to show significant knockdown of TIMP-1 protein compared to the negative control siRNA (data not shown). These experiments were subsequently repeated simultaneously with a different TIMP-1 siRNA supplied by Ambion. The original siRNA was used again. None of the independent experiments showed a significant knockdown effect with TIMP-1 siRNA compared to the negative control at any of the 24 hour time windows up to 96 hours (figure 3.8a). However, with the new sequence siRNA, significant results were achieved, with a reduction of TIMP-1 protein expression at 24 hrs (figure 3.8b), however this effect did not persist at a significant level beyond 24 hrs.

These results have also been confirmed at the mRNA level using quantitative real time PCR. (n=1). The same siRNA caused 79.2% and 80% knockdown at 24 and 48 hours respectively. Further experiments are required to perform statistical analysis.

4.6.3 No excess cytotoxicity due to electroporation with TIMP-1 siRNA

Figure 4-10: Effects of electroporation of TIMP-1 siRNA on cell cytotoxicity

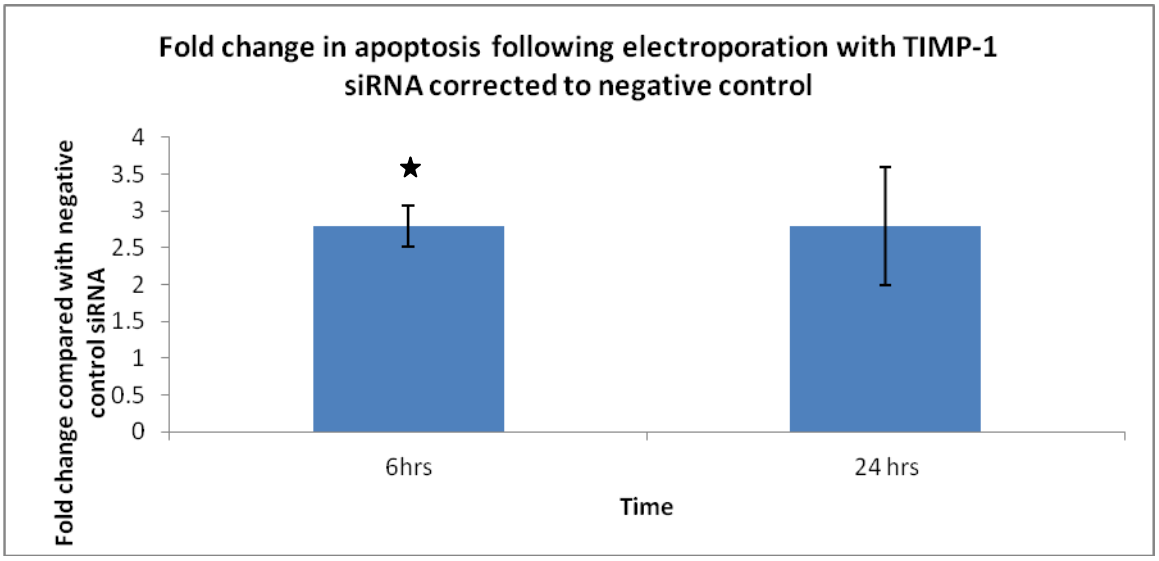


Treatment with siRNA demonstrated no significant increase of cytotoxicity when measured with LDH assay at 24 or 48 hrs compared with non treated controls. Cell lysates were collected after 24 and 48hrs after electroporation, with culture supernate being replaced after 24 hrs in the 48 hr time point. Cytotoxicity estimated from $(LDH)_{supernate}/((LDH)_{supernate}+(LDH)_{lysate})$, absorbance read at 492 nm. Data is representative of 3 independent studies (n=3) and is presented as mean cytotoxicity ± standard error of the mean (SEM).

Cell electroporation and exposure to siRNA are potentially cytotoxic to cells. These results demonstrate that there is no significant adversity cytotoxicity due to either electroporation or siRNA treatment. These results do not show any significant difference in overall cell cytotoxicity of untreated cells (i.e. trypsinised and placed in to 16%FCS) vs. those electroporated with TIMP-1 siRNA.

4.6.4 Increased level of apoptosis following electroporation with TIMP-1 siRNA

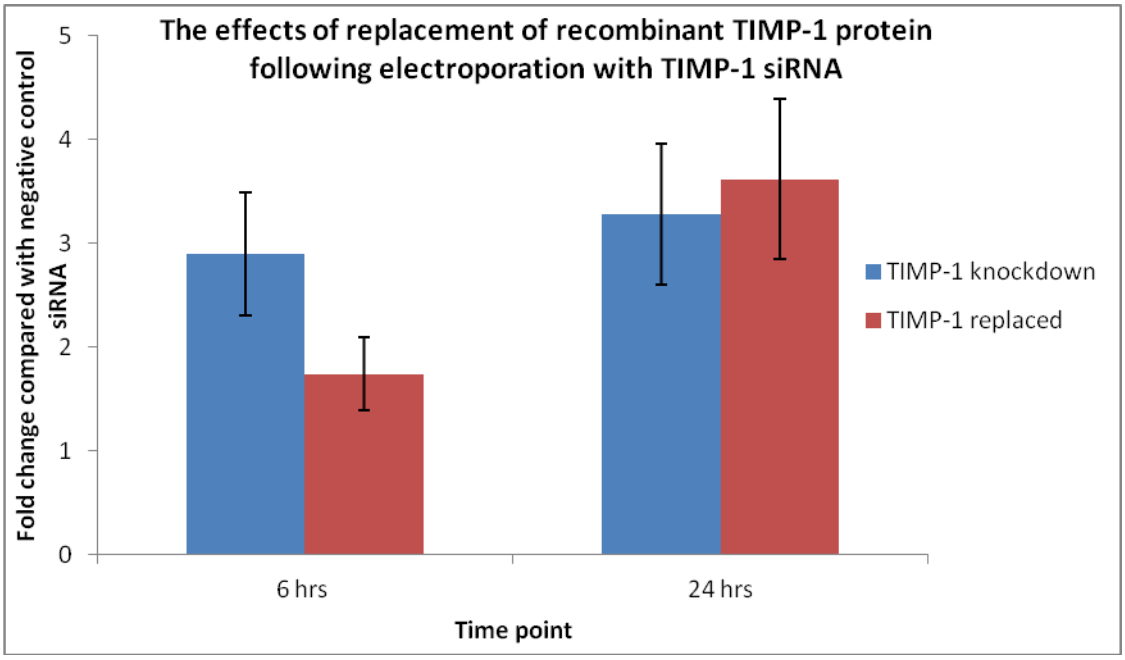
Figure 4-11 Increase in apoptosis following electroporation with TIMP-1 siRNA.



Following electroporation with TIMP-1 siRNA there was a 2.8 fold increase in the level of apoptosis at 6hrs and 24 hrs as determined by measuring caspase 3/7 activity, using the Promega Apo-One caspase 3/7 Assay - luminescence kit.(n=3) . Data is representative of 3 independent studies (n=3) and is presented as mean fold change ± standard error of the mean (SEM).

Following electroporation with TIMP-1 siRNA there was a 2.8 fold increase in the level of apoptosis when determined with the Promega Apo-ONE caspase 3/7 luminescence kit. This increase in apoptosis was seen at both 6hrs and 24 hrs when compared with cells treated with negative control siRNA. Each condition was performed in triplicate, n=3, meaning three separate experiments using cells extracted from different animals. *p=0.02, by one sample T-test.

Figure 4-12 The effects of replacement of recombinant TIMP-1 protein following electroporation with TIMP-1 siRNA



Following electroporation with Timp-1 siRNA, serum free media were supplemented with recombinant rat TIMP-1 200ng/ml. This reduced the level of apoptosis by 1.7 fold when compared to electroporation with negative control siRNA alone. Data is representative of 2 independent studies (n=2) and is presented as mean fold change ± standard error of the mean (SEM).

Following electroporation with TIMP-1 siRNA, serum free media were supplemented with recombinant rat TIMP-1 without carrier protein (the carrier protein was omitted in these experiments so that any potential interference with the apoptosis assay could be avoided).This reduced the fold change of apoptosis by 1.7 at 6 hours. However, this effect was not sustained at 24 hours. There was a return of apoptosis to similar levels as electroporated cells not treated with TIMP-1, at 24 hours. These results do reach statistical significance but require further experiments to allow statistical analysis.

4.7 Summary of in vitro experiments

1. These in vitro experiments showed a dose dependent anti apoptotic effect for TIMP-1 in rat and human PSC.
2. TIMP-1 did not effect proliferation of activated rat PSC.
3. Successful silencing of TIMP-1 at the protein and mRNA level using electroporation of TIMP-1 siRNA.
4. Electroporation with TIMP-1 did not significantly effect PSC cytotoxicity.
5. Electroporation with TIMP-1 significantly increased the level of apoptosis compared with control.
6. Following electroporation with TIMP-1 siRNA, replacement with recombinant TIMP-1 reduces the level of apoptosis.

4.8 Discussion

4.8.1 The role of TIMP-1 in PSC survival and proliferation

Studies of the role of TIMP-1 in PSC have arisen from the observations in liver fibrosis; that on recovery of fibrosis there is a net reduction in activated HSCs and fibrotic matrix, while in progressive fibrosis the activated HSCs and neo-matrix remain. Identification of factors promoting the survival of activated PSC is therefore essential to understanding the pathogenesis of fibrosis.

These results in tissue culture indicate that TIMP-1 has a significant and concentration dependent anti-apoptotic effect on both human and rat PSC induced by cycloheximide. This occurred in human and rat PSC, suggesting that it is a biologically important phenomenon. What is important is that the overall trend of the results were the same and were reproducible. These in vitro studies demonstrate that TIMP-1 directly inhibits apoptosis of activated PSC and that it could be a possible autocrine survival factor. Studies of liver fibrosis have demonstrated an autocrine role for TIMP-1 in preventing apoptosis, by the use of TIMP-1 neutralising antibody (Murphy et al., 2002), applied to HSC in vitro.

TIMP-1 did not have any proliferative effect on activated PSCs. From a biological view, it would seem undesirable for a protein to both inhibit apoptosis and promote proliferation in the same

cell type as expression of such a protein could be potentially carcinogenic. Although this may provide an explanation for high levels of TIMP-1 associated with pancreatic adenocarcinoma.

Current evidence suggests that TIMPs have divergent effects on proliferation and apoptosis in a number of different cell types, which appears independent of their matrix metalloproteinase inhibitory activity. TIMP-1 was originally described to have erythroid potentiating activity(Docherty et al., 1985) . TIMP-1 has also been shown to prevent apoptosis in breast epithelial cells (Li et al., 1999), B lymphocytes (Guedez et al., 1998) and mesangial cells (Lin, Chen, Wang, & Yu, 2002). It appears that TIMP-1 consistently promotes cell survival .

In HSC, a nonfunctional mutated TIMP-1 (T2G mutant) in which all other domains are conserved did not inhibit apoptosis, indicating that inhibition of apoptosis was mediated through MMP inhibition (Murphy et al., 2002). A further recent study has demonstrated that HSC express MMP-2 and the anti-apoptotic glycoprotein N-cadherin. In the presence of pro-apoptotic signals, expression of MMP2 by HSCs induces N-cadherin cleavage, resulting in HSC apoptosis (Hartland et al., 2009).

The high expression of TIMP-1 would appear to alter the balance of ECM deposition within the fibrotic pancreas, by inhibiting the MMP which would otherwise degrade the fibrotic matrix. This data has supported an antiapoptotic effect of TIMP-1, as suggested by the acridine orange experiments. In addition, TIMP-1 has no significant effect on PSC proliferation. It would be of interest to examine whether this effect was dependent or independent of MMP-2 inhibition by the use of a mutant TIMP-1 and synthetic MMP inhibitors that target specific MMPs, as has been demonstrated in HSC (Murphy et al., 2002).

These data I have described in this chapter provide evidence that TIMP-1 is mechanistically important in promoting fibrosis, by inhibiting the apoptosis of activated PSC. Identification of factors promoting the survival of activated PSCs is essential to understanding the pathogenesis of fibrosis.

4.8.2 TIMP-1 Electroporation with siRNA and silencing of TIMP-1

RNA interference (RNAi) is a relatively new technology whereby a long double-stranded RNAs (typically >200nucleotides) can be used to inhibit the expression of target genes. The long

dsRNA is processed by RNAse III –like enzyme into small interfering RNAs (siRNAs). The siRNAs are then assembled into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). Activated RISCs then binds to complementary transcripts by base pairing interactions between the siRNA antisense strand and complementary. The bound mRNA is cleaved and the sequence specific of mRNA results in gene silencing. The use of siRNA technology allows us to identify the specific roles of individual molecules in this process. It therefore provides a useful laboratory tool for studying mechanisms involved in fibrogenesis, and simultaneously identifies a potential therapeutic target.

Utilising the experience gained in transfecting hepatic stellate cells, I have successfully demonstrated TIMP-1 silencing of activated pancreatic stellate cells. Traditionally primary cell cultures have proven to be difficult to transfect. Most importantly this was achieved at the protein level. The effects of this on stellate cell apoptosis have been demonstrated with a significant increase in the level of apoptosis following knockdown of TIMP-1.

These studies demonstrate that siRNA transfection can be used to determine the role of TIMP-1 in pancreatic stellate cell survival in vitro. I have shown here that siRNA, can be successfully transfected into rat primary pancreatic stellate cells, which shows up to 86% knockdown at the protein level. In addition, the protocols that I have used here provide a basis for further work using electroporation in PSC, to target other molecules that may also be involved in cell survival, such as IGF-1 and its receptor.

4.8.3 Off target effects of siRNA

In these studies there appears to be a substantial off target effect of siRNA electroporation in the negative control samples as compared to untreated or electroporated cells in the absence of siRNA. Although the negative control siRNA, have been specifically designed not to target any known human or rodent gene, it is recognised that off target effects are occasionally observed. To combat this problem of non specific or “off-target” effects of negative control siRNA, three different preparations of negative control siRNA were used from Ambion. All of these still showed similar effects of TIMP-1 knockdown, compared with electroporation with buffer alone (data not shown). The effects of TIMP-1 knockdown at the protein level have been

compared to the negative control in each experiment, and it is these results that have been presented.

One of the best ways recommended to increase confidence in data from siRNA experiments is to independently use two or more siRNAs to a single target gene. Different siRNAs to the same gene with comparable gene silencing efficacy should induce similar changes in gene expression profiles or phenotypes. Any changes induced by one siRNA and not the other may be attributed to off-target effects.

The algorithm developed by Cenix Bioscience, available from Ambion for siRNA design, accurately predicts potent siRNA sequences (Ambion, website). The use of effective siRNAs maximises target mRNA reduction and minimise the possibility of off-target effects by allowing the use of lower siRNA concentrations in RNAi experiments. This was attempted in these studies of siRNA, however higher concentrations of siRNA were necessary to achieve the anticipated high levels of silencing required to observe an anticipated phenotypic effect. In the experiments described, 2 different siRNA sequences were used to confirm silencing. They both demonstrated a degree of silencing, although the first sequence designated TIMP 1a, did not reach significance. It is recommended that a scrambled control sequence should be used to discount any changes to the gene expression profile that may result from the siRNA delivery method. To serve as a negative control, it is essential to ensure that the siRNA is not complementary to any gene in the target organism. The negative controls supplied by Ambion, meet these criteria. However, despite using three of the most commonly used negative control sequences, there appeared to be a potential nonspecific effect of TIMP-1 reduction in negative control compared to electroporation with buffer alone. Whilst it is possible to consider the use of a further scrambled siRNA sequence, it was felt that this was unlikely to offer any further information.

A logical step was to attempt a lower concentration of siRNA. Nonspecific silencing effects may be seen when an siRNA is transfected into cells at concentrations of 100 nM or higher (Jackson et al., 2003; Murphy et al., 2002; Persengiev, Zhu, & Green, 2004; Semizarov, Kroeger, & Fesik, 2004). However, this non-specific effect is reduced when siRNAs are used at lower concentrations less than 30nM. It has been suggested that to ensure target specificity it is best to titrate the siRNA and use it at its lowest effective concentration. In my experiments the final

concentration of siRNA used in the culture dish was 25nM. These higher concentrations of siRNA were felt necessary to establish sufficient silencing for there to be observable phenotypic effect.

These non specific effects were seen at all of the concentrations that siRNA was used for electroporation, and therefore does not appear to be a concentration dependent effect. The higher dose of siRNA was chosen as this achieved the highest amount of TIMP-1 protein silencing, which would be desirable given the effects of TIMP-1.

Recent studies indicate that upregulation of the antiviral response may be a useful indicator of nonspecific siRNA effects. The most comprehensive way to monitor the antiviral response is with genome-wide arrays. However, the interferon response can be measured simply, by analysing the upregulation of 2'5' oligoadenylate synthetase(STAT1) mRNA, and activation of RNase L (Chi et al., 2003). Further studies measuring these parameters may be useful in understanding these results.

Ideally, the specificity of an siRNA can only be definitively determined by looking at the global changes in gene expression pattern by using DNA microarrays. In these experiments, it would be possible to determine that siRNAs targeting a particular gene should give rise to “gene-specific” changes in expression profiles. Any off-target effects, will be deemed to be ”siRNA-specific”, rather than gene-specific changes in gene expression pattern. Although these protocols were optimised on HSC which show many similarities with PSC, it still remains a possibility that these effects are specific to PSC.

In this data I have chosen to look at initially at TIMP-1 protein levels for reasons already described. Further experiments are required to confirm these results at the mRNA level(data presented here as n=1), as protein reduction in the absence of mRNA reduction may indicate that siRNA is mediating its effects at the translation level like a micro-RNA(Doench, Petersen, & Sharp, 2003).

5 Chapter 5

5.1 Introduction

Insulin-like growth factor-I and insulin are structurally related polypeptides involved in various metabolic, proliferative, and differentiation processes mediated by endocrine, autocrine, and paracrine mechanisms. Insulin and insulin-like growth factor (IGF-1) are known to be mitogenic for fibroblasts and smooth muscle cells. The biological functions of IGF-1 and insulin are mediated by transmembrane hetero-tetrameric receptors with intrinsic tyrosine kinase activity. The binding of insulin and IGF-1 to their receptors' α-subunit results in autophosphorylation of the receptors' β-subunit on tyrosine residues and propagation of the signals by tyrosine phosphorylation of intracellular substrates, like the insulin receptor substrate-1 (IRS-1). Insulin is under tight control of blood glucose levels and is excreted by the pancreas solely in periods of rising blood glucose levels. IGF-1 is produced under endocrine GH control in the liver as well as in somatic cells.

The expression of insulin by cells is restricted, principally just to β cells of the pancreas. It is stored in secretory granules within pancreatic islets and secreted via regulated pathways in response to stimuli. As described previously the concentrations of insulin within the pancreas are speculated to be 20-50 times that in the circulation, potentially rising tenfold in hyperinsulinaemic states. In contrast, IGFs are widely expressed in tissues throughout the body. Unlike other peptide growth factors, IGFs are not stored within secretory granules, but are secreted as they are produced via the constitutive secretory pathways. IGF-I is a mitogenic growth factor and promotes ECM accumulation in various cell types, including fibroblasts (Johnson et al., 1997; Feld, Hirschberg, Artishevsky, Nast, & Adler, 1995). In experimental diabetic kidney diseases, increased renal IGF-I levels were correlated with pathological alterations consistent with glomerulosclerosis (Segev et al., 1997). However, increases in renal IGF-I levels were not accompanied by an increase in serum IGF-I. Therefore, it is thought that local overproduction of IGF-I may be more pathophysiologically relevant. Additionally, it has been demonstrated that IGF-I expression is increased in renal fibroblasts under hyperglycaemic conditions which contributes to the pathogenesis of diabetic nephropathy (Lam et al., 2003). It has also been demonstrated that IGF-I production seems to be induced by hyperglycaemia

(Pugliese et al., 1996), this may have particular relevance in chronic pancreatitis when functional islets are eventually destroyed leading to diabetes and hyperglycaemia.

Insulin circulates until it encounters its receptor, in contrast to IGFs which bind to IGF binding proteins upon secretion. These are slow in clearance allowing high concentrations of IGFBP protein to build up. Total IGF-1 concentration in the circulation is approx 100nM, which is approximately 1000 time greater than insulin. At the cellular level, optimal IGF-1 concentration is achieved at 1-2nm range, demonstrating that there is vast excess within the circulation. Within local tissues, the concentration of IGF-1 is approximately one third of that in the circulation. Whist IGFs are not stored in cells, there would appear to be a large extracellular store, maintained in complexes with IGFBP. A number of studies have indicated that most of the metabolic and mitogenic effects of IGF-I are mediated by the IGF-I and insulin receptors. IGF-1 actions are determined by the availability of free IGFs to interact with receptors of IGF-1 or insulin. The amount of free IGF is modulated by the level of high-affinity IGF binding proteins (IGFBPs). So far, six distinct IGFBPs have been identified that differ in molecular mass, binding affinities for IGFs, and post translational modifications such as phosphorylation and glycosylation. IGFBPs not only regulate bioavailability of circulating growth factors, but can also inhibit or enhance IGF action on target tissues. Inhibition of IGF action by IGFBPs may result from sequestration of IGF, but the mechanism of potentiating the IGF effects by IGFBP is poorly understood. Abundance of IGFBPs can be regulated at the level of gene expression as well as by limited proteolytic processing. With IGFs the constitutive secretion within a tissue is only one component of total IGF that cells will be exposed to. IGFs are bound to IGFBP with higher affinity than IGF-1R. Circulating proteases make IGF-1 available within tissue.

It has been suggested that insulin and IGF-1 could play a role in liver fibrosis. Several studies have focused on the identification of cytokines and growth factors that are involved in HSC proliferation. Studies have shown that proliferation of HSCs *in vitro* is stimulated in response to IGF-1(Svegliati-Baroni et al., 1999). Studies of HSC biosynthesis of IGF-I, IGFBPs, and the IGF-I-R and IGF-II-R were analysed in both ‘‘early cultured’’ and ‘‘culture-activated’’ HSCs,(Scharf et al., 1998) and shown to be present in both phenotypes, although there was a higher receptor expression in early culture suggesting they are more sensitive to IGF-1 effects during early activation. IGF-1 is released in an autocrine manner by damaged hepatocytes and by stellate cells (Brenzel & Gressner, 1996; Pinzani, Abboud, & Aron, 1990). In addition IGF-1 has been

shown to prevent HSC apoptosis (Issa et al., 2001). IGF-1 is a powerful survival factor for stellate cells and may act together with other soluble factors.

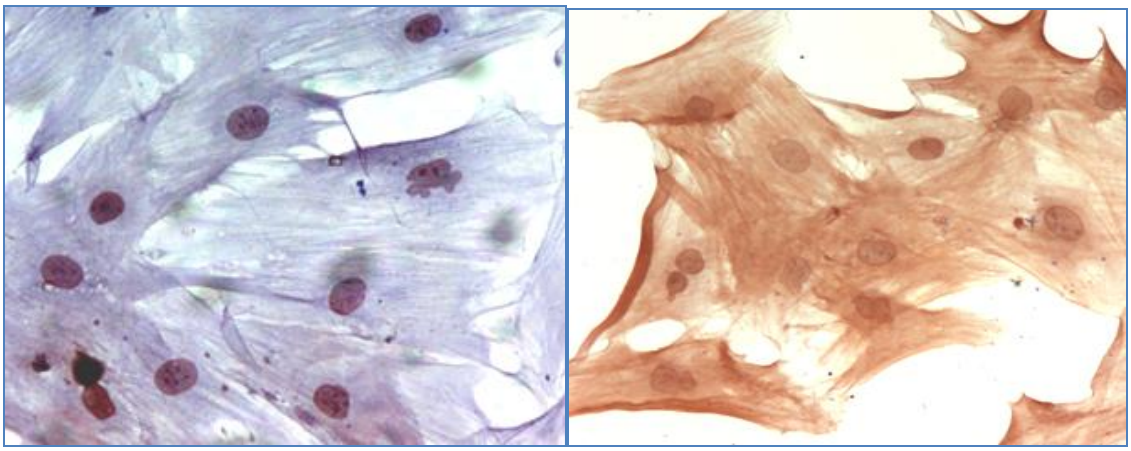
In its phosphorylated form, IRS-1 has been shown to act as a docking protein and influence multiple intracellular signalling enzymes such as PI3-K and ERK. These enzymes have also been implicated in HSC proliferation and collagen synthesis. IGF-1 receptors and insulin receptors have been demonstrated in rat HSC. There are some studies showing the level of IGF-1 in the circulation will affect gene expression of the IGF-1 receptor(Eshet et al., 1993). The mechanism is not clear, but the mRNA receptor expression was decreased with high IGF-1 levels and vice versa.

IGF-1 mRNA is up-regulated in fibroblast-like cells in areas of intense fibrosis. Fibroblast proliferation can also be induced by insulin. In cirrhosis, high plasma insulin levels are detected, which may be secondary to increased production as a result of peripheral insulin resistance and reduced liver degradation.

There is currently little data available data available in chronic pancreatitis and pancreatic fibrosis, with respect to the components of the IGF axis and their actions on PSC.

5.2 Activated pancreatic stellate cells express IGF-1 receptor and insulin receptor

Figure5-1: Insulin and IGF-1 receptor expression was evaluated by immunohistochemistry in cultured rat PSC



a) b)

Activated cultured rat pancreatic stellate cell a) Negative control (treated with non-immune IgG) b)immunostained with IGF-1 receptor-β antibody.

Figure 5-2 IGF-1R expression by western blotting

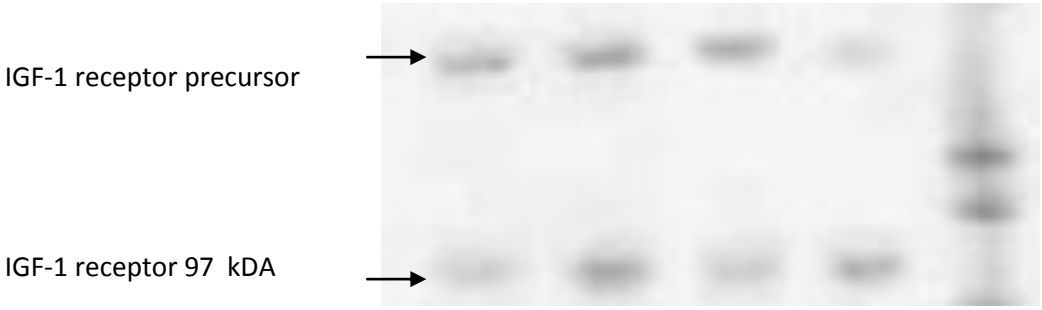
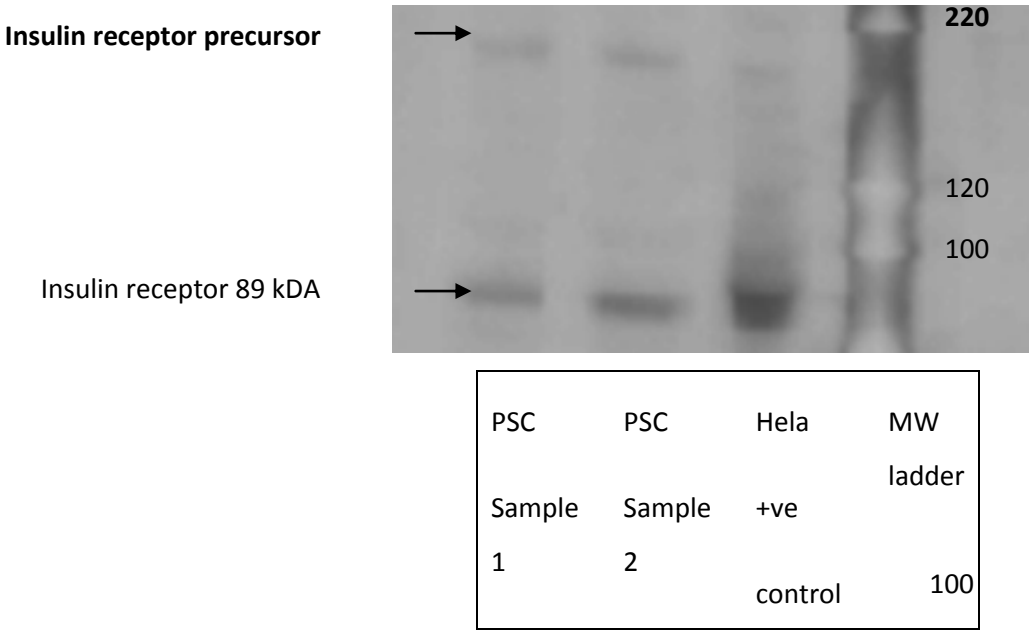


Figure 5-3 : Insulin receptor β subunit expression by western blotting



These experiments represent the presence of IGF-1 receptor and insulin receptor on culture activated pancreatic stellate cells. These representative western blots are from culture activated rat pancreatic stellate cells are from 3 different animals.
Activated pancreatic stellate cells were also stained for IGF-1 receptor and demonstrated positive staining for the receptor.

Figure 5-4: Gel of PCR products demonstrating insulin receptor, IGF-1 receptor and IGF-1

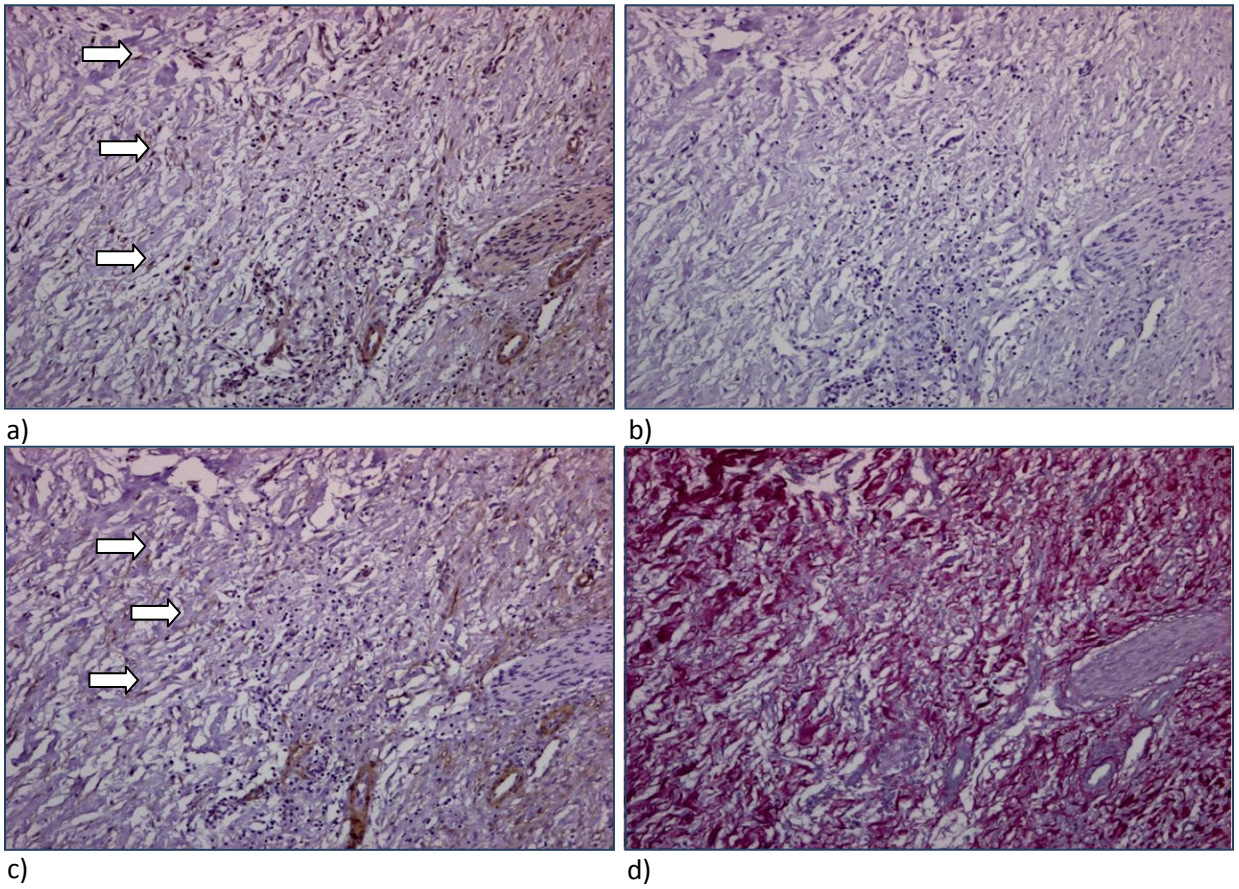
B Actin 98bp InsR 116 bp IGF-1R 113bp IGF-1 89bp

A representative gel of RT-PCR products, demonstrating Insulin receptor, IGF-1 receptor and IGF-1 expression by 3 separate PSC preps

These set of experiments demonstrate that activated pancreatic stellate cells express the mRNA transcript for insulin receptor, IGF-1 receptor and IGF-1 respectively. This representative gel is taken form 3 separate pancreatic stellate cell preps.

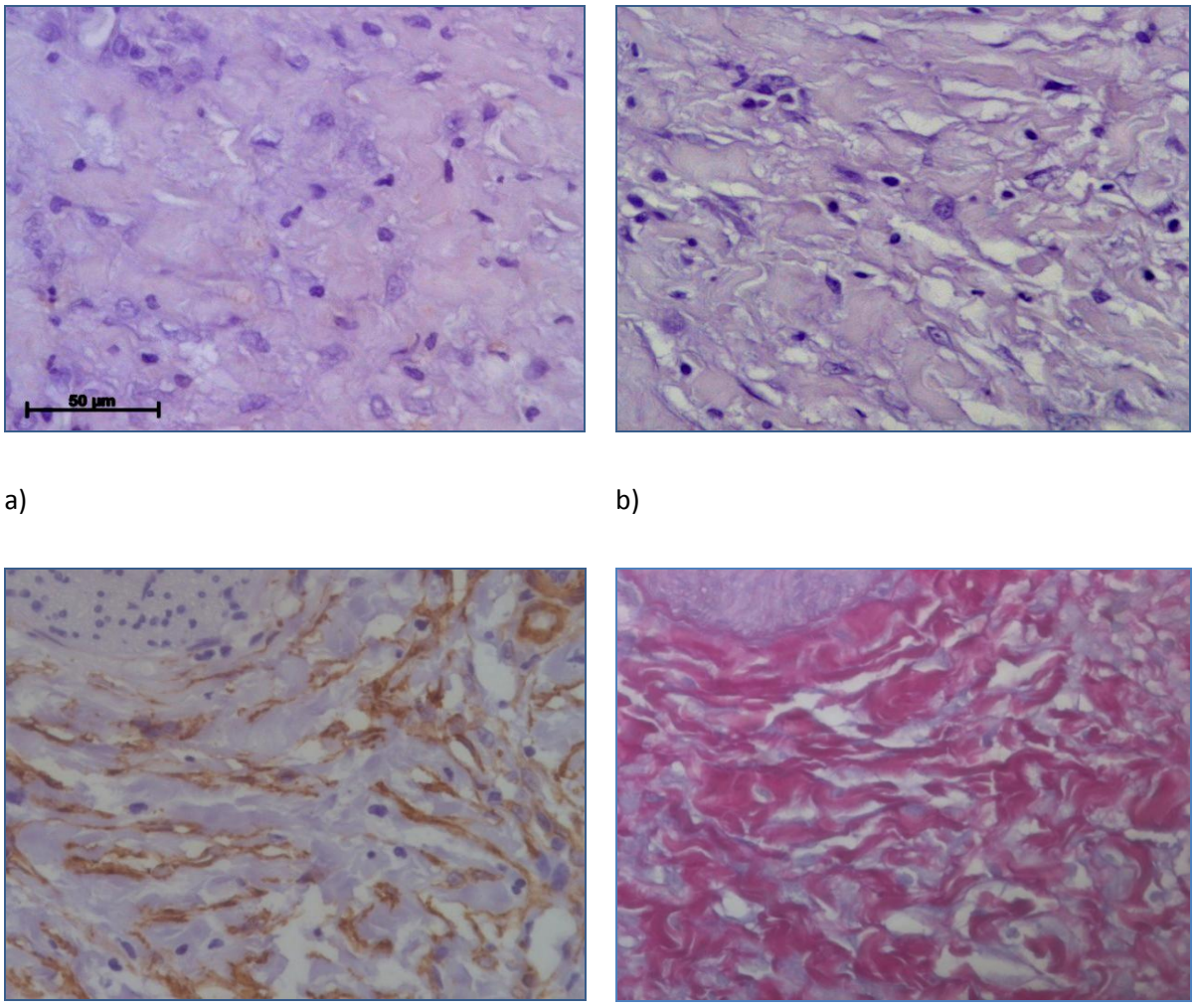
Together these data demonstrate the presence of IGF-1 and insulin receptors suggesting that they have the capacity to be stimulated by their corresponding ligand. However this also provides the possibility that they may also have the capacity to be stimulated by each others ligand. The transcript for IGF-1 mRNA is also present suggesting a potential autocrine role for IGF-1 on pancreatic stellate cells.

Figure 5-5 Low power IGF-1 receptor expression in human chronic pancreatitis resection specimens.



Serial sections of chronic pancreatitis and the extensive fibrosis x10 magnification. The serial IGF-1 receptor sections are immunostained for a)IGF-1r, b)negative control c)α-sma, d)collagen (sirius red). α-sma positive cell are known to be present within pancreatic ducts. Representative sample n=5.

Figure 5-6 High power IGF-1 receptor expression in human chronic pancreatitis resection specimens.



a) b)

c) d)

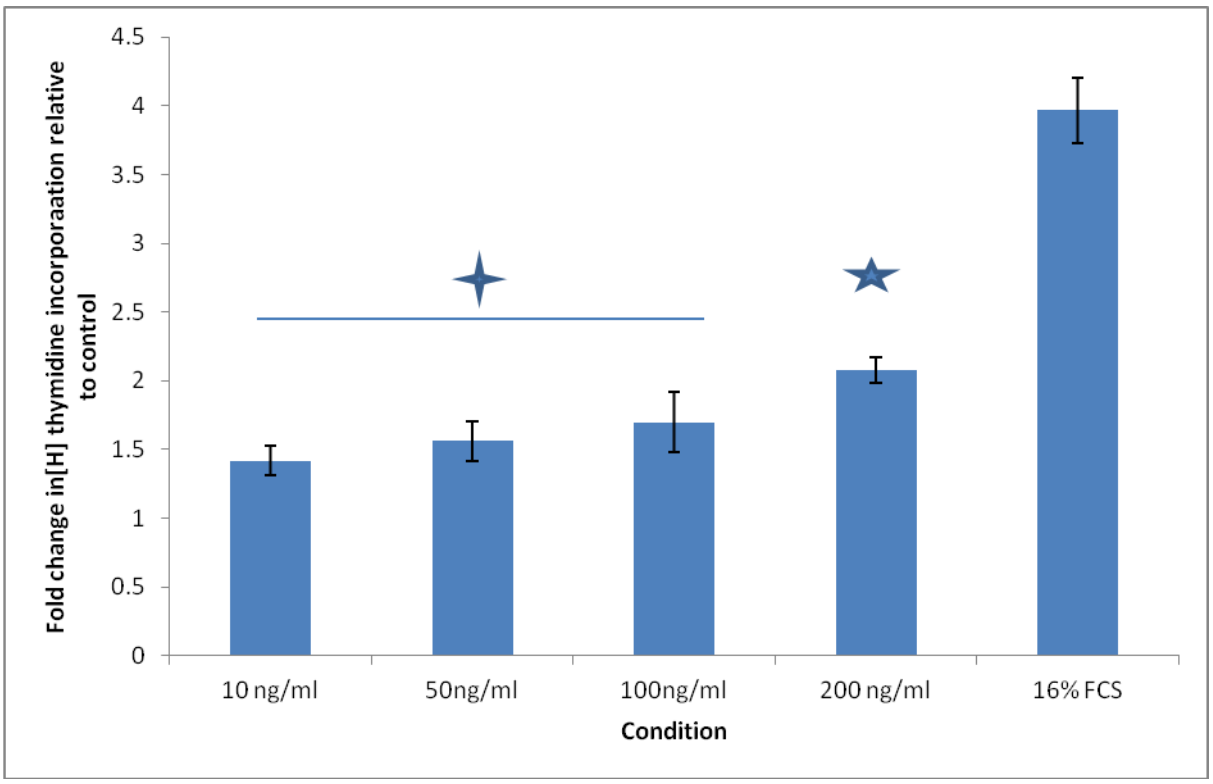
Serial sections of chronic pancreatitis and the extensive fibrosis x40 magnification. The serial IGF-1 receptor sections are immunostained for a)IGF-1r, b)negative control c)α-sma, d)collagen (sirius red). Representative sample n=5.

This specimen shows a representative sample of co-localisation of IGF-1R positive cells and α-SMA positive cells, suggesting that these cells represent PSC within the fibrotic scar. The negative control sample with non-immune IgG , demonstrates no positive staining within fibrotic scar. This suggests that these activated pancreatic stellate cells express IGF-1 receptor. Although these cells stain weakly positive for IGF-1 receptor, it is likely that number of receptors

is limited compared with the α-sma staining which constitutes a major component of the cytoskeleton. Additionally there is relatively paucity of cells within these fibrotic areas.

5.3 IGF-1 has a proproliferative effect on activated rat pancreatic stellate cells

Figure5-7 The effects of IGF-1 on rat PSC proliferation



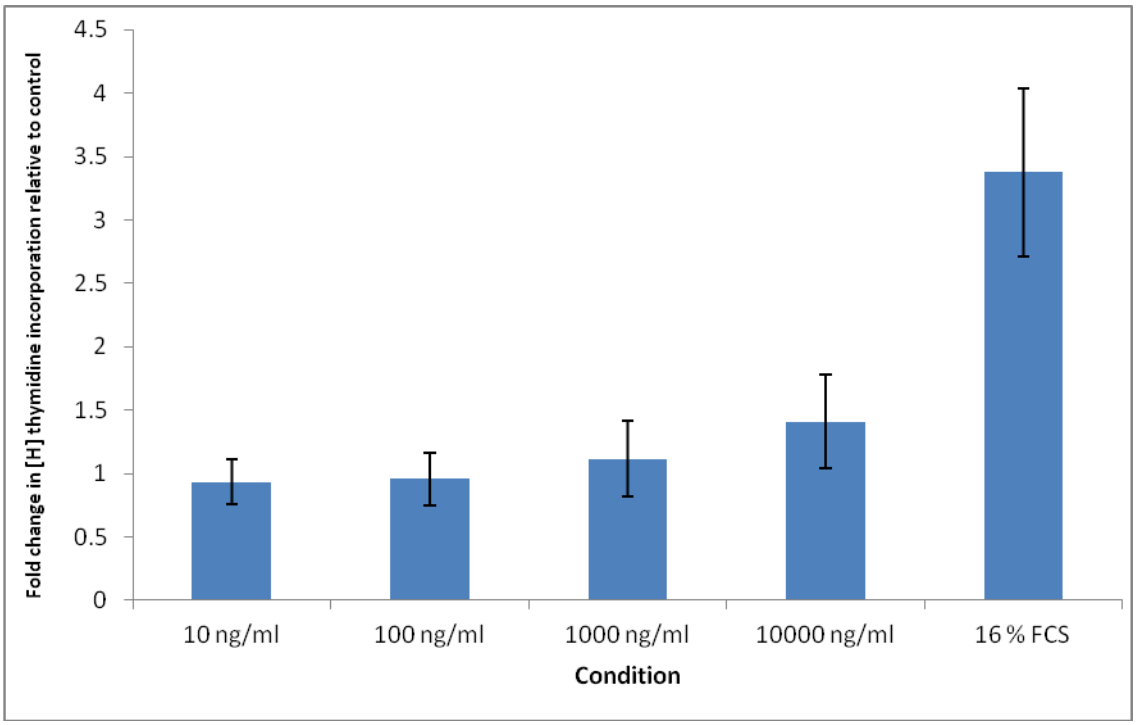
Rat activated PSC cultured on plastic were used to determine if IGF-1 could alter the rate of cellular proliferation. Proliferation was determined by the tritiated thymidine technique as described in the methods. IGF-1 significantly increased the rate of proliferation in the concentration range 10-200ng/ml. (Data are expressed as mean +/- SEM, as normalised to control (serum free conditions), each condition was performed in duplicate, n=4, meaning four separate experiments using cells extracted from different animals. p<0.05 p<0.005, by Student's t test.

Both IGF-1 and PDGF have been reported to promote HSC growth; they and their respective receptors are expressed by HSC (Novosyadlyy, Dudas, Pannem, Ramadori, & Scharf, 2006; Scharf et al., 1998). During liver injury they may act in a paracrine and autocrine manner. I therefore incubated PSCs in the presence of increasing concentrations of these growth factors in the absence of serum. These results demonstrate that IGF-1 promotes PSC proliferation, which

was highly reproducible in concentrations of 10-200ng/ml. A previous report did not show a significant effect on proliferation of PSC when exposed to IGF-1 at concentrations of 50ng/ml, although the average increase in the degree of proliferation in those experiments was similar(Schneider et al., 2001).

5.4 Insulin has no significant effect on proliferation of activated rat pancreatic stellate cells

Figure5-8: The effects of insulin n rat PSC proliferation



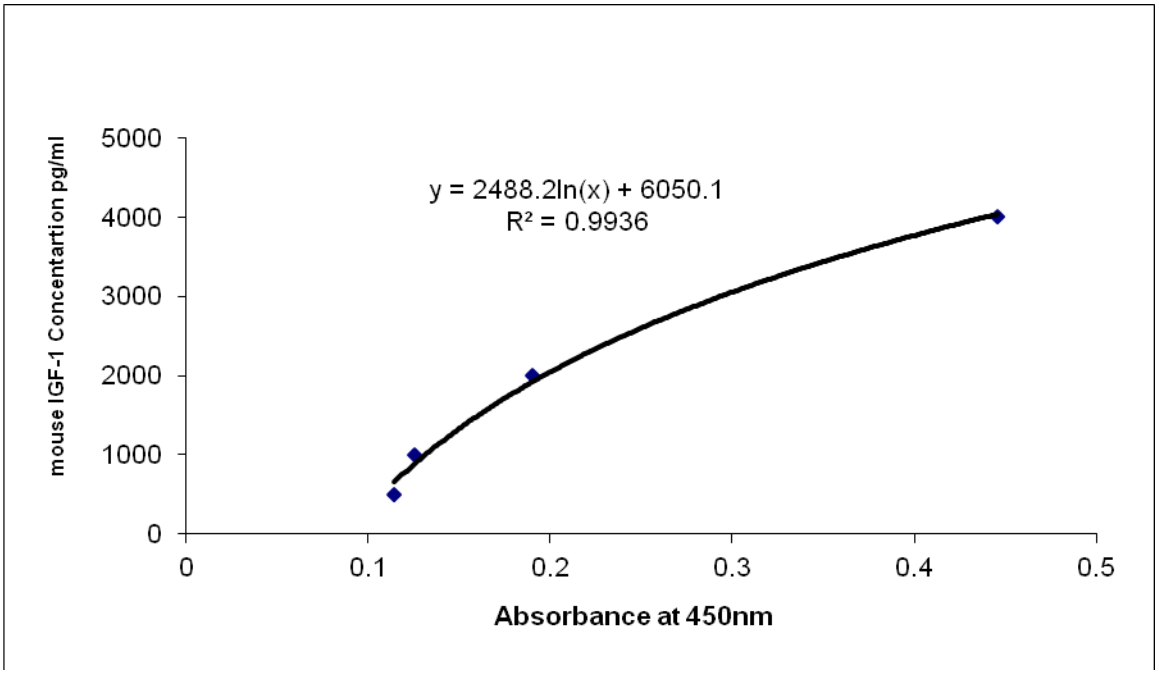
Rat activated PSC cultured on plastic were used to determine if insulin could change the rate of cellular proliferation. Proliferation was determined by the tritiated thymidine technique as described in the methods. Although there was a trend toward increased proliferation, this did not reach statistical significance in the concentration range 10-10000ng/ml. (Data are expressed as mean+/- SEM, as normalised to control (serum free conditions), each condition was performed in duplicate, n=3, meaning three separate experiments using cells extracted from different animals)

Previous studies on HSC have shown that insulin has a proproliferative effect (Svegliati-Baroni et al., 1999) on HSC. Additionally, high concentrations of insulin have been shown to have an effect on PSC proliferation(Hong et al., 2007), although the range used in these this experiment appears to be supraphysiological even when one considers the high concentrations that could be potentially present within the pancreas.

To determine the effect of insulin on PSC proliferation PSC cultures were incubated with increasing concentrations of insulin 1-10000ng/ml (0.17nM-1700nM) growth factors. These higher concentrations of insulin were used due to the potentially high concentration of insulin within the pancreas.

5.5 Determination of IGF-1 concentration by ELISA using a standard curve

Figure5-9 Standard Curve produced by IGF-1 ELISA

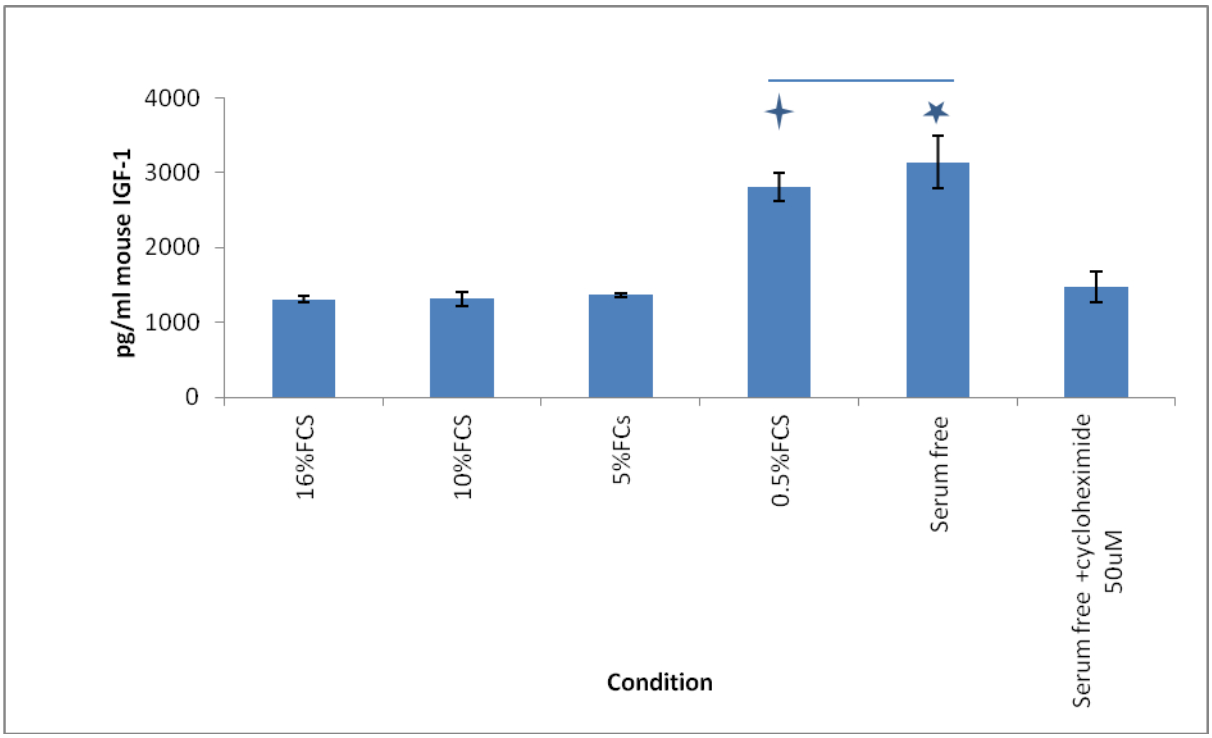


Example of Standard Curve produced by IGF-1 ELISA (R&D DuoSet). The standard curve was plotted using Microsoft Excel using polynomial regression.

A standard curve was produced by serial dilution of a known concentration of IGF-1. Polynomial regression using Microsoft Excel was used to generate the standard curve. This equation was used to calculate the unknown IGF-1 concentrations of the sample supernatants.

5.6 Autocrine IGF-1 production by Rat Pancreatic stellate cells in response to low serum conditions

Figure 5-10: IGF-1 Secretion by rat PSC



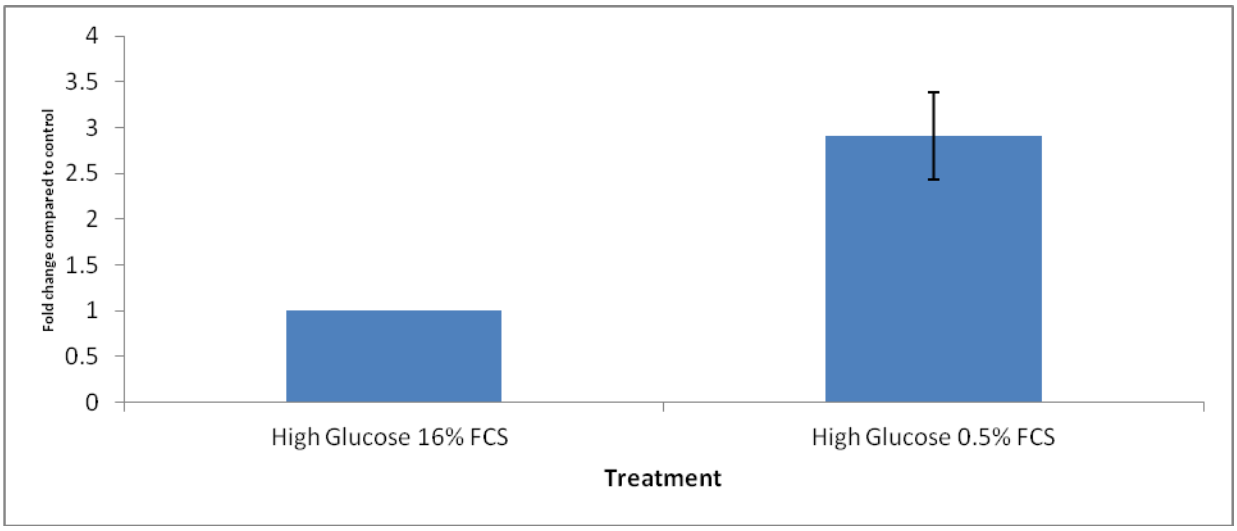
R&D rat IGF-1 DuoSet immunoassay was used to quantify IGF-1 secretion by rat pancreatic stellate cells. Pancreatic stellate cells produce IGF-1, in response to serum deprivation. This effect is inhibited by the presence of cycloheximide 50 µM. n=3, meaning three separate experiments using cells extracted from different animals.

p=0.0007 and p=0.0034.

IGF-1 is known to be produced by HSC and has a possible autocrine role within the liver. During pancreatitis and pancreatic regeneration, IGF-1 levels have been known to increase (Ludwig, Menke, Adler, & Lutz, 1999). I examined PSC supernate using a sandwich ELISA, to detect IGF-1. On reduction of FCS in the culture media, there was a significant increase in the detectable IGF-1 in the culture supernates. This could provide some explanation towards the relative low levels of apoptosis seen on serum deprivation studies (see later).

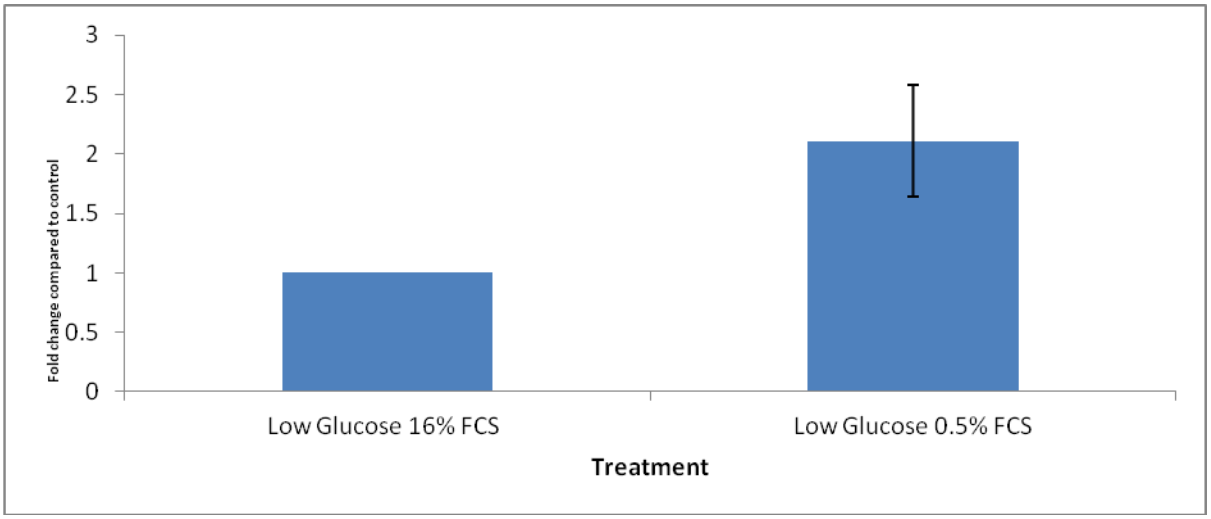
5.7 The effects of serum withdrawal on IGF-1 mRNA synthesis with high and low glucose media

Figure 5-11: The effects of high serum glucose on IGF-1 secretion



TaqMan® Quantitative PCR compared IGF-1 production by PSC, after 24 hours culture in reduced serum medium containing 0.5% FCS. Results are expressed as a percentage (mean±SE) of the control (16 %FCS -normal tissue culture conditions), n=3 meaning three separate experiments using cells extracted from different animals. Whilst this did not reach statistical significance, there was a clear trend towards increased IGF-1 mRNA expression. p=0.0572

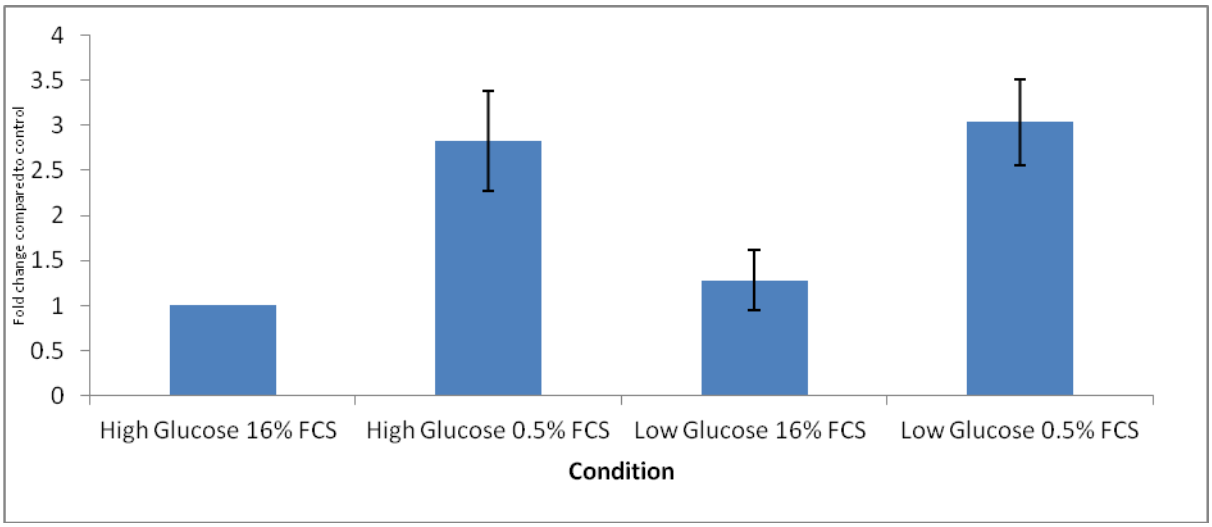
Figure 5-12: The effects of low serum glucose on IGF-1 secretion



TaqMan® Quantitative PCR compared IGF-1 production by PSC, after 24 hours culture in reduced serum medium containing 0.5% FCS. Results are expressed as a percentage (mean±SE) of the control (normal tissue culture conditions) n=3 meaning three separate experiments using cells extracted from different animals. Whilst this did not reach statistical significance, there was a trend towards increased IGF-1 mRNA expression. P=0.1422

5.8 The effects of serum withdrawal on IGF-1 mRNA synthesis

Figure 5-13 The effects of serum withdrawal on IGF-1 mRNA synthesis

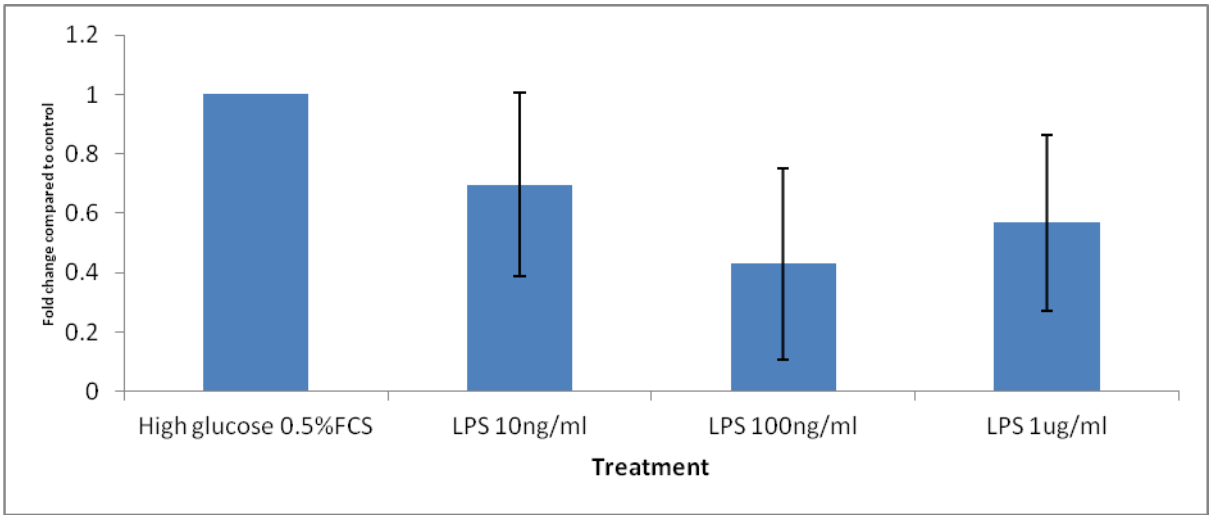


TaqMan® Quantitative PCR compared IGF-1 production by PSC, after 24 hours culture in low or high glucose conditions, with reduced serum medium containing 0.5% FCS. Results are expressed as a percentage (mean±SE) of the control n=3 meaning three separate experiments using cells extracted from different animals. Whilst this did not reach statistical significance, there was a clear trend towards increased IGF-1 mRNA expression with low serum conditions. The concentration of glucose did not effect IGF-1 secretion.

Previous studies have demonstrated that hyperglycaemia causes increased activation and proliferation of PSC (Hong et al., 2007; Ko et al., 2006b). Additionally, IGF-1 expression has been shown to increase following exposure to high glucose conditions (Lam et al., 2003; Segev et al., 1997), I therefore undertook studies to determine if normal or high glucose concentrations in vitro had any effect on the IGF-axis components. These graphs confirm that IGF-1 mRNA expression is increased following a reduction in serum concentration, however there is no apparent effect of hyperglycaemia on expression of IGF-1 by PSC.

5.9 The effects LPS stimulation on IGF-1 mRNA synthesis with high glucose media

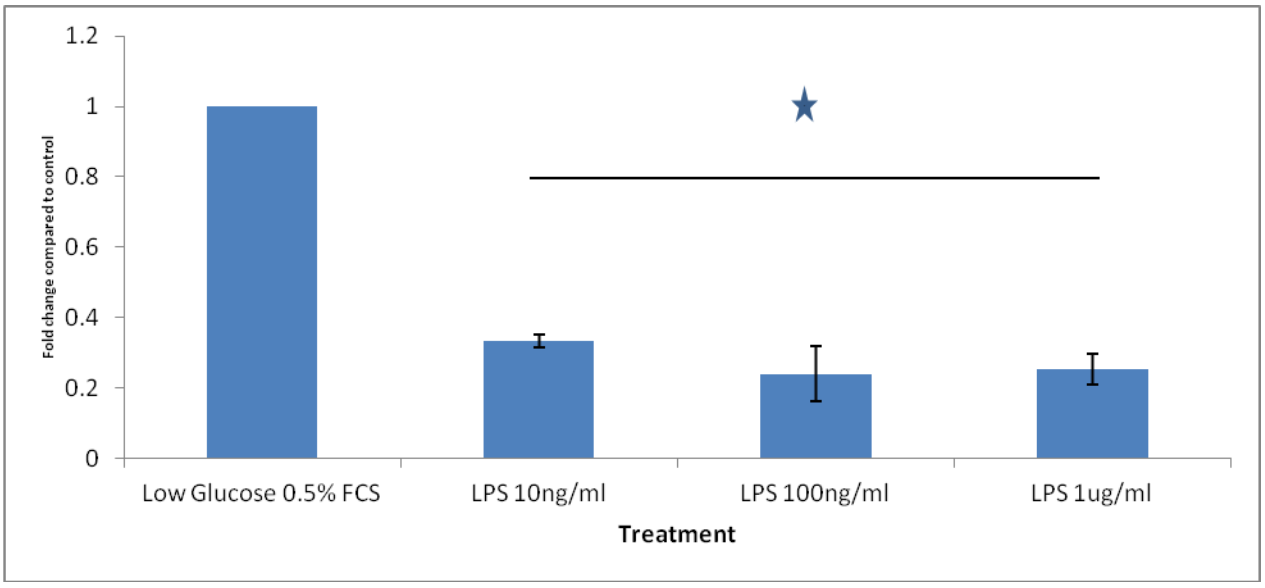
Figure5-14 The effects LPS stimulation on IGF-1 mRNA synthesis with high glucose media



TaqMan® Quantitative PCR compared IGF-1 production by PSC, after 24 hours culture in high glucose conditions, with reduced serum medium containing 0.5% FCS and exposure to LPS 10ng-1000ng/ml. Results are expressed as a percentage (mean±SE) of the control n=3 meaning three separate experiments using cells extracted from different animals. These results do not reach statistical significance.

5.10 The effects of LPS stimulation on IGF-1 mRNA synthesis with low glucose media

Figure5-15 The effects LPS stimulation on IGF-1 mRNA synthesis with low glucose media



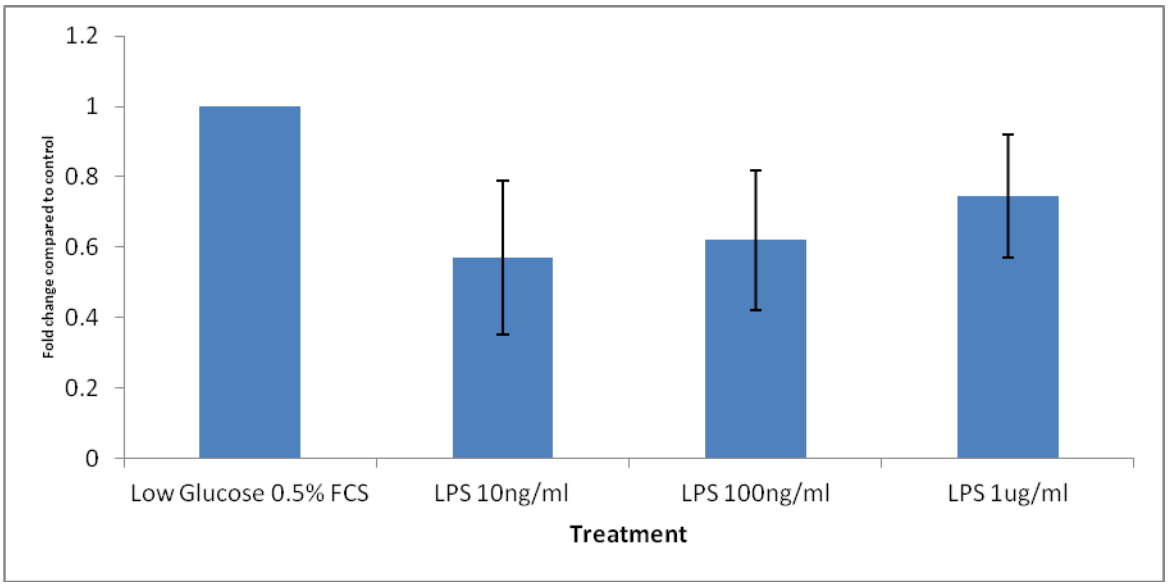
TaqMan® Quantitative PCR compared IGF-1 mRNA transcript by PSC, after 24 hours culture in low or high glucose media, with reduced serum medium containing 0.5% FCS, and exposure to varying concentrations of LPS. Results are expressed as a percentage (mean±SE) of the control n=3, meaning three separate experiments using cells extracted from different animals. This achieved statistical significance when cultured in low glucose media $p<0.05$, however this did not occur with high glucose media.

PSCs have also been shown to be responsive to LPS in a rat model of alcoholic chronic pancreatitis and express the TLR-4 and CD14 receptors (Vonlaufen et al., 2007). Additionally, PSC have a direct cytokine response to LPS producing IL-6 and MCP-1 (Michalski et al., 2007). Recently it has been demonstrated that human mesenchymal stem cells increase their secretion of IGF-1 in response to LPS (Crisostomo et al., 2008). I decided to measure mRNA transcript for IGF-1, in response to exposure to LPS, to determine if there were any possible effects on these cells which may have an effect on apoptosis.

There was a significant reduction in the IGF-1 mRNA transcript of PSC when cultured in low glucose media with LPS. This trend was also observed when cultured under high glucose media, although this was not statistically significant. It is unclear what the mechanisms for this might be. These results have not been confirmed at the protein level.

5.11 IGF-1R in response to LPS Low glucose media

Figure 5-16 IGF-1R in response to LPS stimulation with low glucose media

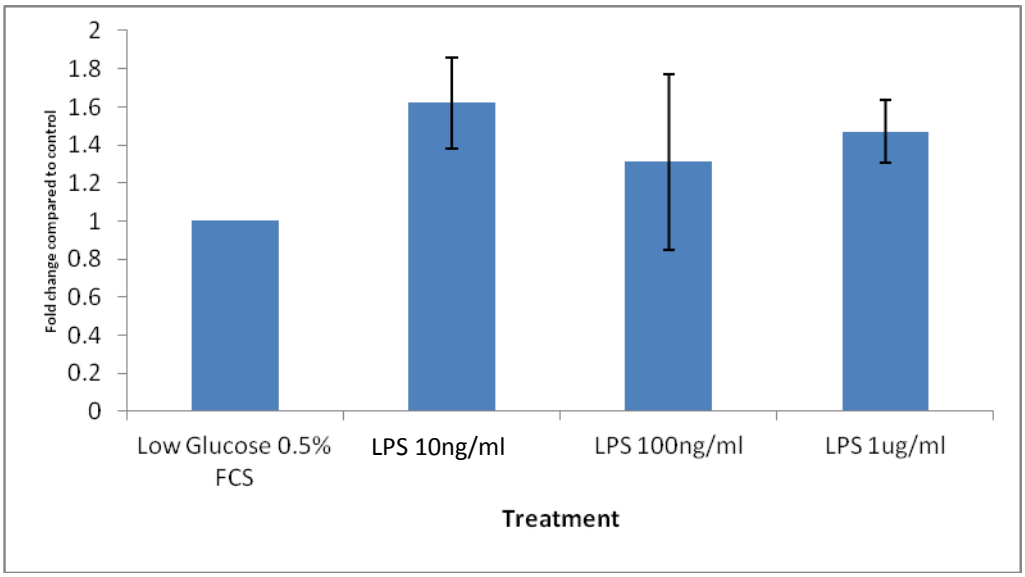


TaqMan® Quantitative PCR compared IGF-1R mRNA transcripts from by PSC, after 24 hours culture in reduced serum medium containing 0.5% FCS. Results are expressed as a percentage (mean±SE) of the control (16 %FCS - normal tissue culture conditions), n=3 meaning three separate experiments using cells extracted from different animals. Whilst this did not reach statistical significance, there was a trend towards decreasedIGF-1 receptor mRNA expression. (p value 0.19, 0.20, 0.28 respectively)

A similar effect of LPS was noted on IGF-1R mRNA transcript. It is possible that this may be a result of a toxic effect on these cells. These findings require further investigation.

5.12 Insulin receptor expression after exposure to LPS response.

Figure 5-17 Insulin receptor expression after exposure to LPS

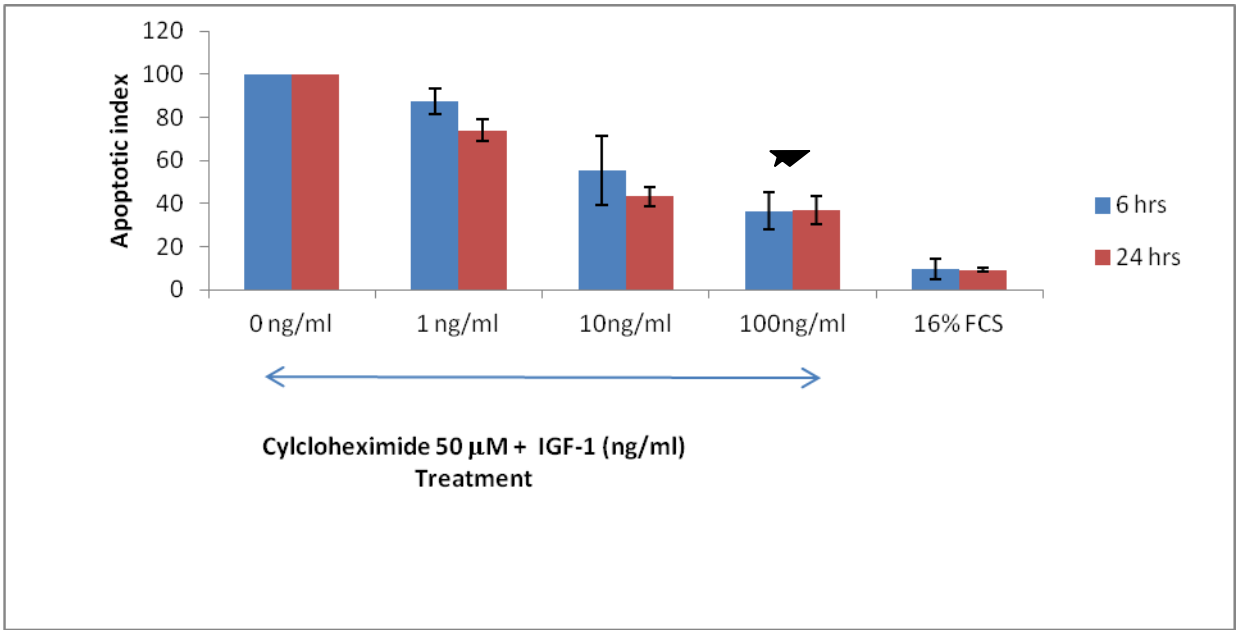


TaqMan® Quantitative PCR compared Insulin receptor transcripts from PSC, after 24 hours culture in reduced serum medium containing 0.5% FCS. Results are expressed as a percentage (mean±SE) of the control , n=3 meaning three separate experiments using cells extracted from different animals. There appeared to be no significant effect of LPS on insulin receptor expression. (p value 0.13, 0.57, 0.10 respectively)

Conversely, there appeared to be an increase in the insulin receptor transcript following exposure to LPS. However, these results did not achieve statistical significance.

5.13 IGF-1 treated rat PSC demonstrates reduced apoptotic index as determined with acridine orange and counting following induction of apoptosis with cycloheximide

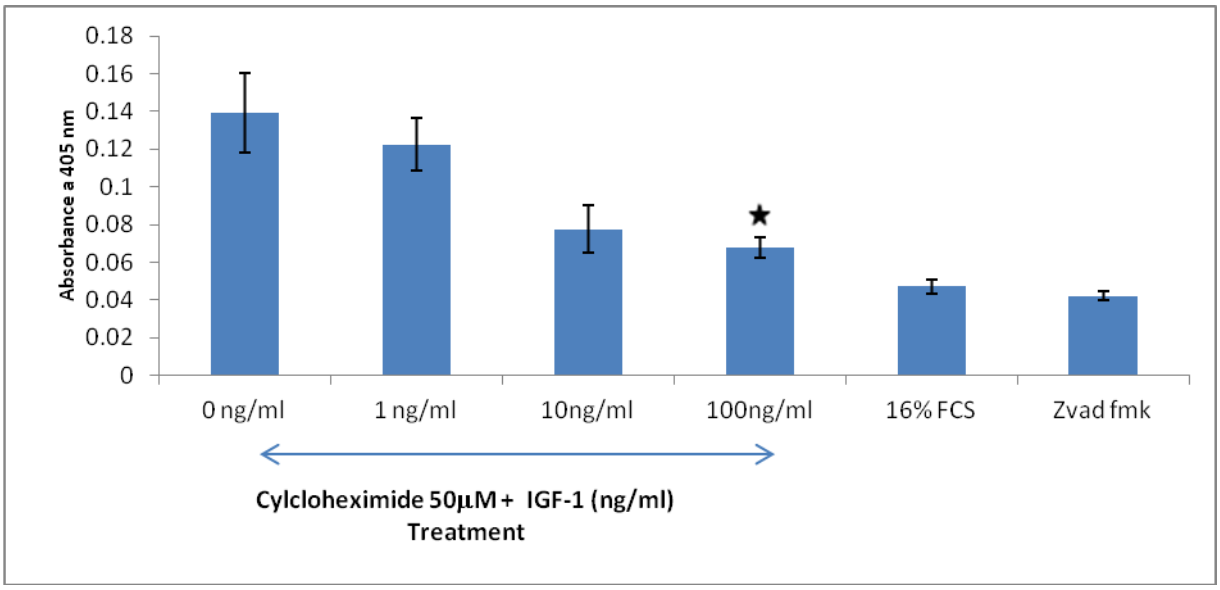
Figure 5-18 IGF-1 treated rat PSC have reduced apoptotic index in response to cycloheximide. Acridine orange



Apoptosis determined by acridine orange staining and counting. Data presented are mean +/-SEM expressed as percentage of control given the arbitrary value of 100%. FCS 16%- DMEM +Foetal calf serum 16%. IGF-1 significantly reduced apoptosis of activated rat pancreatic stellate cells induced by 6 and 24 hours cycloheximide. This occurred in a dose dependent manner over the concentration range 1-100ng/ml. * p<0.005 for cycloheximide versus cycloheximide with 100ng/ml IGF-1 by one sample t-test, n=4

5.14 IGF-1 treated rat PSC have reduced Caspase-3 activity following induction of apoptosis by cycloheximide

Figure 5-19 IGF-1 treated rat PSC have reduced apoptotic index in response to cycloheximide. Caspase 3.

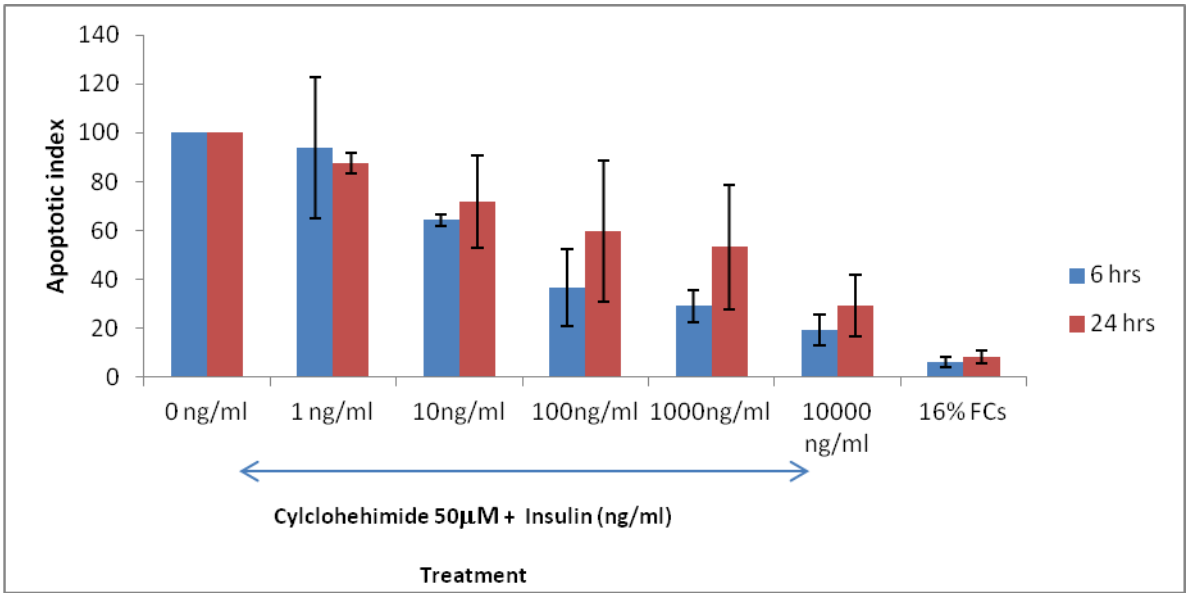


IGF-1 reduced apoptosis of activated rat pancreatic stellate cells induced by 6 hours cycloheximide exposure in a dose dependent manner over the concentration range 1-100ng/ml. (Data presented are mean +/-SEM expressed as percentage of control given the arbitrary value of 100%. *p=0.066 not quite reaching statistical significance for cycloheximide versus cycloheximide with IGF-100 ng/ml at 6hrs by one sample t-test, n=3)

Caspase-3 is a central caspase in the pro apoptotic cascade (Hengartner, 2000)and can be used as an alternative assay to assess apoptosis. PSC cultured in cycloheximide 50 µM with IGF-1 (1-100ng/ml) demonstrated a dose dependent reduction in Caspase-3 activity compared to cycloheximide alone.

5.15 Insulin treated rat PSC demonstrates reduced apoptotic index as determined with acridine orange and counting following induction of apoptosis with cycloheximide

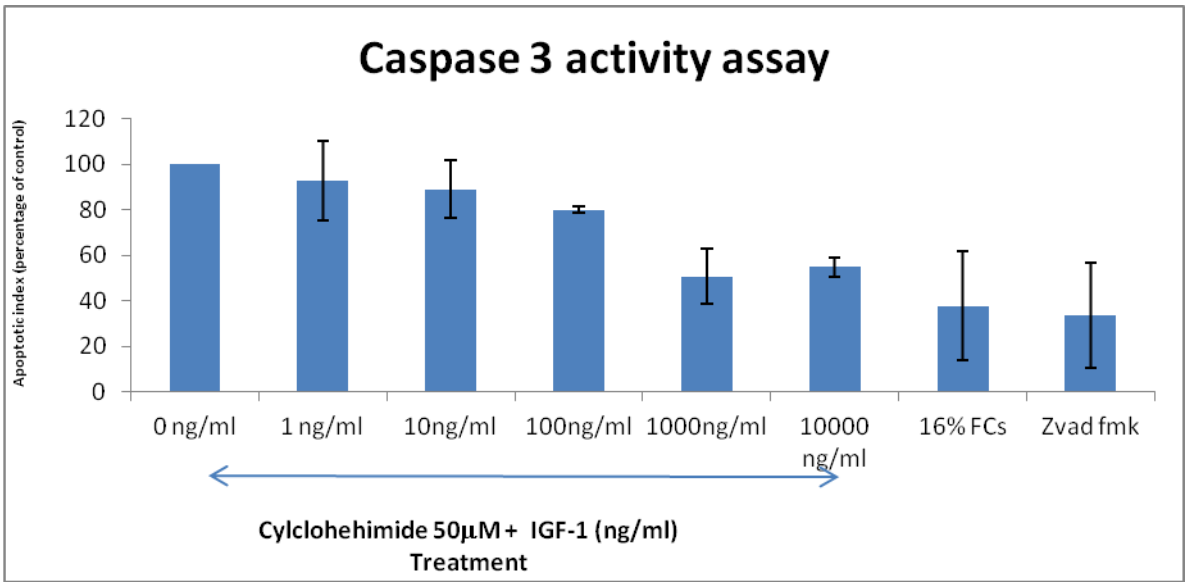
Figure 5-20 Insulin treated rat PSC have reduced apoptotic index in response to cycloheximide. Acridine orange



Apoptosis determined by acridine orange staining and counting. Data presented are mean +/-SEM expressed as percentage of control given the arbitrary value of 100%. FCS 16%- DMEM +Foetal calf serum 16%. Insulin significantly reduced apoptosis of activated rat pancreatic stellate cells induced by 6 and 24 hours cycloheximide. This occurred in a dose dependent manner over the concentration range 1-100ng/ml. $p<0.05$ for cycloheximide versus cycloheximide with 100ng/ml IGF-1 by one sample t-test, n=4

5.16 Insulin treated rat PSC have reduced Caspase-3 activity following induction of apoptosis by cycloheximide

Figure 5-21 IGF-1 treated rat PSC have reduced apoptotic index in response to cycloheximideCaspase 3



Insulin reduced apoptosis of activated rat pancreatic stellate cells induced by 6 hours cycloheximide exposure in a dose dependent manner over the concentration range 10-10000ng/ml (1.7-1700nmol/L) (n=3)

Both insulin and IGF-1 acted as survival factors for cycloheximide induced apoptosis, in acridine orange studies, relative to cycloheximide induced apoptosis. Treatment with IGF-1, 100ng/ml reduced apoptosis by 63% at 6 hrs and 24 hrs. Insulin, 10000ng/ml reduced apoptosis by 81% at 6 hrs and 71% at 24 hrs ($p<0.05$, n=4). There was a concentration dependent effect in both groups. Similar results were also observed in experiments measuring Caspase-3 activity. After 6 hrs incubation with IGF-1, 100ng/ml there was a 56% reduction in caspase activity compared with cycloheximide alone ($p=0.066$). For insulin 10000ng/ml there was a 45% reduction in caspase activity at 6 hrs, although these results did not reach statistical significance ($p=0.058$).

5.17 Discussion

In the first set of studies I set out to determine if IGF-1 and insulin receptors were expressed by in vitro plastic activated pancreatic stellate cells. Immunostaining was performed on passage 1-3 activated rat pancreatic stellate cells. These receptors had previously been shown to be expressed by activated hepatic stellate cells (Scharf et al., 1998). These results confirm that both insulin receptor and IGF-1 receptor are co-expressed by PSC. Immunohistochemistry demonstrated IGF-1R positive staining within areas of fibrosis in chronic pancreatitis and pancreatic cancer, which co-localised to cells which also stained positive for α-SMA, suggesting that these represent PSC.

I also set out to demonstrate that IGF-1 and insulin have a potential anti–apoptotic role in pancreatic stellate cell apoptosis. IGF-1 increases in inflamed and fibrotic tissues and induces proliferation of rat hepatic stellate cells (Svegliati-Baroni et al., 1999). These experiments evaluate the potential roles of these growth factors in the development of pancreatic fibrosis.

IGF-1 induces an increase in PSC numbers by promoting survival as well as increasing proliferation. These data suggest that inhibition of apoptosis has a contribution to the increase in PSCs in culture models.

Importantly, it has been demonstrated that the expression of IGF-1 mRNA is significantly increased following pancreatitis and resection (Calvo, Bernatchez, Pelletier, Iovanna, & Morisset, 1997; Ludwig et al., 1999), indicating that both insulin and IGF may be important in pancreatic regeneration. I have demonstrated in these experiments that IGF-1 can be secreted by PSC, and in particular this response is increased in response to serum withdrawal. This may provide an explanation why there is a relatively low background level of apoptosis within these cells. The level of apoptosis increases following the application of cycloheximide, which is known to inhibit protein translation. This would therefore suggest, that de novo IGF-1 synthesis by PSC possibly prevents PSC apoptosis.

These data support the hypotheses that PSC apoptosis is a general response to serum withdrawal in vitro, which may have relevance to the recovery from fibrosis. The data

presented demonstrate evidence for a key role of PSC apoptosis in the spontaneous recovery from pancreatic fibrosis.

I have determined the potential roles of insulin-like growth factor (IGF)-1, insulin, and TIMP-1 in acting as survival factors for PSCs, and that specific growth factors and cytokines may regulate survival of PSCs.

5.18 Apoptosis studies and proliferation studies

It has been previously hypothesised that apoptosis might be a default pathway for activated HSCs unless specifically signalled to survive by matrix or soluble cytokine and growth factors. These experiments have therefore characterised the response of PSCs to cycloheximide and serum deprivation in the presence and absence of specific growth factors.

Acridine orange staining demonstrated that serum deprivation and incubation with cycloheximide 50 μM, increased the rate of apoptosis above that observed in PSC in serum containing media. This supports the hypothesis that specific growth factors may promote PSC survival. In HSC it has been observed that even in conditions of absolute serum deprivation for up to three days, the absolute level of apoptosis never rises above 25% for HSCs at 72 hours post passage, or 50% for cells 24 hours post passage (Issa et al., 2001). This possibly suggests that other influences may affect HSC apoptosis, and include factors such as stage of the cell cycle, cell-cell, cell-matrix and autocrine factors.

Incubation with cycloheximide has been shown to increase apoptosis in a variety of cell types (Baker et al., 1994; Weil et al., 1996; Issa et al., 2001) and was associated with an increase in PSC apoptosis. In vitro experiments with HSCs, have reported that 100% apoptosis is achieved after 24 hrs after exposure to cycloheximide (Issa et al., 2001). In contrast to studies on HSC, my experiments did not achieve these high levels of apoptosis with serum deprivation alone or exposure to cycloheximide 50 μM. This may reflect the fact that the PSC are inherently more resistant to apoptosis than their liver counterparts. The mechanisms which are responsible for this require further investigation, and include those already discussed.

IGF-1 has been previously assessed for its effects on proliferation of PSCs (Schneider et al., 2001b), although at concentrations up to 50 ng/ml it did not significantly increase PSC

proliferation. It has been reported to promote HSC proliferation (Svegliati-Baroni et al., 1999). I have demonstrated here that PSCs express receptors for both IGF-1 and insulin. During pancreatic injury insulin may exert a proxicrine effect from islets, and IGF-1 an autocrine effect on PSC. It has been previously demonstrated that IGF-1 has an autocrine role in HSC (Pinzani et al., 1990; Brenzel & Gressner, 1996).

These results demonstrate that IGF-1 promotes PSC survival. Incubation of PSC with IGF-1 and cycloheximide was associated with a highly reproducible although modest reduction in apoptosis in concentrations of 10 and 100ng/ml. These data suggest that IGF-1 is a candidate mediator promoting PSC survival in vitro, which may have relevance following pancreatic injury. This effect has been previously demonstrated in liver fibrosis (Issa et al., 2001). IGF-1 at higher concentrations also had an effect on PSC proliferation.

These data are consistent with a model in which PSC apoptosis results from alterations in the balance of pro and antiapoptotic influences which in combination may effect the cell numbers in addition to the effects on proliferation.

It has been demonstrated using IGF1-R antisense strategy, that tumour cells deprived of IGF1-R lose their ability to respond to IGF-1 in vitro, and the change in phenotype was associated with up to a 6-fold decrease in the level of MMP-2 mRNA transcripts in the antisense-treated cells(Long, Navab, & Brodt, 1998). They also showed that ligand mediated activation of the IGF-IR induced MMP-2 synthesis in the wild type tumour cells. These and other lines of investigation have demonstrated that the IGF-1R can play a critical role in the regulation of carcinoma metastasis in vivo and may have relevance for tissue remodelling in fibrotic disease.

Taken together these data suggest that IGF-1 is a candidate mediator promoting PSC survival during injury, an effect which has been documented in HSC(Issa et al., 2001). It also provides a model in wound healing in which an autocrine, paracrine and proxicrine effect may be exerted by IGF-1 and insulin.

Cycloheximide appears to promote PSC apoptosis. One possible explanation is that inhibition of a critical autocrine or intracellular survival factor is necessary to prime highly activated PSCs for apoptosis. These data show that IGF-1 and TIMP are potential candidate factors.

5.19 Further studies

Apoptosis protection occurs principally via IGF induced activation of PI3K and Akt, as well as MAP kinase(Peruzzi et al., 1999). The effect of TIMP-1 is also in part mediated by the Akt pathway(Lee, Yoo, Bae, Kim, & Lee, 2003), and this may represent a common pathway that could be targeted to prevent survival of PSC. These effects can be assessed with the use of a number of commercially available inhibitors of these pathways.

6 Chapter 6

6.1.1 General discussion

Patients with chronic pancreatitis present at a stage where there is established fibrosis.Pancreatic fibrogenesis and its resolution is dependent on the balance between interstitial collagen deposition and its removal. PSCs play a central role in fibrosis as they are the major producers of interstitial collagens. They also have the capacity to remodel this matrix by secreting MMPs and their specific inhibitors TIMPs. Recent evidence suggests the process of fibrogenesis to be dynamic, with a potential for recovery. It has been demonstrated in liver injury there is an increase in HSC numbers through proliferation, increasing the major source of fibrillar collagen. Conversely, recovery from fibrosis is accompanied by loss of HSC through apoptosis (Iredale et al., 1998; Issa et al., 2001; Saile, Matthes, Knittel, & Ramadori, 1999)

Although much knowledge has been gained about the mechanisms that activate and increase proliferation of pancreatic stellate cells, there is currently sparse information on how these processes are terminated. In general it is accepted that cells will either revert to their quiescent state, or undergo apoptosis. Evidence for the former is currently lacking. Examining the effect of various growth factors and TIMP-1, on PSC proliferation and apoptosis is key to understanding this process. Treatments which encourage stellate cell apoptosis could therefore combat pancreatic fibrosis.

Currently, there is limited information about the regulation of apoptosis in PSC, but it appears that this would be one of the mechanisms that may be involved in resolution of fibrosis. Whilst much of our experience on activation of pancreatic stellate cells has been based on studies of hepatic stellate cells in their role in liver fibrosis, it is becoming clearer that genetic differences do exist between these different cell types(Buchholz et al., 2005). Recent studies have also highlighted that human hepatic stellate cells may also have significant differences from their rodent counterparts, particularly with respect to an increased resistance to apoptosis(Novo et al., 2006; Gentilini et al., 2009)

6.1.2 PSC isolation and cell culture

Whilst there have been substantial developments in the isolation of quiescent human pancreatic stellate cells, the extraction and viability remains highly variable. Clearly more work needs to be performed to optimise this technique. However this will remain highly problematic, due to the nature of obtaining so called “normal pancreas”. Unlike the preparation of quiescent hepatic stellate cells, where normal liver is available in large volume from resection of metastatic lesions, no such luxury exists with respect to human pancreas. This problem of scarcity of samples is compounded by the fact that the tissue samples that are available are usually small. Technicalities relating to the time in which pancreatic stellate cells are recovered from tissue specimen will no doubt turn out to be important. Maintaining viability is particularly important, in an organ known to be full of proteolytic enzymes.

An increasing number of centres are beginning to perform pancreatic transplantation and Islet transplantation, this may increase the number of normal or near normal pancreata that are available for pancreatic stellate cell preparation.

Pancreatic stellate cell studies in both rodents and humans remain problematic. This is due to the relatively low yields of cells that are extracted, plus subsequent problems with limited opportunity to passage cells, due to a reduction in proliferation and change in phenotype

It has been observed by many groups that pancreatic stellate cells appear to undergo transformation altering their appearance and reducing their rate of proliferation (Masamune et al., 2003; Sparmann et al., 2004; Jesnowski et al., 2005). These phenotypic changes are observed as the cells undergo repeated passage and has been identified as one of the problems limiting their use. These cells have a completely different phenotype, and appear to be relatively resistant to apoptosis. The mechanisms involved in this transformation are poorly understood. To my knowledge there is little data on the expression of known apoptotic regulators in quiescent, activated and terminally differentiated cells to a more ”fibroblastic” phenotype. One recent study demonstrates the translocation of $p21^{Cip1/WAF1}$ from the nucleus to the cytoplasm, confers a fibroblastic phenotype to PSC(Manapov, Muller, & Rychly, 2005) with increased resistance to apoptosis by inhibition of apoptosis signal regulating kinase 1(ASK1). There appears to be some possible conflicting data, in one study it was shown that the

increased expression of CD95 and TRAIL receptors with prolonged culture increased sensitivity to apoptosis (Klonowski-Stumpe et al., 2002). It is not clear what the explanation for this is.

This fact that rat and human primary cells undergo a significant phenotypic change with repeat passage and prolonged culture has led to the development of human pancreatic stellate cell lines (Masamune et al., 2003; Sparmann et al., 2004; Jesnowski et al., 2005). These have been created via retroviral gene transfer or via conventional subcultivation methods. Use of these cell lines may enable there to be higher throughput of experiments where the numbers of these cells are normally low. They may have greatest benefit for use in the optimisation of experimental protocols, which could then be carried out using primary cells. However, interpretation of experiments looking at the role of apoptosis in resolution of fibrosis would need to be interpreted with caution whilst using these cells. Subcultivation methods select cells which are resilient to apoptotic stimuli, and therefore are questionably resistant to the homeostatic process of apoptosis and morphological changes seen in primary cell culture.

My experience and personnel correspondence with other groups that work with PSC, has suggested that PSC may be more resistant to apoptosis than their counterpart hepatic stellate cells. The expression of anti-apoptotic markers in quiescent and activated cells needs to be studied in further depth. This could be achieved by microarray gene analysis looking at quiescent cells, activated cells and late passage cells. It is surprising that the literature on PSC apoptosis has remained relatively limited, given the substantial role of apoptosis in the resolution of fibrosis in liver disease. This may be a result of the relative paucity of pancreatic stellate cells available for culture, but also the fact that they are clearly distinct cells that may be more resistant to apoptosis than their liver counterparts. Unlike in liver studies, serum deprivation alone did not provide a strong enough apoptotic stimulus, therefore cycloheximide was routinely used. Microarray studies could also be performed comparing hepatic and pancreatic stellate cell apoptosis regulating genes at the same stage of passaging.

6.1.3 Requirement for better animal models of CP

PSC were activated on tissue culture plastic and experiments performed within a controlled system. In vivo, however, PSC are activated by cytokines and growth factors produced by

inflammatory cells in response to injury. Assumptions are made that the activated phenotype is comparable to those cells in vitro. Despite these limitations, these types of study provide us with the opportunity to investigate the effects of different cytokines and growth factors in the absence of any other confounding factors. Ideally these culture results should then confirmed in vivo using an animal model of chronic pancreatitis.

Research into chronic pancreatitis has been hindered by the lack of a reliable and reproducible animal model (reviewed in Aghdassi et al., 2011).

Model	Stimulus
Duct ligation model	Pancreatic duct ligation
	Incomplete duct ligation
	Occlusion with tissue glue, acrylate, glass particles
Repetitive acute pancreatitis	Serial caerulein injections
	Serial L-arginine injections
Alcohol feeding	Lieber-DeCarli formula
Genetic models	Wistar Bonn/Kobori (WBN/Kob) rats
	R122H transgenic mice
	SPINK3-deficient (SPINK3(-/-)) mice
	CFTR-deficient ($cftr^{m1UNC}$) mice and CFTR(-/-) pigs
	Kif3a-deficient mice
	PERK-deficient (PERK(-/-)) mice
	Interleukin 1β transgenic mice (elastase sshIL-1β mice)

CFTR = cystic fibrosis transmembrane conductance regulator; PERK = protein kinase R-like endoplasmic reticulum kinase.

Figure 6.1 Animal models for chronic pancreatitis according to the type of stimulus(taken from Aghdassi et al.Fibrogenesis and tissue Repair 2011)

Fibrosis has been produced in rodents via various approaches. Rat models that have been described include: 1) intravenous injection of dubutyltin chloride (DBTC) (Emmrich et al., 2000); 2) trinitrobenzene sulfonic acid (TNBS) injection into the pancreatic duct (Haber et al., 1999); 3)

repeated intra peritoneal(IP)injections of a superoxide dismutase inhibitor (Matsumura et al., 2001); 4) spontaneous chronic pancreatitis in WBN/Kob rats (Ohashi et al., 1990); 5) intragastric high dose alcohol administration with repeated cerulein injections (Tsukamoto et al., 1988 & Uesugi et al., 2004); 6) severe hyperstimulation obstructive pancreatitis (SHOP), and IP injections of supramaximal doses of cerulein and 7) bile-pancreatic duct ligation (Murayama et al., 1999); 8) chronic alcohol administration with repeated cyclosporin and cerulein injections (Gukovsky et al., 2008) and 9) chronic alcohol administration with repeated endotoxin LPS, injections (Vonlaufen et al., 2007). Mouse models of pancreatic fibrosis include: 1) transgenic mice overexpressing TGFβ or the EGF receptor ligand heparin binding epidermal growth factor-like growth factor (HB-EGF) (Blaine et al., 2009); and 2) repetitive pancreatic injury induced by repeated injections of supramaximal caerulein (Neuschwander-Tetri et al., 2000).Choosing the right animal model is difficult, and not all of the models parallel the classic symptoms of chronic pancreatitis. Repetitive cerulein injection are amongst the most widely used and have a higher reliability and reproducibility, although it is unclear whether the mechanisms are of clinical relevance. Many of the models show rapid resolution of fibrosis following cessation of injury (Aghdassi et el., 2011). Few if any of the models have shown all the features of human disease. Although the mechanisms, have not been formally investigated it is probable that apoptosis plays a role in this scenario. The overall results from animal studies support the concept that PSCs are activated early in the course of the injury, by inflammatory mediators produced by injured acinar cells and inflammatory cells during the acute phase of the injury. Activated PSCs are the major source of collagen in fibrotic areas.

A model was submitted to the ethics committee, however, there were major reservations with respect to the severity of injury that was required. The intention was to perform a mouse model of alcoholic pancreatitis using bacterial endotoxin to stimulate pancreatitis, described by the Apte group in Sydney (Vonlaufen, 2011b). This model has previously been described in the rat (Vonlaufen et al., 2007), and has now been developed in the mouse. This possibly represents the most physiologically relevant model of chronic alcoholic pancreatitis described to date. This model replicates the changes that are seen in chronic pancreatitis including fibrosis and acinar atrophy. The basis of this model consists of feeding the animals a liquid ethanol diet for 8 weeks and subsequently thrice weekly injections of lipopolysacharide for three weeks to induce pancreatic fibrosis.

Although the changes that occur with this model have features consistent with chronic pancreatitis it would appear that these changes have the capacity to resolve on cessation of alcohol and LPS injections. To confirm that pancreatic stellate cell apoptosis plays a central role in the process of resolution of pancreatic fibrosis it would be ideal to study an animal model of resolving chronic pancreatitis. In the rat bacterial endotoxin model of alcoholic chronic pancreatitis (Vonlaufen et al., 2007), it would appear that withdrawal of alcohol significantly inhibits disease progression and reverses pancreatic fibrosis via increased PSC apoptosis (Vonlaufen et al., 2011a). It has been observed, that resolution of fibrosis occurs within a period of a few weeks once pancreatic injury has ceased. This provides a useful tool in determining the events that mediate stellate cell fate in resolution of pancreatic fibrosis.

However, in clinical cases of chronic pancreatitis these studies would be more difficult to confirm. Unlike in studies of progressive liver fibrosis, and its resolution, serial biopsies in CP remain elusive. It is therefore difficult to conceive how these studies can be confirmed in human disease. Also, unlike in liver disease the clinical significance of resolution of fibrosis remains undetermined.

It would also be useful to determine the significance of TIMP-1 by means of utilising a model of TIMP-1 knockout mice. It was unfortunate that a TIMP-1 knockout mouse model was not accomplished in this project.

Unfortunately all animal models of chronic pancreatitis that have been described would appear to fall into the category of “severe” for the purposes of the home office assessment. Most models of chronic pancreatitis rely on the frequent induction of acute pancreatitis, by injection of supraphysiologcal concentrations of the cholecystokinin analogue, cerulein. Although these models of chronic pancreatitis histologically recapitulate the changes seen in human disease, these models appear to bear little relationship to development of clinical cases of pancreatitis that is seen in humans. Any further significant developments in this area, would need to address this. The development of a mouse model of alcoholic chronic pancreatitis represents an exciting development in this area, as this has the benefits of being pathologically relevant to the most common cause of chronic pancreatitis in humans. This would also provide the opportunity to perform studies using knockout mice.The development of an animal model of CP would thus be a priority in the future directions of this project.

6.1.4 Translational research and use of current antifibrotic therapies

Distinct immunological and molecular mechanisms contribute to the progression of fibrotic disease. Dysregulated innate and adaptive immune responses are major contributors to fibrosis. However, cell-intrinsic modifications in myofibroblasts and other structural cells contribute to fibrosis and should be considered in the design and testing of new antifibrotic therapies. Fibrosis is often characterised by the activation of several profibrotic pathway, and therefore targeting multiple pathways will probably be required to reverse fibrosis. Irrespective of the eventual fate of activated PSCs, advances in our knowledge of the processes of PSC activation are aiding our ability to develop potentially useful anti-fibrotic therapies *in vivo*. Several approaches have been used to prevent pancreatic fibrosis in animal models, including:
1) antioxidants: Tasci et al., showed that in chronic pancreatitis induced with intraductal TBS, there was significant histological improvement with allopurinol treatment, with reduced collagen deposition and a reduction in pancreatic atrophy (Tasci et al., 2007).
2) TGFβ suppression:- using TGFβ neutralizing antibodies Menke et al, demonstrated that TGFβ-1 expression in pancreatic cells was suppressed after induction of acute pancreatitis by cerulein in rats (Menke, 2007). Following the application of neutralizing TGF-beta1 antibody, there was a clear reduction of extracellular matrix formation during the regeneration of the pancreas, with reduction in hydroxyproline content and the concentration of collagen types I, III, and fibronectin.
3) anti-inflammatory agents: Camostat mesilate (CM)attenuates DPTC-induced chronic pancreatitis, by inhibiting MCP-1 and TNF-α production by monocytes, and proliferation and MCP-1 production of PSCs (Gibo et al., 2005). CM is currently used in Japan for the treatment of chronic pancreatitis, but evidence for its efficacy is lacking (Motoo, 2007).
4) modulation of signaling: peroxisome proliferator-activated receptor γ(PPARγ)is a member of the nuclear hormone receptor superfamily(Issemann &Green 1990) and its ligands, which are thiazolidinedione derivatives (TZDs), are antidiabetic agents. Modulation of signaling molecules using the PPARγ ligand troglitazone prevents the progression of chronic pancreatitis in the WBN/Kob rat(Shimizu et al., 2004), with attentuation of pancreatic fibrosis and expression of α-SMA. There is no evidence that PPARγ ligands are effective against chronic pancreatitis in

humans, their possible usefulness for the treatment of chronic pancreatitis in humans needs to be evaluated in the future.

5) inhibitors of the renin-angiotensin system: The classical renin–angiotensin system (RAS) is a circulating hormonal system that is known to play a crucial role in regulating blood pressure and fluid and electrolyte homeostasis. Angiotensin (AT) II, an octapeptide produced by proteolytic cleavage of its precursor AT I by angiotensin-converting enzyme (ACE), is the physiologically active mediator of the RAS in the cardiovascular system. Under pathological conditions such as tissue remodelling, AT II acts as a growth factor on myocytes and myofibroblasts(Leung et al., 1997, Tahmasebi et al., 1999 &Leung 2000). Kuno et al., found that the ACE inhibitor lisinopril improved the histological changes of chronic pancreatitis, and reduced myeloperoxidase (MPO) activity, hydroxyproline content, and expression of TGF-β1 mRNA in the pancreas of WBN-Kob rats(Kuno et al., 2003). In a study conducted on a model of chronic pancreatitis induced by repeated injection of cerulein, pancreatic fibrosis was found to be significantly less severe in AT I receptor-deficient mice than in wildtype mice(Nagashio, 2004). There is little involvement of the AT II–AT I receptor pathway in acute pancreatic injury, but that pathway plays a crucial role in the development of pancreatic fibrosis through AT II-mediated PSC activation and proliferation (Nagashio et al., 2004). It was also been demonstrated that AT II stimulates PSC proliferation by transactivating the epidermal growth factor (EGF)- receptor, which leads to ERK activation, and that an EGF-receptor kinase inhibitor reduced AT II stimulated DNA synthesis by PSCs(Hama, 2006) , targeting vitamin A uptake by stellate cells.

An ideal antifibrotic drug should be pancreas specific to avoid adverse effects on extra pancreatic matrix proteins and should selectively attenuate excessive collagen deposition without affecting normal ECM synthesis. It is has been demonstrated that siRNA can be successfully delivered to various organs including the pancreas through intravenous injection (Bradley et al., 2005b; Larson, Jackson, Chen, Rychahou, & Evers, 2007; Bradley et al., 2005a). This presents a potential therapeutic option utilising siRNA to modify the fibrotic process within the pancreas. A novel antifibrotic therapy has been shown to revert pancreatic fibrosis induced by dibutyltin dichloride and cerulein in rats (Ishiwatari et al., 2013). An siRNA targeting collagen-specific chaperone protein gp46 (HSP47 in humans), encapsulated in vitamin A-coupled liposomes (VA-lip-siRNAgp46) was shown to reverse pancreatic fibrosis, and that VA-lip-siRNAgp46 accumulates in activated PSCs and HSCs cells significantly more than in control rats without pancreatic fibrosis. Additionally, there was little nonspecific uptake in organs. This

approach exploits the central pivotal roles of stellate cells in fibrogenesis as well as in the uptake and storage of vitamin A in the target organs.

Removing the injurious agent contributing to pancreatitis remains the intervention of choice. However, since there are varied underlying factors responsible for chronic pancreatitis, is not always possible to achieve this goal. In alcohol dependence the patient may not be psychologically motivated enough to abstain from alcohol. Clinical evidence has demonstrated that in liver disease, cirrhosis not only undergoes histological reversion (Friedman and Bansal, 2006), but can also be associated with improved clinical outcomes (Mallet et al. 2008)]. In contrast with liver disease, outcomes in chronic pancreatitis are for the time being likely to be limited to assessment of clinical outcomes. However, due the heterogeneity of the disease this is also likely to remain problematic.

A more integrated antifibrotic strategy that simultaneously targets important inflammatory mediators, profibrotic cytokines and cell or tissue changes will probably emerge as the most successful way to treat this highly complex and difficult-to-treat pathology.

One of the major obstacles inhibiting the development of antifibrotic drugs is the lack of disease-specific biomarkers that can be used to identify patients who might benefit from a specific therapy. Therefore, it will be important to incorporate genetic and biological phenotyping in the clinical staging of patients diagnosed with fibrosis.

In the liver, advances in noninvasively assessing fibrosis(Baranova et al. , 2011; Castera, 2009 & Hannivoort et al., 2012)have generated enthusiasm towards developing effective antifibrotic drugs. However, the clinical problems of diagnosing chronic pancreatitis remain, and no such fibrosis markers have been evaluated for use in chronic pancreatitis. Common to other fibrotic diseases, accurate evaluation the efficacy of new antifibrotic therapies need to be developed.

However, there are still no currently available drugs that have been approved as an antifibrotic. In reality, there may already be many existing drugs with well-established safety profiles, whose mechanism of action will be also antifibrotic even though they have been developed for other indications. Challenges remain, including the typically decades-long natural history of disease that will require long-term pharmacologic intervention to prevent or reverse pancreatic fibrosis,

and akin to liver disease there are no standardised or accepted noninvasive endpoints for fibrosis assessment.

Emerging concepts suggest that interstitial fibroblasts, perivascular fibroblasts, pericytes and HSCs are all resident mesenchymal precursors of the myofibroblast. They are distinguishable from each other only by their tissue location, a variance in their anatomical connections with endothelial cells, and a limited specialization of function, such as vitamin A storage in the case of HSCs(Kisselva et al., 2012 ; Humphrreys et al., 2010, Goritz et a,l, 2011; Lin et al,, 2008 & Rock et al., 2011). This suggests that any therapeutic antifibrotic, may have a role to be evaluated across the spectrum of fibrotic diseases.

There are a number of therapeutic agents that are under investigation targeting different cells and cytokines that promote fibrosis. Numerous anti-inflammatory drugs, including corticosteroids, immuno-modulatory agents and cytotoxic drugs, have also been tested and found to have little or no therapeutic benefit in other fibrotic diseases including idiopathic pulmonary fibrosis (IPF)(Pereira et al., 2006).

Targeting key inflammatory pathways might also prove beneficial in the treatment of fibrosis. TNF-α has emerged as a key driver of fibrosis in many experimental studies, clinical trials have been initiated to examine whether inhibitors of the TNF-α pathway could be used to treat IPF (Raghu et al., 2008). Imatinib mesylate is a tyrosine kinase inhibitor with activity against the platelet-derived growth factor receptors (PDGFRs), discoidin domain receptors, c-kit, and c-Abl. The proliferative activities of PDGFRs and other tyrosine kinases in IPF pathogenesis has led to in vivo and in vitro investigations to assess imatinib as a potential inhibitor of lung fibrosis (Daniels et al., 2010). Imatinib was identified as a potent inhibitor of lung fibroblast-myofibroblast transformation and proliferation, as well as extracellular matrix production through inhibition of platelet-derived growth factor and transforming growth factor-β signaling. Based on these data, a randomized, double-blind, placebo-controlled trial to investigate the safety and clinical effects of imatinib on survival and lung function in patients with mild-to-moderate idiopathic pulmonary fibrosis was conducted and there are ongoing clinical trials (Richeldi et al, 2011). Additionally, imatinib mesylate treatment decreases fibrosis and results in the relatively rapid and steady improvement of skin changes and knee joint contractures in patients with stage 5 chronic kidney disease (Kay et al., 2008).

Promotion of apoptosis remains an attractive method to treat fibrosis for two reasons. Firstly, it would remove the cells responsible for synthesising collagen type I and TIMP-1 that inhibits collagen degradation. Further research is also required to deepen our understanding of the molecular mechanisms determining stellate cell fate, whether it is ongoing activation and proliferation, apoptosis, or reversion back to a quiescent phenotype. Classical pharmacology aims to target a molecular pathway involved in the disease as specifically as possible to minimize undesirable side effects. Increased specificity of antifibrotic therapies should be the ultimate goal of translational fibrosis research. Allowing us to apply these therapies and individualise anti-fibrotic treatment. Biomarkers that indicate the activation of certain profibrotic pathways need to be developed to guide highly specific targeted therapies.

In common with other fibrotic diseases, improved clinical models that more closely replicate pancreatic fibrosis diseases in humans are also needed.

Thus, to design effective therapeutics for fibrotic disease, we need to begin viewing fibrosis as a pathological process distinct from inflammation. Further, because inflammatory mediators are intimately involved in wound repair and regulate both the initiation and resolution of fibrosis, we need to figure out how to harness the beneficial aspects of inflammation so that fibrosis can be slowed or reversed and normal tissue regenerated, which is the ultimate goal of all fibrosis research.

Whilst biomarker techniques to individualise therapy are anticipated, less selective therapies such as the tyrosine kinase inhibitors may be provide a current option to effectively treat a spectrum of patients with fibrotic disease. Prior to routine clinical use, however, these therapies need careful preclinical and clinical evaluation to minimize the risks of failure. As we have previously learned from experience, what works perfectly in animal models may fail in humans.

6.1.4.1 Mechanistic studies

6.1.4.2 PI3K/Akt signalling pathway

The lipid kinase phosphatidylinositol 3-kinase (PI3K) and its target Akt are important downstream effectors of receptor tyrosine kinases. PI3K/Akt signalling affects proliferation, survival, and resistance to apoptosis. Akt mediates the inhibition of pro-apoptotic proteins, such as BAD and caspase 9. Moreover, it activates the transcription factor nuclear factor (NF)-κB, which promotes survival and resistance to chemotherapy. These are reviewed in (Gukovskaya & Pandol, 2004; Schneider, Siveke, Eckel, & Schmid, 2005)

TIMP-1 can activate AKT in breast cancer cell line (Lee et al., 2003). Apoptosis protection occurs principally via IGF induced activation of PI3K and Akt, as well as MAP kinase (Peruzzi et al., 1999). Further mechanistic studies need to be performed to identify potential common pathways in which TIMP-1, IGF-1 and insulin exert their anti-apoptotic effect. The Akt and ERK pathways would appear to meet this criteria, and investigations blocking this pathway would also be helpful.

6.1.4.3 Interactions between MMP/TIMP and IGF-1

Further studies would be helpful to investigate the complex relationship between MMP/TIMP and IGF-1. MMPs are thought to be important participants in the turnover of ECM in the kidney and other tissues in both normal and disease states. One study of interest shows a potential relationship between MMPs/TIMPs and IGF-1 (Lupia et al., 1999). TIMP levels were slightly decreased in diabetic NOD-MC. The addition of recombinant IGF-1 to non diabetic NOD-MC resulted in a decrease in MMP-2 and TIMP activity. Furthermore, treatment of diabetic NOD-MC with a neutralising antibody against IGF-1 increased the latent form, and restored the active form, of MMP-2. These data demonstrate that the excessive production of IGF-1 contributes to the altered ECM turnover in diabetic NOD-MC, largely through a reduction of MMP-2 activity. These data suggest that IGF-1 could be a major contributor to the development of diabetic glomerulosclerosis, The cells from diabetic NOD mice also showed a constitutive decrease in the activity of TIMP-1 and -2, which are specific endogenous inhibitors of MMP(Lupia et al., 1999).

Exposure of mesangial cells to high glucose concentrations down regulates the expression of MMP-2 in rat (Leehey, Song, Alavi, & Singh, 1995) and human MC (Caenazzo et al., 1997).

These observations may be relevant to the development of pancreatic fibrosis Expression of MMP-2 and MMP-9 has been reported in pancreatic stellate cells (Shek et al., 2002; Phillips et al., 2003a). Further investigations using geltain zymography to determine the effects of IGF-1 on MMPs would lead to greater understanding of these possible interactions.

The breakdown of the ECM by proteinases is an essential step in the processes of cancer invasion and metastasis. Malignant progression is frequently associated with upregulated production and/or activity of one or several MMPs and these enzymes have been implicated in tumour invasion *in vitro* and *in vivo(Johansson, Ahonen, & Kahari, 2000)*. MMP-2, plays a central role due to its degradation of basement membrane type IV collagen. MMP-2 is secreted as an inactive 72kDa zymogen and is activated by proteolytic cleavage, extracellularly. TIMPs can also act as inhibitors of MMP-2 processing and catalytic activity when expressed in excess and their effect on MMP-2-mediated proteolysis ultimately depends on MMP-2:TIMP ratios and the availability of MT1-MMP (Stetler-Stevenson, 2001).

The receptor for the IGF-IR and its ligands IGF-I and IGF-II play critical roles in the regulation of cellular proliferation, apoptosis and transformation. The IGFs have increasingly been recognised as important mitogens in many cell types. *In vivo*, IGF-IR-dependent tumour cell growth can be regulated in a paracrine manner, by serum or stromal cell-derived IGFs, and also via autocrine mechanisms. Increased expression of IGF-IR, IGF-I and IGF-II or a combination therefore have been documented in many animal and human malignancies (Macaulay, 1992), and prospective clinical studies identified a high-plasma IGF-I level as a potential risk factor for carcinomas of the breast, prostate and colon (Samani & Brodt, 2001).

Ligand-dependent activation of the intrinsic IGF-IR tyrosine kinase results in the phosphorylation of several substrates including the insulin receptor substrates (IRS) 1–4 and Shc (Butler et al., 1998; Petley, Graff, Jiang, Yang, & Florini, 1999). This can trigger multiple signal transduction pathways including the MAP kinase (ERK) pathway implicated in receptor-mediated mitogenesis and transformation and the PI 3-kinase-dependent pathway implicated in the transmission of cell survival signals(Samani & Brodt, 2001; Hanahan & Weinberg, 2000)

Although the changes found in response to alterations in IGF-1 levels were modest at the time points studied, one might not expect large changes in ECM turnover, since pancreatic fibrosis develops over a period of decades. These studies show that PSCs have the capacity to synthesise de novo IGF-1 synthesis. There is clearly a complex relationship between MMP/TIMPS IGF-1 and glucose status.

Increased IGF-1 secretion by mesangial cells has been shown to result in a constitutive decrease in MMP-2 activity (Lupia et al., 1999). This group demonstrated the addition of recombinant IGF-1 to non diabetic mesangial cells induced a decrease in MMP-2 activity. Treatment of diabetic MC with IGF-1–neutralising antibody induced an increase of the MMP-2 activity . Thus, the MMP-2 response to the addition of IGF-1 to nondiabetic NOD-MC or the addition of a neutralising antibody to diabetic NOD-MC provides strong evidence that IGF-1 plays a determinative role in the changes found in NOD-MC after the onset of diabetes in vivo.

These results highlight the importance of characterising the nature of this switch at the cellular and molecular level to design new therapeutic approaches.

6.1.5 New insights into senescence and reversion into quiescent phenotype

Two other major mechanisms may be involved in the resolution of fibrosis, namely cell senescence and reversion to a quiescent phenotype. This has been proposed as an additional pathway to apoptosis.

The concept of cell senescence was first described as a state of terminal proliferative exhaustion in fibroblast cell culture (Hayflick, 1965).The phenotype of senescent cells is characterised by a terminal cell cycle arrest, expression of beta galactosidase, and induction of p16, p21, and p53. Studies in other organ systems, suggest that the development of senescence may be a potent mechanism of tumour suppression (Collado, Blasco, & Serrano, 2007).

Senescent cells have a characteristic phenotype that includes alterations in morphology, gene expression patterns, and chromatin structure. However, there are few studies which demonstrate the functional role of senescence in non-cancerous pathologies.

A recent study identified that senescent cells accumulate in the liver, in a carbon tetrachloride mouse model of liver fibrosis (Krizhanovsky et al., 2008). The senescent cells are derived

primarily from activated hepatic stellate cells, which initially proliferate in response to injury and produce the ECM deposited in the fibrotic scar. In mice lacking key senescence regulators, the stellate cells continue to proliferate, leading to excessive liver fibrosis. These cells demonstrate reduced secretion of ECM components, enhanced secretion of ECM-degrading enzymes, and enhanced immune surveillance. Natural killer cells preferentially targeted these senescent activated stellate cells in vitro and in vivo, facilitating the resolution of fibrosis. This intriguing new concept has provided possible further insight into the mechanisms that may be involved in the resolution fibrosis. These data point to senescence as a further mechanism by which the resolution of fibrosis may occur following injury.

It has been demonstrated that senescent human hepatic stellate cells demonstrate a lower BCL2-expressing phenotype compared with activated proliferating HSC. This might explain the higher susceptibility to apoptosis of senescent HSCs (Novo et al., 2006). These observations in human cells are critical, because human cells lack telomerase and after serial passage in culture will develop shortened telomeres and could be considered to develop a classical replicative senescence as originally defined. It remains unclear how the senescence program is initiated and regulated in HSCs. It has been speculated that overstimulation of the cells by cytokines and growth factors could lead to a state comparable to mitogenic over stimulation observed in oncogene transformed cells (Schrader, Fallowfield, & Iredale, 2009). It is yet to be determined, how relevant the observations made in a murine model are to the clinical situation and the relative role of senescence in the development and progression of human liver fibrosis.

In summary, the finding of senescence in HSCs during the development and resolution of fibrosis has identified a new model for the regulation of HSC cell numbers. Moreover, this phenomenon may prove to be important for fibrogenesis in other organs. This may have relevance to the changes in phenotype of activated pancreatic stellate cell, following prolonged culture in vitro and repeated serial passaging. This would be an area of further interest, to define a similar mechanism of pancreatic stellate cell senescence. This could be achieved at looking at various apoptotic regulators during different stages of activation and passaging. Although senescence has been observed by many groups studying pancreatic stellate cells in vivo, there have been few studies which have looked into the mechanisms that may be responsible for this. A recent study has demonstrated increased expression of cell cycle inhibitor cyclin-dependent kinase inhibitor 1A (Cdkn1a, p21/Waf1), in senescent rat PSC (Fitzner

et al., 2013). The functions of Cdkn1a were determined using two approaches, treatment of primary rat PSC with siRNA and tetracycline-regulated overexpression of Cdkn1a in immortalised rat cells. Knockdown of Cdkn1a significantly attenuated the growth-inhibitory effect of doxorubicin and strongly diminished the portion of SA β-Gal-positive cells. Overexpression of Cdkn1a enhanced both the antiproliferative effect of doxorubicin and induction of senescence. In primary PSC, doxorubicin treatment was associated with increased expression of IL-6 , while expression of the activation marker α-SMA, p53 and Cdk1 was diminished. The application of Cdkn1a siRNA specifically antagonised the effects of doxorubicin on the expression of p53, Cdk1 but not IL-6 and α-SMA.

This observation of cell senescence has possibly limited the amount of work in the pancreatic stellate cell field with respects to investigations concerning apoptosis, and this is compounded by the relatively small yields of quiescent cells that are obtained with primary cell isolation techniques. However, it is now emerging as an important area in its own right which requires further study.

Two convergent studies provide compelling experimental evidence supporting in vivo HSC reversion to a quiescent phenotype during fibrosis recovery (Kisseleva et al 2012 & Troeger et al 2012). This was achieved using a combination of genetic cell fate tracking approach, single cell PCR and microarray analysis.

Kisseleva et al. monitored the fate of HSCs/myofibroblasts during recovery from CCl(4)- and alcohol-induced liver fibrosis using Cre-LoxP-based genetic labelling of myofibroblasts. 50% of the myofibroblasts escaped apoptosis during regression of liver fibrosis, with down regulation of fibrogenic genes and acquisition of a phenotype similar to, but distinct from quiescent HSCs. They were more rapidly able to reactivate into myofibroblasts in response to fibrogenic stimuli and contribute to liver fibrosis. There was also an associated inactivation of HSCs with up-regulation of the anti-apoptotic genes Hspa1a/b, which participate in the survival of HSCs in culture and in vivo. Troeger et al, followed the fate of activated hepatic stellate cells using a bacterial artificial chromosome transgenic mice expressing a tamoxifen-inducible CreER driven by the endogenous promoter for vimentin (intermediate filament protein expressed in myofibroblasts), that were crossed to a fluorescent (mGFP) mouse reporter mice. Activated HSC were tracked in mice exposed to carbon tetrachloride, thioacetamide or bile duct ligation during fibrosis progression and recovery. Expression of characteristic fibrogenic markers of activated

HSCs (collagen I, TIMP1, ・ -SMA, TGF- β R) where shown to gradually decrease during the recovery period. In both studies, comparative gene analysis showed that the reverted HSCs reacquire several features of quiescence. Additionally, reverted HSC in culture displayed enhanced susceptibility to subsequent activation by TGF-β1 or mitogens. These data suggest that reverted HSC do not fully revert to a quiescent state, but retain a "primed" , preactivated intermediate state. These studies also broaden our current view of fibrogenic cell heterogeneity based on their diverse origin, including HSC, portal myofibroblasts, bone marrow-derived fibroblasts and pericytes. They further characterise, stellate cells as a plastic population that can adopt a wide range of phenotypes, ranging from a fully quiescent to an activated myofibroblastic state.

Nevertheless, a number of issues remain unresolved. In particular, whether the inactivation process is specific to HSC or also affects other fibrogenic cells remains to be determined. Another major question relates to the characterisation of the mechanisms regulating elimination of activated HSC by apoptosis or phenotype reversion, and the functional consequences of the two pathways on fibrosis resolution.

Determination of the clinical relevance of the current findings is another major issue that might prove challenging, given the current lack of assays available for HSC fate tracking in humans.

Partial reversion to a quiescent phenotype has been described in vitro in pancreatic stellate cells upon exposure to retinol and its metabolites, albumin or culture on matrigel (McCarroll et al., 2003 & Kim et al., 2009), however, there is no in vivo evidence yet to support these findings.

In my experiments, background levels of apoptosis were generally lower than that seen in hepatic stellate cells treated in the same way. Experiments within our group, demonstrate hepatic stellate cell apoptosis appears to peak at 4 to 6 hours and reach levels of approximately 30-40% after exposure to cycloheximide 50 μM. This may reflect inherent differences between pancreatic and hepatic stellate cells, and therefore may have a relevance to studies of apoptosis and its role in resolution of fibrosis in pancreatic disease.

It has also been commented on previously that the background level of apoptosis between cell preparations can be highly variable, 2–10% in cells cultured for 28-days (Klonowski-Stumpe et

al., 2002). Cultures with high apoptotic background were in general more sensitive to apoptotic stimuli independent of the method used to determine the percentage of apoptotic cells. These findings were similar to experiments performed in my studies. However, in each experiment, all conditions were performed with cells from one preparation and are therefore comparable. Subsequent normalisation was performed to account for these differences.

The relative contributions of the processes of apoptosis, senescence and reversion to quiescence in the removal of activated PSCs after pancreatic injury remain to be clarified.

6.2 Conclusions

The primary hypothesis set out in this thesis was that various survival factors may exert an antiapoptotic effect on pancreatic stellate cells. Currently there is sparse literature on the role of pancreatic stellate cell apoptosis in the resolution of pancreatic fibrosis. Resolution of liver fibrosis by hepatic stellate cell apoptosis, has been demonstrated to be critical event in the resolution of liver fibrosis. Identifying key molecules that may be involved in this process would greatly aid our understanding of the events involved in resolution of pancreatic fibrosis. TIMPs inhibit matrix metalloproteinases, which inhibit the degradation of abnormal fibrotic ECM. Therefore targeting molecules that prevent apoptosis would appear to be potential therapeutic targets to aid resolution of fibrosis.

In particular few studies have been performed looking at the role of apoptosis in the resolution of pancreatic fibrosis. I have identified 3 key molecules that have a relevance to fibrosis within the pancreas. Insulin is present in extremely high concentrations within the pancreas and therefore may exert a substantial proxicrine effect, and confer resistance to apoptosis, after they have become activated. These studies have demonstrated that pancreatic stellate cells have the capacity to synthesise IGF-1, and this is increased following withdrawal of serum growth factors. This suggests a possible role for autocrine effects and subsequent persistence of stellate cells despite removal of injury. Further studies are required to determine if this will have a substantial effect on apoptosis in vivo.

Fibrogenesis in chronic pancreatitis is the result of a complex cascade, beginning with acinar cell injury and necrosis. This is followed by an inflammatory reaction with activation of macrophages, aggregation of platelets, release of growth factors and reactive oxygen species, and activation of PSCs. This leads to increased synthesis of connective tissue and subsequently matrix accumulation. PSCs represent the main cellular source of extracellular matrix in chronic pancreatitis and pancreatic adenocarcinoma.

In mild pancreatic injury it would appear that there is adequate regeneration, resulting in restitution of normal tissue architecture. At present, little is known about the termination of PSC activation following acute pancreatitis. Until recently, evidence for redifferentiation of the myofibroblast-like phenotype of PSCs to the quiescent state has been lacking. It is quite likely that apoptosis of activated PSCs is involved in the termination of PSC activation. Two death receptor ligands, CD95 ligand and TRAIL, have been shown to induce apoptosis of activated PSCs. Interestingly, acinar cells and quiescent PSCs are insensitive to CD95 ligand and TRAIL when added at concentrations sufficient to induce apoptosis in activated PSCs. These data suggest that the increased susceptibility of activated PSCs to death receptor ligand-mediated apoptosis might represent an efficient mechanism for eliminating activated PSCs in acute and chronic pancreatitis.

The search for agents that either induce selective apoptosis of activated PSCs or induce redifferentiation from the myofibroblastic phenotype to the quiescent state is a major challenge in fibrosis research.

Valuable insights into the pathogenesis of pancreatic fibrogenesis have emerged in recent years. Both in vivo and in vitro data have demonstrated that PSCs have a key role in fibrogenesis, however it is important to recognise that there are complex interactions between many different cell types and molecular mediators that require further study. As we gain better understanding of these mechanisms, adequate therapies to reduce extracellular matrix deposition might be developed.

6.2.1 Further work

Despite the caveats described with TIMP-1 silencing, it would appear that the degree of silencing compared with negative control represents true TIMP-1 specific silencing. Now that the technique of siRNA transfection has been optimised with the use of TIMP-1 siRNA, further experiments targeting components involved in IGF-1 signalling would be useful to understand their role in stellate cell survival. In the first instance these would include the IGF-1 receptor and IGF-1. Additionally, now that it has been demonstrated that IGF-1 is expressed by stellate cells, it would also be important to determine if IGF binding proteins are also secreted by stellate cells. This family of binding proteins are largely responsible for the regulation of the function of IGF-1. There will also be significant interactions of these binding proteins with the ECM and MMPs and TIMPs secreted by the stellate cells.

7 Appendix

NuPage Buffers (Invitrogen)

NuPAGE® LDS Sample Buffer
Catalogue No. NP0007 (4X) 10ml
- Glycerol 4.00g
- Tris Base 0.682g
- Tris HCl 0.666g
- LDS (lithium dodecyl-sulfate) 0.800g
- EDTA 0.006g
- Serva Blue G250 0.75ml of 1% solution
- Phenol Red 0.25ml of 1% solution
- Ultrapure Water to 10ml

Tris-Acetate Running Buffer
Catalogue No. LA0041 (20X) 500ml
- Tricine 89.5g (1M)
- Tris Base 60.5g (1M)
- SDS 10g (70 mM)
- Ultrapure Water to 500 ml

MOPS SDS Running Buffer
Catalogue No. NP0001 (20X) 500ml
- MOPS 104.6g (1.00M) 3-(N-morpholino) propane sulfonic acid
- Tris Base 60.6g (1.00M)
- SDS 10.0g (69.3mM)
- EDTA 3.0g (20.5mM)
- Ultrapure Water to 500ml

NuPAGE® Transfer Buffer
Catalogue No. NP0006 (20X) 125ml
- Bicine 10.2g (500mM)
- Bis-Tris 13.08g (500mM)
- EDTA 0.75g (20.5mM)
- Ultrapure Water to 125ml

MES SDS Running Buffer
Catalogue No. NP0002 (20X) 500ml
- MES (2-(N-morpholino) ethane sulfonic acid) 97.6g (1.00M)
- Tris Base 60.6g (1.00M)
- SDS 10.0g (69.3mM)
- EDTA 3.0g (20.5mM)
- Ultrapure Water to 500ml

NuPAGE® Reducing Agent
Catalogue No. NP0009
The NuPAGE® Reducing Agent is 0.5 M DTT. It is 98% Research Grade.

Primer sequences for semi-quantitative RT-PCR were designed by PrimerDesign(Southampton, UK).

Rattus norvegicus insulin-like growth factor 1 receptor (Igf1r), mRNA

Accession Number	Official gene symbol	Sequence Length
NM_052807	Igf1r	4,696

Amplicon Details				
Product Length	Tm	Distance from 3'UTR	Single Exon	Structure
113	77.9	1170	ND	NONE

Primer Details					
Sense Primer	Position	Tm	GC%	3'dG	Dimer
ATCTACGAGACGGACTACTACC	3,526	56.5	50	-3.8	-0.7
Anti-sense Primer	**Position**	**Tm**	**GC%**	**3'dG**	**Dimer**
GACCAGACATCGGAATGAGTG	3,638	56.8	52.4	-4.2	-0.6

Rattus norvegicus insulin receptor (Insr), mRNA

Accession Number	Official gene symbol	Sequence Length
NM_017071	Insr	5,397

Amplicon Details				
Product Length	Tm	Distance from 3'UTR	Single Exon	Structure
116	76	2800	ND	NONE

Primer Details					
Sense Primer	Position	Tm	GC%	3'dG	Dimer
CAATGGTGCTGAGGACACTAG	2,597	56.7	52.4	-3.5	-1.7
Anti-sense Primer	Position	Tm	GC%	3'dG	Dimer
CGATGGTGGAGGAGATGTTG	2,712	56.1	55	-4	0

Rattus norvegicus insulin-like growth factor 1 (Igf1), transcript

Accession Number	Official gene symbol	Sequence Length
NM_001082477	Igf1	1,582

Amplicon Details				
Product Length	Tm	Distance from 3'UTR	Single Exon	Structure
89	68.4	105	ND	NONE

Primer Details					
Sense Primer	Position	Tm	GC%	3'dG	Dimer
GGTGCTATTTTGTAGTTTGTTCTATG	1,477	56.4	34.6	-2.9	-0.1
Anti-sense Primer	Position	Tm	GC%	3'dG	Dimer
GGGCTCCAGGCTTTCATTG	1,565	56.5	57.9	-3.5	0

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