Investigating the interaction between SPARC and fibronectin in pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a deadly disease with only 10% of patients surviving 10 years after diagnosis, and it accounts for ~6% of cancer deaths. PDAC is characterised by the formation of a dense stroma providing a significant barrier to drug delivery. However, the presence of the stroma has also been shown to inhibit metastasis. It is therefore important to specifically target tumour promoting aspects of the stroma as a potential way of treating pancreatic cancer.

The matricellular protein SPARC is an important regulator of cell processes and cell-cell interactions and is upregulated in a number of tumours. It has shown to play either tumour-suppressive or tumour-promoting roles depending on tumour type. Overexpression of SPARC in PDAC is correlated to poor patient prognosis. The matrix protein fibronectin provides structural support to cells and overexpression of fibronectin promotes the dense stromal formation. Previous work by Munasinghe et al., (2020) has shown that depletion of fibronectin from serum is able to 'switch' the activity of SPARC from promoting cell proliferation to inducing apoptosis.

In this study, we aimed to investigate the interaction between SPARC and fibronectin. Using a cell proliferation assay (BrdU), the addition of purified fibronectin 'switches' the activity of SPARC from promoting cancer cell proliferation to inhibiting proliferation. Further analysis of the binding of these two proteins using a solid phase binding assay (SBPA) confirmed that there is a degree of direct binding between SPARC and fibronectin, and that domains within fragment 1 of fibronectin binds to SPARC directly, but with a lesser degree.

Whilst the presence of fibronectin was able to regulate the activity of SPARC, it was determined through Coomassie staining and western blotting that it is likely that other proteins are required to fully induce the switch in SPARC activity to promote cell proliferation as seen in 10% medium. Heparin is known to modulate the structure of fibronectin to expose cryptic binding sites however, we have found that the presence of heparin does not regulate the binding of SPARC to fibronectin through a BrdU assay and a solid phase binding assay.

Whilst we have uncovered that SPARC and fibronectin are able to interact directly, further investigation is required to reveal the exact binding sites and form of fibronectin required for modulating the effect of SPARC.

Contents

1. Introduction

1.1. Pancreatic ductal adenocarcinoma (PDAC)

The pancreas is located behind the stomach in the upper abdomen. The anatomy of the pancreas is divided into four parts: the head, neck, body, and tail. The head lies adjacent to the loop of the duodenum with the tail extending across to the spleen.

Pancreatic stellate cells (PSCs) are found in the exocrine region of the pancreas and account for ~4% of the total pancreatic mass (Omary *et al*., 2007). Under normal conditions, PSCs remain in a quiescent state where they store vitamin A droplets in their cytoplasm and secrete metalloproteinases (MMPs) such as MMP-2, MMP-9 and MMP-13 which aid in ECM turnover of normal tissue (Bachem *et al*., 1998; Phillips, 2003). Activation of PSCs results in their transformation into a myofibroblast-like state to express α-SMA. This has implications in the development of fibrosis and cancer (Apte *et al*., 2004; Xu *et al*., 2010) and will be discussed in more depth in Section 1.2.3.

Multiple types of cancer can affect the pancreas: pancreatic ductal adenocarcinoma (PDAC) is the most common, accounting for ~95% of all pancreatic cancer cases. Acinar cell carcinoma, adenosquamous carcinoma and mucinous cystadenocarcinoma are other, less common types of cancer to affect the pancreas (Cancer Research UK, 2018). This study will focus on PDAC.

Pancreatic cancer is the fifth leading cancer related death in the UK and accounts for 6% of all cancer deaths (2015-2017). In the UK alone, there are around 10,300 new cases of pancreatic cancer each year. Almost half of all new cases are diagnosed in those aged 75 and over (Cancer Research UK, 2019). Symptoms of pancreatic cancer are vague and often misdiagnosed. This includes abdominal pain that radiates to the back, loss of appetite/weight loss, jaundice, uncontrollable existing diabetes or new diagnosis of diabetes, fatigue, and dark coloured urine. Symptoms typically do not present until the disease has progressed to later stages which leads to drastically reduced survival rates in patients (Cancer Research UK, 2019).

Full surgical removal of the pancreas can form part of the primary treatment for some patients. Tumours are typically resectable in stages I-II, where the tumour is still localized within the pancreas (Figure 1.1). However, only ~21% of patients are diagnosed within these stages. Due to lack of symptoms during the early stages, patients are commonly diagnosed at stage IV (68-69% of patients) when the cancer has metastasized to distant sites (Cancer Research UK, 2014). After diagnosis, only 25% of patients survive the next 12 months, this drops to 10% after 10 years (American Cancer Society, 2020). Pancreatic tumours can be detected using CT scans, MRI scans or ultrasound imaging though these methods are unable to find small cancerous lesions. Patients can receive chemotherapy treatments alongside surgical removal or alone if the tumour is not resectable.

Figure 1.1: The stages of PDAC. In stages 0-2, the tumour is resectable as the cancer cells have not metastasized to the surrounding healthy tissue. At stage 3, the cancer cells have spread into neighbouring blood vessels and lymph nodes. At stage 4, the tumour has spread to distant organs, such as the lungs or liver. From stages 3-4, the tumour can no longer be surgically resected, and the patient must rely on chemotherapy and radiation (Immunovia, 2018).

The direct cause of pancreatic cancer remains unknown however, there are various factors that increase the risk of development. It has been found that smoking increases the risk by up to 2.2 fold when compared to people who have never smoked (Bosetti *et al*., 2012). Other risk factors include diabetes (Everhart *et al*., 1995), chronic pancreatitis (Lowenfels *et al*., 1993), a family history of pancreatic cancer, obesity (Michaud *et al*., 2001), age and alcohol consumption (Maisonneuve *et al*., 2010). Somatic mutations are present in almost all cases with K-RAS mutations being the most common, present in ~90% of patients (Jones *et al*., 2008) which promotes resistance to anti-EGFR targeted therapies and can indicate poorer prognosis; 50-80% of patients have mutations in TP53 and CDKN2A (tumour suppressor genes), and SMAD4 (regulates the TGF-β signal transduction pathway) inactivating them and enabling cells to proliferate without inhibition (Zeitouni *et al*., 2016).

1.1.1. Tumour microenvironment

The tumour microenvironment is composed of cancer cells, stromal cells, and extracellular components. The stroma, also termed desmoplasia (or desmoplastic reaction, DR), is a fundamental characteristic of PDAC and develops as a result of significant overproduction of ECM proteins and extensive transformation of fibroblast cells to an active myofibroblast-like phenotype (also known as cancer-associated fibroblasts, CAFs) resulting in extensive fibrosis at the tumour site. In desmoplasia, CAFs continuously remodel the ECM resulting in increased tumour stiffness. The stroma can make up to 90% of the tumour volume, a property unique to pancreatic cancer (Xie & Xie, 2015) and is associated with an abnormal vasculature; it is composed of dense collagen (I & III) bundles, and other ECM proteins such as fibronectin, laminin, proteoglycans and SPARC, as well as a range of cells including inflammatory cells, fibroblasts and PSCs.

Figure 1.2.1: The stages of pancreatic cancer in situ. A – normal pancreatic duct. **B** – PanIN-1: cells become elongated. **C** – PanIN-2: loss of cell polarity, nuclear enlargement/crowding, development of excess chromatin and pseudostratification. **D** – PanIN-3: papillary structure, luminal necrosis, severe nuclear abnormalities. Metastasis occurs when the basement membrane is disrupted and cancer cells invade the surrounding tissues (Matthaei *et al*., 2011).

Stromal production is influenced through the activation of cancer cell-derived pathways such as transforming growth factor β (TGFβ), fibroblast growth factors (FGFs), insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF). These activated pathways lead to the secretion of structural matrix proteins such as proteoglycans, collagens and fibronectin (Mahadevan & Von Hoff, 2007). TGFβ1 has a dual nature in cancer: it acts to inhibit neoplastic growth in PanIN1 and PanIN2 lesions, however it promotes cancer cell growth in PanIN3 (Figure 1.2.1) due to the loss/inactivation of SMAD4. Before loss of SMAD4, TGFβ induces EMT and apoptosis of cancer cells through the induction of SNAIL and therefore repression of KLF5 (Padua *et al*., 2009). Conversely, the loss of SMAD4 results in TGF inducing SOX4 which, in conjunction with KLF5, promotes tumour cell proliferation as shown by David *et al*., (2016).

MMPs are involved with ECM turnover and matrix remodelling in both normal and pathological conditions. These enzymes are responsible for degrading various ECM proteins and have major roles in regulating cell proliferation, migration, and apoptosis. In PDAC, the quiescent and active form of gelatinases MMP-2 and MMP-9 are overexpressed, promoting tumour cell migration and invasion through degradation of the ECM. Activated protein C induces MMP-2 activation during angiogenesis (Nguyen *et al*., 2000). Kenny *et al*., (2008) found that inhibition of MMP-2 with antibody treatment in ovarian cancer cells significantly decreased the tumour growth and metastasis and increased the average survival in a mouse model. Other components involved in modulating the ECM which are commonly overexpressed in PDAC include tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) which promotes ECM over-production (Bramhall *et al*., 1996).

1.1.2. Activation of pancreatic stellate cells

In response to injury, PSCs become activated through multiple signalling pathways including MAPK, PI3K and JAK-STAT. Activation leads to a loss of cytoplasmic vitamin A droplets and increased expression of the cytoskeleton protein α-SMA. Expression of α-SMA along with endothelin-1 gives PSCs elasticity and ability to contract. This behaviour is implicated in further stiffening of pathological fibrosis. These activated cells develop functional changes of which include increased proliferation and migration, the synthesis of excessive ECM proteins (such as collagens I and III, and fibronectin) and matrix degrading enzymes such as MMPs and their inhibitors, the secretion of growth factors and cytokines which play roles in cell growth and migration and expression of α-SMA. Continuous activation of PSCs promotes fibrogenesis leading to a highly desmoplastic, hypo-vascular and hypoxic tumour microenvironment (Erkan *et al*., 2009, Apte *et al*., 2011). Activated cells have been shown to change into a spindle-like shape *in vitro* (Figure 1.1.2).

PSC activation is mediated by various growth factors and proinflammatory cytokines which are commonly overexpressed in necroinflammation, including TGFβ1, platelet derived growth factor (PDGF), tumour necrosis factor α (TNFα) and interleukins 1, 6 and 8 (IL-1, IL-6, IL-8). Reactive oxygen species released by damaged inflammatory cells have also been shown to activate PSCs. Numerous studies have reported that TGFβ1 increases α-SMA expression, and the proteins collagen and fibronectin in PSCs. When cultured with PDGF, PSCs increase rate of proliferation and migration (Schneider *et al*., 2001, Apte *et al*., 1999, Kordes *et al*., 2005, Shek *et al*., 2002). Activated PSCs, in turn, secrete TGFβ1, PDGF, IL-1, IL-6 and TNF-related apoptosis inducing ligand resulting in a positive feedback loop of PSC activation and proliferation, and increased stromal formation (Shek *et al*., 2002; Mahadevan & Von Hoff, 2007). Cancer cells themselves also induce PSC activation through the secretion of TGFβ1, PDGF and VEGF thus further increasing the formation of the tumour stroma.

PSCs have also been shown to actively participate in cancer cell proliferation and metastasis; co-injection of PSCs and pancreatic cancer cells in nude mice resulted in larger tumours with significant stromal involvement when compared to tumours developed following injection with pancreatic cancer cells alone (Neesse *et al*., 2007; Xu *et al*., 2010). Inhibition of PSC activation or disruption of the major signalling pathways involved in PSC overexpression or growth factors may provide a potential therapeutic target for investigation.

There have been attempts at targeting the tumour stroma as a way of treating pancreatic cancer or increasing drug assimilation however overall, these attempts have been unsuccessful, with some even having adverse effects. The Sonic Hedgehog (SHH) signalling pathway regulates multiple processes in embryonic development. In adults, dysregulation of this pathway has roles in proliferation and differentiation leading to increased tumour growth (Pasca di Magliano *et al*., 2006, Morton *et al*., 2007). Whilst inhibition of the SHH pathway using IPI-926 treatment decreased stromal formation, it was found that this significantly increased tumour growth, angiogenesis, metastasis, and reduced survival rates. The addition of a VEGFR-blocking antibody was able to selectively improve the survival of mice with SHH-deficient tumours by restraining angiogenesis suggesting that the SHH pathway may act to slow tumour growth (Rhim *et al*., 2014, Lee *et al*., 2014).

In another attempt at targeting the tumour stroma, various studies have reported on the effects of targeting pericytes. Pericytes provide structural support to blood vessels to influence vascular stability (Doore-Duffy and Cleary, 2011) but also play roles in tumour angiogenesis (Raza *et al*., 2010). It was shown that reduction of pericytes increased tumour metastasis hypoxia and expression of HIF1a. These studies have highlighted the need for further study into specific protein-protein interactions within the stroma to inhibit cancer cell proliferation and enhance drug delivery.

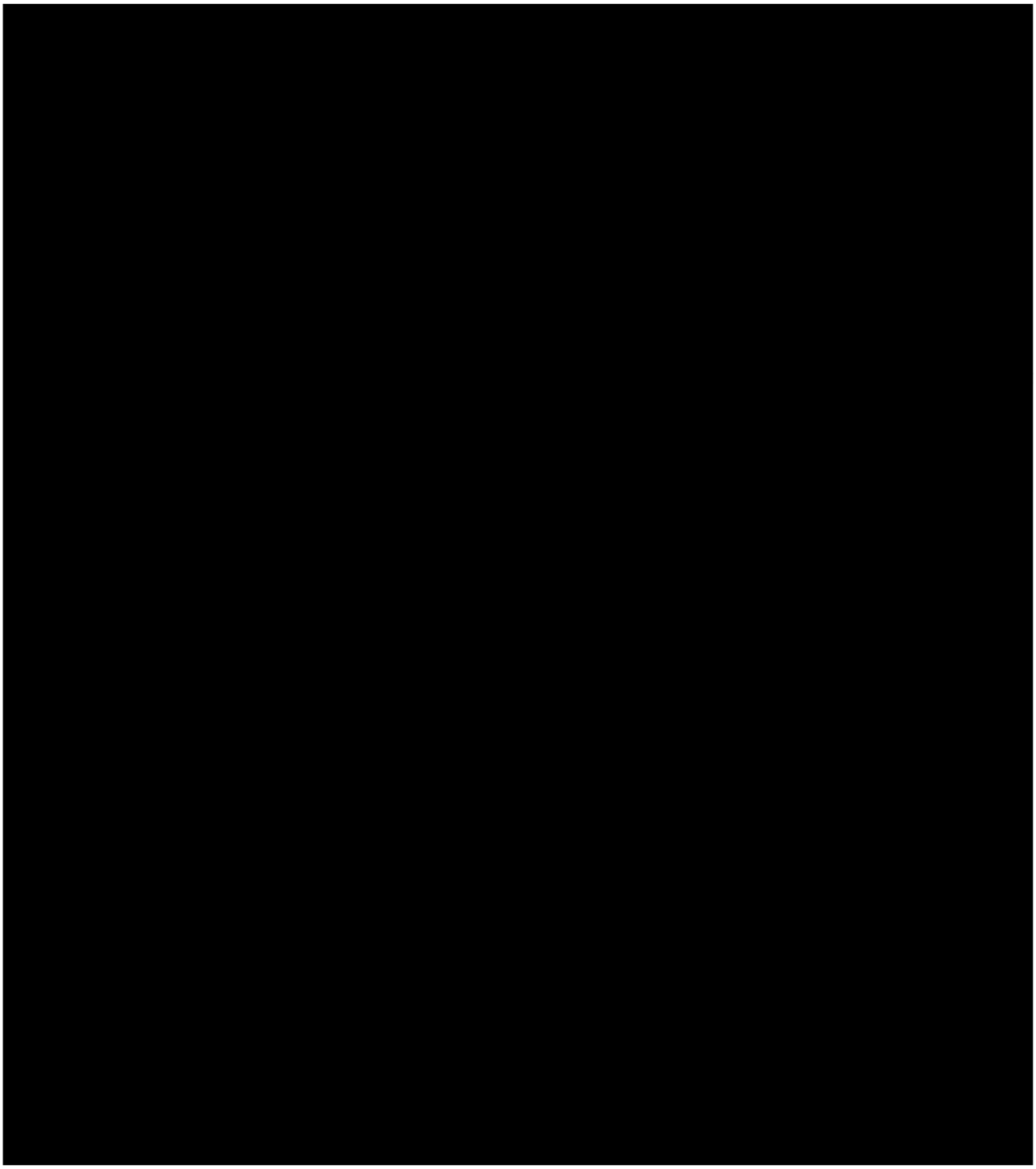


Figure 1.1.2: Activation of PSCs and their role in the tumour stroma. A) Activation of quiescent PSCs occurs through various routes to develop a myofibroblast-like phenotype. These activated PSCs lose their vitamin A droplets and express α-SMA, FAP- α and FSP-1. B) Activated PSCs synthesize collagen I and fibronectin to increase desmoplasia. They promote immunosuppression through the release of cytokines leading to mast cell and MDSC infiltration. PSCs are involved in increased proliferation, invasion, metastasis, and improved cancer cell survival. Figures from Mekapogu *et al*., (2019).

1.1.3. Drug resistance

A subsequent result of the dense stroma in pancreatic cancer is intratumoural hypoxia which also presents a considerable barrier to drug delivery. It is caused by the dense stroma compressing the fine capillary network and excessive ECM deposition. Hypoxia promotes proliferation of PSCs leading to an increase in fibrosis and further hypoxia resulting in a hypoxia-fibrosis cycle (Erkan *et al*., 2009). A study by Komar *et al*., (2009) showed that malignant pancreatic tumours had a 60% reduced blood flow compared to normal tissues and that a high ratio of glucose uptake to blood flow was a predictor of poor patient prognosis. In hypoxic states, hypoxia inducible factor 1α (HIF-1α) is significantly upregulated, which further upregulates genes associated with angiogenesis, enhanced cell survival and EMT, which all contribute to chemoresistance (Akakura *et al*., 2001).

The gold standard treatment for patients with advanced pancreatic cancer is a combination of 5-fluorouracil (5-FU)/leucovorin with irinotecan and oxaliplatin (FOLFIRINOX), and gemcitabine with nab-paclitaxel. Development of chemoresistance to gemcitabine is common with pancreatic cancer cells and severely limits the effectiveness of treatment. In addition to the tumour microenvironment, EMT (Zheng *et al*., 2015), reduction of hENT1 expression (Rauchwerger *et al*., 2000, Mackey *et al*., 1998) and inactivation of dCK (deoxycytidine kinase) (Yuriko *et al*., 2012, Ohhashi *et al*., 2008) all contribute to resistance to gemcitabine, showing that resistance is multifaceted making it more difficult to prevent chemoresistance.

1.2. The extracellular matrix

The ECM is a 3D network comprised of a multitude of components such as proteoglycans (i.e., heparan sulphate, chondroitin sulphate and keratin sulphate), proteins (i.e., collagens, elastins, fibronectin, laminins), water, GFs and MMPs (Figure 1.2). The ECM primarily provides structural and biochemical support to the surrounding cells whilst regulating cell-cell communication. It is essential for growth, wound healing and fibrosis, and influences cell processes (proliferation, migration, differentiation, and apoptosis) (Hadjipanayi *et al*., 2009, Lo *et al*., 2000, Engler *et al*., 2006, Wang *et al*., 2000). The ECM is constantly being remodelled through synthesis, degradation, reassembly, and chemical modification to maintain tissue homeostasis (Lu *et al*., 2011).

The stiffness of the ECM can very between tissues, dependent upon the concentrations of collagen and elastin (Bonnans *et al.,* 2014). In fibrosis and cancer, ECM remodelling is commonly dysregulated, with increased collagen deposition thus increasing matrix stiffness (Frantz *et al*., 2010). Rice *et al*., (2017) found that cancer cell lines grown on gels with increasing matrix stiffness showed increasing expression of vimentin with gel stiffness. Overexpression of vimentin correlates with increased tumour growth and poor patient prognosis (Ivaska *et al*., 2007) although the method of action remains

unknown. Furthermore, matrix stiffness decreases E-cadherin expression leading to a loss of cell-cell adhesion (Rice *et al*., 2017).

There has been increasing interest in the role of matricellular proteins in the development and spread of disease due to their role in maintaining cell homeostasis. One of these proteins will be discussed in further in Section 1.4.

Figure 1.2: A simplified view of the ECM. Collagens constitute the main structural portion of the ECM. Glycoproteins (laminins, fibronectins) act as ligands for cell surface receptors to modulate ECM-cell interaction. The interstitial matrix mainly contains collagen type I and fibronectin to act as a scaffold for tissues. The basement membrane is more compact and serves as a barrier between the cells and the ECM; it consists of collagen IV, laminins, HSPGs and the proteins nidogen and entactin (Bonnans *et al*., 2015).

1.2.1 Collagen

Collagen is the most abundant protein in mammals, with 28 known types. All collagens contain a triple helix, but this can range from between 96% of their structure (collagen I) to less than 10% (collagen XII). Variance between the different types is further increased by the presence of α chains, supramolecular structures and through alternative splicing (Ricard-Blum, 2011). Between 80-90% of collagen in the human body consists of types I, II and III (with type I being the most common). Collagen makes up ~30% of the total body protein.

Collagen can be categorised into 2 distinct groups: fibrillar (types I, II, III, V, XI) and non-fibrillar. The non-fibrillar group can be further subcategorised into smaller groups based upon their structure and function: FACIT (fibril-associated collagens with interrupted triple helices) (types IX, XII, XIV, XIX, XXI), multiplexin (types XV, XVIII), anchoring (type VII), and MACIT (membrane-associated collagens with

interrupted triple helices) (types XIII, XVII) (Franzke *et al*., 2005). In PDAC, collagen I is responsible for the majority of the dense stromal formation (Mollenhauer *et al*., 1987, Imamura *et al*., 1995, Olivares *et al*., 2017). Increasing concentrations of collagen I have been associated with poor patient prognosis (Whatcott *et al*., 2015) indicating that collagen may have an oncogenic function when expression is increased. Collagen IV is also highly expressed in the stroma of PDAC and promotes the proliferation and migration of cancer cells (Öhlund *et al*., 2013).

When cultured in collagen, PDAC cells shows increased ERK1/2 signalling via MT1-MMP, resulting in decreased sensitivity to gemcitabine (Shields *et al*., 2012) highlighting the important role collagen plays in cancer progression and drug resistance.

1.2.2. Heparan sulphate proteoglycans (HSPGs)

As opposed to the structural proteins in the ECM, matricellular proteins are involved in modulating the cell function through interactions with cell-surface receptors, proteases and structural matrix proteins (such as collagens) (Bornstein, 2009).

HSPGs are glycoproteins present in the ECM and at the cell surface and contain one or more heparan sulphate (HS) chains conjugated to amino acids (Esko *et al*., 2009). HSPGs have roles in multiple cellular processes including cell proliferation, migration, survival, and differentiation. In cancers, HSPGs have a tumour-specific role: they promote cancer growth in pancreatic cancer and gliomas through the regulation of fibroblast growth factor-2 (FGF-2) via FGFR1c (Su *et al*., 2006); in lung carcinoma, the HSPG SDC2 works in conjunction with the α5β1 integrin to inhibit metastasis through the formation of actin fibres (Farnedi *et al*., 2015).

Upregulation of the RTK signalling pathway by HSPGs inhibits cancer cell apoptosis through the upregulation of PI3K and MAPK survival pathways (Lemmon and Schlessinger, 2010). HSPGs are also known to increase angiogenesis, thereby promoting cancer survival by interacting with FGF, PDGF and VEGF (Lamorte *et al*., 2012).

1.3. SPARC

1.3.1. Understanding the SPARC family with a focus on SPARC

SPARC (secreted protein acidic and rich in cysteine), SPOCK1, SPOCK2, SPOCK3, SPARCL1, SMOC1, SMOC2 and Fstl1 compose the 8 members of the SPARC protein family. Each SPARC protein contains a conserved EC domain with an E-F hand motif, a follistatin like domain and an N-terminal domain (Figure 1.4); they all play roles in the modulation of the ECM, albeit with a vast variety of functions and interactions.

In contrast to the other members of the SPARC family, SPARC has been the most extensively studied in both pre-clinical and clinical settings. In adult tissues, SPARC expression is normally restricted to tissues that undergo high ECM turnover such as gut, epithelium and bone (SPARC is also commonly referred to as osteonectin or BM40) (Bornstein, 2002) or tissues undergoing repair or remodelling as a result of injury or diseases such as arthritis and cancer (Brekken & Sage, 2001). Conversely, SPARC is highly expressed during embryonic development and during tissue development, indicating that it plays in important role in development. SPARC is also involved in multiple other biological processes such as angiogenesis, matrix cell adhesion, cell differentiation, proliferation, and migration.

SPARC has shown to regulate (both directly and indirectly) the activity of MMPs, ECM proteins and growth factors (TGF-β, PDGF, VEGF and bFGF). Interestingly, one study found SPARC increased the activity of MMPs 1 and 2 and MT1 in glioma cells (McClung *et al*., 2007) but had no effect on MMP-2 activation in melanoma cells (Nischt *et al*., 2001), showing that the role of SPARC in cancer is dependent upon the tumour type and environment.

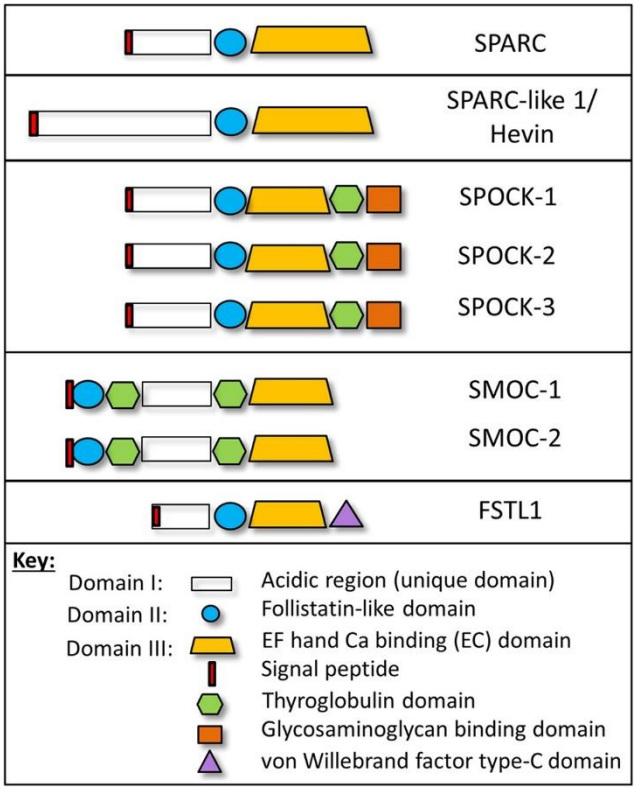


Figure 1.3: The structure of the 8 members of the SPARC family. They all contain a conserved EC domain, a follistatin like domain and a N-terminal domain (not pictured). FSTL1 is the only member of the SPARC family that contains a von Willebrand factor type C domain (taken from Viloria *et al.*, 2016)

1.3.2. SPARC in a clinical setting (with a focus on pancreatic cancer)

SPARC expression is highly dependent on the tumour type, and it can play both tumour suppressive and oncogenic roles. In breast cancer cells, low expression of SPARC leads to early onset of metastasis (Nagai *et al*., 2011) compared to patients with high levels of SPARC within cancer cells. SPARC expression in colon cancer cells is downregulated in primary tumours (Tai at al., 2005) however, exposure to demethylating agent 5-Aza-2'deoxycytidine resulted in increased SPARC expression leading to a greater rate of apoptosis (Cheetham *et al*., 2008).

It is interesting to note that in the stoma of tumours, SPARC is almost always upregulated whereas expression in cancer cells themselves is downregulated in several tumours: pancreatic, breast and ovarian (Vaz *et al*., 2015). SPARC plays an essential role in the desmoplastic reaction and promotes fibronectin unfolding, therefore acting as a mediator for fibrillogenesis, promoting an increase in stroma and ECM deposition. Stromal SPARC overexpression correlates with poor patient prognosis.

In PDAC, the cancer cells induce the overexpression of SPARC in stromal cells. The inability of the cancer cells to express SPARC is attributed to aberrant methylation of the CpG island on their promotor (Sato *et al*., 2003). Pancreatic cancer cells increase inflammatory cell recruitment and promote fibroblast proliferation, thus increasing PSC levels, which in turn release SPARC (Moir *et al*., 2015). SPARC overexpression inhibits VEGF activity and Notch signalling, leading to reduced angiogenesis and a hypoxic stromal environment (Komar *et al*., 2009). Interestingly, Seno *et al*., (2009) found that in hypoxic conditions in malignant glioma cells, SPARC was upregulated suggesting that it favours hypoxic conditions thereby promoting the invasion of hypoxic cells into the surrounding healthy tissue.

Despite the clinical evidence that SPARC overexpression has roles in tumour progression, current published research of *in vitro* experiments indicate that SPARC inhibits pancreatic cell growth and promotes apoptosis, acting as a tumour suppressor (Chen *et al*., 2010). Recently published work by Munasinghe *et al*., (2020) has uncovered this apparent contradiction. Under normal serum conditions, SPARC promotes cancer cell proliferation and survival however, depletion of fibronectin switches the function of SPARC to induce cancer cell apoptosis.

This data shows how SPARC activity is dependent on tumour type and can play both tumour suppressive and oncogenic roles depending on the environment. Due to this, when discussing the role of SPARC 'context' is important and any findings relating to SPARC cannot be applied to all tumour types.

SPARC overexpression can act as a potential biomarker to predict PDAC response to gemcitabine therapy. A study by Von Hoff *et al*., (2011) found that patients with upregulated SPARC expression survived longer when treated with a combination of nab-paclitaxel and gemcitabine compared to patients with lower SPARC expression (17.8 vs 8.1 months). SPARC has a high affinity for albumin

therefore drugs such as nab-paclitaxel which are bound to albumin can accumulate in areas with high SPARC expression. It is important to note that this method of drug delivery is best used for tumours with high perfusion and metabolic activity (Neuzillet *et al*., 2013). However, this opens the possibility of designing a combination of drugs, which can be bound to albumin to treat PDAC.

1.3.3. SPARC in pre-clinical studies

SPARC null ($SP^{-/-}$) mice display numerous abnormalities from birth including lax skin prone to tearing and a distinctive 'curly tail' in addition to severe ECM defects. There is notably a decrease in the amount of cross-linked collagen fibres in the skin of $SP^{-/-}$ mice, with smaller, more uniform collagen fibrils compared to that of wild-type mice (Bradshaw *et al*., 2003). SPARC is known to bind collagens I-V (Brekken *et al*., 2000) suggesting that SPARC has a direct role in mediating fibril formation.

In KC- $SP^{-/-}$ mice (developed using the *LSL-Kras$^{G12D/+}$; p48-Cre* model) there was a significant reduction in collagen deposition in preneoplastic and invasive pancreatic tumours and only 50-60% of the mice developed tumours compared to the KC-$SPARC^{WT}$ mice (Ramu *et al*., 2019).

Conversely, a study by Brekken *et al*., (2003) found that pancreatic tumours injected into $SP^{-/-}$ mice resulted in enhanced tumour growth, reduced collagen deposition, and reduced rates of tumour apoptosis. It is postulated that the reduction in collagen deposition in the stroma resulted in decreased mechanical resistance to tumour growth and metastasis. SPARC is a known promotor of angiogenesis yet surprisingly, there was no difference in microvessel density in $SP^{-/-}$ mice compared to normal mice. The data also showed a reduced number of cells undergoing apoptosis in $SP^{-/-}$ mice.

In human pancreatic tumours, the presence of high levels of SPARC correlates with enhanced tumour growth and poor patient prognosis (Infante *et al*., 2007, Mantoni *et al*., 2008, Shintakuya *et al*., 2018) which is contrary to the data obtained with $SP^{-/-}$ mice. Since $SP^{-/-}$ mice have developed a plethora of abnormalities not seen in humans and see a conflicting role of SPARC in tumour development, it can be reasoned that these models are unable to provide a realistic view of human pancreatic cancer.

1.4. Fibronectin (FN)

1.4.1. Fibronectin in normal physiology

Fibronectin exists as a dimer composed of two almost identical ~250 kDa subunits covalently bound at the C-terminus by a pair of disulphide bonds. Each monomer consists of three types of homologous repeating units: twelve FN type I repeats (FNI), two type II repeats (FNII) and 15 constitutively spliced and two alternatively spliced (commonly referred to extra domain A, EIIIA, and extra domain B, EIIIB) FN type III repeats (FNIII) (To & Midwood, 2011). A non-homologous region (V, variable in length), or type III connecting segment (IIICS) exists between $FNIII_{14\text{-}15}$ which provides flexibility to the fibronectin molecule (Baneyx *et al*., 2001). Splicing within FNIII leads to inclusion or exclusion of two of either EIIIA

(between $FNIII_{11-12}$) or EIIIB (between $FNIII_{7-8}$). EIIIA and EIIIB are primarily expressed during embryogenesis, and their expression in adult tissues is highly conserved. Fibronectins containing EIIIA and EIIIB domains are often termed 'oncofetal FNs' due to their role in embryogenesis and their high expression in cancers and role in progression (Inufusa *et al*., 1995).

Twenty variants of fibronectin exist in humans as a result of alternative splicing of a single mRNA (Wing *et al*., 2011). Two primary forms of fibronectin exist: plasma, (pFN) and cellular fibronectin (cFN). pFN, synthesized by hepatocytes and secreted into the blood plasma, circulates at between 220 µg/ml ±20 in normal conditions, in a compact inactive form (McCafferty *et al*., 1983). The IIICS is present in only one monomer in pFN. pFN commonly follows a relatively simple splicing pattern, with only very low levels of fibronectin containing the EIIIA and EIIIB domains (~1.3-1.4µg/ml) reported to be circulating in blood plasma (Chauhan *et al*., 2008). These isoforms have been shown to be significantly increased during tissue damage and in diseases such as ischaemic heart disease, arthritis and in the stroma of various cancers including in PDAC (Claudepierre *et al*., 1999; Inufusa *et al*., 1995; Song *et al*., 2001).

cFN is synthesized by multiple cell types including fibroblasts, endothelial cells, synovial cells and myocytes (Mao & Schwarzbauer, 2005). It contains more complex splicing of the IIICS domain to allow different forms of fibronectin to be expressed in a tissue dependant manner (Tressel *et al*., 1991). Increased expression of isoforms containing EIIIA and EIIIB are commonly observed during wound healing and tissue repair as they are able to regulate the ECM.

Fibronectin is an essential component of the ECM and under homeostatic conditions has shown to play important roles in cell adhesion, growth, differentiation, and migration.

1.4.2. Fibronectin expression in tumours

Fibronectin plays an essential role in the development of fibrosis. Defined by an excessive deposition of connective tissue, it leads to the impairment of organ structure and function. It is well known that collagen is the main ECM component of fibrosis however, the deposition of fibronectin by PSCs precedes that of collagen. Increased expression of oncofetal fibronectin isoforms is seen in areas of fibrosis (Barnes *et al*., 1999). Binding to integrins results in the extension of the molecule through the reorganisation of the actin cytoskeleton and myosin II-dependant contractility. This exposes the fibronectin binding sites to mediate fibrillogenesis and fibronectin matrix assembly.

Fibronectin upregulation has been identified as an important driver in cancer cell survival, proliferation and invasion and is associated with poor patient prognosis in part due to its chemo resistant properties. It is commonly overexpressed in various cancers including breast, lung and pancreatic (Amrutkar *et al*., 2019; Han *et al*., 2003; Ioachim *et al*., 2002). Fibronectin expression is mostly restricted to the stroma of tumours, with expression limited in the cancer cells themselves (Xiao *et al*., 2018). The presence of fibronectin is a known hallmark of EMT, a process by which

epithelial cells lose their polarity and cell-cell adhesion which precedes metastasis. Cancer cells that undergo EMT upregulate fibronectin, which further promotes EMT and triggers various signalling pathways (ERK/MAPkinase) that increase fibronectin expression. This positive feedback loop provides evidence of a close relationship between fibronectin and cancer cells (Park & Schwarzbauer, 2014).

Fibronectin could potentially serve as a prognostic biomarker. A study (Bae *et al*., 2013) concluded that there was a significant correlation between fibronectin expression and breast cancer tumour stage and metastasis. The survival rate for patients with higher fibronectin expression was considerably lower than those that exhibited negative expression of fibronectin.

In a metastatic niche, fibronectin is commonly overexpressed; this fibronectin can be traced back to the primary tumour indicating that secretion of fibronectin from the primary tumour primes a distant site for tumour growth (Erler *et al*., 2009). Blocking the integrin α5β1 reduced the expression of fibronectin containing EIIIA leading to a reduced metastatic capacity of the tumour (in colon cancer) (Ou *et al*., 2013).

1.4.3. The role of fibronectin in chemotherapy resistance

The role fibronectin plays in resistance to chemotherapy has received particular interest over the past few years. In a recent study by Pontiggia *et al*., (2012) mouse breast cancer cells incubated with fibronectin promoted resistance to tamoxifen. This resistance was induced via activation of PI3K/Akt and MAPK/Erk1/2 signalling pathways through binding with β1 integrin. It was found that inhibiting the interaction between fibronectin and β1 promoted drug-induced apoptosis of the cancer cells.

Tumours overexpressing both β1 and fibronectin have significantly increased tamoxifen resistance in contrast to MCF7 breast cancer cells that do not have fibronectin overexpression and are less resistant to tamoxifen. Increased β1 expression is associated with the EGFR/ERK signalling pathway leading to uncontrolled cell growth. It was found that blocking GPER/EGFR/ERK/ β1 signalling resulted in improved sensitivity to tamoxifen. Interestingly, it was found that culturing tamoxifen-resistant MCF7 breast cancer cells in conditioned medium from CAFs resulted in enhanced cell migration as a result of fibronectin produced by CAFs. This effect was lessened following addition of β1 integrin inhibitor (Yuan *et al*., 2015).

As well as tamoxifen, cancer cells coated atop fibronectin have shown resistance to cetuximab as a result of α5β1-fibronectin engagement. Treatment with cetuximab was also found to increase fibronectin expression from both A549 human lung adenocarcinoma cells and H1299 human non-small lung carcinoma cells from activation of p38-MAPK-ATF2 signalling pathway (Eke *et al*., 2013).

In tumours with high fibronectin expression, there is typically a loss of epithelial targets such as EGFR and HER2 therefore drugs against these targets would elicit little to no effect. It also indicates that fibronectin has a protective quality against apoptosis through the activation of intracellular signalling

pathways (Pontiggia *et al*., 2012). These studies highlight the need to focus on fibronectin as a possible therapeutic target as it plays clear roles in resistance to chemotherapy and inhibition of fibronectin and its associated integrin receptors reversed this effect (Figure 1.4).

Figure 1.4: Integrin-FN interactions in tumour therapy. A – Integrin-FN interaction activates FAK, MAPK/ERK and PI3K/AKT signalling pathways leading to cancer cell therapy resistance. B – inhibiting the interaction between integrins and FN results in increased sensitivity to chemotherapy and cytotoxicity (Wang & Hielscher, 2017).

1.5. Hypothesis and Aims

It has been established that both fibronectin and SPARC are overexpressed in PDAC and have important roles in stroma formation and chemotherapy resistance. Depletion of fibronectin has shown to switch the activity of SPARC from pancreatic cancer cell proliferation to promote cancer cell apoptosis (Munasinghe *et al.,* 2020). Understanding the interaction between SPARC and fibronectin will provide a potential therapeutic target in treating PDAC that will likely be highly tumour-specific reducing drug-induced side effects.

The aims of this project are: to investigate the SPARC-fibronectin interaction by determining the concentration of fibronectin required to induce the switch in SPARC activity and the specific fibronectin domains involved in the interaction; and to use SPARC antibodies to test whether they block the SPARC-fibronectin interaction and whether this can switch the activity of SPARC to promote cancer cell apoptosis.

2. Methods and Materials

2.1. Cell Culture

2.1.1. Media preparation

Gibco RPMI 1640 (Thermo Fisher Scientific #12004997, 500ml, lot: 2176377, qualified, Brazil) supplemented with 5ml L-glutamine and 5ml penicillin-streptomycin was used. 0.5% medium (low serum) was supplemented with 2.5ml FBS (Thermo Fisher Scientific #11573397) (final volume 512.5ml) and 10% medium (complete) was supplemented with 50ml FBS (final volume 560ml). Serum free medium did not contain FBS.

2.1.2. Cell passaging

Cells were typically passaged at 70-80% confluency. Medium was removed from the T75 flask and discarded. The attached cells were washed with 5ml PBS before adding 3ml of trypsin. The flask was returned to the incubator for 3-5 minutes (depending on cell type) until cells were detached. To stop the action of trypsin, 7ml of complete medium was added to the flask and the total volume was taken up into a 25ml Falcon tube. The cell suspension was centrifuged at 1000rpm for 4 minutes. The medium was removed without disrupting the cell pellet. The pellet was then disrupted and resuspended in 1ml fresh 0.5% medium. For a 1:10 split (both PS1 and AsPC-1 cells),100µl of this was transferred into a new T75 flask with 25ml of complete medium. For use the next day, 500µl of the cell suspension was transferred for a 1:2 split.

2.1.3. Cell freezing and storage

Cells with a low passage number were split 1:10 into 4 T75 flasks with 10% medium and left to grow until they reached at least 80% confluency. Cells were trypsinized and centrifuged under the same conditions as in section 2.1.3. Following centrifugation, the supernatant was discarded, and the cell pellet was disrupted and placed on ice. An RPMI solution containing 6ml serum free RPMI 1640, 4ml FBS and 100µl penicillin-streptomycin was filter-sterilised using a syringe fitted with a 0.2mM filter. This solution was added to the cells and the solution was mixed by pipetting up and down. A DMSO solution with 8ml serum free RPMI 1640 and 2ml DMSO was filter sterilized as previously and this was added dropwise to the cell suspension whilst shaking the tube. This shaking allowed the cells to be completely coated in the DMSO solution. From this solution, 1ml was taken up into each cryovial (Thermo Fisher Scientific #5011-0020, 2ml). For 4 T75 flasks, a total of 16 cryovials can be made. The vials were placed in a Mr Frosty (Thermo Fisher Scientific #5100-0001) containing isopropanol and put into a -80°C freezer.

For long term storage, cells were kept in liquid nitrogen (-196°C). For use, cells were part-thawed and 1ml of complete medium was added dropwise. The cells were then transferred to a 25ml Falcon tube

supplemented with 24ml of complete medium. Cell suspension was transferred into a filter-capped T75 cell culture flask (Thermo Fisher Scientific #10364131). Medium in the flask was replaced the next morning without passaging the cells. The cells were kept in an incubator set to 37°C with 5% CO_2 to promote optimal growth.

2.1.4. Cell counting (using a haemocytometer) and plating

Cells were trypsinised and centrifuged as above (2.1.2.). Once the medium was removed, and the cell pellet disrupted, the cells were resuspended in 1ml 0.5% medium. 10µl of this suspension was pipetted into an Eppendorf tube. Trypan blue was added to identify viable cells and act as a diluent. 10µl of this cell suspension was inserted into a chamber of a disposable glass haemocytometer (Immune Systems #BVS100) for cell counting. The concentration of cells/ml was calculated using the below formula:

$$Cells/ml = Average\ number\ of\ cells\ per\ square\ x\ Dilution\ factor\ x\ 10^4$$

AsPC-1 cells were plated at a density of 5,000 cells per well in 100µl per well of a 96 well plate. PS1 cells were plated at 50,000 cells per well in 2ml in a 6 well plate. The formula for calculating the amount of AsPC-1 cell suspension is shown below. The amount of 0.5% medium used was calculated by subtracting the amount of cell suspension required from the total volume needed.

$$Volume\ of\ AsPC\ cell\ suspension\ (\mu l) = \frac{50{,}000\ x\ total\ volume\ needed\ (ml)}{cells/ml}\ x\ 1000$$

2.2. Depleting fibronectin from sera

1ml gelatin sepharose beads (GE Heathcare Life Sciences #11544955) bind 1mg of FN. The ethanol:beads ratio was estimated to be 30:70%. The volume of beads proportional to the amount of FN in 50ml of FBS + 30% were centrifuged for 7 minutes at 12,000rpm. The ethanol layer was removed, and the beads were washed with 500µl PBS. The beads were centrifuged a second time with the same conditions and the clear PBS layer was removed. The beads were transferred to 50ml of FBS and incubated on a rocker for 16 hours – 2 days at 4°C. The FBS was centrifuged for 5 minutes at 4,000rpm. The FBS was removed without disrupting the beat pellet, transferred to a new falcon tube and centrifuged under the same conditions. The FBS was filter-sterilised (using a 0.2µM filter) to remove any remaining beads. An initial validation experiment using 7ml beads was adapted from the protocol described by Liu and Collodi (2002). Used beads were stored in PBS.

2.2.1. Eluting proteins bound to gelatin-sepharose beads

Poly-Prep chromatography columns (Bio-Rad #7311550, 9cm high, 10ml reservoir) were washed with ddH_20. Used beads suspended in PBS were transferred to a column and washed with 2-3 column volumes of PBS. To elute all the proteins adhered to the beads, 4ml of 8M urea solution (Sigma #U5378) was added and the column was placed on a rocker at room temperature for 25 minutes. The urea solution was drained from the column into a sterile 15ml Falcon tube.

2.2.2. Concentrating proteins eluted from gelatin-sepharose beads

The 8M urea solution containing proteins eluted from gelatin-sepharose beads was transferred to a 2-6ml Pierce Protein concentrator with a 10K molecular weight cut-off (MWCO) (Thermo Scientific #88525) and centrifuged at 3,650 x g for 15 minutes. The retentate was aspirated from the chamber and transferred to an eppendorf tube.

2.3. Bradford Assay

A serial dilution of bovine serum albumin (BSA) standards of 0 – 1.4µg/ml were made up from a 10µg/ml stock (0.01g BSA in 1ml 8M urea solution). 5µl of each standard and sample was added in duplicate to a 96 well plate, followed by 25µl of Bio-Rad working solution AS (20µl S and 1ml A). 200µl solution B was added to each well using a multichannel pipette and incubated for 15 minutes at room temperature. The plate was read at 750nm on an Epoch microplate reader.

2.4. SDS – PAGE

Samples prepared from section 2.2 were diluted 4x in sample buffer (312mM Tris-HCL pH6.8, 10% SDS, 10% β-mercaptoethanol, 25% glycerol and 0.015% bromophenol blue) and boiled at 100°C for 6 minutes. Two Mini-Protean Precast gels 4-20% (Bio-Rad #456-1093) were placed in a tank (Mini-Protean Tetra Cell #165-800, Bio-Rad) filled with 1x running buffer (250 mM Tris, 1.92 M glycine, 1% SDS, dH_20, pH 8.3 diluted 10x). 15µg total protein was added to the wells in triplicate, and 4µl of protein ladder (Thermo Scientific #11832124) was added to the end well. 130V was applied and the gel was left to run for 1 hour.

2.4.1. Coomassie Brilliant Blue staining

The gel was stained in Coomassie Brilliant Blue (1g Coomassie Blue R250, 50ml glacial acetic acid, 225ml methanol, 225ml dH_20) for 30 minutes on a rocker and subsequently rinsed with destain (50ml glacial acetic acid, 250ml methanol, 200ml dH_20) twice before leaving to soak for 3 hours with gentle agitation. The destain was discarded and the gel was washed with TBS before imaging using the Li-cor Odyssey CLx Infrared Imaging System set to auto (700nm red channel, 800nm green channel).

2.4.2. Western blot

The gel was placed between 4 sheets of filter paper (7.5x10cm, Bio-Rad #1703965) soaked in 1x Tris/Glycine transfer buffer (25mM Tris, 192mM glycine, pH8.3, Bio-Rad #1610734) with a 0.45µM nitrocellulose membrane and transferred using a Bio-Rad Trans-Blot Turbo machine for 10 minutes. The transfer was confirmed via staining with Ponceau solution then washed with 0.1% Tween-20 TBS (TTBS) and blocked in 5% milk solution for 1 hour on a rocker. This solution was discarded and 4µ rabbit anti-fibronectin with 2µl mouse anti-MMP-2 was diluted in 5ml TTBS (containing 1% BSA) and incubated with the membrane overnight at 4°C. The membrane was washed 3x with TTBS for 15 minutes per wash. IRDye800CW goat anti-mouse and IRDye680RD goat anti-rabbit secondary antibodies were diluted 1:10,000 in TTBS and incubated on a rocker for 1 hour. The membrane was washed as above and imaged using the Li-Cor Odyssey CLx Infrared Imaging System set to auto (700nm red channel, 800nm green channel).

2.4.3. Gelatin Zymography

A 7.5% polyacrylamide gel containing 4mg/ml gelatin was prepared as in table 2.4.3. The samples were diluted 5x in non-reducing sample buffer (4% SDS, 20% glycerol, 0.01% bromophenol blue, 125mM Tris-HCL, 200ml H_2O) and 10µg/well protein was loaded. The gel was run at 120V for 90 minutes then washed for 2x 30-minute periods with agitation with washing buffer (2.5% Triton X-100, 50mM Tris-HCL, 5mM $CaCl_2$, 1µM $ZnCl_2$, 228ml H_2O containing 2ml 2% NaN_3) to remove SDS. The gel was rinsed in incubation buffer (1% Triton X-100, 50mM Tris-HCL, 5mM $CaCl_2$, 1uM $ZnCl_2$, 233ml H_2O containing 2ml 2% NaN_3) for 10 minutes with agitation at 37°C before being replaced with fresh incubation buffer and left at 37°C for 24 hours with agitation. Staining solution (40ml methanol, 10ml acetic acid, 50ml H_2O, 0.5g Coomassie Blue) was added to the gel for 30 minutes at room temperature then rinsed with dH_2O before being soaked in destain solution (200ml methanol, 50ml acetic acid, 250ml H_2O) overnight. The gel was imaged as in sections 2.4.1 and 2.4.2.

Table 2.4.3: Preparation of a 7.5% polyacrylamide gel containing 4mg/ml gelatin

Separating gel		Stacking gel	
1.5M Tris pH8.8	2ml	0.5M Tris pH 6.8	1.25ml
30% acrylamide	2ml	30% acrylamide	0.67ml
H_2O	2ml	H_2O	3.08ml
4mg/ml gelatin	2ml	10% SDS	50µl
10% SDS	80µl	10% APS	50µl
10% APS	80µl	TEMED	10µl
TEMED	10µl		

2.5. Cell proliferation assay (Colourmetric BrdU)

Cell proliferation was measured using Cell Proliferation ELISA, brdU (colorimetric) (Roche #11647229001). Working solutions were prepared as per manufacturer's instructions: BrdU labelling solution was diluted 1:100 with sterile culture medium to provide a final concentration of 100µM; Anti-BrdU POD stock was dissolved in 1.1ml double distilled water for 10 minutes and this was further diluted 1:100 with antibody dilution solution prior to use; washing buffer was diluted 1:10 with dH_20 to achieve a 1x buffer.

AsPC-1 cells were cultured in triplicate in a 96 well plate with 5,000 cells per well for a total of 3 days. Cells were plated in 0.5% medium overnight to synchronise. The medium was discarded, and the treatments were premixed in an eppendorf tube and made up to 330µl (enough for 3.3 wells) before 100µl was added to each well.

For experiments using four SPARC monoclonal antibodies (purchased from DSHB), the cells were cultured in 10% medium to optimise the binding to SPARC, and the antibodies were diluted to 3µg/ml (recommended usage concentration: 2-5µg/ml) with ±10µg/ml SPARC. All other assays were done in 0.5% medium.

Both bovine plasma and human plasma fibronectin were initially titrated between 10-500µg/ml. Fibronectin fragments were diluted to the molar equivalent to 250µg/ml human plasma fibronectin ($1.2077x10^{-6}$M) prior to use and added to the cells ±10µg/ml SPARC. Recombinant human fibronectin was added at 100µg/ml. Heparin was titrated between 0-750µg/ml.

10µl per well prepared BrdU labelling solution was added to each well in the last 18 hours of culture (final BrdU label concentration of 10µM). Cells were placed back into the incubator until the end of the culture period. The medium was removed, and the cells were fixed using 200µl per well of FixDenat for 30 minutes at room temperature. FixDenat was removed and replaced with 100µl per well prepared anti-BrdU-POD solution for 90 minutes at room temperature. Wells were washed with

200µl washing solution 3x. 100µl substrate solution was added to each well with a multichannel pipette and incubated for 3-7 minutes. Absorbance was read using an epoch microplate reader set to 370mn with a reference wavelength at 492nm.

2.6. Protein enzyme-linked immunosorbent assay (ELISA)

2.6.1. SPARC ELISA

Quantikine Human SPARC Immunoassay ELISA (R&D systems #DSP00) is a solid phase ELISA to measure human SPARC in cell culture serum, plasma, and supernatant. FBS was diluted 40-fold with Calibrator Diluent. The human SPARC standard was reconstituted with 1000µl of dH_20 for 10 minutes to produce a stock solution of 500ng/ml. A serial dilution used Calibrator Diluent RD6-59 to obtain a range of concentrations of 0-50ng/ml with the Calibrator Diluent serving as the zero standard (0ng/ml). 100µl of Assay Diluent RD1-60 was added to each microplate well, followed by 100µl of standard, control, or sample per well. The standards were plated in duplicate, and samples plated in triplicate. The wells were covered and incubated for 3 hours at room temperature on a rocker.

Wells were washed and aspirated 6 times using 1x wash buffer. 200µl of cold human SPARC conjugate was added to each well and incubated for 1 hour at 4°C. The wash step was repeated then 200µl of substrate solution was added for 30 minutes at room temperature. 50µl stop solution was added and the plate was read using an epoch microplate reader at 450nm (540nm reference).

2.6.2. Fibronectin ELISA

The bovine fibronectin ELISA (Merck Life Science #RAB1011) is done in its entirety at room temperature. Assay/sample diluent buffer was diluted 5-fold with dH_2O and used to dilute FBS 500-fold. Lyophilized bovine fibronectin protein (item C) was dissolved in 660ul assay diluent to prepare a 300ng/ml standard. A dilution series from 0-300ng/ml was prepared with the assay diluent acting as the zero (0ng/ml). 100ul of each sample and standard was pipetting into each well, covered and incubated for 2.5 hours. The standards were plated in duplicate, and samples in triplicate. The wells were washed 4 times with 1x washing solution. Detection antibody was dissolved in 100µl diluent, and a working solution was prepared by diluting 80-fold. 100µl of this was added to each well and incubated for 1 hour with gentle shaking. The wash step is repeated, followed by 100µl streptavidin diluted 1500-fold incubated for 45 minutes with gentle shaking. The wash step is repeated and 100µl of substrate solution is added for 30 minutes in the dark. The reaction was stopped with 50µl stop solution and read immediately with an epoch microplate reader at 450nm.

2.6.3. Solid-phase binding assays (SPBA)

Human plasma fibronectin and four fibronectin fragments were diluted in 100mM sodium carbonate/bicarbonate buffer (pH 9.6). An untreated 96 well plate (Thermo Fisher Scientific #10216341) was coated in triplicate with 100ul of protein (with buffer as a control) and incubated overnight at 4°C. The wells were washed 4x with 200ul TBS and blocked with 2% casein for 1 hour at room temperature on a rocker. Sage *et al.,* (1989) found that the degree of SPARC binding to collagens was dependent on Ca^{2+} binding. The phosphates present in PBS may inhibit enzymatic reactions as they can sequester cations such as calcium and magnesium, therefore TBS was used instead. Initially, 40mM $CaCl_2$ was added to the TBS to determine if this increased SPARC-fibronectin binding however, this was found to increase background significantly so was not used in further experiments.

Casein was shown to be an effective blocking agent and blocked ~90% of non-specific binding of a peroxidase-conjugated immunoglobulin to a microtiter plate. Casein blocking solution was made by dissolving 2g casein powder (Merck Life Science #C3400) in 100ml 1x TBS solution (250ml dH_2O, 1.5125g Tris, 2.19g NaCl, pH 7.5), adjusting to pH 7 and left to stir overnight. The solution was passed through standard grade filter paper to removed undissolved powder. Resultant solution contained ~1.5% casein (Vogt Jr *et al.,* 1987).

The blocking solution was discarded and replaced with 50µl rhSPARC diluted in TSB to 1µg/well and incubated at 37°C for 1 hour. The wells were washed 4x with 200µl TBS. Rabbit anti-SPARC antibody (Santa Cruz Biological #sc-25574) was diluted 1:500 and incubated for 2 hours at room temperature. Wells were rewashed as before, then incubated with HRP-linked anti-rabbit secondary antibody (Cell Signalling #7074S) diluted 1:350 in TSB for 1 hour at room temperature. Wells were rewashed as before. TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution, 50µl (Merck Life Science #T0440) was added to each well and incubated for 3-5 minutes, before adding 50µl/well 0.5M H_2SO_4 as a stop solution. The plate was read at 450nm using an Epoch microplate reader.

A validation experiment was conducted by binding 0.2µg/well SPARC to the plate overnight. The method remained the same as above but the treatment step at 37°C was excluded. This was done to confirm the binding of SPARC to the anti-SPARC antibody and validate the use of casein as a blocking agent.

It was determined that all four SPARC monoclonal antibodies interfered with the binding of SPARC to the detection antibody therefore for these assays, the SPARC and antibodies were diluted to 0.5µg/well and 4.4µg/well respectably (to promote the binding of all SPARC molecules to the antibodies) and premixed for 15 minutes before being bound to the plate overnight. The wells were blocked and washed as normal, and treated with 0.5µg/well hpFN for 1 hour at 37°C. The wells were rewashed and incubated with a rabbit anti-fibronectin antibody for 2 hours. The rest of the assay remained the same.

2.7. Immunocytochemistry (ICC)

Pancreatic stellate cells (PS1 cells) were plated at 100,000 cells per well onto sterile coverslips (1.5x22x22mm) in a 6 well plate and cultured for either 24, 48 or 72 hours. At the end of the culture period, cells were fixed with 1.5ml per well 4% paraformaldehyde (PFA) for 10 minutes at room temperature. The wells were washed 3x with PBS and blocked with 10% normal horse serum (NHS) for 30 minutes at room temperature. Rabbit anti-SPARC (Santa Cruz #sc-25574), goat anti-collagen I (Abcam #Ab19811) and mouse anti-fibronectin (Abcam #Ab26245) primary antibodies were diluted 1:100 in 10% NHS and 150µl total volume was added to the wells. The plate was incubated overnight at 4°C.

Wells were washed with 2ml PBS x3 and incubated with 150µl of Alexa Fluor-conjugated secondary antibodies chicken anti-rabbit (Thermo Fisher Scientific A-21441, IgG H+L cross absorbed AF488), donkey anti-goat (Abcam #Ab150130, IgG H+L AF555) and donkey anti-mouse (Abcam #Ab150107, IgG H+L AF647), diluted 1:200 in NHS for 1 hour in the dark. Table 2.7 shows which antibodies were added to each coverslip for the sample and controls. Wells were rewashed with PBS and incubated with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, Invitrogen #10184322) diluted 1:1,000 in NHS for 5 minutes to stain the cell nuclei. the coverslips were removed from the plate with sterile tweezers and mounted onto slides using SlowFade™ Gold Antifade Mountant (Invitrogen #S36937). Slides were viewed and imaged using the Zeiss confocal LSM800 microscope.

Table 2.7: The primary and secondary antibodies incubated with the coverslips for sample and controls wells.

	Primary antibodies (1:100)	Secondary antibodies (1:200)
Sample	Rabbit anti-SPARC (Santa Cruz #sc-25574), goat anti-collagen I (Abcam #Ab19811), mouse anti-fibronectin (Abcam #Ab26245)	AF488 Chicken anti-rabbit (Thermo Fisher Scientific A-21441), AF555 donkey anti-goat (Abcam #Ab150130), AF647 donkey anti-mouse (Abcam #Ab150107)
Control SPARC	Goat anti-collagen I (Abcam #Ab19811), mouse anti-fibronectin (Abcam #Ab26245)	AF488 Chicken anti-rabbit (Thermo Fisher Scientific A-21441), AF555 donkey anti-goat (Abcam #Ab150130), AF647 donkey anti-mouse (Abcam #Ab150107)
Control fibronectin	Rabbit anti-SPARC (Santa Cruz #sc-25574), goat anti-collagen (Abcam #Ab19811)	AF488 Chicken anti-rabbit (Thermo Fisher Scientific A-21441), AF555 donkey anti-goat (Abcam #Ab150130), AF647 donkey anti-mouse (Abcam #Ab150107)
Control Collagen	Rabbit anti-SPARC (Santa Cruz #sc-25574), mouse anti-fibronectin (Abcam #Ab26245)	AF488 Chicken anti-rabbit (Thermo Fisher Scientific A-21441), AF555 donkey anti-goat (Abcam #Ab150130), AF647 donkey anti-mouse (Abcam #Ab150107)
Secondary only		AF488 Chicken anti-rabbit (Thermo Fisher Scientific A-21441), AF555 donkey anti-goat (Abcam #Ab150130), AF647 donkey anti-mouse (Abcam #Ab150107)

2.8. Colocalization and statistical analysis

Statistical calculations to determine mean, standard deviation, standard error of the mean and two sample-paired t-tests were carried out in Excel. Images acquired of stained PS1 cells were input into ImageJ software and converted to 8-bit images. The plugin 'JaCoP' was used to analyse colocalization between SPARC and collagen or fibronectin. The thresholds were adjusted manually to optimise the amount of protein in the image to analyse. The Mander's coefficient is expressed as a fraction of SPARC overlapping collagen or fibronectin and shows a combination of correlation and co-occurrence.

3. Results

3.1. Comparing the concentration of SPARC and fibronectin in 3 different batches of serum

The experiments in this project build on the previous study by Munasinghe *et al*. (2020) showing that the fibronectin in serum is important in regulating the activity of SPARC. In order to ensure consistency, it was therefore important to measure the concentrations of both SPARC and fibronectin in serum used by Munasinghe *et al*., (2020) and in the serum used in this project.

The serum was diluted using an appropriate dilution factor and a sandwich ELISA was used to determine the concentrations of fibronectin and SPARC, as shown in Figures 3.1 A and B, respectively. A wider analysis was conducted on a variety of sera from different suppliers however, these differed more significantly compared to the Gibco South American batch (lot 2) and the batch used by Munasinghe *et al*., (data not shown).

The difference in fibronectin concentration between lots 1 and 2 was ~1µg/ml (<17% difference) and therefore unlikely to be a concern in further experiments. The concentration of SPARC was ~40% lower in lot 2 and thus the particular type and batch of serum may impact experimental outcomes in further experiments. It is important to note that this difference is still small compared to the 20-fold dilution between 10% and 0.5% medium.

In later experiments, the depletion of fibronectin from serum was performed using gelatin-sepharose, therefore it was important to measure the extent of fibronectin depletion in the serum. It was confirmed that fibronectin was depleted ~80% (Figure 3.1 A). The concentration of SPARC was also determined in the fibronectin-depleted serum (Figure 3.1 B). Interestingly, ~40% depletion of SPARC was observed (corresponding to ~0.2µg/ml difference). This difference is unlikely to affect overall SPARC activity within the serum as the much higher concentration of 10µg/ml SPARC is added experimentally. Although preliminary, this data does, however, support the idea that there is a direct interaction between SPARC and fibronectin. This relationship is explored further in section 3.9.

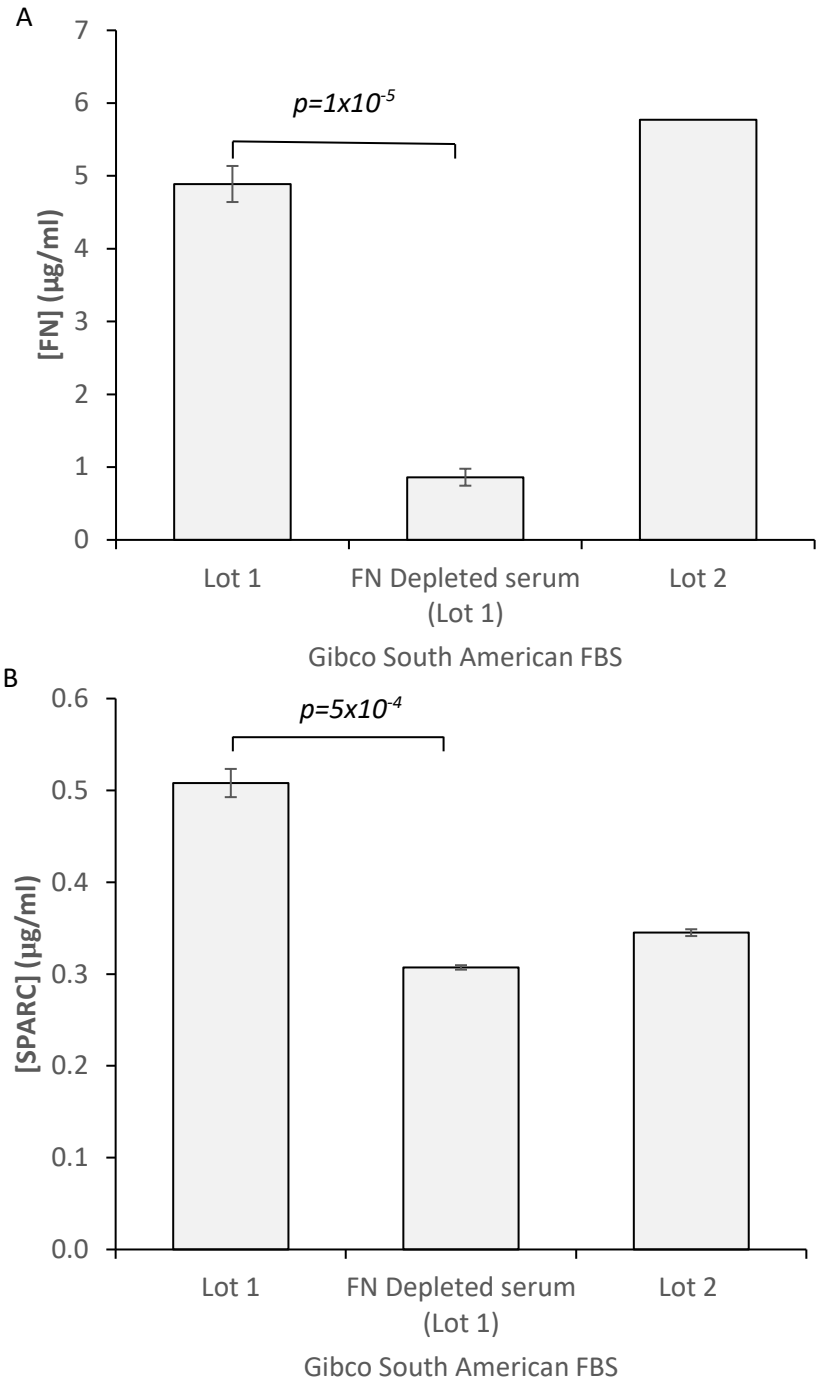


Figure 3.1: Comparing the concentration of FN (A) and SPARC (B) in three different sera: the same batch used in Munasinghe *et al.,* (2020) (lot 1), a version of this batch which has been depleted of FBS using gelatin-sepharose beads, and a current batch of FBS (lot 2). Concentrations of SPARC and FN did not differ majorly between lots 1 and 2. FN was confirmed to be depleted ~82%. Depletion of FN depletes SPARC by ~40%. Graph shows mean concentration, ±SEM (n=3). p-value calculated as two sample t-test. It should be noted that the SPARC ELISA is designed to detect human protein, and although the antibody cross-reacts with bovine proteins the absolute concentrations deduced from the standard curve and shown on the y axis are likely to be an underestimate due to the reduced affinity of the antibody.

3.2. Analysing the proteins depleted by gelatin-sepharose beads

The use of gelatin-sepharose beads is a well-established and widely used approach to depleting and purifying fibronectin (Lui and Collodi, 2002). Fibronectin binds to gelatin with a high affinity. However, there is the potential for other gelatin-binding proteins such as the gelatinases MMP-2 and MMP-9 to be depleted. Additionally, proteins such as heparin that bind to fibronectin may be indirectly depleted (Raitman *et al*., 2018).

To determine whether multiple proteins have been depleted from the serum whilst removing fibronectin, the proteins were eluted off the beads using 8M urea, concentrated and loaded onto a polyacrylamide gel. Coomassie staining was used initially to broadly identify whether multiple proteins are depleted, and subsequently western blotting was used to probe specifically for fibronectin and MMP-2. As shown in the Coomassie stain in Figure 3.2 A, the main protein eluted is around 250kDa, consistent with the molecular weight of fibronectin, and indeed this band was confirmed as fibronectin by western blot (Figure 3.2 B, shown in red).

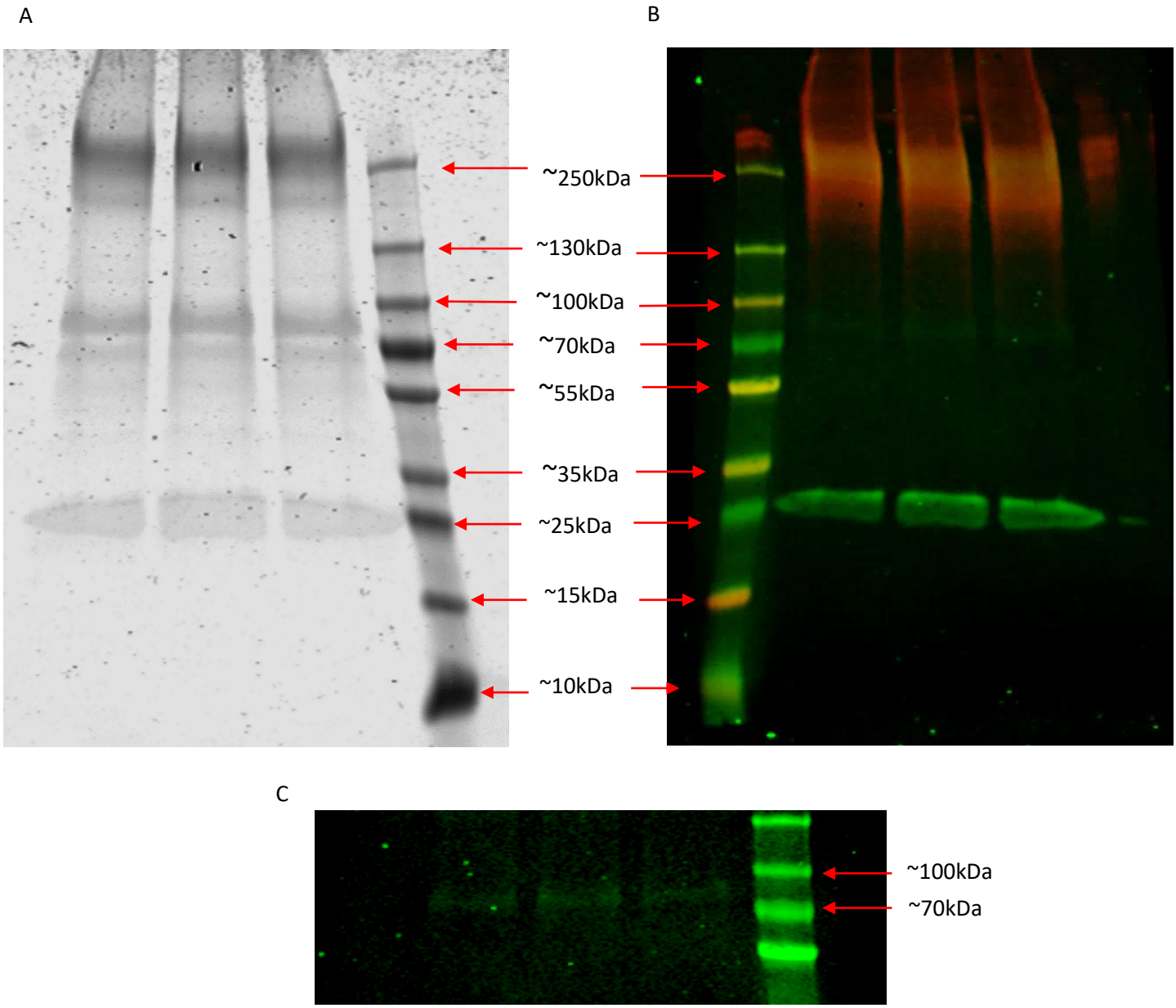


Figure 3.2: Depleting fibronectin from sera using gelatin-sepharose beads removes fibronectin but also multiple other proteins. (A) Coomassie staining of elutant from gelatin-sepharose beads. There is a very bright band around the 250kDa mark indicating a high concentration of FN. There are also bands between 70-100kDa and around 25kDa. (B) Western Blot probing for both fibronectin (red) and MMP-2 (green). The high intensity band at 250kDa confirms removal of fibronectin. (C) Over-exposure of the western blot shows a faint band between 70-100kDa indicating low levels of MMP-2. The sample was added to the wells in triplicate with the ladder on the end. This experiment was performed only once.

Coomassie staining also showed the presence of additional bands between 70-100kDa and at 25kDa suggesting that other proteins have also been removed using this technique. To determine whether the band between 70-100kDa could be MMP-2, a western blot was used probing for both fibronectin and MMP-2. The green bands correlate to staining from the presence of MMP-2 in the sample. Upon increasing the image brightness, a very light band between 70-100kDa can be seen (Figure 3.3 C), indicating that there are very low levels of MMP-2 in the sample.

As MMP-2 has a molecular weight of 72kDa (or cleaved, 64kDa) it is unlikely that the 25kDa band identified is MMP-2 (or a cleaved product of MMP-2). One possibility is that the fluorescent anti-mouse secondary antibody cross-reacts with bovine IgG light chains (~25kDa) present in serum and with affinity for the gelatin column. However, this has not previously been reported, so the 25kDa band may represent an unidentified bovine protein that cross-reacts with the anti-MMP2 antibody. Of note, the antibodies used are designed to target the human proteins and are likely to have lower affinity for the bovine proteins being detected.

There is a faint band visible at ~90kDa in the Coomassie stain which could indicate the presence of MMP-9. To better identify if MMP-2 and MMP-9 have been depleted without a high level of background in imaging, gelatin zymography was used. A 4mg/ml gelatin 7.5% polyacrylamide gel was made to detect the presence of gelatinase proteins in the sample. Active gelatinases in the sample would digest the gelatin within the polyacrylamide gel and followed by staining with Coomassie blue, these areas of degradation would show as clear bands against a blue background. Removal of the urea can, in some cases, allow a degree of protein re-naturation (Neurath *et al.,* 1942). However, we were not able to detect any gelatinase activity, and this may well be due to the lack of activity following elution in 8M urea. Use of arginine instead of urea for elution would be needed to perform zymography; alternatively use of an MMP-9 antibody for western blotting could also be used to confirm whether the 90kDa band eluted indeed corresponds to MMP-9.

In summary, using gelatin-sepharose beads primary depletes fibronectin from the serum as expected however, small amounts of MMP-2 and MMP-9, as well as potentially other proteins, are also likely to be depleted. This may be suggestive that other proteins could be involved in regulating the interaction between SPARC and fibronectin.

3.3. Optimisation of BrdU proliferation assay and validation of the 'switch' in SPARC activity by serum concentration.

Previous work in our lab has shown that while the addition of SPARC increases cancer cell proliferation in complete medium, proliferation is inhibited in low serum conditions and instead SPARC induces cancer cell apoptosis (Munasinghe *et al*., 2020). A BrdU assay was set up as a baseline to confirm this previously published data and validate that the serum and other conditions used provide the same results.

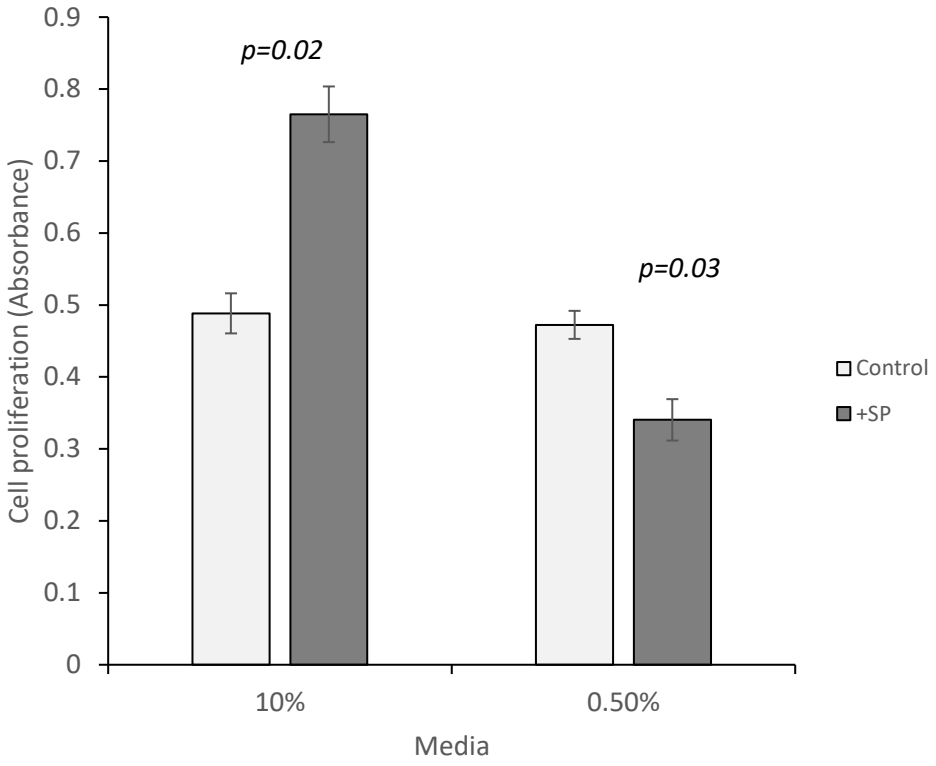


Figure 3.3: Validation of the BrdU assay and the dual nature of SPARC under different serum conditions. AsPC-1 cells were cultured in either 10% or 0.5% media over a period of 5 days ±10µg/ml SPARC. Initially the cells were plated at 5,000 cells/well and left to synchronise overnight in 0.5% media. The media was replaced the following morning and SPARC was added to the relevant wells. In the last 18 hours of culture the BrdU label was added (10µM/well) and the plate returned to the incubator. Cells were fixed and treated with anti-BrdU POD before addition of 100µl/well substrate solution. Cell proliferation is measured as relative absorbance at 370nm. Graph shows mean absorbance ±SEM, n=3. p-value calculated as two sample t-test. This data is representative of 3 individual experiments.

To optimise the assay, the experiment was repeated three times using different incubation times of the BrdU label with the cells: 2, 18 and 24 hours (data not shown). The optimal incubation time was found to be at 18 hours as the difference in ±SPARC was more clearly seen.

As shown in Figure 3.3, the proliferative and anti-proliferative effect SPARC has across 10% and 0.5% medium was validated. In 10% medium, addition of SPARC promotes cancer cell proliferation which correlates to evidence that overexpression of SPARC in PDAC leads to poorer patient prognosis (Mantoni *et al*., 2008, Sin *et al*., 2014, Shintakuya *et al*., 2018). In 0.5% it is seen that the activity of SPARC is 'switched', wherein addition of SPARC leads to inhibition of cell proliferation. The change in SPARC across the two different media indicate that other proteins present in medium interact with SPARC to change its activity.

3.4. Determining the minimal concentration of fibronectin required for the 'switch' in SPARC activity using various forms of fibronectin

We have previously shown that depleting fibronectin switches the activity of SPARC in 10% medium to inhibit cancer cell proliferation and induce apoptosis, demonstrating that the presence of fibronectin in medium is responsible for the switch in SPARC activity (Munasinghe *et al*., 2020). In these previous experiments purified bovine fibronectin was used (to mimic the effect of bovine serum), and we therefore wanted to test whether the same effect is seen with human fibronectin, and whether the effect is recapitulated with recombinant fibronectin. We also wished to determine the minimum concentration of fibronectin at which the 'switch' occurs. In order to achieve these aims, we tested whether the addition of three different forms of fibronectin to AsPC-1 cells cultured ±SPARC in 0.5% medium induces the switch in SPARC proliferative activity and titrated the concentration of fibronectin added in each case.

For the initial titration experiment, bovine plasma fibronectin (bpFN) was used. Following this, the experiment was repeated under the exact same conditions using human plasma fibronectin (hpFN) to determine whether fibronectin exhibits a conserved effect across species. Finally, recombinant human fibronectin (rhFN) was titrated.

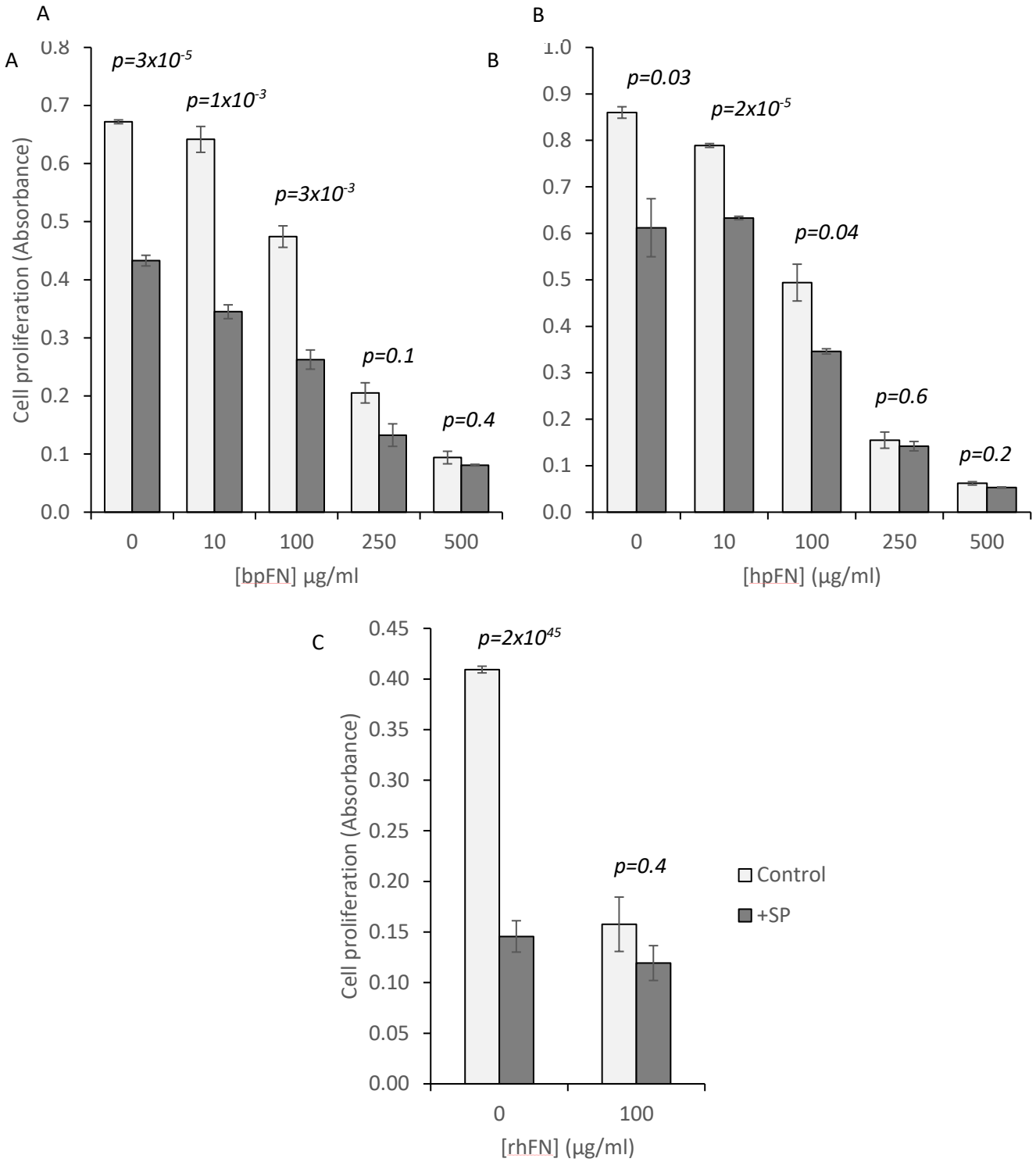


Figure 3.4: Titrating 3 different forms of fibronectin to determine the minimum concentration required for switching the activity of SPARC. Bovine plasma (A – bpFN) and human plasma (B – hpFN) were titrated between 0-500µg/ml with ±10µg/ml SPARC. Recombinant human fibronectin (C – rhFN) was added at 100µg/ml. AsPC-1 cells were cultured in 0.5% media and BrdU added for the last 18 hours of the 72-hour incubation. The key is applicable to all three graphs. Cell proliferation was measured as relative absorbance at 370nm. All graphs show mean absorbance, ±SEM n=3. p-value calculated as two sample t-test. All three experiments were done independently.

As shown in Figure 3.4, the inhibitory effect of SPARC on cell proliferation is lost as increasing amounts of fibronectin are added, and this occurs similarly for bovine and human plasma fibronectin, and for recombinant human fibronectin. The action of fibronectin therefore appears to be conserved across different species and forms of fibronectin. For both bovine and human plasma fibronectin, the concentration of fibronectin required for the switch is between 250-500µg/ml. A further titration of hpFN between 100-250µg/ml was used to identify if the switch happens below 250µg/ml (data not shown) however, <250µg/ml the switch was not clearly seen. The concentration of recombinant human fibronectin needed to block the inhibitory effect of SPARC was slightly lower, at 100 µg/ml (Figure 3.4 C). It is interesting to note that in human serum, the normal concentration of plasma fibronectin is ~220µg/ml ±20µg/ml (McCafferty *et al*., 1983) which correlates to the data obtained here.

In all further experiments, 250µg/ml hpFN will be considered the minimal concentration of fibronectin required to see the switch in SPARC activity.

3.5. Heparin inhibits pancreatic cancer cell proliferation and does not affect the regulation or binding of SPARC and fibronectin

The experiments in Munasinghe *et al*., (2020) show that depletion of fibronectin from serum switches the activity of SPARC from promoting proliferation to inducing apoptosis. In section 3.4, we showed that purified fibronectin can block the inhibition of proliferation by SPARC, but we did not observe increased proliferation in response to SPARC even at the higher concentrations of fibronectin tested. This suggests that additional proteins are also involved in the 'switch' in SPARC activity, consistent with the multiple bands identified by Coomassie staining following elution from the gelatin-sepharose beads (Figure 3.2). Heparin and heparan sulphate proteoglycans (HSPGs) play a diverse role in modulating proteins within the ECM. HSPGs have been shown to modulate the structure of fibronectin to reveal cryptic binding sites to enable direct binding to PDGFs (Smith *et al*., 2009). Treatment of fibronectin with heparin (the active part of HSPGs) results in an irreversible open conformation to expose VEGF binding sites. Heparin acts in a catalytic function wherein roughly 1 molecule of heparin is able to bind and change the structure of 20 molecules of fibronectin (Mitsi *et al*., 2008). We therefore hypothesized that heparin/HSPG may be involved in the SPARC-FN interaction.

To determine whether the addition of heparin affects the activity of SPARC in low serum cultures, heparin was initially titrated 0-750µg/ml with ±10µg/ml SPARC in AsPC-1 cells and the effect on cell proliferation determined by BrdU assay. As shown in figure 3.5 A, addition of >250µg/ml heparin inhibited cell proliferation, and also blocked the inhibition of cell proliferation by SPARC. Interestingly, this is similar to the effect seen when adding high concentrations of fibronectin. The effect of 0-250µg/ml heparin on cell growth was then determined by confluence analysis (live cell imaging) and

cell counting of viable cells. As shown in Figures 3.5.1 B and C, lower concentrations of heparin also inhibited cell growth.

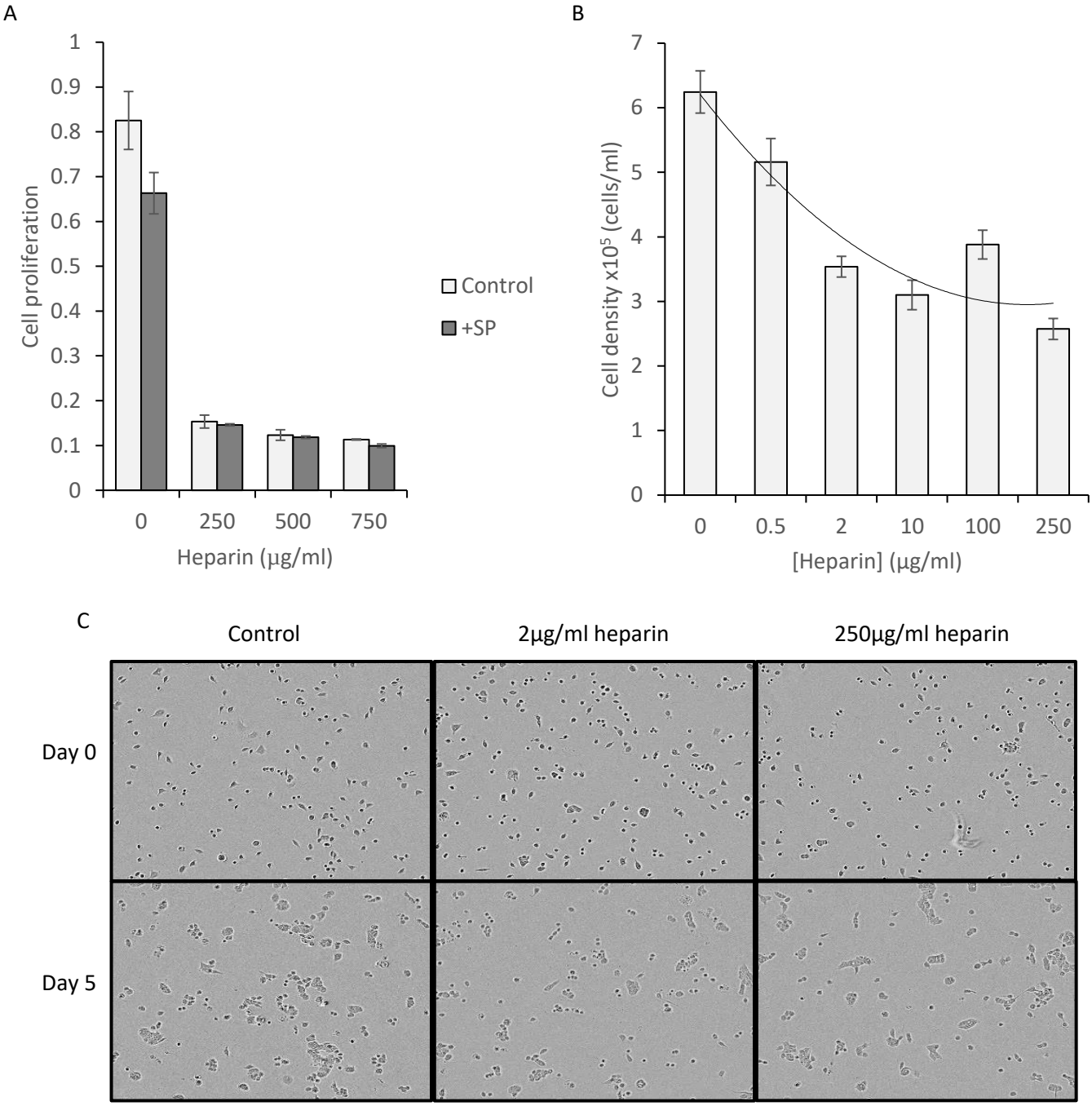


Figure 3.5.1: Testing the effect of heparin on cancer cell proliferation. Heparin was initially titrated between 0-750µg/ml with the addition of ±10µg/ml SPARC in a cell proliferation BrdU assay (A). Cell proliferation was measured as relative absorbance at 370nm. To determine the effect on cell proliferation, AsPC-1 cells were titrated with 0-250µg/ml heparin and placed in an incucyte to image for 5 days (C). The wells were typsinized at the end of the culture period and trypan blue was used to cell count (B).

In order to further test the effect of heparin on fibronectin/SPARC interactions, we examined cell proliferation (BrdU assay) in the presence and absence of heparin, fibronectin and SPARC. As shown in Figure 3.5.2, the switch in SPARC activity occurs at 250µg/ml fibronectin with and without the addition of heparin.

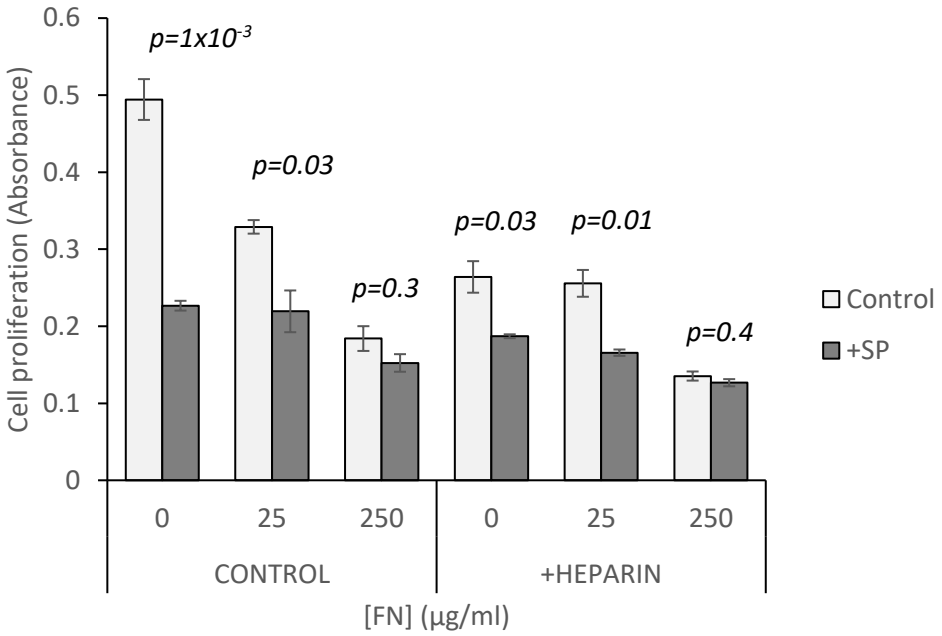


Figure 3.5.2: Determining the effect of heparin on the SPARC-fibronectin interaction. AsPC-1 cells were co-cultured ±10µg/ml SPARC, ±0.5µg/ml heparin and ±0-250µg/ml hpFN and a BrdU assay was performed. The control wells contained no heparin. All wells contained 0-250µg/ml fibronectin. Cell proliferation was measured as relative absorbance at 370nm. Data shows mean absorbance, ±SEM n=3. p-value calculated as two sample t-test.

Culturing ASPC-1s with fibronectin, heparin and SPARC does not regulate the binding of SPARC to FN, and the activity of SPARC remains unchanged between ±heparin.

3.6. SPARC antibodies inhibit the effect of SPARC on pancreatic cancer proliferation in 10% medium

Blocking the proliferative effect of SPARC in 10% medium could provide a potential insight into future therapeutic targets to treat pancreatic cancer. Furthermore, if selective blocking is seen by anti-SPARC antibodies targeting a particular SPARC domain, then this will provide information on the region of SPARC important for promoting cancer cell proliferation.

Four SPARC monoclonal antibodies from DSHB were used to block epitopes in both the follistatin-like and E-C domains (see table 3.6 for more information). AsPC-1 cells were cultured ±SPARC and ±3µg/ml of each monoclonal antibody, and the BrdU assay was used to measure cell proliferation.

Table 3.6: The epitopes targeted by four SPARC antibodies and their known effect on SPARC activity.

Monoclonal Antibody (mAb)	Epitope targeted	Isotype	Additional information
175	Unknown	MIgG2c	Blocks binding of 235 and 303
236	Follistatin domain including SPARC copper binding sequence (KKGHK)	MIgG1, κ-light chain	Blocks the formation of SPARC β1 integrin complex. Does not compete with 293
293	Follistatin domain including SPARC copper binding sequence (KKGHK)	MIgG2b, κ-light chain	Inhibits SPARC binding to Coll I and laminin-1 by 50% Does not compete with 236
303	Extracellular domain	MIgG1, κ-light chain	

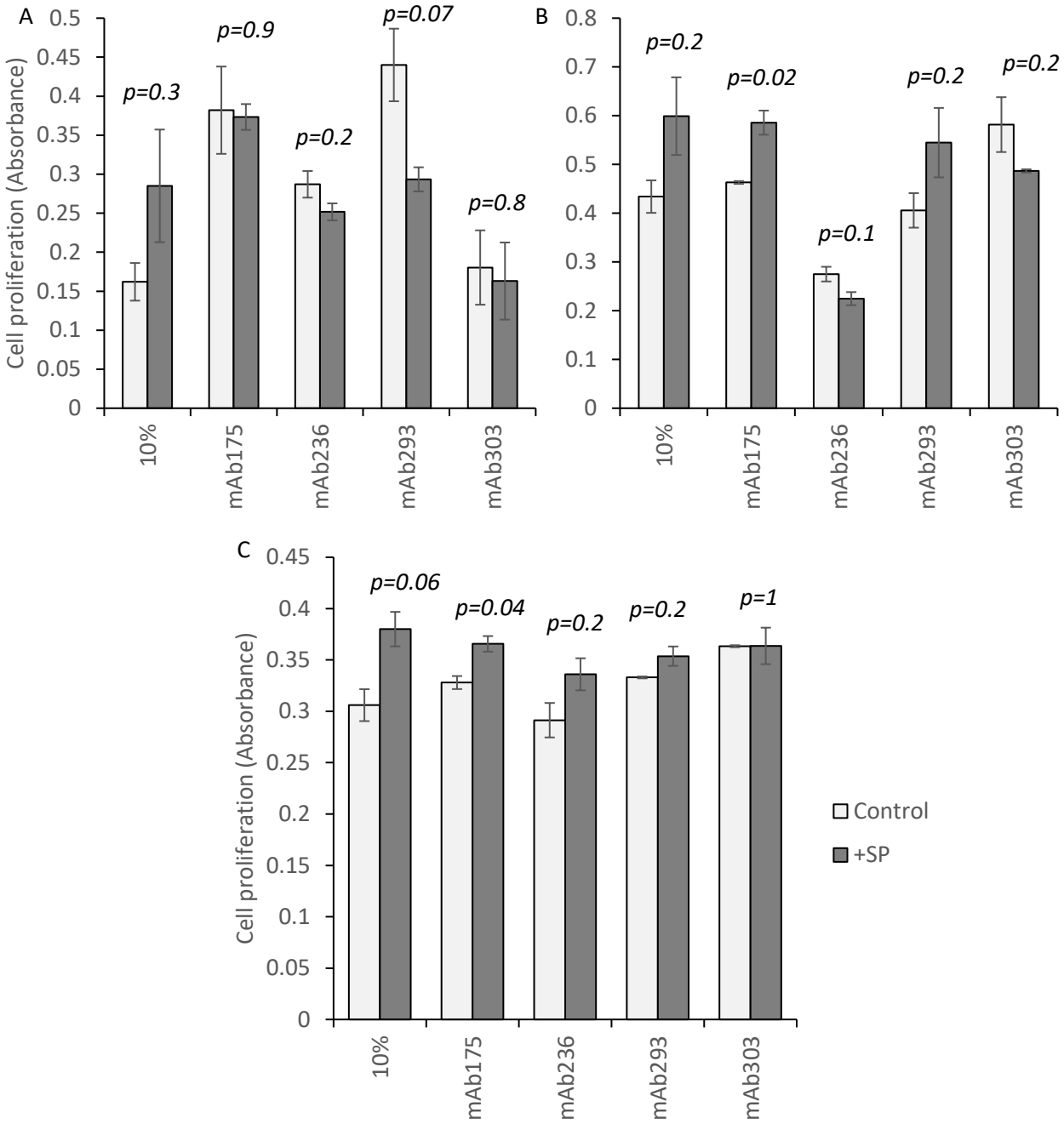


Figure 3.6.: SPARC antibodies inhibit the proliferative effect of SPARC in 10% media. AsPC-1 cells were cultured in 10% media and treated with ±10µg/ml SPARC and ±3µg/ml of each SPARC monoclonal antibody. The figure shows 3 independent experiments. Cell proliferation is a measure of relative absorbance at 370nm. Graphs show mean absorbance, ±SEM n=3. The key is applicable to all three graphs. p-value calculated as two sample t-test.

As shown in Figure 3.6, the effect of individual antibodies varied quite widely between individual experiments, though all antibodies used were able to block or reverse the effect of SPARC in at least one experiment. This may be due to biological differences in the cells; alternatively, more consistent results may be observed with a higher antibody concentration. Therefore, although there is some preliminary evidence from these experiments that anti-SPARC antibodies can inhibit the proliferative effect of SPARC, more testing is required to come to a valid conclusion.

3.7. SPARC is laid down in the extracellular matrix by pancreatic stellate cells

To better understand how SPARC regulates the way collagen I and fibronectin are laid down, PS1 cells were cultured for 24, 48 and 72 hours and stained to show fibronectin, collagen I and SPARC deposition.

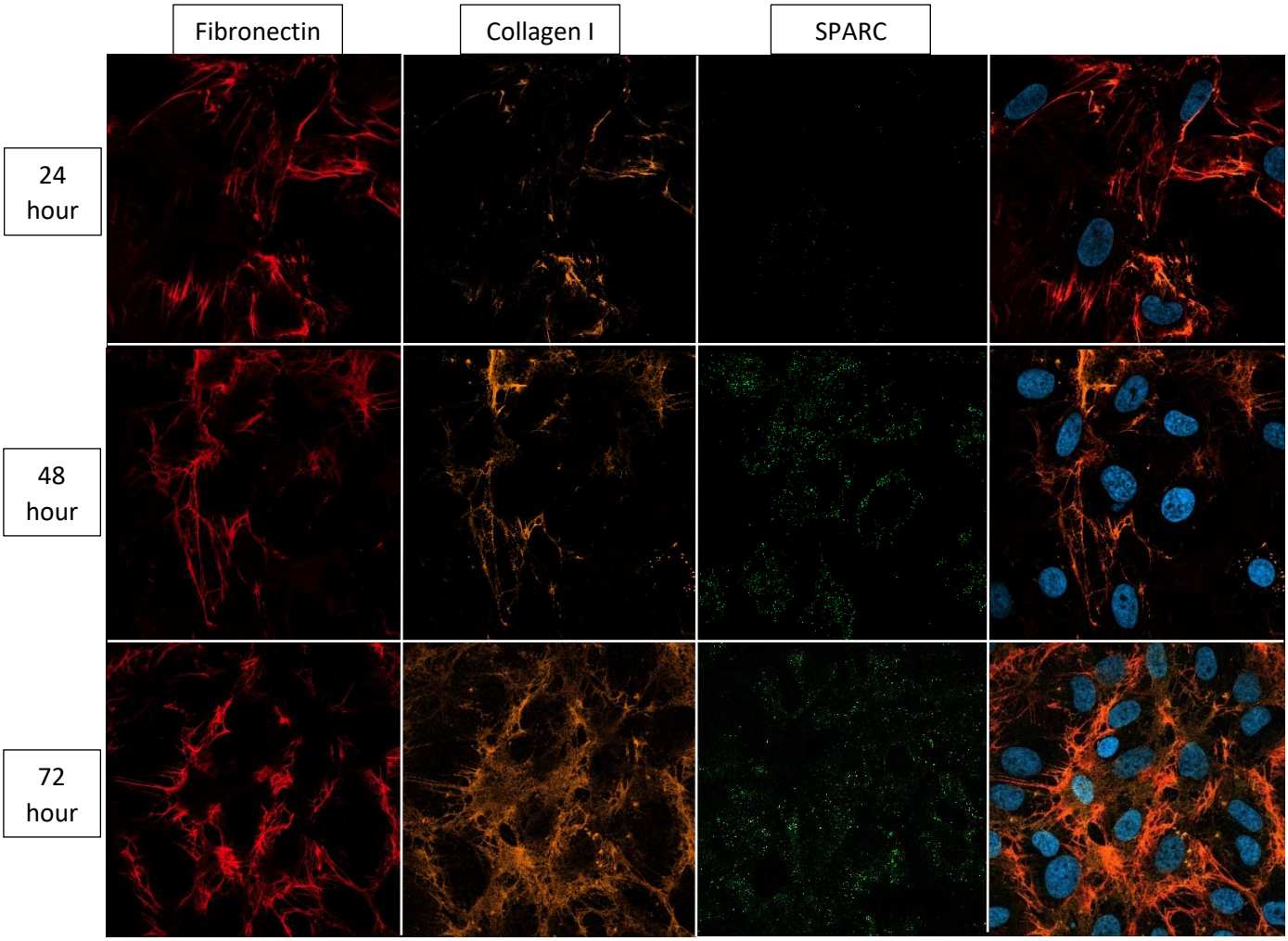
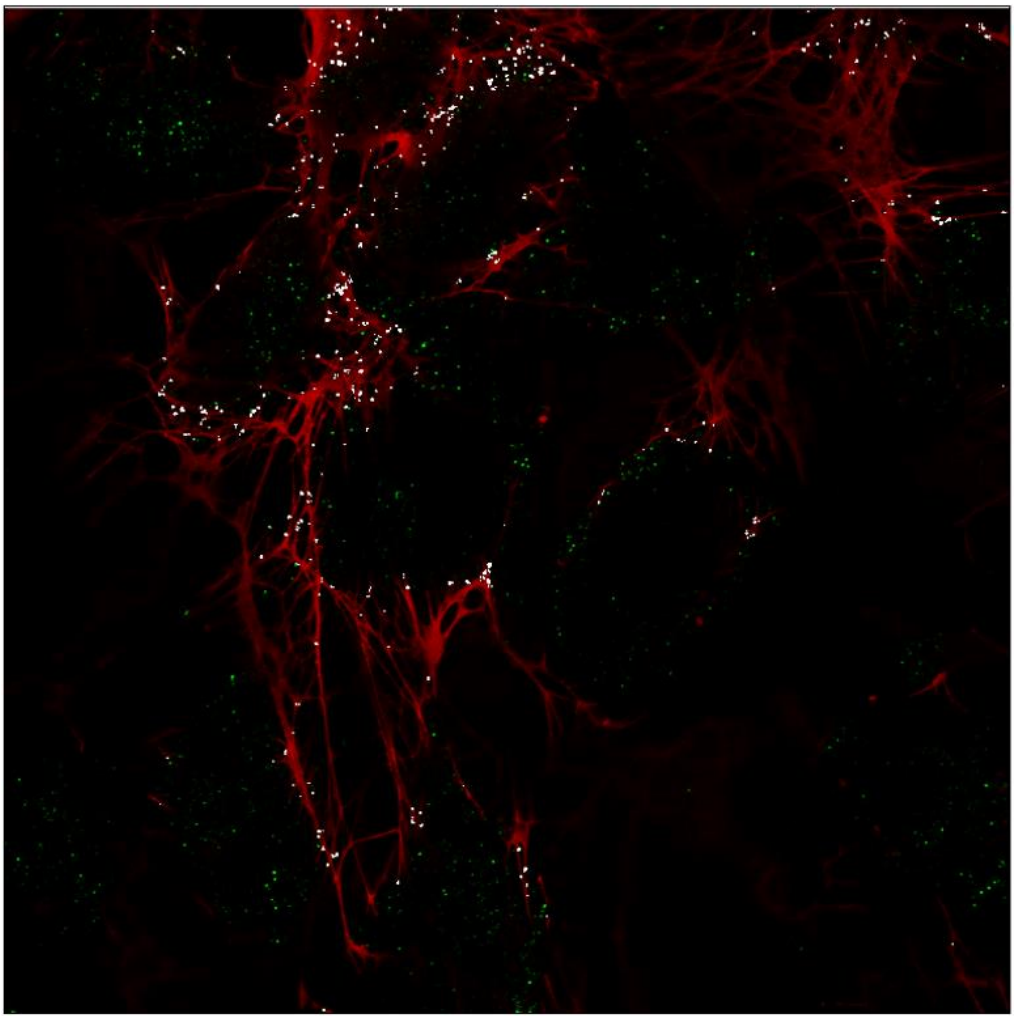


Figure 3.7.1: Pancreatic stellate cells fluorescently stained to show colocalization of SPARC with collagen I and fibronectin. PS1 cells were plated 100,000 cells/well and cultured for 24, 48 or 72 hours on sterile coverslips. Cells were fixed with 4% paraformaldehyde and blocked with 10% natural horse serum. Anti-collagen, SPARC and fibronectin antibodies were incubated with the cells overnight at 4°C then treated with secondary antibodies. Slides were imaged using Zeiss Confocal LSM800.



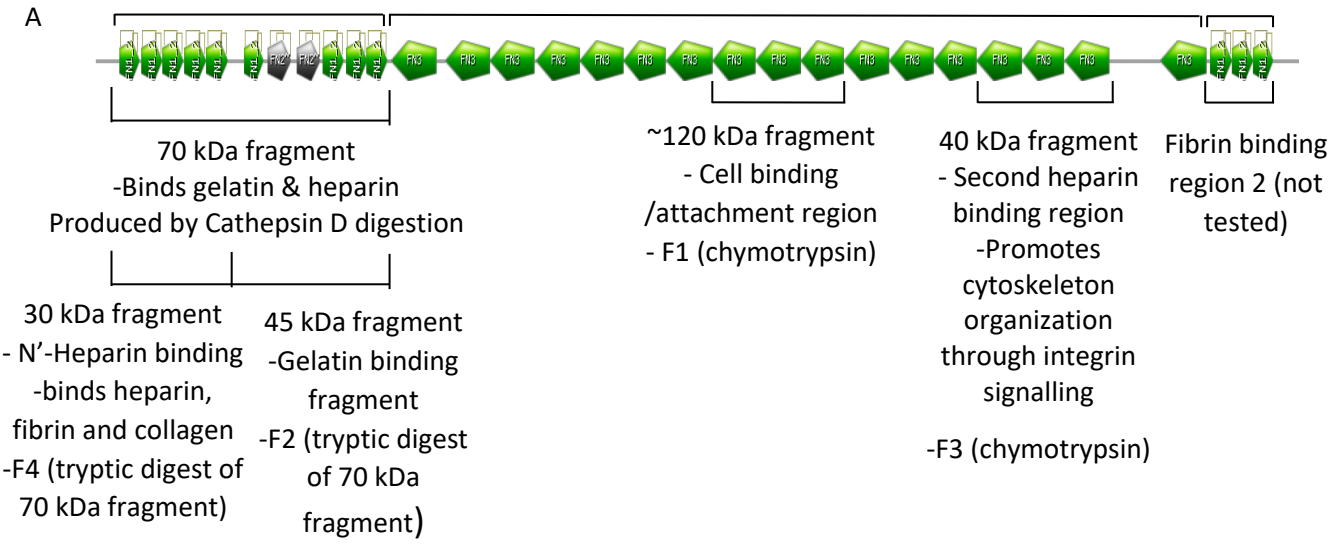
3.7.2: SPARC and fibronectin are colocalised in the matrix. Images acquired from the Zeiss confocal microscope were input into ImageJ. The Costes' mask of confluency highlights the areas of colocalization as white points, between SPARC (green) and fibronectin (red). Image is from PS1 cells cultured over 48 hours.

Collagen and fibronectin deposition are colocalised within the matrix, as seen in Figure 3.7.2. The image thresholds were adjusted manually in ImageJ before the Manders coefficient values were calculated. The fraction of SPARC overlapping fibronectin was 0.916 indicating a high correlation between SPARC and fibronectin deposition. Similar data was obtained for collagen and SPARC deposition (data not shown), with a value of 0.917 of SPARC overlapping collagen, as expected as collagen is deposited over fibronectin.

These experiments show that SPARC is colocalised with both fibronectin and collagen, though further evidence would be required to determine whether protein-protein interactions occur.

3.8. Determining the domain of fibronectin responsible for the 'switch' in SPARC activity

Uncovering the domains in fibronectin which are responsible for binding to SPARC is essential for targeting the interaction between SPARC and fibronectin. Four fragments of fibronectin containing different domains in fibronectin were cultured with AsPC-1 cells ±10µg/ml SPARC in a BrdU assay. The four fragments used were labelled F1-4 based on their relative size with fragment 1 being the largest.



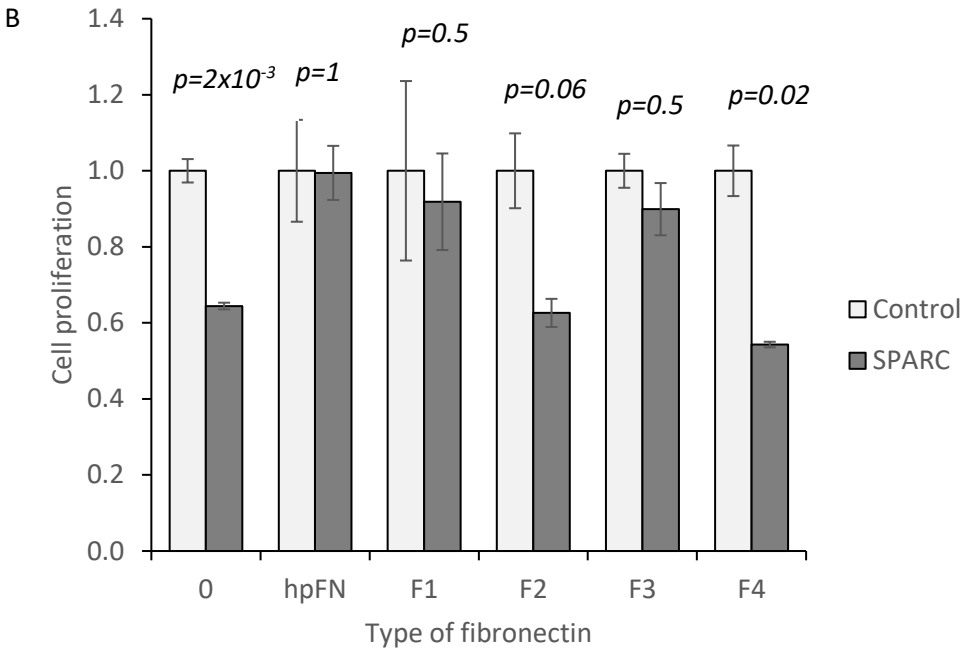


Figure 3.8: Fibronectin fragments 1 and 3 recapitulate the effect of full length hpFN on AsPC-1 cells cultured in 0.5% media. A – schematic of the full-length FN protein. Fragments 1-4 are labelled in approximate regions of the protein. Green domains are either FN1 or FN3. Two FN2 domains are in grey. The fragments were diluted to the molar equivalent of 250µg/ml hpFN and treated with ±10µg/ml SPARC (B). Cell proliferation was measured in absorbance at 370nm. The data obtained from each experiment was pooled and standardised to each untreated sample (data is from 3 independent experiments). p-value calculated as two sample t-test. Graph shows mean absorbance ±SEM n=3.

There is little data provided by the supplier in order to determine the amino acid sequences of the fragments however, each fragment contains 1 known domain: F1 contains the cell binding region; F2 contains the gelatin binding domain; F3 contains the second heparin binding region; and F4 contains the first heparin binding domain.

As shown in Figure 3.8 A, the inhibitory effect of SPARC on cell proliferation in low serum conditions is lost when full length fibronectin is added (consistent with the data in Figures 3.4 and 3.5.2 shown previously). Both fragments 1 and 3 appear to recapitulate the effect hpFN has on the activity of SPARC whereas fragments 2 and 4 have the same effect as no fibronectin added.

The unstandardized data for this Figure is shown in Appendix Figure S1, and, interestingly, it can be seen that addition of fragment 4 increases cell proliferation. There was ~50% increase in cell proliferation of AsPC-1 cells treated with ±SPARC, suggesting that this fragment has additional activity in isolation compared to full length fibronectin.

In summary, these experiments demonstrate that domains within the F1 and F3 fragments, but not F2 and F4 fragments, are responsible for the ability of fibronectin to switch SPARC activity.

3.9. Evaluating direct binding of SPARC to fibronectin and fibronectin fragments

Based on the results in section 3.8 we hypothesized that a direct interaction occurs between the F1 and F3 regions of fibronectin and SPARC. In order to test this hypothesis, we therefore performed solid-phase binding assays with highly purified proteins. Fibronectin and fragments were bound to an ELISA plate overnight and treated with SPARC the following day.

As shown in Figure 3.9A, the presence of fibronectin bound to the plate significantly increases SPARC binding over controls.

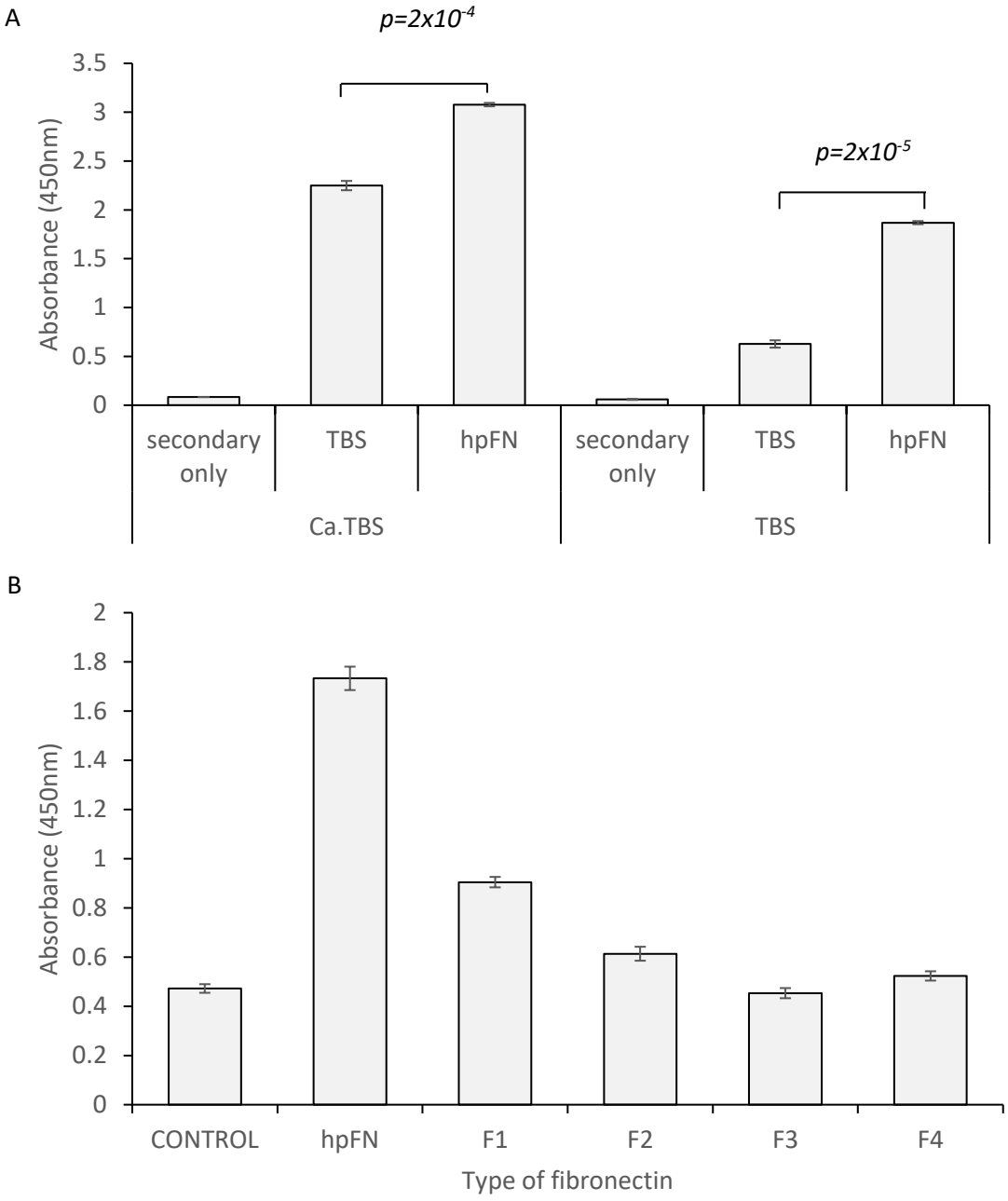


Figure 3.9.1: Testing direct binding between SPARC and fibronectin domains. (A) SPARC binding to full length fibronectin (1µg/well) in the presence or absence of 40mM calcium (Ca.TBS). (B) 1µg/well fibronectin and fragments were bound to the plate overnight. Wells were washed and blocked with 2% casein and treated with 1µg/well SPARC diluted in TBS (data is representative of 2 independent experiments). Anti-SPARC antibody was diluted 1:500. Anti-HRP detection antibody was diluted 1:300. TMB solution was added to develop colour. p-value calculated as two sample t-test.

The presence of calcium ions has been shown to increase SPARC binding to collagens (Sage *et al*., 1989) therefore SPARC-fibronectin binding was initially tested in TBS containing calcium and TBS only (figure 3.9-A). Fibronectin (1µg/well) was bound to the plate overnight and treated with SPARC (1µg/well) the following day (Figure 3.9 A). The presence of calcium in the buffer significantly increases the background absorbance of the negative control well (TSB) so calcium was not used in further experiments.

To identify the regions in fibronectin that bind directly to SPARC, two individual experiments binding fibronectin to the plate were carried out: fibronectin and the fragments were diluted to the molar equivalent of 250µg/ml hpFN and treated with ±10µg/ml SPARC; fibronectin and the fragments were diluted to 1µg/well and treated with ±10µg/ml SPARC. The amount of fibronectin bound to the plate did not affect the binding to SPARC. As shown in Figure 3.9 B, no SPARC binding is observed for fibronectin fragments 2 and 4, consistent with the BrdU data in Figure 3.8.1 showing that the domains within these fragments do not modulate the effect of SPARC on cell proliferation.

In contrast, fibronectin fragment 1 was shown to bind to SPARC as seen in figure 3.9 B. However, it does not bind to SPARC with the same degree as the full-length human plasma protein (~68% reduction in binding). Interestingly, there is no direct binding between fragment 3 and SPARC which is contrary to data shown by the BrdU assay in section 3.8. This suggests that either the full-length fibronectin protein is required for SPARC-fibronectin binding, or alternatively that the presence of cells and/or other proteins in serum are essential for the binding of fragment 3 to SPARC. This also may apply to fragment 1 in that external components are required to enhance the binding to SPARC, since reduced SPARC binding compared to full length fibronectin was observed.

To confirm whether heparin binds SPARC directly, or influences the binding of SPARC to fibronectin, heparin and fibronectin were bound to a plate in a solid-phase binding assay (Figure 3.9.2). Heparin alone appears to bind SPARC just over the control value however, this may be due to an increased background due to the presence of heparin in the well. Co-plating fibronectin with heparin slightly inhibits the binding of SPARC to fibronectin. This effect could be due to the fact that heparin may block some of the binding sites on fibronectin.

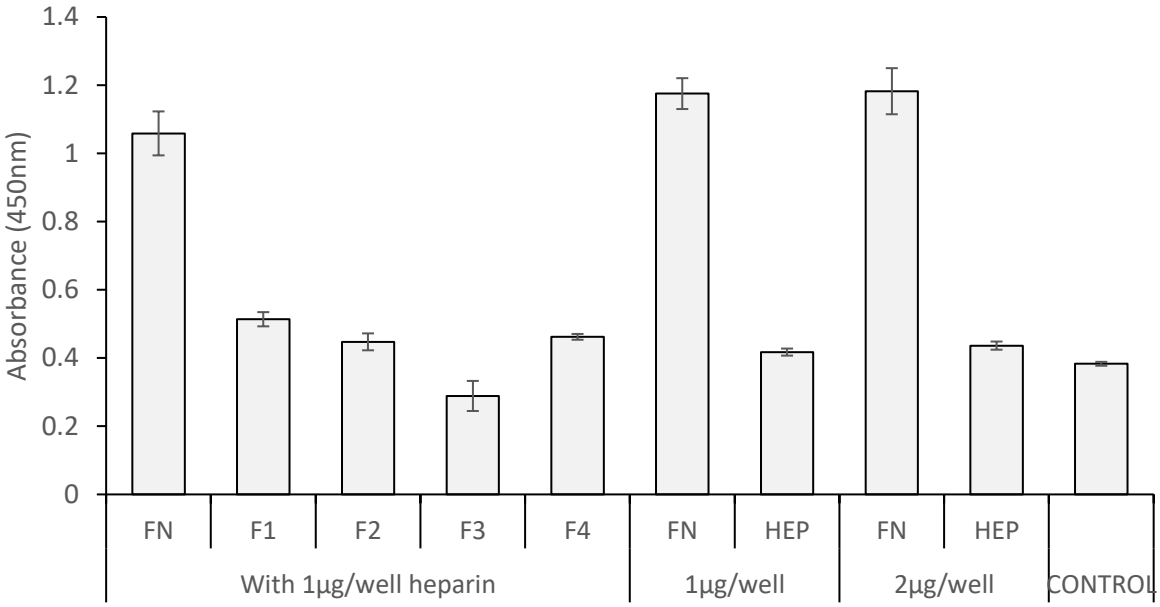


Figure 3.9.2: Heparin does not increase the binding of fibronectin to SPARC. 1µg/well fibronectin and fragments were bound to the plate overnight with 1µg/well heparin (2µg/well total protein). The plate was treated with 1µg/well SPARC protein. Control wells contained either 1 or 2µg/well fibronectin or heparin only, with TBS as the negative control. All data shows mean, ±SEM n=3. SPARC binding was detected using an anti-SPARC antibody.

4. Discussion

This study has confirmed previously published data showing the dual nature of SPARC function in pancreatic cancer cell proliferation. SPARC was shown to have a proliferative effect whereby it induces cancer cell proliferation in 10% medium, but also an anti-proliferative effect and promotes apoptosis in low serum medium. It was discovered that fibronectin was responsible for this switch in SPARC activity (Munasignhe *et al.*, 2020) thus uncovering the mechanism behind this interaction could provide a potential therapeutic target to treating pancreatic cancer.

It has been well reported that SPARC expression is able to influence fibronectin matrix expression (Barker *et al.*, 2005) however to date there has been no data indicating direct binding between SPARC and fibronectin. Here we have shown that without the presence of cells, SPARC is able to directly bind to human plasma fibronectin (Figures 3.8.2 and 3.9). Furthermore, analysing serum depleted of fibronectin showed that SPARC was also depleted, further supporting the evidence that there is direct binding between SPARC and fibronectin.

Heparin, which is known to modulate the conformation of fibronectin to reveal cryptic binding sites to bind VEGF and PDGF, exerts no effect on the binding of fibronectin to SPARC which suggests that the SPARC-fibronectin binding site on fibronectin remains unchanged by the 'open' conformation fibronectin undergoes when influenced by heparin. However, it should be noted that whilst fragment 3 containing the second heparin binding region was able to recapitulate the effect of hpFN in low serum medium, it did not bind SPARC directly under the conditions tested. This conversely suggests that in order for fragment 3 to be able to modulate the effect of SPARC, heparin may be required to bind fibronectin so that it is able to bind growths factors and modulate the activity of SPARC in this way, or alternatively, other proteins or the presence of cells could be required for this binding. Unfortunately, the role of fibrillogenesis on the SPARC-fibronectin interaction remains unknown. In the cell proliferation assays, it could not be determined whether fibronectin underwent fibrillogenesis when in the presence of cells – which it is known to do. To test whether this has an effect on the modulation of SPARC activity, an FUD1 inhibitor could be used to inhibit fibrillogenesis within the cell matrix. The fibrillogenesis of fibronectin may also be induced by binding fibronectin to a plate along with 2M urea solution and evaluating if fibronectin is still able to directly bind to SPARC. Fragment 1 provides the exciting possibility that the greater part of the interaction between SPARC and fibronectin happens within the cell attachment region and adjacent domains. Further analysis is required to accurately determine which domains exactly are present within this fragment (the cell attachment region is estimated to be ~30kDa, this fragment is measured to be ~120kDa).

In normal physiology, fibronectin is able to modulate cell adhesion, migration and signalling through binding to fibronectin receptors – integrins. The main fibronectin receptor is the α5β1 integrin which

binds the cell binding site (RGD) to promote cell proliferation. Inhibition of this integrin resulted in reduced growth of glioma cells and significantly smaller tumour volumes in mice transfected with glioma cells treated with α5β1 integrin inhibitor JSM6427 compared to control mice (Färber *et al*., 2008). It could be postulated that fibronectin binding to SPARC blocks the RGD site and therefore inhibits binding to the α5β1 integrin to prevent cancer cell proliferation. An interesting way to test this theory would be to culture AsPC-1 cells with JSM6427 in 10% medium ±SPARC to determine if this plays a role in modulating the function of SPARC.

One of the major limitations faced when attempting to uncover the binding site between SPARC and fibronectin was the fact that the four available anti-SPARC antibodies purchased from DSHB all interfered with the binding of SPARC to the detection antibody, either by binding the same site, or partially blocking the area (data not shown). One way of potentially determining where the binding site lies would be to repeat the solid phase binding assay, binding hevin to plate (with SPARC as a positive control) and treating with fibronectin. Both hevin and SPARC have similar structures and are over 60% homologous at the C-terminal domains however, they differ significantly at the N-terminus (Hakan *et al*., 2011). If hevin is able to bind fibronectin directly with the same or similar intensity as SPARC, this would suggest that the binding region lays within the C-terminal domains, and if not, the binding region would be within the N-terminal domains.

Despite evidence shown here that there is direct binding of SPARC to fibronectin, it is evident that the interaction is influenced by other proteins. The Coomassie stain in section 3.2 showed that depleting fibronectin removed multiple other proteins which may be required in modulating the activity of SPARC in pancreatic cancer cells.

This study has identified that addition of 250µg/ml hpFN turns the activity of SPARC to inhibit cancer cell proliferation in low serum medium however, a full switch in SPARC activity is not seen (with additional SPARC, cell proliferation is inhibited, but not below cells not treated with SPARC). Furthermore, we have identified specific domains within fibronectin responsible for the ability to regulate SPARC function. It can be concluded that there is a degree of direct binding of fibronectin to SPARC without the presence of other proteins however, more research needs to be undertaken to determine which other proteins present in serum are required for a complete switch in SPARC activity. Identifying this interface and binding mechanism may provide the potential of synergy with existing drugs in the hopes of treating pancreatic cancer.

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7. Appendix

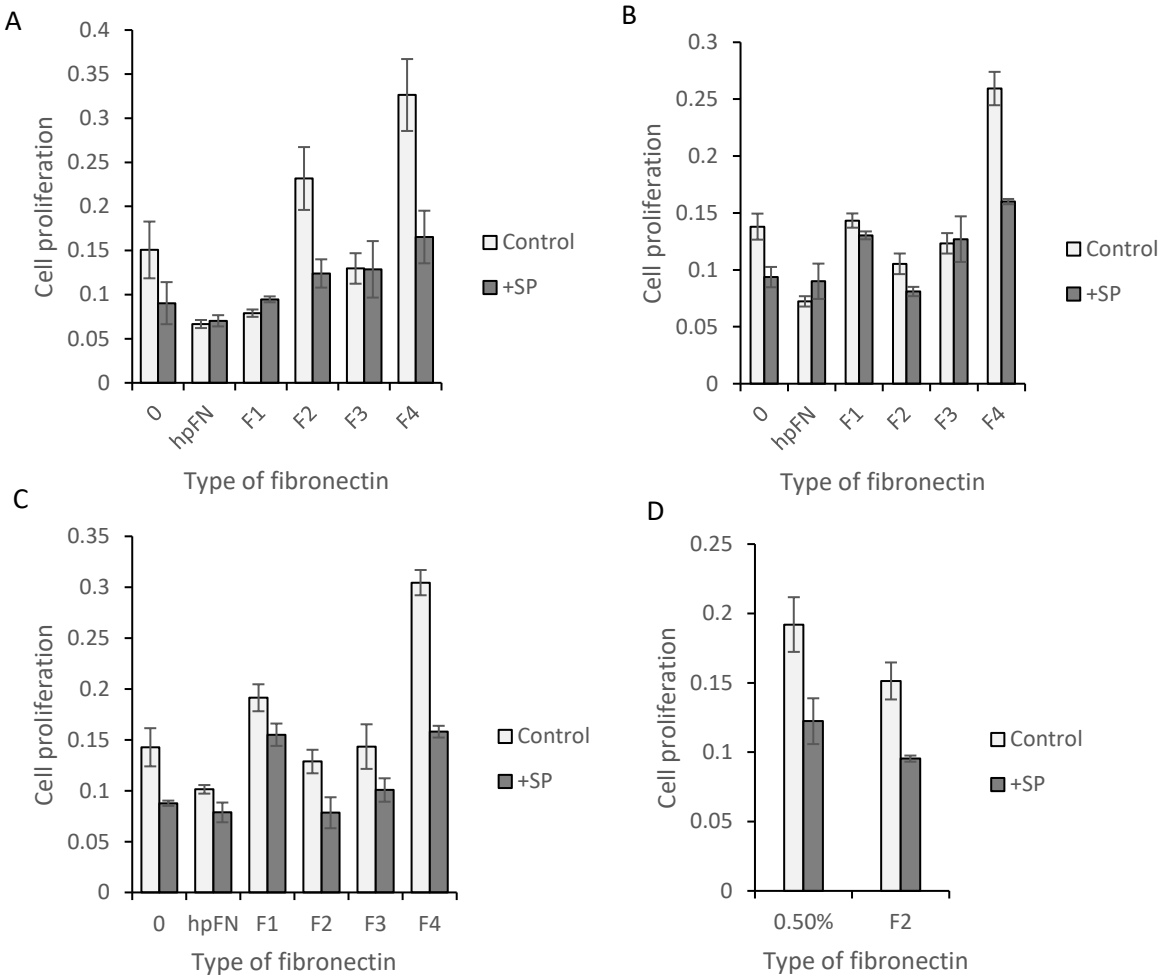

Figure S1: Individual data from section 3.8: AsPC-1 cells cultured in 0.5% media ±SPARC and ±fibronectin and fragments. The experiment was repeated 3 times, with a fourth repeat for fragment 2. Cell proliferation is measured in absorbance at 370nm. Graphs show mean absorbance, ±SEM n=3.

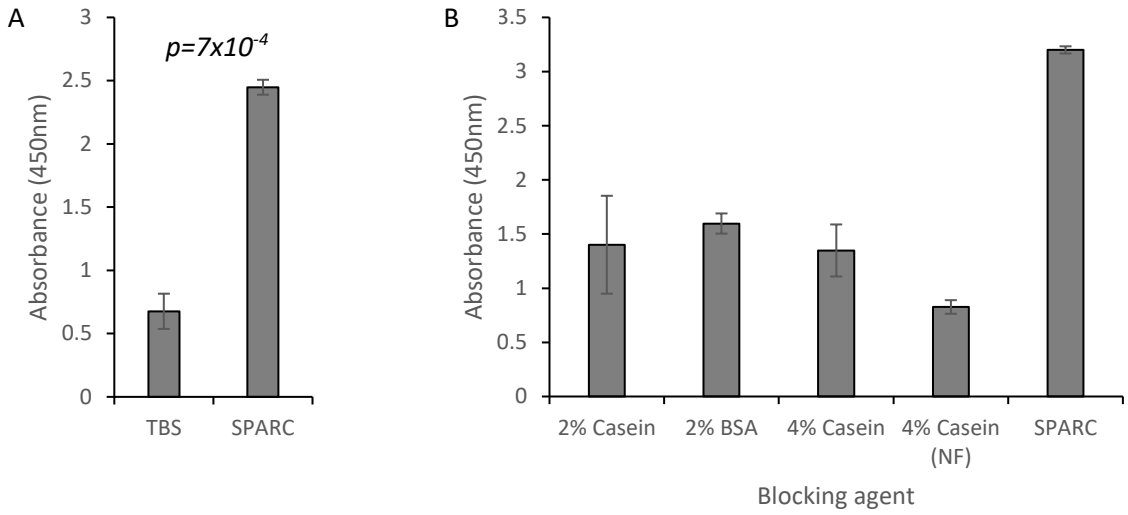

Figure S2: Validating the solid phase binding assay with SPARC: Validation of the SPARC detection antibody in a negative and positive control (A). Comparing the efficacy of different blocking agents in a solid phase binding assay (B). SPARC was bound to the plate overnight and treated with an anti-SPARC antibody the next day after blocking with various blocking agents.