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Investigating SPARC matricellular protein family function in the pancreas

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Abstract

Cellular and matrix interactions are dynamic and bi-directional, creating a suitable environment supportive of growth. The extracellular matrix plays an important role in islet functions essential in maintaining glucose homeostasis. Matricellular proteins such as the SPARC family are powerful regulators of cell-matrix interactions. The SPARC family share structural and functional similarities however their mechanism of action is currently not well understood due to a web of intricate interactions. SPARC is the most well-studied in the family and has been implicated in diabetes and pancreatic cancer. We hypothesized that related SPARC proteins may also be important in regulating β-cell functions and that the SPARC family should be studied systematically and holistically in light of overlapping and contradictory roles.

This study aimed to investigate expression of the wider SPARC family in the pancreas. We found that the proteins of the wider SPARC family are expressed in pancreatic islets and ducts. We characterised multiple cell-type specific isoforms expressed in the pancreas. Bioinformatics analysis identified extensive post translational modifications and alternative splicing for the wider SPARC family. We demonstrate that a holistic approach to studying the SPARC family is essential in uncovering their complex multifunctional roles. Using this approach, we identified alternative isoforms of SPOCK-3 in pancreatic stellate cells and describe FSTL-1 as a novel pancreatic tumour suppressor.

SPARC and its related proteins have largely been studied in 2D culture despite being matricellular proteins. This study aimed to investigate the function of the SPARC family on β-cell and islet growth in a 3D collagen matrix. Using a compressed 3D matrix, β-cells and islets were completely embedded, creating an environment to study the effect of matricellular proteins. We show that the SPARC family inhibits growth and proliferation of β-cells.

This study also aimed to investigate the role of the SPARC family on β-cell adhesion. We identify the SPARC family as novel regulators of glucose-stimulated actin regulation of insulin secretion. We also propose the SPARC family as potential novel regulators of actin-regulated exocytosis. Overall we describe the diversity and complexity of the SPARC family structure

and function. Understanding the role of specific isoforms, including both extracellular and intracellular variants will be essential in realising their clinical importance in islet function.

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"Earnestly, I must exert myself in order to return as much as I have received"

-Albert Einstein

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List of Abbreviations

αSMA – alpha smooth muscle actin
A1C – glycated haemoglobin
ABCC8 – ATP binding cassette subfamily C member 8
ADAMTS4 – a disintegrin and metalloproteinase with thrombospondin motifs 4
ADH – alcohol dehydrogenase
AKT – protein kinase B
ANOVA – analysis of variance
ATP – adenosine triphosphate
BAE – bovine aortic endothelial cell
BCA – bicinchoninic acid assay
bFGF – basic fibroblast growth factor
BiP – binding immunoglobulin protein/ glucose related protein 78
BM40 – basement membrane protein 40/ SPARC
BMP - bone morphogenic protein
BrdU – bromodeoxyuridine
BSA – bovine serum albumin
BSP – bone sialoprotein
CCN - CYR61, CTGF, NOV
Cdc42 – cell division cycle 42
CDKN2 – cyclin dependent kinase inhibitor 2A
cDNA – complementary DNA
CDS – complete coding sequence
CTGF – connective tissue growth factor/ CCN2
CTLA-4 - cytotoxic T lymphocyte – associated protein 4 gene
CVB – coxsackievirus B
CYR61 – cysteine-rich angiogenic protein 61/ CCN1
DAB – 3,3'-diaminobenzidine
DDR – discoidin domain receptor
DMP1 – dentin matrix acidic phosphoprotein 1
DNA – deoxyribonucleic acid
DSPP – dentin sialophosphoprotein
ECGS – endothelial cell growth supplement
ECM – extracellular matrix
EDTA – ethylenediaminetetraacetic acid
EMT – epithelial-mesenchymal transition
EHS – engelbreth-holm-swarm sarcoma
ELISA – enzyme-linked immunosorbent assay
ER – endoplasmic reticulum
ERK – extracellular signal-regulated kinase
EthD1 – ethidium homodimer 1
EtOH – ethanol
FA – fatty acid
FAK – focal adhesion kinase
FBS – fetal bovine serum

FSTL-1 – follistatin-like 1
G6PCS2 – glucose-6-phosphatase catalytic subunit 2
GAG – glycosaminoglycan
GCK - glucokinase
GLT – glucolipotoxic
GSIS – glucose stimulated insulin secretion
GWAS – genome wide association studies
HGF – hepatocyte growth factor
HLA – human leukocyte antigen
HLA-B – major histocompatibility complex class I, B
HMDS – hexamethyldisilazane
HSP47 – heat shock protein 47
HUVEC – human umbilical vein endothelial cell
IDDM1 – insulin-dependent diabetes mellitus locus 1
IFIH1 - interferon-induced helicase 1 gene
IGF – insulin-like growth factor
IGFBP – IGF binding protein
IL – interleukin
IL2RA – interleukin 2 receptor subunit alpha gene
INS – insulin gene
INS-IGF2 – insulin insulin-like growth factor 2 read-through product (insulin isoform 2)
IR – infrared
IRS1 – insulin receptor substrate 1
KCNJ11 – potassium voltage-gated channel subfamily J member 11
KCNQ1 – potassium voltage-gated channel subfamily Q member 1
KLF14 – kruppel like factor 14
KRBH – kreb's ringer bicarbonate HEPES buffer
LAIR – leukocyte associated immunoglobulin like receptor 1
NBF – neutral buffered formalin
NHS- normal horse serum
NLS – nuclear localisation signal
NOV- nephroblastoma overexpressed/ CCN3
MEPE – matrix extracellular phosphoglycoprotein
MHC – major histocompatibility complex
MMP – matrix metalloproteinase
MT-MMP – membrane-type MMP
OPN – osteopontin
p-ERK – phosphorylated ERK
p-FAK – phosphorylated FAK
P4H – prolyl 4-hydroxylase
P53 - tumour protein/ TP53
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PD1 – programmed cell death protein 1
PDAC – pancreatic adenocarcinoma
PDGF – platelet-derived growth factor

PFA - paraformaldehyde
PKA - protein kinase A
PKC – protein kinase C
PPARG – peroxisome proliferator-activated receptor γ
PSC – pancreatic stellate cell
PTK – protein tyrosine kinase
PTPN22 - lymphoid protein tyrosine phosphatase gene
QARS – glutaminyl-TRNA synthetase
Rac – Ras-related C3 botulinum toxin substrate
RAFT – Real Architecture for 3D Tissue
RGD – arginine glycine aspartate peptide
RhoA – Ras homolog gene family member A
RIPA – radioimmunoprecipitation buffer
RNA – ribonucleic acid
RPM- revolutions per minute
RT-PCR – reverse transcriptase PCR
SC1 – synaptic cleft 1/ hevin
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM – scanning electron microscopy
SHH – sonic hedgehog
shRNA- small hairpin RNA
siRNA – short interfering RNA
SMAD4 – Mothers Against Decapentaplegic, Drosophila, Homolog 4
SMOC – SPARC related modular calcium binding
SNARE - soluble N-ethylmaleimide attachment protein receptors
SNAP-25 – synaptosome associated protein
SNP – single nucleotide polymorphism
SPARC – secreted protein acidic and rich in cysteine
SPOCK – SPARC/osteonectin, cwcv and kazal-like domains proteoglycan
TAE – tris base, acetic acid and EDTA
TCF7L2 – transcription factor 7 like 2
TCN – tenascin
TNF-α – tumour necrosis factor α
TGF-β – transforming growth factor β
TRITC - tetramethylrhodamine
TSC-36 – TGF-β 1-stimulated clone 36
TSP-1 – thrombospondin-1
UT - untransfected
UTR – untranslated region
VAMP – vesical associated membrane protein
VEGF – vascular endothelial growth factor

1. Introduction

1.1 The extracellular matrix

Much of the coordinated communication in tissues is organised by interactions occurring in the extracellular space. The extracellular matrix (ECM) is a network of proteins that provide tissue structure and it also regulates cellular interactions. Extracellular signals from the matrix determine gene expression and ultimately cell behaviour. Organs are collectively a network of different cell types that dynamically work together. The dynamic nature of the ECM allows development, tissue remodelling and wound healing throughout life. Bidirectional cross talk between cells and the ECM determines tissue architecture which determines cell differentiation and confers organ specificity (Nelson and Bissell, 2006). Dysregulation of the matrix and tissue architecture can lead to disease pathogenesis (Nelson and Bissell, 2006). There is growing interest in the idea that the ECM can be used as an instruction manual to induce cells to "remember" and revert back into their normal healthy phenotype (Nelson and Bissell, 2006). Deciding which programme a cell will undertake depends on the composition of the surrounding matrix. In pancreatic diseases such as diabetes and cancer, the ECM has been implicated to play a role in the progression of diseased state.

The ECM consists of large fibrillar proteins such as collagens, laminins, fibronectins, and proteoglycans that together function as substrates for cell anchorage [Figure 1.1]. These structural components also act as a migration track or barrier for cells. Not only does the matrix have structural functions, it also has instructional functions. The matrix can sequester growth factors and cytokines acting as a reservoir of signals while at the same time can facilitate presentation of these signals to cell surface receptors, ultimately regulating the degree of interactions. Remodelling and cleavage of matrix proteins can create functional fragments that may have distinct functions. In addition, matrix remodelling can alter elasticity and porosity of the tissue, thereby altering biomechanical forces relayed through integrins and to the cytoskeleton and nucleus. Regulation of the ECM therefore plays an

important role in creating the environment that governs cellular functions such as differentiation, proliferation, and survival.

There are two major types of ECM: stromal and basement membrane. Interstitial or stromal ECM is made of fibrillary proteins and is found between cells. Basement membranes on the other hand are sheet-like and often make up the epithelial lining that separate tissue compartments. The ECM communicates with cells via cell surface receptors namely integrins, (DDR) discoidin domain receptors, dystroglycan and (LAIR) leukocyte associated immunoglobulin like receptor (Huang and Greenspan, 2012). Integrins are the major cell receptors for the matrix which recognise the arginine- glycine- aspartate (RGD) motif on matricellular proteins. Integrin binding to the matrix induces integrins to cluster, which in turn initiates focal adhesion assembly and signalling cascades that transmit the extracellular signal to the nucleus through cytoskeleton remodelling, thus influencing cellular processes. The matrix is produced by stromal cells such as fibroblasts, stellate cells, endothelial and epithelial cells. These supportive cells also produce matricellular proteins – a class of non-structural matrix proteins that regulate the ECM.

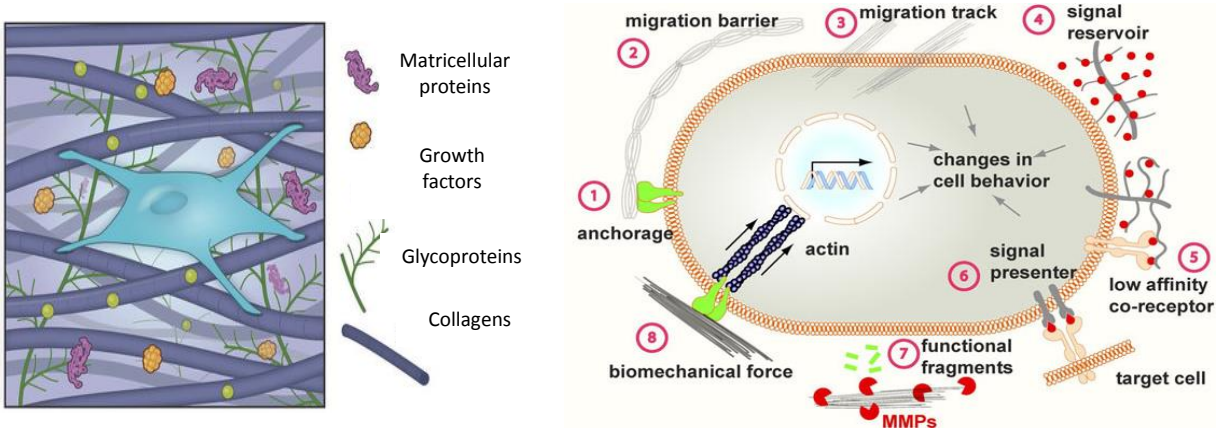


Figure 1.1: The extracellular matrix and its functions. The extracellular matrix consists of large structural proteins such as collagens, laminins and fibronectin that provide structure to tissues. In addition, the matrix also contains growth factors, cytokines and matricellular proteins that together provide instructional signals that determine gene expression, cellular behaviour and function (Image taken from Gaharwar *et al*., 2015).

1.1.1 The matricellular protein family

The matricellular protein family regulates interactions in the matrix. Although they do not contribute to the ECM structure, they play an essential role in matrix production, assembly, and remodelling. Matricellular proteins are defined as secreted proteins that execute their

functions by binding to multiple integrins, growth factors and fibrillary matrix proteins thereby facilitating or inhibiting their interaction (Murphy-Ullrich, 2001; Murphy-Ullrich and Sage, 2014). Matricellular proteins include: periostin, tenascins, thrombospondins, the CCN family, SIBLING family and the SPARC family as shown in Figure 1.2 (Bornstein and Sage 2002; Alford and Hankenson 2006; Murphy-Ullrich and Sage 2014). By mediating matricellular communications, they greatly influence cellular processes such as proliferation, survival, migration, and function. Although they are structurally unrelated, matricellular proteins share many overlapping functions such as modulating cell shape and cytoskeletal organization, often promoting cell rounding and anti-adhesion (Murphy-Ullrich, 2001; Murphy-Ullrich and Sage, 2014). Typically, expression is induced during wound healing, fibrosis, development and tissue remodelling, periods of high cell and matrix turn over. Matricellular proteins such as the SPARC family are important master regulators of matricellular interactions and thereby are essential factors in understanding how the microenvironment is induced into diseased or healthy state.

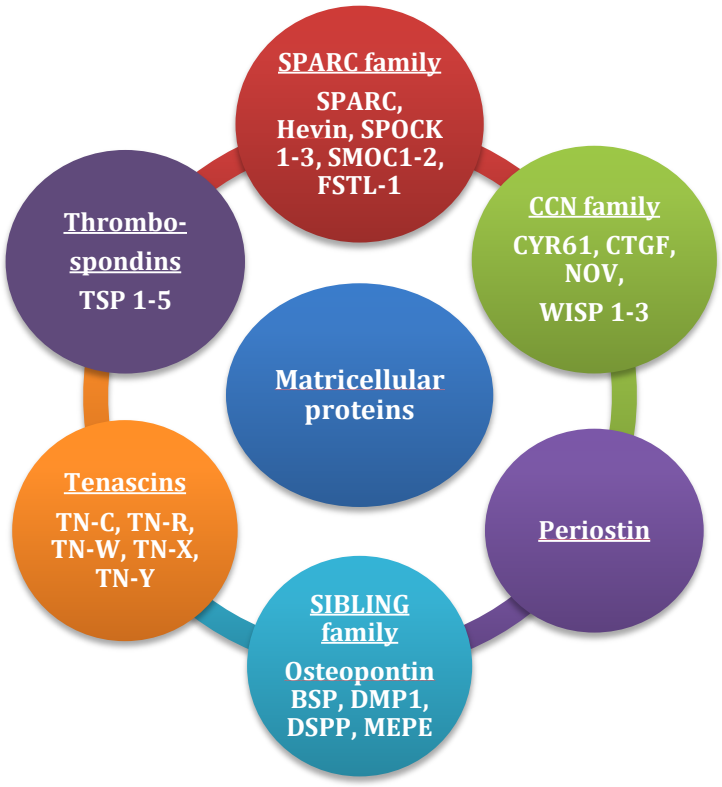


Figure 1.2: Matricellular proteins. Matricellular proteins regulate matrix interactions with cellular receptors. Although they do not contribute to the structure of the matrix, the matricellular protein family are essential in determining cellular responses to signals from the extracellular space. Matricellular proteins include: the SPARC family, CCN family, periostin, SIBLING family, tenascins, and thrombospondins (Based on: Alford and Hankenson 2006; Murphy-Ullrich and Sage 2014).

1.1.2 Extracellular matrix of the pancreas and islet architecture

The islets of Langerhans contain the endocrine component of the pancreas and produce hormones that regulate metabolism. Figure 1.3 A shows that in mouse islets, the central core is composed of insulin producing β-cells while at the outer periphery are the glucagon secreting α cells, somatostatin producing δ cells, and PP cells that produce pancreatic polypeptides. In human islets however, endocrine cells are more interdispersed [Figure 1.3 B] (Stendahl *et al*., 2009). The exocrine pancreas is made up of acinar cells that produce digestive juices secreted into the small intestine (Stendahl *et al*., 2009). The islets of Langerhans are surrounded by a capsule of basement membrane lining (Otonoski *et al*., 2008; Virtanen *et al*., 2008) that is predominantly composed of laminin and collagen IV, but also includes collagens I, III, V, VI, and fibronectin (Stendahl *et al.,*2009). Near the basement membrane of islets are also resident stellate and fibroblast cells that support the islet architecture and function. They secrete growth factors, cytokines as well as components of the ECM (Stendahl *et al.,*2009). There is also direct interaction with the matrix within islets as islets are heavily vascularised to allow immediate release of hormones into the blood stream.

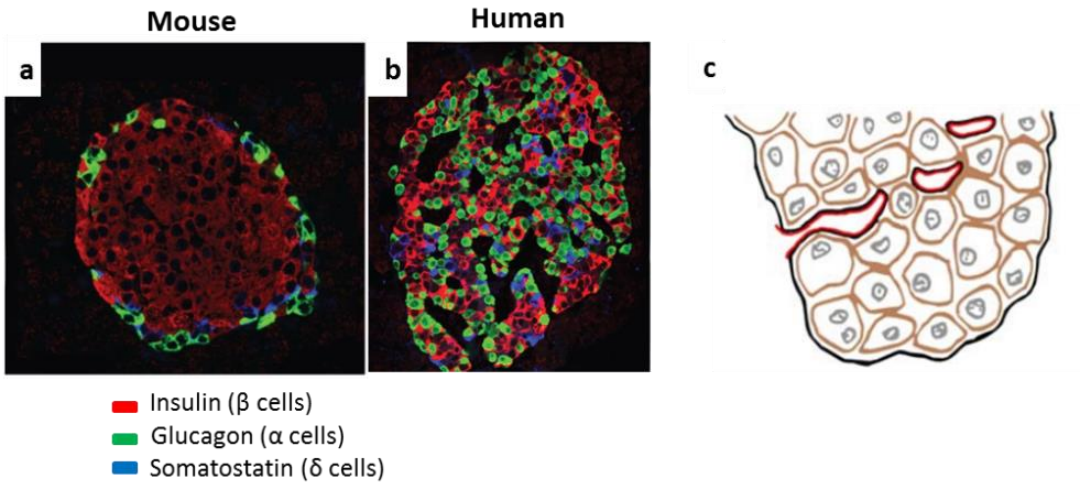


Figure 1.3: Islet architecture. In mice, insulin-producing β-cells are arranged in the inner core of islets while glucagon-producing α cells and somatostatin-producing δ cells are arranged at the outer periphery. In human islets, endocrine cells are more interdispersed (Image from Wang *et al.,* 2015). Islets are surrounded by a supporting basement membrane. Human islets are heavily vascularised and the blood vessels are surrounded by a double basement membrane that separates blood vessels and endocrine compartments (Image from Virtanen *et al.,* 2008).

Human intra-islet blood vessels in particular have been shown to have a double basement lining which have a unique composition [Figure 1.3 C]. For example the outer peri-islet basement membrane contains laminin 511 and 521 while the inner vascular basement membrane additionally contains laminin 411 and 421 (Virtanen *et al*., 2008).

Islets are highly dependent on the matrix and there is growing evidence showing that the ECM signals to improve islet survival, islet mass and insulin secretion. Recently, the role of the ECM in islet function and the pathogenesis of diabetes and pancreatic cancer have been increasingly recognised. Collagens and other ECM proteins have been shown to directly affect islet biology by improving islet survival, insulin secretion, and increasing proliferation and islet mass (Wang and Rosenberg, 1999; Bosco *et al*., 2000; Nagata *et al*., 2001; Beattie *et al*., 2002; Nagata *et al.,* 2002; Zhang *et al*., 2012). Several integrins have been detected in islet β-cells such as α3, α5, α6, αv, and β1 (Wang and Rosenberg, 1999; Ris *et al*., 2002; Virtanen *et al*., 2008; Stendahl, 2009). However, α1β1 integrin has been shown to be the primary receptor utilised by β-cells (Kaido *et al*., 2004). In addition, α1β1 interaction with collagen IV plays a significant role in insulin secretion (Kaido *et al*., 2004). Loss of the ECM during enzymatic harvest of islets is hypothesized to be a major contributor to decreased islet graft function and survival after transplantation (Wang and Rosenberg 1999; Stendahl, 2009; Jalili *et al*., 2010). Proper communication between the ECM and cells is essential for islet development, glucose detection, and insulin secretion (Beattie *et al*., 2002). For example, mice deficient in β1 integrin have shown to have decreased β cell mass, proliferation, glucose tolerance and significantly decreased insulin production (Riopel *et al*., 201; Diaferia *et al*., 2013). Interestingly, islet isolation also results in markedly decreased expression of α5 integrins, which are known to be involved in survival (Wang and Rosenberg, 1999). This decrease in α5 expression is rescued by exposing islets to collagen I and fibronectin (Wang and Rosenberg, 1999). It has therefore been suggested that re-establishment of islet-ECM relationships is essential in improving islet health as demonstrated by increasing studies showing that islets cultured on a matrix have improved survival and glucose stimulated-insulin secretion (Wang and Rosenberg, 1999; Bosco *et al*., 2000; Beattie *et al*., 2002; Nagata *et al*., 2001; Nagata *et al.,* 2002; Zhang *et al*., 2012). In pancreatic cancer on the other hand, the dense and fibrotic stroma is a characteristic of this

aggressive disease. It is therefore important to closely investigate the tightly complex relationship of the ECM to pancreatic diseases.

1.2 Diseases of the pancreas

1.2.1 Diabetes Mellitus

Diabetes is a metabolic disease characterised by chronic hyperglycemia due to loss or dysfunction of insulin-producing β cells, or insulin resistance [Figure 1.4]. Overall, this results in inadequate cellular glucose uptake. Diabetes is an increasingly prevalent problem globally affecting 415 million worldwide and this figure is estimated to increase to 642 million by the year 2040 (IDF, 2015). In the United Kingdom alone, there is an estimated 4.5 million people diagnosed with diabetes and an additional 1.1 million undiagnosed (Diabetes UK Facts And Stats). The life expectancy of those diagnosed is reduced in both types of diabetes (Diabetes UK facts and Stats). In the United Kingdom, it is estimated that the cost of care and treatment is about £23 billion and is predicted to rise by 2035 (Diabetes UK facts and Stats). The pathogenesis of diabetes is still not clearly understood, but appears to manifest as a combination of genetic, lifestyle, and environmental factors.

1.2.2 Type 1 diabetes

Type 1 diabetes results from the autoimmune destruction of β-cells leading to insulin deficiency [Figure 1.4]. Type 1 diabetes accounts for 5-10% of diabetes and was previously known as insulin-dependent or juvenile onset diabetes. It is often diagnosed in younger individuals aged 30 and below, although it can occur at any age (van Belle *et al*., 2011). Genome-wide association studies (GWAS) over the years have identified gene polymorphisms suspected to be involved in the susceptibility to developing Type 1 diabetes. These are regions in the human genome called the IDDM 1-8 (insulin-dependent diabetes mellitus locus) and include: the *HLA* region or IDDM1, the insulin gene or IDDM2, *CTLA4* or *IDDM12*, *PTPN22*, *IL2RA* and *IFIH1* (Ferreira *et al*., 2007; Hakonarson *et al*., 2007; Plagnol *et al*., 2011; Todd *et al*., 2007; Cooper *et al*., 2008; Barrett *et al*., 2009).

HLA or the human leukocyte antigen gene encodes immune response proteins such as the MHC and is linked to many autoimmune diseases (van Belle *et al*., 2011). The HLA class II region in particular is the most influential for genetic risk and certain alleles determine

susceptibility to Type 1 diabetes (van Belle *et al*., 2011; Fu *et al*., 2013). For example, the allele DR2 is protective while alleles DR3 and DR4 determine high risk susceptibility

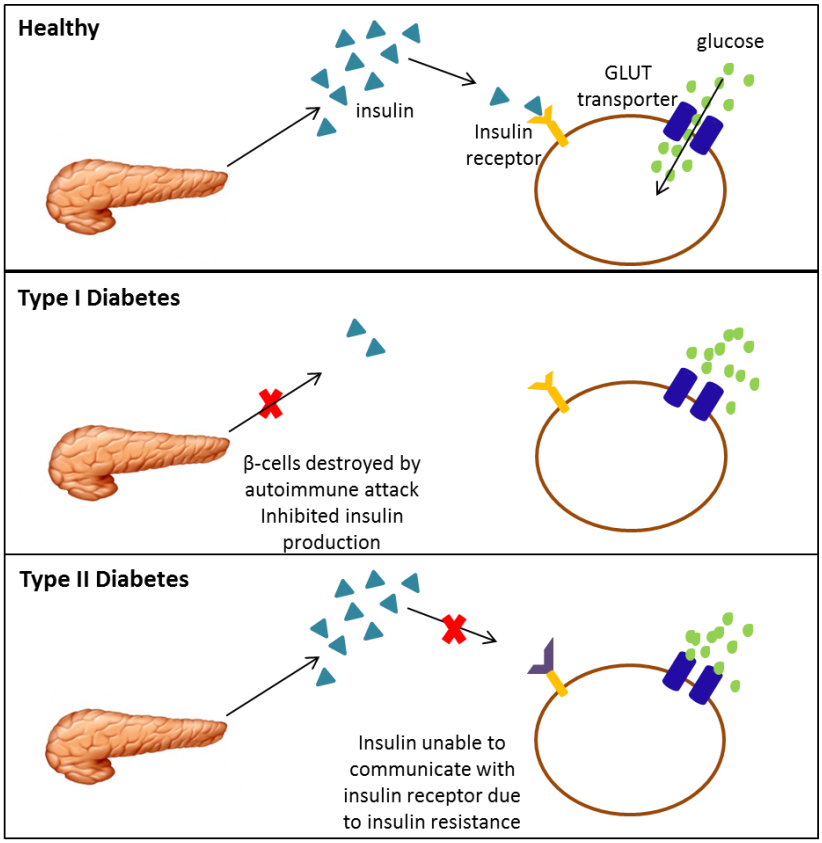


Figure 1.4: Diabetes Mellitus. Diabetes is a metabolic disease that results from inadequate insulin production due to autoimmune attack of β-cells (Type 1) or insulin resistance (Type 2) resulting in insufficient glucose uptake (Based on van Belle *et al*., 2011; American DA, 2010).

(Platz *et al*., 1981; van Belle *et al.,* 2011; Fu *et al*., 2013). *INS* or the insulin gene determines susceptibility by the number of variable tandem repeats in the promoter region. Longer repeats (class III alleles) determine protection while shorter repeats (class I alleles) increase risk (Bennett *et al.,* 1995). The number of tandem repeats likely affect transcription of insulin thus the presence of at least 1 class III allele decreases INS mRNA expression and decreases risk by 3 fold (Vafiadis *et al.,* 2001). The *CTLA4* gene or cytotoxic T lymphocyte 4 encodes proteins needed to inhibit the activation of T cells (van Belle *et al.,* 2011). Although the mechanism of how polymorphisms affect CTLA4 function is still unclear, it is suspected that alternative splicing as well as SNPs in the 3' untranslated region determine protective or susceptible phenotype. For example the G/G homozygous genotype is associated with

decreased production of CTLA4 and increased T cell activation, thus increase risk of Type 1 diabetes while A/A genotypes have a protective phenotype (Ueda *et al*., 2003; Kavvoura and Ioannidis, 2005). *PTPN22* gene encodes the lymphoid protein tyrosine phosphatase which is also a negative regulator of T cell signalling (van Belle *et al*., 2011). SNPs within this gene have been linked to other autoimmune diseases such as Grave's disease and rheumatoid arthritis however, the mechanisms of these polymorphisms are still unclear and contradictory (Bottini *et al*., 2006). The *IL2RA* gene on the other hand encodes the interleukin-2 receptor α chain which is expressed in activated T cells. SNPs at the 5' flanking region of the *IL2RA* gene also determine susceptibility to Type 1 diabetes (Qu *et al*., 2009; Tang *et al.,*2015).

It is also widely accepted that Type 1 diabetes is not purely genetic and that environmental factors play a significant role in triggering disease progression. For instance, only a small population of individuals with genetic susceptibility manifest the disease (van Belle *et al.,* 2011) and more than 85% of people with Type 1 diabetes have no previous family history (Diabetes UK Facts And Stats). The risk for developing type 1 does however increase with first degree family history (Diabetes UK Facts And Stats). In addition, prevalence of the disease varies by country, even between countries in close proximity with similar genetic profiles. For example, Type 1 diabetes is more prevalent in Norway compared to Iceland although distribution and frequency of *HLA* genes are similar (Backman *et al*., 2002).

Several environmental factors have been associated to increased risk. Viral infection has increasingly been shown to be correlated to Type 1 diabetes. *IFIH1*, one of the genes linked to the disease, functions as a sensor for viral infection (van Belle *et al.,* 2011). *IFIH1* is particularly important for the response to coxsackievirus B (CVB), one of the most prevalent virus linked to Type 1 diabetes (Flödstrom *et al*., 2003; Domsgen *et al*., 2016). CVB has been shown to cause insulitis and diabetes in mice (Yoon *et al*., 1978) and additionally was isolated in individuals with Type 1 diabetes (Yoon *et al*., 1979; Vreugdenhil *et al*., 2000). Composition of the intestines such as bacterial immunity, inflammation and variation in gut permeability are also considered variables contributing to Type 1 diabetes (van Belle *et al*., 2011; Chia *et al*., 2017). Other environmental factors hypothesised to have a causal role include cow's milk consumption particularly the protein A1 β-casein, as well as gluten are shown to be

associated with increased autoimmunity (van Belle *et al*., 2011; Chia *et al*., 2017). Additionally vitamin D, which affects dendritic cell differentiation and immune activation, has been linked to Type 1 diabetes as it has been observed to be seasonally onset (Mohr *et al*., 2008). The hours of sunshine is observed to be inversely correlated with Type 1 incidence in that region (Mohr *et al*., 2008). Furthermore, vitamin D metabolism and receptor polymorphisms may also be involved although results have so far been conflicting (Matthieu *et al*., 2002; Guo et al., 2006B; Bailey *et al*., 2007).

Treatment for type 1 diabetes involves insulin injections to compensate for insufficient production. However, it is a difficult disease to manage due to fluctuating levels of glucose throughout the day, and failure to properly control this can lead to hyper or hypoglycaemia. Another option is islet transplantation which however is very limited due to limited availability and graft transplant survival. Currently research is looking into propagating β-cells from stem cells. The ECM may be an important component to induce differentiation of stem cells and support β-cell survival.

1.2.3 Role of the ECM in Type 1 diabetes

The islet basement membrane is suggested to be a barrier that protects β-cells. Studies have shown that loss of matrix and basement membrane are found only at sites of leukocyte infiltration in islets of Type 1 diabetic mice and humans (Irving-Rodgers *et al*., 2008; Korpos *et al*., 2013; Bogdani *et al*., 2014). In addition, insulitis is correlated with loss of the peri-islet basement membrane (Korpos *et al*., 2013; Bogdani *et al*., 2014). Composition of the ECM has also been associated with the activation of immune cells, and therefore dysfunction in ECM composition may be a prerequisite to islet invasion and β-cell destruction (Bogdani *et al*., 2014).

Islet transplantation is a treatment option for severe cases of Type 1 diabetes to replace defective β-cells. However, there is an increasing need for islets due to limited donors and limited survival of islet grafts which usually loses function after 5 years of transplantation (Jalili *et al*., 2010). Enzymatic harvest of the islets strips away the ECM, this causes the islets to lose its native architecture. Interestingly, islet isolation also results in markedly decreased expression of α5 integrin which is known to be involved in survival (Wang and Rosenberg, 1999). Although still not well understood, loss of islet-ECM contact is believed to contribute

to the decline in graft survival. There is therefore an increasing need for viable *ex vivo* culture of islets as well as enhanced grafts to improve transplantation.

1.2.4 Type 2 diabetes

Type 2 diabetes is highly associated with obesity, diet, and lifestyle and is more common than Type 1, accounting for 90-95% of diabetes (American DA, 2010). It results from chronic insulin resistance, when β-cells overcompensate to produce more insulin in order to increase glucose uptake, causing stress on the β-cells and results in β-cell failure [Figure 1.4] (Fu *et al*., 2013). Causes of insulin resistance, or failure to respond to insulin, is not well understood however factors such as obesity, sedentary lifestyle, and stress increase risk (Fu *et al* 2013). Evidence has shown that chronic exposure of islets to fatty acids can impair insulin secretion however, although obesity is associated with insulin resistance, most people with obesity do not develop diabetes (Flier *et al.,* 2001). Decreased islet function and insulin resistance may manifest for 10-12 years prior to diagnosis (Holman, 1998). In highly susceptible individuals, inadequate β-cell compensation persists into β-cell apoptosis and dysfunction and to full blown diabetes. Type 2 diabetes is also strongly associated with genetic predisposition for example a family history increases the risk of developing the disease (Diabetes UK Facts And Stats). In addition, it is more commonly found in South Asians and Africans compared to Caucasians (Diabetes UK Facts And Stats). More than 100 genetic loci have been identified by GWAS to play a role in Type 2 diabetes predisposition (Mohlke and Boehnke, 2015). The most common causal variants are found in genes related to fasting glucose traits such as *G6PC2* [glucose-6-phosphatase catalytic subunit 2], *GCK* [glucokinase], and *TCF7L2* [transcription factor 7 like 2] and additionally, insulin sensitivity genes such as *PPARG* [peroxisome proliferator-activated receptor γ], *KLF14* [kruppel like factor 14], *KCNJ11* [potassium voltage-gated channel subfamily J member 11], *ABCC8* [ATP binding cassette subfamily C member 8], and *IRS1* [insulin receptor substrate 1] (Dupuis *et al*., 2010; Voight *et al*., 2010; Lai *et al*., 2011; Mahajan *et al*., 2014; Ng *et al*., 2014). Risk alleles have been identified to be more common in certain populations, perhaps explaining higher incidence rates. For example, risk alleles for *KCNQ1* gene which encodes a potassium voltage-gated channel, are more highly expressed in East Asians compared to Europeans while risk alleles for *HLA-B* [major

histocompatibility complex class 1, B] and *INS-IGF2* [insulin isoform2] are higher in African Americans (Unoki *et al.,* 2008; Yasuda *et al*., 2008; Ng *et al*., 2011).

Type 2 diabetes is often treated with insulin-sensitising drugs as well as proper diet and exercise. However, management of high glucose levels is difficult and long term hyperglycemia can lead to complications such as cardiovascular disease, impaired wound healing, diabetic retinopathy, nephropathy, and severe nerve damage. Impaired circulation, wound healing, and neuropathy due to Type 2 diabetes is the leading cause of amputation in the UK (Diabetes UK Facts and Stats). Over half of those diagnosed with Type 2 diabetes have manifested complications before the time of diagnosis and therefore the increased importance for improved screening and treatment.

1.2.5 Role of the ECM in Type 2 diabetes

In Type 2 diabetes, fibrosis and ECM remodelling in adipose tissues have been shown to be a prerequisite to obesity and insulin resistance (Khan *et al.,* 2009; Kos and Wilding, 2010; Lin *et al*., 2016). In mice fed with high fat diets, several collagens have increased expression in adipose tissues. Additionally, matricellular proteins such as thrombospondin, MMP9, and SPARC have been shown to mediate fibrosis and insulin resistance (Huber *et al*., 2007; Varma *et al*., 2008; Khan *et al.,* 2009; Kos and Wilding, 2010; Inoue *et al*., 2013; Kang *et al*., 2014). It is hypothesised that ECM remodelling and fibrosis can act as a physical barrier that obstructs substrate (insulin) delivery to muscles and or impairs blood flow to muscles (Williams *et al*., 2015). Insulin resistance in skeletal muscles and the liver has also been linked to impaired integrin and FAK expression and signalling (Williams *et al*., 2015). There is perhaps a link between ECM composition and regulation to normal metabolism (Williams *et al*., 2015).

These studies suggest the important role of the ECM, particularly its remodelling and regulation in diabetes both in Type 1 and Type 2. There is therefore a pressing need to investigate significant regulators of the islet matrix and utilise their potential to induce normal β- cell function. In addition the ECM may also be important in supporting other stromal and endocrine cells in islets.

1.2.6 Pancreatic cancer

Not only is the matrix implicated in diabetes, it is also a key contributor to pancreatic cancer. Pancreatic ductal adenocarcinoma (PDAC) has one of the lowest 5 year survival rates of all cancers (<5%) (Ferlay *et al*., 2013). With a median survival of 6 months it is usually diagnosed in its advanced stages due to lack of specific symptoms in the early stages and effective early diagnostic methods (Siegel *et al*., 2014). Surgical resection in many cases is not an option as the disease has already metastasised, leaving very limited treatment options. In addition, PDAC is highly drug resistant. For example, chemotherapy with gemcitabine increases survival by only 1 month while efforts of combined treatment of gemcitabine with other cytotoxic drugs only marginally improved survival (Neese *et al*., 2015).

PDAC arises from the ductal epithelial cells of the pancreas. Genetic alterations in the K-ras oncogene are present in more than 90% of PDAC cases (Almoguera *et al*., 1988). Other mutations that contribute to the pathogenesis of PDAC include: p53, SMAD4 and CDKN2A (Ying *et al.,* 2016). Previously, studies on the pathogenesis have focused on tumour cells however recently there is also interest in studying stromal interactions. One of the hallmarks of PDAC is the abundant desmoplasia, or extensive stroma, which can constitute up to 80% of tumour mass (Hezel *et al.,* 2006; Helm *et al.,* 2014). The stroma is comprised of activated fibroblasts and stellate cells, inflammatory and immune cells, as well as a very dense ECM of collagens, fibronectins, hyaluronic acid and growth factors that together create a protective capsule around the tumour (Neese *et al.,* 2015).

Pancreatic stellate cells (PSCs) contribute to the normal ECM turnover by producing matrix proteins and degrading enzymes such as MMPs. Chronic inflammation is thought to be a risk factor for PDAC progression. Inflammation sets the stage for chronic pancreatitis to develop which is often a precursor for PDAC. Quiescent PSCs are characterised by their expression of vitamin A stored in lipid droplets [Figure 1.5]. Release of inflammatory signals activates pancreatic stellate cells rendering them more myofibroblast-like in phenotype. Activated PSCs lose their vitamin A stores and express α-smooth muscle actin (α-SMA) and increase their production of ECM proteins leading to fibrosis (Apte and Wilson, 2012; Neese *et al*., 2015; Ying *et al*., 2016). Inactivation of PSCs by retinoic acid is being investigated to treat PDAC (Guo *et al*., 2006A; Froeling *et al*., 2011).

1.2.7 Tumour-stroma cross talk

Pancreatic cancer cells and the tumour stroma have a bidirectional relationship and are interdependent. PSCs increase the proliferation, survival and migration of pancreatic cancer cells (Apte and Wilson, 2012). Likewise, several studies have shown that pancreatic cancer cells also increase the proliferation, migration and ECM production of PSCs. For example, signalling pathways that are highly active in PDAC include stroma-modulating pathways such as the sonic hedgehog (SHH) signalling (Ying *et al*., 2016). SHH ligands expressed by cancer cells activate PSCs to produce MMPs and promote invasion (Ying *et al*., 2016).

The dense stroma around pancreatic cancers increases the pressure in the microenvironment thereby compressing blood vessels. This decreases the flow into the tumour and is a major factor in the highly drug resistant characteristic of PDAC (Apte and Wilson, 2012; Neese *et al*., 2015). The tumour-stroma itself is now considered a therapeutic target to inhibit metastasis and drug resistance. However, current stroma-targeting drugs have so far not been successful in clinical trials (Neese *et al*., 2015). There is growing debate on whether stromal depletion is an effective target for treatment. For example, studies have shown that stromal depletion inhibited pro-stromal SHH signalling and increased perfusion and response to gemcitabine (Olive *et al*., 2009). However, stromal depletion may also favour aggressiveness. For example, some tumour associated fibroblasts may inhibit rather than promote tumour growth (Neese *et al*., 2015; Ying *et al*., 2016). In addition, stromal depletion may also promote the migration of tumour cells from the primary tumour. However, many factors may come into play such as tumour stage and components of the ECM. Rather than stromal depletion, research has turned to stromal reprogramming instead, in which the overall goal is to deactivate fibroblasts to recreate the microenvironment into a quiescent and non-inflammatory state (Neese *et al*., 2015). Thus there is an urgent need to understanding important regulators of the tumour-stroma crosstalk in order to realise a promising therapeutic strategy for PDAC.

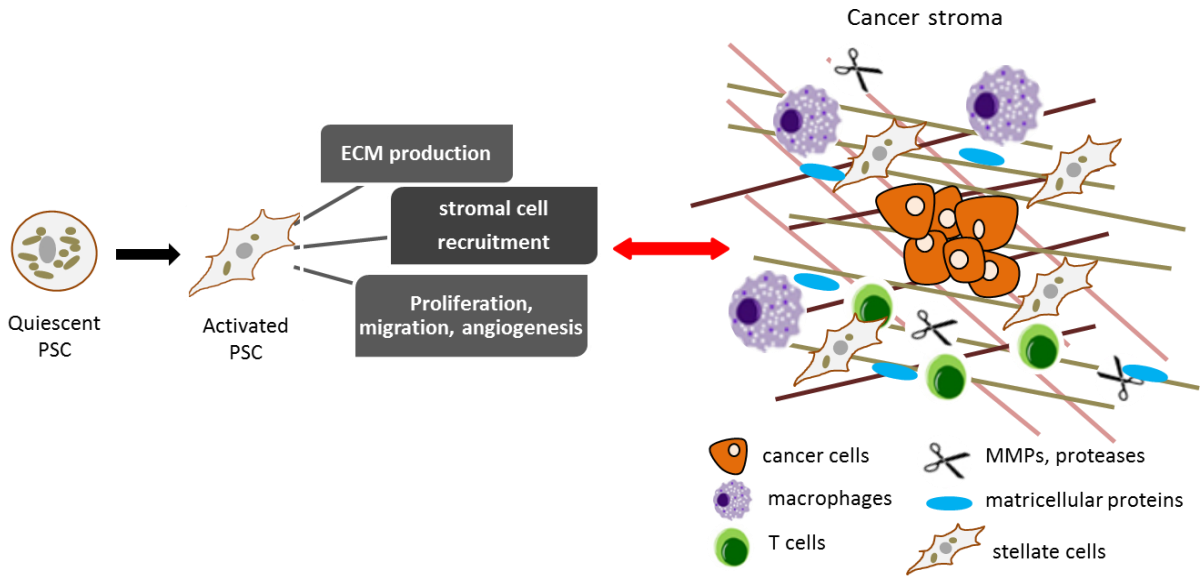


Figure 1.5: Pancreatic cancer stroma. PDAC is characterised by a dense stroma of ECM and activated stromal cells. Quiescent PSCs can become activated by inflammatory signals and consequently produce matricellular proteins to induce fibrosis. This can alter pancreatic ductal architecture and predispose the development of PDAC. The stroma and activated PSCs together create a protective capsule around pancreatic tumours, resulting in drug resistance and increased metastasis (Based on Ying *et al.,* 2016; Apte and Wilson, 2012).

1.3 The SPARC family of matrix proteins

The SPARC family (secreted protein acidic and rich in cysteine) are matricellular proteins that regulate cell-matrix communications. They play a significant role in regulating matricellular interactions and controlling cellular behaviour. Importantly, there is growing evidence indicating that the SPARC family may be essential in the pathogenesis of pancreatic diseases such as diabetes and cancer. Thus, understanding this family of proteins may be essential in understanding the development of pancreatic diseases and developing novel strategies for treatment.

SPARC belongs to the wider SPARC family that together share similar structural and functional roles but also have distinct features. The wider SPARC family also includes: hevin, SPOCK 1, 2 & 3, SMOC 1 & 2, and FSTL-1 (Bradshaw, 2012). These proteins share three main domains: domain I – an acidic low affinity calcium binding domain; domain II – a follistatin-like domain consisting of kazal-like structures; and domain III – a high affinity calcium binding domain with two EF hands (also known as the extracellular calcium binding domain) [Figure 1.6]. Members of the SPARC family also have unique domains. SPOCK proteins have a

thyroglobulin domain at the C terminus while SMOC proteins have 2 thyroglobulin domains on either end of domain I. FSTL-1 contain a von Willebrand factor type C domain at the C terminus (Bradshaw, 2012). The EC domain is well- conserved within the SPARC family. Domain I however is highly variable. For example, SPARC and hevin are highly similar and nearly identical at the follistatin and EC domains which is 63% homologous (Bradshaw, 2012). The two proteins are only different at domain I which is larger in hevin. Interestingly, domain I is also the region primarily affected in alternative splicing and confers structural diversity to the SPARC family indicating that this region may be essential in multi-functionality (Viloria and Hill, 2016).

1.3.1 SPARC

SPARC, also known as BM-40 or osteonectin, is primarily secreted by stromal cells such as fibroblasts, endothelial cells, macrophages, and stellate cells. *In vitro*, it promotes cell rounding and anti-adhesiveness. SPARC has been shown to directly bind to collagens and is involved in collagen processing and fibrillogenesis (Bradshaw *et al.,* 2003; Rentz *et al.,* 2007; Guidici *et al*., 2008; Hohenester *et al*., 2008). Typically it is highly expressed during events that require high collagen expression such as wound healing, remodelling, fibrosis, and also in the stroma of tumours (Bradshaw *et al.,* 2003; Rentz *et al.,* 2007; Guidici *et al*., 2008; Hohenester *et al*., 2008). SPARC is highly conserved among species with ~92% homology between human, rat, and mouse (Clustal Omega). The prototype protein shown in Figure 1.7 has a molecular weight of 34 kDa however, SPARC is often detected at 43 kDa due to glycosylation in the N terminal domain I (Hohenester *et al.,* 1997; Sasaki *et al*.,1998). In addition, SPARC contains sites at its domain I for transglutaminase crosslinking and has been detected crosslinked to cartilaginous matrices and to form SPARC oligomers (Aeschlimann *et al.,* 1995; Hohenadl *et al*., 1995). The follistatin domain contains a kazal-like structure that is similar to follistatin and other protease inhibitors (Bradshaw, 2012). This domain has been shown to be involved in growth factor binding, proliferation and angiogenesis (Funk and Sage 1993; Lane and Sage 1994). The EC domain on the other hand has been shown to bind directly to collagen I and IV in a calcium dependent manner (Mayer *et al.,* 1991; Sasaki *et al*., 1998; Rentz *et al.,* 2007). Furthermore, the FS-EC domains have been shown to be involved in cell shape and focal adhesion formation (Lane and Sage, 1990; Murphy-Ullrich *et al*., 1995).

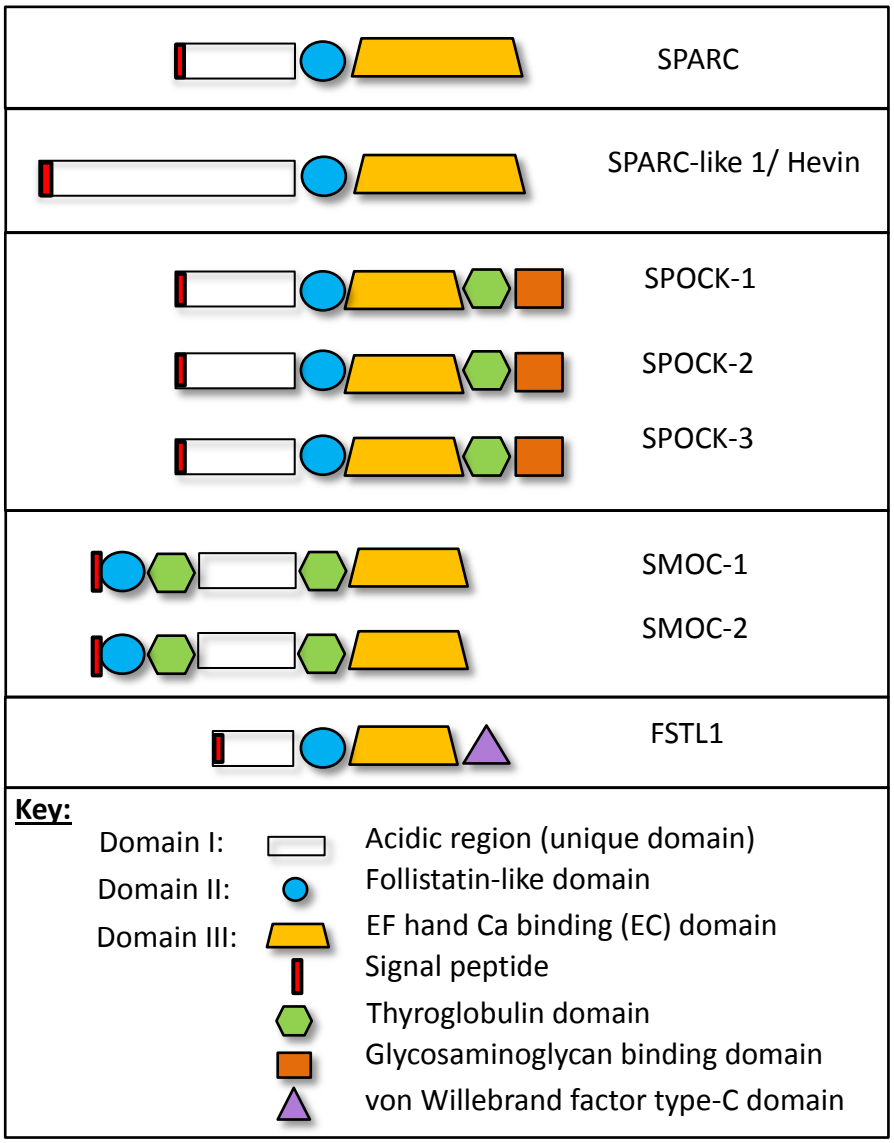


Figure 1.6: Domain structure of the SPARC family of proteins. Proteins of the SPARC family share similar domain structures. Domain I – a highly acidic low affinity calcium binding domain. Domain II- a follistatin-like binding domain. Domain III- a high affinity calcium binding EF hand domain. The signal peptide which directs the proteins into the secretory pathway is typically located in domain I except for the SMOC proteins. Members of the SPARC family also have unique domains: SPOCK proteins contain a thyroglobulin domain while SMOC proteins contain 2 thyroglobulin domains. FSTL-1 contains a von Willebrand factor type-C domain (Image from Viloria *et al.,* 2016).

SPARC regulates growth responses to PDGF, VEGF, bFGF, TGF- β, IGF-1, and HGF (Hasselaar and Sage 1992; Raines *et al*., 1992; Kupprion *et al*., 1999; Motamed *et al*., 2003; Schiemann *et al.,* 2003; Chlenski *et al.,* 2007; Ryall *et al*., 2014). It can regulate growth factor signalling directly, for example SPARC binds to PDGF and VEGF and inhibits interaction with their

receptors (Raines *et al*., 1992; Kupprion *et al*., 1999). SPARC can also influence growth factor signalling indirectly, for example SPARC inhibited bFGF-induced migration and proliferation of endothelial cells although SPARC was not found to bind directly to bFGF (Hasselaar and Sage, 1992; Motamed *et al*., 2003). SPARC and TGF-β have been shown to co-regulate each other. TGF-β, a known modulator of tissue remodelling, increased SPARC mRNA expression while SPARC can also increase TGF-β expression and regulate its activation (Wrana *et al*., 1991; Schiemann *et al.,* 2003; Francki *et al.*, 2004; Chlenski *et al.,* 2007). SPARC is also known to bind to β1 integrins and it is hypothesised that SPARC controls integrin activation, clustering, and cross-talk with growth factor receptor signalling (Weaver *et al.*, 2008; Arnold & Brekken, 2009). SPARC therefore regulates multiple pathways fundamental to cell growth. Furthermore, it has been suggested that the interaction between SPARC and collagen may interfere with integrin activation (Bradshaw, 2009). The ability of SPARC to modify matrix characteristics may therefore be relevant to cell growth as well as cell adhesion.

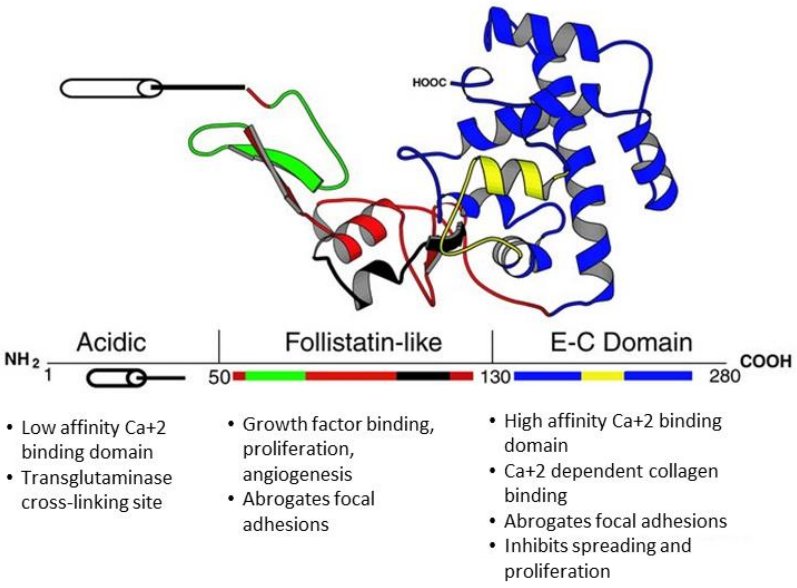


Figure 1.7: The structure of SPARC. The prototype protein of SPARC has a molecular weight of 34 kDa. The acidic domain I of SPARC has been shown to be involved in transglutaminase cross-linking and cell spreading. Follistatin-like domain II has been shown to be involved in proliferation and focal adhesion assembly. Calcium-binding domain III has been shown to be involved in cell spreading, proliferation, focal adhesion assembly and collagen binding (Modified from Bradshaw and Sage 2001).

Interestingly, SPARC also interacts with other matrix proteins such as vitronectin and thrombospondin (Rosenblatt *et al*., 1997; Clezardin *et al*., 1988). Proteolysis of SPARC generates bioactive fragments that have been shown to influence angiogenesis *in vivo* and the physiological effects exerted by these proteolytic fragments are likely to add further to their complexity of interactions (Iruela-Arispe *et al.,* 1995; Sage *et al*., 2003).

SPARC is a multifunctional protein that has been shown to play a role in angiogenesis, tumourigenesis, and fibrosis (Bradshaw, 2012). However, the role of SPARC in cancer appears to be highly tissue dependent. For example, high levels of SPARC have been detected in breast cancer, glioblastomas, and melanomas, while decreased expression was observed in ovarian and colorectal cancers (Ledda *et al.,* 1997; Rempel *et al.,* 1998; Yiu *et al.,* 2001; Jones *et al*., 2004; Lien *et al*., 2007; Cheetham *et al.,* 2008). It has been shown that the *SPARC* gene is hypermethylated in gastric cancers (Chen *et al.,* 2014). In pancreatic cancer, SPARC overexpression in the tumour stroma is strongly associated with poor patient prognosis, although *in vitro,* SPARC is reported to inhibit pancreatic cell growth (Sato *et al*., 2003; Guweidhi *et al*., 2005; Mantoni *et al*., 2008; Chen *et al.,* 2010). The reason for this apparent complexity is not currently known.

Elevated levels of SPARC have also been associated with insulin resistance, diabetes and obesity (Kos *et al.,* 2009; Wu *et al*., 2011, Xu *et al.,* 2013). Furthermore, SPARC is expressed by pancreatic stromal cells and is localised to the islet basement membrane in the pancreas (Ryall *et al.,* 2014). SPARC inhibits β-cell and islet responses to IGF-1 and HGF-1 and can also influence β cell function (Harries *et al.,* 2013; Ryall *et al*., 2014). Production of SPARC by pancreatic stellate cells is regulated by diabetic markers such as glucose, insulin, and leptin suggesting that SPARC may influence β cell loss and dysfunction in Type II diabetes (Ryall *et al*., 2014). In addition, SPARC production in adipocytes is also influenced by glucose, insulin, and leptin and further suggests that SPARC may be involved in insulin resistance (Kos *et al*., 2009). SPARC has therefore been shown to be involved in a number of diseases, including those of the pancreas.

However, the exact mechanism of action of SPARC is not well understood and there are contradictions in literature perhaps due to tissue specific activity. Although SPARC is by far the most well studied protein in the family, little is known of the related SPARC proteins

particularly in pancreatic diseases such as diabetes and cancer. The wider SPARC family may similarly play a role in the pathogenesis of these diseases but however little is understood of their mechanism and have not yet been characterised in the pancreas. Current knowledge for each of the SPARC proteins will be discussed in the following section and is summarised in Table 1.1, particularly on their role in diabetes and pancreatic cancer.

1.3.2 Hevin

SPARC-like 1, SC1 (synaptic cleft 1) or MAST9, was first identified in the rat brain (Johnston *et al*., 1990). The human homologue called hevin was first isolated from high endothelial venule cells (Girard and Springer, 1995). Among the SPARC family, hevin is the most structurally similar to SPARC; the major difference is the much larger acidic domain I in hevin which contains ~400 amino acids compared to ~50 in SPARC. SPARC and hevin have been closely related throughout evolutionary history and arose from two whole genome duplications of an ancestral SPARC gene during vertebrate evolution (Kawasaki *et al*., 2004; Bertrand *et al*., 2013). Functionally, hevin is also very similar to SPARC. It is anti-adhesive, modulates cell shape and is the only other SPARC protein with evidence of collagen binding and influence on collagen assembly (Girard and Springer, 1996; Hambrock *et al*., 2003; Brekken *et al*., 2004; Sullivan et al., 2006). While the role of hevin in diabetes has not yet been examined, hevin has been implicated in many cancers. Similar to SPARC, hevin is also either overexpressed or downregulated depending on the type of cancer. For example, it is down-regulated in brain, prostate, bladder, ovarian, and non-small cell lung cancers (Bendik *et al*., 1998; Nelson *et al*., 1998; Claeskens *et al*., 2000). In pancreatic cancer, hevin mRNA is overexpressed compared to normal tissues, yet was found to inhibit pancreatic cancer cell growth and invasion *in vitro* (Iacobuzio-Donahue *et* al., 2002; Esposito *et al*., 2007). Hevin therefore may play a similar role to SPARC, and in fact cleavage of hevin by matrix metalloproteinases (MMPs) and ADAMTS4 (a disintegrin and metalloproteinase with thrombospondin motifs 4) has been shown to create a "SPARC-like fragment" that may act similarly to SPARC. It has therefore been suggested that hevin may compensate for SPARC and that hevin may act as a SPARC reservoir (Weaver *et al*., 2010; Weaver *et al*., 2011).

	Growth/ Proliferation/Metastasis	Growth Factor activity	ECM activity	KO mice phenotype	Pancreatic cancer	Diabetes
SPARC	Overexpressed in breast, glioblastoma, melanoma (Jones 2004, Lien 2007, Rempel 1998, Ledda 1997) Decreased expression in ovarian and colorectal (Yiu 2001, Cheetham 2008)	PDGF, VEGF, bFGF, TGF-β IGF-1 and HGF signalling in β-cells (Raines 1992, Kupprion 1999, Hasselaar and Sage 1992, Motamed 2003, Schiemann 2003, Chlenski 2007, Ryall 2014)	Binds to Collagen Regulates procollagen processing (Rentz 2007, Bradshaw 2003, Guidici 2008, Hohenester 2008) Binds to β1 integrins Binds to vitronectin, thrombospondin (Rosenblatt 1997, Clezardin 1988, Weaver 2008)	Abnormal ECM assembly in dermis and lens capsule (Bradshaw 2003a, 2009) smaller fibrils and more uniform in diameter (Bradhsaw 2003b) Develop cataracts in lens capsule (Gilmour 1998)	Overexpression in PDAC associated to poor prognosis (Guweidhi 2005; Mantoni 2008) *In vitro*, inhibits pancreatic cancer growth (Chen 2010, Sato 2003)	Elevated levels associated with insulin resistance, diabetes and obesity (Wu 2011; Xu 2013; Kos 2009) Influences insulin production (Harries 2013) Production in adipose and pancreatic stellate cells regulated by insulin, glucose, leptin (Kos 2009)
Hevin	Down regulated in brain, prostate, bladder, ovarian, NSCLC cancers (Nelson 1998, Bendik 1998, Claeskens 2000)		Binds to Collagen I Regulates procollagen processing (Sullivan 2006, Grirard and Springer 1996, Brekken 2004, Hambrock 2003) Cleaved by MMPs (Weaver 2010, Weaver, 2011)	Abnormal collagen structure and decreased fibril diameter (Sullivan 2006) Increased vascular invasion and accelerated wound closure (Sullivan 2008)	Increased mRNA in pancreatic cancers and chronic pancreatitis (Esposito 2007, Iacobuzio-Donahue 2002) *In vitro*, inhibits cancer cell growth and invasion (Esposito 2007)	Unknown
SPOCK-1	Increased expression involved in EMT (Miao 2013) and in invasive cancers (Kawamata 2003)		MMP regulation (Nakada 2001, 2003)	No phenotypic difference in adult mice (Roll 2006) No change in SPOCK-2 or SPOCK-3 expression (Roll 2006)	High expression in stroma of PDAC Associated to poor prognosis	Unknown
SPOCK-2			MMP regulation (Nakada 2001, 2003)	Unknown		Unknown
SPOCK-3	Inhibits glioma invasion (Nakada 2003)		MMP regulation (Nakada 2001, 2003)	Unknown		Unknown
SMOC-1	Gene hypermethylated in breast cancer (Fackler 2011) Stem cell differentiation (Choi 2010)	TGF-β antagonist (Thomas 2009)	Binds to tenascin-C (Brellier 2011)	Unknown	Unknown	Unknown
SMOC-2	Induces proliferation (Liu 2008)	Regulates PDGF-induced cyclin D1 expression (Liu 2008)	Binds to integrins and activates ILK (Barker 2005, Liu 2008)	Unknown	Unknown	Unknown
FSTL-1	Promotes angiogenesis (Ouchi 2008) Hypermethylated in nasopharyngeal cancer (Zhou 2016)	Binds to TGF-β superfamily (Tsuchida 2000)	Inhibits MMP2 expression (Chan 2009)	Perinatal lethality (Sylva 2011) Newborn mice develop lung and skeletal malformations (Sylva 2011)	Unknown	Unknown

Table 1.1: Summary of roles of the SPARC family. Current knowledge of the roles of the SPARC family on cell growth, proliferation, growth factor and matrix interactions, as well in pancreatic diseases.

1.3.3 SPOCK proteins

SPOCK, also known as testican, was first discovered in seminal fluid (Alliel *et al*., 1993). Although found to be expressed in different tissues, it is most highly expressed in the nervous system (Vannahme *et al*., 1999, Marr *et al.,* 2000, Edgell *et al*., 2004). Like SPARC, SPOCK-1 has been shown to inhibit cell attachment and promote cell rounding (Marr and Edgell, 2003). The SPOCK proteins are thought to influence matrix characteristics primarily through protease regulation. For example, both SPOCK-1 and SPOCK-3 were shown to inhibit membrane-type MMP (MT-MMP) activation of pro-MMP2 in gliomas (Nakada *et al*., 2001). SPOCK-2 on the other hand blocked SPOCK-1 and SPOCK-3 inhibition of MT-MMPs thus demonstrating co-regulation within the SPARC family (Nakada *et al*., 2003).

SPOCK and SMOC proteins are widely present throughout the animal kingdom. During metazoan evolution, thyroglobulin domains were incorporated with follistatin and EC domains: SPOCK-like which contained one thyroglobulin, and SMOC-like which contained two (Novinec *et al*., 2006). Subsequent gene duplications of the early SMOC-like ancestor eventually gave rise to SMOC 1 and 2, while the early SPOCK-like ancestor gave rise to SPOCK 1,2, and 3 (Novinec *et al*., 2006). Other members of the thyroglobulin-1 superfamily include: MHCII invariant chain, thyroglobulin and other matrix proteins such as IGFBPs and nidogens which have all been classified to have protease inhibitory functions. SPOCK-1 was shown to inhibit cathepsin L through its thyroglobulin domain (Bocock *et al*., 2003; Meh *et al.,* 2005). However, it is currently unclear whether the thyroglobulin domain is solely responsible for protease inhibition. For example, SPOCK-2 was shown to bind to the C terminus of SPOCK-3 through its domain I while N-tes, a SPOCK-3 variant missing the thyroglobulin domain, has been shown to inhibit MT-MMPs through the unique domain I (Nakada *et al*., 2001; Nakada *et al.,* 2003).

The role of SPOCKs in diabetes is unknown, but high expression of SPOCK-1 in the desmoplastic stoma of PDAC has been associated with poor prognosis (Damhofer *et al*., 2013). In other cancers, the increase in SPOCK-1 expression by TGF-β is thought to be involved in epithelial-mesenchymal transition [EMT] (Miao *et al*., 2013). SPOCK expression was down regulated in highly invasive esophageal cancer cells compared to the non-invasive

types (Kawamata *et al*., 2003). These studies suggest an important role for SPOCK proteins in matrix remodelling and metastasis.

1.3.4 SMOC proteins

SMOC, or secreted modular calcium binding protein, has been detected in several organs such as the brain, lung, kidney, heart, skeletal muscle, and ovaries (Vannahme *et al.,* 2002; Vannahme *et al*., 2003). SMOC-1 expression is typically associated with basement membranes while SMOC-2 also occurs in the connective tissue stroma (Vannahme *et al*., 2002, Srivastava *et al*., 2007, Maier *et al.,* 2008).

The SMOC proteins have been shown to regulate cell responses to growth factors. SMOC-1 is known to be an antagonist for the TGF-β superfamily-BMP proteins, and is important for osteoblast stem cell differentiation (Thomas *et al*., 2009; Choi *et al*., 2010). SMOC-1 may also influence tissue remodelling by interacting with tenascin-C, another matricellular protein (Brellier *et al*., 2011). In breast cancer, the *SMOC-1* gene was found to be hypermethylated however very little is known about the role of SMOC-1 in human disease (Fackler *et al*., 2011).

There is similarly little known about the role of SMOC-2 in disease, although there is growing evidence that SMOC-2 has mitogenic properties and is an important regulator of proliferation. It has been shown to synergise VEGF and bFGF-induced angiogenesis of HUVECs (Rocnik *et al*., 2006). SMOC-2 has also been shown to induce DNA synthesis by increasing cyclin D1 expression during the G1 phase (Liu *et al*., 2008). More specifically, SMOC-2 potentiates PDGF-induced proliferation and appears to be necessary in growth factor induced DNA synthesis and cyclin D1 expression (Liu *et al*., 2008). Although SMOC-2 did not affect PDGF receptor activation, like SPARC, it activated ILK (integrin-like kinase) activity suggesting regulation of integrin-ECM interactions (Barker *et al*., 2005; Liu *et al*., 2008). Furthermore, SMOC-2 has been shown to bind to αvβ1 and αvβ6 integrins promoting cell attachment and focal adhesion formation (Maier *et al*., 2008). In addition, SMOC-2 can influence cell motility of colon cancer cells indicating its importance in regulating cell adhesion (Shvab *et al*., 2015).

1.3.5 FSTL-1

Follistatin-like protein 1 or FRP (follistatin-related protein) is a pro-inflammatory molecule shown to promote the expression of inflammatory cytokines IL-1β, TNF-α, and IL-6 (Miyamae *et al*., 2006). Also known as TSC-36 or TGF-β stimulated clone-36, it is a TGF-β induced protein (Shibanuma *et al.,* 1993) and has been shown to bind directly to TGF-β (Tsuchida *et al*., 2000). This pro-inflammatory molecule may therefore be involved in tissue regeneration and repair (Miyamae *et al*., 2006). Like SPARC, its mechanism of action is still unclear, as it has been demonstrated to be both pro and anti-proliferative. For example, FSTL-1 promoted angiogenesis by activating AKT signalling and inhibited apoptosis of endothelial cells (Ouchi *et al*., 2008). In contrast it also upregulated the expression of apoptotic caspases and inhibited MMP2 expression in endometrial cancers (Chan *et al*., 2009). The FSTL-1 promoter has additionally been shown to be hypermethylated in nasopharyngeal cancers (Zhou *et al.,* 2016).

FSTL-1 is the least similar in structure to SPARC, containing only 12 amino acids in its unique domain I and a C terminal domain homologous to von Willebrand factor type-C. Like other members of the SPARC family, the EC domain of FSTL-1 also contains two EF hands. This highly conserved EC domain has been shown to be the collagen binding domain of SPARC and Hevin (Maurer *et al.,* 1995; Hambrock *et al*., 2003). In keratinocyte-like HaCaT cells, the EC domain of SMOC-1 co-localises with vinculin, while in SMOC-2 it is responsible for cell attachment and focal adhesion formation (Maier *et al*., 2008; Klemencic *et al.,* 2013). However, it is suggested that the EC domain of FSTL-1 is non-functional and therefore may not be involved in collagen binding (Maurer *et al*., 1997).

FSTL-1 has not yet been clinically described in pancreatic cancer or diabetes. However, TGF-β has been shown to regulate β-cell replication which indicates that FSTL-1 may have a role in this signalling (Dhawan *et al*., 2016). Interestingly, FSTL-1 is closely related to other follistatin proteins. FSTL-2, also contains one follistatin domain and has been shown to be a tumour suppressor for different cancers (Suzuki *et al*., 2010; Chen *et al*., 2011). Also known as IGFBP-7, FSTL-2 has been shown to be down-regulated in PDAC and is associated with poor prognosis (An *et al.,* 2012). FSTL-3 on the other hand, has two follistatin domains and has

been shown to bind to the TGF-β family members, activin and myostatin (Mukherjee *et al*., 2007). Furthermore, FSTL-3 knock-out mice have increased pancreatic islet number and improved insulin sensitivity (Mukherjee *et al*., 2007).

1.4 Complexity of studying SPARC proteins

Overall, the exact mechanism of action of the SPARC family is not well understood. There are overlapping and contradictory tissue specific functions that warrant the need for an extensive screening of the entire SPARC family for a unified model. In addition, studying matricellular interactions is challenging as there are numerous complex interactions occurring simultaneously in the matrix [Figure 1.8]. Factors such as matrix composition and growth factor/receptor profile will together influence how the SPARC family behaves. In addition, there is also growing evidence of coordinated regulation within the SPARC family, particularly for SPARC and hevin and within the SPOCK family. Although little is known on the gene mutations of the SPARC family in relation to disease, there has been growing evidence supporting that alternative splicing, proteolysis, and post translational modifications may create functional fragments and reveal cryptic binding sites that determine matricellular interactions and therefore diseased states (Viloria and Hill*,* 2016*)*. Hence there is a need for a comprehensive systematic approach to determine overlapping and non-redundant functions.

The complex microenvironment is additionally difficult to recreate in culture and this itself may add to the controversial mechanisms described for the SPARC family above. Recently our lab has shown that treatment of β-cells with rSPARC inhibits IGF-1 -induced proliferation and also inhibits islet survival (Ryall *et al*., 2014). Interestingly, SPARC, a well-known collagen binding protein, has not been extensively studied in the presence of collagen or an extracellular matrix which may explain the controversial results in literature. The challenge is to understand the dynamic exchange of information from the matrix to intracellular signalling and *vice versa*. There is therefore a need to study SPARC and the SPARC family in a model that mimics the microenvironment of health and disease.

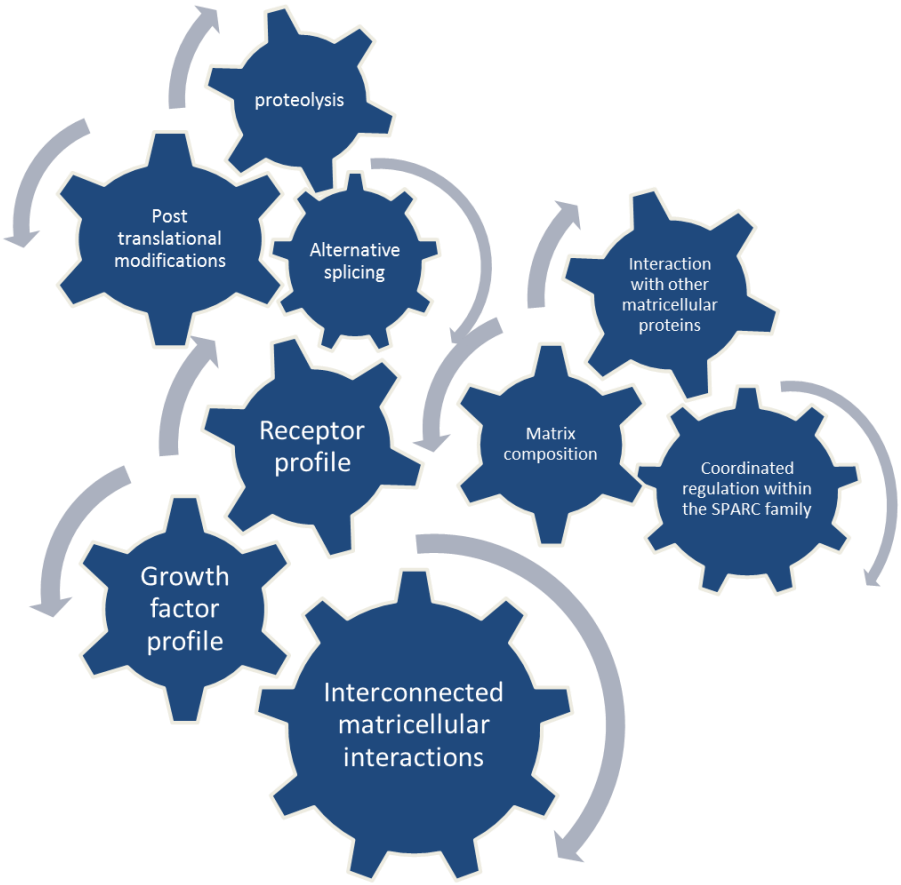


Figure 1.8: Complex and dynamic functions of the SPARC family. Post translational modifications, alternative splicing and enzyme cleavage contribute to the diverse structure of the SPARC family that affect the complexity of its interactions and functions (Based on Bradshaw, 2012). In addition, the composition of the matrix, growth factor and receptor profile overall contribute to the intricate and coordinated interactions of the SPARC family.

1.5 Relevance of 3D Cell Culture

Cells naturally have a constant and dynamic interaction with the matrix *in vivo*. Creating more complex culture systems is therefore essential to mimic and understand disease states. The growing popularity of 3D culture in research initiated a significant advancement in moving towards "closer-to-*in-vivo*" models. The advent of high throughput matrix scaffolds and organ-on-chip systems has improved our understanding of cellular physiology (Ravi *et al*., 2015).

Although 2D culture studies are invaluable, there is growing evidence supporting that culturing cells in 3D induces them to behave as they would in *vivo* (Ravi *et al*., 2015). For example, islets and β-cells cultured in a matrix have increased β-cell mass, proliferation,

survival and improved insulin response compared to those cultured in 2D (Beattie *et al*., 1996; Beattie *et al*., 2002; Nagata et al., 2002; Kaido *et al.,* 2004; Weber *et al.,* 2008; Jalili *et al*., 2010; Zhang *et al*., 2012). Furthermore, integrin β1 has been implicated in regulating β-cell function (Bosco *et al*., 2000; Kaido *et al.,* 2004; Riopel *et al*., 2011; Diaferia *et al*., 2013,). Mice deficient in β1 integrins have impaired β-cell mass and function (Riopel *et al*., 2011; Diaferia *et al*., 2013). There is growing evidence in literature suggesting that it might be possible to reverse the adverse effects of enzymatic harvest of islets by re-establishing islet-ECM interactions using 3D culture (Wang and Rosenberg, 1999). For example, expression of apoptotic caspases in islets decreased in 3D (Zhang *et al*., 2012). Moreover, survival of islets in a collagen gel significantly improved when co-cultured with fibroblasts (Jalili *et al*., 2010). When these islets were transplanted back into mice, those cultured in fibroblast-populated collagen matrices had longer survival and improved function than those cultured in the matrix alone (Jalili *et al*., 2010). Additionally, screening of diabetic drug efficacy has also started to move into 3D islet models (Li *et al*., 2013). It is therefore relevant to investigate the SPARC family in a 3D matrix that more closely mimics the islet matrix to understand their role in diabetes.

1.6 Aims

We therefore aimed to investigate the extended SPARC family in the pancreas and provide a holistic analysis to address the complexity of functions in understanding their role in pancreatic diseases, particularly in diabetes.

1. SPARC has been characterised in the pancreas however there is limited knowledge on the role of the extended SPARC family in diabetes and pancreatic cancer. Our first aim is to identify and characterise the expression pattern of hevin, SPOCK proteins, SMOC proteins and FSTL-1 in the pancreas.
2. Our second aim is to study the SPARC family in a 3D collagen matrix, creating a complex environment that mimics the islet matrix to further understand their role on β-cell and islet growth, proliferation, and survival.
3. We also aim to investigate the role of the SPARC family on β-cell adhesion, cytoskeletal remodelling and their impact on insulin secretion.

2. Methods

2.1 Animals and islet isolation

Pancreas sections for immunohistochemistry and islets were obtained from adult male or female outbred ICR mice (21-25g) from Harlan, Bicester, UK. All animal procedures were carried out according to the UK Home Office Regulations. All animal procedures were approved and maintained by the ethics committee at King's College London. Animals were housed in cages in a clean environment at King's College London. Mice were sacrificed using cervical dislocation. Pancreas tissues were harvested and fixed in 10% NBF.

For islet isolation, a clamp was used to block the Vater's ampulla in order to inject collagenase into the pancreas through the common bile duct. Pancreas were then detached and harvested immediately to be incubated in a water bath at 37°C for 10 minutes to activate the collagenase. Pancreas tissues were re-suspended in MEM supplemented with calf serum, penicillin and streptomycin and then centrifuged (1400 rpm for 1 minute and 15 seconds) and vortexed twice in order to pellet. Islets were separated from tissue debris by sifting through a 425 mm sieve and immediately re-suspended in MEM and centrifuged at 1500 rpm for 1 minute and 30 seconds. Media was poured out and excess moisture was wiped using a chem wipe wrapped around forceps. Once completely dry, islets were isolated by density gradient centrifugation using histopaque (Sigma Aldrich). MEM was slowly added to create two distinct layers. Tubes were centrifuged at 3510 rpm for 24 minutes at 10°C. About 5 mm under the interface, islets were collected using a pipette and transferred into a clean tube. Islets were re-suspended in MEM and washed 3 times by centrifuging at 1500 rpm for 1 minute and 30 seconds, each time taking off the top 25 mL of media and replacing with fresh media. After each round, islets were left to sediment on ice for 4 minutes before repeating. Islets were subsequently washed with RPMI media and cultured at 37°C in petri dishes.

2.2 Immunohistochemistry (IHC)

ICR mouse pancreas tissues were embedded in paraffin. Sections (5 µm) were deparaffinised in either histoclear or histochoice and re-hydrated in 100% and 70% ethanol. Sections

subjected to enzyme treated antigen retrieval were incubated with Proteinase K (Sigma) at 50 µg/mL at 37°C for 20 minutes. Sections subjected to heat-mediated antigen retrieval were microwave-boiled in either citrate-EDTA buffer (pH 6.2, 10 mM citric acid, 2 mM EDTA, 0.5% Tween 20) or EDTA buffer (pH 8, 1 mM EDTA, 0.5% Tween 20) for 10-20 minutes. For sections stained for fluorescence, antigen retrieval was followed by incubation in 0.05 M glycine buffer for 5 minutes to block endogenous aldehydes. All sections were then blocked with 10% normal horse serum (NHS) [Sigma H0146] for 30 minutes in a humidified chamber. SPARC primary antibodies were diluted in NHS and incubated overnight at 4°C followed by PBS washes for 5 minutes, two to three times. Appropriate secondary antibodies were incubated for 1 hour at room temperature. For sections co-stained with glucagon, goat anti-glucagon (C-18 SC779) was used (1:100). To block endogenous peroxidases, sections were incubated in hydrogen peroxide. Antibody binding was detected using a DAB peroxidase kit (Vector labs) and counterstained with haematoxylin. Sections stained with fluorescent antibodies were mounted with Slow-fade DAPI. Digital images were acquired with the Nikon Eclipse 80i microscope. Antibodies used for these experiments are described in Appendix Table 8.1.

2.3 Cell culture

Human PS-1 pancreatic stromal cells and MRC5 fibroblasts were kindly provided by Professor Hemant Kocher from the Barts Cancer Institute, Queen Mary University of London. INS-1 β cells, human AsPC-1, Hpaf, Capan-1, Panc-1 pancreatic cancer cells were kindly provided by Dr. Charlotte Edling from the Blizzard Institute, Barts and the London. MIN-6 β cells were kindly provided by Professor Peter Jones from King's College London. HUVEC endothelial cells were kindly provided by Dr. Andrew Snabaitis at Kingston University, London.

PS-1, AsPC-1, Hpaf, Capan-1, and Panc-1 cells were grown in RPMI-1640 media (Fisher Scientific 12004997) supplemented with 2 mM L-glutamine (Fisher Scientific 11500626), 100 µg/mL penicillin and streptomycin (Fisher Scientific 15140-122), and 10% FBS (Gibco 11573397). INS-1 cells were grown in RPMI-1640 media supplemented with 2 mM L-glutamine, 100 µg/mL penicillin and streptomycin, 1 mM sodium pyruvate (Sigma S8636-100), 10 mM HEPES buffer(Sigma H-0887-100), 0.05 mM β-mercaptoethanol (Sigma M3148)

and 10% FBS. MIN-6 and MRC5 cells were grown in DMEM media supplemented (LGC V30-2002) with 100 μg/mL penicillin and streptomycin and 10% FBS. HUVEC cells were grown in F12K media (Sigma N6658) supplemented with 100 μg/mL penicillin and streptomycin, 0.1 mg/mL heparin, 50 μg/mL ECGS (Millipore 02-102), and 10% FBS. Cells were incubated at 37°C in 5% CO2.

2.4 Growth assays

INS-1 β-cells ($1.5x10^4$ cells/well) and AsPC-1 (5×10^3 cells/well) pancreatic cancer cells were plated in a 96 well plate and syncrhonised in low serum media (0.5% FBS) for 24 hours. Post synchronization, the cells were either treated with fresh complete medium (10% FBS) or with medium containing various concentrations of rFSTL-1 (R&D Systems) [See Appendix Table 8.2] and cultured for a further 72 hours. Cell growth was monitored every 12 hours using the IncuCyteZOOM live cell imaging system (Essen Bioscience). BrdU incorporation was measured during the last 24 hours of the 72 hour culture by ELISA according to the manufacturer's instructions (Roche 11647229001). These experiments were performed in collaboration with Amanda Munasinghe, Kingston University.

2.5 Protein expression analysis by Western blotting

Cells were lysed with RIPA buffer (radioimmunoprecipitation buffer) [Sigma Aldrich] for 20 minutes on ice in the presence of protease inhibitors (Halt™ Protease Inhibitor Single Use Cocktail PN78430). Lysates were subsequently cleared by centrifugation to collect the supernatant. For western blotting of various cell lines to detect the SPARC family, samples were run immediately after lysis to avoid potential issues with protein degradation. For glycosylase experiments, cell lysates were denatured at 100°C for 4 minutes then incubated with PNGase F for one hour at 37°C per manufacturer's instructions (New England BioScience P0704S).

Protein concentration was determined using the BCA (bicinchoninic acid assay) protein assay (Bio-rad 500-0112). Briefly, protein standards were prepared using BSA (bovine serum albumin) [Sigma A2153] in RIPA buffer. Absorbance was detected at 750 nm and protein concentration was calculated using the standard curve.

Sample buffer was added to lysates after which equal protein was loaded onto 12% tris-glycine polyacrylamide gels and subjected to SDS-PAGE (20-25 µg of protein/well). Tris-glycine running buffer (pH 8.3) was used for electrophoresis. Using semi-dry transfer, proteins were then transferred onto nitrocellulose membranes (GE Healthcare 10600001) in Tris-glycine and 20% methanol transfer buffer. Protein transfer was confirmed using Ponceau staining (Sigma). Membranes were next blocked with 5% milk solution (Marvel). Membranes were incubated overnight at 4°C with the relevant rabbit primary antibodies [See Appendix Table 8.1] and mouse β-actin antibody (1:2500, Abcam ab8224). After washing with Tween TBS (pH 8.0), membranes were incubated with anti-rabbit and anti-mouse secondary antibodies conjugated respectively to IR800 (Li-Cor 926-68070) and IR700 (Li-Cor 926-32211) infrared dyes for an hour and a half at room temperature. Membranes were visualized by infrared using the Li-Cor Odyssey CLx scanner. Molecular weight and signal intensity was measured using the Li-Cor Image Studio. Protein bands were standardised to β-actin.

2.6 mRNA expression analysis by qRT-PCR

Total RNA was isolated from PS-1 cells using an RNeasy Mini Kit, including on-column DNase I treatment (Qiagen 74104). Total RNA was quantified using a NanoVue^{TM} Plus Spectrophotometer. RNA integrity was confirmed using an Agilent Bioanalyzer 2100. RNA (700 ng) was reverse transcribed to cDNA using a RevertAid Strand cDNA Synthesis Kit (ThermoFisher Scientific 10387979). PS-1 cell cDNA was amplified using DreamTaq Green PCR Master Mix (ThermoFisher Scientific K1081). Primers were designed to SPOCK-3 splice variants banked in ENSEMBL (Accessed March 2013) using PrimerBLAST [Appendix Table 8.3]. Primer sequences used for DIP2A were: Forward primer- GCAGATGGTGTCCCTGTGAAC; reverse primer-CTGATTTGGATCTGGTTGCTGA. At least one primer in each pair was designed to be exon spanning to avoid amplification of any residual genomic template. RT-PCR products were separated on a 2% agarose gel in TAE. QARS primers (forward - TTCCGGTGTCTCTGCAATGG; rev- CTGCTGAGCCTGAGTAGCG) were used as a loading/positive control. All negative controls were blank (no RT cDNA, no template cDNA, and PCR dH20 control). These experiments were completed in collaboration with Asher, Sharan (Kingston University). Alternative splicing and primer design was completed by Hill, Natasha (Kingston University).

2.7 Bioinformatics

The ENSEMBL database was used to identify alternative splice variants of the human SPARC family of proteins. Only protein- coding transcripts for which the complete coding sequence (CDS) is known were included in the analysis. For these transcripts, protein FASTA sequences were downloaded. The ENSEMBL transcript IDs used are given in Table 3.3 [download date April 2015]. Respective product sizes for each complete transcript were calculated using the Protein Molecular Weight Bioinformatic tool from the Sequence Manipulation Suite. Domain structures for alternative transcripts were determined using the InterPro protein sequence analysis and classification database. Signal peptide expression was determined using Phobius signal peptide predictor. Genecards, UNIPROT, Phosphosite plus, and TRANSDAB were used to identify predicted post-translational modifications of the SPARC family (primary variants). The Human Protein Atlas was accessed July 2016.

2.8 3D Assays

2.8.1 Preparation of 3D cultures

Formulation of the RAFT collagen solution (Lonza 016-1R10, previously TAP Biosystems 016-0R93, 016-0R94) was prepared according to the manufacturer's instructions. MEM, collagen, and neutralising solution were kept on ice and mixed together until the solution was homogenous in colour. RAFT collagen solutions were kept on ice until use. During this time, cells or islets were prepared to the appropriate seeding density and then added to the collagen solution. SPARC, hevin, SPOCK-3, or SMOC-1 (50- 100 µg/mL) were then added to the collagen solution [See Appendix Table 8.2 for protein details]. To make a 100 µm tissue, 240 µL of cell-collagen mixture was distributed per well in a 96 plate. To make a thicker tissue (~200 µm) for islet culture, 400 µL of cell-collagen mixture was distributed per well. The collagen solution was then polymerised for 15 minutes at 37°C. Absorbers provided by the manufacturer were used to take up liquid and compress the collagen gel (1.6 mg/mL) into a tissue (80-90 mg/mL) [Figure4.1].

2.8.2 Fluorescent detection of cells in 3D culture

To validate imaging of the 3D tissue, cells were stained with 15 µM of Cell Tracker Red CMTPX dye (Life Technologies C34552) for 45 minutes at 37°C. Cells were then placed in the

3D collagen solutions as described above and images were acquired using either the EVOS fluorescent microscope, Leica (TCS SP8X) confocal microscope, or the IncuCyteZOOM live cell imaging system (Essen Bioscience). Cell growth was quantified using either the ImageJ software or the IncuCyteZOOM software.

2.8.3 Live/Dead Survival assay

To measure cell survival in the 3D culture, 3D tissues embedded with cells were incubated with 1 µM of ethidium homodimer-1 (EthD-1) and 0.5 µM calcein AM (Life Technologies L-3224) for 45 minutes at room temperature. EthD-1 is a cell-impermeant dye that fluoresces red when bound to the DNA of dead cells. Calcein AM is a cell-permeant green dye that is made fluorescent by esterases of live cells. To validate islet survival in the 3D cultures, 3D tissues were incubated with 2 µM of EthD-1 and 1 µM of calcein AM. Cell survival and growth over time was imaged using the EVOS fluorescent microscope or the Leica (TCS SP8X) confocal microscope. Validation of these experiments were completed in collaboration with Munasinghe, A.

2.8.4 BrdU proliferation assay in 3D

To measure cell proliferation in the 3D culture, BrdU incorporation was measured fluorescently as per manufacturer's instructions (Roche 11296736001). Briefly, 3D tissues embedded with cells were incubated with BrdU (1:1000, Roche) overnight at 37°C for the last 24 hours of a 72 hour culture period. Tissues were then washed with PBS and then treated with NucBlue (Life Technologies R37605) for 20 minutes at room temperature. Ethanol glycine was used to fix tissues for 20 minutes at -20°C. After another PBS wash, cells were permeabilised with 0.1% Triton-X for 5 minutes. Tissues were then incubated with anti-BrdU (1:10, Roche) for 2 hours at 37°C and then incubated with fluorescein conjugated secondary antibody for 1 hour at 37°C. Samples were mounted with Slow-fade Gold (Life Technologies S36937) and coverslips. Images were acquired using the either the FLOID fluorescent microscope or confocal microscope and BrdU incorporation was quantified using ImageJ software. Validation of these experiments were completed in collaboration with Munasinghe, Amanda (Kingston University).

2.8.5 SEM

3D tissues were transferred from the RAFT 96 well plates to 48 well plates and were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide (Agar Scientific AGR1015] for 1 hour each at room temperature. Tissues were then subjected to a series of alcohol dehydration steps in 50%, 70%, 80%, 90%, and 100% ethanol for 10-15 minutes each. Hexamethyldisilazane (HMDS) [Agar Scientific AGR1228] was used to dry the tissues overnight. All procedures above were performed in a fume hood. Fixed tissues were coated with gold and were then imaged in Evo50 Zeiss by SEM at 20 kV. Collagen fiber diameter and porosity were quantified using Image J. These experiments were completed in collaboration with Munasinghe, Amanda (Kingston University).

2.8.6 3D paraffin embedding and sectioning

3D tissues were washed in PBS and fixed in 10% NBF for 15 minutes at room temperature. Tissues were dehydrated through a series alcohol washes and histoclear, for 10 minutes each. These steps were performed manually in 96 well plates and in glass containers for histoclear washes. Tissues were then placed in a paraffin wax bath for 15 minutes. Finally, tissues were embedded in wax. To ensure tissues would be embedded flat on the wax, a wax block was placed on top of the tissue to keep them flat at the bottom of the mold. Samples were stored in 4°C until sectioning. Sections were cut by 5 µm and then stained with DAPI (Fisher Scientific S36939) for fluorescent imaging.

2.9 siRNA knockdown

Transfection complexes were formed using either human SPARC siRNA, rat SPOCK-3 or rat hevin siRNA (Dharmacon) and HiPerFect (Qiagen 301705) in serum-free medium for 20 minutes at room temperature. During this period, PS-1 cells were plated at a density of $1x10^3$ cells/well while INS-1 cells were plated at a density of $1x10^4$ cells/well. Transfection reagents were then added to the cells to give a final concentration of either 80 nM SPARC siRNA or 150 nM SPOCK-3 or Hevin siRNA. Human or rat non-targeting siRNA (Dharmacon) were used controls. For SPOCK-3 and hevin siRNA sequences used in these experiments, see Appendix Table 8.4 and 8.5. Transfection was carried out for 48 hours. For double knockdowns, a second transfection was performed after the first 48 hours. Briefly, media was aspirated and

replaced with fresh media supplemented with new siRNA and transfection was carried out for another 48-72 hours. Knockdown was confirmed by western blotting with SPOCK-3 or hevin antibody. To detect hevin isoform expression following SPARC knockdown in PS-1 cells, hevin was detected using antibodies specific to the N- and the C-terminus.

2.10 Adhesion assays

2.10.1 Attachment assays

INS-1 cells were plated in 96 well plates at a density of $1x10^4$/well. Cells were seeded in either serum free, starving (0.5% FBS) or complete media (10% FBS) supplemented with 5µg/mL of SPARC, hevin or SPOCK-3 [Appendix Table 8.2]. Cells were incubated in 0.5% CO2 at 37°C for 24 hours and attachment was monitored through the IncuCyteZOOM Live Cell Imaging System. Cell rounding and cell area were quantified also using the IncuCyteZOOM analysis software. Cell rounding scores closer to 0 represent rounded cells while scores closer to 1 represent spread cells. Change in cell area over time was standardised to day 0 to account for differences in plating.

2.10.2 Adhesion assays

INS-1 cells were plated in 96 well plates at a density of $1.5x10^4$/well. Cells were seeded in low serum media and allowed to adhere up to 48 hours. The media was aspirated and cells were treated with 5µg/mL of either SPARC, hevin or SPOCK-3 in complete media [Appendix Table 8.2] in fresh complete media for up to 48 hours. Cells were then imaged in the IncuCyteZOOM Live Cell Imaging System before and after cells were washed with PBS. Cell area was quantified using the IncuCyteZOOM analysis software and % cells that detached was calculated as a % change before washing.

2.10.3 Vinculin, insulin, and actin staining

Focal adhesions were detected using the FAK actin cytoskeleton / focal adhesion staining kit from Millipore (FAK100). INS-1 and MIN-6 cells were plated at a density of $4.5x10^4$/well in chamber slides (Nunc Lab-Tek Permanox C7182) and left to adhere and grow for 48 hours in complete media after which cells were treated with either 5µg/mL of SPARC, hevin, or SPOCK-3 [Appendix Table 8.2] for an additional 48 hours. Cells were then washed with PBS and fixed in 4% paraformaldehyde followed by permeabilisation in 0.2% Triton-X. Blocking

with 1% BSA was performed for 30 minutes at room temperature. Samples were then incubated with either mouse anti-vinculin (Millipore) or guinea pig anti-insulin (Abcam ab7842) for 1 hour a room temperature. This was followed by incubation with appropriate secondary antibodies conjugated to AF 488 and phalloidin-TRITC (Millipore) for another 1 hour. Control samples were stained for phalloidin only or insulin only. Finally cells were incubated with DAPI for 4 minutes and then mounted with anti-fade gold (Life Technologies). Cells were imaged using the Leica TCS SP8X confocal microscope. ImageJ analysis software was used to measure total area. For vinculin analysis, the threshold was set so that only strong vinculin staining near the cytoplasm was quantified. The % focal adhesion area was calculated as vinculin area / actin area.

2.11 Glucose-stimulation of β-cells

INS-1 or MIN-6 cells were plated in complete media at a density of $3x10^4$/well or $4.5x10^4$/well respectively in 96 well plates or chamber slides and allowed to adhere for up to 48 hours. Cells were pre-treated with 5µg/mL of SPARC, hevin, or SPOCK-3 in complete media for another 24 hours. Cells were washed with (KRBH) Krebs-Ringer HEPES-buffered solution (Alfa Aesar 15428779) followed by starvation for 2 hours with 2 mM glucose in KRBH at 37°C. Glucose stimulation was performed with 20 mM glucose or 5 mM KCl for 10, 20 or 30 minutes in KRBH at 37°C. Unstimulated control cells were incubated with 0 mM glucose.

For experiments detecting the effect of the SPARC family on insulin secretion, cells were pre-incubated with SPARC, hevin or SPOCK-3 for 24 hours in complete media prior to starvation. Following glucose stimulation, the supernatant was collected and stored in 20°C until used. Ultrasensitive Rat Insulin ELISA (Mercodia 10-1251-01) was used to detect insulin secretion. As per manufacturer's protocol, all samples and calibrators were plated in duplicate in the 96 well plate provided. Absorbance was measured at 450 nm using a plate reader. Insulin secretion µg/L was calculated using the standard curve and standardised to glucose-stimulated controls. Three independent replicates were tested in one 96 well plate. Therefore, each independent experiment was internally standardised before data was pooled.

For cells plated in chamber slides, cells were fixed in 4% paraformaldehyde for actin and insulin staining as described in Section 2.10.3. For experiments detecting the effect of p-ERK and p-FAK activation, cells were treated with SPARC proteins during the 20 minute stimulation period. Following glucose stimulation, cells were lysed for western blotting to detect protein expression using rabbit antibodies for p-ERK (New England Biolabs 9101S) and p-FAK (Abcam ab 81298).

2.12 Glucolipotoxicity assay

To induce glucolipotoxicity in INS-1 and MIN-6 cells either complete or serum free media was supplemented with 2% fatty acid free BSA, 200 µM palmitic acid (Sigma P0500) dissolved in 100% EtOH, and 200 µM of Oleic Acid solution in BSA (Sigma O3008). RPMI-1640 or DMEM was supplemented with additional glucose to make a final concentration of 27 mM. Palmitic acid was first dissolved in EtOH and then added to media supplemented with fatty acid free BSA (Sigma A6003). The solution was mixed and then incubated in a 37°C water bath for 1 hour to conjugate palmitic acid with BSA. Oleic acid and glucose were then added and then the media was filter sterilised using 0.2 µM filters before cell culture use. INS-1 and MIN-6 cells were plated in 96 well plates at a density of $1x\ 10^4$ per well in glucolipotoxic media and growth was monitored using the Incucyte Live Cell Imaging System. Cells plated in chamber slides were seeded at a density of $4.5x\ 10^4$ cells/well and were incubated for a period of 72 hours. Cells were fixed in 4% PFA and stained with phalloidin to detect actin and anti-insulin (Abcam ab 7842) for insulin expression.

2.13 Statistical analysis

Statistical significance was measured using the Student's t-test (unpaired, two-tailed) or one-way ANOVA. Tukey's test was used as a post-hoc test for ANOVA. P-values ≤ 0.05 were considered statistically significant.

3. Results: SPARC family protein complexity in the pancreas

We have previously described the expression of SPARC in the pancreas as well as its role on β-cell response to growth factors (Ryall *et al*.,2014). The extended SPARC family has to this date, not been characterised in the pancreas. In this chapter, we aimed to characterise their expression. Data from this chapter has previously been published and is available online (Viloria *et al*., 2016).

3.1 The SPARC family is highly expressed in pancreatic islets

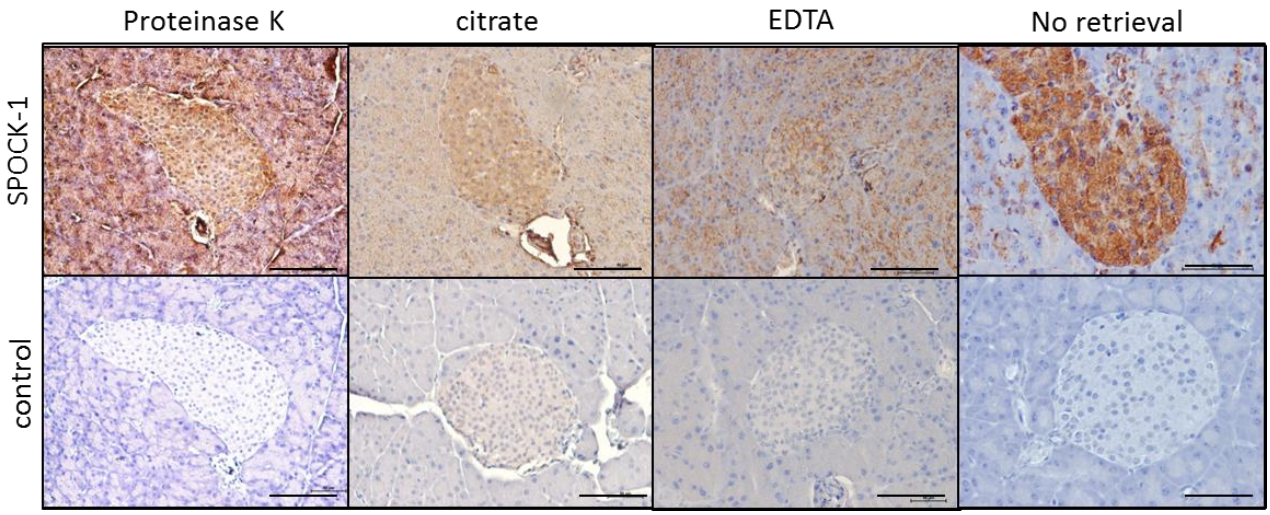
3.1.1 Optimisation of ABC-DAB staining method on pancreatic tissues

To determine the overall expression of SPARC proteins in the pancreas, ICR mouse sections were subjected to ABC-DAB staining. We first titrated the primary antibodies for the SPARC family. Results from this validation determined optimal concentrations i.e., low background and good contrast with haematoxylin, to be used for each antibody for immunohistochemical staining [summarised in Appendix Table 8.1]. It is worth noting that we chose antibodies specific to the N terminus of the SPARC family as this is the domain that is most distinct for each protein. In addition, we also tested several antigen-retrieval techniques to confirm specificity. Figure 3.1 shows representative images for SPOCK-1 as an example to illustrate that proteinase K digestion, as well as heat activated retrieval with citrate or EDTA were unsuccessful as no specific staining was observed. However, sections stained without antigen retrieval showed specific staining and was therefore used for the staining described below. Controls slides were treated with secondary antibody only and showed no staining [Figure 3.1].

3.1.2 Hevin

ICR mouse sections were probed with hevin antibody and subjected to ABC-DAB staining to detect hevin expression. Hevin was detected throughout the islets, with stronger staining in selected cells primarily at the islet periphery, as shown by the solid arrows in Figure 3.2 A

panels (i) & (ii). In these cells, hevin staining was observed primarily in the cytoplasm, although nuclear staining is apparent in some islet cells. Staining of the islet basement membrane was also observed, as indicated by the dotted arrows in (i). Hevin was also expressed in blood vessels (iii), in connective tissues (iv), in ductal cells (v), and in selected cells in the acinar tissue (vi). Hevin expression in the normal mouse pancreas is therefore distinct to that observed in the human pancreas, where expression is much more restricted,



and in islets appears localised specifically to stromal cells within islets (Human Protein Atlas) similar to our previous observation for SPARC in mouse islets (Ryall *et al*., 2014).

3.1.3 SPOCK proteins

ICR mouse sections were probed with SPOCK-1, 2, or 3 antibody and subjected to ABC-DAB staining to detect SPOCK protein expression. As shown in Figure 3.2 B-D**,** the SPOCKs are also detected throughout the islets, with strikingly strong expression of SPOCK-1 and SPOCK-3. The SPOCKs are also expressed in blood vessels, ductal cells and ductal basement membranes, and in selected acinar cells. SPOCK-1 staining was observed in the cytoplasm [Figure 3.2 B i-iii] but could also clearly be observed at the cell surface or extracellularly in

Figure 3.6: Antigen retrievals validated for IHC. IHC staining was validated for each antibody using different antigen retrieval methods. Proteinase K and heat activated retrieval with citrate and EDTA did not yield successful staining. Specific staining was detected without the use of antigen retrieval. Control slides were treated only with secondary antibodies and no staining was detected indicating specificity of antibodies. Scale bar 100 µm.

selected islet cells and in ducts [arrows in Figure 3.2 B i and v], and was largely absent in the

nucleus. A distinct perinuclear staining was observed in selected acinar cells [arrow in Figure 3.2 B vi]. In human islets, SPOCK-1 was strongly expressed in the exocrine acinar tissue and is mostly cytoplasmic. Staining was detected in some dispersed cells in the islets and is also cytoplasmic and nuclear (Human Protein Atlas). Similar to hevin, SPOCK-2 also showed higher

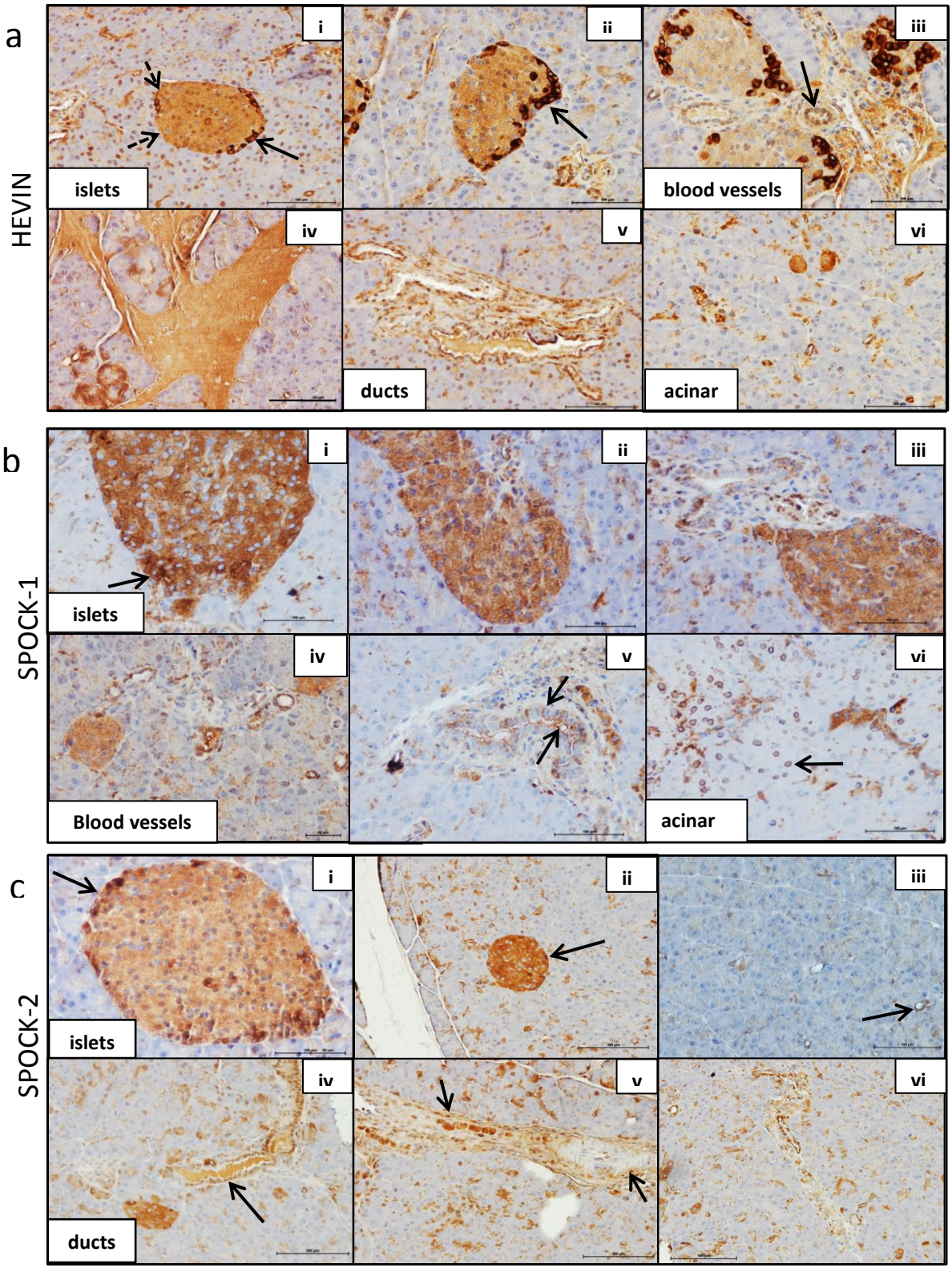


Figure 3.7: The SPARC family of proteins are widely expressed in the pancreas. ICR mouse pancreas sections were probed with antibodies to: (a) hevin, (b) SPOCK-1, (c) SPOCK-2, (d) SPOCK-3, (e) SMOC-1, (f) SMOC-2, (g) FSTL-1 and then stained with ABC-DAB (brown) and counterstained with haematoxylin (blue). Images are representative of 3-5 islets and ducts per section from 3 different mouse pancreas, N=9 (3 replicates from 2-3 independent experiments). Scale bar 100 µm at 20X objective.

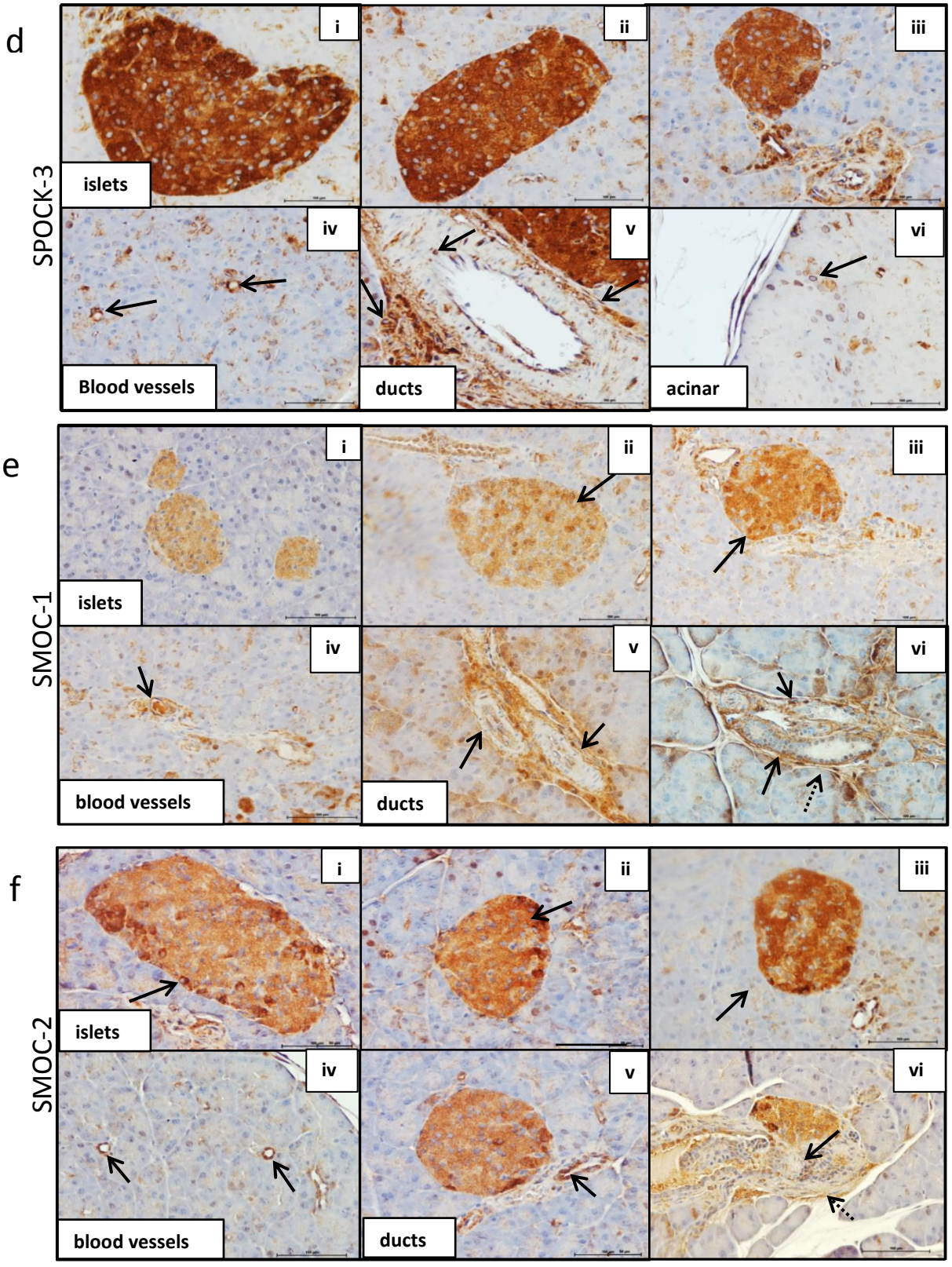


Figure 3.2: The SPARC family of proteins are widely expressed in the pancreas.

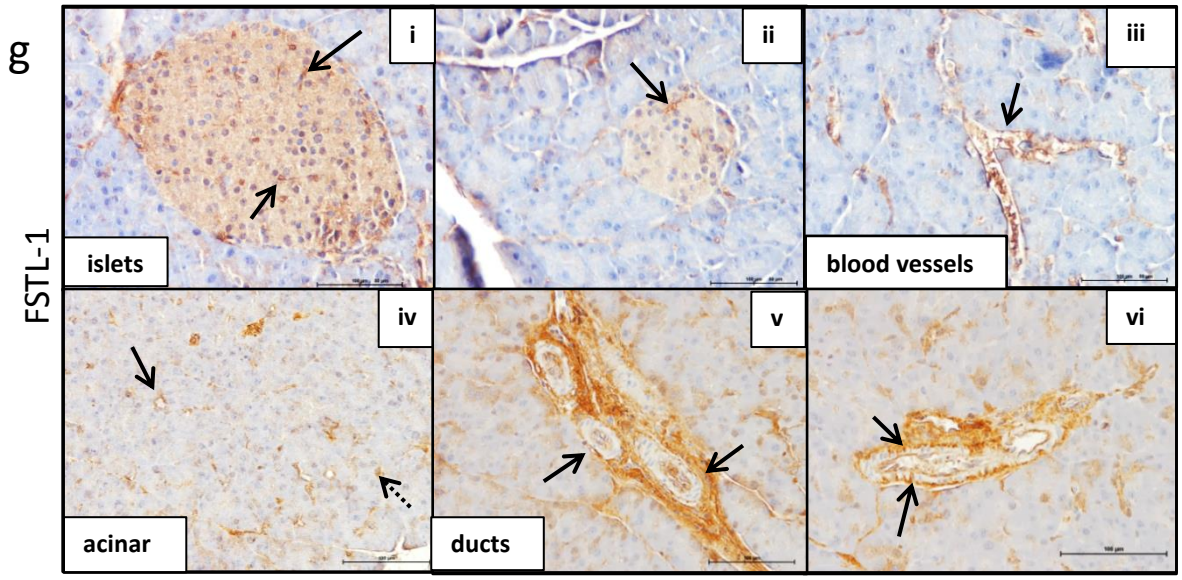


Figure 8.2: The SPARC family of proteins are widely expressed in the pancreas

expression in cells at the islet periphery, and again staining was primarily evident in the cytoplasm as shown in Figure 3.2 C [i & ii]. SPOCK-3 was highly expressed in all islet cells and primarily with a cytoplasmic staining pattern [Figure 3.2 D i-iii], although a more restricted perinuclear staining was observed in scattered cells throughout the acinar tissue [Figure 3.2 D vi]. The high levels of SPOCK expression in islets suggests that these proteins may play an important role in normal islet function, and it will be important for future studies to examine this further. In contrast, there is currently no data on human islets so far for SPOCK-2 and SPOCK-2 (Human Protein Atlas).

3.1.4 SMOC proteins

ICR mouse sections were probed with SMOC-1 or 2 antibody and subjected to ABC-DAB staining to detect SMOC protein expression. Both SMOC-1 [Figure 3.2 E] and SMOC-2 [Figure 3.2 F] were detected throughout the islet. SMOC-1 showed a range of staining intensity in different cells while SMOC-2 had stronger staining at the islet periphery. For both proteins, the staining was again largely cytoplasmic, though strong nuclear SMOC-1 staining could also be observed in selected cells [eg arrow in Figure 3.2 E iii]. The SMOCs were also expressed in blood vessels, in ductal cells [Figures 3.2 E and 3.2 F panels iv, v & solid arrows in vi], as well

as in the surrounding connective tissue [dotted arrows in vi]. In human islets on the other hand, SMOC-1 and SMOC-2 are so far not detected in islets or the exocrine tissue (Human Protein Atlas).

3.1.5 FSTL-1

ICR mouse sections were probed with FSTL-1 antibody and subjected to ABC-DAB staining to detect FSTL-1 protein expression. Within islets, FSTL-1 was expressed primarily in blood vessels and the islet basement membrane, although some weak diffuse staining can be observed throughout the islet [Figure 3.2 G i & ii]. FSTL-1 was also expressed in large and small blood vessels throughout the endocrine and exocrine pancreas as well as in connective tissue (iii & iv). In ducts, FSTL-1 was clearly detected at the cell surface (vi), although some staining was also observed in the cytoplasm of ductal cells (v). In contrast, human islets showed strong expression of FSTL-1 in the cytoplasm of dispersed islet cells and also in the cytoplasm of some cells in the acinar tissue (Human Protein Atlas).

In summary, hevin, SPOCK and SMOC proteins were strongly expressed in islet cells consistent with expression in β cells. Furthermore, hevin, SPOCK-2, and SMOC-2 showed stronger staining in cells at the outer periphery of islets. Indeed, using fluorescent co-staining, we show that these proteins were co-expressed in glucagon expressing cells [Figure 3.3], confirming that α cells strongly express hevin, SPOCK-2, and SMOC-2 in islets. All SPARC family proteins were detected in ductal cells, while SPOCK-1, -2, -3, SMOC-1, and FSTL-1 were found also in ductal basement membranes. Hevin, the SPOCKs, the SMOCs and FSTL-1 were all found in selected acinar cells and in blood vessels throughout the pancreas. FSTL-1 on the other hand was not strongly expressed in islet parenchymal cells, but instead staining was consistent with expression primarily in islet basement membranes and blood vessels.

This suggests that FSTL-1 is likely to be primarily expressed by stromal cells such as fibroblasts and endothelial cells, consistent with SPARC staining pattern in the pancreas (Ryall *et al*., 2014). The SPARC family of proteins are therefore clearly expressed in the pancreas, specifically in islet cells, stromal cells and pancreatic ducts, and need further investigation as to their function within the pancreas.

3.1.6 Intracellular staining of the SPARC family

The SPARC family are defined as secreted matricellular proteins and contain signal peptide sequences to target them to the secretory pathway, as indicated in Figure 1.6 (confirmed by Phobius database). The signal peptide, typically located at the N-terminus, tags proteins into the ER (endoplasmic reticulum) for transport into the secretory pathway (von Heijne, 1990; Emanuelsson *et al*., 2000). SPARC proteins are therefore expected to be observed extracellularly. While hevin, SPOCK-1 and FSTL-1 staining was observed at the cell surface, all members of the SPARC family also demonstrated cytoplasmic staining, and in some cases staining in the perinucleus and nucleus could also be observed. Cytoplasmic staining of SPARC family proteins has been previously described for hevin, SPOCK-1 and SMOC-2 (Vannahme *et al*., 2003; Hausser *et al.,* 2004; Esposito *et al*., 2007 Maier *et al*., 2008). It is likely that all SPARC family proteins are present in the extracellular environment, but this is not easily observed by immunohistochemistry where there is extensive cytoplasmic staining. However, the staining we observe clearly demonstrates the presence intracellularly of all SPARC family proteins. It will therefore be important to consider a possible intracellular role for these proteins in addition to their function in the extracellular matrix. One potential explanation for the intracellular location of these proteins is the expression of splice variants lacking the signal peptide, and this is explored further below.

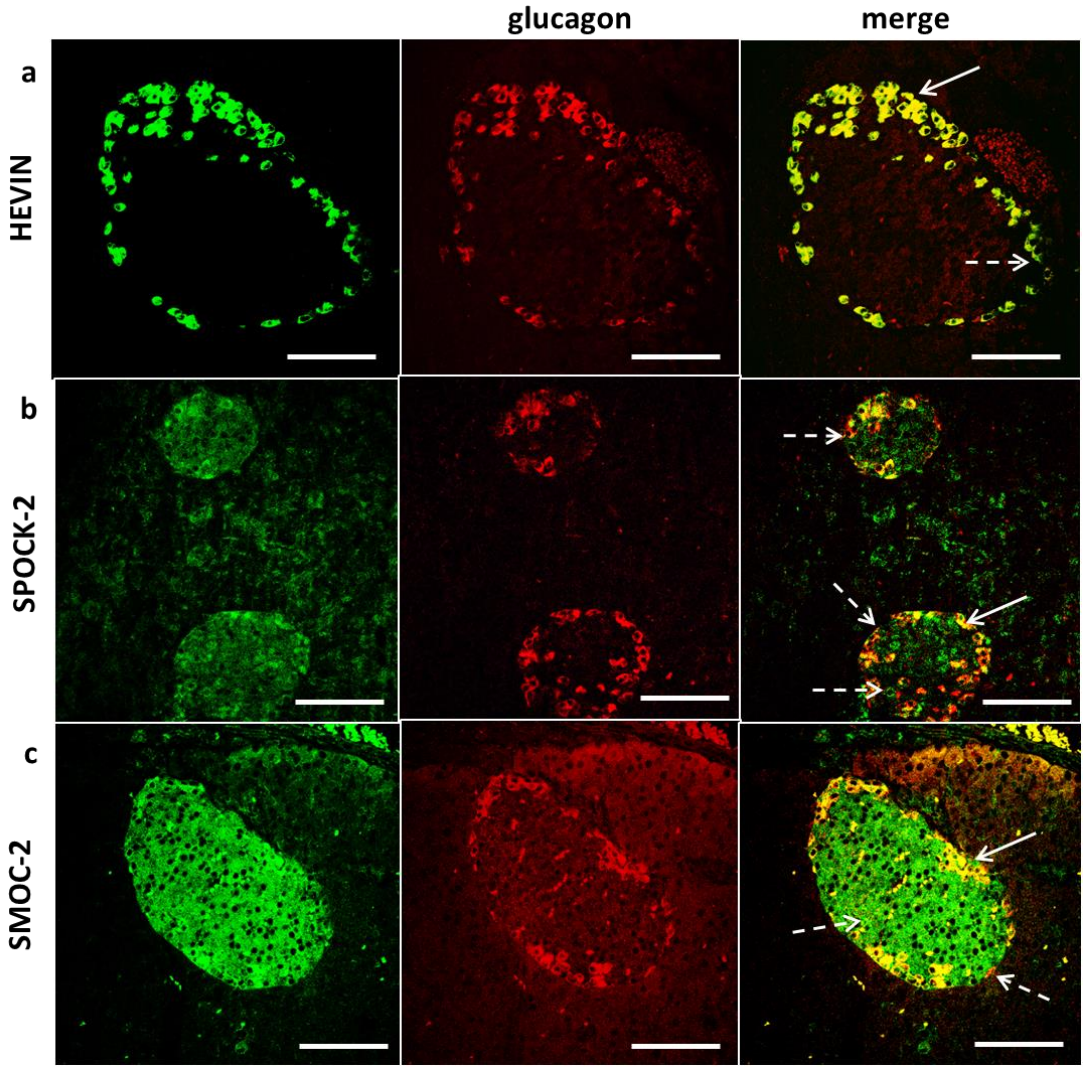


Figure 3.3: Glucagon expressing α-cells also express hevin, SPOCK-2, and SMOC-2. Islets were fluorescently co-stained for (a) hevin, (b) SPOCK-2, (c) SMOC-2 in AF-488 (green) and glucagon in AF-594 (red). Images representative of 3-4 islets, N=1-4 (1-2 independent experiments from 1-2 different mouse pancreas. Single z-scan images were acquired using a confocal microscope at 40X objective. Scale bar 100 µm. Control slides were treated with secondary antibodies only and no staining was detected indicating specificity of antibodies. Arrows pointing to areas of colocalisation of hevin, SPOCK-2 and SMOC-2 with glucagon. Dotted arrows showing where there is no overlap in staining.

3.2 Identification of multiple variants of the SPARC family in pancreatic cell types

3.2.1 Confirming IHC expression using western blotting

We analysed the expression of the SPARC family by western blotting to confirm the expression observed in pancreas sections. In addition, we also compared the expression pattern of the SPARC family in other cell types such as HUVEC and MRC5 lung fibroblasts. Protein quantification was performed to ensure equal loading. Given that cell lysates were

obtained from different cell types with variable actin expression it was not possible to standardise the samples to compensate for small variations in loading, and therefore only a qualitative analysis was performed. It is also worth noting that experiments described below were obtained from fresh cell lysates to avoid problems with protein degradation [Appendix Figure 8.1].

Hevin was expressed in both INS-1 and MIN-6 β cells [Figure 3.4 A], in agreement with the islet staining pattern observed using immunohistochemistry, and was also expressed by stromal cells such as PS-1 stellate cells, MRC5 fibroblasts and HUVEC endothelial cells, consistent with staining in basement membranes and blood vessels. SPOCK-1, -2, and -3 were also clearly detectable in both β cell lines examined [Figure 3.4 B-D], consistent with staining in islet cells shown by immunohistochemistry, and were also expressed in stromal cells such as stellate cells, fibroblasts and endothelial cells. Although only weakly detected in MIN-6 cells, SMOC-1 was strongly expressed in INS-1 β cells [Figure 3.4 E], consistent with the IHC staining. SMOC-1 was also detected in endothelial cells, but is either absent or very weakly expressed in fibroblast and stellate cells. SMOC-2 on the other hand was not detected by western blotting (data not shown). FSTL-1 was not detected in β cells, consistent with the absent or weak staining in majority of islet cells by immunohistochemistry. Instead, FSTL-1 was primarily detected in PS-1 stellate cells, with weak expression in fibroblasts and endothelial cells [Figure 3.4 F] consistent with basement membrane and blood vessel-like staining in pancreas sections.

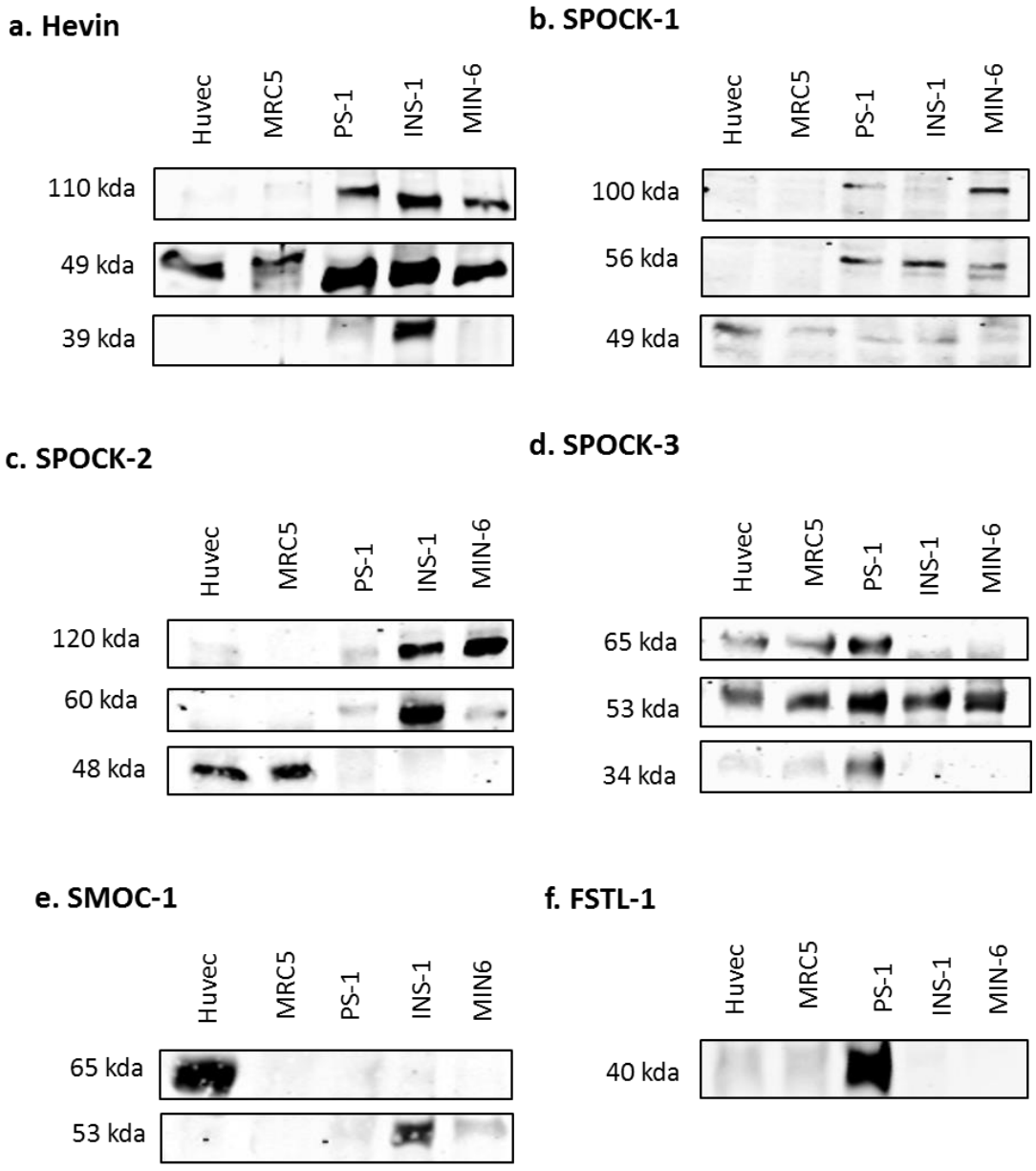


Figure 3.4: Identification of multiple variants of the SPARC family and their expression in specific cell types. Protein lysates (20-25 µg) were analysed by western blotting using antibodies to (a) hevin, (b) SPOCK-1, (c) SPOCK-2, (d) SPOCK-3, (e) SMOC-1, (f) FSTL-1 [See Appendix Table 8.1 for antibodies]. Blots were cropped to show bands consistently observed in at least 2 independent experiments. Full blots shown in Appendix Figure 8.1.

3.2.2 Cell-type specific variant expression of the SPARC family

A further striking observation in the series of western blotting experiments in Figure 3.4 is the presence of multiple bands for all SPARC family proteins, with the exception of FSTL-1 which was only detected as one band in stromal cells. We also observed clear evidence of cell-type specific expression of variants. For example, amongst the cell types examined, endothelial cells uniquely express a 65 kDa variant of SMOC-1 that was not detected in pancreatic cells [Figure 3.4 E]. Similarly, only pancreas-derived cells expressed a 110 kDa variant of hevin, while β cells uniquely expressed high levels of an additional 39 kDa variant [Figure 3.4 A]. SPOCK-2 was specifically expressed by β-cells and in other cell types such as HUVECs and fibroblasts but however was not expressed in pancreatic stellate cells [Figure 3.4 C]. An additional band of 60 kDa was detected in INS-1 β-cells but however was absent in MIN-6 β-cells. On the other hand, multiple bands may well correspond to non-specific binding of the antibodies to non-SPARC proteins. It would be necessary to confirm these variants with either a different antibody that binds to a different epitope or silence the expression of the proteins. siRNA knockdown of SPARC related proteins are further discussed in Chapter 4.4. A summary of molecular weights detected for each protein is shown in Table 3.1. As far as we are aware this is the first systematic analysis of multiple variants of the extended SPARC family of proteins. The identity of these variants and the mechanisms underlying the cell-type specific expression are not known. However, cell type specific expression of variants may well in part explain some of the contradictory and controversial effects of the SPARC family of proteins on cell function, and their often complex association with clinical diseases (Lane *et al*., 1994). For future experiments, it would also be important to compare the expression of the SPARC family in mouse and human islets as variant expression may also be species specific.

Protein	Endothelial cells (HUVEC)	Fibroblasts (MRC5)	Pancreatic stellate cells (PS1)	β cells (INS1)	β cells (MIN6)
Hevin 75 kDa	49	49	110 49	110 49 39	110 49
SPOCK-1 49 kDa	49	49	100 56 49	56 49	100 56 49
SPOCK-2 47 kDa	48	48	120 60	120 60	120 60
SPOCK-3 49 kDa	65 53 34	65 53 34	65 53 34	53	53
SMOC-1 48 kDa	65		53	53	53
SMOC-2 50 kDa	nd	nd	nd	nd	nd
FSTL-1 35 kDa	40	40	40		

Table 3.1: SPARC family variants expressed in pancreatic cell types. Apparent molecular weights of variants detected in western blots from Figure 3.4. Li-Cor Image Studio was used to quantify molecular weight for each band. The table shows consistent bands detected from at least 2 independent blots. For each protein, the predicted molecular weight is indicated in the first column (unmodified protein). nd=not detected.

3.3 Bioinformatic analysis of post translational modifications and splice variants

Possible explanations for the observation of multiple variants include: (1) post-translational modifications such as phosphorylation, glycosylation and addition of glycosaminoglycans, (2) protein cleavage into peptide fragments (Weaver *et al*., 2010; Weaver *et al*., 2011), (3) expression of alternative splice variants (Viloria and Hill, 2016), (4) the use of alternative translational start sites (Shinohara *et al*., 2008) and (5) cross-linking by transglutaminase (Aeschlimman *et al*., 1995; Arjomandi *et al*., 2011). We therefore performed a systematic analysis of these factors for the wider SPARC family of proteins, combining both bioinformatics and experimental approaches to characterised different variant expression in

	Predicted Glycosylation sites			Predicted Phosphorylation sites	
	N-linked	O-linked	Total Glycosylation sites	Phosphorylation	Total Phosphorylation sites
SPARC	Asn 116		1	Tyr145	1
Hevin	Asn169, Asn176, Asn196, Asn280, Asn412, Asn476	Thr31, Thr40, Ser44, Thr116, Thr331, Thr398,	12	Ser92, Ser127, Thr149, Ser182, Ser414, Thr419, Ser420, Ser421	8
SPOCK-1		Ser131, Thr228, Ser383 (GAG), Ser388 (GAG)	4	Tyr65, Ser131, Ser144, Ser150, Thr346, Ser351, Thr352	7
SPOCK-2	Asn225	Ser383 (GAG), Ser388 (GAG)	3	Thr154, Tyr155, Ser156	3
SPOCK-3		Ser384 (GAG), Ser389 (GAG)	2	Ser223, Ser225, Tyr365, Ser372	4
SMOC-1	Asn214, Asn374	Ser37, Thr163, Ser168, Ser172, Thr300, Thr301, Ser351,	9	Thr155	1
SMOC-2	Asn206, Asn362		2	Tyr193	1
FSTL-1	Asn144, Asn175, Asn180		3	Ser165, Ser166, Thr279, Thr284, Tyr286	5

Table 3.2: Predicted post-translational modifications of the SPARC family of proteins. Potential glycosylation and phosphorylation sites for each of the extended family of SPARC proteins were acquired from Genecards, UNIPROT and Phosphosite Plus databases. Positions for known GAG binding sites are also indicated.

pancreatic cells. For this study, the term 'variant' refers to any combination of post-translational modifications and alternative splicing.

3.3.1 Post-translational modification of the wider SPARC family of proteins

Potential glycosylation and phosphorylation sites for the SPARC-related proteins were identified using GeneCards, UniProt, and Phosphosite Plus. As shown in Table 3.2, hevin can be extensively modified, with 12 potential glycosylation sites and 8 phosphorylation sites. Compared to SPARC which only has 1 glycosylation and phosphorylation site, hevin can be considerably modified. Interestingly, majority of the modification sites for hevin are located at the acidic domain I, suggesting the importance of this domain in terms of multifunctionality. The SPOCKs also contain sites for both glycosylation and phosphorylation, and are known to contain O-linked glycosaminoglycans (GAGs) at serine residues in the C' terminal region [Table 3.2 and Figure 1.6]. Glycosaminoglycan linkage can increase the molecular weight by 20 kDa or more (BaSalamah *et al.*, 2001). SMOC-1 can be extensively modified through 9 glycosylation sites, compared to SMOC-2 with only 2 sites. Lastly, FSTL-1 has up to 3 sites for glycosylation and up to 5 for phosphorylation. All proteins in the extended SPARC family can therefore undergo varying degrees of post-translational modification.

3.3.2 Alternative splicing of the SPARC family of proteins

We previously performed an analysis of alternative splice variation in matricellular proteins (Viloria and Hill, 2016). To determine whether the protein variants observed in Figure 3.4 could be due to alternative splicing, we further analysed the SPARC family splice variants banked in the ENSEMBL database. As shown in Table 3.3, there is evidence of a large number of splice variants for the SPARC family of proteins. A total of 17 coding variants were identified for SPOCK-3 but however only 13 were complete coding variants. We therefore restricted our further analysis to coding variants for which the complete coding sequence (CDS) is known.

Protein FASTA sequences were obtained for complete CDS transcripts and domain structures were predicted using the InterPro database. As shown in Figure 3.5, in many cases multiple splice variants encode highly similar proteins. For example, 7 SPOCK-3 variants encode

proteins that are very similar in molecular weight and structure. However, for each of the extended SPARC family of proteins, there was at least one alternative splice variant with distinct protein sequence was identified. For example, SPOCK-3 variant 010 lacks the follistatin domain while variant 005 lacks the thyroglobulin and GAG binding domain and variant 013 contains a second thyroglobulin domain, almost pseudo-SMOC-like. An alternative splice variant of SPOCK-3 missing the thyroglobulin domain and glycosaminoglycan binding sites has been previously described in kidney cells and glioma, referred to as N-Tes, that is likely to correspond to variant 005 in ENSEMBL (Nakada *et al*., 2011). Interestingly, the thyroglobulin domain is involved in the IGF binding properties of IGFBPs, as well as protease inhibitory functions, and it will therefore be interesting to test the function of splice variants either lacking (005) or with additional (013) thyroglobulin domains (Bevec *et al*., 1996; Lenarcic *et al*., 2000; Headey *et al*., 2004; Meh *et al*., 2005).

For hevin, SPOCK-1, SPOCK-3 and SMOC-2 at least one alternative variant lacking the signal peptide was identified, suggesting that both intracellular and extracellular variants of these proteins exist. The intracellular variants may explain the cytoplasmic staining for these proteins described in Figure 3.2 It is also possible that intracellular variants exist for SPOCK-2, SMOC-1 and FSTL-1 for which the complete sequence is not yet known and was therefore not included in this analysis. Nonetheless, these variants indicate a novel intracellular structure and function of the SPARC family.

Perhaps the most striking difference between variants of the extended SPARC family is the size of the acidic domain I, suggesting functional significance of domain I variation. However, the role of this domain is not well understood. Domain I is known to bind to calcium but with low affinity compared to the EC domain, and in SPARC, domain I is involved in the regulation of cell migration (McClung *et al*., 2012). In SPOCK-3, the acidic domain I is involved in MT-MMP inhibition while in SPOCK-2 it is involved in its regulation of SPOCK-3 (Nakada *et al*., 2001; Nakada *et al*., 2003). This domain has diverged and acquired additional acidic residues during evolution (Kawasaki *et al*., 2004; Novinec *et al*., 2006; Martinek *et al*., 2007; Bertrand *et al*., 2013). It is also the least conserved domain between different SPARC family proteins, and is the primary feature that distinguishes SPARC from hevin. As mentioned previously,

domain I is where hevin is highly post translationally modified. Domain I may therefore confer diversity of function to each SPARC family protein, and this diversity is then further expanded by alternative splicing. This analysis therefore suggest the functional importance of domain I variation in the SPARC family of proteins. Characterisation of each variant detected from western blotting in Figure 3.4 will be described below in light of the post translational and alternative splicing data.

Gene name (total no. coding variants)	Transcript Name	ENSEMBL Transcript ID	Base pairs	Amino acids	Predicted Protein size (kDa)
SPARCL1 (Hevin) (10)	201	418378	2994	664	75
	001	282470	2906	664	75
	005	503414	2520	539	62
SPOCK-1 (5)	001	394945	4846	439	49
	201	282223	4488	377	33
SPOCK-2 (4)	004	373109	5445	424	47
	201	317376	5328	424	47
	203	536168	1824	423	47
	202	412663	1284	77	8
SPOCK-3 (17)	001	357154	2986	436	49
	002	502330	2180	436	49
	006	357545	2936	433	49
	012	511269	1768	433	49
	014	506886	2947	436	49
	015	511531	2908	436	49
	016	504953	2900	433	49
	013	510741	1963	393	44
	201	421836	3061	385	44
	017	535728	2007	344	39
	010	512681	1457	338	38
	202	541354	2797	316	37
	005	512648	1456	313	36
SMOC-1 (2)	001	361956	2040	435	48
	002	381280	3666	434	48
SMOC-2 (4)	002	354536	3150	457	51
	001	356284	3117	446	50
	201	535039	1875	136	14
FSTL-1 (4)	001	295633	5943	308	35
	004	424703	1396	273	31

Table 3.3: Bioinformatic analysis of splice variants of the SPARC family of proteins. Predicted alternative transcripts for the extended SPARC family were obtained from ENSEMBL. Only transcripts with protein coding variants with complete CDS were included in the analysis. FASTA protein sequences were downloaded from ENSEMBL and the respective product molecular weight was obtained using Protein Molecular Weight Bioinformatics tool.

		ENSEMBL variant
Hevin	75 kDa	201, 001
	62 kDa	*005
SPOCK-1	49 kDa	001
	33 kDa	*201
SPOCK-2	47 kDa	004, 201, 203
	8 kDa	202
SPOCK-3	49kDa	001, 002, 006, 012, 014, 015, 016
	44 kDa	013
	44 kDa	*201
	39 kDa	*017
	38 kDa	010
	37 kDa	*202
	36 kDa	005/N-Tes
SMOC-1	48 kDa	001, 002
SMOC-2	51 kDa	002
	50 kDa	001
	14 kDa	*201
FST-L1	35 kDa	001
	29 kDa	004

3.3.3 Hevin

As described above, cell-specific expression of 110 kDa and 39 kDa hevin variants was observed in addition to a widely expressed 49 kDa variant. The predicted molecular weight of the hevin precursor protein encoded by the primary transcript is 75 kDa, and the known hevin splice variants are unlikely to explain the

Figure 3.5: Domain structures of predicted alternative splice variants of the SPARC family. For variants with complete protein coding CDS, FASTA sequences were downloaded from ENSEMBL and domain structures were predicted using the InterPro database. Numbers within each domain represent the number of amino acid residues for each domain. Signal peptides are indicated by red boxes. Transcripts lacking the signal peptide are indicated by asterisks.

variants observed [Table 3.3]. However, 39-49 kDa hevin bands have been reported to arise from cleavage by ADAMTS4 and MMP3, and hevin is also a substrate for thrombin and plasmin digestion (Weaver *et al.,* 2010; Weaver *at al*., 2011). These low molecular weight hevin proteins (39 and 49 kDa) are therefore likely to represent the products of enzymatic cleavage. The presence of the 39 kDa fragment exclusively in β cells suggests additional proteolytic cleavage of hevin in these cells, perhaps reflecting a β cell specific role for hevin fragments.

High molecular weight hevin-reactive bands have also been previously observed (Bendik *et al*., 1998; Hambrock *et al*., 2003; Brekken *et al*., 2004; Weaver *et al.,* 2011). Based on the number of predicted glycosylation sites [Table 3.2], we hypothesized that the 110 kDa band is likely to reflect extensive glycosylation. To test this, we digested PS-1 stellate cell lysates with PNGase-F to test for the presence of N-linked glycosylation. As shown in Figure 3.6 A, an additional band at 36 kDa appeared following de-glycosylation. However, only the 49 kDa band and not the 110 kDa band showed any detectable decrease in intensity. The hevin antibody used in these experiments recognises an epitope in the N-terminus and would therefore be predicted to recognise N-terminal cleavage products. These results therefore suggest that the 49 kDa band is a 36 kDa N-terminal cleavage product with approximately 15 kDa of N-linked glycosylation. The full length 110 kDa variant may potentially be conformationally resistant to glycosylase treatment or consist primarily of O-linked glycosylation. Alternatively, hevin is predicted to be a substrate of transglutaminase (TRANSDAB) and the 110 kDa band may therefore represent oligomer formation due to cell-type specific cross-linking (CsoHosz *et al*., 2009). Supporting this hypothesis, SPARC is also known to form oligomers as a result of transglutaminase-mediated cross-linking (Aeschlimann *et al*., 1995; Hohenadl *et al.*, 1995).

Although no direct evidence of hevin splice variants was observed in these experiments, subsequent experiments using an alternative hevin antibody recognising the C-terminus epitope revealed the presence of an additional third variant in PS-1 cells at approximately 60 kDa [Figure 3.6 D], whereas only two variants were detected in PS-1 cells with the N' antibody [Figure 3.4 A]. This observation could suggest the presence of an alternative splice

variant lacking the N-terminus. Consistent with this, hevin variant 005 has a predicted molecular weight of 62 kDa [Table 3.3]. Since this variant lacks a portion of the N-terminal region it may exist in a conformation that is not recognised by the N-terminal antibody used. Variant 005 lacks a signal peptide and is predicted to be an intracellular variant. However, mRNA studies would be required to confirm the detection of this potentially novel intracellular hevin splice variant.

Proteolytic cleavage of hevin has been shown to produce a "SPARC-like fragment" that is likely to correspond to, or be contained within, the approximately 50 kDa band detected using the C-terminal hevin antibody [Appendix Figure 8.2] (Weaver *et al.,* 2010; Weaver *et al*., 2011). It has been suggested that this "SPARC-like fragment" may compensate for the loss of SPARC expression (Brekken *et al*., 2004; Lau *et al*., 2006; Weaver *et al*., 2011). SPARC can be suppressed for example as a result of SPARC promoter methylation during tumourigenesis (Gao *et al*., 2010). Furthermore, SPARC and hevin have overlapping and compensatory roles in angiogenesis inhibition (Barker *et al.,* 2005a). In addition, SPARC and the "SPARC-like fragment" of hevin have both been detected in neovasculature *in vivo* suggesting they may also have similar and synergistic effects (Weaver *et al*., 2011). We therefore tested whether reducing SPARC expression in PS-1 stellate cells by siRNA knockdown results in a compensatory increase in the presence of proteolytic cleavage products detected by both the N'- and C'-antibodies. As shown in Figure 3.6 B-E, despite achieving 90% knockdown of SPARC expression, no significant change in the detection of either the full length hevin or smaller fragments was observed with either the N-terminal or C-terminal antibodies. Therefore, although previous reports have suggested that C-terminal hevin fragments may compensate for loss of SPARC expression, we did not find evidence to support this hypothesis within the cell types examined. However, the various variants of hevin that we have observed are likely to have distinct properties and likely to fulfill specific functions within the particular cell types where they are expressed. Additionally, there is evidence for the importance of additional proteolytic cleavage products specifically in β cells [Figure 3.4 A].

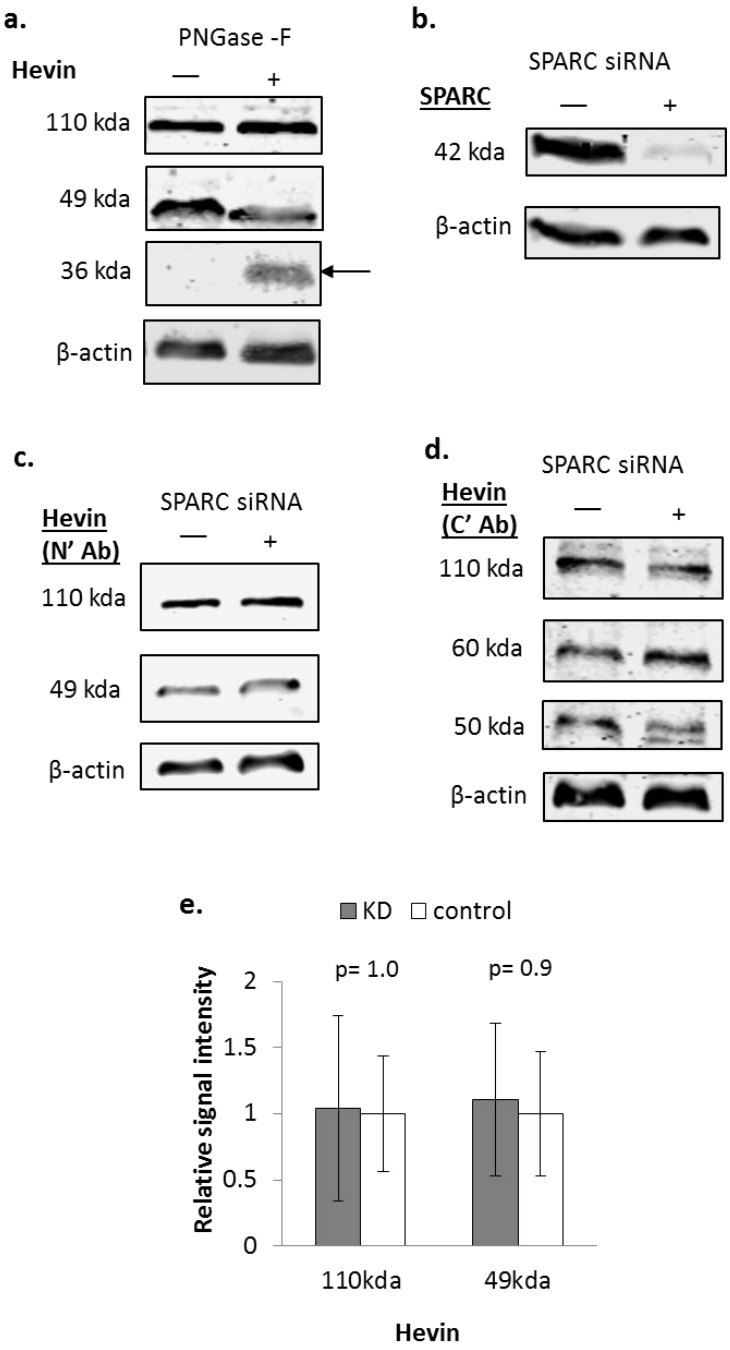


Figure 3.6: Pancreatic stellate cells express N-linked glycosylated hevin. Expression of hevin is not regulated by SPARC knockdown. (a) PS-1 cell lysates were subjected to PNGase-F digestion and analysed by western blot. (b) SPARC expression was silenced in PS-1 cells using siRNA knockdown and hevin expression was detected using western blotting with antibodies to the (c) N-terminus and (d) C-terminus. (e) Quantification of proteins bands detected by the hevin (N-terminus) antibody following SPARC KD. Graphs showing signal intensity for each band relative to the control +/- SEM. N=9 (3 replicates from 3 independent experiments). Statistical significance was measured using student's t-test (unpaired, two-tailed). Full blots available in Appendix Figure 8.3.

3.3.4 SPOCK proteins

For each of the SPOCK proteins three different variants were observed. In the case of SPOCK-1, while an variant of the predicted molecular weight (49 kDa) was observed in all cell lines examined, two additional variants (56 kDa and 100 kDa) were observed specifically in pancreatic stellate cells and β cells [Figure 3.4 B]. SPOCK-1 has previously been detected in the 130-150 kDa range in human plasma and in kidney cells, and the increase in molecular weight is most likely due to the addition of large glycosaminoglycan chains (both chondroitin and heparin sulphate chains) at residues 383 and 388 of the C-terminus [Table 3.2] (Bonnet *et al*., 1992; BaSalamah *et al.*, 2001). The detection of SPOCK-1 at 49 kDa in all cell types suggests a native unglycosylated form of SPOCK-1 is also widely produced, and the addition of glycosaminoglycans in specific cell types may create novel functions. A 33kDa intracellular alternative splice variant was also identified in ENSEMBL [Table 3.3 and Figure 3.5], and it is also possible that the observed 49 kDa and 56 kDa bands represent glycosylated forms of this variant.

SPOCK-2 has 3 highly similar variants at 47 kDa and an additional variant of only 8 kDa [Figure 3.5]. However, proteins of such low molecular weight would not be observed by our western blot analysis. Proteins of around the expected molecular weight of the primary and similar transcripts (47 kDa) were observed in fibroblast and endothelial cells, though not in pancreatic stellate and β cells [Table 3.1 and Figure 3.4 C]. In contrast, in β cells SPOCK-2 was detected at 60 kDa and 120 kDa. As shown in Table 3.2, SPOCK-2 contains both glycan and glycosaminoglycan binding sites, and variable glycosylation is therefore likely to explain the larger variants observed specifically in β cells. Interestingly, pancreatic stellate cells express very low levels of SPOCK-2 indicating a likely specific role of SPOCK-2 in β-cell functions.

Since SPOCK-3 can also be glycosylated [Table 3.2], the 65 kDa stromal variant is likely to correspond to a glycosylated form of the 49kDa protein encoded by the primary or similar transcripts. In order to test whether the variants we observe are due to glycosylation, we subjected PS-1 cell lysates to PNGase-F digestion. As shown in Figure 3.7 the 34 kDa variant increases in intensity by at least four fold following digestion, suggesting that this variant is present in both N-linked glycosylated and unglycosylated forms, and that the 34 kDa variant

is the unglycosylated form. This variant is likely to be similar to variant 005/N-Tes (34-37 kDa) of similar molecular weight (Nakada et al., 2001). Furthermore, a second band of 45 kDa also appears following digestion, suggesting the presence of a second distinct protein variant that is normally N-glycosylated. The size of this band is consistent with the primary transcript, or an alternative transcript of similar size. These experiments demonstrate the presence of at least two variants of SPOCK-3 in pancreatic stromal cells, most likely representing the primary 49 kDa transcript and variant 005/N-Tes (34-37 kDa). In contrast, only a single variant is observed in β cells.

The glycosylation detected by PNGase F treatment may well represent N-linked glycosaminglycan chains attached to non-consensus N-glycosylation motifs. N-linked glycosylation at non-consensus motifs are now known to occur in mammalian genomes (Schwarz and Aebi, 2011). Multiple high molecular weight bands (>90 kDa) were observed in PS-1 and β cell lysates at variable intensities, that are likely to reflect the addition of glycosaminoglycan chains [Appendix Figure 8.1]. Mouse SPOCK-3 was recently shown to contain heparin sulphate proteoglycans, although N-linked glycosylation was not detected in mouse SPOCK-3 (Hartmann *et al*., 2013). These experiments therefore demonstrate the novel finding that SPOCK-3 contains previously undescribed N-linked glycosylation sites.

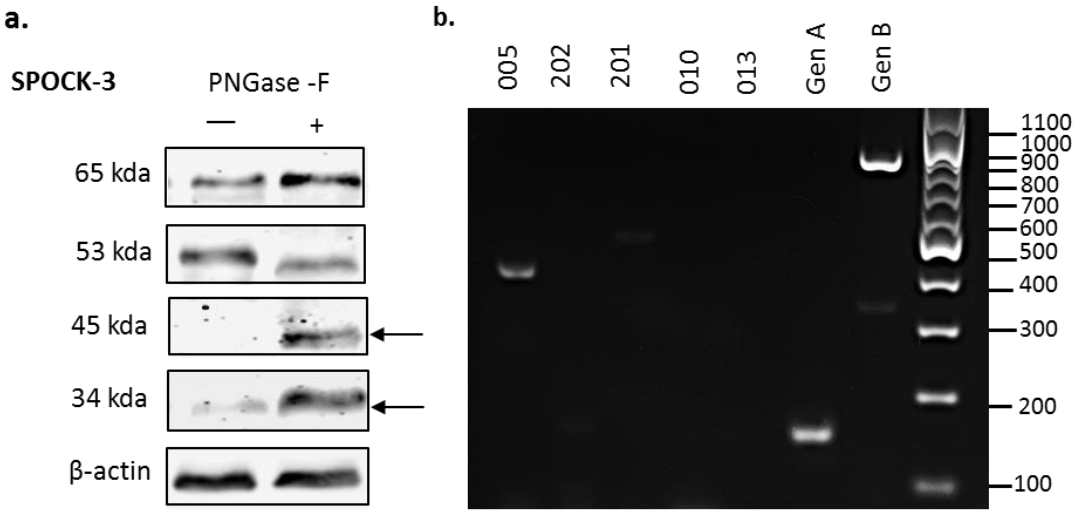


Figure 3.7: Pancreatic stellate cells express two distinct alternative splice variants. (a) PS-1 cell lysates were subjected to PNGase-F digestion and SPOCK-3 variants were detected using western blotting. B-actin was used as a loading control. Figure showing representative blot from 3 independent experiments. Arrows indicating products from cleavage of N-linked glycosylation. Molecular weight of these products is indicated. N=6 (2 replicates from 3 independent experiments). In (b), mRNA of PS-1 cells was isolated and expression of alternative variants of SPOCK-3 were detected using RT-PCR. All negative controls were blank (See methods for details). Alternative transcripts were obtained from ENSEMBL and used for comparison to confirm identity of expressed variants obtained by Sangar sequencing. Full blot available in Appendix Figure 8.4

As described above, the observation that two bands appear following de-glycosylation suggests the expression of two distinct core proteins, and is consistent with the presence of 005/N-Tes (~36kDa) plus the full-length protein corresponding to the primary transcript or similar (~49kDa) in PS-1 cells. However, other explanations are possible, including the presence of digestion products or differential post-translational modification in addition to N-linked glycosylation. We therefore sought to test whether distinct splice variant transcripts could be detected.

The exon structure of all 17 SPOCK-3 alternative splice variants banked in ENSEMBL was examined in order to design primers to detect variants. SPOCK-3 is unusual in that alternative splicing affects multiple regions across the entire coding sequence, as well as the 5'UTR, suggesting that SPOCK-3 has a high degree of tolerance for variability within the protein structure (Viloria and Hill, 2016). Variants truncated at the 5' end (201, 017, 202), at the 3' end (005), and in internal regions (013, 010, 202, 018) are described in the database. As shown in Appendix Figure 8.5, 19 exons are currently identified, the first 4 of which form the 5'UTR. The 7 transcripts that encode proteins highly similar to the primary transcript are shown in blue. It can be seen that these variants differ primarily in the 5'UTR exons, with 001/014, 002/015 and 006/16 pairs having identical coding sequences, differing only in the use of 5'UTR exon 1/exon 2. In contrast, 012 uses 5'UTR exon 4 which in fact contains an upstream translation start producing a predicted protein with an additional 12 amino acids at the N-terminus (translation performed using exPASY). However, it is not clear whether this start codon is used. Interestingly, exons 8 and 9 [as labelled in Appendix Figure 8.5] are identical microexons of 9 bp. The coding sequences of 001, 014, 002 and 015 are therefore identical, and differ from 006/016 by only 3 amino acids. The significance of microexons is not fully understood, but they have been shown to have functional effects, and have been previously identified in murine SPOCKs (Saffell *et al*., 1994; Volfovsky *et al*. 2003; Hartmann *et al*., 2013). Since these 7 transcripts produce highly similar proteins, we focused on transcripts predicted to encode medium length ~44 kDa (201, 013) and short ~36-30 kDa (017, 010, 202, 005) proteins for experimental confirmation.

Specific RT-PCR primers were designed to each of these variants, with the exception of transcript 017 for which unique primers could not be designed. The primer locations are shown in Appendix Figure 8.5 and the sequences and predicted product sizes given in Appendix Table 8.3. Generic primers were designed that should size differentiate between transcript 201 and the remaining transcripts (GenA), and that should amplify all transcripts listed except for 005 and 010 (GenB). As shown in Figure 3.7 B, we detected the 005 transcript in PS-1 stellate cells, and the specificity of the PCR product was confirmed by Sangar sequencing. Transcripts 010, 201 and 202 were not detected using the primers specific to these sequences. The generic primers GenA and GenB detected PCR products of the expected size (158 and 740 bp respectively), and specificity was confirmed by Sangar sequencing. Since the GenB primers could not amplify 005 this confirms the presence of at least one other transcript in addition to 005. The sequence of the GenA PCR product revealed the absence of microexons 8 and 9, and the sequence was instead identical to variants 006/016 (which have identical CDS). We therefore demonstrate that pancreatic stellate cells express two distinct SPOCK-3 splice variants, 005/N-Tes and 006/016, corresponding to the deglycosylated ~34 and ~45 kDa proteins observed by western blot. Pancreatic stellate cells support the islets and are significant in PDAC progression. It will therefore be relevant to further investigate the functions of these variants in terms of islet and ductal regulation.

3.3.5 SMOC proteins

Two distinct variants of SMOC-1 were identified, with highly cell type specific expression [Figure 3.4 and Table 3.1]. The 65 kDa variant was expressed uniquely in endothelial cells, while the 53 kDa variant was present primarily in β cells. Interestingly, SMOC-1 was either not detected or only weakly detected in fibroblasts and stellate cells. Only two splice variants with complete CDS were identified by bioinformatics analysis and both encode for highly similar 48 kDa proteins [Table 3.3 and Figure 3.4], suggesting that alternative splicing is unlikely to account for the two variants observed. However, SMOC-1 has 9 predicted glycosylation sites [Table 3.2] and therefore alternative glycosylation is more likely to explain the two distinct variants identified, as suggested by enzyme digestion in studies elsewhere (Vannahme *et al*., 2002; Liu *et al*., 2008; Choi *et al*., 2010). It will therefore be important to

analyse the effect of alternative glycosylation on SMOC-1 protein function to understand the roles of distinct variants in endothelial cells and β cells.

For SMOC-2, three alternative transcripts were identified in ENSEMBL: transcripts 001 and 002 are predicted to encode proteins of similar molecular weight (~ 50 kDa) and with overall similar domain structure, with the exception of a truncated acidic domain in 001 [Table 3.3 and Figure 3.5] In contrast, variant 201 is predicted to encode a small 14 kDa protein that lacks a signal peptide and any predicted functional domains. The coding region of variant 201 is in fact not overlapping with that of the primary transcript and is therefore likely to encode an intracellular protein of quite distinct function. SMOC-2 has been previously detected between 54 kDa and 60 kDa, and was shown to be glycosylated by PNGase digestion (Vannahme *et al*., 2003; Liu *et al*., 2008).

SMOC proteins have been associated to many cancers and reported to be involved in cellular differentiation, cell-cycle progression and in regulating cell responses to the environment. However, SMOC proteins are largely unstudied in pancreatic diseases such as PDAC and diabetes. We have shown that SMOC-1 and SMOC-2 are widely expressed in the pancreas, and that specific SMOC-1 variants are expressed in endothelial cells and β cells. It will therefore be important to study the function of SMOC variants in these cells and in pancreatic disease.

3.3.6 FSTL-1

Expression of FSTL-1 was the most specific of the SPARC family proteins. We detected strong expression of a single ~40 kDa variant specifically in pancreatic stellate cells, with weaker expression in fibroblasts and endothelial cells [Figure 3.4 and Table 3.1]. This is consistent with the stromal expression pattern and staining in basement membranes observed by immunohistochemistry [Figure 3.2 G]. We identified two alternative transcripts with complete CDS in ENSEMBL that are predicted to share the same functional domains except for a truncated acidic domain in variant 004 [Table 3.3 and Figure 3.5]. Both variants contain a signal peptide sequence and are therefore predicted to be secreted extracellular proteins and confirms the basement-membrane like staining observed in Figure 3.2. The molecular weight of the observed protein (40 kDa) is consistent with the predicted molecular weight of

the primary transcript with minor post-translational modification such as glycosylation, as has been previously reported (Hambrock *et al*., 2004). FSTL-1 is sometimes overlooked as a member of the SPARC family since it has the least structural and sequence homology to SPARC and it has been suggested that the calcium binding EF hand in FSTL-1 may be non-functional (Hambrock *et al.,* 2004). Our data shows that FSTL-1 is expressed by stromal cells within the pancreas with an expression pattern highly reminiscent of SPARC, and is expressed at high levels specifically by pancreatic stellate cells (Ryall *et al*., 2014). This suggests that FSTL-1 may play a related role to SPARC in pancreatic disease and that the function of FSTL-1 needs further study.

3.4 FSTL-1 does not regulate growth and proliferation of β cells

Compared to other SPARC family proteins that we have shown to be highly expressed throughout islets and in β cells, FSTL-1 and SPARC are the only SPARC family proteins detected in stromal cells and not in β cells by western blotting and IHC [Figures 3.2, 3.4 and Ryall *et al*., 2014]. Like SPARC, FSTL-1 has been shown to regulate signalling of the TGF-β superfamily and to regulate growth factor signalling (Shibanuma *et al*., 1993; Tsuchida *et al*., 2000; Geng *et al*., 2011; Xu *et al*., 2013; Ryall *et al*., 2014; Dong *et al*., 2015). We therefore tested whether FSTL-1 can similarly regulate β cell growth. INS-1 β cells were treated with rFSTL-1 in either complete (10% FBS) or low serum (0.5% FBS) media over a period of 3 days. Cell growth was measured using the IncuCyte ZOOM live cell imaging system, and cell proliferation was measured by BrdU incorporation over the final 24 hour period. BrdU or bromodeoxyuridine is a thymine analog that is incorporated into replicating DNA strands. However, as shown in Figure 3.8, the addition of exogenous FSTL-1 had no effect on the growth or proliferation rate of β cells in either serum conditions. Despite a highly similar pattern of expression to SPARC, FSTL-1 is therefore unlikely to be directly involved in the regulation of islet growth and survival.

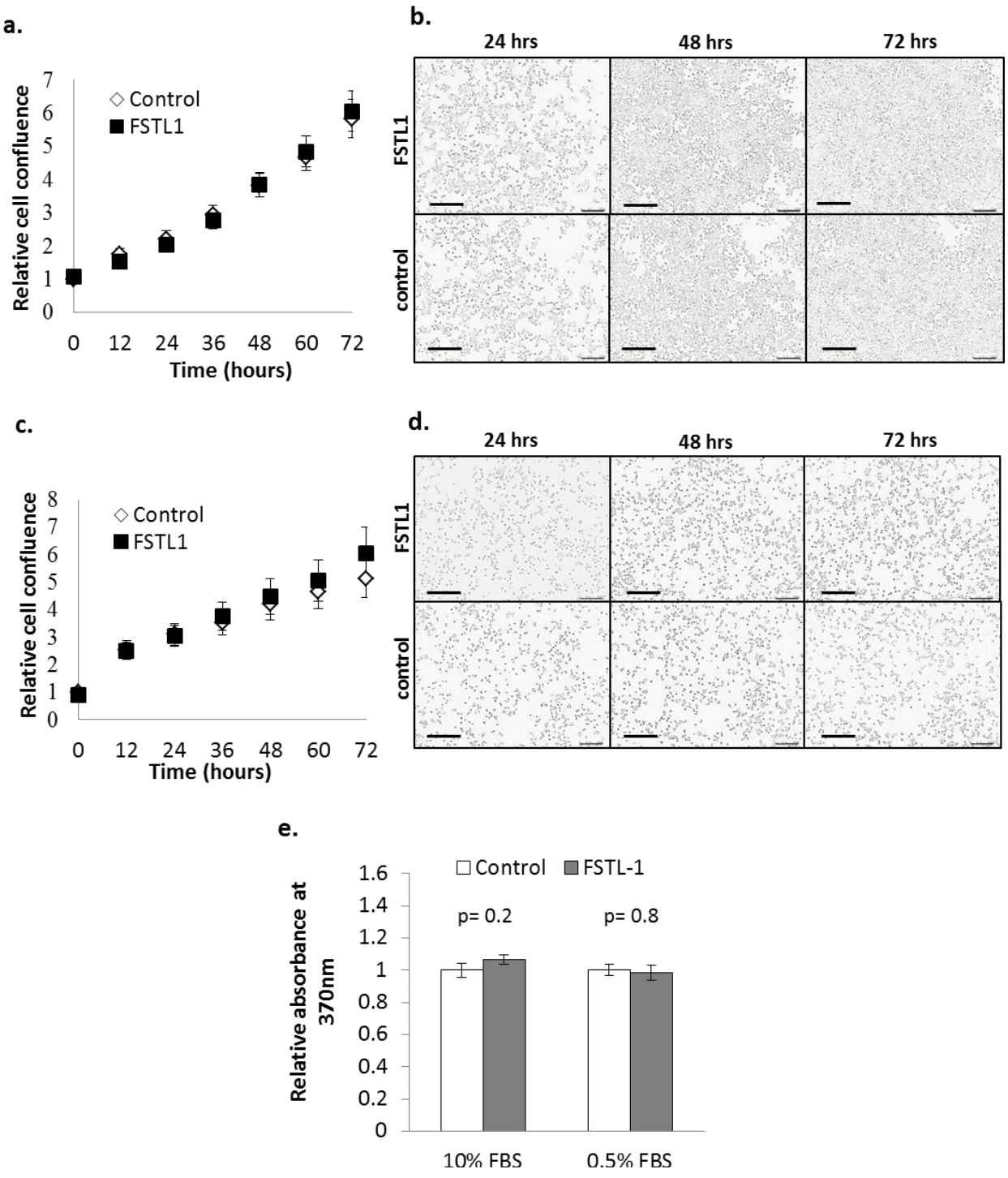


Figure 3.8: FSTL-1 does not regulate β-cell growth or proliferation. INS-1 cells were plated at $1.5x10^4$ cells/well in low serum media. Post synchronisation, cells were treated with rFST-1 (100 ng/mL) in complete media, 10% FBS (a & b) or low serum media, 0.5% FBS (c & d). Cell growth was monitored for 72 hours using the IncuCyte ZOOM imaging system. Scale bars show 300 µm at 10X objective. Cell confluence was measured by quantifying the area and standardising relative to the control +/- SEM. (e) Proliferation of cells was measured using BrdU incorporation between day 2-3 of treatment. Graph showing mean absorbance relative to the control +/- SEM. N=30 (6 replicates from 5 independent experiments). Statistical significance was quantified using student's t-test (unpaired, two-tailed). These experiments were performed in collaboration with Munasinghe, Amanda (Kingston University).

3.5 FSTL-1 inhibits proliferation of pancreatic cancer cells

Although we did not observe any change in the growth or proliferation of β cells in response to FSTL-1, previous reports have suggested that FSTL-1 can act as a tumour suppressor in breast and ovarian cancer (Chan *et al*., 2009). We therefore examined the effect of FSTL-1 on pancreatic cancer cell growth and proliferation. The addition of rFSTL-1 was found to inhibit the growth [Figure 3.9 A & B] and proliferation [Figure 3.9 C] of pancreatic cancer cells. Western blotting revealed that FSTL-1 is only expressed by pancreatic stromal cells and not by cancer cells [Fig 3.9 D].

We then investigated whether pancreatic cancer cells expressed DIP2A, which is known to be a receptor for FSTL-1 (Ouchi *et al*., 2010; Tanaka *et al*., 2010). Figure 3.9 E shows that pancreatic cancer cells express DIP2A and may perhaps mediate FSTL-1 signalling. This however would need to be tested perhaps by siRNA knockdown. Analysis of FSTL-1 immunohistochemistry data in the Human Cancer Atlas database shows that FSTL-1 is expressed at 'medium' levels in the normal pancreas but is not detected in the majority of pancreatic cancer tissues analysed (The Cancer Genome Atlas). Furthermore, FSTL-1 is similarly reduced compared to normal tissue in a range of other cancers including liver, breast, renal and stomach cancer (The Cancer Genome Atlas). Together, this data suggests that FSTL-1 produced by stromal cells may normally act to inhibit pancreatic cancer cell growth, perhaps signalling through the DIP2A receptor, and that FSTL-1 expression is downregulated within pancreatic tumours. Whether this difference is clinically significant would require further analysis that is outside the scope of this study. However, the data suggests that FSTL-1 is a novel tumour suppressor in pancreatic cancer. SPARC on the other hand, is highly expressed in pancreatic tumours (Guweidhi *et al*., 2005; Mantoni *et al*., 2008). SPARC and FSTL-1 derived from stellate cells therefore have opposing effects on pancreatic cancer cell growth and it will therefore be of interest in future studies to test whether the combined signature of SPARC overexpression and FSTL-1 inhibition is useful diagnostically.

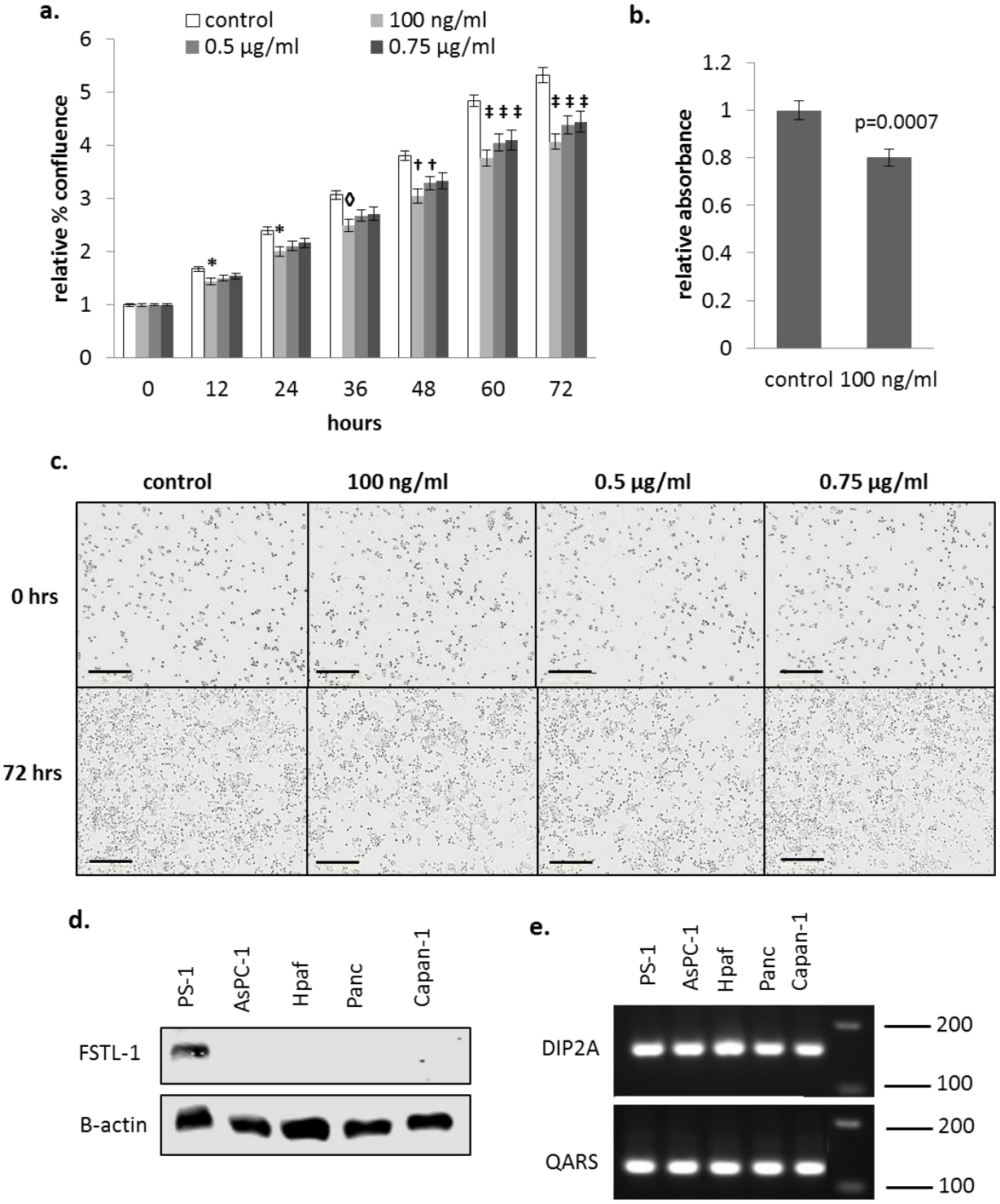


Figure 3.9: FSTL-1 inhibits pancreatic cancer cell growth and proliferation. AsPC-1 pancreatic cancer cells were plated at $5x10^3$ cells/well in low serum media. Post synchronisation, cells were treated with rFSTL-1 at the indicated concentrations in complete media (10% FBS). Growth was monitored using the IncuCyte ZOOM imaging system. (a) Cell confluence was measured by quantifying area and standardised relative to values at time 0 +/- SEM. Statistical significance was measured using one-way ANOVA [*$p < 0.05$, ◊ $p < 0.01$, † $p < 0.001$, ‡ $p < 0.0001$] (b) Proliferation was quantified by detecting BrdU incorporation between days 2-3. Graph showing data standardised relative to the control +/- SEM. Graphs showing data pooled from N=17-18 (5-6 replicates from 3 independent experiments). Statistical significance was measured using student's T-test [unpaired, two-tailed]. (c) Representative images taken at 0 and 72 hours. Scale bars show 300 µm at 10X objective. (d) Expression of FSTL-1 was investigated using western blotting. (e) Expression of DIP2A was analysed by RT-PCR. QARS was used as a housekeeping gene. All negative controls were blank.

3.6 Diversity in structure reflects multifunctionality

In summary, we have shown and described the diverse expression and modifications of the SPARC family in the pancreas. We have shown that the extended SPARC family are highly expressed in islets, specifically by β-cells as well as pancreatic ducts and stromal cells. Multiple variants in the pancreas arise from a complex mixture of post-translational modifications and alternative splicing. We have further identified multiple splice variant expression of SPOCK-3 in pancreatic stellate cells indicating a specific role for stellate cell derived- SPOCK-3 in islets. In addition, intracellular variants of the SPARC family were identified which are likely to have unique intracellular functions and need further investigation. Variation in cell-type specific variant expression therefore reflects the multifunctionality of the SPARC family and implicates important regulatory roles in islet physiology. Furthermore, FSTL-1, like SPARC is specifically expressed by stromal cells and we further have shown that FSTL-1 inhibits pancreatic cancer cell growth suggesting that SPARC and FSTL-1 derived from stromal cells have opposing effects on cancer growth.

4. Results: Investigating SPARC family function in a 3D matrix

SPARC is a collagen-binding matrix protein shown to regulate collagen fibrillogenesis and assembly (Bradshaw *et al*., 2002; Rentz *et al*., 2007; Harris *et al.,* 2011). It has also been shown to regulate interaction of collagen with cell surfaces (Harris *et al.,* 2011). Among the SPARC family, hevin is the only other protein shown to have direct interaction with collagen and regulate its assembly (Sullivan *et al.,* 2006). However, the majority of mechanistic studies to date investigate SPARC and other matricellular proteins in 2D culture, in the absence of the matrix. This may contribute to the underlying contradictory roles depicted throughout the literature. The aim of this chapter is to investigate the function of SPARC and related proteins on β-cell growth, proliferation and survival and further optimise this model to investigate primary islet survival in a 3D matrix environment. Ultimately, the aim is to develop a 3D matrix that can support islet expansion for the treatment of diabetes patients.

Recent technology has enabled the compression of collagen to form a concentrated matrix for culture. RAFT or Real Architecture for 3D Tissue uses a compressed collagen at concentrations of 80-90 mg/mL which closely mimics collagen tissues *in vivo* compared to conventional 3D matrix systems that typically only use 1-3 mg/mL (Ibidi, 2014). RAFT is robust, controlled, and reproducible compared to the conventional Matrigel often used in culture. Matrigel is essentially basement membrane extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells and can give inconsistent results due to its variable composition per batch. In addition, Matrigel contains SPARC and therefore makes it unsuitable to use for our studies. RAFT is purely composed of rat tail collagen I and is a robust high throughput system. RAFT also embeds cells in a 100 µm collagen tissue making it a novel culture system compared to conventional monolayer models seeded on top of matrix-coated plates [Figure 4.1]. We propose that creating a matrix that mimics islet conditions may shed light to the complex and multifunctional role of the matrix-regulating SPARC family on β-cell function.

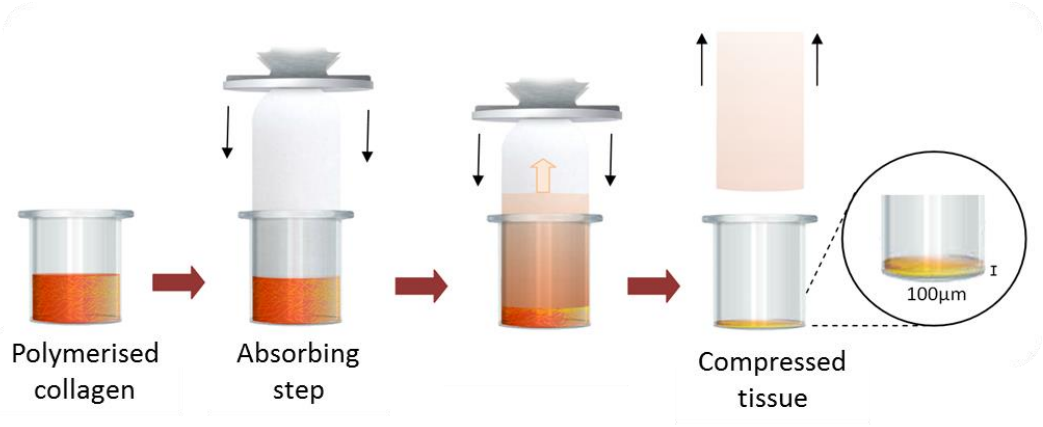


Figure 4.9: RAFT 3D culture. Cells are added into the collagen solution and are embedded into the matrix during polymerisation. Absorbers are used to concentrate the solution and absorb liquid. Gels are compressed into 100 µm tissues composed of 80-90 µg/mL of rat tail collagen I matrix. (Image modified from Larson, 2014).

4.1 Investigating β cells in 3D

4.1.1 The 3D collagen matrix supports β cell survival

We first investigated the survival of cells in the 3D matrix. We used the Live/Dead assay on INS-1 and PS-1 cells. Live cells were stained with Calcein AM, a green cell-permeant dye that is made fluorescent by esterases. Dead cells were stained with EthD-1, a red cell-impermeant dye that binds only to the DNA of cells with a compromised plasma membrane. We found that both INS-1 and PS-1 cells survive up to 10 days with very little to no dead cells detected [Figure 4.2 A]. As expected, growth is slower compared to growth in 2D [Figure 4.2 B] where INS-1 and PS-1 cells would be confluent within 3 days. Nonetheless, these initial experiments show that INS-1 and PS-1 cells survive and grow in the RAFT 3D matrix up to day 10.

PS-1 cells develop their normal elongated stellar-like morphology in the 3D matrix similar to their growth in 2D [Figure 4.2 A & B]. On the other hand, we observed a striking difference in INS-1 morphology when grown in 3D. In 2D, INS-1 cells grow in a monolayer and appear star-like in shape [Figure 4.2 A & B] while in the collagen matrix, INS-1 β-cells congregated together and created islet-like spheroids. This indicates that the 3D matrix supports β-cells to develop their natural spheroid-like islet morphology *in vivo*. In addition, we stained for insulin expression in the 3D matrix as shown in Figure 4.2 C demonstrating that the 3D matrix supports β-cell function.

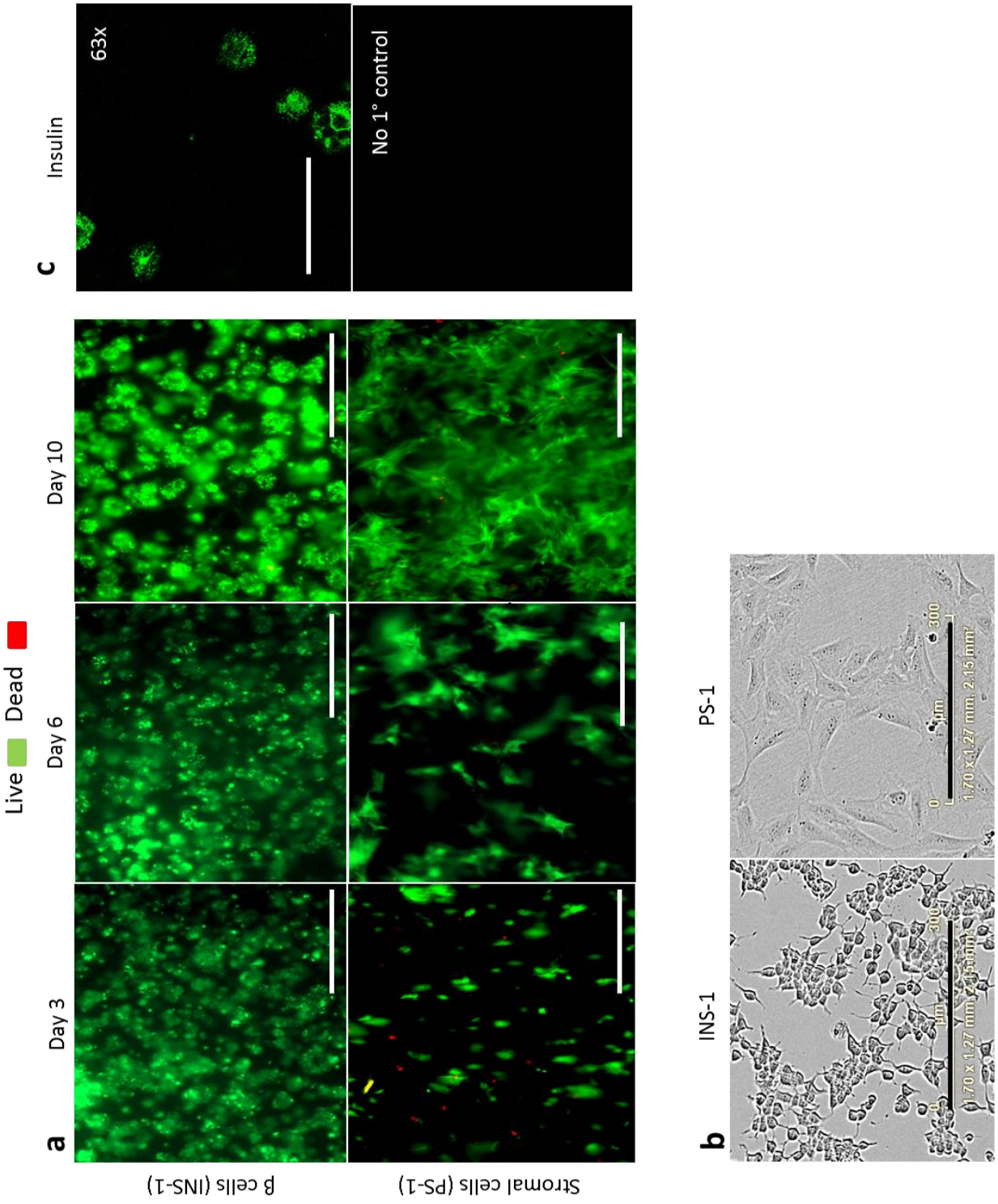


Figure 4.10: INS-1 and PS-1 cells develop their natural morphology in the 3D matrix. INS-1 ($3x10^4$) and PS-1 ($1x10^4$) cells/well were plated into the 3D matrix and cultured over a period of 10 days. (a) Survival of cells was analysed by fluorescent staining using a live/dead assay. Live cells are stained with calcein AM in green while dead cells stained with ethD-1 in red. Images were obtained using the EVOS fluorescent microscope at 20X objective. Scale bar 200 µm. (b) 2D morphology of INS-1 and PS-1 cells plated on plastic ware. Images were obtained using the IncuCyte Live Imaging System Scale bar 300 µm at 10X objective. (c) Insulin staining of INS-1 cells in the 3D matrix using antibody staining for insulin. Images were acquired using a confocal microscope at 63X objective. Scale bar at 100 µm.

4.1.2 Validation of growth assays in 3D

We then investigated the use of CellTracker Red as a means to stain INS-1 cells and measure growth in 3D. CellTracker can be freely taken up by cells and then transformed into cell-impermeant products that are retained in living cells through several generations (Life Tech). Growth was monitored by imaging cells over 13 days and area (μm^2) was quantified through the IncuCyteZOOM Live Cell Imaging System. We first validated this method by culturing INS-1 in the RAFT matrix without the SPARC proteins and monitored their growth. We investigated growth rate, variability between each well, and how long the dye is retained by cells. Area was standardised to the area at day 0 in order to control for differences in plating density. We observed that growth significantly increased by 50% between day 1 and day 5 ($p=0.024$) while this growth rate appeared to plateau between day 5 and day 11 [Figure 4.3 A & B]. However we noticed that variability between wells increased at later time points [Figure 4.3 C]. We therefore decided to set up subsequent experiments with n=6-8 wells per replicate.

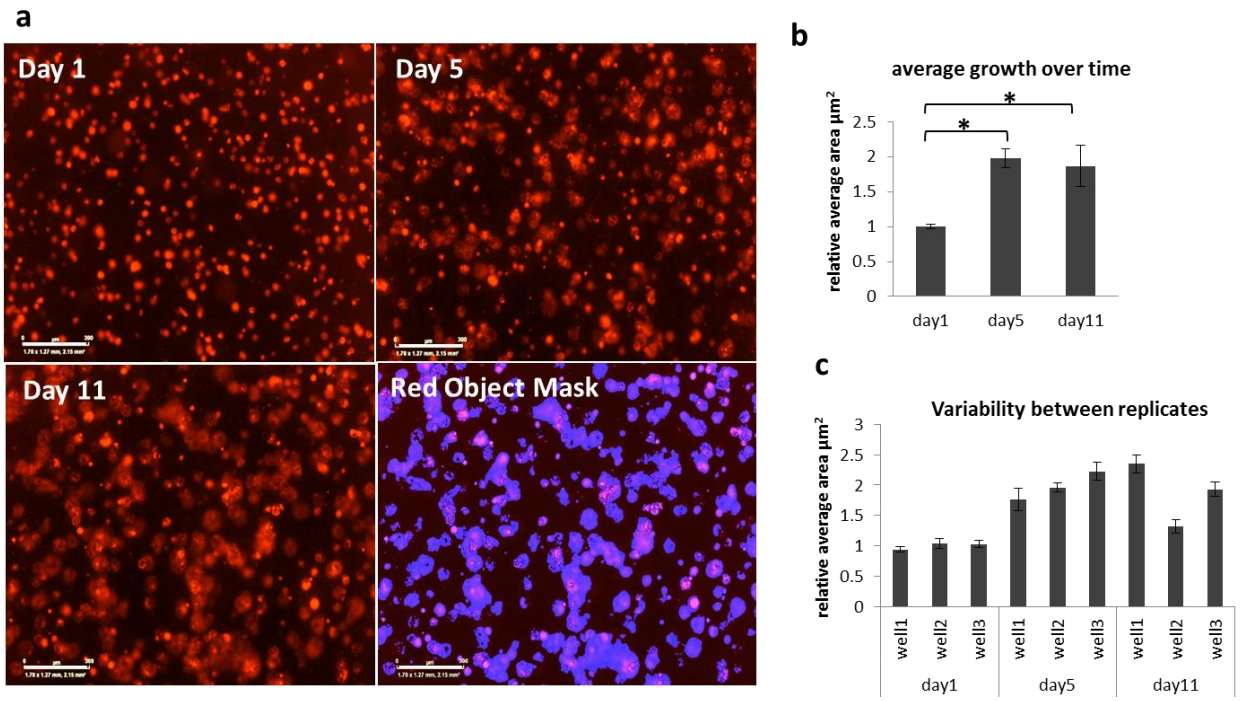


Figure 4.11: Validation of growth assays in 3D using CellTracker Red. INS-1 cells were pre-stained in 15 µM CellTracker Red and then plated at $3x10^4$ cells/well in the 3D matrix. Growth was monitored over a period of 11 days and quantified by area using the IncuCyte Zoom imaging system (10X). (a) Representative images from N=3 (3 replicates from 1 experiment). Scale bar 300 µm at 10X objective (b) Graph showing average area relative to the first time point (day 1) +/- SEM. Statistical significance (*) was measured using student's t-test (unpaired, two-tailed). (c) Area for each individual well showing variability in later time points. Error bars showing +/- SEM.

4.1.3 SPARC and SPOCK-3 inhibit β-cell growth in 3D

We cultured INS-1 cells in the presence of either SPARC, hevin, SPOCK-3, or SMOC-1 in the 3D culture over the course of 13 days. We incorporated 50-100 µg/mL of protein into the collagen solution before the polymerisation step. We validated that proteins were successfully incorporated in the matrix, by fluorescently staining for SPARC as an example shown in Figure 4.4. We found that SPOCK-3 inhibited β-cell growth dose-dependently and was evident from day 5 onwards [Figure 4.5 A & B]. By day 11, growth was significantly inhibited by 27% by treatment with 100 µg of SPOCK-3 ($p= 0.032$). SPARC similarly inhibited growth dose dependently [Figure 4.5 C & D]. By day 13, growth was statistically inhibited by 60% with 100 µg of SPARC ($p=0.008$) compared to the control cells. On the other hand, hevin [Figure 4.5 E & F] overall showed no effect on growth. SMOC-1 [Figure 4.5 G & H] showed a trend of increase however this was only significant at later time points (50 µg, $p=0.049$; 100 µg, $p=0.051$). It is worth noting however, that cells including those in untreated controls of hevin and SMOC-1 experiments did not continue to grow after day 3 which may have affected cell response to the proteins.

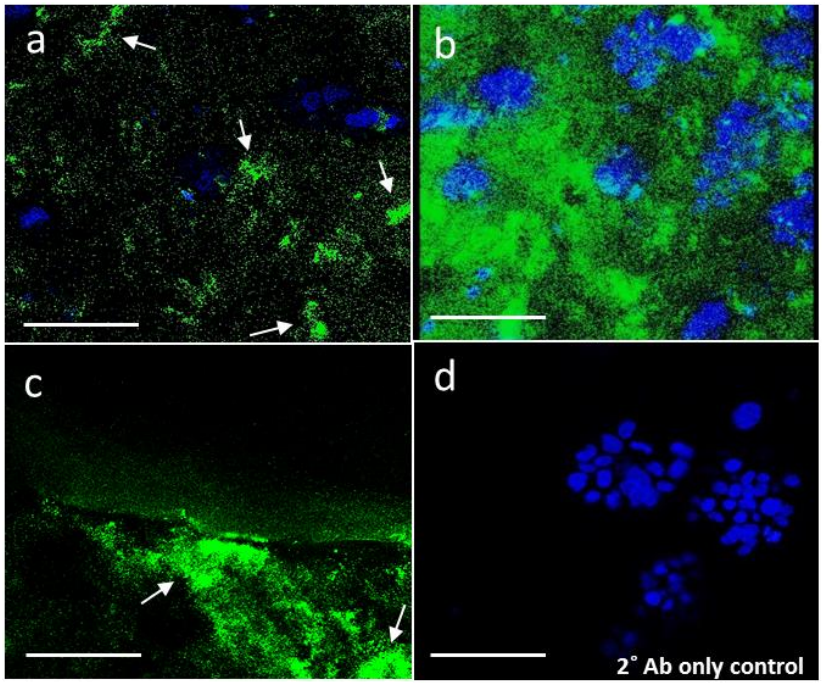


Figure 12.4: SPARC is incorporated into the 3D matrix. SPARC (a & b) 20 µg/mL, (c) (200 µg/mL) was added to the collagen solution before polymerisation in order to incorporate SPARC into the matrix. SPARC was additionally supplemented in the media. PS-1 cells were additionally incorporated into the matrix. NucBlue was used to stain cell nuclei. Tissues were fixed with 4% PFA and stained for SPARC with AF 488 green (ab14174). Images were acquired using confocal microscopy 63X. Scale bar 100 µm (a-b, d) Images show 2D z-stack images from one plane while (c) shows a 3D projection from 192 consecutive z-stack images indicating SPARC incorporation into the matrix. (d) Secondary only control showing no SPARC staining. Validation and images courtesy of Munasinghe, Amanda (Kingston University).

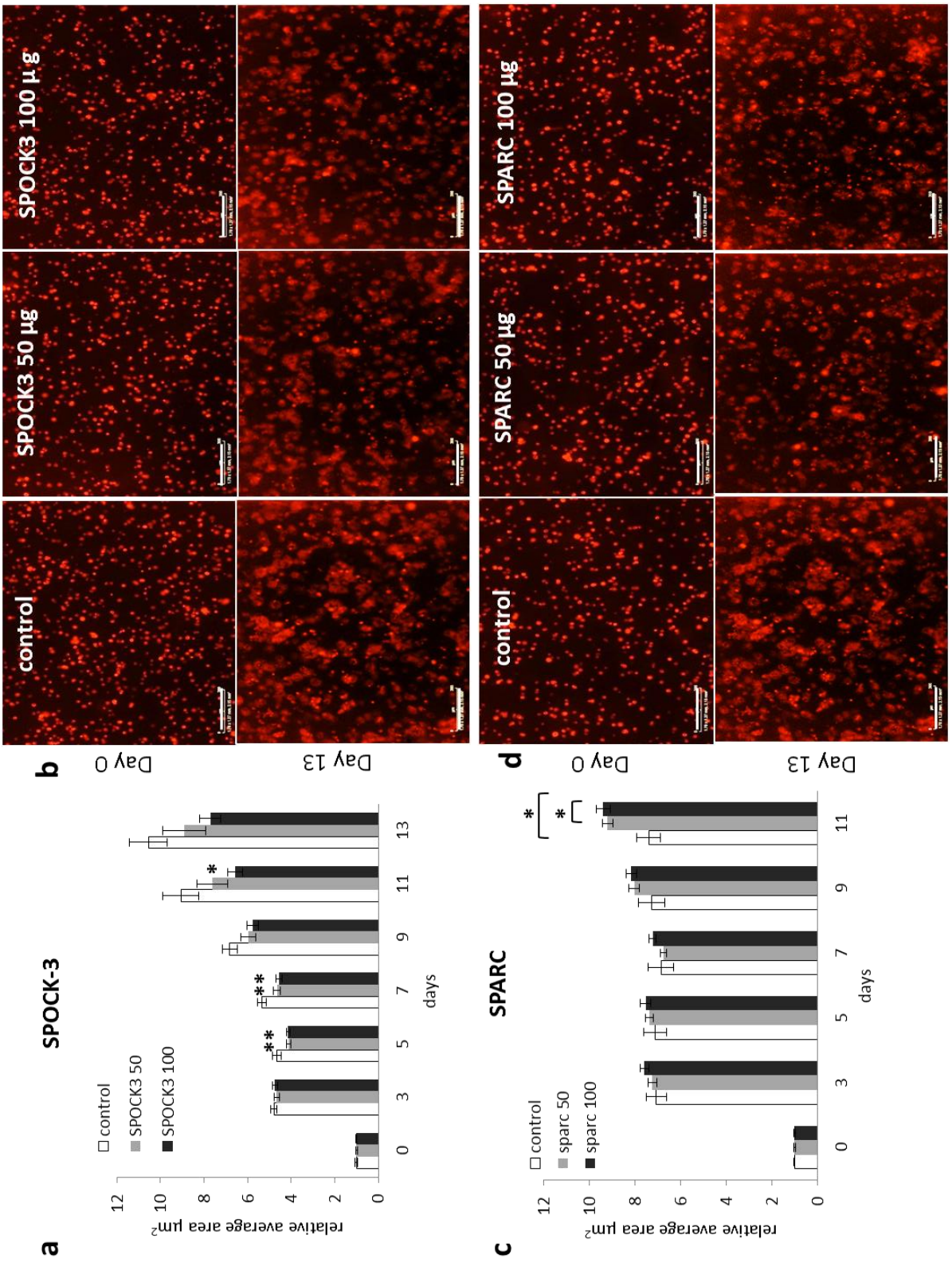


Figure 4.5: Effect of the SPARC family on β-cell growth. Pre-stained fluorescent INS-1 cells ($3x10^4$ cells/well) were embedded in the 3D matrix treated with 50-100 µg/mL of a-b) SPOCK-3 c-d) SPARC e-f) hevin g-h) SMOC-1 for a period for 13 days. Growth was monitored fluorescently using the Incucyte ZOOM live cell imaging. Images representative of 4 images/ well. Scale bar 300 µm at 10X objective. Graphs showing area relative to the first time point (day 0) +/- SEM. Graphs showing representative data of N=23-24 (5-6 replicates from 3-4 independent experiments). [See Appendix Figures 8.6-8.9 for all independent replicates]. Statistical significance (*) was analysed using one-way ANOVA.

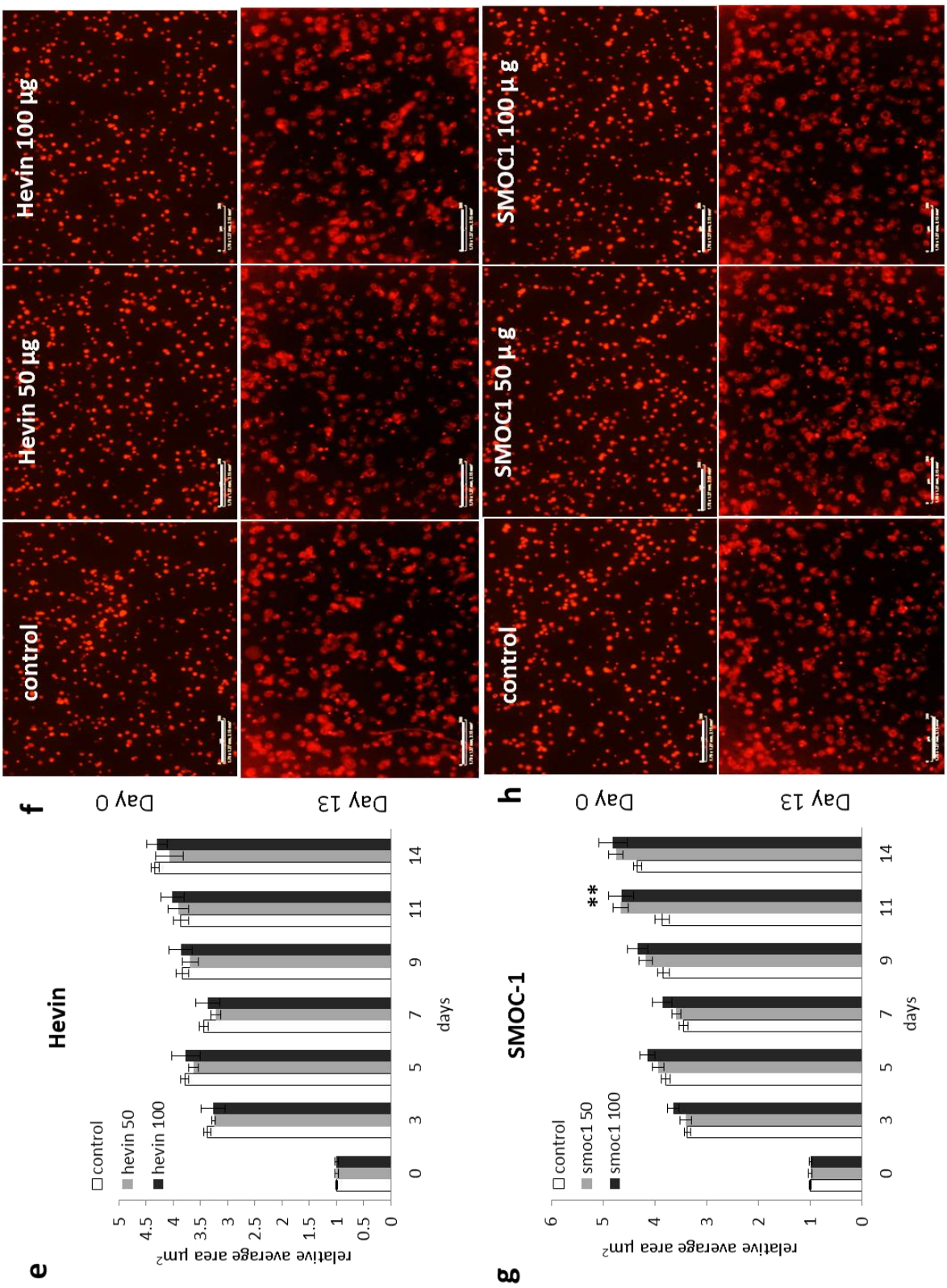


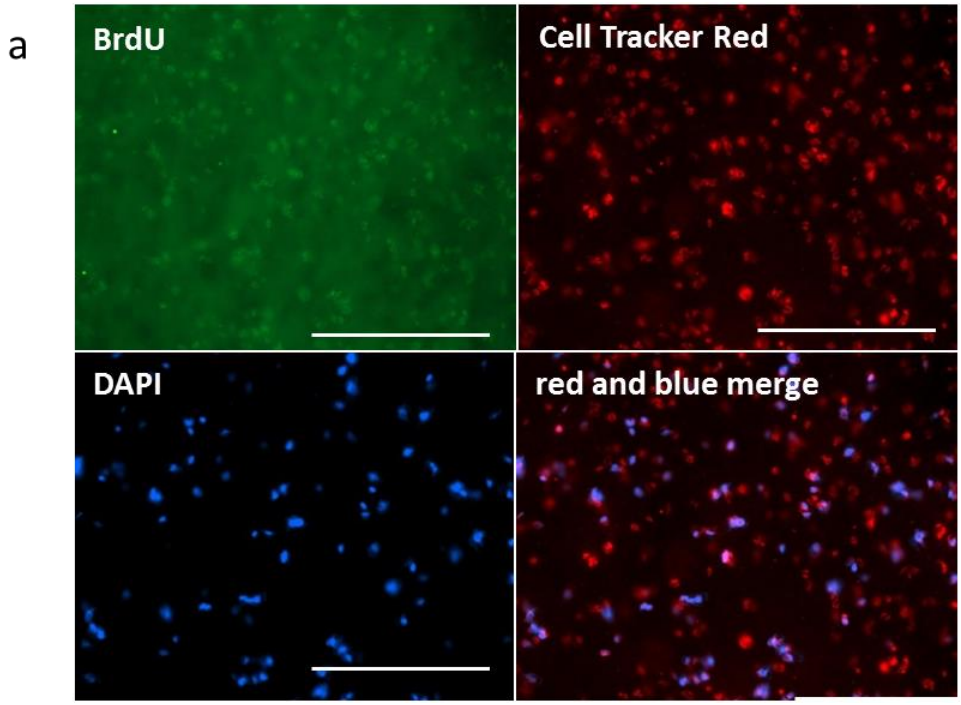
Figure 4.5: Effect of the SPARC family on β-cell growth.

However, we did observe variability between different replicates. SPARC for example showed significant decrease on growth in 1 out of 4 replicates while 3 showed no difference but however had slight increases at later time points [Appendix Figure 8.9]. Although hevin showed no effect on growth overall, 1 of 3 replicates showed significant decrease [Appendix Figure 8.10] while for SMOC-1, 2 of 3 replicates showed slight increases at later time points [Appendix Figure 8.11]. In contrast, SPOCK-3 showed consistent results showing inhibition of growth in all 3 replicates although was only statistically significant in 2 of 3 replicates [Appendix Figure 8.8].

4.1.4 Validation of proliferation assay in 3D

In order to quantify proliferation in the 3D matrix, β-cells were fluorescently stained for BrdU incorporation. CellTracker Red and or DAPI were used as controls to stain both proliferating and non-proliferating cells. Initially, we were not able to detect BrdU staining within the 3D matrix using a standard EVOS fluorescent microscope [Figure 4.6 A]. In addition, DAPI stained less cells than the CellTracker dye, indicating that the DAPI, which was formulated with SLOW-fade gold mountant, was perhaps not able to penetrate through the 3D matrix. This preliminary data warranted further optimising of the BrdU assay, and that investigating appropriate imaging platforms for use in 3D would be necessary.

We initially validated the assay in 2D culture and found that the assay successfully stained proliferating and non-proliferating cells [Figure 4.6 B]. We then proceeded to perform the assay again in the 3D collagen matrix, this time increasing antibody incubation from 30 minutes, as recommended by the manufacturer, to 2 hours as penetration into the matrix depends on tissue composition and thickness (Artym and Matsumoto, 2010). Furthermore, we used NucBlue as a control nuclear stain instead of the DAPI. NucBlue is a live cell stain in solution that may be more able to penetrate the tissue compared to DAPI which was incorporated in mounting solution. As shown in Figure 4.7, this improved BrdU staining and we therefore proceeded to compare imaging using the confocal and FLOID microscopes as well as the automated IncuCyte ZOOM Live Imaging System. A comparison between the imaging platforms is summarised in Table 4.1.



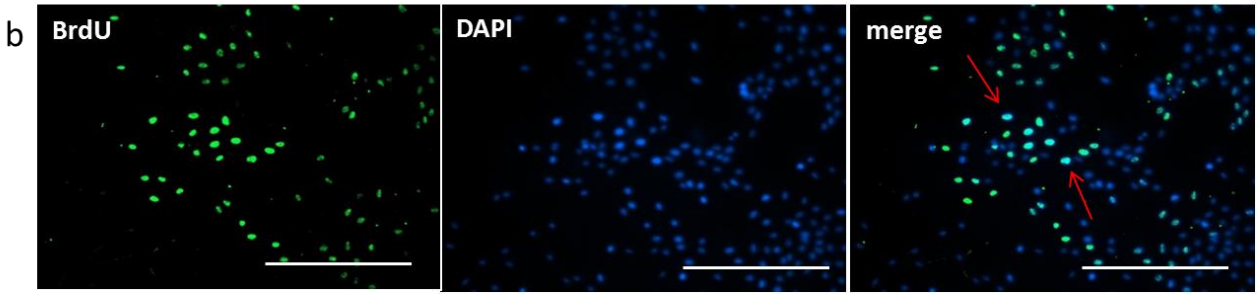


Figure 4.6: Validation of BrdU staining in 3D. (a) INS-1 cells ($3x10^4$ cells/well) were pre-stained with Cell Tracker Red before embedding in the 3D matrix. After 48 hours of culture in 3D, BrdU was added and BrdU incorporation was analysed at 72 hours. Tissues were probed with anti-BrdU and fluorescein-conjugated secondary antibodies (green). Slides were mounted with SLOW-fade gold mixed with DAPI (blue). Images were acquired at 20X using the EVOS fluorescent microscope. Scale bar 200 µm. (b) PS-1 cells were plated on coverslips and stained for BrdU incorporation. Images were acquired using the EVOS fluorescent microscope at 20X magnification. Scale bar 200 µm. [Images from (b) courtesy of Munasinghe, Amanda].

ImageJ analysis software was used to quantify area to calculate % BrdU by dividing BrdU area to nuclear area. We found that imaging with both the FLOID and the confocal microscopes yielded similar results despite imaging from two different platforms and magnifications [Figure 4.7 A-D]. For example, Figure 4.7 B & D shows that slides 3 and 4 had higher %BrdU incorporation compared to slides 1 and 2 from both FLOID and confocal imaging. This indicates that both platforms are likely to yield accurate results [Figure 4.7 G]. The FLOID microscope is a very straightforward imaging platform and is faster to use compared to the

confocal. The background also tends to be generally low. However, the FLOID images through the entire 3D tissue and cannot focus on one plane at a time, cells not in focus therefore would inaccurately have greater area. Furthermore, consistency and accuracy would be compromised since the plane of focus for different channels is not always the same, for example the area in the blue channel may be higher than the green and vice versa. The confocal microscope on the other hand, although takes time to set up and use, is able to accurately image through the 3D matrix using z-stacking and therefore can acquire images from one plane within a 3D tissue. Fluorescent bleeding between channels can also be avoided using sequential scanning. Lastly the IncuCyte, although is an automated and high throughput imaging system, can only image in the red and green channels hence CellTracker Red was used as a control instead of a blue nuclear stain. However, since the IncuCyte has an automated imaging software, the system sometimes is unable to choose the best plane of focus, similar to problems with the FLOID microscope, and has no option to manually choose the plane of focus [Figure E-F]. It therefore sometimes quantifies area to be larger in regions where the image in unfocused, thus values above 100% as seen in Figure 4.7 E & F. We therefore decided to use the confocal microscope in subsequent BrdU 3D experiments and sequential scanning was used to image.

	Pros	Cons
Incucyte	• High throughput • Automated imaging and analysis • Can image within the plate	• Cannot image through one plane at a time • No manual option to choose plane of focus • Only green and red channel, unable to do blue nuclear stain • Ethd-1 red nuclear dye not detected
Evos/FLOID	• Faster than confocal • Low background noise vs confocal	• Cannot image through one plane at a time • Difficult to focus image • Sometimes there is bleeding through the channels
Confocal	• Can image through one plane at a time within the 3D tissue using z-stacking • Can avoid bleeding by sequential scanning	• Struggle to image DAPI below 40x objective • Need to take more images/ sample at higher magnifications • Can have high background • Setting up and imaging takes time, not high throughput

Table 4.1: Pros and Cons of each imaging platform for acquiring 3D images. INS-1 cells embedded in the collagen matrix were fluorescently stained for BrdU incorporation. Imaging was compared between the Incucyte, EVOS/FLOID microscopes and confocal microscopes to determine that best platform for imaging.

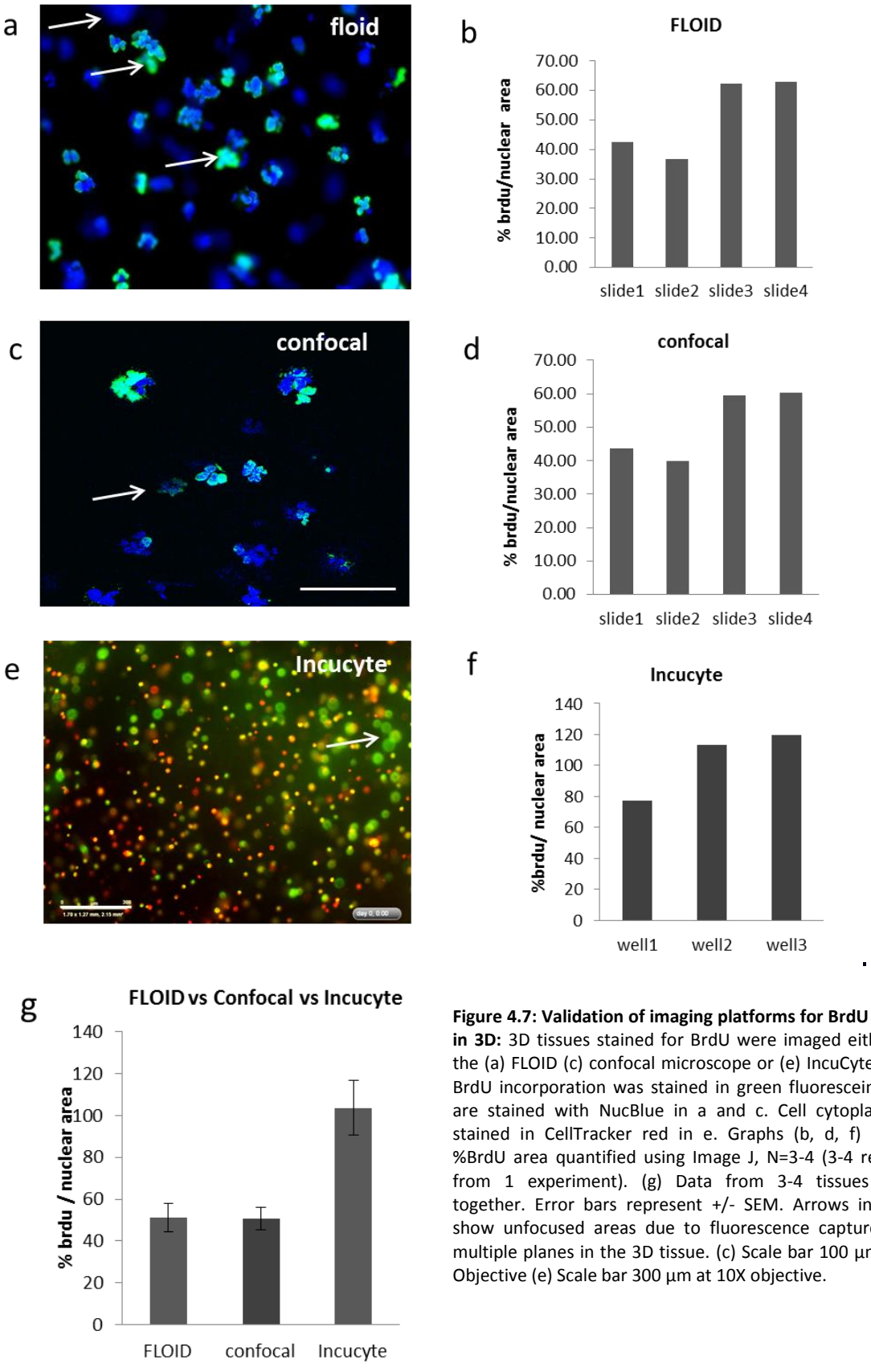


Figure 4.7: Validation of imaging platforms for BrdU imaging in 3D: 3D tissues stained for BrdU were imaged either with the (a) FLOID (c) confocal microscope or (e) IncuCyte ZOOM. BrdU incorporation was stained in green fluorescein. Nuclei are stained with NucBlue in a and c. Cell cytoplasm was stained in CellTracker red in e. Graphs (b, d, f) showing %BrdU area quantified using Image J, N=3-4 (3-4 replicates from 1 experiment). (g) Data from 3-4 tissues pooled together. Error bars represent +/- SEM. Arrows in images show unfocused areas due to fluorescence captured from multiple planes in the 3D tissue. (c) Scale bar 100 µm at 63X Objective (e) Scale bar 300 µm at 10X objective.

4.1.5 SPARC and Hevin inhibited the mean proliferation of β-cells in 3D

We investigated the effect of SPARC, hevin and SPOCK-3 on INS-1 β-cell proliferation in a 3D matrix. INS-1 cells were cultured in the 3D matrix and proteins were incorporated into the matrix during the polymerisation step. BrdU was added at day 2 and BrdU incorporation was investigated at day 3. We first validated SPARC, hevin and SPOCK-3 using a final concentration of 20-50 µg/mL. However, the results were highly variable between replicates [Appendix Figure 8.12]. We then increased the protein concentration to 100 µg/mL. Untreated cells had 50% BrdU area, indicating about half of the cell population is normally actively proliferating between days 2-3 in 3D [Figure 4.8]. SPARC and hevin both decreased the mean BrdU incorporation by up to ~39% [Figure 4.8]. The effect of hevin was not significant (p=0.5) however SPARC was marginally significant with a p-value of 0.067. SPOCK-3 on the other hand did not have an effect on BrdU incorporation. This preliminary data suggest that SPARC and hevin, but not SPOCK-3, promote a trend of decrease in proliferation in the 3D collagen matrix. However, given the variability observed in 3D, this preliminary data would need to be repeated to confirm this conclusion.

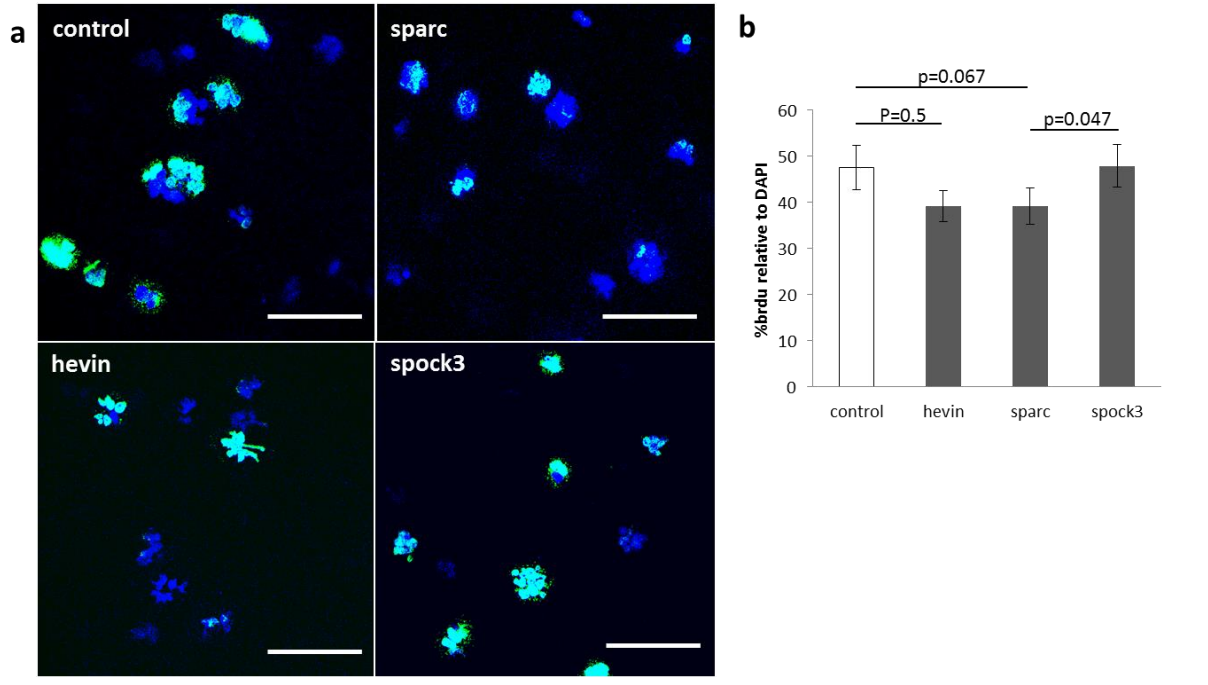


Figure 4.8: SPARC and hevin inhibit the mean β-cell proliferation in 3D: INS-1 cells ($3x10^4$ cells/well) were embedded in the 3D matrix treated with or without 100 µg/mL of either SPARC, hevin, or SPOCK-3 for 3 days. BrdU was added after 48 hours of culture and BrdU incorporation was analysed fluorescently during the last 24 hours of culture. About 10 images per sample were acquired using the confocal microscope (40X). (a) Representative images from N=5-6 (5-6 replicates from 1 experiment) Scale bar 100 µm. (b) %BrdU was quantified by dividing BrdU area by nuclear area. Graphs showing average %BrdU +/-SEM. Statistical significance was measured using one-way ANOVA. P values are indicated in the graph.

4.2 The effect of SPARC on the 3D collagen matrix

We have previously shown that SPARC significantly inhibited β-cell proliferation in 2D (Ryall *et al*.,2014). Our 3D results show that SPARC and hevin did not have consistent effects on growth while both inhibited the mean proliferation but not significantly. In addition, SPOCK-3 significantly inhibited growth but however did not have an effect on proliferation. Perhaps the SPARC family may be acting upon the cells indirectly and instead may be acting upon the matrix. SPARC and hevin have been shown to bind to collagen and regulate fibril assembly (Hambrock *et al.,* 2003; Brekken *et al*., 2004; Sullivan *et al.,* 2006, Rentz *et al*., 2007; Guidici *et al*., 2008, Hohenester *et al.,* 2008;). SPOCK proteins on the other hand are known to regulate MMPs and therefore may also regulate matrix assembly (Nakada *et al.,* 2001; Nakada *et al.*, 2003). We therefore aimed to investigate how SPARC proteins affect collagen assembly in the 3D collagen matrix.

SPARC was incorporated as previously described. Since matricellular components in the serum may influence interaction of SPARC with collagen, we investigated the effect of SPARC in the presence of either low serum media (0.5% FBS) or complete media (10%). After the tissues were polymerised and compressed, tissues were immediately fixed and processed for SEM imaging. Figure 4.9 A shows the dense collagen architecture of the RAFT 3D matrix. We quantified fiber diameter and porosity using ImageJ analysis software. We found that adding SPARC to the collagen solution slightly decreased fiber diameter and porosity in 10% FBS but this difference was not statistically significant [Figure 4.9 B]. On the other hand, low serum media did not have an effect on the matrix [Figure 4.9 B]. However this is only preliminary data. The RAFT 3D matrix is already very concentrated therefore imaging as well as the analysis software may not have been able to detect subtle differences that may have occurred. In addition, the wider SPARC family may have different effects on collagen I fiber assembly and also warrants further investigation. Alternatively, the SPARC family may have roles in long term remodelling of the matrix rather than initial assembly. It is worth noting that in these preliminary experiments, no cells were incorporated into the matrix, and tissues were immediately fixed following assembly. Further study is needed to investigate the long term effects on matrix assembly and remodelling in the presence of stromal cells and the SPARC family.

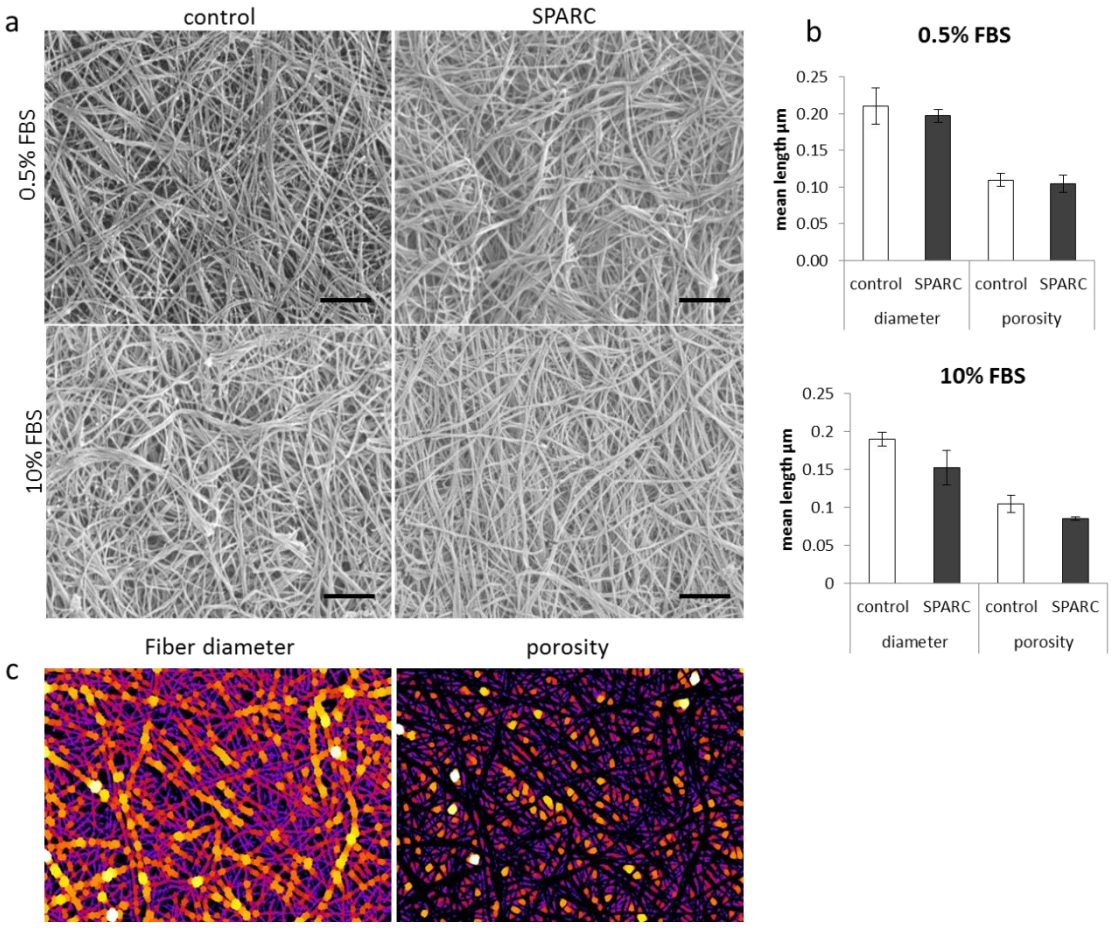


Figure 4.9: SPARC does not affect collagen fiber diameter or porosity during matrix assembly. SPARC was incorporated to the collagen solution before polymerisation. Tissues were immediately fixed after polymerisation for SEM imaging. (a) Representative images from a total of 10 images/ sample were acquired at 20 kv. Scale bar 2 µm. (b) Collagen fiber diameter and porosity was quantified using Image J (c) showing representative quantification of diameter and porosity. Graphs showing average length/ area in µm +/- SEM. N=4 (2 replicates from 2 independent experiment). Statistical significance was measured using Student's T-test (unpaired, two-tailed). Validation and images were performed in collaboration with Munasinghe, Amanda (Kingston University).

4.3 Investigating primary islets in 3D: Islet survival is influenced by matrix tissue thickness

The survival of mouse islets was investigated in the 3D culture over a period of 6 days using the Live/Dead assay. After 1 day in the 3D culture, islets were alive, with some dead cells predominantly on the outer surface of the islet [Figure 4.10 A]. At day 6 however, we observed that more cells die at the outer surface of islets while inner cells survived.

We found that the thickness of the tissue directly impacts survival of the islets in the 3D matrix. Average mouse islet diameter ranges from 50-250 µm (Stendahl, 2009). The RAFT protocol however, is designed to create 3D tissues 100 µm thick. Figure 4.10 A shows that islets embedded in 100 µm of collagen disintegrate by day 3, perhaps as a result of

incomplete embedding. Increasing collagen thickness up to 200 µm supports islet survival and significantly increased survival up to day 6 (p=0.016) [Figure 4.10 B].

It is worth noting that the RAFT 3D matrix is composed of rat tail collagen I whereas the islet basement membrane is predominantly composed of collagen IV and laminin. It will therefore be relevant to optimise this model to incorporate collagen IV and laminin into the matrix to more closely mimic the islet and investigate survival and the role of SPARC proteins. Nonetheless, this is preliminary data demonstrating a model in which survival of islets fully embedded in a matrix can be investigated in 3D.

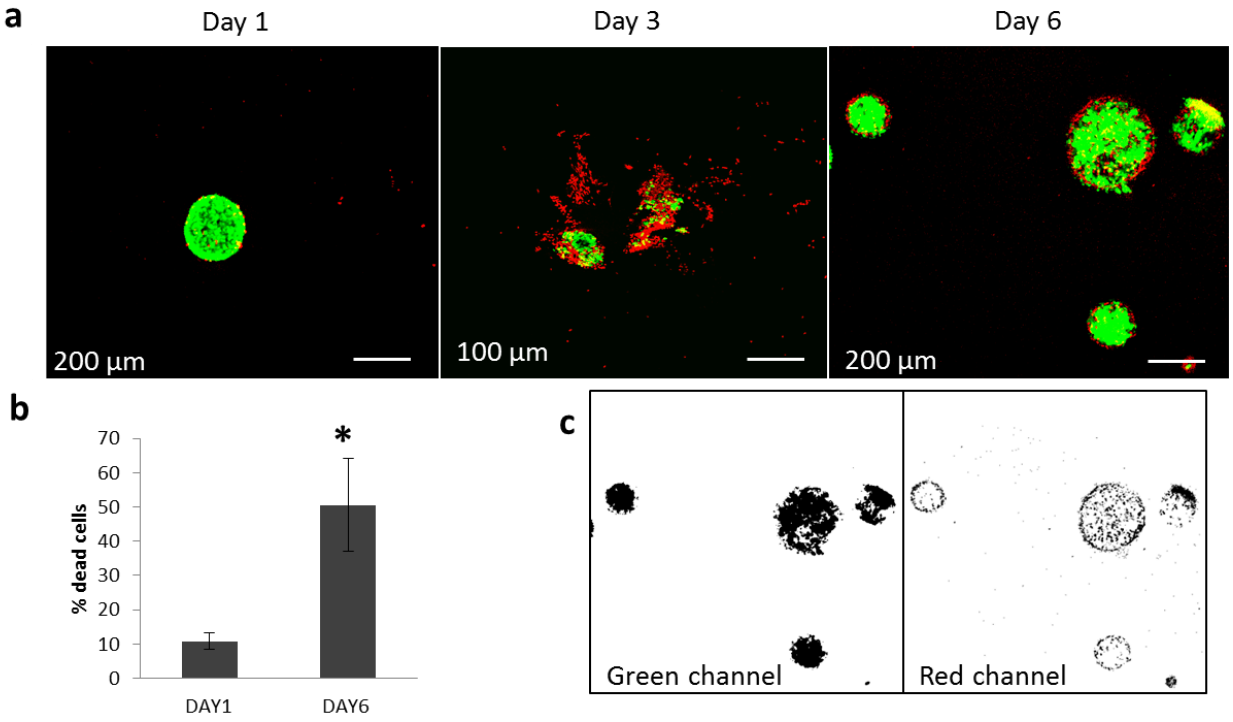


Figure 4.10: The thickness of the collagen matrix influences islet survival in 3D. Primary mouse islets (10-15 islets/ well) were embedded into the matrix composed of either 100 µm or 200 µm of collagen for 6 days. Live cells were stained with calcein AM (green) and dead cells with EthD-a (red). (a) Images representative of 5-10 islets/well . A total of 6 images/ samples were acquired using the confocal microscope, 10X. Scale bar showing 100 µm. Survival was quantified using Image J by measuring area of (c) both green and red channels. (b) Graph showing % dead cells +/- SEM (5-10 islets from 1 experiment). Statistical significance depicted by (*) was measured using Student's T-test (unpaired, two-tailed).

4.4 Investigating sectioning of 3D tissues

It is worth stressing the variability in results acquired from 3D studies, both from growth and proliferation assays. Thus it was important to increase the reagent concentrations, number of N per replicate, and increase the number of images taken per sample. Nonetheless, it was difficult to detect consistent changes in 3D. Whether this is a limitation of the imaging platform or the analysis software needs further investigation. For example, the IncuCyteZOOM may not have been sensitive to small changes in growth because it was not properly able to focus at one plane. In addition, the penetration depth of confocal microscopes is less than 100 µm, shorter than the 3D tissues which are exactly 100 µm (Pawley and Masters, 1996). The thickness and concentration of the tissues, including the cover slip may have been a factor that affected scattering of the beam. Scattering causes the illuminating beam to defocus and thus decreases imaging depth (Graf and Boppart, 2010). This may be a challenge when quantifying BrdU incorporation in islets.

We therefore also investigated sectioning of the 3D tissues in order to avoid issues with *in situ* imaging. Tissues were fixed in formalin and embedded in paraffin wax in order to cut 5 µm sections. Since the tissues are 100 µm thin, it was difficult to cut a flat section of the entire tissue. In addition, it was difficult to visibly detect the tissue within the paraffin block. Only partial sections were obtained, perhaps as a result of slight slanting of the tissue in the paraffin block. We also attempted to embed the tissues in agarose before paraffin embedding but sectioning was unsuccessful. Nonetheless, we found that DAPI staining of 3D sections can be detected at 10X up to 40X using a standard fluorescent microscope [Figure 4.11]. This is however very time consuming and not compatible with high throughput analysis. This preliminary validation nevertheless demonstrates that sectioning may be useful for staining, for example for islet experiments in 3D.

One further point to consider is that although we increased SPARC family concentration, it is worth noting that the proteins were added during collagen polymerisation. At this stage, we have shown that the proteins are incorporated and bound to the matrix [Figure 4.4]. However, some may have been absorbed by the absorbers during the compressing stage, decreasing the effective concentration.

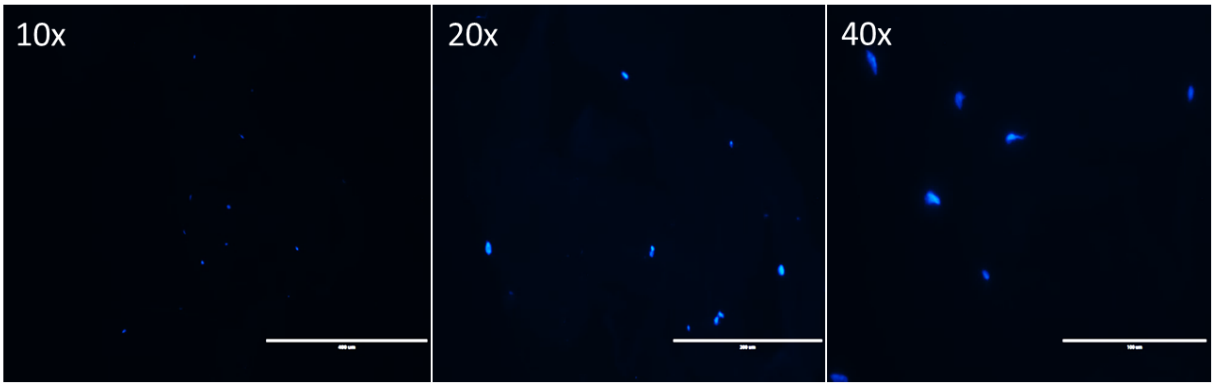


Figure 4.11: DAPI-stained nuclei can be imaged in sectioned 3D tissues. INS-1 cells were embedded into the 3D matrix after which was fixed in 10% NBF. Tissues were then dehydrated through a series of alcohol washes and histoclear. Next tissues were fixed and embedded in paraffin wax. Sections were cut by 5 µm and then stained with DAPI. Images were acquired using the EVOS fluorescent microscope using the indicated objectives. Scale bar 400 µm at 10X. Scale bar 200 µm at 20X. Scale bar 100 µm at 40X. N=1 (1 replicate from 1 experiment).

3D imaging may need more highly specialised platforms such as multi-photon microscopy, which is not affected by high scattering of light (Graf and Boppart, 2010). In addition, multi-photon microscopy can penetrate samples up to a millimetre thick (Graf and Boppart, 2010). Overall, imaging and quantification of assays in 3D culture needs further optimising for sensitivity, robustness, and high throughput procedures. In addition, further optimisation is needed for flexibility to design models with multiple cell types. The growing movement into 3D cultures warrants urgent need to improve models and analysis for research to fully adopt and transition into complex and dynamic 3D cultures.

4.5 Inhibition of SPARC family expression in β-cells by siRNA

4.5.1 Inhibition of protein expression using single and SMARTPOOL siRNA

We have shown in Results Chapter 3 that β-cells and islets strongly express hevin, SPOCK and SMOC proteins. We therefore proposed to silence hevin and SPOCK-3 expression in β-cells by siRNA knockdown to investigate their role on growth and proliferation. Furthermore, our data demonstrates that islet contact with the matrix is essential for survival. Jalili *et al*. have previously shown that survival of islets, particularly cells in the outer periphery improved

when fibroblasts were co-cultured in the matrix (Jalili *et al*., 2010). We first validated our methods by testing rat hevin siRNA on INS-1 cells. For these experiments we tested with single siRNA, namely one sequence of siRNA that binds specifically to one site of the mRNA. We titrated the siRNA up to 150 nM for 48 hours and detected protein expression by western blotting. We found no evidence of reduced protein expression using this siRNA [Figure 4.12 A]. We then tested using Hevin SMARTPOOL siRNA which contains a mixture of 4 different types of individual siRNA that bind to 4 different sites of the mRNA sequence. In addition, we increased siRNA concentration up to 200 nM. However we also did not detect reduced protein expression even at high siRNA concentrations [Figure 4.12 B].

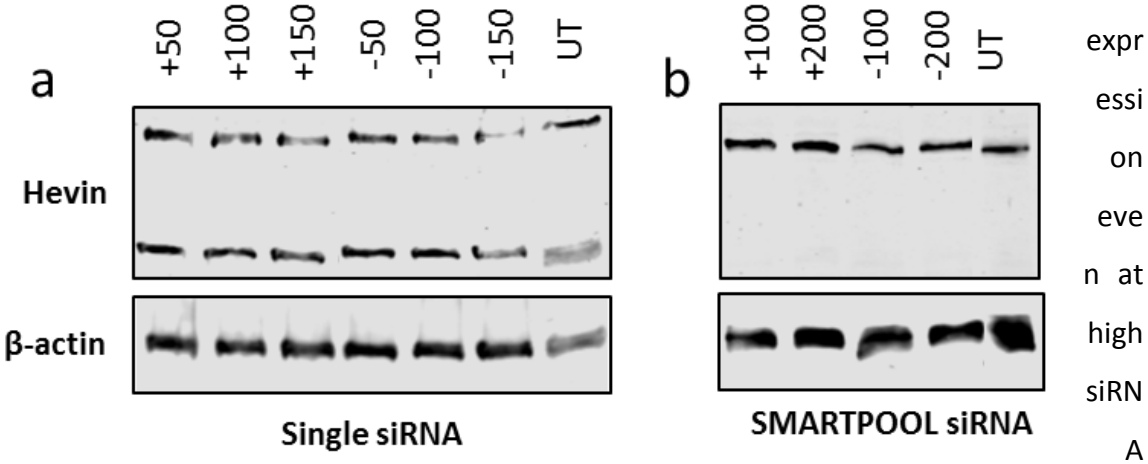


Figure 4.12: Hevin knockdown using single and SMARTPOOL siRNA. INS-1 cells ($1x10^4$ cells/well) were subjected to siRNA knockdown for 48 hours to silence hevin expression using (a) single siRNA and (b) SMARTPOOL– a collection of 4 siRNA (Dharmacon). Transfection complexes were formed using HiPerFect (Qiagen). The concentration (nM) of siRNA used is indicated above. Non-targeting pooled rat siRNA was used as a control. Expression of hevin was detected by western blot. β-actin was used as a loading control. (UT) untransfected.

4.5.2 Inhibition of protein expression using double knockdowns with SMARTPOOL siRNA

We then attempted to perform double knockdown using SMARTPOOL siRNA, in which the media was refreshed with fresh media supplemented new siRNA 48 hours after the first knockdown [Figure 4.13 A]. During this step, we found that cells treated with hevin or SPOCK-3 siRNA became less adhesive compared to cells treated with control siRNA [Figure 4.13 B]. Cells that were left attached however, continued to grow after the second knockdown [Figure 4.13 C]. This was a consistent

observation for up to 3 independent experiments. We also found that after the second knockdown, more cells have detached in hevin or SPOCK-3 siRNA compared to the control siRNA [Figure 4.14 A]. These detached cells were confirmed viable using trypan blue staining [Figure 4.14 B]. However, regardless of the dramatic phenotypic effects of the knockdown, inhibition of protein expression was not detected by western blotting. We hypothesized that perhaps the cells that were successfully inhibited for protein expression were those that detached. Hence we did not observe silencing in cells that were adhered. We then collected detached cells and lysed them for western blot analysis and compared it to attached cells. However, we found that reduced protein expression was still not evident in cells that had

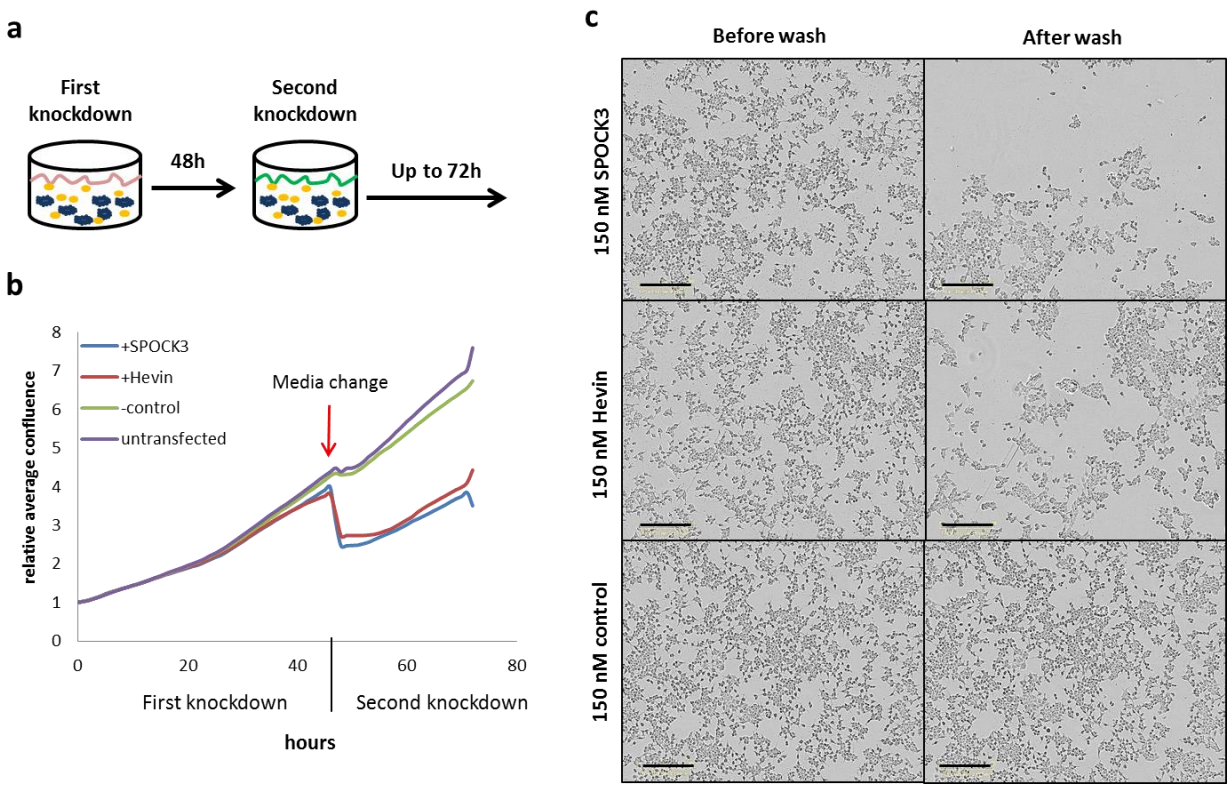


Figure 4.13: Double knockdown using SPOCK-3 or hevin SMARTPOOL siRNA promoted de-adhesion of β cells. INS-1 cells ($1x10^4$) were transfected with SMARTPOOL siRNA for hevin or SPOCK-3 for 48 hours. (a) Media was thereafter aspirated and replaced with fresh media supplemented with new siRNA. The second knockdown was carried out for another 48-72 hours. Cells were monitored using the IncuCyte Zoom imaging system. Non-targeting rat pooled siRNA was used as a control (b) Representative images showing cells before and after the media was changed after 48 hours of the first knockdown. Scale bar 300 µm at 10X objective. (c) Graph showing pooled data of cell confluence over time relative to the first time point. Arrow indicating the second knockdown. N=6 (3 replicates from 2 independent experiments).

detached [Figure 4.15 and Appendix Figure 8.13]. There was

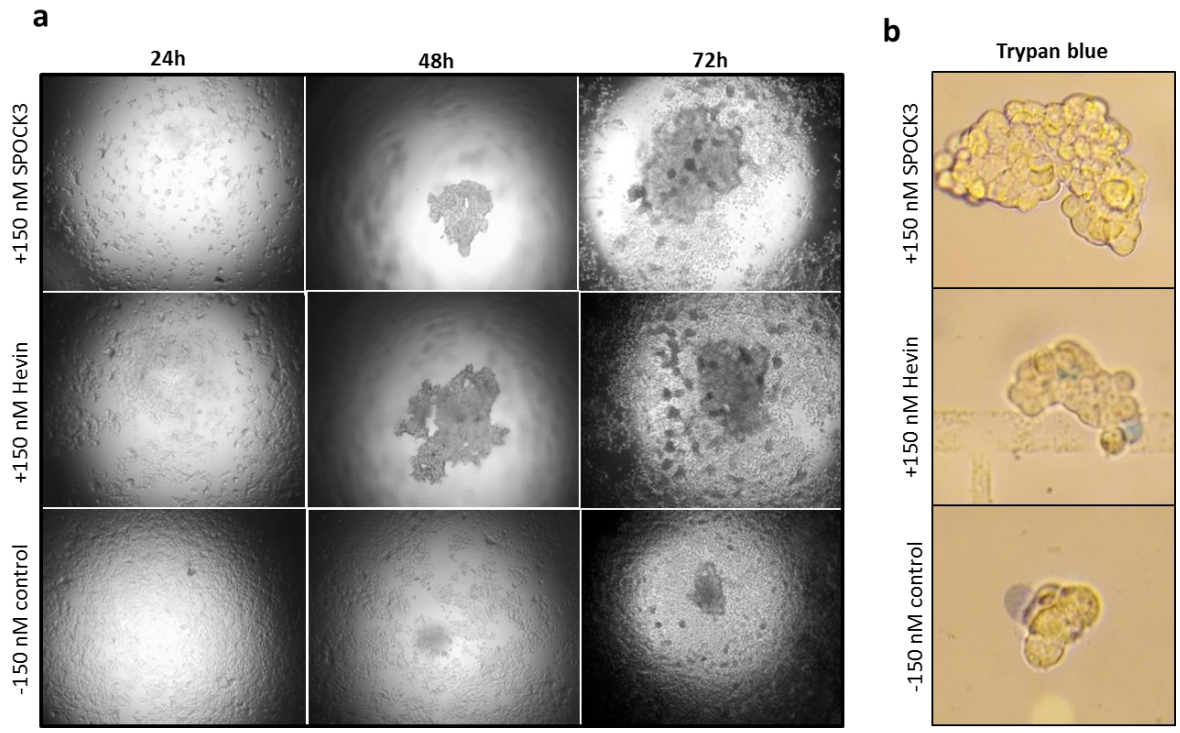


Figure 4.14: β-cells detach following second knockdown using SPOCK-3 and hevin SMARTPOOL siRNA. INS-1 cells ($1x10^4$) were transfected with SMARTPOOL siRNA for hevin and SPOCK-3 for 48 hours. Media was thereafter aspirated and replaced with fresh media supplemented with new siRNA. The second knockdown was carried out for another 48-72 hours. (a) Representative images showing cells after 24-72 hours after the second knockdown from N=9 (3 replicates from 3 independent experiments). (b) Representative images captured using a laboratory camera showing cells stained with trypan blue 24 hours after the second knockdown.

also variable expression of the two SPOCK-3 bands (56 kDa and 65 kDa) which are normally expressed by INS-1 cells that made it difficult to interpret the data [Appendix Figure 8.11 A]. We therefore quantified total protein expression by adding the signal intensity of both the protein isoforms and adding detached cells to adhered cells. Figure 4.15 B shows the quantification of 3 independent replicates pooled together. There was about 27% knockdown 24 hours after the second siRNA treatment and 85% knockdown 48 hours after. However these results were highly variable as reflected by the large error bars. On average, each independent replicate showed about ~30% knockdown at most. Nonetheless, phenotypic effects on adhesion were consistently observed in all 3 independent replicates. Although there was no difference between knockdowns and controls, it is interesting however, that detached cells specifically expressed the 65 kDa band, while in adhered cells, both bands are detected however the 56 kDa band is more strongly expressed [Figure 4.15

A]. Perhaps this indicates a specific function on adhesion for these isoforms and warrants further investigation including regulation of expression and its effect on β-cell adhesion.

We then asked whether mRNA silencing took place but however remaining proteins were still present and therefore reduced protein expression could not be detected by western blotting. We next attempted to detect knockdown by qPCR, a method more specific to mRNA expression. Since we could detect changes in adhesion after the first knockdown, for this experiment, we used SMARTPOOL SPOCK-3 siRNA and lysed cells for RNA extraction 48 hours after a single knockdown. However, we could only detect 0.69% SPOCK-3 knockdown by mRNA quantification [Figure 4.15 C]. It is worth noting that Hevin knockdown was also attempted to be verified by qPCR but however detection of mRNA was unsuccessful with the primers used.

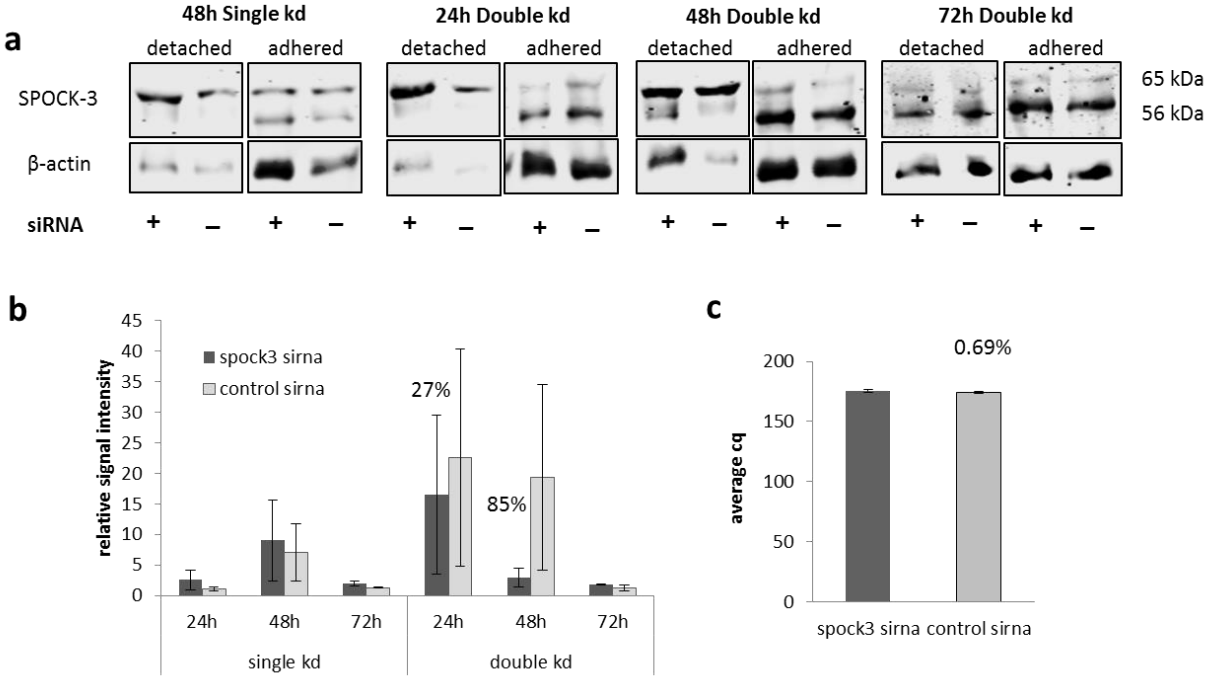


Figure 4.15: SPOCK-3 expression is not silenced by double knockdown using SMARTPOOL siRNA. (a) Cell lysates were subjected to western blotting to detect SPOCK-3 expression following double knockdown. Cells that detached following knockdown were also collected and lysed in parallel with cells that remained adhered. β-actin was used as a loading control. To quantify protein bands, signal intensity of both the 65 and 56 kDa bands were added together since differential expression was observed. In addition, proteins from detached and adhered cells were added together to quantify total protein. (b) Graph showing pooled data from N= 2-6 (2-3 replicates from 2-3 independent experiments) +/- SEM. (c) RNA was extracted following 48 hours of a single knockdown to detect mRNA expression by RT-PCR. Graph showing average cq for SPOCK-3 mRNA from N=3 (3 replicates from 1 experiment). Statistical significance was measured using student's t-test.

4.5.3 Effect of double knockdown using single siRNA on β-cell adhesion

We asked whether the phenotypic effects we observed were the result of SMARTPOOL siRNA and therefore tested the effect of single siRNA on adhesion. We used hevin single siRNA and performed the double knockdown. We observed the same phenotypic effects on adhesion in single siRNA treated cells [Figure 4.16]. It is worth noting that the control siRNA used in all the knockdown experiments were non-targeting SMARTPOOL siRNA, and in these cells, no effect on adhesion was observed indicating an siRNA specific effect.

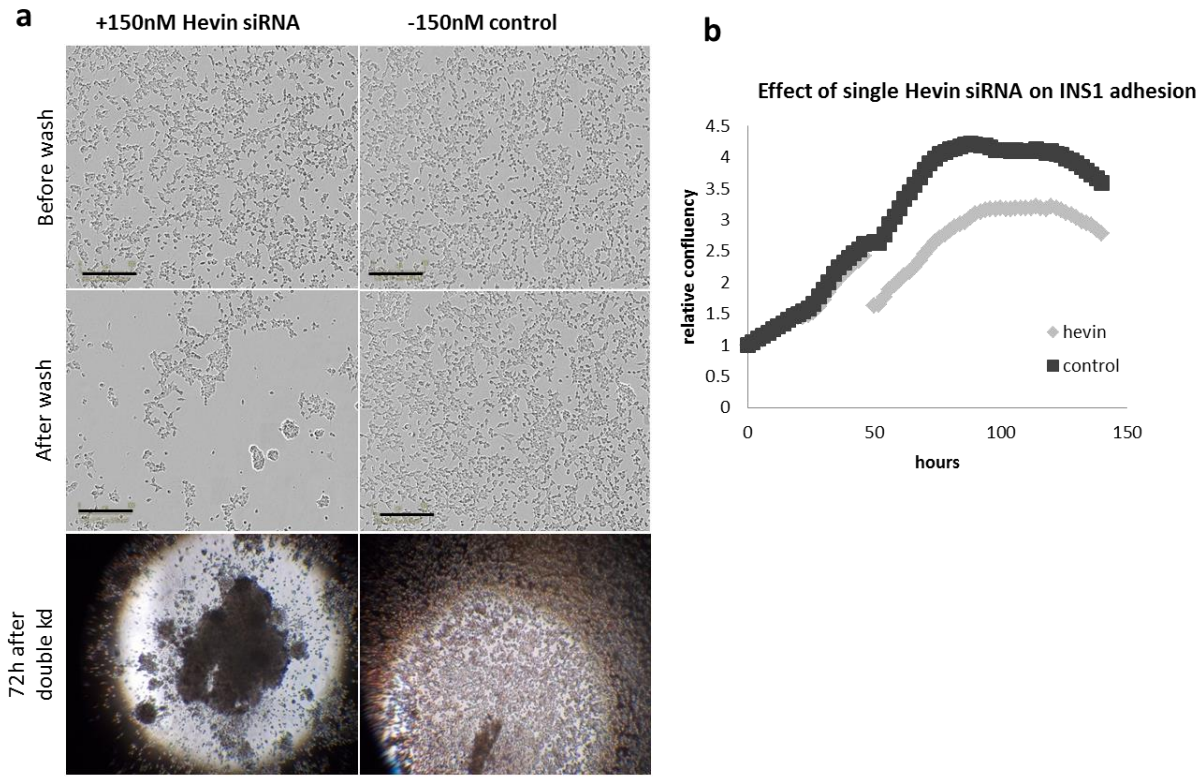


Figure 4.16: Hevin single siRNA also induced β-cell de-adhesion. INS-1 cells ($1x10^4$) were transfected with single siRNA for hevin for 48 hours. Media was thereafter aspirated and replaced with fresh media supplemented with new siRNA. The second knockdown was carried out for another 48-72 hours. Cells were monitored using the IncuCyte Zoom imaging system. Non-targeting rat pooled siRNA was used as a control (a) Representative images showing cells before and after the media was changed after 48 hours of the first knockdown. Scale bar 300 µm at 10X objective. (c) Graph showing average cell confluence over time relative to the first time point. N=3 (3 replicates from 1 experiment).

A summary of SPOCK-3 and hevin siRNA used in this study as well as the parameters tested for knockdown and adhesion are summarised in Appendix Table 8.4 and 8.5. This procedure is a standard routine to achieve knockdown in INS-1 cells performed in our lab, and so this effect on INS-1 adhesion is not a routine technical issue. In addition, SPARC knockdown in PS-

1 cells does not have similar effects on adhesion (Munasinghe, A. unpublished data). Phenotypic effects on adhesion may have been a result of off-target effects. The sequence of the siRNA greatly affects off-target effects as even single pair mismatches can affect siRNA functionality (Saxena *et al.,* 2003). Alternatively, shRNA may be more potent as it is a shorter sequence compared to siRNA. Use of shRNA to silence the SPARC family in β-cells warrants further investigation.

While it is likely that phenotypic effects observed in these studies are the result of off-target effects, it is rather interesting that both siRNA targeting two different transcripts elicit the same effect on adhesion. The sequence of the siRNA used in these studies were checked for compatibility with the mRNA of SPOCK-3 and hevin and indeed, all of the siRNA sequences had 100% complementarity with the mRNA transcript (confirmed through BLAST and Clustal Omega). It is possible that there may be alternative transcripts for which the siRNA was able to silence, both for SPOCK-3 and hevin and therefore why we observed similar phenotypic effects. However out of the isoforms normally expressed in INS-1 cells, there were no consistent obvious changes in either bands following siRNA knockdown that would support this [Figure 4.15 and Appendix Figure 8.11]. Furthermore, the primers used for qPCR were designed to bind generically, and do not target a specific splice variant and therefore should in theory detect multiple variants.

Unfortunately, there are currently no predicted alternative transcripts for the rat SPOCK-3 and hevin transcripts banked in ENSEMBL. Nonetheless, it is likely that there may be unidentified transcripts expressed in rat that the siRNA may have selectively bound to and we were unable to detect at the protein or mRNA level. For example, the antibodies used were specific to the N-terminus, and would not detect a C-terminus variant should the siRNA be specific to this alternative transcript. It has been suggested that novel siRNA design algorithms can be used to design exon-specific RNAi to target individual spliced mRNA isoforms (Celotto and Graveley, 2002; Park *et al*., 2008). Indeed this has been demonstrated in *Drosophila* where specific alternative isoforms were silenced by exon-specific RNAi (Celotto and Gravelely, 2002). Perhaps this is a strategy to investigate silencing the specific isoforms of SPOCK-3 and hevin expressed in rat β-cells and their effect on adhesion. If this is

the case, this may suggest the possibility that SPOCK-3 and hevin are pro-adhesive. However, matricellular proteins in general are known to be anti-adhesive, promoting cell rounding and detachment (Murphy-Ullrich, 2001). We therefore further investigated the role of the SPARC family on β-cell adhesion in Results Chapter 5.

4.6 Chapter Discussion

4.6.1 The role of SPARC in matrix assembly

The extracellular matrix functions to maintain tissue shape and structural support. Indeed we observed that culturing β-cells in the collagen matrix supported the formation of islet-like clusters, a natural morphology not observed in 2D culture. Our data supports other instances where islet cells were cultured in matrices. For example, primary islets grown on a layer of collagen I was shown to grow in monolayers. However when sandwiched between a second layer of collagen I, dissociated cells formed islet-like organoids in which non-β cells arranged at the outer periphery while β-cells arranged in the inner core (Montesano *et al.,*1983). In addition, dispersed embryonic pancreatic progenitors that were cultured in Matrigel formed pancreatic organoids within 7 days. Similarly, cells that expressed PDX1 and SOX9, markers for insulin-producing β-cells, aggregated in the center while epithelial cells remained in the outer layer (Greggio *et al*., 2013). This precise architectural arrangement of β-cells appears to have a functional role in proper islet function. The ECM appears to be an essential component that supports this arrangement, making matrix organisation an important component of the islet microenvironment that establishes cell-cell and cell-matrix relationships.

Our preliminary data has so far shown that SPARC does not affect initial fiber assembly and formation. We added SPARC to the collagen solution before the polymerisation step as it has been suggested previously that SPARC plays a role in the early stages of collagen fibril assembly. SPARC increased the “lag phase” of collagen fibrillogenesis which is the period when fibril precursors form to initiate the growth phase (Guidici *et al*., 2008). However, we did not detect an effect of SPARC on RAFT matrix assembly. It is worth noting that the SPARC used in the above studies is human, while the collagen I is rat tail. However, human and rat SPARC are 92% homologous while human and rat collagen I are also 92% homologous,

therefore the type of species is likely not a contributing factor (verified through Clustal Omega). On the other hand, it is possible that the rSPARC used may not have been compatible or activated to bind to the RAFT collagen I. There is evidence showing that SPARC cleavage and glycosylation affect its binding affinity to collagen. MMP cleavage of SPARC at helix αC results in an increased affinity for collagens I, II, III, IV, and IV (Sasaki *et al*.,1997). Cleavage at this site unmasks helix αA where collagen binding takes place (Sasaki *et al*., 1997). In addition, SPARC with high-mannose structures expressed in bone have higher affinity for collagen I compared to platelet-SPARC which does not contain high-mannose modifications (Kaufmann *et al*., 2004). Our laboratory has shown that the human rSPARC used in our studies showed about 1.5 kDa of N-linked glycosylation after digestion with PNGase-F (in collaboration with Munasinghe, A., unpublished data). Sasaki *et al*. demonstrated about 2 kDa of N-linked high mannose structures in bone-derived SPARC following digestion with endoglycosidase H (Sasaki *et al*., 1997). PNGase-F cleaves all types of N-linked glycosylation including complex glycans while endoglycosidase H is selective only to high mannose N-linked glycosylation (Freeze and Krans, 2008). The true nature of glycosylation in the rSPARC we used in our studies is therefore unknown but may contain different glycan structures that may be a factor in collagen binding. It may be useful for future research to incorporate cells into the matrix, for example stromal cell-derived SPARC may be expressed differently and therefore have a different effect. Furthermore, SPARC binding to collagen is calcium dependent and therefore controlling calcium levels in the media may be relevant.

The most well-known binding site on collagens and other matricellular proteins is the peptide sequence arginine-glycine-aspartic acid (RGD) (Stendahl, 2009). This sequence is detected by integrins which upon binding, activates integrins to transmit the signal into cells through cytoskeletal rearrangement, subsequently transmitting the signal to the nuclear matrix, and ultimately controlling gene expression (Stendahl, 2009; Bissell *et al*., 1982). SPARC binds to several sites on collagen, with two preferred binding sites, one at residues 650-800 on collagen I and to amino acids 100-200 (Wang *et al*., 2005). Interestingly, amino acids 650-800 overlaps with the α1β1 and α2β1 integrin binding site on collagen I and fibronectin (Xu *et al*., 2000; Ingham *et al*., 2002; Wang *et al*., 2005; Martinek *et al.,* 2007). In addition, the SPARC

binding site on collagens II and III is similar to the collagen I domain known to bind to DDR receptors (Guidici *et al.,* 2008, Hohenester *et al.,* 2008). These experiments suggest that SPARC may therefore interfere with integrin- matrix binding. This hypothesis is supported by Yan *et al*. who demonstrated that SPARC-null lens epithelial cells have increased membrane protrusions that extend into the basal lamina composed of collagen IV (Yan *et al.,* 2002). These protrusions are positive for α1β1 integrins which indicates that SPARC may be inhibiting α1β1 integrin- collagen IV binding. In addition, basement membranes in the SPARC-null lens are more disorganized, perhaps as a result of increased cell binding to the matrix (Yan *et al*., 2002). Although it has not yet been shown directly, it has been proposed that SPARC may interfere with receptor binding to collagen, thus regulating collagen fibril organisation and matrix remodelling (Bradshaw *et al*., 2009).

It is possible that SPARC may instead play a role on matrix remodelling and reorganisation by intervening with integrin receptor binding to the matrix. Moreover, the effect of the other members of the SPARC family warrants further investigation. Hevin-null mice have previously been shown to have thinner, more uniform and densely packed collagen fibrils in the dermis, indicating that hevin may affect collagen organisation of the 3D matrix (Sullivan *et al.,* 2006). SPOCK-3 and FSTL-1 have previously been shown to regulate MMP activation and therefore may have a significant role on long term matrix remodelling of the 3D culture (Nakada *et al.,* 2001, Nakada *et al*., 2003; Chan *et al.,* 2009). Further study is needed to investigate whether the wider SPARC family may have different effects on collagen assembly and remodelling.

4.6.2 Intracellular function of SPARC in collagen processing

Although SPARC is expected to act extracellularly in the matrix, there is increasing discussion that SPARC may influence matrix assembly intracellularly. SPARC has been proposed to act as a molecular chaperone that stabilizes procollagen (Martinek *et al*., 2006). Molecular chaperones are proteins that associate with other proteins to protect against unfolding and aggregation during stressed conditions. Other known collagen chaperones include: HSP47 also known as colligin, BiP/ (glucose-related protein 78), P4H (prolyl 4-hydroxylase), PDI (programmed cell death protein 1), and peptidylproline cis-trans isomerase (Martinek *et al*., 2006). HSP47 is well known to stabilize the triple helix of collagen in the endoplastic

reticulum [ER] (Nagata *et al*., 1996; Tasab *et al*., 2000; Matsuoka i., 2004, Martinek *et al.,* 2006). SPARC is proposed to similarly act as a collagen chaperone that stabilizes collagen in the ER (Martinek *et al.,* 2006). Although there is still much to uncover, there is growing evidence that SPARC may be an intracellular collagen chaperone. For example, HSP47 and SPARC are both activated by heat shock and environmental stresses (Kudo *et al.,* 1994). In addition, heat shock response genes are found to be located upstream of HSP47 and SPARC (Sauk *et al*.,1991). HSP47 and SPARC are both co-expressed in tissues undergoing morphogenesis during embryonic development, a period of high collagen synthesis, and therefore may be acting as co-chaperones in the ER [endoplasmic reticulum] (Fisher *et al*., 1987). This may perhaps explain why SPARC-null mice express smaller and more uniform collagen, in which there is still normal expression of HSP47. SPARC may therefore also have a bigger role after HSP47 release of collagen in the ER, the events of transport and maturation in secretory vesicles when procollagen aggregates (Martinek *et al*., 2006). The events of secretory vesicle transport involve microtubules. Interestingly, SPARC has been shown to be expressed in tubulin rich structures during development in the neural tube and microtubular arrays in cilia (Damjanovski *et al.*, 1998; Huynh *et al*., 2004). Perhaps more convincingly, chaperone activity of SPARC was demonstrated when SPARC was shown to act with αB crystalin, a heat shock molecular chaperone, to promote stabilization and folding of the lens ECM (Emerson *et al.,* 2006). In addition, SPARC inhibited the heat aggregation of ADH, a chaperone target protein (Emerson *et al*., 2006). Further investigation is needed to support this novel intracellular role of SPARC in collagen assembly. For example, mapping the localisation of SPARC in relation to procollagens intracellularly, and showing a direct effect of SPARC on collagen degradation in the ER. Furthermore, intracellular matrix processing has not yet been explored for the other members of the SPARC family. We have shown strong cytoplasmic and nuclear staining for hevin, SPOCK and SMOC proteins in islets and therefore warrants further study for intracellular functions in matrix processing.

4.6.3 Potential mechanisms of the SPARC family in islet-matrix interactions

In summary, our data shows that SPARC and SPOCK-3 inhibit growth while SPARC and hevin may inhibit proliferation in the 3D collagen matrix. We describe for the first time that SPARC related proteins have inhibiting roles on β-cells in a 3D matrix. Although growing evidence

has shown that the SPARC family regulates matricellular interactions and cell behaviour, the underlying mechanism of how the SPARC family regulates these interactions is not well understood. To date, no specific receptors for SPARC and its related proteins have been discovered (Arnold and Brekken, 2009). In addition, integrin and growth factor signalling pathways greatly overlap and studies have shown that SPARC regulates cell responses to PDGF, VEGF, bFGF, TGF-β, IGF-1, HGF (Hasselaar and Sage, 1992; Raines *et al*., 1992; Kupprion *et al.,* 1999; Motamed *et al*., 2003; Schiemann *et al.,* 2003; Chlenski *et al.,* 2007; Ryall *et al*., 2014). However, the mechanism of whether SPARC directly or indirectly interacts with these growth factors is currently unclear. Additionally, little is known on growth factor activity of hevin, SPOCK-3 and other related SPARC proteins. However, since we know that SPARC binds directly to integrins, the collagens and growth factors, it is possible that SPARC proteins may be the link that either facilitates or interferes with matrix and growth factor binding with their receptors. SPARC may determine the activating threshold of how extracellular signalling elicits cells responses. SPARC has been proposed as an extracellular "rheostat" [Figure 4.17] (Arnold and Brekken, 2009). SPARC may enhance the binding of the matrix to integrins and likewise growth factors to receptors, therefore amplifying the signal

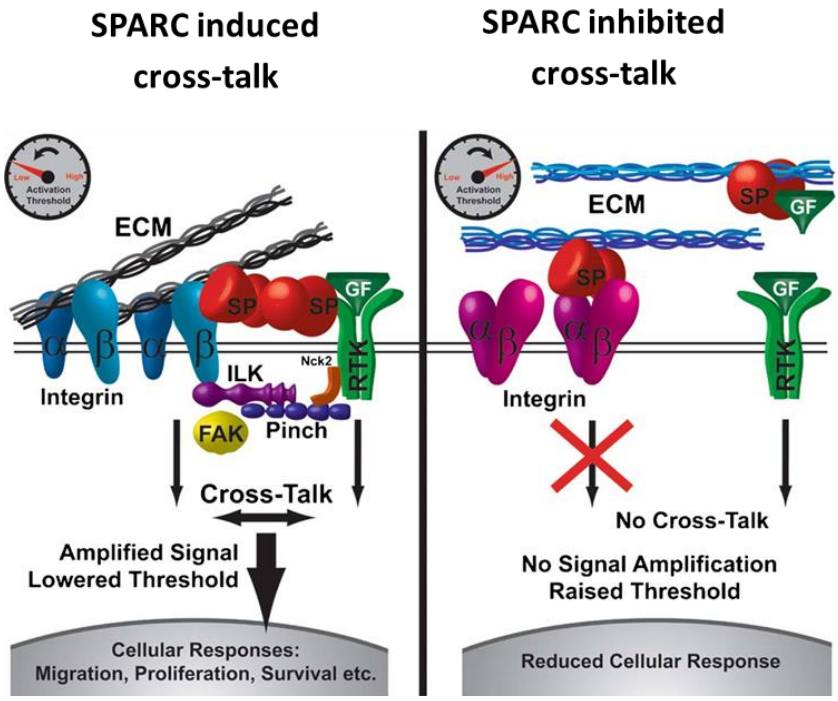


Figure 4.17: Potential mechanism of the SPARC family. SPARC may act as an activation "rheostat" that determines the threshold at which cells respond to extracellular signals. SPARC may facilitate matrix and growth factor binding to their receptors thereby amplifying the signal through integrin-growth factor cross-talk and lowering the activation threshold needed to elicit immediate cell responses to migration, proliferation, and survival. On the other hand, SPARC may also block matrix and growth factor binding to their receptors thereby increasing the activation threshold resulting in reduced cellular responses (Image from Arnold and Brekken, 2009).

resulting in increased growth, proliferation and survival. In addition, coupling of integrin and growth factor receptor activation may lower the threshold signal needed to elicit cell response. On the other hand, it may also do the opposite by inhibiting integrin-growth factor crosstalk thereby raising the signal threshold needed and therefore reducing cellular response. Perhaps SPARC, hevin, and SPOCK-3 inhibit β-cell growth and proliferation by regulating β-cell attachment or integrin binding to the 3D matrix. Additionally, they may perhaps inhibit growth signals by inhibiting growth factor binding with their receptors. This model also agrees with the evidence that SPARC matricellular proteins are anti-adhesive, thereby regulating anchorage-dependent proliferation of cells. However as our results from hevin and SPOCK-3 siRNA knockdown in β-cells have suggested otherwise, we therefore investigated the role of the SPARC family on β-cell adhesion by treatment of recombinant proteins.

5. Results: The effect of the SPARC family on β cell adhesion, actin remodelling and insulin secretion

5.1 Stages of cell adhesion- a reversible process

The matrix relays signals of growth, migration, and proliferation to cells. Through cell surface receptors, outside signals are translated to internal signalling that overall affect cell behaviour. The process of cell adhesion is a reversible process and takes places in three stages (Murphy Ullrich, 2001). The first stage is attachment, the initial interaction of cells with a substrate such as matricellular components like collagen or plastic [Figure 5.1]. Weak adhesion is defined as the state in which cells have weak attachments to the substrate and are still rounded. Binding to the substrate induces integrin activation and clustering which in turn triggers the cells to spread through remodelling of actin fibers, increasing surface contact with the substrate. This is called intermediate adhesion, the state at which cells are attached to the matrix, and although fully spread, do not display stress fibers. Strong adhesion develops after further cytoskeletal reorganisation resulting in the formation of focal adhesions and stress fibers. This stage is observed in non-motile, differentiating or quiescent cells. De-adhesion is the process by which cells transition from strong adhesions to intermediate adhesions, during which focal adhesions and the actin cytoskeleton undergoes remodelling, thus breaking down stress fibers. This stage can be observed in cells that are migrating, responding to injury, and wound healing. Transition to weak adhesions on the other hand is observed only during cytokinesis or apoptosis (Murphy Ullrich, 2001). Matricellular proteins are known to have anti-adhesive effects on cells. We therefore aimed to design experiments that would investigate the role of the SPARC family at different stages of β-cell adhesion [Figure 5.1].

5.2 Proteins of the SPARC family promote intermediate adhesion of β-cells

5.2.1 SPOCK-3 and hevin inhibit β-cell spreading and promote cell rounding

In order to investigate the role of SPARC proteins on β-cell attachment, INS-1 cells were treated with 5 μg/mL of recombinant SPARC, hevin or SPOCK-3 while in suspension before attachment. This concentration was used as SPOCK-1 was previously shown to inhibit

attachment of Neuro-2a cells (Marr and Edgell, 2003). Cells were plated in either serum free, low serum (0.5% FBS) or complete media (10% FBS). Plates were then placed in the Incucyte Zoom live imaging system to monitor cell attachment and spreading over a period of 24 hours. Rounding was quantified by measuring eccentricity. A score of 0 indicates rounded cells while scores closer to 1 indicate spread cells. Area was also quantified to measure spreading.

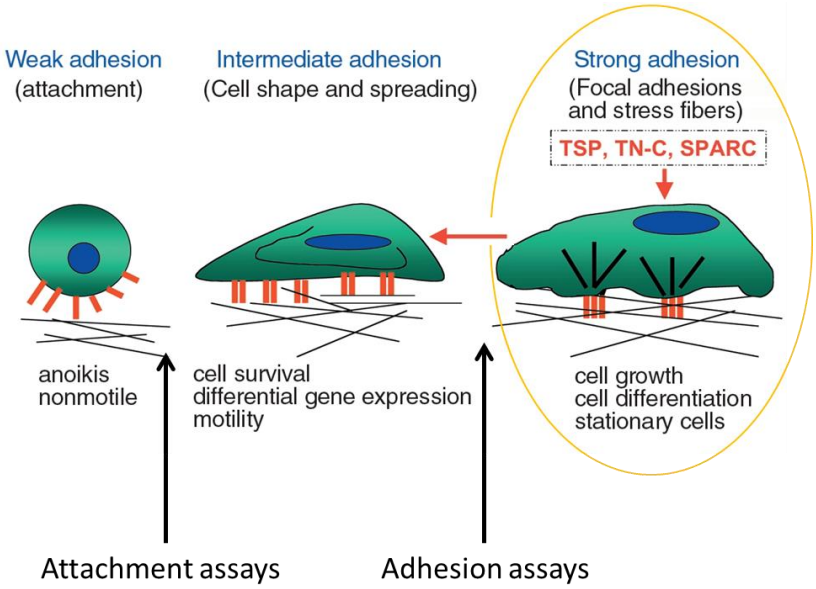


Figure 5.13: The stages of cell adhesion. Cells undergo different stages of adhesion. Weak adhesion takes place during the initial attachment stage of cells in which cells are attached to the substrate but however are still rounded. Intermediate adhesion is the state where cells have spread however do not have stress fibers. Strong adhesion takes place when cells have formed strong focal adhesions and stress fibers that further strengthen their attachment to the substrate. Cell adhesion is a dynamic and reversible process. Matricellular proteins like the SPARC family promote de-adhesion from strong to weaker adhesions (Image taken and modified from Murphy Ullrich, 2001). We have designed experiments to investigate the role of the SPARC family on β-cell attachment and de-adhesion.

In serum free conditions, control untreated cells have started to spread 3 hours after plating, showing short arm-like extensions [Figure 5.2 A]. SPARC-treated cells have also started to spread [Figure 5.2 A]. SPOCK-3 and hevin-treated cells have not yet spread at this point and were significantly more rounded (N= 9; p=0.0002, p=0.0001 respectively) compared to the control and SPARC-treated cells [Figure 5.2 C]. After 24 hours, untreated cells have completely spread showing full star-like conformation [Figure 5.2 B]. SPARC-treated cells similarly have completely spread. However, SPOCK-3 and hevin completely inhibited

spreading of cells with a rounding score significantly lower than SPARC and untreated cells ($p<0.0001$) [Figure 5.2 C]. Interestingly, although SPARC did not have an effect on initial spreading or cell rounding, Figure 5.2 D shows that SPARC significantly inhibited spreading of β-cells after 6 hours ($p=0.004$) of treatment up to 24 hours ($p=0.03$).

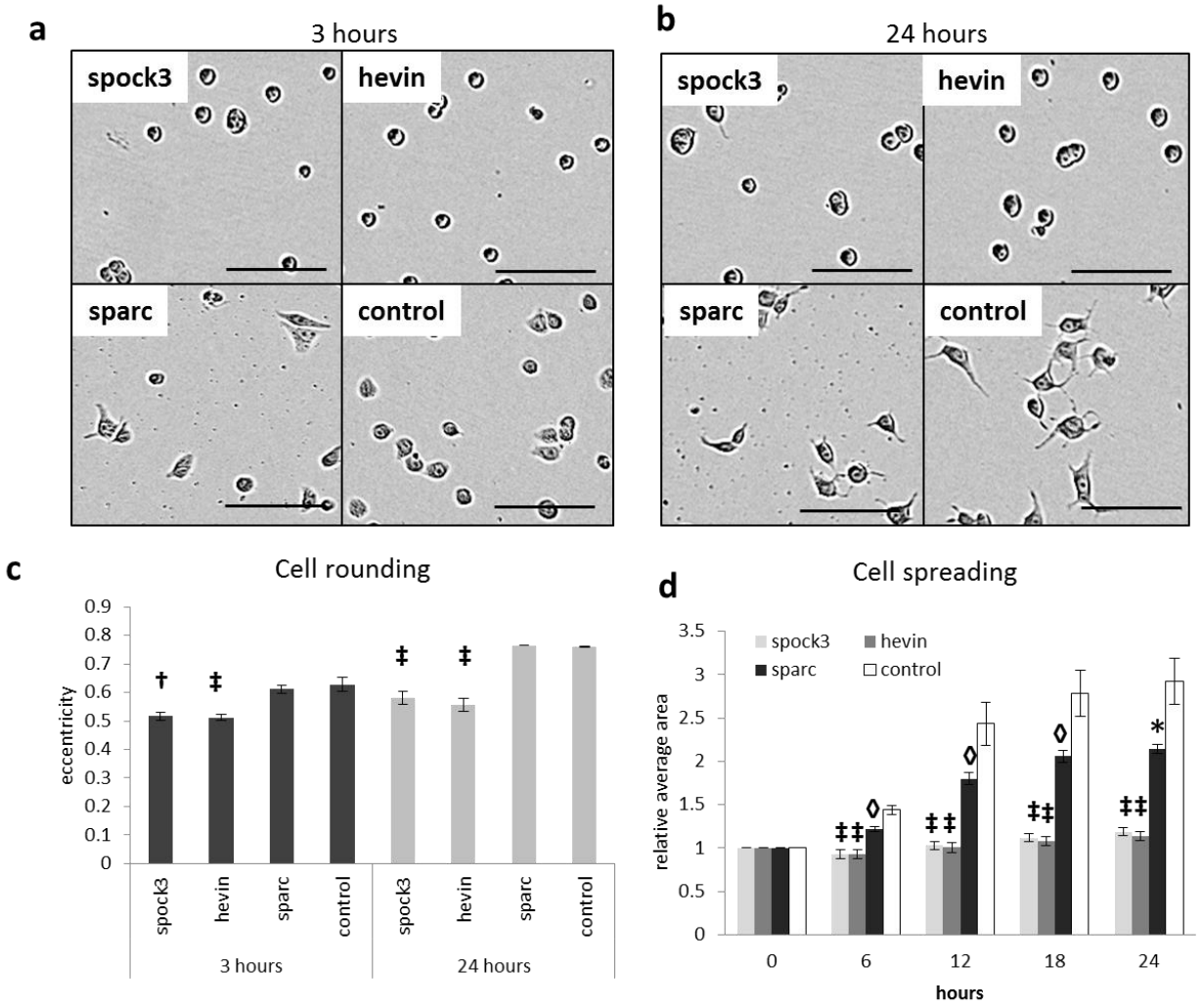


Figure 5.2: SPOCK-3 and hevin promote β-cell rounding while SPARC inhibits spreading in serum free conditions. INS-1 cells ($1x10^4$ cells/well) were seeded in serum free media supplemented with or without 5µg/mL of SPARC, hevin or SPOCK-3. Attachment and spreading of cells were monitored using the IncuCyte ZOOM live cell imaging system for a period of 24 hours. Representative images (a) 3 hours after plating and (b) 24 hours at 10X. Scale bar 300 µm. (c) Eccentricity and (d) area was quantified using the IncuCyte to measure cell attachment and spreading. Graphs showing pooled data from N=9 (3 replicates from 3 independent replicates) +/- SEM. Statistical significance was measured using one-way ANOVA. [* $p< 0.05$, ◊ $p<0.01$, † $p<0.001$, ‡ $p<0.0001$].

Under low serum conditions, untreated cells have started to spread within the first 3 hours [Figure 5.3 A]. SPARC did not affect initial spreading but however inhibited spreading of cells significantly between 6-12 hours of treatment (p=0.003, p=0.023 respectively) [Figure 5.3 C-D]. This suggests that SPARC has a specific role on regulating the spreading of β-cells. SPOCK-3 and hevin on the other hand significantly inhibited initial spreading similar to serum free conditions [Figure 5.3 A]. Cells were significantly more rounded after treatment with SPOCK-3 and hevin compared to untreated cells (p=0.0094, p<0.0001 respectively) [Figure 5.3 C]. In addition, hevin significantly promoted rounding more than SPOCK-3 (p=0.027). After 24 hours, cells treated with SPOCK-3 and hevin had shorter extensions compared to controls

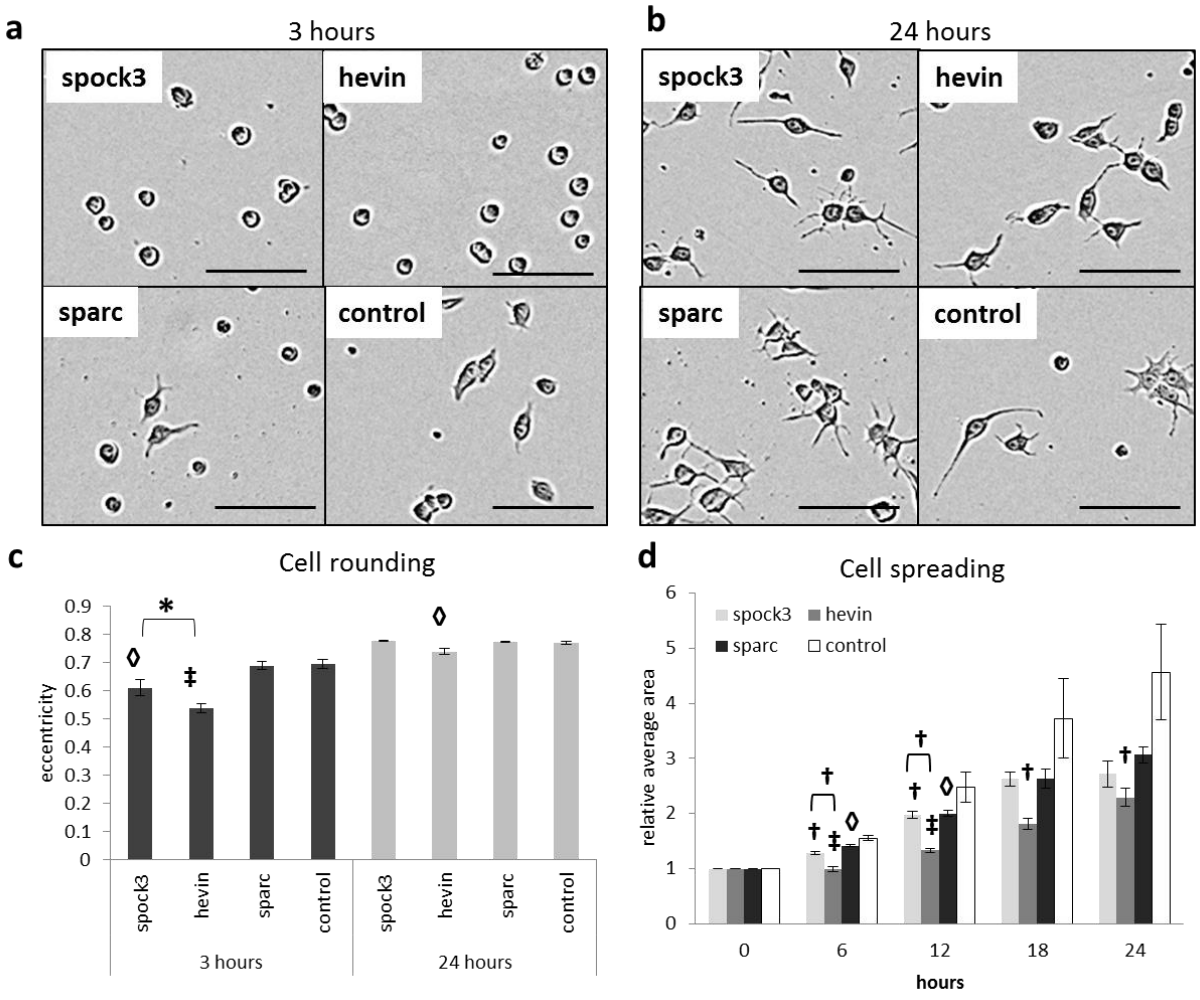


Figure 5.3: SPOCK-3 and hevin promote β-cell rounding and inhibit spreading while SPARC only inhibits spreading in low serum conditions. INS-1 cells ($1x10^4$ cells/well) were seeded in low serum media (0.5% FBS) supplemented with or without 5µg/mL of SPARC, hevin or SPOCK-3. Attachment and spreading of cells were monitored using the IncuCyte ZOOM live cell imaging system for a period of 24 hours. Representative images (a) 3 hours after plating and (b) 24 hours at 10X. Scale bar 300 µm. (c) Eccentricity and (d) area was quantified using the IncuCyte to measure cell attachment and spreading. Graphs showing pooled data from N=9 (3 replicates from 3 independent replicates) +/- SEM. Statistical significance was measured using one-way ANOVA. [* p< 0.05, ◊ p<0.01, † p<0.001, ‡ p<0.0001].

[Figure 5.3 B] and spreading was significantly inhibited by hevin (p=0.002) although this is likely as a result of delayed initial spreading [Figure 5.3 D].

In contrast, in complete media, the SPARC family did not have any effect on initial spreading during the first 3 hours of culture [Figure 5.4 A & C]. After 24 hours, the SPARC family similarly did not affect rounding or spreading [Figure 5.4 B-D] indicating that the SPARC family regulation of β-cell rounding and spreading is highly influenced by components in serum. Interestingly, serum dependence of SPARC on cell adhesion was not observed in pancreatic cancer cells (Munasinghe, A. & Hill, N., unpublished data). It is worth noting that it

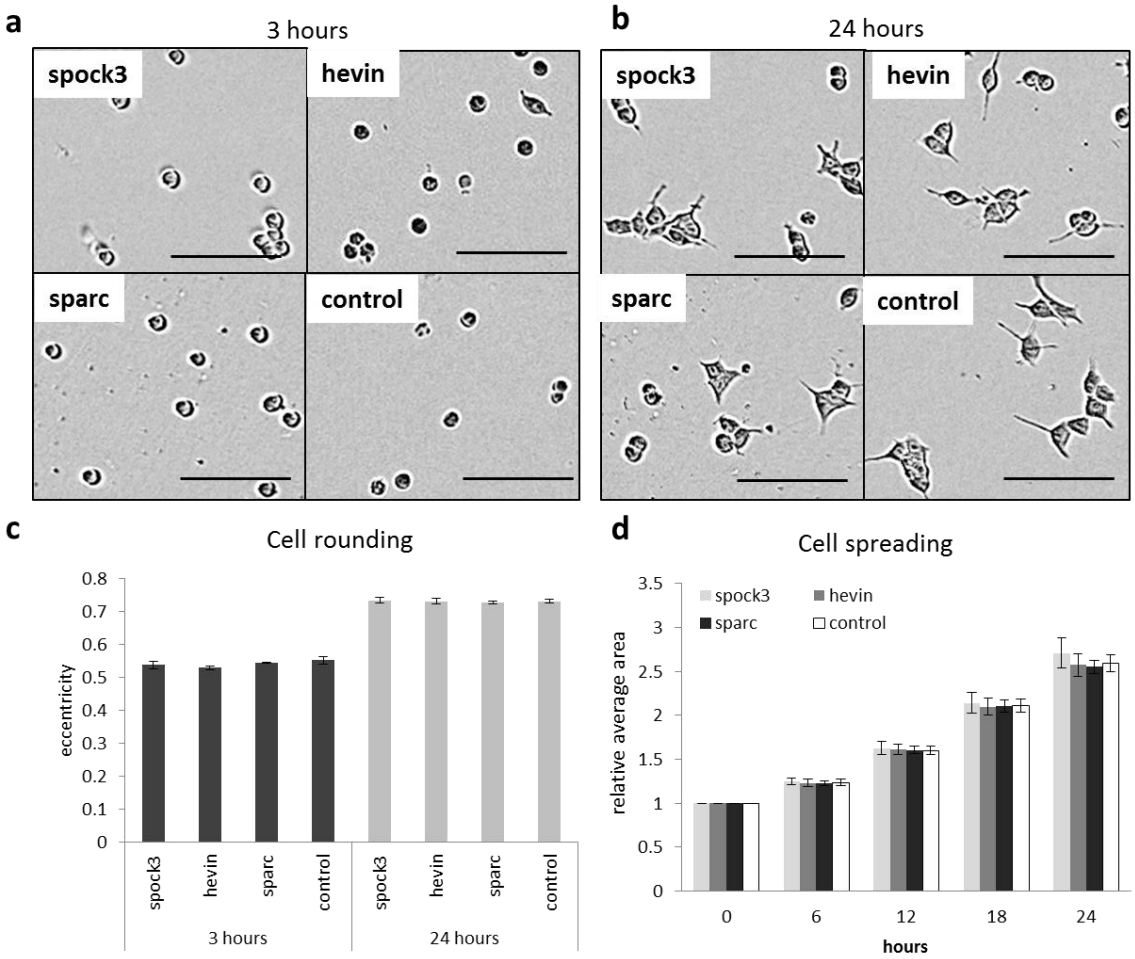


Figure 5.4: The SPARC family had no effect on β-cell rounding and spreading under complete media conditions. INS-1 cells ($1x10^4$ cells/well) were seeded in complete media (10% FBS) supplemented with or without 5µg/mL of SPARC, hevin or SPOCK-3. Attachment and spreading of cells were monitored using the IncuCyte ZOOM live cell imaging system for a period of 24 hours. Representative images (a) 3 hours after plating and (b) 24 hours at 10X. Scale bar 300 µm. (c) Eccentricity and (d) area was quantified using the IncuCyte to measure cell attachment and spreading. Graphs showing pooled data from N=6 (3 replicates from 2 independent experiments) +/- SEM. Statistical significance was measured using one-way ANOVA. [* p< 0.05, ◊ p<0.01, † p<0.001, ‡ p<0.0001].

is unclear whether the rounded cells are attached to the plates as the plates were not washed after treatment. The effect on initial attachment is therefore not known but however the effect is observed on rounding and spreading.

There are many components in the serum that influence cell attachment and adhesion. It is likely that there may be other matricellular components in serum that interfere with the interaction of SPARC with cells and or with plastic. Alternatively, starving and serum free conditions create a stressed environment that may be causing β-cells to respond differently to SPARC proteins compared to normal unstressed conditions (Sauk *et al*., 1991). It has been shown that lens epithelial cells increase the expression of SPARC during serum starved or stressed conditions (Kantorow *et al*., 2000). Interestingly, SPARC-β1 integrin complexes were detected only in cells that were starved or stressed (Weaver *et al*., 2008). In addition, data from our lab has shown that SPARC may either inhibit or promote pancreatic cancer cell growth dependent on serum conditions (Munasinghe, A., unpublished data). Perhaps SPARC family interaction with integrins under stress is a mechanism to detect unfavourable conditions, thereby regulating adhesion, spreading, and growth of cells.

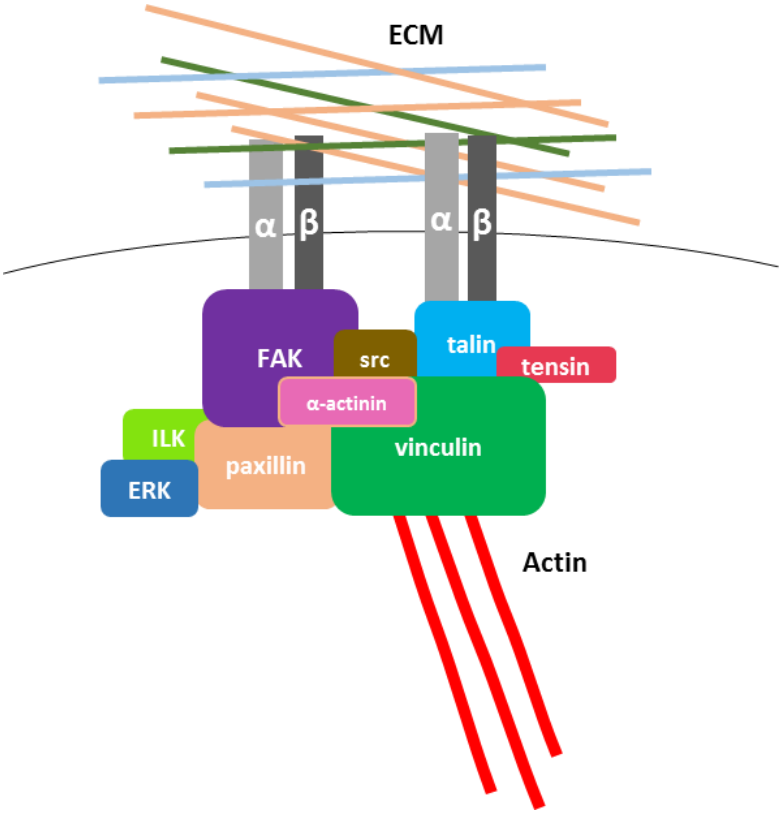
5.2.2 Hevin and SPOCK-3 inhibit focal adhesion formation

We aimed to investigate the effect of the SPARC family on β-cell de-adhesion, specifically the transition from a strong adhesion to intermediate or weak adhesion. In order to investigate this, β-cells were first adhered and then treated with SPARC, hevin, or SPOCK-3. We investigated how these proteins affect the detachment of previously adhered cells. After 24 hours of incubation, media was aspirated and cells were washed with PBS. Images were acquired before and after washes to analyse the effect of the SPARC proteins on de-adhesion. However, after several attempts for this assay, it was difficult to acquire consistent results as INS-1 cells easily detached from the plastic after washing. The percentage of cells that detached varied between replicates for example, control untreated cells also variably detached from <1% to more than 70% [Appendix Figure 8.12]. In addition, unforeseen changes in plastic ware in the lab may have also affected these results as cells adhere differently to different types of plastic. We therefore instead investigated focal adhesion

formation as a means to analyse de-adhesion from strong adhesion to intermediate adhesion.

Focal adhesions are transmembrane complexes comprised of proteins that anchor cells to the matrix and connects the matrix to the actin cytoskeleton. Upon binding to the matrix, integrins cluster together and activate the formation of focal adhesions. The focal adhesion complex as shown in Figure 5.5, consists of focal adhesion kinase (FAK), paxillin, vinculin, src, α-actinin, talin and tensin which together creates the tether where actin filaments attach (Humphries *et al.,* 2007; Campbell *et al*., 2008; Deakin and Turner 2008). Vinculin is the adaptor protein that is crucial for the focal adhesions to form (Humphries *et al*., 2007). Activation of vinculin allows direct interaction with talin which induces integrin clustering, paxillin recruitment, and subsequently recruitment of other proteins for focal adhesion enlargement, allowing strong adhesions and stress fibers to form (Humphries *et al*., 2007). The active conformation of vinculin allows it to bind to talin and incorporate into the focal complexes while the inactive conformation constraints vinculin causing it to be localised in the cytoplasm (Bakolitsa *et al*., 2004; Chen *et al*., 2005).

We therefore investigated whether the SPARC family of proteins affect vinculin incorporation into focal adhesions at the cell surface to detect de-adhesion of β-cells from strong to an intermediate adhesion state. These experiments were designed similarly to the de-adhesion assays described above however cells were plated in chamber slides (Nunc Lab-Tek Permanox C7182) for actin and vinculin staining. We used ImageJ analysis software to quantify focal adhesion area. Vinculin area was measured by setting the threshold so that only strong staining near the cell surface would be quantified while diffuse cytoplasmic staining would be excluded in the analysis [Figure 5.6 A]. Cell area was measured using the overall actin area [Figure 5.6 B]. Focal adhesion area was thereby quantified by dividing vinculin area to actin area.



In control untreated cells, qualitative observation showed that strong vinculin staining was observed near the surface, with overall stronger vinculin expression compared to treated cells [Figure 5.7 A]. SPARC-treated cells expressed vinculin at the surface, but however decreased focal adhesion formation by about 15% compared to the control, although this was not significant [Figure 5.6 A & B]. In contrast, cells treated with hevin and SPOCK-3 displayed diffuse vinculin staining in the cytoplasm and overall less vinculin expression compared to the untreated controls [Figure 5.7 A & B]. Hevin and SPOCK-3 significantly decreased focal adhesion area by 48-50% respectively ($p<0.004$, $p<0.002$) [Figure 5.7 C]. These experiments therefore show that the SPARC family of proteins inhibit vinculin incorporation into focal complexes thereby inhibiting focal adhesion formation. Actin organisation or expression on the other hand, was not different between treated and untreated cells. We further investigated the effect of the SPARC, hevin, and SPOCK-3 on actin organisation in glucose stimulated conditions. It is worth noting that the vinculin staining we observed is specific to INS-1 β-cells in contrast to classic focal adhesion staining observed in adherent cells such as fibroblasts and endothelial cells (Humphries *et al*., 2007).

Overall, SPOCK-3 and hevin inhibited β-cell attachment, spreading and focal adhesion formation. Our data supports previous studies in which related SPARC proteins have been shown to have anti-adhesive properties in other cell types. For example, SPOCK-1 inhibited the attachment of neuro-2a neuroblastoma cells (Marr and Edgel, 2003) while SPOCK-2 prevented spreading of neural cells (Schnepp *et al*., 2005). In addition, anti-adhesiveness is common to the wider matricellular family such as TSP-1 and TN-C which were shown to promote focal adhesion disassembly and loss of stress fibers (Chung *et al*., 1996; Greenwood *et al.,* 1998).

Furthermore, results from our 3D studies suggest that SPOCK-3 regulates growth while hevin may play a role in proliferation. This correlates with inhibiting roles on adhesion in 2D. The counter adhesive activity of hevin, and SPOCK-3 is consistent with the hypothesis that the SPARC family may interfere with cell-ECM binding therefore inhibiting growth and proliferation, i.e. anchorage-dependent growth in 3D. This interference on matrix binding may influence the activation of integrins and focal adhesions consequently regulating growth signalling. SPOCK-3 and hevin may therefore be extracellular regulators of β-cell growth and proliferation potentially by influencing matrix binding.

SPARC on the other hand, did not affect attachment or focal adhesion formation of β-cells but however inhibited their spreading. Our data contradicts previous studies in which SPARC has been shown to have both anti-attachment and anti-spreading properties in other cell types such as in spread fibroblasts and endothelial cells and additionally inhibited focal adhesion remodelling in spread endothelial cells (Sage *et al*., 1989; Lane and Sage, 1990; Murphy Ullrich *et al*., 1995). It does however, agree with our 3D studies in which SPARC inhibited growth. Perhaps SPARC regulation of β-cell adhesion and growth is more complex and involves different pathways. For example, SPARC has been shown to inhibit proliferation of endothelial cells, smooth muscle cells, and fibroblasts but however changes in cell shape or spreading was not observed (Funk and Sage, 1991; Raines *et al*., 1992; Motamed and Sage, 1998). It has been suggested that SPARC may affect growth and proliferation independently of attachment and adhesion through different signalling pathways. SPARC inhibition of cell spreading and focal adhesion assembly has been shown to be through tyrosine-kinase

pathways while inhibition of proliferation is through G protein coupled receptors (Motamed and Sage, 1998).

In summary, our data suggests that SPOCK-3 and hevin function to regulate the transition from weak adhesion to intermediate adhesion. Furthermore, both proteins inhibited focal adhesion formation while cells remained spread and attached [Figure 5.8, Table 5.1]. This suggests that SPOCK-3 and hevin also promotes strong to intermediate de-adhesion of β-cells. We show for the first time in β-cells, that SPOCK-3 and hevin regulates adhesion by inhibiting attachment, spreading and focal adhesion remodelling. In contrast, SPARC plays a specific role in regulating β-cell spreading. We describe for the first time, specific functions for SPARC, hevin and SPOCK-3 in different stages of β-cell adhesion.

Interestingly, focal adhesion remodelling is crucial in the regulation of glucose-stimulated insulin secretion (GSIS). Upon glucose stimulation, β- cells partially spread and remodel the actin cytoskeleton to allow transport of insulin granules to the plasma membrane. We therefore investigated whether the SPARC family regulates glucose-stimulated insulin secretion in β cells.

5.3 Actin-regulated insulin secretion

The cytoskeleton is known to regulate cell shape, motility, cell division, and adhesion. Furthermore, actin is recognised as a key regulator of insulin granule trafficking and plasma membrane docking. This step is considered the rate-limiting step in insulin secretion, particularly in the second phase of secretion whereby the actin network acts as a barrier blocking granule transport and docking (Straub and Sharp, 2002; Wang and Thurmond, 2009; Kalwat and Thurmond, 2013). Upon glucose stimulation, a series of signalling cascades trigger the release of insulin [Figure 5.9]. Glucose stimulation triggers the increase of intracellular ATP which causes K-ATP channels to close, depolarising cells. This voltage change in turn opens Ca+2 channels to open. The increase in intracellular Ca+2 then induces the signalling for insulin release (Straub and Sharp, 2002; Wang and Thurmond 2009).

Insulin secretion occurs in two phases [Figure 5.10 A]; the first phase of insulin secretion takes place with the release of the readily releasable pool of granules that are “pre-docked” at the plasma membrane and “primed” for immediate release (Wang and Thurmond, 2009; Straub and Sharp, 2002; Kalwat and Thurmond, 2013). This phase peaks within the first 5 minutes of stimulation, but however gets rapidly depleted (Straub and Sharp, 2002; Wang and Thurmond, 2009). The second phase of secretion engages after prolonged glucose stimulation wherein β-cells use the reserved pool of insulin granules stored in the cytoplasm. Actin remodelling must take place to reorganise the fibrous web blocking granules from reaching the plasma membrane. Furthermore, actin fibers themselves also mediate the transport of granules. During the second phase, this web of actin disassembles and opens

the way for the reserve pool of granules to dock to the plasma membrane for exocytosis. Underneath the plasma membrane is a ring of cortical actin filaments that additionally acts as a barrier and regulates granule docking. Granule fusion to the plasma membrane is mediated by SNARE proteins (soluble N-ethylmaleimide attachment protein receptors). V-SNAREs (i.e. VAMP-2) on the surface of granules must bind and interact with their target T-SNAREs (syntaxin, SNAP-25) at the plasma membrane to allow exocytosis [Figure 5.10 B] (Kalwat and Thurmond, 2013; Wang and Thurmond, 2009). This process is also regulated by cortical actin which blocks T-SNARE interaction with V-SNAREs at basal conditions. Upon glucose stimulation, the cortical actin disassembles and allows V and T snares to bind for successful granule docking (Thurmond *et al.,* 2003; Wang and Thurmond, 2009; Kalwat and Thurmond, 2013). This second phase of release usually peaks 30 minutes after stimulation and plateaus thereafter (Straub and Sharp, 2002) although can be sustained upon persistent elevated blood glucose (Curry *et al.,* 1968; Henquin *et al*., 2002; Wang and Thurmond, 2009). It can also only be stimulated by glucose compared to other secretagogues such as potassium (Gembal *et al.*, 1992; Straub and Sharp, 2002; Henquin, 2009). This highly regulated second phase is responsible for 99% of insulin release, occurring at a rate of 5-40 granules per minute compared to less than 1% immediately released by the first phase (Barg *et al*., 2002, Wang and Thurmond, 2009). Actin remodelling is a key regulator of the second phase of insulin release. We have shown that the SPARC family regulates attachment and adhesion of β-cells thereby perhaps regulating cell shape, actin remodelling and insulin secretion. We therefore investigated SPARC family regulation of glucose-stimulated actin reorganization and insulin secretion.

5.4 Glucose-dependent SPARC family regulation of actin organisation

5.4.1 Validation of glucose stimulation on the actin network

We first validated glucose stimulation and its effect on the actin network. INS-1 and MIN-6 β-cells were adhered on chamber slides and then starved for 2 hours with 2 mM glucose followed by stimulation with 20 mM glucose or 5 mM KCl for 30 minutes. KCl was used as a control for stimulation. KCl treatment results in the closure of K-ATP channels and therefore induces opening of Ca+2 channels for insulin release. Cells were then fixed and stained for

actin. Images were captured using confocal microscopy at relatively similar settings for non-biased imaging. We observed that actin networks primarily became thinner upon glucose stimulation of INS-1 and MIN-6 cells compared to their unstimulated controls [Figure 5.11].

Stress fibers were less prominent and cortical actin less pronounced. KCl stimulation similarly induced actin remodelling in INS-1 and MIN-6 cells although not as extensively as glucose. This supports previous studies showing that actin remodelling during the second phase of stimulation is specifically glucose induced (Gembal *et al.,* 1992)

5.4.2 SPARC, hevin, and SPOCK-3 regulate glucose-stimulated actin remodelling

In order to investigate the effect of SPARC proteins on glucose-stimulated actin remodelling, we pre-treated adhered INS-1 cells with SPARC, hevin or SPOCK-3 for 24 hours. The cells were then starved and stimulated with 20 mM of glucose as previously described in section 5.4.1. Qualitative observation showed that under basal conditions, actin fibers of β-cells were thick and well-organised [Figure 5.12]. In cells pre-treated with SPARC, hevin or SPOCK-3, actin organisation and expression was relatively unchanged. However upon glucose stimulation, SPARC pre-treatment drastically decreased cortical actin. Actin networks were thinner compared to untreated glucose-stimulated cells indicating that SPARC, hevin and SPOCK-3 specifically regulates glucose-stimulated actin reorganisation of β-cells. These proteins may therefore play a role in regulating signalling pathways that control glucose-stimulated actin remodelling.

5.5 Glucose-dependent SPARC family regulation of insulin secretion

5.5.1 Validation of glucose stimulated insulin secretion

We next tested insulin secretion after 10 and 30 minutes of stimulation in INS-1 and MIN-6 β-cells using an ultra-sensitive ELISA assay. Figure 5.13 A shows that insulin secretion was not induced in INS-1 cells after 10 minutes of glucose or KCl stimulation. However, KCl induced insulin secretion in MIN-6 cells. On the other hand, after 30 minutes of stimulation, glucose and KCl extensively induced increased insulin secretion of INS-1 cells [Figure 5.13 B]. In MIN-6 cells KCl similarly strongly induced secretion while glucose only marginally increased insulin secretion. INS-1 cells appear to be more responsive to glucose stimulation than MIN-6 cells,

secreting 3 fold more insulin compared to the respective controls. We then proceeded to test insulin secretion of INS-1 cells after 30 minutes of glucose stimulation.

5.5.2 Hevin inhibits glucose- stimulated insulin secretion

In order to investigate the effect of SPARC proteins on glucose-stimulated insulin secretion, adhered INS-1 cells were pre-treated with SPARC, hevin, or SPOCK-3 for 24 hours. After 30 minutes of glucose stimulation, the supernatant was processed for ELISA as described in section 5.5.1. Protein concentration was quantified using the BCA protein assay to control for cell density and showed that cell density was relatively consistent between different treatments [Appendix Figure 8.13]. As shown in Figure 5.14, insulin secretion was indeed stimulated by glucose, significantly increasing secretion by more than 2 fold ($p=0.019$). Pre-treatment with hevin significantly inhibited insulin secretion by 60% ($p=0.037$). However, pre-treatment with SPARC or SPOCK-3 only marginally inhibited secretion by about 20% and was not significant. These experiments show that hevin inhibits glucose-stimulated insulin secretion. It will also be important to investigate the effect of these SPARC proteins at basal glucose levels.

5.6 SPARC family regulation of glucose-stimulated phosphorylation of ERK and FAK

5.6.1 Validation of detecting glucose-stimulated p-ERK and p-FAK

Focal adhesion remodelling is essential for glucose-stimulated insulin secretion. This remodelling involves the phosphorylation of FAK, paxillin, and ERK at focal adhesion complexes (Tomas *et al*., 2006; Rondas *et al*., 2011; Rondas *et al*., 2012). It has been shown that inhibition of these proteins decreases focal adhesion formation and insulin secretion after glucose stimulation (Rondas *et al*., 2011). We therefore aimed to investigate whether the SPARC family regulates actin remodelling and insulin secretion through interfering with the signalling of FAK and ERK.

Cells were treated with SPARC proteins during stimulation and then were lysed for western blotting to detect p-ERK and p-FAK. We first validated the time point at which to detect glucose-stimulated phosphorylation of ERK and FAK. Figure 5.15 shows that p-ERK was increased after 10 minutes of glucose stimulation, and further increased after 20 minutes. However, p-ERK was less detectable after 30 minutes. We therefore tested glucose-stimulated p-ERK after 20 minutes.

On the other hand, p-FAK was undetectable at any of the time points tested. We asked whether this was due to the binding dynamics of the antibody to the Y397 phosphorylation

site of FAK or whether FAK was expressed at very low levels in INS-1 cells. We attempted to incubate the antibody at room temperature overnight to promote antibody binding. However, p-FAK was still only detectable at low levels after over exposing the image and there was no indication of stimulation [Appendix Figure 8.14 A]. We then used a different antibody (Cell Signalling TYR 925) that detects phosphorylation at Y935 on FAK and incubated at 4°C overnight. This however, was still undetectable and bands were only visible after overexposing the image [Appendix Figure 8.14 B]. For future studies, it may be useful to test p-FAK expression in MIN-6 β-cells or other adhesive cell types for example fibroblasts, to compare expression levels. Perhaps INS-1 cells express FAK at low levels therefore it might also be useful to induce INS-1 cells to express and activate FAK for example by treating with RGD peptide or investigating phosphorylation upon initial attachment of cells.

5.6.2 SPARC inhibits glucose-stimulated ERK activation

In order to investigate the effect of SPARC proteins on glucose-stimulated phosphorylation of ERK, INS-1 cells were starved and stimulated with glucose for 20 minutes as described previously in section 5.6.1 but were additionally treated with SPARC, hevin, and SPOCK-3 during glucose stimulation. We found that glucose stimulation significantly increased phosphorylation of ERK ($p=0.007$) by 4 folds compared to the unstimulated control [Figure 5.16]. Hevin and SPOCK-3 inhibited p- ERK by about ~30%, although this was not statistically significant. In contrast, SPARC significantly inhibited p-ERK expression by ~80% ($p=0.023$) compared to the glucose stimulated control. Overall, this suggests that SPARC plays a specific role in inhibiting glucose-induced phosphorylation of ERK.

5.7 Glucolipotoxicity

Glucolipotoxicity (GLT), or long term exposure to elevated glucose and fatty acids, is known to be detrimental to β-cells. Increased circulating levels of fatty acids have been shown to

promote insulin resistance, β-cell dysfunction and lipotoxicity (Oliveira *et al*.,2015). GLT induced the activation of STAT1 and NF-κB promoting β-cell death (Bagnati *et al*., 2016). Apoptosis induced by GLT was also shown to be through the ERK pathway (Poitout *et al.*, 2010). In addition, GLT inhibited insulin secretion and insulin gene expression (Olofsson *et al*., 2007; Poitout *et al*.,2010).Furthermore, preliminary data from collaborators indicated that GLT induced changes in expression of genes regulating the ECM and β-cell adhesion (Turner, M., personal communication*)*. We therefore aimed to investigate the effect of GLT on β-cell attachment, adhesion, and glucose-stimulated actin remodelling and insulin secretion.

5.7.1 Effect of glucolipotoxicity on β-cell growth

INS-1 and MIN-6 cells were plated in GLT treated media in order to analyse attachment. GLT conditions were prepared as described previously (Bagnati *et al*., 2016) using palmitic acid and oleic acid however in serum free media as serum contains additional fatty acids. To improve solubility of palmitic acid, 2% fatty acid (FA) free BSA was used to conjugate palmitate before cell culture. We treated cells in complete media (10% FBS), serum free media, and serum free with FA free BSA as controls. Under serum free conditions, INS-1 and MIN-6 cell spreading and growth was inhibited compared to those cultured in complete media, but were able to spread and adhere [Figure 5.17 A & B]. However GLT and serum free media + BSA similarly significantly abrogated β-cell attachment and spreading for both cell types as shown in Figure 5.17 C-E. We then investigated GLT in complete media (10% FBS) to eliminate the influence of serum free conditions on results. Under complete media conditions, INS-1 cells successfully attached and spread, but however GLT conditions promoted β-cell growth [Figure 5.18] contrary to results suggested in literature.

5.7.2 Effect of fatty acid free BSA on β-cell survival

BSA is routinely used to conjugate palmitic acid in cell culture to improve solubility but however, appeared to be detrimental to β-cell attachment and growth. In addition, albumin

concentrations in the serum appeared to influence β-cell response to GLT conditions [Figure 5.17 a & B]. We therefore titrated the BSA concentration in serum free conditions. As shown in Figure 5.19, FA free BSA significantly inhibited β-cell growth dose dependently compared to the untreated control. This suggests that BSA is detrimental to β-cell growth and therefore GLT experiments would need further optimising in order to separate effects of lipotoxicity to albumin toxicity.

The concentration of FA free BSA has been shown to be essential in investigating β-cell death. Increased albumin increases the amount of albumin bound palmitic acid. However, it is actually the unbound palmitic acid that is responsible for inducing β cell death (Warnotte *et al*., 1999, Oliveira *et al*., 2015). Our data supports this which shows that in serum free conditions, where there would be more unbound palmitic acid, GLT significantly inhibited growth [Figure 5.17].

However, in FBS-supplemented media, where a higher percentage of palmitate would be bound to albumin, GLT induced β-cell growth [Figure 5.18]. Serum not only contains albumin but other components that bind free fatty acids and therefore would lower unbound fatty acid concentrations. In addition, the type of BSA may have an influence on β-cell response. Oliveira *et al.* demonstrated that charcoal-absorbed BSA induced more cell death compared to FA free BSA when pre-complexed with palmitic acid. In support of this, there were higher concentrations of unbound FAs in charcoal-absorbed BSA (Oliveira *et al*., 2015). The FA free BSA used in these studies was purified from cold ethanol fractionation (Sigma) although the relevance of this on binding with palmitate remains to be investigated. Alternatively, the type of BSA used in these studies may have high affinity to the plastic ware and affected attachment of β-cells. Routinely, there appears to be a variety of methods used in other lipotoxic studies that investigate both in 10% FBS complete medium (Luo *et al.,* 2012; Bagnati *et al*., 2016) 1% FBS low serum medium (Malhi *et al*.,2006), and serum free conditions (Das *et al*., 2008, Diakogiannaki *et al.,*2008). Nonetheless, factors such as type and concentration of FA free BSA and serum conditions need to be considered for GLT assays. Overall, our data

demonstrates the complexity of setting up this assay and that further optimisation is needed to properly create GLT conditions to investigate β-cell functions.

5.7.3 Effect of glucolipotoxicity on β-cell actin organisation

The effect of GLT on actin organisation was investigated for 72 hours under complete media conditions as cells successfully attached under these conditions. Cells were treated with GLT media during plating. Figure 5.20 shows that exposure to GLT conditions inhibited F-actin polymerization and in addition, the cortical actin was particularly thinner compared to those cultured in normal media. In addition, insulin expression was increased at the surface and in the cytoplasm under GLT conditions compared to the control. This is preliminary data suggesting that long term exposure to GLT induces β-cells to go under constant stress following elevated stimulation to produce insulin and therefore exhibit impaired actin organisation.

5.8 Chapter discussion

5.8.1 Different effects of SPARC, hevin and SPOCK-3 on actin regulated insulin secretion

Attachment and spreading of β-cells on the matrix has been shown to be influenced by glucose (Bosco *et al*., 2000). Cells that are attached to the matrix have increased spreading and insulin secretion after glucose stimulation compared to those cultured in 2D (Bosco *et al*., 2000). β-cells spread in response to glucose to allow cytoskeleton remodelling and insulin granule transport (Rondas *et al*., 2011) and spreading induced by glucose is accompanied by

increased expression of α6β1 integrins (Bosco *et al.,* 2000). Islet contact and interaction with the ECM therefore plays a pivotal role in the regulation of insulin response to glucose.

The SPARC family of proteins are matricellular proteins highly expressed in islets and may play a role in regulating islet function. We have investigated the role of SPARC, hevin, and SPOCK-3 on glucose-stimulated actin remodelling, ERK activation and insulin secretion of β-cells. We show for the first time that all 3 proteins enhance actin remodelling of glucose stimulated β-cells. While enhanced actin remodelling suggests decreased cortical actin and therefore increased insulin secretion, we have observed the opposite and found a trend of inhibition. Additionally, SPARC, hevin and SPOCK-3 showed varying effects on insulin secretion despite dramatic effects on actin remodelling. SPOCK-3 and hevin showed a trend of decrease on ERK activation while only SPARC showed significant inhibiting effects. Furthermore, only hevin showed significant inhibition of insulin secretion. A summary of these results are shown in Table 5.2.

In contrast, our data contradicts previously published data in which Harries *et al.* showed that SPARC increased glucose-stimulated insulin secretion of INS-1 β-cells (Harries *et al.,* 2013). However, SPARC was overexpressed in INS-1 cells which may have created aberrant results as we have previously shown that INS-1 β-cells do not naturally express SPARC but rather SPARC is expressed by pancreatic stellate cells (Ryall *et al*., 2014).

Perhaps the mechanistic role of these SPARC proteins on actin regulated insulin secretion may be more complex and warrant further investigation. One hypothesis is that perhaps SPARC, hevin and SPOCK-3 may inhibit insulin secretion by enhancing actin remodelling i.e., increasing actin depolymerisation therefore inhibiting the ability of β-cells to form new focal adhesions which is required for cells to spread to allow insulin granule transport to the plasma membrane (Rondas *et al*., 2011). It is therefore essential to understand the mechanistic role of the SPARC family in actin regulated insulin secretion to further understand implications in diabetes.

5.8.2 Possible mechanistic roles of the SPARC family in insulin exocytosis

During phase 1 of glucose stimulation, p-ERK has been detected at the tip of actin fibers at focal adhesions within the first 5 minutes of stimulation (Tomas *et al*., 2006). ERK activation seems to take place after actin remodelling as Tomas *et al*. have demonstrated that inhibition of ERK does not affect glucose-stimulated actin remodelling (Tomas *et al*., 2006). FAK and paxillin are subsequently activated by ERK (Rondas *et al.,* 2011). Temporal incorporation of p-ERK at focal adhesions may be activating specific targets located near the plasma membrane upon glucose stimulation to induce insulin secretion such as FAK and paxillin. However, ERK can also work downstream of FAK and paxillin [Figure 5.21] (Rondas *et al*., 2011), further enhancing focal adhesion maturation and thus actin remodelling (Tomas *et al*., 2006; Rondas *et al.*, 2011). In addition, ERK can trigger actin remodelling directly as ERK activation is coupled with the activation of mysolin light-chain kinase (MLCK) protein [Figure 5.21]. (Yu *et al*., 2000; Arous *et al*., 2013).

Whether SPARC, hevin and SPOCK-3 work upstream or downstream of glucose-induced actin remodelling needs further investigation. They may act at different stages of the pathway as although all 3 proteins enhanced actin remodelling, they had different effects on insulin secretion and ERK activation. It has previously been shown that β1 integrin activation of FAK/ERK signalling in β-cells influences insulin expression as well as differentiation and survival (Saleem *et al*., 2009). One hypothesis is that SPARC is known to bind to integrins and therefore influences focal adhesions and ERK. SPARC has been additionally shown to activate FAK and ILK (Shi *et al.,* 2007). However, FAK signalling is also regulated by EGFR, FGFR, and the insulin receptor itself (Schlaepfer *et al*., 1994; Huang *et al*., 2002; Williams *et al.,* 2015). SPARC may perhaps additionally be regulating integrins, FAK and focal complexes through cross-talk with growth factor receptors.

Alternatively, small GTPases have been shown to be involved in glucose signalling (Nevins and Thurmond 2003; Wang *et al*., 2007; Wang and Thurmond 2009; Kalwat and Thurmond, 2013). Rho-family GTPases Cdc42 and Rac1 have been shown to be activated following glucose stimulation and this is temporally correlated with actin remodelling (Li *et al.,* 2004; Nevins and Thurmond 2003,). Activation of Cdc42 and Rac1 is required particularly for phase

2 of insulin secretion where it is essential for actin remodelling to take place as seen in [Figure 5.21] (Wang and Thurmond 2009; Nevins and Thurmond 2003). In addition, Cdc42 has been shown to be directly associated with insulin granules during stimulation where it was detected to form complexes with VAMP-2 SNARE proteins on granule surfaces (Nevins and Thurmond, 2005). Upon stimulation, Cdc42-VAMP-2 complexes were detected to move towards the plasma membrane indicating that Cdc42 may be directly involved in the targeting of insulin granules to the plasma membrane (Nevins and Thurmond, 2005). Cdc42 activation may also indirectly activate ERK through PAK1, and Raf signalling [Figure 5.21] (Kalwat and Thurmond, 2013). Recently, SPARC was shown to induce actin depolymerisation in medulloblastoma cells through inhibition of Rho-Rac-Cdc42 GTPases (Bhoopathi *et al*., 2011). SPARC activated the focal adhesion kinase Src at focal complexes which consequently inhibited the phosphorylation of Rho GTPases, thus inducing cell rounding, decreased proliferation and migration (Bhoopathi *et al*., 2011). While little is known on GTPase regulation of other related SPARC proteins, SMOC-2 on the other hand has been shown to regulate another member of the GTPase family, Ran. Ran-dependent activation of ERK was inhibited when SMOC-2 was knocked down (Milano *et al.,* 2012). It is possible that in β-cells, the SPARC family may also inhibit GTPase family activation thereby regulating actin remodelling and insulin granule transport. Further study is needed to investigate the effect of the SPARC family on GTPase activation such as Cdc42 and Rho to fully understand its mechanistic role on actin regulated insulin secretion.

It is only recently that the role of F-actin has been recognised in insulin regulation. Increasing evidence has shown that the actin network is not passive, but at an active participant of insulin granule trafficking. Actin remodelling must occur simultaneously to allow coordinated and temporally precise insulin release. We have shown that members of the SPARC family, particularly SPARC, hevin, and SPOCK-3 are novel regulators of glucose-stimulated actin remodelling and insulin secretion in β-cells. The significance of these findings would need to be further tested in islets to realise the implications of the SPARC family in islet function and diabetes. In summary, we have shown that the SPARC family are novel regulators of actin-regulated insulin exocytosis.

6. Discussion

6.1 The intermediate state of adhesion- an adaptive state

The intermediate state of adhesion is suggested to be an "adaptive state" of cells, promoting cellular processes and gene expression programs different from the strong adhesive state. The adhesive state of cells can influence response to growth and differentiation factors. It has been suggested that the SPARC family and other matricellular proteins can protect against apoptosis or anchorage dependent anoikis at this state, keeping cells spread and attached to the matrix while focal adhesions are disassembled (Murphy-Ullrich, 2001). Matricellular proteins that promote intermediate adhesion are highly expressed during wound healing and regeneration. Transitioning from strong to weaker adhesions is commonly regarded as a sign of dying cells, however regulation of adhesion is a continuous process and the intermediate state may possibly be an adaptive state and important during regeneration and tissue remodelling. Our studies suggest that SPARC, hevin, and SPOCK-3 may regulate the intermediate state to influence cell adhesion and therefore also regulate insulin secretion signalling of β-cells.

6.2 The matrix-islet relationship is essential in regulated insulin release

We have shown that Hevin and SPOCK-3 inhibit β-cell attachment, spreading and focal adhesion formation while SPARC inhibits spreading. Furthermore, SPARC, hevin and SPOCK-3 regulated actin remodelling during glucose stimulation indicating an important role of these SPARC proteins in regulating the second phase of insulin secretion. We further show for the first time that SPARC inhibits glucose-stimulated phosphorylation of ERK and hevin inhibits glucose-stimulated insulin secretion. Our data supports previous studies showing that regulation of β-cell adhesion plays a pivotal role in regulating insulin exocytosis. For example, attachment and spreading of β-cells to the matrix has been shown to be influenced by glucose (Bosco *et al.,* 2000). β-cells spread in response to glucose to allow cytoskeleton remodelling and insulin granule transport (Bosco *et al*., 2000; Rondas *et al*., 2011). Additionally, it has been shown that spreading induced by glucose is accompanied by increased protein expression of α6β1 integrins and blocking of α1 integrins inhibited insulin

secretion of β-cells suggesting an important role of islet-matrix contact in insulin secretion (Bosco *et al*., 2000 Kaido *et al.,* 2004). We show for the first time that SPARC, hevin and SPOCK-3 are important regulators of β-cell spreading, adhesion and actin regulated insulin secretion.

6.3 Are secreted matricellular SPARC proteins acting internally?

Despite being secreted matricellular proteins, we have shown that the SPARC proteins, namely hevin, SPOCK 1-3, and SMOC 1-2 are highly expressed intracellularly by islet cells. In addition, these proteins were detected in the nuclei of islet and pancreatic ductal cells. We have also described the rich diversity of intracellular splice variants that may have distinct roles from their extracellular variants. Although it is likely that the SPARC family interacts with cell surface receptors such as integrins to regulate proliferation and focal adhesions, alternatively it is also possible that the SPARC family may be acting internally to regulate β-cell functions given that they are so highly expressed intracellularly specifically in islet cells.

Although often detected extracellularly in bone matrices and basement membranes, SPARC has been detected internally in other cells such as kidney, adrenal glands, testicular germ cells, and retinal cells (Vernon and Sage, 1989; Mundlos *et al*., 1992; Porter *et al*., 1995; Yan *et al*., 1998). However, intracellular mechanisms of action have not been fully explored. As mentioned previously, SPARC has been described as a cytoplasmic chaperone in which it is suggested to play a role in procollagen maturation and secretion into the matrix (Martinek *et al*., 2006, Emerson *et al*., 2006). Furthermore, Gooden *et al.* demonstrated that the intracellular expression of SPARC correlated to the stage of cell cycle (Gooden *et al*., 1999. Cells in interphase expressed SPARC primarily in the nucleus while cells undergoing metaphase and anaphase had higher levels of SPARC in the cytoplasm indicating that SPARC may regulate cell proliferation intracellularly (Gooden *et al*., 1999). Interestingly, nuclear internalisation of SPARC has been observed in chicken embryo, bovine aortic endothelial cells (BAE), skeletal muscle progenitor cells, urothelial and testicular germ cells where rSPARC was detected in the cytoplasm and nucleus of cells after 16-17 hours of treatment (Gooden *et al*., 1999; Wilson *et al*., 2006; Kosman *et al*., 2007; Nakamura *et al*., 2014). SPARC was particularly associated to the nuclear matrix (Gooden *et al*., 1999; Kosman *et al*., 2007). The

nuclear matrix itself holds transcription factors and protein attachment to the nuclear matrix influences transcription. This suggests a specific nuclear role for SPARC and further indicates that related SPARC proteins may have a role at the nuclear matrix of β-cells. Intracellular roles for the SPARC family have not yet been described however our data supports the model that the SPARC family may have intracellular functions that regulate signalling and gene expression related to β-cell functions through regulation of the nuclear matrix.

Other secreted proteins have been shown to be internalised into the nucleus such as FGF, proteoglycans, lysyl oxidase, angiogenin as well as insulin, all of which have been shown to have double functions of inducing cell signalling through cell surface receptor interaction as well as through nuclear translocation (Shah *et al*., 1995; Henderson 1997; Li *et al.,* 1997A; Li *et al*., 1997B; Gooden *et al*., 1999). The mechanism of how SPARC is internalised however is not yet fully understood for example, it is unclear whether SPARC contains a nuclear localisation signal [NLS], a sequence of basic residues recognised by importins at the nuclear matrix. Kosman *et al.* demonstrated that alterations in the putative SPARC NLS region did not affect nuclear targeting of SPARC and contradicts the presence of an active NLS region (Kosman *et al*., 2007). On the other hand, internalisation of SPARC was shown to be dependent on α5 integrin translocation suggesting that SPARC may be internalised after binding to integrins (Nakamura *et al.*, 2014). Further study is needed to investigate whether related SPARC proteins are translocated into the nucleus or cytoplasm immediately after translation or internalised from the extracellular matrix. Perhaps different SPARC family variants have different internalisation pathways and further study is needed to understand the mechanism and its relevance to β-cell functions. Nonetheless, it is likely that extracellular and intracellular SPARC family variants may have distinct functions in regulating cell signalling and it is likely that expression may be regulated to modulate β-cells from the extracellular space or intracellularly in the cytoplasm or nucleus.

6.4 SPARC family of proteins: novel regulators of β-cell rest? Implications for diabetes

Insulin secretion is under homeostatic regulation, keeping blood glucose at a constant. During high blood glucose levels, β cells of the islets of Langerhans secrete insulin under

positive regulation to ensure quick responsiveness for glucose uptake (Fu *et al*., 2013). During resting conditions, insulin release is under negative regulation to ensure low glucose uptake (Wang and Thurmond, 2009). We have shown that SPARC, hevin and SPOCK-3 regulates glucose-stimulated actin remodelling and that SPARC inhibits phosphorylation of ERK while hevin inhibits insulin secretion. In addition, hevin, SPOCK and SMOC proteins are highly expressed in islet and β-cells. Together our data suggests that the SPARC family may be novel regulators of glucose stimulated insulin secretion. However, it seems unlikely that insulin producing β-cells would express so much of proteins that inhibit insulin secretion. Potentially, the SPARC family may be a negative feedback mechanism that allows β-cells to rest and replenish their insulin stores.

The concept of β-cell rest has been described as the pharmacologic suppression of insulin release (Hansen *et al.,* 2004; Brown and Rother, 2008). β-cell rest has increasingly been explored as a novel method of improving β-cell function and treatment of metabolic disorders (Hansen *et al*., 2004; Brown and Rother, 2008). Insulin hypersecretion results in overworked and stressed β-cells, thereby leading to decreased ability to respond to glucose and overall β-cell loss and diabetes. Clinical studies have demonstrated the benefits of β-cell rest on β-cell function and diabetes treatment. For example, treatment with exogenous insulin improved insulin secretion and metabolic control of patients with Type 1 diabetes (Bjork *et al.,* 1996). In patients with Type 2 diabetes on the other hand, insulin treatment improved HbA1C levels which persisted after 12 months (Sellers *et al.,* 2004).

At present it is unclear whether β-cell rest is beneficial long term, but several hypotheses have been suggested. Exogenous insulin reduces the demand for β-cells to secrete insulin and therefore allows β-cells to replenish insulin stores. It also immediately reduces glucotoxicity and recovers glucokinase activity which is involved in glucose metabolism (Rizzo *et al*., 2002; Ritzel *et al.,* 2004; Brown and Rother, 2008). β-cell rest is also suggested to improve islet blood flow and decrease islet fibrosis (Jansson *et al., 2003*; Hong *et al.,* 2007). Additionally, β-cell rest may decrease islet antigenicity in Type 1 diabetes as increased work load of β-cells has been associated to antigenicity (Buschard *et al.,* 1988; Karlsson *et al.,*

1997; Brown and Rother, 2008). The potential benefits of β-cell rest in preventing diabetes is further explored in animal and human studies (Brown and Rother, 2008; Hansen *et al*., 2004).

Inhibition of adhesion and actin regulated insulin secretion induced by the SPARC family and their high expression in β-cells may therefore indicate that the SPARC family may promote β-rest. This leads to the question, when do β-cells express the SPARC proteins, particularly hevin and SPOCK-3? Are they expressed during prolonged exposure to glucose or insulin? Under constant glucose stimulation and increased insulin in the extracellular space, the SPARC family may be a mechanism to slow down secretion and allow β-cells to refill insulin stores. Perhaps SPARC proteins may be a normal/ physiologic mechanism for β-cells to induce rest. Dysregulation of their function may have implications in β-cell dysfunction and disease progression to diabetes.

Interestingly, we have previously shown that SPARC production in PS-1 stellate cells is regulated by levels of glucose, insulin, and leptin (Ryall *et al*.,2014). Glucose inhibited SPARC expression while insulin and leptin increased SPARC expression in stellate cells. This may similarly regulate SPARC family expression in β-cells further supporting the negative feedback model, in which constant high glucose levels would decrease the expression of SPARC proteins to induce rapid insulin secretion while on the other hand, increased levels of insulin would increase SPARC family expression to slow down further insulin secretion and maintain homeostasis. Further studies are needed to investigate the role of other members of the SPARC family such as SPOCK 1 and 2, SMOC proteins, and FSTL-1 on insulin secretion and whether the expression of the SPARC family is similarly regulated by metabolic parameters in β-cells. Dysfunction in insulin release and glucose sensitivity of β-cells predetermines Type II diabetes. It would be relevant to investigate when β-cells express the extended SPARC family in relation to glucose stimulation, starvation, and under glucolipotoxic condition. Additionally, β-cell expression of different variants of the SPARC family – and therefore their function may be influenced under these conditions.

6.5 The SPARC family of proteins: novel regulators of actin-regulated exocytosis

Actin regulated exocytosis is a mechanism used not only by β-cells but also by secretory cells such as mammary, salivary and other endocrine cells (Bader *et al*., 2004; Gasman *et al*., 2004; Porat-Shliom *et al*., 2013; Papadopulos, 2017). The cortical actin network is prominent among secretory cells and is now becoming more and more known to be a key regulator of regulated exocytosis (Malacombe *et al.,* 2006, Porat-Shliom *et al.,* 2013). Filopodial extension and membrane shaping affects membrane tension and fusion pore size, thus regulating the amount released by vesicles (Papadopulos, 2017). Cdc42 as well as Rho GTPases have been shown to play a role in the actin-regulated exocytosis of adrenal and neuronal endocrine cells (Ridley, 2006; Bader *et al*., 2004). We have shown that the extended SPARC family are highly expressed in islet cells. Hevin, SPOCK-2, and SMOC-2 in particular, are more highly expressed by islet cells at the periphery [Figure 3.2 A, C, & F] which we have also shown to be glucagon expressing α-cells [Figure 3.3]. Plasma glucagon is increased in Type 2 diabetes and therefore studies are looking into the regulation of glucagon secretion and its impairment in diabetes (Burcelin *et al.,* 2008). There are also other secretory cells in the pancreas such as δ cells and PP cells that potentially express the SPARC family in islets. Our data suggests that strong expression of the SPARC family in endocrine islet cells may indicate their involvement in the regulated secretion of glucagon and other digestive hormones. It would therefore be relevant to investigate the role of the SPARC family in exocytosis of other islet endocrine cells and their implications in homeostasis and diabetes. Additionally, the SPARC family may have importants roles in the regulated exocytosis of other secretory cells. Clinical relevance of the SPARC family function in diseases such as hormonal imbalance and endocrine disorders warrants further studies.

7. References

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8. Appendix

	Immunogen	Stock concentration	IHC titrations	Optimal IHC concentration	WB concentration	Manufacturer
SPARCL1/Hevin	215-244	0.5 mg/mL	1/100-1/800	1/100-1/200	1/300	Abcam (ab107533)
SPARCL1/Hevin	649-664 C terminus	0.5 mg/mL			1/500	LS Bio (LS-C313206)
SPOCK-1	31-61	0.25 mg/mL	1/25-1/200	1/25-1/50	1/500	Abcam (ab174479)
SPOCK-2	200-250	0.4 mg/mL	1/100-1/400	1/100-1/200	1/300	Novus Bio (NBPI-92442)
SPOCK-3	71-365	1 mg/mL	1/200-1/800	1/200-1/400	1/500	Abcam (ab111897)
SMOC-1	140-363	0.9 mg/mL	1/50-1/200	1/100-1/200	1/500	Abcam (ab155776)
SMOC-2	N terminus	0.5 mg/mL	1/25-1/400	1/100-1/200	1/500	Abcam (ab78069)
FSTL-1	Internal	0.3 mg/mL	1/50-100	1/50	1/300	Novus Bio (NBPI-83425)
SPARC	1-90 N-terminus	200 µg/mL			1/1000	Santa Cruz (H-90)

Table 8.2: List of SPARC family antibodies. Table shows the SPARC family antibodies used in IHC and WB studies. Each antibody was specific to the N-terminus region as this is the most unique region for each protein indicated are the epitopes to which each antibody detects and the optimum concentrations used for IHC and WB.

	Species	manufacturer	CLUSTAL % identity to rat (INS-1 cells)
SPARC	Human	R&D Systems 941-SP-050	92.6%
Hevin	Mouse	R&D Systems 4547-SL-050	88%
SPOCK-3	Mouse	R&D Systems 2346-PI-050	97.2%
SMOC-1	Mouse	R&D Systems 5550-SM-050	99.3%
FSTL-1	Mouse	R&D Systems 1738-FN-050	96%

Table 8.2: List of recombinant proteins. Indicated in the table are all recombinant proteins used in the study including their species and homology to rat INS-1 cells. FASTA sequence for human, mouse, and rat proteins were acquired from Uniprot and then aligned for homology using Clustal Omega.

Primer name	sequence	primary target transcript ID	primary PCR prod length	Transcripts with differential product length
GenAFor	TCGGACGGCGGTAATTTTCT	006 (generic)	158	201 (238 bp), long variants with exon 8/9 (167 bp)
GenARev	GGATCTAAAGCCTGATCGAAGG			
GenBFor	CGCACTTGGAGTCCAGGAAA	006 (generic)	740	202 (625 bp), 013 (620 bp), 018 (386 bp)
GenBRev	CAAGGTGGGTCTTGCTGTCT			
005For	AGAAATGTTAAGAGAGCATGCAG	005	386	
005Rev	TTGCGTCTGTAAGGGTCTCA			
202Rev	CCTGCTTCTTTCATCCCTGATCG	202	154	
010For	TTCCGAGACTGCAAACTAGAAT	010	476	
013Rev	GCTGGTATCGAATCCTCTCTTAAC	013	402	
201For	GATCACACAAGATCACATCCACA	201	215	
201Rev	CTTCTTTCATCCTGTGTGTAAGCC			

Table 8.3: Primer sequences used to detect splice variants of human SPOCK-3.

SPOCK3 siRNA sequence	Type of siRNA	species	Cells tested	Mode of testing KD	Effect on adhesion
UAUCAAAUGUGAAGGGCGA	SMARTPOOL L-085546-02	rat	INS1	WB, (single, double KD, compared attached and detached cells) RT-PCR	De-adhesion
GCAAAAUUCAGAAGCGACA	SMARTPOOL L-085546-02	rat	INS1	WB, (single, double KD, compared attached and detached cells) RT-PCR	De-adhesion
GAACAAAUUCCGAGACGUA	SMARTPOOL L-085546-02	rat	INS1	WB, (single, double KD, compared attached and detached cells) RT-PCR	De-adhesion
GCAAACAGACUGCGAGACU	SMARTPOOL L-085546-02	rat	INS1	WB, (single, double KD, compared attached and detached cells) RT-PCR	De-adhesion
GAGAGAAGCAGAUUCGAUA	Individual ON TARGET J-020879-17	Human	PS-1	WB (single, double KD)	No effect

Table 8.4: SPOCK-3 siRNA sequences used in knockdown experiments. Indicated are each siRNA either as individual or part of the SMARTPOOL (Dharmacon). For each siRNA, indicated are the species for which it targets, whether tested for single or double knockdown, how protein expression was detected and effects on de-adhesion of cells.

Hevin siRNA sequence	Type of siRNA	species	Cells tested	Mode of testing KD	Effect on adhesion
ACAAACAAGAUCCGGGUAU	SMARTPOOL L-090046-02	rat	INS1	WB, (single, double KD, compared attached and detached cells)	De-adhesion
				Ordered rat primers but did not work	
	Individual ON-TARGET J-090046-09	rat	INS1	WB, single KD	De-adhesion
GGAUAUUGUUCUUUCGAGA	SMARTPOOL L-090046-02	rat	INS1	WB, (single, double KD, compared attached and detached cells)	De-adhesion
				Ordered rat primers but did not work	
CAACAGGACGAGAGCGGCA	SMARTPOOL L-090046-02	rat	INS1	WB, (single, double KD, compared attached and detached cells)	De-adhesion
				Ordered rat primers but did not work	
	Individual ON TARGET J-090046-11	rat	INS1	WB, single KD	Not tested
AAAGAAUGAUUUCGAGCAA	SMARTPOOL L-090046-02	rat	INS1	WB, (single, double KD, compared attached and detached cells)	De-adhesion
				Ordered rat primers but did not work	

Table 8.5: Hevin siRNA sequences used in knockdown experiments. Indicated are each siRNA either as individual or part of the SMARTPOOL (Dharmacon). For each siRNA, indicated are the species for which it targets, whether tested for single or double knockdown, how protein expression was detected and effects on de-adhesion of cells.

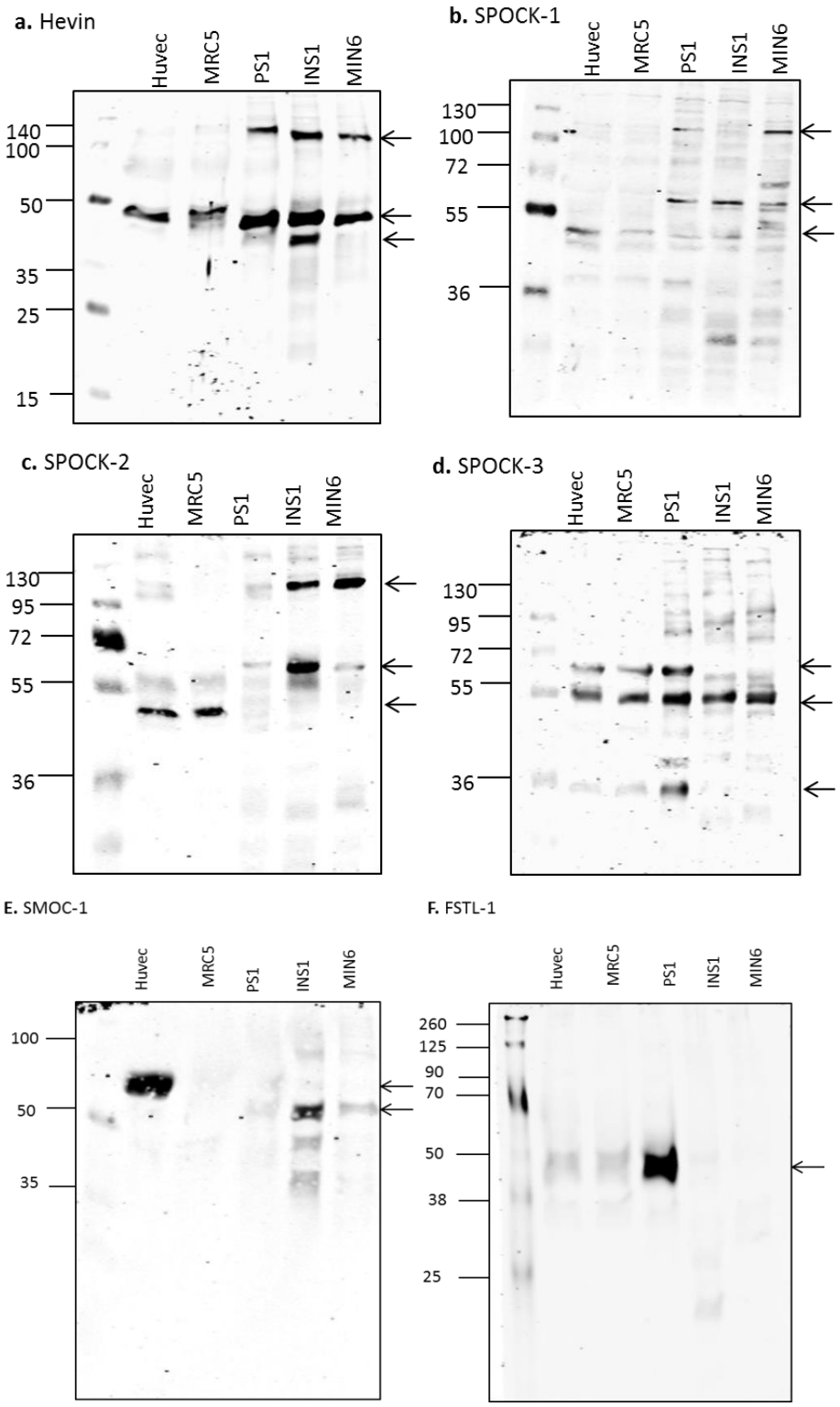

Figure 8.1: Full blots for cropped western blot images in Figure 3.4. Arrows indicate the consistent bands observed from multiple independent experiments.

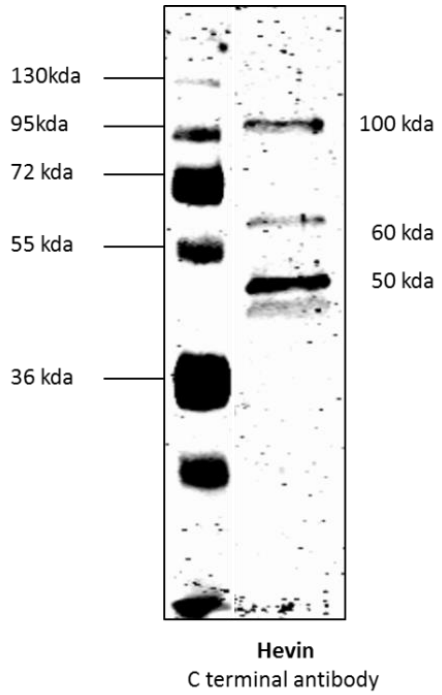

Figure 8.2: Detection of hevin isoforms using a C-terminal antibody. Isoforms of hevin expressed in PS-1 stellate cells were determined by western blotting using anti-hevin antibody specific to the C-terminus of hevin. Images representative of N=3 (3 replicates from 3 independent experiments).

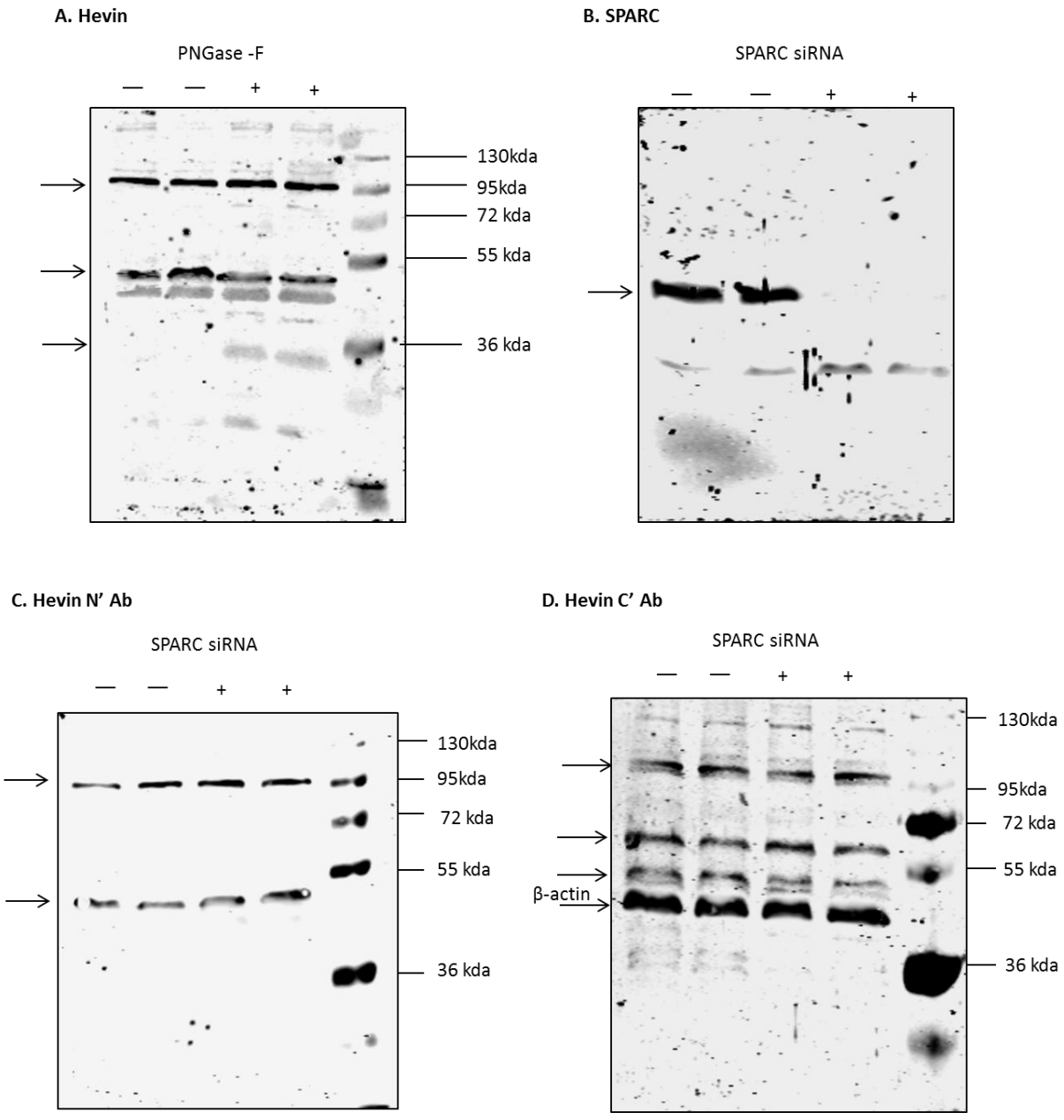

Figure 8.3: Full blots for cropped western blot images in Figure 3.6. Arrows indicate bands/ variants observed for Hevin following (A) PNGase-F digestion or (C-D) SPARC siRNA knockdown (B) Showing SPARC expression inhibition. Smaller secondary band is a non-target band detected by the antibody but however is not silenced by SPARC siRNA.

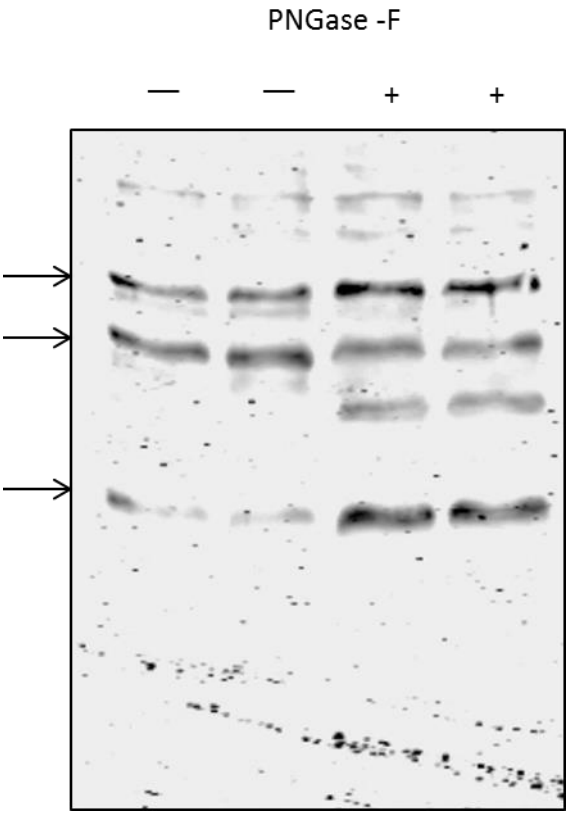

Figure 8.4: Full blots for cropped western blot images in Figure 3.7. Arrows indicate bands/ variants observed for SPOCK-3 following PNGase-F digestion .

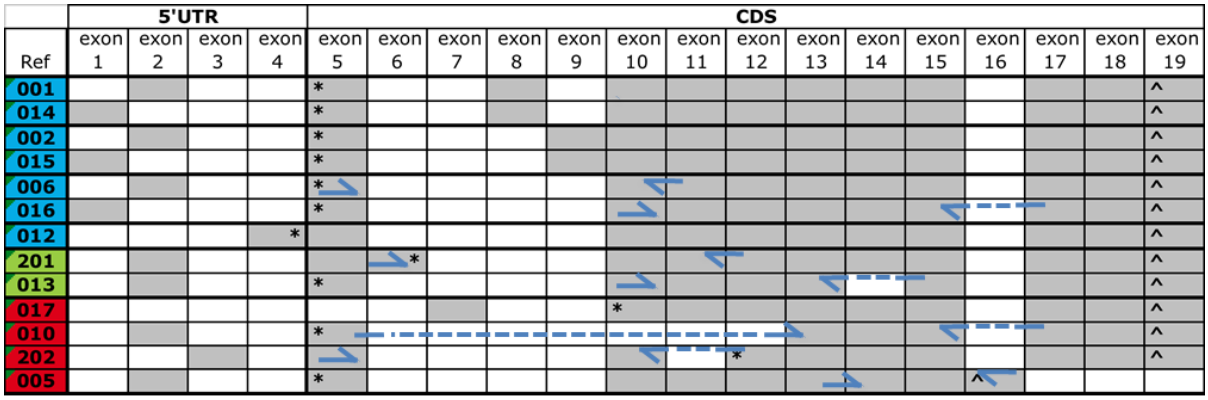

Figure 8.5: Exon structure of SPOCK-3 splice variants. Splice variants banked in ENSEMBL (Accessed March 2013) for SPOCK-3 (ENSG 196104), showing 5'UTR and CDS exons. The first column indicates the ENSEMBL transcript reference. Shaded boxes indicate exons present in each variant. Long variants are designated in blue (49 kDa), medium in green (44 kDa), small in red (36-39 kDa). Shading indicates the presence of the particular exon. * indicates translational start site, while ^ indicates translation termination site. Diagram is not to scale. Blue arrows indicate position of forward and reverse primers. The primers shown on transcript 006 and 016 indicate position of GenA and GenB primers, respectively. All other primers correspond to the indicated transcript. Primer sequences and predicted PCR product sizes are given in Table 7.3. (Analysis and primer design by Hill, N.).

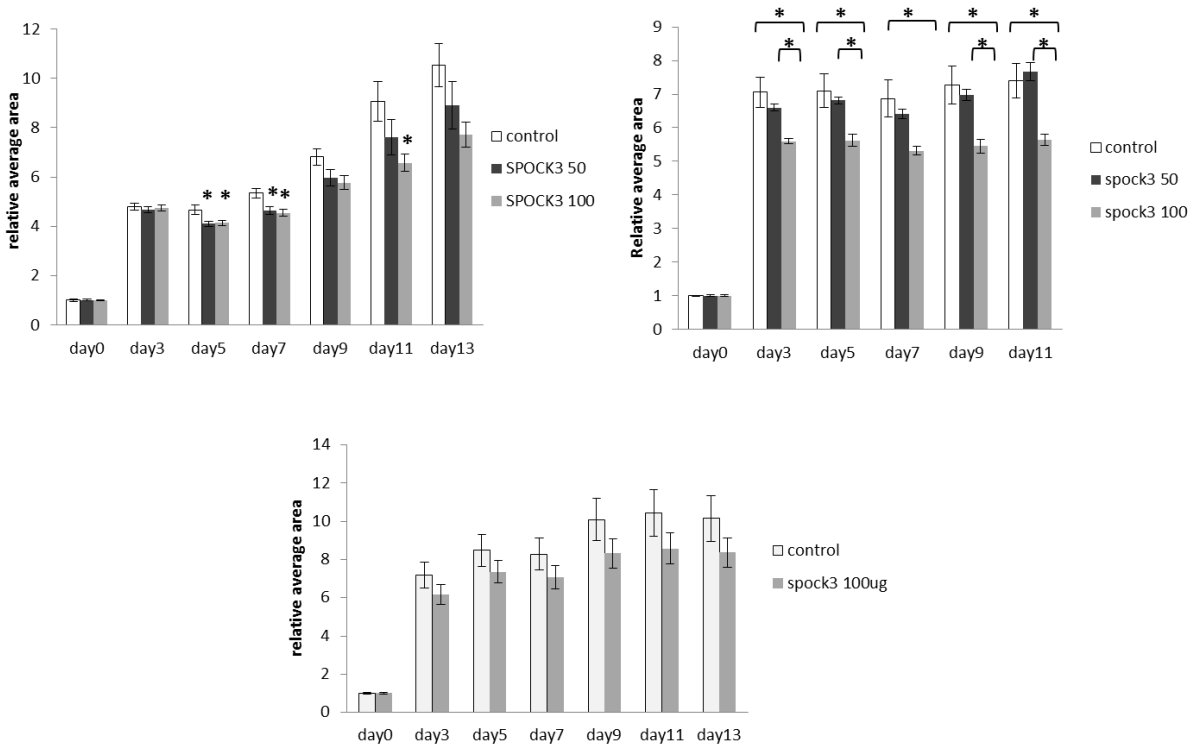

Figure 8.6: Individual experiments showing the effect of SPOCK-3 on β-cell growth in 3D. Each individual replicate represented in Figure 4.5 A. Pre-stained fluorescent cells ($3x10^4$ cells/well) were embedded in the 3D matrix treated with 50-100 µg/mL of SPOCK-3 for a period of 13 days. Growth was monitored fluorescently using the Incucyte ZOOM live cell imaging. Graphs showing area relative to the first time point (day 0) +/- SEM. N=23-24 (6- 8 replicates for each of 3 independent experiments). Statistical significance (*) was analysed using one-way ANOVA.

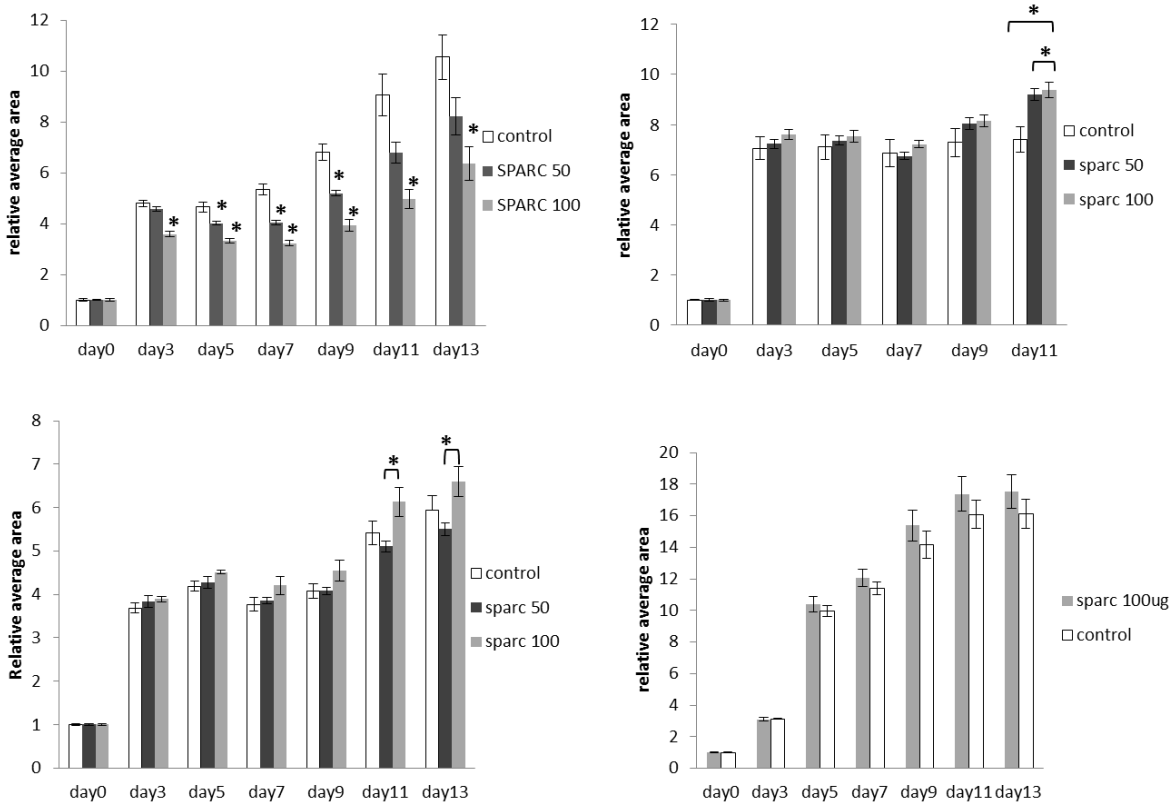

Figure 8.7: Individual experiments showing the effect of SPARC on β-cell growth in 3D. Each individual replicate represented in Figure 4.5 B. Pre-stained fluorescent cells ($3x10^4$ cells/well) were embedded in the 3D matrix treated with 50-100 µg/mL SPARC for a period of 13 days. Growth was monitored fluorescently using the Incucyte ZOOM live cell imaging. Graphs showing area relative to the first time point (day 0) +/- SEM. N=23-24 (6- 8 replicates for each of 3 independent experiments). Statistical significance (*) was analysed using one-way ANOVA.

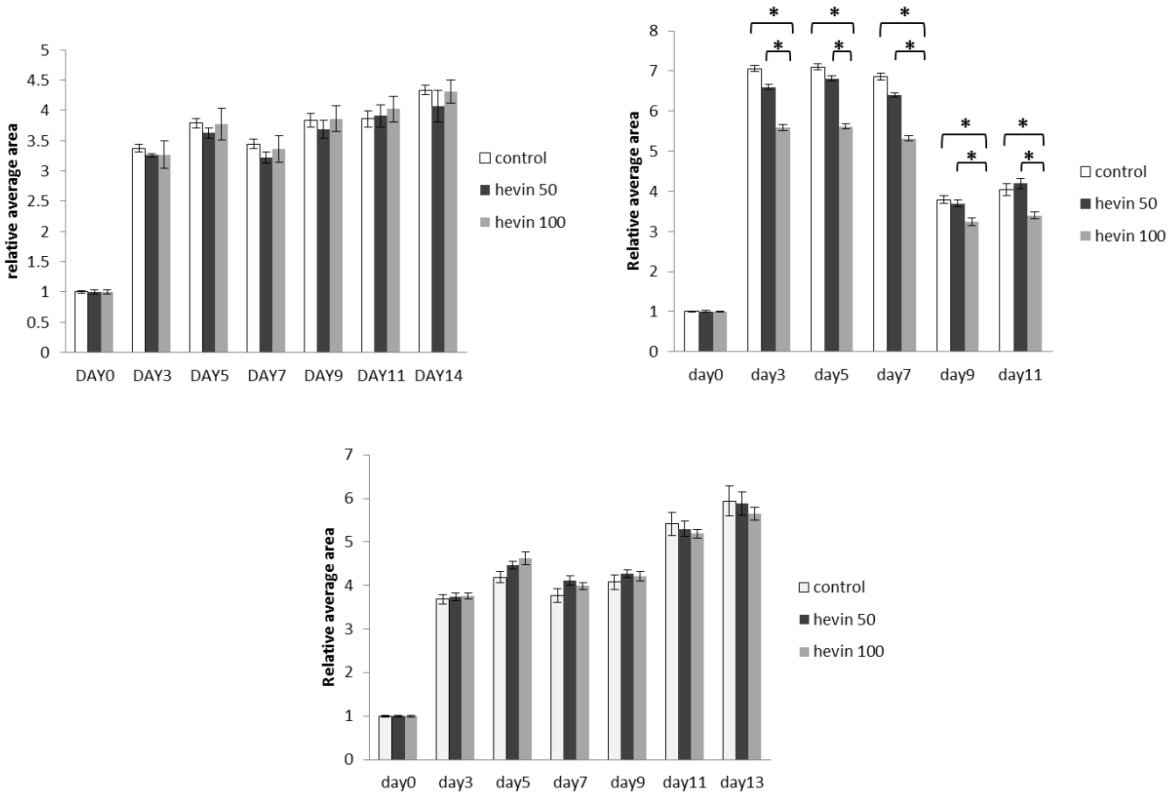

Figure 8.8: Individual experiments showing the effect of hevin on β-cell growth in 3D. Each individual replicate represented in Figure 4.5 C. Pre-stained fluorescent cells ($3x10^4$ cells/well) were embedded in the 3D matrix treated with 50-100 µg/mL of hevin for a period of 13 days. Growth was monitored fluorescently using the Incucyte ZOOM live cell imaging. Graphs showing area relative to the first time point (day 0) +/- SEM. N=23-24 (6- 8 replicates for each of 3 independent experiments). Statistical significance (*) was analysed using one-way ANOVA.

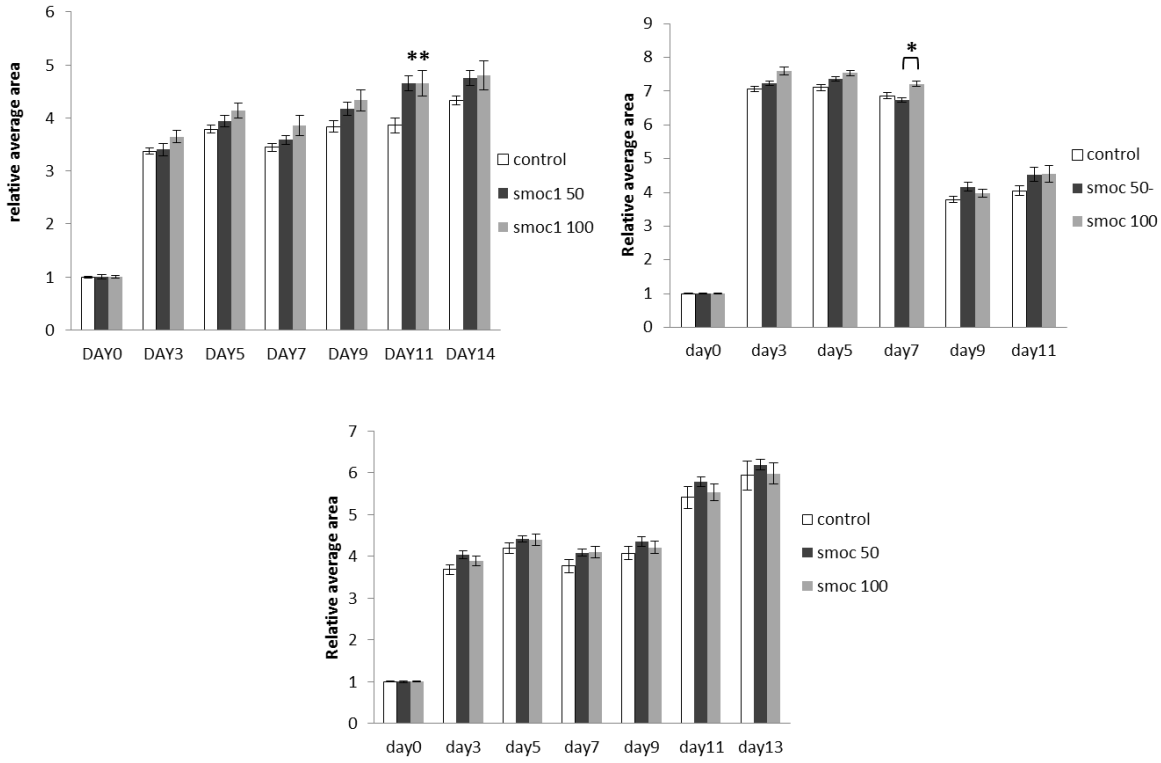

Figure 8.9 Individual experiments showing the effect of SMOC-1 on β-cell growth in 3D Each individual replicate represented in Figure 4.5 D. Pre-stained fluorescent cells ($3x10^4$ cells/well) were embedded in the 3D matrix treated with 50-100 µg/mL of SMOC-1 for a period of 13 days. Growth was monitored fluorescently using the Incucyte ZOOM live cell imaging. Graphs showing area relative to the first time point (day 0) +/- SEM. N= 23-24 (6-8 replicates for each of 3 independent experiments. Statistical significance (*) was analysed using one-way ANOVA.

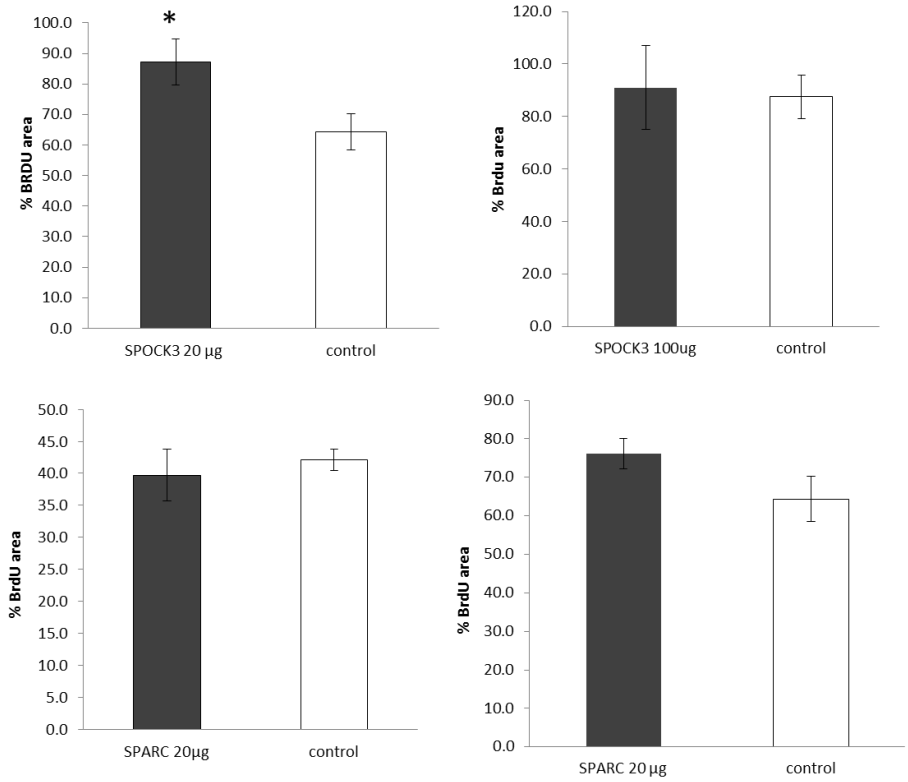

Figure 8.10: Individual experiments showing the effect of SPOCK-3 and SPARC on β-cell proliferation in 3D. Initial experiments showing variable results from testing 20 and 100 µg/mL of protein in 3D. INS-1 cells ($3x10^4$ cells/well) were embedded in the 3D matrix treated with or without SPOCK-3 or hevin at the indicated concentrations for 3 days. BrdU was added after 48 hours of culture and BrdU incorporation was analysed fluorescently during the last 24 hours of culture. About 10 images per sample were acquired using the FLOID microscope (20X). %BrdU was quantified by dividing BrdU area by nuclear area. Graphs showing average %BrdU +/-SEM. Statistical significance was measured using student's t-test. P values are indicated in the graph

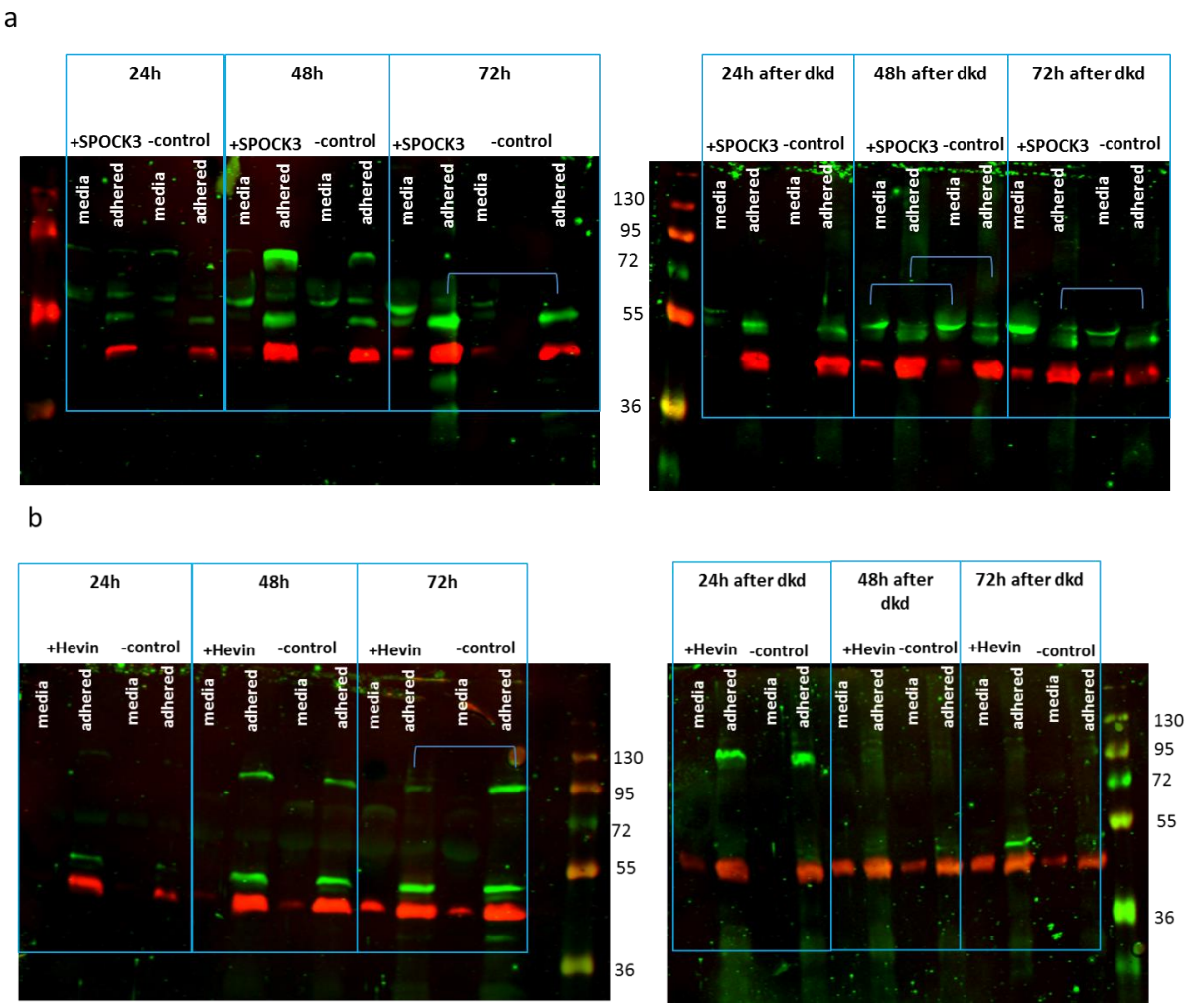

Figure 8.11: Full blots showing SPOCK-3 and hevin expression in INS-1 cells following SMARTPOOL siRNA double knockdown. Shown above are full blots representative of 3-4 independent experiments for the knockdown of (a) SPOCK-3 and (b) hevin using SMARTPOOL siRNA. INS-1 cells ($1x10^4$) were transfected with SMARTPOOL siRNA for hevin and SPOCK-3 for 48 hours. Media was thereafter aspirated and replaced with fresh media supplemented with new siRNA. The second knockdown was carried out for another 48-72 hours. Non-targeting rat pooled siRNA was used as a control. Detached and adhered cells were collected and lysed for western blot analysis. β-actin was used as a loading control. Green in IR800-conjugated secondary antibodies detecting either (a) SPOCK-3 or (b) hevin. Red in IR700-conjugated secondary antibody detecting β-actin. Dkd=double knockdown

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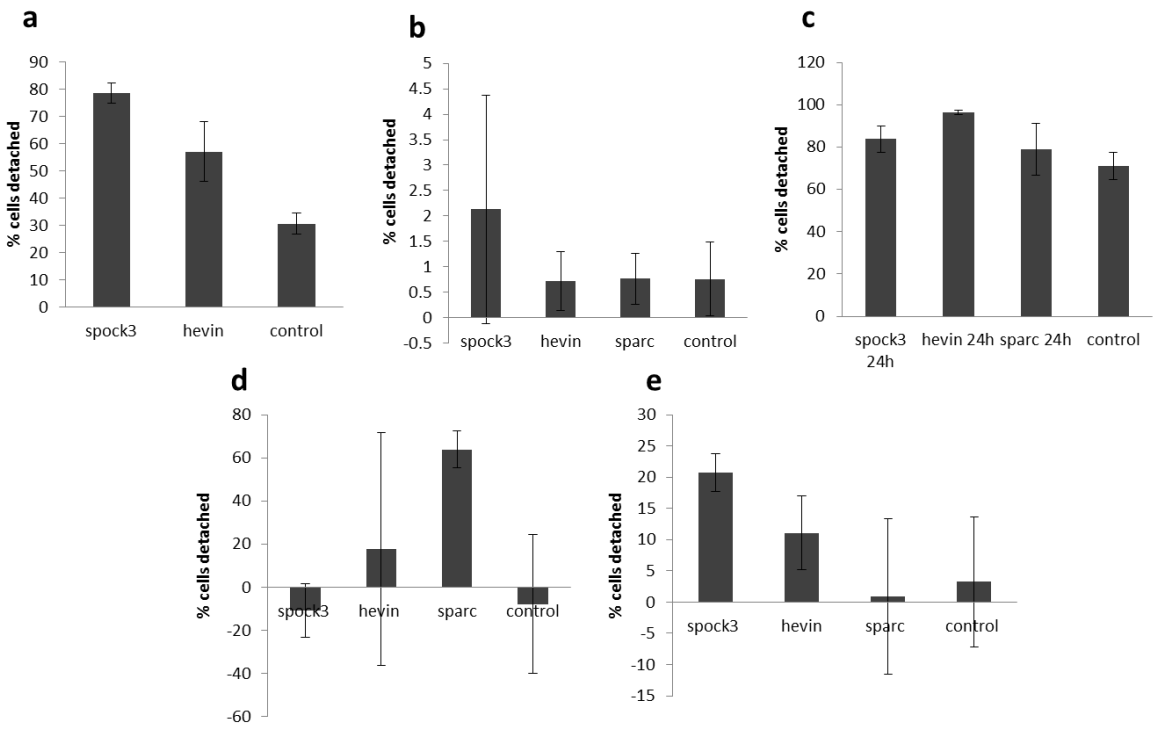

Figure 8.12: Individual experiments showing the effect of the SPARC family on β-cell adhesion. Individual replicates showing variability for the de-adhesion assays described in Section 5.2.2. INS-1 cells ($3x10^4$ cells/well) were plated in 96 well plate to adhere for up to 48 hours. Media was aspirated and cells were treated with fresh media supplemented with 5 µg/mL of SPARC, hevin, or SPOCK-3. Cells were then treated for a further period of 48 hours. Cells were imaged before and after PBS washing to analyse cell detachment. De-adhesion was measured as the percent area of cells detached after washes. Graphs showing +/- SEM from N=3 replicates for each of 5 independent experiments.

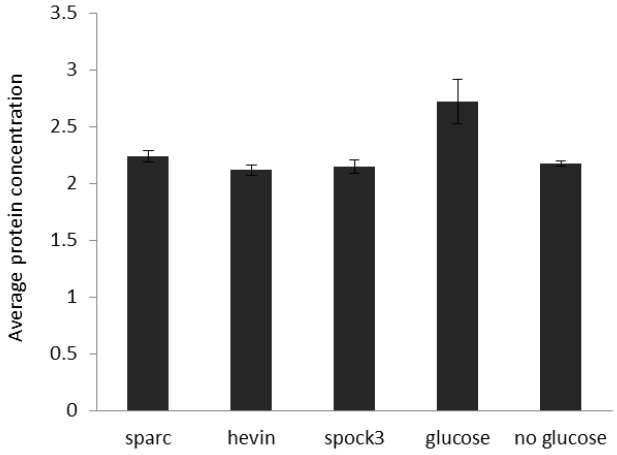

Figure 8.13: Protein concentration of cells subjected to glucose stimulation for insulin ELISA. INS-1 cells ($3x10^4$ cells/well) were plated on to 96 well plates and adhered for up to 48 hours. Cells were then washed in PBS and starved in 2 mM glucose in KRBH for 2 hours at 37°C. Cells were stimulated with or without 20 mM glucose in KRBH for 30 minutes at 37°C. The supernatant was collected and subjected to an ELISA. Cells were lysed and subjected to a BCA protein quantification assay to measure protein content as a control for cell density. Graph showing average protein concentration +/-SEM, N=8-9 (2-3 replicates pooled from 3 independent experiments).

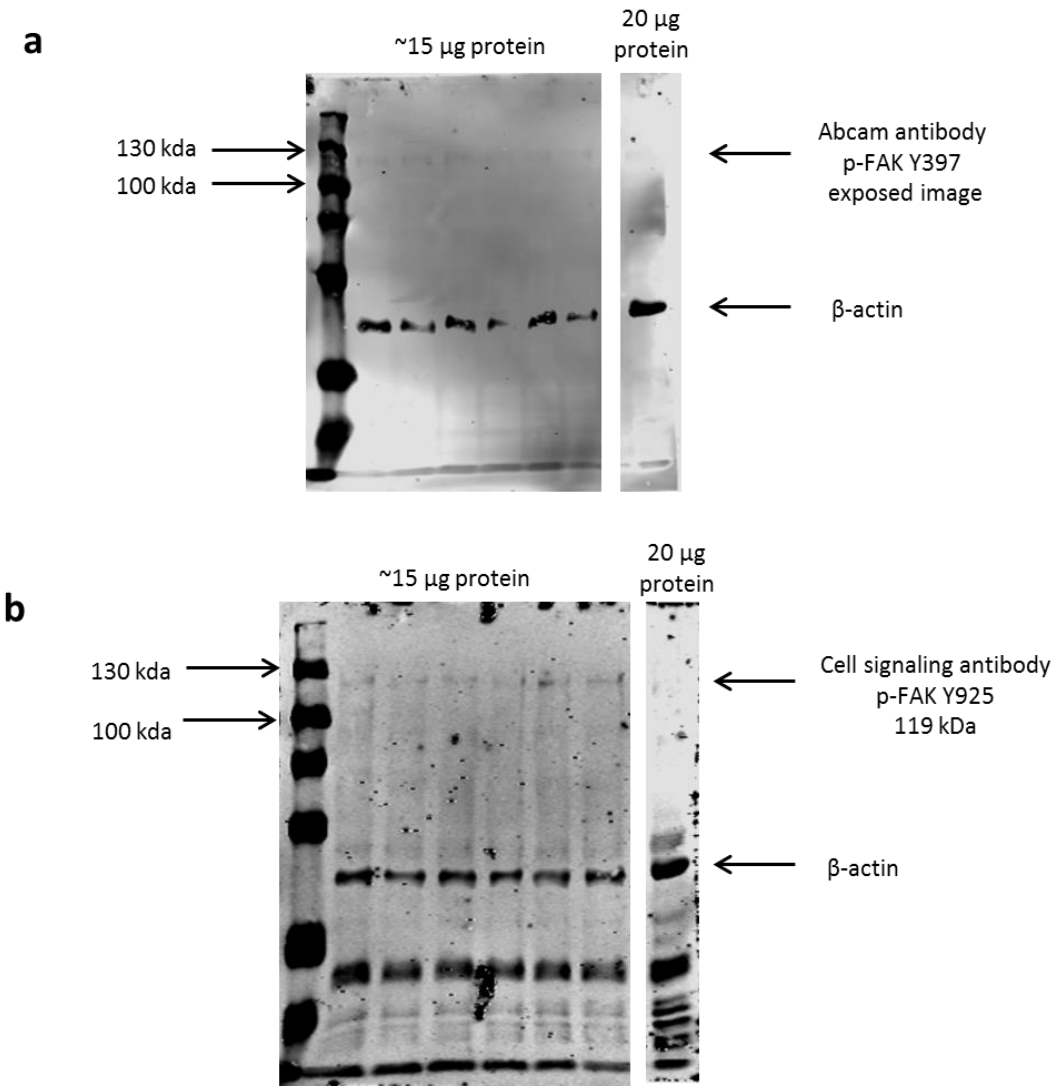

Figure 8.14: Validation of glucose-stimulated p-FAK expression. INS-1 cells ($3x10^4$ cells/well) were plated on to 96 well plates and adhered for up to 48 hours. Cells were then washed in PBS and starved in 2 mM glucose in KRBH for 2 hours at 37°C. Cells were stimulated with or without 20 mM glucose in KRBH for at the indicated times at 37°C. Cells were lysed and p-FAK expression was measured using western blotting with (a) antibodies for P-FAK Y37, Abcam or (b) P-FAK Y925, Cell Signaling. β-actin was used as a loading control. About 20 µg of INS-1 lysate was also loaded as a control. (a) Image was over-exposed to reveal bands for p-FAK indicated by arrows (~119 kDa).
