Novel Culture of Human Islets with Pancreatic Matrix Proteins

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Abstract

Background

The islet basement membrane (BM) is dissociated during the digestion phase of human islet isolation. A number of studies have demonstrated the beneficial effects conferred by extracellular matrix proteins (ECMP) of the BM on non-human islet function, but data are lacking regarding primary human islets. The aim of this study was to investigate the protective effects of supplementation of Connaught Medical Research Laboratories culture medium (CMRL) with Nidogen-1 (NID), Laminin-521 (L521), Perlecan (PLC), and Collagen-IV (C-IV), individually and in combination, on islets in normoxic and a subsequent hypoxic culture simulating the enhanced stresses encountered during islet transplantation.

Methods

With appropriate consent and ethical approval, human islets were isolated from donor pancreases using standard techniques. Firstly, a titration of ECMPs ranging from 25 to 100nM was performed, with the most effective concentration used for subsequent experiments. Next, isolated islets were pre-cultured in standard clinical CMRL or CMRL supplemented with 25nM NID, 25nM L521, 25nM PLC, or 100nM C-IV, for 24-48 hours in normoxia (21% O_2, 37^{o}C). Islets subsequently underwent culture in an *in vitro* transplant simulation where islets were cultured in ECMP-free CMRL for 48-72 hours in hypoxic conditions (1% O_2, 37^{o}C) and were assessed pre- and post-hypoxic culture for survival and function. ECMPs were then combined and the effects on islet survival and function were compared both to individual ECMPs and CMRL in an identical procedure.

Results

Titration experiments (n=8) demonstrated that 100nM of C-IV increased recovery of islets and reduced fragmentation versus 25nM whereas, all other ECMP showed no significant differences across concentrations. Individual protein experiments (n=12) revealed that, after normoxic culture, all ECMPs decreased fragmentation. NID and PLC also demonstrated increased islet recovery, while C-IV, L521, and PLC increased VEGF expression. Post-hypoxic culture, PLC and NID reduced fragmentation and improved recovery. NID and C-IV increased INS gene expression, in addition to C-IV and PLC decreasing DDIT3 expression. When ECMPs were combined (n=6), after normoxic culture, islets were less fragmented and demonstrated increased BCL2 expression, VEGF expression, recovery and stimulation index (SI), although viability was reduced. After hypoxia, MPC-preconditioning decreased fragmentation of islets as well as increasing recovery, viability, and SI.

Conclusions

This study suggests that ECMP supplementation of culture of media improves human islet survival and *in vitro* function. Pre-culture of islets in PLC, L-521 and NID increases islet survival and *in vitro* function after hypoxia. Combining these proteins further augmented individual benefits and hence, may therefore enhance islet graft function leading to improved outcomes post-transplant. These findings have potential application to both intraportal islet transplants and islets placed within macrocapsules.

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Abbreviations

ACTB – β-Actin gene

BAX – Bax (Bcl-2- Associated X Protein) gene

BCL2 - Bcl-2 (B-cell lymphoma 2) gene

BM – Basement Membrane

BMI – Body Mass Index

CASP3 – Caspase 3 gene

CIT – Cold Ischaemia Time

C-IV – Collagen-IV

C-VI – Collagen-VI

CMRL – CMRL-1066 (Connaught Medical Research Laboratories Medium 1066)

DBD – Donor after Brain Death

DCD – Donor after Circulatory Death

DDIT3 – Ddit3 (DNA damage-inducible transcript 3) gene – also known as Chop (C/EBP homologous protein)

DNA – Deoxyribonucleic Acid

DTZ – Dithizone

ER – Endoplasmic Reticulum

FCS – Foetal Calf Serum, also known as Foetal Bovine Serum (FBS)

GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase gene

GSIS – Glucose-Stimulated Insulin Secretion

KRB – Krebs Ringer Buffer

HIF1A – Hif-1α (Hypoxia- Inducible Factor 1 – Alpha Subunit) gene

HSPG2 – Heparan-Sulphate Proteoglycan 2 (Perlecan)

IBMIR – Instant Blood-Mediated Inflammatory Response

IEQ – Islet Equivalent Number

IN – Islet Number (absolute)

INS – Insulin Gene

L521 – Laminin-521

MPC – Matrix Protein Combination

mRNA – Messenger Ribonucleic Acid

NID – Nidogen-1

PBS – Phosphate-Buffered Saline

PLC – Perlecan (Fragment V of full protein)

qRT-PCR – Quantitative Polymerase Chain Reaction

RGD - Arginine-Glycine-Aspartic acid (amino acid sequence)

RNA – Ribonucleic Acid

rRNA – Ribosomal Ribonucleic Acid

SEM – Standard Error of the Mean

SI – Stimulation Index

T1DM – Type 1 Diabetes Mellitus (T1DM)

UW – University of Wisconsin Solution

VEGF – Vegfa (Vascular Endothelial Growth Factor) Gene

WIT – Warm Ischaemia Time

WPT – Whole-Organ Pancreas Transplantation

Chapter 1: Background and Aims

1.1 Type 1 Diabetes Mellitus

1.1.1 Prevalence and Aetiology

Type 1 Diabetes Mellitus (T1DM) is a chronic condition characterised by loss of normal blood-glucose regulation. Previously known as 'Insulin-Dependent' or 'Juvenile-Onset' diabetes, T1DM affects millions of people around the globe and it has been estimated that approximately 42 million people are living with the condition, of which 542000 are children (IDF, 2015; World Health Organization, 2016). T1DM is characterised by elevated blood glucose levels resulting from a loss of insulin production and is caused by autoimmune-mediated destruction of the insulin-producing β-cells found within the islets of Langerhans. Named after the German anatomist Paul Langerhans, who discovered them in 1869, islets of Langerhans (or islets) are clusters of cells located within the pancreas. An average healthy pancreas contains approximately three million islets (Ionescu-Tirgoviste *et al.*, 2015) and each islet contains several thousand hormone-producing cells. Whilst the insulin-producing β-cells are a key component, other important cell-types include glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing γ-cells. These different cell types act to regulate blood glucose levels synergistically through the varying actions of the peptides they secrete. In T1DM, the body's own natural killer and cytotoxic T-cells selectively target and destroy pancreatic β-cells (Dotta *et al.*, 2007; de Beeck and Eizirik, 2016). Although the precise aetiology is currently unknown, data demonstrating expression of viral RNA in newly-diagnosed patients suggest that infection, particularly enteroviral, might be the trigger for immune destruction in genetically predisposed individuals (Gamble and Taylor, 1969; Schulte *et al.*, 2010).

1.1.2 Current Treatment

Until the 1920s, T1DM was largely untreatable. However, following the discovery of insulin by Canadian scientists Banting and Best (Banting *et al.*, 1922), exogenous insulin became the mainstay treatment for T1DM. This transformed the condition from one that was largely fatal to one in which those afflicted can lead a relatively normal life. Since then, insulin products and regimes have improved dramatically. Until the 1980s, porcine or bovine insulin were universally used, but advances in treatment have seen their replacement with recombinant human insulin and insulin analogues. In parallel with this, technological developments have led to many patients using a continuous insulin pump, rather than intermittent insulin injections. More recently, the production of sophisticated insulin pumps linked to glucose sensors has offered a more refined 'physiological' method of management, with real-time control of blood-glucose levels. However, while these devices have improved dramatically over the last few decades, there remain a number of challenges preventing these from truly mimicking normal glucose homeostasis. These include mechanical factors such as blockages, leaks, battery issues, and pump failure (Guilhem *et al.*, 2009; Ross *et al.*, 2015) as well as limitations arising from cost.

Overall, for many patients, insulin therapy for the treatment of diabetes leads to short term stability and control. However, the challenge now is to prevent the long-term complications from developing and reversing those that have already become established. In some patients, blood glucose levels fluctuate dramatically on a daily basis, despite a strict insulin regime, leading to hyper- and hypoglycaemia. Hypoglycaemia occurs when insulin levels are too high or glucagon levels too low, the resultant low blood glucose levels leads to 'warning signs' including fatigue, sweating and dizziness, amongst other symptoms, as the central nervous system is starved of glucose. In extreme cases there is a danger of coma and death. With frequent and recurrent episodes of hypoglycaemia these classic 'warning signs' become less

detectable and patients can develop dangerous and life-threatening hypoglycaemic unawareness. Whilst it is possible to run blood glucose levels higher in these patients to try to prevent severe hypoglycaemia, this then makes them vulnerable to the dangers and complications of hyperglycaemia.

Hyperglycaemia on the other hand, occurs where insufficient insulin or excessive glucagon produce high blood glucose levels. Adipose tissue releases free fatty acids and tissues begin to metabolise amino acids and triglycerides instead of glucose. Free fatty acids are transported into the mitochondrial matrix, a process normally inhibited by insulin, and converted into ketones. Excretion of ketones through urine leads to increased loss of potassium and, in extreme cases, this can lead to hypokalaemia, resulting in muscle weakness and, if left unchecked, paralysis, and respiratory failure. Prolonged mild hyperglycaemia is the primary cause of many of the long-term complications of T1DM (The Diabetes Control and Complications Trial Research Group, 1993). It initiates the formation of advanced glycation end-products through the Maillard reaction, where sugars undergo non-enzymatic reduction with the amino groups within proteins, lipids, and nucleic acids. Most notably this causes the glycation of haemoglobin and the production of haemoglobin A1c, the presence of which is used to diagnose T1DM. Crosslinks are also formed within the extracellular matrix and vessel walls, reducing flexibility and impairing function of the vasculature (Vlassara, Brownlee and Cerami, 1986). As a consequence, blood delivery to tissues and organs may be significantly reduced, resulting in ischaemic damage (Forbes *et al.*, 2004, Soldatos and Cooper, 2006), as well as increasing the rate of heart disease and stroke to twice that of the non-diabetic population (Emerging Risk Factors Collaboration *et al.*, 2010). Furthermore, the loss of blood flow to the periphery increases the lifetime risk of amputations by up to 30 times (Ebskov and Ebskov, 1996; Moxey *et al.*, 2011). Concurrent with loss of blood flow, nephropathy is one of the most severe long-term complications of T1DM, with patients 15 times more likely than the general population to develop this condition. This can lead to renal failure and the need for dialysis or renal

transplantation (Go *et al.*, 2004; Einollahi *et al.*, 2008). Overall, it has been estimated that premature death related to the complications of diabetes affected almost four million people worldwide in 2012 (World Health Organization, 2016) and even in high-income countries, where a wide range of treatments is available, the life expectancy of those affected with T1DM is reduced by approximately 12 years (Huo *et al.*, 2016).

Therefore, the ideal treatment for T1DM would be one that maintains entirely normal glucose homeostasis by allowing release of islet hormones in a truly physiological manner, without relying on exogenous insulin or mechanical devices. Additionally, this treatment would be achieved soon after diagnosis, most likely during childhood, rather than once complications have developed. One way to achieve this is to replace the islet cells that have been lost thorough autoimmune destruction either through transplantation of a whole pancreas or by specifically transplanting the islets alone.

1.2 Whole Organ Pancreas Transplantation

Whole-organ pancreas transplantation (WPT), utilising an intact pancreas, is employed for patients with poor glycaemic control leading to acute metabolic complications, such as hypoglycaemia, hyperglycaemia, or ketoacidosis. It is also used to treat those for whom insulin therapy causes a significant emotional problem (American Diabetes Association, 2004). Often transplanted either simultaneously with or after a kidney transplant, patients with diabetes and end-stage renal disease are the primary recipients of WPT. First successfully implemented in 1966 (Kelly *et al.*, 1967), development of WPT accelerated throughout the 1980s after the discovery of cyclosporine (Dholakia *et al.*, 2016), an immunosuppressant drug that prevents organ rejection in transplant patients, and improvements in surgical techniques. Since then, this has become the clinical standard treatment for poorly-controlled T1DM with over 40 000

transplant performed (Dean *et al.*, 2017). While it has a high success rate, WPT is a majorly invasive surgical undertaking with a number of inherent risks and secondary complications (Humar *et al.*, 2004a; Finger *et al.*, 2013). In 2003, a study by Venstrom *et al.* (2003) showed a more than 10% higher mortality rate after transplant compared with patients on the waiting list in the USA. More recent data have demonstrated a reversal of this statistic, with a 21% lower mortality rate after transplant compared to those remaining on the waiting list (Van Dellen *et al.*, 2013). Even so, given that surgery-related mortality remains at approximately 4% and the likelihood of re-surgery at 29% (Gruessner and Gruessner, 2012; Rudolph *et al.*, 2015), a whole pancreas transplant does not always represent the most suitable option for the patient and is unlikely ever to become applicable to children soon after diagnosis.

1.3 Islet Transplantation

Islet transplantation on the other hand, is a minimally invasive treatment where only the isolated endocrine portion of the pancreas is transplanted.

1.3.1 History of Islet Transplantation

The first attempt to transplant processed pancreatic tissue was performed in Bristol by Watson-Williams in the 1890s. A subcutaneous graft of homogenised sheep's pancreas was used to treat a 13-year-old boy with severe ketoacidosis, although, while there was a temporary improvement in glycosuria, the boy died 3 days later rejecting the xenograft (Watson-Williams, 1894). Almost 80 years later, the first successful islet transplant in a non-human model was achieved by Ballinger and Lacy (1972). Streptozotocin-induced diabetic rats were made normoglycaemic by transplantation of isolated rat islets into the peritoneal cavity. It was a further eight years

before the first human islet transplant was accomplished by Najarian *et al.* (1980), with autotransplantion of islets isolated from patients with pancreatitis after total pancreatectomy. Success further improved into the 1990s with several patients receiving autotransplants and remaining insulin independent for up to 7 years (Pyzdrowski *et al.*, 1992).

Progress was also being made at this time towards allogeneic transplants. A number of groups demonstrated temporary insulin independence (Scharp *et al.*, 1990), or reduction in insulin requirement (Warnock *et al.*, 1989), in small numbers of patients transplanted with islets isolated from individual or pooled donors (Warnock *et al.*, 1991). While only around 12% of patients maintained insulin independence post-transplant for more than 1 week (Close *et al.*, 2004), success in eliminating hypoglycaemic episodes was achieved longer-term (Alejandro *et al.*, 1997). In 2000, clinical islet transplantation was dramatically advanced by the introduction of the 'Edmonton Protocol' of immunosuppression consisting of sirolimus, tacrolimus, and daclizumab (Shapiro *et al.*, 2000). This enabled the routine, and lasting, reversal of diabetes in the non-human models to be translated to the bedside. Since 2005 in the UK, those requiring islet transplantation have had the therapy funded by the NHS.

1.3.2 Practical Aspects of Islet Transplantation

Clinical islet transplantation consists of 2 stages: first, extraction of isolation of islets from the human donor pancreas and second, the transplantation of the isolated islets.

1.3.2.1 Islet Isolation

Islet transplantation currently relies on the efficient extraction of high numbers of quality islets from a donor pancreas. Isolating thousands of intact 'mini-organs' from within a major organ

is a unique challenge. This is achieved through a combination of enzymatic and mechanical digestion of the pancreas, followed by separation of the islets from associated digested exocrine tissue. With appropriate consent and regulation, the pancreas is procured from either a 'Donor after Brain Death' (DBD) or a 'Donor after Circulatory Death' (DCD).

After retrieval, organs are shipped from the operating theatre to the Human Islet Isolation Facility in a cold-storage medium, usually University of Wisconsin solution (UW), at 4°C. This aims to preserve cellular functions through suppression of metabolism in addition to aiding in prevention of reperfusion injury. Upon reaching the isolation facility, the pancreas is dissected away from the attached visceral fat, spleen, and duodenum. The pancreas is cannulated through the main pancreatic duct and infused with a mixture of bacterial collagenase, derived from *Clostridium histolyticum*, and neutral protease in order to fully disperse enzymes around the pancreas (Lakey et al., 1999). It is subsequently cut into small pieces before being sealed into a Ricordi digestion chamber containing ball bearings. This is then placed into a digestion circuit, as part of the semi-automated method of digestion developed by Ricordi *et al.* (1988). The chamber is then shaken, either automated or by hand, to break-down the pancreas mechanically while media is circulated through the chamber at a temperature of 37°C to facilitate optimal enzymic activity. The combination of mechanical tissue disruption and enzymic digestion of the extracellular matrix allows dissociation of endocrine and exocrine tissue. Liberation of islets from the surrounding tissue is confirmed through sampling and microscopic analysis of the circulating medium. Once a good number of 'free' islets are visualised, the circuit can be opened to allow collection, washing, and pooling of the pancreatic digest. In order to then separate islets from the digested exocrine tissue, the islets are purified using a continuous density gradient. This involves centrifugation of tissue within a pre-loaded density gradient media using a COBE 2991 cell processor (London and Bell, 1992). Achieving an islet purity of >50% at this stage is important as infusion of the portal vein with large amounts of unwanted exocrine tissue poses a significant risk of portal vein thrombosis (Mittal *et al.*, 1981; Toledo-

Pereyra *et al.*, 1984; Hyon *et al.*, 2004). The overall result of the isolation is then formally assessed at this stage by performing an islet count, assessing islet purity, and calculating percentage viability. Successful islet preparations are then cultured in a CMRL-based culture medium prior to transplantation. This allows recovery from the stresses of the isolation process and ensures the islet preparation does not fall below the transplant criteria. Any dramatic decline in islet number or viability is also critically assessed as this may be an indicator of poor graft survival post-transplant.

Unfortunately, not all isolations successfully lead to a transplant. While there is a loss of islets during the isolation process, a further 60% of islets are lost post-transplant (Biarnes *et al.*, 2002; Eich *et al.*, 2007). This increases the required graft size and so 5000 IEQ per kilogram of recipient body weight (McCall and Shapiro, 2012) is currently the minimum islet requirement, along with good viability, above 70% viable cells, and islet purity above 50%. To ensure that these strict criteria are met, islets are cultured for 24-48 hours. Even with these standards, patients will usually require further 'top-up' grafts to ensure maximal restoration of glycaemic control and restoration of hypoglycaemic awareness.

1.3.2.2 Islet Transplant

After the 24-48 hour culture period has elapsed, islets are recounted and their viability is assessed. If these parameters have remained fairly consistent, with minimal deterioration in number or viability they are subsequently prepared for transplant. Once an islet isolation is deemed as successfully meeting the transplant criteria, the patient is prepared for the transplant through induction of the immunosuppressive regime. Islets can then be transplanted with minimal risk of rejection. Being minimally invasive, the transplant is performed in radiology under local anaesthesia. The patient's portal vein is cannulated by percutaneous transhepatic

catheterization, with a contrast medium infusion used to confirm successful cannulation (Weimar *et al.*, 1999). The islet can then be infused gently under gravity into the liver.

1.3.2.3 Indications for Islet Transplantation

Currently in the UK, islet transplantation is performed in order to treat and reverse life-threatening hypoglycaemic episodes due to the dangers of immunosuppression. As such, islet transplantation is considered for patients with a history of recurrent severe hypoglycaemia for more than 1 year, despite compliance with an optimised insulin regime. This required clear documentation of the patient having experienced multiple hypoglycaemic episodes, with documented evidence of blood glucose levels below 2mmol/L, resulting in third party intervention to administer dextrose or glucagon injection. In many cases, these hypoglycaemic episodes have resulted in seizures or even comas. For islet transplant alone, patients must have normal renal function or, for islet after kidney transplant, a functioning renal graft. Furthermore, recipients must be over 18, with established T1DM for over 5 years, in addition to presenting no evidence of insulin resistance, requiring an insulin dose of more than 0.7U/Kg.

1.3.2.4 Recent Clinical Outcomes

With over 1500 transplants performed worldwide since 2000, approximately 50% of patients retaining insulin independence over 5 years, and over 70% retaining good glycaemic control 10 years later (Shapiro, Pokrywczynska and Ricordi, 2016), islet transplant can be considered very successful. Indeed in the UK, after the first 3 years of availability on the NHS-funded integrated islet transplant program, graft function was 80% at 2 years post-transplant with hypoglycaemic episodes reduced by 98.5%. Furthermore, 45% of UK patients achieved a bonus of insulin

independence, with an overall 60% decrease in insulin requirement over 2 years (Brooks *et al.*, 2013). However, the immediate focus, in order to further enhance the outcomes of transplantation, are increasing resistance of islets to hypoxia and protecting them from the immediate blood-mediated immune reaction (IBMIR).

1.3.3 Ongoing Challenges

1.3.3.1 Limited Donor Pool

One of the primary factors limiting the number of islet transplants that may be carried out is the limited availability worldwide of donor organs. In addition, not all donor pancreases are suitable for islet isolation. Donor pancreases are divided between islet and whole-organ transplantation depending on donor variables as well as tissue-matching to the recipient. While whole-organ transplant has had more success with younger, leaner donors (Humar *et al.*, 2004b; Salvalaggio *et al.*, 2007), islet transplant tends to be more successful utilising older donors with a higher Body Mass Index (BMI). This bias is attributable to increased success found in isolation of islets organs from these older, high BMI patients (Lakey *et al.*, 1996; Nano *et al.*, 2005; Brandhorst *et al.*, 2009; Berney and Johnson, 2010). The improved islet yield is extremely important for the success of islet transplantation, although the exact mechanisms of this benefit are still to be determined. There is still, however, some overlap in criteria and so, where this competition does occur, compromise must be found. Ideally, a new, more abundant source of islets, such as xenogeneic, stem cells, or stem-cell derived, is required in order to meet the demand. However, given the additional hurdles that these sources currently present, at this time maximising the outcomes of allogenic isolation and transplantation is a more critical avenue of research.

1.3.3.2 Sub-Optimal Islet Engraftment

Transplantation of islets is normally achieved via infusion of tissue into the portal vein. However, this does not necessarily provide the most ideal site for the transplantation of islets. The initial post-transplant period of engraftment is characterised by severe hypoxia and local inflammation, which leads to the death of a large proportion of islets. This is due to a variety of factors, the most important of which is hypoxia and IBMIR. IBMIR is the primary cause of islet loss, the complement immune system and coagulation cascade trigger the destruction of transplanted cells (Bennet *et al.*, 2000; Johansson *et al.*, 2006; Sakata *et al.*, 2009). Secondary is the relatively hypoxic environment. Due to their high metabolic demand, islet receive 15-20% of the pancreatic blood flow, although they only represent 1-2% of the total tissue volume of the pancreas (Lifson *et al.*, 2017, Jansson and Hellerström, 1983). Partial oxygen pressure of native islets is ~40mmHg, however, this drops to below 10mmHg post-transplant in the portal vein (Olsson *et al.*, 2011; Suszynski, Avgoustiniatos and Papas, 2014). As a consequence, this hypoxic situation can have a dramatically detrimental effect on islet energy generation (Hellman *et al.*, 1975) and insulin secretory capacity via ATP-sensitive potassium channels (Ashcroft and Rorsman, 1990). In addition to the harmful effects of immunosuppression on the patient, long-term immunosuppression has been shown to be toxic to beta cells, (Drachenberg *et al.*, 1999; Tanemura *et al.*, 2009), leading to cell death and impairing graft function.

During the isolation process, islets are extracted from their normal pancreatic environment, becoming devascularised and dependant on the passive diffusion of nutrients and oxygen. These conditions, present both during *in vitro* culture and persisting after transplantation (Jansson and Carlsson, 2002) further exacerbate the hypoxic challenge faced by islets. Full revascularisation of implanted islets takes approximately 7-14 days, although this still does not necessarily restore perfect oxygen supply (Carlsson, Palm and Mattsson, 2002). Furthermore, due to the critical

nature of the process of angiogenesis, the growth of new blood vessels, to islet function and morphological integrity, islets poorly vascularised beyond this point exhibit reduced function.

1.3.3.4 Eliminating the Need for Immunosuppression

In the UK currently, islet transplantation is only considered for those with severe and potentially life-threatening hypoglycaemic unawareness, an inability to detect dangerously low blood-glucose levels. This is primarily due to the dangers of immunosuppression. While immunosuppressants allow the toleration of transplanted allograft tissue, they allow opportunistic infection by pathogens that do not normally cause disease in a healthy host to occur (Huurman *et al.*, 2006). Examples of this include pneumonia or herpes. Moreover, immunosuppressants can also induce side-effects similar to those found in patients treated with chemotherapy including mouth ulceration, anaemia, leukopenia, diarrhoea, headache, neutropenia, nausea, and fatigue (Shapiro *et al.*, 2006). In addition, the risk of skin, and other cancer, development is greatly increased with prolonged immunosuppression (Vajdic *et al.*, 2006). In order to expand the criteria for transplantation these severe unwanted effects would have to be removed, thereby improving the quality of life for many T1DM patients. A number of strategies have been considered to circumvent this issue, such as induction of immune tolerance by co-transplanting islets with T-regulatory cells or immune-modulating mesenchymal stem cells (Li *et al.*, 2010; Kerby *et al.*, 2013), or protecting the islets from the immune system altogether using islet encapsulation.

1.3.4 Islet Encapsulation

Islet encapsulation is an adjunctive technology for islet transplantation whereby islets are shielded from the immune system by encasement within protective capsule, while insulin secretion is still permitted through capsular pores. The attraction of such technology is that it could render the use of immunosuppression unnecessary. This would not only remove the potentially dangerous side-effects previously described, but would also increasing the eligibility criteria to patients for whom, currently, the hazards outweigh the benefits. This could pave the way for islet transplantation to be used in newly diagnosed children from the beginning, ensuring tight glycaemic regulation when it can be challenging (Daneman *et al.*, 1999). Furthermore, as such technology isolates cells from the recipient's immune system and safely contains implanted cells, it opens up the opportunity of utilising cells derived from sources other than donated human organs, such as xenogenic and stem cells. This in turn could dramatically extend the transplantable cell resource enabling much greater numbers of patients to benefit from this important treatment. Finally, shielding islets from the harmful effects of IBMIR and the immunosuppressive agents could present advantages to graft function or reduce the number of islets required for transplantation.

Successful encapsulation of islets must not only offer protection from immune-mediated destruction, but must also allow the cells uninhibited access to oxygen, glucose, and essential nutrients, in addition to efflux of hormones and waste products.

There are two major strategies employed in bringing encapsulation to fruition; microencapsulation and macroencapsulation:

Microencapsulation encases single, or sometimes several, islets in individual capsules of a bio-compatible material prior to implantation. The most common material for this is alginate, an anionic polysaccharide produced by brown algae (Leung *et al.*, 1983; Omami *et al.*, 2017).

Other approaches have shown great success modifying alginate with polyethylene glycol or ploy-L-lysine (Goosen *et al.*, 1985; Chandy, Mooradian and Rao, 1999; Desai *et al.*, 2000; Strand *et al.*, 2002) or using alternate materials such as polysulphone (Lembert *et al.*, 2001; Petersen *et al.*, 2002). Once coated, islet-containing capsules may be transplanted, however there is a greater chance of full or partial blockage of the portal vein, due to their enlarged size. Portal embolism and resultant interruption of blood-flow can lead to subsequent graft failure. Explantation of these failed grafts is not practically feasible and therefore capsule size reduction (Kobayashi *et al.*, 2006) or alternative transplant sites, such as the kidney capsule (Dufrane *et al.*, 2006) and omentum (Kobayashi *et al.*, 2006), are being explored.

Macroencapsulation, by comparison, encloses an entire, or multiple, islet preparations within a single device. The device can be implanted in one of two ways; intravascularly or extravascularly, each with their own advantages and disadvantages. Intravascular devices are connected directly to the vasculature and allow fast diffusion of small molecules and nutrients through the device while filtering out the larger molecules and immune cells. Whilst this approach generally allows good oxygenation and diffusion of insulin (Kobayashi *et al.*, 2006), some differences found by Monaco *et al.* (1991) in xenograft and allograft efficacy results indicate that complete protection from immune-mediated islet destruction may require further development. Further disadvantages lie, primarily, in the risks of surgical implantation by anastomosis and subsequent blockage of the vasculature connection by thrombosis (Zhu *et al.*, 2015). Blockage leads to ischaemic cell death requiring secondary surgery to unblock or remove the device. By comparison, extravascular devices are implanted in either a subcutaneous, sub muscular or intra-peritoneal site and are not directly connected to the vasculature. These devices, such as the MAILPAN device produced by Defymed (see Figure 1.3.4.1), require vascular growth over the outer membrane for passive diffusion of molecules. They can be transplanted and explanted with minimal risk to the patient. However, there is limited transmission of nutrients, insulin, and oxygen across the membrane (Colton, 1995;

Avgoustiniatos and Colton, 1997). Furthermore, inflammatory-mediated fibrotic growth can impair transplant function (Algire and Legallais, 1949; Prehn, Weaver and Algire, 1954), however advances in biocompatible materials and selection of immune-privileged locations provide simple solutions.

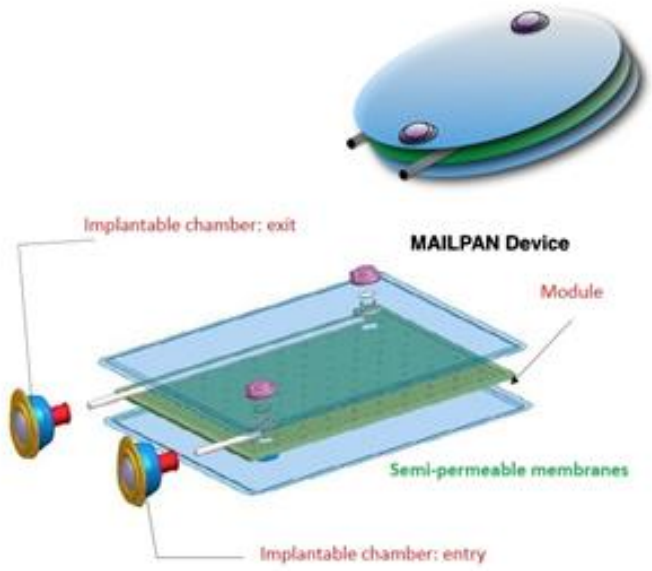


Figure 1.3.4.1: MAILPAN extra-vascular macroencapsulation device (courtesy of Defymed)

While there has been much success utilising macroencapsulation in numerous animal models (Strautz, 1970; Brauker *et al.*, 1995; Storrs *et al.*, 2001; Lamb *et al.*, 2011; Ludwig *et al.*, 2013; Bowers *et al.*, 2017), the slow rate of oxygen transfer, and the resulting hypoxic environment, represents a significant challenge for these islets. There have been a number of solutions proposed to combat the severe hypoxia within the macroencapsulation devices, for instance, the Beta-O_2 device utilises a direct infusion of an O_2/CO_2 mix into the device (Ludwig *et al.*, 2010, 2012). However, infusions must be made daily and neglect leads to graft failure. Other solutions include co-transplantation of oxygen generating microalgae (Bloch *et al.*, 2006),

mesenchymal stem cells (Vériter *et al.*, 2011; Vaithilingam *et al.*, 2017), or oxygen generating biomaterials (Pedraza *et al.*, 2012). Further consideration must be made toward mechanical stresses, such as sheer frictional and collisional forces, impacted upon islets free-floating within devices. Simple resolutions employ matrices or adherent inner membranes within the device. This additionally prevents sedimentation of islets, which further exacerbates hypoxic conditions.

Maintaining islet survival and function is the key to the success of these devices. Advancing our understanding of the islet-matrix relationship and the disruptions to this interaction that occur as a result of the islet isolation process is one of the fundamental steps to improving islet survival and function. Illuminating the mechanisms involved, and how they may be manipulated for advantage, will provide key areas with which islet transplantation may be augmented.

1.4 Islet Basement Membrane

1.4.1 Structure and Function

The extracellular matrix is a protein scaffold that surrounds cells in tissue providing support, segregation between tissues, and also relays molecular signals back to the cells. Secreted by the cells themselves the signals conveyed by the extracellular matrix influence a number of physiological functions including proliferation, migration, apoptosis, and differentiation (Miner and Yurchenco, 2004; Yurchenco and Patton, 2009). The extracellular matrix can be divided into two broad categories; the interstitial matrix, conferring flexibility to tissues, and the basement membrane (BM) a sheet-like structure that acts as a barrier to molecules and cells. Unique to human islets, a double layer BM is present surrounding islet vasculature (Virtanen *et al.*, 2008). Each layer has a distinct structural make-up, but like all extracellular matrices is

primarily composed of collagens, laminins, nidogens, and proteoglycans (Paulsson, 1992; van Deijnen *et al.*, 1992; Bix *et al.*, 2004; Yurchenco and Patton, 2009; see Figure 1.4.1). Each protein performs a discrete function, both as part of cellular interaction and within the architecture of the matrix.

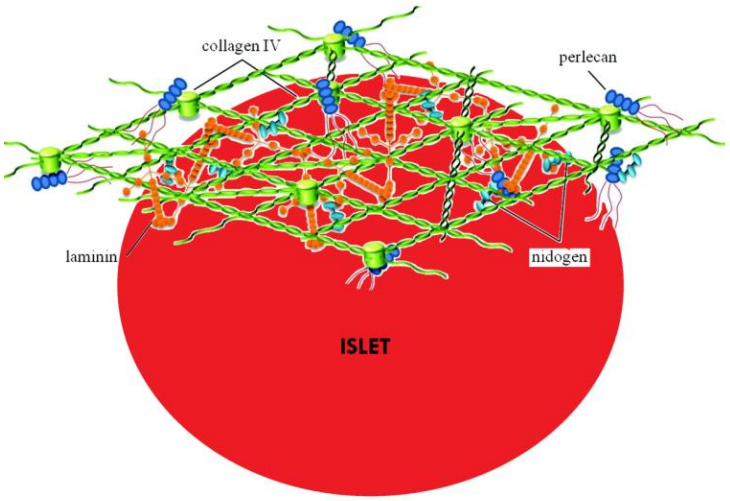


Figure 1.4.1: Islet basement membrane (adapted from Chang et al. 2011)

Structurally, collagens, particularly collagen IV (C-IV), and laminins form the basic foundations of the BM, they are able to self-aggregate to form a matrix structure (Yurchenco and Furthmayr, 1984; Hussain, Carafoli and Hohenester, 2011; Hohenester and Yurchenco, 2013) and from this other proteins associate to enhance functional capabilities further. Nidogens have affinity for both C-IV and the laminin γ1 chain and therefore acts as a link between these two molecules (Fox *et al.*, 1991) while proteoglycans, such as perlecan and agrin, tend to influence the physiochemical function of the matrix as well as influencing cellular signalling (Yanagishita, 1993). Interactions with cells are mediated through integrins, heterodimeric transmembrane receptors composed of an alpha and a beta subunit and the specific composition of the two determines the specificity of the receptor. These are extremely important in cellular function as integrin matrix interactions are fundamental to the prevention of anoikis, a form of

apoptosis that occurs in non-adhered cells (Meredith, Fazeli and Schwartz, 1993; Frisch and Screaton, 2001). Determining the exact integrin expression for islets is complicated and has been the subject of some discrepancy, however, there has been some consensus on the presence of subunits α1,α3, α5, αv, β1, and β3 (Cirulli *et al.*, 2000; Kaido, Yebra, *et al.*, 2004; Virtanen *et al.*, 2008). The heterodimers formed by these subunits are specific for collagen, fibronectin and laminins (Pierschbacher and Ruoslahti, 1984). Although there is some evidence that fibronectin is present in human islets during development (Wang *et al.*, 2005), there is little to suggest it remains during adulthood. Fibronectin interaction with integrins occurs on the arginine-glycine-aspartic acid (RGD) binding motif present on the protein (Pierschbacher and Ruoslahti, 1984). This motif is also present on the heparan sulphate proteoglycan, perlecan, as well as nidogens found in the adult islet BM and therefore these proteins may replace fibronectin within the adult islet protein architecture.

1.4.2 Extracellular Matrix Proteins and Islets

Enzymatic degradation of the extracellular matrix is used to separate endocrine from exocrine tissue during the islet isolation process. This process leads to degradation of the islet basement membrane (Irving-Rodgers *et al.*, 2014; Cross *et al.*, 2017) and can induce anoikis in islets (Thomas *et al.*, 2001). Indeed, it has been shown that mantled islets that retain a 'halo' of exocrine tissue, and therefore retain an intact BM, have significantly improved function (Ricordi *et al.*, 1995). Moreover, gene expression studies in freshly isolated human islets demonstrate amplified expression of pro-apoptotic Bax, and minimal, or no, expression of anti-apoptotic Bcl-2 (Schwartz, Schaller and Ginsberg, 1995; Campbell *et al.*, 2012). Disruption to the balance of apoptosis-regulating factors after disruption of the integrin-matrix association further implicates the role of the BM in islet survival (Martin and Vuori, 2004). Multiple *in vitro* studies have demonstrated the benefits of the co-culture of BM protein with rat (Nagata *et*

al., 2001, 2002; Pinkse *et al.*, 2006), dog (Wang and Rosenberg, 1999), and pig islets (Edamura *et al.*, 2003) as well as in MIN6 β-cell lines (Pinkse *et al.*, 2006; Weber, Hayda and Anseth, 2008) and primary β-cells (Kaido, Yebra, *et al.*, 2004). However, given the unique nature of the human islet BM and integrin expression patterns (van Deijnen *et al.*, 1992), in order for observed benefits to be translated to a clinical setting, it is first necessary for primary human islets to be utilised. Furthermore, as the islet is a formed of a heterogeneous combination of cells, including α cells which may be critical in T1DM (Brown, Sinaii and Rother, 2008), beneficial effects of BM co-culture may not be limited to β cells. There is not a vast array of data in human islets, however two studies have been performed. The first, by the Montréal group (Daoud *et al.*, 2010) who demonstrated seeding of islets onto culture plates coated with collagen, laminin, and fibronectin. They established fibronectin was the most effective at maintaining islet structural integrity, while collagen adhered islets expressed insulin mRNA most highly. The second study, by Johansson *et al.* (2015), investigated spider silk as a structural support for *in vitro* human islet culture. They demonstrated that silk scaffolds, enriched with RGD binding motifs, improved islet survival by more than twice that of the control islets after 1 month in culture. Furthermore, human islets were more likely to bind to the silk matrix than mouse islets, further confirming the importance of studying human islets over animal models. While promising, neither study is immediately clinically applicable, requiring either extensive safety studies for the co-transplantation of the matrices, or enzymatic separation of islets and matrix, further damaging the BM.

1.5 This Thesis

1.5.1 Aims and Hypothesis

The research reported in this thesis is based on the following hypothesis:

'Primary human islet culture in media supplemented with proteins of the basement membrane improves survival and function of islets *in vitro* during a hypoxic challenge'

Therefore this project had four main aims.

1) To define the most effective concentration of the matrix proteins Collagen IV (C-IV), Laminin 521 (L521), Perlecan (PLC), and Nidogen (NID) across a range of concentrations.

2) To examine whether supplementation of these proteins, at their most effective concentration, into the islet culture medium confers benefits to survival and function after normoxic culture.

3) To examine whether supplementation of normoxic culture medium with these proteins further enhances survival and function after a subsequent culture period in a hypoxic challenge.

4) To test whether the proteins found to be effective are able to further enhance islet function and survival if combined together as in the native islet basement membrane.

1.5.2 Thesis Overview

The hypoxic environment that islets are subjected to post-transplantation, both within the portal vein and within a macroencapsulation device, represents a major hurdle to be overcome. This is particularly important given the high oxygen requirement of islets. Necrosis and pro-apototic signals triggered by hypoxia are further exacerbated by the loss of the survival-promoting islet-matrix interactions, resulting in cell death and reduced graft function. Reinstatement of these interactions may enhance islet survival and improve graft function. Improved islet survival could also reduce the threshold for minimum islet requirement, thereby increasing transplant numbers and minimising waiting time for patients. Although there have been a number of studies demonstrating the benefits of extracellular matrix proteins on islet survival in animal models, there have been few performed with primary human islets (Wang and Rosenberg, 1999; Nagata *et al.*, 2001, 2002; Pinkse *et al.*, 2006). This therefore provides a fruitful area of research.

Shown to be lost during the isolation process, C-IV, L521, PLC, and NID were investigated as potential enhancers of islet function (Irving-Rodgers *et al.*, 2014; Cross *et al.*, 2017). Given the novel nature of this study in human islets, a set of titration experiments were first required due to the lack of previous data providing optimal concentrations. These experiments are described in Chapter 2. Once optimal concentration was discerned, experiments in Chapter 3 compared the effects of each individual protein to an unsupplemented CMRL control, firstly in a normoxic environment and subsequently in a hypoxic environment. In Chapter 4, the effective proteins were then combined and tested against both individual proteins and the clinical CMRL control. Chapters 3 and 4 were designed to mirror the current clinical islet transplant protocol as closely as possible in order to maximise translational potential for the improved graft function. Therefore, islets were initially cultured post-isolation for 24-48 hours in clinical culture medium in normoxic conditions, mimicking the pre-transplant culture period. The culture medium was, at this point, supplemented with the proteins of the extracellular matrix. Subsequently, the islets

were cultured to simulate the hypoxic challenge encountered by islets in the portal vein or inside a macroencapsulation device in media supplemented with foetal calf serum (FCS) and without the extracellular matrix proteins. Islets were assessed for survival, viability, glucose-stimulated insulin secretion, and gene expression after both normoxic and hypoxic culture periods. Figure 1.5.1 demonstrates the procedure for Chapters 3 and 4. Finally, Chapter 5 concludes the study, drawing together the findings before discussing limitations and future work.

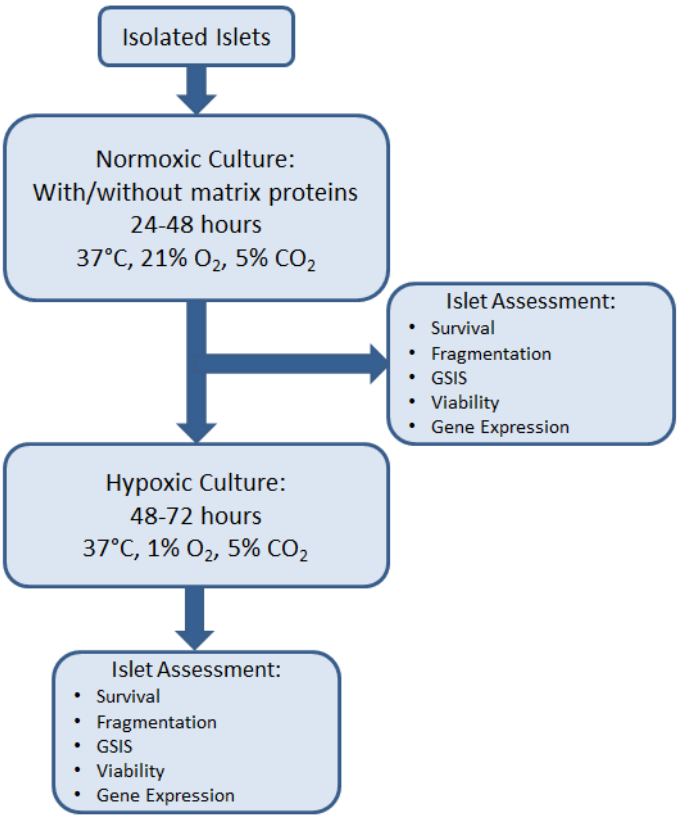


Figure 1.5.1: Basic flow chart of the transplant-simulating experimental procedures followed in Chapters 3 and 4.

Chapter 2: Determining the Optimal Concentrations of Extracellular Matrix Proteins for Supplementation of Islet Culture Medium

2.1 Introduction

Given the lack of available data regarding incubation of human islets with Collagen IV (C-IV), Laminin 521 (L521), Perlecan (PLC), and Nidogen-1 (NID; Lemos *et al.*, 2017), before being able to compare the effects of co-culture of islets with these proteins objectively it was necessary to perform a titration experiment. The aim was to determine the most effective concentration of the aforementioned extracellular matrix proteins across a range of concentrations for use as supplementary factors suspended within an islet culture medium. A study by Daoud *et al.* (2010) had previously investigated coating culture plates with extracellular matrix proteins – specifically C-IV, collagen I, and non-specific laminin - at a concentration of 6.25μg/cm^2, however these values do not translate to a 3-dimensional suspension culture. Furthermore, optimal concentration may not be identical for both 3-dimensional and 2-dimensional culture. For these reasons, this chapter describes the experiments used to elucidate the optimal concentration with which to supplement islet culture medium.

2.2 Methods

Isolated islets (isolated as described in Section 2.2.1) were cultured in CMRL-1066 (CMRL) supplemented with matrix proteins at 0nM, 25nM, 50nM, or 100nM for 48-72 hours in normoxic conditions (as described in Section 2.2.3) and were assessed post-culture for viability (as described in Section 2.2.4.1), fragmentation (as described in Section 2.2.4.2), recovery (as described in Section 2.2.4.2), and glucose-stimulated insulin secretion (as described in Section 2.2.4.3). The lowest concentration of extracellular matrix proteins utilised was 25nM in accordance with L521 manufacturer information (BioLamina, 2015). This was then increased

two-fold, to 50nM, and four-fold, to 100nM, with a concentration of 0nM employed for reference.

2.2.1 Islet Isolation

With appropriate consent and ethical approval, donor pancreases were retrieved from donors after brain death (DBD) and donors after circulatory death (DCD) using standard techniques. All donors met our clinical inclusion criteria and were less than 60 years of age, with a BMI below 37. In-situ perfusion was with University of Wisconsin solution (UW; Bridge to Life, London, UK). Pancreases were then placed in UW and transported to the Oxford DRWF Human Islet Isolation Facility at 4°C. Pancreases were subsequently processed and islets isolated using the well-established 'semi-automated method' (Ricordi *et al.*, 1988). Processed pancreases had a cold ischemia time (CIT) of less than 9 hours, defined as the interval between pancreas placement in the ice box and injection of the collagenase blend into the pancreatic duct. Isolated islets were counted and assessed for viability. A representative aliquot of islets was allocated for this project and stored by suspending the islets in UW solution at 4°C.

2.2.1.1 Donor Details

Human pancreases were retrieved from 8 human (5 male and 3 female) multi-organ DBD donors (see Table 2.2.1.1). For this study, 9868.3 ± 1893.2 islet equivalents (IEQ), with a viability of 79.2 % ± 2.2 and purity of 61.9% ± 4.2 were used. Individual islet preparations, from a single pancreas, were assessed separately and represented one technical replicate. This allowed for control of differences in donor factors across treatment groups.

Table 2.2.1.1: Islet donor details

8 DBD Donors	Median	Range
BMI	25.9	21.0 - 29.0
Age (Years)	40.0	40.0 – 59.0
CIT (Hours)	6.5	5.0 – 7.5
WIT (Mins)	32.5	0.0 – 98.0

BMI – Body mass index, CIT – Cold ischaemia time, WIT – Warm ischaemia time

2.2.2 Extracellular Matrix Proteins

The major components of the islet basement membrane (BM) collagens, laminins, nidogens, and heparan sulphate proteoglycans are digested and lost during islet isolation (Irving-Rodgers *et al.*, 2014; Cross *et al.*, 2017). These proteins therefore provide the most promising candidates for the improvement of islet function and, in order to ensure maximal benefit and clinical relevance, all proteins were sourced as recombinant human or human-derived. The specific proteins investigated were C-IV from human placenta (Sigma-Aldrich, Dorset, UK), recombinant human L521 (BioLamina, Sundbyberg, Sweden), recombinant human perlecan domain V fragment (PLC; R&D Systems, Minneapolis, USA), and recombinant human NID (Cloud-Clone Corp., Katy, USA).

2.2.2.1 Matrix Protein Concentrations

The proteins of interest, C-IV, L521, PLC, and NID were tested across a range of concentrations. A concentration of 25nM, as recommended by the manufacturers of L521 (BioLamina, 2015), was chosen as the lowest concentration investigated and subsequently increased 2-fold and 4-fold, to 50nM and 100nM respectively, to discern the optimal concentration (see Table 2.2.2.1.1).

Table 2.2.2.1.1: Matrix protein titration concentrations

	Concentration (nM)	0	25	50	100
Concentration (µg/mL)	**Collagen IV (440 kDa)**	0	11.00	22.00	44.00
	Laminin 521 (762 kDa)	0	19.05	38.10	76.20
	Perlecan (28 kDa)	0	0.70	1.40	2.80
	Nidogen-1 (150 kDa)	0	3.75	7.50	15.00

2.2.3 Islet Culture

2.2.3.1 Culture Medium

Islet culture was performed in CMRL supplemented with 2mM L-glutamine (Sigma-Aldrich), 23.8g/L Penicillin-Streptomycin (Sigma-Aldrich), 20mM HEPES (Sigma-Aldrich), and 2% Human Serum Albumin (HSA; Bio Products Laboratory, Elstree, UK) according to the clinical protocol. Islets were cultured at a seeding density >300 IEQ/cm^2 (Brandhorst *et al.*, 2016).

2.2.3.2 Matrix Protein Supplementation

Extracellular matrix proteins were additionally supplemented into the medium and added to 24-well suspension culture plates (Greiner Bio-One, Kremsmünster, Austria) at 500 µL per well according to Figure 2.2.3.2.1. The experimental groups were organised in rows and the wells were designated according to the assays for islet recovery, viability, and glucose-stimulated insulin secretion (GSIS). The culture period was for 48-72 hours at 37°C, 21% O_2, 5% CO_2.

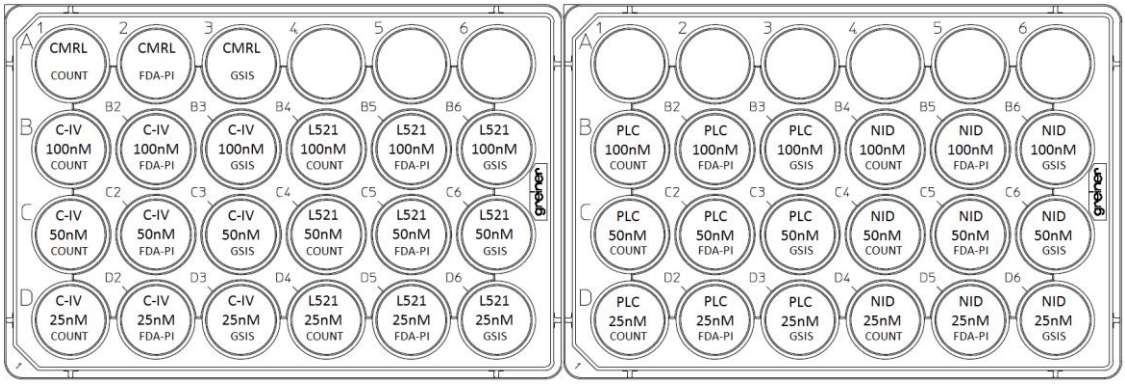


Figure 2.2.3.2.1: Islet culture plate map for titration experiment

2.2.4 Islet Assessment

Islets for all assessment parameters throughout this thesis, except for the islet count, were hand-picked and size-matched. Hand-picking allows the size-matching of islets and minimises the analysis of unwanted exocrine tissue. Size-matching of islets is important as this reduces variations in tissue volume, which can impact parameters such as GSIS where an increase in β-cell number will increase insulin secretion. Furthermore, different sized islets often have different properties, for instance large islets may secrete more insulin, as previously discussed, but have a lower viability, as oxygen is unable to permeate to the centre of the islet due to devascularisation. Therefore, size matching, across a range of islet sizes gleans a more representative assessment of the islet population.

2.2.4.1 Viability

40 size-matched islets per experimental group were hand-picked and assessed for viability by fluorescent staining with 0.67µmol/L of fluorescein diacetate (FDA; Sigma- Aldrich) for metabolically-active cells and 4.0µmol/L of propidium iodide (PI; Sigma-Aldrich) for necrotic cells. Fluorescence values were then quantified with a fluorometric plate reader (for full

protocol see Appendix 1.1) and used to calculate the percentage of live cells within the islets. Percentage of live cells was calculated by dividing the fluorescence of FDA by the combined fluorescence of FDA and PI.

2.2.4.2 Islet Count

A representative aliquot of islets was taken from each experimental group and stained with dithizone (DTZ; Sigma- Aldrich), a Zn^{2+} specific stain, which allows distinguishing of islets from exocrine tissue. Stained islets were counted and their size was estimated under a 5x magnification microscope using a standard protocol (Ricordi *et al.*, 1990). Islet number (IN) was converted to IEQ, by relating islet size to that of a 'standard islet' of 150 µm diameter (for full protocol see Appendix 1.2). Islet recovery after culture was calculated as the percentage of IEQ cultured in one well that remain post culture. Islet fragmentation was calculated as IN/IEQ. Viable IEQ, the percentage of viable islets recovered, was calculated as the IEQ*(viability/100).

2.2.4.3 Glucose-Stimulated Insulin Secretion

2.2.4.3.1 Sequential Glucose-Stimulated Insulin Secretion

GSIS was assessed during sequential static glucose incubation of 20 size-matched, hand-picked islets per experimental group. Islets were incubated for 45 mins in Krebs Ringer Buffer (KRB) supplemented with 2mM glucose (initial 2mM incubation) before transfer into a glucose concentration of 20mM for a second incubation of 45 mins. Glucose incubation was finalised by a third incubation for 45 mins at 2mM glucose (secondary 2mM incubation). Islets were subsequently lysed by sonication and treated with acidified ethanol to extract intracellular insulin (for full protocol see Appendix 1.3).

2.2.4.3.2 Insulin Determination

Secreted and intracellular insulin were measured by enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden) and normalised to the number of islets incubated per insert or tube (for full protocol see Appendix 1.4).

2.2.4.3.3 Glucose-Stimulated Insulin Secretion Analysis

The glucose-stimulation index was calculated by dividing insulin secretion at 20mM glucose by the average secretion of insulin of both 2mM glucose incubation stages. Where percentage of insulin secreted is discussed, values were calculated by dividing the insulin secreted at the incubation stage of the GSIS protocol defined, by the total amount insulin secreted at every incubation step plus islet insulin content and then multiplied by one hundred (see Figure 2.2.4.3.3.1).

$$Pecentage\ insulin\ secretion\ at\ specific\ incubation\ step = \left(\frac{Absolute\ insulin\ secretion\ at\ specific\ incubation\ step}{(Total\ insulin\ secreted\ at\ every\ incubation\ step + intracellular\ insulin\ content)}\right) \times 100$$

Figure 2.2.4.3.3.1: Percentage of insulin secreted calculation

2.2.5 Statistical Analyses

All assessment parameters, where paired results were available, were analysed using Prism 7 software (GraphPad Software, California, USA) by applying the Friedman test followed by Dunn's multiple comparison test, utilising islets without matrix protein treatment as controls.

All unpaired results were analysed using Prism 7 by applying Kruskal-Wallis test with the CMRL treated islets (0nM) as the control. Statistical differences in figures are indexed by * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, and **** for $p<0.0001$.

2.3 Results

2.3.1 Collagen IV

After incubation, islet fragmentation (see Figure 2.3.1.1.A) was significantly reduced in islets cultured with increasing concentrations of C-IV ($p=0.0012$). 100nM C-IV (1.19 ± 0.19 IN/IEQ) had significantly decreased fragmentation compared to those cultured in 25nM C-IV (1.90 ± 0.62 IN/IEQ; $p=0.004$), as well as those cultured in 50nM C-IV (1.52 ± 0.33 IN/IEQ; $p=0.0485$). However, there was no significant difference in fragmentation between islets cultured in the 0nM control group and the other three conditions. There was also a significant increase in islet recovery ($p=0.0329$; see Figure 2.3.1.1.B) of islets cultured in media supplemented with C-IV. 100nM C-IV (58.48 ± 14.02 %) increased recovery in comparison to 25nM (33.77± 9.72 %; $p=0.0099$) and 0nM control (21.92 ± 7.42%; $p=0.0185$).

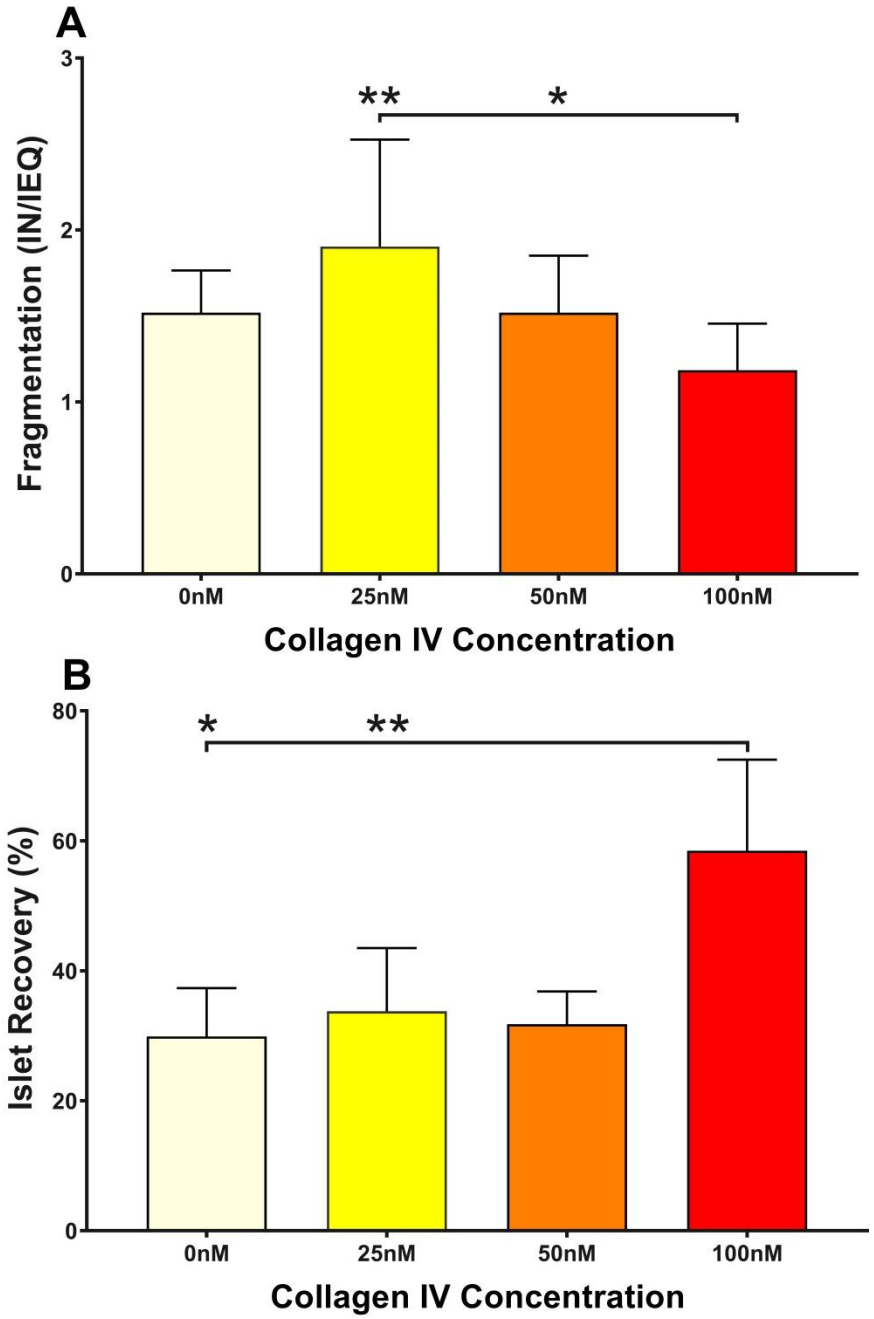


Figure 2.3.1.1: Islet fragmentation (A) and islet recovery (B) after culture in in media supplemented with differing concentrations of C-IV (mean ± SEM). Islet recovery is normalised to islet yield pre-culture (n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM).

Differences in viability of islets cultured with C-IV were not statistically significant, however, the viable IEQ (see Figure 2.3.1.2), a combined measure of viability and islet equivalent number retrieved after culture, was significantly correlated with C-IV concentration (p=0.0162). It was significantly higher in islets cultured with 100nM C-IV (317.1 ± 76.7 IEQ) than those cultured

with 25nM C-IV (184.6 ± 49.8 IEQ; p=0.0226) or the 0nM control (148.4 ± 50.3 IEQ; p=0.0411). 100nM C-IV (317.1 ± 76.7) had a marginally higher viable IEQ compared to 50nM (161.4 ± 29.6 IEQ; p=0.0975), however this did not quite reach statistical significance.

Table 2.3.1.1: Islet viability and secretion data (Mean ± SEM)

Collagen IV Concentration (nM)	Viability (%)	Islet Insulin Content (µg/islet)	Total Insulin Secreted (%)	Stimulation Index
0	70.7 ± 7.0	5.4 ± 1.7	22.9 ± 10.7	1.3 ± 0.2
25	60.2 ± 3.6	4.4 ± 2.1	53.6 ± 11.3	1.0 ± 0.1
50	58.2 ± 2.5	8.9 ± 3.6	42.2 ± 12.6	1.0 ± 0.1
100	62.6 ± 3.6	3.6 ± 1.5	57.2 ± 11.0	1.3 ± 0.3

n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM

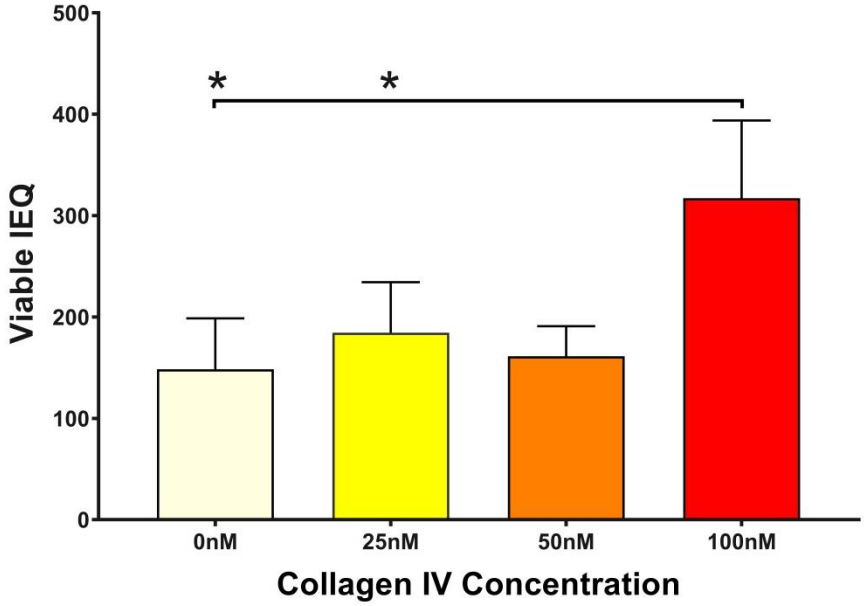


Figure 2.3.1.2: Number of viable islet equivalents recovered after culture in media supplemented with differing concentrations of C-IV (Mean ± SEM; n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM 4).

The glucose stimulation index (SI; see Table 2.3.1.1) was not significantly different across different concentrations of C-IV. There was also no significant change in the overall percentage of total insulin secreted (see Table 2.3.1.1) across all concentrations of C-IV. This is further demonstrated in the insulin secretion data (see Figures 2.3.1.3.A, 2.3.1.3.B).

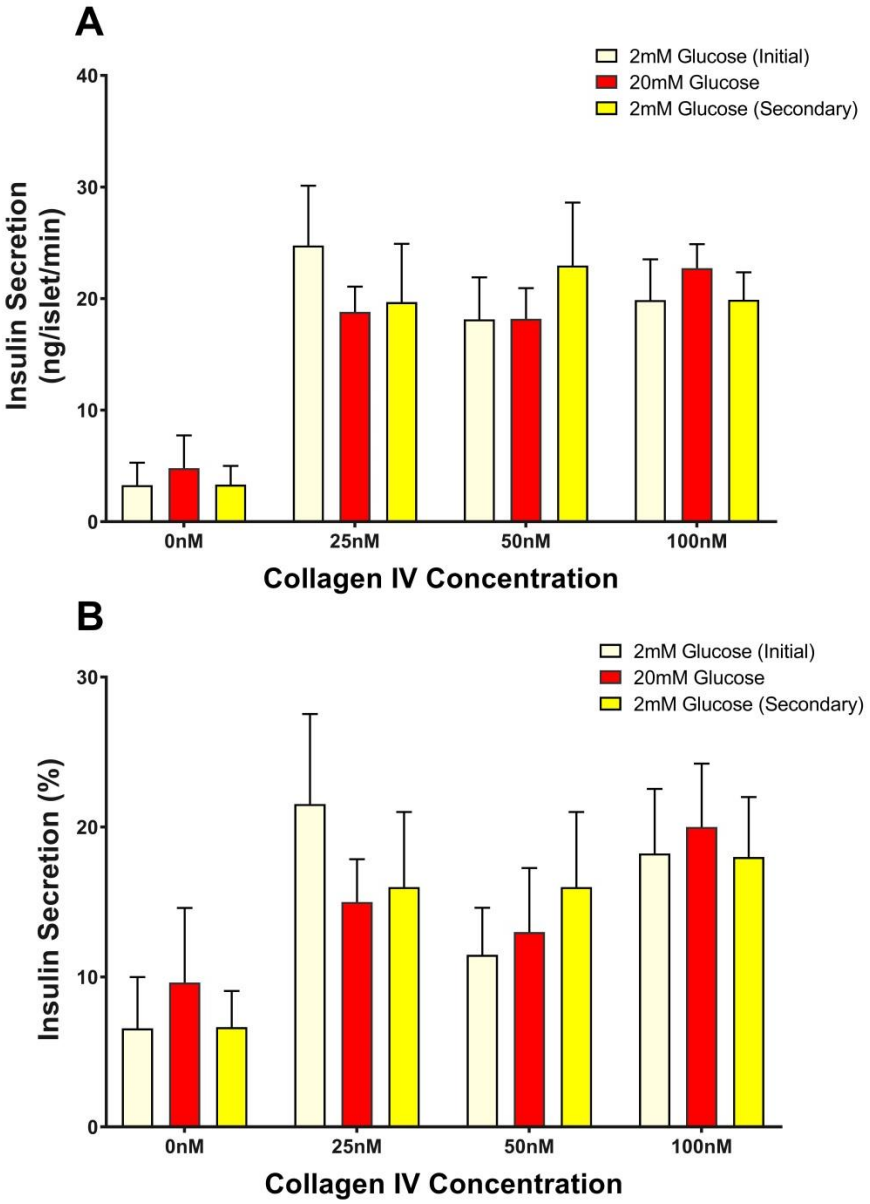


Figure 2.3.1.3: Glucose-stimulated insulin secretion of islets from each stage of GSIS after culture in media supplemented with differing concentrations of C-IV determined (A) as absolute secretion and (B) as percentage of total islet insulin content (mean ± SEM; n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM)

2.3.2 Laminin 521

After incubation, fragmentation of islets (see Figure 2.3.2.1.A) and recovery of islets (see Figure 2.3.2.1.B) were not significantly altered by changes in L521 concentration.

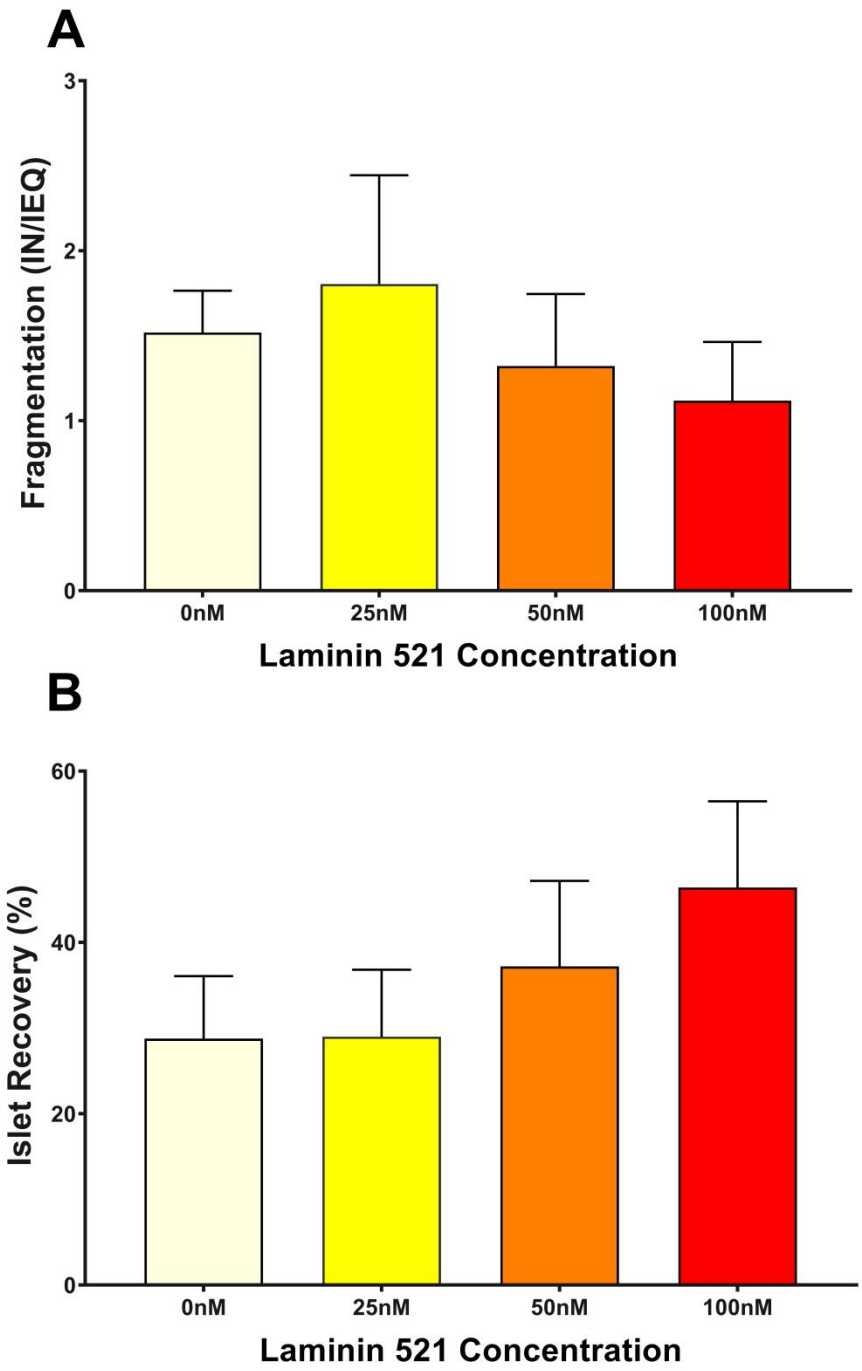


Figure 2.3.2.1: Islet fragmentation (A) and islet recovery (B) after culture in media supplemented with differing concentrations of L521 (mean ± SEM). Islet recovery is normalised to islet yield pre-culture (n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM).

Islet viability (see Table 2.3.2.1) was not significantly different across different concentrations of L521. Viable IEQ (see Figure 2.3.2.2), however, demonstrates a statistically significant trend towards enhanced recovery of viable islets with increasing concentrations of L521 (p=0.0084).

Table 2.3.2.1: Islet viability and insulin secretion data (Mean ± SEM)

Laminin 521 Concentration (nM)	Viability (%)	Insulin Content (µg/islet)	Total Insulin Secreted (%)	Stimulation Index
0	70.7 ± 7.1	5.4 ± 1.7	22.9 ± 10.7	1.3 ± 0.2
25	64.7 ± 3.3	3.6 ± 1.1	50.8 ± 0.1	1.2 ± 0.2
50	69.6 ± 5.3	3.5 ± 1.0	49.1 ± 9.9	1.0 ± 0.1
100	74.5 ± 7.5	2.7 ± 0.6	49.4 ± 10.3	1.1 ± 0.2

n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM

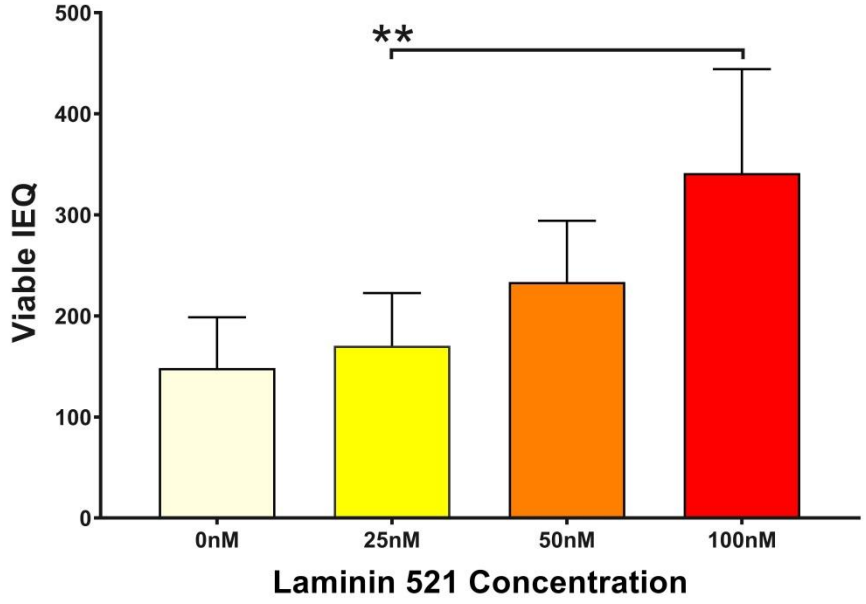


Figure 2.3.2.2: Number of viable islet equivalents recovered after culture in media supplemented with differing concentrations of L521 (mean ± SEM; n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM).

Islet SI was not significantly different across treatment groups (see Table 2.3.2.1) and percentage of islet insulin secreted (see Table 2.3.2.1) was not significantly different across concentrations of L521. While there are no significant changes in the absolute insulin secretion data (see Figure 2.3.2.3.A), when the percentage insulin secretion is interrogated, the secondary incubation of islets at 2mM glucose (3.3 ± 1.7 %) leads to a significantly higher secretion of insulin in all L521 treated islets: 25nM (320.1 ± 4.3 %; p=0.0448), 50nM (18.9 ± 4.0 %; p=0.0374), and 100nM L521 (18.3 ± 4.0 %; p=0.0488) indicating insulin secretion dysfunction. Percentage insulin secreted at 20mM glucose was also significantly higher (p=0.0001) in islets

cultured in media supplemented with L521 at 25nM (16.9 ± 4.0 %; p=0.0213), 50nM (16.3 ± 3.5 %; p=0.0295), and 100nM (18.3 ± 4.0 %; p=0.0305) compared to CMRL alone (4.8 ± 2.9 %).

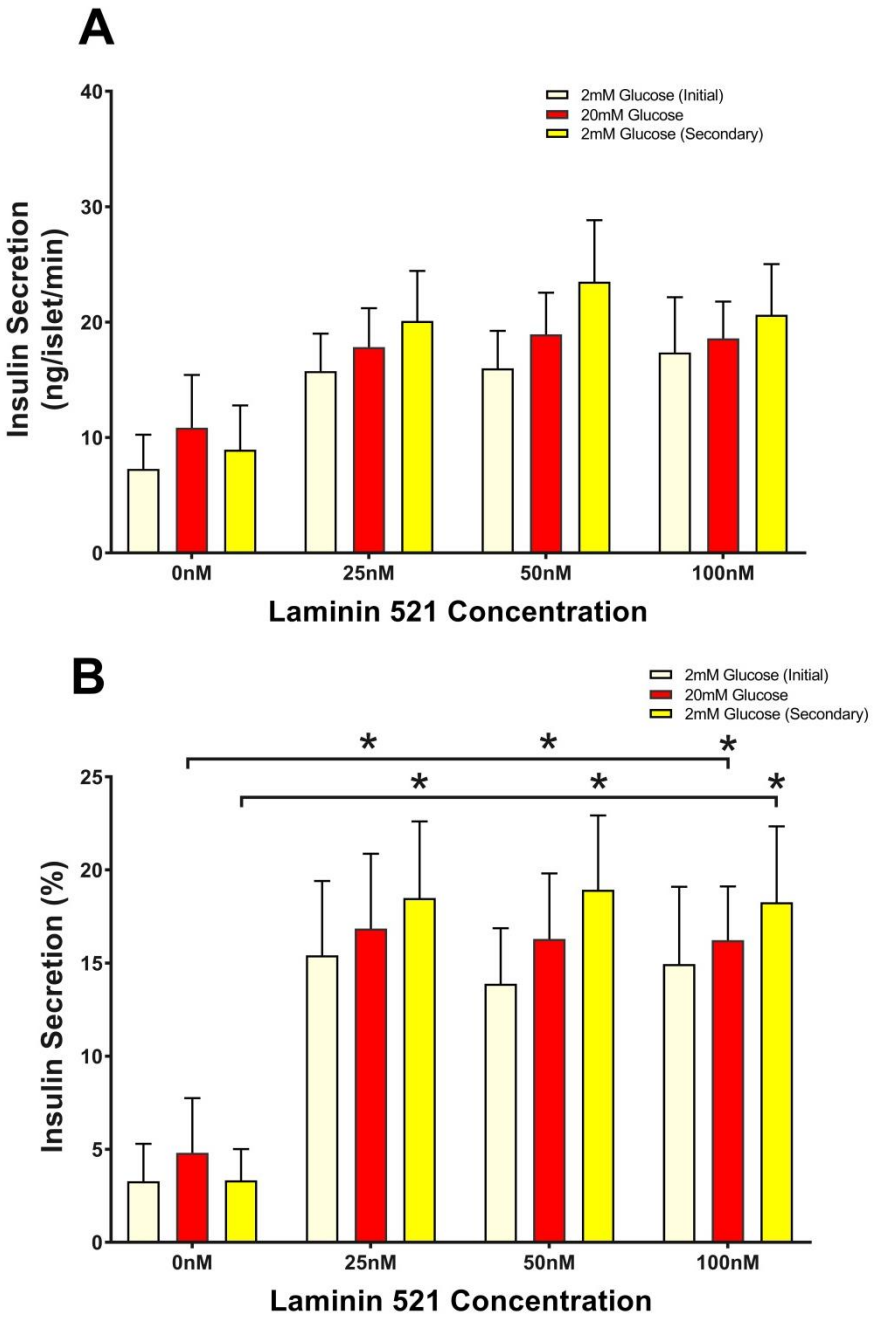


Figure 2.3.2.3: Glucose-stimulated insulin secretion of islets from each stage of GSIS after culture in media supplemented with differing concentrations of L521 determined (A) as absolute secretion and (B) as percentage of total islet insulin content (mean ± SEM; n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM).

2.3.3 Perlecan

After culture, islet fragmentation and viability (see Table 2.3.3.1) were not significantly different across PLC concentrations. Similarly, there was no significant difference in recovery (see Table 2.3.3.1) of islets cultured in media supplemented with different concentrations of PLC.

Table 2.3.3.1: Morphology, viability and recovery of islets treated with different concentrations of Perlecan (Mean ± SEM).

Perlecan Concentration (nM)	Islet Fragmentation (IN/IEQ)	Recovery (%)	Viability (%)	Viable IEQ
0	1.5 ± 0.3	29.2 ± 7.3	70.7 ± 7.1	148.4 ± 50.3
25	1.4 ±0.3	42.1 ± 10.5	67.2 ± 3.4	242.7 ± 59.0
50	1.4 ± 0.3	37.5 ± 7.8	65.0 ± 2.5	215.1 ± 51.6
100	1.65 ± 0.5	33.0 ± 9.0	68.7 ± 2.1	205.5 ± 51.4

n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM

Increasing PLC concentration in islet culture medium did not significantly alter islet SI (see Table 2.3.3.2). There was also no significant change in either the overall percentage of insulin secreted (see Table 2.3.3.2) across all concentrations of PLC or in the insulin secretion at each stage of the GSIS assay (see Figure 2.3.3.1.A and Figure 2.3.3.1.B).

Table 2.3.3.2: Insulin secretion data (Mean ± SEM)

Perlecan Concentration (nM)	Insulin Content (µg/islet)	Total Insulin Secreted (%)	Stimulation Index
0	5.4 ± 1.7	22.9 ± 10.7	1.3 ± 0.2
25	4.4 ± 1.7	54.9 ± 12.7	1.1 ± 0.2
50	4.8 ± 1.9	46.4 ± 10.0	1.2 ± 0.1
100	5.1 ± 1.7	47.3 ± 10.9	1.4 ± 0.2

n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM

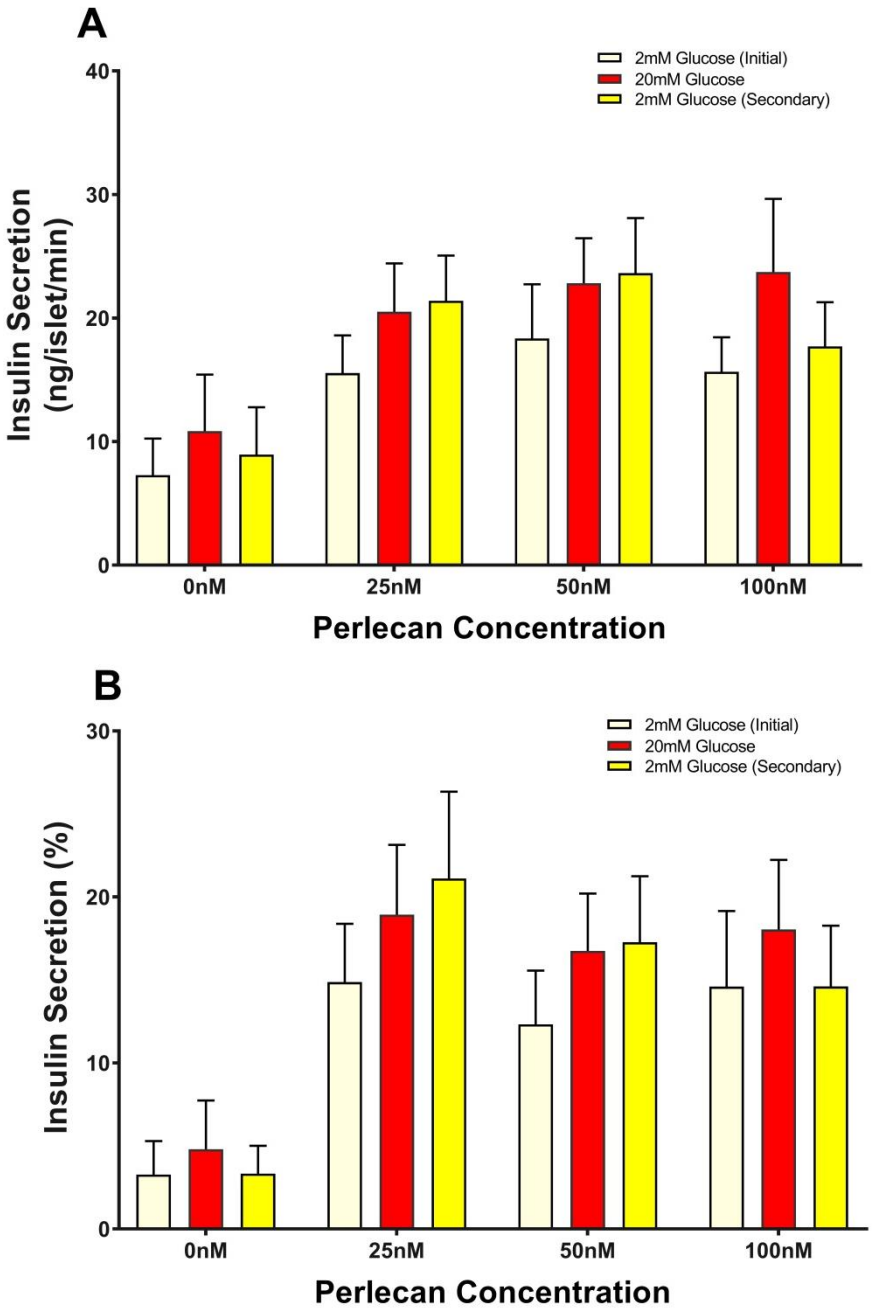


Figure 2.3.3.1: Glucose-stimulated insulin secretion of islets from each stage of GSIS after culture in media supplemented with differing concentrations of PLC determined (A) as absolute secretion and (B) as percentage of total islet insulin content (mean ± SEM; 25nM, 50nM, and 100nM n=8; for 0nM n=4).

2.3.4. Nidogen

After incubation, there was no statistically significant change in either viability or viable IEQ of islets across concentrations of NID (see Table 2.3.4.1). Similarly, there was no significant difference in islet recovery (see Table 2.3.4.1.).

Table 2.3.4.1: Morphology, viability and recovery of islets treated with different concentrations of Nidogen (Mean ± SEM).

Nidogen Concentration (nM)	Islet Fragmentation (IN/IEQ)	Islet Recovery (%)	Viability (%)	Viable IEQ
0	1.5 ± 0.3	29.2 ± 7.3	70.7 ± 7.1	148.4 ± 50.3
25	1.6 ± 0.4	28.6 ± 6.2	63.4 ± 2.9	159.9 ± 34.0
50	1.5 ± 0.4	38.3 ± 8.9	55.7 ± 4.5	196.0 ± 49.7
100	1.2 ± 0.3	36.6 ± 7.9	62.0 ± 3.4	207.6 ± 52.6

n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM

Islet SI did not significantly differ across concentrations of NID (see Table 2.3.4.2) and no significant change in the overall percentage of insulin secreted (see Table 2.3.4.2) was observed. There was also no significant change in the absolute insulin secretion at each stage of the GSIS assay (see Figure 2.3.4.1.A) however, when the secretion is measured as a percentage of total insulin, the secondary incubation of islets at 2mM glucose secreted a significantly higher percentage of insulin at 50nM (22.9 ± 4.5 %; p=0.0481) and 100nM NID (25.6 ± 5.6 %; p=0.0410) compared with the CMRL control (9.0 ± 3.8 %).

Table 2.3.4.2: Insulin secretion data (Mean ± SEM)

Nidogen Concentration (nM)	Insulin Content (µg/islet)	Total Insulin Secreted (%)	Stimulation Index
0	5.4 ± 1.7	22.9 ± 10.7	1.3 ± 0.2
25	5.4 ± 1.6	43.9 ± 10.3	1.2 ± 0.1
50	5.1 ± 2.1	45.9 ± 10.0	1.1 ± 0.1
100	4.8 ± 1.6	46.1 ± 10.5	1.1 ± 0.1

n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM

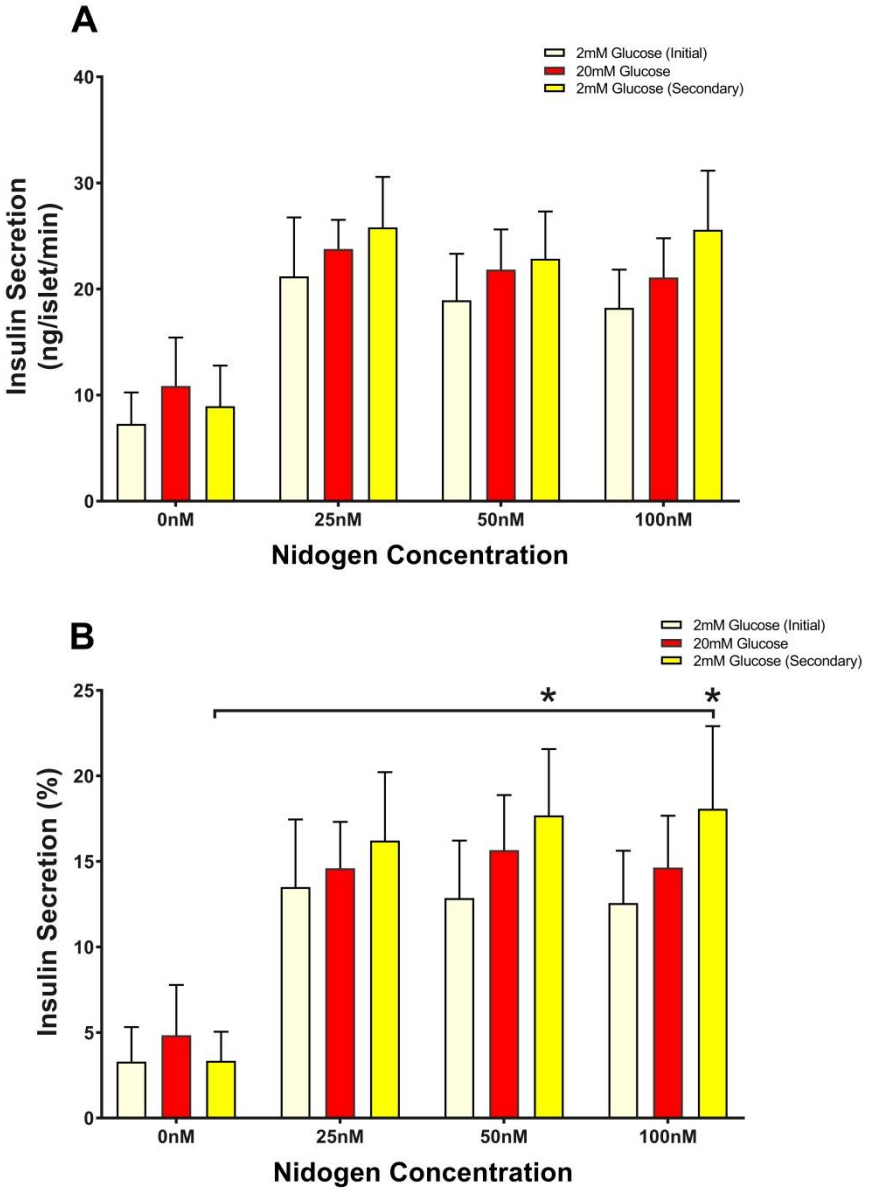


Figure 2.3.4.1: Glucose-stimulated insulin secretion of islets from each stage of GSIS after culture in media supplemented with differing concentrations of NID determined (A) as absolute secretion and (B) as percentage of total islet insulin content (mean ± SEM; n=8 for 25nM, 50nM, and 100nM; n=4 for 0nM).

2.4 Discussion

2.4.1 Collagen IV

It can be seen (from Figure 2.3.1.1) that 100nM of C-IV showed significantly improved islet survival and preserved islet integrity compared to those cultured at 50nM, 25nM, or in the C-IV-free medium. Whilst there was no discernible effect on viability, the overall viable IEQ was significantly improved over the islets cultured in 25nM C-IV as well as compared to the CMRL control. There was however, no significant difference in the function of islets across the different concentrations of C-IV. 100nM was therefore deemed as the most effective concentration for the supplementation of islet culture medium due to this higher survival and lower fragmentation.

Given that 100nM was the highest concentration tested, it may be that further increasing the concentration would similarly increase the beneficial effects of C-IV. However, data published by Llacuna *et al.* (2016) demonstrated that concentration of C-IV higher than 50µg/ml (approximately 311nM) did not confer any additional significant improvement in islet function. Therefore, it may be that the saturation point lies somewhere between 100nM and 311nM. It must be mentioned that Llacuna *et al.* utilised C-IV combined into an alginate microcapsule and free-floating C-IV, as in this study, may still be more effective at higher concentrations. This may have particular relevance if polymerised into a lattice, as in the native BM of islets. Therefore further investigation into the effects of supplementation of higher C-IV concentrations into islet culture medium should be conducted.

2.4.2 Laminin 521

No significant differences in survival, viability, fragmentation or function were apparent in islets cultured in CMRL supplemented across the range of L521. The difference between high

and low L521 concentrations was able to reach statistical significance when the recovery and viability were combined to give the viable IEQ. However, this measure alone is not robust enough to give a reliable indication of the pro-survival effects of L521. Given that there were no significant differences in islet insulin secretory function across the concentrations utilised, 25nM L521 seemed the most appropriate concentration for supplementation of islet culture media.

As laminins are such a key component in the formation of the BM, it may be expected that higher concentrations of L521 would demonstrate significant benefits to islet survival and function. However, while there was a trend towards reduced fragmentation and increased recovery of islets with higher concentrations of L521, this was not statistically significant. Given that there is still some laminin remaining on the islet surface after isolation (Cross *et al.*, 2017), it may be that the additional benefits conferred by supplementation of media are limited. However, there are a number of factors that may have obscured the true result. The stock concentration of L521 from the manufacturer was at 100μg/ml, approximately 131nM. Consequently, the culture medium at 50nM and 100nM was predominantly composed of the L521 buffer, consisting of phosphate-buffered saline with 10% glycerol. Therefore the vital compounds within CMRL, such as glucose, amino acids, and vitamins, were much more diluted, potentially negating the positive effects of the increased L521 content. A potentially stronger confounding effect may have been caused by the osmolality of the L521-supplemented medium. CMRL 1066 has an osmolality of approximately 300mOsm/kg, whereas the L521 buffer lies close to 370mOsm/kg and at 100nM the osmolality remained high at 360mOsm/kg. Even the lowest concentration of L521, at 25nM, the osmolality was 340mOsm/kg, much higher than that of CMRL. Hyperosmolar media is extremely detrimental to islet viability, firstly causing cell shrinkage, which may further obscure islet recovery and fragmentation results, but it can also induce apoptosis through oxidative stress pathways (Qin *et al.*, 1997; Schliess and Häussinger, 2002). Replacement of the L521 stock medium with CMRL or an increase in L521

stock concentration would eliminate this effect, maintaining optimal osmolality and minimising dilution of culture media, thereby allowing true determination of the most advantageous L521 concentration.

While high concentrations have been shown to be effective in both human and non-human 2-dimensional islet culture (Daoud *et al.*, 2010; Yamashita *et al.*, 2015), these studies were performed with non-specific laminins and therefore do not represent the overall concentration of L521. Other groups have utilised the specific amino acid sequences known to interact with integrins, such as PSDGR and RGD (Llacua *et al.*, 2016). These were employed at concentrations spanning those used here 10nM, 100nM and 1mM, all demonstrating significant improvements to islet insulin secretion compared to the control. However, effects at the different concentrations were not directly compared. Therefore, the optimal L521 concentration, as determined in this study, is 25nM.

2.4.3 Perlecan

Supplementation of islet culture medium with PLC suggested a trend towards reduced survival and a deterioration of morphological integrity with increasing concentrations. However, this observation was not found to be a statistically significant. There was also no significant change observed in islet viability or insulin secretory function. 25nM was therefore decided to be the concentration at which maximal effect could be reliably observed given the lack of statistical significance along with the in survival, fragmentation, and function observed. While there have been some studies into the effects of the domain V fragment (endorepellin), utilised here, have been performed on non-endocrine cells (Goyal *et al.*, 2011; Lee *et al.*, 2011; Clarke *et al.*, 2012), there have been few or no investigations as to the effects of proteoglycans on isolated islets,

human or in an animal model. These results may therefore, provide a guideline for future investigations.

2.4.4 Nidogen

The survival of islets was not significantly enhanced by supplementing culture medium with higher concentrations of NID. Similarly, the integrity of islets was not significantly altered by the change in NID concentration. There was no obvious trend found in the viability of islets cultured in the varied concentrations and this was also the case regarding the insulin secretory function of the islets. Given the lack of significant variation in islets cultured in media supplemented within the range of concentrations, 25nM NID was taken to be the most appropriate concentration.

The n-terminal globular domain binds to C-IV, laminin γ1 chain, and domain IV of PLC (Mayer *et al.*, 1993; Hopf *et al.*, 1999; Ries *et al.*, 2001). This allows NID to act as a bridge between the various constituents of the BM stabilising and solidifying the macromolecular structure. Undigested C-IV and laminins remaining within the BM on the surface of islets after isolation may preferentially sequester NID and therefore may mean that the efficacy of NID is limited by the amount of protein remaining on the islet surface. Nevertheless, as there is little or no data regarding the effects of NID on isolated islets, though other cell types have been investigated at concentration within the range investigated here (Mishima *et al.*, 1996; Lee *et al.*, 2007). These results may therefore, provide a guideline for future investigations.

2.4.5 Chapter Conclusion and Next Step

In this chapter 100nM C-IV exhibited enhanced islet recovery compared to lower concentrations, whereas PLC, L521, and NID, did not significantly improve islet function or survival at higher concentrations. 100nM C-IV, 25nM L521, 25nM PLC, and 25nM NID were therefore the concentrations used for the subsequent experiments described in chapters 3 and 4. This experiment only demonstrated one statistically significant benefit conferred by the supplementation of CMRL culture medium with matrix proteins, i.e. that 100nM C-IV enhanced islet survival. The other 3 proteins tested presented no statistically significant advantage in this context. Nevertheless, these proteins may still contribute an advantage to islets intended for transplant by providing protection in hypoxic conditions, although they may also prove to be detrimental, and will continue to be investigated in future experiments.

Chapter 3 - Defining the Effects of Supplementation of Individual Extracellular Matrix Proteins into Islet Culture Medium

3.1 Introduction

The aim of this chapter was to investigate the effects conferred on islet function and survival by supplementing CMRL with extracellular matrix proteins during the post-isolation culture period and a subsequent pseudo-transplant hypoxic culture period. The titration experiments described in Chapter 2 demonstrated reduced fragmentation and increased islet recovery in islets cultured in normoxic atmosphere and suspended in CMRL supplemented with 100nM C-IV compared to islets cultured in lower concentrations of C-IV, or the unsupplemented CMRL. L521, NID, and PLC conferred no significant benefits to islet survival, viability, or function across concentrations. Therefore, in this *in vitro* simulation of the clinical islet transplant process, the clinical standard CMRL control during the post-isolation recovery culture period was compared to CMRL additionally supplemented with 100nM C-IV, 25nM L521, 25nM PLC, or 25nM NID. This simulation involved a 24-48 hour post-isolation recovery culture period in normoxic conditions followed by a hypoxic 'post-transplant' culture period.

BM proteins were added during the post-isolation culture period for two theoretical reasons. Firstly, reinstatement of the islet-matrix interaction at this point minimised the time where this interaction is broken and could prevent the irreversible commitment of the cells to apoptosis. It also may enhance cell survival prior to transplant, thereby maximising graft cell numbers. Secondly, while BM proteins may be clinically effective when co-transplanted within a macroencapsulation device, co-transplantation of islets and BM proteins is unlikely to be effective after portal vein infusion as hydraulic pressure of the circulatory system would islets would separate islets from un-adhered proteins. Furthermore, direct supplementation of transplant media would require extensive feasibility studies to ensure patient safety. Supplementation pre-culture reduces direct risk potential from addition of these proteins.

3.2 Methods

Initially, isolated islets (isolated as described in Section 2.2.1) were cultured in CMRL either supplemented with or without individual matrix proteins at the aforementioned concentrations for 24-48 hours in normoxic conditions (as described in section 3.2.1). Half were assessed post-culture for viability (as described in Section 2.2.4.1), fragmentation (as described in Section 2.2.4.2), recovery (as described in Section 2.2.4.2), glucose-stimulated insulin secretion (as described in Section 2.2.4.3), and gene expression (as described in Section 3.2.2). The remaining islets were cultured in CMRL supplemented with FCS and without any matrix proteins in hypoxic conditions for 48-72 hours (as described in Section 3.2.1.2) before undergoing similar assessment.

3.2.1 Donor Details

Human pancreases were retrieved from 12 human (8 male and 4 female), deceased multi-organ donors (2 DCD and 10 DBD; see Table 3.2.1.1) and, for this study, 12430.74 ± 2591.2 islet equivalents (IEQ), with a viability of 74.2 ± 1.9 %and purity of 68.8 ± 3.9 % post-purification were used. Individual islet preparations, from a single pancreas, were assessed separately and represented one technical replicate.

Table 3.2.1.1: Islet Preparation Details

12 Donors	Median	Range
BMI	29.0	18.0 – 36.0
Age (Years)	46.0	33.0 – 55.0
CIT (Hours)	5.8	4.5 – 8.5
WIT (Mins)	0.0	0.0 – 154.0

BMI – Body mass index, CIT – Cold ischaemia time, WIT – Warm ischaemia time

3.2.2 Islet Culture

3.2.2.1 Culture Medium

Islet culture was performed in CMRL as described in Section 2.2.3.1.

3.2.2.2 Matrix Protein Supplementation

CMRL was supplemented with the extracellular matrix proteins of interest according to the most suitable concentration found during the matrix protein titration experiments in Chapter 2. C-IV was applied at 100nM (44.00 µg/mL), L521 at 25nM (79.05 µg/mL), PLC at 25nM (0.70 µg/mL), and NID-1 at 25nM (3.75 µg/mL).

3.2.2.3 Normoxic Culture

Islets were cultured in CMRL (as described in Section 3.2.2.1) alone or with matrix proteins added individually (as described in Section 3.2.2.2). Islets were seeded in 25cm^2 culture flasks (Sarstedt AG & Co, Nümbrecht, Germany) for 24-48 hrs at 37°C, 21% O_2, 5% CO_2. Atmosphere and culture vessels were selected according to the clinical protocol for islet culture prior to islet transplantation.

3.2.2.4 Hypoxic Culture

Islets were removed from the culture flasks, washed with CMRL and cultured in 24-well suspension culture plates utilising 500µl culture medium CMRL, as described in Section 3.2.2.1, additionally supplemented with 5% FCS. Islets not used for normoxic assessment were

distributed in the plate according to Figure 3.2.2.4.1 before being cultured for 48-72 hrs at 37°C, 1% O_2, 5% CO_2.

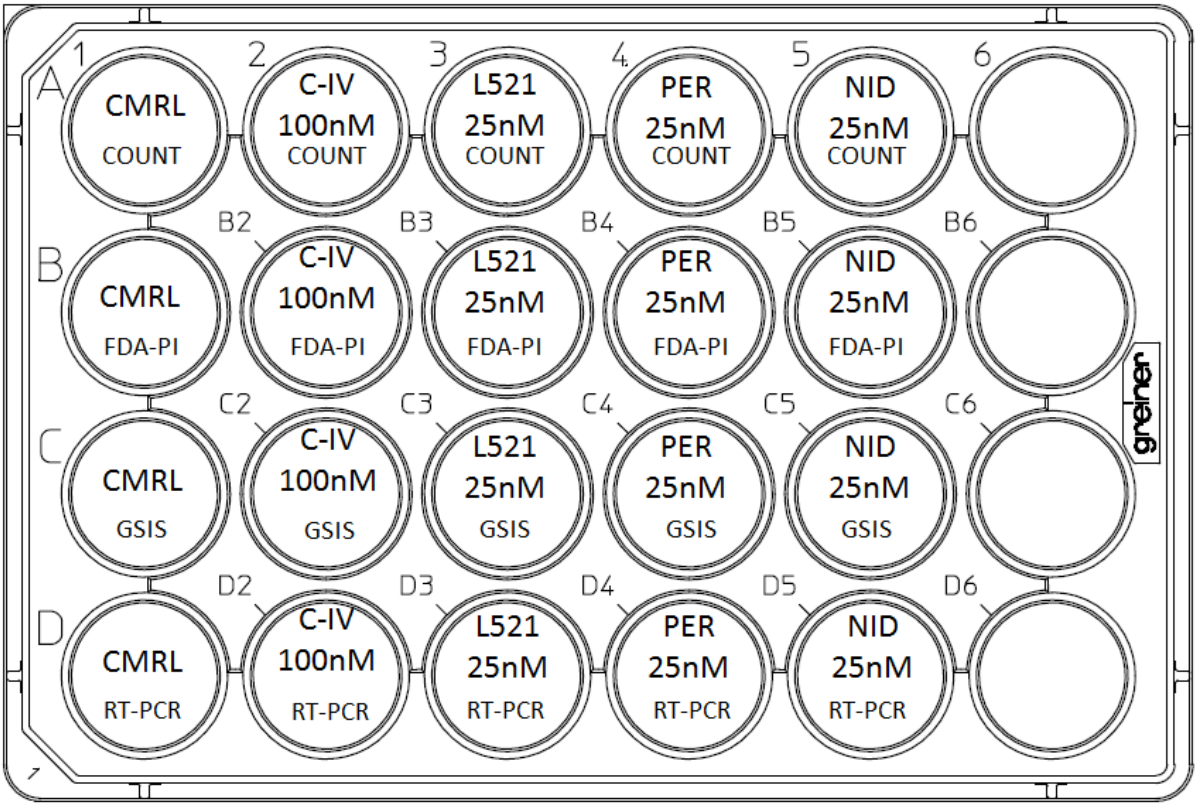


Figure 3.2.2.4.1: Islet culture plate map for individual matrix protein supplementation.

3.2.3 Gene Expression

3.2.3.1 RNA extraction

100 islets were hand-picked in 20µl PBS before isolation of the total RNA using the Qiagen RNeasy Micro Kit (For full protocol see Appendix 1.5) and stored at -20°C before assessment.

3.2.3.2 qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on each sample in triplicate, using Invitrogen (Carlsbad, California, United States) CellsDirect One-Step qRT-PCR Kit (for full protocol see Appendix 1.6) for 40 amplification cycles. TaqMan gene expression assay (Applied Biosystems, Foster City, California, United States) for 18s rRNA (VIC/MGB; Hs99999901_s1) was used as the endogenous control, with BAX (FAM/MGB; Hs00180269_m1), BCL2 (FAM/MGB; Hs00608023_m1), INS (FAM/MGB; Hs00355773_m1), VEGFa (FAM/MGB; Hs00900055_m1), HIF1A (FAM/MGB; Hs00153153_m1), DDIT3 (FAM/MGB; Hs00358796_g1), and CASP3 (FAM/MGB; Hs00234387_m1) used for determination of relative mRNA expression. All expression was normalised to the unsupplemented CMRL control.

3.2.3.3 Genes of Interest

18s rRNA constitutes the structural RNA portion of the small subunit of the eukaryotic ribosome. Gene expression studies require a stable control gene with consistent expression in order to normalise the amount of RNA used for each reaction. In isolated islets, the most commonly utilised endogenous control genes, GAPDH and ACTB, have been demonstrated to be highly variable, whereas 18s rRNA remains stable (Rodriguez-Mulero and Montanya, 2005). Therefore, in order to effectively normalise to tissue mass used, 18s rRNA was used as the endogenous control in this study.

The BCL2 gene codes for the B-cell lymphoma 2 (Bcl-2) protein, a promoter of cellular survival (Vaux, Cory and Adams, 1988). Associated with the mitochondrial membrane, it acts to inhibit the release of cytochrome c from the mitochondrial matrix, thereby preventing activation of apoptotic proteins of the caspase family (Kluck *et al.*, 1997). Another member of the Bcl-2

family, Bcl-2-associated X protein (Bax) encoded by the BAX gene, is a pro-apoptotic factor that, when levels become amplified, forms a homodimer and becomes inserted to the mitochondrial membrane allowing the release of cytochrome c, activating apoptosis (Gross *et al.*, 1998; Jurgensmeier *et al.*, 1998). Bax-mediated apoptosis is inhibited under normal cellular conditions by dimerization with Bcl-2 (Yin, Oltvai and Korsmeyer, 1994), therefore the expression levels of Bax and Bcl-2 are important indicators of the apoptotic state of the cell. In particular, the ratio of Bax to Bcl-2 is important as it determines whether Bax forms a heterodimer or a homodimer (Salakou *et al.*, 2007). Further along this pathway, caspase-3, encoded by the CASP3 gene, is a terminal protein in the apoptosis cascade initiating chromatin condensation and DNA fragmentation (Wolf *et al.*, 1999). As this protein is linked to the extrinsic death pathway, as well as the intrinsic pathway, activity is therefore, a good indicator of cell fate determined by matrix-cell interactions. While specific activity of the protein is regulated by proteolytic cleavage by caspase-9, expression of CASP3 has been shown to be indicative of cell susceptibility to apoptosis (Droin *et al.*, 1998; Sabbagh *et al.*, 2004).

The HIF1A gene encodes the α-subunit of the transcription factor hypoxia-inducible factor- 1 (Hif-1). Hif-1 manages the response of cells to hypoxia, promoting survival at low oxygen concentrations (Wenger and Gassmann, 1997; Carmeliet *et al.*, 1998). It is therefore one of the genes investigated here, particularly considering that it has been shown to improve outcomes in islet transplantation (Stokes *et al.*, 2013). The actions of Hif-1 are mediated through induction of expression of important genes, such as vascular endothelial growth factor (VEGF), a promoter of angiogenesis (Forsythe *et al.*, 1996), as well as those encoding glycolytic enzymes, allowing ATP synthesis in an oxygen-independent manner, and reactive oxygen species-attenuating enzymes (Kim *et al.*, 2006). Given the importance of vascularisation of islet post-transplant as discussed in Section 1.2.3.2, VEGF expression was also measured. In contrast to Hif-1, C/EBP homologous protein (Chop), encoded by DNA damage-inducible transcript 3 (DDIT3), is a pro-apoptotic protein induced by severe hypoxia or anoxia (Zheng *et al.*, 2012).

It acts through induction of the apoptotic pathway via Ero-1-mediated release of calcium from the endoplasmic reticulum (ER), in response to ER-stress and the unfolded protein response (Oyadomari and Mori, 2004; Tabas and Ron, 2011). Along with Hif-1, DDIT3 expression demonstrates the hypoxic effects on islet intracellular environment.

Finally, increased oxidative stress reduces insulin (encoded by INS gene) expression (Kaneto *et al.*, 2002). Given the importance of insulin production on the function of islets after transplantation in T1DM patients, it is important to evaluate not only the function of insulin secretion but also the ability of the cell to replenish stores of the protein through assessment of β-cell mRNA production.

3.2.4 Statistical Analyses

All assessment parameters were analysed with Prism 7 using Friedman's test followed by Dunn's multiple comparison test, utilising islets without matrix protein treatment as controls. Statistical differences in figures are indexed by * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, and **** for $p<0.0001$.

3.3 Results

3.3.1 Post-Normoxic Culture

After normoxic incubation, islet fragmentation (see Figure 3.3.1.1.A) was significantly lower in islets cultured in CMRL supplemented with C-IV (1.1 ± 0.2 IN/IEQ; p=0.0005), L521 (1.3 ± 0.3 IN/IEQ; p=0.0067), PLC (1.1 ± 0.2 IN/IEQ; p=0.0002), and NID (1.2 ± 0.3 IN/IEQ; p=0.0030) versus the CMRL-only control (1.7 ± 0.4 IN/IEQ). Islet recovery after culture (see

Figure 3.3.1.1.B) was significantly improved when CMRL (30.9 ± 9.5 %) was additionally supplemented with PLC (46.8 ± 5.2 %; p=0.0045) and NID (38.1 ± 6.5 %; p=0.0282).

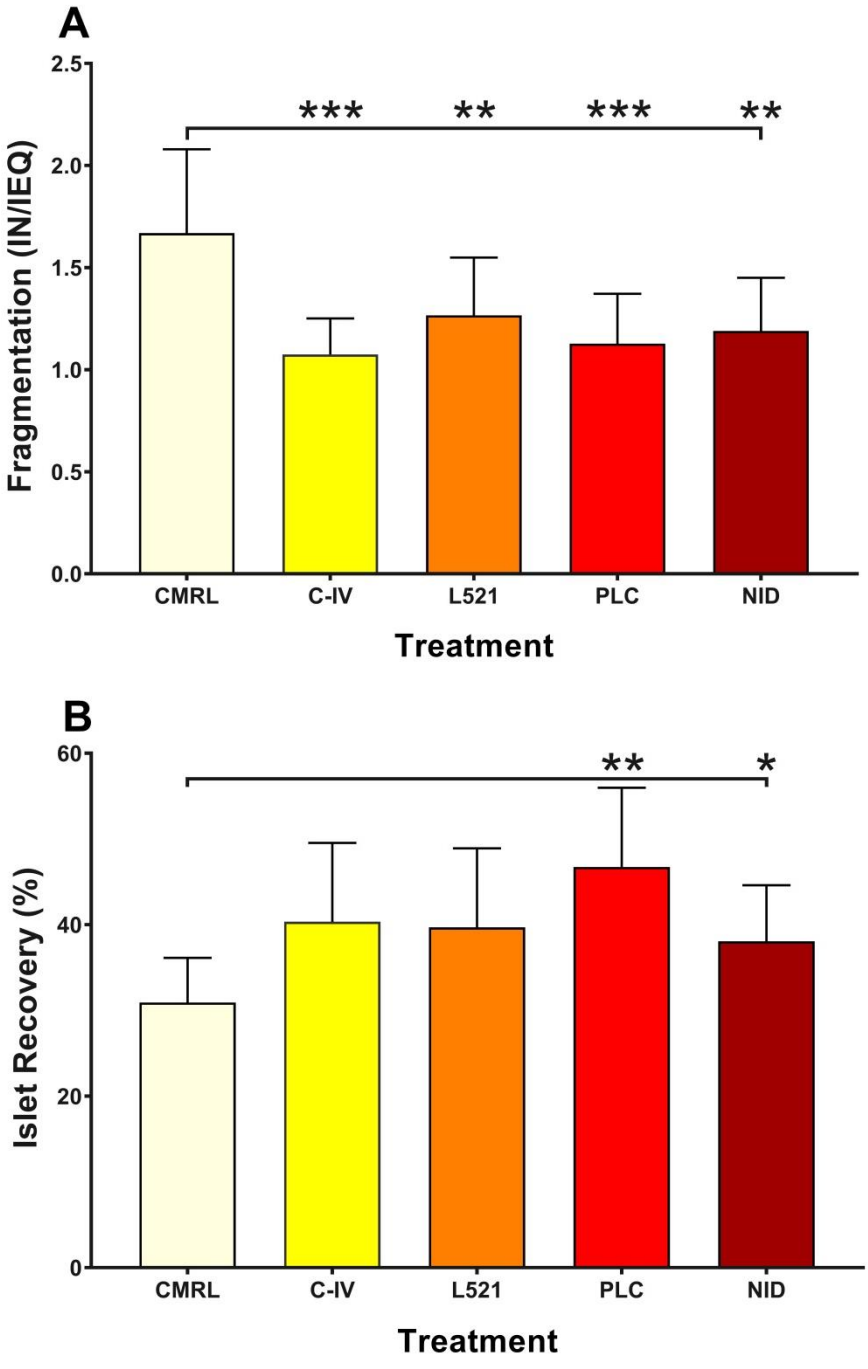


Figure 3.3.1.1: Islet fragmentation (A) and recovery (B) after normoxic culture in media supplemented with or without individual matrix proteins (Mean ± SEM; n=12)

Differences in the post-normoxic culture viability of islets (see Table 3.3.1.1) were not statistically significant. The viable IEQ (see Figure 3.3.1.2), was only statistically significantly improved by the addition of PLC (719.5 ± 262.2 IEQ; p=0.0030) to CMRL (395.8 ± 99.6 IEQ), although C-IV treated islets approached significantly higher levels (457.9 ± 82.8 IEQ; p=0.0707).

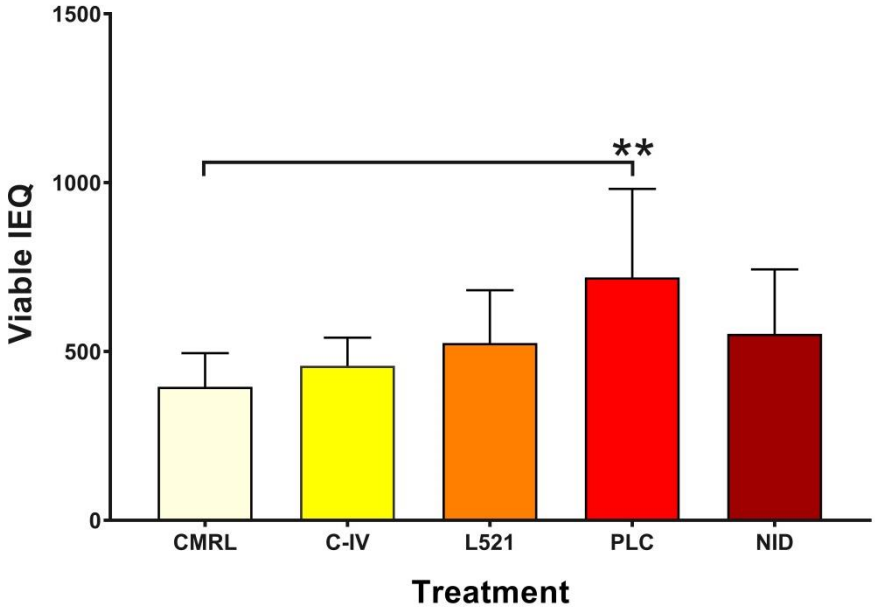


Figure 3.3.1.2: Viable IEQ recovered after normoxic culture in media supplemented with or without individual matrix proteins (Mean ± SEM; n=12)

Table 3.3.1.1: Islet recovery, viability and insulin secretion data after normoxic incubation with or without matrix proteins (Mean ± SEM; n=12)

	Recovery (%)	Viability (%)	Insulin Content (µg/islet)	Total Insulin Secreted (%)	Stimulation Index
CMRL	37.9 ± 5.8	53.3 ± 4.2	3.5 ± 1.3	38.6 ± 6.1	0.7 ± 0.1
C-IV (100nM)	44.7 ± 9.9	55.2 ± 4.2	2.2 ± 1.0	49.9 ± 7.4	1.7 ± 0.8
L521 (25nM)	42.8 ± 8.8	57.1 ± 5.0	3.9 ± 1.5	42.2 ± 6.3	0.9 ± 0.3
PLC (25nM)	47.3 ± 9.5	57.2 ± 4.0	3.7 ± 1.1	39.8 ± 7.4	1.0 ± 0.2
NID (25nM)	44.0 ± 7.0	56.7 ± 3.6	3.8 ± 1.1	42.3 ± 6.1	1.2 ± 0.3

Islet SI (see Figure 3.3.1.3.A) was not improved by the supplementation of CMRL with each of the individual matrix proteins. Similarly, the total percentage of insulin secreted (see Table 3.3.1.1) was not significantly increased by the addition of matrix proteins and, while islet INS expression (see Figure 3.3.1.1.B) was significantly increased only by supplementation of culture medium with NID (1.9 ± 0.5 fold; p=0.0369), the absolute secretion and percentage insulin secreted (see Figure 3.3.1.3.C and Figure 3.3.1.3.D) also did not demonstrate any significant differences.

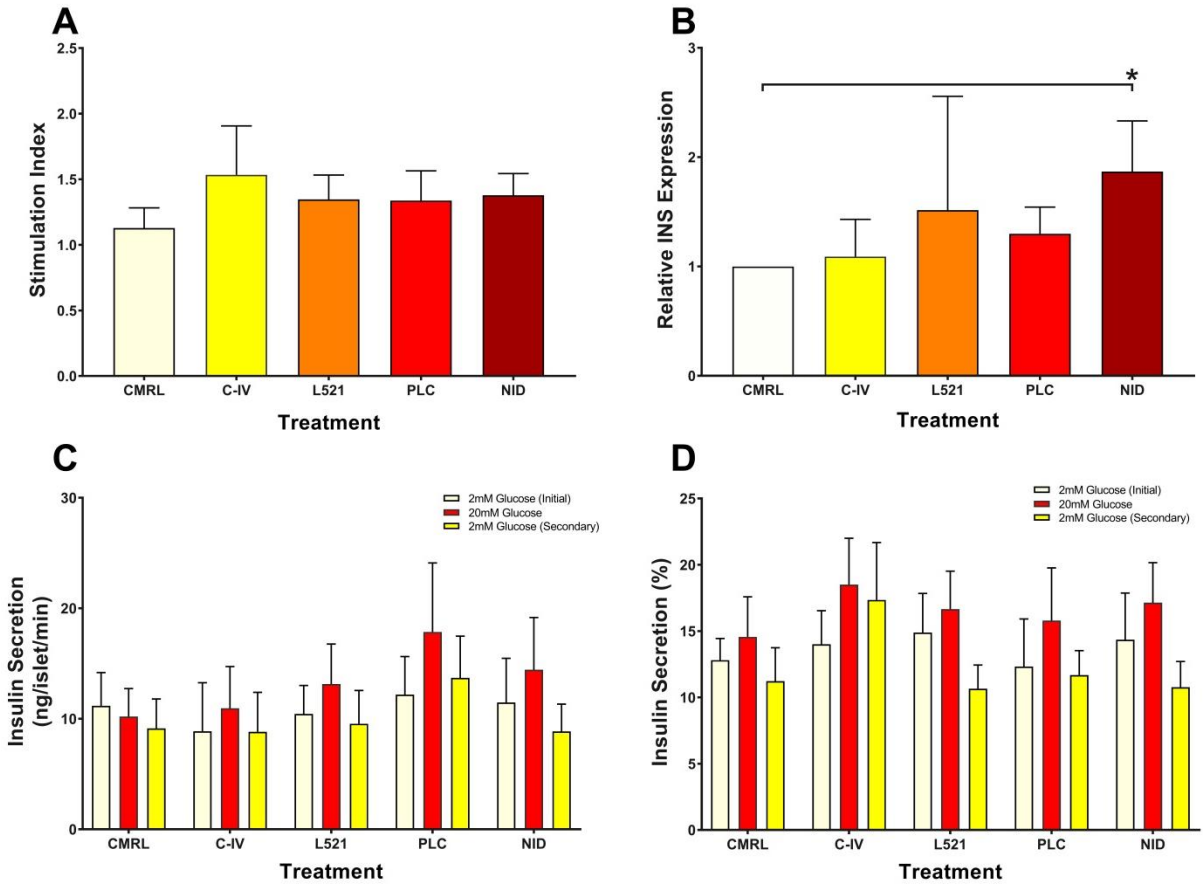


Figure 3.3.1.3: Stimulation index (A), INS gene expression (B; normalised to CMRL control), and glucose-stimulated insulin secretion of islets from each stage of GSIS after normoxic culture in media supplemented with or without individual matrix proteins determined as (C) absolute secretion and (D) percentage of total islet insulin content (mean ± SEM; n=12).

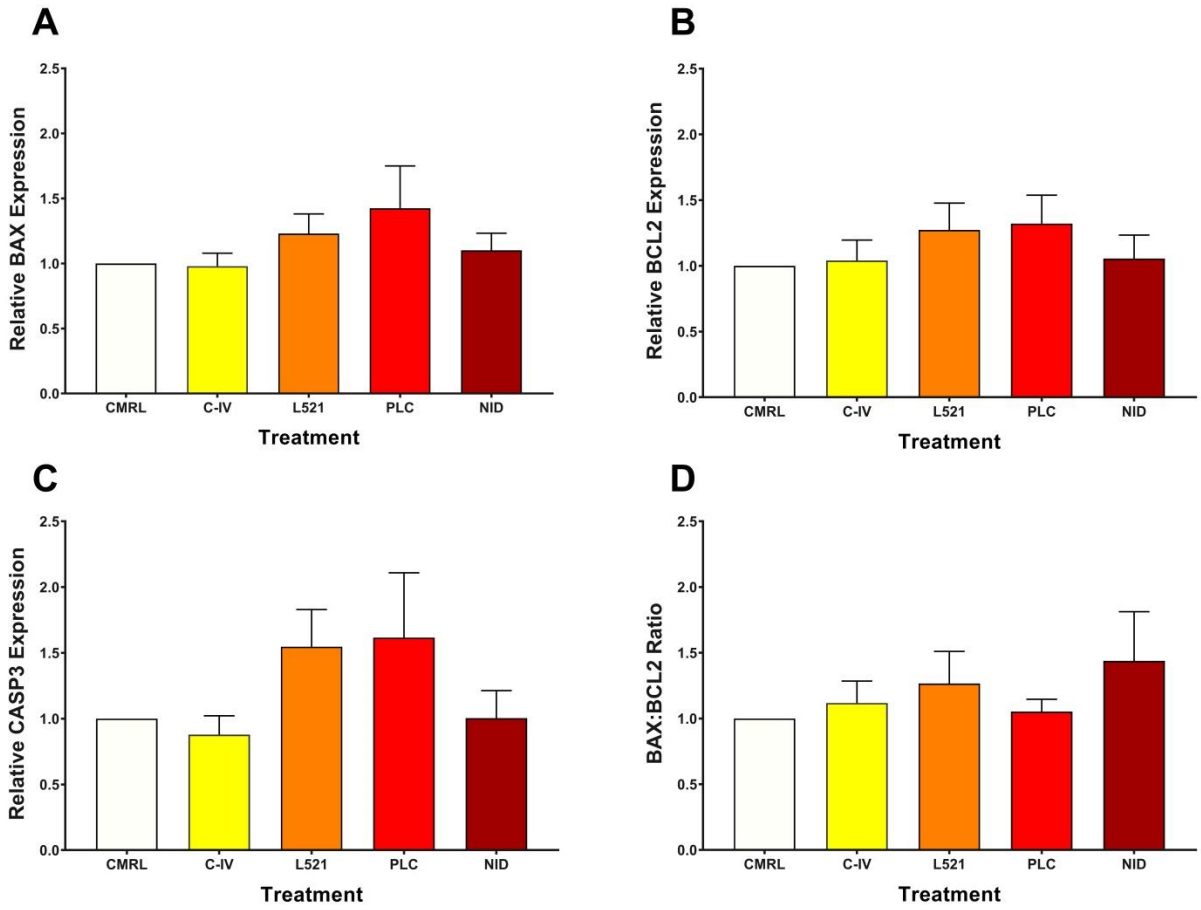


Figure 3.3.1.4: Gene expression of (A) BAX, (B) BCL2, and (C) CASP3 as well as (D) BAX:BCL2 ratio of islets cultured in media supplemented with or without individual matrix proteins (values normalised to CMRL control; mean ± SEM n=12).

Gene expression analysis of islets demonstrated that, after normoxic culture, BAX (see Figure 3.3.1.4.A), BCL2 (see Figure 3.3.1.4.B) and CASP3 expression (see Figure 3.3.1.4.C) were not significantly altered by the addition of the BM proteins. The BAX:BCL2 ratio also did not significantly change. Furthermore, islet HIF1A (see Figure 3.3.1.5.A) and DDIT3 (see Figure 3.3.1.5.C) expression were not significantly different. VEGF expression (see Figure 3.3.1.5.B), however, was significantly increased when CMRL was supplemented with C-IV (1.7 ± 0.3 fold; p=0.0273), L521 (2.0 ± 0.6 fold; p=0.0030), and PLC (1.9 ± 0.5 fold; p=0.0477).

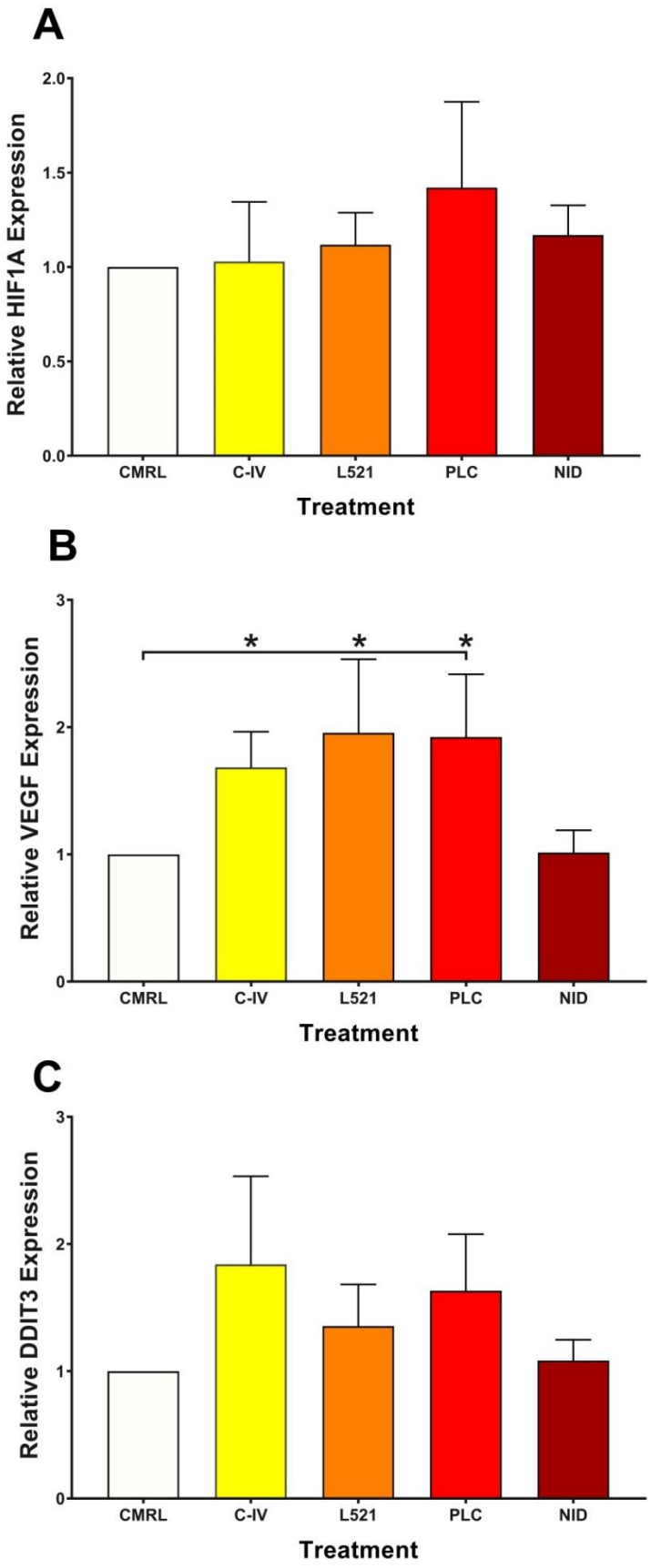


Figure 3.3.1.5: Gene expression of (A) HIF1A, (B) VEGF, and (C) DDIT3 in islets cultured in media supplemented with or without individual matrix proteins (values normalised to CMRL control; mean ± SEM; n=12).

3.3.2 Post-Hypoxic Culture

After hypoxic incubation, islet fragmentation (see Figure 3.3.2.1.A) was significantly lower in L521 (1.2 ± 0.2 IN/IEQ; p=0.0050), PLC (1.1 ± 0.1 IN/IEQ; p<0.0001), and NID (1.2 ± 0.2 IN/IEQ; p=0.0001) conditioned islets in comparison to those conditioned in the standard clinical medium (2.0 ± 0.3 IN/IEQ). Islet recovery (see Figure 3.3.2.1.B) after hypoxic culture was significantly improved by conditioning with L521 (16.8 ± 5.6 %; p=0.0201), PLC (22.3 ± 7.3 %; p<0.0001) or NID (20.5 ± 6.9 %; p=0.0045) compared to those conditioned in the standard clinical medium (9.9 ± 2.9 %).

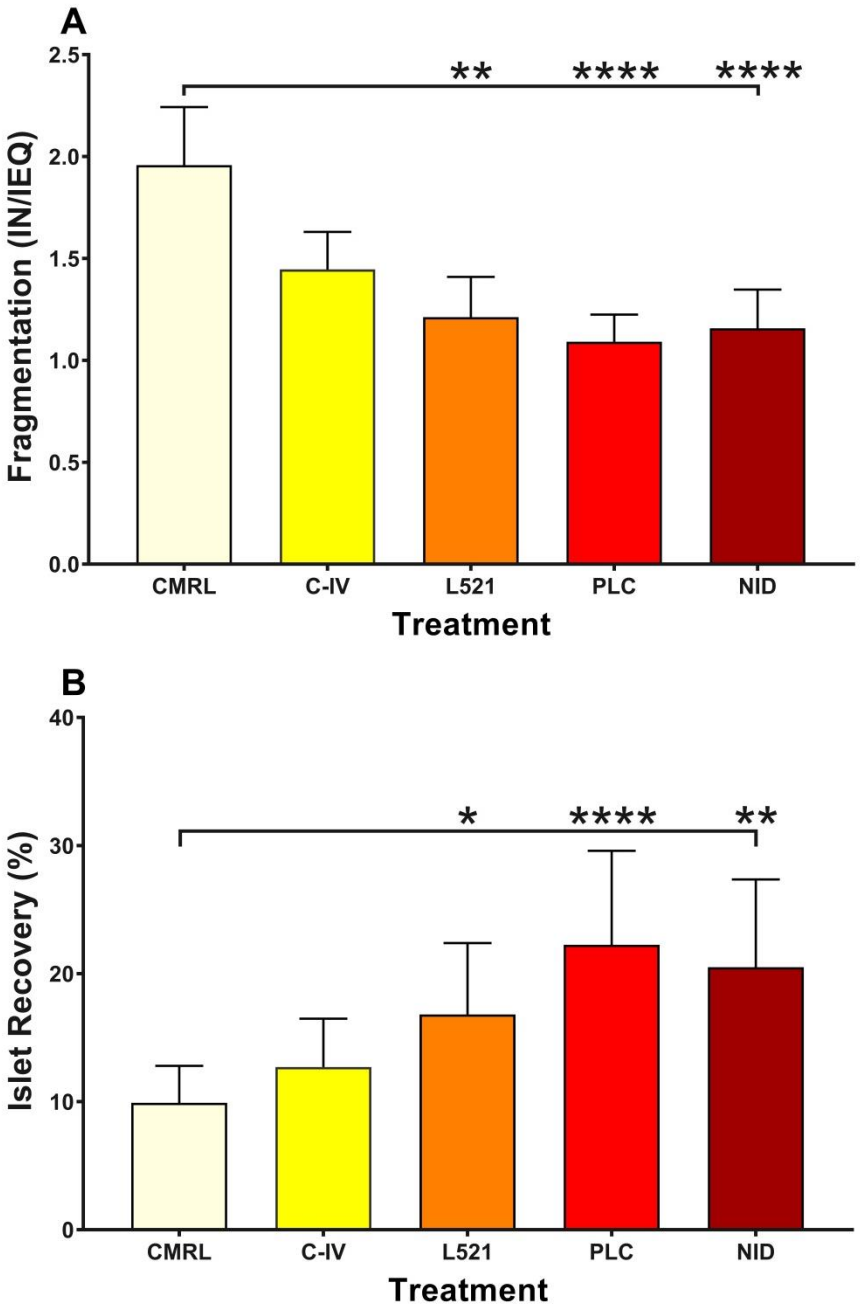


Figure 3.3.2.1: Islet fragmentation (A) and recovery (B) after hypoxic culture in media supplemented with or without different matrix proteins (mean ± SEM; n=6).

The viability of islets (see Table 3.3.2.1) after hypoxic culture was not altered by preconditioning islets with extracellular matrix proteins. However, the viable IEQ (see Figure 3.3.2.2) was significantly improved by conditioning of islets with L521 (199.9 ± 46.0 IEQ; p=0.0201) and PLC (462.0 ± 175.0 IEQ; p=0.0005) compared to those conditioned in CMRL alone (137.5 ± 50.6 IEQ).

Table 3.3.2.1: Post-hypoxia viability and secretion data of islets preconditioned with or without individual matrix proteins (Mean ± SEM; n=12)

	Viability (%)	Insulin Content (µg/islet)	Total Insulin Secreted (%)	Stimulation Index
CMRL	61.6 ± 4.8	3.0 ± 0.6	40.7 ± 6.5	1.3 ± 0.2
C-IV (100nM)	61.6 ± 3.6	2.5 ± 0.5	35.6 ± 4.7	1.3 ± 0.2
L521 (25nM)	62.1 ± 4.2	4.7 ± 1.5	33.8 ± 6.7	1.8 ± 0.5
PLC (25nM)	62.9 ± 3.1	3.1 ± 0.6	29.2 ± 6.1	1.6 ± 0.2
NID (25nM)	60.2 ± 2.1	2.9 ± 0.7	52.3 ± 12.3	1.9 ± 0.4

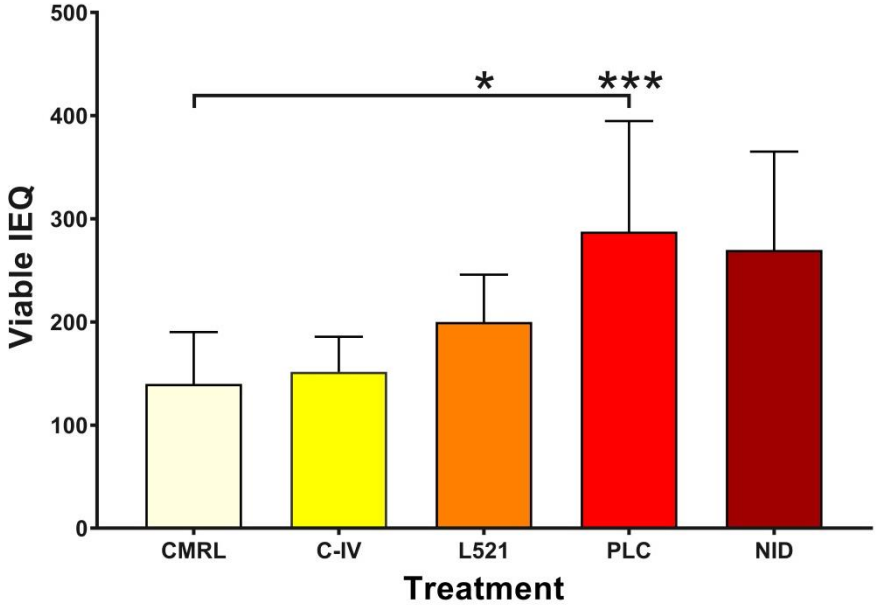


Figure 3.3.2.2: Viable IEQ recovered after hypoxic culture in media supplemented with or without different matrix proteins (mean ± SEM; n=12).

Islet SI and percentage of total insulin secreted (see Table 3.3.2.1) were not significantly improved by conditioning with matrix proteins. The absolute insulin secretion of islets (see Figure 3.3.2.3.A) demonstrates that PLC (13.1 ± 3.1 vs. 9.5 ± 3.7 ng/islet/min; p=0.0038), NID (14.1 ± 3.3 vs. 7.3 ± 2.6 ng/islet/min; p=0.0123), and CMRL-only (15.5 ± 4.7 vs. 11.4 ± 5.0 ng/islet/min; p=0.0123) conditioned islets exhibited significant down-regulation of insulin secretion between the 20mM stage and the secondary 2mM stage. Insulin secretion of L521 conditioned islets was also down-regulated when the glucose concentration was reduced. However, this did not reach statistical significance (9.7 ± 3.3 vs. 3.7 ± 0.9 ng/islet/min;

p=0.0892). The percentage of total insulin secreted at each stage of the secretion assay (see Figure 3.3.2.3.B) reveals that L521 (15.3 ± 4.0 vs. 8.4 ± 2.3 %; p=0.0070), NID (24.6 ± 8.6 vs. 11.0 ± 2.6 %; p=0.0123), and CMRL-only (15.5 ± 4.7 vs. 10.2 ± 2.8 %; p=0.0070) conditioned islets demonstrated significant down-regulation of insulin secretion between the 20mM stage and the secondary 2mM stage.

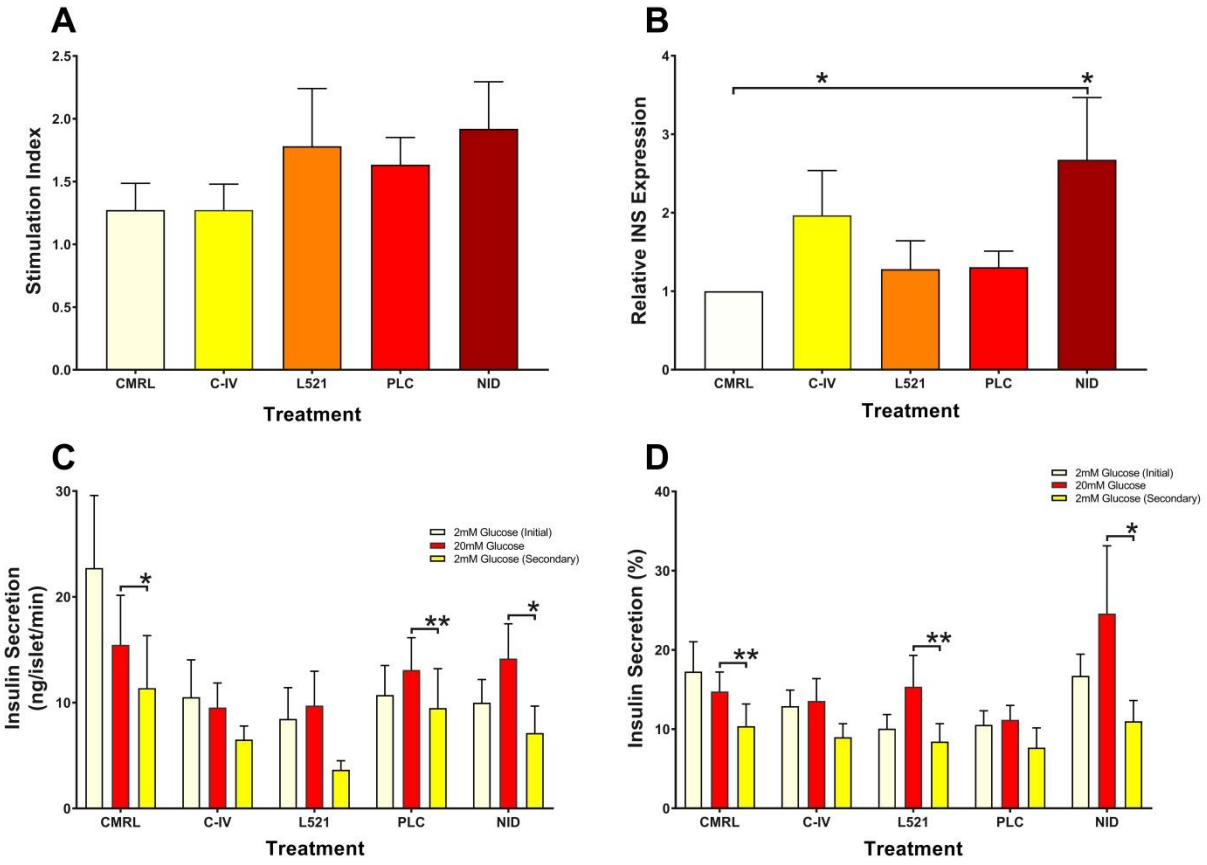


Figure 3.3.2.3: Stimulation index (A), INS gene expression (B; normalised to CMRL control) and glucose-stimulated insulin secretion of islets preconditioned in media supplemented with or without different matrix proteins from each stage of GSIS after hypoxic culture, determined as (C) absolute secretion and (D) percentage of total islet insulin content (mean ± SEM; n=12).

Gene expression analysis of islets demonstrated that, after hypoxic culture, BAX expression (see Figure 3.3.2.4.A) was significantly higher in islets pre-cultured in media supplemented with L521 (1.3 ± 0.1 fold; p=0.0162) compared with CMRL alone. Similarly, the BAX:BCL2 ratio (see Figure 3.3.2.4.D) was also significantly higher in islets pre-cultured in media

supplemented with L521 (1.7 ± 0.2 fold; p=0.0339) compared with CMRL alone. BCL2 (see Figure 3.3.2.4.B) and CASP3 (see Figure 3.3.2.4.C) islet expression was not significantly altered when preconditioned in media supplemented with any of the individual proteins.

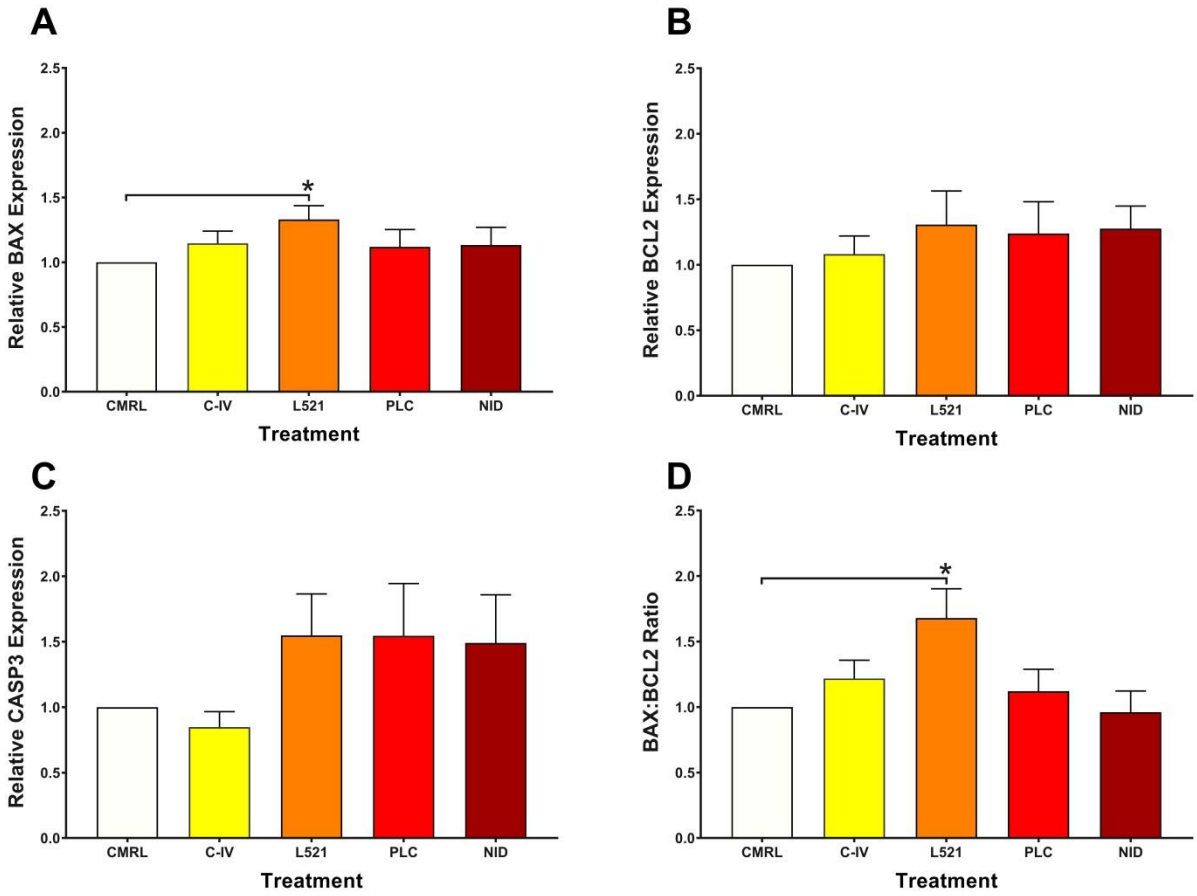


Figure 3.3.2.4: Gene expression of (A) BAX, (B) BCL2, and (C) CASP3, as well as (D) BAX:BCL2 ratio, in islets preconditioned in media supplemented with or without individual matrix proteins after hypoxic culture (values normalised to CMRL control; mean ± SEM; n=12).

Further gene expression analysis demonstrated that, after hypoxic culture islet HIF1A expression (see Figure 3.3.2.5.A) was significantly lower in islets pre-cultured in media supplemented with C-IV (0.8 ± 0.1 fold; p=0.0369) and PLC (0.8 ± 0.1 fold; p=0.0369) compared with CMRL alone. Similarly, DDIT3 expression (see Figure 3.3.2.5.C) was also lower in islets pre-cultured in media supplemented with C-IV (0.6 ± 0.1 fold; p=0.0180) and PLC (0.5 ± 0.1 fold; p=0.0112) compared with CMRL alone. Interestingly, unlike HIF1A,

VEGF expression (see Figure 3.3.2.5.B) was not significantly altered by preconditioning with individual matrix proteins.

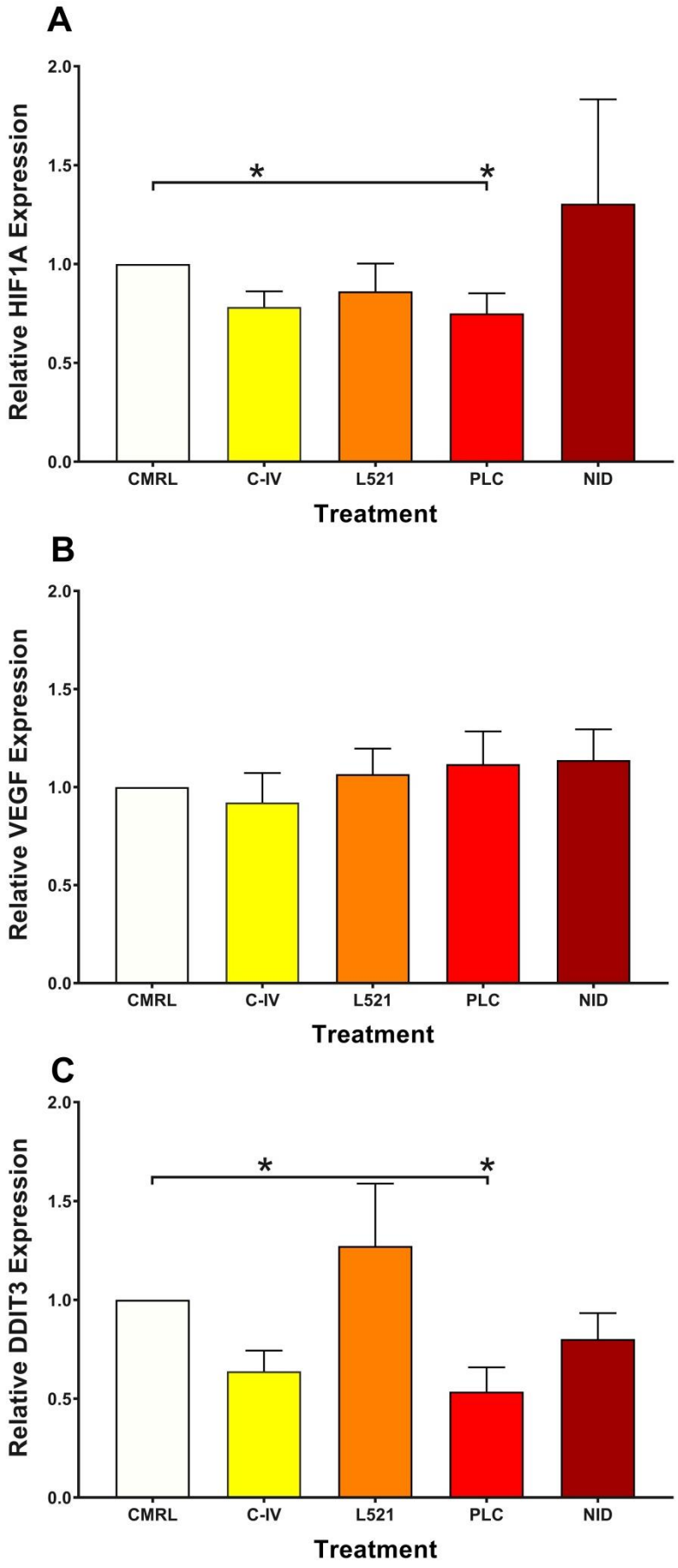


Figure 3.3.2.5: Gene expression of (A) HIF1A, (B) VEGF, and (C) DDIT3 in islets preconditioned in media supplemented with or without individual matrix proteins after hypoxic culture (values normalised to CMRL control; mean ± SEM; n=12).

3.4 Discussion

3.4.1 Collagen IV

After normoxic culture, supplementation of culture medium with 100nM CIV led to significantly reduced fragmentation and increased VEGF expression. Recovery, viability and stimulation index of islets were not increased in a statistically significant manner. After hypoxic culture, there were no significant differences in survival or function although the viable IEQ recovered did indicate some improvement. Both HIF1A and DDIT3 expression were significantly lower compared to those conditioned with CMRL alone.

Abundant in all extracellular matrices, collagens are not only important structurally, but also have an important role in signalling. A trimeric helical molecule, C-IV is unique among collagens in that it polymerises into a lattice rather than the more fibrillar structures formed by other isoforms (Yurchenco and Furthmayr, 1984; Kern *et al.*, 1993). It predominantly binds to the α1β1 integrin on the cell membrane, thought to mediate reactive oxygen species generation and proliferation (Pozzi *et al.*, 1998; Chen *et al.*, 2007). C-IV has also been shown to bind to α3β1 in islets and through this interaction it has been suggested to regulate insulin secretion (Kaido, Yebra, *et al.*, 2004; Wang *et al.*, 2005).

In this study, C-IV provided a minor benefit to the sensitivity of islets to changes in glucose concentration after normoxic and hypoxic incubation. While insulin gene expression was unaltered after normoxic culture, there was significant up-regulation after hypoxic culture. Previously, Daoud *et al.* (2010) demonstrated that islets cultured on plates coated with C-IV increased insulin expression. Interestingly, Daoud *et al.* observed upregulation of insulin gene expression in islets directly in contact with C-IV. In this study insulin upregulation occurred only when islets were cultured in unsupplemented media, not containing C-IV, after preconditioning with C-IV. It may be therefore, that C-IV becomes attached to the islet, remaining associated through binding to integrin receptors and other matrix proteins remaining

on the cell surface, thereby continuing to exert beneficial effects in a C-IV-free medium. By comparison, Kaido *et al.* (2006) observed reduced insulin expression and significant glucose-independent insulin secretion, with a resultant reduction in insulin content, on a C-IV-coated surface. These experiments, however, were performed in individual adult and foetal β-cells. Within the islet, other endocrine cell-types interact with β-cells, influencing function and thus, investigating β-cells alone does not fully represent their function within the islet micro-organ. In addition to the aforementioned effects on insulin expression, Daoud *et al.* also observed maintenance of islet morphology on C-IV, similar to those outcomes demonstrated here. The convergent results described both in this study and by Daoud *et al.*, combined with the observed reduction in pro-apoptotic DDIT3 expression and enhanced islets recovery, highlight the pro-survival benefits of C-IV.

Even utilising the highest concentration of any protein in this study, in addition to the benefits observed during the titration experiments, in this chapter, C-IV was the least effective of the proteins tested. The improvements to islet function and survival conferred by C-IV were minor compared with those cultured in CMRL alone. There is some evidence that the collagen VI (C-VI) content of the native pancreas is higher than that of C-IV (Hughes *et al.*, 2006). Accordingly, C-VI may exert greater influence on islet cells than C-IV. C-VI is more resistant to degradation by collagenases than C-IV (Heller-Harrison and Carter, 1984) and may therefore remain present on the islet surface, rendering it a poor candidate for culture medium supplementation. There is however no published data, as of yet, to confirm this. An alternative explanation for the minimal benefits conferred by C-IV is that, while C-IV is able to self-assemble into a matrix (Yurchenco and Ruben, 1987) it may not directly be directly in contact with islet cells. As laminins are integral for C-IV organisation in the BM and association with the islet (Form, Pratt and Madri, 1986; Graham *et al.*, 1997; Huang, 2003) C-IV may therefore be dependent on the laminin remaining in the islet surface after isolation (Cross *et al.*, 2017). Without laminin, C-IV may be unable to exert maximal effect.

Another benefit conferred by C-IV was to significantly increase VEGF expression, as a promoter of angiogenesis, this suggests positive outcomes for islet engraftment. This is supported by a number of *in vivo* studies demonstrating that vascular regrowth is improved by C-IV co-culture, allowing transplants to reach euglycaemia faster (Salvay *et al.*, 2008; Yap *et al.*, 2013). Unfortunately this effect was not observed after hypoxic culture. Indeed, HIF1A expression, a promoter of VEGF production was significantly lower, although expression VEGF expression was not its self significantly altered. This change may be indicative of decelerated vascular growth long term, which may indicate that C-IV is required in direct concert with islets for beneficial effects to be maintained as demonstrated by Daoud *et al.* (2010).

3.4.2 Laminin 521

Supplementation of culture medium with 25nM L521 led to a significantly reduction in islet fragmentation and an increase in VEGF expression. After hypoxic culture, L521 pre-cultured islets were significantly less fragmented and showed a small increase in recovery and viability compared to CMRL alone. Additionally, L521 treated islets demonstrated significant down regulation of insulin secretion when islets passed from high to low glucose concentration.

Laminins, in particular those containing α4 and α5 chains (Virtanen *et al.*, 2008), are a major part of the islet BM and knocking out the γ1 subunit is embryonically lethal (Smyth *et al.*, 1999; Miner *et al.*, 2004). They are vital as, unlike other proteins of the BM, they are able to self-assemble into sheet-like matrices on the surface of cells (McKee *et al.*, 2007). This polymerisation, orchestrated between the short arm of adjacent proteins and binding of the α-chain long arm to α3β1 integrins (Nishiuchi *et al.*, 2006; Otonkoski *et al.*, 2008; Virtanen *et al.*, 2008), are the starting point for the formation of the BM. Here, supplementation of culture

medium with L521 reduced fragmentation of islets both after normoxic and hypoxic culture. This implies rescue of apoptotic cells from anoikis potentially through the binding of arginine-glycine-aspartic acid (RGD) elements within the L521 amino acid sequence to the α3β1 integrin (Pinkse *et al.*, 2006). Although not the most effective of the proteins examined in this study, the aforementioned organisational role provided by laminins in BM formation (Form, Pratt and Madri, 1986; Graham *et al.*, 1997; Huang, 2003) may lead to enhanced benefits when applied in combination with other extracellular matrix proteins.

3.4.3 Perlecan

Supplementation of 25nM PLC into islet culture media significantly increased viable IEQ, VEGF expression, recovery of islets, and reduced fragmentation, after normoxic culture, although viability was unchanged. PLC also demonstrated a significant down regulation of insulin secretion from high to low glucose concentration. After hypoxic culture, these islets exhibited a further increase in viable IEQ, reduced fragmentation, and increased recovery of islets, while HIF1A and DDIT3 expression was significantly reduced. Viability of islets, once again, was unchanged.

As the most effective of the proteins tested, PLC improved islet survival and reduced fragmentation, likely mediated through two mechanisms of action. The first may be via RGD domains binding to integrins. All integrins containing the αv subunit, as well as the α5β1 and α3β1 integrins, bind the RGD sequence (Koivunen *et al.*, 1994; Kaido, Perez, *et al.*, 2004) most commonly associated with fibronectin. While fibronectin is an integral part of the extracellular matrix in many tissues (Proctor, 2017), where it mediates cellular activity through the aforementioned RGD sequence (Bachman *et al.*, 2015), there is minimal evidence for its presence in the BM of adult human islets. The laminin A chain-like domains of PLC (Haaparanta *et al.*, 1991; Iozzo *et al.*, 1994) also contained RGD sequences and binding of these

to integrins α3β1, α5β1, or αvβ3 prevents anoikis (Pinkse *et al.*, 2006), rescuing the cell from apoptosis. The second mechanism by which PLC may exert its benefit is through the endothelial growth factor (EGF)-like sequences within its primary structure (Noonan *et al.*, 1991; Iozzo *et al.*, 1994). EGF binding to cell surface receptors represses apoptosis through phosphorylation of the pro-apoptotic protein BAD (Humphrey *et al.*, 2008) and in islets, expression of a dominant-negative EGF receptor lead to decreased function and apoptosis of β-cells resulting in diabetes (Miettinen *et al.*, 2006). While this indicates a large potential benefit for islets, EGF has also been shown to induce dedifferentiation of isolated human islets (Hanley *et al.*, 2011). However, given that INS expression was not shown to be lower than in the unsupplemented control, it is unlikely that this occurred in these experiments. To further confirm this it may be of interest to investigate PDX-1 expression, a marker of β-cell identity (Gao *et al.*, 2014). The significant increase in islet survival as well as reduced fragmentation and DDIT3 expression, in addition to a small increase in islet function, is most likely due to a mixture of both RDG-integrin interaction and the beneficial effects of EGF signalling.

Interestingly, the fragment V region of the full proteoglycan (HSPG2), also known as endorepellin, is a potent inhibitor of angiogenesis (Bix *et al.*, 2004). This effect is mediated through the α1β2 integrin which is not present on islet endocrine cells (Cirulli *et al.*, 2000; Bix *et al.*, 2004; Kaido, Yebra, *et al.*, 2004; Virtanen *et al.*, 2008). Endothelial cells, however, do express α1β2. Some data suggests directly conflicting pro-angiogenic action through α5β1 integrin (Clarke *et al.*, 2012), although the brain endothelial cells used do not express the α2 integrin subunit (Lee *et al.*, 2011). This α1β2-mediated inhibition of angiogenesis may also be responsible for some of the benefits conferred by PLC seen in this study through the inhibition of endothelial cell proliferation, thereby directing more nutrients to endocrine cells. Although potentially advantageous *in vitro*, in a transplant environment inhibiting angiogenesis can lead to prolonged hypoxia, islet cell death, and ultimately, reduced graft function or failure (Hellman *et al.*, 1975). Yet, islets in culture with PLC demonstrate significantly increased VEGF

expression, similar to that shown in neural endothelial cells, as observed by Clarke *et al.*, (2012), and it may be that pro-angiogenic αvβ3 integrin activation by the RGD sequence of PLC (Montenegro *et al.*, 2012) outweighs any anti-angiogenic actions. Interestingly though, the reduction of HIF1A expression after hypoxia may result in a reduction of VEGF secretion in the long term. Furthermore, endorepellin has been shown to block VEGF receptor activation in endothelial cells (Goyal *et al.*, 2011) and this may attenuate the positive effects of increased VEGF production. Given these divergent results, it would be interesting to determine the outcome of these opposing effects in an *in vivo* setting, focussing on angiogenesis within the islet graft.

Given that these results only determine the effect of the domain V fragment of HSPG2, employing the entire protein may confer additional benefits. In the native BM, HSPG2 is orientated in a unidirectional manner (Iozzo *et al.*, 1994) with N-terminal domain I, with connected heparan sulphate chains, in contact with the cell surface and the C-terminal domain V directed outward. Given the difficulty in synthesising a large protein such as this, domain I and heparan sulphates may be the next logical step in improving islet function as an alternative to the full protein.

3.4.4 Nidogen

Supplementation of 25nM NID into islet culture media significantly reduced fragmentation as well as increasing recovery of islets after normoxic culture. After hypoxic culture, preconditioning with NID significantly reduced fragmentation and increased recovery of islets compared to those preconditioned with CMRL alone. The islets also exhibited a marginal improvement in regulation of insulin secretion, significantly reducing their in insulin secreted when transferred from a high to a low glucose concentration.

The vital role of NID has been demonstrated in animal models. While the absence of a single nidogen isoform does not necessarily prevent laminin or C-IV from developing into a BM (Kang and Kramer, 2000; Bader *et al.*, 2005), the loss of both isoforms results in lethality shortly after birth (Willem *et al.*, 2002). The beneficial role played by NID in cellular health has been further confirmed here in islets. Supplementation of culture medium with NID demonstrated similar positive effects on islets survival as PLC, though less potent. Fragmentation of islets was significantly reduced after both normoxic and hypoxic culture, and islet recovery was increased after hypoxic culture compared to the CMRL control. The similarities in effect observed are potentially due to the presence of RGD binding element found in the amino acid sequence of both proteins. Indeed, studies in other cell types have elucidated NID-mediated adhesion occurs through interaction with this motif (Yelian *et al.*, 1993; Dong, Hsieh and Chung, 1995) However, unlike PLC, the NID c-terminal exhibits a globular domain which binds with high affinity to the laminin y1 subunit (Timpl, 1999; Takagi *et al.*, 2003). Additionally, the n-terminal globular domain binds to both C-IV and domain IV of PLC (Hopf *et al.*, 1999; Ries *et al.*, 2001). This allows NID to act as a bridge between the various constituents of the BM stabilising and solidifying the macromolecular structure. The linking role may explain the disparity between the effects of RDG-integrin binding of the two proteins. Undigested C-IV and laminins remaining within the BM on the surface of islets after isolation may preferentially sequester NID, preventing RDG binding to integrins on the cell surface, thereby inhibiting the response. Conversely, NID demonstrated a significant increase in insulin expression, compared to CMRL alone and the improved sensitivity to change in glucose concentration may be a consequence of the bridging role of the NID protein (Koivunen *et al.*, 1994), indirectly influencing islet function.

3.4.5 Chapter Conclusion and Next Step

Supplementation of CMRL with each of the four proteins; C-IV, L521, NID, and PLC, demonstrated some benefit to islet survival, fragmentation, or function over the CMRL-only control. Therefore, all four proteins were to be combined in the next set of experiments, described in Chapter 4, to ascertain whether the effects of the individual proteins on islets were synergistically augmented by co-supplementation.

Chapter 4: Defining the Effects of Supplementation of a Combination of Extracellular Matrix Proteins into Islet Culture Medium

4.1 Introduction

Chapter 3 demonstrated the beneficial effects of C-IV, L521, PLC, and NID on islet fragmentation, insulin secretion and gene expression, after both normoxic and hypoxic culture. This chapter reports the investigation into whether combining these proteins enhances the individual benefits conferred to the function, morphology, and integrity of islets. As with the previous chapter, islets were cultured post-isolation in the standard CMRL supplemented with, or without, individual matrix proteins, in addition to a further group applying these proteins in combination. Islets were then assessed after 24-48 hours of normoxic culture and placed in the *in vitro* 'transplant-simulating' hypoxic culture for 48-72 hours before undergoing further assessment. As previously, the proteins were implemented at the optimal concentration determined by the titration experiments: 100nM C-IV, 25nM L521, 25nM PLC, and 25nM NID. The combination also used these concentrations. The results of the islet assessments compared the combination of proteins to each of the individual proteins and the unsupplemented CMRL control.

4.2 Methods

Initially, isolated islets (isolated as described in Section 2.2.1) were cultured in either unsupplemented CMRL, CMRL supplemented with individual matrix proteins, or CMRL supplemented with a combination of the proteins (MPC), at the aforementioned concentrations (as described in Section 4.2.2.2) for 24-48 hours in normoxic conditions (as described in section 3.2.1). Islets were assessed post-culture for viability (as described in Section 2.2.4.1), fragmentation (as described in Section 2.2.4.2), recovery (as described in Section 2.2.4.2), glucose-stimulated insulin secretion (as described in Section 2.2.4.3), and gene expression (as described in Section 3.2.2). The remaining islets were cultured in CMRL supplemented with

FCS, and without any matrix proteins, in hypoxic conditions for 48-72 hours (as described in Section 3.2.1.2), before undergoing similar assessment.

4.2.1 Donor Details

Human pancreases were retrieved from 6 human (4 male and 2 female), multi-organ DBD (see Table 4.2.1.1) and, isolated as described in Section 2.2.1. For this study, 17395.6 ± 5926.9 IEQ, with a viability of 75.4 ± 2.7 % and purity of 72.5 ± 5.0 % post purification were used. Individual islet preparations, from a single pancreas, were assessed separately and represented one technical replicate.

Table 4.2.1.1: Islet Preparation Details

6 Donors	Median	Range
BMI	30.4	27.0 – 36.0
Age (Years)	48.5	46.0 – 55.0
CIT (Hours)	5.3	4.5 – 6.3
WIT (Mins)	22.0	0.0 – 154.0

BMI – Body mass index, CIT – Cold ischaemia time, WIT – Warm ischaemia time

4.2.2 Islet Culture

4.2.2.1 Culture Medium

Islet culture was performed in CMRL as described in Section 2.2.3.1.

4.2.2.2 Matrix Protein Supplementation

CMRL was supplemented with the matrix proteins of interest according to the most suitable concentration found during the titration experiments in Chapter 2 either individually (as described in Section 3.2.2.2) or in combination. MPC consisted of 100nM C-IV (44.00 µg/mL), 25nM L521 (79.05 µg/mL), 25nM PLC (0.70 µg/mL), and 25nM NID-1 (3.75 µg/mL).

4.2.2.3 Normoxic Culture

Islets were cultured in CMRL (as described in Section 4.2.2.1) alone, with matrix proteins added individually (as described in Section 3.2.2.2) or in combination (as described in Section 4.2.2.2). Islets were seeded in 25cm^2 culture flasks (Sarstedt AG & Co, Nümbrecht, Germany) for 24-48 hrs at 37°C, 21% O_2 5% CO_2. Atmosphere and culture vessels were selected according to the clinical protocol for islet culture prior to islet transplantation.

4.2.2.4 Hypoxic Culture

Islets were cultured in hypoxia as described in Section 3.2.2.4 and were distributed in the plate according to Figure 4.2.2.4.1 before being cultured for 48-72 hrs at 37°C, 1% O_2, 5% CO_2.

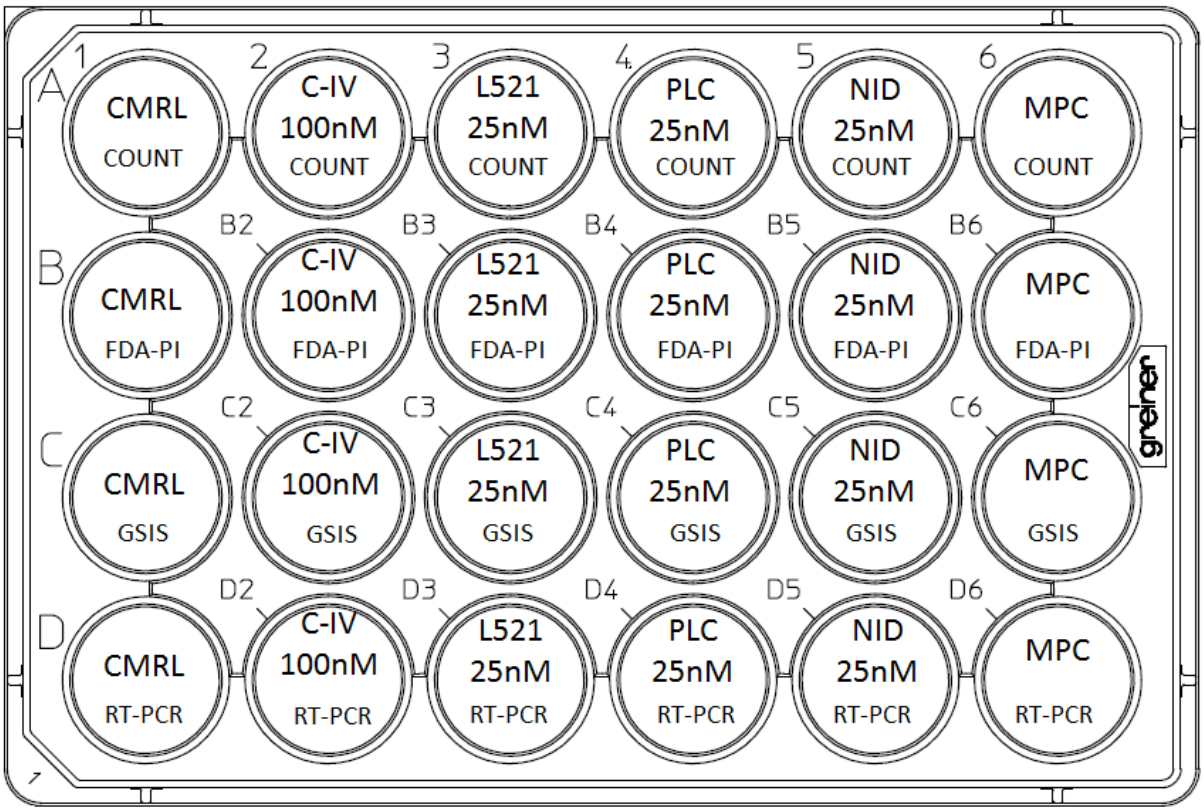


Figure 4.2.2.4.1: Islet culture plate map for individual and combined matrix protein supplementation.

4.2.3 Statistical Analyses

All assessment parameters were analysed with Prism 7 using Friedman's test followed by Dunn's multiple comparison test, utilising islets without matrix protein treatment as controls. Statistical differences in figures are indexed by * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, and **** for $p<0.0001$ when compared with MPC. Statistical differences in figures are indexed by + for $p<0.05$, ++ for $p<0.01$, +++ for $p<0.001$, and ++++ for $p<0.0001$ when compared with CMRL.

4.3 Results

4.3.1 Post-Normoxic Culture

After normoxic incubation, islet fragmentation (see Figure 4.3.1.1.A) was significantly reduced in islets cultured in MPC (0.5 ± 0.1 IN/IEQ) compared with those cultured in CMRL alone (1.1 ± 0.2 IN/IEQ; p=0.0001) as well as CMRL supplemented with L521 (0.8 ± 0.1 IN/IEQ; p=0.0308), and NID (0.8 ± 0.2 IN/IEQ; p=0.0206). Supplementation of CMRL with the individual proteins C-IV (0.7 ± 0.1 IN/IEQ; p=0.0136) and PLC (0.7 ± 0.1 IN/IEQ; p=0.0136) also decreased fragmentation compared with CMRL alone (1.1 ± 0.2 IN/IEQ). Addition of MPC further increased islet recovery (see Figure 4.3.1.1.B) compared to CMRL alone (45.0 ± 6.9 vs. 23.0 ± 1.7 %; p=0.0087). Recovery of islets, compared with those cultured in CMRL alone, was significantly increased by the addition of PLC (37.8 ± 6.8 vs. 23.0 ± 1.7 %; p=0.0087).

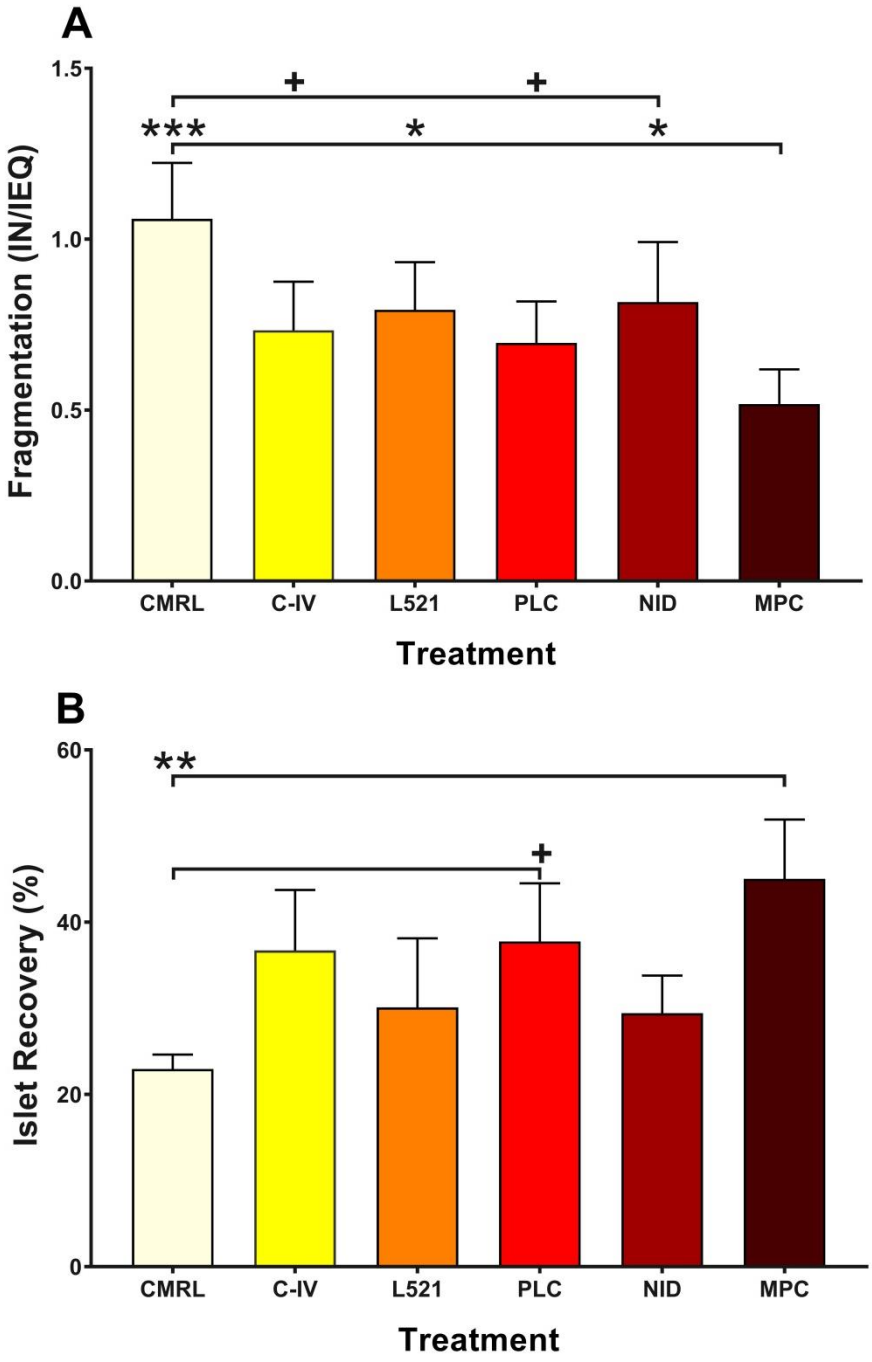


*Figure 4.3.1.1: Islet fragmentation (A) and islet recovery (B) after normoxic culture in media supplemented with or without different matrix proteins (mean ± SEM; ***** significance versus MPC, + significance versus CMRL; n=6).*

The post-normoxic culture viability of islets (see Figure 4.3.1.2) was not significantly improved when CMRL was supplemented with matrix proteins, although when compared with PLC (51.9 ± 6.1 %; p=0.0087) and C-IV (50.5 ± 6.5 %; p=0.0308), MPC demonstrated significantly lower

islet viability (38.1 ± 6.5 %). The viable IEQ (see Table 4.3.1.1) was not significantly changed by the addition of individual or combined matrix proteins to the culture medium.

Table 4.3.1.1: Post-normoxia viability and secretion data of islets in culture medium supplemented with or without matrix proteins (mean ± SEM; n=6).

	Viable IEQ	Insulin Content (µg/islet)	Total Insulin Secreted (%)
CMRL	315.8 ± 115.6	6.3 ± 2.6	23.4 ± 7.6
C-IV (100nM)	460.7 ± 110.3	4.5 ± 1.9	30.8 ± 14.0
L521 (25nM)	331.9 ± 98.6	7.8 ± 2.7	22.3 ± 6.2
PLC (25nM)	555.8 ± 200.6	6.7 ± 2.0	25.3 ± 7.5
NID (25nM)	386.1 ± 100.8	6.6 ± 2.2	26.7 ± 7.2
MPC	403.7 ± 94.2	6.1 ± 1.6	28.0 ± 6.0

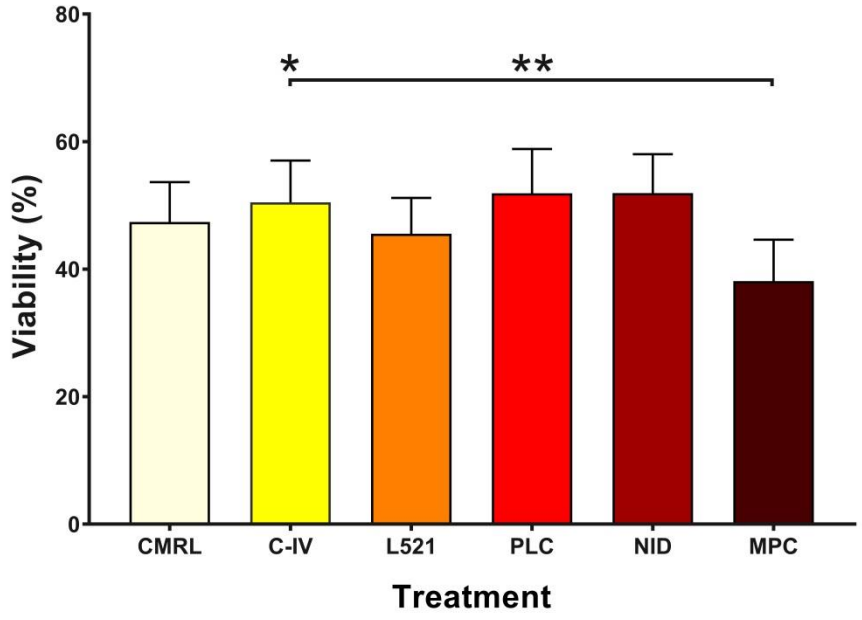


Figure 4.3.1.2: Islet viability after normoxic culture in media supplemented with or without different matrix proteins (Mean ± SEM).

Islet SI (see Figure 4.3.1.3.A) was significantly higher with supplementation of CMRL with MPC (4.0 ± 2.1 vs. 0.7 ± 0.1 ng/islet/min; p=0.0136). This however did not correspond to any significant alteration in INS gene expression (see Figure 4.3.1.3.B). The absolute insulin

secretion results (see Figure 4.3.1.3.B) showed a marked improvement in the secretory profile of the islets incubated with MPC, there was a significantly higher insulin secretion at 20mM glucose (26.6 ± 7.4 ng/islet/min) compared with the initial 2mM step (14.9 ± 4.7 ng/islet/min; p=0.0209). In addition, there was a reduction in insulin secretion when islets were transferred from the 20mM glucose incubation (12.4 ± 5.2 ng/islet/min) to the secondary 2mM incubation (26.3 ± 7.4 ng/islet/min; p=0.0433). Furthermore, the MPC treated islets have a distinctly higher secretion at 20mM (26.3 ± 7.4 ng/islet/min) compared with those cultured with L521 (14.8 ± 7.2 ng/islet/min; p=0.0449) and in the CMRL-only control (10.2 ± 5.1 ng/islet/min; p=0.0136). Islets cultured with NID (19.0 ± 9.8 ng/islet/min; p=0.0449) and PLC (15.4 ± 6.1 ng/islet/min; p=0.0308) also showed significantly higher secretion of insulin at 20mM glucose compared with those cultured in CMRL alone (10.2 ± 5.1 ng/islet/min). Percentage of total insulin secreted (see Figure 4.3.1.3.D) further demonstrates the benefits of MPC supplementation on islet insulin secretion.

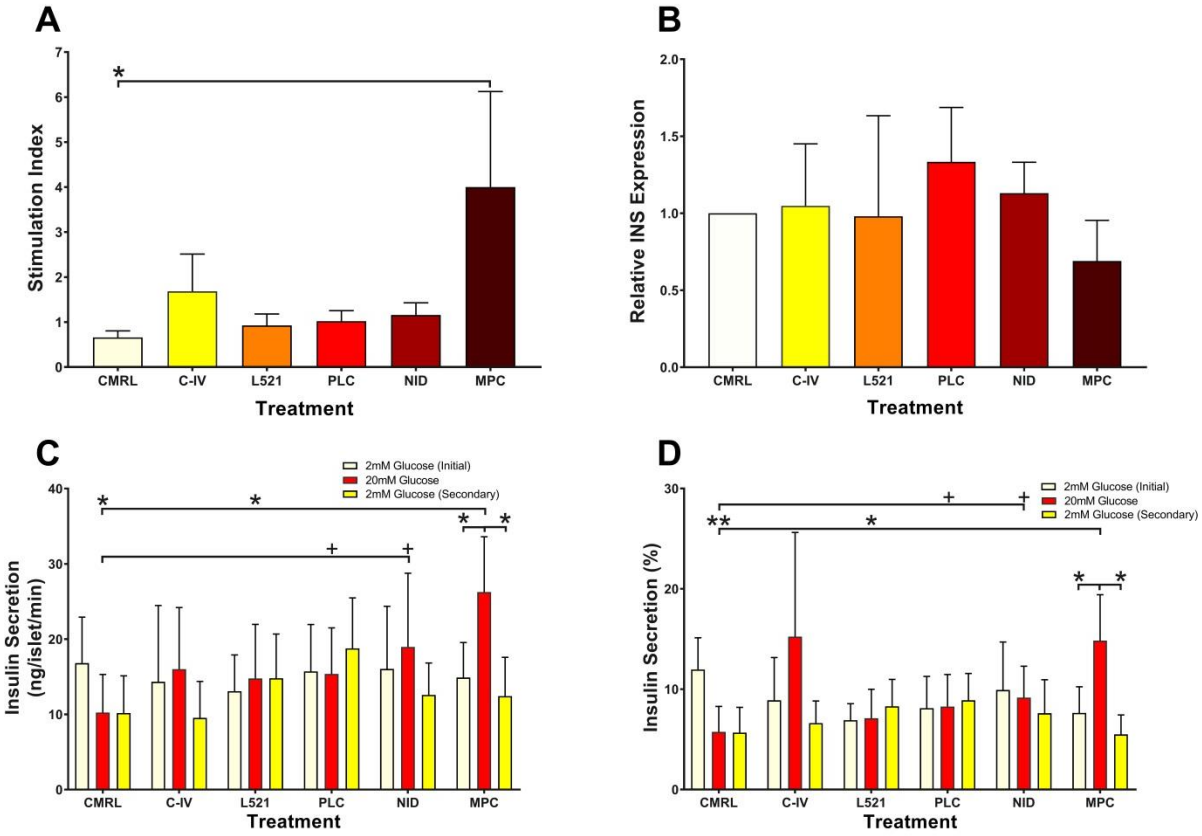


*Figure 4.3.1.3: Stimulation index (A), INS gene expression (B; normalised to CMRL control), and glucose-stimulated insulin secretion of islets from each stage of GSIS after normoxic culture in media supplemented with or without different matrix proteins determined as (C) absolute secretion and (D) percentage of total islet insulin content (mean ± SEM; ***** significance versus MPC (20mM Glucose), + significance versus CMRL (20mM Glucose); n=6).*

Pro-apoptotic BAX expression (see Figure 4.3.1.4.A) was not significantly altered by MPC supplementation of culture medium. However, expression of the anti-apoptotic BCL2 gene (see Figure 4.3.1.4.B) was significantly higher in islets culture in media supplemented with MPC (2.0 ± 0.3 fold) compared with C-IV (0.8 ± 0.1 fold; p=0.0004), PLC (1.3 ± 0.4 fold; p=0.0136), and NID (0.9 ± 0.1 fold; p=0.0007). MPC BCL2 expression appeared higher than in CMRL control, however this did not quite reach statistical significance (2.0 ± 0.3 fold; p=0.0641). Consequently, the BAX:BCL2 ratio (see Figure 4.3.1.4.D), a measure of the apoptotic state of islets, was significantly lowered by culture with MPC (0.7 ± 0.1 fold) in comparison with C-IV (1.3 ± 0.2 fold; p=0.0004), PLC (1.1 ± 0.1 fold; p=0.0136), and NID (1.3 ± 0.2 fold; p=0.0007).

While demonstrating a similar expression profile to BCL2, islet CASP3 expression (see Figure 4.3.1.4.C) was not significantly altered by MPC supplementation.

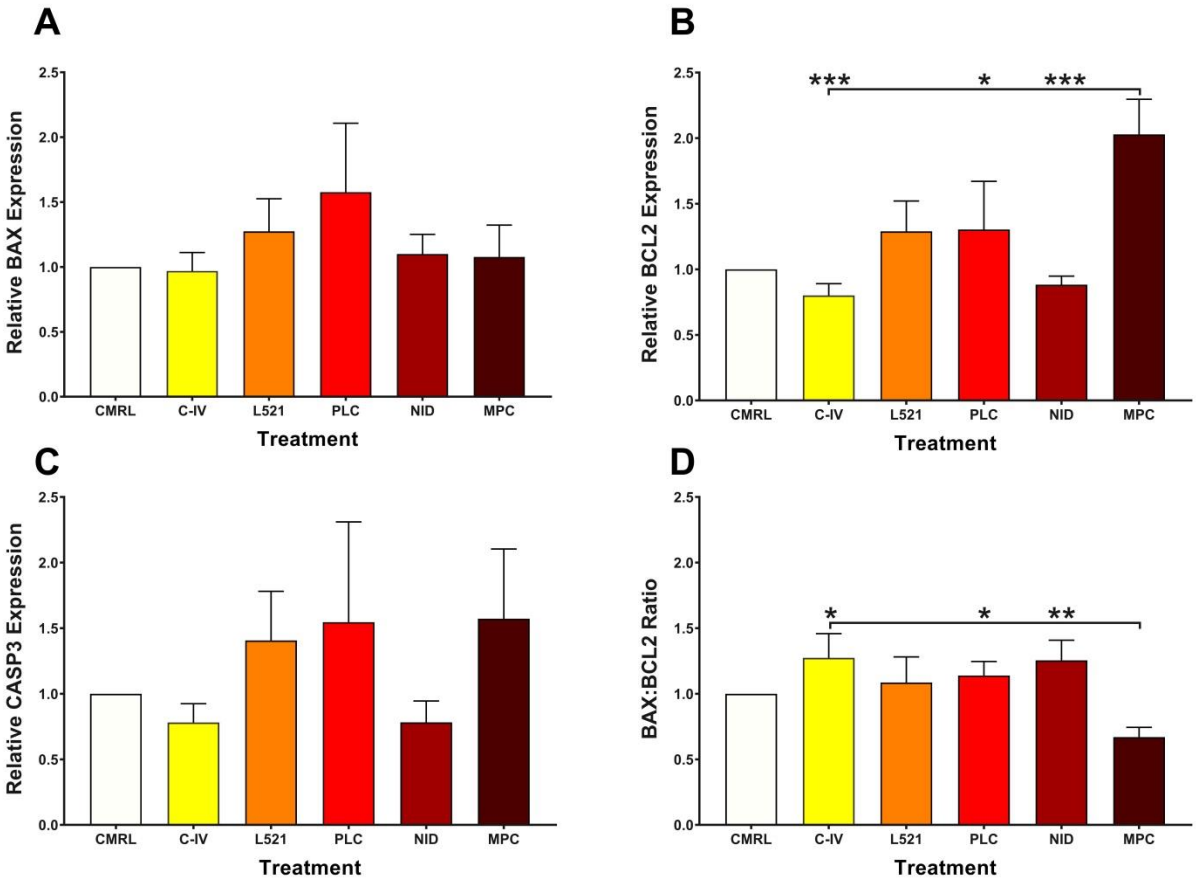


Figure 4.3.1.4: (A) BAX, (B) BCL2, and (C) CASP3 expression as well as (D) BAX:BCL2 ratio of islets cultured in media supplemented with or without different matrix proteins (values normalised to CMRL control; mean ± SEM; n=6).

Islet HIF1A expression (see Figure 4.3.1.5.A) and DDIT3 expression (see Figure 4.3.1.5.C) was not significantly altered by MPC supplementation of culture media. VEGF (see Figure 4.3.1.5.B) however, was expressed at significantly higher levels in islets cultured in MPC supplemented media than those cultured in the CMRL control (2.1 ± 0.3 fold; p=0.0068).

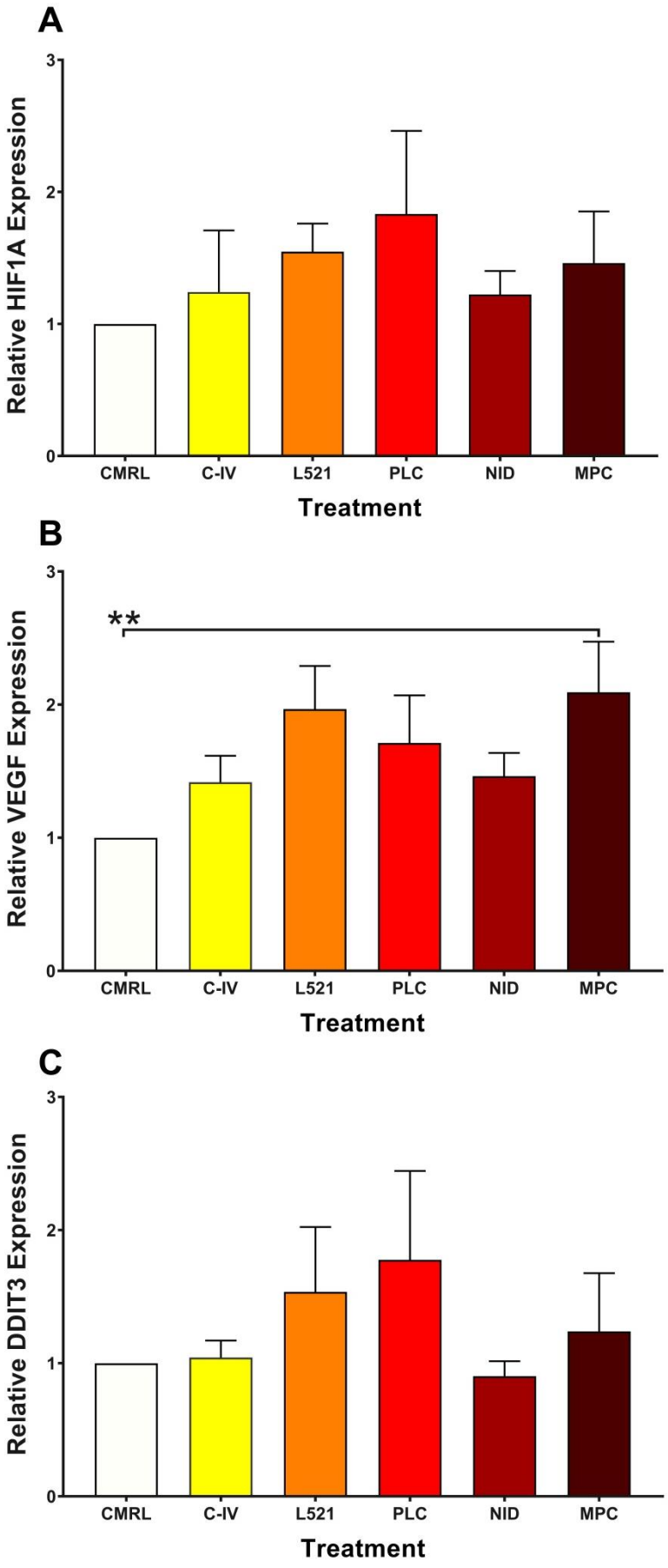


Figure 4.3.1.5: Post-normoxic (A) HIF1A, (B) VEGF, and (C) DDIT3 expression of islets cultured in media supplemented with or without different matrix proteins (values normalised to CMRL control; mean ± SEM; n=6).

4.3.2 Post-Hypoxic Culture

After hypoxic incubation, islet fragmentation (see Figure 4.3.2.1.A) was significantly lower in L521 (0.9 ± 0.1 IN/IEQ; p=0.0087), PLC (1.0 ± 0.2 IN/IEQ; p=0.0055), NID (1.0 ± 0.2 IN/IEQ; p=0.0020), and MPC (0.8 ± 0.1 IN/IEQ; p=0.0012) treated islets in comparison to those conditioned in the standard clinical medium (1.6 ± 0.2 IN/IEQ). In comparison to C-IV (0.8 ± 0.1 IN/IEQ), MPC significantly reduced islet fragmentation (1.3 ± 0.2 IN/IEQ; p=0.0449) and in addition, islet recovery (see Figure 4.3.2.1.B) after hypoxic culture was significantly increased by conditioning with MPC (15.2 ± 3.1 %; p=0.0308), PLC (13.6 ± 3.5 %; p=0.0206), and NID (14.9 ± 3.3 %; p=0.0206) compared with islets conditioned in CMRL alone (8.3 ± 2.8 %).

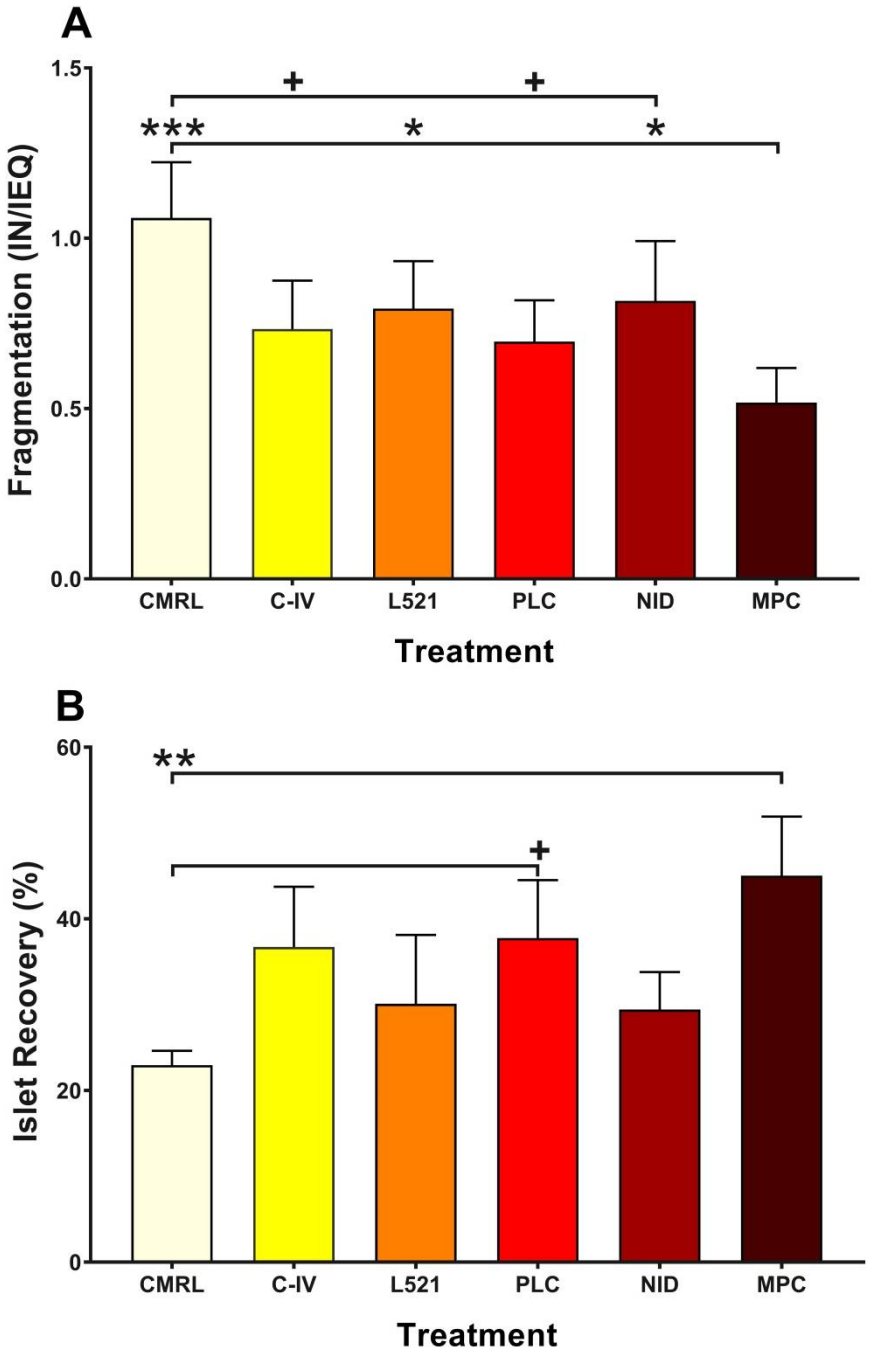


*Figure 4.3.2.1: Fragmentation (A) and recovery (B) post-hypoxia of islet pre-cultured in media supplemented with or without different matrix proteins (mean ± SEM; ***** significance versus MPC, + significance versus CMRL; n=6).*

The viability of islets (see Figure 4.3.2.2.A) after hypoxic culture was not significantly altered by preconditioning islets with extracellular matrix proteins, although the MPC treated islets (70.6 ± 4.4 %) had a higher viability compared with those conditioned with NID (60.7 ± 3.9 %; p=0.0136). The viable IEQ (see Figure 4.3.2.2.A) was significantly increased by

preconditioning of islets with MPC (232.3 ± 38.6 IEQ) compared with both C-IV (142.6 ± 43.0 IEQ; p=0.0206) and CMRL alone (109.0 ± 23.9 IEQ; p=0.0409).

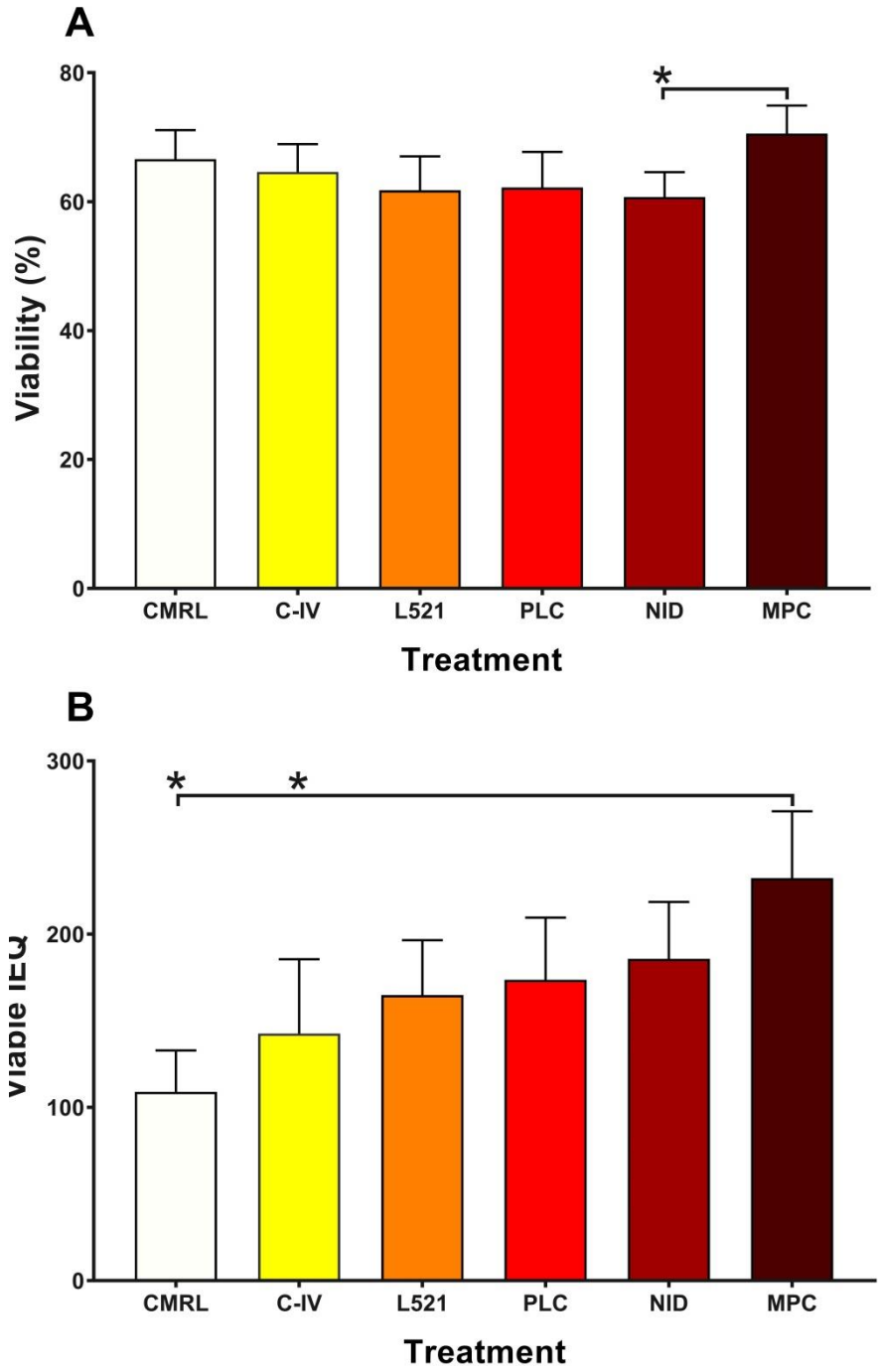


Figure 4.3.2.2: Viability (A) and viable IEQ recovered (B) post-hypoxia of islet pre-cultured in media supplemented with or without different matrix proteins (mean ± SEM; n=6).

Table 4.3.2.1: Post-hypoxia insulin data of islets preconditioned with or without individual matrix proteins (mean ± SEM).

	Insulin Content (µg/islet)	Total Insulin Secreted (%)
CMRL	3.9 ± 1.2	10.7 ± 3.5
C-IV (100nM)	4.0 ± 0.7	6.8 ± 2.0
L521 (25nM)	7.6 ± 2.2	4.7 ± 2.0
PLC (25nM)	4.8 ± 1.0	4.8 ± 1.3
NID (25nM)	4.3 ± 0.9	6.0 ± 1.4
MPC	7.8 ± 3.3	3.7 ± 1.7

Islet SI (see Figure 4.3.2.3.A) was significantly improved by MPC supplementation (4.0 ± 0.6) during pre-culture compared with those conditioned with L521 (1.5 ± 0.3; p=0.0135), C-IV (1.0 ± 0.1 vs.; p=0.0030), and CMRL alone (1.0 ± 0.2; p=0.0030). This corresponded to a significantly higher INS gene expression (see Figure 4.3.2.3.B) in MPC treated islets (2.5 ± 0.8 fold; p=0.0206) compared with both CMRL and PLC (1.3 ± 0.3 fold; p=0.449). Absolute islet insulin secretion (see Figure 4.3.2.3.C) further demonstrates the benefits conferred by MPC pre-conditioning. MPC preconditioned islets had significant up-regulation of insulin secretion when going from 2mM (9.5 ± 5.7 ng/islet/min) to 20mM glucose (20.8 ± 7.5 ng/islet/min; p=0.0066) and down-regulation from 20mM (20.8 ± 7.5 ng/islet/min) to 2mM glucose (2.8 ± 1.0 ng/islet/min; p=0.0151). None of the other treatment groups reacted to changes in insulin concentration in this way. Although CMRL (8.8 ± 2.5 vs. 3.0 ± 1.4 ng/islet/min; p=0.0323), L521 (9.7 ± 3.9 vs. 2.7 ± 1.3 ng/islet/min; p=0.0323), and PLC (9.4 ± 1.7 vs. 2.3 ± 0.9 ng/islet/min; p=0.0323) conditioned islets did demonstrate significant down-regulation of secretion from 20mM to 2mM glucose. Furthermore, MPC had both significantly higher secretion at 20mM glucose (20.8 ± 7.5 ng/islet/min) compared with CMRL alone (8.8 ± 2.5 ng/islet/min; p=0.0383) and lower secretion at 2mM glucose (9.5 ± 5.7 ng/islet/min) compared with CMRL alone (8.8 ± 2.5 ng/islet/min; p=0.0152). Similar results may be observed in the percentage insulin secretion (see Figure 4.3.2.3.D), although percentage secretion of insulin at 20mM across treatment groups was no significantly different.

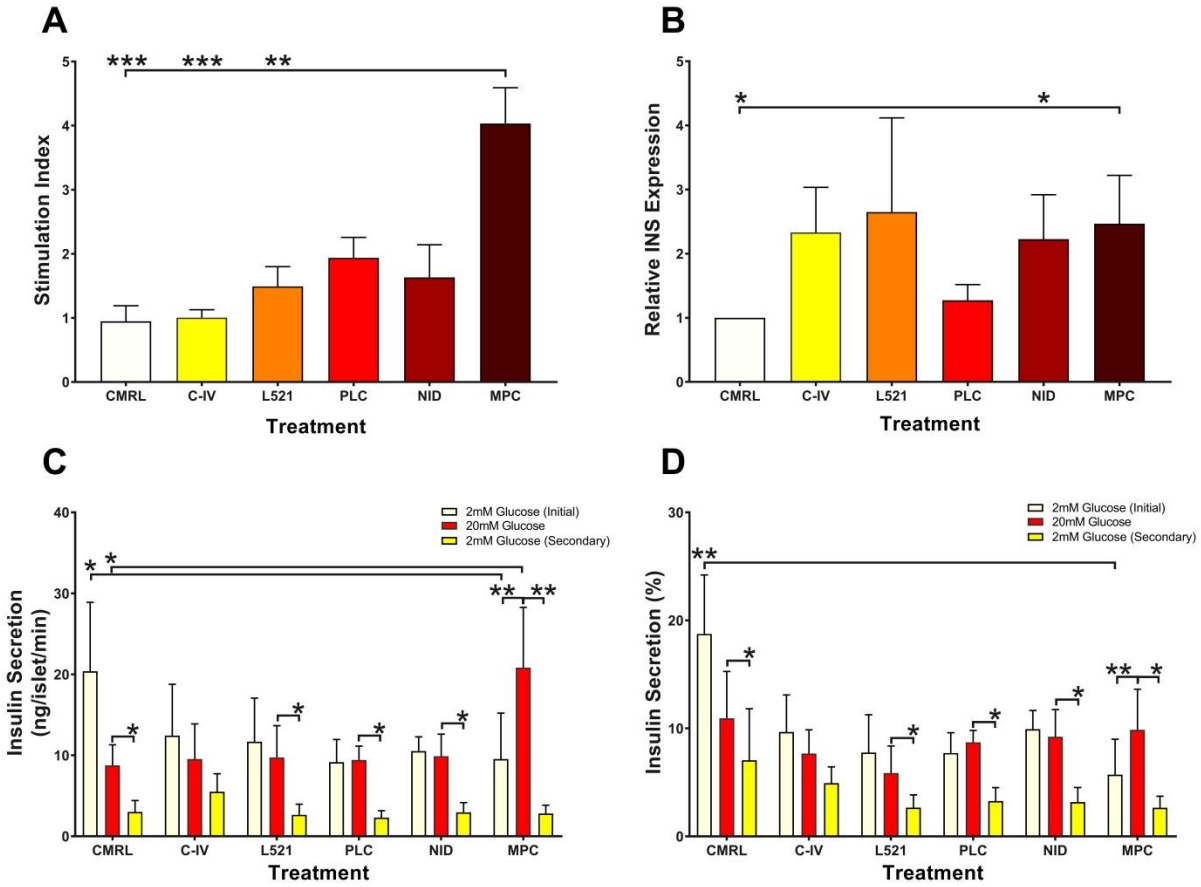


Figure 4.3.2.3: Stimulation index (A), INS gene expression (B; normalised to CMRL control) and glucose-stimulated insulin secretion of islets preconditioned in media supplemented with or without different matrix proteins from each stage of GSIS after hypoxic culture, determined as (C) absolute secretion and (D) percentage of total islet insulin content (mean ± SEM).

Gene expression analysis of islets demonstrated that, after hypoxic culture, there were no significant differences seen in expression of BAX (see Figure 4.3.2.4.A), BCL2 (see Figure 4.3.2.4.B), CASP3 (see Figure 4.3.2.4.C), or the ratio of BAX:BCL2 expression (see Figure 4.3.2.4.D). Furthermore, pre-culture of islets with MPC did not significantly alter HIF1A (see Figure 4.3.2.5.A), VEGF (see Figure 4.3.2.4.B), or DDIT3 (see Figure 4.3.2.4.C) expression after normoxic culture.

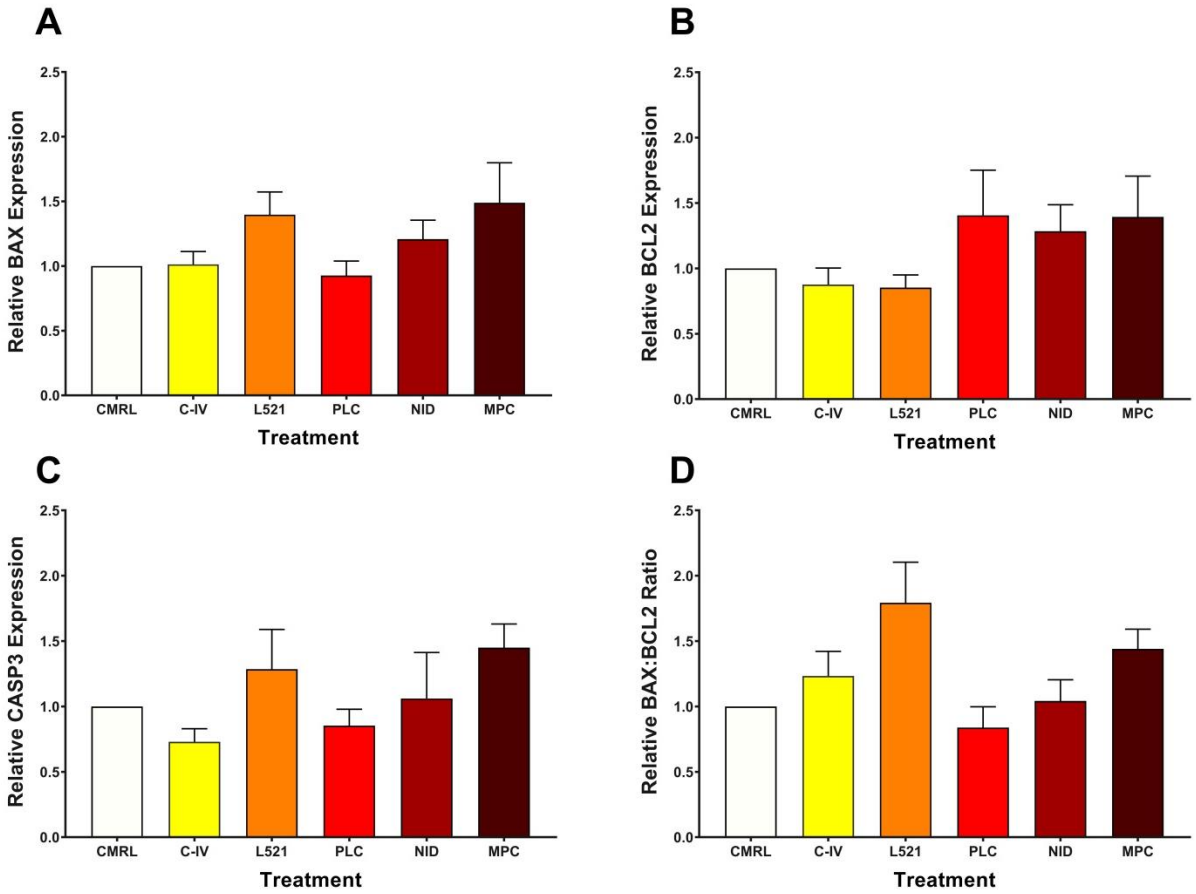


Figure 4.3.2.4: Post-hypoxic (A) BAX, (B) BCL2, and (C) CASP3 expression as well as (D) BAX:BCL2 ratio of islets pre-cultured in media supplemented with or without different matrix proteins (values normalised to CMRL control; mean ± SEM; n=6).

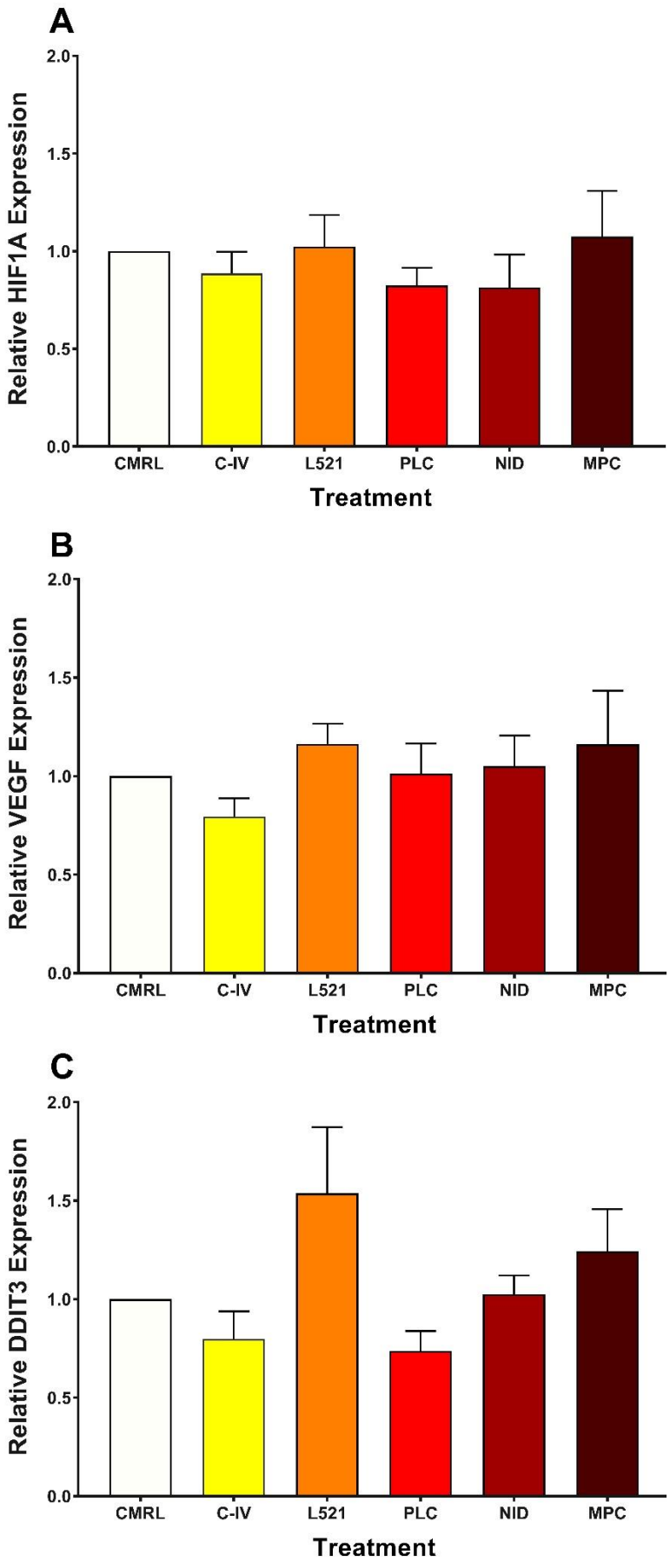


Figure 4.3.2.5: Post-hypoxic (A) HIF1A, (B) VEGF, and (C) DDIT3 expression of islets pre-cultured in media supplemented with or without different matrix proteins (values normalised to CMRL control; mean ± SEM n=6).

4.4 Discussion

After culture in normoxic atmosphere, supplementation of culture medium with a combination of matrix proteins significantly increased recovery and decreased fragmentation of islets compared to those cultured in CMRL alone. A combination of matrix proteins also increases the SI and sensitivity to glucose, responding in a physiological manner towards a change in glucose concentration, in spite of lower viability. The lower viability was not statistically significant however when compared to the CMRL control, though this may be the result of a lack of statistical power caused by a low number of technical replicates. Viability was higher in C-IV and PLC treated islets treated with MPC. Nevertheless, *in vitro* function of the islets, along with their higher overall survival, implies that viability is not necessarily indicative of overall outcomes. Furthermore, MPC-cultured islets were less fragmented than those cultured with L521 or NID, had higher insulin secretion at 20mM glucose than those cultured with L521, and expressed BCL2 more highly than those cultured with C-IV, PLC, and NID. After the subsequent 48-72 hours of hypoxic culture, pre-conditioning of islets with a combination of matrix proteins significantly decreased fragmentation of islets, increased stimulation index and INS gene expression as well as demonstrating a more physiological secretory profile compared to those preconditioned in CMRL alone. Islet recovery and viable IEQ were also increased. Pre-conditioning with a combination of matrix proteins significantly lowered fragmentation preconditioning in C-IV, in addition, preconditioning with improved islet stimulation index compared to CMRL, C-IV, and L521 after hypoxic culture. Islets also clearly demonstrated a strict response to changes in glucose concentrations, unlike any of the islet pre-conditioned with individual proteins. Finally, islet preconditioned with a combination of matrix proteins had higher viability and INS expression than NID after hypoxic culture.

Formation of the BM is thought to be initiated by laminin, recruiting NID and collagens as well as proteoglycans such as HSPG2 (Form, Pratt and Madri, 1986; Graham *et al.*, 1997; Huang,

2003; McKee *et al.*, 2007). Each protein performs a distinct function with C-IV forming the scaffold, laminins anchoring to the cell surface, nidogens linking these together, and heparan sulphate proteoglycans providing additional structural support (Hohenester and Yurchenco, 2013). This synergistic partnership is potentially the basis for the improved effects demonstrated in these experiments when the matrix proteins were combined.

Recovery of islets was significantly higher when islets were cultured with MPC and the fragmentation was lower both after the normoxic and subsequent hypoxic cultures compare to those cultured without any matrix proteins. Improved structural integrity may originate, not only from intracellular effects discussed in chapter 3, but also from a formation of a new BM surrounding the islet. This structural envelope may be securing endocrine cells together within the islet and preventing apoptosis. Furthermore, this may also explain the lower viability in MPC cultured than those cultured in PLC and C-IV, whereby formation novel BM binds apoptotic or necrotic cells within the islet. Conversely, this was reversed after culture in 1% O_2 with higher viability in MPC conditioned islets, thereby demonstrating the reduced susceptibility to hypoxia-induced apoptosis, which could lead to a greater likelihood of graft survival.

A dramatic improvement was observed in the insulin secretory function of MPC conditioned islets compared with any of the other experimental group. Not only did these islets demonstrate increased stimulation index compared to other islets, their expression of the INS gene was significantly higher, thus the potential for sustained insulin secretion is much increased as insulin stores are replenished. Furthermore, the islets secreted insulin in a more physiological manner, with significant up and down regulation of insulin secretion with respective changes in glucose concentrations, following both normoxic and hypoxic culture. This was not the case in any of the other groups investigated, further demonstrating the beneficial defects of matrix interactions. While the effect observed may be purely a result of accumulation of the beneficial effects of each of the 4 proteins, given that the proteins associate closely with the native BM,

these functional gains may have been further enhanced by positive interactions between the proteins.

Cellular stress, particularly induced by low O_2 conditions can increase the expression of CASP3. In islets cultured with MPC, BCL2 expression is increased after normoxic culture and this may have an effect in counteracting increased CASP3 expression, leading to inhibition of caspase activation, and may be the mechanism which results in the enhanced recovery and reduced fragmentation observed when islet culture medium is supplemented with MPC. Similarly, amplified expression of BCL2 and VEGF may also have a role negating damage caused by oxidative stress.

The significant morphological and functional benefits of MPC strongly endorse a potential avenue for maximisation of islet survival both after post-isolation culture and during the hypoxic stresses experienced subsequent to transplantation in the portal vein.

Chapter 5: Conclusions and Future Work

5.1 Study Conclusions

The first aim of this study was to define the most effective concentration of the matrix proteins C-IV, L521, PLC, and NID across the range examined. This was achieved in Chapter 2 through islet culture in media supplemented with each protein across a range of concentrations from 25nM to 100nM, in addition to a CMRL-only control. C-IV demonstrated benefits to both islet function and function at 100nM compared with 25nM, whereas none of the other proteins conferred any additional improvements to islet survival or function at concentrations higher than 25nM.

The second aim of this study was to examine whether supplementation of the proteins, at their most effective concentration, into the islet culture medium confers benefits to survival and function after normoxic culture. This was achieved in Chapter 3 by simulating the 24-48 hour pre-transplant culture period. Islets were cultured in the clinical culture standard CMRL medium supplemented with, or without, each of the proteins C-IV, L521, PLC, or NID individually. C-IV and L521 reduced islet fragmentation, increased VEGF expression. PLC increased islet recovery, viable IEQ, and VEGF expression as well as reducing fragmentation. Finally, NID increased islet recovery and INS expression as well as reducing fragmentation. These results reveal that islet survival in culture is improved by supplementation of culture media with extracellular matrix proteins of the BM.

The third aim of this study was to examine whether culture of islets in media supplemented with extracellular matrix proteins further enhances survival and function during a subsequent culture period in a hypoxic environment. This was addressed in Chapter 3 and was accomplished by culture of islets for 48-72 hours in 1% O_2 to simulate the hypoxic challenge encountered by islets post-transplant. C-IV did not demonstrate any statistically significant differences in survival or function, although, HIF1A and DDIT3 expression was lower with and higher in INS expression than islets treated with CMRL-alone. L521 significantly reduced

fragmentation. PLC increased viable IEQ, reduced fragmentation, and increased recovery of islets, while HIF1A and CHOP expression was significantly reduced. NID-treated islets were less fragmented and recovery was increased, they also exhibited a significant down regulation of insulin secretion when transferred from a high to low glucose concentration. These results reveal that islet survival in hypoxia is improved by pre-culture in culture media supplemented with extracellular matrix proteins of the BM.

The fourth, and final, aim of this study was to test whether the extracellular proteins found to be beneficial and are able to further enhance islet function and survival if combined together, in a manner similar to that found within the native islet BM. This was addressed in Chapter 4, implemented by comparing survival and function of islets firstly after culture identical to that performed clinically pre-transplant and secondly, after a hypoxic culture period simulating the low oxygen conditions encountered by islets post-transplant. After normoxic culture the matrix protein combination significantly enhanced islet stimulation index, sensitivity to change in glucose concentration, and recovery, in addition to decreasing fragmentation of islets compared to islets co-cultured with CMRL alone. Furthermore, antiapoptotic BCL2 expression was increased resulting in an rise in the BAX:BCL2 ratio and viability was reduced. After the subsequent hypoxic culture, islets pre-conditioned with a combination of matrix proteins were significantly less fragmented, stimulation index was higher, as was INS gene expression. Islets also demonstrated a more physiological secretory profile compared to those preconditioned in CMRL alone. Islet recovery and viable IEQ were also increased. These results reveal that islet survival in both normoxia and hypoxia is improved by supplementation of culture media supplemented with extracellular matrix proteins of the basement membrane in combination.

In summary, here it has been demonstrated that supplementation of islet culture medium with the extracellular matrix proteins C-IV, L521, PLC, and NID, provide significant benefit to islet survival, integrity, or function in culture both in normoxia and in subsequent hypoxia. Combining these proteins further enhanced effects exerted by the individually, most likely due

to the re-instatement of the integrin-matrix interaction, lost during the digestion phase of islet isolation. Restoration of this association may counteract anoikis signalling prevalent in pre-apoptotic cells within isolated islets (Thomas et al., 1999, 2001), thereby inducing protection from apoptosis. The hypothesis 'Primary human islet culture in media supplemented with proteins of the basement membrane improves survival and function of islets *in vitro* during a hypoxic challenge' was not disproved.

5.2 Limitations of the Study

A number of limitations have been identified within the structure of this study. Some of those have been previously discussed, such as the difficulties in the titration experiments, and others, such as donor variables, are and unavoidable consequence of the use of primary human islets.

Firstly, the range of concentrations tested as part of this titration was relatively narrow and not exhaustive, as well as only occurring in normoxia. While an exhaustive study such as this may be difficult to achieve, particularly due to the lack islet availability, time, and parameters measured – each may be the subject of individual projects themselves, this would be the most effective way of discovering the optimal concentrations for each protein. Furthermore, only four of the main proteins within the BM were tested. There is a large array of proteins that were not investigated, including other isoforms of laminin, collagen, nidogen, and perlecan. Again, this is due to the difficulties of performing an exhaustive study and those investigated here have been demonstrated to be lost during the isolation process. Therefore these proteins were considered to be the most likely candidates to prevent anoikis, providing benefit to islet function and survival. Further proteins not present in the adult human BM may also provide benefit and a prospective study investigating this would be extremely interesting. In addition to the narrow range of the study, there was some obfuscation of results of the titration study by factors not

controlled for, such as the osmolality of supplemented media and dilution of important factors within CMRL culture medium. To account for this it would be ideal to have each protein in an identically-composed high concentration stock solution. This would control for the volume of protein stock added to the clinical culture medium. Finally, the titration study was only conducted on islets cultured in normoxic conditions. Given that the main objective of this thesis was to investigate benefits conferred by BM proteins to islets in the hypoxic conditions present post-transplant, it may have advantageous to test the range of concentrations hypoxia. While this would have been ideal, primary human islets are a scarce resource and adding this culture condition would have required more islets than were available, doubling the number used. It was therefore decided that normoxic culture of islets, performed in the presence of the proteins, was the most representative portion of the clinical protocol.

Some of the limitations within the study are unavoidable within the field of islet transplantation. Variability in both donor organs and in the islet isolation process are a major factor influencing deviations in islet quality. It is close to impossible to fully account for the inconsistency in donor organs as these are dependent on the life and cause of death of each individual donor, although some factors, such as drug use and cardiac downtime, are regulated within the clinical programme. These factors have influence not only on tissue quality, but also on the outcomes of islet isolation. They alter the efficacy of the digestion and purification, as well as the purity, viability, and yield of islets. This was controlled to some degree in this study by setting a minimum viability of 70% and a minimum purity of 50%, the latter preventing sequestering of proteins by exocrine tissue. Furthermore, all treatment groups were assessed with islets from a single donor, producing a single technical replicate. This meant that variations between donors did not produce variability between treatment groups. Donor variation was an issue between technical replicates and may account for a lack of statistical power seen in some experiments and this is discussed in greater detail later in the section. Given that differences in donors will always be an issue when using donated organs, it would be false to not allow this variability in

the study. All islet preparations used here did not meet the criteria for a clinical transplantation, either in number or viability. This is another unavoidable constraint of the field. Indeed as one of the prevailing issues of islet transplantation is the limited donor pool, improving the quality of islets that do not currently meet clinical criteria will increase the number of successful transplants and is therefore a vital area of study.

One of the effects of the aforementioned variability in donor organs leads to inconsistencies in results between islets sourced from different donors. In order to counteract this variation it is necessary to have a large sample size, the magnitude of which may be estimated with power calculations. However, as this study has investigated novel supplementations of culture medium, it is not possible to perform these calculations because the lack of prior knowledge means that the possible effect size is unknown. This may have led to some of the analyses being underpowered, in particular those performed during the titration experiments. Increase in the number of organs used may elucidate further, smaller, benefits or detriments conferred by these proteins. However, as previously stated, primary human islets are a scarce resource and this study was limited in the number of preparations available. Future experiments investigating C-IV, L521, PLC, and NID would be able to use the results presented here as a guide for performing power calculations to determining the required number of replicates to confer effective analysis of results.

It has been demonstrated that BCL2 expression has been shown to be severely reduced immediately after islet isolation, most likely due to loss of the matrix-integrin interaction (Thomas *et al.*, 2002). While the BAX:BCL2 ratio is considered a useful indicator of the susceptibility of cell to apoptosis (Raisova *et al.*, 2001), higher expression of the anti-apoptotic Bcl-2 family members Bcl-xl and Mcl-1 post isolation may indicate that these genes are more important in islet survival (Campbell *et al.*, 2012). In the future therefore, determination of relative expression of these factors, in addition to BCL2, may provide a more complete picture of the apoptotic state of a cell as well as giving a better indication of cell fate. Another way to

improve the gene expression studies would be to investigate activated protein levels in conjunction with qRT-PCR analysis of the islets. In particular, HIF expression levels were not significantly different across treatment groups and there is evidence that a rapid reduction in HIF expression levels occurs once cells are returned to normoxic conditions (Wang *et al.*, 1995) which may account for this. Within the methodology of the study return to normoxic conditions was unavoidable, as islet were picked and lysed in normoxia. Therefore activated protein levels may not only be a more reliable measure of HIF production but would also give the degree to which activation was occurring within the cells (Huang *et al.*, 1996).

It is interesting to note that islets in all experimental groups demonstrated a higher viability after hypoxic than after normoxic culture. This phenomenon is particularly evident in MPC treated islets. It may be that this is due to the addition of FCS in the culture medium which contains a number of growth factors beneficial to cell survival. Avgoustiniatos *et al.* (2012) demonstrated the superiority of research grade islets, cultured with foetal bovine serum (FBS), over clinical grade islets, cultured with HSA, in reversal of diabetes in mice. Furthermore, islets from the same preparations had, on average, 27% higher oxygen consumption rates, a measure of the metabolic state of cells, when FBS was supplemented into the culture medium rather than HSA. Another potential issue with the use of HSA is that it may also inhibit the binding of matrix proteins to islets, in a similar manner to use as a blocker in adhesion assays (Kramer, 1994) and therefore may reduce their potency. A comparative study investigating differences in islet survival with or without HSA, both in the presence and in the absence of matrix proteins could elucidate the blocking effects.

5.3 Future Work

As previously discussed in Chapter 4 and above, while there was some evidence that the combination extracellular matrix proteins conferred greater benefits to islet survival and function than the individual proteins, increasing the number of technical replicates in these experiments would allow further definition of synergistic effects. Another interesting extension would be to investigate how other hormones, glucagon in particular, are affected by supplementation of culture media with the matrix proteins. The role of glucagon is vital to glucose homeostasis. It is particularly relevant to T1DM and islet transplantation as prevents hypoglycaemic episodes through upregulation of blood glucose levels (Gerich *et al.*, 1974; Reinhardt *et al.*, 1993). One of the advantages of islet transplantation over pure β-cell replacement or insulin therapy as it delivers more refined regulation of blood glucose levels through the combined actions of all islet cells. Therefore, while the α-cells are indirectly examined during fragmentation and viability assays, analysis of their secretory capacity during the glucose–stimulated insulin secretion assays, where an inverse correlation to glucose concentration is expected, would provide a more complete view of islet function.

Given that PLC in these experiments was a fragment of the whole HSPG2 protein, it would potentially be more effective to utilise the complete proteins in place of PLC. As there are diverse functional domains contained within the core protein (Iozzo *et al.*, 1994), in addition to the heparan sulphate chains, the full molecule may provide further synergistic advantage through associations with both the cell and other component of the BM, such as NID, concurrently. Furthermore, the potentially anti-angiogenic actions of the domain V fragment used here may be circumvented.

During the titration phase of this study the aim was to investigate the optimal concentration for each individual protein, these may not represent the ideal concentrations when applied combination. For instance, the presence of exogenous C-IV and L521 may necessitate an

increase in the NID concentration to allow NID-mediated unification between the two proteins, thereby achieving maximal benefit for islets. Knowledge of the relative abundance of these proteins within the native islet BM would be an extremely useful resource on which to base these concentrations. However, firstly, there is no current data available on the concentrations in the native BM and this would be a complex set of values to determine. Secondly, this may still not represent the optimum concentration for recombinant proteins. A secondary titration of proteins in combination may therefore be necessary.

Embedding islets within a 3-dimensional gel scaffold has been shown to enhance islet survival and improve transplant outcomes (Beattie *et al.*, 2002). This has expanded the feasibility of alternative site to portal vein transplantation, such as subcutaneous transplantation, to become viable (Juang *et al.*, 1996; Kim *et al.*, 2012; Najjar *et al.*, 2015). Enriching these gel scaffolds with matrix proteins further augments beneficial effects to murine islets (Weber and Anseth, 2008; Beenken-Rothkopf *et al.*, 2013) and therefore, incorporation of the protein combination demonstrated in this thesis into a supporting gel matrix may yield increased advantage to human islets. While enriched gel scaffolds are not practically implementable for the current islet transplantation procedure, they allow further development of aforementioned alternative transplant sites. Moreover, this has enormous potential for advancement of macroencapsulation. Scaffolds such as these allow islets to resist hypoxia, enhancing survival and function, as well as immobilising the islets within the device. This also prevents islet sedimentation, which can exacerbate hypoxic conditions, and limits sheer mechanical stress. Consequently, this is an exciting and important avenue of research where the findings presented in this thesis may lead.

An alternative immobilisation technique applied to prevent islet sedimentation in a macroencapsulation device involves coating the inner device membrane with adherent molecules or proteins of the islet basement membrane. While there has been some investigation into 2-dimentional islet culture using collagens and laminins (Daoud *et al.*, 2010; Johansson *et al.*, 2015), this was not examined in this thesis due to the impracticality of implementing this

technique into the current islet transplantation protocol. Removal of the islets from the coated plates, as would be necessary, requires further enzymic, or ionic, dissociation of the islet-matrix interaction thereby negating any benefits gained from the matrix-intergrin interaction. However, 2-dimensional islet culture remains another enticing prospect for application of the results exhibited in this thesis towards the realisation of a macroencapsulation device.

Finally, if implementing preconditioning of islet with these proteins, whether individually or in combination, into the clinical transplantation protocol is to be achieved in any capacity, the next stage of the process must be to investigate the outcomes of transplantation into an *in vivo* model. Successful demonstration of the advantages provided by the proteins in this manner would facilitate the conduction of a clinical trial, the final proof of concept, and ultimately, lead to routine clinical application.

5.4 Final Remarks

In conclusion, this study delivers a positive step forward towards improving the outcomes of islet transplantation for patients with poorly controlled diabetes. In addition to providing improved islet survival and function in the hypoxic conditions present after transplantation into the portal vein, this work further enhances the progression and development of macroencapsulation towards production of a bio-artificial pancreas. Creation of a functioning macroencapsulation device would allow islet transplantation to become applicable to a wider demographic of patients living with T1DM, potentially including children soon after diagnosis, thereby circumventing the need for insulin therapy and alleviating complications later in life. Improving these two methods of islet transplantation utilising the research performed in this thesis, therefore, have the potential to immensely improve the quality of life for millions of people who live with T1DM.

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Appendix: Standard Operating Procedures

Appendix 1.1: FDA/PI Viability

Assay: **FDA-PI Viability Staining**

Aim: To measure the membrane integrity of isolated islets using a fluorometric staining.

Materials:

	Reagent	Order No.	Company	Temp.
1.	Fluorescein diacetate (FDA)	F7378-5G	Sigma	4°C
2.	Propidium iodide (PI)	81845-100MG	Sigma	4°C
3.	Dulbecco´s PBS with Ca, Mg	H15-001	PAA	4°C
4.	Acetone (99.8%)	10417440	Fisher Scientific	RT

	Equipment	Order No.	Company
1.	Scale		
2.	50 mL-Centrifuge tubes (conical)	210261	Greiner Bio-One
3.	250 mL-Storage bottles	BC776	Appleton Woods
4.	500 mL-Storage bottle	BC777	Appleton Woods
5.	1000 µL-Variopipette		Eppendorf
6.	200 µL-Variopipette		Eppendorf
7.	1000 µL-Wide bore filter tips	AXF032	Appleton Woods
8.	200 µL-Wide bore filter tips	AXF031	Appleton Woods
9.	Pipetting aid	HTL540	Appleton Woods
10.	10 mL-Plastic pipette	CC114	Appleton Woods
11.	2 mL-Microcentrifuge tubes	AXM9025	Appleton Woods
12.	15 mL-Centrifuge tube (conical)	188271	Greiner Bio-One
13.	96-Well microtest plate (black)	655076	Greiner Bio-One
14.	Microplate reader F200	F200-Pro	Tecan
15.	Fluorescence filter FDA	485/535 nm	Tecan
16.	Fluorescence filter PI	510/595 nm	Tecan

Procedure:

1. **Staining reagents:**

1.1 <u>67 µM FDA stock</u>:

- weigh out 0.020 g (20 mg) of FDA in a 50 mL-centrifuge tube
- add 16 mL of acetone and dissolve FDA carefully by using a 10 mL-pipette
- add 20 mL of acetone (=1340 µM)
- transfer exactly 1.0 mL into a fresh 50 mL-centrifuge tube
- add 19 mL of acetone (**=67 µM**)
- cover tube with aluminium foil and aliquot into 500 µL-aliquots
- label and store at -20°C for a **maximum of 3 months**

1.2 <u>400 µM PI stock</u>:

- weigh out 0.010 g (10 mg) of PI in a 50 mL-centrifuge tube
- add 17 mL of PBS and dissolve PI carefully by using a 10 mL-pipette
- add 20 mL of PBS **(=400 µM**)
- cover tube with aluminium foil and aliquot into 500 µL-aliquots
- label and store at -20°C for a **maximum of 3 months**

2.0 **Islet staining:**

2.1 Collect duplicate islet samples in exactly 1000 µL of PBS in 2 mL-microcentrifuge tubes.

2.2 Add 10 µL of FDA stock (**=0.67 µM**) and 10 µL of PI stock (**=4.0 µM**).

2.3 Protect samples from light.
2.4 Allow islets to completely sediment at 1 x g.
2.5 Aspirate islets in a maximum of 100 µL volume.
2.6 Transfer islets as duplicate samples into a **black** 96-well plate.
2.7 Pipette 2 x 100 µL of supernatant as background samples (BG) into the same plate.

3. **Measurement using the Tecan F200 plate reader:**
3.1 Switch on the Tecan F200 plate reader before starting the software.
3.2 Start ***Magellan***.
3.3 ***Create/Edit Measurement***.
3.4 Open ***FDA-PI*** script.
3.5 If needed, select ***Measurement Parameters***:
- change ***Plate Layout***
- modify ***Concentration***, ***Dilution*** or ***Reference Values***

3.6 Place black 96-well plate without lid into the frame.
3.7 Click ***Start***.
3.8 Select ***Data Export***.
3.9 Rename file using following information: FDA-PI-Pancreas ID-Date.xlsx.
3.10 Enter and mark all relevant information into the worksheet:
- pancreas ID
- parameter measured
- sample location according to well number

3.11 Calculate the percentage of viable cells:

$$\frac{(((FDA\ FI_1 + FDA\ FI_2)/2) - ((FDA\text{-}BG_1 + FDA\text{-}BG_2)/2)) \times 100}{(((FDA\text{-}FI_1 + FDA\text{-}FI_2)/2) - ((FDA\text{-}BG_1 + FDA\text{-}BG_2)/2) + ((PI\text{-}FI_1 + PI\text{-}FI_2)/2) - ((PI\text{-}BG_1 + PI\text{-}BG_2)/2))}$$

Appendix 1.2: Islet Count

Assay: **Islet Count**

Aim: To determine number of islet equivalent in defined samples.

Materials:

	Reagent	**Order No.**	**Company**	**Temp.**
1.	Dithizone-stock solution			4°C
2.	Dulbecco´s PBS with Ca, Mg	H15-001	PAA	RT
	Equipment	**Order No.**	**Company**	
3.	200 µL-Variopipette		Eppendorf	
4.	200 µL-Wide bore filter tips	AXF031	Appleton Woods	
5.	35 x 10 mm-Petri dishes	627102	Greiner	
6.	2 mL Microcentrifuge tube	657185	Greiner	
7.	Inverted microscope	DM IL LED	Leica	

Procedure:

1. **Islet sampling:**

1.1 Take two representative 100 µL-samples from each treatment group.

1.1a Ensure mixing by using rapid pipette-mediated aspiration and ejection of the fluid.

1.2 Transfer the samples into separate microcentrifuge tubes.

1.3 Add 20 µL of dithizone stock to each sample.

1.4 If islets have been treated in wells of 6, 12- or 24-well plates add dithizone stock in an amount of 10% of the initial incubation volume.

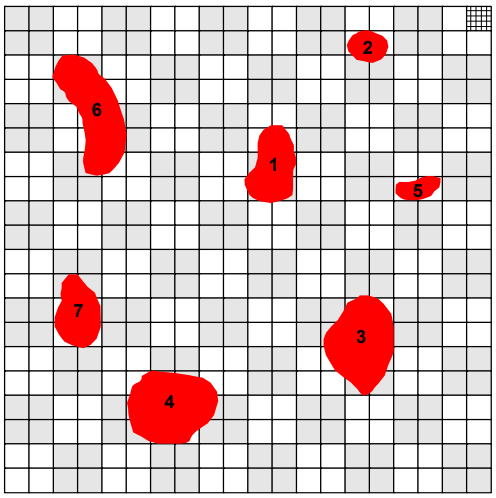
1.5 Spread each sample as two or three fluid lines in a separate 35 x 15 mm-petri dish.

1.6 Incubate for 1 to 2 min at room temperature.

2. **Islet counting:**

2.1 Place dithizone-stained samples onto the stage of the inverted Leica microscope.

2.2 Estimate the diameter of each dithizone-positive particle using the grid and the 5.0x-objective (see figure):



5.0x-Objective (µm):

Islet:	**1**	**2**	**3**	**4**	**5**	**6**
Size (µm):	300x150	175x125	400x300	375x300	175x150	500x150
Range:	201-250	151-200	301-350	301-350	151-200	301-350

2.3 Categorise each assessed dithizone-positive particle according to its size using the table shown below:

Diameter ranges (µm):	50-100	101-150	151-200	201-250	251-300	301-350	351-400	>400	∑
Sample 1:									
Sample 2:									
Sample 3:									
Sample 4:									
Column totals:									
Volume factor:	0.167	0.667	1.685	3.5	6.315	10.352	15.833	22.75	
IEQ:									
IEQ total:					Islet purity (%):				
IC total:					Islets embedded (%):				

Appendix 1.3: Glucose-Stimulated Insulin Secretion

Assay: **Static Glucose Stimulation (Krebs Ringer Buffer-based)**

Aim: To measure the glucose-stimulated insulin release of isolated islets.

Materials:

	Reagent	Order No.	Company	Temp.
1.	Sterile water	15230097	Life Technologies	4°C
2.	Pen-Streptomycin (10,000 U/mL)	15140122	Life Technologies	-20°C
3.	HEPES (1 M)	15630106	Life Technologies	4°C
4.	Human serum albumin (20%)	C11-096	PAA	4°C
5.	Calcium chloride (1 M)	21115	Sigma	4°C
6.	Sodium chloride	S7653	Sigma	RT
7.	Potassium chloride	P9333	Sigma	RT
8.	Potassium phosphate monobasic	P0662	Sigma	RT
9.	Magnesium sulfate	M7506	Sigma	RT
10.	Sodium hydroxide (1 N)	10745141	Fisher Scientific	RT
11.	Glucose solution (100 mg/mL)	G8644-100ML	Sigma	4°C
12.	HCl (concentrated; 38%)	H1758-100ML	Sigma	4°C
13.	Ethanol (99%)	E7023-500ml	Sigma	4°C
14.	Sterile alcohol wipes	VJT061	NHS supply	RT

	Equipment	Order No.	Company
1.	1000 mL-Glass beakers (Duran)	DC011	Appleton Woods
2.	Magnet bars (25 x 6 mm)	AC506	Appleton Woods
3.	Stirrer		
4.	Scale		
5.	50 mL-Syringe	FWC034	NHS Supply
6.	10 mL-Syringe	FWC068	NHS Supply
7.	0.22 µM-Syringe filter	102684401	Fisher Scientific
8.	Pipetting aid	HTL540	Appleton Woods
9.	25 mL-Plastic pipette	CC116	Appleton Woods
10.	50 mL-Centrifuge tubes (conical)	210261	Greiner Bio-One
11.	pH-Tester pHep 5	PH0004	Appleton Woods
12.	15 mL-Centrifuge tubes (conical)	188271	Greiner Bio-One
13.	200 µL-Variopipette		Eppendorf
14.	200 µL-Pipette tips	CD017	Appleton Woods
15.	1000 µL-Variopipette		Eppendorf
16.	1000 µL-Pipette tips	CD019	Appleton Woods
17.	250 mL-Storage bottle	BC776	Appleton Woods
18.	35 x 10 mm-petri dishes	627102	Greiner Bio-One
19.	200 µL-Wide bore filter tips	AXF031	Appleton Woods
20.	2 mL-Microcentrifuge tubes (round bottom)	GZ025	Appleton Woods
21.	Microplate orbital shaker	PMS-1000i	Grant
22.	Warming cabinet		
23.	1.5 mL-Microcentrifuge tubes	KC126	Appleton Woods
24.	Ultrasonic dismembrator (50 W)	12961151	Fisher Scientifc
25.	0.5 mL-Microcentrifuge tubes	KS123	Appleton Woods

Procedure: **1. Media**:

1.1 Pour 200 mL of a freshly opened 1000 mL-bottle of sterile water in a sterile 1000 mL-glas beaker equipped with a sterile magnet bar.

1.2 Krebs Ringer Buffer (KRB) stock solution:

- 200 mL sterile water
- add 7540 mg of NaCl
- add 360 mg of KCl
- add 165 mg of KH_2PO_4
- add 145 mg of $MgSO_4$
- mix on a stirrer until all reagents are dissolved
- aliquot stock solution using 50 mL-syringes

1.3 KRB working solution:

- 800 mL sterile water
- add all reagents using a 0.22 µm-syringe filter:
  - 200 mL stock solution
  - 2.5 mL $CaCl_2$
  - 10 mL Pen-Strep
  - 10 mL HEPES
  - 10 mL HSA
  - adjust to pH 7.2 – 7.4 by adding dropwise NaOH (1 N)
- prepare 20 x 50 mL-aliquots and freeze at -20°C

1.4 KRB glucose-free (0 mM):

- thaw a frozen 50 mL-aliquot of KRB
- transfer exactly 9.0 mL into a labeled 15 mL-centrifuge tube

1.5 KRB basal glucose (2.0 mM):

- transfer exactly 30.6 mL of thawed KRB into a labeled 50 mL-centrifuge tube
- add 110 µL of glucose solution (100 mg/mL) to obtain a concentration of 2 mmol/L

1.6 KRB stimulating glucose (22.0 mM):

- transfer exactly 10.1 mL of thawed KRB into a labeled 15 mL-centrifuge tube
- add 400 µL of glucose solution to obtain a concentration of 22.0 mmol/L

1.7 Acid ethanol:

- pipette 1.5 mL of concentrated HCl into a 250 mL-plastic bottle
- dilute with 98.5 mL of 99% ethanol
- label and store at 4°C

2. Islet collection:

2.1 Equilibrate all media at 37°C.

2.2 Use duplicate samples for the assay.

2.3 Collect treated and control islets in a minimum volume of culture medium from the 24-well plate and place into a 35 mm-petri dish.

2.4 Add 2000 µL of 2 mM-KRB.

2.5 Collect 2 x 10 islets and transfer those in 15 µL of 2 mM-KRB into two 2 mL-microcentrifuge tubes (round bottom).

3. Islet equilibration:

3.1 Add 985 µL of 2 mM-KRB to each 2 mL-microcentrifuge tube placed on an orbital plate shaker at 37ºC.

3.2 Equilibrate islets at 37ºC for 42 min at continuous orbital shaking at position 3.

3.3 Stop orbital shaking for 3 min to sediment islets.

3.4 Collect 900 µL of the supernatant in labeled 1.5 mL-microcentrifuge tubes and store at -20ºC.

4. Basal phase:

4.1 Refill 2 mL-microcentrifuge tubes with 900 µL of pre-warmed/-gassed **2 mM-KRB**.
4.2 Re-start incubation at 37ºC for 42 min at continuous orbital shaking at position 3.
4.3 Stop orbital shaking for 3 min to sediment islets.
4.4 Collect 900 µL of the supernatant in labeled 1.5 mL-microcentrifuge tubes and store at -20ºC.

5. Up-regulation phase:

5.1 Refill 2 mL-microcentrifuge tubes with 900 µL of pre-warmed/-gassed **22 mM-KRB**.
5.2 Re-start incubation at 37ºC for 42 min at continuous orbital shaking at position 3.
5.3 Stop orbital shaking for 3 min to sediment islets.
5.4 Collect 900 µL of the supernatant in labeled 1.5 mL-microcentrifuge tubes and store at -20ºC.

6. Down-regulation phase:

6.1 Refill 2 mL-microcentrifuge tubes with 900 µL of pre-warmed/-gassed **0 mM-KRB**.
6.2 Re-start incubation at 37ºC for 42 min at continuous orbital shaking at position 3.
6.3 Stop orbital shaking for 3 min to sediment islets.
6.4 Collect 900 µL of the supernatant in labeled 1.5 mL-microcentrifuge tubes and store at -20ºC.

7. Cell disintegration:

7.1 Refill 2 mL-microcentrifuge tubes with 900 µL of ice-cold distilled sterile water.
7.2 Sonicate samples for 30 s.
7.3 Clean the tip of the sonicator each time after processing a sample using alcohol wipes.
7.4 Store samples at -20ºC or continue with insulin extraction.

8. Insulin extraction:

8.1 Collect 2 x 50 µL from each sonicated sample in two labeled 0.5 mL-microcentrifuge tubes.
8.2 Add 150 µL of ice-cold acid ethanol (see 1.7) to each 0.5 mL-microcentrifuge tube.
8.3 Incubate samples overnight at 4°C.
8.4 Perform insulin ELISA (SOP 2.7) or store samples at -20°C until measurement.

Appendix 1.5: Insulin Determination

Assay: **Human Insulin Determination (ELISA)**

Aim: To measure the glucose-stimulated insulin release and intracellular insulin content of isolated islets using ELISA techniques

Materials:

	Reagent	Order No.	Company	Temp.
1.	Human Insulin ELISA Kit	10-113-01	Mercodia	4°C
2.	Sterile water	15230097	Life Technologies	4°C
3.	Sodium chloride	S7653	Sigma	RT
4.	Disodium hydrogen phosphate (anhydrous)	S5136	Sigma	RT
5.	Human serum albumin (20%)	C11-096	PAA	4°C
6.	Sodium azide (NaN_3)	S8032	Sigma	RT
	Equipment	**Order No.**	**Company**	
1.	1000 mL-Glass beakers (Duran)	DC011	Appleton Woods	
2.	250 mL-Glass measuring cylinder (Duran)	DC159	Appleton Woods	
3.	Magnet bars (25 x 6 mm)	AC506	Appleton Woods	
4.	Stirrer			
5.	Scale			
6.	50 mL-Syringe	FWC034	NHS Supply	
7.	10 mL-Syringe	FWC068	NHS Supply	
8.	0.22 µM-Syringe filter	102684401	Fisher Scientific	
9.	1000 µL-Variopipette		Eppendorf	
10.	1000 µL-Pipette tips	CD019	Appleton Woods	
11.	50 mL-Centrifuge tubes (conical)	210261	Greiner Bio-One	
12.	Pipetting aid	HTL540	Appleton Woods	
13.	25 mL-Plastic pipette	CC116	Appleton Woods	
14.	96-Well microtest plate	655185	Greiner Bio-One	
15.	200 µL-Variopipette		Eppendorf	
16.	200 µL-Pipette tips	CD017	Appleton Woods	
17.	Multipette		Eppendorf	
18.	5 mL-Dispenser tips	0030.089.669	Eppendorf U.K.	
19.	Microplate orbital shaker	PMS-1000i	Grant	
20.	Automatic Plate Washer	TE-HFE	Tecan	
21.	Microplate reader F200	F200-Pro	Tecan	
22.	Absorbance filter Insulin	450 nm	Tecan	

Procedure:

1. **Insulin kit components**:
   - 1.1 Allow assay components to equilibrate to room temperature.
   - 1.2 Prepare Enzyme Conjugate 1x Solution by diluting 10 µL of Enzyme Conjugate 11x with 100 µL Enzyme Conjugate Buffer, per well of kit used.
   - 1.3 Prepare Wash Buffer Working Solution by diluting 1.5 mL of Wash Buffer 21x with 30 mL of deionised water, per coated strip used, in a glass beaker.
   - 1.4 Assemble the required number of coated strips in the plate holder.
   - 1.5 Any strips not used must be resealed in the foil pouch with silica gel.

1.6 Insulin dilution buffer stock solution:

- 200 mL sterile water
- add 6000 mg of NaCl
- add 5680 mg of anhydrous Na_2HPO_4
- add 200 mg of NaN_3
- mix on a stirrer until all reagents are dissolved
- aliquot stock solution using 50 mL-syringes

1.7 Insulin dilution working buffer:

- 775 mL sterile water
- add all reagents using a 0.22 µm-syringe filter:
  - 200 mL stock solution
  - 15 mL HSA solution (20%)
  - 10 mL Pen-Strep
- prepare 20 x 50 mL-aliquots and freeze at -20°C

2. **Insulin standard and controls**:

2.1 Pipette 2 x 25 µL of each insulin standard (duplicates) to column 1 and 2 of the 96-well plate as shown in the table below:

Well	Insulin Conc. (mU/L)*	Volume (µL)
1-2 A	0	25
1-2 B	3.27	25
1-2 C	10.9	25
1-2 D	33.2	25
1-2 E	107	25
1-2 F	211	25

2.2 *Keep in mind that the concentration of the standards and controls is lot-dependent.

3. **Insulin samples**:

3.1 Remove samples from the freezer and thaw at room temperature.

3.2 If out-of-range insulin values are expected, dilute samples with the insulin dilution buffer (see 1.7).

3.3 Pipette 2 x 25 µL of each sample (duplicates) in a horizontal arrangement to column.

3.4 Prepare a map of the sample location.

4. **Subsequent assay procedure**:

4.1 Add 100 µL of Enzyme Conjugate 1x solution to each well occupied by standards or samples.

4.2 Incubate on orbital shaker at 700-900 rpm for 1 hour at 37°C.

4.3 Perform six wash cycles with 700 µL of Wash Buffer Working Solution each time using the automatic plate washer.

4.4 Invert the plate and pat it on a dry paper towels as absorbent surface to minimise enzyme carry-over and imprecision.

4.5 Add 200 µL of Substrate TMB to each well occupied by standards or samples.

4.6 Incubate at room temperature for 15 min.

4.7 Add 50 µL of Stop Solution to each well occupied by standards or samples.

4.8 Place plate on orbital shaker for 5 seconds to ensure mixing.

4.9 Check all wells for the presence of air bubbles affecting optical density.

5. **Measurement using the Tecan F200 plate reader:**

5.1 Switch on the Tecan F200 plate reader before starting the software.

5.2 Start ***Magellan***.

5.3 ***Create/Edit Measurement***.

5.4 Open ***Insulin ELISA*** script.

5.5 If needed, select ***Measurement Parameters***:

- change ***Plate Layout***
- modify ***Concentration***, ***Dilution*** or ***Reference Values***

5.6 Place 96-well microtest plate without lid into the frame.
5.7 ***Start***.
5.8 Select ***Data Export***.
5.9 Rename file using following information: Insulin-Pancreas ID-Date.xlsx.
5.10 Enter and mark all relevant information into the worksheet:

- pancreas ID
- parameter measured
- sample location according to well number

5.11 If insulin values are out of range, dilute samples with the insulin dilution buffer (see 1.7).

6. **96-Well plate sample location**:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Donor: Treatment:											
B	Donor: Treatment:											
C	Donor: Treatment:											
D	Donor: Treatment:											
E	Donor: Treatment:											
F	Donor: Treatment:											
G	Donor: Treatment:											
H	Donor: Treatment:											

Appendix 1.6: RNA Extraction

Assay: **RNA Extraction**

Aim: To extract total RNA from isolated islets.

Materials:

	Reagent	Order No.	Company	Temp.
1.	RNeasy Micro Kit	74004	Qiagen	
2.	PBS (Mg^{2+} and Ca^{2+} free)	14190169	Life Technologies	
3.	β-Mercaptoethanol	M6250-10ML	Sigma	4°C
	Equipment	**Order No.**	**Company**	
1.	Pipetting aid	HTL540	Appleton Woods	
2.	25 mL-Plastic pipette	CC116	Appleton Woods	
3.	10 mL-Plastic pipette	CC114	Appleton Woods	
4.	2 mL-Microcentrifuge tubes	GZ025	Appleton Woods	
5.	Ultrasonic dismembrator (50 W)	12961151	Fisher Scientifc	
6.	35 x 10 mm-petri dishes	627102	Greiner Bio-One	
7.	Centrifuge		Heraeus	
10.	20 µL-Variopipette			
11.	20 µL Filtered Pipette tips	0030077539	Eppendorf	
12.	200 µL-Variopipette			
13.	200 µL Filtered Pipette tips	0030077547	Eppendorf	
14.	1000 µL-Variopipette			
15.	1000 µL Filtered Pipette tips	0030077571	Eppendorf	
18.	35 x 10 mm-petri dishes	627102	Greiner Bio-One	

Procedure:

1 **Set-up**:

1.1 Add 3.5 µL β-Mercaptoethanol to 350 µL RLT buffer per sample (+10%).
1.2 Add 44 mL 100% Ethanol to Buffer RPE (if not added already).
1.3 Dissolve lyophilised DNase I in 550 µL RNase-free water, mix by inversion.
1.4 Add 10 µL DNase I stock solution to 70 µL RDD Buffer per sample (+10%).
1.5 Add 105 µL RNase-free water to 245 µL Ethanol per sample (+10%) to create 70% Ethanol.
1.6 Add 100 µL RNase-free water to 400 µL Ethanol per sample (+10%) to create 80% Ethanol

2. **Cell Lysis**:

2.1 Collect islets in a minimum volume of culture medium from the 24-well plate and place into 35 mm-petri dish.
2.2 Add 1500 µL of PBS.
2.3 Collect 100 islets from each group in 20 µL of PBS into 2 mL microcentrifuge tubes.
2.4 Add 350 µL RLT buffer to each tube.
2.5 Sonicate samples for 20 to 30 sec.
2.6 Add 350 µL Ethanol to each tube pipetting up and down to mix.
2.7 Transfer lysate (approx 700 µL) to Spin Column.
2.8 Centrifuge for 15 s at 10 000 rpm.
2.9 Discard flow-through.
2.10 Add 350 µL Buffer RW1 to Spin Column.
2.11 Centrifuge for 15 s at 10 000 rpm.
2.12 Discard flow-through.
2.13 Add 80 µL DNase Incubation Mix to Spin Column.
2.14 Incubate for 15 mins at room temperature.
2.15 Add 350 µL Buffer RW1 to Spin Column.
2.16 Centrifuge for 15 s at 10 000 rpm.

2.17 Discard flow-through and collection tube.
2.18 Place Column in new collection tube and add 500 µL Buffer RPE to Spin Column.
2.19 Centrifuge for 15 s at 10 000 rpm.
2.20 Discard flow-through.
2.21 Add 500 µL 80% Ethanol to Spin Column.
2.22 Centrifuge for 2 mins at 10 000 rpm.
2.23 Discard flow-through and collection tube.
2.24 Place Column in new collection tube and centrifuge at full speed (13 000 rpm)
for 5 mins with lid open.
2.25 Discard flow-through and collection tube.
2.26 Place Spin Column in a new 1.5 mL collection tube.
2.27 Add 30 µL RNase-free water to each Spin Column and incubate for 5 mins.
2.28 Centrifuge at full speed (13 000 rpm) for 1 minute.
2.29 Store at -20°C for short-term or -80°C for long-term storage.

Appendix 1.7: qRT-PCR

Assay: **Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Aim: To measure the gene expression of isolated islets using qRT-PCR.

Materials:

	Reagent	Order No.	Company	Temp.
1.	CellsDirect One-Step qRT-PCR kit	11753500	Invitrogen	-20°C
2.	Microsol			
3.	Ice			
4.	18S rRNA (Ref) Primer/Probe (VIC/MGB_PL)	4319413E	Applied Biosystems	-20°C
5.	Gene of Interest Primer/Prober (FAM/MGB)			

	Equipment	Order No.	Company
1.	PCR Hood with UV light		
2.	Ice Bucket		
3.	2 mL Microcentrifuge Tube	0030120094	Eppendorf
4.	QuantStudio 7 qPCR Machine	4485693	Applied Biosystems
5.	MicroAmp Fast Optical 96-Well Reaction Plate, 0.1 mL	4346907	Applied Biosystems
6.	MicroAmp Clear Adhesive Film	4306311	Applied Biosystems
7.	Centrifuge (with 96-well rotor)		
8.	10 µL-Variopipette		
9.	10 µL Filtered Pipette tips	0030077768	Eppendorf
10.	20 µL-Variopipette		
11.	20 µL Filtered Pipette tips	0030077539	Eppendorf
12.	200 µL-Variopipette		
13.	200 µL Filtered Pipette tips	0030077547	Eppendorf
14.	1000 µL-Variopipette		
15.	1000 µL Filtered Pipette tips	0030077571	Eppendorf
16.	Plate cover sealing tool		

	Software	Order No.	Company
1.	QuantStudio Real-Time PCR software		Applied Biosystems

Procedure:

1 **Set-up**:

1.1 Clean PCR hood, ice bucket, and equiptment with Microsol.

1.2 UV-sterilise PCR hood, with equiptment inside, for 15 mins.

1.3 Fill ice bucket with ice.

1.4 Place reagents on ice to thaw.

1.5 Create Plate Map, ensuring samples are run in triplicate and 4 no-template controls (NTC) are designated.

1.6 Ensure QuantStudio qPCR machine has 96-well adapter installed and turn on.

1.6a Install 96-well adapter if necessary before turning on.

1.7 Run QuantStudio Software.

1.7a Run ‘New Experiment’ from Template according to experiment.

1.7b Check ‘Experiment Properties’ Section is correct.

1.7c Check ‘Define Section’, defining samples and genes of interest.

1.7d Fill in Assign Section according to plate map.

1.7e Ensure correct Run Method is selected.

2. **Master Mix**:

2.1 On ice in PCR hood, create master mix according to table below in sterile 2 mL microcentrifuge tube:

Reagent	Volume per Well*
Taq mix	0.5
2x Reaction Mix	10
Ref Primer/probe	1
Gene of Interest Primer/probe	1
ROX	0.5
DEPC treated water	5

*Ensure 10% added on top of actual wells used.

3. **Assay Proceedure**:

3.1 All steps performed on ice in PCR hood.

3.2 Pipette 18 µL of Master Mix into 96-well plate wells according to plate map.

3.3 Pipette 20 µL of Master Mix into NTC wells.

3.4 Pipette 2 µL of sample into designated wells, pipetting up and down to mix.

3.5 Cover plate with film cover using covering tool ensuring all wells have a consistent seal around the edges.

3.6 If there are bubbles at the bottom of the wells, spin plate briefly at 2000rpm at 4°C.

3.7 Load plate into QuantStudio qPCR machine.

3.8 Click ‘Start Run’ on QuantStudio qPCR software.

6. **96-Well plate sample location**:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Donor: Treatment:											
B	Donor: Treatment:											
C	Donor: Treatment:											
D	Donor: Treatment:											
E	Donor: Treatment:											
F	Donor: Treatment:											
G	Donor: Treatment:											
H	Donor: Treatment:											