The Effects of Microgravity Cell Culture on Extracellular Matrix Expression, Islet Remodelling, and Insulin Secretory Function Following Enzyme Digestion

A thesis submitted in fulfilment of the requirements of the University of Brighton for the degree of Doctor of Philosophy

Nouf AL-Hasawi

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ABSTRACT

Insulin producing cells are vulnerable to damage at several key stages during clinical islet transplantation. This damage is caused by a number of factors; enzymatic-digestion during islet isolation, post-transplant exposure to hypoxic condition, and toxicity from immunosuppressant therapy. The enzymatic isolation process disrupts islet cell membranes, damages cell-to-cell interactions and inhibits vital functions. Extracellular Matrix (ECM) is a complex 3D-network of proteins and polypeptides and an essential component of the islet cell basement membrane (BM). We have developed a novel bioreactor, rotary cell culture system (RCCS) in order to study the molecular remodelling of Min6-Pseudoislets (PIs) following enzymatic digestion. The initial aim was to study the biology of PIs within our controlled cell culture environment RCCS and then to investigate the effects of digestive enzymes on PIs-ECM contacts and to determine if the novel 3D- microgravity cell culture system could restore and enhance ECM expression. Thus, PIs-ECM expression, localization and remodelling was analysed pre-and post-enzymatic digestion using qRT-PCR (for gene expression), western blotting (for protein expression) and immunocytochemistry (for cellular localization) techniques. Results showed that our RCCS system had a beneficial effect on PIs structure and function compared to standard culture conditions. Also, qRT-PCR gene analysis and immunoblotting of protein content of PIs detected alterations in ECM expression levels. Observed changes included increased expression of Fibronectin, Collagen IV, and Laminin V in PIs cultured in the RCCS system. PIs digestion with collagenase-accutase enzyme was optimized such that cell viability was not affected but cellular stress and subsequent remodelling of ECM expression was achieved. Insulin gene expression and insulin release in PIs were significantly increased in response to high glucose. These responses were significantly decreased following enzymatic digestion but recovered following subsequent culture in the RCCS-bioreactor system. This recovery of insulin secretory function was directly associated with increased ECM expression as demonstrated by enhanced cytoplasmic staining in the remodelled RCCS-cultured PIs. Our results confirmed that a controlled microenvironment optimized to promote cell survival and recovery is able to control the detrimental effects of enzymatic and mechanical stress. The ECM is a very good indicator of cellular integrity and function in PIs and it has a vital role in sustaining glucose-responsive insulin release. Our RCCS-bioreactor system has the potential to restore the lost ECM expression in PIs following enzymatic digestion. We believe that ECM interaction is an accurate biomarker for functional islets integrity and by maintaining high levels of its expression we hope to be able to improve islet cells graft function for current clinical islet transplantation programmes.

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List of Abbreviation

α	Alpha
AMV	Avian Myeloblastosis Virus
ATP	Adenosine Triphosphate
β	Beta
BM	Basement membrane
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic Acid
Coll IV	Collagen IV
CO_2	Carbone Dioxide
Ct	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
δ	Delta
ΔCt	Delta Cycle threshold
ΔΔCt	Delta Delta Cycle threshold
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
ds.DNA	Double strand Deoxyribonucleic Acid
ECL	Enhanced Chemi-luminescence

ECM	Extracellular Matrix
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylene di-amine tetra-acetic Acid
FBS	Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FN	Fibronectin
GAD	Glutamin Acid Decarboxylax
γ	Gamma
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GHRH	Growth Hormone-Releasing Hormone
GLUT-4	Glucose Transporter
HARV	High Aspect Ration Vessel
HbA_1C	Glycated hemoglobin
H&E	Haematoxylin and Eosin
hESCs	human Embryonic Stem Cells
H_2O_2	Hydrogen Peroxide
HEPES	4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid
HPI	Hoechst-Propidium iodide
HRP	Horseradish Peroxide
ICC	Immunocytochemistry
IM	Interstitial Matrix

IE	Islet Equivalents
IRS	Insulin Receptor Signalling pathway
L	Liter
LM	Laminin
LmV	Laminin V
MHC	Major Histocompatibility Complex
Min6	Mouse insulinoma cell line 6
mRNA	messenger Ribonucleic Acid
MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2, 5 DiphenyltetrazoliumBromide)
Nacl	Sodium Chloride
NASA	National Aeronautics and Space Administration
N-CAD	Neural Adhesion Molecules
OCT	Optimal Cutting Temperature compound
O_2	Oxygen
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PHHI	Persistent Hyperinsulinemic Hypoglycemia of Infancy
PI-B	Pseudoislets in Bioreactor
PI-D	Pseudoislets in Dish
PI-M	Pseudoislets membrane
PIs	Pseudoislets
PI-3 kinase	Phosphoinositide 3-kinase

qRT-PCR	quantitative Reverse Transcriptase- Polymerase Chain Reaction
R24-B	Recovery 24 hours in Bioreactor
R48-B	Recovery 48 hours in Bioreactor
R24-D	Recovery 24 hours in Dish
R48-D	Recovery 48 hours in Dish
RCCS	Rotating Cell Culture System
RCCV	Rotating Cell Culture Vessel
RGD	Arg–Gly–Asp
rpm	Revolution per minute
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TEMD	Tetramethylethylnedamine
T1DM	Type 1Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
3D	Three Dimensional
2D	Two Dimensional
U	Units
v/v	Volume per volume
w/v	Weight per volume

This thesis is lovingly dedicated to;

My beloved Parents….

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The successful completion of this project titled;

"The Effects of Microgravity Cell Culture on Extracellular Matrix Expression, Islet Remodelling, and Insulin Secretory Function Following Enzyme Digestion."

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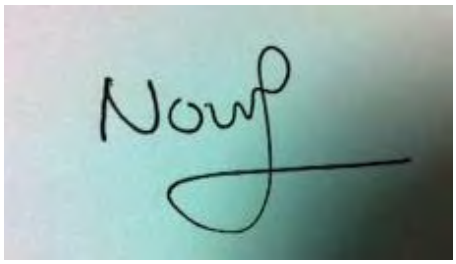
Author's Declaration

All of investigations and data presented in this thesis are the original work of the author. The thesis has not been previously submitted for the award of any degree, and does not incorporate any material already submitted for a degree.

Signature: 

Name: Nouf AL-Hasawi

Date: 01/06/2016

Chapter 1. **INTRODUCTION**

1.1. **Diabetes Mellitus**

Diabetes mellitus is a metabolic disorder characterised by chronic hyperglycaemia which results from a disturbance in insulin secretion, insulin action, or both [1]. Diabetes is classified according to the aetiology of glycaemia: type 1, or insulin-dependent; type 2, or non-insulin-dependent; and gestational diabetes. Type 1 diabetes mellitus (T1DM) is an autoimmune disorder characterised by the destruction of insulin-secreting pancreatic β-cells leading to complete insulin deficiency [1]. Type 2 diabetes mellitus (T2DM) is a condition that ranges from defective insulin secretion, with or without resistance to insulin, to insulin deficiency in combination with insulin resistance. Gestational diabetes is glucose intolerance that is initially detected during pregnancy [2]. Diabetes is now considered a major health problem worldwide. By the year 2025, it is expected to affect about 334 million people around the world with India, China, and United States expected to report the highest estimated number of cases [3]. In developed countries, it is estimated that approximately 382 million people live with diabetes, a figure which is predicted to increase to 592 million by 2035 [4].

Most children affected with T1DM experience polydipsia (increased thirst), polyphagia (excessive eating), polyuria (frequent urination), weight loss, and recurrent infections [5]. These symptoms may be mild, absent, or persistent, prior to confirming a diagnosis of T1DM. It is estimated that 30–50% of T1DM patients exhibit signs and symptoms after the age of 20 years [6]. T1DM diagnosis in adulthood has also been reported, with a longer symptomatic period prior to diagnosis. Insulin is usually produced by the β-cells in the islets of Langerhans, the endocrine portion of the pancreas [7].

Insulin is normally released in response to food intake and acts on receptors across multiple sites in the body, including the liver and the brain, to regulate glucose uptake,

storage, and release, as well as the release of other neuropeptides and hormones [7]. Insulin levels also play an important role in the metabolism of lipids and carbohydrates, typically used as fuel sources by the body in the fasting state [**Figure 1-1**] [8].

1.1.1. Type 1 Diabetes Mellitus

Causative factors and epidemiology

T1DM is a disease caused by the deficiency of insulin, a hormone which promotes the uptake of glucose into cells. Usually diagnosed in children, it is characterised by abnormal glucose metabolism resulting from the inability of the pancreas to secrete insulin [9]. Insulin is normally produced by the pancreas, a gland with both exocrine- and endocrine-secreting cells. The loss of insulin production in people with T1DM is caused by the destruction of β-cells, endocrine cells found in the pancreatic islets of the pancreas, through a defective autoimmune response **[Figure 1-2].** In this abnormal immune action, the body's own immune system recognises the β-cells as foreign bodies and mounts an abnormal immune response directed at destroying the β-cells through the production of autoantibodies [10].

In contrast, causes of T2DM range from insulin resistance with relative insulin deficiency to secretory defects along with insulin resistance [11]. Of note, however, is that T2DM features both insulin deficiency and some degree of insulin resistance, while T1DM is exclusively caused by insulin deficiency.

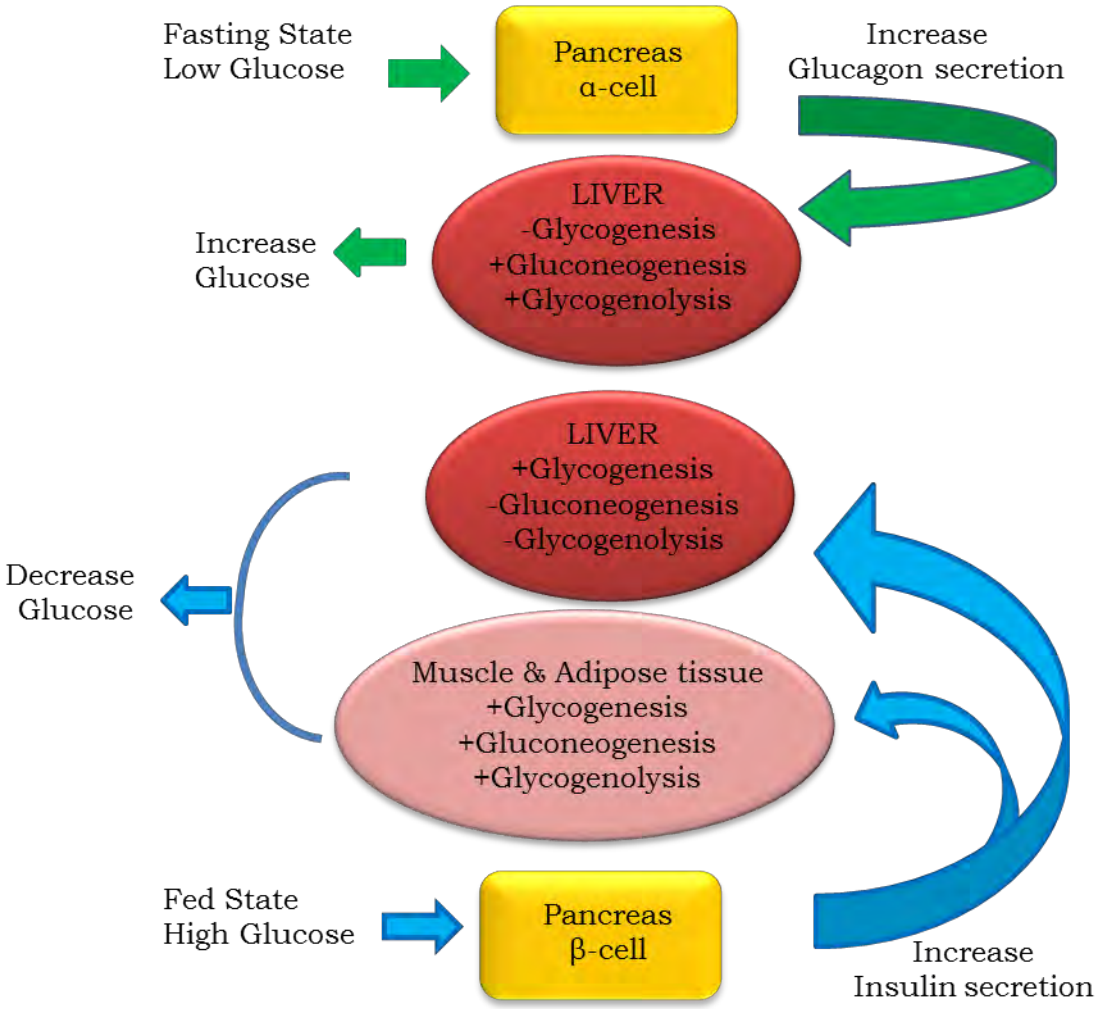


Figure 1-1: **Schematic diagram of insulin and glucagon action in the body**.

In the fasting state, insulin levels are low (green arrows), so insulin acts on the liver to stimulate glycogenolysis and gluconeogenesis increasing glucagon secretion; in the fed state (blue arrows), insulin suppresses glucose production by the liver and promotes its uptake by muscle and adipose tissue.

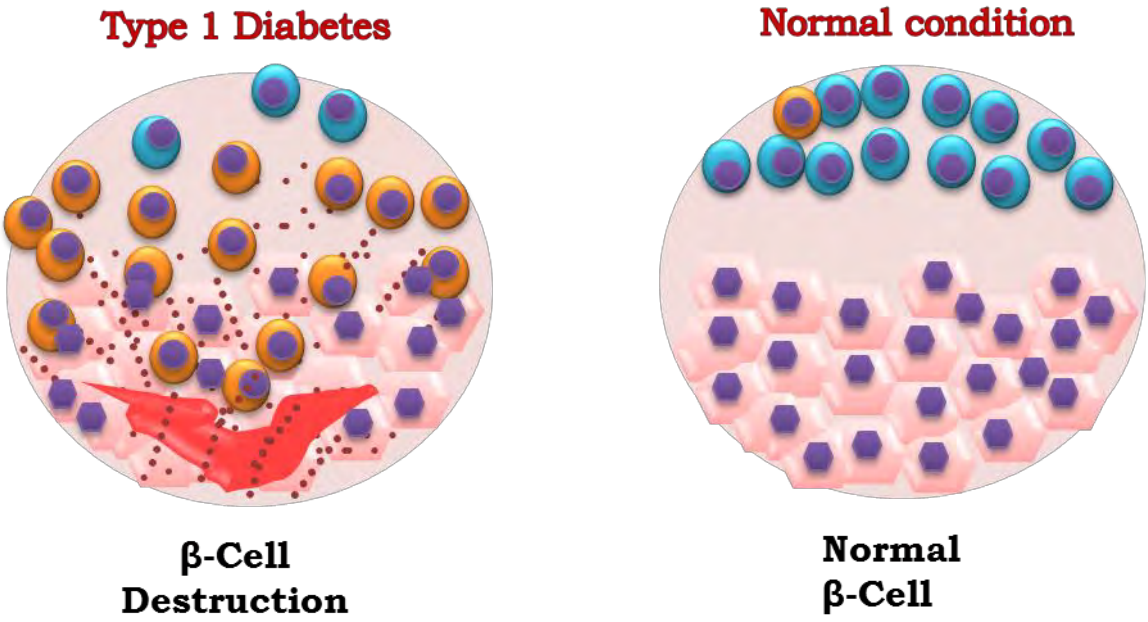


Figure 1-2: Beta cell (β-cell) destruction in Type 1 Diabetes (T1D).

In T1D, an autoimmune reaction activates immune cells known as T-effector cells (yellow) which release toxic cytokines causing destruction to β-cell; while in healthy immune system, T-regulator cells (blue) prevent effector T-cells from attacking β-cell.

In the UK, approximately 8.5% of all individuals with diabetes have T1DM [12]. This disorder has been identified as belonging to a family of HLA-associated autoimmune diseases of a polygenic nature. Autoantibodies generated in the body destroy the β-cells in the pancreas, leading to the insulin deficiency observed in these patients [13]. Although this form of diabetes is less prevalent than T2DM, it is considered equally important in terms of pursuing improved treatment options, particularly as the congenital nature of the condition means that prevention is less appropriate than in T2DM.

1.1.2. **Complications of T1DM**

T1DM is associated with long-term complications such as retinopathy, nephropathy, and neuropathy [14]. Effective management reduces the risk of developing these complications; however, serious complications can arise when the condition is not well managed. Complications frequently develop at an early stage in patients with poor metabolic control [15]. People with diabetes are approximately twice as likely to develop various cardiovascular diseases compared to people without the disease [16]. Disorders of the cardiovascular system are the main causes of disability and death amongst individuals with diabetes, and it is estimated that 44% of individuals with T1DM die from cardiovascular-related illnesses [17]. Thus, it is quite difficult to maintain a long term glucose homeostasis if isn't controlled. Because T1DM is characterised by lack of endogenous insulin, exogenous insulin is supplemented via either via injection or pump therapy [18].

1.1.3. **Treatments of T1DM**

Current treatment options for patients with T1DM include conventional insulin therapy, whole pancreas transplantation, or islet cell transplantation [19].Good glycaemic control

delays the onset and progression of diabetes-related complications amongst adults and children with T1DM.

T1DM requires a long-term therapeutic strategy in order to achieve glucose homeostasis, which is the balance between insulin and glucagon to maintain stable blood glucose levels [14]. As patients with T1DM can no longer produce enough insulin, insulin therapy becomes a 'life-sustaining' treatment [20].

Insulin Therapy

Insulin remains the mainstay of treatment for T1DM. An anabolic polypeptide hormone, it enables cells to promote energy substrates such as glycogen, fat, and protein and its synthesis and secretion are mediated by β-cells in the pancreas. The effects of insulin in the body are complex, and the major sites for insulin action are the liver, muscle, and adipose tissue [21]. Insulin stimulates the uptake of glucose, only in muscle and adipose tissue, thus regulating blood glucose levels [21]. Prandial or bolus administration of insulin involves giving a short- or rapid-acting insulin analogue at each meal in an attempt to mimic normal insulin secretion from the pancreas. Although intensive insulin therapy has been shown to prevent the progression of T1DM complications, a long term prevention was quite difficult to achieve [22]. Therefore, a replacement of β-cells is the only effective treatment that can maintain normoglycemia [23].

Transplantation

Replacement of β-cells as a means of restoring normal insulin secretion, either by pancreatic (whole organ) or islet cell transplantation, has been found to be more advantageous than insulin therapy in treating individuals with T1DM [24].

β-cell replacement also provides better glycaemic control and the opportunity for insulin independence as compared to insulin therapy[25]. Compared with whole organ transplantation, islet cell transplantation is a less invasive procedure which involves the

infusion of islet cells from cadaveric donors through the portal vein into the liver of the patient [26] with an overall goal of achieving adequate glycaemic control [27]. Although the treatment has been shown to be safe and effective, current research is focused on improving tolerance, including reducing the level of immunosuppression therapy required and prolonging graft function [19]. The likelihood of rejection of implanted cells means that patients currently require immunosuppressive drugs for the remainder of their lifetime [28]. Prior to the establishment of current protocols, as little as 8% of islets transplant patients remained insulin therapy-independent at one year post-treatment [29]. Since 2000, a protocol for the use of steroid-free immunosuppressive drugs has allowed insulin independence to be achieved by many previously insulin-dependent diabetic patients [30]. Although insulin independence cannot yet be achieved in all transplant recipients, evidence suggests that approximately 80% regain independence from insulin therapy, recovering functional glucose, lipid and protein metabolism [19]. Another study has shown that seven patients with uncontrolled T1DM (i.e., a history of severe hypoglycaemic episodes) remained insulin-free at one year when islet cell transplantation was accompanied by administration of glucocorticoid-free immunosuppressive drugs [29].

There is evidence that some of the short-term benefits of islet cell transplantation may not be maintained in the long term. Insulin independence is lost over time in a considerable number of patients who did initially achieve it, as β-cell function begins to decline after as little as one year post-treatment [30]. These patients may maintain some benefit, however, as despite the requirement to reinitiate insulin treatment they may still retain glycaemic stability [28]. Evidence also supports the observation that islet cell transplantation is free from major side effects and preserves liver function [19]. Even in patients who continue to require insulin therapy after treatment, there remain some benefits including improved HbA1c control, reduced risk of recurrent hypoglycaemia

and reduced risk of long-term complications associated with T1DM [19]. Other reported benefits of islet cell transplantation include improved quality of life and reduced risk of complications such as neuropathy and nephropathy [31]. Transplants are generally associated with low rates of complications, of which those reported appear to be minor [29].

Based on the outcomes of the studies discussed thus far, it appears that, while the use of islet cell transplantation in T1DM may offer some hope of a short-term cure, this may not be the case for many recipients of the intervention. The main limitations of the treatment are not necessarily related to the success of the transplant itself, as the 80% success rate in achieving initial insulin independence can be considered significant. The shortcomings instead appear to be associated with maintenance of the initial short-term improvements, as in order to be considered a true 'cure' for T1DM, islet transplantation must provide a long-term reduction in insulin-dependence.

Various methods for prolonging the survival of ex *vivo* pancreatic islet cells have been discussed in the literature, but one which appears to have gained substantial support is the use of 3-D matrices, as these may be used to culture cells prior to transplant.

In the following section, the physiological characteristics of the pancreatic islets of Langerhans, types of cells in islet cells and their function, and the specific extracellular matrix components involved in islets survival and functionality will be discussed.

1.2. **Physiology of the pancreatic Islets of Langerhans**

1.2.1. **Pancreas**

The pancreas, a retroperitoneal gland approximately 12–15 cm long and 2.5 cm thick, is located behind the greater curvature of the stomach [32]. It consists of a head, an uncinated process, a neck, a body, and a tail, and is usually attached to the second portion of the duodenum via two ducts. The head comprises the expanded portion that

lies next to the C-shaped concavity of the duodenum. The uncinate process is the projection forming the inferior section of the pancreatic head, and it extends medially to the left. The neck is the constricted part between the head and the body. Left of the superior mesenteric vessel is the body, which passes over the aorta and the second lumbar vertebrae. Lastly, anterior to the left kidney is the tail, which terminates as it passes between the layers of the splenorenal ligament [32].

Small clusters of glandular epithelial cells comprise the pancreas. Almost 99% of these clusters (acini) make up the exocrine portion of the organ [33], with the remaining 1%, the pancreatic islets (islets of Langerhans), constituting the endocrine portion of the pancreas. These cells produce the hormones insulin, glucagon, somatostatin, and pancreatic polypeptide [34]. Acinar cells are pyramidal in form and possess extensive secretory machinery, including zymogen granules which secrete digestive enzymes such as proteases, lipases, and amylases into the duodenum[35]. The endocrine function of the pancreas, however, is performed by a number of cell types in the islets of Langerhans, aggregates or clusters of cells embedded in the surrounding exocrine tissue [36]. The majority of islets contain 3,000–4,000 cells of five major cell types: α, β, δ γ, and Epsilon cells **[Figure 1-3].**

Constituting 20% of islet cells, the function of α-cells is to secrete the hormone glucagon. β-cells are the most numerous cell types, comprising 50–80% of cells in the islet with the main function of insulin secretion. δ-cells, which account for 10% of islet cells, secret the hormone somatostatin which plays a role in maintaining the balance between insulin and glucagon hormone levels. γ-cells constitute only 5% of cells, and their function is to secrete pancreatic polypeptide (PP) hormone that controls the secretion of somatostatin. Epsilon cells constitute less than 1% of islets and their function is to secrete ghrelin hormone [37].

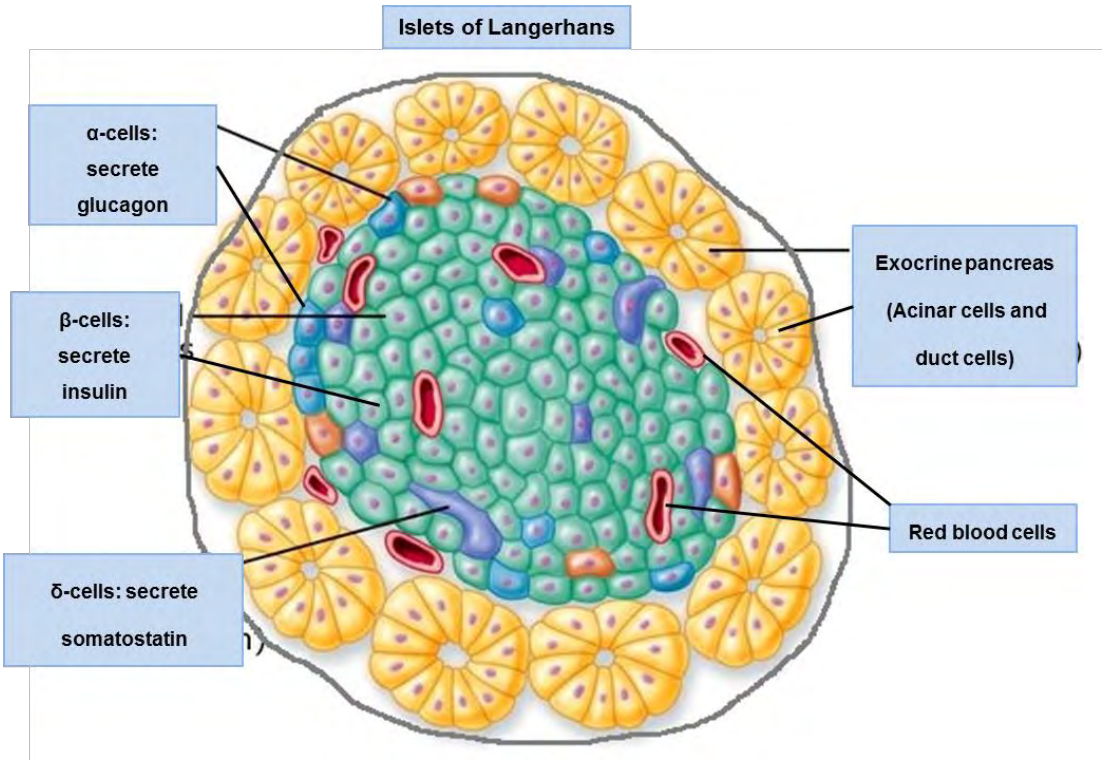


Figure 1-3: Islets of Langerhans.

Islet cells constitute the endocrine part of the pancreas. The figure shows α, β, and δ hormone-producing cells. Modified from reference [37].

1.2.2. **Anatomy of Islets of Langerhans**

There are approximately one million pancreatic islets in the human pancreas, comprised of intertwined polygonal endocrine cells surrounded by acinar cells [36]. In most mammals, regardless of the species, the size of the islets of Langerhans is between 100–200 μm [38]. Bigger islets are found near key arterioles while smaller islets are located deep in the parenchyma of the pancreas. In addition to a system of arteries which delivers blood straight to the exocrine pancreas, rich networks of capillaries surround each islet, arranged in a portal system, and deliver blood to the acinar cells [39]. The portal system allows the confined action of hormones from the islets, especially insulin, on the exocrine pancreas [40]. The endocrine cells (islets of Langerhans) are infused with a dense network of capillaries and partly surrounded by a thin collagen capsule and glial sheet that separates the cells from the exocrine component [41] [**Figure 1-4**]. The organisation of β-cells is distinctive of pancreatic islets that are adapted to synthesise and secrete proteins [42].

The uniqueness of β-cells derives first from the observation that they are programmed to synthesise insulin, via its precursors proinsulin and preproinsulin, and in sophisticated mechanisms that they have developed to regulate the rate of insulin secretion in response to glucose [42]. Insulin, a 51-amino acid peptide, is essential for cellular nutrient uptake and therefore vital to the survival of an organism [42].

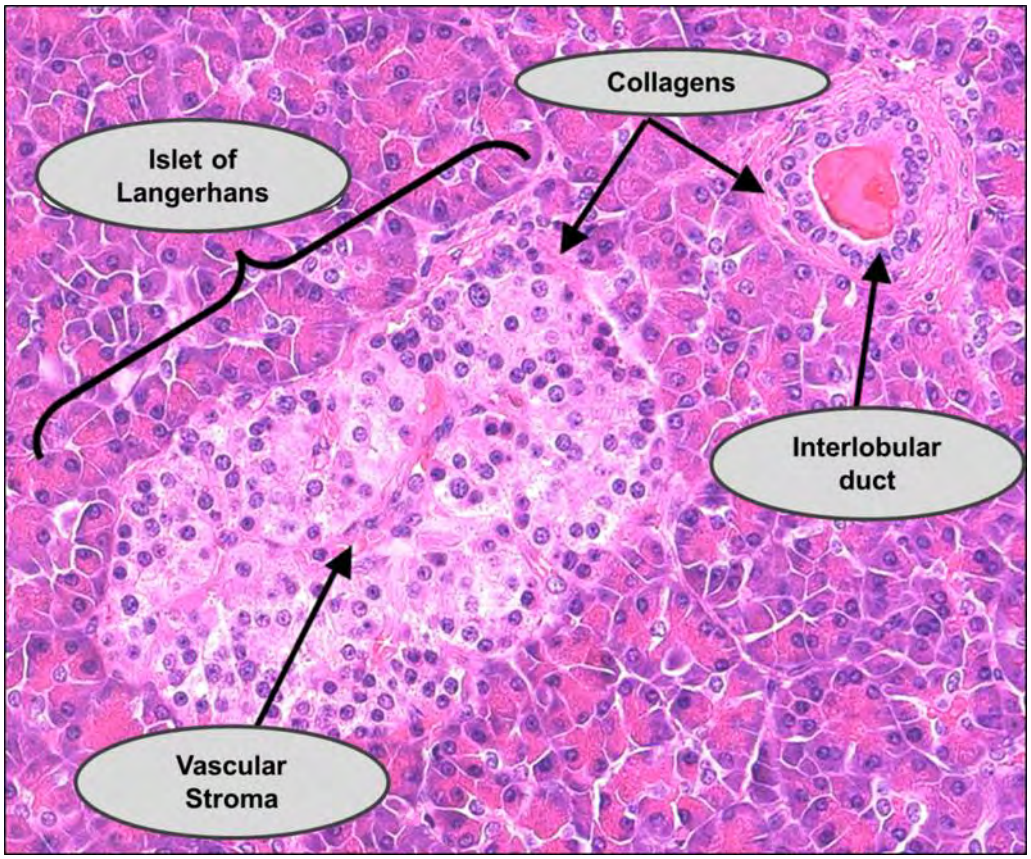


Figure 1-4: Histology-Islets of Langerhans.

The endocrine part of the pancreas, or Islets of Langerhans, is surrounded by a thin collagen capsule and glial sheet that separates the endocrine cells from the exocrine component. Modified from reference [41].

The major function of proinsulin, the biosynthetic precursor of insulin, is to permit correct orientation of the interchain disulphide bridges in the insulin structure in order to correctly complete the polypeptide chains A and B **[Figure 1-5]** [43]. Within 30 minutes of its synthesis in the β-cell endoplasmic reticulum, enzymes in the Golgi complex cleave the proinsulin molecule at specific residues to form insulin [42]. The excised part of proinsulin, the connecting peptide C or C-peptide, is stored in the β-cell along with insulin and secreted at the same time [44]. It has no clear physiological function outside the β-cell, but may be of clinical significance as the existence of circulating C-peptide in the blood indicates the presence of residual β-cell function. This is particularly relevant in patients receiving insulin injections in whom blood insulin measurements have no meaning as a measure of endogenous β-cell activity [42]. Briefly, the mechanism of insulin action is initiated by the binding of insulin to its protein receptor, stimulating intrinsic tyrosine kinase and leading to the activation of autophosphorylation reactions and occupation of signalling molecules such as insulin receptor substrates (IRS) **[Figure 1-6]** [21]. The activation of IRS initiates a cascade of phosphorylation and dephosphorylation reactions leading to the metabolic effects of insulin [21]. There are two phosphorylation pathways activated by IRS, one of which is the phosphatidylinositol-3-kinase (PI-3 kinase) pathway which stimulates the translocation of GLUT-4, a glucose transporter, to the cell membrane, thus inducing the synthesis of glycogen, protein, and lipids. The other pathway is MAP-kinase, which is involved in cellular proliferation, growth, and gene expression [21].

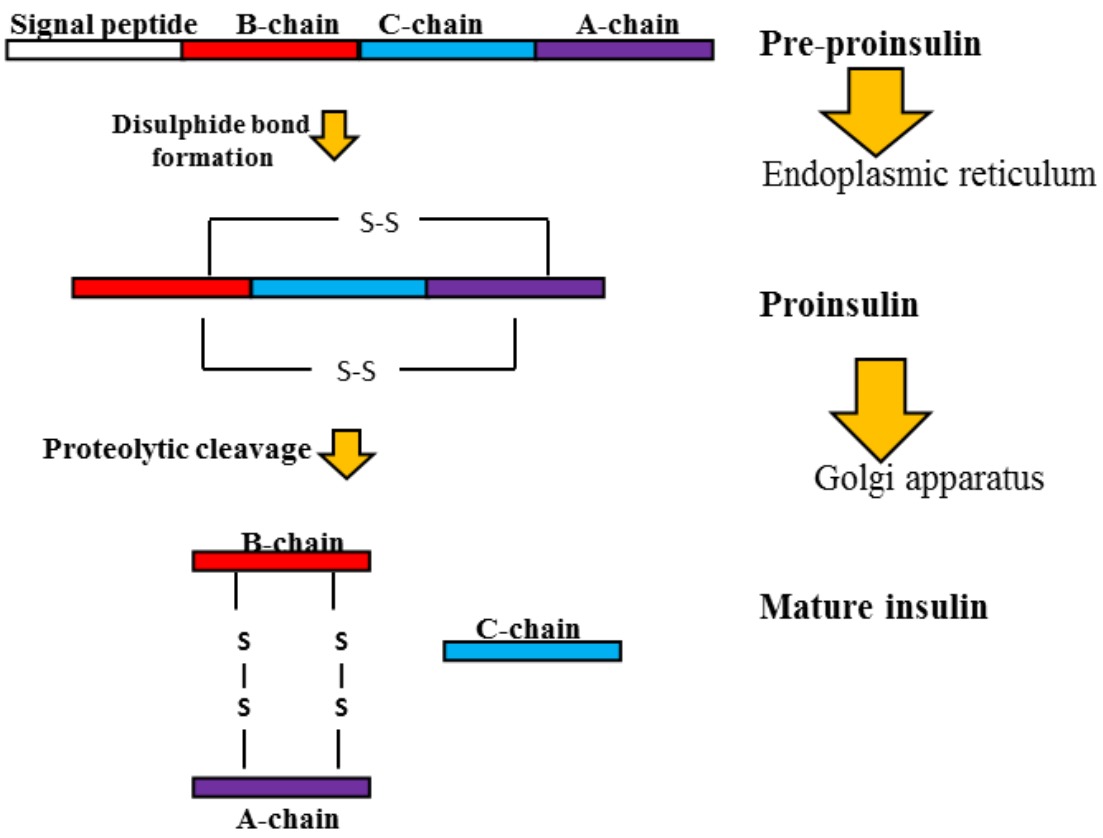


Figure 1-5: Schematic diagram of the biosynthesis of insulin hormone.

The diagram illustrates the biosynthesis of insulin hormone. Insulin is synthesized in β-cells as pre-proinsulin. It consists of single peptide. In the endoplasmic reticulum, a disulphide bond is generated, where it is translocated into lumen of Rough endoplasmic reticulum generating proinsulin. Proinsulin is then transported to Golgi apparatus where immature granules are formed. A proteolytic cleavage of the proinsulin is occurred releasing a fragment of C-chain and leaving 2-peptides; A and B-chains. After cleavage, C-chain became at the central portion of proinsulin. The final product of the biosynthesis is mature and active insulin.

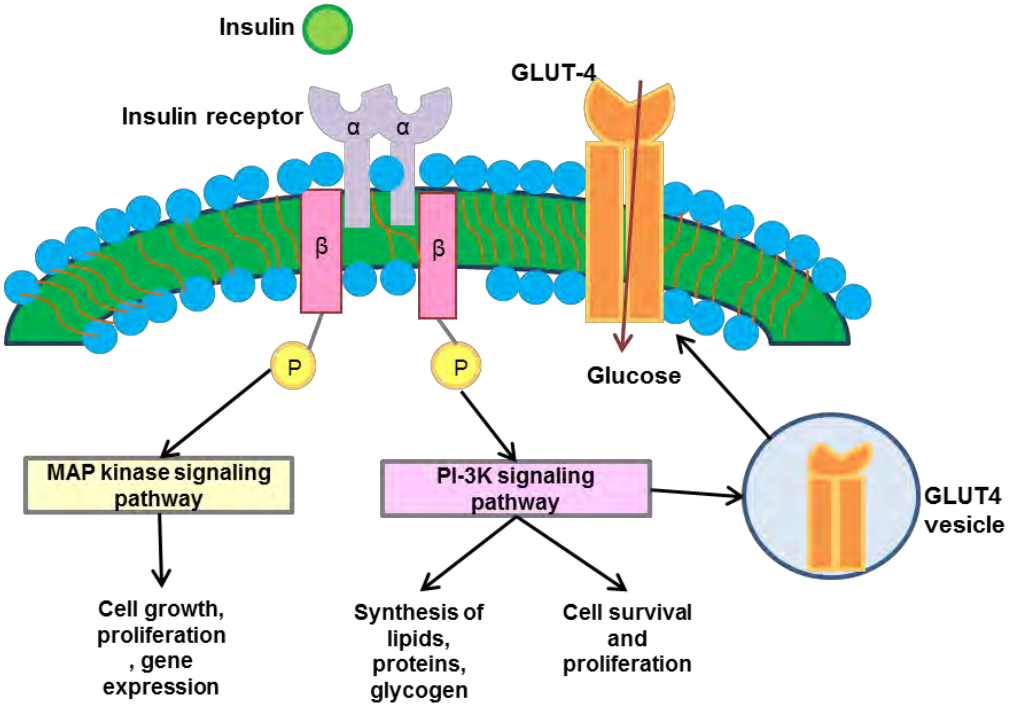


Figure 1-6: Schematic diagram of the mechanism of insulin action.

Once insulin hormone binds to its insulin receptor, phosphorylation of IRS-1 takes place. IRS-1 activates the PI-3 kinase signalling pathway which mediates the glycogen synthesis. This action recruits the glucose receptor to the cell membrane.

β-cells adhere closely to their neighbours by a diversity of cell surface proteins [45]. These proteins interact discriminately within the cell membrane and form intracellular junctions or channels that are permeable to a wide range of metabolites, ions, and secondary messengers [46]. Some junctions establish adhesive links between neighbouring cells, promoting islet structural cohesiveness; other junctions facilitate the anchoring of endocrine cells to components of the extracellular pancreas [47]. Extensive direct cell-to-cell communication is enabled through a mixture of glycoproteins called cell adhesion molecules (CAMs) [48]. Some communication is also mediated by connexins, which join cytoplasm to allow the two-way exchange of cytosolic molecules [49]. The form of communication in endocrine cells is of particularly paracrine, communicate via gap junctions. Therefore, this communication is important for the survival and functioning of cells. Moreover, the cells are surrounded by extracellular matrix (ECM), and the micro-organs found in islet cells influence its processes [50].

1.2.3. **The Extracellular Matrix of Islets of Langerhans**

Islet ECM is divided into basement membranes (BMs) and interstitial matrix (IM). BM is an extremely tight and complex network of glycoproteins which function in the separation of compartments while also directing cellular processes and whereas the IM provides strength and elasticity to pancreatic tissue through fibrin-collagen interactions[51]. The BMs are comprised of distinct layers of laminin (LM) and non-fibrillar collagen, linked together by nidogen-1 (or entactin), one of the four major elements of BM, the other three being collagen IV, LM, and perlecan.

Collagen IV is a nonfibrillar collagen which comprises 50% of all BM and differs from connective tissue fibrillar collagens by featuring globular or rod-like collagenous domains [52].

LM is the most abundant BM non-collagenous protein. There are eleven chains in the LM protein family, with each chain designated as an α, β, or γ chain, based on protein domain organisation. The G domain is the major site for cell adhesion of LM [53], although these BM proteins possess several binding sites for the adhesion of cells, and various motifs provide ligands for cell surface receptors.

Receptor binding to BM proteins triggers intracellular signalling pathways that influence cell behaviour, differentiation, proliferation, and migration [54]. In general, different integrins function as receptors for different components of the ECM, although different integrins may also recognise identical ligands and integrins with similar subunits may show diverse ligand binding specificities [55]. Integrins modulate the adhesion of cells to the basal lamina and ECM of epithelial cells, on which they proliferate [55].

In most tissues, cells are enclosed by a complex system of macromolecules comprising the ECM **[Figure 1-7],** which includes various polysaccharides and proteins [56]. The cells themselves secrete these proteins locally and assemble them in an organised meshwork adjacent to the surface of the cell that produced them. The ECM in connective tissues is relatively abundant compared to the cells it surrounds, and it determines the physical properties of the tissue [57].

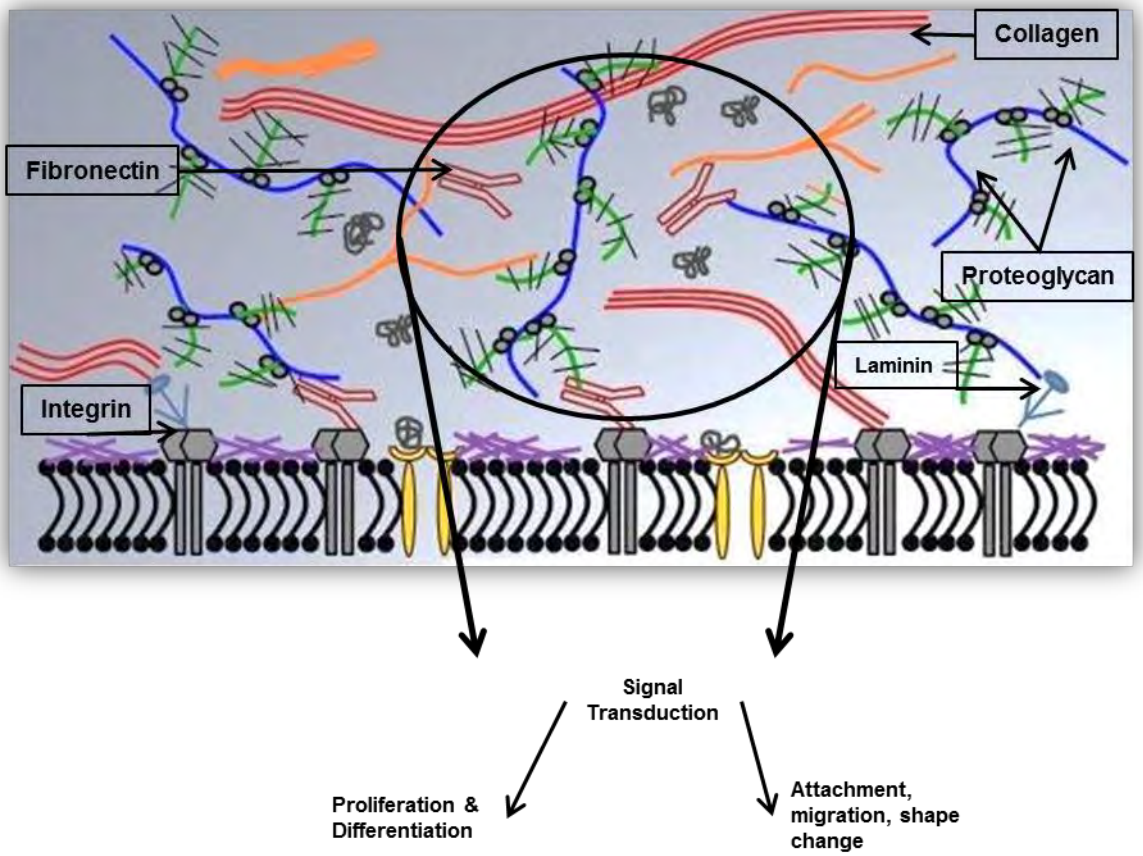


Figure 1-7 : Macromolecular components of extracellular matrix (ECM).

An illustration of ECM components showing the major proteins collagen, laminin, fibronectin, and proteoglycan. The interactions of these proteins with integrins result in signal transduction which regulates numerous dynamic cellular processes such as proliferation, differentiation, migration, and attachment. Modified from [58].

1.2.4. **ECM composition of Pancreatic Islets**

1.2.4.1. ***Collagens***

Collagens are structural proteins that provide mechanical strength to the islet and typically exist in a triple helical structure **[Figure 1-8]**. There are six types of collagens such as I, III, II, IV, V, and type VI predominantly found in islets [59]. Of these, Coll-I, III, and V form the fibrillar structures of the ECM and the long fibres of connective tissue, whereas Coll-VI forms beaded filaments and Coll-IV forms planar hexagonal networks. The most abundant of these types is Coll-IV as it is most commonly associated with islets. Coll-IV links to other tetramers through N-terminus extensions and forms antiparallel triple helical tetramers. The integrins that bind Coll-IV tetramers are α1β1, and (less frequently) α3β1 and α2β1. To date, only study has reported the binding of α1β1 integrins to Coll-IV [51].

Pancreatic ECM is primarily composed of collagen-IV (Coll-IV) and LM layers, although collagen I, III, V, VI, and fibronectin are components also found in the ECM [57]. Studies also support the evidence that the β-cell phenotype is maintained by LM [58]. Pancreatic islets have increased levels of BM, occurring around almost every acinar cell of the exocrine pancreas and encasing each pancreatic islet cell and duct. A recent study indicates that both human and murine perivascular BMs are in close proximity to β-cells and are rich in LM α4 and α5, whereas peri-islet BM is in close contact to endocrine tissues and consists of collagen and LM isoforms [59].

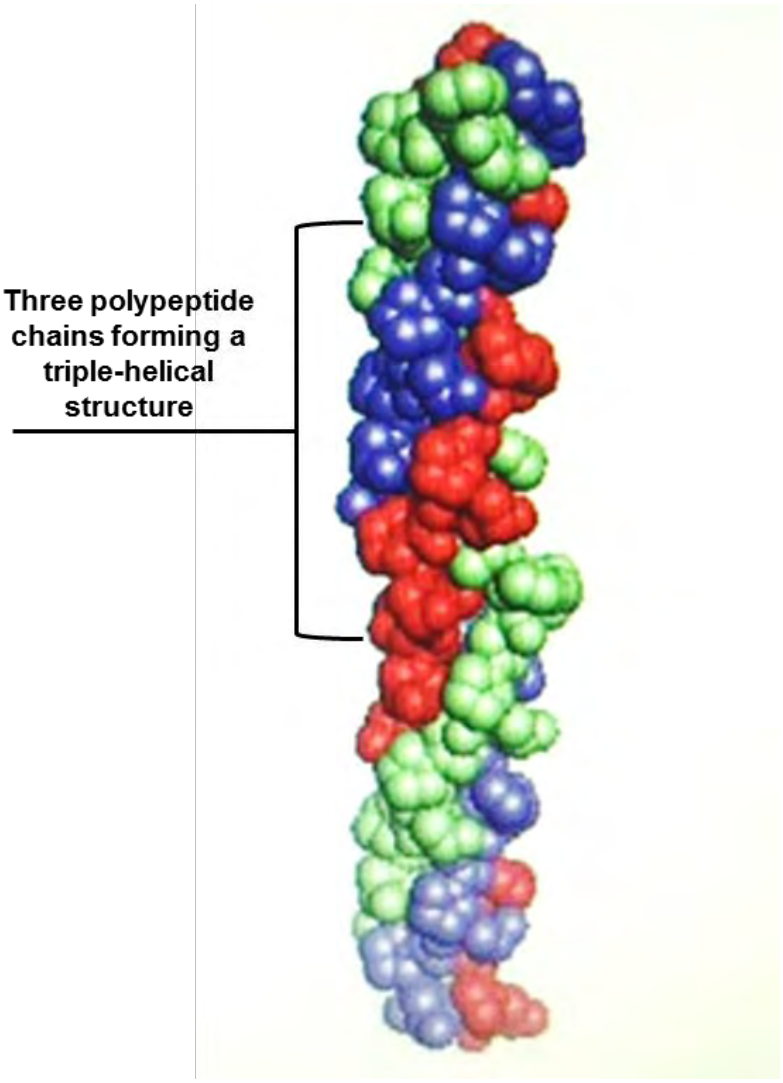


Figure 1-8: Arrangement of collagen structure.

Three polypeptides, coloured green, red, and blue, are folded together to form a triple-helical structure. Modified from [60].

Moreover, Coll-IV can bind to other elements in the matrix such as heparin sulphate or LM via direct interaction or through glycoprotein channels. The role of Coll-IV in islet physiology remains unclear, despite its abundance in the pancreas [61]. Type I and IV collagen provide structural support for islets which stimulate other cell surface receptors essential for pancreatic cell processes [61]. Additionally, Coll-IV increases islets survival and contributes to the enhancement of insulin secretory function [62].

1.2.4.2. ***Laminins***

Laminins (LMs) are a type of glycoprotein which forms triple polypeptide chains in a cross-shaped structure which is linked with di-sulphide bonds **[Figure 1-9].** To date, 12 types of LM isoforms have been identified. Several studies support the evidence that perivascular matrix and peripheral matrix components consist of LM [51]. LM molecules allow the adhesion of molecular receptors such as α3, α6, β4, and β1 as well as other non-integrin factors; LM interactions might therefore not always be regulated by integrins. Similarly, the interaction of LM with islet cells may not necessarily be mediated through integrins [63]. In a recent study, the co-expression of LM with α6 integrins in the developing pancreas was reported [64]. Furthermore, an *in vitro* study by Pinske *et al*. (2006) demonstrated LM involvement in islet survival [65] while several studies have reported the expression of LM in specific regions of islet cells [66]. Moreover, Parnaud *et al*. (2006) showed that the expression of specific LM isoform LM-V was mainly observed in the α-cells of rat and human islets [67].

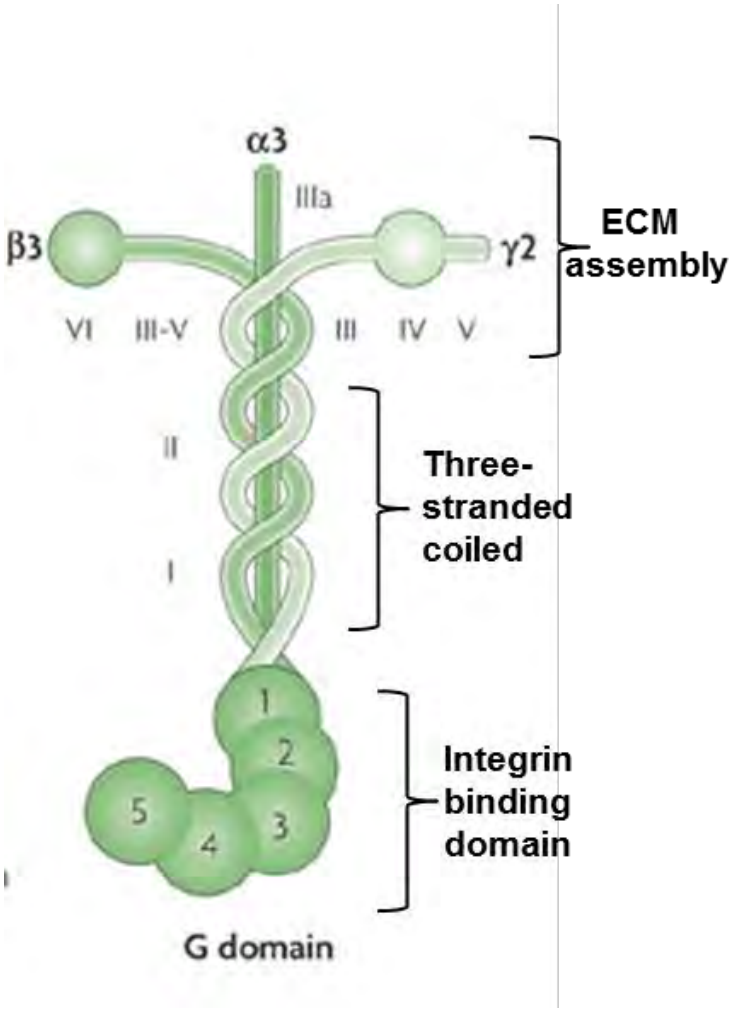


Figure 1-9: Laminin (LM) structure.

Representation of LM as a cross-shaped structure consisting of three chains, each of which associates with a specific binding site. Modified from reference [68].

1.2.4.3. *Fibronectin*

The most prevalent ECM component is fibronectin [69], a high molecular weight glycoprotein which structurally consists of a dimer of two identical peptides [**Figure 1-10**]. The length of the two peptide chains is between 60–70 nm, and 2–3 nm in thickness. Each chain consists of six folded domains with high affinity binding sites for different substrates such as heparin, fibrin, and collagen [70]. Cell-matrix interactions are most commonly associated with fibronectin and integrins, whereas pancreatic fibronectin is found beneath the endothelial cells and epithelial channels [71].

RGD, a tripeptide motif found within fibronectin which mediates cell attachment, is an extended, flexible loop of proteins. Fibronectins attach themselves to integrins through RGD motifs, enabling integrins to transfer biochemical signals throughout the cell and regulating cell integrity and motility processes. Furthermore, fibronectin protects cells against apoptosis. Pro-His-Ser-Arg-Asn (PHSRN), a peptide sequence that binds the RGD motif, is found on fibronectin [43] along with several other elements that act as adhesive motifs for RGD Recent studies have indicated that islet cells exhibit increased adhesion to fibronectin substrates, but that this property is unrelated to any improvement in cell function [51]. Another study reported improved fibronectin adherence in purified β-cells; cells lacked cell spreading, migration, and survival properties in both foetal and adult cells [59]. Therefore, fibronectin is considered critical in cell migration and islet development. Several studies suggest that interactions between fibronectin/ and RGD are responsible for these processes. However, islet structure may be maintained by the interactions between non-RGD and BM molecules such as LM [51]. Therefore, evidence suggests that RGD has an important role in the pancreatic developmental process in adult islets and may have potential applications in transplantation and culture technologies.

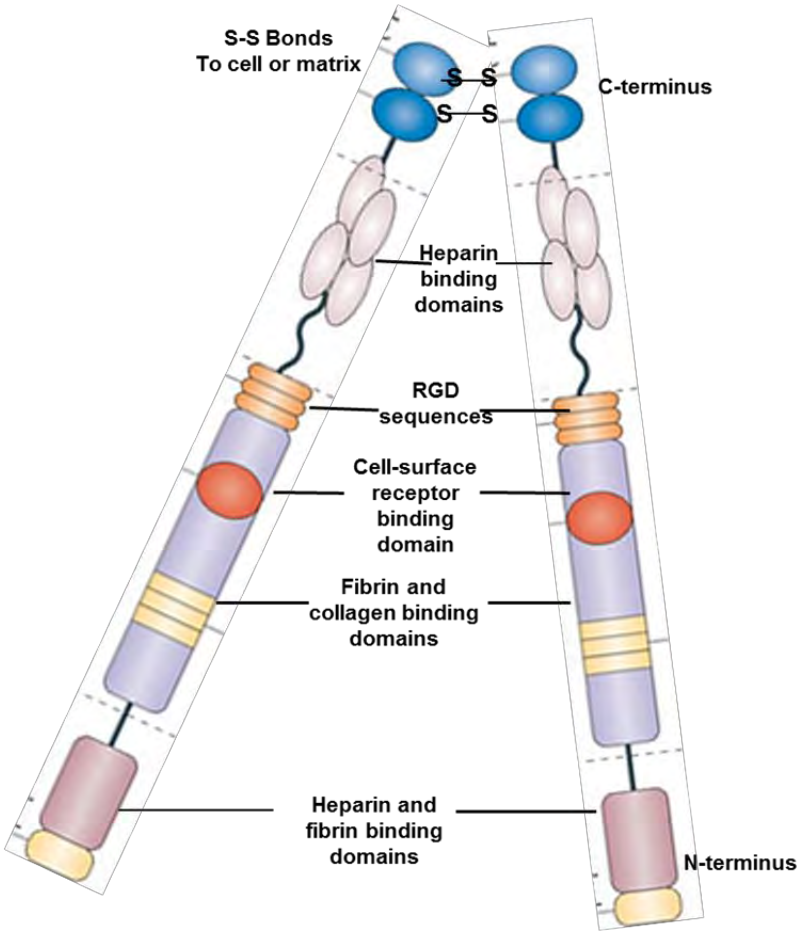


Figure 1-10: Fibronectin molecular structure.

Fibronectin structure comprising two identical polypeptide chains joined by disulphide bonds. The chains are folded into globular domains containing binding sites for other ECM components and for specific binding receptors on the cell membrane. Modified from [70].

1.2.5. **Islets-ECM interactions.**

Islets were found to be heavily influenced, like other tissues, by cells-ECM interaction [51]. There is a significant evidence showing that in mature islets, the interaction with either ECM or synthetic matrix has a great influence on survival, insulin secretion, and proliferation [72, 73]. The ECM functions as a substrate for cell attachment and also influences maturation and growth of β-cells. In a study conducted by Daoud *et al*. (2010), ECM components such as collagen I and IV, and fibronectin were shown to induce islet cell adhesion, whereas fibronectin alone was shown to maintain the structural integrity of islet cells as well as regulate insulin content distribution [57].

The islets themselves do not form the BM; ECM is formed by neighbouring endothelial cells, supporting the rationale behind the culturing of islets on ECM. Furthermore, when islets were cultured on different ECMs, as demonstrated by Kanayama *et al.* (2009), islets grown on collagen I and V contributed to high insulin release [74]. The interactions between ECMs are also important in signalling, cell differentiation, propagation, relocation, and suppression of apoptosis. Thus, the advantage of direct contact between islets with ECM may increase vitality and function in cells. Although the mechanism of action remains unclear, the interior part of islets has an indirect contact with the peripheral ECM through intracellular junction interaction which transmit signals from peripheral cells [51].]. Gap junctions and cell adhesion molecules, such as integrins, E-cadherin, and neural adhesion molecule (N-CAD), are also found in islet cells [75, 76] and these linkages and connections are important for islet functions such as glucose homeostasis and development of islet cells.

Regulation of ECM levels is important in term of islets transplant, as islet ECM can be degraded by enzymatic digestion induced during islet isolation. Islet transplantation can result in early rejection as donor islets faces challenging conditions within the host's body, including various mechanical stresses, differences in osmolarity, absence of C-

peptide production, and exposure to digesting enzymes which leads to peri-insular BM loss [77]. Implanted cells are also exposed to immune attack, hypoxia, toxins, and effects of immunosuppressive therapy [78]. Transplanted islets are thus required to adapt to new peripheral ECM and surroundings without support from appropriate vascularisation and innervations [79]. It is therefore important that the functional and structural viability of transplanted grafts is maintained, in order to provide structural support to cells while maintaining extracellular cell interactions crucial for islet function [77]. These perivascular BMs not only maintain the vascularity of islet cells, but also provide the cell with a platform for attachment and migration. However, during enzymatic digestion, peri-insular BM, along with interstitial matrix, is digested during islet isolation. This leads to the induction of apoptosis as the cell matrix interaction is lost and BM is destroyed [51].

In pancreatic islet isolation, large numbers of cells are lost as a result of disruption of islets into solitary cells [80]. Islets are highly vascularised structures; their isolation from their environment disrupts islets-matrix interaction and leads to ischaemia [41]. Islet cell behaviour can also be disrupted with respect to the paracrine influences important to their function. According to Hughes *et al*. (2006), purified β-cells or isolated islet cells are typically less responsive to glucose stimulation in culture, compared with whole islets [41].

In addition to that, BM membrane loss during islet isolation has been reported, although its effect on islet cell survival remains unclear [81]. For example, pancreatic digestion to free islets from the surrounding ECM was shown to cause apoptosis in islet cells [82]. Therefore, apoptosis may be initiated by stress induced during islet isolation, and some studies have reported that the process continues during transplantation [83]. A study by Wang and Rosenberg (1999) indicated that apoptotic cell death was most significant in post-isolation islets that were separated from matrix proteins, as matrix proteins can

improve the survival and function of islets. However, association with matrix proteins was shown to only prevent apoptosis for up to three days [77].

Evidence suggests that, in order to improve the survival process, integrin mediated adhesion to matrix proteins is crucial [77]. However, that the mechanism by which matrix contact restoration can improve the functioning and survival of cells remains unclear, since β-cells are located on the inner curvatures of the islet and are not directly exposed to the matrix itself [81].

One possible mechanism is that ECM may release certain growth factors that attach themselves to the interior of islets. Therefore, it is important to select a transplant site that is relatively similar, in terms of ECM composition, to the host pancreas [51]. Another key factor may be the identification of isolation end points in order to reduce the extent of damage and exposure to cells [84].

It is possible to construct a similar ECM to the donor ECM to protect cells from isolation and apoptosis, and to maintain function. Materials such as proteins, ligands, and polymers can be assembled in degradable scaffolds and delivered to the graft site. Hence, scaffolds which contain material similar to the host ECM are more likely to facilitate successful transplantation [85]. Short peptide chains are preferable to intact matrix proteins as they offer higher purity and less immunogenicity [86]. Therefore, ECM islet replacement can enable the integration of matrix based signals, acting in a controlled manner and similar to the host cell [83].

To conclude, islet-ECM interactions can influence the success of the transplantation process and promote cell functioning and survival. However, these interactions require further research to determine the precise mechanism of action.

Other factors, including vascularity of the site of transplantation, are of critical importance in the grafting process. It has been reported that islet isolation can lead to apoptosis if cells are not placed in a similar environment to the host organ [51].

The grafting process is thus significantly affected if cells are not provided with appropriate attachment sites.

In the next section, the process of islet transplantation will be discussed more in detail including the challenges and potential solutions were used to improve the outcome of the procedure.

1.3. **Islet transplantation**

Transplantation of functioning β-cells is the most physiological treatment for diabetes. The predilection for technical complications within pancreas transplants is the motivation for islet transplantation [87]. Compared with whole-pancreatic grafts, isolated islet transplantation is minimally invasive since the islets represent only 1–2% of the entire pancreatic mass. Furthermore, the native pancreatic bed is relatively inaccessible and efforts to bring islet grafts into the splenic vasculature have brought about considerable morbidity, including rupture, infarction, and gastric perforation [88].

In current protocols, islets are generally introduced under local anaesthesia into the hepatic portal vein by measured infusion directly into the liver, the site which has been shown to provide the greatest clinical success [29]. Regardless of the apparent advantages linked with islets compared with whole organs, however, risks of potential immune reactions are similar.

In 1977, the first islet cell transplantation was reported [89]. Early experiences with islet cell transplants were disheartening, however, with only a small number of people effectively cured of their diabetes. In 2000, however, James Shapiro and colleagues demonstrated outstanding success in transplantation rates among seven individuals who remained insulin-free at one year after islet cell transplantation [29].

These successes were attributed to several factors. The researchers realised that individuals were receiving inadequate amounts of islet cells, and decided to inject cells

from two to three donors into each individual. It was also discovered that previously utilised immunosuppressant therapies contained steroids, which in fact were diabetogenic. The group decided to use newer therapeutic agents and forgo steroid treatment. Lastly, the researchers determined that the reagent used to harvest islet cells from the pancreas was actually toxic to the cells, so they modified the islet cell isolation process to preserve cell function [89].

1.3.1. **Procedure**

In summary, islet cells are acquired from donors who are brain-dead. The donors should preferably be aged 20–50 years with negligible warm ischaemia, which is the period before and during [90], since long-standing cold ischaemia (greater than 12 hours) has been shown to damage the pancreas [91]. To isolate islet cells from the donor pancreas, collagenase enzyme is used. A collagenase solution is injected via the pancreatic duct, which runs throughout the pancreas, resulting in distension of the organ [92]. When islet transplants started, a recipient requires at least 10,000 islet cells per kilogram of body weight, achieved by extracting cells from an average of 2–3 donor pancreases[93]. The protocol was performed on seven diabetic patients diagnosed with brittle T1DM, with an infusion of 2-3 of deceased donors being applied. All the seven subjects were treated with glucorticoid free immunosuppressive therapy consisting of sirolimus, tacrolimus, and daclizumab, and with a follow-up up to a year all subjects were insulin independent, showing tight glycemic control with stable glycated haemoglobin (HbA1C) [29].

Transplantation is performed in the hospital by a radiologist, who utilises X-rays and ultrasound imaging to direct the placement of a catheter into the portal circulation (portal vein) via the upper abdomen using a laparoscopic or trans-hepatic approach under local anaesthesia [92]. The isolated islet cells can then be slowly infused via the catheter into the liver. In some patients, transplantation may be performed through a small incision under general anaesthesia. The isolated islets lodge in the smaller portal

vein, where they engraft and receive their blood supply from the patient's vessel for growth [94]. The injected islet cells will immediately start to release insulin, although their full function and corresponding blood vessel growth takes additional time to develop. Medications are provided to maintain islet cell function and prevent rejection. Insulin administration may be required until the cells regain their full function [95]. **Figure 1-11** illustrates the islet cell transplantation procedure.

1.3.1.1. *Islet Cell isolation*

The pancreas is treated with collagenase enzyme to isolate the islet cells [94]. Liberase HI, a blend of collagenase isoforms 1 and 11, has been used as the standard digestive enzyme for islet isolation [96]. The enzyme allows separation of the adjacent acinar cells by degrading the pancreatic ECM. Although digestion is vital, it is an unpredictable stage of islet cell isolation [91]. The collagenase preparation and donor variables such as age, ischaemia (cold or warm) throughout the procurement procedure, and discrepancies in islet purification are some of the factors contributing to variability in this phase. The establishment of Ricordi's automated process for isolation of pancreatic islets marked a significant improvement in islet cell isolation [97]. In this automated pancreas dispersion method, collagenase is injected into the pancreatic duct and the pancreas is positioned in a compartment comprised of steel spheres. The compartment is gently agitated, enabling steady separation of the pancreas.

This procedure takes approximately 45 minutes until the islets are completely devoid of adjoining acinar tissues (cleaved islets) and ready for density gradient purification [91].

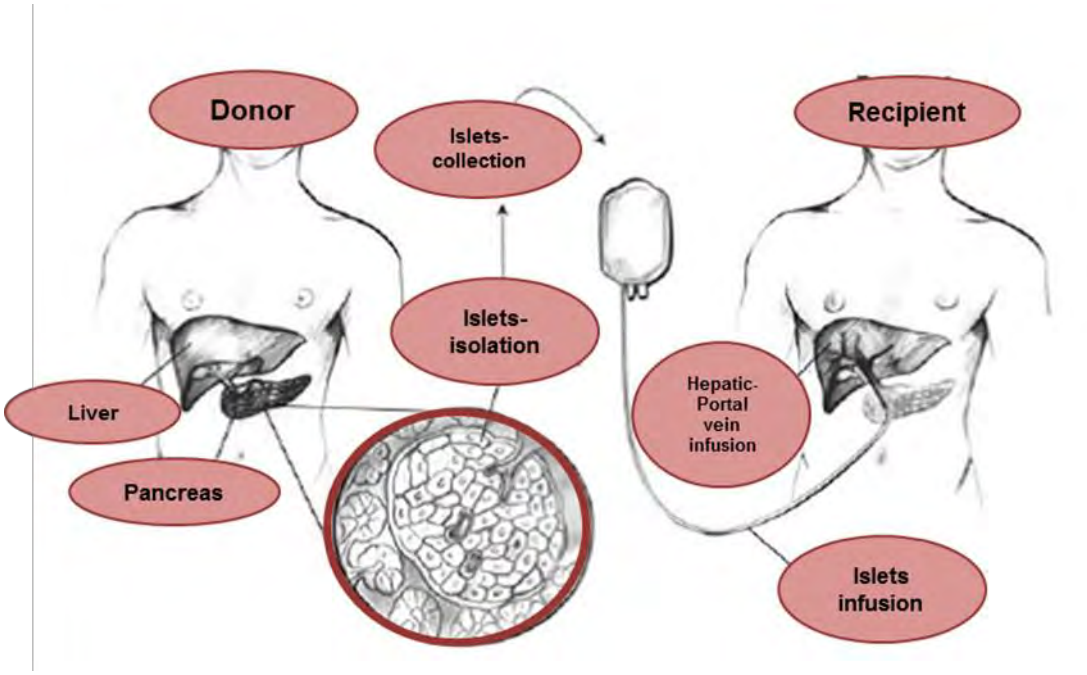


Figure 1-11: **Islet isolation and transplantation from donor to recipient.**

The donor pancreas is infused with collagenase digestive enzyme to separate islets cells from exocrine tissue. A purification process is followed by gradient centrifugation in order to harvest the most viable cells. Islet cells are infused via catheter into the hepatic portal vein of the recipient, under local anaesthesia, using a very fine thin needle and X-rays to locate the portal vein. Islets then travel to the liver and start to vascularise and produce insulin**.** Modified from [98].

About 15-40 mL of pancreatic digest is generally amassed, including cleaved and uncleaved islets, lymphoid and ductal remnants, and acinar tissues, and the tissue is then purified [97]. Acinar and islet tissues have varying densities, enabling partition using a density gradient [99]. Purification allows optimal islet acquisition by decreasing the amount of unnecessary contaminating acinar tissues and resulting in a safer transplantation process. Islet revascularisation is also enhanced by purification [23].

Following islet isolation, significant mechanical stress and trauma is introduced to cells as indicated by the presence of apoptosis, necrosis, and proinflammatory cytokines [100, 101]. Staining procedures are applied to specifically identify islet cells, and only those fractions which show more than 30% purity are further processed. These fractions are concentrated further by centrifugation and suspended in the culture media of choice [94].

Islet Equivalents (IE) index is then calculated by staining islets with diphenythiocarbazone dye then counting them under scaled microscope looking for a size equivalent of 150μm in diameter [102], following that cells are either transplanted directly into the recipient or cultured for a further 48 hours [94].

Thus, the ultimate goal of isolating islets, is to maintain viable islets whether in clinical *in vivo* islets isolation or in researching *in vitro* studies. So, one of the major key elements that aid to a successful islet isolation is by culturing cells in a control proper culture condition [103].

1.3.1.2. ***Culturing of Cells***

The first successful use of cell cultures was reported in the 1950s, when human cell lines were grown *in vitro* using cells taken from a specimen of cervical cancer. Since then, cell culture techniques have developed and improved, and rigorous protocols designed, subsequently leading to the successful growth of many varieties of human cell lines. The importance of cell culturing was initially highlighted in the context of

research, as it provided a means of ensuring potentially limitless supplies of specific types of human cells for scientific study. In more recent years, cell culturing has, however, been identified as having a role in the design of novel therapeutic approaches to disease, including islet cell transplantation [104].

After the islet cells are mechanically or enzymatically harvested from the pancreas of the human donor, they may therefore be transplanted directly to the recipient or cultured for a period of time before the transplant procedure [105]. Islet culture prior to transplantation ensures that only the healthiest cells are transplanted [106] and also allows the procedure to be performed under elective conditions, with prior administration of immunosuppressive therapy and reduced travel burden for recipients [27]. Furthermore, islets are cultured to guarantee the quality of islet cells, while also permitting additional time for patient preparation and pre-transplant interventions, allowing the procedure to take place at a given location and facilitating the shipment of islets to remote sites.

Thus, the main purposes of culturing islet cells within most protocols devised for islet cell transplantation is two-fold; firstly, this is perceived to be an effective method for reducing the number of damaged cells prior to infusion [104], with the benefit of reducing the number of non-viable cells included in the original mass of transplanted cells. However, one of the limitations noted in clinical studies of islet cell transplantation is that, very soon after transplant, a significant number of cells fail to graft into the patient, therefore reducing the number of cells which can ever become fully functional [107]. This observation therefore appears to suggest that cell culture is unable to fully eliminate damaged or non-viable cells from the transplanted islet cell mass. This could, however, be due to deterioration of cells in culture if not adequately protected [108].

Some previous studies appear to support the arguments above. A number of researchers have explored protocols which forgo the need for culture yet still provide evidence of similar levels of success, appearing to indicate little benefit in including the additional culture step. Shapiro *et al*. (2006), for example, conducted a clinical trial using the Edmonton protocol for islet cell transplantation in 36 participants [109]. Importantly, this protocol did not include cell culture, and islet cells were transplanted directly to the recipient within two hours of isolation and purification. The authors concluded from the results that this protocol was successful in restoring endogenous insulin production and control in the majority of patients [109]. Overall, 44% of participants were able to achieve full insulin independence, although only five participants maintained this independence at the 2-year follow-up assessment.

In all of these studies, both those using conventional culturing techniques and those forgoing this step in the procedure, it is clear that the effectiveness of grafting can be greatly improved overall. It can be further argued that, at present, the similarities in results between these two approaches may be due to a trade-off between protection of cells and improvement of the quality of cells grafted.

At the same time, however, culturing cells undoubtedly provides a better opportunity to assess the quality of cells prior to transplant. One issue of particular significance, which is highlighted by Street *et al*. (2004), is that the outcomes associated with islet cell transplantation may vary markedly depending on the composition of the transplanted cell mass [110]. The authors found that differences in composition regarding cell layers, and the number of ductal or progenitor cells contained in the transplant, all had significant effects on the insulin-producing activity demonstrated by cells at 2-year post-transplant [110].

The authors suggested that many previous clinical trials had not rigorously monitored the composition of the transplants, although they argued that, in many cases, it was unlikely that more than 50% of the total cell mass would be comprised of β-cells [110].

The other main argument for the use of cell culture prior to transplantation is to reduce the risk of non-specific immune responses once the cells are transplanted. Lacy *et al.* (1991) suggested that the type of culture protocol most often adopted is inadequate for removal of resident passenger leukocytes from isolated cells, the likely main culprits responsible for triggering the immune response in the cell recipient [111]. Other authors have presented evidence, however, that long-term culture is an effective method to significantly reduce the numbers of these leukocytes [112]. There is more support for the argument in that it may be an effective method to reduce the level of cell-surface antigenic markers, specifically the major histocompatibility complex (MHC) class II markers [104, 112]. This should therefore reduce the level of the immune response; leading to a lower requirement for the use of immunosuppressant agents in transplant recipients. An ideal approach would thus be to design a protocol which includes cell culture, in which additional steps were taken to protect islet cells during this stage of the process and beyond.

The benefits of culturing islets prior to transplantation can allow the recovery of cells post-isolation by culturing with oxygen and nutrients to prevent further damage and loss of cells [23]. Additionally, other practical advantages of pre-transplant culturing of islet cells include providing manageable time to arrange transportation of the patient, coordination of islet infusion, preparation of immunosuppression drugs, and antimicrobial testing for contamination [23]. There are a number of additional factors which may be added to the culture media which have been shown to offer protection to islet cells, thereby improving viability and prolonging graft survival once transplanted

[113]. This is discussed in further detail later in the chapter with respect to the challenges associated with generating sufficient cells for transplant.

1.3.1.3. *Islet cell infusion*

The transplantation procedure is performed under local anaesthesia and involves the injection of purified islets directly into the portal vein via a percutaneous hepatic approach [114]. The infusion procedure of islets takes between 15–40 minutes depending on two factors, the tissue volume to be infused and the portal pressure induced during islet isolation [23]. Prior to the establishment of homeostasis, post infusion portography and pressure are determined at the completion of islet infusion [23]. There is, however, one limitation associated with transplantation at this site—inflammatory processes can lead to destruction of many of the transplanted islets [30]. Therefore, this step of the islet transplantation process is also a point at which the β-cells are vulnerable.

1.3.1.4. *Immunosuppression*

Immunosuppressive therapy administration, post-transplantation, is necessary to prolong islet graft survival. In 2000, an update to the Edmonton protocol in the use of immunosuppression regimens was applied in order to reduce the toxicity of corticosteroids on β-cells [29]. Shapiro *et al*. (2000) used sirolimus, daclizumab, and tacrolimus as immunosuppression treatments to provide additional immediate immunosuppressive protection [29]. However, some studies have reported that sirolimus can have serious long-term side effects on islet cell function [115].

Therefore, it is necessary for future clinical trials to investigate the most effective immunosuppressive drugs with the least morbidity to islet cells [23].

In the next section, challenges face islet transplant and possible strategies that could improve the outcome of the procedure.

1.3.2. **Challenges and potential solutions**

The transplantation of islets has been explored and its effectiveness as a treatment for T1DM has been demonstrated [116]. However, several challenges face this promising intervention. The first is the effect of immunosuppressive drugs on the patient. Rejection remains the main limitation to any transplant procedure. The immune system is programmed to overcome any microorganisms or tissue it recognises as foreign in the body, including transplanted islet cells. Moreover, the recurrence of the autoimmune process that destroyed the recipient's own islet cells is a possibility [86]. Hence, immunosuppressive drugs are required to prevent rejection of the transplanted islets cells. These drugs must be taken daily for the rest of the patient's life to prevent rejection, although they are known to exert long-term side effects. Balamurugan *et al*. (2006) reported several adverse effects of some immunosuppressive drugs, including infection, nephrotoxicity, neurotoxicity, hypertension, hyperlipidaemia, hypercholesterolemia, diarrhoea, proteinuria, joint pain, anaemia, mouth ulcers, and carcinomas [105]. For this reason, most clinicians favour the use of long-term insulin therapy over maintaining the patient on immunosuppressive drugs [107].

Another major barrier in the widespread use of islet transplantation for the management of T1DM is the shortage of donor islets. Today, the only acceptable human source of cells capable of sensing glucose and secreting insulin are β-cells from islets of Langerhans [117]. Regardless of how islets are packaged for transplantation, the source must be a brain-dead human donor, and the numbers of such donors are very limited.

Although the process of islet transplantation is relatively well established, there remain several challenges. Specific limitations identified in the literature include increasing the transplanted islet mass and findings ways to preserve β-cell function [30], the same issues identified earlier in this review.

The following sections discuss these issues in further detail, starting with islet cell mass considerations before moving on to discuss issues of cell quality which may influence on-going β-cell function.

1.3.2.1. *Islet Cell Mass*

One of the main issues of current transplantation procedures is maintaining cell masses at the level required for optimal functioning [118, 119]. There are numerous reasons for this limitation and there appears to be no agreement in the literature regarding which is the most important factor, possibly as the precise mechanism of β-cell growth and survival, including signalling pathways, is not yet fully understood [120]. Studies reporting patients as insulin-independent at one year post-transplantation have shown that islet cell masses of approximately 10,000 to 11,500 IE per kilogram of body weight are required in order to have any chance of achieving this outcome [29, 118]. Other authors have reported using even greater cell masses—up to 16,000 IE per kilogram of body weight [116].

It is difficult to generate this number of cells, as it is unlikely that a single donor pancreas would permit culture of this amount of viable functioning islet cells. Yet, if insufficient cells are transplanted, insulin production levels are initially lower and transplanted cells are overworked, thereby increasing the risk of damage and cell death. As more cells continue to die, the remaining cells become even more overworked until a critical level is reached.

This process can be overcome by using cell from two or more donors [119]. Finding new sources of pancreatic islets has been specifically cited by Madsen (2005) as a major challenge of islet transplantation [121]. A number of different solutions have been investigated, although the most popular solutions proposed include the proliferation of β-cells, use of human embryonic stem cells (hESCs), and use of pancreatic stem cells. Each of these potential solutions is discussed in further depth below.

Proliferation of Islet Cells

In spite of the observation that human islet cells do not normally proliferate, laboratory experiments have shown that adult differentiated β-cells can be stimulated to proliferate and produce up to a 30,000-fold increase in cell numbers. The major limitation of this technique is that the β-cells lose their insulin expression after a small number of cycles [104], indicating that this approach may have limited utility in relation to islet transplantation for the treatment of diabetes. There have, however, been a number of studies which suggested that the use of additional factors may not only address this problem, but also significantly improve proliferation itself. For example, Ludwig *et al.* (2010) reported the use of synthetic growth hormone receptor agonists to stimulate proliferation in pancreatic islet cell lines. The results indicated that, not only was this associated with an increased level of proliferation, but that it may also offer some protective benefits by reducing cell apoptosis [122]. The results were favourable, with regard to insulin-producing ability of the cells derived by this method once transplanted, which were shown to be functionally similar to those in healthy individuals. This study was based, however, in rat models; it remains to be seen whether the results can be replicated when in human subjects.

Human Embryonic Stem Cells

hESCs have been proposed as a suitable method to generate sufficient cell masses for islet transplantation in humans. Authors including Liew *et al*. (2005) argued that they provide an effective and viable alternative to current whole organ, synthetic organ, or animal-derived organ transplantation [123]. These cells have a replicative capacity which is virtually unlimited, and can differentiate into most types of body cell, including pancreatic islet cells, while maintaining a normal karyotype [124]. hESCs have been shown to produce β-cells by transformation of a mixed cell population of immature pancreas cells to islet-like endocrine clusters [125].

There are two key characteristics of β-cells required for the effective control of blood glucose: they must be able to detect glucose levels, and release insulin in response. Both of these traits are unique features of pancreatic β-cells [121]. Studies have demonstrated that the required characteristics of these cells are attainable using hESCs, even though the cells generated may not wholly resemble true β-cells [126]. There was previously an assumption that this process was non-directed, meaning that the potential exists for cells to instead differentiate into other cell types, although protocols have been developed for directing differentiation through the normal stages of human pancreatic development [127]. These protocols have been demonstrated to generate cells efficient in producing insulin [128]. There are, however, some remaining issues with this approach to generating pancreatic islet cells. For example, it could be described as an inefficient method due to the additional steps of differentiation required after replication.

Pancreatic Progenitor Cells

Although some authors have suggested that pancreatic progenitor cells can be stimulated to proliferate and generate islet cell mass, their very existence appears to be a source of controversy within the literature. Laboratory studies from mouse models suggest that, in the adult pancreas, β-cells are renewed from existing β-cells rather than from pluripotent stem-cell differentiation [129]. These progenitor cells may be isolated and stimulated *in vitro* to differentiate to multiple pancreatic cell lineages, including hormone-producing islet-like cells, under the influence of fibroblast-like growth factor (FGF)-2 [130]. Although the existence of these cells has only been supported by research evidence in recent years, the implications for the treatment of diabetes have already been identified in the literature [131]. The failure of some researchers to demonstrate the presence of these cells within the pancreatic cell mass may, however, be due to their apparent dependence on the expression of certain transcription factors. The necessity of specific transcription factors such as STRO-1 and CD146 was

demonstrated by Xu *et al*. (2008) in a study which appeared to support the argument for these progenitor cells [132]. Their findings showed that these factors can be utilised for regeneration of the periodontium to a greater extent [132]. Similarly, other studies have demonstrated the need for transcription factors such as cf2, Onecut1, and Foxa2 [133] but have only demonstrated their apparent existence in mouse models and not in human pancreatic tissue. Furthermore, a lack of evidence exists to demonstrate that this approach results in cells which are efficient in producing insulin, a necessary feature for application to clinical practice.

1.3.2.2. ***Islet Cell Quality***

It may not only be the islet cell mass which determines the outcomes of transplantation. Islet cell quality may also be highly important, as this is associated with maintenance of islet functions including cell growth and survival [28]. It remains unclear as to the precise elements which determine the quality of islet cells in regard to their continued functioning once grafted. Prediction of the functional capacity of isolated islet cells prior to transplantation has therefore been suggested as a major challenge in islet cell transplantation [30]. This is not a limitation specific to islets, but to any cells which have been cultured for transplant. Measures of quality which are commonly used in pre-transplantation assessments include cell viability, attachment efficiency, and metabolic testing, all of which provide objective criteria [134].

In human islet cells, for example, these assessments may involve testing for glucose-stimulated oxygen consumption and cytochrome C reduction as a measure of metabolic activity [135]. However, there is a lack of studies available to demonstrate that this objective *in vitro* measures directly correlate with continued functioning of cells once transplanted into human subjects.

1.3.2.3. *Islet Cell Encapsulation*

Islet cell encapsulation is the process of placing a group of islets within a selective membrane constructed from immune-protective biomaterial, creating a type of artificial pancreas. Microencapsulated cells were first used to successfully correct diabetes in a rat model in 1980 [136]. The benefit this process is that the membrane excludes proteins and some of the larger cells of the immune system, thereby protecting the islet cells encapsulated within by reducing the risk of an immune response.

This reduces the need for immunosuppression, which may be a major risk associated with the procedure in its original form [30].

The membrane is designed from materials selected to have a molecular weight threshold such that the membrane still permits small molecules to enter. The cells within are thus able to receive glucose and other nutrients; waste materials can be removed so the risk of toxicity is eliminated. Insulin is also permitted to exit the capsule, allowing the islet cells to continue to regulate blood glucose via hormone release. Beck *et al.* (2007) described three different methods of encapsulation: intravascular or extravascular macrocapsules, and microcapsules. The authors concluded, from their review of the literature, that there are benefits and limitations associated with all methods, and were unable to determine which provided the best approach in practice [28].

1.3.2.4. *Endocrine Tissue Engineering*

As an alternative to encapsulation, it is possible that whole endocrine tissue may be engineered through the use of decellularised ECM as a natural scaffold. This relatively new technique has been described by a number of researchers in relation to the engineering of different human organs, and has been made possible due to new perfusion decellularisation technologies which allow ECM scaffolds to be generated that retain their 3D anatomical structure and vasculature [137].

Conrad *et al.* (2010) reported favourable results from experimental tissue engineering trials in which mesenchymal stem cells and islet cells were placed inside decellularised ECM. Tissue was maintained inside a bioreactor under perfused conditions [138], and histological and immunofluorescence analyses showed that the cells formed a 3D tissue structure with close relationships between mesenchymal and islet cells, as would be expected when examining intact tissue derived from a living host [138].

Islet cells were also shown to be structurally and functionally preserved, and to demonstrate a continued insulin response. The researchers also demonstrated, in a rat model, that the tissue could be successfully transplanted whole into a living animal [138].

This approach offers an effective means of protecting islets, as the ECM provides a means of repair and physiological regeneration which may not be provided by synthetic scaffolds [137]. This approach was therefore selected for further investigation in this study, as it was deemed to offer the best potential means of protecting the cells, not only during the culture phase, but also during the transplant phase. If effective, islet cells will receive additional protection during two of the phases at which they are most vulnerable to damage. It was therefore anticipated that this process should significantly improve the long-term functionality of the cells once transplanted.

Alternatively to decellularisation technologies, pancreatic β-cell lines were established and became valuable tools for the study of molecular and biological events underlying β-cell function. Also, they could presented a potential source of "transplantable source" in the clinical field [139].

Pancreatic β-cell lines

The utilisation of primary β-cells in molecular and biochemical research has been limited by accessibility to endocrine tissue/cells as well as by the complexity of the

procedures necessary to separate pancreatic islets, disseminated islet cells, and isolate and decontaminate β-cells from other types of islet cells [140]. A number of investigators have endeavoured to establish a cell line that secretes insulin and preserves the normal parameters of insulin secretion [141]. Several approaches have been evaluated to achieve this, including stimulation of pancreatic tumours by infection of viruses or irradiation, immortalisation of *in vitro* β-cells, and the development of genetically altered mice with targeted expression of β-cells via recombinant oncogene methodology [94].

This study sought to investigate the effects of ECM under microgravity culture conditions on a pancreatic β-cell line derived from a mouse model. As discussed previously, several obstacles hinder the success of islet transplant outcomes, so the generation of an *in vitro* β-cell model was essential in understanding the biology of islets in order to address the factors influencing the functionality of cells.

This study therefore elected to use the Min6 β-cell line, an insulinoma cell line derived from a transgenic mouse model [142]. This cell line exhibits characteristics of β-cells, displaying glucose-stimulated insulin secretion at levels similar to native pancreatic β-cells. This activity has also been shown to exceed that of other similar cell lines such as Min7 [143], and Min6 β-cells have also been shown to produce other endocrine hormones including glucagon, somatostatin and ghrelin [144]. Min6 β-cells were thus considered an appropriate model of pancreatic islets for this experimental study [143]. These cells have also been specifically used in studies which evaluated factors involved in islet functioning, including 2D or 3D matrix structures in which cells were allowed to proliferate [145].

The βTC group of insulinoma cell lines was created from genetically modified mice by targeted expression of the SV40 large T-antigen in β-cells [141]. Additional cell lines, βCT3 and βCT6, displayed half the normal reaction to sugar (0.5 mM D-glucose). βTC

cells produce both proinsulin I and proinsulin II, and can convert both into mature insulin [146]. Using electron microscopy, insulin was shown to be stored within secretory granules in these cells. Insulin production with a lower threshold for maximal stimulation compared with normal β-cells was also shown, and the characteristics of differentiated β-cells were preserved for about 50 passages in culture [146].

Correct anatomical cell structure is vital for cellular and physiological cell processes such as insulin production [147]. It was therefore shown that the separation of islet cells from their native environment resulted in the dramatic decline of cell functionality [147].

Several recent studies have shown that the generation of 3D-islet like structures from Min6 β-cells *in vitro* are a beneficial and useful model to study and understand important cellular processes such as cell-to-cell and cell-to-matrix interactions and their roles in insulin expression and secretion. These 3D-structures, known as "pseudoislets" (PIs) are generated when Min6 β-cells are cultured under non-adherent static culture conditions for 6–8 days [148]. Several studies reported that Min6-Pseudoislets (PIs) recapitulated the morphology, functionality, and size of primary β-cells *in vitro;* with glucose responsiveness which is significantly higher compared with Min6 β-cells cultured as monolayer [148]. Thus, the generation of highly functional islet cells *in vitro* requires the correct anatomical configuration in order to resemble the cell-to-cell communication seen in islet cells *in vivo*, affecting the understanding of islet cell biology in diabetes [149]. Many studies have shown promising data using PIs as a model for studying cell-to-cell interactions in response to appropriate nutrients [149]. Luther *et al*. (2006) reported that Min6-PIs displayed high insulin secretion, not only in response to glucose, but also to non-nutrients such as carbachol, tolbutamide, PMA and forskolin [150], which means insulin secretion of islet-like structures is highly regulated by both; intracellular signals generated during metabolism of glucose, and by the neurohormonal agonists that act through cell surface receptors [151]. Their findings

suggested that correct anatomical structure has the potential to influence insulin secretory function [150].

However, when PIs are generated under 2D culture conditions, poor viability, uncontrolled cell size, and growth arrest were exhibited during the first week of incubation [152, 153]. Insufficient diffusion of essential metabolites and oxygen through the cells inside PI clusters potentially contributed to apoptosis. Thus, to overcome these limitations, 3D cell culture methods, such as bioreactors (i.e., spinner flasks and rotational cell culture system (RCCS)), have been used instead of conventional static culture methods. The environment inside these systems is homogenous due to the continuous diffusion of nutrients, metabolites and oxygen [153].

Lock *et al.* (2011) reported that the generation of Min6-PIs under simulated microgravity culture conditions leads to high viability, superior cell survival, and maintenance of controlled growth, unlike PIs generated from static culture conditions [153]. Thus, this scalable culture system has the potential to generate aggregates with superior survival and high insulin secretory function. A further study has reported that culturing islets under RCCS reduces immunogenicity and preserves the functionality of cells [154]. Additionally, polymer scaffolds were used by several studies and shown to provide a highly significant improvement in cell culture methods [112].

It was further shown that embedding PIs within multiple layers of nanofil encapsulation did not damage the cellular structure of clusters, and has the potential to enhance insulin secretory function [155]. In a recent study, Song *et al.* (2013) combined polymer scaffold with simulated microgravity culture conditions to investigate insulin secretion and islet viability. The researchers cultured islets under three different culture conditions; static, embedded with polymer scaffold, and embedded with polymer combined with a simulated microgravity culture method [156]. They reported that culturing islets under 3D culture conditions combined with polymers had the potential

to increase cell viability, enhance functionality, and reduce immunogenicity, thus prolonging the life of the graft.

Therefore, a proper cell culture condition has a great influence on cellular behaviour and function. In the following section, a more detailed of cell culture conditions will be discussed.

1.3.2.5. *Cell culture conditions*

Traditional cell culture methods have been extensively used in biological studies for some time. The technique involves equipment such as petri dishes, tissue flasks, and cell culture well-plates which are used to generate 2D cultures of adherent cells known as monolayers. The principle of conventional 2D cell culture methods is the culture of cells at a specific density below a layer of culture medium [157]. Cell biology and biochemistry has conventionally relied on culturing cells on 2D surfaces, although this has been cited as a potential limitation in some research where these 2D surfaces were inadequate [30]. In traditional cell culture, cells are thus removed from their native environment which in turn influences morphological structure and cell behaviour in terms of gene expression, protein translation, cell-to-cell/ matrix interactions, and other important cellular signalling processes [157]. In particular, the suitability of conventional 2D culture methods may require re-evaluation in the light of more recent developments. Other parameters such as morphology, long-term viability and functionality must be achieved to prolong the life of islet engraft.

There are a number of additional factors which can be added to the media and have been proposed to offer protection to the islets, thereby improving viability and prolonging graft survival once transplanted [27]. Growth factors, such as growth hormone (GH) and prolactin (PRL), may have the potential to enhance β-cell mass for example [158]. Surprisingly, little attention has been given to the potential role of growth hormone-releasing hormone (GHRH) or its agonists in β-cell survival since its characterisation

in 1982 [159]. GHRH has been found to potentially stimulate the release of GH from the pituitary gland. A recent study reported a significant increase in β-cell proliferation and survival following the addition of a synthetic GHRH agonist to insulinoma INS-1 cells. Furthermore, a factor called JI-36 has been used to promote β-cell survival prior to transplantation into diabetic animal models [122].

Although these factors promote the survival of β-cells and islet proliferation *in vitro*, suggesting a therapeutic promise for diabetes, some challenges remain such as the generation of a suitable microenvironment and conditions that mimic that native environment of primary islet cells. Thus, culturing islet cells under 3D culture conditions, where cells tend to be cultured in a free-floating manner, could have the potential to influence cellular behaviour and prolong the life of islet engraftments. The idea that pancreatic islets could be cultured in a free-floating form was derived from studies conducted in the 1970s [104]. A challenge associated with culturing pancreatic islet cells, however, is that they have a tendency to clump together, with the overall result of this that cells located in the core of the clump die as they are unable to receive sufficient nutrition [28]. As this is a natural progression of cell proliferation, it suggests that this process may be an inevitable consequence of allowing cells to be cultured under 3D culture conditions rather than as a static culture. Whether or not this is improved by microgravity conditions, and that reduction in clumping is the reason behind improved functionality, remains to be seen.

In recent years, there have been further developments in the design of 3D matrices (which were previously unavailable), resulting in a decreased focus on understanding the most natural and favourable aggregation patterns displayed by islets. These patterns may hold various benefits, however, for 3D culturing of islet cells over conventional methods, given that this more closely resembles the patterns exhibited by cells growing *in vivo*. The main benefit associated with 3D matrices is that they more closely resemble

the natural ECM in which cells grow *in vivo* [30, 160]. The 3D freedom which cells in these matrices have also promotes cell-cell and cell-matrix interactions.

Widely used ECM-derived materials have been demonstrated to control proliferation [161], survival [162], and insulin secretion [163] in purified β-cell cultures. Interestingly, islets which were incompletely isolated and retained some of their original ECM were demonstrated to have a strikingly lower rate of apoptosis and showed considerably greater functionality in terms of insulin response *in vitro* compared with purified, fully isolated islet cells [164]. London *et al.* (1998) further enumerated several limitations of the traditional islet cell culture, including the size of islet cells, their non-prolific characteristics, and the variations of islets from species to species [165].

Simulated Microgravity 3D-Cell Culture

Based on the discussion presented previously, the use of 3D-ECM culture techniques for pancreatic islets was selected for further investigation. This approach was postulated to offer the greatest potential for improvement of islet transplantation outcomes of the different approaches to culturing and transplantation reviewed [166]. This section presents further analysis of the literature surrounding the application of this technique, including the use of a bioreactor to create microgravity conditions. The characteristics of simulated microgravity culture conditions that modulate numerous biological processes are hydrostatic conditions in a fluid-filled compartment, the sedimentation status of tissues/cells, and the continuous diffusion of oxygen and metabolites under low-shear forces. These parameters have a significant influence, either directly or indirectly, on essential cellular communication and signalling [167]. Different types of bioreactor, designed by the United States National Aeronautics and Space Administration (NASA), have been widely used by researchers; these include the

continuous stirred tank, the perfusion system, and the rotating-cell culture system (RCCS) **[Figure 1-12]** [168].

Stirred-tank reactors, or spinner flasks, are characterised by a continuous steady state of flow of the culture medium, due to a magnetic stirrer, and by other products such as scaffolds. In spinner flasks, cell are either cultured as suspensions or embedded with scaffold [169]. Some disadvantages of this reactor include poor mass transfer with uncontrolled shear stress, which might generate poor quality tissue [170].

This was confirmed by a study showing that high shear stress applied to bovine calf chondrocytes cells seeded into a stirred culture system led to an alteration in the synthesis of matrix elements [171]. Thus, uncontrolled shear forces in stirred tank bioreactors have limited the success of this method.

Therefore, the supply of continuous nutrients provides optimal growing conditions, and the removal of metabolic waste reduces toxicity. This system has been widely used in cultivating bone tissue [172]. The third type of bioreactor, RCCS, is also known as a rotational cell culture vessel (RCCV).

This apparatus is capable of rotating a number of autoclavable or disposable vessels, depending on the design of the system. The high aspect ratio vessel (HARV) is equipped with a membrane for gas exchange to optimise gas and oxygen supply [173]. The RCCS is characterised by adjustable rotational speeds which may reduce the hydrodynamic damage induced by shear stress [173]. The difference between RCCS and stirred-suspension applications is that cells which aggregated into 3D clusters under stirred-suspension systems are larger in size than cells growing under RCCS [173]. This is because the shear force under a stirred-suspension system is uncontrolled and can lead to the development of a necrotic core. RCCS differs from the unique culture conditions by allowing sufficient nutrient and oxygen supply which appear to be the most important factors to enhance tissue growth, survival, and functionality.

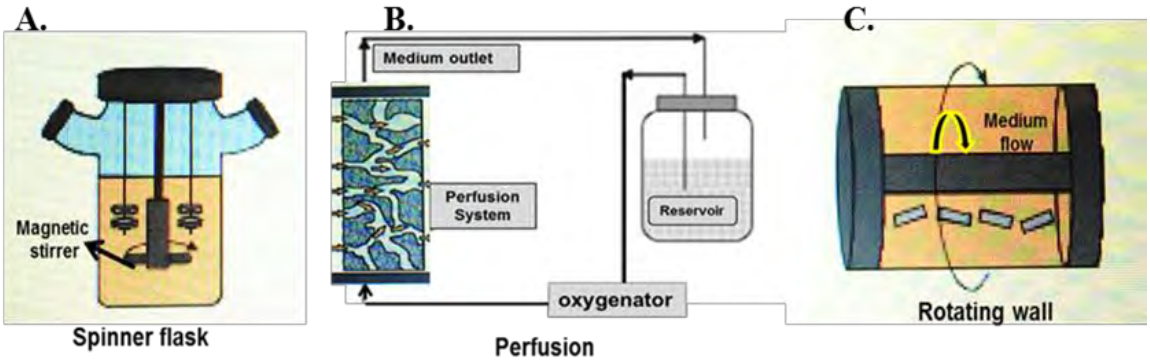


Figure 1-12: Types of bioreactor.

Three common types of bioreactor: spinner flask (A), perfusion system (B), and rotating cell culture system (RCCS) (C). Modified from [168].

Therefore, these characteristics obtain a valuable model that can benefit research and the clinical field [168].

Briefly, the RCCS is a rotating clinostat that horizontally rotates a fluid-filled (with culture medium) vessel, equipped with a gas-exchange membrane to control the oxygen supply [167]. Under these conditions, cells are in a state of simulated free-fall which presents a sufficient nutrient supply for islet cells *in vitro* and lengthens survival time [174].

Furthermore, the microenvironment generated inside the vessel includes free-air bubbles where fluid dynamics generate a laminar flow state, maintaining low shear-force conditions and reducing the risk of cell damage [167]. Moreover, the rotational speed of the vessel can be modified to the required conditions needed for aggregates (3D cell cultures) where rotation can be on the same axis. In addition, the speed can be adjusted over the duration needed for cell aggregates to grow in order to compensate for sedimentation rates [175].

In a further study, Samuelson and Gerber (2012) demonstrated that a microgravity 3D culture system improved the development and function of pancreatic progenitor cells. Specifically, the cells exhibited healthy proliferation as well as enhanced transcriptional signalling [176], and also demonstrated improved transcription of pancreatic genes.

The results of a study by Song *et al.* (2004) indicated that culturing islets under these conditions was associated with improved cell survival and improved secretory functions compared with cells grown in a flask under normal conditions [174].

The equipment selected for use in this study allows these conditions to be generated; this approach was therefore expected to further improve upon results obtained through the use of ECM culturing alone.

The main limitation of culturing islet cells under simulated microgravity conditions is the risk of developing anoxic necrosis when human islets are cultured at higher densities. However, Murray *et al.* (2005) reported the opposite, in that culturing human islet cells under these condition at high densities of between 500–1500 islets/mL caused cells to exhibit less necrosis, preserved structural integrity, and increased insulin secretory function over 10 days of culture incubation [177].

Further improvements and experimentation were evaluated by incorporating the use of polymer scaffold for islet culture to further enhance culture conditions *in vitro*. For some time, an interest in transplantation of cells using artificial polymers as a matrix scaffold has existed. The theory behind this method is that placing cells into a matrix structure provides additional protection to that which would be afforded to individual cells transplanted directly into the tissue or vascular system. Often, these matrices are designed to be biodegradable, so that they remain in the body only as long as is necessary to provide the required level of protection to transplanted cells [178]. Matrix-integrin interactions have been shown by researchers to play a key role in survival and functioning of human β-cells both *in vitro* and *in vivo* [46, 77]. This indicates that placing isolated cells within a 3D ECM may not only be beneficial, but may in fact be a necessary step in ensuring the long-term survival of cells, both during the culture stage and initial implantations. This may be particularly true while cells remain in culture where they cannot graft into ECM independently.

However, this may also indicate failure to adequately graft cells into ECM within the islets which may be associated with the deterioration in cell survival and function observed after transplant. This observation is further attributed to oxidative stress, according to Nagatani *et al*. (2011), who found that administering edaravone to mop up free radicals was effective at improving rates of engraftment and subsequent insulin

control in rats. However, ECM has been suggested to enhance attachment and proliferation of cells [179].

This observation further supports the importance of ECM in enabling islet cells to engraft within the recipient. Evidence presented by Hammar *et al*. (2004) suggests that the main overriding mechanism by which ECM supports β-cells is by switching on regulatory pathways which inhibit apoptosis, thereby promoting survival of the cells in the absence of direct damage [180]. There also exists empirical evidence that the use of ECM may be associated with improved survival of pancreatic islet cells when compared with other methods. Nagata *et al*. (2002) investigated the survival of rat islet cells in different types of 3D cell cultures, including collagens type I, III, IV and LM, and found that all were associated with suppressed cell death and continued functioning of cells [181]. A further study by Beattie *et al.* (2002) specifically compared the use of this culture technique to conventional 2D techniques and demonstrated that the use of a 3D matrix was associated with improved proliferation and morphology, indicating better quality cells where insulin production was improved three-fold [182]. Although this study examined cells from a mouse model, the findings appear to indicate potential for use with human cells. Further studies by Webb *et al*. (2007) also demonstrated that cells grown in 2-D culture lost hormone expression upon differentiation, and that this functionality was not re-established. In comparison, the use of a bioreactor was shown to lead to the re-establishment of hormone expression, although this finding was based on a very small sample size [183]. Furthermore, some studies have been reported which examined cell culture in 3D matrices under microgravity conditions in a bioreactor.

Song *et al*. (2004) cultured rat islet cells and found that, while no difference between flask-cultured and bioreactor cultured cells was observed after 3 days, there was a significant difference in survival rates by day 7 ($p < 0.01$). Only 72% of islet cells survived to day 7 when flask-cultured, and this was reduced to 59% by day 14 [174].

In contrast, 90% of islet cells cultured in the bioreactor survived to day 7, with an 80% survival rate at day 14. These findings were based on samples of approximately 4,400 cells initially, taken from eight separate experiments [174].

Song *et al.* (2004) also showed that bioreactor cultured cells maintained a higher level of insulin production than flask-cultured cells, up to 30 days after isolation from pancreatic tissue. By the 30th day, flask-cultured cells had reduced their insulin production from 67.48 ± 0.27 μM/L to less than 5 μM/L. The cells cultured in the bioreactor had also drastically reduced their insulin production over this time, from 72.35 ± 0.30 μM/L to 12.59 ± 0.45 μM/L, but were still observed to be producing more insulin than the flask-cultured cells [174]. These results have been supported by other studies; Hou *et al*. (2009), for example, showed that insulin production and blood glucose control were superior in islets from a rat model cultured in a 3D matrix under microgravity conditions in the bioreactor [184]. The rat islets were found to be normal in morphology and in their ECM structure which may indicate that they have the potential to remain in a functional state in the long-term. Furthermore, a microgravity environment affects ECM protein and gene expression in islet cells. In a 3D environment, cells connect to each other and form their natural cell-to-cell attachments which allow them to exert forces on one another to move/migrate as *in vivo* [176].

In other words, 3D cell culture replicates the intricate structures and morphology of *in vivo* conditions, resulting in better cell differentiation, polarisation, cell behaviour, and cell-to-cell interaction. In a study by Webb and colleagues (2007), the creation of cell aggregates of islets was reported from persistent hyperinsulinemic hypoglycaemia of infancy (PHHI) patients in the microgravity environment of a HARV, resulting in reactivation of insulin, glucagon, somatostatin, and GAD expression that was previously hindered [183]. The unique environment of the HARV promoted the up-regulation of endocrine expression in the islet-derived cells.

In this study, Min6-PIs, considered a useful experimental model, were cultured directly under a simulated microgravity environment (in a bioreactor) after being generated from static conditions. The purpose behind this approach was to improve the characteristics of PIs as a model in order to investigate the effect of enzymatic digestion and recovery on PIs and determine whether they are robust enough to preserve their structural integrity, viability, ECM expression, and insulin secretory function.

1.4. **Aims of This Study**

The overall aim of this study was to improve insulin-producing cell function post-digestion, *in vitro*, using a Min6-Pseudoislet (PIs) model in combination with 3D-microgravity cell culture conditions.

1. Investigate the effects of cell culture in static and microgravity conditions on morphological structure, viability, and ECM expression of PIs.
2. Investigate enzymatic digestion with aacutase enzyme on morphological appearance, viability, and ECM expression of PIs.
3. Investigate the recovery effects of culture in static and microgravity conditions post-digestion on PIs remodelling, viability and ECM expression.
4. Investigate the insulin secretory functions of PIs post-digestion and remodelling.

Chapter 2. **MATERIALS & METHODS**

2.1. **Materials**

The materials used for this project were obtained from the following suppliers:

Tissue culture media: Dulbecco Modified Eagle Medium (DMEM) and foetal bovine serum (FBS) from PAA, UK; sterile tissue culture flasks, 75cm², 250 mL, red filter cap from Greiner Bio-One; general laboratory plastic ware from Fisher Scientific, UK.

Chemicals (Hoechst, propidium iodide, Mayer's haematoxylin, and Eosin Y) from Sigma-Aldrich, UK.

RCCS-4D Bioreactor-rotary cell culture system (manufactured by NASA, US) and disposable sterile culture vessels were purchased from Cellon, UK.

cDNA synthesis kit from Invitrogen, UK; SYBR green qPCR mix and protein assay reagents were purchased from Bio-Rad laboratories, UK; PCR primers from Integrated DNA Technologies, UK; Western blotting materials and nitrocellulose membranes purchased from Amersham Biosciences UK..

Cryotome cryostat microsystem-LEICA CM 1900 (manufactured in UK); cryo-embedding matrix x 125 mL media, from LAMB, UK; Superfrost slides, from ThermoScientific, UK; bovine serum albumin (BSA), Fisher scientific, UK; Unconjugated primary antibodies (Fibronectin, Collagen IV, and LM V), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from AbCam, UK; for fluorescence ICC, FITC-conjugated anti-rabbit secondary antibody , from DAKO, Denmark; Vectashield DAPI, from Vectashield, UK; Goat serum (G9023-10 mL), from Sigma-Aldrich, UK; ImmEdge hydrophobic barrier pen (H-4000), from Vector Laboratories, UK, Phalloidin, goat serum, from Sigma, UK.

2.2. Methods

2.2.1. Cell Culture

Cell Growth

Cell culture was performed by initially culturing Min6 β-cells, a mouse pancreatic β-cell line [133], into 75 cm^2 tissue culture flasks. Min6 β-cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and used between passage numbers 31 and 34. Cells were grown in 15 mL of DMEM media (containing 5 mmol/L glucose) per flask, and supplemented with 10% FBS. All cell culture experiments were performed under sterile conditions in a laminar flow hood.

Cell Passage

Throughout culture, cells were examined under an inverted microscope and sub-cultured when 70–80% confluence was reached. First, cells were washed with phosphate buffered saline (PBS) and incubated with 1 mL trypsin for 5 minutes at 37ºC. The cells were checked under the microscope to monitor detachment, and then gently tapped to ensure complete detachment of remaining cells. Next, 9 mL of fresh media was added to halt further trypsin digestion of cells. The detached cells were centrifuged at 300 g for 5 minutes; supernatant was removed and cells were re-suspended in 1 mL of media. Cells were counted using a haemocytometer slide **[Figure 2-1]** and the following formula:

(Average cell count per highlighted square/4)X10^4 = cells / mL.

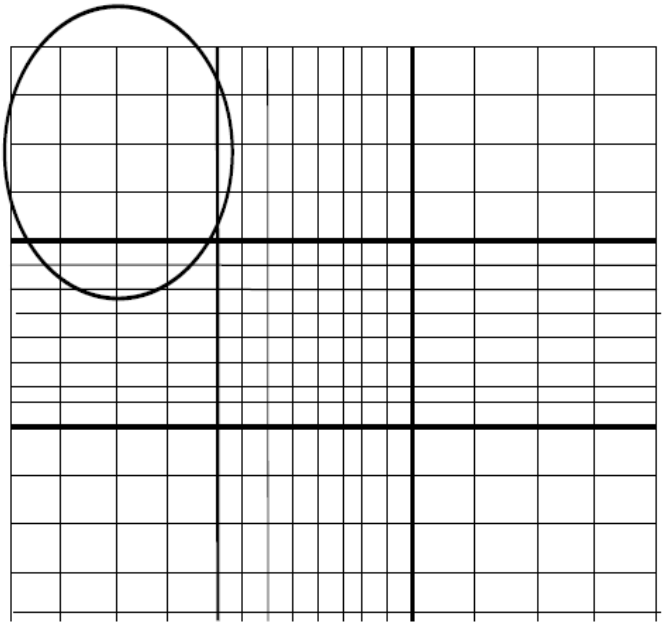


Figure 2-1: Cell counting using a haemocytometer slide.

This apparatus consists of a microscopic chamber slide with a small square (3 mm × 3 mm) on the surface. Cell suspensions are pipetted through a corner of the slide and a coverslip, which rests at 0.1 mm above the slide, is placed on the slide. Number of cells is determined using the formula (Average cell count per highlighted square/4) X104 = cells / ml.

2.2.2. **Formation of Min6-Pseudoislet**

In order to transform Min6 β-cells into Pseudoislet (PIs), cells were seeded at 40,000 cells/ml into non-adherent dishes. After 48 hours of incubation, dishes were examined by inverted microscope for aggregated clusters. These aggregations appeared in the form of 3D islet-like clusters known as PIs **[Figure 2-2].** Further incubation for 6–8 days resulted in the formation of well-characterised PIs which were selected for experimental use by using a dissecting microscope.

2.2.3. **3D Microgravity Cell Culture.**

For microgravity studies, a Rotary Cell Culture System (RCCS-4D) was used **[Figure 2-3].** Cells were cultured in a rotating bioreactor. A re-circulating loop designed to maintain composition within the target range of culture conditions required for living cells, namely, a combination of gas exchange and continuous supplement of all essential metabolites required. The cells were allowed to float at low shear forces to maintain close cell-to-cell interactions. Experiments were performed by selecting PIs (80–100 clusters), which were previously generated in static dishes at day 4 of formation, using a dissecting microscope to transfer cells by pipette into 10 mL HARVs through the filling port **[Figure 2-3, panel B]** for re-culturing at 8 rpm.

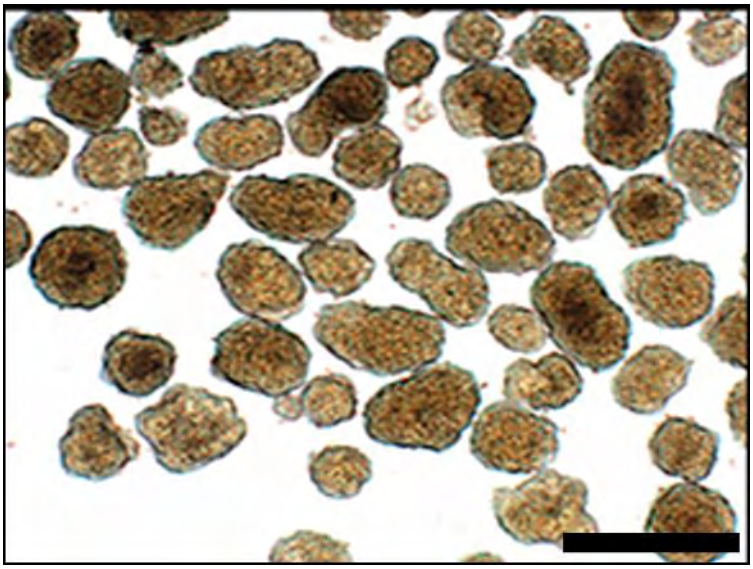


Figure 2-2: Pseudoislet (PIs) formation in static dishes.

Min6 β-cells were seeded at a density of 40,000 cells/ml into non-adherent (static) dishes in order to generate PIs. After 48 hours of incubation, cells started to aggregate, giving rise to 3D islet-like clusters. Light microscopy was performed to analyse morphology and image was taken at x5 magnification. Scale bar: 400µm.

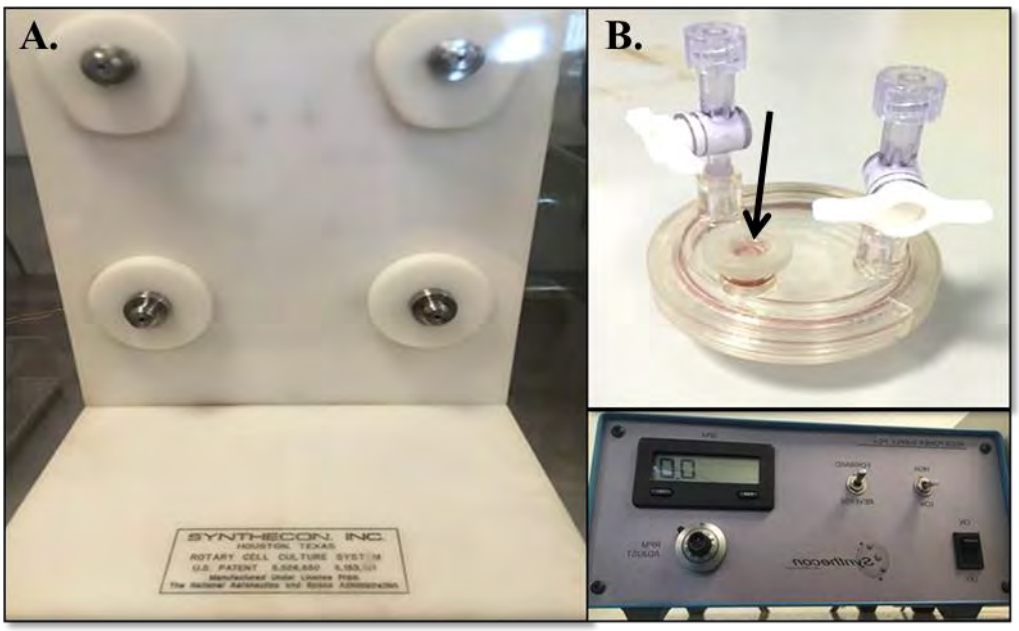


Figure 2-3: Rotary Cell Culture System (RCCS)

The RCCS is placed inside a cell culture incubator at 37℃ and 5% CO_2 which is capable of rotating four vessels (RCCS-4D) (Panel A) at the same speed; the rotation speed was adjusted to 8 rpm. PIs were transferred into 10 mL of high aspect ratio vessel (HARV) vessels through the filling port (Panel B, see arrow).

2.2.4. **Optimisation of enzymatic digestion.**

In order to optimise the digestion experiments, PI clusters were digested using a collagenase digestive enzyme (accutase) in a separate reaction for each cluster. Ten PI clusters were selected from the RCCS and incubated with 200 μL accutase at 37˚C for 0, 30 seconds, 2 minutes, and 5 minutes. Digested clusters were selected and placed directly onto a microscope slide, and a cover slip was gently lowered over the cluster. The cluster was examined at x40 magnification and photographed using a light microscope equipped with a digital camera (Canon). Two minutes of digestion was determined as the optimal time for digestion in this study because of the reduced effect on cell membranes of clusters. Experiments were repeated in triplicate.

2.2.5. **Recovery process (remodelling) of Min6-Pseudoislets.**

Twenty PI clusters were incubated in 200 μL accutase for 2 minutes, transferred into 6 mL fresh media in a non-adherent dish, and incubated for 24 or 48 hours. After 24 hours, 10 clusters were examined and photographed under light microscopy at x40 magnification. The remaining clusters were incubated further for 48 hours in total. After incubation, some clusters were examined and photographed as before. Additionally, 20 PIs, digested for 2 minutes, were transferred in 10 mL of fresh media into a rotary cell culture vessel (RCCS-bioreactor) and also incubated for 24 or 48 hours. The recovery experiment was repeated in triplicate.

2.2.6. **Morphological analysis**

2.2.6.1. ***Histological Analysis***

Preparation of cell block

PI clusters (50–70 in total) were selected from the static dishes and from the RCCS-bioreactor using a dissecting microscope. Clusters were transferred to 1.5 mL Eppendorf tubes and washed 3 times with PBS. Clusters were fixed with 3.7% formalin in a 200 µL volume and incubated at room temperature for 20–30 minutes. Formalin was gently aspirated to avoid disturbing the clusters, and clusters were washed three times with PBS.

Embedding

200 µL of a pre-prepared 1% agarose solution was added gently to the clusters. A small wire loop was inserted into the pre-prepared agarose gel to facilitate the removal of the block following solidification. The gel was allowed to set for 15–30 minutes at room temperature **[Figure 2-4].** A frozen embedding medium known as optimum cutting temperature (OCT) was poured onto the cell block, which was then placed into liquid nitrogen for complete freezing and stored overnight at −80°C for later sectioning.

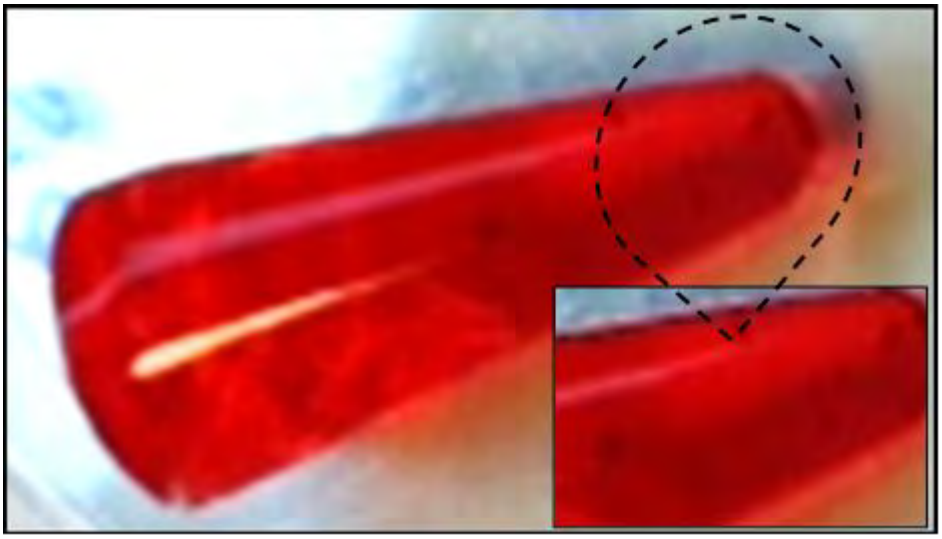


Figure 2-4: Agarose gel embedding medium.

The block represents a tissue block containing the PIs (dotted circle) inside the gel in order to visualise clusters and facilitate sectioning.

Cryostat Sectioning.

The frozen cell block was placed inside a cryostat chamber at temperatures between −20 and −22 °C **[Figure 2-5].** Blocks were sectioned to 5–10 μM thicknesses and cluster sections were placed onto Superfrost slides. The slides were dried at room temperature for 20–30 minutes followed by a rehydration step with serial washing in ethanol (100, 90, 80, 75, and 70% for 20 seconds each).

2.2.6.2. *Haematoxylin and eosin staining (H&E).*

In order to define the morphology of PIs, H&E staining was performed. H&E is a common histological staining protocol comprising a combination of acid and base dyes. Haematoxylin (H) acts as a basic dye whereas eosin (E) acts as an acidic dye [185]. A chemical reaction occurs in the nucleus where haematoxylin binds to the acidic nucleic acid resulting in blue staining. The eosin dye, however, binds to basic components in the cytoplasm of the cell, staining them pale pink. Rehydrated PIs sections on Superfrost slides were stained with haematoxylin for 10 seconds followed by gentle rinsing under tap water 1 minute. Sections were covered with 1% acetic acid for 17 seconds followed by rinsing in tap water for 20 seconds. 1% eosin was added to the sections and incubated for 10 seconds; excess eosin was removed by rinsing in water for 3 minutes. Slides were dehydrated through four changes of ethanol (70%, 75%, 80%, 90%, and 100% for 20 seconds each). Cluster sections were cleared with Histo-clear solution for 20 seconds. The slides were dried at room temperature, mounted with vector-permanent mounting medium, and covered with a cover slip (22 × 50 mm) which was placed gently on the slide and sealed with colourless nail varnish. Microscopic examination was performed by light microscopy and images were captured at x40 magnification.

Figure 2-5: Cryosectioning of Pseudoislet blocks

PI blocks (coloured pink colour within the OCT medium) were sectioned into 5–10 μM thicknesses and placed on Superfrost slides for analysis.

2.2.6.3. *Phalloidin staining (Actin staining)*

Cluster sections on treated slides were circumscribed with an Immedge pen and allowed to air dry before incubation with aqueous solution. Cluster sections were incubated with 30 µL of diluted phalloidin in PBS (0.1 µg/mL in DMSO) for 1 hour at room temperature under dark, humidified conditions. Slides were then washed three times in washing buffer (0.4% Tween 20, 0.7% glycerol in PBS) for 5 minutes before being mounted with cover slips using Vectashield mounting medium 4', 6-diamidino-2-phenylindole (DAPI). It is a fluorescent dye which binds to A-T site in DNA. The excitation/emission wavelengths are at 358nm/461nm [186]. Excess Vectashield was removed and the edges of coverslips were sealed with colourless nail varnish. Analysis was performed by confocal microscopy (Leica TCS SP5) and images were taken at x40 and x100 The DAPI setting was used for nuclear staining and TRITC settings were used for phalloidin staining (actin staining). Sequential scans were performed between the two frames in order to avoid false signals.

2.2.6.4. *Scanning electron microscopy (SEM)*

SEM was performed on PIs that had been cultured under different culture conditions (see section 2.2.1). Clusters were fixed with 2.5% of glutaraldehyde (0.1 M sodium cacodylate buffer) for 30 minutes inside a fume hood. The fixative solution was then discarded followed by washing of clusters with 0.1 M sodium cacodylate buffer (pH 7.4) three times, for 5 minutes each time. A dehydration step was performed by 60 second immersions into a graded ethanol series (5%, 10%, 20%, 50%, 70%, 80%, 90% and 100% v/v).

Clusters were frozen at −80°C and freeze dried by vacuum 0.42 mbar (Christ® alpha labs) overnight. Samples were examined using a Zeiss sigma field emission gun (Zeiss NTS) at an accelerating voltage (EHT) of 3 kV. Images were captured at x1.00K and x5.00K.

2.2.7. **Cell Viability**

Cell viability of Min6-PIs was determined as follows: Min6 β-cells were seeded at a density of 4×10^4 cells/static dish for 7 days in order to generate PI clusters. The cell density per dish was approximately equal to 80–100 clusters. As described previously, 80 clusters were transferred to HARV in RCCS at day 4 of cluster formation for further incubation. Cell viability was determined following a number of different culture conditions. Initially, viability of cells grown in static and microgravity was measured; cells were cultured as described in section 2.2.1 and the viability was determined at 7 days. Additionally, viability of digested PIs (section 2.2.2) and recovered PIs (section 2.2.3) was evaluated. Cell viability was determined using the following methods: MTT assay, and Hoechst Propidium iodide (HPI) staining.

2.2.7.1. ***MTT assay***

MTT is a colorimetric assay in which NAD(P)H-dependent cellular oxidoreductase enzyme, produced by mitochondria, correlates to the viability of cells by converting tetrazolium enzyme into purple insoluble formazan, indicating that cells are viable. The insoluble crystals dissolve in DMSO and absorbance (O.D) of samples was measured at 540 nm on a plate reader (Multiscan, Thermoscientific®. MTT assays were performed following harvesting PIs cultured according to the given experimental conditions (section 2.2.5) and selected using a dissecting microscope.

The method was carried out as follows: Clusters were washed with PBS 3 × 5 minutes each, and transferred into 12-well plates.

Prepared MTT solution (0.5 mg/mL) was added to each well and incubated for 1 hour. MTT solution then was aspirated carefully and 200µl of DMSO was added to each well to dissolve the formazan with incubation for 5 minutes on an orbital shaker. Aliquots of dissolved formazan solution were transferred to a 96-well plate and analysed at 540 nm on a plate reader. A blank sample was included, which was DMSO. Samples were measured in triplicate and average absorbance was subtracted from the blank value. Results were interpreted as absorbance relative to control (PIs cultured in static dishes), with higher absorbance indicative of more mitochondrial activity, while lower absorbance indicated a decrease in mitochondrial activity.

2.2.7.2. *Hoechst and Propidium Iodide (HPI) staining*

HPI staining is a rapid technique for examining cell viability using fluorescence imaging with colorimetric differentiation of viable, apoptotic, and necrotic cells. HPI stain was prepared by adding the following chemicals to a 1.5mL Eppendorf tube: 50 µL propidium iodide (1 mg/mL) and 50 µL Hoechest dye (5mg/mL), which is sensitive to light; 900 µL fresh media was added to the mixture. The experiment was performed following the selection of PIs using a dissecting microscope. Clusters were harvested as according to the given experimental conditions (section 2.2.5). PIs were transferred into 6-well plates and the culture medium was removed by aspiration. 200 µL of prepared HPI staining mixture was added to each well and incubated for 5 minutes. Excess staining was removed carefully and cells were immediately analysed by confocal microscopy under x40 magnification.

As Hoechst and propidium iodide are nuclear stains, the filters used were DAPI/Hoechst filter, with excitation by 405 diode laser emitting blue colour, and propidium iodide (PI) filter, at maximum excitation was 535 nm and maximum emission of 617 nm; the laser line emitted was primarily red.

2.2.8. **Quantitative analysis**

2.2.8.1. ***Quantitative Reverse Transcription Polymerase-Chain Reaction (qRT-PCR).***

Reverse-Transcription polymerase-chain reaction (RT-PCR) is used for the quantification of gene expression amplified by a PCR process. It is a reliable and sensitive technique for messenger RNA (mRNA) detection and quantification. The technique involves several steps in order to generate PCR products. In some experiments, cells were disrupted using digestion enzyme and allowed to recover at specific time (see sections 2.2.4 and 2.2.5). So, gene expression was expressed as mRNA content because the current paradigm of gene expression suggests that the transcription process takes hours.

mRNA extraction

mRNA was extracted from cells using the GeneElute^{TM} Mammalian total RNA Miniprep kit from Sigma according to the manufacturer's protocol. Briefly, mRNA was extracted from cells seeded and cultured as according to the stated experimental conditions. Pelleted cells, Min6 β-cells as a monolayer and as PIs from dishes and RCCS, were collected, re-suspended in lysis solution and filtered to an approximate volume of 50 µL. Extracted samples were frozen at −20ºC until use.

DNase treatment

To avoid genomic contamination, mRNA samples were treated with DNase. A solution of 1 µL of DNase enzyme, 1 µL of DNase buffer, and 8µL of RNA extract was prepared. This mixture was incubated at 37ºC for 30 minutes and the reaction was terminated by adding 1 µL of stop buffer and incubating at 65ºC for 10 minutes.

RNA quantification (Nanodrop)

Nanodrop 2000 from ThermoScientific® was used to quantify mRNA in samples at 260 nm. This technique was also used to determine the purity of mRNA samples by using the 260/280 ratio. Ratios of 1.9–2.1 were considered optimal, so samples outside of this range were excluded.

Reverse transcription (RT)

RT is a key method for the analysis of mRNA and is used to convert single stranded RNA into double stranded cDNA which can then be amplified by PCR. The cDNA synthesis reaction solution was prepared as listed in **Table 1**. The mixture was denatured by incubating at 65ºC for 5 minutes. A master mix was prepared for each reaction tube **[Table 2].** Cloned Avian Myeloblastosis Virus (AMV), which is a RNA-directed DNA polymerase, is used to synthesise complementary DNA (cDNA) and was omitted from the negative samples. The mixture solution used for negative samples was 7 µL of master mix and 1 µL of DEPC water per tube. Positive sample tubes contained 1 µL of cloned AMV. All tubes were incubated at 55˚C for 45 minutes followed by 85˚C for 5 minutes.

Table 1: Components for cDNA synthesis

Component	1 Reaction tube
(50 mM) Oligo-dT	1 µL
(5 µg) RNA sample	2 µL
(10 mM) dNTP mix	2 µL
DEPC-treated water	4 µL

Table 2: Preparation of Master Mix

Component	1 Reaction tube
5× cDNA synthesis buffer	4 µL
(0.1 M) DTT	1 µL
(40 U/µL) RNase out	1 µL
DEPC-treated water	1 µL
Cloned AMV RT	1 µL

qRT-PCR

Amplification was performed on reverse transcribed RNA samples and determined using Rotor-gene (Qiagene) software. SYBR green fluorescence dye, which emits bright green fluorescence when it binds to double strand DNA (ds DNA) **[Figure 2-6],** was used to quantify the end products of PCR reactions. The following components were added to the PCR reaction tube: cDNA from the RT experiments, DNA polymerase, specific primers, and SYBR green dye **[Table 3]**; each sample was run in triplicate.

Table 3: Preparation of Master Mix

Component	Volume per 1 reaction tube
SYBR Green	12.5 µL
Forward primer	1 µL
Reverse primer	1 µL
DEPC-treated water	8.5 µL

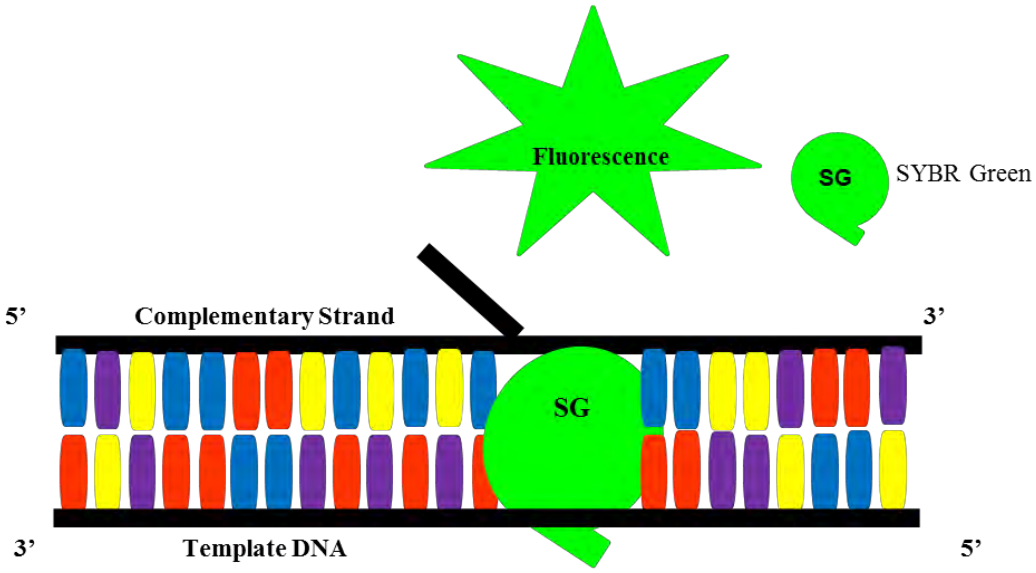


Figure 2-6: SYBR green reaction.

SYBR green fluorescence dye binds only to double strand (ds) DNA during the amplification process and emits a bright green light.

qRT-PCR calculation

Calculation of relative gene expression was determined using the Livak calculation method ($-2^{\Delta\Delta Ct}$ calculation), assuming that equal efficiencies of 100% (+/- 5%) were achieved [187]. Relative calculation of gene expression was performed as opposed to absolute gene expression, as an unknown quantity of gene of interest with a housekeeping gene to provide a standard curve.

From this, the efficiency was determined and a ratio of gene expression was performed accordingly using the method below:

- ΔCt control: gene of interest (GOI) - house-keeping gene (GAPDH).
- ΔCt Test: Ct (GOI) - Ct (Ref.G).
- $-2^{\Delta\Delta C}$t: 2 - (ΔCt (control) - ΔCt (Test)).

qRT-PCR conditions were as follows

- **ECM primers**
  - *Fibronectin F (mouse forward): 5'ATCACAGTAGTTGCGGCAGGA 3'*
  - *Fibronectin R (mouse reverse): 5' TGTCATAGTCAATGCCAGGCT 3'*
  - *Collagen IV F (mouse forward): 5'GCCAACGCACTTCCTGGAAT3'*
  - *Collagen IV R (mouse reverse): 5'CGGTGTTGCCCATGAATCCTT3'*
  - *Laminin V F (mouse forward): 5'TTCATGCTGGACACGGCAGTA3'*
  - *Laminin V R (mouse reverse): 5' CTTCTCAAAGCTGCCGGC 3'*

Temperature	Time	Cycles
95ºC	5 minutes	1
95ºC	30 seconds	
60ºC	30 seconds	40
72ºC	30 seconds	
72ºC	5 minutes	1

- **GAPDH primers**

  GAPDH F (mouse forward): 5' AAGGGCTCATGACCACAGTCCAT 3'

  GAPDH R (mouse reverse): 5' TGTCAGATCCACGACGGACACATT 3'

Temperature	Time	Cycles
95ºC	5 minutes	1
95ºC	30 seconds	40
60ºC	30 seconds	
72ºC	30 seconds	
72ºC	5 minutes	1

- **Insulin primers**

  Insulin F (mouse forward): 5'TCCGCTACAATCAAAAACCAT3'

  Insulin R (mouse reverse): 5'GCTGGGTAGTGGTGGGTCTA3'

Temperature	Time	Cycles
95ºC	5 minutes	1
95ºC	15 seconds	40
60ºC	15 seconds	
72ºC	20 seconds	
72ºC	5 minutes	1

2.2.9. **Protein extraction and quantification**

Protein expression of Min6 β-cells were analysed using western blot technique. The expression was determined by measuring protein content of cells because in some experiments cells were disrupted using digestion enzyme and allowed to recover at specific time (see sections 2.2.4 and 2.2.5). The current paradigm of protein expression suggests that the transcription and translation processes take hours. Therefore, protein expression is expressed as protein content of the cells.

2.2.9.1. *Whole Cell Extracts*

Protein was extracted from three sources of Min6 β-cells, cells cultured as monolayers, cells cultured in static dishes as PIs, and cells cultured in RCCS as PIs. Experiments were performed to quantify specific ECM proteins (fibronectin, collagen IV, and laminin V) of interest. Whole cell extracts were selected for complete extracting of membrane-bounded and cellular proteins of the cell. The experiment was initiated by culturing Min6-cells, as monolayers into 10 cm cell culture plates. Once cells reached 50% confluence, media was aspirated and 1 mL of PBS was added. Cells were scraped and transferred to 1.5 mL Eppendorf tubes for centrifugation. For cells grown as PIs, 70–100 PIs were selected for protein extraction at day 8 of culture. All cells were incubated with 400 µL of lysis buffer (Tris buffer 10mM at pH 7.4, β-glycerophosphate 20mM, NP-40 1%, 1× protein inhibitor cocktail) and incubated on ice for 15 minutes, followed by addition of 25 µL of 10% triton X-100 for 30 minutes on ice.

Samples were centrifuged for 1 minute at 13,000 rpm and supernatants were collected. Samples were immediately snap frozen in liquid nitrogen.

2.2.9.2. *Bradford Assay*

The Bradford assay is used to measure protein concentration in a given sample. BIO-RAD, a protein assay dye, was used in the ratio 800 µL of water to 200 µL of dye. The experiment was initiated by constructing a standard curve using various known dilutions of BSA (1mg/mL) in 1 mL of protein assay dye, i.e.. 0, 1, 2, 4, 6, 8, 10, 16 and 20 µg.

For measuring protein concentration per sample, 5 µL of sample was added to a cuvette and mixed with 1 mL of 1× Bradford dye. Cuvettes were inverted several times to ensure complete mixing, followed by incubation for 5 minutes at room temperature.

The absorbance of each sample was measured at 595 nm using a spectrophotometer (Eppendorf Biophotometer®, 8.5 mm).

2.2.9.3. *Western Blot Analysis*

SDS-PAGE

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) procedure was used to separate proteins of a given sample based on their mobility on a polyacrylamide gel. Equal amounts of protein extracts were mixed in a 1:1 ratio with SDS-buffer (SDS 20%, bromophenol blue 0.1%, Tris-HCL 1M at pH 6.8, β-mercaptoethanol 10%). All SDS-PAGE used 10% polyacrylamide gels (Mini-PROTEAN TGX Precast gels, BIO-RAD UK). ECL rainbow marker was loaded in the first lane of the gel to determine the approximate size of protein. Protein samples were loaded in the remaining lanes, and gels were run for 1 hour at 150 V in running buffer (Tris-base 3g/L, SDS 1g/L).

Transfer of proteins from PAGE to nitrocellulose membrane

Transferring of proteins was performed using a TRANS-BLOT semi-dry blotting apparatus (BIO-RAD, UK). A transfer sandwich was prepared which consisted of pre-soaked, thick filter paper, nitrocellulose membrane, SDS-PAGE gel and another sheet of thick filter paper. The transfer sandwich was prepared by first pre-soaking the filter papers and nitrocellulose membrane in transfer buffer (Tris-base 0.3%, glycine 1.44%).

The gel was removed from the cassette using the opening lever, and the transfer sandwich was assembled. The transfer process was performed for 1 hour at 15 V, after which the nitrocellulose membrane to which the proteins were transferred was blocked in 10% blocking buffer (semi-skimmed dried milk, 10g; washing buffer 1×: 200mM Tris-HCL pH7.6; 29.2g/l Nacl; 500µl Tween 20) for 1 hour on an orbiter shaker at room temperature. The membrane was then washed three times for five minutes each time on the shaker.

Immunodetection

Preparation for immunodetection, specific concentrations of primary antibodies against the proteins of interest (collagen IV 1:1000, laminin V 1:1000, fibronectin 1:3000, GAPDH 1:1000) were diluted. Membranes were incubated with primary antibody at 4^{o}C overnight with rotation. The following day, membranes were washed in 1x wash buffer, with two quick washers followed by 15 minutes once and 5 minutes 6 times on an orbital shaker. HRP-linked secondary antibody at 1:5000, diluted in 1x wash buffer, was added to the membrane solution and incubated for 1 hour with rotation at room temperature followed by the washing steps described previously. Immunodetection was performed by incubating membranes with ECL-plus solution (Amersham Bioscience, UK). An ECL detection solution was prepared by mixing 500 µL of solution A with 500 µL of solution B. Excess washing buffer was drained from membranes which were then placed gently on saran wrap using forceps. ECL-plus mixture was added for 1 minute, membranes were enclosed with saran wrap, air bubbles were removed, and membranes were placed inside a film cassette. X-ray film (Amersham, Bioscience, UK) was exposed to membranes in a dark room, and radioactive signals were detected using a Konika SRX101A automatic developer.

2.2.9.4. ***Densitometry Analysis***

Densitometry analysis was performed using Image J software to normalise experimental conditions using the following formula: ratio of experimental densitometry values divided by loading control densitometry values.

2.2.10. **Determination of Insulin Release and Content**

A Mercordia mouse insulin ELISA kit was used to quantify the amount of insulin released from Min6 β-cells. Three sources of Min6 β-cells were used for this

experiment, cells grown as monolayers, PIs cultured in static dish, and PIs cultured in RCCS.

The experiment was divided into two parts; the analysis of insulin secretion and the quantification of insulin content of the cells. Cells were seeded at a density of 4×10^4 cells/ml and grown as monolayer in 6-well plates; PIs, were cultured in petri dishes and RCCSs using 5 mM basal glucose. For monolayer cells, the experiment was initiated once cells reached 50% confluence. Similar sized PIs were selected from dishes and RCCS was accurately determined, and PIs of similar size were chosen. All three sources of cells were harvested at 0 times for basal glucose levels, and centrifuged. Supernatants were collected for insulin release analysis and pellets were retained for measuring insulin content.

2.2.10.1. *Insulin release analysis*

This experiment was conducted at 15 minutes, 30 minutes and 1 hour time points. Samples were collected from each source, and cells were incubated with 0 mM glucose overnight followed by quick washing and culture with 25 mM glucose at the time points (mentioned above). Cells were harvested and centrifuged and supernatants were collected for analysis; pellets were retained the next part of the experiment.

2.2.10.2. *Insulin content analysis*

Following glucose stimulation, , pellets from each source were incubated with 1 ml acid ethanol (15 mL of 12 M HCL/L, 70% ethanol). Cells were then disrupted by vigorous pipetting and incubated at $4^{\circ}C$ overnight for complete extraction. Next day, samples were centrifuged for 3 minutes at 2000 g and supernatants were collected for insulin content quantification.

Insulin ELISA was performed by adding 10 µL of calibrators and samples to each well of the coated plate provided with the ELISA kit. 100 µL of enzyme 1× conjugate (1 plate: 1.2 mL enzyme conjugate 11×, 12 mL washing conjugate buffer) was added to each well and the plate was incubated on a plate shaker for 2 hours at room temperature. A washing step was performed by adding 350 µL 1× washing buffer (1 plate: 35 mL washing buffer 21×, 700 mL re-distilled water) to each well six times. 200 µL of substrate TMB was added to each well and incubated for 15 minutes at room temperature. The reaction was terminated by adding 50 µL of stopping solution and the plate was shaken for 10 seconds for complete mixing. The plate was then read at 450 nm on a MultiScan Plate Reader (Thermo Scientific®).

2.2.11. **Immunocytochemistry analysis (ICC)**

ICC involves the use of specific antibodies to identify or localise proteins of interest in tissue sections viewed by light or fluorescence microscopy. ICC is typically used in animal research, unlike immunohistochemistry (IHC) which is primarily used in human pathology using antibodies against proteins in paraffin tissue sections with fixed tissue sections.

In order to perform ICC experiments, an optimised method was required using high affinity purified antibodies for labelling and as controls. Performing ICC experiments is useful in determining morphological appearance and localising the targeted protein within the tissue section. In this study, we sought to examine the immunoreactivity of specific targeted proteins present in PI cells, cultured under different culture environments, to a range of specific antibodies. Thus, antibodies were tested at different dilutions to determine the specificity of the experiment, and an optimal dilution was determined [see **Table 4**]. The principle of this experiment was the use of frozen sectioning preparation of pre-embedded cell blocks of PIs, cultured under different

culture conditions (section 2.2.2.1). PI blocks were sectioned using a cryo-sectioning method to 5-7 μM thicknesses. Sections were placed on Superfrost slides in preparation for the blocking step.

The experiment was performed using indirect fluorescence and enzyme-labelled antibody ICC analysis.

Indirect Fluorescence-labelled antibody method

This method was used in addition to the previous method as some targeted proteins were difficult to visualise by light microscopy using enzyme-labelled antibody analysis.

Table 4: ECM primary antibodies that have been used in ICC experiment:

Primary Ab	Clonality	Raised in	Target species	Ig-Isotype	Dilution
Anti-Fibronectin	Polyclonal	Murine	Mouse	IgG	1:950
Anti- Laminin V	Polyclonal	Human	Mouse	IgG	1:850
Anti-Collagen IV	Polyclonal	Mouse	Mouse	IgG	1:850

This experiment was therefore useful in localising proteins of interest from frozen sections. The premise of this method is the conjugation of secondary antibody with a fluorochrome which can be detected by fluorescence microscopy [**Figure 2-7].**

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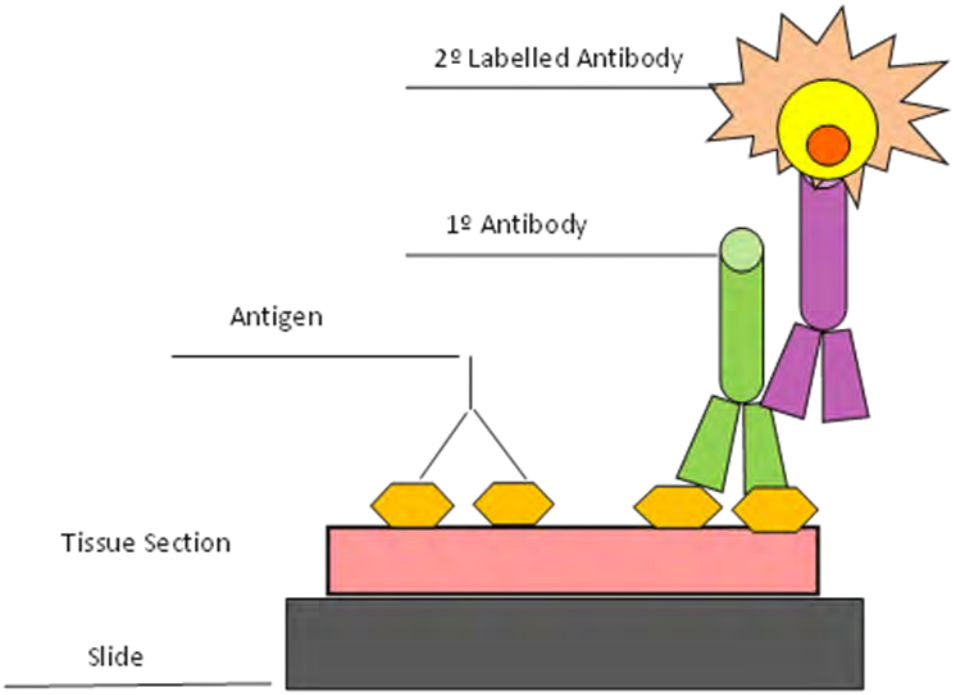

Figure 2-7: Indirectly-tagged ICC analysis.

The schematic illustrates the secondary-tagged antibody with either a fluorochrome or enzyme attached to the FC region of the unlabelled primary antibody.

Non-Specific Binding

In order to achieve successful reactions, there are some potential issues can arise during tissue and cell section preparation which can lead to incorrect results. Normally, antibodies bind specifically via Fab binding regions to specific epitopes of the targeted antigen (protein).

However, non-specific binding site exist where the antibody can potentially bind, leading to incorrect reactions. For example, charged groups, Fc- receptors, and endogenous antibodies are the causes of non-specific reactions **[Figure 2-8].**

Thus, it is important to block these sites using various blocking agents. In this study, a BSA blocking agent, commonly used in ICC experiments, was used as the cell sections of interest produce charged groups during the fixation process

2.2.11.1. ***Blocking incubation***

100 µL of blocking buffer (glycerol 0.7%, Tween-20 0.4%, BSA 2%, goat serum 5%, in PBS) was added to tissue sections and incubated in a humidified chamber at room temperature for 1 hour. Blocking buffer was removed and slides were washed three times with PBS for 5 minutes each time

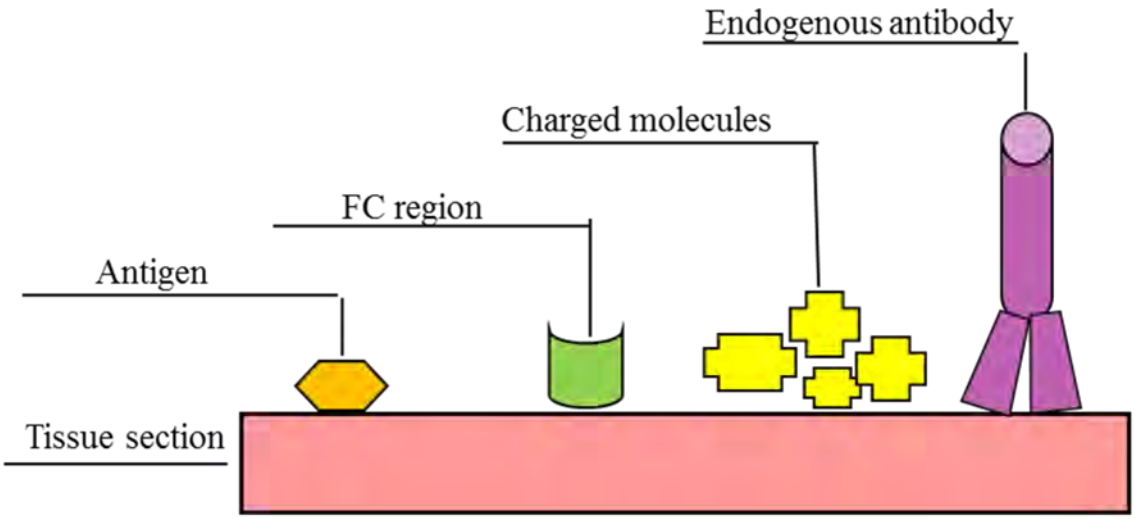

Figure 2-8: Schematic illustrating non-specific binding sites that might be found on cell sections.

Non-specific binding sites such as FC region receptors, charged molecules, and endogenous antibody can produce unwanted back ground staining once the tissue section is incubated with primary antibody. To prevent non-specific binding sites, bovine Serum Albumin (BSA) is the most common blocking agent used to reduce non-specific hydrophobic binding.

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2.2.11.2. *Primary and Secondary Antibody incubation*

35 µL of diluted primary antibody [previous **Table 4**] in blocking buffer was added to the sections and incubated overnight in a humidified chamber at 4℃. Slides were washed three times with PBS for 5 minutes each time. Appropriate diluted secondary antibody (FITC-conjugate polyclonal anti-mouse, dilutions of 1:80) in blocking buffer was added to sections and incubated in a humidified chamber at room temperature for 1 hour. Slides were rinsed three times with a PBS washing buffer (glycerol 0.7%, Tween-20 0.4%, BSA 2%) for 5 minutes each time.

2.2.11.3. *Visualisation of the staining reaction*

In this experiment, a FITC-conjugated antibody was used to visualize the staining reaction using a confocal microscope equipped with krypton/argon lasers. After embedded-clusters section had been incubated with optimum diluted secondary antibody, slides were washed three times with washing buffer for 5 minutes each time. Slides were then mounted with Vectashield-DAPI medium and sealed with colourless nail varnish.

2.2.12. **Data and Statistical Analysis**

Data are presented as the mean ± standard deviation (mean ± SD). For all ECM gene and protein expression studies, the statistical significance of the difference between means was calculated using one way Analysis of Variance (ANOVA). For ELISA studies, the difference of two factors was calculated using two-way ANOVA. Probability values of less than 0.05 were considered significant.

Chapter 3. **Characterisation of Min6-Pseudoislets.**

Abstract: Pancreatic β-cell lines are a valuable resource to study the biology of β-cells *in vitro*. The most widely used pancreatic β-cell line is Min6 β-cells, which is derived from transgenic mice [116]. These cells have the ability to aggregate into 3D islet-like structures known as Pseudoislets (PIs), a useful model in many areas of research. The aim of this chapter was to evaluate the characterisation of PIs cultured under two different culture environments, static and simulated microgravity. PIs characterisation was determined by investigating the morphological appearance, histological structure, and expression of specific ECM proteins fibronectin (FN), collagen IV (Coll IV) and laminin V (LmV) under two different culture conditions. PIs were initially generated in static dishes followed by further culturing in our novel RCCS bioreactor. Morphological appearance was assessed using light microscopic analysis and histological characteristics were evaluated using H&E and actin staining. Overall, the structural appearance was examined using SEM. The expression of ECM elements was analysed by qRT-PCR, western blotting, and ICC in Min6-PIs cultured under static and RCCS culture conditions. Results showed that Min6 β-cells typically grew as adherent monolayers, while PIs generated from static dishes showed irregular shapes of clusters; PIs cultured in RCCS, however, showed a retained and unified shape of clusters. H&E staining of PIs cultured in RCCS showed that clusters grew to larger sizes compared with PIs cultured in static dishes. Actin structures were observed both in PIs cultured in static dishes and RCCS. SEM analysis showed different structural surfaces between PIs cultured in static dishes and RCCS; the surface of PIs in dishes was rough compared with PIs cultured in RCCS. Specific ECM gene and protein expression was significantly increased in PIs cultured in RCCS compared with those cultured in static dish for FN (p<0.05), Coll IV (p<0.0009), and LmV (p<0.001). ICC analysis confirmed these findings. It was thus concluded that the RCCS bioreactor can enhance the morphological characteristics and ECM expression of the PIs model used in this study.

3.1. **Introduction**

Min6 β-cell lines are widely used in biological research as an insulin-secreting pancreatic cell line. Importantly, they are capable of re-aggregation in static culture conditions (e.g., petri dishes), forming islet-like clusters known as Pseudoislets (PIs), which are akin to native islet cells [188-191]. The behaviour of PIs is very close to native islet cells, which makes them a useful model to study the biological function of β-cells and insulin secretory responsiveness [188]. PIs are usually generated from static dishes, retaining most of the characteristics of native β-cells such as insulin secretory function [189, 190]. However, several shortcomings affect the quality of the clusters, such as poor transportation of metabolites leading to uncontrolled cluster size [191]. This limitation can have a negative impact on the glucose responsiveness of PIs. Thus, it was important to develop a PI model more analogous to islet cell characteristics *in vivo*. Development of 3D simulated microgravity cell culture techniques have highlighted the shortcomings of static culture conditions by providing an environment that more closely mimics native tissue by forming natural cell-to-cell attachment. The culture conditions in 3D techniques are homogenous, maintaining a continuous transfer of nutrients, metabolites and oxygen [192]. Bioreactors are one of the most commonly used 3D culture systems in many research areas. The bioreactor is essentially a closed system used to study biological and physiological mechanisms of cells in a 3D culture environment [193]. In this study, a rotating cell culture system (RCCS) was used to improve the characteristics of PIs as a model. The principle behind the RCCS bioreactor is the culture of a cell suspension in vessels where the suspension is kept in motion [192]. Continuous motion means that cells do not adhere to the vessel wall but instead form cell-to-cell attachments [193].

Furthermore, one of the most important features of bioreactors is the provision of low-shear stress conditions to mimic cellular microenvironments *in vivo* [193].

In this study, two culture methods were used: static dishes for generating PIs and RCCS bioreactor for further culturing PIs in order to grow clusters in a controlled microenvironment where the transportation of nutrients and oxygen is distributed equally. This functionality may have the potential to improve the cellular characteristics of PIs as a model in this study. The most important parameters for improving the characteristics of Min6-derived PIs investigated in this study were ECM proteins. The basic function of ECM is to provide support to the cell by maintaining physical attachment from cell to cell through its biomechanical structure [194]. The ECM of native islet cells plays a crucial part in their functional and structural development, especially in tissue development and homeostasis. ECM acts as a scaffold to hold tissue components together. This polymerised scaffolding consists of fibrous proteins such as collagen fibrils and elastin, and non-fibrous proteins such as the glycoproteins fibronectin and LM. Collagen, considered to be the structural support of multicellular mechanisms [195], is involved in many cellular processes such as migration, differentiation and proliferation [196]. Thus, any alteration in the composition of ECM can damage tissue and promote the loss of normal functioning of the cell along, with alterations in the structure of the tissue [194].

Similarly, the relationship between ECM and islet cells is complex, and the interactions between them involve the regulation of islet physiology such as insulin secretion [194]. This framework produces various signals that determine tissue function and structure. ECM occurs as a very thin layer outside the BM, and surrounds only large ducts and blood vessels. Islet ECM is divided into BM and interstitial matrix (IM).

BM is a tight and complex network of glycoproteins that separate compartments and also manage and direct cellular processes and loose interstitial matrix, whereas IM provides strength and elasticity to pancreatic tissue through fibrin-collagen interactions [51]. Glycoprotein components present in BM are mainly fibronectin and LM.

Fibronectin plays a role in cell attachment and inhibition of apoptosis [197], while LM is involved in intracellular signalling, thus preserving culture conditions [198]. Thus, the presence of cell-matrix is critical for islet integrity and functionality.

The study first determined cluster morphology, evaluated histological characteristics, and analysed specific ECM proteins such as FN, Coll IV, and LmV expression by PIs cultured under static and simulated microgravity culture conditions.

Therefore, the aim of the work described in this chapter was to enhance the characterisation of Min6-PIs for use as a tool to examine the effects of enzymatic digestion on cellular components and functionality, as addressed in subsequent chapters.

3.1. **Results**

3.1.1. **Morphological appearance of Min6-Pseudoislets**

Cell morphology examination, i.e., shape, size, and structural appearance, remains an increasingly essential tool in controlled cell culture methods. Cells are surrounded by an outer membrane, the plasma membrane, which acts as a barrier separating the cell interior. Through this membrane, several essential biological and cellular processes take place, such as cell adhesion and signalling, allowing the interaction with the extracellular environment [199, 200]. Thus, cellular morphology is considered an important measurement of cellular organisation and the physiological state of cells.

In this study, the morphological appearance of PIs cultured in static dishes and in RCCS was examined. The examination was performed by assessing the cluster surface structure of PIs using light microscopic analysis.

Results showed that Min6 β-cells were grown as monolayer as standard. PIs were formed by culturing dispersed Min6 β-cells in non-adherent petri dishes; after 48 hours of sub-culturing, cells started to aggregate. At day 4 of PIs incubation in static dishes, PIs were selected and further cultured in RCCS. Microscopic analysis was performed

for the three sources of Min6 β-cell [**Figure 3-1]**. Min6 β-cells normally grow as flat island-like structures on adherent treated tissue culture flasks, giving the morphological appearance of monolayer cells **[Figure 3-1,** panel A**].** PIs cultured in static dishes aggregated as clusters after 48 hours of sub-culturing, and 6 to 7 days of incubation were required to produce well-characterised clusters **[Figure 3-1,** panel B**].** However, after 7 days of culture, irregular sizes were observed in static dishes. **Figure 3-1** (panel C) shows the morphological appearance of PIs from day 4 of generation, cultured in RCCS for a further 3 days. After a total of 7 days of incubation in RCCS, the morphology of PIs showed almost unified cluster sizes compared with clusters in static dishes **[Figure 3-1, panel C].**

3.1.2. **Histological analysis of Min6-Pseudoislets**

H&E staining

Histological analysis is the scientific study of biological cell compartments that have been carefully prepared using histological techniques such as cryo-sectioning, used in this study. Cryo-sectioning is carried out by examining frozen thin slices, or 'sections' of tissue, under light or confocal microscopy.

The frozen section method was used here because of its rapid performance; because of the small size of PIs it was also more appropriate to use this technique. H&E staining, the most commonly used stain in histology [185], was used to provide distinguishable details of biological compartments such as the nucleus and cytoplasm of PIs cultured under static and simulated microgravity e-nvironments. Haematoxylin dye highlights nuclear definition through the oxidation of haematoxylin into active, 'haematic' ingredients by staining the nucleus a blue colour.

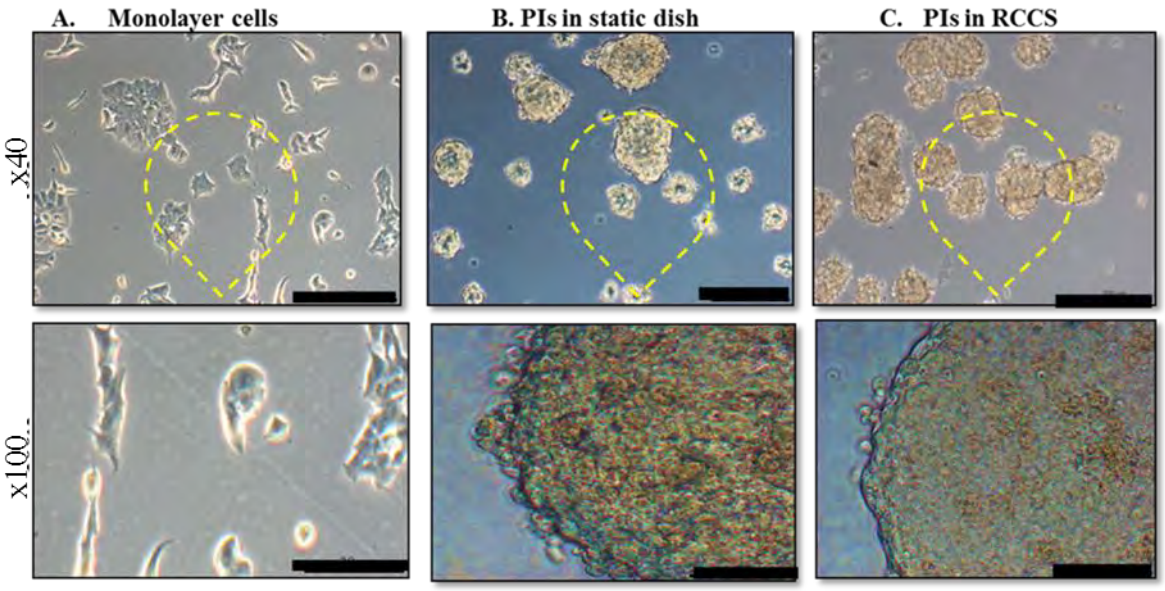

Figure 3-1: Morphological appearance of Min6 β-cells under different culture conditions.

Min6 β-cells are typically cultured as monolayers (panel A) and under x40 objectives they have the appearance of flat island-like structures. PIs were generated by culturing Min6 β-cells in non-adherent dishes (panel B), and they aggregated as clusters after 48 hours of sub-culturing. Incubation for 6 to 7 days was required to produce a well-characterised PI cluster. PIs in static dishes showed different shapes with irregular outer surfaces. At day 4 of PI culture in static dishes, PIs were further cultured in an RCCS bioreactor (panel C), showing a more refined smooth cluster surface. Scale bar: panel B, x40 100 μM; x100 50 μM; panel C: x40 150 μM; x100 70 μM.

For cellular detail, the cluster section was counterstained with eosin dye, which colours the cytoplasmic proteins pink. The purpose of histological H&E staining is to define the morphological structure of PIs cultured under different conditions, examining more specifically the size of the cluster. Therefore, the aim of analysing the histological characteristics of PI cells is to evaluate the quality of the cell culture systems used in this study, and to determine whether culture conditions have the potential to change the size of PIs. Thus, H&E staining techniques showed that PIs cultured in RCCS bioreactor exhibited bigger size (150 μM) **[Figure 3-2].** Furthermore, PIs cultured in RCCS tended to include more cells within the cluster **[Figure 3-2, panel B]** compared with PIs cultured in static dishes **[Figure 3-2, panel A]**.

3.1.3. **Cytoskeletal examination of Min6-Pseudoislets.**

Cytoskeletal structure is located under the cell membrane, providing mechanical support to the cell and participating in cellular and biological signalling processes [201]. The cellular cytoskeleton components include actin filaments, intermediate filaments, and microtubules. An actin filament marker was selected in this study to examine PI morphology. Clusters were cultured under static and simulated microgravity conditions and PIs were harvested and stained with phalloidin.

Analysis was performed using confocal microscopy at x65 magnification **[Figure 3-3].** Results showed that PIs cultured in RCCS demonstrated a numerous distribution of actin fibers all over the cluster **[Figure 3-3, panel B].** For more detailed study of the structural surface of PIs cultured under different culture conditions, SEM was used to study the smallest details of cluster surfaces.

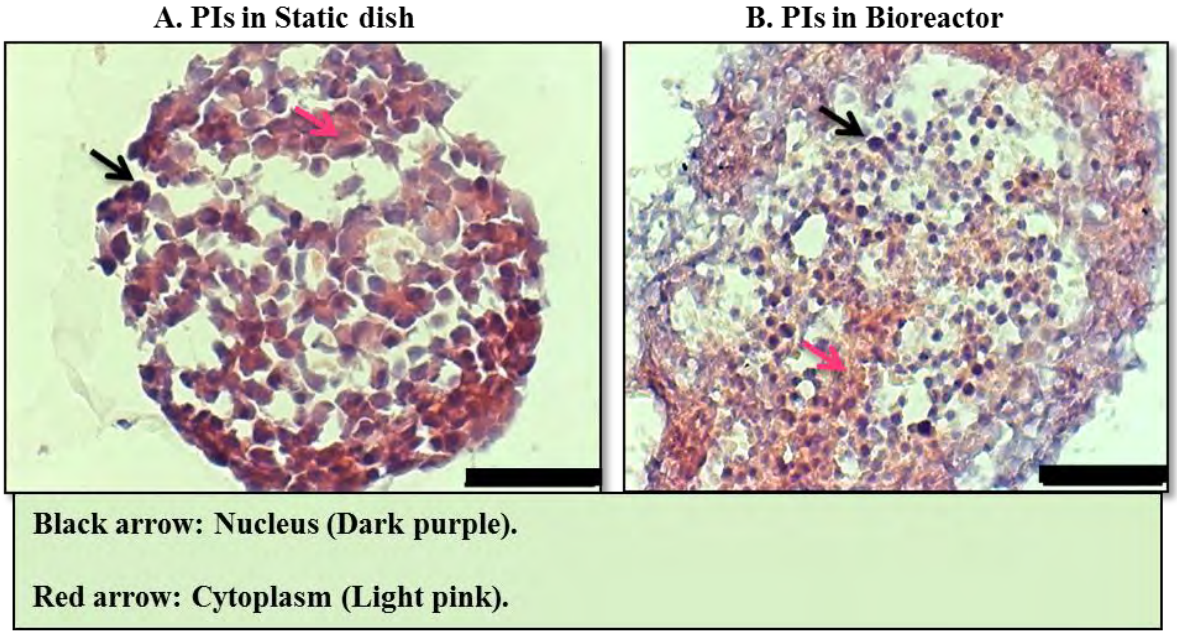

Figure 3-2: Histological structure of Min6-pseudoislets (PI) cultured in static dishes and RCCS.

Ten PI clusters were selected, embedded with freezing medium (OCT), and sectioned (5 µM) using cryosectioning techniques. Cluster sections were stained using H&E, and morphology was examined by light microscopy at x40 magnification. PIs cultured in RCCS (panel B) exhibited a bigger size compared with PIs in static dishes (panel A). More cells were observed in PI clusters cultured in RCCS, compared with PIs cultured in static dishes. Scale bar: panel A: 100 µm; panel B: 150 µm.

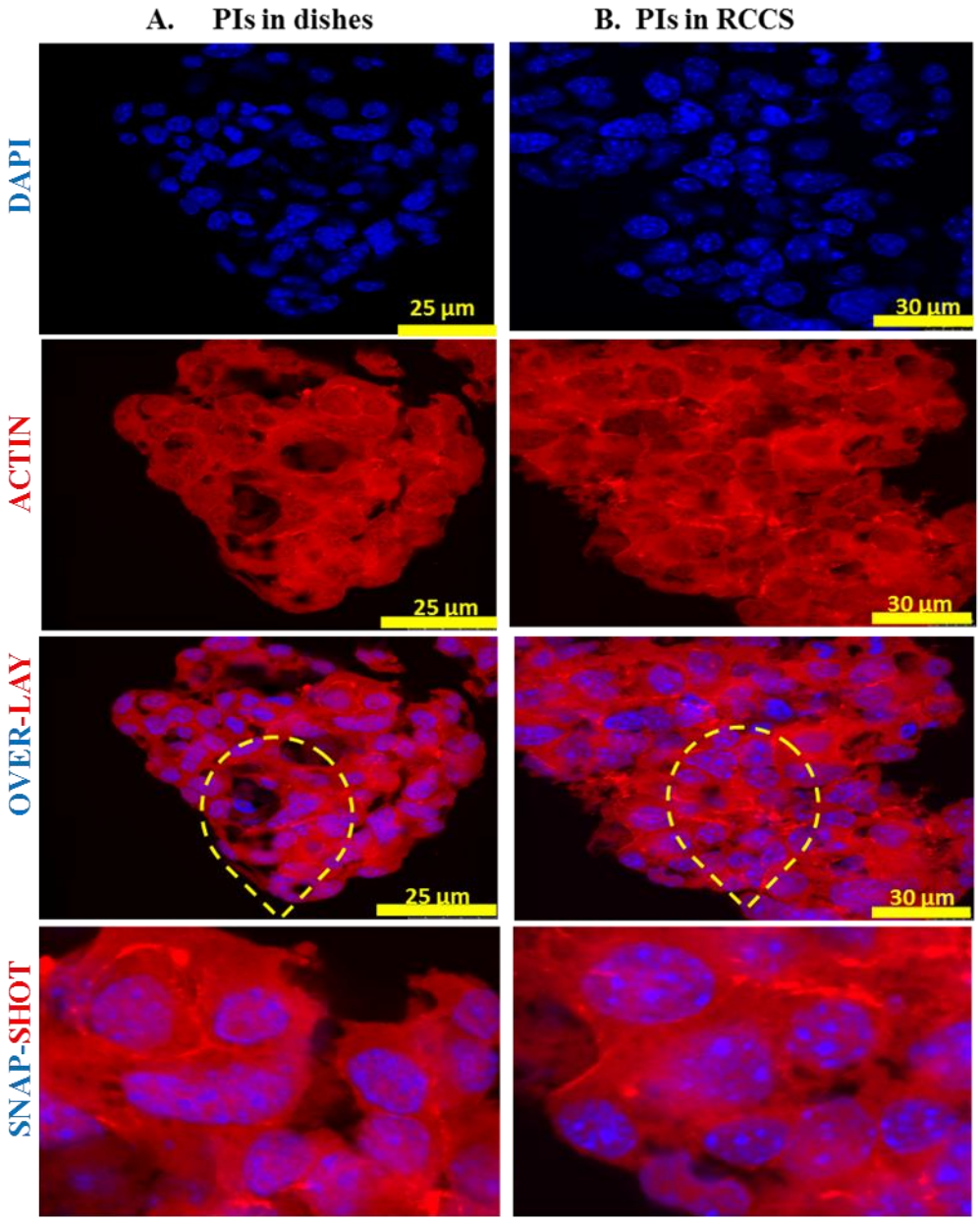

Figure 3-3: F-Actin staining of Min6-Pseudoislets (PIs).

Ten PIs from both static dishes and bioreactor were fixed with 3.7% formalin for 30 minutes at room temperature. OCT-embedded cryo-sections were incubated with phalloidin antibody to stain actin filament of cells. Slides were mounted with DAPI and cells were visualised by confocal microscopy with images captured at x65 magnification. Results shown are representative of three separate experiments, and images are representative of six separate fields. Results showed that PIs cultured in dishes and in RCCS proliferated in groups. PIs in RCCS exhibited a larger size compared with PIs cultured in dishes. Actin structures (red colour) were observed both in PIs cultured in static dishes and in bioreactors. SNAP-SHOT is a selected area, closed capture, of over-lay image.

3.1.4. **Scanning electron microscopy (SEM) analysis of Min6-Pseudoislets.**

The purpose of using SEM is to study the structural surface of small specimens, thus giving greater detail. This is facilitated by passing a beam of electrons across the surface of the specimen so those electrons are imaged by an electron detector. Thus, the aim of using SEM in this study was to examine the structural surface of clusters cultured in static dishes and in RCCS. Clusters were selected from culture types and fixed with glutaraldehyde, dehydrated with graded concentrations of ethanol, and coated with platinum to a coating size of 4 nm. Zeiss Sigma field emission gun SEM was used to examine the clusters, and images were captured at magnifications of 1K, 5K, and 10K. PI cells were also coated with platinum and examined using Zeiss Sigma field emission gun SEM (Zeiss NTS). Results showed that the structural surface of PIs cultured in static dishes was rough compared with those cultured in RCCS, which showed a smooth surface **[Figure 3-4,** dish (panel A), RCCS (panel B)]. Furthermore, the size of PIs cultured in the RCCS was observed to be larger **[Figure 3-4**, panel B] than PIs cultured in dishes **[Figure 3-4**, panel A]. Therefore, these findings confirmed the findings of previous experiments that examined morphological surface using light or confocal microscopic analysis. From these results, it was concluded that the RCCS microenvironment has the potential to influence cell growth inside the cluster, an observation which is further investigated in the next chapter by analysing PI cell viability using an MTT assay.

Following analysis of the morphological appearance of PIs cultured under static and simulated microgravity culture conditions, it was clear that an investigation of specific biological markers essential to cell development *in vivo* and to the functionality of adult islet cells was important. In the following section specific extracellular matrix (ECM) proteins were analysed.

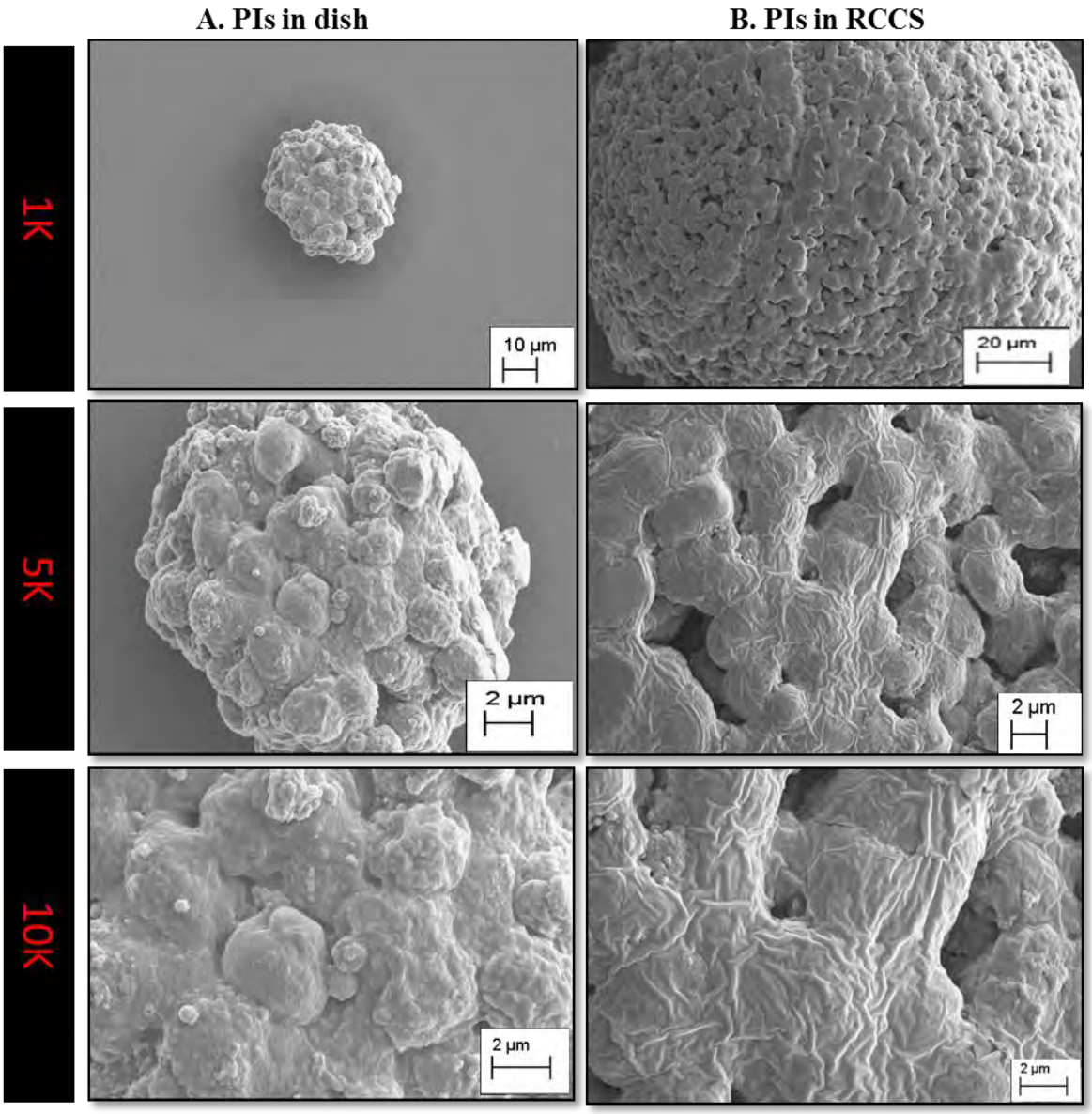

Figure 3-4: Scanning electron microscopy (SEM) of the structural surface of Min6-Pseudoislets (PIs).

PIs were selected from static dishes and from RCCS and fixed with 2.5% of glutaraldehyde, dehydrated, and coated with platinum coating (4 nm). PIs were examined using Zeiss Sigma field emission gun SEM (Zeiss NTS). Images were captured at magnifications of 1K, 5K, and 10K. The structural surface of PIs in dishes (A) was rough, while PIs cultured in RCCS (B) showed a smooth surface by comparison. The observed size of PIs cultured in RCCS (B) was bigger than that of PIs cultured in dishes (A).

3.1.5. **Extracellular matrix (ECM) expression of Min6-Pseudoislets.**

Specific ECM proteins i.e. fibronectin (FN), collagen IV (Coll IV), and laminin V (LmV), were selected for investigation. The following sections describe how ECM expression was determined by qRT-PCR to analyse gene expression, western blotting to analyse protein expression, and ICC to determine the localisation of ECM.

ECM gene expression of Min6-Pseudoislets.

Gene expression was analysed by using a qRT-PCR method in order to examine the effect of different culture conditions on specific ECM gene expression. The experiment was performed by extracting mRNA from three sources of Min6 β-cells, cells cultured as monolayers, cells configured as PIs in static dishes, and PIs cultured in an RCCS bioreactor. After extraction, samples were treated with a DNase treatment assay and cDNA was synthesised. GAPDH was employed as a housekeeping gene and specific primers were used to target the expression of the gene of interest. All ECM genes (FN, Coll IV, and LmV) expression was measured relative to GAPDH expression. Monolayer cells were used as controls in this experiment. Results showed that there was a significant difference of FN gene expression between PIs cultured in RCCS and in static dishes **[Figure 3-5,** panel A, **P*<0.05**]**.

There was no significant difference between PIs cultured in static dishes and cells cultured as monolayers [**Figure 3-5,** panel A, *P*>0.05]. In contrast, a significant increase in FN expression in PIs cultured in the RCCS was observed compared to monolayer cells **[Figure 3-5,** * *P*<0.05**].** With respect to the other ECM genes, Coll IV **[Figure 3-5,** panel B, ***P*<0.001**]** and LmV **[Figure 3-5,** panel C, ****P*<0.0007**]** showed a significant increase in PIs cultured in RCCS compared to control cells. Similarly, a significant increase in both Coll IV and LmV gene expression was observed in PIs cultured in RCCS compared to monolayer cells.

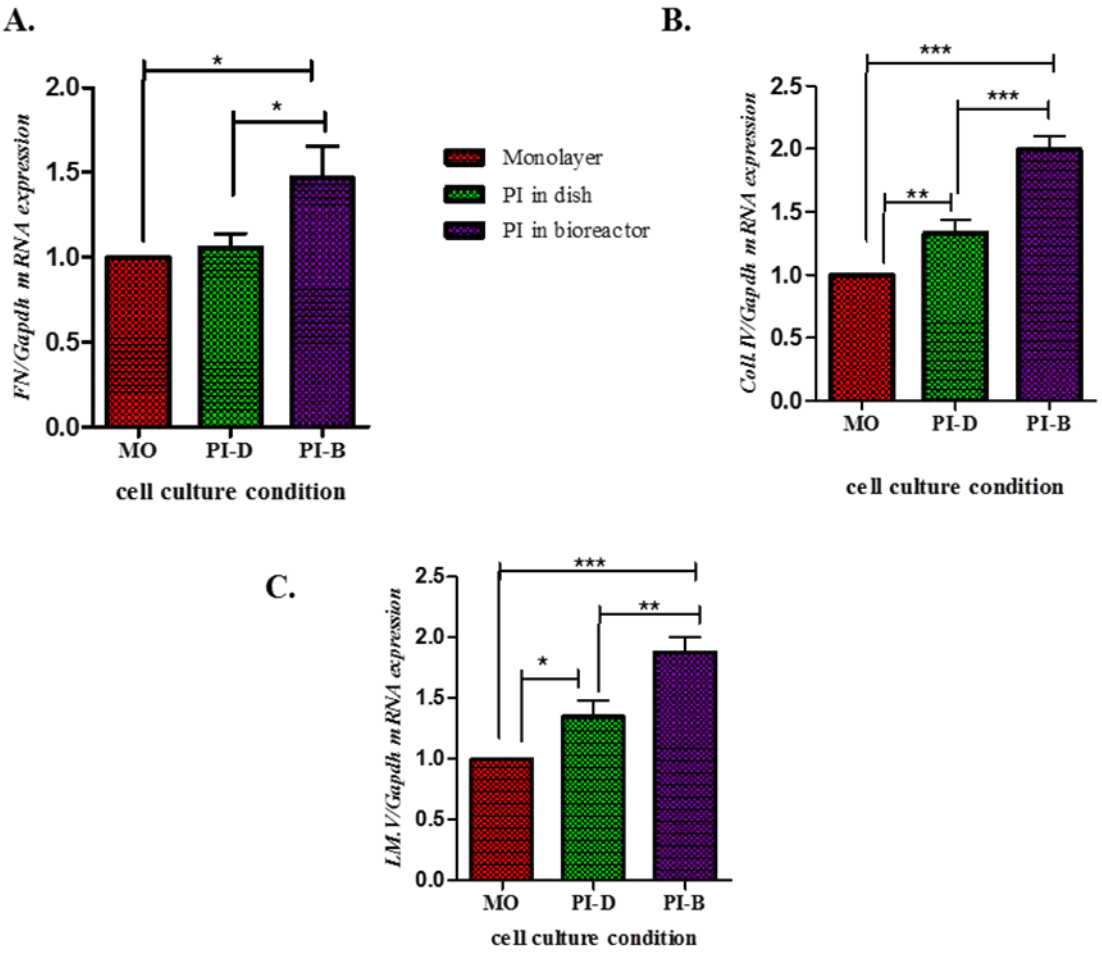

Figure 3-5: ECM gene expression in Min6-Pseudoislets.

mRNA was extracted from three different sources of Min6 β-cells, cells as monolayers, PIs in static dishes, and PIs in an RCCS bioreactor. DNase treatment and reverse transcription using specific primers to ECM genes (FN, Coll IV, and LmV) was then performed. ECM expression was measured relative to GAPDH, and a negative control was included. Results are representative of three separate experiments and expressed relative to monolayer cell gene expression. A significant increase in fibronectin gene expression (A) in PIs cultured in RCCS compared to those cultured in static dish or control cells was observed (*P<0.01). Coll IV (B) and LmV (C) gene expression was significantly higher in PIs in RCCS compared to other cells (B, P<0.001; C, P<0.0007). Error bar values represent mean ± standard deviation.

This inferred that our RCCS bioreactor was capable of upregulating gene expression of all ECM, consistent with the observation that the most abundant ECM proteins expressed in the Min6-PIs model are collagens and LMs. The following section describes the analysis of ECM protein expression using western blotting in order to confirm the gene expression findings.

ECM protein expression of Min6-Pseudoislets.

In the previous section, ECM gene expression was analysed and the findings showed that the RCCS enhanced ECM expression, specifically Coll IV and LmV, in the PIs model used in this study. In order to confirm these findings, protein expression of all three ECM components was investigated using western blotting. Whole cell proteins were extracted from the three sources of Min6 β-cells and concentrations of protein samples were measured by Bradford assay. Protein samples were blotted, immunodetected with specific ECM antibodies to targeted proteins, and detected with chemiluminescent substrate (see chapter 2, section 2.2.7.3). Densitometry analysis was performed using Image J software and monolayer cells were used as a calibrator.

ECM protein expression results are detailed in **Figure 3-6**. Results showed that there was significant difference in FN protein expression between PIs cultured under static and RCCS culture conditions [**Figure 3-6,** panel B-I, $P<0.05$**].** Also, a significant increase was observed in FN expression in PIs cultured in RCCS compared to monolayer cells. Furthermore, a significant increase in Coll IV (panel B-II) and LmV (panel B-III) protein expression was seen in PIs cultured in RCCS compared to those cultured in dishes or monolayer cells **[Figure 3-6,** panel B-II, $^{**}P<0.001$; panel B-III, $^{***}P<0.001$**].** According to these findings, it can be confirmed that gene and protein expression of ECM components Coll IV and LmV are increased significantly in PIs cultured in RCCS.

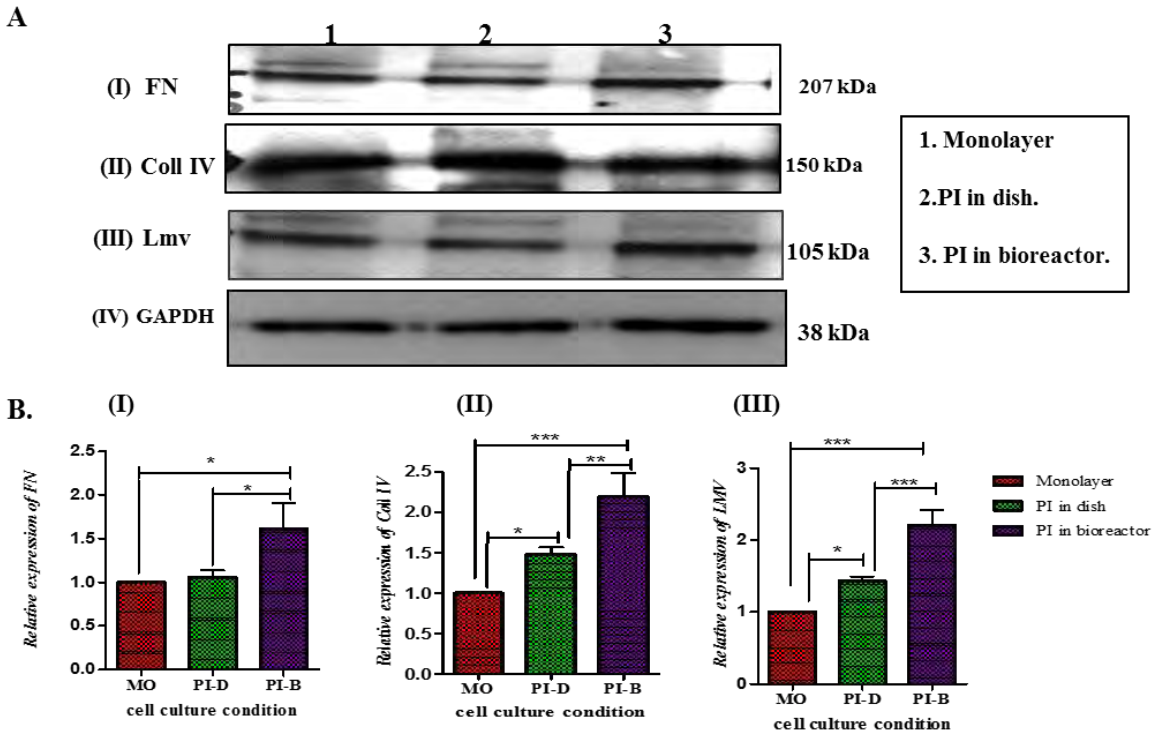

Figure 3-6: ECM protein expression in Min6-Pseudoislets.

Whole cell protein was extracted from three sources of Min6 β-cells, monolayers, PIs in static dishes, and PIs in a RCCS bioreactor. Whole cell extract (20 µg) was separated on 10% SDS-PAGE. ECM content in cell lysates was analysed by western blotting and bands were quantified (A) with results expressed relative to GAPDH. FN protein expression in PIs cultured in RCCS was significantly increased compared to cells cultured as monolayers (panel B-I, $P<0.0163$). A significant increase of FN expression was also observed in PIs cultured in RCCS compared to PIs cultured in dishes (panel B-I, $P<0.05$). Coll IV and LmV protein expression was significantly higher in PIs cultured in RCCS compared to PIs cultured in a dish and to monolayers (panel B-II, $P<0.0009$; panel B-III, $P<0.0010$). These results were reproduced in three separate experiments. Error bar values represent mean ± standard deviation.

In the following section, ECM protein localisation was performed in PIs cultured under static and simulated microgravity culture conditions, using ICC to confirm our previous quantitative analysis and to determine the subcellular localisation of ECM proteins, whether extracellular or cytoplasmic.

ECM protein localisation of Min6-Pseudoislets.

ICC was used to investigate subcellular localisation and expression in6tensity of ECM proteins in PIs cultured in static dishes and in RCCS bioreactor. This allowed us to confirm the previous quantitative analysis of ECM gene and protein expression in these cultures. ICC analysis was performed on frozen cluster sections of PIs from dishes and RCCS (Chapter 2, section 2.2.9). Cluster sections of PIs from both cell culture methods were incubated overnight at 4^{o}C with specific antibodies targeting ECM proteins of interest (FN, Coll IV, and LmV) followed by incubation with FITC-conjugated secondary antibody for 1 hour at room temperature.

Results revealed that ECM protein expression of PIs cultured in dishes and in RCCS was cytoplasmic. **Figure 3-7** shows that FN expression was seen in both PIs cultured in dishes and in the RCCS. Furthermore, the expression of Coll IV **[Figure 3-8]** and LmV **[Figure 3-9]** was higher in PIs cultured in RCCS compared to PIs cultured in dishes. Therefore, it was concluded that ECM proteins were expressed at higher level in PIs cultured in the RCCS.

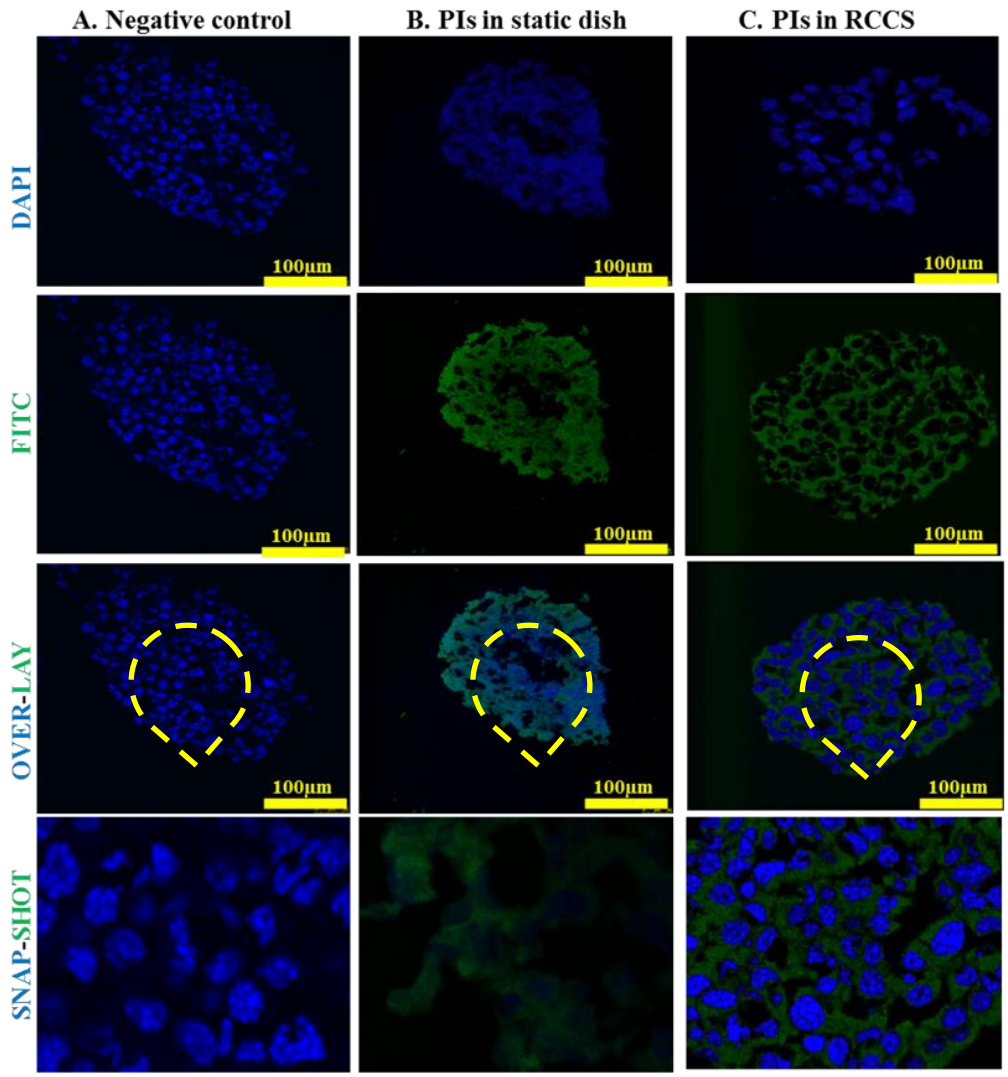

Figure 3-7: Fibronectin localisation in Min6-Pseudoislets.

Immunostaining of FN protein was performed using a specific primary antibody. The reaction was visualised by incubating slides with a FITC-conjugated secondary antibody and mounting with DAPI. Results are representative of three separate experiments, and images are representative of six separate fields. A negative control, showing no expression of FN protein, was included (A). Results showed that PIs cultured in dish and in RCCS both displayed cytoplasmic staining of FN. The immunostaining intensity of FN in PIs was approximately similar in PIs under static conditions and in RCCS. SNAP-SHOT is a selected area, closed capture, of over-lay image.

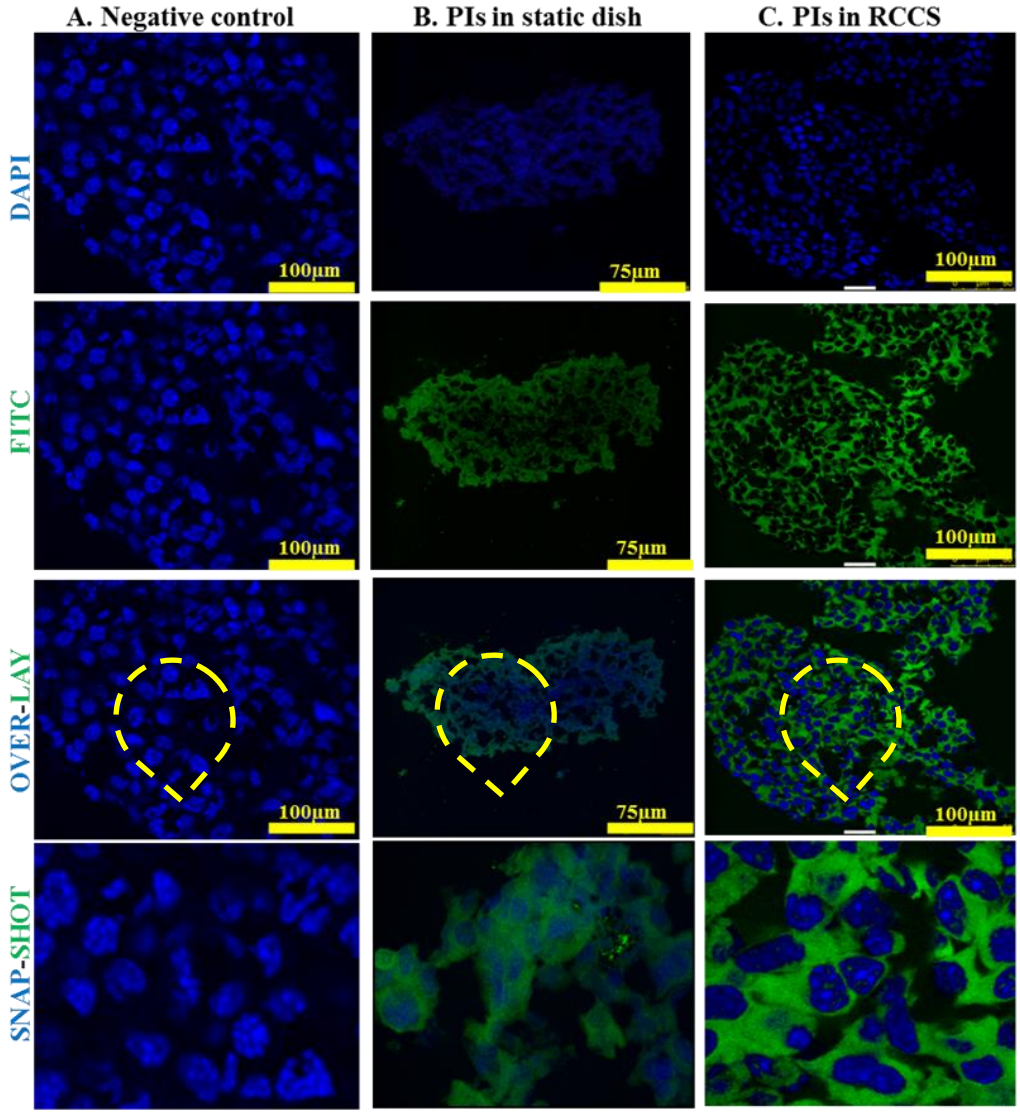

Figure 3-8: Collagen IV localisation in Min6-Pseudoislets.

Immunostaining of Coll IV protein was performed using a specific primary antibody. The reaction was visualised by incubating slides with FITC-conjugated secondary antibody and mounting with DAPI. Results are representative of three separate experiments, and images are representative of six separate fields. A negative control was included (A). Results showed a significant increase of Coll IV expression in PIs cultured in RCCS (C) compared to PIs cultured in static dishes. The localization of Coll.IV exhibited cytoplasmic staining in both PIs cultured I dishes and in RCCS. SNAP-SHOT is a selected area, closed capture, of over-lay image.

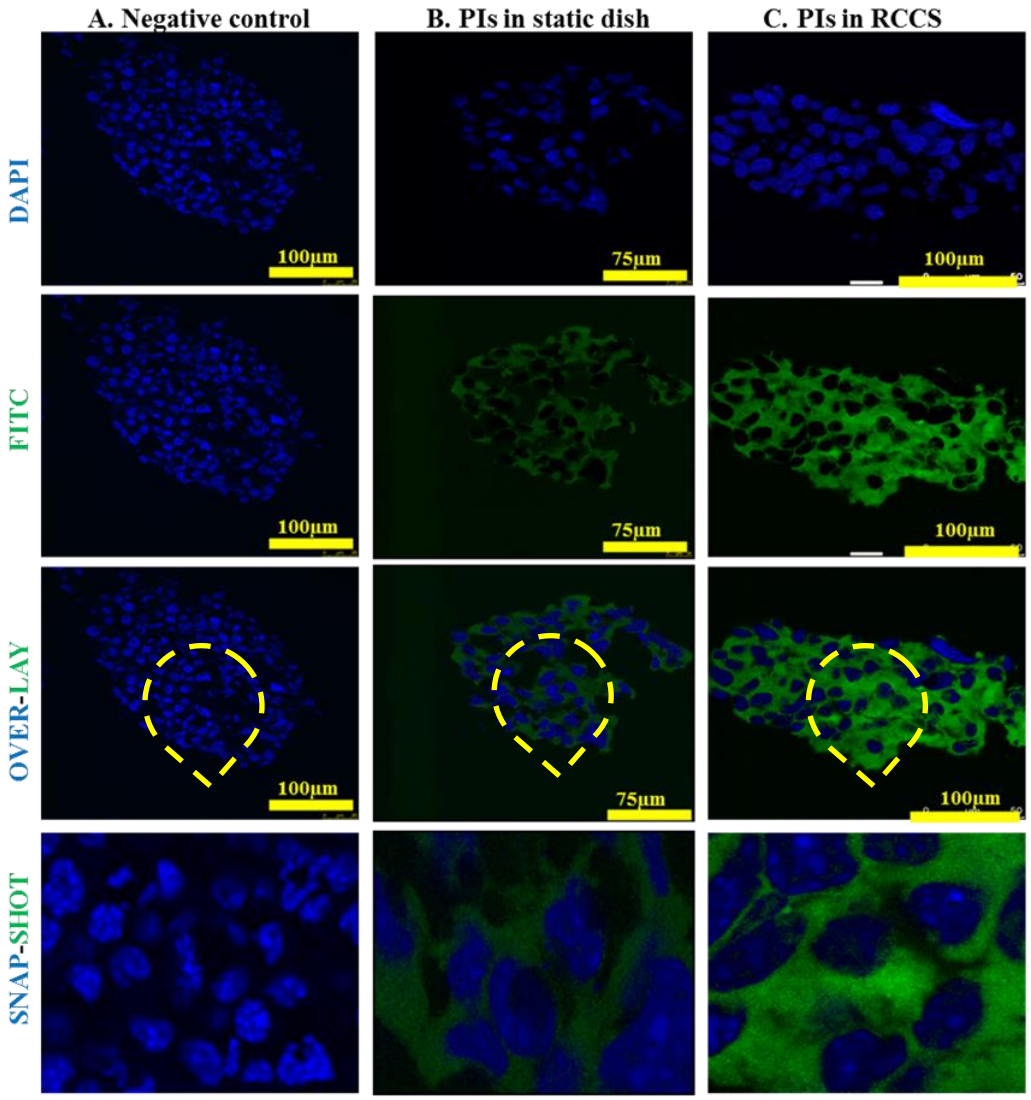

Figure 3-9: Laminin V localisation in Min6-Pseudoislets.

Immunostaining of LmV protein was performed using a specific primary antibody. The reaction was visualised by incubating slides with FITC-conjugated secondary antibody. Slides were mounted with DAPI. Results are representative of three separate experiments and images are representative of six separate fields. A negative control was included. Results indicated that PIs cultured in dishes and in RCCS exhibited cytoplasmic staining of LmV, with higher immunostaining intensity seen in PIs cultured in RCCS (C). SNAP-SHOT is a selected area, closed capture, of over-lay image.

3.2. **Discussion**

The objective of the work described in this chapter was to characterise PIs as a research model. In this study, we cultured Min6 β-cells as PIs in static dishes **[Figure 3-1, panel B]** and in an RCCS bioreactor **[Figure 3-1, panel C],** as a more physiological representation of the primary pancreatic islet. PIs grow as a 3D cluster generated from the insulinoma cell line, with many researchers reporting that Min6 β-cells behave in the same way as islet β-cells *in vivo* [149]. The advantage of using PIs is that animal ethical committee approval is not required as the work is performed *in vitro*. PIs mimic the primary islet β-cell function of glucose-induced insulin secretion. Thus, the use of Min6 β-cell lines configured as PIs may prove a useful model with relevance for islet transplantation [149].

For a better understanding of the characteristics and morphological appearance of PIs cultured under both static and stimulated microgravity environments, clusters were stained with H&E dyes **[Figure 3-2]**. Both types of PIs cultured cells exhibited a similar morphological appearance. In addition to this, PIs cultured in the RCCS visually showed greater numbers of cells and in size compared with PIs cultured in static dishes. Similar to other forms of cells, β-cells adhere closely to their neighbours by a diversity of cell surface proteins [45]. These proteins interact discriminately within the cell membrane and form intercellular junctions or channels that are permeable to a wide range of metabolites, ions, and secondary messengers [46]. Thus, the simulated microgravity culture of the RCCS method may enhance the function of PIs by improving cell growth in a way that mimics primary islet cell behaviour *in vivo*. Researchers have suggested that 3D cell culture is a way of growing cells in an environment closely resembling that of the body [202]. Thus, the benefit of this type of cell culture is that cells can grow in a more natural environment, thus preserving their normal cellular responses.

These efforts are important in fields of research involving the study of live intact cells, such as drug research and cellular transplantation. Expanding the generation of fully-characterised insulin-producing cell lines could be a promising approach towards modelling islet transplant procedures [190]. In addition to this, providing a controlled *in vitro* microenvironment with an abundant amount of highly functional β-cells may improve strategies aimed at β-cell functionality, growth, and islet engraft post-transplantation.

The cytoskeletal structure of PIs from static dishes and RCCS was next examined using actin staining of PIs actin filaments. The investigation was performed to confirm our previous examination of the morphological and histological appearance of PIs in assessing their overall structure. The significance of the presence of actin fibres in cells is because actin protein participates in many cellular processes such as cell signalling and cell junctions present on cell membranes required for cell survival [203]. Furthermore, actin fibres are involved in framing the shape of cells by acting as a scaffold to maintain structural integrity [203]. **Figure 3-3** showed that actin protein in PIs cultured in RCCS was distributed throughout the cluster, compared to PIs cultured in static dishes. This confirmed our previous observation that culturing PIs under simulated microgravity conditions enhances the formation of cytoskeleton in cells within the cluster and explains that proper transportation of nutrients required during culture has a great influence in preserving the morphological integrity of PI cultured cells in the bioreactor. Moreover, all living cells are constructed in 3D microenvironments *in vivo*, so static cell culture conditions have a limited ability to provide the optimal microenvironment required for cells on a large scale.

Thus, scientists have changed cell culture practices by modifying 2D methods to culture cells with ECM components to improve the status of cell cultures.

For a more detailed analysis of the morphological structure of PIs cultured under different culture environments, SEM was performed. The structural surface of clusters cultured in the RCCS revealed a smooth appearance with intact cells observed **[Figure 3-4,** panel B**]** compared to clusters cultured in static dishes that showed rough and uneven surface structure. This observation was indicative of the high mass of transportation of all essential metabolites required for living cells, maintained in simulated microgravity culture conditions which involved suspending clusters in continuous free-fall with low shear stress. In agreement with previous work, Hou Y *et al*. (2009) showed that 3D culture conditions significantly improved the morphology of pancreatic islet cells, reflecting positively on insulin secretory function [184]. In terms of evaluating the morphological appearance of PIs cultured in different environments, it can be concluded that RCCS bioreactor had the ability to improve the morphological appearance of PIs by promoting a unified cell size with a defined, smooth structural surface. The hypothesis of whether the improved structural appearance of PIs has the potential to enhance ECM expression is discussed later. The RCCS bioreactor method reconstitutes PIs into a morphological appearance that may show superior survival and biological functions of PIs ECM, which is the support for mammalian tissue *in vivo*, acts as a network 'glue' by connecting cells to each other in order to provide the biological communication and signalling needed for cell survival [190].

Furthermore, ECM plays an essential role in regulating cell behaviour by facilitating cell-cell/matrix interactions [51].

Thus, it was deemed essential to investigate normal ECM expression in our model, which is PIs cultured in dishes and RCCS bioreactor. The next part in this chapter details the analysis of specific ECM component (FN, Coll IV, and LmV) expression using qRT-PCR, western blotting, and ICC techniques.

ECM gene expression was performed using qRT-PCR relative to reference gene (GAPDH) expression. A previous study showed that ECM proteins were involved in islet cell functions such as insulin secretion and expression, and cell adhesion [51]. In addition to this, LM was found in another study to be involved in BM formation in islet cells [204]. Therefore, these specific ECM components were chosen for investigation in this study. From our findings, ECM gene expression was highly increased in PIs cultured in RCCS compared with cells cultured as PIs in dishes and as monolayer cells **[Figure 3-5,** panel A-C**].** More specifically, FN gene expression in PIs cultured in RCCS was significantly higher compared to PIs cultured in static dishes and monolayer cells **[Figure 3-5,** panel A, **P*<0.05**]**. FN is categorised under glycoprotein components of ECM that binds to membrane receptors called integrins [205]. More specifically, integrin α1 β1 is expressed by β-cells, close to peri-insular BM. Indeed, islet cells require signals from BM to survive and maintain the structural integrity essential for their secretory function. Moreover, a study by Maillard *et al*. (2009) showed that FN was major ECM component in the formation of PIs and also in the stability of cell matrix fibrillar adhesion [195]. Thus, this can give an insight into FN-islets interaction that induces appropriate cellular signalling mechanism needed for functional islets.

Further to the analysis of ECM gene expression, a significant increase in Coll IV expression was observed in PIs cultured in the RCSS, compared with control cells and PIs cultured in dishes **[Figure 3-5,** panel B, ***P*<0.001**]**. Collagens are considered ubiquitous structural proteins which provide mechanical support and are responsible for biological communication in living organisms [196]. Coll IV, investigated in this study, is one of the precursors of collagen fibres which are likely to be responsible for organisation of β-cells during neogenesis [198]. Previous reports suggested that collagens can potentially have an impact on β-cell development [206]. Thus, from these findings, our RCCS bioreactor has a positive impact on the reconstruction of PIs during

culture by preserving the structural integrity of ECM components where collagen fibres are located.

Additionally, a significant increase in LmV gene expression was observed in PIs cultured in the RCCS bioreactor, compared with PIs in dishes and monolayers **[Figure 3-5,** panel C,***P<0.0007]. Laminin is a non-collagenous protein that is also associated with the BM of the cell and involved in cell differentiation [198]. Thus, according to our findings, culture in both static dishes and the RCCS bioreactor enhanced gene expression of Coll IV and LmV compared with monolayer cells. This observation was supported by a previous study in which the gene expression of Coll IV was similar between PIs cultured in dishes and adherent cells at day 1, but that an increase in PIs Coll IV expression was observed after day 5 of culture [195]. Furthermore, Maillard *et al*. (2009) found that culturing Min6 β-cells on treated plate with ECM in monolayers and as PIs in dishes caused the gene expression of Lm 5 to significantly increase in PIs compared to monolayers; suggesting that Lm5 might be involved in PIs formation [195]. According to our findings, the gene expression of LmV was slightly higher in PIs cultured in dishes compared to monolayer cells. This observation may be due to the loss of the cells' capacity to express more LmV, a membrane-bound protein, because of the limitations of static cell culture conditions including insufficient transportation of nutrients and oxygen to all cells within the PI cluster, leading decreased formation of a complete matrix protein.

The present study findings confirmed that PIs, especially when cultured in RCCS bioreactor, expressed more ECM components compared to PIs cultured in dishes**.** The increase of the ECM expression was attributed to the effect of micro environmental factors existing in the bioreactor. This controlled microenvironment was adopted because of the presence of stable culture conditions required for living cells, such as constant nutrient concentration and sufficient supply of oxygen along with other gases.

Although a static cell culture environment lacks these conditions, rendering it close to insufficient to meet the demands of cell, PIs that have been cultured in this environment still expressed more ECM genes compared to monolayer cells. Therefore, simulated microgravity cell culture environments influenced the growth of cells in terms of structural appearance and biomolecular development, including the formation of ECM specifically present within the BM of cells from which it can response to environmental stimuli or changes affecting expression.

Following identification of increased ECM gene expression in simulated microgravity culture conditions, it was important to investigate this at the level of protein expression. ECM protein expression was determined using western blotting and quantified by densitometry analysis of visualised bands. Immunoblotting analysis revealed that all ECM proteins were highly expressed in PIs cultured in the RCCS bioreactor compared with cells cultured as monolayers. **Figure 3-6**, panel B-I, showed that PIs cultured in RCCS exhibited a significant increase in FN content compared with monolayer cells. Also, these was a significant difference was observed in FN protein expression between PIs cultured in static dishes and RCCS, supporting our previous finding that FN gene expression showed a slight differences in gene expression between these two culture methods. Thus, configuration of Min6 cells as PIs enhances the expression of FN correlating to the anatomical structure of cells that facilitates the cell-matrix interactions.

In addition to this, maintaining optimal microenvironment culture conditions, as seen in the RCCS bioreactor, significantly increases the expression of FN. There was a significant increase observed in Coll IV protein expression in PIs cultured in RCCS, compared with PIs cultured in dishes or cells cultured as monolayers **[Figure 3-6,** panel B-II, $**P<0.0009$**]**. Initial analysis of the PIs has shown an overall improvement of structure of clusters grown under RCCS conditions [**Figure 3-1, panel C]**. As collagen proteins are located in the BM, the increased collagen protein expression identified here

[Figure 3-6, panel B-II**]** maybe contributing to this overall improvement in structure seen.

An increase in LmV protein expression was also seen in PIs cultured in RCCS compared with PIs in dishes and cells cultured as monolayers **[Figure 3-6,** panel B-III, $^{**}P<0.0010$**].** Thus it can be concluded from these findings that culturing PIs in a simulated microgravity environment has the potential to increase PIs integrity and production of ECM proteins by providing conditions that mimic primary islet cells *in vivo.*

In order to further elucidate the role of ECM in PIs under two different culture environments, the subcellular localisation and expression of ECM components was determined by ICC. The ECM proteins of interest are found in two locations in the islet: the IM and the BM, the latter of which is arguably the most important of the two [77]. Experiments were performed using indirect immunofluorescence with polyclonal antibodies specific to the ECM proteins of interest. ECM proteins were mostly localised in the cytoplasm of PIs cultured both in static dishes and the RCCS bioreactor. ICC detection of all ECM proteins was observed in the cytoplasm and at the extracellular regions of cluster sections. **Figure 3-7** showed cytoplasmic staining of the FN protein, exhibiting similar expression intensity in PIs cultured in both environments. This result complements the data from qRT-PCR and western blotting whereby the expression of fibronectin showed a slight significant difference between the two systems.

Furthermore, Coll IV protein expression exhibited strong cytoplasmic staining in PIs cultured in the RCCS compared with PIs in static dishes **[Figure 3-8].** Strong cytoplasmic expression of LmV was seen in PIs cultured in the RCCS bioreactor compared with PIs cultured in static dishes **[Figure 3-9]**. These results further complemented our previous PCR and western blotting analyses. From these findings, it

can be conclude that the distribution of matrix proteins appears to be more abundant in the cytoplasm of PIs.

In pancreatic islet cells, the ECM surrounding the β-cells is produced by local endothelial vascular cells [207], forming an incomplete capsule made up of a single layer of fibroblast and collagen fibres. A supplementary matrix protein is attached to this capsule and known as peri-insular BM [208].

Chemical and mechanical signalling communication between β-cells and the ECM is known to regulate various physiological features such as survival, proliferation, and insulin secretion in islets [72, 209, 210]. Inside the pancreas, islet cells synthesise ECM components and deposit them to form a niche [211]. This niche determines the fate of cells as well as affecting their mechanical properties and tissue composition. However, this function is not replicated in vitro and β-cells rarely proliferate once this environment is removed. Therefore, attempts have been made to discover the composition of these niches that are necessary for β-cell expansion. Similarly, the relationship between islet ECM and cells is complex and co-dependent.

This framework provides various signals that determine tissue function and structure and occurs as a very thin layer outside the BM surrounding large ducts and blood vessels. The composition, thickness, and continuity of ECM differ between species [160]. Moreover, variation in ECM thickness is also influenced by age of the individual. Some studies have reported that the distribution of matrix proteins appears to differ in developing human islet cells. In early precursor islet tissue, both FN and Coll IV have been identified, while in developing islets, Coll IV and LmV have been identified [160]. Furthermore, other studies have supported our findings by reporting strong cytoplasmic staining of Coll IV protein expression in Min6-PI cells cultured in static dishes on day 3 of cluster formation [81].

Several studies have demonstrated the importance of re-establishing the ECM microenvironment as a key factor in improving β-cell survival and insulin secretion. In a study conducted by Weber *et al.* (2008), controlled matrix interactions were presented to Min6 β-cells in three-dimensional cultures within polyethylene glycol hydrogels [85] In this study, β-cells were encapsulated in gel environments containing several ECM proteins such as collagen type I, collagen type IV, LM, fibronectin, fibrinogen, and vitronectin. Results from this study revealed that β-cell survival was considerably higher than in gels without ECM proteins over a period of 10 days [85]. Additionally, β-cells in these encapsulated microenvironments were less likely to die, suggesting the capability of individual matrix interactions to prevent matrix signalling-related apoptosis. Specifically, β-cells cultured with Coll IV or LM proteins secreted more insulin in response to glucose stimulation compared with β-cells cultured in other ECM proteins. Overall, this study demonstrated that specific, realistically designed ECM microenvironments promote isolated β-cell survival and function.

Traditional techniques of cell manipulation and growth on 2D surfaces have been shown to be inadequate for cell biochemistry and biology, including islet transplantation. At present, 3D cell culture matrices with improved chemistry, geometry, and signalling environment of natural ECM have been possible with advancements in materials chemistry, fabrication materials, and processing technology [211]. Thus, a better understanding of communication between pancreatic β-cells and components of their local microenvironment may lead to progression in cell replacement therapies for the treatment of insulin-dependent diabetes. The capability to re-establish critical ECM and β-cell signalling may improve the islet culture techniques currently utilised in islet isolation and transplantation.

Clear conclusions can be drawn from the findings of this study, in that improved re-establishment of microenvironment culture conditions of islet cells has a positive impact

on maintenance of high structural integrity and on ECM expression, as these factors influence cell differentiation, survival, and function. In β-cells, cell matrix communication activates intracellular signalling pathways that regulate cell survival, proliferation, and many other cell processes. Therefore, this much-improved microenvironment is crucial for Min6-Pseudoislets growth and survival as well as for normal insulin secretion [**Figure 3-10**].

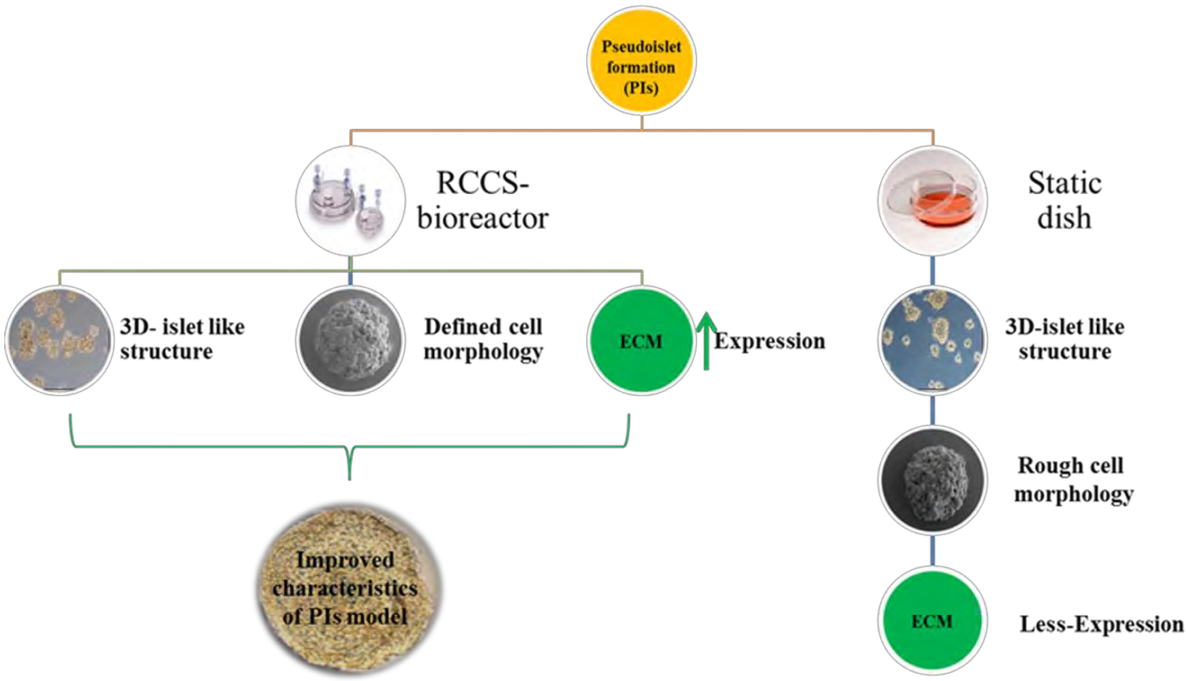

Figure 3-10: Schematic diagram, summarising the effect of rotary cell culture system (RCCS) on Min6-Pseudoislets.

RCCS-bioreactor has a positive effect on Min6-Pseudoislets model by maintaining high structural integrity and upregulate ECM expression, as these factors influence cell differentiation, survival, and function.

Chapter 4. **Cell Viability**

Abstract: Cell viability plays a fundamental role in all biological studies for evaluation of cell culture methods. Assays can be used as a monitoring tool for counting the number of cells, live or dead, and can determine the metabolic activity of cells. Cell death is an event whereby biological cells end their cellular functionality and can be caused by internal or external stressful stimuli. The most common assays for cell viability are the MTT and Hoechst propidium iodide (HPI) staining assays. This chapter reports the cell viability of Min6-Pseudoislets (PIs) cultured under static and simulated microgravity culture conditions, digested with accutase digestive enzyme at various time points, and recovered in static and simulated microgravity environments. The viability of PIs cultured under static and microgravity culture conditions was assessed. The digestion process at different time points (0, 30 seconds, 2 minutes, and 5 minutes) was evaluated using accutase and the viability of digested PIs was assessed. Digested PI clusters were allowed to recover in static dishes and in a RCCS bioreactor for 24 and 48 hours culture. Cell viability of recovered PIs was then examined. HPI results of PIs cultured in static dishes and in RCCS revealed viable cells; however, the metabolic activity of PIs cultured in RCCS was significantly increased compared with PIs cultured in static dishes as measured by MTT. Accutase had less effect on cell viability after 2 minutes digestion and a significant effect after 5 minutes. MTT results revealed that the metabolic activity of PIs digested for 2 minutes was significantly decreased compared with control cells and that at 5 minutes digestion, the metabolic activity of PIs was decreased. For recovery PIs, the metabolic activity of PIs recovered for 24 hours and 48 hours in static dishes was increased compared with control cells, while a significant increase was noted in PIs recovered under RCCS culture conditions. For HPI results, some apoptosis with necrosis was seen in PIs digested for 2 minutes, although more apoptotic and necrotic cells were noted in PIs at 5 minutes of digestion. Thus, 2 minutes of digestion was taken as the optimal time for digestion in this study to model the digestion of islets pre-transplant. HPI results for recovery cells in static dishes revealed some apoptotic cells and necrosis at the cell periphery compared with none in PIs recovered in our RCCS bioreactor. From these findings, it can be concluded that our novel RCCS bioreactor has a positive impact on PIs viability under mechanical stress caused by digestive enzyme and post-digestion.

4.1. **Introduction**

Cell viability is an essential parameter in many research studies. It has been used to determine whether cells are able to recover viability under culture conditions [212]. It can be used as a monitoring tool for counting numbers of live or dead cells, and can also be used to determine the anabolic activity of cells; thus, viability assays encompass the evaluation of living or dead cells [212]. Viability assays also serve to measure the number of healthy cells in a given culture prior to their use for various purposes. Different assay technologies are available to help researchers estimate the number of viable cells in a given culture medium [213]. The most common assays of cell viability are the MTT and Hoechst propidium iodide (HPI) staining assays [212]. The MTT assay is a colorimetric assay which makes use of the mitochondrial potential consumption rate of cells in order to estimate cell viability [212]. The HPI staining assay, mainly used to identify living or dead cells, is a rapid technique for examining cell viability using fluorescence analysis with colorimetric differentiation of viable, apoptotic, and necrotic cells.

Cell viability is a fundamental factor, not only in research, but also in the clinical field. In this study, islet viability is one of the most crucial factors in clinical islet transplantation [24]. Islet cell transplantation has produced significant effects on the quality of life of diabetic patients, especially those with brittle T1DM [116]. Although this therapeutic procedure has been recently considered as a promising cure, some obstacles limit the success of islet engraftment in which the key factor that influences the success of islet transplant is the isolation process [214, 215]. Ryan *et al.* (2005) proposed a clinical islet transplant procedure following the Edmonton protocol [116].

Sixty-five patients with hypoglycaemia were evaluated, of which 44 completed the transplant procedure and became insulin independent. After the 5-year follow up, 80% of subjects returned to insulin injection.

The reasons behind the failure to achieve long-term insulin independence included sub-optimal islet isolation and the toxicity of immunosuppressant medications [96]. Therefore, it was proposed that the proper selection of enzymes with an appropriate incubation time may greatly influence post-isolation outcomes. However, the selection of the most appropriate enzymes is not yet clear [216]. Thus, the optimisation of the islet isolation process is a critical step in obtaining high islet integrity with high cell viability.

Achieving highly effective islet transplantation relies on proper optimisation of islet isolation procedures. It is essential to perform this optimisation in order to achieve high efficacy in islet integrity and functionality [217, 218]. There are two major factors affecting islet yield, the selection of a suitable donor and the enzyme characteristics [214]. Balamurugan *et al.* (2012) reported that a mixture of neutral protease with controlled concentrations of collagenase class 1 enzyme showed increased islet yields while preserving islet functionality [219]. Thus, successful islet isolation has a marked impact on islet transplantation outcomes because preserving the characteristics of high quality and purity in the collagenase enzyme has a great influence on islet viability. Moreover, another factor which influences the efficiency of collagenase enzyme activity is the optimum time for incubating islet cells with the enzyme. A study showing that prolonged incubation in cold storage of the pancreas during islet isolation results in the loss of islet integrity and promotes cell death [220]. Therefore, uncontrolled islet isolation could potentially affect islet viability, thus limiting the success of islet engrafting post-transplantation.

The loss of islet cell viability is contributed to by the activation of a cascade of stress responses inside the cell.

The activation of these cascades in turn activates caspases along with other inflammatory mediators such as NF-κB, triggering apoptosis [101]. Programmed cell

death or apoptosis could result due to physiological or pathological stimuli [221]. Cells can sense either external or internal cell death signals in order to control cell growth and development. However, if cells fail to receive that controlled death signal pathological cell death will take place [222]. Therefore, programmed cell death is considered a critical step in cell development by which the physiological state of cells can be determined.

Culturing islet cells post-isolation is an important process for storing cells prior to transplantation. However, some studies have reported that culturing islets before transplantation affects the viability of cells by causing necrosis at the central core of islets; researchers proposed that this was due to inadequate nutrient or oxygen supply for the islet size [165]. However, other studies have supported the idea of culturing islet cells prior to transplantation as they found that islet culture could be a valuable preparation step for cells to be purified and protected against rejection after transplantation [223]. Traditionally, studies tended to culture islet cells post-isolation in conventional static conditions. Although this type of environment maintained islet viability, some factors limited islet survival in the long-term and contributed to the shortage of nutrients and oxygen transportation resulting in cell death. Holmes *et al.* (1995) showed that such shortages in static cell culture methods had a huge effect on the hormonal functionality and on the morphological structure of rat, porcine, and human islet cells [224]. Also in term of using Min6-Pseudosilets (PIs), the shortages of an adequate transportation had led to enlarged or irregularly-sized PIs which could potentially induce apoptosis and affect cell proliferation mechanisms [152, 225]. These observations indicate that the microenvironment culture condition is critical in terms of maintaining islet survival.

3D cell culture methods using bioreactors are a new technology developed by NASA [226]. There are many types of bioreactors available, but the RCCV is most commonly

used in biological studies and was adopted in this study. The environment inside an RCCV tends to be a favourable condition for cells to live in because of low-shear modelled microgravity found there and the abundant transportation of nutrients and oxygen to cells during vessel rotation [167]. This homogenous microenvironment provides tight control over cell size, enhancing the efficiency and viability of cells [153]. Primary pancreatic islet cells typically do not proliferate within *in vitro* culture systems, so recently transformed islet cells from different species have been used as alternative models [188]. Brit et *al.* (1981) and Halban *et al.* (1987) reported that cultured transformed islet cells in static dishes were capable of aggregating to generate islet-like clusters known as PIs [188, 189]. PIs were originally generated using static cell culture methods. This static environment has a marked impact on PIs biology in terms of cell viability. In PIs characterised after 1 week of culture, uncontrolled size was observed and this contributed to insufficient nutrient and oxygen supply [153]. Although 3D clusters have been successfully generated from 2D culture environments, their efficiency is far removed from *in vivo* states. Thus, 3D culture methods have been applied in a wide range of cellular research projects as they permit the long-term maintenance of tissue. Accordingly, Lock *et al.* (2011) cultured Min6 β-cell structures in stirred-suspension cultures in order to generate PIs [153]. They concluded that the stirred-suspension culture environment preserved morphology and functionality of PIs which subsequently exhibited superior cell viability with improved insulin secretory mechanisms [153]. Although their study reported improved outcomes of culturing Min6 β-cells under 3D culture environments, some limitations remained and were investigated in this current study.

Here, the cell viability of Min6-PIs, cultured in static cultures and rotational culture system (RCCS) conditions, was investigated with respect to the effect of culture conditions on PIs viability. Accutase digestive enzyme, which is a mixture of

collagenase and proteolytic enzymes, was subsequently used to mimic the islet isolation process *in vitro* by stressing PIs viability at specific times and assessing that viability. The benefit of using accutase in this study came from its negligible effects on cell viability [227]. Stressed PIs were then allowed to recover, under the same culture conditions in which they were cultured initially, after which cell viability was again measured at suitable intervals. The cell viability of PIs was assessed using the common tests such as the HPI staining assay, which accounts for the membrane integrity of cells to provide information about cell viability [228]. Additionally, an MTT assay which makes use of mitochondrial potential and oxygen consumption rate of cells to estimate cell viability was used in this study [229].

4.2. **Results**

Viability Assays

The purpose of assessing cell viability is to measure the number of healthy cells in a given culture and to relate the behaviour of cells to their numbers and when measuring metabolic capacity. PIs viability was determined by selecting 70 clusters, cultured under normal static or simulated microgravity (RCCS) culture conditions. Clusters were digested with accutase enzyme at 0, 30 second, 2 minute and 5 minute time points, were re-cultured under static and RCCS culture conditions for 24 and 48 hours for recovery.

4.2.1. **MTT assay**

MTT, a colorimetric assay using NAD (P) H-dependent cellular oxidoreductase enzyme produced by mitochondria, assesses the viability of cells by converting tetrazolium enzyme into purple insoluble formazan, indicating that cells are alive. The insoluble crystals were dissolved in DMSO and the absorbance of samples was measured at 540 nm on a plate reader (Multiscan plate reader, Thermoscientific®). MTT assays were performed by selecting PI clusters according to the stated experimental conditions

(section 4.2). Mean absorbance of triplicate samples with standard deviation was calculated as a percentage of viability of each stressed concentration against the average absorbance of the positive control, assuming that the viability of the control sample was 100%. Statistical analysis was performed using Graph Pad Prism 5 (version 5.03) software and two-way ANOVA with a Bonferroni post-test applied.

4.2.1.1. *Viability of Min6-Pseudoislets.*

Pseudoislet clusters were selected using a dissecting microscope from 6 mL static dish volumes and from 10 mL RCCS-bioreactor volumes cultured under normal conditions. Clusters were washed three times with PBS for 5 minutes each, and then transferred into 12-well plates. MTT solution (0.5 mg/mL) was added to each well and plates were incubated for 1 hour. DMSO was then added to dissolve the formazan and plates were incubated for 5 minutes on an orbital shaker. The well contents were analysed at 540 nm on a plate reader, with a DMSO blank included. **Figure 4-1** shows a two-fold increase in metabolic activity was measured in PI cluster cultured in RCCS compared with control cells (**P<0.0033). PI cells cultured in static dishes were used as controls in this experiment. After determining the viability of PIs cultured under different conditions, PIs were selected from static dishes and used as a control to test the viability of clusters which had been digested with accutase enzyme, as discussed in the next section.

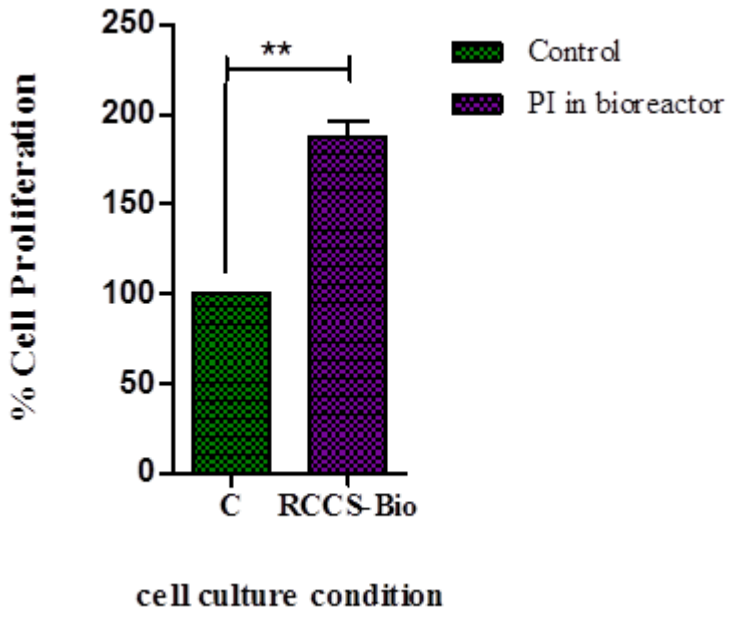

Figure 4-1: Cell proliferation of Min6-PIs in static dishes and an RCCS bioreactor, evaluated by MTT assay.

Ten PIs were selected from static dishes and the RCCS bioreactor, and incubated with MTT solution for 1 hour. DMSO was added to dissolve formazan and absorbance was measured at 540 nm. Results were representative of three separate experiments and error bars represent mean ± standard deviation. A two-fold increase in metabolic activity was observed in PIs cultured in the bioreactor compared to control cells (** $P<0.0033$).

4.2.1.2. *Viability of Digested Min6-Pseudoislets.*

The viability of digested PIs was assessed in order to investigate the effect of accutase digestive enzyme and examine whether enzymatic digestion has an effect at specific incubation time points. In this experiment, the viability of PIs digested at different times (0 minute, 30 second, 2 minute, and 5 minute time points) was examined. Seventy clusters were selected from static dishes and an RCCS using a dissecting microscope. Enzymatic digestion was achieved using accutase enzyme for different times and clusters were incubated with MTT solution (Chapter 2, section 2.2.7.1) followed by DMSO. The dissolved formazan solution was analysed at 540 nm on a plate reader and a DMSO blank was included. **Figure 4-2** shows that no significant difference was observed between PIs digested for 30 seconds and control cells. However, the cell number, as indicated by metabolic activity of PIs digested for 2 minutes was decreased approximately 1.5-fold compared with control cells **[Figure 4-2,** D2**].** In contrast, a significant decrease (two-fold) was noticed in PIs digested for 5 minutes compared with control cells. PIs were used as a control in this experiment. From these findings, the 2 minute digestion time point was selected because of the reduced effects on cell viability observed under these conditions.

4.2.1.3. *Viability of Recovered Min6-Pseudoislets.*

In order to determine whether re-culturing PIs post-digestion may improve viability, PIs digested for 2 minutes were re-cultured in static dishes in a RCCS bioreactor for 24 and 48 hours. After 2 minutes of digestion, PIs were allowed to recover for 24 or48 hours by transferring the digested PIs into fresh medium under static and RCCS culture conditions (Chapter 2, section 2.2.5). The viability of recovered PIs was assessed by MTT assay as described in section 4.2.1.

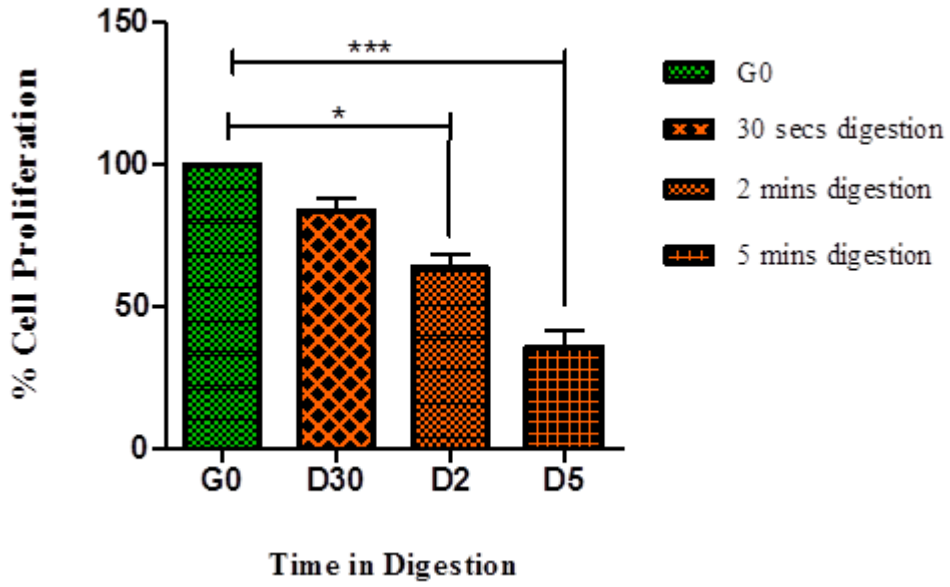

Figure 4-2: Cell proliferation of digested Min6-Pseudoislets (PIs) assessed by MTT assay.

Ten PIs from static dishes were selected using a dissecting microscope and transferred to 12-well plates. Clusters were washed three times with PBS and then incubated with 200 µL accutase enzyme for different time; zero (G0), 30 second (D30), 2 minute (D2), and 5 minute (D5) time points. The reaction was terminated by adding fresh media and then washed three times with PBS. Clusters were incubated with MTT solution for 1 hour and DMSO was then added to dissolve formazan. The contents of well were transferred into a 96-well plate for absorbance reading at 540 nm. Results are representative of three separate experiments and error bars represent mean ± standard deviation. No significant difference was observed between PI digested for 30 seconds and control cells. A significant decrease of approximately 1.5-fold in metabolic activity was observed in PIs digested for 2 minutes compared with control cells (*P<0.05). Furthermore, a 2-fold decrease in metabolic activity was seen in PIs digested for 5 minutes compared to control cells (***P<0.0001).

Figure 4-3 shows that the metabolic activity of PI cells recovered in static dishes for 24 and 48 hours (*P<0.05) increased by approximately 1.5-fold compared with control cells. By contrast, a significant, 2-fold increase (***P<0.0001**)** was seen in PIs recovered in the RCCS for 24 and 48 hours, compared with control cells. From these findings, it can be concluded that re-culturing PIs post-digestion can potentially increase cell viability, especially in cells recovered in the RCCS bioreactor.

Following assessment of PI viability by MTT assay, the findings were confirmed using an HPI staining assay to identify living or dead cells inside PI cluster cultured according to the stated experimental conditions.

4.2.2. **Hoechst Propidium Iodide (HPI) staining**

MTT viability results were confirmed using HPI staining assay to identify living or dead PIs cultured in static dishes or in RCCS-bioreactor; PIs evaluated were digested with accutase enzyme or were post-digestion, as discussed in the following sections. The HPI assay is a rapid technique for examining cell viability using fluorescence analysis with colorimetric differentiation of viable, apoptotic, and necrotic cells. HPI staining was performed using the following reagents: propidium iodide (1 mg/mL), Hoechst dye (5 mg/mL), and fresh culture medium (Chapter 2, section 2.2.7.2). The experiment was initiated by harvesting clusters according to the experimental conditions mentioned previously. PIs were then incubated with HPI solution for 5 minutes in a cell culture incubator. The analysis was performed using confocal microscopy under x40 magnification. As both Hoechst and propidium iodide are nuclear stains, the results were represented as blue staining (indicating live/viable cells), red staining (indicating necrotic/dead cells), or bright blue staining (indicating apoptotic cells).

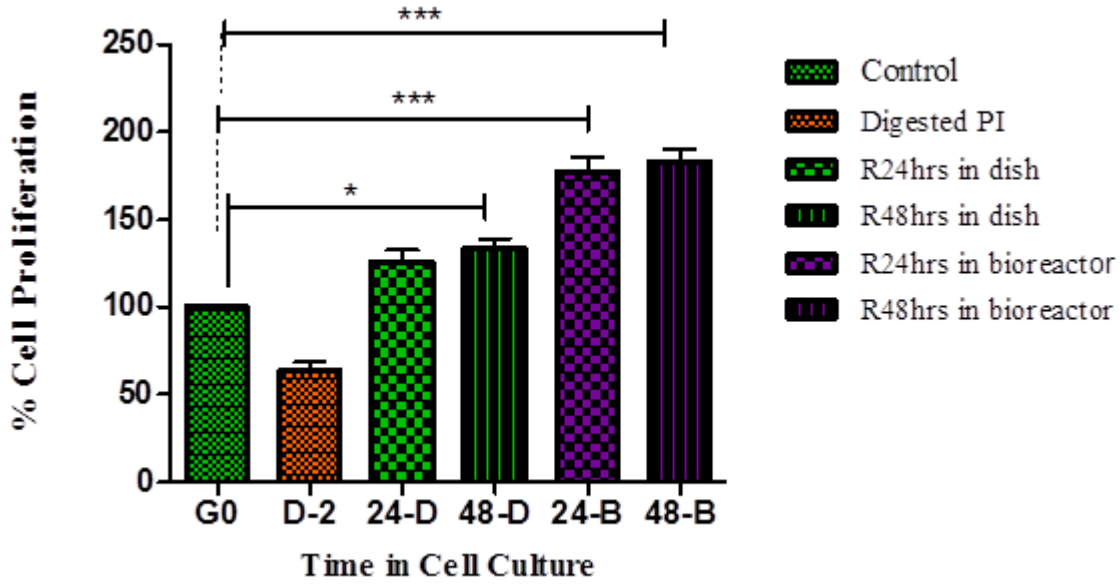

Figure 4-3: Cell Proliferation of Min6-Pseudoislets (PIs) post-digestion.

Ten PIs were digested for 2 minutes and then re-cultured in static dishes or in an RCCS-bioreactor for 24 and 48 hours for recovery. Similar numbers of clusters were selected from types of cultures and incubated with MTT solution. DMSO was then added to dissolve formazan, and absorbance was read at 540 nm. Results were representative of three separate experiments and error bars represent mean ± standard deviation. A significant increase, approximately-1.5 fold , was observed in the metabolic activity of PIs recovered in static dishes for 48 hours compared with control cells (*P<0.05). However, a 2-fold increase in metabolic activity was seen in PIs recovered in an RCCS bioreactor for 24 hours and 48 hours (***P<0.0001) compared with control cells.

4.2.2.1. *Viability of Min6-Pseudoislets.*

Viability testing of Min6-PIs cultured under static or RCCS culture conditions showed the presence of viable cells **[Figure 4-4].** The previous MTT results were confirmed by HPI staining, indicating that both culture systems produced viable cells. In the following section, the viability of PIs digested with accutase enzyme at different time points is described.

4.2.2.2. *Viability of Digested Min6-Pseudoislets.*

This experiment aimed to confirm the optimisation of the digestion process performed previously. The viability of digested PIs was examined by HPI at similar digestion time points (section 4.2.1.2) with accutase enzyme **[Figure 4-5].** The purpose of this experiment was to confirm previous MTT results and to examine the effect of a digestive enzyme on PIs by identifying living or dead cells. Results showed that accutase enzyme digestion affected PI viability at specific incubation time points. After 30 seconds **[Figure 4-5,** D30**],** cell viability was not affected. However, after 2 minutes of digestion, cell viability was slightly affected as indicated by the presence of apoptotic cells **[Figure 4-5,** D2**].** The digestive enzyme exerted a detrimental effect on PIs viability after 5 minutes of digestion **[Figure 4-5,** D5**]** as indicated by the presence of necrotic cells and apoptotic cells (especially at the peripheral edges). The previous MTT results were supported by HPI findings confirming that prolonged incubation times with accutase enzyme have the potential to affect cell viability and metabolic activity. The following section discusses the viability of PIs post-digestion.

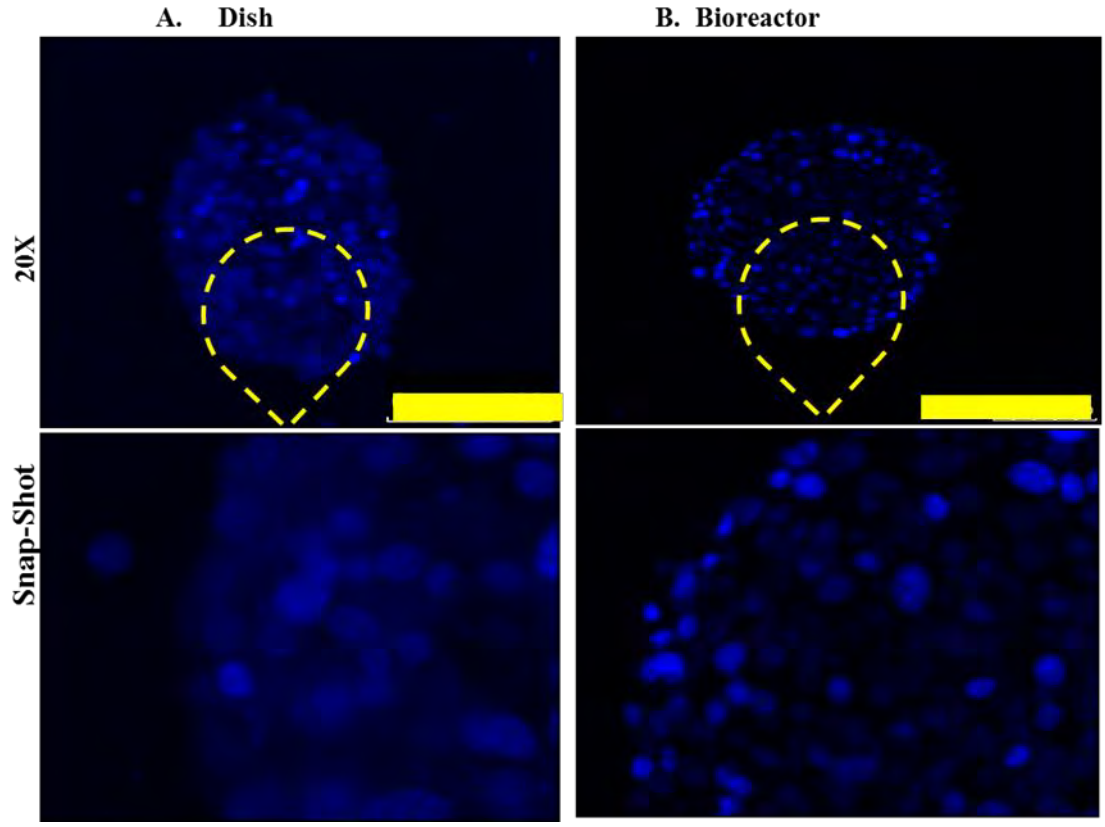

Figure 4-4: Viability of Min6-Pseudoislets (PIs).

Ten PI clusters were selected from both static dishes and an RCCS and incubated with the HPI staining mixture. Images were obtained using confocal microscopy using DAPI/Hoechst filters and three separate experiments. PIs in dishes were used as controls in this experiment (A). Images were captured at x20 magnification. The majority of cells in PI clusters cultured in dishes and in the RCCS were shown to be viable (blue staining). Scale bar: panel A =100 μM; panel B = 150 μM. **SNAP-SHOT** is a selected area, closed capture, of over-lay image.

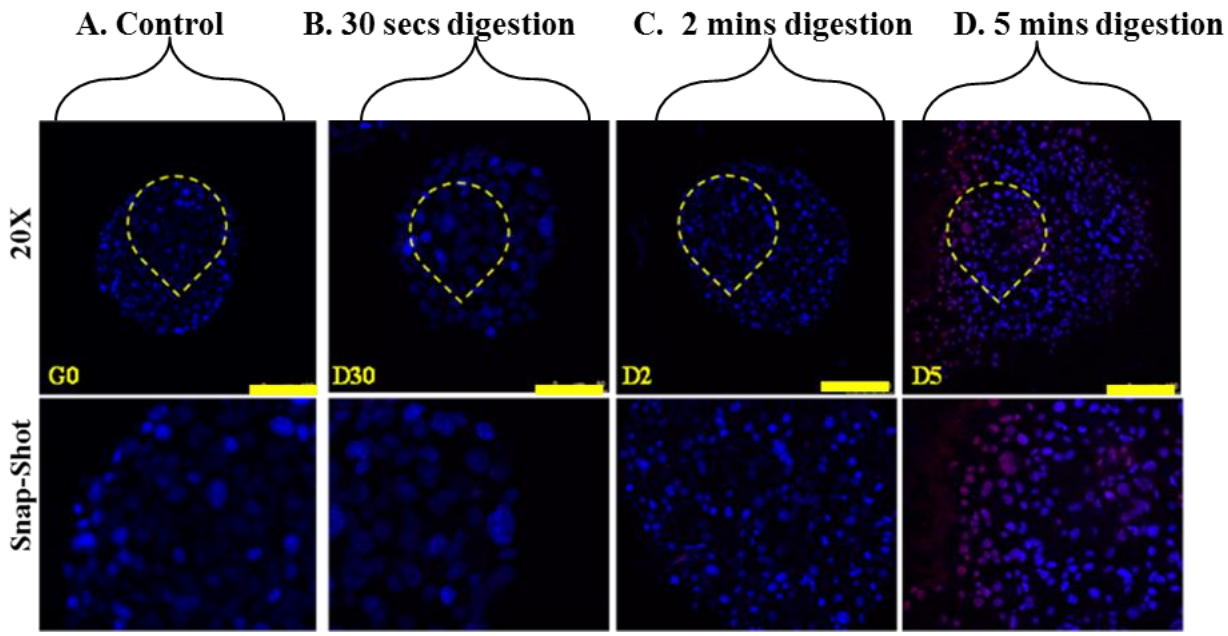

Figure 4-5: Viability of digested Min6-Pseudoislets (PIs) by HPI staining.

Ten PI clusters were selected from the RCCS and digested with accutase enzyme for zero (G0), 30 second (D30), 2 minute (D2), and 5 minute (D5) time points. Images were examined using confocal microscopy and DAPI/Hoechst filters. Three separate experiments were performed. Control cells used were PIs in dishes (A), and images were captured at x20 magnification. Digestive enzyme had no effect on PIs digested for 30 seconds (panel B-D30). Following 2 minutes of digestion (panel C-D2), the viability of cells was slightly affected as indicated by the presence of some apoptotic cells at the periphery. At 5 minutes of digestion (panel D-D5), however, apoptosis and necrosis were apparent in PIs. Prolonged incubation with accutase digestive enzyme therefore triggers apoptosis and necrosis in PIs. Scale bar =100 μM**. SNAP-SHOT** is a selected area, closed capture, of over-lay image.

4.2.2.3. *Viability of recovered Min6-Pseudoislets.*

The cell viability of PIs that were recovered in static dishes or the RCCS bioreactor was examined using HPI staining. The viability examination of PIs post-digestion was done in order to evaluate the recovery process performed at suitable incubation times under different culture conditions, and to confirm our previous MTT results. Error! Reference source not found. shows that the cell viability of PIs recovered in static dishes for 24 and 48 hours was affected **[Figure 4-6,** R24-D & R48-D**]**, as indicated by the presence of some necrotic cells at the centre of the cluster. The viability of PIs re-cultured in the RCCS bioreactor was not affected, however; viable cells were observed after 24 and 48 hours of recovery time **[Figure 4-6,** R24-B & R48-B**].**

Therefore, it can be concluded that the culture of PIs in our RCCS bioreactor pre- and post-digestion can preserve the viability of cells and enhance it post-digestion. After characterising Min6-PIs (Chapter 3) and optimising PI viability pre- and post-digestion, it was considered essential to investigate the effects of enhanced morphological structure and increased cell viability on specific biological markers crucial for Min6-PI functionality post-digestion.

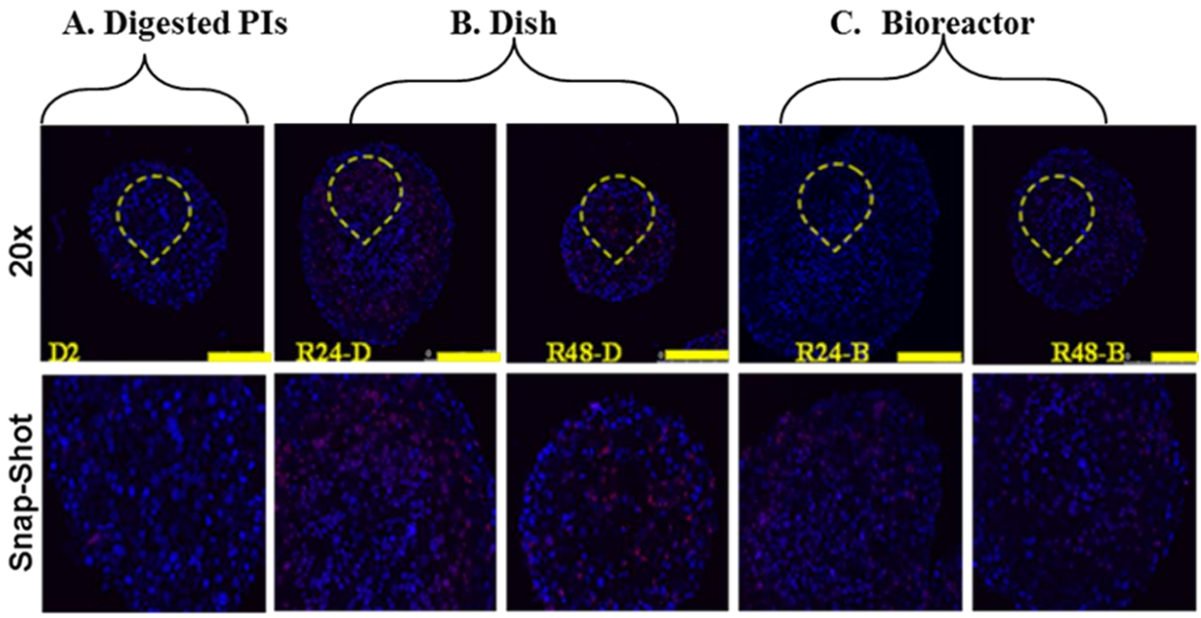

Figure 4-6: Viability of recovered Min6-Pseudoislets.

Ten Pseudoislet clusters were digested for 2 minutes (A) and then re-cultured under static (B) and RCCS bioreactor (C) culture conditions for 24 and 48 hours. Three separate experiments were performed. Confocal microscopy was performed and images were captured at 20× magnification. Recovery PIs in static dishes showed some apoptosis and necrosis (panel B, R24-D and R48-D). However, PIs recovered in the RCCS bioreactor were viable at 24 and 48 hours (panel C, R24-B and R48-B). Scale bar: panel A-B = 100 µM; panel C= 150 µM**. SNAP-SHOT** is a selected area, closed capture, of over-lay image.

4.3. **Discussion**

In this chapter, the cell viability of Min6-PIs cultured under different experimental conditions was investigated. PIs were cultured in static dishes and an RCCS bioreactor before digesting with collagenase (Accutase) digestive enzyme at different time points, and re-culturing post-digestion in static dishes and the RCCS for 24 and 48 hours. The aim of assessing the viability of PIs was; one to investigate the effects of different culture conditions on PIs viability; second to investigate the effects of digestive enzyme on PIs viability; and third to investigate the effects of the recovery period on PIs viability following re-culture under static and RCCS culture conditions. Thus, the overall aim of this work was to preserve the viability of the PIs model. In the previous chapter, the morphological structure and ECM interactions with PIs was shown to improve after culture in our RCCS bioreactor, so the hypothesis was whether an improved morphological appearance along with increased ECM expression has an impact on PIs viability or not. The standard methods of assessing cell viability used in this study were the MTT assay and the HPI assay. Both MTT and HPI assays were performed by selecting 70 PI clusters, cultured at different experimental conditions as previously described. MTT assay results showed that the metabolic activity of PIs cultured by RCCS was significantly increased (2-fold) compared with control cells, which were PIs cultured in a static dish **[Figure 4-1]** (***P*<0.0033). This observation was confirmed by HPI staining assay which indicates the capacity of cells to absorb specific dyes. Results showed that the majority of cells in PI clusters cultured in dishes and in the RCCS were viable (blue staining) [**Figure 4-4]**.

A previous study has reported that the culturing of cells in a rotary suspension bioreactor enhances the viability, efficiency and homogeneity of embryonic stem cells [230]. Moreover, Murray *et al*. (2005) reported that human islet cells cultured under a rotational culture system showed decreased necrosis, likely related to a high level of gas

exchange that preserves islet integrity [177]. Thus, the present findings supported these previous studies.

In terms of clinical transplantation, islet cells are vulnerable to death during islet isolation and prior to transplantation, and factors such as the health and age of the donor, duration between the donor's death and islet harvest, and organ ischemia reduce their viability [231]. Nutrition and an oxygen supply are two basic essentials for viable cells, and a compromised oxygen supply or inadequate nutrition can lead to cell death by causing irreversible damage to the cell [232]. Both factors are in turn dependent upon the blood supply, which is maintained through the circulatory system. Microcirculation in the immediate vicinity of cells is a major determinant of viability. In addition to these factors, the structural support provided by tissue matrix is crucial as it not only holds cells in place but also maintains a microenvironment that contributes to cell viability [233].

Additionally, islet cells undergo a significant cascade of stressful events such as apoptosis, necrosis and activation of inflammatory responses that ultimately causes damage to islet functionality [101]. These stressful factors may potentially threaten islets viability and lead to cell death [234]. Contributing to these damaging events, several studies have reported that β-cells do not produce enough endogenous antioxidants naturally, making them vulnerable to oxidative stress induced during the isolation process [235].

In this study, an *in vitro* isolation process was developed which mimicked islet isolation. Accutase digestion enzyme, a mixture of collagenase and proteolytic enzymes, was used for the digestion procedure. The digestion process was optimised to investigate the effect of the enzyme on PI viability at different time points (30 seconds, 2 minutes and 5 minutes). The overall aim of this experiment was to preserve the viability of PIs post-digestion.

The results presented in **Figure 4-2** showed that the metabolic activity of PIs digested for 30 seconds with accutase enzyme was not affected; however, after 2 minutes of digestion, the metabolic activity of PIs decreased approximately 1.5-fold compared with control cells (*P<0.05). A significant decrease (***P<0.0001) was observed in the metabolic activity of PIs digested for 5 minutes (2-fold) compared with control cells **[Figure 4-2].** This may be indicative of a reduction in cell number. These findings were confirmed by HPI staining assay which showed that the viability of PIs digested for 30 seconds was not affected while after 2 minutes the viability was slightly affected as shown by the presence of some apoptotic cells at the PIs periphery **[Figure 4-5,** D30 & D2**].** By contrast, the viability of PIs digested for 5 minutes was significantly affected, showing both apoptosis and necrosis **[Figure 4-5,** D5**].** This increased cell death correlates with the decreased metabolic activity measured by MTT assay, indicating that prolonged incubation with accutase digestive enzyme can reduce the number of viable PIs. This negative effect of accutase enzyme on PIs is only seen at longer treatment. Two minutes was thus chosen as the optimal digestion time in this study as the 30 second time point had no effect on cell viability but 5 minutes showed increased damage to cells. A study on a canine islets model showed that the islet isolation process destroyed the islet-matrix relationship, leading to structural and functional changes which resulted in cell death [77]. Additionally, it has been reported by other researchers that peri-insular BM, one of the ECM components, was lost during the isolation process [234]. Thus, any damage to BM, where cell-matrix interactions take place, has the potential to induce apoptosis or necrosis in cells. Barshes *et al.* (2005) reported that apoptosis occurs due to stressful events and activates a significant cascade of pro-inflammatory mediators that are stimulated by inflammatory cells [236].

These intracellular inflammatory mediators are involved in islet dysfunction. Once islet cells become stressed during the isolation process, the activation of inflammatory cells

(such as macrophages) takes place. As a result, pro-inflammatory mediators, notably interlukin-1 β (IL-1β), are released, causing apoptosis and cell death [237]. Additionally, the activation of IL-1β during the isolation process can affect insulin biosynthesis and secretion from islet β-cells [238]. Thus, the activation of inflammatory cascades induced by mechanical stress is most likely sensed by receptors interacting within the cell-matrix. This interaction is essential in order for cells to respond to external stimuli, regardless of whether that response is normal or abnormal.

The findings presented in this chapter show that accutase digestive enzyme has the potential to induce apoptosis in PIs, even after a short incubation time for digestion. To address the factors influencing islet viability post-isolation, many studies have focussed on how to optimise cell culture conditions in order to preserve islet integrity and functionality. A mouse model study by Rutsky *et al.* (2002) cultured mouse islets, post-isolation, under an RCCS or conventional culture followed by transplantation into diabetic mice. Islet cells were shown to survive for over 100 days when cultured under the RCCS condition, unlike cells cultured in dishes which survived for only 15 days [239]. In order for transplants to be successful, islet cells must be viable in culture in order for them to start secreting insulin upon injection into a transplant patient. According to the viability results presented in this chapter, the findings were in agreement with the previous studies showing that microgravity culture conditions have the potential to improve cell viability even after mechanical stress.

The RCCS and conventional culture dishes were chosen for re-culturing PIs that were digested for 2 minutes and then allowed to recover for 24 and 48 hours. The purpose of this experiment was to identify suitable recovery culture conditions at an appropriate time point in order to investigate the effect of the recovery process on PIs viability. Results showed that the cell proliferation of PIs recovered in static dishes was significantly increased (*P<0.05), approximately 1.5-fold (especially after 48 hours)

compared with control cells **[Figure 4-3,** R24-D and R48-D]. However, HPI staining results showed that the viability of PIs after recovery was affected, as some necrosis and apoptosis was observed under static culture conditions **[Figure 4-6,** R24-D and R48-D**]**. This observation could be due to the limitation of the static dish microenvironment, where poor nutrient transportation causes cessation of PIs growth after one week, which was seen in a study by Lock et al. (2011), and is likely the main cause of necrosis and apoptosis [153]. PIs allowed to recover under RCCS conditions for 24 and 48 hours showed significantly increased cell proliferation (*** $P<0.0001$) by 2-fold compared with control cells **[Figure 4-3,** R24-B and R48-B**].** Furthermore, HPI results showed that PIs recovered for 24 hours and 48 hours under RCCS culture conditions exhibited more viable cells than PIs recovered in static dishes **[Figure 4-6,** R24-B and R48-B**].** It can thus be concluded that the RCCS bioreactor presents a preferable environment for PIs recovery from mechanical stress caused by accutase digestive enzyme. In accordance with several previous studies, this may be due to the simulated microgravity environment which is characterised by the continuous transportation of nutrients, metabolites and oxygen, essential factors for cell survival (unlike the microenvironment found in conventional culture dishes). Furthermore, other studies have indicated that culturing human islets with rotational cell culture systems compared to static dishes sustains islet integrity and functional viability [177]. Thus, optimising conditions to be more suitable for islet cell post-isolation culture is crucial to allow cells restore their fragile construction and functionality in order to prolong islet engraftment.

This chapter concluded that our RCCS bioreactor exerted a positive impact on PIs by promoting increased survival of Min6-PIs by enhancing cell viability under normal culture and post-digestion conditions. Therefore, the RCCS bioreactor may be used as a dynamic culture environment, capable of producing highly viable cells.

Figure 4-7 illustrates a summary of the overall culture processes described in this chapter.

Having determined the effect of our novel RCCS bioreactor on PIs viability before and after the digestion process, the next step was to investigate whether PIs-matrix attachment was degraded during enzymatic digestion and post-digestion. This objective is addressed in the next chapter.

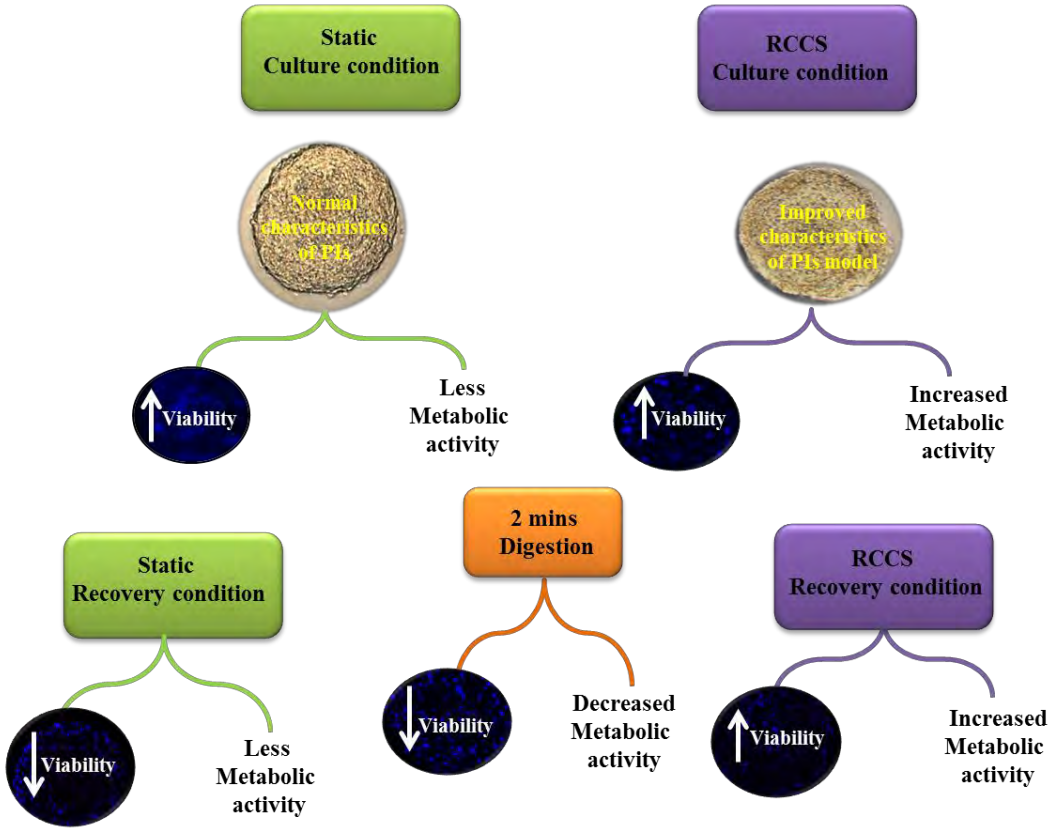

Figure 4-7: Schematic diagram summarising the optimisation of Min6-PI viability.

From the diagram, an improved morphological structure of PIs cultured under RCCS is presented, with the ability to enhance cell viability compared to static culture conditions. After 2 minutes of enzymatic digestion, both the viability and metabolic activity were significantly decreased; following recovery, however, a significant increase in PI metabolic activity and improved cell viability was observed after 48 hours of recovery, compared to PIs cultured in static dishes.

Chapter 5. **The effect of enzymatic digestion and remodelling on Min6-Pseudoislets.**

Abstract: Islet cells are exposed to significant mechanical stress during and after the isolation process, causing damage to islet structural integrity and affecting cell-ECM interactions. In this chapter, two *in vitro* models were assessed by enzymatic digestion that mimicked the islet-isolation and remodelling process and re-culturing. These were Min6-Pseudoislets (PIs) cultured in static dishes and in an RCCS bioreactor. The appropriate time required to repair the damage caused to PIs by digestion was evaluated. Thus, this chapter examines the effects of the digestion and recovery processes on the morphological structure and ECM expression of PIs. Specific ECM components fibronectin (FN), Collagen IV (CollIV), and Lamanin V (LmV) were investigated. Previously, the effect of increasing digestion times on cell viability was determined. Here in this chapter, it was important to consider the effect of these incubations on the structural surface and ECM expression of PIs. In this study, two minutes of digestion was taken as the optimal time because of the minimum degradation effect observed at this time point. Digested PIs were transferred to static dishes and RCCS to recover for 24 hours and 48 hours. The structural surface of digested and recovery PIs was examined using light microscopy and SEM. ECM expression of digested and recovery PIs was determined using qRT-PCR, western blotting, and ICC. Results showed that the outer surface of PI clusters (PIs 'coat') was partially degraded by 2 minutes of digestion, while a complete loss of the PI coat was seen following 5 minutes of digestion. SEM analysis of PIs digested for 2 minutes showed a degraded structural surface and ECM gene and protein expression was significantly decreased. Following digestion for 2 minutes, clusters were re-cultured in static dishes and RCSS and microscopic analysis showed restoration of the PI coat. Improved restoration was observed in PIs allowed to recover in an RCCS. ECM gene and protein expression was significantly increased in PIs recovered in an RCCS compared to static dishes. ECM localisation of PIs digested for 2 minutes and recovery PIs was cytoplasmic, with decreased expression seen in digested PIs and increased expression in recovery PIs. From these findings, it was concluded that maintenance of optimised culture conditions for Min6-PIs post-digestion can preserve cell structural integrity and enhance PI-ECM interactions.

5.1. **Introduction**

The success of islet transplant outcomes is dependent on identifying a viable method to repair the damage caused during and after islet isolation, in order to achieve improved islet survival [166]. It has been observed that, post-isolation of β-cells, a disruption of islet integrity and cell-matrix interaction was induced, leading to apoptosis [215, 234]. Furthermore, Rosenberg *et al.* (1999) reported that the peri-insular BM of islet cells was lost, immediately after the isolation process, leading to the activation of apoptotic and inflammatory pathways [234]. Therefore, in order to preserve the viability of islet cells and re-establish lost ECM-islet interactions, a preservative strategy should be adopted. To alleviate the damage caused by digestive effects post-isolation, it is important to first optimise the isolation method and re-establish a viable culture environment in order to successfully maintain islet survival. Many researchers have sought to optimise the isolation process in order to minimise the damage caused to islets and prolong the life span of cells post-transplantation. The major issue hindering the quality of islet isolation is the gel-like structure which builds up during collagenase digestion and surrounds islets, damaging islets yield [240]. Typically, the dissociation of pancreatic tissue is performed using *Clostridium histolyticum* collagenase, a mixture of collagenotic and proteolytic enzymes [241]. Well-optimised and controlled characteristics of collagenase enzymes, such as concentration and pH, are essential for improving islet yields. A study by Amoli *et al.* (2005) was conducted on rat pancreatic islets [240], where several factors were optimised that could hinder the quality of collagenase enzymatic digestion, such as optimal concentration of enzymes, pH ranges (between 7.7 and 7.9 was determined as ideal), and incubation temperature; 38±1°C was considered an ideal temperature for rat islets. Furthermore, these researchers added calcium ions and glycerol to the collagenase mixture which reduced the formation of the gel-like structure [240].

Moreover, a recent study on increasing the yield and transplant rate of human islets was performed by Balamurugan *et al*. (2012), where a new collagenase enzyme mixture for enzymatic digestion was evaluated [242]. The new combination of enzymes included two types of collagenase, class 1 and 2 from *C. histolyticum,* and neutral protease (NP) from Bacillus *Thermoproteolyticus rokko*. Higher islets yields (>200,000 IEQ) were reported with class 1 collagenase and NP, with islet quality and functionality retained [242]. An appropriate optimisation of collagenase digestion is therefore crucial to this process. An additional consideration in the optimisation of the islet isolation process is the provision of a protective mechanism for cells post-digestion, thus allowing them to recover from the induced mechanical stress. Typically, the recovery mechanism following post-isolation involves culturing islets under 2D cell culture conditions. Although culturing islets under conventional static culture condition post-isolation has been shown to successfully repair cell damage, several factors can limit complete and long-term graft functioning post-transplantation such as insufficient oxygen and nutrient supply. The 2D culture environment is lacking normal physiological organisation because the native *in vivo* microenvironment for cells is 3D [177]. Once islets are isolated from their native environment, a disruption to the 3D structure or islets-matrix is induced which causes islet fragmentation and damage to cell functionality [234, 243]. Thus, studies have focused on modifying 2D culture condition by maintaining a 3D environment for post-isolation islets. A study of human islets in a 3D matrix culture has been performed, and showed superior survival in islets with adequate insulin responsiveness following transplantation into nude mice [244]. Therefore, providing an adequate 3D structural organisation for islets is of critical importance. Several reports have focussed on tissue engineering principles by culturing islet cells in 3D scaffolds to achieve cell-cell/matrix interactions [245]. Protein scaffolds have been engineered to mimic the physiological properties of native ECM present in

primary tissues [246]. One such study, conducted by Riopel *et al*. (2014), demonstrated that the expansion and survival of islets was improved by using collagen proteins, specifically types I and IV, which provide structural support and mediate signalling when embedded within islets [61]. They concluded that collagen scaffolds promoted islets survival post-isolation, a feature which could potentially be a beneficial protective mechanism to prolong islet engraftment [61]. Additionally, a study by Cheng *et al.* (2011) used ECM components as a scaffold and promoted islet survival and enhanced functionality post-transplantation [247]. Furthermore, 3D models of islet cells have been generated from conventional 2D culture methods. PIs spheroids are typically generated from Min6 β-cells cultured under conventional static conditions. PIs have been widely used by many researchers as an optimal model to mimic native islet cells *in vivo*. Although 3D models have been successfully developed, the limitations of culture conditions has meant that cell growth usually ceased after 7 days of incubation [153]. Recently, several micro-engineering methods, such as simulated microgravity culture systems, have been developed with the aim of generating 3D islet models in vitro which mimic native islets in the pancreas. These models exhibit improved cellular function, thus providing a better understanding of islet behaviour and biology. 3D-microgravity cell culture methods demonstrated islets with a superior structural integrity and improved insulin functionality. Tanaka *et al.* (2013) developed a new model of Min6-PIs, generated from a clinostat suspension [248]. They showed that PIs generated from 3D-simulated microgravity culture conditions exhibited a superior insulin secretory function compared to PIs generated from 2D static culture conditions [248]. Thus, the optimisation of the cell culture microenvironment can potentially have a positive impact on islet cells.

In this study, Min6-PIs were used as a model for culturing under a simulated microgravity environment which has not been used previously. Pseudoislet clusters

were generated initially from static dish then they were transferred to RCCS (see chapter 2 section 2.2.3). The aim of this work was to enhance the functionality of PIs and improve matrix interactions, without embedding PIs with ECM components. Furthermore, PIs were used as a model for islet isolation *in vitro* by using collagenase digestive enzyme to mimic the isolation process *in vivo*. In the previous chapter, the viability of Min6-PIs during digestion with accutase enzyme and post-digestion was assessed. According to the previous findings, 2 minutes of digestion was taken as the optimal time point because of the reduced effects caused to cluster viability. Thus, following assessment of the viability PIs pre- and post-digestion, it was deemed essential to further investigate the effects of the digestion enzyme and recovery process at the structural surface level. The work described in this chapter aimed to investigate the effects of enzymatic digestion and of simulated microgravity and conventional culture conditions on the morphological structure and ECM expression of Min6-PIs post-digestion. The targeted ECM proteins that were analysed were FN, Coll IV and LmV. A previous study has shown that these ECM proteins were involved in islet cell functions such as insulin secretion, expression, and cell adhesion, and also found that they were localised on the BM of islets [195].

As in the previous chapter, the effect of increasing digestion times on cell viability was determined. Here in this chapter, it was important to consider the effect of these incubations on the structural surface and ECM expression of PIs. PIs have then allowed to recover under conventional static and RCCS conditions for a suitable duration. The specific parameters of interest tested were morphological structure and ECM expression.

5.2. **Results.**

5.2.1. **Light microscopic analysis of digested Min6-Pseudoislets.**

Initially the outer surface (cluster coat) of PIs was analysed in order to investigate the effect of collagenase digestive enzyme on PIs morphological structure. Enzymatic digestion was performed using accutase digestive enzyme. The experiment was performed by digesting PI clusters, selected from RCCS, for zero, 30 second, 2 minute, and 5 minute time points (Chapter 2, section 2.2.4). **Figure 5-1** showed the effects of accutase enzyme on PIs digested at different time points. Control cells, which were PIs cultured in a static dish, were included in the experiment. No affect was observed within the cluster coat of PIs digested for 30 seconds **[Figure 5-1,** D30**]**, while PIs digested for 2 minutes displayed partial degradation of the cluster coat **[Figure 5-1,** D2**].** In contrast to the 5 minute digestion time point, the cluster coat was completely lost **[Figure 5-1,** D5**]**. According to these findings, the 2 minute digestion time point was chosen as the optimal digestion time in this study. For a more detailed microscopic examination, the structural surface of PIs digested for 2 minutes was evaluated using SEM, as discussed in the following section.

5.2.2. **Scanning electron microscopic analysis of digested Min6-Pseudoislets.**

The surface structure of PIs digested for 2 minutes was determined using scanning electron microscopy (SEM). The purpose of this experiment was to study in greater detail the morphological structure of PIs digested for 2 minutes. Pseudoislet clusters were selected from RCCS, digested with accutase enzyme for 2 minutes, and washed with 0.1 M cacodylate buffer. Clusters were then fixed with 2.5% of glutaraldehyde followed by a graded dehydration of ethanol (Chapter 2, section 2.2.6.4).

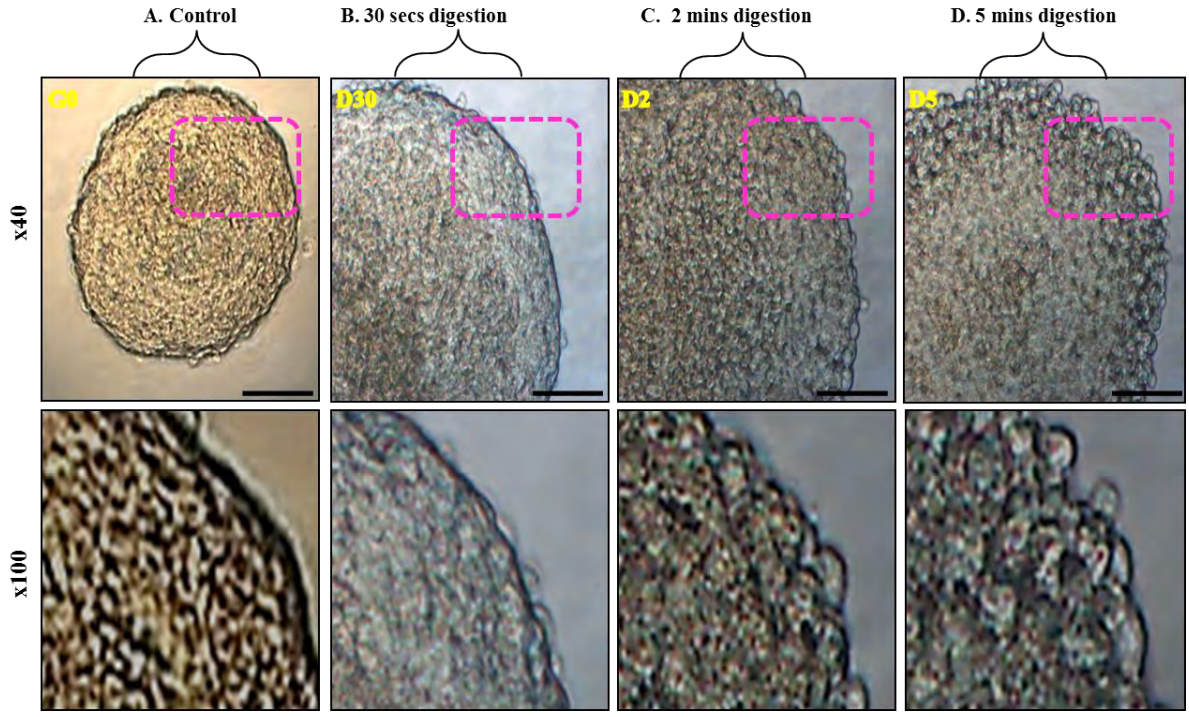

Figure 5-1: **Microscopic analysis of the surface structure of digested Min6-Pseudoislets.**

Enzymatic digestion with accutase was performed in 10 clusters from RCCS for 0 minute (G0), 30 second (D30), 2 minute (D2), and 5 minute (D5) time points. Control cells were included (A). Results are representative of three separate experiments and images were taken under x40 and x100 magnification. No degradation was observed within the cluster surface of PIs digested for 30 seconds (Panel B, D30). In contrast, panel D2 minor degradation within the cluster coat with an uneven surface structure was observed. Clusters that were digested for 5 minutes (Panel D5), however, showed major degradation within the cluster coat with a rough surface. Scale bar = 150 μM.

Clusters were coated with platinum and images were taken at magnifications of 1K, 5K, and 10K. Control cells were included in the experiment. Results showed that the structural surface of digested PIs was slightly degraded [**Figure 5-2]**. The cells inside the cluster were exposed, revealing an absence of surrounding cluster coat compared with control cells. These findings confirmed our previous results (obtained by light microscopic analysis), indicating that accutase digestive enzyme exerts a detrimental effect on the structural surface of PIs following a short period of incubation.

For further examination of the effects of accutase digestive enzyme on PIs, ECM expression was determined using qRT-PCR and western blotting in PIs digested for 2 minutes. Next, it was essential to investigate the effect of digestion enzyme on ECM gene and protein expression in Min6-PIs to determine whether 2 minutes of digestion had the potential to affect ECM expression.

Analysis of ECM expression of digested Min6-Pseudoislets.

The purpose of analysing the ECM expression of digested PIs was to examine whether it was affected by the degraded surface structure observed. Specific ECM components, commonly expressed in islets/ with important functioning roles in islets, FN, Coll IV, and LmV, were selected for analysis. ECM expression was determined using different techniques: qRT-PCR, western blotting, and ICC analysis.

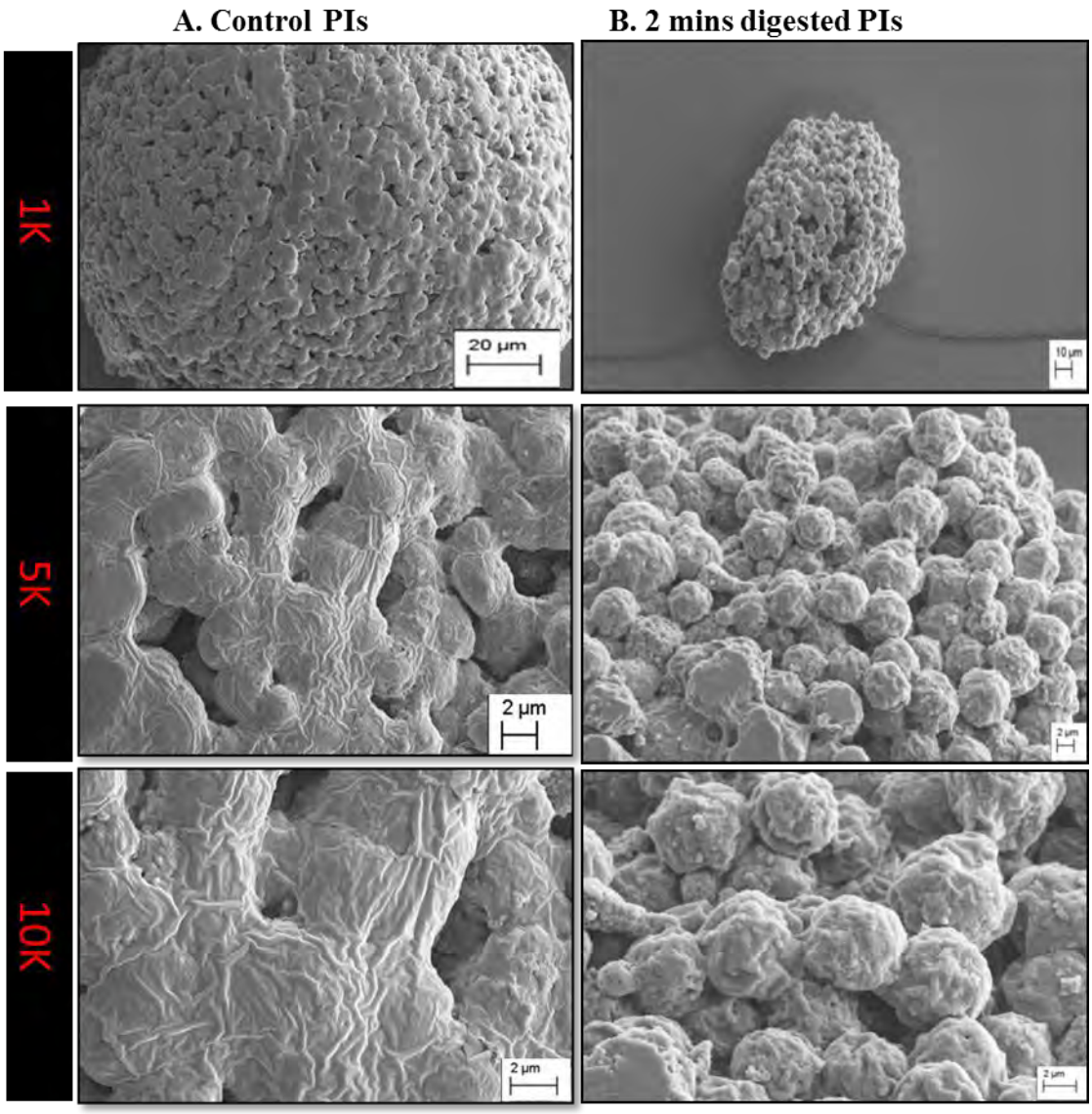

Figure 5-2: Scanning electron microscopy (SEM) of the morphological structure of digested Min6-Pseudoislets.

The morphological structure of digested PIs was examined by selecting 10 clusters from RCCS and digested for 2 minutes using accutase enzyme. Clusters were washed in buffer 0.1M cacodylate and fixed with 2.5% of glutaraldehyde, dehydrated, and coated with platinum (4 nm). Control cells were included (A). Results are representative of three separate experiments. PIs were examined using Zeiss Sigma field emission gun SEM (Zeiss NTS). Images were captured at the magnifications 1K, 5K, and 10K. The morphological structure of digested PIs (B) showed a rough/degraded surface compared to control cells. Scale bar: 1K = 10 µM, 5K and 10K = 2 µM.

5.2.3. **ECM gene expression of digested Min6-Pseudoislets.**

ECM gene expression was analysed using a qRT-PCR method. Briefly, the experiment was conducted by extracting mRNA from PIs under different conditions, cultured in static dishes and digested with accutase for 2 minutes. Following extraction, samples were treated with DNase and reverse transcribed into cDNA (Chapter 2, section 2.2.8.1). ECM gene expression of digested PIs in this experiment was expressed mRNA content of the clusters (see chapter2 section 2.2.8). GAPDH was used as a reference gene and all ECM gene expression was related to its expression. Negative controls, which were PIs cultured in static dishes and digested for 2 minutes but not reverse transcribed, were included in the experiments. Specific primers were added to ECM genes to be analysed. Results showed that all ECM gene expression in digested PIs was altered. A significant decrease in PIs digested for 2 minutes compared with control cells (***P<0.0001) **[Figure 5-3].** Overall, accutase digestion enzyme exerted a significantly negative effect on all ECM gene expression measured in these cells. ECM protein expression was then investigated to determine whether the digestion has an effect on the protein level of ECM.

5.2.4. **ECM protein expression of digested Min6-Pseudoislets.**

ECM protein expression, protein content, of PIs digested for 2 minutes was determined using Western blotting in order to determine whether the enzymatic degradation within cluster coat has the potential to affect protein level of ECM. ECM protein expression in this experiment is expressed as protein content of clusters (see chapter2 section 2.2.9). Whole cell extracts were selected for complete extraction of membrane-bounded and cellular proteins of PIs. Briefly, protein was extracted from two sources of Min6-PIs: those cultured under normal conditions in static dishes and those digested for 2 minutes.

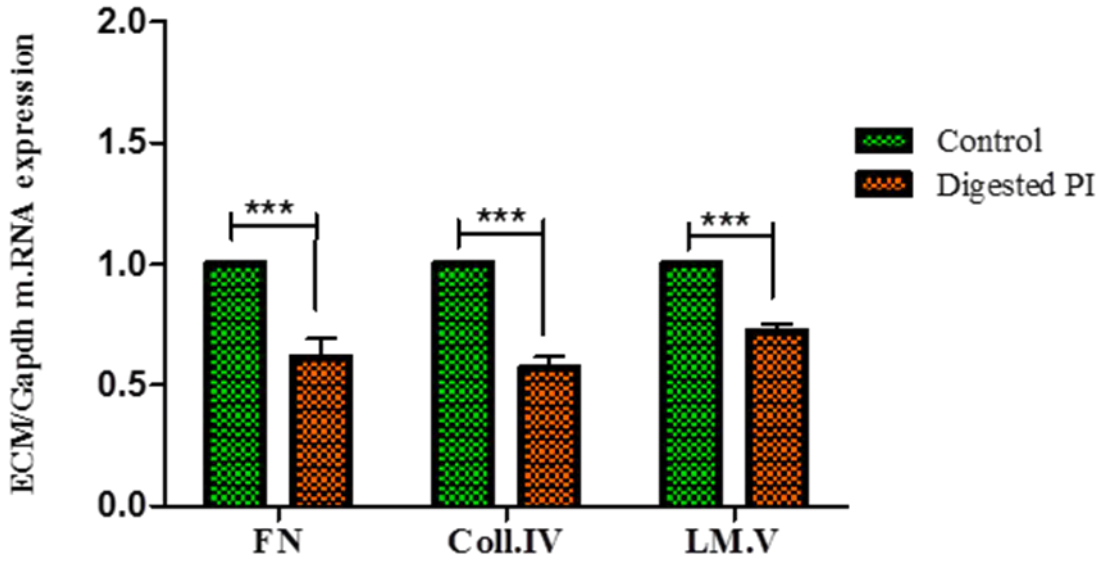

Figure 5-3: ECM gene expression of digested Min6-Pseudoislets.

ECM gene expression was performed by qRT-PCR. mRNA was extracted from two sources of Min6-PIs, static dishes and PIs digested for 2 minutes. Samples were treated with DNase and reverse transcribed into cDNA. Specific primers were added to the reaction. All ECM gene expression i.e. FN, Coll IV, and LmV was relative to GAPDH expression. Results are representative of three separate experiments. Error bar values represent mean ± standard deviation. PIs cultured in static dishes were used as a calibrator. Expression of all ECM genes from digested PIs was significantly decreased compared with control cells (***P<0.001).

Samples were then subjected to immunoblotting using a western blotting method (Chapter 2, section 2.2.9.3).

Results showed a significant decrease in all ECM protein expression from PIs digested for 2 minutes compared with control cells [**Figure 5-4,** panel B]. This outcome indicated that digestion with accutase has a significant effect on ECM protein expression.

Following quantitative analysis of ECM gene and protein expression, qualitative analysis of ECM protein expression was performed in order to confirm the previous data and to determine if ECM digested with accutase could affect expression.

5.2.5. **Analysis of ECM localisation within digested Min6-Pseudoislets.**

ICC analysis was used to determine the localisation of specific ECM proteins within digested PIs. This experiment aimed to qualitatively identify whether ECM protein expression and localisation were affected by enzymatic digestion, and also to confirm the previous quantitative findings. Briefly, the procedure was performed by sectioning a cluster block of PIs, digested for 2 minutes, by a cryostat-sectioning method (Chapter 2, section 2.2.6.1). Cluster sections were stained with specific ECM antibodies to target proteins of interest. A negative control was included. Results showed that all ECM protein expression of digested PIs was cytoplasmic, with low expression **[Figure 5-5,** panel B**].**

Therefore, to analyse the effect of accutase digestive enzyme on structural integrity and ECM expression, it can be hypothesised that despite the minor effects observed within the structural surface of PIs digested for 2 minutes, a significant alteration in ECM-PI interactions occurred. In order to restore cells from the damage to the structure and degradation of ECM expression observed in digested PIs, a recovery mechanism was instigated using static culture and RCCS for suitable durations.

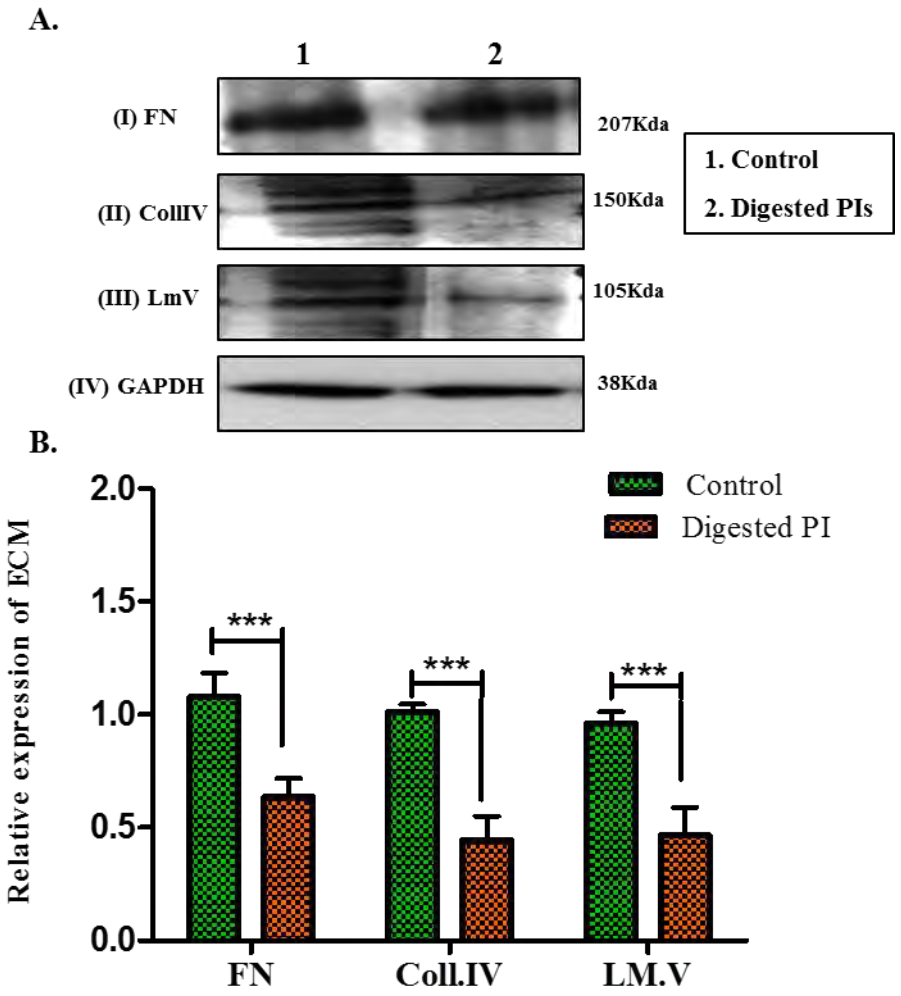

Figure 5-4: Expression of ECM proteins in digested Min6-Pseudoislets.

Whole protein was extracted from two sources of Min6-PIs, those cultured in static dishes and those digested for 2 minutes. 20 µg of whole cell extract was separated by 10% SDS-PAGE (A). Western blotting showed that expression of all ECM proteins was significantly decreased in digested PIs compared with control cells (B) (***P<0.001). These results were reproduced in three separate experiments. Error bar values represent mean ± standard deviation.

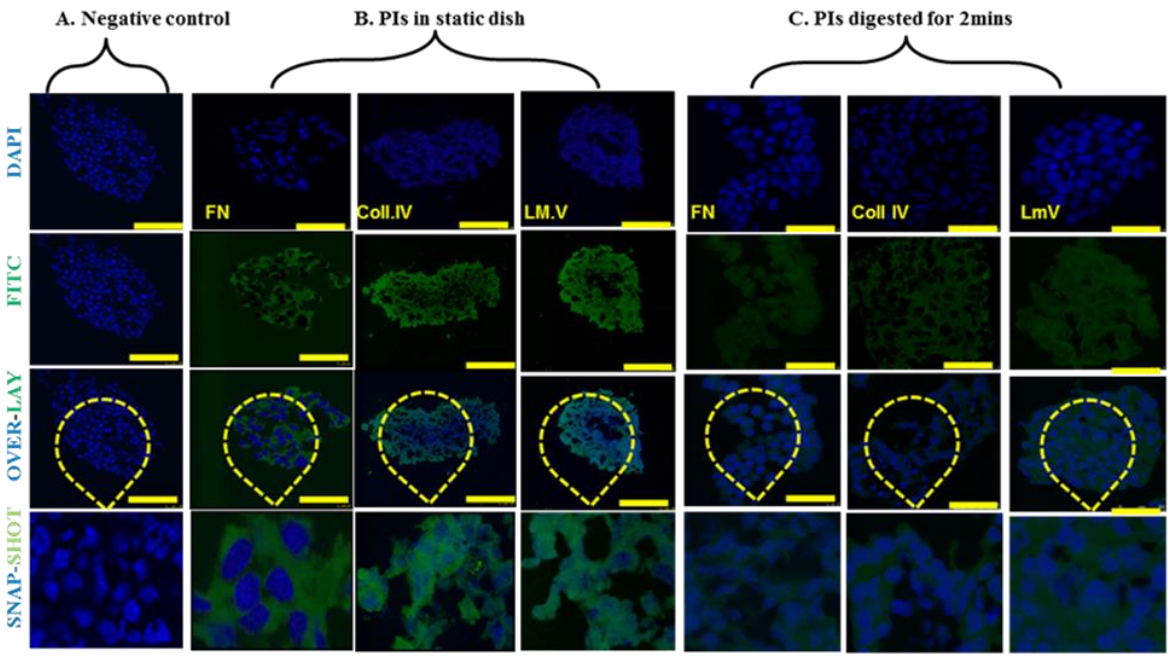

Figure 5-5: ECM localisation within digested Min6-Pseudoislets.

Min6-PIs were digested for 2 minutes, fixed with 3.4% formalin, and frozen with OCT medium in liquid nitrogen. Cluster sections were incubated with specific antibodies (against FN, Coll IV, or LmV). Images were captured using a confocal microscope. Results are representative of three separate experiments and images are representative of six separate fields. All ECM protein localisation and expression in digested PIs; was shown to be cytoplasmic and expressed at very low levels (panel C) compared with control cells (panel B). Overall, accutase digestive enzyme was shown to have an effect on ECM protein expression. . SNAP-SHOT is a selected area, closed capture, of over-lay image.

5.2.6. **Light microscopic analysis of Min6-Pseudoislets post-digestion.**

Effects of the digestion process were identified; the ECM coat surrounding PI clusters is clearly reduced following digestion with accutase. It was therefore considered essential to next investigate the recovery of PIs post-digestion by re-culturing them under two different culture conditions for selected durations. Static dishes and an RCCS were used to investigate the recovery effect for 24 and 48 hours. The purpose of these experiments was to re-establish the PI clusters coat lost during digestion. Briefly, experiments were performed by re-culturing PIs digested for 2 minutes into fresh medium under static and RCCS culture conditions for 24 and 48 hours. The morphological appearance of PIs was analysed using light microscopy. Results showed that the loss of the cluster coat in digested clusters was re-built to a certain extent under conventional static conditions **[Figure 5-6,** R24-D and R48-D**],** but that PIs recovered in RCCS displayed superior restoration of the cluster coat **[Figure 5-6,** R24-B and R48-B**].** In the following section, a more detailed analysis of the structural surface of recovery PIs was performed using SEM analysis.

5.2.7. **Scanning electron microscopic analysis of Min6-Pseudoislets post-digestion.**

The key purpose of performing SEM analysis on recovery PIs was to obtain a greater understanding of the restoration process within the cluster coat that was previously observed. Similar methods were used for SEM as described previously (section 5.2.2). Following 2 minutes of digestion, clusters were transferred to static dishes or RCCS for 24 and 48 hours of recovery.

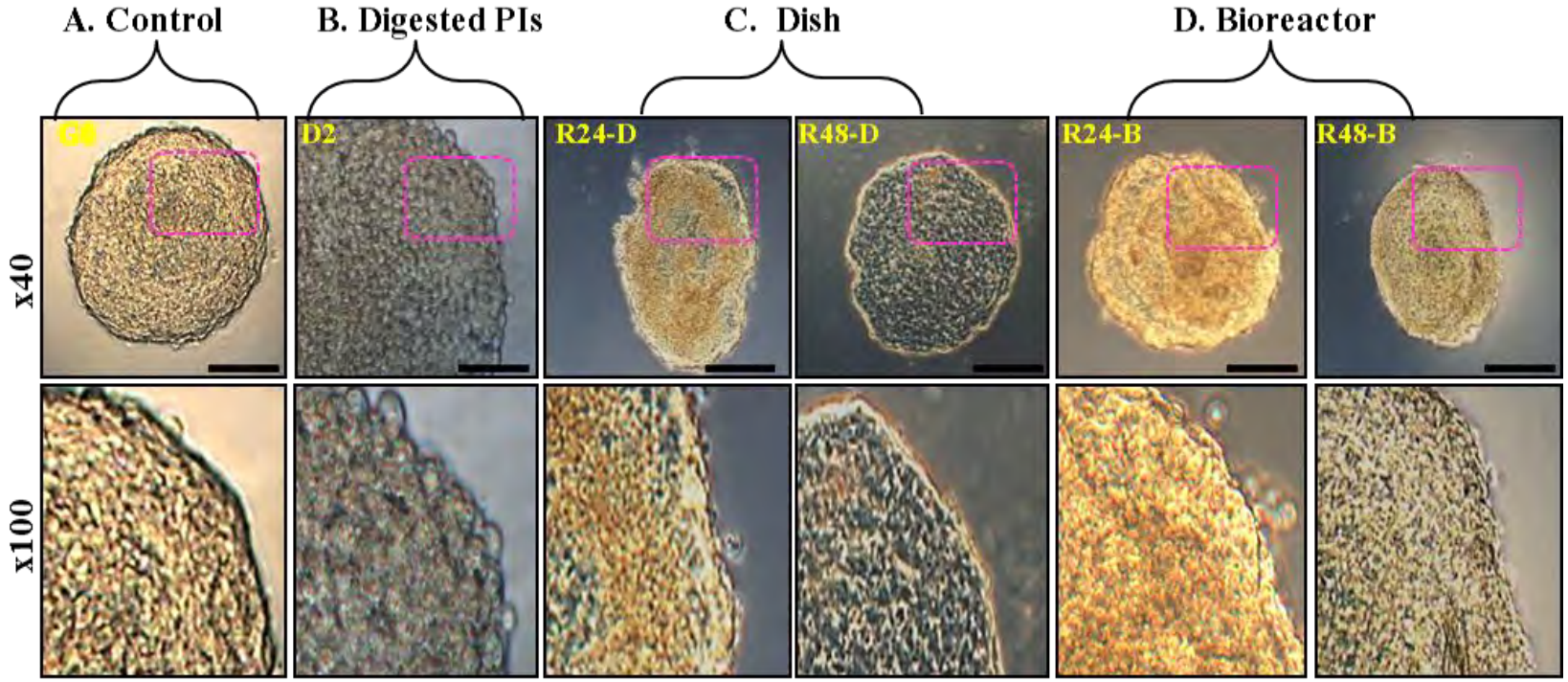

Figure 5-6: Microscopic appearance of the structural surface of Min6-Pseudoislets post-digestion.

PIs were digested for 2 minutes (B-D2) and then allowed to recover under static (R24-D & R48-D) and RCCS bioreactor (R24-B and R48-B) conditions. Results shown are representative of three separate experiments and images are representative of six separate fields evaluated. Panel C- R24-D, shows a clear restoration of the cluster membrane after 24 hours, compared with the digested cluster (B). After 48 hours, further recovery within the cluster membrane was observed (Panel C-R48-D). As shown in Panel D-R24-B and R48-B, clusters showed superior restoration within the cluster membrane. Overall, the recovery process in the bioreactor showed increased restoration within the cluster membrane compared with the static culture at the same time points. Scale bar: A-B = 150 μM; C= 125 μM; D= 150 μM

Results showed that the structural surface of PIs recovered in static dishes was restored [**Figure 5-7,** R24-D and R48-D**]** compared with digested PIs**.** Above and beyond this effect, the restoration within the cluster surface of PIs re-cultured in RCCS was completely recovered **[Figure 5-7,** R24-B and R48-B**]** compared with PIs recovered in static dishes.

These findings confirmed our previous results (under light microscopic analysis) that re-culturing clusters post-digestion shows better restoration in a 3D culture environment compared with conventional static conditions.

Analysis of ECM expression of Min6-Pseudoislets post-digestion.

Following microscopic examination of cluster coat restoration, it was essential to investigate the effects of cluster coat recovery on ECM gene and protein expression.

5.2.8. **ECM gene expression of recovered Min6-Pseudoislets.**

These experiments aimed to investigate the effect of RCCS culture conditions on ECM gene expression post-digestion. mRNA was extracted from six sources of Min6-PIs; PIs in static dishes, PIs digested for 2 minutes, PIs recovered for 24 and 48 hours in static dishes and in RCCS bioreactor. PIs cultured under normal static culture conditions were used as a calibrator. GAPDH was used as a housekeeping gene and ECM gene expression of PIs was determined, relative to its expression.

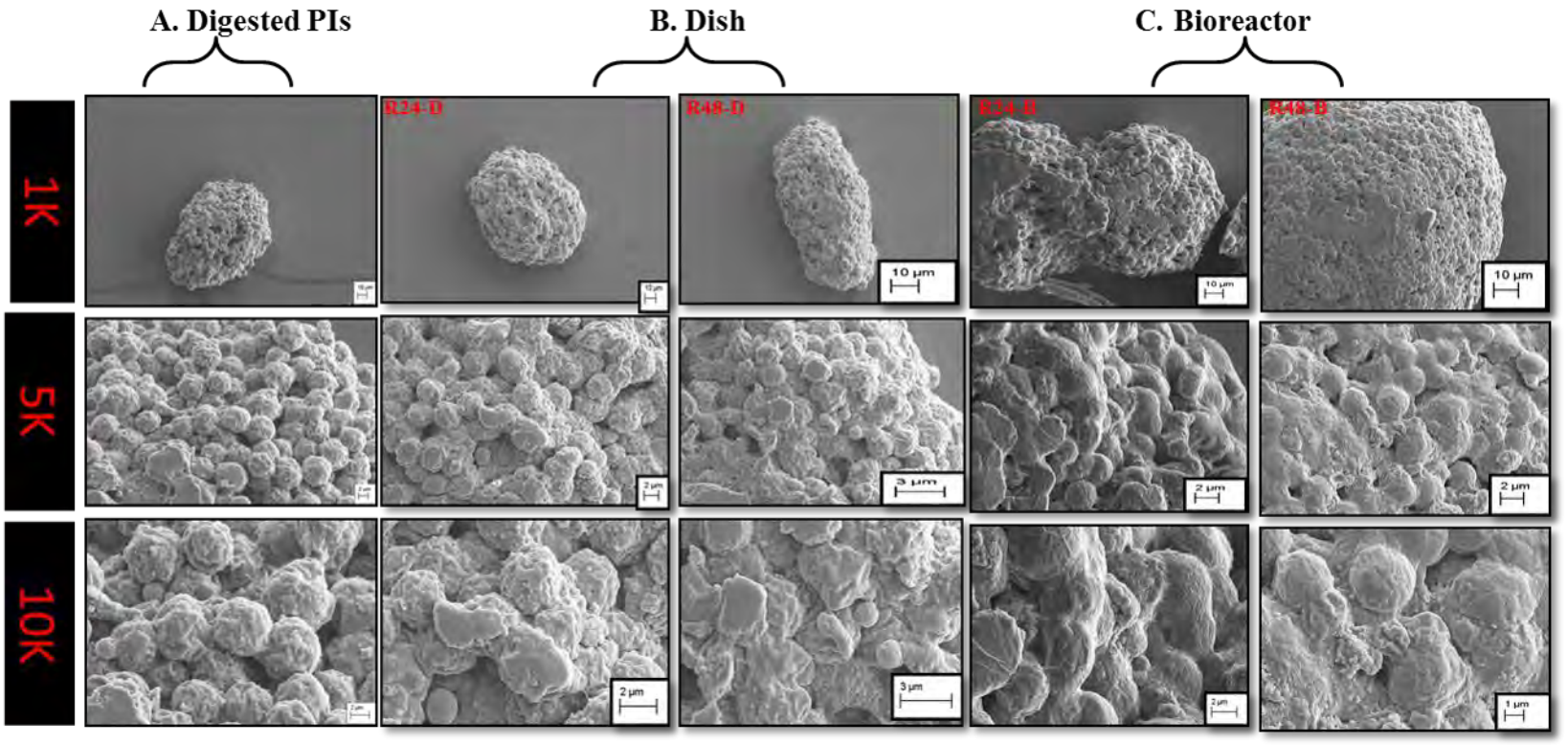

Figure 5-7: Scanning electron microscopy (SEM) of the morphological structure of Min6-Pseudoislets post-digestion.

Recovery PIs from static dishes and from an RCCS were fixed with 2.5% of glutaraldehyde, dehydrated, and coated with platinum (4 nm). PIs were examined using Zeiss Sigma field emission gun SEM (Zeiss NTS). Images were captured at 1K, 5K, and 10K magnifications and were representative of six separate fields. The surface structure of digested PIs (A) was observed to be degraded. The recovery PIs from static dishes (B) showed a restoration as indicated by the presence of some intact cells within the cluster. Increased restoration was observed in PIs recovered under RCCS conditions (C) with a smooth surface. Scale bars: 1K = 10 µM, 5K and 10K = 2 µM).

Results showed that the expression of all ECM genes measured was upregulated in PIs recovered in an RCCS for 24 and 48 hours compared with PIs recovered in static dishes**[Figure 5-8,** panel A, B and C**]** (***P<0.0001). Furthermore, the recovery of PIs in RCCS showed significant up-regulation of all ECM genes compared with digested PIs, while PIs recovered under static conditions showed no significant difference in ECM expression compared with digested PIs. Interestingly, the expression of ECM genes in PIs recovered under RCCS conditions was significantly higher than in control cells, although no significant difference in ECM expression was observed between PIs recovered under static dish and control conditions. The recovery mechanism at 24 and 48 hours under RCCS culture conditions was shown to exert a significant positive effect on remodelling by modulating ECM gene expression. To support these findings, ECM protein expression was analysed as described in the following section.

5.2.9. **ECM protein expression of recovered Min6-Pseudoislets.**

Western blotting was used to support the previous findings, and similar procedures were applied as described previously (section 5.1.1). The aim of this experiment was to support the previous ECM gene expression levels observed in post-digestion PIs, in order to determine whether the increase in gene expression was reflected by an increase in protein expression. Results showed that all ECM proteins was increased in PIs recovered in both static dishes and in a RCCS bioreactor for 24 and 48 hours **[Figure 5-9,** panel B I-III] compared with digested PIs (***P<0.001). However, a significant increase was observed in PIs recovered in RCCS compared to PIs recovered in static dishes (FN ***p<0.001, CollIV *p<0.05, LmV **p<0.001) **[Figure 5-9].** From these observations, it was concluded that the recovery of PIs in a RCCS bioreactor can potentially up-regulate post-digestion PI ECM expression.

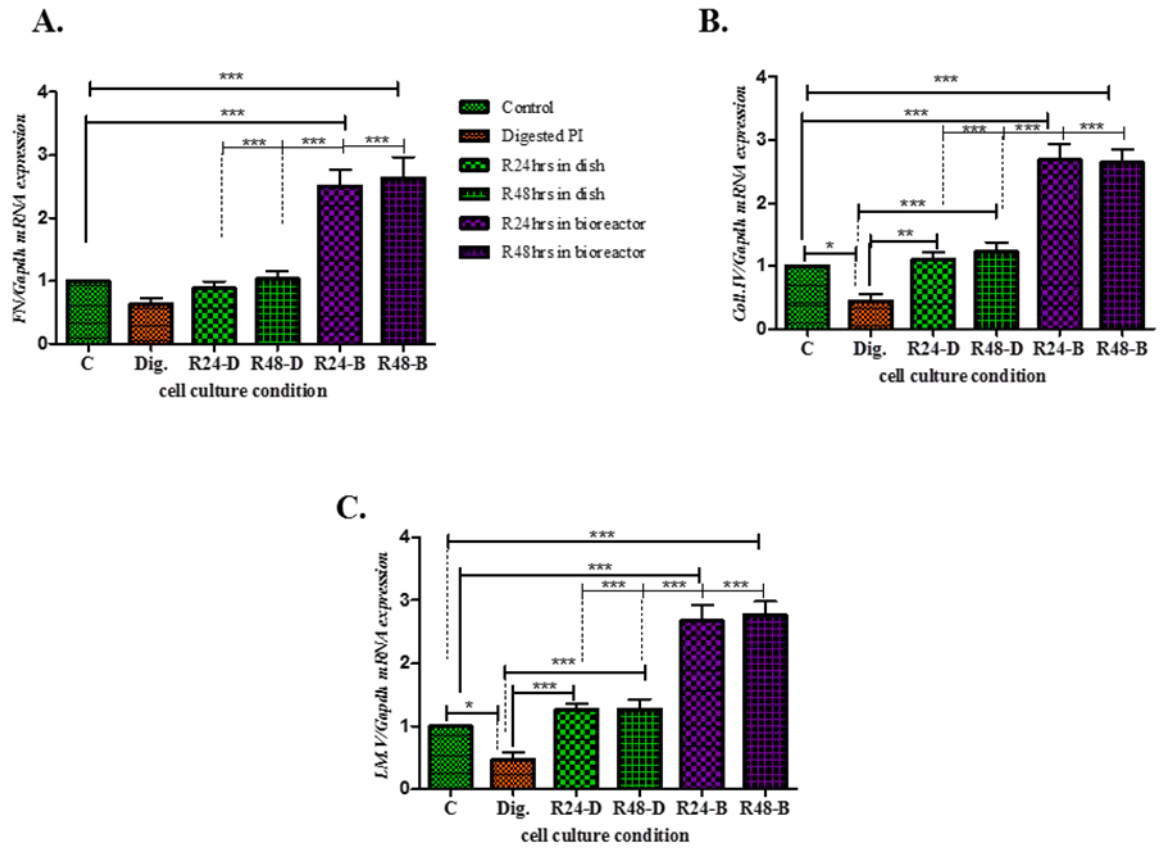

Figure 5-8: ECM gene expression in recovered Min6-Pseudoislets.

ECM gene expression was determined by qRT-PCR relative to the reference gene GAPDH. Results are representative of three separate experiments. The Ct values were relative to Ct values of PIs cultured in static dishes (control). Results are representative of three separate experiments. FN (panel A), Coll IV (panel B), and LmV (panel C) expression in PIs recovered in RCCS for 24 and 48 hours was significantly increased compared with PIs recovered in static dishes, control cells, and digested PIs (***P <0.001). No significant differences in ECM gene expression were seen between PIs recovered in static dishes and control cells. Error bar values represent mean ± standard deviation. Overall, the RCCS bioreactor was shown to have the ability to upregulate expression of all three ECM genes in post-digestion PIs.

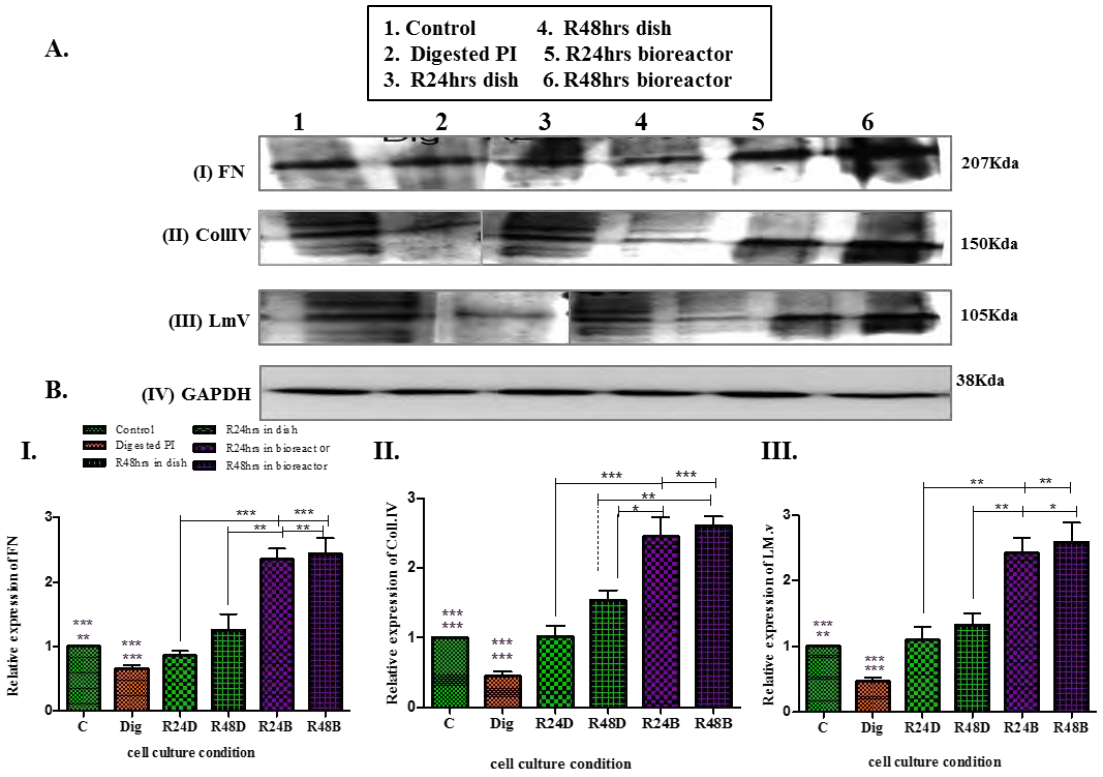

Figure 5-9: ECM protein expression in recovered Min6-Pseudoislets.

ECM protein expression was analysed by Western blotting. Proteins were extracted from six sources of PIs cultured under different experimental conditions: PIs cultured in dishes, PIs digested for 2 minutes, PIs recovered for 24 and 48 hours in static dishes and in a RCCS bioreactor. 20 µg of whole cell extract was separated by 10% SDS-PAGE (A). Western blotting showed that fibronectin (panel B, I) protein expression in PIs recovered in an RCCS was significantly upregulated compared with PIs recovered in static dishes and control cells after 24 (*** P<0.0001) and 48 hours (**P<0.001). Furthermore, a significant difference in fibronectin expression was observed between PIs under RCCS conditions and digested PIs (***P<0.0001). Coll IV expression (panel B, II) was significantly upregulated in PIs under RCCS conditions compared with PIs under static conditions after 24 (***P<0.0001) and 48 hours (**P<0.001). A significant difference was also seen in Coll IV expression between PIs under RCCS conditions for 24 and 48 hrs and control cells or digested PIs (***P<0.0001). LMV protein expression (panel B, III) was significantly increased in PIs under RCCS compared with PIs under static conditions for 24 (**P<0.001) and 48 hours (*P<0.05). Finally, there was a significant difference in LM V expression between PIs under RCCS conditions for 24 and 48 hours and control cells or digested PIs (***P<0.0001). These results were reproduced in three separate experiments. Error bar values represent mean ± standard deviation.

5.2.10. **ECM localisation of recovered Min6-Pseudoislets.**

ECM protein expression in recovery PIs was confirmed using ICC to localise ECM protein expression using a similar method to that described previously (section 5.2.4). **Figure 5-10** showed that localisation of ECM proteins in PIs recovered for 24 hours in static dishes and a RCCS bioreactor was extracellular and cytoplasmic. The expression of all ECM proteins in PIs recovered under RCCS conditions (B) was noticeably higher compared to PIs recovered under static dish conditions (A). Increased cytoplasmic expression of ECM proteins was also seen in PIs recovered for 48 hours under RCCS conditions (B) compared to PIs recovered under static dishes (A) **[Figure 5-11].**

Therefore, the recovery of Min6-PIs in RCCS bioreactor post-digestion conditions had a remarkable effect on ECM-PIs interactions by restoring the loss of cluster membrane integrity and significantly upregulating ECM expression.

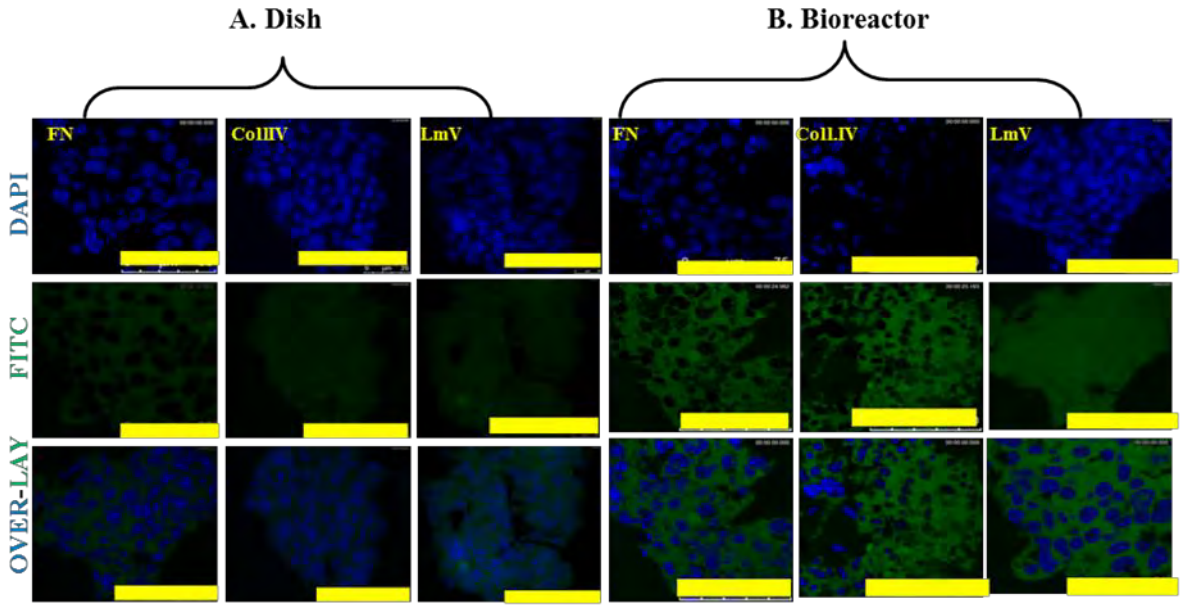

Figure 5-10: ECM protein expression in recovered Min6-Pseudoislets after 24 hours.

ICC was performed on recovery of Min6-PIs under static and RCCS culture conditions for 24 hours. Specific antibodies were against ECM proteins of interest were used (FN, Coll IV, and LmV). Results are representative of three separate experiments and images are representative of six separate fields. ECM protein localisation and expression of PIs recovered for 24 hours was shown to be cytoplasmic with very low expression in PIs from static dishes (panel A) and cytoplasmic with high expression in PIs cultured in a bioreactor (panel B).

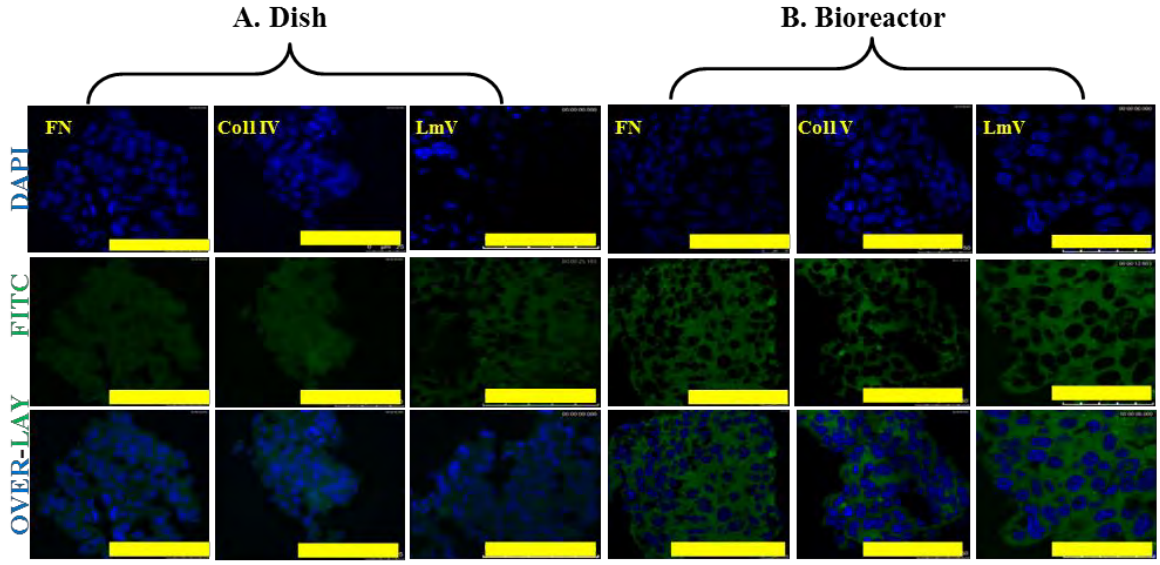

Figure 5-11: ECM protein expression in recovered Min6-Pseudoislets after 48 hours.

ICC was performed on recovery of Min6-PIs under static and RCCS culture conditions for 48 hours. Specific antibodies were against ECM proteins of interest were used (FN, Coll IV, and LmV). Results are representative of three separate experiments and images are representative of six separate fields. ECM protein localisation and expression of PIs recovered for 24 hours was shown to be cytoplasmic with very low expression in PIs from static dishes (panel A) and cytoplasmic with high expression in PIs cultured in a bioreactor (panel B).

5.3. **Discussion**

Islet transplantation shows promise as a treatment for T1DM; however, the success of this procedure is hindered by several obstacles, such as a shortage of islet donors, mechanical stress caused by the islet isolation process, and the toxicity of immunosuppressant post-transplantation therapy. Various studies have shown that islet isolation has a negative impact on islet survival and functionality due to mechanical stress caused by digestive enzymes during isolation which disrupts islet-matrix interactions, leading to apoptosis [234]. Irving-Rodgen *et al.* (2014) reported that a complete loss of basement membrane (BM), a sensitive biomarker of islet damage, was noticed after mouse-islet isolation [249]. Isolated islet cells cultured for 4 days showed no evidence of BM restoration, although the re-establishment of BM was observed following transplantation [249]. From this study, islet isolation was considered to have a negative impact on islet functionality due to the removal of islets from their native environment. Thus, the isolation process dramatically changes islet cell interactions with the ECM.

ECM proteins are crucial for functional islet cells **[Figure 5-12]** and are evident in two forms, the interstitial matrix (IM) and BM. Both forms are composed of Coll IV, LM, and FN [249], proteins which interact with integrins found in islet cells. These integrins are groups of receptors which transmit biochemical signals in and out of cells and form the basis of cell adhesion. Wang and Rosenberg *et al*. (1999) also reported that the loss of islet cell BM during the isolation process prevented islet cells from properly adhering to each other [[77]. They proposed that preservation of the interactions between these matrix proteins and integrins prevented the decrease in islet cell function and insulin content and also delayed islet cell death or apoptosis [77].

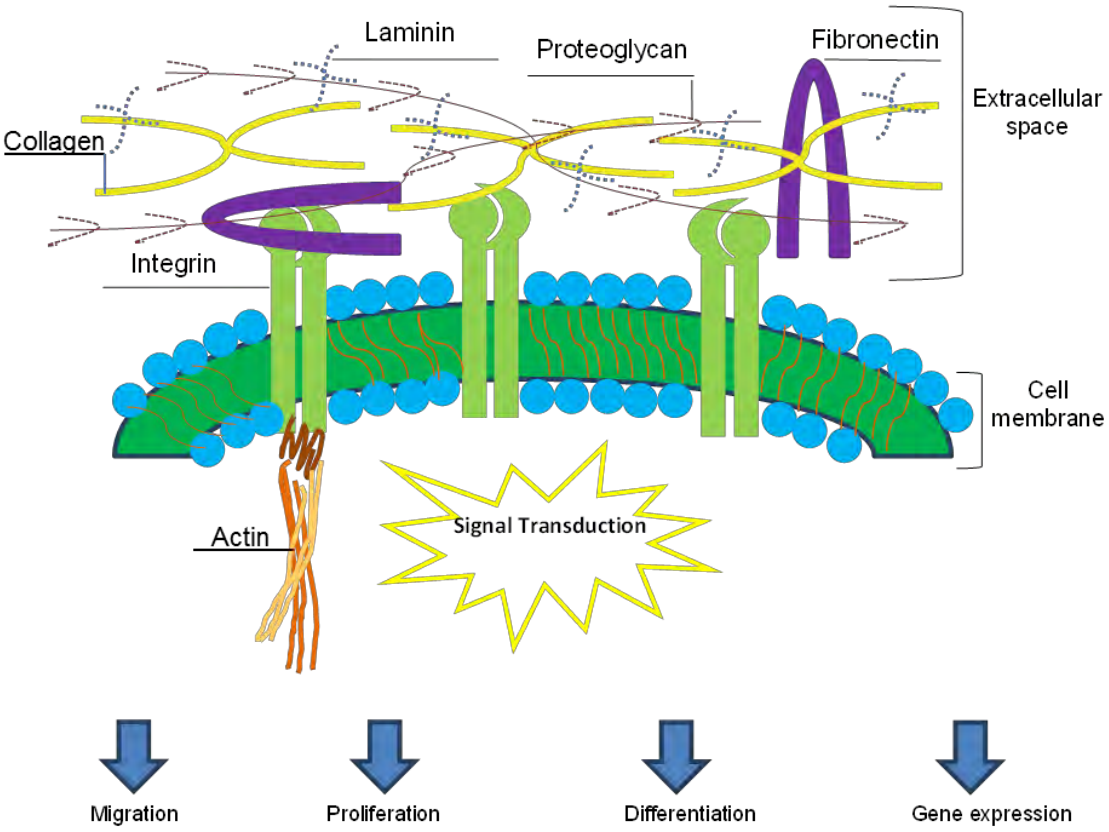

Figure 5-12: Schematic diagram showing the composition of ECM proteins and their roles within the cell.

The structure of ECM is made up of collagen, which is the most abundant protein found in the cell, elastin, proteoglycans, and structural glycoproteins such as fibronectin and LM. Proteins such as collagen, FN, and LM are found at the extracellular space of the cell. Through FN ECM proteins are attached to the cell membrane via receptor proteins known as integrins which are embedded in the cell membrane. Integrins function to transmit signals between extracellular spaces of cell and its interior side. This signal transduction process regulates several dynamic cellular behaviours such as migration, proliferation, differentiation, and gene expression. Depending on the signal detected by integrins, cells are instructed to activate or deactivate.

In general, cells are constantly subjected to either internal or external stresses which lead to alterations in ECM expression [250]. Mechanical stress is sensed by transmembrane receptors known as integrins**,** which link ECM components to the cytoskeleton of cells. Thus, any degradation within ECM components will be detected by integrins and signals will be sent accordingly to the nucleus to report the abnormal expression of ECM [251]. In terms of islet transplantation, islet β-cells are introduced post-isolation to significant mechanical stress such as that exerted in the vasculature and by hypoxia leading to apoptosis [215]. Further reports have shown that enzymatic digestion causes disruption to the cell-matrix relationship which also lead to apoptosis [234]. These cellular stresses are sensed by specific integrins (such as α3, α5, αV and β1) where expressed by human islets [252]. An indication of cellular stresses and apoptosis induced in islets during the isolation process was investigated by Wang *et al*. (1999) by analysing the expression of integrins and the distribution of peri-insular BM in the human, porcine, canine, and hamster pancreas [88]. This study identified that alteration within islets-matrix interactions were caused by the loss of BM and decreased expression of integrins following the isolation process [88]. Thus, it was deemed crucial to alleviate that damage to islet cells in order to prolong the life-span of transplanted islets post-transplantation. Several reports subsequently suggested that allowing islet cells, post-isolation, to interact with specific ECM components during culture had the potential to enhance insulin secretory mechanisms and reduce apoptosis [65]. Since Coll IV, FN and LM were shown to regulate survival, insulin secretion, and proliferation in mature islet cells [182, 253, 254], artificial ECM components were considered significant for post-isolation islets survival.

Several reports have shown improved islet survival and enhanced insulin secretion in rat models [180] along with reduced apoptotic rates [164].Therefore, a greater understanding of islets-ECM interactions could provide an insight into appropriate mechanisms to reduce the stress induced by the isolation process, in order to develop successful remodelling methods for cells to re-build the loss of ECM-interactions.

In this chapter, the effect of collagenase and subsequent remodelling of Min6-PIs post-digestion was investigated. Specifically, the effect of digestion and recovery under static culture and RCCS bioreactor conditions on PIs structural integrity and ECM expression was analysed. In the previous chapter, the effect of digestion on PIs viability was investigated showing that 2 minutes incubation with accutase has minor effect on cell viability. However, 5 minutes digestion caused a major effect on the viability, thus in this chapter it was necessary to investigate the effect of digestion on pseudoislets structure and ECM expression. The findings from this study showed that accutase digestive enzyme has an effect on the cluster coat of PIs at 2 minutes and 5 minutes. It was observed that, at 2 minutes of digestion, degradation within the cluster coat of PIs took place **[Figure 5-1];** however, at 5 minutes of digestion, a complete loss of the cell coat was observed **[Figure 5-1**, D5**].** Furthermore, the cluster coat of PIs digested for 2 and 5 minutes was uneven and rough compared to that of control PIs **[Figure 5-1,** panel A**]** and to PIs digested for 30 seconds **[Figure 5-1,** panel B**].** As previously demonstrated in Chapter 4 (section 4.2.1.2), the viability of PIs was affected after digestion for 2 minutes as indicated by the presence of apoptotic cells, whereas PIs showed more necrosis and apoptosis after 5 minutes digestion compared to 2 minutes digestion. The 2 minute digestion time point was thus selected as the optimal digestion duration in this study. For a more detailed examination of the effect of digestion enzyme on the structural surface of PIs, SEM analysis was performed on PIs digested for 2 minutes.

Results showed that the structural surface of PIs was degraded **[Figure 5-2]**. The cells inside the cluster were exposed, showing no surrounding cluster coat compared with control cells [**Figure 5-2**, panels A-B]. Thus, from these observations, it can be inferred that the degradation or the minor loss of cluster coat after 2 minutes of digestion can potentially affect presence of ECM. Following analysis of the structural degradation of PIs, ECM expression was determined using qRT-PCR, Western blotting, and ICC. The purpose of these analyses was to investigate the effect of enzymatic digestion on ECM expression after 2 minutes. Specific ECM components FN, CollIV, and LmV were chosen for analysis. The purpose of choosing these specific ECM proteins was that previous studies showed their involvement in the formation and enhancement of PI functionality [195]. Results showed a significant decrease in expression of all ECM genes in PIs digested for 2 minutes **[Figure 5-3,** FN ***P<0.001, CollIV ***P<0.001, LmV **P<0.01**]**. This degradation observed and alterations in ECM gene expression of digested PIs was in agreement with a previous study in which the BM of mouse islet cells was completely lost immediately after the isolation process [249]. Moreover, Kragl and Lammert (2010) showed in mouse and human islet models that the key factor in correctly isolating islet cells depended on the presence of BM, which acts as a biomarker of highly functional β-cells [59]. Thus, the presence of vascular BM has a great influence on ECM components, insulin transcription and secretory function, as well as β-cell proliferation [59]. Any alteration within BM caused by isolation stress could thus impact the efficiency of the islet microenvironment and function. Therefore, the findings of this chapter are in agreement with those of previous studies in which the damage caused by digestion enzyme was shown to influence the structure integrity of PIs along with ECM expression.

However, no previous studies have evaluated this in an *in vitro* isolation mechanism on PIs model as shown here. Previous research has revealed that the process of human islet

isolation triggers a cascade of stressful events involving the activation of apoptosis and necrosis and the production of pro-inflammatory molecules.

These stressful events have a negative impact on islet yields and function [101]. How cells sense these stressful events and translated them into a biological response was a point of interest in this work. Normally, cells adhere to each other through cell surface receptors known as integrins, consisting of two subunits α and β [255]. Recent evidence has indicated that integrins, which act as membrane receptors on the cell surface, play an important role in the process of 'mechano-transduction'. Through this process, cells have the ability to sense mechanical stress and convert it into altered gene expression [250]. Thus, according to previous studies, it can be hypothesised from our results that PIs express these integrins; hence, the stressful stimulus induced by enzymatic digestion may be sensed by PIs and translated into an altered ECM gene expression profile. Previous studies showed that Min6-PIs have the ability to rapidly sense mechanical stress because of the presence of specific integrins targeting BM-matrix components, such as β1-integrin [256].

In addition to ECM gene expression analysis, protein expression was determined using Western blotting. The aim of this work was to confirm the previous gene expression data and find out whether the digestion enzyme also has an effect on protein expression. Results showed that the expression of all ECM proteins was significantly decreased compared to control cells **[Figure 5-4,** panel B, ***P<0.0001**].** More specifically, Coll IV protein expression was decreased more significantly compared to the other ECM proteins.

This may be explained by a study by Irving-Rodgers (2014) that reported the loss of Coll IV protein, one of the BM matrix proteins, during the isolation process [249].

Given that collagen protein is predominantly located on the BM of islet cells, any alteration within the BM may lead to the degradation of collagen.

Therefore, in order to overcome and restore the loss and damage caused to PIs structural surface and ECM expression, an *in vitro* recovery mechanism was performed allowing PIs to be re-cultured under static and simulated microgravity environments. The purpose here was to evaluate the effect of our recovery process on PI structural integrity and ECM expression. The principle of using RCCS in this study was the ability to adjust vessel rotation at constant speeds of 8 rpm, where PIs float freely in a low shear environment and are not influenced by gravitational field as seen in static culture conditions. Other studies have shown that culturing cells under a simulated microgravity environment can influence gene expression and the protein contents of ECM, for example in thyroid cancer cells [164].

The re-establishment of the cluster coat that was lost and the increase of ECM gene and protein expression were achieved within 24 and 48 hours of incubation. Microscopic analysis of recovery PIs under RCCS conditions showed the complete restoration of the surface structure **[Figure 5-6,** R24-B and R48-B**]** compared with PIs recovered under static conditions **[Figure 5-6,** R24-D and R48-D**].** For a more detailed analysis, SEM was used and results showed an increased restoration with a smooth surface structure on PIs recovered under RC CS **[Figure 5-7,** R24-B & R48-B**]** compared with those recovered in static dishes. Thus, an appropriate time point was chosen to allow stressed PIs to recover, and a positive impact on surface structure was observed.

In clinical islet transplant studies, it is important to culture islet cells post-isolation in order to preserve islet quality prior to transplantation [165]. Traditionally, researchers tended to use conventional static cell culture as a method for culturing islet cells post-isolation; however, some limitations to islet culture exist under these conditions such as

inadequate nutrients/oxygen transportation to cells causing a necrotic core to develop [165]. Thus, improving islet culture methods has proved challenging in order to maintain islet viability, recovery, and survival [166]. Recent advances in 3D cell culture technologies have shown great potential in maintaining a suitable microenvironment for islet cells post-isolation [257]. The study showed that culturing islet cells under 3D environments gave rise to up-regulated insulin expression and preserved the ECM of cells, leading to the maintenance of cellular function and survival in culture compared with conventional culture methods [257].

According to this chapter's findings, RCCS culture conditions had a positive impact on the surface structure of PIs, leading to up-regulation of ECM gene and protein expression in Min6-PIs **[Figure 5-8, Figure 5-9].** Expression was significantly increased compared to control cells and recovery PIs in static dishes (***P<0.001). Although 3D clusters were generated from 2D culture conditions (static dishes), microgravity culture conditions dramatically enhanced gene and protein expression. This may be explained by the observation that cells settle on the bottom of the culture when cultured using static culture methods (2D) unlike microgravity culture where cells tend to float freely, with no shear forces, maintaining close cell-to-cell interactions [258]. Therefore, the up-regulation of ECM gene and protein expression was considered characteristic and confirmative of the complete recovery of PI membrane. Our findings supported those of a previous study which showed restoration within *in vitro* β-cell-matrix interactions in a mouse model following isolation and transplantation, thus conferring major benefits on islet survival and function [249].

Moreover, Sheyn and colleagues (2010) hypothesised that important signalling pathways were stimulated in human mesenchymal stem cells (MSCs) during culture in a rotating wall vessel (RWV); they tested their hypothesis through stimulation of MSCs

neovascularisation and showed the formation of a capillary network [259]. Microgravity cell cultures have attracted much attention in biological studies. Aleshcheva *et al.* (2013) reported similar findings to previous studies in a study of chondrocyte cells cultured on a random positioning machine, a form of microgravity culture [260]. They showed that a microgravity culture environment influenced chondrocyte cell morphology and gene expression, with protein content positively up-regulating TGF-β1 after 24 hours of culture incubation [260].

In this study, ICC was performed on digested PI cells and recovery PIs. The purpose of this qualitative analysis was to confirm the previous quantitative analysis data on ECM gene and protein expression and to investigate whether the localisation of ECM proteins was influenced by digestion and recovery mechanisms. **Figure 5-5** showed the ECM expression of PIs digested for 2 minutes. The degradation of the membrane of digested PIs was characterised by discontinuous staining of all ECM proteins, indicating an alterations in ECM gene and protein expression as observed previously. Despite this, the deposition of Coll IV, FN and LmV proteins was observed in PIs recovered in static dishes for 24 hours **[Figure 5-10,** panel A**]** and associated with the continuous staining of all ECM proteins at 48 hours, indicating further ECM remodelling **[Figure 5-11,** panel A**].** More interestingly, the ECM staining in particular was strong and widespread in PIs recovered in an RCCS bioreactor for 24 and 48 hours [**Figure 5-10, Figure 5-11**]. Taken together, these findings suggested that the reestablishment of cluster membranes and remodelling of ECM were most enhanced in PIs cultured under RCCS conditions. Thus, the molecular remodelling of Min6-PI ECM could lead to rebuilding of the lost cluster membrane. Moreover, ECM remodelling would be potentially beneficial to PIs functionality in terms of insulin secretion as addressed in the next chapter.

Thus, the data determined from this study revealed that the cluster membrane of PIs was damaged during digestion at 2 minutes as indicated by alterations in ECM expression. The recovery mechanism was shown to have a positive impact on PIs by restoring the cluster membrane with up-regulation of ECM expression following PIs culture in both static dishes and RCCS. However, a remarkable restoration within BM and a significant increase in ECM expression was noted in cells cultured in a bioreactor, thus indicating the re-establishment of PI-ECM interactions **[Figure 5-13**].

It was therefore concluded that the cluster membrane acts as an essential biomarker for PI integrity. By analysing it after enzymatic digestion and remodelling processes, it can predict the effectiveness and quality of the digestive enzyme and the optimal culture conditions for remodelling which could be used for improving PIs survival as a model in this study.

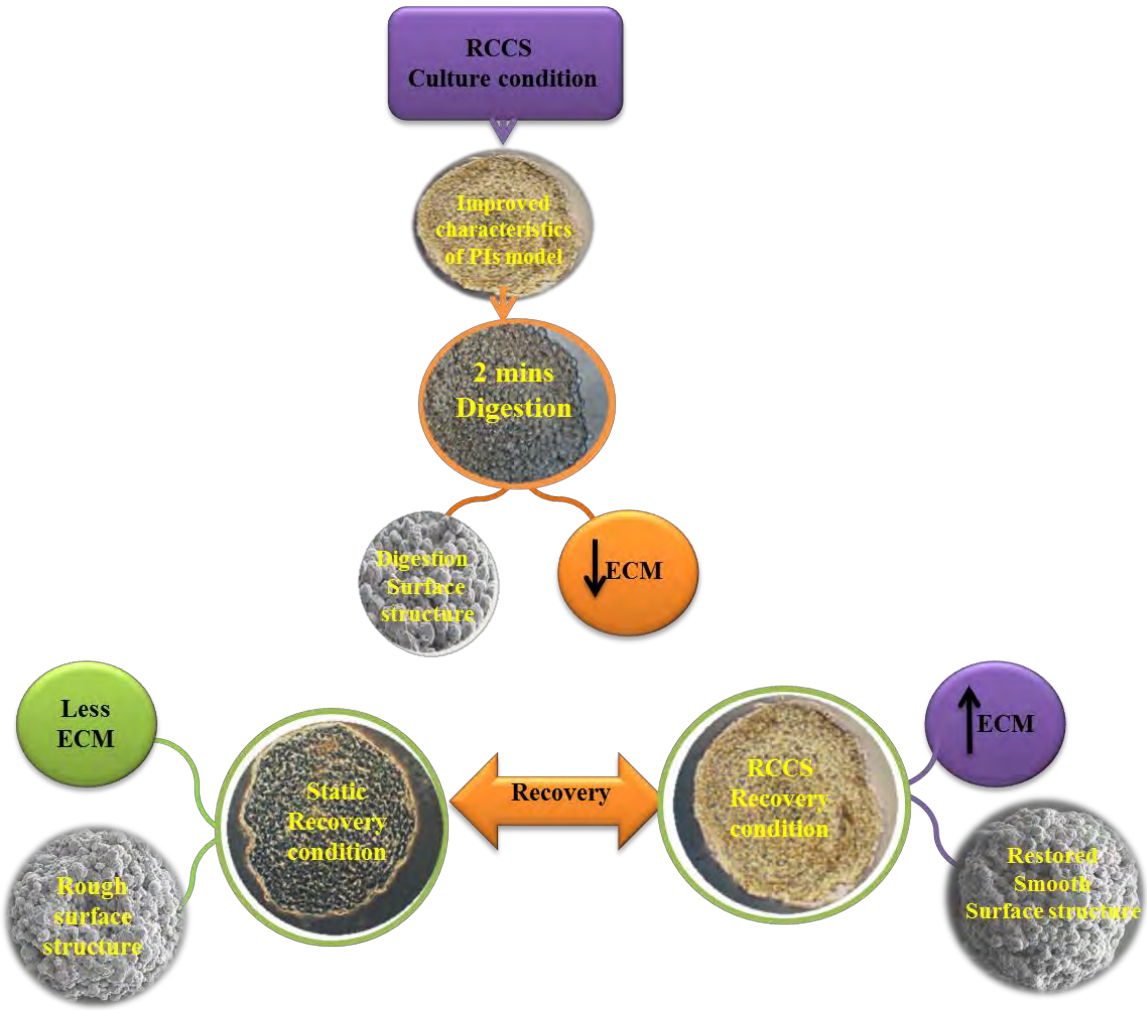

Figure 5-13: Schematic diagram of the digestion and recovery processes on Min6-Pseudoislets.

The diagram illustrated the effect of the two *in vitro* processes i.e. digestion and recovery on the morphological structure and ECM expression of Min6-Pseudoislets**.** Two minutes digestion had a great effect on the structural integrity leading to a significant decrease of ECM expression. Post-digestion, an enhancement of the structural integrity with an up-regulation of ECM expression was observed in PIs recovered under RCCS culture conditions compared to static conditions.

Chapter 6. **Effect of Simulated microgravity culture conditions on insulin secretion, content, and expression in Min6-Pseudoislets pre- and post-enzymatic digestion.**

Abstract: Achievement of glucose-stimulated insulin secretion is the primary goal in generating highly functional β-cell models *in vitro.* In this study, Min6-PIs were used to evaluate insulin secretory function. This chapter describes the evaluation of insulin gene expression, secretion and content in PIs cultured in static dishes and an RCCS bioreactor. Additionally, insulin gene expression and content following stress caused by enzyme digestion and subsequent remodelling of PIs under static and RCCS culture conditions is evaluated. mRNA was extracted from Min6 β-cells, cultured under different experimental culture conditions, and was converted to cDNA. Insulin gene expression was identified and analysed by specific primers using qRT-PCR. Insulin release was initiated first by culturing cells at 5 mM glucose for 24 hours to achieve a basal level of insulin release, and then by incubating cells without glucose overnight to switch off insulin synthesis. Insulin secretion in response to 25 mM glucose stimulation was measured by ELISA at 0 minute, 15 minute, 30 minute, and 1 hour time points. Insulin content was determined by extracting protein from PIs that had been previously stimulated and measuring content by ELISA. Results showed that basal insulin gene expression was significantly increased compared to monolayer cells in PIs cultured in both dishes and an RCCS, while insulin gene expression in response to glucose stimulation was significantly higher in PIs cultured in an RCCS compared to those cultured under static conditions. Basal insulin gene expression of PIs digested for 2 minutes was significantly decreased, and an up-regulation of insulin gene expression in PIs recovered under RCCS conditions compared to static culture conditions was observed. Insulin secretion of PIs cultured under RCCS conditions was significantly higher, especially after 1 hour of stimulation, compared to cells cultured as a monolayer and PIs in static dishes. The insulin content of PIs cultured in an RCCS was significantly increased after 15 minutes of stimulation compared to other cells. The insulin content of digested PIs was dramatically reduced, whereas an increase was seen in recovery PIs cultured in an RCCS compared to PIs cultured in static dishes. From these findings, it was concluded that simulated microgravity culture conditions have a significant effect on up-regulating insulin gene expression at basal levels and in response to glucose stimulation, thus enhancing insulin secretion and content in post-digestion PIs following recovery.

6.1. **Introduction**

Over the past three decades, researchers have invested significant resources into the establishment of insulin-secreting β-cell lines that exhibit normal characteristics of insulin secretion *in vivo*. The main purpose of generating these cell lines was to maintain a useful surrogate of pancreatic β-cells to understand the biology of primary islets in order to enhance the their functionality [27]. An increased understanding of the biology of islet cells *in vitro* could contribute to the provision of an alternative source of primary islet cells for cellular therapy [261]. The most widely-used pancreatic β-cell line by researchers internationally is the Min6 β-cell line, which was established from insulinomas obtained by the expression of T antigen of SV40 gene in transgenic mice [142]. Min6 β-cells exhibit the characteristics of native pancreatic β-cells with respect to insulin secretory function [148]. Typically, Min6 β-cells are maintained as a monolayer, although they tend to lose their insulin responsiveness over time [148]. The configuration of Min6 β-cells into 3D clusters, or PIs, enables the cells to remain glucose-responsive over longer periods of time. PIs have been widely used by researchers due to the relatively anatomical islet-like structures that they produce, a feature essential to their functioning [142]. These clusters demonstrate close cell-to-cell interactions essential for maintaining high levels of cell survival, viability, and insulin secretion. [148]. Although PIs have become a useful tool to study the insulin secretory mechanism, some obstacles limit the usefulness of this model including insufficient transportation of nutrients and oxygen during culture, leading to cell growth arrest. This growth arrest may, in part, be due to the irregular size and shape of PIs generated from static culture conditions which can cause apoptosis [153]. This observation was described by Lock *et al.* (2011), who showed that PIs generated from static dishes exhibited irregular shape and size. Conversely, PIs generated in a stirred-suspension bioreactor showed increased cell viability with high levels of insulin secretion [153].

Thus, culture conditions have a significant influence on the physiology and functionality of PIs.

Recently, 3D microgravity culture methods have been explored by researchers seeking to improve culture conditions between the islet isolation and transplantation procedures [85]. Different varieties of bioreactor, of which there are various types (as detailed in Chapter 1, section 1.3.2.5), have been used in several biological studies. The most commonly used bioreactor is of the RCCS type, which has two particularly beneficial features. The first of these is low shear stress which provides close cell-to-cell and cell-to-matrix contact because of the adjustable speed, whereby cells in culture medium inside the vessels rotate synchronously to generate low shear stress and sufficient transportation of nutrients along with oxygen. The second feature is the randomisation of gravitational vectors that enhance cell differentiation and promote the rapid establishment of 3D aggregates [173]. The achievement of cell-to-cell and cell-to-matrix interactions is essential, as both initiate crucial cues for biological and cellular processes such as morphogenesis, differentiation, proliferation, and homeostasis [262]. ECM determines the microenvironment of pancreatic islet cells *in vivo* [254]. The important roles of ECM in pancreatic islet biology involve the maintenance of islet structure and the regulation of the insulin secretory function [263]. Therefore, the re-establishment of the islet-matrix relationship, following islet isolation, is essential to improve the loss of matrix caused by enzymatic digestion [254]. Several reports have thus focused on developing an *in vitro* re-establishment mechanism to improve the functionality of islet cells, for example, by culturing insulin-producing cells, from rat models, on 2D culture platforms coated with specific ECM components such as collagen type I mixed with collagen type III, type IV, and LM [209]. These studies reported a significant enhancement in islet viability and insulin secretion.

Further studies have demonstrated highly improved islet functionality when cells were cultured under 3D culture conditions such as the use of ECM-coated hydrogels, perfusion bioreactors, or RCCS bioreactors. One study demonstrated the effect of two different culture methods, 2D static dishes and a 3D stirred suspension system, on cell viability, morphological structure and insulin secretion from Min6-Pseudoislets (PIs) [153]. Lock *et al.* (2011) generated PIs from static dishes and stirred-suspension cultures and showed an improvement in structural integrity, increased cell viability, and highly increased insulin secretion in PIs generated in a 3D culture microenvironment [153]. From these studies, it was concluded that improved culture conditions can contribute to improve functionality of islet cells and their interactions with ECM.

In this chapter, the mechanism of insulin secretory function was evaluated using Min6-PIs as a model, cultured under different experimental conditions. The effects of RCCS on the structural integrity, viability, and ECM expression of PIs were tested; enhanced morphological structure, increased cell viability and an upregulation of ECM expression was observed. From these findings, it was next deemed interesting to analyse the effect of the improved PIs model on insulin secretion following culture in an RCCS. Thus, the overall aim of this chapter was to investigate the effect of RCCS culture conditions on insulin gene expression, secretion, and content.

6.2. **Results**

Analysis of insulin expression and production in Min6-Pseudoislets cultured under different conditions.

6.2.1. **Insulin gene expression of Min6-Pseudoislets.**

Analysis of gene expression is considered a powerful tool in assessing the behaviour of cells under normal or disease states [264]. Therefore, the purpose of these experiments was to analyse the effect of static and RCCS culture conditions on PIs

by measuring insulin gene expression at basal levels and in response to a glucose challenge. The objective was to determine the speed of the insulin responsiveness to glucose stimulation and to determine the levels of insulin synthesised by PIs. GAPDH primers were used as a house-keeping gene and insulin primers were used to specifically target the insulin gene (Chapter 2, section 2.2.8.1). Briefly, mRNA was extracted from three sources of Min6 β-cells; cells cultured as monolayers, PIs cultured in static dishes, and PIs cultured in an RCCS. For basal insulin gene expression, the three sources of Min6 β-cells were cultured with 5 mM glucose for 24 hours before harvesting. A proportion of non-harvested cells were cultured without glucose in order to completely switch off insulin synthesis. For the glucose challenge, all three sources of Min6 β-cells were cultured with 25 mM glucose for 12 or 24 hours. Negative controls were included in the experiment, which were mRNA extracted from the three sources of Min6 β-cells that had not been reverse transcribed.

Results showed that basal insulin gene expression was increased significantly in cells cultured as PIs under simulated microgravity culture conditions, compared with cells cultured under static conditions and as monolayers **[Figure 6-1,** PIs in RCCS: 2.75±0.05; PIs in dishes: 1.63±0.11; monolayer cells: 1.00±0.00, means ±SD, *** $P<0.0001$**].** A significant difference in basal insulin gene expression was observed between PIs cultured in static dishes and cells as monolayers **[Figure 6-1,** PIs in dishes: 1.63±0.11; monolayer cells: 1.00±0.00, means ±SD, ***$P<0.0001$**].**

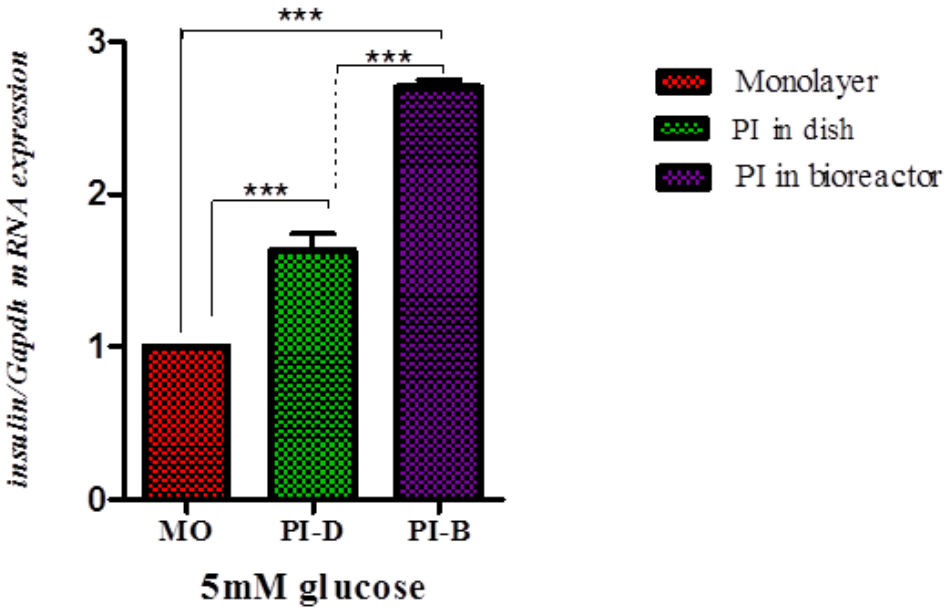

Figure 6-1: Basal insulin gene expression in Min6-Pseudoislets.

Min6 β-cells were cultured as monolayers, as PIs in static dishes, and as PIs in RCCS bioreactor with a basal glucose concentration of 5 mM for 24 hours. Data represent the ratio of insulin relative to GAPDH in each sample. Results are representative of three separate experiments. Error bar values represent mean ± standard deviation, and expression was relative to monolayer cells. Rotor-gene (Qiagen) software was used to calculate threshold Ct values, and the relative amount of gene present was calculated using the ΔΔCt method. Insulin gene expression in PIs cultured in an RCCS was significantly higher than those cultured in static dishes and as monolayers (*** $P<0.0001$). There was also a significant difference observed between PIs cultured in dishes and monolayer cells (*** $P<0.0001$).

The expression of insulin in response to high glucose was increased significantly in PIs cultured in an RCCS after 24 hours of stimulation **[Figure 6-2,** RCCS: 1.10±0.13 gene/0h, 2.51±0.19 gene/24h, means ±SD,****P*<0.0001**],** compared to PIs cultured under static conditions and a monolayer cells **[Figure 6-2,** PIs in RCCS: 2.51±0.19 gene/24h; PIs in dishes: 1.50±0.19 gene/24h; monolayer cells: 1.0±0.0 gene/24h, means ±SD,****P*<0.0001, ****P*<0.0001**].** Furthermore, a significant increase was observed in insulin gene expression in response to glucose stimulation after 12 hours in PIs cultured in an RCCS compared to PIs cultured in static dishes and monolayer cells **[Figure 6-2,** PIs in RCCS: 1.10±0.13 gene/0h, 1.75±0.34 gene/12h; PIs in dishes: 0.80±0.14 gene/0h, 1.18±0.20 gene/12h; monolayer cells: 1.0±0.0 gene/0h, 1.0±0.0 gene/12h, means ±SD,**P*<0.001, ****P*<0.001**].** There was also a significant difference in insulin expression between PI cells in static dishes and cells cultured as monolayers after 12 hours of stimulation with 25 mM glucose **[Figure 6-2,** PIs in dishes: 0.80±0.14 gene/0h, 1.18±0.20 gene/12h; monolayer cells: 1.0±0.0, 1.0±0.0 gene/12h, means ±SD, **P*<0.05**].** Furthermore, the basal insulin gene expression in PIs cultured under RCCS conditions was significantly higher than when stimulated with high glucose concentration. This could explained that at low glucose level, insulin expression is at the peak level, that's why after glucose challenge insulin expression could not exceed that level. From these findings, it can be hypothesised that PIs recruit more neighbouring cells to an active state which in turn increases the utilisation of glucose; furthermore, the growth of PIs could be influenced by micro-environmental factors. To test this hypothesis, the evaluation of PI basal insulin expression and in response to glucose stimulation in static dishes and an RCCS bioreactor was determined, with the simulated microgravity environment shown to enhance insulin gene expression compared with other cells cultured under different conditions.

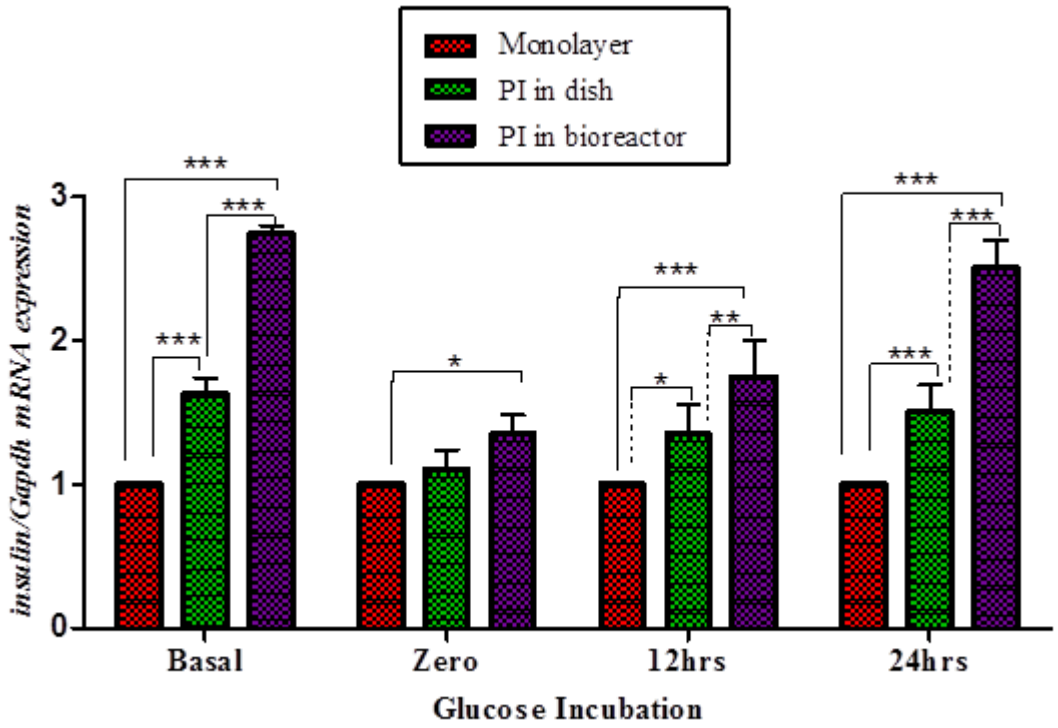

Figure 6-2: Insulin gene expression in response to glucose stimulation.

Min6 β-cells cultured as monolayers, PIs in static dishes, or PIs in an RCCS bioreactor were cultured at a basal glucose concentration of 5 mM for 24 hours, and basal insulin cells were harvested at a 0 hour time point. In order to fully switch off insulin synthesis, cells were incubated in zero glucose overnight, after which they were stimulated with 25 mM glucose for 12 or 24 hours. mRNA samples were extracted, and qRT-PCR was performed for insulin and GAPDH; the data represented the ratio of insulin relative to GAPDH in each sample. Results are representative of three separate experiments. Error bar values represent mean ± standard deviation, and gene expression was relative to that of PIs cultured in dishes. A significant increase in insulin gene expression was observed in response to 25 mM glucose between cells cultured as monolayers and PIs in static dishes at 12 hours (*P<0.05). The amount of insulin gene expression in response to 25 mM glucose for 12 hours in PIs cultured in the bioreactor was significantly higher than that seen for monolayers (***P<0.001). After 24 hours of 25 mM glucose stimulation, there was a significant increase in insulin gene transcription in PIs cultured in a bioreactor compared to those cultured in dishes or monolayers (**P<0.01; ***P<0.001).

The following section describes how insulin secretion from Min6-PIs cultured in static dishes and in an RCCS was determined using an ELISA method.

6.2.2. **Insulin secretion from Min6-Pseudoislets.**

Insulin secretion from Min6-Pseudoislets was analysed using a Mercodia mouse insulin ELISA kit. The aim of these experiments was to analyse the effect of culture conditions on the glucose responsiveness of PIs. These experiments were performed to quantify the amount of insulin released from three sources of Min6 β-cells, cells grown as monolayers, cells cultured in static dishes as PIs, and cells cultured in an RCCS bioreactor as PIs. Briefly, the experiment was initiated by culturing the three sources of Min6 β-cells at 5 mM glucose for 24 hours. Cells were harvested and media was collected for basal insulin release analysis. Non-harvested cells were incubated in glucose-free media, without FBS for 24 hours. All cells were then subjected to a 25 mM glucose challenge for 15 minutes, 30 minutes, and 1 hour (Chapter 2 section 2.2.10.1) at which media was collected. Monolayer cells were used as a control in these experiments.

Basal insulin release from monolayer cells and from PIs in dishes showed no significant differences in basal insulin secretion **[Figure 6-3,** PIs in dishes: 4.92±0.63 ng insulin/µg, monolayer cells: 4.05±0.29 ng insulin/µg protein/h, means ±SD, $P>0.05$**]**. By contrast, basal insulin release from PIs cultured in RCCS was significantly higher compared with cells cultured as PIs in dish and as monolayers **[Figure 6-3,** PI in RCCS: 6.65±0.18 ng insulin/µg, PIs in dishes: 4.92±0.63 ng insulin/µg, adherent cells: 4.05±0.29 ng insulin/µg protein/h, means ± SD,$^{***}P<0.0007$**].** To investigate the glucose responsiveness of Min6-PIs, cells were cultured under static and RCCS culture conditions, and a glucose challenge was performed for 15 minutes, 30 minutes, and 1 hour with 25 mM glucose.

Figure 6-3 showed that there was a significant increase in insulin release in PIs cultured in static dishes compared with monolayer cells in response to glucose at 30 minutes and 1 hour only **[Figure 6-3:** PIs in dishes: 1.81±0.71 ng insulin/μg protein/30 mins, 3.90±0.35 ng insulin/μg, protein/1 h; monolayer cells: 0.96±0.18 ng insulin/μg protein/30 mins, 1.98±0.12 ng insulin/μg, protein/1 h, means ±SD, $^{**}P<0.01$, $^{***}P<0.001$**].** By comparison, PIs cultured under RCCS conditions showed a significant increase in insulin secretion at all-time points of glucose stimulation compared with PIs cultured in static dishes and monolayer cells **[Figure 6-3:** PIs in RCCS: 1.94±0.32 ng insulin/μg, protein/15mins; 3.90±0.22 ng insulin/μg, protein/30 mins; 5.64±0.29 ng insulin/μg, protein/1 h; PI in dish: 0.93±0.17 ng insulin/μg, protein/15 mins, 1.81±0.71 ng insulin/μg protein/30 mins, 3.90±0.35 ng insulin/μg, protein/1 h; monolayer cells: 0.63±0.02 ng insulin/μg, protein/15 mins, 0.96±0.18 ng insulin/μg protein/30 mins, 1.98±0.12 ng insulin/μg, protein/1 h, means ± SD, $^{***}P<0.001$**].**

From these findings, previous results on insulin gene expression at basal levels and in response to glucose stimulation were confirmed; it could thus be inferred that simulated microgravity culture conditions have the ability to enhance insulin synthesis in the improved PI model when cultured under these condition. Following this work, the insulin content of Min6-PIs was determined using ELISA, in order to validate the previous insulin secretion findings.

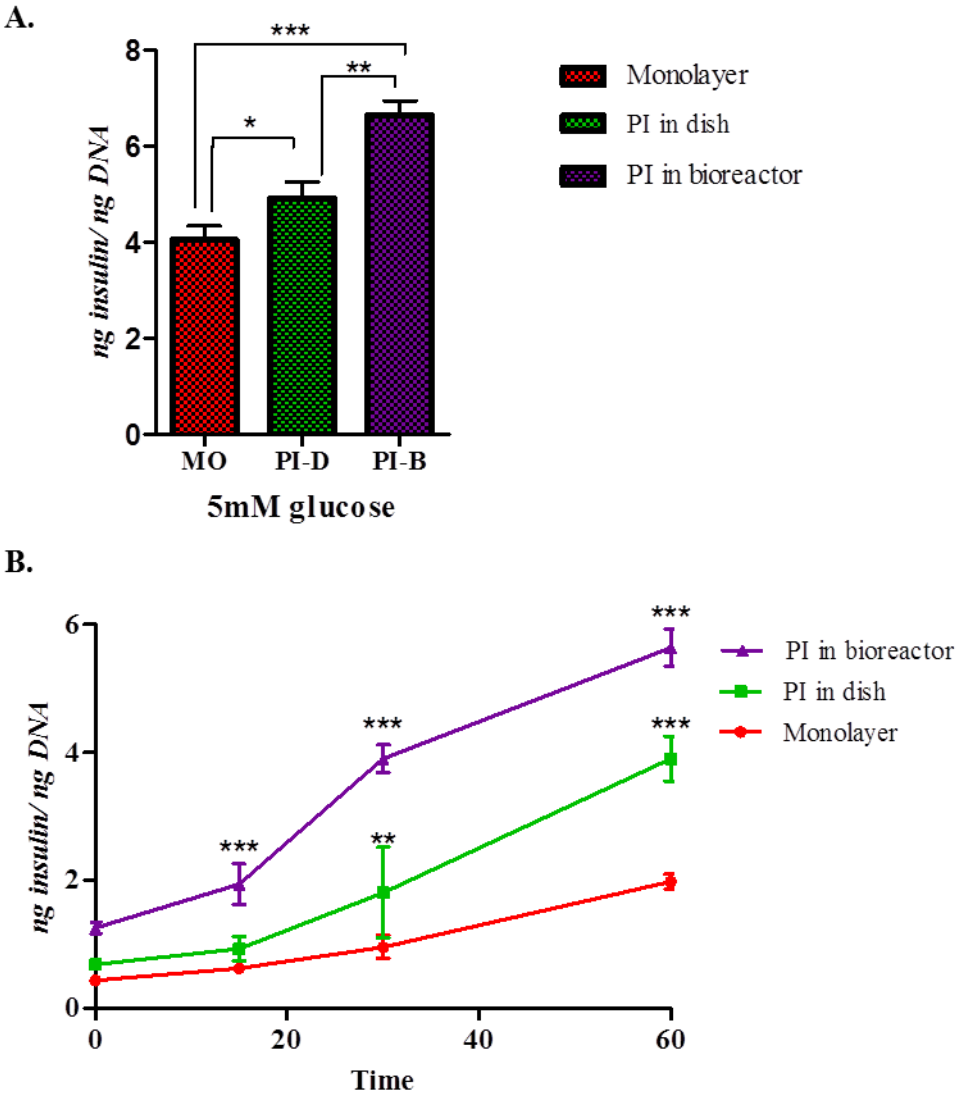

Figure 6-3: Insulin secretion from Min6-PIs in response to 25 mM glucose.

Insulin release was determined from three sources of Min6 β-cells: monolayer cells, PIs in static dishes, and PIs in an RCCS bioreactor. Cells were cultured with 5 mM glucose for 24 hours and harvested for basal insulin release in culture media (A). In order to fully switch off insulin synthesis, all three sources of cells were incubated with zero glucose overnight and media was collected. Non-harvested cells were stimulated with 25 mM glucose for 15, 30, or 60 minutes before media was removed (B). Results are representative of three separate experiments. Error bars represent ± standard deviation. Results show that basal insulin secretion was significantly greater in PIs cultured in an RCCS than PIs in dishes and cells cultured as monolayers (** $P<0.01$; ***$P<0.001$ (A)). There was a significant increase in insulin release in response to 25 mM glucose in PIs cultured in an RCCS after 1 hour of stimulation, compared to PI cells cultured in static dishes (***$p<0.001$).

6.2.3. **Insulin content of Min6-Pseudoislets.**

Intracellular insulin content was determined by using acid-ethanol extraction method. (See chapter 2 section 2.2.10.2). Briefly, for basal insulin level and following glucose stimulation, pellets from each source were incubated with acid-ethanol. The cells were then disrupted by vigorous pipetting, followed by incubation overnight at 4°C for complete extraction. Insulin content was measured by also using a Mercodia mouse insulin ELISA kit as described in Chapter 2 section 2.2.10.2. The purpose of this experiment was to validate the previous data on insulin secretion. Results showed that basal insulin content was significantly increased in PIs cultured under static and RCCS culture conditions compared to monolayer cells [**Figure 6-4,** panel A: PIs in dishes: 4.90±0.30 µg protein/h; PIs in RCCS: 6.58±0.29 µg protein/h; monolayer cells: 4.03±0.32 µg protein/h, means ± SD, **P*<0.05, ****P*<0.0001**].** In response to glucose stimulation, PIs cultured in an RCCS showed a significant increase in insulin content from zero to 25 mM glucose compared with other cells **[Figure 6-4,** panel B, ****P*<0.0001**].** In a comparison between PIs cultured in static dishes and PIs cultured in an RCCS, a significant increase in insulin content was seen from zero to 25 mM glucose for up to 30 minutes of stimulation **[Figure 6-4,** panel B**,** PIs in RCCS: 2.62±0.38 µg, protein/0 h, 3.31±0.49 µg, protein/15 mins, 6.28±0.35 µg, protein/30 mins; PIs in dishes: 0.65±0.11 µg, protein/0 h, 0.75±0.15 µg, protein/15 mins, 3.22±0.30 µg, protein/30 mins, means ± SD ****P*<0.001**].** Furthermore, a significant difference in insulin content was observed from 0 to 25 mM glucose between PIs cultured in an RCCS and in monolayers at all specified time points of stimulation **[Figure 6-4,** panel B**,** PIs in RCCS: 2.62±0.38 µg, protein/0 h, 3.31±0.49 µg, protein/15 mins, 6.28±0.35 µg, protein/30 mins, 5.01±0.30 µg, protein/1 h; monolayer cells: 0.53±0.06 µg, protein/0 h, 0.62±0.06 µg, protein/15 mins, 1.97±0.48 µg, protein/30 mins, 3.02±0.60 µg, protein/1 h, Means ± SD, ****P*<0.001**].** However, there was no significant

difference in insulin content between PIs cultured in static dishes and in monolayers from zero to 25 mM of glucose at 15 minutes of stimulation **[Figure 6-4,** panel B**,** PIs in dishes: 0.65±0.11 μg, protein/0 h, 0.75±0.15 μg, protein/15 mins; monolayer cells: 0.53±0.06 μg, protein/0 h, 0.62±0.06 μg, protein/15 mins, Means ± SD, $P>0.05$**].**

Therefore, it was suggested from these findings that intracellular insulin content of Min6 β-cells was greater in cells cultured as PIs than in cells cultured as monolayers at specific time points of stimulation. PIs cultured under RCCS culture conditions showed a significant increase in both insulin secretion and content compared with other cells cultured in a conventional 2D microenvironment. This outcome confirmed our hypothesis that creating a suitable microenvironment to improve the characteristics of Min6-PIs as a model has the potential to preserve and enhance the mechanism of insulin secretion

In the following section, the effects of digestion and recovery on insulin gene and protein expression, and the insulin content of Min6-PIs were investigated**.**

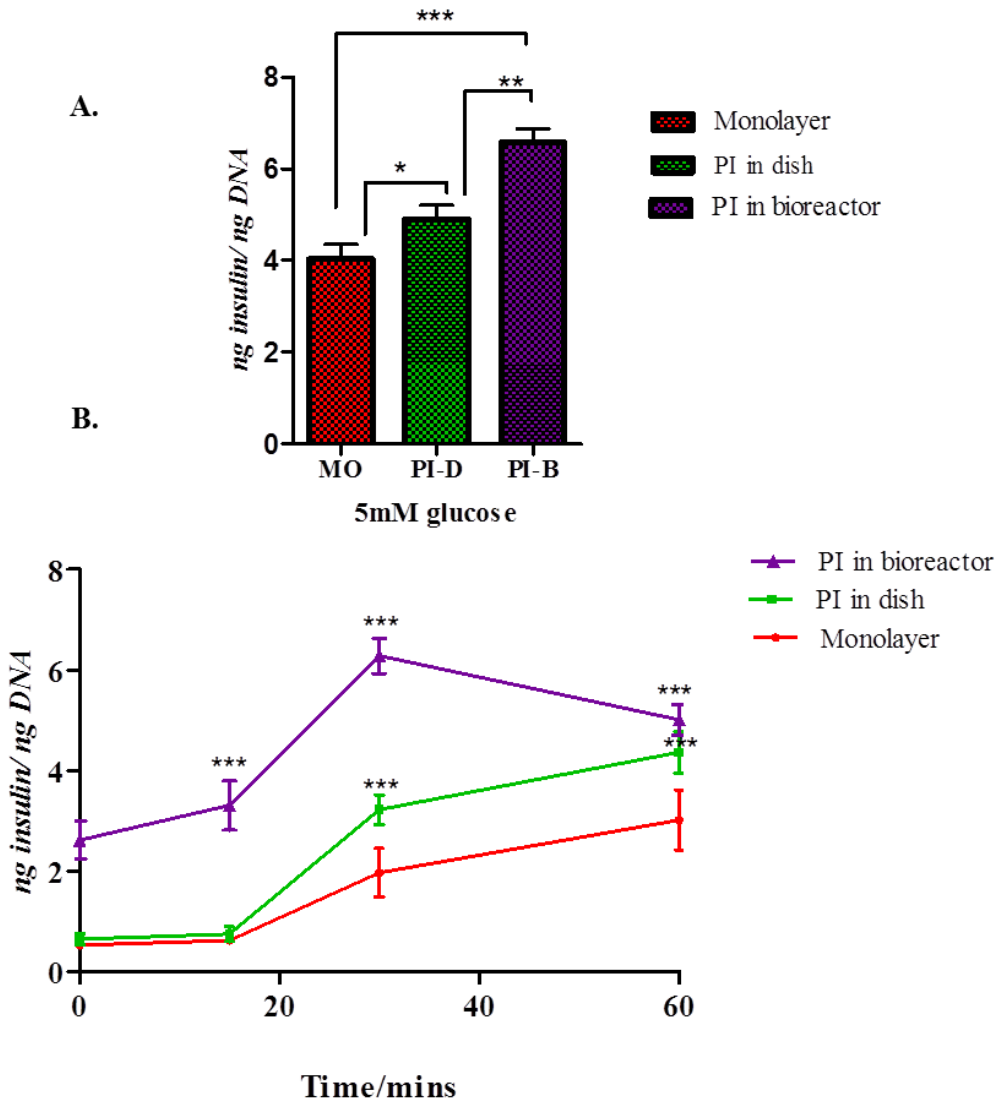

Figure 6-4 : Insulin content of Min6-Pseudoislets.

Insulin content was determined from the three sources of Min6 β-cells described previously. All cell types were cultured with 5 mM glucose for 24 hours and harvested for basal insulin release by collecting cell pellets (A). In order to fully switch off insulin synthesis at the zero time point, all sources of cells were incubated at zero glucose overnight before media was collected. Non- harvested cells were stimulated with 25 mM glucose for 15, 30, and 60 minutes, and media was removed (B). Pellets from stimulated samples were collected. Results are representative of three separate experiments. Error bars represent ± standard deviation. Results showed that basal insulin content was significantly greater in PIs cultured in RCCS compared to monolayer cells (** $P<0.01$; ***$P<0.001$ (A)). There was a significant increase in insulin content in response to 25 mM glucose in PIs cultured in an RCCS after 15 and 30 mins of stimulation, compared with PIs cultured in static dishes (***$P<0.001$). Also, a significant increase in insulin content in response to 25 mM glucose in PIs cultured in RCCS was observed, compared to monolayer cells (***$P<0.001$).

Analysis of insulin expression and production of digested and recovered Min6-Pseudoislets.

Previously in chapters 4 and 5, the effect of accutase digestion on Min6-PIs, as well as the effect of remodelling post-digestion under static and simulated microgravity culture conditions in terms of the cell viability, structural surface and ECM expression, was investigated. Digestion of ECM caused a significant effect on the cell viability, structural integrity of PIs in addition to a disruption in ECM expression. However, restoration of the cell viability, structural surface and up-regulation of ECM expression was observed in PIs which were allowed to recover in static dishes and in an RCCS. This chapter aimed to determine the effect (if any) that ECM digestion and remodelling had on insulin gene expression and protein content in PIs. Experiments were performed by analysing insulin gene expression and content in PIs digested for 2 minutes, and in PIs recovered for 24 and 48 hours under static and RCCS culture conditions.

6.2.4. **Insulin gene expression of digested Min6-Pseudoislets.**

mRNA samples were extracted from PIs cultured under different experimental conditions: PI cells under static culture conditions, PIs digested for 2 minutes with accutase enzyme, PIs recovered for 24 and 48 hours in static dishes and in an RCCS (similar steps of extraction, DNase treatment, reverse transcription, and qRT-PCR amplification were followed as described previously in section 6.2.1.). All samples included a negative control with non-reverse transcribed mRNA. The GAPDH house-keeping gene was used as a reference to which the Ct values of all samples were related. PIs cultured under normal static culture conditions were used as controls, as the experiments described in this section focussed only on PIs cultured in static dishes and RCCS. Similar calculations were performed as mentioned in section 6.2.1. Results showed that there was a significant decrease in insulin gene expression in PIs digested

for 2 minutes compared to control cells **[Figure 6-5:** control cells: 1±0.0; digested PIs: 0.44±0.11, means ± SD, ***P<0.0001**].**

However, for PIs which were allowed to recover, no significant difference in insulin gene expression was shown between control cells and PIs recovered in static dishes for 24 and 48 hours **[Figure 6-6:** control cells: 1±0.0 ng, gene/h; recovery PI cells for 24 and 48 hrs: 1.55±0.23, 1.70±0.28, means ± SD, P>0.05**].** This inferred that the recovery duration after 48 hours showed the highest expression levels in PIs cultured under static conditions. By comparison, PIs allowed to recover in an RCCS showed an up-regulation of insulin gene expression compared to control and to PIs recovered under static culture conditions **[Figure 6-6:** recovered PIs in RCCS for 24 and 48 hrs: 4.8±0.66, 5.4±0.51; recovery PIs in dishes for 24 and 48 hrs: 1.55±0.23, 1.70±0.28 ng, means ± SD, ***P<0.0001**].** These results indicated that the remodelling process under RCCS conditions in digested PIs exerted a rapid response in insulin gene expression.

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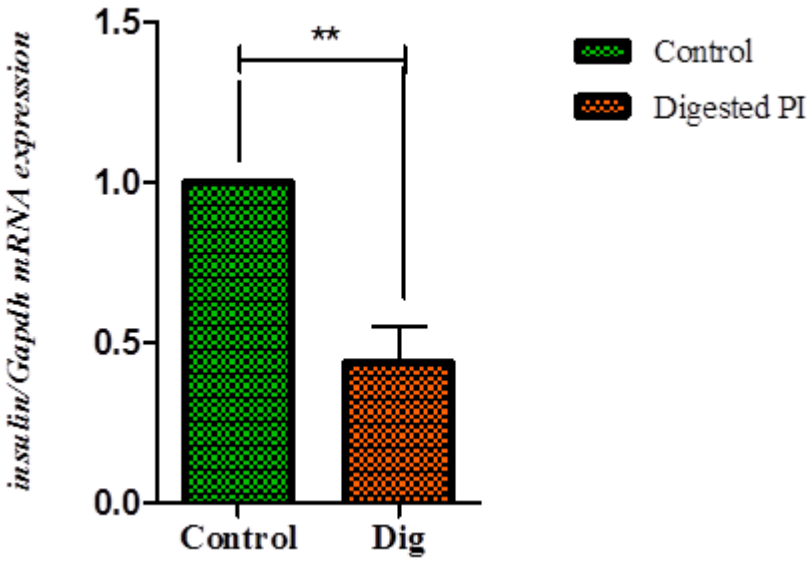

Figure 6-5: Insulin gene expression in digested Min6-Pseudoislets.

Basal insulin gene expression was determined using qRT-PCR. mRNA extraction was performed from two sources of Min6-PIs, PIs cultured in static dishes and PIs digested for 2 minutes with accutase enzyme. The data represent the ratio of insulin expression relative to GAPDH expression in each sample. Results are representative of three separate experiments. Error bar values represent mean ± standard deviation. Expression was relative to PIs cultured in static dishes (control cells). Insulin gene expression of digested PIs was shown to be significantly decreased compared to control PIs (***P<0.0019).

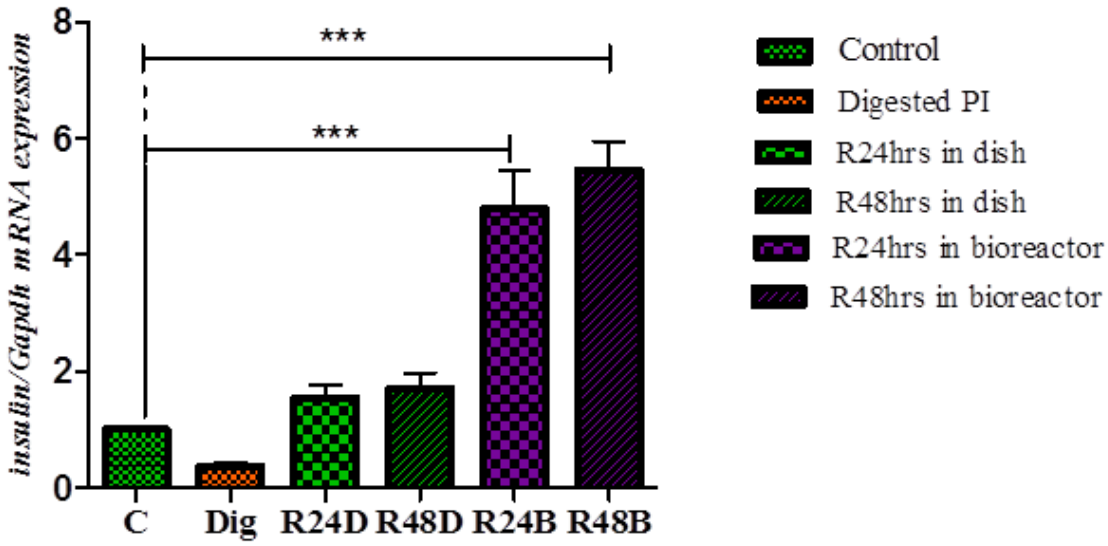

Figure 6-6: Insulin gene expression in recovery Min6-Pseudoislets.

Basal insulin gene expression in PIs which were allowed to recover in static dishes and RCCS for 24 and 48 hours was determined using qRT-PCR. mRNA extraction was performed from six sources of Min6-PIs, PIs cultured in static dishes (control PIs), digested PIs, and PIs allowed to recover in static dishes and RCCS for either 24 hours or 48 hours. The data represent the ratio of insulin relative to GAPDH in each sample. Results are representative of three separate experiments. Error bar values represent mean ± standard deviation. The expression of each sample was relative to control cells. Insulin gene expression of PIs recovered in an RCCS for 24 and 48 hrs was significantly increased compared to control cells and to PIs recovered in static dishes (*** $P<0.0019$).

From these results, it was clear that the RCCS bioreactor microenvironment had a positive impact on insulin gene expression of PIs over 24 and 48 hours. Following this work, insulin content was determined and the aim of these experiments was to confirm the previous quantitative analysis of insulin gene expression

6.2.5. **Insulin content of digested and recovered Min6-Pseudoislets.**

Insulin content of digested PIs and recovery PIs under static and RCCS culture conditions for 24 and 48 hours was measured using a Mercodia mouse Insulin ELISA kit as described in section 6.2.1.3. PIs cultured in static dishes were considered as the control group. These experiments were performed to support the previous findings of insulin gene expression by measuring intracellular insulin content inside PIs that had been subjected to digestion and recovery processes. Results showed that the total insulin content of digested PIs was significantly decreased **[Figure 6-7**: control cells: 4.53±0.17 µg protein/h; digested PIs: 0.77±0.13 µg protein/h, means ± SD, ***P<0.0001**].** PIs that were allowed to recover under static culture conditions showed no significant difference from the control cells **[Figure 6-7:** control cells: 4.53±0.17 µg protein/h; PIs recovered in dish for 24 and 48 hours: 4.19±0.21 µg protein/R24 hr, 4.27±0.34 µg protein/R48 hr, means ± SD, P>0.5**]**. However, compared with digested PIs, the insulin content of recovery PIs in static dishes showed a significant increase of 4 µg/L [**Figure 6-7:** PIs recover in dish for 24 & 48 hours: 4.19±0.21 µg protein/R24hs,: 4.27±0.34 µg protein/R48hrsh; Digested PIs: 0.77±0.13 µg protein/h, means ± SD, ***P<0.0001]. In comparison, the insulin content of PIs recovered in RCCS showed a significant increase to 5.3 µg/L after 24 hours of recovery **[Figure 6-7:** PIs recovered in RCCS for 24 and 48 hrs: 5.27±0.26 µg protein/R24hr, 6.13±0.19 µg protein/R48hr; control cells: 4.53±0.17 µg protein/h; PIs recovered in dish for 24 and 48 hours: 4.19±0.21 µg protein/R24hr, 4.27±0.34 µg protein/R48hr, means ± SD, ***P<0.0001**]**, and a significant increase of 6.13 µg/L after 48 hours of recovery compared with control cells.

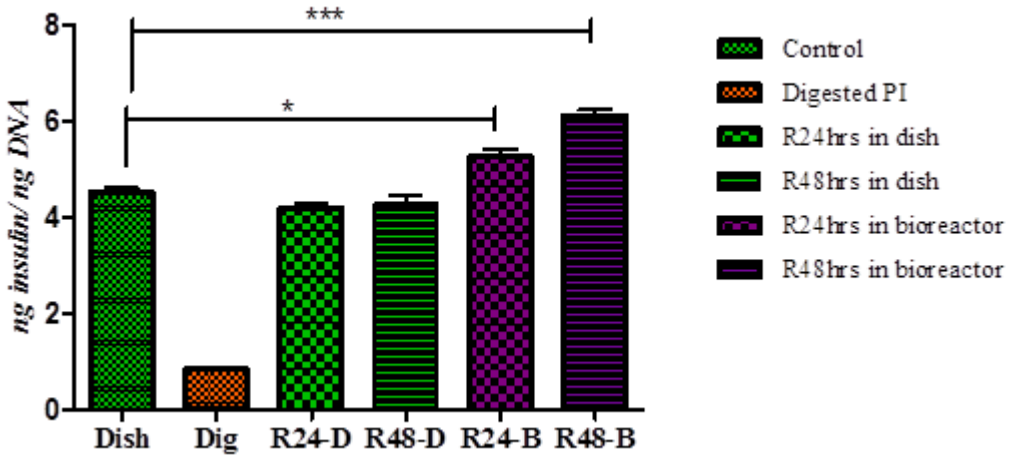

Figure 6-7: Insulin content of digested and recovered Min6-Pseudoislets.

Insulin content was determined using an ELISA method. All six sources of Min6-PIs, control PIs, digested PIs, and PIs allowed to recover for 24 hours or 48 hours under static and RCCS bioreactor culture conditions before protein was extracted for assay measurement. Results are representative of three separate experiments. Error bar values represent mean ± standard deviation. There was a significant increase in insulin content of PIs recovered in static dishes and RCCS observed at 24 and 48 hrs, compared with PIs digested for 2 minutes (***P<0.001). Insulin content of recovery PIs after 48 hrs in the bioreactor was significantly greater than PIs recovered in static dishes for 24 and 48 hrs (***P<0.001; **P<0.01).

6.2.6. **Insulin protein expression in Min6-Pseudoislets under different culture conditions.**

ICC analysis was performed to evaluate insulin protein expression in PIs cultured under static culture conditions, PIs digested for 2 minutes, and recovery PIs in static dishes and RCCS. These experiments aimed to investigate the effect of the experimental conditions mentioned above on insulin localisation and expression. Experiments were conducted according to the ICC method described in Chapter 2 (section 2.2.11). Control cells, which were PIs cultured in static dishes, were included in the experiment [**Figure 6-8**, panel A**]**. Results showed weak cytoplasmic insulin expression in digested PIs **[Figure 6-8,** panel B**]** compared with control cells. Recovery PIs exhibited up-regulated insulin expression [**Figure 6-8**, panels: C-F**].** However, the expression of insulin in PIs that had recovered in RCCS was higher than in PIs recovered in static dishes **[Figure 6-8,** R24-B; R48-B**].** Restoration of insulin protein was observed in PIs which had been allowed to recover under static and RCCS culture conditions.

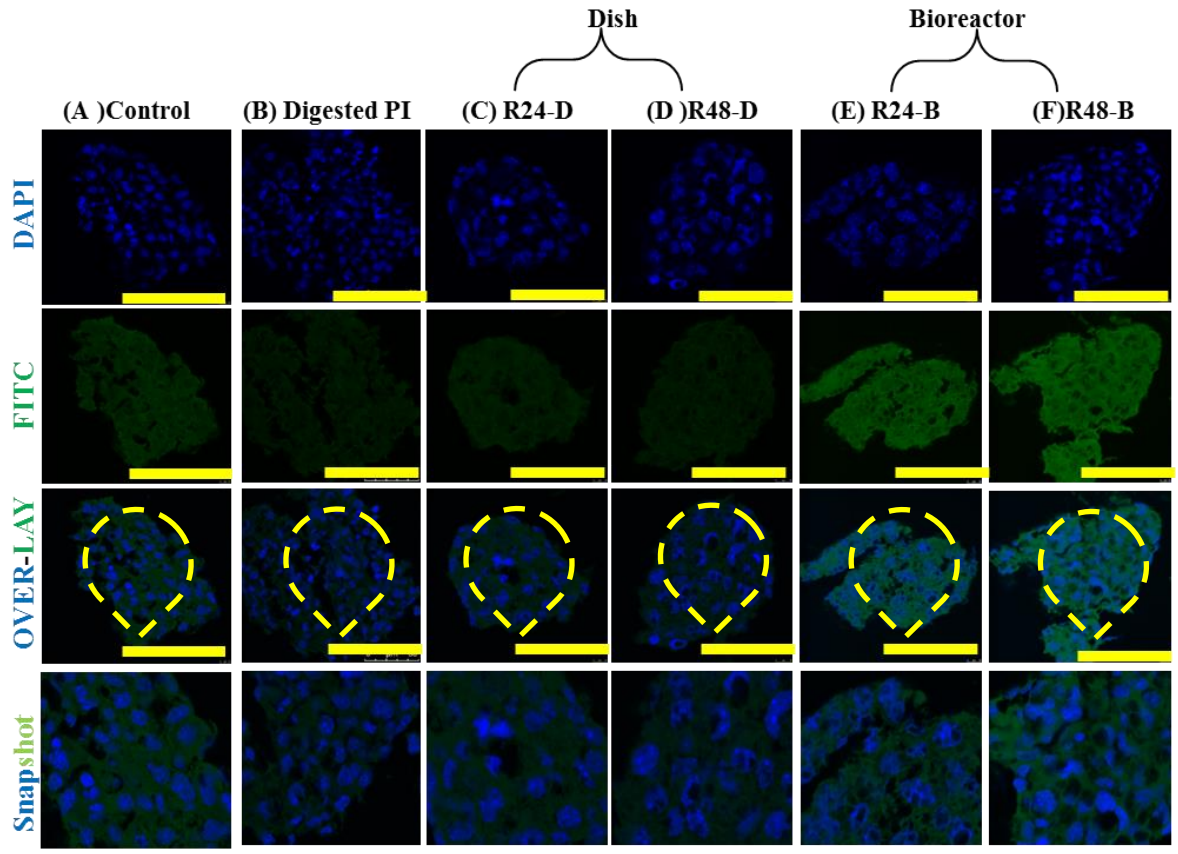

Figure 6-8: Insulin protein expression in digested and recovered Min6-Pseudoislets.

Insulin protein expression in digested and recovery PIs in static dishes and RCCS was determined using ICC. PI sections were obtained using a cryosectioning method and sections were stained with a specific insulin antibody. Results are representative of three separate experiments and images are representative of six separate fields. Insulin localisation and expression in digested PIs was cytoplasmic with weak expression (panel B). Insulin localisation and expression of PIs recovered for 24 or 48 hours under both static and RCCS conditions (panels: C-D; E-F) was cytoplasmic with up-regulated of insulin expression compared with digested PIs. However, high insulin expression was seen in PIs recovered under RCCS conditions compared with PIs cultured in dishes. Overall, PIs recovered in an RCCS bioreactor showed greater up-regulation of insulin protein expression. Scale bar: 100 µM. SNAP-SHOT is a selected area, closed capture, of over-lay image.

6.3. **Discussion**

In islet transplantation, isolated islet cells are subjected to culture for 1-2 days prior to transplantation to improve cell viability and mitigate the damage caused to basement membrane (BM) due to enzymatic digestion during the isolation process [265]. Thus, many researchers have invested significant effort in developing a protective culture mechanism to restore the damage caused to islet cells [265]. Modification of culture conditions such as coating petri dishes with specific ECM components, seeding insulin-producing cells into scaffolds composed of specific ECM, or using simulated 3D culture methods such as bioreactor have been applied. The most significant site of damage during islet isolation is BM or the peri-islet capsule where ECM is located. The major components of ECM found in islet cells are collagen type I and IV, LM, and fibronectin. These proteins play a major role in islet biology by providing physical stability and a protective barrier against any damage [266, 267].

According to transplantation studies, the presence of ECM in islets biology is essential for their functionality, communication and attachment [77]. Thus, the reestablishment of destroyed ECM components post-islet isolation is crucial in order to preserve and restore islet functionality post-transplantation. An indication of the importance of ECM in improving islet transplant outcomes has been demonstrated in mouse models transplanted with ECM-coated scaffolds. In a study by Salvay *et al.* (2008), implantation of Coll IV with lactide and glycolide polymer scaffold led to improved outcomes [268]. Also, similar results were obtained from a human islet model where islet cells were embedded in a synthetic matrix made up of nanofibers [73]. The resulting 3D structures were transplanted into diabetic mice, leading to significant insulin secretion [73].

Thus, the provision of a suitable microenvironment and preservation of the 3D organisation of islet cells prior to transplantation could potentially maintain the initial morphology of cells which, in turn, could lead to an increase in insulin secretory function for several weeks of culture [269].

Achievement of appropriate insulin secretion is vital as this mechanism is dependent on how β-cells communicate, either directly with other cells or indirectly with ECM molecules that simultaneously signal a multitude of cells. Therefore, the effective insulin secretory function of β-cells is associated with their 3D anatomical configuration [270]. An *in vitro* β-cell line model that has been used widely in several research studies is the; Min6 β-cell lines. These cells were derived from transgenic mice, and were developed as an ideal model to study the biology of β-cells *in vitro* [142]. These cell lines exhibit normal characteristics of native β-cells of insulin secretory function, especially when re-configured into 3D structures known as PIs [148]. This 3D anatomical configuration has been shown to induce a significant enhancement in insulin secretory responses [150].

The findings of the present study of improved insulin secretion in Min6 β-cells from monolayers to PIs are in line with previous research. This chapter sought to investigate the effect of simulated microgravity culture conditions, an RCCS bioreactor; on thee insulin secretory function of Min6-PIs to determine whether culturing PIs in RCCS further improves this insulin secretion. Beyond this the insulin secretion post-digestion and recovery was also evaluated. As a starting point, it was important to investigate the mechanism of insulin gene expression and secretion in Min6-PIs cultured under static and RCCS culture conditions, and to evaluate how the culture microenvironment has the ability to influence insulin synthesis.

It was clearly shown in Chapters 4 and 5 that the culture microenvironment influences the cell viability, structural appearance and expression of specific ECM proteins in PIs.

Accordingly, there was a significant increase in insulin gene expression at a basal level **[Figure 6-1]** and in response to glucose stimulation for 12 and 24 hours **[Figure 6-2]** in PIs cultured in RCCS compared to those cultured under static conditions and cells cultured as monolayers (**P<0.01; ***P<0.001). This indicates that the improved anatomical structure and enhanced ECM expression in PIs cultured in RCCS positively influenced insulin gene expression at basal levels and maintained a rapid response to a glucose challenge compared with PIs cultured under static conditions. A glucose challenge was applied in Min6β-cells at different time points, i.e., 0 minute, 15 minutes, 30 minutes, and 1 hour, in order to analyse insulin secretion. The insulin secretory response to glucose stimulation was significantly higher in cells configured as clusters (PIs) compared to monolayer cells **[Figure 6-3].** By comparison, PIs cultured under static conditions released less insulin compared to those cultured under simulated microgravity conditions where insulin release was significantly increased after 30 minutes and 1 hour of glucose stimulation **[Figure 6-3].** By comparison, PIs cultured under RCCS culture conditions showed a significant increase in insulin secretion at all-time points of glucose stimulation compared with PIs cultured under static culture conditions and monolayer cells. The significant rise in insulin secretion in PIs under RCCS conditions reflected the considerable amount of newly-synthesised insulin found inside the clusters. An indication of insulin secretion depletion in PIs cultured under static culture conditions was reported in a study testing cell-to-cell interactions in Min6-PIs. The study was demonstrated by Luther *et al*. (2005) which they reported that the growth of PIs was arrested after 1 week of incubation [225]. It was noticed that this growth arrest contributed to the irregular size of PIs and influenced insulin secretion and cell viability [225]. Inhibited cell growth was related to the physical culture environment found in conventional static conditions (such as poor transportation of oxygen and nutrients) rather than to the PIs self-regulatory mechanisms. Thus, a study

by Lock *et al*. (2010) tested this hypothesis by generating PIs from both static dishes and a stirred-suspension bioreactor [153]. PIs maintained under spinner flask conditions for two weeks exhibited high levels of insulin release, increased proliferation, and improved viability, unlike PIs maintained under static dish conditions which showed a necrotic core after 1 week of incubation, indicating growth arrest whereby the functionality of cells was decreased [153]. Considering the findings of these studies, no study has previously demonstrated the effect of a 3D microgravity culture environment directly on PIs by culturing clusters directly under these conditions. This study has therefore demonstrated a novel mechanism by testing 3D culture environment effects on PIs that were initially generated from static dishes. The purpose of this work was to improve the functionality and characteristics of the Min6-PI regulatory mechanism.

From these findings, the hypothesis behind testing the effect of a 3D RCCS bioreactor on insulin synthesis in Min6-PIs was validated. This was achieved by maintaining intact cells, due to favourable culture conditions in the RCCS, which recruited more neighbouring cells to an active state which in turn increased PI utilisation of glucose. In addition to this, the suitable culture microenvironment presented by the RCCS is beneficial in creating close cell-to-cell interactions essential for cell integrity as well as providing a viable environment for highly functional PIs. Thus, the maintenance of PIs-matrix attachments under a simulated microgravity environment may improve the PIs insulin secretory function.

Furthermore, a recent study has reported that a simulated microgravity culture environment had a positive impact on islet transplantation of streptozotocin-induced diabetic mice by generating a large number of highly functional β-cells from a mouse model *in vitro* using 3D clinostat [248]. The 3D clinostat approach is classified as a simulated microgravity culture condition. Tanaka *et al*. (2013) managed to generate spheroids of size 250 μM of under clinostat conditions, a size considered optimal for the

induction of successful engrafting [248]. They reported a significant increase in insulin gene expression at basal levels and in response to glucose stimulation, compared with Min6-PIs maintained under static dish conditions [248].

Insulin content was measured in this chapter and it was considered essential to evaluate the ability of PIs to produce insulin in different culture environments. In this study, PIs cultured under simulated microgravity conditions showed a significant increase (***P<0.001) in insulin content compared with cells cultured under static conditions as PIs and as monolayers **[Figure 6-4].** Notably, after 15 minutes of glucose stimulation, the insulin content in PIs cultured under RCCS produced more insulin compared to other incubation time points, as compared with cells cultured under static conditions **[Figure 6-4].** Although 15 minutes of glucose challenge is considered a short duration for new protein production, this observation may be attributed to the homogenous environment found in RCCS which most likely improved the rapid response of glucose stimulation via PIs-matrix interactions. Therefore, 15 minutes of glucose-induced insulin release was considered the peak incubation time for PIs in this study in order to optimise insulin production. Moreover, many studies have concluded that significant evidence exists to suggest that islet cells are highly influenced by cell-ECM interactions. Therefore, intact islet cells that interact with ECM have shown significant cell survival [181], increased insulin secretion [254], and increased proliferation [182].

Thus, from the results presented above, significant conclusions can be drawn. As cells naturally grow in a 3D environment *in vivo*, a novel 3D microenvironment culture condition was maintained *in vitro* in this study. These novel culture conditions have the ability to improve the characteristics and functionality of Min6-PIs. Therefore, it can be proposed that proper transport of nutrients and oxygen was likely the main reason for enhanced insulin gene expression, secretion, and content of PIs. To that end, the 3D microenvironment cell culture method has the potential to maintain the functionality of

insulin-producing cells which regulate β-cell insulin secretion, and may also be scaled up for the generation of highly physiological and cellular responses in cells to the benefit of clinical applications.

In order to replicate the conditions that islet cells are exposed to prior to islet transplantation, it was necessary to evaluate insulin functions in PIs that were stressed with accutase enzyme and then allowed to recover under static dish and RCCS bioreactor conditions. Insulin gene expression in digested PIs (for 2 minutes with accutase enzyme) was analysed using qRT-PCR and compared to control cells. A significant decrease in insulin gene expression was observed in digested PIs as compared with control cells [**Figure 6-5].** In addition, the insulin content was measured in digested PIs [**Figure** 6-7] and also showed a significant decrease in insulin content compared with control cells. The decreases in both insulin gene expression and production indicate that, during the digestion process, significant damage was exerted on the PI-ECM, disrupting the mechanism of insulin synthesis as described in Chapter 5. Thus, complete detachment between islet cells and ECM could thus affect insulin response to glucose stimulation [164]. A study in a goat model, conducted by Hani *et al.* (2015), showed that a huge mechanical stress was induced during islet isolation which affected the structure and functionality of isolated islet cells [271]. Isolated islets were purified pre-transplantation in order to limit the damage caused by enzymatic digestion. Although the purified islets exhibited minor alterations in structure and viability, this did not affect the functionality of the cells [271]. This indicates that the damage induced by enzymatic digestion can be restored through the use of a purification step leading to the re-establishment of the islet-matrix relationship post-isolation [77].

In this study, an *in vitro* model of islets isolation was successfully implemented in order to mimic the actual *in vivo* isolation procedure, indicated by the significant decrease both in insulin gene expression and production which was attributed to the loss of

cluster membrane and the disruption of PI-matrix interactions. Following an enzymatic digestion step, PIs were allowed to recover under static and simulated microgravity culture conditions for 24 and 48 hours to determine whether culture conditions had the ability to restore damage caused to insulin function.

The remodelling process in Min6-PIs was determined by investigating its effect on insulin gene expression and content. Previously (Chapter 5), the PI cluster membrane was re-established *in vitro* after re-culturing PIs under static dish and RCCS conditions. The assembly of cluster membranes was well underway at 48 hours of incubation and was characterised by restored morphological structure, increased cell viability, and up-regulation of ECM expression. According to the re-establishment process, ECM (in particular) and insulin gene expression and content were up-regulated in PIs. **Figure 6-6** showed that insulin gene expression was up-regulated in PIs under both static and simulated microgravity culture conditions compared with digested PIs. Interestingly, insulin gene expression in recovery PIs under RCCS conditions showed a significant increase compared with control cells and PIs recovered under static conditions **[Figure 6-6,** ***P<0.001**].** Furthermore, a significant increase in insulin content was observed in recovery PIs under RCCS conditions compared to those under static conditions and to monolayer cells **[Figure 6-7].** However, there was no significant difference in insulin gene expression or content between PIs recovered under static dish conditions and control cells [**Figure 6-6; Figure 6-7,** P>0.05**].** This observation explained that, for PIs re-cultured under 2D-culture conditions, a 48 hour recovery period is insufficient for PIs to restore the damage caused to their structure and ECM. Furthermore, insulin synthesis can potentially require more time in order to response to glucose in damaged cells. Because of the poor culture conditions presented by static dishes (insufficient oxygen and nutrient supply to allow cells to recover), the restoration of insulin function may be delayed. According to these findings, success in maintaining a suitable

microenvironment for PIs under simulated microgravity conditions that can modify ECM expression post-digestion is a crucial aspect in mediating the intracellular interactions required for cell growth, adhesion and insulin secretory function. Recent reports have considered the importance of collagen-based scaffolds on achieving long-term glucose homeostasis [61]. Riopel and Wang (2014) reviewed a study conducted by Nagata *et al.* (2002) that showed a significant increase in insulin function and suppressed cell death in a rat model, once islets were coated with a mixture of collagen and LM-scaffold post-isolation for revascularisation [61, 181]. Thus, the maintenance of islet-ECM relationships post-isolation is crucial for achieving highly-functional islets.

The use of simulated microgravity culture techniques post-isolation was considered one of the optimal methods of culturing cells *in vitro*. A previous study has shown that the morphology and cell growth of Min6-PIs was enhanced with regulated insulin secretion at basal level and in response to glucose once cells were cultured in a stirred-suspension bioreactor compared to static dishes [153]. However, this study focused on re-establishing an improved, protective mechanism for Min6-PIs as a model that has been cultured directly under RCCS conditions.

Moreover, the re-establishment process used in this study, and the assembly of Min6-PIs post-digestion, was maintained and characterised by continuous staining of insulin protein [**Figure 6-8**]. Recovery PIs showed continuous staining of insulin protein under static and RCCS culture conditions [**Figure 6-8:** panels C-D and E-F] compared with digested PIs, where insulin staining was decreased showing weak protein expression [**Figure 6-8**, panel B]. However, insulin staining was strongly expressed in PIs recovered under RCCS conditions [**Figure 6-8:** panels E-F], compared to those recovered under static conditions.

Our model RCCS bioreactor therefore preserves the functionality of Min6-PIs in terms of ECM interactions and insulin secretory function pre- and post-digestion, a process

which can benefit from a useful model in order to develop innovative therapeutic approaches to diabetes. Therefore, the potential benefit of the remodelling process under 3D RCCS culture could include improved insulin secretory function, mediated by maintenance of PI-matrix attachments **[**Error! Reference source not found.**].** Based on current research, the Min6-PIs model is capable of re-establishing the natural ECM environment *in vitro.*

Therefore, this approach needs to be tested on human islets and may thus prove valuable for the improvement of islet transplant outcomes, a process which remains ineffective at maintaining long-term glucose control. Thus, by using similar techniques to those used in this study, the preservation of long-term islet function and survival may prove successful.

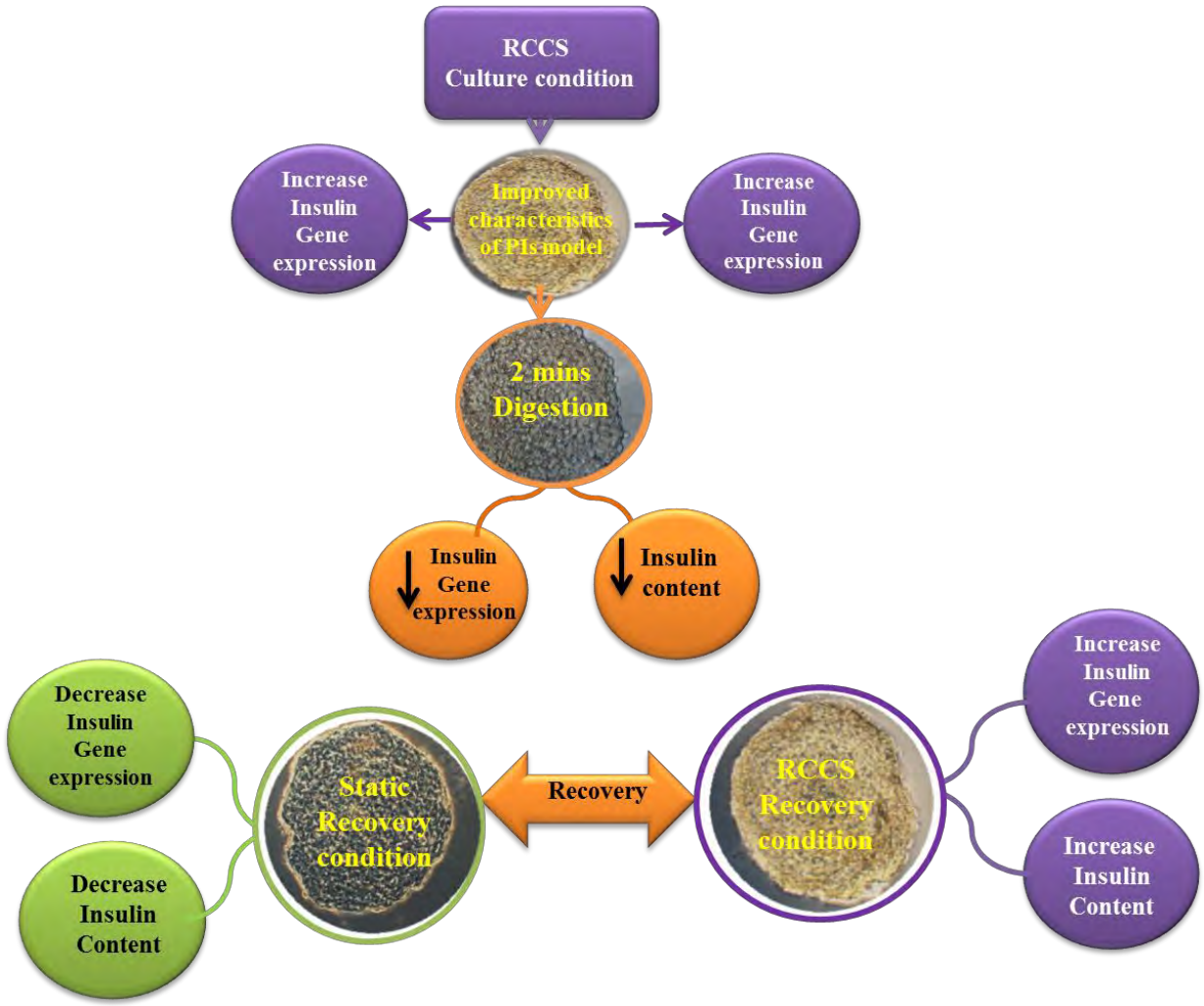

Figure 6-9: Schematic diagram showing the effect of microgravity cell culture condition on insulin secretory function of Min6-Pseudoislets.

Culturing Min6-Pseudoislets under RCCS-bioreactor culture conditions have enhanced the characteristics of Pseudoislets i.e. the morphological structure and ECM expression that improved insulin gene expression and secretory function in response to glucose stimulation. The enzymatic stress that has been induced to PIs has decreased insulin gene expression and content. However, the recovery process under RCCS culture conditions has up-regulated insulin gene expression and increased insulin content.

Chapter 7. **Conclusions and Future Work**

The work described in this study may be considered an optimistic first step towards the generation of highly improved Min6-Pseudoislets (PIs) which could provide an attractive model for expanding the knowledge on the important of maintaining a long-term suitable culture environment using rotary cell culture system (RCCS) that has the ability to preserve β-cells in PIs physiology.

In this chapter, a summary of the main aims and research findings, along with their conclusions, is presented. Furthermore, future research work is proposed, thus illustrating the next experimental steps to be investigated.

7.1. Aims, main findings and conclusions

- **To investigate the effect of static and RCCS cell culture conditions on the morphological structure, cell viability, ECM expression, and insulin expression and secretory function of Min6-PIs.**

1. PIs were found to be well adapted by RCCS culture conditions, showing enhanced normal cellular organisation and morphological features by maintaining a refined-smooth cluster membrane.
2. The static culture environment also exhibited preserved cellular morphology of PIs, although irregular surfaces with rough membranes were observed.
3. The culture conditions presented by RCCS preserved PIs viability and increased their metabolic activity.
4. The viability of PIs cultured under static dish conditions was affected by the presence of some apoptotic cells, with less metabolic activity observed compared with RCCS culture conditions.

5. A significant increase in ECM gene and protein expression in PIs cultured under RCCS conditions compared to static conditions was observed.
6. A significant increase in insulin gene expression at basal level and in response to glucose stimulation was seen in PIs cultured under RCCS conditions compared with those cultured under static conditions.
7. Insulin secretion and content at basal level and in response to a glucose challenge was significantly increased in PIs cultured under RCCS conditions compared to those cultured in static dishes

Conclusion: Min6-PIs have the ability to adapt in a period of 7 days culture to the effect of a simulated microgravity culture environment. Demonstrating enhanced cellular organisation, and evoking superior survival of Min6-PIs by preserving cell viability and enhancing metabolic activity of cells. An up-regulated ECM gene and protein expression was seen in PIs cultured under RCCS conditions which contributed to enhanced insulin gene expression, secretory response and content, as compared with static culture conditions

- **To investigate the effect of digestion enzyme on Min6-PIs morphological structure, cell viability, ECM expression, and insulin expression and production.**

1. Enzymatic digestion of PIs for 2 minutes caused minor degradation within the structure membrane.
2. The cell viability of digested PIs was affected, as measured, by the presence of apoptotic cells with some necrosis at the periphery of the cluster.
3. A significant decrease on ECM gene and protein expression was observed in digested PIs.

4. Enzymatic digestion down-regulated insulin gene expression and content at a basal level in digested PIs

Conclusion: Enzymatic digestion, similar to that which pre-transplant islets are subjected, had a noticeable effect on the cluster membrane and viability of PIs. A significant decrease in ECM gene and protein expression and a significant decrease in insulin gene expression and content were measured.

- **To investigate the effect of static and RCCS cell culture recovery conditions on of Min6-PIs in terms of morphological structure, cell viability, ECM expression, and insulin expression and secretory function post-digestion.**

1. Complete restoration within the structure membrane of PIs recovered post-digestion under RCCS culture conditions compared to static dishes was observed.
2. The viability of recovered PIs under RCCS conditions was preserved compared with static culture conditions where the viability was affected indicated by the presence of apoptotic and necrotic cells.
3. ECM gene and protein expression was up-regulated in recovered PIs under RCCS conditions compared with static culture conditions after 24 and 48 hours of incubation.
4. A significant up-regulation of basal insulin gene expression and content was observed in PIs recovered under simulated microgravity culture conditions compared to static conditions.

Conclusion: A simulated microgravity culture environment had the ability to promote an effective remodelling mechanism for PIs post-digestion. An enhanced morphological structure with preserved cell viability of PIs recovered under RCCS conditions was observed. The restoration of the structure and cell viability lead to an up-regulation of ECM expression and increased insulin secretory function of PIs recovered under RCCS conditions compared with static culture conditions.

7.2. **Final Conclusion**

The improved morphological structure with preserved cell viability and ECM expression of PIs cultured under a simulated microgravity environment contributed to enhanced insulin gene expression, secretory response and content, compared with static culture conditions. Enzymatic digestion had a negative effect on the morphological structure resulting in decreased cell viability, and significant decrease to ECM expression and insulin gene expression and production. However, restored morphological structure linked with an increase in cell viability, up-regulated ECM expression and improved insulin expression and secretion, followed remodelling under RCCS culture conditions. Therefore from these findings, the novel RCCS culture condition had the ability to improve the characteristics of Min6-Pseudoislets model, and make the clusters robust against the enzymatic stress by the quick recovery response. This describes a novel approach to enhance ECM expression and insulin secretory responsiveness in Min6-PIs **[Figure 7-1].**

In conclusion, this study has developed an attractive model that mimic the digestion of pancreatic islets pre-transplant. By using a novel cell culture method, successful restoration of PIs structure post-digestion was achieved. This in return preserved the viability of the cells and enhanced PIs-ECM interaction leading to greatly enhanced insulin secretory function. This suggests that the novel of RCCS could present a protective mechanism for islet cells post-isolation in clinical transplants.

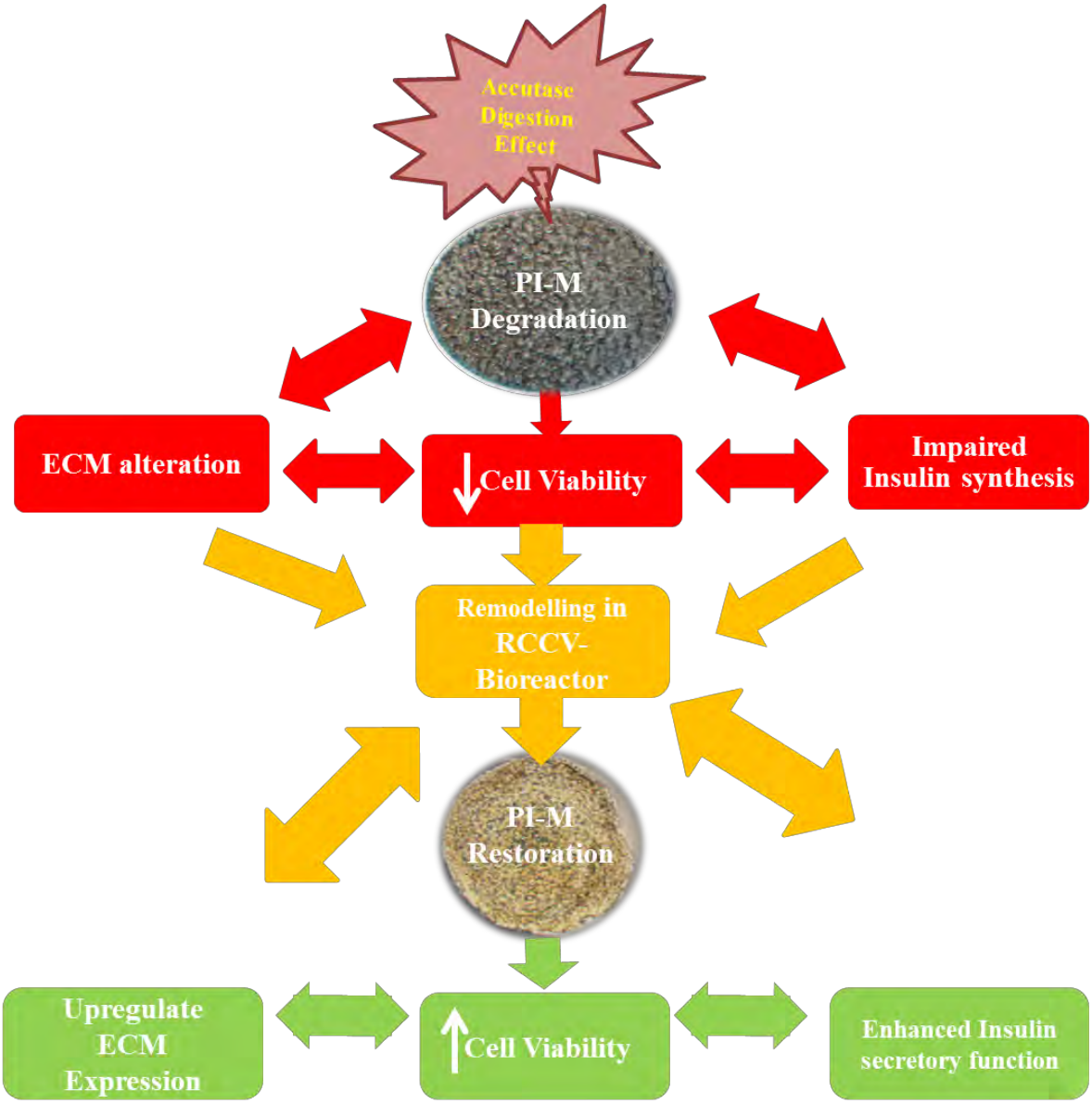

Figure 7-1: Schematic diagram of the effect of microgravity cell culture on Pseudoislet membrane (PI-M), extracellular matrix (ECM) expression, Pseudoislets remodelling and insulin secretory function following enzyme digestion.

The novelty of 3D-microgravity cell culture (RCCS) had the ability to restore cell viability, upregulate specific ECM expression and enhance insulin secretory responsiveness in Min6-PIs, which could present a successful remodelling mechanism the PIs post-enzymatic digestion.

7.3. **Proposed future work**

Significant limitations, such as a shortage of donors and the mechanical stress induced in islet cells during the isolation process, have hindered the success of islet transplants to date [94]. Researchers have therefore investigated various approaches to address these obstacles by developing alternative techniques to generate sufficient highly-functional islet surrogates. Development of protective mechanisms for isolated islets have also been investigated by culturing islets under simulated microgravity conditions or embedding them in polymer scaffolds in order to re-build the matrix loss induced by enzymatic digestion. Current studies have focused on the use of the rodent Min6-PIs model to study cellular communication and matrix interactions of β-cells. These biological interactions are required for a normal pattern of insulin release for which an alternative surrogate could be potentially beneficial in the clinical field. The novel Min6-PIs model presented here produced effective and promising data, although a number of future recommendations are suggested to improve this approach.

- **Investigation of the effect of recovery under perfusion system on Min6-Pseudoislets.**

The recovery process that was performed in this study was under RCCS culture conditions i.e. for 24 hours and 48 hours. So, it would be interestingly to test the recovery effect on Min6-Pseduoislets under perfusion bioreactor system culture conditions for longer periods of time. In a perfusion system, a continuous feeding of fresh cell culture medium is maintained from an external media bottle. Thus, continuous transportation of nutrients, metabolites, and O_2 is achieved with a continuous removal of waste products. As a results, a highly increase in cell aggregates and productivity is achieved which could provide an attractive *in vitro* method for large-scale production [272]. Therefore, it would be interesting to investigate if further enhancement to the

effect of this controlled environment on Min6-Pseduoislets ECM interaction and insulin secretory function.

❖ Investigation of the effect of hypoxic conditions on Min6-Pseudoislets.

During islet isolation, cells are exposed to hypoxia. Islet cells are thus known to be highly sensitive to hypoxic conditions [273]. The loss of BM has a significant effect on specific ECM components (such as collagen type I) and on cell-matrix interactions, specifically the expression of integrin receptors α3, α5, αV and β1, found to be highly expressed in human islet cells *in vivo.* Thus, to mimic the entire process of islet isolation *in vitro*, effects of hypoxic conditions could be evaluated using a hypoxia chamber that permits controlled humidity, temperature, O_2, and CO_2 concentrations. The optimal selected O_2 concentration for hypoxic conditions, previously determined by our research group, is 3%. [Kumar, S. unpublished observation]. Furthermore, it would be interesting to investigate specific biological parameters such as the expression of vascular endothelial growth factor (VEGF). VEGF plays a major role in maintaining normal islet blood vessels and increase insulin secretory function [274], hence the survival of islet graft, pre-islet transplant, with proper islet function depends on revascularization. Therefore, VEGF considered an essential key factor for improving islets functionality, and thus it would be interesting to investigate the expression of VGEF in Min6-Pseudoislets under 3D-cell culture conditions.

To sum up, this study has developed a novel model help to understand and improve islet transplant. A number of perspectives have been offered; improved characteristics of Min6-PI model, enhanced physiological properties of PI i.e. viability, ECM expression and insulin secretory function under in our novel 3D-cell culture system. Additionally, the optimised *in vitro* methods; digestion and remodelling under RCCS bioreactor conditions, enabled our attractive Min6-PI model to be recovered and demonstrate improved insulin glucose responsiveness.

From a clinical point of view, to improve the outcome of islet transplants it is essential to understand how to maintain long-term survival of islets engrafting. The achievement of enhanced β-cell function is dependent on maintaining the optimum environment for cells post-digestion, which has a great impact on cellular organisation and ECM interactions that are needed for β-cell survival.

This study has established and optimised a novel system, which could be a key in remodelling pre-transplant islets to improve clinical outcomes for patients with Type 1 diabetes.

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