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## The Role of Integrins in Support of Pancreatic Function, Survival and Maturation

Matthew Riopel, *The University of Western Ontario*

Supervisor: Dr. Rennian Wang, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pathology

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**THE ROLE OF INTEGRINS IN SUPPORT OF PANCREATIC FUNCTION,  
SURVIVAL AND MATURATION**

(Thesis format: Integrated Article)

by

Matthew Riopel

Graduate Program in Pathology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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## Abstract

The pancreas is a glandular organ composed of endocrine and exocrine compartments. Integrins are cell adhesion molecules that connect cells to the extracellular matrix (ECM). Integrins modulate a variety of cellular effects, yet their mechanism of action in the developed pancreas is not well understood. Fibrin is a provisional ECM protein that contains ligands for integrin receptors. Fibrin is capable of supporting islet health, but it is unclear how fibrin exerts its effects. The objective of this thesis is to understand the role of integrin receptors on *in vivo* pancreatic cell function, survival, and proliferation. In addition, this thesis investigates how fibrin promotes pancreatic cell maturation, function, and survival in culture. Pancreatic tissues from transgenic adult mice lacking  $\beta 1$  integrin were harvested at 4 and 7 weeks post-induction. The rat insulinoma cell line, INS-1, and human fetal-islet epithelial cells were cultured on tissue culture polystyrene plates or with fibrin in two or three dimensions for up to 4 weeks, then collected for analyses.

$\beta 1$  integrin knockout mice demonstrated significant glucose intolerance and reduced glucose-stimulated insulin secretion (GSIS).  $\beta 1$  integrin knockout mice also had reduced beta cell expression of Pdx-1 and Nkx6.1, as well as reduced acinar cell expression of amylase, lipase and Reg-II. Alternatively, to investigate the effects of supplementing pancreatic cells with ECM in culture, INS-1 cells and human fetal islet-epithelial clusters were cultured with fibrin. Culturing these cells with fibrin led to significantly increased integrin  $\alpha \beta 3$  expression. INS-1 cells cultured with fibrin had significantly increased GSIS and significantly reduced caspase-3 cleavage, which was reversed by antibody blockade of integrin  $\alpha \beta 3$ . Human fetal islet-epithelial cells cultured with fibrin had increased PDX-1 expression, which was mediated, in part, by the mTOR/p70<sup>s6k</sup> pathway. Transplantation of fibrin-mixed human fetal-islet epithelial cells led to maintained differentiation and improved graft vascularization. Integrins are essential for maintaining glucose homeostasis and acinar cell function. Furthermore, culturing pancreatic cells with fibrin leads to increased integrin expression and improved cell maturation, function, survival and proliferation. Understanding how integrins modulate the pancreas and associated islet cells will lead to more effective protocols for the culture of isolated islets.

Keywords: integrins, beta cells, diabetes, extracellular matrix, fibrin, islet, glucose metabolism, exocrine tissue, pancreas

## Co-Authorship Statement

The studies in chapters 2, 3, 4 and 5 were performed by Matthew Riopel in the laboratory of Dr. Rennian Wang, with assistance of the co-authors named below.

Chapter 2: Mansa Krishnamurthy performed half the intraperitoneal glucose and insulin tolerance tests, conducted morphometric analyses (Pdx-1 and Nkx6.1 cell counting) and assisted with manuscript writing. Jinming Li provided technical help with staining and western blots. Shangxi Liu assisted with mouse breeding and genotyping. Andrew Leask provided assistance with manuscript revisions and data interpretation. Rennian Wang designed the project, assisted with data interpretation and analysis and revised the manuscript.

Chapter 3: Jinming Li provided technical assistance with real-time RT-PCR. Shangxi Liu assisted with mouse breeding and genotyping. Andrew Leask provided assistance with manuscript revisions and data interpretation. Rennian Wang designed the project, assisted with data interpretation and analysis and revised the manuscript.

Chapter 4: William Stuart assisted with data collection (cell counting) and interpretation. Rennian Wang contributed to experimental design, data interpretation and manuscript revisions.

Chapter 5: Jinming Li assisted with real-time RT-PCR. Mark Trinder has a role in data analysis, while Rennian Wang designed the project, assisted with data interpretation and helped with manuscript construction.

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## List of Abbreviations

|                     |                                       |
|---------------------|---------------------------------------|
| Akt                 | v-akt murine thymoma viral oncogene   |
| ANOVA               | Analysis of variance                  |
| AUC                 | Area under the curve                  |
| $\beta$ 1KO         | $\beta$ 1 integrin knockout mice      |
| BSA                 | Bovine serum albumin                  |
| CK-19               | Cytokeratin-19                        |
| Colla1              | Collagen type Ialpha1                 |
| Colla2              | Collagen type Ialpha2                 |
| Cre+ Ctrl           | Cre+ control mice                     |
| Cre- Ctrl           | Cre- control mice                     |
| CRE-ER <sup>T</sup> | Tamoxifen-inducible cre recombinase   |
| Ctrl                | Control                               |
| DAPI                | 4'-6-Diamindino-2-phenylindole        |
| DM                  | Diabetes mellitus                     |
| ECM                 | Extracellular matrix                  |
| ELISA               | Enzyme-linked immunosorbent assay     |
| ERK                 | Extracellular signal-regulated kinase |
| FAK                 | Focal adhesion kinase                 |
| FBS                 | Fetal bovine serum                    |



|       |   |
|-------|---|
| Fn1   | Fibronectin 1   |
| Gcg   | Glucagon  |
| GFAP  | Glial fibrillary acidic protein                       |
| GSIS  | Glucose-stimulated insulin secretion                  |
| H&E   | Haematoxylin and eosin stain                          |
| Endo  | Endocrine cell  |
| HMS   | Human mitochondrial surface protein                   |
| HRP   | Horseradish peroxidase                                |
| ILK   | Integrin-linked kinase                                |
| i.p.  | Intraperitoneal                                       |
| IPGTT | Intraperitoneal glucose tolerance test                |
| IPITT | Intraperitoneal insulin tolerance test                |
| Ins   | Insulin   |
| JNK   | c-Jun N-terminal kinase                               |
| KO    | Knockout  |
| Lama1 | Laminin $\alpha$ 1                                    |
| Lamb1 | Laminin $\beta$ 1                                     |
| Lamc1 | Laminin $\gamma$ 1                                    |
| MAPK  | Mitogen-activated protein kinase                      |
| MafA  | v-maf avian musculoaponeurotic fibrosarcoma homolog A |

|          |  |
|----------|--|
| mTOR     | mammalian target of rapamycin                                    |
| PSC      | Pancreatic stellate cell   |
| qRT-PCR  | real-time reverse transcriptase PCR                              |
| Ngn3     | Neurogenin3  |
| Nkx6.1   | Nk6 homeobox 1   |
| P        | Phosphorylated   |
| PCR      | Polymerase chain reaction  |
| Pdx1     | Pancreatic and duodenal homeobox 1                               |
| PECAM-1  | Platelet endothelial cell adhesion molecule-1                    |
| Ptf1a    | Pancreas transcription factor 1a                                 |
| RGD      | Arg-Gly-Asp  |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis       |
| SE       | Standard error of the mean                                       |
| SEM      | Scanning electron microscopy                                     |
| Sox9     | Sry-related HMG box 9  |
| Sox17    | Sry-related HMG box 17   |
| TCPS     | Tissue culture polystyrene                                       |
| TEM      | Transmission electron microscopy                                 |
| TUNEL    | Terminal deoxynucleotidyl transferase mediated nick end-labeling |
| STZ      | Streptozotocin   |

VEGF

Vascular endothelial growth factor

# Chapter 1

## 1 General introduction<sup>1</sup>

---

<sup>1</sup> Parts of this work have been published and submitted in the following manuscripts:

- Riopel M, Trinder M, Wang R. Fibrin, a scaffold material for islet transplantation and pancreatic endocrine tissue engineering. Submitted to Tissue Engineering Part B (MS# TEB-2014-0188)
- Riopel M, Wang R. Collagen matrix support of pancreatic islet survival and function. Front Biosci 2014;19:77-90
- Riopel M, Wang R. Stem cells: a solution for cell replacement therapies in the treatment of diabetes? In Your Lab: Stem Cell Research Edition. CEDARLANE 2012;20-26

## 1.1 Significance of this PhD thesis

Cell-based therapies for diabetes are currently the most promising treatment modality to render diabetics insulin-independent. Universal availability of these techniques are limited by a comprehensive understanding of islets. Integrins, a group of cell adhesion molecules, are critical for integrating the extracellular environment with interior cellular processes. However, the mechanisms behind these processes in the pancreas are not completely understood. My studies are significant because I demonstrate the necessity of cell-extracellular matrix (ECM) interactions in maintaining islet cell health and provide insight on improving cell-based therapies for diabetes.

## 1.2 Pancreas anatomy and development

The pancreas is a glandular organ that regulates blood glucose as well as aids in food digestion. It lies inferior to the stomach, attaches to the duodenum via the pancreatic duct and is vascularized via the superior mesenteric artery and vein. The pancreas itself consists of exocrine and endocrine tissue, the latter composing only 2-3% of the total pancreas mass. The exocrine compartment is made up of acinar cells that produce digestive enzymes and pH neutralizing bicarbonate through an intricate network of ducts that lead to the duodenum. The endocrine pancreas is composed of scattered cell clusters known as the Islets of Langerhans, or more conventionally, islets. Islets are made up of cells that secrete insulin (beta cells), glucagon (alpha cells), somatostatin (delta cells), pancreatic polypeptide (PP cells) and ghrelin (epsilon cells). In human islets, beta and alpha cells make up most of the islet (54% and 36%, respectively), with a few delta, epsilon and PP cells [1]. Meanwhile, mouse islets are primarily made up of beta cells (~87%), where alpha cells (7%) make up the periphery of the islet, and a few delta, epsilon and PP cells [1]. The role of beta cells is to secrete insulin when blood glucose becomes too high. Subsequently, this insulin will stimulate insulin receptors on hepatocytes, adipocytes and myocytes. Stimulation of the insulin receptor allows glucose uptake and promotes glycogen or fatty acid synthesis. Conversely, alpha cells secrete glucagon in response to low blood glucose, which will stimulate the liver to produce glucose.

Development of the pancreas is very complex and requires well-timed expression of transcription factors and signaling pathways. The pancreas is derived from the

endoderm germline during development. It begins budding at embryonic day 8.5 in the mouse [2] or gestational day 25 in humans [3]. A transcription factor essential to the development of definitive endoderm is the Sry-related HMG box 17 protein (Sox17) [4]. Two parts of this definitive endoderm evaginate as primitive pancreatic buds, and express the transcription factors pancreatic duodenal homeobox 1 (Pdx1) and pancreas transcription factor 1a (Ptf1a) [5], which are essential for pancreatic development. Distinct signaling pathways dictate the specific expression and repression of transcription factors, which induce transient expression of neurogenin3 (Ngn3) and initiate endocrine cell differentiation [6]. Microarray analysis of Ngn3 knockout mice shows that Ngn3 regulates a large subset of genes required for progression of progenitor cells into endocrine cells [7]. Neurogenic differentiation 1 and islet-1 are downstream of Ngn3 and have been explicitly shown to be essential for endocrine lineage determination [8, 9]. These endocrine precursor cells then split into separate differentiated endocrine lineages; alpha, beta, delta, epsilon and PP cells, and begin to organize into islets by embryonic day 16 (for a full review on pancreatic development see [3, 10]). A set of transcription factors including Pdx1, Nk2 homeobox 2 and Nk6 homeobox 1 (Nkx6.1), Paired box gene 4 and 6, and v-maf avian musculoaponeurotic fibrosarcoma homolog A (MafA) are required not only for beta cell development, but also for beta cell maturation and function (reviewed in [11]). If there is a defect during pancreatic development or the adult pancreas is unable to regulate blood glucose sufficiently, Diabetes Mellitus (DM) occurs.

### 1.3 Diabetes Mellitus and treatment options

Endocrine cells make up only 2-3% of cells of the total pancreas mass. This population plays a critical role in regulating glucose homeostasis. Importantly, impaired glucose homeostasis is associated with DM; a condition of absolute or relative insulin deficiency. Type 1 DM refers to the autoimmune condition whereby host T cells attack beta cells of the pancreas, which results in a state where little to no insulin is produced. Type 2 DM has a multi-factorial pathogenesis, whereby initial compensatory insulin production/secretion is insufficient to reduce blood glucose due to peripheral insulin resistance, reduced beta cell mass, and increased body weight. In both conditions, the eventual treatment regimen is daily exogenous insulin injections aimed to maintain blood glucose levels normalized to that of unaffected individuals. This regimen has

significantly lowered the mortality and morbidity rates of DM. However, it has been suggested that the blood glucose variability under exogenous insulin treatment may contribute to long-term complications in diabetic patients such as cardiomyopathies, neuropathies or retinopathies [12, 13]. Therefore, it is imperative that researchers discover a curative therapy that leads to physiologic glycemic control, preventing the need for exogenous insulin and reducing long-term complications.

One proposed DM cure is islet transplantation, a cell-based therapy that removes islets from a donor pancreas and transplants them into a diabetic individual. Pioneered at the University of Alberta, the Edmonton protocol for islet transplantation uses a glucocorticoid-free immunosuppression regimen as islets are transplanted into type 1 DM patients [14]. Patients are initially insulin-independent after transplantation, but most revert back to insulin injections within 5 years to maintain glycemia due to graft failure [15]. This graft failure is associated with increased islet cell death and loss of islet cell function. As such, islet transplantation techniques need to be improved so that patients can be insulin-independent for their entire lifetimes. One limitation in mastering these techniques is the unavailability of human islets [16]. Therefore, methods that can increase the number of islets (specifically beta cells) and improve the survival and function of existing islets will be critical for the success of islet transplantation. However, ex vivo islet mass expansion has been a difficult task, since senescence and loss of islet integrity and insulin expression occur after extended culture [17]. Moreover, the islet micro-environment plays an important role in maintaining islet health and the mechanisms behind cell-ECM interactions are largely unknown [18]. In particular, integrins are the major cell receptor type that integrates signals from the ECM to the interior of the cell.

## 1.4 Integrin receptors

Integrins are a class of cell and extracellular matrix (ECM) binding proteins that integrate the exterior environment to the inside of the cell. Twenty-four combinations of  $\alpha$  and  $\beta$  integrin subunits have been discovered, each with a unique range of affinity to different ligands (Figure 1-1) [19]. Integrins have been implicated in a large variety of cellular processes in almost all cell types [20]. They can be categorized based on their ligand of preference. For example,  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 10$ , or  $\alpha 11$  associate with  $\beta 1$  to bind

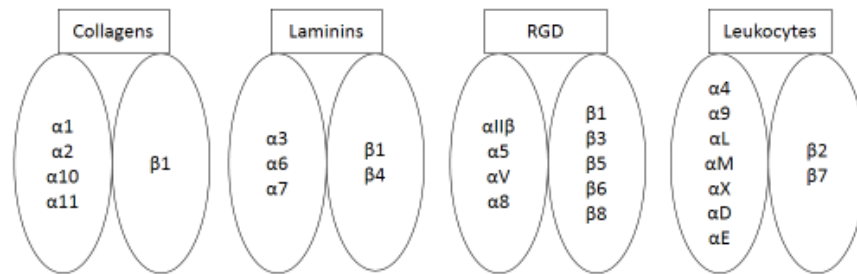


Figure 1-1 Mammalian integrin receptors and their preferential binding partners.

Integrin  $\beta 1$  is the most promiscuous receptor, associating with  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\alpha 8$ ,  $\alpha 9$ ,  $\alpha 10$  and  $\alpha 11$  to bind with many different ligands.  $\beta 2$  integrin primarily binds to cell surface receptors and associates with  $\alpha L$ ,  $\alpha M$ ,  $\alpha X$ ,  $\alpha D$ . The same is true for  $\alpha E\beta 7$ . Integrin  $\alpha v$  pairs with  $\beta 3$ ,  $\beta 5$ ,  $\beta 6$  and  $\beta 8$  to bind to Arg-Gly-Asp domains, while  $\beta 4$  integrin binds to laminins with  $\alpha 6$  integrin [19-22].



to collagen motifs [21]. Meanwhile,  $\alpha_v$  associates with  $\beta_3$ ,  $\beta_5$ ,  $\beta_6$  or  $\beta_8$  to preferentially bind to fibronectin type proteins that contain Arg-Gly-Asp (RDG) domains in their structure (Figure 1-1) [22]. It should be noted, however, that integrins are not restricted to binding only one ligand and have low affinity to many ligands [22]. Before integrins can be bound by ligands, integrins must be activated to a high-affinity conformation by a process termed inside-out signaling [23]. Inside-out signaling is orchestrated by proteins such as talin [24, 25] and those of the kindlin family [26, 27], which bind to the intracellular cytoplasmic tails of integrins. Once integrins are in this open, high-affinity state, binding of ligands to integrin receptors leads to a cascade of events, collectively known as outside-in signaling. The clustering of ligand-bound receptors into focal complexes or adhesions causes significant downstream effects including recruitment and activation of second messengers and signaling pathways [28]. Focal adhesion kinase (FAK) is an important protein that interact with integrin-binding proteins to mediate intracellular signaling pathways such as mammalian target of rapamycin (mTOR), v-akt murine thymoma viral oncogene (AKT) and extracellular signal-regulated kinase 1/2 (ERK1/2) [29-31]. Integrin-linked kinase (ILK) is another key protein recruited to link integrins to the cytoskeleton and modulate cell migration [32]. Knockout studies indicate that ILK plays a role in apoptosis [33, 34] and proliferation via AKT and p27 [35, 36]. Many other proteins are involved in outside-in signaling that mediate cellular effects, but it is outside the scope of this thesis to discuss them.

#### 1.4.1 Integrin receptors and signaling pathways in the pancreas

Despite being ubiquitous, the expression of integrins in each organ and cell type varies tremendously. Wang and colleagues demonstrated that adult human islets express integrins  $\alpha_3$ ,  $\alpha_5$ ,  $\alpha_v$  and  $\beta_1$  [37]. Furthermore, endocrine cells of the developing human pancreas commonly express integrins  $\alpha_3$ ,  $\alpha_5$ , and  $\alpha_6\beta_1$  [38]. Integrins  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  are also expressed in early human pancreatic progenitors and play a major role in adhesion and migration [39]. In the rodent pancreas, the integrin receptors  $\alpha_3$ ,  $\alpha_5$ , and  $\alpha_6\beta_1$  are localized to duct and islet cells [40]. Meanwhile, another report demonstrated expression of integrins  $\alpha_6$  and  $\alpha_3$  in acinar cells,  $\alpha_6\beta_4$  in the duct,  $\alpha_6\beta_1$  in acinar and ductal cells, and  $\alpha_3\beta_1$  in all pancreatic cells [41]. Indeed, the most promiscuous and well-studied integrin is  $\beta_1$ . In human fetal pancreatic cells and adult rat islets,  $\beta_1$

integrin is critical for the maintenance of cell adhesion, survival and insulin expression [38, 40]. In the developing mouse pancreas,  $\beta 1$  integrin regulates beta cell expansion [42]; whereas in the developed mouse pancreas, it is critical for maintaining glucose homeostasis, beta cell proliferation and survival [43; Chapter 2], as well as exocrine tissue architecture and function [44, 45; Chapter 3]. In fact, when  $\beta 1$  integrin is ablated in mouse beta cells, there is a significant reduction in the number of beta cells and dysregulation in the expression of genes associated with cell cycle progression [42]. In addition, integrins  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha v$ ,  $\beta 1$ ,  $\beta 3$ , and  $\beta 4$  have abnormal expression in pancreatic cancer (reviewed in [46]).  $\beta 1$  integrin has also been shown to play an important role in the invasiveness of different pancreatic carcinoma cell lines [47]. Taken together, these studies demonstrate that the integrins, especially  $\beta 1$  integrin, play a critical role in maintaining pancreatic endocrine and exocrine health.

It was previously mentioned that integrin receptor activation mediates intracellular signaling pathways in order to affect cellular processes. One of the first molecules to be activated after integrin stimulation is FAK. In beta cells, loss of FAK leads to decreased beta cell survival and proliferation, along with reduced activation of AKT and ERK1/2 [48]. Both AKT and ERK1/2 have important roles in regulating beta cell function, proliferation and survival. In general, AKT is critical for the regulation of cell growth, survival, and proliferation (reviewed in [49]). In beta cells, activation of AKT leads to cell cycle progression by regulating cyclin dependent kinase 4, cyclin D1, cyclin D2 and p21 [50]. Increasing active AKT during development significantly increases beta cell replication and beta cell mass [51]. Similar to AKT, ERK1/2 activation mediates cell proliferation and survival in multiple cells types [52]. However, phosphorylation of ERK1/2 in beta cells is sufficient to stimulate insulin expression [53]. In vitro, ERK1/2 activation leads to phosphorylation of Pdx-1 and MafA to promote beta cell function [54]. In this manner, ERK1/2 plays multiple roles in beta cells, including promoting cell survival, proliferation and function. Meanwhile, AKT activation is important for beta cell survival and proliferation.

Another intracellular signaling pathway important in the pancreas is the mTOR pathway. Nutrient sensing has been described as one of the important roles of mTOR (reviewed in [55]). In beta cells, however, two roles of mTOR have been described. Firstly, mTOR pathway stimulation promotes beta cell growth and proliferation [56].

Alternatively, chronic activation of the mTOR pathway activity (by high glucose) leads to inhibition of AKT, and eventually beta cell apoptosis [57]. Thus, an important balance of mTOR stimulation is required to maintain proper beta cell survival and growth. In order for integrins to activate any of these intracellular signaling pathways, ligand stimulation is required. The primary ligands of integrin receptors are the proteins of the ECM.

## 1.5 The pancreatic extracellular matrix

ECM proteins present in the pancreas include collagen I, collagen IV, fibronectin, and laminin [58]. Many of these ECM proteins are components of the basement membrane, which is necessary for the maintenance of islet architecture and acinar cell clusters. The basement membrane acts as a scaffold for cell attachment and modulates endocrine cell survival, insulin secretion, proliferation, differentiation, and migration (reviewed in [58]). However, over production of ECM adversely affects the exocrine pancreas, potentially causing fibrosis, inflammation, as well as cancer (reviewed in [59]). Therefore, it appears that an optimal amount of ECM protein is essential for pancreas survival and function. Two prominently expressed ECM proteins in the pancreas are collagen I and collagen IV.

### 1.5.1 Collagen I and IV

In the native rat and human pancreas, collagen I is present in perivascular islet capillaries. However, the human pancreas also contains collagen I immunoreactivity in and around islets [60]. Therefore, numerous studies have investigated whether in vitro replacement of collagen I could maintain islet differentiation, proliferation, survival, and function [61-73]. In particular, culturing isolated rat islets in a collagen I matrix led to a significant reduction in islet cell death after 5 days [61] and improved glucose-stimulated insulin secretion (GSIS) after 11 days [62]. Using the rat insulinoma cell line, INS-1, our research group has found that INS-1 cells exposed to collagens I or IV demonstrated significant increases in cell adhesion, spreading, and proliferation when compared to cells cultured on either fibronectin, laminin, or tissue-culture polystyrene [63]. Perturbation of  $\alpha 3\beta 1$  integrin interactions with collagen I and IV led to significantly decreased FAK, ERK1/2, and Akt phosphorylation, and significantly increased cleaved caspase-3 levels. This indicates that binding between collagen I and

IV to  $\alpha 3\beta 1$  integrin is essential to support the survival of INS-1 cells, human fetal pancreas, and human adult islets [63, 64]. Analysis of canine islets cultured on collagen I showed a significant improvement in adhesion, GSIS, and insulin content, all while significantly reducing islet cell death when compared to controls [65]. Likewise, human adult islets cultured on a collagen I matrix exhibited significantly improved insulin response to acute glucose challenge after 3 days and were viable for longer than 8 weeks [66]. Meanwhile, studies in human fetal pancreatic islet cells demonstrated that  $\beta 1$  integrin blockade resulted in significantly decreased expression of essential islet genes (insulin, glucagon and PDX-1), along with down-regulation of the FAK/mitogen-activated protein kinase (MAPK)/ERK signaling cascade when cells were plated on collagen I gels [67]. Islet survival, however, was significantly increased when isolated islets were cultured on a combination of collagen IV, laminin, and fibronectin when compared to just collagen I alone [68]. Furthermore, adding a combination of soluble laminin or fibronectin to the culture media of islets cultured on collagen I prevented islet-epithelial transformation [69]. Culturing neonatal islets on a collagen I gel led to significantly increased cell proliferation and expansion of c-Kit-expressing cells, which was maintained for up to 8 weeks [70]. Past work has also shown that collagen I supports differentiation and maturation of pancreatic precursor cells into beta cells when cultured in 3D polyethylene glycol hydrogels [71]. The use of a collagen I-based 3D entrapment method improved adult islet morphology, viability, and function with no defects in oxygen consumption when compared to controls [72]. Collagen I gels supplemented with other ECM proteins also supported long-term human islet culture demonstrating similar insulin stimulation indexes to freshly isolated islets [73]. In summary, these results suggest that collagen I plays a role in regulating islet cell proliferation, function, and survival.

Another collagen that has an essential role in the basement membrane surrounding islets is collagen IV [74]. Many studies have investigated the importance of collagen IV in vivo, and as a supportive gel in vitro. Injecting streptozotocin (STZ) into neonatal Wistar rats had a significant impact on glucose homeostasis, and after recovery, collagen IV expression did not recover to normal, suggesting that collagen IV may be required to facilitate beta cell growth and function [75]. Collagen IV is also crucial for beta cell motility and insulin secretion via  $\alpha 1\beta 1$  integrin [76]. Likewise, collagen IV improved adult beta cell insulin secretion in an ERK-dependent manner [77], which

likely occurs through increased fibroblast growth factor receptor 1 protein expression [78]. Culturing islets on collagen IV also prevented apoptosis when compared to controls [68]. We have demonstrated that collagen IV improves human islet and INS-1 cell adhesion, spreading, survival, and function by interacting with integrin  $\alpha 3\beta 1$  [63]. However, another immortalized beta cell line, RIN-5F, showed minimal changes in cell adhesion and proliferation when cultured on plates coated with either collagen I or IV compared to controls [79]. Using encapsulation methods, collagen IV was able to improve GSIS, and demonstrated a synergistic improvement when mixed with laminin [80]. Three dimensional culture of human adult islets in collagens I and IV led to higher insulin gene expression compared to fibronectin or laminin [81]. Another 3D culture system was developed using poly(lactic-co-glycolic acid)-collagen hybrid scaffolds, which supported RIN-5F cell proliferation, function, and differentiation [82]. These studies indicate that collagen I and collagen IV are able to improve beta cell function, proliferation, and survival after short term culture.

### 1.5.2 Collagen-associated islet transdifferentiation

Despite the observed improvements in islet health associated with collagen culture, reports show that islets cultured on collagen gels end up losing their insulin secretion function, express markers of ductal cells (CK-19), and form cystic structures [83, 84]. When isolated adult male Sprague-Dawley islets were cultured in collagen gels (produced from rat tails) with growth factors, there was a time-dependent decrease in insulin secretion as well as increased CK-19 expression [85]. These results suggest that collagen may not be able to maintain the differentiated state of islets, and instead supports the transdifferentiation of islets to a ductal-cell phenotype. Indeed, a similar finding was observed when culturing isolated adult human [83] and canine [84] islets in three-dimensional rat tail collagen gels. Development of these duct-like epithelial cystic structures is initially associated with a significant increase in islet cyclic adenosine monophosphate, c-Jun N-terminal kinase (JNK) signaling, and cleaved caspase-3 levels, with reduced ERK/Akt signaling, giving a net effect of increased apoptosis. Following ductal-like structure formation, there is a consequent reduction in JNK signaling and cleaved caspase-3 levels with an increase in ERK/Akt signaling resulting in duct-like cell proliferation [69, 86]. Interestingly, this transdifferentiation could be reduced, in a dose-dependent manner, by the addition of transforming growth

factor  $\beta$  [87]. Another research group cultured human islets in a collagen matrix and showed a concentration-dependent effect of epidermal growth factor on islet transdifferentiation via the AKT and ERK signaling pathways [88]. As well, when collagen I-cultured islet-derived duct-like epithelial cells were treated with the islet neogenesis-associated protein, they re-differentiated back to islet-like structures, including all four endocrine cell types [89]. Conversely, culturing human islets with adhered collagen molecules did not lead to duct cell transdifferentiation, but rather to maintenance of insulin gene expression [83]. This suggests that the negative effects of collagen culture on islets can be mediated by the addition of growth factors, but that collagen culture by itself is limited in its usefulness in promoting islet health. In contrast, a provisional ECM protein that does not promote islet transdifferentiation during culture is fibrin.

## 1.6 Fibrin(ogen) and the pancreas

### 1.6.1 Fibrin structure and function

Fibrinogen is a soluble 340kDa protein that is 45nm in length [90]. Two D domains make up the outer part of the molecule that is connected by disulfide bridges to a central E domain. Each D domain is made up of a polypeptide trimer, named  $A\alpha$ ,  $B\beta$ , and  $\gamma$  (Figure 1-2) [91]. Fibrinogen also contains the Arg-Gly-Asp (RGD) amino acid sequence, which acts as a ligand for a number of integrin receptors [92]. Fibrinogen is produced in the liver of rodents and humans, and circulates in the blood until activation by thrombin [93, 94]. The enzymatic activity of thrombin cleaves four fibrinopeptides from both  $\alpha$  and  $\beta$  subunits, which allows the resultant fibrin monomers to conglomerate and polymerize [95]. Fibrin polymerization occurs because fibrinopeptide cleavage exposes high affinity binding sites on the  $\alpha$  and  $\beta$  chains ( $E_A$  and  $E_B$ ), which bind to  $D_A$  and  $D_B$  sites located on an adjacent fibrin monomers (Figure 1-2) [91]. This process leads to double stranded fibrin fibrils with staggered 22.5nm longitudinal repeats that eventually branch via bilateral and equilateral junctions [96, 97]. A bilateral junction occurs when two double-stranded fibrils bind adjacently to one, while equilateral junctions occur when three fibrin monomers interact together. Most frequently, the combination of both types of junctions leads to a complex mesh network [91, 98, 99].

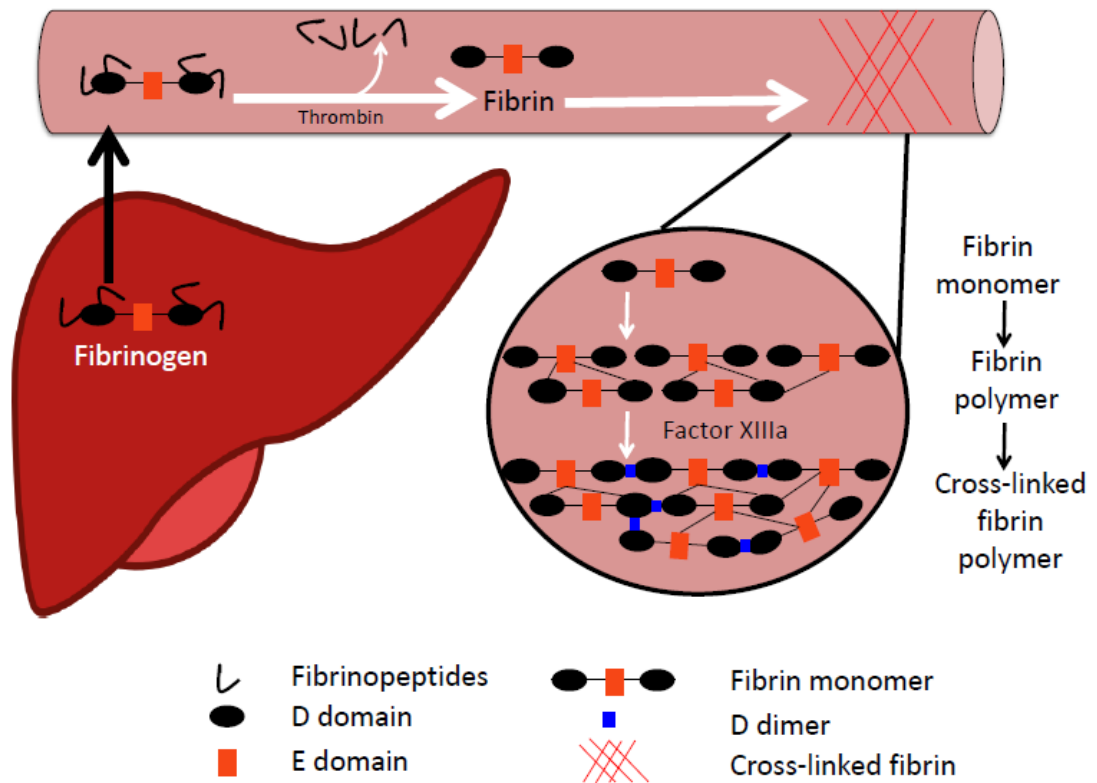


Figure 1-2 Fibrin structure and formation.

Fibrinogen, made by the liver, circulates in the blood stream until activation of the coagulation cascade, whereby thrombin eventually cleaves fibrinopeptides from fibrinogen. The resulting molecule is then able to polymerize and form a fibrin polymer by allowing binding between the D and E fragments. After exposure to factor XIIIa, D dimers cross-link fibrin polymer to form a fibrin network mesh. Scanning electron micrograph shown in bottom left to illustrate complex fibrin mesh network.

The native function of fibrin is to form a clot to promote wound healing. As wound healing occurs, the fibrin clot is slowly degraded. In this way, fibrin is a biodegradable provisional ECM protein [100]. High blood fibrinogen concentration has been associated with elevated risk of stroke and myocardial infarction [101]. However, overexpression of native murine fibrinogen in mice did not increase the risk of occlusive platelet thrombus formation [102]. But, when human fibrinogen was infused into mice, vessel occlusion occurred more quickly due to abnormally high fibrin content in the plasma [103]. These findings suggest that reducing native fibrinogen levels in humans could reduce the risk of thrombosis.

Clot formation is also affected by fibrin's structure, which can be modified by alternative splicing of the  $\gamma$  fibrinogen domain. Additionally, the concentration of fibrinogen and thrombin, pH, porosity, permeability and other factors have been shown to influence the conformation of fibrin [104-106]. Factors such as diabetes [107], smoking [108], and homocysteine concentration [109] also have dramatic effects on fibrinogen's activity.

### 1.6.2 Fibrin and integrin receptors

Two means by which fibrin exerts its effects are by acting as a 3D scaffold emulating the natural extracellular environment and/or by stimulating receptors on the cell surface. One receptor that is stimulated by fibrin and has a well-established role in cancer and angiogenesis is integrin receptors  $\alpha\nu\beta 3$  [110, 111]. Interestingly, fibrinogen domains  $\alpha$ ,  $\gamma$ , and  $\alpha_{EC}$  are able to bind to integrin  $\alpha\nu\beta 3$  [112]. Given this, fibrin is able to mediate various effects on different cells. For instance, endothelial cells cultured in fibrin gels form luminal structures, which depend on integrins  $\alpha\nu\beta 3$  and  $\alpha\nu\beta 5$  [113]. These pseudo-capillaries vary based on the relationship between integrin  $\alpha\nu\beta 3$  distribution and the stretch resistance of fibrin [114]. Fibrin-integrin  $\alpha\nu\beta 3$  interactions induce morphological changes of endothelial cells, which includes enlarging the intracellular space [115]. In wound healing, human fibroblast expression of integrin  $\alpha\nu\beta 3$  promotes is essential for adhesion to fibrin and thus migration [116]. Integrin  $\alpha\nu\beta 3$  also mediates fibrin gel retraction during wound repair [117]. Spreading and migration of smooth muscle cells into fibrin gels is an important step in wound healing and integrin  $\alpha 5\beta 1$  and  $\alpha\nu\beta 3$  are required for this process [118]. Furthermore, the  $\alpha IIb\beta 3$  integrin receptor, which binds fibrin, plays a role in clot formation [119]. Other cell



surface receptors that bind to fibrin are vascular endothelial cadherin [120], vascular endothelial growth factor receptor [121], integrin  $\alpha$ M $\beta$ 2 [122], and others [91]. In these regards, integrin  $\alpha$ v $\beta$ 3 is a key player involved in mediating the cellular effects of fibrin. An ECM protein related to fibrin in structure and function is fibronectin.

### 1.6.3 Contrasting fibrin and fibronectin

Fibronectin is a large glycoprotein with essential structural and functional roles. It is alternatively spliced such that one isoform circulates as plasma fibronectin, and the other isoform remains as cellular fibronectin [123]. Structurally, fibronectin contains domains that are able to mediate binding of other ECM proteins, as well as stimulate integrin receptors [123]. Like fibrin, fibronectin contains RGD domains that are a ligand for integrins  $\alpha$ v $\beta$ 3 and  $\alpha$ 5 $\beta$ 1, among others [124]. This interaction causes a conformational change in the integrin receptor and allows outside-in signaling to occur [125].

Plasma fibronectin, like fibrin, plays a role in the coagulation cascade during wound healing. Fibronectin and fibrin can interact during clot formation [126]. In the clot, plasma fibronectin's presence increases platelet aggregation and stabilizes the thrombus [127]. Indeed, mice deficient for plasma fibronectin had significantly larger infarct size after focal cerebral ischemia and reperfusion [128]. Furthermore, in a model of murine atherosclerosis, loss of plasma fibronectin reduced the number of atherosclerotic plaques [129].

After these initial steps in wound healing, fibroblasts deposit cellular fibronectin, which allows other ECM molecules to adhere and thus regulate a variety of cellular processes through receptor binding [130]. This differs from fibrin because as wound repair occurs, fibrin is degraded and lost from the wound site. Deposition of this native fibronectin matrix architecture increases cell proliferation, compared to a mutant fibronectin that contained similar binding motifs, but an altered matrix, which inhibited cell cycle progression [131]. This suggests that the entire fibronectin structure, and not just specific binding motifs, is required for proper receptor activation. Furthermore, altered fibronectin matrices induced a reduction in FAK phosphorylation and p53 levels, which led to apoptosis induction [132]. Fibrin and fibronectin have similar functions, yet, the

characteristics of fibrin are such that it can easily form a 3D scaffold and will support long-term islet cell growth, function and angiogenesis.

#### 1.6.4 Fibrin use in islet cell culture and transplantation

Fibrin is a provisional matrix protein that can support islet architecture, improve islet health and promote islet vascularization [133, Chapter 4]. Most importantly, islets embedded in 3D fibrin can be expanded *in vitro* without the loss of beta cell function that is seen with traditional culture techniques [134]. In fact, when human islets were cultured in fibrin, proliferation, insulin content, and GSIS increased significantly compared to controls (Figure 1-3) [134]. These improvements to beta cell function are believed to be associated with the ability of 3D fibrin to maintain cell-cell contacts and enable cellular response to growth factors both *ex vivo* and *in vivo* [133, Chapter 4; 134].

Improvements in GSIS, proliferation, and survival were observed when beta cells were cultured in 3D fibrin for up to 4 weeks [133, Chapter 4]. Enhancement of fibrin-cultured beta cell insulin secretion can also be achieved by co-culture with human umbilical vein endothelial cells [135]. The aforementioned study also showed that the distance between co-culture cell populations was critical for promoting beta cell functionality [135]. Fibrin also has a protective effect against apoptotic stimuli, which was demonstrated by subjecting fibrin-cultured young porcine islets to hydrogen peroxide treatment [136].

Binding of extracellular matrix proteins, such as fibrin, to integrin receptors is critical for the maintenance of islet function, differentiation, and survival *in vitro* and *in vivo* [42, 66, 137, 138]. Our previous study demonstrated that mice with a deficiency in  $\beta 1$  integrin had significantly reduced beta cell mass, proliferation, and Pdx-1 and Nkx6.1 expression, as well as reduced signaling through the FAK/MAPK/ERK pathway [42, Chapter 2]. Culture of beta cells within 2D or 3D fibrin scaffolds showed islet-like cluster formation with significantly increased glucose-stimulated insulin secretion and cell proliferation, as well as decreased cell apoptosis for up to 4 weeks [133, Chapter 4]. Fibrin improves beta cell function and survival by increasing integrin  $\alpha v \beta 3$  protein levels and increasing the phosphorylation of FAK, Erk1/2 and Akt [133, Chapter 4].

Taken together, fibrin binds to integrin receptors, promotes and maintains islet cluster formation, and improves islet health.

Besides binding to integrin receptors, fibrin may promote *in vivo* islet health by increasing blood flow to islets via angiogenesis. Embedding islets in 3D fibrin is associated with significant increases in vessel cord branching from islets [139, 140]. Fibrin scaffolds may promote more effective angiogenesis by inducing the endothelial cells and endothelial progenitor cells that reside within islets to proliferate and/or differentiate [140]. Alternatively, the vessel changes may be in response to hypoxia generated from embedding islets in fibrin gels, which was demonstrated after overnight culture of human islets in 3D fibrin [141]. Fibrin-embedded human islets that were supplemented with an oxygen-diffusing medium acted synergistically to promote improved islet function and survival *in vitro* [141]. Therefore, utilization of an oxygen-diffusing medium seems to be a simple strategy to reduce hypoxic damage to islets caused by islet isolation and 3D fibrin scaffolds [141].

#### 1.6.5 Fibrin support of islet cell function and survival *in vivo*

Fibrin has been used as a carrier for many cell types with great success [142]. With respect to islet transplantation, fibrin improved islet function and survival, whereby circulating human c-peptide levels were significantly increased in diabetic mice that received fibrin-cultured islets as opposed to free floating ones [134]. Indeed, the use of fibrin reduced the required marginal islet mass by up to 90% in a subcutaneous xenogeneic porcine islet transplantation model [143]. This finding could be explained by the observation that grafts of fibrin-embedded islets gave rise to larger islets with more populated endocrine cells than grafts of free-floating islet controls [134].

Mice that received fibrin-cultured porcine islets had highly vascularized islet structures, along with improved islet viability compared to controls [143]. It has been demonstrated that the delay in reforming vessel contacts has a significant impact on islet transplantation outcomes [144]. Therefore, fibrin's pro-angiogenic effects may be a major factor in the observed improvements *in vivo*. In particular, the transplantation of islets to regions of poorer vascularization, such as subcutaneous sites, may benefit considerably from incorporation of pro-angiogenic fibrin in the graft [145]. Incorporation of isolated islets into extracellular matrix scaffolds for culture or

transplantation have also shown to be protective against intraislet endothelial cell death or dedifferentiation [90]. One experiment discovered that 8 days after fibrin-enriched human islets were subcutaneously transplanted into mice, there was a significant increase in islet vessel density and neovascularization [146]. If fibrin scaffolds can be further confirmed to provide sufficient vascularization to subcutaneously transplanted islet grafts, the more invasive and complicated kidney subcapsule or portal transplantation routes may become extinct [147]. However, before this procedure can enter clinical trials, future studies still need to decipher the mechanism and importance of donor intraislet endothelial cell versus host endothelial cell contribution to successful engraftment following transplantation.

The use of fibrin also overcomes technical difficulties associated with islet transplantation by allowing islets to be embedded in a fibrin carrier system that does not interfere with islet function or impose any short-term harm on surrounding tissues (Figure 1-3) [148]. Subcutaneous transplantation of fibrin-embedded islets was as effective as transplantation of free-floating islets under the kidney capsule in diabetic mice [149]. This study's results further support the notion that fibrin is an effective carrier [149]. In addition, fibrin reduces islet fragmentation into lone cells that is often seen in free-floating transplantation procedures [145]. This observation stresses the utility of fibrin for islet transplantation because dispersed islets have been shown to undergo an integrin-mediated form of apoptosis known as anoikis [145]. Concentrating transplanted islet cells also ensures that these cells can generate a high enough concentration gradient of VEGF and other growth factors in order to promote effective graft angiogenesis and thus graft survival [145]. As such, fibrin is a great candidate to use during islet transplantation to improve transplantation outcomes.

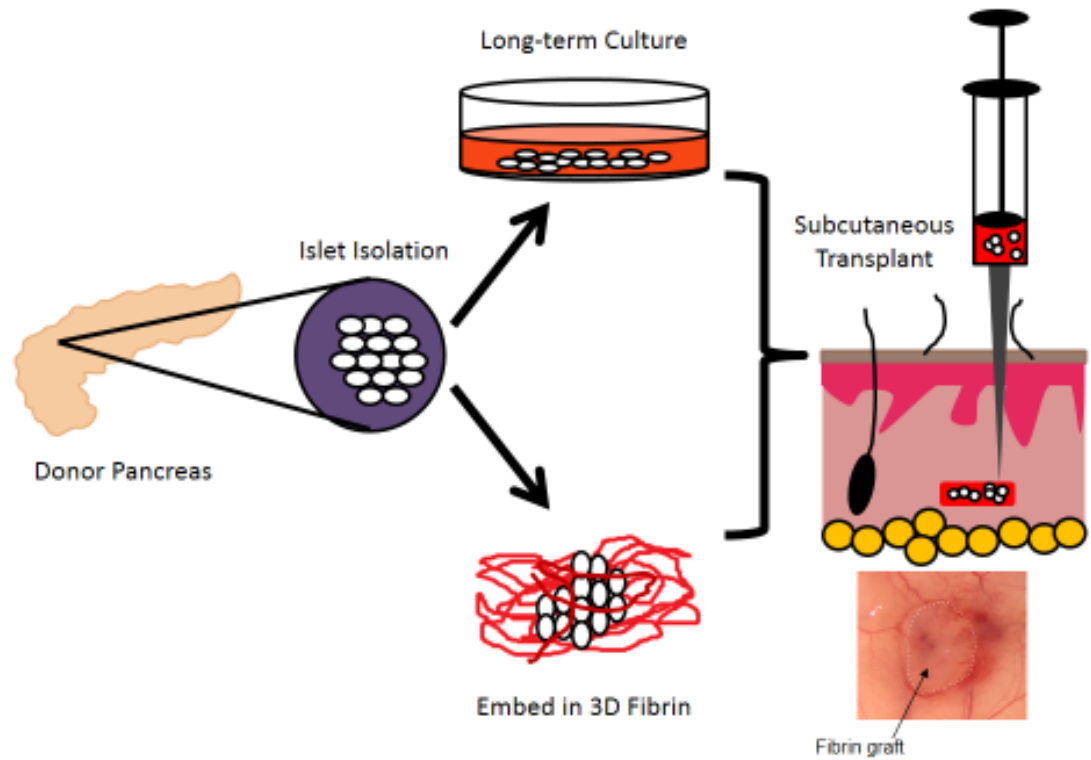


Figure 1-3 Strategies for fibrin use during cell-based therapies for diabetes treatment.

Schematic diagram for subcutaneous islet transplantation using fibrin. Isolated islets could be either cultured for long-term or embedded in 3D fibrin, then subcutaneously transplanted [134, 148]. A gross image of subcutaneously transplanted fibrin-wrapped human fetal pancreatic cells is shown. The dash arrow indicates the fibrin graft with vasculature surrounding it.

## 1.7 Rationale for thesis and objectives

The pancreas is enriched by ECM proteins that stimulate integrin receptors to mediate cellular processes. However, the mechanisms behind the dynamics of integrin-ECM interactions in the pancreas are not clear. The overall objective of this thesis is to demonstrate the necessity of integrin receptors and ECM proteins in promoting both in vivo and in vitro proliferation, survival, function, and maturation of pancreatic cells, especially beta cells. The overall hypotheses in this thesis are: 1) loss of pancreatic  $\beta 1$  integrin leads to glucose intolerance, with reduced proliferation and increased apoptosis of pancreatic beta and acinar cells and 2) culturing pancreatic cells with fibrin will improve beta cell function and survival, and maintain differentiation.

The objective of Chapter 2 is to determine the in vivo role of  $\beta 1$  integrin in the adult murine endocrine pancreas using a conditional  $\beta 1$  integrin knockout mouse model.

The objective of Chapter 3 is to investigate the in vivo role of  $\beta 1$  integrin on the adult exocrine pancreas using the same mouse model as Chapter 2.

The objective of Chapter 4 is to analyze the role of fibrin-integrin interactions in support of beta cell function, proliferation, and survival using INS-1 cells.

The objective of Chapter 5 is to discover the beneficial effects of fibrin-culture on human fetal islet-epithelial clusters and mechanisms behind these effects.

Taken together, these studies will illustrate the requirement of integrin receptors and ECM to promote and maintain adult and fetal cell function, survival, proliferation, and maturation. Results from this work have implications for cell-based therapies for diabetes treatment, especially islet transplantation, as this procedure breaks the essential connections between cells and their native ECM environment.

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## Chapter 2

### 2 Conditional $\beta 1$ integrin-deficient mice display impaired pancreatic beta cell function<sup>2</sup>

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<sup>2</sup> This work has been published in the following manuscript:

- Riopel M, Krishnamurthy M, Li J, Liu S, Leask A, Wang R. Conditional  $\beta 1$  integrin-deficient mice display impaired pancreatic  $\beta$  cell function. *J Path* 2011;224(1):45-55

## 2.1 Introduction

Integrin-ECM interactions are well known regulators of beta cell survival and function [1, 2]. Integrin receptors associate with ECM components, enabling cell–cell and cell–matrix contacts and influence numerous cellular processes, including differentiation, proliferation and function [3 - 5]. The most extensively studied integrin in islet biology is  $\beta 1$ . Its association with 12  $\alpha$  subunits [5] involved in the regulation of morphogenesis [6, 7], differentiation, proliferation [8, 9] and survival [5] has been well-documented in beta cells. Integrins  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  mediate the attachment and spreading of rat beta cells on collagen I and 804G matrices, respectively, improving cell survival and insulin secretion [10, 11]. Moreover, our and other studies have shown that perturbing  $\beta 1$  integrin function in isolated islets from pre- and post-natal rat and human fetal pancreas caused decreased adhesion on ECM and increased islet cell apoptosis [1, 2, 12, 13]. Recently we also demonstrated that  $\beta 1$  integrin collagen I and IV interactions in rat insulinoma cell line, INS-1, is essential for survival, proliferation and insulin secretion [14], and that  $\beta 1$  integrin activation of the FAK/MAPK/ERK signalling pathway enables the differentiation and survival of human fetal islet cells [15].

We have used mice homozygous for a loxP - integrin  $\beta 1$  allele and hemizygous for tamoxifen-dependent cre recombinase expressed under the control of a collagen type I promoter, to test whether a conditional knockout of  $\beta 1$  integrin in collagen I-producing cells would have physiological and functional implications in pancreatic endocrine cells in vivo. Our results show that male  $\beta 1$  integrin-deficient mice demonstrate significant alterations in glucose tolerance and beta cell mass by 4 weeks post-tamoxifen injection (7 weeks of age), along with significantly reduced phospho-FAK/ERK1/2 signals and increased caspase-3 cleavage in the pancreas.



## 2.2 Materials and methods

### 2.2.1 Conditional $\beta$ 1 integrin-deficient mice

C57BL/6 mice with a tamoxifen inducible cre recombinase controlled by the *coll1a2* promoter were crossed with floxed  $\beta$ 1 integrin mice to generate conditional  $\beta$ 1 integrin knockout mice in collagen I producing cells [16]. The mice used for experiments were genotyped by polymerase chain reaction (PCR) to detect  $\beta$ 1 integrin and Cre as described previously [16]. The  $\beta$ 1 integrin knockout was induced in 3 week old mice by intraperitoneal (i.p.) tamoxifen injection (4-hydroxytamoxifen; Sigma, St. Louis, MO, USA) [17]. Deletion of  $\beta$ 1 integrin in the pancreas was determined by real-time reverse transcriptase PCR (qRT-PCR; Appendix 5), western blot (Rabbit anti- $\beta$ 1 integrin, Millipore, Billerica, MA, USA) and immunofluorescence staining (Goat anti- $\beta$ 1 integrin, R&D Systems, Minneapolis, MN, USA; Figure 2-1). Controls include corn oil-injected Cre<sup>+</sup> mice [Cre<sup>+</sup> Ctrl] and tamoxifen-injected Cre<sup>-</sup> mice [Cre<sup>-</sup> Ctrl]. All protocols were approved by the Animal User Subcommittee at the University of Western Ontario, in accordance with the guidelines of the Canadian Council of Animal Care (Appendix 1).

### 2.2.2 RNA extraction and real-time RT-PCR

Total RNA was extracted from pancreata of  $\beta$ 1 integrin-deficient and control mice at 1 week post-injection using the miRNeasy kit (Qiagen, Germantown, MD, USA). For each reverse transcription reaction, 2 mg of total RNA from whole pancreatic tissue were used with oligo(dT) and random primers, as well as Superscript reverse transcriptase (Invitrogen, Burlington, ON, CAN). Sequences of PCR primers used for qRT-PCR with expected size of product are listed in Appendix 5. A master mix of primers, water and SYBR green was made and then added to separate tubes with each cDNA sample. Real-time RT-PCR analyses were conducted using the CFX manager software (Bio-Rad Laboratories, Mississauga, ON, Canada) and CFX Connect Real-Time System (Bio-Rad Laboratories). Data were normalized to levels of 18S RNA subunit and relative gene expression was calculated based on the  $2^{-\Delta\Delta CT}$  method as PCR signals from  $\beta$ 1 integrin-deficient pancreata relative to control pancreata.

### 2.2.3 Immunofluorescence and morphometric analyses

Body weight, intraperitoneal glucose (IPGTT) and insulin (IPITT) tolerance tests were performed in  $\beta 1$  integrin knockout ( $\beta 1$ KO), Cre<sup>+</sup> Ctrl and Cre<sup>-</sup> Ctrl mice at 4 and 7 weeks post-injection [18]. For the IPGTT, glucose [D-(+)-glucose; dextrose; Sigma] a dosage of 2 mg/g of body weight was administered (i.p.) and blood glucose levels were examined at 0, 15, 30, 60 and 120 minutes after injection. Area under the curve (AUC) was used to quantify glucose responsiveness [18, 19]. For the IPITT, normal human insulin (Humalin, Eli Lilly, Toronto, Ontario, Canada) at 1 U/kg of body weight was injected [18]. GSIS tests were performed on 7 week post-tamoxifen injected male  $\beta 1$  integrin-deficient mice and age-matched controls. Blood samples were collected before (0 minutes) and after glucose loading at 5 and 35 minutes [18]. Insulin secretion levels were assessed by enzyme-linked immunosorbent assay (ELISA).

### 2.2.4 In vivo metabolic studies and glucose-stimulated insulin secretion

Pancreatic tissues collected from  $\beta 1$  integrin-deficient, Cre<sup>+</sup> and Cre<sup>-</sup> Ctrl mice at 4 and 7 weeks post-injection were fixed in 4% paraformaldehyde. Pancreatic tissue sections (4  $\mu$ m thick) were prepared from the entire length of the pancreas and stained with haematoxylin and eosin (H&E), Masson's trichrome (for total collagen analysis), picrosirius red (for birefringent collagen I staining [20]) or with appropriate dilutions of primary antibodies, as listed in Appendix 4. Fluorescent secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Areas of alpha and beta cells in sections from all groups were traced manually with a minimum of five pancreata per age group per experimental group. Total alpha and beta cell mass was determined using previously described methods [21]. The percentage of Ki67, Pdx-1 and Nkx6.1 labelled in beta cells was determined by double immunostaining and counting beta cells from at least 12 islets randomly per pancreatic section (Appendix 4).

To measure islet capillary area, sections were double stained with anti-mouse platelet endothelial cell adhesion molecule (PECAM-1) antibody and insulin (Appendix 4).

At least 12 islets per section were selected at random, and islet capillaries were traced manually. The ratio of islet capillary area to the total islet area was calculated to determine the proportion of vessels occupying the islet area [22]. A minimum of five pancreata per age group per experimental group was analysed [18].

### 2.2.5 Insulin enzyme-linked immunosorbent assay (ELISA)

Pancreatic insulin was extracted in an ethanol – acid solution (165 mM HCl in 75% ethanol). Plasma and pancreatic insulin content were measured using a mouse ultrasensitive insulin ELISA kit (ALPCO, Salem, NH, USA) with a sensitivity of 0.15 ng/ml, according to the manufacturer’s instructions. Insulin release and content was expressed as ng/ml and ng/mg protein, respectively.

### 2.2.6 Protein extraction and western blot analysis

Protein extraction and western blot analyses were conducted as previously described [15]. Briefly, protein from  $\beta 1$  integrin-deficient and Cre<sup>+</sup> control pancreata was extracted in Nonidet-P40 lysis buffer. Equal amounts (35  $\mu$ g) of lysate proteins from each experimental group was separated by either 5%, 10% or 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were incubated with appropriate dilutions of primary antibodies, as listed in Table 2 (see Appendix 4), followed by the application of appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Proteins were detected using ECL-Plus western blot detection reagents (Perkin Elmer, Wellesley, MA, USA) and exposed to BioMax MR Film (Kodak, Rochester, NY, USA). Densitometric quantification of bands at subsaturation levels was performed using Syngenetool gel analysis software (Syngene, Cambridge, UK) and normalized to appropriate loading controls. Data are expressed as the relative expression levels of phosphorylated proteins to total protein levels or protein levels to the loading control (calnexin) [15].

### 2.2.7 Statistical analysis

Data are expressed as mean  $\pm$  standard error of the mean (SE). Statistical significance was determined using either the unpaired Student’s *t*-test or one-way analysis of

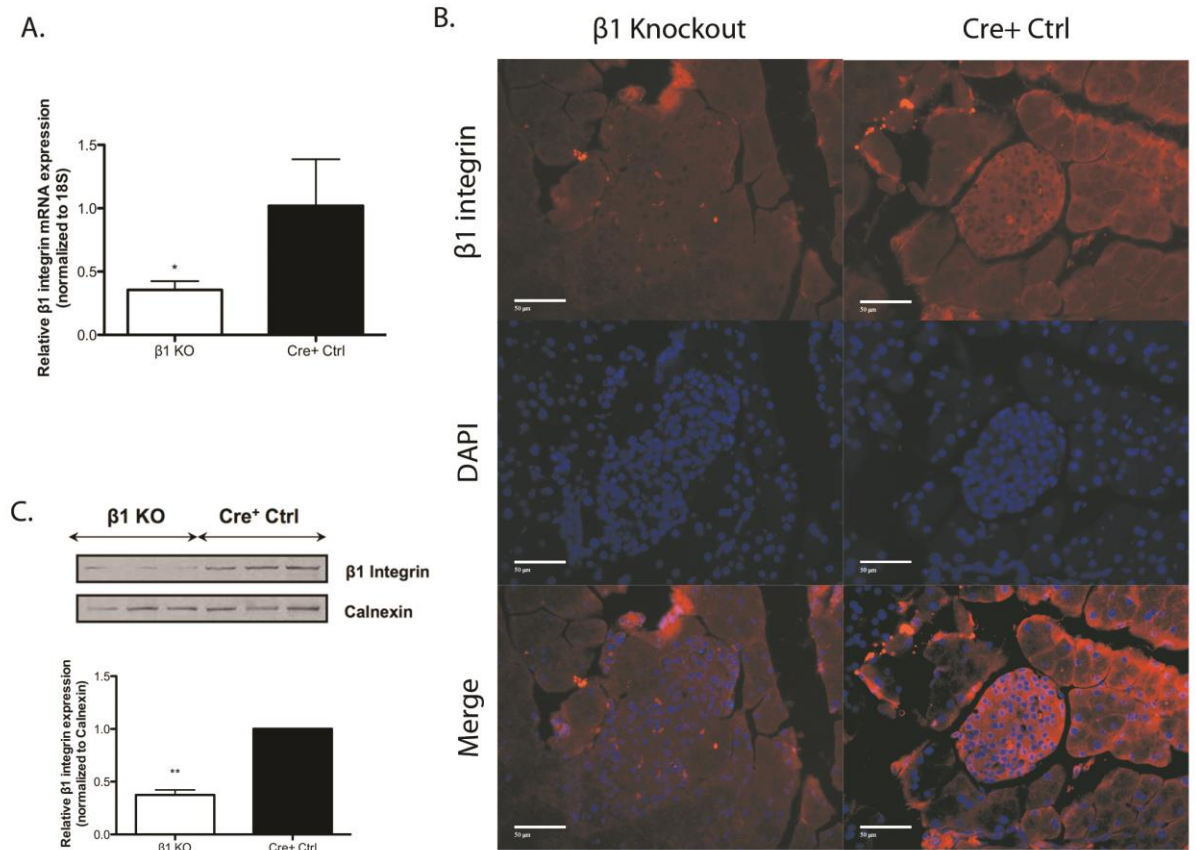
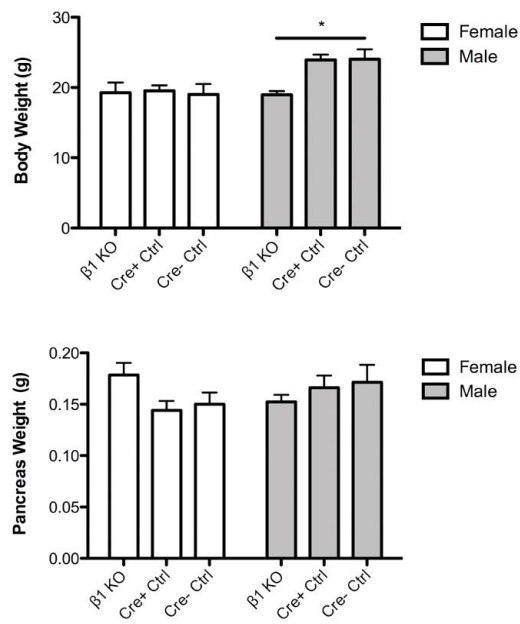


Figure 2-1 Decreased  $\beta 1$  integrin expression in the pancreas of knockout mice

(A) qRT – PCR analysis of  $\beta 1$ -*integrin* mRNA ( $n = 5$ ) and (B) immunofluorescence staining for  $\beta 1$  integrin in pancreata from 1 week post-injected male  $\beta 1$  integrin-deficient and Cre+ control mice. (C) Western blots and quantitative analysis of  $\beta 1$  protein ( $n = 3$ ) in pancreata from 4 weeks post-injected male  $\beta 1$  integrin-deficient and Cre+ control mice. Data are expressed as means  $\pm$  SE. \* $p < 0.05$ , \*\* $p < 0.01$  versus Cre+ control mice. Scale bar = 50  $\mu$ m. KO, knockout; Ctrl, control



Supplemental Figure 2-i Effect of  $\beta 1$  integrin deficiency on the pancreas of 4 week post-injected  $\beta 1$  integrin knockout mice.

Body and pancreas weight of  $\beta 1$  integrin knockout male and female mice with respective controls. Data are expressed as means  $\pm$  SE ( $n=5-10$ ). \* $p < 0.05$  vs. Cre<sup>+</sup>- and Cre<sup>-</sup>-control mice.

variance (ANOVA), followed by post hoc least significant differences and Bonferroni comparison test. Differences were considered statistically significant when  $p < 0.05$ .

## 2.3 Results

### 2.3.1 Decreased $\beta 1$ integrin expression observed in the pancreas of $\beta 1$ integrin-deficient mice

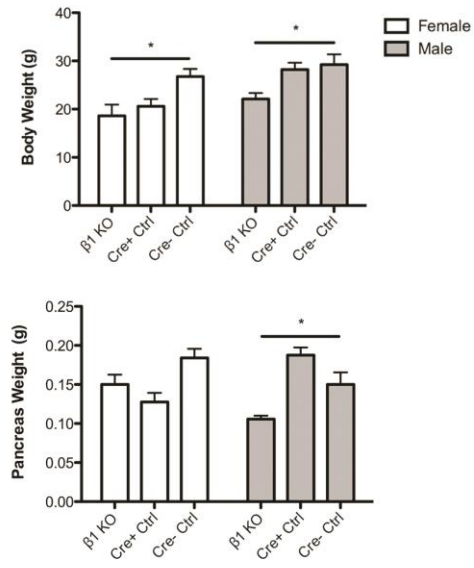
By 1 week post-tamoxifen treatment, the expression of  $\beta 1$  integrin mRNA in the pancreas of  $\beta 1$  integrin-deficient mice was significantly reduced by 50%, determined by qRT – PCR ( $p < 0.05$ , Figure 2-1A), and showed decreased  $\beta 1$  integrin staining in pancreatic exocrine and islet cells (Figure 2-1B). Total  $\beta 1$  integrin protein in the pancreas was significantly reduced after 4 weeks post-tamoxifen injection ( $p < 0.01$ , Figure 2-1C).

### 2.3.2 $\beta 1$ integrin-deficient mice showed a reduction of body and pancreas weight

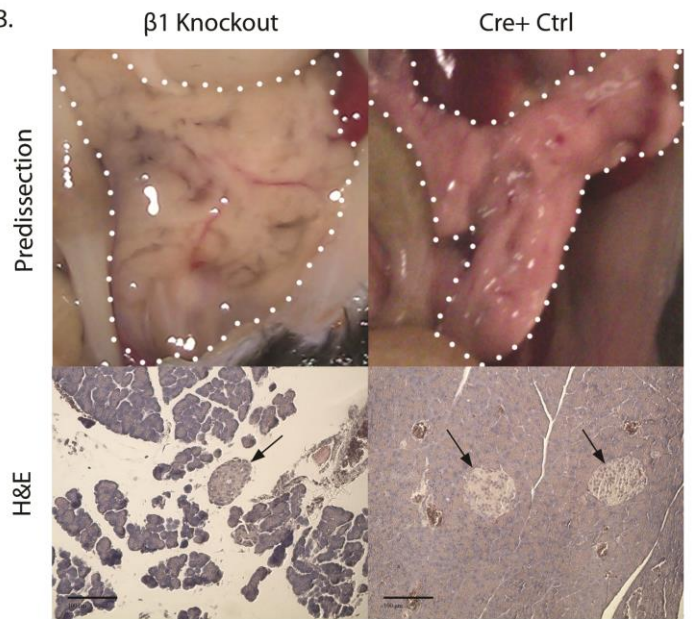
The body weight of male  $\beta 1$  integrin-deficient mice displayed significant decreases at 4 and 7 weeks post-tamoxifen injection when compared to Cre<sup>+</sup> and Cre<sup>-</sup> controls ( $p < 0.05$ , Figure 2-2A, upper panel; Supplementary Figure 2-i). However, reduced weight in female  $\beta 1$  integrin-deficient mice was only observed at 7 weeks post-tamoxifen injection when compared to female Cre controls ( $p < 0.05$ , Figure 2-2A, upper panel; Supplementary Figure 2-i). A significant reduction in pancreatic weight was found in male  $\beta 1$  integrin-deficient mice 7 weeks post-tamoxifen injection when compared to corresponding controls ( $p < 0.05$ , Figure 2-2A, lower panel; Supplementary Figure 2-i).

It was noted that the pancreas of  $\beta 1$  integrin-deficient mice at 7 weeks post-tamoxifen injection appeared edematous (Figure 2-2B, upper panel). H&E staining of pancreatic sections revealed highly fragmented and torn tissue, as well as islets isolated from surrounding exocrine cells (Figure 2-2B, lower panel). Furthermore, Masson's trichrome (Figure 2-2C, upper panel) and picrosirius red (Figure 2-2C, lower panel) staining revealed a relative reduction in collagen fibres and connective tissue in the pancreas, especially around pancreatic islets (Figure 2-2C).

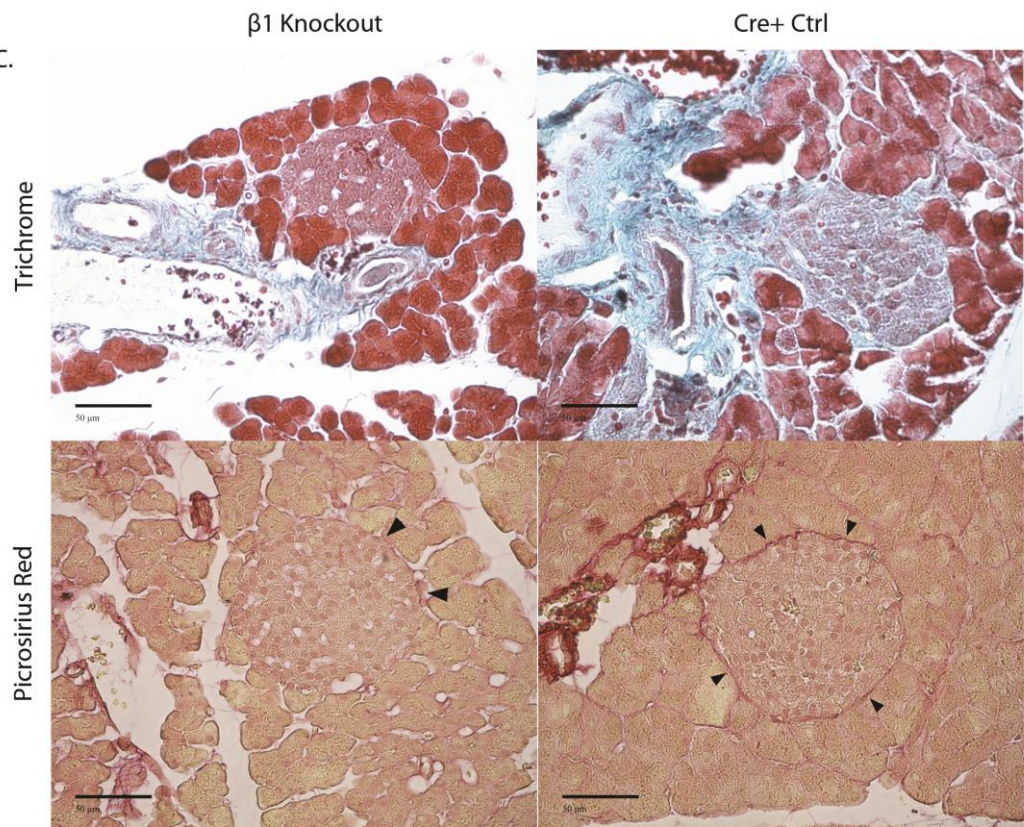
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B.



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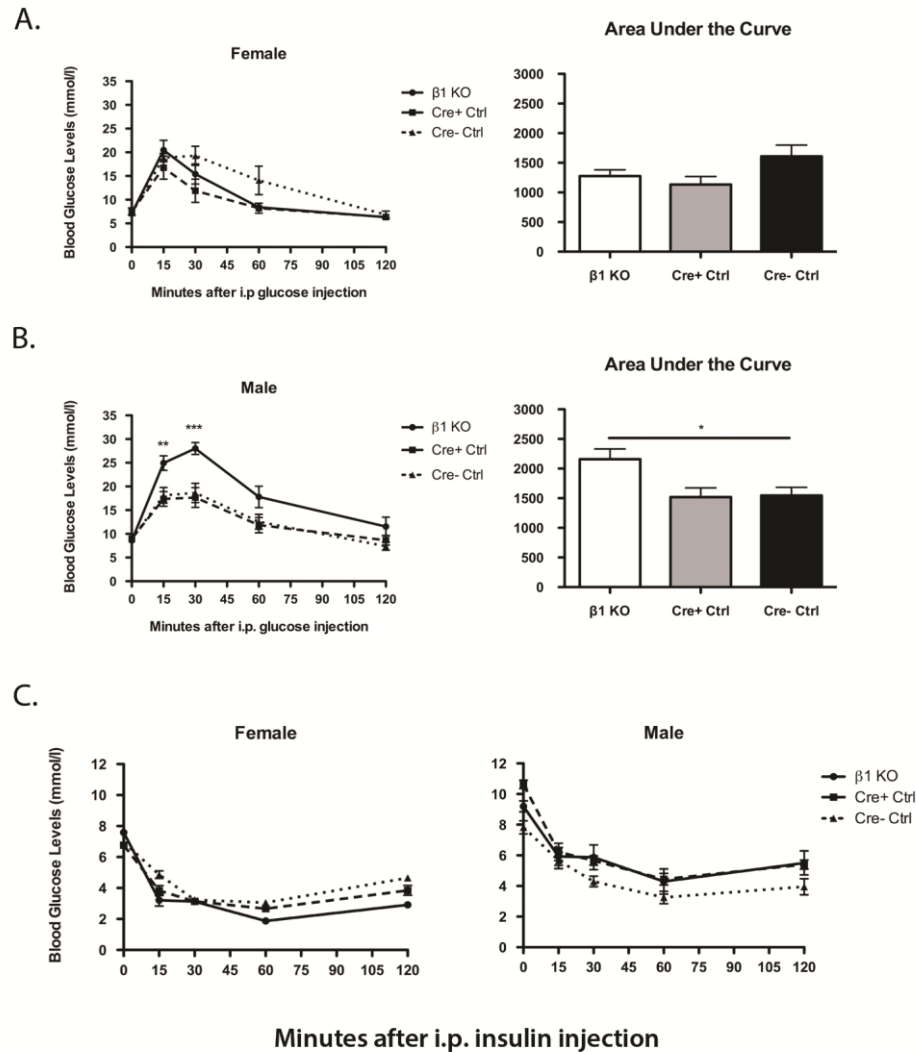




## Figure 2-2 Histology and effects of $\beta 1$ integrin-deficient pancreas

Effect of  $\beta 1$  integrin deficiency on the pancreas of 7 week post-injected  $\beta 1$  integrin-deficient mice. (A) Body and pancreas weight of  $\beta 1$  integrin-deficient male and female mice with respective controls. Data are expressed as mean  $\pm$  SE ( $n = 4-8$ ).  $*p < 0.05$  versus Cre<sup>+</sup> and Cre<sup>-</sup> control mice. (B) Photographs of the male mouse pancreas (dotted line) before dissection (top) and haematoxylin and eosin-stained (bottom). (C) Masson's trichrome stain shows collagen fibres in blue (top) and picosirius red stain shows birefringent collagen I in dark red (bottom) of pancreatic sections of male  $\beta 1$  integrin-deficient mice and respective controls. Representative images are shown; arrows indicate the islets, arrowheads indicate the basement membrane. Scale bars = 100  $\mu\text{m}$  (B); 50  $\mu\text{m}$  (C). KO, knockout; Ctrl, control.





Supplemental Figure 2-ii

Intra-peritoneal glucose tolerance test (IPGTT) in female (A) and male (B)  $\beta 1$  integrin knockout,  $Cre^+$  and  $Cre^-$  mice at 4 weeks post-injection. Glucose responsiveness of the corresponding experimental groups is shown as a measurement of the area under the curve of the IPGTT graphs with units of (mmol/L). Data are expressed as means  $\pm$  SE ( $n=6-14$ ). \* $p < 0.05$  vs.  $Cre^+$ - and  $Cre^-$ -controls. (C) Intra-peritoneal insulin tolerance test in  $\beta 1$  integrin knockout,  $Cre^+$  and  $Cre^-$  control mice at 4 weeks post-injection showed no changes in insulin sensitivity in all experimental groups ( $n=6-14$ ).

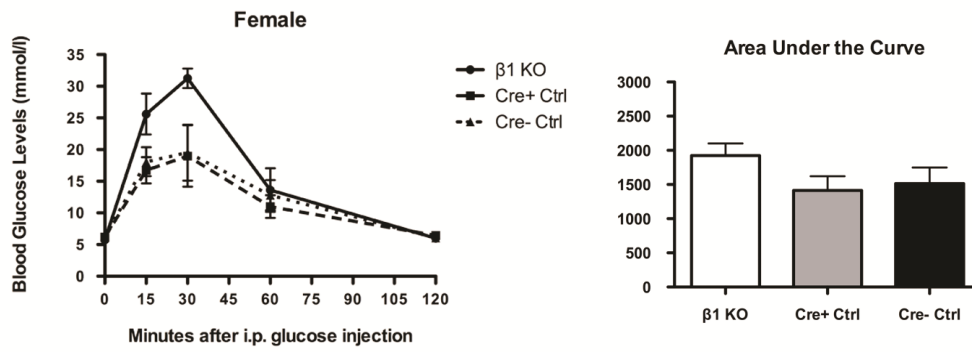
### 2.3.3 Impaired glucose tolerance and insulin secretion in $\beta 1$ integrin-deficient male mice

To evaluate the effects of  $\beta 1$  integrin deficiency on glucose metabolism, glucose/insulin tolerance tests were examined at 4 and 7 weeks post-injection. No significant changes in IPGTT were observed in female  $\beta 1$  integrin-deficient mice, despite the comparably higher peak level at 30 minutes to the controls (Figure 2-3A, Supplementary Figure 2-iiA). However, significantly impaired glucose tolerance was observed in male  $\beta 1$  integrin-deficient mice at 4 and 7 weeks post-tamoxifen injection ( $p < 0.05$ , Figure 2-3B; Supplementary Figure 2-iiB). A significant increase in blood glucose levels at 15 ( $p < 0.05$ ) and 30 ( $p < 0.05$ ) minutes following intraperitoneal glucose load was observed in male  $\beta 1$  integrin-deficient mice at 4 (Supplementary Figure 2-iiB) and 7 (Figure 2-3B) weeks post-injection, along with significantly increased area under the curve (AUC) of the IPGTT ( $p < 0.05$ , Figure 2-3B; Supplementary Figure 2-iiB). Furthermore, insulin sensitivity, as determined via IPITT, was unaltered among the experimental groups at 4 and 7 weeks post-injection (Figure 2-3C; Supplementary Figure 2-iiC). Assessment of GSIS showed less insulin secretion in 7 week post-injected male  $\beta 1$  integrin-deficient mice compared to Cre<sup>+</sup> control littermates (Figure 2-3D).

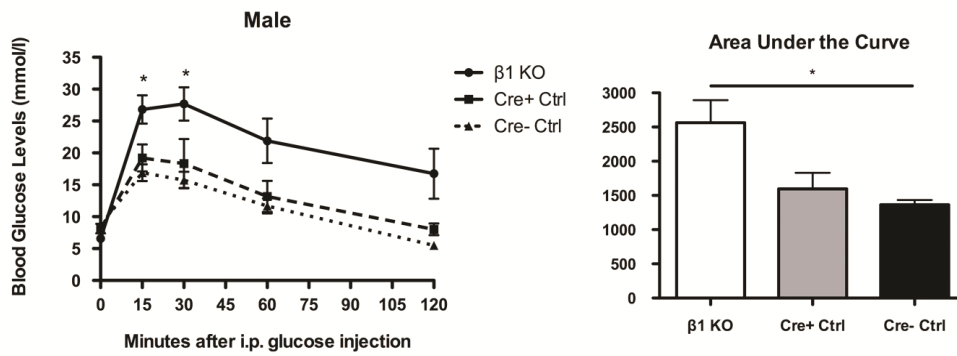
### 2.3.4 $\beta 1$ integrin-deficient mice showed a reduction of beta cell mass along with reduced pancreatic insulin content

Our results showed that male  $\beta 1$  integrin-deficient mice display diabetic symptoms at 4 weeks post-tamoxifen injection. Therefore, morphometric analyses were performed to further examine effects of lacking  $\beta 1$  integrin. The number of islets in the pancreas revealed no difference between the genders or between the  $\beta 1$  integrin-deficient mice and their controls at 7 weeks post-injection (Figure 2-4A). However, partitioning islets by size showed a significant increase in small islets ( $< 500 \mu\text{m}^2$ ), with decreases in large ( $2501 - 10\,000 \mu\text{m}^2$ ) and extra-large ( $> 10\,000 \mu\text{m}^2$ ) islets in male  $\beta 1$  integrin-deficient mice pancreas when compared to controls at 7 weeks post-injection (Figure 2-4B). No difference in islet size was observed in female  $\beta 1$  integrin-deficient mice. Morphometric analysis revealed no changes in alpha cell mass between the genders and among study groups at 4 (Supplementary Figure 2-iiiA) and 7 (Figure 2-4C) weeks

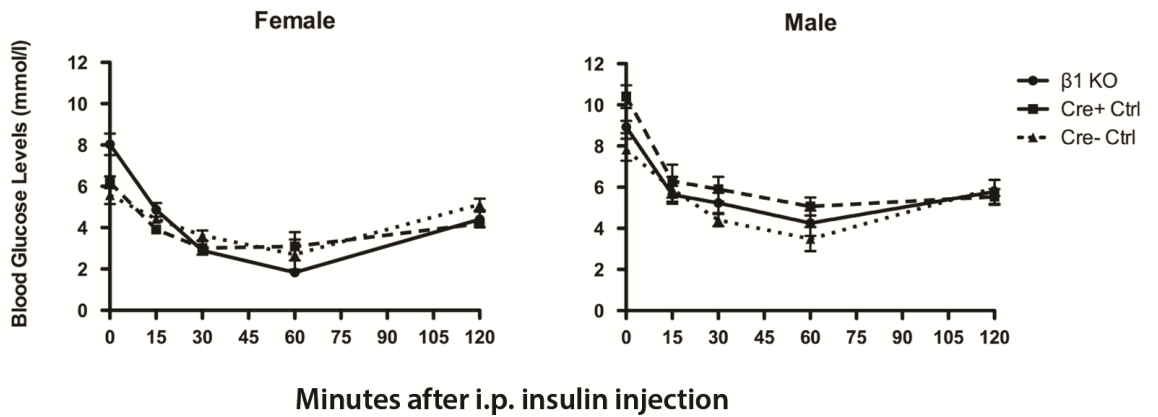
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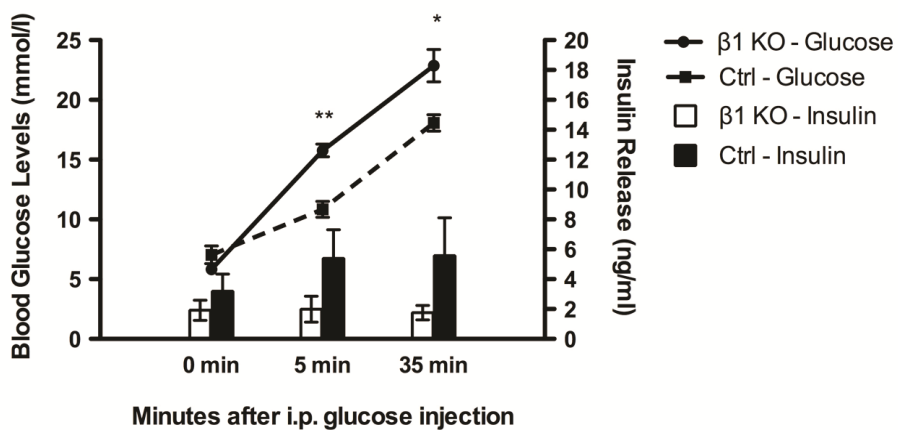
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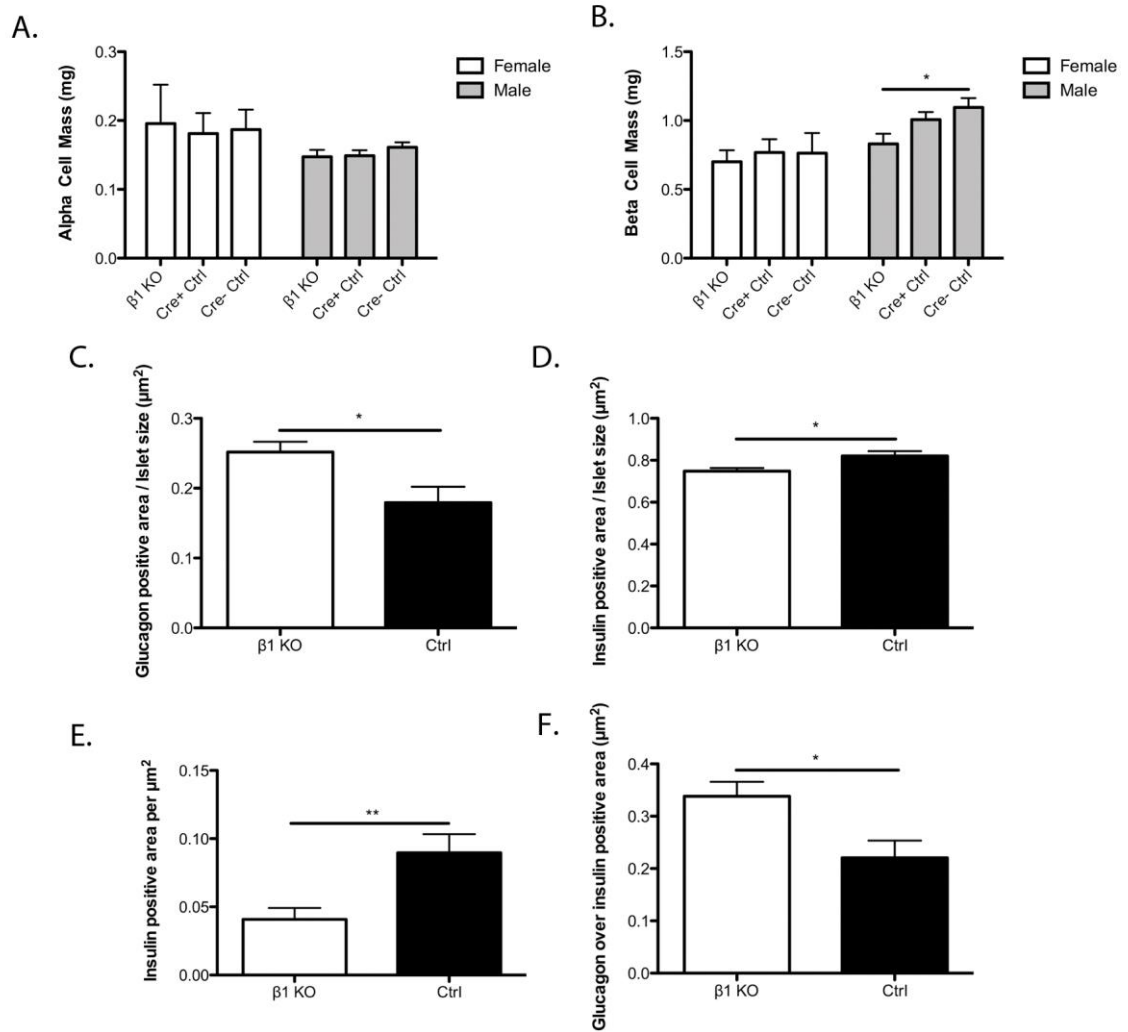


D.



### Figure 2-3 Metabolic studies in $\beta$ 1KO mice and controls

Intraperitoneal glucose tolerance test (IPGTT) in (A) female and (B) male  $\beta$ 1 integrin knockout, Cre<sup>+</sup> and Cre<sup>-</sup> mice at 7 weeks post-injection. Glucose responsiveness of the corresponding experimental groups is shown as a measurement of the area under the curve of the IPGTT graphs with units of (mM). Data are expressed as mean  $\pm$  SE ( $n = 5 - 10$ ). \* $p < 0.05$  versus Cre<sup>+</sup> and Cre<sup>-</sup> controls. (C) Intraperitoneal insulin tolerance test in  $\beta$ 1 integrin knockout, Cre<sup>+</sup> and Cre<sup>-</sup> control mice at 7 weeks post-injection showed no changes in insulin sensitivity in all experimental groups ( $n = 3 - 12$ ). (D) Glucose-stimulated insulin secretion of 7 week post-injected male  $\beta$ 1 integrin knockout and Cre<sup>+</sup> control mice.  $\beta$ 1 integrin-deficient mice demonstrate a reduction in plasma insulin release after glucose loading ( $n = 3 - 6$ ). KO, knockout; Ctrl, control.



Supplemental Figure 2-iii Morphometric analysis of alpha (A) and beta cell (B) mass in  $\beta 1$  integrin knockout and Cre control mice at 4 weeks post-injection ( $n=3-6$ ). Glucagon (C) and insulin (D) positive area over islet area in male  $\beta 1$  integrin knockout pancreas compared to controls (Cre<sup>+</sup>/Cre<sup>-</sup>) at 7 weeks post-injection ( $n=3-4$ ). (E) Insulin positive area per pancreatic section area and (F) ratio of alpha to beta cells in the pancreas of male  $\beta 1$  integrin knockout and control mice at 7 weeks post-injection ( $n=3-7$ ). Data are expressed as means  $\pm$  SE. \* $p < 0.05$ , \*\* $p < 0.01$  vs. Cre<sup>+</sup>- and Cre<sup>-</sup>- controls.

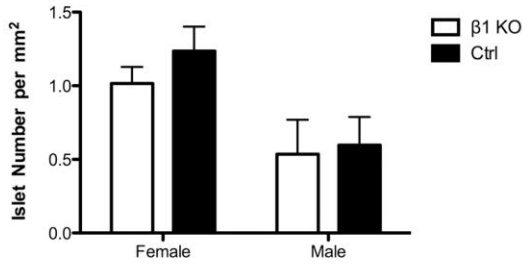
post-injection. beta cell mass of 4 (Supplementary Figure 2-iiiB) and 7 (Figure 2-4D) weeks post-tamoxifen injected  $\beta 1$  integrin-deficient male mice showed a significant reduction when compared to controls ( $p < 0.05$  for 4 weeks,  $p < 0.001$  for 7 weeks, Figure 2-4D).

To confirm that the reduction in beta cell mass was not only due to the reduction in pancreatic weight, we examined the ratio of beta cell area to either islet (Supplementary Figure 2-iiiD) or pancreatic section area (Supplementary Figure 2-iiiE) and found an absolute reduction of total beta cell area in male  $\beta 1$  integrin-deficient mice ( $p < 0.05 - 0.01$ ). Islets of 7 week post-injected  $\beta 1$  integrin knockout males had significant beta cell loss, which resulted in a relative increase in alpha cell area in islets ( $p < 0.05$ , Supplementary Figure 2-iiiC). Furthermore, the ratio of alpha cell area to beta cell area was increased significantly in  $\beta 1$  integrin knockout males when compared to controls ( $p < 0.05$ , Supplemental Figure 2-iiiF). Although female  $\beta 1$  integrin-deficient mice did not show a statistically significant glucose metabolic disorder at either 4 or 7 weeks post-tamoxifen injection, a significant reduction in beta cell mass at 7 weeks post-tamoxifen injection was observed when compared to controls ( $p < 0.05$ , Figure 2-4D). Furthermore, pancreatic insulin content was significantly decreased in  $\beta 1$  integrin-deficient male ( $p < 0.01$ ) and female ( $p < 0.05$ ) mice at 7 weeks post-tamoxifen injection when compared to controls (Figure 2-4E).

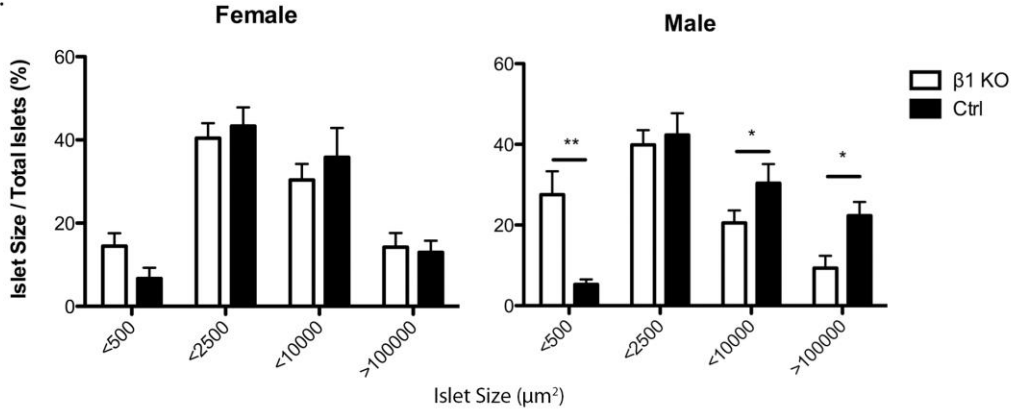
### 2.3.5 Reduced beta cell proliferation, Pdx-1 and Nkx6.1 expression in $\beta 1$ integrin-deficient mice

A significant reduction in Ki67<sup>+</sup>/insulin<sup>+</sup>-labelled cells was observed in 4 ( $p < 0.05$ ; Supplementary Figure 2-ivA) and 7 ( $p < 0.05$ ; Figure 2-5A) week post-tamoxifen injected male  $\beta 1$  integrin-deficient mice when compared to controls. Although female  $\beta 1$  integrin-deficient mice showed lower proliferative capacity than that of the controls, no statistical significance was observed (Figure 2-5A). Furthermore, we found that Pdx-1 expression, a transcription factor important in regulating insulin gene expression, was significantly decreased in beta cells of both male ( $p < 0.001$ ) and female ( $p < 0.05$ )  $\beta 1$  integrin-deficient mice at 4 and 7 weeks post-tamoxifen injection when compared to their respective controls (Figure 2-5B; Supplementary Figure 2-ivB). Moreover, a significant decrease in Nkx6.1 expression was observed in 7 week

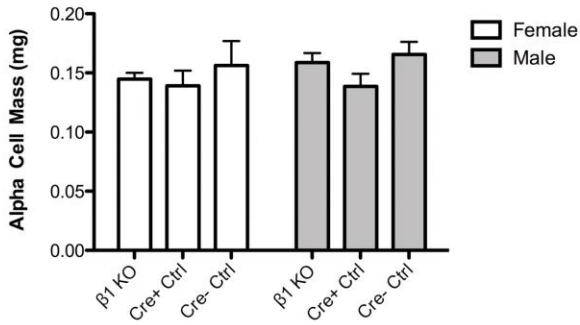
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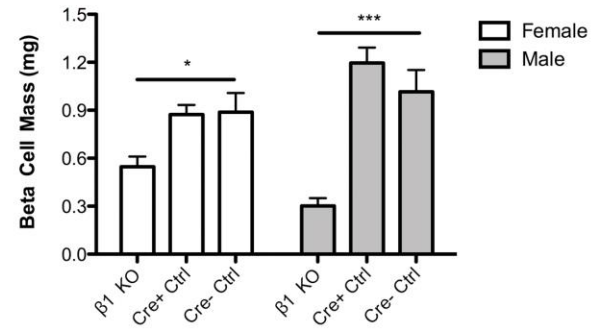
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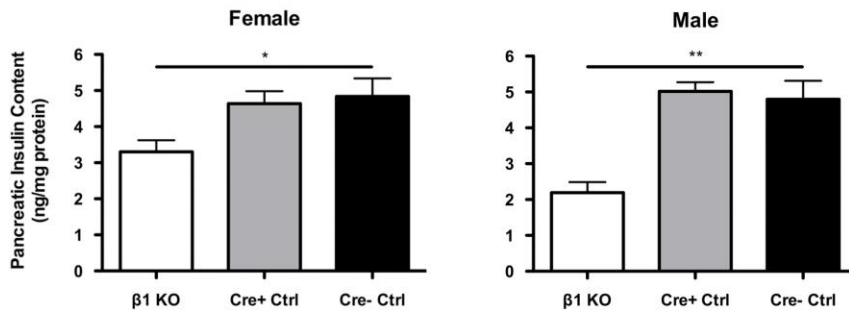
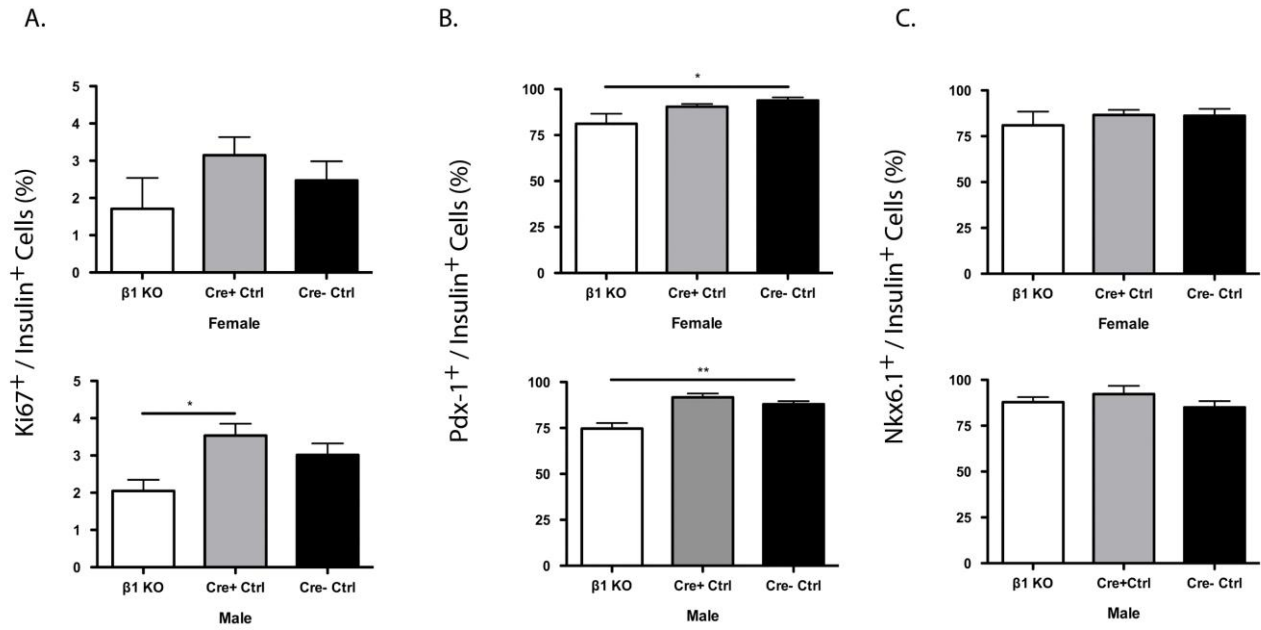


Figure 2-4 Morphometric analysis of  $\beta$ 1KO mice and controls

Morphometric analysis of (A) islet numbers, (B) islet size, (C) alpha cell mass and (D) beta cell mass in  $\beta$ 1 integrin-deficient and Cre control mice at 7 weeks post-injection ( $n = 4 - 9$ ). (E) Insulin content in the pancreas of  $\beta$ 1 integrin-deficient and Cre controls at 7 weeks post-injection ( $n = 5$ ). Data are expressed as mean  $\pm$  SE. \* $p < 0.05$ , \*\* $p < 0.01$  \*\*\*  $p < 0.001$  versus Cre<sup>+</sup> and Cre<sup>-</sup> controls. KO, knockout; Ctrl, control.





Supplemental Figure 2-iv Quantitative analysis of Ki67 (A), Pdx-1 (B), Nkx6.1 (C) expression in beta cells of  $\beta 1$  integrin-deficient and control mice at 4 weeks post-injection. Data are expressed as mean percent of positive cells over insulin<sup>+</sup> cells  $\pm$  SE ( $n=3-8$ ) \* $p < 0.05$  and \*\* $p < 0.01$  vs. Cre<sup>+</sup>- and Cre<sup>-</sup>-controls.

post-tamoxifen injected male and female  $\beta 1$  integrin-deficient mice when compared to controls ( $p < 0.05$ , Figure 2-5C).

### 2.3.6 Reduced islet capillary area in male $\beta 1$ integrin-deficient mice

To understand whether pancreatic  $\beta 1$  integrin deficiency had an affect on islet vasculature, a measurement of islet capillary area was performed in both  $\beta 1$  integrin-deficient and control mice at 7 weeks post-injection. Double immunofluorescent staining for PECAM-1 and insulin showed less PECAM-1+ area present in islets of  $\beta 1$  integrin-deficient mice (Figure 2-6A). Male  $\beta 1$  integrin-deficient mice demonstrated 40% less islet capillary area per islet when compared to controls ( $p < 0.05$ , Figure 2-6B), while  $\beta 1$  integrin-deficient females displayed only a slight reduction when compared to controls (Figure 2-6B).

### 2.3.7 Reduction of phospho-FAK, phospho-ERK1/2 and cyclin D1 expression along with increased caspase 3 cleavage in $\beta 1$ integrin-deficient male mice

Our previous studies have demonstrated that  $\beta 1$  integrin signals primarily via the FAK – MAPK – ERK1/2 pathway to regulate proliferation and function in isolated beta cells [15]. Here, we revealed the in vivo pancreatic  $\beta 1$  integrin signalling cascade and found a 40% reduction in phospho-FAK ( $p < 0.05$ , Figure 2-7A), with a 75% reduction of phospho-ERK1/2 levels ( $p < 0.001$ , Figure 2-7B) in 4 week post-tamoxifen injected  $\beta 1$  integrin-deficient male mice compared to Cre+ controls. Furthermore, an increase in cleaved caspase 3 ( $p < 0.01$ , Figure 2-7C) and a reduction of cyclin D1 expression ( $p < 0.001$ , Figure 2-7D) was detected in  $\beta 1$  integrin-deficient male mice, while there was no alteration in phospho-Akt expression in the pancreas (Figure 2-7F). Finally, western blot analysis showed a 50% reduction in Pdx-1 expression in  $\beta 1$  integrin-deficient male pancreas when compared to controls ( $p < 0.05$ , Figure 2-7E).

## 2.4 Discussion

We have shown that  $\beta 1$  integrin in the pancreas is a critical regulator of beta cell proliferation and function in vivo. Significant reduction of  $\beta 1$  integrin expression in

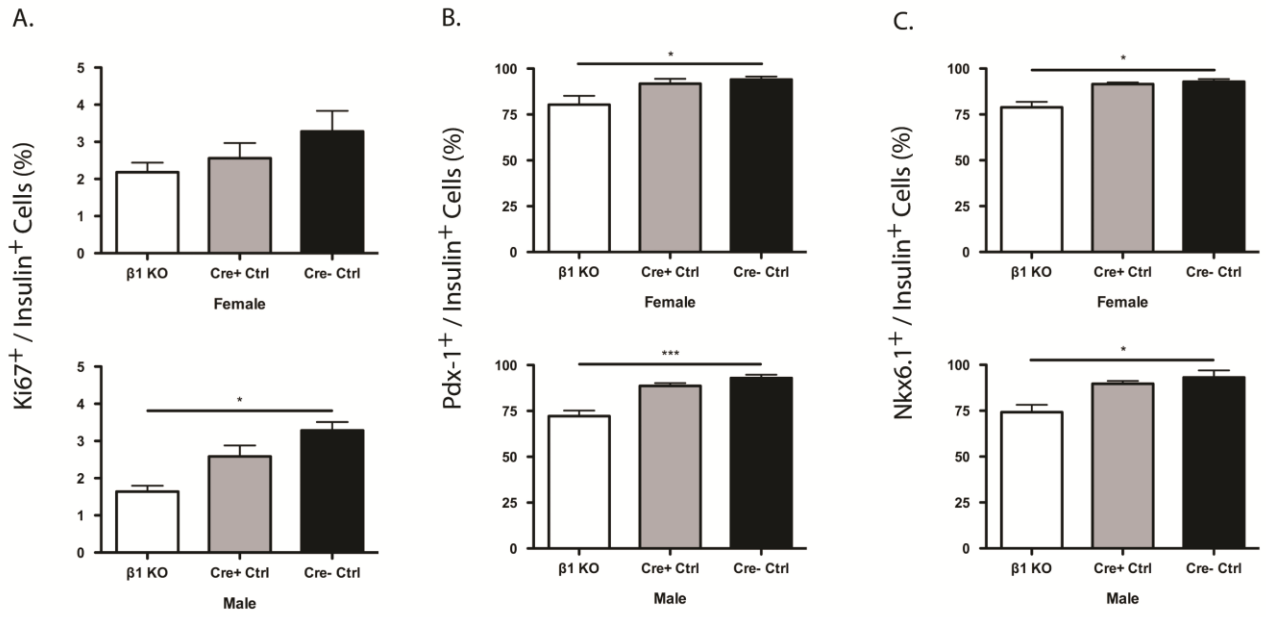


Figure 2-5 Transcription factor expression in  $\beta$ 1KO and control mice

Quantitative analysis of (A) Ki67, (B) Pdx-1 and (C) Nkx6.1 expression in beta cells of  $\beta$ 1 integrin-deficient and control mice at 7 weeks post-injection. Data are expressed as mean percentage of positive cells over insulin<sup>+</sup> cells  $\pm$  SE ( $n = 4-7$ ) \* $p < 0.05$  and \*\*\* $p < 0.001$  versus Cre<sup>+</sup> and Cre<sup>-</sup> controls. KO, knockout; Ctrl, control.

the pancreas resulted in disrupted pancreatic morphology, including a reduction of total pancreatic weight and pancreatic collagen matrices. ECM proteins are well known to be essential for cell – cell interactions and promoting cell survival and function through integrin receptors [23]. As such, loss of  $\beta 1$  integrin in pancreatic ECM-producing cells contributed to the observed reduction in pancreas weight and altered pancreatic function, which may also be associated with the loss of body weight in  $\beta 1$  integrin-deficient mice. These results were in correlation with a study using mice with a pancreas-specific knockout of  $\beta 1$  integrin [24]. Furthermore, altered glucose metabolism in  $\beta 1$  integrin-deficient mice was observed, which was associated with a 50 – 70% reduction in beta cell mass and cell proliferation, along with a significant decrease in pancreatic insulin content. These results indicate that  $\beta 1$  integrin deficiency is responsible for the loss of blood glucose control as well as changes in pancreatic morphology.

It is well established that Pdx-1 and Nkx6.1 expression are integral to normal development of the pancreas and maintenance of beta cell function [25 – 27]. Reduced Pdx-1 and Nkx6.1 expression in  $\beta 1$  integrin-deficient mice suggests that not only is  $\beta 1$  integrin required for normal beta cell development and function but that it may be involved in mediating Pdx-1 and Nkx6.1 protein expression. Mice containing only one functional allele of Pdx-1 (Pdx-1<sup>+/-</sup>) demonstrated increased islet cell apoptosis and reduced beta cell mass [28]. Thus, reduced beta cell mass and insulin content, as well as glucose intolerance observed in  $\beta 1$  integrin-deficient mice, may also be due to low levels of Pdx-1 and Nkx6.1 in their pancreatic islets.

Besides transcription factor expression, islet capillary area was also significantly reduced in  $\beta 1$  integrin-deficient males. Nikolova *et al* have demonstrated the requirement for adequate islet endothelial cells for production of basement membrane components (primarily laminins), which stimulate  $\beta 1$  integrins to promote beta cell proliferation and insulin gene expression [29]. Also, proper vessel function is necessary to supply enough blood to islets for ideal glucose sensing and insulin response [30]. This suggests that  $\beta 1$  integrin loss in islets has an effect on endothelial cell function, which may further contribute to beta cell dysfunction by restricting blood flow or impairing the production of basement membrane.

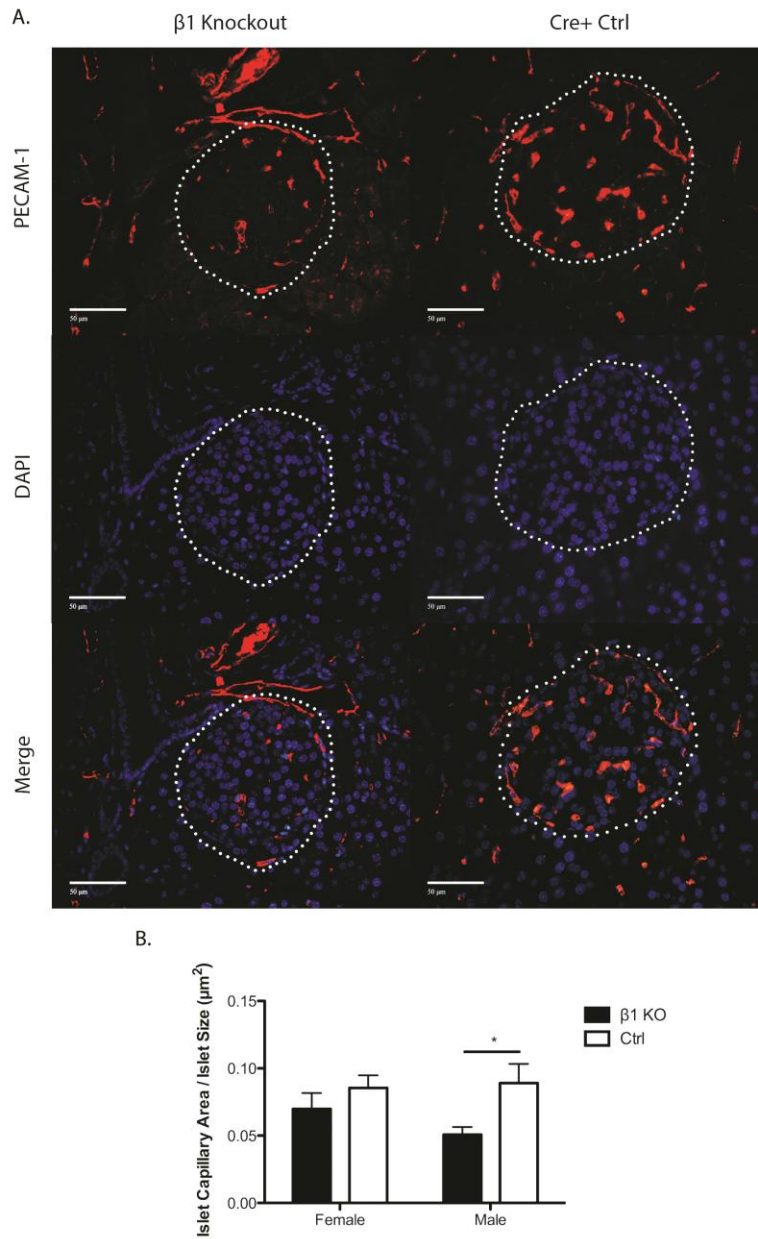


Figure 2-6 Effect of  $\beta$ 1 integrin knockout on islet capillary area

(A) Immunofluorescence staining for PECAM-1 (red) of a 7 week post-injected  $\beta$ 1 integrin-deficient and control islet (dotted lines). (B) Morphometric analysis of islet capillary area at 7 week post-injected  $\beta$ 1 knockout and control mice. Data are expressed as means  $\pm$  SE ( $n = 3-6$ )  $*p < 0.05$  versus Cre+ and Cre- controls. KO, knockout; Ctrl, control. Scale bar = 50  $\mu\text{m}$ .

In several model systems,  $\beta 1$  integrin has been shown to activate the FAK/MAPK/ERK cascade to enhance cell survival and function [31, 32]. Hammer *et al* determined that 804G matrix protects against rat adult beta cell apoptosis through the  $\beta 1$  integrin – FAK signalling pathway [33], while our previous *in vitro* studies have demonstrated that  $\beta 1$  integrin signalling through the FAK/MAPK/ERK pathway regulates the differentiation and survival of human fetal islet cells [15]. To investigate the signalling cascade of  $\beta 1$  integrin *in vivo*, alterations in phospho-FAK, ERK1/2 and Akt were examined, along with cyclin D1 expression and caspase 3 cleavage. Significant decreases in phospho-FAK and ERK1/2 were observed, along with reduced cyclin D1 expression and increased caspase 3 cleavage, but no effect on Akt pathway, indicating that  $\beta 1$  integrin signals primarily through the FAK/MAPK/ERK cascade *in vivo* to regulate glucose metabolism and beta cell survival and function.

Our data indicate that loss of  $\beta 1$  integrin affects pancreatic structure and function in male, but less in female, mice. The observed differences in the requirement for integrin  $\beta 1$  in glucose metabolism are consistent with previous results indicating that there are gender differences in glucose metabolism. Our previous characterization of cKit<sup>W<sup>v</sup></sup> mutant mice revealed severe glucose intolerance, reduced beta cell mass and low cell proliferation rates in insulin+ cells, primarily in male mice [18]. Moreover, oestrogen administration has been shown to protect mice from beta cell ablation from STZ administration by preventing beta cell apoptosis [34].

In summary, this study is the first, using a conditional  $\beta 1$  integrin-deficient mouse model, to demonstrate the physiological and functional effects of  $\beta 1$  integrin deficiency on the endocrine pancreas *in vivo*.  $\beta 1$  integrin plays an important role in maintaining beta cell mass, glucose metabolism, islet capillary size as well as Pdx-1 and Nkx6.1 expression. Moreover, our data showed that  $\beta 1$  integrin primarily signals through the FAK/MAPK/ERK cascade to regulate these physiological and morphological parameters *in vivo*. This study increases our understanding of integrin – ECM interactions and should prove very useful for cell-based therapies for the treatment of diabetes.

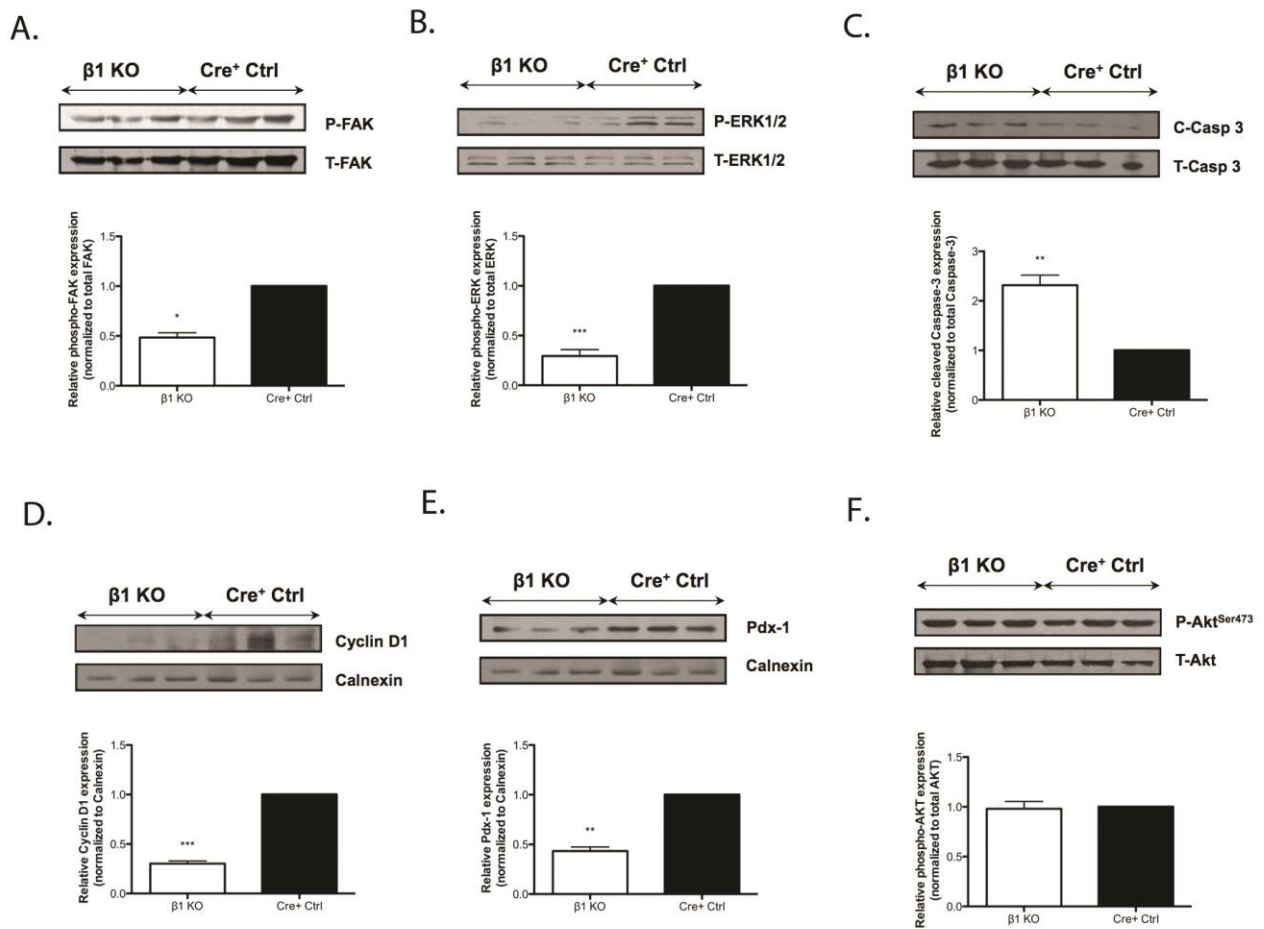


Figure 2-7 Signaling pathway activity in  $\beta 1$ KO and control mice

Down-regulation of phospho-FAK/ERK1/2 signalling in  $\beta 1$  integrin-deficient mouse pancreata. Western blot analyses of: (A) phosphorylated and total FAK; (B) phosphorylated and total ERK1/2; (C) cleaved and total caspase-3; (D) cyclin D1; (E) Pdx-1; and (F) phosphorylated and total Akt expression in the pancreas of male  $\beta 1$  integrin-deficient and control mice at 4 weeks post-injection. Representative blots are shown. Data are normalized to the Cre<sup>+</sup> controls (set at 1) and expressed as mean  $\pm$  SE ( $n = 3$ ) \* $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  versus Cre<sup>+</sup> controls. P, phosphorylated; T, total; C, cleaved; KO, knockout; Ctrl, control.

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## Chapter 3

### 3 $\beta$ 1 integrin-extracellular matrix interactions are essential for maintaining exocrine pancreas architecture and function<sup>3</sup>

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<sup>3</sup> This work has been published in the following manuscript:

Riopel M, Li J, Liu S, Leask A, Wang R.  $\beta$ 1 integrin-extracellular matrix interactions are essential for maintaining exocrine pancreas architecture and function. *Lab Invest* 2013;93(1):31-40

### 3.1 Introduction

Integrin receptors have a significant role in cell–cell and cell–ECM contacts in many different tissue types. There are 18  $\alpha$  and 8  $\beta$  receptors capable of forming 24 heterodimeric interactions; yet, half these interactions are made up by  $\beta$ 1 integrin receptors [1]. This  $\beta$ 1 integrin receptor group is largely responsible for attachment to the ECM [2]. Upon stimulation,  $\beta$ 1 integrin has been shown to mediate cell motility, survival, proliferation and differentiation [2-5].

Pancreatic acini are enclosed round structures that produce digestive enzymes [6]. Basal lamina covers the acini at the basal surface, stimulating acini integrin receptors [6]. Pancreatic stellate cells (PSCs) are identified as periacinar fibroblast-like cells of the pancreas that express glial fibrillary acidic protein (GFAP) and produce ECM proteins in support of surrounding tissue [7]. PSCs are also, in part, responsible for the fibrosis observed in chronic pancreatitis [8, 9]. Previous studies have shown that  $\alpha$ 3 $\beta$ 1 integrin is essential for proper apical/basolateral cell surface receptor organization and basement membrane formation in the submandibular gland [10]. As well,  $\beta$ 1 integrin deficiency has shown to interfere with laminin-1 expression and basement membrane synthesis and assembly in embryoid bodies [11] and teratoma [12], as well as collagen IV expression in the lens fiber of mice [13]. This is also true for proper fibronectin assembly, which requires  $\alpha$ 5 $\beta$ 1 integrin [14].  $\beta$ 1 integrin has been studied in many tissues, yet the role of  $\beta$ 1 integrin in the postnatal exocrine pancreas is almost wholly unknown. One recent study examined the effect of loss of  $\beta$ 1 integrin expression in acinar cells during development; 6-week-old  $\beta$ 1 integrin-deficient mice were susceptible to pancreatitis and displayed aberrant acinar cell polarity and necrosis [15]. More recently, we have begun to probe the role of  $\beta$ 1 integrin expression in the postnatal, developed pancreas, using a tamoxifen-dependent cre recombinase expressed under the control of a collagen I-specific promoter/enhancer.

We demonstrated that adult mice deficient in  $\beta$ 1 integrin showed impaired glucose tolerance with a significant reduction in pancreatic beta cell function, consistent with the onset of diabetes. Furthermore, these  $\beta$ 1 integrin-deficient mice displayed a significant decrease in pancreatic FAK and ERK1/2 activation, along with increased caspase-3

cleavage and decreased cyclin D1 expression [16]. However, the effects of  $\beta 1$  integrin deficiency on pancreatic exocrine morphology and function in conditional  $\beta 1$  integrin-deficient mice have still to be determined.

Here, we used mice homozygous for a loxP- $\beta 1$  integrin allele and hemizygous for tamoxifen-dependent cre recombinase expressed under the control of a collagen type I promoter to analyze pancreatic exocrine morphology and function in vivo [16]. We found that mice with  $\beta 1$  integrin deficiency controlled by the collagen I promoter had a primary defect in PSCs and islets in the pancreas, which lead to a significant decrease in ECM products in the exocrine compartment.  $\beta 1$  integrin-deficient mice displayed significantly decreased food intake with a loss of body weight, which was associated with reduced pancreatic amylase, carboxypeptidase A and regenerating islet-derived protein II expression. These  $\beta 1$  integrin-deficient mice also demonstrated decreased exocrine cell proliferation and increased apoptosis. Interestingly, cultured acinar cell clusters isolated from  $\beta 1$  integrin-deficient mouse pancreata in the presence of ECM proteins showed an improved acinar cell apoptosis. This study indicates that sufficient ECM and  $\beta 1$  integrin interactions are essential for maintaining exocrine pancreatic integrity and function.

## 3.2 Materials and methods

### 3.2.1 Conditional $\beta 1$ integrin-deficient mice

To generate conditional  $\beta 1$ KO in collagen I-producing cells, floxed  $\beta 1$  integrin mice were crossed with C57BL/6 mice containing CRE-ER<sup>T</sup> (tamoxifen-inducible cre recombinase) gene downstream of the collagen type Ia2 (Col1a2) promoter, as described previously [17]. Progeny mice with positive genotype, as analyzed by PCR [17], were induced by intraperitoneal injection of 1 mg tamoxifen (4-hydroxytamoxifen; Sigma, St Louis, MO, USA) per mouse per day for 5 days at 3 weeks of age [16]. Corn oil-injected Cre- positive and tamoxifen-injected Cre-negative mouse groups were merged as controls and experiments were carried out on male mice at 4 and 7 weeks post-injection. Deletion of  $\beta 1$  integrin was confirmed by qRT-PCR, western blot and immunofluorescence as described previously [16]. A Rosa26loxP-STOP-lacZ mouse (Jackson Laboratories), which does not

express the  $\beta$ -galactosidase reporter gene unless cre recombinase is expressed in the nucleus, was crossed with Col1a2-Cre-ER<sup>T</sup> to identify the cells within the pancreas that expressed cre under control of the Col1a2 promoter [17]. All protocols were approved by the Animal Use Subcommittee at the University of Western Ontario in accordance with the guidelines of the Canadian Council of Animal Care (Appendix 1).

### 3.2.2 Body and pancreas weight and food intake studies

The body and pancreas weight of  $\beta$ 1 integrin-deficient and control mice were measured at 4 and 7 weeks post-injection. At 4 weeks post-injection, mice from both  $\beta$ 1 integrin-deficient and control groups were separated individually and had their food weighed daily at 0900 hours and monitored their food intake for a week. Data were expressed as average food intake per mouse per day.

### 3.2.3 Acinar cell culture experiments

To isolate acinar cell clusters, both  $\beta$ 1 integrin-deficient and control mouse pancreata at 3 weeks post-injection were dissected and digested with collagenase XI (1 mg/ml; Sigma). Acinar cell clusters were cultured in modified RPMI 1640 media [18] with either 1% BSA (Sigma) or 10% FBS (Invitrogen, Burlington, ON, Canada), reported to be enriched for ECM proteins [19, 20], for 24 h. Cell clusters from four experimental groups ( $\beta$ 1KO-BSA, Ctrl-BSA,  $\beta$ 1KO-FBS, and Ctrl-FBS) were harvested and processed for immunofluorescence with at least four mouse pancreata per experimental per group used.

### 3.2.4 RNA extraction and real-time RT-PCR

Total RNA was extracted from pancreata of  $\beta$ 1 integrin-deficient and control mice at 1 week post-injection using the miRNeasy kit (Qiagen, Germantown, MD, USA) [16]. For each reverse transcription reaction, 2 mg of total RNA from whole pancreatic tissue were used with oligo(dT) and random primers, as well as Superscript reverse transcriptase (Invitrogen). Sequences of PCR primers used for RT-PCR with expected size of product are listed in Appendix 5. A master mix of primers, water and SYBR green was made and then added to separate tubes with each cDNA sample. Real-time RT-PCR analyses were

conducted using the CFX manager software (Bio-Rad Laboratories, Mississauga, ON, Canada) and CFX Connect Real-Time System (Bio-Rad Laboratories). Data were normalized to levels of 18S RNA subunit and relative gene expression was calculated based on the  $2^{-\Delta\Delta CT}$  method as PCR signals from  $\beta 1$  integrin-deficient pancreata relative to controls.

### 3.2.5 Protein extraction and western blot analysis

$\beta 1$  integrin-deficient and control pancreata, as well as acinar cell clusters, were sonicated in Nonidet-P40 lysis buffer to extract protein. Equal amounts (2mg) of lysate pancreatic proteins from each experimental group were separated by 10 or 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated with appropriate diluted primary antibodies listed in Appendix 4. Proteins were detected using ECL<sup>TM</sup>-Plus Western blot detection reagents (Perkin Elmer, Wellesley, MA, USA) and exposed using the Versadoc Imaging System (Bio-Rad Laboratories). Densitometric quantification of bands at subsaturation levels was performed using Quantity One software (Bio-Rad Laboratories) and normalized to appropriate loading controls. Data are expressed as relative expression level of protein to the loading control [16].

### 3.2.6 Serum assays for amylase and pancreatic lipase

Serum was collected from 7 week post-injected  $\beta 1$  integrin-deficient and control mice. To measure serum amylase levels, a Phadebas Test tablet (Magle Life Sciences, Lund, SWE) was added to diluted serum samples, based on the manufacturer's instructions. Amylase activity was measured with a spectrophotometer and determined by comparing absorbance values to standard curve [21]. To examine the serum level of pancreatic lipase, serum samples were mixed with lipase enzyme substrate and enzyme activator as per the manufacturer's instructions. Pancreatic lipase activity was measured using a spectrophotometer and a formula offered by Genzyme Diagnostics (Charlottetown, P.E.I., CAN) [22].



### 3.2.7 Extracellular matrix protein analysis

Pancreata from  $\beta 1$  integrin-deficient and control mice at 7 weeks post-injection were dissected and fixed in 4% paraformaldehyde. Four  $\mu\text{m}$  thick pancreatic tissue sections were prepared from the entire length of the pancreas and stained with hematoxylin and eosin, Masson's trichrome (for total collagen analysis), picosirius red (for birefringent collagen I staining), as described previously [16]. To quantitatively evaluate fibronectin concentration in the pancreas of  $\beta 1$  integrin-deficient and control mice, a fibronectin mouse sandwich ELISA kit was used (Abcam, Cambridge, MA, USA).

### 3.2.8 Immunofluorescence and TUNEL assay

Sets of pancreatic tissue sections (4  $\mu\text{m}$  thick) were immunofluorescently stained with appropriately diluted primary antibodies listed in Appendix 4. Fluorescent secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and 4'-6-Diamidino-2-phenylindole (DAPI, Sigma) was used for nuclear counterstaining [16].

To assess the structural organization of focal adhesion contacts in pancreatic acinar cells, an Actin Cytoskeleton and Focal Adhesion Staining kit (Chemicon, Temecula, CA, USA) containing mouse anti-vinculin monoclonal antibody and TRITC-conjugated Phalloidin was used. Cell proliferation was examined using Ki67 labeling (Abcam).

To examine the cells undergoing apoptosis, the terminal deoxynucleotidyl transferase mediated nick end-labeling (TUNEL) assay was performed [23]. Briefly, pancreatic sections were pretreated with 0.1% trypsin, then incubated with the TUNEL reaction mixture conjugated with fluorescein-dUTP (Roche, Montreal, QUE, CAN). Percent cell proliferation and apoptosis were calculated by counting Ki67 or TUNEL labeled cells in exocrine tissue from at least 12 randomly selected fields of view per pancreatic section [16].

### 3.2.9 Statistical analysis

Data are expressed as means  $\pm$  SE. Statistical significance was determined using the unpaired student's *t*-test. Differences were considered to be statistically significant when  $p < 0.05$ .

## 3.3 Results

### 3.3.1 Loss of $\beta 1$ integrin observed in pancreatic stellate cells and islets with a significant reduction in ECM expression in the pancreas of $\beta 1$ integrin-deficient mice

To determine the pancreatic cell types that would be directly affected by the knockout of  $\beta 1$  integrin, transgenic Rosa26loxP-STOP-lacZ mice were crossed with Col1a2-CRE(ER)<sup>T</sup> mice [17]. The resultant mice were injected with tamoxifen to allow expression of  $\beta$ -galactosidase in cells specifically expressing cre recombinase under control of the *Col1a2* promoter. Cells within the pancreas positive for  $\beta$ -galactosidase were detected with an anti- $\beta$ -galactosidase antibody (Figure 3-1A). Double labeling using an antibody directed against GFAP, a marker of PSCs [24] revealed that the *Col1a2* promoter was active in PSCs (Figure 3-1A). Moreover,  $\beta$ -galactosidase expression was also found in islets of the pancreas (Figure 3-1A). These results indicate that these two cell populations express cre recombinase under the control of the *Col1a2* promoter.

To examine the effects of  $\beta 1$  integrin loss in PSCs and islets on pancreas function, mice hemizygous for Col1a2-CRE-ER<sup>T</sup> and homozygous for loxP- $\beta 1$  integrin were generated. The resultant mice were injected with either tamoxifen or corn oil to generate mice deleted or not for  $\beta 1$  integrin in PSCs and islets. To assess whether  $\beta 1$  integrin-deficient mice pancreas showed altered ECM expression, quantitative RT-PCR (qRT-PCR) analysis of collagen type I $\alpha$ 1 (*Col1a1*), collagen type I $\alpha$ 2 (*Col1a2*), fibronectin 1 (*Fn1*), laminin  $\alpha$ 1 (*Lama1*), laminin  $\beta$ 1 (*Lamb1*) and laminin  $\gamma$ 1 (*Lamc1*) mRNA was performed at 1 week post-injection. We found a significant decrease in the expression of *Col1a2* ( $p < 0.001$ ), *Fn1* ( $p < 0.05$ ) and *Lamc1* ( $p < 0.05$ ) mRNA in  $\beta 1$  integrin-deficient mice pancreata when

compared to controls (Figure 3-1B-G). These results indicate that  $\beta 1$  integrin expression in PSCs and islets regulates ECM gene expression.

### 3.3.2 Disturbed acinar cell-cell contacts and focal contact complex observed in the $\beta 1$ integrin-deficient mouse pancreas

Loss of  $\beta 1$  integrin in pancreatic PSCs and islets resulted in decreased ECM expression; therefore, we set to investigate any alterations in pancreatic morphology. Based on hematoxylin and eosin stain, we found that pancreatic acini similarly organized between  $\beta 1$  integrin-deficient and control mice. However, the detachment of acini clusters from one another was observed along with very basophilic stain in the periphery of the acinus in  $\beta 1$  integrin-deficient pancreata (Figure 3-2A). Masson's trichrome (Figure 3-2B; upper panel) and picosirius red (Figure 3-2B; lower panel) staining revealed a reduction in collagen fibers and connective tissue between the acini clusters. Furthermore, analysis of pancreatic fibronectin protein levels in  $\beta 1$  integrin-deficient mice showed a significant decrease compared to controls ( $p < 0.05$ ; Figure 3-2C). Actin cytoskeleton and focal adhesion staining showed more robust cytoskeletal fibers along with more pronounced and continuous vinculin stain in control, but not  $\beta 1$  integrin-deficient, mouse pancreas at 7 weeks post-tamoxifen injection (Figure 3-2D). Likewise, there was less co-localization of F-actin and vinculin suggesting fewer focal adhesions in 7 week post-tamoxifen injected  $\beta 1$  integrin-deficient mice when compared to controls (Figure 3-2D). Finally, anti-laminin immunofluorescence showed a dramatic loss of laminin in 7 week post-tamoxifen injected  $\beta 1$  integrin-deficient mouse pancreas whereas controls show clear laminin at cell-cell contacts (Figure 3-2E).

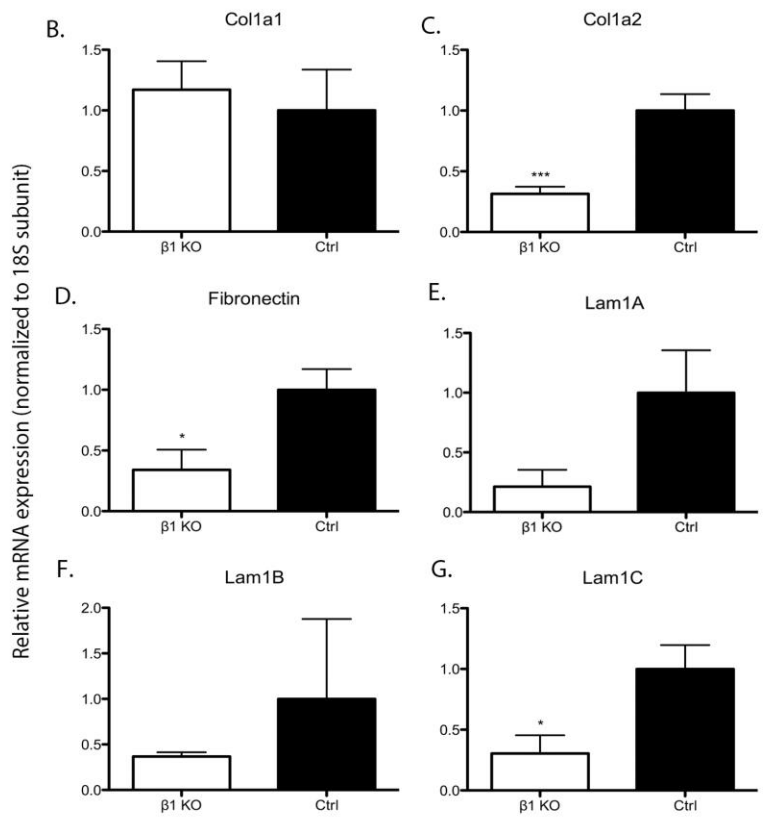
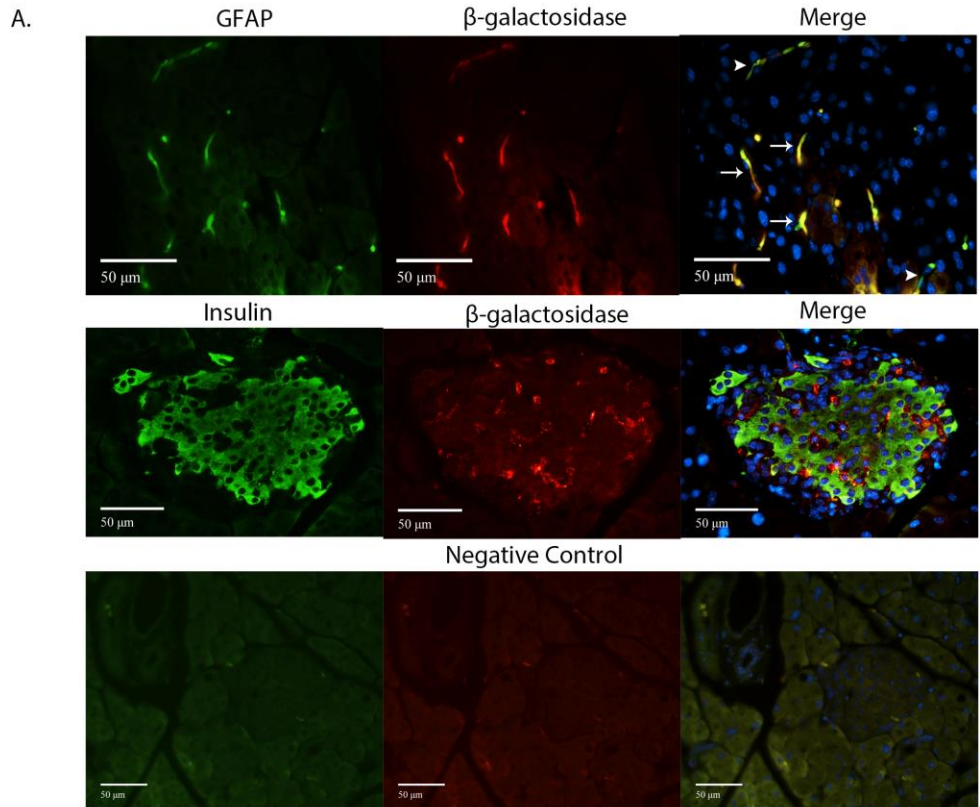


Figure 3-1 Confirmation of  $\beta$ 1 integrin knockout and extracellular matrix protein expression

(A) Representative immunofluorescence images of the pancreas of a transgenic Rosa26loxP-STOP-lacZ mouse crossed with a Col1a2-CRE-ER<sup>T</sup> mouse whereby the  $\beta$ -galactosidase reporter gene is expressed only if cre recombinase is present using anti-GFAP, anti- $\beta$ -galactosidase, and anti-insulin antibodies (arrows indicate colocalization of GFAP and  $\beta$ -galactosidase, while arrowheads show no colocalization). (B) Relative mRNA expression (normalized to 18S subunit) of *Colla1*, *Colla2*, *Fn1*, *Lama1*, *Lamb1*, *Lamc1* of  $\beta$ 1 integrin-deficient and control mouse pancreas at 1 week post-tamoxifen injection ( $n=3-5$ ). Data are expressed as means  $\pm$  SE. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs. controls.

### 3.3.3 Reduced $\beta$ 1 integrin in acinar cells, along with significantly reduced pancreas weight and food intake observed in $\beta$ 1 integrin-deficient mice

Although the  $\beta$ -galactosidase stain showed a direct effect on PSCs and islets, but not acinar cells in the  $\beta$ 1 integrin-deficient mouse pancreas, reduced  $\beta$ 1 integrin expression stain in the acinar cell population was observed in 1 week post-tamoxifen injected  $\beta$ 1 integrin-deficient mice when compared to controls, but not at 3 days (Figure 3-3AB). Protein expression analyses confirm these results, indicating a significant reduction in  $\beta$ 1 integrin-deficient mice at 7 days, but not 3, when compared to controls ( $p < 0.05$ ; Figure 3-3CD). This suggests that loss of  $\beta$ 1 integrin expression in the acinar cells may be secondary to the loss of ECM proteins. Analysis of body and pancreas weight in 4 and 7 week post-tamoxifen injected  $\beta$ 1 integrin-deficient mice showed a significantly reduced ratio of pancreas to body weight at 7 weeks post-injection when compared to control mice ( $p < 0.05$ ; Figure 3-3E). As well, a significant decrease in food intake was observed in 4 week post-tamoxifen injected  $\beta$ 1 integrin-deficient compared to controls ( $p < 0.001$ ; Figure 3-3F).

### 3.3.4 Significantly reduced amylase expression observed in $\beta$ 1 integrin-deficient mice

To assess the defect of  $\beta$ 1 integrin deficiency on exocrine pancreatic products, the expression of amylase mRNA and protein, as well as serum amylase levels were examined. We found that  $\beta$ 1 integrin-deficient mice compared to controls had significant decreases in amylase mRNA expression ( $p < 0.05$ ; Figure 3-4A), with 50% reduction of serum amylase level ( $p < 0.01$ ; Figure 4B) and total amylase protein expression in the pancreas ( $p < 0.05$ ; Figure 3-4D). Furthermore, serum pancreatic lipase activity was significantly reduced in  $\beta$ 1 integrin-deficient mice, but not controls ( $p < 0.05$ ; Figure 3-4C). Protein expression level of CPA and Reg-II was also significantly decreased in  $\beta$ 1 integrin-deficient pancreata when compared to controls at 7 weeks post-injection ( $p < 0.05-0.01$ ; Figure 3-4EF), confirming reduced pancreatic exocrine cell function in  $\beta$ 1 integrin-deficient mice.

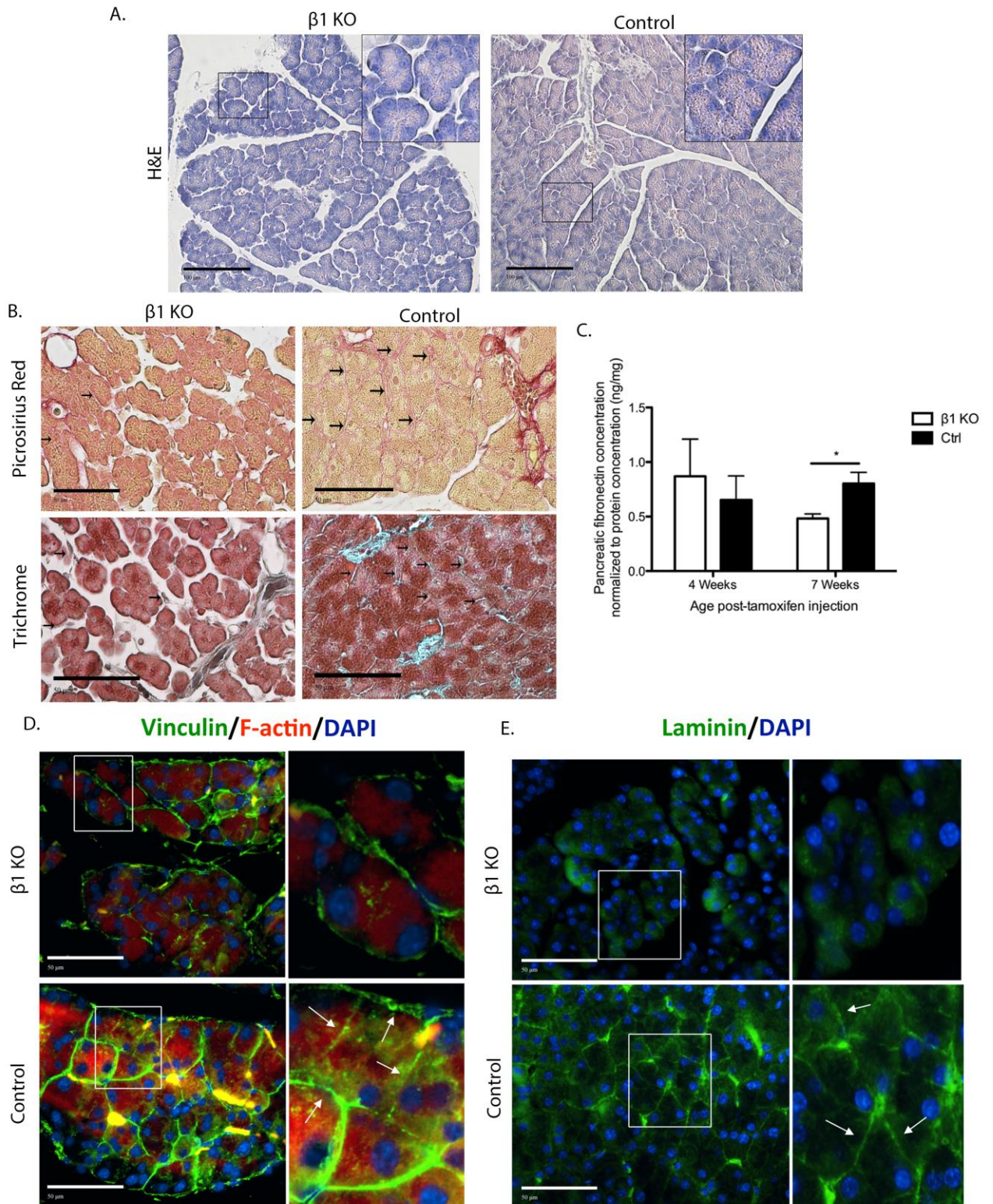




Figure 3-2 Histological analysis of  $\beta 1$  integrin-deficient and control mice

(A) Hematoxylin and eosin, (B) picrosirius red (upper panels), and trichrome (lower panels) staining of  $\beta 1$  integrin-deficient and control mouse pancreas at 7 weeks post-injection. Black arrows indicate positive collagen and connective tissue stain in controls with fewer in  $\beta 1$  integrin-deficient mice. (C) Relative fibronectin concentration between  $\beta 1$  integrin-deficient and control mice at 4 and 7 weeks post-injection ( $n=3-4$ ). Data are expressed as means  $\pm$  SE. \* $p < 0.05$  vs. control. Immunofluorescence was used to investigate focal adhesions (D) and laminin expression (E). Focal contacts are defined by vinculin (green) and actin filaments by TRITC-conjugated phalloidin (red). Nuclei are counterstained by DAPI (blue). Boxed regions display zoom, while arrows display cell-cell contacts in focal adhesion and laminin immunofluorescence in control but not  $\beta 1$  integrin-deficient mouse pancreas. Scale bar = 100 $\mu$ m (A) and 50 $\mu$ m (B, D and E).



### 3.3.5 Reduced $\beta 1$ integrin in acinar cells led to significantly increased cell apoptosis, which was partially rescued by culturing acinar cell clusters with ECM proteins

To analyze the proliferative and apoptotic status of  $\beta 1$  integrin-deficient mouse pancreas, Ki67 immunofluorescence staining and TUNEL was conducted. The percent of Ki67<sup>+</sup> proliferating acinar cells was significantly decreased  $\beta 1$  integrin-deficient at 7 weeks post-tamoxifen injection when compared to control mice ( $p < 0.05$ ; Figure 3-5A). Furthermore, a significant increase in acinar cells undergoing apoptosis was observed in  $\beta 1$  integrin-deficient mice ( $p < 0.001$ ; Figure 3-5B).

Since the primary  $\beta 1$  integrin defect is in PSCs and islets, we hypothesized that the pancreatic exocrine dysfunction is due to problematic ECM stimulation of acini. To investigate this, we isolated acinar cell clusters from control and  $\beta 1$  integrin-deficient pancreata and cultured them with or without FBS that contains rich ECM proteins [19, 20].  $\beta 1$  integrin-deficient acinar cell clusters cultured with BSA showed significantly increased cell apoptosis when compared to the control cell clusters ( $p < 0.05$ ; Figure 3-5C). However, the number of TUNEL-positive cells in  $\beta 1$  integrin-deficient acinar cell clusters was reduced and reached control levels when cultured with FBS medium ( $p < 0.05$ ; Figure 3-5C).

## 3.4 Discussion

The present study analyzed the effects of  $\beta 1$  integrin deficiency on pancreatic exocrine tissue. Our results demonstrate that  $\beta 1$  integrin deficiency under control of the collagen I promoter directly effected PSCs and islets, and this lead to significantly reduced ECM protein production in the pancreas. PSCs support parenchyma by secreting ECM components that integrins use as ligands. Indeed, PSCs in human pancreatic acini stain positive for collagen I, III and IV, laminin, fibronectin, and other ECM proteins [7]. Furthermore, there is a necessity of  $\alpha 5\beta 1$  integrin in connective tissue growth factor stimulation of PSC collagen I synthesis [25]. It has also been reported that PSC secretion of growth factors and ECM components are, in part, responsible for fibrosis in chronic

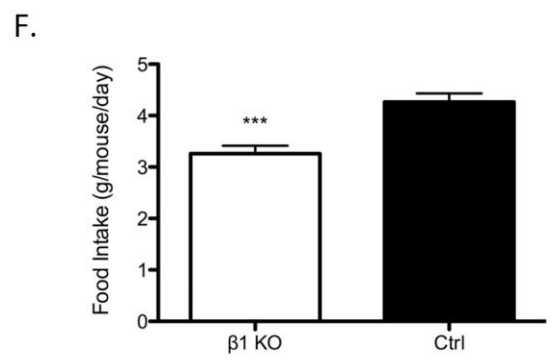
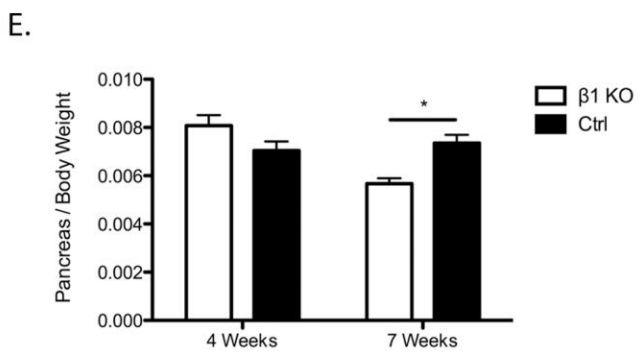
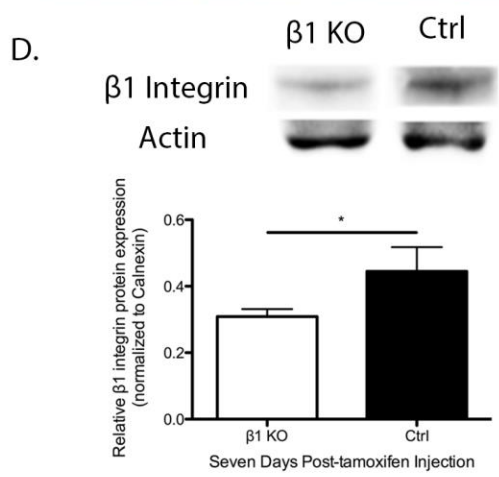
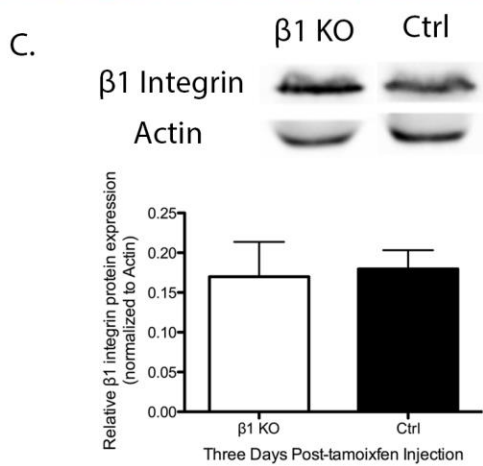
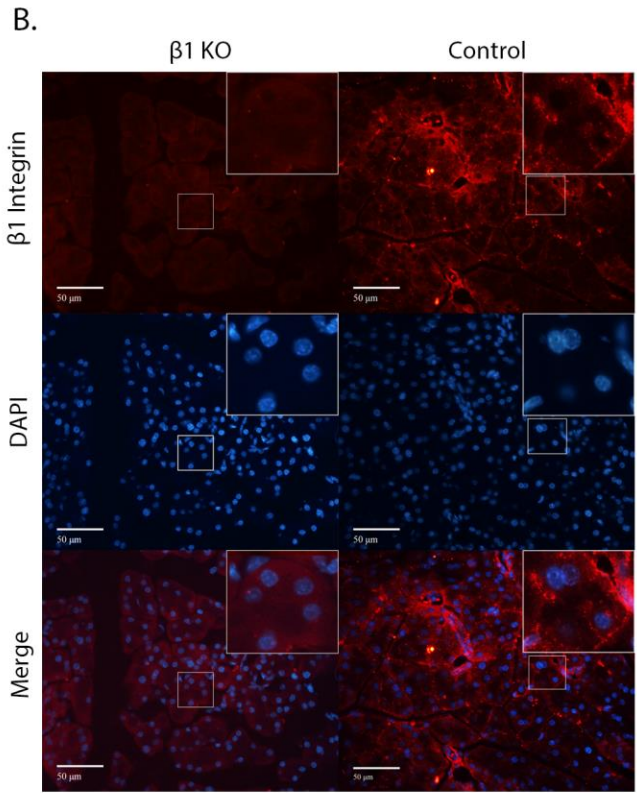
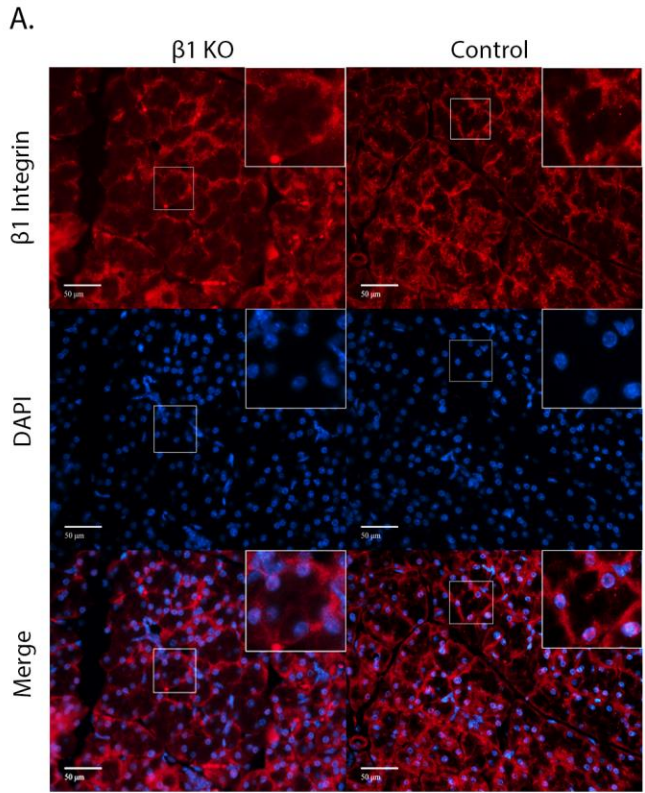


Figure 3-3  $\beta 1$  integrin expression is reduced in acinar cells

Representative immunofluorescence images of  $\beta 1$  integrin-deficient and control mouse pancreas at 3 (A) and 7 days (B) post-tamoxifen injection using an anti- $\beta 1$  integrin antibody. Western blot analyses of  $\beta 1$  integrin-deficient and control mouse  $\beta 1$  integrin expression at 3 (C) and 7 days (D) post-tamoxifen injection ( $n=4$ ). (E) Pancreas to body weight ratio in  $\beta 1$  integrin-deficient and control mice at 4 and 7 weeks post-injection ( $n=7-17$ ). (F) Food intake data from 4 week post-tamoxifen injected  $\beta 1$  integrin-deficient mice with respective controls ( $n=7-8$ ). Data are expressed as means  $\pm$  SE. \* $p < 0.05$ ; \*\*\* $p < 0.001$  vs. controls. Scale bar =  $50\mu\text{m}$ .

pancreatitis [8, 9]. Our results support previous research, in that  $\beta 1$  integrin is required to maintain PSC expression of certain ECM proteins, including *Colla2*, *Fn1* and *Lamc1*.

Our observation of reduced pancreatic ECM mRNA was concomitant with disrupted cell-cell contacts between acini along with reduced connective tissue and collagen fibers, laminin immunoreactivity and acinar cell expression of  $\beta 1$  integrin. It is unclear precisely which process preceded the other, but studies have demonstrated that  $\beta 1$  integrin loss lead to improper basement membrane assembly and laminin expression in teratoma [12] and embryoid bodies [11], implicating  $\beta 1$  integrin as an essential part of ECM maintenance. It has also been demonstrated that ECM proteins can regulate integrin expression in human fibroblasts [26]. Ablation of  $\beta 1$  integrin by inactivating monoclonal antibody treatment disrupted cell-cell contacts of keratinocytes [27]. Likewise, mouse mammary gland alveoli deficient of  $\beta 1$  integrin could not attach to the basement membrane laminin substratum [28]. This study also showed decreased focal adhesion contacts in  $\beta 1$  integrin-deficient mammary epithelium when compared to controls [27]. Taken together, the findings of this study demonstrate that reduced  $\beta 1$  integrin in PSCs had a direct effect on ECM expression, which in turn, effected acinar cell  $\beta 1$  integrin expression, cell-cell interactions, and subsequently cell proliferation and death.

$\beta 1$  integrin has been well-established as upstream of the mitogen-activated protein kinase (MAPK) pathway, which has profound effects on cell survival and proliferation [16, 23]. Our previous analysis of  $\beta 1$  integrin-deficient mice demonstrated a significant reduction in FAK and ERK1/2 phosphorylation, indicating reduced MAPK signaling [16]. The present results corroborate these studies by demonstrating a reduction in pancreatic  $\beta 1$  integrin, along with significantly decreased proliferation and increased apoptosis. To elucidate if the observed effects of pancreatic exocrine dysfunction were due to  $\beta 1$  integrin or ECM protein deficiency, isolated exocrine cell clusters from  $\beta 1$  integrin-deficient and control mouse pancreas were cultured in ECM-enriched medium. Our data indicate that apoptosis was significantly reduced in  $\beta 1$  integrin-deficient cell clusters when cultured in media with FBS, suggesting that supplementing ECM proteins to  $\beta 1$  integrin-deficient cell clusters can improve their survival status. This strengthens our working model, whereby pancreatic

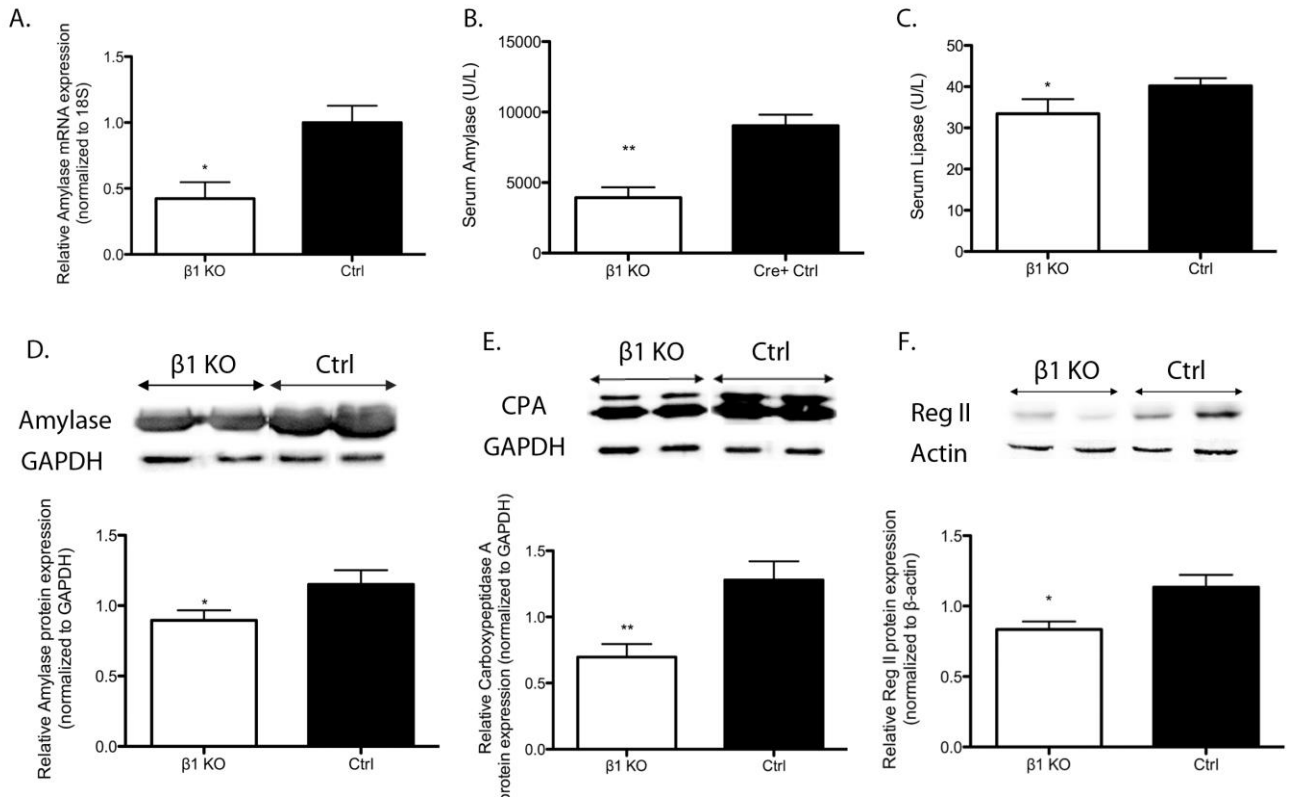


Figure 3-4 Exocrine protein expression in  $\beta 1$  integrin-deficient and control mice

(A) qRT-PCR analysis of amylase mRNA expression in 1 week post-injected  $\beta 1$  integrin-deficient and control mouse pancreas ( $n=3$ ). Analysis of serum (B) and pancreatic protein (D) amylase and serum pancreatic lipase (C) in 7 week post-injected  $\beta 1$  integrin-deficient and control mice ( $n=3-10$ ). Relative carboxypeptidase-A (D) and Reg-II (E) protein expression in the pancreas of 7 week post-injected  $\beta 1$  integrin-deficient and control mice pancreas ( $n=4$ ). Data are expressed as means  $\pm$  SE. \* $p < 0.05$ , \*\* $p < 0.01$  vs. controls.

exocrine defects associated with  $\beta 1$  integrin deficiency may be largely due to insufficient ECM proteins.

It was noted that food intake was significantly reduced in  $\beta 1$  integrin-deficient at 4 weeks post-tamoxifen injection along with a significant decrease in body weight [16]. Many studies have established a positive correlation between food consumption and weight gain [29, 30]. Food intake is controlled by a brain-gut axis associated with multiple factors including pancreatic enzyme secretion [31-33]. Our data demonstrated a significant reduction in pancreatic lipase, amylase and CPA expression, major enzymes produced by the exocrine pancreas, in  $\beta 1$  integrin-deficient mice when compared to controls, suggesting that the pancreatic insufficiency occurred due to the loss ECM proteins and  $\beta 1$  integrin in the pancreas. The pancreas of  $\beta 1$  integrin-deficient mice became significantly underweight at 7 weeks post-tamoxifen injection [16], which was more drastic than the drop in body weight as shown by the ratio of pancreas to body weight. This ratio was significantly decreased in  $\beta 1$  integrin-deficient mice when compared to controls further indicating a severe pancreatic defect. Meanwhile, other research has shown that reduction in food intake decreases serum [32] and pancreatic [33] amylase, which may be related to lower insulin levels. These studies are in line with our research, whereby  $\beta 1$  integrin-deficient mice display reduced pancreatic insulin along with reduced serum amylase activity and pancreatic amylase protein and mRNA expression [16]. Therefore, the lack of pancreatic ECM proteins or  $\beta 1$  integrin affected pancreatic enzyme levels, which may have negatively affected food intake, and thus body weight.

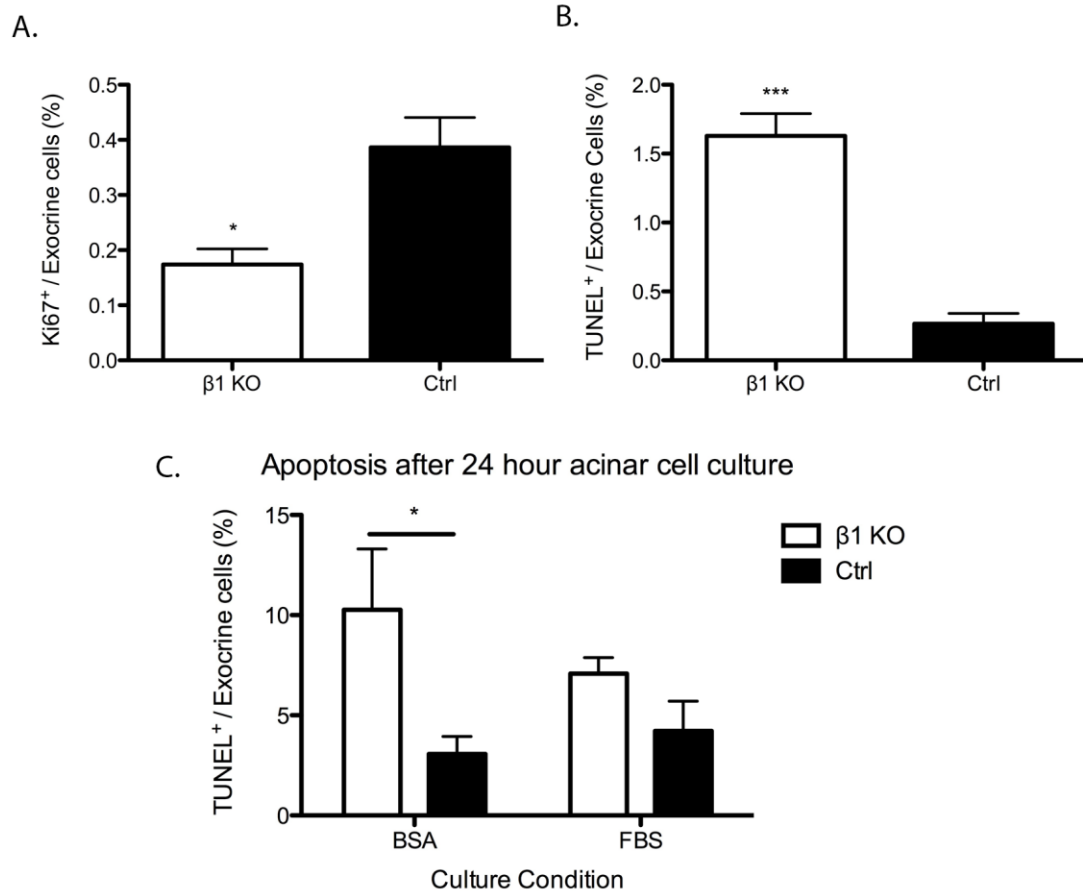


Figure 3-5 Cell proliferation and death is altered in  $\beta 1$  integrin-deficient mice

Quantitative analysis of cell proliferation (A) and apoptosis (B) in the exocrine pancreas of  $\beta 1$  integrin-deficient and control mice at 7 weeks post-injection. (C) Apoptosis in exocrine cell clusters cultured in either BSA or FBS for 24 hours. The percentage of Ki67 or TUNEL positive cells over total number of cells counted was calculated and data are expressed as means  $\pm$  SE ( $n=4$ ). \* $p < 0.05$ , \*\*\* $p < 0.001$  vs. controls.

In summary,  $\beta 1$  integrin-deficient mice showed significant pancreatic exocrine dysfunction compared to controls. The decrease in pancreas to body weight ratio, essential pancreatic digestive enzymes and ECM matrix expression all suggest that  $\beta 1$  integrin and ECM proteins have an important role in maintaining pancreatic function and differentiation. Furthermore, we observed increased cell death and reduced cell proliferation, indicating disturbed cell survival/apoptosis homeostasis. A recovery in apoptosis was observed after  $\beta 1$  integrin-deficient cell clusters were provided ECM proteins. Taken together, these results implicate  $\beta 1$  integrin as an essential component to maintain ECM expression along with exocrine pancreatic structure and function.



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## Chapter 4

### 4 Fibrin improves beta (INS-1) cell function, proliferation and survival through integrin $\alpha\beta3$ <sup>4</sup>

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<sup>4</sup> This work has been published in the following manuscript:

Riopel M, Stuart W, Wang R. Fibrin improves beta (INS-1) cell function, proliferation and survival through integrin  $\alpha\beta3$ . *Acta Biomater* 2013;9(9):8140-8148

## 4.1 Introduction

Diabetes Mellitus is a debilitating disease of glycemic dysfunction with increasing prevalence. Islet transplantation is a viable means to cure diabetes, however, donor shortage is a significant limitation due to the low yearly number of available donor pancreata [1]. As well, the current islet transplant regimen requires two donor pancreases to reverse diabetes [2]. To overcome such an obstacle, researchers have looked towards using other types of donors [3], generating beta cells from different cell types (either fully differentiated cells [4], or beta cell precursors [5]), or methods to maintain existing beta cell proliferation and function [6].

Of critical importance to islets and beta cells is the maintenance of ECM-integrin interactions. Integrins are a group of cell adhesion receptors that have profound influences on beta cell survival [7], differentiation [8] and function [9]. Upon engagement by ECM proteins, including fibronectins, collagens, and/or laminins, integrins activate and mediate intracellular signaling cascades [10]. Isolated islets cultured with an RDG peptide, type IV collagen or laminin significantly reduced cell death [11]. As well, canine islets cultured with type I collagen improved GSIS and islet insulin content while increasing cell survival [7]. Likewise, laminins are as an essential part of the islet basement membrane that mediates islet  $\beta 1$  integrin activity [12]. Culture of facultative liver stem cells (hepatic oval cells) with laminin induced the expression of Pdx-1, Pax-6, insulin and glucagon, indicating that laminin may influence islet cell differentiation [13]. Antibody inhibition of  $\beta 1$  integrin and laminin-5 interactions led to a significant reduction in beta-cell spreading and GSIS when compared to controls [14]. Fibronectin contains RGD domains that are preferentially bound by a large group of integrins. When immortalized rat insulinoma cells (INS-1) were cultured on fibronectin and RGD peptides, GSIS was significantly enhanced compared to controls [15]. While each of these ECM proteins is a viable means to improve short-term islet health in vitro, they are unable to maintain this benefit for long-term, nor can they easily form a 3D scaffold for in vivo transplantation [16].

Fibrin is a provisional ECM protein essential for clotting in mammals. Originally from the liver, fibrinogen is cleaved to fibrin by thrombin and undergoes polymerization (reviewed

in [17]). This can be taken advantage of, in vitro, to form gels that are biocompatible [18], biodegradable [19], able to support 3D scaffolds [20] and promote angiogenesis [21]. Fibrin also contains RGD amino acid motifs that are essential for binding integrin receptors such as  $\alpha v\beta 3$  [22]. As well, fibrin can enhance adult human islet function and survival [23] and subcutaneous transplantation of fibrin-cultured islets in diabetic mice significantly enhanced glucose tolerance [24]. However, the mechanism behind fibrin's ability to promote beta cell health is unclear. The current research analyzed the necessity of integrins involved in promoting beta cell function, proliferation and survival under fibrin culture.

## 4.2 Materials and methods

### 4.2.1 INS-1 cell culture and fibrin gel preparation

One million INS-1 (832/13, a gift from Dr. Christopher Newgard, Duke University Medical Center, USA) cells per well were cultured in RPMI-1640 containing 10% fetal bovine serum (Invitrogen, Burlington, ON, CAN) [25]. A fibrinogen solution (1.8mg/ml) was mixed with thrombin (Sigma, St Louis, MO, USA), to initiate polymerization. This resultant solution was then quickly administered to wells of a 24-well plate. INS-1 cells plus medium were then added on top of fibrin gel for the 2D experimental group, while the 3D fibrin-cultured group had INS-1 cells added to the fibrinogen solution pre-polymerization. Three INS-1 cell experimental groups, tissue culture polystyrene (TCPS), 2D fibrin and 3D fibrin, were cultured for 1, 2 and 4 weeks, then samples were collected for analyses.

### 4.2.2 Glucose-stimulated and basal insulin secretion

Overnight media samples were collected from duplicates of INS-1 cells from each experimental group for basal insulin secretion levels. Meanwhile, duplicates of INS-1 cells from each experimental group were incubated with RPMI1640 culture media with 1% bovine serum albumin containing either 2.2 mmol/l or 22 mmol/l glucose for 2 hours to analyze GSIS. All collected samples were analyzed by a highly sensitive rat insulin ELISA

(Alpco, Salem, NH, USA). A static GSIS index was calculated [26], while basal insulin secretion was expressed as nanogram per ml.

#### 4.2.3 Scanning electron microscopy

Fibrin-cultured INS-1 cells were fixed with 2.5% glutaraldehyde at 4°C overnight, then dehydrated with an increasing concentration gradient of ethanol. Three-dimensional fibrin samples were cut with scissors to expose cells. Samples were subjected to critical point drying and gold coating, and then visualized by a Hitachi 3400-N Variable Pressure Scanning Electron Microscope.

#### 4.2.4 Integrin $\alpha\beta 3$ blockade using an immunoneutralizing antibody

INS-1 cells were preincubated with either mouse anti- $\alpha\beta 3$  integrin antibody or IgG isotype-matched negative control (10 $\mu$ g/ml; Abcam, Cambridge, MA, USA) for 1 hour then plated on 2D fibrin gels for 24 hours. Cells were then photographed and harvested for analyses.

#### 4.2.5 RNA extraction and real-time RT-PCR

Total RNA was extracted from 1 week fibrin-cultured INS-1 cells and controls using the miRNeasy kit (QIAGEN, Germantown, MD, USA). Briefly, samples were washed then added to a volume of QIAzol lysis reagent. After vortexing, an equal volume of ethanol was added, and samples were spun through the RNeasy Mini Spin Column. After a series of washes, RNA on the column was eluted using hot elution buffer.

For each reverse transcription reaction, 2  $\mu$ g of DNA-free RNA was used with random hexamers/oligo-deoxythymidine primers and Superscript reverse transcriptase (Invitrogen). Sequences of PCR primers used for RT-PCR with expected product size are listed in Appendix 5. A master mix of primers, water and SYBR green was made and then added to separate tubes with each cDNA sample. Real-time RT-PCR analyses were conducted using the CFX manager software (Bio-Rad Laboratories, Mississauga, ON, Canada) and CFX Connect Real-Time System (Bio-Rad Laboratories). Data were



normalized to levels of 18S rRNA subunit and relative gene expression was calculated based on the  $2^{-\Delta\Delta CT}$  method as PCR signals from 2D and 3D fibrin-cultured INS-1 cells normalized to TCPS controls.

#### 4.2.6 Immunofluorescence and TUNEL assay

INS-1 cells at the end of the culture period were collected and fixed in 4% paraformaldehyde overnight, then agarose embedding and wax processing was undertaken. The sections (4  $\mu\text{m}$  thick) were prepared and stained with appropriate dilutions of primary antibodies listed in Appendix 4. Fluorescent secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Percent co-localization of Pdx-1 or Ki67 with insulin was calculated by double immunofluorescence staining, and then cell counting of Pdx-1 or Ki67 positivity in insulin positive cells.

The TUNEL assay was performed to examine cells undergoing apoptosis [28]. INS-1 cell sections were incubated with the TUNEL reaction mixture conjugated with fluorescein-dUTP (Roche, Montreal, QC, Canada) and anti-insulin antibodies (Zymed). The percent cell apoptosis was calculated by counting TUNEL-labeled cells in insulin-positive cells.

#### 4.2.7 Protein extraction and western blot analysis

Fibrin-cultured and control INS-1 cell protein was extracted in Nonidet-P40 lysis buffer. Equal amounts (15  $\mu\text{g}$ ) of lysate protein from each experimental group was separated by either 7.5, 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Mississauga, ON, Canada). Membranes were incubated with appropriate dilutions of primary antibodies listed in Appendix 4. The application of appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) then followed. Proteins were detected using ECL<sup>TM</sup>-Plus Western blot detection reagents (Perkin Elmer, Wellesley, MA, USA) and exposed using the Versadoc Imaging System (Bio-Rad Laboratories). Densitometric quantification of bands at subsaturation levels was performed using Quantity One software (Bio-Rad Laboratories) and normalized to appropriate

loading controls. Data are expressed as the relative expression level of phosphorylated proteins to total protein levels or protein levels to the loading control [29].

#### 4.2.8 Statistical analysis

Data are expressed as means  $\pm$  SE using at least 3 to 4 different cell passages per experimental group, representing  $n = 3-4$ . Statistical significance was determined using either the unpaired student's *t*-test or one-way ANOVA followed by post hoc least significant differences and Bonferroni comparison test. Differences were considered statistically significant when  $p < 0.05$ .

### 4.3 Results

#### 4.3.1 INS-1 cells form islet-like clusters and interact with fibrin when cultured with fibrin gels

INS-1 cells were cultured on TCPS, 2D and 3D fibrin for periods of 1, 2, and 4 weeks. After 1 week, INS-1 cells began to form small clusters on 2D and in 3D fibrin gels and expanded significantly at 2 weeks of culture, while cells cultured on TCPS grew as a monolayer (Fig. 4-1A). After 4 weeks, robust islet-like clusters in fibrin-cultured INS-1 cells were often observed (Fig. 4-1A). It was noted that both TCPS and 2D fibrin gel-cultured INS-1 cells had more dark spots associated with the clusters compared to 3D fibrin after 4 week of the culture (Fig. 4-1A).

Scanning electron microscopy revealed contacts between fibrin gels and INS-1 cells in both 2D and 3D cultures after 1 week (Fig. 4-1B). Cells cultured in 3D fibrin were enveloped in fibrin fibers and showed frequent contacts between the cells and fibrin; however, cells on 2D fibrin were only connected to fibrin at the basal surface (Fig. 4-1B, white arrows).

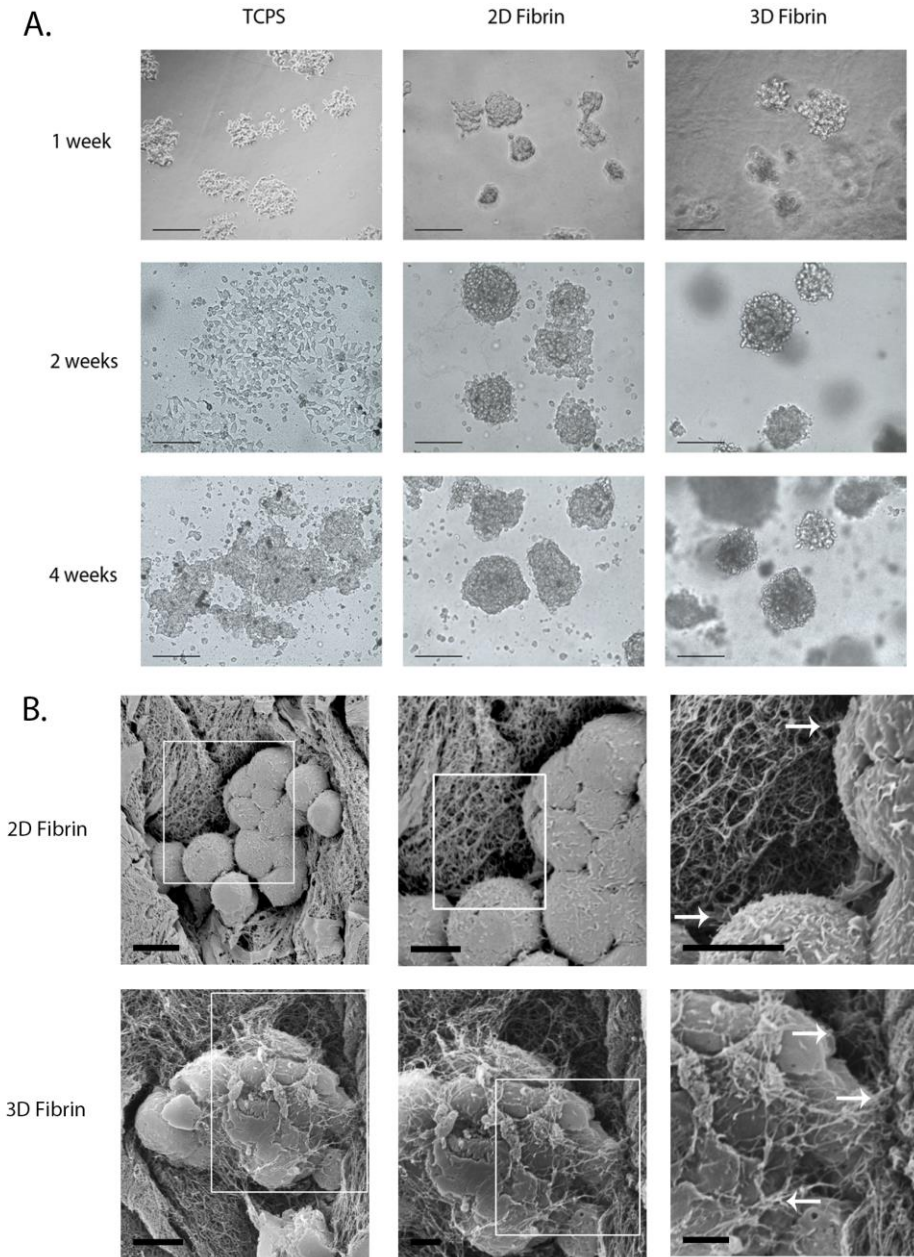


Figure 4-1 Fibrin promoted islet-like cluster formation.

(A) INS-1 cells cultured on TCPS, on 2D and in 3D fibrin for 1, 2 and 4 weeks. Scale bars: 100 $\mu$ m (B) Scanning electron micrographs of fibrin-cultured INS-1 cells. White arrows indicate examples of areas of fibrin-cell contacts. Scale bars: 5 $\mu$ m, 2 $\mu$ m, 2 $\mu$ m for 2D fibrin, 3 $\mu$ m, 1 $\mu$ m and 1 $\mu$ m for 3D fibrin.

### 4.3.2 Significantly improved insulin secretion function observed in INS-1 cells when cultured with fibrin

Basal insulin secretion of INS-1 cells was significantly increased in 2D fibrin after 4 weeks of culture, with a relative increase in 3D fibrin culture (Fig. 4-2A). A significant increase in GSIS was observed in 2D and 3D fibrin-cultured cells 2 weeks (Fig. 4-2B). Furthermore, GSIS of 3D fibrin-cultured INS-1 cells was 4-fold higher compared of TCPS controls after 4 weeks (Fig. 4-2B). While there was a similar effect in the 2D fibrin group, statistical significance was not attained when compared to TCPS culture (Fig. 4-2B).

INS-1 cells also maintained their differentiated state when cultured in fibrin as opposed to TCPS controls. Real-time RT-PCR analyses of important beta cell genes indicated a significant increase in the *Ins1* gene after 1 week of 3D-fibrin culture (Fig. 4-2C). The protein level of Pdx-1 was significantly higher in INS-1 cells cultured with fibrin for 4 weeks, but not 1 week (Fig. 4-2D). This increase was associated with higher co-localization of Pdx-1 and Insulin in INS-1 cells cultured with fibrin at 1 and 4 weeks compared to controls (Fig. 4-2E).

### 4.3.3 Integrin $\alpha_v$ and $\beta_3$ expression plus intracellular pathway signaling was significantly increased in fibrin-cultured INS-1 cells

Since fibrin contains RGD amino acid motifs, integrins receptors that preferentially bind to RGD domains such as  $\alpha_v$  associated with  $\beta_1$ ,  $\beta_3$  and  $\beta_5$ , as well as  $\beta_1$  associated with  $\alpha_3$ ,  $\alpha_5$  and  $\alpha_8$  were examined. Real-time RT-PCR analysis of integrin mRNA showed a relative increase in a number of integrin receptors in fibrin-cultured INS-1 cells after 1 week, yet statistical significance was not reached (Fig. 4-3A). Fibrin-cultured INS-1 cells showed significantly higher protein levels of integrins  $\alpha_v$  and  $\beta_3$ , at 1 and 4 weeks of culture when compared to TCPS controls (Fig. 4-3B). However, protein expression of integrin  $\alpha_3$  and  $\beta_1$  was relatively unchanged (Fig. 4-3C). The increases in integrin protein expression in fibrin-cultured INS-1 cells was associated with a significant increase in

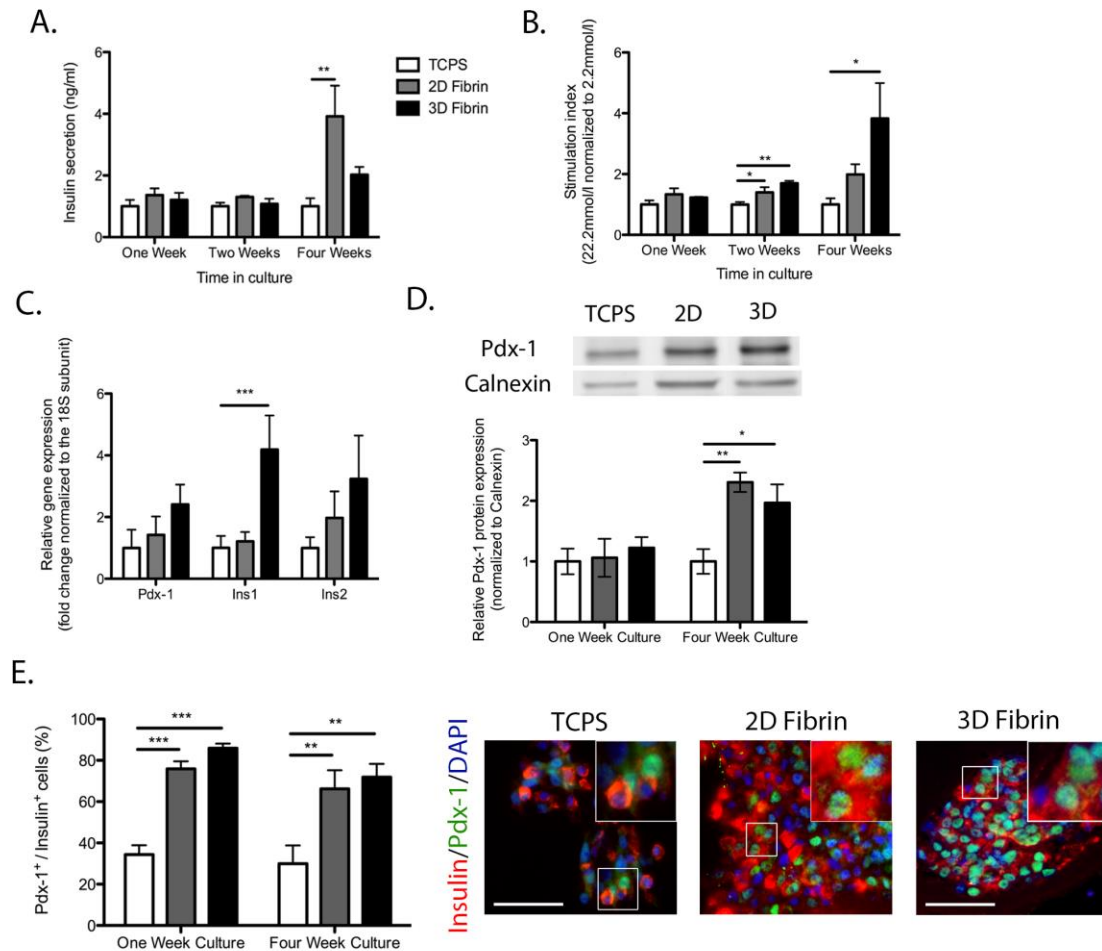


Figure 4-2 Fibrin improved INS-1 cell function and increased Pdx-1 expression.

(A) Basal insulin secretion of INS-1 cells cultured with fibrin or on TCPS. (B) INS-1 cell function as measured by glucose-stimulated insulin secretion in fibrin-culture of up to 4 weeks. (C) Relative mRNA expression of important beta cell genes in INS-1 cells cultured with fibrin or on TCPS for 1 week. Western blot analysis (D) and the percent of Pdx-1/insulin positive cells labeled by double immunofluorescence stain of Pdx-1 (green) with insulin (red) (E) in fibrin or on TCPS cultured INS-1 cells. Magnified images for each corresponding image are shown in the insert. Data are expressed at means  $\pm$  SE (n=3-5). Representative blots are shown. Arrows indicate double positive cells and scale bar: 50 $\mu$ m. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 vs. TCPS control.

phosphorylated focal adhesion kinase (FAK), extracellular-signal regulated kinase 1/2 (Erk1/2) and Akt activity at 4 weeks compared to controls (Fig. 4-3DEF).

#### 4.3.4 Significantly improved cell proliferation and decreased cell death observed in INS-1 cells when cultured with fibrin

INS-1 cells cultured with fibrin at 1 and 4 weeks showed a significant increase in beta cell proliferation as labeled by Ki67 and insulin (Fig. 4-4A). As well, cyclin D1 was significantly increased in fibrin-cultured INS-1 cells at 4 weeks of culture (Fig. 4-4B). Cleaved caspase 3 level was significantly reduced in both 2D and 3D fibrin-cultured INS-1 cells at 1 and 4 weeks of culture (Fig. 4-4C), and this was associated with a decrease in the number of insulin positive cells labeled by TUNEL (Fig. 4-4D).

#### 4.3.5 Blockade of integrin $\alpha\beta3$ increased cell death observed in INS-1 cells when plated on 2D fibrin

To understand the necessity of integrin  $\alpha\beta3$  and fibrin interactions for cell survival, INS-1 cells were treated with an anti-integrin  $\alpha\beta3$  blocking antibody then plated on 2D fibrin. After 24 hours, anti- $\alpha\beta3$  treated INS-1 cells displayed a decrease in spreading on the fibrin gel (Fig. 4-5A). Activated FAK was downregulated by about 50% (Fig. 4-5B) but there were no changes in Erk1/2 and Akt phosphorylation, cyclin D1 or Pdx-1 expression (data not shown). There was, however, a significant increase in cleaved caspase-3 compared to both controls (Fig. 4-5C).

## 4.4 Discussion

This study investigated how fibrin can promote beneficial effects in beta cells. Long-term fibrin culture of INS-1 cells led to improved insulin secretion in response glucose stimulation and increased Pdx-1 protein expression compared to controls. Fibrin-cultured INS-1 cells also showed significantly improved cell proliferation and reduced cell death. This was associated with increased integrin  $\alpha\beta3$  expression and activity of downstream signal transduction pathways (FAK, Erk 1/2 and Akt). Finally, blockade of integrin  $\alpha\beta3$  led to significant decreases in activated FAK along with significantly increased caspase-3

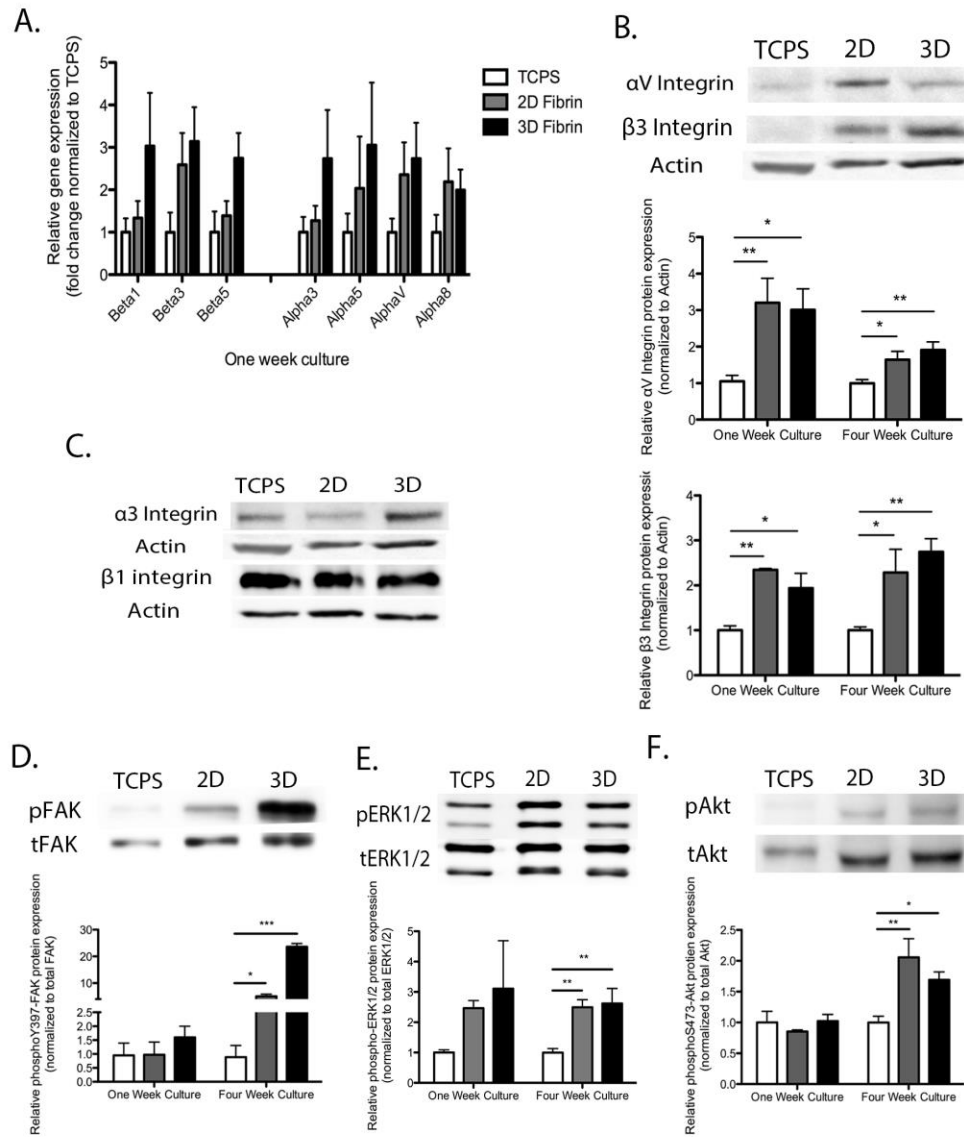


Figure 4-3 Fibrin induced integrin  $\alpha v \beta 3$  expression.

(A) Relative mRNA expression of integrin receptors in INS-1 cells cultured for 1 week with fibrin or on TCPS. Western blot analysis of integrins  $\alpha v$  and  $\beta 3$  (B),  $\alpha 3$  and  $\beta 1$  (C) and phosphorylated FAK (D), Erk1/2 (E) and Akt (F) in INS-1 cells cultured with fibrin or on TCPS for 1 and 4 weeks. Data are expressed at means  $\pm$  SE (n=3-5). Representative blots are shown. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 vs. TCPS control. p: phosphorylated; t: total.

cleavage in fibrin-cultured INS-1 cells. Fibrin culture of INS-1 cells led to significantly increased insulin secretion in response to glucose challenge at 2 and 4 weeks of culture. Our study is unique from other research that showed a significant improvement in GSIS after 7 days [23]. However, that study used adult human islets, and also cultured them with hepatocyte growth factor, which may have assisted beta cells to function optimally while cultured with fibrin [23]. Another study cultured human pancreatic islets in 3D gels supplemented or not with ECM components for 10 days and observed improvements to islet gene expression [30]. Furthermore, when INS-1 cells were cultured with fibronectin or RGD peptides for 7 days, there was an improvement in cell growth and GSIS [15]. We did not observe significant changes in INS-1 cell function after one week culture on either 2D or 3D when compared to TCPS culture, but phospho-FAK and ERK signaling molecules were slightly increased. These results suggest that: (i) fibrin may not directly influence beta cells, but rather maintains their functional status during long-term culture better than TCPS culture and/or (ii) INS-1 cells cultured on TCPS do not require ECM support for short-term culture due to its cancer cell phenotype. We also observed that 2D fibrin-cultured INS-1 cells had significantly higher basal insulin secretion, as opposed to GSIS, in which INS-1 cells cultured in 3D fibrin had the greatest increase. A major difference between 2D and 3D cultured cells is their morphology and polarization. Cells grown in 2D are flat, proliferate into a monolayer and are forced into an apical-basal polarity, while 3D-cultured cells form stellate-like clusters [31]. These changes in cell shape and polarity are able to modulate cellular activity, including protein synthesis and function [32]. There have also been reports that a 3D ECM environment can sequester and limit the transport of soluble factors [33]. In this manner, it is possible that under basal conditions, insulin is either held within fibrin, or that the 3D cell morphology is not conducive to insulin release without stimuli. However, under high glucose conditions, 3D fibrin-cultured INS-1 cells are able to overcome the barrier associated with fibrin and release insulin in a significant manner.

In the short-term, we observed increases in integrin expression with slight increased phospho-FAK and ERK1/2; however, significant differences in signaling pathway activation were detected during long-term 4 week culture. This lag may be due to the



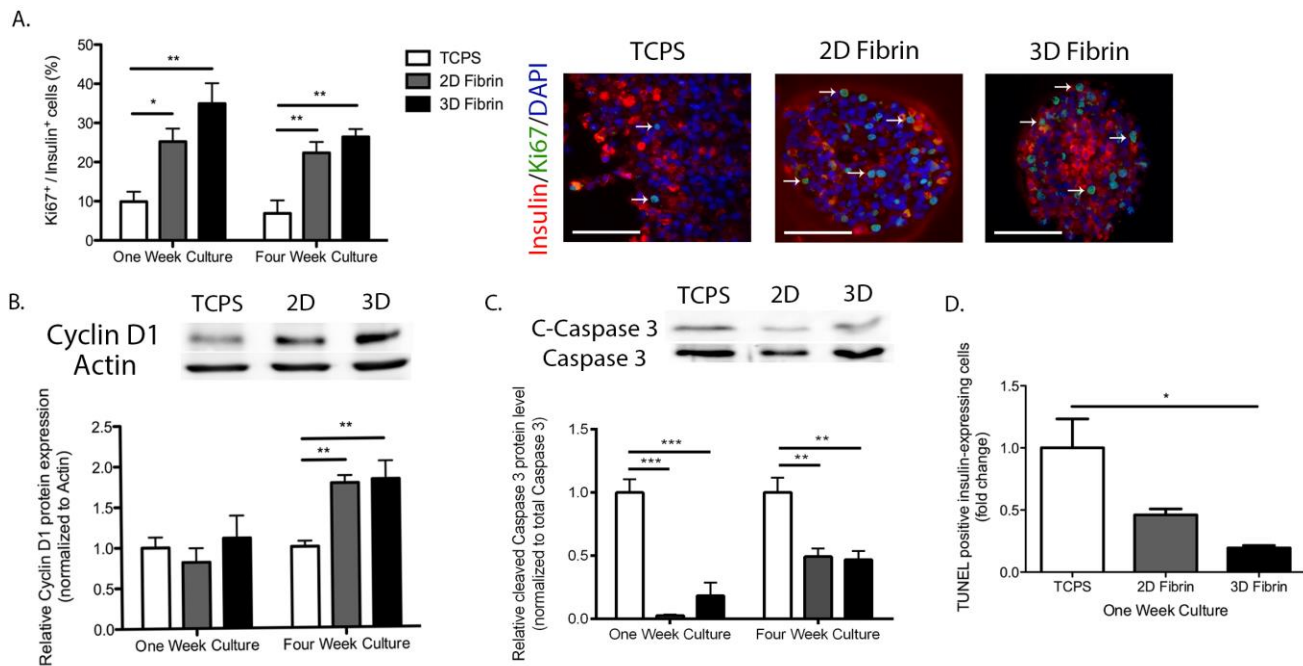


Figure 4-4 Fibrin improved cell proliferation and survival

The percent of Ki67/insulin positive cells determined by double immunofluorescence staining for Ki67 (green) and insulin (red) (A), western blot analysis of cyclin D1 (B) and cleaved caspase-3 (C) in INS-1 cells cultured with fibrin or on TCPS for 1 and 4 weeks. (D) The percent of beta cell death labeled by TUNEL (green) with insulin (red) staining in INS-1 cells cultured with fibrin or on TCPS for 1 week. Arrows indicate proliferating beta cells, scale bar: 50µm. Data are expressed at means  $\pm$  SE (n=3-4). Representative blots are shown. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 vs. TCPS control.

intricate nature of integrins, which have their activation dictated by the talin protein [34]. As well, there could be a threshold of integrin receptors required before a substantial signal can be transduced to downstream protein kinases, which we observed after long-term fibrin culture. Finally, it is also possible that after 4 weeks of culture, maintenance of integrin receptor expression is greatly diminished in TCPS-cultured INS-1 cells. This may have been prevented in fibrin culture, which allowed cells to thrive functionally and maintain expression of Pdx-1, cyclin D1 and phosphorylated signaling members.

In our study, improvements in beta cell function were associated with increased integrin expression, signaling pathway activity, cell proliferation and reduced cell death after 4 weeks of culture. INS-1 cells cultured on TCPS form a monolayer after 4 weeks, as opposed to fibrin, which stimulates an islet-like clustering. This clustering itself may have been a factor that enhanced function [35]. One group has reported successful islet culture for up to 6 months in a specialized 3D suspension serum-free media [36] and demonstrated reversal of diabetes when post-cultured islets were transplanted into streptozotocin-treated mice [37]. These cultures are without ECM supplementation and it is unknown how this media functions to improve islet cell viability; however, culture in 3D suspension has been demonstrated to improve islet viability [38]. Integrin receptors besides  $\alpha\beta3$  may have been involved, as well as other receptors that have been shown to bind fibrin, yet not been characterized in beta cells such as CD44 [39]. The complete mechanism of action of fibrin's effects on INS-1 cells is still unknown, since blocking integrin  $\alpha\beta3$  immediately showed a change in cleaved caspase-3. This suggests that changes in INS-1 cell function and proliferation are due to long-term stimulation of integrin  $\alpha\beta3$  or mediated by other cell receptors.

The up-regulation of integrin receptors was associated with significantly increased phosphorylated FAK and Erk/2, which can activate the insulin promoter, as well as important beta cell transcription factors like Pdx-1, Beta2 or MafA [40, 41]. INS-1 cells cultured with 2D and 3D fibrin showed increased Pdx-1 expression, suggesting that it might be due to active Erk1/2 modulation. Akt was also increased in fibrin-cultured INS-1 cells. The PI3K/Akt pathway has been shown to activate Pdx-1, promote beta cell survival,

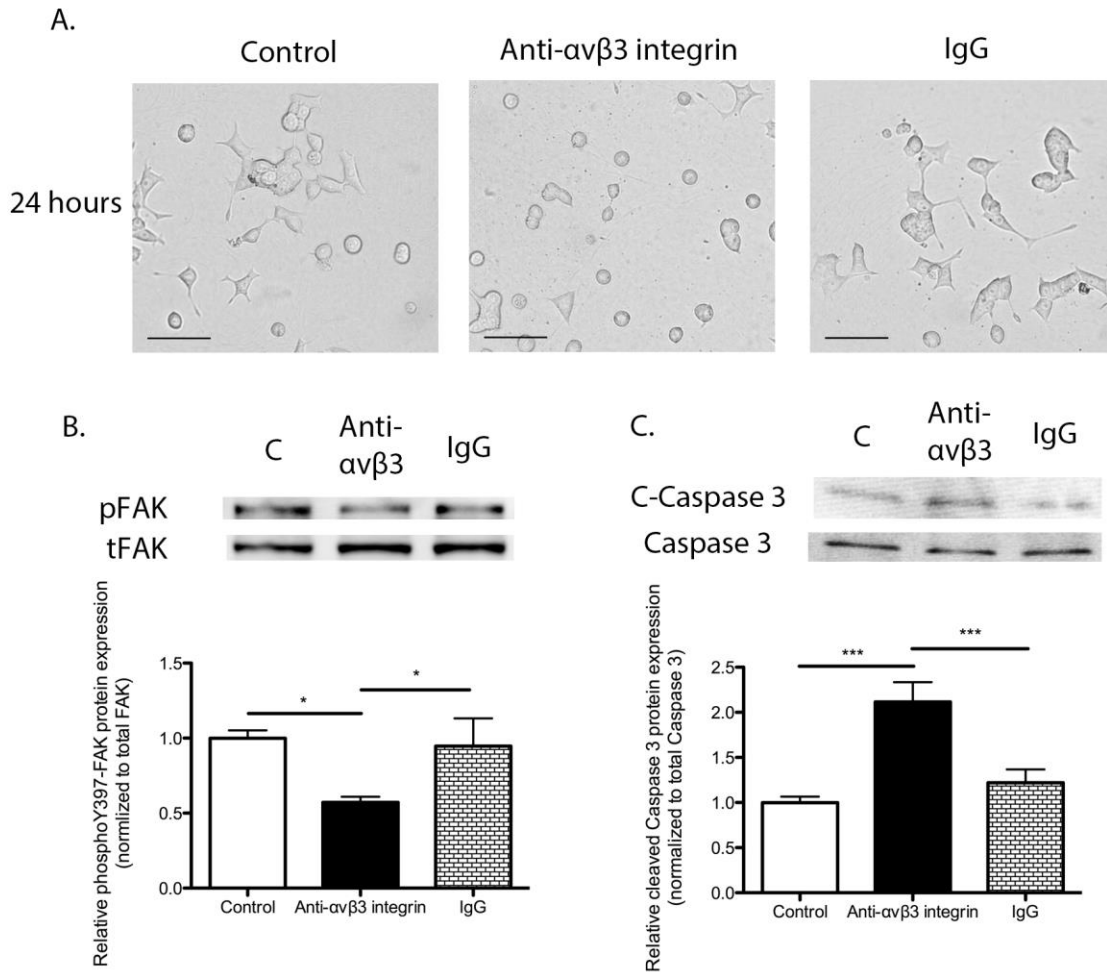


Figure 4-5 Integrin  $\alpha\text{v}\beta\text{3}$  blockade led to increased cell death when INS-1 cells were cultured on 2D fibrin.

(A) Brightfield microscope images and (B) western blot analysis of focal adhesion kinase activity and caspase-3 cleavage in INS-1 cells treated with or without an anti- $\alpha\text{v}\beta\text{3}$  blocking antibody for 24 hours. Scale bar: 100 $\mu\text{m}$ . Data are expressed at means  $\pm$  SE (n=4). Representative blots are shown. \* $p < 0.05$  and \*\*\* $p < 0.001$  vs. untreated control.

and increase proliferation by increasing cyclin D1 expression [42-44]. Fibrin culture of INS-1 cells led to up-regulation of certain integrins, namely  $\alpha\beta3$ . Fibrin contains RGD domains, which can be bound by integrin  $\alpha\beta3$  [45] and this ECM-mediated integrin regulation has previously been shown in vascular smooth muscle cells [46]. Scanning electron microscopy revealed clear contacts between INS-1 cells and fibrin fiber projections (Fig. 4-1B), suggesting that cell adhesion receptors including integrins are stimulated. We sought to block  $\alpha\beta3$  integrin-ECM interactions, in hopes of determining a mechanism behind fibrin-mediated improvements to beta cell function, proliferation and survival. In  $\alpha\beta3$  integrin-blocked cells, there was a significant reduction in phospho-FAK expression, suggesting that FAK signaling pathways are mediated by integrin  $\alpha\beta3$ ; however, there were no changes in Erk1/2 and Akt activation. The reasons for this could be that the blockade was not long enough to affect these pathways in a significant way. Also, other integrins could be compensating for reduced  $\alpha\beta3$  activity by maintaining Erk1/2 and Akt activity through a different means such as integrin-linked kinase, observed in cardiomyocytes and mammary epithelial cells [47, 48]. Cleaved caspase-3 was significantly increased in integrin  $\alpha\beta3$ -blocked INS-1 cells, suggesting that interactions between integrin  $\alpha\beta3$  and fibrin may have a primary role in maintaining INS-1 survival via FAK signaling pathways. The other signaling pathway members that contribute to beta cell health via fibrin-integrin interactions remain to be investigated.

Previous work has shown that blockade of a more promiscuous integrin,  $\beta1$ , led to a significant decrease in human fetal pancreatic cell phospho-FAK, phospho-Erk1/2 with increased cleaved caspases 9 and 3 [49]. Likewise, blocking  $\beta1$  integrin receptors on INS-1 cells during collagen culture reduced cell adhesion as well as Pdx-1 and insulin expression [25].  $\beta1$  integrin can pair with various  $\alpha$  integrin subunits, which makes it much more detrimental to cells when blocked. When  $\alpha\beta3$  integrin was blocked in endothelial cells and fibroblasts, there were inhibitions in VEGF signaling and cell motility [50]. The role of  $\alpha\beta3$  integrin in angiogenesis has been well-characterized [51]; yet, in the context of other integrins, there is complex cross-talk between integrins that influences how cells will behave [52]. This cross-talk may be an important factor to consider with respect to the effects of fibrin-cultured beta cells.

## 4.5 Conclusions

Fibrin is a provisional ECM protein that improves beta cell function, proliferation and survival. Fibrin also increases integrin expression, which may be the mechanism behind the beneficial effects we observed in beta cells. At least one integrin receptor,  $\alpha v\beta 3$ , plays a role in mediating beta cell survival when cells are cultured on fibrin. Understanding how fibrin promotes beta cell health is essential to improve upon therapies for diabetes treatment. Treatments such as islet transplantation are only effective if there is a sufficient supply of beta cells and this supply can be increased if beta cell integrin-ECM interactions are understood.

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## Chapter 5

### 5 Fibrin enhances human fetal islet-epithelial cell differentiation via p70<sup>s6k</sup> and promotes vascular formation during transplantation<sup>5</sup>

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<sup>5</sup> This work has been submitted for peer-review as the following manuscript:

Riopel M, Li J, Trinder M, Fellows G, Wang R. Fibrin enhances human fetal islet-epithelial cell differentiation via p70<sup>s6k</sup> and promotes vascular formation during transplantation. Biomater (MS#jbmt27573)

## 5.1 Introduction

Islet transplantation for the treatment of diabetes is currently limited by a shortage of available donor pancreata [1]. In order to surpass this problem, generating beta cells from progenitor cell sources is a viable option [2]. However, this procedure and others like it produce low numbers of effective, functional beta cells [2]. A greater understanding of the factors, especially those in the microenvironment, which regulate pancreatic development and islet cell differentiation are required to significantly increase the yield of beta cells that these procedures generate for islet transplantation.

Human pancreatic development begins at day 26 post-conception, with tubular structures protruding from the ventral and dorsal side of the foregut endoderm. Two transcription factors that are required for the initiation of pancreatic development are PTF-1A and PDX-1 [3], in which the latter also promotes beta cell maturation and function [4]. By 8 weeks of age, Sry-related HMG box 9 (SOX9) is expressed in the pool of pancreatic progenitors [5] and is essential to support their proliferation and survival as well as maintain a transcriptional network with other factors [6]. SOX9 is also an important regulator of pancreatic endocrine cell differentiation [5]. Insulin positivity emerges around 7.5 weeks post-conception, followed by glucagon and somatostatin one week later [7]. Islet-like structures eventually form where cells commonly express more than one hormone [7]. Subsequently, clustering occurs leading to construction of the islet of Langerhans [8, 9].

Cell receptors in the human fetal pancreas that promote interactions with the ECM are integrins. Integrins are a family of highly expressed heterodimeric alpha and beta receptors that bind to motifs of laminin, collagen, fibronectin and other cell surface receptors [10]. Integrins modulate multiple cellular processes in a variety of tissues. In the mouse pancreas, the conditional removal of  $\beta 1$  integrin leads to dysfunction of the endocrine [11] and exocrine [12] compartments. Removal of  $\beta 1$  integrin in developing pancreatic beta cells leads to a massive reduction in insulin positive cells and cell cycle progression genes, suggesting that beta cell expansion relies upon  $\beta 1$  integrin and ECM interactions [13]. Meanwhile, studies with human fetal pancreas indicate that integrins  $\alpha 3$ ,  $\alpha 5$   $\alpha 6$  and their associated  $\beta 1$  counterpart are present by 8 weeks of age, and increase as development

continues [14, 15]. Also, integrins  $\alpha_v$  with  $\beta_1$ ,  $\beta_3$  and  $\beta_5$  are important for human fetal beta cell adhesion and motility [16-18]. Knockdown of integrin receptors in human fetal pancreatic cells show that  $\beta_1$  integrin is required for maintenance of insulin expression, cell survival and adhesion [14, 18]. Likewise, loss of  $\alpha_3$  integrin function leads to decreased intracellular signaling, adhesion to ECM proteins and insulin expression in human fetal pancreatic cells [19]. In this way, integrin receptors are important components of the human fetal pancreas, especially in regards to insulin expression and islet development.

Fibrin is a protein found in blood clots. Derived from fibrinogen following activation of the coagulation cascade and cleavage by thrombin, fibrin forms crosslinks to form a clot [20]. Fibrin also contains the amino acid motif RGD, a ligand to a large group of integrin receptors [21]. Addition of an RGD peptide to culture has been shown to significantly reduce islet cell death [22]. Culture of the adult rat insulinoma cell line, INS-1, with RGD peptides, fibronectin and fibrin gels lead to significant improvements in glucose-stimulated insulin secretion and proliferation [23, 24]. Blockade of  $\alpha_v\beta_3$  integrin by a neutralizing antibody resulted a significantly increased beta-cell apoptosis, indicating that functional  $\alpha_v\beta_3$  integrin is required for beta-cell survival [24]. Furthermore, 3D fibrin culture of isolated human islets significantly enhanced beta cell function and survival [25]. Fibrin is able to support a 3D cellular environment [26] and promote angiogenesis [27], which may be beneficial for human fetal pancreatic islet cell growth and maturity during culture and/or transplantation [7].

Transplantation of human fetal pancreata has been suggested as a treatment alternative for diabetes due to its reduced immunogenic nature [28]. Indeed, transplantation of human fetal pancreatic cells into athymic diabetic mice leads to normo-glycemia [29] and the use of fibrin may assist in graft survival and function [25, 30]. Therefore, the objective of this study is to analyze whether fibrin can promote human fetal pancreas differentiation and proliferation in vitro and in vivo as well as the potential mechanisms involved. We hypothesize that fibrin will promote differentiation of human fetal pancreatic progenitor cells into insulin producing cells through integrin  $\alpha_v\beta_3$  receptor up-regulation, angiogenesis stimulation and promotion of cell proliferation.

## 5.2 Materials and methods

### 5.2.1 Human fetal islet-epithelial cell culture and fibrin gel preparation

Human fetal pancreata (15-21 weeks of age) were collected according to protocols approved by the Health Sciences Research Ethics Board at Western University in accordance with guidelines of the Canadian Council on Health Sciences Research Involving Human Subjects. After dissociation by collagenase V, islet-epithelial cell clusters were cultured in CMRL media containing 10% fetal bovine serum (Invitrogen, Burlington, Ontario, CAN). To initiate polymerization, thrombin (Sigma, St Louis, MO, USA) was added to fibrinogen, as previously described [24]. The resultant solution was quickly inserted into wells of a 24-well plate to form 0.1-0.2mm thickness gel. Human fetal islet-epithelial cell clusters plus medium were then added on top of the fibrin gels for the 2D experimental group or TCPS for the control group. The 3D fibrin-cultured group had human fetal islet-epithelial cell clusters added to the fibrinogen solution before polymerization to form 0.3mm 3D scaffold. Cell clusters plated on TCPS, 2D fibrin or 3D fibrin were cultured for 1 and 2 weeks, and then collected for analyses. For the mTOR inhibition study, dissociated human fetal pancreatic cells were pretreated with 20ng/mL of rapamycin (LC Laboratories, Woburn, MA, U.S.A.) for 30 min then plated on 2D fibrin for 48 hours. Samples were collected for protein and double immunofluorescence analysis.

### 5.2.2 RNA extraction and real-time RT-PCR

Total RNA was extracted from 1 week fibrin-cultured human fetal islet-epithelial cell clusters and controls using the miRNeasy kit (QIAGEN, Germantown, MD, USA). For each reverse transcription reaction, 2 µg of DNA-free RNA was used with random hexamers/oligo-deoxythymidine primers and superscript reverse transcriptase (Invitrogen). Sequences of PCR primers used for RT-PCR with expected product size are listed in Appendix 5. A master mix of primers, water and SYBR green was made and then added to separate tubes with each cDNA sample. Real-time RT-PCR analyses were conducted using the CFX manager software (Bio-Rad Laboratories, Mississauga, ON,

Canada) and CFX Connect Real-Time System (Bio-Rad Laboratories). Data were normalized to levels of 18S rRNA subunit and relative gene expression was calculated based on the  $2^{-\Delta\Delta CT}$  method as PCR signals from 2D and 3D fibrin-cultured human fetal islet-epithelial cell clusters relative normalized to TCPS controls.

### 5.2.3 Scanning electron, transmission electron and immunofluorescence microscopy

For SEM, human fetal pancreata or fibrin-cultured human fetal islet-epithelial cell clusters were fixed with 2.5% glutaraldehyde at 4°C overnight, then dehydrated with an increasing concentration gradient of ethanol. Samples were subjected to critical point drying and gold coating, and then visualized by a Hitachi 3400-N Variable Pressure Scanning Electron Microscope.

For transmission electron microscopy (TEM), human fetal pancreata were dissected, cut into small pieces, then fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer. Post-fixation was done with 1% osmium tetroxide. After dehydration through a graded ethanol series and propylene oxide treatment, samples were embedded in araldite medium and cured at 65°C overnight. Ultra-thin sections were cut at 60nm and examined with a Philips 410 electron microscope (Philips Electron Optics, Hillsboro, OR, USA) at 60kV.

For immunofluorescence microscopy, human fetal pancreata and cultured human fetal islet-epithelial cell clusters were collected and fixed in 4% paraformaldehyde overnight, followed by agarose embedding and wax processing. Sections (4µm thick) were prepared and stained with appropriate dilutions of primary antibodies listed in Appendix 4. Fluorescent secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

### 5.2.4 Protein extraction and western blot analysis

Fibrin-cultured and control cell protein was extracted in a Nonidet-P40 lysis buffer. Equal amounts (15µg) of lysate protein from each experimental group was separated by either 7.5, 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to



a nitrocellulose membrane (Bio-Rad Laboratories, Mississauga, ON, Canada). Membranes were incubated with appropriate dilutions of primary antibodies listed in Appendix 4. The application of appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies) then followed. Proteins were detected using ECL™-Plus Western blot detection reagents (Perkin Elmer, Wellesley, MA, USA) and exposed using the Versadoc Imaging System (Bio-Rad Laboratories). Densitometric quantification of bands at subsaturation levels was performed using Quantity One software (Bio-Rad Laboratories) and normalized to appropriate loading controls. Data are expressed as the relative expression level of phosphorylated proteins to total protein levels or protein levels to the loading control [11].

### 5.2.5 Transplantation of cultured human fetal islet-epithelial cell clusters

Freshly isolated human fetal islet-epithelial cell clusters either mixed with fibrin or type I rat tail collagen were injected subcutaneously. Alternatively, cell clusters cultured for 1 week in either 3D fibrin or 3D type I rat tail collagen were used for subcutaneously transplantation. Briefly, 8 week-old male nude mice were anesthetized with isoflurane and either empty gels or gels containing cultured human fetal islet-epithelial cell clusters were implanted subcutaneously. Mice were sacrificed at 1 week post-transplantation, where grafts were harvested for histological analyses.

### 5.2.6 Statistical analysis

Data are expressed as means  $\pm$  SE using 4-5 different pancreata preparations per experimental group, representing  $n = 4-5$ . Statistical significance was determined using either the unpaired student's *t*-test or one-way ANOVA followed by post hoc Bonferroni comparison test. Differences were considered statistically significant when  $p < 0.05$ .

## 5.3 Results

### 5.3.1 The native human fetal pancreas contained robust extracellular matrix and integrin $\alpha\beta3$ expression

Human fetal pancreas at 16 weeks of age, analyzed by SEM, revealed very prominent ECM fibers (Fig. 5-1A). These fibers surround cell clusters, and are also present in between individual cells (Fig. 5-1A). Under the TEM, ECM fibers were often perpendicular to the cell membrane (Fig. 5-1B). The RGD-domain containing ECM protein fibronectin, was frequently associated with insulin positive cells (Fig. 5-1C), as determined by double immunofluorescence staining.

Due to the profound expression of fibronectin in the fetal pancreas, we investigated the expression of one of its receptors, integrin  $\alpha\beta3$ . Integrin  $\alpha\beta3$  was highly expressed in the developing human pancreas, and co-localized with PDX-1, insulin and CK-19 (Fig. 5-1DEF). These findings suggest that the interactions between the ECM microenvironment and pancreatic integrin receptors is essential for normal human fetal islet development.

### 5.3.2 Fibrin enhanced PDX-1 and VEGF-A expression while promoting endocrine cell differentiation of human fetal islet-epithelial cell clusters

Human fetal islet-epithelial cell clusters were cultured in 2D or 3D fibrin and TCPS for up to 2 weeks. Smaller clusters were maintained in fibrin cultured cells when compared to TCPS cultured group (Fig. 5-2A). Scanning electron microscopy revealed connections between cells and fibrin gels in both 2D and 3D cultures (Fig. 5-2B). Quantitative RT-PCR analysis of human fetal islet-epithelial cell clusters after 1 week of fibrin culture showed significantly higher expression of *PDX-1*, *INS* and *GCG* in 3D culture (Fig. 5-2C). Protein levels of PDX-1 in 2D and 3D fibrin-cultured human fetal islet-epithelial cell clusters were significantly increased during the culture period compared to TCPS controls (Fig. 5-2D). Fibrin-cultured human fetal islet-epithelial cell clusters also showed significantly higher expression of VEGF-A at 2 weeks of culture (Fig. 5-2E). Double immunofluorescence

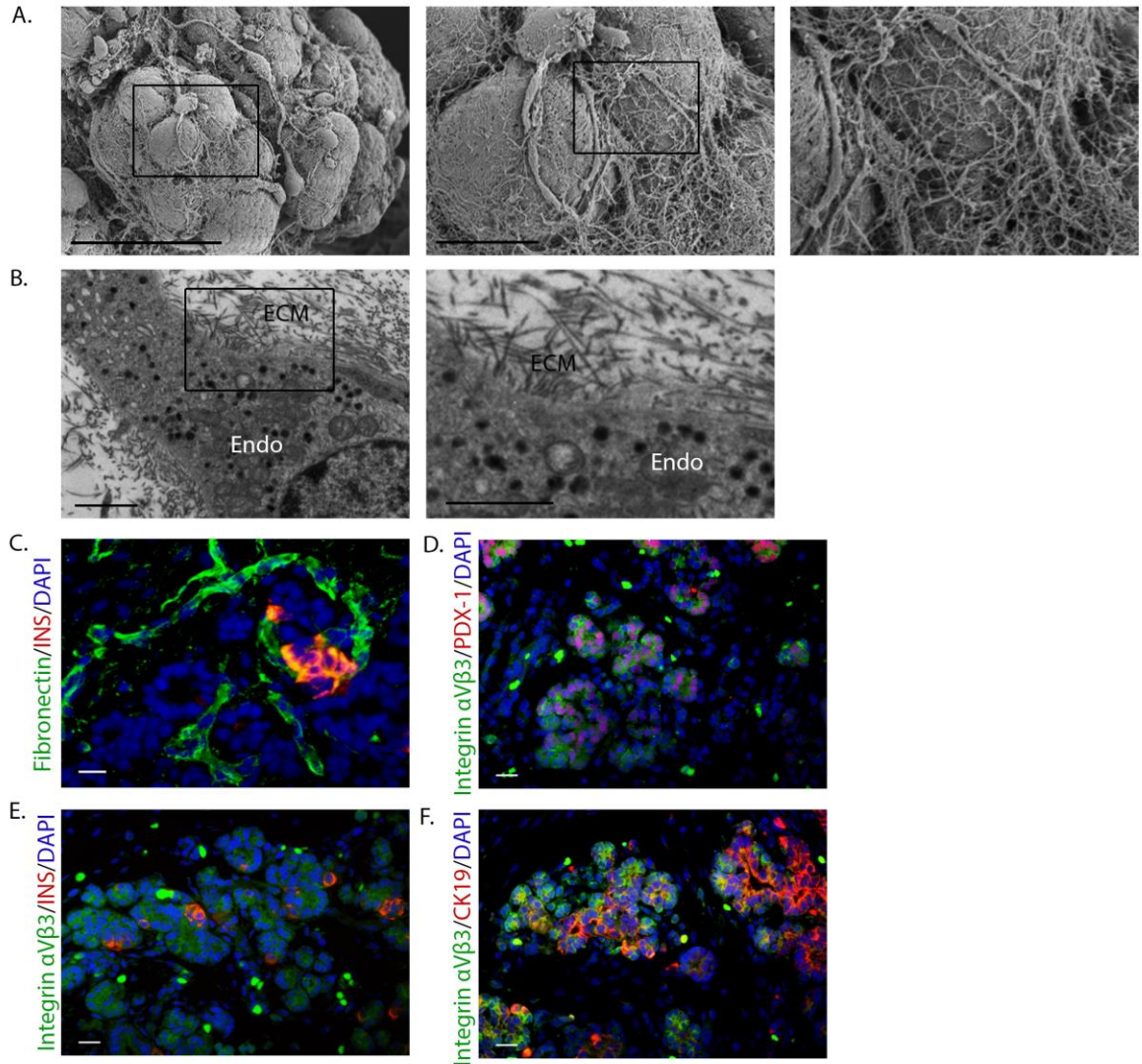


Figure 5-1 The human fetal pancreas contains ECM fibers and expresses integrin  $\alpha\beta3$ .

(A) Scanning electron micrographs of human fetal pancreas at 16 weeks of age. Scale bar: 50 $\mu$ m (left) and 10 $\mu$ m (middle). (B) Human fetal pancreas under the transmission electron microscope at 16 weeks of age. Scale bar: 1 $\mu$ m. Double immunofluorescence staining of human fetal pancreas at 16 weeks of age for fibronectin (green)/ insulin (red) (C) and integrin  $\alpha\beta3$  (green) with PDX-1 (red) (D), insulin (red) (E) and CK19 (red) (F). Scale bar: 50 $\mu$ m and nuclei are stained with DAPI (blue). ECM: extracellular matrix; Endo: endocrine cell.

staining revealed scattered clusters of insulin and glucagon positive cells in all culture conditions with a slight increase in both 2D and 3D fibrin-cultured groups relative to TCPS controls (Fig. 5-2F top). The majority of cells in all cultured groups were SOX9 and CK19 positive (Fig. 5-2F middle). Higher VEGF-A expression in fibrin-cultured human fetal islet-epithelial cell clusters was associated with prominent CD31 positive cells and maintenance of Ki67 positive cells compared to the TCPS controls (Fig. 5-2F lower).

### 5.3.3 Fibrin increased integrin $\alpha$ v and $\beta$ 3 expression in human fetal-islet-epithelial cell clusters

To investigate the mechanism behind fibrin's enhancement of islet differentiation, we analyzed the expression of various integrin receptors. At the mRNA level, 1 week cultured human fetal islet-epithelial cell clusters had significantly increased  $\alpha$ v and  $\beta$ 3 integrin expression (Fig. 5-3A). We further confirmed this observation by western blot and found that the protein level of integrins  $\alpha$ v and  $\beta$ 3 were significantly higher in human fetal islet-epithelial cell clusters cultured with fibrin (Fig. 5-3BC). Expression of the most prominent integrin,  $\beta$ 1, was unchanged by fibrin culture (Fig. 5-3B)

### 5.3.4 Fibrin induced PDX-1 expression via increased phospho-focal adhesion kinase and p70<sup>s6k</sup> activity in human fetal islet-epithelial cell clusters

Downstream of integrin receptors, phosphorylated focal adhesion kinase (FAK) was significantly up-regulated in human fetal islet-epithelial cell clusters during 2D and 3D fibrin culture compared to controls (Fig. 5-4A). No changes were observed in pathways further downstream the FAK pathway, including phosphorylated AKT (Fig. 5-4B) and activated ERK1/2 (Fig. 5-4C). However, phosphorylated p70<sup>s6k</sup>, a member of the mTOR pathway, revealed significant up-regulation in human fetal pancreatic cells cultured with fibrin compared to controls (Fig. 5-4D).

Using the mTOR pathway inhibitor, rapamycin, to treat human fetal islet-epithelial cell clusters cultured on 2D fibrin, a significant decrease in phosphorylated p70<sup>s6k</sup> was observed

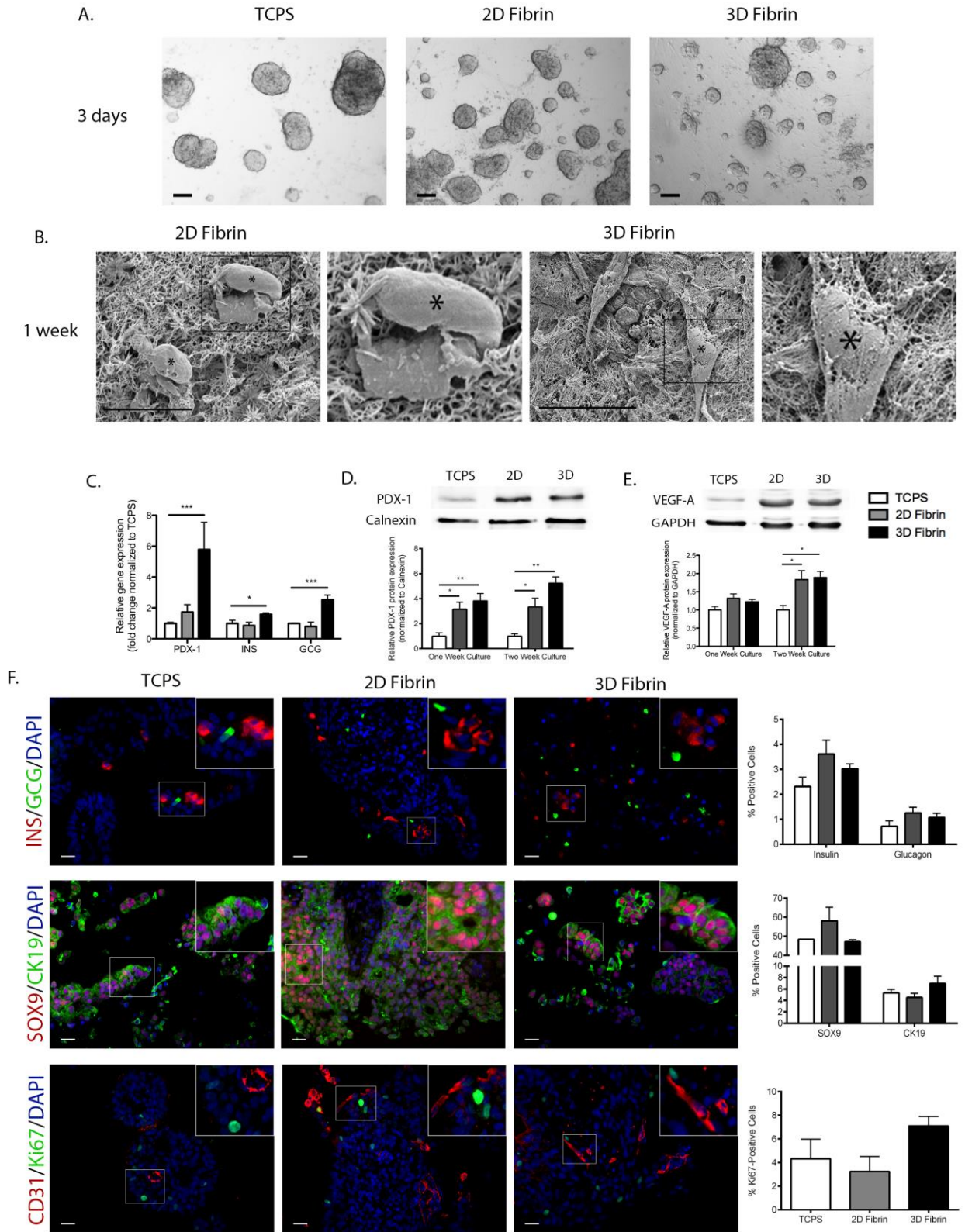


Figure 5-2 Fibrin improves human fetal pancreatic cell differentiation and increases VEGF-A expression.

(A) Human fetal pancreatic cells cultured on tissue-culture polystyrene (TCPS), on 2D and in 3D fibrin for 3 days. Scale bars: 100 $\mu$ m. (B) Scanning electron micrograms of fibrin-cultured human fetal pancreatic cells. Scale bars: 20 $\mu$ m. (C) Real time RT-PCR analysis of PDX-1, insulin and glucagon mRNA expression in human fetal pancreatic cells cultured for 1 week with fibrin or on TCPS. Western blot analysis of PDX-1 (D) and VEGF-A (E) in human fetal pancreatic cells during 1 and 2 weeks of fibrin culture. (F) Representative immunofluorescence images of human fetal pancreatic cells cultured with fibrin being stained for insulin (red)/ glucagon (green) (top row), SOX9 (red)/ CK19 (green) (middle row) and CD31 (red) / Ki67 (green) (bottom row) and the percentage of the positive cells. Nuclei are stained with DAPI in blue. Scale bars: 50 $\mu$ m. Data are expressed as means  $\pm$  SE (n=3-5). Representative blots are shown. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 vs. TCPS control.

(Fig. 5-4E). Two dimensional fibrin cultures were used because the cells were affected to the same extent as 3D fibrin culture with respect to PDX-1 protein expression and signaling pathway activity. Inhibition of the mTOR pathway resulted in a significant decrease in PDX-1 expression, observed by western blot (Fig. 5-4F). Furthermore, the effects of rapamycin treatment on fibrin-cultured human fetal islet-epithelial cell clusters caused a reduction in cell proliferation, noted by cyclin D1 expression and Ki67 immunofluorescence (Fig. 5-4GH).

### 5.3.5 Subcutaneous injection of human fetal islet-epithelial cell clusters with fibrin improved vascularization

To investigate how fibrin could be used as a vehicle to transplant cells subcutaneously, male nude mice were injected with freshly isolated human islet-epithelial cell clusters cells mixed with fibrin immediately before polymerization (Fig. 5-5A). Cells mixed with collagen I (Fig. 5-5B), empty fibrin and empty type I collagen (Fig. 5-5C) were used as controls. A fibrin-wrapped cell graft was clearly observed under the skin of the nude mice (Fig. 5-5A left). A cell core was clear, with the presence of vasculature around the fibrin-wrapped graft interface (Fig. 5-5A right). A collagen-wrapped cell graft was also present under the skin of nude mice; however, prominent vascular development was not observed (Fig. 5-5B). At the cellular level, fibrin- and collagen I-wrapped human fetal islet-epithelial cell grafts after 1 week implantation in vivo had relatively similar levels of insulin and glucagon positivity (Fig. 5-5D 1<sup>st</sup> row). Both fibrin- and collagen I-wrapped human fetal islet-epithelial cell grafts showed higher a number of SOX9/CK19 positive cells compared to pre-implantation samples (Fig. 5-5D 2<sup>nd</sup> row). An antibody raised against human mitochondrial surface (HMS) protein allowed for identification of the implanted human fetal cells. These HMS positive cells showed a high level of cell proliferation as marked by Ki67 in both the fibrin- and collagen-wrapped groups (Fig. 5-5D 3<sup>rd</sup> row). Implanted human cells in the fibrin-wrapped group contained a high number of CD31 positive cells with visible host CD31 positive cells around the graft (Fig. 5-5D 4<sup>th</sup> row). The collagen-wrapped group had large HMS-positive vessels with minimal small vessels (Fig. 5-5D 4<sup>th</sup> row).



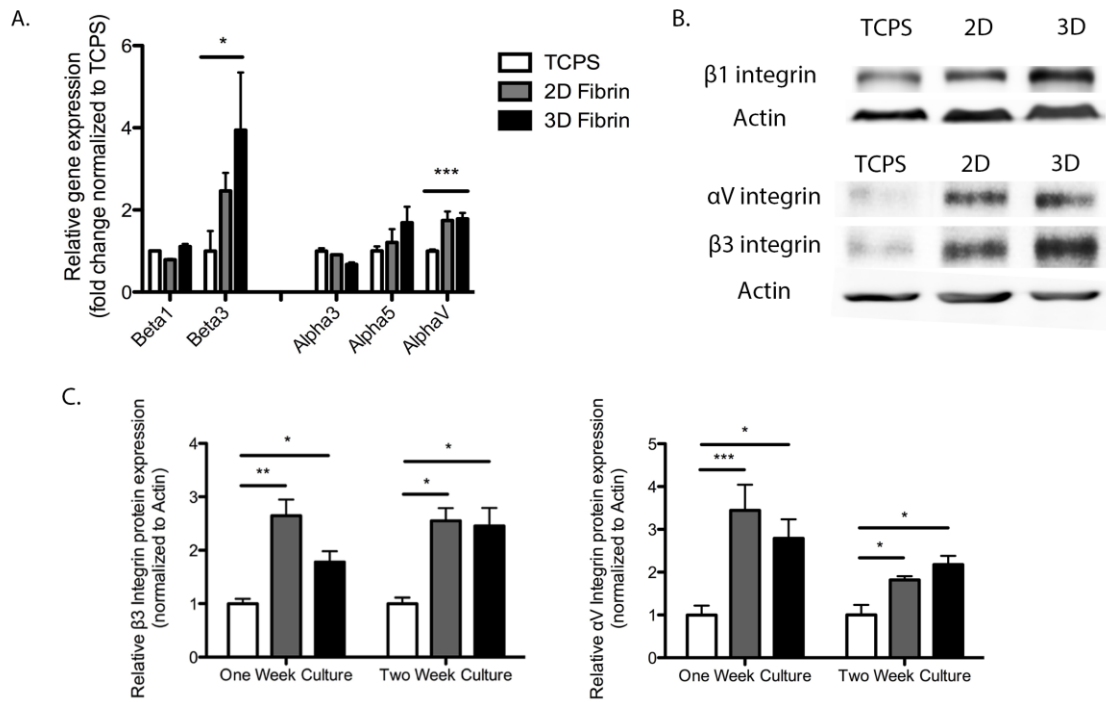


Figure 5-3 Fibrin induces integrin  $\alpha\beta3$  expression in human fetal pancreatic cells.

(A) Relative mRNA expression of integrin receptors in human fetal pancreatic cells cultured for 1 week with fibrin or on TCPS. (B) Representative western blots for  $\beta1$ ,  $\alpha\text{v}$  and  $\beta3$  integrin expression. Quantitative analysis of integrins  $\alpha\text{v}$  (C) and  $\beta3$  (D) in human fetal pancreatic cells during the culture period. Data are expressed at means  $\pm$  SE (n=3-5). \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001 vs. TCPS control.



### 5.3.6 Subcutaneous transplantation of cultured human fetal islet-epithelial cell clusters with fibrin maintained insulin and glucagon expression and improved vascularization

Human fetal islet-epithelial cell clusters were cultured in 3D fibrin or 3D collagen for 1 week, then transplanted subcutaneously into nude mice to investigate the potential of either ECM protein to promote differentiation and vascular formation. As controls, empty fibrin and empty collagen were cultured and transplanted adjacently. Grossly, transplantation of fibrin-cultured cells led to a graft with vessels surrounding it, while an empty fibrin graft can be observed immediately next to it, with markedly less vascularity (Fig. 5-6A left). Conversely, cells cultured with collagen and then transplanted led to a graft with less vascularity, very similar to both the empty fibrin and empty collagen grafts (Fig. 5-6A right). Double immunofluorescence staining of insulin and glucagon was similarly maintained in fibrin and collagen after 7 days of culture. However, after 1 week subcutaneous transplantation, the fibrin group had slightly more insulin and glucagon immunoreactivity than cells cultured and transplanted with collagen (Fig. 5-6B top row). Cells cultured and transplanted with fibrin also had maintained expression of CK19, which was lost in the collagen grafts (Fig. 5-6B 2<sup>nd</sup> row). Cell proliferation in the transplanted groups was also maintained compared to the groups after 7 days in culture (Fig. 5-6B 3<sup>rd</sup> row). Finally, blood vessels were prominent and elongated, similar to native pancreatic vessels, when fibrin-cultured human fetal islet-epithelial cell clusters were transplanted compared to the collagen group (Fig. 5-6B 4<sup>th</sup> row).

## 5.4 Discussion

This study investigated how fibrin can promote differentiation and proliferation of human fetal islet-epithelial cell clusters *in vitro* and *in vivo*. The native human fetal pancreas contains many ECM proteins, including fibronectin that may associate with integrin  $\alpha\text{v}\beta\text{3}$ . Culture of human fetal pancreatic cells in 2D and 3D fibrin for one week significantly increased PDX-1, insulin and glucagon expression. This was associated with up-regulation of integrins  $\alpha\text{v}$  and  $\beta\text{3}$ , VEGFA, phosphorylated FAK and phosphorylated p70<sup>s6k</sup>. The

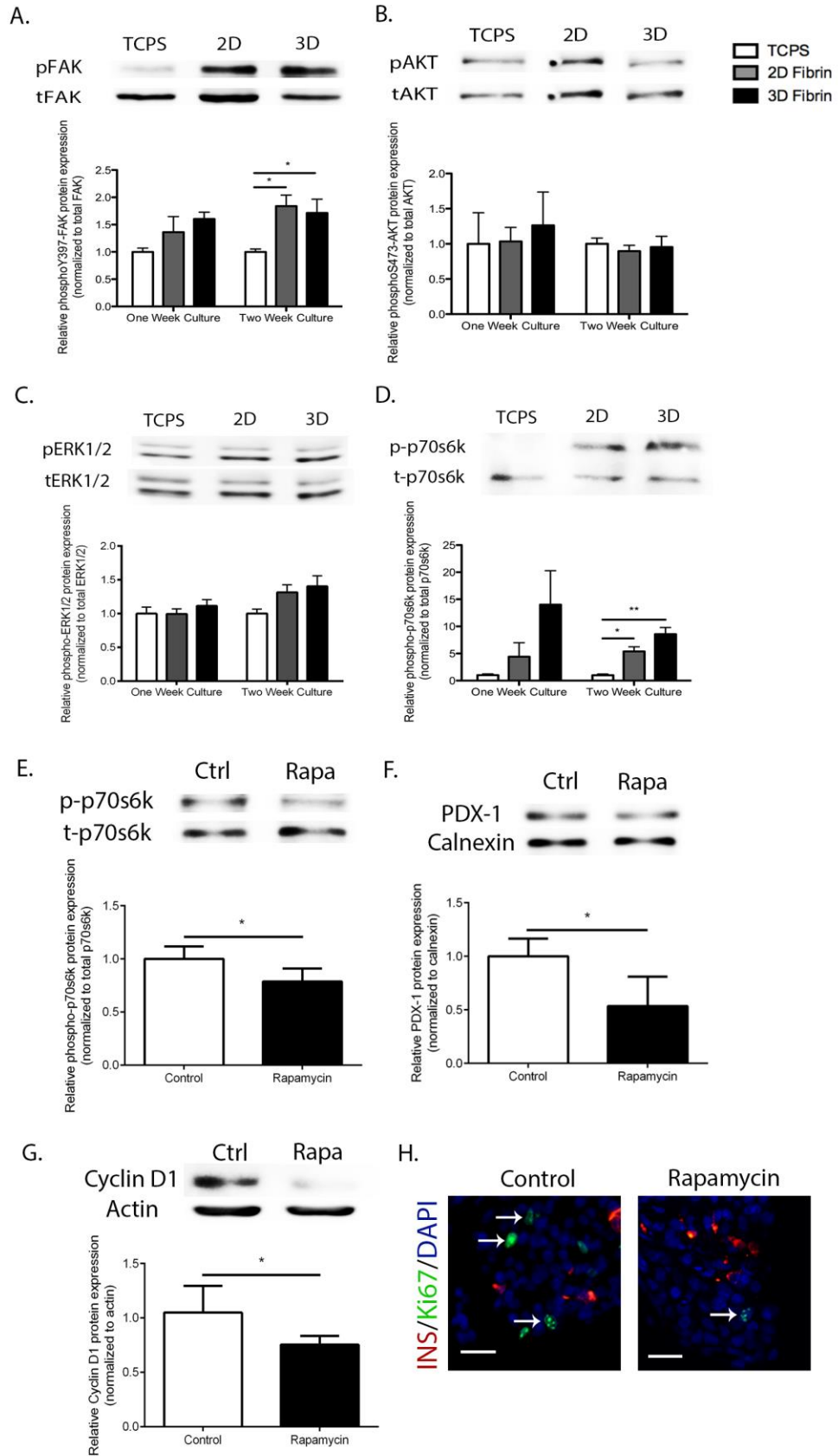


Figure 5-4 PDX-1 expression is regulated by mTOR signaling pathway activity.

Quantitative analysis of phosphorylated FAK (A), AKT (B), ERK1/2 (C) and p70<sup>s6k</sup> (D) in human fetal pancreatic cells during the culture period. Western blot analysis of phosphorylated p70<sup>s6k</sup> (E), PDX-1 (F) and cyclin D1 (G) expression, and (H) immunofluorescence staining of Ki67 in 2D fibrin-cultured pancreatic cells with or without rapamycin treatment. Arrows indicate proliferating cells, scale bar: 50 $\mu$ m and nuclei are stained by DAPI (blue). Data are expressed at means  $\pm$  SE (n=3-5). Representative blots are shown. \*p<0.05, \*\*p<0.01 and \*\*\*p<0.001 vs. TCPS control. p: phosphorylated; t: total.

reduction in p70<sup>s6k</sup> phosphorylation by rapamycin treatment led to significant decreases in PDX-1 expression and cell proliferation. Subcutaneous injection of fibrin mixed with human fetal islet-epithelial cell clusters into nude mice showed remarkable vascularization compared to collagen controls. We extended these results by subcutaneously transplanting pre-cultured human fetal islet-epithelial cell clusters with fibrin, which led to maintained expression of insulin and glucagon, but also more pronounced vascularization in the graft compared to collagen. This study shows that fibrin can maintain human fetal islet-epithelial clusters and enhance vessels formation in vitro and in vivo.

Robust ECM proteins are present in the developing human pancreas along with integrin  $\alpha\beta3$ . Previous studies have indicated the presence of multiple integrin receptors in the human fetal pancreas, which bind to the ECM to mediate cellular responses [14]. In particular, integrin  $\beta1$  stimulates FAK and ERK1/2 to promote human islet-epithelial cell differentiation and survival [18]. Alongside, human islet development requires integrins  $\alpha\beta3$  and  $\alpha\beta5$  [17] and integrin  $\alpha\beta1$  [16] for adhesion and migration of cells. In mice, the ECM component laminin-1 promotes differentiation of pancreatic beta cells [31], likely through  $\alpha6$  integrin [32]. Culture of beta cells in hydrogels with ECM proteins improves survival and function [33]. However, loss of beta cell phenotype including diminished insulin expression occurs during long-term culture [34, 35]. In current study, fibrin culture of human islet-epithelial cells replaced, at least in part, the ECM lost during pancreatic dissociation. This led to improved expression of integrin receptors, signaling molecules and differentiation of islet cells.

The present study demonstrated that culture of human fetal islet-epithelial cell clusters with fibrin increased the expression of integrins  $\alpha\upsilon$  and  $\beta3$  and downstream activity of FAK and p70<sup>s6k</sup> signaling molecules. Many integrin receptors on different cell types have been shown to bind fibrin, including integrins  $\alpha\upsilon\beta3$  and  $\alpha\text{IIb}\beta3$  [36]. We have recently shown that fibrin-culture of INS-1 cells increased expression of integrins  $\alpha\upsilon$  and  $\beta3$ , but not  $\beta1$  [24]. These results support the notion that stimulation of integrins with ECM can promote cellular expression and activation of downstream signaling pathways [12, 24]. At one week of culture, no differences in signaling pathway activity changes were observed. However,

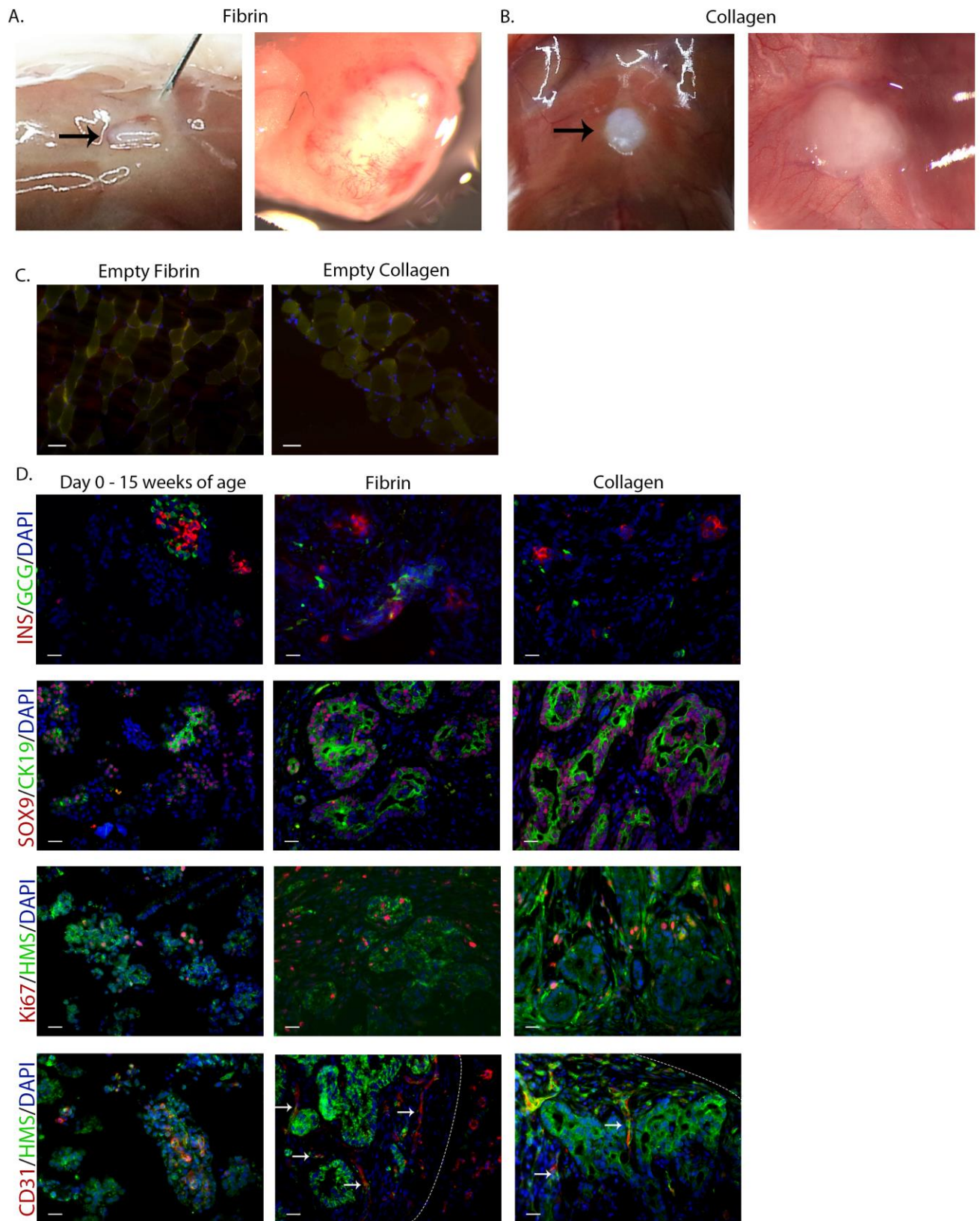


Figure 5-5 Subcutaneous injection of human fetal pancreatic cells with fibrin improves vasculature.

Gross demonstrations of subcutaneously injected human fetal pancreatic cells mixed with fibrin (A) or collagen (B) in low (left) and high (right) magnification. (C) Histological image of control empty injections of fibrin and collagen. (D) Double immunofluorescence labeled human fetal pancreatic cells either before injection (left column) or after 1 week in vivo with fibrin (middle column) or collagen (right column). Cells were stained with insulin (red)/ glucagon (green) (top row), SOX9 (red)/ CK19 (green) (2<sup>nd</sup> row), Ki67 (red)/ human mitochondrial surface protein (HMS, green) (3<sup>rd</sup> row) and CD31 (red)/ HMS (green) (4<sup>th</sup> row). Representative images are shown with DAPI staining the nuclei (blue). Black arrows point to graft location, while white arrowheads indicate donor derived blood vessels. Scale bars: 50 $\mu$ m

there was a trend towards increasing pathway phosphorylation in the fibrin-cultured group. A reason for this could be that, despite having significantly more integrin receptor expression, their activation did not reach a threshold to activate enough downstream signaling molecules to cause an effect. Yet, after one week of fibrin culture, the expression of insulin, glucagon and PDX-1 was increased, suggesting that these markers were being modulated by molecules other than integrins, AKT and ERK1/2.

Aberrant mTOR pathway signaling occurs in multiple diseases states [37]. We observed significant increases in phosphorylated p70<sup>s6k</sup> when human fetal pancreatic cells were cultured with fibrin, which was AKT-independent (Fig. 5-4). Despite many studies confirming that the mTOR pathway relies upon AKT, research in a pancreatic beta cell line (INS-1) showed that beta cell proliferation mediated by active mTOR/p70<sup>s6k</sup> signaling is AKT-independent [38, 39]. We expand on these results, suggesting that mTOR activity was stimulated without active AKT to modulate differentiation of human fetal pancreatic cells. However, leucine stimulation of the mTOR pathway in fetal rat pancreatic cells led to inefficient differentiation of Pdx-1 positive cells into neurogenin-3 positive cells [40]. This finding suggests that mTOR activity has a negative effect on pancreatic differentiation. The major difference between our study and previous research is the use of human cells. Numerous articles have outlined signaling pathway differences between the rodent and human pancreas [41-43]. Also, the mTOR pathway has different effects on islet cell cycle depending on animal species [44, 45]. In particular, stimulation of rodent islets with palmitate led to increased DNA synthesis (in an mTOR-dependent manner) in rodent islets, but not those isolated from humans [45]. Therefore, mTOR pathway activity may have different effects on islet cell differentiation based on whether cells are human or rodent. Our results suggest that the mTOR pathway has a positive effect on differentiation and proliferation of human fetal pancreatic cells when these cells were cultured with fibrin.

When human fetal islet-epithelial cell clusters were cultured with fibrin, we observed significant increases in VEGF-A expression (Fig. 5-2). We also observed robust vascular presence when cells were transplanted with fibrin. Two transplantation methods are used in the current study: (1) transplantation of human fetal islet-epithelial cell clusters with fibrin just after polymerization initiation and (2) one week culture of cells in fibrin then



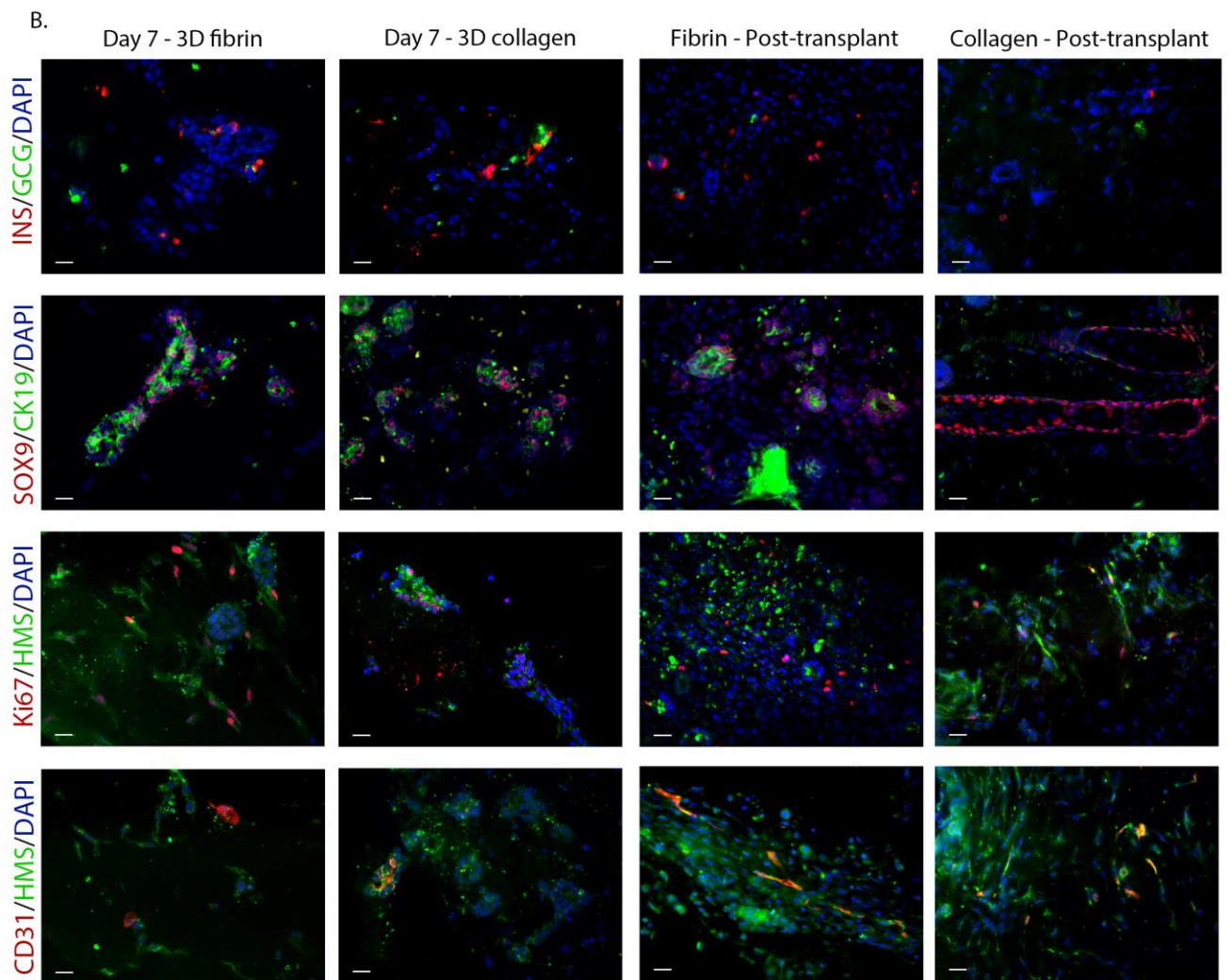
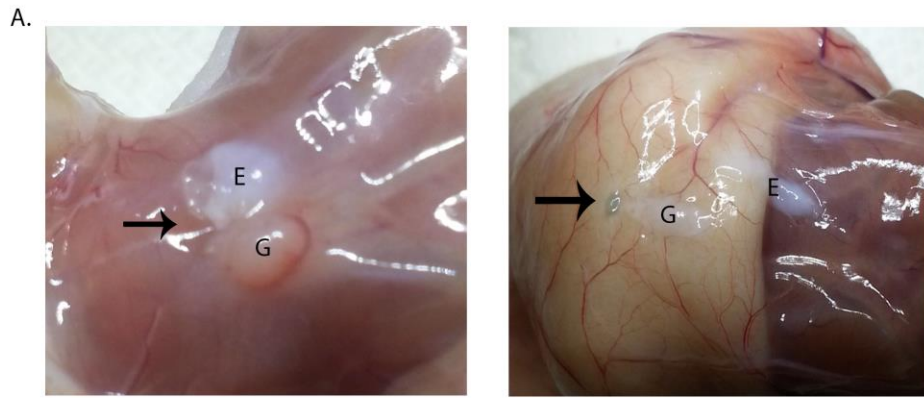




Figure 5-6 Subcutaneous transplantation of fibrin-cultured human fetal pancreatic cells maintains expression of insulin, glucagon, CK19 and improves vasculature.

(A) Human fetal pancreatic cells cultured for 1 week with fibrin (left) or collagen (right) then transplanted subcutaneously into nude mice with an empty gel for 1 week. E represents the empty gel, while G is the graft. (B) Double immunofluorescence staining of human fetal pancreatic cells after 1 week of 3D culture with fibrin (left column) or collagen (2<sup>nd</sup> column); or after 1 week culture and 1 week post-transplantation in nude mice (fibrin – 3<sup>rd</sup> column, collagen – 4<sup>th</sup> column). Cells were stained for insulin (red)/ glucagon (green) (top row), SOX9 (red)/ CK19 (green) (2<sup>nd</sup> column), Ki67 (red)/ HMS (green) (3<sup>rd</sup> column) and CD31 (red)/ HMS (green). Representative images are shown with DAPI staining the nuclei in blue. Scale bars: 50µm.

transplantation of the combination subcutaneously for one week. Both of these experiments demonstrated that transplanted fibrin-wrapped human fetal islet-epithelial cell clusters could promote vasculature formation and maintain cell differentiation and proliferation. Previous reports indicate that culturing human adult islets in fibrin for one week followed by transplantation of the cells resulted in improved graft size, reduced ductal structures and significantly increased c-peptide levels compared to animals that received free-floating cultured islets [25]. It was postulated that the angiogenic-promoting effects of fibrin are what led to such results. These effects have also been observed when a fibrin hydrogel-islet composite was subcutaneously transplanted in mice [46]. Fetal porcine islet-like cell clusters were transplanted under the kidney capsule of nude mice and sufficient angiogenesis occurred to develop an autonomous microcirculation supporting these islet-like clusters [47]. Likewise, normalization of glycemia occurred when fibrin-encapsulated VEGF-treated rat islets were transplanted into diabetic mice [48]. Results from these studies in combination with our own confirm that blood vessel formation is important for the maintenance of islet graft function and survival.

Fibrin has been used in many tissue-engineering specialties as a carrier [49, 50]; however, as an encapsulation vehicle for islet transplantation, fibrin has not been well characterized. Encapsulating rat islets with fibrin, then transplanting them into diabetic nude mice normalized glycemia with a minimum of only 100 islet equivalents [51]. Another study found that transplantation of islets in a fibrin glue reduced diabetic mouse blood glucose to normal levels [30]. Our results show that human fetal pancreatic cells can be cultured with fibrin and transplanted to yield a successful graft. Taken together, fibrin may be used as a microencapsulation scaffold for islet transplantation to enhance outcomes.

## 5.5 Conclusions

The human fetal pancreas contains rich ECM fibers and expression of integrin  $\alpha\beta3$ . Fibrin improves human fetal pancreatic cell differentiation via the mTOR pathway that is associated with increases in integrin  $\alpha\beta3$  expression. Fibrin also improves graft vascularization when human fetal pancreatic cells are transplanted subcutaneously with or without a pre-culture period. Understanding how fibrin promotes human pancreatic

progenitor differentiation will enhance existing protocols that aim to generate functional beta cells for the treatment of diabetes.

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## Chapter 6

### 6 General discussion, limitations and future direction

## 6.1 Summary of discussion

Integrin-ECM interactions mediate a variety of cellular processes in the pancreas. The goal of this thesis was to investigate the role of  $\beta 1$  integrin in the adult murine pancreas and the role of fibrin-integrin interactions in support of pancreatic cell function, proliferation, survival and maturation.

My first and second objectives were to investigate the *in vivo* role of  $\beta 1$  integrin on the murine pancreas. Primary loss of  $\beta 1$  integrin on pancreatic stellate and beta cells had a detrimental effect on ECM expression and tissue architecture. As well, a decrease in pancreatic  $\beta 1$  integrin expression occurred, which was associated with glucose intolerance, significantly reduced protein levels associated with exocrine tissue function and decreased FAK/MAPK/ERK1/2 signaling pathway activity. Importantly, loss of  $\beta 1$  integrin led to significantly decreased pancreatic cell proliferation and increased cell death.

The significance of this work lies in the clinical relevance for cell-based therapies for diabetes treatments. As mentioned previously, islet transplantation requires isolation of islets from the donor pancreas. The process of islet isolation destroys connections between the ECM and integrin receptors, which I have shown to be essential for pancreatic cell integrity. In particular, the loss of islet cell-cell and islet cell-endothelial cell, and islet cell-basement membrane connections leads to progressive loss of insulin expression and increased cell death [1, 2]. The combination of these consequences leads to low quality islets for transplantation and increases the susceptibility of graft failure. To avoid this, transplanting islets immediately after isolation is ideal in order to re-establish islet cell contacts with surrounding cells or ECM. However, this removes all opportunities to expand beta cells *in vitro*, which has been met with limited success due to the high incidence of cell death. For this reason, researchers have been culturing islets with various stimulating factors or ECM proteins in an attempt to improve *in vitro* islet health and expand beta cells.

My third and fourth objectives were to investigate the role of fibrin-integrin interactions in support of pancreatic cell function, proliferation, survival and maturation. Culturing INS-1 cells with fibrin significantly increased integrin  $\alpha \beta 3$  expression, improved GSIS, and

prevented cell death through integrin  $\alpha\text{v}\beta\text{3}$ . Meanwhile, when human fetal pancreatic cells were cultured with fibrin, significant increases in islet gene expression occurred, including increased expression of integrin  $\alpha\text{v}\beta\text{3}$ , phosphorylated FAK and phosphorylated p70<sup>s6k</sup>. Subcutaneous injection of human fetal pancreatic cells wrapped in fibrin led to robust vascularization compared to collagen.

Previous studies have cultured pancreatic islets in a variety of other ECM matrix proteins, in an attempt to recapitulate the native pancreas environment. However, culturing islets in these conditions leads to either cystic formation [3], transdifferentiation [4], or loss of function [5]. It appears that without stimulation of a variety of cell surface receptors, islets are doomed to a less-than-ideal fate. Indeed, when islets are cultured with multiple ECM proteins or matrigel (a gel derived from tumor cells), islet cells have improved insulin secretion [6, 7]. Therefore, ensuring stimulation of cell surface receptors after islet isolation, especially integrins, is critical to maintain or expand beta cells in vitro. Fibrin was able to support mature beta cells with respect to function, proliferation, and survival. There were also improvements to pancreatic progenitor differentiation when human fetal islet-epithelial clusters were cultured with fibrin. These effects were, in part, mediated by fibrin's binding to integrin receptors. However, to obtain ideal maintenance of islet cell health, it is likely that a combination of factors and ECM proteins are required during culture.

When pancreatic cells were cultured with fibrin, integrin  $\alpha\text{v}\beta\text{3}$  was increased with no change in other integrin receptor expression, such as  $\beta\text{1}$ . It has been previously mentioned that fibrin contains RGD domains that preferentially bind to integrin  $\alpha\text{v}\beta\text{3}$ . However, the mechanism by which integrins mediate their own specific expression is not clear. Cells are able to make their own ECM matrix, which may autostimulate receptors for continued survival [8]. This feedforward mechanism may stimulate integrin receptor expression, noted in fibroblasts [9, 10]. This, however, does not explain why only certain integrin receptors had increased expression and others did not.

The non-receptor tyrosine kinase, FAK, had significantly decreased activity in mice lacking  $\beta\text{1}$  integrin, and significantly increased activity in pancreatic cells cultured with

fibrin suggesting that FAK is a mediator of integrin activation. Indeed, mice lacking beta cell-expression of FAK had reduced viability and function due to FAK signaling pathway down-regulation and impaired cytoskeletal dynamics [11]. The level of FAK phosphorylation is correlated to beta cell survival, due to either cyclin-dependent kinase 5 activation [12] or after ECM culture [13]. In this way, the deleterious effects of  $\beta 1$  integrin loss and beneficial effects of fibrin culture may be mediated through FAK activation.

## 6.2 Limitations

Common to all basic science research are the limitations associated with the use of animal models as surrogates for humans. Rodents are the primary animal used in the studies presented here and despite sharing a large percent of their DNA with humans, significant differences exist. Above all, the organization of pancreatic islets differ, with significantly more alpha cells being in contact with beta cells in humans compared to mice [14]. As well, the glucose-induced electrical activity and insulin release of beta cells is significantly different between mice and humans [15]. These differences should be considered when translating these studies to humans.

One limitation of the conclusions drawn from Chapter 2 and 3 is that the promoter used to control Cre recombinase expression has activity in multiple cell types. The  $\beta 1$  integrin knockout mouse model used here has Cre expression restricted to cells that express collagen 1 $\alpha 2$ . Staining for  $\beta$ -galactosidase showed immunoreactivity in islets and stellate cells, with an eventual loss of  $\beta 1$  integrin in most of the pancreas. Despite this latency, mice lacking  $\beta 1$  integrin developed significant blood glucose dysregulation associated with decreased beta cell mass, improper insulin secretion, and reduced FAK/MAPK/ERK1/2 signaling pathway activity. A recently published study demonstrated that beta cell specific knockout of  $\beta 1$  integrin using the rat insulin promoter showed significantly reduced beta cell mass [16]. This suggests that the lack of specificity of Cre expression, in our mouse model, did not dramatically affect the islet phenotype observed here. However, since  $\beta 1$  integrin loss in the current study was not specific to beta cells, secondary effects that might explain the findings presented here cannot be ruled out.

The main limitation of Chapter 4 is the use of the INS-1 cell line as a surrogate for primary adult beta cells. This cell line is derived from rat insulinoma cells and has been immortalized. It is reminiscent of normal beta cells with respect to propensity to form clusters, secrete insulin in response to glucose, and express the majority of beta cell-associated transcription factors. However, it has significant genetic alterations that render it categorically different than primary beta cells isolated from mice and especially humans.

Finally, two important limitations must be considered for Chapter 5. The first is the use of human samples, which have dramatic diversity between individuals. Inbred mice have minimal inherent differences because they are nearly genetically identical, whereas tissue obtained from different humans have a wide range of gene expression and DNA composition. This causes each human biological replicate to behave differently to stimuli. In the laboratory, this leads to data that is largely insignificant unless a biological replicate number is high enough. Another limitation of our studies using human fetal pancreatic tissue is the timing of sample acquisition. We restricted our studies to the 15-21 week old fetal pancreas, but may have observed more profound effects if we restricted our time point to only a single week, or used one later in development. Unfortunately, there is a significant lack of human fetal pancreata for research purposes, which leads to skewed data.

### 6.3 Future direction

The studies associated with this thesis do not present an entire story. The field of islet biology with respect to cell adhesion receptors has much to be discovered in order to improve upon current islet transplantation techniques.

To improve upon the first study presented in Chapter 2, future experiments should use mice with an inducible Cre-loxP system where the  $\beta 1$  integrin gene is floxed and Cre expression is restricted to beta cells of the pancreas. Mice are commercially available that have tamoxifen-inducible Cre expression regulated by the mouse insulin promoter. Crossing this mouse with the floxed  $\beta 1$  integrin mouse would yield mice that only lose  $\beta 1$  integrin expression in beta cells after tamoxifen injection. This prevents any confounding factors and better elucidates the direct role of  $\beta 1$  integrin in the developed endocrine pancreas.

Alongside, it will be beneficial to isolate islets from these mice and perform in vitro GSIS tests on them. This experiment determines the primary defect in islets and whether or not it is due to dysfunctional insulin secretion.

Future studies for Chapter 3 of this thesis should include use of a mouse model that has Cre expression specified to acinar cells of the exocrine pancreas. Promoters such as amylase or carboxipeptidase-A would specify  $\beta 1$  integrin loss to pancreatic acinar cells, and thus allow investigation of the direct effects of  $\beta 1$  integrin deficiency.

To further investigate the results of Chapter 4, the use of primary adult beta cells or islets isolated from rodents or humans would generate more representative data. As well, it would be of great interest to transplant fibrin-cultured adult beta cells or islets into diabetic mice, to investigate the effects of long-term culture on the in vivo function of endocrine cells.

Finally, future studies for Chapter 5 should focus on increasing the replicate number to ensure the data is a true representation of the human population. Alternatively, sorting cells into various different cell populations before culture would allow for more precise data collection since the effects of fibrin may be specific to cells expressing  $\alpha v \beta 3$ . Alternatively to cell sorting, experiments should use specific differentiation media that drive progenitor cells towards islet-specific gene expression during fibrin culture. These effects could then be analyzed in vivo by transplanting cells into diabetic mice.

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# Appendices

## Appendix 1 Approved animal use protocol

[https://iwc.uwo.ca/iwc\\_static/layout/shell.html?lang=en&2-4.01\\_...](https://iwc.uwo.ca/iwc_static/layout/shell.html?lang=en&2-4.01_...)

Subject: **eSirius Notification - New Animal Use Protocol is APPROVED2008-038-04::5**  
To:  
Cc:

Date: 10/29/12 12:13 PM  
From:



**AUP Number:** 2008-038-04

**PI Name:** Wang, Rennian

**AUP Title:** Pancreatic Beta Cell Development: The Role Of The C-kit And Integrin Receptors

**Approval Date:** 10/29/2012

**Official Notice of Animal Use Subcommittee (AUS) Approval:** Your new Animal Use Protocol (AUP) entitled "Pancreatic Beta Cell Development: The Role Of The C-kit And Integrin Receptors" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2008-038-04::5

This AUP number must be indicated when ordering animals for this project. Animals for other projects may not be ordered under this AUP number. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura  
on behalf of the Animal Use Subcommittee  
University Council on Animal Care

*The University of Western Ontario*  
Animal Use Subcommittee / University Council on Animal Care  
Health Sciences Centre. • London, Ontario • CANADA – N6A 5C1  
PH: .  
Email: • <http://www.uwo.ca/animal/website/>

## Appendix 2 Approved use of human participants



### Use of Human Participants - Ethics Approval Notice

**Principal Investigator:** Dr. Rennie Wang  
**Review Number:** 10060  
**Review Level:** Delegated  
**Approved Local Adult Participants:** 100  
**Approved Local Minor Participants:** 0  
**Protocol Title:** Development of Human Fetal Pancreas  
**Department & Institution:** Physiology, London Health Sciences Centre  
**Sponsor:** Natural Sciences and Engineering Research Council

**Ethics Approval Date:** December 22, 2011      **Expiry Date:** April 30, 2016  
**Documents Reviewed & Approved & Documents Received for Information:**

| Document Name                            | Comments                   | Version Date |
|--|----------------------------|--------------|
| Revised Study End Date                   |                            |              |
| Increase in number of local Participants | Increased from 100 to 200. |              |

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The UWO HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 0000940.

Signature \_\_\_\_\_

#### Ethics Officer to Contact for Further Information

|                   |             |                 |
|-------------------|-------------|-----------------|
| Janice Sutherland | Grace Kelly | Shantel Walcott |
|-------------------|-------------|-----------------|

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Office of Research Ethics

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## Appendix 3 Approval for biosafety



**Researcher: Dr. R. Wang**

**Biosafety Approval Number: BIO-LHRI-0046**

**Expiry Date: May 29, 2014**

May 31, 2011

Dear Dr. Wang:

Please note your biosafety approval number listed above. This number is very useful to you as a researcher working with biohazards. It is a requirement for your research grants, purchasing of biohazardous materials and Level 2 inspections.

Research Grants:

- This number is required information for any research grants involving biohazards. Please provide this number to Research Services when requested.

Purchasing Materials:

- This number must be included on purchase orders for Level 1 or Level 2 biohazards. When you order biohazardous material, use the on-line purchase ordering system ([www.uwo.ca/finance/people/](http://www.uwo.ca/finance/people/)). In the "Comments to Purchasing" tab, include your name as the Researcher and your biosafety approval number.

Annual Inspections:

- If you have a Level 2 laboratory on campus, you are inspected every year. This is your permit number to allow you to work with Level 2 biohazards.

To maintain your Biosafety Approval, you need to:

- Ensure that you update your Biohazardous Agents Registry Form at least every three years, or when there are changes to the biohazards you are working with.
- Ensure that the people working in your laboratory are trained in Biosafety.
- Ensure that your laboratory follows the University of Western Ontario Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories.
- For more information, please see: [www.uwo.ca/humanresources/biosafety](http://www.uwo.ca/humanresources/biosafety).

Please let me know if you have questions or comments.

Regards,

Jennifer Stanley  
Biosafety Coordinator for Western

#### Appendix 4 Antibodies used for experiments

| Primary antibody                                | Dilution | Company, Location   |
|---|----------|---|
| Mouse anti-actin                                | 1:5000   | Sigma; St. Louis, MO, U.S.A.  |
| Rabbit anti-AKT                                 | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Mouse anti-AKT (phospho-Ser473)                 | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Mouse anti-amylase                              | 1:1000   | Santa Cruz; Santa Cruz, CA, U.S.A.                                      |
| Mouse anti-calnexin                             | 1:1000   | BD Biosciences; Mississauga, ON, CA                                     |
| Mouse anti-carboxypeptidase A                   | 1:1000   | AbD serotec; Raleigh, NC, U.S.A.  |
| Mouse anti-caspase-3                            | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Mouse anti-cleaved caspase-3                    | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Mouse anti-cyclin D1                            | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Mouse anti-cytokeratin 19                       | 1:100    | Dako; Glostrup, DEN   |
| Rabbit anti-ERK1/2                              | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Rabbit anti-ERK1/2 (phospho-Thr202/Tyr204)      | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Rabbit anti-FAK                                 | 1:2000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Rabbit anti-FAK (phospho-Tyr397)                | 1:2000   | Abcam; Cambridge, MA, U.S.A.  |
| Rabbit anti- $\beta$ -galactosidase             | 1:100    | Abcam; Cambridge, MA, U.S.A.  |
| Mouse anti-GFAP                                 | 1:100    | PharMingen; Mississauga, ON, CA   |
| Rabbit anti-glucagon                            | 1:100    | Santa Cruz; Santa Cruz, CA, U.S.A.                                      |
| Mouse anti-human mitochondrial surface protein  | 1:50     | Millipore; Billerica, MA, U.S.A.  |
| Guinea pig anti-insulin                         | 1:50     | Zymed; San Francisco, CA, U.S.A.  |
| Rabbit anti-Ki67                                | 1:200    | Abcam; Cambridge, MA, U.S.A.  |
| Mouse anti-laminin                              | 1:50     | DSHB; Iowa City, IO, U.S.A.   |
| Mouse anti-nkx6.1                               | 1:100    | DSHB; Iowa City, IO, U.S.A.   |
| Rabbit anti-p70 <sup>s6k</sup>                  | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Rabbit anti-p70 <sup>s6k</sup> (phospho-Thr389) | 1:1000   | Cell Signaling; Temecula, CA, U.S.A.                                    |
| Rabbit anti-pdx-1                               | 1:1500   | Dr. Christopher Wright; University of Vanderbilt, Nashville, TN, U.S.A. |
| Rabbit anti-PECAM-1                             | 1:100    | Santa Cruz; Santa Cruz, CA, U.S.A.                                      |

|                                  |        |                                      |
|----------------------------------|--------|--------------------------------------|
| Mouse anti-reg-II                | 1:1000 | R&D System; Minneapolis, MN, U.S.A.  |
| Rabbit anti-SOX9                 | 1:50   | Abcam; Cambridge, MA, U.S.A.         |
| Rabbit anti- $\alpha$ v integrin | 1:1000 | Abcam; Cambridge, MA, U.S.A.         |
| Rabbit anti- $\beta$ 3 integrin  | 1:1000 | Abcam; Cambridge, MA, U.S.A.         |
| Rabbit anti- $\beta$ 1 integrin  | 1:1000 | Millipore; Billerica, MA, U.S.A.     |
| Goat anti- $\beta$ 1 integrin    | 1:150  | R&D Systems; Minneapolis, MN, U.S.A. |

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### Appendix 5 Primers used for experiments

| Primer Name | Accession & Definition | Primer Sequence  | Size (bp) |
|-------------|------------------------|--|-----------|
| Amy1        | NM_007446.2            | F: 5'- CAA GTG GAG GTA TCG AGA AC -3'<br>R: 5'- CCC TGC TAC GCC AAT GTC AA -3'   | 149       |
| Colla1      | NM_007742.3            | F: 5'- CCT CCC AGT GGC GGT TAT GAC -3'<br>R: 5'- GGG TTC GGG CTG ATG TAC CAG -3' | 383       |
| Colla2      | NM_007743.2            | F: 5'- AGG GTG AAA CTG GTC TCC GA -3'<br>R: 5'- CCC TCC ATC ACC ACG ACT TC -3'   | 373       |
| Fn1         | NM_101233.1            | F: 5'- AAC TAC GAT GCC GAT CAG AA -3'<br>R: 5'- CCC TCC TCG TGA CGC TGG TG -3'   | 263       |
| GLUCAGON    | NM_002054.2            | F: 5'- GAT GAA CGA GGA CAA GCG CC -3'<br>R: 5'- CCT TTC ACC AGC CAA GCA AT -3'   | 240       |
| INSULIN     | NM_000207.1            | F: 5'- TAC CTA GTG TGC GGG GAA CG -3'<br>R: 5'- CTG CGG GCT GCG TCT AGT TG -3'   | 229       |
| Insulin I   | NM_019129.1            | F: 5'- ACA CCC AAG TCC CGT CGT GA -3'<br>R: 5'- TGA TCC ACA ATG CCA CGC TT -3'   | 131       |
| Insulin II  | NM_019130.1            | F: 5'- ATG GCC CTG TGG ATC CGC TT -3'<br>R: 5'- TGC CAA GGT CTG AAG GTC AC -3'   | 243       |
| ITGA3       | NM_002204.1            | F: 5'- AGA TGC GGG CAG CCT TCG TG -3'<br>R; 5'- GTT TTC ATG CCA GAC TCA CC -3'   | 484       |
| Itga3       | NM_001108292.1         | F: 5'- CTG CTA GTA GGG AGC CTG TCG -3'<br>R: 5'- TGT CAG CCT CCA CTG TGT AAG -3' | 223       |
| ITGA5       | NM_002205.2            | F: 5'- GGC CAG CCC TAC ATT ATC AG -3'<br>R: 5'- GGT TCA CGG CAA AGT AGT CA -3'   | 303       |
| Itga5       | NM_001108118.1         | F: 5'- ACC AGA GCA AGA GCC GGA TAG -3'<br>R: 5'- GGT TCA CCG CGA AGT AGT CA -3'  | 288       |
| Itga8       | NM_001173972.1         | F: 5'- CCC GTG TCT CTG GCG TTC AA -3'<br>R: 5'- GAT ATC CGG CTG GCT AGT GT -3'   | 162       |
| ITGAV       | NM_002210.2            | F: 5'- GGA GCA ATT CGA CGA GCA CT -3'<br>R: 5'- ACT AAT GTT AGC AGG CGT GA -3'   | 246       |

|       |                |  |     |
|-------|----------------|--|-----|
| ItgaV | NM_001106549.1 | F: 5'- TTA TGC CAA AGA TGA CCC ACT -3'   | 287 |
|       |                | R: 5'- CGG GAC CTC CAA GAA GTA CTC -3'   |     |
| ITGB1 | NM_002211.2    | F: 5'- AGA GAG CTG AAG ACT ATC CCA T -3' | 313 |
|       |                | R: 5'- CGC TGT TTT CCA ACA AGT TC -3'    |     |
| Itgb1 | NM_017022.2    | F: 5'- GTT GGT CAG CAG CGC ATA TC -3'    | 369 |
|       |                | R: 5'- GCC CAC TGC TGA CTT AGG AA -3'    |     |
| ITGB3 | NM_000212.2    | F: 5'- AAG ATT GGA GAC ACG GTG AG -3'    |     |
|       |                | R: 5'- AGT ACT TGC CCG TGA TCT TG -3'    |     |
| Itgb3 | NM_153720.1    | F: 5'- TGG CAA GAT CAC CGG CAA GT -3'    | 120 |
|       |                | R: 5'- CAG TCC GAG TCA CAC ACG CA -3'    |     |
| Itgb5 | NM_47139.2     | F: 5'- TGG TGT TCA CGA CGG ACG AC -3'    | 148 |
|       |                | R: 5'- AGC AAG GCA AGC GAT GGA TA -3'    |     |
| Lama1 | NM_008480.2    | F: 5'- GGC GGT ATA ACA ACG GAA CC -3'    | 180 |
|       |                | R: 5'- GCA ATC CAC CCA CGT AAA TC -3'    |     |
| Lamb1 | NM_010721.2    | F: 5'- CGA GAG TAT GAG GCG GCA CT -3'    | 285 |
|       |                | R: 5'- CAC TTC CAC CAA GCG GGT CT -3'    |     |
| Lamc1 | NM_010683.2    | F: 5'- TAG CCA AAT TAG CCG ACT GC -3'    | 122 |
|       |                | R: 5'- GCT CCC TGG AGG CGA TCT CA -3'    |     |
| PDX1  | NM_001108292.1 | F: 5'- CTG CTA GTA GGG AGC CTG TCG -3'   | 223 |
|       |                | R: 5'- TGT CAG CCT CCA CTG TGT AAG -3'   |     |
| Pdx1  | NM_022852.3    | F: 5'- GGC TTA ACC TAA ACG CCA CA -3'    | 175 |
|       |                | R: 5'- AGA GTC CCA GAG GCA GAC CT -3'    |     |
| 18S   | NR_046237.1    | F: 5'- GTA ACC CGT TGA ACC CCA TTC -3'   | 151 |
|       |                | R: 5'- CCA TCC AAT CGG TAG TAG CG -3'    |     |

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## Appendix 6 Curriculum Vitae

### Matthew Riopel

#### Education:

##### Western University, London, ON

- Ph.D. Pathology Graduate Program 2009-2014
- B.M.Sc. Biochemistry and Medical Sciences 2004-2009

#### Research Experience:

##### Western University, London, ON

**Project:** Investigating the role of  $\beta$ 1 integrin in the endocrine and exocrine murine pancreas

- Articles published in *J Path*, 2011 and *Lab Invest*, 2012

**Project:** Examining fibrin-integrin interactions in support of  $\beta$  cell function, differentiation, survival and proliferation

- Articles published in *Acta Biomater*, 2013 and submitted to *Biomaterials* (MS#jbmt27573)

#### Publications:

##### Peer-reviewed Articles

1. **Riopel M**, Li J, Trinder M, Fellows G and Wang R. Fibrin enhances human fetal islet-epithelial cell differentiation via p70<sup>s6k</sup> and promotes vascular formation during transplantation. *Submitted to Biomaterials* (MS#jbmt27573)

2. **Riopel M**, Stuart W and Wang R. Fibrin improves beta (INS-1) cell function, proliferation and survival through integrin  $\alpha\beta 3$ . *Acta Biomater* 9(9):8140-8148 (2013)
3. **Riopel M**, Li J, Liu S, Leask A and Wang R.  $\beta 1$  integrin-extracellular matrix interactions are essential for maintaining exocrine pancreas architecture and function. *Lab Invest* 93:31-40 (2013)
4. Feng ZC, **Riopel M**, Li J, Donnelly L and Wang R. Down-regulation of Fas activity rescues early onset of diabetes in c-Kit<sup>Wv/+</sup> mice. *Am J Physiol Endocrinol Metab* 304(6):E557-565 (2013)
5. Feng ZC, Donnelly L, Li J, Krishnamurthy M, **Riopel M** and Wang R. Inhibition of GSK3 $\beta$  activity improves  $\beta$ -cell function in c-Kit<sup>Wv/+</sup> male mice. *Lab Invest* 92:543-555 (2012)
6. Feng ZC, Li J, Turco B, **Riopel M**, Yee S-P and Wang R. Overexpression of c-Kit protects against diabetes through direct effects on pancreatic beta cells in mice. *Diabetologia* 55(8):2214-2225 (2012)
7. **Riopel M**, Krishnamurthy M, Li J, Liu S, Leask A and Wang R. Conditional  $\beta 1$  integrin-deficient mice display impaired pancreatic  $\beta$ -cell function. *J Pathol* 224:45-55 (2011)

#### **Peer-reviewed Review Articles**

8. **Riopel M** and Wang R. Collagen Matrix Support of Pancreatic Islets. *Front Biosci* (Landmark Ed). 19:77-90 (2014)

#### **Manuscripts in Preparation**

9. **Riopel M**, Li J, Fellows G, Goodyer C and Wang R. Human fetal pancreatic ultrastructural development from 8 to 21 weeks.
10. **Riopel M**, Trinder M and Wang R. Fibrin in tissue engineering and the future of islet transplantation. *Review*.
11. Feng ZC, **Riopel M** and Wang R. Role of c-Kit/Stem Cell Factor in Pancreatic Islet Development and Function. *Review*.

## Peer-reviewed Abstracts

12. **Riopel M**, Li J, Stuart W and Wang R. Fibrin modulates  $\alpha v\beta 3$  integrin expression and FAK signalling to mediate islet cell differentiation, function, proliferation and survival. *Diabetologia* 56: Suppl 1:S222 (2013)
13. **Riopel M**, Li J, Stuart W and Wang R. Fibrin-integrin support of  $\beta$ -cell differentiation, function and proliferation. *Diabetologia* 55: Suppl 1:S179 (2012)
14. Feng ZC, **Riopel M**, Li J, Donnelly L and Wang R. Improved  $\beta$ -cell proliferation and function in c-Kit<sup>Wv/+</sup>;Fas<sup>lpr/lpr</sup> double mutant mice. *Diabetes* 60: Suppl 1:A28 (2011)
15. **Riopel M**, Krishnamurthy M, Liu S, Li J, Leask A and Wang R. Conditional  $\beta 1$  integrin knockout affects glucose metabolism and Pdx-1 expression and FAK/MAPK/ERK signaling. *Diabetes* 59:Suppl1, A449 (2010)

## Professional Experience:

### Teaching Assistant, Department of Pathology, Western University, ON 2011-2014

- **Courses:** Pathology 4400B (Environmental Pathology), Pathology 4200A (Concepts in the Pathogenesis of Human Diseases)
- Had an essential role in marking assignments, responding to student questions and proctoring exams

### Supervisory Experience, Children's Health Research Institute, ON 2011-2014

- Supervised undergraduate students by organizing and delegating tasks for a project in support of the honours project courses Pathology & Toxicology 4980E and Physiology and Pharmacology 4980E
- Lectured for the Schulich School of Medicine and Dentistry 5161 course Gastrointestinal Physiology

### Manuscript Reviewer

2010-2014

- Revised manuscripts considered for publication by commenting on research quality and experimental design of studies for the academic journals: *Diabetes, American Journal of Physiology – Endocrinology and Metabolism, Current Medicinal Chemistry, Health Science Inquiry* and *Islets*

### **Committee Membership**

**2010-2014**

- Candidate Selection Subcommittee – Schulich School of Medicine and Dentistry, Western University
  - Evaluated prospective executive candidates by mediating group discussions and conducting interviews
- Graduate Education Committee – Department of Pathology, Western University
  - Improved the Pathology graduate education program by mediating course changes and setting evaluation criteria for bursaries
- Western Pathology Association – Chair
  - Drafted the Association’s Terms of Reference and organized social, academic and professional events in support of graduate student development
- Society of Graduate Students – Bursaries and Subsidies Committee Chair
  - Allocated funds up to \$10,000 to graduate students by setting and evaluating specific criteria
- Society of Graduate Students – Pathology Councilor
  - Represented students in the Pathology Department and improved programs like the the graduate student health plan, bus pass and others
- Lawson Association of Student and Fellows President
  - Organized social and professional events for students and fellows of the institute

### **Awards and Honours**

- 2<sup>nd</sup> place Poster Presentation, 4<sup>th</sup> Annual Diabetes Research Day, ON, CAN 2013
- Cameron Wallace Award, Department of Pathology, Western University, ON 2013

- Ontario Graduate Scholarship, Ministry of Training, Colleges & Universities,  
ON 2012-2013
- 2<sup>nd</sup> place Platform Presentation, London Health Research Day, ON, CAN 2013
- Western Graduate Research Scholarship, Western University, ON, CAN 2009-2014
- Graduate Thesis Research Award, Western University, ON, CAN 2012
- 1<sup>st</sup> place Poster Presentation, London Health Research Day, ON, CAN 2012
- 1<sup>st</sup> place Poster Presentation, 1<sup>st</sup> Annual Diabetes Research Day, London, ON 2010