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Collagen IV-β1Integrin Regulation of Exocytotic Machinery in Pancreatic Beta-Cell Insulin Secretion

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Abstract

Diabetes is a prevalent metabolic disease characterized by impaired insulin secretion, action, or both. β1-integrin is a key receptor that regulates cell-ECM interactions and is important in maintaining beta-cell functions, including insulin secretion. However, little is reported about the relationship between β1-integrin and the exocytotic proteins involved in insulin secretion. This study examined the influence of ECM-mediated β1-integrin activation on exocytotic machinery involved in insulin secretion using rat insulinoma (INS-1) cells. Collagen IV (COL IV) promoted INS-1 cell adhesion, spreading, and insulin secretion. Additionally, these cells displayed changes in levels and localization of exocytotic proteins involved in insulin secretion. β1-integrin antibody blocking on cells cultured on COL IV showed significantly reduced adhesion, spreading, and insulin secretion along with reduced exocytotic protein levels. Additionally, β1-integrin blocking influenced the localization of exocytotic proteins at varied time points of glucose stimulation. These results indicate that specific ECM-integrin interactions are critical for proper beta-cell function.

Keywords: β1-integrin, exocytotic proteins, extracellular matrix, collagen IV, INS-1 cells, beta-cell, glucose-stimulate insulin secretion

Summary for Lay Audience

Diabetes is defined as chronic high blood sugar resulting from impaired insulin release, action, or both. Insulin is a hormone produced by beta-cells, a pancreatic cell type, which functions to decrease blood sugar. Islet transplants and bioartificial pancreas replacements are techniques under investigation for the treatment of diabetics. However, there are many shortcomings with these technologies which have been suggested to be, in part, due to the loss of the normal external cellular environment. The environment of a cell plays a major role in its function. Integrins are a family of proteins found on the outside of cells which communicate information about the environment to the cell and vice versa. β1-integrins comprise a major family of integrin receptors, and β1-integrin signalling in beta-cells is well known to be important for insulin secretion. However, it is not known how β1-integrin influences the machinery required for insulin secretion. This study aimed to determine how β1-integrin signaling influences the machinery involved in insulin secretion to enhance insulin secretion. The INS-1 rat beta-cell line was used as it expresses β1-integrin and secretes insulin similar to normal physiology.

INS-1 cells were examined following culture on different protein ligands of β1-integrin. Of the different proteins, collagen IV was found to provide the greatest enhancement of glucose stimulated insulin secretion (GSIS). We then looked specifically at the machinery involved in insulin secretion and found increases in both overall protein amounts and where they were located within cells. To validate that the changes we were observing were due to interactions between collagen IV and β1-integrin, β1-integrin was blocked prior to culturing on collagen IV. Cells that had β1-integrin blocked had decreased GSIS. Furthermore, these cells have alterations in the machinery involved in insulin secretion. Overall, this adds a novel method by which β1-integrin

influences insulin secretion and underscores the importance of collagen IV-β1-integrin interactions in the proper functioning of beta-cells.

Co-Authorship Statement

The methodology described in Chapter 2 was conducted primarily by Malina Barillaro in the lab of Dr. Rennian Wang. Meg Schuurman provided assistance with immunofluorescence staining, ELISA, and INS-1 cell maintenance. Gurleen Sahi assisted with immunofluorescence staining. Studies were designed by Dr. Rennian Wang, who also provided insight in data interpretation, and thesis edits.

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List of Abbreviations

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Bag3	Bcl2-associated athanogene 3
BM	Basement membrane
BSA	Bovine serum albumin
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
GLUT	Glucose transporter
GSIS	Glucose stimulated insulin secretion
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IgM	Immunoglobulin M
IM	Interstitial matrix
KO	Knock-out
mRNA	Messenger RNA
ºC	Degrees Celsius
PBS	Phosphate buffered saline

PFA	Paraformaldehyde
PM	Plasma membrane
ROI	Region of interest
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNAP	Soluble NSF attachment protein
SNARE	Soluble N-ethylmaleimide sensitive-factor attachment protein receptor
t-SNARE	Target membrane SNARE
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TRITC	Tetramethyl rhodamine isothiocyanate
v-SNARE	Vesicle membrane SNARE
VAMP	Vesicle-associated membrane protein

Chapter 1

1. Introduction

1.1. Significance of thesis

β1-integrin has proven to be important in many aspects of beta-cell function and survival (17, 26, 85), and it has been shown that β1-integrin plays a multifaceted role in the regulation of insulin secretion (39, 51, 90). There is a great deal of research focusing on the influence of insulin secretion by β1-integrin, the cytoskeleton, and Soluble N-Ethylmaleimide Sensitive-Factor Attachment Protein Receptor (SNARE) proteins individually (43, 89, 90, 107, 113). However, little to no reports reveal information regarding interplay between β1-integrin and SNARE proteins in regulating insulin exocytosis. Our lab has previously shown that blocking β1-integrin on INS-1 cells cultured on collagen IV caused reduced insulin secretion (51). Additionally, mice with beta-cell specific β1-integrin knockout (KO) display impaired insulin secretion and decreased islet SNARE mRNA expression (78). This project aimed to explore the relationship between β1-integrin activation by distinct extracellular matrix (ECM) protein ligands and SNARE proteins in the regulation of insulin secretion. The findings from this study expand on the understanding of how the extracellular environment influences beta-cell activity, specifically how ECM-β1-integrin interactions regulate exocytotic machinery involved in insulin exocytosis. Understanding the effects of extracellular interactions on insulin secretion can aid development of islet transplantation and bioartificial endocrine pancreas therapies, and thus seeks to improve islet-cell based treatments for diabetes.

1.2. The Pancreas

The pancreas is a critical organ with roles in digestion and metabolic homeostasis. These roles are regulated by specialized exocrine and endocrine tissue compartments that synthesize and secrete digestive enzymes or glucose homeostasis hormones, respectively. Although both functions are critical for normal physiology, majority of the pancreas is comprised of exocrine tissue while only around one percent of the pancreas is endocrine tissue. This endocrine tissue is found in clusters, known as islets of Langerhans, scattered throughout the pancreas which increase in density from head to tail (proximal to distal) (94). Within each islet, five different hormone-producing cell types exist; glucagon producing alpha-cells, insulin producing beta-cells, pancreatic polypeptide producing PP-cells, somatostatin producing delta-cells, and ghrelin producing epsilon-cells (88). The cell type ratio and organization within islets varies between species, however, across species beta-cells are conserved as the major islet cell type (10, 79). The main role of beta-cells is to secrete insulin in response to hyperglycemia. Once in circulation, insulin acts on peripheral tissues to promote the uptake of glucose, thus facilitating a decrease in blood glucose levels.

1.2.1. *Diabetes Mellitus*

Diabetes mellitus is a metabolic disease characterized by hyperglycemia due to impaired insulin secretion, action, or both. According to the International Diabetes Federation, 451 million people lived with diabetes in 2017, a number expected to rise to 692 million by 2045 (15). Type 1 diabetes (T1DM) is an autoimmune disease whereby pancreatic beta cells are destroyed and accounts for approximately 5-10% of diabetic patients (124). Type 2 diabetes (T2DM) is a

progressive disease often associated with obesity and is characterized by insulin resistance that can ultimately lead to beta cell failure (124).

In addition to the rigorous monitoring of hyperglycemic events in patients, hypoglycemia and secondary complications remain major concerns for diabetic patients even with well-controlled glycemia. Therefore, current research efforts work to develop treatments that can maintain euglycemia in a manner similar to normal physiology (80). One rigorous treatment protocol currently available to T1DM patients that have complications with blood glucose regulation is pancreatic islet transplantation. Although this treatment is successful, its shortcomings include a shortage on donors, suboptimal long-term efficacy, and the requirement of immunosuppressive therapy (7). To circumvent these limitations, research is also being done to develop a bioartificial endocrine pancreas, a device of islets, stem-cell derived or xenogeneic, encapsulated in a semipermeable immune-protective membrane (80). Ideally this would provide long-term euglycemia, in a less invasive manner and without immunosuppression or donor requirement (7). Unfortunately, current attempts at a bioartificial endocrine pancreas have revealed several challenges including diffusional distance, sufficient vascularization, and continued requirement of immunosuppression (80). Many factors influence the shortcomings of both islet isolation and bioartificial pancreas procedures. However, it has been hypothesized that the lack of a proper ECM is a major contributor and that the restoration of these components can recapitulate the physiological endocrine pancreas (12).

1.3. Insulin Secretion

The production and release of insulin from pancreatic beta cells, and its major role in glucose homeostasis was discovered 100 years ago by Sir Frederick Banting and Charles Best. The process

of glucose-stimulated insulin secretion (GSIS) begins with increased blood glucose levels and glucose uptake into beta-cells through the glucose transporter (GLUT) 1 (human) or 2 (rodent). Next, glycolysis ensues resulting in an increased ATP:ADP ratio (62). ATP acts on the ATP-sensitive potassium (K_{ATP}) channel, a channel which normally functions to maintain resting membrane potential, leading to its closure and subsequent membrane depolarization (18). This depolarization activates L-type voltage-gated Ca^{2+} channels, which increases intracellular Ca^{2+} levels and stimulates fusion of insulin-containing granules with the plasma membrane (PM) via SNARE complexes (119) (**Figure 1.1**). Insulin secretion occurs in a biphasic pattern consisting of a transient robust first phase lasting around 10 minutes followed by a second phase of sustained release lasting 30 minutes to a few hours (24).

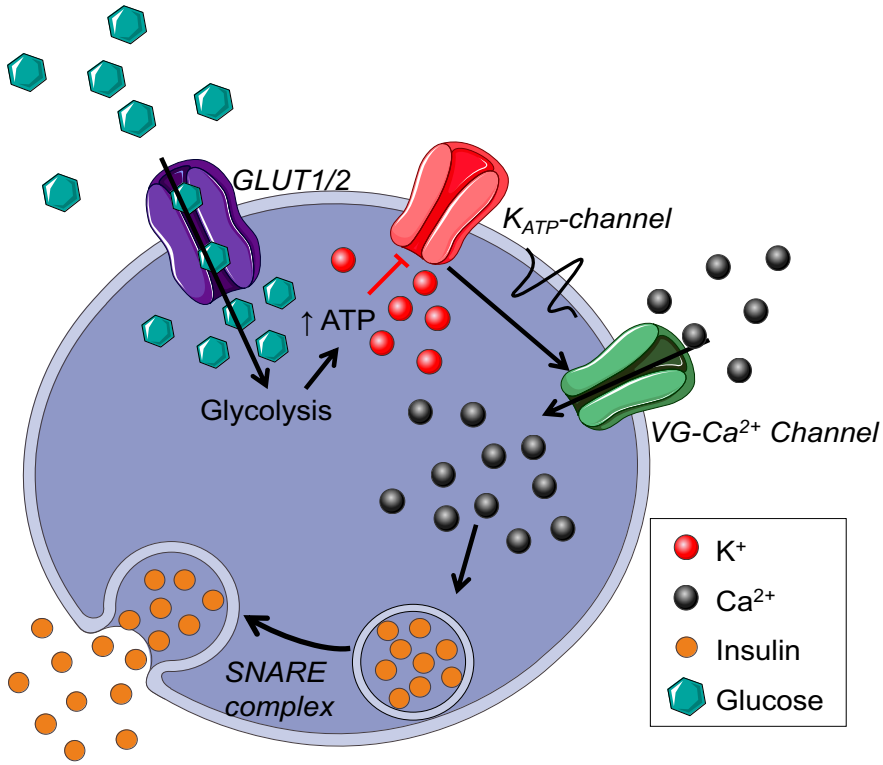


Figure 1.1: A diagrammatic representation of glucose stimulated insulin secretion from pancreatic beta cells.

The figure was adapted from Wang and Thurmond 2009 (113) and modified by MB using Servier Medical Art Templates (https://smart.servier.com/).

1.3.1. *SNARE Proteins*

SNARE proteins are transmembrane and membrane-associated proteins involved in mediating membrane fusion. They can be categorized into two groups based on the membrane they associate with: target-SNARES (t-SNARES) associated with the PM, or vesicle-SNARES (v-SNARES) associated with the vesicle membrane (96). There are many types of SNARE proteins found in mammalian cells; they share a common SNARE motif involved in formation of the SNARE complex which mediates membrane fusion (118). In the complete SNARE core complex there are two t-SNARE proteins (a SNAP and a Syntaxin) and one v-SNARE protein (vesicle-associated membrane protein (VAMP)), that interact with another via SNARE motifs (113). The major SNARE proteins involved in beta-cell insulin exocytosis are SNAP25, Syntaxin1A, and VAMP2 (118) (**Figure 1.2**).

In addition to the core SNARE complex proteins, the Munc18 family of proteins are associated with Syntaxins and play a critical role in the formation of the SNARE-complex (86). Beta-cells express three Munc18 isoforms Munc18-1, -2, and -3 (70, 71). Munc18 proteins are well documented to interact with Syntaxins. Munc18-1 and Munc18-2 pair with PM-localized Syntaxins 1-3, while Munc18-3 only pairs with Syntaxin4 (101, 102). Studies of beta-cell specific Munc18-1 deficiency in mice demonstrated impaired first phase of insulin secretion (70), and depletion of Munc18-2 in rat islets disabled SNARE complex formation causing dysfunctional beta-cell insulin exocytosis (55). Additionally, Munc18-1 is suggested to play a role in assisting the conformational change of Syntaxin1A from its closed to open state that occurs following glucose stimulation (4, 118).

In a basal state, the key SNARE proteins remain unattached, and Munc18-1 is in a complex with Syntaxin1A in its closed state (**Figure 1.2A**). Under glucose stimulation the Syntaxin1A-Munc18-1 interaction is altered and Syntaxin1A undergoes a conformational change to its open state (19). Additionally, insulin vesicles are transported to the PM thus facilitating SNARE complex formation and membrane fusion (**Figure 1.2B**).

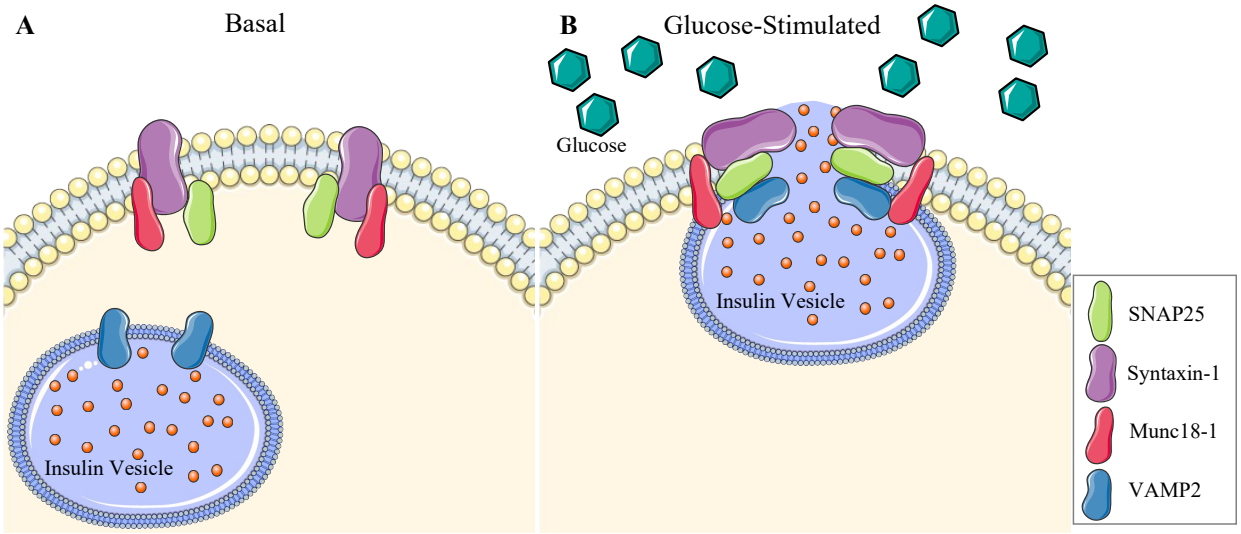


Figure 1.2: A simplified schematic of SNARE-mediated insulin vesicle exocytosis in beta-cells.

(**A**) In a basal state, SNARE proteins SNAP25, Syntaxin1A, and VAMP2 remain unattached, and Munc18-1 is bound to Syntaxin1A in its closed conformation. (**B**) With glucose stimulation, Syntaxin is released from its closed conformation and the insulin vesicle is brought to the membrane. This allows for Syntaxin1A, SNAP25, and VAMP2 to join, resulting in membrane fusion and subsequent insulin exocytosis. The figure was adapted from Rorsman and Renström 2003 (91) and modified by MB using Servier Medical Art Templates (https://smart.servier.com/).

1.3.2. *Cytoskeleton Role in Insulin Secretion*

The association between insulin granule secretion and the cytoskeleton has been observed as early as the 1960s (54). Prior to exocytosis, insulin granules are translocated to the membrane, a process which relies on microtubules and F-actin remodeling (69). In an unstimulated state, cortical F-actin in beta-cells sits below the PM in a dense network where it is suggested to block granule movement (90, 106) (**Figure 1.3A**). This network is disrupted with glucose stimulation allowing insulin secretion to occur through SNARE-mediated vesicle exocytosis (43, 72, 106) (**Figure 1.3B**). In support of this, insulin granule secretion is observed when cells are treated with latrunculin B, an actin disrupting agent (106). Furthermore, in MIN6 cells, glucose, but not KCl, administration diminished cortical actin localization, a finding that remained even with jasplakinolide treatment, an actin polymerizing agent (69). Thus, glucose plays a major role in actin cytoskeleton alterations observed during insulin secretion.

Insulin granule localization and secretion has also been suggested to be influenced by cytoskeleton-SNARE protein interactions. Microtubules are responsible for transporting insulin granules from the cell interior to the periphery where F-actin, but not G- actin, brings the granules in close proximity to t-SNARE complexes (113) (**Figure 1.3A**). In MIN6 cells and isolated mouse islets, jasplakinolide treatment increased insulin granule localization to the PM, and only with the addition of glucose, not KCl, was a visible diminishment in cortical actin and decrease in Syntaxin1A and F-actin interactions observed (69). Thus, during beta-cell activation and insulin secretion, signaling upstream of the K_{ATP} channel appears to be required for mediating changes in the actin cytoskeleton and its interaction with Syntaxin1A. Once this occurs the subsequent increase in intracellular Ca^{2+} can further enhance insulin granule secretion.

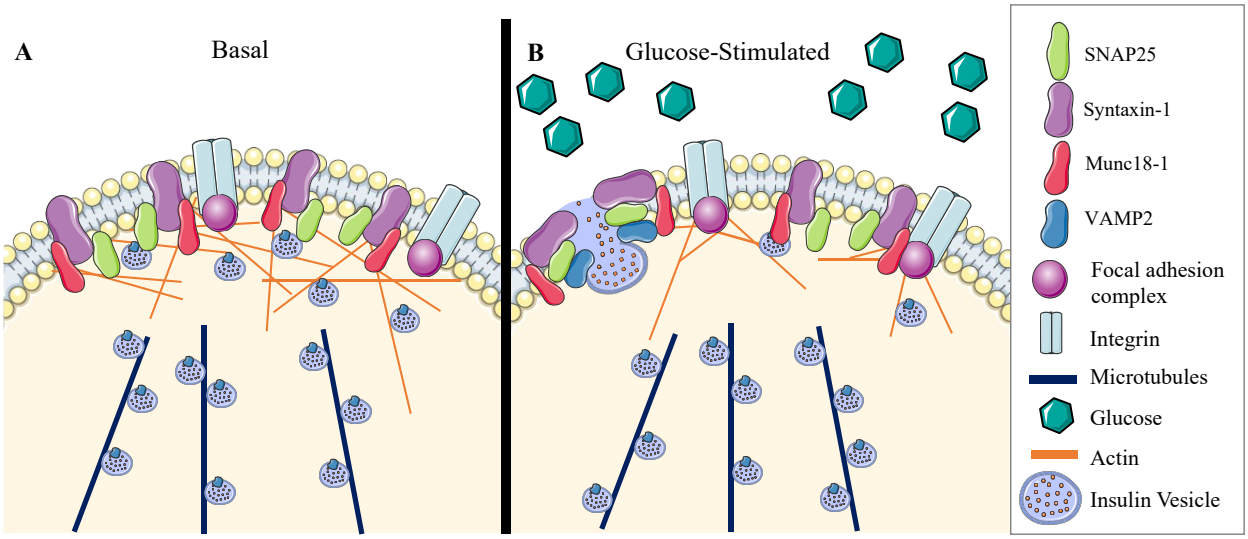


Figure 1.3: A simplified schematic of cytoskeletal involvement in beta-cell insulin secretion

(**A**) In the basal state, microtubules are responsible for transporting insulin granules from the cell interior to the periphery. F-actin sits in a dense network at the plasma membrane where it has been demonstrated to form a complex with Syntaxin1A and block granules from reaching the PM. (**B**) Upon stimulation, the actin network is disrupted and Syntaxin1A is released making SNARE complexes at the PM available for vesicles to bind to and facilitating insulin secretion. The figure was adapted from Wang and Thurmond 2009 (113) and modified by MB using Servier Medical Art Templates (https://smart.servier.com/).

Actin remodeling is commonly regulated by small Rho-family GTPases. This family of proteins includes Rho, Rac, and Cdc42. Cdc42 has been found to be of particularly importance during GSIS, localizing with insulin granules, and associating with Syntaxin 1A and VAMP2 where it is hypothesized to be involved in targeting granules to "active sites" at the PM for exocytosis (1, 65, 69, 113). Cdc42 is also a protein recruited to integrin activation sites (1, 65) providing evidence of a link between integrins and SNARE proteins in the regulation of insulin secretion.

Another mechanism by which F-actin is thought to mediate insulin secretion is through its association with Syntaxin1 and Syntaxin4, both of which have been shown to be involved in insulin secretion (97). Specifically, F-actin associates with Syntaxin1 and Syntaxin4 at basal conditions, and following glucose stimulation and actin changes, this interaction is significantly reduced (43, 97, 106). Selective competitive disruption of F-actin-Syntaxin4 binding in MIN6 cells enhances GSIS by increasing PM granule accumulation and Syntaxin4 accessibility (43). It is hypothesized that the binding of Syntaxin4 to F-actin prevents granule docking, while glucose-induced binding of Syntaxin4 to granules promotes SNARE complex formation and vesicle fusion (43). Interestingly, simply releasing Syntaxin4 from F-actin linkage is not sufficient for SNARE complex formation, indicating involvement of other regulatory mechanisms in the assembly of the SNARE complex (43).

1.4.Extracellular Matrix

Tissues and organs contain a non-cellular component known as the extracellular matrix (ECM) (103). Broadly, the ECM has the following main functions: (1) act as a scaffold to support cells, (2) communicate information about the environment to the cell and (3) influence cell behaviours

such as development, cell adhesion, and homeostasis (103). There are two classes of ECM: the interstitial matrix (IM) which surrounds cells, and the pericellular matrix which is in close contact with cells (i.e., basement membranes (BMs)). ECM composition varies between tissue types, demonstrating specialized roles in cell function. Commonly found ECM components include collagens, elastin, fibronectin, laminins, glycoproteins, proteoglycans and glycosaminoglycans (103). In addition to structural proteins, the ECM houses hormones, growth factors, and cytokines all of which influence cellular behavior (5).

1.4.1. *Pancreatic ECM*

Within the pancreas, there is a great deal of BM surrounding islets, vessels and acinar cells, with minimal IM present (8). At the endocrine-exocrine interface, the BM consists of a peri-insular capsule which has roles in directing morphogenesis during embryonic development as well as tissue regeneration after injury (38). Both human and mouse peri-islet BMs have been reported to contain collagen IV, agrin, perlecan, and laminins, although specific isoforms vary between species (8). The interstitial ECM is a highly variable network of ECM proteins and polysaccharides located between islet cells, containing collagens I, II, III and VI, fibronectin, fibrillin-2 and matrilin-2 (8). Additionally, a laminin-rich ECM is also present in the endothelial BM of islet capillaries (2, 8). Thus, pancreatic islets are in a complex extracellular environment that influences cellular activity.

The ECM facilitates several functions within beta-cells during development and post-natal life. In support of its various functions, ECM composition has been found to vary at different stages of development (111, 121). Islet precursors express vitronectin, fibronectin and collagen IV and when islet formation begins, collagen IV and laminin act as the major ECM components (2).

Functionally, ECM components have been shown to improve cell adhesion, survival and insulin release in mature beta-cells (112). The ECM has also been suggested to be important for maintenance of beta-cell polarity, aiding in providing insulin secretion toward the vasculature (2, 26).

1.4.2. *Fibronectin*

Fibronectin is a glycoprotein found as a soluble component of plasma and an insoluble portion of the ECM (74). It has numerous interactions with integrins and its major receptor is α5β1 integrin (60, 74). Fibronectin is an important ECM component that supports pancreatic islets. Isolated porcine islets incubated with a fibronectin-mimicking peptide designed to bind to α5β1 was found to increase fibronectin production indicating a potential for isolated islets to generate their own ECM with appropriate integrin receptor activation (6). Additionally, culture on a fibronectin substrate increased expression of islet cell markers in the differentiation of hepatic oval cells, which demonstrates that fibronectin may also be important for maintaining an islet endocrine cell phenotype (56).

1.4.3. *Laminins*

Laminins comprise a family of glycoproteins most prominently found in BMs (20). Evidence of laminin isoform expression within pancreatic vessel BMs has been found, thus interaction with islet cells can occur (44). Laminin appears to play a role in promoting the development of islet cells (25, 111). Additionally, human mesenchymal stem cells cultured in insulin-producing cell induction and differentiation medium had increased expression of pancreatic precursor cell markers and genes governing insulin expression in the presence of laminin 411 (82). Moreover,

when these cells were transfused into streptozotocin (STZ)-treated rats, a treatment that eliminates the majority of endogenous beta-cells and induces severe hyperglycemia, a rapid and significant drop in fasting blood glucose, improved symptoms and survival, and significantly decreased HbA1c was observed (82). These findings may indicate that enriching developing endocrine cells with laminin can help specify beta-cell fate. It has also been demonstrated that laminin improves islet function and survival. Human islets encapsulated in collagen IV combined with specific laminin sequences had improved survival compared to islets in capsules without ECM proteins, even when exposed to inflammatory cytokines which are known to impair beta-cell function (57).

1.4.4. *Collagens*

Collagens are the most abundant ECM protein in vertebrates (92). As with the other ECM proteins, collagens have been shown to play a major role in numerous cell functions. There are two collagens of focus in this thesis: collagen I and collagen IV.

Collagen I is the most highly expressed collagen throughout the body and is notably found in bone, ligaments, skin and arteries (27, 76). It forms organized fibrils that provide strong structural support as well as a point of cellular attachment (76). Collagen I has been suggested to play a role in the organization of islets during development. Islets isolated from neonatal rat pancreases were cultured on collagen I-coated dishes and covered with an overlay of gelling collagen I solution. This induced reorganization of endocrine cells into three-dimensional islet-like organoids in a manner which maintained the characteristic distribution of cell types found *in vivo* (64). Collagen I has also been found to promote function in developed islets. The incubation of isolated Wistar rat islets with collagen I alone, or with collagens I and IV, was found to improve glucose stimulated insulin secretion for up to 11 days *ex vivo* (66).

Collagen IV is the major collagen found in BMs where it forms complex networks that have been demonstrated to influence cell adhesion, migration, and differentiation (49, 76). Collagen IV has been noted to act as a binding substrate for multiple cell types via integrin and non-integrin receptors (49). In pancreatic islets, collagen IV has been found to be present within the peri-islet capsule and can directly interact with islet cells (40). This interaction has been demonstrated to be important for beta-cell function. Islets isolated from mice and seeded on collagen IV-modified scaffolds were found to significantly reduce the time required to achieve post-transplantation euglycemia when transplanted in STZ-induced diabetic mice (120). Additionally, these scaffolds were found to reduce early-stage apoptosis and improve viability (120). Islets isolated from human fetal and adult pancreases were found to have significantly increased migration when cultured on collagen IV compared to other ECM proteins (47). Human fetal islets also demonstrated a significant increase in insulin secretion when cultured on collagen IV-coated plates compared to poly-L-lysine and bovine serum albumin (BSA)-coated surfaces. This increase was determined to be mediated in part through $\alpha 1\beta 1$ signaling (47). Our lab has previously shown INS-1 cells cultured on collagen IV-coated plates display increased cell adhesion, viability, and insulin secretion as a result of $\beta 1$-integrin signaling, further supporting that specific collagen-integrin interactions are important for beta-cell survival and function (51).

1.5. Integrins and ECM

For the ECM to influence cellular fate, cells require specialized receptors that provide the connection between the external and internal environments. One major family of receptors that provides such interactions are integrins. Integrins are dimeric cell adhesion molecules consisting of α and β subunits that mediate cell-ECM and cell-cell interactions (37). Having both extracellular

and cytoplasmic domains allows integrins to mediate “inside-out” and “outside-in” signaling (53). They link extracellular components to the cytoskeleton and stimulate intracellular signaling pathways imperative for function. Signaling through integrins is often achieved via focal contacts/adhesions where focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, is recruited and interacts with various signaling molecules (89). In beta-cells, integrin signaling through FAK has been demonstrated to have a role in numerous functions including glucose-stimulated insulin secretion (GSIS), survival, and cell adhesion, demonstrated that ECM-induced integrin signaling regulates beta-cells (23).

1.5.1. *β1-Integrin*

β1-integrin is the most prevalent β-integrin subunit and has been shown to pair with numerous α subunits enabling their binding to different ligands including ECM proteins as listed in **Table 1.1** (2, 11, 35, 81, 98, 111). As a result, β1-integrin has roles in various cellular processes for a number of cell types including pancreatic beta-cells (98, 111). In human and rodent fetal islets, β1-integrin has been shown to be involved in cell adhesion, survival, development, and insulin expression (111, 121). In developed islets, β1-integrin has been shown to be important for beta-cell survival, insulin secretion, and adhesion (17, 53, 77). These findings demonstrate that β1-integrin is important throughout beta-cell development into their mature state within islets. Research has also demonstrated roles for specific α subunits in complex with β1-integrin. α3β1 was shown to be involved in ECM attachment and spreading in isolated rat islets and RIN-2A cells (48), and also acts as a regulator for the migration of CK19+/ PDX-1+ putative pancreatic progenitors of human fetal pancreatic epithelial cells on netrin-1 (122). α5 has also been suggested to be involved in signaling pathways protecting against cell death as decreased α5 expression

during culture of rat islets was paralleled by increased islet apoptosis (112). In hESCs, differentiation to insulin-secreting beta-cells was achieved by seeding cells on an acellular ECM with conditioned media (68). During this process, expression of α1, αV, and β1 subunits were found to increase; α2, α3, α5 and α6 subunits fluctuated in cells but ultimately were higher than control conditions, while β5 expression decreased during differentiation (68). Implantation of these cells led to recovery in STZ-induced diabetic mice after four weeks, where the mice displayed glucose levels and glucose tolerance that was similar to mice not treated with STZ (68). Emphasizing the direct and overall role of β1-integrin as a regulator in insulin secretion, beta-cell specific β1-integrin KO mice display impaired insulin secretion in response to *in vivo* glucose challenge (17, 77). Overall, β1-integrin activation has been demonstrated to be critical for beta-cell development as well as mature function.

Table 1.1: β1-integrin subunits and their associated ligands

Integrin	Ligand
α1β1	collagen, laminin
α2β1	collagen, fibronectin, laminin
α3β1	collagen, fibronectin, laminin
α4β1	fibronectin
α5β1	fibronectin
α6β1	laminin
α7β1	laminin
α8β1	fibronectin
α10β1	collagen
α11β1	collagen
αvβ1	fibronectin

Adapted from Stupack and Cheresh (2002) and Plow et al. (2000).

1.5.2. *β1-integrin, FAK and Insulin Secretion*

Integrin receptors do not contain kinase domains and rely on other proteins to initiate their signaling pathways. Focal adhesions are a diverse group of proteins found at sites of adhesion between integrins (or proteoglycans) and the cytoskeleton (115). Focal adhesion protein composition varies with receptor and cell type. A common protein, and primary signaling molecule, found at integrin focal adhesions is FAK which is activated via tyrosine phosphorylation (115). A major result of FAK activation is changes in the cytoskeleton (**Figure 1.4**) (89, 93). In addition to integrin activated FAK, there is ample evidence of glucose stimulated FAK activation mediating cytoskeletal changes observed in insulin secretion. Glucose stimulation in primary rat beta-cells activates focal contacts containing FAK, paxillin, and ERK1/2 (89). Mice with beta-cell specific FAK KO fail to demonstrate changes in cortical actin density and have impaired *in vivo* insulin secretion in response to glucose stimulation (14). These beta-cells also had reduced insulin granules near the PM and failed to increase membrane-associated granules with glucose stimulation (14). This suggests that FAK regulates actin dynamics to control insulin granule trafficking as well as insulin secretion.

β1-integrin expression and activation has been demonstrated to influence FAK/ERK signaling in beta-cells. Mice with a beta-cell specific β1-integrin KO displayed reduced FAK phosphorylation and impaired insulin secretion (77). Isolated fetal human islets displayed reduced FAK^{Tyr398} with anti-β1-integrin treatment (93) and β1-integrin siRNA transfection also reduced *INSULIN* mRNA that was able to be rescued by FAK overexpression (93). Previous work by our lab demonstrated that INS-1 cells cultured on collagens I and IV and blocked with a β1-integrin antibody significantly decreased insulin content, GSIS, and FAK phosphorylation (51).

Cumulatively, this supports a signaling link between β1-integrin and FAK activity that is required to induce insulin production and beta-cell function.

Another downstream effector of FAK is BCL-2-associated athanogene 3 (BAG3), a multifunctional protein shown to be involved in several cellular processes including cytoskeletal arrangement and insulin secretion (99). Iorio et al. examined BAG3 in β-TC-6 cells and found that in basal conditions, BAG3 is a co-chaperone protein that interacts with SNAP25 to inhibit it from interacting with v-SNARE complexes, thus preventing insulin secretion (39). Upon glucose-stimulation, BAG3 is phosphorylated and it's interaction with SNAP25 is lost, promoting SNARE complex formation (39). Although, a direct link between integrins and BAG3 has yet to be established in beta-cells, FAK has been suggested to be responsible for phosphorylating BAG3 as FAK inhibition was found to abolish BAG3 phosphorylation and reduced SNAP25-Syntaxin1A interactions (39). BAG3 was also identified in epithelial cancer cells as an important factor in cell adhesion to the integrin-dependent ligand fibronectin versus adhesion to poly L-ornithine, a proteoglycan-dependent ligand (41). Iwasaki et al. also found BAG3 to have roles in regulating activity of Rac1, a small Rho GTPase linked to actin rearrangement (1, 41). Overall, these findings suggest that BAG3 may have a critical role in insulin secretion, and that FAK can affect BAG3 activity, but further investigation is required to establish a link between beta-cell integrin signaling and BAG3.

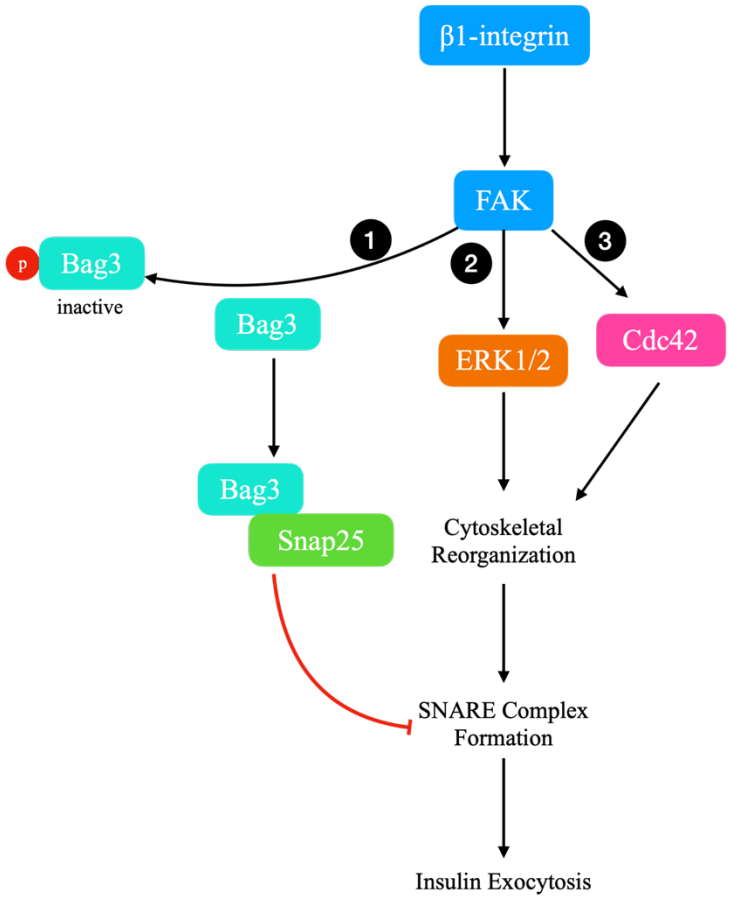


Figure 1.4: Pathway of FAK signaling leading to insulin exocytosis

FAK activation, whether by glucose stimulation or integrin activation, has been shown to facilitate insulin secretion via multiple pathways. (1) FAK phosphorylation of BAG3 releases it from SNAP25, allowing SNAP25 to participate in the formation of SNARE complexes utilized in insulin exocytosis. (2) FAK activation of ERK1/2 is involved in cytoskeletal rearrangement utilized in insulin exocytosis. (3) FAK is an upstream modulator of Cdc42, a common actin remodeling protein found to be involved in targeting insulin vesicles to the membrane for exocytosis.

1.5.3. *Link Between Integrins and SNARE Proteins*

Evidence of a relationship between integrins and exocytotic machinery has been described in neurons. Laminin-mediated β1-integrin activation was shown to be responsible for cytoskeletal rearrangement and controlling VAMP7-regulated exocytosis and neurite development of cortical neurons in mice (29). Following inhibition of upstream signaling molecules involved in the β1 integrin-signaling pathway, neuritogenesis was able to be rescued by over expressing VAMP7 (29). This provides evidence for a direct interaction between integrin signaling and the exocytotic machinery involved in vesicle release. However, despite commonalities in machinery, these relationships have not been reported in pancreatic beta-cells.

Integrin activation has also been demonstrated to alter SNARE-mediated exocytosis in human platelets (123). LPS-activated platelets have increased release of α-granules, an abundant platelet granule type (123). SNAP23, VAMP8, Syntaxin2A, and Munc18a have been shown to be major factors involved in α-granule release and are influenced by LPS-activation in platelets (123). αIIbβ3 integrin, the most abundant platelet surface integrin, is also involved in platelet activation (123). PKCθ, a known outside-in mediator of αIIbβ3 integrin signaling, had increased expression with LPS activation. Upon PKCθ inhibition, decreased Munc18a expression, phosphorylation and association with SNAP23, Syntaxin2A, and VAMP was observed as well as decreased PM localization for Munc18c and VAMP8 (123). This provides evidence of integrin-mediated regulation of exocytosis via modulation of SNARE proteins.

1.6. Insulin Secreting Cell Line (INS-1 Cells)

Although islets can be isolated from animals models for the *ex vivo* investigation of integrin-induced insulin release, the isolation procedure is time-consuming and resulting samples are limited by low proliferation rates (79). Because of this, a great deal of research has been done to develop proliferative cell lines which display beta-cell-like characteristics, most notably the ability to secrete insulin in response to glucose challenge.

One of the major beta-cell lines studied is the rat insulinoma cell line (INS-1). INS-1 cells were introduced by Asfari et al. in 1992 from cells isolated from an x-ray induced rat transplantable insulinoma (3). The major advantage of this cell line is its responsiveness to glucose within the normal physiological range (95). From the original INS-1 parent line, subsequent clones were developed to further improve cell function by transfecting the original INS-1 cells with the human insulin gene (34). Hohmeier et al. demonstrated that the INS-1 832/13 clone developed in this work displayed characteristics comparable to islets. It was the most responsive to glucose challenge, had effective K_{ATP} channel-dependent and -independent GSIS, contained high insulin content, and was responsive to numerous known potentiators of insulin secretion in islets (34). Furthermore, the INS-1 832/13 clone demonstrated stable insulin secretion for longer than six months of culture (34). Studies by our laboratory found that INS-1 832/13 cells express a variety of integrins including α1-6, αV, and β1 (51). Thus, INS-1 832/13 is a good line to use in the proposed study to examine the precise mechanisms of β1-integrin and SNARE protein interplay in the regulation of insulin secretion. However, it is important to keep in mind the potential for differences between a cancer cell line compared to primary beta-cells or *in vivo* islets.

1.7. Objectives and hypothesis of present study

Objective: To determine how beta-cell β1-integrin-ECM interactions promote exocytotic proteins for insulin secretion.

Hypothesis: In pancreatic beta-cells, β1-integrin enhances protein level and alters cellular localization of exocytotic machinery to facilitate insulin secretion.

Specific Aims

1. Determine which β1-integrin-ECM interactions influence exocytotic protein levels and localization in beta-cells
2. Examine how β1-integrin-ECM interactions alter focal adhesions and SNARE protein localization in beta cells during GSIS
3. Examine if blocking β1-integrin could change beta cell adhesion and spreading, insulin secretion, and alterations in exocytotic protein level and localization

Specific Questions:

1. Which ECM protein(s) most influences INS-1 cell adhesion and spreading?
2. Which ECM protein(s) promote insulin secretion and the exocytotic machinery involved?
3. How does collagen IV influence exocytotic proteins and focal adhesions during GSIS?
4. What role does β1-integrin play in the collagen IV-mediate changes observed in INS-1 cell insulin secretion and exocytotic machinery?

Chapter 2

2. Methods

2.1. INS-1 Cell Culture and ECM Coating of Tissue Culture Plates

INS-1 832/13 cells (a gift from Dr. Christopher Newgard, Duke University Medical Center, USA) were cultured to 90% confluency prior to passage and experimentation in RPMI-1640 media with L-glutamine (Gibco, Amarillo, Texas, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Burlington, ON, Canada), 10mmol/L HEPES (Sigma, St. Louis, MO, USA), 1 mmol/L sodium pyruvate (Invitrogen), and 50µmol/L β-mercaptoethanol (Sigma). Cells were incubated at 37ºC in 5% CO_2.

For experimentation, 12- or 96-well tissue culture plates (Fisher Scientific, Ottawa, ON, Canada), or 4- or 8-wells cell culture chamber slides (Nunc Labtek, Fisher Scientific) were precoated with one of the ECM proteins listed in **Table 2.1** or 1% bovine serum albumin (BSA; Sigma) as control. ECM protein precoating concentrations and incubation times listed in **Table 2.1** were based on previous work from our lab and as indicated by the manufacturer (52). INS-1 cells were incubated on pre-coated plates for 24 hours at 37ºC plus 5% CO_2 in serum-free media. Serum-free media (SFM) consisted of RPMI-1640 media with L-glutamine (Gibco), 23.8mM $NaHCO_3$ (Fisher Scientific), 1% BSA (Sigma), 25mM HEPES (Sigma), 5µg/mL transferrin, and 0.1µL/mL IGF-1. Three to six cell passages were used for each set of experiments (n=3-6).

2.2. Functional Blocking of β1-integrin

INS-1 cells were incubated with hamster anti-rat β1-integrin antibody (CD29, 5μg/ml, BD Biosciences, Mississauga, ON, Canada) (anti-β1), hamster IgM isotype-matched negative control (5μg/ml, BD Biosciences) (IgM) or untreated in serum free media (control) for 1 hour at 37ºC, 5% CO_2 prior to being plated on collagen IV pre-coated plates or chamber slides and cultured for 24 hours.

2.3. Cell Adhesion and Spreading Assay

For the cell adhesion assay, $5x10^4$ INS-1 cells were plated on 96-well tissue culture plates pre-coated with different ECM proteins or BSA (control) as listed in **Table 2.1**. For the β1-integrin blocking experiments, INS-1 cells were pretreated with β1 integrin blocking antibody, IgM or control prior to plating on collagen IV pre-coated tissue culture plates. Cells were cultured in serum-free media for 3 hours. All wells were rinsed twice using 1x PBS to remove non-adhered cells. Six random fields were imaged per well using a Leica DMIRE2 microscope (Leica Microsystems) at 40x magnification. After 24 hours in culture, cells were analyzed for the cell spreading assay. Imaging was captured at six random fields per well at 40x magnification using a Leica DMIRE2 microscope. Cells that adhered or spread were counted and normalized to the control groups. Data is expressed is as fold change versus control. Each experiment was performed in triplicate with six repeats per group.

2.4. Glucose Stimulated Insulin Secretion (GSIS) Assays

2.4.1. 96-well Tissue Culture Plate GSIS

$1x10^5$ INS-1 cells were cultured on 96-well tissue culture plates pre-coated with different ECM proteins or BSA (control) as listed in **Table 2.1**. For the β1-integrin blocking experiments, INS-1

cells were pretreated with β1 integrin blocking antibody, IgM or control prior to plating on collagen IV pre-coated tissue culture plates. Cells were cultured in serum-free media for 24 hours. Overnight media was collected to determine basal insulin secretion. Wells were gently rinsed twice with no glucose RPMI-1640 (Sigma) plus 0.5% BSA. Cells were then incubated in RPMI-1640 (Sigma) plus 0.5% BSA with 2.2mmol/L glucose for 1 hour followed by 1 hour with 22mmol/L glucose to analyze glucose-stimulated insulin secretion. Media was collected after each treatment to analyze insulin secretion in response to glucose stimulation. Cells were then harvested to determine cellular insulin content. Insulin concentrations were determined by a Stellux chemiluminescent high range rodent insulin ELISA kit (Alpco, Salem, NH, USA). Static GSIS stimulation index was calculated and expressed as the ratio of insulin secretion at 22mM over 2.2mM glucose stimulation (51). Insulin content was normalized to protein concentration. INS-1 cell protein concentration was determined via Bradford assay using Bradford dye (Bio-Rad Laboratories, Mississauga, ON, Canada) and BSA (0-0.5mg/mL) as a standard. Each experiment was performed in technical triplicates with 4-5 biological repeats per group.

2.4.2. Chamber slide time-dependent insulin secretion during INS-1 cell GSIS

$1.5x10^5$ INS-1 cells were cultured on 4- or 8-well cell culture chamber slides (Nunc Labtek, Fisher Scientific) pre-coated with BSA or collagen IV. For the β1-integrin blocking experiments, INS-1 cells were pretreated with β1 integrin blocking antibody, IgM or control prior to plating on collagen IV pre-coated chamber slides. Cells were cultured in serum-free media for 24 hours, then media was harvested, and cells were immediately fixed for the basal condition. For glucose treated cells, cells were first rinsed twice with no glucose RPMI plus 0.5% BSA followed by glucose stimulation in RPMI plus 0.5% BSA media containing of one of four conditions: (1) 2.2mmol/L

glucose for 30 minutes (L30), or 22mmol/L glucose for (2) 5 minutes (H5), (3) 30 minutes (H30), or (4) 60 minutes (H60). Media was collected at each time point and insulin secretion was determined using a Stellux chemiluminescent high range rodent insulin ELISA kit (ALPCO). Cells were then fixed for immunofluorescence staining as described in **2.6.1 Chamber Slide Immunofluorescence Analysis**.

2.5.Protein Extraction and Western Blotting

INS-1 cell protein was extracted by incubating cells in Nonident-P40 lysis buffer (Nonident-P40, phenylmethylsolfonyl fluoride, sodium orthovanadate [Sigma] and complete protease inhibitor cocktail tablet [Roche; Mississauga, ON, Canada]) for 20 minutes on ice followed by sonication and centrifugation at 13 000 rpm (12 879 x g) for 20 minutes at 4ºC. The supernatant was collected and frozen at -80ºC. Protein concentrations were determined via Bradford assay using Bradford dye (Bio-Rad Laboratories, Mississauga, ON, Canada) and BSA (0-0.5mg/mL) as a standard.

An equal amount (25µg) of lysate proteins from each experimental group was separated by either 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-page). Following separation, proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories Inc.; Mississauga, ON, Canada) by running on ice for 2 hours at 250mA. Transfer was confirmed using Ponceau S dye (Sigma-Aldrich). Membranes were washed in Tris buffer-saline with 0.1% Tween-20 (TBST; Sigma) followed by blocking in 5% non-fat dry milk (2.5mL TBS, 50µL NP-40 [Sigma] in 50 mL double distilled H_2O with pH adjusted to 7.4) at room temperature (RT) for 1 hour or overnight at 4ºC. Primary antibodies were incubated overnight at 4ºC and concentrations are listed in **Table 2.2**. Membranes were washed 3 times for 5 minutes in TBST and subsequently

incubated in anti-rabbit or anti-mouse IgG, HRP-linked secondary antibodies (Cell Signaling Technology, Whitby, ON) for 1 hour at RT. Cells were then washed 3 times for 5 minutes in TBST. Chemiluminescent detection (ECL, PerkinElmer, Waltham, MA) was used to visualize proteins of interest and membranes were imaged on a Versadoc version 4.6.9 (Bio-rad Laboratories Inc.) imaging system. Reference protein (GAPDH) and total proteins were used to normalize protein bands of interest and protein phosphorylation levels, respectively. Probing for total protein levels was completed after immersing the membrane in a mild stripping buffer (200mM glycine [Fisher Scientific], 3.5mM SDS [Sigma], and 10µL/mL Tween-20 in double distilled water with pH adjusted to 2.2) for 10 minutes at RT, washing in TBST 3 times for 5 minutes, and incubating for 1 hour in milk blocking solution. Densitometric quantification of bands was determined by Image Lab software (Bio-Rad Laboratories). Data is expressed as fold-change from control, all protein levels are normalized to loading control or total protein (51).

2.6.Immunofluorescence Analysis

INS-1 cells were cultured on 12-well plates pre-coated with ECM proteins, or BSA (control), as listed in **Table 2.1** in serum-free medium for 24 hours. Cells were harvested and fixed in 4% paraformaldehyde for one hour at RT before being washed with 1x PBS. Cells were then embedded in 2% agarose gel and treated with a standard protocol for dehydration and paraffin embedding (52).

4µm sections from each group were taken and placed on slides. Sections were deparaffinized and rehydrated followed by blocking in 10% normal goat serum for 1 hour at RT. If indicated in **Table 2.2**, sections were treated with heat-induced antigen retrieval solution (citrate pH 6.0) to remove PFA-induced cross-links and improve antibody detection. Sections were then probed with

primary antibodies at the appropriate dilutions (**Table 2.2**) and incubated overnight at 4ºC. Sections were incubated with secondary antibodies (**Table 2.2)** conjugated with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) (Jackson Immunoresearch, West Grove, PA) for 1 hour at RT the following day. Nuclei were counterstained with 4'-6'-diamidino-2-phenylindole (DAPI) (1:1000 dilution; Sigma-Aldrich). Positive staining images were captured using a Nikon Eclipse Ti2 confocal microscope (Nikon, Mississauga, ON) set to 60x magnification with oil (Nikon). Imaging was captured at 6-7 random areas per section with a minimum of 3 repeats per group. Displayed images are representative of staining found in each group. Negative controls were determined by adding secondary antibodies alone to rule out non-specific binding.

2.6.1. Chamber Slide Immunofluorescent Analysis

After time-dependent chamber slide GSIS, INS-1 cells cultured on chamber slides were fixed in 4% paraformaldehyde (PFA) for 20 minutes at RT. Cells were then treated with 0.2% Triton for 30 minutes followed by appropriate dilution of primary antibodies (**Table 2.2**), with incubation performed as described above. Stained images were captured using a Nikon Eclipse Ti2 confocal microscope set to 60x magnification with oil. Imaging was captured at 6-7 random areas and visually analyzed for changes in protein localization within the cell and intensity. Displayed images are representative of staining found in each group.

2.6.2. Colocalization Analysis

Double immunofluorescence staining for SNAP25 (green) and VAMP2 (red) was performed to examine colocalization, and co-stained images were captured using a Nikon Eclipse Ti2

confocal microscope set to 60x magnification with oil. A minimum of 50 cells per experimental group were imaged. For each image, regions of interest (ROI) were generated by manually tracing around groups of cells to limit background noise. Colocalization analysis was performed on each ROI using NIS-Elements software (Nikon) using the co-localization function. Manders coefficents were determined to examine changes in overlap irrespective of intensity. Manders overlap coefficient, which analyzes the overall overlap between proteins of interest, and Manders colocalization coefficients (M1 and M2), which describe the above-background pixels of one protein of interest that overlap with another protein of interest amount of overlap, were reported, and averaged for all ROI in each experimental group.

2.7.Fluo-4 Ca^{2+} Imaging

$1x10^5$ INS-1 cells were pretreated for 1 hour with β1-integrin blocking antibody, IgM, or untreated control followed by culture on collagen IV pre-coated 35mm glass bottom dishes (Cedarlane, Burlington, ON, Canada) for 24 hours in serum-free media. Cells were rinsed twice with HBSS and incubated with 4 μM Fluo-4 AM (Abcam) in HBSS (Sigma) for 30 min at 37ºC, 5% CO_2 followed by 30 min at RT. Cells were then imaged using a Nikon Eclipse Ti2 confocal microscope set to 60x magnification with oil. Cells were imaged for 15 seconds in order to determine basal Fluo-4 levels. High glucose (22mM) was then added, and cells were imaged for 15-minute time series with images taken every 8 seconds.

FIJI was used for analysis, where ROIs were drawn around stimulated cells and mean grey value was plotted over time. To account for differences in Fluo-4 uptake, mean grey values during glucose stimulation were normalized to the mean grey values in the first 15 seconds of imaging

prior to glucose addition. Area under curve (AUC) was measured for each trace and averaged for the cells in each image using GraphPad Prism.

2.8. Statistical Analysis

Data are expressed as means ± SD. Statistical significance was determined using a one-way ANOVA followed by Tukey's post-hoc test, if significant. For examining differences in rate of insulin secretion, a two-way ANOVA was used. Differences were considered statistically significant when $p<0.05$. Analysis was performed using GraphPad Prism 7.0 (GraphPad Software, Inc.).

Table 2.1: List of ECM Proteins

ECM Protein	Dilution	Dilutant	Incubation	Company
Fibronectin	5µg/mL	Serum-free media	1 hour	Fisher Scientific, Ottawa, ON, Canada
Laminin	6µg/mL	1x PBS	2 hours	Sigma, St. Louis, MO, USA
Rat tail Collagen I	5µg/mL	0.02M Acetic Acid	1 hour	Wang Lab (51)
Collagen IV	19.8 µg/mL	0.05M HCl	1 hour	Santa Cruz Biotechnology Inc, Dallas, TX, USA

Table 2.2: List of Antibodies

Primary Antibody	**Dilution**	**Company**
Mouse Anti-BAG3	1:50/1:1000[W]	Santa Cruz Biotechnology Inc, Dallas, TX, USA
Rabbit Anti-pBAG3 (Tyr457)	1:50	Antibodies-online Inc., Limerick, PA, USA
Rabbit Anti-β1-integrin	1:1000[W]	Millipore Sigma, Saint Louis, MO, USA
Mouse Anti-β1-integrin	1:50	Abcam Inc., Cambridge, MA, USA
Rabbit Anti-FAK	1:2000[W]	Invitrogen, Burlington, ON, CA
Rabbit Anti-pFAK (Tyr397)	1:1000[W]	Invitrogen, Burlington, ON, CA
Mouse Anti-GAPDH	1:2000[W]	Santa Cruz Biotechnology Inc, Dallas, TX, USA
Rabbit Anti-Munc18	1:100*/1:1000[W]	Abcam Inc., Cambridge, MA, USA
Phalloidin	1:25 000	Abcam Inc., Cambridge, MA, USA
Mouse Anti-SNAP25	1:50/1:1000[W]	Santa Cruz Biotechnology Inc, Dallas, TX, USA
Rabbit Anti-VAMP2	1:200/1:500[W]	Abcam Inc., Cambridge, MA, USA
Anti-Vinculin	1:50	Millipore Sigma, Saint Louis, MO, USA
Secondary Antibody	**Dilution**	**Company**
Goat Anti-Mouse	1:50	Jackson Immunoresearch, West Grove, Pam USA
Goat Anti-Rabbit	1:50	Jackson Immunoresearch, West Grove, Pam USA
Anti-Mouse HRP-linked	1:500-3000[W]	Cell Signaling Technology, Whitby, ON, CA
Anti-Rabbit HRP-linked	1:500-3000[W]	Cell Signaling Technology, Whitby, ON, CA

* Citrate Antigen Retrieval (pH 6.0) used

[W]Used in western blot probing

Chapter 3

3. Results

3.1. Collagen IV enhances INS-1 cell adhesion and spreading

Cell adhesion and subsequent spreading are early indicators of cell-ECM interactions (87). To examine how β1-integrin ligands; fibronectin (FIB), laminin (LAM), collagen I (COL I), and collagen IV (COL IV) influence INS-1 cell adhesion and spreading, cells were plated and analyzed after 3 and 24 hours, respectively. After 3 hours of culture on ECM-coated tissue culture plates, INS-1 cells displayed varied levels of increased adhesion, depending on the ECM protein, compared to the BSA-coated control (CTRL) (**Figure 3.1A**). Quantitative analysis demonstrated that COL IV provided the greatest increase in adhesion out of all experimental groups and was significantly higher than CTRL and LAM conditions ($p<0.01$, $p<0.05$, respectively) (**Figure 3.1B**). After 24 hours of culture, it was found that all ECM proteins improved cell spreading compared to CTRL conditions (**Figure 3.1A**) with mean fold changes from CTRL of 8.1, 40.4, 26.9, and 62.2 for FIB, LAM, COL I, and COLIV groups, respectively (**Figure 3.1C**). Statistically significant differences in cell spreading were observed between COL IV and CTRL ($p<0.01$), and COL IV and FIB ($p<0.01$).

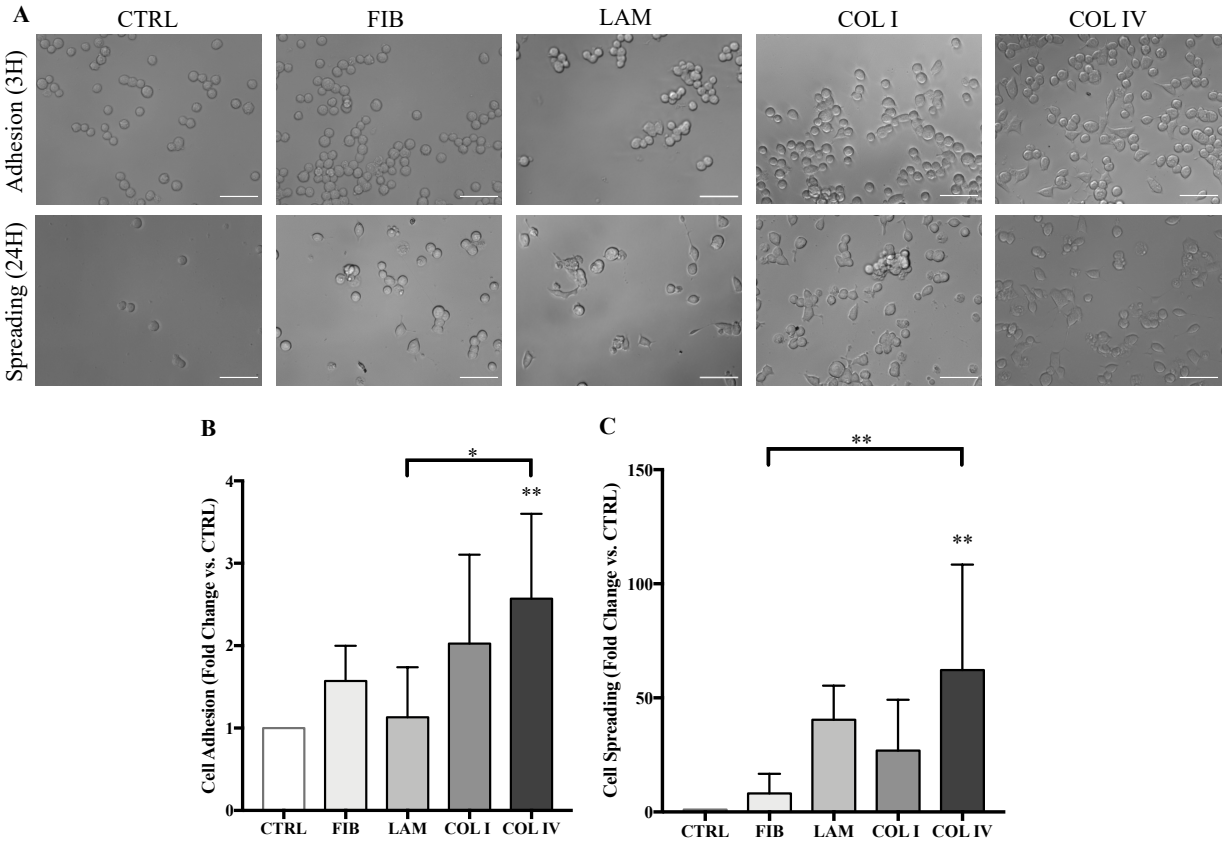

A
CTRL
FIB
LAM
COL I
COL IV
Adhesion (3H)
Spreading (24H)
B
Cell Adhesion (Fold Change vs. CTRL)
0
1
2
3
4
*
**
CTRL
FIB
LAM
COL I
COL IV
C
Cell Spreading (Fold Change vs. CTRL)
0
50
100
150
**
**
CTRL
FIB
LAM
COL I
COL IV

Figure 3.1: Collagen IV enhances INS-1 cell adhesion and spreading

(**A**) Phase contrast images taken 3- and 24-hours following INS-1 cell plating on BSA (CTRL), fibronectin (FIB), laminin (LAM), collagen I (COL I), or collagen IV (COL IV) pre-coated tissue culture plates to determine cell adhesion (3 hours) and spreading (24 hours), respectively. Scale bar: 50μm. Quantification of INS-1 cell (**B**) adhesion and (**C**) spreading. Data are expressed as fold-change vs. CTRL (mean ± SD, n=6 experiments/treatment group, all done in triplicate). *$p<0.05$, **$p<0.01$ vs. CTRL or as indicated. Determined by one-way ANOVA followed by Tukey's multiple comparisons test.

3.2.Collagen IV enhances INS-1 cell glucose stimulated insulin secretion and insulin content

The major function of beta cells is to secrete insulin. We examined if different ECM proteins can influence the amount of insulin secreted under basal conditions and in response to glucose stimulation. Under basal conditions, no differences were found between experimental groups for insulin secretion (**Figure 3.2A**).

The effects of ECM on INS-1 cell GSIS was next examined. INS-1 cells were washed with glucose-free media and incubated in 2.2 mM glucose (low glucose) for 1 hour followed by 1 hour of 22 mM glucose (high glucose). From this, the stimulation index, the ratio of insulin secretion from high over low glucose stimulation, was determined. Consistent with the adhesion and spreading results, COL IV had the greatest stimulation index compared to other ECM groups, with a mean index 3.4-fold higher than CTRL ($p<0.05$) (**Figure 3.2B**).

Finally, insulin content within the cells was examined. Although all experimental groups were found to have increased insulin content compared to CTRL, the COL IV group displayed the largest increase, with a 3.2-fold difference compared to CTRL ($p<0.05$) (**Figure 3.2C**).

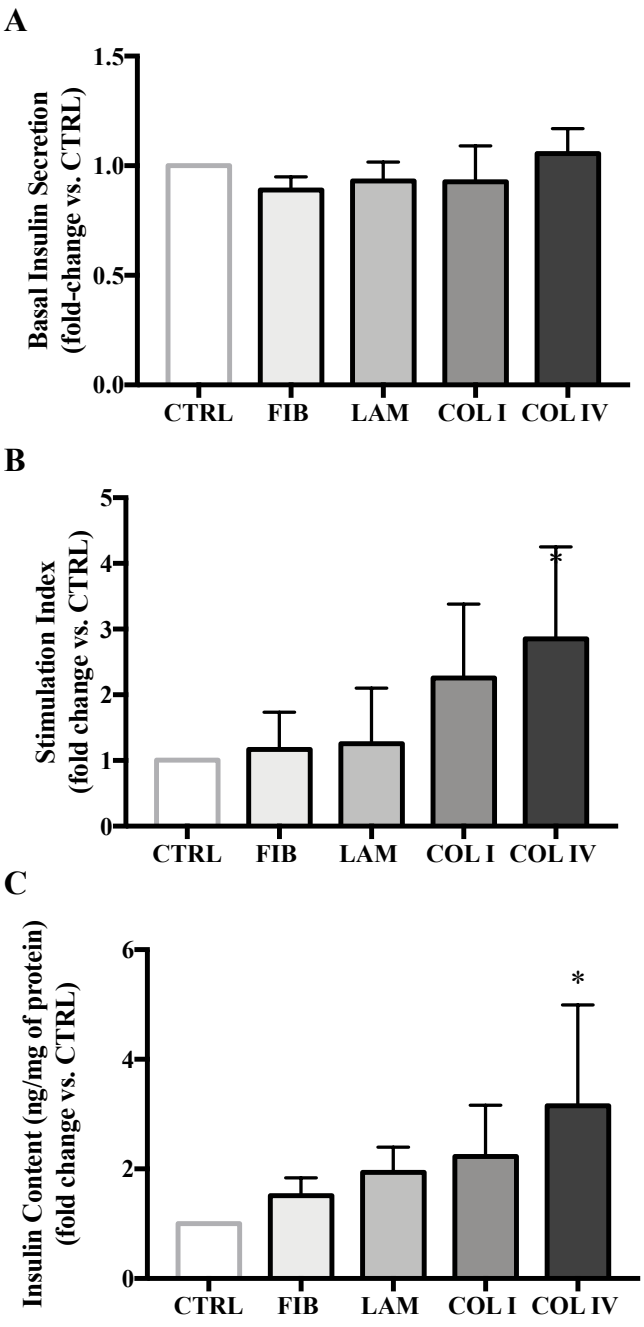

A
Basal Insulin Secretion (fold-change vs. CTRL)
1.5
1.0
0.5
0.0
CTRL
FIB
LAM
COL I
COL IV
B
Stimulation Index (fold change vs. CTRL)
5
4
3
2
1
0
*
CTRL
FIB
LAM
COL I
COL IV
C
Insulin Content (ng/mg of protein) (fold change vs. CTRL)
6
4
2
0
*
CTRL
FIB
LAM
COL I
COL IV

Figure 3.2: Collagen IV enhances glucose-stimulated insulin secretion (GSIS) and content in INS-1 cells

(**A**) Basal insulin secretion, (**B**) GSIS stimulation index (ratio of insulin secretion from high glucose: low glucose stimulation), and (**C**) insulin content of INS-1 cells cultured on BSA, FIB, LAM, COL I, or COL IV pre-coated plates. Insulin content was measured and normalized to protein content determined by a Bradford assay. Each experiment was performed in triplicate and data are expressed as fold-change vs. CTRL (mean ± SD, n=3-6 experiments/treatment group) *$p<0.05$ vs. CTRL determined by one-way ANOVA followed by Tukey's post-hoc test.

3.3. ECM proteins impact INS-1 cell exocytotic protein level and localization

To determine whether the effect of ECM protein enrichment on INS-1 cell insulin secretion is associated with changes in exocytotic proteins, Munc18-1 and SNARE total protein levels and localization were examined using western blot and immunofluorescence microscopy.

Munc18-1, a SNARE-associated protein involved in orchestrating the assembly of SNARE complex formation, was found to be influenced by cell-ECM interactions. Immunofluorescent staining revealed a notable increase in Munc18-1 cytoplasmic puncta with COL IV cells (**Figure 3.3A, B**). INS-1 cells cultured on COL IV were found to have significantly increased Munc18-1 protein levels when compared to the FIB group ($p<0.05$), and a notable increase compared to CTRL ($p=0.1026$) (**Figure 3.3C, D**).

Interestingly, SNAP25, a SNARE complex protein involved in insulin exocytosis, was found to have increased membrane protein localization and intensity in LAM and COL IV compared to other groups (**Figure 3.4A, B**). When examining total protein level, SNAP25 was found to be significantly higher in cells cultured on LAM when compared to CTRL cells ($p<0.05$) and although increased in the COL IV group, no statistically significant differences were found ($p=0.3961$) (**Figure 3.4C, D**).

Finally, VAMP2, another SNARE protein involved in INS-1 cell insulin exocytosis, did not appear to visually change when cultured on different ECM proteins (**Figure 3.5A, B**). Although protein levels varied, no significant differences in VAMP2 were observed between groups when examined via western blot analysis (**Figure 3.5C, D**).

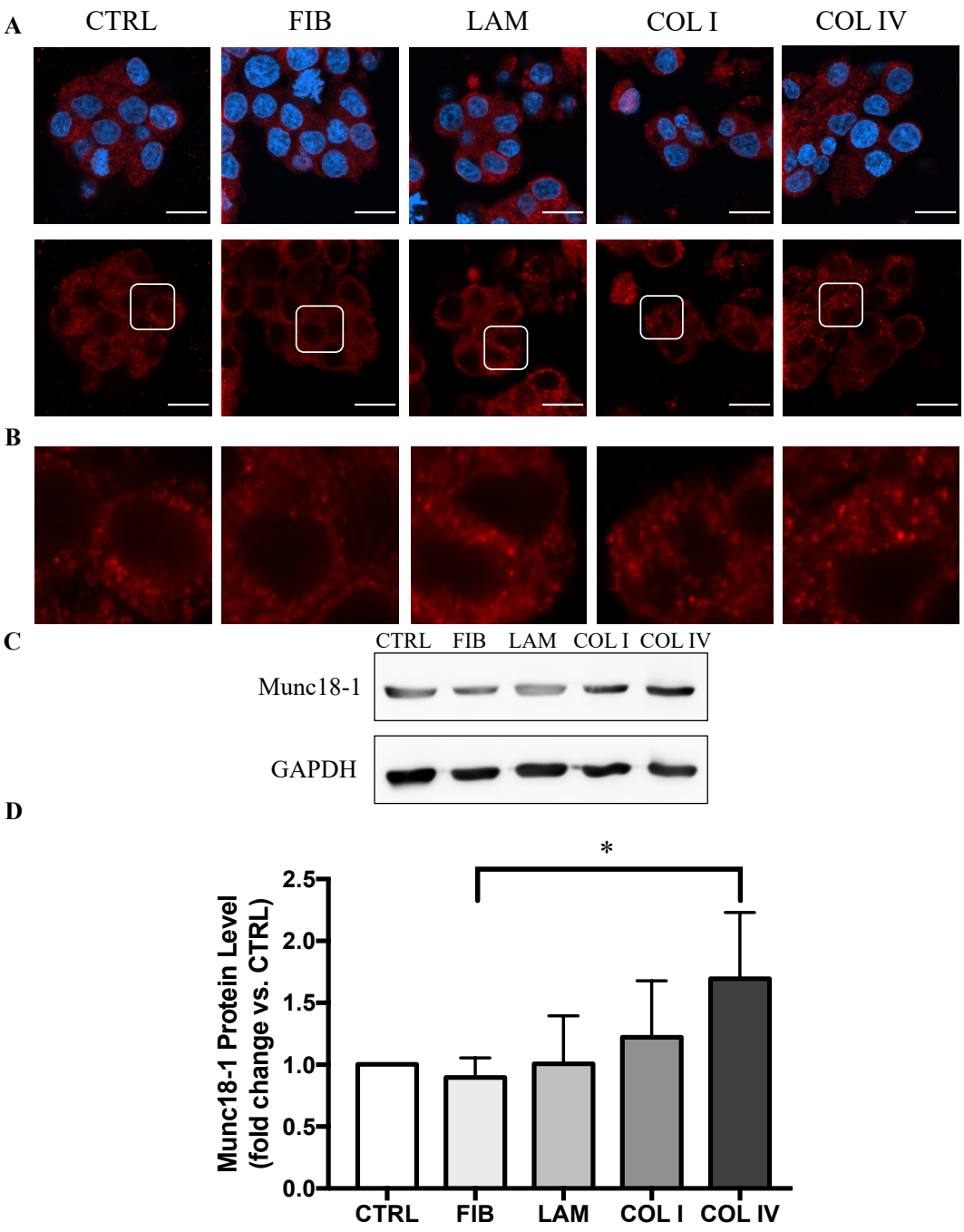

A
CTRL
FIB
LAM
COL I
COL IV
B
C
CTRL FIB LAM COL I COL IV
Munc18-1
GAPDH
D
*
Munc18-1 Protein Level
(fold change vs. CTRL)
2.5
2.0
1.5
1.0
0.5
0.0
CTRL
FIB
LAM
COL I
COL IV

Figure 3.3: Collagen IV enhances Munc18-1 expression and protein levels in INS-1 cells

(**A**) Representative confocal images of the SNARE protein Munc18-1 (red) and nuclei (blue) in INS-1 cells cultured on BSA, FIB, LAM, COL I, or COL IV pre-coated plates. (**B**) Magnified images of the highlighted region for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of Munc18-1 protein levels. Data are normalized to GAPDH, a loading control, and expressed as fold-change vs. CTRL (mean ± SD, n=4 experiments/treatment group). *$p<0.05$ determined by one-way ANOVA followed by Tukey's multiple comparisons test.

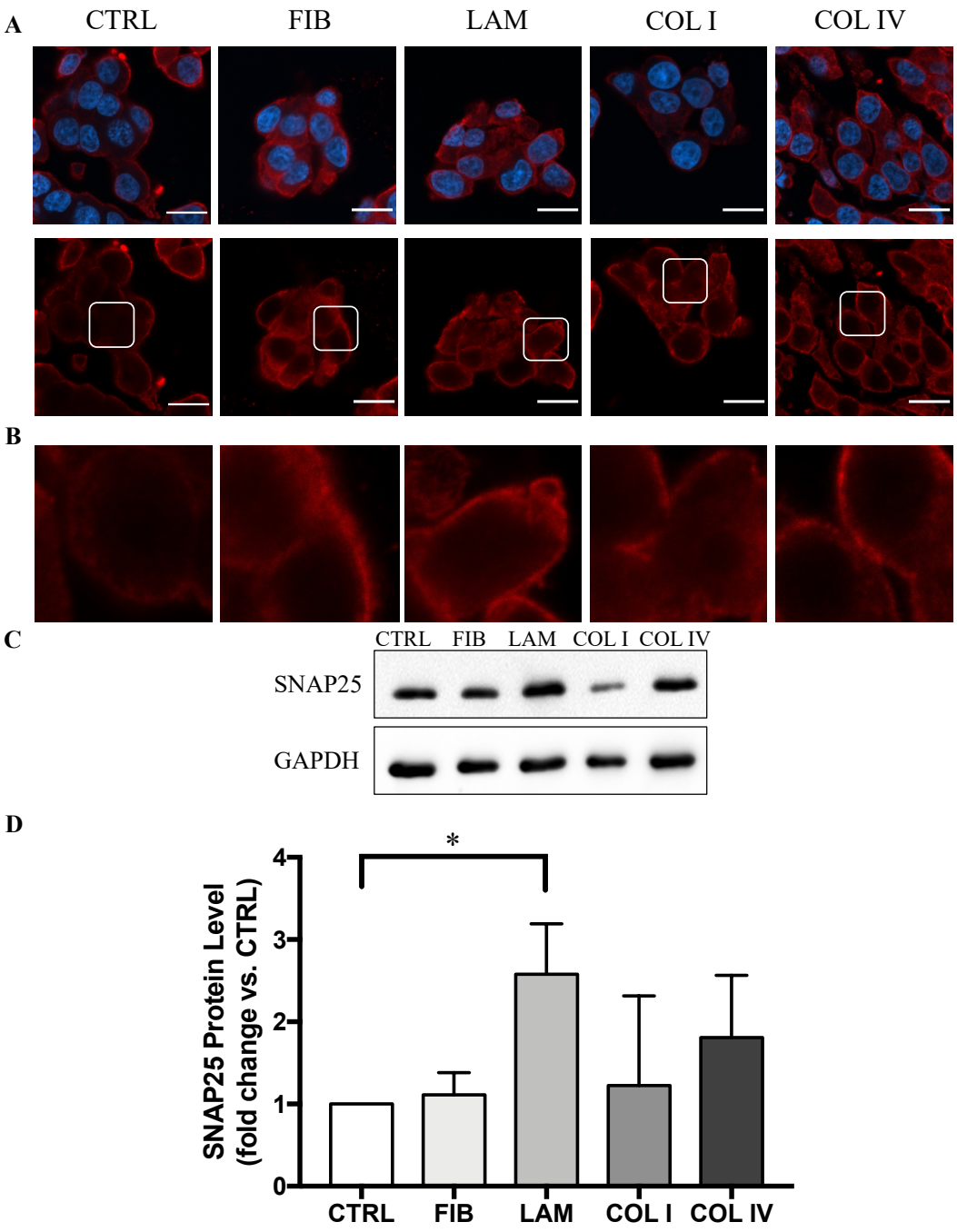

A
CTRL
FIB
LAM
COL I
COL IV
B
C
CTRL FIB LAM COL I COL IV
SNAP25
GAPDH
D
*
SNAP25 Protein Level
(fold change vs. CTRL)
4
3
2
1
0
CTRL
FIB
LAM
COL I
COL IV

Figure 3.4: Some ECM proteins influence SNAP25 expression and protein level in INS-1 cells

(**A**) Representative confocal images of the SNARE protein SNAP25 (red) and nuclei (blue) in INS-1 cells cultured on BSA, FIB, LAM, COL I, or COL IV pre-coated plates. (**B**) Magnified images of the highlighted region for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of SNAP25 protein levels. Data are normalized to GAPDH, a loading control, and expressed as fold-change vs. CTRL (mean ± SD, n=3-5 experiments/treatment group). *$p<0.05$ determined by one-way ANOVA followed by Tukey's multiple comparisons test.

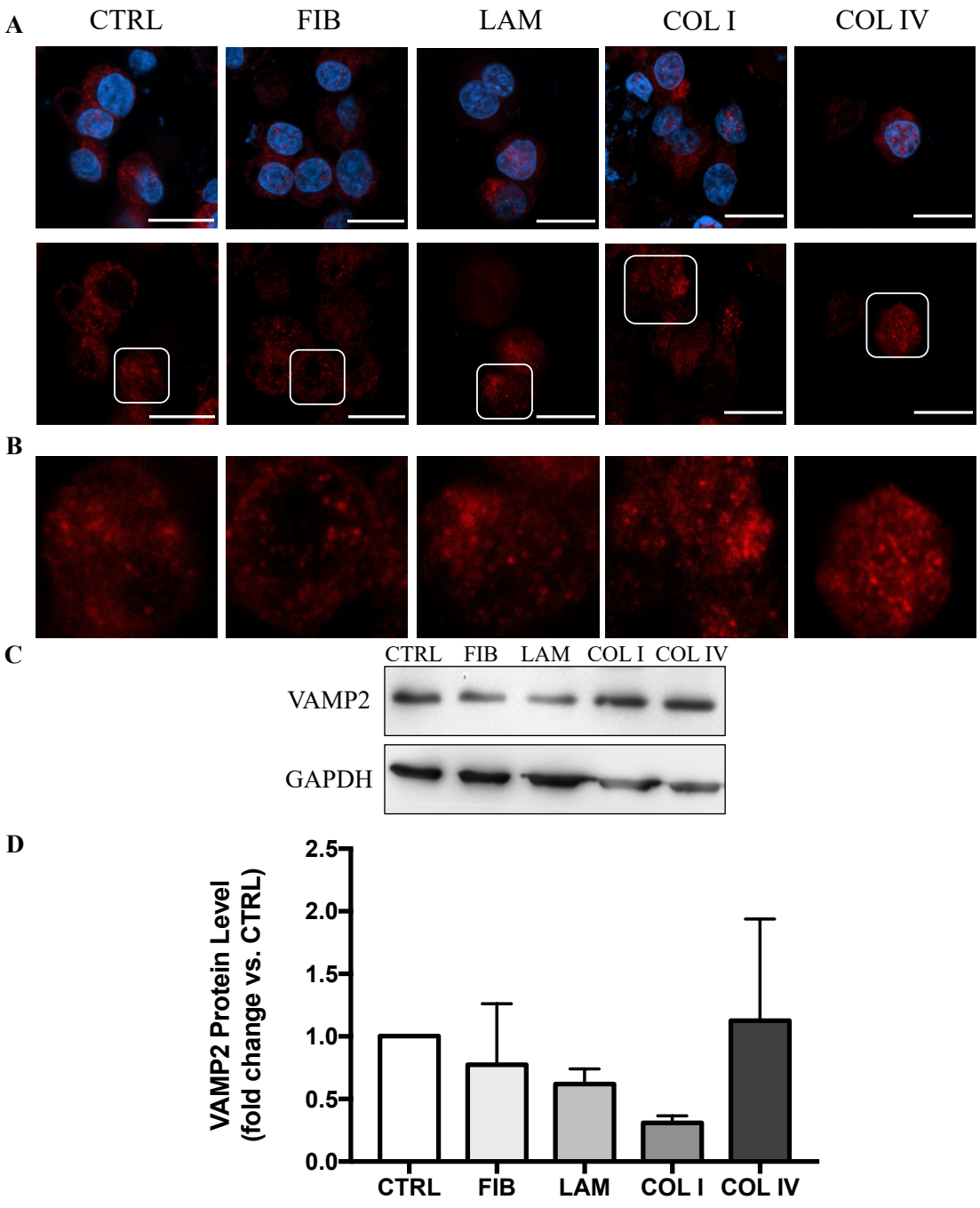

A
CTRL
FIB
LAM
COL I
COL IV
B
C
CTRL FIB LAM COL I COL IV
VAMP2
GAPDH
D
VAMP2 Protein Level
(fold change vs. CTRL)
2.5
2.0
1.5
1.0
0.5
0.0
CTRL
FIB
LAM
COL I
COL IV

Figure 3.5: ECM proteins do not appear to influence VAMP2 expression and protein levels in INS-1 cells

(**A**) Representative confocal images of the SNARE protein VAMP2 (red) and nuclei (blue) in INS-1 cells cultured on BSA, FIB, LAM, COL I, or COL IV pre-coated plates. (**B**) Magnified images of the highlighted region for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of VAMP2 protein levels. Data are normalized to GAPDH, a loading control, and expressed as fold-change vs. CTRL (mean ± SD, n=3-5 experiments/treatment group). *$p<0.05$ determined by one-way ANOVA followed by Tukey's multiple comparisons test.

3.4.Collagen IV increases visual overlap of SNAP25 and VAMP2

Analysis of SNAP25 and VAMP2 immunofluorescent co-staining revealed COL IV produces minor changes in colocalization between the two proteins. Manders coefficient, a measure of the degree of overlap between proteins of interest irrespective of intensity, was used to quantify the degree of overlap between the two proteins. Manders overlap coefficient, which examines the overall overlap between the two proteins of interest, did not differ between groups, with mean values ranging from 0.75 to 0.78 (**Figure 3.6A**). Next, Manders colocalization coefficients, the values which describe the above-background pixels of one protein of interest that overlap with another protein of interest, were examined. M1, the coefficient describing the amount of SNAP25 which overlaps with VAMP2, was not found to differ (**Figure 3.6B**). However, M2, the coefficient describing the amount of VAMP2 which overlaps with SNAP25, was increased in COL IV cells compared to other groups, albeit not significantly (**Figure 3.6C**). Specifically, mean M2 values were 2.76, 1.54, 2.21, 1.92, and 3.56 for CTRL, FIB, LAM, COL I, and COLIV, respectively. Representative colocalization images for each group were displayed in **Figure 3.6D**. An increase in the visual colocalization, seen in yellow, of SNAP25 and VAMP2 was observed in COL IV compared to other groups (**Figure 3.6E**).

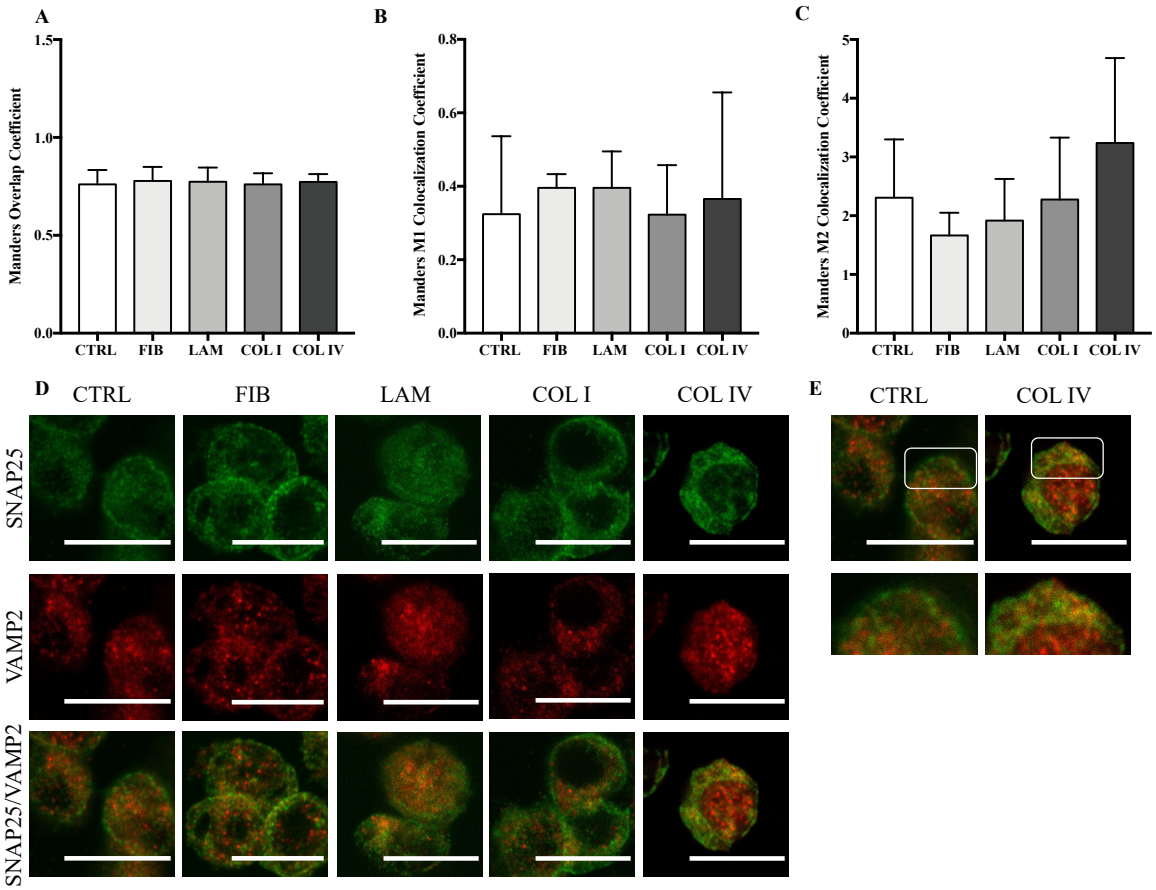

A
Manders Overlap Coefficient
1.5
1.0
0.5
0.0
CTRL
FIB
LAM
COL I
COL IV
B
Manders M1 Colocalization Coefficient
0.8
0.6
0.4
0.2
0.0
CTRL
FIB
LAM
COL I
COL IV
C
Manders M2 Colocalization Coefficient
5
4
3
2
1
0
CTRL
FIB
LAM
COL I
COL IV
D
CTRL
FIB
LAM
COL I
COL IV
SNAP25
VAMP2
SNAP25/VAMP2
E
CTRL
COL IV

Figure 3.6: Collagen IV increases visual overlap of SNAP25 and VAMP2

(**A**) Manders overlap coefficient, (**B**) Manders M1 colocalization coefficient and (**C**) Manders M2 colocalization coefficient of INS-1 cells cultured on BSA (CTRL), FIB, LAM, COL I, COL IV pre-coated plates (mean ± SD, n=3-5). (**D**) Representative confocal images of the SNARE proteins SNAP25 (green) and VAMP2 (red) in INS-1 cells cultured on ECM and CTRL. (**E**) Magnified images for CTRL and COL IV groups. Scale bar: 10μm.

3.5.ECM proteins promote INS-1 cell β1-integrin, FAK, and BAG3 protein levels

β1-integrin was found to be increased in intensity and membrane-associated puncta in COL I and COL IV groups (**Figure 3.7A, B**). Both COL I and COL IV also displayed slight increases in protein levels, although these changes were not statistically significant (**Figure 3.7C, D**). FAK, a major signaling modulator for β1-integrin, phosphorylation was also examined. No significant differences between groups were found although LAM, COL I, and COL IV groups had higher phosphorylation compared to CTRL (1.27, 1.14, and 1.29, fold-increases vs. CTRL, respectively) (**Figure 3.7E, F**).

BAG3, an accessory protein associated with insulin exocytosis, was found to be influenced by cell-ECM interactions. Cytoplasmic total BAG3 staining was visually increased in FIB, COL I, and COL IV vs. CTRL (**Figure 3.8A**). Particularly, a notable increase in COL IV intensity was observed compared to CTRL (**Figure 3.8B**). No significant changes were observed in protein levels of BAG3 between groups (**Figure 3.8D**).

Phosphorylated BAG3, which has been demonstrated in previous research to be involved in SNARE complex formation (39) was also changed. In CTRL, sparse cytoplasmic localization with less density of staining of phosphorylated BAG3 staining is observed in contrast to the ECM groups (**Figure 3.8E, F**). No major changes in localization between experimental groups is observed with only a minimal increase in puncta observed for the COL IV group (**Figure 3.8 E, F).**

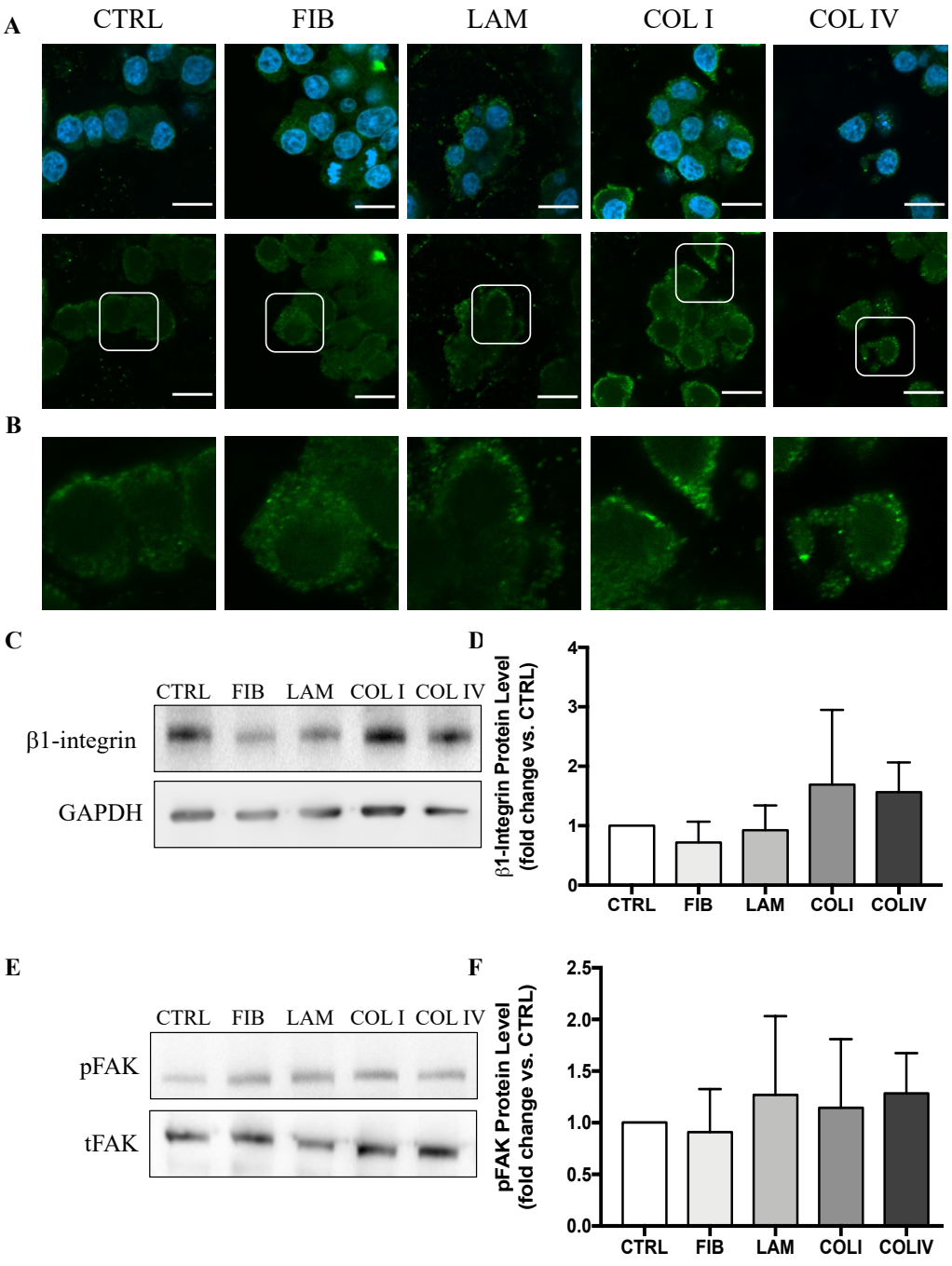

A
CTRL
FIB
LAM
COL I
COL IV
B
C
CTRL FIB LAM COL I COL IV
β1-integrin
GAPDH
D
β1-Integrin Protein Level (fold change vs. CTRL)
0
1
2
3
4
CTRL
FIB
LAM
COLI
COLIV
E
CTRL FIB LAM COL I COL IV
pFAK
tFAK
F
pFAK Protein Level (fold change vs. CTRL)
0.0
0.5
1.0
1.5
2.0
2.5
CTRL
FIB
LAM
COLI
COLIV

Figure 3.7: ECM proteins impact β1 Integrin localization but not protein levels or FAK phosphorylation in INS-1 cells

(**A**) Representative confocal images of β1-integrin (green) and nuclei (blue) in INS-1 cells cultured on BSA, FIB, LAM, COL I, or COL IV pre-coated plates. (**B**) Magnified images of the highlighted region for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of β1-integrin protein levels. (**E**) Representative blotting images and (**F**) western blot analysis of phosphorylated FAK (pFAK). β1-integrin protein levels are normalized to GAPDH, a loading control, and pFAK levels are normalized to total FAK (tFAK) levels. Data are expressed as fold-change vs. CTRL (mean ± SD, n=3-4 experiments/treatment group). Data was analyzed using a one-way ANOVA followed by Tukey's multiple comparisons test.

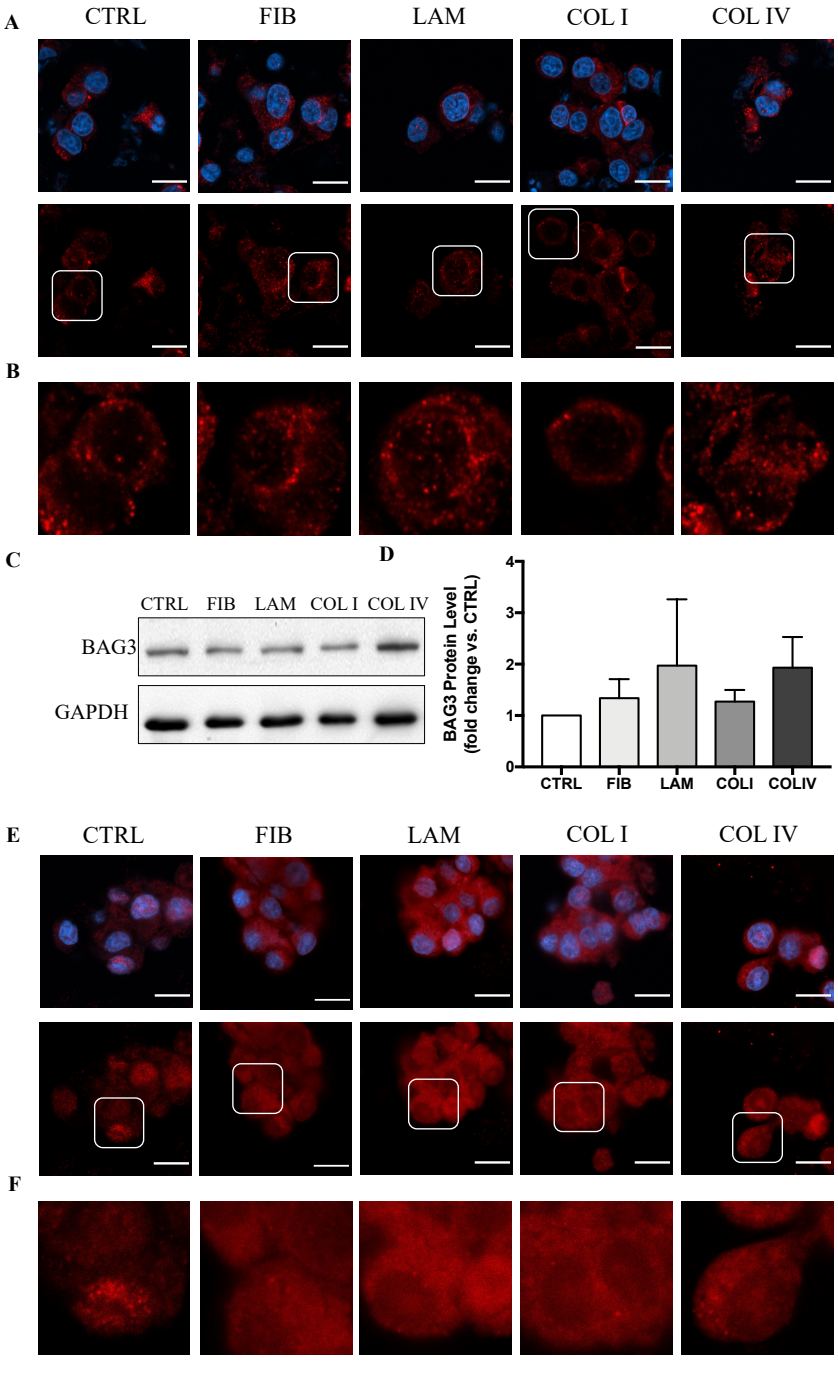

A
CTRL
FIB
LAM
COL I
COL IV
B
C
CTRL FIB LAM COL I COL IV
BAG3
GAPDH
D
BAG3 Protein Level (fold change vs. CTRL)
0
1
2
3
4
CTRL
FIB
LAM
COLI
COLIV
E
CTRL
FIB
LAM
COL I
COL IV
F

Figure 3.8: Collagen IV enhances BAG3 localization and ECM proteins impact pBAG3 localization

(**A**) Representative confocal images of BAG3 (red) and nuclei (blue) in INS-1 cells cultured on BSA, FIB, LAM, COL I, or COL IV pre-coated plates. (**B**) Magnified images of the highlighted region for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of BAG3 protein levels. Data are normalized to GAPDH, a loading control, and expressed as fold-change vs. CTRL (mean ± SD, n=3-4 experiments/treatment group). (**E**) Representative confocal images of phosphorylated BAG3 (red) and nuclei (blue) in INS-1 cells cultured on BSA, FIB, LAM, COL I, or COL IV pre-coated plates. (**F**) Magnified images of the corresponding highlighted area for each group. Scale bar: 10μm.

3.6.Collagen IV promotes focal adhesion complex formation in INS-1 cells

Focal adhesion complexes are complex PM-associated macromolecular assemblies that interact with ECM-integrin activation sites and physically connect with the actin cytoskeleton to recruit focal adhesion-associated proteins such as vinculin. To determine the influence of collagen IV on focal adhesion complexes, INS-1 cells were cultured on chamber slides precoated with collagen IV (COL IV) or BSA (CTRL) for 24 hours, and then stimulated with different glucose conditions. In general, CTRL cells remained rounded with weak actin staining and less vinculin at the cell periphery compared to COL IV cells (**Figure 3.9A**). COL IV cells also demonstrated intense actin stress fibers with increased amount of positive vinculin staining at the cell periphery were observed when compared to CTRL (**Figure 3.9B**) consistent with the idea that collagen IV promotes focal adhesion complex formation. The differences between groups remained consistent across all tested glucose stimulation conditions (**Figure 3.9Ab-e, Bb-e**). Actin in CTRL cells appears to be relatively unchanging in CTRL cells regardless of glucose treatment (**Figure 3.9Ab-e**). However, actin staining in COL IV cells is generally homogenous across the cell periphery under basal conditions, with only some areas of intensity while with glucose stimulation induced some areas with intense actin staining and others without (**Figure 3.9Bb-e**). This indicates that collagen IV support in INS-1 cells induces alterations in the actin cytoskeleton in response to glucose.

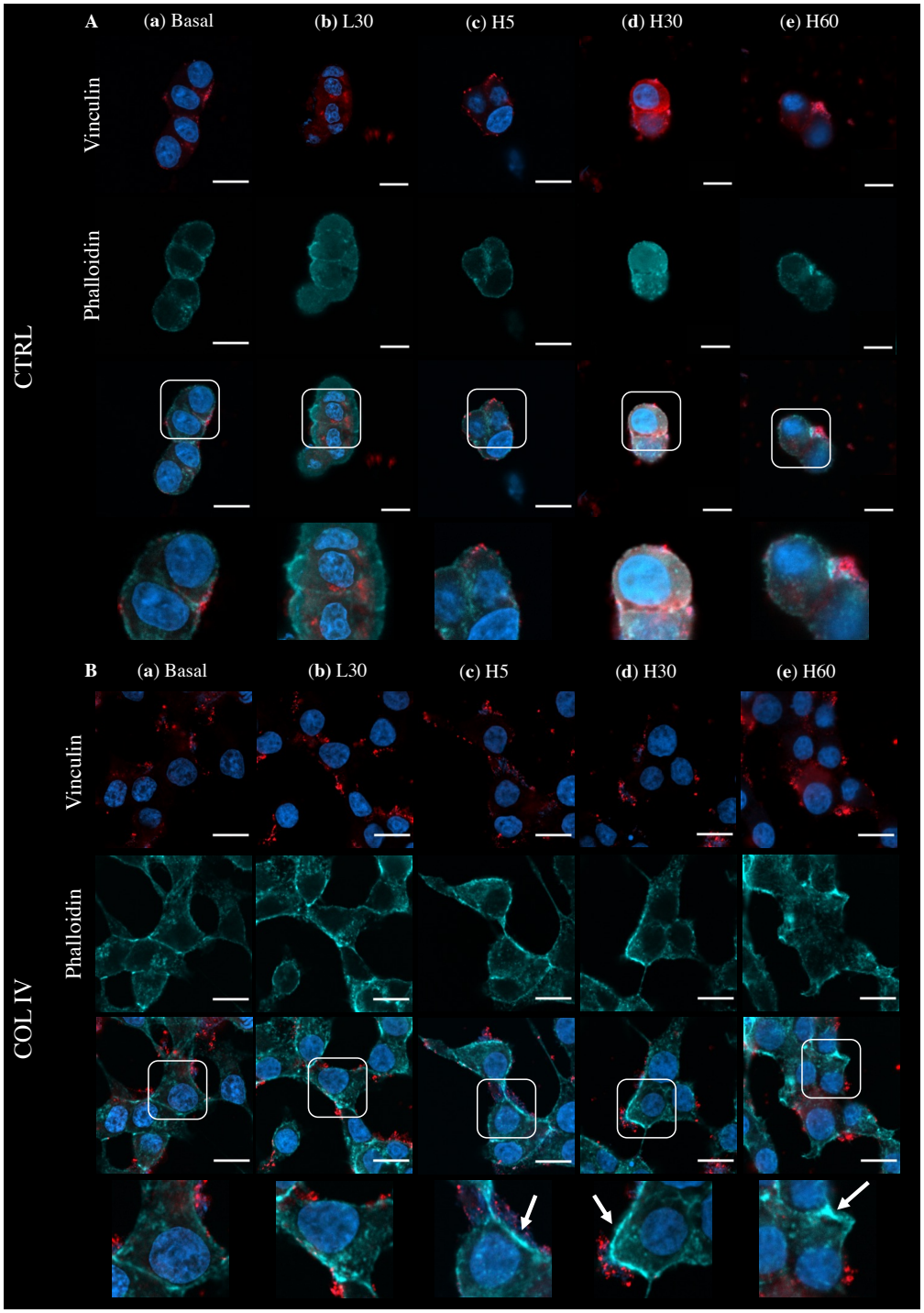

A
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Vinculin
Phalloidin
CTRL
B
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Vinculin
Phalloidin
COL IV

Figure 3.9: Collagen IV promotes focal adhesion complex formation during GSIS

Representative confocal images of vinculin (red) and phalloidin (cyan) in INS-1 cells cultured on (**A**) CTRL or (**B**) COL IV coated chamber slides. Five groups are shown (**a**) no treatment following overnight incubation (basal), (**b**) low glucose (2.2mmol) for 30 minutes (L30), high glucose (22mmol) for (**c**) 5 minutes (H5), (**d**) 30 minutes (H30), or (**e**) 60 minutes (H60). Magnified images of the highlighted region for each group are shown at the bottom of each panel. Arrows indicate areas of interest. Scale bar: 10μm.

3.7.Collagen IV alters SNARE and exocytotic proteins staining in INS-1 cells during GSIS

SNARE proteins SNAP25 and VAMP2, both involved in beta-cell insulin secretion, were also examined at different time points and concentrations of glucose stimulations. In CTRL conditions, cells at basal showed SNAP25 located in both the membrane and cytoplasm (**Figure 3.10a**). In the COL IV group, membrane staining is more defined, suggesting increased t-SNARE complexes at the membrane under basal conditions (**Figure 3.10Ba**). At L30, both groups showed increased SNAP25 cytoplasmic staining in increased compared to basal, and while CTRL cells lost membrane-associated localization, COL IV had some areas of intense membrane staining (**Figure 3.10Ab, Bb**). At H5, COL IV cells showed weak membrane-associated localization, while by H30 there are areas with intense membrane-associated localization (**Figure 3.10Bc, Bd**). In contrast, the CTRL group showed increased SNAP25 membrane localization at H5 which is lost at H30 (**Figure 3.10Ac, Ad**). At H60, CTRL membrane localization is regained, and cytoplasmic staining is also intense (**Figure 3.10Ae**). In COL IV at H60, cells maintained SNAP25 membrane localization between cell-cell and cell-ECM contacts (**Figure 3.10Be**).

VAMP2 was also examined during GSIS. At basal conditions both CTRL and COL IV cells had areas of dense staining located in the cytoplasm and near the membrane (**Figure 3.11Aa, Ba**). At L30, COL IV cells displayed slight increased intensity of cytoplasmic staining compared to CTRL (**Figure 3.11Ab, Bb**). At H5, CTRL cells showed areas of dense cytoplasmic localization similar to that observed under basal conditions, and this localization was more dispersed at H30 (**Figure 3.11Ac, Ad**). In COL IV cells, H5 and H30 conditions were similar, and VAMP2 was less dense and intense than CTRL cells (**Figure 3.11Bc, Bd**). At H60, CTRL had consistent VAMP2 localization throughout the whole cell with no areas of denser staining (**Figure 3.11Ae**).

Contrastingly, COL IV maintained the areas of denser VAMP2 staining, and gained some membrane-associated localization (**Figure 3.11Be**).

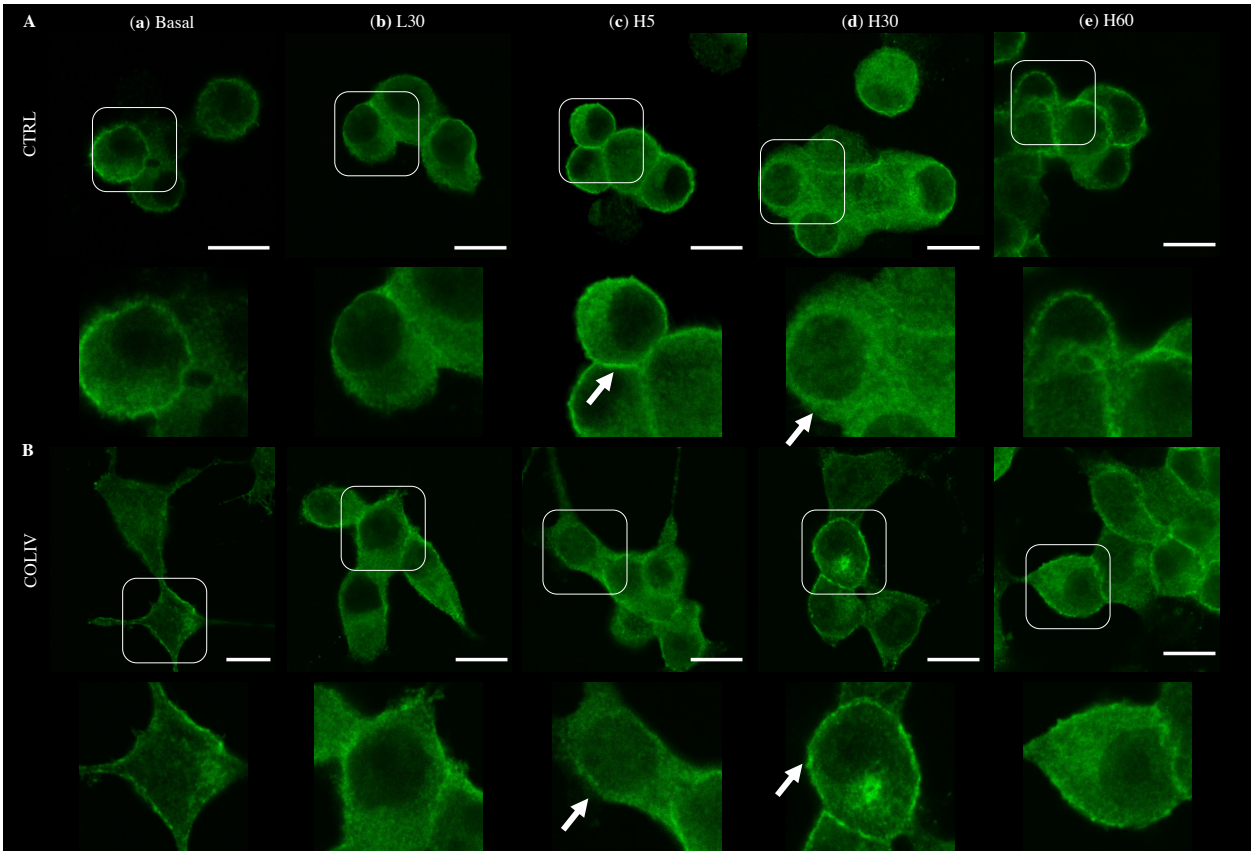

A
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
CTRL
B
COLIV

Figure 3.10: SNAP25 staining in INS-1 cells during GSIS

Representative confocal images of SNAP25 (green) in INS-1 cells cultured on (**A**) CTRL or (**B**) COL IV coated chamber slides. Five groups are shown (**a**) no treatment following overnight incubation (basal), (**b**) low glucose (2.2mmol) for 30 minutes (L30), high glucose (22mmol) for (**c**) 5 minutes (H5), (**d**) 30 minutes (H30), or (**e**) 60 minutes (H60). Magnified images of the highlighted regions for each group are shown at the bottom of each panel. Arrows indicate areas of interest. Scale bar: 10μm.

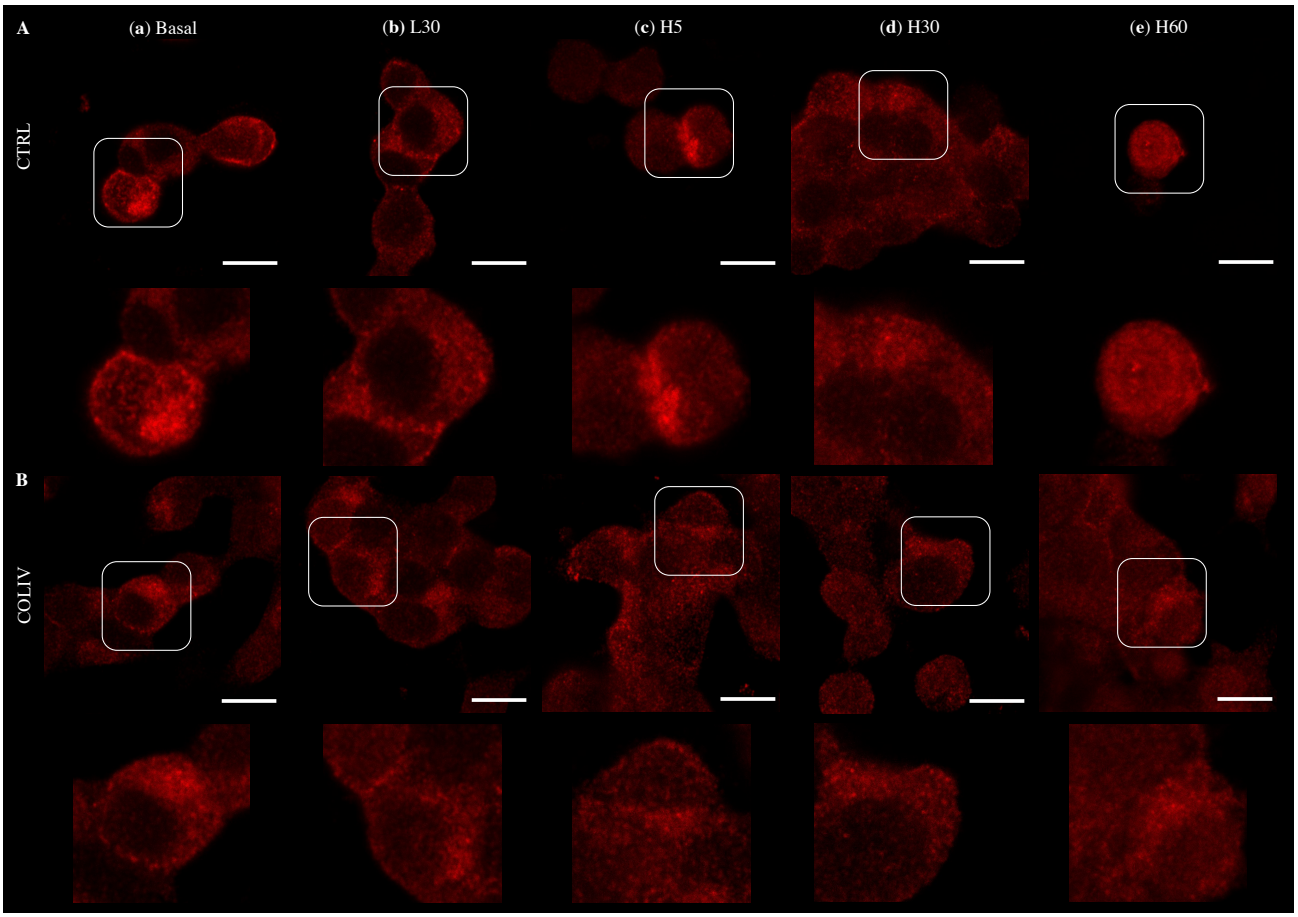

A
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
CTRL
B
COLIV

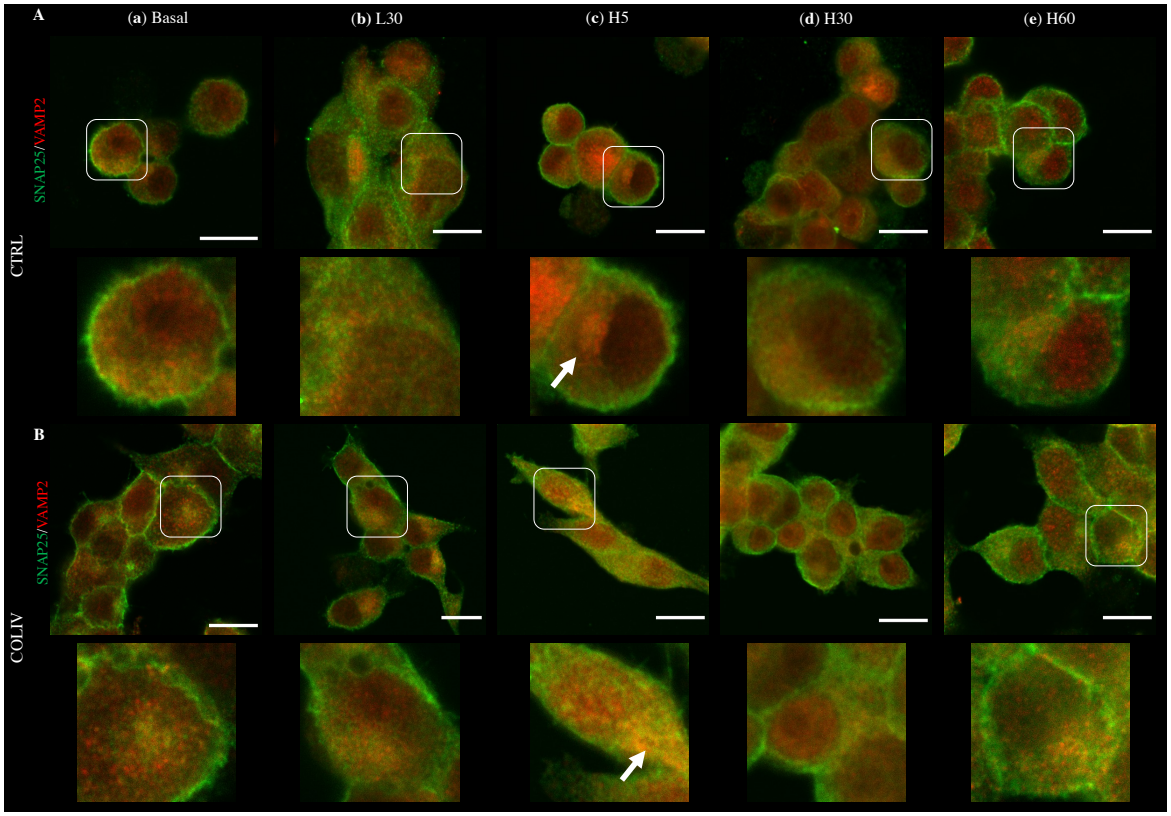
Figure 3.11: VAMP2 staining in INS-1 cells during GSIS

Representative confocal images of VAMP2 (red) in INS-1 cells cultured on (**A**) CTRL or (**B**) COL IV coated chamber slides. Five groups are shown (**a**) no treatment following overnight incubation (basal), (**b**) low glucose (2.2mmol) for 30 minutes (L30), high glucose (22mmol) for (**c**) 5 minutes (H5), (**d**) 30 minutes (H30), or (**e**) 60 minutes (H60). Magnified images of the highlighted regions for each group are shown at the bottom of each panel. Scale bar: 10μm.

3.8. Collagen IV impacts SNAP25 and VAMP2 visual colocalization and rate of insulin secretion during GSIS in INS-1 cells

Colocalization of SNAP25 and VAMP2 during GSIS were examined in the conditions described above. Across the varied glucose stimulation conditions, the amount of overlap does not vary in CTRL cells compared to the basal condition (**Figure 3.12Ab-e**). In COL IV cells, basal, L30, H30, and H60 conditions all appear to have similar levels of overlap and don't significantly vary compared to CTRL (**Figure 3.12Aa-b, d-e, Ba-b, d-e**). In contrast, at H5, COL IV cells displayed increased SNAP25 and VAMP2 staining and enhanced overlap compared to other groups and CTRL (**Figure 3.12Bc**).

In addition to examining SNARE proteins during varied glucose stimulation conditions, the rate of insulin secretion was also determined for each condition. It was found that the rates of insulin secretion (ng/mL/minute) at basal and L30 conditions did not differ between groups (**Figure 3.12C**). However, COL IV displayed higher rates of insulin secretion compared to CTRL at H5 (COL IV 7.82 vs. CTRL 6.14 ng/mL/minute) and H60 (COL IV 1.16 vs. CTRL 0.68 ng/mL/minute) but not at H30 (COL IV 1.40 vs. CTRL 1.6 ng/mL/minute) (**Figure 3.12C**).


A
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
SNAP23/VAMP2
CTRL
B
SNAP23/VAMP2
COLIV

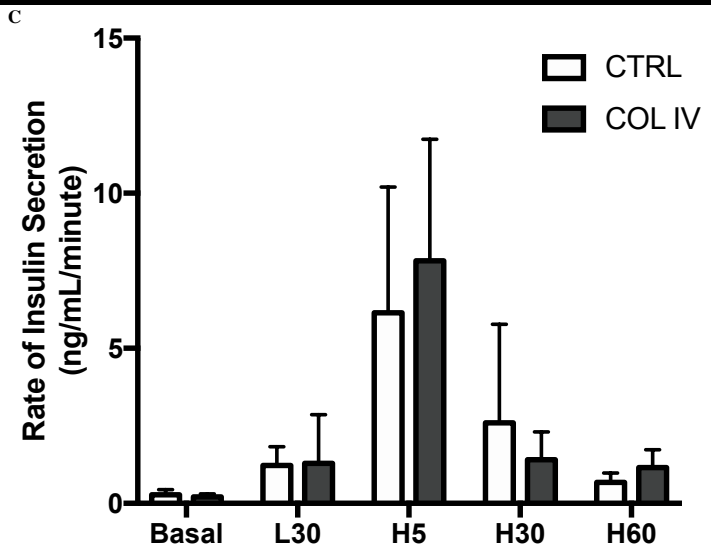

C
Rate of Insulin Secretion (ng/mL/minute)
15
10
5
0
CTRL
COL IV
Basal
L30
H5
H30
H60

Figure 3.12: Collagen IV impacts SNAP25 and VAMP2 visual colocalization and rate of insulin secretion during GSIS in INS-1 cells

Representative confocal images of SNAP25 (green) and VAMP2 (red) in INS-1 cells cultured on (**A**) CTRL or (**B**) COL IV coated chamber slides. Five groups are shown (**a**) no treatment following overnight culture (basal), (**b**) low glucose (2.2mmol) for 30 minutes (L30), high glucose (22mmol) for (**c**) 5 minutes (H5), (**d**) 30 minutes (H30), or (**e**) 60 minutes (H60). Magnified images of the highlighted regions for each group are shown at the bottom of each panel. Scale bar: 10μm. (**C**) Rates of insulin secretion of INS-1 cells cultured on BSA (CTRL) or collagen IV (COL IV) after one of five treatments described above. Data are expressed as mean rate of insulin secretion (ng/mL/minute) ± SD (n=4-6 experiments/group).

3.9. Blocking β1-integrin impacts INS-1 cell β1-integrin localization but not total protein level

β1-integrin localization and protein levels were examined in INS-1 cells following treatment with β1-integrin blocking antibody and compared to control groups. A notable decrease in membrane-associated and cytosolic puncta was observed in the anti-β1 group compared to controls (**Figure 3.13A, B**). However, no effect on was seen on total β1-integrin protein levels (**Figure 3.13C, D**). FAK, a major signaling molecule for β1-integrin-mediated downstream signaling, was also examined. Anti-β1 treated cells reduced FAK phosphorylation compared to controls, although not significantly ($p=0.1127$) (**Figure 3.13E, F**).

3.10. Blocking β1-integrin reduces INS-1 cell adhesion and spreading on collagen IV

To determine if β1-integrin plays a role in adhesion and spreading, INS-1 cells were pretreated with either a β1-integrin blocking antibody (anti-β1), IgM-matched (IgM) control, or untreated control and cultured on collagen IV-coated plates for 24 hours. Anti-β1 INS-1 cells displayed decreased cell adhesion and spreading on collagen IV-coated plates (**Figure 3.14A**). Quantification of adhesion revealed a significant decrease in adhesion for anti-β1 vs. control groups ($p<0.0001$) (**Figure 3.14B**). Additionally, at the 24-hour mark, INS-1 cells spreading was found to be significantly lower in cells treated with the β1-integrin antibody block compared to controls ($p<0.001$, $p<0.05$, respectively) (**Figure 3.14C**).

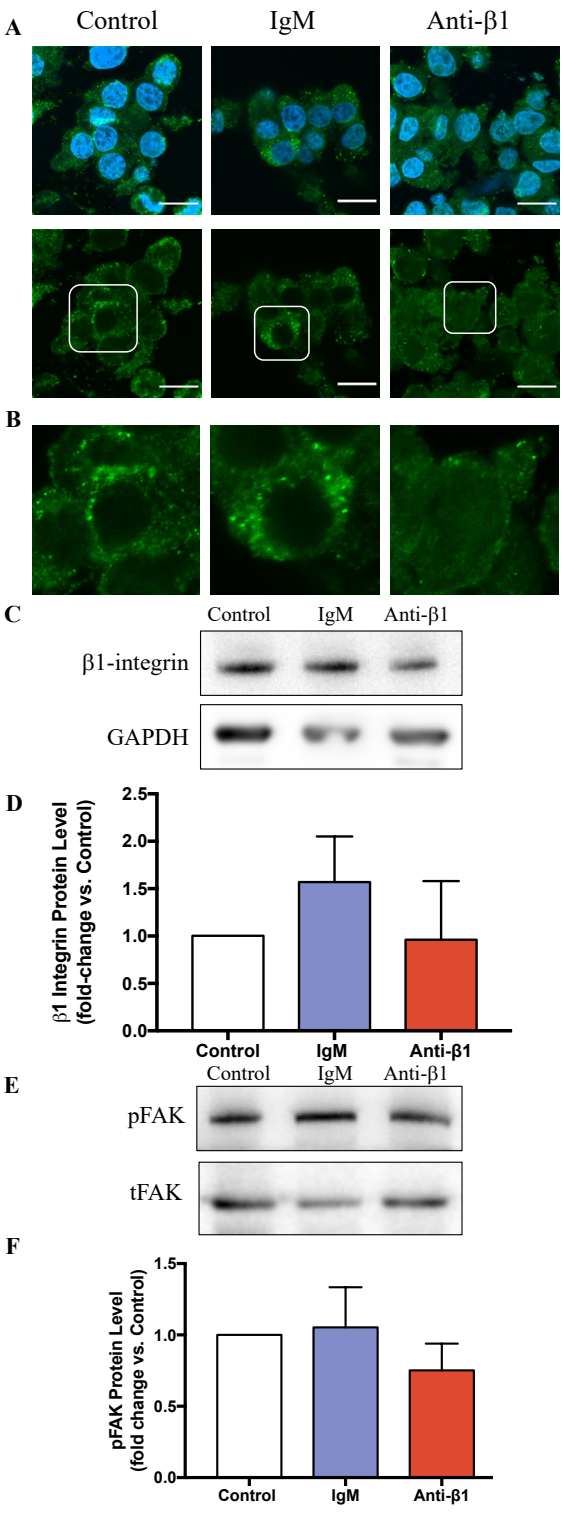

A
Control
IgM
Anti-β1
B
C
Control
IgM
Anti-β1
β1-integrin
GAPDH
D
β1 Integrin Protein Level
(fold-change vs. Control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
IgM
Anti-β1
E
Control
IgM
Anti-β1
pFAK
tFAK
F
pFAK Protein Level
(fold change vs. Control)
1.5
1.0
0.5
0.0
Control
IgM
Anti-β1

Figure 3.13: Blocking β1-integrin impacts INS-1 cell β1-integrin localization but not protein level

(**A**) Representative confocal images of β1-integrin (green) and nuclei (blue) in INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates. (**B**) Magnified images of the corresponding highlighted area for each group. Scale bar: 10μm. (**C, E**) Representative blotting images and (**D, F**) western blot analysis of β1-integrin protein levels and phosphorylated FAK (pFAK), respectively. β1-integrin protein levels are normalized to GAPDH, a loading control, and pFAK levels are normalized to total FAK (tFAK) levels. Data are expressed as fold-change vs. control (mean ± SD, n=3 experiments/treatment group).

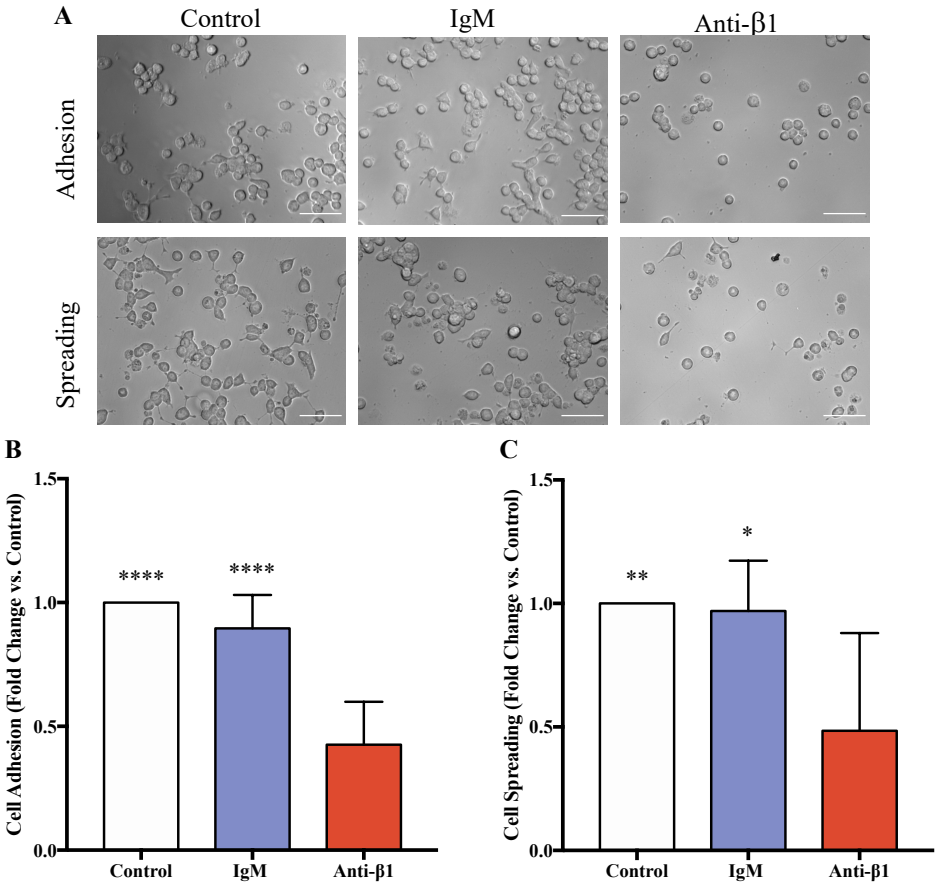

A
Control
IgM
Anti-β1
Adhesion
Spreading
B
Cell Adhesion (Fold Change vs. Control)
1.5
1.0
0.5
0.0
****
****
Control
IgM
Anti-β1
C
Cell Spreading (Fold Change vs. Control)
1.5
1.0
0.5
0.0
**
*
Control
IgM
Anti-β1

Figure 3.14: Blocking β1-integrin decreases INS-1 cell adhesion and spreading on collagen IV

(**A**) Phase contrast images taken 3- and 24-hours following INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates, to determine cell adhesion and spreading, respectively. Scale bar: 50μm. Quantification of INS-1 cell (**B**) adhesion and (**C**) spreading. Data are expressed as fold-change vs. control (mean ± SD, n=6 experiments/treatment group, all done in triplicate). *$p<0.05$, **$p<0.01$, ****$p<0.0001$ vs. anti-β1. Determined by one-way ANOVA followed by Tukey's post-hoc test.

3.11. Blocking β1-integrin reduces INS-1 cell insulin secretion when cultured on collagen IV

To determine if β1-integrin played a role in insulin secretion, INS-1 cells were pretreated with either a β1-integrin blocking antibody (anti-β1), IgM-matched (IgM) control, or untreated control and cultured on collagen IV-coated plates for 24 hours. The examination of basal insulin secretion revealed that anti-β1cells displayed an approximate 40% significant decrease in basal insulin secretion compared to both controls ($p<0.05$) (**Figure 3.15A**). The GSIS stimulation index was reduced approximately 60% in anti-β1 cells, significantly lower than controls ($p<0.05$) (**Figure 3.15B**). Finally, anti-β1 cells displayed an approximate 35% decrease in cellular insulin content compared to controls, but statistical significance was not reached (**Figure 3.15C**) ($p=0.06$).

3.12. Blocking β1-integrin did not influence Ca^{2+} signaling during GSIS

Fluo-4 staining was used to examine whether reduced GSIS in the anti-β1 group is associated with alterations in Ca^{2+} levels. No notable changes in intensity traces (**Figure 3.16A**) or visual differences in intensity (**Figure 3.16B**) of Fluo-4 following glucose stimulation between anti-β1 and control groups (untreated and IgM) were observed. Quantification of intensity traces AUC further demonstrated no significant differences between groups (**Figure 3.16C**).

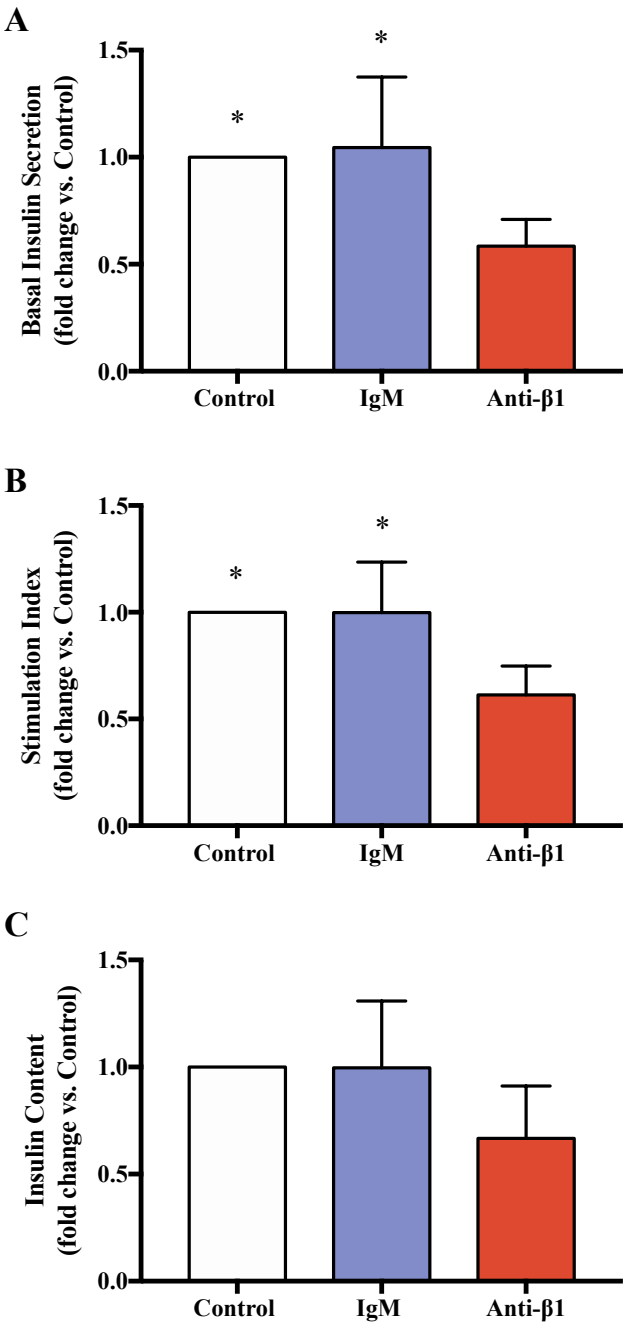

A
Basal Insulin Secretion
(fold change vs. Control)
1.5
1.0
0.5
0.0
*
*
Control
IgM
Anti-β1
B
Stimulation Index
(fold change vs. Control)
1.5
1.0
0.5
0.0
*
*
Control
IgM
Anti-β1
C
Insulin Content
(fold change vs. Control)
1.5
1.0
0.5
0.0
Control
IgM
Anti-β1

Figure 3.15: Blocking β1-integrin decreased INS-1 cell glucose-stimulated insulin secretion**.**

(**A**) Basal insulin secretion, (**B**) GSIS stimulation index (ratio of insulin secretion from high glucose: low glucose), and (**C**) insulin content ± SD of INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates. Insulin content was first normalized to protein content determined by a Bradford assay. Each experiment was performed in triplicate and data are expressed as fold-change vs. anti-β1. (mean ± SD, n=3-6, *$p<0.05$ vs. anti-β1, one-way ANOVA, Tukey's post-hoc test).

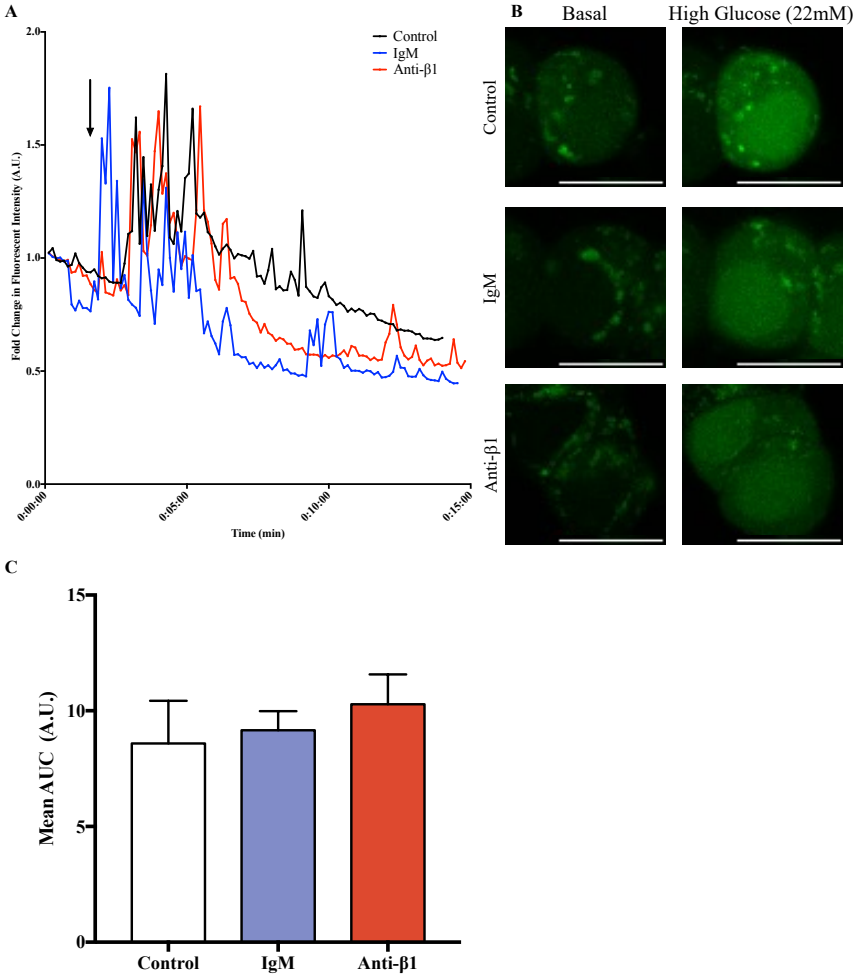

A
Control
IgM
Anti-β1
Fold Change in Fluorescent Intensity (A.U.)
2.0
1.5
1.0
0.5
0.0
0:00:00
0:05:00
0:10:00
0:15:00
Time (min)
B
Basal
High Glucose (22mM)
Control
IgM
Anti-β1
C
Mean AUC (A.U.)
15
10
5
0
Control
IgM
Anti-β1

Figure 3.16: Blocking β1-integrin did not influence glucose-stimulated Ca2+ signaling

(**A**) Representative intensity traces of Ca^{2+} intensity (Fluo-4) in INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates, normalized to basal conditions Arrow indicates timing of glucose addition to reach final concentration of 22mM. (**B**) Representative z-stack images of Fluo-4 treated cells at basal and high glucose conditions (22mM). Scale bar: 10μm. (**C**) Mean area under the curve (AUC) (arbitrary units [A.U.]) of intensity traces ± SD for control, IgM, and anti-β1 cells (n=3 experiments/treatment group).

3.13. Blocking β1-integrin impacted some, but not all, INS-1 cell SNARE protein localization and protein levels

To determine the influence of β1-integrin signaling on exocytotic proteins involved in insulin secretion, Munc18-1 and SNARE proteins localization and protein levels were examined using immunofluorescence and western blot analysis. Munc18-1 was not found to be influenced by β1-integrin signaling. Indeed, no changes in localization were observed, and cells maintained intense cytoplasmic and membrane puncta in all groups (**Figure 3.17A, B**). Munc18-1 protein levels also were not found to significantly differ between groups (**Figure 3.17C, D**). Interestingly, SNAP25 was found to be influenced by β1-integrin signaling. With anti-β1 treatment, membrane-associated SNAP25 localization was found to be reduced compared to both control groups, although localization at cell-cell contacts was still observed (**Figure 3.18A, B**). This was complemented by a significant reduction of SNAP25 protein levels in anti-β1 treated INS-1 cells compared to both controls ($p<0.05$) (**Figure 3.18C, D**). Finally, VAMP2 was also found to be impacted by the β1-integrin blocking antibody. A notable decrease in visual cytoplasmic localization was observed in anti-β1 treated INS-1 cells compared to controls **(Figure 3.19A, B**). Additionally, a significant reduction in VAMP2 protein levels was found in anti-β1 treated INS-1 cells compared to control conditions ($p<0.01$, $p<0.05$) (**Figure 3.19C, D**).

To further our analysis, BAG3 and pBAG3 were also examined. Visually, there was a decrease in total BAG3 intensity in the cytoplasm and a decrease in the size of clusters observed in anti-β1 cells compared to controls (**Figure 3.20A, B**). However, no significant changes in BAG3 protein levels were found ($p=0.1891$) (**Figure 3.20C, D**). When examining phosphorylated BAG3, less

cytoplasmic staining intensity in anti-β1 treated INS-1 cells was noted, and no major changes were observed between control treatments (**Figure 3.20E, F**).

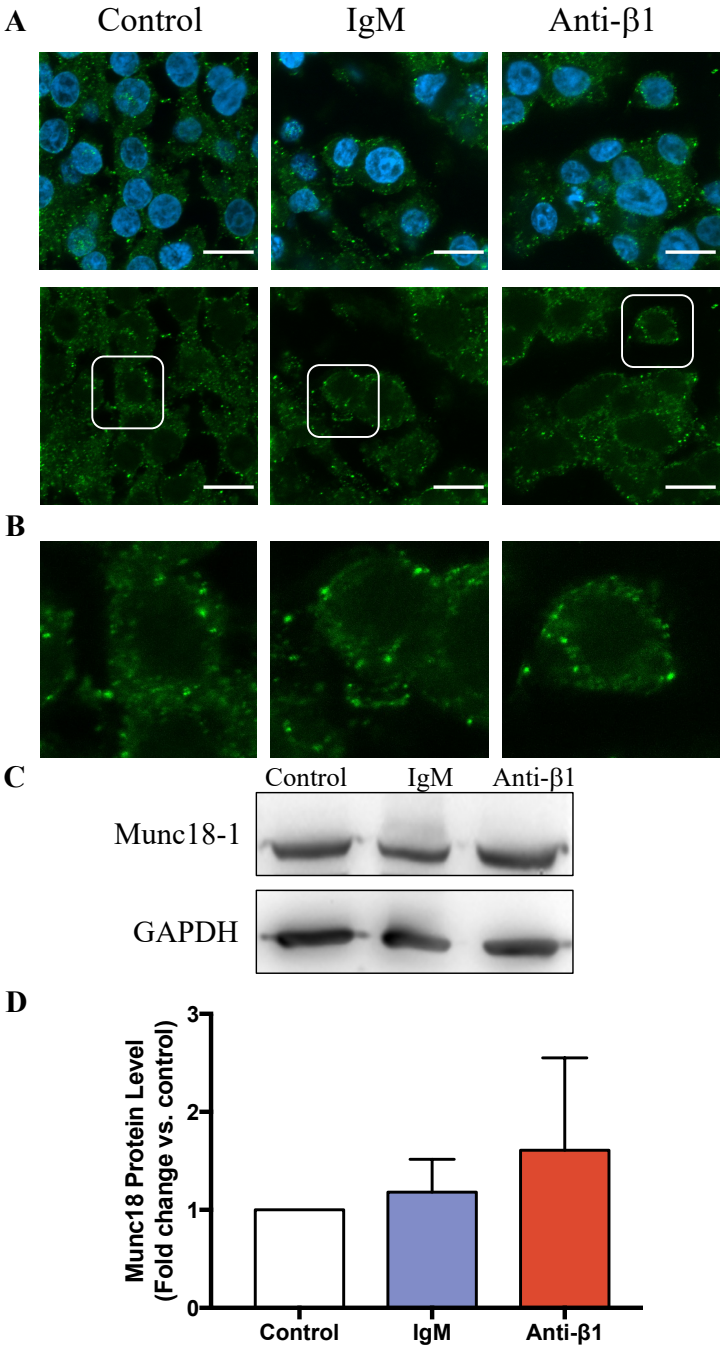

A
Control
IgM
Anti-β1
B
C
Control
IgM
Anti-β1
Munc18-1
GAPDH
D
Munc18 Protein Level
(Fold change vs. control)
0
1
2
3
Control
IgM
Anti-β1

Figure 3.17: Blocking β1-integrin did not influence INS-1 Munc18-1 protein levels and localization

(**A**) Representative confocal images of Munc18-1 (green) and nuclei (blue) in INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates. (**B**) Magnified images of the highlighted region for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of Munc18-1 protein levels. Protein levels are normalized to GAPDH, a loading control. Data are expressed as fold-change vs. control (mean ± SD, n=3-4 experiments/treatment group).

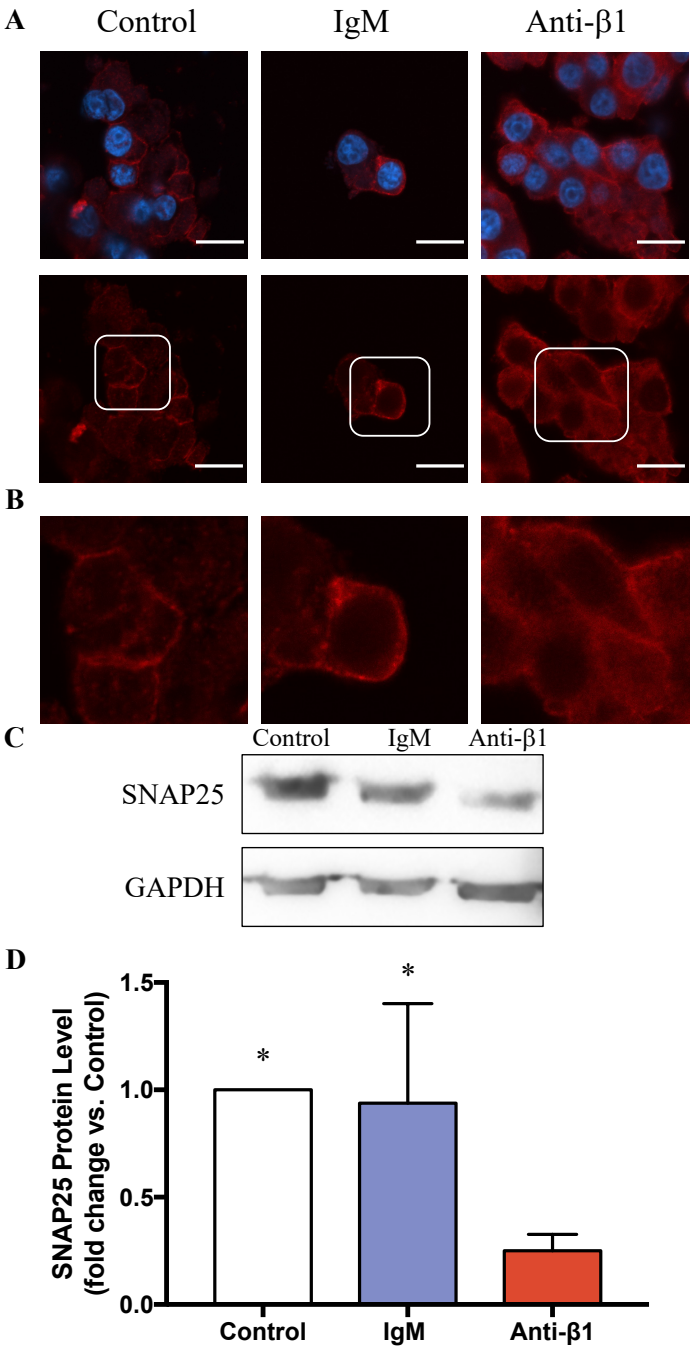

A
Control
IgM
Anti-β1
B
C
Control
IgM
Anti-β1
SNAP25
GAPDH
D
SNAP25 Protein Level
(fold change vs. Control)
1.5
1.0
0.5
0.0
*
*
Control
IgM
Anti-β1

Figure 3.18: Blocking β1-integrin decreased INS-1 cell SNAP25 protein levels and localization

(**A**) Representative confocal images of SNAP25 (red) and nuclei (blue) in INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates. (**B**) Magnified images of the corresponding highlighted area for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of SNAP25 protein levels. Protein levels are normalized to GAPDH, a loading control. Data are expressed as fold-change vs. control (mean ± SD, n=3-4 experiments/treatment group). *$p<0.05$ vs. anti-β1 determined by one-way ANOVA followed by Tukey's post-hoc test.

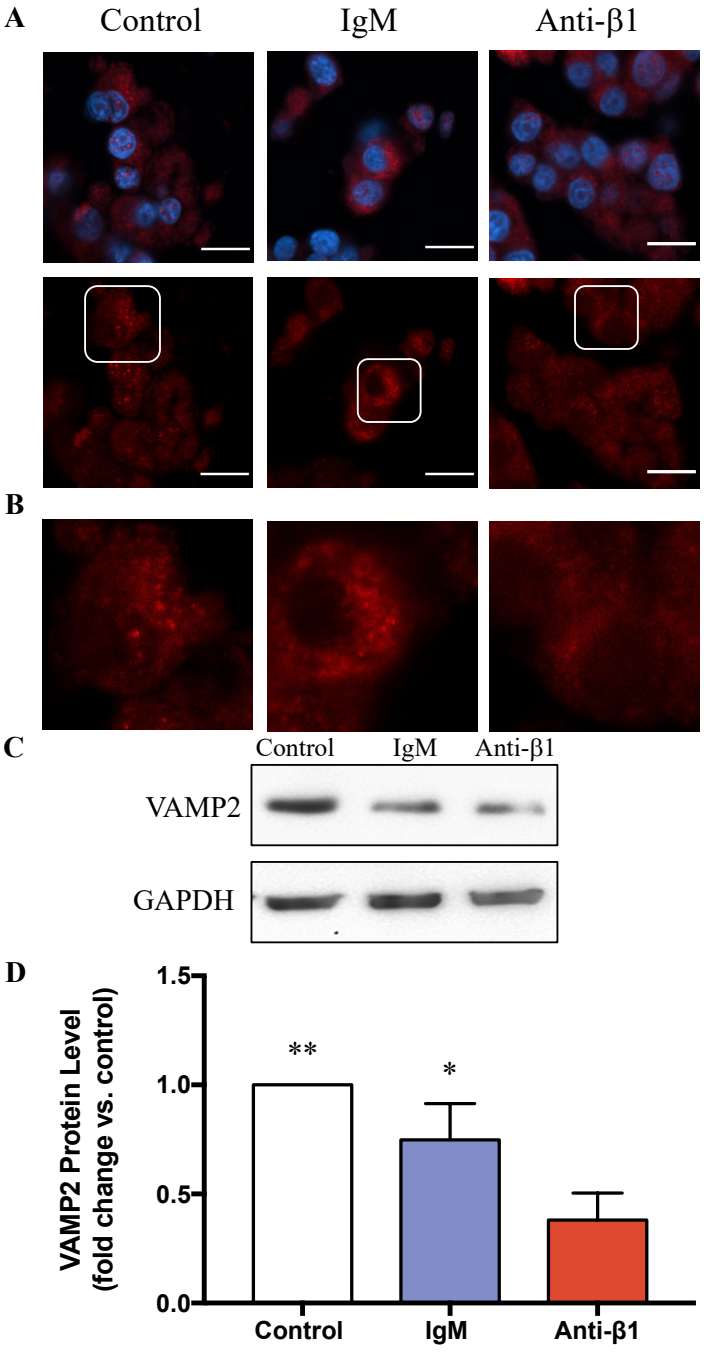

A
Control
IgM
Anti-β1
B
C
Control
IgM
Anti-β1
VAMP2
GAPDH
D
VAMP2 Protein Level
(fold change vs. control)
1.5
1.0
0.5
0.0
**
*
Control
IgM
Anti-β1

Figure 3.19: Blocking β1-integrin decreased INS-1 cell VAMP2 protein levels and localization

(**A**) Representative confocal images of VAMP2 (red) and nuclei (blue) in INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates. (**B**) Magnified images of the corresponding highlighted area for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of VAMP2 protein levels. Protein levels are normalized to GAPDH, a loading control. Data are expressed as fold-change vs. control (mean ± SD, n=3-4 experiments/treatment group). *$p<0.05$, **$p<0.01$ vs. anti-β1 determined by one-way ANOVA followed by Tukey's post-hoc test.

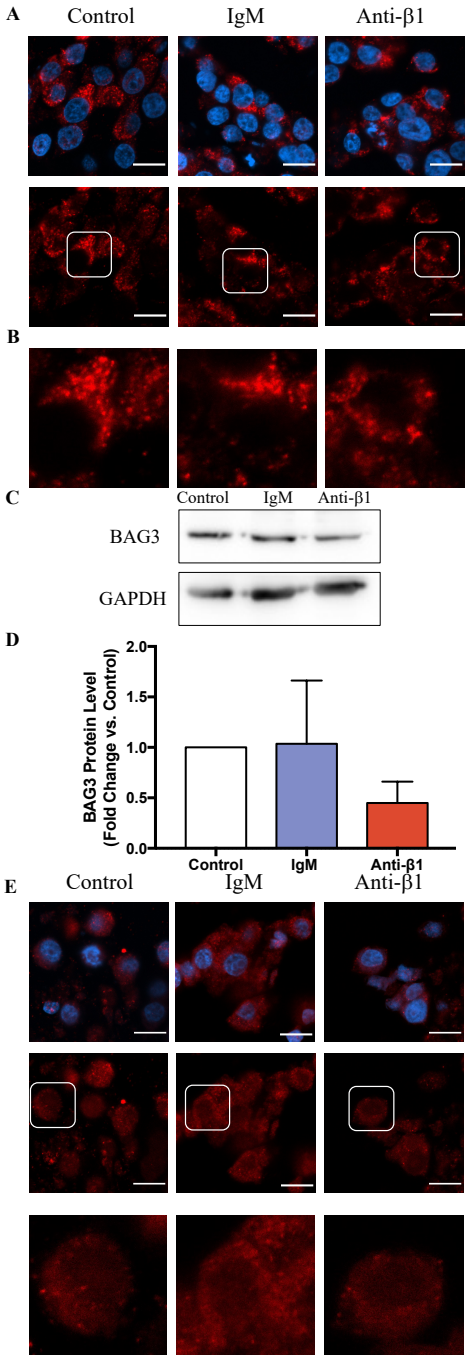

A
Control
IgM
Anti-β1
B
C
Control
IgM
Anti-β1
BAG3
GAPDH
D
BAG3 Protein Level
(Fold Change vs. Control)
2.0
1.5
1.0
0.5
0.0
Control
IgM
Anti-β1
E
Control
IgM
Anti-β1

Figure 3.20: Blocking β1-integrin decreased BAG3 staining intensity, and slightly diminishes pBAG3 intensity

(**A**) Representative confocal images of BAG3 (red) and nuclei (blue) in INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates. (**B**) Magnified images of the highlighted region for each group. Scale bar: 10μm. (**C**) Representative blotting images and (**D**) western blot analysis of BAG3 protein levels. (**E**) Representative confocal images of pBAG3 (red) and nuclei (blue) in INS-1 cells pretreated with anti-β1-integrin (anti-β1) or IgM-matched control (IgM), or untreated (control) and cultured on collagen IV coated plates. (**F**) Magnified images of the highlighted region for each group. Scale bar: 10μm. Protein levels are normalized to GAPDH, a loading control. Data are expressed as fold-change vs. control (mean ± SD, n=3-4 experiments/treatment group).

3.14. Blocking β1-integrin weakened focal adhesion complex formation during GSIS

In the basal condition, control cells have intense actin filament stress fibers and clusters of vinculin at the cell periphery while anti-β1 cells have weaker actin staining, a round cell shape, and membrane-associated vinculin (**Figure 3.21Aa, Ba**). When exposed to low glucose (L30), control cells had increased actin intensity and increased clusters of vinculin at the periphery (**Figure 3.21Ab, Bb**). With anti-β1 treatment in L30, there were alterations in actin cytoskeleton with some areas of intense staining at the periphery, and there was an increase in clusters of vinculin compared to basal conditions (**Figure 3.21Cb**). At high glucose conditions H5 and H30, actin and vinculin were similar to L30 conditions in control cells (**Figure 3.21Ac, d, Bc, d**). With anti-β1 cells at these time-points, the intensity of vinculin was increased with slightly more clusters, while actin remained the same (**Figure 3.21Cc, d**). At H60, all groups had an increase in vinculin intensity and cluster localization at the periphery along with areas of strong actin staining (**Figure 3.21e**).

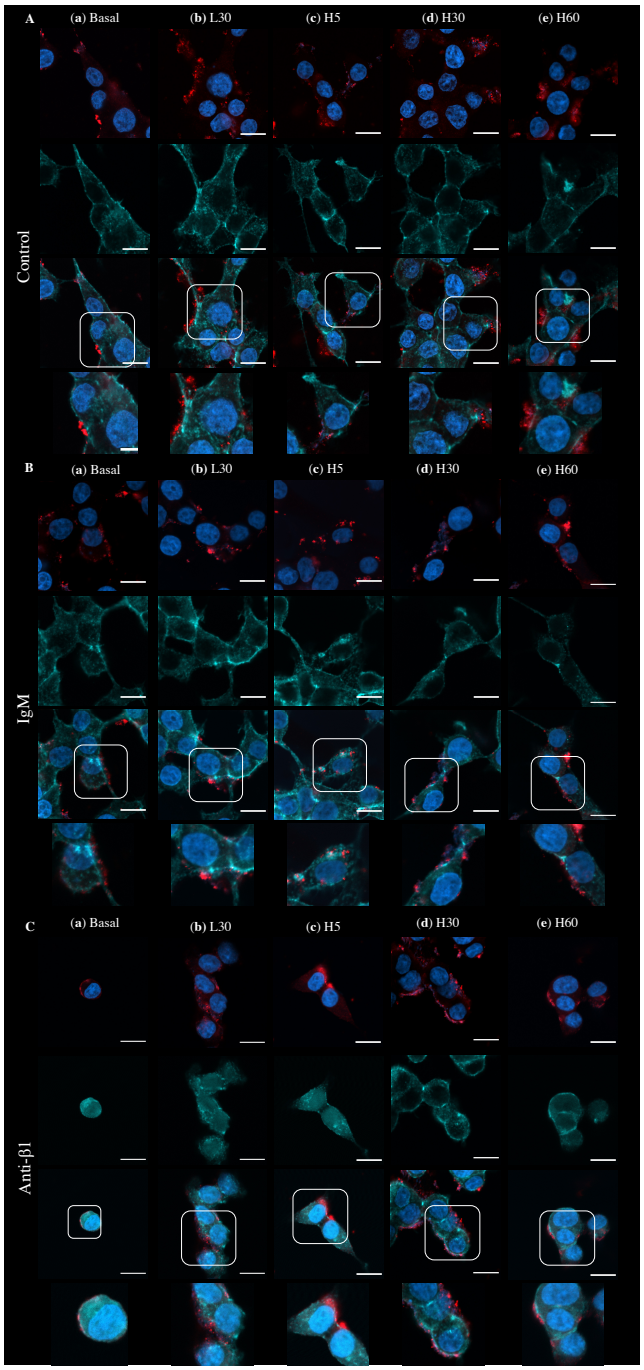

A
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Control
B
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
IgM
C
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Anti-β1

Figure 3.21: Blocking β1-integrin reduced INS-1 cell focal adhesions complex formation during GSIS

Representative confocal images of vinculin (red) and phalloidin (cyan) in INS-1 cells that were (**A**) untreated (control) or pretreated with (**B**) IgM-matched control (IgM) or (**C**)anti-β1-integrin (anti-β1). All cells were cultured on collagen IV-coated chamber slides. Five groups are shown (**a**) no treatment following overnight incubation (basal), (**b**) low glucose (2.2mmol) for 30 minutes (L30), high glucose (22mmol) for (**c**) 5 minutes (H5), (**d**) 30 minutes (H30), or (**e**) 60 minutes (H60). Magnified images of the corresponding highlighted for each group are shown at the bottom of each panel. Scale bar: 10μm.

3.15. Blocking β1-integrin impacted SNARE localization during GSIS

To determine the role of β1-integrin during GSIS, SNARE proteins were examined after β1-integrin blocking using the previously described concentrations and timings of glucose-stimulation. At basal conditions, SNAP25 membrane localization, outside of cell-cell contacts, was decreased in the anti-β1 group compared to controls (**Figure 3.22a**). At L30, no notable differences were observed between groups (**Figure 3.22b**). At H5, all groups had a decrease in membrane staining but, this change was least evident in the anti-β1 group that also displayed high intensity and localization at cell-cell contacts, a finding not observed in controls (**Figure 3.22c**). By H30, control groups regained membrane localization while anti-β1 membrane localization remained weak, and cell-cell contact localization and intensity remained high (**Figure 3.22d**). At H60, all groups appear to lose the defined membrane staining (**Figure 3.22e**).

VAMP2 was also examined during at the above time-points during GSIS. At basal conditions, control cells had some areas of dense staining while anti-β1 did not (**Figure 3.23a**). With glucose stimulation, no major changes were observed with between groups (**Figure 3.23b, c, d)**. However, control cells show areas with increased density at H60 that is not present in anti-β1 conditions (**Figure 3.23e**).

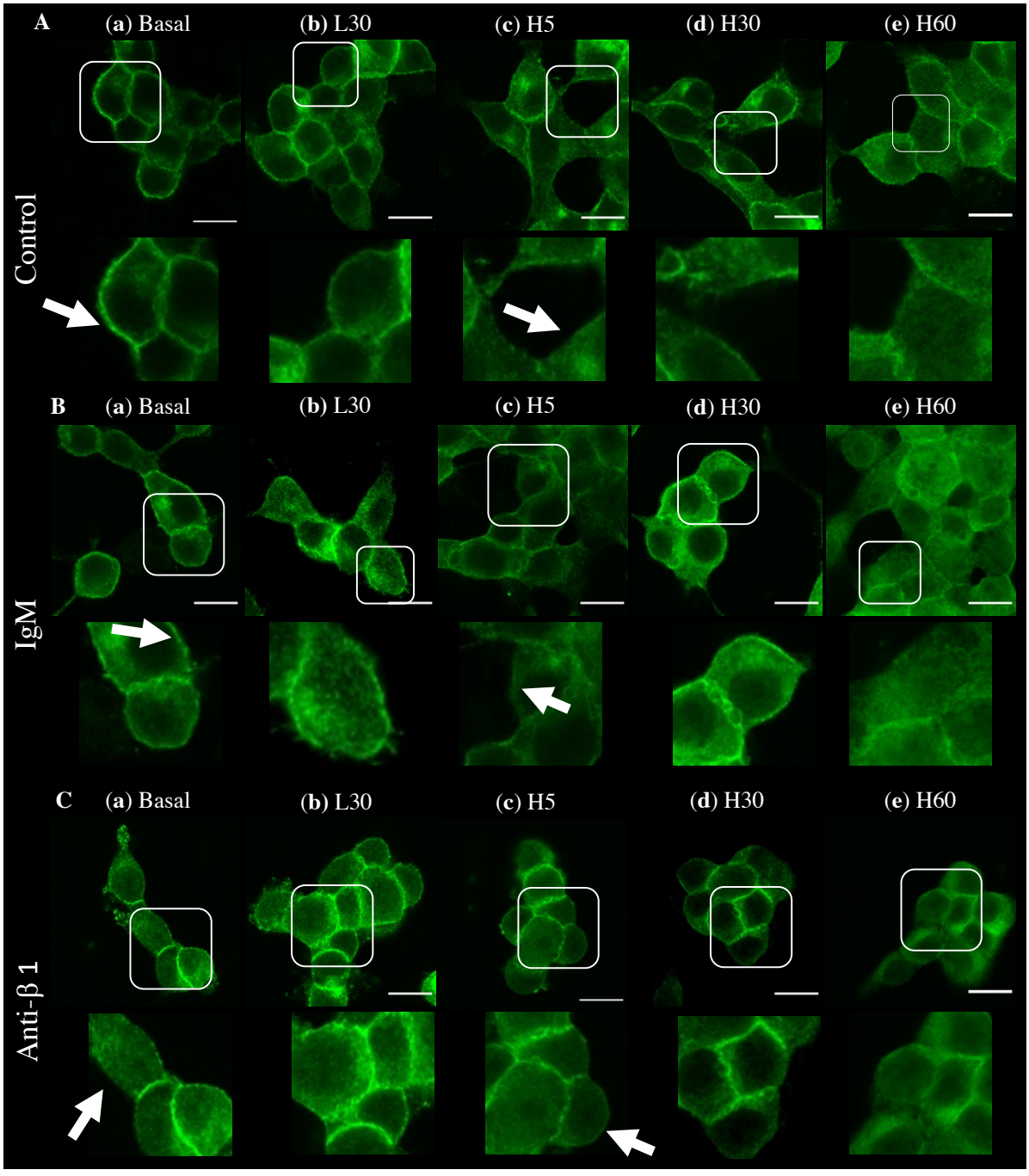

A
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Control
B
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
IgM
C
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Anti-β 1

Figure 3.22: Blocking β1-integrin impacted INS-1 cell SNAP25 localization during GSIS

Representative confocal images of SNAP25 (green) in INS-1 cells that were (**A**) untreated (control) or pretreated with (**B**) IgM-matched control (IgM) or (**C**) anti-β1-integrin (anti-β1). All cells were cultured on collagen IV-coated chamber slides. Five groups are shown (**a**) no treatment following overnight incubation (basal), (**b**) low glucose (2.2mmol) for 30 minutes (L30), high glucose (22mmol) for (**c**) 5 minutes (H5), (**d**) 30 minutes (H30), or (**e**) 60 minutes (H60). Magnified images of the highlighted regions for each group are shown at the bottom of each panel. Arrows indicate areas of interest. Scale bar: 10μm.

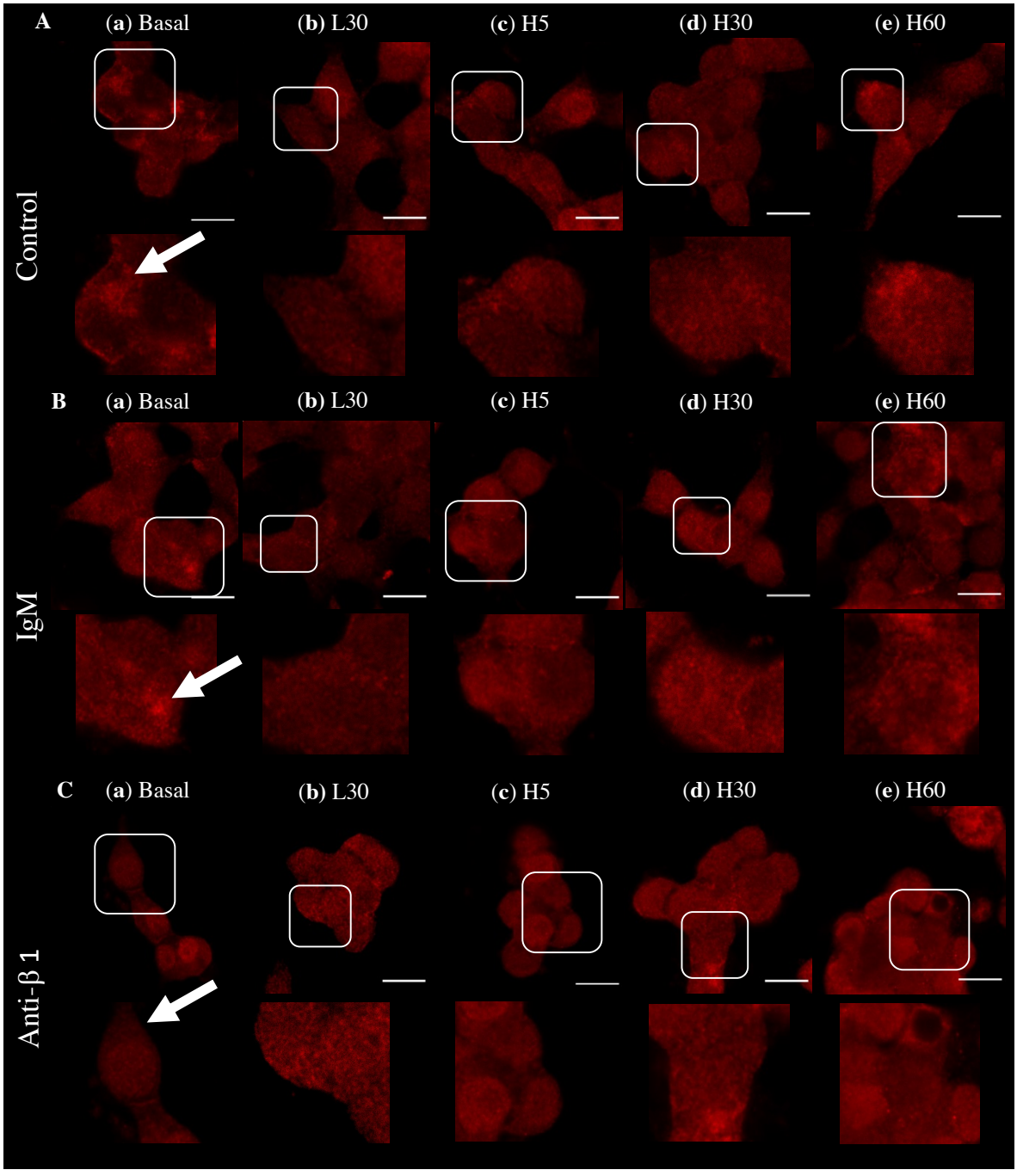

A
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Control
B
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
IgM
C
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Anti-β1

Figure 3.23: VAMP2 was minimally influence by β1-integrin block

Representative confocal images of VAMP2 (red) in INS-1 cells that were (**A**) untreated (control) or pretreated with (**B**) IgM-matched control (IgM) or (**C**)anti-β1-integrin (anti-β1). All cells were cultured on collagen IV-coated chamber slides. Five groups are shown (**a**) no treatment following overnight incubation (basal), (**b**) low glucose (2.2mmol) for 30 minutes (L30), high glucose (22mmol) for (**c**) 5 minutes (H5), (**d**) 30 minutes (H30), or (**e**) 60 minutes (H60). Magnified images of the highlighted regions for each group are shown at the bottom of each panel. Arrows indicate areas of interest. Scale bar: 10μm.

3.16. Blocking β1-integrin reduces SNAP25 and VAMP2 visual colocalization and rate of insulin secretion during GSIS in INS-1 cells

Colocalization of SNAP25 and VAMP2, observed as yellow fluorescence, during GSIS were examined in the conditions described above. Across all conditions, control groups displayed some overlap of SNAP25 and VAMP2 while anti-β1cells showed little to no overlap (**Figure 3.24A, B, C**). When comparing different glucose treatments, control groups displayed the same pattern as described above (see **Results section 3.8**) where a major increase in overlap was observed in H5 compared to other conditions (**Figure 3.24A, B**). However, no changes in overlap were observed in the anti-β1 group when comparing between any conditions (**Figure 3.24C**).

In addition to examining SNARE proteins during varied glucose stimulation conditions, the rate of insulin secretion was determined for each condition. Anti-β1 treatment was found to decrease the rate of insulin secretion 25-50% in all treatment groups compared to control, except for the H30 treatment (**Figure 3.24D**). The largest decrease was observed in H5 treatments (Control 3.47 and IgM 4.69 vs. anti-β1 2.14 ng/mL/min).

Overall, this thesis demonstrates that interaction of collagen IV and β1-integrin were found to augment INS-1 cell insulin secretin, in part due to alterations in the exocytotic proteins involved. Taken together, these results add to the growing body of knowledge regarding the critical role of β1-integrin in beta-cell function.

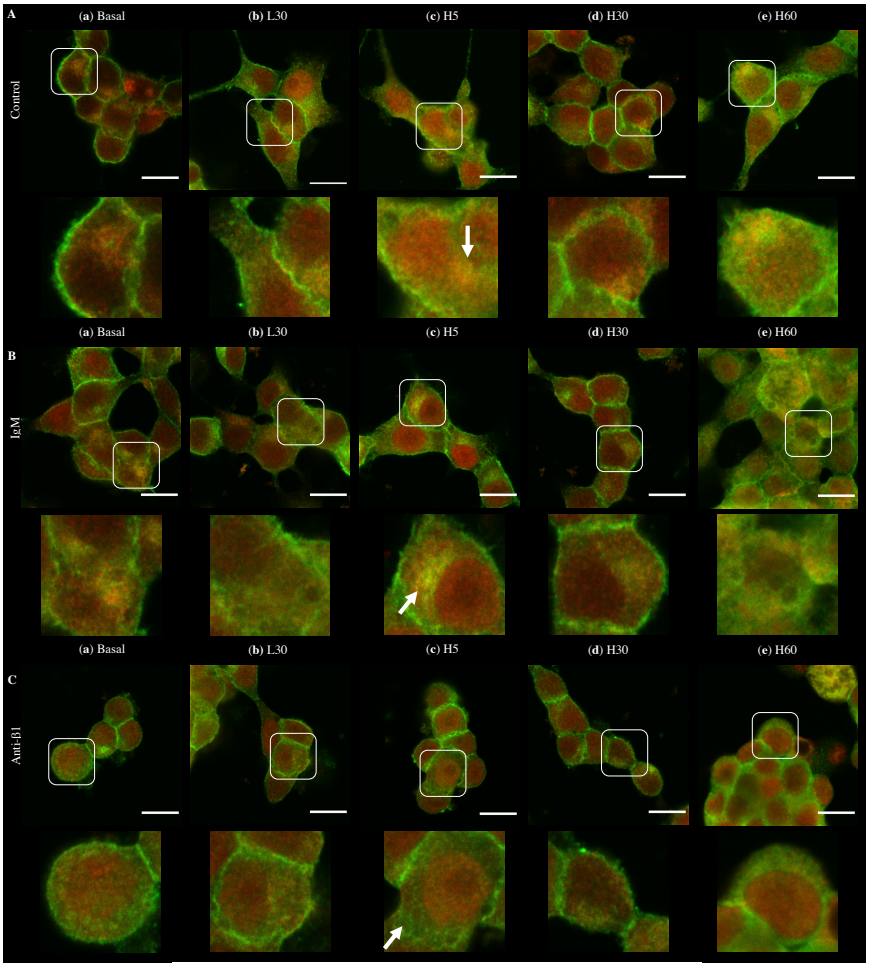

A
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Control
B
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
IgM
C
(a) Basal
(b) L30
(c) H5
(d) H30
(e) H60
Anti-B1
D
Control
IgM
Anti-B1
Rate of Insulin Secretion (ng/mL/minute)
0
2
4
6
8
Basal
L30
H5
H30
H60

Figure 3.24: Blocking β1-integrin impacted SNAP25 and VAMP2 colocalization and rate of insulin secretion in INS-1 cell GSIS

Representative confocal images of SNAP25 (green) and VAMP2 (red) in INS-1 cells that were (**A**) untreated (control) or pretreated with (**B**) IgM-matched control (IgM) or (**C**)anti-β1-integrin (anti-β1). All cells were cultured on collagen IV-coated chamber slides. Five groups are shown (**a**) no treatment following overnight incubation (basal), (**b**) low glucose (2.2mmol) for 30 minutes (L30), high glucose (22mmol) for (**c**) 5 minutes (H5), (**d**) 30 minutes (H30), or (**e**) 60 minutes (H60). Magnified images of the highlighted region for each group are shown at the bottom of each panel. Arrows indicate areas of interest. Scale bar: 10μm. (**C**) Rates of insulin secretion of INS-1 cells untreated or pretreated with IgM-matched control or anti-β1 and cultured on collagen IV after one of five treatments described above. Data are expressed as mean rate of insulin secretion (ng/mL/minute) ± SD (n=3-6 experiments/group).

Chapter 4

4. Discussion

Islet transplantation and bioartificial pancreas treatments for diabetics are limited by several factors including the number of eligible donors required, immunosuppression, and longevity of treatment (28, 36). Identifying factors that enhance the function of beta-cells can be advantageous in efforts to improve these treatments. This study highlights the importance of collagen IV-activated β1-integrin and subsequent changes in exocytotic proteins that lead to augmented insulin secretion in INS-1 cells. Not only was collagen IV found to alter overall protein levels and localization of exocytotic proteins, but it was also found to change exocytotic protein localization throughout glucose-stimulated insulin secretion (GSIS). β1-integrin was demonstrated to be responsible for some of the changes observed as they were diminished with β1-integrin antibody blocking. This underscores the importance of the collagen IV-β1-integrin interaction regarding the mechanics of insulin secretion from beta cells. In reference to cell-based therapies for diabetics, this work suggests specific integrin-ECM interactions should be taken advantage of to optimize these procedures.

4.1. Collagen IV enhanced INS-1 cell adhesion and spreading

Integrins are known mediators of cell-ECM interactions and it is well described that integrin activation can provide increased adhesion and spreading (46, 61). The β1-integrin ligands, fibronectin, laminin, collagen I and collagen IV, which are all expressed in pancreatic islets, were tested to establish cell-ECM interactions in the INS-1 cell line. It was determined that collagen IV

provided the greatest enhancement in adhesion and spreading compared to other experimental groups (**Figure 3.1**).

Given the important of adhesion sites *in vivo* and the disruption of ECM that occurs during islet isolation procedures used in islet transplantation, exploration in the impact of ECM factors on beta cell function has been done with the goal of improving graft survival. Many ECM proteins have been demonstrated to promote adhesion in islets and beta-cells. Laminin has been demonstrated to be essential for isolated rat beta-cell adhesion on 804G matrix (75) and was found to promote adhesion of human islets (73). Culturing on fibronectin has been demonstrated to produce the greatest amount of adhesion in porcine islet cells compared to laminin, collagen I, gelatin, poly-L-lysine, and control (21). Our study agrees with previous findings of our lab stating that collagen IV is an optimal ECM protein for INS-1 cell adhesion and spreading (51). In support of this, Kaido et al. demonstrated collagen IV promoted beta-cell mobility in both fetal and adult human islets (47). Additionally, in porcine islets, adhesion was found to be the greatest following 3 hour culture on collagens I or IV compared to fibronectin, laminin, fibrinogen, and BSA (67). This underscores the importance of collagen IV-beta-cell interactions within beta-cells.

4.2. Collagen IV enhanced INS-1 cell insulin secretion and influenced protein level and localization of exocytotic proteins

Once integrin-ECM interactions are established, downstream signaling mediates functional changes within cells. Given the major function of beta-cells is insulin secretion, basal and glucose-stimulated insulin secretion and insulin content were examined. INS-1 cells cultured on collagen IV were found to have statistically significant increased insulin secretion compared control (**Figure 3.2**), which matches previous work from our lab (51). Collagen IV has been demonstrated

to augment insulin secretion by other studies as well. For example, primary human islets cultured on collagen IV microwells were found to have increased glucose-stimulated insulin secretion compared to control conditions (30). Fetal human islets also displayed significant increased insulin secretion for cells cultured on collagen IV-coated plates compared to poly-L-lysine and BSA (47). Isolated mouse islets cultured for 24-hours on collagen IV-modified scaffolds had increased stimulation index compared to fibronectin, laminin, and control (120).

Given the alterations in insulin secretion, INS-1 cells cultured on the different ECM proteins were then further examined for protein level and localization changes in exocytotic proteins and cell-ECM signaling proteins. Overall, collagen IV consistently provided the greatest impact on the examined proteins. This group displayed increased protein levels and intensity of cytoplasmic puncta of Munc18-1 as well as increased membrane localization of SNAP25 (**Figures 3.3-3.5**). Taken together, these results indicate that cell-ECM interactions impact SNARE protein(s) expression which, in turn, influences insulin secretion from INS-1 cells. There is a great deal of research supporting alterations in exocytotic proteins impacting exocytosis (42, 70, 97). However, there is little research on the impact of cell-ECM interactions on exocytotic proteins. One study examined the impact of culturing isolated rat islets on fibronectin and found that after six days, a significant increase in Munc18-1, SNAP25, and Syntaxin1A expression was observed (22). Thus, our research provides novel information in the relationship between collagen IV and exocytotic proteins.

In addition to exocytotic proteins, β1-integrin, FAK, and BAG3 were examined since they are all signaling proteins demonstrated to impact insulin secretion. In the collagen IV group, β1-integrin, BAG3, and FAK phosphorylation protein levels appeared to be trending to increase but

did not reach statistical significance (**Figures 3.7-3.8**). However, immunofluorescent staining changes were still observed. β1-integrin had increased intensity and membrane associated puncta and BAG3 had increased intensity in cells cultured on collagen IV and cytoplasmic staining of phosphorylated BAG3 was increased in all ECM groups compared to control (**Figures 3.7-3.8**). Given the inhibitory role of BAG3 and promotive role of phosphorylated BAG3 on insulin secretion (39) it is interesting that no major changes of phosphorylated BAG3 were observed in the collagen IV group compared to the other experimental groups. However, when describing the role of BAG3 phosphorylation in insulin secretion Iorio et al. observed that phosphorylation was a result of glucose stimulation. Thus, it seems that collagen IV increases total BAG3, but the regulation of BAG3 phosphorylation is achieved through other means such as the presence of glucose. Not only were changes in individual proteins found, but an increase in the amount of VAMP2 that colocalized with SNAP25 was found in the collagen IV group. This research demonstrated collagen IV-β1-integrin signaling – likely mediated via FAK – is also involved in mediating changes in exocytotic protein levels, localization, and interaction as well as influencing regulatory proteins such as BAG3. Understanding the relationship between cell-ECM signaling and the components of insulin secretion is critical for determining the optimal ECM environment for pancreatic beta-cell function.

4.3.Collagen IV impacted the mechanics of exocytotic proteins during GSIS

Focal adhesion contact sites are initiated by interactions of integrins with the ECM. They connect integrins with actin and are sites where cytoskeletal proteins bind to act as scaffolds for signaling proteins that mediate effects on cell behaviour (115, 116). In beta cells, focal adhesions have been demonstrated to have a role in many processes including fetal islet differentiation, beta-cell mass, improved beta-cell survival, and glucose stimulated insulin secretion (31, 90, 93, 108). Our study demonstrated major differences between INS-1 cells cultured on collagen IV or BSA (control) with regards to focal adhesion complex formation. Across all conditions, INS-1 cells cultured on collagen IV were highly spread with intense actin stress fibers and vinculin staining at the cell periphery compared to control cells which remained round and had weak actin and vinculin staining (**Figure 3.10**). These findings indicate increased formation of focal adhesion complexes with collagen IV that supports the previously described enhanced adhesion and spreading (**Figure 3.1**) in INS-1 cells cultured on collagen IV. Signaling through focal adhesions is well documented to impact glucose stimulated insulin secretion, most notably via FAK, a protein commonly associated with focal adhesion complexes. For example, glucose stimulation alone has been demonstrated to significantly increase FAK phosphorylation (89) which has also been shown to mediate actin remodeling (89) and to phosphorylate BAG3(39), both of which are involved in insulin secretion.

Although there is a great deal of research examining the role of exocytotic proteins in beta-cell insulin secretion, there is little, if any, research examining localization changes of these proteins at different points of glucose stimulation. In general, SNAP25 membrane localization was increased and some minor differences in VAMP2, specifically areas of density, were observed in

collagen IV cells compared to control (**Figure 3.10, 3.11**). Interestingly, when comparing five-minutes post high glucose stimulation, opposing effects were observed between control and collagen IV cells. Intense membrane associated SNAP25 localization and areas of dense cytoplasmic VAMP2 are observed after five minutes of high glucose stimulation in control and thirty minutes of high glucose stimulation in collagen IV cells (**Figure 3.10Ac, Bc, 3.11Ac, Bc**). This may indicate differences in the spatial timing of insulin secretion that are influenced by external ECM support.

Overlap of SNAP25 and VAMP2 was also examined. Overall, collagen IV cells displayed increased overlap between SNAP25 and VAMP2 compared to control cells (**Figure 3.12A, B**), and this was especially prominent following five minutes of high glucose stimulation (**Figure 3.12Bc**). Although the interaction between SNAP25 and VAMP2 is well described to occur at the plasma membrane to mediate insulin granule exocytosis (104), the majority of overlap observed is found in the cytoplasm. Interestingly, SNAP25, traditionally associated with the plasma membrane, has been demonstrated to be localized in neuronal synaptic vesicles along with Syntaxin1A and VAMP2 (110). There are multiple hypotheses for the function of vesicle-associated t-SNAREs including protein recycling, sequential/compound exocytosis, and fusion between vesicles (32, 110). Sequential exocytosis, referred to as the "most massive mode of exocytosis" (50), has been documented to occur in mouse pancreatic islets and was associated with redistribution of SNAP25 onto membrane of vesicles (100). Thus, it is possible collagen IV increases insulin secretion via increased sequential exocytosis.

Finally, when examining insulin secretion, differences in the rate of insulin secretion were observed between groups. Little research displays time course data of insulin secretion in INS-1

cells (63). However, it is widely accepted that there are two phases of insulin secretion, where the first phase consists of a transient insulin spike lasting approximately ten minutes followed by a second phase of sustained insulin release typically at a slower rate than the first phase (105). This study demonstrates a notable increase in the rate of insulin secretion in response to five and sixty minutes of high glucose stimulation in INS-1 cells cultured on collagen IV compared to control cells, indicating the augmentation provided by collagen IV-INS-1 cell interactions influences both phases of insulin secretion with an emphasis in phase I.

In summary, these findings suggest that collagen IV-INS-1 cell interactions induce focal adhesion contacts as well as alterations in SNARE protein localization and colocalization. These results appear to influence the rate of insulin secretion and further emphasizes the importance of ECM-cell interactions in the enhancement of beta-cell function.

4.4. β1-integrin is mainly responsible for changes in exocytotic proteins observed in INS-1 cells cultured on collagen IV

Integrins containing a β1-integrin subunit comprise the largest subgroup of integrin receptors (13). β1-integrin is known to be expressed in INS-1 cells (51), and collagen IV is a known β1-integrin ligand (109). Thus, our lab next aimed to determine if the effects observed from INS-1 cells cultured on collagen IV described above were mediated via signaling from collagen IV-β1-integrin interactions. To do this, INS-1 cells received a β1-integrin blocking antibody pre-treatment (anti-β1), IgM-matched control (IgM) or no treatment (control).

First, β1-integrin protein levels and localization were examined to validate the β1-integrin blocking antibody. Anti-β1 cells displayed a major decrease in β1-integrin puncta but no change

in protein levels (**Figure 3.13A-D**). Integrins cluster at points of ECM-integrin interactions (114) so, a decrease in puncta indicate deceased collagen IV-β1-integrin interactions in the anti-β1 cells. It is unsurprising that no change was determined in total β1-integrin protein as the blocking antibody acts to prevent interactions between β1-integrin and its ligands rather than alter protein levels.

Blocking β1-integrin resulted in significant decreases in adhesion and spreading (**Figure 3.14)**, and basal insulin secretion and GSIS stimulation index (**Figure 3.15)** when compared to controls. This indicates collagen IV-β1-integrin interactions have a key role in promoting INS-1 cell insulin secretion. To ensure changes in insulin secretion were not a result of alterations in Ca^{2+} signaling, Fluo4 was used to measure Ca^{2+} concentrations before and during high glucose. No changes were found between groups (**Figure 3.16**). These findings match previous work from our lab demonstrating that collagen IV-β1-integrin interactions promote cell adhesion, spreading, and insulin secretion in INS-1 cells (51, 52). Additional evidence of β1-integrin's role in these beta-cell functions has also been demonstrated in other model systems (17, 77, 84). Overall, our results taken with others support that β1-integrin is important for beta-cell adhesion, spreading, and insulin secretion. However, the influence of β1-integrin on the exocytotic machinery involved in insulin secretion has yet to be established and is examined in this study.

When examining some of the SNARE proteins involved in insulin secretion, it was determined that β1-integrin signaling impacts SNAP25 and VAMP2, but not Munc18-1. Anti-β1 cells displayed no changes in Munc18-1 cellular localization or protein levels compared to controls (**Figure 3.17**). This indicates the altered localization and increased protein levels observed in collagen IV cells compared to control are not due to collagen IV-β1-integrin interactions but to

one of the several other collagen IV-receptor interactions (9). Thus, the collagen IV-β1-integrin interactions impacting insulin secretion are not mediated via changes in Munc18-1.

Contrastingly, SNAP25 was found to have significantly decreased protein levels and membrane localization (**Figure 3.18**) and VAMP2 was found to have a notable decrease in visual cytoplasmic localization and significantly reduced protein levels in anti-β1 cells compared to controls **(Figure 3.19**). This suggests a link between collagen IV-β1-integrin mediated signaling and these SNARE proteins supporting previous research from our lab which found decreased SNAP25, VAMP2, and Syntaxin1A mRNA expression and immunofluorescent staining in islets of beta-cell specific β1-integrin knockout mice (77). Although this relationship has yet to be explored in beta-cells outside of our lab, some research has linked the external environment with exocytotic proteins in neurons. Reelin, an extracellular matrix molecule, was found to impact SNAP25 protein levels as reelin mutant mice had significantly decreased SNAP25 protein levels in hippocampal and cortical tissue (33). In addition, laminin engagement via β1 integrin was found to be responsible for cytoskeletal rearrangement controlling VAMP7-mediated exocytosis and neurite development (29).

Finally, BAG3, a protein demonstrated to have regulatory roles in insulin secretion, was examined (39). Total BAG3 was found to be decreased in staining intensity, and phosphorylated BAG3 was found to be of less cytoplasmic staining intensity in anti-β1 cells compared to controls (**Figure 3.20**). BAG3 has been shown to bind to SNAP25 and prevent SNARE complex formation and upon phosphorylation by FAK, this interaction is disrupted freeing SNAP25 and allowing for SNARE-mediated exocytosis to ensue (39). β1-integrin blocking may result in a two-fold influence on BAG3 activity: (1) decreased total BAG3 availability impacting the proper regulation

of insulin secretion in beta cells, and/or (2) decreased β1-integrin signaling which may prevent FAK-mediated BAG3 phosphorylation and thus SNAP25 release. Overall, these results along with alterations in insulin secretion provide ample evidence of a novel role for β1-integrin signaling on SNARE-mediated insulin exocytosis in beta-cells.

To further explore this relationship, focal adhesions, SNAP25, and VAMP2 were examined during GSIS in INS-1 cells. Unsurprisingly, focal adhesion sites are greatly impaired in the anti-β1 group. Although minor changes are observed with glucose stimulation, overall anti-β1 cells have weaker actin staining, a round cell shape, and membrane-associated vinculin compared to controls (**Figure 3.21**). Focal adhesions are dynamic protein clusters recruited to activated integrin sites in order to facilitate cell signaling and influence changes in the actin cytoskeleton (89). Most research linking focal adhesions to insulin secretion demonstrates the role of focal adhesions or FAK in mediating changes in the actin cytoskeleton (14, 89). However, focal adhesions have also been demonstrated to be involved in targeting the location of insulin secretion. *In vivo* insulin is secreted into the blood stream, and in mice, the vascular face of beta cells are enriched with β1-integrin and proteins of focal adhesions (26). These sites were suggested to be involved in targeting insulin secretion to the vasculature as preincubation with a FAK inhibitor disrupted targeted granule fusion in isolated mouse islets (26).

In anti-β1 cells, SNAP25 was found to have decreased membrane localization in basal conditions and only minor changes in localization with glucose stimulation compared to controls (**Figure 3.22**). For VAMP2, anti-β1 cells did not display the increased areas of density observed in controls at basal and H60 (**Figure 3.23**). This suggests effects downstream of collagen IV-β1-integrin interactions also mediate changes that effect exocytotic protein localization during insulin

secretion. Interestingly, under all glucose conditions, anti-β1 cells displayed considerably decreased overlap between SNAP25 and VAMP2 compared to controls. As previously mentioned, SNAP25 localization in neurons was found in VAMP-rich vesicles and this was suggested to be involved in membrane-associated protein recycling, sequential exocytosis, and/or fusion between vesicles (110). Sequential exocytosis has also been documented to occur in mouse pancreatic islets and was associated with redistribution of SNAP25 onto the membrane of vesicles (100). Together this provides preliminary data supporting the idea that collagen IV-β1-integrin interactions lead to alteration in sequential exocytosis.

In support of the alterations in exocytotic machinery during GSIS, the rate of insulin secretion was found to be decreased 25-50% in all treatment conditions compared to control groups except for H30 (**Figure 3.24**). This further emphasizes the link between enhanced insulin secretion observed in INS-1 cells cultured on collagen IV and the effects of collagen IV-β1-integrin signaling.

In summary, the results of the β1-integrin antibody blocking studies showed that β1-integrin, through many methods, impacted insulin secretion. A summary of these findings can be found in **Figure 4.1**. Overall, this adds to the growing body of evidence demonstrating the importance of β1-integrin signaling on pancreatic beta-cell function.

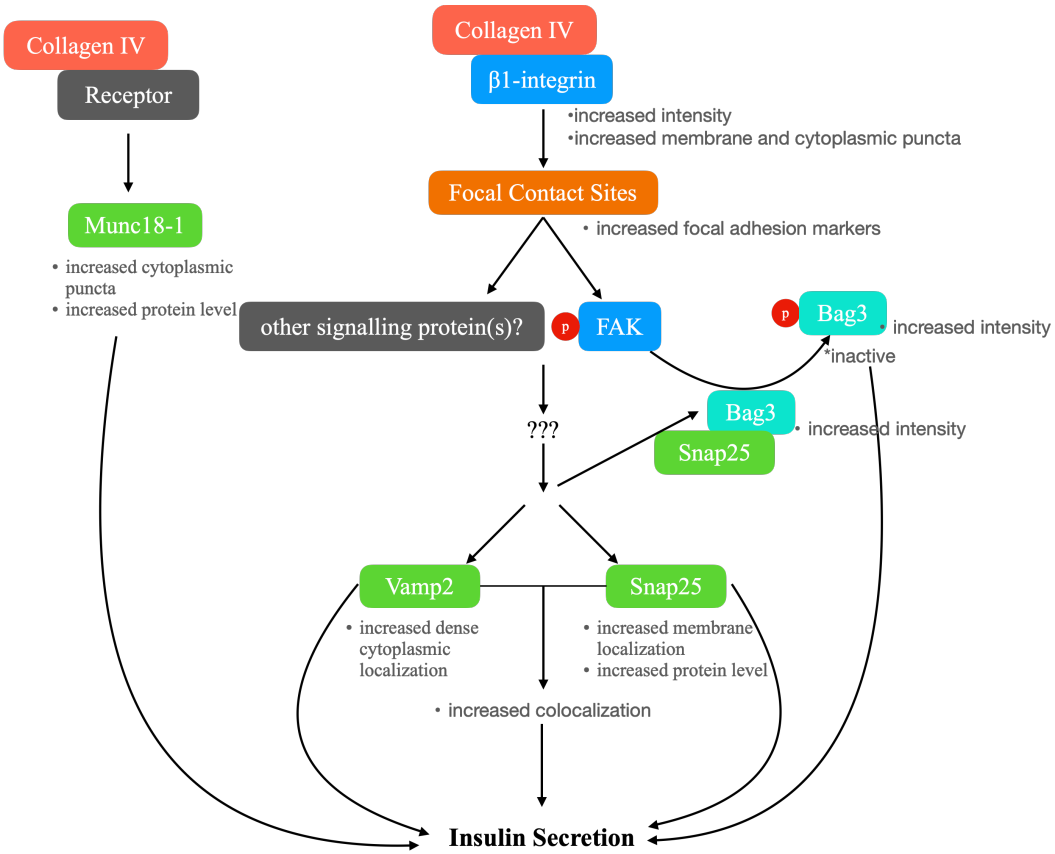


Figure 4.1: Summary of the effects of collagen IV and β1-integrin on regulation of SNARE protein distribution during GSIS in INS-1 cells

A number of factors involved in the machinery of INS-1 cell insulin secretion were found to be impacted by β1-integrin signaling in this study. Collagen IV-β1-integrin signaling initially increases focal adhesion sites and FAK phosphorylation. Activated FAK leads to phosphorylation of BAG3, leading to the release of SNAP25, as determined by Iorio et al. Resulting from a currently unknown signaling pathway VAMP2 localization changed, SNAP25 increased membrane localization and protein levels, and colocalization of the two proteins was increased. However, Munc18-1 had increased cytoplasmic puncta and protein levels due to collagen IV, which was not influence by blocking β1-integrin signaling. Together, all of these effects work to augment insulin secretion in INS-1 cells.

4.5.Limitations

INS-1 cells are a good model for pancreatic beta-cell insulin secretion as their secretion patterns are similar to what is observed *in vivo* (79). However, this is a cell line developed from x-ray induced insulinoma in rats (79). This is disadvantageous for three main reasons: (1) differences exist between human and rodent pancreatic islets (59); (2). INS-1 cells are cancer derived and therefore possess neoplastic characteristics not observed *in vivo* limiting the application of results to human beta cells; and (3) cancer cell types have been demonstrated to create their own unique ECM environments which impact cell-ECM interactions (117). Additionally, this study looked at the isolated effects of singular ECM proteins on INS-1 cell functions. In reality, a number of other extracellular factors as well as different cell types are present *in vivo* and in cell-based diabetic therapies, both of which would impact the function of beta-cells. Finally, the signaling pathway(s) initiated by collagen IV-β1-integrin interactions were not elucidated in this project. Although a relationship was determined between β1-integrin signaling and exocytotic machinery, how these changes occur was not. β1-integrin associated with a number of α subunits, and utilizes multiple signaling pathways to achieve functional changes in cell behavior including MAPK/ERK and PI3K/AKT pathways (52, 84, 89, 93)

4.6.Conclusions and Significance

β1-integrin and its downstream signaling is well described to be important to beta-cell insulin secretion (14, 51, 77, 89, 93). The results of the presented study add to this body of knowledge by providing additional mechanisms by which β1-integrin signaling impacts insulin secretion. First, collagen IV-β1-integrin interactions are shown to augment INS-1 cell adhesion and spreading which subsequently results in development of focal contact sites where β1-integrin is activated.

These interactions were demonstrated to be directly linked to insulin secretion. Further exploration revealed collagen IV-β1-integrin signaling impacts multiple components of the machinery involved in insulin secretion. Specifically, SNAP25 and VAMP2 protein levels, localization, and their colocalization was impacted by collagen IV-β1-integrin signaling. Overall, this underscores the importance of the external environment on optimal beta-cell function.

Diabetes is currently a disease with no cure. Although islet transplantations are performed, and bioartificial pancreas technology continues to improve, both treatment options have serious shortcomings. Understanding the optimal external environment that promotes essential integrin-ECM interactions will allow for further enhancements in these treatments, bringing us one step closer to a cure.

4.7. Future Studies

Characterization of the effects modulated by cell-ECM interactions in beta functions are critical for developing an optimal extracellular environment in cell-based therapies for diabetics. This study has demonstrated collagen IV to be an essential component of the beta-cell ECM due to its direct effects on insulin secretion, specifically the exocytotic proteins involved. However, important questions still need to be answered. For example, by which signaling pathways are the changes in exocytotic proteins achieved? β1-integrin can signal through different signaling pathways, thus identifying which are utilized to achieve these results is important. Could a cyclical relationship exist between integrins and exocytotic proteins? Integrins depend on exocytotic machinery to be delivered to the plasma membrane and thus, impacting exocytotic machinery could impact their own transport. This work is supported by previous work on cancer cells which revealed a role for SNARE proteins in the delivery of integrins to the plasma membrane, although

specific SNARE proteins involved in this process appear to vary (16, 58, 83). How does the collagen IV-β1-integrin interaction influence cells long term? A major drawback of islet transplantation is its efficiency long term, where only 25-50% of patients are insulin dependent five years following transplantation (45). This study only examined effects of the collagen IV-β1-integrin interaction after 24 hours of culture. Therefore, future studies need to examine long-term effects. Additionally, chamber slide studies used in this study only examine proteins at a certain timepoint of glucose stimulation. Since changes in proteins can occur within seconds, it would be interesting to observe time-course data. This may elucidate minute changes that occur on a much smaller time scale than investigated in this study.

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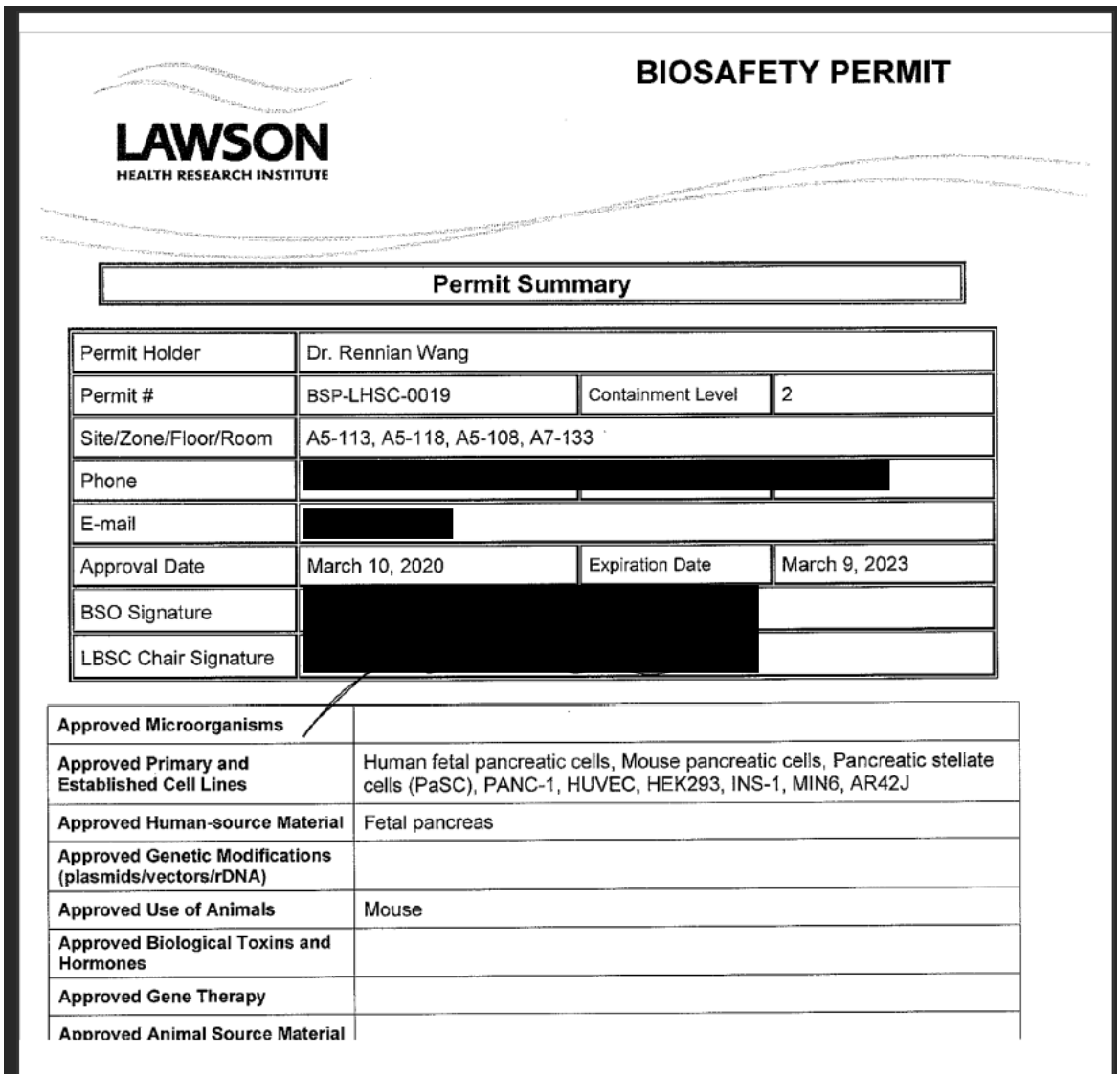
Appendix

Appendix A: Biosafety Approval Form



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BIOSAFETY PERMIT

Permit Summary

Permit Holder	Dr. Rennian Wang		
Permit #	BSP-LHSC-0019	Containment Level	2
Site/Zone/Floor/Room	A5-113, A5-118, A5-108, A7-133		
Phone	[redacted]		
E-mail	[redacted]		
Approval Date	March 10, 2020	Expiration Date	March 9, 2023
BSO Signature	[redacted]		
LBSC Chair Signature	[redacted]		

Approved Microorganisms	
Approved Primary and Established Cell Lines	Human fetal pancreatic cells, Mouse pancreatic cells, Pancreatic stellate cells (PaSC), PANC-1, HUVEC, HEK293, INS-1, MIN6, AR42J
Approved Human-source Material	Fetal pancreas
Approved Genetic Modifications (plasmids/vectors/rDNA)	
Approved Use of Animals	Mouse
Approved Biological Toxins and Hormones	
Approved Gene Therapy	
Approved Animal Source Material	

Curriculum Vitae

Malina Barillaro

Education

MSc Candidate: Physiology and Pharmacology, University of Western Ontario

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Dissertation: Collagen IV-β1Integrin Regulation of Exocytotic Machinery in Pancreatic Beta-Cell Insulin Secretion

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Graduate Student: Dr. Rennian Wang Laboratory, Children's Health Research Institute, University of Western Ontario

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Undergraduate Research: Dr. Wataru Inoue Laboratory, University of Western Ontario

June 2018 – April 2019

Topic: Astrocyte Plasticity during Chronic Stress

Graduate Teaching Assistantships

Physiology 2130: September 2019 – April 2020

Physiology and Pharmacology 3000: September 2020 – April 2021

Awards

Western Graduate Research Scholarship: Graduate and Postdoctoral Studies, University of Western Ontario

Duration: September 2019-August 2021

Highlight Trainee: Canadian Islet Research and Training Network

Duration: July 2021

Presentations

Physiology and Pharmacology Research Day: University of Western Ontario, 2019, 2020, 2021

EASD (European Association for the Study of Diabetes) Annual Meeting: Virtual, 2020

Title: Preventative N-acetyl-L-cysteine treatment improved metabolic and beta-cell function in HFD-T2DM model

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