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# a3p1 INTEGRIN REGULATION OF BETA CELL SURVIVAL AND FUNCTION

Mansa Krishnamurthv
*Western University*

# α3β1 INTEGRIN REGULATION OF BETA CELL SURVIVAL AND FUNCTION

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**Mansa Krishnamurthy**

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THE UNIVERSITY OF WESTERN ONTARIO
FACULTY OF GRADUATE STUDIES

**CERTIFICATE OF EXAMINATION**

Supervisor

Dr. Rennian Wang

Supervisory Committee

Dr. Subrata Chakrabarti

Dr. Martin Sandig

Examiners

Dr. Andrew Leask

Dr. Zia Khan

Dr. Wei-Ping Min

The thesis by

**Mansa Krishnamurthy**

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Date_____

Chair of Thesis Examination Board

## Abstract

Integrin-extracellular matrix (ECM) interactions are important determinants of beta cell behaviours. The β1 integrin is a well known regulator of beta cell activities, however, little is known of its associated α subunits. In the present study, αβ1 integrin expression was examined in rat insulinoma cell line, INS-1 cells, to identify their role in beta cell survival and function. Seven α subunits were identified, of which α3β1 was most highly expressed. Remarkable increases in adhesion, proliferation and insulin secretion were observed in cells cultured on collagen I or IV. To investigate relationships between α3β1 integrin and these matrices, cells were treated with immunoneutralizing antibodies for β1 or α3 integrin and cultured on collagen I or IV. Anti-β1 treatments caused marked decreases in adhesion and proliferation on both collagens, while perturbed α3 function led to changes only on collagen IV. Cellular insulin contents and secretion were greatly impaired on collagen I and IV upon anti-β1 treatment, while α3 integrin blockade caused similar changes only on collagen IV. Furthermore, perturbing either β1 or α3 integrin function decreased FAK and ERK1/2 phosphorylation and increased caspase3 cleavage. Blocking α3 integrin function reduced Akt and GSK3-β phosphorylation and decreased XIAP expression; however, these changes were not observed upon anti-β1 treatments. These results suggest that dynamic integrin-ECM interactions are critical for modulating beta cell survival and function through highly specialized signaling cascades.



**Keywords:** α3 integrin, β1 integrin, extracellular matrix, AKT/XIAP/GSK3-β signaling pathways, FAK/MAPK/ERK signaling pathways, INS-1 cells

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| | |
|---|---|
| °C | degrees Celsius |
| % | percent |
| µg | microgram |
| µl | microliter |
| AKT | protein kinase B |
| AP-1 | activation protein |
| Apaf-1 | apoptotic protease activating factor 1 |
| Bcl-2 | B-cell lymphoma 2 |
| bHLH | basic helix loop helix |
| BM | basement membrane |
| bp | base pairs |
| BrdU | bromodeoxyuridine |
| BSA | bovine serum albumin |
| CAMs | cell adhesion molecules |
| cDNA | complimentary DNA |
| $CO_2$ | carbon dioxide |
| CI | collagen I |
| CIV | collagen IV |
| Ctrl | control |
| DAPI | 4'-6' diamidino-2-phenylindole |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| e | embryonic |
| ECL | enhanced chemiluminescence |
| ECM | extracellular matrix |
| ERK | extracellular signal-regulated kinase |
| ERK1/2 | extracellular signal-regulated kinase 1 and 2 |
| FAK | focal adhesion kinase |
| FAT | focal adhesion-targeting |
| FBS | fetal bovine serum |
| FITC | fluorescein isothiocyanate |
| FN | fibronectin |
| g | G-force |
| GDP | guanosine 5'-diphosphate |
| Grb2 | growth factor receptor-bound protein 2 |
| GSK3-α | glycogen synthase kinase 3-alpha |
| GSK3-β | glycogen synthase kinase 3-beta |
| GTP | guanosine 5'-triphosphate |
| hr (s) | hour (s) |
| ICAM | intercellular cell adhesion molecule |
| ICM | inner cell mass |
| ILK | integrin-linked kinase |
| INS-1 | rat insulinoma cell line-1 |
| JNK | Jun N-terminal kinase |

| | |
|---|---|
| kDa | kilodalton |
| L | laminin |
| mol | molar |
| MAPK | mitogen activated protein kinase |
| MEK | mitogen activated protein kinase kinase |
| min (s) | minute(s) |
| ml | milliliter |
| MMP | matrix metalloproteinase |
| mRNA | messenger RNA |
| NCAM | neural cell adhesion molecule |
| NFκB | nuclear factor-kappa B |
| nmol | nanomolar |
| PAGE | polyacrylamide gel electrophoresis |
| PI3-K | Phosphatidylinositol-3-Kinase |
| PIP2 | PI, 4,5 biphosphate |
| PIP3 | PI 3,4,5 triphosphate |
| PKC | protein kinase C |
| PBS | phosphate buffered saline |
| Pdx-1 | pancreas/duodenal homeobox-1 or insulin promoter factor-1 |
| PFA | paraformaldehyde |
| PP | pancreatic polypeptide |
| PTEN | phosphatase and tensin homolog |
| qRT-PCR | quantitative reverse transcription-polymerase chain reaction |
| Rb | retinoblastoma |
| RGD | arginine-glycine-aspartic acid |
| RIP | receptor interacting protein |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RT-PCR | reverse transcription polymerase chain reaction |
| SDS | sodium dodecyl sulphate |
| SEM | standard error of the mean |
| SH2 | Src homology 2 |
| SH3 | Src homology 3 |
| SIS | Small intestinal submucosa |
| Src | Src family of non-receptor protein kinases |
| TBS | tris buffered saline |
| TBST | tris buffered saline + Tween-20 |
| TRITC | tetramethyl rhodamine isothiocyanate |
| VCAM-1 | vascular cell adhesion molecule-1 |
| VN | vitronectin |
| WHO | World Health Organization |
| wpc | weeks post conception |
| XIAP | X-linked inhibitor of apoptosis |

Chapter 1

# Introduction

Losely based on the manuscript entitled "Integrins and Extracellular Matrix Proteins in Pancreatic Tissue Engineering" by Krishnamurthy, M. and Wang, R. Submitted to **Frontiers in Bioscience**.

# 1 INTRODUCTION

The studies included in this thesis focus on the characterization of interactions between αβ1 integrins and their respective extracellular matrix proteins which influence beta cell behaviours including survival and insulin secretion. The aim of this introductory chapter is to briefly describe the problem addressed by my studies, my rationale and significance.

## 1.1 Purpose of the thesis

The cohesive nature of the islet of Langerhans, highly specialized aggregates of cells, suggests that cell-cell and cell-matrix interactions are critical for survival, development and function of the endocrine pancreas. Although numerous receptors and cellular molecules have been shown to facilitate islet cell development, survival and function, of particular importance are the integrins. As part of a superfamily of cell adhesion receptors which bind to the extracellular matrix (ECM), cell-surface and soluble ligands, integrins regulate a plethora of cellular programs including proliferation, migration, differentiation, survival and function (Boudreau et al., 1999; Bouvard et al., 2001; Brakebusch et al., 2005; Coppolino et al., 1999; Danen et al., 2003; French-Constant et al., 2004; Juliano et al., 2004; Lee et al., 2004).

In the field of islet biology, a large body of evidence supports the notion that interactions between the ECM and integrins provide a physical substratum for the spatial organization of cells and their downstream signaling pathways play critical roles in islet hormone regulation. Particular importance has been placed on the β1 subfamily of

integrins, as they orchestrate the majority of islet cellular changes, and heavily influence alterations in hormonal gene expression (Wang et al., 2005; Yashpal et al., 2005). While possessing diverse ligand binding capabilities, the β1 integrin and its associated α subunits have been shown to maintain islet architecture, by preventing anoikis and conferring a degree of stability (Hammer et al., 2004; Wang et al., 2005; Yashpal et al., 2005). Relationships between the β1 integrin and ECM proteins also promote increased cell survival and gene expression of critical islet cell markers. Studies examining the effects of ECM-β1 integrin interactions in purified populations of beta cells have reinforced the notion that these relationships prolong viability under *in vitro* conditions and also heavily influence the transcription, secretion and storage of insulin (Kaido et al., 2004).

The ability of integrins to coordinate events of cellular morphogenesis and regulate tissue homeostasis makes them ideal receptors to study and manipulate for greater remedial purposes such as islet transplantation and pancreatic tissue engineering. Therefore, the aim of this thesis was to identify relationships between αβ1 integrins and their respective ECM proteins which support beta cell viability, survival and function. Furthermore, downstream signaling mechanisms transduced by these integrin-ECM interactions, were examined. Given that the ultimate goal is to generate an abundant supply of islets for therapeutic purposes, integrin-ECM interactions in the microenvironment can be manipulated in future investigations which seek to improve islet-cell based treatments for diabetes.

## 1.2 **The Pancreas**

### 1.2.1 **The Pancreas**

The term pancreas is derived from Greek roots – "pan" meaning all and "creas" meaning flesh (Slack, 1995). An accessory digestive gland, in humans the pancreas is found posterior to the stomach, between the duodenum and spleen (Moore and Dalley, 1999), and measures 15-25 cm in length and weighs in the range of 70-150 grams (Slack, 1995). The pancreas is divided into head, neck, body and tail parts to define specific anatomical regions from proximal to distal (Slack, 1995). The head portion of the organ is attached to the descending and horizontal part of the duodenum, while the neck is adjacent to the pylorus of the stomach (Moore and Dalley, 1999). The body forms part of the stomach bed and the tail lies in front of the left kidney and is closely associated with the spleen (Moore and Dalley, 1999). The main pancreatic duct runs from tail to head of the pancreas, where it joins the common bile duct and forms the ampulla of Vater, which opens into the duodenum (Moore and Dalley, 1999; Slack, 1995).

The pancreas is comprised of two main classes of cells – epithelial and mesenchymal (Hao et al., 2006). Ductal cells and the two major types of glandular tissue, endocrine and exocrine, are epithelial in origin (Hao et al., 2006), while pancreatic fibroblasts and endothelial cells form the mesenchymal component (Hao et al., 2006).

The exocrine tissue, responsible for secreting enzymes for digestion, forms the majority of the pancreas (98%). The endocrine component, which maintains glucose homeostasis, comprises only 2% of the total cellular population and can be found in aggregates of cells termed islets of Langerhans. Neatly embedded within the exocrine

tissue, the islets of Langerhans contain five major cell types: alpha (α), beta (β), delta (δ), pancreatic polypeptide (PP), and a recently discovered sub-population, termed epsilon (ε) cells (Heller et al., 2005). The insulin secreting beta cells, located centrally, are the most predominant cell type in the islet of Langerhans, making up 60% of the cellular population, while the glucagon secreting alpha cells, found towards the periphery, form approximately 25% of each islet (Fox, 1999). Proper coordination of beta and alpha cell function is critical for maintaining control of blood glucose levels - beta cells secrete insulin in response to a rise in blood glucose concentrations, while the alpha cells secrete glucagon in response to a fall (Fox, 1999). Sub-populations of δ cells produce somatostatin (Fox, 1999), PP-cells secrete pancreatic polypeptide (Slack, 1995), while the ε cells secrete the peptide hormone ghrelin (Heller et al., 2005).

### 1.2.2 Development of the Pancreas:

Although the studies conducted in this thesis do not directly examine development of the pancreas, the ontogenic processes are described to provide background on the origins of endocrine pancreatic cells, and the differentiation and maturation processes of islet cell types.

#### Development of the Rodent Pancreas:

The mouse pancreas begins to develop between embryonic days 8.5 and 9.5 (e8.5, e9.5) from a pre-patterned endodermal epithelium in the region of the forgut, as dorsal and ventral buds of cells (Habener et al., 2005). The formation of these two primordial organs consists mainly of undifferentiated ductal epithelium and is termed the first

developmental transition (Habener et al., 2005). At e10.5, the partially differentiated epithelium undergoes extensive branching (Habener et al., 2005; Hill, 2005). These highly branched structures give rise to the exocrine pancreas via extensive processes of differentiation between e14.5 and e15.5 (Hill, 2005). While the acini and ducts appear at this point of development, the endocrine components appear much earlier in development, representing 10% of the pancreas by e15.5 (Hill, 2005). At e16 and e17, the dorsal and ventral buds rotate and fuse to form one single organ.

**Development of the Human Pancreas:**

Similar to mouse, the human pancreas also originates from two buds, which develop from dorsal and ventral outgrowths of the foregut endoderm (Piper et al., 2004; Slack, 1995). The dorsal bud, visible by 26 days post-conception, is derived from one side of the gut tube, while the ventral bud arises adjacent to the hepatic diverticulum (Slack, 1995). During embryogenesis, both ventral and dorsal buds extend into the surrounding mesenchyme. The rotation of both buds facilitates their fusion into one organ. The ventral bud forms the posterior portion of the head of the pancreas, while the dorsal bud forms the remainder of the organ (Slack, 1995).

### 1.2.3 **The Endocrine Pancreas:**

**Development of the Rodent Endocrine Pancreas:**

Endocrine cells are derived from stem/progenitor cells located in the gut endoderm. These cells can be found as early as e9.5. At e14, this stem cell population remains as single cells dispersed within the ductal epithelium, which subsequently

undergoes a secondary transition characterized by extensive proliferation (Habener et al., 2005). While by e16, the endocrine cells begin to organize themselves into islet-like clusters, islet cells are not formed until a few days prior to birth (Hill, 2005).

The first endocrine cells are detectable by e9.5, and express glucagon (Habener et al., 2005). By e10.5, the endocrine cells co-express glucagon and insulin, marking a stage when these cells are about to differentiate into either the alpha or beta cell lineage (Habener et al., 2005). By e11.5, well defined clusters of insulin$^+$ and glucagon$^+$ cells are detected, representing the precursor populations for mature beta and alpha cells (Slack, 1995). At e14, well defined δ-cells expressing somatostatin are detected.

The third developmental transition begins prior to e19 and is characterized primarily by the remodeling and maturation of islets for 2-3 weeks after birth (Hill, 2005). Postnatal days 7-21, are marked by a transient wave of apoptosis required for the destruction of a certain population of beta cells (Hill et al., 2005; Petrik et al., 1998). This remodeling process is especially necessary for ensuring that beta cells develop into well adapted robust cells, capable of managing and responding to high glucose challenge for proper maintenance of blood glucose homeostasis (Hill, 2005).

**Development of the Human Endocrine Pancreas:**

Islets cells in the human fetal pancreas typically undergo two developmental phases: primary and secondary islet formation. Rare epithelial cells expressing insulin are first apparent at 52 days post-conception (7.5 weeks), after the initial outgrowth of the human pancreatic buds (Piper et al., 2004). Glucagon$^+$ and somatostatin$^+$ cells are first detected at 8.5 weeks into development, while PP cells begin to emerge at 10 weeks. By

10 weeks, insulin$^+$ cells appear as distinct cell clusters and begin to associate with glucagon$^+$ cells (Lyttle et al., 2008; Piper et al., 2004). By 15 weeks, large primitive islet structures begin to appear, as endocrine cells aggregate into adult-like islet structures composed of all four endocrine cell types (Lyttle et al., 2008). By the end of the second trimester, capillary networks begin to associate with islet aggregates indicating the point at which the endocrine pancreas begins to function (Lyttle et al., 2008). Ontogenic changes from this time point onwards involve expansion and growth.

### 1.3 **Insulin Secreting Cell Line (INS-1 cells)**

The studies performed in this thesis made use of a newly created beta cell line. New Insulin-Secreting Cell Lines (INS-1) were established in the early 1990s, as a means to create beta cells which retain prominent features including morphology, insulin biosynthesis and the ability to secrete insulin in response to glucose challenge for a prolonged time in culture (Asfari et al., 1992).

The parental cell lines, INS-1 and INS-2 were isolated from an x-ray induced rat transplantable insulinoma and when compared to other cell lines, are extremely adaptable (Asfari et al., 1992). The INS-1 and INS-2 cell lines retain more differentiated characteristics than the widely used RINm5F cells, and express both proinsulin I and proinsulin II peptides. Unfortunately, they possess a limited capacity for insulin secretion following glucose stimulation, similar to the native tumor from which they are derived.

From these parental cells, several daughter cell lines were derived, through selective clonal expansion, including INS-1 832, INS-1 832/2 INS-1 832/13, INS-1 D and INS-1 E cells. The INS-1 832 cells possess much higher insulin contents in

comparison to parental cell lines, but still demonstrate poor insulin secretion upon glucose stimulation, due to low levels of cAMP. Similarly, insulin release from INS-1 832/2 cells maximally rose 1.5 fold upon glucose challenge, although these cells possess adequate insulin contents (Yang et al., 2004). In contrast, the INS-1 832/13 cells are characterized by high activity of the cAMP secondary messenger system, critical for their ability to maintain high insulin secretion levels as well as insulin contents within cells. In addition to increased sensitivity to cAMP, the exocytotic response, examined through patch-clamp studies, was much higher for INS-1 832/13 cells in comparison to INS-1 832/2 (Yang et al., 2004). Given that the metabolic and physiological capabilities of INS-1 832/13 cells most closely resemble the behaviours of human adult beta cells, the following studies presented in this thesis made use of the INS-1 832/13 cells.

It is well established that all of the INS-1 cell lines mentioned above, exhibit a certain degree of plasticity, simultaneously expressing several islet hormones including insulin, glucagon, somatostatin and pancreatic polypeptide. Transcription factors specific for alpha, beta, delta and PP cell lineages such as Pdx-1 (Keller et al. 2007; Wang et al., 2000), NeuroD/Beta2 (Moates et al., 2003; Wang et al., 2000), Isl-1 (Wang et al., 2001), HNF1α (Wang et al., 2000; Yang et al., 2002), HNF4α (Wang et al., 2000), Pax4 (Wang et al., 2000), Pax6 (Wang et al., 2000), Nkx2.2 and Nkx6.1 (Schisler et al., 2004) are also expressed in INS-1 cells. A pluoripotent tumor cell line, the INS-1 cells are often used to study islet cell differentiation and inter-conversion between islet cell types. Specifically, the INS-1 832/13 cells are optimal for studying integrin-ECM interactions as well as growth factor signaling. Studies in our laboratory have demonstrated that INS-1 cells express a wide variety of ligands and receptors including integrins α1-6, αV, β1 and c-Kit

(Chapter 2). Moreover, INS-1 cells also express EGFR, FGFR1, FGFR2, IGF1R (Asfari et al., 1995; Buteau et al., 2003; Le Bras et al., 1998; Rachdi et al., 2001). Lastly, given that they are a tumor cell line, the INS-1 832/13 cells are capable of creating their own microenvironment, through the secretion of several extracellular matrix proteins including fibronectin, laminin and collagen IV.

## 1.4 **Integrins and ECM**

### 1.4.1 **Integrin family of receptors**

Integrins orchestrate a variety of cellular processes and functions including regulation of cell phenotype, adhesion, spreading, migration, differentiation, survival and cell cycle progression (Clark and Brugge, 1995; Hynes, 1987; Schwartz, 1994; Schwartz, 1995). The distinct structure of these heterodimers allows them to function as integrators of exterior and interior environments of a cell as they possess extracellular domains which bind to external ligands and cytoplasmic domains which facilitate interaction with the actin cytoskeleton and other affiliated proteins (Boudreau et al., 1999). As a result, integrins exhibit unique and dynamic signaling capabilities, which allow them to coordinate and integrate extracellular events and intracellular changes (Boudreau et al., 1999; Coppolino et al., 1999; Danen et al., 2003; Lee et al., 2004). These bidirectional mechanisms described as "outside-in" and "inside-out" signaling, include the activation of numerous intracellular molecules which propagate conformational changes from cytoplasmic tails, across the membrane, toward ligand binding regions. Downstream signaling cascades can, in turn, subsequently induce increased expression of integrins at the cell membrane as well as changes in receptor affinity and avidity, which allow for

further alterations including the redistribution of integrins and clustering, facilitating ECM binding (Danen et al., 2003; Lee et al., 2004). These interactions allow tissues to withstand mechanical load as integrins act as mechanosensors to detect and respond to physical stresses within their microenvironement (Shyy and Chen 1997; Stupack and Cheresh 2002).

Integrin receptors also demonstrate a considerable amount of overlap in their ligand binding specificities (**Table 1.1**) (Boudreau et al., 1999; Lee et al., 2004). The redundancy in integrin-ECM interactions suggests that specific relationships are capable of eliciting highly specialized signaling functions and that a certain degree of compensation whereby other integrins or receptor types can still allow for the development of normally functioning tissues. Recent developments of murine loss of function phenotypes in either constitutive or cell type-specific models have been especially important to our understanding of integrin/ECM signal transduction pathways which affect development and maintenance of tissues (Bouvard et al., 2001). The wide range of phenotypes, from either early lethality to apparently normal mice, reinforces the notion that 1) a certain hierarchy must exist among the integrin family of receptors, and that 2) compensation between integrins and perhaps other receptor types allow for the development of normal functioning tissues. Furthermore, integrins are also involved in morphogenetic decisions throughout development, contributing to the normal functioning of all tissues and organs. They are critical for maintaining homeostasis throughout adulthood and have been implicated in the pathogenesis of various diseases (Horwitz, 1997; Taddei et al., 2003).

**Table 1.1.** Ligand properties of β1 integrins and corresponding amino acid recognition sites, shown in letter code. Adapted from Berman et al., 2003; Brakebusch and Fassler, 2005; Eble, 1997; Mizjewski, 1999; Oxvig and Springer, 1998; Stupack and Cheresh, 2002.

| Integrin | Ligands | Recognition Site (aa) |
|---|---|---|
| α1β1 | collagen, laminin | YIGR, RHDS |
| α2β1 | collagen, laminin | DGEA, RHDS |
| α3β1 | collagen, laminin, fibronectin, thrombospondin | RGD |
| α4β1 | fibronectin, VCAM-1, MadCAM-1 | EILDV, IDAPS, REDV |
| α5β1 | fibronectin, fibrin | LDV, RGD, PHSRN |
| α6β1 | laminin | May bind VIGSR |
| α7β1 | laminin | VGVAPG, YIGSR |
| α8β1 | fibronectin, vitronectin | IDG,LDV, IDA |
| α9β1 | tenascin, VCAM-1 | IDG, LDV, IDA |
| α10β1 | collagen | |
| α11β1 | collagen | |
| αvβ1 | fibronectin, vitronectin, osteopontin | RGD |

### 1.4.2 **Integrin structures and their respective ligands**

Integrin transmembrane receptors are heterodimeric, composed of distinct α (120-180kDa) and β (90-100kDa) subunits, which associate non-covalently (Berman et al., 2003). To date, 18α and 8β subunits have been identified in mammalian model systems, generating 24 distinct integrin receptors (Berman et al., 2003). The diversity of integrins is increased by numerous splice variant isoforms of individual subunits. The binding partners of various integrin subunits are depicted in **Figure 1.1**.

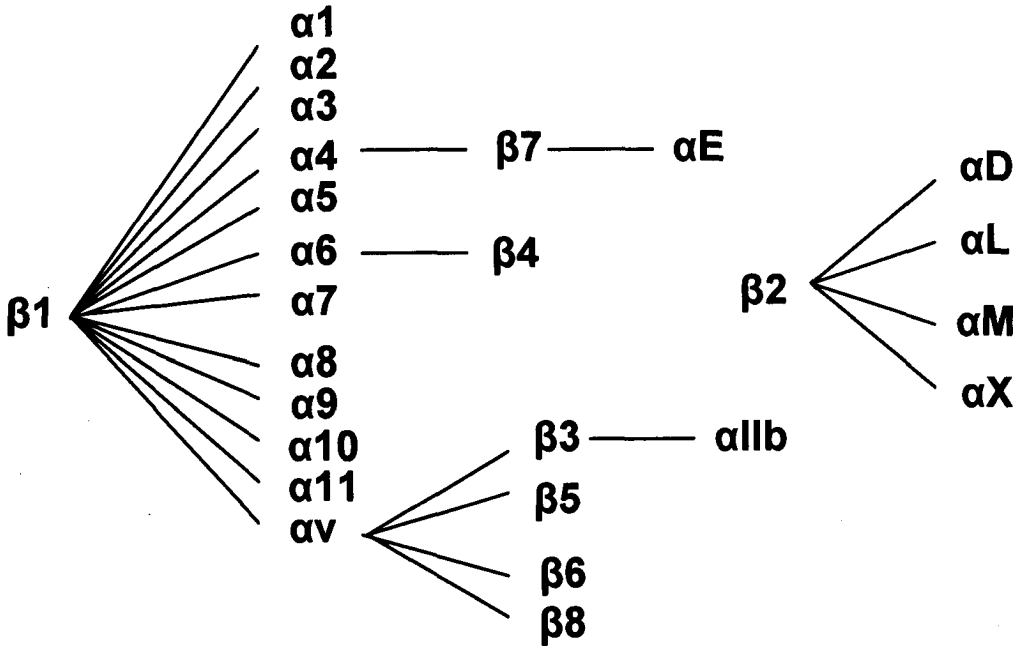


**Figure 1.1**. Pairing of β and α integrin subunits. Adapted from Bouvard et al., 2001.

Integrin receptors typically have a large extracellular domain (~1200 amino acids for α subunits and 800 amino acids for β subunits), one transmembrane segment and a short cytoplasmic tail consisting of 50 amino acids or less. In the β heterodimers, the cytoplasmic domain consists of regions associated with various cytoskeletal elements critical for mediating integrin signaling through the activation of mitogen-activated protein kinases, small GTPases, protein kinase C as well as the modulation of phosphoinositide levels (Berman et al., 2003; Hynes, 1987, Mechai et al., 2005). Given that the cytoplasmic tail is devoid of enzymatic features, integrins heavily rely on

associative adaptor proteins which link the receptors to the actin cytoskeleton, cytoplasmic kinases and transmembrane growth factor receptors (Clark and Brugge, 1995; Liu et al., 2000). Although the specificity of integrin binding to various ligands appears to be dependent primarily on the extracellular domain of the α subunit, both α and β heterodimers are necessary for the proper functioning of these receptors (Berman et al., 2003; Clark and Brugge, 1995; Hynes, 1987; Liu et al., 2000).

### 1.4.3 **Integrins as receptors of the extracellular matrix**

Integrin-dependent attachment to ECM proteins, fibronectin, laminin, collagen I and collagen IV were examined in this thesis. Therefore, integrin receptors which commonly interact with these matrix proteins are described.

#### **Integrins as Fibronectin Receptors**

Fibronectin (FN), a ubiquitous and abundant extracellular matrix protein, is secreted as a soluble dimer and assembled into an insoluble fibrillar network (Wierzbicka-Patynowski and Schwarzbauer, 2003). Assembly of FN into a dense meshwork is a multi-step process that interestingly, requires interactions with integrin receptors at the cell surface (Wierzbicka-Patynowski and Schwarzbauer, 2003).

Integrin α5β1 has been identified as a key FN receptor in several cell types. Its critical role in activating FN for its assemblage into fibrils is due to the classic RGD cell-binding sequence (Arg-Gly-Asp) in the type $III_9$ module along with synergy with the type $III_{10}$ module (Bowditch et al., 1994; Hynes, 1992; Nagai et al., 1991; Ruoslahti and Pierschbacher, 1987; Wierzbicka-Patynowski and Schwarzbauer, 2003). As a ligand, FN

causes clustering of integrin receptors at the cell surface, further bringing together these matrix dimers, increasing its concentration at specific sites and subsequently promoting fibril formation (Wierzbicka-Patynowski and Schwarzbauer, 2003). Lastly, the binding of FN to integrins allows for the expansion of the compact dimer by mediating FN-FN interactions (Wierzbicka-Patynowski and Schwarzbauer, 2003).

Binding of FN to integrin α5β1 contributes to the full activation of Rho and influences changes in morphology, survival and function through FAK, Src and PI3K signaling (Miranti and Brugge, 2002; Schwartz et al., 1995). These downstream effectors have been identified as critical for the assembly of fibronectin fibrils. In fact, a feedback loop has been proposed whereby mutual activation and reinforcement of these kinases results in FN matrix assembly (Ohashi et al., 2002; Pankov et al., 2000; Zamir et al., 2000). This loop is also thought to stimulate changes in cell survival, proliferation and gene expression (Wierzbicka-Patynowski and Schwarzbauer, 2003).

**Integrins as Laminin Receptors**

A fundamental extracellular matrix protein, laminin is critical for assembly and architectural integrity of the basement membrane (BM) (Tzu and Markinovich, 2008). Composed of three different polypeptide chains α, β and γ (Takagi et al., 2007), there are currently five α, three β and three γ chains known to make up 15 different laminin trimeric isoforms in mammals (Aumailley et al., 2005). In addition to forming laminin polymers, laminin binds to other extacellular components of the BM including collagen IV, nidogen and fibulin (Tzu and Markinovich, 2008). The importance of laminins in maintaining tissue integrity has been well exemplified through several diseases where the

structure or presence of laminin has been compromised due to genetic mutations (Tzu and Markinovich, 2008).

Integrins and several other cell surface receptors have been shown to bind to the laminin family of proteins (Tzu and Markinovich, 2008). Eight common integrin receptors have been identified to associate with laminin proteins, which include α1β1, α2β1, α3β1, α5β1, α6β1, α7β1, α9β1, α6β4, αvβ3, αvβ5 and αvβ8 (Burkin and Kaufman, 1999; Colognato et al., 1997; Kuhn and Eble, 1994; Mercurio, 1995; Mizushima et al., 1997; Nomizu et al., 1995; Tzu and Marinkovich, 2005). Integrin binding sequences are found in the laminin α chains, which contain highly specialized binding/recognition sites (Tzu and Markinovich, 2008). More specifically, the laminin α1, α2, α3 and α5 chains have been shown to possess integrin-binding sites (Colognato et al., 1997; Kikkawa et al., 1998; Mizushima et al., 1997; Orian-Rousseau et al., 1998). Through extensive mapping using proteolytic fragments and peptides, it has been identified that the G domain (encoding a glutamic acid) at the C-terminal region of the laminin α chain mediates laminin-integrin interactions (Belkin and Stepp, 2000; Ido et al., 2007).

Once integrins bind to laminin, intracellular effectors including focal adhesion kinase, small rhoGTPases, Src and mitogen-activated protein kinase pathways become activated to influence cellular activities including proliferation, migration and gene transcription (Giancotti, 2000; Givant-Horwitz, 2005).

**Integrins as Collagen Receptors**

Collagen is an essential ECM protein shown to be involved in various cellular responses including proliferation, differentiation, migration and morphogenesis (Heino,

2000; Ortega and Werb, 2002; Vogel, 2001). Collagens are ubiquitous proteins, consisting of 20 genetically distinct types (Bosman and Stamenkovic, 2003), responsible for maintenance of structural support in different organisms (Kaido et al., 2004).

Collagen binding integrins are glycoproteins of ~130kDa α subunits and ~90kDa β subunits (White et al., 2004). Of the 12 members of the β1 integrin family, 5 of them are known to bind to collagen matrices. Integrins α1β1, α2β1, α3β1, α10β1 and α11β1 (White et al., 2004) are thought of as collagen receptors. Four of these integrins α1β1, α2β1, α10β1 and α11β1 are structurally and functionally similar to each other, as they possess an αI-domain in the α subunit. The αI-domain is typically found in the head region of the integrin, where it plays a central role in ligand binding activities (Emsley et al, 2000). Moreover, the α subunits of integrins, α1β1, α2β1, α10β1 and α11β1 contain high sequence similarities in their cytoplasmic domains, but differ in their C termini (White et al., 2004). This variation facilitates specific interactions with cytoplasmic signaling factors. Since the α3β1 integrin does not contain this unique domain, it is thought to be an accessory receptor (White et al., 2004).

**a) Collagen I**

Collagen I is widely distributed in the body, contributing to the structure of skin, bones, blood vessels, gingiva, placenta and muscle (Pope et al., 1983), but not to cartilageous tissues (Kadler et al., 1996). Type I collagen, is made up of three 2-residue peptides, which fold into a straight, right-handed triple helix, approximately 65 Angstroms in length and 12 Angstroms in diameter (Kadler et al., 1996). Its long fibrillar composition enables it to act as a scaffold for cell attachment and anchorage of

macromolecules, allowing the shape of tissues to be defined and maintained (Kadler et al., 1996). Collagen fibril formation is largely dependent on processes of self-assembly, but can also be mediated through cell-regulatory processes, especially in young and healing tissues, as well as states of disease (Kadler et al., 1996).

Type I collagen contains a signature bend; however, this does not affect interactions with αI-domains found on connecting integrin receptors (Emsley et al., 2000). The collagen triple helix contains one residue between each respective strand, allowing each strand to remain unique and be designated specific names including "leading", "middle" and "trailing" depending on the N-terminus (Emsley et al., 2000).

The collagen I peptide binds across the upper surface of the α-I domain found on the corresponding integrin. This interface requires three metal ion-dependent adhesion site (MIDAS) loops, found at the integrin end, to coordinate and stabilize the interactions, so that downstream signals can be transduced. The integrin connects mostly with the middle strand of the collagen triple helix, with minimal interactions along the trailing strand (Emsley et al., 2000).

**b) Collagen IV:**

Type IV collagen is recognized as a unique member of the mammalian collagen family. Unlike other collagen proteins, collagen IV is only expressed in basement membranes, and consists of six genetically distinct α chains designated as α1(IV) to α6(IV). Although there are many potential combinations, the collagen IV α chains interact and assemble with great specificity to form three heterotrimers of α1α1α2, α3α4α5, and α5α5α6 (Khoshnoodi et al., 2008). Collagen IV acts as a scaffold, conferring

mechanical stability and allowing for assembly of tissues into organs. Its interactions with numerous cell types through both integrin and non-integrin receptors are essential for cell adhesion, migration, proliferation, survival and differentiation.

The recognition of collagen IV through integrin receptors is dependent on the classic RGD (Arg-Gly-Asp) sequence, which is largely found within the triple helix (Khoshnoodi et al., 2008). Integrins α1β1 and α2β1 have been identified as the classic collagen receptors. Both integrins have an affinity for collagen IV; however, α1β1 exemplifies a stronger preference for collagen IV binding (Kern al., 1993; Tulla et al., 2001). The major interface for integrin binding is located on the CB3 fragment of the N-terminus of collagen IV. In addition to α1β1 and α2β1, integrin α3β1 also mediates adhesion to collagen IV (Elices et al., 1991; Khoshnoodi et al., 2008). The binding site for α3β1 has been identified on α1 chain of collagen IV, on residues 531-543 (Lauer et al., 1998; Miles et al., 1994; Miles et al., 1995). Recently, integrins α10β1 and α11β1 were reported to associate with collagen IV (Tiger et al., 2001; Tulla et al., 2001). Although integrin α10β1 demonstrates an increased affinity for collagen IV in comparison with α11β1, the binding sites on these integrin receptors remains to be elucidated (Khoshnoodi et al., 2008).

## 1.5 **Integrins: The Signaling Platform**

The initial function of integrins involves an "inside out" signaling mechanism, whereby an integrin assumes an active state upon recognition of a ligand (Hynes 20002; Pozzi and Zent, 2003). Cellular activation by a variety of non-integrin extracellular signals, including the binding of a chemoattractant, generates a signal within a cell,

resulting in a conformational change in the integrin heterodimers, and thus heightening the affinity for ligand binding. The β subunit is especially critical for regulating the "inside-out" signaling activity in cells. Mutations and truncations of the cytoplasmic domain of the β subunit can either cause constitutive activation or inactivation (Aplin et al., 1998; Loftus et al., 1994; Springer, 1997). Monoclonal antibodies against the β1 subunit have been shown to either stimulate or inhibit integrin activity and act as allosteric regulators (Diamond and Springer, 1994; Faull and Ginsberg, 1995; Mould et al., 1998; Takada and Puzon, 1993). The key amino acid sequence responsible for activity of the β1 subunit contains residues 207-218 - a target for antibody inhibition and in a few cases, activation (Diamond and Springer, 1994; Faull and Ginsberg, 1995; Mould et al., 1988; Takada and Puzon, 1993). Several ligand binding sites are close to these residues, suggesting that changes in conformation of this region are critical for regulating exposure of ligand-binding sites (Fernandez et al., 1998).

Through a mechanism referred to as "outside-in" signaling, the binding of the extracellular regions of the integrin to a ligand leads to activation and generation of biochemical signals within the cell (Hynes, 2002; Pozzi and Zent, 2003). Like the majority of cellular receptors, the activation of an integrin, triggers downstream signaling cascades, including the induction of calcium fluxes, the activity of tyrosine and serine/threonine kinases and changes in function of RhoGTPases. This process of activation subsequently causes changes in cytoskeletal contraction, and the regulation of gene expression leading to alterations in cell proliferation, differentiation and survival (Berman et al., 2003; Danen et al., 2003; Giancotti and Ruoslahti, 1999; Hynes 2002; Stupack and Cheresh, 2002). The focal adhesion complex is a major assembly of proteins

which undergoes activation as a result of cell adhesion through integrin receptors (Schaller et al., 2001). Integrin-ECM interactions at focal contacts cause clustering of integrins and the recruitment of signaling molecules and actin filaments to the cytoplasmic domain of the receptor (Danen et al., 2003, Lee et al., 2004). These plaques are essential for integrins to establish connections to numerous signaling molecules including FAK, c-Src, PI-3-kinase, RhoGAP, paxillin, talin, p130CS, integrin-linked kinase and phosphorylated Caveolin-1 (Schoenwaelder et al., 1999). The recruitment of these cell signaling proteins are critical for actin remodeling, maintaining polarity and movement, as well as controlling cell survival, proliferation and differentiation (Clarke and Brugge, 1995; Giancotti and Ruoslahti, 1999).

The well known enzymes focal adhesion kinase (FAK), integrin-linked kinase (ILK) and the Src family of kinases are essential to integrin signaling because of their ability to bind several adaptor proteins and thus integrate cell-ECM interactions with cell phenotype (Clark and Brugge, 1995; Guan, 1997; Li et al., 1999). In fact, the importance of FAK and ILK has been revealed in studies examining targeted gene deletions, in which embryonic lethality occurs (Schwartz. 2001). Mechanisms by which integrins regulate cellular responses involve both the organization of the cytoskeleton through cytoskeletal linker proteins, including talin or paxillin, as well as the activation of tyrosine and serine/threonine protein kinases, inositol lipid metabolism and the Rho family of small GTPases (Rho, Rac and Cdc42) (Berman et al., 2003; Clarke and Brugge, 1995; Hynes, 2002; Stupack and Cheresh, 2002).

In addition to traditional signaling mechanisms, integrins can also be activated in the presence of soluble growth factors or ligands for other receptors, thereby influencing

downstream cascades and mediating changes in cell survival and function. Since development of several tissues can proceed normally without the presence of widely expressed integrins, it is highly likely that these receptors participate in a much larger network of signaling processes. In fact, studies have demonstrated that cellular responses to soluble growth factors including epidermal growth factor (EGF), platelet derived growth factor (PDGF), lysophosphatidic acid (LPA) and thrombin require the adhesion of cells on substrates via integrins (Hynes et al., 2002). Furthermore, it is well known that the activation of parallel pathways by integrins and growth factors, synergize at the level of phosphorylation of downstream signaling proteins (Danen et al., 2003, Schwartz et al., 2002). For example, integrins and growth factors can independently trigger a weak activation of the extracellular signal-regulated kinase, but their combined activity leads to strong and more sustained ERK activity (Chen et al., 1996). Integrins can also increase the signals generated by growth factor receptors by bringing kinases and substrates in close proximity. This is often achieved by ECM bound integrin organization of focal adhesions, bringing proteins like FAK, Src, p130Cas, ILK and ERK close to cell-matrix adhesion sites so that signals from various pathways may be amplified (Chen et al., 1996; Hynes, 2002). The ECM, therefore, can be thought of as a niche which simultaneously provides a physical substratum and houses numerous growth factors, cytokines and mitogenic signals, allowing for integrins to influence numerous cellular behaviours including survival and function.

## 1.6 **The β1 Integrin Subfamily**

The β1 integrin and its associated α subunits, constitute the largest subfamily of integrins, forming 12 distinct receptors with different ECM ligand binding properties (Brakebusch et al., 2005). Their dynamic role in controlling intracellular signaling pathways, and thus influencing cell morphogenesis, proliferation, differentiation and survival has been well established (Aoudjit and Vuori, 2000; Bagutti et al., 1996; Carroll et al., 1995; Howlett et al., 1995; Naylor et al., 2005; Strueli et al., 1991; Zhang et al., 2004). Integrins are devoid of enzymatic activities and do not posses an actin binding domain; however, their interactions with integrin associated molecules including adaptor and linker protein complexes still enables them to transduce downstream signals and influence a variety of cellular functions (Brakebusch et al., 2005).

### 1.6.1 **General Structure of the β Heterodimer**

The β subunit has several distinctive features, including tandem repeats of four cysteine-rich extracellular regions, which are thought to be critical for maintaining conformation of integrin molecules (Eble, 1997; Humphries, 2000). The β subunit also contains a "conserved domain" in the proximal membrane region, also known as the βA-domain, which encompasses the region shown to be essential for ligand and cation binding (Berman et al., 2003; Eble, 1997; Green et al., 1998). The βA domain contains two critical insertions: one that forms the core of the interface with the α-subunit's propeller domain and one known as the SDL loop (Arnaout et al., 2005). The SDL loop is primarily involved in ligand binding and contributes to the intersubunit interface. Another distinct feature of the β subunit is a new cation site known as ADMIDAS, which falls

adjacent to the MIDAS site on the α subunit (Arnaout et al., 2005). The $Ca^{2+}$ ion found at this site is especially important for stabilizing the β subunit, so that it can effectively associate with the adjacent α subunit and form an αβ heterodimer (Arnaout et al., 2005).

### 1.6.2 **The β1 Heterodimer:**

The cytoplasmic tail of the β1 integrin is capable of interacting with more than 10 molecules, of which 5 can also interact with the β1 integrin associated α subunits (Liu et al., 2000). Protein complexes including alpha-actinin, talin, filamin and ILK have all been shown to mediate attachment of the β1 subunit to the actin cytoskeleton (Brakebusch et al., 2005). These interactions serve two purposes: 1) they provide critical connections between the ECM and the intracellular cytoskeleton and 2) provide a binding platform for signaling molecules and additional cytoskeletal components for the propagation of downstream signals.

Highly conserved motifs located in the cytoplasmic domain of the β1 integrin are especially critical for illiciting downstream signaling. The β1A and β1D are two spliced variants which contain two conserved NPxY motifs (Cordes et al., 2006) in the cytoplasmic domain, which facilitate the inside-out activation of integrins, (Cordes et al., 2006) and outside-in activation of the focal adhesion kinase (FAK) (Wennerberg et al., 2000). Although a β1B integrin variant has been identified, it lacks the carboxy terminal half containing the NPxY motif of the β1A variant, thus representing an inactive conformation which confers the adhesive properties of integrins, but is devoid of signaling capabilities (Cordes et al., 2006). The physiological significance of β1 integrin

mediated signaling has been verified both *in vivo* and *in vitro* in several model and organ systems.

### 1.6.3 General Structure of the α Heterodimer

The α-subunit consists of an extracellular portion which interacts with divalent cations, consistent with the idea that all integrin interactions are divalent cation-dependent and is also implicated in ligand binding (Berman et al., 2003). The α subunits contain seven tandem repeats of approximately 60 amino acids at their N-terminus. Within these repeats there are four or five metal binding regions of the typical structure DxDxDGxxD. The α subunits can be subdivided into two groups: those which contain an A-domain, and those with a proteolytic cleavage site.

**a) A domain alpha subunits:**

The A domain is approximately 200 amino acids long and is found on subunits α1, α2, αD, αE, αL, αM and αX (Fernandez et al., 1998). The A domain is composed of five parallel beta strands and one anti-parallel beta strand surrounded by seven alpha helices (Fernandez et al., 1998). Integrin A domains contain one MIDAS motif. A series of mutation, ablation and peptide blocking experiments have revealed that the A domain is sufficient for integrin-ligand binding (Fernandez et al., 1998).

**b) Proteolytic Clevage site:**

The proteolytic cleavage site of the α3, α5, α6, α7, α8, αIIb and αV subunits is located close to the transmembrane domain and bridged by disulphide bonds (Fernandez

et al., 1998). The α4 subunit also contains a centrally located cleavage site which does not contain a disulphide bridge (Fernandez et al., 1998). Less is known about ligand binding sites for integrins which lack the A domain. The majority of chimera, substitution and epitope mapping studies have revealed that the N-terminal repeats in the α subunits facilitates ligand binding (Fernandez et al., 1998).

### 1.6.4 **Cytoplasmic tails of α and β integrins:**

The use of NMR technology has helped decipher the exact structures of α and β integrins and how changes in receptor activity and affinity result in altered structure and conformation of cytoplasmic tails (Li et al. 2001; Ulmer et al. 2001; Vinogradova et al. 2002; Vinogardova et al. 2004; Weljie et al. 2002). Although there is incongruency in the literature as to the interactions between respective cytoplasmic tails of the α and β integrins, several studies have reported that electrostatic and hydrophobic interactions link the two tails together (Vinogardova et al., 2002). Disrupting these hydrophobic and electrostatic interactions destabilizes the cytoplasmic complexes and perturbs downstream signaling (Arnoaut et al., 2005). Lastly the cytoplasmic tails of certain integrins have been identified to have highly conserved phosphorylation sites which enable the transduction of downstream signals.

A schematic diagram illustrating the general biochemical motifs of αβ integrins is presented as **Figure 1.2**.

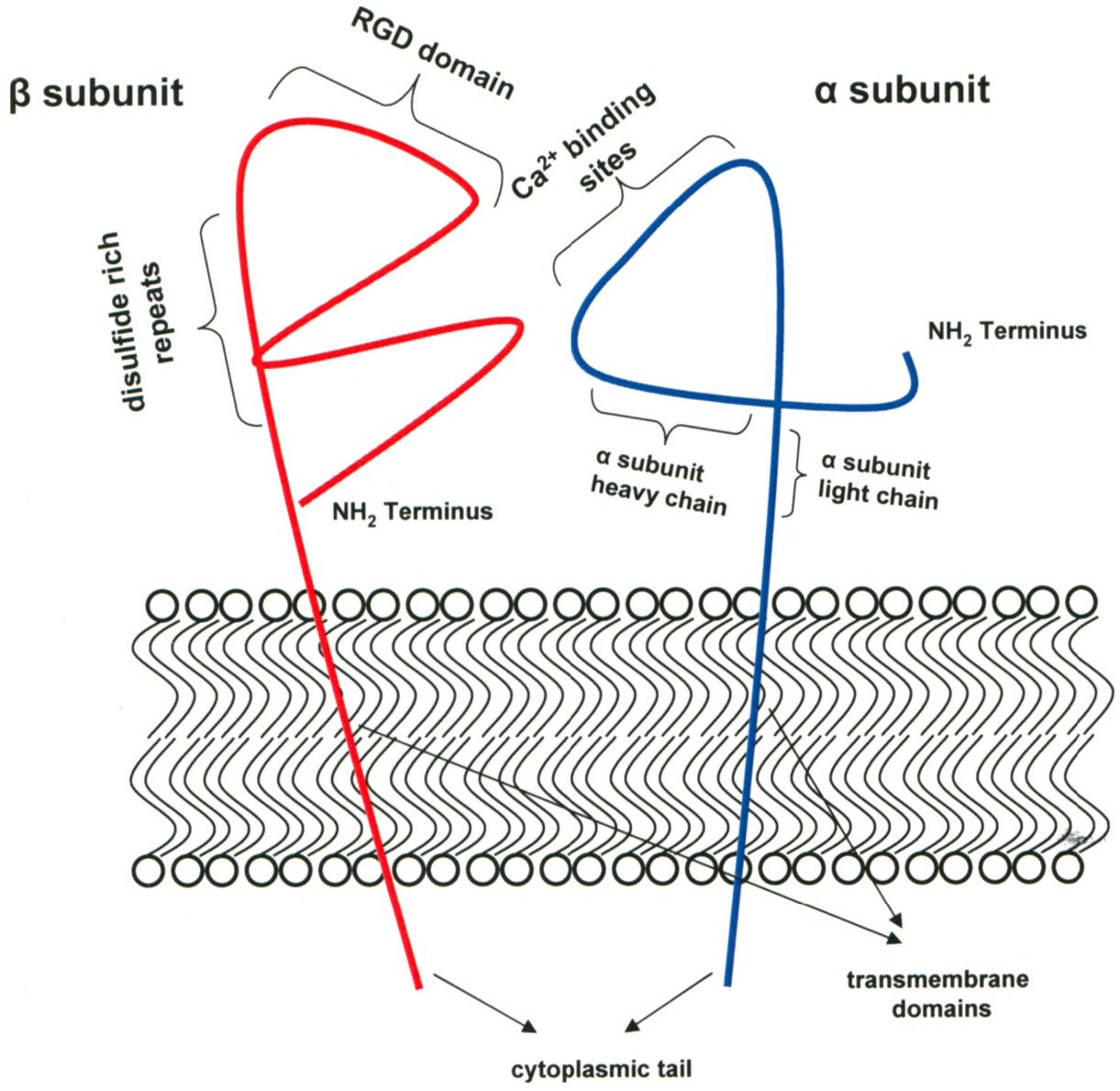


**Figure 1.2:** A schematic diagram illustrating motifs of the α and β integrin subunits, which are critical for their function. Adapted from Berman et al., 2003; Eble 1997; Green et al., 1998; Humphries, 2000.

### 1.6.5 **The α3β1 integrin**

The studies performed in this thesis have revealed that integrin α3β1 is important for beta cell survival and function. This section aims to generally describe functions of the α3β1 integrin in various cell types.

An enigmatic member of the integrin family, α3β1 has conventionally been associated with laminin. However in recent studies, α3β1 has been shown to be pleiotrophic, as it associates with several matrix proteins including FN, collagen I, collagen IV, nidogen and thrombospondin (Coppolino et al., 1995; Delwel et al., 1994). Although its localization has been limited to the basolateral surface of epithelial cells, cells and tissues deficient for this integrin reveal that this receptor is involved in much more than just interactions with the basement membrane. Its expression pattern and function has been well characterized in several model systems. In keratinocytes, α3β1 is essential for skin development and wound closure (DiPersio et al., 1997; Hertle et al., 1991; Kriedberg 2000). Integrin α3β1 forms cytoskeletal attachments in glomerular podocytes mediating attachment to the glomerular basement membrane (Kreidberg et al., 1996; Kreidberg 2000; Miner et al., 2000). Lastly, α3β1 integrin deficient mice display a high level of neural disorganization (Anton et al., 1999; Kreidberg 2000; Mechai et al., 2005), indicating that this integrin is essential for neurite growth and migration (Kreidberg 2000).

## 1.7 **Signaling Pathways Regulated by Integrins:**

In the following section, a few major integrin signaling pathways will be discussed. These downstream signaling cascades regulate beta cell survival and function.

### 1.7.1 **The MAPK pathway: Extracellular Signal-Regulated Kinase (ERK)**

It is now well established that integrin-mediated adhesion to the ECM activates the MAPK pathway resulting in the phosphorylation of a wide variety of cellular proteins. The activation of transcription factors, cytoskeletal proteins, kinases and several other enzymes can greatly influence gene expression, metabolism, cell division, cell morphology and cell survival (Qi and Elion, 2005). An intact actin cytoskeleton, which requires integrin dependent complexes, is especially important for the activation of MAPK (Clark and Brugge, 1995; Qi and Elion, 2005).

The MAPK family is three-tiered, composed of MAPK, MAPK kinase (MAPKK, MKK or MEK) and MAPKK kinase or MEK kinase (MAPKKK or MEKK) (Chang and Karin, 2001). The MAPKs exist as five families in mammalian cells: i. Extracellular signal-regulated kinases1 and 2 (ERK1 and ERK2) ii. Jun N-terminal kinases (JNK1, JNK2 and JNK3) iii. p38 kinase isoenzymes (p38α, p38β1, p38γ, p38δ) and iv. ERK3/ERK4 and v. ERK5 (Qi and Elion, 2005).

ERK1and ERK2 are critical regulators of gene expression, cell survival, proliferation and differentiation through their association with: i. transcription factors including activating protein-1 (AP-1), NFκB, Myc ii. Kinases such as Rsk, Bcl-2, cPL2 and iii. cytoskeletal scaffold protein paxillin (Qi and Elion, 2005). The activation of ERK1 and ERK2 occurs in the following way: i. ligands bind to cell-surface receptor

tyrosine kinases or G-protein coupled receptors ii. Tyrosine phosphorylation of ERK creates a binding site for adaptor protein Grb2 (Puente and Ostergaard, 2003) iii. Grb2 recruits the Ras guanylnucleotide-exchange factor mSOS1, which converts inactive Ras-guanosine 5'-diphosphate (GDP) to active Ras-guanosine 5'-triphosphate (GTP) (Qi and Elion, 2005) iv. Ras-GTP recruites the kinase Raf to the plasma membrane where it subsequently becomes activated v. Activated Raf phosphorylates MEK1/MEK2, which phosphorylates and activates ERK1/ERK2 (Qi and Elion, 2005).

### 1.7.2 **FAK/MAPK/ERK Signaling: Mediator of Cell Survival and Death**

The binding of cells to the ECM allows the transmission of signals from the FAK complex, which is critical for cell survival (Reddig and Juliano, 2005). This is best exemplified by FAK expressing cells, which undergo growth arrest due to a loss of matrix adhesion, and subsequent accumulation of hypophosphorylated Rb (Giancotti and Ruoslahti, 1999). Consequently, there is inactivity of cyclinD-Cdk4/6 and cyclinE-Cdk 2, both of which are essential for cell cycle progression (Giancotti and Ruoslahti, 1999).

Cell death mediators, including members of the caspase family, have the ability to perturb FAK survival signals (Allan et al., 2003). The cleavage of FAK by caspases results in the release of the FAT domain (found in FAK's carboxy terminus) which inhibits FAK signaling (Zou et al., 1997) and enhances apoptosis. FAK also directly interacts with RIP (receptor interacting protein), a death domain containing serine threonine kinase (Kurenova et al., 2004). RIP mediates pro-apoptotic signals in response to disrupted adhesion and survival signals of FAK, by interacting with proteins in the death receptor complex, thus mediating increased apoptosis through NFκB signaling

(Hsu et al., 1996; Stanger et al., 1995). Lastly, increased expression of cytoskeletal protein vinculin is also associated with disrupted FAK survival signaling. The absence of vinculin enhances pro-survival FAK/paxillin signaling and subsequently activates ERK (Reddig and Juliano, 2005).

### 1.7.3 **The PI3-K Pathway: Akt (Protein Kinase B)**

Signaling through the PI3K pathway as a result of integrin-mediated adhesion to the ECM is less studied. Although the PI3K pathway is critical for the activation of kinases and enzymes which influence gene expression, metabolism, cell survival and proliferation, the majority of cellular changes have been identified as a result of growth factor signaling and not integrin-ECM interactions.

The binding of a growth factor to its receptor triggers the activation of phosphatidylinositol (PI) 3-kinase, a lipid kinase, through the activation of the p110 catalytic subunit, which in turn, results in the phosphorylation of the D-3 position (Zdychova and Komers, 2004). The subsequent activation of this enzyme causes the conversion of PI 4,5-biphosphate (PIP2) to PI 3,4,5-triphosphate (PIP3), a putative second messenger which stimulates Akt activity (Cantley, 2002).

Several studies have demonstrated that PI3K is necessary and sufficient for growth factor dependent activation of Akt. Mutants deficient for growth factor receptors fail to activate PI3K and thus are unable to mediate the phosphorylation of Akt (Burgering and Coffer, 1995; Franke et al., 1995). Dominant inhibitory alleles of PI3K also prevent the activation of Akt (Burgering and Coffer, 1995) and PI3K inhibitors wortmannin and LY294002 block Akt activation via growth factor signaling

(Andjelkovic et al., 1996; Burgering and Coffer, 1995; Franke et al., 1995; Franke et al., 1997; Kohn et al., 1995).

### 1.7.4 **Akt Signaling Pathway: Cell survival versus apoptosis**

Akt, a serine/threonine kinase with a pleckstrin homology domain in its N-terminus and catalytic domain (Zdychova and Komers, 2004), is also known as PKB and Rac and has been shown to regulate cell survival and apoptosis (Burgering and Coffer, 1995; Franke et al., 1995; Franke et al., 1997). Its activation, results from the binding of PI3P (activation of PI3K) at the pleckstrin homology domain, and subsequent translocation into the nucleus. Moreover, the increasing levels of phosphoinositides function as second messenger molecules, activating PDK1 and PDK2. These PI-dependent kinases phosphorylate Akt upon its membrane translocation at sites serine 473 and threonine 308 (Alessi et al., 1996; Alessi et al 1997; Bellacossa et al. 1998). Activated Akt subsequently phosphorylates its downstream targets, thus promoting cell viability and preventing apoptosis through the inhibition of Bad (Cardone et al., 1998), forkhead transcription factors (Brunet et al., 1999), c-Raf (Zimmerman and Moelling, 1999), caspase-9 and glycogen synthase kinase 3-α/β (GSK3-α/β) (Cross et al, 1995; Hajduch et al, 2001) as well as the indirect inactivation of caspase-3 cleavage through increased expression of XIAP (Deveraux and Reed, 1998; Deveraux et al., 1999). The inactivation of Akt is accomplished by protein phosphatases including protein phosphatase 2 and Akt antagonists, ceramides and PTEN (Ozes et al., 2001, Teruel et al., 2001).

Akt includes a family of three highly conserved and closely related homologues known as Akt1, Akt2 and Akt3 or PKBα, PKBβ and PKBγ, respectively (Testa and Tsichlis, 2005). These three isoforms are ubiquitously expressed in all cell types and tissues; however, Akt3 displays a more restricted and specialized expression pattern (Toker and Yoeli-Lerner, 2006). The proteins encoded by these proto-oncogenes are serine/threonine kinases which mediate the signal transduction pathways of PI3K through the phosphorylation of several substrates, thus regulating growth and survival mechanisms (Toker and Yoeli-Lerner, 2006).

The pleiotrophic effects of Akt in regulating normal physiology and various diseases, has been well established. Its diverse signaling cascades primarily regulate cell proliferation and survival; however Akt signaling has also been implicated in cell size and response to nutrient availability, glucose metabolism, cell invasiveness, genome stability and angiogenesis (Testa and Tsichlis, 2005).

## 1.8 **Integrins in Islet Cell Biology**

### 1.8.1 **Integrins and ECM in Islet Formation**

ECM proteins and cell adhesion receptors have been well known to contribute to the organogenesis, morphogenesis and cytodifferentiation of several organ systems including the pancreas (Theiry et al., 2003). Recent findings have demonstrated that integrin adhesion systems are implicated in numerous developmental stages and regulate diverse processes including cell positioning, tissue patterning, compartmentalization and cell polarity (Theiry et al., 2003).

In the pancreas, many integrin-ECM interactions have been characterized in rodent and human systems. The migration of islet precursors from the ductal epithelium into the surrounding mesenchyme is governed by the activities of matrix metalloproteinases (MMPs) (Miralles, 1998). In fact, interactions between MMP2 and MMP9 and their inhibitors, tissue inhibitors of MPs (TIMPs), are largely responsible for processes of aggregation. Moreover, transforming growth factor-β (TGF-β) signaling can also regulate matrix metalloproteinase activity and thus, further support the role of MMPs in islet cell morphology and development. EGFR signaling is also a positive regulator of MMP activity; EGFR mutant mice display a decrease in pancreas size due to reduced epithelial branching and islet migration (Miettinen et al., 2000). Thus, matrix degradation is critical for the remodeling and epithelial morphogenesis and migration of endocrine precursors. Interestingly, ectopic expression of EGF in beta cells leads to dramatic increases in islet size, when compared to age-matched control animals without impairment of islet function or glucose homeostasis (Krakowski et al., 1999).

Studies have identified the basement membrane, rich in laminin, as a critical mediator of ductal and tubular morphogenesis and differentiation, giving rise to either endocrine or exocrine precursors. The epithelial-mesenchymal interface, established by the BM, is rich in biologically active components including collagens, heparin and chondroitin sulfate, laminin and FN (Crisera et al., 2000). Specifically, the role of laminin in the regulation of ductal morphogenesis from undifferentiated pancreatic epithelium has been well investigated (Gittes et al., 2000). Organization and concentration of laminin-1 in the BM is critical for its development and subsequent influence over epithelial cell differentiation into either the endocrine or exocrine pancreas (**Figure 1.3**).

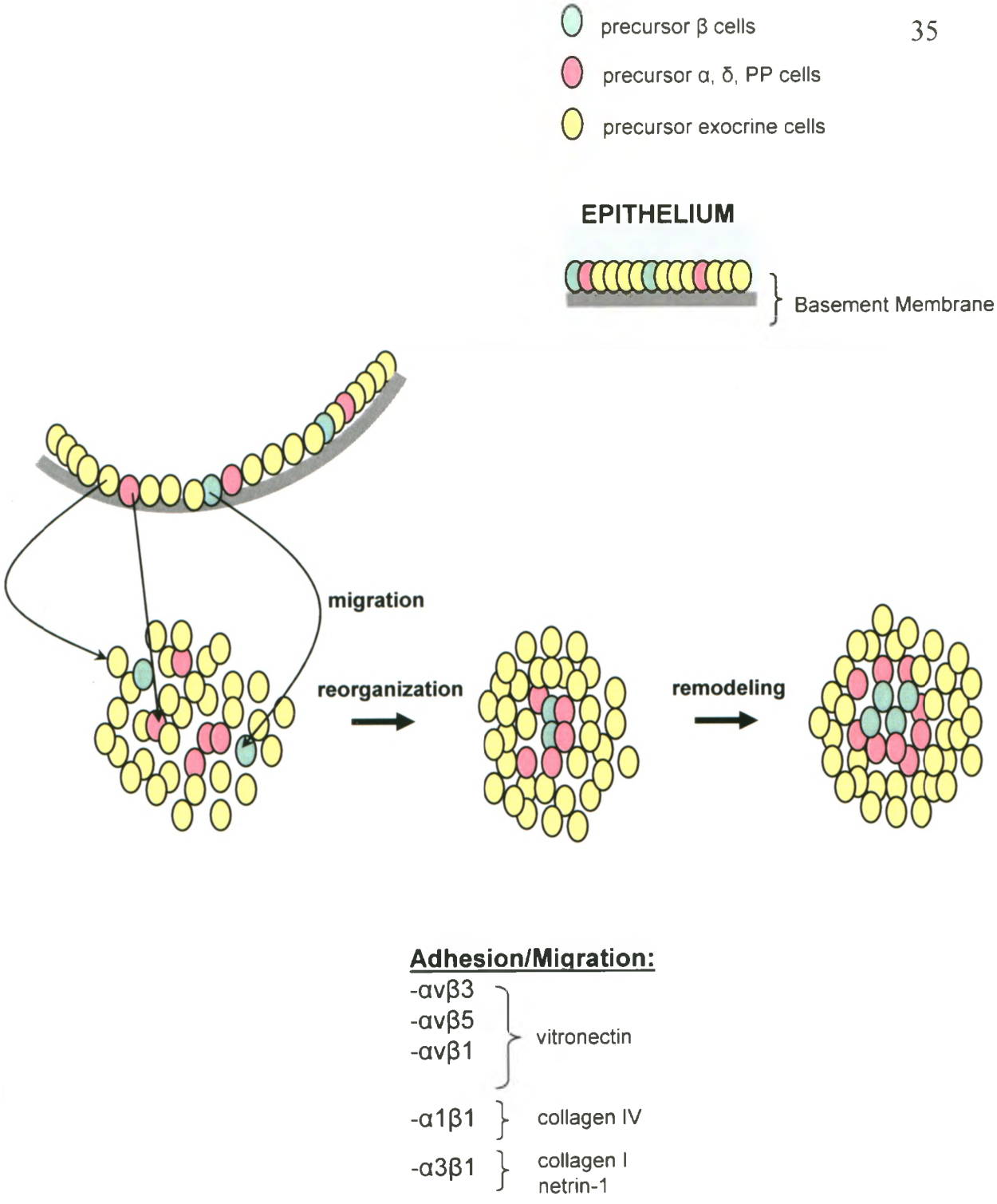


**Figure 1.3. Adhesive and migratory cycles leading to islet formation.** Precursor epithelial cells attach to and migrate through the basement membrane, leading to their differentiation into ductal, islet or exocrine cells. ECM and integrin interactions allow for adhesion and migration of endocrine cells to form mature islet-like structures.

Direct contact of pancreatic epithelium with laminin-1 allows for the formation of the exocrine pancreas, whereas those cells which are spared this interaction organize into endocrine cell clusters and eventually form islets by the end of gestation (Gittes et al., 2000). Highly specialized experimental models, in which early pancreatic epithelium is grown under laminin-poor renal capsules, resulted in the default selection of endocrine lineage, demonstrating that the absence of laminin is necessary for endocrine cell formation (Gittes et al., 2000). *In vitro* experiments have further solidified the notion that interactions between laminin-1 and integrin receptors allow for epithelial cell differentiation. Crisera and colleagues demonstrated that isolated pancreatic epithelium requires α6β1 for the formation of ductal structures (Crisera et al., 2000).

The ECM also confers migratory properties essential for islet formation. Endocrine precursors located in pancreatic ducts, migrate through the basement membrane and cluster with the aid of integrin receptors during early stages of islet assembly (**Figure 1.3**) (Kim et al., 2001; Perez et al., 2005). Studies by Cirulli and colleagues have demonstrated that integrins αvβ3 and αvβ5 contribute to the migration of putative endocrine progenitor cells (Cirulli et al., 2000). The treatment of human fetal islet-like clusters with anti-αvβ3 and anti-αvβ5 antibodies resulted in the detachment of cells and three-dimensional clustering. Furthermore, when human fetal pancreatic fragments were transplanted into the kidney capsule of NOD/SCID mice along with RGD blocking peptides, a significant disruption in islet architecture and size was observed (Cirulli et al., 2000).

Another migratory cue which regulates islet cell development is netrin (Yebra et al., 2003). A well known contributor to axon guidance, netrin is expressed in discrete

pancreatic populations within fetal and adult ductal epithelia and exocrine tissue. Although it is not found in adult islets, it is localized to the basal surfaces of pancreatic cells. Integrins α6β4 and α3β1 (Yebra et al., 2003) mediate the adhesion and migration of fetal pancreatic epithelial cells to netrin-1. The majority of cells which migrate on netrin-1 are Pdx-$1^+$ suggesting that netrin-1 supports the migration of ductal progenitors (Yebra et al., 2003).

Once islets form, islet-ECM and cell-cell interactions become essential for proper coordination of events including development and maturation. Vitronectin and its association with several integrin receptors have been shown to contribute significantly to human fetal islet development. Its association with epithelial and insulin$^+$ cells combined with its restricted expression near progenitor populations, suggests that its synthesis and deposition is involved in the regulation of morphogenetic events needed for islet neogenesis (Cirulli et al., 2000). Moreover, *in vitro* experiments have identified relationships between vitronectin and integrins αvβ1 and αvβ5 as important mediators of human beta cell adhesion, spreading and motility (Cirulli et al., 2000). In particular, αvβ1 was responsible for migration of human fetal beta cells. The developmental loss of this integrin in adult beta cells resulted in the lack of migration, suggesting that this receptor is critical for motile processes required for fetal islet assembly. Given that expression of the αVβ1 integrin is lost in adult beta cells, it is most likely that this receptor aids in static cell-matrix interactions (Cirulli et al., 2000).

Numerous other studies have also demonstrated the importance of the β1 integrin in mediating motile processes for islet development. This receptor has been shown to support the migration of human fetal beta cells, in turn, facilitating islet formation. The

spatial and temporal expression of the β1 integrin and its associated α subunits has been well characterized in fetal and pre- and post-natal rat pancreas, indicating the importance of the β1 integrin family during development and suggesting that these heterodimers contribute substantially to islet restructuring (Wang et al., 2005; Yashpal et al., 2005). Assessment of whole pancreata mRNA and protein revealed significant increases in α3β1 and α6β1 by one month after birth, while expression of α5β1 remained relatively constant. However, a decrease in integrin expression in rat pancreata was noted in postnatal life, possibly since the majority of pancreatic construction occurs by e18 with remodeling occurring until the first three weeks of postnatal life. The co-expression of ductal marker cytokeratin 20 and the β1 integrin was frequent throughout pre- and postnatal life, while glucagon$^+$ and insulin$^+$ cells also expressed integrins β1, α3, α5 and α6 (Wang et al., 2005; Yashpal et al., 2005).

Similarly, in the human fetal pancreas, the importance of α3β1, α5β1 and α6β1 has been well demonstrated since these integrins exhibit specific expression patterns during progressive pancreatic development (Wang et al., 2005). Although the expression of all three integrin heterodimers was noted before the budding of glucagon$^+$ and insulin$^+$ cells from pancreatic ducts, α3β1 and α6β1 demonstrated significant increases in expression by 16-20 weeks of development. These studies suggest that the formation and maturation of the islet of Langerhans requires increasing α3β1, α5β1 and α6β1 expression to contribute adhesive and migratory properties (Wang et al., 2005; Yashpal et al., 2005).

Collagen IV and its interactions with integrin α1β1 have also been shown to support the adhesion and migration of human fetal and adult beta cells (Kaido et al.,

2004). However, the migratory response of fetal beta cells was much higher in comparison with adult beta cells, suggesting that this critical basement membrane protein provides the necessary motile processes required for islet neogenesis during development. The strong adhesion rate and maturational loss of migration, proposes that collagen IV is essential for maintenance of architecture and integrity in adult islets.

Co-localization studies of these integrins with specific ECM proteins have further solidified the importance of integrin-ECM relationships in the developmental process of the islet of Langerhans. Association of collagen IV, laminin and fibronectin with the β1 integrin family suggests that the multiplicity of this receptor is critical for islet development and biology (Wang et al., 2005; Yashpal et al., 2005).

Lastly, cell adhesion molecules (CAMs) and cadherins have also been shown to contribute to proper islet formation and maintenance of architecture (Dahl et al., 1996; Esni et al., 2001). Cadherins E and N are expressed in areas of cell contact between beta cells and other cell types (Dahl et al., 1996; Esni et al., 2001), while R-cadherin is prominently expressed only on beta cells (Dahl et al., 1996; Esni et al., 1999). Neural cell adhesion molecule (N-CAM) is expressed on both beta cells and other cell types in the pancreas and is required for proper islet cell aggregation and organization into typical islet structures, with beta cells concentrated in the center and alpha cells remaining in the periphery (Cirulli et al., 1994; Esni et al., 1999).

### 1.8.2 **Integrins and ECM in Islet Survival and Function**

Several studies have implicated integrin-matrix relationships as critical determinants of islet survival, proliferation, differentiation and function. Given that the

ECM confers stability and support to islets, destruction of matrix proteins would undoubtedly increase cell necrosis and apoptosis. In fact, freshly isolated canine islets were shown to undergo apoptosis, due to loss of the perinsular-basement membrane (Wang and Rosenberg, 1999). However, when cultured in or on matrix proteins such as collagen I and fibronectin, these islets exhibited improved survival. These islets were also shown to undergo a transdifferentiation process, characterized by a switch from islet to ductal phenotype indicating of loss in stability, as a result of perturbed islet-matrix relationships (Wang et al., 2001). Moreover, the aggregatory nature of pancreatic islets also contributes to the survival of individual islet cells. Cell sorting experiments, whereby human islets are dispersed into single cells resulted in reduced survival and increased apoptosis (Ris et al., 2002). However, matching ECM proteins to specific integrin receptors expressed on islet cells improved cell survival.

Perturbation studies using immunoneutralizing antibodies or siRNA have been valuable for the identification of key integrin receptors which mediate adhesion, spreading and proliferation when interacting with specific matrix proteins. Again, recent evidence has highlighted the importance of the β1 integrin family and its associated α subunits in the regulation of numerous islet cell activities. Both primary and transformed rat islet cells were shown to form aggregates resembling native islets when cultured on collagen I, in vitro (Montesano et al., 1983). Interactions between matrices and the β1 integrin were further solidified when a decrease in adhesion and spreading were observed upon treatment of rat islet cells with anti-β1 integrin, and subsequently cultured on bovine corneal endothelial cell extracellular matrix (BCEM) (Kantengwa et al., 1997). Isolated rat islets and human fetal islet epithelial clusters demonstrated similar decreases

in adhesion and spreading when cultured on fibronectin, laminin and collagen I after treatment with an immunoneutralizing antibody for β1 (Wang et al., 2005; Yashpal et al., 2005). Blocking the β1 integrin resulted in increased apoptosis of beta cells in rat and human fetal primary cells, as well as reduced insulin and glucagon gene expression. Taken together, these studies indicate the importance of the β1 integrin in mediating cellular events and conferring anti-apoptotic properties.

Relationships between α6β1 and 804G matrix have also been shown to affect beta cell function *in vitro*. The increased insulin secretory response noted when rat beta cells adhered to this matrix was shown to be mediated through the α6β1 integrin (Bosco et al., 2000). Furthermore, adhesion of cells to this matrix and treatment with insulin secretagogues allowed for increased expression of the integrin itself, suggesting that both "inside out" and "outside in" signaling mechanisms are active and critical for islet function. Laminin, the primary constituent of the 804G matrix, is thought to be responsible for the majority of positive effects on islet survival and function (Pernaud et al., 2006). Both beta cell spreading and insulin secretion in response to glucose stimulation, on 804G matrix, was significantly inhibited when cells were treated with anti-laminin-5 antibody and complementary results were noted with anti-β1 integrin treatments.

The above studies clearly demonstrate that integrin-matrix interactions are critical for the viability and function of islets, and that in most cases "inside out" and "outside in" signaling is necessary for facilitating these events. Studies exploring changes in downstream signaling cascades have identified FAK, MAPK and ERK-activation as critical mediators of islet cellular events (**Figure 1.4**).

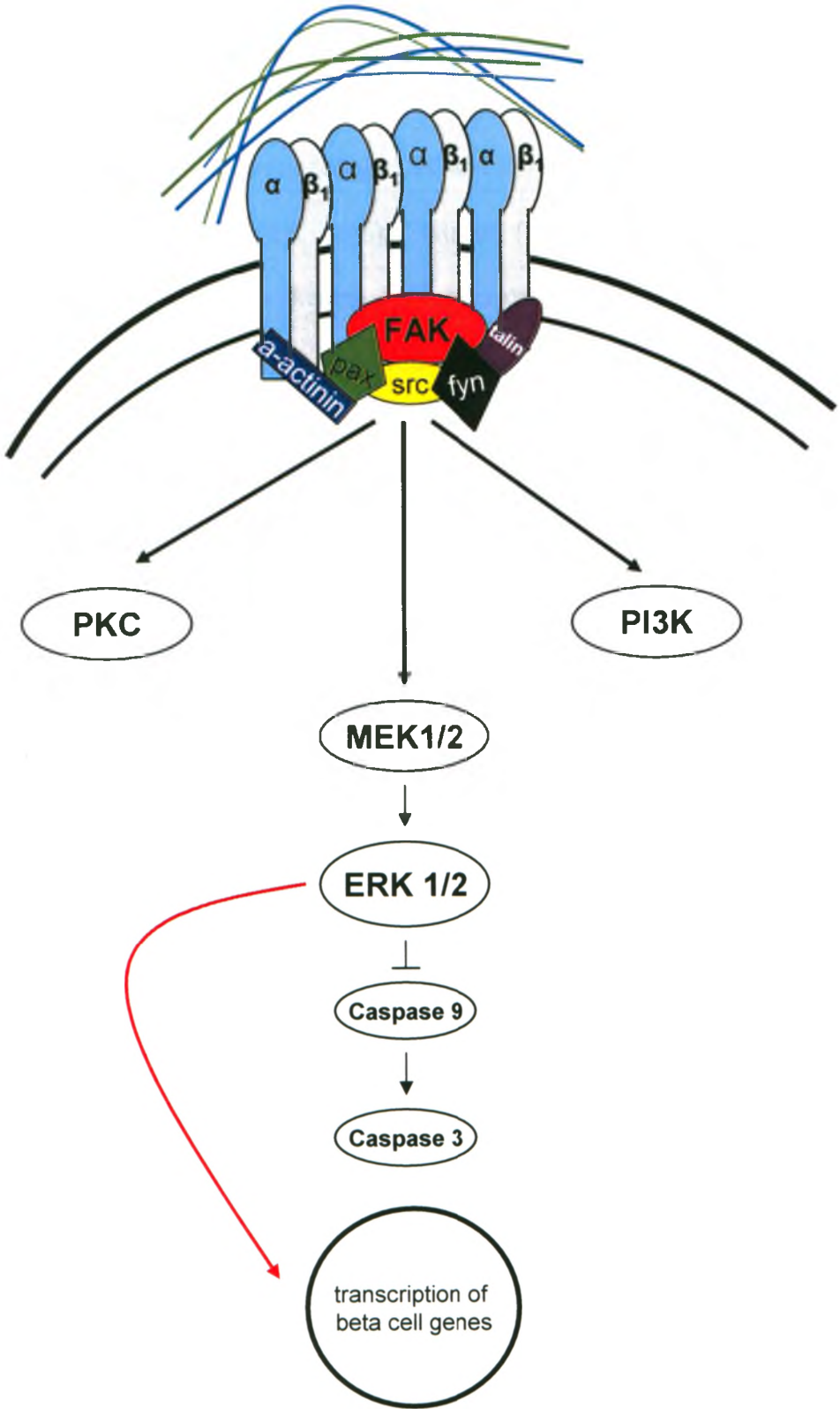


**Figure 1.4. αβ1 Integrin signaling in pancreatic beta cells.**
Cell adhesion to ECM proteins leads to integrin clustering, activation of cytoskeletal proteins in focal contacts and increases transcription of beta cell genes through ERK1/2 signaling pathway. (Hammer et al., 2004; Unpublished data from Dr. Wang's lab)

A decrease in insulin content and subsequent increase in insulin release as a result of human fetal beta cell adhesion to collagen IV and vitronectin was shown to be dependent on ERK signaling (Kaido et al., 2006). Collagen IV was also shown to induce ERK-dependent insulin secretion in human adult beta cells (Kaido et al., 2006). Moreover, laminin-5 and β1 interactions resulted in increases in FAK phosphorylation (Hammer et al., 2004). Metabolic changes triggering the release of intracellular calcium are also suspected to be involved in the functional changes noted in integrin-matrix interactions.

Although the majority of integrin-matrix interactions have been shown to potentiate islet survival and function, it is important to note that a loss in function can be caused by adhesion to certain ECM substrates. Fetal and adult beta cells demonstrated significant decreases in insulin gene expression when cultured on collagen IV and vitronectin (Kaido et al., 2006). Moreover, blockade studies of isolated rat islets with anti-β1 integrin antibodies and RGD peptides showed a decrease in apoptosis and an increase in pro-survival Akt phosphorylation (Pinske et al., 2006).

## 1.9 **Diabetes Mellitus and Islet Transplantation:**

### 1.9.1 **Diabetes Mellitus: Type 1 and 2**

Diabetes mellitus, a major disease of the pancreas, is typified by fasting hyperglycemia and the presence of glucose in urine (Fox, 1999). It is estimated by the World Health Organization (WHO) that 194 million people are afflicted with diabetes. These numbers are expected to grow exponentially – by 2025, 300 million people are thought to have to deal with this disease (Merani and Shapiro, 2006). The two main forms of this disease are categorized as Type 1 and 2. The absolute or relative lack of

number or function of beta cells is the major cause of both forms of this disease (Donath and Halban, 2004).

Type 1 disease (juvenile diabetes or insulin dependent diabetes) is most often found in children, and results from auto-immune destruction of pancreatic beta cells, leading to permanent insulin deficiency (Slack, 1995). Apoptosis has been identified as the major underlying mechanism responsible for beta cell death, in this form of diabetes. The release of cytokines from inflammatory cells which enter the islets subsequently destroys beta cells (Eizirik and Mandrup-Poulsen, 2001), leading to insulin deficiency and causing poor maintenance of blood glucose levels (Lernmark and Falroni, 1998). Individuals with Type 1 diabetes lead a life with daily dependence on insulin injections (Lernmark and Falroni, 1998). The etiology of Type 1 diabetes remains unknown and factors such as genetic disposition, viruses, chemicals, diet/nutrition and stress have been proposed (Knip and Akerblom, 1999).

Type 2 diabetes (non-insulin dependent diabetes) develops slowly, is hereditary and most often occurs as a result of obesity (Slack, 1995). Although Type 2 diabetics have elevated blood insulin levels, there exists a degree of non-responsiveness in target tissues. Thus, larger than normal amounts of insulin are required to allow for normal regulation of blood glucose levels (Fox, 1999). Individuals with type 2 diabetes develop this disease over a wide age range and etiologies of this form of diabetes include obesity, excessive caloric intake, and physical inactivity (Fox, 1999).

### 1.9.2 **Islet Transplantation: Problems**

Although exogenous insulin therapy is currently the most common mode of treatment, normal physiological glycemic control can only be achieved through beta cell replacement. There are several advantages to islet transplantation, including a simple administration route which is minimally invasive and the possibility of repeating transplantation procedures without major patient discomfort (Balamurugan et al., 2006; Ryan et al., 2006). The Edmonton protocol was initially successful, allowing for 80% insulin independence for one year (Balamurugan et al., 2006; Ryan et al., 2006). However, long-term function of islet grafts failed with only 10% of patients maintaining insulin independence for 5 years (Balamurugan et al., 2006). Other issues including excessive cell death due to isolation, inadequate immunosuppression, and beta cell toxicity result in the loss of long term viability and islet mass (Shapiro et al., 2000). A shortage in islet supply is also considered a major limitation of islet transplantation. Given that isolation procedures expose islets to a variety of stresses, the induction of apoptosis in beta cells accounts for the bulk of graft loss (Bhonde et al., 2007; Gao et al., 2007; Kin et al., 2007; Shapiro et al., 2000; Wang et al., 1999). Moreover, immunosuppressive drugs also complicate matters as they only partially prevent auto-immunity and their effectiveness depends on several other factors including the quality of isolated islets, implantation sites and the transplantation procedures itself (Balamurugan et al., 2006). Recent research has shown that certain immunosuppressive treatments greatly affect the long-term survival of islet grafts by inhibiting the natural process of neogenesis and negatively impacting beta cell survival, proliferation and differentiation (Gao et al., 2007). Although several issues still need to be addressed through clinical and

basic science research, there has been immense progress in the field of islet transplantation.

### 1.9.3 **Islet Transplantation: Promising Studies**

The *in vitro* generation and expansion of islets with maintenance of viability and function is a possible alternative that can circumvent donor shortage limitations (Bonner-Weir and Sharma, 2002; Heit et al., 2004). Several expansion programs worldwide are currently investigating mechanisms of beta cell growth, by focusing on either beta cell neogenesis or replication (Banerjee et al., 2003; Bonner-Weir et al., 1992; Bonner-Weir et al., 2000; Dor et al., 2004; Kanitkar et al., 2004; Katdare et al., 2004; Ramiya et al., 2000; Rosenberg et al., 1998). The neogenesis of islets, or the budding of pancreatic epithelium or intra-islet precursors to form new islets, occurs primarily during fetal or perinatal stages of development, and has been noted in the adult pancreas to a lesser extent (Smith et al., 1991; Swenne et al., 1992). The duplication of existing beta cells and their ability to maintain function in culture is another mechanism which is being heavily investigated.

For purposes of beta cell neogenesis or replication, the culturing of islets on substrates which mimic natural microenvironments seems to be a logical prospect. Islet-ECM interactions have been shown to inhibit apoptosis and induce prolonged survival of beta cells after isolation. These interactions also allow for extensive manipulation for successful *in vitro* survival and proliferation.

To address the inadequate supply of human islets, several studies have focused on the expansion and differentiation of beta cells. Bonner-Weir and colleagues examined the

proliferation and differentiation of human ductal tissue into islet cells, as well as their function *in vitro* (Bonner-Weir et al., 2000). The expansion of these cells and subsequent overlay with Matrigel resulted in the formation of three dimensional ductal cysts, eventually budding into islet like clusters expressing beta cell hormones and responding well to glucose challenge. This study highlights two key areas of research: 1) matrix proteins have the ability to support and stimulate three dimensional organization of pancreatic precursors into islet cells, which in turn, facilitates survival and function and 2) that the shortage in donor material can be alleviated by inducing ductal precursors to differentiate into an islet population through three dimensional assays.

Furthermore, several studies have examined the effects of ECM proteins on the establishment and maintenance of isolated islets *in vitro*. Long-term culture studies of human islets embedded in type I collagen maintained their architecture and high secretory capacities. Overlaying this *in vitro* system with additional collagen I resulted in the formation of a three-dimensional islet like structure and enhanced insulin secretion rates (Lucas-Clerc et al., 1993). Moreover, culturing isolated rat islets on a collagen I, II, IV and laminin hydrogel resulted in significant decreases in cell death, but no changes in insulin, glucagon and somatostatin expression (Nagata et al., 2004). A study by Montessano and colleagues demonstrated the ability of dispersed neonatal rat islet cells to reorganize into islet like organoids within a three dimensional collagen matrix (Montessano et al., 1983). Islet cells seeded on collagen matrix remained in monolayer, while the addition of a second layer of collagen I resulted in a dramatic reorganization of islets. The typical architecture noted of islets *in vivo*, was re-established in this three

dimensional model, as beta cells remained concentrated in the center of these organoids and alpha and delta cells were located in the periphery.

A relatively novel and interesting area of research is focused on using naturally occurring matrices for culture and inducing expansion of islets. The small intestinal submucosa (SIS), a cell free matrix extracted from porcine intestines, is rich in collagens, glycoproteins, proteoglycans and glycosamineglycans (Xiaohui et al., 2005; Xiohui et al., 2006). Its use as a scaffold in the remodeling and regeneration of a variety of other tissues suggests that it may be useful in potentiating islet survival and function. The *in vitro* tissue culture of isolated rat islets on SIS resulted in the maintenance of morphology, increased insulin secretion upon glucose challenge and a significant reduction in cell death, suggesting that the SIS confers both protection and support to islets (Lakey et al., 2001; Xiaohui et al., 2005; Xiohui et al., 2006). Moreover, intrinsic mechanical properties make this matrix highly compliable and histocompatible while its porous nature allows for the diffusion of cellular nutrients.

Recent islet isolation methods have significantly improved over the last three decades; however the current method of collagenase digestion still destroys the basement membrane and results in reduced islet viability and function (Merani et al., 2006). In order to improve current transplantation methods, an understanding of the peri-insular membrane, islet-exocrine interface and general distribution of matrix proteins in the islet and surrounding periphery is required.

### 1.9.4 **Islet Transplantation: Important points of Consideration**

Expression patterns of the laminin, fibronectin and collagen family of proteins has been thoroughly examined in both human and rodent pancreas. Collagens I, IV and V are abundantly expressed in the peri-insular region of the human pancreas, while collagen V and VI expression was significant around adult islets (McShane et al., 2003). Moreover, collagen VI demonstrated consistent levels of expression in the islet-exocrine interface throughout the head, body and tail regions of the pancreas (Hughes et al., 2005). Laminin was found to be expressed in the acinar basement membrane and the surrounding ductal epithelium but no significant expression was found surrounding adult murine islets (Geutskens et al., 2004; Jiang et al., 2002). Interestingly, fibronectin expression was predominant underneath endothelial cells, in the perivascular regions, the islet periphery and intralobular regions (Cirulli et al., 2000; Geutskens et al., 2004). The less studied proteoglycan, Lumican, was found to be localized in alpha cells (Lu et al., 2002). The corresponding integrin receptors expressed in islets include α3, α5, αV and β1 (Wang et al., 1999). In fact, β1, α2 and α6 were found in the parenchyma, whereas α3 was expressed in ductal cells. The laminin receptor α6 demonstrated high levels of expression in exocrine acini, while expression of fibronectin receptors, α4 and α5, was primarily noted in the ECM surrounding ducts and vessels (Lu et al., 2002). After islet isolation, the disruption of islet-matrix relationships results in a decrease in integrin expression which may be due to perturbation of the peri-insular basement membrane (Wang et al., 1999). The critical information provided by immunohistology studies of matrix and integrin expression should be taken into consideration during islet isolation, and methods of preserving such relationships should be devised.

A recent finding by Nikolova et al (2006) demonstrated that beta cells rely on adjacent capillary endothelial cells to produce basement membrane ECM proteins (Nikolova et al., 2006). As a result, a depletion of islet capillaries demonstrated lower levels of insulin gene expression and secretory granules (Lammert et al., 2001). Developmental studies also showed that a rich vasculature is necessary for the induction of insulin in embryos, indicating that the ECM is involved in ensuring intact beta cell function (Lammert et al., 2001; Yoshitomi et al., 2004). These results indicate that the existing dynamic between endothelial and hormone producing cells which reside within the islet must also be preserved in order to ensure success of islet transplantation procedures.

From this extensive literature review, it is quite evident that integrin-ECM interactions are essential for islet cell development, survival and function. The majority of studies have focused on the role of the β1 integrin subfamily, and their regulation of islet cell adhesion, spreading, proliferation, gene expression and insulin secretion, along with their protection against cell death. Although the contributions of the β1 integrin have been extensively characterized, the role of its associated α subunits have not been thoroughly examined. In light of this, this thesis aims to elucidate the independent functions of the β1 integrin and its associated α subunits and to characterize signaling mechanisms downstream of these subunits, which regulate beta cell survival and function

## 1.10 **Objectives of present study**

The objectives of the present study were:

1) to investigate the expression patterns of αβ1 integrins on beta (INS-1) cells
2) explore how interactions between these integrins and their respective ECM proteins contribute to increased survival and function and
3) identify and characterize downstream signaling mechanisms of these αβ1 integrins in their regulation of beta (INS-1) cell survival and function

*Hypothesis:*

- interactions between α3β1 integrin and collagen I and IV matrix proteins are critical for beta (INS-1) cell survival and function.
- α3β1 integrin signals through both the PI3K and MAPK pathway to mediate beta (INS-1) cell survival and function.

***Specific Questions:***

- Which αβ1 integrins are most highly expressed in beta (INS-1) cells?
- Which matrix proteins best support the survival and function of beta (INS-1) cells?
- How do interactions between the individual subunits of the α3β1 integrin and collagen I and IV matrix proteins allow for survival and function of beta (INS-1) cells?
- Which signaling mechanisms do the individual subunits α3 and β1 integrin transduce to increase beta (INS-1) cell survival and function?

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Chapter 2

# Interactions between αβ1 Integrins and Extracellular Matrices promote INS-1 Cell Proliferation and Function

**Authors:** Krishnamurthy M., Li J., Al-Masri M., and Wang R.

All experiments in Chapter 2 were performed by Mansa Krishnamurthy with assistance from Dr. Rennian Wang. Technical support, solutions and assistance for RT-PCR and real-time RT-PCR analysis were provided by Jinming Li. Assistance for RNA extraction and RNA quality analysis was provided by Maia Al-Masri. This manuscript was prepared by Mansa Krishnamurthy and edited by Dr. Rennian Wang. Submitted to **Matrix Biology**.

## 2.1 Introduction

Pancreatic beta cells are essential for rapid and precisely controlled responses to changes in blood glucose levels. Disturbance of their function leads to diabetes mellitus, a disease characterized by hyperglycemia and several other complications. Given that diabetes affects millions of people worldwide, recent research efforts have focused on devising cell-based strategies to promote beta cell survival and function, for either organ regenerative purposes or islet transplantation.

One such research focus is the extracellular matrix (ECM) and their receptors, the integrins. In addition to controlling morphogenetic decisions during development, maintaining homeostasis and providing a physical basis for the spatial organization of cells (Thiery et al., 2003), integrin-ECM interactions influence a variety of cellular behaviours including growth, differentiation and function (Boudreau and Jones, 1999; Bouvard et al., 2001; Brakebusch and Fassler, 2005; Coppolino and Dedhar, 1999; Danen and Sonnenberg, 2003; French-Constant and Colognato, 2004; Lee and Juliano, 2004). A family of glycoproteins, integrins are composed of individual α and β subunits which non-covalently link to form distinct heterodimeric receptors (Berman et al., 2003; Boudreau and Jones., 1999; Coppolino and Dedhar, 1999; Danen and Sonnenberg, 2003; Juliano, 2004). Their unique bi-directional signaling properties enable them to integrate exterior and interior environments of a cell, and thus they influence a plethora of cellular processes including changes in migration, differentiation, proliferation and gene expression (Berman et al., 2003; Boudreau and Jones., 1999; Bouvard et al., 2001; Brakebusch and Fassler, 2005; Coppolino and Dedhar, 1999; Danen and Sonnenberg, 2003; French-Constant and Colognato, 2004; Juliano et al., 2004; Lee and Juliano, 2004).

In the field of islet cell biology, several studies have demonstrated that integrin-ECM interactions are critical for survival, differentiation and function. In isolated canine islets, disruption of integrin-ECM relationships induces some apoptosis, subsequently leading to the transdifferentiation of islets to a ductal phenotype (Wang and Rosenberg, 1999; Wang et al., 2001). However, culturing these islets on fibronectin and collagen I matrices restored islet survival (Wang and Rosenberg, 1999). Embedding isolated human adult islets in type I collagen gels increased insulin secretory responses (Montesano et al., 1983), while culturing human beta cells on bovine corneal endothelial matrix increased basal and stimulated insulin secretion levels (Kantengwa et al., 1997). Similarly, the culturing of rat islets on laminin-5 also increased insulin secretory responses (Hammer et al., 2005), while human fetal beta cells demonstrated significant increases in insulin secretion upon glucose stimulation and a reciprocal decline in insulin content when exposed to matrices vitronectin and collagen IV (Kaido et al., 2006). Taken together, these studies emphasize the importance of specific integrin-ECM relationships in potentiating beta cell survival and function.

The β1 integrin and its associated α subunits constitute the largest integrin subfamily (Brakebusch and Fassler, 2005). Particular importance has been placed on these integrins as they orchestrate the majority of changes in beta cell behaviours and heavily influence alterations in hormone expression and secretion (Bosco et al., 2000; Kantengwa et al., 1997, Kaido et al., 2004; Wang et al., 2005; Yashpal et al., 2005). We have previously demonstrated the spatial and temporal expression patterns of the β1 integrin and its associated α3, α5 and α6 subunits in the developing human fetal and pre- and post-natal rat pancreas (Wang et al., 2005; Yashpal et al., 2005). Furthermore,

integrins αvβ1 and α1β1 have been shown to facilitate human fetal beta cell adhesion, motility and insulin secretion on vitronectin and collagen IV matrices, respectively (Cirulli et al., 2000; Kaido et al., 2004). Similarly, integrins α3β1 and α6β1 increase rat and human adult beta cell adhesion and spreading, respectively, augmenting cell survival and insulin secretion (Bosco et al., 2000; Kantengwa et al., 1997).

Based on the above findings, the goal of the present study was to characterize the αβ1 integrin expression pattern in pancreatic beta cells, INS-1, and examine alterations in beta cell behaviours, including adhesion, spreading, viability, proliferation and function, upon exposure to various matrix proteins. Through immunofluorescence, qRT-PCR and western blotting, we identified seven α subunits, α1-6 and αV, of which α1 and α3-6 co-immunoprecipitated with the β1 integrin. Among these heterodimers, α3β1 was most highly expressed; thus common ligands for this integrin including fibronectin, laminin and collagen I and IV were all tested. Our results revealed that both collagen matrices increased integrin-dependent attachment and spreading and also enhanced INS-1 cell viability and proliferation, when compared to fibronectin, laminin and control. Moreover, culturing INS-1 cells on these collagen matrices enhanced insulin expression and increased basal insulin release as well as insulin secretion upon high glucose challenge. These observations provide us with a better understanding of integrin-ECM relationships which can be manipulated for improving beta cell-based therapies for the treatment of diabetes.

## 2.2 Materials and Methods

### 2.2.1 Cell Culture:

INS-1 (832/13) cells (a gift from Dr. Christopher Newgard, Duke University Medical Center, USA) were cultured in RPMI-1640 with L-glutamine containing 10% fetal bovine serum (FBS, Invitrogen, Burlington, ON, Canada), 10mmol/l HEPES (Sigma), 1mmol/L sodium pyruvate (Invitrogen) and 50 μmol/l β-mercaptoethanol (Sigma) (Jensen et al., 2001; Pederson et al., 2007)

### 2.2.2 Adhesion/Spreading Assay:

1 x $10^5$ cells were plated on 96-well tissue culture plates (Fisher Scientific, Ottawa, ON, Canada) pre-coated with 5μg/ml of either human fibronectin, human laminin (Chemicon, Temecula, CA, USA), rat tail collagen I, human collagen IV (BD Biosciences, Mississauga, ON, Canada) or control (BSA, Sigma) in serum-free medium and cultured for 3 hours. Non-adhered cells were removed by washing wells twice with phosphate buffer saline [PBS]. Six random fields were imaged per well using a Leica DMIRE2 fluorescence microscope with Openlab image software (Improvision, Lexington, MA, USA). Cells which attached and spread were counted and normalized to control groups, and expressed as fold changes. Each experiment was conducted in triplicate with at least six repeat experiments per group.

### 2.2.3 MTT Assay:

Cell viability was examined using an MTT assay (Wang et al., 2005). 1x$10^4$ cells were plated per well in 96-well tissue culture plates (Fisher Scientific) pre-coated with 5μg/ml

of either fibronectin, laminin, rat tail collagen I, collagen IV or control, in serum-free medium for 24 hours, in triplicate. Cells were then incubated in 50µl serum-free medium and 5µl of stock of 5mg/ml (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) solution for 2 hours at 37°C. Cells were harvested and lysed using 50 µl DMSO, as previously described (Li et al., 2006). Samples were assayed for absorbance at 595nm using a Multiskan® Spectrum spectrophotometer (Thermo Labsystems, Franklin, MA, USA). Each experiment was conducted in triplicate with at least six repeat experiments per group.

### 2.2.4 **RNA isolation and qRT-PCR:**

To determine the expression levels of β1 and associated α subunits as well as Pdx-1 and insulin mRNA, total RNA was extracted from cells cultured in serum containing medium or cells cultured on different matrix proteins, for 24 hours in serum free medium, in 12 well culture plates (Fisher Scientific), using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions (Li et al., 2006; Wang et al., 2005). For each RT reaction, 2µg of DNA-free RNA were used with oligo(dT) primers and Superscript reverse transcriptase. Primers used are listed in **Table 2.1**. Real-time RT-PCR analyses were performed using 0.1µg cDNA using iQ SYBR Green Supermix kit in Chromo4 Real time PCR (Bio-rad). Data were normalized to the 18S RNA subunit, with at least three repeats per experimental group (Wang et al., 2005; Yashpal et al., 2005). Relative gene expression of αβ1 integrins and the expression of Pdx-1, insulin I and insulin II was calculated using the arithmetic formula "$2^{-\Delta\Delta C}{}_{T}$" and normalized to control data (Livak and Schmittgen, 2001). Controls involved omitting reverse transcriptase or

cDNA and no amplified fragments were detected (Wang et al., 2005; Yashpal et al., 2005).

**Table 2.1:** RT-PCR and qRT-PCR primer sequence information

| Primer | Primer Pair Sequence | Accession # | Fragment Size (bp) |
|---|---|---|---|
| β1 Integrin | TGG TCA GCA GCG CAT ATC TGG A<br>GCC CAC TGC TGA CTT AGG AAT | NM_017022.1 | 367 |
| α1 Integrin | GGC CCT GGT CAC TAT TGT TA<br>CAT GAC CAC AGT TCC GTT CC | NM_030994.1 | 184 |
| α2 Integrin | AAG TAA CAT GCC AGA TTG GT<br>CTA TGA GGC TGA CCG AAT TG | XM_345156.3 | 204 |
| α3 Integrin | GAA CGA TTG TGA ACG CAT GG<br>GCC AGG GTC GAG CTG TAG GT | XM_340884.3 | 223 |
| α4 Integrin | CAG CAT TGA TGA AAG CGA AC<br>GTC ACT TCC GAC GAG CAC TC | XM_230033.4 | 235 |
| α5 Integrin | ACC AGA GCA AGA GCC GGA TA<br>TGG TTC ACC GCG AAG TAG TCA | XM_235707.4 | 289 |
| α6 Integrin | CGC GCA CAG AGG CCG TAG CA<br>CAC GTT GTC CTC GCG GGT AT | XM_215984.4 | 150 |
| αV Integrin | TGA ACC TCC AGT GGC CTT AC<br>GCG CTC TTC CCT CTA TCC AG | XM_230950.4 | 286 |
| Pdx-1 | GGC TTA ACC TAA ACG CCA CA<br>AGA GTC CCA GAG GCA GAC CT | NM_022852.3 | 175 |
| Insulin I | CCC AAG TCC CGT CGT GAA GT<br>TGA TCC ACA ATG CCA CGC TTC T | NM_019129.1 | 128 |
| Insulin II | ATG GCC CTG TGG ATC CGC TT<br>TGC CAA GGT CTG AAG GTC AC | NM_019130.1 | 243 |
| 18S | GTA ACC CGT TGA ACC CCA TTC<br>CCA TCC AAT CGG TAG TAG CG | M11188.1 | 151 |

### 2.2.5 **Immunofluorescence and Morphometric Analysis:**

To determine the expression of α subunits associated with the β1 integrin, INS-1 cells were cultured on coverslips (Fisher Scientific) coated with poly-L-lysine (Sigma). Immunofluorescence staining was carried out using the antibodies as listed in **Table 2.2**. For quantitative analysis of Pdx-1 and insulin expression and BrdU labelled cell proliferation, cells cultured on different matrix proteins for 24 hours. Three hours prior to

collection, BrdU was added to each well. Cells were harvested and fixed in 4% paraformaldehyde, embedded in 2% agarose gel and processed into tissue blocks (Wang et al., 2005). Five μm sections from experimental groups were deparaffinized and incubated with appropriately diluted primary antibodies listed in **Table 2.2**, overnight at 4°C. The secondary antibodies conjugated with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) were obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Nuclei were counterstained with DAPI. The percentage of immunoreactive cells was obtained by counting at least 500 cells per section per experimental group, with a minimum of six repeat experiments per group.

**Table 2.2:** List of antibodies/antisera used for immunofluorescence

| Primary Antibody | Dilution | Company, Location |
|---|---|---|
| Mouse anti-β1 | 1:200 | Chemicon, Temecula, CA, USA |
| Goat anti-α1 | 1:100 | Santa Cruz, Montreal, QC, Canada |
| Rabbit anti-α2 | 1:100 | Santa Cruz, Montreal, QC, Canada |
| Rabbit anti-α3 | 1:200 | Chemicon, Temecula, CA, USA |
| Mouse anti-α4 | 1:200 | Santa Cruz, Montreal, QC, Canada |
| Rabbit anti-α5 | 1:500 | Chemicon, Temecula, CA, USA |
| Rabbit anti-α6 | 1:200 | Santa Cruz, Montreal, QC, Canada |
| Rabbit anti-αV | 1:200 | Santa Cruz, Montreal, QC, Canada |
| Mouse anti-human insulin | 1:1000 | Sigma, Saint Louis, Missouri, USA |
| Rabbit anti-Pdx-1 | 1:1000 | Gift from Dr. Wright, Vanderbilt University, USA |
| Mouse anti-BrdU | 1:500 | Sigma, Saint Louis, Missouri, USA |

### 2.2.6 **Immunoprecipitation:**

INS-1 lysates were precleared by incubation with 40 μl of 50 mg/ml BSA in lysis buffer and 5 μg of β1 integrin antibody (Chemicon, Temecula, CA, USA). Lysates were incubated at 4°C for 4 hours with gentle agitation. 40 μl of Protein-G beads (Santa Cruz Biotechnologies) were added and incubated for 3 hours at 4°C with gentle agitation. The

immune complexes were pelleted and the pellets washed three times with lysis buffer (Nonidet-P40, phenylmethylsulfonyl fluoride, sodium orthovanadate [Sigma] and complete protease inhibitor cocktail tablet [Roche, Montreal, QC, Canada]) as previously described (Davis et al., 1998). After the final wash, each pellet was resuspended in 50 µl of Laemmli sample buffer containing DTT and incubated at 100°C for 6 min (Davis et al, 1998). Samples were subsequently analyzed through western blotting analysis.

### 2.2.7 **Protein extraction and Western Blotting:**

To determine the expression of αβ1 integrins, INS-1 cells were extracted by sonicating in Nonidet-P40 lysis buffer (Nonidet-P40, phenylmethylsulfonyl fluoride, sodium orthovanadate [Sigma] and complete protease inhibitor cocktail tablet [Roche]), as described previously (Wang et al., 2005), and centrifuged at 13,000 rpm (12,879 x g) for 20 mins at 4 °C. The supernatant was recovered and frozen at -80°C. Protein concentrations were measured by a protein assay using Bradford dye (Bio-Rad Laboratories, Mississauga, ON, Canada) and bovine serum albumin (fraction V) as the standard. An equal amount (50µg) of lysate proteins from each experimental group was separated by either 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). After transfer, the membranes were washed in Tris buffer-saline (TBS) containing 0.1% Tween-20 and blocked with 5% nonfat dry milk overnight at 4°C. Immunoblotting was performed with appropriate dilutions of primary antibodies as listed in **Table 2.3** for 1 hour at room temperature. Blots were subsequently washed 3 times for 5 minutes in TBS-T followed by the application of appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies. Proteins were detected using ECL™-Plus

Western blot detection reagents (Perkin Elmer, Wellesley, MA, USA) and exposed to BioMax MR Film (Kodak, Rochester, NY, USA), according to the manufacturers instructions.

**Table 2.3:** List of antibodies/antisera used for western blotting

| Primary Antibody | Dilution | Company, Location |
|---|---|---|
| Mouse anti-β1 | 1:800 | Chemicon, Temecula, CA, USA |
| Goat anti-α1 | 1:1000 | Santa Cruz, Montreal, QC, Canada |
| Rabbit anti-α3 | 1:2000 | Chemicon, Temecula, CA, USA |
| Mouse anti-α4 | 1:2000 | Santa Cruz, Montreal, QC, Canada |
| Rabbit anti-α5 | 1:2000 | Chemicon, Temecula, CA, USA |
| Rabbit anti-α6 | 1:2000 | Santa Cruz, Montreal, QC, Canada |
| Rabbit anti-αV | 1:2000 | Santa Cruz, Montreal, QC, Canada |

### 2.2.8 **Glucose-Stimulated Insulin Secretion Assay:**

INS-1 cells were cultured on different matrix proteins for 24 hours, in triplicate batches, which underwent 1 hour preincubation in prewarmed oxygenated Krebs-Ringer bicarbonate HEPES buffer containing no glucose and 0.5% BSA, followed by 1 hour in Krebs-Ringer bicarbonate HEPES buffer containing 2.2mmol/l or 22mmol/l glucose. Insulin secretion in response to glucose challenge was determined using a high range rat insulin ELISA kit (Alpco, Salem, New Hampshire, USA). Data are expressed as ng/ml/hr. Cellular insulin content was also measured, and data are expressed as ng/mg protein (Moibi et al., 2007). INS-1 cell protein content was measured by a protein assay using Bradford dye (Bio-Rad) and bovine serum albumin (fraction V) as the standard. Each experiment was conducted in triplicate with at least 3 repeat experiments per group.

2.2.9 **Statistical analysis:**

Data are expressed as means ± SEM. Statistical significance was determined using one-way ANOVA followed by Least Significant Difference (LSD) or Bonneferoni comparison tests. Differences were considered to be statistically significant when $p<0.05$.

## 2.3 Results

### 2.3.1 *Expression of αβ1 integrins in INS-1 cells*

To identify the most highly expressed αβ1 integrin in the INS-1 cell line, we first screened for several possible α subunits. Using immunofluoresence microscopy, we observed that integrin β1 and its associated subunits α1, α2, α3, α4, α5, α6 and αV are all expressed (**Fig. 2.1A**). Analysis of gene expression through qRT-PCR demonstrated that integrins α3 and β1 are most highly expressed when compared to α1, α2, α4, α5, α6, and αV (**Fig. 2.1B**). Western blotting analysis confirmed the expression of integrin subunits α1, α3, α4, α5, α6, αV and β1 (**Fig. 2.1C**), while our co-immunoprecipitation results revealed the association of α3, α4 and α6 with β1 (**Fig. 2.1D**).

### 2.3.2 *Collagen I and IV matrix proteins enhance INS-1 cell adhesion and spreading*

The most common ligands for integrin α3β1 are fibronectin, laminin, collagen I and collagen IV (Coppolino et al., 1995; Delwel et al., 1994). To test the effects of these matrices on various cellular behaviours, adhesion and spreading of these cells on fibronectin (**FN**), laminin (**L**), collagen I (**CI**), collagen IV (**CIV**) or control (**Ctrl**) (BSA blocked plastic) were examined at the 3 hr time point. INS-1 cells demonstrated marked increases in adhesion on all matrices tested in comparison to control ($p<0.01$-$0.001$, **Fig. 2.2A, Bi**). Collagen I and IV allowed for maximum cell spreading in comparison to control ($p<0.001$, **Fig. 2.2A, Bii**), although culturing on fibronectin caused a marginal increase ($p<0.05$ vs. control), while exposure to laminin failed to demonstrate any increases in cell spreading.

Figure 2.1. **αβ1 integrin expression in INS-1 cells.** (A) immunofluorescence and (B) qRT-PCR analysis (n=6 experiments/group). Scale bar: 10μm. (C) Western blotting analysis of αβ1 integrin expression in INS-1 cells (n=4-6 experiments/group). (D) Co-immunoprecipitation of integrins α3, α4 and α6 with the β1 integrin (n=4-6 experiments/group).

Fig. 2.1

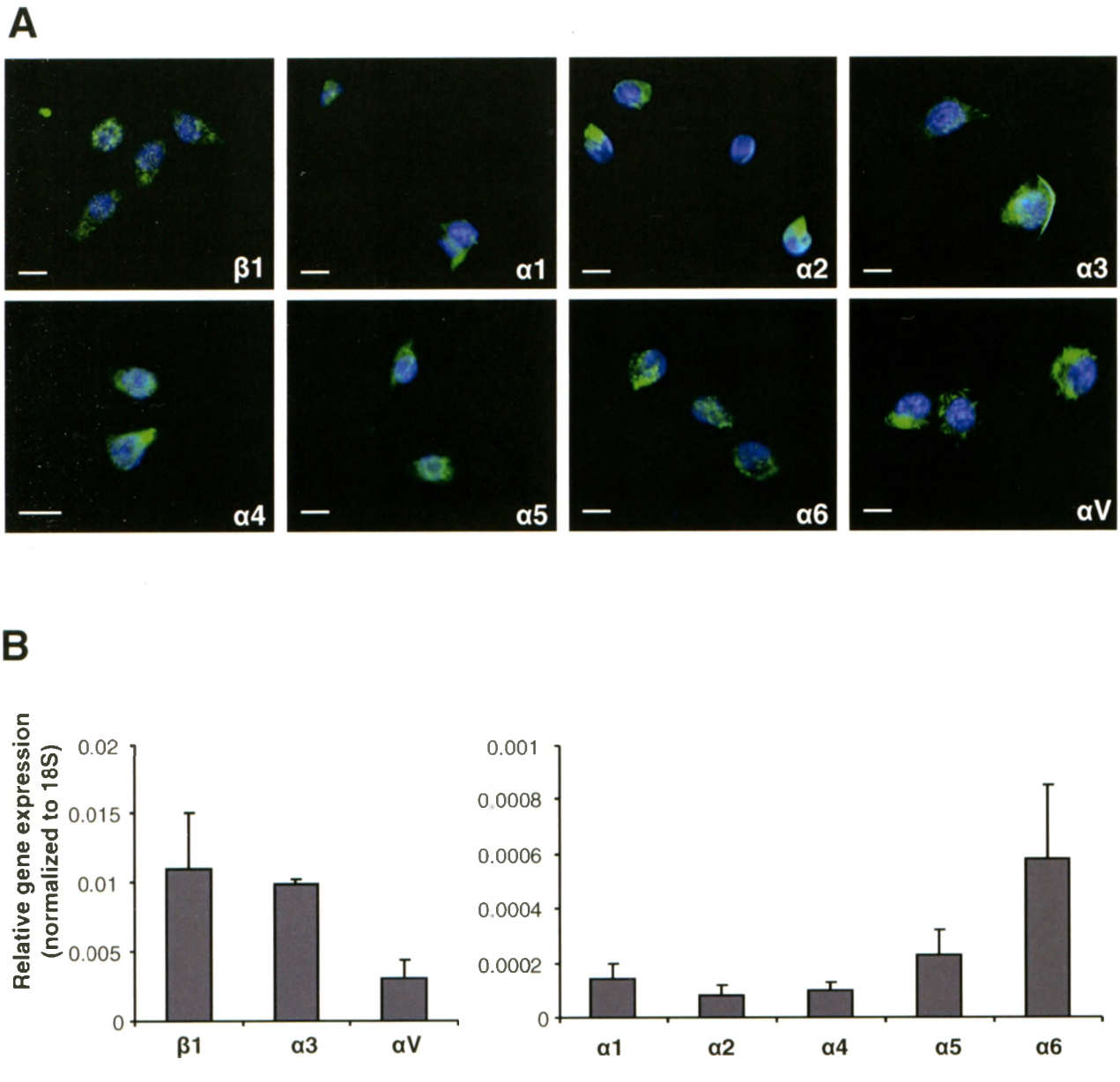
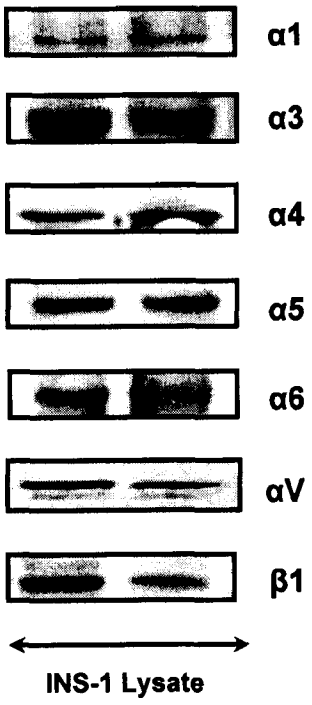

A
β1
α1
α2
α3
α4
α5
α6
αV
B
Relative gene expression (normalized to 18S)
0.02
0.015
0.01
0.005
0
β1
α3
αV
0.001
0.0008
0.0006
0.0004
0.0002
0
α1
α2
α4
α5
α6

Fig. 2.1

C


α1
α3
α4
α5
α6
αV
β1
INS-1 Lysate

D

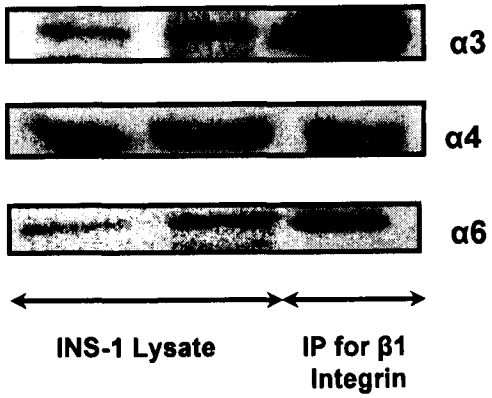
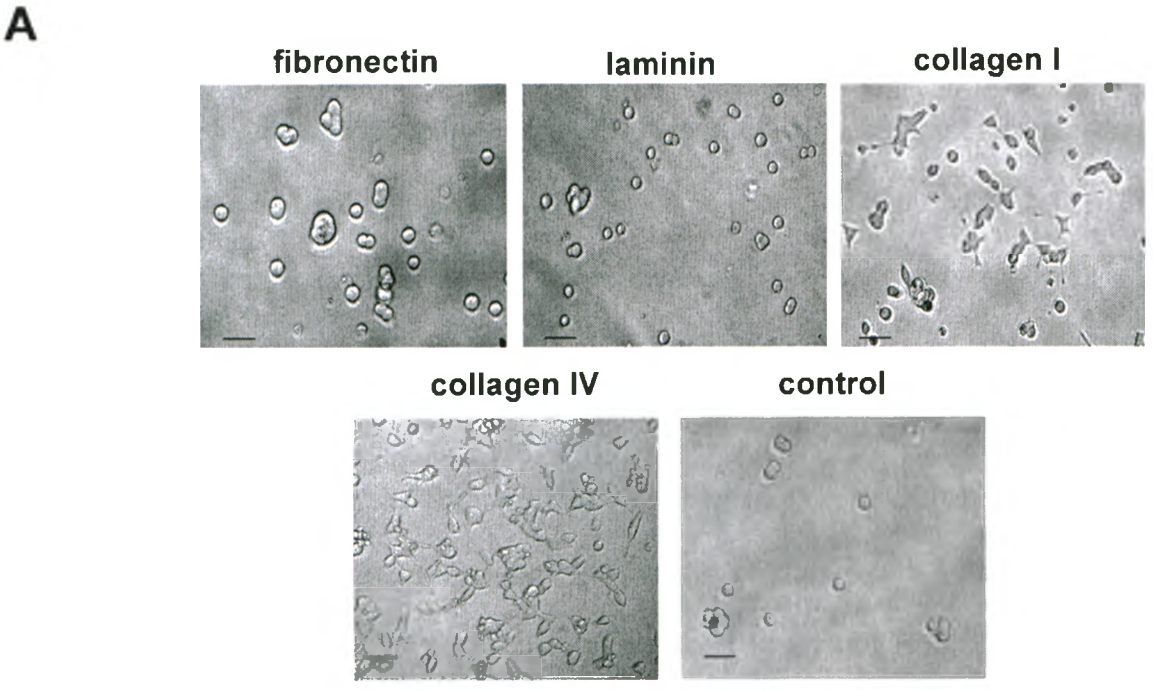

α3
α4
α6
INS-1 Lysate
IP for β1 Integrin

Figure 2.2. **Collagen I and IV enhance INS-1 cell adhesion and spreading.**

(A) Phase contrast micrographs of INS-1 cells cultured on fibronectin, laminin, collagen I and collagen IV, or BSA coated control for 24hrs. Scale bar: 40μm. (B) Fold change of i) cell adhesion and ii) spreading after 3hrs of culture. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control. (n=6 experiments/group).

Fig. 2.2


A
fibronectin
laminin
collagen I
collagen IV
control
B
i
Cell Adhesion (Fold Change)
0
1
2
3
4
***
**
***
***
*
FN
L
CI
CIV
Ctrl
ii
Cell Spreading (Fold Change)
0
1
2
3
4
*
***
***
*
FN
L
CI
CIV
Ctrl

### 2.3.3 *Collagen I and IV increase INS-1 cell survival and proliferation*

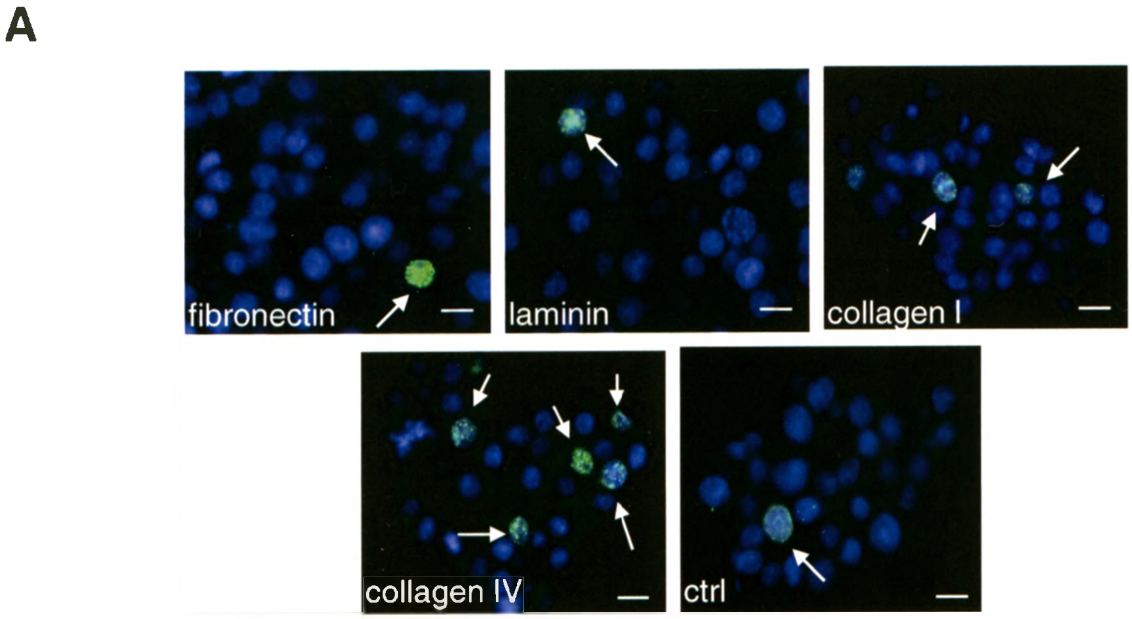
To assess changes in cell viability upon contact with fibronectin, laminin, collagen I, collagen IV or control, MTT assays were performed. Exposure to collagen I or collagen IV for 24 hours revealed remarkable increases in viability (**Fig. 2.3Bi**). However, when cultured on collagen IV, cells demonstrated the greatest increases in cell viability when compared to other matrices tested and control ($p<0.001$, **Fig. 2.3Bi**). Cells cultured on fibronectin demonstrated slight increases in cell viability ($p<0.05$), while contact with laminin demonstrated no change when compared to control (**Fig. 2.3Bi**).

To corroborate the noted alterations in cell viability, cell proliferation upon contact with fibronectin, laminin, collagen I, collagen IV or control were examined through BrdU labelling. An increase in the number of $BrdU^+$ cells was noted on both collagen I (3.5-fold) and IV (6-fold) in comparison to control (**Fig. 2.3A**), while no change was noted in the number of immuno-labelled cells on fibronectin and laminin (**Fig. 2.3Bii**). In concordance with the changes in cell viability, we also noted remarkable increases in cell proliferation on collagen IV when compared to the other matrices tested (**Fig. 2.3Bii**).

Figure 2.3. **Collagen I and IV increase INS-1 cell viability and proliferation.**

(A) Immunostaining for BrdU of INS-1 cells cultured on fibronectin, laminin, collagen I and collagen IV, as well as BSA coated control for 24 hrs. Proliferating cells are labeled green (arrows) and nuclei are counterstained with DAPI (blue). Scale bar: 10μm. (B) i) MTT assay and ii) BrdU labeling index (n=5-6 experiments/group) *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control.

Fig. 2.3


A
fibronectin
laminin
collagen I
collagen IV
ctrl
B
i
Cell Viability (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
*
***
***
***
FN
L
CI
CIV
Ctrl
ii
Cell Proliferation (Fold Change)
8
6
4
2
0
**
***
***
FN
L
CI
CIV
Ctrl

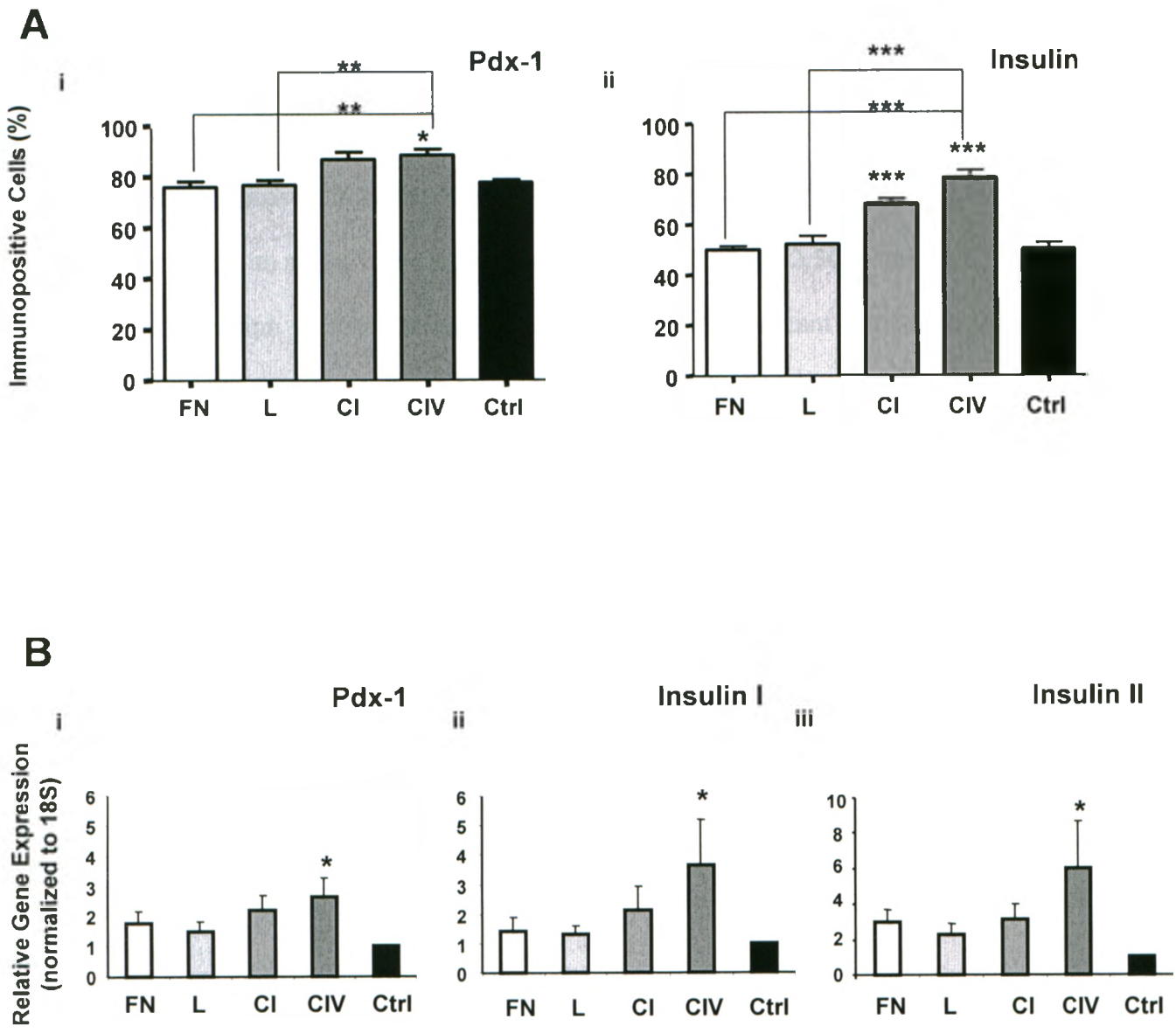
### 2.3.4 *Collagen I and IV increase Pdx-1 and insulin expression in INS-1 cells*

Pdx-1 is an essential transcription factor expressed in beta cells, responsible for maintaining phenotype and ensuring adequate insulin expression (Piper et al., 2004). Protein expression of Pdx-1 was analyzed following 24 hr culture of INS-1 cells on matrix proteins fibronectin, laminin, collagen I, collagen IV and control (**Fig. 2.4 Ai**). INS-1 cells cultured on collagen IV demonstrated increased Pdx-1 expression when compared to control ($p<0.05$), fibronectin and laminin ($p<0.01$), with no significant difference to those cultured on collagen I (**Fig. 2.4 Ai**). qRT-PCR analysis revealed with an increase in Pdx-1 expression in cells exposed to collagen IV when compared to control ($p<0.015$, **Fig. 2.4Bi**). The number of insulin$^+$ cells on both collagen I and collagen IV groups were also observed to be significantly higher than that of controls ($p<0.001$, **Fig. 2.4Aii**). However, cells cultured on collagen IV matrix showed the greatest increase in insulin expression at both the mRNA ($p<0.016$) and protein ($p<0.001$) level when compared to controls or other matrices tested (**Fig. 2.4Bii,iii** ).

Figure 2.4. **Collagen I and IV enhance Pdx-1 and insulin gene and protein expression in INS-1 cells.**

(A) Morphometric analysis of (i) Pdx-1 and (ii) insulin expression following 24 hour culture. (n=5-6 experiments/group) *$p<0.05$, ***$p<0.001$ vs. control. (B) qRT-PCR analysis of (i) Pdx-1, (ii) Insulin I and (iii) Insulin II following 24 hour culture. (n=5-6 experiments/group).

Fig. 2.4


A
i
Pdx-1
Immunopositive Cells (%)
100
80
60
40
20
0
**
**
*
FN
L
CI
CIV
Ctrl
ii
Insulin
***
***
***
***
FN
L
CI
CIV
Ctrl
B
Pdx-1
Insulin I
Insulin II
Relative Gene Expression (normalized to 18S)
i
ii
iii
6
5
4
3
2
1
0
10
8
6
4
2
*
FN
L
CI
CIV
Ctrl

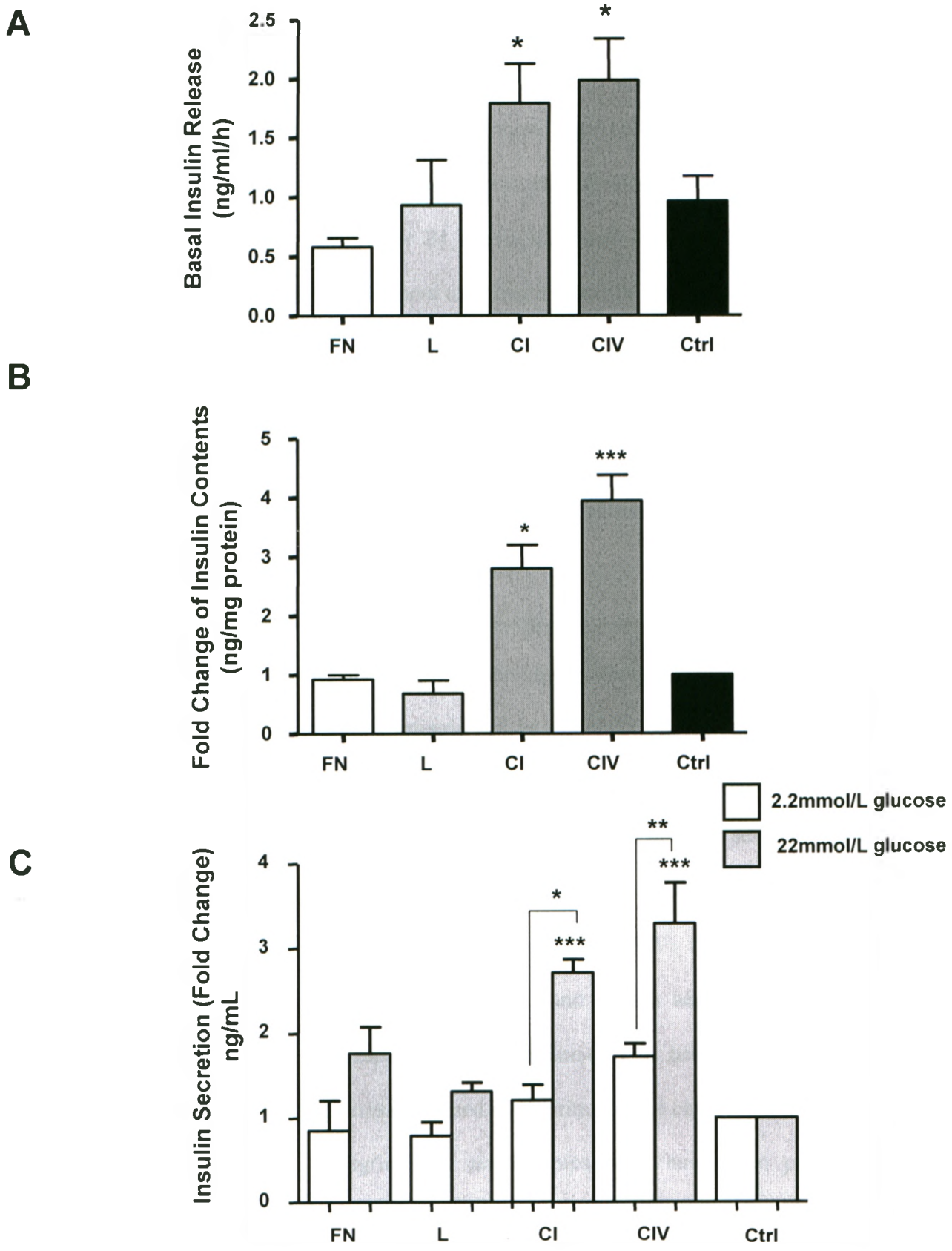
### 2.3.5 *Collagen I and IV enhance insulin secretion from INS-1 cells*

INS-1 cells were cultured on different matrix proteins for 24 hrs and assessed for basal insulin secretion levels. Cells exposed to collagen I or IV enhanced basal insulin release when compared to control ($p<0.05$, **Fig. 2.5A**), displaying a 2 and 2.3 fold increase, respectively. The observed increase in basal insulin release on both collagen I and IV was associated with an increase in cellular insulin content (**Fig. 2.5B**). Furthermore, a glucose-stimulated insulin secretion test revealed no significant change in insulin secretion at a low dose of glucose (2.2mmol/L, **Fig. 2.5C**); however, cells challenged with high glucose (22mmol/L) demonstrated a significant increase in insulin secretion on both collagen I (1.5-fold, $p<0.05$) and IV (1.6-fold, $p<0.01$) groups when compared to the corresponding matrix groups challenged with a 2.2mmol/L dose of glucose (**Fig. 2.5C**). Upon high glucose challenge, INS-1 cells demonstrated a 2.7 and 3.3 fold increase in insulin release on collagen I and IV, respectively, when compared to control ($p<0.001$, **Fig. 2.5C**). Cells cultured on fibronectin and laminin demonstrated a lower insulin secretory response to high glucose challenge (**Fig. 2.5C**).

Figure 2.5. **Collagen I and IV increase basal insulin release and enhance insulin secretion upon acute glucose challenge.**

(A) Basal insulin release, (n=6 experiments/group). (B) cellular insulin content and (C) glucose-stimulated insulin secretion from INS-1 cells cultured on fibronectin, laminin, collagen I, collagen IV or control for 24 hours. (n=3 experiments/group) *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control.

Fig. 2.5


A
Basal Insulin Release (ng/ml/h)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
FN
L
CI
CIV
Ctrl
B
Fold Change of Insulin Contents (ng/mg protein)
5
4
3
2
1
0
*
***
FN
L
CI
CIV
Ctrl
2.2mmol/L glucose
22mmol/L glucose
C
Insulin Secretion (Fold Change) ng/mL
4
3
2
1
0
*
***
**
***
FN
L
CI
CIV
Ctrl

## 2.4 Discussion

The present study characterizes the αβ1 integrin expression pattern in INS-1 cells and demonstrates that integrin-dependent attachment to collagen I and IV increase survival and function of these beta cells. The three hour culture of INS-1 cells on collagen I and IV led to remarkable increases in cell adhesion and spreading, while exposure to these collagen matrices for 24 hours, increased INS-1 cell viability and proliferation. Integrin dependent attachment to collagen I and IV also enhanced beta cell function, as increases in Pdx-1 and insulin mRNA and protein were observed. Culturing these beta cells on collagen I and IV augmented basal insulin release, and also increased insulin content and secretion upon high glucose challenge. Taken together, these findings indicate that interactions between integrin and collagen matrices are essential for the survival and function of beta cells.

The essential role of the β1 subfamily of integrins in regulating beta cell survival and function has been well established. We have previously characterized the spatial and temporal expression patterns of the β1 integrin and its associated subunits α3, α5 and α6 in the pre- and post-natal rat pancreas and during human fetal pancreatic development (Wang et al., 2005; Yashpal et al., 2005). In the field of islet cell biology, several studies have demonstrated that the α3 integrin is of particular importance for the regulation of islet cell survival and function. A study by Wang et al (1999) demonstrated high expression levels of α3 in canine, porcine, hamster and human adult isolated islets. Moreover, Kantengwa et al (1997) reported that expression of α3β1 heterodimer accounts for almost half of the total β1 integrins expressed in rat primary islet cells. Although these studies point toward critical integrin-ECM relationships, they have been primarily

conducted using a heterogenous islet cell population. In the current study, we report the direct behavioural and functional effects of integrin dependent attachment on beta cell survival and function.

Through immunohistochemical approaches and western blotting, we identified seven α subunits associated with the β1 integrin in INS-1 cells, and determined that α3β1 is most highly expressed through qRT-PCR. Pleitrophic binding of the α3β1 integrin to various matrix proteins suggests that its associations with the ECM would be highly influential on beta cell activities and function. Matrix proteins fibronectin, laminin, collagen I and collagen IV have been previously described as ligands of the integrin α3β1 (Coppolino et al., 1995; Delwel et al., 1994), and therefore INS-1 cell adhesion, spreading and proliferation on these matrices were tested. Here, we report that INS-1 cells preferentially adhere and spread on collagen I and IV in comparison to fibronectin and laminin. Although both collagen matrices augment cell viability and proliferation, exposure to collagen IV maximally increased the number of viable and BrdU-immunolabelled cells.

Functional analyses of these integrin-ECM interactions demonstrated increased insulin expression when INS-1 cells were exposed to collagen I and IV in comparison to fibronectin, laminin and control. Moreover, we note remarkable increases in insulin secretion at high doses of glucose on collagen I and IV. Although both collagen matrices enhance insulin release at low and high doses of glucose, the insulin secretory response is much higher when cells are cultured on collagen IV. In support of our findings, Kaido et al., reported that collagen IV greatly stimulates insulin release from human fetal beta cells in comparison to collagen I, fibronectin and laminin. The findings of this study,

along with our results demonstrate that attachment of beta cells to collagen matrices promote beta cell function.

The current study suggests that tight regulation of integrin-matrix interactions would be required to prevent anoikis (Greek word meaning "homelessness") and a loss of function in beta cells. The majority of studies have highlighted the importance of traditional matrix proteins found in the islet including fibronectin, collagen I and laminin. Fibronectin has been shown to aid in islet attachment following isolation, increase basal insulin release and reduce islet cell apoptosis (Wang et al., 1999). Laminin, on its own, or as part of matrix constituent, 804G, has been shown to increase beta cell spreading and greatly augment insulin release from isolated islets (Hammer et al., 2005; Kaido et al., 2006). Interactions between laminin and the $\alpha 3\beta 1$ integrin protect against cell death and increase cell attachment in several other model systems. Interestingly, fibronectin and laminin did not stimulate changes in INS-1 cell survival and function, suggesting that highly specialized integrin-ECM interactions are essential for the survival and function of this beta cell line.

The positive effects of embedding islets in collagen I gels has been well demonstrated in numerous studies (Lucas-Clerc et al., 1993; Montesano et al., 1983). Montesano et al. (1983) developed a three-dimensional culture module where dispersed islet cells were seeded on collagen I matrices and subsequently overlaid with an additional layer of collagen I. Exposure to collagen I allowed the islet cells to re-aggregate into organoids which resembled native islets. Similarly, culturing islets on collagen I increased cell adhesion, stimulated basal insulin secretory responses and reduced cell death (Wang et al., 1999). These findings are congruent with the results in

our study, which reinforce the beneficial effects of collagen I on beta cell survival and function.

Although a major basement membrane constituent, collagen IV is a less well studied matrix protein in islet cell biology. In situ, collagen IV is found in the basement membrane of intra-islet blood vessels (Kaido et al., 2004, Van Deijnen et al., 1994), and beta cells closely reside among and associate with these structures (Lammert et al., 2003). Islet cells, typically, express low levels of collagen binding integrins; however the importance of collagen IV has been demonstrated by Kaido et al (2004), who reported increased adhesion and migration of human fetal beta cells on this matrix protein. Our own studies have matched collagen IV expression in human fetal pancreatic islets with α3 integrin, suggesting that interactions between α3β1 and collagen IV have developmental and functional implications *in vivo* (Wang et al., 2005).

In summary, we characterize here the αβ1 integrin expression in the pancreatic beta (INS-1) cell line and demonstrate how integrin-dependent adhesion to various ECM constituents including fibronectin, laminin, collagen I and collagen IV regulate beta cell survival and function. Our analyses provide some insight into integrin-ECM associations which augment beta cell function, *in vitro*. The identification and manipulation of such critical factors may be important in deriving islet-based therapies for the treatment of diabetes.

## 2.5 **Acknowledgements**

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Chapter 3

# α3β1 integrin regulation of beta cell survival and function through Akt and Erk1/2 Signaling

**Authors:** Krishnamurthy M., Li J, and Wang R

All experiments in Chapter 3 were performed by Mansa Krishnamurthy with assistance of Dr. Rennian Wang. Technical support, solutions and reagents were provided by Jinming Li. This manuscript was prepared by Mansa Krishnamurthy and edited by Dr. Rennian Wang. Submitted to **Diabetes.**

## 3.1 Introduction

The extracellular matrix (ECM), a dynamic complex of peptides, hormones, growth factors and cytokines (Aszodi et al., 2006; Borojevic, 1999; Streuli 1999), is one of the most critical regulators of beta cell survival and function. The ECM interacts with the classic cell adhesion molecules, the integrins, enabling cell-cell and cell-matrix contact, thus influencing a plethora of cellular processes (Boudreau et al., 1999; Bouvard et al., 2001; Brakebusch et al., 2005; Coppolino et al., 1999; Danen et al., 2003; French-Constant et al., 2004; Juliano et al., 2004; Lee et al., 2004). Integrin receptors are a large family of heterodimeric transmembrane adhesion molecules, composed of distinct α and β subunits, which associate non-covalently (Berman et al., 2003; Boudreau et al., 1999). Unlike other receptors, integrins possess unique signaling properties which integrate the external and internal environments of a cell, allowing for dynamic co-ordination of extracellular events with intracellular changes (Hynes, 2002; Pozzi and Zent, 2003). Their bi-directional signaling mechanisms have been shown to regulate multiple beta cell behaviours including changes in cell migration, differentiation, proliferation and function (Berman et al., 2003; Boudreau and Jones., 1999; Bouvard et al., 2001; Brakebusch and Fassler, 2005; Coppolino and Dedhar, 1999; Danen and Sonnenberg, 2003; French-Constant and Colognato, 2004; Juliano et al., 2004; Lee and Juliano, 2004).

The most extensively studied integrin subunit, in the field of islet cell biology, is the β1 integrin. Its association with 12 α subunits (Bouvard et al., 2001), and its distinct ability to regulate morphogenesis (Bagutti et al, 1996; Fassler et al., 1995), cell differentiation and proliferation (Carroll et al, 1995; Streuli and Bissell, 1991), as well as cell survival (Bouvard et al., 2001) has been well documented in several studies. Integrins

levels of integrins β1 and α3 and preferentially bind to matrices collagen I and IV (Chapter 2). Based on these findings, the goal of the present study was to examine the distinct contributions of integrin α3 and β1 in the regulation of beta cell survival and function, and their downstream signaling pathways. Here, we demonstrate that the β1 integrin is a key regulator of beta cell activities, influencing adhesion, spreading, proliferation and insulin secretion, while the α3 integrin affects these beta cell behaviours and functions to much lesser extent. Examination of downstream signaling mechanisms activated by the β1 integrin was consistent with the literature: our results revealed that the β1 integrin signals primarily through the MAPK/ERK pathway to modulate changes in cell survival and function. However, the α3 integrin is capable of activating both MAPK/ERK and PI3K pathways to regulate alterations in beta cell survival and function. These results demonstrate that dynamic interactions between integrin heterodimers and ECM proteins are critical for modulating beta cell adhesion, survival and function through highly specialized signaling cascades. Increased understanding and manipulation of beta cell microenvironments may be useful for future investigations which seek to improve beta cell-based therapies for the treatment of diabetes.

## 3.2 Materials and Methods

### 3.2.1 Cell Culture and Treatments:

INS-1 (832/13) cells (a gift from Dr. Christopher Newgard, Duke University Medical Center, USA) were cultured in RPMI-1640 with L-glutamine (GIBCO, Burlington, ON, Canada), containing 10% fetal bovine serum (FBS, Invitrogen, Burlington, ON, Canada), 10mmol/l HEPES (Sigma, Sigma, St. Louis, MO, USA), 1mmol/L sodium pyruvate (Invitrogen) and 50 μmol/l β-mercaptoethanol (Sigma) (Jensen et al., 2001; Pederson et al., 2007).

*Functional Blocking β1 or α3 integrin Assays:*

INS-1 cells were incubated with either hamster anti-rat β1(CD29, 5μg/ml) [**anti-β1**], hamster IgM isotypye-matched negative control (5μg/ml, BD Biosciences, Mississauga, ON, Canada) [**IgM**] or mouse anti-rat α3 (CD49c, 10μg/ml) [**anti-α3**], mouse IgG isotypye-matched negative control (10μg/ml, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) [**IgG**], or untreated in serum free medium (**ctrl**) for 1 hour at 37°C in a tissue culture incubator at 5% $CO_2$ prior to being plated on either type I collagen (rat; 5μg/ml) or type IV collagen (BD Biosciences; 5μg/ml) coated 96 or 12-well culture plates (Fisher Scientific, Ottawa, ON, Canada).

*Pharmacological Inhibition:*

INS-1 cells were incubated with either MEK inhibitor U0126 (20μM; Promega, Madison, WI, USA), PI3K inhibitor Wortmannin (20μM; Sigma), or untreated with serum free medium (control) for 1 hour at 37°C in a tissue culture incubator at 5% $CO_2$ prior to being plated on either type I collagen (rat; 5μg/ml) or type IV collagen (BD Biosciences; 5μg/ml) coated 96 or 12-well culture plate (Fisher Scientific, Ottawa, ON, Canada).

At the end of the culture period, cells were used for either adhesion/spreading or MTT assays or harvested and processed for either protein preparation or fixed for histology studies. All culture experiments were repeated at least six times per experimental group.

### 3.2.2. **Adhesion/Spreading Assay:**

1 x $10^5$ cells were plated on 96-well tissue culture plates (Fisher Scientific, Ottawa, ON, Canada) pre-coated with 5μg/ml of either rat tail collagen I or collagen IV (BD Biosciences) in serum-free medium for 3 hours. Non-adhered cells were removed and adhered cells were thoroughly washed with Phosphate Buffer Saline (PBS). Six random fields were imaged per well using a Leica DMIRE2 fluorescence microscope with Openlab image software (Improvision, Lexington, MA, USA). Each experiment was conducted in triplicate with at least six repeat experiments per group.

### 3.2.3 **MTT Assay:**

Cell viability was examined using an MTT assay as previously described (Li et al., 2006). 1x$10^4$ cells per well were plated in 96-well tissue culture plates pre-coated with 5μg/ml of either rat tail collagen I or collagen IV (BD Biosciences) in serum-free medium for 24 hours in triplicate. Cells were then harvested in 50μl serum-free culture medium and 5μl of stock of 5mg/ml (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) solution were added for 2 hour incubation at 37°C. Cells were washed and lysed using 50 μl DMSO. Samples were assayed for absorbance at 595nm using a Multiskan® Spectrum spectrophotometer (Thermo Labsystems, Franklin, MA, USA). Each experiment was conducted in triplicate with at least six repeat experiments per group.

### 3.2.4 **Immunofluorescence and Morphometric Analysis:**

For analysis of Pdx-1, insulin and cell proliferation, after 24hr of culture, cells were embedded in 2% agarose and fixed in 4% paraformaldehyde followed by paraffin embedding. 5 μm sections from experimental groups were deparaffinized and incubated with appropriately diluted primary antibodies overnight at 4°C as listed in Table 1. Sections were then incubated with either fluorescein isothiocyanate (FITC) anti-rabbit secondary antibody or tetramethyl rhodamine isothiocyanate (TRITC) anti-mouse secondary antibody obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA) for 1 hour at room temperature. Nuclei were counterstained with DAPI. The percentage of immunoreactive cells was obtained by counting at least 500 cells per section per experimental group, with a minimum of six repeat experiments per group.

**Table 3.1:** List of antibodies/antisera used for immunofluorescence

| **Primary Antibody** | **Dilution** | **Company, Location** |
|---|---|---|
| Mouse anti-human Insulin | 1:1000 | Sigma, Saint Louis, Missouri, USA |
| Rabbit anti-Pdx-1 | 1:1000 | Gift from Dr. Wright, Vanderbilt University, USA |
| Mouse anti-BrdU | 1:500 | Sigma, Saint Louis, Missouri, USA |

### 3.2.5 **Protein extraction and Western Blotting:**

To determine the expression levels of signaling molecules, INS-1 cells from both treated and non-treated groups were harvested at 24 hours of culture in Nonidet-P40 lysis buffer (Nonidet-P40, phenylmethylsulfonyl fluoride, sodium orthovanadate [Sigma] and complete protease inhibitor cocktail tablet [Roche]), as described previously (Wang et al., 2005), and centrifuged at 13,000 rpm (12,879 x g) for 20 mins at 4 °C. The supernatant was recovered and frozen at -80°C. Protein concentrations were measured by a protein assay using Bradford dye (Bio-Rad Laboratories, Mississauga, ON, Canada) and bovine

serum albumin (fraction V) as the standard. Equal amounts (50μg) of lysate proteins from each experimental group were separated by either 5%, 7.5%, 10% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). After transfer, the membranes were washed in Tris buffer-saline (TBS) containing 0.1% Tween-20 and blocked with 5% nonfat dry milk overnight at 4°C. Immunoblotting was performed with appropriate dilutions of primary antibodies as listed in Table 2 for 1 hour at room temperature. Blots were subsequently washed 3 times for 5 minutes in TBS-T followed by the application of appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies. Proteins were detected using ECL™-Plus Western blot detection reagents (Perkin Elmer, Wellesley, MA, USA) and exposed to BioMax MR Film (Kodak, Rochester, NY, USA), according to the manufacturers instructions. Densitometric quantification of bands at subsaturation levels was performed using Syngenetool gel analysis software (Syngene, Cambridge, UK) and normalized to appropriate loading controls which included either total expression levels of signaling or housekeeping proteins. Data are expressed as the relative expression level of phosphorylated proteins to total protein levels or loading controls.

**Table 3.2:** List of antibodies used for Western Blotting

| Primary Antibody | Dilution | Company, Location |
|---|---|---|
| Mouse anti-phosphorylated Akt (Ser 473) | 1:2000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-phosphorylated Akt (Thr 308) | 1:2000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-Akt | 1:2000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-phosphorylated Thr202/Tyr204 ERK12 | 1:2000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-ERK1/2 | 1:1000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-XIAP | 1:800 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-Cleaved Caspase 3 | 1:1000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-Caspase 3 | 1:1000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-phosphorylated GSK3-β | 1:2000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-GSK3-β | 1:2000 | Cell Signaling, Danvers, MA, USA |
| Mouse anti-cyclin D1 | 1:1000 | Cell Signaling, Danvers, MA, USA |
| Rabbit anti-Pdx-1 | 1:5000 | Gift from Dr. Wright, Vanderbilt University, USA |
| Mouse anti-Calnexin | 1:2000 | BD Biosciences, Mississauga, ON, Canada |

### 3.2.6 **Glucose Stimulated Insulin Secretion Assay:**

Triplicate batches of INS-1 cells from both treated and non-treated groups underwent 1 hour preincubation in prewarmed oxygenated Krebs-Ringer bicarbonate HEPES buffer containing no glucose and 0.5% BSA, followed by 1 hour in Krebs-Ringer bicarbonate HEPES buffer containing either 2.2mmol/L or 22mmol/L glucose. The glucose-stimulated insulin secretion and cellular insulin content after stimulation were measured by high range rat insulin ELISA (Alpco, Salem, New Hampshire, USA). Cell protein content of each sample was measured by a protein assay using Bradford dye (Bio-Rad Laboratories, Mississauga, ON, Canada) and bovine serum albumin (fraction V) as the standard. A minimum of five repeats per experimental group were performed.

### 3.2.7 **Statistical analysis:**

Data are expressed as means ± SEM. Statistical significance was determined using either a two-tailed unpaired Student's t test or one-way ANOVA followed by the Bonneferoni comparison test. Differences were considered to be statistically significant when $p<0.05$.

## 3.3 Results

### 3.3.1 *β1 Integrin blockade decreases INS-1 cell adhesion and spreading on both collagen I and IV matrices.*

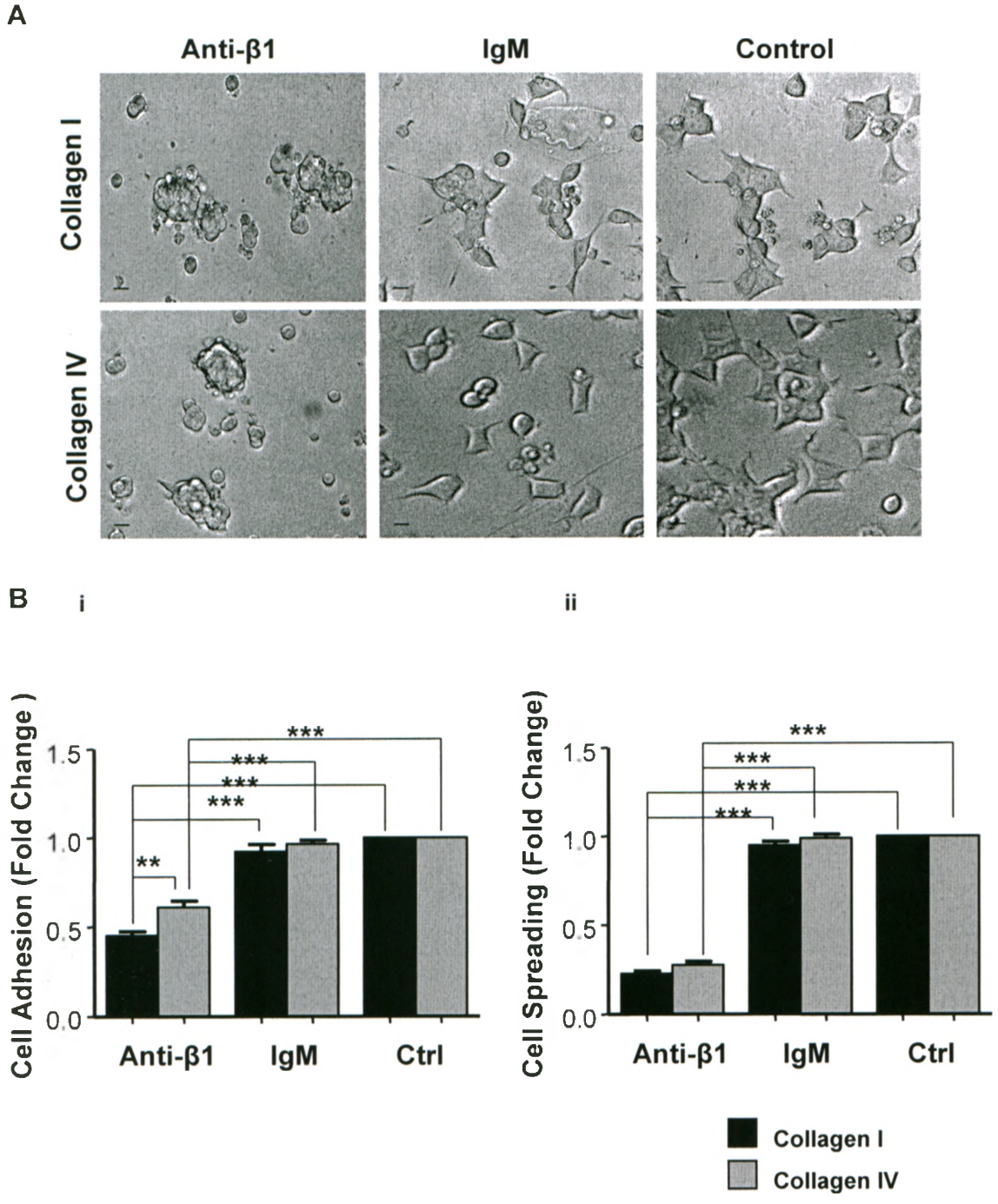
Several studies have demonstrated that functional blockade of the β1 integrin results in reduced adhesion and spreading on various matrices. INS-1 cell adhesion and spreading, on collagen I and IV, were examined upon functional blockade of the β1 integrin. Anti-β1 integrin groups loosely attached to collagen I and IV, and remained in tight spherical clusters when compared to control groups (untreated or IgM) (**Fig. 3.1A**). Quantitative analysis demonstrated a significant decrease in cell adhesion ($p<0.001$) on collagen I and IV upon β1 integrin blockade when compared to control groups (untreated or IgM) (**Fig. 3.1B**). Moreover, INS-1 cell spreading was also significantly reduced on collagen I ($p<0.001$) and collagen IV ($p<0.001$) upon anti-β1 treatment when compared to controls. Lastly, cells pre-treated with the anti-β1 integrin demonstrated increased attachment to collagen IV when compared to those cultured on collagen I ($p<0.01$) (**Fig. 3.1B**).

Figure 3.1. **β1 integrin blockade decreases INS-1 cell adhesion and spreading on collagen I and IV.**

(A) Phase contrast micrographs of INS-1 cells treated with either anti-β1 integrin or IgM or untreated and cultured on collagen I or IV for 24 hours. Scale bar: 20μm

(B) Quantitative analysis of INS-1 cell i) adhesion and ii) spreading after 3 hours of culture. (n=6 experiments/group). **$p<0.01$, ***$p<0.001$ vs. IgM or control

Fig. 3.1


A
Anti-β1
IgM
Control
Collagen I
Collagen IV
B
i
ii
Cell Adhesion (Fold Change )
Cell Spreading (Fold Change)
1.5
1.0
0.5
0.0
***
**
Anti-β1
IgM
Ctrl
Collagen I
Collagen IV

### 3.3.2 *Perturbing β1 Integrin function reduces INS-1 cell survival and proliferation on both collagen matrices.*

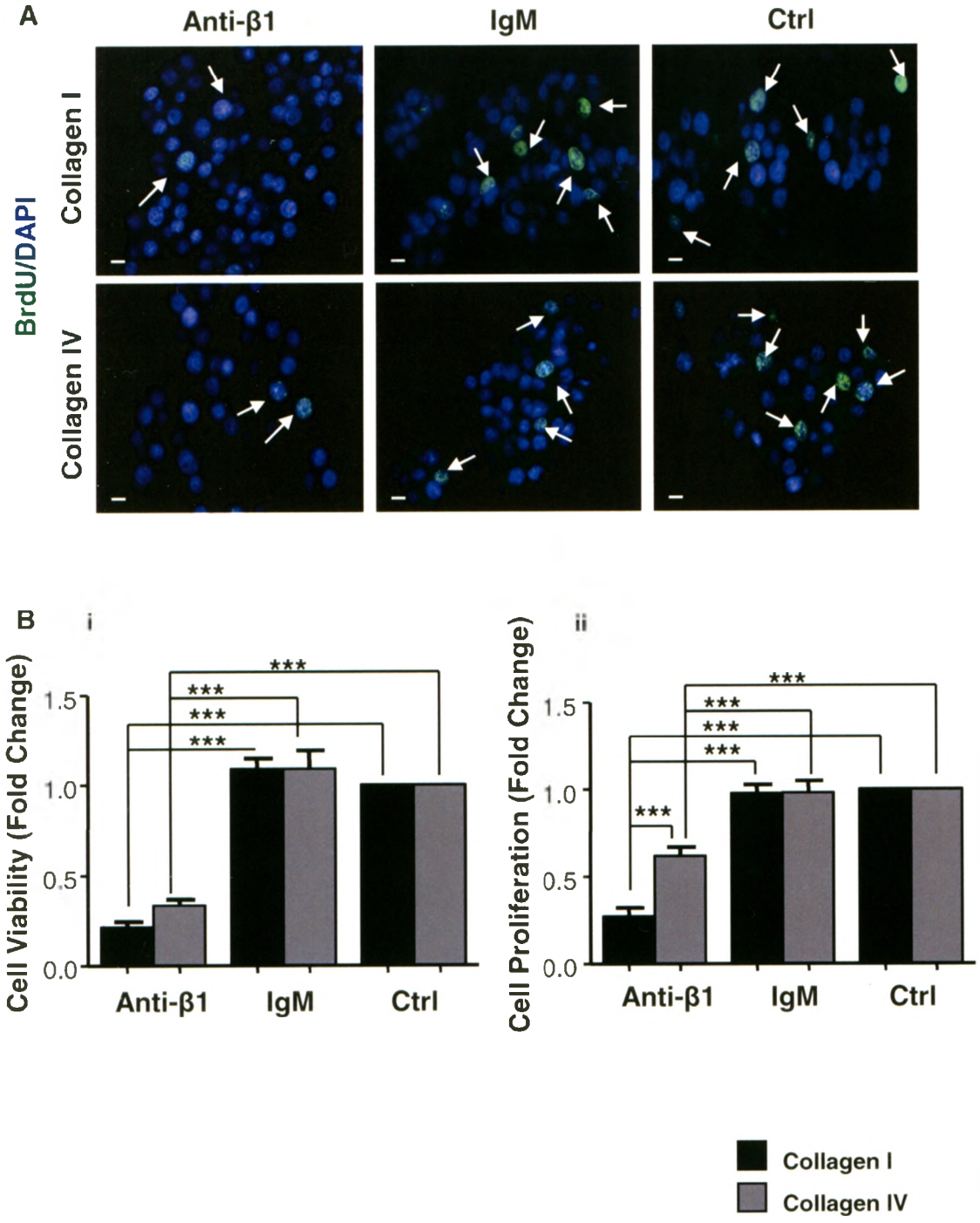
INS-1 cells treated with the anti-β1 integrin demonstrated reduced cell viability on collagen I ($p<0.001$) and collagen IV ($p<0.001$) matrices when compared to control groups (untreated and IgM) (**Fig. 3.2Bi**). To verify if the β1 integrin is a mediator of cell proliferation, BrdU expression was examined in anti-β1 and control groups (untreated and IgM) (**Fig. 3.2A**). Functional blockade of the β1 integrin reduced BrdU expression on both collagen I ($p<0.001$) and IV ($p<0.001$) when compared to controls (untreated and IgM) (**Fig. 3.2Bii**). Interestingly, fewer BrdU-immunolabelled cells were observed on collagen I when compared to collagen IV ($p<0.001$), upon anti-β1 treatment (**Fig. 3.2Bii**).

Figure 3.2. **β1 integrin blockade decreases INS-1 cell viability and proliferation on collagen I and IV.**

(A) Immunostaining for BrdU of INS-1 cells either treated with anti-β1 integrin or IgM or untreated and cultured on collagen I or IV for 24 hours. Proliferating cells are labeled green (arrows) and nuclei are counterstained with DAPI (blue). Scale bar: 10μm.

(B) (i) MTT and ii) quantitative analyses of BrdU expression after 24 hours of culture. Nuclei are counterstained with DAPI. (n=5-6 experiments/group). ***$p<0.001$ vs. IgM or control

Fig. 3.2


A
Anti-β1
IgM
Ctrl
Collagen I
Collagen IV
BrdU/DAPI
B
i
Cell Viability (Fold Change)
1.5
1.0
0.5
0.0
***
***
***
***
Anti-β1
IgM
Ctrl
ii
Cell Proliferation (Fold Change)
1.5
1.0
0.5
0.0
***
***
***
***
Anti-β1
IgM
Ctrl
Collagen I
Collagen IV

### 3.3.3 *β1 Integrin blockade affects endocrine cell protein expression on collagen I and IV*

Pdx-1 is an essential transcription factor expressed in beta cells, responsible for maintaining phenotype and ensuring adequate insulin contents (Piper et al., 2004). In order to assess changes in beta cell function, expression of Pdx-1 and insulin were analyzed upon anti-β1 integrin treatment. Morphometric analysis revealed reduced Pdx-1 expression when INS-1 cells were treated with anti-β1 integrin and subsequently cultured on collagen I ($p<0.001$) and IV ($p<0.01$), when compared to controls (untreated and IgM) (**Fig. 3.3Ai**). The observed decrease was also verified through western blotting (**Fig. 3.3B**). Although INS-1 cells also displayed reduced insulin expression upon anti-β1 integrin treatment on both collagen matrices ($p<0.001$) in comparison to control groups (untreated and IgM) (**Fig. 3.3Aii**), anti-β1 groups cultured on collagen IV demonstrated marginal increases in insulin expression when compared to corresponding collagen I groups ($p<0.05$) (**Fig. 3.3Aii**).

### 3.3.4 *β1 blockade reduces cellular insulin content and secretion from INS-1 cells on both collagen I and IV matrices.*

To assess the functional consequences of blocking the β1 integrin, cellular insulin contents and insulin release from anti-β1 integrin groups, cultured on collagen I or IV, were compared to corresponding control groups (untreated and IgM). Upon anti-β1 treatment, INS-1 cells demonstrated reduced cellular content of insulin on both collagen I ($p<0.001$) and IV ($p<0.01$) (**Fig. 3.3 C**). Insulin release following glucose stimulation was also examined. Anti-β1 integrin groups cultured on both collagen I and IV demonstrated a 4.5 fold and 2.5 fold decrease in insulin release, respectively, at a dose of 2.2mmol/L glucose (**Fig. 3.3 D**). A similar decrease in insulin secretion was also observed upon β1 integrin blockade on collagen I and IV, upon high glucose challenge, when compared to corresponding controls (IgM and untreated) (**Fig 3.3 D**).

Figure 3.3. **β1 integrin blockade decreases INS-1 cell function on collagen I and IV.**

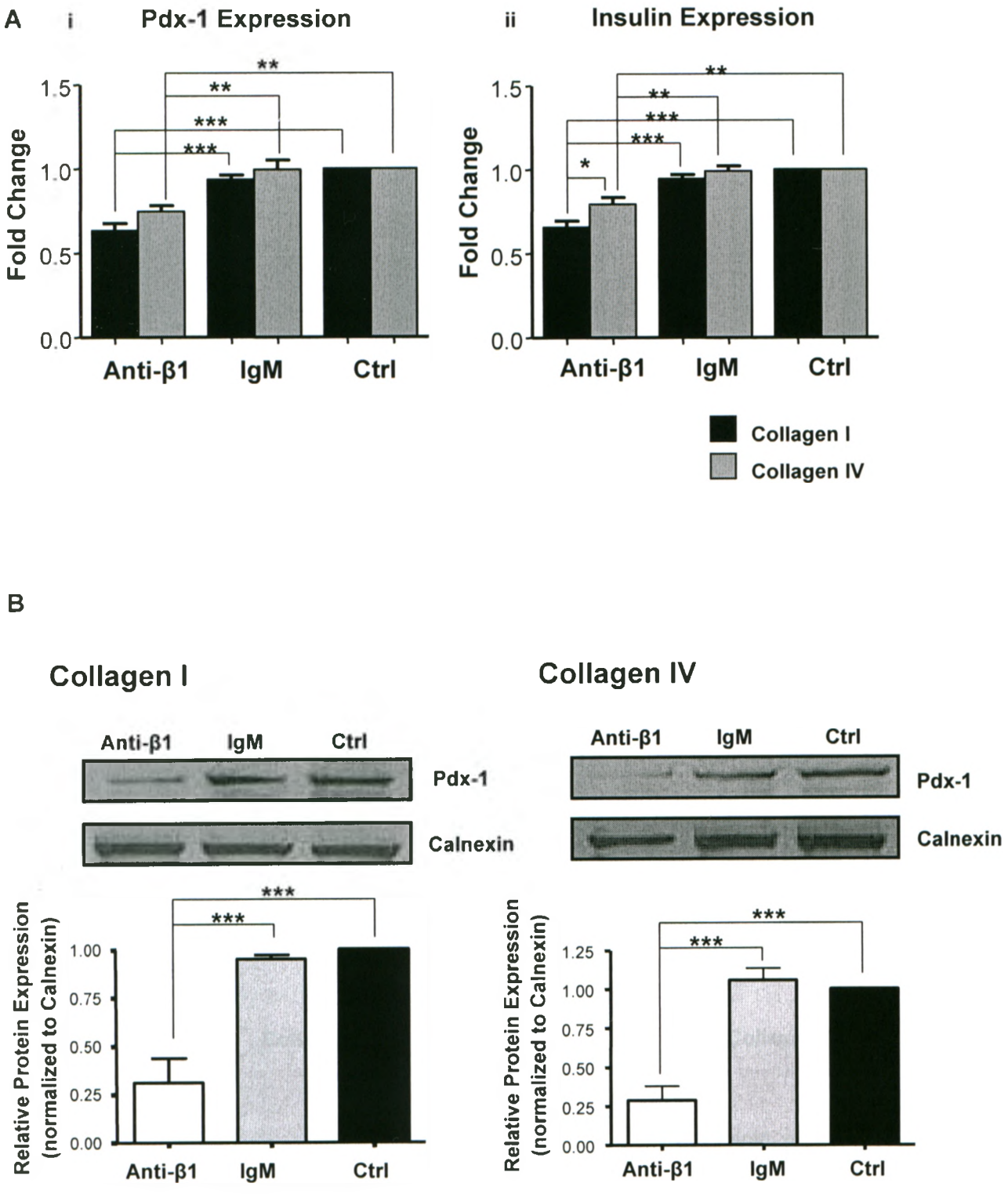
(A) Morphometric analysis of i) Pdx-1 and ii) insulin expression following 24 hours of cell culture. Nuclei are counterstained with DAPI. (n=5-6 experiments/group). *p<0.05, **p<0.01, ***p<0.001 vs. IgM or control

(B) Western blot analysis reveals reduced Pdx-1 expression on both i) collagen I and ii) collagen IV upon anti-β1 integrin treatments when compared to controls (IgM and untreated). (n=3 experiments/group) ***p<0.001 vs. IgM or control

(C) INS-1 cells demonstrate reduced cellular content of insulin (n=3 experiments/group)

(D) insulin secretion following low and high glucose challenge. (n=5 experiments/group). **p<0.01, ***p<0.001 vs. IgM or control

Fig. 3.3


A
i
Pdx-1 Expression
ii
Insulin Expression
Fold Change
1.5
1.0
0.5
0.0
**
**
***
***
*
Anti-β1
IgM
Ctrl
Collagen I
Collagen IV
B
Collagen I
Collagen IV
Anti-β1
IgM
Ctrl
Pdx-1
Calnexin
Relative Protein Expression
(normalized to Calnexin)
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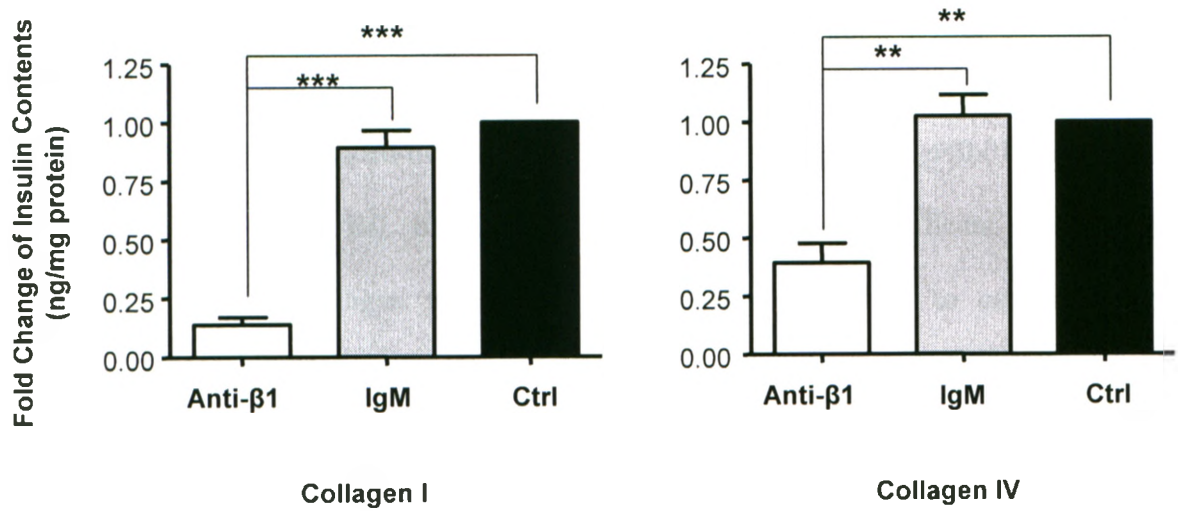
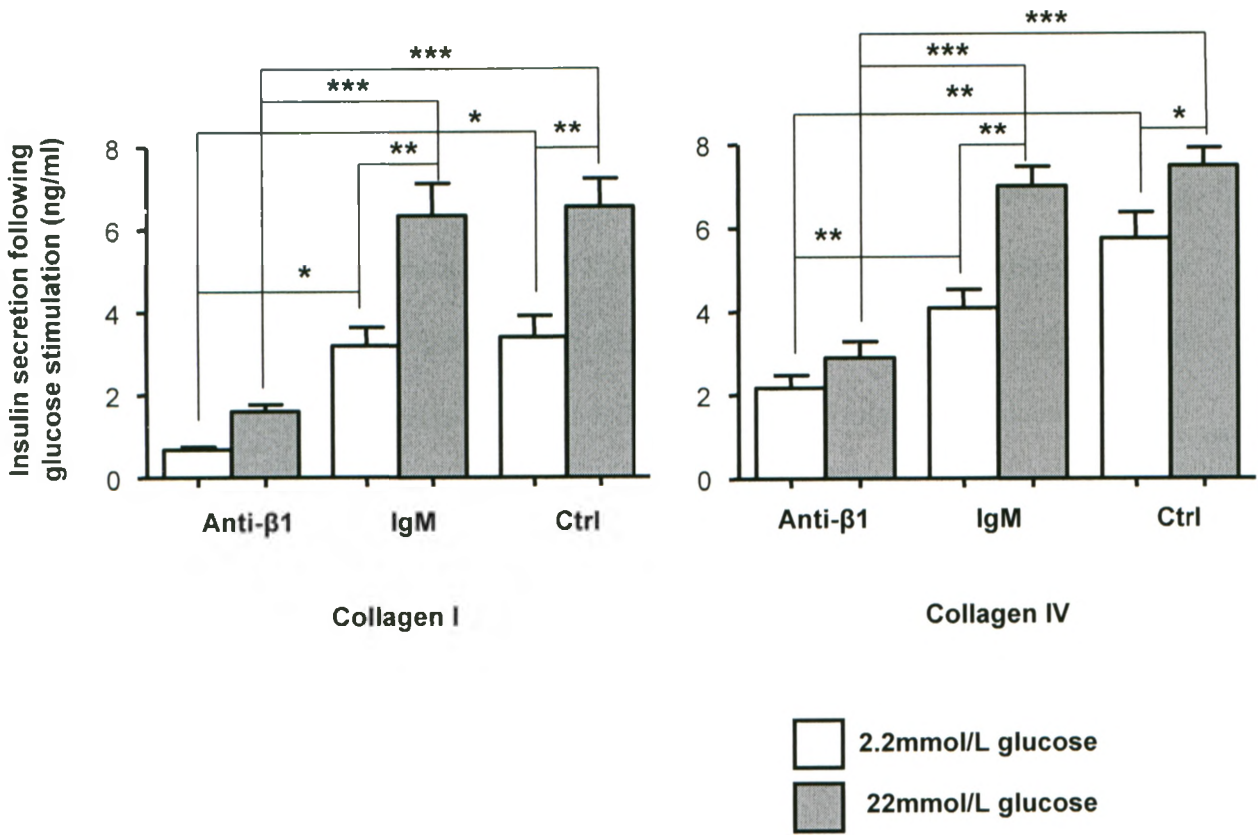
1.00
0.75
0.50
0.25
0.00

C


Fold Change of Insulin Contents (ng/mg protein)
1.25
1.00
0.75
0.50
0.25
0.00
***
***
Anti-β1
IgM
Ctrl
Collagen I
**
**
Anti-β1
IgM
Ctrl
Collagen IV

D


Insulin secretion following glucose stimulation (ng/ml)
8
6
4
2
0
***
***
*
**
**
*
Anti-β1
IgM
Ctrl
Collagen I
***
***
**
**
*
**
Anti-β1
IgM
Ctrl
Collagen IV
2.2mmol/L glucose
22mmol/L glucose

### 3.3.5 *α3 Integrin blockade decreases INS-1 cell adhesion on collagen IV and spreading on both collagen I and IV matrices.*

In order to investigate the effects of the α3 integrin on INS-1 cell morphology, cells were treated with anti-α3 integrin and cultured on collagen I or IV for 3 hours. Anti-α3 groups displayed reduced number of attachments on collagen IV ($p<0.01$), but not on collagen I (**Fig. 3.4A, Bi**). Interestingly, cells demonstrated significant decreases in spreading on both collagen matrices ($p<0.001$) when compared to control groups (untreated or IgG) (**Fig. 3.4Bii**).

### 3.3.6 *Perturbing α3 Integrin function reduces INS-1 cell survival and proliferation only on collagen IV.*

Our results demonstrated that the β1 integrin is a key mediator of INS-1 cell viability and proliferation. To better understand if the α3 subunit regulates cell viability and proliferation, MTT incorporation and BrdU expression were examined following anti-α3 treatments and subsequent cell culture on collagen I or IV. Anti-α3 groups demonstrated reduced cell viability only on collagen IV ($p<0.05$) (**Fig. 3.5Bi**), when compared to controls (untreated or IgG). INS-1 cells also displayed a reduction in cell proliferation capacity only on collagen IV ($p<0.01$) (**Fig. 3.5Bii**). Furthermore, fewer BrdU-immunolabelled cells were observed on collagen IV ($p<0.01$) upon anti-α3 treatment when compared to corresponding collagen I groups (**Fig. 5A, Bii**).

Figure 3.4. **α3 integrin blockade decreases INS-1 cell adhesion on collagen IV and spreading on both collagen matrices.**

(A) Phase contrast micrographs of INS-1 cells treated with either anti-α3 integrin or IgG or untreated and cultured on collagen I or IV. Scale bar: 30μm

(B) Quantitative analysis of INS-1 cell i) adhesion and ii) spreading after 3 hours of culture. (n=6 experiments/group). **p<0.01, ***p<0.001 vs. IgG or control

Fig. 3.4

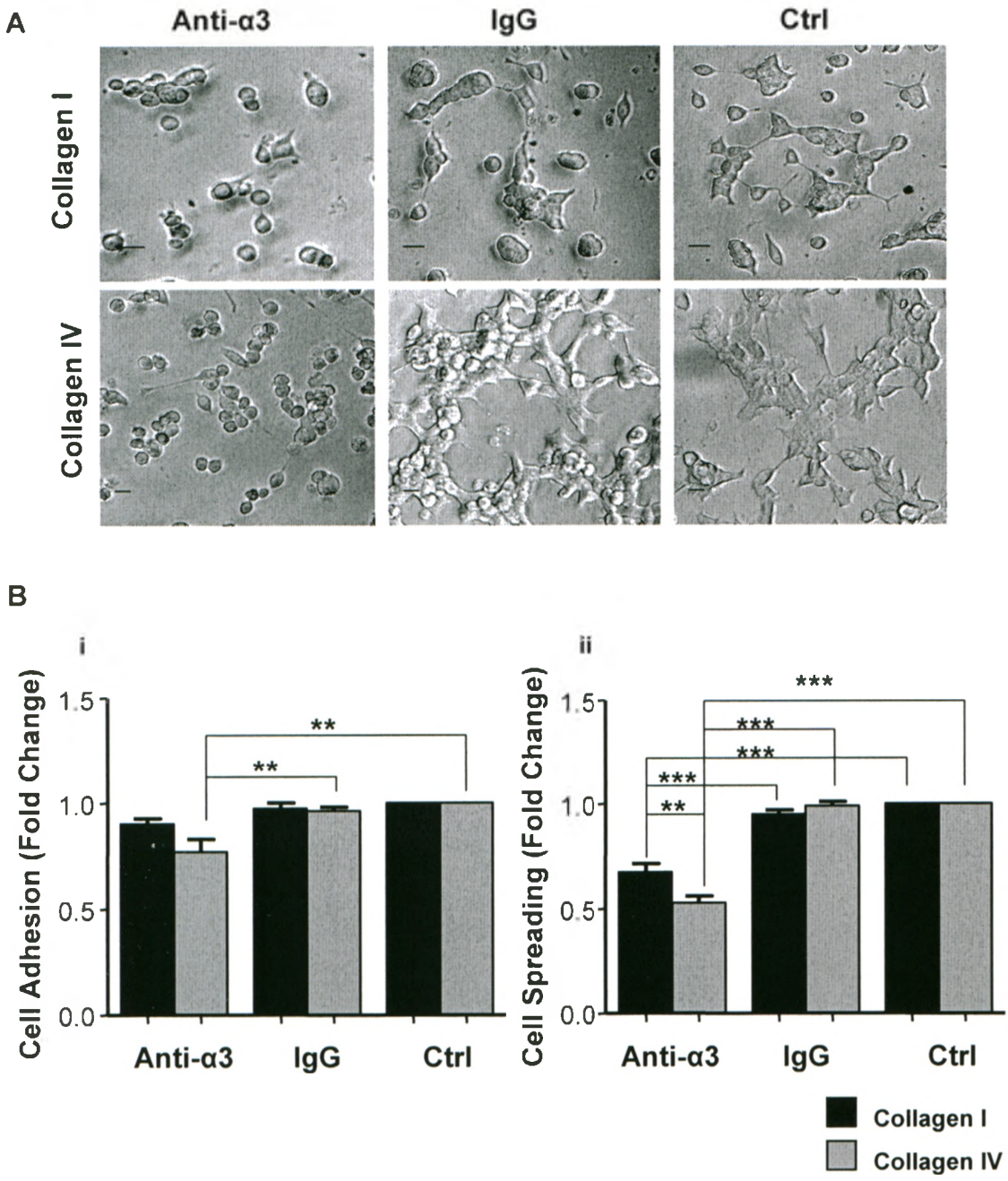
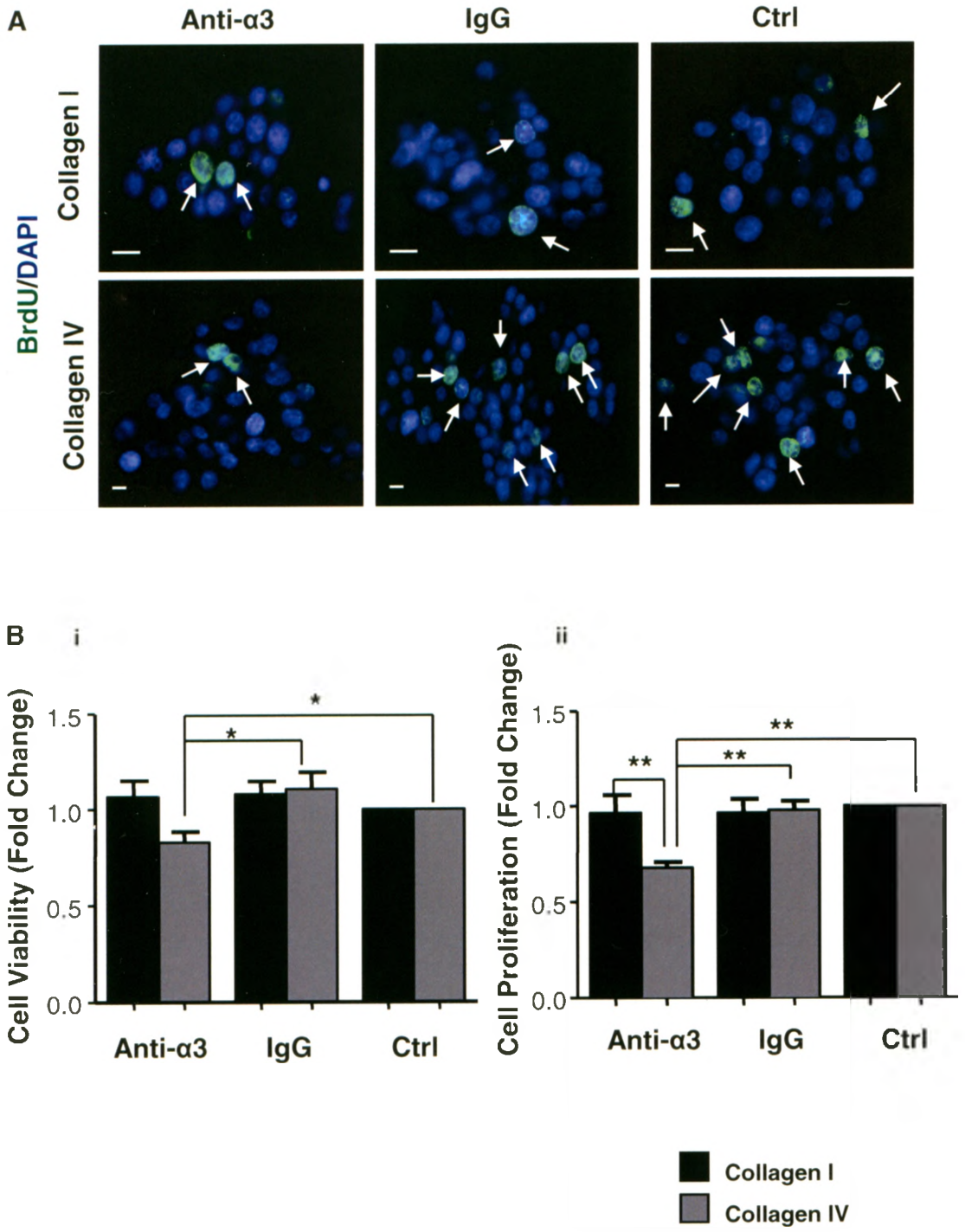

A
Anti-α3
IgG
Ctrl
Collagen I
Collagen IV
B
i
Cell Adhesion (Fold Change)
1.5
1.0
0.5
0.0
**
**
Anti-α3
IgG
Ctrl
ii
Cell Spreading (Fold Change)
1.5
1.0
0.5
0.0
***
***
***
***
**
Anti-α3
IgG
Ctrl
Collagen I
Collagen IV

Figure 3.5. **α3 integrin blockade decreases INS-1 cell viability and proliferation on collagen IV.**

(A) Immunostaining for BrdU of INS-1 cells either treated with anti-α3 integrin or IgG or untreated and cultured on collagen I or IV for 24 hours. Proliferating cells are labeled green (arrows) and nuclei are counterstained with DAPI (blue). Scale bar: 10μm.

(B) (i) MTT and ii) quantitative analyses of BrdU expression after 24 hours of culture. Nuclei are counterstained with DAPI. (n=5-6 experiments/group). *$p<0.05$, **$p<0.01$ vs. IgG or control.

Fig. 3.5


A
Anti-α3
IgG
Ctrl
Collagen I
Collagen IV
BrdU/DAPI
B
i
Cell Viability (Fold Change)
1.5
1.0
0.5
0.0
*
*
Anti-α3
IgG
Ctrl
ii
Cell Proliferation (Fold Change)
1.5
1.0
0.5
0.0
**
**
**
Anti-α3
IgG
Ctrl
Collagen I
Collagen IV

### 3.3.7 *α3 Integrin blockade affects endocrine cell protein expression primarily on collagen IV.*

To assess the role of the α3 integrin in beta cell function, Pdx-1 and insulin expression were examined in anti-α3 integrin and control groups (untreated or IgG). Morphometric analysis following the blockade of α3 integrin function, resulted in reduced Pdx-1 expression only on collagen IV ($p<0.05$) when compared to control groups (untreated of IgG) (**Fig. 3.6Ai**), while our western blotting analyses revealed a significant decrease in Pdx-1 expression on both collagen I ($p<0.05$) and IV ($p<0.01$) (**Fig. 3.6B**). Lastly, reduced insulin expression was observed on both collagen I ($p<0.05$) and IV ($p<0.001$) upon anti-α3 treatments in comparison to controls (untreated or IgG) (**Fig. 3.6C**).

### 3.3.8 *α3 blockade reduces cellular insulin content only on collagen IV and release from INS-1 cells on collagen I and IV upon high glucose challenge.*

Blockade of the β1 integrin resulted in significant decreases in cellular insulin contents and insulin release upon glucose stimulation. To better understand the functional consequences of perturbing α3 function, anti-α3 integrin and control groups (untreated or IgG) were analyzed for cellular insulin content and insulin release upon low and high glucose challenge. Upon anti-α3 treatment, INS-1 cells demonstrated decreases in cellular insulin content only on collagen IV ($p<0.001$), when compared to controls (IgM and untreated) (**Fig. 3.6C**). Anti-α3 groups demonstrated decreases in insulin secretion only upon high glucose challenge, revealing a 2.5 fold and 4.8 fold reduction in insulin release on collagen I and IV respectively (**Fig. 3.6 D**).

Figure 3.6. **α3 integrin blockade decreases INS-1 cell function primarily on collagen IV.**

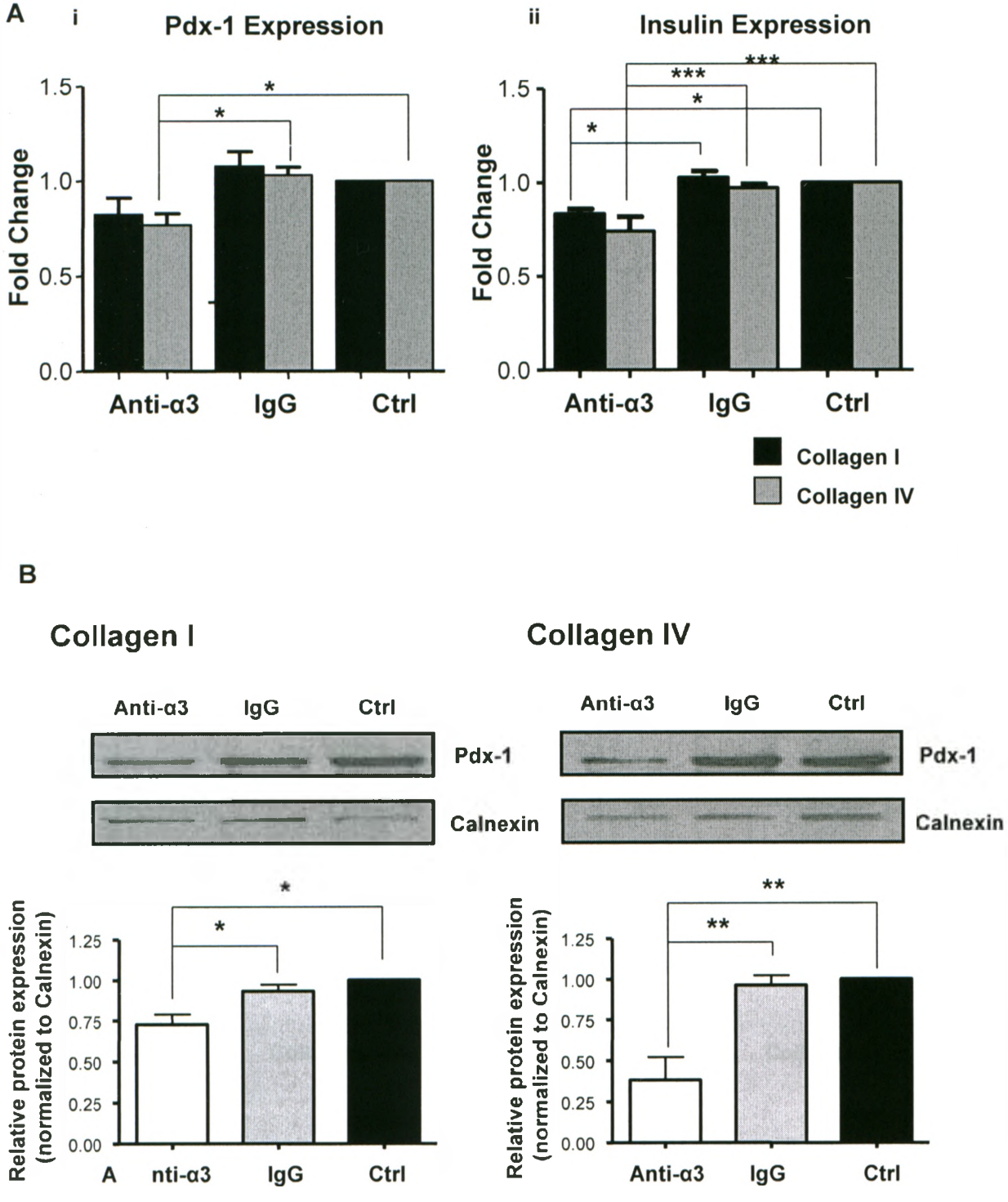
(A) Morphometric analysis of i) Pdx-1 and ii) insulin expression following 24 hours of cell culture. (n=5-6 experiments/group). *$p<0.05$, ***$p<0.001$ vs. IgG or control.

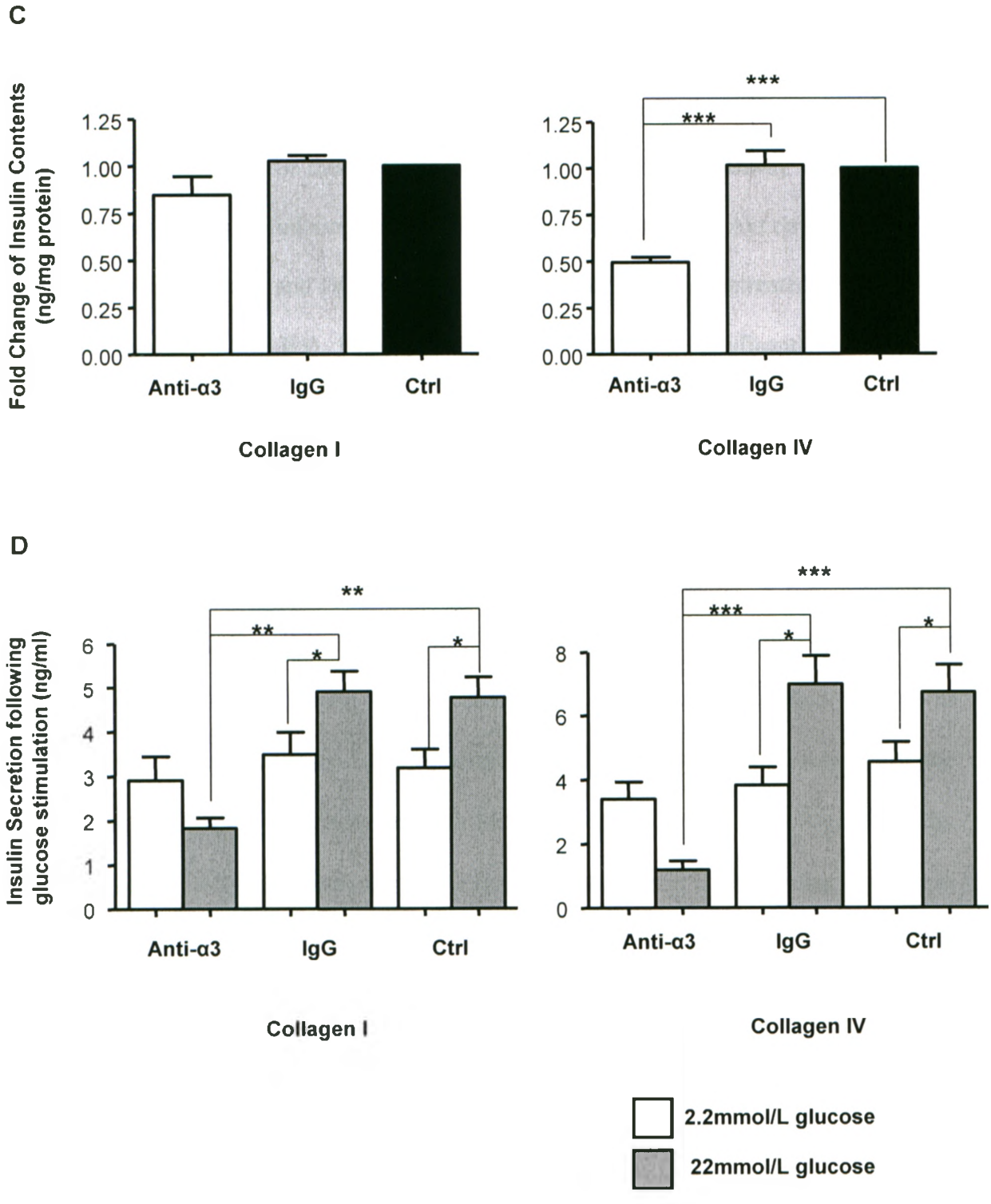
(B) Western blot analysis reveals reduced Pdx-1 expression on both collagen I and collagen IV upon anti-α3 integrin treatments when compared to controls (IgM and untreated). (n=3 experiments/group). *$p<0.01$, **$p<0.05$ vs. IgG or control

(C) INS-1 cells demonstrate reduced insulin content only on collagen IV. (n=3 experiments/group) ***$p<0.001$ vs. IgG or control.

(D) Insulin secretion upon high glucose challenge on both collagen I and IV. (n=3 experiments/group). **$p<0.01$, ***$p<0.001$ vs. IgG or control. *$p<0.05$ vs. 22mmol/L glucose groups.

Fig. 3.6


A
i
Pdx-1 Expression
Fold Change
1.5
1.0
0.5
0.0
*
*
Anti-α3
IgG
Ctrl
ii
Insulin Expression
***
***
*
*
Collagen I
Collagen IV
B
Collagen I
Collagen IV
Anti-α3
IgG
Ctrl
Pdx-1
Calnexin
Relative protein expression
(normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
**
Anti-α3
IgG
Ctrl


C
Fold Change of Insulin Contents (ng/mg protein)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-α3
IgG
Ctrl
Collagen I
***
***
Collagen IV
D
Insulin Secretion following glucose stimulation (ng/ml)
**
**
*
*
6
5
4
3
2
1
0
Collagen I
***
***
*
*
8
6
4
2
0
Collagen IV
2.2mmol/L glucose
22mmol/L glucose

### 3.3.9 *Perturbing α3 integrin function decreases activation of Akt and GSK3-β and reduces XIAP expression.*

Our results suggest that integrin heterodimers α3 and β1 can independently mediate INS-1 cell survival and function. To characterize downstream signaling pathways responsible for these effects, alterations in the PI3K pathway were explored. Perturbing β1 integrin function did not alter the phosphorylation of Akt on Ser 473 or Thr 308 on both collagen I and IV when compared to control groups (untreated or IgM) (**Fig. 3.7Ai, B i, Fig. 3.8Ai, B i**). However, α3 integrin blockade significantly reduced the phosphorylation of Akt on both sites when compared to controls (untreated or IgG) (**Fig. 3.7Aii, Bii, 3.8Aii, Bii**). Downstream mediators of the PI3K pathway including GSK3-β and XIAP were also examined. Perturbing β1 integrin function revealed no changes in the expression of XIAP (**Fig. 3.9Ai, Bi**) or the phosphorylation of GSK3-β at Ser 9 (**Fig. 3.10Ai, Bi**), on both collagen I and IV, when compared to control groups (untreated or IgM). Blockade of α3 integrin function demonstrated a 3.5 fold and 3 fold decrease in XIAP expression on collagen I and IV, respectively (**Fig. 3.9Aii, Bii**). Interestingly, the reduction in GSK3-β phosphorylation on Ser 9 was only observed on collagen IV (**Fig. 3.10Aii, Bii**). Lastly, examination of cell cycle regulator Cyclin D1, following β1 integrin blockade, demonstrated reduced expression levels on both collagen I and IV (**Fig. 3.11Ai, Bi**), while perturbing α3 integrin function reduced Cyclin D1 expression only on collagen IV (**Fig. 3.11 Aii, Bii**).

Figure 3.7. **α3 integrin blockade decreases Akt phosphorylation at Ser 473.**

(A) i. Perturbing β1 integrin function reveals no change in the phosphorylation of Akt at Ser 473, in comparison with control groups (IgM and untreated) on collagen I. (n=3 experiments/group).

(A) ii. Blockade of the α3 integrin reveals reduced Akt phosphorylation at Ser 473 on collagen I, when compared to control groups (IgG and untreated). (n=3 experiments/group). ***$p<0.001$ vs. IgG or control.

(B) i. Blocking β1 integrin function does not alter phosphorylation levels of Akt at Ser 473 on collagen IV, in comaprison with control groups (IgM and untreated). (n=3 experiments/group).

(B) ii. α3 integrin blockade reduces Akt phosphorylation at Ser 473, in comparison with control groups (IgG and untreated) on collagen IV. (n=3 experiments/group). ***$p<0.001$ vs. IgG or control.

Fig. 3.7

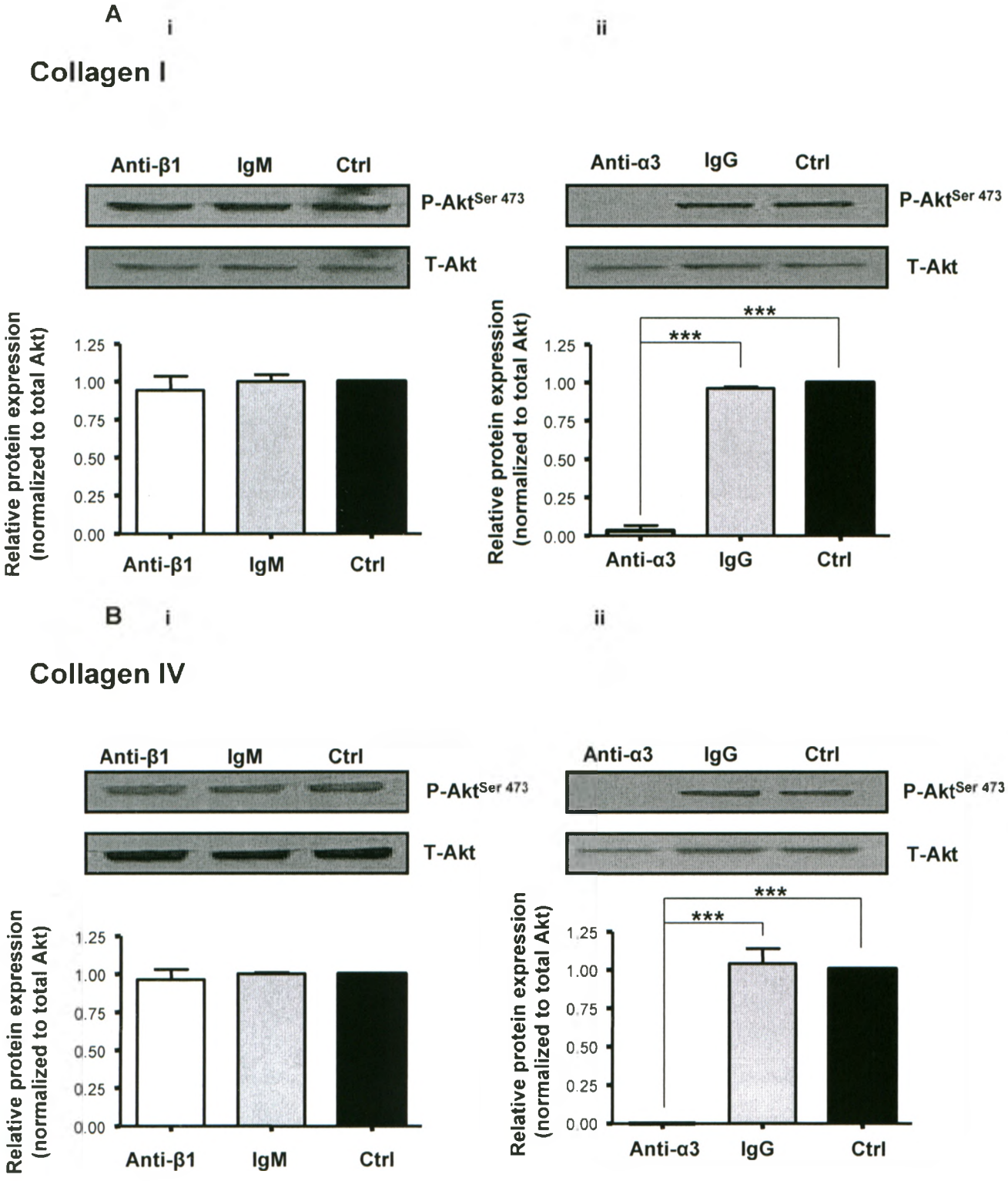

A
i
ii
Collagen I
Anti-β1
IgM
Ctrl
P-Akt Ser 473
T-Akt
Relative protein expression (normalized to total Akt)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-β1
IgM
Ctrl
Anti-α3
IgG
Ctrl
***
***
B
i
ii
Collagen IV

Figure 3.8. **α3 integrin blockade decreases Akt phosphorylation at Thr 308.**

(A) i. Perturbing β1 integrin function reveals no change in the phosphorylation of Akt at Thr 308, in comparison with control groups (IgM and untreated) on collagen I. (n=3 experiments/group).

(A) ii. Blockade of the α3 integrin reveals reduced Akt phosphorylation at Thr 308 on collagen I, when compared to control groups (IgG and untreated). (n=3 experiments/group). ***$p<0.001$ vs. IgG or control.

(B) i. Blocking β1 integrin function does not alter phosphorylation levels of Akt at Thr 308 on collagen IV, in comaprison with control groups (IgM and untreated). (n=3 experiments/group).

(B) ii. α3 integrin blockade reduces Akt phosphorylation at Thr 308, in comparison with control groups (IgG and untreated) on collagen IV. (n=3 experiments/group). *$p<0.01$ vs. IgG or control.

Fig. 3.8

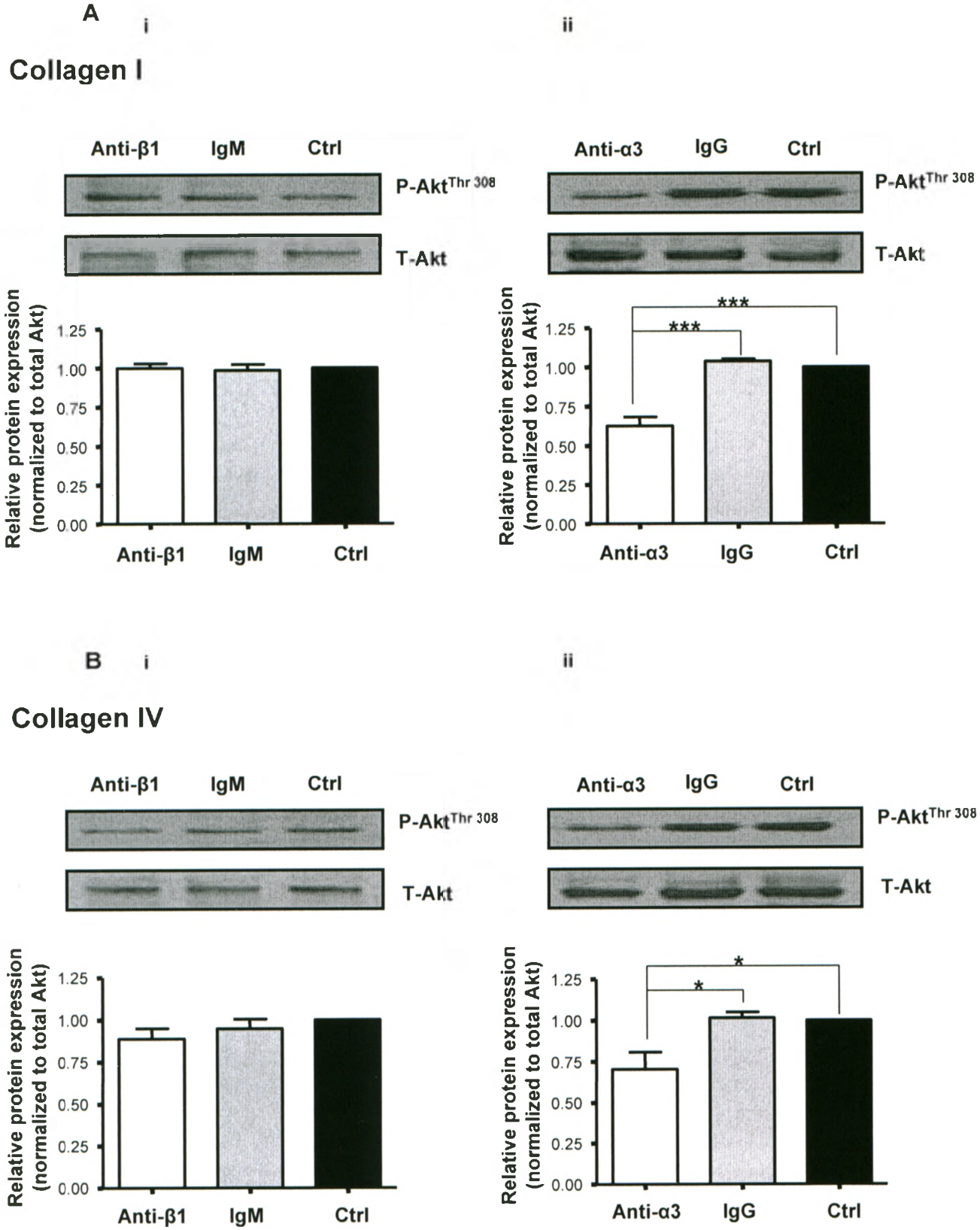

A
i
ii
Collagen I
Anti-β1
IgM
Ctrl
P-Akt Thr 308
T-Akt
Relative protein expression (normalized to total Akt)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-β1
IgM
Ctrl
Anti-α3
IgG
Ctrl
***
***
B
i
ii
Collagen IV
*
*

Figure 3.10. **Blockade of the α3 integrin decreases GSK3-β phosphorylation at Ser 9.**

(A) i. β1 integrin blockade does not alter the phosphorylation of GSK3-β in comparison with control groups (IgM and untreated) on collagen I. (n=3 experiments/group).

(A) ii. Blockade of the α3 integrin does not decrease GSK3-β phosphorylation on collagen I, when compared to control groups (IgG and untreated). (n=3 experiments/group).

(B) i. Blocking β1 integrin function reveals no change in GSK3-β phosphorylation on collagen IV, in comaprison with control groups (IgM and untreated). (n=3 experiments/group).

(B) ii. Functional blockade of the α3 integrin reduces GSK3-β phosphorylation, in comparison with control groups (IgG and untreated) on collagen IV. (n=3 experiments/group). *$p<0.05$ vs. IgG or control.

Figure 3.9. **Blockade of the α3 integrin reduces XIAP expression.**

(A) i. Functional blockade of the β1 integrin function reveals no change in XIAP expression, in comparison with control groups (IgM and untreated) on collagen I. (n=3 experiments/group).

(A) ii. Blockade of the α3 integrin decreases XIAP expression on collagen I, when compared to control groups (IgG and untreated) (n=3 experiments/group). ***$p<0.001$ vs. IgG or control.

(B) i. Blocking β1 integrin function does not alter XIAP expression levels on collagen IV, in comaprison with control groups (IgM and untreated). (n=3 experiments/group).

(B) ii. α3 integrin blockade reduces XIAP expression, in comparison with control groups (IgG and untreated) on collagen IV. (n=3 experiments/group). **$p<0.01$, ***$p<0.001$ vs. IgG or control.

Fig. 3.9

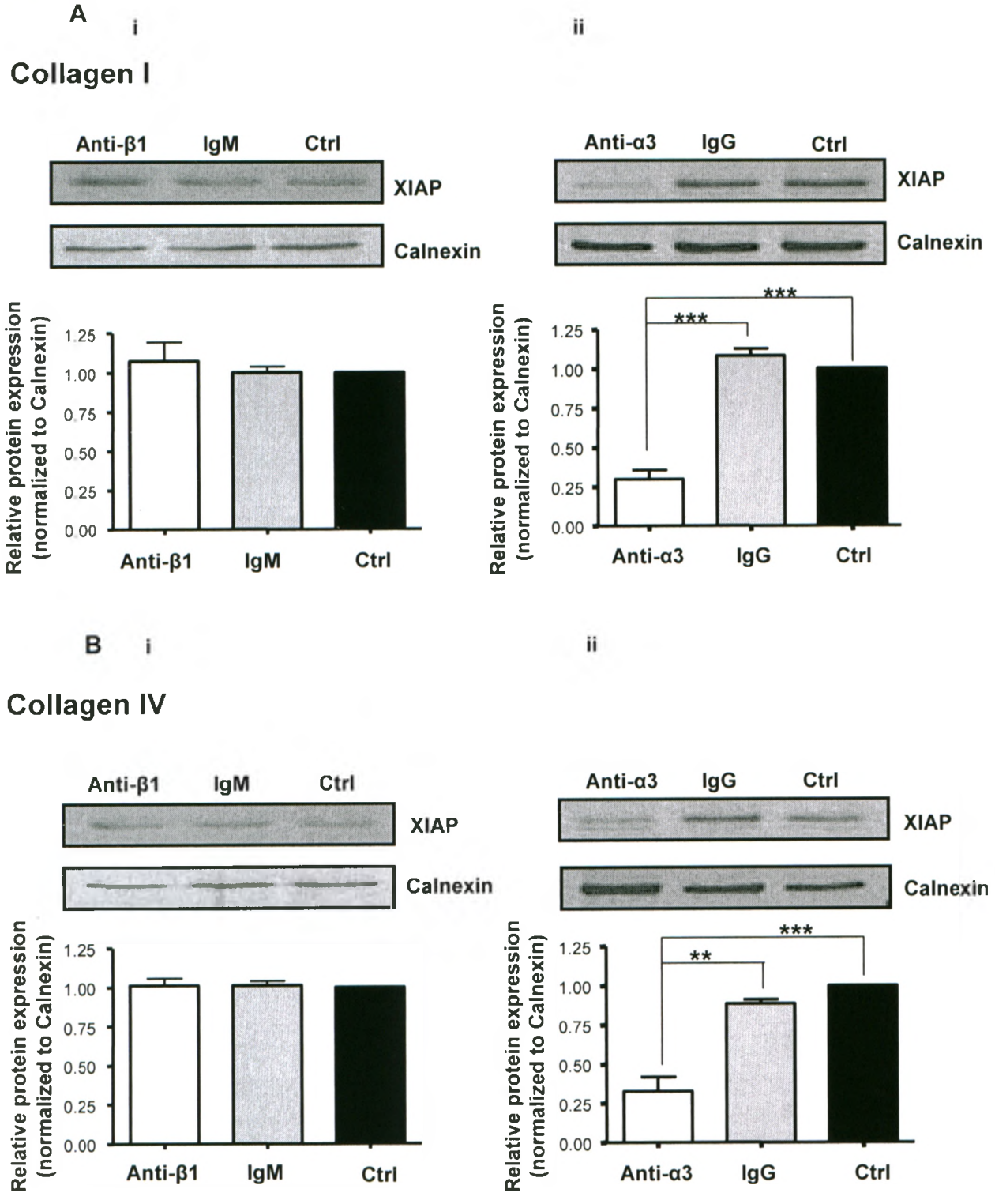

A
i
ii
Collagen I
Anti-β1
IgM
Ctrl
XIAP
Calnexin
Anti-α3
IgG
Ctrl
XIAP
Calnexin
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-β1
IgM
Ctrl
***
***
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-α3
IgG
Ctrl
B
i
ii
Collagen IV
Anti-β1
IgM
Ctrl
XIAP
Calnexin
Anti-α3
IgG
Ctrl
XIAP
Calnexin
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-β1
IgM
Ctrl
**
***
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-α3
IgG
Ctrl

Figure 3.10. **Blockade of the α3 integrin decreases GSK3-β phosphorylation at Ser 9.**

(A) i. β1 integrin blockade does not alter the phosphorylation of GSK3-β in comparison with control groups (IgM and untreated) on collagen I. (n=3 experiments/group).

(A) ii. Blockade of the α3 integrin does not decrease GSK3-β phosphorylation on collagen I, when compared to control groups (IgG and untreated). (n=3 experiments/group).

(B) i. Blocking β1 integrin function reveals no change in GSK3-β phosphorylation on collagen IV, in comaprison with control groups (IgM and untreated). (n=3 experiments/group).

(B) ii. Functional blockade of the α3 integrin reduces GSK3-β phosphorylation, in comparison with control groups (IgG and untreated) on collagen IV. (n=3 experiments/group). *$p<0.05$ vs. IgG or control.

Fig. 3.10

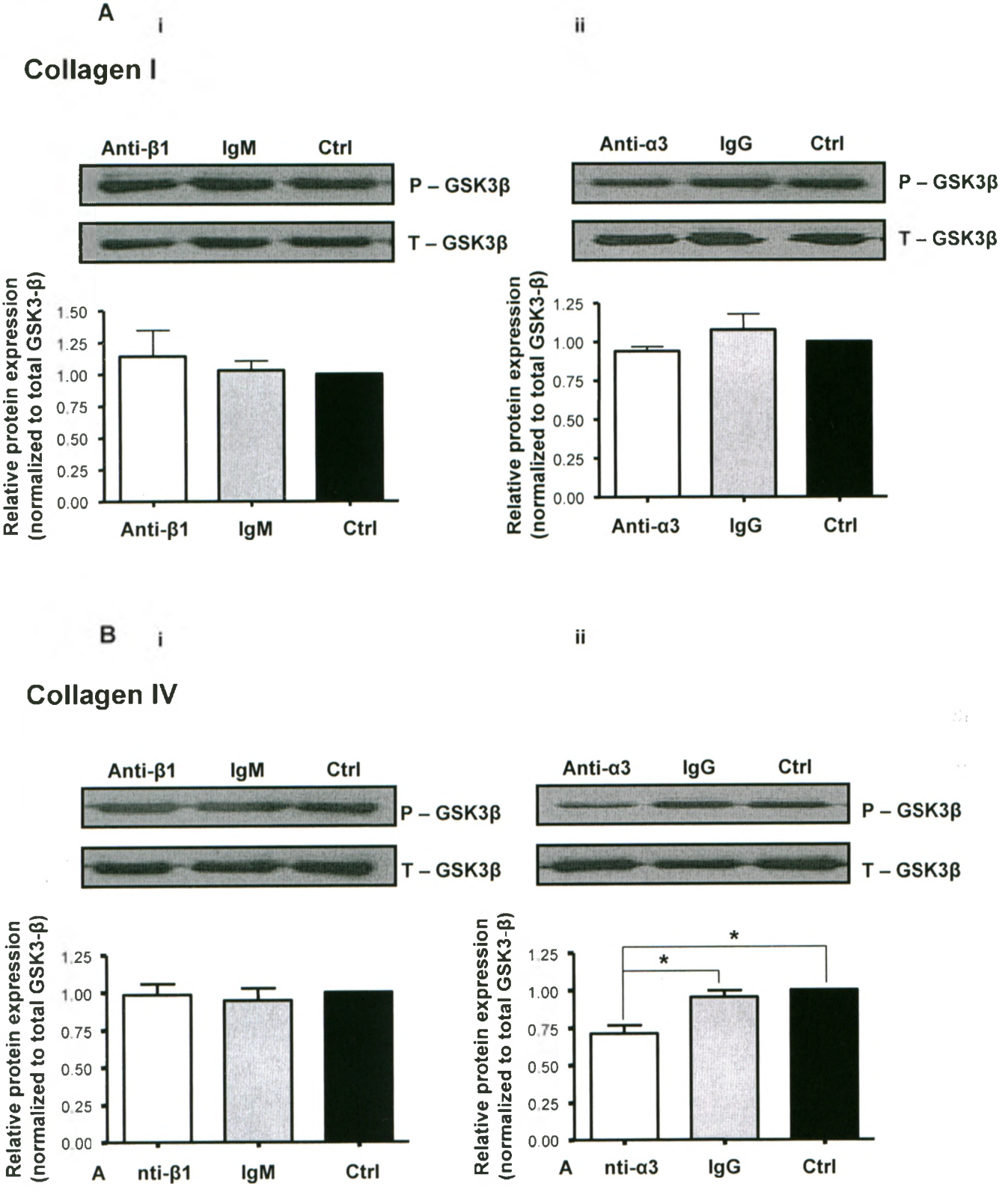

A
i
ii
Collagen I
Anti-β1
IgM
Ctrl
P – GSK3β
T – GSK3β
Anti-α3
IgG
Ctrl
Relative protein expression (normalized to total GSK3-β)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Anti-β1
IgM
Ctrl
Anti-α3
IgG
Ctrl
B
i
ii
Collagen IV
Anti-β1
IgM
Ctrl
P – GSK3β
T – GSK3β
Anti-α3
IgG
Ctrl
Relative protein expression (normalized to total GSK3-β)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-β1
IgM
Ctrl
*
*
Anti-α3
IgG
Ctrl

Figure 3.11. **Blockade of the β1 integrin reduces cyclinD1 expression on both collagen I and IV. Functional blockade of the α3 integrin decreases cyclinD1 expression only on collagen IV.**

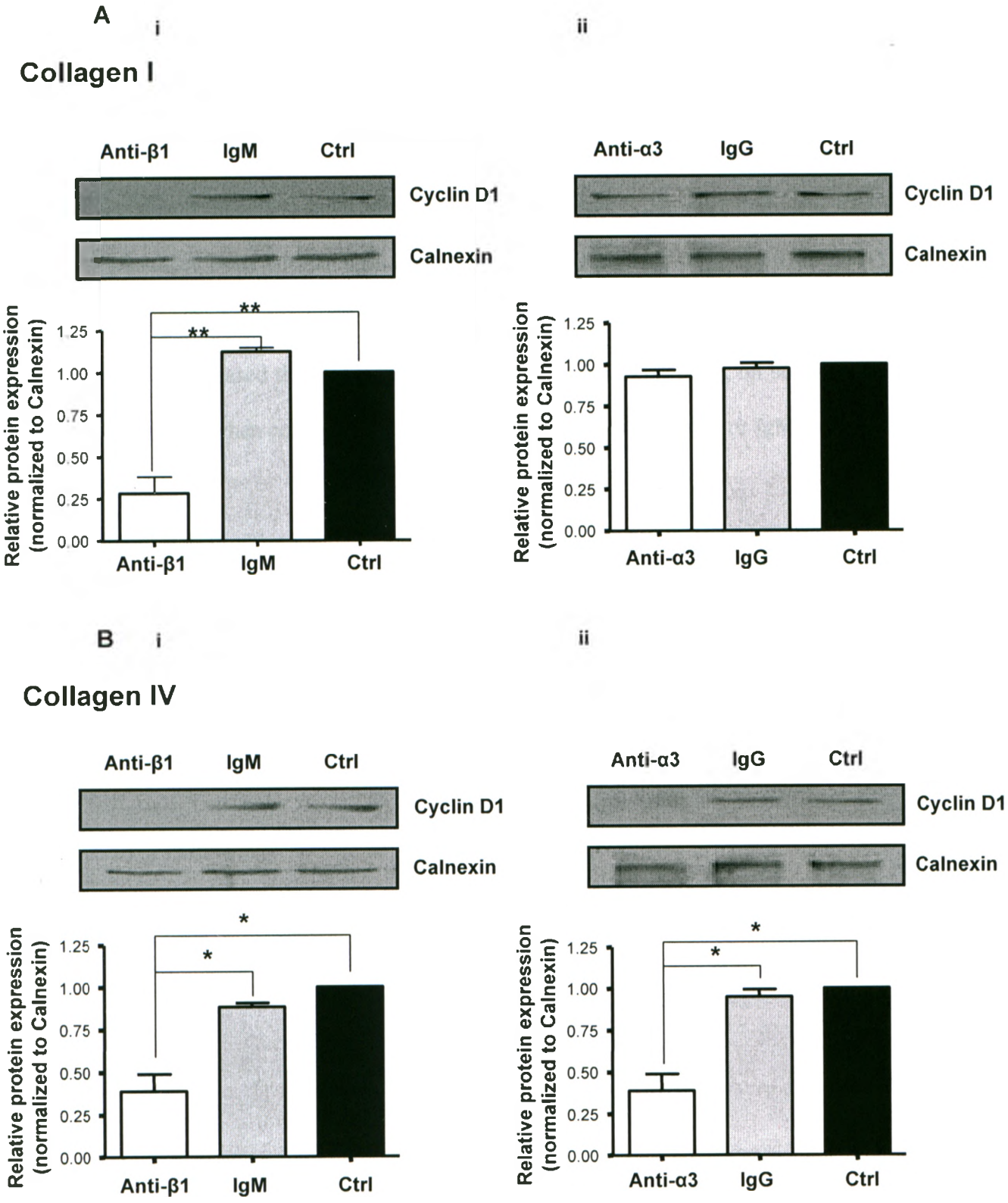
(A) i. Blocking β1 integrin function decreases cyclinD1 expression on collagen I, in comparison with control groups (IgM and untreated). (n=3 experiments/group). **$p<0.01$ vs. IgM or control.

(A) ii. Blockade of the α3 integrin does not alter cyclinD1 expression on collagen I, when compared to control groups (IgG and untreated). (n=3 experiments/group).

(B) i. β1 integrin blockade reduces cyclinD1 expression levels on collagen IV, in comaprison with control groups (IgM and untreated). (n=3 experiments/group). *$p<0.05$ vs. IgM or control.

(B) ii. Perturbing α3 integrin function reduces cyclinD1 expression, in comparison with control groups (IgG and untreated) on collagen IV. (n=3 experiments/group). *$p<0.05$ vs. IgG or control.

Fig. 3.11


A
i
ii
Collagen I
Anti-β1
IgM
Ctrl
Cyclin D1
Calnexin
Anti-α3
IgG
Ctrl
Cyclin D1
Calnexin
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
**
**
Anti-β1
IgM
Ctrl
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
Anti-α3
IgG
Ctrl
B
i
ii
Collagen IV
Anti-β1
IgM
Ctrl
Cyclin D1
Calnexin
Anti-α3
IgG
Ctrl
Cyclin D1
Calnexin
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
*
*
Anti-β1
IgM
Ctrl
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
*
*
Anti-α3
IgG
Ctrl

### 3.3.10 *Perturbing β1 or α3 Integrin function reduces FAK and ERK1/2 phosphorylation and increases caspase 3 cleavage.*

Numerous studies have identified that MAPK signaling is critical for integrin function (Chen et al., 2006; Choma et al., 2006; Gonzalez et al., 1999; Hammer et al., 2004; Manohar et al., 2004). To investigate if integrins β1 and α3 signal through the MAPK pathway in INS-1 cells, alterations in the phosphorylation of FAK and ERK1/2 were examined. Both anti-β1 and anti-α3 treatments decreased FAK ($p<0.05$-$0.001$) as well as ERK1/2 phosphorylation ($p<0.01$-$0.001$) (**Fig. 3.12Ai,ii Bi,ii 3.13Ai,ii Bi,ii**) and subsequently increased the cleavage of caspase 3 (**Fig. 3.14Ai,ii Bi,ii**), on both collagen I and IV matrices, when compared to corresponding controls (untreated or IgM or IgG).

Figure 3.12. **Blockade of β1 and α3 integrin function reduces FAK phosphorylation.**

Perturbing β1 integrin function decreases the phosphorylation of FAK on (A) i. collagen I and (B) i. collagen IV, when compared to controls (IgM and untreated). (n=3 experiments/group). ***$p<0.001$ vs. IgM or control.

Blockade of the α3 integrin reduces FAK phosphorylation on both (A) ii. collagen I and (B) ii. collagen IV when compared to controls (IgG and untreated). (n=3 experiments/group). *$p<0.05$, **$p<0.01$ vs. IgG or control.

Fig. 3.12

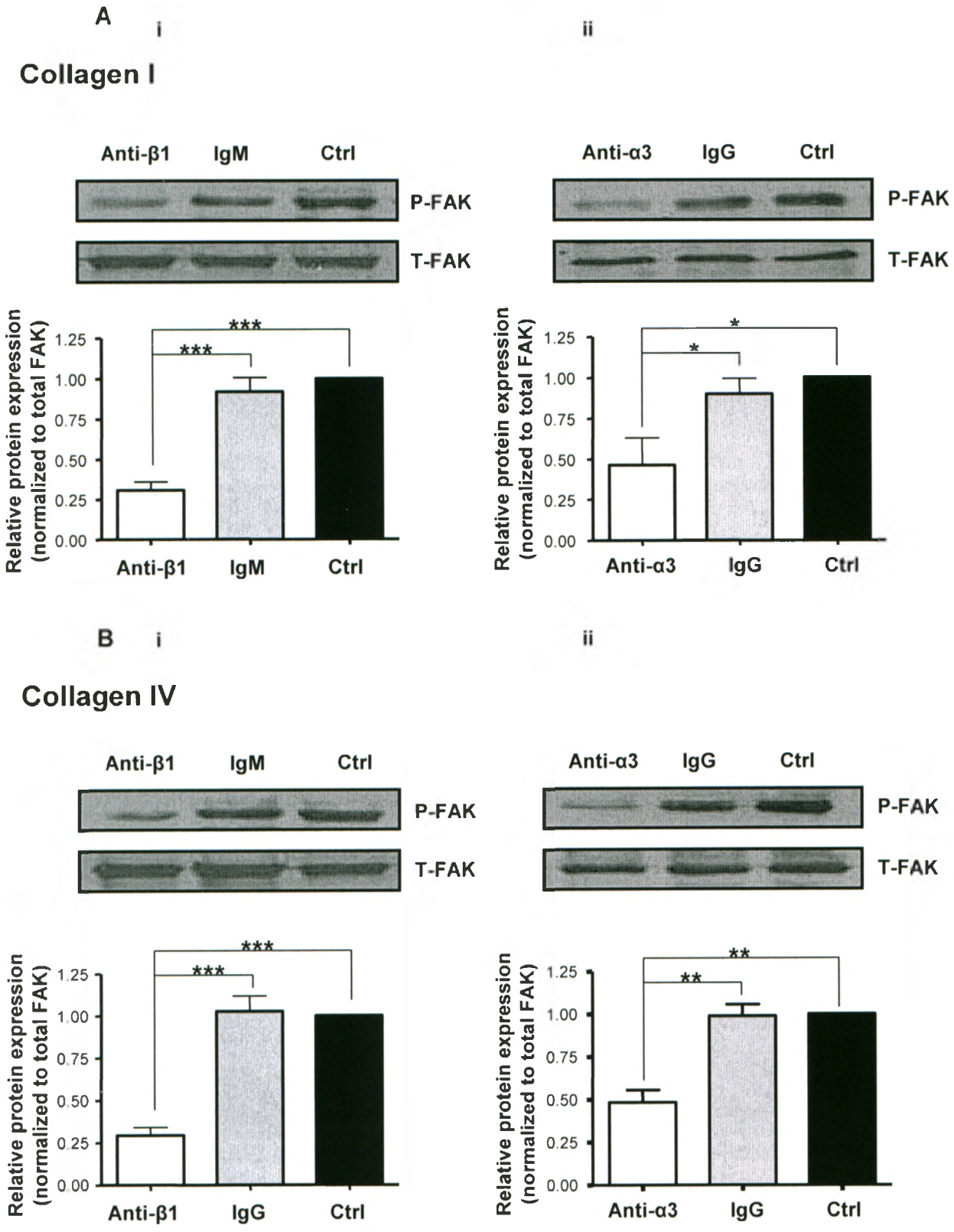

A
i
ii
Collagen I
Anti-β1
IgM
Ctrl
P-FAK
T-FAK
Anti-α3
IgG
Ctrl
Relative protein expression (normalized to total FAK)
1.25
1.00
0.75
0.50
0.25
0.00
***
***
*
*
Anti-β1
IgM
Ctrl
Anti-α3
IgG
Ctrl
B
i
ii
Collagen IV
Anti-β1
IgM
Ctrl
P-FAK
T-FAK
Anti-α3
IgG
Ctrl
Relative protein expression (normalized to total FAK)
1.25
1.00
0.75
0.50
0.25
0.00
***
***
**
**
Anti-β1
IgG
Ctrl
Anti-α3
IgG
Ctrl

Figure 3.13. **Blockade of β1 and α3 integrin function reduces ERK1/2 phosphorylation.**

β1 integrin blockade reduces ERK1/2 phosphorylation on (A) i. collagen I and (B) ii. collagen IV when compared to controls (IgM and untreated). (n=3 experiments/group). **p<0.01, ***p<0.001 vs. IgM or control.

Blocking α3 integrin function decreases ERK1/2 phosphorylation on both (A) i. collagen I and (B) ii. collagen IV when compared to controls (IgG and untreated). (n=3 experiments/group). ***p<0.001 vs. IgG or control.

Fig. 3.13

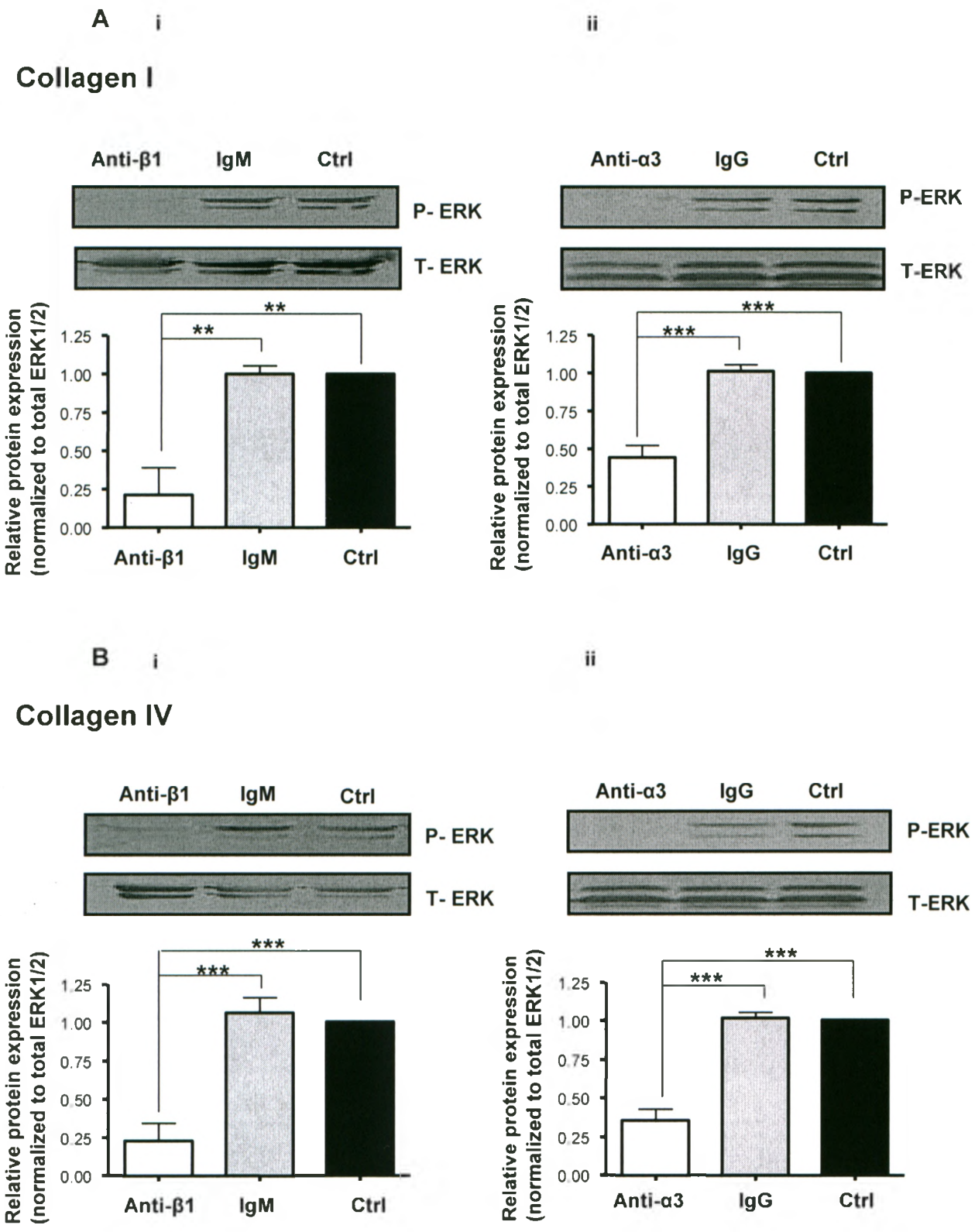
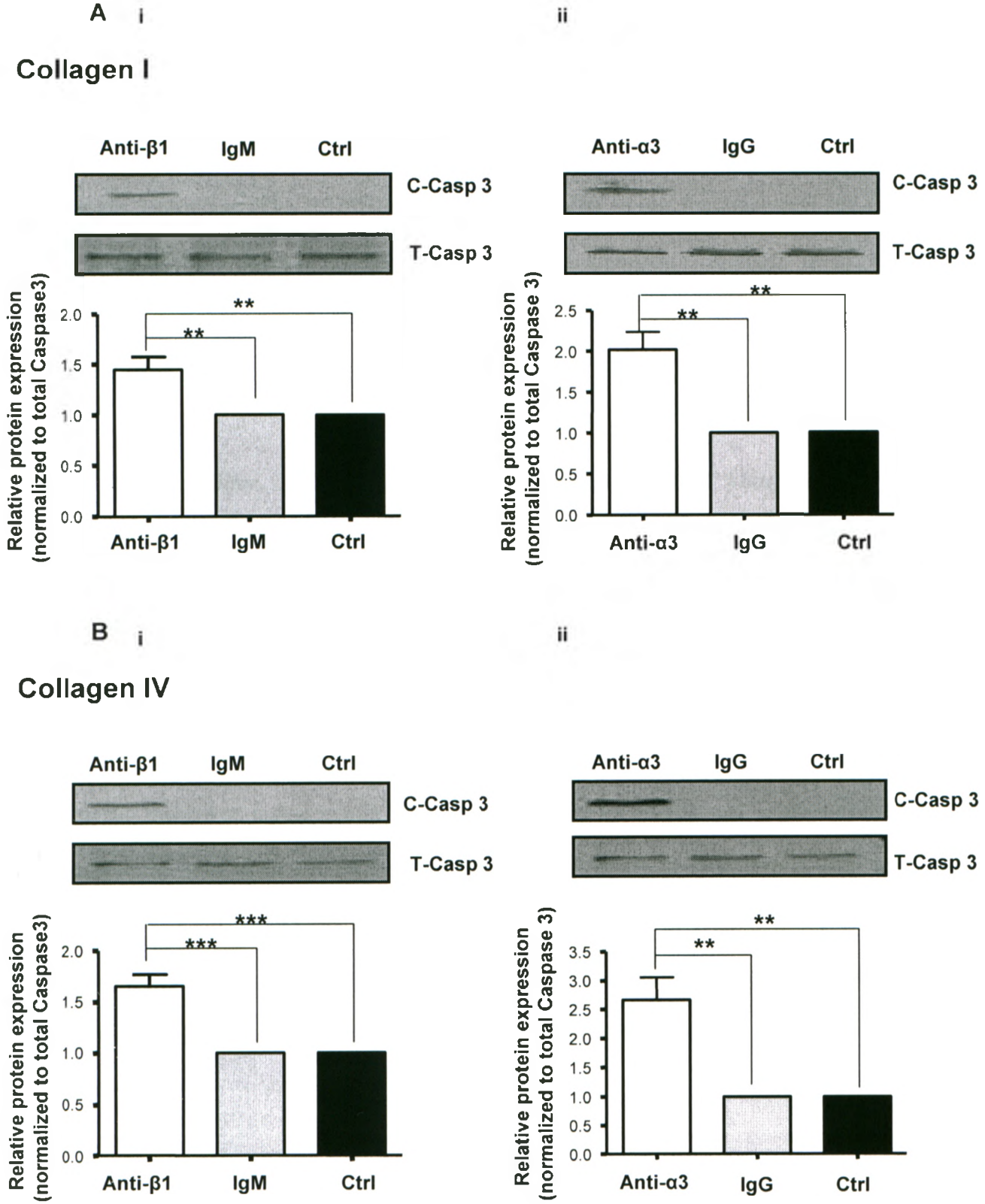

A
i
ii
Collagen I
Anti-β1
IgM
Ctrl
P- ERK
T- ERK
Relative protein expression (normalized to total ERK1/2)
1.25
1.00
0.75
0.50
0.25
0.00
**
**
Anti-β1
IgM
Ctrl
Anti-α3
IgG
Ctrl
P-ERK
T-ERK
***
***
Anti-α3
IgG
Ctrl
B
i
ii
Collagen IV
Anti-β1
IgM
Ctrl
P- ERK
T- ERK
***
***
Anti-β1
IgM
Ctrl
Anti-α3
IgG
Ctrl
P-ERK
T-ERK
***
***
Anti-α3
IgG
Ctrl

Figure 3.14. **Blockade of β1 and α3 integrin function increases the cleavage of Caspase 3.**

Perturbation of β1 integrin function increases cleavage of caspase 3 on both (A) i. collagen I and (B) ii. collagen IV, when compared to controls (IgM and untreated). (n=3 experiments/group). **p<0.01, ***p<0.001 vs. IgM or control.

α3 integrin blockade increases cleavage of caspase 3 on (A) ii. collagen I and (B) ii. collagen IV, when compared to controls (IgG and untreated). (n=3 experiments/group). **p<0.01 vs. IgG or control.

Fig. 3.14


A
i
ii
Collagen I
Anti-β1
IgM
Ctrl
C-Casp 3
T-Casp 3
Relative protein expression (normalized to total Caspase3)
Anti-α3
IgG
Ctrl
Relative protein expression (normalized to total Caspase 3)
B
i
ii
Collagen IV

### 3.3.11 *MEK inhibitor, U0126, decreases ERK1/2 activation, increases cleavage of caspase 3 and reduces Pdx-1 expression.*

To confirm that both the β1 and α3 integrin signal through the MAPK pathway, INS-1 cells were treated with MEK inhibitor U0126, and subsequently cultured on collagen I or IV. A decrease in ERK1/2 activation (**Fig. 3.15A**) and a subsequent increase in caspase 3 cleavage (**Fig. 3.15B**) were observed, similar to our findings upon anti-β1 integrin or anti-α3 integrin treatments. Moreover, a significant decrease in Pdx-1 expression was noted on both collagen I and IV upon U0126 treatment, when compared to control (**Fig. 3.16**).

### 3.3.12 *PI3K inhibitor, Wortmannin decreases Akt and GSK3-β phosphorylation, reduces XIAP and cyclinD1 expression and increases caspase 3 cleavage, but does not reduce Pdx-1 expression.*

The treatment of INS-1 cells with wortmannin demonstrated a decrease in Akt phosphorylation at both Ser473 and Thr308 (**Fig. 3.17A,B**) and reduced XIAP expression (**Fig. 3.18A**), on both collagen I and IV, similar to that observed upon anti-α3 integrin treatments. Moreover, a decrease in GSK3-β activation (**Fig. 3.18B**), and reduced cyclin D1 expression was observed on both collagen matrices (**Fig. 3.19A**), along with an increase in the cleavage of caspase 3 (**Fig. 3.19B**). Interestingly, we observed no changes in Pdx-1 expression upon treatment with Wortmannin on both collagen I and IV (**Fig. 3.20**), indicating that the PI3K pathway is more involved in the regulation of cell survival and apoptosis.

Figure 3.15. **Treatment with MEK inhibitior U0126 reduces ERK1/2 phosphorylation and increases cleavage of Caspase 3.**

On both collagen I and IV, U0126 treated INS-1 cells demonstrate (A) reduced ERK1/2 phosphorylation and (B) a subsequent increase in caspase 3 cleavage, when compared to controls (untreated). (n=3 experiments/group) *$p<0.05$, **$p<0.01$ vs. control.

Fig. 3.15

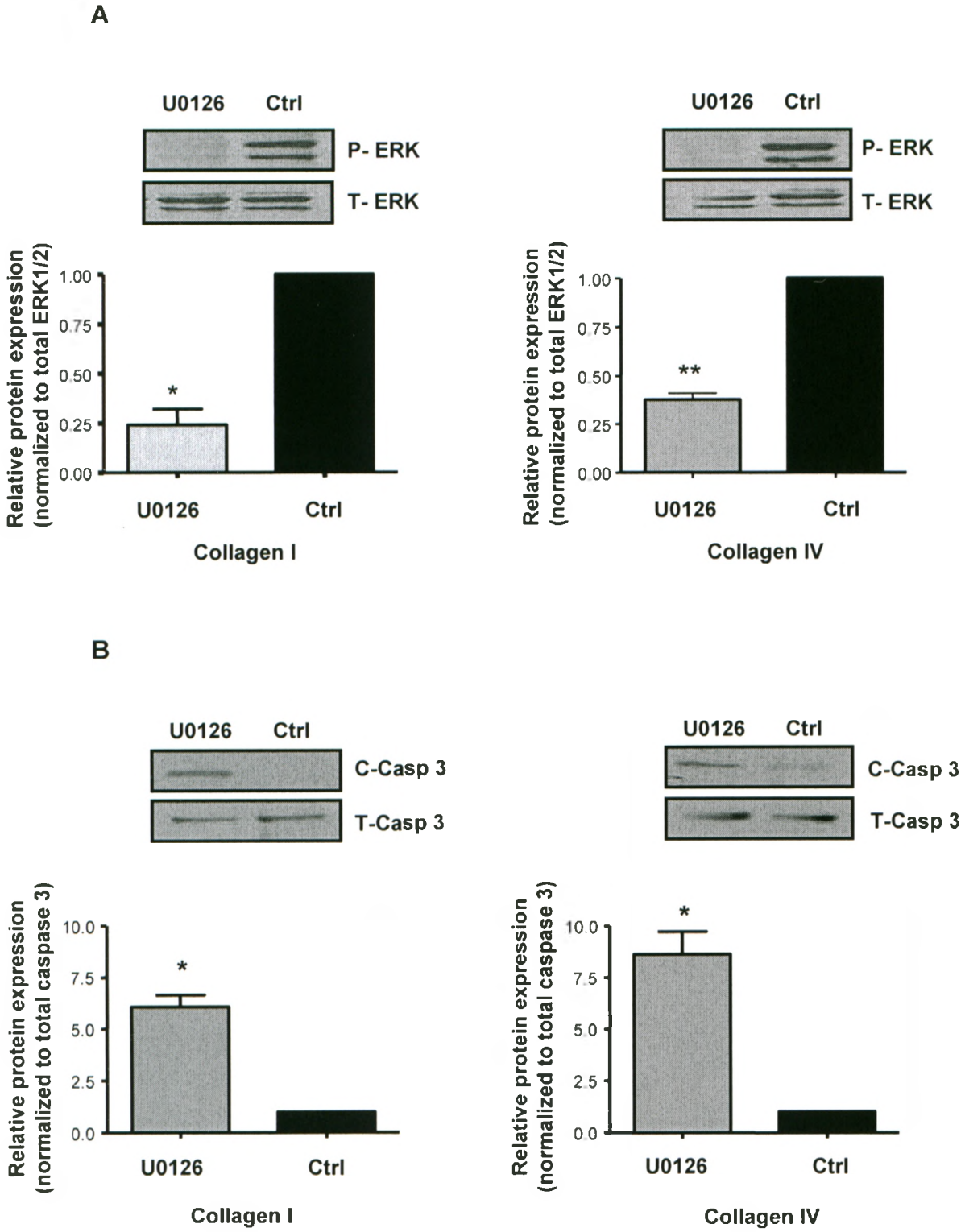

A
U0126
Ctrl
P- ERK
T- ERK
Relative protein expression (normalized to total ERK1/2)
1.00
0.75
0.50
0.25
0.00
*
U0126
Ctrl
Collagen I
U0126
Ctrl
P- ERK
T- ERK
Relative protein expression (normalized to total ERK1/2)
1.00
0.75
0.50
0.25
0.00
**
U0126
Ctrl
Collagen IV
B
U0126
Ctrl
C-Casp 3
T-Casp 3
Relative protein expression (normalized to total caspase 3)
10.0
7.5
5.0
2.5
0.0
*
U0126
Ctrl
Collagen I
U0126
Ctrl
C-Casp 3
T-Casp 3
Relative protein expression (normalized to total caspase 3)
10.0
7.5
5.0
2.5
0.0
*
U0126
Ctrl
Collagen IV

Figure 3.16. **Treatment with MEK inhibitior, U0126, reduces Pdx-1 expression.**

On both collagen I and IV, U0126 treated INS-1 cells display reduced Pdx-1 expression when compared to controls (untreated). (n=3 experiments/group)*$p<0.05$ vs. control.

Fig. 3.16

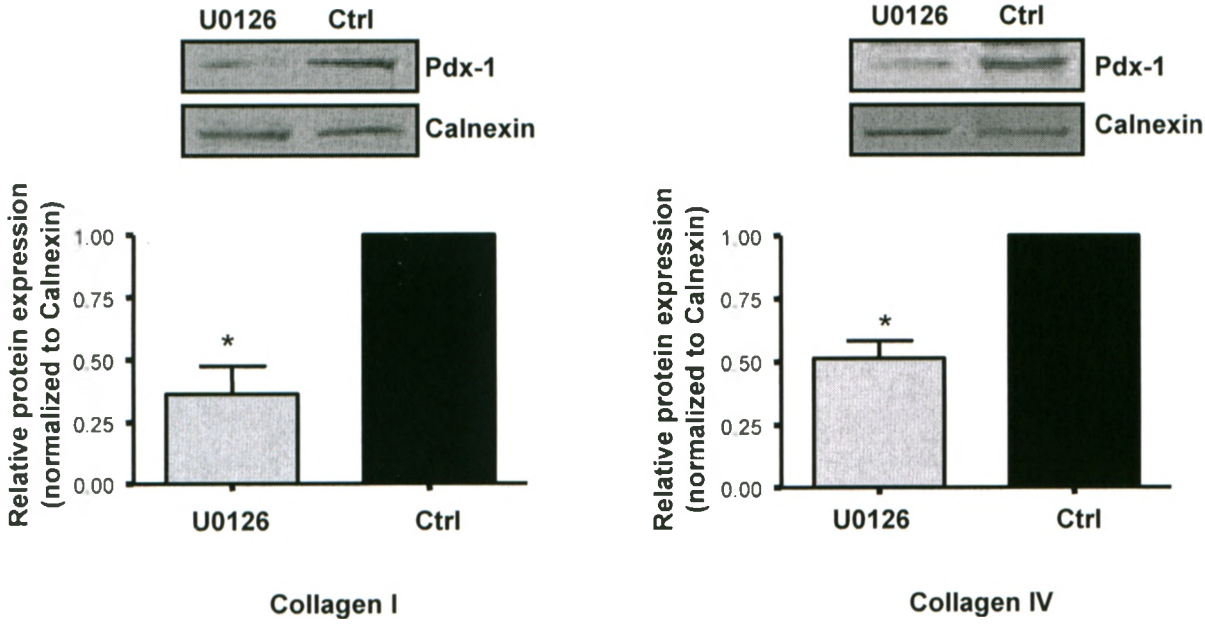

U0126
Ctrl
Pdx-1
Calnexin
Relative protein expression (normalized to Calnexin)
1.00
0.75
0.50
0.25
0.00
*
U0126
Ctrl
Collagen I
U0126
Ctrl
Pdx-1
Calnexin
Relative protein expression (normalized to Calnexin)
1.00
0.75
0.50
0.25
0.00
*
U0126
Ctrl
Collagen IV

Figure 3.17. **Treatment with PI3K inhibitor, Wortmannin, reduces Akt phosphorylation at Ser 473 and Thr 308.**

INS-1 cells treated with Wortmannin, demonstrate reduced Akt phosphorylation at both (A) Ser 473 and (B) Thr 308, on both collagen I and IV, when compared to controls (untreated). (n=3 experiments/group) **p<0.01 ***p<0.001 vs. control.

Fig. 3.17

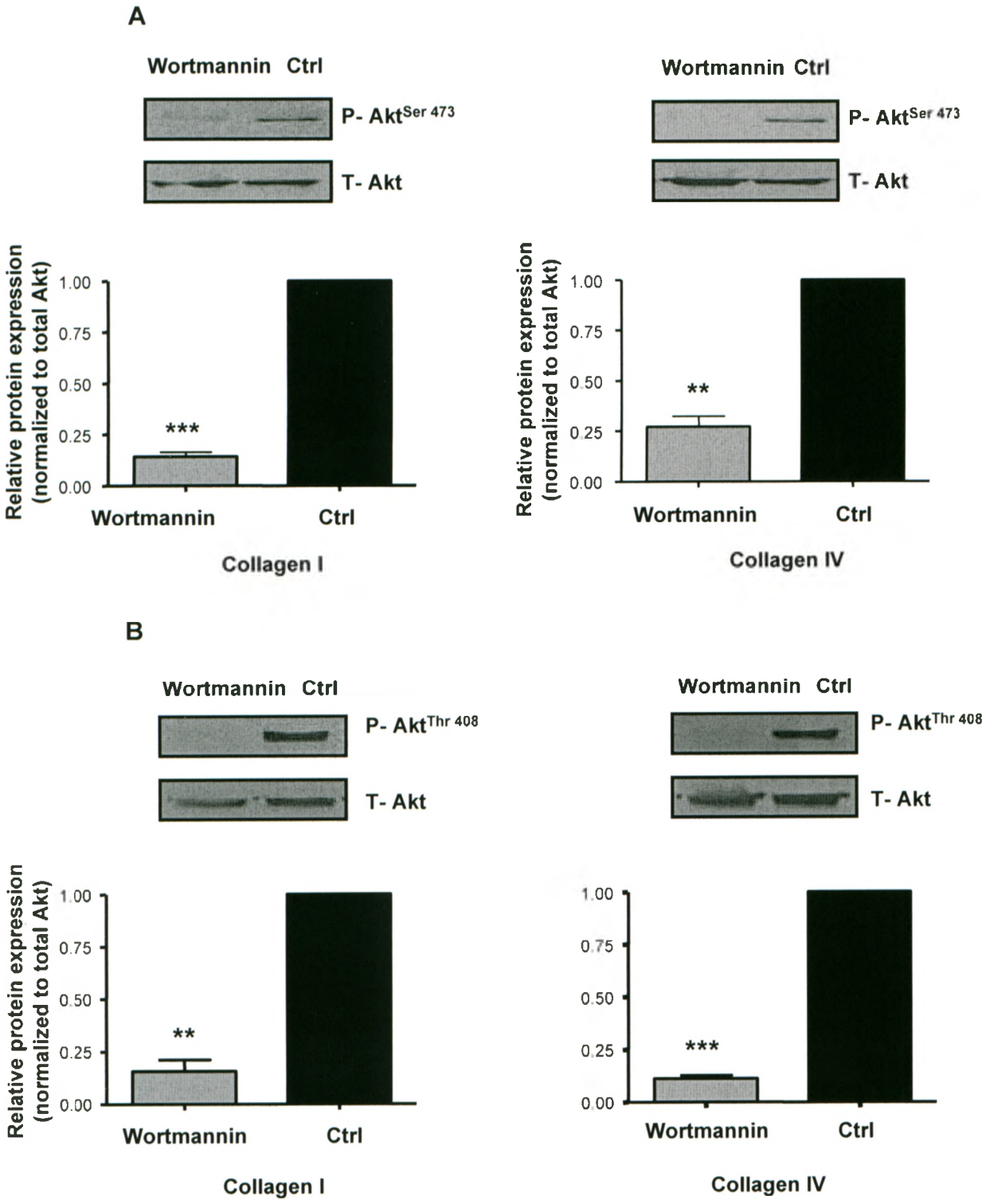

A
Wortmannin Ctrl
P- Akt Ser 473
T- Akt
Relative protein expression (normalized to total Akt)
1.00
0.75
0.50
0.25
0.00
***
Wortmannin
Ctrl
Collagen I
Wortmannin Ctrl
P- Akt Ser 473
T- Akt
Relative protein expression (normalized to total Akt)
1.00
0.75
0.50
0.25
0.00
**
Wortmannin
Ctrl
Collagen IV
B
Wortmannin Ctrl
P- Akt Thr 408
T- Akt
Relative protein expression (normalized to total Akt)
1.00
0.75
0.50
0.25
0.00
**
Wortmannin
Ctrl
Collagen I
Wortmannin Ctrl
P- Akt Thr 408
T- Akt
1.00
0.75
0.50
0.25
0.00
***
Wortmannin
Ctrl
Collagen IV

Figure 3.18. **Treatment with PI3K inhibitor, Wortmannin, reduces XIAP expression and decreases GSK3-β phosphorylation at Ser 9.**

Reduced expression of (A) XIAP and (B) a decrease in GSK3-β phosphorylation at Ser 9, is observed upon Wortmannin treatment, when compared to controls (untreated). (n=3 experiments/group) *$p<0.05$ **$p<0.01$ ***$p<0.001$ vs. control.

Fig. 3.18

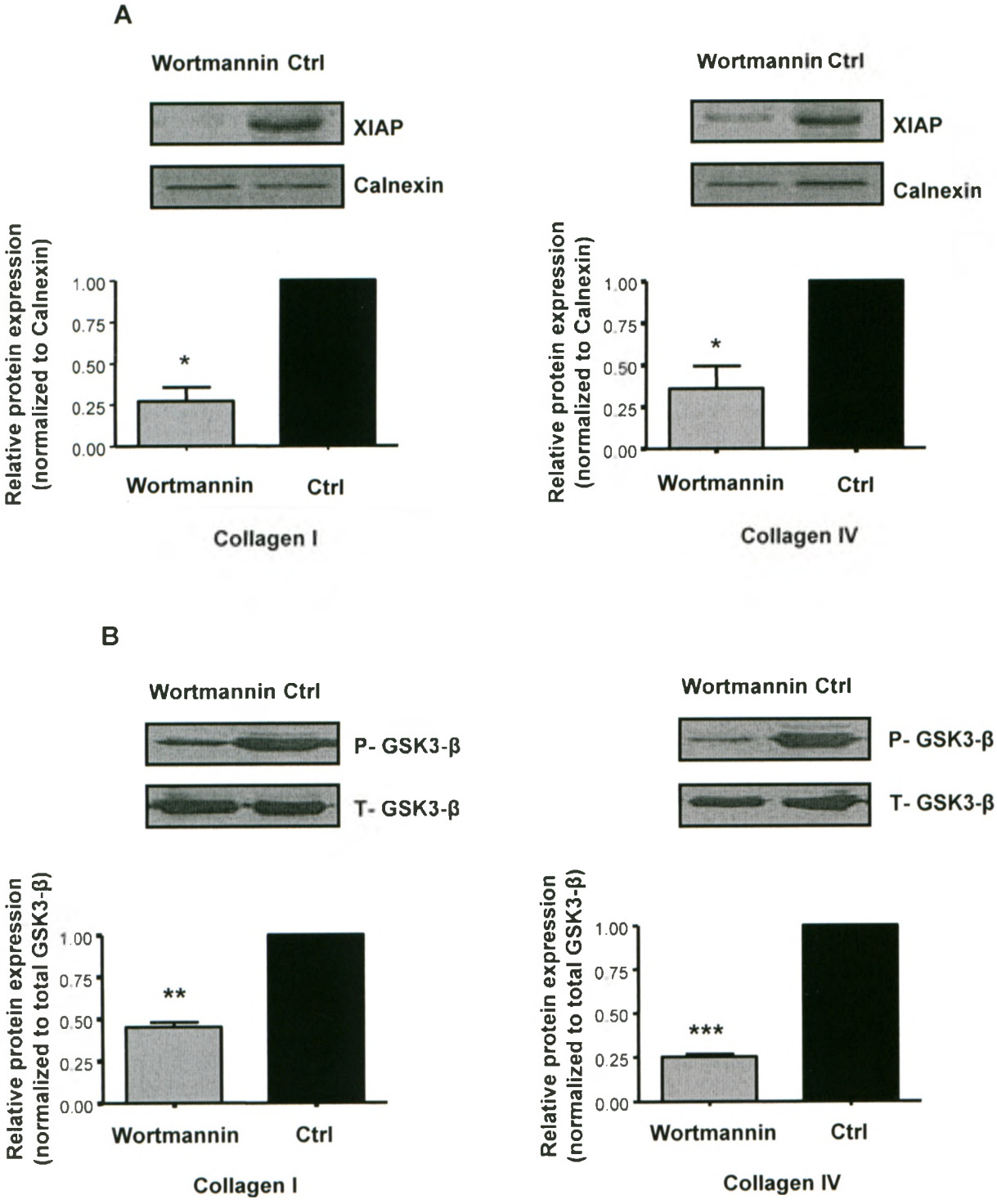

A
Wortmannin Ctrl
XIAP
Calnexin
Relative protein expression (normalized to Calnexin)
1.00
0.75
0.50
0.25
0.00
*
Wortmannin
Ctrl
Collagen I
Wortmannin Ctrl
XIAP
Calnexin
Relative protein expression (normalized to Calnexin)
1.00
0.75
0.50
0.25
0.00
*
Wortmannin
Ctrl
Collagen IV
B
Wortmannin Ctrl
P- GSK3-β
T- GSK3-β
Relative protein expression (normalized to total GSK3-β)
1.00
0.75
0.50
0.25
0.00
**
Wortmannin
Ctrl
Collagen I
Wortmannin Ctrl
P- GSK3-β
T- GSK3-β
Relative protein expression (normalized to total GSK3-β)
1.00
0.75
0.50
0.25
0.00
***
Wortmannin
Ctrl
Collagen IV

Figure 3.19. **Treatment with PI3K inhibitor, Wortmannin reduces cyclin D1 expression and increases cleavage of caspase 3.**

A significant decrease in (A) cyclin D1 and an increase in (B) cleaved caspase 3 was observed upon Wortmannin treatment, when compared to controls (untreated). (n=3 experiments/group). *$p<0.05$ **$p<0.01$ vs. control.

Fig. 3.19

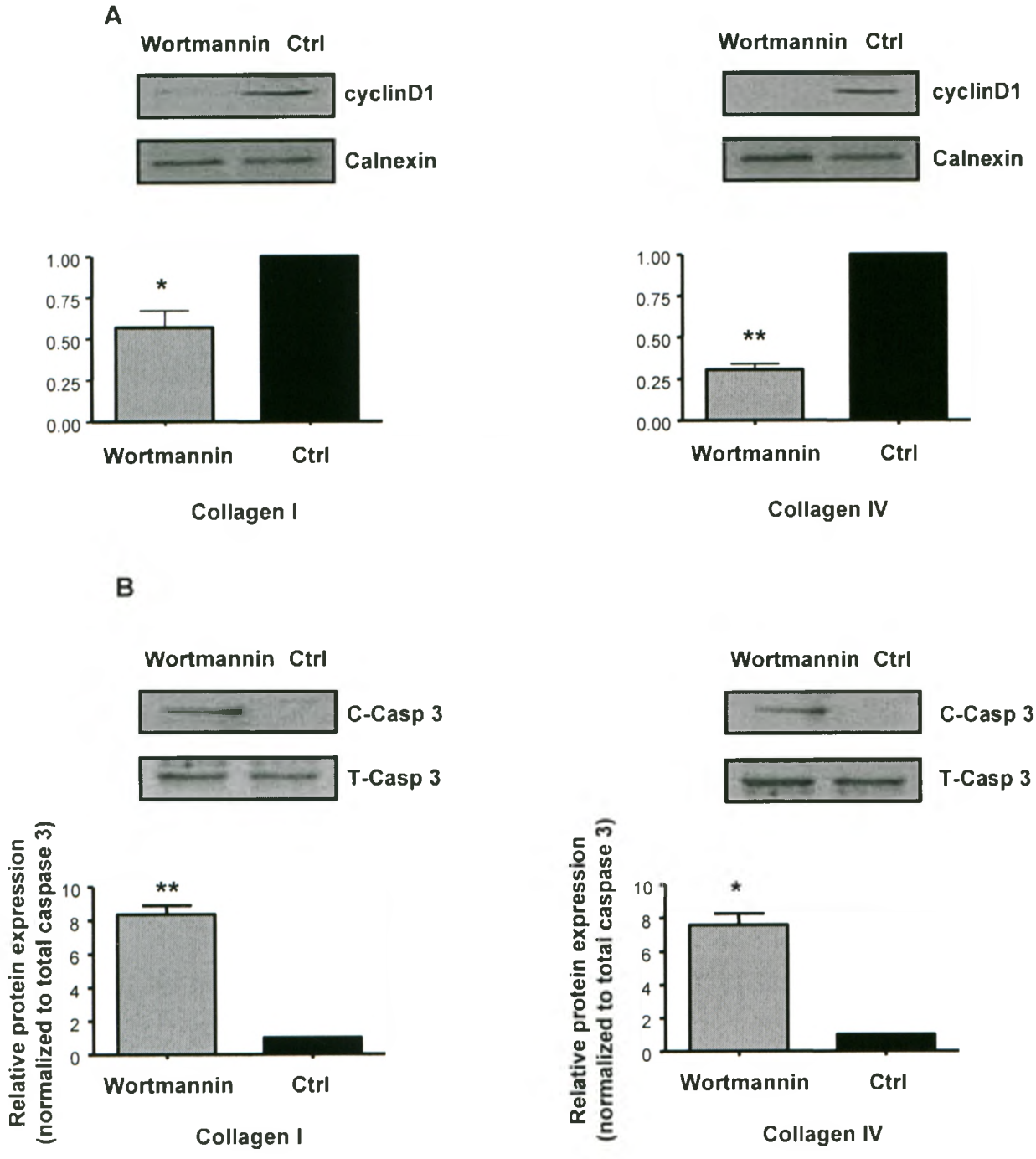
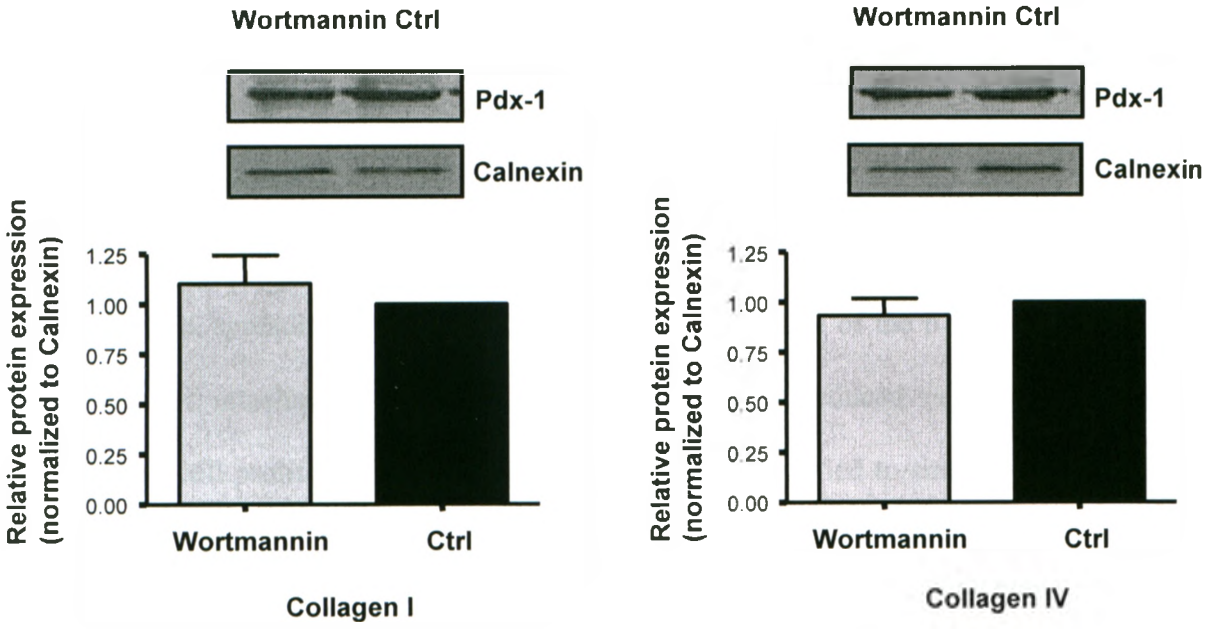

A
Wortmannin Ctrl
cyclinD1
Calnexin
1.00
0.75
0.50
0.25
0.00
*
Wortmannin
Ctrl
Collagen I
Wortmannin Ctrl
cyclinD1
Calnexin
1.00
0.75
0.50
0.25
0.00
**
Wortmannin
Ctrl
Collagen IV
B
Wortmannin Ctrl
C-Casp 3
T-Casp 3
Relative protein expression
(normalized to total caspase 3)
10
8
6
4
2
0
**
Wortmannin
Ctrl
Collagen I
Wortmannin Ctrl
C-Casp 3
T-Casp 3
Relative protein expression
(normalized to total caspase 3)
10
8
6
4
2
0
*
Wortmannin
Ctrl
Collagen IV

Figure 3.20. **Treatment with PI3K inhibitor, Wortmannin, does not reduce Pdx-1 expression.**

INS-1 cells treated with Wortmannin do not demonstrate reduced Pdx-1 expression, on either collagen matrix, when compared to controls (untreated). (n=3 experiments/group).

Fig. 3.20


Wortmannin Ctrl
Pdx-1
Calnexin
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
Wortmannin
Ctrl
Collagen I
Wortmannin Ctrl
Pdx-1
Calnexin
Relative protein expression (normalized to Calnexin)
1.25
1.00
0.75
0.50
0.25
0.00
Wortmannin
Ctrl
Collagen IV

## 3.4 Discussion

We have previously shown that INS-1 cells express high levels of integrin α3β1 and demonstrate an innate preference for matrices collagen I and IV (Chapter 2). In the present study, we dissected the integrin receptor into its respective subunits to identify and characterize the individual contributions of the β1 and α3 subunits for INS-1 cell survival and function. Furthermore, we examined signaling cascades downstream of the β1 and α3 subunits to define the intracellular mechanisms responsible for modulating beta cell behaviours.

The present study demonstrates that the β1 integrin is a primary regulator of beta cell adhesion, spreading, viability and proliferation. Blockade of the β1 integrin resulted in a loss of attachment to collagen I and IV, greatly reduced cell spreading and diminished cell proliferation. Perturbing α3 integrin function led to similar alterations in beta cell behaviours, but to a much lesser extent, suggesting that the α3 subunit is important for beta cell survival, but remains subordinate to the β1 integrin. High expression levels of both α3 and β1 subunits in islet cell types, has been demonstrated by several groups and suggests that these integrin subunits may be of particular importance for the regulation of islet cell survival and function (Kantengwa et al., 1997; Wang et al., 1999; Wang et al., 2005). Functional blockade of the β1 integrin has been conducted in numerous studies, which all report consistent findings: the β1 integrin is essential for mediating attachment, spreading and viability of islets to various extracellular matrices in several model systems (Kaido et al., 2004a; Kaido et al., 2004b; Kantengwa et al., 1997; Wang et al., 2005; Yashpal et al., 2005). These islet studies, parallel our results in INS-1 cells, indicating a major role for the β1 integrin in beta cell proliferation and survival. For

the first time, we demonstrate that the α3 subunit also contributes to beta cell survival and function, but to a much lesser extent.

To determine if interactions between collagen matrices and the β1 or α3 integrin influence beta cell function, Pdx-1 and insulin expression and secretion were examined upon functional blockade of the β1 or α3 integrin. Remarkable decreases in Pdx-1 and insulin expression along with insulin secretion were observed on both collagen I and IV upon blockade of the β1 integrin. Interestingly, perturbing α3 integrin function affected Pdx-1 expression only on collagen IV, while insulin expression and release were diminished on both collagen matrices. These results are consistent with findings in the literature, that have shown that exposure of islets to ECM proteins greatly improves insulin expression and release, a response potentially mediated through integrin-ECM interactions (Bosco et al., 2000; Lucas-Clerc et al., 1993; Thivolet et al., 1985). Furthermore, we have demonstrated that β1 integrin perturbation in both the rat and human fetal pancreas decreases Pdx-1 and insulin gene and protein expression (Saleem et al., 2008; Wang et al., 2005; Yashpal et al., 2005). The functional consequences of β1 integrin blockade in INS-1 cells are congruent with previous reports; however, we also demonstrate that collagen matrix interactions with the α3 integrin are essential for beta cell function.

Integrin-dependent adhesion to the surrounding ECM is essential for cell anchorage and maintenance of tissue integrity (Brakebusch et al., 2003; Chen et al., 2006). ECM ligand binding to the cell surface of integrins, stimulates integrin clustering leading to the formation of focal adhesion contacts (Danen and Sonnenberg, 2003, Lee et al., 2004). These plaques act as a platform, enabling integrins to establish connections

with downstream signaling molecules including focal adhesion kinase (FAK) and phosphoinositide 3-Kinase (PI3-K) (Schoenwaelder et al., 1999), thus facilitating actin remodeling, maintenance of cell polarity and control of cell survival and differentiation (Clarke and Brugge, 1995; Giancotti and Ruoslahti, 1999).

In several model systems, the β1 integrin has been shown to activate the FAK/MAPK/ERK cascade in order to mediate changes in cell survival and function (Chen et al., 2006; Choma et al., 2006; Gonzales et al., 1999; Manohar et al., 2004). A previous report by Hammer et al determined that the 804G matrix constituent protects against beta cell apoptosis through interactions with the β1/FAK signaling pathway (Hammer et al., 2004). To further investigate signaling mechanisms of the α3 and β1 integrins in INS-1 cells, alterations in the PI3K and MAPK cascades were examined upon anti-β1 or anti-α3 antibody treatments. Here we report that the β1 integrin primarily activates the FAK/MAPK/ERK cascade to increase Pdx-1 expression and protect against cleavage of caspase 3. While the α3 integrin also signals through this pathway, it has the ability to activate the PI3K/Akt/XIAP and PI3K/Akt/GSK3-β axes to protect against cell death and stimulate cell proliferation. While, the majority of the observed changes in α3 and β1 integrin signaling were consistent between the two collagen matrices, the reduction in phospho-GSK3-β and the decrease in cyclin D1 expression were only observed on collagen IV upon anti-α3 treatment, suggesting that ligand binding interactions of this integrin stimulate specific cascades to mediate cellular changes.

Many recent studies have demonstrated that the intracellular cytoplasmic tail of α subunits physically associate with adaptor and signaling proteins in order to bring about changes in cell signaling, motility, spreading and function (Mechai et al., 2005; Qin et al.,

2004). The cytoplasmic tail of the α4 integrin was one of the first to be identified which activated signaling cascades independent of the β1 integrin (Liu et al, 1999; Liu and Ginsberg 2000). The α4 cytoplasmic tail was shown to contain a highly conserved domain which enables interactions with adaptor proteins, including paxillin, to regulate cell spreading (Liu et al., 1999). These studies taken together with the results presented here demonstrate an increasingly important role of α integrins in the regulation of multiple cellular processes. Given the prominent expression of the α3 integrin in beta cells, it is highly likely that this integrin subunit signals through highly specialized downstream cascades, which is indicative of its multifaceted role in regulating beta cell survival and function.

In summary, this study provides insight into the individual roles of the α3 and β1 integrins in their regulation of beta cell survival and function. These integrins were shown to possess dynamic ligand binding capabilities which modulated numerous beta cell behaviours through highly specialized signaling cascades. Increased understanding of integrin-ECM interactions may prove useful for future investigations which seek to manipulate and improve islet microenvironments for cell-based therapies for the treatment of diabetes.

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Chapter 4

# General Discussions and Conclusions

# 4 General Discussion and Conclusions

Islet transplantation and other cell-based therapies for the treatment of diabetes are limited by the shortage in availability of insulin-producing tissue. Identifying factors which promote survival and enhance function of beta cells offers the possibility of re-populating and restoring damaged or functionally compromised insulin-producing cells. However, the success of such an application will depend on precise identification and characterization of factors which control survival, growth and function. In light of this, the results presented in this thesis highlight two important findings. Firstly, the thorough characterization of alterations in beta cell behaviours and function, as a result of integrin inhibition, demonstrate that individual integrin subunits partake in the regulation of both beta cell survival and function. Moreover, the individual subunits possess specific ECM ligand binding properties, which add another dimension to integrin regulation of beta cell activities. Secondly, integrin subunits participate in highly specialized signaling cascades in order to modulate beta cell survival and function. The activated biochemical pathways become important when examining the functional capacities of beta cells as well as their ability to proliferate and survive long term. Taken together, these findings suggest that integrin receptors are multi-faceted and their interactions with ECM proteins influence a variety of cellular behaviours, from morphology to function, through distinct signaling mechanisms. Thus, integrin-ECM relationships can be manipulated in future investigations which seek to improve cell-based therapies for the treatment of diabetes.

## 4.1 α3β1 integrin is highly expressed in pancreatic (INS-1) beta cells

In the field of islet cell biology, there is paramount evidence that the subfamily of β1 integrins orchestrate changes in islet cell development, growth, survival and function

while maintaining cellular integrity, architecture and protecting against "anoikis" and cell death (Chen et al., 2006; Kantengwa et al., 1997; Wang et al., 2001; Wang et al., 2005; Yashpal et al, 2005). However, the majority of studies examining β1 integin-ECM interactions have been conducted using isolated islets, a heterogenous cell population. Thus the direct effects of the β1 integrin on beta cell survival and function have not yet been identified. Using a relatively new beta cell line, the first part of my thesis was to characterize the αβ1 integrin expression in INS-1 cells. Through immunhistochemical, qRT-PCR and western blotting approaches, it was determined that integrins α1-6, αV and β1 are all expressed in this cell line (**Chapter 2, Figure 2.1A-D**). Among these heterodimers, integrin subunits α3 and β1 were most highly expressed (**Chapter 2, Figure 2.1B**).

The expression of integrin α3β1 in islet cell types has been confirmed by several groups. Wang et al. (1999) demonstrated that isolated islets from canine, porcine, hamster and human pancreas, highly express the α3 integrin. Moreover, Kantengwa et al. (1997) also reported that the α3β1 integrin constitutes approximately half of the αβ1 integrins expressed in rat primary islet cells. Lastly, our previous studies revealed that α3 integrin expression increases during development of the human fetal and rat pancreas, and is often expressed in insulin$^+$ cells (Wang et al., 2005; Yashpal et al., 2005). Taken together, these studies suggest that integrin α3β1 is essential for islet cell development and possibly plays a critical role in the survival and function of beta cells.

**4.2 Collagen I and IV greatly enhance survival and function of INS-1 cells**

Given the high level of expression of integrin α3β1, common ligands which bind to this heterodimer were tested in order to identify the most suitable matrix protein for INS-1 cells. Moreover, fibronectin, laminin, and collagen I and IV are all matrices found within or near islet cells, *in situ*, as either critical constituents of the basement or perinsular membrane (Kaido et al., 2004; Lammert et al., 2003; Van Deijnen et al., 1994). In INS-1 cells, matrices collagen I and IV maximally increased cell adhesion, spreading, viability and proliferation when compared to fibronectin, laminin and control (**Chapter 2, Figure 2.2 A,B; Figure 2.3 A,B**). Between the two collagen matrices, type IV collagen enhanced these changes in beta cell behaviours to a much greater extent (**Chapter 2, Figure 2.2 A,B; Figure 2.3 A,B**). Both collagen matrix proteins increased insulin expression within INS-1 cells and stimulated insulin release upon high glucose challenge (**Chapter 2, Figure 2.4 A,B; Figure 2.5**). Taken together, these studies demonstrate that integrin-dependent adhesion on collagen matrices are critical in the regulation of beta cell survival and function.

It has been well established that integrins facilitate the adhesion of cells to the surrounding extracellular matrix (ECM) and to neighbouring cells for the maintenance of tissue integrity and architecture (Brakebusch and Fassler, 2005). Integrin-ECM ligation determines multiple cellular responses, including cell spreading and survival (Chen et al., 2006). Moreover, cell contact with the ECM stimulates the attachment of cells to surrounding substrates, internal extension of filopodia (Giancotti and Ruoslahti, 1999), formation of focal adhesions and further generation of actin-rich lamellipodia (Giancotti and Ruoslahti, 1999). The common mediators of cell spreading are the Rho family of

proteins, including Rac, which establish a positive feedback loop by promoting integrin clustering and further stimulating Rac activation, cell spreading and integrin clustering (Giancotti and Ruoslahti, 1999). Although the signal transduction pathways activated in INS-1 cells upon exposure to collagen I or IV were not explored, it is highly likely that the integrin-dependent adhesion and spreading responses on the collagen matrices were due to the activation of the Rho-family of proteins.

The majority of studies which have characterized the beneficial role of matrix proteins have identified that laminin, either on its own or as part of a larger matrix constituent, is especially important for providing a physical substratum for islet cells thus, promoting growth, survival, differentiation and insulin gene and hormone expression (Choma et al., 2006; Gonzales et al., 1999; Hammer et al., 2004; Manohar et al., 2004). Interestingly, the results presented in this thesis have identified that integrin-dependent adhesion to collagen IV also has beneficial effects on beta cells, supporting cell survival, proliferation and function. In support of the current findings, Kaido et al (2006) have demonstrated that collagen IV increases adhesion of human fetal beta cells and also supports insulin release upon glucose stimulation when compared to fibronectin, laminin and collagen I. This study, taken with ours, suggests that interactions between integrins and collagen IV should also be examined, to gain a better understanding of factors which can be manipulated for improving islet cell based therapies.

**4.3 α3 and β1 integrin interact independently with collagen matrices to enhance INS-1 cell survival and function**

The majority of studies examining the role of the α3β1 integrin in islet cell survival and function have studied the heterodimer in its entirety. In order to identify the individual roles of the subunits and characterize their contributions to beta cell survival and function, the α3β1 integrin was dissected into α3 and β1 parts, and functional blockade studies of each subunit were performed. Our studies revealed that the β1 subunit is a key regulator of beta cell behaviours and function, affecting cell attachment, spreading, viability and proliferation on both collagen I and IV matrices (**Chapter 3, Figure 3.1 A,B; Figure 3.2 A,B**). Interestingly, perturbing β1 integrin function affected adhesion, viability and proliferation to a greater extent on collagen I when compared to collagen IV (**Chapter 3, Figure 3.1 A,B; Figure 3.2 A,B**). Moreover, we also noted decreases in Pdx-1 expression and insulin expression upon functional blockade of the β1 integrin (**Chapter 3, Figure 3.3 A-C**). Lastly, perturbing β1 integrin-collagen interactions had a functional consequence. INS-1 cells demonstrated reduced insulin secretion, upon functional blockade of the β1 integrin, when challenged with low and high doses of glucose (**Chapter 3, Figure 3.3 D).**

Interestingly, functional blockade of the α3 integrin altered beta cell behaviours and function to a much lesser extent. We noted reduced cell attachment, viability and proliferation only in INS-1 cells cultured on collagen IV (**Chapter 3, Figure 3.4 A,C,D**), while cell spreading was significantly reduced on both collagen matrices (**Chapter 3, Figure 3.4 B**). Pdx-1 expression and insulin contents were decreased upon perturbation of the α3 integrin on collagen I and IV, while insulin release was reduced on both

collagen matrices, upon high glucose challenge (**Chapter 3, Figure 3.6 A-D**). These results suggest that the β1 integrin is a key regulator of beta cell survival and function, possibly because of its ability to partner with multiple α subunits. Although subordinate to the β1 integrin, our results demonstrate that the α3 subunit also has a role in the regulation of beta cell survival and function, and possibly has greater affinity for type IV collagen.

The results presented in this thesis have identified that integrins function as regulators of cell survival and enhance expression of beta cell markers, Pdx-1 and insulin, which maintain phenotype and function. Anchorage to the ECM via integrin receptors, confers stability to cells, prevents cell death or "anoikis" and enables proliferation and differentiation (Giancotti and Ruoslahti, 1999). Cell anchorage has also repeatedly been shown to have substantial effects on gene expression by activating highly specialized signaling pathways (Juliano and Haskill, 1993). Specifically, co-operation between Ras-ERK pathways and the actin cytoskeleton have resulted in the regulation of gene expression in response to extracellular stimuli (Chang and Karin, 2001). Although activation of the PI3K pathway has beneficial effects on beta cell function and reinforces the expression of beta cell markers including Pdx-1 and Glut-2 (Li et al., 2007; Lowe 2003; Wente et al., 2006), the majority of these changes are due to growth factor stimulation and not integrin-ECM interactions. The studies presented in this thesis demonstrate that both integrin subunits α3 and β1 modulate beta cell survival and function. The signaling pathways responsible for altering beta cell behaviours, as a result of functional inhibition of integrins, were then subsequently characterized.

**4.4 Identification of highly specialized signaling mechanisms of the α3 integrin which regulate beta cell survival and function.**

By thoroughly examining the molecular signals controlling beta cell survival and function, it was determined that the β1 integrin primarily signals through the FAK/MAPK/ERK pathway to regulate cell survival, promote the expression of beta cell markers and increase insulin secretion upon glucose stimulation (**Chapter 3, Figures 3.7-3.13**). Similarly, the α3 integrin also signals through this pathway to enable beta cell survival and function (**Chapter 3, Figures 3.7-3.13**). Interestingly, the α3 subunit also activates the PI3K pathway to modulate pro-survival and anti-apoptotic signals (**Chapter 3, Figures 3.7-3.13**); however, inhibition of this pathway, through the use of Wortmannin, did not decrease the expression of transcription factor Pdx-1 (**Chapter 3, Figure 3.19**). Since the Akt/XIAP/Caspase 3 and Akt/GSK3β/Cyclin D axes were inactivated upon functional blockade of α3, Akt and its downstream mediators are possibly more involved in the promotion of cell survival and proliferation, and less concerned with cell function and maintenance of phenotype. The use of MEK inhibitor, U0126, confirmed a decrease in the activation of ERK1/2, subsequent increase in the cleavage of caspase 3 and a reduction in Pdx-1 expression, reinforcing the notion that activation of the MAPK pathway in integrin-ECM interactions is essential for both the survival and function of beta cells (**Chapter 3, Figure 3.14-3.15**). These results indicate that the individual integrin subunits have the ability to activate highly specialized signaling cascades for the modulation of beta cell survival and function, *in vitro*. The signaling mechanisms identified in this thesis are depicted in **Figures 4.1 and 4.2**.

## α3 signaling

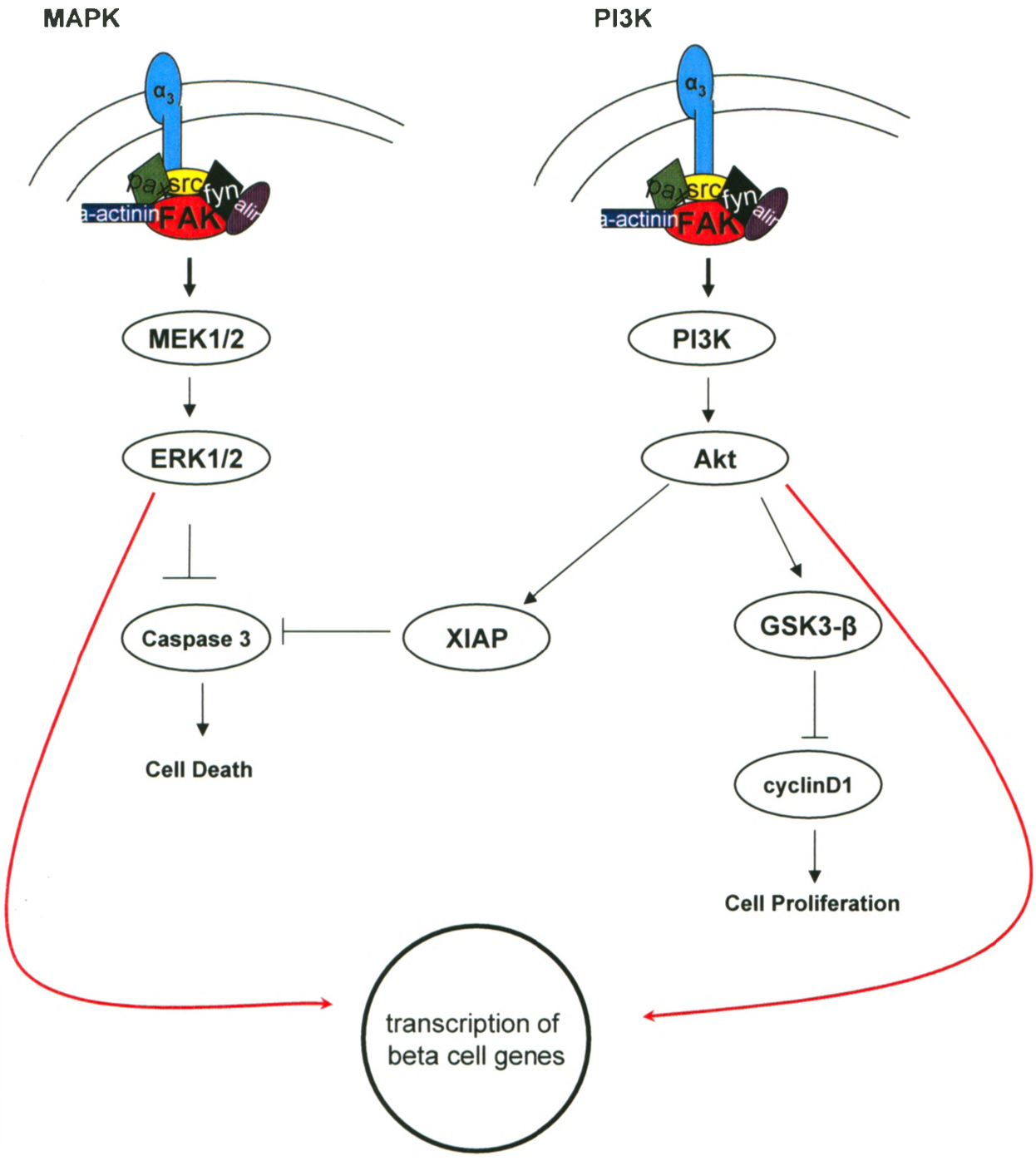


**Figure 4.1** Schematic demonstrating α3 integrin signaling in pancreatic beta cells

## β1 signaling

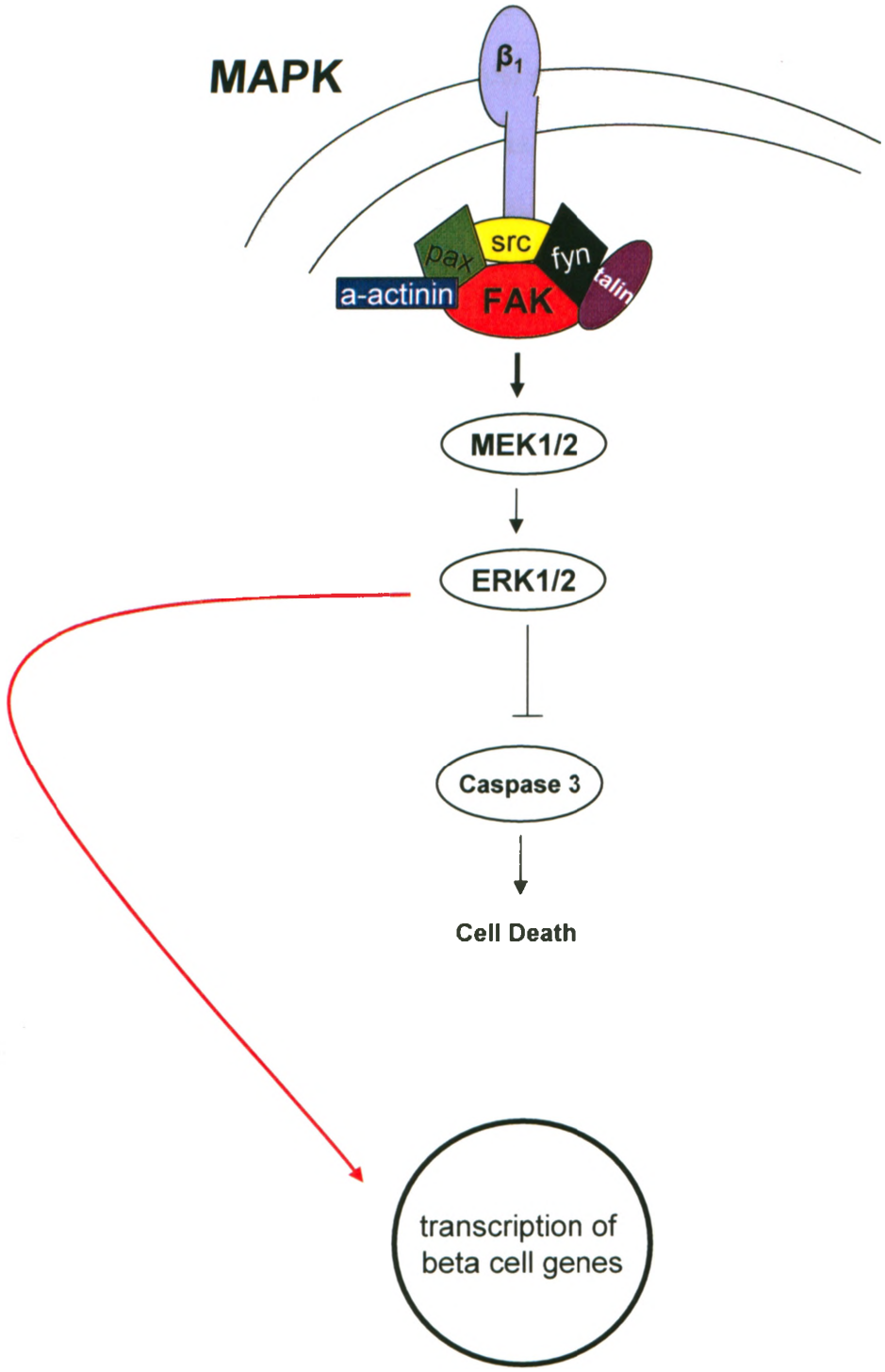


**Figure 4.2** Schematic demonstrating β1 integrin signaling in pancreatic beta cells

Ligands bind to the extracellular surface of integrins, enabling phosphorylation of specific sites on the intracellular cytoplasmic tail, leading to actin cytoskeletal connection and the propogation of downstream signals (Brakebusch and Fassler, 2005). Integrins lack an actin binding domain and are devoid of enzymatic activity, thus, they heavily rely on adaptor proteins and associated molecules to transduce downstream signals. The focal adhesion complex, which directly links to both cytoplasmic tails of the β1 and α3 integrin, serves as a signaling center whereby changes in cell polarity, migration, growth and survival can be regulated (Brakebusch and Fassler, 2003). The cytoplasmic domain of the β1 integrin contains highly conserved motifs essential for the modulation of downstream signals. The NPxY motif, a 16 amino acid stretch (Mulrooney et al., 2001), is found in both β1A and β1D variants and is necessary for the inside-out activation of integrins (Cordes et al., 2006) and outside-in activation of the focal adhesion kinase (Wennerberg et al., 2000). Specific regions within the NPxY domain are necessary for ligand binding, protein-protein-interactions, targeting adaptor proteins to focal adhesions and integrin mediated signaling (Burridge and Chrzanowska-Wodnicka, 1996; Dedhar and Hannigan, 1996; LaFlamme et al., 1997; Schwartz et al., 1995). Interestingly, several studies have revealed that the phosphorylation of specific sites on the β1 integrin cytoplasmic tail can simultaneously enhance and inhibit specific cellular changes. The phosphorylation of S785 promotes the attachment of cells but inhibits cell spreading and migration (Mulrooney et al., 2001). Sequences of amino acid residues, which constitute the cytoplasmic tails, must also be conserved for maintenance of β1 integrin function. *In vivo* replacement of β1 cytoplasmic tyrosines with alanines resulted in the loss of interactions with phosphotyrosine-binding domains and thus, caused a complete loss in

β1 integrin function (Chen et al., 2006). However, replacing β1 integrin cytoplasmic tyrosines with phenylalanine conserved its function, *in vivo* (Chen et al., 2006). Taken together, these results suggest that conservation of specific sites on integrin cytoplasmic tails have critical implications for molecular mechanisms and integrin function.

Similar phosphorylation sites have been recently identified in some α integrins (Liu and Ginsberg, 2000; Liu et al., 1999; Zhang et al., 2001). The paxillin binding site of the α4 integrin was the first to be mapped, and its functional contribution to integrin signaling as well as cellular processes was identified (Liu and Ginsberg, 2000). Similarly, the α3 subunit also contains a recently identified phosphorylation site within its cytoplasmic tail, mapped to S1042 (Zhang et al., 2001), which occurs within a QPSXXE motif. Phosphorylation of S1042 is especially critical for the regulation of signaling, motility, and cytoskeletal engagement (Zhang et al., 2001), since mutations of this site abrogated tyrosine phosphorylation of FAK and paxillin and decreased cell spreading and migration on laminin. These findings, in combination with those presented in this thesis, demonstrate that α3 phosphorylation is functionally relevant, and most likely contributes to the regulation of cell survival and function.

## 4.5 Significance and Future Studies

The characterization of integrin regulation of various cellular behaviours and function in the beta cell is a fundamental starting point for future studies which may involve the manipulation of either precursor or functionally compromised cells into islet cell types. From these studies, the high level of α3 and β1 integrin expression, their interactions with collagen I and IV and their independent signaling mechanisms can be

manipulated for the development of novel cell-based therapies for the treatment of diabetes.

For instance, these integrin-ECM interactions can be applied to stem cell biology. A large body of evidence, along with the findings of the current study, supports the notion that under the appropriate trophic support, stem cells can be induced to differentiate into beta cells, and that integrin dependent adhesion can maintain viability and promote long term survival and insulin release.

Similarly, the relationship between integrin and growth factor receptors can be explored. The co-activation of these receptor types has resulted in their physical association at the plasma membrane, phosphorylation of downstream signaling molecules and synergistic increases in gene expression. In this model, ECM proteins can be thought as a niche, housing multiple molecular signals which can act synergistically to promote the survival and function of islet cell types.

The ultimate goal for alleviating the shortage in donor material, for purposes of islet transplantation or tissue engineering, is to develop a culture protocol, whereby beta cells can be constantly regenerated. Whether the initiatives involve stem cell differentiation or the rescue of functionally compromised tissue, a thorough understanding of integrin/ECM interactions will allow both the derivation and employment of different methods for generating beta cells or manipulation of natural expansion programs for the treatment of diabetes.

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