Role for cell-to-cell communication in stem cell specification toward pancreatic progenitors: relevance to the design of novel therapies for diabetes.

Wendy Yang

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2016

Reading Committee:

Vincenzo Cirulli, Chair

Laura Crisa

Paul D. Lampe

Program Authorized to Offer Degree:

Pharmacology

University of Washington

Abstract

Role for cell-to-cell communication in stem cell specification toward pancreatic progenitors: relevance to the design of novel therapies for diabetes.

Wendy Yang

Chair of the Supervisory Committee:
Vincenzo Cirulli
Metabolism, Endocrinology & Nutrition

Pancreatic islets of Langerhans, responsible for the production of hormones such as insulin and glucagon, develop from pluripotent pancreatic progenitors following their specification toward an endocrine phenotype. Based on the established role of cell-to-cell communication as an important mechanism regulating developmental decisions during embryonic life, I investigated the expression pattern of Connexins (Cxs), the building blocks of Gap Junction channels, in the developing human pancreas, and in an *in vitro* model of pancreatic progenitor differentiation from human embryonic stem cells (hESC). I also investigated the role of β1 integrins and an associated downstream effector, integrin-linked kinase (ILK), on islet development in mice.

In a first series of experiments, I investigated the expression pattern of Cxs in the developing human pancreas. Results from these studies revealed that while Cx32 is predominantly expressed in the acinar tissue, Cx36 is primarily expressed in developing islet β-cells. Cx43 exhibited the most interesting expression pattern, being primarily detected in putative islet cell progenitors that delaminate from the pancreatic ductal epithelium and aggregate with developing islet cell clusters. Building on these exciting findings, and based on the growing interest in defining mechanisms that drive islet cell progenitor development from populations of

progenitors and stem cells, I decided to focus my subsequent studies primarily on the role of Cx43 during stem cell differentiation toward the islet cell lineage.

Consequently, I focused on the analysis of Cx43 expression and function in stem cells during their differentiation along the pancreatic cell lineage, with a specific emphasis on its functional requirement for the induction of early pancreatic islet progenitors, as defined by the acquisition of the transcription factor Pdx1 (**Chapter 3**). The results from these experiments showed that Cx43 is expressed at high levels in Definitive Endoderm (DE) cells, as defined by expression of E-cadherin, FoxA2, and Sox17. Building on these results, I adopted a gain-of-function approach using AAP10, a Cx43 specific peptide that promotes Cx43 phosphorylation and causes constitutive activation (i.e. opening) of Cx43-Gap Junction channels.

Results from these experiments show that AAP10 treatment positively affects the propensity of hESCs to adopt a DE phenotype. Gene expression analysis revealed significantly higher levels of FoxA2 and Sox17 transcripts in DE populations treated with AAP10, compared to untreated controls. This effect was correlated with larger Cx43 Gap Junction plaques. Finally, to investigate the developmental consequences of increasing Cx43 Gap Junction plaques by AAP10 treatment, I monitored the expression of Pdx1 and Nkx6.1, two transcription factors required for islet cell development and differentiation, at the end of the differentiation protocol. My results indicated that AAP10 treatment during the early stages of hESC differentiation lead to a robust increase in the number of $Pdx1^{+}$ pancreatic progenitors, when compared to untreated cells. Collectively, these results demonstrate that the purposeful activation of Cx43 Gap Junction channels at the early stages of hESC commitment toward DE cells leads to higher yields of pancreatic progenitors during the directed differentiation of hESCs toward pancreatic cell lineages.

My next set of experiments focused on the knockdown of Cx43 in the derivation of pancreatic progenitors from hESCs (**Chapter 4**). Since gain-of-function studies showed a role of Cx43 in increasing the derivation of DE cell types, I hypothesized that knockdown of Cx43 would have the opposite effect. A targeted siRNA approach did show knockdown of Cx43, however, I observed multiple off target effects in mock and scramble transfections with this approach. Therefore, I moved to an shRNA approach specifically targeting Cx43. In these studies, I observed

that targeted knockdown of Cx43 led to inhibition of Pdx1, Nkx6.1 and Insulin gene expression. Hence, these studies validated the requirement for Cx43 expression at the DE and PGT stages for hESC to effectively progress toward pancreatic endocrine progenitors.

In parallel studies, based on the notion that Cx43 can also affect integrin-mediated cell adhesion and migration[1], and building on our laboratory's established experience on the role of integrin receptors as regulators of pancreatic progenitors cell adhesion, growth and differentiation[2], I studied the role of β1 integrins in islet cell development and function (**Chapter 5**). For these investigations, I focused on a loss-of-function approach using a Cre-lox strategy that made use of an insulin promoter-driven DNA Cre recombinase, cross-bred to β1 integrin-floxed mice[3]. The results of these studies showed that ablation of the β1 integrin gene in developing β-cells resulted in a dramatic reduction of the pancreatic β-cell mass. Based on the observation that ablation of β1 integrin caused a reduced expression of genes regulating cell cycle progression, we interpreted these results as being caused by a defective β1 integrin signaling which in turn negatively impacted β-cell expansion. Surprisingly, these mice were not diabetic, and their islet clusters showed normal architectural organization, and normal expression of mature β-cell markers. Collectively, these results demonstrated that β1 integrin receptors function as crucial positive regulators of β-cell expansion.

Building on these results, I investigated the role of downstream effectors of β1 integrin signaling, and focused my efforts on the function of integrin linked kinase (ILK) on pancreatic development (**Chapter 6**). The first set of experiments determined the localization of ILK within pancreatic cells. Immunostaining showed co-expression of ILK and β1 integrins in the pancreatic epithelium. Next, I examined the impact of ILK inhibition by Cpd22 on pancreatic ductal cell proliferation using the G3LC and SU86 cell lines. ILK inhibition by Cpd22 resulted in a dramatic decrease in BrdU incorporation. Interestingly, inhibition of ILK by Cpd22 positively impacted the gene expression levels of insulin, Pdx1, and Cx36. Next, I conducted *in vivo* experiments focusing on the generation of knockout mice in which I targeted the ablation of ILK to early pancreatic progenitors using a Pdx1-Cre transgenic mice crossed with ILK-floxed mice. Preliminary results from these studies revealed that deletion of ILK in $Pdx1^{+}$ pancreatic progenitors phenocopied our earlier animal model of β1 integrin deletion in β-cells[3]. I noticed that Pdx1-Cre/$ILK^{-/-}$ mice had a

significantly reduced islet cell mass. Interestingly, Pdx1-Cre/ILK$^{-/-}$ mice exhibited a mild glucose intolerance in spite of a dramatic reduction in β-cell mass. This is at variance with the β1 integrin mutant mice which did not show a diabetic phenotype. These results open up an avenue to investigate if the Pdx1-Cre/ILK$^{-/-}$ phenotype is to be attributed to the reduced islet cell mass, or to a direct effect of loss on ILK function on insulin secretion.

Together, these studies illuminate an important role for mechanisms of cell-to-cell communication in the development and function of pancreatic islet cells. Furthermore, the new approaches tested in our experiments of stem cell differentiation toward islet cells may contribute a significant improvement of current protocols for the derivation of islet β-cells to be used as a possible cell replacement therapy for type 1 diabetes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Full name
Cx	Connexin
DE	Definitive Endoderm
EP	Endocrine Progenitors
GJ	Gap Junction
hESC	Human Embryonic Stem Cell
hGH	Human Growth Hormone
ILK	Integrin Linked Kinase
iPSC	Induced Pluripotent Stem Cell
ME	Mesendoderm
PGT	Primitive Gut Tube
PP	Pancreatic Progenitors
RIP	Rat Insulin Promoter
STZ	Streptozotocin
T1D	Type 1 Diabetes

ACKNOWLEDGEMENTS

"We have done the impossible and that makes us mighty." –Malcolm Reynolds, *Firefly*

I am forever grateful for the support network I had during my tenure as a graduate student. This work would not have been completed without my mentors Vincenzo Cirulli and Laura Crisa. Thank you both for setting me down a path of discovery, feeding me real Italian food, and countless espressos and chocolates. I also need to recognize Pat Kensel-Hammes, Stephanie Pardike, and Jeff Vercollone for their hours of discussion, camaraderie, and baked goodies over the years.

I would like to thank my thesis committee: Stan McKnight, John Scott, Bill Mahoney, and Paul Lampe for their years of guidance. In addition, I never could have graduated without the expertise of the Stem Cell Core headed by Carol Ware, and the support of administration in both ISCRM and Pharmacology: Kris Vosk, Leena Pranikay, and Diane Schulstad.

I am fortunate to have great friends who have helped me along this path in a myriad of ways. Becca Minich, Jenn Deem, Darragh Kerr, and Jamie Kuhar for teaching me to be brave. Nick Strand for being a near daily sounding board for science and fantasy football. My sailing family and Zephyr crew for racing in Lake Union and the Puget Sound. Serena Wang for being my constant cheerleader and forever friend. And, Erick Thompson for being my teammate, my unwavering bastion of support, and a constant source of mirth.

Finally, I have to thank my family. My parents, King and Jane, have always encouraged me to pursue my interests no matter how farfetched and far flung they would take me. My brother, Wayne, will always be my psychic twin despite our 6 year age difference. My sister-in-law, Becky, has shared a love for figure skating outfits and tiny adorable things, and my nephew, Malcolm, is an adorable delight.

DEDICATION

To my grandfather who would have been delighted to crown another Dr. Yang.

Chapter 1.

INTRODUCTION

1.1 OVERVIEW

This chapter provides a literature review on studies that have defined basic mechanisms of cell-to-cell communication as crucial regulators of cell fate specification and maturation during development. Specifically, I will focus on the role of Connexins, the building blocks of Gap Junctions, as regulators of an exquisite form of direct cell-to-cell communication, both in the developing pancreas and in stem cell differentiation toward the pancreatic islet cell lineage. Topics covered also include the role of integrins and their downstream effectors in pancreatic development and an overview of Type 1 Diabetes as a disease model that could one day benefit from stem cells as a source of insulin-producing cells for transplantation.

1.2 GAP JUNCTION CONNEXINS

1.2.1 *Gap Junctions Lifecycle*

Gap junctions (GJs) form channels between contacting cells that allow for a direct route of cell-to-cell exchange of biochemical signals, providing a specialized mechanism for adjacent cells to communicate with one another while bypassing the lipid bilayer and the extracellular space[4,5]. GJs are made up of connexins (Cxs), a family of proteins which regulate the assembly and function of these channels. Cxs are highly conserved across species[6], with 20 types found in mice and 21 in humans[7].

Cxs are synthetized in the endoplasmic reticulum as monomers. Distinct Cxs are named after their molecular weight (e.g. Cx32, Cx36, Cx43 have molecular weights of 32kDa, 36kDa and 43kDa, respectively). Cxs monomers have a structure of 4 transmembrane domains, with both the N and C terminal tails in the cytoplasm[8]. The C-terminal tail harbors multiple phosphorylation sites that are important for the regulation of internal signaling cascades such as PKC, MAPK, and Src kinase[9], whereas the N-terminal tail is the site of the gap junctional voltage sensor[10]

important for voltage gating of the channel (i.e. opening and closing of the channel). Upon synthesis, Cxs monomers assemble into hexameric hemichannels termed Connexons with oligomerization occurring in the Golgi/trans-Golgi network[11]. Connexons from adjacent cells dock together to form full homomeric GJ channels[7]. Building further, individual GJ channels can subsequently dock together in a *cis* fashion and form clusters of channels referred to as "plaques". Through these tertiary and quaternary levels of protein assembly, GJ plaque size, determined by the number of GJ channels in *cis* aggregation, determines the amount of signal that can be exchanged between contacting cells[12]. The size of the channel itself can also determine what kind of signals may pass between cells[13,14].

Fully formed GJs can have a turnover time of between 2 and 4 hours[15–19]. Cxs are pulled as full GJs into one cell or the other as opposed to adjacent cells pulling apart the gap junction into hemichannels again[20]. They are then degraded within multivesicular endosomes[21] by proteasomes and lysosomes[18].

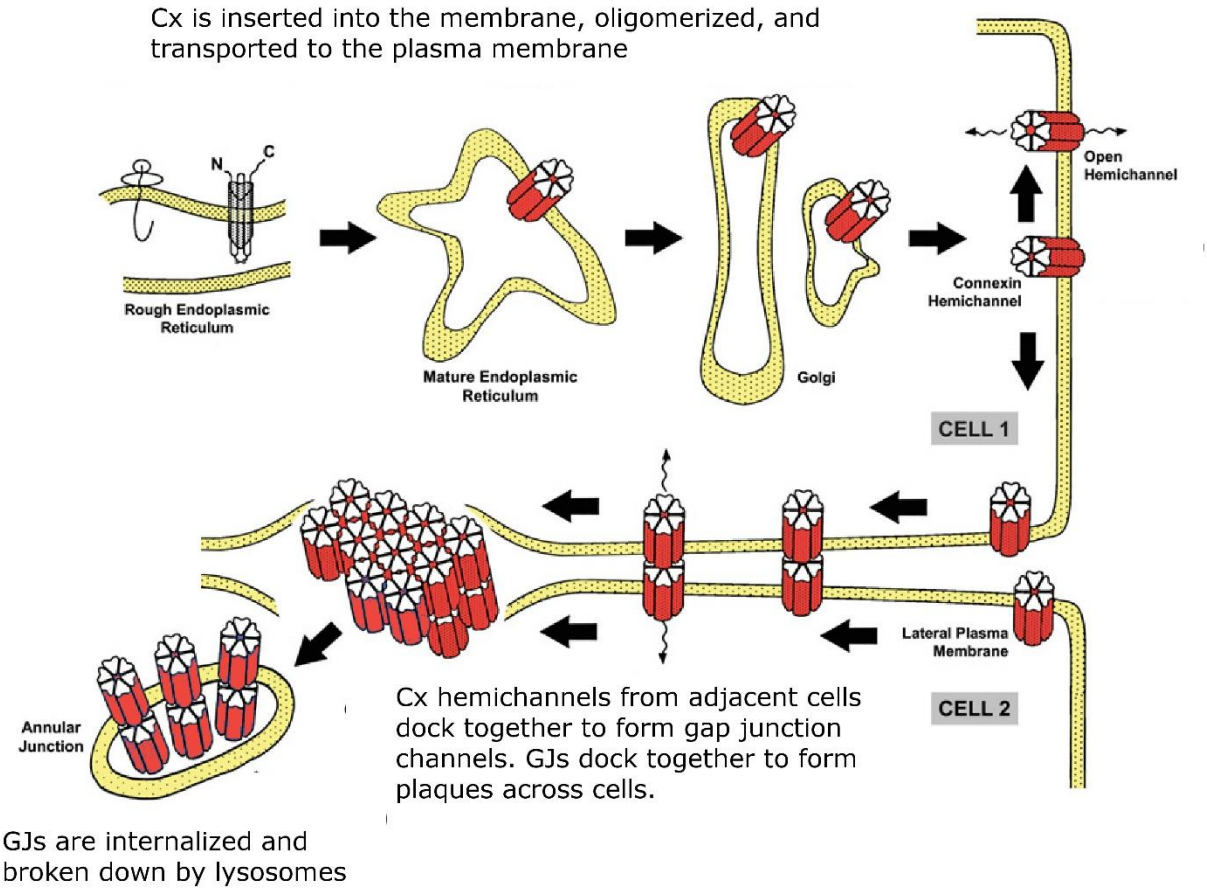


Figure 1-1 *Connexins lifecycle*

Cxs are synthesized in the endoplasmic reticulum as monomers. They mature and oligomerize into connexon hemichannels. These hemichannels are transported to the plasma membrane where they are then available to dock with hemichannels from adjacent cells in a homomeric fashion. Fully formed Gap Junctions are able to aggregate into plaques in a cis manner. When GJs are recycled, they are engulfed as a whole into one of the adjacent cells and internalized and broken down by multivesicular lysosomes (adapted from Evans 2006[5]).

1.2.2 *Gap Junctions Function and Expression*

GJs are expressed throughout the body in virtually all tissues in tissue-specific spatio-temporal patterns. **Table 1-1** summarizes tissue specific localization of Cxs 26, 32, 36, and 43 which are most important for pancreatic development.

Table 1-1 *Pancreatic specific Cxs cell type expression, functions, and related diseases*

Cx	Cell Type	Function	Disease
26	Keratinocyte[22,23], Hepatocyte[24], Parietal cell, Acinar cell, Alveolar cell, Pinealocyte, Neuron, Thyrocyte	Exocrine secretion, Peptide hormones, Glycoproteic hormones, blood-transported molecules	Deafness, Skin disease[25]
32	Hepatocyte[24], Schwann cells[26], Acinar tissue[27], Parietal cell, Alveolar cell, Pinealocyte, Neuron, Thyrocyte	Enhances secretion of amylase from exocrine pancreas[27], exocrine secretion, peptide hormones, amine hormones, blood-transported molecules	X-linked Charcot-Marie-Tooth[28], acute pancreatitis/reduced acinar cell apoptosis[29]
36	Retina[30], Neurons[31], □-cells[32]	□-cell synchronization[32,33], amine hormones	Inhibition can protect against glaucoma[34]
43	Keratinocyte, Cardiomyocyte, Astrocyte, Endothelial cells, Undifferentiated embryonic stem cells[15], Leydig cells, Sertoli cell, Spongiocyte, Chief cell, C cells, Basophil cell, Acidophil cell, Neuron, Granulosa cell, Luteal cells, Alveolar cell	Maintenance of pluripotency[35], Cardiac synchronicity[36], Steroid hormones, peptide hormone glycoproteic hormone	Cardiac arrythmia[37], Oculodentodigital dysplasia[38]

(adapted from Serre-Beinier et al 2002[39])

Intercellular channels formed by Cxs allow for the passage of many signals between cells, including ions, cell survival and death signals[40], as well as siRNAs and small peptides[41,42]. Some GJs are also highly selective for charged particles[43]. For example, Cx36 in the pancreas and Cx46 in Xenopus appear to have high selectivity for cations[44,45].

A number of Cxs mutations have been linked to tissue dysfunction. Specific examples demonstrating the integral importance of Cxs' function in different cell type development, survival, and function include Cx46 and Cx50 in which single point mutations can lead to cataracts[46], Cx26 in which loss of function leads to deafness[25], and Cx43 in which defects lead to

cardiac arrythmias[47]. Overexpression of Cxs can be equally problematic. Overexpression of Cx26 in melanoma cells may lead to increased levels of metastasis[48] and overexpression of Cx43 in glioma cells can lead to suppression of tumor growth.

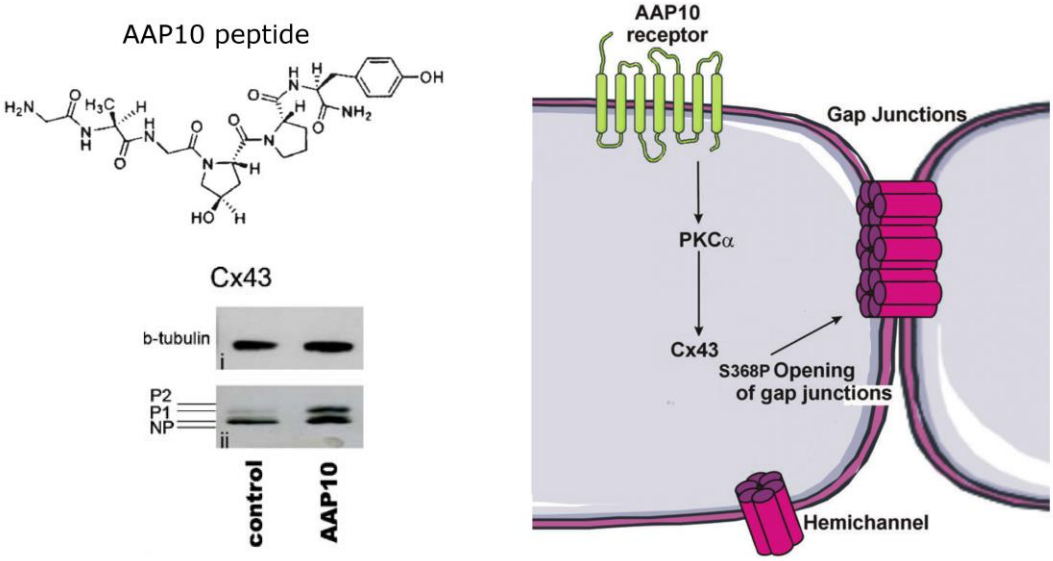
Interestingly, Cx43 function has also been linked to the maintenance of a pool of dividing cells during fin regeneration in zebra fish[49]. Finally, of significance of the subject of study of my thesis project, Cx43, and to a lesser degree Cx45, have been shown to be required for the maintenance of stem cell pluripotency[50].

1.2.3 *Gap Junction Manipulation*

Cxs function can be manipulated in a multitude of fashions. However, many of these approaches (e.g. knock-down) present significant challenges due to either redundancy in polypeptide sequences in multiple Cxs, and/or non-specific reactivity with other regulatory proteins. Consequently, beyond gene knock-out approaches, the use of chemicals such as oleic acid and arachidonic acid have been successfully used to change voltage gating of Cxs[51,52]. Similarly, 18 and 18 glycyrrhetinic acid, carbenoxolone, and heptanol[53] can be used as global Cxs inhibitors[54]. Another example is Tamoxifen which can be used as an inhibitor of gap junctions in a reversible manner, although some off target effects on other types of channels have been noted[55].

On the other end, significant attention has been devoted to the identification of "gain of function" reagents that could be used to promote GJ function. These reagents include rotigaptides[56], anti-arrythmic peptides[57], and Cx mimetic peptides[58–60]. Among them, the AAP10 peptide has been shown to positively regulate Cx43 phosphorylation, and Gap Junction channel opening, thus enhancing cell-to-cell communication and increasing Cx43-mediated cardiomyocyte synchronization (**Figure 1-2**). Thanks to these properties, this peptide has been used with success as an anti-arrhythmic drug. These applications have led to the notion that the purposeful manipulation of Cxs function in various cell types can be exploited to modulate cell function. Thus, it has been observed that these peptides can influence adoption of specific cell fates during development, survival and differentiation[40,61]. Conversely, pharmacological

blockade of Cxs leads to de-synchronization of cells and apoptosis[62,63]. Other examples include experimental conditions in which blockade of Cxs can also have cell protective effects in cells during mechanical injury[64]. In this case, blocking Cxs from passing death signals between coupled cells lead to greater cell survival. Thus, it appears that the spatio-temporal expression pattern of select Cxs plays important roles in supporting the correct development, survival, and functional maturation of a number of cell types.



Adapted from De Vuyst et al 2011 *Br J Pharmacol,* Easton et al 2009 *Naunyn-Schmied Arch Pharmacol*

Figure 1-2 *AAP10 structure and function*

The AAP10 sequence is H-Gly-Ala-Gly-Hyp-Pro-Tyr-NH(2). Its effects can be visualized by Western Blot with clear phospho-laddering seen in the peptide treated sample. AAP10 works via a PKC mechanism that leads to phosphorylation of Cx43 channel at S368 residue resulting in constitutive channel opening. (Adapted from De Vuyst et al 2011[57], Easton et al 2009[65])

1.2.4 *Non-Conventional Gap Junction Assembly and Functions*

Although for the most part, Cxs assemble into GJ channels in a homomeric fashion, (i.e. each channel is made by a single Cx type), there are some examples of heterotypic GJ formation. Examples of these rare instances include chimeric heterotypic channels[41,66–68] in a number of cell

types, such as Cx30/Cx32 in the brain[69], Cx26/Cx32 in the liver[70], Cx26/Cx30 in the cochlea[71], and Cx46/Cx50 channels in lens cells[72].

Also, just recently recognized, is the ability of some Cxs hemichannels (CxHcs) to mediate intracellular signaling prior to their assembly into fully functional Gap Junction channels. For example, CxHcs have been shown to be indispensable for the proper growth, differentiation, and functional maturation of many cell types in different tissues, both during embryonic development and in postnatal life[40,61]. CxHcs have also been shown to play an important role in the facilitation of intracellular ATP concentration and in cell decisions between apoptosis *versus* necrosis[73,74]. Finally, CxHcs have been shown to be activated by ATP depletion which could lead to transference of apoptotic signals across cells[75].

1.3 A ROLE FOR CONNEXINS IN THE PANCREAS

Significant evidence has demonstrated that Cxs 26, 32, 36, and 43 are important for proper development toward a fully mature pancreas, and for its secretory functions[32,33,76] (**Figure 1-3**). For example, Cx32 has been shown to control proper secretion in exocrine cells[76]. Accordingly, Cx32 knockout mice were shown to exhibit increased basal levels of amylase secretion from acinar tissue[27]. It has also been shown that mice deficient in Cx32 experienced a severe form of acute pancreatitis and exhibited a decreased sensitivity to apoptotic signals in acinar tissue[29]. These results suggest that Cx32 regulates growth in acinar tissue.

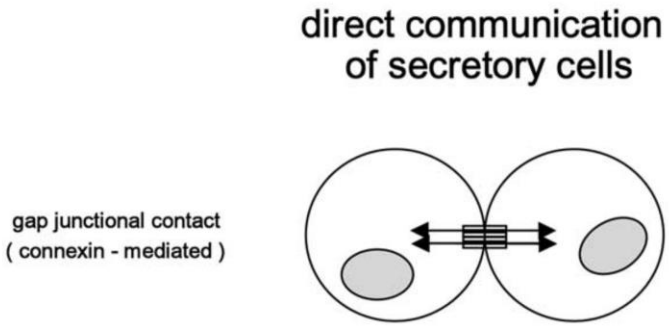


Figure 1-3 *Connexins allow for direct communication between secretory cells.*

Gap Junctions are able to bypass extracellular space. Connexins mediate this communication by linking the surfaces of adjacent cells. Adapted from Serre-Beinier et al 2002[39]*.*

In yet another set of experiments, Cx32 was overexpressed in mice using an RIP driven transgene[76]. Transgenic mice expressed Cx32 in islet cells and showed increased coupling of pancreatic β-cells. However, these mice also showed decreased output of glucose stimulated insulin secretion compared to normal mice despite similar numbers of islets between groups. These results suggested that Cx32 may positively regulate islet cell expansion during the first two weeks of perinatal life. This also suggests that Cxs are tightly and delicately regulated within the pancreas to control β-cell secretion.

Previous work in Dr. Cirulli's lab has established that Cxs 26, 32, 36, and 43 can all be found expressed in adult pancreatic tissue. Of special interest is the expression of Cxs 36 and 43 in islet cells, representing only ~2% of total pancreatic cell mass (**Figure 1-4**). Research has shown that Cx43 is expressed in α-cells[77] and Cx36 is expressed in β-cells[78], pointing to the cell type specificity of Cxs expression.

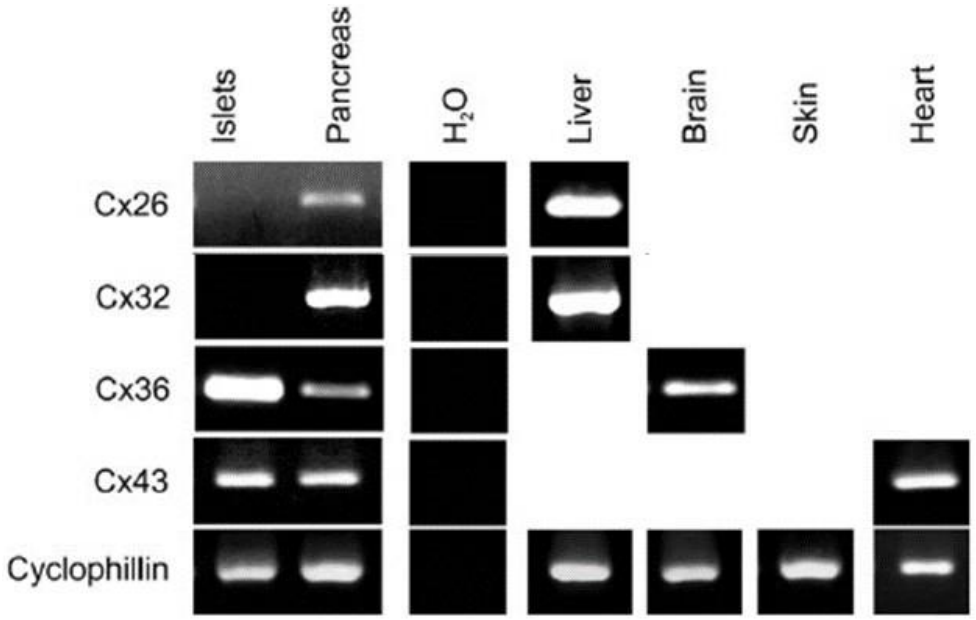


Figure 1-4 *Cxs expression in adult tissue*

Different cell types exhibit distinct Cxs expression patterns. Islet cells harbor a distinct Cxs expression profile when compared to total pancreas. Cx43 is expressed in islet cells as well as other cell types which populate the pancreas such as endothelial cells (Adapted from Serre-Beinier et al 2009[78]).

These results show that Cx36 is the most prominently expressed GJ protein in islet β-cells[79] (**Figure 1-5**). Cx36 has also been shown to control secretion in endocrine cells[33,80] and is required for synchronization of islet β-cells[78,81]. Other studies have reported that overexpression of Cx36 can protect islet β-cells against toxic insult[82]. In these experiments WT rats and rats overexpressing Cx36 were treated with streptozotocin (STZ), a drug that shows a selective uptake and toxicity in islet β-cells and is therefore used to generate a surrogate model of insulin-dependent diabetes. Rats expressing a higher copy number of Cx36 were able to survive the toxic insult and maintain normal levels of insulin production. It has been suggested that this is due, in part, to survival signals passed between β-cells via Cx36[82]. In agreement with these properties attributed to Cx36, it has also been shown that this GJ protein can exert anti-apoptotic and regenerative functions in models of β-cell toxicity and pancreatic injury[83]. These results demonstrate that Cx36 regulates important functions in pancreatic β-cell biology.

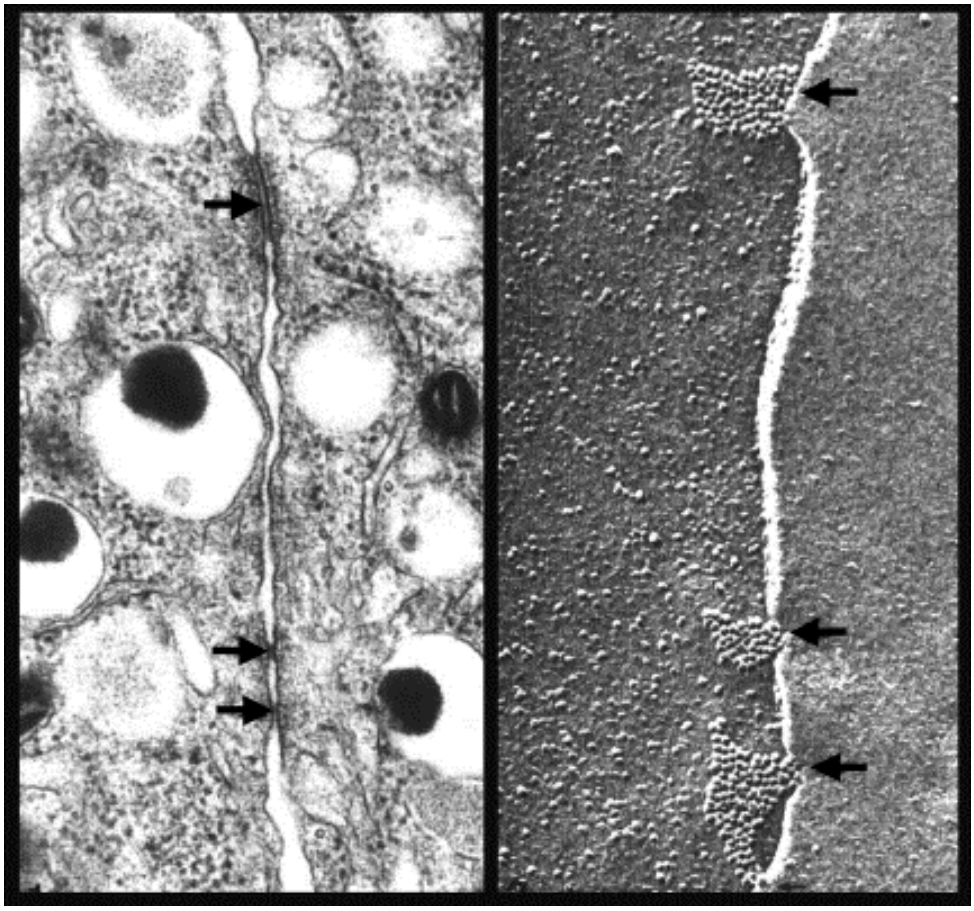


Figure 1-5 *Islet β-cells are connected by Cx36*

The left panel shows a TEM image of islet β-cells side by side. The arrows point to the 'gaps' that gap junctions are named for. The right panel shows a cryo-fracture SEM image of Cx36 in β-cells. The arrows point to gap junction plaques in a top down view. Courtesy of Paolo Meda, Geneva, Switzerland.

Building on these observations, previous work in Dr. Cirulli's lab has also shown that overexpression of Cx32 and Cx36 have significant effects on pancreatic hormonal expression. Thus, in human fetal pancreas, lentiviral-mediated overexpression of Cx32 and Cx36 revealed distinct functional involvement in the regulation of insulin and glucagon gene expression. While overexpression of Cx32 led to a decrease in islet hormone expression, overexpression of Cx36 led to a significant increase in these hormone specific transcripts (**Figure 1-6**). These results point to

divergent functions of these two Cxs as regulators of the acquisition and maintenance of an endocrine phenotype in immature pancreatic progenitors.

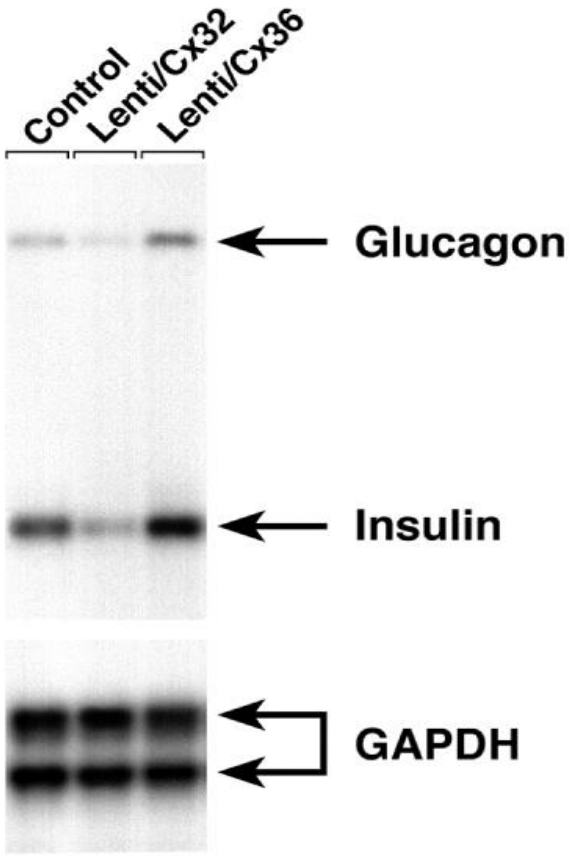


Figure 1-6 *Lentiviral overexpression of Cx32 and Cx36 in human fetal pancreatic progenitors*

Human fetal pancreatic progenitors were infected with lentiviruses expressing either Cx32 or Cx36. Overexpression of these two Cxs had distinct effects on islet hormone expression. Thus, while Cx32 led to a decrease in insulin, glucagon specific transcripts, Cx36 led to an increase in these hormones.

Beyond the aforementioned effects of Cxs found expressed in select pancreatic cell lineages, Cx43 is perhaps the most interesting, as our laboratory has found that it is primarily expressed in pancreatic islet progenitors that appear to delaminate from the pancreatic ductal epithelium[79]. Intriguingly, following endocrine differentiation, these islet progenitors down-regulate Cx43 and acquire Cx36. Equally interesting is the fact that Cx43 expression increases

during pancreatic cancer progression[84]. Collectively, these observations point to an important role of Cx43 in the development, delamination and possibly the migration of islet cell progenitors from ductal domains into the surrounding mesenchyme, where they proceed with their differentiation toward an endocrine cell phenotype upon acquisition of Cx36. **Figure 1-7** shows examples of the distinct expression patterns of Cx32, primarily detected in acinar tissue (**Figure 1-7**A); Cx36 found mostly in developing islet b-cell clusters (**Figure 1-7**B, arrowheads); and Cx43 detected in putative islet progenitors as soon as they arise from the ductal epithelium and associate with developing islet clusters (**Figure 1-7**C).

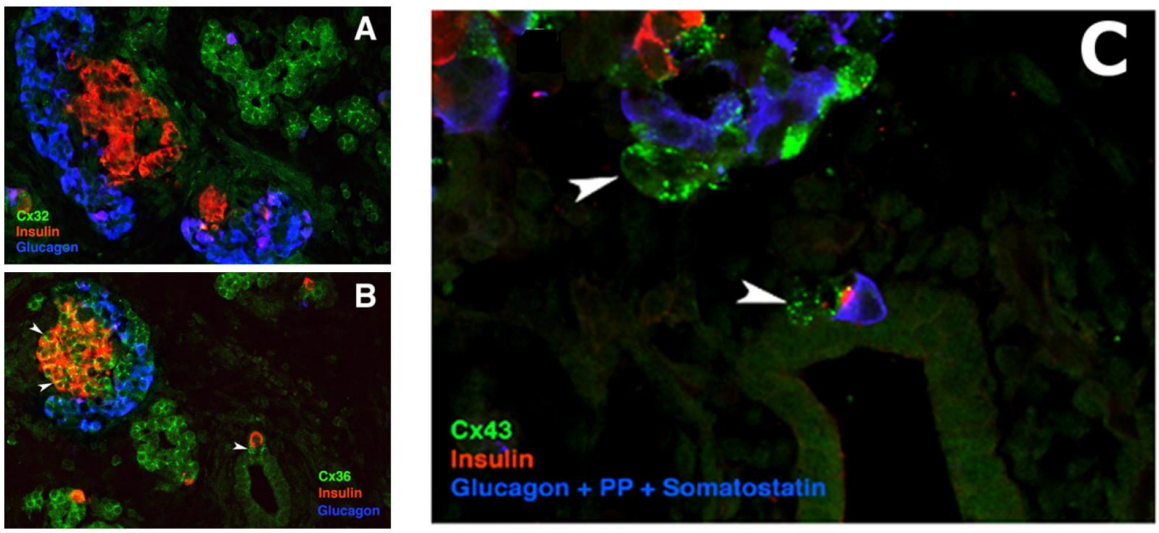


Figure 1-7 *Cx32, 36, and 43 expression in human fetal pancreas*

*Cxs are labeled in green, Insulin in red, Glucagon in blue. (**A**) Cx32 is expressed in acinar tissue and not in islet cells. (**B**) Cx36 is primarily expressed in developing □-cells (arrowheads). Cx36 expression becomes progressively restricted to insulin producing cells with maturation. (**C**) Cx43 is detected in hormone-negative islet progenitors either delaminating from the ductal epithelium or associated with developing islet clusters (arrowheads).*

1.4 Other Cell Adhesion and Communication Molecules

Besides Cxs, the developing pancreatic epithelium expresses a number of other cell surface proteins that regulate homotypic and heterotypic cell interactions and communication with the tissue microenvironment. Of particular interest to our lab are integrins which comprise a family of α/β heterodimeric receptors (**Figure 1-8**) that mediate the recognition of ECM components and elicit the activation of bidirectional signaling from both the inside and the outside of cells. 'Inside-out' signaling occurs when intracellular biochemical signals induce integrins to bind to their matrix ligands[85,86], whereas 'outside-in' signaling results from the binding of a given ECM component by cognate integrin receptors[87]. This ECM binding, in turn, promotes integrin association with the actin cytoskeleton and activates signaling pathways inside the cell[88]. While the α subunit regulates specificity for the recognition of ECM ligands, the β subunit functions as a signal transduction module[85,87–90]. Through this dual partnership of ligand specificity and signaling properties, each α/β integrin heterodimer transduces extracellular cues into distinct cellular responses as diverse as cell adhesion, migration, proliferation, differentiation and cell survival[88,89,91,92].

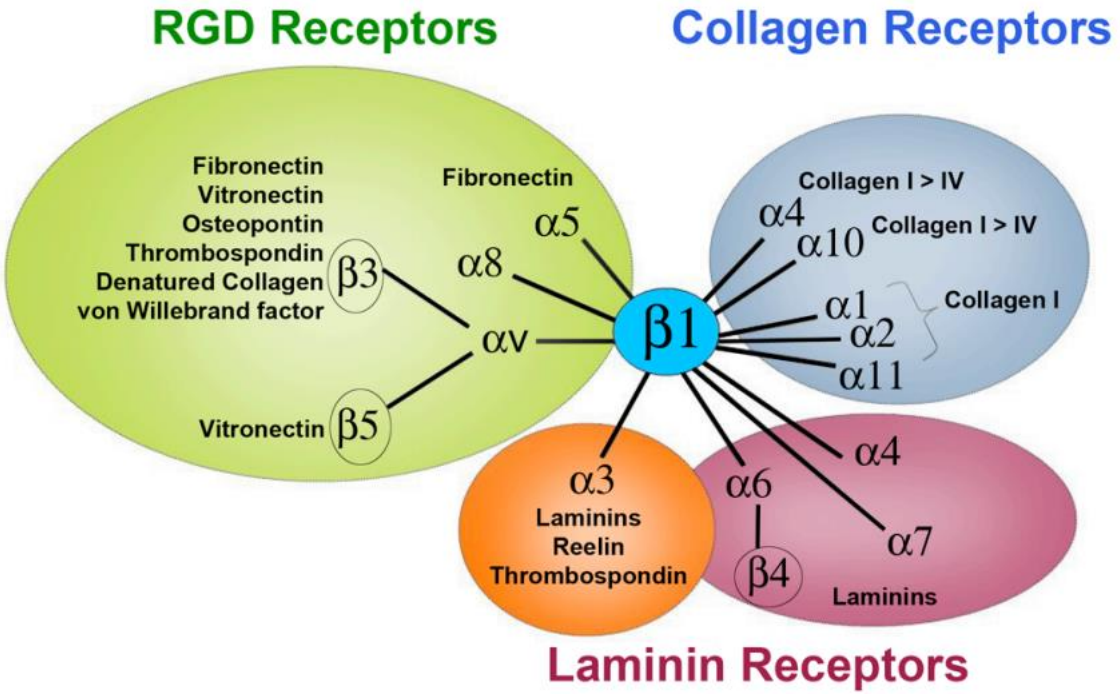


Figure 1-8 *Integrin receptors and cognate ECM ligands expressed in epithelial tissues*

1.4.1 *Role of Integrins Within the Developing Pancreas*

Several *in vitro* studies using embryonic pancreatic epithelium have shown that integrins regulate cell adhesion and migration[2,93–95], cell differentiation and proliferation[93,96,97], as well as secretory functions in pancreatic endocrine cells[97,98]. Specifically, whereas integrins αvβ3, αvβ5 and α6β4 regulate cell attachment to specific ECMs and the migration of undifferentiated pancreatic epithelial cells from ductal compartments[2,94], β1 integrin functions encompass regulation of cell proliferation and differentiation[93,95,97,99]. A few studies have addressed the function of β1 integrins in the developing pancreas *in vivo* by targeting either collagen type I-producing cells[100] or acinar cells[101]. However, virtually nothing is known about the requirement of β1 integrins in the development of the endocrine cell lineage, as represented by the islets of Langerhans[102] (P. Langerhans, PhD thesis, Friedrich-Wilhelms Universität, Berlin, Germany, 1869). Development of the endocrine compartment of the pancreas occurs through a series of highly regulated events involving branching of the pancreatic epithelium, specification and delamination of islet progenitors from ductal domains, followed by their differentiation, expansion, and three-dimensional organization into islet clusters[103]. Among these processes, mechanisms regulating islet cell expansion are crucial for the establishment of a suitable β-cell mass that will ensure adequate insulin secretion in response to normal and modified metabolic demands throughout life.

1.4.2 *Downstream Effectors of Integrin Signaling*

Integrin adhesion complexes function as signaling platforms that transduce biochemical information from the extracellular environment into the cell. Many elements of this outside-in signaling network have been identified[87,104] including integrin linked kinase (ILK).

ILK (**Figure 1-9**) is perhaps one of the most interesting effectors of integrin signaling. It is a protein that plays a major role as a signaling scaffold at sites of integrin adhesion, with a significant and preferential interaction with β1 integrins[105–110]. ILK forms a heterotrimeric complex with the LIM-domain protein PINCH and the actin- and Paxillin-binding protein Parvin.

This complex serves as a "hub" in integrin signaling networks and, in mammalian cells, formation of this complex precedes, and is required for, correct targeting of its components to integrin-mediated adhesions. ILK-integrin complex formation also protects its components from proteosomal degradation[105–107], thus making this signaling module a spatially and temporally stable transducer of integrin signaling. ILK also contains an N-terminal Ankyrin-repeat domain that mediates protein interactions with PINCH1 or PINCH2, and a C-terminal kinase domain that supports interactions with α-, β- or γ-Parvin, Paxillin and β integrin tails[110,111]. Importantly, while ILK signaling can be activated by a number of adhesion receptors, growth factors, and RTKs, its ability to directly interact with β1 integrins[105–107,112] allows this kinase to function as a critical switch of "outside-in" growth and survival signals mediated by this class of adhesion receptors[88,90].

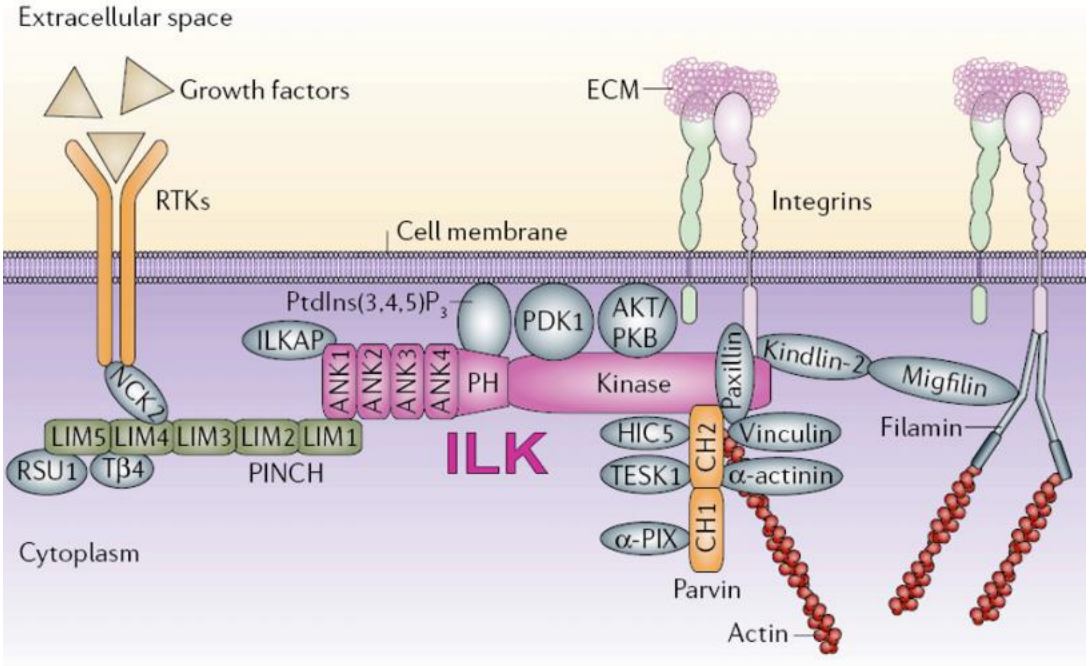


Figure 1-9 *Integrin-linked kinase (ILK) is a downstream effector of β1 integrin signaling. Adapted from Legate et al 2006[109].*

Although lacking the catalytic residues that are normally conserved among protein kinases, ILK plays a central role in integrin signaling and cytoskeletal connections. Hence, ILK activity can be placed directly downstream of ECM-integrin initiated functions such as adhesion,

migration, proliferation, differentiation and survival[105–107], including regulation of pancreatic development.

1.5 Type 1 Diabetes as a Disease Model for the Translational Application of Mechanisms of Cell Adhesion and Communication

1.5.1 *Type 1 Diabetes Pathology and Treatment Options*

Type 1 diabetes (T1D), also referred to as insulin-dependent diabetes, is a metabolic disease that affects about 3,000,000 children in the U.S. alone. On average, ~20,000 new cases diagnosed every year. Collectively, beyond the devastating toll on the quality of life, there is a staggering economic burden that has been estimated at about $174B per year[113].

T1D results from an autoimmune destruction of insulin-producing β-cells found within the pancreatic islets of Langerhans. Loss of insulin producing cells leads to an inability to manage blood glucose, a critical homeostasis necessary for living a normal life. Without tight control, this can lead to acute life-threatening complications such as severe hyperglycemia leading to ketoacidosis and coma, or episodes of lethal hypoglycemia resulting from insulin injection overdoses. In addition, a large number of patients with T1D, despite tight metabolic control will eventually develop long-term complications that include an increased risk for cardiovascular disease[114,115], retinopathy[116,117], nephropathy[118], and peripheral neuropathies[119].

Islet β-cells have a very limited regenerative capability[120] which is one reason that T1D persists throughout an entire lifetime. Currently, the most widely used effective treatment is multiple daily injections of insulin. However, recent advances also consider the use of smart insulin patches[121] and even a bionic pancreas[122].

Islet transplant is another option for replacing lost β-cell function[123,124]. Some of the earliest islet transplant recipients were able to achieve insulin independence for up to 3 years[125], with a modest yearly increase in success rate since the establishment of the Edmonton protocol in 2000[126]. However, of all the patients thusly treated, so far only 58% have been able to attain insulin independence during the trials. These results have been attributed to fraft failure, and 76% of subjects had to restart insulin therapy after 2 years[127]. In addition, 5 year follow-ups

showed that while many patients maintained the presence of C-peptide (~80%), indicating endogenous insulin production by remnant islet β-cells, few (~10%) were actually insulin independent. Overall, this data suggests that many transplants will have to be repeated within 2-5 years of the original surgery[128] leading to an increased demand on the already strained donor supply of islet cells for transplant.

Considering the limited supply of organ donors and variability in quality[129] of donor tissue for transplantation, recent progress in stem cell biology and regenerative medicine has fueled renewed enthusiasm for the development of cell-based tissue replacement therapies. Hence, significant efforts are currently being devoted to optimized *ex vivo* conditions that are suitable for the generation of an unlimited number of insulin producing cells from either embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), and/or expansion of tissue derived progenitor cell populations[130–134].

1.5.2 *Stem Cells as a Possible Cell-based Therapy for the Treatment of Type I Diabetes*

The key to stem cell therapy for T1D is the concerted differentiation of pluripotent stem cells towards a given cell fate. The idea to develop a stem cell-based treatment of diabetes was first proposed in the 1990s[135–137]**.** More recently, the use of mesenchymal stem cells to generate islet cells *in vitro* has been shown to reverse insulin-dependent diabetes[131,138,139]. Another strategy has been to reprogram duct cells to replenish the endogenous β-cell mass in rodent animal models[140]. Realistically, however, there are not enough donors of ductal tissue for this to be a viable process. Therefore, the majority of efforts are currently focusing on strategies to promote the *de novo* derivation of islet cell types from embryonic stem cells (ESC), or induced pluripotent stem cells (iPSC).

At present, differentiation protocols based on the use of embryonic or pluripotent stem cells aim to recapitulate the *in vivo* signaling pathways of normal pancreatic development by adding growth factors or transcription factors to stem cell growth media. This development is induced in a step-wise fashion where cells progress from undifferentiated cells through mesendoderm, definitive endoderm (DE), primitive gut tube (PGT), pancreatic progenitors (PP) and finally endocrine progenitors (EP)[141–144] stages. Progression through these stages can be

followed by monitoring the expression of stages specific surface markers and transcription factors[145–150]. Although effective, most differentiation protocols remain inefficient, leading to only ~25% derivation of insulin producing cells[145,151–155]**.** In addition, many of these cells prove to be polyhormonal and non-glucose responsive, rendering them still unusable as a functional transplant for the effective treatment of diabetes.

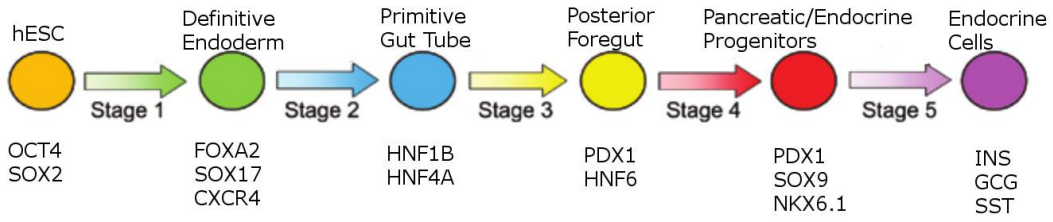


Figure 1-10 *Stage specific markers of differentiation*

Each stage of differentiation can be identified by the presence of specific cell markers. Some representative markers are shown in the figure. The stages are as follows: embryonic stem cell (hESC), definitive endoderm (DE), primitive gut tube (PG), posterior foregut (PF), pancreatic progenitors (PP), endocrine progenitors (EP), and β-cells (EC) (adapted from Liu et al 2014[150]).

From these initial studies, most recent efforts have focused on discovering additional molecular cues that may increase the percentage of useable cells. Some methods to increase differentiation efficiency may include culture conditions such as optimizing media, growth factor combinations, co-culture with feeder cell types, supplementation of select extracellular matrices, and replacement of animal-derived fetal bovine serum (FBS) with xeno-free reagents such as recombinant serum replacements (KSR)[156]. An additional option that has recently been used with significant success is exploitation of 3D culture set ups, a configuration favorable to the growth and endocrine differentiation of islet like cell clusters[157–162]. Our lab has a long-standing interest in the study of mechanisms that regulated cell-to-cell communication mechanisms and its function in hastening development of differentiating stem cells. In this regard, a specialized form of intercellular communication is the one mediated by Gap Junction (GJ) channels. GJ channels

are unique in that they allow for the rapid exchange of biochemical signals between cells in contact bypassing the extracellular space.

Building on these notions, a major tenet of my thesis work is that Connexins (Cxs), the building blocks of GJ channels, contribute to stem cell differentiation toward select cell lineages. Specifically, my work tested the hypothesis that the targeted manipulation of select Cxs at specific stages of stem cell differentiation may be exploited to enhance the *ex vivo* derivation of the pancreatic islet cell types.

1.6 THESIS AIMS

The studies presented here summarize our findings on the role of cell-to-cell communication and their impact on pancreatic development and function with a primary focus on Connexins, and corollary studies on the role of integrin-mediated cell adhesion and signaling. In ***Chapter 3***, we report on the role of Cx43 in human embryonic stem cell development toward pancreatic progenitors. We used gain-of-function strategies to assess the role of this Cx in fostering the development of stem cells toward pancreatic progenitors. ***Chapter 4*** describes a loss-of-function strategy probing at Cx43 function throughout development toward pancreatic progenitors. ***Chapter 5*** reports on integrins and their impact in proper pancreatic islet development. ***Chapter 6*** describes the role of ILK, a downstream effector of 1 integrin, in pancreatic development and glucose control in mice.

Together, this body of work provides evidence that both connexins and integrins can positively regulate the development of pancreatic cell types. Furthermore, a corollary resulting from these studies supports the concept that the purposeful manipulation of proteins regulating cell-to-cell communication and cell interaction with the microenvironment may provide innovative strategies to recapitulate *in vivo*-like conditions that can be exploited to develop more efficient protocols for the derivation of pancreatic islet cell types from stem cells. Hence, we anticipate that this work may have significant implications for the future development of cell-based therapy for Type 1 Diabetes.

Chapter 2.

MATERIALS AND METHODS

2.1 STEM CELL EXPERIMENTS

Cell differentiation experiments were carried out using the H1 cell line (WiCell Alias WA01) and a modified version of the D'Amour 2005[141] protocol, as shown in **Table 2-1** and **Table 2-2**. Undifferentiated cells were passaged as cell rafts using Dispase (ThermoFisher Scientific) and grown in mTeSR1 media (Stem Cell Technologies). H1 cells were plated on Matrigel Matrix (BD) coated plates and grown to 80% confluency before beginning differentiation. Initial expansion and differentiation were carried out in hypoxic incubators at 37°C.

Table 2-1 *Protocol for Stem Cell Differentiation for AAP10 experiments*

Stage	**Medium**	**Factors**
Day 1 Mesendoderm	RPMI (NO Serum)	Activin A (100 ng/ml) Wnt3a (30 ng/ml)
Day 2-3 Def. Endoderm	RPMI (0.2% SR)	Activin A (100 ng/ml)
Day 4-6 Primitive Gut Tube	RPMI (2% SR)	KGF (50 ng/ml)
Day 7-9 Posterior Foregut	DMEM (1% B27)	KAAD Cyclopamine (0.25 µM) All-trans Retinoic Acid (2µM) Noggin (50 ng/ml)
Day 10-12 Endocrine Precursor	DMEM (1% B27)	Noggin (50 ng/ml) KGF (50 ng/ml) EGF (50 ng/ml)
Day 13-16 Endocrine Progenitor	DMEM (1% B27)	No growth factors

Table 2-2 *Protocol for Stem Cell Differentiation for shRNA experiments*

Stage	**Medium**	**Factors**

Days 1-2 Mesendoderm	RPMI (NO Serum)	Activin A (100 ng/ml) Wnt3a (25 ng/ml)
Days 3-5 Def. Endoderm	RPMI (0.2% SR)	Activin A (100 ng/ml)
Days 6-9 Prim. Gut Tube	RPMI (2% SR)	KGF (25 ng/ml)
Days 10-12 Post. Foregut	DMEM (1% SR)	KAAD Cyclopamine (0.25 μM) All-trans Retinoic Acid (2μM) Noggin (50 ng/ml)
Days 13-15 Panc. Progenitors	DMEM (1% SR)	
Days 16-19 Endocrine Progenitors	DMEM (1% SR)	Exn4 (10nM)

2.1.1 *Generation of Embryoid Bodies*

H1 cells were expanded as undifferentiated cells for 5 days. Cells were then lifted using 0.01% Trypsin (Gibco) and cultured overnight in mTESR media (Stem Cell Technologies) in non-adherent plates in the presence of 5μM Rock Inhibitor (Stem Cell Technologies). The next day, cell aggregates were harvested and replated in media containing F12/DMEM (Gibco), 16.67% SR (Thermo Scientific), and 1x each of Penstrep (Gibco), Sodium Pyruvate (Gibco), and NEAA (Gibco), plus 0.1mM βME. Embryoid bodies (EBs) were further grown up to 3 weeks to observe spontaneous differentiation. EBs were collected and mounted in Histogel (Thermo Scientific) as cell pellets and processed into paraffin blocks for histologic analysis.

2.1.2 *AAP10 Peptide and Non-peptide Experiments*

Non-peptide controls were grown following the protocol for stem cell differentiation (**Table 2-1**). For peptide experiments, AAP10 (H-Gly-Ala-Gly-Hyp-Pro-Tyr-CONH(2)) was obtained from Genscript, reconstituted in 10mM HEPES, 0.2% BSA. Media changes were made daily, with factors added fresh that day. For “Peptide Thru” experiments, 400nM AAP10 was added daily during media changes. For “Peptide Stop” experiments, 400nM AAP10 was added daily during media changes for Days 1-3.

2.1.3 *Targeted shRNA Infection Against Cx43*

AAV-sh43 with a GFP reporter, Ad-GFP-U6-hGJA1-shRNA, was obtained from Vector Biolabs (shAAV-209911). The shRNA sequence is as follows: CCGG-GCCCAAACTGATGGTGTCAAT-CTCGAG-ATTGACACCATCAGTTTGGGC-TTTTTG (VBLKO#059773).

Cells were grown to PGT and then lifted with 0.01% Trypsin (Gibco) and replated into 6-well plates at 500k cells per well. Cells were infected with 0.1 MOI and left to continue growth following the differentiation protocol. Cells were monitored for GFP expression by fluorescence microscopy to determine time of shRNA expression and determine frequency of successfully infected cells within the cultures.

2.1.4 *qPCR of Markers of Stem Cell Differentiation*

RNA was isolated from pelleted cells collected at the end of each stage of differentiation using the Aurum™ total RNA Mini Kit (Biorad), and transcribed into cDNA using the iScript™ cDNA synthesis kit (Biorad). RNA was incubated at 25° for 5 minutes, 42° for 25 minutes, and 85° for 5 minutes. Specific transcripts were amplified using the primers listed in **Table 2-3** and iTaq™ Universal SYBR® Green Supermix (Biorad). qPCR was carried on at 95°C 3', (95°C 15s, 57°C 30s, 72°C 20s)x39 cycles, 95°C 15s, melt curve 55°C -> 95°C using a Biorad CFX96 machine and associated software (Biorad). Gene expression was normalized to 18s levels. Changes in the transcript level were calculated as fold change compared to undifferentiated cells.

Table 2-3 *Primers Used for qPCR in stem cell experiments*

Target	**Forward**	**Reverse**
18s	CCTGCGGCTTAATTTGACTC	GACAAATCGCTCCACCAACT
Cx26	CAAACCGCCCAGAGTAAAGA	CTTTCCAATGCTGGTGGAGT

Cx32	TGAGACCATAGGGGACCTGT	TCATCACCCCACACACTCTC
Cx36	GCAGCAGCACTCCACTATGA	AAGCAAAGACTGGGGGTACA
Cx43	GTGCCTGAACTTGCCTTTTC	CCCTCCAGCAGTTGAGTAGG
CxCR4	CCGTGGCAAACTGGTACTTT	GACGCCAACATAGACCACCT
Ecad	GAACGCATTGCCACATACAC	ATTCGGGCTTGTTGTCATTC
FoxA2	ATTGCTGGTCGTTTGTTGTG	TACGTGTTCATGCCGTTCAT
Glucagon	AAGTTCCCAAAGAGGGCTTG	AGCTGCCTTGTACCAGCATT
GSC	GAACCTCTTCCAGGAGACCA	CGTTCTCCGACTCCTCTGAT
Hnf1b	CAACCAGACTCACAGCCTGA	TGCCATGGTGACTGATTGTT
Hnf4a	GAATGCGACTCTCCAAAACC	TGAGGTTGGTGCCTTCTGAT
Hnf6	AACCCTGGAGCAAACTCAAA	GAGTTCGACGCTGGACATCT
Insulin	CTACCTAGTGTGCGGGGAAC	GCTGGTAGAGGGAGCAGATG
MafA	GCGGAGAACGGTGATTTCTA	AGGAAAGGGAGGCTGAGAAG
Nanog	GCCGAAGAATAGCAATGGTG	ACTGGATGTTCTGGGTCTGG
Ncad	AGCCAACCTTAACTGAGGAGT	GGCAAGTTGATTGGAGGGAT
Ngn3	CCCTCTACTCCCCAGTCTCC	CCTTACCCTTAGCACCCACA
Nkx2.2	TCTACGACAGCAGCGACAAC	TTGTCATTGTCCGGTGACTC
Nkx6.1	CCCGGGCTCTACTTCAGC	TCTTCCCGTCTTTGTCCAAC
Oct4	TGCTCCAGCTTCTCCTTCTC	CGCCGTATGAGTTCTGTGG
Pdx1	CCTTTCCCATGGATGAAGTC	TTCAACATGACAGCCAGCTC
Sox17	AGCAGAATCCAGACCTGCAC	TTGTAGTTGGGGTGGTCCTG

2.1.5 *FACS Analysis for H1 Cells at Definitive Endoderm*

H1 cells were differentiated to Definitive Endoderm (Days 1-3 of diff protocol) in plates coated with Matrigel Matrix (BD). Cells were collected using non-enzymatic cell dissociation buffer (Sigma-Aldrich) and stained with CXCR4 and ECad. If continuing with nuclear staining, cells were then fixed using cytofix/cytoperm buffer and perm buffer from the BD Pharmingen BrdU Flow kit (BD), then stained with Sox17 and FoxA2. Antibodies used for FLOW are listed in **Table 2-4.** Cells were then analyzed using FACSCalibur (BD) or sorted at a FACS ARIA. Analysis was completed using FlowJO software.

Table 2-4 *Antibodies used for Flow Cytometry*

Target	**Fluorophore**	**Vendor**	**Cat #**
CD184 (CXCR4)	PE	Biolegend	306506
CD324 (Ecad)	PerCP/Cy5.5	Biolegend	324114

Hnf3/FoxA2	Alexa Fluor ®488	R&D Systems	IC2400G
Sox17	PE	R&D Systems	IC19241P

2.1.6 *Western Blotting*

Cells were collected by scraping at the end of each stage of differentiation and lysed in RIPA buffer (150mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH8.0) with protease inhibitors (Complete mini tab, Sigma) and phosphatase inhibitors (2mM orthovanadate, 5mM NaF, 1mM PMSF) for 30' on ice. Alternatively, the NE-PER™ Kit (Thermo Scientific), with inhibitors added, was used for extraction of cytoplasmic and nuclear fractions for western blot. Protein concentration of cell extracts was determined using a micro BCA kit (Thermo Scientific). Lysates were boiled in 2x SDS loading buffer and 7-20ug were loaded into gels (Biorad). Gels were transferred to Immuno-Blot® PVDF membranes (Biorad). Blots were developed using Lumigen substrate (Lumigen, Inc.). Antibodies used for Western Blot are listed in **Table 2-5**.

Table 2-5 *Antibodies used for Western Blot*

Antigen	**Host**	**Vendor**	**cat #**
Cx36	Rabbit	U.S. Biological	C7855-14
Cx43 373	Rabbit	Paul Lampe	
Cx43 CT1	Mouse	Paul Lampe	
Cx43 NT1	Mouse	Paul Lampe	
Hnf3β/FoxA2	Goat	R&D Systems	AF2400
p44/42 MAPK (ERK1/2)	Rabbit	Cell Signaling	#4695
phospho p44/42 MAPK (ERK1/2)	Rabbit	Cell Signaling	#9101S
Sox17	Goat	R&D Systems	AF1924
α-Tubulin	Mouse	ABCAM	AB7291
β-catenin	Mouse	BD Transduction Laboratories	610153

CT1, NT1, 373 generously donated by Paul Lampe. Secondary HRP conjugated antibodies were obtained from Jackson Immunoresearch.

2.1.7 *Immunofluorescence Staining in H1 Cells*

H1 cells were seeded and differentiated on glass coverslips coated with Matrigel Matrix (BD). Coverslips were fixed at the end of each stage of differentiation in 4% PFA for 10' at room temperature and stored in PBS at 4° until ready for staining. Cells were post-fixed in frozen (-20°C) MeOH/Acetone, blocked in 50mM glycine, than blocked in blocking solution (1xPBS with 1% BSA, 5% Donkey Serum). Coverslips were then stained with primary antibodies, washed with 1xPBS + 0.1% BSA and stained with secondary antibodies for 1 hour at room temperature, then mounted in ProLong® (ThermoFisher Scientific). Rhodamine-donkey anti-rabbit and anti-mouse, FITC-donkey anti-guinea pig, and Cy5-donkey anti-guinea pig antibodies were from Jackson ImmunoResearch.

Paraffin slides underwent antigen retrieval via boiling in citrate buffer for 30', and cooled to room temperature. Staining followed as above starting from blocking in 50mM Glycine.

Stained slides were imaged on a fluorescence microscope (Nikon Eclipse 800) using NIS-Elements AR software. Measurements of Nkx1, Pdx1, or Sox9 positive cells were counted in GNU Image Manipulation Program, and expressed as a percentage of DAPI positive cells.

Table 2-6 *Primary antibodies used for IHC*

Antibody	**Host**	**Vendor**	**cat #**
Cx43 IF1	Mouse	Paul Lampe	
E-cadherin	Mouse	BD Transduction Labs	610182
FoxA2	Goat	R&D Systems	AF2400
Glucagon	Rabbit	Cell Signaling	#2760s
Insulin	Guinea pig	DAKO	A0564
Nanog	Rabbit	Stemgent	09-0020
Oct 4	Rabbit	Stemgent	09-0023
PCNA	Mouse	Santa Cruz	sc-56
Pdx1	Goat	BCBC	
pH3	Rabbit	MILLIPORE	06-570
Sox17	Goat	R&D Systems	AF1924
Sox9	Rabbit	Millipore	AB5535

2.2 ANIMAL STUDIES

2.2.1 *Mouse Genotyping*

The use of animal subjects was reviewed and approved by the University of Washington Institutional Animal Care and Use Committee. RIP-Cre (RIP, rat insulin 2 promoter) transgenic mice[163] were crossed with β1-integrin$^{flox/flox}$ mice[164] to generate conditional knockout mice lacking β1 integrin in pancreatic β-cells. Genotyping was performed by PCR using primers as previously described[163,164] (**Table 2-7**).

Table 2-7 *Primers used in Animal Studies*

Primer	Forward	Reverse
loxP5'	CGCAGAACAATAGGTGCTGAAATTAC	CTGACACTGAGAACCACAAACGGC
loxP3'	CGGCTCAAAGCAGAGTGTCAGTC	CCACAACTTTCCCAGTTAGCTCTC
Ag2	CTGCTAACCATGTTCATGCCT	
Cre1	CCTGTTTTGCACGTTCACCG	
Cre3	ATGCTTCTGTCCGTTTGCCG	
P21	TTGTCGCTGTCTTGCACTCT	AATCTGTCAGGCTGGTCTGC
Cyclin D1	TCGTGGCCTCTAAGATGAAGGA	CCATTTGCAGCAGCTCCTC
GAPDH	GTGGAAGGGCTCATGACCA	GGATGCAGGGATGATGTTCT
ILK	CCAGGTGGCAGAGGTAAGTA	CAAGGAATAAGGTGAGCTTCAGAA

2.2.2 *BrdU Proliferation Studies*

For proliferation studies, adult mice were injected intraperitoneally with BrdU (Sigma-Aldrich) at 0.1 g/kg body weight every other day for 1 week before harvesting the pancreas. The glucose tolerance test was performed after an overnight fast by intraperitoneal injection of glucose (1 mg/kg body weight) and blood samples were obtained from the tail vein at different time points. Blood glucose was measured with a glucometer (LifeScan) and plasma insulin levels were measured by ELISA (Alpco Diagnostic).

2.2.3 *Embryonic Pancreas Dissection and Collagenase Digestion*

E14.5–E15.5 embryos were obtained from time-dated pregnant WT females. Embryonic pancreatic tissue was dissected and digested for 1 hour at 37°C in HBSS+0.1% collagenase A+20 μg/ml DNase. Tissue was pipetted at 15 minute intervals during digestion to facilitate dissociation of the mesenchyme from the epithelial component of the pancreas. Resulting epithelial clusters were separated from the mesenchymal fraction via 3 rounds of gravity sedimentations in sterile medium. This procedure consistently resulted in >80% enrichment of epithelial cells, as measured by immunostaining and flow cytometric analysis of cell suspensions for the epithelial marker E-cadherin. Resulting clusters were grown on Matrigel Matrix (BD) coated plates.

2.2.4 *Generation of ILK Transgenic Mice*

$ILK^{flox/flox}$ mice[165] provided by Dr. R. St-Arnaud (McGill University, Montreal, Canada) were bred with Pdx1-Cre mice (obtained from Dr. Doug Melton, Harvard University, Cambridge, MA) to study the functional requirement of ILK in the development of the pancreatic epithelium. The progeny from this first cross, Pdx1-Cre/$ILK^{flox/-}$, was backcrossed to $ILK^{flox/flox}$ mice to generate homozygous Pdx1-Cre/$ILK^{-/-}$ mutants. Genotyping was performed by PCR on tail DNA using primers specific for Cre and ILK-floxed sequences, as described[165,166] (**Table 2-7**). KO mice were selected to undergo a glucose tolerance test, pancreata was then dissected out for tissue analysis.

2.2.5 *Glucose Tolerance Test*

Mice were given a bolus of glucose (300mg/kg) via IP injection and blood glucose was measured at 15', 30', 60', 90', and 120' using a glucometer (LifeScan).

2.2.6 *FACS Analysis for Pancreatic Islets*

Pancreatic islets were dissociated into a cell suspension, fixed, permeabilized, and stained by two-color immunofluorescence with PE-conjugated anti-β1 integrin (Biolegend) and Alexa

488-conjugated sheep anti-insulin antibodies, and analyzed using a FACSVantage cell sorter (Becton Dickinson).

2.2.7 *Adhesion and Proliferation Assays*

Islets were isolated by intraductal injection of 0.5 mg/ml Liberase (Roche), purified on a Ficoll gradient and either cultured overnight in RPMI containing 10% fetal bovine serum (Gibco) or dissociated into a single-cell suspension with a non-enzymatic dissociation medium (Sigma-Aldrich) and plated onto different ECMs as previously described[93]. After 1 hour, cells were fixed then stained for insulin or glucagon by indirect immunocytochemistry and positive cells counted under the microscope. For *in vitro* proliferation assessment, whole islets or single-cell suspensions were plated onto 804G-coated coverslips in RPMI with 10% FBS supplemented with 20 ng/ml hepatocyte growth factor [HGF; also known as scatter factor (SF)][167,168]. 48 hours after plating, cells were pulsed with 10 μM BrdU (SigmaAldrich) and cultured for an additional 24 hours. After staining for BrdU and insulin, double-positive cells (BrdU+/insulin+) were counted under a fluorescence microscope and results expressed as a percentage of total β-cells.

2.2.8 *Immunofluorescence Staining and Morphometric Analysis of Pancreatic Islets*

Two- and three-color immunofluorescence and confocal analysis were performed on paraffin sections of fetal and adult mouse pancreas or isolated mouse pancreatic islet cells, as previously described[94]. Primary antibodies are listed in **Table 2-8**. The species-specific fluorophore-labeled F(ab)2 secondary antibodies LRSC donkey anti-rabbit and anti-mouse IgGs, FITC-donkey anti-rat and anti-sheep IgGs, and Cy5-donkey anti-sheep IgGs were from Jackson ImmunoResearch. Stained sections were viewed on a Zeiss Axiovert 35M microscope equipped with a laser scanning confocal attachment (MRC1024, Bio-Rad) or on a fluorescence microscope (Nikon Eclipse 800), and morphometric measurement performed using Image Pro Plus software (Media Cybernetics). Measurements of insulin- and glucagon-positive areas were measured as a percentage of total pancreatic area. At least 50 non-consecutive sections (5 μm) at intervals of 100 μm were analyzed per adult pancreas (~5% of the pancreas), collected from five to eight animals per group. For P4 pancreas, we analyzed 30 sections at intervals of 50 μm (~10% of the

pancreas), collected from six animals per group. For E17.5 pancreas, we analyzed 20 sections at intervals of 25 µm (~20% of the pancreas), collected from eight embryos per group.

Table 2-8 *Antibodies used In Animal Studies*

Target (host)	Vendor	Application and dilution
PE-anti-□1 integrin (CD29) (rat)	Biolegend, #102207	FACS, 1:50
Alexa 488 anti-insulin (sheep)	Conjugated in house	FACS, 1:100
Anti-□1 integrin (CD29) (rat)	Millipore, #MAB1997	IHC, 1:50
Anti-□1 integrin (CD29) (mouse)	BD Transduction Laboratories, #610467	WB, 1:1000
Anti-insulin (sheep)	Binding Site, #PC059.X	IHC, 1:1000
Anti-glucagon (rabbit)	Millipore, #AB932	IHC, 1:200
Anti-BrdU (mouse)	Sigma-Aldrich, #8434	IHC, 1:100
Anti-phospho ERK1/2 (rabbit)	Cell Signaling, #4370	WB, 1:500
Anti-phospho S473-AKT (rabbit)	Cell Signaling, #4060	WB, 1:500
Anti-□-tubulin (rabbit)	Santa Cruz, #sc-5546	WB, 1:500
Anti-PTEN (rabbit)	Cell Signaling, #9552	WB, 1:200
Anti-□-actin	Sigma-Aldrich, #A1978	WB, 1:1000

FACS: fluorescence activated cell sorting, IHC: immunohistochemistry, WB: western blot

2.2.9 *Western Blotting, Quantitative PCR (qPCR) and Microarray Analysis*

Protein extracts from mouse islet lysates were resolved by PAGE, transferred to a PDVF membrane, blocked and incubated with the antibodies described in **Table 2-8**. Membranes were incubated with HRP-conjugated secondary antibody and visualized by ECL (Pierce).

Total RNA from sorted β-cells was extracted using TRIzol reagent (Life Technologies) and 1µg total RNA was reverse transcribed using random primers and SuperScript III reverse transcriptase (Life Technologies) following the manufacturer's instructions. SYBR Green qPCR was performed using the ABI Prism 7900HT system (Life Technologies) and the primers listed in ***Table 2-7***. For microarray analysis the Illumina mouse-6 v2 Expression BeadChip array was used as previously described[93]. Briefly, biotinylated cRNA was prepared using the Illumina RNA Amplification Kit (Thermo AMIL1791) according to the manufacturer's directions starting with 100 ng total RNA. Hybridization of labeled cRNA to the BeadChip, and washing and scanning were

performed according to the Illumina BeadStation 500× manual. The arrays were scanned on the Illumina BeadArray Reader, a confocal-type imaging system with 532 (cye3) nm laser illumination. Data analysis and QC was carried out using the BeadStudio software (Illumina). Data presented are MIAME compliant and raw data have been deposited in the EBI ArrayExpress Database (accession number: E-MEXP-3736).

2.3 ILK INHIBITION WITH CPD-22

2.3.1 *Cell Culture*

SU86 and G3LC were grown in normoxia in RPMI (Lonza) with 10% FBS (Gibco), 2.5 g/L glucose (22mM), and 1% each of Penstrep (Gibco), NEAA (Gibco), and NaPy (Gibco). SU86 and G3LC cells were grown for 2 days before being exposed to the Cpd-22 ILK inhibitor (EMD Millipore) for 24 hours at 1 or 2μM concentration before harvest.

Min6 cells were cultured in DMEM, 15% FBS, 1x each Sodium Pyruvate, Sodium Bicarbonate, β-me, Glutamate, PenStrep (Gibco). Cells were pulsed overnight with 1 or 2μM Cpd22 or BrdU. Cells were analyzed by FACS for BrdU incorporation. Another subset of cells was collected for RNA extraction and qPCR.

2.3.2 *BrdU Incorporation*

Cells were pulsed with BrdU 6-8hrs prior to collection. Cells were collected as described in the included protocol from the BD Pharmingen BrdU Flow Kit. Cells were analyzed using a FACSCalibur (BD) and FlowJO software.

2.4 STATISTICS

The statistical significance of differences in data values was validated by analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, analysis of *p*-trend, or by two-tailed Student's t-test, using the Prism 4 statistical package (GraphPad), with significance limit set at $P<0.05$.

Chapter 3.

CX43 ACTIVATION ALLOWS FOR GREATER INDUCTION OF DEFINITIVE ENDODERM AND PANCREATIC ISLET PROGENITORS FROM HUMAN EMBRYONIC STEM CELLS

3.1 OVERVIEW

This chapter explores the role of Cx43 in stem cell differentiation toward pancreatic progenitors. Experiments were carried out using a novel peptide, AAP10, to constitutively activate Cx43 channels during stem cell differentiation. My results show that addition of AAP10 to the culture media during the directed differentiation of stem cells toward pancreatic cell lineages leads to an increased yield of derived Definitive Endoderm (DE) cells, and subsequently $Pdx1^+$ and $Nkx6.1^+$ cells, as well as $Pdx1^+/Sox9^+$ co-expressing cells. In addition, it appears that to achieve this enhanced differentiation of stem cells toward DE, and later into pancreatic progenitors, treatment with AAP10 is only required during the early stages of differentiation (i.e. up to DE stage), when Cx43 expression levels are most prominent. These studies reveal a novel method for increasing the yields of pancreatic cell types from undifferentiated stem-cell preparations, a milestone that will have significant implications for perfecting protocols for the derivation of insulin-producing cells from stem cells for the treatment of Type 1 Diabetes.

3.2 INTRODUCTION

3.2.1 *A stem cell based therapy for the treatment of Type 1 Diabetes*

Recent advancements in stem cell biology have renewed enthusiasm for the design of innovative cell-based replacement therapies that would no longer depend on the isolation of cells of interest from organ donors In the case of type 1 diabetes, recent success in the derivation of insulin-producing cells from human embryonic stem cells (hESCs) has the potential to generate an unlimited number of insulin-producing cells that could be used for trasplantation[133,134].

Current protocols of directed differentiation of hESCs are designed to recapitulate *in vivo* signaling pathways that have been shown to be important for normal pancreatic islet development[141–144,148]. However, for this strategy to be clinically relevant, the efficiency of these protocols of hESC differentiation must significantly improve above the current yields of 25% of islet cells in a typical differentiation procedure[145,151–154]. In addition, about 15% of these cells will still form teratomas post transplantation[169]. Thus, the critical problem with *de novo* generation lies in the heterogeneity of the finished product. The studies presented in this section seek to identify strategies to increase the homogeneity of stem cell preparations differentiated along the islet cell lineage. Hence, increasing the homogeneity of islet cell preparations that can be used for transplantation is a key milestone for the establishment of a stem cell-based therapy for diabetes.

3.2.2 *Exploiting the function of Cxs for driving pancreatic islet cell differentiation*

One method to overcome heterogeneity of stem cell derived cells may lie in studying cell-to-cell communication methods to drive stem cell differentiation toward the cell types of interest. Mechanisms of cell-to-cell communication allow for the exchange of signaling molecules which may restrict cell development toward specific cell types.

One specialized form of direct cell communication between adjacent cells is mediated by Connexins (Cxs), the building blocks of Gap Junctions. There are 21 different types found in humans and they are named for their molecular weight (kDa) size of each monomer[7]. These channels can allow for direct cytoplasmic exchange between cells as well as the passage of growth and survival factors between them[4,5,170]. Cxs can impart functional and developmental cues in their tissue types, and contribute to controlling cell growth[61].

Of particular interest to my thesis work is Cx43, which was found to be expressed at high levels in early stages of stem cell differentiation and later in pancreatic islet progenitors. In addition, Cx43 has been shown to play an important role in the maintenance of pluripotency in human embryonic stem cells[49]. Earlier studies have also shown that Cx43 plays a pivotal role in the maturation of β-cells in the pancreas. My work identifies Cx43 as the most prominent Cx found in putative islet cell progenitors delaminating from ductal epithelium, followed by a

significant downregulation during β-cell maturation[79]. Together, these studies provide evidence that proper expression of Cx43 throughout development is crucial for forming islet cell progenitors.

The studies presented here aimed to uncover the role of specific Cxs during stem cell differentiation toward pancreatic progenitors. First, we defined the temporal dynamics of Cxs expression during stem cell differentiation, and then we tested whether manipulation of Cxs expression and function at distinct stages of differentiation affected the propensity of stem cells to adopt a pancreatic progenitor phenotype, and eventually insulin production. Specifically, herein, we describe the manipulation of Cx43 via a Cx43 activating peptide through stem cell differentiation using a stepwise protocol for development towards pancreatic progenitors.

3.3 RESULTS

3.3.1 *Cxs Expression in the Developing Human Pancreas*

We first set out to determine the distribution of Cxs expression in the developing human pancreas. To survey Cxs expression patterns by PCR, we isolated specific cell types from cryo-sections of human embryonic pancreas at 18 weeks of gestation using laser capture microdissection. In these experiments we isolated ductal tissue, developing islet clusters, blood vessels, acinar cells, whole pancreas, and pancreatic epithelium. This revealed unique expression profiles of Cxs 32, 36, and 43 segregated by cell types (**Figure 3-1**A). We found that Cx32 was expressed in ducts, acinar cells and whole pancreas. Cx36 expression was readily detected in developing islet clusters, whole pancreas, and faintly in acinar cells. Conversely, Cx43 was mainly detected in developing islet clusters, blood vessels, whole pancreas, and cultures of pancreatic epithelium. We further investigated Cx43 immunoreactivity in human fetal pancreatic sections. As shown in **Figure 3-1**B, Cx43-specific immunoreactivity identified a discrete population of cells that appeared to delaminate from ductal domains. Cx43 appeared to also be expressed near developing islet cell clusters. The association of these Cx43 positive cells with the ductal epithelium and developing endocrine cells suggests that they may represent a population of islet progenitors.

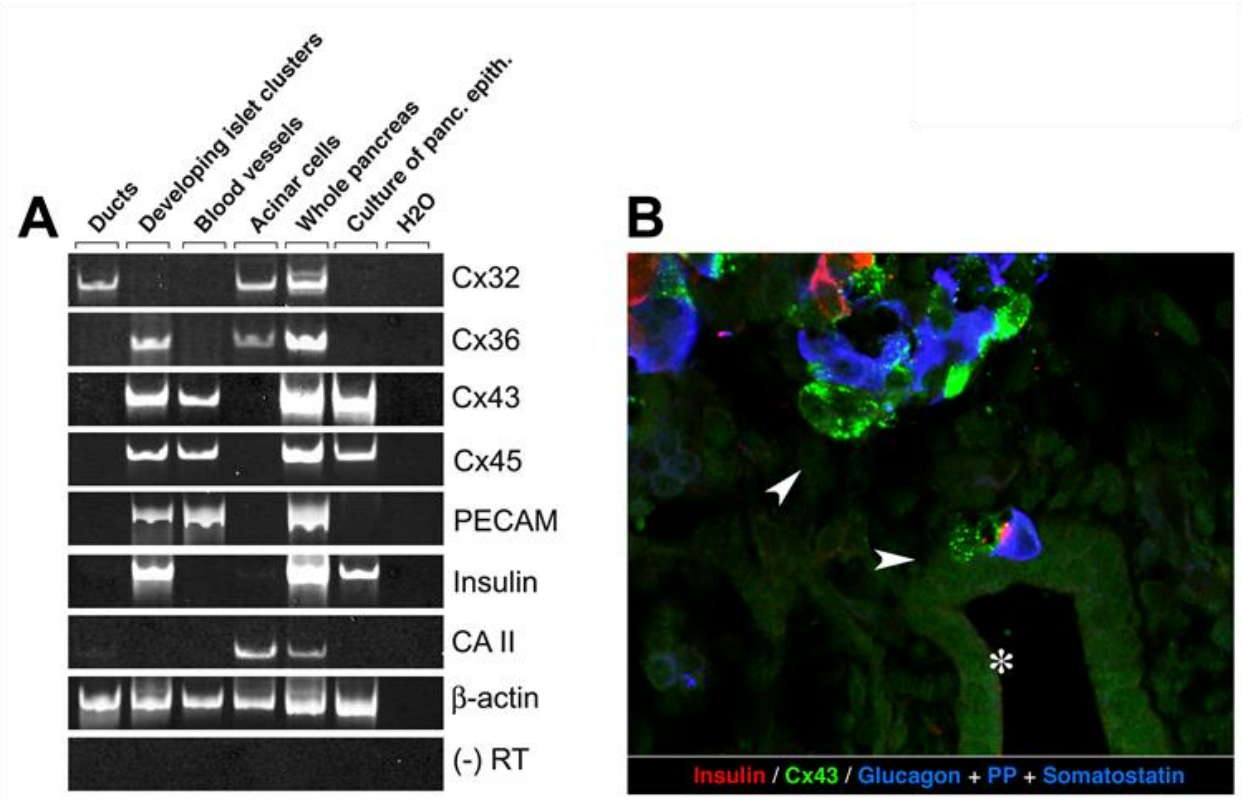


Figure 3-1 *Distribution of Cxs expression in the developing human pancreas*

*(**A**) Isolation by laser-capture microdissection of distinct cell types from the embryonic human pancreas (18 weeks of gestation) reveals unique Cxs expression profiles. Note that Cx43 is the most prominent zcx expressed in developing islet cell clusters. (**B**) Cx43-specific immunoreactivity identifies a discrete population of cells that appear to delaminate from ductal domains (*), and cluster into developing islet cell clusters (arrowheads). The association of these Cx43^{+} cells with the ductal epithelium and developing endocrine cells suggests that they may represent a population of islet progenitors.*

3.3.2 *Cx43 Expression Pattern in Developing hESC*

We next sought to determine the expression pattern of Cx43 in embryoid bodies (EBs), an *in vitro* model of hESCs spontaneous differentiation into the three main embryonic germ layers: Endoderm, Mesoderm and Ectoderm. Three-color immunostaining for E-cadherin, FoxA2, and Sox9 allowed for the identification of Neuro/Ectoderm (ECad^{-}/Sox9^{+}), DE (ECad^{+}/FoxA2^{+}), and

Mesoderm (Ecad$^-$/FoxA2$^-$/Sox9$^-$) cells (**Figure 3-2**A and B). Serial sections of EBs were immunostained for Cx43, Sox17, and Vimentin and revealed enriched expression of Cx43 in DE cells, as defined by the co-expression of E-cadherin with FoxA2, Sox9, and Sox17 (**Figure 3-2**C and E). Significant Cx43 specific immunoreactivity was also detected in ME cells, identified in a sequential section by expression of E-cadherin and Sox9, but not Sox17 (**Figure 3-2**C and D). Interestingly, although at later stages of embryonic development Cx43 was found prominently expressed in Mesoderm derivatives such as cardiomyocytes, early Mesoderm did not express significant levels of Cx43, as defined by Vimentin expression and lack of E-cadherin immunoreactivity.

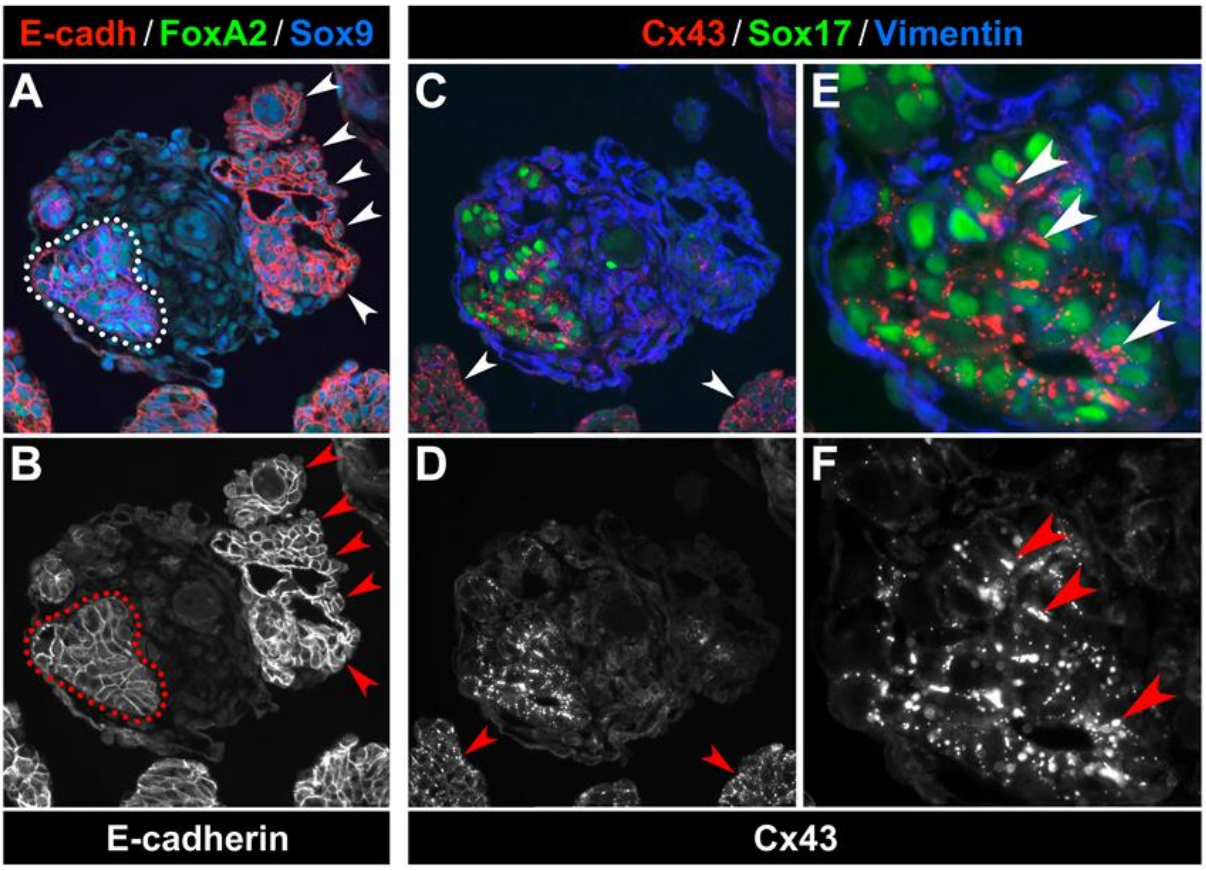


Figure 3-2 *Cx43 is preferentially expressed in Mesendoderm and Definitive Endoderm developing from hESC in vitro*

Embryoid Bodies (EBs), used as an in vitro *model of spontaneous hESC differentiation, and immunostained for E-cadherin (red), FoxA2 (green), and Sox9 (blue) identified all three germ layers: Ectoderm (**A**, arrowheads), Endoderm (**A**, dotted line) and Mesoderm (**A**, cells negative for E-cadherin). (**B**) Monochromatic imaging from the microscopic field shown in panel A allows better identification of E-cadherin^{+} cells. (**C**) Serial sections from the same EBs immunostained for Cx43 (red), Sox17 (Green) and Vimentin (blue) revealed that Cx43 expression was enriched in Definitive Endoderm cells, as defined by the co-expression of E-cadherin, FoxA2, and Sox9 (**A**, dotted line), and Sox17 (**C** and **E**, green). Significant Cx43-specific immunoreactivity was also detected in ME cells, identified in a sequential section by the expression of E-cadherin and Sox9, but not Sox17 (**C** and **D**, arrowheads). Panels **E** and **F** show higher magnification of Definitive Endoderm cells identified in **C** and **D**. Note that immunoreactivity specific for Cx43 is not detected at significant levels in Mesoderm cells, identified by Vimentin expression and lack of E-cadherin immunoreactivity. Images are representative of n=12 fields (Cx43-Sox17-Vimentin) and n=10 fields (Ecad-Foxa2-Sox9).*

To determine the expression profile of Cx43 in stem cell development toward pancreatic progenitors, we profiled Cx43 transcript and protein in stem cells collected at specific stages of differentiation. hESCs were developed into pancreatic cell lineages following a step-wise protocol for directed differentiation (**Figure 3-3**A). We found that gene expression of Cx43 steadily rose throughout the first three stages of differentiation: Mesendoderm (ME), Definitive Endoderm (DE), and Primitive Gut Tube (PGT). After these stages, e.g. Posterior Foregut (PF), Cx43 gene expression sharply declined (**Figure 3-3**B). Western blot for Cx43 showed a large increase in expression during the first three stages of differentiation followed by a drop off at later stages. Cx36 protein was upregulated at DE and slowly dropped off from there (**Figure 3-3**C).

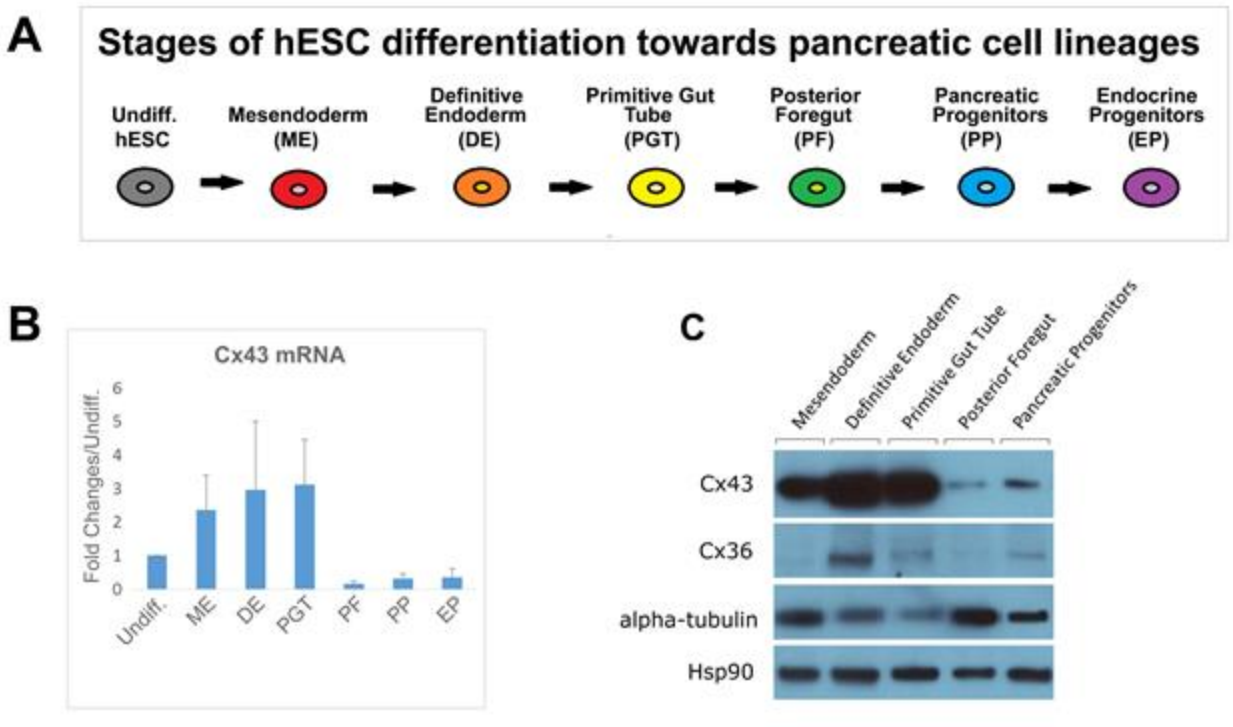


Figure 3-3 *Expression profile of Cx43 in hESC directed to differentiate towards pancreatic cell lineages.*

*(**A**) Schematic of step-wise protocol for the directed differentiation of hESC into pancreatic cell lineages. (**B**) Gene expression of Cx43 throughout all stages of hESC differentiation (n=4). Expression of Cx43, normalized to levels present in undifferentiated hESC, progressively increases during early stages of differentiation into ME, DE, and PGT, followed by a sudden decline following the specification of downstream cell lineages (i.e., PF). (**C**) Gene expression data for Cx43 was validated at the protein level by Western blotting using α-tubulin and Hsp90 as a loading control.*

3.3.3 *Cx43 Gain of Function using AAP10*

To determine the effects of Cx43 gain-of-function in hESCs differentiation, we applied AAP10, a Cx43 activating peptide, to enhance Cx43 conductance throughout stem cell differentiation. AAP10 was originally reported as an antiarrhythmic peptide due to its ability to constitutively open Cx43 channels and synchronize beating heart cells. We utilized this

functionality as a gain-of-function manipulation in our experiments of hESCs differentiation toward pancreatic cell lineages.

To determine the effects of AAP10, we conducted gene expression analysis of DE markers FoxA2 and Sox17 (**Figure 3-4**A). Gene expression of FoxA2 and Sox17 was significantly increased at the DE and PGT stages in cultures treated with 400ng/mL AAP10 compared to untreated cells ($p<0.01$). FoxA2 and Sox17 steadily declined through the later stages of differentiation. This result was recapitulated by western blotting where there was an induction of Sox17 in DE nuclear lysates of cells treated with peptide (**Figure 3-4**D). This indicated a greater induction of DE cells as a result of Cx43 activation by AAP10.

To determine the percent population of DE cells generated in culture, cells were differentiated to DE and then analyzed by FACS based on the expression of E-cadherin and CXCR4. E-cad$^+$/CXCR4$^+$ co-expression marks the DE population, while CXCR4$^+$/E-cad$^-$ cells characterizes a mesoderm population[141]. In these experiments we observed that addition of AAP10 to cell culture increased the percentage of DE cells from an average of 36% to 51.5% (i.e. a 52% increase in DE cells) (**Figure 3-4**B). The induction of DE with AAP10 was further quantified by immunostaining cells fixed at the DE stage. Cells were stained using intracellular markers for DE such as FoxA2 and Sox17 in combination with Cx43 and Nanog. In these experiments, we observed that cells treated with AAP10 displayed a marked increase in FoxA2 nuclear immunoreactivity demonstrating an induction of DE type cells. In addition, while AAP10 did not increase total protein content of Cx43, peptide treated cells were found to exhibit much larger Cx43 plaques (**Figure 3-4**C).

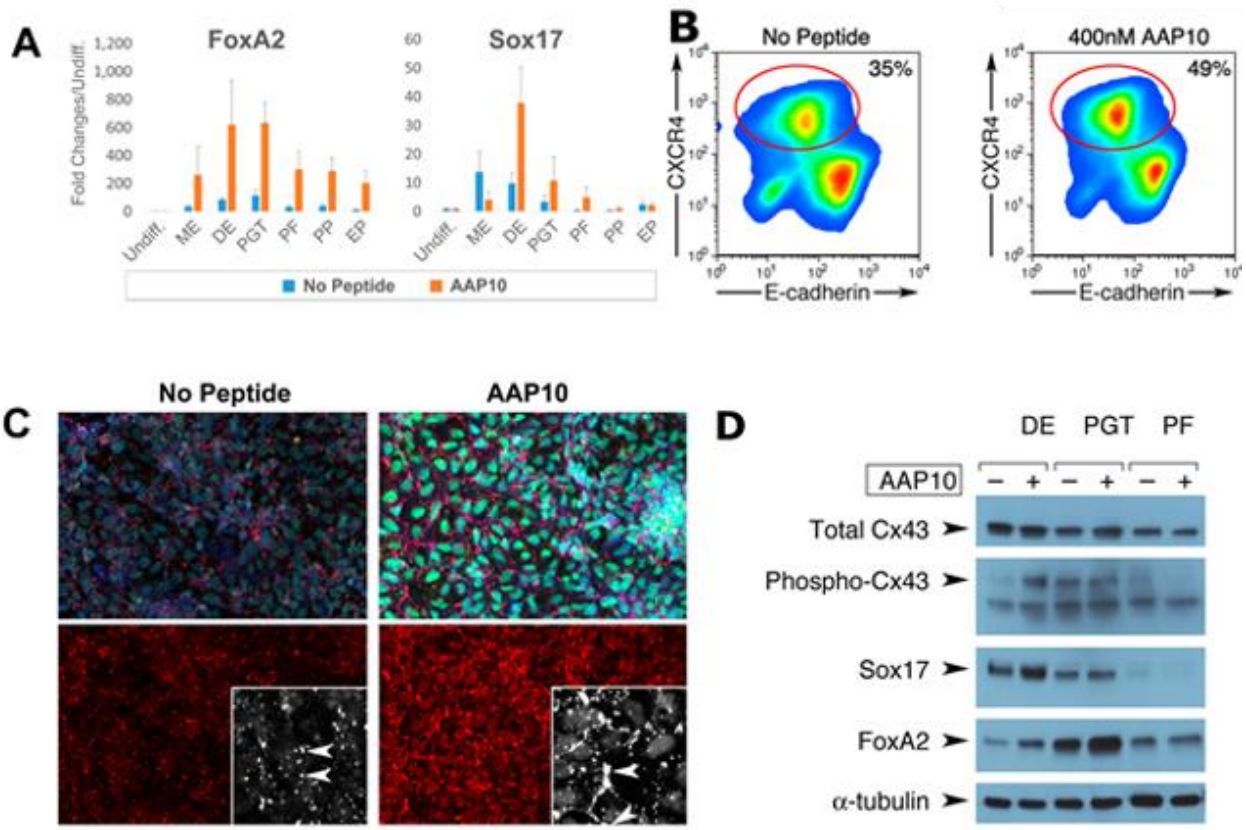


Figure 3-4 *Induction of Definitive Endoderm is enhanced by the Cx43 agonist AAP10 peptide.* (***A***) *Gene expression of FoxA2 and Sox17 throughout all stages of differentiation. Gene expression is significantly increased at DE and PGT stages in cultures treated with 400ng/ml AAP10 ($p < 0.01$), followed by a decline during subsequent stages. This indicates a greater induction of DE cells as a result of Cx43 activation by the AAP10 peptide. (No peptide n=4, Peptide Thru n=3)* (***B***) *FACS analysis of DE cells. Cells were differentiated to the DE stage and stained for E-cadherin and CXCR4. Addition of AAP10 resulted in a marked increase of double positive cells from $\bar{x} = 36\%$ to $\bar{x} = 51.5\%$ double positive cells (i.e., 52% increase). (FACS is representative of n=4)* (***C***) *Corresponding immunostaining of H1 cells at DE with and without peptide treatment. Cells treated with AAP10 show a marked increase in frequency and levels of expression of FoxA2 demonstrating an induction of DE cells. In addition, while AAP10 did not increase total protein content of Cx43, peptide treated cells were shown to have much larger Cx43 plaques (insets, arrowheads). (Staining is representative of n=3 fields).* (***D***) *Western blot in differentiated H1 cells showed a greater induction of Sox17 with addition of peptide compared to untreated cells at the DE stage.*

3.3.4 *AAP10 is only necessary during early stages of differentiation*

To determine the effects of AAP10 on later stages of differentiation, we surveyed end-stage (e.g. Endocrine Progenitors (EP)) cells for expression of endocrine progenitor specific transcription factors Pdx1, Sox9, and Nkx6.1. Cells grown on coverslips were fixed at the end of differentiation and then immunostained by two-color immunofluorescence for Pdx1/Nkx6.1 and Sox9/Pdx1. In these studies we observed that AAP10-treated cells were significantly enriched for pancreatic progenitors as defined by co-expression of Pdx1 and Sox9. Interestingly, while treatment with AAP10 throughout the entire duration of the differentiation resulted in a significant increase in the number of $Pdx1^+$ pancreatic progenitors, compared to control cultures (i.e., no peptide), when AAP10 treatment was applied only up to the DE stage (referred to as Pepstop) we observed a higher number of cells co-expressing Pdx1 and Sox9 (One way ANOVA $p = 0.0664$, post test for linear trend $p = 0.0069$), with a concomitant increase of the total number of $Sox9^+$/$Ecadherin^+$ cells (One way ANOVA $p = 0.0048$, post test for linear trend $p = 0.0007$) (**Figure 3-5**A). Collectively, there was a significant increase in $Pdx1^+$ cells in later stages of differentiation in both peptide-treated conditions compared to no peptide (One way ANOVA $p = 0.0246$, post test for linear trend $p = 0.0017$) (**Figure 3-5**B). These results indicated that activation of Cx43 gap junction channels by AAP10 was an effective treatment for the derivation of a significantly larger number of $Pdx1^+$ progenitors compared to conventional protocols of differentiation. Furthermore, based on the observation that AAP10 treatment was effective in producing significantly higher numbers of $Pdx1^+$/$Sox9^+$ progenitors when applied up to the DE stage of differentiation (Pepstop), we speculated that these results may be explained by the requirement of Cx43-mediated cell-to-cell communication, or signaling, during early induction of DE cells that holds a higher propensity to further differentiate toward pancreatic progenitors. Hence, our results suggest that AAP10 drives the specification of a larger number of DE cells toward pancreatic progenitors. We conclude that cell-to-cell communication through Cx43 gap junctions is required to recruit heterogeneous hESC populations into differentiation programs toward Definitive Endoderm and Pancreatic Progenitors.

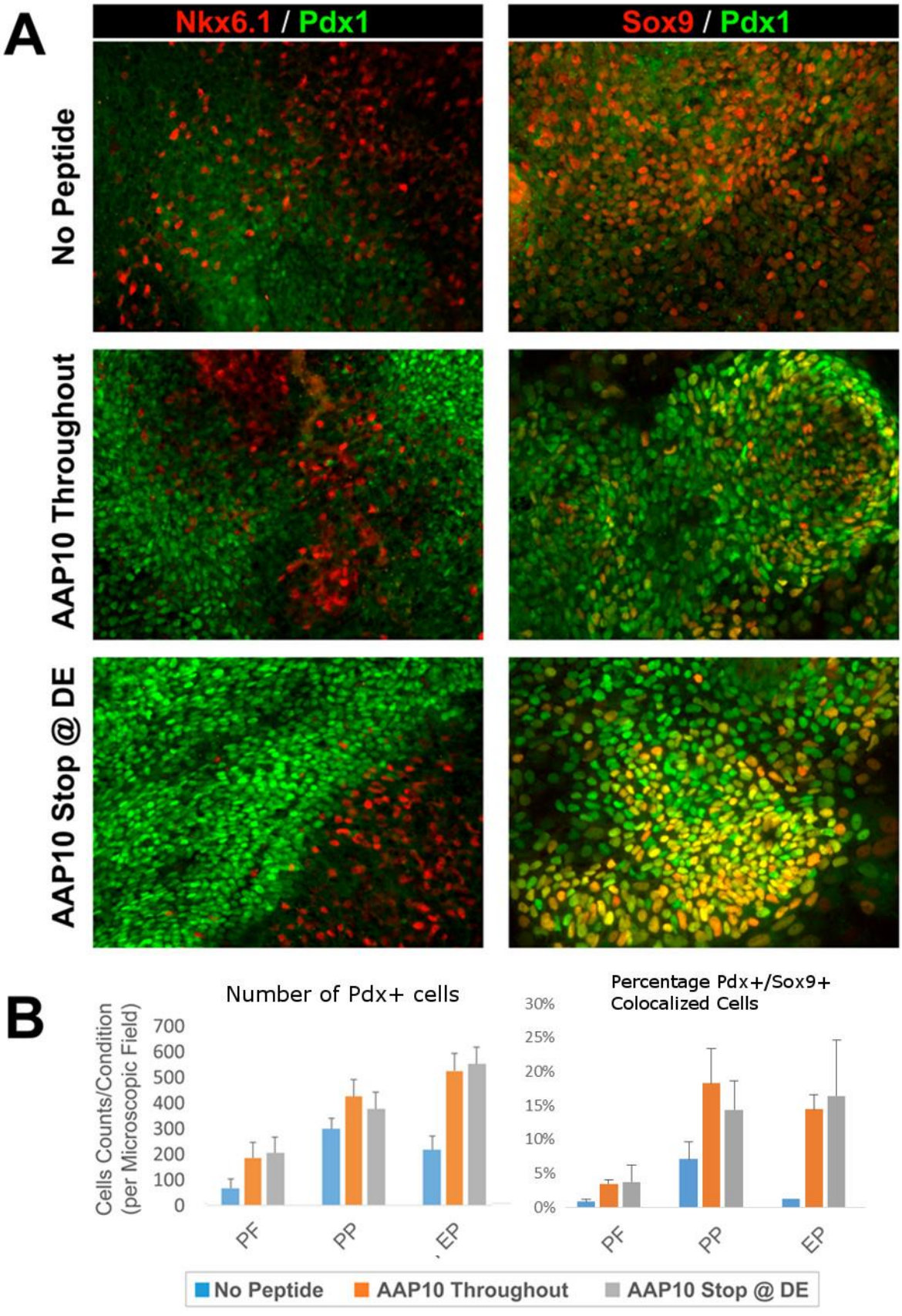

A
Nkx6.1 / Pdx1
Sox9 / Pdx1
No Peptide
AAP10 Throughout
AAP10 Stop @ DE
B
Number of Pdx+ cells
Cells Counts/Condition (per Microscopic Field)
700
600
500
400
300
200
100
0
PF
PP
EP
Percentage Pdx+/Sox9+ Colocalized Cells
30%
25%
20%
15%
10%
5%
0%
PF
PP
EP
No Peptide
AAP10 Throughout
AAP10 Stop @ DE

Figure 3-5 *Addition of AAP10 during early stages results in greater induction of Pdx1⁺ cells.*

*(**A**) Immunostaining of end-stage cells (i.e. Endocrine Progenitors; EP) for Pdx1/Nkx6.1 and Sox9/Pdx1. In "no peptide" condition (n=3), there is very little Pdx1 and a greater number of Nkx6.1 and Sox9 indicating a possible pancreatic population but few cells that will further develop into endocrine cells. In samples treated with AAP10 throughout the duration of the differentiation (n=3), there is a greater induction of Pdx1 cells compared to no peptide. Interestingly, when the AAP10 peptide was added to the cultures only up to the DE stage (i.e., Peptide stop @ DE) (n=2), we observed an even higher induction of Pdx1 positive cells, suggesting that the purposeful activation (i.e., phosphorylation) of Cx43 by AAP10 during stages of highest Cx43 expression (see Figures 2 and 3) plays an important role in early commitment towards islet cell lineage, as opposed to a steady activation of Cx43 throughout all stages of differentiation. This possibility is suggested not only by a significant increase in the number of Pdx1 positive cells, (**B**) but also by the higher frequency of Sox9/Pdx1 co-expressing cells, known to represent endocrine-committed progenitors likely to further develop into beta cells. There is also a significant difference in the number of Sox9 cells.*

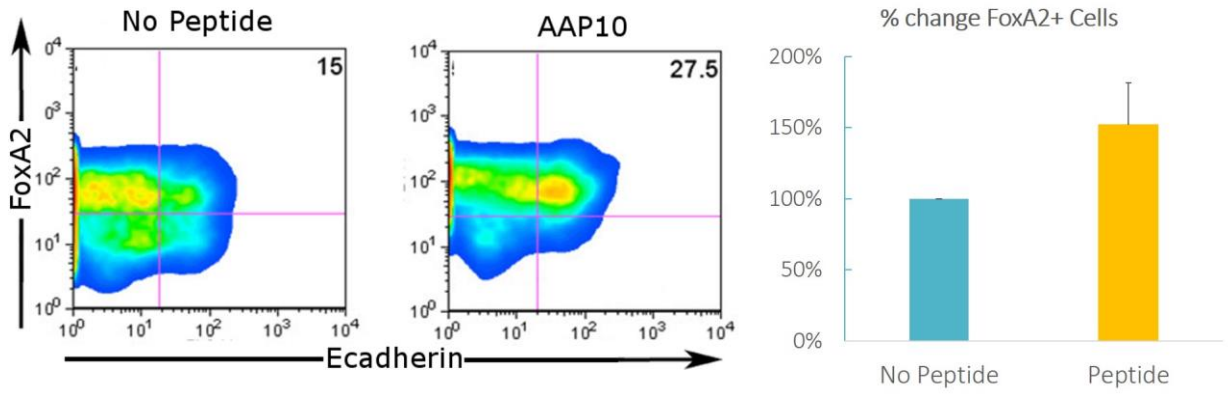


Figure 3-6 *Flow for FoxA2 in DE cells with and without peptide.*

Addition of peptide led to 50% increases in expression of FoxA2 as analyzed by Flow. This enrichment in cell markers for DE indicates that addition of peptide increases the population of DE cells found in culture.

3.4 DISCUSSION

Cx43 is one of the most ubiquitously expressed Cxs found in the human body. Importantly, it functions to help maintain stem cell pluripotency, but is also found in newly developing pancreatic cells. Our studies followed a gain-of-function strategy to determine the importance of Cx43 throughout development.

With addition of the Cx43-activating peptide AAP10 we found much larger gap junction plaques between DE type cells. These larger gap junction plaques may allow for greater communication between cells allowing for more growth-promoting signals to be transferred between adjacent cells, leading to the derivation of desired cell types. This interpretation is supported by the observation that AAP10 induced phosphorylation of Cx43 in our cultures, compared to non-treated cells, as determined by Western blotting for phospho-Cx43. Interestingly, this protein was found in the nuclear fraction rather than the cytosolic. One explanation for this is that the phospho-protein may be anchored in the cytoskeleton and was pulled down into the nuclear fraction. The enrichment of Cx43 at the DE stage also indicated a prominent role for this Cx during the development of this cell lineage.

Our observation that addition of AAP10 resulted in larger gap junction plaque size is consistent with previous studies showing that AAP10 reduces ischemia-induced internalization of Cx43[171]. Thus, while AAP10 phosphorylation at S368 leads to opening of the channel, phosphorylation at S297 or S330 leads to internalization of the channel[57]. AAP10 works via a PKC mechanism coupled with a hypothesized GPCR as evidenced by less activity in culture with GPCR inhibitors[172].

Experiments showing that addition of AAP10 led to enrichment for DE markers FoxA2 and Sox17 are also very interesting as these two transcription factors have been shown to regulate Sox9 expression[173]. Therefore, it is possible that DE cells developing from cultures containing AAP10 may have an enhanced propensity to proceed toward a $Pdx1^{+}/Sox9^{+}$ pancreatic progenitor cell phenotype. This is further supported by evidence demonstrating that Pdx1 and Sox9 both cooperatively regulate pancreatic lineage induction, and that their co-expression identifies cells endowed with a higher ability to develop into endocrine cells.

In further stages of differentiation, we noticed an upregulation of Pdx1, Sox9, and Nkx6.1 in peptide treated samples. Sox9 has been shown to be required for maintenance of pancreatic progenitor cell pools[174] as well as coordinating the transcriptional network in pancreatic progenitor cells[173]. It has also been shown to have a dosage dependent requirement for pancreatic endocrine cell formation[175]. In addition, the cooperation of Sox9 and Pdx1 is necessary for the lineage allocation of posterior foregut cells[176]. Given the importance of Sox9 in regulating the development and homeostasis of pancreatic lineages, our results support the concept that cells treated with AAP10 have a higher propensity of becoming pancreatic islet cells.

Although significant advances have been made in the generation of islet-like cells from hESCs, most stem cell preparations still yield heterogeneous cell preparations. These results point to the need of identifying strategies that can promote higher homogeneity in endocrine cell content, a requirement that remains a high priority before these stem cell-derived cell preparations can be used for clinical applications. Collectively, our results demonstrate that the activation of Cx43 during early stages of hESC commitment towards DE leads to higher yields of pancreatic progenitor cells that are likely to further differentiate into islet cells. Hence, our approach provides a significant improvement of current protocols for the derivation of islet cells to be used as a possible cell replacement therapy for type 1 diabetes.

Chapter 4.

CXS KNOCKDOWN IN STEM CELL DIFFERENTIATION TOWARD PANCREATIC PROGENITORS

4.1 OVERVIEW

In this chapter, I present evidence that further supports an important role of Cx43 in stem cell development towards pancreatic progenitors. Specifically, results presented here demonstrate that by using a "loss of function" approach for Cx43 using an adeno-associated virus expressing a targeted shRNA against Cx43, hESCs lose their ability to develop into pancreatic progenitors. These studies were designed as a logical series of experiments following the results described in **Chapter 3**, in which a "gain-of-function" approach of Cx43 function was shown to enhance the derivation of DE cell types and downstream pancreatic progenitors. Therefore, we hypothesized that loss-of-function of Cx43 would have the opposite effect on stem cells. Effectively, we expected that knockdown of Cx43 would lead to an inability to generate DE cell types or other stages within the pancreatic cell lineage. Results from these studies suggest that Cx43 is required for the development of pancreatic progenitors.

4.2 INTRODUCTION

In the developing pancreas, Cx43 is found abundantly expressed in newly developing islet progenitors delaminating from the ductal epithelium. Subsequently, as these progenitors acquire hormone expression, Cx43 is down-regulated while Cx32 and Cx36 are acquired by exocrine and endocrine lineages, respectively[177]. Building on these notions, and on my observation that activation of Cx43 by AAP10 fosters the development of DE and subsequently pancreatic progenitors, I pursued a "loss-of-function" approach to establish the requirement of Cx43 for these developmental outcomes in protocols of stem cell differentiation toward the pancreatic cell lineage. The underlying hypothesis for this experimental strategy was that knocking down

the expression of Cx43 would have the opposite effect as AAP10, and lead to a reduced ability of hESCs to differentiate into pancreatic progenitors.

4.3 RESULTS

4.3.1 *Expression of αV integrins in H1 cells*

As a first step for the implementation of the aforementioned experimental strategy making use of an adeno-associated virus (AAV) expressing a targeted shRNA against Cx43, we determined the expression levels of the integrin subunit αV, which in association with a β3 and β5 subunit forms the αVβ3 and αVβ5 integrins known to function as a receptors for Adeno viruses entry into cells[178,179]. These studies revealed that undifferentiated hESC H1 cells cannot be infected with AAV because they do not express significant levels of these integrin receptors. However, αV expression became readily detected in Mesendoderm cells, and increased through the DE stage, followed by a progressive downregulation from PGT toward PF and subsequent stages (**Figure 4-1)**.

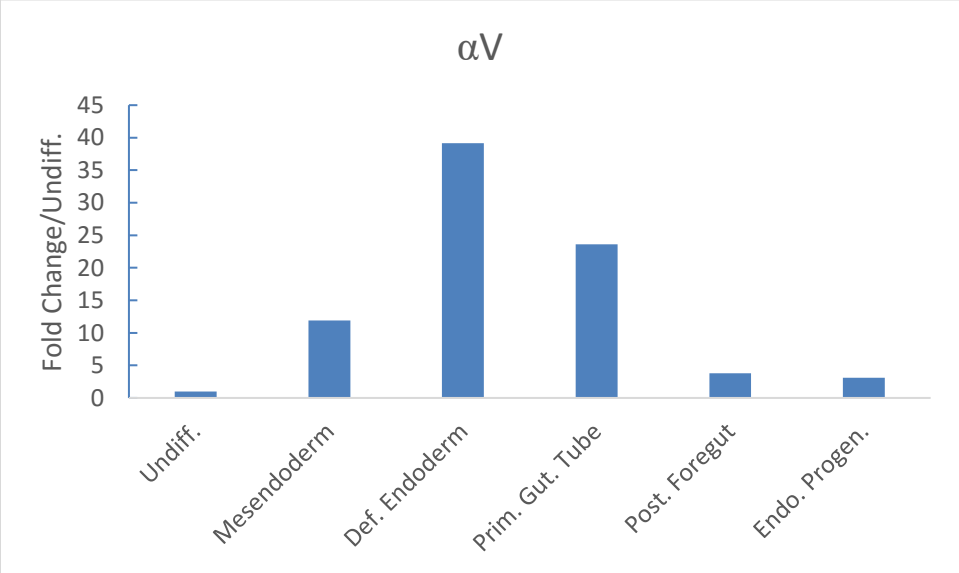


Figure 4-2 *Levels of αV integrins in stem cells differentiating toward pancreatic progenitors αV integrins are upregulated in the DE and PGT stages of cell differentiation. The expression of this family is essential for AAV infection.*

4.3.2 *Knockdown of Cx43 in stem cell culture using AAV-shRNA-Cx43*

Based on the results presented on the expression of αV integrin, we performed experiments in which the AAV expressing an shRNA against Cx43 was added to differentiating hESC cultures at the PGT stage and left to continue their differentiation program from there on (**Figure 4-3**A). We reasoned that at this stage, there would still be enough residual Cx43 to see a functional effect of Cx43 knockdown. Infected cells, identified by expression of a GFP reporter, were seen starting 3 days post infection and throughout to the end of differentiation (**Figure 4-3**B).

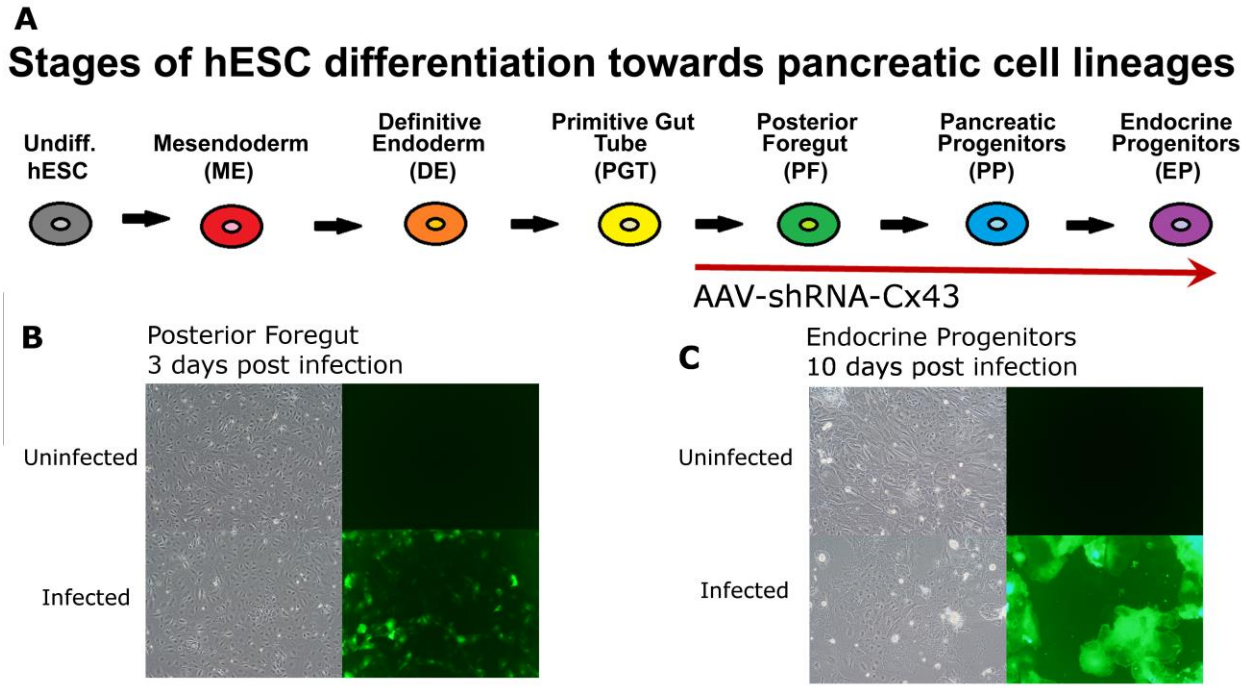


Figure 4-3 *Schematic of protocol for AAV infection during stem cell differentiation toward pancreatic cell lineages.*

*(**A**) Cells were infected with virus after being fully grown through the PGT stage. (**B**) Cells begin to express GFP as soon as 3 days post infection, indicating viral infection. (**C**) Cells show increased expression of GFP up through the end of differentiation.*

To determine the effects of Cx43 knockdown on the derivation of pancreatic progenitors from stem cells, we evaluated gene expression data for pancreatic markers. Infected cells showed reductions in Pdx1, Nkx6.1 and Insulin gene expression (**Figure 4-4**).

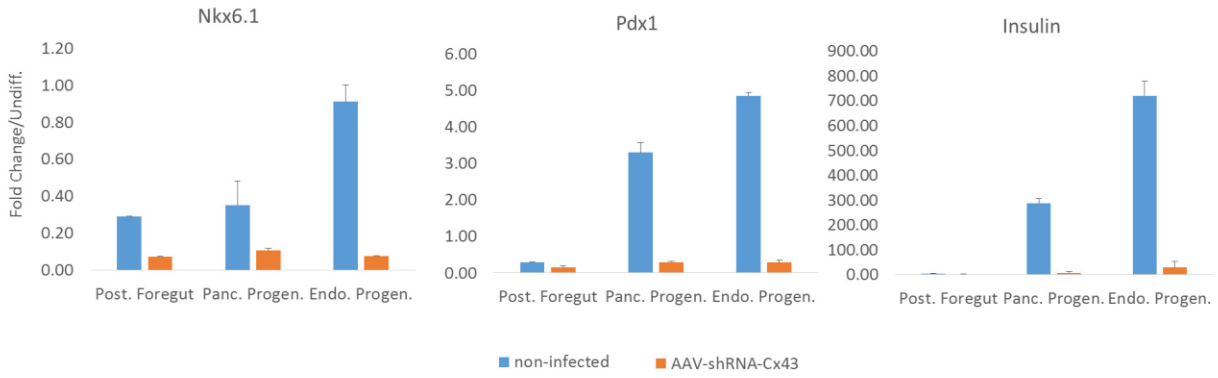


Figure 4-4 *Gene expression analysis for markers of endocrine differentiation in AAV-shRNA/Cx43-infected cells.*

Infected cells showed a marked decrease of expression of Pdx1, Nkx6.1, and Insulin gene expression throughout the last 3 stages of differentiation. This is likely due to prevention of induction rather than a knockdown.

Cultures of hESCs infected with AAV-shRNA-Cx43 showed a marked decrease of Pdx1, Nkx6.1, and Insulin gene expression throughout the last 3 stages of differentiation, indicating that Cx43 played a positive regulatory role in the induction of the islet cell lineage. Western blotting analysis of Cx43 protein levels in these AAV-shRNA-Cx43-infected cells confirmed the efficient downregulation of Cx43, thus validating the notion that Cx43 is required for efficient development of the islet cell lineage. Interestingly, infected cells also revealed decreased levels of pERK1/2 and cyclinD1, especially at the Pancreatic Progenitor (PP) stage, suggesting that Cx43 may also function as a positive regulator of signaling pathways and expression of genes that are involved in the proliferation and survival of cell lineages developing from DE. Importantly, these effects appeared to be fairly selective since other important genes regulating cell-to-cell interactions such as E-cadherin and β-catenin were not affected (**Figure 4-5)**.

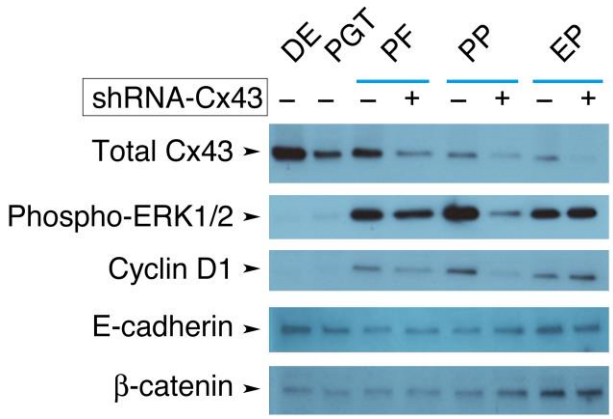


Figure 4-5 *Western Blotting in AAV-shRNA Treated Cells*

Western blot of total cell lysates showed that AAV-shRNA-Cx43-infection was able to effectively knockdown Cx43. Infected cells showed a reduction of phospho-ERK1/2 and CyclinD1, suggesting a positive regulatory role of Cx43 in cell growth. Importantly, the expression level of E-cadherin and β-catenin, two other proteins regulating cell-cell interactions, was not affected.

4.4 DISCUSSION

We initially set out to study knockdown of Cx43 using a targeted siRNA approach. Using this model, we discovered that hESCs are extremely sensitive to transfection reagents used for siRNA interference. In addition, we also noted that multiple siRNA reagents for Cx43 exhibited a series of "off target" effects. Therefore, we moved to an AAV approach expressing shRNAs specific for Cx43. This approach, although more compatible with cell viability, could only be used to infect hESCs at either the DE stage, or PGT stages, since undifferentiated hESCs and Mesendoderm did not express sufficient levels of the αVβ3 and αVβ5 integrins known to function as a receptors for AAVs. Despite this limitation, since PGT cells express relatively high levels of Cx43 (see ***Figure 3-3***C), we reasoned that knockdown of Cx43 at this later stage of differentiation would still be informative in determining the function of this gap junction protein in the specification of downstream cell lineages. As anticipated, in these studies I was able to effectively downregulate Cx43 protein levels, and analyze the developmental propensity of PGT cells at later stages of differentiation. Results from these experiments revealed that

Cx43 is required for PGT cells to acquire a pancreatic progenitor phenotype, as determined by the expression levels of Pdx1, Nkx6.1, and ultimately insulin (***Figure 4-4***). Interestingly, the effect of Cx43 knockdown on PGT developmental propensity can be detected as early as the PF stage, suggesting that Cx43 is required for the commitment of these early gut tube progenitors to acquire Pdx1 and commit toward a pancreatic epithelium. In addition, the observation of a reduced expression of CyclinD1 may be consistent with previous data showing that Cx43 may have a role in helping cells enter the cell cycle[49].

Infected cells also showed a reduced phosphorylation of ERK1/2 in AAV-shRNA-Cx43-infected cells. In the pancreas, blockade of ERK1/2 activation partially impairs Pdx1 stimulated proliferation[180]. Therefore, the lower expression of phospho-ERK1/2 suggests that Cx43 may also be required for cell expansion, an event that is equally important for the emergence of PF and PP from the *in vitro* directed differentiation of hESC cultures.

Together, the reduced expression levels of CyclinD1 and reduced pERK1/2 indicate that knockdown of Cx43 is negatively impacting the ability of stem cells to proliferate. Furthermore, the reduced availability of Cx43 may also impair the responsiveness of stem cells to our *in vitro* differentiation protocol and lead to a stochastic differentiation toward multiple cell lineages rather than toward the islet cell lineage.

Acknowledgements

Prerana Ranjitkar conducted experiments of integrin subunits expression profiling, including the αV integrins gene expression analysis.

Chapter 5. B1 INTEGRINS AS REGULATORS OF B-CELL MASS

5.1 OVERVIEW

This chapter discusses the importance of β1 integrins as regulators of β-cell mass during pancreatic development. Cellular interactions with the extracellular matrix (ECM) mediated by receptors of the integrin family are postulated to regulate key functions in endocrine development processes. In this chapter, we show that ablation of the β1 integrin gene in developing pancreatic β-cells reduces their ability to expand during embryonic life, during the first week of postnatal life, and thereafter. Mice lacking β1 integrin in insulin-producing cells exhibit a dramatic reduction of the number of β-cells to only ~18% of wild-type levels. Despite the significant reduction in β-cell mass, these mutant mice are not diabetic. A thorough phenotypic analysis of β-cells lacking β1 integrin revealed a normal expression repertoire of β-cell markers, normal architectural organization within islet clusters, and a normal ultrastructure. Global gene expression analysis revealed that ablation of this ECM receptor in β-cells inhibits the expression of genes regulating cell cycle progression. Collectively, our results demonstrate that β1 integrin receptors function as crucial positive regulators of β-cell expansion.

5.2 INTRODUCTION

Many types of cell surface signaling receptors contribute to proper growth and development in the pancreas. Specifically, development of the islet cell lineage depends on a series of highly regulated processes that include proliferation, delamination, migration and differentiation of trunk ductal progenitors into the surrounding mesenchyme where they organize into cell clusters. Cellular interactions with the extracellular matrix (ECM) are of critical importance during development and in postnatal life as they affect a variety of events including mitogenesis, differentiation, cell type-specific functions, and survival[88,91,92,104,181]. Over the past two decades it has become clear that the extracellular matrix (ECM) represents an important "informational unit" affecting a large spectrum of cellular functions through the interaction with cell surface receptors of the integrin family[91,92,104,181,182]. These cellular responses to the

extracellular microenvironment are imposed not only by the type of ECM presented to the cell, but also by cell adhesion receptors of the integrin family that mediate the recognition of distinct ECMs.

In this study, we investigated the function of β1 integrins in developing islet β-cells by targeting the deletion of exon 3 of the mouse β1 integrin gene (Itgb1) using a Cre-lox approach. Accordingly, we demonstrated that ablation of β1 integrin gene in developing pancreatic β- cells results in a dramatic reduction of the number of β-cells, down to about 18% of that measured in wild type littermates. Despite the significant reduction in β-cell mass, these mutant β-cells harbor a normal repertoire of β-cell markers, proper architectural organization within islet clusters, and a normal ultrastructure. Global gene expression analysis reveals that ablation of this ECM receptor in β-cells negatively affects expression of genes regulating cell cycle progression. Our results demonstrate that this class of integrin receptors is required for proper β-cell expansion during development and in postnatal life.

5.3 RESULTS

5.3.1 *Cre-mediated deletion of β1 integrin in pancreatic β-cells*

In the pancreas, β1 integrin has been documented to mediate important functions spanning from adhesion and migration of progenitor cell populations, to cell survival and secretory functions of adult islet cells [93–97,100,111]. In situ immunolocalization reveals that the β1 integrin subunit is broadly expressed in both the developing and postnatal pancreas (**Figure 5-1**). In E17.5 pancreas, the brightest levels of β1 integrin- specific immunoreactivity are in ductal cells (**Figure 5-1**A-D, cyan arrows) and in vascular structures colonizing developing islet cell clusters (**Figure 5-1**A-D, cyan arrowheads). Significant β1 integrin- specific immunoreactivity is readily detected at cell-cell and cell- matrix boundaries of developing β-cells (**Figure 5-1**A-D, yellow arrowheads). This expression pattern remains relatively unaltered in postnatal life at P4 (**Figure 5-1**E-H), when β1 integrin remains expressed at high levels in the ductal epithelium (cyan arrows) and in endothelial cells (cyan arrowheads), and at lower levels in β-cells (yellow arrowheads) and in acinar cells, as determined by both qualitative assessment (**Figure 5-1**A-H) and quantitative measurement of the pixel intensity of β1 integrin-specific immunoreactivity (**Figure 5-1**I).

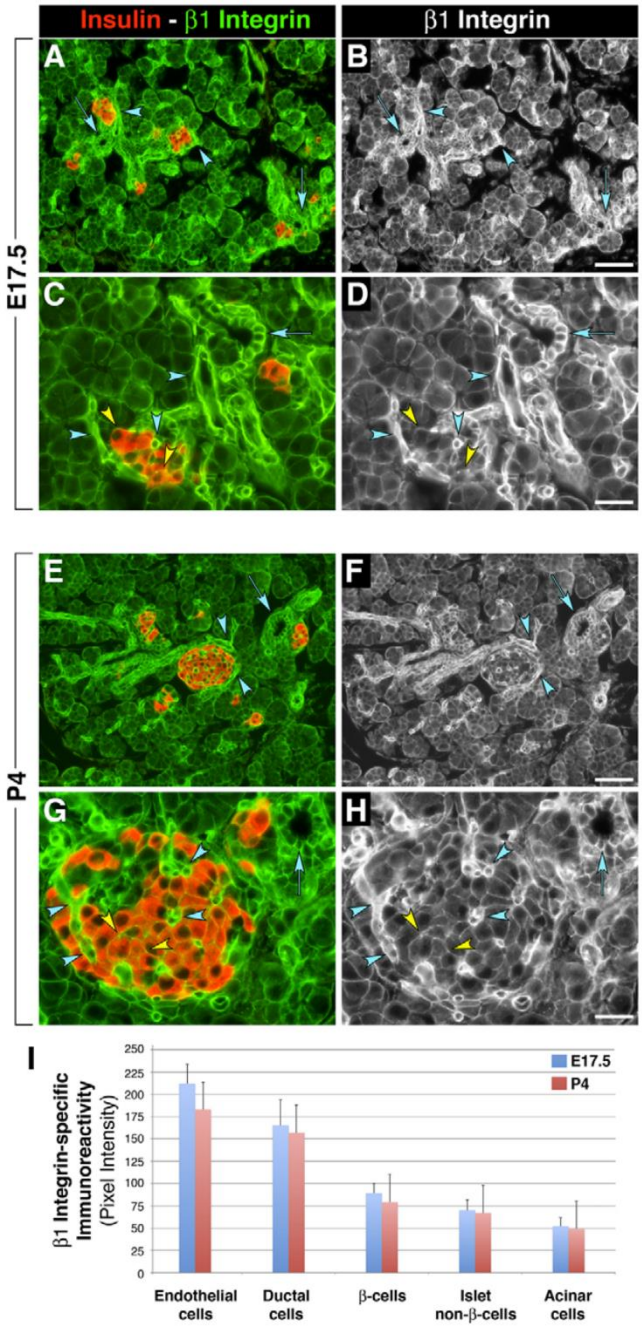


Figure 5-1 *Expression pattern of β1 integrin in the developing and postnatal mouse pancreas.* ***(A-H)*** *Immunolocalization of β1 integrin and insulin in E17.5 (**A-D**) and P4 (**E-H**) pancreas. (**B,D,F,H**) Monochromatic images to better visualize cell-cell and cell-matrix boundaries immunostained for β1 integrin. The brightest β1 integrin-specific immunoreactivity is detected in ductal cells (cyan arrows) and in endothelial cells (cyan arrowheads). Significant*

*β1 integrin expression is also detected at cell-cell and cell-matrix boundaries of developing β-cells (yellow arrowheads). (**I**) Quantitative assessment of the pixel intensity of β1 integrin-specific immunoreactivity in E17.5 and P4 pancreas. Values are presented as mean±s.e.m. Scale bars: 50 µm in B,F; 25 µm in D,H.*

Due to the relative lack of fidelity and/or temporal expression of Cre in pancreatic cell types used in these earlier studies, it had remained unclear whether β1 integrin is also important for β-cell development and function. We addressed this question using a Cre-lox approach to delete β1-integrin in β-cells[3]. We investigated the functional requirement of β1 integrin during β- cell development by means of a conditional gene knockout approach. We chose RIP-Cre mice, which have been used previously to successfully target floxed DNA sequences in pancreatic β-cells[163]. **Figure 5-2**A shows X-Gal staining of islets of Langerhans in a pancreas from a heterozygous RIP- Cre;129S-Gt(ROSA)26Sortm1Sor/J mouse[183]. Following this control experiment demonstrating Cre activity in islet cells, we crossed RIP-Cre mice with homozygous B6;129- Itgb1tm1Efu/J mice that harbor loxP sites flanking exon 3 of the β1 integrin gene[164]. **Figure 5-2**B shows the PCR genotyping of β1 integrin heterozygous/RIP-Cre+ (ht/Cre+) and β1 integrin homozygous/RIP-Cre+ (hm/Cre+, henceforth referred to as RIP-Cre/β1KO). RIP-Cre/β1KO mice were viable and were used for biochemical and morphological characterization.

Western blotting analysis of islet protein extracts from RIP-Cre/β1KO mice demonstrates efficient ablation of β1 integrins (**Figure 5-2**C), with only a faint β1 integrin-specific immunoreactive band in the RIP-Cre/β1KO sample, which is likely to be due to the non-insulin-expressing endocrine cells or other non-endocrine cells present within the islets. Fig. 3D shows immunofluorescent staining of isolated islet cells for β1 integrin (red) and insulin (green). Note that whereas significant β1 integrin-specific immunoreactivity is detected in wild-type (WT) insulin-positive and insulin-negative cells (**Figure 5-2**D, left), in RIP-Cre/β1KO mice the β-cells (green) lack β1 integrin-specific staining (**Figure 5-2**D, right). Flow cytometry analysis on islet cells immunostained for insulin and β1 integrin demonstrates ~95% recombination in RIP-Cre/β1KO mice (**Figure 5-2**E,F). Collectively, these experiments demonstrate that β1 integrin is efficiently deleted in islet β-cells of RIP-Cre/β1KO mice.

5.3.2 *Defective β-cell adhesive properties in RIP- Cre/β1KO mice*

The ability of integrin receptors to support cell adhesion depends on the recognition of specific ECM ligands present in the extracellular environment. To determine whether pancreatic β- cells from RIP-Cre/β1KO mice exhibit impaired cell adhesion properties, intact islet clusters or isolated islet cells were tested for their ability to adhere to a cell-assembled basal membrane-like matrix (804G)[167,168] or to select ECM components. We found that islet cell clusters isolated from WT mice promptly adhered and spread onto 804G matrix, and established monolayer colonies within 72 hours in culture (**Figure 5-2**G, top left). By contrast, RIP-Cre/β1KO islet cells, cultured under the same conditions, remained loosely attached to the 804G matrix and failed to establish cell monolayers (**Figure 5-2**G, top right). When islets were dissociated into a single-cell suspension and plated on the 804G matrix, WT islet cells adhered and established small colonies (**Figure 5-2**G, bottom left), whereas islet cells isolated from RIP-Cre/β1KO mice were unable to do so (**Figure 5-2**G, bottom right).

To examine the adhesive property of β-cells and non-β-cell types present in islet cell preparations, islet cells isolated from either WT or RIP-Cre/β1KO mice were tested for their ability to adhere to select ECM proteins using a short-term adhesion assay under serum- free conditions, as previously described[93,94]. At the end of the assay, adherent cells were fixed, stained for insulin or glucagon by immunocytochemistry to identify α- and β-cells, respectively, and counted. Fewer β-cells from RIP-Cre/β1KO islets adhered to collagen IV and fibronectin when compared with WT cell preparations (**Figure 5-2**H). By contrast, β-cell adhesion to laminin and vitronectin was unaffected, which was likely to be due to the ability of other integrin receptors, such as α6β4 and αvβ5, to mediate cell adhesion to these ECMs[2,184]. Glucagon-producing α-cells, which were not targeted in RIP-Cre/β1KO mice, displayed comparable adhesion to all ECM substrates tested in both animal groups (**Figure 5-2**I).

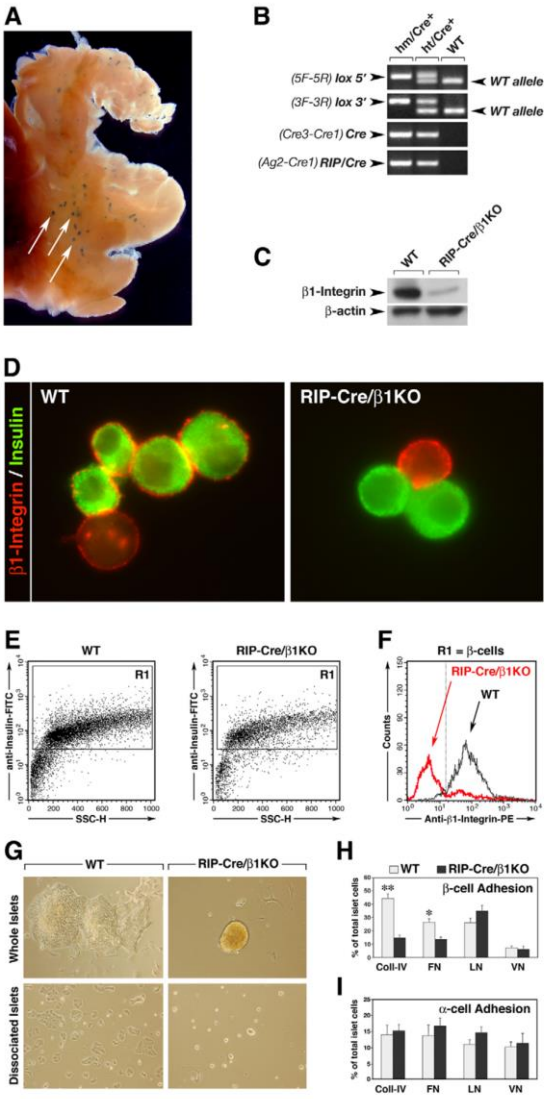


Figure 5-2 *Efficient deletion of β1 integrin in pancreatic β- cells.*

*(**A**) Whole-mount of X-Gal-stained (blue) adult pancreas from a RIP-Cre/ROSA26 mouse demonstrating targeting of Cremediated recombination to pancreatic islet clusters (arrows). (**B**) PCR genotyping of tail DNA showing the presence of the loxP sequences flanking exon 3 of Itgb1 (lox 5 and lox 3); Cre3-Cre1 primers were used to detect the presence of the Cre gene band and Ag2-Cre1 to detect the correct integration of the Cre gene with the RIP sequence. (**C**) Western blot analysis of isolated pancreatic islets from WT or RIP-Cre/β1KO mice probed for β1 integrin. β-actin was used as loading control. (**D**) Immunofluorescence analysis of dissociated islet cells from WT or RIP-Cre/β1KO pancreas stained for β1 integrin (red) and insulin (green). (**E**) Flow cytometry analysis of islet cells isolated from WT or RIP-Cre/β1KO*

*pancreas. (**F**) Levels of β1 integrinspecific immunoreactivity in insulin-positive cells from the R1 gate in E. (**G**) Long-term adhesion and spreading assay of whole islets (top row) or dissociated islet cells (bottom row). (**H,I**) Quantitative assessment of β-cell and α-cell adhesion to the ECM proteins collagen IV (Coll-IV), fibronectin (FN), laminin (LN) and vitronectin (VN). Values are expressed as a percentage ± s.d. of total number of cells seeded. (H) n=5, *P<.001, ** P<.05.*

Reduced β-cell numbers in RIP-Cre/β1KO mice Pancreatic sections from 8-week-old WT or RIP-Cre/β1KO mice were used for immunofluorescence and morphometric studies to determine whether the deletion of β1 integrin affected the phenotype and architecture of the islets of Langerhans. These experiments revealed that islet clusters are significantly smaller in RIP- Cre/β1KO mice when compared with WT (**Figure 5-3**A-D). A representative example of this phenotype is shown in **Figure 5-2**A, where immunostaining for insulin and glucagon identifies islet clusters. Within islets of RIP-Cre/β1KO mice, β1 integrin-specific immunoreactivity is only detected in α-cells (red) and in capillary endothelial cells (red arrows), but not in β-cells. Extensive morphometric measurements revealed that the area occupied by β- cells is reduced by ~80% in RIP-Cre/β1KO mice compared with either WT or control RIP-Cre transgenics (**Figure 5-3**B), whereas the number of α-cells is unaffected (**Figure 5-3**C). In addition, analysis of the islet size distribution revealed that whereas the frequency of small islet clusters (50-100 cells) does not differ significantly between WT and RIP-Cre/β1KO mice, the number of larger islets (>100 cells) is dramatically reduced in RIP-Cre/β1KO mice, suggesting that β-cells, following their specification and differentiation, are unable to expand in the absence of β1 integrin (**Figure 5-3**D). Morphometric assessment of apoptotic events as a possible mechanism for the reduction in β-cell numbers revealed no differences between RIP-Cre/β1KO and WT pancreata (data not shown). Further phenotypic characterization revealed that β-cells from RIP-Cre/β1KO mice exhibit a normal expression pattern of the transcription factors MAFA, PAX6, NKX6.1 and PDX1 (**Figure 5-3**E), suggesting that loss of β1 integrin does not affect their ability to express these markers of endocrine differentiation.

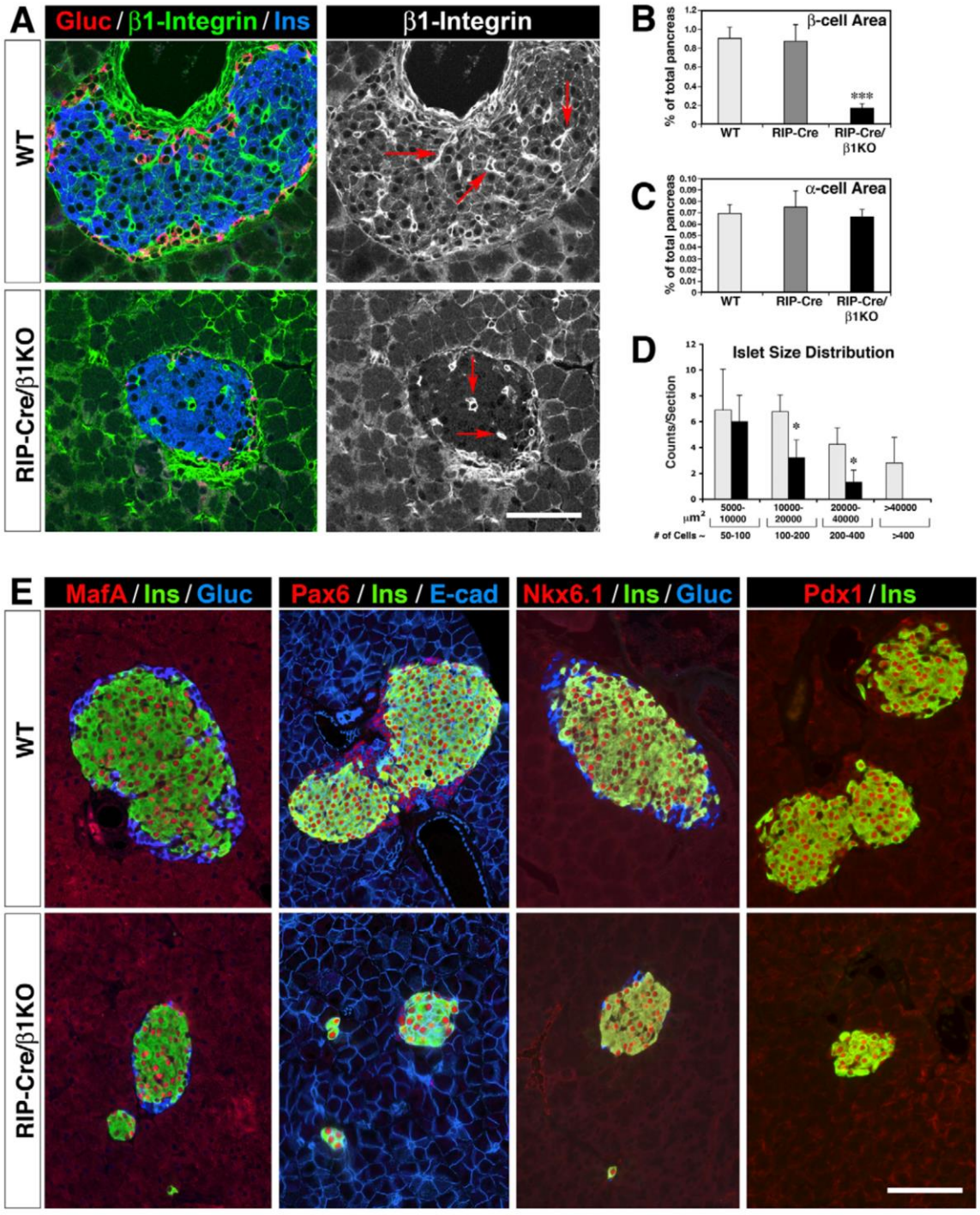


Figure 5-3 *Characterization of β1 integrin-deficient pancreatic islets.*

*(**A**) Representative confocal images of pancreatic sections from 8-week-old WT (top row) and RIP-Cre/β1KO mice (bottom row) immunostained for β1 integrin (green), glucagon (red) and insulin (blue). Red arrows in monochromatic images point to intra-islet endothelial cells*

*strongly immunoreactive for β1 integrin. (**B,C**) Quantification of insulin-positive (**B**) and glucagon-positive (**C**) area in WT (n=5), RIP-Cre (n=3) and RIP-Cre/β1KO (n=5) pancreas. Values are expressed as a percentage of total pancreatic area ± s.d. ***P<0.001. (**D**) Islet cell number and size distribution in WT and RIP-Cre/β1KO mice. Values are expressed as mean ± s.d. of the number of islets per section (n=3 animals). *P<0.05. (**E**) Immunolocalization of transcription factors MAFA, PAX6, NKX6.1 and PDX1 in WT and RIP-Cre/β1KO pancreas. Scale bars: 50 μm in A; 70 μm in E.*

5.3.3 *Loss of β1 integrin does not affect the differentiation or function of β-cells*

To fully define the phenotype of RIP-Cre/β1KO β-cells we performed ultrastructural studies by transmission electron microscopy (TEM). Representative TEM images of β-cells from WT (**Figure 5-4**A,C) and RIP-Cre/β1KO (**Figure 5-4**B,D) mice show morphologically normal insulin granules in both cell preparations, thus demonstrating that ablation of β1 integrin does not negatively affect the cellular machinery responsible for the biosynthesis of these subcellular compartments. Interestingly, despite previous evidence indicating that β1 integrin plays important roles in the assembly and maintenance of basal membranes[86,185], we find that β-cells from RIP-Cre/β1KO mice exhibit electron-dense basal membrane structures that are indistinguishable from those observed in WT (**Figure 5-4**D versus C, brackets). In addition, microcapillary endothelial cells lining basal membranes adjacent to RIP-Cre/β1KO β-cells exhibit normal fenestrations (**Figure 5-4**C,D, arrowheads), suggesting that ablation of β1 integrin in β-cells has no functional consequences for the morphology of the adjacent vascular endothelium. In these studies we also observed that β-cells from RIP-Cre/β1KO mice appear to contain an increased number of insulin granules.

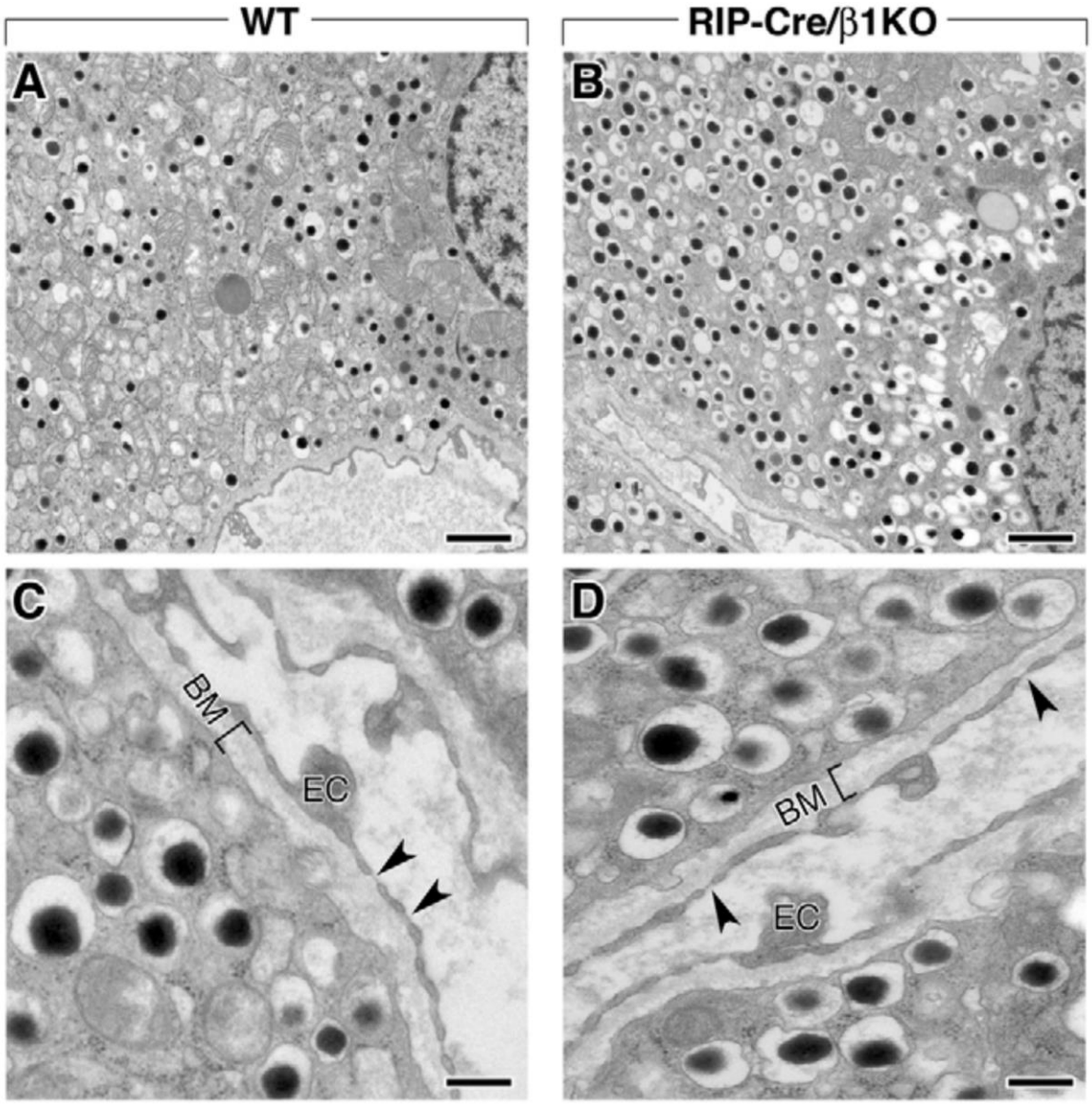


Figure 5-4 *Ultrastructural analysis of WT and RIP-Cre/β1KO pancreatic islets.*

*(**A,B**) Transmission electron microscopy (TEM) of β-cells in WT (A) and RIP-Cre/β1KO (**B**) islets showing insulin-containing granules with their typical electron-dense core. (**C,D**) Higher magnification TEM images of WT (**C**) and RIP-Cre/β1KO (**D**) islets identifying the presence of a basal membrane (BM, bracket) at the interface between endothelial cells (EC) and β-cells. Normal endothelial cell fenestrae (**C,D**, arrowheads) can be identified in both WT and RIP-Cre/β1KO samples. Scale bars: 1 µm in A,B; 0.3 µm in C,D.*

This observation was validated biochemically by the demonstration that β-cells in RIP-Cre/β1KO mice contain close to 3-fold more insulin than in WT (**Figure 5-5**A). Despite the significant reduction in β-cell numbers (**Figure 5-3**), adult RIP-Cre/β1KO mice did not display abnormal fasting glycaemia, nor altered response in a glucose tolerance test (**Figure 5-5**B). These results are not surprising in consideration of the fact that, in most rodent models, overt diabetes occurs only upon loss of more than 80% of the total pancreatic β-cell number[186]. The only functional difference we observed in RIP-Cre/β1KO mice is a reduced insulin output in response to glucose, although both the first and second phases of insulin release are preserved (**Figure 5-5**C). RIP-Cre/β1KO β-cells also display a normal membrane-targeted expression of the glucose transporter GLUT2 (SLC2A2 – Mouse Genome Informatics) (**Figure 5-5**D,E), and Glut2 expression appears significantly upregulated in RIP-Cre/β1KO β- cells when compared with WT (**Figure 5-5**F). These results indicate that RIP-Cre/β1KO β-cells harbor a functional glucose-sensing machinery that allows for sufficient insulin secretion to maintain normoglycemia. It is possible that, despite the reduced β-cell mass and decreased levels of circulating insulin (**Figure 5-5**C), RIP-Cre/β1KO mice may develop peripheral insulin hypersensitivity as an adaptive counter-regulatory mechanism to maintain normoglycemia.

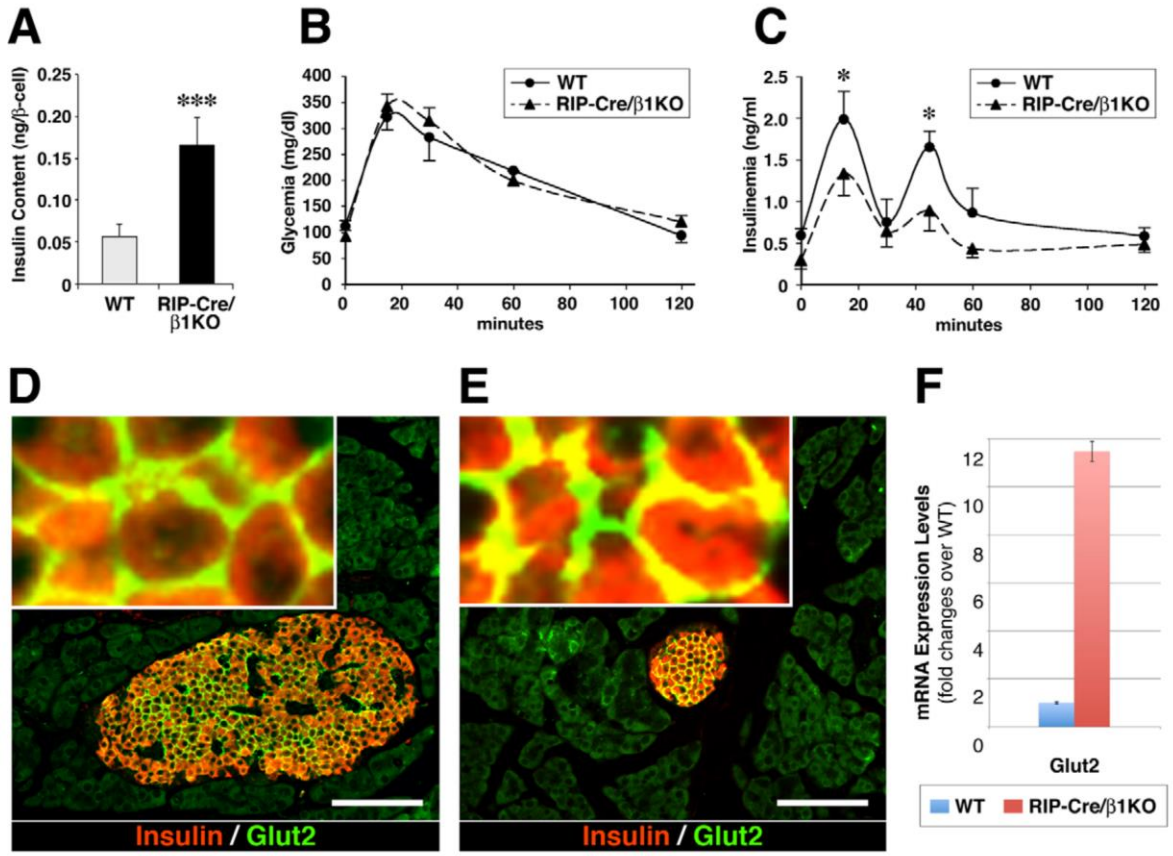


Figure 5-5 *Insulin secretory function of WT and RIP-Cre/β1KO pancreatic islets.*

*(**A**) Insulin content measured in WT and RIP-Cre/β1KO β-cells (n=4). *P<0.001 (B,C) Blood glucose (**B**) and plasma insulin (**C**) quantification during a glucose tolerance test in 4-month-old WT and RIP-Cre/β1KO mice. Values are expressed as mean ± s.e.m. (n=5 animals for each genotype). **P<0.05. (**D,E**) GLUT2-specific immunoreactivity detected in RIP-Cre/β1KO (E) β-cells. (**F**) mRNA expression levels of Glut2 measured by zPCR in β-cells isolated from WT and RIP-Cre/β1KO pancreas. Scale bars: 70 μm.*

5.3.4 *β1 integrin is required for β-cell expansion*

Based on the evidence that β1 integrins may function as positive regulators of cell cycle progression and survival[187,188], we postulated that the reduced number of β-cells in RIP-Cre/β1KO mice might result from defective β-cell expansion and/or survival during the developmental and neonatal periods. Indeed, immune-morphometric assessment of pancreata stained for markers of cell proliferation demonstrated that at E17.5 numerous proliferating β-cells can be identified

in the pancreas of WT but not RIP-Cre/β1KO mice (**Figure 5-6**A, arrowheads). This analysis revealed a significant reduction of the number of proliferating β-cells, as determined by double immunostaining for insulin and phospho-histone H3 (**Figure 5-6**B), or double staining for insulin and PCNA (**Figure 5-6**C). These results correlated with a reduction of β-cell area already at this relatively early stage of islet cell cluster expansion (**Figure 5-6**D). This phenotype becomes even more striking by P4, when the number of proliferating β-cells increases dramatically in WT but not in RIP-Cre/β1KO mice (**Figure 5-6**E-G), an effect that correlates with a dramatic reduction of the β-cell area (**Figure 5-6**H). Eventually, by 8 weeks of age, although occasional proliferation of β-cells can still be detected in WT mice, BrdU+ (or PCNA+) β-cells become extremely rare in RIP-Cre/β1KO mice (**Figure 5-6**I-K). Accordingly, RIP-Cre/β1KO mice exhibit a β-cell area that is only ~18% of that measured in WT or in RIP-Cre transgenic mice used as additional controls (**Figure 5-6**L).

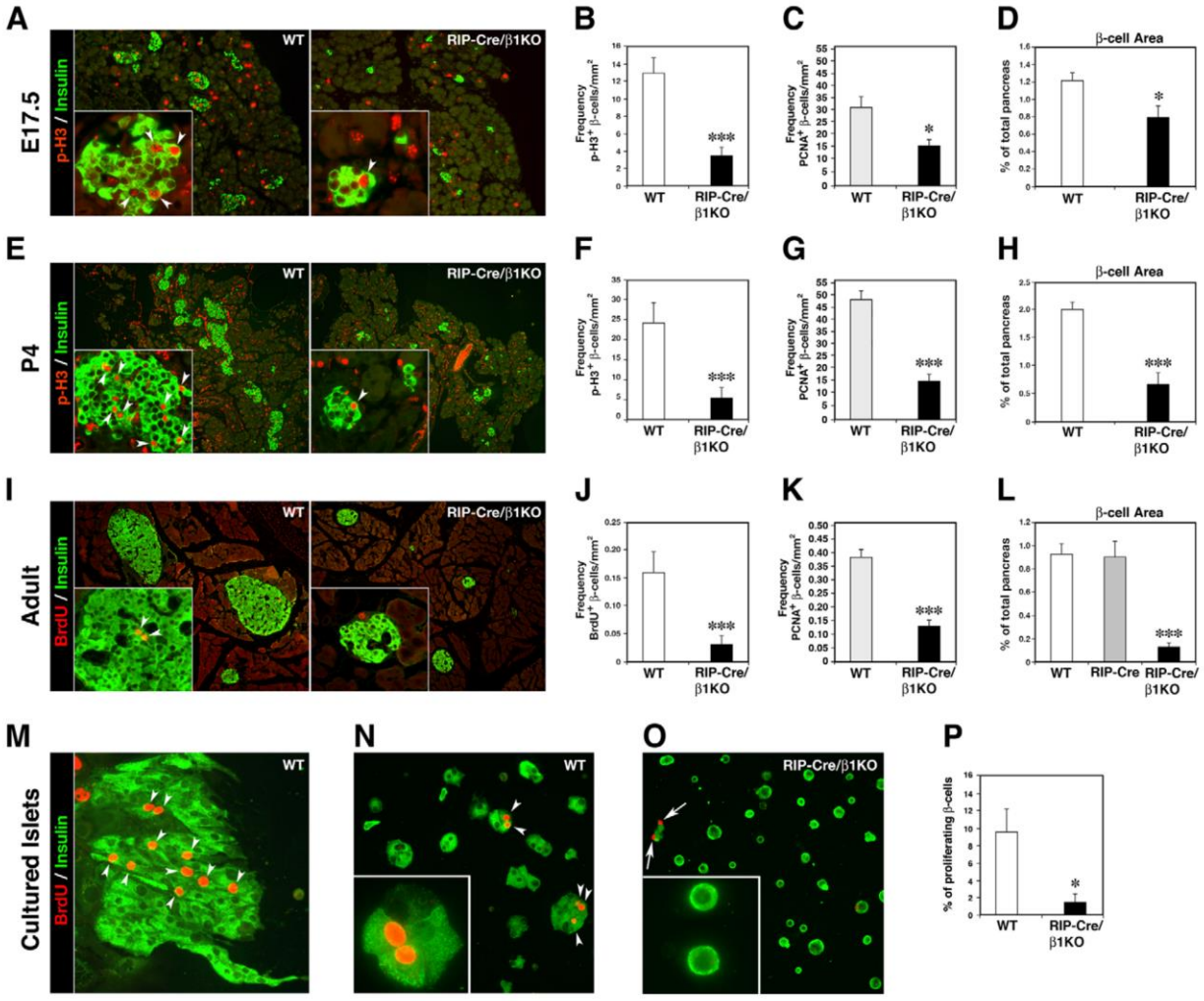


Figure 5-6 *Defective β-cell expansion in RIP-Cre/β1KO mice during pancreas development.*

*(**A**) Representative images of pancreatic sections from WT and RIP-Cre/β1KO E17.5 embryos immunostained for phospho-histone H3 (p-H3) and insulin. (**B-D**) Frequency of p-H3+ (**B**) and PCNA+ (**C**) β-cells and measurement of β-cell area (**D**) in WT and RIP-Cre/β1KO E17.5 pancreas. (**E**) Pancreatic sections of P4 WT and RIP-Cre/β1KO mice immunostained for pH3 and insulin. (**F-H**) Frequency of p-H3+ (**F**) and PCNA+ (**G**) β-cells and measurement of β-cell area (**H**) in P4 WT and RIP-Cre/β1KO mice. (**I**) Adult pancreatic sections from WT and RIP-Cre/β1K mice stained for BrdU and insulin. (**J-L**) Frequency of BrdU+ (**J**) and PCNA+ (**K**) β-cells and measurement of β-cell area (**L**) in pancreatic sections from adult WT and RIP-Cre/β1K mice. (**M-O**) Immunostaining for BrdU and insulin in cultures to assess the induction of β-cell proliferation by 804G matrix and HGF in WT whole islets (**M**), partially dissociated WT (**N**) or RIP-Cre/β1KO (**O**) islet cells. Insets in **A,E,I,N,O** show higher magnification of islet cells.*

*Arrowheads in **A,E,I,M,N** indicate nuclei of proliferating β-cells. Arrows in O indicate proliferating insulin-negative cells. (**P**) Quantification of BrdU+ β-cells measured for WT and RIP-Cre/β1KO islet cell preparations. (**A-D**) n=8 pancreata for each genotype; (**E-L**) n=5 pancreata for each genotype; (**M-P**) representative of n=4 independent experiments using both WT and RIP-Cre/β1KO pancreatic islets. Values in graphs represent mean±s.e.m. (**B**) ***P<0.001.*

To investigate whether β-cells from RIP-Cre/β1KO mice can be induced to proliferate in vitro, we isolated islets from both WT and RIP-Cre/β1KO mice, and cultured them on 804G extracellular matrix-coated coverslips in the presence of HGF as previously described[168]. WT islets established colonies that comprised numerous BrdU-incorporating insulin+ cells over a 3- day culture period (**Figure 5-5**M). By contrast, islets isolated from RIPCre/β1KO mice failed to adhere, spread or generate colonies (**Figure 5-2**F) and did not show any significant cell proliferation (not shown). In similar experiments, plating of partially dissociated islets onto the 804G extracellular matrix gave similar results, with numerous BrdU-incorporating β-cells detected in WT (**Figure 5-6**N,P) but not in RIP-Cre/β1KO islet cell preparations (**Figure 5-6**O,P). Hence, ablation of β1 integrin in β-cells severely impacts on their ability to respond to mitogenic stimuli provided by ECM cues and by growth factors such as HGF.

5.4 DISCUSSION

It is well established that β1 integrin receptors regulate multiple functions, encompassing cell adhesion, migration, differentiation, growth and survival[85,88,189]. In this study, we provide original evidence for a crucial role of the β1 integrin subunit in the in vivo expansion of pancreatic β-cells following their endocrine specification. Our results indicate that 'pro-proliferative' signaling cues transduced by β1 integrins in pancreatic β-cells are uncoupled from 'pro-differentiative' signals attributed to these receptors in other cell types, suggesting that alternative integrins, and cell-cell adhesion receptors, are likely to be responsible for mediating

the interaction of β-cells with their extracellular microenvironment and with neighboring cells, and for regulating the maintenance of their differentiated endocrine phenotype.

The signaling properties of integrin receptors are dictated primarily by their state of activation resulting from ECM ligand occupancy and/or cis-transactivation through interaction with growth factor receptors harboring tyrosine kinase functions, rather than by their levels of expression[85,88,189–194]. Nevertheless, it is reasonable to infer that the levels of β1 integrin expression detected in different pancreatic epithelial cell types correlate with cell type-specific proliferative capabilities. Accordingly, ductal cells expressing high levels of β1 integrin exhibit a higher propensity to respond to mitogenic stimuli than islet cells and acinar cells[195–199]. Conversely, acinar cells, which we find to express low levels of β1 integrin, are unable to undergo significant proliferation unless they enter an acinar-to-ductal transdifferentiation[200–202]. Islet cells have been reported to undergo epithelial-to mesenchymal transition when replicating[99,203], a phenomenon that has been described in many epithelial cell types and that is associated with significant upregulation of β1 integrin[204,205]. This provides a possible mechanism by which cells about to enter the cell cycle can enhance their propensity to use this integrin subunit to respond to microenvironmental cues and activate pro-proliferative signaling cascades.

Following their endocrine specification and insulin expression, pancreatic β-cells enter a spatiotemporally regulated expansion that starts to become evident at ~E17.5, continues to increase during the last week of embryonic life, reaches a plateau during the first 2 weeks of postnatal life[206,207], and gradually declines thereafter[208–210]. Based on this well-established timeline of islet cell mass development, and on the established pro-proliferative functions of β1 integrin described in other epithelia, our finding that RIP-Cre/β1KO mice exhibit significantly reduced numbers of β-cells led us to postulate that the ablation of β1 integrin in β-cells might negatively impact on their replication and/or survival. Accordingly, our analysis of RIP-Cre/β1KO pancreas reveals that the frequency of proliferating β-cells is significantly decreased already at E17.5, a defect that becomes even more dramatic during the first week of postnatal life, and in adult mice. This proliferative defect leads to a severe reduction in β-cell numbers in adult RIP-Cre/β1KO pancreas as compared with WT mice. Collectively, these results clearly demonstrate

that β1 integrin functions as a crucial positive regulator of β-cell expansion during development and in postnatal life.

A detailed phenotypic analysis of β-cells from RIP-Cre/β1KO mice revealed that, despite defective cell adhesion to fibronectin and collagen type IV, they retain all of the essential hallmarks of endocrine differentiation, normal architectural organization within islet clusters, and glucose-stimulated secretory function. Accordingly, we found that the expression pattern of the transcription factors PDX1, PAX6, NKX6.1 and MAFA appears indistinguishable from that of WT β-cells. Interestingly, the insulin content of β-cells from RIP-Cre/β1KO mice is increased 3-fold, suggesting that maintenance of endocrine differentiation might be regulated by alternative mechanisms of cell-cell and/or cell-matrix interaction. Notably, we found that the expression of the canonical cadherins Cdh1 and Cdh2 (E-cadherin and N-cadherin), which are the primary adhesion receptors responsible for cell-cell aggregation of islet cells[211], is unaffected in β-cells from RIP-Cre/β1KO, suggesting that their pro-differentiative function is preserved in the absence of β1 integrin.

Previous work has proposed that laminins produced by endothelial cells play an important role in regulating insulin gene expression, and that blockade of β1 integrin laminin receptors in β- cells interferes with insulin secretion[98,167,212]. At variance with these earlier in vitro studies, we find that the genetic ablation of β1 integrin in β- cells in vivo does not affect their insulin production or secretion in response to glucose. These results suggest that, in the absence of β1 integrin, β-cells might activate alternative mechanisms of ECM recognition that support their endocrine phenotype and function Hence, our findings might be explained by the fact that pancreatic islet cells express not only β1 but also β3, β4 and β5 integrins[2,93–97,99,100,213]. These integrins might provide alternative mechanisms of cell-matrix recognition and might contribute/transduce pro-differentiative signaling cues allowing for the development and functional maturation of β-cells. In support of the possible compensatory function of these alternative integrins, there is evidence that β3 and β5 integrins (i.e. αvβ3 and αvβ5) mediate a number of functions ranging from stationary cell adhesion to motility and invasion, as well as cell proliferation or differentiation depending on the cellular context. In addition, it was demonstrated that β1, β3 and β5 integrins share a reciprocal regulatory cross-talk at the

transcriptional, translational and posttranslational levels, such that significant β3 and/or β5 compensatory functions can be elicited when β1 is either blocked or genetically ablated, resulting in the maintenance or enhancement of cell differentiation programs[214–218]. Further supporting a compensatory function by alternative integrins, the β4 subunit, partnering with α6 to form the α6β4 laminin 5 receptor, has been shown to support a multitude of signaling pathways depending on the cellular context and the complement of other adhesion receptors[88,94,219–225]. Our full-genome Illumina array shows that β- cells from RIP-Cre/β1KO mice exhibit significant upregulation of ECM components, such as vitronectin (Vtn) and laminin 5 β-chain (Lamb3), that serve as ligands for αvβ3 and α6β4, respectively. Among other genes upregulated in β-cells as a result of β1 integrin deletion are those that encode netrin 1 and netrin 4 (Ntn1 and Ntn4), two proteins that share significant homology with laminins and that we have previously reported to be expressed in the developing pancreas in distinct cellular compartments, where they support cell adhesion, migration and endocrine differentiation[93,94,226]. Based on evidence demonstrating that netrins may also contribute to signaling that supports β-cell survival[227], it is possible that ablation of β1 integrin might trigger counter-regulatory mechanisms that protect β-cells from apoptosis.

Maintenance of cell differentiation is constantly at balance with mechanisms regulating cell proliferation. This is achieved through the interplay between positive and negative regulators of the cell cycle that are controlled by both cell-autonomous and nonautonomous mechanisms of gene expression[206,207,228]. Integrins provide an example of multimodular receptors that, depending on their αβ heterodimeric composition, can function as highly specialized transducers of extracellular cues capable of activating outside-in signaling pathways ultimately affecting cellular decisions. Our experiments clearly demonstrate how ablation of β1 integrin in β- cells can dramatically alter their ability to respond to growth stimuli provided by ECMs and growth factors, both in vivo and in vitro. Thus, our full-genome microarray analysis shows that whereas positive regulators of the cell cycle are downregulated, genes that prevent or interfere with cell replication, and thus foster cell differentiation, are upregulated (Fig. 8). The upregulation of genes promoting cell cycle arrest might be explained by mechanisms of derepression invoked as a result of the ablation of β1 integrin, whereas the upregulation of genes supporting cell cycle

progression might reflect an attempt to overcome the proliferative defect caused by the loss of β1 integrin signaling. Accordingly, loss of β1 integrin signaling is supported by the observed downregulation of some of the cyclins or cyclin kinases, as well as of MAPK activity, which are known downstream effectors of β1 integrin signaling[213,229–232]. Also of interest is the downregulation of Ovol2 and Tuba1. Ovol2 downregulation has been shown to interfere with c-MYC and NOTCH1 activity, thus causing cell cycle arrest in G1/G0 phase[233]. Similarly, blockade of α- tubulin has been reported to inhibit cyclin B accumulation and ERK2 activation, leading to arrest in interphase[234]. Both ERK and AKT are known to be directly regulated by integrins[213,229], and their activity has been shown to regulate cell proliferation and survival[235,236]. Hence, reduced activation of the ERK and AKT pathways may impact negatively on the regulation of cyclin D1 transcription. Based on evidence that c-MYC, NOTCH1 and ERK2 are regulated by β1 integrin activation, our results suggest that Ovol2 and Tuba1 might function as novel effectors of β1 integrin utilization in islet β-cell expansion.

Collectively, mounting evidence points to an important role of β1 integrins in the regulation of pancreatic cell interactions with the extracellular microenvironment and in directing crucial developmental decisions such as growth and/or differentiation. To exploit this in regenerative strategies, the availability of β1 integrins agonists would be ideal. However, while pharmacological treatments aiming to negatively regulate integrins' ligand binding has made significant progress, strategies to activate integrin function remain limited. In contrast, modulation of downstream effectors of integrins' activation is gaining significant traction for therapeutic interventions [105–107,237]. Building on this notion, future studies will be built to investigate developmental stage specific effects of ILK-mediated function(s) that have been placed immediately downstream of β1 integrins signaling.

Acknowledgements

My contribution to these studies consisted of performing experiments of qPCR, gene expression analysis and manuscript editing. Giuseppe R. Diaferia performed most of the experiments with pancreatic tissues, analyzed data and contributed to the writing of the manuscript. Antonio J. Jimenez-Caliani performed experiments of immunostaining and morphometric analysis. Laura

Crisa performed flow cytometry experiments and helped in the analysis of results. Vincenzo Cirulli conceived most of the experiments, performed studies of transmission electron microscopy, analyzed data and wrote the manuscript.

Chapter 6.

INTEGRIN LINKED KINASE: A DOWNSTREAM EFFECTOR OF B1 INTEGRIN SIGNALING IN PANCREATIC DEVELOPMENT

6.1 OVERVIEW

This chapter presents data supporting the importance of integrin linked kinase (ILK), a downstream effector of β1 integrin signaling, in pancreatic development. We report the preliminary results on the generation of ILK knockout mice and characterization of their pancreatic phenotype and glucose homeostasis. Specifically, we investigated the effects of ILK ablation in $Pdx1^{+}$ pancreatic progenitors and found an islet phenotype that, in part phenocopies the loss of β1 integrin function, as shown by the development of a reduced islet cell mass. These observations point to an *in vivo* function of ILK as a "master switch" of pancreatic β-cell growth, survival, and function.

6.2 INTRODUCTION

In recent years, ILK has become the target of intense drug discovery efforts that have already lead to clinical trials in which ILK-blocking compounds are being tested to halt cancer cell growth and metastasis [105–107]. Conversely, other efforts are focusing on the identification of drugs that can selectively activate ILK to promote cell survival, as well as to foster cell growth and regeneration in damaged tissues [238–240]. More recently, significant alterations in ILK signaling have been reported to be associated with gestational diabetes, both in rodents and humans [241], and in models of β-cell injury [242]. Current knowledge indicates that ILK function(s) may be cell context-specific [105,109,110,243–246]. This notion underlines the importance of defining the function of ILK in the β-cell lineage, and specifically at distinct developmental and functional stages. These studies raise important questions on the role of ILK as a possible regulator of β-cell development and homeostasis.

The purpose of these studies were two-fold: First, our studies aimed to dissect ILK function in developing β-cells, and determine if this signaling effector of β1 integrin is required for their differentiation, expansion, survival, and function. Second, we aimed to assess whether ILK regulates the establishment of a functional β-cell mass in postnatal life.

6.3 RESULTS

6.3.1 *ILK expression within pancreas*

To determine the localization of ILK expression within the pancreas, we dissected embryonic pancreatic epithelium from E14.5 embryos. We found that ILK was expressed within embryonic pancreatic epithelium and that it co-localized with β1 integrin (**Figure 6-1**, Yellow arrows). β-cells delaminating from ductal domains also co-expressed ILK and β1 integrin (**Figure 6-1**, White arrowheads).

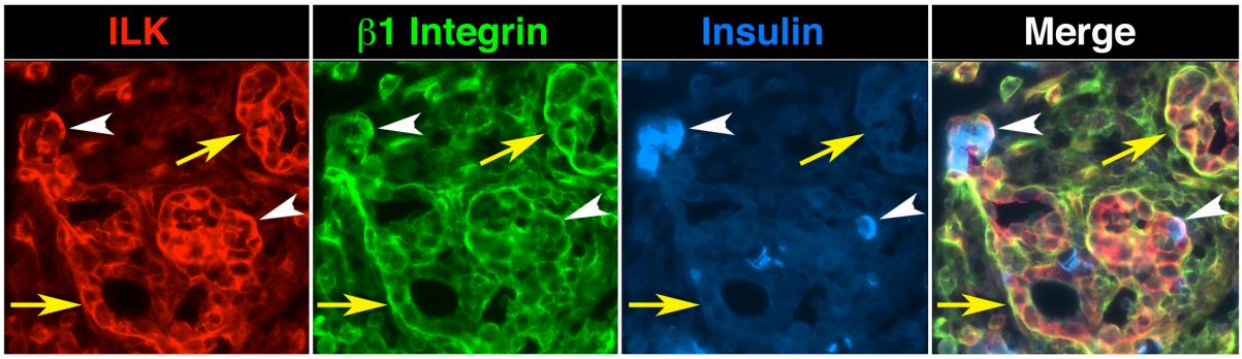


Figure 6-1 *Co-expression of ILK and β1 integrin in the E14.5 pancreas.*

These panels show expression of ILK within embryonic pancreas. The yellow arrows correspond to areas of the pancreatic epithelium that show both ILK and β1 integrin expression. The white arrowheads point to β-cells delaminating from ductal domains as evidenced by Insulin-positive staining.

6.3.2 *ILK inhibition by Cpd-22 in ductal cell proliferation*

To determine if ILK function is required for ductal cell proliferation, we cultured two pancreatic ductal cell lines in the presence of a well characterized ILK-specific inhibitor, i.e. Cpd-

22[108]. For these experiments, we used a moderately differentiated and slow-growing pancreatic ductal line called SU86[247], and a subclone of this line (G3LC) that exhibits a more rapidly growing phenotype (Dr. L. Crisa, personal communication), and applied Cpd-22 after 2 days of growth. Flow cytometric analysis of cells pulsed with BrdU demonstrated that treatment with the Cpd-22 ILK inhibitor decreases BrdU incorporation in a dose-dependent manner in both cell lines (**Figure 6-2**). No toxic effects of this inhibitor were noted at the concentrations used under these experimental conditions.

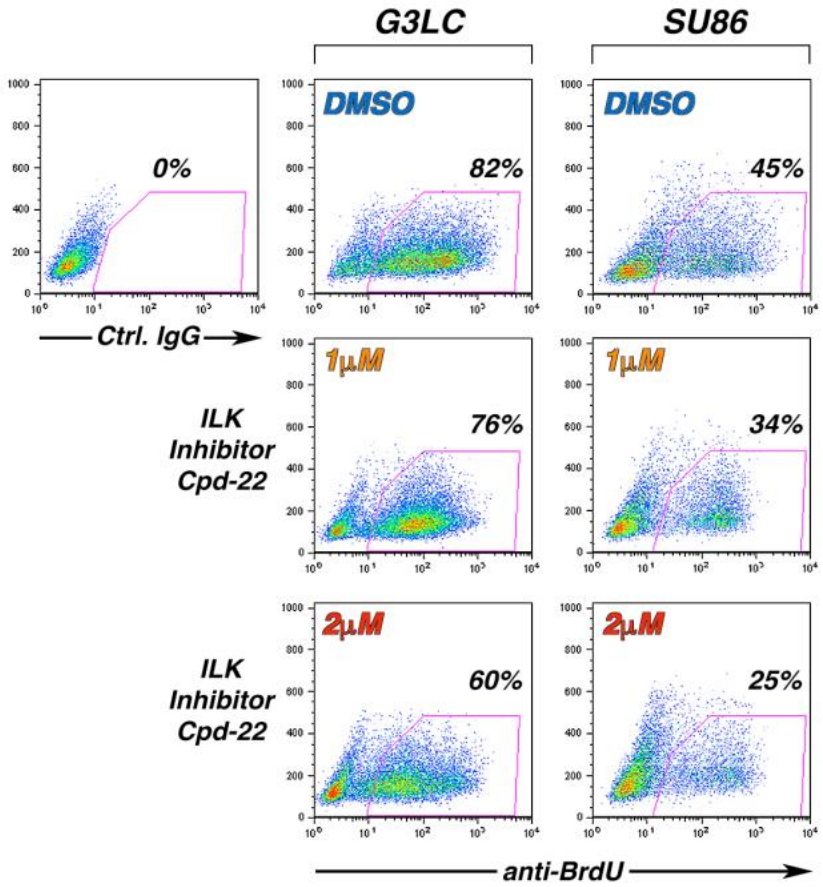


Figure 6-2 *Impact of ILK inhibition by Cpd-22 on pancreatic ductal cell proliferation. Inhibition of ILK by Cpd-22 decreased BrdU incorporation in pancreatic ductal cell lines. This is seen across both SU86 and G3LC cell lines. Note the dose dependent decrease in proliferation.*

6.3.3 *ILK inhibition by Cpd-22 in embryonic pancreatic epithelial cells*

To determine whether ILK regulates the proliferation of primary embryonic pancreatic epithelial cells, embryonic pancreatic epithelium (E15.5) was isolated and cultured on matrigel-coated plates to foster proliferation in response to ECM and HGF/SF (10ng/ml). These culture conditions elicited significant BrdU incorporation over 18 hours (**Figure 6-3**B). Again, addition of the ILK inhibitor Cpd-22 resulted in a dose-dependent inhibition of BrdU incorporation (**Figure 6-3**C).

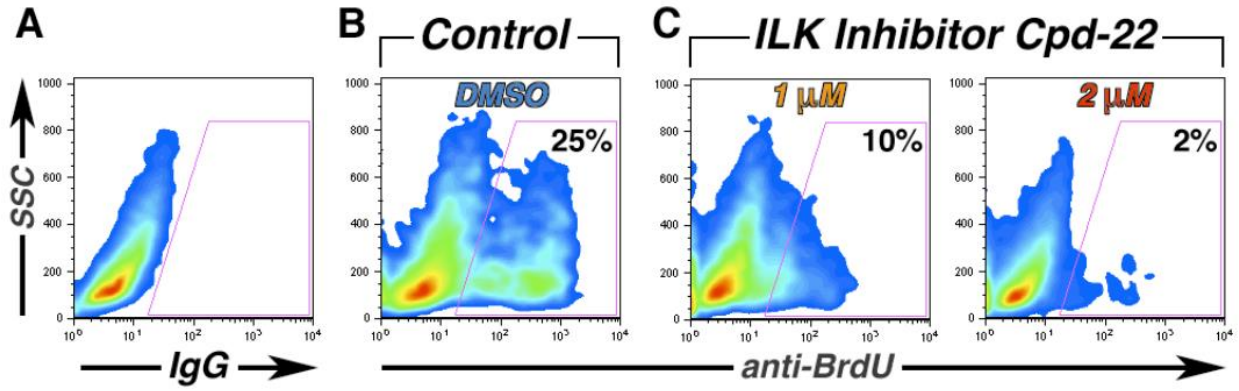


Figure 6-3 *Impact of ILK inhibition by Cpd-22 on the proliferation of the pancreatic epithelium at E15.5.*

Compared to control treated pancreas, ILK inhibition resulted in a decrease in BrdU incorporation. This effect was dose-dependent for the inhibitor and mirrored results from SU86 and G3LC ductal cell line tests.

6.3.4 *ILK as a regulator of β-cell proliferation*

To establish preliminary proof of principle on the function of ILK in β-cell proliferation, we performed similar ILK-inhibition studies using the mouse β-cell line Min6. We found that the ILK inhibitor Cpd-22 caused a significant decrease in BrdU incorporation, demonstrating that ILK functions as a positive regulator of β-cell proliferation. Cpd-22 suppressed ILK-mediated phosphorylation of Akt at Ser473 site, thereby down-regulating the downstream targets, such as

GSK-3β and myosin light chain. ILK also caused a transcriptional repression of the transcription factor Y-Box binding protein 1.

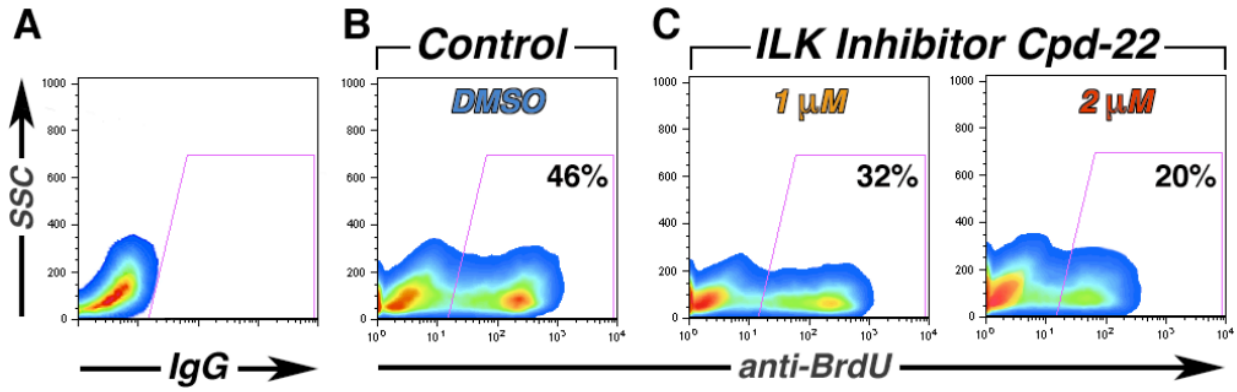


Figure 6-4 *ILK inhibition by Cpd-22 negatively regulate β-cell (Min6) proliferation.*

6.3.5 *Effects of ILK inhibition on the expression pancreatic islet genes*

To further determine if ILK inhibition affects the expression of genes regulating the endocrine phenotype of β-cells, Cpd-22-treated and vehicle-treated Min6 cells were analyzed by qPCR for the expression of insulin, the transcription factor Pdx1, and Cx36, a gap junction protein whose expression has been linked to the acquisition of a functional β-cell phenotype[78,80,248]. ILK blockade resulted in a significant upregulation of Insulin, Pdx1, and Cx36 (**Figure 6-5**).

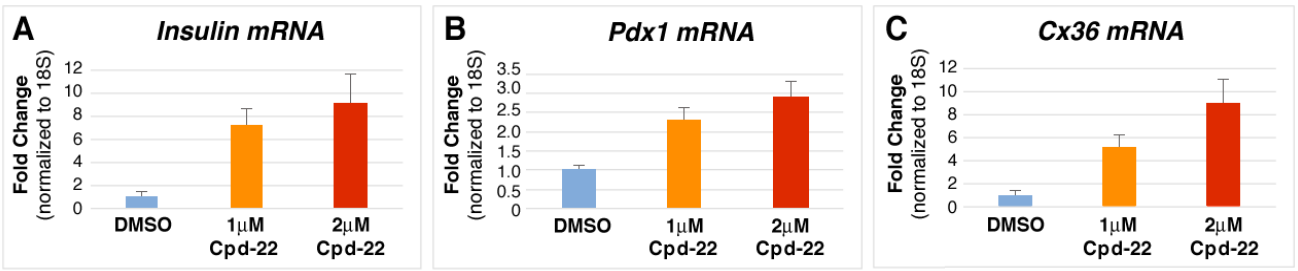


Figure 6-5 *ILK inhibition by Cpd-22 positively impacts islet-specific gene expression.*

Gene expression analysis for endocrine markers showed enrichment of Insulin, Pdx1, and Cx36 with inhibition of ILK in a dose dependent manner. N=3.

6.3.6 In vivo *function of ILK studied in a mouse model*

To gain knowledge on the *in vivo* function of ILK during the development of the pancreatic epithelium, we conducted preliminary studies focusing on the ablation of a floxed ILK allele in $Pdx1^{+}$ pancreatic progenitors. Preliminary analysis of these recombinant mice revealed a significant reduction of the islet cell mass (**Figure 6-6**A-B), and mild glucose intolerance at weaning age. We also found that ablation of ILK in $Pdx1^{+}$ pancreatic progenitors lead to a redistribution of the β-catenin pool normally restricted to cell-cell contacts to the cytosol (**Figure 6-6**D).

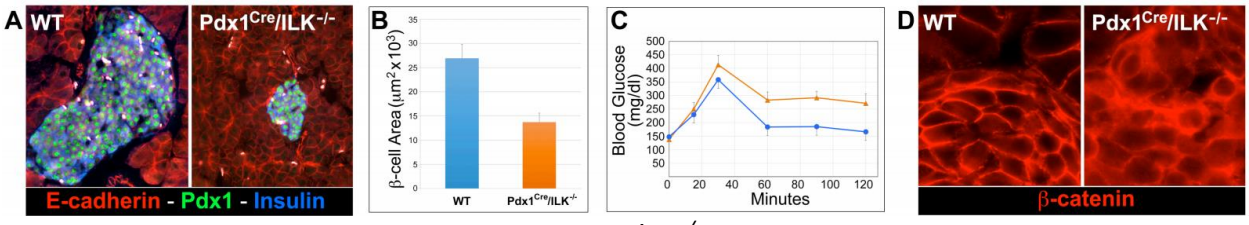


Figure 6-6 *Pancreatic islet phenotype in Pdx1Cre/ILK$^{-/-}$ mice.*

*Morphometric analysis of Pdx1Cre/ILK$^{-/-}$ pancreata (**A**) revealed a significant reduction of the β-cell mass compared to WT mice (**B**; n=4). This phenotype resulted in a mild glucose intolerance, as determined by glucose tolerance test, by the time they reached weaning age (**C**; n=4). Islet cells in Pdx1Cre/ILK$^{-/-}$ mice also exhibited a redistribution of β-catenin from cell-cell domains to the cytosol (**D**).*

6.4 DISCUSSION

Our previous work has demonstrated the importance of β1 integrins for the proper development and expansion of the pancreatic β-cell mass[3]. Building on this work, we studied the effects of integrin linked kinase (ILK), a downstream effector of β1 integrin. We first showed that ILK is co-expressed with β1 integrin within the embryonic pancreatic epithelium as well as delaminating ductal cells (**Figure 6-1**). This expression pattern suggests a functional partnership between these two molecules during processes of β-cell development.

Our next experiments explored the role of ILK in proliferation. By studying ILK inhibition in ductal cell lines, SU86 and G3LC (**Figure 6-2**), as well as in embryonic pancreatic epithelium (**Figure 6-3**), we were able to observe a marked decrease in BrdU incorporation with inhibition of ILK by Cpd-22. These results indicate a role for ILK-mediated signaling in the proliferation of pancreatic type cells. Specifically, our results suggest that there is a functional requirement for ILK in the expansion of the pancreatic epithelium, and support our hypothesis that ablation of ILK in $Pdx1^{+}$ pancreatic progenitors *in vivo* may affect the growth potential of the pancreatic epithelium.

We further studied the effects of ILK inhibition in Min6 cells. The results from these experiments clearly show a significant up-regulation of Insulin, Pdx1, and Cx36 gene expression indicating that blockade of ILK function enhances endocrine-specific gene expression networks that regulate the acquisition and/or maintenance of β-cell identity (**Figure 6-5**). Interestingly, these results are consistent with the increased insulin content that we reported in the studies of β1 integrin ablation in β-cells[3]. Importantly, in all cases (experiments presented in **Figure 6-2** through **Figure 6-5**), cell surface levels of β1 integrin were not altered by the treatment with the ILK inhibitor Cpd-22, indicating that the effects on cell proliferation and on endocrine gene expression depends on the functional blockade of ILK signaling rather than on secondary effects linked to a down-regulation of β1 integrin expression.

Interestingly, in agreement with the reported function of ILK as a signaling effector of β-catenin stabilization[105–107,239,249], ILK inhibition resulted in a redistribution of the pool of β-catenin seen in the islet cells of ILK-KO mice. Although this morphological evidence remains to be validated biochemically, it suggests that loss of ILK signaling negatively impacts the β-catenin/Wnt pathway. Since previous studies demonstrated that the purposeful stabilization of β-catenin, or disruption thereof, can have distinct effects on pancreatic cell growth and differentiation depending on the stage of development[187,238,239,250], our observations underline the importance of investigating ILK function at select time points in β-cells, i.e. during their development as well as during their functional maturation in postnatal life.

Acknowledgements

For these studies, I was responsible for the generation of mutant *Pdx1Cre/ILK*$^{-/-}$ mice, experiments of immunolocalization of ILK, morphometric analysis of their pancreata, analysis of the metabolic phenotype, analysis gene expression by qPCR, and editing the manuscript. Laura Crisa performed flow cytometry experiments and helped in the analysis of results. Vincenzo Cirulli conceived experiments, analyzed data, and wrote the manuscript.

Chapter 7. CONCLUSIONS AND FUTURE DIRECTIONS

7.1 FUTURE WORK WITH CXS RESEARCH

My work has shown that manipulation of Cxs can be useful tools to foster the differentiation of stem cells toward pancreatic progenitors. So far, we have shown that addition of AAP10 can enrich the development of Pdx1 positive cells during stem cell differentiation. In addition, we have shown that addition of AAP10 is only necessary during the early stages of differentiation to foster this growth. However, addition of AAP10 is still not sufficient to drive derivation of insulin producing cells.

For future work studying Cxs in pancreatic development, it would be important to define the exact stage that is most responsive to manipulation of Cx43 activation. For these studies, I plan to complete experiments of differentiation in which the addition of the AAP10 peptide will be monitored at distinct stages of maturation toward the pancreatic islet cell lineage. By this strategy, we will be able to determine if the efficacy of the AAP10 treatment can be enhanced by selecting specific stages of differentiation (e.g. at DE, or PGT stages).

A second set of experiments will focus on the maturation of peptide treated cells. In this case, it will be important to test multiple growth conditions, including optimizing media or suspension culture, to determine if these changes can affect the propensity of cells to differentiate toward insulin-producing cells. Based on evidence that a number of stem cell lines can efficiently differentiate into insulin-producing cells, we will test new lines, such as the $MEL1^{INS\text{-}GFP}$ cells[143], to determine if alternative protocols of differentiation based on 3-dimensional aggregation of stem cells during the entire duration of the differentiation protocol can enhance the yield of insulin-producing cells upon treatment with the AAP10 peptide. Finally, we will also determine if implantation of AAP10-treated stem cell preparations into NOD/SCID mice will improve their *in vivo* functional maturation into glucose responsive insulin-producing cells.

A parallel study should also look at whether mice transplanted with these AAP10-treated cells will be able reverse a model of diabetes in mice. These steps are of crucial importance for the translation of our *in vitro* studies to the eventual clinical application to treat type 1 Diabetes.

I would also like to further explore Cx43 knockdown using a CRISPR/Cas9 approach. This would allow us to knockdown Cx43 at earlier stages of differentiation, an experimental approach that has been impossible to implement with siRNAs or shRNAs in undifferentiated stem cells.

7.2 FUTURE WORK WITH ILK

For studies aimed at completing the work on the function of ILK in the developing pancreas, we would like to target ILK ablation to the islet β-cells using Ins1-Cre mice. Completion of these studies will address a significant gap in knowledge on the function(s) of this signaling partner of ECM-integrin interactions in β-cells, both during development and in postnatal life. For this new series of experiments two new Cre knock-in mouse models have been recently generated. The first one allows for the constitutive expression of Cre in β-cells as soon as they activate the *Ins1* gene (Ins1-Cre mice), whereas the second model is based on an inducible strategy that allows for the induction of Cre activity upon administration of Tamoxifen (Ins1-Cre^{ERT2})[166]. Hence, while the Ins1-Cre model will useful for the generation of a constitutive ILK ablation in β-cells during embryonic life, the Ins1-Cre^{ERT2} mice will be useful to investigate the functional requirement of ILK in post-natal β-cells.

The rationale for conducting these studies is that by restricting Cre-mediated ablation of ILK to β-cells we will be able to determine possible cell autonomous mechanisms of ILK function *versus* possible effects of heterotypic cell-cell interactions with other islet cell types (i.e., α, δ, γ, ε) contributing to the islet architecture and function. We also anticipate that ablation of ILK in β-cells will not entirely phenocopy our previous results obtained in the RIP-Cre/$\beta1^{-/-}$ mice in which all signaling downstream of β1 integrin is abrogated since the receptor itself is no longer expressed[3]. Thus, deletion of ILK may result in a more measured phenotype, since the proposed approach targets a selective "signaling node" downstream of β1 integrin. This view is supported by the notion that some level of β1 integrin signaling may still take place through alternative signaling pathways such as those initiated by growth factors receptor tyrosine kinases (RTKs) that

we have previously reported to also regulate the balance between islet cell replication and differentiation through the PI3K[251]. Based on the notion that the repertoire of pancreatic β-cell-specific genes continue to change as they transition from fetal to adult life[103,252], it is possible that the impact of ILK loss-of-function on cell survival, expansion and differentiation will be different depending of their stage of development and/or competency to functionally respond to the metabolic milieu of the postnatal life. There are many other precedents for this, where loss-of-function of a given molecule has very different outcomes depending on the cell type and/or developmental stage at which deletion occurs[239,250,253]. In addition, opposing effects of ILK ablation (i.e., poor anti-proliferative) have been linked to cell context-specific microenvironments[165,240,243–246].

A second set of experiments will focus on the conditional ablation of the floxed ILK allele by crossing Ins1-Cre^{ERT2} knock-in mice with ILK$^{flox/flox}$ mice to generate Ins1-Cre^{ERT2}/ILK$^{-/-}$ animals. These studies would allow for selecting the time window of postnatal life when the most significant expansion of β-cells is known to occur. We postulate that targeting the ablation of ILK to β-cells during the first two weeks of life using a drug inducible conditional approach (i.e. Ins1-Cre^{ERT2} mice) may impact β-cells' ability to expand, and/or survive. A corollary to this hypothesis is that Ins1-Cre^{ERT2}/ILK$^{-/-}$ mutant β-cells may be unable to maintain a functional phenotype in face of increasing metabolic stress, and/or fail to regenerate following toxic injury. The experiments proposed with the inducible Ins1-Cre^{ERT2} mice will address the important question of ILK function in postnatal β-cells, a time when these endocrine cells must adapt to increased metabolic demands and acquire their fully functional competency.

We anticipate that ablation of ILK in β-cells during the first two weeks of postnatal life may lead to a defective β-cell expansion. This phenotype may become evident by weaning age, when, under a normal conditions, a fully functional β-cell mass should be established. As a result of significant reduction in β-cell mass, we may observe that Ins1-Cre^{ERT2}/ILK$^{-/-}$ will exhibit glucose intolerance. Alternatively, it is possible that in the context of the β-cell machinery or microenvironment present in perinatal life, ablation of ILK will primarily affect insulin secretion rather than β-cell expansion. This is because ILK is known to regulate the activity of Akt and ERK signaling [111,254], two pathways that we and others have shown to positively regulate insulin

production and secretion[95–97]. Similarly, given the evidence that Akt can be phosphorylated by ILK[107], and that an active Akt pathways positively regulate β-cell survival in addition to proliferation[238,254–257], it is likely that the removal of ILK signaling in Ins1-Cre/ILK$^{-/-}$ mice will render postnatal β-cells more prone to undergo apoptosis, and more sensitive to metabolic stressors, which in turn may decrease their ability to maintain proper homeostatic functions.

After generation, these mice will be tested for their ability to compensate metabolic demands following exposure to metabolic stressors such as high fat diet, and ability to regenerate cells following β-cells injury by STZ. We anticipate that the proposed studies will uncover novel mechanisms of islet cell neogenesis, growth and differentiation, as well as homeostasis and regeneration, and may ultimately contribute to the identification of novel "signaling nodes" that could be pharmacologically targeted to promote β-cell survival and/or elicit regeneration. We postulate that ablation of ILK in β-cells during embryonic life will negatively impact on their expansion, differentiation and functional maturation. Results from these studies have significant translational implications as they provide the groundwork for pharmacologically targeting this signaling module to elicit β-cell expansion, survival and/or regeneration, or to expand stem cell-derived progenitors *ex vivo*.

Chapter 8. BIBLIOGRAPHY

1. Kameritsch, P., Pogoda, K. & Pohl, U. Channel-independent influence of connexin 43 on cell migration. *Biochim. Biophys. Acta - Biomembr.* **1818,** 1993–2001 (2012).
2. Cirulli, V. *et al.* Expression and function of alpha(V)beta3 and alpha(V)beta5 integrins in the developing pancreas: Roles in the adhesion and migration of putative endocrine progenitor cells. *J. Cell Biol.* **150,** 1445–1459 (2000).
3. Diaferia, G. R. *et al.* B1 Integrin Is a Crucial Regulator of Pancreatic B-Cell Expansion. *Development* **140,** 3360–72 (2013).
4. Goodenough, D. A., Goliger, J. A. & Paul, D. L. Connexins, Connexons and Intercellular Communication. *Annu. Rev. Biochem.* **65,** 475–702 (1996).
5. Evans, W. H., De Vuyst, E. & Leybaert, L. The gap junction cellular internet: connexin hemichannels enter the signalling limelight. *Biochem. J.* **397,** 1–14 (2006).
6. Willecke, K. *et al.* Structural and functional diversity of connexin genes in the mouse and human genome. *Biol Chem* **383,** 725–37 (2002).
7. Söhl, G. & Willecke, K. Gap junctions and the connexin protein family. *Cardiovasc. Res.* **62,** 228–232 (2004).
8. Yeager, M. & Gilula, N. B. Membrane topology and quaternary structure of cardiac gap junction ion channels. *J. Mol. Biol.* **223,** 929–48 (1992).
9. Vinken, M. *et al.* Connexins: Sensors and regulators of cell cycling. *Biochim. Biophys. Acta - Rev. Cancer* **1815,** 13–25 (2011).
10. Solan, J. L. & Lampe, P. D. Connexin phosphorylation as a regulatory event linked to gap junction channel assembly. *Biochim. Biophys. Acta - Biomembr.* **1711,** 154–163 (2005).
11. Gaietta, G. *et al.* Multicolor and Electron Microscopic Imaging of Connexin Trafficking. *Science* **296,** 503–507 (2002).
12. Segretain, D. & Falk, M. M. Regulation of connexin biosynthesis, assembly, gap junction formation, and removal. *Biochim. Biophys. Acta - Biomembr.* **1662,** 3–21 (2004).
13. Wei, C.-J., Xu, X. & Lo, C. W. Connexins and Cell Signaling in Development and Disease. *Annu. Rev. Cell Dev. Biol.* **20,** 811–838 (2004).

14. Kumar, N. M. & Gilula, N. B. The Gap Junction Communication Channel. *Cell* **84,** 381–388 (1996).
15. Laird, D. W. Life cycle of connexins in health and disease. *Biochem. J.* **394,** 527–543 (2006).
16. Laird, D. W., Puranam, K. L. & Revel, J. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem J* **273,** 67–72 (1991).
17. Fallon, R. F. & Goodenough, D. A. Five-hour half-life of mouse liver gap-junction protein. *J Cell Biol* **90,** 521–526 (1981).
18. Laing, J. G., Tadros, P. N., Westphale, E. M. & Beyer, E. C. Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. *Exp. Cell Res.* **236,** 482–492 (1997).
19. Beardslee, M. A., Laing, J. G., Beyer, E. C. & Saffitz, J. E. Rapid turnover of connexin43 in the adult rat heart. *Circ Res* **83,** 629–635 (1998).
20. Jordan, K., Chodock, R., Hand, a R. & Laird, D. W. The origin of annular junctions: a mechanism of gap junction internalization. *J. Cell Sci.* **114,** 763–73 (2001).
21. Leithe, E., Brech, A. & Rivedal, E. Endocytic processing of connexin43 gap junctions: a morphological study. *Biochem. J.* **393,** 59–67 (2006).
22. Salomon, D. *et al.* Topography of mammalian connexins in human skin. *J Invest Dermatol* **103,** 240–7 (1994).
23. Kretz, M. *et al.* Altered connexin expression and wound healing in the epidermis of connexin-deficient mice. *J. Cell Sci.* **116,** 3443–3452 (2003).
24. Zhang, J. & Nicholson, B. The topological structure of connexin 26 and its distribution compared to connexin 32 in hepatic gap junctions. *J Membr Biol* **139,** 15–29 (1994).
25. Kelsell, D. P., Di, W. L. & Houseman, M. J. Connexin mutations in skin disease and hearing loss. *Am. J. Hum. Genet.* **68,** 559–568 (2001).
26. Kleopa, K. A. & Sargiannidou, I. Connexins, gap junctions and peripheral neuropathy. *Neurosci. Lett.* **596,** 27–32 (2015).
27. Chanson, M. *et al.* Enhanced secretion of amylase from exocrine pancreas of connexin32-

deficient mice. *J. Cell Biol.* **141,** 1267–1275 (1998).

28. Omori, Y., Mesnil, M. & Yamasaki, H. Connexin 32 mutations from X-linked Charcot-Marie-Tooth disease patients: functional defects and dominant negative effects. *Mol. Biol. Cell* **7,** 907–916 (1996).
29. Frossard, J. L. *et al.* Severe acute pancreatitis and reduced acinar cell apoptosis in the exocrine pancreas of mice deficient for the Cx32 gene. *Gastroenterology* **124,** 481–493 (2003).
30. Kantor, O. *et al.* Characterization of connexin36 gap junctions in the human outer retina. *Brain Struct Funct* (2015).
31. Swayne, L. A. & Bennett, S. A. L. Connexins and pannexins in neuronal development and adult neurogenesis. *BMC Cell Biol.* **17,** 10 (2016).
32. Meda, P. Gap junction involvement in secretion: the pancreas experience. *Clin Exp Pharmacol Physiol* **23,** 1053–7 (1996).
33. Bavamian, S. *et al.* Islet-cell-to-cell communication as basis for normal insulin secretion. *Diabetes, Obes. Metab.* **9,** 118–132 (2007).
34. Akopian, A. *et al.* Gap Junction-Mediated Death of Retinal Neurons Is Connexin and Insult Specific: A Potential Target for Neuroprotection. *J. Neurosci.* **34,** 10582–10591 (2014).
35. Huettner, J. E. *et al.* Gap junctions and connexon hemichannels in human embryonic stem cells. *Stem Cells* **24,** 1654–67 (2006).
36. Bupha-Intr, T., Haizlip, K. M. & Janssen, P. M. L. Temporal changes in expression of connexin 43 after load-induced hypertrophy in vitro. *Am. J. Physiol. Heart Circ. Physiol.* **296,** H806–14 (2009).
37. Boulaksil, M. *et al.* Spatial Heterogeneity of Cx43 is an Arrhythmogenic Substrate of Polymorphic Ventricular Tachycardias during Compensated Cardiac Hypertrophy in Rats. *Front. Cardiovasc. Med.* **3,** 5 (2016).
38. Paznekas, W. A. *et al.* Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am. J. Hum. Genet.* **72,** 408–18 (2003).
39. Serre-Beinier, V. *et al.* Connexins and secretion. *Biol. Cell* **94,** 477–92 (2002).
40. Krysko, D. V., Leybaert, L., Vandenabeele, P. & D'Herde, K. Gap junctions and the

propagation of cell survival and cell death signals. *Apoptosis* **10,** 459–469 (2005).

41. Valiunas, V. *et al.* Connexin-specific cell-to-cell transfer of short interfering RNA by gap junctions. *J. Physiol.* **568,** 459–68 (2005).
42. Neijssen, J. *et al.* Cross-presentation by intercellular peptide transfer through gap junctions. *Nature* **434,** 83–88 (2005).
43. Charpantier, E., Cancela, J. & Meda, P. Beta cells preferentially exchange cationic molecules via connexin 36 gap junction channels. *Diabetologia* **50,** 2332–2341 (2007).
44. Bukauskas, F. F. Neurons and β-cells of the pancreas express connexin36, forming gap junction channels that exhibit strong cationic selectivity. *J. Membr. Biol.* **245,** 243–253 (2012).
45. Ebihara, L. & Steiner, E. Properties of a nonjunctional current expressed from a rat connexin46 cDNA in Xenopus oocytes. *J. Gen. Physiol.* **102,** 59–74 (1993).
46. Retamal, M. A., Evangelista-Martinez, F., Leon-Paravic, C. G., Altenberg, G. A. & Reuss, L. Biphasic effect of linoleic acid on connexin 46 hemichannels. *Pflugers Arch. Eur. J. Physiol.* **461,** 635–643 (2011).
47. Fontes, M. S. C., Van Veen, T. A. B., De Bakker, J. M. T. & Van Rijen, H. V. M. Functional consequences of abnormal Cx43 expression in the heart. *Biochim. Biophys. Acta - Biomembr.* **1818,** 2020–2029 (2012).
48. Ito, A. *et al.* A role for heterologous gap junctions between melanoma and endothelial cells metastasis. *J. Clin. Invest.* **105,** 1189–1197 (2000).
49. Hoptak-Solga, A. D. *et al.* Connexin43 (GJA1) is required in the population of dividing cells during fin regeneration. *Dev. Biol.* **317,** 541–548 (2008).
50. Assou, S. *et al.* A meta-analysis of human embryonic stem cells transcriptome integrated into a web-based expression atlas. *Stem Cells* **25,** 961–73 (2007).
51. Lavado, E., Sanchez-Abarca, L. I., Tabernero, A., Bolaños, J. P. & Medina, J. M. Oleic acid inhibits gap junction permeability and increases glucose uptake in cultured rat astrocytes. *J. Neurochem.* **69,** 721–8 (1997).
52. Schmilinsky-Fluri, G., Valiunas, V., Willi, M. & Weingart, R. Modulation of cardiac gap junctions: the mode of action of arachidonic acid. *J Mol Cell Cardiol* **29,** 1703–1713

(1997).

53. Schilling, A. F. *et al.* Gap junctional communication in human osteoclasts in vitro and in vivo. *J. Cell. Mol. Med.* **12,** 2497–2504 (2008).

54. Guan, X., Wilson, S., Schlender, K. & Ruch, R. Gap-junction disassembly and connexin 43 dephosphorylation induced by 18 beta-glycyrrhetinic acid. *Mol Carcinog* **16,** 157–64 (1996).

55. Verrecchia, F. & Herve, J. Reversible inhibition of gap junctional communication by tamoxifen in cultured cardiac myocytes. *Pflugers Arch* **434,** 113–6 (1997).

56. Clarke, T. C., Thomas, D., Petersen, J. S., Evans, W. H. & Martin, P. E. M. The antiarrhythmic peptide rotigaptide (ZP123) increases gap junction intercellular communication in cardiac myocytes and HeLa cells expressing connexin 43. *Br. J. Pharmacol.* **147,** 486–495 (2006).

57. De Vuyst, E. *et al.* Pharmacological modulation of connexin-formed channels in cardiac pathophysiology. *Br. J. Pharmacol.* **163,** 469–483 (2011).

58. Braet, K., Vandamme, W., Martin, P. E. M., Evans, W. H. & Leybaert, L. Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26. *Cell Calcium* **33,** 37–48 (2003).

59. Braet, K. *et al.* Pharmacological Sensitivity of ATP Release Triggered by Photoliberation of Inositol-1,4,5-Trisphosphate and Zero Extracellular Calcium in Brain Endothelial Cells. *J. Cell. Physiol.* **197,** 205–213 (2003).

60. Evans, W. H., Bultynck, G. & Leybaert, L. Manipulating connexin communication channels: Use of peptidomimetics and the translational outputs. *J. Membr. Biol.* **245,** 437–449 (2012).

61. Vinken, M. *et al.* Connexins and their channels in cell growth and cell death. *Cell. Signal.* **18,** 592–600 (2006).

62. Yulyana, Y. *et al.* Carbenoxolone enhances TRAIL-induced apoptosis through the upregulation of death receptor 5 and inhibition of gap junction intercellular communication in human glioma. *Stem Cells Dev.* **22,** 1870–82 (2013).

63. Yu, J. *et al.* Gap Junction Blockade Induces Apoptosis in Human Endometrial Stromal

Cells. *Mol. Reprod. Dev.* **81,** 666–675 (2014).

64. Paschon, V., Higa, G. S. V., Resende, R. R., Britto, L. R. G. & Kihara, A. H. Blocking of Connexin-Mediated Communication Promotes Neuroprotection during Acute Degeneration Induced by Mechanical Trauma. *PLoS One* **7,** (2012).
65. Easton, J. A., Petersen, J. S. & Martin, P. E. M. The anti-arrhythmic peptide AAP10 remodels Cx43 and Cx40 expression and function. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **380,** 11–24 (2009).
66. Bukauskas, F. F., Bukauskiene, A., Verselis, V. K. & Bennett, M. V. L. Coupling asymmetry of heterotypic connexin 45/ connexin 43-EGFP gap junctions: properties of fast and slow gating mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 7113–7118 (2002).
67. Rackauskas, M. *et al.* Gating properties of heterotypic gap junction channels formed of connexins 40, 43, and 45. *Biophys. J.* **92,** 1952–1965 (2007).
68. Cottrell, G. T. & Burt, J. M. Heterotypic gap junction channel formation between heteromeric and homomeric Cx40 and Cx43 connexons. *Am. J. Physiol. Cell Physiol.* **281,** C1559–67 (2001).
69. Orthmann-Murphy, J. L., Freidin, M., Fischer, E., Scherer, S. S. & Abrams, C. K. Two Distinct Heterotypic Channels Mediate Gap Junction Coupling between Astrocyte and Oligodendrocyte Connexins. *J. Neurosci.* **27,** 13949–13957 (2007).
70. Sosinsky, G. Mixing of connexins in gap junction membrane channels. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9210–9214 (1995).
71. Sun, J. *et al.* Cochlear gap junctions coassembled from Cx26 and 30 show faster intercellular Ca2+ signaling than homomeric counterparts. *Am. J. Physiol. Cell Physiol.* **288,** C613–C623 (2005).
72. Jiang, J. X. & Goodenough, D. A. Heteromeric connexons in lens gap junction channels. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1287–1291 (1996).
73. Braet, K., Mabilde, C., Cabooter, L., Rapp, G. & Leybaert, L. Electroporation loading and photoactivation of caged InsP3: Tools to investigate the relation between cellular ATP release in response to intracellular InsP3 elevation. *J. Neurosci. Methods* **132,** 81–89 (2004).

74. Nicotera, P., Leist, M. & Ferrando-May, E. Intracellular ATP, a switch in the decision between apoptosis and necrosis. *Toxicol Lett* **102-103,** 139–42 (1998).
75. Vergara, L., Bao, X., Cooper, M., Bello-Reuss, E. & Reuss, L. Gap-junctional Hemichannels Are Activated by ATP Depletion in Human Renal Proximal Tubule Cells. *J. Membr. Biol.* **196,** 173–184 (2003).
76. Charollais, A. *et al.* Junctional communication of pancreatic β cells contributes to the control of insulin secretion and glucose tolerance. *Strategy* **106,** 235–243 (2000).
77. Charollais, A. *et al.* Loss of alpha 1 connexin does not alter the prenatal differentiation of pancreatic beta cells and leads to the identification of another islet cell connexin. *Dev Genet* **24,** 13–26 (1999).
78. Serre-Beinier, V. *et al.* Cx36 makes channels coupling human pancreatic beta-cells, and correlates with insulin expression. *Hum. Mol. Genet.* **18,** 428–439 (2009).
79. Carvalho, C. P. F. *et al.* Beta cell coupling and connexin expression change during the functional maturation of rat pancreatic islets. *Diabetologia* **53,** 1428–1437 (2010).
80. Bosco, D., Haefliger, J. A. & Meda, P. Connexins: Key mediators of endocrine function. *Physiol. Rev.* **91,** 1393–1445 (2011).
81. Ravier, M. a *et al.* Loss of Connexin36 Channels Alters β-cell Coupling, Islet Synchronization of Glucose-Induced Ca2+ and Insulin Oscillations , and Basal Insulin Release. *Diabetes* **54,** 1798–1807 (2005).
82. Klee, P. *et al.* Connexins protect mouse pancreatic β cells against apoptosis. *J. Clin. Invest.* **121,** 4870–9 (2011).
83. Klee, P. *et al.* Connexin implication in the control of the murine beta-cell mass. *Pediatr. Res.* **70,** 142–147 (2011).
84. Solan, J. L., Hingorani, S. R. & Lampe, P. D. Changes in Connexin43 Expression and Localization During Pancreatic Cancer Progression. *J Membr Biol* **245,** 255–262 (2012).
85. Liddington, R. C. & Ginsberg, M. H. Integrin activation takes shape. *J. Cell Biol.* **158,** 833–839 (2002).
86. Aumailley, M., Pesch, M., Tunggal, L., Gaill, F. & Fässler, R. Altered synthesis of laminin 1 and absence of basement membrane component deposition in (beta)1 integrin-deficient

embryoid bodies. *J. Cell Sci.* **113 Pt 2,** 259–268 (2000).

87. Hynes, R. O. Integrins: Bidirectional, allosteric signaling machines. *Cell* **110,** 673–687 (2002).
88. Giancotti, F. G. & Ruoslahti, E. Integrin Signaling. *Science* **285,** 1028–1032 (1999).
89. Schoenwaelder, S. M. & Burridge, K. Bidirectional signalling between the cytoskeleton and integrins. *Curr.Opp.Cell Biol.* **11,** 274–286 (1999).
90. Ginsberg, M. H., Partridge, A. & Shattil, S. J. Integrin regulation. *Curr. Opin. Cell Biol.* **17,** 509–516 (2005).
91. Howe, A., Aplin, A., Alahari, S. & RL, J. Integrin signaling and cell growth control. *Curr Opin Cell Biol* **10,** 220–31 (1998).
92. Miyamoto, S., Katz, B. Z., Lafrenie, R. M. & Yamada, K. M. Fibronectin and integrins in cell adhesion, signaling, and morphogenesis. *Ann. N. Y. Acad. Sci.* **857,** 119–129 (1998).
93. Yebra, M. *et al.* Endothelium-derived Netrin-4 supports pancreatic epithelial cell adhesion and differentiation through integrins α2β1 and α3β1. *PLoS One* **6,** 1–12 (2011).
94. Yebra, M. *et al.* Recognition of the neural chemoattractant Netrin-1 by integrins and α6β4 and α3β1 regulates epithelial cell adhesion and migration. *Dev. Cell* **5,** 695–707 (2003).
95. Kaido, T. *et al.* αv-Integrin Utilization in Human β-Cell Adhesion, Spreading, and Motility. *J. Biol. Chem.* **279,** 17731–17737 (2004).
96. Kaido, T., Yebra, M., Cirulli, V. & Montgomery, A. M. Regulation of human β-cell adhesion, motility, and insulin secretion by collagen IV and its receptor α1β1. *J. Biol. Chem.* **279,** 53762–53769 (2004).
97. Kaido, T. *et al.* Impact of defined matrix interactions on insulin production by cultured human β-cells: Effect on insulin content, secretion, and gene transcription. *Diabetes* **55,** 2723–2729 (2006).
98. Parnaud, G. *et al.* Blockade of β1 integrin-laminin-5 interaction affects spreading and insulin secretion of rat β-cells attached on extracellular matrix. *Diabetes* **55,** 1413–1420 (2006).
99. Kaido, T. J. *et al.* Impact of integrin-matrix interaction and signaling on insulin gene

expression and the mesenchymal transition of human β-cells. *J. Cell. Physiol.* **224,** 101–111 (2010).

100. Riopel, M. *et al.* Conditional beta1-integrin-deficient mice display impaired pancreatic beta cell function. *J Pathol* **224,** 45–55 (2011).
101. Bombardelli, L. *et al.* Pancreas-Specific Ablation of beta1 Integrin Induces Tissue Degeneration by Disrupting Acinar Cell Polarity. *Gastroenterology* **138,** 2531–2540 (2010).
102. Orci, L. & Unger, R. Functional subdivision of islets of Langerhans and possible role of D cells. *Lancet* **2,** 1243–4 (1975).
103. Pan, F. C. & Wright, C. Pancreas organogenesis: From bud to plexus to gland. *Dev. Dyn.* **240,** 530–565 (2011).
104. Ekblom, P., Vestweber, D. & Kemler, R. Cell matrix interactions and cell adhesion during development. *Ann Rev Cell Biol* **2,** 27–47 (1986).
105. Hannigan, G., Troussard, A. a & Dedhar, S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. *Nat. Rev. Cancer* **5,** 51–63 (2005).
106. Wu, C. & Dedhar, S. Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. *J. Cell Biol.* **155,** 505–510 (2001).
107. Qin, J. & Wu, C. ILK: a pseudokinase in the center stage of cell-matrix adhesion and signaling. *Curr. Opin. Cell Biol.* **24,** 607–613 (2012).
108. McDonald, P. C., Fielding, A. B. & Dedhar, S. Integrin-linked kinase--essential roles in physiology and cancer biology. *J. Cell Sci.* **121,** 3121–3132 (2008).
109. Legate, K. R., Montañez, E., Kudlacek, O. & Fässler, R. ILK, PINCH and parvin: the tIPP of integrin signalling. *Nat. Rev. Mol. Cell Biol.* **7,** 20–31 (2006).
110. Elad, N. *et al.* The role of integrin-linked kinase in the molecular architecture of focal adhesions. *J. Cell Sci.* **126,** 4099–107 (2013).
111. Teo, Z. L. *et al.* Integrin linked kinase (ILK) is required for lens epithelial cell survival, proliferation and differentiation. *Exp. Eye Res.* **121,** 130–142 (2014).
112. Pan, L. *et al.* β1-Integrin and Integrin Linked Kinase Regulate Astrocytic Differentiation of

Neural Stem Cells. *PLoS One* **9,** (2014).

113. American Diabetes Association. The Cost of Diabetes. Available at: http://www.diabetes.org/advocacy/news-events/cost-of-diabetes.html.
114. Diabetes Control and Complications Trial (DCCT)-Epidemiology of Diabetes Interventions and Complications (EDIC) Research Group *et al.* Risk factors for Cardiovascular Disease in Type 1 Diabetes. *Diabetes* (2016).
115. Katz, M., Giani, E. & Laffel, L. Challenges and Opportunities in the Management of Cardiovascular Risk Factors in Youth With Type 1 Diabetes: Lifestyle and Beyond. *Curr Diab Rep* **15,** 119 (2015).
116. Yau, J. W. Y. *et al.* Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care* **35,** 556–564 (2012).
117. Tapp, R. J. & Shaw, J. E. The Prevalence of and Factors Associated With Diabetic Retinopathy in the. *Diabetes Care* **26,** 1731–1737 (2003).
118. Cao, Z. & Cooper, M. E. Pathogenesis of diabetic nephropathy. *J. Diabetes Investig.* **2,** 243–247 (2011).
119. Kanikarla-Marie, P. & Jain, S. K. Hyperketonemia and ketosis increase the risk of complications in type 1 diabetes. *Free Radic. Biol. Med.* **95,** 268–277 (2016).
120. Khalaileh, A. *et al.* Determinants of pancreatic beta-cell regeneration. *Diabetes. Obes. Metab.* **10 Suppl 4,** 128–135 (2008).
121. Ye, Y. *et al.* Microneedles Integrated with Pancreatic Cells and Synthetic Glucose-Signal Amplifiers for Smart Insulin Delivery. *Adv. Mater.* (2016). doi:10.1002/adma.201506025
122. Russell, S. *et al.* Day and night glycaemic control with a bionic pancreas versus conventional insulin pump therapy in preadolescent children with type 1 diabetes: a randomised crossover trial. *Lancet Diabetes Endocrinol* **4,** 233–43 (2016).
123. Slavin, S. *et al.* New approaches for control of anti-self reactivity in type 1 diabetes and transplantation of pancreatic islets. *Transplant. Proc.* **29,** 1929–31 (1997).
124. Sutherland, D. E. R., Gruessner, R. W. G. & Gruessner, A. C. Pancreas transplantation for treatment of diabetes mellitus. *World J. Surg.* **25,** 487–496 (2001).
125. Brennan, D. C. *et al.* Long-term (>30 months) follow-up of a single successful kidney-islet

transplant recipient. *Transplant. Proc.* **29,** 2231–2233 (1997).

126. Shapiro, A. M. J. *et al.* Islet Transplantation in Sevent Patients with Type 1 Diabetes Mellitus Using a Glucocorticoid-Free Immunosuppressive Regimen. *N. Engl. J. Med.* **343,** 230–238 (2000).
127. Shapiro, M. J. *et al.* International trial of the Edmonton protocol for islet transplantation. *N. Engl. J. Med.* **355,** 1318–30 (2006).
128. Ryan, E. A. *et al.* Five-Year Follow-Up After Clinical Islet Transplantation. *Diabetes* **54,** 2060–2069 (2005).
129. O'Gorman, D. *et al.* The Standardization of Pancreatic Donors for Islet Isolations. *Transplantation* **80,** 801–6 (2005).
130. Katuchova, J. *et al.* Impact of different pancreatic microenvironments on improvement in hyperglycemia and insulin deficiency in diabetic rats after transplantation of allogeneic mesenchymal stromal cells. *J. Surg. Res.* **178,** 188–95 (2012).
131. Wu, C. *et al.* Engineered hair follicle mesenchymal stem cells overexpressing controlled-release insulin reverse hyperglycemia in mice with type l diabetes. *Cell Transplant.* **24,** 891–907 (2015).
132. Yaochite, J. *et al.* Therapeutic efficacy and biodistribution of allogeneic mesenchymal stem cells delivered by intrasplenic and intrapancreatic routes in streptozotocin-induced diabetic mice. *Stem Cell Res. Ther.* **6,** 31 (2015).
133. Johannesson, B., Sui, L., Freytes, D. O., Creusot, R. J. & Egli, D. Toward beta cell replacement for diabetes. *EMBO J.* **34,** 841–55 (2015).
134. Serup, P., Madsen, O. D. & Mandrup-Poulsen, T. Islet and stem cell transplantation for treating diabetes. *BMJ* **322,** 29–32 (2001).
135. Ikehara, S. *et al.* Autoimmune diseases as stem cell disorders. *Tohoku J Exp Med* **173,** 141–155 (1994).
136. Bochan, M. R., Shah, R., Sidner, R. A. & Jindal, R. M. Reversal of diabetes in the rat by injection of hematopoietic stem cells infected with recombinant adeno-associated virus containing the preproinsulin gene. *Transplant. Proc.* **31,** 690–1 (1999).
137. Marmont, A. Stem Cell Transplantation For Sever Autoimmune Diseases: Progress and

Problems. *Haematologica* **83,** 733–43 (1998).

138. Baetge, E. E. Production of beta-cells from human embryonic stem cells. *Diabetes. Obes. Metab.* **10 Suppl 4,** 186–194 (2008).
139. Aali, E. *et al.* A comparative study of mesenchymal stem cell transplantation with its paracrine effect on control of hyperglycemia in type 1 diabetic rats. *J. Diabetes Metab. Disord.* **13,** 76 (2014).
140. Al-Hasani, K. *et al.* Adult duct-lining cells can reprogram into β-like cells able to counter repeated cycles of toxin-induced diabetes. *Dev. Cell* **26,** 86–100 (2013).
141. D'Amour, K. a *et al.* Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat. Biotechnol.* **23,** 1534–1541 (2005).
142. Kroon, E. *et al.* Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat. Biotechnol.* **26,** 443–452 (2008).
143. Russ, H. a *et al.* Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro. *EMBO J.* **34,** e201591058 (2015).
144. Lee, D. H. & Chung, H. M. Differentiation into endoderm lineage: Pancreatic differentiation from embryonic stem cells. *Int. J. Stem Cells* **4,** 35–42 (2011).
145. Kelly, O. G. *et al.* Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. *Nat. Biotechnol.* **29,** 750–6 (2011).
146. Bose, B., Shenoy P, S., Konda, S. & Wangikar, P. Human embryonic stem cell differentiation into insulin secreting β-cells for diabetes. *Cell Biol. Int.* **36,** 1013–1020 (2012).
147. Keil, M. *et al.* Transcriptional expression profile of cultured human embryonic stem cells in vitro and in vivo. *Vitr. Cell Dev Biol Anim* **48,** 165–174 (2012).
148. Pagliuca, F. W. *et al.* Generation of functional human pancreatic beta cells in vitro. *Cell* **159,** 428–439 (2014).
149. Bruin, J. E. *et al.* Characterization of polyhormonal insulin-producing cells derived in vitro from human embryonic stem cells. *Stem Cell Res.* **12,** 194–208 (2014).
150. Liu, H. *et al.* Systematically labeling developmental stage-specific genes for the study of

pancreatic β-cell differentiation from human embryonic stem cells. *Cell Res.* **24,** 1–20 (2014).

151. Nostro, M. C. *et al.* Stage-specific signaling through TGFβ family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. *Development* **138,** 861–71 (2011).
152. Kunisada, Y., Tsubooka-Yamazoe, N., Shoji, M. & Hosoya, M. Small molecules induce efficient differentiation into insulin-producing cells from human induced pluripotent stem cells. *Stem Cell Res.* **8,** 274–284 (2012).
153. Rezania, A. *et al.* Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. *Diabetes* **61,** 2016–2029 (2012).
154. Schulz, T. C. *et al.* A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. *PLoS One* **7,** (2012).
155. Hua, H. *et al.* iPSC-derived b cells model diabetes due to glucokinase deficiency. *J. Clin. Invest.* **123,** 3146–3153 (2013).
156. Skottman, H. & Hovatta, O. Culture conditions for human embryonic stem cells. *Reproduction* **132,** 691–698 (2006).
157. Bhandari, D. R. *et al.* The simplest method for in vitro β-cell production from human adult stem cells. *Differentiation* **82,** 144–152 (2011).
158. Guo-Parke, H. *et al.* Configuration of electrofusion-derived human insulin-secreting cell line as pseudoislets enhances functionality and therapeutic utility. *J. Endocrinol.* **214,** 257–265 (2012).
159. Liu, X. *et al.* Involvement of RhoA/ROCK in insulin secretion of pancreatic ??-cells in 3D culture. *Cell Tissue Res.* 359–369 (2014). doi:10.1007/s00441-014-1961-2
160. Villa-Diaz, L. G., Ross, A. M., Lahann, J. & Krebsbach, P. H. Concise review: The evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings. *Stem Cells* **31,** 1–7 (2013).
161. Pelto, J. *et al.* Novel polypyrrole-coated polylactide scaffolds enhance adipose stem cell proliferation and early osteogenic differentiation. *Tissue Eng. Part A* **19,** 882–92 (2013).

162. Wang, X. & Ye, K. Three-dimensional differentiation of embryonic stem cells into islet-like insulin-producing clusters. *Tissue Eng. Part A* **15,** 1941–52 (2009).
163. Herrera, P. L. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* **127,** 2317–2322 (2000).
164. Raghavan, S., Bauer, C., Mundschau, G., Li, Q. & Fuchs, E. Conditional ablation of β1 integrin in skin: Severe defects in epidermal proliferation, basement membrane formation, and hair follicle invagination. *J. Cell Biol.* **150,** 1149–1160 (2000).
165. Terpstra, L. *et al.* Reduced chondrocyte proliferation and chondrodysplasia in mice lacking the integrin-linked kinase in chondrocytes. *J. Cell Biol.* **162,** 139–148 (2003).
166. Thorens, B. *et al.* Ins1Cre knock-in mice for beta cell-specific gene recombination. *Diabetologia* **58,** 558–565 (2014).
167. Bosco, D., Meda, P., Halban, P. a & Rouiller, D. G. Importance of cell-matrix interactions in rat islet beta-cell secretion in vitro: role of alpha6beta1 integrin. *Diabetes* **49,** 233–243 (2000).
168. Hayek, A. *et al.* Growth Factor/Matrix-Induced Proliferation of Human Adult 0-Cells. **44,** 1458–1460 (1995).
169. Mfopou, J. K., Chen, B., Sui, L., Sermon, K. & Bouwens, L. Recent advances and prospects in the differentiation of pancreatic cells from human embryonic stem cells. *Diabetes* **59,** 2094–2101 (2010).
170. Dbouk, H. A., Mroue, R. M., El-Sabban, M. E. & Talhouk, R. S. Connexins: a myriad of functions extending beyond assembly of gap junction channels. *Cell Commun. Signal.* **7,** 4 (2009).
171. Jozwiak, J. & Dhein, S. Local effects and mechanisms of antiarrhythmic peptide AAP10 in acute regional myocardial ischemia: Electrophysiological and molecular findings. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **378,** 459–470 (2008).
172. Weng, S. *et al.* Pharmacological modification of gap junction coupling by an antiarrhythmic peptide via protein kinase C activation. *FASEB J.* **16,** 1114–6 (2002).
173. Lynn, F. C. *et al.* Sox9 coordinates a transcriptional network in pancreatic progenitor cells. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 10500–5 (2007).

174. Seymour, P. A. *et al.* SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 1865–70 (2007).
175. Seymour, P. A. *et al.* A dosage-dependent requirement for Sox9 in pancreatic endocrine cell formation. *Dev. Biol.* **323,** 19–30 (2008).
176. Shih, H. P. *et al.* A gene regulatory network cooperatively controlled by Pdx1 and Sox9 governs lineage allocation of foregut progenitor cells. *Cell Rep.* **13,** 326–336 (2015).
177. Pérez-Armendariz, E. M. *et al.* Connexin 36 is Expressed in Beta and Connexins 26 and 32 in Acinar Cells at the End of the Secondary Transition of Mouse Pancreatic Development and Increase During Fetal and Perinatal Life. *Anat. Rec.* **295,** 980–990 (2012).
178. Summerford, C., Bartlett, J. S. & Samulski, R. J. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat. Med.* **5,** 78–82 (1999).
179. Goldman, M. J. & Wilson, J. M. Expression of αvβ5 integrin is necessary for efficient adenovirus-mediated gene transfer in the human airway. *J. Virol.* **69,** 5951–5958 (1995).
180. Hayes, H. L. *et al.* Pdx-1 activates islet α- and β-cell proliferation via a mechanism regulated by transient receptor potential cation channels 3 and 6 and extracellular signal-regulated kinases 1 and 2. *Mol. Cell. Biol.* **33,** 4017–29 (2013).
181. Hynes, R. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69,** 11–25 (1992).
182. Ruoslahti, E., Yamaguchi, Y., Hildebrand, A. & Border, W. Extracellular matrix/growth factor interactions. *Cold Spring Harb Symp Quant Biol* **57,** 309–315 (1992).
183. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21,** 70–71 (1999).
184. Belkin, A. & Stepp, M. Integrins as receptors for laminins. *Microsc Res Tech* **51,** 280–301 (2000).
185. Ruoslahti, E. Integrin signaling and matrix assembly. *Tumour Biol* **17,** 117–24 (1996).
186. Bonner Weir, S., Trent, D. F. & Weir, G. C. Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. *J. Clin. Invest.* **71,** 1544–1553 (1983).
187. Novak, A. *et al.* Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-

catenin signaling pathways. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4374–9 (1998).

188. Sherr, C. J. & Roberts, J. M. Inhibitors of mammalian cyclin-dependent kinases. *Genes Dev.* 1149–1163 (1995). doi:10.1101/gad.9.10.1149
189. Wang, F. *et al.* Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 14821–14826 (1998).
190. Fujita, M., Takada, Y. K. & Takada, Y. Insulin-like growth factor (IGF) signaling requires αvβ3-IGF1- IGF type 1 receptor (IGF1R) ternary complex formation in anchorage independence, and the complex formation does not require IGF1R and Src activation. *J. Biol. Chem.* **288,** 3059–3069 (2013).
191. Ju, L. & Zhou, C. Association of integrin beta1 and c-MET in mediating EGFR TKI gefitinib resistance in non-small cell lung cancer. *Cancer Cell Int* **13,** 15 (2013).
192. Margadant, C., Monsuur, H. N., Norman, J. C. & Sonnenberg, A. Mechanisms of integrin activation and trafficking. *Curr. Opin. Cell Biol.* **23,** 607–614 (2011).
193. Mccall-Culbreath, K. D., Li, Z. & Zutter, M. M. Crosstalk between the alpha 2 beta 1 integrin and c-met/HGF-R regulates innate immunity. *Blood* **111,** 3562–3570 (2008).
194. Mitra, A. K. *et al.* Ligand-independent activation of c-Met by fibronectin and α(5)β(1)-integrin regulates ovarian cancer invasion and metastasis. *Oncogene* **30,** 1566–76 (2011).
195. Beattie, G. M. *et al.* Sustained proliferation of PDX-1+ cells derived from human islets. *Diabetes* **48,** 1013–9 (1999).
196. De Lisle, R. & Logsdon, C. Pancreatic acinar cells in culture: expression of acinar and ductal antigens in a growth-related manner. *Eur J Cell Biol* **51,** 64–75 (1990).
197. Githens, S. *et al.* Mouse pancreatic acinar/ductular tissue gives rise to epithelial cultures that are morphologically, biochemically, and functionally indistinguishable from interlobular duct cell cultures. *Vitr. Cell Dev Biol Anim* **30A,** 622–35 (1994).
198. Means, A. L. *et al.* Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates. *Development* **132,** 3767–76 (2005).
199. Rooman, I., Heremans, Y., Heimberg, H. & Bouwens, L. Modulation of rat pancreatic

acinoductal transdifferentiation and expression of PDX-1 in vitro. *Diabetologia* **43,** 907–14 (2000).

200. Fukuda, A., Morris, J. P. & Hebrok, M. Bmi1 is required for regeneration of the exocrine pancreas in mice. *Gastroenterology* **143,** 821–831.e2 (2012).
201. Kopp, J. L. *et al.* Identification of Sox9-Dependent Acinar-to-Ductal Reprogramming as the Principal Mechanism for Initiation of Pancreatic Ductal Adenocarcinoma. *Cancer Cell* **22,** 737–750 (2012).
202. Reichert, M. *et al.* The Prrx1 homeodomain transcription factor plays a central role in pancreatic regeneration and carcinogenesis. *Genes Dev.* **27,** 288–300 (2013).
203. Montgomery, A. M. & Yebra, M. The epithelial-to-mesenchymal transition of human pancreatic β-cells: inductive mechanisms and implications for the cell-based therapy of type I diabetes. *Curr Diabetes Rev* **7,** 346–55 (2011).
204. Lim, J. & Thiery, J. P. Epithelial-mesenchymal transitions: insights from development. *Development* **139,** 3471–3486 (2012).
205. Yeh, Y.-C. *et al.* Transforming growth factor-β1 induces Smad3-dependent β1 integrin gene expression in epithelial-to-mesenchymal transition during chronic tubulointerstitial fibrosis. *Am. J. Pathol.* **177,** 1743–1754 (2010).
206. Georgia, S. & Bhushan, A. P27 Regulates the Transition of β-Cells From Quiescence To Proliferation. *Diabetes* **55,** 2950–2956 (2006).
207. Georgia, S., Soliz, R., Li, M., Zhang, P. & Bhushan, A. p57 and Hes1 coordinate cell cycle exit with self-renewal of pancreatic progenitors. *Dev. Biol.* **298,** 22–31 (2006).
208. Dhawan, S., Tschen, S. & Bhushan, A. Bmi-1 regulates the Ink4a / Arf locus to control pancreatic β -cell proliferation service Bmi-1 regulates the Ink4a / Arf locus to control pancreatic b -cell proliferation. 906–911 (2009). doi:10.1101/gad.1742609
209. Teta, M., Long, S. Y., Wartschow, L. M., Rankin, M. M. & Kushner, J. a. Very slow turnover of beta-cells in aged adult mice. *Diabetes* **54,** 2557–2567 (2005).
210. Tschen, S., Dhawan, S., Gurlo, T. & Bhushan, A. Age-dependent Decline in β-cell Proliferation Restricts the Capacity of β-Cell Regeneration in Mice. *Diabetes* **58,** 1312–1320 (2009).

211. Rouiller, D. G., Cirulli, V. & Halban, P. A. Uvomorulin mediates calcium-dependent aggregation of islet cells, whereas calcium-independent cell adhesion molecules distinguish between islet cell types. *Dev. Biol.* **148,** 233–242 (1991).
212. Nikolova, G. *et al.* The vascular basement membrane: A niche for insulin gene expression and β cell proliferation. *Dev. Cell* **10,** 397–405 (2006).
213. Saleem, S. *et al.* beta1 integrin/FAK/ERK signalling pathway is essential for human fetal islet cell differentiation and survival. *J. Pathol.* **219,** 816–26 (2009).
214. Brunetta, I., Casalotti, S. O., Hart, I. R., Forge, A. & Reynolds, L. E. β3-integrin is required for differentiation in OC-2 cells derived from mammalian embryonic inner ear. *BMC Cell Biol.* **13,** 5 (2012).
215. Guan, K., Czyz, J., Furst, D. & Wobus, A. Expression and cellular distribution of alpha(v)integrins in beta(1)integrin-deficient embryonic stem cell-derived cardiac cells. *J Mol Cell Cardiol* **33,** 521–32 (2001).
216. Hirsch, E., Lohikangas, L., Gullberg, D., Johansson, S. & Fässler, R. Mouse myoblasts can fuse and form a normal sarcomere in the absence of beta1 integrin expression. *J. Cell Sci.* **111,** 2397–2409 (1998).
217. Jeanes, A. I. *et al.* Specific β-containing integrins exert differential control on proliferation and two-dimensional collective cell migration in mammary epithelial cells. *J. Biol. Chem.* **287,** 24103–24112 (2012).
218. Retta, S. F. *et al.* Beta1-Integrin Cytoplasmic Subdomains Involved in Dominant Negative Function. *Mol. Biol. Cell* **9,** 715–731 (1998).
219. Dowling, J., Yu, Q.-C. & Fuchs, E. B4 Integrin Is Required for Hemidesmosome Formation, Cell Adhesion and Cell Survival. *J. Cell Biol.* **134,** 559–572 (1996).
220. Giancotti, F. G., Suzuki, S., Jolla, L. & Angeles, L. Proteolytic Processing of Endogenous. **118,** (1992).
221. Niessen, C. M. *et al.* Deficiency of the integrin beta 4 subunit in junctional epidermolysis bullosa with pyloric atresia: consequences for hemidesmosome formation and adhesion properties. *J. Cell Sci.* **109,** 1695–1706 (1996).
222. Sonnenberg, A. *et al.* Integrin α6/β4 complex is located in hemidesmosomes, suggesting

a major role in epidermal cell-basement membrane adhesion. *J. Cell Biol.* **113,** 907–917 (1991).

223. van der Neut, R. *et al.* Partial rescue of epithelial phenotype in integrin beta4 null mice by a keratin-5 promoter driven human integrin beta4 transgene. *J. Cell Sci.* **112,** 3911–3922 (1999).
224. van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C. M. & Sonnenberg, A. Epithelial detachment due to absence of hemidesmosomes in integrin beta 4 null mice. *Nat. Genet.* **13,** 366–369 (1996).
225. Xia, Y., Gil, S. G. & Carter, W. G. Anchorage mediated by integrin α6β4 to laminin 5 (epiligrin) regulates tyrosine phosphorylation of a membrane-associated 80-kD protein. *J. Cell Biol.* **132,** 727–740 (1996).
226. Cirulli, V. & Yebra, M. Netrins: beyond the brain. *Nat. Rev. Mol. Cell Biol.* **8,** 296–306 (2007).
227. Yang, Y. H. C. *et al.* Paracrine signalling loops in adult human and mouse pancreatic islets: Netrins modulate beta cell apoptosis signalling via dependence receptors. *Diabetologia* **54,** 828–842 (2011).
228. Miller, J. P., Yeh, N., Vidal, A. & Koff, A. Interweaving the cell cycle machinery with cell differentiation. *Cell Cycle* **6,** 2932–2938 (2007).
229. Velling, T., Nilsson, S., Stefansson, A. & Johansson, S. β1-Integrins induce phosphorylation of Akt on serine 473 independently of focal adhesion kinase and Src family kinases. *EMBO Rep.* **5,** 901–905 (2004).
230. Chiang, J. K. *et al.* Homocysteine induces smooth muscle cell proliferation through differential regulation of cyclins A and D1 expression. *J. Cell. Physiol.* **226,** 1017–1026 (2011).
231. D'Amico, M. *et al.* The integrated-linked kinase regulates the cyclin D1 gene through glycogen synthase kinase 3β and cAMP-responsive element-binding protein-dependent pathways. *J. Biol. Chem.* **275,** 32649–32657 (2000).
232. Azreq, M.-A. El, Naci, D. & Aoudjit, F. Collagen/β1 integrin signaling up-regulates the ABCC1/MRP-1 transporter in an ERK/MAPK-dependent manner. *Mol. Biol. Cell* **23,** 3473–

3484 (2012).

233. Wells, J. *et al.* Ovol2 suppresses cell cycling and terminal differentiation of keratinocytes by directly repressing c-Myc and Notch1. *J. Biol. Chem.* **284,** 29125–29135 (2009).
234. Vée, S. *et al.* Evidence for a role of the (alpha)-tubulin C terminus in the regulation of cyclin B synthesis in developing oocytes. *J. Cell Sci.* **114,** 887–98 (2001).
235. Chambard, J. C., Lefloch, R., Pouysségur, J. & Lenormand, P. ERK implication in cell cycle regulation. *Biochim. Biophys. Acta - Mol. Cell Res.* **1773,** 1299–1310 (2007).
236. Lawlor, M. A. & Alessi, D. R. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci.* **114,** 2903–2910 (2001).
237. Cox, D., Brennan, M. & Moran, N. Integrins as therapeutic targets: lessons and opportunities. *Nat. Rev. Drug Discov.* **9,** 804–820 (2010).
238. Blandino-Rosano, M. *et al.* Enhanced beta cell proliferation in mice overexpressing a constitutively active form of Akt and one allele of p21 Cip. *Diabetologia* **55,** 1380–1389 (2012).
239. Rulifson, I. C. *et al.* Wnt signaling regulates pancreatic beta cell proliferation. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 6247–6252 (2007).
240. Sekine, S., Gutiérrez, P. J. A., Lan, B. Y. A., Feng, S. & Hebrok, M. Liver-specific loss of β-catenin results in delayed hepatocyte proliferation after partial hepatectomy. *Hepatology* **45,** 361–368 (2007).
241. Petropoulos, S. *et al.* Gestational Diabetes Alters Offspring DNA Methylation Profiles in Human and Rat: Identification of Key Pathways involved in Endocrine System Disorders, Insulin Signaling, Diabetes Signaling and IL-K Signaling. *Endocrinology* **156,** en20141643 (2014).
242. Jiang, Y., Mizisin, A. P., Rearden, A. & Jolivalt, C. G. Diabetes induces changes in ILK, PINCH and components of related pathways in the spinal cord of rats. *Brain Res.* **1332,** 100–109 (2010).
243. Gkretsi, V. *et al.* Liver specific ablation of integrin linked kinase in mice results in abnormal histology, enhanced cell proliferation, and hepatomegaly. *Hepatology* **48,** 1932–1941 (2008).

244. Donthamsetty, S. *et al.* Excessive hepatomegaly of mice with hepatocyte-targeted elimination of integrin linked kinase following treatment with 1,4-bis [2-(3,5-dichaloropyridyloxy)] benzene. *Hepatology* **53,** 587–595 (2011).
245. Pontier, S. M. *et al.* Integrin-linked kinase has a critical role in ErbB2 mammary tumor progression: implications for human breast cancer. *Oncogene* **29,** 3374–85 (2010).
246. Nakrieko, K. *et al.* Impaired hair follicle morphogenesis and polarized keratinocye movement upon conditional inactivation of integrin linked kinase in the epidermis. *Mol. Biol. Cell* **19,** 1462–73 (2008).
247. Drucker, B. *et al.* A new human pancreatic carcinoma cell line developed for adoptive immunotherapy studies with lymphokine-activated killer cells in nude mice. *Vitr. Cell Dev Biol* **24,** 1179–87 (1988).
248. Nlend, R. N. *et al.* Cx36 is a target of Beta2/NeuroD1, which associates with prenatal differentiation of insulin-producing β cells. *J. Membr. Biol.* **245,** 263–273 (2012).
249. Tan, C. *et al.* Inhibition of integrin linked kinase (ILK) suppresses beta-catenin-Lef/Tcf-dependent transcription and expression of the E-cadherin repressor, snail, in APC-/- human colon carcinoma cells. *Oncogene* **20,** 133–40 (2001).
250. Heiser, P. W., Lau, J., Taketo, M. M., Herrera, P. L. & Hebrok, M. Stabilization of β-catenin impacts pancreas growth. *Development* **133,** 2023–2032 (2006).
251. Ptasznik, A. *et al.* Phosphatidylinositol 3-Kinase Is a Negative Regulator of Cellular Differentiation. **137,** 1127–1136 (1997).
252. Hang, Y. & Stein, R. MafA and MafB activity in pancreatic β cells. *Trends Endocrinol. Metab.* **22,** 364–373 (2011).
253. Talchai, S. C. & Accili, D. Legacy Effect of Foxo1 in Pancreatic Endocrine Progenitors on Adult β-Cell Mass and Function. *Diabetes* **64,** 2868–2879 (2015).
254. Tang, Q. *et al.* Inhibition of integrin-linked kinase expression by emodin through crosstalk of AMPKα and ERK1/2 signaling and reciprocal interplay of Sp1 and c-Jun. *Cell. Signal.* **27,** 1469–1477 (2015).
255. Elghazi, L., Rachdi, L., Weiss, A. J., Cras-Meneur, C. & Bernal-Mizrachi, E. Regulation of beta-cell mass and function by the Akt/protein kinase B signalling pathway. *Diabetes*

Obes.Metab **9 Suppl 2,** 147–157 (2007).

256. Elghazi, L., Balcazar, N. & Bernal-Mizrachi, E. Emerging role of protein kinase B/Akt signaling in pancreatic beta-cell mass and function. *Int. J. Biochem. Cell Biol.* **38,** 157–163 (2006).

257. Srinivasan, S., Bernal-Mizrachi, E., Ohsugi, M. & Permutt, M. A. Glucose promotes pancreatic islet {b}-cell survival through a PI3-kinase/Akt signaling pathway. *Am. J. Physiol. Endocrinol. Metab.* **283,** 784–793 (2002).

VITA

Wendy Yang is a stem cell biologist from Farmington Hills, MI. Her parents set her on a STEM career early on when they sent her to a robotics camp where she built self-driving LEGO cars. While fun, Wendy's passions truly lay in biological sciences. She nurtured this interest at the University of Michigan where she earned her B.S. in Neuroscience and learned to love college football. Since then, Wendy has been pursuing a career focused on patient-centered healthcare. This pursuit brought her to the University of Washington where she joined the lab of Dr. Vincenzo Cirulli to study stem cells and diabetes. She chose this lab to engage in cutting edge research with direct patient impact. Concurrently, Wendy also developed an interest in the business side of science. She completed the Technology Entrepreneurship Certificate from the Foster School of Business, and worked with the UW Coulter Translational Research Program managing teams, headed by engineers and clinicians, to help commercialize their research. She also found the opportunity to work with an additional cohort of entrepreneurial research labs as a fellow with the UW CoMotion Innovation Fund. Post-PhD, she is kickstarting a career in life science commercialization with the commercialization team at Life Science Washington in Seattle, WA. Her ultimate goal is to create value in the world around her by bridging the gap between basic research and new medical technology to improve patient centric healthcare.

Outside of lab, Wendy enjoys ballet classes, knitting, sailing-especially Tuesday nights, and managing her fantasy football team (there's always next year).