THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Pancreatic extracellular communication

Applications to beta cell cultures and islet amyloid polypeptide aggregation

DIANA RIBEIRO

Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY

Translational Genomics Department,

Innovative Medicines and Early Development Biotech Unit

AstraZeneca R&D

Gothenburg, Sweden 2018

Pancreatic extracellular communication

Applications to beta cell cultures and islet amyloid polypeptide aggregation

DIANA RIBEIRO

ISBN 978-91-7597-643-3

Doktorsavhandlingar vid Chalmers tekniska högskola

Ny serie 4324

ISSN 0346-718X

Department of Biology and Biological engineering

Chalmers University of Technology

SE-412 96 Gothenburg

Sweden

Telephone + 46 (0)31-772 1000

Cover:

The illustration shows a zoom-up of the exocrine and endocrine compartments of the pancreas and, it highlights the islets of Langerhans and extracellular vesicles released by the cells that constitute the islets. The illustration is not in scale.

Printed by Chalmers Reproservice

Gothenburg, Sweden 2018

Pancreatic extracellular communication
Applications to beta cell cultures and islet amyloid polypeptide aggregation

DIANA RIBEIRO

Department of Biology and Biological Engineering
Chalmers University of Technology

Translational Genomics Department,
Innovative Medicines and Early Development Biotech Unit
AstraZeneca R&D

Abstract

Diabetes mellitus (DM) is a disease with epidemic proportions due to increased incidences worldwide, persistence of undiagnosed cases, uncontrolled forms of the disease, association with cardiovascular complications, and lack of definitive treatment options. In particular, type 2 DM (T2DM) is placing an enormous financial burden on worldwide healthcare systems that will increase in the future

There is a need to develop physiological assay platforms that provide functionally relevant models of diabetes in order to accelerate the discovery of new treatments. In this thesis I will focus on the biological relevance of pancreatic extracellular communication, by addressing the implications of extracellular vesicles (EVs), and the pancreatic extracellular matrix (ECM) on different *in vitro* models.

First, I present new insights on how pancreatic EVs modulate the aggregation of the hormone amylin (also known as islet amyloid polypeptide, IAPP), which is the major component of amyloid deposits found in pancreatic islets of patients with T2DM. Here, I demonstrate that EVs secreted from healthy (but not T2DM) pancreatic islets efficiently reduced amyloid formation *in vitro* (Paper I). Additionally, I showed that these pancreatic EVs can regulate insulin expression in stem cell-derived pancreatic cells, differentiating in a 3D-culture system *in vitro* (Paper II). I further addressed the significance of pancreatic ECM for the development of complex 3D-culture systems as a mean to improve the *in vitro* differentiation and commitment of stem cell-derived pancreatic cells. In this work, I tested stem cell-derived pancreatic cells cultured in a pancreatic decellularized scaffold with endothelial cells (Paper III). Through a collaboration I also helped to show that 3D-culturing improved the adipogenic

differentiation of adipose-derived stem cells (Paper IV). Lastly, I summarized the current knowledge of 3D-strategies used to increase the functional relevance of *ex vivo* primary pancreatic islets, and generation of stem cell-derived pancreatic beta cells (Paper V).

Keywords

Diabetes mellitus, type 2 diabetes mellitus, extracellular vesicles, insulin, islet amyloid polypeptide, protein aggregation, stem cell differentiation, induced pluripotent stem cells, adipose-derived stem cells, glucose-stimulated insulin secretion, extracellular matrix, 3D-culture, *in vitro* pancreatic models.

Preface

This dissertation is submitted for the fulfilment of the degree of doctor of philosophy. It is based on work carried out from February 2014 to February 2018 at AstraZeneca R&D, Gothenburg, Sweden under the supervision of Anna Forslöw, and at Chalmers University of Technology under the supervision of Professor Pernilla Wittung Stafshede. The research was funded by the by the People Program (Marie Curie Actions) of the European Union's Seventh Framework Program FP7, under REA grant agreement 607842, and AstraZeneca R&D, Sweden.

List of publications and contributions

The thesis is based on the work described on the following research manuscripts:

I. Extracellular Vesicles from Human Pancreatic Islets Suppress Human Islet Amyloid Polypeptide (IAPP) Amyloid Formation.

Diana Ribeiro, Istvan Horvath, Nikki Heath, Ryan Hicks, Anna Forslöw, and Pernilla Wittung-Stafshede

Proc Natl Acad Sci U S A. 2017 Oct 2. pii: 201711389. doi: 10.1073/pnas.1711389114.

I conceived the idea, planned and performed all the experiments together with Istvan Horvath and Pernilla Wittung-Stafshede. I analysed the results and helped writing the paper together with Istvan Horvath and Pernilla Wittung-Stafshede.

II. Human pancreatic islet-derived extracellular vesicles modulates insulin expression in 3D-differentiating iPS clusters.

Diana Ribeiro, Eva-Marie Andersson, Nikki Heath, Anette Persson-Kry, Richard Collins, Ryan Hicks, N. Dekker, Anna Forslöw

PLOS One. 2017 Nov 8;12(11):e0187665. doi: 10.1371/journal.pone.0187665.

I conceived the idea, planned and performed all the experiments together with Eva-Marie Andersson and Nikki Heat. I analysed the results and wrote the paper together with Nikki Heat, Ryan Hicks and Anna Forslöw.

III. 3D co-culture with HUVECs do not drive functional differentiation of iPS-derived pancreatic progenitor cells.

Diana Ribeiro, Duong Nguyen, Ryan Hicks, Anna Forslöw.

Manuscript.

I conceived the idea, planned and performed all the experiments together with Duong Nguyen. I analysed the results and wrote the paper together with Duong Nguyen, Ryan Hicks and Anna Forslöw.

IV. Increased Adipogenesis of Human Adipose-Derived Stem Cells on Polycaprolactone Fiber Matrices.

Cecilia Brännmark, Alexandra Paul, Diana Ribeiro, Björn Magnusson, Gabriella Brolén, Annika Enejder and Anna Forslöw.

PLOS One. 2014 Nov 24;9(11):e113620. doi: 10.1371/journal.pone.0113620

I helped with the gene expression experiments and data analysis, and continued the optimization of ADSCs isolation protocol.

V. 3D-models of insulin-producing β-cells: from primary islet cells to stem cell-derived islets.

Diana Ribeiro, Alexander J. Kvist, Pernilla Wittung-Stafshede, Ryan Hicks, Anna Forslöw

Stem Cell Reviews and Reports (2017), 1-12, doi: 10.1007/s12015-017-9783-8

I conceived the idea. All authors contributed to concept development and writing of this text.

Contents

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by progressive hyperglycemia, caused by insufficient production of, or resistance to, insulin which is produced by beta cells (β cells) within the pancreatic islets [1]. Type 1 diabetes mellitus (T1DM) occurs when the body's immune system destroys the β cells, resulting in insufficient insulin supply or insulin resistance leading to elevated blood-glucose levels. Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance where different organs no longer fully respond to insulin. In response, β cells increase insulin production but overall β cell function and mass continues to decline. Several secondary metabolic disorders can develop from this disease, including neuropathy, retinopathy, nephropathy, stroke and heart failure [2, 3].

Common treatment for T1DM involves rigorous blood-glucose control through daily injections of insulin or through an insulin pump [4]. An alternative to administration of exogenous insulin is the transplantation of allogenic islets, isolated from cadaveric sources. However, in this scenario, the amount of islets recovered from a single donor is often insufficient for a full transplantation treatment [5]. Additionally, supply of human tissue is sporadic and dependent on organ donors, where the quality and timing of delivery of the tissue is variable. The physical structure of islets is commonly lost due to post-isolation stress, leading to reduced functionality [6]. For T2DM, a variety of anti-diabetic drugs are prescribed aiming to preserve or rejuvenate the β cells [7, 8]. Nevertheless, current treatments do not alter the progressive nature of the insulin secretory deficit and the islets decreased health.

The alarming projection for the increase in prevalence of diabetes has lead the World Health Organization to predict that diabetes will become the leading cause of fatality by 2030 (Reviewed June 2016, [9]). The prevalence of diabetes, primarily T2DM, is already placing a financial burden on worldwide healthcare systems [10] with a predicted rise of 5-20% in total health expenditure, as stated by the International Diabetes Federation in its 7th edition review (2015, [11]).

A key goal is therefore development of improved *in vitro* models to drive developments in drug discovery and to diminish reliance on cadaveric islets. This thesis will focus on the relevance of pancreatic extracellular communication, by addressing the implications of extracellular vesicles (EVs), and the pancreatic extracellular matrix (ECM) on different *in vitro* model systems.

The dissertation begins with a background on the topics relevant to the thesis subject, divided in 3 chapters, followed by a description of my original work (chapter 4). The first chapter is an introduction to pancreatic islets of Langerhans in relation to T2DM. Chapter 2 reviews stem cell differentiation towards pancreatic islets, while chapter 3 explores extracellular signalling communication, via EVs, and its implications on diabetes-related biological processes. The original work is presented in chapter 4 and it is divided into two sections. The first summarizes the results on the modulation of IAPP aggregation by pancreatic EVs and IAPP-induced cytotoxicity in human islet cultures. The second part is devoted to *in vitro* differentiation of stem cells, of both induced pluripotent stem cells (iPSCs) and adipose-derived stem cells (ADSCs), towards pancreatic endocrine cells. The effect of EV supplementation and 3D-culturing using pancreatic ECM motifs are explored in the *in vitro* differentiating cell models.

Chapter 1: Human pancreatic islets

Chapter 1 will introduce pancreatic islets and the main hormone-producing cells. The insulin-producing cells (β cells) will be given special attention, with respect to their functional properties and relevance to T2DM.

1.1 Endocrine cell types and hormones

The adult pancreas is a heterogeneous gland with both exocrine and endocrine compartments, **Figure 1** [12]. The exocrine portion of the pancreas is composed of secretory cells that produce digestive enzymes, which are released into the pancreatic ducts [13]. The endocrine portion is composed of islets of Langerhans (Islets) that produce and release hormones to regulate glucose homeostasis [14]. The human islet is described to have 50%–60% insulin-producing cells (β cells), 30%–45% glucagon-producing cells (α cells), less than 10% somatostatin-producing cells (δ cells) and pancreatic polypeptide-producing cells (γ cells), and less than 1% ghrelin-producing cells (ε cells) [15, 16]. Human islets have been shown to be quite heterogeneous in terms of cell composition but also to have a substantially different architecture from widely studied murine islets. In isolated murine islets α and γ cells resided at the periphery of the β-cell core. However, human islets were markedly different in that α, γ, and β cells were dispersed throughout the islet [17]. Furthermore, human islets contained proportionally fewer β cells and more α cells than murine islets. These interspecies differences translate into different functional properties, in fact Cabrera et al [15] also found that cell oscillatory activity was not coordinated throughout the human islet as in mouse islets. Furthremore, human islets responded with an increase in [$Ca2^{+}$] when lowering the glucose concentration [15].

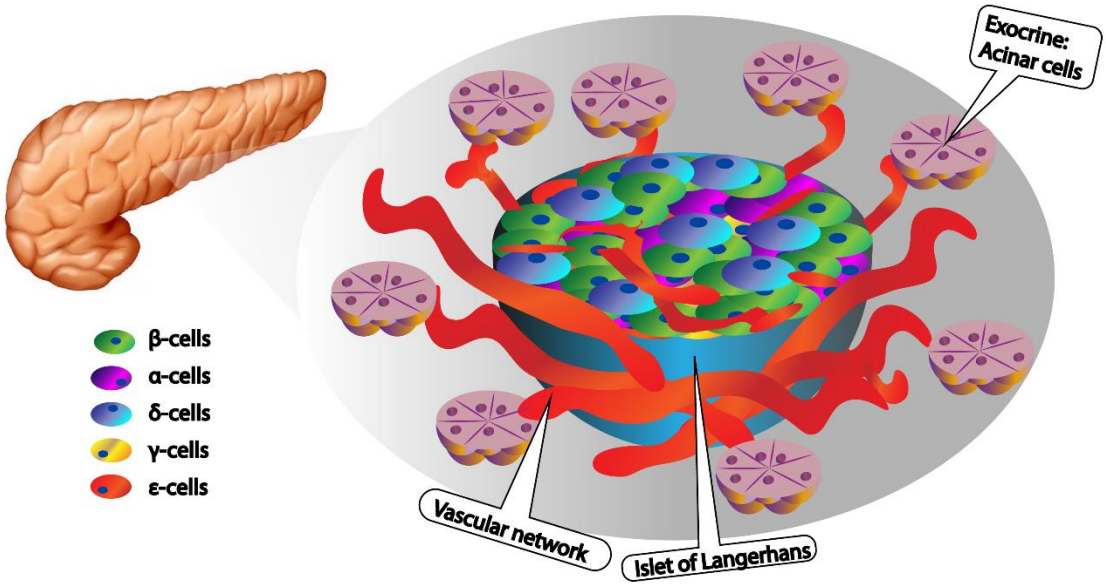


Figure 1 – Schematic representation of pancreas. *View of pancreatic exocrine and endocrine compartments. Blood vessels are also represented in this scheme as the pancreas is also a highly vascularized tissue. The Islets of Langerhans scheme showing all the main hormone-producing cells: β cells, α cells, δ cells and other hormones. Representations are not in scale.*

1.2 β cells

β cells are highly specialized cells that dedicate up to half of their protein synthesis to insulin production, with newly translated insulin packaged into specific insulin granules (**Figure 2A**) [18]. Insulin biosynthesis starts with the translocation of preproinsulin (110 residues, **Figure 2B**) to proinsulin (81 residues), from the ER to the Golgi apparatus where the N-terminus sequence is removed. Proinsulin is then packaged into the secretory granules, where there is a Zn^{2+} and Ca^{2+} rich environment, which propitiate the formation of a Zn^{2+} and Ca^{2+} hexameric specie of the proinsulin. C-peptide excision occurs through endo-peptidase cleavage resulting in a single C-peptide of 31 residues, being produced for every proinsulin that is cleaved [19]. Hence, the C-peptide contributes the second most abundant cargo in the insulin granule. C-peptide removal significantly alters the solubility of the insulin hexamer, which must be converted to insulin monomer for biological activity [20]. The insulin hexamer is stabilized by the coordination of one calcium and two zinc ions in addition to a variety of soluble proteins and ions [20]. This inactive form is thought to stabilize insulin by preventing degradation within the storage vesicle and to segregate proinsulin from newly formed insulin as the conversion to insulin occurs. The dissociation of the hexamer to the functional insulin monomer, the 51

residue peptide, is believed to occur somewhere along the transport of insulin from pancreas to the liver, where binding of insulin to liver cell receptors triggers the uptake of glucose from the blood. C-peptide is also secreted into blood, but has no known biological activity [21].

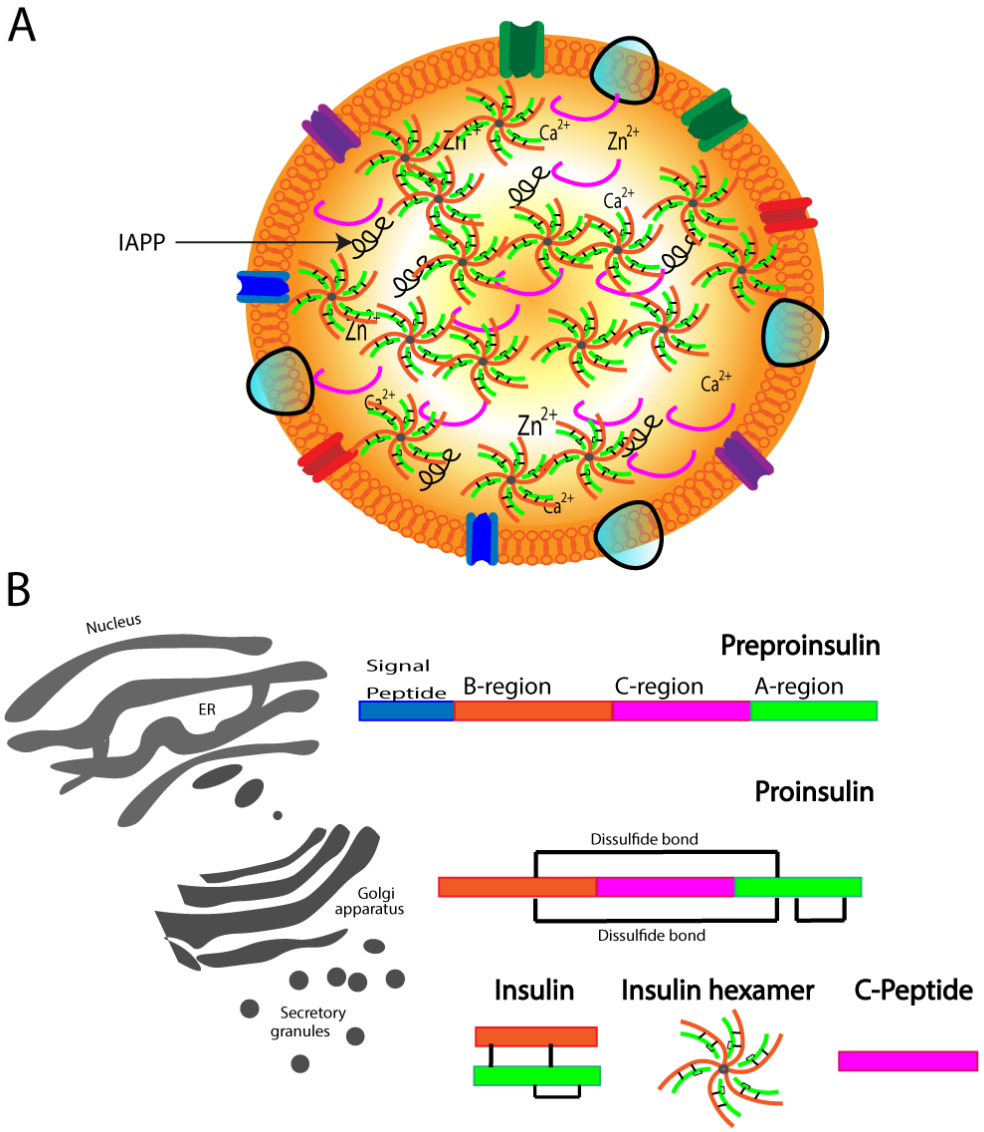


Figure 2 – Overview of the insulin synthesis and granule cargo. A) *Scheme of the insulin granule and main components such as proteins, small molecules and several transmembrane proteins and membrane associated proteins are represented but not specified. Main pancreatic hormones, insulin, C-peptide and IAPP are represented. Adapted from [22].* ***B)*** *Scheme of posttranslational modifications of the human insulin through the secretory pathway in β cells. Preproinsulin (110 residues) conversion into insulin (51 residues) and C-peptide (31 residues) starts with the cleavage of the N-terminal signal peptide at the ER (S: MALWMRLLPLLALLALWGPDPAAA). The protein is then transported to the trans-Golgi network where three disulfide bridges are formed, 1st) Cys-7 at B-region and Cys-7 A-region; 2nd) Cys-19 at B-region and Cys-50 A-region; and 3rd) Cys-6 and Cys-41 at A-region. The cleavage of the C-Peptide fragment occurs within the secretory vesicles.*

Islet amyloid polypeptide (IAPP, also known as amylin) can also be found in insulin granules in the β cells, but at smaller amounts. IAPP is a 37 residue peptide co-secreted with insulin, and is known to inhibit glucagon release and influence insulin secretion depending on concentration [23]. More information about IAPP is provided in section **1.3**

The insulin granule is an organelle in which many regulatory pathways cross and converge, and furthermore is the origin of several signals that regulate both β cell activity and affect neighbouring and distant cell types associated with glucose metabolism (**Figure 3,** [22]). Overall, β cells use changes in plasma-membrane potential to couple variations in the blood glucose concentration to stimulation or inhibition of insulin secretion [24, 25].

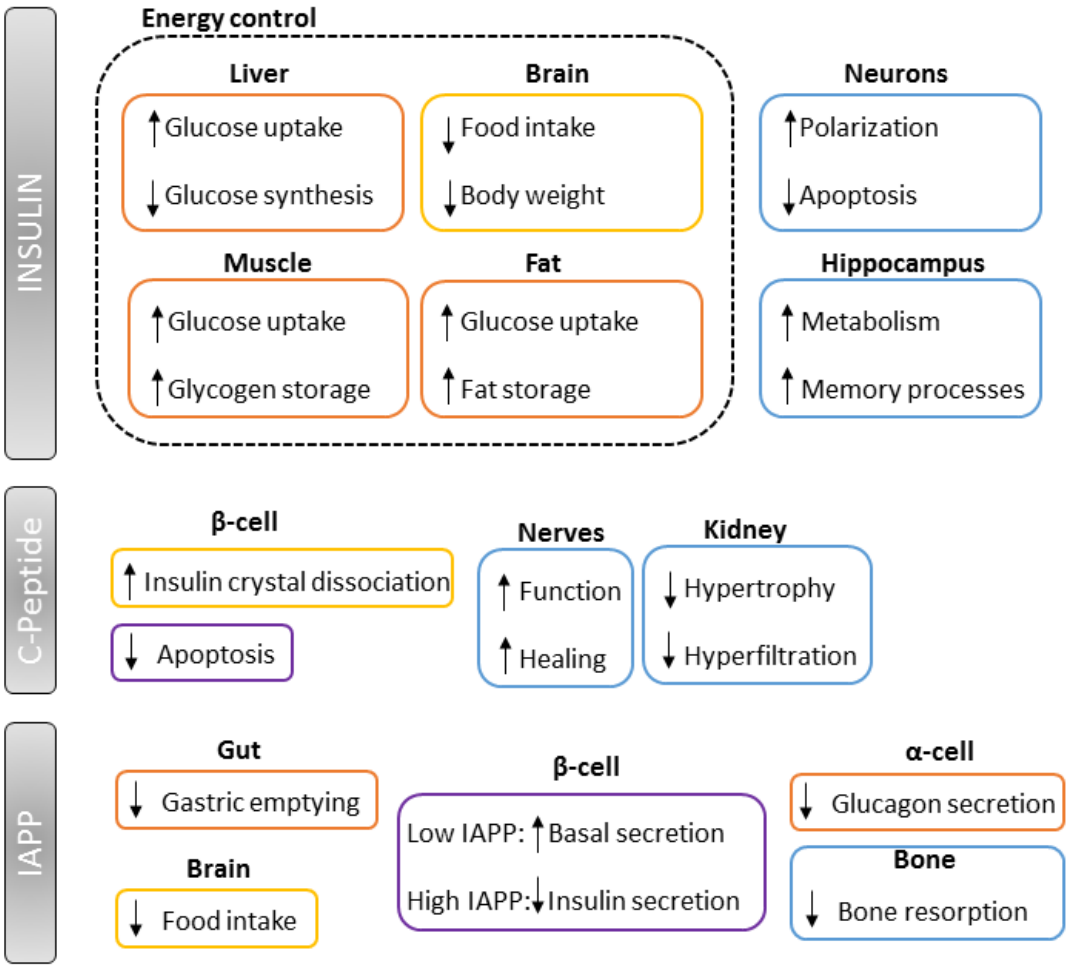


Figure 3 – Summary of pancreatic hormone-mediated regulatory pathways. *The cargo proteins of the insulin granule have a wide variety of effects on the β cell itself and on neighbouring cells related to glucose metabolism. In this scheme, signals regulating energy control are coloured orange for direct effects, yellow for indirect effects, unrelated effects are in blue, and autocrine effects are in purple. It is important to consider that several regulatory pathways result from the signalling network of several of these*

hormones and other peptides, and this complexity is not represented in this scheme. Adapted from [22] © TRENDS in Endocrinology & Metabolism.

1.2.1 Glucose-stimulated Insulin secretion (GSIS)

The β cell is electrically excitable and uses changes in membrane potential to couple variations in the blood glucose concentration to stimulate or inhibit insulin secretion (**Figure 4,** reviewed here [24, 26, 27]).

Central to glucose sensing by β cells is the stimulation of glycolytic and oxidative metabolism of sugar [28], ultimately causing enhanced mitochondrial ATP synthesis. GLUT1 and GLUT2 are believed to be the predominant glucose transporters in β cells [29, 30]. At non-stimulatory glucose concentrations (<3 mM), the activity of ATP-sensitive potassium (K_{ATP}) channels and inwardly rectifying potassium channels (K_{ir}) channels maintain a negative membrane potential in the β cell (approximately −70 mV) [31]. Increasing glucose to 6 mM or more reduces the resting conductance and triggers membrane depolarization, and once a threshold potential of is exceeded (approximately −60 Mv), regenerative electrical activity is observed. Eventually, the depolarization is large enough to activate several Ca^{2+} channels, followed by the opening of Na^{+} channels. At the peak of the action potential (−20 mV and above), the P/Q-type Ca^{2+} channels open and trigger exocytosis of insulin granules [32, 33].

At a higher glucose concentration, the β cell may undergo stronger depolarization because of more complete inhibition of K_{ATP} channels. This stronger depolarization in turn results in voltage-dependent inactivation of Na^{+} channels, lowered action potential amplitude, and reduced opening of P/Q-type Ca^{2+} channels. Accordingly, action potential firing and insulin secretion at high glucose principally involve L-type Ca^{2+} channels [24].

The objective of β cell electrical activity is to produce the $[Ca^{2+}]$ elevation that triggers insulin exocytosis of granules. GSIS follows a biphasic time course [34]. The basal rate of secretion corresponds to < 0.01% of insulin per min , transiently increases to $0.15\%.min^{-1}$ ~ 5 min after increasing glucose (first-phase secretion), and finally (after about 10 min) settles at > $0.05\%.min^{-1}$ (second-phase secretion) [35]. Human β cells contain approximately 10,000 secretory granules, each containing ~200,000 or more molecules of insulin [24, 26, 36]. In terms of secretion per β cell, the above rates therefore correspond to 1 $granule.min^{-1}$ (basal), 15 $granule.min^{-1}$ (peak first phase), and 5 $granule.min^{-1}$ (steady-state second phase) [24, 35]. Biphasic insulin secretion is required for proper insulin action and is observed not only *in vivo*, but also in isolated pancreatic islets [37, 38].

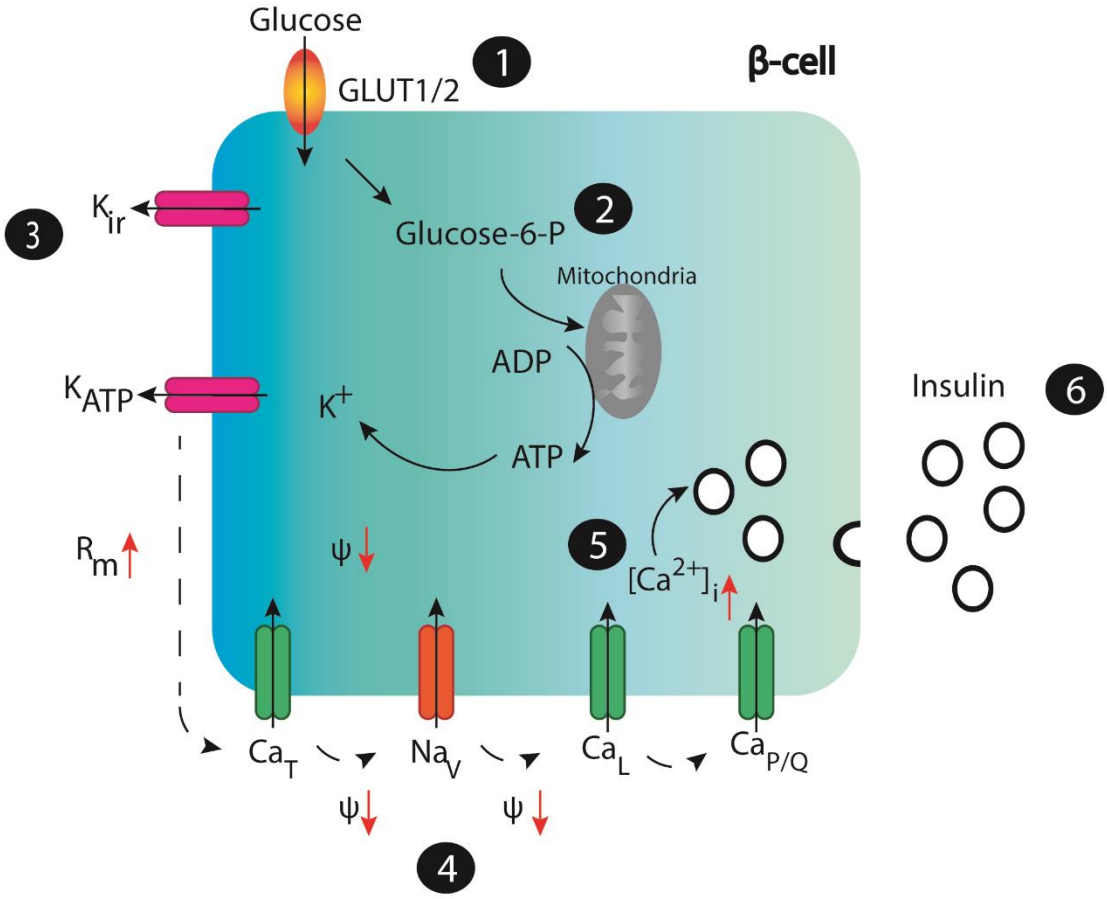


Figure 4 - Glucose-stimulated insulin secretion and electrical activity in the human β cell. *At no stimulatory glucose concentrations (<3 mM), the activity of K_{ATP} channels and inwardly rectifying K^+ channels (Kir) keeps a negative membrane potential in the β cell (approximately −70 mV). When glucose is increased, to concentrations that are just above the threshold for stimulation of insulin secretion [**(1)**; approximately 3.5–6 mM], an increase in the glucose uptake (via GLUT1/2 transporters) across the plasma membrane is observed. As a consequence of the previous step, the glucose metabolism is stimulated **(2)**, which leads to increased membrane resistance (R_m ↑), due to the closure of the K_{ATP} channels resulting from the metabolically induced elevation of the cytosolic ATP/ADP ratio **(3)**. When the membrane resistance is high, spontaneous openings of T-type Ca^{2+} channels (Ca_T) depolarize (Ψ↓) the β cell sufficiently to initiate opening of additional T-type Ca^{2+} channels, producing further depolarization leading to the opening and the activation of L-type Ca^{2+} channels (Ca_L) followed by Na^+ channels (Na_V) **(4)**. At the peak of the action potential (−20 mV and above), P/Q-type Ca^{2+} channels ($Ca_{P/Q}$) open **(5)**, and the increase in the concentration of the intracellular Ca^{2+} ($[Ca^{2+}]_i$). The result of this increased in the $[Ca^{2+}]_i$ triggers exocytosis of insulin from the secretory granules **(6)**. Solid lines indicate flux of a metabolite or an ion, while dashed lines indicate a change in membrane potential. This scheme was adapted from [24].*

1.3 IAPP and Diabetes

Protein failure to achieve a functional folded structure may be due to misfolding. A possible biological consequence of polypeptide misfolding is the development of so-called misfolding diseases, including amyloid formation diseases where peptides aggregate into ordered long amyloid fibrils. Typically, amyloid fibrils have a cross-β structure that is composed of double-layered β-sheets perpendicularly orientated to the fibril orientation axis [39]. The phenomenon of amyloid formation and accumulation of insoluble fibrillar protein aggregates in tissues and organs, has become the subject of rapidly increasing research activities across a wide range of diseases. Amyloid formation has been implicated in several debilitating medical disorders, such as Parkinson´s disease, Alzheimer's disease and T2DM, many of which are major threats to human health and welfare in the modern world [40, 41]. It has become clear that the ability of proteins to form amyloid structures is more general than previously imagined, and there is a need to understand the mechanisms by which the cellular protein homeostasis systems fail to correct or prevent misfolding and aggregation. However, it is important to highlight that amyloid formation has been recently discovered and associated with beneficial functions [42], such as antimicrobial activity [43].

T2DM is a multifactorial disease characterized by dysfunctional secretion of insulin by the β cells of the pancreas, eventually leading to β cell death. The relative contribution of different abnormalities varies among patients, as well as during the course of the disease [44]. The peptide hormone IAPP is thought to play a central role in diabetes pathology [40, 45], and challenges in the treatment of T1DM through islet cell transplantation have also been attributed to the amyloidogenic properties of human IAPP [46].

1.3.1 IAPP protein

Alike insulin, IAPP is derived from a larger propeptide precursor (preproIAPP) that in humans is an 89-amino acid peptide. In the endoplasmic reticulum (RE), a disulfide bound is formed and the signal peptide is enzymatically cleaved, leading to the formation of proIAPP (67 residue). ProIAPP is transported through the Golgi network to the insulin granule, where the N-terminal and C-terminal fragments are cleavaged to form the mature 37-amino acid peptide IAPP [47-49], **Figure 5.**

IAPP is coproduced and cosecreted with insulin by the pancreatic β cells, and is released in response to the stimuli that drives insulin secretion [50, 51]. The physiological roles of soluble

IAPP (**Figure 3**) are not completely understood, but IAPP is believed to play a role in controlling food intake and energy homeostasis [52], in the suppression of glucagon release and influence insulin secretion depending on concentration [23, 53]. Several reviews of the function of IAPP have recently appeared and provide more in depth discussions [54-56].

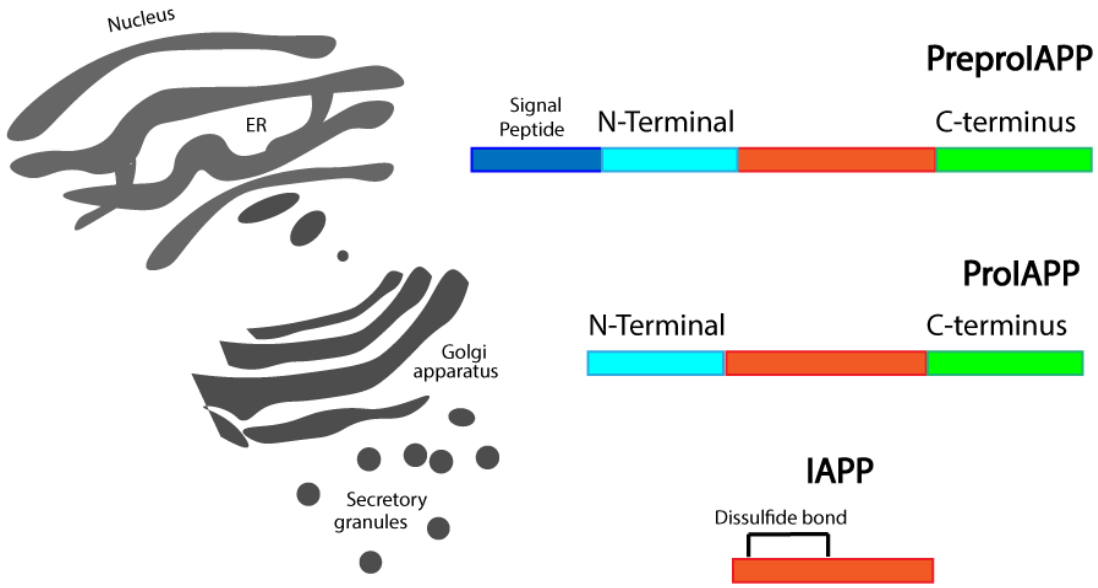


Figure 5. Posttranslational modification of human IAPP through the secretory pathway in β cells. *Once expression of the primary sequence of the 89-residue, preproIAPP, a disulfide bond is formed at the ER and the N-terminal signal peptide (S:MGILKLQVFLIVLSVALNHLKA) is cleaved and the protein transported to the trans-Golgi network. The cleavage of the C-terminus fragment (C:NAVEVLKREPLNYLPL) and the N-terminal fragment (N: TPIESHQVEKR) from proIAPP (67-residue) occurs within the secretory vesicles. The mature 37-residue human IAPP has an intramolecular disulfide bridge between Cys-2 and Cys-7 and an amidated C-terminus.*

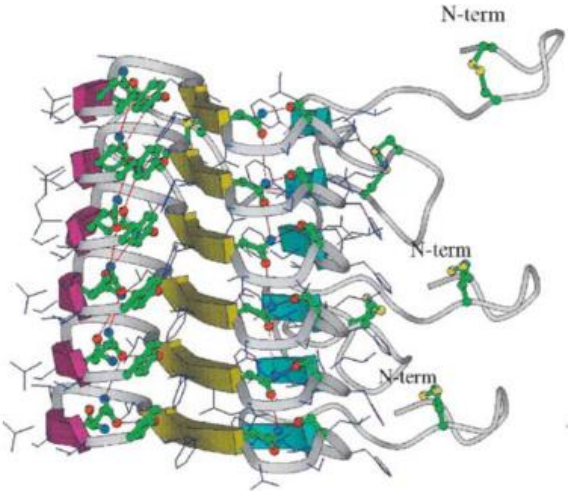
The ratio of IAPP to insulin in the granule can vary, but IAPP is always found at lower levels than insulin [51, 57]. Some evidence suggests that IAPP secretion or fibrillar IAPP is increased in diabetics [58], however IAPP can be found in healthy islets as well as T1DM [59] and T2DM [60]. Nevertheless, IAPP is thought to be localized at high concentrations in the secretory granule, higher than the level required to promote rapid amyloid formation *in vitro* [51, 54], and in close proximity to both the lipid membrane and the insulin crystal [61]. Thus, there must be factors which inhibit the premature, irreversible aggregation of IAPP in the granule. Mature human IAPP is natively unfolded in its monomeric state but forms amyloids in T2DM, leading to an excessive extracellular and intracellular accumulation of toxic species that may be soluble oligomers or insoluble fibrils [60, 62]. The degree of amyloid formation often reflects the severity of DM, but a causal relationship between amylin aggregation and disease progression is less clear.

1.3.2 IAPP aggregation in vitro

Human IAPP amyloid fibrils are described to consist of two β-sheets that twist around each other and run perpendicular to the fibril axis, with the hydrogen bonds oriented parallel to the long axis of the fibril (**Figure 6A**, [63-65]). Experiments have shown that there is three key parts within the IAPP sequence, which contribute to the fibril formation process (**Figure 6B**). Even though the exact amino acid residue position can vary between studies, the 8-17 region is believed to be responsible for IAPP-membrane interactions and to weakly contribute for the fibril aggregation in solution [66], the second (18-22 region) is thought to be the amylogenic part [67], although only the third part (23-37 region) is described to be essential for the formation of amyloid-like fibrils in aqueous media [68-70]. In addition the N-terminal domain (residue 1 to 7), is not essential for fibril formation [67], but might be involved in the protein folding and overall kinetics of fibril formation [71]. Another characteristic of the N-terminal region is the intramolecular disulfide bridge between Cys2 and Cys7, which was shown to be essential for IAPP biological activity measured as insulin-stimulated rates of glycogen synthesis [72]. Other *in vitro* studies highlight that the disulfide bond is not involved in the amyloid fibril core, however, it does contribute to the assembly mechanism since loss of the disulfide bond reduces fibril formation [73].

The kinetics of fibril formation is often monitored as a function of time by the use of the fluorescent molecule Thioflavin T (ThT) that binds to amyloid fibrils, which results in increased fluorescence at 480nm [74]. Fibril formation often involves a lag phase followed by a sigmoidal transition to high fluorescence (**Figure 7**). In the lag phase, small soluble oligomers form what is called "nuclei" (nucleation phase); in a second step, called the elongation (expansion) phase, involves propagation of fibril growth from nuclei with consumption of monomer. Finally a plateau in the ThT emission is reached when the amount of fibril remains constant (stationary phase) [41]. The initial step is called primary nucleation, which differs from secondary nucleation that involves interaction with existing fibrils, and it is dependent on the behaviour of the aggregates that form during the polymerization reaction [75]. Fibril fragmentation is a type of a secondary process, where each fragmentation event increases the number of fibril ends that generate growth through the attachment of monomers [75]. Human IAPP amyloid formation is described to occur via a nucleation-dependent polymerization process, although secondary nucleation is also observed [76].

A



B

Figure 6. Structure of IAPP. *A) Model of the parallel β-sheet structure of IAPP described by [77] © J. Mol. Biol.,Elsevier. The model shows the three β-sheets twist around each other, having the N-terminal protruding from the core of the amyloid fibril. B) The amino acid sequence of human IAPP and distinction of the different amyloidogenic parts. Residues 8–17 are colour coded in red, and described to not take part in β-sheet structure in existing models of IAPP amyloid. Residues 18–22, coloured in green, and residues 23–37, coloured in blue, take part in an intermolecular β-sheet, but only that last ones are described to be essential for the fibril formation in solution. The colour coding corresponds to the IAPP amyloid structural model developed by the UCLA group [64]. The first 7 residues do not take part. Adapted from [63] © Current Opinion in Structural Biology.*

1.3.3 Factors affecting IAPP fibrillation in vitro

Several factors have been described to affect amyloid formation of IAPP *in vitro*. Of particular interest are the β cell granule components, such as proinsulin, insulin, and C-peptide, which are stored together with IAPP in the secretory granule.

Insulin is described to slow down the fibrillation kinetics of IAPP in solution [23, 78]. The magnitude of the inhibitory effect is dependent on the concentration of insulin [23], yet independent of the concentration of IAPP [79]. Others reported that as long as the insulin monomer is stable in solution, monomeric human IAPP is also stabilized through the formation of protein complexes of insulin and IAPP. One IAPP monomer binds up to three insulin monomers, but no complexes with more than one IAPP monomer have been found [80]. It has been reported that *in vitro*, insulin keeps its inhibitory effect for a limited time(4h), while

promoting amylin aggregation after long-term incubation (72h) [81]. *In vivo*, insulin is in a semicrystalline state in the granule, and IAPP has been shown to interact with insulin crystal surfaces *in vitro* [61]. Proinsulin showed a weaker inhibitory effect, while C-peptide, Ca^{2+} and Zn^{2+} each enhanced IAPP fibril formation in one study [78], but inhibited IAPP aggregation in another study when added in excess [23].

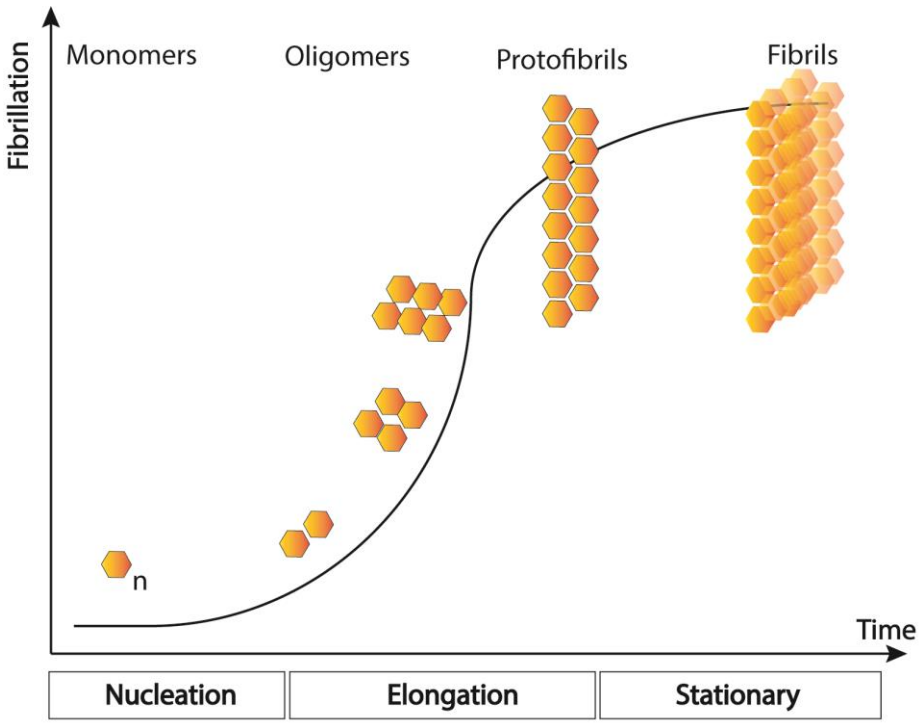


Figure 7. Schematic representation of fibrillation of human IAPP over time. *During the nucleation phase IAPP monomers associate in order to form oligomers of various sizes (Nucleation). As the nucleation phase extends to the elongation phase it is observed the formation of protofibrils, which build blocks of the mature fibrils. Finally a plateau in the fibril formation kinetics is reached and the amount of fibril remains constant (stationary phase) Adapted from [82].*

The plasma membrane is another key factor with implications on IAPP fibril formation. Insulin β cell line lipid content was quantified as 68.9±3.5% phosphatidylcholine (PC), 24.0±3.4% phosphatidylethanolamine (PE), 4.6±0.2% sphingomyelin (SM), 1.6±0.4% phosphatidylinositol (PI), 0.6±0.1% phosphatidylserine (PS), 0.1±0.03% phosphatidic acid (PA), and 0.2±0.02% phosphatidylglycerol (PG) [83]. Although, there are 10% more lipids with a negatively charged head group (PI, PS, PA, and PG) in isolated rat islets, while similar levels of zwitterionic phospholipids PC and PE between the cell line and rat islets [84].

The β cell plasma membrane is described to be asymmetric, with the anionic lipids predominantly localized to the inner leaflet and the outer leaflet enriched with sphingomyelin [85]. Human IAPP is a relatively hydrophobic peptide, but contains several positively charged

residues, Lys-1, Arg-11, and, depending upon the pH, His-18; and has an isoelectric point of 9.8 and thereby a net positive charge at biologically relevant pH values [49, 67]. The charge of IAPP may be of importance for interactions with the lipid membrane, and modulate the fibril formation kinetics.

It has been shown that *in vitro* IAPP fibril formation can be accelerated by the presence of phospholipids isolated from whole pancreas of T2DM patients, and by negatively charged phospholipid bilayers composed of dioleoylphosphatidylglycerol (DOPG) [86]. By contrast, no fibril formation was observed in the presence of a zwitterionic lipid (dioleoylphosphatidylcholine, DOPC) or a cationic (dioleoyltrimethylammoniumpropane, DOTAP). The same authors reported that the lipid interactions occur at the N- but not the C-terminus of IAPP [86]. Additionally, human IAPP monomers have been found to insert in lipid monolayers composed of DOPC:DOPS, while mature fibrillar IAPP was not able to insert in the lipid monolayer [66, 87].

Furthermore, cholesterol (CH) and sphingomyelin (SM) are important membrane components affecting membrane fluidity and formation of lipid rafts [88], and it has been reported that the presence of SM in PC-based liposomes slows down [85], while CH added to synthetic lipid membranes inhibits IAPP amyloid formation [85, 89, 90].

Since the presence of lipid membranes can strongly affect the kinetics of IAPP aggregation and fibrillation, an IAPP-mediated membrane disruption mechanism has been suggested (**Figure 8**, [91]). Brender *et al* suggest a mechanism mediated by a membrane pore formation and/or augmentation to a severe membrane fragmentation scenario [91].

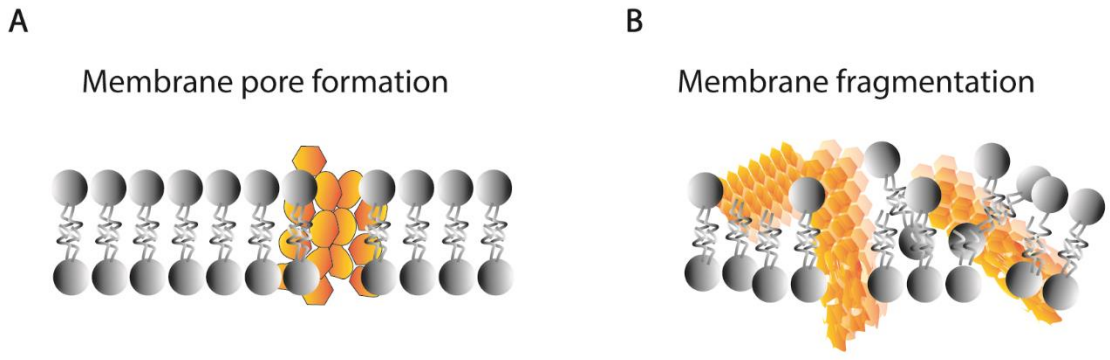


Figure 8. Hypothetical mechanism for membrane disruption mediated by IAPP. *In solution, the monomeric peptide can bind to the membrane in a cooperative manner where it can then aggregate to form stable, possibly toroidal, pores* ***(A)****. The continued aggregation and growth of amyloid fibers of IAPP on the membrane leads to complete fragmentation of the membrane* ***(A)****. Drawing is not to scale. Adapted from [92] © J. Acc. Chem Res.*

1.3.4 IAPP-mediated β cell cytotoxicity

β cell dysfunction, as a result of impaired GSIS, defective prohormone processing and exacerbated by metabolic stress, can either propitiate or cause the process of islet amyloid formation to occur in T2DM. Declining β cell mass is predicted to increase metabolic demand on remaining β cells, promoting a feed-forward cycle of β cell decline [93]. Others have reported that amyloid deposition in human pancreatic islets is associated with decreased β cell area and increased β cell apoptosis [62], confirming an association between islet amyloid deposition and T2DM. IAPP induced toxicity in pancreatic β cells is often related to T2DM disease development [40, 94].

IAPP-mediated cytotoxic effects, in *in vitro* cell lines, are often observed at micro molar range. Increased cell death and decreased cell proliferation has also been attributed to IAPP accumulation (0.5 – 8 μM) in insulinoma cells [95]. The IAPP-mediated effects (10 to 1000 pM) on pancreatic cells proliferation has also been shown to be glucose dependent. There was an increase of β cell proliferation at low glucose (5.5 mM) conditions, whereas glucose reduced β cell proliferation at high glucose (25 mM) levels [96]. Additionally, in an earlier report it was showed that IAPP fibrils (8 to 20 μM) can induce β cell apoptosis in both rat and human islets [97]. However, it was not clearly described how the authors excluded the effects of monomers and oligomers in solution, since the IAPP was dissolved in water and immediately added to cells culture media. In fact, it has been revealed that toxic human IAPP oligomers, rather than human IAPP fibrils (20-80 μM), initiate β cell apoptosis [98, 99]. In pancreatic rat insulinoma cells, it has been shown that IAPP monomer and oligomer uptake (10 μM), via endocytic compartments, is dependent on both time and concentration [100]. Likewise, IAPP fibrils (10 μM) are not toxic in rat insulinoma INS-1 cells but elicit toxicity when combined with freshly dissolved IAPP [101]. The authors identified that this secondary toxicity was quantitatively stronger than the toxicity observed with freshly dissolved IAPP (primary toxicity) alone. Nevertheless, it was reported that IAPP (10 μM), both in soluble form and as aggregates, induced mouse islet inflammation [102], which often occurs in β cells with impaired GSIS.

Chapter 2: Stem cell-derived β-cells

It has been shown that the unique organization of islet cells observed within human pancreata is not maintained in cultured isolated islets [103]. Others reported critical post-isolation effects of human islets such as cell death involving primarily β-cells [6], and loss of mature β-cell phenotype [104]. Thereby, there is a need to develop physiologically relevant cell-derived assay platforms for diabetes modelling.

Extensive work in the field has successfully demonstrated embryonic stem cell (ESCs) and induced pluripotent stem cell (iPSC) differentiation to pancreatic β-cells *in vitro*, indicating the feasibility of ESC/iPSC-based cell therapy for diabetes [105-107]. A renewable and close to unlimited source of native-like pancreatic cells would reduce reliance on the finite and variable source of human islets that are available through donation of cadaveric islets.

2.1 Stem cells

Stem cells are primal cells common to all multicellular organisms that retain the ability to renew themselves through cell division and can be differentiated into a wide range of specialized cell types. The rigorous definition of a stem cell requires that it possesses two properties: self-renewal and unlimited potency. Self-renewal means the ability to go through numerous cycles of cell division while maintaining the undifferentiated state. Unlimited potency means the capacity to differentiate into any mature cell type. All stem cells are unspecialized (undifferentiated) cells, though totipotency is shown only very early on embryonic stem cells, the adult stem cells possess multipotency and differential plasticity (**Figure 9**, [108-110]).

Totipotent stem cells are produced from the fusion of an egg and a sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent (e.g. Morula). These cells can differentiate into embryonic and extraembryonic cell types [108-110], in particular ESCs are derived from the inner cell mass of the blastocysts [109, 111]. They proliferate *in vitro* and are able to form all three germ layers, endoderm, ectoderm and mesoderm, and many of their downstream derivatives, such as hematopoietic, neural, and cardiovascular lineages [111, 112]. Furthermore, ESCs can form teratomas (noncancerous tumours) when injected into immune deficient mice [113, 114]. These pluripotency features distinguishes ESCs from multipotent cells found in adults, which can only form a limited number of different cell types [115].

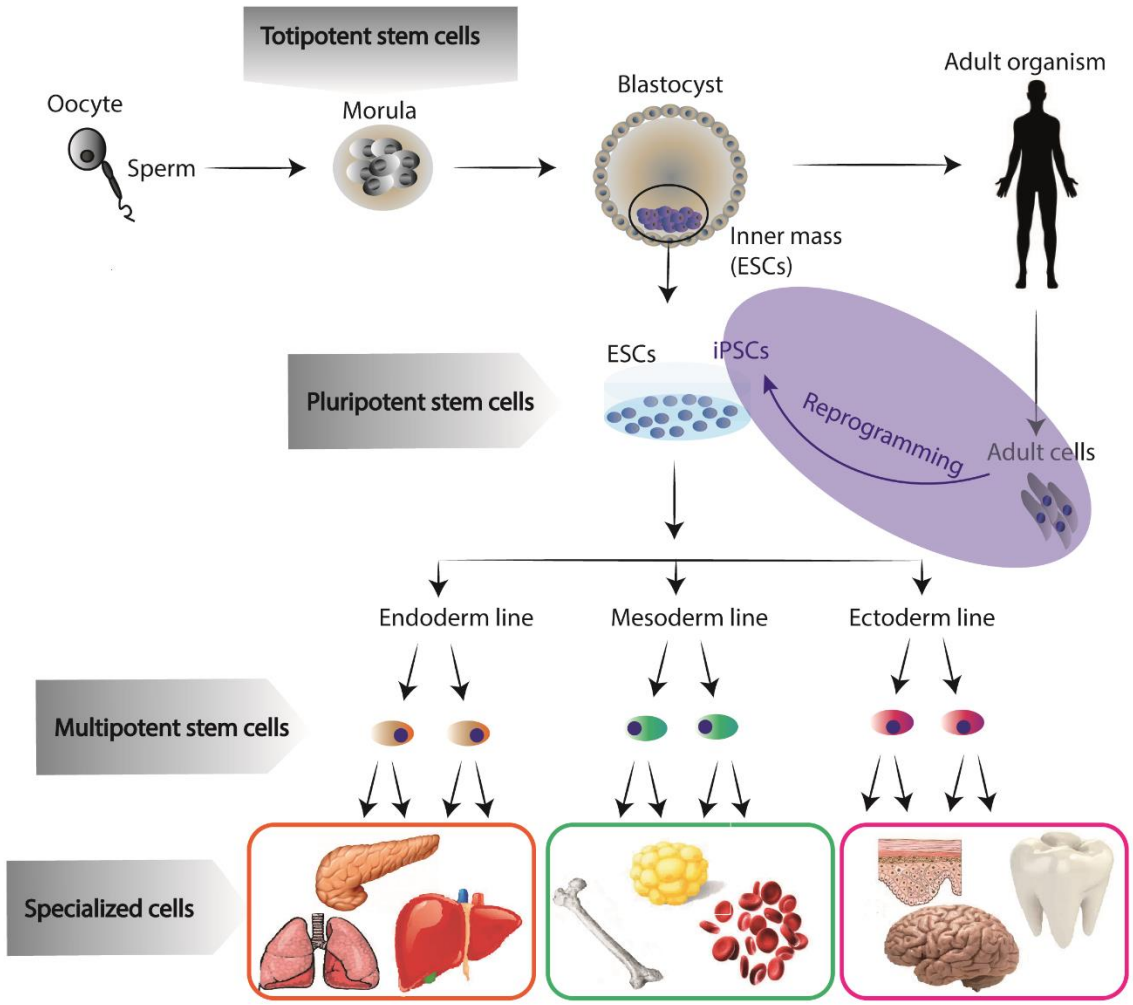


Figure 9 – Hierarchy of stem cells potency and differentiation. *The fusion of the oocyte and sperm will form a fertilized egg (zygote) which evolve to the formation of totipotent complex called Morula. The cells from morula have the ability to generate a new organism. However, as the development proceeds a decrease in the stem cell potency decreases and the inner cell mass of the blastocyst (next development stage) contains only pluripotent stem cells called embryonic stem cells (ESCs). The ESCs can give rise to all the all three germ layers, endoderm, ectoderm and mesoderm and downstream derivatives. Adult cells can be reprogrammed to become pluripotent using key transcription factors (Yamanaka factors: Oct4, Sox2, Klf4, and c-Myc, [116]). These are called induced pluripotent stem cells (iPSCs) and behave an in vitro comparable to ESCs. Adult stem cells are localized in niches in the adult organism, such as bone marrow, central nervous, skin and others. These stem cells are multipotent and have limited differentiation ability.*

Several key transcription factors are described as master agents that control ESCs regulatory circuitry underlying pluripotentcy - *OCT3/4*, *NANOG* and *SOX2* [117]. A different line of research suggests that concomitant expression of these transcription factor specifiers within pluripotent cells enables differentiation into every fetal lineage [118].

Nevertheless in 2006 a research group used four masters transcription factor, known to be involved in the maintenance of pluripotency, *OCT4*, *SOX2*, *KLF4*, and *c-MYC*. Expression of these specific transcription factors was used to reprogram mouse fibroblasts (cells found in the skin and other connective tissue) to an embryonic stem cell–like state, maintaining the defining properties of ESCs. These adult cells, called induced pluripotent stem cells (iPSCs), were found to exhibit the morphology and growth properties of ESCs and express ESCs marker genes [116]. Furthermore, subcutaneous transplantation of iPSCs into nude mice resulted in tumours containing a variety of tissues from all three germ layers. Following injection into blastocysts, iPSCs contributed to mouse embryonic development. In 2007, a new milestone was achieved by showing the generation of iPSCs from human cells [119, 120].

Since the discovery of iPSC technology, enormous progress has been made in stem cell biology and regenerative medicine. Human iPSCs have been widely used for disease modelling, drug discovery and cell therapy development [121]. In 2012, Professor Shinya Yamanaka was recognized for the discovery that mature cells can be reprogrammed to become pluripotent with the Nobel Prize in Physiology or Medicine.

Adult stem cells are undifferentiated and multipotent stem cells found throughout the body, localized in stem cell niches, which create unique microenvironments and have the dominant role in controlling stem cell behaviour. Signals from the niche influence the cycling status of stem cells and also help to maintain their undifferentiated state [122, 123].

Bone marrow is the major source of adult stem cells, also known as somatic or mesenchymal stem cells (MSCs). There are mainly two types of marrow stem cells: bone marrow hematopoietic stem cells and bone marrow MSCs. Hematopoietic stem cells are stem cells and the early precursor cells which give rise to all the blood cell types and can reconstitute the bone marrow after depletion caused by disease or irradiation [124]. MSCs have the remarkable capacity to replenish themselves through self-renewal and to give rise to either unipotent (adult cells) or more (multipotent) downstream differentiated cell lineages [122].

MSCs can also be isolated from other non-marrow tissues, such isolated from adipose tissue (fat tissue) following surgical-procedures - adipose derived stem cells (ADSCs) [125]. This cell population seems to be similar in many ways to bone marrow MSCs and have been shown to differentiate into multiple mesodermal cell types, adipogenic, chondrogenic, or osteogenic differentiation [126-128]. In addition, MSCs have also been described to trans-differentiate towards endoderm and pancreatic phenotypes as well [129, 130], making them a possible source for future application in the clinic [126, 131]. Overall, the ability to isolate and propagate human

stem cells including ESCs, ADSCs and iPSCs provides powerful means for modelling human development in a dish [110].

2.2 Pancreatic differentiation *in vitro*

Several strategies have been developed for obtaining human differentiated and functional pancreatic islets. At present, several groups have reported stepwise protocols for mimicking the mechanism of *in vivo* pancreas development, some of the most commonly cited protocols are summarized in **Figure 10A**, [132]). These protocols induce definitive endoderm differentiation at the first stage, then pancreatic specialization and maturation. Monitoring of differentiation commitment is performed by checking for the expression of precise pancreatic markers in a temporal way (**Figure 10B**).

The optimization of ESC differentiation to the definitive endoderm lineage has been described as a key step and pre-requisite for efficient differentiation to mature endoderm derivatives [133]. Others have established differentiation protocols that drive stem cells to insulin-producing cells using chemically-defined culture media while avoiding the use of feeders, stroma cells or serum, all of which can interfere with experimental outcomes [134]. The interplay of several factors has been found to be crucial for further endocrine commitment. EGF is reported to enrich for $PDX1^+$ pancreatic progenitors [135], while others have reported the combined effects of retinoic acid and FGF4 in direct differentiation towards $PDX1^+$ foregut endoderm [136]. In addition, the combined actions of βFGF, ActivinA and BMP-induced signaling have been shown to promote formation of endoderm and subsequently pancreatic cells from ESCs [137].

Limited hormonal functionality of monolayer-based cell culture systems has driven the need to develop suspension cluster culture systems, aiming to mimic islets 3D-architecture and morphology. In developing this methodology it was shown that expression of $PDX1^+/NKX6.1^+$ cells correlated with cell density in adherent cultures which was further increased by aggregation of cells into islet-like clusters [138]. Cell clusters generated in suspension cultures under multistep differentiation protocols have been shown to express the hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin [139-141]. These cell clusters release insulin in response to multiple secretory stimuli, but with sub-physiological levels of response to glucose as compared to adult islets. However, the potential of these culture systems becomes clearer in the context of transplantation into animal models to further drive maturation of cells. In this setting, the stem cell derived β cells develop improved insulin secretion in

response to glucose and normalization of blood glucose levels in models of induced hyperglycemia [142-144].

A

Rezania et al, 2014 [143]	Pagliuca et al, 2014 [145]	Russ et al, 2015 [146]
Matrigel coating	*Spinner flasks (suspension)*	*Low-adherence plates (suspension)*
2d: BSA, GDF8, MCX-928	**1d:** VitC, BSA, ITS, ActA, CHIR	**1d:** ActA, Heregulin b-1
1d: BSA, GDF8, CHIR-99021	**2d:** VitC, BSA, ITS, ActA	**1d:** FBS, ITS, Wnt3, ActA
2d: BSA, AA, FGF7	**3d (SOX17$^+$):** VitC, BSA, ITS, FGF7	**1d:** FBS, ITS, ActA
2d: BSA, AA, FGF7, SANT-1, RA, LDN193189, ITS, TPB	**2d (PDX1$^+$):** VitC, BSA, ITS, FGF7, RA,SANT-1, LDN193189, PdbU	**1d:** FBS, ITS, TGFb$_i$, FGF7
3d (PDX1$^+$/NKX6.1$^+$): BSA, AA, FGF7, SANT-1, RA, LDN193189, ITS, TPB*	**5d (PDX1$^+$/NKX6.1$^+$):** VitC, BSA, ITS, FGF7, RA, SANT-1	**2d:** FBS, ITS, FGF7
Air-liquid interface (inserts)	**3d:** VitC, BSA, ITS, Heparin, RA, SANT-1, T3, XXI, BETACELLULIN, ALK5$_i$II	**2d:** High Glucose, B27, TTNBP
3d (PDX1$^+$/NKX6.1$^+$/ NEUROD1$^+$): BSA, SANT-1, RA, LDN193189, ITS, T3, ALK5$_i$II, Zinc sulfate, heparin	**3d:** VitC, BSA, ITS, Heparin, RA, XXI, T3, BETACELLULIN, ALK5$_i$II	**1d:** High Glucose, B27, TTNBP, EGF
7-15d (NKX6.1$^+$/INSULIN$^+$): BSA, LDN193189, ITS, T3, ALK5$_i$II, Zinc sulfate, γ-secretase$_i$ II, heparin	**13d (C-Peptide$^+$/NKX6.1$^+$):** High BSA, T3, ALK5$_i$II	**1d (PDX1$^+$/NKX6.1$^+$):** High Glucose, B27, EGF, FGF7
7-15d (NKX6.1$^+$/MAFA$^+$/ INSULIN$^+$): High glucose, BSA, ITS, T3, ALK5$_i$II, Zinc sulfate, N-Cys, Trolox, R428, heparin		**5d:** High Glucose, B27, LDN193189, TBP, ALK5$_i$II, FGF7
		7d (Insulin$^+$/NKX6.1$^+$): High Glucose

B

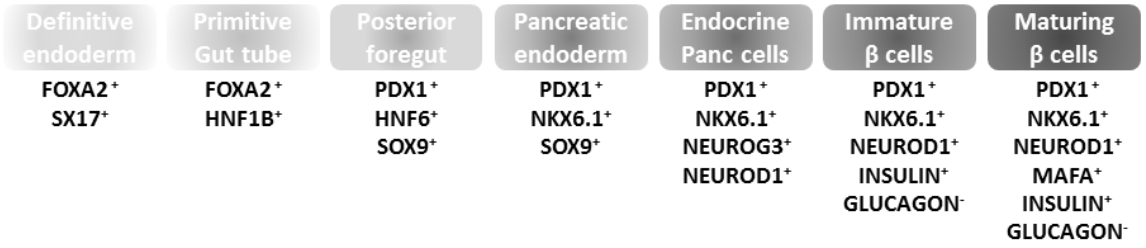


Figure 10. Multistep differentiation protocols and expression profiles of differentiate ESC and iPSCs to insulin-producing cells. A)*. Published protocols commonly cited on the literature and respective (main) induction agents added to differentiation media and time of differentiation supplementation. d: day/s; some factors are abbreviated as indicated here, ActA: Actvin A; ITS: Insulin-Transferrin-Selenium; AA: ascorbic acid; RA: retinoic acid; T3: 3,3',5-Triiodo-L-thyronine sodium salt; VitC: vitamin C. * states for media with the same composition but different concentrations of one or more compounds. B) Sequence of*

differentiation stages and the temporal pattern of expression of key pancreatic genes/proteins is indicated under each developmental stage. Adapted [147] © Nature Biotechnology.

Russ and colleagues have demonstrated the importance of precise temporal activation of endocrine differentiation in pancreatic β cell differentiation [146]. They show that *PDX1*$^{+}$*/NKX6.1*$^{+}$ progenitor cells convert to glucose-responsive and insulin-expressing β-like cells *in vitro* and *in vivo*. Furthermore, the application of the same stimuli of differentiation to a developmental pre-cursor in the form of *PDX1*$^{+}$*/NKX6.1*$^{-}$ cells results in precocious endocrine induction and generation of poly-hormonal, functionally immature cells. Pagliuca et al have optimized the cluster suspension protocol in order to deliver cluster suspension cultures capable of generating hundreds of millions of glucose-responsive β cells from human iPSC *in vitro* [145]. Characterization of their stem-cell-derived β cells revealed functional responses and morphological features characteristic of mature β cells. The features described herein included Ca^{2+} flux in response to glucose, packaging of insulin into secretory granules and secretion of insulin at physiologically relevant levels comparable to adult β cells in response to multiple sequential glucose challenges *in vitro*. Furthermore, transplantation of these cells into diabetic mice normalized experimentally induced hyperglycemia [145].

Adult multipotent stem cells, isolated from mesenchymal sources, are often overlooked in favour of pluripotent ESCs or iPSCs for β-cell differentiation and diabetic therapy. Nevertheless, an increased understanding of the utility of adult stem cells has demonstrated their potential and proved them to be a promising alternative for β cell generation with the aim of cell-therapy [148]. MSCs isolated from adipose tissue [129, 130], umbilical cord [149, 150] and bone marrow [151] have all demonstrated the capacity to differentiate into physiologically functional islet-like cells or aggregates. These cell clusters showed *in vitro* functional responses with insulin secretion in response to glucose stimulation and transplantation of MSCs-derived clusters restored near normo-glycemia in a diabetic model [129, 130, 149, 150].

2.3 The role of pancreatic extracellular matrix (ECM)

Extracellular matrix (ECM) is a non cellular component present in all tissues and organs, composed of proteins, proteoglycans, polysaccharides and other molecules secreted by cells. Their main role it to provide structural and biochemical support to the surrounding cells and tissue [152, 153]. The tissue ECM composition is variable and supports the biological properties and function, such as cell adhesion, cell-to-cell communication and differentiation. Collagens

(COLs) and laminins (LNs) are examples of fibrillar or network ECM proteins commonly associated with the pancreatic environment [154-156].

There has in recent years been an increase in reports using 3D-scaffolds as a means of further improving the physiological mimicry of the cellular ECM microenvironment, while preserving and/or increasing *in vitro* longevity of islets and prolonging functionality in the form of insulin secretion [157-160]. Scaffolds combined with different pancreatic ECM motifs have also shown additional relevance for islets culture and functionality [161-172]. Taken together, these studies demonstrate the importance of creating a native-like 3D support for *in vitro* culture of viable and functional pancreatic islets, independently of species source. They further highlight the importance of using ECM proteins from the microenvironment of pancreatic islets as tissue-specific factors. **Table 1** highlights the most abundant pancreatic ECM-proteins identified in the literature. It is likely that there are developmentally driven, spatially and temporally coordinated changes to the ECM that contribute to cellular signalling and β cell development. Strategies that reestablish the ECM micro-architecture may further improve stem cell-derived β cells function through formation of a more native-like cellular micro-environment.

ESCs or iPSC-derived differentiation models under adherent culture conditions often use feeder layers [140] or Matrigel® [135, 143], as active coatings to support cell culture [173]. A simplified protocol was reported using Synthemax as an active coating for human ESCs pancreatic differentiation, in order to overcome some of the batch-to-batch variations of Matrigel® [174]. The authors of this study reported a comparable quantity and quality of the derived insulin-producing cells on surfaces coated with either matrix. As a tradeoff between defined culture conditions and biological relevance, it is possible to use coatings of specific ECM proteins.

The relevant ECM niche of the given tissue is thereby represented, notionally with the aim of more robustly driving cells towards the cell types that would normally populate this specific niche. An example of such an approach is the use of fibronectin (FN) and/or LN (isoform not specified) [151] as a matrix to support improved differentiation of MSCs towards insulin-producing cells. The authors observed an increase in proinsulin and insulin protein levels and an increase in insulin release in response to glucose concentration. Human umbilical cord MSCs (UC-MSCs) cultured on LN-411 coated surfaces showed a significant increase in the expression and secretion of insulin. Moreover, transfusion of MSCs differentiated on the different surfaces demonstrated superiority for LN-411 cultured cells in relation to survival of a rat experimental model of T1DM [150].

Table 1 – Summary of ECM proteins observed in pancreatic tissue.

Tissue	ECM		Detection assay	Ref.
	Protein	**Subtype**		
Mice Fetal pancreatic epithelium	LN	α1β1γ1	ICC*	[175]
Human fetal pancreas	FN	N/A	IHC**	[176]
	COL-IV	N/A	IHC	[176]
	VN	N/A	IHC	[176]
Human fetal pancreas	LN	α1, α2, α4, α5	IHC	[156]
Mice pancreas	COL	N/A	SHG***	[177]
Mice pancreas	LN	α2β1γ1 α2β2γ1 α2β1γ3 α5β1γ1 α5β2γ1	IHC	[178]
Mice pancreas	LN	α2β1γ1 α2β2γ1 α4β1γ1 α4β2γ1	IHC	[179]
Mouse pancreas	LL	α1, α2, α3, α4, α5	IHC	[155]
Mouse pancreas	COL-IV	α1, α2	IHC	[155]

N/A not available

* Immunocytochemistry

** Immunohistochemistry

*** Second Harmonic Generation; label-free

LN – Laminin

FN - Fibronectin

VN - Vitronectin

COL - Collagen

In addition to the biochemical cues provided by ECM surfaces, the mechanical properties of nanopatterned surfaces have also been shown to promote human ESCs and iPSCs differentiation into pancreatic progenitors that can give rise to pancreatic endocrine cells [180]. This study points to the fact that differentiation signals may also be triggered by nanotopographical patterns and physical cues of proteins, such as ECM.

2.3.1 Decellularized pancreas: the most biomimetic scaffold

The incorporation of every key (known and as yet unidentified) ECM factor into a biomaterial substrate, to create an ideal platform for differentiation and maintenance of islet-like cells, is a challenge of high complexity. Decellularized scaffolds have been reported as a source of native ECM structures, for applications in regenerative medicine [181]. These ECM scaffolds are prepared by decellularization of mammalian tissues leading to a scaffold composed of natural

biologic cues with preserved 3D-ultrastructure and biochemical composition. A decellularization protocol generally begins with lysis of the cell membrane using physical treatments, followed by separation of cellular components from the ECM using enzymatic treatments, and finally removal of cellular debris from the tissue [182, 183]. The ECM-based materials, either from a xenogeneic or allogeneic origin, elicit a variety of favorable cellular responses that improve stem cell differentiation, maturation and/or functional tissue reconstruction [184-186].

In the pancreatic arena, rat-islets cultured on decellularized pancreatic slices showed a constant GSIS during long-term *in vitro* incubation [187]. Similarly, lyophilized and solubilized decellularized pancreatic matrix, combined with microbeads, enhanced the survival and function of rat pancreatic β-cells, by creating close-packed 3D structures that include cell-to-cell and cell-to-ECM interaction [188]. Adult human liver cells and human MSCs, induced to differentiate into β-like cells, showed increased insulin regulation when encapsulated in hydrogels derived from decellularized pancreas tissue. *In vivo*, the encapsulated cells were shown to be non-immunogenic, and to significantly improve the glycemic control in a diabetic mouse model [189].

Decellularization followed by re-cellularization of whole mouse pancreas has been reported [190]. The authors successfully removed the cellular material, while preserving the 3D-ECM architecture, and then re-cellularized with a β cell line (MIN-6). This resulted in a significant cellular engraftment within the decellularized pancreas, and gave rise to strong up-regulation of insulin expression. Similar results were observed, upon the reintroduction of primary pig islets in whole organ decellularization of pig pancreas [191, 192].

2.4 Clinical translation

Translation into clinical applications for stem cell derived β cells is currently being pioneered through the development of macro-encapsulation devices. Agulnick et al have established that human ESC-derived insulin-producing cells can mature and function *in vivo* in medical devices [193]. Others have characterized the glycemic correction in animal models upon transplantation [194]. In particular, Vegas et al described long-term glycemic correction in a diabetic immunocompetent animal model, using human mature β cells derived from human ESCs. Implants retrieved after 174 days contained viable insulin-producing cells and the human C-peptide concentrations and *in vivo* glucose responsiveness demonstrated a therapeutically relevant glycemic control [195]. Interestingly, a first-in-human phase 1/2 study was recently

initiated to test a stem cell-derived, encapsulated cell replacement therapy (VC-01™, Viacyte) in subjects with T1DM. Information can be found at https://clinicaltrials.gov (Identifier: NCT02939118 and NCT02239354). Further developments in the macro-encapsulation field may include 3D-printing devices that have already demonstrated some suitability for long-term culture of MSC-derived islet-like clusters [196].

Chapter 3: Pancreatic extracellular communication

The body depends on a variety of receptors and endogenous extracellular signaling in order to maintain health and tissue homeostasis. Understanding the signaling mechanisms behind the pancreatic development and regulation can be influential to the development of functional stem cell differentiation protocols. In fact, previous literature suggests that factors released by the developing pancreas may be instrumental in engineering stem cells [197]. In this chapter special attention will be given to paracrine signaling such as extracellular signaling mediated by extracellular vesicles (EVs) and the contribution of angiogenic cells for the regulation and functionality of the pancreatic islets.

3.1 Cell-cell signaling

Cellular phenotype is heavily modulated by the extracellular environment. Cells constantly create and respond to external signals, including soluble secreted factors, direct cell-cell communication, and extracellular matrix–based signals [198]. These external signals, in turn, drive signaling cascades that lead to maintenance or alteration of the cellular phenotype. In animals, cell-cell signaling can be classified into three types: endocrine, paracrine, or autocrine, based on the distance over which the signal acts [199], **Figure 11**.

In endocrine cell-cell communication, signalling molecules, called hormones are transported by the blood, and act on target cells distant from their site of synthesis by cells of endocrine organs. For example the insulin signalling by pancreatic β cells provides glucose control in the target tissues[200]. In paracrine signalling, the signalling molecules released by a cell affects the target cells in close proximity. Many signalling molecules regulating development in multicellular organisms also act at short range, such as neurotransmitters – a classic example of paracrine cell-cell signalling [201]. Nevertheless, paracrine communication via release of specific growth factors and cytokines has been implied in several tissues, like cardiac regeneration and in neovascularization [202]. In autocrine signalling, cells respond to substances that they themselves release. Tumour cells are a typical example of autocrine signalling, since they continuously release autocrine growth factors that stimulate their own growth and proliferation [203].

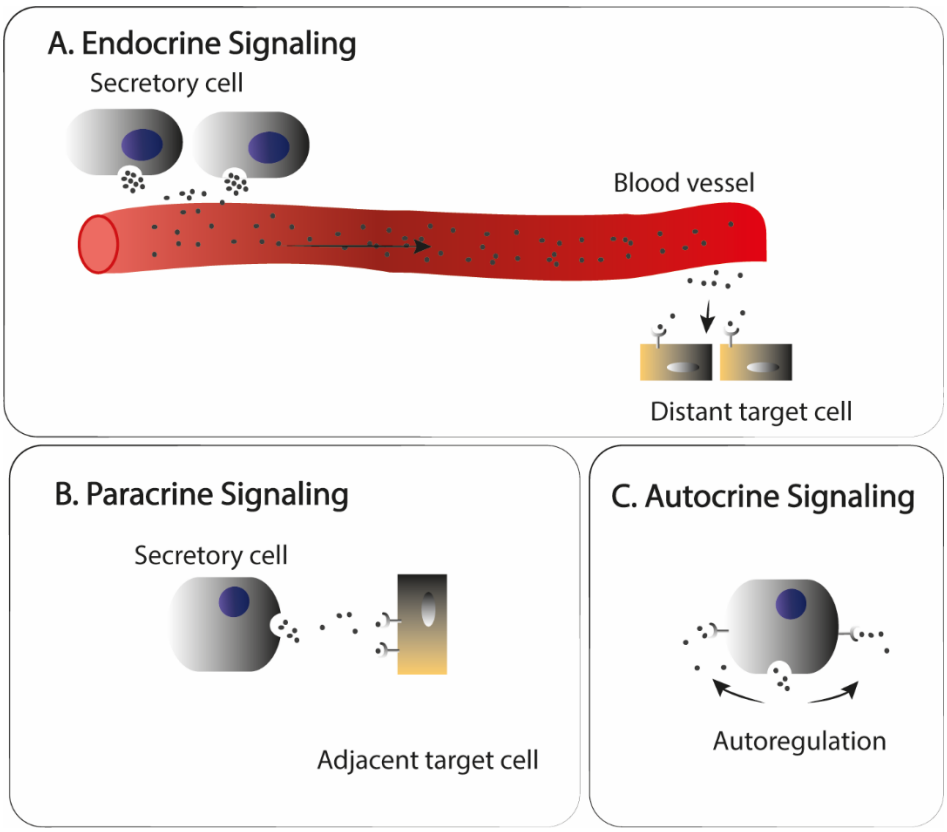


Figure 11. Scheme of intercellular signaling pathways. A) *In endocrine signaling, hormones are carried through the circulatory system to act on distant target cells.* ***B)*** *In paracrine signaling, a molecule released from one cell acts locally to affect nearby target cells.* ***C)*** *In autocrine signaling, a cell produces a signaling molecule to which it also responds.*

Some compounds can act in two or even three types of cell-to-cell signalling, e.g. epidermal growth factor receptor ligands [204]. Often, several types of cell-cell signalling happen simultaneously during the cell life cycle. Autocrine and paracrine regulation of ESCs is known to act on self-renewal and early differentiation (revised here [198]). Intercellular signalling through the TGF-β, Notch, and Hedgehog pathways are crucial for appropriate specification of endocrine and exocrine pancreas cell fates [205]. Moreover, some factors are difficult to exclusively separate into one of the specific class of signalling, such as Ca^{2+}. Ca^{2+} is a very important and versatile messenger and exerts its effects on numerous locations inside (intracellular) and outside (extracellular) the cells [206]. Other type of extracellularly released agents are EVs (described in section 3.2). EVs from macrophages residing in adipose tissue have shown to modulate systemic insulin responses via miRNAs through mechanisms of paracrine or endocrine regulation [207].

3.2 Extracellular Vesicles (EVs)

First described in the 1980s, exosomes are circulating, membrane-bound nanovesicles secreted from the endosomal pathway of cells [208]. Originally these kind of vesicles were considered a discarding mechanism for membrane proteins. However nowadays they are well recognized as cellular messengers. In early 2007, pioneering work by Valadi et al [209] showed that exosomes/EVs, can mediate communication between cells. Nowadays, it is well accepted that transcriptional regulators and secreted RNA molecules, within membrane vesicles, modify the phenotype of target cells by transferring signals capable of altering cell function and/or reprogramming of targeted cells [210, 211].

Generally two classes of EV are often named in the literature: exosomes [intraluminal vesicles (ILVs) when not yet released to the extracellular space] and a broad class of lager vesicles which is often named as ectosomes, shedding vesicles, microparticles and microvesicles (revised here [208, 212, 213], **Figure 12**). This second classed will be called microvesicles from this point on for simplification purposes. Exosomes are vesicles of endosomal origin and are released via multivesicular bodies (MVBs) that fuse with the plasma membrane. These have a characteristic size ranging from 50 to 100 nm, and express key tetraspanin surface proteins such as CD63 and CD81. Tetraspanins are a very important class of transmembrane proteins involved in protein traffic and membrane fusion, and are abundant on various types of endocytic membranes [214]. Microvesicles are derived from the plasma membrane, often have a larger size (150-1000 nm) and do not contain the mentioned tetraspanin proteins [215]. However, nomenclature of released vesicles is often mixed in the literature and the use of the term ‘‘extracellular vesicle’’ (EV) is encouraged as a generic term for all secreted vesicles [216, 217]. Additionally the field still lacks a standardization of the isolation and characterizing techniques of the EV [217, 218], which make it difficult to determine/distinguish the real biogenic population of EVs.

The original and gold standard technique used for the separating and collection of secreted vesicles is differential centrifugation [219]. This technique is also used as a way to define the microvesicle (pellet at ~10,000 x g) and exosome (pellet at ~70,000-100,000 x g) as separate classes of secreted vesicle [219]. However, different exosome (or EV) isolation kits (miRCURY, ExoQuick, and Invitrogen Total Exosome Isolation Reagent), have emerged over time as faster and less labored techniques, while showing the similar EV properties as the differential ultracentrifugation method [220]. These kit-based techniques rely on polymer-based precipitation or immunocapture by antibody-coated beads. Nevertheless, all the available

isolation techniques have pros and cons. Often, different vesicles of similar size as well as protein aggregates can cosediment or precipitate in the same preparations, and some methods lead to an artificial enrichment of EV subtypes. Steps for standardization have been made with coordination of International Society for ExtracellularVesicles [221].

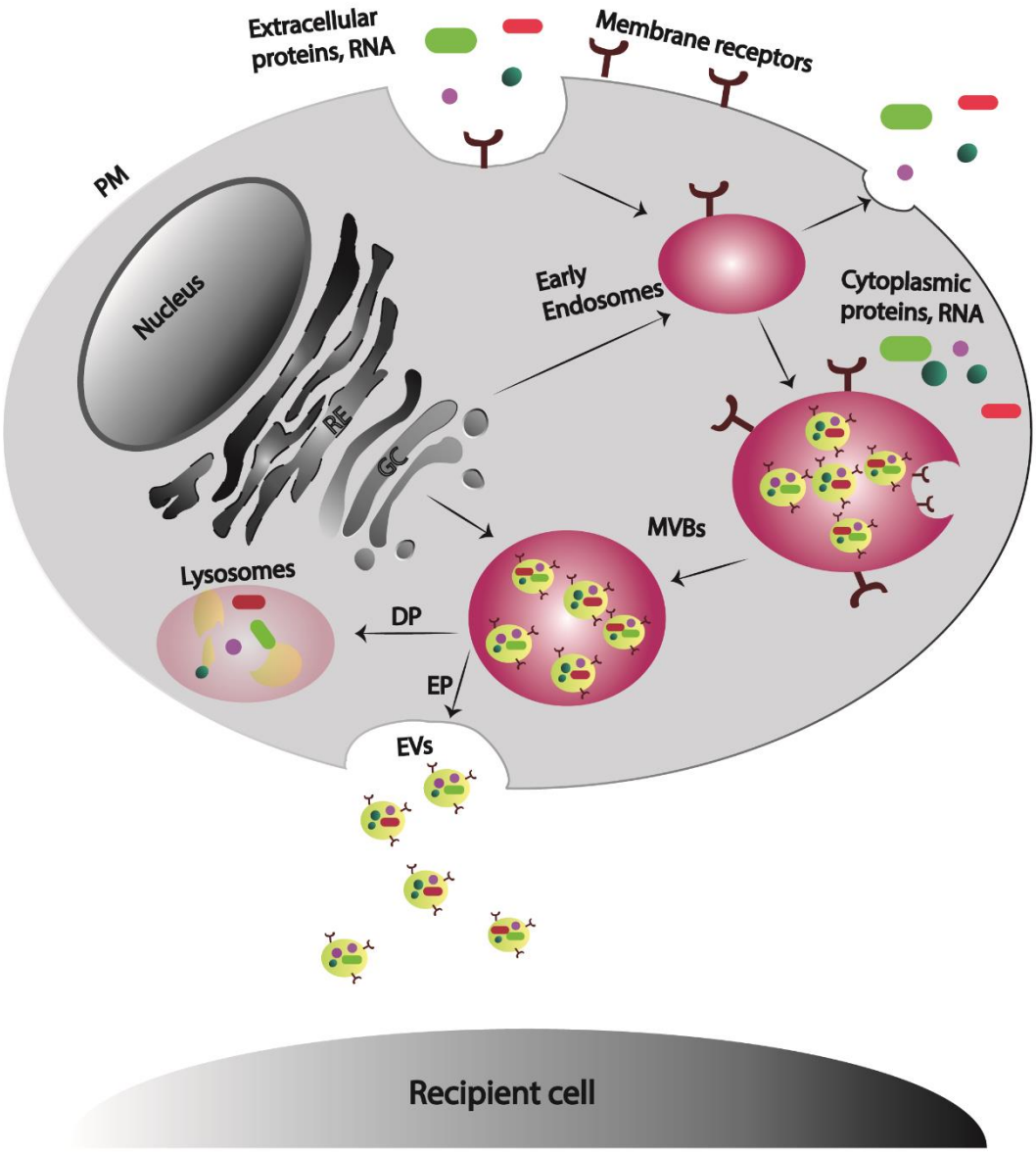


Figure 12. Pathways involving EV formation and secretion. *Various types of cargo, e.g., proteins, RNA, is taken up by the cell via endocytosis (receptor-mediated) and formation of early endosomes. In early endosomes, proteins are either recycled to the PM or sequestered into the intraluminal vesicles of MVBs. Formation of EVs starts with inward budding of the early endosome's membrane and subsequent formation of MVBs. In the exocytic pathway (EP), MVBs fuse with the PM to release their contents (EVs) into the extracellular space; In the degradative pathways (DP), signaling molecules and proteins are restricted and lead to degration via lysosomes. Exosomal cargo delivery to the recipient cell can occur through various mechanisms, i.e., direct fusion with the recipient cell's membrane, pinocytosis/phagocytosis, or ligand–receptor binding. Adapted from [222].*

In spite of above described limitations with respect to isolation and identification, in general all EVs are loaded with various proteins, lipids and RNA (**Figure 13**, revised here [212, 222, 223]). Specific classes of RNAs have been found present in EVs, both coding RNAs, such as messenger RNAs and noncoding RNAs, such as long noncoding RNAs, microRNAs, and circular RNAs [224].Extensive research has been carried out to characterize the content of different populations of EVs, although, the exact composition of each EV cargo might differ depending on the cell of origin. This has resulted in the generation of different databases of open access such as ExoCarta ([225-228], Available online: http://www.exocarta.org), Vesiclepedia ([229], Available online: http://microvesicles.org) and EVpedia ([230], Available online: http://student4.postech.ac.kr/evpedia2_xe/xe/).

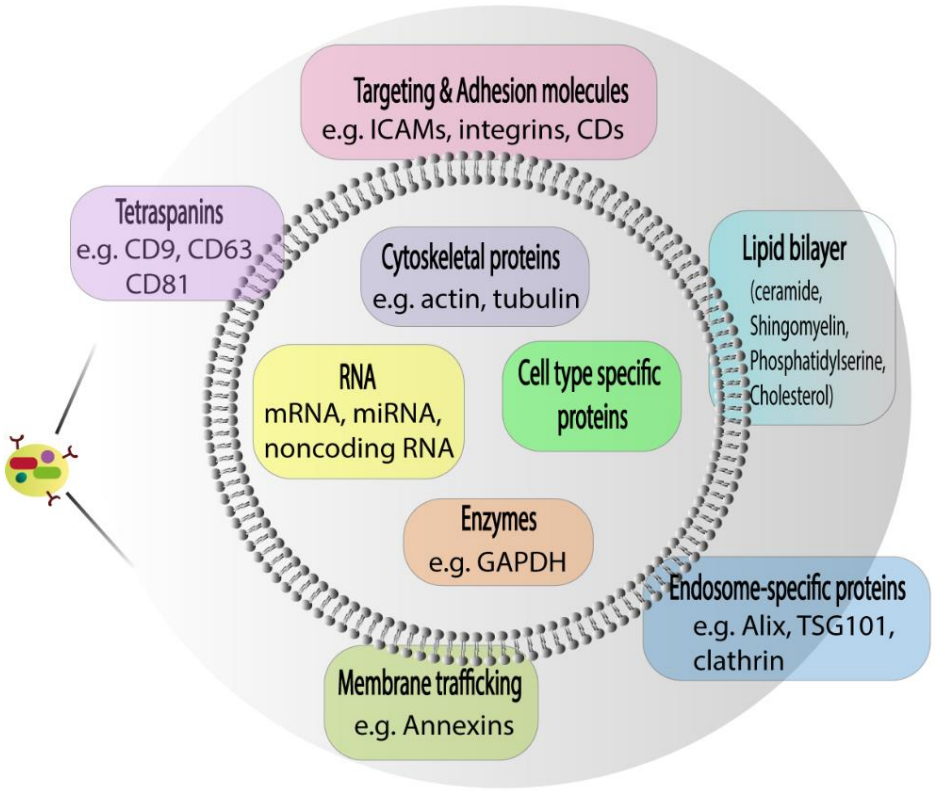


Figure 13. Schematic representation of the composition EVs. *Exosomal luminal cargo predominantly consists of mRNA, miRNA and gDNA fragments, and several different proteins depending on the cell of origin. Generally, proteins involved in MVB formation, tetraspanins, membrane transport and fusion, transmembrane proteins, cytoskeletal components and proteins of cytosolic origin are part of exosomes are also found in the EVs. Note that each listed component may in fact be present in some subtypes of EVs and not in others. Adapted from [212].*

3.2.1 Implications on amyloidogenic disorders

The link between EVs and amyloid diseases are largely unexplored, but some studies have suggested that EVs play a role in Parkinson's and Alzheimer's diseases. EVs have been demonstrated to transport amyloidogenic proteins and amyloid fiber seeds or oligomers from cell to cell, thus potentially providing a prion-like disease spreading [231-233]. In particular, exosomes have been show to spread throughout different regions of the brain tissue and clustered around amyloid-β (Aβ) plaques in an Alzheimer's disease animal model [234]. Others showed that exosomes secreted into mouse brain extracellular space accumulate Aβ precursor proteins [235]. Exosomes from a neuron-derived population can also drive conformational changes in Aβ to form nontoxic aggregates and promoted uptake and clearance via microglia cells [236]. It was reported that cerebrospinal fluid exosomes from patients with Parkinson's disease contained α-synuclein species that readily induced aggregation of α-synculein in a reporter cell line [237]. EVs isolated from neuroblastoma cells reduced the lag time of α-synuclein aggregation *in vitro*, via a process that depended on certain ganglioside lipids in the neuronal exosomes [238]. However, investigations of the putative effects on IAPP amyloid formation by EVs secreted from pancreatic islets in the context of T2DM are lacking. Since IAPP amyloid formation can be affected by synthetic lipid vesicles *in vitro* [63, 239-241] one may predict that also EVs may have effects. In fact, the medium used to culture rat insulinoma INS-1 cells was shown to suppress IAPP fibril formation.

3.2.2 Implications on stem cell differentiation

Pancreatic differentiation has often focused on optimization of stepwise cocktails that are thought to mimic developmental stages of human pancreatic β cells. Yet, deciphering the key factors necessary to drive the differentiation events in a temporal and synergistic way might not be easily reproduced *in vitro*. Understanding the role of pancreatic EVs in pancreas development and homeostasis might lead to the development of new tools for stem cell differentiation protocols.

EVs produced by pancreatic β cells have been found to affect β cell functionality, β cell autoimmunity and overall development of INS resistance, all of which have implications in development of T1DM and/or T2DM [242]. Microvesicles isolated from pancreatic β cell lines were found to secrete microRNAs (miRNA) that can be transferred to neighbouring β cells, and induce apoptotic signals [243]. The profile of the miRNAs in these vesicles changed when the

donor cells were exposed to pathophysiological conditions commonly associated with diabetes. Others have reported an up-regulation of TNF receptors in microvesicles released from β cell lines after cytokine stimulation [244]. β cells from nondiabetic mice can also transfer their secretory cargo to phagocytes, via a process susceptible to glucose concentration and the presence of intracellular Ca^{2+} [245]. Likewise, human islets release biologically active EVs which are able to shuttle specific mRNAs and miRNAs into human islet endothelial cells, inducing INS mRNA over-expression, protection from apoptosis and enhancement of angiogenesis [246].

Other sources of EVs also affect pancreatic islets or contribute to the development of diabetes and/or other diseases related to the pancreas. Pancreatic cancer-derived EVs have been implicated in the development of autoimmunity [247], β cell dysfunction [248] and overall pancreatic cancer communication and biology [249]. MSCs exosomes, isolated from human BMSCs, improved islet transplantation in a mouse model, by inhibiting immune rejection [250]. Adipose-derived EVs are thought to interplay in the communication that drives the development of cardiovascular and metabolic diseases, in particular obesity [251] and insulin sensivity [207]. In line with previous findings, *in vivo* administration of EVs from high fat diet (HFD)-fed mice induced insulin resistance and glucose intolerance compared to EVs from regular diet fed mice [252]. Detailed characterization of gut microbe-derived EVs revealed the dominance of *Pseudomonas panacis* EV in the HFD-fed mice. Exosomes released form skeletal muscle of mice were shown to be taken up *in vivo* by pancreatic β cells and deliver functional cargo [253]. Briefly, exosomes isolated from the muscle of mice fed with a palmitate enriched diet affected islets size *in vivo*, as well as the expression of genes involved in development such as Ptch1. *In vitro*, an induced specific transcriptional signature in MIN6B1 cells was reported. These data suggested that muscle exosomes might have an endocrine effect and could contribute to the regulation of β cell mass during insulin resistance.

Stem cells, in particular MSCs, have also been show to release and to be affected by exosomes from other cellular sources. MSCs treated with exosomes derived from a neuronal progenitor cell, developed neuron-like morphology, and gene and protein expressions of neuronal markers were upregulated. The authors correlated the differentiation enhancement with an exosomal delivery of miRNAs [254]. MSC-derived exosomes can elicit hepato-protective effects against toxin-induced injury in a mouse model [255]. Similarly, EVs derived from a murine pancreatic β-cell line (MIN6) combined with a Matrigel platform containing bone marrow cells, elicited a long-term control of glucose levels over 60 days in diabetic immunocompromised mice [256].

3.3 Endothelial signaling

The endocrine tissue of the pancreas has a dense microvascular structure [257, 258]. In fact, β cells are not more than one cell away from arterial blood, due to a polarized organization of the cells towards a capillary [259]. The microvasculature structure is known to be tightly regulated by the expression of vascular endothelial growth factor (VEGF)-A, which is indispensable for the formation of this structure in β cells [260]. Moreover, the islet capillaries are highly permeable, and the endothelial cells have about 10 times more fenestrations than those of the exocrine tissue [258, 261]. The islet endothelial cells function not only as a cellular barrier, but also produce a number of vasoactive substances, angiogenic substances and growth factors [262], and thereby can play a critical role in the development of diabetes. In fact, the islet vascular system is essential for normal insulin secretion into the blood stream [257, 260-262], and also in the regain of islet function following transplantation [263, 264].

The cross-talk between β cells and endothelial cells has been shown to be mediated by paracrine action, including EVs. EVs released from the endothelial cells enhanced human islet revascularization transplanted in a mice model, alongside with insulin secretion and reduced islet apoptosis [265]. Reversely, islet-derived EVs have been shown to induce a pro-angiogenic effect on human islet endothelial cells [246].

The need to promote the development of microvasculature in *in vitro* cell systems, by co-culture with endothelial cells, remains unexplored in the context of pancreatic 3D-models. However, it was shown that mice islets transplanted using pro-vascularization hydrogels had superior glycemic function in diabetic animals when compared to islets delivered intrahepatically [266].

Chapter 4: Original work

In this section of the thesis, I will summarize and discuss the experimental work performed over four years of PhD studies. The results are organized into two main topics: 1) IAPP aggregation and *in vitro* cytotoxic effects on human islets, and 2) stem cells pancreatic differentiation.

4 *In vitro* models to study IAPP and relation to T2DM

4.1.1 Pancreatic EVs constrain IAPP aggregation

Protein assembly into amyloid fibrils underlies neuro-degenerative disorders such as Alzheimer's and Parkinson's disease. T2DM also involves amyloid formation, although in the pancreas. Because there are no cures for amyloid diseases and T2DM is on the rise due to an increase in prevalence of obesity, identifying involved mechanisms and control processes is of utmost importance. EVs can mediate physiological and pathological communication both locally and at a distance, although their roles in metabolic diseases have not been well explored. To begin to address this question, we isolated and characterized EVs from pancreatic islets of healthy patients and patients with T2DM, and investigated the effects of these EVs on IAPP amyloid formation *in vitro* (**Paper I**). In summary, we found the EVs from healthy islets to have approximately fivefold less insulin than C-Peptide, whereas for T2DM EVs and serum EVs, the amounts of insulin and C-Peptide were roughly equal. The absolute amount of C-Peptide was lower in T2DM and serum-derived EVs than in EVs from healthy islets, while IAPP amounts were similar in all three EV types. Moreover, a comparison of the lipid contents in the three different EV samples showed different lipid profiles in serum EVs and pancreatic EVs. No significant differences between healthy and T2DM pancreatic EVs were found. The aggregation assay, monitored by the ThT dye, showed a significant retardation of amyloid formation only when the healthy EVs were incubated with IAPP, but not T2DM or serum-derived EVs. The inhibition mechanism appears to result from the interaction between IAPP–healthy EV. Using size exclusion chromatography and atomic force microscopy (**Figure 14**), we observed the formation of lager EV-IAPP particles that can be explained either by IAPP-induced fusion of several EVs or by surface binding of many IAPP peptides per EV.

4.1.2 IAPP supplementation affects human islets viability

IAPP induced toxicity in pancreatic β cells is often related to T2DM disease development in the literature. Human IAPP added externally to immortalized β cell line cultures has shown in other experiments to become internalized and to kill β cells along with internal amyloid formation [95, 101]. To further address this effect in a human *in vitro* cell model, we performed

a series of studies where exogenous IAPP or IAPP+EVs were added to cultures of human islets and viability and β-cell function were measured.

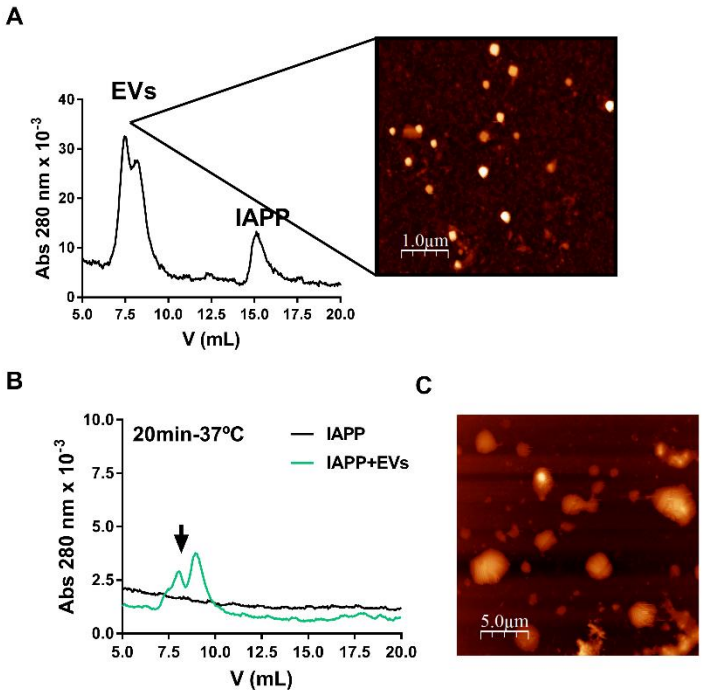


Figure 14. Assessment of IAPP interaction with healthy pancreatic EVs by SEC and AFM. *A) SEC of a mixture of IAPP and EVs without incubation (elutes as monomeric IAPP and EVs in the void), with AFM imaging of the EVs at the indicated fraction. B) SEC of IAPP (which forms amyloids and is filtered away before SEC injection) and a mixture of IAPP and EVs preincubated for 20 min at 37 °C before SEC analysis. The eluate contains no monomeric IAPP; instead, absorption of EVs in void is decreased, which may be explained by the formation of larger particles that are partially filtered away before SEC. The arrow points to reduced absorption of EVs after incubation with IAPP. C) AFM image of an incubated mixture of IAPP and EVs (larger particles than in the AFM image in A). Adapted from [267].*

Considering the results described in **Paper I**, we aimed to test the effect of healthy pancreatic EVs on the potential IAPP-mediated toxic processes. For this purpose, human pancreatic islets were incubated with IAPP or IAPP+EVs solutions. In our conditions, we observed no toxic effects of IAPP addition to human islets, as probed by apoptosis assay after 5h of incubation (**Figure 15A**). GSIS assay after 72h of incubation (**Figure 15B**) was also not disturbed by the addition of IAPP alone or pre-incubated mixtures of IAPP and EVs.

Nonetheless, performing these experiments in the presence of ThT allowed us to visualize IAPP amyloids in the islet cultures (**Figure 16A**). By analysis of ThT mean intensity per image, we observed that IAPP added alone showed a significant higher ThT intensity than upon addition

of IAPP pre-incubated with EVs, in agreement with the *in vitro* result of EV-mediated amyloid inhibition in aggregation assays (**Paper 1**).

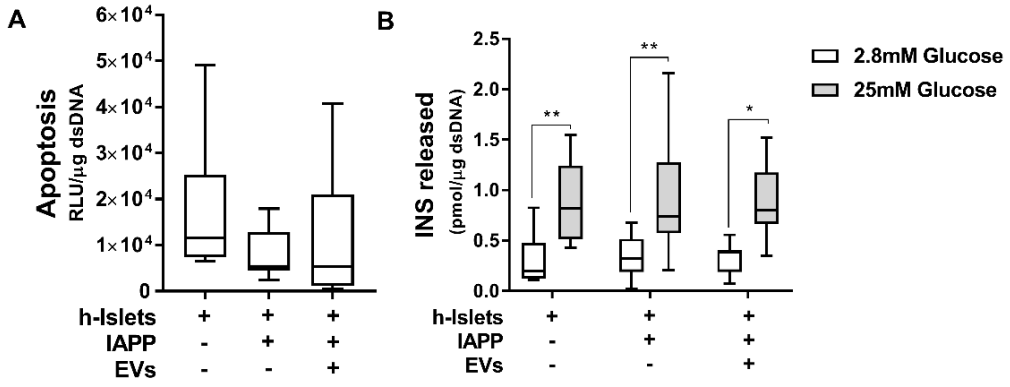


Figure 15. Human pancreatic islets culture in the presence of IAPP and IAPP+EVs. *A) Apoptosis (relative luminescence units – RLU per µg of dsDNA). B) GSIS assay measured using an Insulin (INS) ELISA and normalized for the total µg of dsDNA content, at low (2.8 mM) and high (25 mM) glucose challenge. * represent statistical significance, and corresponding p-value description is the following: *<0.05, **<0.01.*

Human islets incubated only with media containing ThT showed significantly lower fluorescence as compared to IAPP and IAPP+EVs conditions (**Figure 16B**). However, this assay does not allow for conclusions about the internalization of IAPP by the islet cells, since it is not possible to discriminate if the fluorescent IAPP conjugates are present on the cells surface or internally.

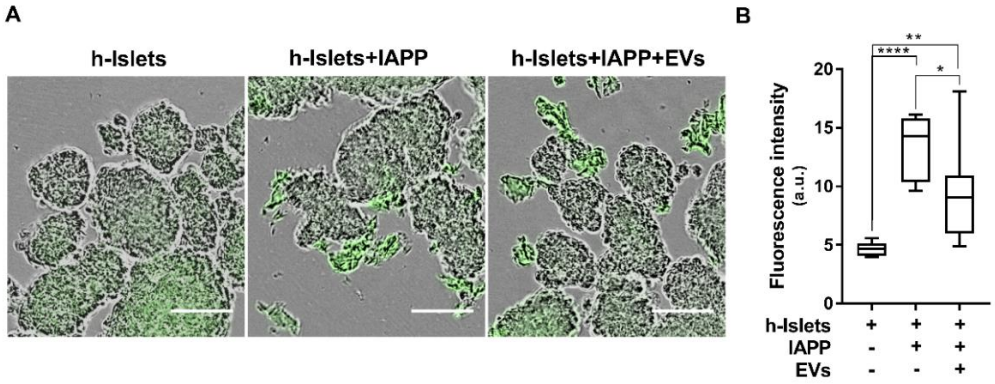


Figure 16. Imaging of human pancreatic islets culture in the presence of IAPP and IAPP+EVs. *A) Imaging of pancreatic islets (bright field) incubated with ThT dye (green fluorescence) and IAPP conditions. Bar = 100 µm. B) Automatic quantification of ThT signal measured by the mean fluorescence intensity, performed in an IncuCyte® ZOOM instrument. * represent statistical significance, and corresponding p-value description is the following: *<0.05, **<0.01 and ****<0.0001.*

Next, I aimed to assess if IAPP internalization and cytotoxic effects require islets cell cluster structure dissociation followed reassembly, in order to expose cells surface within the islet cluster structure. Furthermore, the dissociation of primary rat and human donor islets followed by controlled cellular reassembly has shown to result in islet microtissues with an average 2.5 to 4-fold GSIS, as compared with freshly isolated islets [268].

Human islet cells were dissociated into single cells and seeded in AggreWell™400 plates with or without different concentrations of IAPP in the culture media (freshly prepared). The cultures were left for 5 days without any further manipulation to induce reassembly. At day 5 samples were collected to assess cells viability and phenotype.

IAPP exogenous supplementation to islet microtissues induced significant cytotoxic changes, as observed in **Figure 17A**. Quantification of the fluorescence signal for the cell viability dye (calcein-AM, green signal) did not show significant decreases comparing the IAPP stimulated conditions to control, without IAPP stimuli (**Figure 17B**). The fluorescence signal for the dead dye (ethidium homodimer-1, red) showed a significant increase associated with increased IAPP concentration supplemented (10 µM, **Figure 17C**).

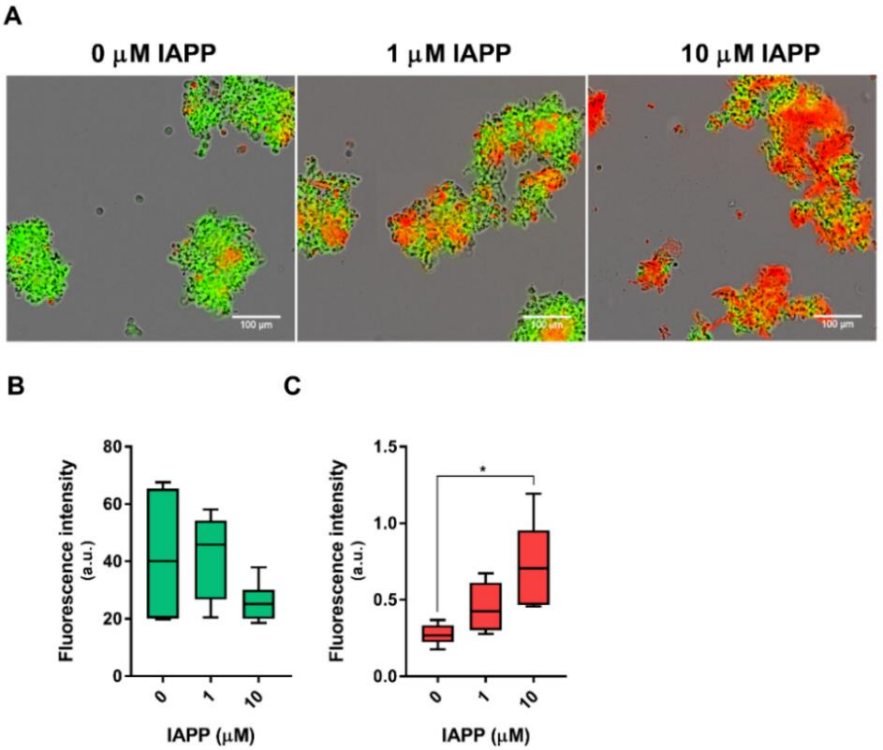


Figure 17. Islet microtissues dual color LIVE/DEAD Cell Viability/Cytotoxicity Assay after IAPP supplementation. *A) live/dead imaging using an IncuCyte® ZOOM instrument, and automatic quantification of green (live, B) and red (dead, C) signal using mean fluorescence intensity per well. * represent statistical significance and p-value<0.05.*

Gene (**Figure 18**) and protein (**Figure 19**) analysis were performed on the same groups, and compared to freshly isolated human islets. mRNA expression analysis of several pancreatic hormone markers and glucose transporters revealed an overall decreased in expression when comparing fresh samples and microtissues, with the exception of mRNA for *IAPP* which remained unaltered (**Figure 18**). mRNA for the main pancreatic hormones, insulin (*INS*), glucagon (*GCG*), somatostatin (*SOM*) and *IAPP* were not affected by IAPP supplementation during islet cells reassembly into microtissues. The expression of the *GLUT1* gene however, increased significantly in microtissues supplemented with the 10 µM of IAPP as compared to control (0 µM of IAPP).

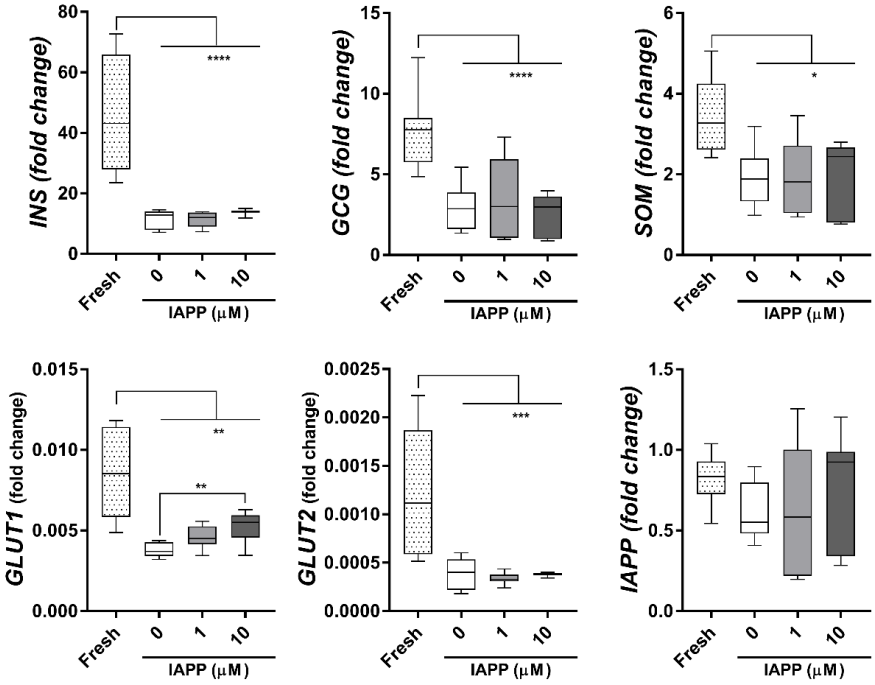


Figure 18. Gene expression profile of islet microtissues exposed to IAPP. *mRNA levels of pancreatic hormone markers (INS, GCG, SOM and IAPP) and glucose transporters (GLUT1 and GLUT2) in fresh isolated islets and islets microtissues with and without IAPP stimuli. Fold change normalized against mRNA expression of the GAPDH housekeeping gene. * represent statistical significance, and corresponding p-value description is the following: *<0.05, **<0.01, ***<0.001 and ****<0.0001.*

Total hormonal protein content of Insulin, C-Peptide and IAPP was measured by ELISA and normalized against the total dsDNA content (**Figure 19**). Islet reassembly protocol induced a significant decrease in the total content of Insulin as observed on **Figure 19B**, although this change was mostly observed in the non-IAPP supplemented condition or low IAPP

concentration (1 µM). C-Peptide and IAPP remained unchanged independently of the condition (**Figure 19A&C**).

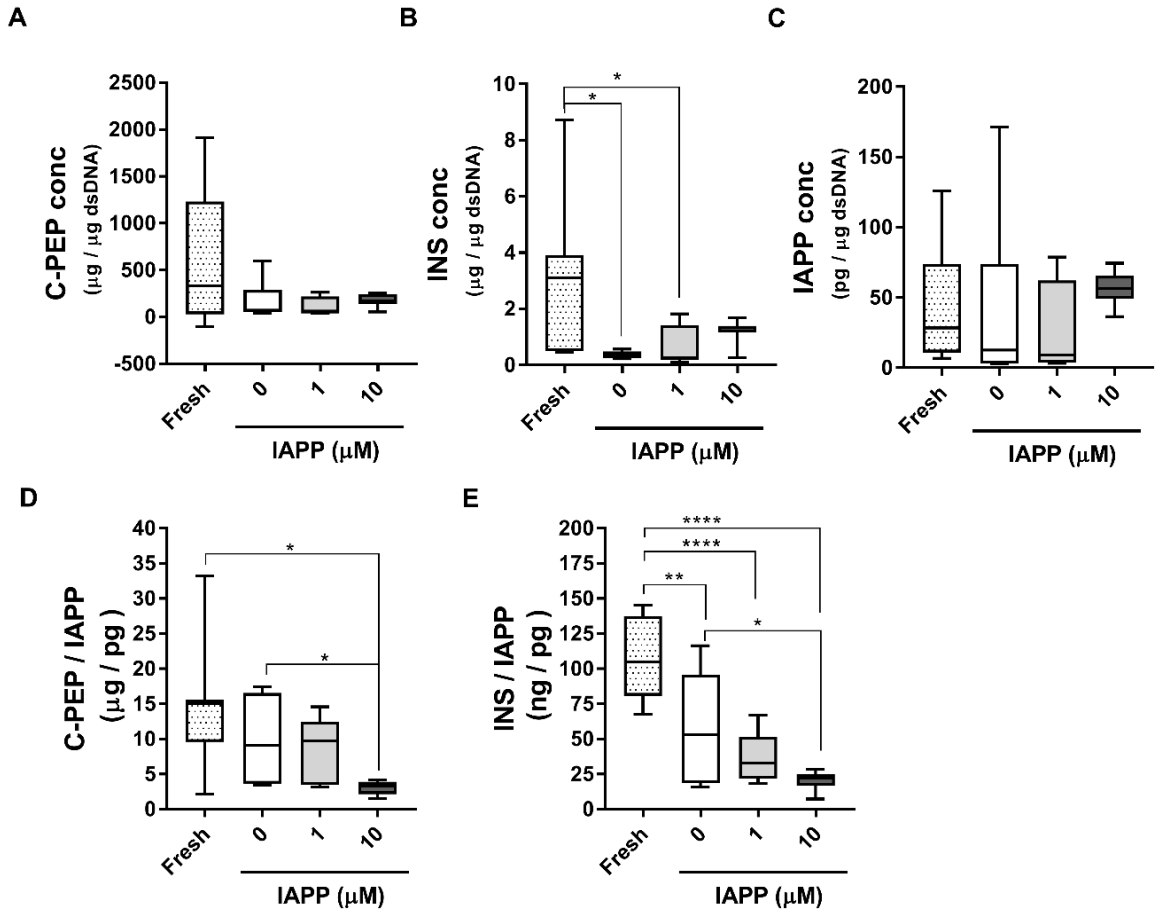


Figure 19. Hormone protein content of islet microtissues exposed to IAPP. *A) C-Peptide total content. B) Insulin total content. C) IAPP total content. All hormones quantification was normalized against dsDNA concentration. Ratio of C-Peptide/IAPP (D) and Insulin/IAPP (E). * represent statistical significance, and corresponding p-value description is the following: *<0.05, **<0.01 and ****<0.0001.*

It is often suggested in the literature that changes in the ratio of Insulin/IAPP can be a sign of metabolic phenotype changes and a relation with T2DM is often associated with increase in IAPP intracellular levels [55]. In **Figure 19D&E** we compared the ratio of C-Peptide/IAPP and Insulin/IAPP, and one can observe a significant decrease in both ratios C-Peptide/IAPP and Insulin/IAPP at the higher concentration of IAPP supplementation (10µM). This data suggests that intracellular accumulation of IAPP leads to a downregulation of insulin synthesis, and further characterization of this conditions could highlight the mechanism of IAPP accumulation and toxicity in T2DM patients. As a disclosure note, islet microtissues were washed several

times in PBS before lysed in H_2O, in order to assess only the intracellular levels of hormones and not those released or supplemented externally.

In section 4.1.1 we showed that EVs reduce IAPP aggregation in solution. In this section we aim to assess if pancreatic EVs from healthy donors can also reduce cytotoxic effects associated with IAPP accumulation in human islets. Similar to results described in **Figure 17**, islet microtissue viability and cytotoxicity was evaluated when exposed to IAPP and/or EVs (**Figure 20**).

Imaging of islets microtissue dual LIVE/DEAD assay showed that EVs did not induce an increase in the red signal (cytotoxicity), while all IAPP conditions induced intense red stain (**Figure 20A**). Automatic quantification of the LIVE/DEAD mean fluorescence stain showed an unclear message, the quantification of the green stain (live; **Figure 20B**) increased by the addition of IAPP and IAPP-EVs contradicting the fluorescence images and the quantification of the red stain (dead, **Figure 20C**). The quantification of the dead stain shown the same trend as presented in the images in **Figure 20A**, all IAPP conditions induced significant cytotoxic effects on islet microtissues. Nevertheless, the apoptosis assay showed that only IAPP supplementation induced a significant increase in the apoptosis levels but not IAPP-EVs, as compared to control (islet microtissues). This result might suggest that IAPP induces islets toxicity mediated by an apoptotic mechanism, which is reduced in the presence of IAPP incubated with healthy EVs.

We next assessed changes in β cell phenotype and functionality (**Figure 21**). IAPP supplementation induced a significant downregulation of *INS* mRNA expression (**Figure 21A**), although this significance was not observed in **Figure 18** with similar IAPP stimulus (10μM). Expression of *IAPP* mRNA remained unchanged independently of the stimuli (**Figure 21B**), although a decreased trend was observed when IAPP was supplemented exogenously. Overall IAPP and/or EVs did not induce changes in the GSIS profile of the islet microtissues (**Figure 21C**). Furthermore, islet reassembly reduced islet functionality, since microtissues no longer responded to an increase in glucose changeling which was observed in fresh islets (**Figure 15B**). Microtissues generated here only showed a significant GSIS in the presence of KCL, contradicting the GSIS results reported in the literature describing islet microtissues functionality [268].

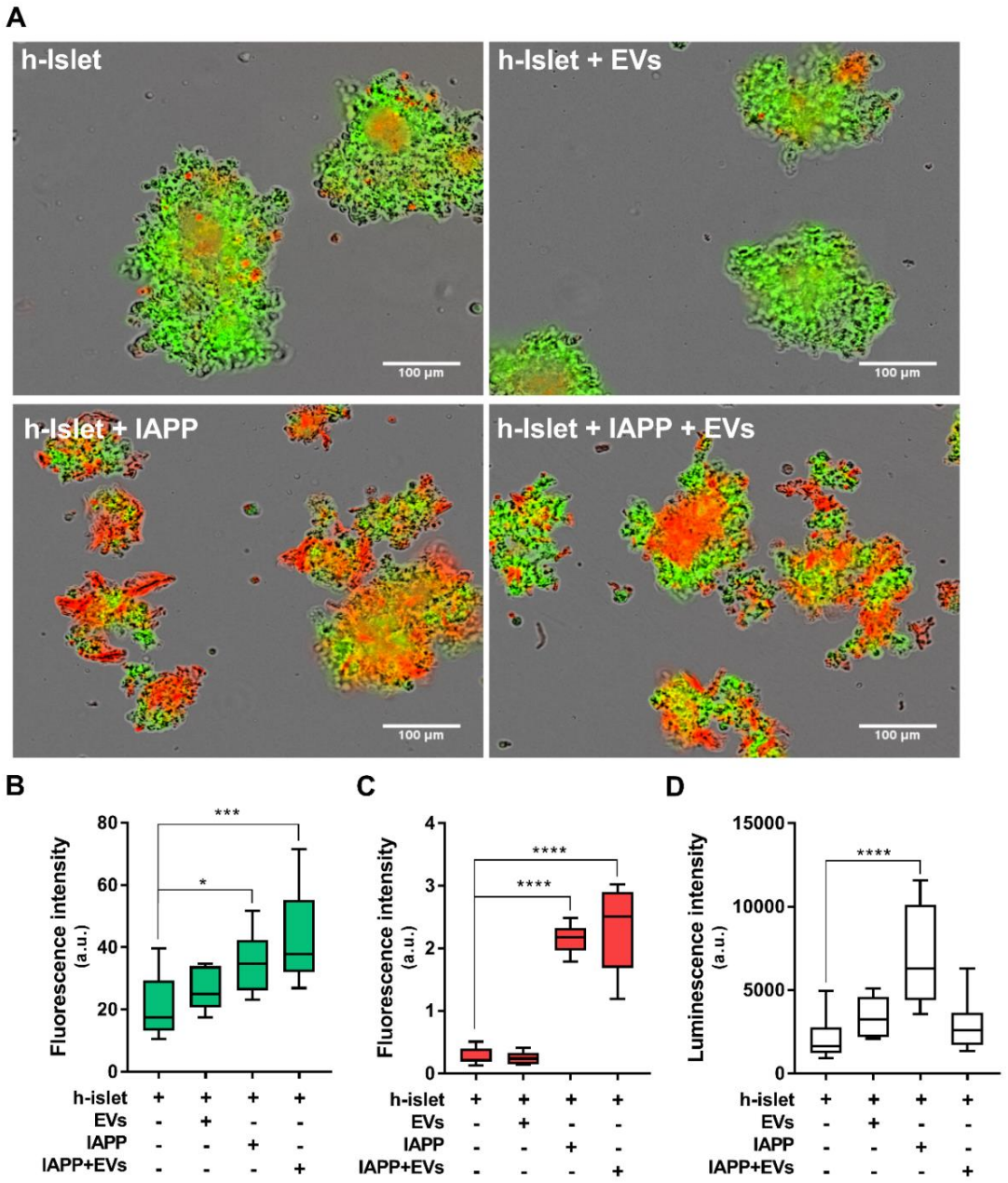


Figure 20. Islet microtissue dual color LIVE/DEAD Cell Viability/Cytotoxicity Assay after IAPP and/or EVs supplementation. *A) live/dead imaging using an IncuCyte® ZOOM instrument, and automatic quantification of green (live, B) and red (dead, C) signal using the mean fluorescence intensity per well. D) Apoptosis assay (luminescence units). * represent statistical significance, and corresponding p-value description is the following: *<0.05, ***<0.001 and ****<0.0001.*

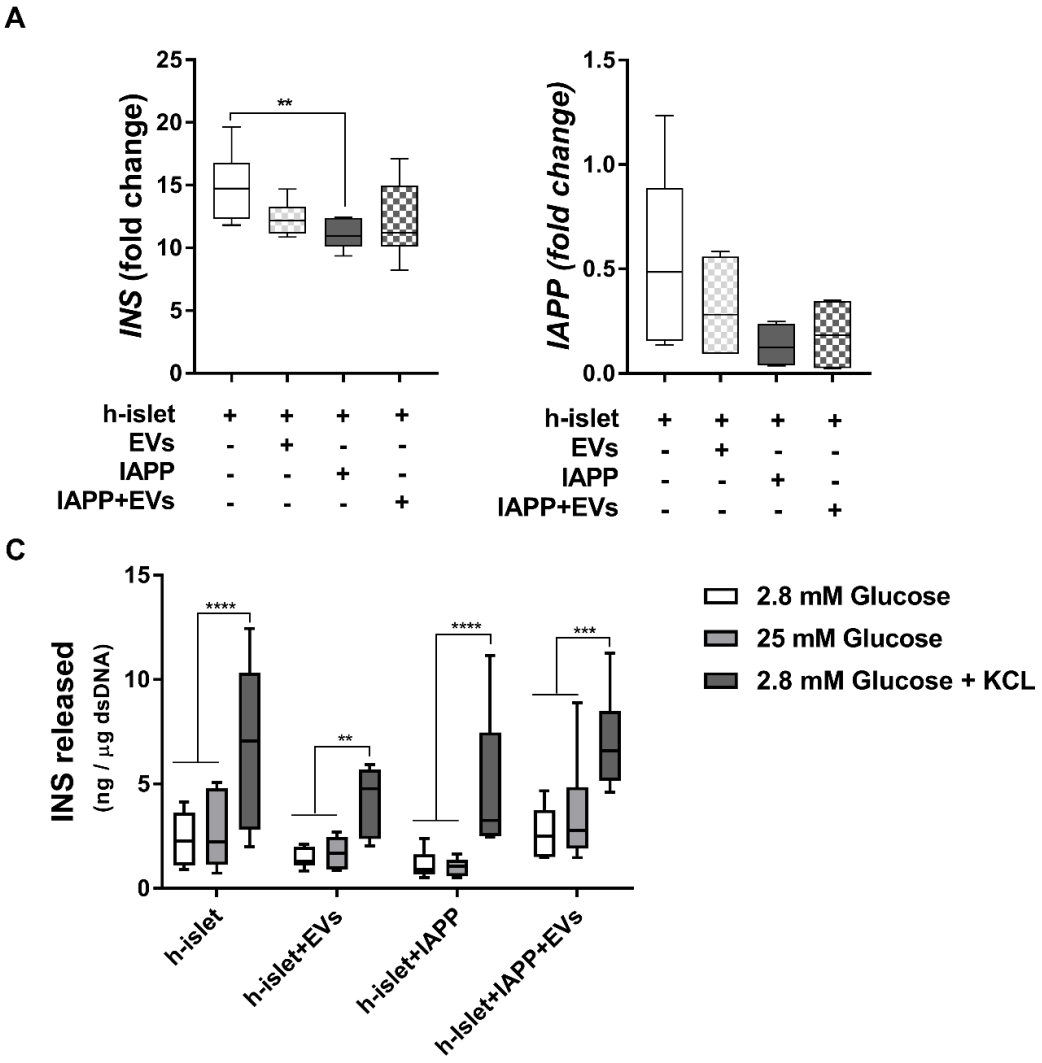


Figure 21. Gene and functional analysis islet microtissues after IAPP and/or EVs supplementation. *mRNA expression for INS gene (A) and IAPP gene (B). GSIS at low glucose (2.8 mM), high (2.8mM) and in the presence of KCL (3mM). * represent statistical significance, and corresponding p-value description is the following: **<0.01, ***<0.001 and ****<0.0001.*

4.2 Stem cell-derived pancreatic models

4.2.1 iPSCs differentiation towards β-cells

Common differentiation protocols use complex and sequential cocktails of growth factors and/or small molecules to direct endocrine differentiation of stem cells [133, 134, 140]. Recently, differentiation protocols have been used in combination with stem cell clusters cultured in suspension [143-146]. These clusters resemble islet 3D-architecture and show increased functional insulin release properties *in vitro* [193-195]. Despite increased efforts in the development of complex *in vitro* 3D-stem cell culture systems, previously mentioned differentiation protocols may not provide the dosage, temporal and synergistic signals that constitute the pancreatic islet *in vivo* niche.

In this section we will assess the effect of pancreatic extracellular communication on iPSCs differentiating *in vitro* towards pancreatic β cells, by the delivery of pancreatic EVs, and use of pancreatic ECM bioscaffolds composed of collagen or rat pancreas decellularized tissue.

It has been suggested that EVs can mediate crosstalk between hormones and metabolites within the pancreatic tissue. However, the possible effect of pancreatic EVs on stem cell differentiation into pancreatic lineages remains unknown. Here, we test the hypothesis that EVs isolated from human pancreatic islets can deliver RNA and/or proteins to iPS-derived differentiating cells, and influence the outcome of the differentiation towards insulin producing cells (**Paper II**).
Briefly, I found that the expression of key pancreatic transcription factor mRNA, such as NGN3, MAFA and PDX1, and pancreatic hormone proteins such as C-peptide and glucagon, were confirmed in h-Islet-EVs. Furthermore, h-Islet-EVs, supplemented to the iPSC clusters in the later stages of differentiation, led to an up-regulation of the intracellular levels of C-peptide in iPSC clusters in a concentration-dependent manner (**Figure 22**). However, h-Islet-EVs delivered to iPSC clusters differentiating in 3D-collagen hydrogels did not affect the insulin or C-peptide intracellular content.

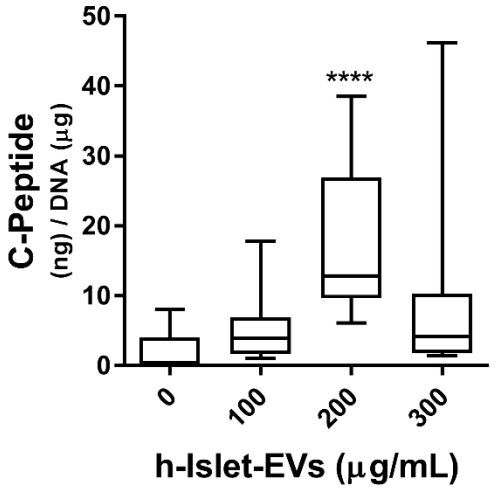


Figure 22. Dosage effect of h-islet-EV supplementation in iPSC clusters differentiated in suspension. *Quantification of C-peptide by ELISA, normalized by total DNA content. ***<0.001 as compared with 0 µg/mL condition. Adapted from [267].*

The next step aimed to assess the contribution of the 3D co-culture with HUVECs on iPSCs pancreatic differentiation.

The incorporation of every key (known and as yet unidentified) ECM factor into a biomaterial substrate, to create an ideal platform for differentiation and maintenance of islet-like cells, is a challenge of high complexity. Decellularized scaffolds have been reported as a source of native ECM structures, and their usefulness to increase pancreatic islets and cells functionality *in vitro* has been reported [187, 188, 191, 192, 269]. Furthermore, the incorporation of more complex models by co-culture with endothelial cells remains poorly explored in the context of pancreatic 3D-models, in particular regarding potential synergistic effects on stem cell differentiation. Here, we assess the effect of 3D-culture with pancreatic decellularized tissue in combination with HUVECs co-culture and their effects on iPSCs pancreatic differentiation (**Paper III**).

In summary, an NKX6.1-GFP-iPSC reporter line was used to monitor the pancreatic progenitor commitment over time in live cells. NKX6.1 expression was observed by day 16 and significantly increased by day 22. The differentiated cells, by the end of the differentiation protocol, expressed insulin at gene and protein level, as well as other pancreatic transcription factors and hormones. However, these differentiated cells did not exhibited a glucose responsiveness *in vitro*. The glucose responsiveness profile remained unaltered by 3D-culture in Matrigel^{TM} coatings or decellularized scaffolds, or by pre-vascularization with stimulated HUVECs on the previously mentioned 3D-cultures.

Overall the results reported herein show that the iPSC differentiation protocol provided a temporal control over the generation of pancreatic progenitor and insulin-producing cells, yet the establishment of β-like cells that exhibit glucose responsiveness *in vitro* remains unaccomplished. It would be recommended to repeat the experiments assessing the effect of both EVs and 3D-culutre differentiation in differentiated stem cells with functional properties.

4.2.2 ADSCs differentiation towards pancreatic progenitors

The interest in developing 3D-physiologically relevant assay platforms is increasing. In fact, 3D-strategies have been used to increase the functional relevance of ex *vivo* human primary pancreatic islets and the generation of stem cell-derived pancreatic β cells (Reviewed in **Paper V**). *In vitro* pancreatic islet differentiation is typically attempted by adding complex media cocktails to stem cells [145, 146] without taking into account the effect of the 3D pancreatic microenvironment. The re-establishment of the 3D-environment within *in vitro* differentiation protocols may be vital to deciphering the mechanism of endocrine pancreatic cell differentiation, while providing cellular support and expose cells to key tissue-specific ECM proteins. It is known that human islets are involved in double-layered basement membrane organization, in close contact with the vascular channels [156]. The inner vascular membrane of the duplex contains Laminins (LN)-411 and LN-511 and the outer membrane facing the endocrine cells contains only LN-511. The presence of collagen proteins in human islets ECM has also been described previously; collagen type I, type III, type IV and type V are the main sub-types associated with the pancreatic ECM [154].

Adult multipotent stem cells, isolated from mesenchymal sources, are often overlooked in favour of pluripotent ESCs or iPSCs for β cell differentiation and diabetic therapy. Nevertheless, these multipotent stem cells have been shown to trans-differentiate towards endoderm and pancreatic phenotypes as well [129, 130]. In particularly, human adipose-derived stem cells (ADSCs) are widely distributed in the human body and easily isolated from adipose tissue segmental resections following surgical-procedures [125].

This investigation aimed to create conditions that mimic the natural stimuli within an islet ECM microenvironment and assess the outcomes on the *in vitro* differentiation of ADSC clusters. To achieve this, we cultured ADSC clusters in a LN suspension composed of LN411 and/or LN511 and evaluated the integration and organization of laminins into cluster spheroids. Finally, we

compared the extent of differentiation of the ADSC clusters in suspension and in 3D-collagen hydrogels.

First, ADSCs were characterized following the guidelines of the International Society for Cellular Therapy statement [127]. Briefly, the ADSCs were analysed for the expression of mesenchymal stem cell positive markers CD73, CD90 and CD105, and negative expression of embryonic stem cells marker OCT4, **Figure 23**. Furthermore, ADSCs ability to differentiate into adipocytes was investigated on 3D-fiber scaffolds made of PCL and compared functionality to conventional 2D-cultures (**Paper IV**). ADSCs cultured in 3D-fibers, as compared to 2D-cultures, showed an increased sensitivity in insulin-stimulated glucose uptake, and increased cellular lipid accumulation, and hormone sensitive lipase content.

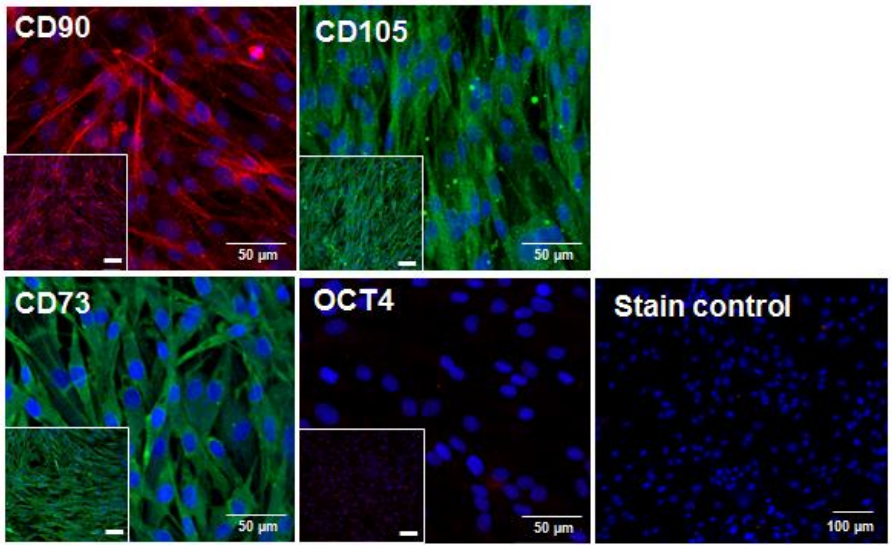


Figure 23. ADSCs characterization. *Undifferentiated ADSCs, at passage 2, immunolabeled for the typical MSCs surface markers: CD90 (red) CD105 (green), and CD73 (green); negative markers such as OCT4 (red). Nuclear stain (blue). Stain control image is shown on the right side – no primary antibody was used. The scale of the zoom-out picture corresponds to 100µm.*

ADSCs ability to differentiate towards endocrine lineages was performed by culture the ADSCs as suspension clusters. Cell clusters morphology and differentiation potential were characterized.

Clusters formed under the presence of LNs showed significantly lower cluster diameter and size (**Figure 24A & B**), suggesting laminins affect the remodelling of cell clusters' and their organization. LN supplementation did not affect cluster cells viability. **Figure 24C** shows the dual-colour live/dead staining, confirming the viability of cells in all conditions after 48h of culture in cluster suspensions.

Laminin protein expression was increased in clusters formed under LN-supplementation (**Figure 25A**). In clusters formed with mixed LN411/511 supplementation cells predominantly expressed LN511. Moreover, all conditions showed a cytoplasmic-like expression pattern of laminins, aggregating together the individual cells within the cluster. It was also possible to observe occasionally dot-like expression pattern indicating extracellular deposition. Furthermore, LN-supplemented clusters had significantly reduced expression of COL1 mRNA and to some extent reduced expression of LN mRNA genes (**Figure 25B**). Together, these results suggest external laminin supplementation relieves cells of ECM protein production during the clustering process.

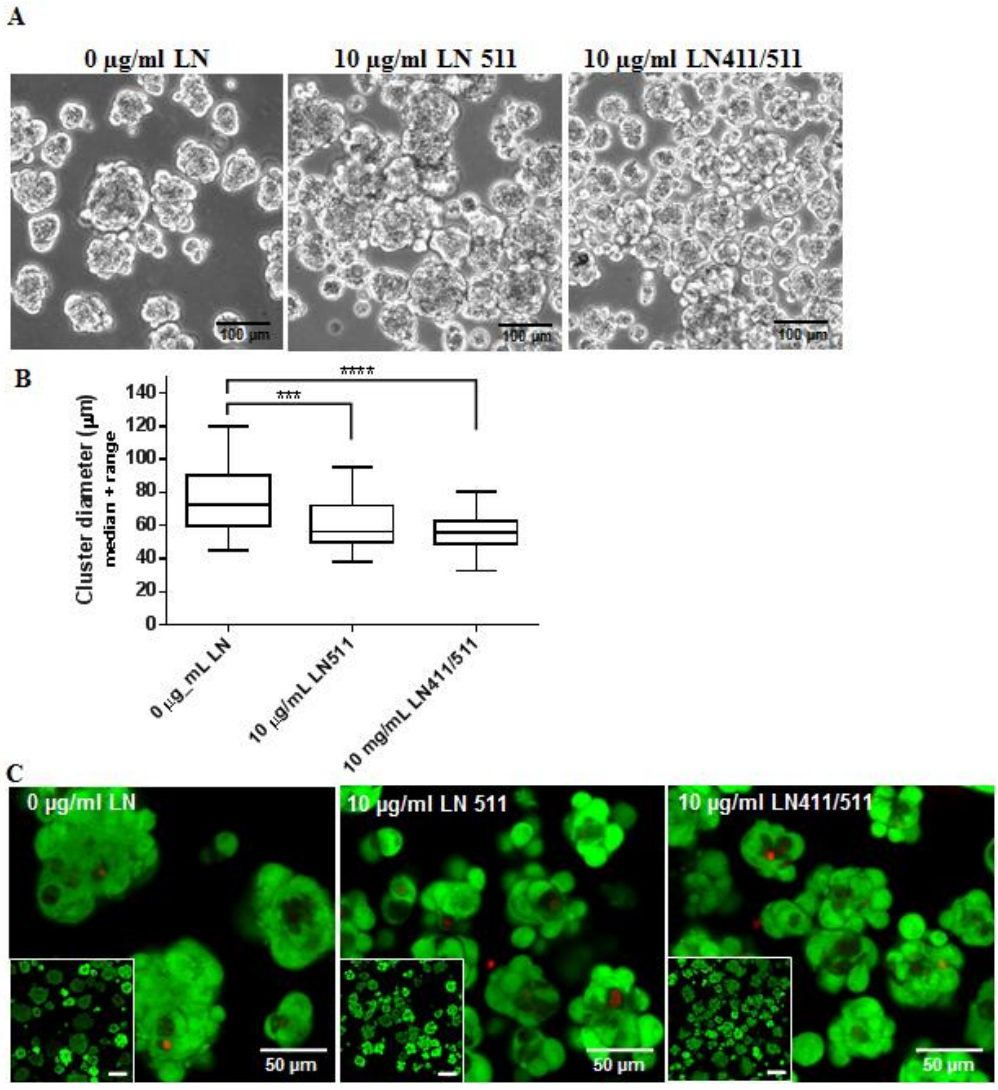


Figure 24. Undifferentiated ADSCs clusters characterization, after 48h of culture. *A) Bright field images. B) Clusters diameter quantification. C) Dual color LIVE/DEAD Cell Viability/Cytotoxicity Assay D. The scale of the zoom-out picture corresponds to 100µm. * represent statistical significance, and corresponding p-value description is the following: ***<0.001 and ****<0.0001.*

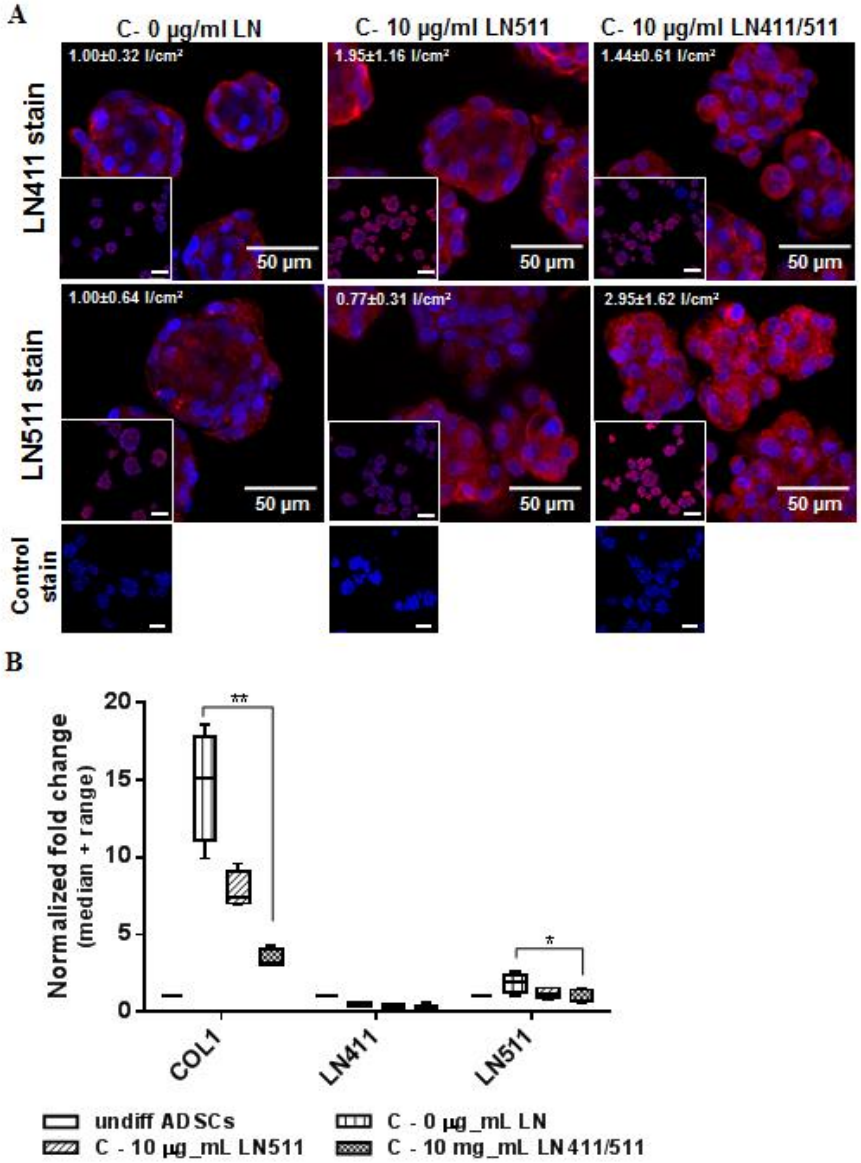


Figure 25. ECM formation during ADSCs clustering. *A) Expression of Laminin proteins (red) on undifferentiated ADSCs clusters in suspension. Nuclear stain (blue). Mean intensity (I) / cluster area (cm^2). The scale of the zoom-out picture corresponds to 100µm. B) ECM mRNA expression on ADSCs clusters. * represent statistical significance, and corresponding p-value description is the following: *<0.05 and **<0.01.*

Next, the concentration of laminin and the effect of single or combined supplementation of LN411 and LN511 was optimized and changes in the mRNA expression of mesenchymal (*CD105* and *CD73*) and endoderm (*SOX17*) markers was evaluated (**Figure 26**). LN411 resulted in a decrease in expression of *SOX17*, while LN511 led to an increased expression. An increase was also observed when LN411 and LN511 were supplemented simultaneously. Moreover, all differentiated conditions showed significantly decreased mRNA expression of

mesenchymal stem cell markers *CD105* and *CD73* in comparison to undifferentiated cells confirming differentiation into endoderm lineage.

Based on this data, three conditions were selected for further studies: clusters formed without Laminin supplementation, clusters formed with supplementation of LN511 alone and LN411/511 at 10 μg/mL concentration.

The expression of a key factor, *NKX6.1*, in cells from clusters differentiated in suspension or 3D-ECM hydrogels was evaluated (**Figure 27**). The NKX6.1-controlled gene regulatory network is known to be essential for maintaining the functional and molecular traits of mature beta cells in pancreatic islets [270]. mRNA expression levels of *NKX6.1* (**Figure 27A**) significantly increased in in LN-clusters as compared to non-supplemented clusters, while no differences were found regarding the supplementation of LN511 alone or the LN411/511 combination. Clusters cultured without LN supplementation and encapsulated in ECM hydrogels, displayed a trend of increased of *NKX6.1* mRNA expression in all encapsulated conditions (**Figure 27B**). However, only cells in the COL+LN511 hydrogels had a significantly higher levels than the suspension culture cells. Conversely, no differences were observed by encapsulating laminin supplemented clusters in COL1 hydrogels (**Figure 28**).

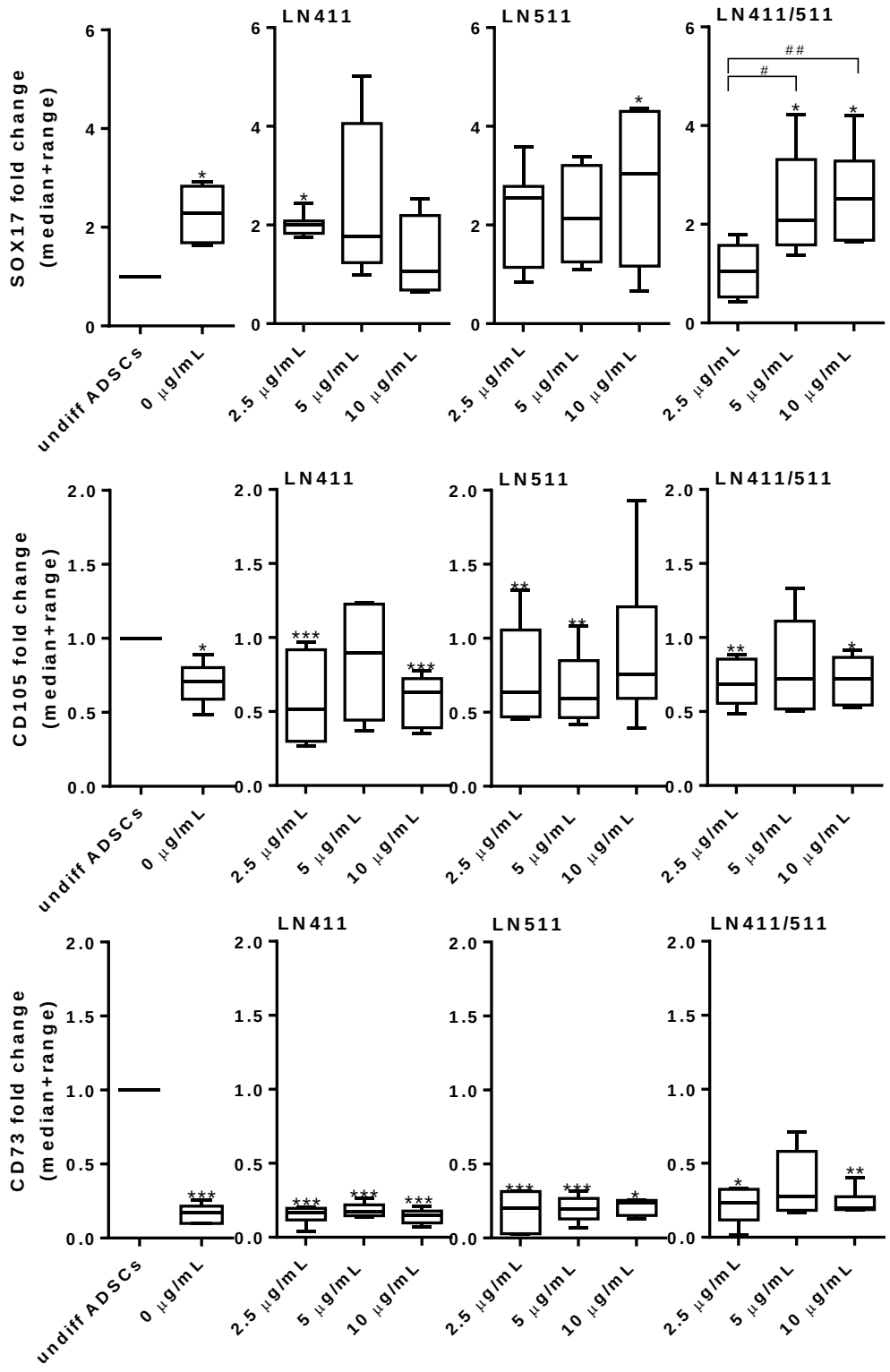


Figure 26. The Effect of laminin supplementation on ADSCs clusters differentiation to endoderm lineage. *mRNA expression of SOX17, CD105 and CD73 genes normalized to undifferentiated cells.* denotes significant difference compared to undifferentiated ADSCs; * represent statistical significance as compared to undifferentiated (undiff) ADSCs, and corresponding p-value description is the following: *<0.05, **<0.01 and ***<0.001.# denotes significant difference within each laminin supplementation group.*

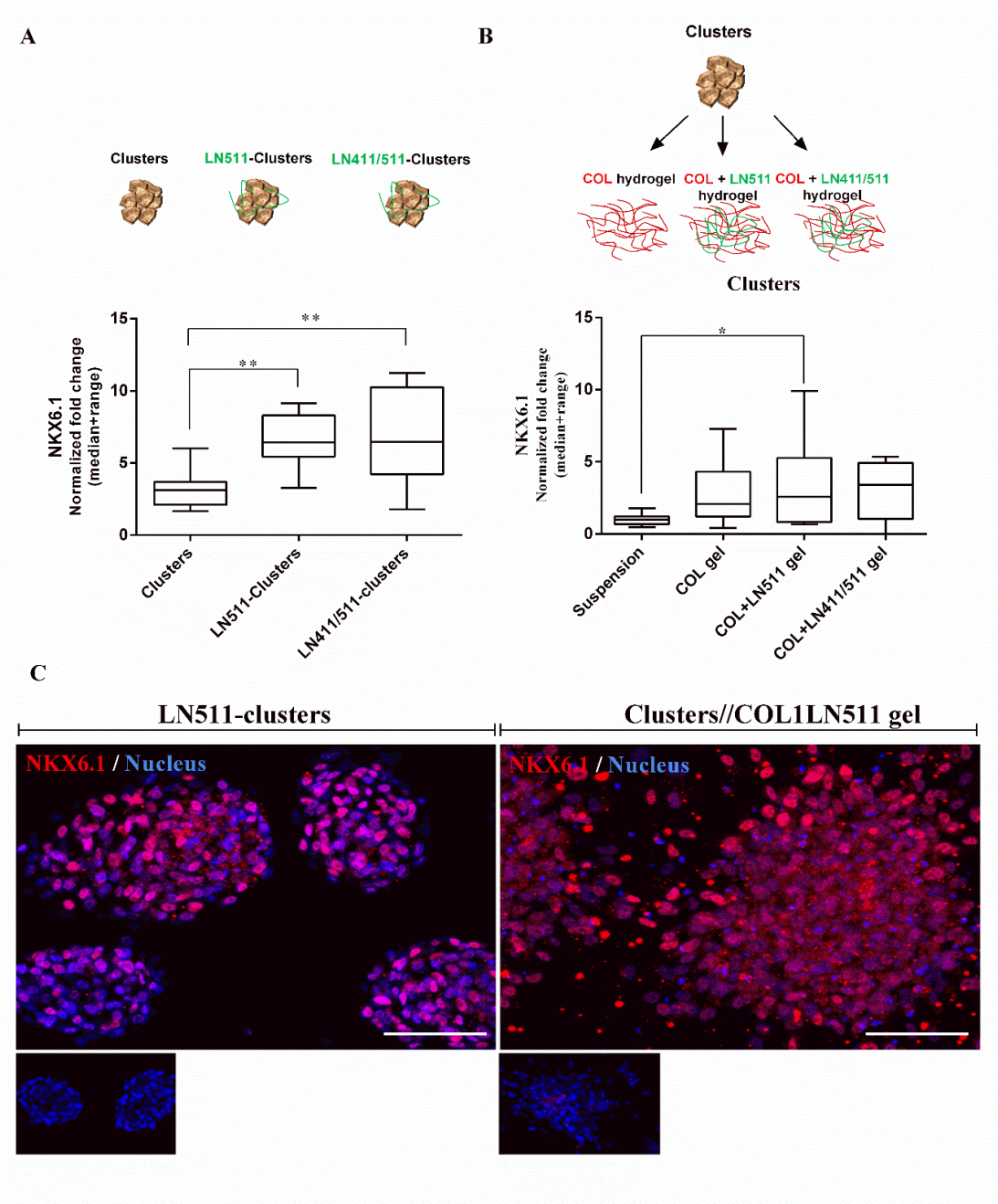


Figure 27. Expression of NKX6.1 in clusters differentiated in suspension or in 3D-ECM hydrogels culture. *NKX6.1 mRNA expression in A) Clusters cultured in suspension with or without LN511 or LN411+-511 supplementation. B) Clusters encapsulated in collagen or collagen/laminin hydrogels. * denotes significant difference as compared to suspension culture. C) Confocal image of NKX6.1 protein expression in ADSCs LN511-clusters under suspension differentiation and in clusters encapsulated in COL1+LN511 hydrogel. Nuclear stain (blue) was performed for nuclear identification. Bar = 100 µm. * represent statistical significance, and corresponding p-value description is the following: *<0.05 and **<0.01.*

The expression of NKX6.1 was further evaluated at protein level in both suspension clusters and in encapsulated clusters, where the mRNA was found to be significantly upregulated in both (**Figure 27C**). In clusters differentiated in suspension culture, there was a visible mixed population of either NKX6.1 expression positive or negative cells. Interestingly, in COL+LN511 encapsulated clusters, intense expression of NKX6.1 protein was detected. Overall, the NKX6.1 protein expression was uniformly distributed across the cells within the cluster, with a typical nuclear expression pattern as described in previous studies [145, 146].

The expression of other key factors known to drive endocrine development were evaluated and expression confirmed at the mRNA levels (data not shown), such as NGN3 and its downstream target NEUROD1 [12, 271, 272], MAFA, known to be exclusively expressed in β-cells [270] and MAFB in α-cells [273]. However, the expression of PDX1, a key pancreatic transcription factor [274], and pancreatic hormones (insulin, glucagon and somatostatin, were not detected in ADSCs differentiated cells, suggesting that the ADSCs pancreatic commitment was not successfully drive and the differentiation protocol requires further optimization.

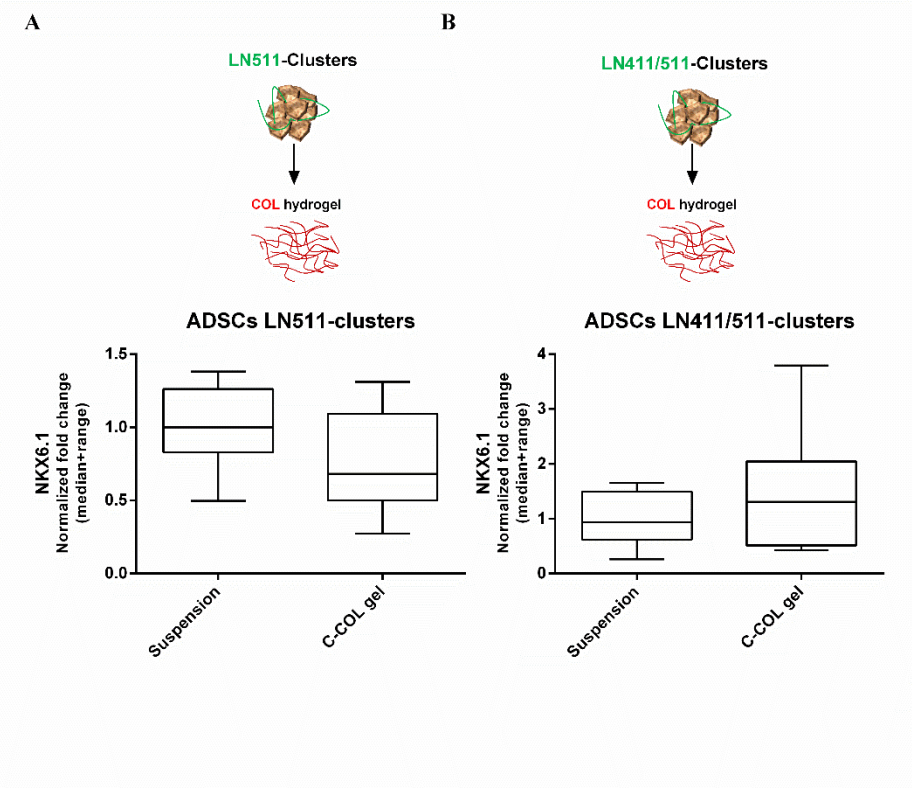


Figure 28. Expression of NKX6.1 in LN-clusters differentiated in 3D-COL1 hydrogels culture. *NKX6.1 mRNA expression in A) LN511 supplemented clusters cultured in suspension or encapsulated in collagen hydrogels. B) LN411/511 supplemented clusters cultured in suspension or encapsulated in collagen hydrogels. No significant differences between the conditions.*

Concluding remarks

Tissue development, functional differentiation, and homeostasis are orchestrated and sustained by a dynamic balance of biochemical and biophysical cues from the organ's microenvironment. The interaction of ECM proteins, hormones, neighbouring cells and soluble local and systemic cues, is crucial to determining cell behaviour and directing tissue development, structure and function.

In this thesis, I have discussed how different extracellular signalling factors coordinate pancreas-specific function, and how the understanding of these cues could be applied to develop complex *in vitro* models of relevance for T2DM research. First, I assessed the effect of pancreatic EVs on IAPP aggregation *in vitro* and on the *in vitro* differentiation of iPSCs. Our findings shown that healthy pancreatic EVs, of negative charge, limited IAPP amyloid formation *in vitro*. This data is in accordance with work showing that IAPP amyloid formation is strongly reduced by the extracellular environment and by plasma membranes of pancreatic β cells [101]. These authors speculated on the presence of particular lipids or proteins in the plasma membranes and/or secreted molecules that mediated this effect. The results described in this thesis showed that EVs secreted from pancreatic islets may have mediated the observed inhibitory effects, via a mechanism that may involve IAPP–EVs binding. In h-islet cultures, I observed a significant decrease in both ratios C-Peptide/IAPP and Insulin/IAPP, when islets were supplemented with 10μM of IAPP supplementation. This data suggests that intracellular accumulation of IAPP leads to a downregulation of insulin synthesis, and could be of potential interest for T2DM research. IAPP and/or EVs did not induce changes in the GSIS profile of the islet microtissues, but IAPP-EVs mixtures lead to a significant decrease on the apoptosis levels in the islet microtissues, as compared to IAPP supplemented condition. This preliminary result might suggest that IAPP induces islets toxicity mediated by an apoptotic mechanism, which is reduced in the presence of IAPP incubated with healthy EVs.

We further investigated the effect of h-Islet-EVs on insulin expression in differentiating iPSC clusters. Human islets released biologically active EVs that were able to induce a significant increase in iPSC clusters intracellular levels of C-peptide, in a concentration-dependent way. 3D-culture of iPSC clusters in collagen hydrogels supplemented with h-Islet-EVs did not affect insulin expression, however a regulation of cell apoptosis was observed.

Finally, I studied the role of mimicking the natural stimuli from an islet ECM microenvironment during *in vitro* differentiation of iPSCs and ADSCs, aiming to create a more physiologically and relevant pancreatic cell models. 3D-ECM hydrogels composed of collagen, and rat

decellularized scaffolds further advanced endocrine differentiation of both ADSCs and iPSCs. Although other studies with the same differentiation protocol [146] or different [143, 145], have reported iPSC or ESCs-derived β cells with functional GSIS response, in our hands the differentiation protocol did not induce significant changes in functionality of the differentiated cells. Overall, the identified regulatory effects may be of significance for future pancreatic differentiation models, but there is still a need to identify relevant targets for efficient conversion of differentiated progenitor cells into functional β cells *in vitro*.

The pancreatic extracellular communication mediated by EVs is an area of research that I would like to keep developing in my career, and I have many ideas *incubating* for future research. As a next step a detailed transcriptomic and proteomic analysis of the h-Islet-EVs may lead to the identification of novel molecular pathways underlying pancreatic regulation and commitment. A extensive omics characterization approach of the pancreatic EVs isolated from healthy and T2DM-derived donors may also lead to the identification of mechanisms contributing to disease progression, and be of relevance for the generation of disease biomarkers for drug screening.

Isolation of EVs from pancreatic islets kept in culture during different time points, such as post-isolation and longer cultures, may also bring more knowledge about the temporal release of active factors and association with apoptotic signaling. EVs isolated from these cultures could stimulate different signaling pathways and affect iPSCs differentiation, functionality and viability *in vitro*. Stimulation of isolared human islets with Ca^{2+} or other small molecules relevant on the functional GSIS process could help to further decipher the missing signalling steps needed to generate functional differentiated stem cells.

In a T2DM model, using IAPP as biological tool, it would be of great interest to understand and recapitulate the effects of native healthy EVs on the IAPP aggregation, using model lipid-protein systems. Generation of synthetic EV-like particles containing cargo proteins and lipid composition variations, based on omics characterization, may lead to the identification of targets that potentially could inhibit IAPP aggregation *in vitro* and also in clinical diabetic patients.

Another line of research may involve the investigation of EV-mediated processes in T2DM development. Addressing the long-term loss or gain of viability and functionality of T2DM pancreatic islets and as a function of external EV-mimicking cues, with the purpose of identifying the mechanism(s) for switching from a healthy to a T2D phenotype and vice versa.

Overall, EV-mediated actions in the pancreas and diabetes, as well as other tissues and diseases, is a relatively new science and this thesis barely touch the surface of this topic. It is, nonetheless,

very rewarding to see how this little contribution can open so much lines of research and hopeful have an impact beyond the basic science.

Acknowledgements

I have been very luck to work in two amazing groups during the four years of my PhD, the AZ iPS group and the group from Pernilla at Chalmers. Both have wonderful people that made my stay in Sweden and PhD full of rich experiences. I could not be more thankful to all of these people, and others that already left our groups. I will only mention one person in particular, Anette our iPS goddess! Without Anette´s daily hugs it would be impossible to bear all the iPS differentiation frustrations, you are the best! =)

I joined Pernilla´s group later in my PhD and it was crucial for my PhD studies. I am not sure if I would be defending my thesis or even if I would have a thesis to defend without Pernilla's help. She boosted my PhD motivation in a time of need and I learned so much from watching her developing a scientific story into a manuscript, all the help that she gave me on all my scientific writings and the million revisions to my thesis. Thank you for always including me in the group and division even though I was not at the University, and making sure that we have funny team activities as well as working times.

At AZ, I would like to start to thank Anna, which is a wonderful person that always gave me freedom to follow the science that I wish to work on. Ryan for being the best team leader ever! You changed our fragile iPS group to an amazing and super iPS group, where all of us love to come to work every day! We all have so much fun at work now! Moreover, thank you for taking over my research manuscripts when I gave up on them and turning them upside-down as many times as needed and yepi we published it!!!! =D and because it is never enough, thank you for making sure that all the members of the iPS group feel recognized and valuable.

Finally and most important my PhD buddies, Alex and Michael, Juris and our FINON fellows. We struggled so much but always together!

And of course, again to Michael and to our very little Peach (even fluffy Tiger) for remembering me that life is so much more than work ♥

Bibliography

[1] American Diabetes Association, Diagnosis and classification of diabetes mellitus, Diabetes care 33 Suppl 1 (2010) S62-9.
[2] N. Tiwari, Therapeutic Targets for Diabetes Mellitus: An Update, Clinical Pharmacology & Biopharmaceutics 3(1) (2014).
[3] D. Daneman, Type 1 diabetes, The Lancet 367(9513) (2006) 847-858.
[4] D.M. Nathan, B. Zinman, P.A. Cleary, J.Y. Backlund, S. Genuth, R. Miller, T.J. Orchard, Modern-day clinical course of type 1 diabetes mellitus after 30 years' duration: the diabetes control and complications trial/epidemiology of diabetes interventions and complications and Pittsburgh epidemiology of diabetes complications experience (1983-2005), Arch Intern Med 169(14) (2009) 1307-16.
[5] A.C. Croon, R. Karlsson, C. Bergström, E. Björklund, C. Möller, L. Tydén, A. Tibell, Lack of donors limits the use of islet transplantation as treatment for diabetes, Transplantation proceedings 35(2) (2003) 764.
[6] Paraskevas S, Maysinger D, Wang R, Duguid TP, R. L, Cell loss in isolated human islets occurs by apoptosis, Pancreas 20(3) (2000) 270-6.
[7] B.L. Wajchenberg, β-Cell Failure in Diabetes and Preservation by Clinical Treatment<beta Cell Failure in Diabetes and Preservation by Clinical.pdf>, Endocrine Reviews 28(2) (2007) 187–218.
[8] Z.X. He, Z.W. Zhou, Y. Yang, T. Yang, S.Y. Pan, J.X. Qiu, S.F. Zhou, Overview of clinically approved oral antidiabetic agents for the treatment of type 2 diabetes mellitus, Clinical and experimental pharmacology & physiology 42(2) (2015) 125-38.
[9] W. World Health Organization, Global report on diabetes, (2015).
[10] W.R. Rowley, C. Bezold, Creating public awareness: state 2025 diabetes forecasts, Population health management 15(4) (2012) 194-200.
[11] I. International Diabetes Foundation, Diabetes Atlas, Seventh Edition, (2015).
[12] A. Rojas, A. Khoo, J.R. Tejedo, F.J. Bedoya, B. Soria, F. Martin, Islet cell development, in: M.S. Islam (Ed.), The Islets of Langerhans, Advances in Experimental Medicine and Biology, Springer Science+Business Media B.V.2010, pp. 59-75.
[13] R.M. Case, Synthesis, intracellular transport and discharge of exportable proteins in the pancreatic acinar cell and other cells, Biol Rev Camb Philos Soc 53(2) (1978) 211-354.
[14] The Islets of Langerhans. Advance in experimental medicine and Biology., Springer Netherlands.
[15] O. Cabrera, D.M. Berman, N.S. Kenyon, C. Ricordi, P.O. Berggren, A. Caicedo, The unique cytoarchitecture of human pancreatic islets has implications for islet cell function, Proceedings of the National Academy of Sciences of the United States of America 103(7) (2006) 2334-9.
[16] S. Bonner-Weir, B.A. Sullivan, G.C. Weir, Human Islet Morphology Revisited: Human and Rodent Islets Are Not So Different After All, J Histochem Cytochem 63(8) (2015) 604-12.
[17] Brissova M, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM, P. AC, Assessment of Human Pancreatic Islet Architecture and Composition by Laser Scanning Confocal Microscopy, J Histochem Cytochem 53(9) (2005) 1087-97.
[18] F C Schuit, P A In't Veld, D.G. Pipeleers, Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells, Proc Natl Acad Sci 85(11) (1988) 3865–3869.
[19] Ashcroft FM, A. SJH, Insulin Molecular Biology to Pathology, New York: Oxford University Press 5–92., 1992.

[20] M.F. Dunn, Zinc-ligand interactions modulate assembly and stability of the insulin hexamer -- a review, Biometals 18(4) (2005) 295-303.
[21] Yu SS, K. AE, Biological activity of proinsulin and related polypeptides in the fat tissue, J Biol Chem. 11(248) (1973) 3753-61.
[22] J. Suckale, M. Solimena, The insulin secretory granule as a signaling hub, Trends in endocrinology and metabolism: TEM 21(10) (2010) 599-609.
[23] S. Janciauskiene, S. Eriksson, E. Carlemalm, B. Ahrén, B Cell Granule Peptides Affect Human Islet Amyloid Polypeptide (IAPP) Fibril Formationin Vitro, Biochemical and biophysical research communications 236(3) (1997) 580-585.
[24] P. Rorsman, M. Braun, Regulation of insulin secretion in human pancreatic islets, Annual review of physiology 75 (2013) 155-79.
[25] P.E. MacDonald, J.W. Joseph, P. Rorsman, Glucose-sensing mechanisms in pancreatic beta-cells, Philosophical transactions of the Royal Society of London. Series B, Biological sciences 360(1464) (2005) 2211-25.
[26] G.A. Rutter, T.J. Pullen, D.J. Hodson, A. Martinez-Sanchez, Pancreatic beta-cell identity, glucose sensing and the control of insulin secretion, The Biochemical journal 466(2) (2015) 203-18.
[27] S. Barg, A. Lindqvist, S. Obermuller, Granule docking and cargo release in pancreatic beta-cells, Biochem Soc Trans 36(Pt 3) (2008) 294-9.
[28] W.F. Pralong, C. Bartley, C.B. Wollheim, Single islet beta-cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca2+ and secretion, The EMBO journal 9(1) (1990) 53-60.
[29] L. Chen, T. Alam, J.H. Johnson, S. Hughes, C.B. Newgard, R.H. Unger, Regulation of beta-cell glucose transporter gene expression, Proceedings of the National Academy of Sciences of the United States of America 87(11) (1990) 4088-92.
[30] M. Tal, Y. Liang, H. Najafi, H.F. Lodish, F.M. Matschinsky, Expression and function of GLUT-1 and GLUT-2 glucose transporter isoforms in cells of cultured rat pancreatic islets, The Journal of biological chemistry 267(24) (1992) 17241-7.
[31] F.M. Ashcroft, D.E. Harrison, S.J. Ashcroft, Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells, Nature 312(5993) (1984) 446-8.
[32] S.N. Yang, P.O. Berggren, The role of voltage-gated calcium channels in pancreatic beta-cell physiology and pathophysiology, Endocr Rev 27(6) (2006) 621-76.
[33] R. Jahn, D. Fasshauer, Molecular machines governing exocytosis of synaptic vesicles, Nature 490(7419) (2012) 201-7.
[34] J.C. Henquin, D. Dufrane, M. Nenquin, Nutrient control of insulin secretion in isolated normal human islets, Diabetes 55(12) (2006) 3470-7.
[35] P. Rorsman, E. Renstrom, Insulin granule dynamics in pancreatic beta cells, Diabetologia 46(8) (2003) 1029-45.
[36] S.L. Howell, The mechanism of insulin secretion, Diabetologia 26(5) (1984) 319-327.
[37] Henquin JC, Nenquin M, Stiernet P, A. B., In vivo and in vitro glucose-induced biphasic insulin secretion in the mouse: pattern and role of cytoplasmic Ca2+ and amplification signals in beta-cells., Diabetes care 55(2) (2006) 441-56.
[38] Zawalich WS, Yamazaki H, Z. KC., Biphasic insulin secretion from freshly isolated or cultured, perifused rodent islets: comparative studies with rats and mice., Metabolism 57(1) (2008) 30-9.
[39] R.N. Rambaran, L.C. Serpell, Amyloid fibrils: abnormal protein assembly, Prion 2(3) (2008) 112-7.
[40] A. Abedini, A.M. Schmidt, Mechanisms of islet amyloidosis toxicity in type 2 diabetes, FEBS Lett 587(8) (2013) 1119-27.
[41] T.P. Knowles, M. Vendruscolo, C.M. Dobson, The amyloid state and its association with protein misfolding diseases, Nature reviews. Molecular cell biology 15(6) (2014) 384-96.

[42] D. Otzen, Functional amyloid, Prion 4(4) (2014) 256-264.
[43] P. Bergman, N.R. Roan, U. Romling, C.L. Bevins, J. Munch, Amyloid formation: functional friend or fearful foe?, J Intern Med 280(2) (2016) 139-52.
[44] E. Ferrannini, Insulin Resistance versus Insulin Deficiency in Non-Insulin-Dependent Diabetes Mellitus: Problems and Prospects, Endocrine Reviews 19(4) (1998) 477-490.
[45] J. Höppener, B. Ahrén, C. Lips, Islet Amyloid and Type 2 Diabetes Mellitus, The New England Journal of Medicine 343 (2000) 411-419.
[46] K.J. Potter, A. Abedini, P. Marek, A.M. Klimek, S. Butterworth, M. Driscoll, R. Baker, M.R. Nilsson, G.L. Warnock, J. Oberholzer, S. Bertera, M. Trucco, G.S. Korbutt, P.E. Fraser, D.P. Raleigh, C.B. Verchere, Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts, Proceedings of the National Academy of Sciences of the United States of America 107(9) (2010) 4305-10.
[47] Sanke T, Bell GI, Sample C, Rubenstein AH, S. DF., An islet amyloid peptide is derived from an 89-amino acid precursor by proteolytic processing, J Biol Chem. 263(33) (1988) 17243-6.
[48] C. Betsholtz, V. Svensson, F. Rosman, U. Engström, G.T. Westermark, E. Wilander, K. Johnson, P. Westermark, 1989, Experimental cell research 183(2) (Islet amyloid polypeptide (IAPP): cDNA cloning and identification of an amyloidogenic region associated with the species-specific occurrence of age-related diabetes mellitus) 484-493.
[49] R. Akter, P. Cao, H. Noor, Z. Ridgway, L.H. Tu, H. Wang, A.G. Wong, X. Zhang, A. Abedini, A.M. Schmidt, D.P. Raleigh, Islet Amyloid Polypeptide: Structure, Function, and Pathophysiology, J Diabetes Res 2016 (2016) 2798269.
[50] A.R. Lomasney, L. Yi, M.G. Roper, Simultaneous monitoring of insulin and islet amyloid polypeptide secretion from islets of Langerhans on a microfluidic device, Anal Chem 85(16) (2013) 7919-25.
[51] Gedulin B, Cooper GJ, Y. AA., Amylin secretion from the perfused pancreas: dissociation from insulin and abnormal elevation in insulin-resistant diabetic rats., Biochem Biophys Res Commun. 180(2) (1991) 782-9.
[52] T.A. Lutz, The role of amylin in the control of energy homeostasis, Am J Physiol Regul Integr Comp Physiol 298(6) (2010) R1475-84.
[53] B. Åkesson, G. Panagiotidis, P. Westermark, I. Lundquist, Islet amyloid polypeptide inhibits glucagon release and exerts a dual action on insulin release from isolated islets, Regulatory Peptides 111(1-3) (2003) 55-60.
[54] R.L. Hull, G.T. Westermark, P. Westermark, S.E. Kahn, Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes, J Clin Endocrinol Metab 89(8) (2004) 3629-43.
[55] P. Westermark, A. Andersson, G.T. Westermark, Islet amyloid polypeptide, islet amyloid, and diabetes mellitus, Physiol Rev 91(3) (2011) 795-826.
[56] E.T.A.S. Jaikaran, A. Clark, Islet amyloid and type 2 diabetes: from molecular misfolding to islet pathophysiology, Biochimica et Biophysica Acta (BBA) 1537(3) (2001) 179-203.
[57] T. Hanabusa, K. Kubo, C. Oki, Y. Nakano, K. Okai, T. Sanke, K. Nanjo, Islet amyloid polypeptide (IAPP) secretion from islet cells and its plasma concentration in patients with non-insulin-dependent diabetes mellitus, Diabetes Res Clin Pract 15(1) (1992) 89-96.
[58] Iki K, P. PM., Distribution of pancreatic endocrine cells including IAPP-expressing cells in non-diabetic and type 2 diabetic cases., J Histochem Cytochem 55(2) (2007) 111-8.
[59] T. Tomita, Islet amyloid polypeptide in pancreatic islets from type 1 diabetic subjects, Islets 3(4) (2014) 166-174.
[60] T. Tomita, Islet amyloid polypeptide in pancreatic islets from type 2 diabetic subjects, Islets 4(3) (2012) 223-32.
[61] J.D. Knight, J.A. Williamson, A.D. Miranker, Interaction of membrane-bound islet amyloid polypeptide with soluble and crystalline insulin, Protein Sci 17(10) (2008) 1850-6.

[62] C.A. Jurgens, M.N. Toukatly, C.L. Fligner, J. Udayasankar, S.L. Subramanian, S. Zraika, K. Aston-Mourney, D.B. Carr, P. Westermark, G.T. Westermark, S.E. Kahn, R.L. Hull, beta-cell loss and beta-cell apoptosis in human type 2 diabetes are related to islet amyloid deposition, Am J Pathol 178(6) (2011) 2632-40.
[63] P. Cao, A. Abedini, D.P. Raleigh, Aggregation of islet amyloid polypeptide: from physical chemistry to cell biology, Curr Opin Struct Biol 23(1) (2013) 82-9.
[64] J.J. Wiltzius, S.A. Sievers, M.R. Sawaya, D. Cascio, D. Popov, C. Riekel, D. Eisenberg, Atomic structure of the cross-beta spine of islet amyloid polypeptide (amylin), Protein Sci 17(9) (2008) 1467-74.
[65] S. Bedrood, Y. Li, J.M. Isas, B.G. Hegde, U. Baxa, I.S. Haworth, R. Langen, Fibril structure of human islet amyloid polypeptide, The Journal of biological chemistry 287(8) (2012) 5235-41.
[66] L. Khemtemourian, M.F. Engel, R.M. Liskamp, J.W. Hoppener, J.A. Killian, The N-terminal fragment of human islet amyloid polypeptide is non-fibrillogenic in the presence of membranes and does not cause leakage of bilayers of physiologically relevant lipid composition, Biochimica et biophysica acta 1798(9) (2010) 1805-11.
[67] Jaikaran ET, Higham CE, Serpell LC, Zurdo J, Gross M, Clark A, F. PE., Identification of a novel human islet amyloid polypeptide beta-sheet domain and factors influencing fibrillogenesis., J Mol Biol. 308(3) (2001) 515-25.
[68] Westermark P, Engström U, Johnson KH, Westermark GT, B. C., Islet amyloid polypeptide: pinpointing amino acid residues linked to amyloid fibril formation, Proc Natl Acad Sci USA 87(13) (1990) 5036-40.
[69] Nilsson MR, R. DP., Analysis of amylin cleavage products provides new insights into the amyloidogenic region of human amylin., J Mol Biol. 294(5) (1999) 1375-85.
[70] Padrick SB, M. AD, Islet amyloid polypeptide: identification of long-range contacts and local order on the fibrillogenesis pathway., J Mol Biol 308(4) (2001) 783-94.
[71] C. Goldsbury, K. Goldie, J. Pellaud, J. Seelig, P. Frey, S.A. Muller, J. Kistler, G.J. Cooper, U. Aebi, Amyloid fibril formation from full-length and fragments of amylin, Journal of structural biology 130(2-3) (2000) 352-62.
[72] Roberts AN, Leighton B, Todd JA, Cockburn D, Schofield PN, Sutton R, Holt S, Boyd Y, Day AJ, e.a. Foot EA, Molecular and functional characterization of amylin, a peptide associated with type 2 diabetes mellitus, Proc Natl Acad Sci U S A. 86(24) (1989) 9662-6.
[73] B.W. Koo, A.D. Miranker, Contribution of the intrinsic disulfide to the assembly mechanism of islet amyloid, Protein Sci 14(1) (2005) 231-9.
[74] C. Xue, T.Y. Lin, D. Chang, Z. Guo, Thioflavin T as an amyloid dye: fibril quantification, optimal concentration and effect on aggregation, R Soc Open Sci 4(1) (2017) 160696.
[75] T.P.J. Knowles, C.A. Waudby, G.L. Devlin, S.I.A. Cohen, A. Aguzzi, M. Vendruscolo, E.M. Terentjev, M.E. Welland, C.M. Dobson, An Analytical Solution to the Kinetics of Breakable Filament Assembly, Science 326(5959) (2009) 1533-1537.
[76] Padrick SB, M. AD, Islet amyloid: phase partitioning and secondary nucleation are central to the mechanism of fibrillogenesis., Biochemistry 41(14) (2002) 4694-703.
[77] A.V. Kajava, U. Aebi, A.C. Steven, The parallel superpleated beta-structure as a model for amyloid fibrils of human amylin, J Mol Biol 348(2) (2005) 247-52.
[78] Westermark P, Li ZC, Westermark GT, Leckström A, S. DF., Effects of beta cell granule components on human islet amyloid polypeptide fibril formation., FEBS Lett. 379(3) (1996) 203-6.
[79] J.L. Larson, A.D. Miranker, The Mechanism of Insulin Action on Islet Amyloid Polypeptide Fiber Formation, Journal of Molecular Biology 335(1) (2004) 221-231.

[80] A.C. Susa, C. Wu, S.L. Bernstein, N.F. Dupuis, H. Wang, D.P. Raleigh, J.E. Shea, M.T. Bowers, Defining the molecular basis of amyloid inhibitors: human islet amyloid polypeptide-insulin interactions, Journal of the American Chemical Society 136(37) (2014) 12912-9.
[81] W. Cui, J.W. Ma, P. Lei, W.H. Wu, Y.P. Yu, Y. Xiang, A.J. Tong, Y.F. Zhao, Y.M. Li, Insulin is a kinetic but not a thermodynamic inhibitor of amylin aggregation, FEBS J 276(12) (2009) 3365-71.
[82] L. Caillon, A.R. Hoffmann, A. Botz, L. Khemtemourian, Molecular Structure, Membrane Interactions, and Toxicity of the Islet Amyloid Polypeptide in Type 2 Diabetes Mellitus, J Diabetes Res 2016 (2016) 5639875.
[83] J. Seeliger, K. Weise, N. Opitz, R. Winter, The effect of Abeta on IAPP aggregation in the presence of an isolated beta-cell membrane, J Mol Biol 421(2-3) (2012) 348-63.
[84] J. Turk, B.A. Wolf, J.B. Lefkowith, W.T. Stump, M.L. McDaniel, Glucose-induced phospholipid hydrolysis in isolated pancreatic islets: quantitative effects on the phospholipid content of arachidonate and other fatty acids, Biochimica et biophysica acta 879(3) (1986) 399-409.
[85] X. Zhang, J.R. St Clair, E. London, D.P. Raleigh, Islet Amyloid Polypeptide Membrane Interactions: Effects of Membrane Composition, Biochemistry 56(2) (2017) 376-390.
[86] J.D. Knight, A.D. Miranker, Phospholipid catalysis of diabetic amyloid assembly, J Mol Biol 341(5) (2004) 1175-87.
[87] K. Sasahara, K. Morigaki, T. Okazaki, D. Hamada, Binding of islet amyloid polypeptide to supported lipid bilayers and amyloid aggregation at the membranes, Biochemistry 51(35) (2012) 6908-19.
[88] L.J. Pike, Lipid rafts: bringing order to chaos, Journal of Lipid Research 44(4) (2003) 655-667.
[89] W.J. Cho, S. Trikha, A.M. Jeremic, Cholesterol regulates assembly of human islet amyloid polypeptide on model membranes, J Mol Biol 393(3) (2009) 765-75.
[90] S. Singh, S. Trikha, D.C. Bhowmick, A.A. Sarkar, A.M. Jeremic, Role of Cholesterol and Phospholipids in Amylin Misfolding, Aggregation and Etiology of Islet Amyloidosis, Advances in experimental medicine and biology 855 (2015) 95-116.
[91] J.R. Brender, S. Salamekh, A. Ramamoorthy, Membrane disruption and early events in the aggregation of the diabetes related peptide IAPP from a molecular perspective, Acc Chem Res 45(3) (2012) 454-62.
[92] R. Soong, J.R. Brender, P.M. Macdonald, A. Ramamoorthy, Association of Highly Compact Type II Diabetes Related Islet Amyloid Polypeptide Intermediate Species at Physiological Temperature Revealed by Diffusion NMR Spectroscopy, J. Am. Chem. Soc. 131(20) (2009) 7079–7085.
[93] J. Montane, A. Klimek-Abercrombie, K.J. Potter, C. Westwell-Roper, C. Bruce Verchere, Metabolic stress, IAPP and islet amyloid, Diabetes, obesity & metabolism 14 Suppl 3 (2012) 68-77.
[94] P. Cao, P. Marek, H. Noor, V. Patsalo, L.H. Tu, H. Wang, A. Abedini, D.P. Raleigh, Islet amyloid: from fundamental biophysics to mechanisms of cytotoxicity, FEBS Lett 587(8) (2013) 1106-18.
[95] N. Shigihara, A. Fukunaka, A. Hara, K. Komiya, A. Honda, T. Uchida, H. Abe, Y. Toyofuku, M. Tamaki, T. Ogihara, T. Miyatsuka, H.J. Hiddinga, S. Sakagashira, M. Koike, Y. Uchiyama, T. Yoshimori, N.L. Eberhardt, Y. Fujitani, H. Watada, Human IAPP-induced pancreatic beta cell toxicity and its regulation by autophagy, J Clin Invest 124(8) (2014) 3634-44.
[96] M. Visa, G. Alcarraz-Vizan, J. Montane, L. Cadavez, C. Castano, M.L. Villanueva-Penacarrillo, J.M. Servitja, A. Novials, Islet amyloid polypeptide exerts a novel autocrine action in beta-cell signaling and proliferation, FASEB J 29(7) (2015) 2970-9.

[97] A. Lorenzo, Bronwyn Razzaboni, Gordon C. Weir, B.A. Yankner, Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus, Nature 368 (1994) 756 - 760.
[98] J.J. Meier, R. Kayed, C.Y. Lin, T. Gurlo, L. Haataja, S. Jayasinghe, R. Langen, C.G. Glabe, P.C. Butler, Inhibition of human IAPP fibril formation does not prevent beta-cell death: evidence for distinct actions of oligomers and fibrils of human IAPP, Am J Physiol Endocrinol Metab 291(6) (2006) E1317-24.
[99] Janson J, Ashley RH, Harrison D, McIntyre S, B. PC., The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles, Diabetes 48(3) (1999) 491-8.
[100] S. Trikha, A.M. Jeremic, Distinct internalization pathways of human amylin monomers and its cytotoxic oligomers in pancreatic cells, PloS one 8(9) (2013) e73080.
[101] D.E. Schlamadinger, A.D. Miranker, Fiber-dependent and -independent toxicity of islet amyloid polypeptide, Biophysical journal 107(11) (2014) 2559-66.
[102] C. Westwell-Roper, H.C. Denroche, J.A. Ehses, C.B. Verchere, Differential Activation of Innate Immune Pathways by Distinct Islet Amyloid Polypeptide (IAPP) Aggregates, The Journal of biological chemistry 291(17) (2016) 8908-17.
[103] Bosco D, Armanet M, Morel P, Niclauss N, Sgroi A, Muller YD, Giovannoni L, Parnaud G, B. T., Unique arrangement of alpha- and beta-cells in human islets of Langerhans., Diabetes 59(5) (2010) 1202-10.
[104] Negi S, Jetha A, Aikin R, Hasilo C, Sladek R, P. S., Analysis of beta-cell gene expression reveals inflammatory signaling and evidence of dedifferentiation following human islet isolation and culture, PloS one 7(1) (2012) e30415.
[105] J. Dominguez-Bendala, G. Lanzoni, D. Klein, S. Alvarez-Cubela, R.L. Pastori, The Human Endocrine Pancreas: New Insights on Replacement and Regeneration, Trends in endocrinology and metabolism: TEM 27(3) (2016) 153-62.
[106] E.M. Abdelalim, M.M. Emara, Advances and challenges in the differentiation of pluripotent stem cells into pancreatic beta cells, World journal of stem cells 7(1) (2015) 174-81.
[107] F.W. Pagliuca, D.A. Melton, How to make a functional beta-cell, Development 140(12) (2013) 2472-83.
[108] S. Avasthi, R.N. Srivastava, A. Singh, M. Srivastava, Stem cell: past, present and future--a review article, Internet Journal of Medical Update, Vol. 3 3(1) (2008) 22-30.
[109] R. Jaenisch, R. Young, Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming, Cell 132(4) (2008) 567-82.
[110] J. Wu, J.C. Izpisua Belmonte, Stem Cells: A Renaissance in Human Biology Research, Cell 165(7) (2016) 1572-1585.
[111] M. Thomson, S.J. Liu, L.N. Zou, Z. Smith, A. Meissner, S. Ramanathan, Pluripotency factors in embryonic stem cells regulate differentiation into germ layers, Cell 145(6) (2011) 875-89.
[112] C.E. Murry, G. Keller, Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development, Cell 132(4) (2008) 661-80.
[113] Nishikawa S, Jakt LM, E. T., Embryonic stem-cell culture as a tool for developmental cell biology, Nat Rev Mol Cell Biol. 8(6) (2007) 502-7.
[114] M. Evans, Discovering pluripotency: 30 years of mouse embryonic stem cells, Nature reviews. Molecular cell biology 12(10) (2011) 680-6.
[115] Orkin SH, M. SJ., Stem-cell competition, Nature. 418(6893) (2002) 25-7.
[116] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126(4) (2006) 663-76.
[117] R.A. Young, Control of the embryonic stem cell state, Cell 144(6) (2011) 940-54.
[118] K.M. Loh, B. Lim, A precarious balance: pluripotency factors as lineage specifiers, Cell stem cell 8(4) (2011) 363-9.

[119] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131(5) (2007) 861-72.
[120] J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin, II, J.A. Thomson, Induced pluripotent stem cell lines derived from human somatic cells, Science 318(5858) (2007) 1917-20.
[121] Y. Shi, H. Inoue, J.C. Wu, S. Yamanaka, Induced pluripotent stem cell technology: a decade of progress, Nat Rev Drug Discov 16(2) (2017) 115-130.
[122] Y.C. Hsu, E. Fuchs, A family business: stem cell progeny join the niche to regulate homeostasis, Nature reviews. Molecular cell biology 13(2) (2012) 103-14.
[123] S.J. Morrison, A.C. Spradling, Stem cells and niches: mechanisms that promote stem cell maintenance throughout life, Cell 132(4) (2008) 598-611.
[124] C. Verfaillie, Hematopoietic stem cells for transplantation., Nat Immunol. 3(4) (2002) 314-7.
[125] D.M. Minteer, K.G. Marra, J.P. Rubin, Adipose stem cells: biology, safety, regulation, and regenerative potential, Clinics in plastic surgery 42(2) (2015) 169-79.
[126] V. Feisst, S. Meidinger, M.B. Locke, From bench to bedside: use of human adipose-derived stem cells, Stem cells and cloning : advances and applications 8 (2015) 149-162.
[127] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, E. Horwitz, Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement, Cytotherapy 8(4) (2006) 315-7.
[128] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284(5411) (1999) 143-7.
[129] V. Chandra, G. Swetha, S. Muthyala, A. Jaiswal, J. Bellare, P. Nair, R. Bhonde, Islet-Like Cell Aggregates Generated from Human Adipose Tissue Derived Stem Cells Ameliorate Experimental Diabetes in Mice, PloS one 6(6) (2011) e20615.
[130] V. Chandra, S. G, S. Phadnis, P.D. Nair, R.R. Bhonde, Generation of pancreatic hormone-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells, Stem cells 27(8) (2009) 1941-53.
[131] S.K. Kapur, S. Dos-Anjos Vilaboa, R. Llull, A.J. Katz, Adipose tissue and stem/progenitor cells: discovery and development, Clinics in plastic surgery 42(2) (2015) 155-67.
[132] J.V. Schiesser, J.M. Wells, Generation of beta cells from human pluripotent stem cells: are we there yet?, Annals of the New York Academy of Sciences 1311 (2014) 124-37.
[133] K.A. D'Amour, A.D. Agulnick, S. Eliazer, O.G. Kelly, E. Kroon, E.E. Baetge, Efficient differentiation of human embryonic stem cells to definitive endoderm, Nature biotechnology 23(12) (2005) 1534-41.
[134] C.H. Cho, N.R. Hannan, F.M. Docherty, H.M. Docherty, M. Joao Lima, M.W. Trotter, K. Docherty, L. Vallier, Inhibition of activin/nodal signalling is necessary for pancreatic differentiation of human pluripotent stem cells, Diabetologia 55(12) (2012) 3284-95.
[135] D. Zhang, W. Jiang, M. Liu, X. Sui, X. Yin, S. Chen, Y. Shi, H. Deng, Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells, Cell research 19(4) (2009) 429-38.
[136] M. Johannesson, A. Stahlberg, J. Ameri, F.W. Sand, K. Norrman, H. Semb, FGF4 and retinoic acid direct differentiation of hESCs into PDX1-expressing foregut endoderm in a time- and concentration-dependent manner, PloS one 4(3) (2009) e4794.
[137] X. Xu, V.L. Browning, J.S. Odorico, Activin, BMP and FGF pathways cooperate to promote endoderm and pancreatic lineage cell differentiation from human embryonic stem cells, Mechanisms of development 128(7-10) (2011) 412-27.

[138] T. Toyoda, S. Mae, H. Tanaka, Y. Kondo, M. Funato, Y. Hosokawa, T. Sudo, Y. Kawaguchi, K. Osafune, Cell aggregation optimizes the differentiation of human ESCs and iPSCs into pancreatic bud-like progenitor cells, Stem cell research 14(2) (2015) 185-97.
[139] N. Lumelsky, O. Blondel, P. Laeng, I. Velasco, R. Ravin, R. McKay, Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets, Science 292(5520) (2001) 1389-94.
[140] K.A. D'Amour, A.G. Bang, S. Eliazer, O.G. Kelly, A.D. Agulnick, N.G. Smart, M.A. Moorman, E. Kroon, M.K. Carpenter, E.E. Baetge, Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells, Nature biotechnology 24(11) (2006) 1392-401.
[141] J. Jiang, M. Au, K. Lu, A. Eshpeter, G. Korbutt, G. Fisk, A.S. Majumdar, Generation of insulin-producing islet-like clusters from human embryonic stem cells, Stem cells 25(8) (2007) 1940-53.
[142] T.C. Schulz, H.Y. Young, A.D. Agulnick, M.J. Babin, E.E. Baetge, A.G. Bang, A. Bhoumik, I. Cepa, R.M. Cesario, C. Haakmeester, K. Kadoya, J.R. Kelly, J. Kerr, L.A. Martinson, A.B. McLean, M.A. Moorman, J.K. Payne, M. Richardson, K.G. Ross, E.S. Sherrer, X. Song, A.Z. Wilson, E.P. Brandon, C.E. Green, E.J. Kroon, O.G. Kelly, K.A. D'Amour, A.J. Robins, A scalable system for production of functional pancreatic progenitors from human embryonic stem cells, PloS one 7(5) (2012) e37004.
[143] A. Rezania, J.E. Bruin, P. Arora, A. Rubin, I. Batushansky, A. Asadi, S. O'Dwyer, N. Quiskamp, M. Mojibian, T. Albrecht, Y.H. Yang, J.D. Johnson, T.J. Kieffer, Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells, Nature biotechnology 32(11) (2014) 1121-33.
[144] Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J, Gauvin R, Narayan K, Karanu F, O'Neil JJ, Ao Z, Warnock GL, K. TJ., Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice, Diabetes 61(8) (2012) 2016-29.
[145] F.W. Pagliuca, J.R. Millman, M. Gurtler, M. Segel, A. Van Dervort, J.H. Ryu, Q.P. Peterson, D. Greiner, D.A. Melton, Generation of functional human pancreatic beta cells in vitro, Cell 159(2) (2014) 428-39.
[146] H.A. Russ, A.V. Parent, J.J. Ringler, T.G. Hennings, G.G. Nair, M. Shveygert, T. Guo, S. Puri, L. Haataja, V. Cirulli, R. Blelloch, G.L. Szot, P. Arvan, M. Hebrok, Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro, The EMBO journal 34(13) (2015) 1759-72.
[147] N. Hanley, Closing in on pancreatic beta cells, Nature biotechnology 32(11) (2014) 1100-2.
[148] R.R. Bhonde, P. Sheshadri, S. Sharma, A. Kumar, Making surrogate beta-cells from mesenchymal stromal cells: perspectives and future endeavors, The international journal of biochemistry & cell biology 46 (2014) 90-102.
[149] Chao KC, Chao KF, Fu YS, L. SH., Islet-Like Clusters Derived from Mesenchymal Stem Cells in Wharton's Jelly of the Human Umbilical Cord for Transplantation to Control Type 1 Diabetes, PloS one 3(1) (2008) e1451.
[150] Qu H, Liu X, Ni Y, Jiang Y, Feng X, Xiao J, Guo Y, Kong D, Li A, Li X, Zhuang X, Wang Z, Wang Y, Chang Y, Chen S, Kong F, Zhang X, Zhao S, Sun Y, Xu D, Wang D, Z. C, Laminin 411 acts as a potent inducer of umbilical cord mesenchymal stem cell differentiation into insulin-producing cells, Journal of translation medicine May(20) (2014) 12:135.
[151] H.Y. Lin, C.C. Tsai, L.L. Chen, S.H. Chiou, Y.J. Wang, S.C. Hung, Fibronectin and laminin promote differentiation of human mesenchymal stem cells into insulin producing cells through activating Akt and ERK, Journal of biomedical science 17 (2010) 56.
[152] F.T. Bosman, I. Stamenkovic, Functional structure and composition of the extracellular matrix, J Pathol 200(4) (2003) 423-8.

[153] C. Frantz, K.M. Stewart, V.M. Weaver, The extracellular matrix at a glance, J Cell Sci 123(24) (2010) 4195-200.
[154] J.C. Stendahl, D.B. Kaufman, S.I. Stupp, Extracellular Matrix in Pancreatic Islets: Relevance to Scaffold Design and Transplantation, Cell transplantation 18(1) (2009) 1-12.
[155] G. Nikolova, N. Jabs, I. Konstantinova, A. Domogatskaya, K. Tryggvason, L. Sorokin, R. Fassler, G. Gu, H.P. Gerber, N. Ferrara, D.A. Melton, E. Lammert, The vascular basement membrane: a niche for insulin gene expression and Beta cell proliferation, Developmental cell 10(3) (2006) 397-405.
[156] T. Otonkoski, M. Banerjee, O. Korsgren, L.E. Thornell, I. Virtanen, Unique basement membrane structure of human pancreatic islets: implications for beta-cell growth and differentiation, Diabetes, obesity & metabolism 10 Suppl 4 (2008) 119-27.
[157] M. Zhao, C. Song, W. Zhang, Y. Hou, R. Huang, Y. Song, W. Xie, Y. Shi, C. Song, The three-dimensional nanofiber scaffold culture condition improves viability and function of islets, J Biomed Mater Res A. 94(3) (2010) 667-672.
[158] S. Schneider, P.J. Feilen, V. Slotty, D. Kampfner, S. Preuss, S. Berger, J.r. Beyer, R. Pommersheim, Multilayer capsules: a promising microencapsulation system for transplantation of pancreatic islets, Biomaterials 22 (2001) 1961-1970.
[159] D. Gallego-Perez, N. Higuita-Castro, R.K. Reen, M. Palacio-Ochoa, S. Sharma, L.J. Lee, J.J. Lannutti, D.J. Hansford, K.J. Gooch, Micro/nanoscale technologies for the development of hormone-expressing islet-like cell clusters, Biomedical microdevices 14(4) (2012) 779-89.
[160] N.D. Shalaly, M. Ria, U. Johansson, K. Avall, P.O. Berggren, M. Hedhammar, Silk matrices promote formation of insulin-secreting islet-like clusters, Biomaterials 90 (2016) 50-61.
[161] B.R. Lee, J.W. Hwang, Y.Y. Choi, S.F. Wong, Y.H. Hwang, D.Y. Lee, S.H. Lee, In situ formation and collagen-alginate composite encapsulation of pancreatic islet spheroids, Biomaterials 33(3) (2012) 837-45.
[162] Y. Jun, M.J. Kim, Y.H. Hwang, E.A. Jeon, A.R. Kang, S.H. Lee, D.Y. Lee, Microfluidics-generated pancreatic islet microfibers for enhanced immunoprotection, Biomaterials 34(33) (2013) 8122-30.
[163] Y. Jun, A.R. Kang, J.S. Lee, G.S. Jeong, J. Ju, D.Y. Lee, S.H. Lee, 3D co-culturing model of primary pancreatic islets and hepatocytes in hybrid spheroid to overcome pancreatic cell shortage, Biomaterials 34(15) (2013) 3784-94.
[164] L.M. Weber, K.S. Anseth, Hydrogel encapsulation environments functionalized with extracellular matrix interactions increase islet insulin secretion, Matrix biology : journal of the International Society for Matrix Biology 27(8) (2008) 667-73.
[165] N.E. Davis, L.N. Beenken-Rothkopf, A. Mirsoian, N. Kojic, D.L. Kaplan, A.E. Barron, M.J. Fontaine, Enhanced function of pancreatic islets co-encapsulated with ECM proteins and mesenchymal stromal cells in a silk hydrogel, Biomaterials 33(28) (2012) 6691-7.
[166] J.T. Daoud, M.S. Petropavlovskaia, J.M. Patapas, C.E. Degrandpre, R.W. Diraddo, L. Rosenberg, M. Tabrizian, Long-term in vitro human pancreatic islet culture using three-dimensional microfabricated scaffolds, Biomaterials 32(6) (2011) 1536-42.
[167] J. Liu, S. Liu, Y. Chen, X. Zhao, Y. Lu, J. Cheng, Functionalized self-assembling peptide improves INS-1 beta-cell function and proliferation via the integrin/FAK/ERK/cyclin pathway, Int J Nanomedicine 10 (2015) 3519-31.
[168] H.P. Shih, D. Panlasigui, V. Cirulli, M. Sander, ECM Signaling Regulates Collective Cellular Dynamics to Control Pancreas Branching Morphogenesis, Cell reports 14(2) (2016) 169-79.
[169] T. Kaido, M. Yebra, V. Cirulli, C. Rhodes, G. Diaferia, A.M. Montgomery, Impact of defined matrix interactions on insulin production by cultured human beta-cells: effect on insulin content, secretion, and gene transcription, Diabetes 55(10) (2006) 2723-9.

[170] J. Daoud, M. Petropavlovskaia, L. Rosenberg, M. Tabrizian, The effect of extracellular matrix components on the preservation of human islet function in vitro, Biomaterials 31(7) (2010) 1676-82.
[171] J.H. Ko, Y.H. Kim, S.H. Jeong, S. Lee, S.N. Park, I.K. Shim, S.C. Kim, Collagen esterification enhances the function and survival of pancreatic beta cells in 2D and 3D culture systems, Biochemical and biophysical research communications 463(4) (2015) 1084-90.
[172] S. Yamashita, K. Ohashi, R. Utoh, T. Okano, M. Yamamoto, Human Laminin Isotype Coating for Creating Islet Cell Sheets, Cell Med 8(1-2) (2015) 39-46.
[173] L.G. Villa-Diaz, A.M. Ross, J. Lahann, P.H. Krebsbach, Concise review: The evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings, Stem cells 31(1) (2013) 1-7.
[174] P.Y. Lin, S.H. Hung, Y.C. Yang, L.C. Liao, Y.C. Hsieh, H.J. Yen, H.E. Lu, M.S. Lee, I.M. Chu, S.M. Hwang, A synthetic peptide-acrylate surface for production of insulin-producing cells from human embryonic stem cells, Stem cells and development 23(4) (2014) 372-9.
[175] Jiang FX, Cram DS, DeAizpurua HJ, H. LC., Laminin-1 promotes differentiation of fetal mouse pancreatic beta-cells., Diabetes. 48(4) (1999) 722-30.
[176] Cirulli V, Beattie GM, Klier G, Ellisman M, Ricordi C, Quaranta V, Frasier F, Ishii JK, Hayek A, S. DR, Expression and function of alpha(v)beta(3) and alpha(v)beta(5) integrins in the developing pancreas: roles in the adhesion and migration of putative endocrine progenitor cells, J Cell Biol. 150(6) (2000) 1445-60.
[177] W. Hu, G. Zhao, C. Wang, J. Zhang, L. Fu, Nonlinear optical microscopy for histology of fresh normal and cancerous pancreatic tissues, PloS one 7(5) (2012) e37962.
[178] Jiang FX, Naselli G, H. LC., Distinct distribution of laminin and its integrin receptors in the pancreas, J Histochem Cytochem 50(12) (2002) 1625-32.
[179] Miner JH, P.B. Li C, Laminins alpha2 and alpha4 in pancreatic acinar basement membranes are required for basal receptor localization., J Histochem Cytochem. 52(2) (2004) 153-6.
[180] J.H. Kim, H.W. Kim, K.J. Cha, J. Han, Y.J. Jang, D.S. Kim, J.H. Kim, Nanotopography Promotes Pancreatic Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells, ACS Nano 10(3) (2016) 3342-55.
[181] B.N. Brown, S.F. Badylak, Extracellular matrix as an inductive scaffold for functional tissue reconstruction, Transl Res 163(4) (2014) 268-85.
[182] T.W. Gilbert, T.L. Sellaro, S.F. Badylak, Decellularization of tissues and organs, Biomaterials 27(19) (2006) 3675-83.
[183] T.J. Keane, I.T. Swinehart, S.F. Badylak, Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance, Methods 84 (2015) 25-34.
[184] B.M. Sicari, J.P. Rubin, C.L. Dearth, M.T. Wolf, F. Ambrosio, M. Boninger, N.J. Turner, D.J. Weber, T.W. Simpson, A. Wyse, E.H. Brown, J.L. Dziki, L.E. Fisher, S. Brown, S.F. Badylak, An acellular biologic scaffold promotes skeletal muscle formation in mice and humans with volumetric muscle loss, Sci Transl Med 6(234) (2014) 234ra58.
[185] G.K. Bejjani, J. Zabramski, Safety and efficacy of the porcine small intestinal submucosa dural substitute: results of a prospective multicenter study and literature review, J Neurosurg 106(6) (2007) 1028-1033.
[186] L.J. Ning, Y.J. Zhang, Y. Zhang, Q. Qing, Y.L. Jiang, J.L. Yang, J.C. Luo, T.W. Qin, The utilization of decellularized tendon slices to provide an inductive microenvironment for the proliferation and tenogenic differentiation of stem cells, Biomaterials 52 (2015) 539-50.
[187] E. De Carlo, Baiguera S, Conconi MT, Vigolo S, Grandi C, Lora S, Martini C, Maffei P, Tamagno G, Vettor R, Sicolo N, P. PP, Pancreatic acellular matrix supports islet survival and function in a synthetic tubular device: In vitro and in vivo studies, International Journal of Molecular Medicine 25(2) (2009).

[188] W. Li, S. Lee, M. Ma, S.M. Kim, P. Guye, J.R. Pancoast, D.G. Anderson, R. Weiss, R.T. Lee, P.T. Hammond, Microbead-based biomimetic synthetic neighbors enhance survival and function of rat pancreatic beta-cells, Scientific reports 3 (2013) 2863.
[189] D. Chaimov, L. Baruch, S. Krishtul, I. Meivar-levy, S. Ferber, M. Machluf, Innovative encapsulation platform based on pancreatic extracellular matrix achieve substantial insulin delivery, Journal of Controlled Release (2016).
[190] S.K. Goh, S. Bertera, P. Olsen, J.E. Candiello, W. Halfter, G. Uechi, M. Balasubramani, S.A. Johnson, B.M. Sicari, E. Kollar, S.F. Badylak, I. Banerjee, Perfusion-decellularized pancreas as a natural 3D scaffold for pancreatic tissue and whole organ engineering, Biomaterials 34(28) (2013) 6760-72.
[191] S.H. Mirmalek-Sani, G. Orlando, J.P. McQuilling, R. Pareta, D.L. Mack, M. Salvatori, A.C. Farney, R.J. Stratta, A. Atala, E.C. Opara, S. Soker, Porcine pancreas extracellular matrix as a platform for endocrine pancreas bioengineering, Biomaterials 34(22) (2013) 5488-95.
[192] Y. Katsuki, H. Yagi, T. Okitsu, M. Kitago, K. Tajima, Y. Kadota, T. Hibi, Y. Abe, M. Shinoda, O. Itano, S. Takeuchi, Y. Kitagawa, Endocrine pancreas engineered using porcine islets and partial pancreatic scaffolds, Pancreatology 16(5) (2016) 922-30.
[193] A.D. Agulnick, D.M. Ambruzs, M.A. Moorman, A. Bhoumik, R.M. Cesario, J.K. Payne, J.R. Kelly, C. Haakmeester, R. Srijemac, A.Z. Wilson, J. Kerr, M.A. Frazier, E.J. Kroon, K.A. D'Amour, Insulin-Producing Endocrine Cells Differentiated In Vitro From Human Embryonic Stem Cells Function in Macroencapsulation Devices In Vivo, Stem Cells Transl Med 4(10) (2015) 1214-22.
[194] G.H. Mao, G.A. Chen, H.Y. Bai, T.R. Song, Y.X. Wang, The reversal of hyperglycaemia in diabetic mice using PLGA scaffolds seeded with islet-like cells derived from human embryonic stem cells, Biomaterials 30(9) (2009) 1706-14.
[195] A.J. Vegas, O. Veiseh, M. Gurtler, J.R. Millman, F.W. Pagliuca, A.R. Bader, J.C. Doloff, J. Li, M. Chen, K. Olejnik, H.H. Tam, S. Jhunjhunwala, E. Langan, S. Aresta-Dasilva, S. Gandham, J.J. McGarrigle, M.A. Bochenek, J. Hollister-Lock, J. Oberholzer, D.L. Greiner, G.C. Weir, D.A. Melton, R. Langer, D.G. Anderson, Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice, Nature medicine (2016).
[196] O.M. Sabek, M. Farina, D.W. Fraga, S. Afshar, A. Ballerini, C.S. Filgueira, U.R. Thekkedath, A. Grattoni, A.O. Gaber, Three-dimensional printed polymeric system to encapsulate human mesenchymal stem cells differentiated into islet-like insulin-producing aggregates for diabetes treatment, J Tissue Eng 7 (2016) 2041731416638198.
[197] P. Vaca, F. Martin, J.M. Vegara-Meseguer, J.M. Rovira, G. Berna, B. Soria, Induction of differentiation of embryonic stem cells into insulin-secreting cells by fetal soluble factors, Stem cells 24(2) (2006) 258-65.
[198] L. Przybyla, J. Voldman, Probing embryonic stem cell autocrine and paracrine signaling using microfluidics, Annu Rev Anal Chem (Palo Alto Calif) 5 (2012) 293-315.
[199] G. Cooper, Functions of Cell Surface Receptors., in: S. Associates (Ed.), The Cell: A Molecular Approach., Sunderland (MA)2000.
[200] I.B. Leibiger, B. Leibiger, P.O. Berggren, Insulin signaling in the pancreatic beta-cell, Annu Rev Nutr 28 (2008) 233-51.
[201] Chiu SY, K. S., Neurotransmitter-mediated signaling between axons and glial cells., Glia 11(2) (1994) 191-200.
[202] M. Gnecchi, Z. Zhang, A. Ni, V.J. Dzau, Paracrine mechanisms in adult stem cell signaling and therapy, Circulation research 103(11) (2008) 1204-19.
[203] J.H. Walsh, W.E. Karnes, F. Cuttitta, A. Walker, Autocrine growth factors and solid tumor malignancy, West J Med 155(2) (1991) 152-63.

[204] A.B. Singh, R.C. Harris, Autocrine, paracrine and juxtacrine signaling by EGFR ligands, Cell Signal 17(10) (2005) 1183-93.
[205] T. Doerks, R.R. Copley, J. Schultz, C.P. Ponting, P. Bork, Systematic identification of novel protein domain families associated with nuclear functions, Genome Res 12(1) (2002) 47-56.
[206] A.M. Hofer, E.M. Brown, Extracellular calcium sensing and signalling, Nature reviews. Molecular cell biology 4(7) (2003) 530-8.
[207] W. Ying, M. Riopel, G. Bandyopadhyay, Y. Dong, A. Birmingham, J.B. Seo, J.M. Ofrecio, J. Wollam, A. Hernandez-Carretero, W. Fu, P. Li, J.M. Olefsky, Adipose Tissue Macrophage-Derived Exosomal miRNAs Can Modulate In Vivo and In Vitro Insulin Sensitivity, Cell (2017).
[208] G. Raposo, W. Stoorvogel, Extracellular vesicles: exosomes, microvesicles, and friends, The Journal of cell biology 200(4) (2013) 373-83.
[209] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nature cell biology 9(6) (2007) 654-9.
[210] P.J. Quesenberry, J. Aliotta, M.C. Deregibus, G. Camussi, Role of extracellular RNA-carrying vesicles in cell differentiation and reprogramming, Stem cell research & therapy 6 (2015) 153.
[211] S.E.L. Andaloussi, I. Mager, X.O. Breakefield, M.J. Wood, Extracellular vesicles: biology and emerging therapeutic opportunities, Nat Rev Drug Discov 12(5) (2013) 347-57.
[212] M. Colombo, G. Raposo, C. Thery, Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles, Annu Rev Cell Dev Biol 30 (2014) 255-89.
[213] M. Simons, G. Raposo, Exosomes--vesicular carriers for intercellular communication, Curr Opin Cell Biol 21(4) (2009) 575-81.
[214] Z. Andreu, M. Yanez-Mo, Tetraspanins in extracellular vesicle formation and function, Frontiers in immunology 5 (2014) 442.
[215] E. Cocucci, J. Meldolesi, Ectosomes and exosomes: shedding the confusion between extracellular vesicles, Trends in cell biology 25(6) (2015) 364-72.
[216] S. Kourembanas, Exosomes: vehicles of intercellular signaling, biomarkers, and vectors of cell therapy, Annual review of physiology 77 (2015) 13-27.
[217] S.J. Gould, G. Raposo, As we wait: coping with an imperfect nomenclature for extracellular vesicles, Journal of extracellular vesicles 2 (2013).
[218] M. Franquesa, M.J. Hoogduijn, E. Ripoll, F. Luk, M. Salih, M.G. Betjes, J. Torras, C.C. Baan, J.M. Grinyo, A.M. Merino, Update on controls for isolation and quantification methodology of extracellular vesicles derived from adipose tissue mesenchymal stem cells, Frontiers in immunology 5 (2014) 525.
[219] Théry C, Amigorena S, Raposo G, C. A., Isolation and characterization of exosomes from cell culture supernatants and biological fluids, Current Protocols in Cell Biology, John Wiley & Sons, Inc2006.
[220] I. Helwa, J. Cai, M.D. Drewry, A. Zimmerman, M.B. Dinkins, M.L. Khaled, M. Seremwe, W.M. Dismuke, E. Bieberich, W.D. Stamer, M.W. Hamrick, Y. Liu, A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents, PloS one 12(1) (2017) e0170628.
[221] K.W. Witwer, E.I. Buzas, L.T. Bemis, A. Bora, C. Lasser, J. Lotvall, E.N. Nolte-'t Hoen, M.G. Piper, S. Sivaraman, J. Skog, C. Thery, M.H. Wauben, F. Hochberg, Standardization of sample collection, isolation and analysis methods in extracellular vesicle research, Journal of extracellular vesicles 2 (2013).
[222] H. Kalra, G.P. Drummen, S. Mathivanan, Focus on Extracellular Vesicles: Introducing the Next Small Big Thing, International journal of molecular sciences 17(2) (2016) 170.

[223] E.R. Abels, X.O. Breakefield, Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake, Cell Mol Neurobiol 36(3) (2016) 301-12.
[224] K.M. Kim, K. Abdelmohsen, M. Mustapic, D. Kapogiannis, M. Gorospe, RNA in extracellular vesicles, Wiley Interdiscip Rev RNA (2017).
[225] S. Mathivanan, C.J. Fahner, G.E. Reid, R.J. Simpson, ExoCarta 2012: database of exosomal proteins, RNA and lipids, Nucleic Acids Res 40(Database issue) (2012) D1241-4.
[226] R.J. Simpson, H. Kalra, S. Mathivanan, ExoCarta as a resource for exosomal research, Journal of extracellular vesicles 1 (2012).
[227] S. Mathivanan, R.J. Simpson, ExoCarta: A compendium of exosomal proteins and RNA, Proteomics 9(21) (2009) 4997-5000.
[228] S. Keerthikumar, D. Chisanga, D. Ariyaratne, H. Al Saffar, S. Anand, K. Zhao, M. Samuel, M. Pathan, M. Jois, N. Chilamkurti, L. Gangoda, S. Mathivanan, ExoCarta: A Web-Based Compendium of Exosomal Cargo, J Mol Biol 428(4) (2016) 688-692.
[229] H. Kalra, R.J. Simpson, H. Ji, E. Aikawa, P. Altevogt, P. Askenase, V.C. Bond, F.E. Borras, X. Breakefield, V. Budnik, E. Buzas, G. Camussi, A. Clayton, E. Cocucci, J.M. Falcon-Perez, S. Gabrielsson, Y.S. Gho, D. Gupta, H.C. Harsha, A. Hendrix, A.F. Hill, J.M. Inal, G. Jenster, E.M. Kramer-Albers, S.K. Lim, A. Llorente, J. Lotvall, A. Marcilla, L. Mincheva-Nilsson, I. Nazarenko, R. Nieuwland, E.N. Nolte-'t Hoen, A. Pandey, T. Patel, M.G. Piper, S. Pluchino, T.S. Prasad, L. Rajendran, G. Raposo, M. Record, G.E. Reid, F. Sanchez-Madrid, R.M. Schiffelers, P. Siljander, A. Stensballe, W. Stoorvogel, D. Taylor, C. Thery, H. Valadi, B.W. van Balkom, J. Vazquez, M. Vidal, M.H. Wauben, M. Yanez-Mo, M. Zoeller, S. Mathivanan, Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation, PLoS Biol 10(12) (2012) e1001450.
[230] D.K. Kim, J. Lee, S.R. Kim, D.S. Choi, Y.J. Yoon, J.H. Kim, G. Go, D. Nhung, K. Hong, S.C. Jang, S.H. Kim, K.S. Park, O.Y. Kim, H.T. Park, J.H. Seo, E. Aikawa, M. Baj-Krzyworzeka, B.W. van Balkom, M. Belting, L. Blanc, V. Bond, A. Bongiovanni, F.E. Borras, L. Buee, E.I. Buzas, L. Cheng, A. Clayton, E. Cocucci, C.S. Dela Cruz, D.M. Desiderio, D. Di Vizio, K. Ekstrom, J.M. Falcon-Perez, C. Gardiner, B. Giebel, D.W. Greening, J.C. Gross, D. Gupta, A. Hendrix, A.F. Hill, M.M. Hill, E. Nolte-'t Hoen, D.W. Hwang, J. Inal, M.V. Jagannadham, M. Jayachandran, Y.K. Jee, M. Jorgensen, K.P. Kim, Y.K. Kim, T. Kislinger, C. Lasser, D.S. Lee, H. Lee, J. van Leeuwen, T. Lener, M.L. Liu, J. Lotvall, A. Marcilla, S. Mathivanan, A. Moller, J. Morhayim, F. Mullier, I. Nazarenko, R. Nieuwland, D.N. Nunes, K. Pang, J. Park, T. Patel, G. Pocsfalvi, H. Del Portillo, U. Putz, M.I. Ramirez, M.L. Rodrigues, T.Y. Roh, F. Royo, S. Sahoo, R. Schiffelers, S. Sharma, P. Siljander, R.J. Simpson, C. Soekmadji, P. Stahl, A. Stensballe, E. Stepien, H. Tahara, A. Trummer, H. Valadi, L.J. Vella, S.N. Wai, K. Witwer, M. Yanez-Mo, H. Youn, R. Zeidler, Y.S. Gho, EVpedia: a community web portal for extracellular vesicles research, Bioinformatics 31(6) (2015) 933-9.
[231] D.K. Sarko, C.E. McKinney, Exosomes: Origins and Therapeutic Potential for Neurodegenerative Disease, Front Neurosci 11 (2017) 82.
[232] L. Barile, G. Vassalli, Exosomes: Therapy delivery tools and biomarkers of diseases, Pharmacology & therapeutics (2017).
[233] J. Ngolab, I. Trinh, E. Rockenstein, M. Mante, J. Florio, M. Trejo, D. Masliah, A. Adame, E. Masliah, R.A. Rissman, Brain-derived exosomes from dementia with Lewy bodies propagate alpha-synuclein pathology, Acta Neuropathol Commun 5(1) (2017) 46.
[234] T. Zheng, J. Pu, Y. Chen, Y. Mao, Z. Guo, H. Pan, L. Zhang, H. Zhang, B. Sun, B. Zhang, Plasma Exosomes Spread and Cluster Around beta-Amyloid Plaques in an Animal Model of Alzheimer's Disease, Front Aging Neurosci 9 (2017) 12.
[235] R. Perez-Gonzalez, S.A. Gauthier, A. Kumar, E. Levy, The exosome secretory pathway transports amyloid precursor protein carboxyl-terminal fragments from the cell into the brain extracellular space, The Journal of biological chemistry 287(51) (2012) 43108-15.

[236] K. Yuyama, H. Sun, S. Mitsutake, Y. Igarashi, Sphingolipid-modulated exosome secretion promotes clearance of amyloid-beta by microglia, The Journal of biological chemistry 287(14) (2012) 10977-89.
[237] A. Stuendl, M. Kunadt, N. Kruse, C. Bartels, W. Moebius, K.M. Danzer, B. Mollenhauer, A. Schneider, Induction of alpha-synuclein aggregate formation by CSF exosomes from patients with Parkinson's disease and dementia with Lewy bodies, Brain 139(Pt 2) (2016) 481-94.
[238] M. Grey, C.J. Dunning, R. Gaspar, C. Grey, P. Brundin, E. Sparr, S. Linse, Acceleration of alpha-synuclein aggregation by exosomes, The Journal of biological chemistry 290(5) (2015) 2969-82.
[239] H. Wang, P. Cao, D.P. Raleigh, Amyloid formation in heterogeneous environments: islet amyloid polypeptide glycosaminoglycan interactions, Journal of molecular biology 425(3) (2013) 492-505.
[240] P. Cao, A. Abedini, H. Wang, L.H. Tu, X. Zhang, A.M. Schmidt, D.P. Raleigh, Islet amyloid polypeptide toxicity and membrane interactions, Proceedings of the National Academy of Sciences of the United States of America 110(48) (2013) 19279-84.
[241] J. Seeliger, F. Evers, C. Jeworrek, S. Kapoor, K. Weise, E. Andreetto, M. Tolan, A. Kapurniotu, R. Winter, Cross-amyloid interaction of Abeta and IAPP at lipid membranes, Angew Chem Int Ed Engl 51(3) (2012) 679-83.
[242] A.J. Lakhter, E.K. Sims, Minireview: Emerging Roles for Extracellular Vesicles in Diabetes and Related Metabolic Disorders, Molecular endocrinology 29(11) (2015) 1535-48.
[243] C. Guay, V. Menoud, S. Rome, R. Regazzi, Horizontal transfer of exosomal microRNAs transduce apoptotic signals between pancreatic beta-cells, Cell communication and signaling : CCS 13 (2015) 17.
[244] Palmisano G, Jensen SS, Le Bihan MC, Lainé J, McGuire JN, Pociot F, L. MR., Characterization of membrane-shed microvesicles from cytokine-stimulated β-cells using proteomics strategies, Mol Cell Proteomics 11(8):230-43(8) (2012) 230-43.
[245] Vomund AN, Zinselmeyer BH, Hughes J, Calderon B, Valderrama C, Ferris ST, Wan X, Kanekura K, Carrero JA, Urano F, U. ER, Beta cells transfer vesicles containing insulin to phagocytes for presentation to T cells, Proc Natl Acad Sci U S A. 6(112(40)) (2015) E5496-502.
[246] F. Figliolini, V. Cantaluppi, M. De Lena, S. Beltramo, R. Romagnoli, M. Salizzoni, R. Melzi, R. Nano, L. Piemonti, C. Tetta, L. Biancone, G. Camussi, Isolation, characterization and potential role in beta cell-endothelium cross-talk of extracellular vesicles released from human pancreatic islets, PloS one 9(7) (2014) e102521.
[247] R. Bashratyan, H. Sheng, D. Regn, M.J. Rahman, Y.D. Dai, Insulinoma-released exosomes activate autoreactive marginal zone-like B cells that expand endogenously in prediabetic NOD mice, European journal of immunology 43(10) (2013) 2588-97.
[248] N. Javeed, G. Sagar, S.K. Dutta, T.C. Smyrk, J.S. Lau, S. Bhattacharya, M. Truty, G.M. Petersen, R.J. Kaufman, S.T. Chari, D. Mukhopadhyay, Pancreatic Cancer-Derived Exosomes Cause Paraneoplastic beta-cell Dysfunction, Clinical cancer research : an official journal of the American Association for Cancer Research 21(7) (2015) 1722-33.
[249] S. Heiler, Z. Wang, M. Zoller, Pancreatic cancer stem cell markers and exosomes - the incentive push, World journal of gastroenterology 22(26) (2016) 5971-6007.
[250] D. Wen, Y. Peng, D. Liu, Y. Weizmann, R.I. Mahato, Mesenchymal stem cell and derived exosome as small RNA carrier and Immunomodulator to improve islet transplantation, J Control Release 238 (2016) 166-75.
[251] E. Milbank, M.C. Martinez, R. Andriantsitohaina, Extracellular vesicles: Pharmacological modulators of the peripheral and central signals governing obesity, Pharmacology & therapeutics 157 (2016) 65-83.

[252] Y. Choi, Y. Kwon, D.K. Kim, J. Jeon, S.C. Jang, T. Wang, M. Ban, M.H. Kim, S.G. Jeon, M.S. Kim, C.S. Choi, Y.K. Jee, Y.S. Gho, S.H. Ryu, Y.K. Kim, Gut microbe-derived extracellular vesicles induce insulin resistance, thereby impairing glucose metabolism in skeletal muscle, Scientific reports 5 (2015) 15878.
[253] A. Jalabert, G. Vial, C. Guay, O.P. B.Wiklander, J.Z. Nordin, H. Aswad, A. Forterre, E. Meugnier, S. Pesenti, R. Regazzi, E. Danty-Berger, S. Ducreux, H. Vidal, S. El-Andaloussi, J. Rieusset, S. Rome, Exosome-like vesicles released from lipid-induced insulin-resistant muscles modulate gene expression and proliferation of beta recipient cells in mice, Diabetologia. 59(5) (2016) 1049-58.
[254] Y.S. Takeda, Q. Xu, Neuronal Differentiation of Human Mesenchymal Stem Cells Using Exosomes Derived from Differentiating Neuronal Cells, PloS one 10(8) (2015) e0135111.
[255] Tan CY, Lai RC, Wong W, Dan YY, Lim SK, H. HK., Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models, Stem Cell Reseach & Therapy 10(5(3)) (2014).
[256] Oh K, Kim SR, Kim DK, Seo MW, Lee C, Lee HM, Oh JE, Choi EY, Lee DS, Gho YS1, P. KS., In Vivo Differentiation of Therapeutic Insulin-Producing Cells from Bone Marrow Cells via Extracellular Vesicle-Mimetic Nanovesicles., ACS Nano 9(12) (2015) 11718-27.
[257] I. Konstantinova, E. Lammert, Microvascular development: learning from pancreatic islets, BioEssays : news and reviews in molecular, cellular and developmental biology 26(10) (2004) 1069-75.
[258] Henderson JR, M. MC, A morphometric study of the endocrine and exocrine capillaries of the pancreas., Q J Exp Physiol 70(3) (1985) 347-56.
[259] S. Bonner-Weir, Morphological evidence for pancreatic polarity of beta-cell within islets of Langerhans., Diabetes 37(5) (1988) 616-21.
[260] H. Watada, Role of VEGF-A in Pancreatic Beta Cells, Endocrine Journal 57(3) (2010) 185-191.
[261] M.M. Zanone, E. Favaro, G. Camussi, From Endothelial to beta Cells: Insights into Pancreatic Islet Microendothelium, Current Diabetes Reviews 4 (2008) 1-9.
[262] D. Eberhard, M. Kragl, E. Lammert, 'Giving and taking': endothelial and beta-cells in the islets of Langerhans, Trends in endocrinology and metabolism: TEM 21(8) (2010) 457-63.
[263] R. Olsson, P.O. Carlsson, The pancreatic islet endothelial cell: emerging roles in islet function and disease, The international journal of biochemistry & cell biology 38(4) (2006) 492-7.
[264] P. Quaranta, S. Antonini, S. Spiga, B. Mazzanti, M. Curcio, G. Mulas, M. Diana, P. Marzola, F. Mosca, B. Longoni, Co-transplantation of endothelial progenitor cells and pancreatic islets to induce long-lasting normoglycemia in streptozotocin-treated diabetic rats, PloS one 9(4) (2014) e94783.
[265] V. Cantaluppi, L. Biancone, F. Figliolini, S. Beltramo, D. Medica, M.C. Deregibus, F. Galimi, R. Romagnoli, M. Salizzoni, C. Tetta, G.P. Segoloni, G. Camussi, Microvesicles derived from endothelial progenitor cells enhance neoangiogenesis of human pancreatic islets, Cell transplantation 21(6) (2012) 1305-20.
[266] E.A. Phelps, D.M. Headen, W.R. Taylor, P.M. Thule, A.J. Garcia, Vasculogenic bio-synthetic hydrogel for enhancement of pancreatic islet engraftment and function in type 1 diabetes, Biomaterials 34(19) (2013) 4602-11.
[267] D. Ribeiro, I. Horvath, N. Heath, R. Hicks, A. Forslöw, P. Wittung-Stafshede, Extracellular vesicles from human pancreatic islets suppress human islet amyloid polypeptide amyloid formation, Proceedings of the National Academy of Sciences (2017) 201711389.
[268] R.A. Zuellig, G. Cavallari, P. Gerber, O. Tschopp, G.A. Spinas, W. Moritz, R. Lehmann, Improved physiological properties of gravity-enforced reassembled rat and human

pancreatic pseudo-islets, Journal of tissue engineering and regenerative medicine 11(1) (2017) 109-120.
[269] X. Wang, K. Wang, W. Zhang, M. Qiang, Y. Luo, A bilaminated decellularized scaffold for islet transplantation: Structure, properties and functions in diabetic mice, Biomaterials 138 (2017) 80-90.
[270] B.L. Taylor, F.F. Liu, M. Sander, Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells, Cell reports 4(6) (2013) 1262-75.
[271] S. Bernat, In vitro differentiation of pancreatic beta-cells, Differentiation 68 (2001) 205-219.
[272] H. Liu, H. Yang, D. Zhu, X. Sui, J. Li, Z. Liang, L. Xu, Z. Chen, A. Yao, L. Zhang, X. Zhang, X. Yi, M. Liu, S. Xu, W. Zhang, H. Lin, L. Xie, J. Lou, Y. Zhang, J. Xi, H. Deng, Systematically labeling developmental stage-specific genes for the study of pancreatic beta-cell differentiation from human embryonic stem cells, Cell research 24(10) (2014) 1181-200.
[273] Artner I, Le Lay J, Hang Y, Elghazi L, Schisler JC, Henderson E, Sosa-Pineda B, S. R., MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells, Diabetes 55(2) (2006) 297-304.
[274] H. Xu, K.S. Tsang, J.C. Chan, P. Yuan, R. Fan, H. Kaneto, G. Xu, The combined expression of Pdx1 and MafA with either Ngn3 or NeuroD improves the differentiation efficiency of mouse embryonic stem cells into insulin-producing cells, Cell transplantation 22(1) (2013) 147-58.