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Transcriptomic analysis of pancreatic islet cells derived from stem cells

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Master's Degree in Biotechnology – Molecular Biology

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Abstract

Diabetes mellitus type 1 is an autoimmune disease leading to the destruction of the pancreatic beta-cells. The beta-cells are responsible for secreting the hormone insulin which lowers the blood sugar after a meal. Dysfunction and destruction of the beta-cells leads to an abnormal blood sugar regulation and hyperglycemia. Some papers have also put focus on the dysfunction of alpha cells in type 1 diabetes. The alpha cells are another cell type found in the pancreas and its job is to secrete the hormone glucagon to raise blood sugar levels to avoid hypoglycemia. Both cell types are found clustered together in the islet of Langerhans in the pancreas. From previous research we know that cytokines released by immune cells entering the islets play an important role in the pathogenesis of type 1 diabetes. Less is known on how each of the cell types react to them.

In this study we will differentiate SC-alpha and SC-beta cells from human induced pluripotent stem (iPS) cells as a source for human pancreatic endocrine cells. Then we will treat them and human islets from donors with cytokines, extract RNA and run qPCR on pre-selected genes. This will allow us to compare differences in gene expression between the cell types. We will use an immunoprecipitation (IP) protocol to pull out mRNA-ribosome complexes and compare this to full lysate samples to see the actively translated genes compared to the total transcriptome. Information on the transcriptome is a popular tool to observe the total RNA in a cell, and one can observe changes in the total gene expression in different states such as disease and drug-treatment. However, it fails to capture the fact that the total mRNA transcripts in the cell do not necessarily correlate with the mRNA levels translated. The translation profile, showing actively translated mRNAs, can reveal regulatory variations and can provide insight on the cells current physiological state. RNA-sequencing will be performed on all samples to see the active and the total gene expression profile for each cell type with and without cytokine treatment. A flowchart of the study design can be seen in Figure 1.

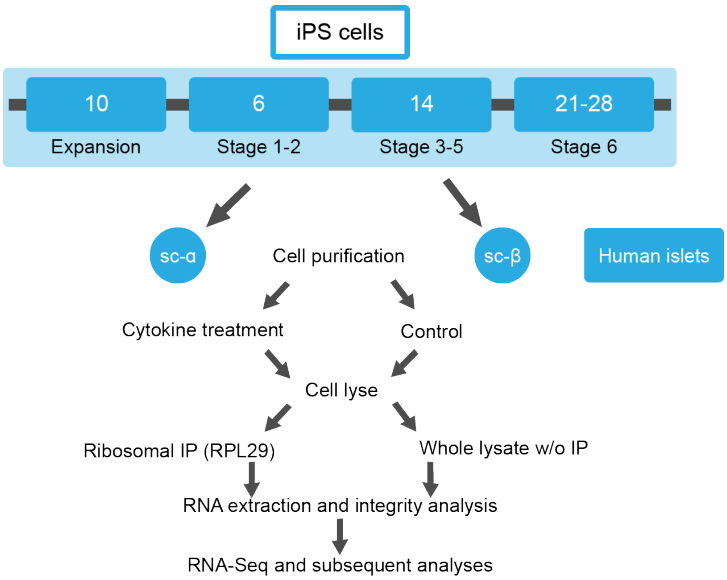

Figure 1: Flowchart of the study design. Study starts on top with differentiation of iPS cells, cell purification, cytokine treatment, IP, RNA extraction and qPCR and RNA-sequencing as the last step.

Sammendrag

Diabetes mellitus type 1 er en autoimmun sykdom som resulterer i ødeleggelse av beta-cellene i pankreas. Beta-cellene er ansvarlige for sekresjon av hormonet insulin som senker blodsukkeret etter et måltid. Dysfunksjonen og ødeleggelse av beta-celler fører til unormal blodsukker-regulering og hyperglykemi. Noen forskningsartikler har også satt fokus på dysfunksjonen av alfa-celler i diabetes type 1. Alfa cellene er en annen celletype som eksisterer i pankreas. Alfa cellens jobb er å sekrere hormonet glukagon som øker blodsukkeret for å avverge hypoglykemi. Begge celletypene er samlet i klaser i Langerhans' øyer i pankreas. Fra tidligere forskning vet vi at cytokiner frigjort fra immun cellene som bryter inn i Langerhans' øyene spiller en viktig rolle i patogenesen til type 1 diabetes. Mindre er kjent om hvordan hver av celletypene responderer på de.

I denne studien vil vi differensiere SC-alfa og SC-beta celler fra humane induserbare pluripotente stam (iPS) celler som en kilde til humane endokrine pankreas celler. Deretter vil vi behandle de og humane Langerhans' øyer fra donorer med cytokiner, ekstrahere RNA og utføre qPCR med forhåndsutvalgte gener. Dette vil gi oss mulighet til å sammenlikne forskjeller i gen ekspresjonen mellom de ulike celletypene. Vi vil bruke en immunpresipitering (IP) protokoll for å trekke ut mRNA-ribosom komplekser og sammenlikne dette med full-lysat prøvene for å observere de aktivt translaterte genene sammenliknet med det totale transkriptomet. Kunnskap om transkriptomet er ett populært verktøy for å observere alt RNA i cellen, og endringer i den totale gen ekspresjonen kan observeres som følge av endringer i situasjon slik som sykdom eller medikament-behandling. Den feiler imidlertid på å fange opp det faktum at den totale mengden mRNA i cellen ikke nødvendigvis korresponderer til mengden mRNA som blir translatert. Translasjonsprofilen, som viser aktivt translaterte mRNA, kan vise variasjoner i regulering og kan gi innsikt i cellens nåværende fysiologiske status. RNA-sekvensering vil bli utført på alle prøvene for å studere det aktive og den totale gen ekspresjonsprofilen for hver celletype både med og uten cytokin behandling. Et flytskjema som viser studiedesignet kan ses i Figur 1.

Table of Contents

Acknowledgments 2

Abstract 3

Sammendrag 5

1.0 Introduction 1

1.1 Diabetes Mellitus 1

1.2 Pancreas 2

1.2.1 Development of the pancreas 2
1.2.2 Islets of Langerhans 3
1.2.3 Alpha cells and glucagon secretion 4
1.2.4 Beta cells and insulin secretion 5
1.2.5 The other endocrine cells in Langerhans 7
1.2.6 Exocrine cells of the human pancreas 8

1.3 Diabetes mellitus type 1 8

1.3.1 Onset and diagnosis 8
1.3.2 Causes and mechanisms 9
1.3.3 Alpha cells in DM1 10
1.3.4 Current therapies 12

1.4 Immune cells and cytokines in diabetes 12

1.4.1 Cytokines impact on pancreatic cells 12
1.4.2 Other stress factors (ER stress) 14

1.5 Stem cell-derived pancreatic endocrine cells for diabetes research 15

1.5.1 Advantages of using stem cell-derived cells 15
1.5.2 Progress in the field 15

2.0 Scientific questions to be addressed in this project 20

3.0 Materials and methods 21

3.1 Thawing and expansion phase of iPS cells 21

3.1.1 iPS cells used in the study 21

3.2 Differentiation of alpha and beta cells 24

3.3 Quality control 29

3.3.1 Cell fixation 29

3.3.2 QC analysis 29
3.4 Cell sorting 31
3.5 Human islets 32
3.6 Cytokine treatment 33
3.7 Cell lysis 33
3.8 Immunoprecipitation of mRNA-ribosome complex 34
3.9 RNA-extraction 35
3.9.1 Extraction 35
3.9.2 Reverse transcription 36
3.10 Gene expression profile 37
3.11 Calculation of changes in gene expression 37
4.0 Results 39
4.1 Differentiation of SC-α/β cells 39
4.2 Quality control during the differentiation 39
4.2.1 SC-α cells 40
4.2.2 SC-β cells 42
4.3 Effect of cytokines on pancreatic cells 44
4.4 Gene expression data 45
4.5 Calculation of gene expression changes 49
4.5.1 Alpha cells 49
4.5.2 Beta cells 50
4.5.3 Human islets 53
5.0 Discussion 57
References 63
Appendix 71

1.0 Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus type 1 (DM1) is an insulin deficiency disease. It is an autoimmune disease where cytotoxic immune cells enter the islets of Langerhans and attack the insulin-producing beta-cells (β-cells) (Bluestone, Herold, & Eisenbarth, 2010). According to the International Diabetes Federation (IDF) Diabetes atlas the number of people suffering from diabetes was 463 million in 2019, with an expected projection of 700 million people suffering from diabetes in 2045 if following the current trend (International Diabetes Federation, 2019). It is estimated that 5-15 percent of diagnosed diabetes cases are type 1 diabetes, and patients are usually diagnosed before age 30 (Jenkins, O'Neal, Nolan, & Januszewski, 2016). Around 128,900 of the newly diagnosed cases each year are children under 19 years of age suffering from type 1 diabetes (International Diabetes Federation, 2019). Diabetes was the 8th leading cause of deaths in 2012, with 1.5 million deaths due to high blood glucose. This number is even higher if deaths due to other diseases related to high blood glucose concentrations is included, which caused another 2.2 million deaths in 2012. Diabetes is also a primary risk factor for other health complications like cardiovascular diseases, retinopathy, and neuropathy. The global diabetes prevalence had increased from 4.7% in 1980 to 8.5% in 2014. There is a variation in the prevalence of diabetes across the world with numbers rising the most in low- and middle-income countries (WHO, 2016).

The β-cells function is to control blood glucose levels to keep it at a narrow, healthy range. The blood glucose level in the body is regulated by sensing the glucose concentration within the islet of Langerhans, where the β-cells are located and releasing the hormone insulin in response to a hyperglycemic environment (high blood sugar), i.e., after a meal. In DM1, these β-cells are destroyed by the body's immune system. Since the immune system is attacking the patients' cells, it is called an autoimmune disease. DM1 is caused by a mixture of genetic predisposition and environmental factors. The environmental factors affecting DM1 is still uncertain, but there are observations that factors like increased hygiene, environmental stress and toxins, or viral infections can affect the development or progression of DM1 (Bluestone et al., 2010). Patients presenting with DM1 symptoms already has an estimated loss of about 50-60% off their total β-cells mass. β-cells cell destruction causes insulin deficiency making patients dependent on exogenous insulin injections permanently (Jenkins et al., 2016). The majority of later studies on viral infections as a activator for DM1 has focused in the

coxsackievirus as a contributor to a starting interferon (IFN) -based inflammation (Mark A. Atkinson, von Herrath, Powers, & Clare-Salzler, 2015).

1.2 Pancreas

1.2.1 Development of the pancreas

The pancreas is a gland organ comprised of two different cell types. A set of exocrine and endocrine cells together make up the pancreas. The organ develops from the endoderm during organogenesis and is vital for nutrient digestion and blood glucose regulation. The exocrine portion consists of acinar cells that secrete enzymes, and duct cells responsible for facilitation of the passage of enzymes to the small intestine. The endocrine portion of cells consists of five different cell types that produce and secrete important hormones. The endocrine cells clump together to form the islets of Langerhans (see chapter 1.2.2) (Pan & Wright, 2011). The pancreas consists of around 90% acinar cells while the islets of Langerhans make up around 1-2% of the pancreatic cell mass (Larsen & Grapin-Botton, 2017).

There is still a lack of knowledge on the developmental steps of the human pancreas, and research still heavily leans on the knowledge we have gained from pancreas development in the mouse (Pan & Brissova, 2014). Many of the important genes involved in the pancreatic development come from transgenic mouse experiments. From these experiments we have found important steps for the development of pancreatic cells. Some examples are: Pancreatic duodenal homeobox 1 (Pdx1) is a vital transcription factor in the pancreatic development, and elimination of this factor leads to a complete lack of pancreas. Pax6 deficient mice lack alpha-cells (α-cells), and a combination of Pax6 and Pax4 absence leads to a failure in development for all pancreatic endocrine cells. Elimination of the homeobox gene Nkx6.1 disrupts the β -cell precursor stage and is therefore a vital β cell marker (Poudel, Savari, Tekin, & Hara, 2016). MafA is a marker of mature β-cell function as it is a transcription factor regulating the expression of insulin (NCBI, 2020). Sox17 is an important regulator needed for the endoderm formation in early development. Sox17 expression seem to be important in regulation of the formation and segregation of the pancreas and the biliary system (Pan & Wright, 2011; Spence et al., 2009). The pancreas organogenesis starts out with a population of pancreatic progenitor cells expressing pancreatic cell markers at varying levels. These cells have the potential to differentiate into all cell types found in the pancreas. Signaling during development leads to segregation of the cell lines (Figure 2) (Larsen & Grapin-Botton, 2017).

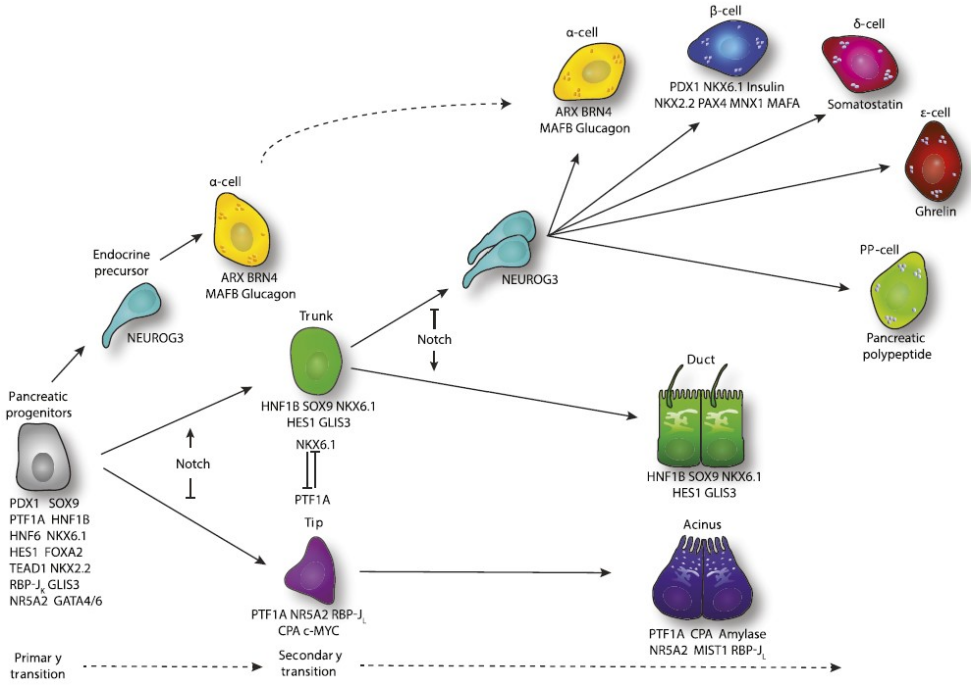

Figure 2:Lineage hierarchy in the pancreas organogenesis. Borrowed from: (Larsen & Grapin-Botton, 2017).

1.2.2 Islets of Langerhans

The human pancreas has an endocrine portion of cells responsible for producing and secreting hormones. The cells are responsible for the regulation of blood glucose and other resources of energy in the body. The islets of Langerhans consist of five different cell types (Figure 3):

- Alpha (α)–cells producing glucagon
- Beta (β)-cells producing insulin
- Delta (δ)–cells producing somatostatin
- Pancreatic polypeptide (PP) cells (γ-cells) producing the pancreatic polypeptide
- Epsilon (ε)-cells producing ghrelin

(Jenkins et al., 2016).

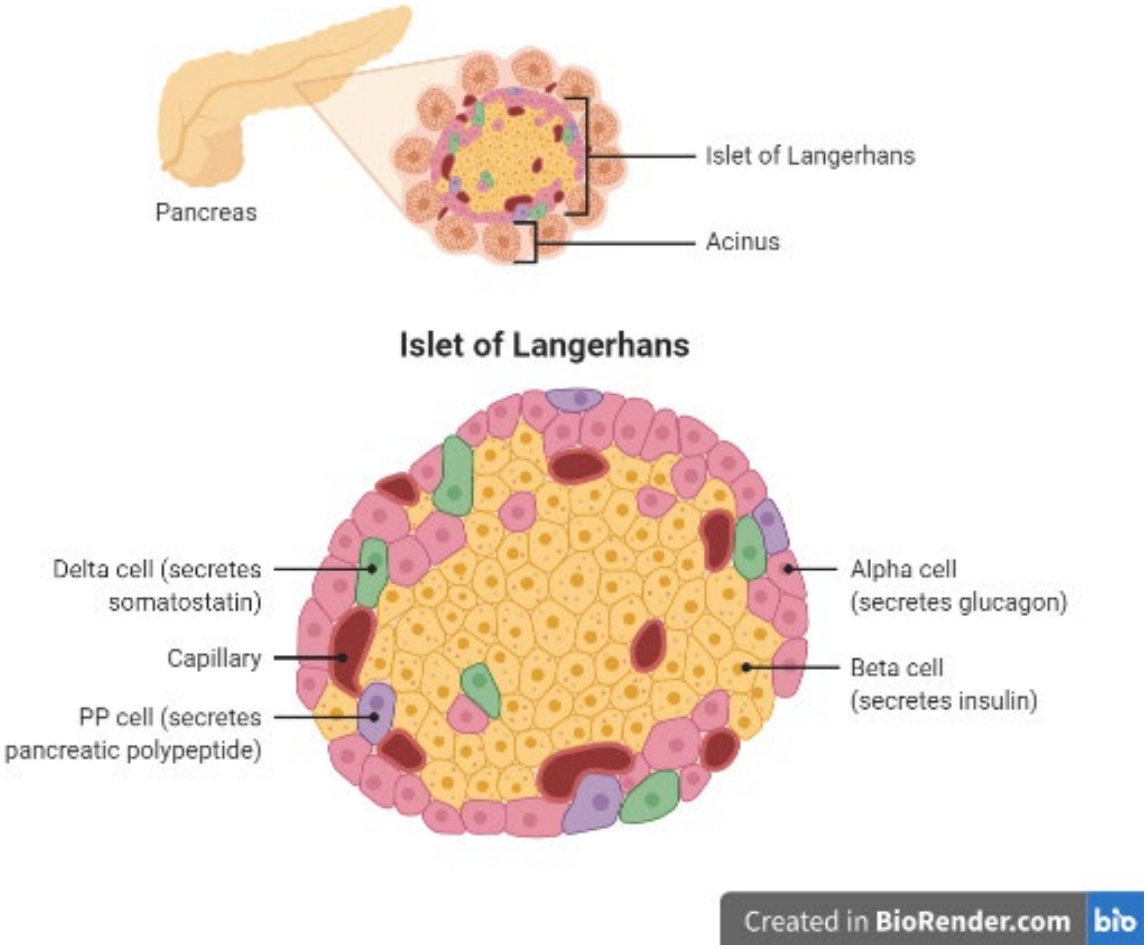

Figure 3: Illustration of the islets of Langerhans containing delta cells, capillaries, PP-cells, alpha cells and beta cells. (Created with BioRender.com)

The blood glucose regulation is strictly maintained to be within a healthy range in non-diabetic individuals. In a fasted state the blood glucose should range from 3.5 to 5.5 mmol/L, and between 5.0 to 7.5 mmol/L in the fed state (Jenkins et al., 2016).

1.2.3 Alpha cells and glucagon secretion

α-cells secrete the hormone glucagon to rectify a hypoglycemic event (low blood sugar) and is the counter-regulatory hormone for insulin and mainly functions in the liver. The release of glucagon stimulates the liver to create glucose via breakdown of stored glycogen (glycogenolysis) and creation of glucose (gluconeogenesis) in order to increase the blood glucose level (Jenkins et al., 2016). Glucagon release in a normoglycemic or hyperglycemic environment is inhibited by insulin and other bi-products released from the nearby β-cells in

the islet. In a hypoglycemic state, the β-cells will decrease their insulin secretion, which in turn stimulates glucagon secretion (Figure 4). The glucagon secretion can also be inhibited by the secretion of somatostatin from δ-cells in the Langerhans (Da Silva Xavier, 2018; Kulina & Rayfield, 2016).

1.2.4 Beta cells and insulin secretion

The β-cell is the most studied cell-type in connection to diabetes research. The cell releases the hormone insulin in response to high levels of glucose or by stimulation from neurotransmitters. Insulin triggers cells to take up glucose from the blood and is important for the glucose homeostasis to lower the blood glucose. It binds to the insulin receptor on cells for example in the liver, adipose tissue, skeletal- and cardiac muscles. In the liver insulin activates the synthesis of glycogen from excess glucose and inhibits glycogenolysis and gluconeogenesis (Figure 4). This is the opposite effect on blood glucose as the glucagon secreted by α cells (Jenkins et al., 2016).

Insulin secretion from β-cells is inhibited by certain hormones or signaling molecules, such as somatostatin, ghrelin and leptin (Da Silva Xavier, 2018). Insulin secretion is boosted by the hormones released by the gut after a meal, incretins. Incretins hormones secreted by enteroendocrine cells found in the gut. After a meal the release of incretins will help upregulate the secretion of insulin to help lower the blood glucose level. The effects of incretin of insulin release can be seen in healthy individuals where the insulin release is much higher after a dose of oral glucose compared to an intravenous glucose delivery. This phenomenon is called the incretin effect (Jenkins et al., 2016; Kim & Egan, 2008).

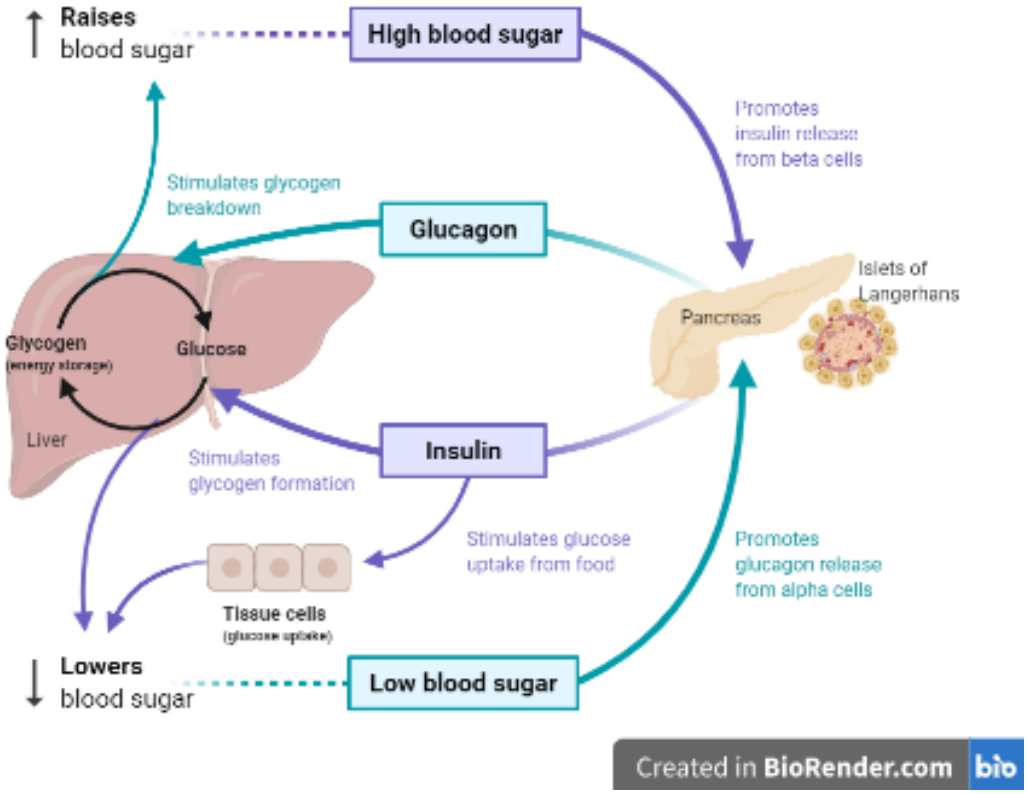

Figure 4: Illustration of blood sugar regulation. (Created with BioRender.com)

Insulin is primary translated with 4 chains where the first is a signal sequence. This state is called preproinsulin. After cleavage of the signal sequence the insulin is in its proinsulin step. These two stages are inactive versions of insulin. The C-chain called C-peptide is cleaved when the proinsulin has reached the secretory granule. C-peptide is therefore secreted together with the insulin (Figure 5) (Jenkins et al., 2016). The co-secretion of the two means that measurement of the presence of C-peptide can be associated with the secretion of insulin.

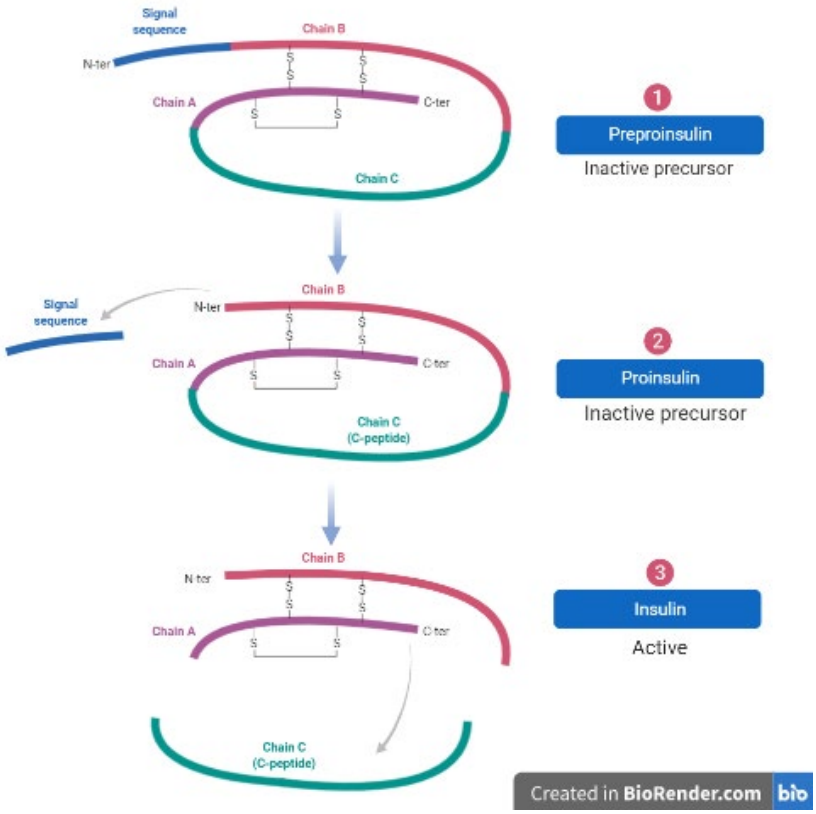

Figure 5: The structure of insulin. 1) preproinsulin containing a signal sequence. 2) proinsulin. 3) active form of insulin and cleaved off C-peptide chain (Created with Bioender.com)

1.2.5 The other endocrine cells in Langerhans

The islet of Langerhans contains other endocrine cells, but less is known about their functions in blood glucose regulation. δ-cells produce and secrete the hormone somatostatin. This hormone is also expressed elsewhere in the body, and one of its known functions is as an inhibitor for growth hormone. δ-cells and somatostatin's interactions in the islet are understudied but are gaining some attention now (Huising, Meulen, Huang, Pourhosseinzadeh, & Noguchi, 2018). δ-cells partakes in the intra-islet control of insulin, glucagon and PP secretion as a paracrine inhibitor and plays a role in regulating blood glucose and setting the glucose homeostatic set point. Somatostatin secretion is promoted by the hormone ghrelin and the protein urocortin (Da Silva Xavier, 2018). It is suggested that diabetes can affect δ-cells or somatostatin release, further complicating the glucose regulation (Arrojo E Drigo et al., 2019). Research on diabetic rat models suggests that excess somatostatin secretion during insulin-induced hypoglycemia may cause decreased counter-regulatory glucagon response. Experiments on the rat model show that the treatment addition

of a somatostatin-inhibitor might help restore the glucagon counter-regulatory response (Rorsman & Huising, 2018).

Pancreatic polypeptide (γ) cells release pancreatic polypeptide triggered by nervous stimulation after a meal and its main function seems to be a satiety hormone. Studies show that it's a part of regulating glucagon secretion from near-by α-cells (Aragon et al., 2015; Batterham et al., 2003).

ε-cells secrete the hormone ghrelin. Little research has been done on this hormone, but it appears to be an inhibitor of insulin as well as a regulator for glucagon, PP and somatostatin secretion in the other islet cell types (Da Silva Xavier, 2018).

1.2.6 Exocrine cells of the human pancreas

The majority of the pancreatic cell mass contains exocrine cells. These cells produce and secrete inactive digestive enzymes into the pancreatic ducts. By secreting inactivated digestive enzymes, the cells in the pancreas are protected from the digestive effects (autodigestion). These enzymes travel through the ducts and are released into the duodenum, the first section of the small intestine. When they reach the duodenum, the enzymes are activated by the change in pH. The production and secretion of digestive enzymes are stimulated by intake of food and changes in the distension of the stomach and intestine, gut hormones and the nervous system (Jenkins et al., 2016). The active enzymes catalyze the breakdown of carbohydrates, proteins and lipids from the diet (Larsen & Grapin-Botton, 2017).

1.3 Diabetes mellitus type 1

1.3.1 Onset and diagnosis

Type 1 diabetes most commonly has its onset in children or young age. Noticeable signs of diabetes are high level of thirst and hunger, increased urination (some experience bed-wetting), fatigue, blurry vision and weight-loss. Some patients may also experience diabetic ketoacidosis. This is a dangerous condition where insulin levels are low and blood glucose levels are high. Because the body cannot utilize the glucose due to insulin deficiency it starts

to break down fat for energy. This fat breakdown causes an increase in ketones making the blood acidic (American Diabetes Association, 2020a, 2020b).

1.3.2 Causes and mechanisms

Most studies on DM1 has been performed on animal models, often mice or rats (murines). Although they are not a match to the disease picture seen in humans, they share a lot of similarities. Non-obese diabetic (NOD) mice studies have shown that the autoimmune attack in DM1 is caused by an error in the regulation of the immune system. This leads to a destruction of β-cells by a combination of the innate immune system, B-lymphocytes and $CD4^+$ and $CD8^+$ T-cells. In NOD mice dendritic cells and macrophages are the first to enter the islet of Langerhans. A short time after this T-cells can be found around the islets, potentially after activation in the lymph nodes surrounding the pancreas. Increased β-cell death leads to new activated T-cells as they are presented with new β-cell epitopes. The major histocompatibility complex (MHC) class I and II T-cells have been discovered in blood-samples from DM1 patients and found in mouse models. Studies show that the cells attacking the islets create an inflammatory response with cytotoxicity-inducing molecules (cytokines) (M. A. Atkinson et al., 2011; Bluestone et al., 2010).

Newer studies add other aspects to why and how DM1 develops. Earlier it was assumed that humans had a set number of β-cells and that a loss of these resulted in diabetes. More recent studies show that the number of β-cells can differ greatly between people independent on factors like age, sex and BMI. The timeline from DM1 onset to clinical presentation of DM1 symptoms can then be differ between patients based on their individual starting β-cell mass. There is also new data showing that there are variations in the β-cells susceptibility to the cytokines and immune cells. This can affect the progression timeline of DM1, and explain why some examinations still discover remaining insulin-positive β-cell post-mortem in advanced-stage DM1 patients (Mark A. Atkinson et al., 2015; Keenan et al., 2010).

The Joslin Medalist study showed that patients who had been suffering from DM1 for more than 50 years still showed signs of low insulin secretion. The study included 411 patients who had been dependent on exogenous insulin for more than 50 years. They found that several of the patients still had measurable C-peptide levels in their blood, suggesting some quantity of β-cell survival and function through secretion. Post-mortem examinations of 9 of the patients

pancreases showed the presence of insulin positive cells regardless if the patient had presented with detectable C-peptide levels on earlier examinations (Keenan et al., 2010).

1.3.3 Alpha cells in DM1

Research into both the molecular background of diabetes and the blood glucose regulation has focused mainly on the β-cells, and less is known about the α-cells, especially in the context of diabetes (Bru-Tari et al., 2019). Most α-cell studies have been performed on animals, usually murine models, that that have a different islet structure than humans. Murines have an islet with β-cells in the center (60-80%) with α-cells surrounding them (15-20% of cells). Humans have a random distribution of β-and α-cells with a more equal portion of each cell type (50-60% β, and 30-45% α). This can affect the cell-signaling within the islet, thereby effecting our knowledge on the α-cell regulation (Yosten, 2018).

A research paper from 2019 found that using a new mouse model, RIP-B7.1, for experimental autoimmune diabetes (EAD) showed a disease pattern closer to the one found in humans than earlier mouse models. They found that the α-cell mass and cell number was decreased in what they defined as advanced-stage diabetes, while it was unaffected during the early-stage. The proliferation rate had increased, and they saw signs of neogenesis, there was also a lack of apoptosis at the advanced stage. Since these findings are contradictory, it suggests a complex regulation of the total α-cell mass. They also found bi-hormonal (glucagon and insulin expressing) and MafA positive cells in both the early-stage and the advanced-stage supporting other papers that suggest cell plasticity by α- to β-cell transdifferentiation as a β-cell regeneration pathway (Bru-Tari et al., 2019). The same transdifferentiation with bihormonal cells has not been proved in naturally humans. Researchers have found that DM1 changes the expression dictating α-cell identity and that some cells express Nkx6.1 that is a β cell-specific marker. This knowledge could mean that α- to β-cell conversion might be possible with added signal cues to force transdifferentiation (Brissova et al., 2018). Another group tried to force transdifferentiation of human α-cells and PP-cells to β-cells using the β-cell gene expression markers Pdx1 and MafA. Their reprogramming protocol leads to β cell-like cells that were able to secrete insulin in response to a stimulus with glucose. The converted α-cells could reverse diabetes when transplanted into mice, and transcriptomic analysis of the cells showed that they continued expression with α-cell markers as well as the β-cell markers. The cells also appeared to be less immunogenic compared to native β-cells (Furuyama et al., 2019).

Some researchers believe that hyperglycemia in diabetes is caused by unregulated α-cells rather than purely β-cell loss (Unger & Cherrington, 2012). This is supported by the discovery that hyperglucagonemia is a common finding in diabetes patients with high blood glucose concentrations. They found that high glucagon concentrations are caused by an upregulation of glucagon secretion rather than an increase in α-cell mass. They also found that exogenous insulin could not regulate the glucagon expression suggesting that intra-islet insulin is needed for paracrine control to help suppress the glucagon expression (Meier, Ueberberg, Korbas, & Schneider, 2011). Exogenous insulin is used in a concentration that is safe for the peripheral tissues, resulting in the concentration within the islet being lower than with β-cell expression. Experiments have been done using somatostatin, pramlintide (amylin analog), or leptin as glucagon suppressors to lower hyperglucagonemia with better glycemic control as a result. This suggests that monotherapy with insulin is not satisfactory in controlling hyperglycemia alone (Unger & Cherrington, 2012). However, an experiment on mice found that glucagon inhibition as a supplementary treatment to exogenous insulin may only be beneficial in patients with some remainder of endogenous insulin secretion. For mice, without endogenous insulin, and with glucagon inhibition, the hypoglycemic state could not be rectified (Damond et al., 2016). The reason behind the hyperglucagonemia is uncertain in DM1, but researchers believe it can be connected to the loss of communication between α-cell and β-cells. It is also believed that it can be influenced by the α-cells higher resistance to stress. While the β-cells are negatively impacted by different stress signals, glucagon expression is enhanced by the same signals (Yosten, 2018). It is believed that one of the reasons α-cells are more resistant to oxidative stress than the β-cells is because they have a higher expression of antioxidant enzymes, particularly catalase (Bloch, Shichman, Vorobeychik, Bloch, & Vardi, 2007).

DM1 patients with advanced-stage diabetes can often also have an increase in hypoglycemic events. This is caused by the α-cells incorrect response to low glucose levels where glucagon seems to be produced but not secreted (Yosten, 2018). A research paper show that the DM1 α-cells display a change in genes directly connected to α-cell identity. It is believed that these changes might, directly and indirectly, affect the glucagon secretion pathway making the α-cells abnormally express glucagon. It is uncertain why the α-cells are affected by DM1. It is believed that the α-cells could also be affected by the attacking immune cells like the β-cells are. It could also be caused by the lack of α- to β cell contact, the lack of intra-islet insulin, or the hyperglycemic environment (Brissova et al., 2018).

1.3.4 Current therapies

Currently there is no cure for diabetes. In some countries one can get a pancreas or islet transplantation, but there is a scarcity of donors and patients will be forced to a life on immunosuppressive medication which comes with its own risks. Newer research is looking into the potential of using stem cells or induced pluripotent stem (iPS) cells as an unlimited resource for cells that can be differentiated into pancreatic cells and used in treatment. Transplantations also come with the risk of graft rejection. There are current research looking into methods to minimize the likelihood of graft rejections including encapsulation techniques and targeted immunosuppression treatments (Thomas, Graham, Loudovaris, & Kay, 2016).

Existing treatments for diabetes often involve exogenous supply of the pancreatic hormones to help rectify the glucose instability. Most used is exogenous insulin to lower blood sugar, either through syringes or external/internal pumps. Drugs to treat a severe hypoglycemic occurrence might also be needed on occasion through glucagon injections. Patients need to monitor their blood sugar levels constantly and control it with insulin or with intake of food. Patients can use glucose testing instruments to check their blood sugar regularly. In some countries one can get constant glucose monitoring instruments. These can also be connected to pumps that can respond to high blood sugar with the release of an appropriate dose of insulin. Some diabetics also require other drugs to treat medical problems that arise as a complication of their diabetes i.e. medicines to help with vascular diseases (Jenkins et al., 2016).

1.4 Immune cells and cytokines in diabetes

1.4.1 Cytokines impact on pancreatic cells

The pathogenesis of DM1 and the involvement of cytokines in the disease initiation and progression in humans is still understudied. Histological samples from humans with DM1 is limited to patients that are already presenting with clinical symptoms. This means that it is difficult to study the pathogenesis before disease reaches the level of severity where the clinical symptoms are already present. At this time the disease will already be in the later stages with major β cell damage (Rabinovitch & Suarez-Pinzon, 2003).

Some studies have shown a potential link between DM1 and previous Coxsackievirus infections. One study found that there was an increase in production of the cytokine INF-γ in

newly diagnosed DM1 patients compared to healthy individuals. The INF-γ production from T-cells were discovered using a coxsackievirus antigen, suggesting that coxsackievirus infection and islet autoimmunity might be connected (Varela-Calvino, Ellis, Sgarbi, Dayan, & Peakman, 2002).

A study showed that a experimental autoimmune diabetes (EAD) model could be created in mice by immunizing them with DNA-vaccines created from preproinsulin (Karges et al., 2007). Preproinsulin is the complete translated mRNA strand for insulin (Jenkins et al., 2016). About 3-4 weeks after the vaccination the mice had developed diabetes, and the islets showed invasion of both $CD8^+$ - and $CD4^+$ - T cells. The EAD mouse model is dependent on $CD8^+$ T-cells, but not the $CD4^+$ T-cells according to the study according to their T-cell depletion experiments (Karges et al., 2007). A large histopathology study of donor islets from patients with DM1 ranging from 1 week to 8 years after diagnosis show $CD8^+$ T-cells that recognize islet antigens. This is an indication that the T-cells likely play a major role in the establishment of DM1. Newly diagnosed patients show specificity for one T-cell, while later stage DM1 patient islets have T-cells with more than one specificity (Coppieters et al., 2012).

Regulatory T cells (Treg) are a specialized group of T cells that help suppress the immune response by having the opposite function of an autoreactive T effector cell (attacking T-cell). A theory is that the Treg cells protect the β-cells from the T effector cells associated with autoimmune cell destruction, and that a disproportion between these cell types are one of the explanations for the β-cell death. A study by Wang et. al. showed that DM1 patients have significantly lower amounts of the protecting Treg cells compared to healthy individuals (Wang, Yan, Xu, Yin, & Hui, 2019). DM1 patients had a higher expression of pro-inflammatory cytokines, and a lower expression of anti-inflammatory cytokines. A transfusion of Treg cells into DM1 model rats show that a increased number of Treg cells lowered the expression of pro-inflammatory cytokines and increased the expression of anti-inflammatory cytokines (Wang et al., 2019).

Expression of the cytokine IFN-α is seen in the islets of DM1 patients. IFN-α plays a major role in the earlier stages of DM1, and previous research has shown that blocking of the IFN-α receptors can prevent diabetes in mice models. IFN-α and its role in DM1 is understudied in human β-cells. A study has found the expression of IFN-α to be a cause of upregulation of the human leukocyte antigen (HLA) class 1, inflammation markers and ER (endoplasmic reticulum) stress in β cells (Marroqui et al., 2017). Several papers have confirmed that

cytokines affect the gene expression and the following proteins in β-cells. A new study shows the dynamics of the chromatin after exposure to pro-inflammatory cytokines. Around 2600 regulatory elements in the β -cell are cytokine responsive. Their study also indicated that exposure to cytokine induce three-dimensional changes in the chromatin allowing other target gene promotors to be available (Ramos-Rodríguez et al., 2019).

1.4.2 Other stress factors (ER stress)

The endoplasmic reticulum (ER) is responsible for the proper folding if newly synthesized proteins. The β-cells can produce up to 1 million insulin molecules per minute to rectify a hyperglycemic state. The unfolded protein response (UPR) is a quality control system in the ER to make sure that all the proteins are properly folded before they can escape the ER. An imbalance in the relationship between folding capacity and the amount of unfolded protein causes ER stress and triggers the UPR as a response. The calcium concentration is higher in the ER than in the cytosol, and is needed to retain the proper environment for protein folding. This gradient is generated by protein pumps that pump calcium into the ER lumen. In DM1 there is a hyperactivation of the UPR that causes dysfunction and apoptosis in β-cells. Factors like cytokines and hyperglycemia induce ER stress and can cause ER calcium depletion leading to progressive ER stress (Cardozo et al., 2005; Clark & Urano, 2016). The ER calcium depletion has been shown to affect the number of insulin granules that are transferred to the antigen presenting cells. These cells are presented with a higher number of misfolded insulin proteins. These misfolded proteins are unknown to the immune system and can potentially be classified as neoantigens. As they are not recognized by the immune cells they can be triggers of autoimmunity, leading the immune cells to kill the β-cells (Clark & Urano, 2016). A constant hyperactivation of the UPR triggers apoptosis for the β-cells. It is uncertain how the cytokines induce ER stress. Studies suggest that in rats the cytokines induce nitric oxide (NO) production that affects the ER stress. The study found that the mouse had induction in ER stress that is mostly independent from NO, and that human β-cells have a ER stress induction that is not dependent on NO production (Brozzi et al., 2015).

1.5 Stem cell-derived pancreatic endocrine cells for diabetes research

1.5.1 Advantages of using stem cell-derived cells

There is a need for treatment options for diabetes other than exogenous insulin injections. One treatment option that has been tested is replacement therapy. Options like full pancreas transplants, or islet transplants have been achieved and show benefitting effects on diabetes patients. However, these treatments depend on cadaveric donors, of which there is a shortage (Ryan et al., 2005). It is, therefore, a need for therapeutic replacement options that are limitless in cell numbers. Pluripotent stem cells are an option to create unlimited numbers of cells due to their renewal capabilities. Both embryonic stem (ES) cells and induced pluripotent stem (iPS) cells can be used as options, but the use of either of them needs to be evaluated based on safety and ethical considerations (Schroeder, 2012). There is also research into how transplanted islets, both from donors and created from stem cell therapies can be protected from the patients' immune system using encapsulation techniques (Orlando et al., 2014).

The differentiation of ES cells or iPS cells into pancreatic cells requires an in-depth knowledge of the transcription factors needed to recapitulate the *in vivo* pancreatic cell development. Successful differentiation protocols for pancreatic cells can provide a tool both for clinical research and potential treatments (Randolph, Bhattacharyya, & Lian, 2019). Access to a sustainable and consistent cell source would help the problem of limited access to human islets for research purposes. It could create a new opportunity for possible drug screenings, and help give insight into disease modeling (Pagliuca et al., 2014).

1.5.2 Progress in the field

The first paper showing the successful differentiation of β-like cells was published in 2006. The group used human ES cells in their five-stage differentiation protocol, where stage 1 pushed the ES cells toward the definitive endoderm fate. Stage 2 and 3 forced the expression towards the primitive gut tube and then the posterior foregut development, and stage 5 was endocrine hormone expressing cells (endocrine precursor cells). The cells in this experiment were polyhormonal. They focused their further studies on the cells that expressed insulin and found that the cells did not have all the β cell-specific markers. The C-peptide secretion following glucose stimulation showed minimal response giving the cells closer resemblance

to immature fetal β-cells than mature β-cells (D'Amour et al., 2006). In 2008 the same group published a new paper where they used an optimized version of their previous protocol. In this experiment, they made implants of the unpurified cells during stage 4 and implanted them in immunocompromised mice. The SC-β cells secreted insulin after glucose stimulation at the same level as adult human islets engrafted into mice, and 92% of the mice had high enough insulin levels to protect them from streptozotocin (STZ) – induced hyperglycemia. This, proving that the cells had differentiated and matured *in vivo* (Kroon et al., 2008).

Most differentiation protocols on pancreatic cells that are published have focused on the differentiation of β-cells. In 2011, a paper was published on an α-cell differentiation protocol from hES cells. They based their protocol on a similar differentiation as with earlier β-cell protocols and created a 6-stage method to mimic the natural α-cell development. Glucagon secretion was detected from the cells in stage 5. However, they were not monohormonal cells as they also expressed insulin. They kept the stage 6 cells in culture for an extended time to let the cells mature. After some time, they saw a decrease in insulin-positive cells and an increase in glucagon expressing cells proving *in vitro* maturation. They also looked for maturation *in vivo* by transplanting immature stage 6 cells into mice. The same signs of maturation were seen in the grafts four months after transplantation as with the *in vitro* maturated cells (Rezania et al., 2011).

The potential to create pluripotent cells from differentiated cells created a new source of cells after its discovery in 2006. The Takahashi and Yamanaka group found that cells could be forced back to their pluripotent state by induction of the pluripotency markers Oct4, c-Myc, Sox2 and Klf4 (iPS cells) (Takahashi & Yamanaka, 2006). As this method has been further developed the iPS cells have been popular in research as an unlimited cell source. The earlier β-cell differentiation protocols used did not manage to create cells that are distinctly similar to true human β-cells and were still missing either one or more distinct β-cell abilities. This changed in 2014 when a paper was published reporting monohormonal SC-β cells with key expression markers that were able to respond to glucose with insulin secretion both *in vitro* and *in vivo*. They managed to create these cells using both hES cells, and with human iPS cells. The 6 stage protocol involved a new scalable 3D culturing system where the cells were differentiated in suspension and used a modified version of older published differentiation protocols (Pagliuca et al., 2014). Another group published a 7 stage β-cell differentiation protocol the same year. They made a different protocol based on earlier publications. The differentiation resulted in cells with high β-cell resemblance. The cells secreted insulin after

glucose stimulation and showed the expression of vital β-cell markers. They also tried their protocol on both hES cells and hiPS cells and achieved good results with both. The β-cells were similar to true β-cells and were able to secrete insulin after implantation into mice. They did, however, discover during extensive analysis that some traits of their cells were not identical to human islets. Their cells had a slower insulin release after glucose stimulation compared to human islets and concluded that their stage 7 cells are less functionally mature when compared to human islets (Rezania et al., 2014).

Experiments on performing differentiation protocols on hiPS cells from type 1 diabetic patients were performed by the D. Melton and F.W. Pagliuca group in 2014. For the starting cell, they used fibroblasts from DM1 patients. They found that the SC-β cells created from DM1 patients showed a great resemblance to the SC-β cells created from non-diabetics they published in 2014, but neither of them is identical to true adult β-cells (Millman et al., 2016; Pagliuca et al., 2014). Both SC-β cell types produced insulin *in vivo*, and the researchers did not find any differences between the two cell lines in the time-period they studied them. Exposure to a cocktail of cytokines to incuse stress response consisting of tumor necrosis factor (TNF) -α, interferon (IFN) -γ and interleukin (IL)-1β showed that both SC-β cell lines lost β-cell expression markers with no clear differences in cytokine sensitivity. This suggests that iPS cells from DM1 patients could also potentially be a source for replenishing β-cells in DM1 therapy (Millman et al., 2016).

A paper was published in 2019 that used single-cell sequencing to look at the different cell-types that can arise from the same β-cell protocol. The SC-β cells created were highly similar to cadaveric β-cells in their insulin secretion abilities, and their cell-specific markers like Nkx6.1. However, they found that there are still some differences in gene expression between SC-β cells and cadaveric β-cells. The researchers believe that these missing β-cell markers i.e. MafA might be expressed after maturing further *in vivo*. The single-cell sequencing also discovered SC-α like cells from their SC-β cell differentiation, but with polyhormonal abilities. These cells are believed to help regulation of β-cells if transplanted together possibly (Veres et al., 2019).

Some experiments following differentiation of SC-β cells require a collection of as pure as possible cells. Sorting out the positive SC-β cells can be done in different ways. Veres et al. showed that magnetic sorting utilizing the surface marker CD49a works to sort out SC-β cells to a purity of about 80% (Veres et al., 2019). Micallef et al. showed a method where they

inserted a green fluorescent protein (GFP) into the insulin locus of hES cells. This made them able to look at the GFP signal as a marker for cells expressing insulin. This made it easier to follow the SC-β cell development, as well as sorting out insulin-positive cells using the GFP combined with fluorescence-activated cell sorting (FACS) (Micallef et al., 2012).

iPS cells have the potential to be used in diabetes research to advance the knowledge off the initiation and progression off the disease. A recent paper differentiated β cells from iPS cells to look at their interactions with the cytokines IFN-γ and IL-1β, or INFα alone. They used a mixture of seeded β-cells, α-cells and polyhormonal cells in their study. They found that the cells expressed receptors for the cytokines at comparable levels to human islet cells. They also performed an experiment to look at cell death in their cells. The IFN-γ + IL-1β cytokine cocktail showed a higher induction of apoptosis compared to the INF-α alone. They also looked at expression markers of apoptosis to see which cells were dying. Both the α-cells and the β-cells showed markers of apoptosis where the β-cells seemed to be more sensitive to the cytokine treatment. The iPSC derived β-cells responded to IFN-γ and IL-1β in the same way as human islets, while they seem to have a slight difference in their reaction to INF-α (Demine et al., 2020). B-cells differentiated from iPS cells derived from fulminant DM1 patients also show upregulation of apoptosis markers after cytokine exposure. The SC- β cells from DM1 patients might be more sensitive to cytokine exposure compared to SC- β cells derived from healthy donors, however the SC- β cells in the study were not functionally mature and that might also affect the sensitivity to cytokines (Hosokawa et al., 2018).

α -like cells can rise from β-cell protocols. Most articles have referred to them as pre-α cells or bihormonal cells, as they are not mature α cells and some might express insulin as well as glucagon. A new 6 stage SC-derived α cell protocol made changes to their existing SC- β cell protocol to induce α cells from the pre- α cell population (Peterson et al., 2020; Veres et al., 2019). Changes were made in the factors added to the medium to avoid induction of the β-cell marker Nkx6.1. After stage 5 they found that a large proportion of the cells expressed insulin as well as glucagon. They performed a compound screening to look at molecules involved in the signaling pathway that convert pre- α cells to SC- α cells. The protein kinase c (PKC) activator (PdbU) molecule proved to decrease the proportion of insulin expressing cells. The addition of PdbU was later used in stage 6 for 28 days. The resulting SC- α cells from using this protocol demonstrated electrophysiology patterns, gene transcription profile and glucagon granule morphology as primary α cells. The SC- α cells were also transplanted into mice to

evaluate their function in vivo. The cells accomplished to protect from hypoglycemia, but there was also an increase in glucagon in the fed state (Peterson et al., 2020).

Differentiation of pancreatic cells from pluripotent stem cells have great potential, but higher efficiency and consistency in creating them is needed. Differencing results between different cell lines mean that variability is still a problem. This and batch-to-batch inconsistency needs to be improved. Most protocols use high-cost components during differentiation. These issues need to be improved to be used as a successful tool in research and therapy. To be used for therapeutic purposes in DM1, a way to protect the cells from attacking immune cells is also needed (Jacobson & Tzanakakis, 2017; Randolph et al., 2019). Some test has been done using encapsulating techniques to protect transplanted β-cells. These capsules protect the cells from the immune cells in DM1, but they also change the glucose, oxygen, and nutrient delivery to the cells (Zhou & Melton, 2018). The cells are also at risk of dying from ischemia if the encapsulation method does not allow for correct blood-flow through the capsule. The material chosen for the capsule is an important step as it needs to allow for good blood-flow for factors like nutrients and oxygen, but also not induce aggravated foreign-body response from the immune system (Tang & Desai, 2016). A study from 2016 using SC- β cells encapsulated in a specially made polymer showed potential for long-term glycemic control after implantation in mice. The cells were taken out 174 days after implantation and the cells were still producing insulin. The capsulation technique had protected the β cells from the immune system proving that the method has potential as a cell replacement technique, and is a potential treatment option compared to pancreas donation (Vegas et al., 2016).

2.0 Scientific questions to be addressed in this project

I. Do SC- α / β cells derived from iPS cells respond to cytokines differentially from human islets?

To answer this question, we differentiated SC- α and β cells from iPS cells, purified insulin and glucagon positive cells and then treated them with a cytokine cocktail consisting of IL-1β (1 ng/mL), TNF-α (5ng/mL) and IFN-γ (5ng/mL). The differentiation protocol used was a modified 2D version of the protocols published by the Douglas Melton group (Peterson et al., 2020; Veres et al., 2019). Human islets were treated with the same cytokine regime. To compare the gene expression changes between the cell-types we chose genes that have previously been reported to be responsive to cytokines relevant for pancreatic cells. We extracted RNA from the samples and performed RT-qPCR using probes corresponding to the chosen genes to investigate changes in gene expression following the cytokine treatment.

II. What are the major changes in the total transcriptome following the cytokine treatment, and in the actively translated genes?

To answer this question, we will compare the transcriptome from cytokine treated cells as well as untreated cells using RNA-sequencing. To study the actively translated genes we will use a ribosomal immunoprecipitation (IP) protocol to pull out RNA-mRNA complexes in order to fish out the active translation profile and compare it to the total RNA transcriptome. Next generation sequencing (NGS) will be used in the form of RNA-sequencing to look for changes in the active translation following cytokine treatment.

3.0 Materials and methods

3.1 Thawing and expansion phase of iPS cells

3.1.1 iPS cells used in the study

The inducible pluripotent stem (iPS) cell line 1016 was used in this experiment to generate functional pancreatic alpha (SC- α) and beta (SC- β) cells (Peterson et al., 2020; Veres et al., 2019). In order to obtain the purest SC- α / β cells, two transgenic cell lines 1016-INSULIN Red Nucleus (IRN) and 1016-GLUCAGON Red Nucleus (GRN), had been made from the original 1016 iPS cell line (unpublished data). The mCherry reporter gene is placed in the 3'untranslated region (UTR) of each hormone, so that it co-expresses with the insulin/glucagon hormone, without affecting the hormone itself. The goal of the differentiation protocol is to achieve mature cells that express glucagon/insulin. The advantage of using these transgenics lines is to monitor insulin/glucagon expression as the cells mature and allow the positive cells to be sorted out based on their mCherry signal using FACS to achieve pure insulin or glucagon positive SC-α/β cells for further experiments.

3.1.1.1 Day 0

To support the growth of iPS cells, the culture vessels (cell flasks and wells) were coated with Biolaminin 521 to mimic basal lamina prior to thawing the iPS cells. To do this, Biolaminin 521 was diluted 1:10 in DPBS +/+ (with calcium and magnesium) and distributed evenly onto the surface of the culture vessels and left in the incubator 37°C for 2 hours before the seeding the cells (Table 1).

Culture vessel	Surface Area cm^2	Coating volume, Biolaminin	Coating volume, Matrigel	Total volume expansion	Total volume differentiation
6 well plate (per well)	9,5	1mL	700µL	3mL	3mL
T25	25	2,5mL	-	5mL	-
T75	75	7,5mL	6mL	15mL	20mL
T175	175	12,5mL	-	30mL	-

Table 1: Overview of the different culture vessels and volumes used during cell expansion and differentiation. – means it was not used in this experiment. The coating volume is the total volume with diluted Biolaminin/Matrigel.

Two cell lines were taken out from a liquid nitrogen tank and thawed quickly and carefully in the 37°C water-bath by moving the vial around in the water. The thawed cells were quickly transferred to 9 mL pre-warmed 37°C mTeSR1 (Stemcell technologies, Catalog #85870) full medium (mTeSR1 basal medium + supplement) plus Rock inhibitor (10µM. Y27632). The mTeSR1 full medium is an optimal medium used for the culturing of undifferentiated human ES cells and iPS cells. The addition of Rock inhibitor prevents apoptosis and boost survival of single-cells. The cell suspension was centrifuged at 200g for 5 minutes to pellet the cells. The supernatant was gently removed, and the cell pellet was resuspended in warm mTeSR1 full medium and counted using a Cedex instrument. Equation 1 was used to calculate the volume of the cell suspension needed for the reseeding depending on the surface area of the culture vessel and the total number of cells in the suspension.

Equation 1: Calculating the cells needed for reseeding.

$$Number\ of\ cells\ for\ reseed = Number\ of \frac{cells}{cm^2} \times surface\ area\ cm^2$$

$$Cells\ for\ reseed\ in\ mL = \frac{number\ of\ cells\ for\ reseed}{number\ of \frac{cells}{mL}}$$

The calculated volume of cell suspension was pipetted into each culture vessel, and more medium was added to get the right volume for the culture vessel. Here the cells were reseeded at 300K cells/cm^2 in one well in a 6-well plate, as shown in Table 2. The culture vessel was

moved carefully in a cross-motion to disperse the cells evenly in the culture vessel. The cells were then incubated at 37°C, with 5% CO_2.

Passage	**Culture vessel**	**Reseed at K cells/cm^2**
Thawing	One well in 6wp	300K
Passage 1	1xT25	80K
Passage 2	1xT75	50K
Passage 3	1xT175	80K
Passage 4	1xT75 + Full 6wp	520K

Table 2: Overview of culture vessels and cell-densities to reseed.

3.1.1.2 Day 1

The cells were checked under a microscope to observe the cell morphology before the medium change. Medium change was performed by aspirating the old medium, and carefully pipetting new pre-warmed medium to the culture vessel.

3.1.1.3 Day 2 (passage 1)

Cell morphology and confluence were checked under a microscope. After 48 hours culture, the cells should be dense and reached ~100% confluence, meaning that they were ready for passage. New culture vessels were coated in the same way as on Day 0 and incubated before stating the passage.

The old medium was aspirated from the cell cultures. The cells were then washed 2x with equal medium volume of pre-warmed 37°C PBS -/- (without calcium and magnesium), by adding and removing the PBS (i.e. 3mL for a well in 6wp). After the last round of PBS washing, ½ volume amount of pre-warmed trypLE Select was added to the cells to dissociate them from the culture vessel. The cells were incubated for ~5 minutes and checked in a microscope to see if cells were detached from the culture vessels.

The cells were resuspended enough to get a single cell solution by carefully pipetting up and down. After getting a single-cell suspension, the cells were transferred to a falcon tube and quenched with mTeSR1+10µM ROCKi (Y27632) with a volume 2x the TrypLE Select volume. The cells were pelleted by centrifuging at 200g for 5 minutes. The supernatant was discarded, and the pellet was resuspended with 1mL pipette, and an adequate amount of medium was added before counting the cells in the Cedex. The cells were reseeded at 80K cells/cm^2 in the new culture vessels.

3.1.1.4 Day 3-9 (passage 2 and 3)

The cells were observed under the microscope every day to check cell growth and morphology. On day three, the medium was changed. On day four, the cells were ready for a new passage the same way as on Day 2 (passage 2, reseeded at 50-60K cells/cm^2). On days 5 and 6, the medium was changed. On day 7, the cells were ready for a new passage (passage 3, reseeded at 70-80K cells/cm^2, followed by medium change on days 8 and 9.

3.1.1.5 Day 10

Day 10 was the last passage and the last day of the expansion phase. The new culture vessels were coated with Matrigel (Corning, Ref.#356230) diluted to a final concentration of 100µg/mL in mTeSR1 medium with volume according to Table 1. The matrigel is pre-aliquoted and has a concentration of 8,7mg/mL. Matrigel was kept cool (on ice) until it was mixed with the medium. The medium was fridge cold, and pipette tips were flushed with cold medium to be cooled down before pipetting the matrigel. After coating, the vessels were incubated for 1 hour at 37°C. The passage was performed in the same way as on Day 2, and the cells were reseeded at 520K cells/cm^2.

3.2 Differentiation of alpha and beta cells

After the expansion phase, the iPS cells were differentiated into SC- α and SC-β cells. This is a six-stage process (Figure 6), and we adhered to an established protocol (Veres et al., 2019). The first two stages were the same for both cell types, and the last four stages were different. The differentiation process uses different combinations of basal mediums (Table 3) and factors to force the cells towards α cell identity or β-cell identity. These combinations can be viewed in Table 4 and Table 5 respectively. During the differentiation, the medium was changed daily with a pre-warmed medium. The factors for that specific day was thawed on the bench and added to the warm medium. On day 1 of a new stage, the culture vessels were washed one time with DPBS -/- (same volume as used for medium), before adding the new stage medium. A quality control (QC) sample was taken on the first day of each stage, except for stage 3, to determine the expression of specific transcription markers by using flow cytometry.

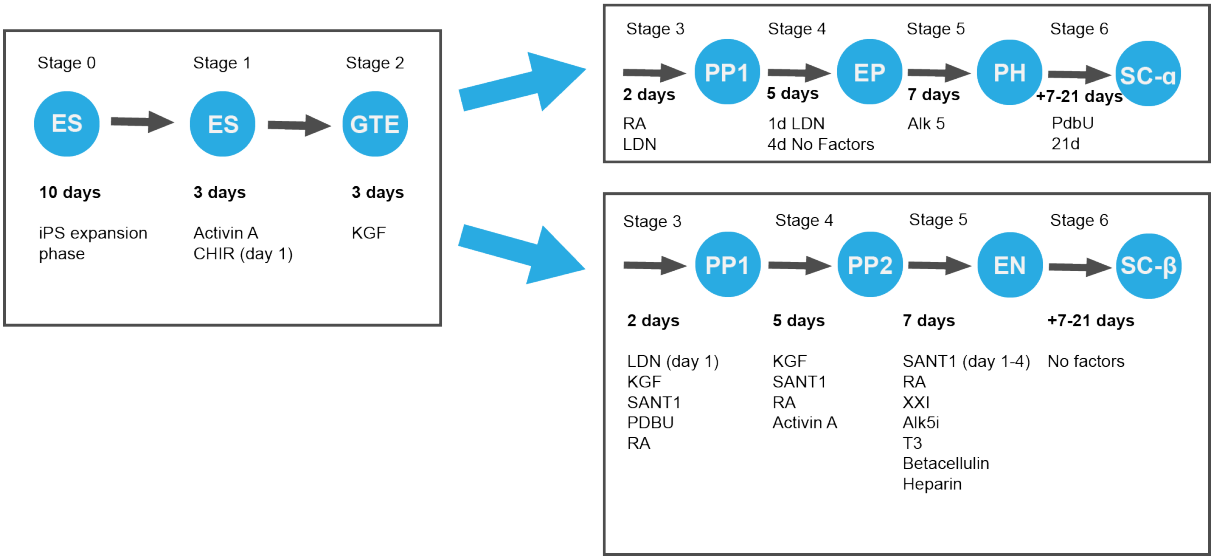

Figure 6: A flowchart of the differentiation process for generation of human SC- α and SC-β cells using iPS cells.ES, embryonic stem-cell. GTE, gut tube endoderm. PP1/2, pancreatic progenitor. EP, endocrine progenitor. PH, polyhormonal. EN, endocrine.

During the differentiation, four different mediums were used. The full mediums were made by following the instructions in Table 3. The medium was prepared by weighing up and adding the solid supplements to a 500mL bottle of MCDB131. The medium was then left in the fridge overnight to let the supplements dissolve. The next day the medium was warmed in a 37°C water-bath for about 30 minutes before the liquid supplements were added to the bottle. After adding all supplements, the medium was filtered using a sterile filter. The full medium was stored in the fridge.

	Manufacturer	S1 media	S2 media	S3 media	BE5 media
MCDB131	Gibco 10372-019	1 bottle (500 mL)	1 bottle (500 mL)	1 bottle (500 mL)	1 bottle (500 mL)
Glucose		0,22g	0,22g	0,22g	1,3g
$NaHCO_3$		1,23g	0,615g	0,615g	0,877g
FAF-BSA (2%)		10g	10g	10g	10g
ITS-X		10µL	10µL	2,5mL	2,5mL
Glutamax (2mM)	Gibco 35050-038	5mL	5mL	5mL	5mL
Vitamin C (0,25 mM)		22mg	22mg	22mg	22mg
Heparin		-	-	-	5mg
P/S (1%)	Gibco 15140-122	5mL	5mL	5mL	5mL

Table 3: Medium and supplements used to make the four differentiation mediums. Abbreviations: Fatty acid-free bovine serum albumin (FAF-BSA), Insulin transferrin selenium ethanolamine (ITS-X), Penicillin-Streptomycin (P/S)

Alpha Cell Protocol			
3 days	**Stage 1d1: S1 media**	**Dilution**	**70 mL**
	CHIR (4,65mg/mL)	1:3333	21 µL
	Activin-A (10ug/mL)	1:100	700 µL
	Stage 1d2-3: S1 media	**Dilution**	**70 mL**
	Activin-A (10ug/mL)	1:100	700 µL
3 days	**Stage 2d1-3: S2 media**	**Dilution**	**70 mL**
	KGF (50ug/mL)	1:1000	70 µL
2 days	**Stage 3d1-2: S3 media**	**Dilution**	**70 mL**
	RA (10mM)	1:5000	14 µL
	LDN(5mM)	1:5000	14 µL
5 days	**Stage 4d1: S3 media**	**Dilution**	**70 mL**
	LDN (5mM)	1:5000	14 µL
	Stage 4d2-5: S3 media	**Dilution**	**70 mL**
	No Factors		
7 days	**Stage 5d1-7: S3 media**	**Dilution**	**70 mL**
	Alk5 inh (100mM)	1:10000	7 µL
21-28 days	**Stage 6d1: S3 media**	**Dilution**	**70 mL**
	PdbU (1mM)	1:2000	35 µL

Table 4: Factors needed, and dilutions needed at each stage of the α cell differentiation. Example for 70mL medium.

Beta Cell Protocol			
3 days	**Stage 1d1: S1 media**	**Dilution**	**70 mL**
	CHIR (4,65mg/mL)	1:3333	21 µL
	Activin-A (10ug/mL)	1:100	700 µL
	Stage 1d2-3: S1 media	**Dilution**	**70 mL**
	Activin-A (10ug/mL)	1:100	700 µL
3 days	**Stage 2d1-3: S2 media**	**Dilution**	**70 mL**
	KGF (50ug/mL)	1:1000	70 µL
2 days	**Stage 3d1: S3 media**	**Dilution**	**70 mL**
	KGF (50ug/mL)	1:1000	70 µL
	Sant-1 (1mM)	1:4000	17.5 µL
	LDN (1mM)	1:5000	14 µL
	PdbU (1mM)	1:2000	35 µL
	RA (10mM)	1:5000	14 µL
	Rock inh	1:500	140 µL
	Stage 3d2: S3 media	**Dilution**	**70 mL**
	KGF (50ug/mL)	1:1000	70 µL
	Sant-1 (1mM)	1:4000	17.5 µL
	PdbU (1mM)	1:2000	35 µL
	RA (10mM)	1:5000	14 µL
	Rock inh	1:500	140 µL
5 days	**Stage 4d1-5: S3 media**	**Dilution**	**70 mL**
	Sant-1 (1mM)	1:4000	17.5 µL
	RA (10mM)	1:100000	0.7 µL
	KGF (50ug/mL)	1:1000	70 µL
	Activin-A (10ug/mL)	1:2000	35 µL
	Rock inh	1:500	140 µL
7 days	**Stage 5d1-4: BE5 media**	**Dilution**	**70 mL**
	Sant-1 (1mM)	1:4000	17.5 µL
	RA (10mM)	1:100000	0.7 µL
	XXI (10mM)	1:10000	7 µL
	Alk5 inh (100mM)	1:10000	7 µL
	T3 (10mM)	1:10000	7 µL

	Betacellulin (100ug/mL)	1:5000	14 µL
	Stage 5d5-7: BE5 media	**Dilution**	**70 mL**
	RA (10mM)	1:400000	0.175 µL
	XXI (10mM)	1:10000	7 µL
	Alk5 inh (100mM)	1:10000	7 µL
	T3 (10mM)	1:10000	7 µL
	Betacellulin (100ug/mL)	1:5000	14 µL
14-21 Days	**Stage 6d1: S3 media**	**Dilution**	**70 mL**
	No factors		

Table 5: Factors needed, and dilutions needed at each stage of the β-cell differentiation. Example for 70mL medium.

3.3 Quality control

3.3.1 Cell fixation

To verify that the cells showed crucial gene expression markers indicating that they were following the differentiation correctly, we performed quality controls (QC) on the two cell lines. To do this, we sampled cells at stages 1, 2, 4, 5, and 6 and fixed the samples in paraformaldehyde (PFA). We then stained the cells with antibodies and analyzed them with a fluorescence-activated cell sorting (FACS) instrument.

The sampling was performed similarly to the passage described above. The single cells were pelleted and fixed in 1 mL 4% PFA for 30 minutes at room temperature. The cells were pelleted again at 600g for 6 minutes. 900µL of the PFA suspension was removed, and 900µL of PBS-/- was added to the cells to leave them in a 0,4% PFA solution. The fixated cells were kept in the fridge until further use.

3.3.2 QC analysis

The α- and β-cells were stained to look at marker protein expressions according to Table 6. For each stained sample, one isotype control was used accordingly. The isotype control is a matching antibody with the same conjugated fluorophore as the primary antibody, but it lacks specificity for the target. The isotype control is run as a negative control to look at potential

disturbances caused by unspecific antibody binding. Due to the mCherry co-expression with glucagon/insulin, fluorophores interfering with the mCherry signal was avoided.

Alpha-cell staining		
Stage	**Staining**	**Isotype control**
1	OCT4 Cy5.5 + SOX17 AF488	PerCP Cy5.5 + AF488
2	OCT4 Cy5.5 + SOX17 AF488	PerCP Cy5.5 + AF488
4	Goat-PDX1 AF488 + Mouse-NKX6.1 AF647	Anti-goat AF488 + anti-mouse AF647
5	Goat-PDX1 AF488 + Mouse-NKX6.1 AF647	Anti-goat AF488 + anti-mouse AF647
6	Mouse-Glucagon AF647 + Rat-C-peptide AF488	Anti-mouse AF647 + anti-rat AF488
Beta-cell staining		
Stage	**Staining**	**Isotype control**
1	OCT4 Cy5.5 + SOX17 AF488	PerCP Cy5.5 + AF488
2	OCT4 Cy5.5 + SOX17 AF488	PerCP Cy5.5 + AF488
4	Goat-PDX1 AF680 + SOX17 AF488	Anti-goat AF680 + AF488
5	Goat-PDX1 AF488 + mouse-NKX6.1 AF647	Anti-goat AF488+ anti-mouse AF647
6	1: Goat-PDX1 AF488 + mouse-NKX6.1 AF647 2: mouse-NKX6.1 AF647 + Rat-C-peptide AF488	Anti-mouse AF647 + anti-rat AF488

Table 6: Overview of the gene-marker and isotype control staining for each stage QC control.

The fixed cells were homogenized, and about 1 million cells were used for either positive staining or isotype control. The aliquoted cells were pelleted, washed once with 500µL 1x BP Perm/wash buffer (diluted with autoclaved water), and then incubated in 500µL 1x BP Perm/Wash buffer for 20 minutes to permeabilize the cells.

During the incubation, the primary antibody and isotype control mixes were prepared following the dilutions in Table 7. After 20 minutes incubation, the cells were centrifuged at 800g for 3 mins, and the supernatant was discarded. 200µL of the primary antibody mix was added to the antibody-staining samples, and isotype control mix was added to the control

samples. The sample was mixed with the pipette and left in the dark at 4°C overnight to incubate.

OCT3/4 PerCP Cy5.5	1:20
SOX17 AF488	1:20
Goat-PDX1	1:200
Mouse-NKX6.1	1:200
Rat-C-peptide	1:100
Mouse-glucagon	1:500
Perm/Wash buffer	200µL x samples
Isotype PerCP Cy5.5	
Isotype anti-mouse	
Isotype anti-rat	
Perm/Wash buffer	200µL x samples

Table 7: Staining dilutions

The following day the cells were centrifuged at 800g for 3 mins, and then the supernatant was removed. For the secondary conjugated antibody staining, these next few steps could be skipped. For the stages that required secondary staining, the cells were washed two times after removing the primary antibody mix. This was done by adding 1mL 1X BD Perm/Wash Buffer, spinning down at 800g for 3 mins and removing the supernatant. After that, 200µL of secondary antibody mix prepared the same way as the primary mix, was added to the samples. The antibody/cell suspension was mixed with a pipette and left in the dark for 1 hour at room temperature. After 1 hour, the cells were centrifuged at 800g for 3 mins, and then the supernatant was removed.

All the samples were washed two times with 1mL 1X BD Perm/Wash buffer and resuspended in 400µL FACS Buffer (PBS -/-, two %FCS, and 5mM EDTA). Samples were filtered (40µm) to remove potential aggregates that could clot the FACS instrument before being analyzed using a BD FACS Fortessa.

3.4 Cell sorting

According to previous reports and our QC analyses at the last stage, we could obtain a fraction of cells that are glucagon or insulin (C-peptide/Nkx6.1) positive. This could be

confirmed by looking at the mCherry signal under a fluorescent microscope. Because our study needed a pure population of α or β- cells, we sorted out the positive cells by using a SONY SH800 cell sorter according to a positive mCherry signal.

The differentiated cells were dissociated the same way as for a passage to get single cells. The cells were pelleted, resuspended in 2-3 mL chilled FACS sorting buffer, and then passed through a filter cap into a new falcon tube to remove aggregates. The cells were kept on ice and passed through another filter before the sorting.

After sorting, the positive single cells were pelleted and resuspended in the S3+Rocki medium. The cells were counted and re-plated at about 400 000 cells per well in a 12-well plate pre-coated with matrigel. Regular medium changes were performed until cytokine treatment.

For some batches of α and β -cell, due to the poor adherence to the Matrigel-coated surface, we chose to form clusters from the sorted cells by using Aggrewell plates. The clusters were then cultured in suspension 6-well plates with constant shaking.

3.5 Human islets

In order to investigate whether SC- α and SC-β respond to cytokines differently from human islets, we included human cadaveric pancreatic islets. Human islets for the experiment were ordered from Prodo Laboratories Inc. Information on the donors can be viewed in Table 8. The islets arrived in standard Prodo islet medium (PIM(S) + 5% Human AB Serum + PIM(G) + 1%P/S). After arriving at the laboratory, the islets were maintained in a fresh Prodo islet medium (containing 5,6mM glucose), and were kept for about 1-2 days prior to the cytokine treatment. Roughly 6000 islet equivalent (IEQ) islets from each donor were used for the experiment (1500 IEQ for each experimental condition).

Donor 1	Non-diabetic. 64-year-old Caucasian male, 74 inches tall, 163 lbs., BMI of 20,9. The donor died from a stroke. HbA1c was 5,5%
Donor 2	Non-diabetic. 52-year-old Caucasian male, 72 inches tall, 218 lbs., BMI of 29,6. The donor died of a stroke. HbA1c was 5,4%
Donor 3	Non-diabetic. 30-year-old Hispanic male, 70 inches tall, 159 lbs., BMI of 22,7. The donor died of an anoxic event. HbA1c was 5,2%

Table 8: Description of the human islet donors

3.6 Cytokine treatment

In this study, we chose a combination of for IL-1β, TNF-α, and IFN-γ to treat the pure α , β cells, and human islets (Eizirik et al., 1994; Farnsworth, Walter, Hemmati, Westacott, & Benninger, 2015). A previous study at AstraZeneca determined the dose and period of the cytokine treatment. Accordingly, the cells were treated with a mixture of 1 ng/mL IL-1β, 5ng/mL TNF-α and 5ng/mL IFN-γ added to the medium for 16 hours. For the experiment, half of the cells were treated with cytokines to induce a stress response in the cells. The other half of the cells did not get treated with cytokines to be used as a control. Roughly 750 000 cells were used for each condition.

3.7 Cell lysis

The cells were lysed by using Ribo IP lysis buffer (100mM KCl, 12mM MgCl2, 50mM Tris, and 1mM Sodium orthovanadate, pH 7,4). The cells were harvested and washed twice with chilled PBS. Half of the cell pellet was then lysed in 500µL complete lysis buffer (Ribo IP lysis buffer + 1% tween-20/1% NP-40, protease inhibitor tablet, 1mM Dithiothreitol (DTT), 100ug/mL Cycloheximide (CHX) and 20u/mL RiboLock) on ice for 20 minutes, with mixing every 5 minutes. After 20 minutes, the cells were centrifuged at 4°C at 13000g for 15 minutes to extract the total protein. The other half, which was used as un-immunoprecipitation control, was lysed with 350µL RLT buffer +DTT (40µL DTT/mL RLT buffer) and put in the freezer.

3.8 Immunoprecipitation of mRNA-ribosome complex

The IP was performed by using Dynabeads Protein A beads (ThermoFisher). The beads were homogenized by mixing prior to use. 120 µL beads needed, with 60 µL for pre-clearing the sample, and 60 µL for antibody binding. The antibody RA0279 targets the ribosomal protein subunit RPL29. The flowchart in Figure 7 shows the workflow for the IP protocol.

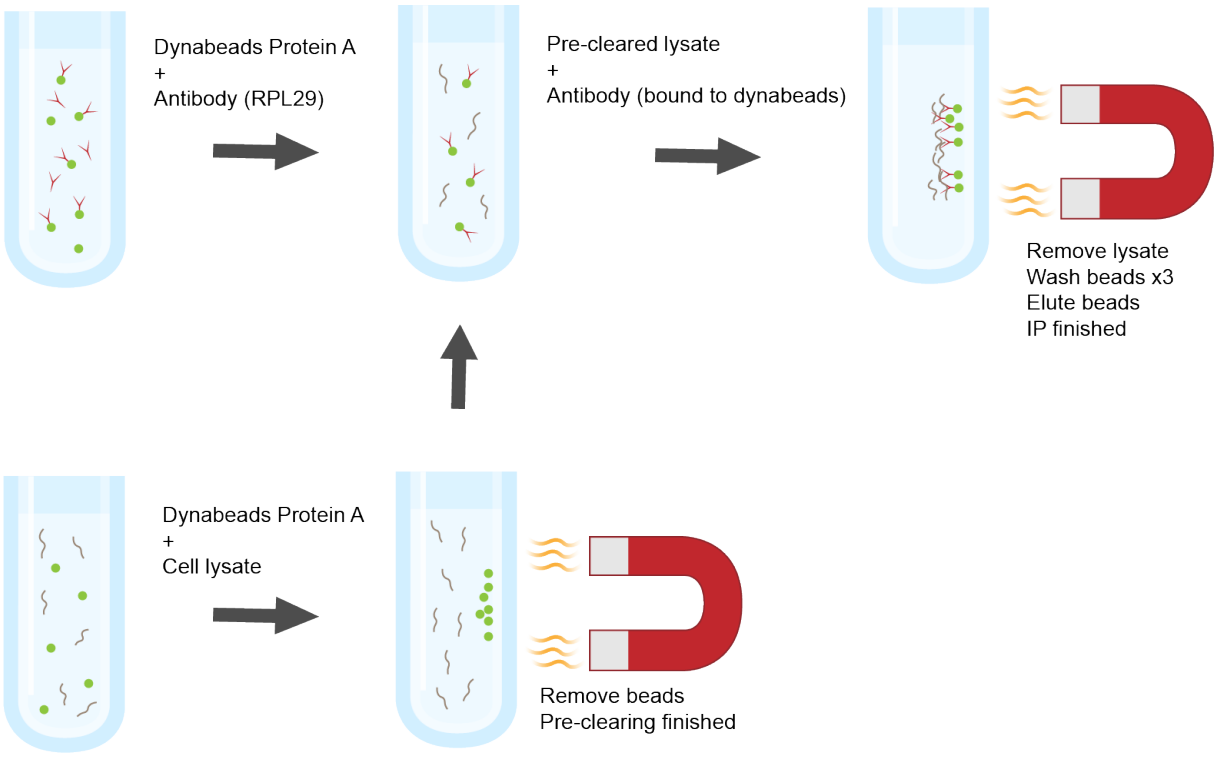

Figure 7: A flowchart showing the steps of the IP protocol.

Beads pre-wash: The tube with beads were put on a magnetic stand for 1 minute, and the supernatant was removed. An equal volume of Ribo IP lysis buffer, with protease inhibitor and 1% tween 20, was added to the beads and mixed to wash the beads. The beads were put back on the stand to remove the lysis buffer. This washing step was repeated three times. The beads were resuspended in the same volume of complete lysis buffer after removal of lysis buffer in the last washing step.

Lysate pre-clearing and antibody conjugation: 60 µL of beads were added to each sample tube to pre-clear the samples. 120 µL of the beads go in one Eppendorf together with 10uL antibody (against RPL29, 1mg/mL stock) to conjugate. Extra complete lysis buffer was added to get a large enough volume (400-500 µL) to ensure proper mixing on the rotor. Both beads+cell-lysate and beads+ antibody tubes were put on a rotor in 4 degrees overnight.

IP incubation: The next day, the tubes with cell-lysate were put on a magnetic stand after the pre-clearing was finished. The pre-cleared lysates were transferred to new Eppendorf tubes. The bead+antibody mix was split evenly between the two cell lysates. The tubes with beads and lysate were then put back on the rotor in 4 degrees for 6-8 hours to incubate.

Washing: The lysate+ab/beads were put back on the magnetic stand after incubation. The lysate was removed, and the beads were washed three times with 1mL lysis buffer + protease inhibitor and 1% tween 20. The beads were eluted with 350 µL RLT buffer+DTT. The samples were put in the freezer until RNA-extraction.

3.9 RNA-extraction

3.9.1 Extraction

RNA extraction was performed by using the RNeasy Micro Kit (Qiagen, 2016) according to the manufacturer's instructions.

The two IP samples still contained the magnetic beads from the IP-protocol. After thawing, the IP samples were put on a magnetic stand, and the cell lysate was transferred to new tubes to remove the magnetic beads before RNA extraction. 350 µL 70% ethanol was added to each sample and mixed. The samples were transferred to RNeasy MinElute spin columns placed in 2mL collection tubes and centrifuged at 10000g for 15 seconds. The flow-through in the collection tubes were discarded. 350 µL RW1 buffer was added to the spin columns and centrifuged again. The remaining DNA was removed by incubating with 80 µL DNase I incubation mix for 15 minutes.

350 µL RW1 buffer was added to each column after incubation and centrifuged at 10000g for 15 sec. The collection tubes were discarded and replaced with new 2mL tubes. The column was further washed by 500 µL RPE buffer, followed by 500 µL 80% ethanol and centrifuging for 2 min at 10000g. The collection tubes were discarded and replaced with new 2 mL tubes. The spin columns were centrifuged with open lids for 5 min. at max RCF to dry the spin column membrane. The collection tubes were discarded and replaced with 1,5mL collection tubes.

14 µL RNase-free water was added close to the membrane in the spin columns. For the human islets' samples, 20µl RNase-free water was used because a higher RNA amount was

expected. The samples were centrifuged for 1 min. at max speed to elute the RNA. After the RNA extraction, the RNA quantity was measured using a Nanodrop instrument. 1 µL was used to measure the RNA amount in ng/ µL. The RNA-samples were stored in a -80-degree freezer until the reverse transcription step.

3.9.2 Reverse transcription

To determine the gene expression in the samples, cDNA was synthesized using reverse transcriptase (High-Capacity cDNA Reverse Transcription Kit (appliedbiosystems, 2016)). The mastermix was made following the volumes in Table 9. One per sample + one extra for excess volume. 1 µL Ribo-Lock was added to the finished mix.

Reagent	**Volume µL**
10X RT buffer	2,0
25X dNTP Mix (100mM)	0,8
10X RT random primers	2,0
MultiScribe reverse transcriptase	1,0
Nuclease-free water	4,2
Total	**10,0**

Table 9: Reagents to make RT-mastermix

10 µL RT-mastermix was added to a 0,2mL tube for each sample. The RNA amount measured after RNA extraction was used to calculate how much of the RNA was needed for the reverse transcription. 50-90ng RNA was used for reverse transcription. The calculated volume of RNA was added to the RT-mastermix, and RNase-free water was added to reach a total reaction volume of 20 µL per sample. The tubes were quickly spun down to make sure there were no drops on the tube walls. The samples were put in a thermal cycler and run on the following settings. 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes. Followed by a hold step at 4°C until the samples are collected and put in the -20-degree freezer.

3.10 Gene expression profile

The qPCR was set up with 12 primers for different genes. Two housekeeping genes, *TBP* and *HPRT1,* were run as a reference point for the experiment as they should stay constant even after cytokine treatment. The other primers in the experiment were for the genes *NF-κB1, NF-κB2, STAT1, STAT3, ISL1, IRF1, SOD2, EIF4EBP2, CXCL2,* and *CCL20.* The TaqMan PCR-mastermix was made by mixing the components in Table 10.

Reagent	**Volume µL**
TaqMan Fast Advanced Master Mix	5
TaqMan Assay primers	0,5
Nuclease-free water	3,5
Total	**9**

Table 10: Reagents to make qPCR-mastermix

9 µL PCR-mastermix was added to wells on a 384-well plate according to a setup drawn before starting. All samples were run in triplicate for each gene. cDNA samples were thawed on ice and diluted with nuclease-free water. 1 µL sample was added to each corresponding well. The plate was sealed and centrifuged to remove potential droplets and air bubbles. The plate was then run on a QuantStudio PCR instrument. The following conditions were used for the qPCR: 2 min hold at 50°C, 2 min hold at 95°C, followed by 40 cycles with denaturing at 95°C for 1 sec and annealing/extending at 60°C for 20 secs. Data analysis was performed on the qPCR results.

3.11 Calculation of changes in gene expression

To analyze the gene expression, the ΔΔCt Livak method was chosen. The average was calculated from the Ct values of the triplicates for each sample. The *HPRT1* housekeeping gene was an internal reference. To normalize the Ct data, the formulas in Equation 2 were used. This was done on both the IP group and the no-IP group individually. The two housekeeping genes, *TBP* and *HPRT1,* were run as a reference point for the experiment. The *HPRT1* C_T values were used as the housekeeping gene (hkg) in the ΔΔCt Livak method.

$$\Delta Ct\ (control) = Ct\ (target\ gene, control) - Ct\ (hkg, control)$$

$$\Delta Ct\ (cytokine) = Ct\ (target\ gene, cytokine) - Ct(hkg, cytokine)$$

Equation 2: Formula to normalize target gene expression data against housekeeping-gene (hkg)

To look at the difference between the cytokine treated samples and the control group, the next step was to normalize the cytokine treated Ct values to the control Ct values. This was done by using the formula in Equation 3. One for the IP group and one for the no-IP group.

$$\Delta\Delta Ct = \Delta Ct\ (cytokine) - \Delta Ct\ (control)$$

Equation 3: Formula to calculate the ΔΔCt

The gene expression was shown as fold change by using the function $2^{-\Delta\Delta Ct}$. The number is an indication of the upregulation or downregulation of the target gene expression after cytokine treatment compared to the control group. The IP and the no-IP group were compared to look at the difference between the total gene expression and the active gene expression in the cell after cytokine treatment. The *TBP* gene was used as an internal control for the experiments as the expression of this housekeeping gene should be stable (fold change value around 1). The fold change was calculated for the group with IP (w/IP), and the group without the IP protocol (w.o/IP). The fold change is a description of the ratio between the original gene expression (control) and the gene expression under experimental settings (cytokine treated). A positive fold change value would mean the gene expression has increased in the treated group compared to the control. A negative number would mean the gene expression is down regulated after treatment.

4.0 Results

4.1 Differentiation of SC-α/β cells

The differentiation protocol took ~6 weeks from start to finish. We observed the red fluorescent protein under the microscope at different time-points during stage 6 to look for clusters of positive cells to indicate that the differentiation was successful. The differentiation protocol led to varying numbers of positive glucagon and insulin expressing cells between the batches of differentiation we performed. The positive cells were sorted out using the Sony SH800 based on their mCherry expression as described in 3.4. The first two batches of cell differentiations did not lead to enough positive cells after sorting to continue with the rest of the experiment. After the third batch of differentiation we achieved enough positive cells, but only for the β-cells. The sorted out positive cells were seeded on a 12 well plate pre-incubated with matrigel. Forty-eight hours after seeding, most of the β -cells had not adhered to the matrigel and could not be used for the following experiments. Therefore, we decided to change the protocol to form clusters of the sorted positive cells and leave them under constant movement for the next batch of cells.

The fourth differentiation had high quality and quantity of positive β-cells, and roughly 1,5 million positive cells were sorted out. The positive cells were divided into four different groups in the experiment and had roughly 350 000 cells in each group before the lysis. The α-cell batch from the same differentiation resulted in high quality and quantity of the α-cells but the number reduced rapidly during medium change in stage 6 and therefore was discarded. α -cells from a differentiation performed simultaneously where the we changed the protocol to creating clusters the first day of stage 6 resulted in better quantity of α-cells that could be used in the following experiments after cell-sorting.

4.2 Quality control during the differentiation

To confirm that the cells presented with the right markers indicating successful stages in the differentiation we performed quality controls in some stages following the protocol in chapter 3.3. The cell plots from the FACS instrument show a cell scatter based on the fluorophore used in the staining and the laser settings.

4.2.1 SC-α cells

In Figure 8 we can see the cell scatter plots from the FACS instrument performed on the QC-samples taken from the α-cells during differentiation. In stage 1 we saw a high percentage of Oct4 positive cells. This was expected as the cells from this sample is iPS cells before the initiation of stage 1 and Oct4 expression is an indication that the cells are pluripotent (Takahashi & Yamanaka, 2006). In stage 2 and 3 we saw a high percentage of Sox17 positive cells. Sox17 expression is a part of the regulation of the endoderm formation in embryonic development and the cell fate in pancreas initiation (Pan & Wright, 2011; Spence et al., 2009). A smaller population of the cells expressed Oct4. This is what we wanted as the differentiation protocol should force them towards pancreatic fate and away from pluripotency. In later stages we saw cells expressing Pdx1. This is an expression marker for cell fate leading to pancreatic cells and is vital for the differentiation of pancreatic cell identity (Veres et al., 2019). We saw an induction of Nkx6.1 in a population of cells, but most of the cells did not express it. We wanted a low induction of Nkx6.1 as this is a β-cell identity marker (Peterson et al., 2020). In stage 6 day 1, we saw that a high proportion of cells expressing both glucagon and C-peptide. These are polyhormonal cells, meaning that they are not mature α -cells. After the presence of PdbU (a PKC activator in stage 6) (Peterson et al., 2020), we a proportion of cells expressing glucagon only. Still a large portion of cells express C-peptide and are not monohormonal after 3 weeks maturation in stage 6.

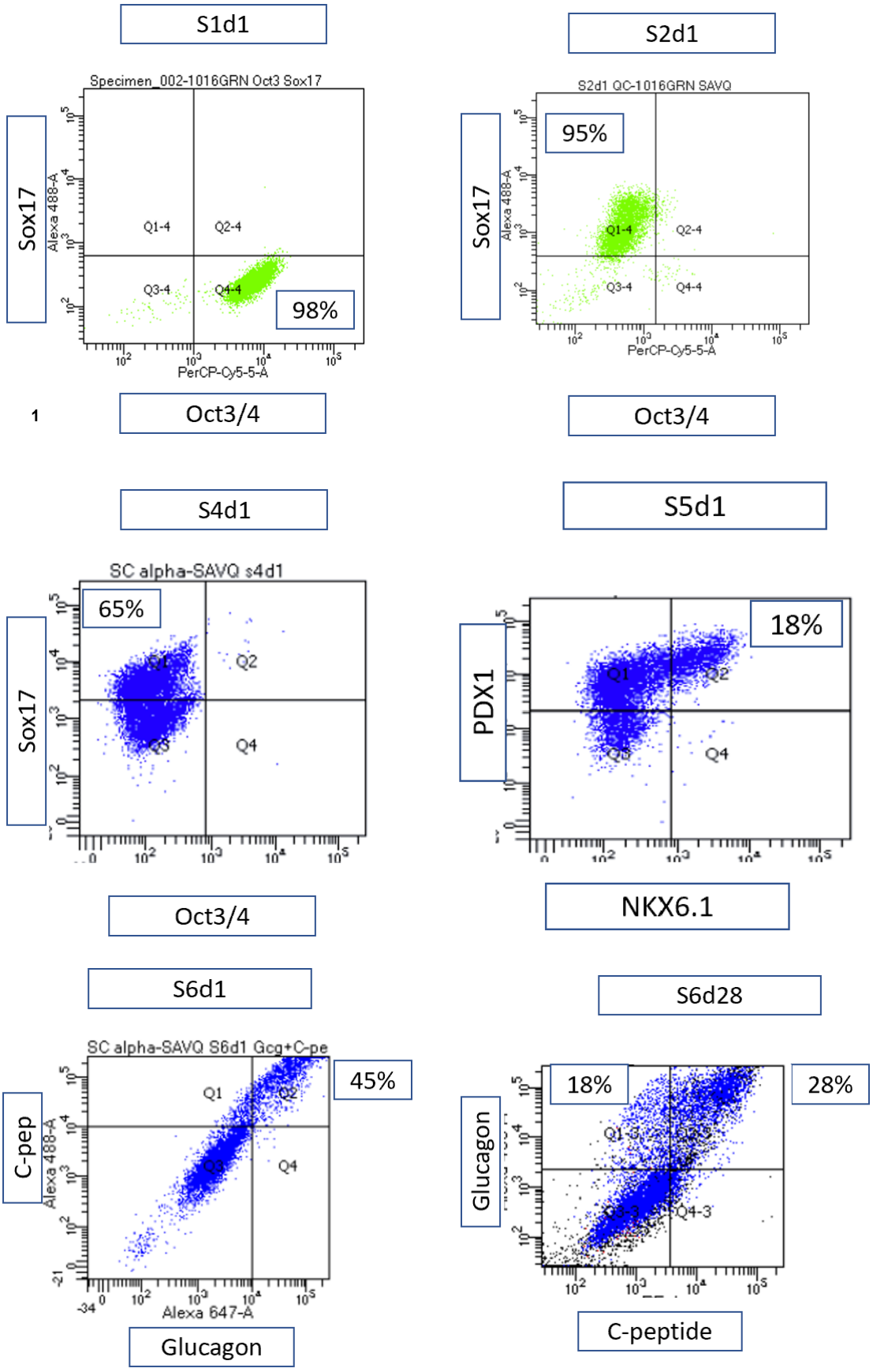

Figure 8: Cell plots from the QC samples in stage 1,2,4,5 and 6 for the α-cell differentiation.

4.2.2 SC-β cells

In Figure 9 we can see the cell scatter plots from the FACS instrument performed on the QC-samples taken from the β-cells during differentiation. Since the β -cell protocol shares common conditions and factors as the α -cell protocol, we expected to have the same cell populations in stage 1 and 2. And the FACS showed that the cells from the β- cell differentiation are the same as in the α-cell differentiation. In stage 4 we saw a high proportion of Pdx1 positive cells, and in stage 5 we saw a high proportion of cells expressing Pdx1 and Nkx6.1. These are important markers for pancreatic cell fate and β-cell precursor fate (Poudel et al., 2016). In stage 6 we saw a high proportion of cells positive for both C-peptide and Pdx1 as this is the mature β -cell identity (Veres et al., 2019). The cell population positive for both markers were higher on day 30 than on day 1 of stage 6 showing that this step helps the cells mature further.

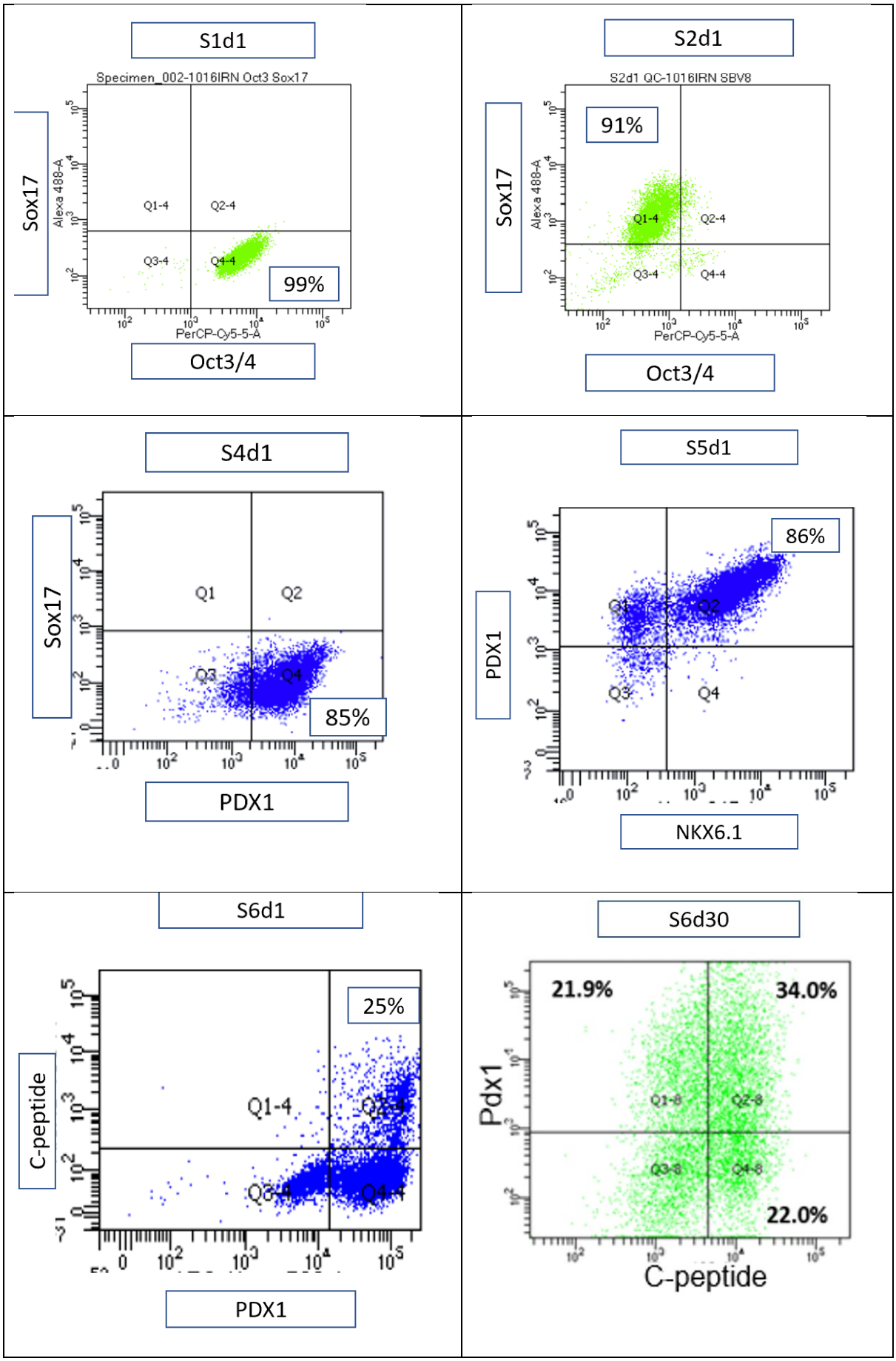

Figure 9: Cell plots from the QC samples in stage 1,2,4,5 and 6 for the β-cell differentiation.

4.3 Effect of cytokines on pancreatic cells

To investigate the effect of the cytokines on the gene expression of α cell, β cells and human islets, we treated the cells with a cytokine cocktail containing IL-1β, TNF-α and IFN-γ leaving half of the cells untreated as a control as described in chapter 3.6. The cytokine treated cells were left for 16 hours in order to look at changes in gene expression before apoptosis. After 16 hours all the cells were lysed according to the protocol in chapter 3.7. On half of the cytokine treated cell lysate and half of the control cell lysate we performed IP according to chapter 3.8 to pull out the actively translated genes. After the cytokine treatment and IP protocol was completed, we extracted RNA from all samples following the protocol in chapter 3.9. The RNA quantity measured in ng/µL and the RNA quality measured by the A260/280 ratio can be seen below in Table 11, 12 and 13 for the different cell types.

RNA-extraction from alpha cells			
Batch	**Experimental conditions**	**ng/µL RNA**	**A260/280 ratio**
1	Cytokine treated w/IP	19,9	1,48
	Cytokine treated w.o/IP	39,6	1,69
	Control w/IP	33,4	1,45
	Control w.o/IP	17,7	1,92

Table 11: RNA quantity and quality from the α-cell samples

RNA-extraction from beta cells			
Batch	**Experimental conditions**	**ng/µL RNA**	**A260/280 ratio**
1	Cytokine treated w/IP	5,9	2,32
	Cytokine treated w.o/IP	34,8	1,61
	Control w/IP	5,3	2,24
	Control w.o/IP	22,5	1,73

Table 12: RNA quantity and quality from the β-cell samples

RNA-extraction from human islets			
Batch	**Experimental conditions**	**ng/µL RNA**	**A260/280 ratio**
1	Cytokine treated w/IP	17,7	2,00
	Cytokine treated w.o/IP	71,5	1,90
	Control w/IP	53,1	1,75
	Control w.o/IP	84,9	2.02
2	Cytokine treated w/IP	30,9	1,37
	Cytokine treated w.o/IP	44,4	1,90
	Control w/IP	20,6	1,45
	Control w.o/IP	47,7	1,75
3	Cytokine treated w/IP	9,8	1,32
	Cytokine treated w.o/IP	85,6	1,62
	Control w/IP	32,5	1,44
	Control w.o/IP	40,5	1,74

Table 13: RNA quantity and quality from the human islet samples

From Table 11, 12 and 13 we see that the RNA quality, which should be 2, is not optimal with numbers both higher and lower than this. We cannot observe an obvious pattern in the quality of RNA based on A260/280 ratio in connection to cytokine treatment or the use of IP from the values above. The RNA quantity we achieved in the experiment was not optimal, and there was not enough RNA leftover to perform RNA-sequencing after the qPCR. The IP protocol appears to provide a lower RNA quantity in all samples compared to the w.o/IP sample with the same condition. This is true for all the samples above except for the α-cell control condition where the IP protocol provided 33,4 ng/µL, while the sample w.o/IP only provided 17,7 ng/µL.

4.4 Gene expression data

After the RNA-extraction the samples were analyzed with qPCR according to the setup in chapter 3.10. The Tables 13, 14, 15, 16, 17 and 18 are representatives of the RT-qPCR data collected from the study. The C_T value (cycle threshold) represents the number of cycles needed to produce a fluorescent signal strong enough to cross the signal threshold. Each gene was analyzed in triplicates, and the average of the three values is presented in the following Tables 14-19.

Alpha cells w.o/IP		
Gene	Avg. Ct control	Avg. Ct value cytokine
TBP	26,73	27,22
HPRT1	25,54	26,22
NF-κB1	26,51	25,68
NF-κB2	27,52	25,80
STAT1	24,10	20,28
STAT3	26,37	25,37
ISL1	23,34	24,77
IRF1	27,55	22,49
SOD2	24,41	19,85
EIF4EBP2	25,49	26,60
CXCL2	29,22	23,25
CCL20	29,30	22,17

Table 14: Average Ct values for control and cytokine treated cells w.o/IP

Alpha cells w/IP		
Gene	Avg. Ct value control	Avg. Ct value cytokine
TBP	33,37	30,66
HPRT1	32,75	29,17
NF-κB1	33,38	28,27
NF-κB2	34,84	28,87
STAT1	31,92	23,35
STAT3	33,54	28,18
ISL1	30,68	27,86
IRF1	34,13	25,46
SOD2	31,75	22,67
EIF4EBP2	33,09	29,53
CXCL2	37,62	27,69
CCL20	37,32 (no curve, high value)	27,87

Table 15: Average Ct values for control and cytokine treated cells w/IP

Beta cells w.o/IP		
Gene	Avg. Ct value control	Avg. Ct value cytokine
TBP	28,23	28,23
HPRT1	27,40	27,48
NF-κB1	28,42	26,46
NF-κB2	28,74	26,51
STAT1	24,80	21,85
STAT3	28,07	26,40
ISL1	25,93	26,74
IRF1	28,57	22,66
SOD2	26,73	20,74
EIF4EBP2	27,30	27,05
CXCL2	27,76	23,71
CCL20	28,48	22,24

Table 16: Average Ct values for control and cytokine treated cells w.o/IP

Beta cells w/IP		
Gene	Avg. Ct value control	Avg. Ct value cytokine
TBP	32,97	29,68
HPRT1	33,31	29,15
NF-κB1	32,43	27,59
NF-κB2	28,53	28,04
STAT1	29,77	22,87
STAT3	32,04	27,34
ISL1	28,94	27,63
IRF1	33,10	23,62
SOD2	30,65	21,73
EIF4EBP2	32,48	28,08
CXCL2	33,42	25,39
CCL20	35,12	24,55

Table 17: Average Ct values for control and cytokine treated cells w/IP

Human islets w.o/IP						
Gene	Avg. Ct value control			Avg. Ct value cytokine		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
TBP	26,54	26,68	-	28,07	26,88	-
HPRT1	23,87	24,64	24,69	24,88	24,10	24,92
NF-κB1	26,16	27,03	26,75	24,69	23,97	25,22
NF-κB2	26,84	29,02	27,93	25,35	24,86	24,86
STAT1	21,04	24,85	24,68	20,92	20,13	20,67
STAT3	24,49	25,85	25,22	24,31	23,61	24,10
ISL1	23,69	24,54	24,45	26,34	25,64	25,39
IRF1	24,61	27,87	27,70	21,19	20,76	21,64
SOD2	22,78	23,63	23,62	18,46	17,09	17,46
EIF4EBP2	25,02	26,16	25,77	26,94	26,36	26,54
CXCL2	28,53	28,28	28,19	23,89	22,56	22,99
CCL20	29,74	28,83	29,31	24,31	22,42	23,68

Table 18: Average Ct values for control and cytokine treated cells w.o/IP

Human islets w/IP						
Gene	Avg. Ct value control			Avg. Ct value cytokine		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
TBP	28,88	34,20	-	28,53	37,72	-
HPRT1	26,06	31,47	33,11	25,62	30,21	33,51
NF-κB1	29,10	33,81	35,85	25,68	30,24	33,69
NF-κB2	29,50	28,53	28,53	25,86	30,97	33,53
STAT1	22,80	31,56	32,90	21,09	26,43	28,97
STAT3	26,89	32,05	34,36	25,30	29,66	32,72
ISL1	26,71	31,09	32,41	27,63	32,11	33,51
IRF1	26,96	34,49	35,39	21,31	26,76	29,91
SOD2	25,47	30,99	32,39	19,12	22,90	25,84
EIF4EBP2	27,32	33,88	33,14	26,49	33,11	33,27
CXCL2	31,99	36,21 (value from re-run)	36,17	24,63	30,58	30,62
CCL20	34,01	37,00 (value from batch 3)	37,00	26,39	31,12	31,66

Table 19: Average Ct values for control and cytokine treated cells w/IP

4.5 Calculation of gene expression changes

As mentioned earlier only one batch of each of the differentiated SC-α and SC-β cells were used for the RT- qPCR analysis. The replicates of Ct-values and the following determination of fold-change are thus calculated from one sample source and hence limits thee possibility to do statistical analyses. Therefore, we regarded fold changes of 5 or above as significant changes in accordance with previous experiences in our lab. The average C_T values shown above were used to calculate the fold changes of the different genes using the ΔΔCt Livak method.

4.5.1 Alpha cells

The α-cells treated with the cytokine coctail showed an upregulation of *CXCL2, CCL20, IRF1, SOD2* and *STAT1* expression. There was also a small increase in *NF-κB2* expression both with and without the use of IP. This can be seen in Table 20, and visually presented in Figure 10.

Gene	Fold change w.o/IP	Fold change w/IP
TBP	1,14	0,55
NF-κB1	2,85	2,88
NF-κB2	5,28	5,25
STAT1	22,55	20,85
STAT3	3,19	3,43
ISL1	0,59	0,59
IRF1	53,80	33,92
SOD2	37,70	45,40
EIF4EBP2	0,74	0,98
CXCL2	99,91	81,94
CCL20	223,98	72,36

Table 20: Overview of the fold change values for each gene without the IP (w.o/IP) protocol and with (w/IP) the IP protocol.

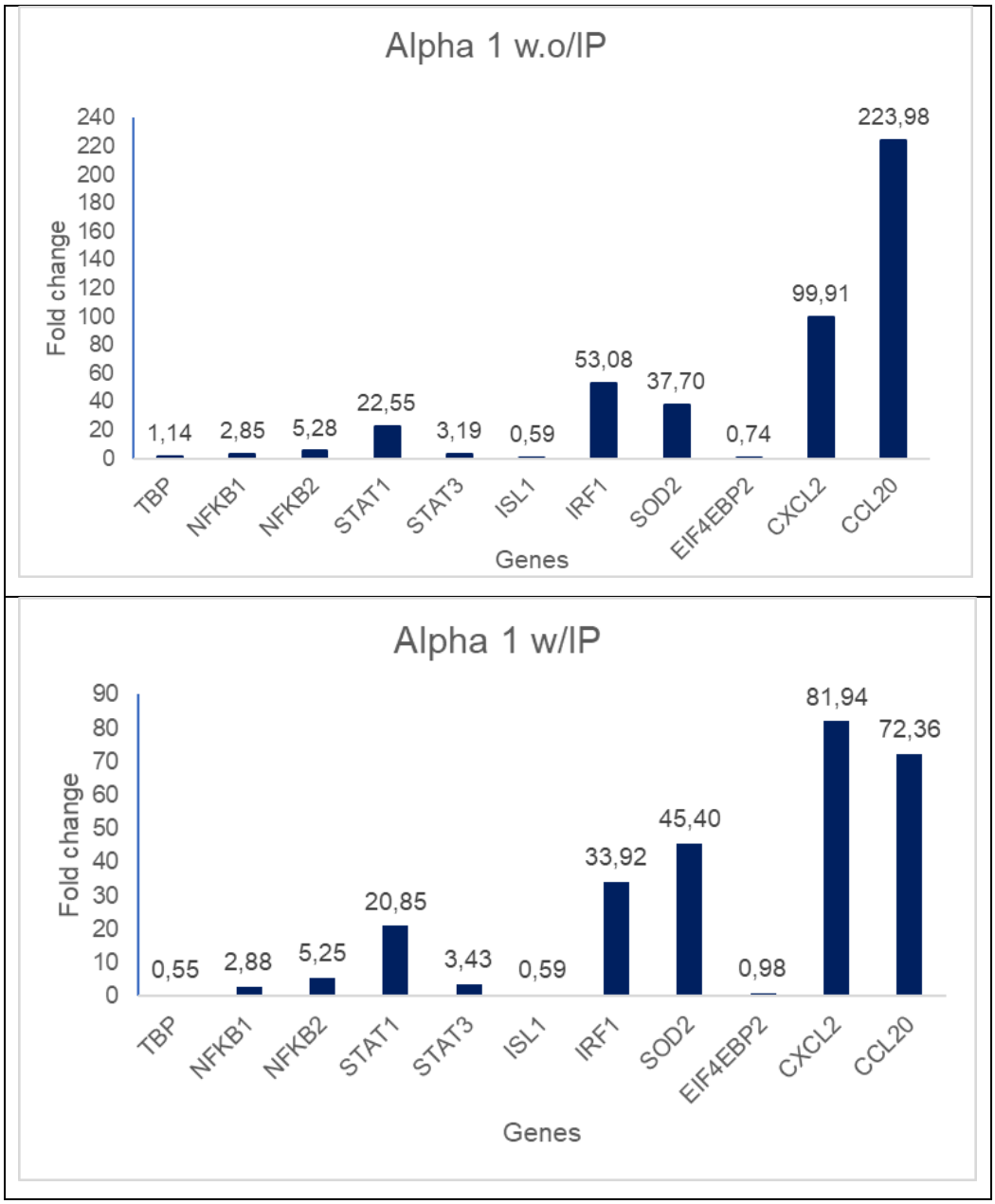

Figure 10: Diagrams illustrating the fold change. Top diagram, w.o/IP. Bottom diagram, w/IP.

4.5.2 Beta cells

As for α-cells, the β-cells also displayed increases of *CXCL2*, *CCL20*, *IRF1*, *SOD2,* but the increase in *STAT1* expression was slightly less pronounced. *NF-κB1* and *NF-κB2* had a slight increase in fold change but this was only seen without the IP protocol. This can be seen in Table 20, and visually presented in Figure 11.

Gene	Fold change w.o/IP	Fold change w/IP
TBP	1,06	0,55
NF-κB1	4,10	1,59
NF-κB2	4,96	0,08
STAT1	8,12	12,75
STAT3	3,36	1,44
ISL1	0,60	0,14
IRF1	63,62	39,94
SOD2	67,16	26,94
EIF4EBP2	1,26	1,18
CXCL2	17,42	14,56
CCL20	79,75	84,70

Table 20: Overview of the fold change values for each gene without the IP (w.o/IP) protocol and with (w/IP) the IP protocol.

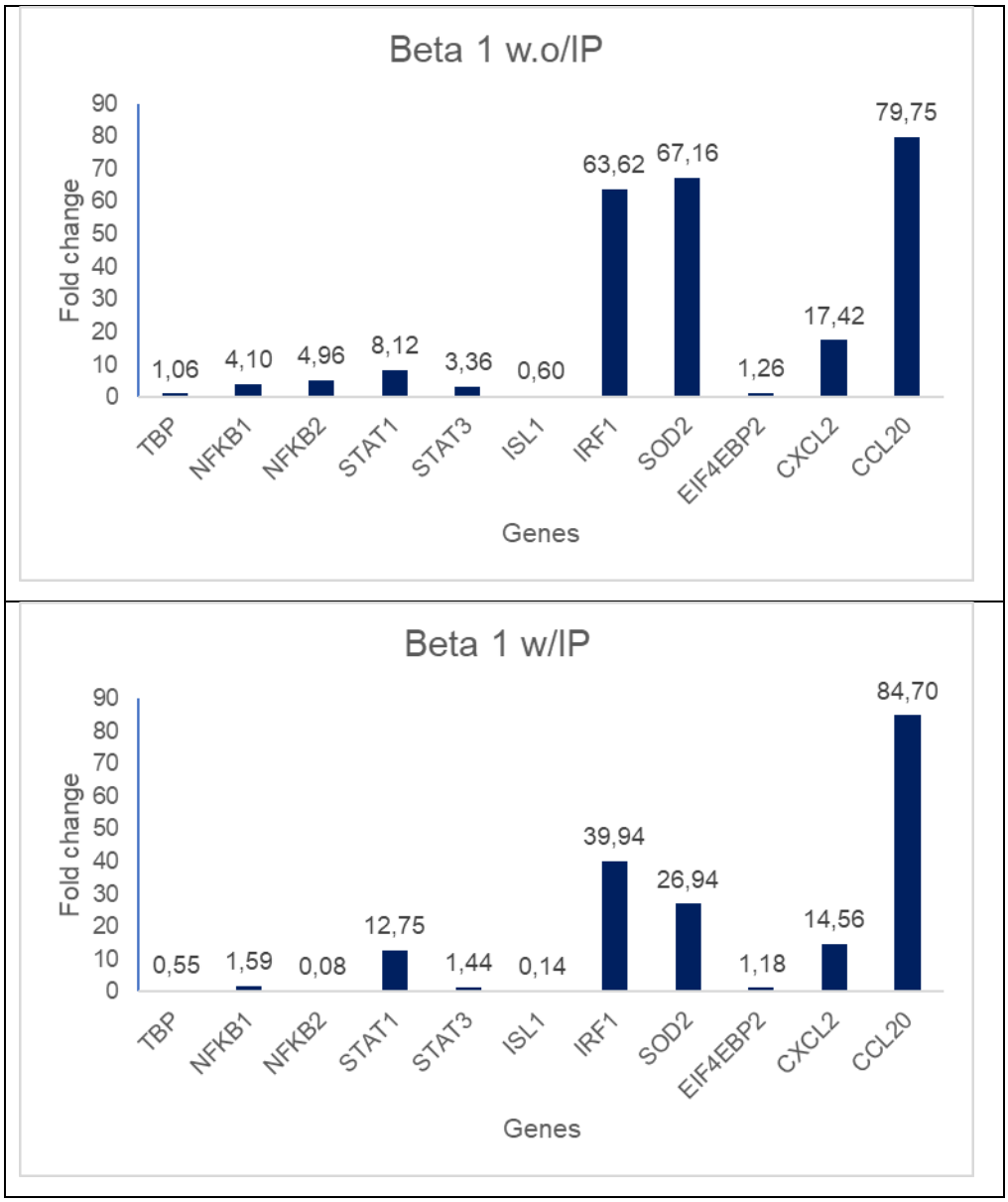

Figure 11: Diagrams illustrating the fold change. Top diagram, w.o/IP. Bottom diagram, w/IP.

4.5.3 Human islets

The human islets showed an upregulation of *CXCL2*, *CCL20*, *IRF1* and *SOD2* both with and without the IP protocol. *STAT1* had an upregulation in donor 2 and 3, but not in donor 1. *NF-κB1* had a slight upregulation in all donors. *NF-κB2* had a small upregulation in the samples without IP for all donors. With the IP protocol there was only an upregulation in donor 1, while donor 2 and 3 showed no fold change after cytokine treatment. The other genes showed no change in either donor. This can be seen in Table 21 and visually presented in Figure 12, 13 and 14.

Gene	Fold change w.o/IP			Fold change w/IP		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
TBP	0,70	0,60	-	0,94	1,17	-
NF-κB1	5,60	5,73	3,36	7,89	4,97	5,93
NF-κB2	5,67	12,28	9,80	9,20	0,08	0,04
STAT1	2,19	18,28	18,77	3,24	15,91	23,01
STAT3	2,28	3,27	2,55	2,22	2,18	4,11
ISL1	0,32	0,32	0,61	0,39	0,21	0,61
IRF1	21,62	95,80	78,52	37,15	89,08	59,21
SOD2	40,24	64,05	83,79	60,27	114,00	141,27
EIF4EBP2	0,53	0,60	0,69	1,32	0,71	1,21
CXCL2	50,18	36,28	43,22	120,80	20,76	61,81
CCL20	86,52	58,70	57,96	144,94	24,56	53,19

Table 21: Overview of the fold change values for each gene without the IP (w.o/IP) protocol and with (w/IP) the IP protocol.

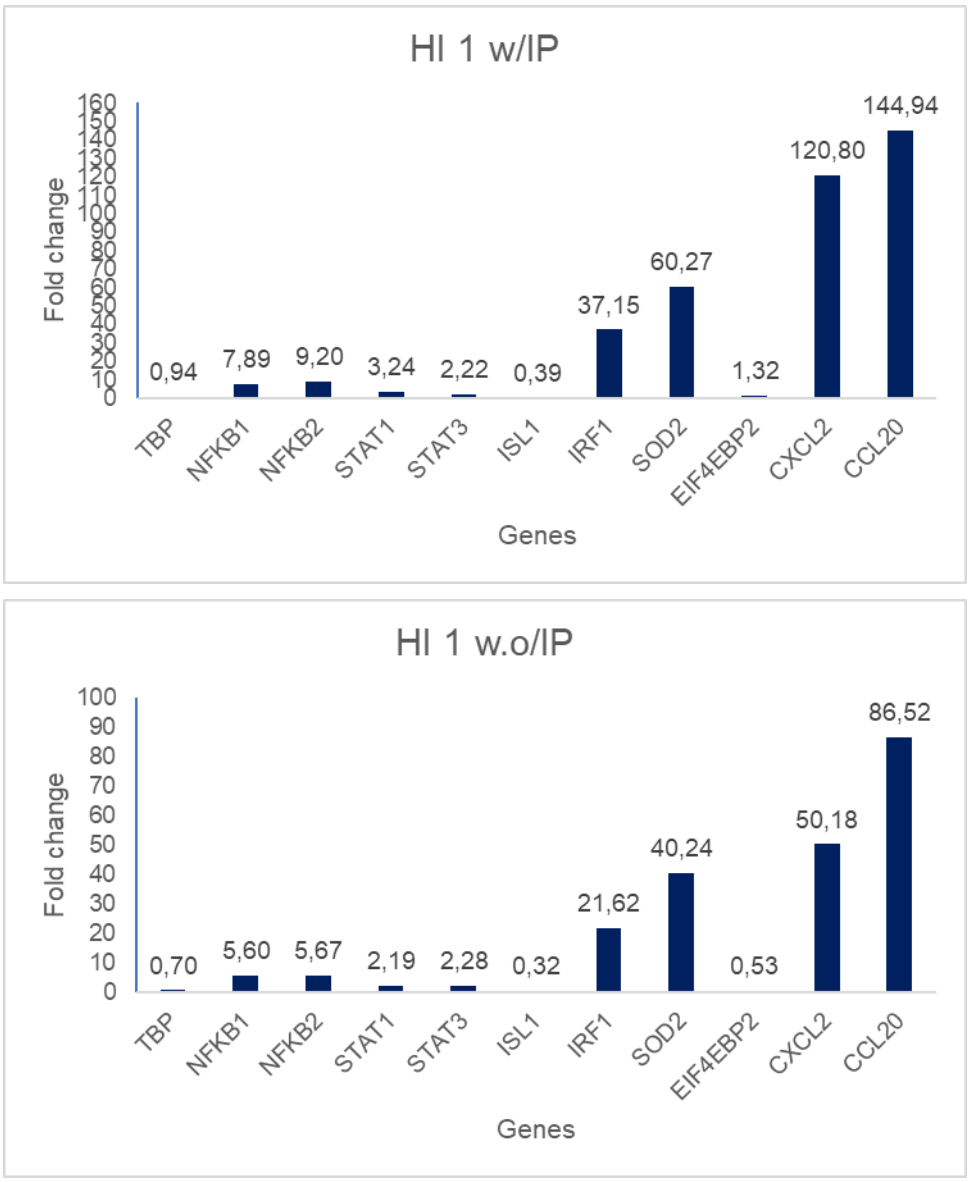

Figure 12: Diagrams illustrating the fold change for human islets donor 1. Top diagram, w.o/IP. Bottom diagram, w/IP.

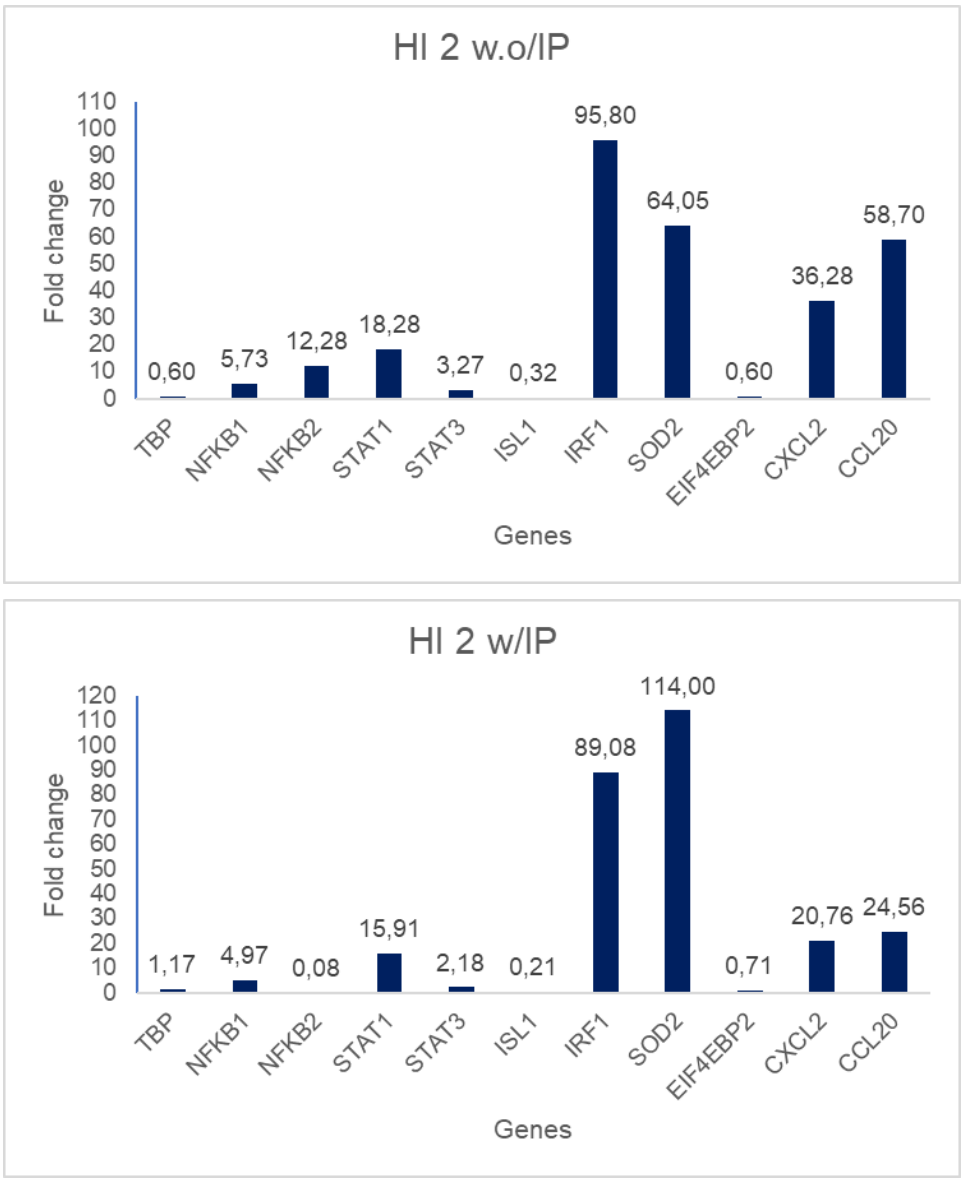

Figure 13: Diagrams illustrating the fold change for human islets donor 2. Top diagram, w.o/IP. Bottom diagram, w/IP.

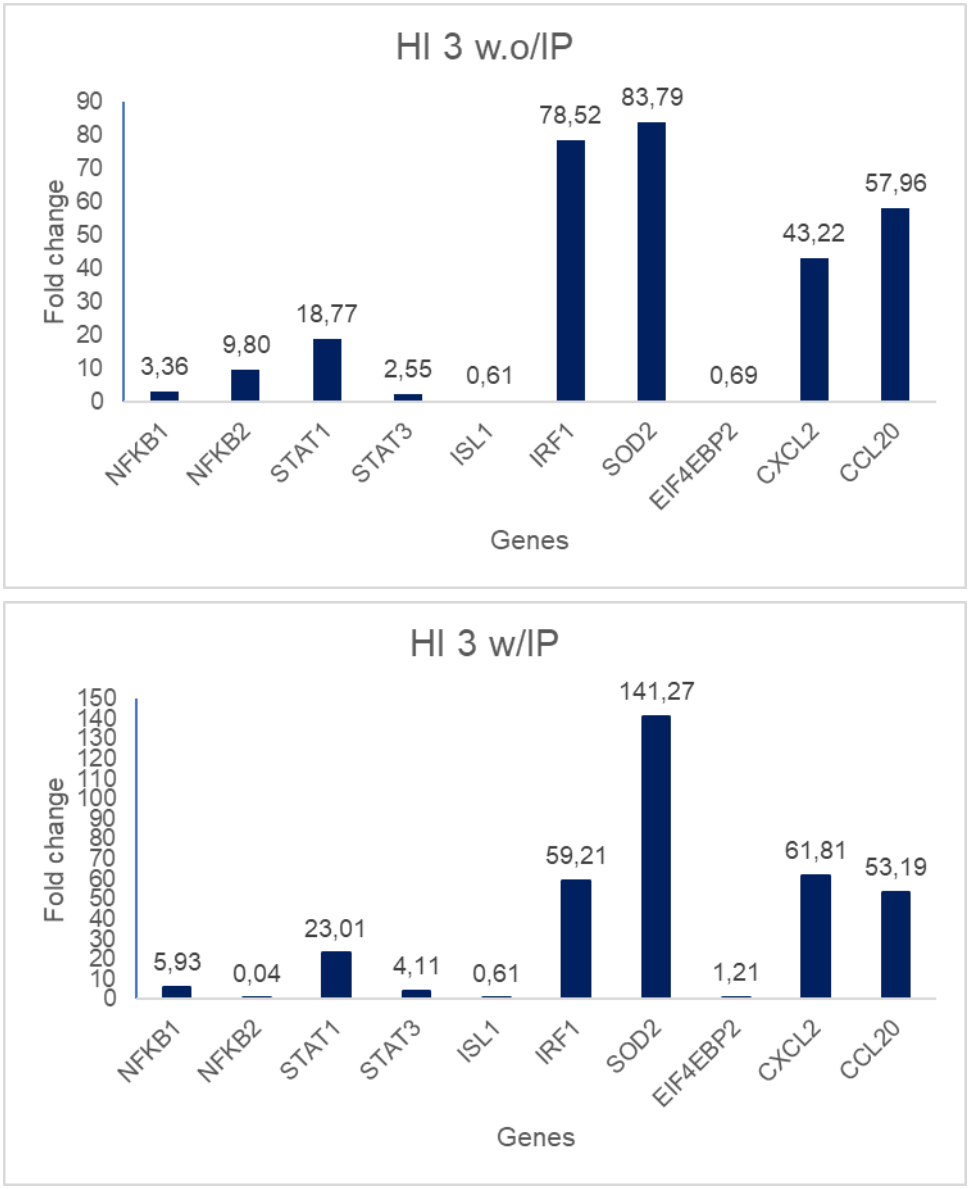

Figure 14: Diagrams illustrating the fold change for human islets donor 3. Top diagram, w.o/IP. Bottom diagram, w/IP.

Diagrams illustrating a comparison of the use of IP protocol compared to without the use of IP protocol can be seen in the Appendix.

5.0 Discussion

The aim of this study was to compare differences and investigate changes in gene expression for SC-α cells, SC-β cells and human pancreatic islets following cytokine treatment with IL-1β, TNF-α and IFN-γ. To answer our first question differentiated SC-α cells and SC-β cells from iPS cells and treated them, and donor human islets, with the prementioned cytokines. We chose a set of genes that have been reported to be responsive to cytokines (only IL-1β and IFN-γ) in β -cells (Lopes et al., 2014) to compare the different cell types' response to cytokine treatment. Most published articles show how these genes function only in β-cell. More research is needed on their function in α-cells and how or if they contribute to the dysfunction seen in α-cells. To answer our second question to further investigate the actively translated genes, we employed a recently developed ribosomal immunoprecipitation approach (Knight et al., 2012). Here, we used a previously validated antibody against the ribosomal protein RPL29 to pull out ribosome-mRNA complexes from the cell lysates. In parallel, we also investigated the transcripts from whole cell lysates, aiming to validate our ribosomal IP methodology. The aim was to perform RNA-sequencing on the samples to look further into the changes in the actively translated genes following cytokine treatment.

In this study we have successfully differentiated iPS cells into insulin positive β-cells and glucagon positive α-cells. An efficient differentiation protocol, that is also scalable, provides a stable cell resource of pancreatic endocrine cells. These cells have potential to be used in diabetes research, and other research involving the pancreatic α- and β-cells. Some researchers also believe that they could potentially be a source of cells used for treatment purposes in conditions like DM1 in the future (Vantyghem, de Koning, Pattou, & Rickels, 2019). The α-cell differentiation protocol is especially exiting as there is very little published research on the subject. The iPS-derived α-cells have potential to be used as a tool to broaden our knowledge on the α-cell function and electrophysiology in disease and in healthy individuals and expand the knowledge on diabetes.

The iPS cells used in this study were reporter lines for insulin (β- cells) and glucagon (α-cells). The reporter lines were created by inserting a red fluorescent protein, mCherry, into the DNA sequences for insulin and glucagon respectively. This meant that the mCherry is expressed with the hormone insulin/glucagon. Since the hormones are expressed when the cells are mature endocrine cells, the positive mCherry signal could be used as a reporter for a mature α- or β- cell. The mature cells could then be sorted out from the total cell population

based on the positive mCherry signal using a FACS machine. Examining the cells under the microscope after sorting proved that most of the sorted-out cells have a positive mCherry signal. This ability to sort out mature cells easily will give potential for the use of reporter lines in other research project. The use of reporter lines gives an advantage in *in vitro* studies and can be used in the future for other studies like pancreatic cell plasticity studies looking at dedifferentiation and transdifferentiation.

This study included an IP protocol to fish out the actively translated genes. The use of an IP protocol would allow us to compare the active translation with the total transcripts in the cell. In this IP protocol we used an antibody targeting the ribosomal subunit RPL29. After comparing the qPCR results from the controls without the IP protocol to the ones with the IP protocol, we can assume that the IP protocol did not falter the expression profile of the genes tested. This is an indication that the IP protocol was successful. A successful IP protocol is promising for future studies where information on the actively translated genome is useful.

The exposure to cytokines leads to an upregulation of genes that lead to attraction of immune cells that start or continue an inflammatory state in the islets. In this study the cytokine cocktail concentrations and the time the cells were exposed to cytokines were decided based on data from a previous master student at AstraZeneca. The 16-hour time-point was chosen to look at the early changes in expression before the cells underwent apoptosis. The qPCR was used to get an expression profile for the cells that could be compared to previously published data. The intention was to further to look at the early global changes in the cells using RNA-sequencing, but due to time constraints on the project this was not completed and is potential for a future study.

Overall, the qPCR data showed that several genes were upregulated following the cytokine treatment for the α-cells, β-cells and human islets, however, there was no sign of downregulation for any of the genes examined at in the study for either cell type. Compared to the study performed by Lopes et al. (Lopes et al., 2014), there are some differences in the expression patterns we saw for the human islets. Their group found a down-regulation of the *ISL1* (within 24h) and *EIF4EBP2* (before and after 24h), while this study shown no change in expression for these genes with or without the use of the IP protocol. This could be caused by aspects like alterations in the cytokine treatment, time-points, sensitivity or other experimental differences.

The human islets came from three different donors. There were some differences in the gene expression patterns between the three donors. It is uncertain why that is, but it might be caused by the three donors themselves. One aspect could be variations in α-cell to β-cell ratio between the donors. Donor 2 and 3 seem to have more similarities in their gene expression patterns. The differences could also be caused by factors like ethnicity, age or status of health. Issues during the experiment could also be the cause of differences in the results.

Both the sample with and without the use of IP displayed a strong upregulation of *CXCL2, CCL20, IRF1* and *SOD*2 in the α-cells, β-cells and the human islets. *STAT1* expression was increased in the α-cells, and in human islets donor 2 and 3. The β-cells had a small increase in STAT1 expression, while human islets donor 1 did not show a clear increase. *NF-κB1* had a slight increase in expression in the human islets both with and without the use of IP protocol. In the β-cells the slight increase could only be seen without the IP protocol. The α-cells did not have an increase in expression of *NF-κB1* in either protocol. *NF-κB2* had a slight increase in the β-cells that could only be seen without the IP protocol, in the α-cells it could be seen with both protocols. For the human islets it could be seen in all donors without the IP protocols, but only in donor 1 with the IP protocol.

We also compared our data to that generated by a previous master student who used the same methodologies. There were some small differences in the data, but they followed similar patterns for upregulated genes. The comparison of data show that the expression patterns are similar between the pervious study and this study. Both experiments show *SOD2, IRF1, CXCL2* and *CCL2*0 as the most differentially expressed genes for both cell-types. More batches of differentiation are needed to draw a firm conclusion. iPS cells from more than one donor would also give more reliable results. This study shows that the human islets follow the same general expression pattern for the studied genes as found in the SC-α and SC-β cells.

It has previously been demonstrated that *STAT1* is upregulated in rat primary β-cells by IFN-γ (Moore et al., 2011). This upregulation promotes apoptosis for the cells. *STAT1* is also a mediator for cytokine induced loss of β-cell identity like loss of insulin expression or other β-cell identity markers (Moore et al., 2011). Previous research show that *CXCL1* and *CXCL2* expression is enhanced by exposure to IL-1β (Burke et al., 2014). *CXCL1* and *CXCL2* are two of the ligands that can bind to the CXCR2 receptors on CXCR2 positive cells like neutrophils and chemoattract them to the site of inflammation. IL-1β regulates the *CXCL1* and *CXCL2* expression through the NF-κB and *STAT1* pathway (Burke et al., 2014). *CXCL1* was not

chosen as one of the genes in this stud. An RNA-sequencing would show if this was expressed as well after treatment with the cytokine cocktail.

CCL20 is shown to be induced within 1h after exposure to IL-1β through the NF-κB pathway according to research on human islets and β-cell lines (Burke et al., 2015). *CCL20* upregulation is found in several inflammatory conditions including DM1. The expression of *CCL20* is shown to chemoattract immune cells. Expression of *CCL20* is viewed as a contributor to the inflammation seen in insulitis in DM1 and other inflammatory diseases. Research has also shown that *CCL20* is overexpressed in both diabetes mice and in obese mice. The inflammatory cytokines present in DM1 also promote expression of the surface receptor for *CCL20* called CCR6 on neutrophils (Burke et al., 2015).

IRF1 has been shown to down-regulate inflammatory mediators. *IRF1* expression is regulated by IFN-γ through binding of *STAT1* to the *IRF1* promotor region. It may also be induced by other transcription factors (Moore et al., 2011). *SOD2* is one of the cells antioxidant defense systems. Stress related to free oxygen radicals is proven to be related to the destruction of β-cells in diabetes (Pourvali, Abbasi, & Mottaghi, 2016).

As few studies have been performed on the α-cells response to cytokines, we do not have a lot of data to compare our results to. The data set from the previous master student and this study may indicate that the α-cells are also sensitive to the cytokine treatment, same as the β-cells. This could mean that the α-cells in the human islets also respond to the inflammatory process seen in DM1. α-cells sensitivity to cytokines can be a possible explanation for the hyperglucagonemia and defective glucagon regulation seen in some DM1 patients. α-cells have been shown to be more robust compared to β-cells when it comes to apoptosis in DM1 (Barbagallo et al., 2013). More research on α-cells and their involvement in DM1 is needed as this cell type has been understudied in diabetes research so far. An RNA-sequencing would give more information on the α-cells response to the cytokines. This would provide a new insight into the complete gene expression changes seen in α-cells. This data could be compared to the changes seen in β-cells to possibly point out why the β-cells are more sensitive to inflammation.

We had some problems during the differentiation leading to a low number of positive cells after the cell sorting. This meant we did not have enough cells for the following experiments. After the RNA-extraction of the first batch of α- and β-cells the amount of RNA in the samples were too low to be performed with RNA-sequencing on the samples. On the

following differentiations we used a larger scale of cells to hopefully get enough RNA to complete both qPCR and RNA-sequencing. Unfortunately, the differentiations were not completed due to time-constraints caused by the restrictions following the Corona-virus outbreak. In the project we had planned more differentiations, following experiments, and RNA-sequencing data for all samples. The RNA quality in this study was also not optimal. The ratio of A260/280 should be around 2 indicating good quality and purity of RNA. Since the values here deviated from 2 this means the RNA samples are not pure or may be degraded to some extent. This could be due to contamination of the samples from DNA or protein left-over after the RNA-extraction. The quality of the RNA-samples in this study might have affected the differences in expression seen between the human islet donors, and between this study and some other studies. As we only have qPCR data from one set of SC-α and SC-β cells we do not have enough information to draw any conclusions on whether the cells respond to the cytokine treatment differentially than the human islets. The qPCR results indicate that the SC-β cells respond to cytokines mostly in line with earlier publications, but we need more than one batch of cells to confirm the results. It also indicates that the SC-α cells respond to cytokines and that this may lead to their observed dysfunction in diabetes which we cannot find any literature to compare it to. Due to the time-constraints mentioned above we also did not have time to attempt RNA-sequencing on the samples where we had good quantity of RNA. A follow-up study with additional differentiations and following RNA-sequencing should be performed on samples with better RNA quality and quantity. Some further experiments to optimize this should be performed to get the best RNA-samples for a future study. We have demonstrated some genes where we can assume changes in gene expression with qPCR that can be used as a basis for genes to study further in a future RNA-sequencing experiments to look at the total active gene expression profile.

In summary we show the following:

- Glucagon positive SC-α cells and insulin positive SC-β cells were differentiated according to the differentiation protocols and showed key cell markers during the differentiation steps.
- Cell were successfully sorted to purify positive cells based on their fluorescent mCherry expression by FACS.
- The cytokine mediated changes in gene expression in this study matches the data obtained by a former master student indicating that the protocol can provide comparable cells between differentiations.

- We observed similarities in the gene expression changes for the SC- α cells, SC- β cells and the human islets. This could in the future potentially lead to a better understanding of the pathogenesis of DM1 and the implications of α-cell dysfunction in the disease.

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Appendix

Figure 1-5 visualize the effect of fold change values using the IP protocol (blue) and without the use of IP protocol (orange). From these we can see that the IP protocol did not loose any of the genes of interest and can be used to pull out the active transcriptome.

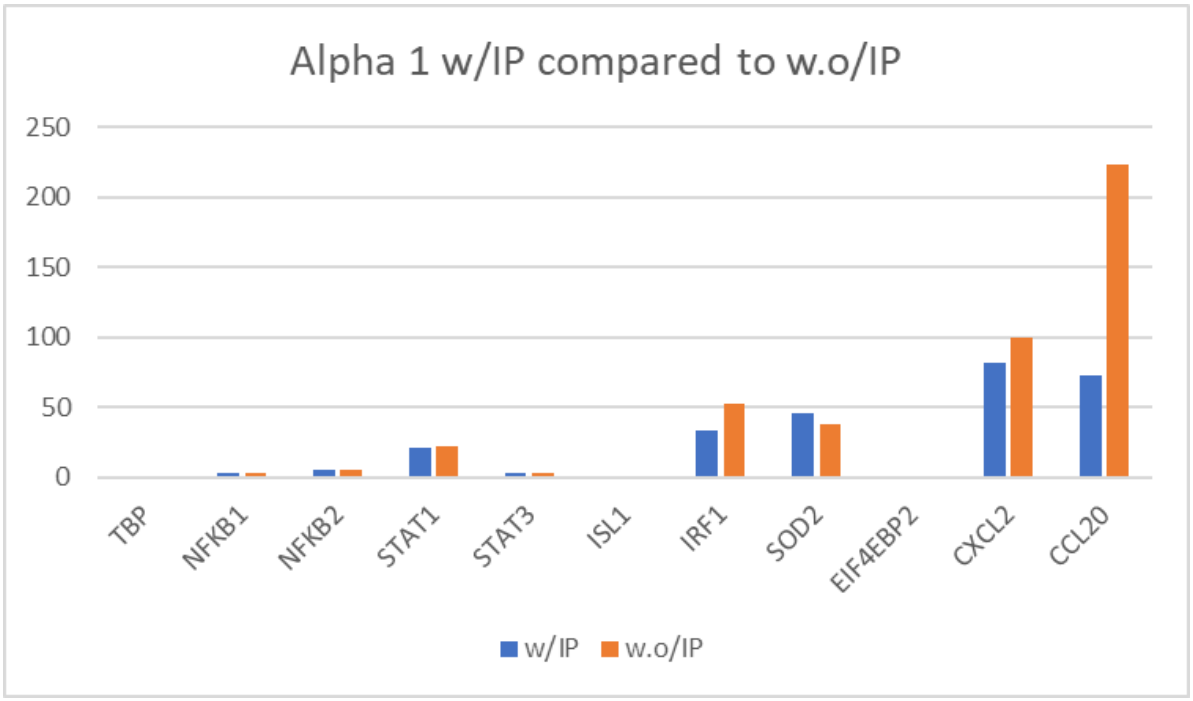

Figure 1:Comparison of fold change values for α-cells with and without the IP protocol.

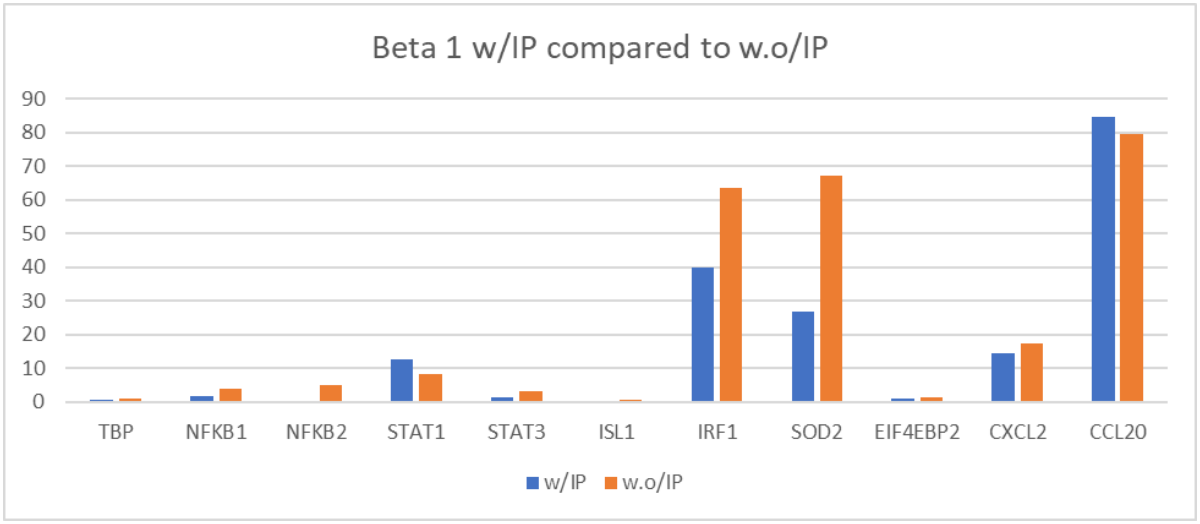

Figure 2: Comparison of fold change values for β-cells with and without the IP protocol.

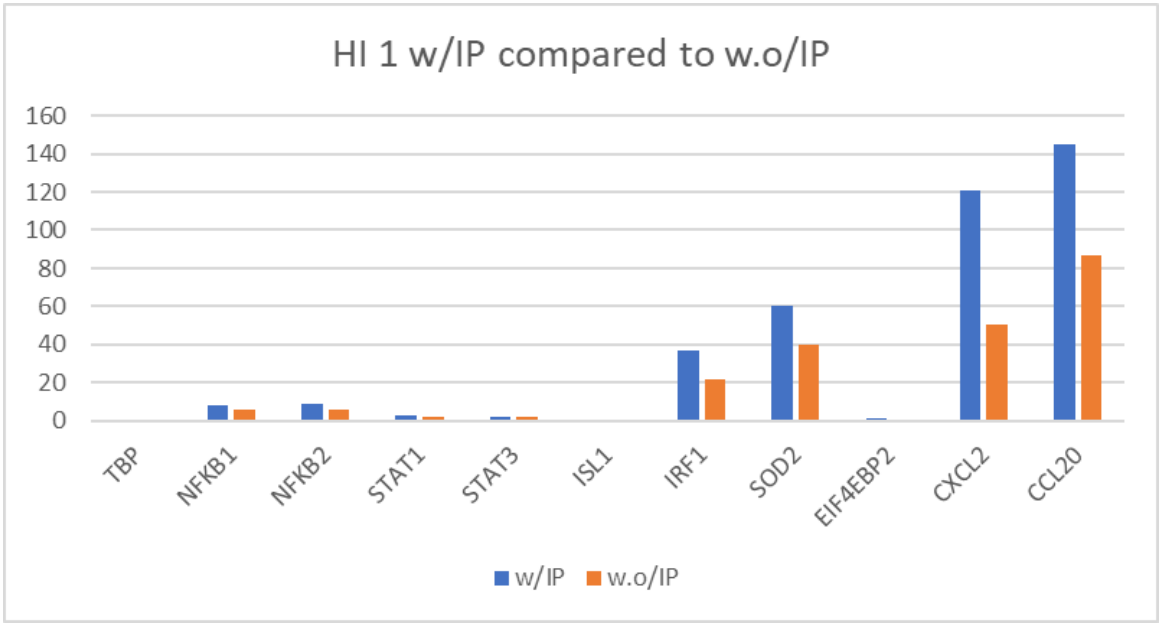

Figure 3: Comparison of fold change values for the donor 1 human islets with and without the IP protocol.

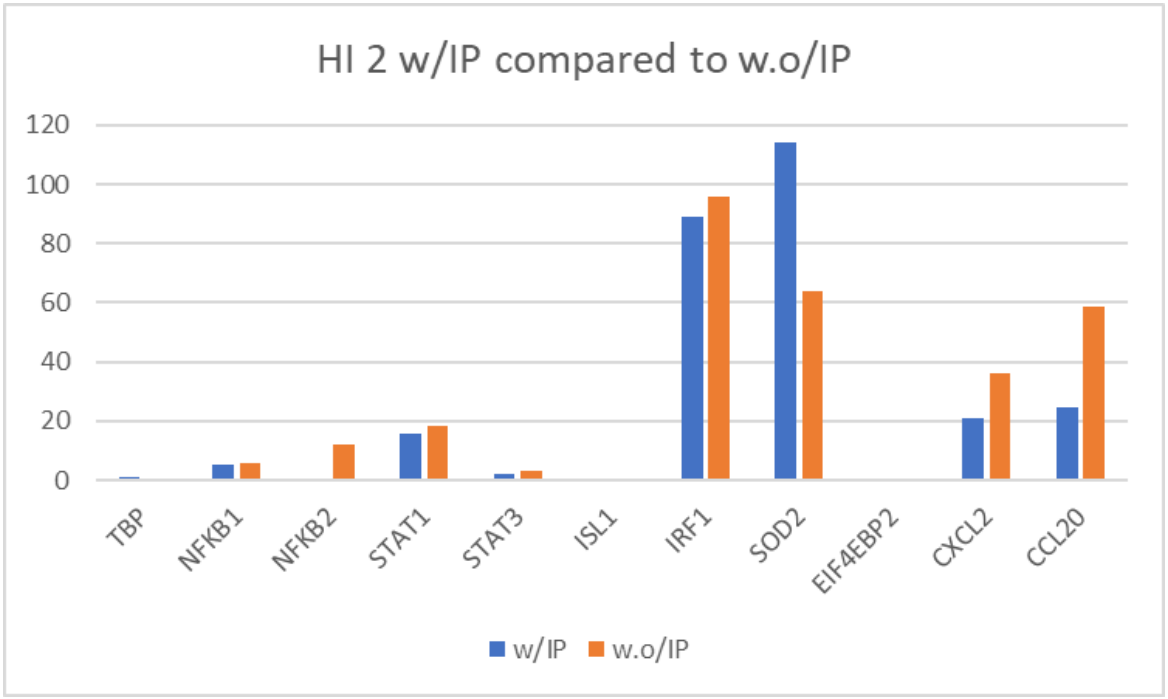

Figure 4: Comparison of fold change values for the donor 2 human islets with and without the IP protocol.

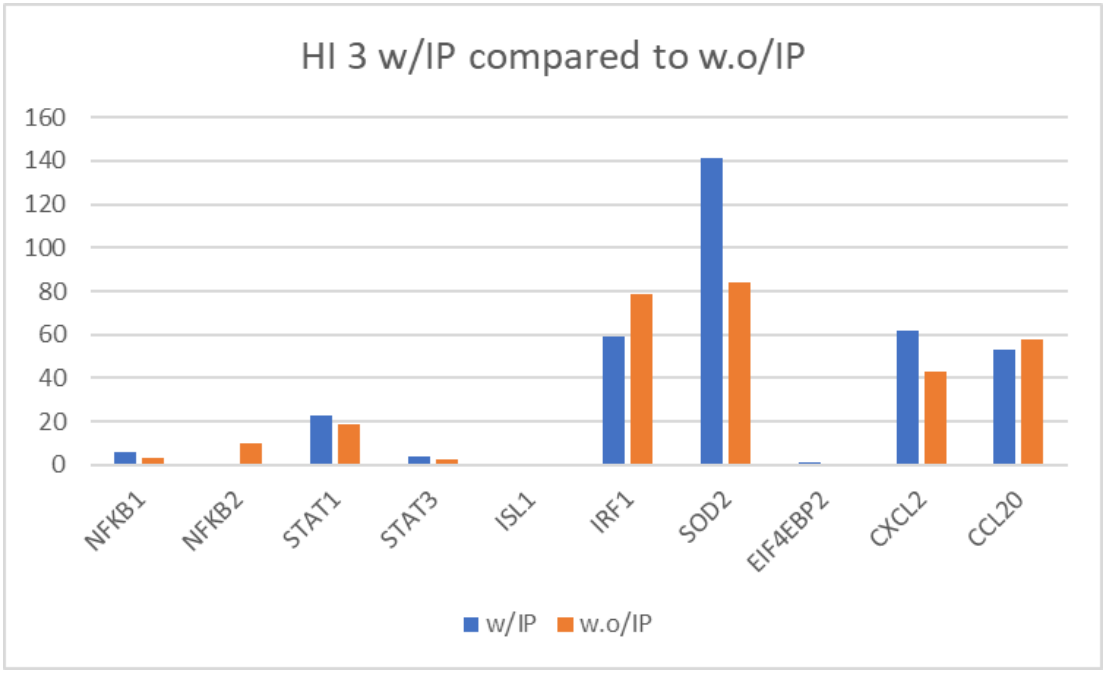

Figure 5: Comparison of fold change values for the donor 3 human islets with and without the IP protocol.

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