MASTER THESIS

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Reprogramming human gastrointestinal stem cells into insulin-secreting cells: developing a cell therapy for diabetes

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Kurzfassung

Eine erfolgreiche Behandlung von Typ 1 Diabetes benötigt das Regenerieren verlorener insulinproduzierender Zellen und das Umgehen der Autoimmunreaktion. Tierstudien haben gezeigt, dass die Reprogrammierung von adultem Gewebe die neu regenerierten Zellen großteils immunisiert. In dieser Arbeit reprogrammieren wir menschliche Stammzellen isoliert aus Teilen des Magens und des Dünndarms.

Transduktion von adenoviralen Plasmiden ermöglicht die Reprogrammierung durch Aktivierung von Ngn3, PDX1 und MafA. Die transiente Expression dieser Gene wird durch 4-Hydroxytamoxifen und Doxycyclin gesteuert. 4-Hydroxytamoxifen im Zellkulturmedium ermöglicht die Expression von Ngn3 und die Umwandlung der Stammzellen zu entero-endokrinen Zellen. Um die Expression von PDX1 und MafA und die Reprogrammierung zu Insulin produzierenden Zellen zu aktivieren, muss Doxycyclin zum Medium hinzugefügt werden. Die Reprogrammierung dauert 10 Tage, bis die Stammzellen Insulin ausschütten. Um die Funktion und das Überleben der Zellen in Organismen zu testen, werden die Zellen in immun-geschwächte Mäuse transplantiert.

C-peptid, ein Nebenprodukt von Insulin, kann schon nach vier Tagen festgestellt werden. Langzeit-Reprogrammierung der Stammzellen erhöht die Menge und Qualität der beta-ähnlichen Zellen. MafA, ein wichtiges Betazellgen, ist co-lokalisiert mit C-peptid. Dies bestätigt die erfolgreiche Reprogrammierung erneut. *In vitro* werden eine Reprogrammierungsrate von 30% bis 40% erreicht. Eine Transplantation von Stammzellen und darauf folgenden Reprogrammierung in Mäusen zeigt wenig überlebende Zellen und keine messbare Reprogrammierung. Optimierung der Reprogrammierung *in vitro* resultiert in einer Änderung des Mediums und der Reprogrammier-Prozedur. Reduzierung des fetalen Kälberserums im Medium erhöht die Insulin Expression. Um das Überleben der Stammzellen in Mäusen zu verbessern, werden die Stammzellen gemeinsam mit ETV2-überexprimierenden Endothelzellen aggregiert. Stammzellen des Magens, genauer des Korpus, zeigen keine messbare Reprogrammierung nach der Aggregation mit Endothelzellen. Um die Reprogrammierung *in vivo* zu verbessern, wurde ein neuer Adenovirus hergestellt. Dieser nutzt das TetOff Expressionssystem anstatt des TetOn Systems. *In vitro* konnte keine Reprogrammierung der neu kreierten Zelllinie festgestellt werden.

Reprogrammierung von Stammzellen des Magen-Darm-Trakts ist eine vielversprechende Methode, um Insulin produzierende Zellen herzustellen. Die Optimierung des gesamten Vorganges ist schwierig, allerdings hat das Reprogrammieren dieser Stammzellen viele Vorteile.

Schlagwörter: Typ 1 Diabetes, Stammzelle, Reprogrammierung, Betazelle, Beta Zellregeneration

Abstract

Successful treatment of type 1 diabetes requires renewal of insulin secreting cells and protection against autoreactive T-cells. In mice, reprogramming adult tissue related to beta cells shows promise in evading the autoimmune reaction. Here, we successfully reprogram adult gastro intestinal stem cells into insulin secreting cells and evaluate their potential for clinical applications.

Adeno-associated viral vectors are transduced into the corpus, duodenum and antrum stem cells to initiate the reprogramming. These vectors carry gene expression systems activated by 4-Hydroxytamoxifen and Doxycycline. The reprogramming process starts after adding 4-Hydroxytamoxifen to the cell culture medium. This induces Ngn3 expression and converts the gastro intestinal stem cells into entero-endocrine cells. For further reprogramming into insulin secreting cells, Doxycycline is added, which activates MafA and PDX1 expression. Ten days of Doxycycline reprograms the cells and functioning insulin secreting cells are detected *in vitro*. To evaluate the cells survival and function, the reprogrammed beta-like cells are aggregated and transplanted into immune-deficient NSG mice.

Immuno-staining of reprogrammed stem cells show that C-peptide (CPPT), a biproduct of insulin, can be detected after 4 days of Doxycycline reprogramming. Long term reprogramming of cells for 10 days or longer result in more and better insulin secreting cells. MafA, a gene associated with beta cells, is co-localized with CPPT and confirms the reprogramming. A reprogramming efficiency of 30%-40% can be reached *in vitro*. Transplantation of stem cells and reprogramming confirm low survival and no detectable reprogramming *in vivo*. *In vitro* reprogramming optimization shows that the medium and process used have to be altered. Reduction of fetal bovine serum in the medium increases the insulin expression. Co-aggregation of reprogrammed stem cells with ETV2-overexpression HUVECs is hypothesized to increase survivability *in vivo* and insulin expression. Corpus stem cells show no detectable reprogramming when co-aggregated with HUVECs. Using the TetOff expression system instead of the TetOn system is meant to enable the reprogramming *in vivo*. *In vitro* no stem cell reprogramming can be detected.

Reprogramming gastro-intestinal stem cells into insulin secreting cells shows great promise. This approach is difficult and requires constant optimization but reprogramming autologous gastro-intestinal tissue and transplanting it has many advantages.

Keywords: Type 1 Diabetes, Stem Cell, Reprogramming, Beta Cell Regeneration

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1 Introduction

Diabetes mellitus is a metabolic disease present worldwide. Though the percentage of diagnosed diabetes mellitus varies depending on the region, estimates suggest that the number of affected patients is increasing. In regions like the Middle East and North Africa less people are reported to have diabetes, while the Western Pacific region shows a high prevalence of diabetes in adults. [1]

The epidemic proportions of diabetes can be explained due to the fact that it is not specific to any age group. T1D can occur at any age. Comparing the two subtypes of diabetes, type 1 (T1D) is usually diagnosed in children or young adults, while type 2 (T2D) is more frequently documented in adults or elderly people. 90-95% of the diabetic population suffer from T2D, while 5-10% have T1D. [2]

Diabetes is a metabolic disease, which is characterized by hyperglycemia. This hyperglycemia can be explained by defects in insulin secretion by pancreatic beta cells or insulin resistance by peripheral organs. The main difference between T1D and T2D is that T1D is an autoimmune disease, whereas T2D is not. While T2D is called adult-onset diabetes and can be controlled with several drugs, T1D is diagnosed in children and require insulin injections for survival. If drug treatment shows no improvement, insulin injections can be used to treat T2D.

Patients suffering from T2D acquire a peripheral resistance to insulin. This means that the peripheral tissues like liver and fat cannot extract insulin from the blood due to insensitivity or resistance to insulin signalling. By frequent observation and blood glucose control, this disease can be well managed.

T1D is characterized by the autoimmune destruction of the pancreatic β-cells. The cells of the adaptive immune system destroy the β-cells through inflammation and humoral response. Without β-cells, the pancreas cannot produce insulin and the blood glucose starts to rise. To counter the effects of this destruction, frequent insulin-injections are necessary. People with specific genetic anomalies are more prone to develop diabetes but this field of research requires much more attention. [3]

These insulin injections and the control of the blood sugar are currently the only viable treatment options but since the prevalence of diabetes is increasing, a lot of resources are employed in search of deeper insight into diabetes and easier and better treatment alternatives.

1.1 Current research

There are a lot of different approaches towards curing or preventing T1D or other autoimmune diseases nowadays. The mechanisms of these approaches are different but the desired result is the same. Cell-therapeutic experiments try to modulate the beta cells to increase proliferation, insulin secretion and resistance against T-cells. Many mitogenic agents and transcription factors are researched in hope of finding a viable proliferation method. Controlling and manipulating the autoreactive T-cells is a different cell-based approach. Many different genes and molecules have the potential the modulate the immune system. The focus of bio-engineers lies in developing biomaterials which increase beta cell survival and shield the cells from autoreactive immune cells. By enveloping the beta islets in specific biomaterials, the immune cells cannot reach their target. Stem cell experts are researching ways to reprogram existing human tissue into insulin secreting cells. Many different tissues are researched for reprogramming purposes. While many scientists put their hope into induced pluripotent stem cells, others focus on pancreatic or closely related tissue. Papers on the current research are described in the following paragraphs.

A recent study conducted by Ouaamari et al. suggests SerpinB1 as an enhancer for β-cell differentiation and therefore partial resistance to diabetes.[4] In this study a liver insulin receptor knock-out is used to proliferate β-cells until islet hyperplasia. They show that the liver-derived protease inhibitor SerpinB1 is responsible for the islet hyperplasia observed in these mice. Furthermore SerpinB1 is shown to increase the islet mass in mice as well as humans and zebrafish. These data suggest SerpinB1 to be a potential treatment option for T1D. [4]

Loh et al. present a way to pharmacologically and genetically increase the insulin production of β islets. [5] They observed that the Y1 receptor and its resulting pathway inhibits insulin secretion in murine islets. By knocking out the insulin secretion in murine islets. By knocking out the 1 receptor, this inhibitory pathway can be deactivated and the islets secret increased amounts of insulin. Alternatively, to the genetic manipulation, pharmacological blocking of the Y1 receptor leads to an increased release of insulin in murine and human islets as well. Thus, the manipulation of this receptor could potentially decrease the severity of diabetes. [5]

Proliferating the β-cells before the autoimmune attack is another way to decrease the efficiency of the destruction. In their study Dirice et al. claim that an enhanced beta cell mass prior to the onset of diabetes changes the β-antigens, which in term weakens or prevents the autoimmunity. [6] The $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells generated in mice subjected to an expansion of beta cell mass show reduced abilities at killing beta cells. This supports the hypothesis that expansion results in a change of the β-cell identity and therefore a weaker depletion of β-cells. [6]

In the last years a lot of drugs used to treat T1D in mice have been tested in clinical trials. These drugs mostly target antigens expressed by beta cells. An immunization with these antigens show a prevention of T1D or similar beneficial effects in mice. [7], [8], [9], [10]
One of these antigens is GAD65. This glutamate decarboxylase isoform is known as an autoantigen expressed by beta cells. After immunizing non-obese diabetic (NOD) mice with this antigen a prevention of T1D was achieved. However, clinical studies demonstrated no significant effects on beta cell function loss in diabetic patients. This is most likely due to the high variability in the dose and timing of the treatment. [7], [8], [9], [10]
Another important autoantigen in T1D is insulin. Preclinical studies in NOD mice have shown that oral administration of insulin prevents further development of T1D. In clinical studies insulin proved to have beneficial effects on a subset of patients though not as significant as in a mouse model. Again the timing and dose is a crucial variable in these trials. [10]
Using the $Foxp3^+$ regulatory T cells (Treg) as another way to treat T1D has been discovered. These Tregs are partly responsible for the suppression of autoreactive T-cells. This pathway can be exploited by inducing these cells and therefore diminish the autoimmune destruction of beta cells. Although this method is still in its infancy, Serr et al show that these regulatory T-cells are stable and express Treg specific genes like Foxp3 and CTLA4 *in vivo*. [11] In future this concept could be used to vaccinate patients. Keeping in mind that this prevents an autoimmune attack, it is useless if the beta cells are already destroyed. [11]
Clinical studies revolving around dipeptidyl peptidase-4 (DDP4) were conducted to investigate its effect on T1D. DDP4 is inhibited which inhibits glucagon release. Specifically, DDP4-inhibitors increase incretin levels. These incretins inhibit glucagon release and reduce blood glucose. Wang et al researched these studies and reviewed their results. They conclude that insulin supplemented with DDP4 inhibitors improved the pathogenesis of T1D. After reviewing several clinical studies they report that while this effect does exist, it is not significant. [12]
Fattah et al hypothesise that some treatment options used in T2D patients are applicable for T1D as well. One of these drugs inhibits the sodium glucose cotransporter 2 (SGLT2). By inhibiting this pathway, the glucose retention ability of the kidneys is diminished. This solves the problem of hyperglycaemia without risking hypoglycaemia which is the case during insulin treatment. The usage of this drug to treat T2D is approved by the US Food & Drug Association (FDA), which should increase the chance of approval for T1D treatment. [13]
In the last 2 decades, pharmacological advancements in the field of T1D allowed for a lot of new drugs. These drugs use different dosages, delivery systems or pharmacologically enhanced active substances to regulate the blood glucose level. Just looking at insulin as active substance, there are drugs like Insulin Degludec, an ultra-long-acting insulin compound, or BioChaperone, an ultrarapid insulin. Dosing and mixing drugs can achieve promising study results but if not followed to the latter, they can be very problematic. Hypoglycaemia and increased cardiac problems are just two side effects of such drugs. An alternative to insulin is the commonly used drug Metformin.

By increasing the glucose uptake of skeletal muscles as well as glucose sensitivity the blood glucose levels can be kept in check. Studies in T1D patients show conflicting results. While some studies produce significant data that show positive effect with Metformin, other studies fail to reproduce these results. This reveals another big problem in T1D patients: every patient can react differently to the same treatment, which makes finding a pharmacological cure very challenging. [14]

To develop a universal treatment, scientists concentrate on regenerating the pancreatic tissue. To regenerate endocrine tissue from the pancreas, the most desired possibility are pancreatic stem cells.

Xu X. et al suggest in their paper, that beta cell progenitors exist in adult mice. [15] By creating an injury model in adult mice, parts of the pancreas were destroyed. Ngn3-positive cells inside the lining of the pancreatic duct started to proliferate and differentiate into insulin+ cells. This shows that in adult mice an Ngn3-positive progenitor of endocrine tissue is present. [15]

Regenerating specifically beta cells is a challenging task. In the last twenty years several methods replenishing destroyed beta cells in mice were developed. Different approaches include dedifferentiation of other pancreatic endocrine cells like alpha cells, or by generating new pancreatic tissue *in vitro* by reprogramming.

Thorel et al investigated the conversion of pancreatic alpha cells into functioning beta cells in mice. [16] They provide insight into the regeneration of beta cells after near total ablation by selective diphtheria toxin treatment. Injecting treated mice with insulin keeps them alive long enough to experience beta cell renewal over a span of 6 months. Long term experiments for 10 months showed that after 6 months of insulin injection, the mice survived on their own due to beta cell regeneration. Lineage tracing of alpha cells prior to the experiments revealed the dedifferentiation of alpha cells into insulin secreting beta cells. These results suggest plasticity between cell types of certain pancreatic endocrine cells. [16]

Reprogramming adult tissue into insulin secreting cells has been researched for many decades. Recent transcription factor screening revealed three key regulators for beta cell identity. Ngn3, PDX1 and MafA (referred to as NPM factors) are overexpressed in an adult mouse pancreas, which leads to reprogramming of pancreatic acinar cells into insulin secreting cells. An adenoviral vector can be used to introduce the genes into the pancreas. [17] The generated beta cells show the same characteristics as endogenous beta cells in terms of shape, size and ultrastructure. This direct reprogramming approach has advantages over reprogramming pluripotent cells. [17]

Alpha cells can be reprogrammed into beta-like cells by Arx inactivation. Courtney et al provide information on the relationship between Arx, Pax4 and the beta-like cell conversion. [18] Deletion of the Arx gene in pancreatic tissue of mice resulted in the conversion of alpha cells into functional beta-like cells. While Arx is reported to be the main regulator of this conversion, Pax4 can contribute to this reprogramming as well. Diabetes can be reverted in Arx mutated mice after beta cell depletion. This makes Arx, its targets and cofactors possible strategies for *in vitro* testing on human cells. [18]

To find other types of tissues prone to reprogramming into beta cells, whole body overexpression of the NPM factors are necessary. Ariyachet et al show that certain intestinal tissues are capable of reprogramming. Antrum cells can be reprogrammed into insulin secreting cells and hyperglycaemia can be suppressed for 6 months. [19] Organoids generated from reprogrammed antrum cells suppressed hyperglycaemia in diabetic mice after transplantation. The high turnover rate of the intestine increases the yield of insulin secreting cells significantly. [19]

Genetic modifications are not well suited for clinical application. Preferentially cytokines and other molecules are used to regenerate beta cells. Baeyens et al developed a reprogramming approach without using genetic modification. [20] Transiently administering epidermal growth factor and ciliary neurotrophic factor to adult pancreatic tissue depleted of beta cells leads to differentiation of acinar cells into beta-like cells. These cells are glucose responsive and function like beta cells. Normal glucose levels are established and remain for 248 days. Though this process creates beta-like cells without modifying genes, the methods takes a long time and the number of acinar cells limits the efficacy of the method. [20]

Proof of concept studies in animal models generate a large number of desirable and possible methods for beta cell regeneration. The translation from animal to human is the big challenge in beta cell renewal. Many factors used in animal research does not work on human cells. The conversion of human pancreatic alpha cells into beta-like cells provides the best results. Conversion of mouse pancreatic alpha cells into beta-like cells is accomplished by inducing PDX1 and MafA. [21] Research by Xiao et al suggests that human alpha cells can be converted in similar fashion. Introducing PDX1 and MafA into human alpha cells in absence of human beta cells starts the alpha- to beta-like cell conversion. These reprogrammed human insulin secreting cells can reverse hyperglycaemia in mice after xenograft transplantation. This provides proof that human beta cell fate can be artificially induced in non-beta cells. Alpha cell conversion is a possible therapeutic approach for treating T1D. [21] Other human cell types reliably reprogrammable into beta-like cells are pancreatic duct cells. If the duct cells are cultured in a 3D spheroid and treated with Ngn3, PDX1 and MafA, the gene profile shifts to an endocrine progeny state, resembling a beta cell line. [22] The beta-like cells have similarities in function, insulin production and secretion post glucose challenge. This study shows that other cell types than alpha cells are able to reach beta-like cell fate. [22]

Pancreatic exocrine tissue can be reprogrammed into insulin secreting cells according to recent findings. [23] The factors responsible for the reprogramming are mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3). Expression of these factors in exocrine tissue leads to Ngn3 expression in 50%-80% of the cells. The transduced exocrine cells start to express PDX1 and Pax4 as indicators of beta-cell like conversion. Due to the vast number of available pancreatic exocrine tissue, this approach could be further developed into clinical applications. [23]

Beside reprogramming tissues similar to beta cells, approaches using human embryonic stem cells or induced pluripotent stem cells have emerged in the last years.
Kroon et al study the differentiation method of beta-like cells from human embryonic stem cells. [24] They show that human embryonic stem cell derived pancreatic endoderm reliably generate glucose-responsive beta cells. After engraftment in mice the beta-like cells exhibit beta cell gene expression, maturation and secretion of insulin. Hyperglycemia can be prevented by transplanting the pancreatic endoderm into mice. This data suggests that human pancreatic tissue can be artificially generated *in vitro* and remains its function *in vivo*. [24]
Pancreatic endodermal tissue derived from human embryonic stem cells are rejected by the immune system of mice. To counter this immune reaction Szot et al researched treatment options that improve graft survival and function. [25] In immunodeficient mice the human pancreatic endodermal tissue matures and survives. The adaptive immune system of mice attacks foreign tissue and destroys it. CTLA4Ig and anti-CD40L antibody treatment of mice resulted in minimal rejection of the xenograft. Further, the treatment modulated the immune system to produce T-cells not reactive to human pancreatic endodermal tissue. Studies showed that upon injection of splenocytes isolated from treated mice, xenograft survival was significantly increased. The same results were observed when injecting human peripheral blood mononuclear cells (PBMC) into mice post xenograft. CTLA4Ig and anti-CD40L antibody treated cells reacted less to allogenic tissue. This shows that differentiating human embryonic stem cells and treating human PBMCs with CTLA4Ig and anti-CD40L antibodies provides an approach for clinical research. [25]

Islet transplantation is another currently used and researched treatment option for T1D patients. This procedure uses allogenic beta cell islets to replenish the destroyed islet population. The main problems with this treatment is the immunogenicity and survivability of the allograft. Even though new and functional beta islets are transplanted into patients, the immune system destroys these cells as well. This means that immune suppressing drugs are a necessity for these patients which has its own problems and risks. Additionally, the vascularization and survivability of the graft can be problematic.
Song et al. developed an *ex vivo* genetic engineering approach to improve the survival rate of transplanted islets. They transduce pancreatic islets with an adenovirus expressing betacellulin, a growth factor ligand important for beta cell growth and differentiation. Pancreatic islets transplanted together with betacellulin-expressing islets show enhanced differentiation, survival and insulin secretion. [26]
NK1.1-positive cells were shown by Tripathi et al. to be important factors for graft survivability. By inducing IL-22 production through NKG2A, these cells enhance the survivability and insulin secretion of the beta cells. Furthermore, NKG2A plays a role in the reduction of the immune response to allogenic materials. These liver-derived NK1.1-positive cells and their response to allografts could decrease the need for immunosuppressive drugs and therefore improve the health of the patient. [27]

Donor islets necessary for islet transplantations do not have to be isolated from cadavers. Studies into xenograft transplantations raised the promise of interspecies transplants. Animals size equivalent to humans can be used as pancreas donors if the pancreas is functional and insulin has the same effect on humans. Dufrane et al. studied the effects of pig islets encapsulated in alginate in primates. [28] They transplanted encapsulated and non-encapsulated pig islets in optimal and non-optimal conditions into primates and measured the pig insulin levels. Capsule integrity, cellular growth, anti-pig antibodies, islet survival and pig CPPT levels were analysed for up to 6 months post transplantation. As expected non-encapsulated islets and islets in non-optimal conditions were quickly destroyed. Without administering immunosuppressants, in all of the primates 86% of the transplanted islets survived for 6 months and showed no capsule fibrosis. Explanted islet capsules had residual pig insulin and showed glucose responsiveness. This study demonstrates that encapsulated xenograft islets can survive and produce insulin in primates for 6 months. [28]

Another study conducted by Sun et al. focused on the function of encapsulated porcine islets in diabetic primates. [29] Insulin independency was reached for these primates after several islet transplantations. The independency ranged from 120 to 804 days. Increased insulin levels and faster glucose clearance rates were measured after the transplantations. Isolation of the transplanted tissue 3 months after transplantation revealed intact islet capsules without cellular overgrowth. Inner organs of the primates were analysed 2 years after the study and no untoward effects were found. [29]

Another xenograft related approach to treating T1D is growing human pancreas in suitable host organisms. Proof-of-concept studies in animal models like rodents show that generating chimeric animals and isolating tissue can overcome induced diabetes. Kobayashi et al. showed that injecting wild type pluripotent stem cells into PDX1-KO mouse blastocysts, the pancreas is almost completely composted of wild type tissue. [30] PDX1 is the gene determining the pancreatic development. Inter species chimeras were created by injecting mouse or rat pluripotent stem cells into rat or mouse blastocysts, respectively. This experiment confirmed that interspecies pluripotent stem cells contribute to the embryonic development. The creation of xenogenic pancreata was accomplished by injecting rat pluripotent stem cells into PDX1 KO mice. These data show that pluripotent stem cells can be used to generate xenogenic pancreata in animals. [30]

Yamaguchi et al. researched the generation of rat-sized mouse pancreata. [31] Earlier studies of their lab successfully created mouse-sized rat pancreata, which are too small to isolate islets and treat diabetes in rats. Here they do the opposite and inject mouse pluripotent stem cells into rat PDX1-deficient blastocysts, creating rats with mouse pancreata. The islets isolated from these pancreata were used to treat and reverse diabetes in mice. Normoglycemia was restored and stabilized for more than 370 days. No immunosuppression was necessary to keep the transplant and host healthy. These data provide insight into xenogenic organogenesis and proof of concept. [31]

The recent advances in the generation of biomaterials and the development of immunosuppressants advance therapies for T1D constantly. Encapsulation in biomaterial solves the main problem of autoimmunity in patients but has drawbacks. The physical barrier between islets and host tissue blocks the T-cells but restricts the glucose sensing and insulin secretion. Further, survival of grafts can be impaired by oxygen supply. Improved biomaterial encapsulation can solve most of these issues. [32]
New clues pointing to other treatment options come from studies of diabetic patients. Keenan et al. describe that even after 50 years of T1D a population of beta cells secreting insulin exists in the pancreas. [33] They conclude that enhancing the endogenous beta cells regeneration could be a viable solution for many T1D patients. [33]
Similar results were obtained by Liu et al. They found that even after many years of T1D, the pancreas secrets insulin. [34] They propose that by administering only immunosuppressants the autoimmune attack on the pancreatic islets is diminished and the endogenous beta cells start to recover. Euglycemia is believed to help recover beta cell function and regeneration as well. By artificially creating unfavourable conditions like high concentrations of blood insulin the natural regeneration of the pancreas is weakened. [34]
These studies suggest that the regenerative ability of the human pancreas is higher than expected and that less invasive and aggressive therapies could improve beta cell survival and renewal.

1.2 Zhou Lab

The Zhou Lab is dedicated to discover ways to diminish the severity of T1D. Described above, there are numerous different approaches to reach this goal. Specializing in stem cells, the Zhou Lab plans to reprogram stem cells into insulin-secreting cells. In the sections below, their previous work will be explained.

1.2.1 Reprogramming of stem cells

Reprogramming of tissue into insulin+ cells can be very challenging. The transcription factors commonly used for the reprogramming are the NPM factors.[35] These factors consist of Neurogenin3 (Ngn3), Pancreatic and duodenal homeobox 1 (PDX1) and MafA.
Ngn3 is known to be essential in differentiation and functionality of a subset of gastric endocrine cells as well as all the intestinal and pancreatic endocrine cells. [36] The development of pancreatic and gastric endocrine cells is controlled by the endocrine progenitor cells Ngn3 expression.
PDX1 is responsible for pancreatic development.[37] This includes beta cell maturation and duodenal differentiation.
MafA is a transcription factor responsible for the insulin expression post glucose challenge. Other beta cell genes are induced through MafA overexpression. [38]

Current research focuses on reprogramming acinar cells into insulin+ cells. Acinar cells, derived from the same progenitors as beta cells, are common targets of reprogramming approaches. Their similarity with beta cells increases the successful reprogramming drastically. [39] The big issue with using acinar cells for cell therapy is the non-autologous transplantation. Allogenic tissues bear the risk of rejection by the host.
Different tissues prone to reprogramming into insulin+ cells are needed. To achieve this goal, mice with conditional global overexpression of Ngn3, PDX1 and MafA were designed and bred. Injection of Dox induced the overexpression and reprogramming of tissues. The results showed that stem cells located in the stomach and intestine have the potential to be reprogrammed to insulin+ cells. The comparability of murine and human tissue was tested *in vitro* and *in vivo*. The most promising stem cells were discovered to be antrum, corpus and duodenum cells.

1.2.2 Different reprogramming approaches

Trying to reprogram the stem cells without the need of gene modification, a compound library screening was conducted. The results indicated that it is not possible to reprogram human gastro-intestinal stem cells (hGISCs) into insulin+ cells using chemical compounds.
The new strategy was to create a plasmid with conditional expression of Ngn3, PDX1 and MafA. This plasmid was packaged into a viral particle and the stem cell lines were infected. Results after transplantation showed that the stem cells did not survive *in vivo*.
To overcome this problem, the stem cells are first reprogrammed into enteroendocrine cells. This can be done by inducing Ngn3 expression without PDX1 and MafA. Two plasmids were constructed, one expressing Ngn3, the other PDX1 and MafA.

1.2.2.1 Engineering of cells

The conditional induction of gene expression in the plasmids differ in terms of the mechanism to activate them. The plasmid expressing Ngn3ER carries the EF1 alpha promotor, activated by addition of Tam. 4-Hydroxytamoxyfen activates the Cre expression. Once Cre is expressed, the lox-stop-lox site, which is upstream of the gene of interest, is removed and Ngn3ER is induced. The TetOn gene expression system was used to design the second plasmid. Doxycycline binds to the rtTA molecule, activates it and the complex then binds the TetOn promotor. The expression of PDX1 and MafA follows the activation of the promotor.

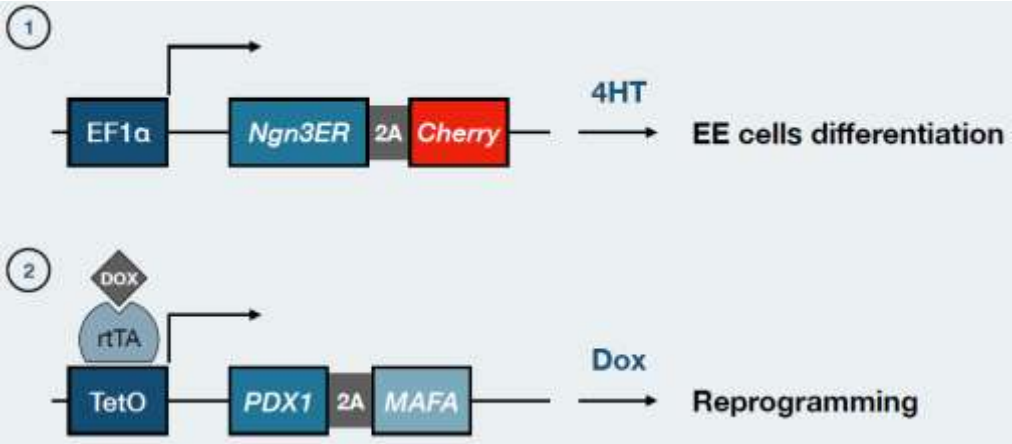

Figure 1: Graph showing the Ngn3ER and rtTA-TetOn expression systems. The top graph shows the Ngn3ER expression system induced by 4-Hydroxytamoxyfen (4HT). The bottom graph shows the PDX1 and MafA expressing rtTA-TetOn system.

To create a cell line carrying both plasmids, the cells need to be infected twice. Each plasmid carries its individual antibiotic resistance gene. These two genes are Puromycin and Blasticidin.
Selecting the cells for three days resulted in the generation of cell lines with both plasmids, which were used for the experiments.

1.2.3 Previous experiments

These cell lines were reprogrammed into EE cells by induction of Ngn3. The reprogramming was evaluated by staining the tissue for Glucose-dependent insulino-tropic polypeptide (GIP). GIP is a marker for specific EE cells in human duodenum stem cells. [40] Stainings showed that after Tam is added to the medium, the stem cell clusters express GIP.
These EE cells are then reprogrammed into insulin+ cells. C-peptide, MafA, MafB, PDX1 and NKX6.1 are indicators of a successful reprogramming. C-peptide (CPPT) is a byproduct of insulin processing. Pro-insulin is cleaved, resulting in CPPT and activated insulin. [41]
MafB is an important transcription factor during pancreatic development. During maturation of beta cells, MafB expression is decreased continuously. [42]
NKX6.1 is responsible for the maintenance of the beta cells and drives early progenitors towards beta cell fate. [43]

2 Methods

2.1 Culturing of hGISCs

The cultivation of human gastrointestinal stem cells (hGISCs) requires special medium, special environmental conditions and special culture dish coating. 50mL of the general culture medium, referred to as 2D medium, used for all of the different hGISCs is composed of 45 mL base medium, 5mL R-spondin2 conditioned medium, 10mM Nicotinamide, 25µM Primocin, 1µM A8301, 5µg/mL Insulin, 10µM Y-27632, 1µM DMHI, 50ng/mL EGF and 2µM T3 hormone. R-spondin2 conditioned medium activates Wnt signaling, Nicotinamide modulates stem cell differentiation, Primocin is an antibacterial chemical, A8301 improves *in vitro* stem cell (SC) self-renewal, Y-27632 is a Rho-inhibitor and enhances SC survival, EGF promotes cell proliferation and the T3 hormone regulates the differentiation.
The base medium itself is a mixture of 27mL DMEM high glucose medium, 9mL F12K Kaighn´s Modification medium and 10mL FBS/FCS.
The R-spondin2 conditioned medium has to be prepared by culturing the R-spondin2 cell line for 2 days. Then, Zeocin to a final concentration of 300µg/mL is added. At roughly 60% confluency, the cells are washed twice with PBS and the medium is changed back to regular DMEM complete medium. Incubate the cells until full confluency and collect the media everyday up to 4 times.
Culturing hGISCs requires a different CO2 concentration in the air than regular cell culture. While the vast majority of cell lines need 5% CO2, hGISCs grow better in 7.5% CO2.
The culture dish used for cultivating the stem cells needs to be prepared beforehand. One day before seeding the hGISCs, the dish has to be coated with 0.1% gelatin for at least 30 minutes. After the coating, the feeder layer necessary for stem cells culturing needs to be seeded. Usually MEF at a confluency of 90-99% are used for this task.

2.1.1 Preparation of MEF

To be able to seed MEFs for such a purpose they need to be inactivated. After the MEF were seeded and amplified, they are cultured until over-confluency to increase the MEF to their maximum. The medium used for their culturing is DMEM high glucose medium supplemented with 1% penicillin/streptomycin, 10% FBS, 1% sodium pyruvate and 1% non-essential amino acids. Adding mitomycin C at a concentration of 10µg/mL into the culture medium for 1.5h will stop their proliferation. These MEFs can be frozen at any required concentration, usually 2 cryovials per 15-cm cell culture dish. To evaluate their number and viability, one vial is thawed onto a 10-cm dish. Their confluency should be around 90-95%.

2.1.2 Splitting of hGISCs

After seeding human gastro-intestinal stem cells onto the feeder layer, it usually takes 4 days for them to grow to large cell colonies. Before the cell colonies touch and merge, the cells need to be passaged to keep them proliferating. These cell colonies consist of mainly stem cells at the outer parts and possibly some differentiated cells in the center. This is why the washing step before adding the TrypLE is very important. These differentiated cells produce mucus once the media is removed. Removing this mucus is important for the TrypLE to be able to detach the cells from the dish. After the washing, the cells are incubated with TrypLE at 37C for 12 minutes. After 7 minutes of incubation, regular observation of the colonies is advised to not over-digest them.
The TrypLE is deactivated with media containing 10% FBS and the cells are centrifuged at 1000 rpm. Following the resuspension in 2D medium, the cells can be seeded onto a new feeder layer. The regular splitting ratio is 1:3.

2.1.3 Aggregation of hGISCs

The detachment of hGISCs is different if the purpose is to aggregate the cells afterwards. Aggregation is done after the first four days of reprogramming, meaning the cells transform and are more easily detachable. The washing step is the same, but the incubation time with TrypLE differs. 2-4 minutes is the estimated time the cells need to detach from the dish. Observing the cell colonies regularly is advised. After the digestion, deactivating TrypLE and washing with PBS remains the same. Afterwards the cells can be seeded into low attachment plates for aggregation.

2.2 Culturing of beta cells

To have a realistic control during comparative molecular methods, a beta cell line has to be cultured. In this case, the EndoC-BH1 cell line was used. For them to attach and proliferate, the dish has to be coated overnight with a special coating medium. 5mL of this coating medium is required for a 10-cm dish and contains 4.88mL DMEM low glucose, 50µL pen/strep, 50µL Matrigel and 10µg fibronectin.
500mL of the special EndoC-BH1 cell line medium consists of 495mL DMEM low glucose, 10g BSA fraction V, 1.75µL Beta-mercaptoethanol, 0.611g Nicotinamide, 2.75mg transferrin, 3.35µg Sodium selenite and 5mL pen/strep. The splitting ratio is usually 1:2.

2.3 Culturing of HUVECs

To culture the ETV2 HUVEC cell line, a medium supplemented with 2% FBS, 0.04% Hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, and 0.1% heparin is used. The endothelial basal medium together with all of the supplements can be bought from Lonza. Coating the cell culture dish prior to seeding increases HUVEC viability and attachment on the dish. For this purpose 0.1% gelatine is used.

2.4 Culturing of HEK293FT

The HEK293FT cell line uses DMEM high glucose medium supplemented with 10% FBS, 1% pen/strep, 1% non-essential amino acids and 1% sodium pyruvate. Changing the medium every 2 or 3 days is suggested because HEK293FT grow rapidly.

2.5 Reprogramming of hGISCs

The reprogramming of human gastro-intestinal stem cells requires the addition of 4-Hydroxyamoxifen (Tam) and Doxycycline (Dox) at specific time points. Due to the introduction of the plasmids and the expression pattern of beta cell genes, Ngn3 needs to be expressed first. After culturing the stem cells for roughly 2 days post splitting, add 4-Hydroxytamoxifen at a ratio of 1:10000.

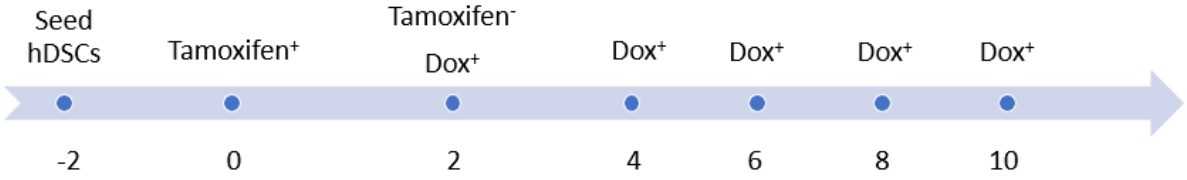

Figure 2: Graph depicting the reprogramming process.

At day 2 post Tam-addition, the medium needs to be changed and Dox is added at a ratio of 1:1000 to the medium instead of Tam. This induces the expression of murine or human PDX1 and MAFA. Depending on the experiment, the duration of Tam and Dox can vary but the regular protocol requires 2 days of Tam treatment and at least 8 days of Dox treatment. During the Dox treatment, depending on the goal of the experiment, different media are used to culture the stem cells. For the purpose of immune-staining the stem cells are cultured 8 days post Dox-addition and then fixed and stained. If the experiment requires qPCR, the stem cells are aggregated 2 days post Dox-addition. After the aggregation, the Dox treatment continues for additional 6 days.
The different media used for the treatments can vary depending on the experimental set-up.

2.6 Immunostaining

Staining the cells with antibodies require a few very important steps. First the cells need to be washed with PBS. Then the cells are fixed with 4% paraformaldehyde (PFA) at room temperature for 10 minutes. To get rid of the PFA, the cells are washed twice with PBS. The cells are blocked with blocking solution consisting of 10% donkey serum in PBST at room temperature for 1 hour. Afterwards the cells can be stained with the primary antibody diluted in blocking solution at 4 degrees overnight. The dilution ratio depends on the antibody and the intensity of the signal. The next day the primary antibody needs to be washed away with PBST twice. Then the secondary antibody diluted in blocking solution can be applied. The incubation with this antibody takes an hour at room temperature or overnight at 4 degrees. From this step onwards, the cells should be protected from direct light to minimize bleaching. After the secondary antibody incubation, the cells can be stained with DAPI diluted in PBST at a concentration of 1:3000 at room temperature for 5 minutes. To get rid of the DAPI and residual secondary antibody wash the cells twice with PBST for 15 minutes each. Maybe rock the plate while washing to maximize the efficiency. Then change the PBST one last time and evaluate the staining. The plates can be kept for a long time at 4 degrees if light is avoided.

2.7 RT-PCR

To be able to analyse the gene expression of cell aggregates, a RT-PCR and qPCR is necessary. After aggregating the cells and treating them, the aggregates are collected and lysed. To 9µL lysate, 10L RT buffer and 1µL RT enzyme is added to transcribe the mRNA into cDNA. For this purpose the samples are heated to 37 degrees for 1 hour, then further heated to 95 degrees for 5 minutes and then incubated at 4 degrees until the qPCR can be performed.

2.8 qPCR

Depending on the purpose different reaction amounts need to be used. Here, a 5µL reaction includes 2.5µL TaqMan Fast Advanced MasterMix, 1.25µL Ultra-Pure water, 0.25µL TaqMan probe and 1µL cDNA from the RT PCR. The used ΔΔCt program consists of a hold stage at a temperature of 50 degrees for 2 minutes, another hold stage at 95 degrees for 20 seconds and the PCR stage with 95 degrees for 1 second followed by 60 degrees for 20 seconds.
After the qPCR run the results can be viewed and exported into an Microsoft Excel file.

2.9 Kidney Capsule Transplantation

Kidney capsule transplantation is a live operation, meaning the animal is alive and anesthetized. The start of the procedure is the anesthesia of the animal.

2.9.1 Anesthesia

Isoflurane or ketamine can be used to anesthetize the animal. Usually isoflurane is used due to its low mortality rate and side effects. The animal is put into an airtight box connected to the isoflurane evaporator. 3% isoflurane was used to anesthetize the animals. The animal was closely observed until its heart rate is low and steady. To make sure the animal is asleep a toe pinch was done. To start the procedure, the animal was transferred to the operating area and isoflurane was continuously administered through a nose cone. The isoflurane concentration was reduced to 1.5 – 2%. After making sure the animal is ready for the operation, the incision area was shaved rigorously. The incision site is below the ribcage and to the left of the spine. For this purpose the animal was positioned on its belly. To keep the wound clean, the incision site was wiped with alcohol patches. Painkillers were administered to cause as little pain as possible.

2.9.2 Procedure

The incision was made along the spine, a few millimeters to its left. The skin was cut 1 – 1.5 cm to have a big visual field. The kidney was located through the muscle by checking the intestine, spleen and other organs. The muscle was cut above the kidney.
This cut needs to be smaller than the kidney; roughly 0.5 cm. The kidney was pushed through the cut in the muscle by gently putting pressure on the intestine. If the cut in the muscle was too big and the kidney slipped back under the muscle, a clamp was used to hold back the muscle. Sodium chloride solution was prepared and administered to keep the kidney wet. After successfully uncovering the kidney, a needle was used to prick the kidney capsule at its dorsal side. Pricking the kidney capsule at the anterior was important to have a lot of room for the transplanted tissue. A small plastic tube was prepared and carefully pushed between the kidney capsule and the kidney. If at this point the kidney started to bleed a lot, the animal was euthanized and a different animal was used to continue. Using a pipette, the tissue ready for transplantation was pushed through the tube into the space between kidney and kidney capsule. If necessary, moving the tube carefully increased the transplantation space and it was possible to transplant more tissue. After successfully transplanting the tissue, the tube was removed while putting pressure on the pipette to prevent leakage. A cauterizing tool was prepared to close the prick of the kidney capsule. The kidney was then pushed back into the body cavity very carefully. The muscle cut was closed using a bio-absorbable suture. After every knot, the suture was cut and applied again to prevent wound reopening by the animal. The skin incision was closed using a wound clip applier and wound clips. The animal was placed onto a heat pad to keep it warm until it regained consciousness.

For the next three days, the animal was monitored daily to check for pain indicators or other complications.

2.10 qPCR analysis

To visualize the results of the qPCR, the program GraphPad is used. Before entering the data into GraphPad, the raw data is converted by using a few mathematical functions.
First, the internal control needs to be subtracted from the sample data. If statically significant data is generated, at least three replicates are necessary. Here, the average of the external control is determined and subtracted from the sample value. To calculate the fold change of the gene expression compared to the external control, 2 to the power of the negative sample value has to be determined. This value can then be visualized using a lot of different software like GraphPad.

2.11 Generation of transgenic cell line

Generating a new gene manipulated cell line requires the generation of a viral plasmid and the introduction into human cells. The exact steps necessary to achieve this are listed below.

2.11.1 Generation of plasmid

Depending on the purpose, the viral plasmid backbone has to be chosen wisely. Here, a Lentiviral plasmid backbone with antibiotic resistant genes, specifically Puromycin, was used to create the plasmid. Into this plasmid, the DNA of two inducible expression systems and three genes was ligated. The first expression system uses the EF1 alpha promotor and is inducible via 4-Hydroxytamoxifen while the second expression system utilizes the TetOff promotor. This promotor is activated and expresses the downstream genes if no Doxycycline is present.
The Ngn3ER gene followed by an MCherry fluorescent gene is ligated downstream of the EF1 alpha promotor. The fluorescent gene enables easy tracking of expression. Downstream of the TetOff promotor, the genes PDX1 and MafA are inserted to induce reprogramming.
The insertion and ligation can be achieved by cutting the viral backbone or existing plasmid with restriction enzymes and adding the DNA and ligase.
After constructing the plasmid, competent cells are transformed. The plasmid was purified by MiniPrep and correct cloning was verified by sequencing.

2.11.2 Viral packaging

HEK293FT cells are transfected with the plasmid to package the plasmid into lentiviral particles. HEK293FT cells are cultured on a 6-cm cell culture dish to 92-99% confluency.
On the day before the HEK293FT are transfected the medium has to be changed to packaging medium. This medium is 1% GlutaMAX, 1mM Sodium Pyruvate, 5% FBS and 1% Pen/Strep added to 50mL Gibco Opti-MEM 1.
On the day of the lipofection 15µL Lipofectamine 3000 is diluted with 500µL Opti-MEM 1. At the same time 13µL P3000 reagent, 0.87µL MD2.9, 2.69µL PSP and 3.19µg plasmid is diluted with 500µL Opti-MEM 1. The diluted Lipofectamine 3000 is added to the diluted reagents, mixed well and incubated for 15 minutes at room temperature. When the 15 minutes are up, half of the cell culture medium is removed, in this case 2mL. Then the 1mL DNA-lipid complexes are added to the cells and incubated for 6 hours at 37 degrees Celsius and 5% oxygen. After adding the complexes, it is very important to shake and distribute the DNA-lipid complexes everywhere on the dish.
The cell culture medium is removed and 4mL fresh packaging medium is added after the 6 hour incubation. Then the cells are incubated overnight and the medium is collected 24 hours post transfection. 4mL fresh packaging medium is added and the cells are again incubated for 28 hours. The collected medium is kept on 4 degrees Celsius. 52 hours post transfection the medium is collected again and the cells are destroyed and discarded. To concentrate the virus in the collected medium the 8mL medium is centrifuged in a 100K centrifugal filter. The remaining medium is used to resuspend the virus before aliquoting and freezing it.

2.11.3 Infection of cells and selection

Prior to infecting and creating the cell line the concentration of virus in the collected medium has to be determined. The easiest way is to seed cells into a 12-well plate and use different concentrations of viral medium. After incubating the cells with the virus 48 hours the medium has to be changed and the selection period starts. Selection is done by adding the appropriate amount of antibiotic to the cell culture medium. The amount necessary depends on the cells and the antibiotic used. Evaluating different Puromycin and Blasticidin concentrations prior to the experiment is necessary to determine the correct concentration. A kill curve for the specific cell line should be created by titrating the antibiotics. Transfected cells carrying the plasmid express the resistance gene to the antibiotics and remain healthy. Not transfected cells become sick and die. The minimum duration of selection is 72 hours. After 72 hours the wells are observed and evaluated. Choosing the viral concentration with roughly 50% cell death after selection should be considered. Overloading the cells with virus can decrease their proliferation while using too little virus will result in too few infected cells. The surviving cells are passaged and frozen to have stocks of freshly infected cells. If the backbone of the plasmid contains a fluorescent protein the ratio of infected cells can be evaluated with a fluorescent microscope.

3 Results

3.1 Immuno-staining of reprogrammed hDSCs

The reprogramming duration of gastro-intestinal stem cells varies depending on the experiment and desired outcome of the experiment. The usual minimal reprogramming time for human duodenal stem cells is 10 days. To confirm this duration and to evaluate the stem cell´s gene expression, an experiment was designed to reprogram hDSCs for 10 days.

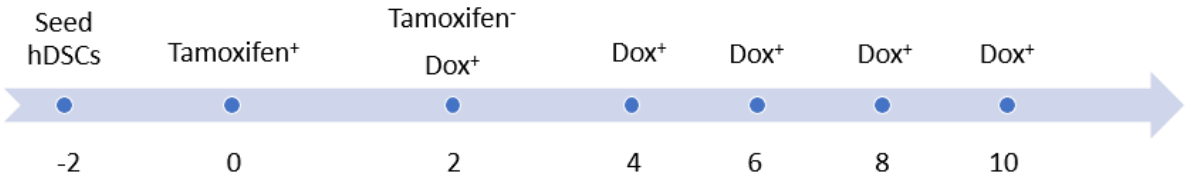

Figure 3: Regular timeline used to reprogram hGISCs. After seeding the hGISCs they should be incubated 2 days for them to get accustomed to the new culturing conditions. At day 0, after the medium change 4-Hydroxy-Tamoxifen is added to induce the reprogramming. After 2 days of Tamoxifen treatment, the medium has to be changed and Doxycycline is added. The medium was changed every 2 days and Doxycycline was added to the medium every time until the cells were stained or harvested.

Figure 3 shows the timeline of the reprogramming of regular hGISCs. After seeding the cells and waiting until they start growing again, Tamoxifen treatment for 2 days was initiated. Tamoxifen is removed after 2 days and Doxycycline treatment for the remainder of the culturing time is set up.

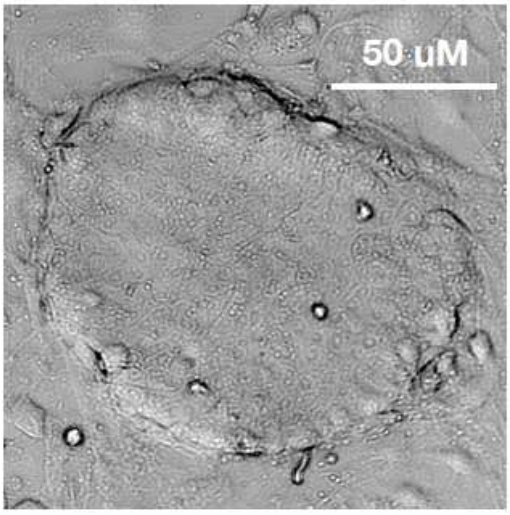

Figure 4: Gastrointestinal stem cells growing on a MEF feeder layer. The stem cells form cell clusters.

Figure 4 shows gastrointestinal stem cells growing on a MEF feeder layer. The cells grow together and form a few big cell clusters. Cells on the inside of the clusters are prone to differentiation while the outer layers of cells retain their stem cell status. This figure shows the cells on day 0 of the reprogramming.

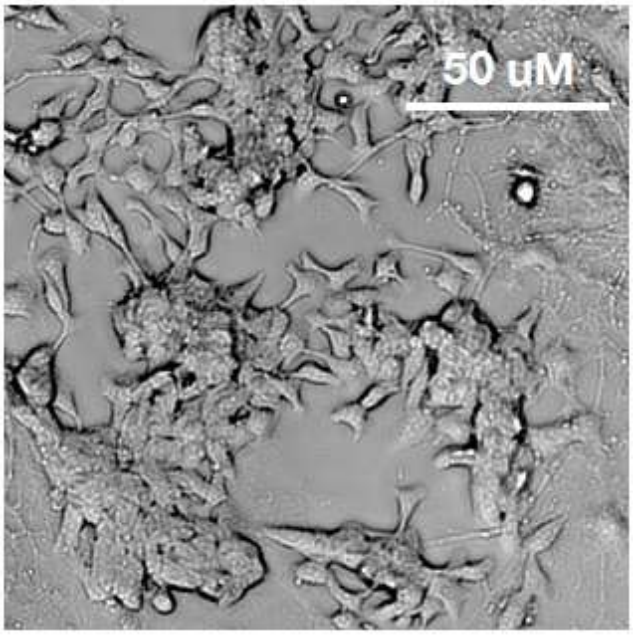

Figure 5: Gastrointestinal enteroendocrine (EE) cells growing on a MEF feeder layer. After induction of Ngn3 expression, the cell clusters collapse into single cells. These single EE cells start forming cell clusters.

In figure 5, the collapse of the stem cell clusters can be seen. After differentiating the stem cells into EE cells, the clusters collapse into single cells. These single cells start forming new clusters. This figure shows day 2 of the reprogramming. At day 2, Doxycycline treatment is started and the expressions of MafA and PDX1 is activated.

MafA, a protein associated with beta cells, GIP, a marker for duodenum-derived gastro-intestinal cells, and C-peptide (CPPT), a biproduct of insulin are the proteins visualized by staining. According to the hypothesis, GIP and CPPT should not be expressed in the same cells or aggregates while MafA and CPPT should be co-localized and expressed in the same cells. The GIP-CPPT co-staining can be seen in figure 6.

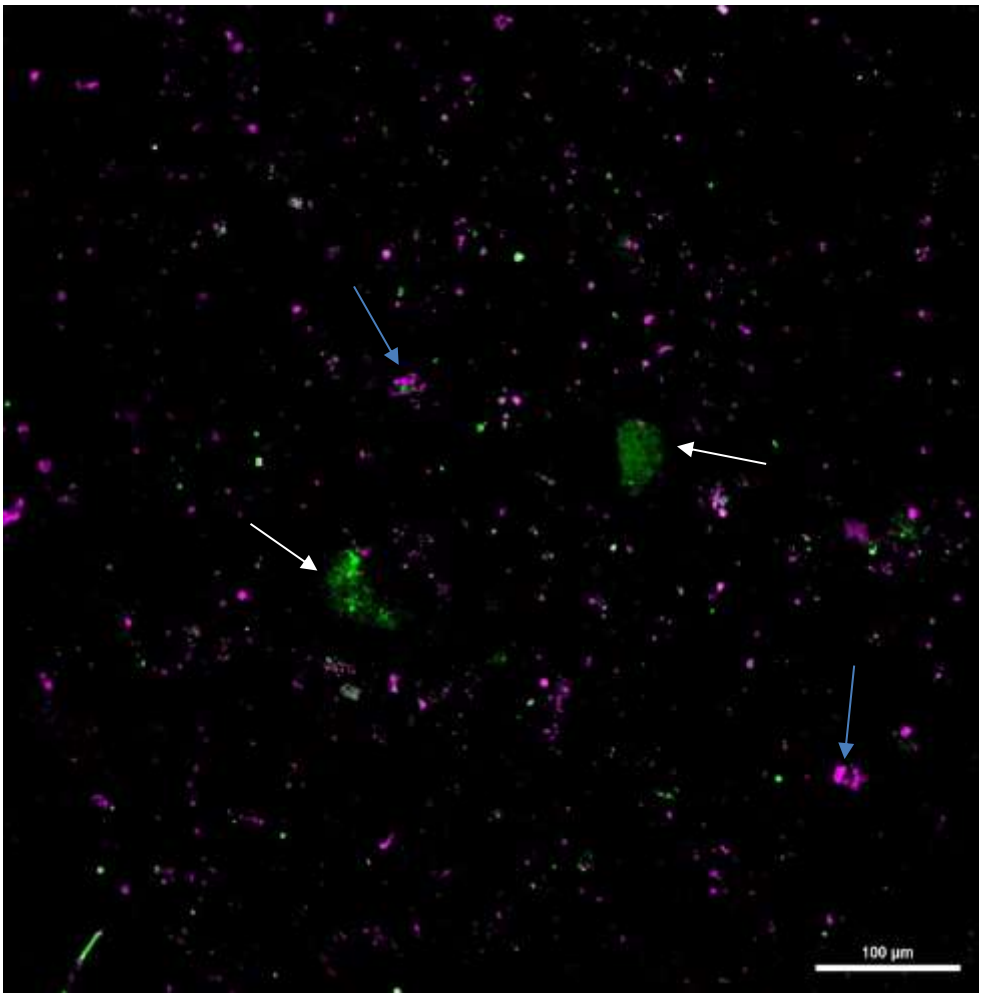

Figure 6: Co-staining of CPPT and GIP of hDSCs after 4 days of Doxycycline treatment. Green cells and aggregates show C-peptide; purple cells represent GIP expression. White arrows point to highly CPPT positive cell clusters. Blue arrows point to highly GIP positive cells. The magnification used was 10x.

In figure 6, human duodenum stem cell (hDSC) aggregates after 4 days of Doxycycline can be seen. Analysing their expression shows that CPPT is not expressed in cell clusters which express GIP and vice versa. This points to the conclusion that the C-peptide positive cell clusters were reprogrammed, therefore do not express GIP anymore but were differentiated into insulin+ cells. GIP positive cells were resistant to the reprogramming cocktail and remained duodenum-derived entero-endocrine cells which is why they do not show CPPT expression.

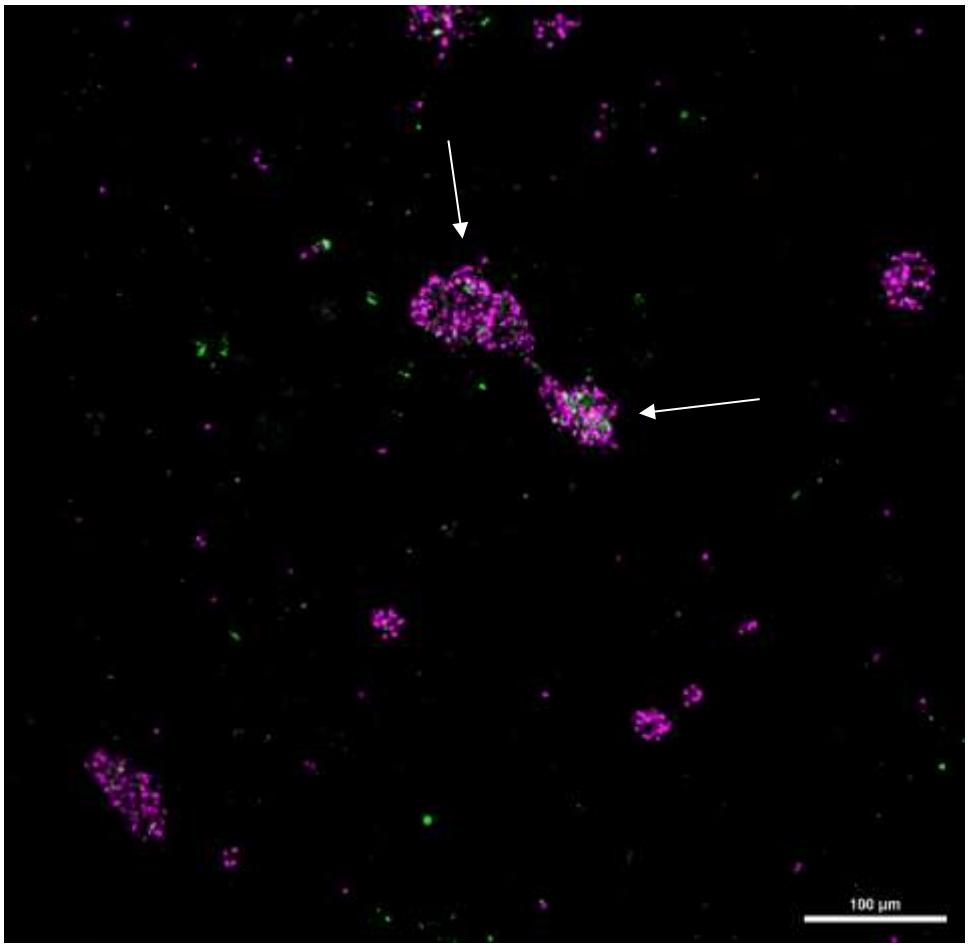

Figure 7: Co-staining of MAFA and CPPT of hDSCs after 4 days of Doxycycline treatment. Green cells show CPPT expression; purple cells represent MAFA expression. White arrows point to cell clusters where MAFA and CPPT is highly co-localized. The magnification used was 10x.

In figure 7 hDSCs co-stained with CPPT and MAFA are depicted. The green GFP signal represents presence of CPPT. The purple CY7 signal shows MAFA expression. In this figure the co-localization of MAFA and CPPT can be observed. Most of the big hDSC cell clusters, which express MAFA, additionally express Insulin. MAFA is used during the reprogramming to induce insulin+ cell differentiation. Cell clusters expressing MAFA but not Insulin are coloured in purple, but not green do to the absence of CPPT.

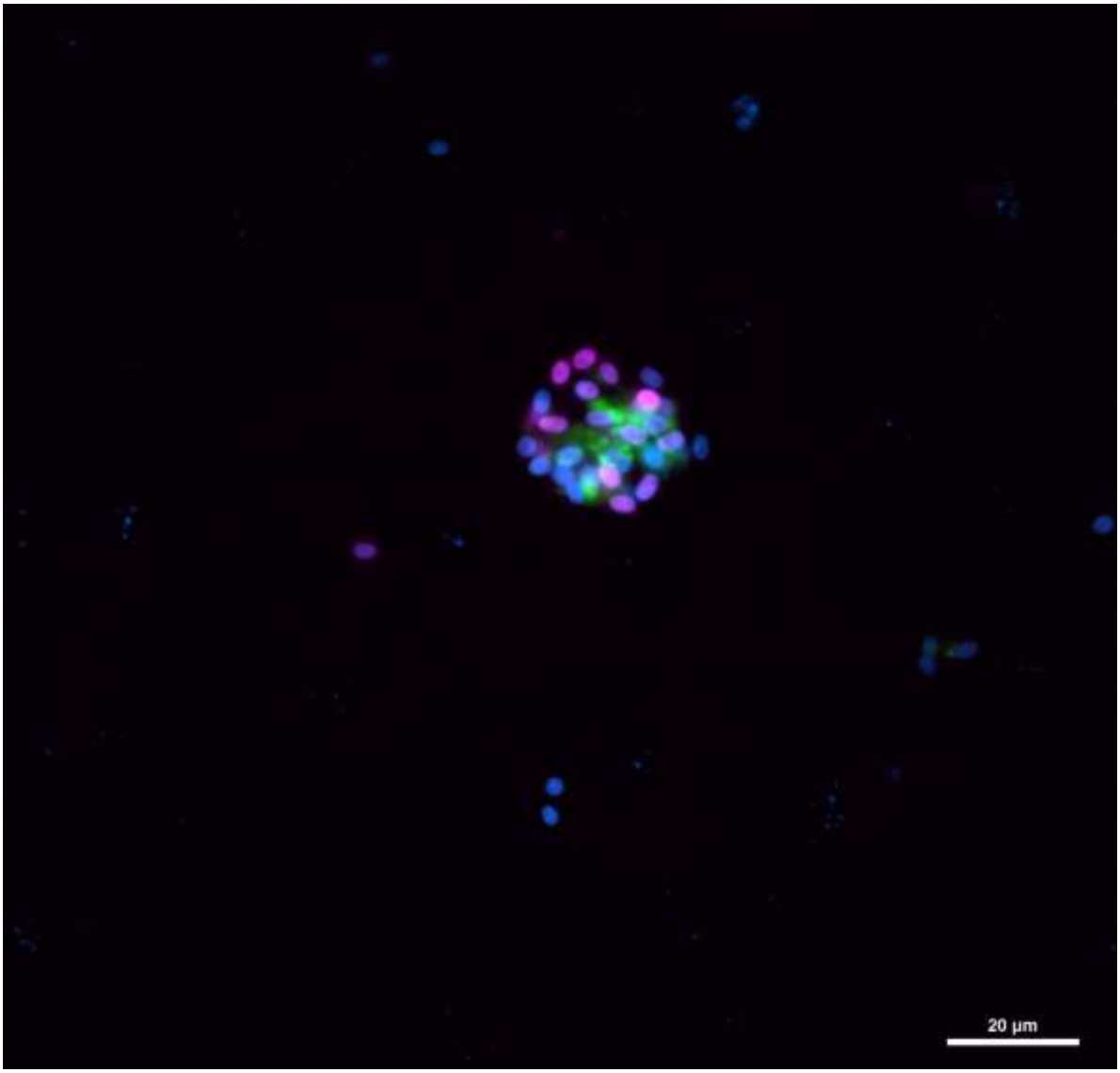

Figure 8: hDSC cell cluster treated with Doxycycline for 8 days is co-stained with CPPT, MAFA and DAPI. MAFA expression can be seen in purple located in the nucleus; CPPT can be observed in green located in the cytoplasm; DAPI is colored in blue and is located in the nucleus. Co-localization of MAFA in the nucleus and CPPT in the cytoplasm is a clear indicator for reprogrammed insulin+ cells. Incomplete reprogramming of cells is indicated by only MAFA expression and absence of CPPT. The magnification used was 40x.

In figure 8 human duodenum stem cells are reprogrammed and treated with Doxycycline for 8 days before fixing and staining them for MAFA and CPPT. To visualize the nucleus DAPI was used to stain DNA. Indications of a successful reprogramming of hDSCs are MAFA expression in the nucleus and CPPT presence in the cytoplasm. By counting these successfully reprogrammed cells and dividing their number by the number of total cells in the cell cluster the reprogramming efficiency of the cell line can be determined. In the case of hDSCs the reprogramming efficiency equals to 30-40%. Cells that are not reprogrammed either need more time to express Insulin or are resistant to reprogramming.

3.2 Transplantation of hGISCs into mice

To evaluate the cell lines different potential for reprogramming and survival, duodenum and antrum cells were transplanted. For this purpose large cell numbers are necessary. These numbers were achieved by culturing the cells in a 10cm dish each. When a sufficient number of stem cells was reached, the reprogramming process was initiated. The timeline of the reprogramming can be seen in figure 1.

On day 0, Tam was added to initiate Ngn3 expression. After 2 days of expression, Tam was removed and Dox was added to the medium for 2 days. On the fourth day, the cells were aggregated in 96-well plates. The aggregates were transplanted on day 6 into the kidney capsule of NSG mice. To continue the reprogramming of the stem cells, Dox was added to the mice´s drinking water.

5 weeks post transplantation the mice were euthanized, the kidney was harvested and analysed by fluorescence microscopy.

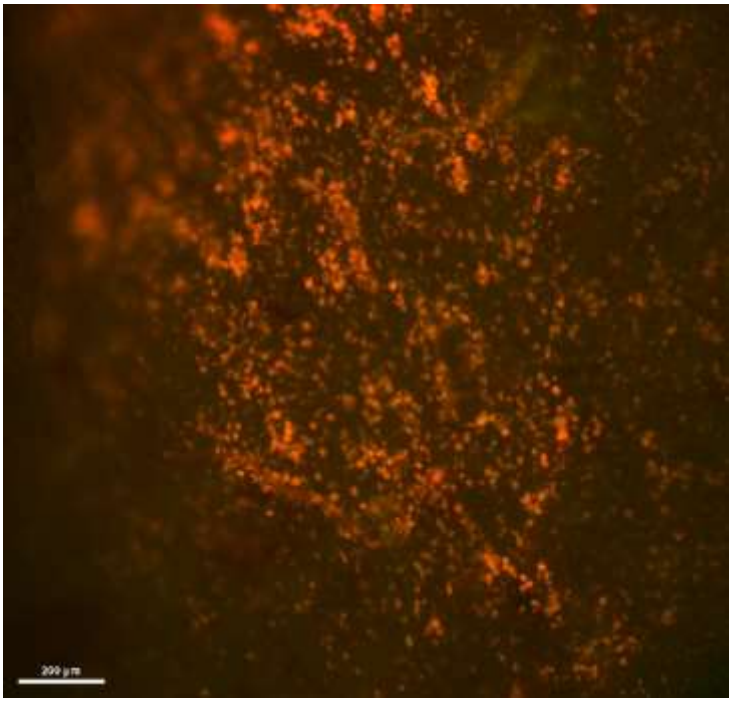

Figure 9: Duodenum stem cells 5 weeks post transplantation. By design the stem cells express mCherry. In this figure, the mCherry of the stem cells and the autofluorescence of apoptotic cells can be seen. The orange color represents dead stem cells. The magnification used was 4x.

In figure 9, dead and apoptotic duodenum stem cells can be seen. The used stem cell lines are designed to express an mCherry fluorophore constantly. The co-colouring of the mCherry fluorophore and the autofluorescence of dead cells was orange. Evidently the survival of duodenum stem cells after transplantation is bad.

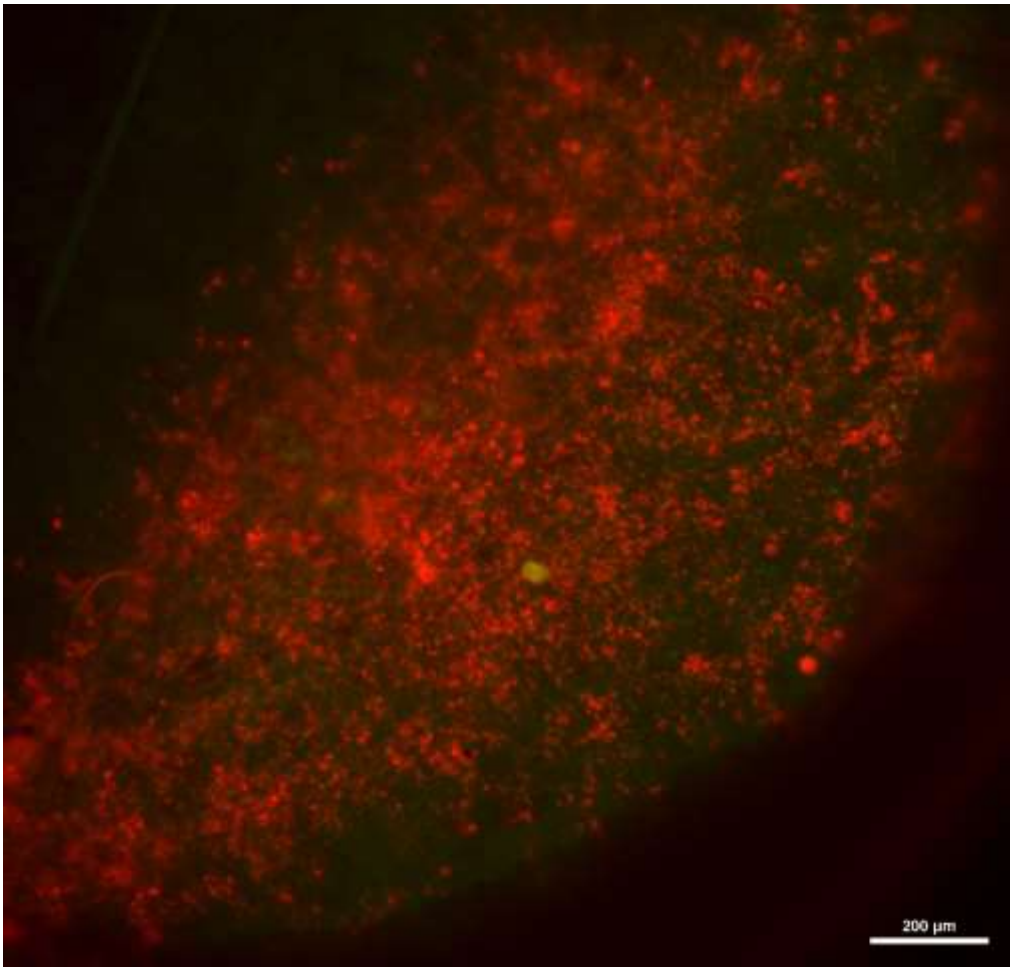

Figure 10: Antrum stem cells 5 weeks post transplantation. The red fluorescence of the stem cells can be observed in this picture. Survival of antrum stem cells was better in comparison to duodenum cells. Low number of orange fluorescence corresponds to low number of dead cells. The magnification used was 4x.

Figure 10 depicts the antrum stem cells inside the kidney capsule.
5 weeks after transplanting the antrum aggregates into the mice, the insulin levels of these mice were observed. No significant changes compared to the control was recorded. Harvesting the tissue of the mice revealed that antrum aggregates survived well under the kidney capsule. Additional staining of the tissue showed no MafA, PDX1 and Insulin expression in the surviving cells.

These results lead to the conclusion that the transplantation into the kidney capsule is problematic. Missing MafA expression indicated that the Dox in the drinking water could not reach the cells. This fact lead to experiments concentrating on generating better cell lines and improving survival.

3.3 Medium examination for reprogramming

The 2D stem cell culture medium as described in the Methods section has, apart from providing the optimal growing conditions for intestinal stem cells, major limitations when considering *in vivo* experiments. Studies conducted by colleagues in the Zhou Lab showed that the survivability of reprogrammed stem cells *in vivo* is very low. Partly this problem is generated by the FBS in the medium. To further optimize the medium used for reprogramming, the necessity for every component was tested. Human duodenum stem cells were cultured in regular 2D medium, while after reprogramming different media were used.
10 different media were used to test the importance of different medium components. These media were:

1. Regular 2D medium with FBS reduced from 20% to 2%
2. Regular 2D medium with R-spondin 2 conditioned medium substituted by MEF medium
3. Regular 2D medium without Nicotinamide
4. Regular 2D medium without A8301
5. Regular 2D medium without Insulin
6. Regular 2D medium without Y-27632
7. Regular 2D medium without DMHI
8. Regular 2D medium without EGF
9. Regular 2D medium without T3 hormone
10. Regular 2D medium

Primocin was included in every medium to destroy possible contaminations.
The timeline for this project can be seen in figure 11.

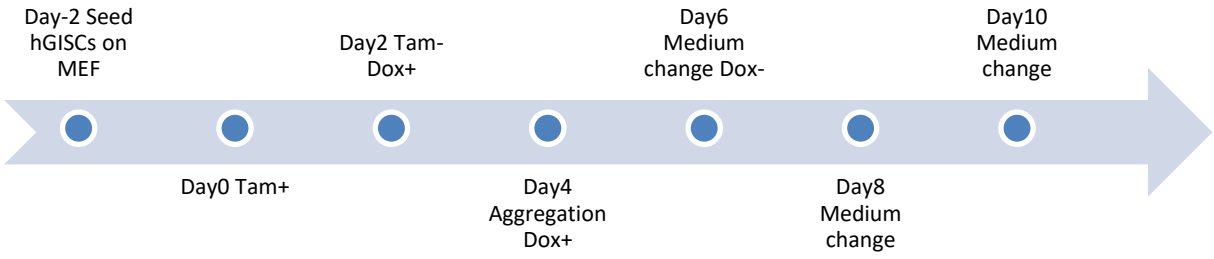

Figure 11: Timeline for Medium examination experiment. This figure shows the timeline used in the Medium examination experiment. 2 days prior to the reprogramming, hDSCs were seeded onto MEF. On day 0, the cells were treated with Tamoxifen to induce Ngn3 expression. After 2 days, Tamoxifen was removed and Doxycycline was added to induce PDX1 and MAFA expression. 2 days after Doxycycline addition, the cells were aggregated. On day 6, Doxycycline was removed. On day 12, aggregates were collected and analyzed to inspect gene expression.

2 days before reprogramming the stem cells, hDSCs were trypsinized and seeded into 3 wells of a 6-well plated prepared with a dense monolayer of MEF. After letting the cells grow accustomed to the medium, which takes 2 days, the reprogramming was initiated by adding 4-Hydroxytamoxyfen to the medium. After second day the medium was changed and thus 4-Hydroxytamoxyfen was removed. At the same time Doxycycline was added to express the necessary genes to achieve reprogramming. 2 days later, the cells were trypsinized and aggregated into 6 aggregates per condition. Every second day post aggregation half of the medium was removed and the same amount of conditioned medium was added to slowly increase the conditioned medium percentage. On day 10, all of the medium was changed to conditioned medium. 12 days after starting the reprogramming, the cells were collected and checked for expression of certain target genes. The expression of 6 different genes were analyzed. The data can be seen in figure 12.

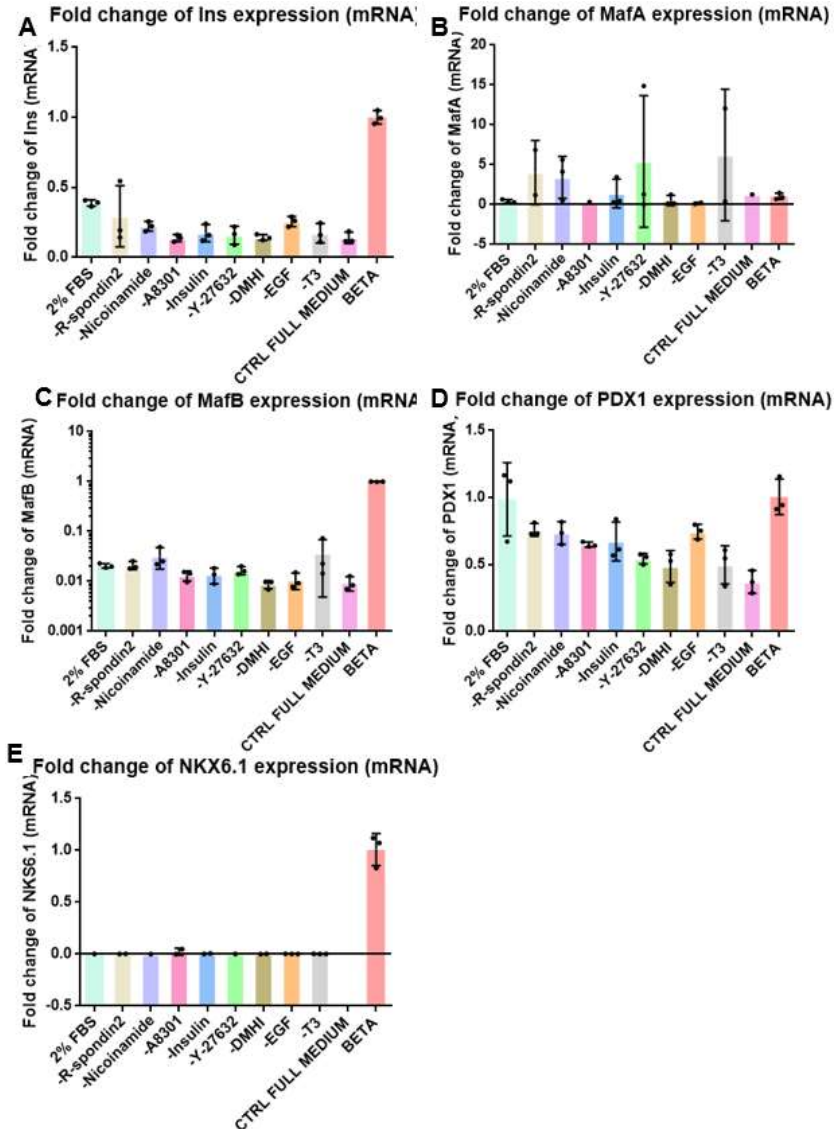

Figure 12: Fold change of expression of Insulin, MafA, MafB, NKX6.1 and PDX1. All samples show variable and different gene expressions. No clear conclusion can be drawn. Beta cells were chosen as a control sample. The different aggregate conditions are depicted on the x-axis. All panels show mean ± SD; n=3.

In panel A of figure 12, the fold change of the insulin expression in all of the samples can be observed. With beta cells as control, the reduced FBS conditioned showed increased insulin expression compared to the other conditions. In panel B, the MafA fold change is depicted. Aside the 2% FBS condition, other conditions display similar MafA expression. In panel C can be observed, that no condition has significant MafB expression. NKX6.1 expression in panel D divulges, that the reprogrammed cells do not express this gene. The gene expression of PDX1 in panel E shows that depending on the medium they are reprogrammed the cells express PDX1 comparable to human beta cell expression. The condition close to the beta cell gene expression is the 2% FBS condition.

3.4 Reprogramming optimization

Considering the results of the previous experiment and the high amount of insulin production after only 4 days of Doxycycline treatment this experiment was devised to investigate the different lengths of reprogramming. Additionally to the reprogramming length, this experiment determined, if the O2 concentration in the incubator can be used to prepare the cells for the low oxygen concentrations after kidney capsule transplantation into mice.
The different reprogramming lengths were then additionally divided into two different groups based on their medium. All of the reprogramming conditions are as follows:

1. Reduced O_2-concenration to 5% - 4 days of Dox - Ctrl 2D medium
2. Reduced O_2-concenration to 5% - 4 days of Dox - 2% FBS 2D medium (with supplements)
3. Reduced O_2-concenration to 5% - 4 days of Dox – 2% FBS wo (without supplements)
4. Regular environmental conditions (7.5% CO_2 and 20% O_2) - 4 days of Dox - Ctrl 2D medium
5. Regular environmental conditions - 4 days of Dox - 2% FBS 2D medium
6. Regular environmental conditions - 4 days of Dox - 2% FBS wo
7. Regular environmental conditions - 6 days of Dox - 2% FBS 2D medium
8. Regular environmental conditions - 6 days of Dox - 2% FBS wo
9. Regular environmental conditions - 8 days of Dox - 2% FBS 2D medium
10. Regular environmental conditions - 8 days of Dox - 2% FBS wo
11. Regular environmental conditions - 10 days of Dox - 2% FBS 2D medium
12. Regular environmental conditions - 10 days of Dox - 2% FBS wo
13. Regular environmental conditions - 4 days of Dox - 2% FBS 2D medium (long-term culture to investigate changes)
14. Regular environmental conditions - X days of Dox - 2% FBS 2D medium (long-term culture to investigate changes)

The media entitled 2D are regular media with all supplements while WO represents media without any supplements other than 2% FBS. All of these conditions contained Primocin to avoid possible contaminations. The gene expression of insulin, MafA, MafB, NKX6.1 and PDX1 was measured.

The timeline for this experiment is very similar to Figure 11. The only difference is that not every condition has different Doxycycline removal dates. The medium changes to the conditioned media was conducted in the following way:

- On day 6, 50% of the medium in the wells was removed and the conditioned medium was added.
- On day 8, 75% of the medium in the wells was removed and substituted for conditioned medium.
- On day 10, 100% of the medium was carefully exchanged for conditioned medium.

This method was used to continuously reduce the concentration of stem cells 2D culturing medium and at the same time increase the conditioned medium concentration. The insulin expression of the cells reprogrammed with 2D medium can be seen in figure 13.

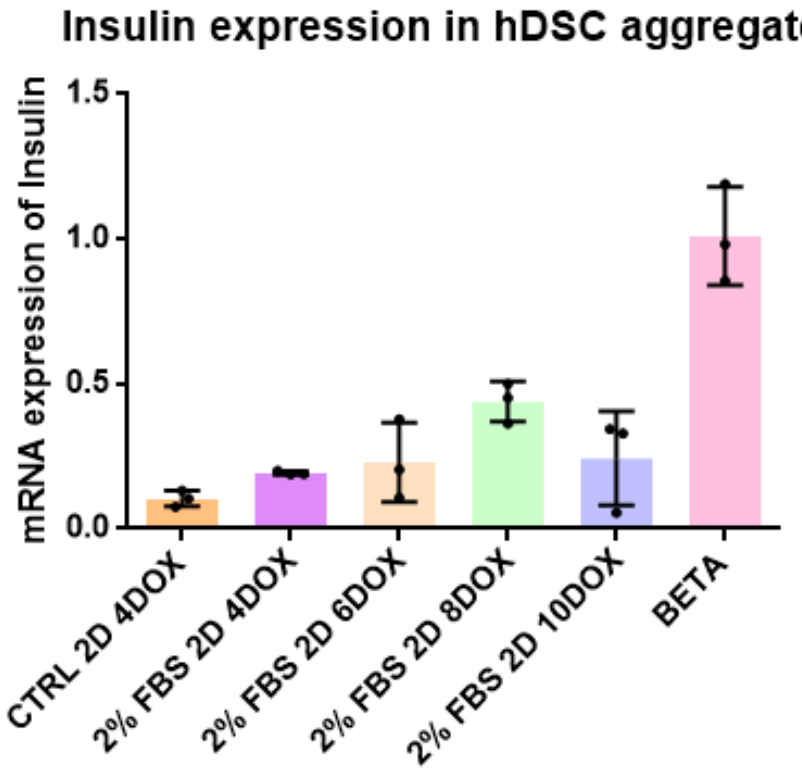

Figure 13: Insulin expression in reprogrammed aggregates compared to control beta cells. Cells were reprogrammed with reduced FBS concentration. Highest insulin expression was measured after 8 days of Dox treatment. 4DOX represents the length of Doxycycline incubation, respectively. The panel shows mean ± SD; n=3.

Figure 13 shows the insulin expression in reprogrammed duodenum stem cells reprogrammed with reduced serum medium. The results show that a longer duration of reprogramming is beneficial to the insulin production. The insulin expression continuously increases with the length of Doxycycline treatment until day 8. Samples with 10 days of Doxycycline treatment have a lower expression of insulin which is uncharacteristic and unexpected. Even though the insulin expression increases it is significantly less compared to a beta cell control.

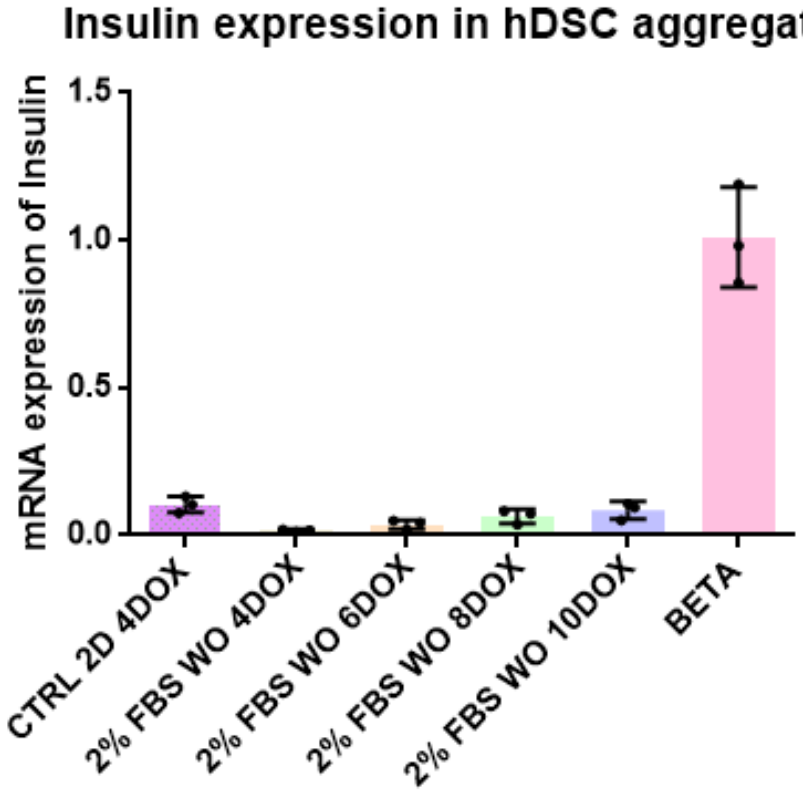

Figure 14: Insulin expression of duodenum stem cells reprogrammed with reduced serum medium and no medium supplements. Insulin expression decreases after removal of the supplements. 4DOX represents the length of Doxycycline treatment; 4DOX is 4 days of treatment, 6DOX is 6 days of treatment, respectively. The panel shows mean ± SD; n=3.

In Figure 14, the insulin expression of aggregates reprogrammed in medium without supplements can be seen. Significantly less insulin is expressed in aggregates subjected to supplement-less reprogramming medium. This can be observed by comparing bar 1, entitled CTRL 2D 4DOX, and bar 2, marked 2% FBS WO 4DOX. The length of the Doxycycline treatment increases the insulin expression, but its level is significantly lower than comparable samples reprogrammed with regular medium.

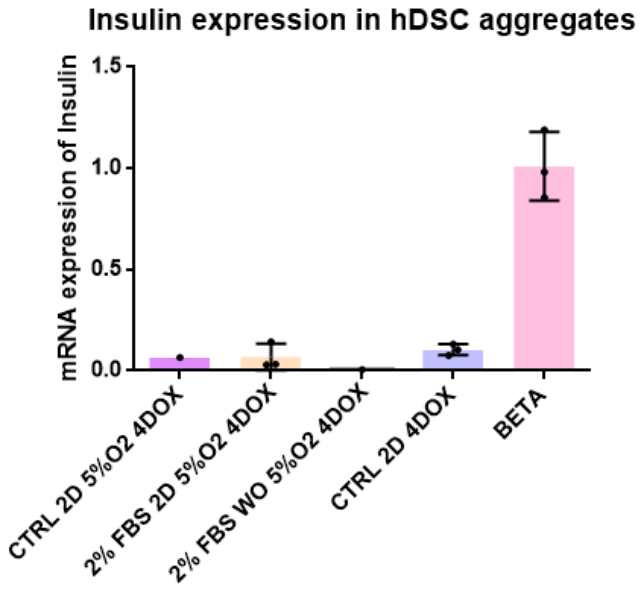

Figure 15: Insulin expression of aggregates reprogrammed in oxygen reduced conditions. Low levels of insulin are measured in 5% O2 conditions. The environment is unfavorable and reduces reprogramming efficiency. 4DOX represents 4 days of Doxycycline treatment; 5%O2 corresponds to the oxygen concentration in the incubator during reprogramming. The panel shows mean ± SD; n=3.

Figure 15 displays the expression of insulin in oxygen reduced conditions. The expression levels show that a low oxygen environment has negative effects on the reprogramming of duodenum stem cells. Every condition cultured in 5% oxygen has significantly lower expression of insulin than the beta cell control and lower concentration than a control sample cultured in a regular 7.5% CO2 environment.

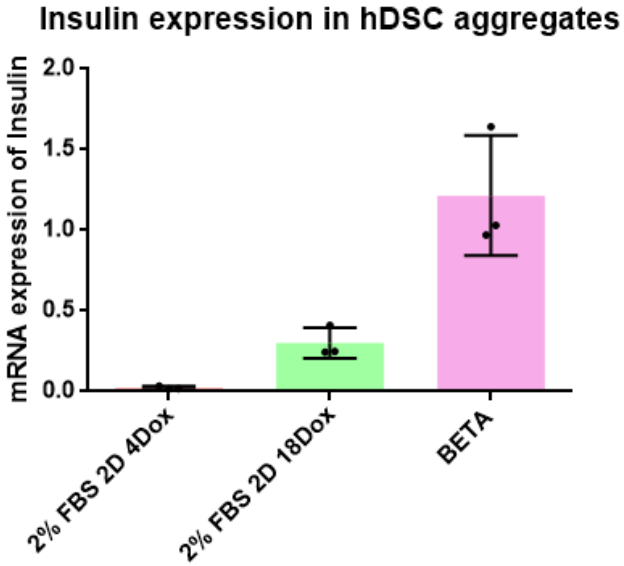

Figure 16: Insulin expression of long term aggregates. Long term experiments show that longer reprogramming times increase reprogramming efficiency and insulin secretion. 4DOX represents 4 days of Doxycycline treatment, but the cells were aggregated for 14 days after Doxycycline removal. 18DOX equals 18 days of Doxycycline treatment. The panel shows mean ± SD; n=3.

In figure 16 the insulin expression of long term cultured hDSC aggregates can be observed. Aggregates reprogrammed for 4 days with Doxycycline show significantly less insulin expression compared to aggregates treated with Doxycycline for 18 days. The total culturing time of both sample groups is 16 days after aggregation. Both samples display significantly less insulin expression than the control beta cells.
The expression of MafA, MafB, PDX1 and NKX6.1 can be observed in figure 17.

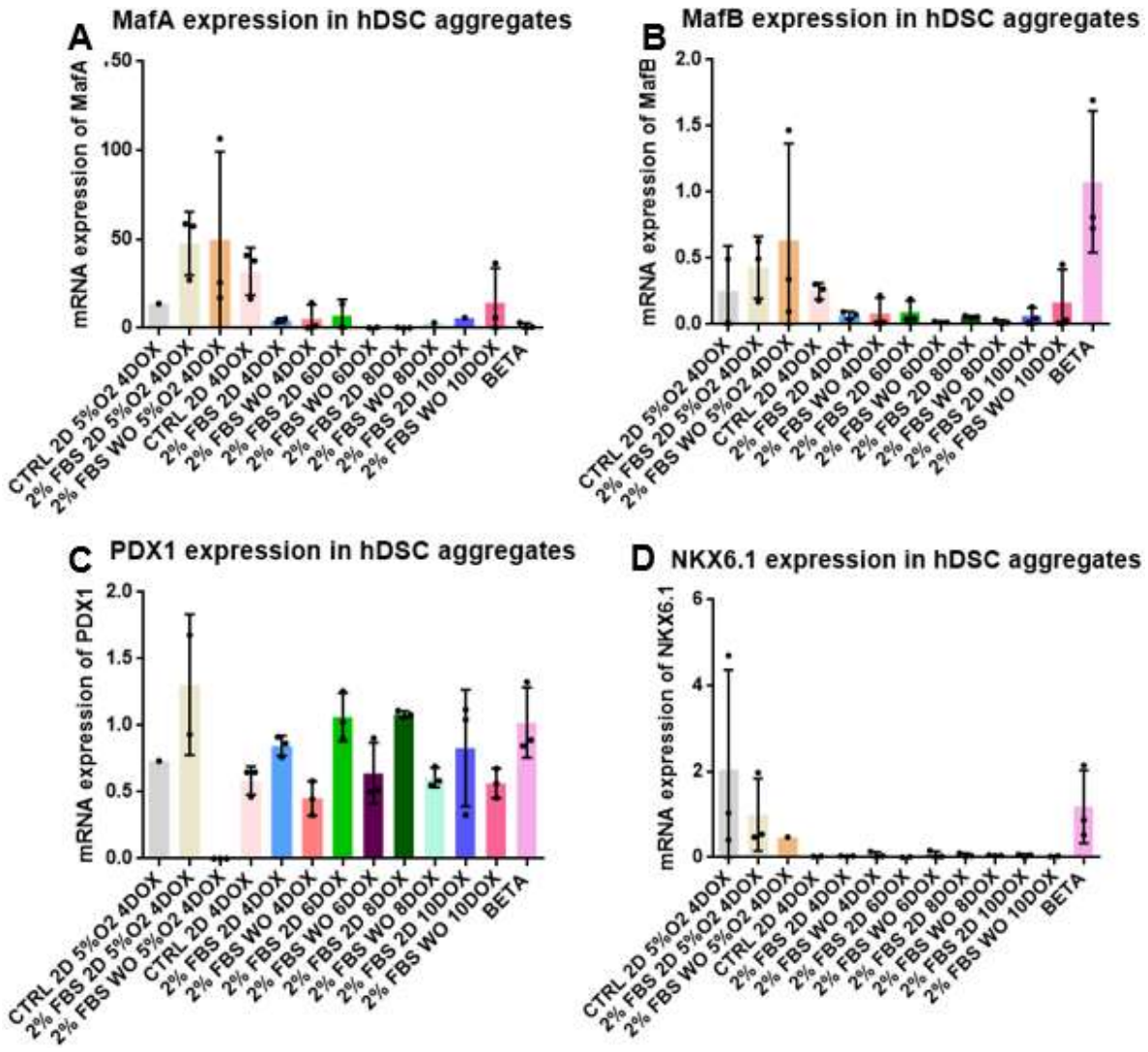

Figure 17: Gene expression of MafA, MafB, PDX1 and NKX6.1 in hDSC aggregates. Gene expression patterns of every sample is different than the control. No clear functional beta cell reprogramming was possible. CTRL 2D, 2% FBS 2D and 2% FBS WO represent different medium conditions. 4DOX, 6DOX, 8DOX and 10DOX equals to the length of the Doxycycline treatment. All panels show mean ± SD; n=3.

Figure 17 shows hDSC gene expression of the target genes MafA, MafB, PDX1 and NKX6.1. The different medium conditions were named CTRL 2D, 2% FBS 2D and 2% FBS WO. 4DOX, 6DOX, 8DOX and 10DOX represented the length of the Doxycycline reprogramming. The MafA expression was highly variable throughout all of the samples. Most of them had significantly higher expression. MafB has a highly variable expression too which is why no verified conclusion can be made. The expression of PDX1 for most of the samples was similar to the control. NKX6.1 gene expression showed significant changes in most of the samples.
No sample expressed similar mRNA values across all of the target genes. This means that the reprogrammed failed to induce the complete beta cell-like state.

3.5 Reprogramming of hCSCs and HUVEC

Taking into consideration that vascularization and survival *in vivo* is the biggest challenge, options to improve vessel formation were considered. This experiment used the human gastro-intestinal stem cells with the highest survivability together with HUVECs overexpressing ETV2. Aggregating these two cell types together is hypothesized to significantly increase the viability of the stem cells *in vivo*. For this purpose reprogramming parameters were changed and evaluated.
These parameters were the length of the 4-Hydroxy-Tamoxyfen treatment and the start of the Doxycycline treatment. The different conditions were as following:

1. 2 days of Tamoxifen treatment - Doxycycline treatment starting on day 0
2. 2 days of Tamoxifen treatment - Doxycycline treatment starting on day 1
3. 2 days of Tamoxifen treatment - Doxycycline treatment starting on day 2
4. 1 day of Tamoxifen treatment - Doxycycline treatment starting on day 0
5. 1 day of Tamoxifen treatment - Doxycycline treatment starting on day 1
6. 1 day of Tamoxifen treatment - Doxycycline treatment starting on day 2

At day 2 every condition was cultured in the same medium. The medium change schedule can be seen in figure 18.

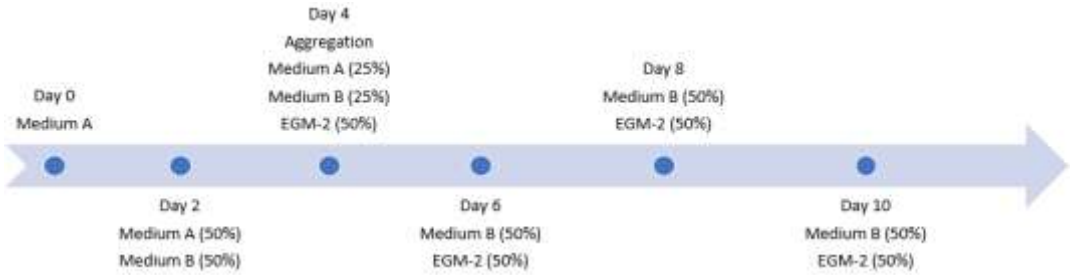

Figure 18: Schedule of medium changes and mixture of media. Medium A is 2D complete meium; Medium B represents Advanced DMEM/F12 plus primocin, 10mM HEPES, GlutaMax, B27 N2, 10mM Nicotinamide, 10uM Y, 1mM NAC and 1uM A8301; EGM-2 medium can be bought from Lonza.

Three different media are used during this experiment. Medium A is regular 2D complete medium used for culturing hGISCs. Medium B consists of Advanced DMEM/F12 supplemented with primocin, 10mM HEPES, GlutaMax, B27, N2, 10mM Nicotinamide, 10uM Y, 1mM NAC and 1uM A8301. EGM-2 can be bought from Lonza and is regular endothelial growth medium.

Starting on day 2 the concentration of Medium A is continuously reduced by diluting it 1:1 with Medium B. During the aggregation on day 4, 5000 human corpus stem cells (hCSCs) per condition were aggregated with 5000 ETV-2 cells per aggregate. As a control 5000 hCSCs alone were aggregated as well. This leads to a total of 12 conditions and 6 aggregates per condition; half of them were aggregated with ETV-2, the other half was reprogrammed as control. The cells were aggregated in 25% Medium A, 25% Medium B and 50% EGM-2. From day 6 onwards the culturing medium consisted of 50% Medium B and 50% EGM-2.

On day 10, the aggregates were harvested, lysed and their gene expression was analysed. The gene expression of insulin can be observed in figure 19.

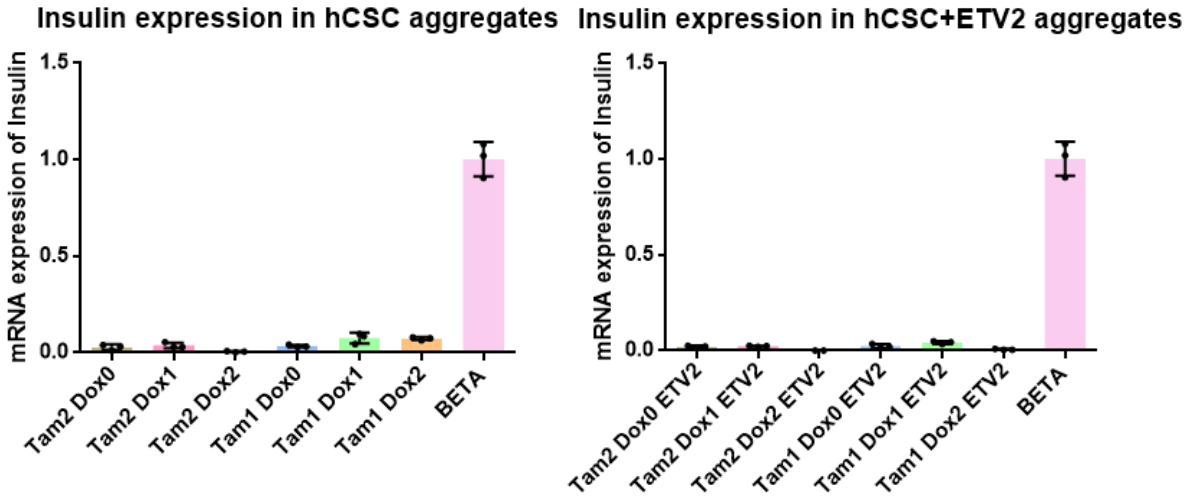

Figure 19: Insulin expression of hCSC aggregates cultured in different conditions. In the left panel, the insulin expression of the control hCSC aggregates is depicted, while in the right panel the hCSCs were aggregated with ETV2 at a concentration of 1:1. Tam1 and Tam2 represent the length of the Tamoxifen treatment; Dox0, Dox1 and Dox2 shows the start day of the Doxycycline treatment. No sample shows a significant advantage over the others. Every sample has similarly low insulin expression. BETA is the control beta cell sample. All panels show mean ± SD; n=3.

In figure 19 the insulin expression of hCSC aggregates can be observed. In the right panel, the hCSC+ETV2 aggregates showed significantly less insulin expression compared to the beta cell control. The same observation can be made on the left side with hCSC aggregates. No condition displayed significant advantages over other conditions. These results lead to the conclusion that the reprogramming failed to induce insulin expression.

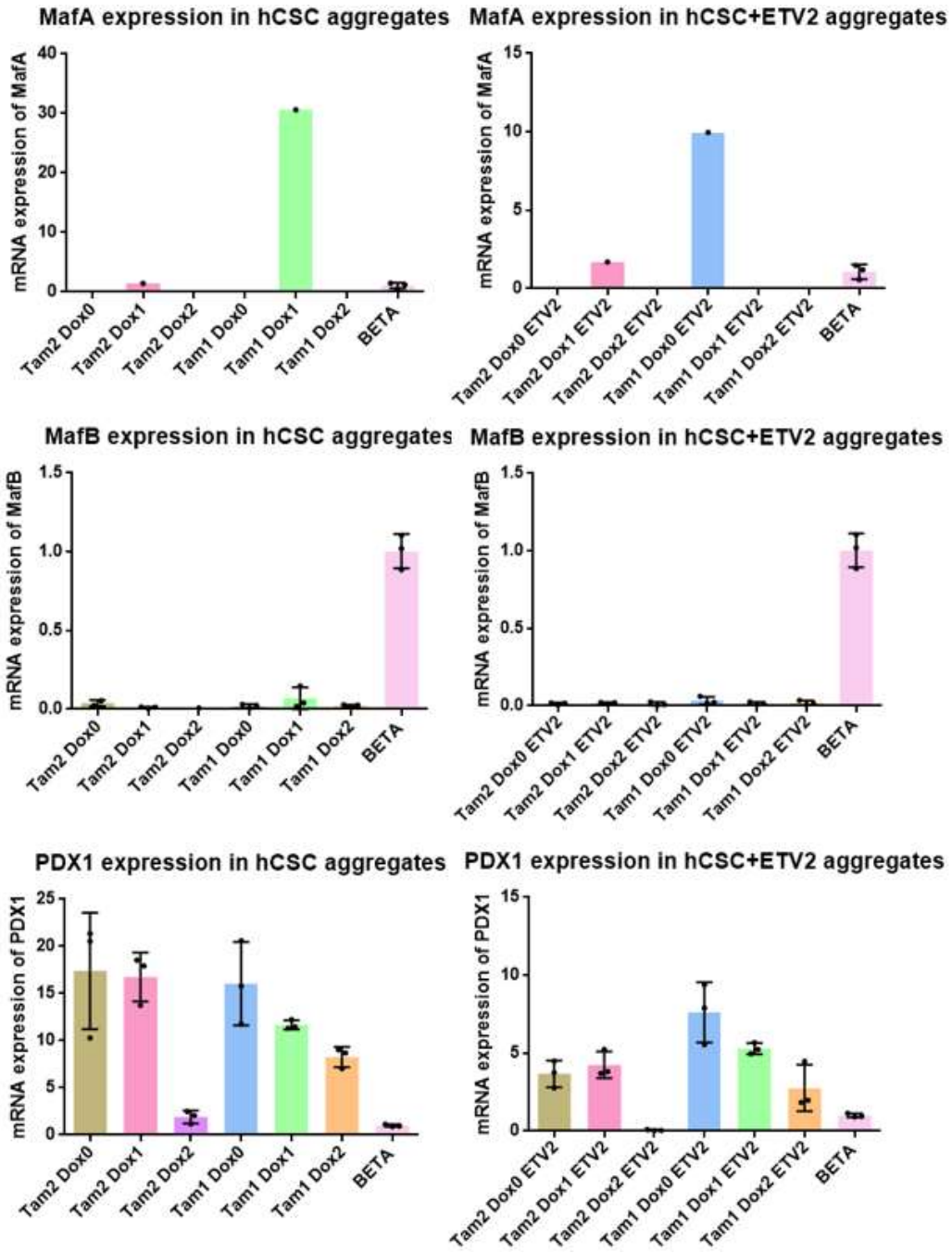

Figure 20: Expression of MafA, MafB and PDX1 in hCSC aggregates. In the right panels the gene expression of hCSC+ETV2 aggregates can be observed. On the left side, the gene expression of control aggregates can be seen. Tam1 and Tam2 represent the length of the Tamoxifen treatment; Dox0, Dox1 and Dox2 shows the start day of the Doxycycline treatment. No gene expression pattern of any sample is comparable to the control. BETA is the control beta cell sample. All panels show mean ± SD; n=3.

In figure 20 the gene expression of MafA, MafB and PDX1 of hCSC and hCSC+ETV2 aggregates can be seen. The expression of MafA is very variable. Some samples have significantly higher expression than the control while others have no detectable expression. MafB has a significantly lower expression in hCSC and hCSC+ETV2 aggregates than in the control sample. In both hCSC and hCSC+ETV2 aggregates, the PDX1 gene expression is variable and significantly higher compared to the beta cell control.
Looking at the gene expression profile of both hCSC and hCSC+ETV2 aggregates the conclusion can be drawn that the hCSCs were not reprogrammed.

3.6 Reprogramming of TetOff hCSCs

Improving reprogramming efficiency and survival of reprogrammed aggregates *in vivo* are two major challenges. *In vivo* reprogramming was conducted by transplanting human gastro-intestinal stem cells into mice and treating their drinking water with the necessary reagents. The results of the experiment were analysed by measuring the insulin levels of the blood and staining the mouse tissue after removing the kidney. No reprogramming was reported, which lead to the conclusion that the Doxycycline was not able to reach the cells. Therefore a new cell line with the TetOff system, replacing the TetOn system, was established. The human corpus stem cells were selected because of their superior survivability *in vivo*. In contrast to the TetOn system, the TetOff promotor expresses the genes in absence of Dox. After proliferating this cell line to get sufficient stock, *in vitro* test were conducted to evaluate their reprogramming.
Different lengths of Tam treatment were tested beside the cell line´s ability of reprogramming. The conditions were as follows:

1. 1 days of Tamoxifen treatment - Doxycycline treatment stopping on day 1
2. 2 day of Tamoxifen treatment - Doxycycline treatment stopping on day 2

Additionally, two wells of a 48-well plate were reprogrammed to stain for CPPT and MafA.

The reprogramming timeline can be seen in figure 5. On day 4 the cells were aggregated into 96-well plates. After 12 days of reprogramming, the two wells of the 48-well plate were stained and analysed. The results can be seen in figure 21.

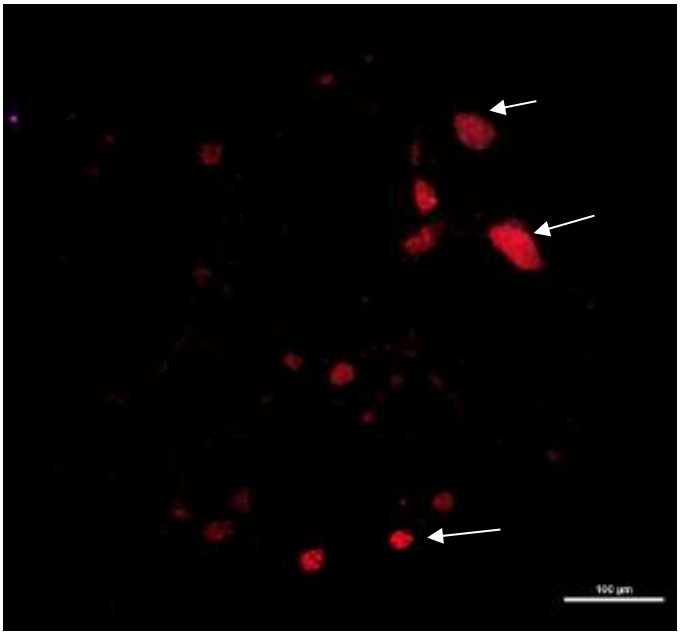

Figure 21: Staining of TetOff hCSCs after 12 days of reprogramming. The length of Tam treatment was one day. White arrows point to highly mCherry positive cells. No CPPT or MafA signal is visual. The magnification use was 10x.

Figure 21 shows the CPPT and MafA staining of reprogrammed TetOff hCSCs. The white arrows point to mCherry positive stem cell clusters. No CPPT or MafA signal can be seen. This means that the reprogramming failed and no insulin+ cells were generated.

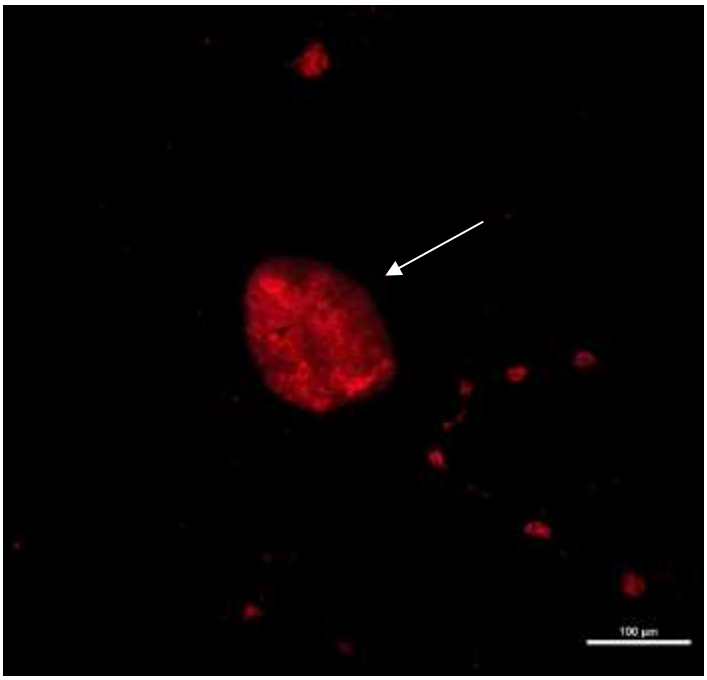

Figure 22: Staining of TetOff hCSCs after 12 days of reprogramming. The length of Tam treatment was two days. White arrow points to highly mCherry positive stem cell cluster. No CPPT or MafA signal is visual. The magnification used was 10x.

In figure 22 the CPPT and MafA expression of reprogrammed TetOff hCSCs can be seen. One day of additional Tam treatment does not change the outcome of the reprogramming. These cells do not express Insulin or MafA. This led to the conclusion that the tTA background is too strong. In order to correct this mistake, the process of creating the plasmid and cell line had to be repeated.

4 Discussion

4.1 Reprogramming of hDSCs

We show in this experiment that the reprogramming of hDSCs into insulin+ cells is possible. The Tam treatment for two induces a reprogramming to enteroendocrine cells, which are then reprogrammed into insulin+ cells. This approach is gentler than the previous reprogramming method. First reprogramming the hDSCs into enteroendocrine cells has a beneficial effect on their viability and survival *in vivo*. Enteroendocrine (EE) cells are the intermediate between gastro-intestinal cells and insulin secreting beta cells. Both EE cells and beta cells are endocrine cells. Not every stem cells can be reprogrammed into EE cells and further into insulin+ cells.

Our results show that the plasmids carrying the genes and their quantity are essential to the reprogramming. mCherry was used as a reporter fluorophore to signal the presence of the generated plasmids. Stronger mCherry fluorescence is related to an easier reprogramming. This phenomenon is due to the quantity of the plasmids. Preferentially very strong mCherry positive cells are reprogrammed. This was controlled by adding antibiotics into the cells culture medium. Cells with no plasmid or just one plasmid die in contrast to cells with many plasmids. We quantified the number of reprogrammed cells versus the number of total cells and reach 30-40%. This percentage is consistent with other publications. [44]

Our staining show that a longer reprogramming time with Dox can increase the number of CPPT positive hDSC clusters.

Our results confirm that there is no co-localization of GIP and CPPT. This means that CPPT is exclusively expressed in hDSC clusters that are fully reprogrammed. On the other hand a co-localization of MafA and CPPT was confirmed. Cell clusters that express CPPT are always MafA positive as well.

4.2 Transplantation

Every cell type and cell line has different properties in terms of reprogramming and survival. Duodenum stem cells are easier to reprogram but high mortality *in vivo* can be observed. Antrum stem cells have better survivability but are harder to reprogram. Duodenum stem cells derived from different patients show a different mortality and *in vivo* survival. This fact increases the difficulty of developing a generalized reprogramming approach for all hGISCs. The transplantation itself is not an issue due to the survival of the antrum cells.

Our staining shows no CPPT expression during *in vivo* reprogramming of hGISCs. We hypothesise that ether the reprogramming is blocked after transplantation or the Dox cannot reach the tissue in time. After transplantation the stem cells need to form new blood vessels to survive. If this procedure takes too long, the reprogramming will be impaired. Cell death occurs during this neovascularization and a loss of reprogramming factors impairs the reprogramming.

4.3 Reprogramming optimization

Our previous results suggest that the reprogrammed stem cells die *in vivo* very quickly. Culturing and reprogramming the stem cells in 20% FBS medium was hypothesised to be one of the reasons of the mortality rate. Lowering the percentage of FBS during reprogramming increased the insulin levels measured by qPCR. The other conditions showed no improvement over each other. Through this experiment the necessary supplements for the medium were investigated. Following experiments determined which factors benefit to the reprogramming. No definitive conclusion can be drawn from the MafA gene expression. MafA is highly variable most of the time and a comparison is very difficult. Technical replicates show high variability as well. The other beta cell markers, MafB, NKX6.1 and PDX1, have different expressions throughout the samples. No sample resembles the positive control good enough to call it a success.

Previous results indicated that four days of Dox treatment were sufficient for a complete reprogramming. The new experiment disproved this result by showing that the peak of insulin expression is after eight days of Dox treatment. This indicates that treating the stem cells too long with Dox could be disadvantageous.

Our current result confirm previous results that many of the supplementary factors are necessary for stem cell reprogramming. hDSCs reprogrammed in supplement free medium expressed very low levels of insulin compared to its counterpart with supplements. Nicotinamide, Y 27632 and A8301 are essential, while the other factors are important for proliferation. For reprogramming purposes the non-essential supplements were removed.

MafA, MafB, PDX1 and NKX6.1 expression levels show that no sample´s gene expression is comparable to the beta cell expression. Studies show that NKX6.1 is essential to beta cell function in mice [45], while its role in human beta cells is largely unknown.

The 5% oxygen reprogramming approach resulted in no significant insulin expression. During the reprogramming, oxygen is too essential to prepare the cells for low oxygen environments. This lead us to plan experiments which increase the neovascularization of the stem cells drastically.

4.4 New reprogramming approach

Aggregation with ETV2-overexpressing HUVECs was tested to increase tissue vascularization. ETV2 is important during the early stages of development and can induce neovascularization. [46] This hypothesis was confirmed through *in vivo* transplantation by colleagues.

Using the TetOff induction system was planned to activate MafA and PDX1 after removing Dox in the culturing medium. [47] The results showed that this activation was not successful. Analysing every step of the creation of the virus and reprogramming, the problem was the tTA. Tests revealed that the suppression of tTA and therefore the activation of the transgenic genes was not completely possible. Some tTA was present after administering Dox. This led to the generation of a plasmid with better background suppressing properties. This approach was meant to simplify the *in vivo* reprogramming.

4.5 Difficulties

During the course of the experiments I met a lot of different problems I had to work around or try to help fix. The first and biggest problem is the reprogramming efficiency. In order to produce large numbers of insulin+ cells, the reprogramming has to be optimized. Several experiments were conducted to examine the perfect reprogramming length, medium and other parameters. This led to the next obstacle which was the variability of reprogramming among the cell types and cell lines. Antrum, corpus and duodenum stem cells behaved very differently when being treated the same way. Antrum cell lines isolated from different donors presented with different reprogramming potential and properties as well. One such property was the ability to aggregate. Many cell lines did not aggregate, while others aggregates perfectly overnight. I started concentrating on one cell line to simplify the experiments.
Another big problem was the *in vivo* vascularization and survival. Reprogrammed stem cells showed low survivability. Those which survived were not reprogrammed. To overcome this challenge, the cells were prepared to survive unfavourable conditions by reprogramming them in 2% FBS medium. Further, stem cells were aggregated with ETV2-overexpressing HUVEC. This increased both their aggregation ability and vascularization.
Analysing the gene expression of the reprogrammed cells proved to be another critical part of the experiments. The beta cell control was very sensitive. The conclusion drawn from the qPCR results change drastically with the amount of beta cell lysate. Lower amounts of RNA lead to false positive insulin measurements.
The other analysed genes have very variable expression, which makes it hard to draw clear conclusions.

4.6 Future plan

The future plans of this project revolve around successfully increasing the reprogramming efficiency, the transplantation and *in vivo* survival has to be optimized. The functional analysis of reprogrammed stem cells will follow.
To enhance the reprogramming, further screens for transcription factors have to be conducted. The current reprogramming cocktail seems unable to reprogram hGISCs into insulin+ cells. By searching and discovering more transcription factors, the cocktail can be improved.

Another big issue that needs future attention is the *in vivo* insulin expression. Until now, no human insulin can be detected in xenografted mice. One possible explanation is the number of reprogrammed cells. In the future, experiments transplanting more reprogrammed cells will be conducted. Further, different transplantation methods will be tested. One such is to reprogram the aggregates stem cells in Matrigel. After reprogramming, the Matrigel will be transplanted into the fat pad of NSG mice.
The secretion of insulin will be tested in future experiments. Our theory is, that the insulin+ cells express insulin but are incapable of secreting it into the blood stream.
The ability of the reprogrammed cells to evade auto-immune destruction will be tested as well.

Considering all of the difficulties and achieved goals, this project shows a lot of promise and will hopefully result in a reproduceable method to create large numbers of insulin-secreting human cells evading auto-immune destruction.

Bibliography

[1] A. T. Kharroubi, “Diabetes mellitus: The epidemic of the century,” *World J. Diabetes*, vol. 6, no. 6, p. 850, 2015.

[2] R. A. DeFronzo, E. Ferrannini, and P. Zimmet, “International Textbook of Diabetes Mellitus. Vol. 1 (4th edition),” *Diabetes Care*, 2010.

[3] C. Solis-Herrera, C. Triplitt, C. Reasner, R. A. DeFronzo, and E. Cersosimo, *Classification of Diabetes Mellitus*. 2000.

[4] A. El Ouaamari *et al.*, “SerpinB1 Promotes Pancreatic β Cell Proliferation,” *Cell Metab.*, vol. 23, no. 1, pp. 194–205, Jan. 2016.

[5] K. Loh *et al.*, “Inhibition of Y1 receptor signaling improves islet transplant outcome,” *Nat. Commun.*, vol. 8, no. 1, Dec. 2017.

[6] E. Dirice *et al.*, “Increased β-cell proliferation before immune cell invasion prevents progression of type 1 diabetes,” *Nat. Metab.*, vol. 1, no. 5, pp. 509–518, May 2019.

[7] J. Tian *et al.*, “Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice,” *Nat. Med.*, vol. 2, no. 12, pp. 1348–1353, Dec. 1996.

[8] R. Tisch, R. S. Liblau, X. D. Yang, P. Liblau, and H. O. McDevitt, “Induction of GAD65-specific regulatory T-cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice.,” *Diabetes*, vol. 47, no. 6, pp. 894–9, Jun. 1998.

[9] J. M. Pleau, F. Fernandez-Saravia, A. Esling, F. Homo-Delarche, and M. Dardenne, “Prevention of autoimmune diabetes in nonobese diabetic female mice by treatment with recombinant glutamic acid decarboxylase (GAD 65).,” *Clin. Immunol. Immunopathol.*, vol. 76, no. 1 Pt 1, pp. 90–5, Jul. 1995.

[10] J. E. Tooley, F. Waldron-Lynch, and K. C. Herold, “New and future immunomodulatory therapy in type 1 diabetes,” *Trends in Molecular Medicine*, vol. 18, no. 3. pp. 173–181, Mar-2012.

[11] I. Serr *et al.*, “Type 1 diabetes vaccine candidates promote human Foxp3+ Treg induction in humanized mice,” *Nat. Commun.*, vol. 7, Mar. 2016.

[12] Q. Wang *et al.*, “DPP-4 Inhibitors as Treatments for Type 1 Diabetes Mellitus: A Systematic Review and Meta-Analysis,” *Journal of Diabetes Research*, vol. 2018. Hindawi Limited, 2018.

[13] H. Fattah and V. Vallon, “The Potential Role of SGLT2 Inhibitors in the Treatment of Type 1 Diabetes Mellitus,” *Drugs*, vol. 78, no. 7, pp. 717–726, May 2018.

[14] S. Sabet, M. E. Condren, A. F. Boston, L. C. Doak, and L. J. Chalmers, “Evolving pharmacotherapeutic strategies for type 1 diabetes mellitus,” *Journal of Pediatric Pharmacology and Therapeutics*, vol. 23, no. 5. Pediatric Pharmacy Advocacy Group, Inc., pp. 351–361, 01-Sep-2018.

[15] X. Xu *et al.*, “β Cells Can Be Generated from Endogenous Progenitors in Injured Adult Mouse Pancreas,” *Cell*, vol. 132, no. 2, pp. 197–207, Jan. 2008.

[16] F. Thorel *et al.*, “Conversion of adult pancreatic α-cells to B-cells after extreme B-cell loss,” *Nature*, vol. 464, no. 7292, pp. 1149–1154, Apr. 2010.

[17] Q. Zhou, J. Brown, A. Kanarek, J. Rajagopal, and D. A. Melton, “In vivo reprogramming of adult pancreatic exocrine cells to β-cells,” *Nature*, vol. 455, no. 7213, pp. 627–632, Oct. 2008.

[18] M. Courtney *et al.*, “The Inactivation of Arx in Pancreatic α-Cells Triggers Their Neogenesis and Conversion into Functional β-Like Cells,” *PLoS Genet.*, vol. 9, no. 10, Oct. 2013.

[19] C. Ariyachet *et al.*, “Reprogrammed Stomach Tissue as a Renewable Source of Functional β Cells for Blood Glucose Regulation,” *Cell Stem Cell*, vol. 18, no. 3, pp. 410–421, Mar. 2016.

[20] L. Baeyens *et al.*, “Transient cytokine treatment induces acinar cell reprogramming and regenerates functional beta cell mass in diabetic mice,” *Nat. Biotechnol.*, vol. 32,

no. 1, pp. 76–83, Jan. 2014.
[21] X. Xiao *et al.*, “Endogenous Reprogramming of Alpha Cells into Beta Cells, Induced by Viral Gene Therapy, Reverses Autoimmune Diabetes,” *Cell Stem Cell*, vol. 22, no. 1, pp. 78-90.e4, Jan. 2018.
[22] J. Lee *et al.*, “Expansion and conversion of human pancreatic ductal cells into insulin-secreting endocrine cells,” *Elife*, vol. 2013, no. 2, Nov. 2013.
[23] M. Lemper *et al.*, “Reprogramming of human pancreatic exocrine cells to β-like cells,” *Cell Death Differ.*, vol. 22, no. 7, pp. 1117–1130, Jul. 2015.
[24] E. Kroon *et al.*, “Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo,” *Nat. Biotechnol.*, vol. 26, no. 4, pp. 443–452, Apr. 2008.
[25] G. L. Szot *et al.*, “Tolerance induction and reversal of diabetes in mice transplanted with human embryonic stem cell-derived pancreatic endoderm,” *Cell Stem Cell*, vol. 16, no. 2, pp. 148–157, Feb. 2015.
[26] M. Y. Song, U. J. Bae, K. Y. Jang, and B. H. Park, “Transplantation of betacellulin-transduced islets improves glucose intolerance in diabetic mice,” *Exp. Mol. Med.*, vol. 46, no. 5, 2014.
[27] D. Tripathi *et al.*, “A TLR9 agonist promotes IL-22-dependent pancreatic islet allograft survival in type 1 diabetic mice,” *Nat. Commun.*, vol. 7, p. 13896, Dec. 2016.
[28] D. Dufrane, R.-M. Goebbels, A. Saliez, Y. Guiot, and P. Gianello, “Six-Month Survival of Microencapsulated Pig Islets and Alginate Biocompatibility in Primates: Proof of Concept,” *Transplantation*, vol. 81, no. 9, pp. 1345–1353, May 2006.
[29] Y. Sun, X. Ma, D. Zhou, I. Vacek, and A. M. Sun, “Normalization of diabetes in spontaneously diabetic cynomologus monkeys by xenografts of microencapsulated porcine islets without immunosuppression,” *J. Clin. Invest.*, vol. 98, no. 6, pp. 1417–1422, Sep. 1996.
[30] T. Kobayashi *et al.*, “Generation of Rat Pancreas in Mouse by Interspecific Blastocyst Injection of Pluripotent Stem Cells,” *Cell*, vol. 142, no. 5, pp. 787–799, Sep. 2010.
[31] T. Yamaguchi *et al.*, “Interspecies organogenesis generates autologous functional islets,” *Nature*, vol. 542, no. 7640, pp. 191–196, Feb. 2017.
[32] A. J. Vegas *et al.*, “Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice,” *Nat. Med.*, vol. 22, no. 3, pp. 306–311, Mar. 2016.
[33] H. A. Keenan *et al.*, “Residual insulin production and pancreatic β-cell turnover after 50 years of diabetes: Joslin medalist study,” *Diabetes*, vol. 59, no. 11, pp. 2846–2853, Nov. 2010.
[34] E. H. Liu *et al.*, “Pancreatic beta cell function persists in many patients with chronic type 1 diabetes, but is not dramatically improved by prolonged immunosuppression and euglycaemia from a beta cell allograft,” *Diabetologia*, vol. 52, no. 7, pp. 1369–1380, Jul. 2009.
[35] “In Vivo Reprogramming in Regenerative Medicine - Google Books.” [Online]. Available: https://books.google.com/books?id=bA5ADwAAQBAJ&pg=PA21&lpg=PA21&dq=reprogramming+npm+factors&source=bl&ots=YRl0yLZDtT&sig=ACfU3U2PZgeju2bd5jGxz26sgtajiM48kQ&hl=de&sa=X&ved=2ahUKEwizpJXw7Z7mAhVwn-AKHbPfCSEQ6AEwB3oECAsQAQ#v=onepage&q=reprogramming npm factors&f=false. [Accessed: 05-Dec-2019].
[36] C. S. Lee, N. Perreault, J. E. Brestelli, and K. H. Kaestner, “Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity,” *Genes Dev.*, vol. 16, no. 12, pp. 1488–1497, Jun. 2002.
[37] “[PDF] PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. | Semantic Scholar.” [Online]. Available: https://www.semanticscholar.org/paper/PDX-1-is-required-for-pancreatic-outgrowth-

and-of-Offield-Jetton/54515a52ee9b4e711b187d3d2dc78bb0e8aceec2. [Accessed: 05-Dec-2019].
[38] Y. Zhu, Q. Liu, Z. Zhou, and Y. Ikeda, “PDX1, Neurogenin-3, and MAFA: critical transcription regulators for beta cell development and regeneration,” *Stem Cell Res. Ther.*, vol. 8, no. 1, p. 240, Dec. 2017.
[39] C. L. Pin, J. F. Ryan, and R. Mehmood, “Acinar cell reprogramming: A clinically important target in pancreatic disease,” *Epigenomics*, vol. 7, no. 2. Future Medicine Ltd., pp. 267–281, 01-Apr-2015.
[40] A. R. Gunawardene, B. M. Corfe, and C. A. Staton, “Classification and functions of enteroendocrine cells of the lower gastrointestinal tract,” *International Journal of Experimental Pathology*, vol. 92, no. 4. pp. 219–231, Aug-2011.
[41] “Insulin Synthesis and Secretion.” [Online]. Available: http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/pancreas/insulin.html. [Accessed: 05-Dec-2019].
[42] G. Xiafukaiti *et al.*, “MafB Is Important for Pancreatic β-Cell Maintenance under a MafA-Deficient Condition,” *Mol. Cell. Biol.*, vol. 39, no. 17, Jun. 2019.
[43] A. E. Schaffer *et al.*, “Nkx6.1 Controls a Gene Regulatory Network Required for Establishing and Maintaining Pancreatic Beta Cell Identity,” *PLoS Genet.*, vol. 9, no. 1, p. e1003274, Jan. 2013.
[44] H. M. Shahjalal, A. Abdal Dayem, K. M. Lim, T. Il Jeon, and S. G. Cho, “Generation of pancreatic β cells for treatment of diabetes: Advances and challenges,” *Stem Cell Res. Ther.*, vol. 9, no. 1, Dec. 2018.
[45] B. L. Taylor, F. F. Liu, and M. Sander, “Nkx6.1 Is Essential for Maintaining the Functional State of Pancreatic Beta Cells,” *Cell Rep.*, vol. 4, no. 6, pp. 1262–1275, Sep. 2013.
[46] C. Park, “Mechanisms of ER71/ETV2-regulated Vascularization.”
[47] A. T. Das, L. Tenenbaum, and B. Berkhout, “Tet-On Systems For Doxycycline-inducible Gene Expression,” *Curr. Gene Ther.*, vol. 16, no. 3, pp. 156–167, Jun. 2016.

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List of Abbreviations

T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
hGISCs	Human Gastro-Intestinal Stem Cells
MEF	Mouse Embryonic Fibroblasts
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
CO2	Carbon dioxide
HUVEC	Human Umbilical Vein Endothelial Cells
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Triton X
Tam	4-Hydroxytamoxyfen
Dox	Doxycycline
CPPT	C-peptide
EE cells	Enteroendocrine cells
PDX1	Pancreatic and Duodenal Homeobox 1
MafA	V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog A
Ngn3	Neurogenin 3
NKX6.1	Homeobox Protein NK-6 Homolog A
BSA	Bovine Serum Albumin
hCSC	Human Corpus Stem Cell
hDSC	Human Duodenum Stem Cell
MafB	V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog B
GIP	Glucose-Dependent Insulinotropic Polypeptide
ETV2	ETS Translocation Variant 2