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Gene therapies and their potential application in Type 1 Diabetes

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ABSTRACT

Type 1 diabetes (T1D) is an autoimmune metabolic disorder, that arises due to autoimmune destruction of the β-cells in the islets of Langerhans in the pancreas. As a result, there is insufficient insulin production and ineffective glucose uptake, resulting in hyperglycemia. Currently available therapies are the widely used insulin injections and the not so common pancreatic islet transplantation. Even though, Insulin has been able to increase the life expectancy of T1D individuals, it is still shorter than the healthy population and the need for chronic uptake of immunosuppressive drugs, as well as the limited supply of healthy pancreatic islet cells that are needed for the islet transplantation, are some of the limiting factors of these two therapies. Therefore, scientists have been interested in developing other, more permanent treatments. Gene therapy has emerged as a potential therapeutic alternative to treat patients with T1D, even though its safety has not yet been established in humans. This thesis will focus on explaining the basics of gene therapy, as well as presenting the current status and future perspectives through analysis of various in vitro and in vivo studies, these include overexpression of genes or proteins involved in the molecular pathways leading to T1D.

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BIO 680 Scientific Methodology in Molecular Biology

Student Presentation

Friday, 14 May 2021 at 10:45

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Type 1 diabetes (T1D) is an autoimmune metabolic disorder, that arises due to autoimmune destruction of the β-cells in the islets of Langerhans in the pancreas. As a result, there is insufficient insulin production and ineffective glucose uptake, resulting in hyperglycemia. Currently available therapies are the widely used insulin injections and the not so common pancreatic islet transplantation. Even though, Insulin has been able to increase the life expectancy of T1D individuals, it is still shorter than the healthy population and the need for chronic uptake of immunosuppressive drugs, as well as the limited supply of healthy pancreatic islet cells that are needed for the islet transplantation, are some of the limiting factors of these two therapies. Therefore, scientists have been interested in developing other, more permanent treatments. Gene therapy has emerged as a potential therapeutic alternative to treat patients with T1D, even though its safety has not yet been established in humans. This thesis will focus on explaining the basics of gene therapy, as well as presenting the current status and future perspectives through analysis of various in vitro and in vivo studies, these include overexpression of genes or proteins involved in the molecular pathways leading to T1D.

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INTRODUCTION

Diabetes mellitus is a chronic, metabolic disease and is described by high levels of blood glucose over a prolonged period. Some of its more common symptoms include increased thirst and frequent urination, also known as polydipsia and polyuria, respectively. If diabetes is left untreated, over time it can lead to complications, including diabetic ketoacidosis or even death. Diabetic ketoacidosis is considered to be the most acute hyperglycemic complication in T1D patients and is characterized by the presence of high blood or urinary ketoacids and a high anion gap metabolic acidosis (Dhatariya, Glaser et al. 2020). High blood and urine concentrations of ketone bodies, acetone, acetoacetate, and β-hydroxybutyrate, are present when this metabolic state is emerging (Ghimire, Dhamoon 2021).

Diabetes diagnosis is based on plasma glucose criteria and it can be classified in four categories (American Diabetes Association 2017, American Diabetes Association 2019). Type 1, T1D, that is characterized by total insulin inadequacy because of autoimmune β-cell demolition, type 2, T2D, that often has the background of insulin resistance and is distinguished by the progressive loss of β-cell insulin secretion, the gestational diabetes mellitus, GDM, that is diagnosed after the second trimester of pregnancy and it is not conspisuous prior to gestation and, finally, specific types of diabetes due to other explanations (American Diabetes Association 2017). The classification between the two main categories of diabetes, T1D and T2D, is important, in order to determine the therapy that is going to be used (American Diabetes Association 2019). The differentiation of the two main forms of diabetes is made by the use of biomarkers of β-cell autoantibodies (Pociot, Lernmark 2016). T1D differs from T2D pathogenically, one is immune mediated and the other is mediated by metabolic mechanisms, respectively (Eizirik, Pasquali et al. 2020).

Type 1 Diabetes

As mentioned previously, in T1D the pancreas is unable to produce the amount of insulin that is needed to break down blood glucose, because of the autoimmune demolition of β-cells in the islets of Langerhans, resulting in hyperglycemia (Katsarou, Gudbjörnsdottir et al. 2017). The loss of β-cells activity is a result of a decreased abundance of β-cells, as well as the dysfunction of them, which in manifested by insulitis (Fløyel, Kaur et al. 2015). Insulitis is considered the hallmark of

T1D and is the inflammatory lesion on the pancreatic islets which is defined by immune and inflammatory cells in the periphery and inside the pancreatic islets (Pugliese 2016). In simple words, insulitis is the protestation of the autoimmunity against β-cells (Pugliese 2016). Normally, β-cells synthesize and secrete insulin, because of differences in blood glucose levels (Handorf, Sollinger et al. 2015). Insulin, then, operates on other cells to advocate the uptake of glucose from the blood and consequently it lowers the blood glucose levels back to normal (Handorf, Sollinger et al. 2015).

According to Eizirik et al, T1D is an aftermath of perplexing interactions between infecting or resident macrophages and T cells, which then discharge cytokines and chemokines in the islet microenvironment and distribute cell-cell proapoptotic signals and β-cells, by signals that are generated physiologically or via stressed, damagd or dying β-cells that attract and activate immune cells to the islets (Eizirik, Pasquali et al. 2020). These interactions are the results of the genetic background of the patient, as wells as environmental factors (Blanter, Sork et al. 2019, Eizirik, Pasquali et al. 2020, Fløyel, Kaur et al. 2015). A common hypothesis is that the genes provide the predisposition of a person to develop diabetes, while the environment provides the cause for the onset of it (Blanter, Sork et al. 2019). The use of biomarkers, for the prediction of T1D, is important, both, for the hosts health and for the disease path (Pociot, Lernmark 2016).

Usually, T1D symptomatic onset is during childhood or adolescence but symptoms can, also, develop at an older age (Katsarou, Gudbjörnsdottir et al. 2017). During the first stage of the pathogenesis of T1D, the patients develop two or more T1D biomarkers and are normoglycemic (Insel, Dunne et al. 2015). In the second stage, the patients are glucose intolerant, or dysglycemic due to the loss of functional β-cell mass (Insel, Dunne et al. 2015). In the third and final stage, the manifestation of the clinical symptoms and indications of diabetes begin (Insel, Dunne et al. 2015).

Genetic causes for T1D

Approximately 50 genetic regions, loci, that affect the risk of T1D development have been identified by Genome Wide Association Studies (GWAS) (Fløyel, Kaur et al. 2015). GWAS aims to showcase the causative genes and their function in disease etiology, and to discover mechanistic pathways (Heinonen, Moulder et al. 2015). 80% of the heritability of T1D is elucidated by these loci (Fløyel, Kaur et al. 2015). A great amount of the genes is expressed in pancreatic islets and β-cells (Fløyel, Kaur et al. 2015, Blanter, Sork et al. 2019).

The human leukocyte antigen (HLA) region is responsible for approximately 40% of the genetic risk of T1D, with the class II alleles exhibiting the strongest association with the disease (Santin, Eizirik 2013). HLA region affects the speed of loss of the functional β-cells succeeding the presence of autoantibodies (Eizirik, Pasquali et al. 2020). HLA region is characterized with extreme polymorphism, as class I (A, B and C) molecules are expressed as single chain proteins and can produce intracellular antigen to CD8+ T cells, whereas class II (DP, DQ and DR) molecules are heterodimers that are expressed on professional antigen-presenting cells and produce extracellular antigen to CD4+ T cells (Xie, Chang et al. 2014). HLA class II loci is responsible to approximately 40-50% risk to the T1D susceptibility, playing a main role in the early stages of the autoimmune process (Xie, Chang et al. 2014, Lempainen, Ilonen 2012). Both susceptible and protective alleles can be found at the DRB1, DQA1 and DQB1 loci, of which DRB1 and DQB1 are correlated with type 1 diabetics from all around the world (Xie, Chang et al. 2014). However, differences have been found between the protective and susceptible alleles from people of different origins (Lempainen, Ilonen 2012, Xie, Chang et al. 2014). Specific combinations of alleles, in the three loci mentioned previously, contribute to the risk of developing this disease (Xie, Chang et al. 2014).

Other genes, from non-HLA regions play, also, a role in T1D susceptibility. INS is the first strong non-HLA gene that is linked with T1D (Xie, Chang et al. 2014). INS risk genotype has been linked with the display of insulin autoantibodies, that is usually the first autoantibody that is detected, and humoral β-cell immunity (Lempainen, Ilonen 2012, Fløyel, Kaur et al. 2015). INS gene polymorphisms, as well as the variable number of tandem repeats (VNTRs), that are based 596 bp upstream of the INS gene, are linked with T1D risk (Lempainen, Ilonen 2012, Xie, Chang et al. 2014). The VNTRs that are located in the INS gene are classified in three forms, class I alleles, that are related with susceptibility to T1D, class II alleles and class III alleles, that are related with protection to T1D (Xie, Chang et al. 2014). Class I alleles are, also, related with high insulin mRNA expression levels in the pancreas and low levels in the thymus, whereas class III alleles are related with lower levels of insulin mRNA in the pancreas and higher levels in the thymus (Xie, Chang et al. 2014). Proinsulin, a precursor of insulin, and insulin are potential target autoantigens for β-cell demolition (Xie, Chang et al. 2014). Low levels of proinsulin in the thymus, can influence the positive selection of T cells in the thymus and induce migration of CD4+ proinsulin-specific T lymphocytes to the periphery, rising the risk for T1D (Xie, Chang et al. 2014). In

contradiction, high levels of proinsulin in the thymus can advocate the negative selection of T cells of insulin-specific autoreactive T lymphocytes, contributing to immune tolerance and a lower risk for T1D (Xie, Chang et al. 2014).

Moreover, Protein Tyrosine Phosphatase non-receptor 22 (PTPN22), another non-HLA gene, has risk polymorphisms that amplify the risk of establishing T1D (Blanter, Sork et al. 2019). PTPN22 encodes lymphoid tyrosine phosphatase (LYP), which is expressed in T cells and plays a preventative role in the activation of the immune system, by blocking T cell receptor (TCR) signaling, by dephosphorylation of three kinases int the TCR signaling pathway, and preventing the expansion of effector T cells (Blanter, Sork et al. 2019, Xie, Chang et al. 2014). It, also, positively regulates TLR-triggered IFN production in myeloid cells (Blanter, Sork et al. 2019). These two functions of PTPN22, demonstrate that it contributes to, both, adaptive and innate immune functions (Blanter, Sork et al. 2019).

Environmental causes for T1D

Environmental factors may contribute to the disease, as well, and collaborate with genetic factors to influence the development and progression of T1D (Xie, Chang et al. 2014). Dietary and microbial triggers are some of the environmental factors that might contribute to T1D (Rak, Bronkowska 2018). Viral infections are considered as probable triggers for T1D, especially those from the enterovirus family, that might initiate the process that leads to β-cell damage (Blanter, Sork et al. 2019, Rak, Bronkowska 2018, Xie, Chang et al. 2014). Risk factors from diet are wheat and cow's milk, but protective roles from breastfeeding and numerous nutrients have been demonstrated as well (Xie, Chang et al. 2014, Heinonen, Moulder et al. 2015). The environmental triggers can cause epigenetic changes, that regulate gene expression and affect immune cell function (Xie, Chang et al. 2014).

Treatment for T1D

As T1D patients' numbers increase there is an insurmountable need for discovering the treatment of this disease, in order to prevent or even reverse it. A cure for diabetes is not currently available, rendering the patient dependent on insulin injections forever. Diabetics have been relying in insulin injections for years upon years and even though it has increased their life expectancy, it is still briefer in comparison to that of the healthy population (Li, W., Huang et al. 2017).

Current treatments

Type 1 diabetics are, mostly, relying in insulin treatments, in conjunction with a healthy diet and daily exercise to keep their blood glucose in normal levels. Another therapy that is used, not so commonly, for the treatment of T1D is the pancreatic islet transplantation.

Insulin Treatments

People with T1D have the obligation to control their blood glucose, for them to be able to calculate the insulin dosage they need. The correct dosage will help them stay on healthy levels of blood glucose, without leading them to hypoglycemia or hyperglycemia, and in the long run it will keep them from developing comorbidities. For that purpose, scientists have developed and improved the glucose monitoring systems over time (Tauschmann, Hovorka 2018). There are two main types of glucose monitoring systems, the capillary blood glucose measurements and the continuous glucose monitoring systems (Tauschmann, Hovorka 2018). The first, is a hand-held portable meter that diabetics use multiple times per day, in conjunction with lancets and glucose test strips, to optimize diabetes control (Tauschmann, Hovorka 2018). The second type are small sensors that are implanted in the diabetics' bodies and measure interstitial glucose concentrations subcutaneously at small time intervals, using enzyme-tipped electrodes or fluorescence technology (Tauschmann, Hovorka 2018). HbA_{1C} is a clinically useful biomarker of glucose homeostasis that provides an overview of average blood glucose levels of approximately three months and is used to observe the blood glucose monitoring of the patients (Campbell, Pepper et al. 2019, Schnell, Crocker et al. 2017).

Insulin administration is made either with insulin pens or insulin pumps (Tauschmann, Hovorka 2018). Insulin pumps are usually used in combination with the continuous glucose monitoring systems. It is found that individuals that use insulin pumps over insulin pens, as well as individuals that use continuous glucose monitoring systems over the capillary way of measuring blood glucose, have lower HbA_{1C} (Tauschmann, Hovorka 2018).

Pancreatic Islet Transplantation

Another therapeutic strategy that has been on development since early 1970s, is the pancreatic islet transplantation or simply islet transplantation (Shapiro, A. M., Pokrywczynska et al. 2017, Rickels, Robertson 2019). Islet transplantation is a therapy that replaces β-cells (Shapiro, A. M., Pokrywczynska et al. 2017, Rickels, Robertson 2019). After forty years of trial and error,

pancreatic islet transplantation has become a routine clinical procedure with predictable results (Shapiro, A. M., Pokrywczynska et al. 2017). This technique is offered mainly to unstable type 1 diabetics, that have hypoglycemia unawareness, as well as multiple hypoglycemic episodes and thus are unable to control their diabetes with the classic methods that we discussed previously (Shapiro, A. M., Pokrywczynska et al. 2017). The technique that is used is that the doctors use islets with healthy β-cells from the pancreas of a deceased organ donor, that they then inject into a vein that carries blood to the liver of a person with type 1 diabetes (Alejandro, Baidal et al. 2018). The transplanted islets begin to create and discharge insulin in the patient's body (Alejandro, Baidal et al. 2018). For the patient to stop using insulin, multiple injections of the transplanted islet cells are needed (Alejandro, Baidal et al. 2018).

Islet transplantation is characterized as one of the safest and least invasive organ transplantation techniques (Shapiro, A. M., Pokrywczynska et al. 2017). However, islet transplantation has its disadvantages. One of the adverse events, and maybe the most serious, of the islet transplantation, is the need for chronic immunosuppression drugs receival by the patients (Shapiro, A. M., Pokrywczynska et al. 2017). Moreover, the supply of healthy pancreas islet cells from deceased donors is, also, limited (Shapiro, A. M., Pokrywczynska et al. 2017).

Emerging therapies

In consequence, the research community has resorted on new avenues to treat T1D, as several scientific teams are now fixated on establishing techniques for treating diabetes with the use of gene therapy, a route that seems to be very promising. Predominantly, gene therapy refers to the introduction of functional genes into the body for the treating of genetic disorders (Shu, Wang et al. 2015). According to the US Food and Drug Administration (FDA), "human gene therapy seeks to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use" (FDA 2018).

The main mechanisms that are utilized for gene therapy reasons, are replacing the unhealthy gene with a healthy copy, inactivating the unhealthy gene or introducing a new or modified gene into the body to help treat a disease (FDA 2018, Shu, Wang et al. 2015). The unhealthy gene usually causes the disease and, thus, it is not functioning properly. To facilitate this process, plasmid DNA, viral and bacterial vectors are used to deliver the functioning gene to the patient, as well as DNA and oligonucleotides, that are called delivery vehicles or vectors (Shu, Wang et al. 2015, FDA

2018). The optimal gene delivery vehicle should not interact with any vascular endothelial cells, it must be small enough to pass through the cell membrane and stable enough to reach the nucleus, to help the therapeutic gene to reach its target cells without the involvement of biodegradation, and be expressed (Shu, Wang et al. 2015).

Furthermore, gene therapy can be classified into two categories germ line and somatic gene therapy (Wirth, Parker et al. 2013). In the first, germ line gene therapy, the delivered gene will be passed on to the next generation, in contradiction with the somatic gene therapy where the delivered gene will not be passed on to the next generation (Wirth, Parker et al. 2013).

As diabetes type 1 is considered an autoimmune disease, gene therapy is considered to be the next potential intervention for the target specific and controllable characteristics (Shu, Wang et al. 2015). As discussed previously, many genes and proteins become under expressed so that T1D occurs. Gene therapy often tries to overexpress these proteins and genes in order to facilitate homeostasis of a T1D patient, as it is impossible to express genes via surgical or instrumental approaches (Chellappan, Sivam et al. 2018). Gene therapy's aim is to maintain amnear-normal blood glucose level, through an efficient, safe, and specific way (Chellappan, Sivam et al. 2018). Klotho, glucose-6-phosphatase, hepatocyte growth factor, regenerating islet-derived protein3 γ and insulin growth factor 1 genes are some of the genes that are researched as gene therapy targets throughout the years. Some of the important proteins that are, also, considered great targets for gene therapy of T1D, are Neurogenin-3, Betacellulin, pancreas duodenal homeobox-1 and leptin.

According to, *Shu, Wand et al*., animal models treated with gene therapy has proven to be reliable and competent research tool to cure multiple autoimmune diseases. Non-obese diabetic (NOD) mouse is one of the most frequently used animals to study Type 1 diabetes, as it seems to be very analogous to human T1D (Rees, Alcolado 2005). Its first symptom is insulitis, which is followed by subclinical β-cell destruction and a reduce in the circulating insulin concentrations (Rees, Alcolado 2005, Brito-Casillas, Melián et al. 2016). However, ketoacidosis in NOD mice is not as severe as in human T1D and the animals are able to survive without the use of insulin for weeks (Rees, Alcolado 2005). Another mouse that is used is the ob/ob that has a monogenic, autosomal, recessive mutation on chromosome 6 (Brito-Casillas, Melián et al. 2016). Ob/ob mouse is an overtly obese mouse due to the leptin mutation, that renders it hyperglycemic and mildly glucose intolerant (Brito-Casillas, Melián et al. 2016). Additionally, there is the use of induced diabetes

models that are generated via the administration of toxic substances with β-cell tropism, like streptozotocin (STZ) (Brito-Casillas, Melián et al. 2016). STZ can be administered at different doses, intervals and paths, intraperitoneal or intravenous (Brito-Casillas, Melián et al. 2016). When STZ is administered in several, small doses for a few days it causes a process that mirrors human T1D, with immune destruction and no insulin production, but without the autoimmune features (Brito-Casillas, Melián et al. 2016). Other models that are used are the RAG-1 and Akita mouse, also.

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OVERVIEW

Gene Therapy for Diabetes Type 1

As discussed previously, in order for T1D to occur several proteins and genes are underexpressed or overexpressed. Gene therapy aims to overexpress or delete, respectively, these affected proteins and genes, creating novel treatment methods for patients affected with T1D. After the discovery of potential genes and proteins that affect diabetes, methods for the delivery of those proteins and genes, in the patient's system are needed for the body to not reject the inserted proteins and genes.

The two main problems for developing such techniques, as to replace daily insulin injections via gene therapy, are efficacy and safety (Chen, Meseck et al. 2001). A need of an efficient and safe vector, along with the normal expression of insulin gene is fundamental (Chen, Meseck et al. 2001). The mechanisms that control the insulin expression need to be (a) adequate via having a sufficient production of insulin to induce normoglycemia and (b) safe via stopping excessive insulin supply to avert hypoglycemia (Chen, Meseck et al. 2001).

Overexpression of genes and proteins and their potential application of type 1 diabetes

Overexpression of genes

Insulin-Like Growth Factor-1

Insulin-Like Growth Factor (IGF) axis, and especially IGF-1 and IGF-2 are candidates for correcting the deficient immunoregulation and the impaired β-cell viability and function (Shapiro, M. R., Wasserfall et al. 2020). IGFs are hormones that are mainly produced by the liver and they induce cellular proliferation by the broadly expressed IGF-1 receptor (Shapiro, M. R., Wasserfall et al. 2020). IGF-1 and IGF-2 have very similar structure with insulin and, thus, can mediate homologous metabolic effects, even though they are unable to make up for the loss of insulin production in T1D patients (Shapiro, M. R., Wasserfall et al. 2020). The difference between insulin and IGF-1, is that the seconds C chains are not separated, and it has an extension of the A chain, called the D domain (Raman, Singal et al. 2019). IGF-1 mediates the growth-promoting effects of growth hormone (GH) to children and the anabolic effects of GH in adults (Chisalita, Ludvigsson 2018). The upregulated GH receptors as well as the increased IGF-1 production causes insulin to

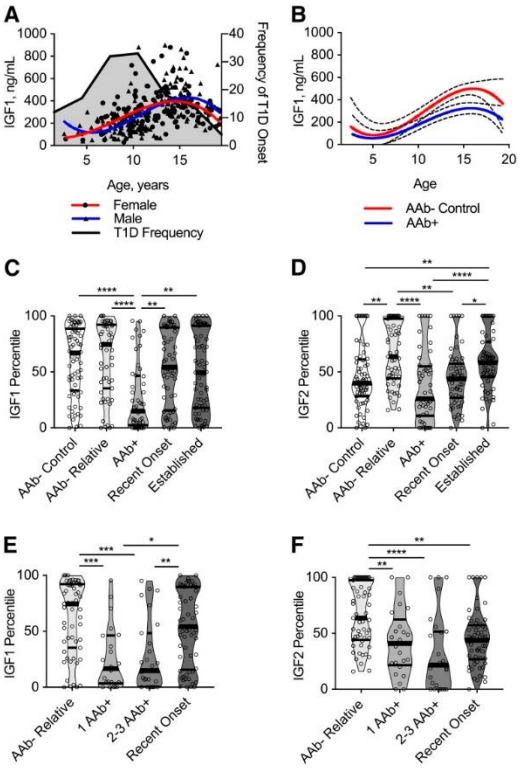


Figure 1 IGF1 and IGF2 levels decline significantly in AAB+ individuals at high risk for T1D onset. (Shapiro, Wasserfall et al. 2020)

enhance the sensitivity of the liver to the growth hormone (Chisalita, Ludvigsson 2018). IGF-1 is bound by the six IGF binding proteins (IGFBP 1-6), that modulate its biological activity (Chisalita, Ludvigsson 2018, Nambam, Schatz 2018). IGFBP-3's, the major binding protein, role is to prolong IGF-1 half-life, acting as a natural store (Chisalita, Ludvigsson 2018). IGFs promote the regulation of T-cell mediated inflammation and they, also, support endocrine and exocrine pancreatic growth (Shapiro, M. R., Wasserfall et al. 2020).

In a study conducted by Shapiro, Wasserfall et al., in 2020, the hypothesis that IGF1 levels or bioavailability, that are related to IGFBPs, are changed during the progression to T1D was tested. Pre-T1D can be separated in two steps, the first being seroconversion to two or more disease-related autoantibodies, called AAb, against β-cell antigens, whereas the second occurs when many AAb+ subjects exhibit dysglycemia (Shapiro, Wasserfall et al. 2020). The scientific team found that IGF-1 levels peaked during puberty (Figure 1A), but the AAb+ subjects had lower levels of IGF-1 in comparison with control groups of AAb- individuals (Figure 1B) (Shapiro, M. R., Wasserfall et al. 2020). When the bioavailability of the IGF was tested and normalized for age and sex, the results showed low levels of IGF1 and IGF2 in AAb+ individuals, whereas a recovery was observed in individuals with established T1D (Figure 1C and D) (Shapiro, M. R., Wasserfall et al. 2020). Unexpectedly, these low levels of IGF1 and IGF2 were observed not only in high risk individuals, with multiple AAB, but, also, within the low risk subjects, only positive for single AAB (Figure 1E and F) (Shapiro, M. R., Wasserfall et al. 2020). Later on, they wanted to check how IGF1 levels responded through the progression of T1D, in both children and adults (Figure 2A) (Shapiro, M. R., Wasserfall et al. 2020). In

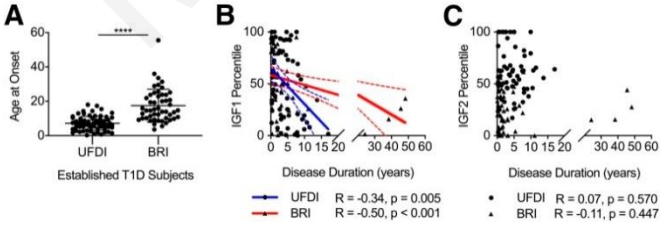


Figure 2 IGF1 levels decreases with the increased duration of T1D. UFDI is the children's group and BRI the adult's group. (Shapiro, Wasserfall et al. 2020)

the lower-aged cohort the loss of IGF-1 was very abrupt, whereas in the BRI cohort it was a gradual one (Figure 2B) (Shapiro, M. R., Wasserfall et al. 2020). Nonetheless, in both cohorts IGF-1 levels dropped as the disease was progressing (Shapiro, M. R., Wasserfall et al. 2020). C-peptide levels, as well as IGF-1 levels decreased after the diagnosis in the patients, associating them (Shapiro, M. R., Wasserfall et al. 2020). However, IGF2 levels did not seem to have an association with the disease duration (Figure 2C) (Shapiro, M. R., Wasserfall et al. 2020).

In 2017, a study by Mallol, Casana et al. showcased that local expression of IGF-1 in β-cells, in non-obese diabetic (NOD) mice can help suppress the development or onset of T1D. Firstly, in their study it was clearly shown that in the transgenic NOD-IGF1 mice, the mass and functionality of β-cells are preserved via the reduction of the population of apoptotic β-cells (Mallol, Casana et al. 2017). Later on, the scientists experimented with IGF1 and gene therapy in NOD mice (Mallol, Casana et al. 2017). They delivered the IGF1 gene, with the help of Adeno-Associated Viruses (AAV) of serotype 8 and used microRNAs (miR) (AAV8-IGF1-dmiRT), in order to deliver the gene only in the pancreas and not in the liver and heart (Mallol, Casana et al. 2017). The gene therapy, with the created vector, outcomes demonstrated that NOD mice were rescued from development of autoimmune diabetes, they had preserved their β-cell mass and had normal levels of circulating insulin (Figure 3) (Mallol, Casana et al. 2017). The therapeutic results could stop the disease onset even when the therapy was done at 11 weeks of age (Figure 3D and E), when the mice begun to turn

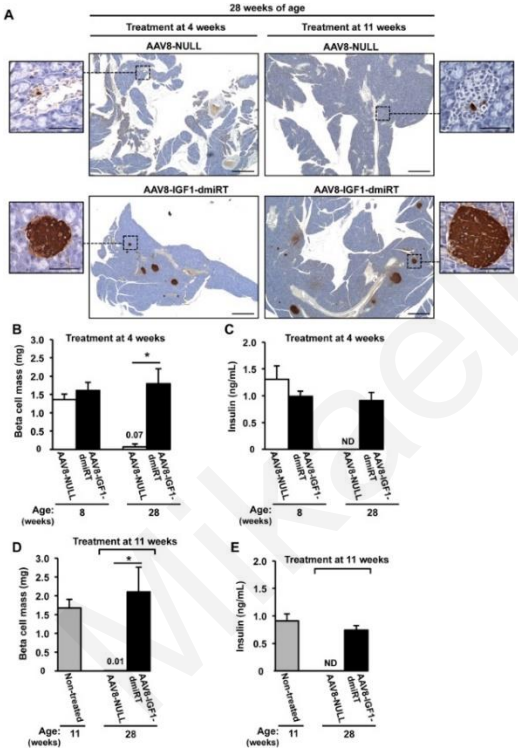


Figure 3 IGF1 expression in the pancreas maintains circulating insulin levels and β-cell mass. (Mallol, Casana et al. 2017)

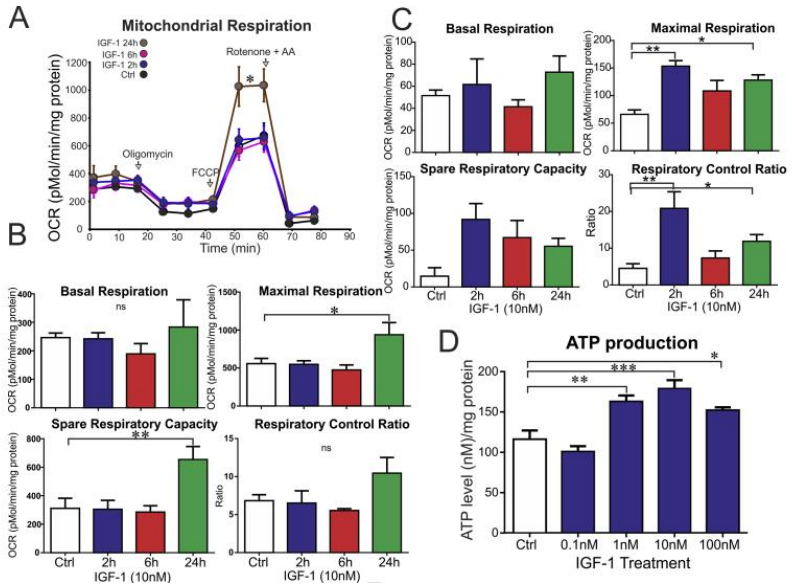


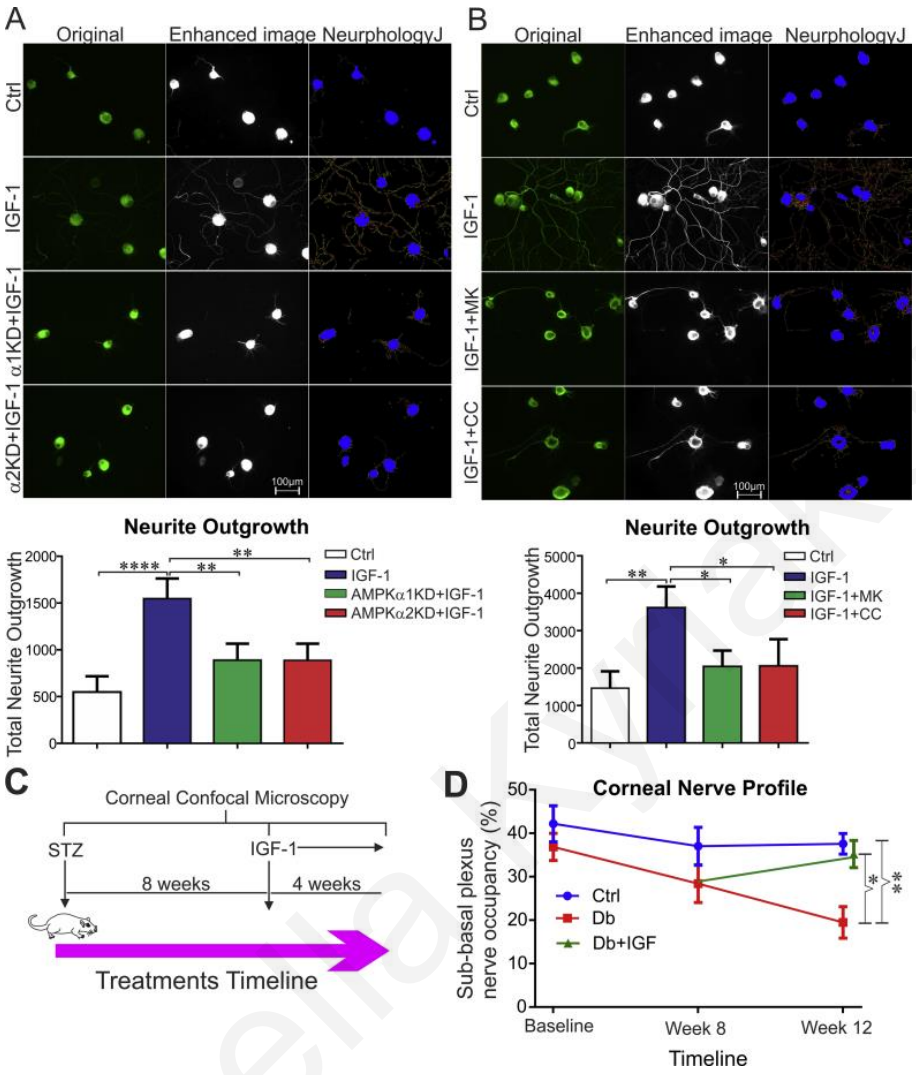
Figure 4 IGF1 upregulates ATP production and mitochondrial respiration. (Aghanoori, Smith et al. 2019)

hyperglycemic, even though the therapeutic benefits were greater at the younger age of 4 weeks (Figure 3B and C) (Mallol, Casana et al. 2017).

IGF-1 receptor mobilizes the Akt/ phosphoinositide-3 kinase (PI-3K) and the mitogen-activated protein kinase (MAPK) pathways, mediated by insulin receptor substrate (IRS) 1 and 2 phosphorylation after the ligand binding (Aghanoori, Smith et al. 2019). The role of the Akt/PI-3K pathway is to activate the mammalian target of rapamycin pathway, that leads direct protein synthesis and cell growth via downstream effectors (Aghanoori, Smith et al. 2019). Whereas MAPK pathway role is to activate extracellular signal-regulated kinase (ERK)-1/2 and to target transcription factors in order to regulate the survival of the cells (Aghanoori, Smith et al. 2019).

In the study of Aghanoori, Smith et al., exogenous IGF1 was used as treatment in diabetic rats and their cells in in vitro and in vivo experiments. Their results exhibited that IGF-1 treatment could activate and upregulate AMPK pathway to amplify the mitochondrial function (Figure 4A, B and C), ATP production (Figure 4D), mtDNA copy number, as well as the expression of electron transport system proteins in the dorsal root ganglia neurons of the rats, that were cultured (Aghanoori, Smith et al. 2019). The topical delivery of IGF1 in the diabetic rat's eye, blocked the

Figure 5 IGF1 prevents the loss of corneal nerves and AMPK /AKt inhibitors abolish the regeneration caused by IGF1. (A) DRG neurons from rats transfected with AMPKα1 or α2- specific siRNAs, cultivated for 24hours or (B) pretreated with inhibitors for 2 hours and treated with or without IGF1. (C) timeline for topical delivery of IGF1 to the eye of STZ-diabetic mice. (D) line charts indicating nerve density. (Aghanoori, Smith et al. 2019)



progressive loss of the corneal nerve (Figure 5A and B) (Aghanoori, Smith et al. 2019). Moreover, in their research Aghanoori, Smith et al., have found that IGF1 treatment of the cultured dorsal root ganglia enhanced the expression of some dysregulated genes of the area and it, also, upregulated the AMPK and Akt functional isoforms. However, in this study some of the effects of the exogenous IGF-1 were found to be blocked by several factors, like the Akt inhibitor, MK-2206, that abolished the enhancement of mitochondrial function and the neurite outgrowth (Figure 5) (Aghanoori, Smith et al. 2019).

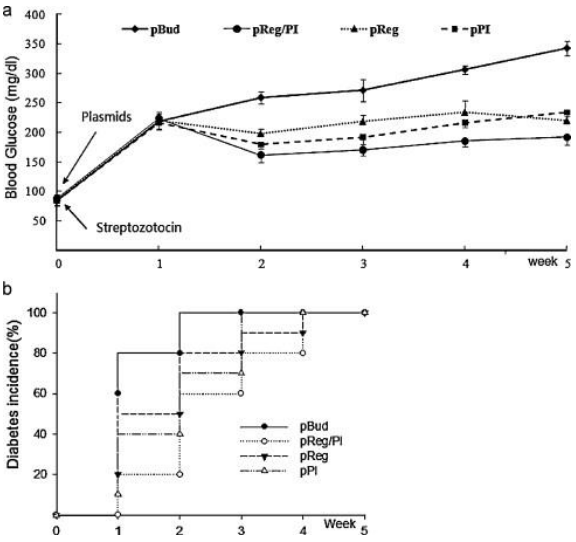


Figure 6 The constructed plasmids improved hyperglycemia. (top panel) blood glucose levels, (bottom panel) diabetes incidence. (Hou, Xie et al. 2011)

Regenerating islet-derived protein 3 γ gene

According to Xia, Cao et al., Reg gene's role in the development of diabetes is to encode an endogenous lectin that is implicated in the regeneration or the growth of β-cells. Reg gene forms a Reg family, Reg1-4, that is classified by the primary structures of the proteins, that all have a conservative sequence with C-peptide leptins (Xia, Cao et al. 2016, Parikh, Stephan et al. 2012). It is, also, crucial in the regeneration of islet β-cells during pancreatic inflammation damage (Xia, Cao et al. 2016).

A research that delivered regenerating islet-derived protein 3 γ gene (Reg3g) and proinsulin intramuscular, via naked Plasmid DNA, has shown a reduction in diabetes incidence as well as a postponed onset of T1D (Figure 6) (Hou, Xie et al. 2011). Furthermore, the therapy resulted in promotion of CD4+CD25+Foxp3+Treg cells, downregulation of Th1 cytokines and upregulation Th2 cytokines (Figure 7) (Hou, Xie et al. 2011). Th1 cytokines play a role in β-cell demolition, while Th2 cytokines involve in protecting β-cells (Hou, Xie et al. 2011). In addition, apoptosis and insulitis in the pancreas were languished in mice treated with Reg3g and proinsulin (Figure 8) (Hou, Xie et al. 2011). Lastly, this gene therapy led to lower levels of NF-κB in the pancreas and higher insulin contents (Figure 11, Figure 12) (Hou, Xie et al. 2011).

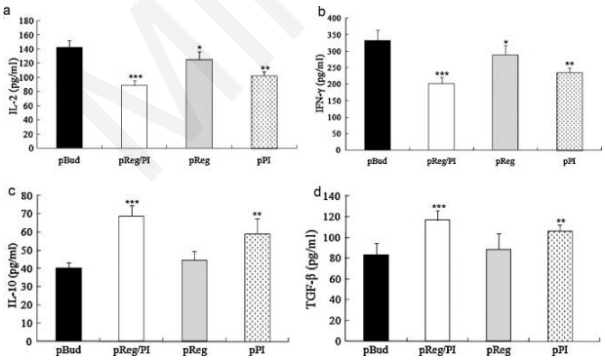


Figure 7 Levels of Th1 and Th2 cytokines in plasmid treated mice. (a) levels of IL2, (b) IFN-γ, (c) IL10 and (d) TGF-β in the serum. (Hou, Xie et al. 2011)

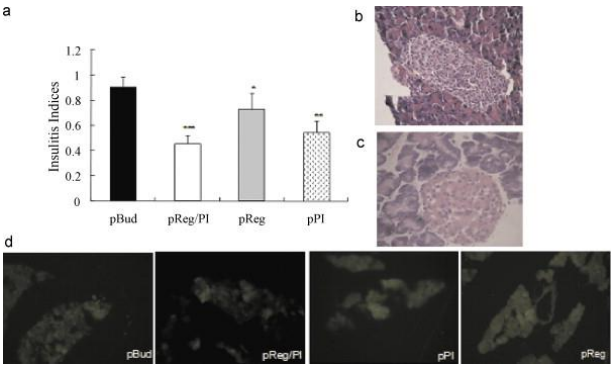


Figure 8 Reg3γ and proinsulin rescue pancreatic cells from apoptosis and insulitis. (Hou, Xie et al. 2011)

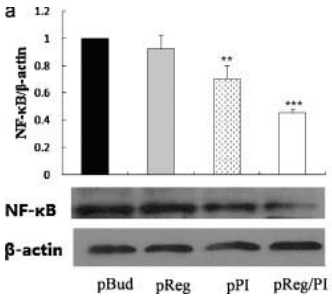


Figure 12 NF-κB levels in plasmid treated mice. (Hou, Xie et al. 2011)

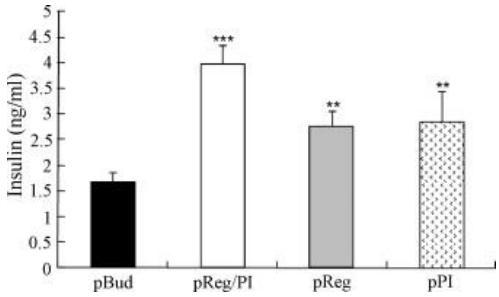


Figure 11 Serum insulin levels of plasmid treated mice. (Hou, Xie et al. 2011)

In 2016, a research showed evidence that treatment with Reg3g had delayed the onset of hyperglycemia in NOD mice (Figure 9) (Xia, Cao et al. 2016). Xia, Cao et al. expressed Reg3g with lentivirus vector to pancreatic cells and with intraperitoneal injections to augment the delivery to the pancreas, which is considered as a site-directed delivery (Xia, Cao et al. 2016). Moreover, the Reg3g treatment helped to decrease the lymphocytic infiltrate and augment β-cell regeneration, driving to rehabilitated insulin production (Figure 10) (Xia, Cao et al. 2016). Additionally, CD4+CD25+FoxP3+Treg cells abundance seemed enhanced in NOD mice treated with Reg3g (Xia, Cao et al. 2016). CD4+CD25+T cells protect target organs from autoimmune disease and sustain the immune tolerance (Xia, Cao et al. 2016). The JAK2, STAT3, NF-κB pathway was enhanced, when Reg3g was overexpressed, leading to the increased regeneration of islets (Xia, Cao et al. 2016).

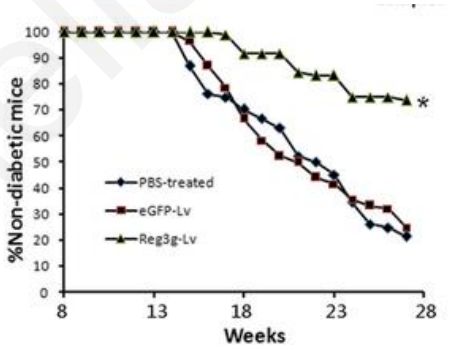


Figure 9 Reg3g prolonged the onset of diabetes in female NOD mice) (Xia, Cao et al. 2016).

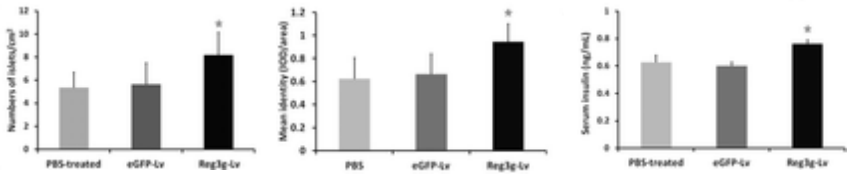


Figure 10 Islet numbers and insulin levels were increased in Reg3g treated mice) (Xia, Cao et al. 2016).

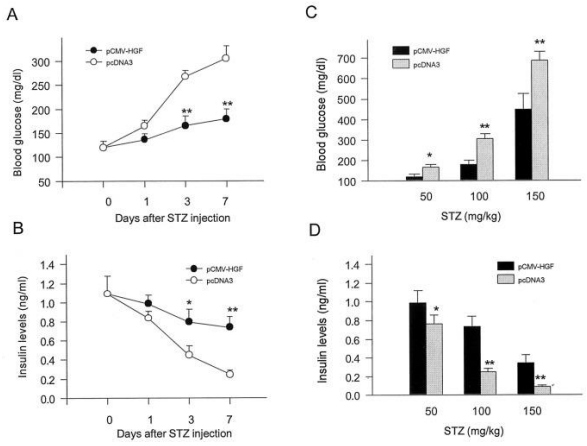


Figure 13 HGF rescued mice from high blood glucose and induced insulin production (Dai, Li et al. 2003).

Hepatocyte growth factor gene

Hepatocyte growth factor (HGF) is a multifunctional cytokine, which displays several glucoregulatory and anti-diabetic properties, as it is greatly involved in the physiology of the pancreas (Gaddy, Riedel et al. 2012, Matsuda, Obama et al. 2021). Moreover, HGF is known for its antiapoptotic effects, as it activates AKT kinase to inhibit the apoptotic pathway (Wu, Yoon et al. 2011).

A therapy encoding HGF gene driven under a cytomegalovirus promoter was conducted in 2003 by Dai, Li et al. In this study, HGF treated mice had lower hyperglycemia and higher expression of insulin in their islets in comparison with control diabetic mice (Figure 13) (Dai, Li et al. 2003). Moreover, HGF treatment seemed to positively affect the β-cell numbers that could produce insulin (Figure 15) and prevented cell apoptosis (Figure 14) (Dai, Li et al. 2003). Proliferating cell nuclear antigen, a marker for G1 to S phase transition, was increased a few hours after STZ injection in mice, indicating the islet proliferation that HGF injection caused (Dai, Li et al. 2003). The pro-survival protein Bcl-xL, β-cell lymphoma extra-large, was overexpressed in a time-dependent manner in diabetic mice treated with HGF plasmid, preventing cell apoptosis (Dai, Li et al. 2003).

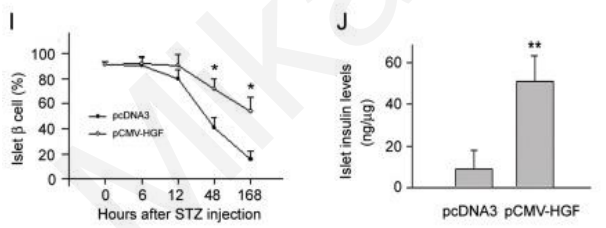


Figure 15 HGF preserves β-cell mass and insulin production (Dai, Li et al. 2003).

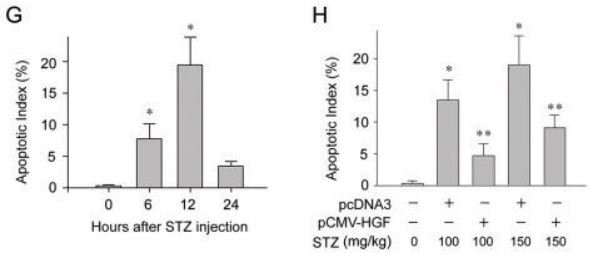


Figure 14 HGF ameliorates apoptosis (Dai, Li et al. 2003).

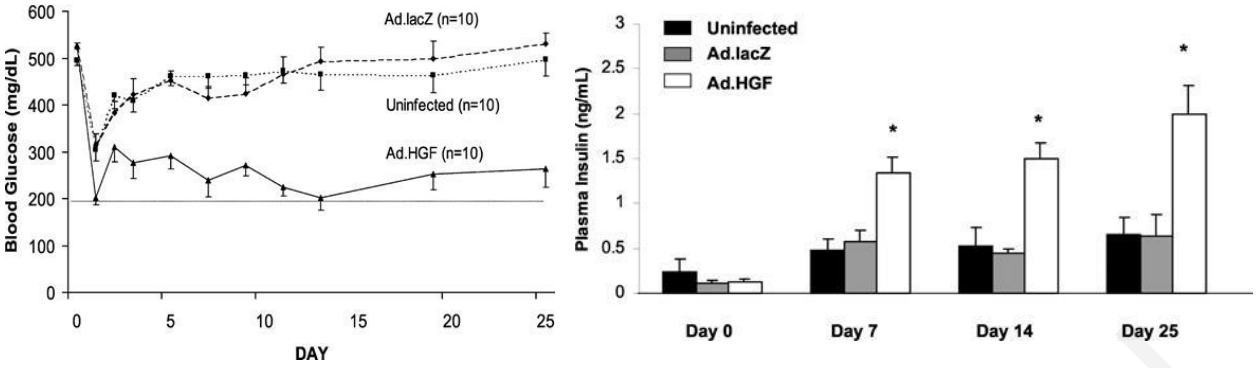


Figure 18 Blood glucose levels (left panel) and plasma insulin levels (right panel) of HGF gene therapy of pancreatic islets in islet transplanted and immunosuppressed rat models (Lopez-Talavera, Garcia-Ocaña et al. 2004).

In 2004, Lopez-Talavera, Garcia- Ocaña et al., used adenoviral vector to deliver HGF gene to rats that developed diabetes after the combinational use of immunosuppressive drugs. Their results showed that the rat's transplanted islets had enhanced function (Figure 17), as well as better glucose control (Figure 18) (Lopez-Talavera, Garcia-Ocaña et al. 2004). A treatment with HGF prior to the transplantation, had lowered the blood glucose values of the rats almost to normal levels (Figure 18) (Lopez-Talavera, Garcia-Ocaña et al. 2004). Their results clearly exhibited the possibility of HGF gene therapy to improve the effectiveness of portal pancreatic islet allografts for the treatment of T1D (Figure 16) (Lopez-Talavera, Garcia-Ocaña et al. 2004).

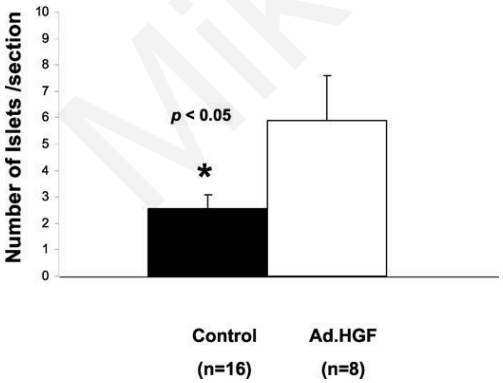


Figure 17 Number of islets per liver section in control vs. HGF treated rats (Lopez-Talavera, Garcia-Ocaña et al. 2004).

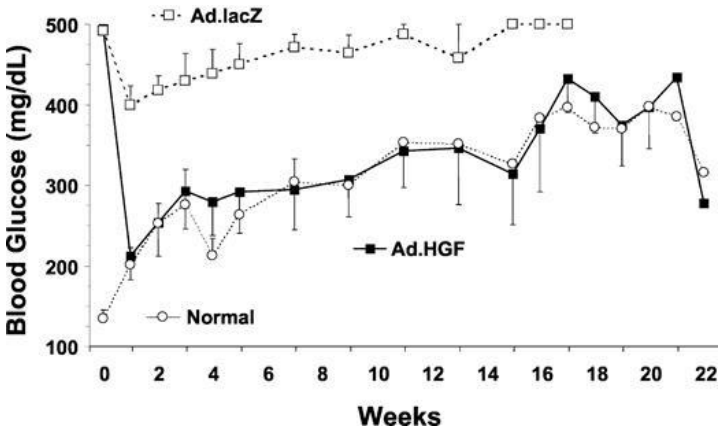


Figure 16 Long-term function of islet allografts as assessed by blood glucose values in normal, HGF-treated and empty vector treated rats, treated with Edmonton immunosuppressive drugs (Lopez-Talavera, Garcia-Ocaña et al. 2004).

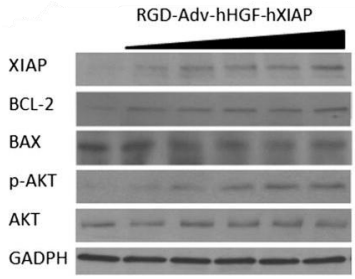


Figure 19 HGF and XIAP expression led to upregulation of BCL-2 and p-AKT in a dose dependent manner (Wu, Yoon et al. 2011).

Wu, Yoon et al., constructed an Arginine-Glycine-Asparagine modified adenovirus that delivered X-linked inhibitor of apoptosis (XIAP) and HGF genes, into human islets (Wu, Yoon et al. 2011). Gene therapy with these two genes increased the expression of both genes in the transduced islets, implying a better transduction ability, with their expression after therapy being higher than their endogenous expression prior to the therapy (Wu, Yoon et al. 2011). In western blots, it was shown an upregulation of p-AKT and BCL2, antiapoptotic markers, in a dose-dependent manner, indicating the antiapoptotic impact of the two genes on the human islets (Figure 19) (Wu, Yoon et al. 2011). Additionally, the overexpression of HGF and XIAF, rescued the cells and the islets from the apoptotic death that was induced by the addition of inflammatory cytokines, revealing the enhanced function and viability of the β-cells that produce insulin in the transduced human islets (Figure 20) (Wu, Yoon et al. 2011). Moreover, the transplantation outcomes were greater via the gene therapy, with better mean blood glucose levels, extended period of normoglycemia and, lastly, the diabetes being reversed in mice (Figure 22) (Wu, Yoon et al. 2011). After a glucose tolerance test, it became clear that the transduction of islets with HGF and XIAP genes, helped the islets to better respond to high blood glucose (Figure 21) (Wu, Yoon et al. 2011). After an extended period of time, the transduced islets seemed to be stable morphologically and the function of the islets remained intact (Wu, Yoon et al. 2011).

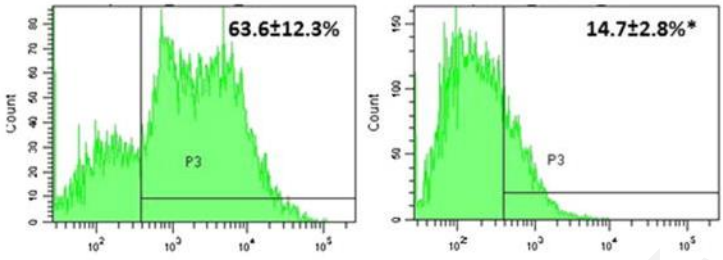


Figure 20 HGF and XIAP expression protects human islets from apoptotic cell death. P3 indicates the percentage of apoptotic cells. Left are the untransduced and right are the RGD-Adv-hHGF-hXIAP islets (Wu, Yoon et al. 2011).

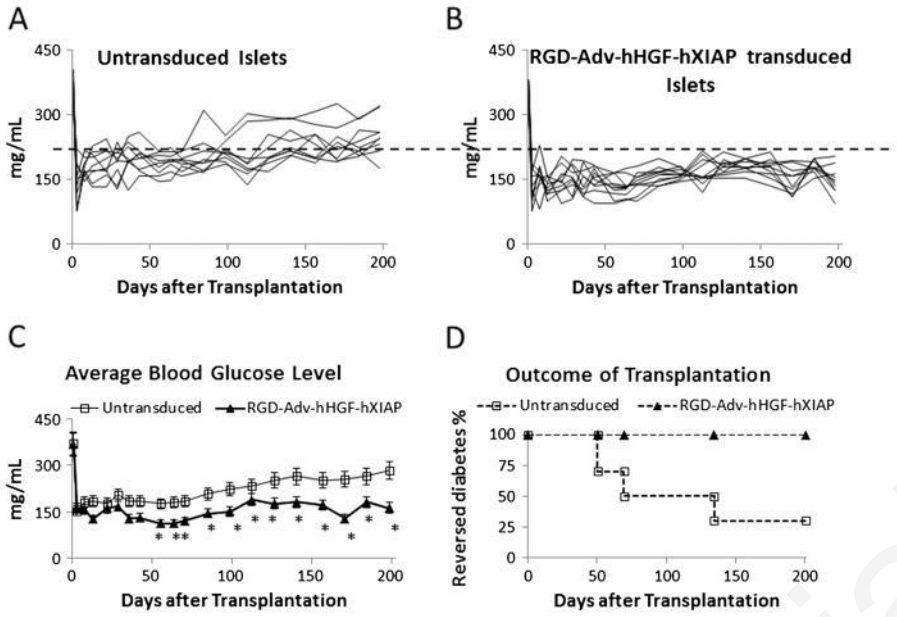


Figure 22 The transduction impact of RGD-Adv-hHGF-hXIAP on islet transplantation. Blood glucose level of mice receiving (A) untransduced or (B) RGD-adv-hHGF-hXIAP islets, (C) average blood glucose levels of mice and (D) reversed-diabetes ratio, ≤200 mg/dl, of mice. (Wu, Yoon et al. 2011).

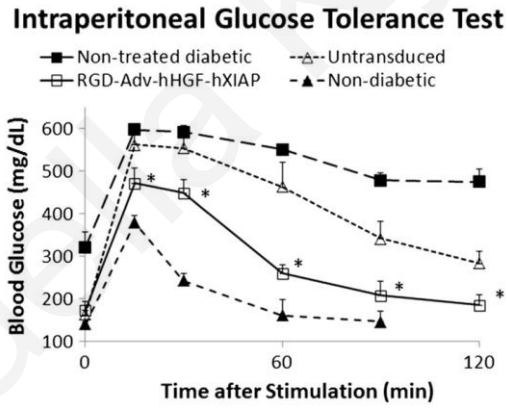
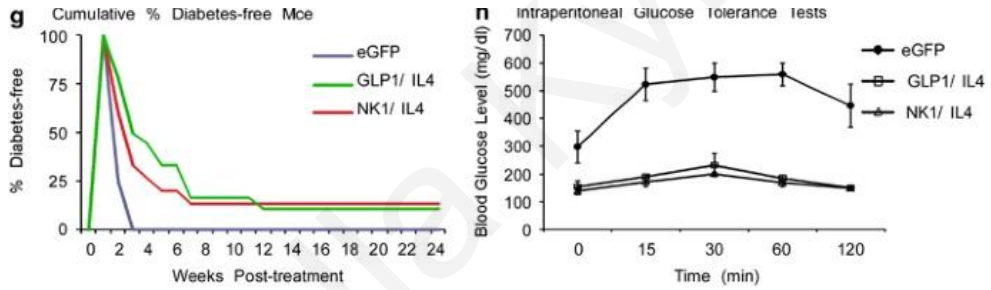


Figure 21 Glucose tolerance test 30 days after transplantation (Wu, Yoon et al. 2011).

In a study performed in 2012 by Gaddy, Riedel et al., a multigene transfer therapy was explored (Gaddy, Riedel et al. 2012). The scientists delivered the N-terminal NK1 domain of HGF, which exhibits partial activity of the full HGF without being associated with neoplasia formations, in conjunction with IL-4 cytokine with the help of adeno-associated virus serotype 8 (dsAAV8) (Gaddy, Riedel et al. 2012). Approximately 10% of the diabetic mouse that were treated had been cured of diabetes and 29% had shown extended disease-free responses (Figure 23) (Gaddy, Riedel et al. 2012). Moreover, insulitis incidence was reduced in NOD mice treated with NK1 and IL-4, with only 9% of the population displaying severe insulitis (Figure 24a, b) (Gaddy, Riedel et al. 2012). The NOD mice had augmented β-cell mass after the combined gene treatment (Figure 24c), which was attributed to higher proliferation of β-cells and/or suppression of β-cell apoptosis (Gaddy, Riedel et al. 2012).

Figure 23 NK1/IL4 combinational therapy, rescues diabetic NOD mice from hyperglycemia. (g) Diabetes-free mice percentage of, <280mg/100ml blood glucose levels. (h) Glucose tolerance test on 42-week-old NOD mice that were euglycemic after the co-therapy. (Gaddy, Riedel et al. 2012)



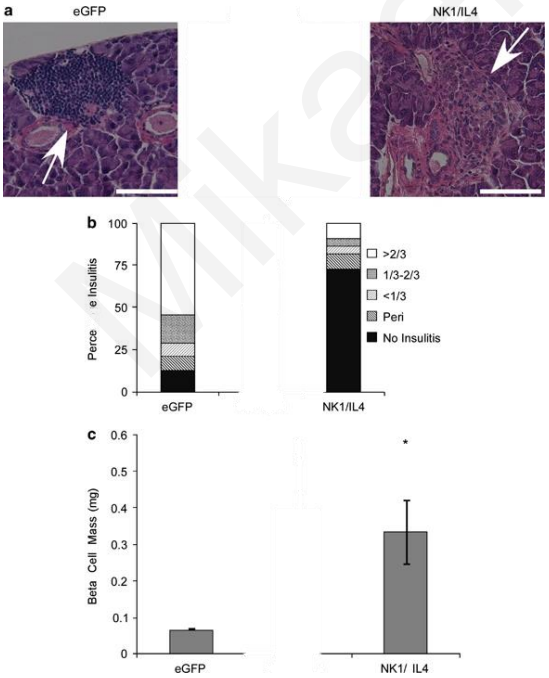


Figure 24 Immunohistochemical analysis of infiltrating leukocytes in NOD mice. (a) H&E staining of pancreatic sections, non-age-matched diabetic control mice treated with dsAAV-eGFP (left panel), combination treated mice (right panel), arrows indicate individual islets. (b) Insulitis scores of dsAAV-eGFP (left panel), combination treated mice (right panel). (c) β-cell mass of the mice. (Gaddy, Riedel et al. 2012)

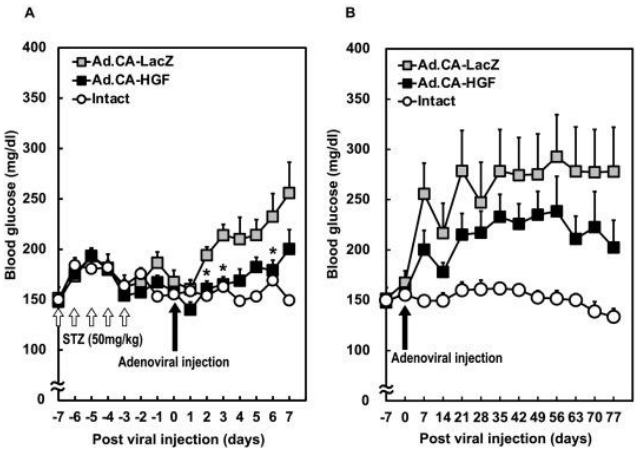


Figure 26 HGF treatment corrects STZ-induced hyperglycemia. (A) Blood glucose levels 7 days before and 7 days after adenoviral injection. Ad.CA-HGF significantly corrected blood glucose levels compared to empty vector treated mice. (B) The effect remained but it was not statistically significant. (Matsuda, Obama et al. 2021)

In 2021, a study examined the impact of treatment with adenoviral vectors that expressed the HGF gene in diabetic mice (Matsuda, Obama et al. 2021). The treatment seemed to decrease the blood glucose concentration of the mice and this effect remained in some extend (Figure 26) (Matsuda, Obama et al. 2021). As expected, it was shown that the therapeutic effect was because of the survival of insulin producing β-cells (Figure 25 left) (Matsuda, Obama et al. 2021). Lastly, the scientists wanted to check whether the low dose of HGF therapy was safe and would not induce hepatotoxicity to the liver. Indeed, one injection of an adenoviral mediated HGF treatment was both safe and effective (Figure 25 right) (Matsuda, Obama et al. 2021).

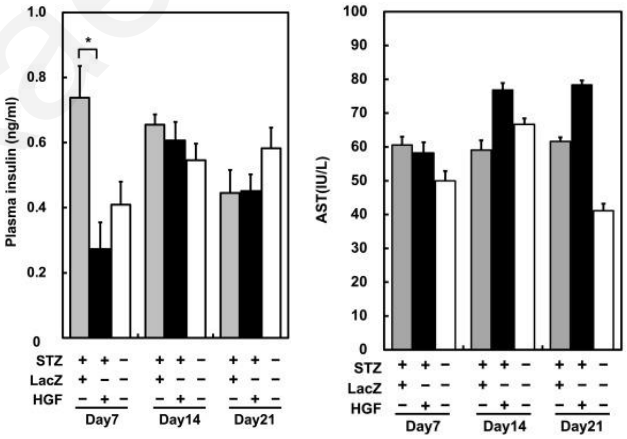


Figure 25 Plasma insulin levels (left panel) and AST levels (right panel) in STZ-injected Ad-treated mice. (left panel) On day 7 HGF was able to attenuate the increase in plasma insulin but not on days 14 and 21. (right panel) HGF did not induce any hepatotoxicity compared to the other two groups. (Matsuda, Obama et al. 2021)

Glucose-6-phosphate gene

Glucose-6-phosphatase (G6Pase) is an enzyme, that produces endogenous glucose in the liver, kidney and intestines, and catalyzes the hydrolysis of G6P to glucose in the last step of gluconeogenesis and glycogenolysis (Kim, Lee et al. 2015). It is formed by a kinase that acts on glucose (ROSE, O'CONNELL 1964).

In vitro experiments with transduced rat hepatoma cells, G6Pase activity seemed to be enhanced when aldolase B enhancer was incorporated and its insulin and glucose responsiveness were maintained (Chen, Meseck et al. 2001). When G6Pase was introduced in diabetic rats, in vivo, via adenoviral vectors their hyperglycemia decreased, even though it did not reach normal levels, while their body weight increased, reaching that of the nondiabetic control rats (Chen, Meseck et al. 2001). Later, comparing non-diabetic rats with diabetic rats and G6Pase treated animals, the scientists had observed that in the vector-treated rats, insulin was responsive to the blood glucose levels, although not at the same level as the non-diabetic controls' (Figure 28) (Chen, Meseck et al. 2001).After a glucose tolerance testing, the treated rats had not proceed to hypoglycemia levels and they reached normal glycemic stage after approximately 8 hours of fasting, better results than the diabetic rats but worse than the control rats (Figure 27) (Chen, Meseck et al. 2001).

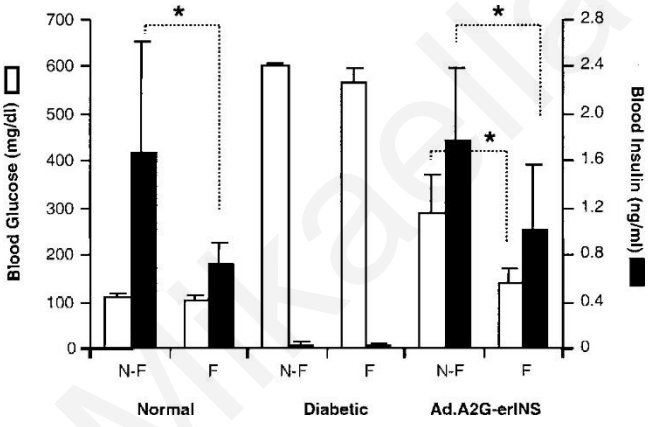


Figure 28 Insulin production in response to glucose levels. N-F, non-fasting conditions; F, fasting conditions. (Chen, Meseck et al. 2001)

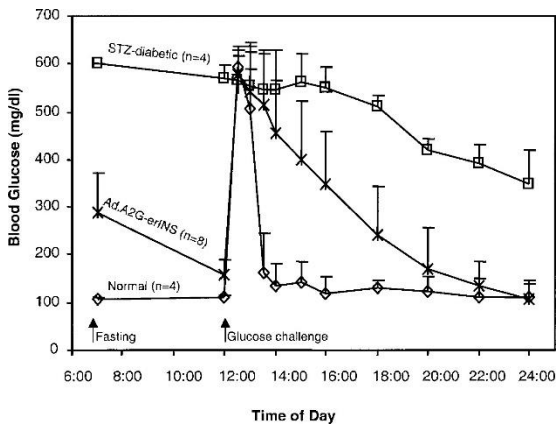


Figure 27 Blood glucose levels after a glucose tolerance test. Normal non-diabetic rats, Ad.A2G-erINS rats and diabetic controls. (Chen, Meseck et al. 2001)

Klotho gene

Klotho gene was recently recognized as a putative antiaging-suppressor gene (Lin, Sun 2015). This gene is expressed mainly in the kidneys and brain choroid plexus (Lin, Sun 2015, Lin, Sun 2012). In kidneys, Klotho gene is generated in full length of 130kDa and short-form of 65kDa, with only the short-form being expressed in pancreatic β-cells (Lin, Sun 2015).

Lin and Sun in 2015, explored how Klotho gene delivery could affect β-cells in NOD mice. When they compared wild type mice with half Klotho deficiency mice, that were treated with multiple low doses of STZ, they discovered that Klotho half deficiency led to higher blood glucose and lower glucose tolerance, lower plasma insulin and higher glucose levels in urine (Lin, Sun 2015). Furthermore, Klotho deficient mice had less insulin positive cells and higher percentage of apoptotic cells, in the pancreatic islets, in comparison to wild type mice (Lin, Sun 2015). Later on, a treatment of wild type mice with Klotho gene, that were post injected with STZ, prevented the hyperglycemia, the glucose intolerance and stopped the decrease of plasma insulin levels, in comparison with wild mice injected with STZ (Figure 29) (Lin, Sun 2015). Moreover, the increased klotho protein in the pancreas exhibited the effective gene delivery of Klotho (Lin, Sun 2015). The gene therapy prevented the aggravation of insulin storage and population of β-cells in the islets (Lin, Sun 2015). The expression of Klotho gene in MIN6 β-cells stopped apoptosis, that was caused from STZ and TNF-α, and augmented cell adhesion to collagen IV (Lin, Sun 2015). Klotho therapy upregulated the phosphorylation of FAK and Akt pathway, an adhesion signaling and a survival signaling respectively (Figure 30) (Lin, Sun 2015). Klotho, also, suppressed the expression of integrin β1 and constrained caspase 3 activity, an apoptotic pathway (Figure 30) (Lin, Sun 2015). Integrin β1 is a receptor of extracellular matrix, and blocking it stopped the

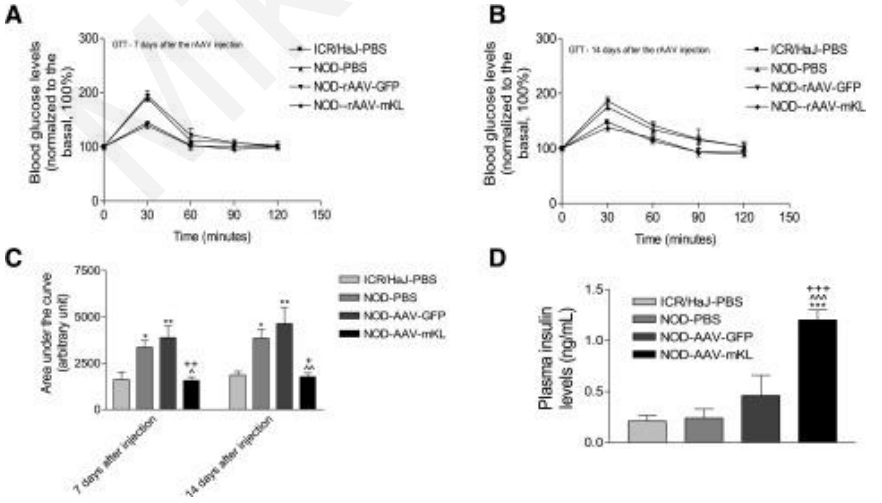


Figure 29 Expression of Klotho augmented glucose tolerance and increased plasma insulin levels. Glucose Tolerance Test at (A) 7 days and (B) 14 days. (C) Area under the curve for the glucose tolerance tests results. (D) Plasma insulin levels. (Lin, Sun 2015)

antiapoptotic action of Klotho as well as the promotion of cell adhesion to collagen IV, explaining the requirement of integrin β1 by Klotho gene for these two activities in MIN6 cells (Lin, Sun 2015). Furthermore, integrin β1 is needed by Klotho for phosphorylation of Akt and FAK, as well as for inhibition of caspase 3 cleavage (Lin, Sun 2015). Gene therapy with Klotho in NOD mice annulled glucose intolerance and increased plasma insulin levels (Lin, Sun 2015). Finally, it decreased the percentage of CD3 cells and apoptotic cells in NOD mice (Lin, Sun 2015).

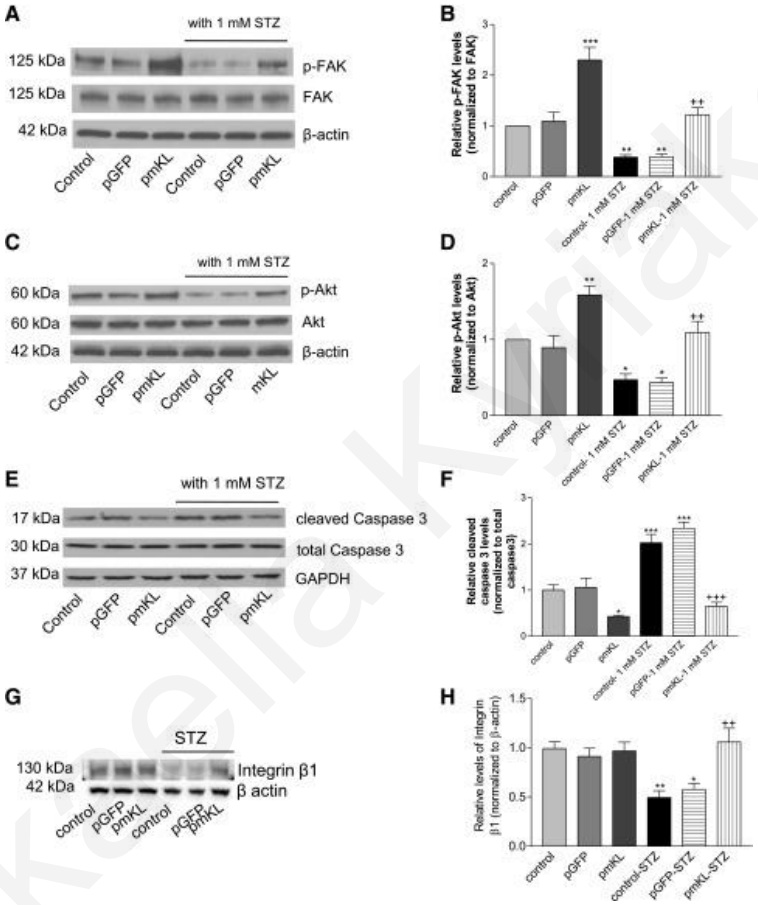


Figure 30 Expression of Klotho augmented phosphorylation of Akt and FAK and diminished caspase 3 schism. (A) Western blot of FAK. (B) Quantification of phosphorylation of FAK (C) Western blot of Akt. (D) Quantification of phosphorylation of Akt. (E) Western blot of Caspase 3 cleavage. (F) Quantification of caspase 3 cleavage. (G) Western blot of Integrin β1. (H) Quantification of Integrin β1. (Lin, Sun 2015)

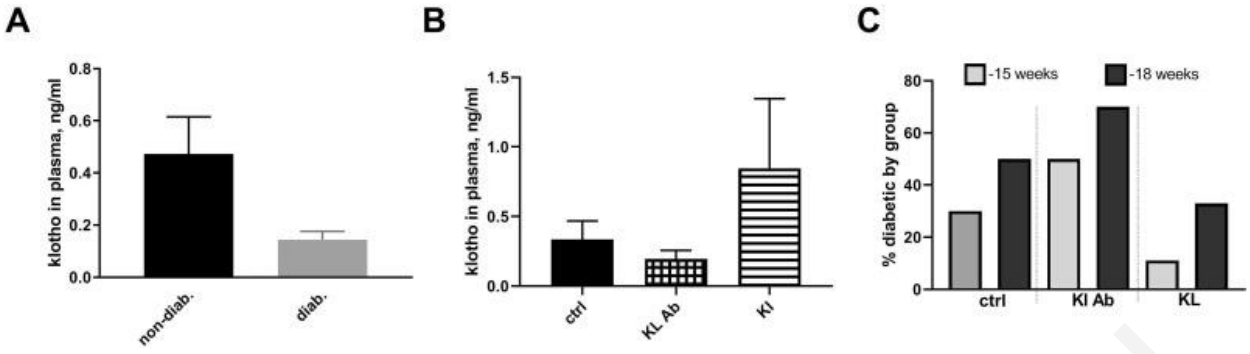


Figure 33 s-Klotho levels in plasma and diabetes incidence. (A) s-Klotho levels of diabetic and non-diabetic NOD mice. (B) s-Klotho administration increased klotho levels, while Klotho-Ab decreased Klotho levels. (C) Diabetes incidence at 15- and 18-weeks old mice. (Prud'homme, Glinka et al. 2020)

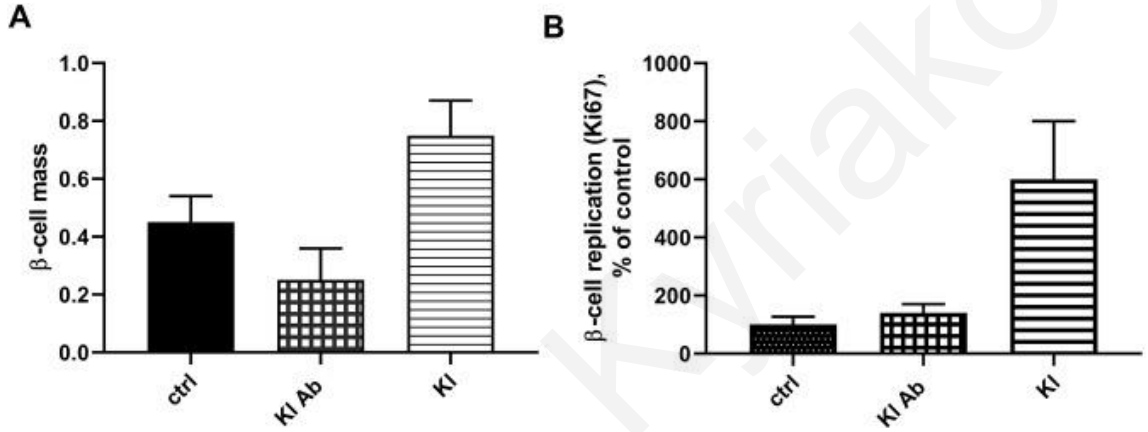


Figure 32 β-cell mass and β-cell replication in treated NOD mice. (A) β-cell mass in control mice vs Klotho Ab treated and s-Klotho treated. (B) β-cell replication. (Prud'homme, Glinka et al. 2020)

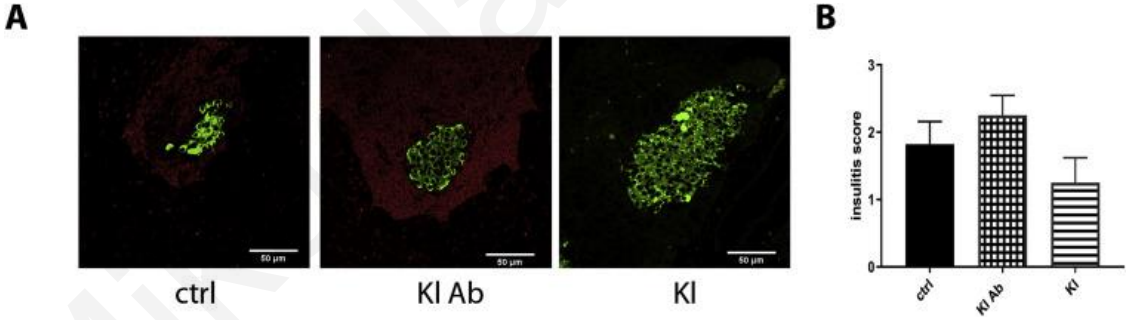


Figure 31 s-Klotho treatment mitigated insulitis in NOD mice. (A) Islet images, red staining indicates peri-insulitis. (B) Comparison of insulitis scores. (Prud'homme, Glinka et al. 2020)

A research published in 2020, exhibited the difference of injecting NOD mice with soluble form of Klotho (s-Klotho) and antibody of Klotho (ab-Klotho) (Prud'homme, Glinka et al. 2020). The incidence of diabetes was decreased when NOD mice were injected with s-Klotho, whereas when the NOD mice were injected with ab-Klotho the diabetes incidence was increased (Figure 33, Figure 32) (Prud'homme, Glinka et al. 2020). Treatment with s-Klotho enlarged the islets of the diabetic mice, by increasing the proliferation of β-cells and reduced insulitis, while ab-Klotho treatment had the opposite results (Figure 31) (Prud'homme, Glinka et al. 2020).

Overexpression of proteins

Neurogenin-3

Neurogenin-3 (Ngn3) is a pancreatic transcription factor that is expressed upstream of NeuroD in the endocrine differentiation cascade (Phillips, Kay 2014, Samson, Chan 2006). The expression of Ngn3 is temporary during embryogenesis and decreases to undetectable levels until birth (Samson, Chan 2006). According to Samson and Chan, islet cells derive from Ngn3-positive precursor cells and Ngn3 is a required factor for the activation of the expression of downstream transcription factors in the endocrine developmental cascade.

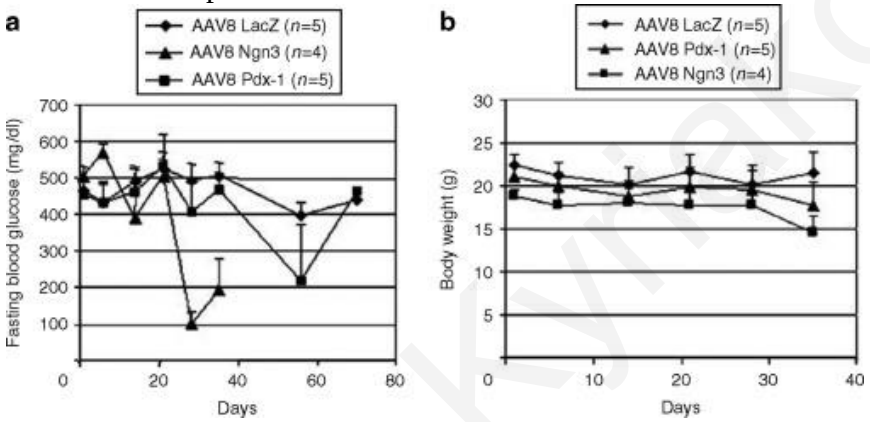


Figure 34 Blood glucose levels and body weight of STZ diabetic mice injected with AAV-Pdx1 or AAV-Ngn3. (a) 6 hours fasting blood glucose levels of STZ-diabetic mice treated with AAV-Pdx1, AAV-Ngn3 or empty-vector. (b) Body weight of STZ-diabetic mice treated with AAV-Pdx1, AAV-Ngn3 or empty-vector. (Wang, Ehrhardt et al. 2007)

In 2007, Wang, Erhardt et al., used adeno-associated virus serotype 8 to transduce Ngn3 in the livers of mice that were made diabetic by a dose of STZ. The treated mice developed hyperglycemia and lost weight as if they were not treated (Figure 34) (Wang, Ehrhardt et al. 2007). In addition, in an experiment that was conducted in order to check whether the gene therapy with adeno-associated delivery of Ngn3 caused liver damage, high levels of bilirubin were displayed, showcasing liver dysfunction (Figure 35) (Wang, Ehrhardt et al. 2007). However, when the diabetic mice were treated with both, an unrelated adenovirus expressing the human coagulation factor IX gene (AdVhFIX) and plasmid expressing Ngn3 (pNgn3), displayed lower blood glucose as well as higher body weight values than what mice that were injected with

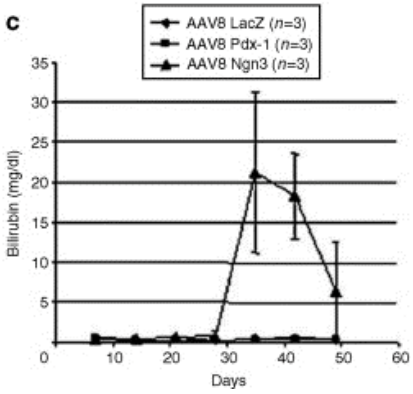


Figure 35 Liver toxicity parameters in treated mice. (c) Bilirubin levels from STZ-treated diabetic mice injected with AAV-Pdx1, AAV-Ngn3 or empty-vector (Wang, Ehrhardt et al. 2007)

pNgn3 only (Figure 36 bottom panels) (Wang, Ehrhardt et al. 2007). Moreover, after glucose tolerance testing the mice displayed near normal glucose blood levels and higher insulin levels (Figure 37 top panels) (Wang, Ehrhardt et al. 2007). Later on, when the scientists co-delivered plasmids that expressed adenoviral genes in conjunction with Ngn3 in mice, the mice remained diabetic with no improvement in their glycemic state (Figure 37 a bottom panel) (Wang, Ehrhardt et al. 2007). Nevertheless, mice that were treated with plasmid Ngn3 and transducing units of an irrelevant helper-dependent adenoviral vector expressing canine coagulation factor IX (HDAVcFIX)), that have similar transduction efficiency as first-generation adenoviral vectors but without the adenoviral genes, had lowered the hyperglycemia to an almost euglycemic range, demonstrating the ability of the adenoviral capsid and Ngn3 to cure STZ caused diabetes (Figure 37 b bottom panel) (Wang, Ehrhardt et al. 2007). In Rag-1 deficient mice that were injected with STZ, therapy with Ngn3 and adenoviral capsid did not show any correction of their glycemic state (Wang, Ehrhardt et al. 2007). Diabetic mice that were treated with Ngn3 or/and adenovirus, both

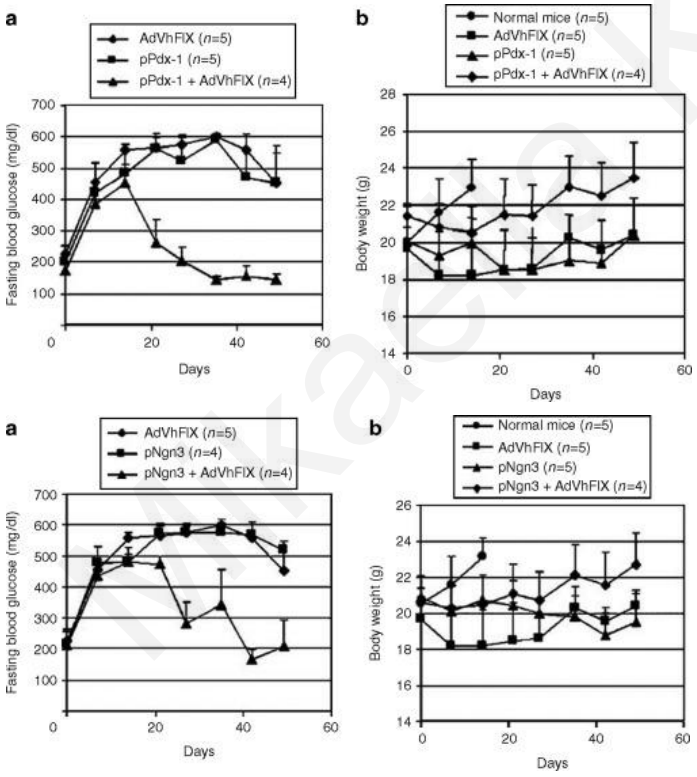


Figure 36 Blood glucose and body weight in STZ-diabetic mice treated with various gene transfer protocols. (a top panel) 6 hours fasted blood glucose levels of pPdx1/ AdVhFIX. (b top panel) body weight of STZ mice treated with pPdx1/ AdVhFIX.. (a bottom panel) 6 hours fasted blood glucose levels of pNgn3/ AdVhFIX. (b top panel) body weight of STZ mice treated with pNgn3/ AdVhFIX. (Wang, Ehrhardt et al. 2007)

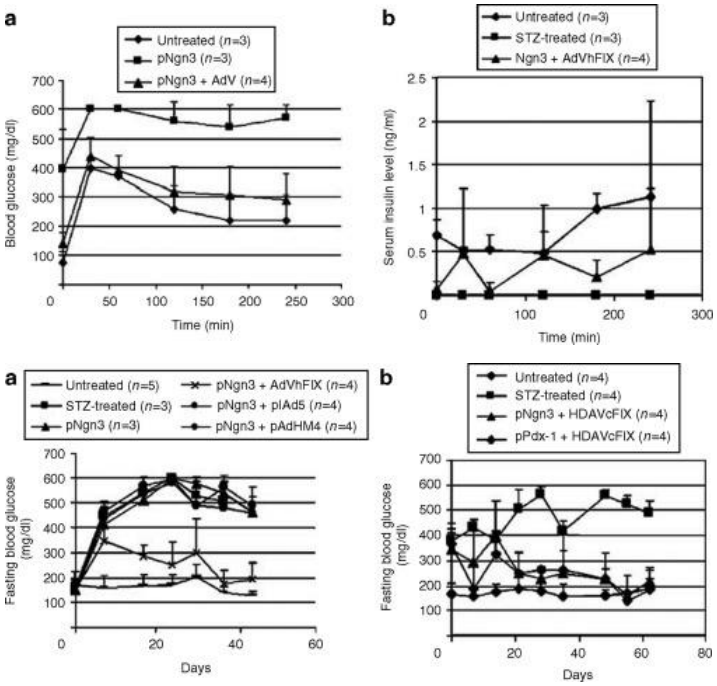


Figure 37 Glucose tolerance tests and fasting glucose levels in STZ-diabetic mice subjected to various gene transfer protocols. (a top panel) Glucose tolerance test on pNgn3+AdVhFIX treated mice. (b top panel) Insulin levels during the glucose tolerance test from treated mice. (a bottom panel) Fasting blood glucose of mice treated with various plasmid combinations and Ngn3. (b bottom panel) Fasting blood glucose levels of mice treated with pNgn3 or pPDX1 and HDAVcFIX. (Wang, Ehrhardt et al. 2007)

endogenous and exogenous Ngn3 was detected, as well as the insulin-1 and insulin-2 (Figure 38) (Wang, Ehrhardt et al. 2007).

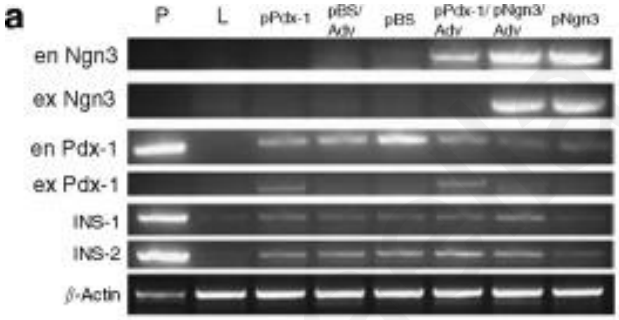


Figure 38 Gene expression profiles in the liver of treated mice. Untreated mouse pancreas (P); untreated mouse liver (L); insulin 1 (INS-1); insulin 2 (INS-2). RT-PCR analyses of total RNA from P, L, or mice treated with pPdx1, pNgn3, pBS/AdV, pPdx1/AdV, pNgn3/AdV. (Wang, Ehrhardt et al. 2007)

In a follow up research, in 2014, Philips and Kay, introduced Ngn3 gene with an adenoviral vector to STZ diabetic mice. The mice that received the treatment had improved their fasting blood glucose levels and increased their body weight (Figure 40a, b) (Phillips, Kay 2014). Moreover, the injected mice had higher insulin levels than the diabetic mice but lower levels than the control mice (Figure 40c) (Phillips, Kay 2014). After a glucose tolerance test the mice that were treated had lower blood glucose levels than the diabetic mice but higher blood glucose than the treated mice, that showcased the effect of Ngn3 in the glucose homeostasis (Figure 40d) (Phillips, Kay 2014). When the scientists depleted Kupffer cells, which are responsible for the first immune defense against bacterial infection, in order to observe how the treatment with Ngn3 would be affected, they noticed that the blood glucose was unstable and was significantly increased in the

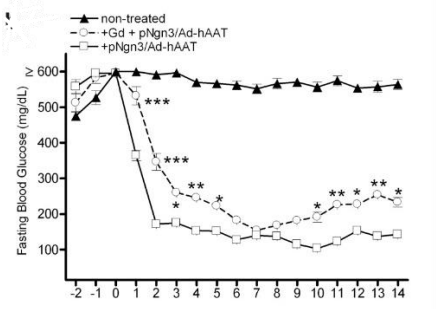


Figure 39 Depletion of Kupffer cell. Fasting blood glucose levels of STZ-diabetic mice treated with pNgn3/Ad-hAAT with or without $GdCl_3$, which mediated the Kupffer cell depletion. (Phillips, Kay 2014)

first five weeks and in the last four weeks of the experiment in comparison with the unaffected Kupffer cells (Figure 39) (Phillips, Kay 2014). That indicated the partial role of Kupffer cells in the treatment with Ngn3. The next question that raised up was whether the addition of dimethyldioctadecylammonium bromide (DDA) or polyinosinic-polycytidylicacid (poly-IC) could assist the delivery of Ngn3 gene via adeno-associated virus to treat diabetes. Indeed, the addition of either DDA or poly-IC had decreased the blood glucose levels, however the results were temporary, and they did not reach the low blood glucose levels that the adenoviral vector induced (Phillips, Kay 2014). Interestingly, when mice were injected for a second time with STZ, after being treated with adenovirus expressing Ngn3, the time of the second injection had a significant role in the disease evolvement. Mice that were injected with STZ, 5 and 8 weeks after the gene therapy, had developed high blood glucose levels, while mice that were exposed at 25 weeks had significantly lower blood glucose levels (Figure 41) (Phillips, Kay 2014). These observations revealed that treatment with Ngn3 and adenoviral vector needs time to provide protection. Finally, the scientists treated mice before exposing them to STZ and 47% partially responded to the treatment developing later diabetes,

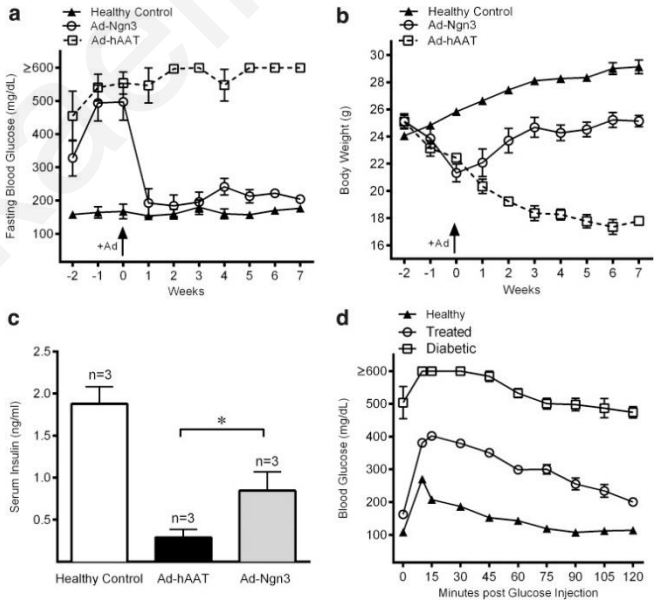


Figure 40 STZ- diabetic mice treated with Ngn3 had improved blood glucose levels, body weight, serum insulin and glucose tolerance. (a) Fasting blood glucose. (b) Body weight. (c) Serum insulin levels 3 weeks post injection. (d) Blood glucose tolerance test. (Phillips, Kay 2014)

40% had normal blood glucose levels and only 13% developed severe diabetes (Figure 42) (Phillips, Kay 2014).

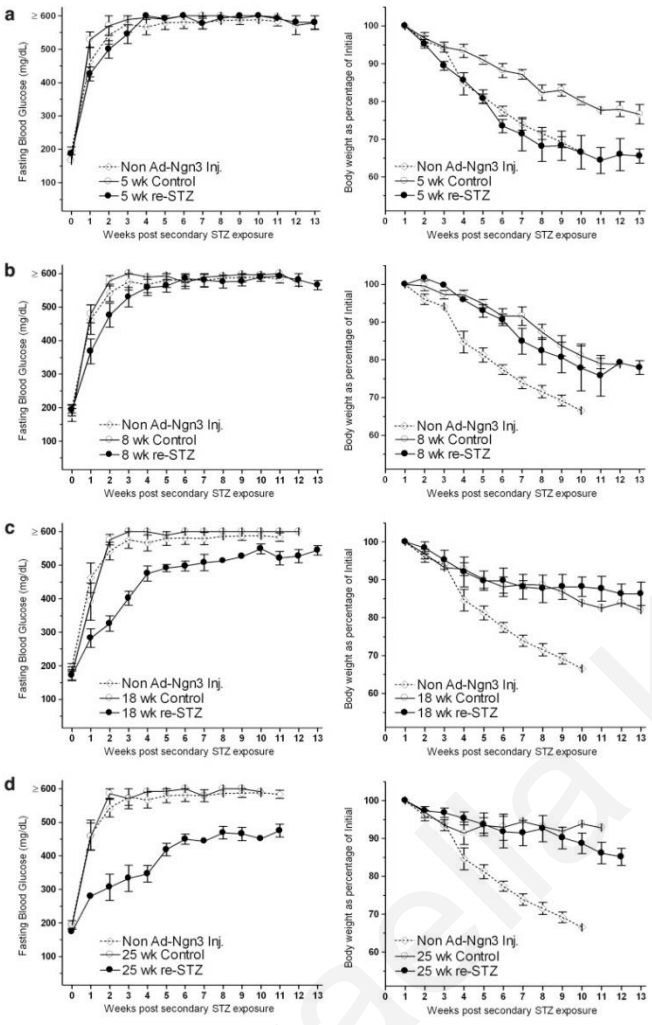


Figure 41 Secondary dose of STZ in Ad-Ngn3 treated mice. Fasting blood glucose (left) and body weight measurements (right) of mice that were exposed to a secondary STZ injection at (a) 5 weeks, (b) 8 weeks, (c) 18 weeks and (d) 25 weeks after the initial treatment with Ad-Ngn3. (Phillips, Kay 2014)

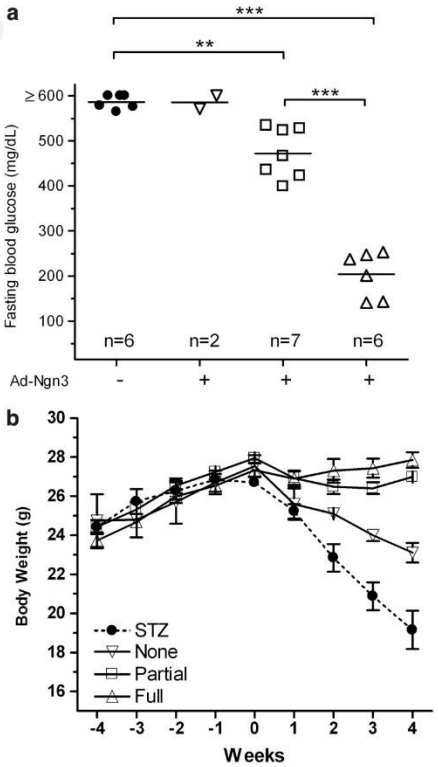


Figure 42 Pre-treatment with Ad-Ngn3 protects against STZ-induced hyperglycemia. (a) Fasting blood glucose levels 4 weeks after STZ treatment. (b) Body weight measurements for the duration of the experiment. (Phillips, Kay 2014)

Betacellulin

Betacellulin (Btc) is an epidermal growth factor, which signals through the epidermal growth factor receptor (Song, Bae et al. 2014). At first is composed as a transmembrane protein and is divided by specific proteases to discharge the circulating form (Song, Bae et al. 2014). Btc is expressed in pancreatic α and β cells, and duct cells, as well (Song, Bae et al. 2014). It has an important part in inhibiting apoptosis, promoting the neogenesis of β cells and conversion of non-β cells into insulin-producing ones (Song, Bae et al. 2014). Btc is often used in conjunction with Ngn3, to amplify its effects (Yechoor, Liu, Espiritu et al. 2009).

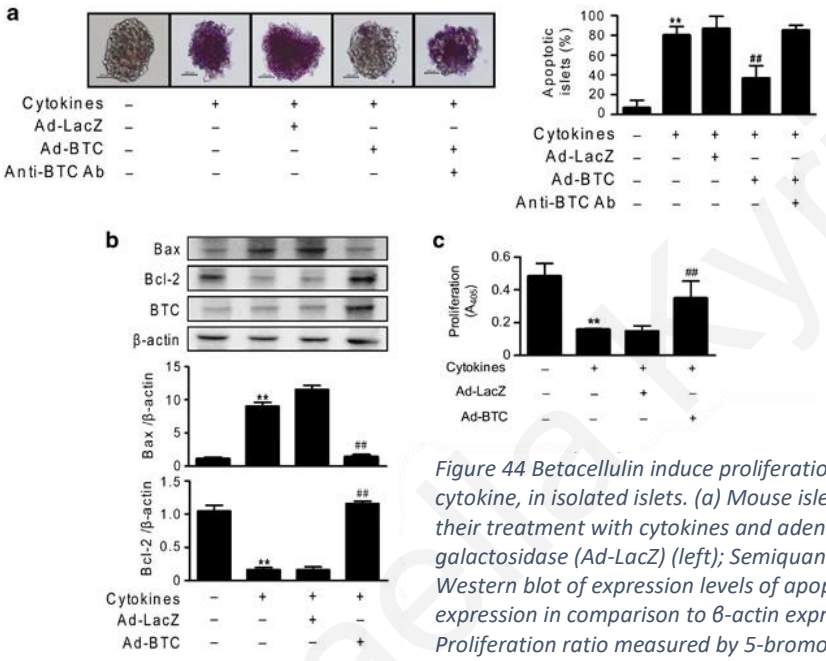


Figure 44 Betacellulin induce proliferation and prevents apoptosis, induced by cytokine, in isolated islets. (a) Mouse islets infused with ApoPercentage dye after their treatment with cytokines and adenoviruses expressing BTC (AdBTC) or β-galactosidase (Ad-LacZ) (left); Semiquantification of apoptotic staining (right). (b) Western blot of expression levels of apoptosis-related proteins (top); Ratio of the expression in comparison to β-actin expression (middle and bottom). (c) Proliferation ratio measured by 5-bromo-2-deoxyuridine (BrdU) of AdLacZ and AdBTC. (Song, Bae et al. 2014)

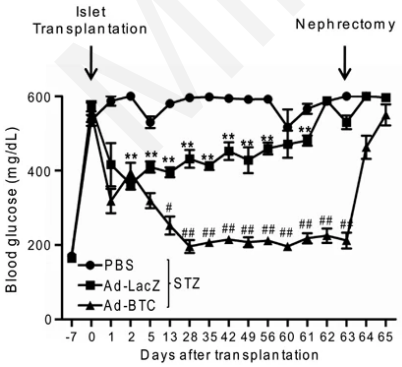


Figure 43 BTC treatment improves islet allograft. (Song, Bae et al. 2014)

Yechoor, Liu et al. observed that the introduction of Btc alone was to be unable to induce euglycemia (Yechoor, Liu, Espiritu et al. 2009). Mice livers treated with Btc alone, could not produce insulin, glucagon, pancreatic polypeptide and somatostatin, that are major islet hormones (Yechoor, Liu, Espiritu et al. 2009).

In 2014, Song, Bae et al. transduced Btc via adenoviral vector into mouse pancreatic islets. When the islets were exposed to cytokine toxicity, treatment with Btc inhibited the apoptotic effects of the cytokines, significantly (Figure 44a) (Song, Bae et al. 2014). The proapoptotic protein Bax levels were decreased in the Btc treated islets, whereas the antiapoptotic protein Bcl-2 were increased (Figure 44b), explaining the apoptosis inhibition caused by Btc (Song, Bae et al. 2014). Proliferation levels were increased in Btc-treated islets, in comparison to cytokine treated islets (Figure 44c) (Song, Bae et al. 2014). Btc treated islets and control islets had similar morphology and strong insulin immunoreactivity, while cytokine treated islets exhibited severe apoptosis and weak insulin immunoreactivity (Figure 45A) (Song, Bae et al. 2014). Moreover, Btc inhibited the effects of cytokines on insulin secretion, preserving glucose-stimulated insulin secretion (Figure 45B) (Song, Bae et al. 2014). When Btc was transduced via the Adenoviral vector to STZ-induced diabetic mice, reduced blood glucose levels were exhibited until the removal of kidney, where hyperglycemia occurred again (Figure 43) (Song, Bae et al. 2014). After a glucose tolerance test on transplanted diabetic mice, Btc therapy decreased the blood glucose levels and augmented the plasma insulin levels (Song, Bae et al. 2014). Furthermore, insulin immunoreactivity was stronger in Btc treated islet engraftments in comparison to empty-vectors, and Btc mice engraftments displayed more newly formed blood vessels, this explained the previously discussed differences of blood glucose levels and plasma insulin levels (Figure 46) (Song, Bae et al. 2014).

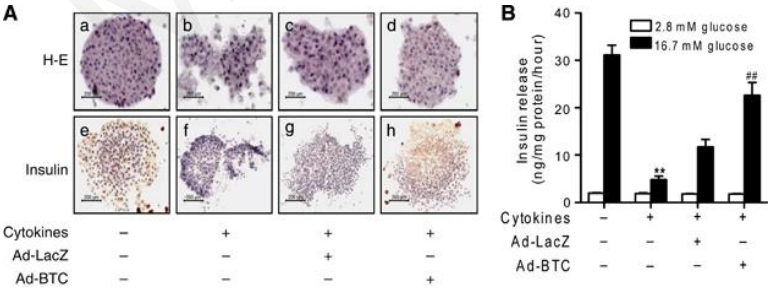


Figure 45 Btc preserves glucose-stimulated insulin secretion. (A) Ad-LacZ or Ad-BTC islets were treated with cytokines and stained with H&E or anti-insulin antibody. (B) Quantification of the staining and normalized to total protein content. (Song, Bae et al. 2014)

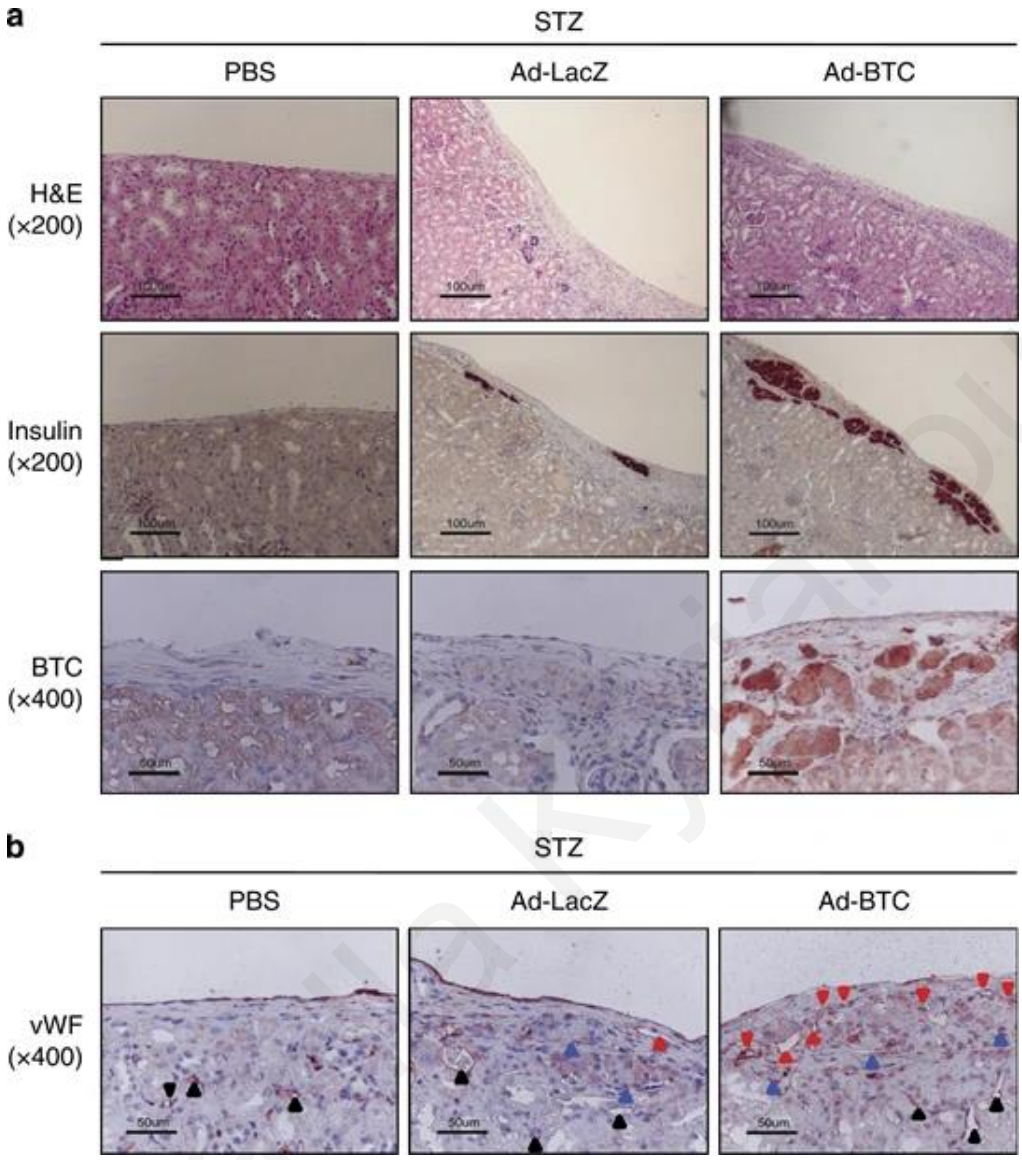


Figure 46 Betacellulin induces blood vessel development in transplanted pancreatic islets. (a) Stained islet grafts with H&E or anti-insulin or anti-BTC antibody. (b) Immunostaining of islet grafts with anti-vWF antibody. Internal positive blood vessels (black arrowheads); newly formed blood vessels (red arrowheads); transplanted islets (blue arrowheads). (Song, Bae et al. 2014)

Pancreas duodenal homeobox-1

Pancreas duodenal homeobox-1 (Pdx1) is a pancreas-defining transcription factor, located upstream of Ngn3 (Yechoor, Liu, Espiritu et al. 2009). The homeodomain transcription factor Pdx1 is expressed in the pancreatic endoderm, where is needed for its early development (Bahrebar, Soleimani et al. 2015). Pdx1 is expressed in mature β-cells, normally, and is needed for normal insulin secretion (Yechoor, Liu, Espiritu et al. 2009).

In a previously discussed research by Wang, Ehrhardt et al., used adeno-associated virus serotype 8 to transduce Pdx1 in the livers of mice that were made diabetic by a dose of STZ, and compared the results with the transduction of Ngn3 (Wang, Ehrhardt et al. 2007). The expression of Pdx1 in the liver of STZ diabetic mice did not reduce hyperglycemia but reduced the body weight (Figure 34) (Wang, Ehrhardt et al. 2007). Moreover, the levels of aspartate aminotransferase, alanine aminotransferase and bilirubin were in the same level of the control vector, thus indicating that Pdx1 expression did not cause liver dysfunction, as opposed to the Ngn3 results (Figure 35) (Wang, Ehrhardt et al. 2007). When the diabetic mice were treated with both, an unrelated adenovirus expressing the human coagulation factor IX gene and plasmid expressing Pdx1, displayed lower blood glucose and an improvement in body weight values, than what mice that were injected with plasmid expressing Pdx1 only (Figure 36 top panels) (Wang, Ehrhardt et al. 2007). Moreover, the co-expression of Pdx1 and an irrelevant helper-dependent adenoviral vector expressing canine, this time, coagulation factor IX, established the correction of hyperglycemia that was induced by STZ-caused diabetes (Figure 37 b bottom panel) (Wang, Ehrhardt et al. 2007). However, when the delivery of these two factors was made in diabetic immunodeficient RAG mice there was not an improvement in the glycemic state of the mice, indicating that the adenoviral capsid elicited an immune response that was accountable for the improvement of the glycemic state of the diabetic mice (Wang, Ehrhardt et al. 2007). Endogenous Pdx1 was expressed by all the diabetic liver samples, treated and untreated (Figure 38) (Wang, Ehrhardt et al. 2007). Notwithstanding, co-delivery of Pdx1 and the irrelevant adenovirus, but not Pdx1 alone, caused the expression of endogenous Ngn3 (Figure 38) (Wang, Ehrhardt et al. 2007).

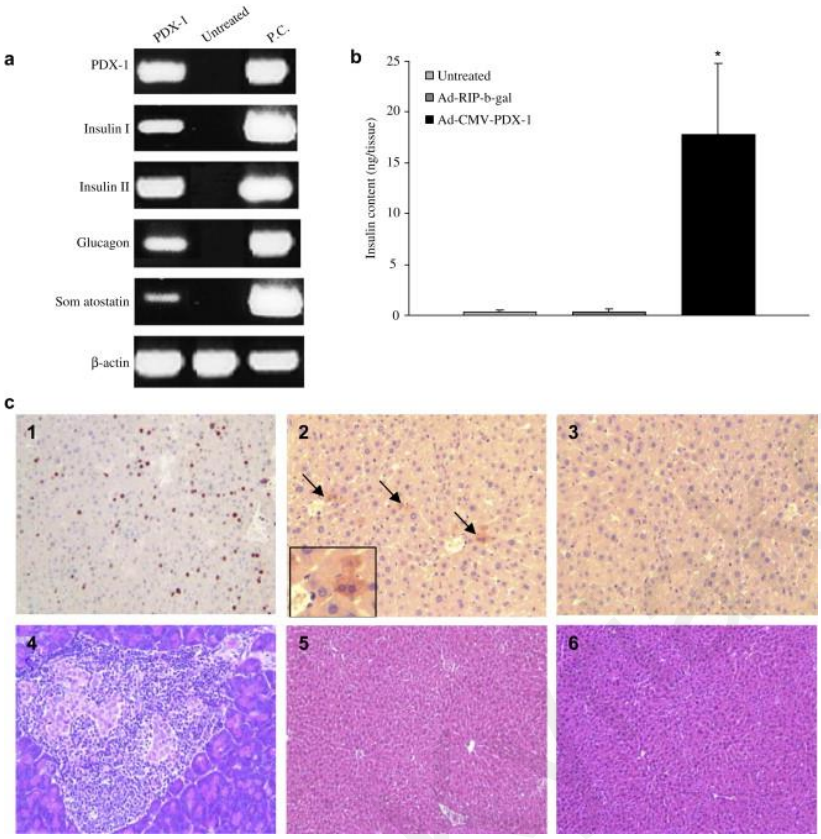


Figure 47 Therapy with PDX1 induces pancreatic cell morphology into hepatic cells of NOD mice. (a) RT-PCR analyses of pancreatic gene expression in PDX1 treated and untreated mouse liver and positive control (P.C.). (b) Hepatic insulin content. (c) Immunohistological analyses of liver sections. (1) PDX1 staining; (2) insulin staining; (3) Insulin immunostaining of Ad-Rip-β-gal treated liver section. (4) Hematoxylin-eosin staining of pancreas and (5) Hematoxylin-eosin staining of liver of Ad-Ripβ-gal mice or (6) normoglycemic Ad-CMV-PDX1 treated mice liver. (Shternhall-Ron, Quintana et al. 2007)

In the same year, a research paper by Shternhall-Ron, Quintana et al. used a recombinant adenovirus to administer Pdx1 to livers of overtly diabetic NOD mice. The treated mice exhibited insulin, glucagon and somatostatin genes in their liver (Figure 47a), which are major pancreatic hormones as mentioned earlier (Shternhall-Ron, Quintana et al. 2007). Insulin content was present in the liver of Pdx-1 treated mice, in contradiction to untreated control mice livers and empty vector livers (Figure 47b) (Shternhall-Ron, Quintana et al. 2007). Moreover, immunohistological analyses of liver sections treated with Pdx1, exhibited Pdx1 and insulin positive cells (Figure 47c) (Shternhall-Ron, Quintana et al. 2007). The treated mice became euglycemic and remained at that state, while they excreted insulin almost at the same level of non-diabetic control mice and maintained stable weight (Figure 48a, b, c) (Shternhall-Ron, Quintana et al. 2007). A glucose tolerance test showcased the similarities of Pdx1 treated and normoglycemic mice in blood glucose levels (Figure 48d) (Shternhall-Ron, Quintana et al. 2007). Splenocytes retrieved from Pdx1 treated mice had significantly lower proliferative responses to antigens GAD, HSP60 and insulin,

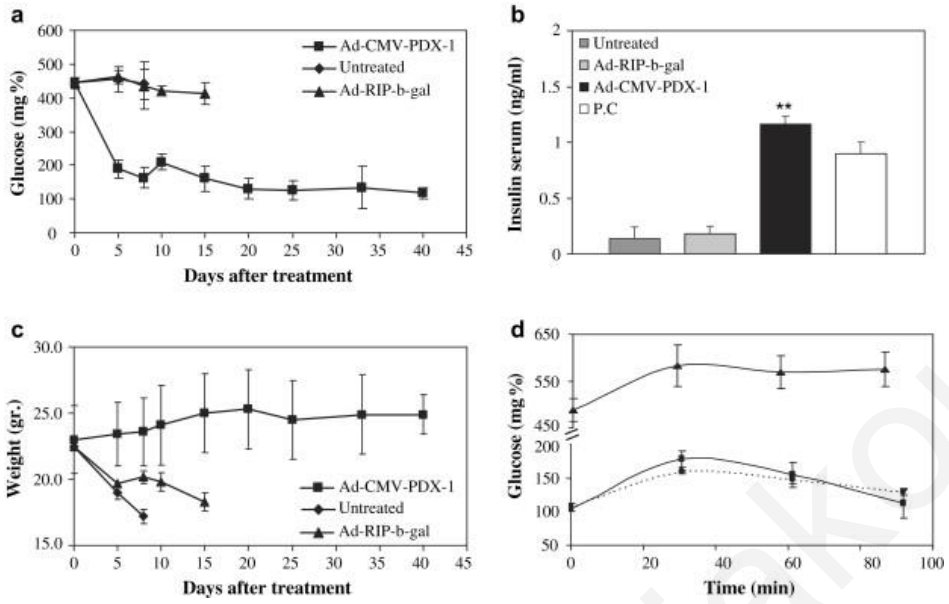


Figure 48 Ad-CMV-PDX1 alleviates diabetes in NOD mice. (a) Blood glucose levels; (b) Serum insulin levels; (c) Body weight; (d) Glucose tolerance test. (Shternhall-Ron, Quintana et al. 2007)

indicating the decrease of the diabetogenic proliferation of T-cells (Shternhall-Ron, Quintana et al. 2007). Moreover, there was a decrease in IFNγ release, connected with secretion to Th1 cytokines, and an increase in IL10 release, connected to Th2 cytokine secretion, in insulin HSP60 and GAD, in treated mice splenocytes (Shternhall-Ron, Quintana et al. 2007). When splenocytes from treated NOD mice were transplanted to NOD/SCID mice diabetes incidence was delayed, but eventually the NOD/SCID mice became hyperglycemic (Shternhall-Ron, Quintana et al. 2007).

Bahrebar, Soleimani et al., investigated the impact of the overexpression of Pdx1, via lentivirus vector, in human adipose tissue-derived stem cells (hAMSCs). hAMSCs were found positive for CD90 and CD105, that are markers for mesenchymal cells, while they were negative for CD34 and CD45, that are markers for hematopoietic cells (Bahrebar, Soleimani et al. 2015). Moreover, hAMSCs were confirmed by oil red O-staining and Alizarin red staining to have the capability to differentiate into adipogenic and osteogenic lineages, respectively (Figure 49) (Bahrebar, Soleimani et al. 2015). In the oil red O-staining, fat droplets were seen, whereas in the Alizarin red staining, calcium deposits were observed (Figure 50) (Bahrebar, Soleimani et al. 2015). hAMSCs in the control group were spindle-shaped and were not in clusters in both 0 and 10 days, as it was in the backbone group (Bahrebar, Soleimani et al. 2015). However, their morphology changed at day 5 and 10, when they were treated with Pdx1, they took a similar morphology to

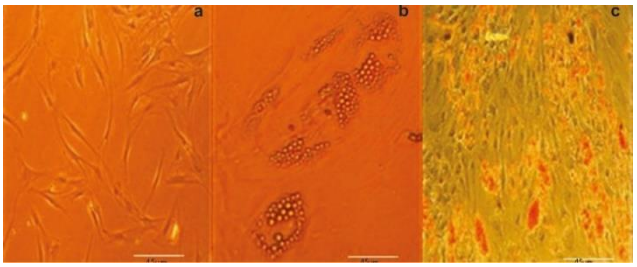


Figure 49 Differentiation to adipocytes. (a) hAMSCs in normal medium. (b) Non-staining. (c) Oil red O-staining. (Bahrebar, Soleimani et al. 2015)

islets, round, and they were clustering (Figure 51) (Bahrebar, Soleimani et al. 2015). In addition, the treated cells expressed pancreatic genes, such as Pdx1, Ngn3, Nkx2-2, Insulin and B2M, in similar levels at day 10 and 14 as what pancreatic cells were expressingFigure 54(Figure 54) (Bahrebar, Soleimani et al. 2015). Pdx1, Ngn3, Nkx2-2 and insulin were significantly upregulated in the treated cells in comparison to the empty vector treated cells (Figure 54) (Bahrebar, Soleimani et al. 2015). Pdx1 treated hAMSCs were found positive in insulin after an immunofluorescence test (Bahrebar, Soleimani et al. 2015). On day 14, the Pdx1 treated cells seemed to be fully matured and differentiated, they were transformed into insulin-producing cells, as the dithizone-staining (DTZ) indicated (Figure 52) (Bahrebar, Soleimani et al. 2015). When the cells were tested for the amount of secreted insulin, the Pdx1 treated cells had significantly higher values of insulin than the empty vector treated cells (Figure 53a) (Bahrebar, Soleimani et al. 2015). Pdx1 treated hAMSCs in response to glucose they excreted a higher amount of insulin than the empty vector group (Figure 53b) (Bahrebar, Soleimani et al. 2015). This study showcased the ability of hAMSCs to transform into insulin producing cells with the induction of Pdx1 in order to treat diabetes.

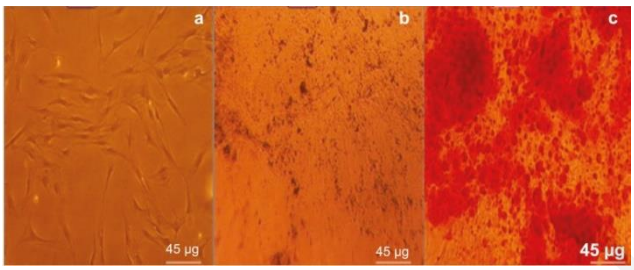


Figure 50 Differentiation to osteoblasts. (a) hAMSCs in normal medium. (b) Non-staining. (c) Alazarin red staining. (Bahrebar, Soleimani et al. 2015)

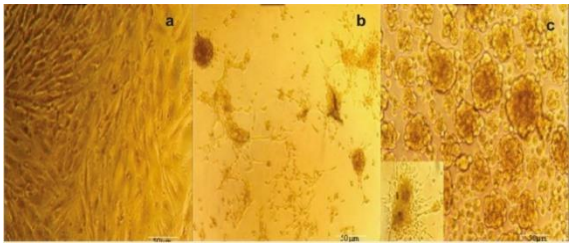


Figure 51 Morphologic changes in test group. (a) day 0. (b) day 5. (c) day 10. (Bahrebar, Soleimani et al. 2015)

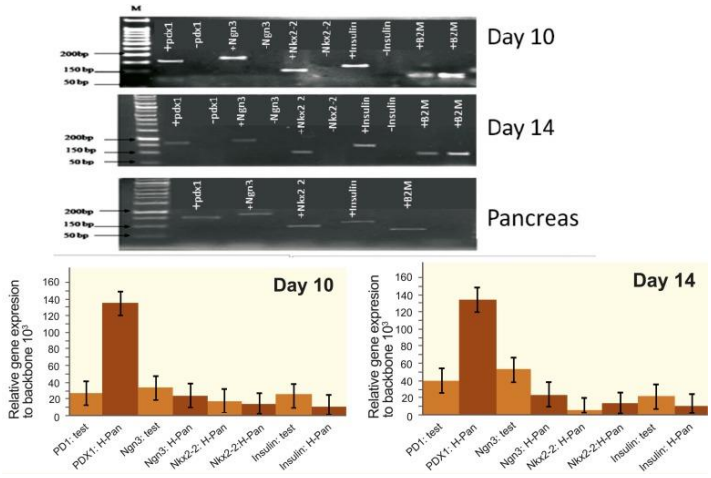


Figure 54 Expression of pancreatic related genes. (a) RT-PCR at 10th and 14th day. (b) real-time PCR at day 10th and 14th. (+) positive expression in the test group or pancreas tissue sample; (-) negative expression in the backbone group; (h-Pan) human pancreas. (Bahrebar, Soleimani et al. 2015)

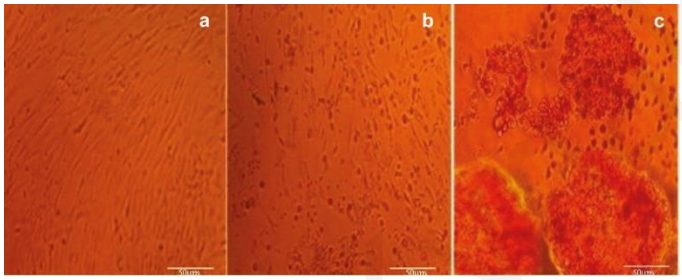


Figure 52 DTZ staining of HAMSCs on day 14 to identify insulin-producing cells. (a) control group. (b) backbone group. (c) test group. (Bahrebar, Soleimani et al. 2015)

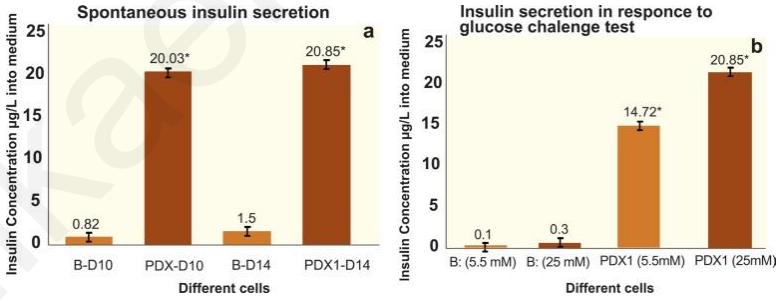


Figure 53 Secretion of insulin in a medium. (a) Spontaneous insulin secretion in day 10 and 14 of differentiation. (b) Insulin secretion in response to glucose tolerance teston day 14 of differentiation. (B-D10) Backbone group/hAMSCs + Lv-null on day 10 of protocol; (PDX1-D10) test group/hAMSCs + LvPDX-1 on the 10th day of differentiation; (B-D14) Backbone group/hAMSCs + Lv-null at day 14 of protocol; (PDX1-D14) Test group/hAMSCs + LvPDX-1 on day 14 of differentiation. (Bahrebar, Soleimani et al. 2015)

Leptin

Leptin is a peptide hormone, and it has a crucial role in the regulation of glucose homeostasis independently of weight and food intake (Fernández-Formoso, Pérez-Sieira et al. 2015). Moreover, leptin is considered an important regulator of pancreatic β-cell function, e.g. apoptosis, cell growth, insulin gene expression and insulin secretion (Marroquí, Gonzalez et al. 2012).

In 2009, Kojima, Asakawa et al. induced leptin via an adeno-associated virus into STZ diabetic mice. Mice that were treated had an increase in the food intake at the first 4 weeks, causing them to gain weight, that later decreased until it got to a plateau at 12 weeks, when the weight plateaued as well, evident of health (Figure 56A, B) (Kojima, Asakawa et al. 2009). Blood glucose levels moved to similar pace, with an increase until week 4, a decrease until week 12 and plateau until the end of the experiment, with the plateau being at similar levels with control non-diabetic mice (Figure 56C) (Kojima, Asakawa et al. 2009). Moreover, the insulin levels of the leptin treated mice were approximately to 100pg/ml and at 52 weeks, the end of the experiment, were increased to approximately 230pg/ml, while the food intake was stable, and the control non-diabetic mice insulin levels were approximately 500pg/ml and 1600pg/ml respectively (Figure 55) (Kojima, Asakawa et al. 2009). That small increase of insulin secretion in leptin treated mice might be pancreatic β-cells attempt of insulin synthesis reestablishment (Kojima, Asakawa et al. 2009).

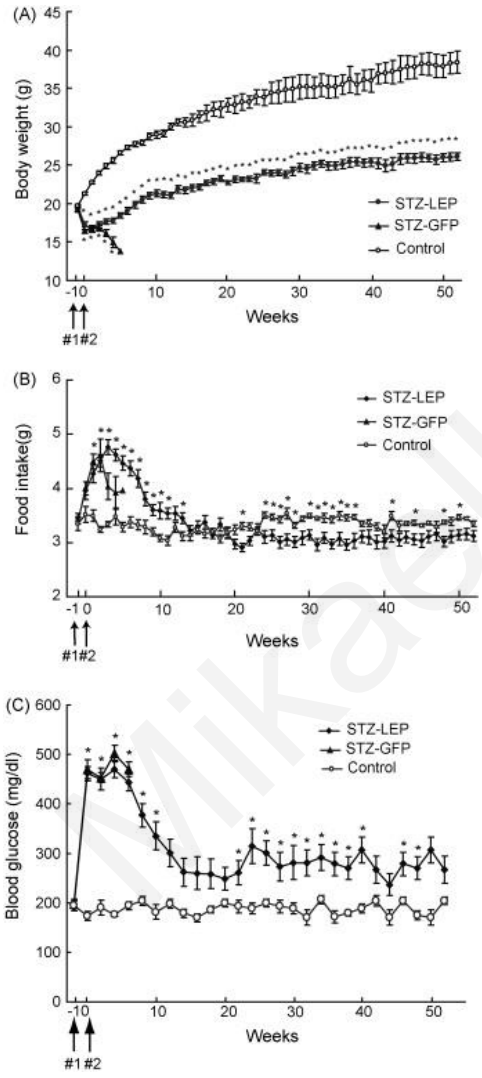


Figure 56 Leptin treatment effects in mice. (A) body weight measurements. (B) food intake measurements. (C) blood glucose levels. (Kojima, Asakawa et al. 2009)

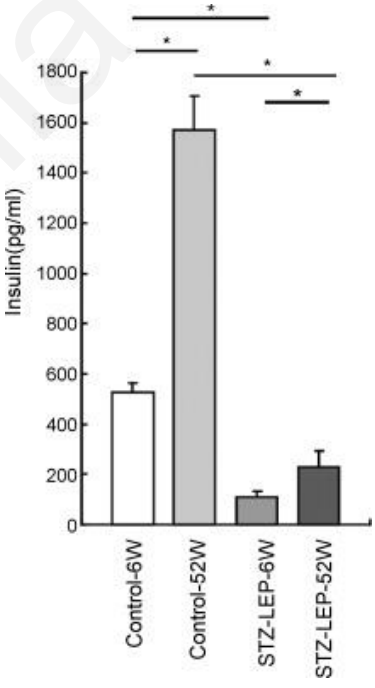


Figure 55 Leptin treatments effects on insulin levels at week 6 and 52. (Kojima, Asakawa et al. 2009)

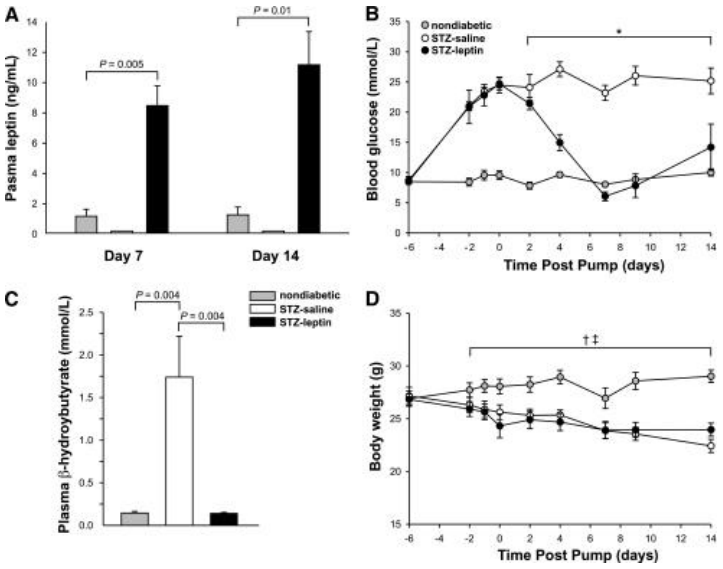


Figure 58 Leptin therapy effects on blood glucose, ketone production and bosyweight. (A) Leptin levels on day 7 and 14. (B) Blood glucose levels. (C) Levels of plasma β-hydroxybutyrate on day 14. (D) Body weight measurements. (Denroche, Levi et al. 2011)

A 2011 research of Denroche, Levi et al. delivered 10μg daily of mouse recombinant leptin for 14 days to STZ-diabetic mice. Levels of plasma leptin were elevated in mice treated with leptin compared to nondiabetic mice, while in STZ mice plasma leptin was depleted (Figure 58A) (Denroche, Levi et al. 2011). Levels of blood glucose of STZ-leptin mice were normalized at day 7 and were kept at normal levels throughout the duration of the study (Figure 58B) (Denroche, Levi et al. 2011). Moreover, plasma β-hydroxybutyrate levels, which is a predominantly ketone body, were increased in STZ diabetic mice and leptin therapy completely rescued it (Figure 58C), while leptin therapy was unable to rescue the loss of body weight caused by STZ injections (Figure 58D) (Denroche, Levi et al. 2011). The blood glucose levels were gradually decreasing when 5μg of leptin were administered in the mice for 14 days, when the leptin pump was removed hyperglycemia returned rapidly (Denroche, Levi et al. 2011). Control diabetic mice had to be killed because of their bad health condition due to uncontrolled diabetes by day 19 (Denroche, Levi et al. 2011). On day 24, another 7-day osmotic pump was implanted on the STZ- leptin group, delivering 24 μg of leptin daily (Denroche, Levi et al. 2011). Hyperglycemia declined within 3 days of treatment (Denroche, Levi et al. 2011). That was an indication that leptin needs to be administered continuously to keep euglycemic levels, on

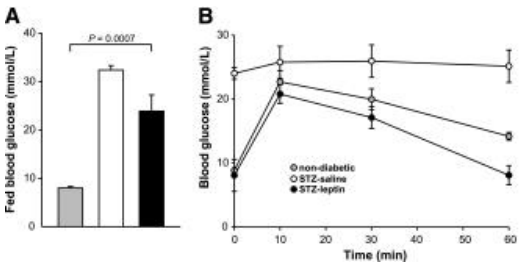


Figure 57 Leptin ameliorates non-fasting blood glucose. (A) Random fed blood glucose on day 13. (B) Blood glucose tolerance test. (Denroche, Levi et al. 2011)

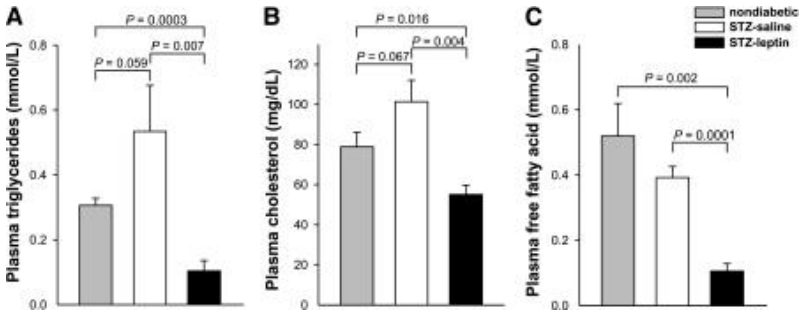


Figure 59 Leptin reduces lipid plasma levels in STZ-diabetic mice. (A) Plasma triglycerides. (B) Plasma cholesterol. (C) Plasma free fatty acids. (Denroche, Levi et al. 2011)

this mice model, and that the glucose lowering is dose dependent (Denroche, Levi et al. 2011). On day 13, the scientists measured the blood glucose of fed mice, STZ-diabetic mice were

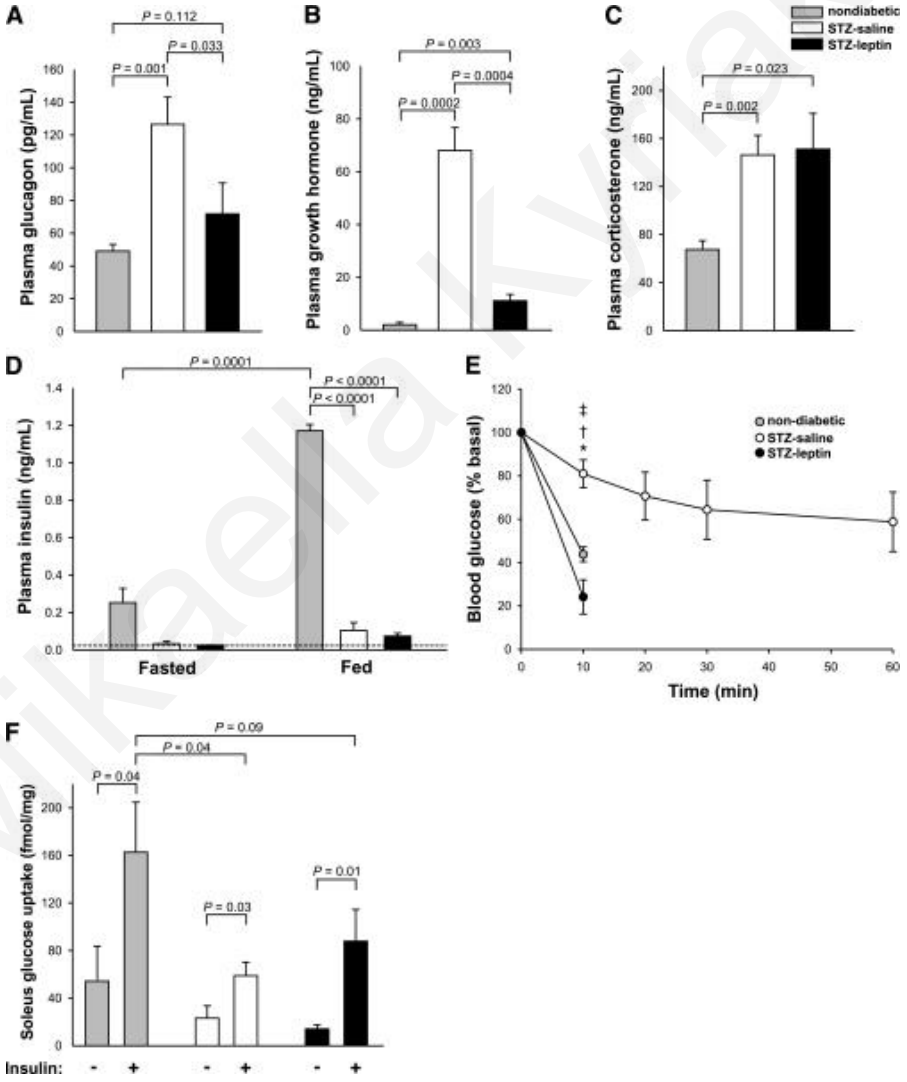


Figure 60 Leptin reduces plasma glucagon and growth hormone, while it increases insulin action in STZ-diabetic mice. (A) Plasma glucagon. (B) Growth hormone. (C) Plasma cholesterol. (D) Plasma insulin. (E) Insulin tolerance test. (F) Glucose uptake in soleus muscle. (Denroche, Levi et al. 2011)

approximately 33 mmol/L, while STZ-leptin mice were approximately 24 mmol/L and nondiabetic were 9 mmol/L, showcasing that leptin treatment decreased blood glucose by 26% approximately (Figure 57A) (Denroche, Levi et al. 2011). On day 9, the scientists performed a glucose tolerance test, were the control diabetic mice had poor glycemic control and leptin treatment had attenuated the hyperglycemia caused by STZ, to almost similar levels as the non-diabetic mice (Figure 57B) (Denroche, Levi et al. 2011). Plasma lipids were measured 4 hours fasted, STZ increased the levels of triglycerides and cholesterol but did not alter the levels of free fatty acids, 10 μg of leptin per day reversed the effect of STZ and the tree lipids were at significantly lower levels than in control non-diabetic mice (Figure 59), correcting the diabetic dyslipidemia caused by STZ (Denroche, Levi et al. 2011). STZ diabetic controls displayed an increase in their 4 hours fasted plasma glucagon, in comparison to the nondiabetic controls and leptin seemed to rescue the increase in plasma glucagon (Figure 60A) (Denroche, Levi et al. 2011). Likewise, fasted plasma growth hormone levels were increased due to STZ and leptin decreased the levels significantly, but not at the level of non-diabetic mice (Figure 60B) (Denroche, Levi et al. 2011). On the contrary, plasma

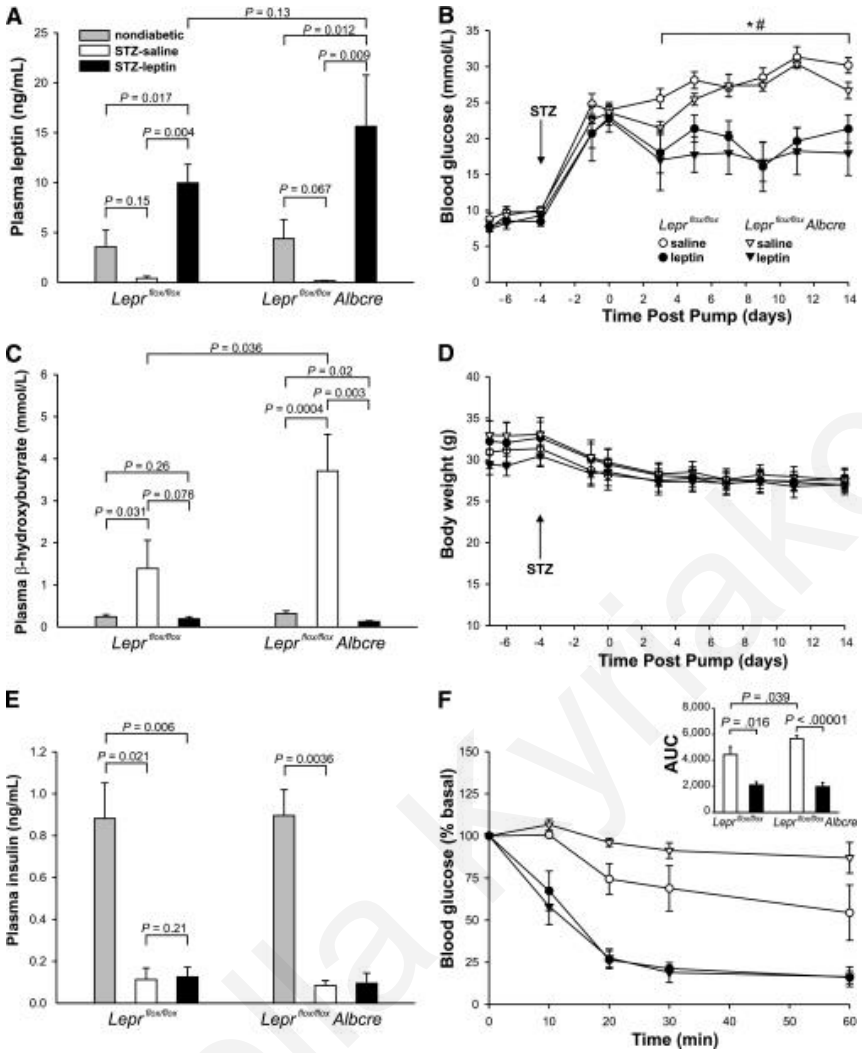


Figure 61 Leptin therapy ameliorates diabetes symptoms in Lepr$^{flox/flox}$ Albcre mice. (A) Plasma leptin, (B) Blood glucose levels. (C) Plasma β-hydroxybutyrate levels. (D) Body weight measurements. (E) Plasma insulin levels. (F) Insulin tolerance test on day 11. (Denroche, Levi et al. 2011)

corticosterone levels were augmented in STZ mice in comparison to non-diabetic ones and leptin therapy was unable to decrease its levels, making glucagon and growth hormone liable for the glucose lowering by leptin (Figure 60C) (Denroche, Levi et al. 2011). Plasma insulin levels were below detection point for STZ mice whether they were treated with leptin or not, confirming that leptin therapy does not reverse diabetes by increasing insulin secretion (Figure 60D) (Denroche, Levi et al. 2011). After an insulin tolerance test, STZ leptin treated mice and non-diabetic mice had glucose levels of less than 4mmol/L at 10 minutes and had to be administered exogenous glucose, thus only one measurement was collected (Figure 60E) (Denroche, Levi et al. 2011). However, STZ mice were more resistant to insulin than nondiabetic controls and leptin therapy

seemed to make the mice even more sensitive to insulin in comparison to nondiabetic controls (Figure 60E) (Denroche, Levi et al. 2011). The glucose uptake in soleus muscle was proportionate in both STZ groups, whether they were treated or not with leptin, an intravenous bolus of insulin was able to increase the uptake of glucose in the muscle but in a non-distinguishing matter between the two groups, making it obvious that skeletal muscle cannot be the reason for the effect of leptin therapy on glucose-lowering and for the augmented insulin sensitivity (Figure 60F) (Denroche, Levi et al. 2011). Later on, the scientists used mice with disturbed hepatic leptin signaling ($Lepr^{flox/flox}$ Albcre), that in its livers the majority of leptin receptors did not contain the JAK-STAT signaling domain, to inspect the possibility that leptin signaling could be implicated in the therapeutic effects of leptin therapy (Denroche, Levi et al. 2011). The therapy included of osmotic pumps that delivered 10 μg of leptin daily for 14 days in diabetic $Lepr^{flox/flox}$ Albcre and in diabetic control $Lepr^{flox/flox}$ mice (Denroche, Levi et al. 2011). Nondiabetic $Lepr^{flox/flox}$ Albcre and

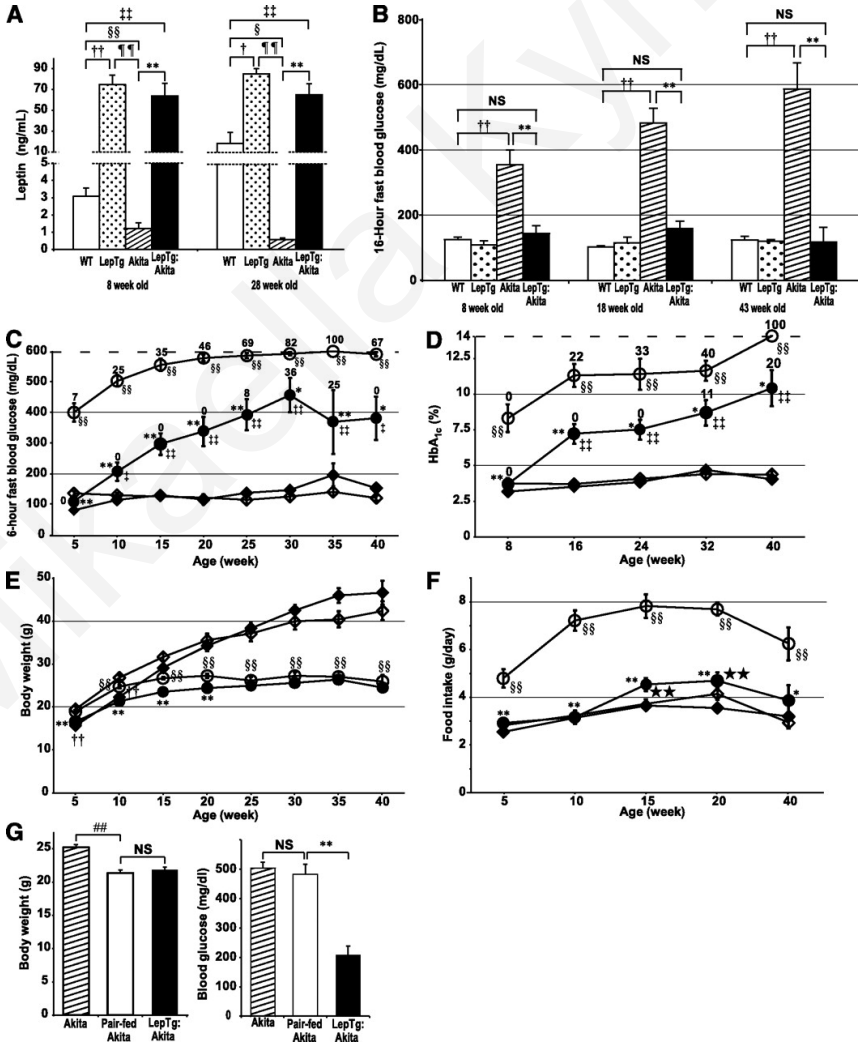


Figure 62 Leptin therapy effects in plasma leptin, blood glucose, HbA1c, body wight and food intake. (A) Leptin levels. (B) 16-hour fasting blood glucose levels. (C) 6-hour fast blood glucose levels. (D) HbA1C. (E) Body weight. (F) 24-hour food intake. (G) Body weight at the end of 3 week of pair feeding. (H) 6-hour fasting blood glucose levels. WT (◇); LepTg (◆); Akita (○); and LepTg:Akita (●) mice (Naito, Fujikura et al. 2011)

Lepr$^{flox/flox}$ mice had similar plasma leptin, blood glucose and body weight (Figure 61) (Denroche, Levi et al. 2011). Plasma leptin was reduced due to STZ administration in both types of mice, while leptin therapy rescued the depletion and significantly enhanced plasma leptin levels in both types of mice in comparison to non-diabetic controls (Figure 61) (Denroche, Levi et al. 2011). Both groups of STZ mice developed hyperglycemia, that were rescued via leptin therapy and was equally effective in both mice groups (Figure 61) (Denroche, Levi et al. 2011). Additionally, β-hydroxybutyrate levels were significantly increased in Lepr$^{flox/flox}$ Albcre and Lepr$^{flox/flox}$ mice, 10- and 5- fold respectively, due to STZ injections (Figure 61C) (Denroche, Levi et al. 2011). However, leptin therapy was able to reverse the high ketone levels by 96% and 85% in Lepr$^{flox/flox}$ Albcre and Lepr$^{flox/flox}$ controls, respectively, showcasing that leptin therapy is not mediated through leptin signaling (Figure 61C) (Denroche, Levi et al. 2011). As expected, leptin treatment did not rescue the effect of STZ in insulin levels in neither of the mice groups (Figure 61E) (Denroche, Levi et al. 2011). After an insulin tolerance test, both groups that were leptin-treated had significantly augmented insulin sensitivity in comparison to their not treated counterparts, indicating that hepatic leptin signaling is not necessary for the enhancement of insulin action gained via leptin therapy (Figure 61F) (Denroche, Levi et al. 2011).

Another study was published by Naito, Fujikura et al. at the same year investigating the consequences of leptin in Akita mice with physiological hyperleptinemia, via cross-mating Akita mice and leptin-expressing transgenic mice. Leptin levels of Akita mice were decreased from 1,2 ng/ml at 8 weeks to 0,58 ng/ml at 28 weeks old, whereas the wild type mice had 3,1 ng/ml and 18,8ng/ml, respectively (Naito, Fujikura et al. 2011). Transgenic expression of leptin on leptin-transgenic mice and the Akita-leptin mice were considerably increased and balanced both at 8 and at 28 weeks old, indicating that Akita-leptin transgenic mice was indeed overexpressing leptin (Figure 62A) (Naito, Fujikura et al. 2011). In continuation to the experiment, 16 hour fasting plasma blood glucose measurements were taken at 8-, 18- and 43-week-old mice (Figure 62B) (Naito, Fujikura et al. 2011). Akita mice displayed extreme hyperglycemia at 8 weeks old, that was increased as he weeks passed by (Naito, Fujikura et al. 2011). However, wild type mice, leptin expressing mice and Akita-leptin mice were at normoglycemia levels, without significant

difference between their blood glucose levels (Naito, Fujikura et al. 2011). Unlike the 16-hour fasting blood glucose, the 6-hour one indicated that the Akita-leptin mice had significantly higher blood glucose than the wild type one and significantly lower than the Akita (Figure 62C) (Naito, Fujikura et al. 2011). Its glycemic levels were escalated gradually after 10 weeks of age as time passed by but did not overcome the 400 mg/dl at 40 weeks of age (Naito, Fujikura et al. 2011). HbA1c levels of Akita-leptin transgenic mice at 8 weeks old were the same as the wild type mice, while the Akita mice had significantly increased HbA1c (Figure 62D) (Naito, Fujikura et al. 2011). After 16 weeks of age, the Akita-leptin mice established higher values of HbA1c, in a time-

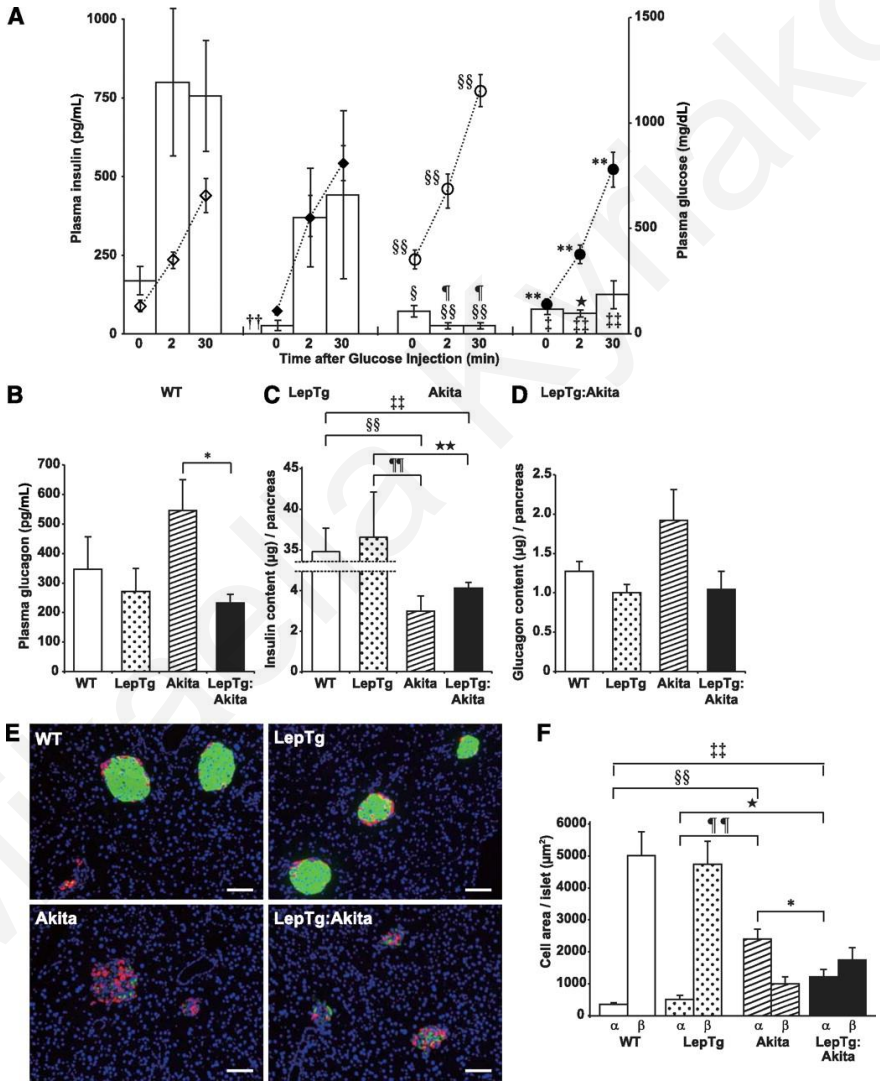


Figure 63 Glucose-stimulated insulin secretion, plasma glucagon levels, and pancreatic hormone contents. A: Plasma insulin (open bars) and glucose (black lines) content. B: Plasma glucagon content in ad libitum–fed mice at 22 weeks of age. (C) Pancreatic insulin and (D) glucagon content at 18 weeks of age. E: Double immunofluorescent staining against insulin (green) and glucagon (red) in pancreatic sections from mice at the age of 18 weeks. F: α-Cell and β-cell areas per islet of mice. (Naito, Fujikura et al. 2011)

dependent manner, than the wild type one but it was still at lower value than the Akita mice (Figure 62D) (Naito, Fujikura et al. 2011). All four mice groups had gained weight gradually, with the Akita and Akita-leptin mice gaining significantly less body weight in comparison to the two other groups (Figure 62E) (Naito, Fujikura et al. 2011). Notwithstanding, Akita mice ate in a significantly higher manner than the wild type, Akita-leptin and leptin mice, that had similar food intake throughout the study (Figure 62F) (Naito, Fujikura et al. 2011). When Akita mice were pair-fed to reach the ad libitum of Akita-leptin mice, its weight decreased and reached the Akita-leptin mice's weight, while its blood glucose levels were stable (Figure 62G, H)(Naito, Fujikura et al. 2011). Glucose tolerance tests that were performed showcased that Akita had increased glucose intolerance at 8 weeks and it increased further at 16 weeks of age, while Akita-leptin mice displayed normal glucose tolerance at 8 weeks that became glucose intolerance at 16 weeks of age, although not as severe as that of the Akita mice displayed at 8 or 16 weeks old (Naito, Fujikura et al. 2011). The insulin tolerance test indicated that Akita-leptin mice enhanced sensitivity in insulin

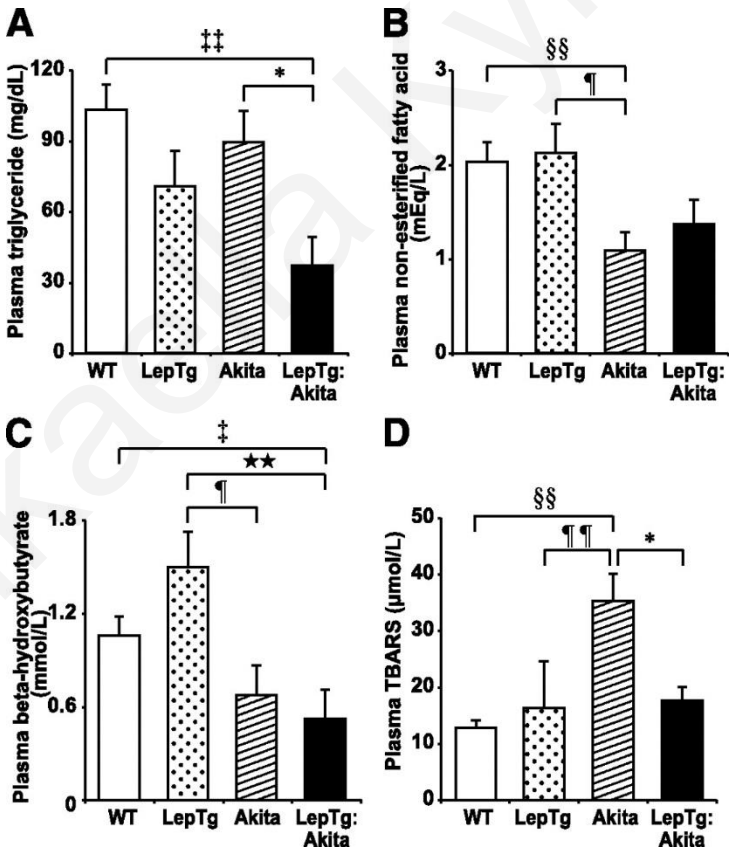


Figure 64 Plasma levels of triglycerides, non-esterified fatty-acids, β-hydroxybutyrate, and TBARS. Fasting plasma levels of triglycerides (A) and non-esterified fatty-acids (B) concentrations in mice at 18 weeks of age. (C) Fasting plasma levels of β-hydroxybutyrate concentrations in mice at 11 weeks of age. (D) Fasting plasma levels of TBARS concentrations in mice at 18 weeks of age. (Naito, Fujikura et al. 2011)

remained stable throughout the study, while the wild type mice and leptin mice sensitivity was deteriorating in an age dependent manner (Naito, Fujikura et al. 2011). The fasting insulin levels of Akita, Akita-leptin and leptin mice were similar among the three groups and significantly lower than wild type mice (Naito, Fujikura et al. 2011). However, when they were administered glucose, Akita-leptin mice had a slow and slight insulin response, Akita mice did not respond and wild-type and leptin mice had an intense insulin response (Figure 63A) (Naito, Fujikura et al. 2011). The plasma glucagon concentration at 22 weeks of age in Akita-leptin mice was normal, while the glucagon of Akita mice was in a significantly higher concentration (Figure 63B) (Naito, Fujikura et al. 2011). In the pancreas, the insulin content of Akita and Akita-leptin mice was similar among the two groups and was approximately 10% lower than the wild type and leptin mice, while the glucagon content of Akita mice was almost increased twice in comparison with the other three groups (Figure 63C, D) (Naito, Fujikura et al. 2011). Akita and Akita-leptin mice had abnormal islet histology, with the first group exhibiting higher proportion of α cells and fewer active β cells and the second group displaying fewer active β cells, but it abolished the α cell hyperplasia, compared to wild type and leptin mice (Figure 63E, F) (Naito, Fujikura et al. 2011). Akita-leptin mice had lower levels of triglyceride in comparison to the other three groups, while the levels of non-esterified fatty acids were decreased but not significantly from the wild type and leptin mice and increased compared to Akita mice (Figure 64A, B) (Naito, Fujikura et al. 2011). The levels of β-hydroxybutyrate were lower in both Akita groups compared to wild type and leptin mice, indicating less ketone bodies in those two groups (Figure 64C) (Naito, Fujikura et al. 2011). Akita mice displayed the higher oxidative stress between the four genotypes and the levels of the other three were similar (Figure 64D) (Naito, Fujikura et al. 2011). The expression of leptin in Akita mice attenuated the effect of extreme albumin excretion (Naito, Fujikura et al. 2011). Lastly, Akita mice displayed much less life span than the Akita-leptin mice, that survived 1 year, while the first Akita mice died at 27 weeks old and at 40 weeks 50% of their population died (Naito, Fujikura et al. 2011).

A research published in 2013 by Denroche, Quong et al., administered leptin to inspect the theory that fewer transplanted islets are nessecary to reverse STZ-induce diabetes in mice. STZ injected diabetic mice were administered leptin at different doses daily, 1, 3, 5, 10 μg per day, for four weeks (Denroche, Quong et al. 2013). The doses of 5 and 10 μg made the diabetic mice normoglycemic by day 5, while 3μg normalized blood glucose by day 15 (Figure 65A) (Denroche, Quong et al. 2013). The smaller dose of 1 μg had not corrected hyperglycemia to a nondiabetic mice level, but the blood glucose levels were lower than the diabetic mice treated with empty vector ((Denroche, Quong et al. 2013). The mice that received 10 μg of leptin daily had a setback at 15 days, resulting in hyperglycemia, the same happened with the 5 μg dosage but to a lesser extend (Figure 65A) (Denroche, Quong et al. 2013). An area under the curve analysis, of 5 to 12 days, showcased that blood glucose was lowered at a dose dependent way, indicating that 1 μg was the ideal dosage to test their hypothesis (Figure 65**Error! Reference source not found.**A) ADDIN RW.CITE{{120 Denroche,H.C. 2013}}(Denroche, Quong et al. 2013). The treated mice, independently of the leptin dosage they received, had lower insulin levels than the control nondiabetic mice and almost the same levels as that of the untreated diabetic mice (Figure 65C) (Denroche, Quong et al. 2013). However, the plasma leptin levels were dose-dependent for 1, 3, 5 μg, but the 10 μg dose induced less plasma leptin than the 5 μg, both in days 12 and 25 of the experiment (Figure 65D) (Denroche, Quong et al. 2013). Interestingly enough, the lowest dosage that caused normoglycemia, 5 μg, had higher levels of plasma leptin than the nondiabetic control mice, indicating that the higher levels of leptin are needed to induce euglycemia (Figure 65D) (Denroche, Quong et al. 2013). In day 25 the plasma leptin levels were lower in the two higher

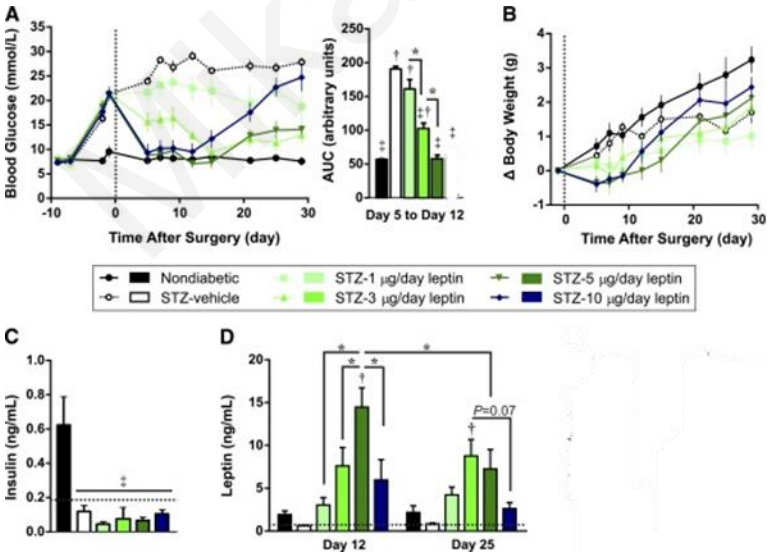


Figure 65 Leptin reverses STZ-induced diabetes in a dose-dependent manner. (A) Fasting blood glucose. (B) Change in body weight. (A) AUC analysis of blood glucose levels from day 5 to day 12. (C) 4-hour fasted plasma insulin levels on day 25. (D) 4-hour fasted plasma leptin levels on days 12 and 25. (Denroche, Quong et al. 2013)

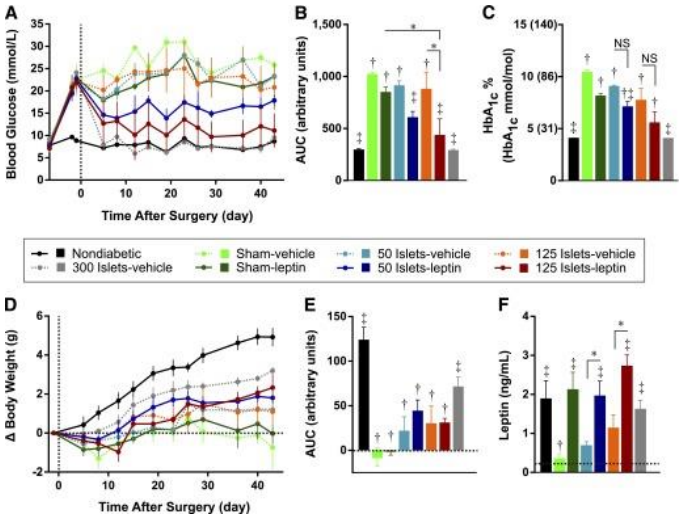


Figure 66 Islet transplantation efficacy is enhanced by leptin administration for the treatment of STZ-induced diabetes. (A) 4-hour fasted blood glucose levels. (B) Blood glucose data from days 5 to 43 analyzed by area under the curve. C: 4-hour fasted HbA1c levels in whole blood as percentages. (D) 4-hour fasted body weight gain normalized to day −1 and net area under the curve from days 5 to 43 (E). (F) 4-hour fasted plasma leptin. (Denroche, Quong et al. 2013)

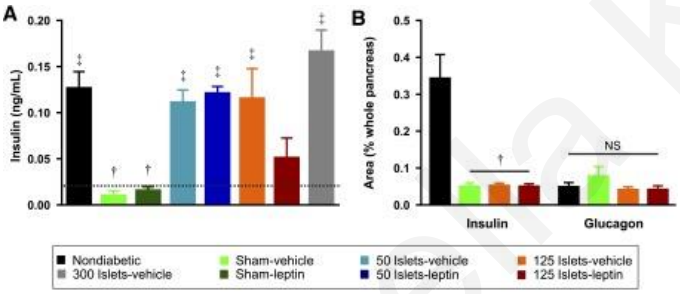


Figure 67 Leptin co-therapy does not induce the secretion of circulating insulin neither β-cell recovery. (A) 4-hour fasted plasma insulin levels. (B) Immunofluorescent quantifications of β-cell and α-cell areas. (Denroche, Quong et al. 2013)

dosages, whereas the 1 and 3 μg dosages maintained the same level of plasma leptin in both days (Figure 65D) (Denroche, Quong et al. 2013). This might be contributed to the fact that after day 15, the mice treated with 5 and 10 μg started to develop hyperglycemia, while mice treated with 1 and 3 μg maintained their effect blood glucose levels (Figure 65A) (Denroche, Quong et al. 2013). After a glucose tolerance test, the mice treated with 3 and 5 μg leptin daily followed the trend of the nondiabetic mice in glucose tolerance, with a hyperglycemia trend at glucose peak and hypoglycemia at the recovery phase, while the mice treated with 10 and 1 μg showed a trend toward glucose intolerance (Denroche, Quong et al. 2013). These results indicated that 1 μg of leptin per day does not reverse either hyperglycemia, neither glucose intolerance, thus this dose is the perfect match to inspect if leptin can improve the performance of islet transplantation (Denroche, Quong et al. 2013). Later, the scientists performed sham surgery, they transplanted syngeneic islets under the left kidney capsule, and simultaneously delivered 1 μg of leptin per day in STZ induced diabetic mice (Figure 66) (Denroche, Quong et al. 2013). Mice that were transplanted with 300 islets were back to normoglycemia levels quickly, this indicated that donor islets were both functional and viable (Figure 66A) (Denroche, Quong et al. 2013). However, the transplantation of 50 islets and 125 islets alone could not induce normoglycemia, neither could leptin treatment alone (Figure 66A) (Denroche, Quong et al. 2013). The combination treatment of both leptin and 50 or 125 islet transplantation was able to lower the

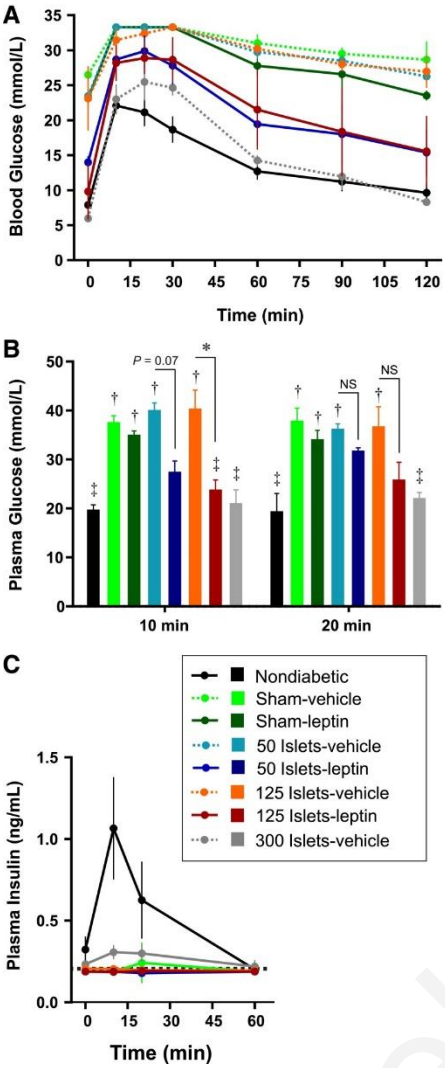


Figure 68 Co-therapy with leptin improves glucose tolerance. (A) Glucose tolerance test on day 20 after a 6-hour fast in mice. (B) Plasma glucose levels at 10 and 20 minutes. C: Plasma insulin levels. (Denroche, Quong et al. 2013)

blood glucose levels significantly, in comparison to untreated diabetic mice (Figure 66A) (Denroche, Quong et al. 2013). The mean blood glucose levels of mice treated with leptin and 125 transplanted islets, was nearly as low as mice that were transplanted with 300 islets (Figure 66A, B) (Denroche, Quong et al. 2013). HBA1C levels revealed the same effects on the long run, with the combination of 125 or 50 islets and leptin having reduced levels of HBA1C in comparison to untreated diabetic mice (Figure 66C) (Denroche, Quong et al. 2013). Plasma leptin levels were similar in mice co-treated with leptin and transplanted islets, nondiabetic controls and mice treated with 300 islets (Figure 66F) (Denroche, Quong et al. 2013). Insulin levels were not increased in mice treated with leptin alone, while mice treated with islets only showcased an increase in insulin production (Figure 67A) (Denroche, Quong et al. 2013). Co-treatment with leptin did not further increase insulin production, even though it decreased blood glucose, on the contrary the co-therapy of leptin and 125 islets decreased insignificantly the secretion of insulin (Figure 67A) (Denroche, Quong et al. 2013). In addition, the islet transplantation did not increase insulin cells in the pancreas, indication that the insulin increase was due to the graft (Figure 67B) (Denroche, Quong et al. 2013). After an oral blood glucose tolerance test, control diabetic mice had enhanced glucose intolerance, as well as the mice treated only with 50 or 125 islets or mice treated only with leptin (Figure 68A) (Denroche, Quong et al. 2013). In another note, mice that were treated with both leptin and islet grafts, were displaying great glucose tolerance, almost at the same level of the 300-islet transplanted diabetic mice and the nondiabetic controls (Figure 68A) (Denroche, Quong et al. 2013). Nonetheless, samples from plasma insulin levels 10 and 20 minutes after the glucose administration were taken (Denroche, Quong et al. 2013). The co-treatment with leptin and 50 islets had a nonsignificant decrease of plasma glucose in comparison to treatment

with 50 islets alone, counter to 125 islets and leptin that displayed a significant decrease at 10 minutes (Figure 68B) (Denroche, Quong et al. 2013). Nondiabetic mice had similar levels of plasma glucose with 300 islet transplanted mice and co-treated 125 islets mice at 10 minutes (Figure 68B) (Denroche, Quong et al. 2013). However, the plasma insulin levels were not increased in neither of the therapies used (Figure 68C) (Denroche, Quong et al. 2013). Lastly, plasma triglycerides, free fatty acids and β-hydroxybutyrate were measured to inspect the effect of leptin in lipid homeostasis (Denroche, Quong et al. 2013). In all three lipid metabolites, combinational therapy of leptin and islets allografts caused their normalization (Denroche, Quong et al. 2013).

Denroche, Kwon et al. injected mice with STZ to render them diabetic and they, then, treated them with recombinant murine leptin to understand what metabolic pathways leptin use to lower the blood glucose (Denroche, Kwon et al. 2015). The STZ diabetic mice developed hyperglycemia

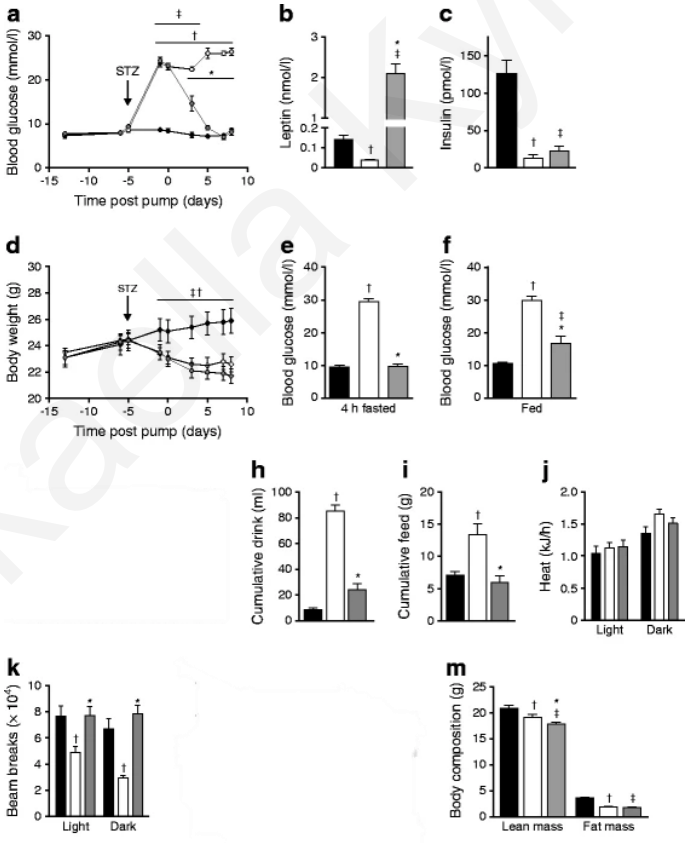
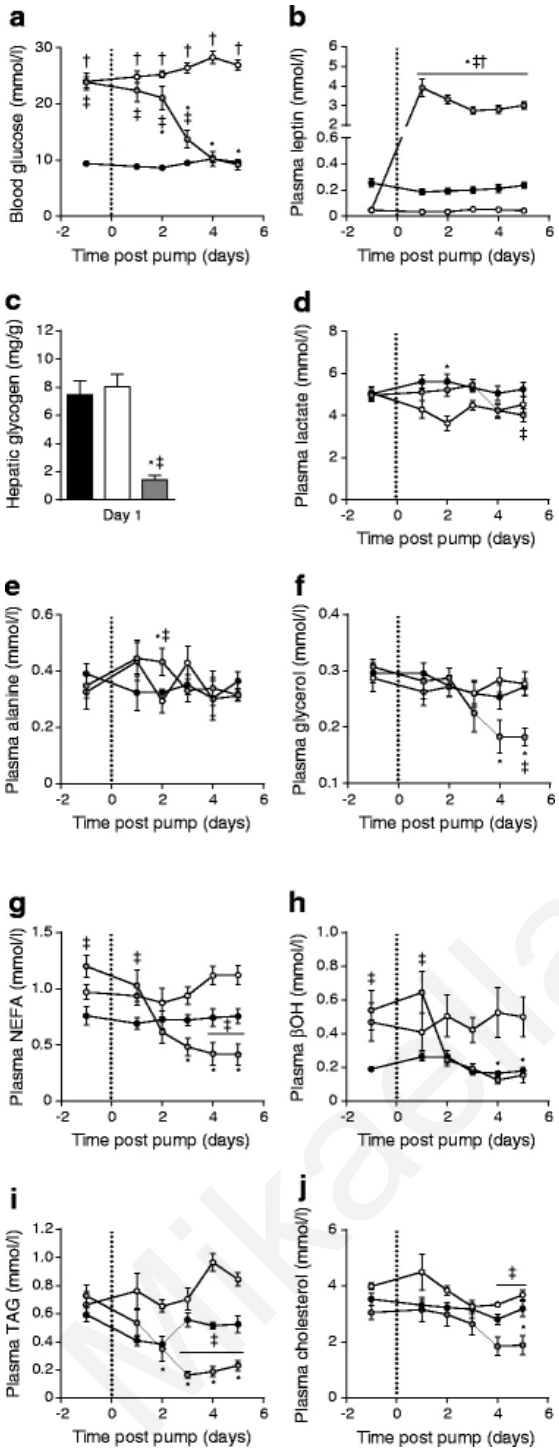


Figure 69 Leptin therapy reverses the disturbances of insulin-deficient diabetes. (a) 4- hour fasted blood glucose. (b, c) day 8 plasma leptin and insulin. (d) body weight. (e, f) 4-hour fasted and fed blood glucose levels on days 6 and 8 in a separate cohort, respectively. (h) Cumulative water intake, (i) food intake, (j) average energy expenditure, (k) average locomotor activity and (m) body composition. STZ–leptin (grey circles/bars); STZ–vehicle (white circles/bars); non-diabetic (black circles/bars) mice. (Denroche, Kwon et al. 2015)



and low plasma leptin levels and plasma insulin levels (Figure 69a, b, c) (Denroche, Kwon et al. 2015). However, treatment with leptin attenuated hyperglycemia to normal levels in day 8, where it increased plasma leptin levels more than normal and kept plasma insulin levels at the same levels of diabetic controls (Figure 69a, b, c) (Denroche, Kwon et al. 2015). Moreover, leptin did not change the weight of mice in comparison to STZ diabetic mice that were not administered leptin (Figure 69d) (Denroche, Kwon et al. 2015). Fasted blood glucose levels of leptin treated mice at 4 hours were near the levels of nondiabetic mice, whereas when fed the mice had higher blood glucose levels than nondiabetic controls and lower than STZ empty vector treated mice (Figure 69e, f) (Denroche, Kwon

et al. 2015). Leptin therapy corrected the need of mice to eat, drink and urinate excessively, as well as the decreased locomotor activity that were caused by the uncontrolled diabetes (Figure 69h, i, j, k) (Denroche, Kwon et al. 2015). On the other hand, leptin therapy reduced lean mass and fat mass in comparison to STZ diabetic mice (Figure 69m) (Denroche, Kwon et al. 2015). Transcript levels of Pck1, a control gene for the regulation of gluconeogenesis, were elevated in STZ mice whether they were treated with leptin or not and Slc2a2, the encoder of glucose transporter GLUT2, transcript levels were elevated in STZ diabetic mice with leptin treatment reducing its levels (Figure 70a) (Denroche, Kwon et al. 2015). After a pyruvate tolerance test blood glucose did not differ between leptin treated mice whether or not they were pyruvate injected, indicating that leptin does not constrain gluconeogenic activities (Figure 70b) (Denroche, Kwon et al. 2015). Hepatic glycogen, glucose and triacylglycerol were reduced significantly in leptin treated mice compared to STZ diabetic mice and nondiabetic controls (Figure 70c, d, e) (Denroche, Kwon et al. 2015). Hepatic cholesterol, however, remained at the same levels, as did free CoA levels (Figure 70f, h) (Denroche, Kwon et al. 2015). On the other hand, Acetyl-CoA levels raised in STZ diabetic mice and leptin therapy was able to decrease them, showcasing the higher glucose oxidation

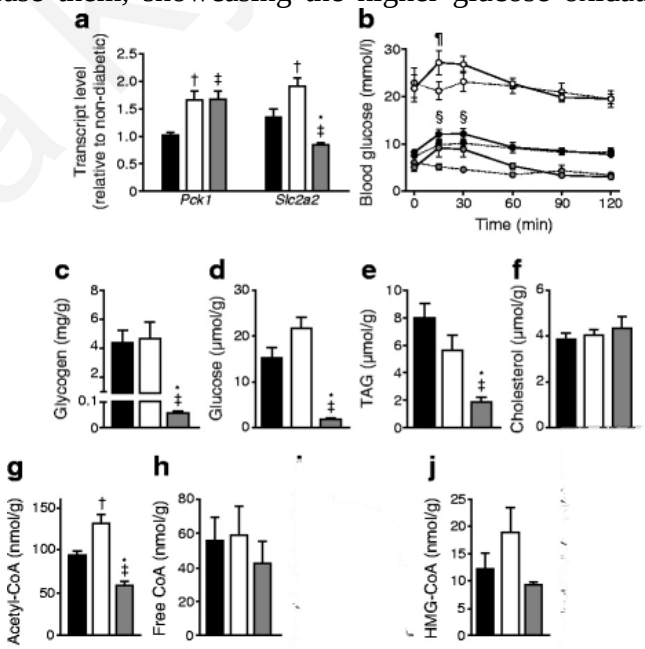


Figure 71 Reversal hyperglycemia kinetics by leptin correlates with reduced plasma glycerol. (a) 4-hour fasted blood glucose and (b) plasma levels of leptin, (c) hepatic glycogen, (d) lactate, (e) alanine, (f) glycerol, (g) NEFA, (h) β-hydroxybutyrate, (i) TAG and (j) cholesterol. STZ–leptin (grey circles), STZ–vehicle (white circles) and non-diabetic (black circles) mice. (Denroche, Kwon et al. 2015)

Figure 70 Leptin therapy depletes hepatic energy-yielding substrates. (a) RT-qPCR for the abundance of hepatic Pck1 and Slc2a2 transcripts. (b) Pyruvate tolerance test on day 5 in a separate cohort (pyruvate injection, solid line; vehicle injection, dotted line). (c) Hepatic glycogen, (d) glucose, (e) TAG and (f) cholesterol content, (g, h) acetyl-CoA and free CoA levels, (j) HMG-CoA. STZ–leptin (grey circles/bars), STZ–vehicle (white circles/bars) and non-diabetic (black circles/bars) mice. (Denroche, Kwon et al. 2015)

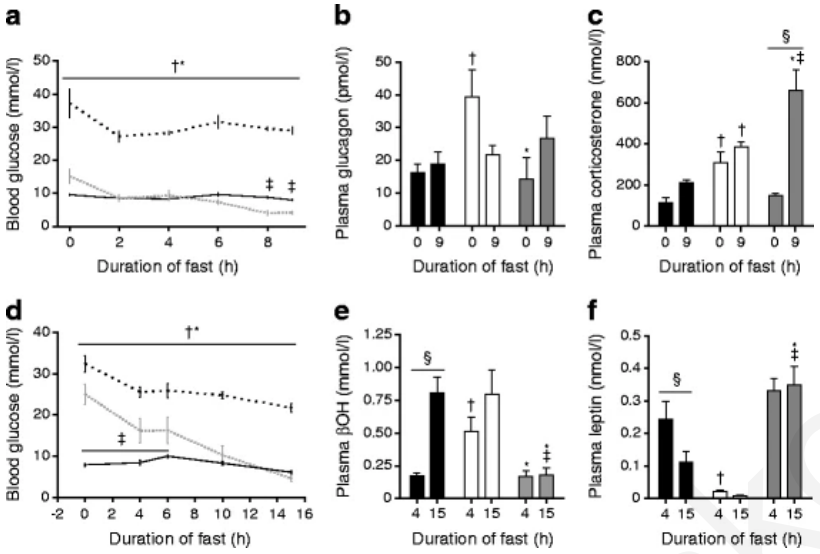


Figure 72 Leptin-treated STZ-diabetic mice are sensitive to prolonged fasting. One STZ–leptin mouse was saved at 8 h (a) and 14 h (d) because of hypoglycemia (blood glucose <2 mmol/l). Plasma glucagon (b), corticosterone (c), β-hydroxybutyrate (e) and leptin (f) were measured at the indicated times. STZ-diabetic mice were treated with leptin (grey bars/grey dotted line) at a dose of 20 μg/day (a–c) or 10 μg/day (d–f) and compared with STZ-vehicle (white bars/black dotted line) and non-diabetic controls (black bars/solid black line) on day 7 or 11 of treatment, respectively. (Denroche, Kwon et al. 2015)

compared to lipid oxidation (Figure 70g) (Denroche, Kwon et al. 2015). Hepatic 3-hydroxy-3-methykglutaryl (HMG)-CoA levels of leptin treated mice were reduced relative to diabetic mice, indicating the normalization of ketogenesis (Figure 70j) (Denroche, Kwon et al. 2015). Leptin therapy normalized blood glucose levels gradually and by day 4 it reached nondiabetic levels, while plasma leptin levels were elevated immediately after therapy (Figure 71a, b) (Denroche, Kwon et al. 2015). The hepatic glycogen levels were depleted in STZ-leptin mice in day 1 (Figure 71c) (Denroche, Kwon et al. 2015). Plasma lactate and alanine remained at the same levels (Figure 71d, e), while glycerol was decreased in leptin treated mice in comparison to diabetic and nondiabetic mice (Figure 71f) (Denroche, Kwon et al. 2015). Plasma TAG, NEFA and β-hydroxybutyrate, energy yielding substrates, levels were reduced in a similar way with blood glucose and glycerol (Figure 71g, h, i) (Denroche, Kwon et al. 2015). 2 hours after fasting STZ-leptin mice, administered 20μg of leptin daily, reached normoglycemia and at 6 hours they developed hypoglycemia (Figure 72a), plasma glucagon was at normal levels in non-fasting state and 9 hours after fasting it increased non significantly (Figure 72b), while corticosterone levels

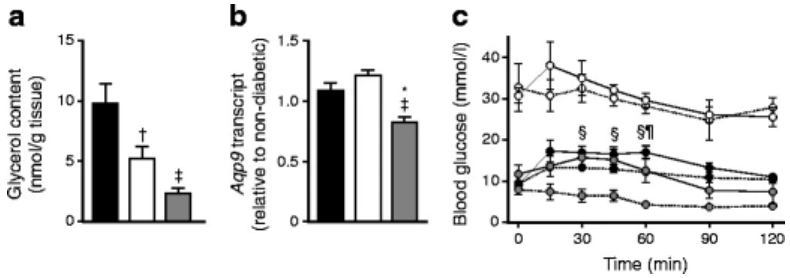


Figure 74 Glycerol injection prevents hypoglycemia in STZ–leptin-treated mice. Hepatic glycerol content (a) and transcript abundance of Aquaglyceroporin 9 (Aqp9) (b) in livers of 4 h fasted STZ–leptin (grey bars), STZ–vehicle (white bars) and non-diabetic mice (black bars), on day 8 of treatment. (c) Mice were injected with either glycerol (solid lines) or vehicle (dotted lines). (Denroche, Kwon et al. 2015)

were at nondiabetic levels at first, 9 hours of fasting significantly increased corticosterone (Figure 72c), indicating that corticosterone is not implicated in the decrease of glucose (Denroche, Kwon et al. 2015). When STZ mice were treated with 10μg of leptin daily, hyperglycemia was higher when the mice were fed, compared to the higher dose of leptin, and it decreased rapidly when the mice were at a fasting state, by 15 hours of fasting mice developed hypoglycemia (Figure 72d) (Denroche, Kwon et al. 2015). β-hydroxybutyrate levels were normalized at the leptin treated mice at 4 hours and remained at the same levels at 15 hours, while in non-diabetic controls it increased significantly (Figure 72e) (Denroche, Kwon et al. 2015). The leptin levels were diminished in STZ mice, while they maintained their high expression in leptin treated mice (Figure 72f) (Denroche, Kwon et al. 2015). On day 7, at 9 hours of fasting, β-hydroxybutyrate levels of STZ-leptin mice

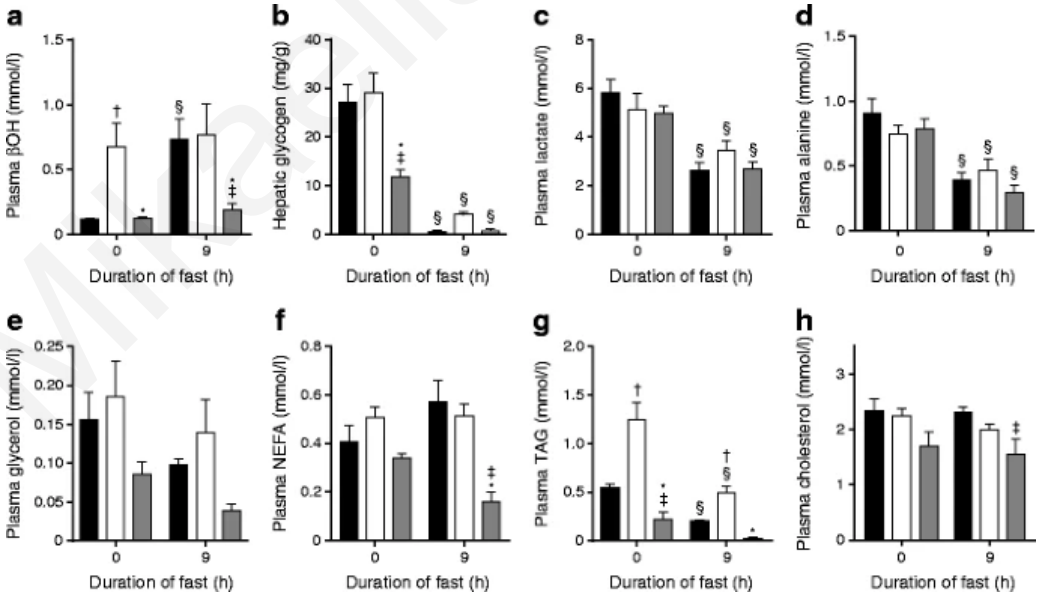


Figure 73 After a prolonged fasting of leptin-treated mice, the gluconeogenic substrate availability is altered. Plasma β-hydroxybutyrate (a), hepatic glycogen (b), plasma lactate (c), alanine (d), glycerol (e), NEFA (f), TAG (g) and cholesterol (h) were measured, STZ–leptin (grey bars), STZ–vehicle (white bars) and non-diabetic (black bars) mice. (Denroche, Kwon et al. 2015)

were low in comparison to non-diabetic and diabetic controls (Figure 73a) (Denroche, Kwon et al. 2015). Hepatic glycogen was decreased when the leptin mice were subjected to prolonged fasting (Figure 73b), showcasing the use of that glycogen by leptin mice (Denroche, Kwon et al. 2015). Plasma lactate and alanine was significantly reduced in all groups due to prolonged fasting, while leptin treatment did not cause further alternations (Figure 73c, d) (Denroche, Kwon et al. 2015). Moreover, glycerol levels were decreased in leptin mice when fasted, but not significantly (Figure 73e) (Denroche, Kwon et al. 2015). Fasted NEFA levels of leptin treated mice were decreased in comparison to the other two groups and triacylglycerol levels were also reduced, in both fasting and fed state (Figure 73f, g) (Denroche, Kwon et al. 2015). Glycerol and Aquaglyceroporin 9, which facilitates hepatic glycerol uptake, levels were low in STZ-leptin mice at 4 hours of fasting, compared to non-diabetic controls (Figure 74a, b) (Denroche, Kwon et al. 2015). When glycerol was added via injections at 7 hours of fasting to the three groups, STZ-leptin mice seemed to ameliorate hypoglycemia temporarily (Figure 74c), implying that glycerol plays a role in the leptin-induced fasting hypoglycemia (Denroche, Kwon et al. 2015).

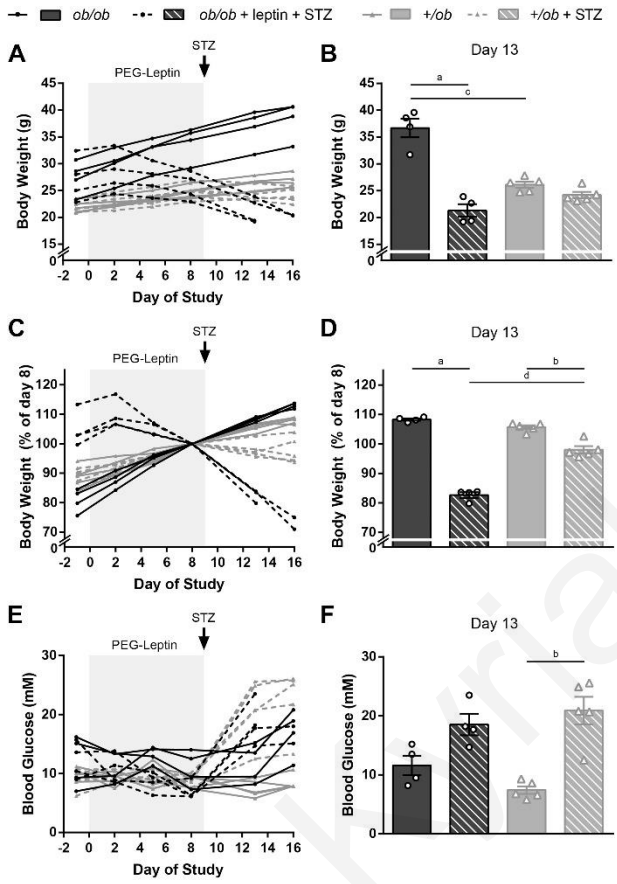


Figure 75 STZ-injected ob/ob mice experience extreme weight loss and mortality. From day 0 to 9 ob/ob mice were administered daily injections of PEGylated leptin. Raw body weight (A), body weight normalized to day 8 to observe the effect of STZ injection (C), and blood glucose levels (E) were measured after a 4-hour fast or at humane end point. Raw body weight (B), body weight normalized to day 8 (D), and blood glucose levels (F) on day 13. (Neumann, Kwon et al. 2018)

In 2018, Neumann, Kwon et al. developed STZ-induced diabetic ob/ob mice that were leptin-deficient to understand the action of insulin when leptin is absent and they, then, used a leptin antagonist, to block leptin increase, in insulin administered STZ diabetic mice to comprehend how leptin affects insulin therapy. The ob/ob mice were injected 5μg of PEGylated leptin daily until they reached the body weight of control +/ob mice and then at day 9 they were injected with 180mg/kg STZ (Figure 75A) (Neumann, Kwon et al. 2018). The STZ and PEG-leptin treated mice lost weight rapidly, by day 13 they lost approximately 20% of their body weight and by day 16 all mice reached a humane end point, while the +/ob mice treated or not with STZ did not suffer from significant weight loss, since they lost approximately 2% of their body weight by day 13 (Figure 75C and D) (Neumann, Kwon et al. 2018). Moreover, STZ and leptin treated mice had insignificantly higher blood glucose levels on day 13 than untreated mice controls, while STZ treated +/ob mice had significantly higher values of blood glucose than +/ob mice (Figure 75E, F) (Neumann, Kwon et al. 2018). In addition, ob/ob treated mice were producing less insulin, close

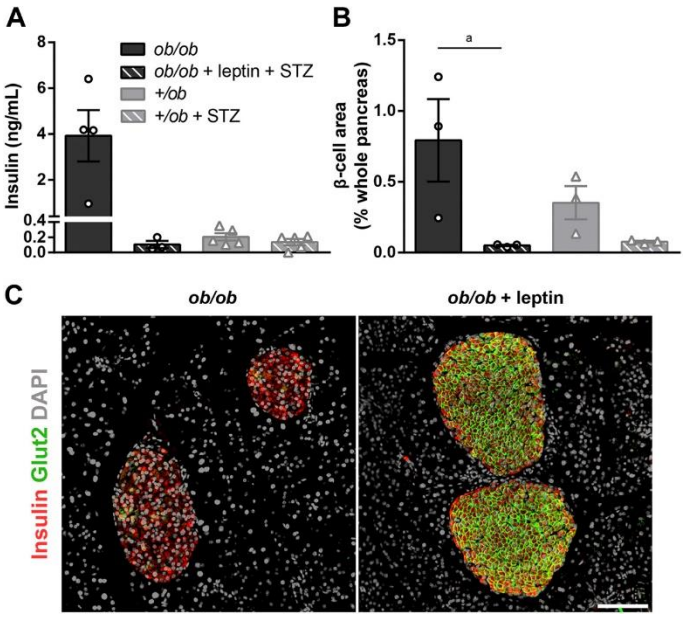


Figure 76 Leptinized ob/ob mice have increased GLUT 2 in β-cells, and injection of STZ leads in reduced β-cell area. (A) Plasma insulin, (B) β-cell area. (C) Representative images of pancreata from 1 ob/ob and 1 ob/ob + leptin mouse before STZ injection stained for cytoplasmic insulin (red), plasma membrane-associated GLUT 2 (green), and DAPI (gray). (Neumann, Kwon et al. 2018)

to the limit of detection, than the ob/ob mice, but similar levels to +/ob STZ mice (Figure 76A) (Neumann, Kwon et al. 2018). Similarly, the β-cell area of ob/ob leptin and STZ treated mice were significantly smaller than the ob/ob mice (Figure 76B) (Neumann, Kwon et al. 2018). However, ob/ob mice that were treated with leptin had high expression of GLUT2 in comparison to ob/ob untreated mice (Figure 76C) (Neumann, Kwon et al. 2018). High doses of STZ can act via GLUT2 and cause toxicity and death in mice, justifying the not so high blood glucose levels and the extreme weight loss observed previously (Neumann, Kwon et al. 2018). Thus, the same experiment was conducted but with lower dose of 140mg/kg STZ to not cause toxicity (Neumann, Kwon et al. 2018). With this dose, ob/ob mice lost weight rapidly but did not reach humane end point like the higher dose treated mice, while the blood glucose had higher value, suggesting less STZ toxicity (Figure 77A, B) (Neumann, Kwon et al. 2018). At day 14 a slow-release insulin pellet was implanted to STZ treated mice, ob/ob leptin treated ones had higher blood values in comparison to +/ob mice, especially on day 17 and 21, showcasing that leptin plays some kind of role in insulin therapy via mediating in glucose (Figure 77B) (Neumann, Kwon et al. 2018). Moreover, the scientists used a plasmid to deliver a leptin antagonist in STZ diabetic mice, that significantly increased the body weight of the mice comparing with control STZ diabetic mice (Neumann, Kwon et al. 2018). The leptin

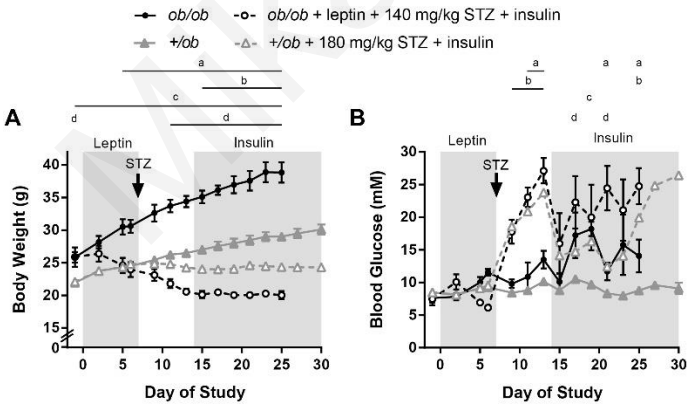


Figure 77 STZ diabetic ob/ob mice respond less robustly to insulin therapy than STZ diabetic controls. 4-hour-fasted body weight (A) and blood glucose (B). (Neumann, Kwon et al. 2018)

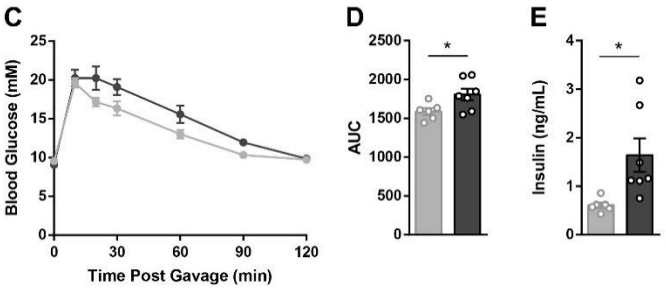


Figure 78 Wild-type mice expressing leptin antagonist exhibit increased body weight, glucose intolerance, and elevated plasma insulin levels. On day 9, mice were force-fed with glucose following a 4-h fast, blood glucose levels were monitored (C), and area under the curve was calculated (D). On day 15, 4-hour-fasted plasma insulin levels were analyzed (E). Plasmid encoding a leptin antagonist (pLA, dark grey); empty plasmid (pEmpty, light grey). (Neumann, Kwon et al. 2018)

antagonist managed to increase glucose intolerance, while it significantly increased insulin levels (Figure 78), rendering this method of delivering the leptin antagonist, successful (Neumann, Kwon et al. 2018). When leptin antagonist was injected in mice 3 days before insulin treatment, the weight remained the same as the empty vector treated mice, while the fasting blood glucose was improved in insulin treated mice whether they received leptin antagonist or not (Figure 78) (Neumann, Kwon et al. 2018). However, during days 8 to 12 the empty vector treated mice had significantly lower values of blood glucose than the mice treated with leptin antagonist (Figure 80B) (Neumann, Kwon et al. 2018). On day 6 and day 14 glucose tolerance test was performed and as expected STZ worsened the tolerance to glucose, but insulin had corrected it, with therapy or not with leptin antagonist did not make a difference in the values (Figure 80C-F) (Neumann, Kwon et al. 2018). Then the scientists treated the mice with PEGylated form of leptin antagonist, there were no differences between insulin treated mice that received or not the PEGylated antagonist (Figure 79A) (Neumann, Kwon et al. 2018). At the same time, STZ caused higher values of fasting blood glucose, with insulin therapy lowering them and PEGylated leptin antagonist debilitating the action of insulin therapy (Figure 79B) (Neumann, Kwon et al. 2018). After a glucose tolerance test, blood glucose of mice treated with the PEGylated antagonist had higher values than the mice treated with an empty vector (Figure 79C) (Neumann, Kwon et al. 2018). The leptin antagonist caused an increase in the epididymal fat pad weight, while STZ caused a decrease and insulin treatment caused a mild increase (Figure 81A) (Neumann, Kwon et al. 2018). Leptin levels were rescued when STZ diabetic mice were treated with insulin almost at control nondiabetic mice (Figure 81B) (Neumann, Kwon et al. 2018). These outcomes indicated that leptin has a role in glucose homeostasis.

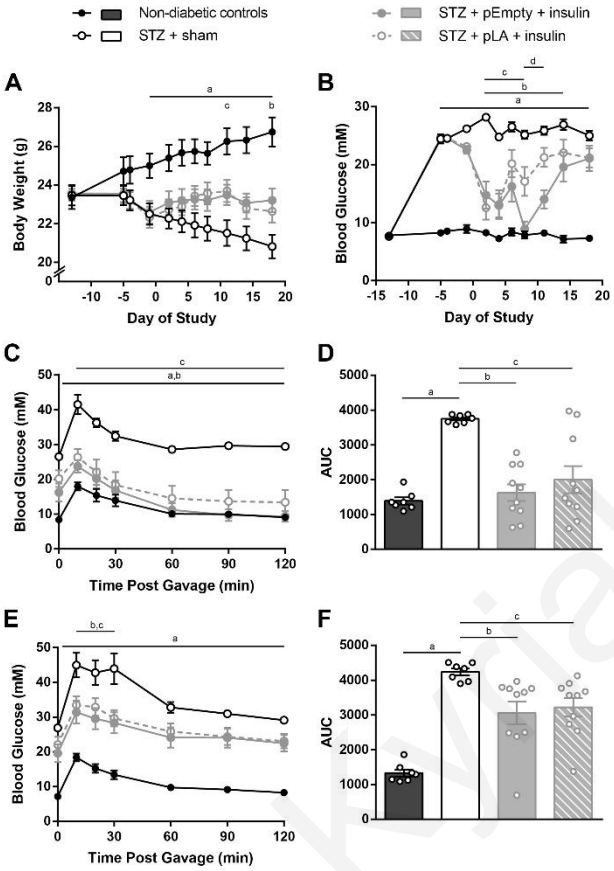


Figure 80 STZ diabetic mice experience an attenuated insulin-mediated lowering of blood glucose when expressing a leptin antagonist. 4-hour-fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 6 (C and D) and day 14 (E and F) an oral glucose tolerance test was performed and area under the curve was calculated. (Neumann, Kwon et al. 2018)

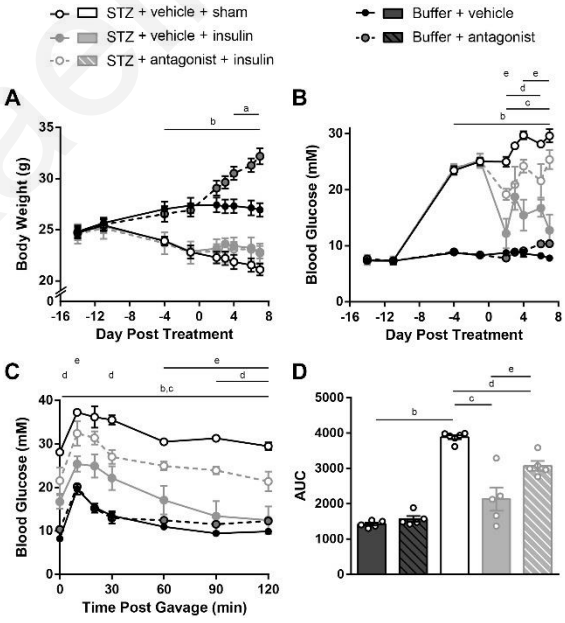


Figure 79 STZ diabetic mice respond less robustly to insulin therapy when leptin action is blocked. 4-hour-fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 6, a glucose tolerance test was performed (C) and area under the curve was calculated (D). (Neumann, Kwon et al. 2018)

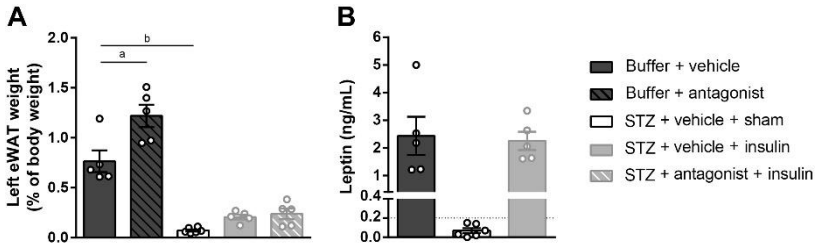


Figure 81 Leptin antagonist peptide augments epididymal white adipose tissue (WAT) weight in nondiabetic mice, and insulin therapy reestablishes leptin levels in STZ diabetic mice. 4-h fast, epididymal WAT (eWAT) weight was measured (A), and plasma leptin levels were measured (B), on day 7. (Neumann, Kwon et al. 2018)

Combinational therapies

The previously discussed publishments were only concerning at one protein treatment at a time; however, two or three proteins have been found to work synergistically in order to treat T1D.

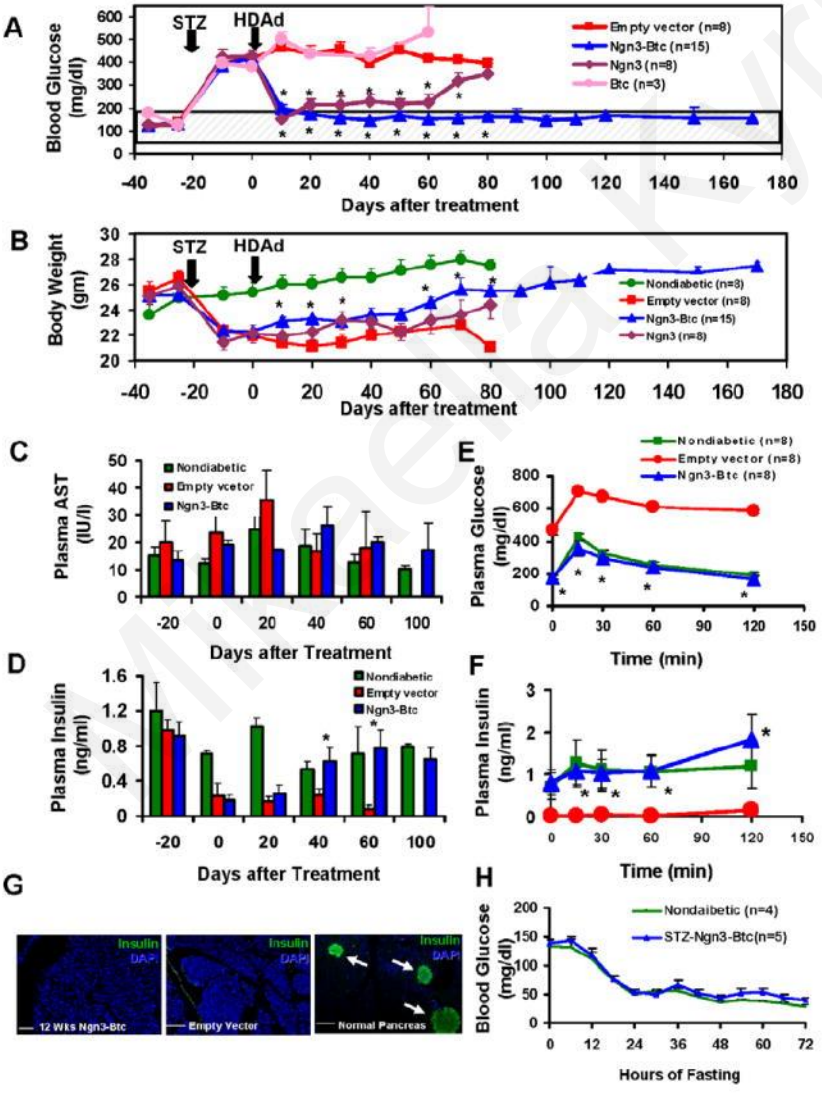


Figure 82 Co-therapy with Ngn3 and BTC reverse diabetes effectively. (A) Fasting blood glucose, (B) body weight, (C) Plasma aspartate aminotransferase (AST), (D) fasting insulin, (E) Plasma glucose, (F) insulin during a glucose tolerance test, (G) Representative pancreas sections of STZ-diabetic mice treated with either Ngn3-Btc or empty vector are shown along with nondiabetic control by insulin IF (green) and by DAPI nuclear stain (blue), (H) Blood glucose during a 72 h fast. (Yechoor, Liu et al. 2009)

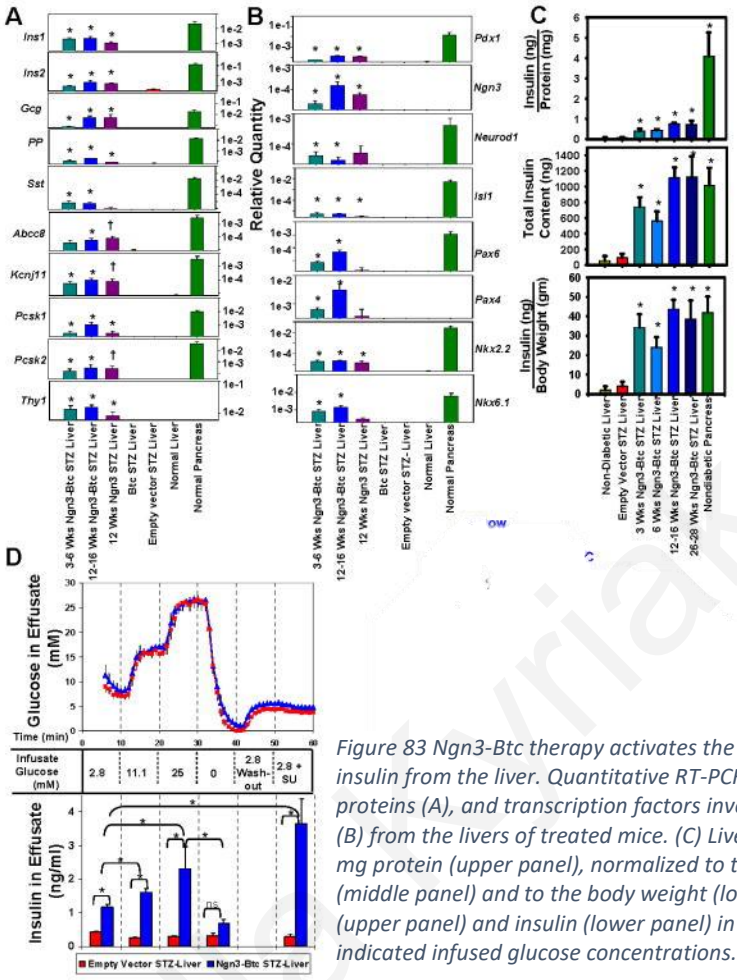


Figure 83 Ngn3-Btc therapy activates the production of regulated insulin from the liver. Quantitative RT-PCR for islet hormones, proteins (A), and transcription factors involved in islet development (B) from the livers of treated mice. (C) Liver insulin content (ng) per mg protein (upper panel), normalized to the whole organ weight (middle panel) and to the body weight (lower panel (D) Glucose (upper panel) and insulin (lower panel) in effusate are shown at indicated infused glucose concentrations. (Yechoor, Liu et al. 2009)

In a research that was conducted in 2009, Yechoor, Liu et al., used helper dependent adenoviral vectors to deliver Ngn3 and Btc in to diabetic STZ mice. When Ngn3 was injected into the mice, a rapid correction of blood glucose was achieved, however these results were temporary (Figure 82A) (Yechoor, Liu, Espiritu et al. 2009). Btc was added to augment the Ngn3 results, with the outcome of lasting six months (Figure 82A) (Yechoor, Liu, Espiritu et al. 2009). Ngn3-Btc treated animals displayed high aspartate aminotransferase, a biomarker for liver health, and gained weight (Figure 82B, C) (Yechoor, Liu, Espiritu et al. 2009). Moreover, their insulin levels were approximately at the same level of non-diabetic mice (Figure 82D) (Yechoor, Liu, Espiritu et al. 2009). The glucose tolerance test indicated that Ngn3-Btc treated mice responded well, with near normal glucose absorption and insulin secretion (Figure 82E, F) (Yechoor, Liu, Espiritu et al. 2009). After three days of fasting, the treated mice stopped secreting insulin, without resulting to a hypoglycemia state (Figure 82H) (Yechoor, Liu, Espiritu et al. 2009). In Ngn3-Btc, as well as Ngn3 treated mice insulin mRNA was expressed, while the first insulin secretion could be detected

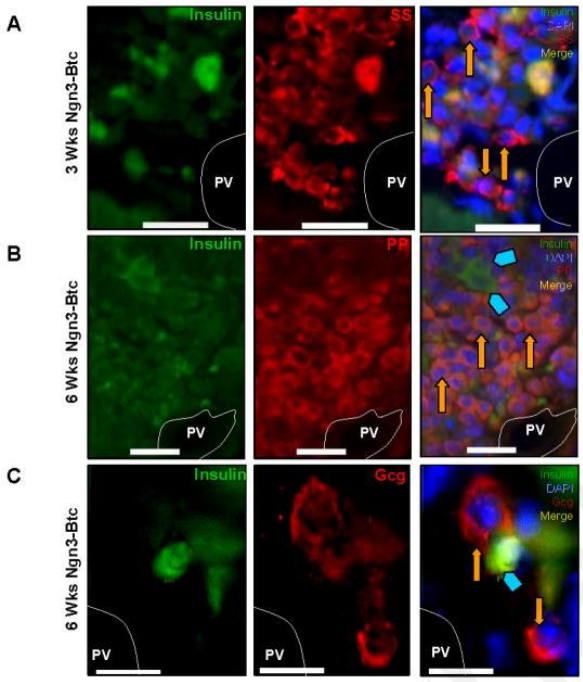


Figure 84 Ngn3-Btc-induced neo-islets express individual islet hormones. Representative sections of Ngn3-Btc treated STZ-diabetic mouse liver stained by IF for insulin – left panels; pancreatic polypeptide (PP), somatostatin (SS) or glucagon (Gcg) - middle panels; or merged images - right panels. Individual hormone-producing cells can be seen for each of the hormones (blue arrows for insulin and orange arrows for others). (Yechoor, Liu et al. 2009)

3 weeks after treatment and it plateaued at 12-16 weeks (Figure 83A, C) (Yechoor, Liu, Espiritu et al. 2009). When the scientists provided glucose at different concentrations to the liver of the Ngn3-Btc treated mice, in situ, the insulin production seemed to respond accordingly (Figure 83D) (Yechoor, Liu, Espiritu et al. 2009). Islet hormones and β-cell transcripts were expressed in livers of mice treated with Ngn3-Btc or Ngn3 alone (Figure 83B) (Yechoor, Liu, Espiritu et al. 2009). Moreover, Ngn3-Btc treated mice, expressed high levels of insulin, in parenchymal hepatocytes, 3 weeks after treatment and 6 weeks after treatment, insulin expression faded (Yechoor, Liu, Espiritu et al. 2009). When insulin expression in the hepatocytes diminished, cell clusters, that were expressing immunoreactive insulin, emerged in the periportal region in both Ngn3 and Ngn3-Btc treated mice (Yechoor, Liu, Espiritu et al. 2009). In the periportal area islet hormone, e.g. somatostatin, pancreatic polypeptide and glucagon, producing cells were expressed (Figure 84) (Yechoor, Liu, Espiritu et al. 2009). The periportal neo-islets seemed to derive from oval cells and to cluster closer with pancreatic β-cells rather than hepatocytes or oval cells (Yechoor, Liu, Paul et al. 2009).

Like the previous research, a combination of Ngn3 and Btc treatment in mice was executed. When diabetic mice, that were administered STZ, were treated with helper dependent adenoviral vectors that expressed Ngn3 and Btc, the blood glucose levels decreased to normal levels and the insulin secretion increased to normal levels (Yechoor, Liu, Paul et al. 2009). In addition, this combination treatment corrected the ketonemia, by decreasing the levels of plasma β-hydroxybutyrate (Figure 85A) (Yechoor, Liu, Paul et al. 2009). When Ngn3 and Btc treated mice's hepatocytes were isolated, the glucose that was secreted was approximately the same as the control nondiabetic mice, in contrast with the empty vector's which was higher (Figure 85B, C) (Yechoor, Liu, Paul et al. 2009). The liver glycogen, in Ngn3 and Btc treated mice, was restored and approximately at the same level of the control mice (Figure 85D) (Yechoor, Liu, Paul et al. 2009). Moreover, this treatment seemed to correct the nonesterified fatty acids and triglycerides back to normal levels

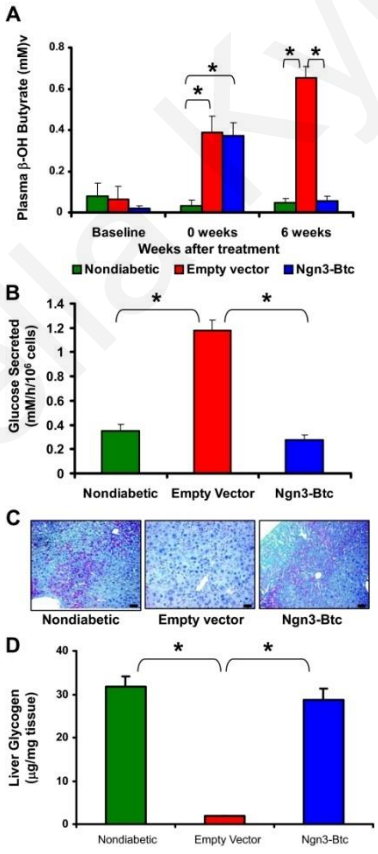


Figure 85 Ngn3-Btc reverses ketonemia and rescues glucose metabolism. (A) Fasting plasma β-hydroxybutyrate. (B) In vitro glucose secretion from hepatocyte. (C) Representative sections of mouse liver stained for glycogen with periodic acid-Schiff staining. (D) Liver glycogen content. (Yechoor, Liu, Paul et al. 2009)

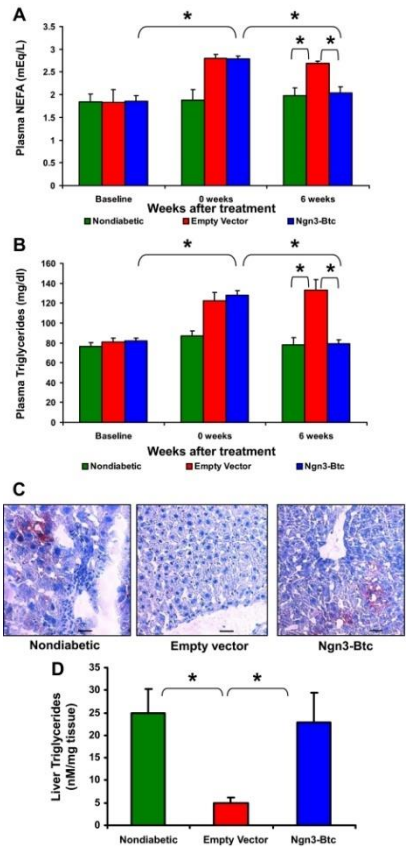


Figure 86 Ngn3-Btc therapy normalizes hepatic lipid metabolism. Fasting plasma nonesterified fatty acid (NEFA) (A) and triglycerides (B). (C) Representative sections of mouse liver stained for neutral lipid with Oil Red O. (D) Liver triglyceride content. (Yechoor, Liu, Paul et al. 2009)

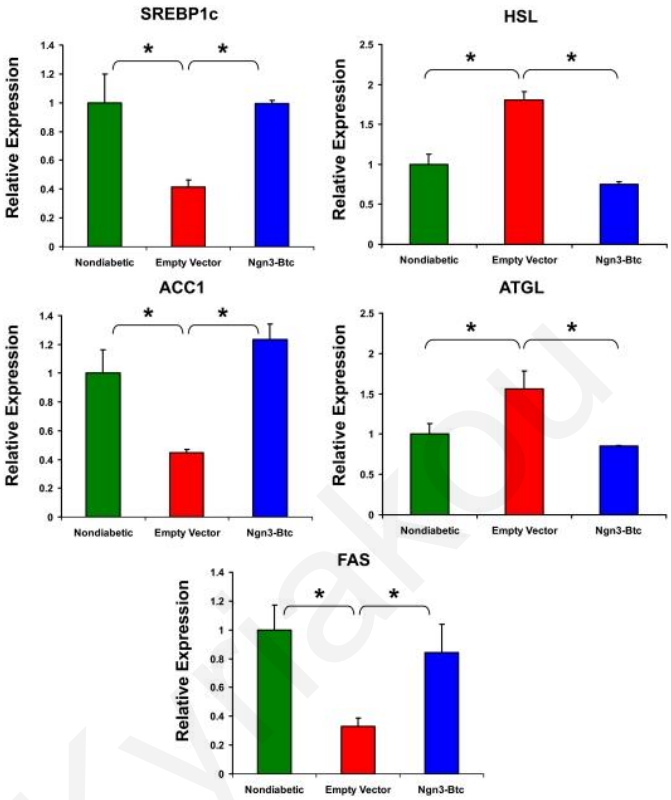


Figure 87 Ngn3-Btc therapy normalizes hepatic lipogenesis and lipolysis-related gene transcripts. Quantitative RT-PCR for key lipogenesis and lipolysis-related gene transcripts in the liver from STZ-diabetic mice 6 weeks after the indicated treatment. (Yechoor, Liu, Paul et al. 2009)

(Figure 86A, B), since when diabetes occurs there is a disruption of lipid metabolism, with higher lipolysis rates and lower lipogenesis, that is why there is of weight loss in T1D patients (Yechoor, Liu, Paul et al. 2009). Ngn3 and Btc treatment, also, upregulated the liver triglycerides back to normal levels (Figure 86D) (Yechoor, Liu, Paul et al. 2009). Genes that are involved in lipogenesis, like Srebp1c, ACC1 and FAS, were upregulated and corrected in the treated mice, while genes that are involved in lipolysis, like HSL and ATGL, are downregulated and corrected in comparison to untreated diabetic mice (Figure 87) (Yechoor, Liu, Paul et al. 2009). After a transcriptome analysis of RNA in the neo-islets, the transcripts of insulin, pancreatic polypeptide and somatostatin, seemed to be in similar levels as the native pancreatic islets, while the transcript of glucagon was at a lower level (Figure 88A) (Yechoor, Liu, Paul et al. 2009). The qPCR results in the genes confirmed the previous results, with insulin, glucose transporter 2, pancreatic and duodenal

homeobox 1 and FFA receptor 1 being at the same levels as the native pancreatic cells, while glucagon was downregulated (Figure 88B) (Yechoor, Liu, Paul et al. 2009). The similarity between transcriptome of the induced neo-islets and the native pancreatic islets was extreme (Figure 88C) (Yechoor, Liu, Paul et al. 2009). Lastly, the Ngn3-Btc neo-islets shared similar morphology with the native pancreatic islets (Figure 89) (Yechoor, Liu, Paul et al. 2009). This strategy seems to normalize both glucose and lipid metabolism in STZ mice.

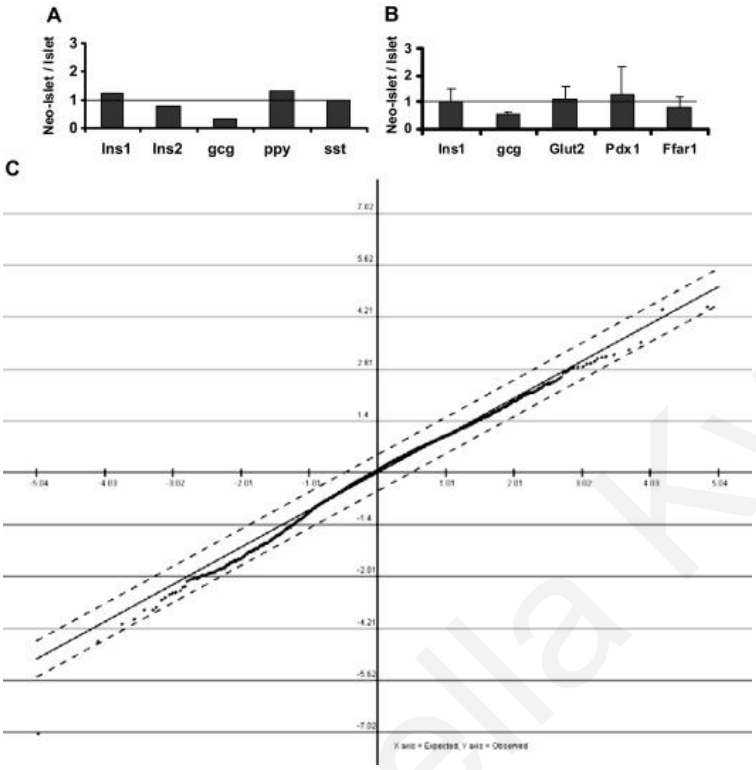


Figure 88 Ngn3-Btc-induced neo-islets express individual islet hormones. (A) Microarray-derived expression of pancreatic hormone transcripts from Ngn3-Btc induced neo-islets compared with that of native pancreatic islets. (B) Quantitative RT-PCR for islet-related genes from neo-islets. (C) A SAM plot showing the close similarity of the expressed transcript profiles of Ngn3-Btc-induced neo-islets and normal pancreatic islets. (Ins1) Insulin1; (Ins2) insulin2; (gcg) glucagon; (ppy) pancreatic polypeptide; (sst) somatostatin; (Glut2) glucose transporter 2; (Pdx1) pancreatic and duodenal homeobox 1 (Ipf1); (Ffar1) FFA receptor 1 (GPR40). (Yechoor, Liu, Paul et al. 2009)

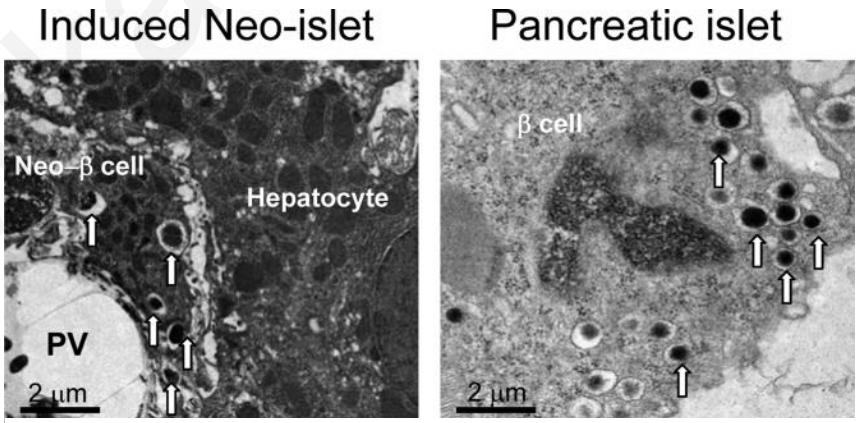


Figure 89 Ngn3-Btc-induced neo-islets have a similar structure to pancreatic β-cells. Electron micrograph of an induced neo-islet in the periportal region from a Ngn3-Btc-treated mouse liver (left panel). Arrows, Electron-dense granules with a halo typical of mature insulin secretory granules seen in a normal pancreatic islet (right panel). PV, Portal vein. (Yechoor, Liu, Paul et al. 2009)

In a follow-up research by Li, Buras et al., a gene therapy treatment using Ngn3-Btc and suppressors of cytokine signaling (SOCS1) was used in NOD mice. Gene therapy with the delivery of Ngn3 and Btc led to a non-permanent improvement of hyperglycemia and variable body weight in Nod mice opposed to the permanent diabetes symptoms of STZ diabetic mice (Li, R., Buras et al. 2015). These transient results were due to infiltration of the insulin expressing cell clusters, that were located in the periportal regions of the liver, with lymphocytes, revealing an autoimmunity response (Figure 90) (Li, R., Buras et al. 2015). In an effort to abolish this autoimmune response, the scientists used SOCS1 c-DNA, led by the rat insulin promoter using helper-dependent adenovirus vector (RipSOCS1), which would only express the SOCS1 to the insulin-expressing neo-islets in order to protect them from cytokine mediated cell apoptosis, while in the non-insulin cells it would not be expressed (Li, R., Buras et al. 2015). Approximately 50% of the NOD mice

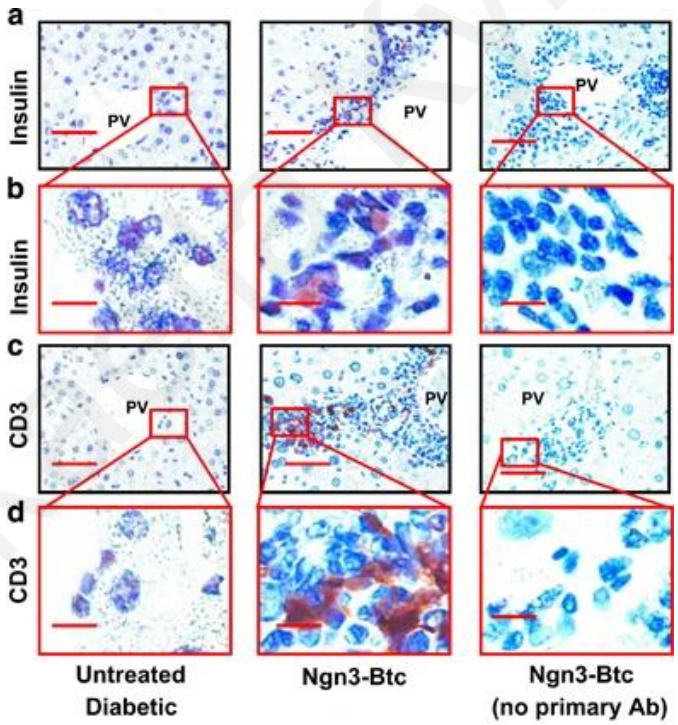


Figure 90 Ngn3-Btc treatment leads to infiltration in the insulin-expressing cell clusters in the periportal regions of the liver. (A) Immunochemistry for insulin-positive cells. (C) CD3 immunostaining for insulin-positive cells and infiltration. (B, D) magnifications. Portal vein (PV). (Li, R., Buras et al. 2015)

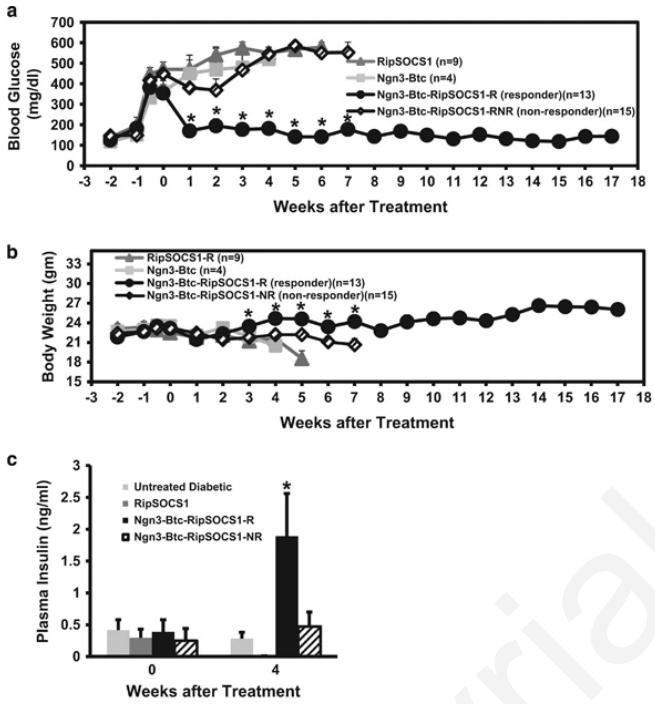


Figure 91 Ngn3-Btc-RipSOCS1 treatment leads to diabetes reversal 50% of the times. (a) blood glucose, (b) body weight, (c) plasma insulin levels. (Li, R., Buras et al. 2015)

that were treated with SOCS1 had achieved normoglycemia and maintained it, gained body weight, and had upregulation of their insulin, while the other 50% remained hyperglycemic but they had detectable insulin levels (Figure 91) (Li, R., Buras et al. 2015). The treated mice that were hyperglycemic lived for linger time than the Ngn3-Btc treated mice and the control mice (Figure 91) (Li, R., Buras et al. 2015). After a glucose tolerance test, the treated NOD mice that were responsive had normalized blood glucose levels and insulin levels, maintaining glucose homeostasis (Figure 92), and indicating the emergence of the functioning insulin secreting β-cells

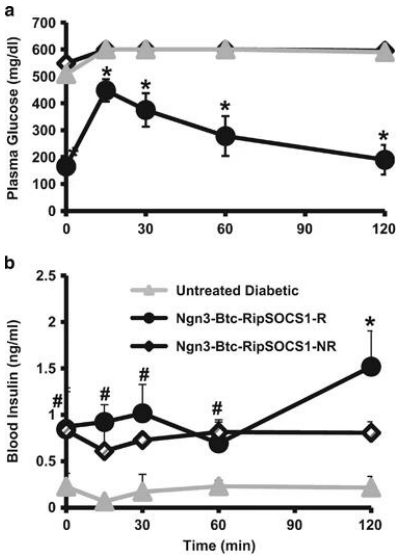


Figure 92 Ngn3-Btc-RipSOCS1 co-therapy leads to glucose tolerance. (a)blood glucose levels after a glucose tolerance test, (b) blood insulin levels. (Li, R., Buras et al. 2015)

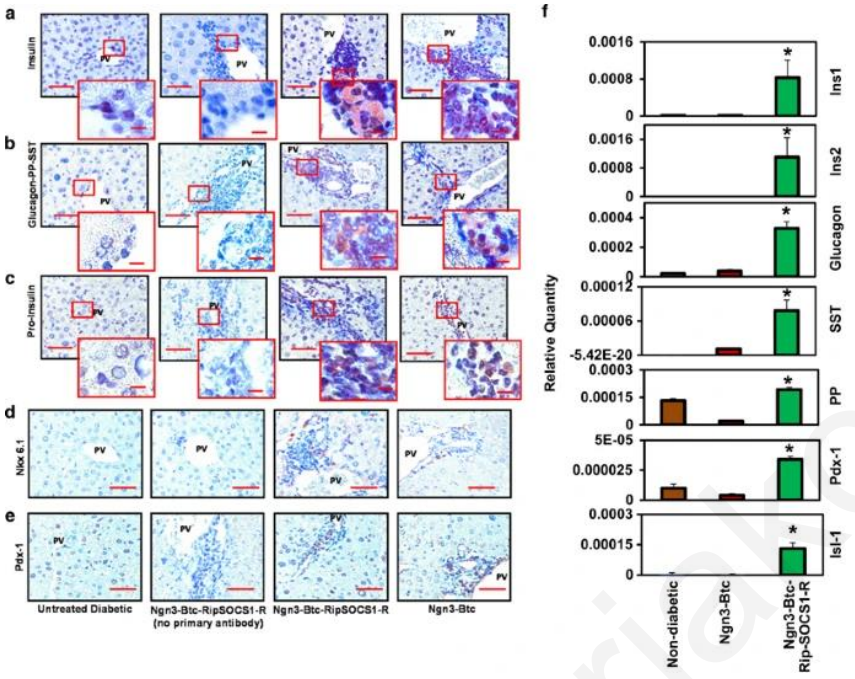


Figure 93 Co-therapy of Ngn3-Btc-RipSOCS1 leads to hormone-positive periportal "neo-islets". Immunochemistry for (A) insulin, (B) other islet hormones, (C) proinsulin, (D) Nkx6.1 and (E) Pdx1. (F) RT-qPCR for islet hormones and transcription factors from mice livers. (Li, R., Buras et al. 2015)

(Li, R., Buras et al. 2015). In the treated mice insulin-producing cells were found in the periportal areas of the liver, while in the Ngn3-Btc treated mice these cells were found rarely and in the untreated NOD mice they did not exist (Figure 93a), indicating the fact that SOCS1 had rescued the neo-islets from autoimmunity (Li, R., Buras et al. 2015). The neo-islets expressed islet hormones and islet specific transcription factors (Figure 93b-f) (Li, R., Buras et al. 2015). In the treated with Ngn3-Btc and Ngn3-Btc-SOCS1 mice, there was CD3 positive expression, suggestive of insulitis, in the periportal areas and expression of TNF-α, suggestive of activation of lymphocytes (Li, R., Buras et al. 2015). In a final testing, splenocytes from treated euglycemic and hyperglycemic mice were transferred to NOD-Scid mice that were driven to hyperglycemia, to confirm that the SOCS1 gene therapy does not induce peripheral immunosuppression (Li, R., Buras et al. 2015).

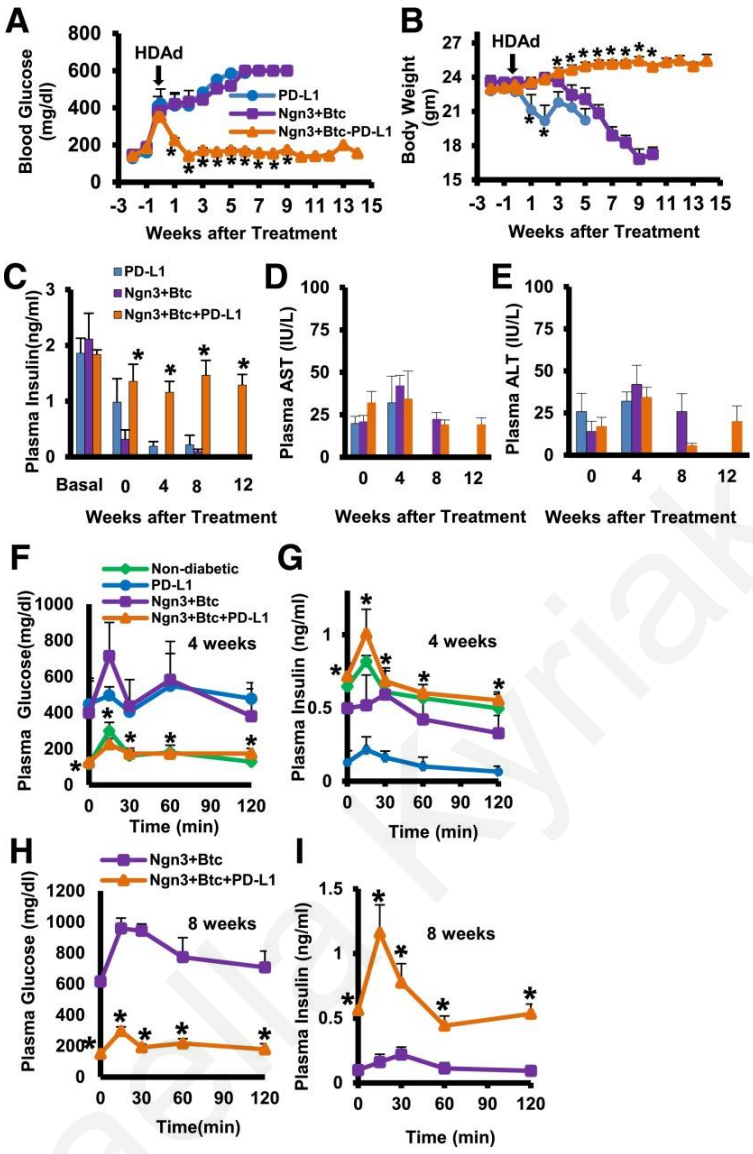


Figure 94 Ngn3-Btc and PD-L1 restores glucose homeostasis. (A) blood glucose, (B) body weight, (C) plasma insulin levels, (D) Plasma Aspartate aminotransferase, € plasma Alanine aminotransferase, (F, H) plasma glucose and (G, I) plasma insulin after a glucose tolerance test. (Li, R., Lee et al. 2015)

In another study of 2015, Li, Lee et al., used combinational therapy of Ngn3 and Btc, with the overexpression of programmed death ligand-1 (PD-L1), to be expressed only in insulin-expressing cells, in NOD mice (Li, R., Lee et al. 2015). Gene therapy, in overtly diabetic mice, that combined all three factors ameliorated blood glucose levels and kept the body weight of NOD mice stable opposed to Ngn3-Btc or PD-L1 treatment (Figure 94A, B) (Li, R., Lee et al. 2015). Moreover, this triple gene therapy caused the normal circulation of insulin without any toxicity on liver function, shown by aspartate aminotransferase and alanine aminotransferase (Figure 94D, E) (Li, R., Lee et al. 2015). Ngn3-Btc and PD-L1 treatment's blood glucose levels and insulin levels after a glucose

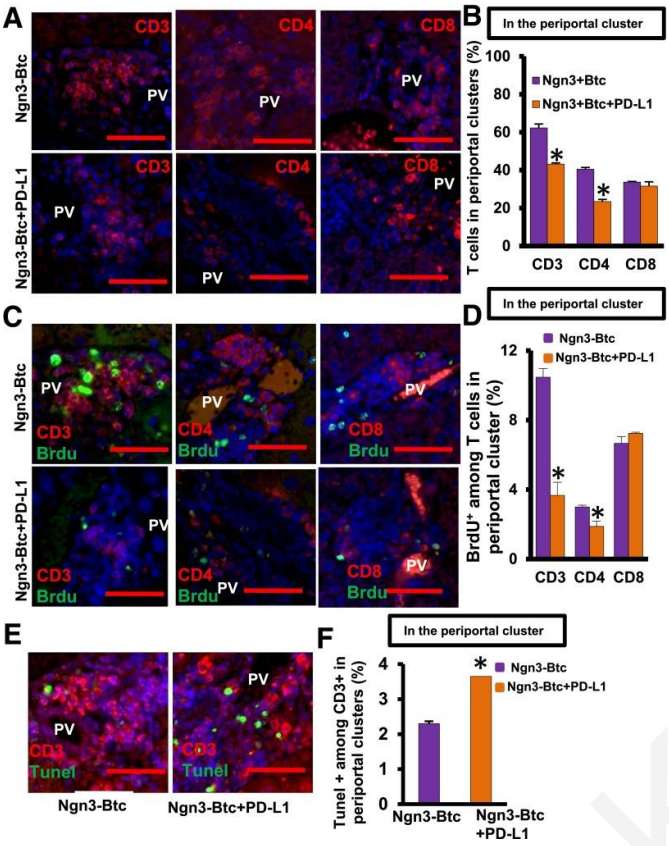


Figure 95 Ngn3-Btc and PD-L1 reduces the local number of CD4 T cells. (A) Livers stained for CD3, CD4 and CD8. (B) percentage of T cells. (C) Livers stained for BrdU. (D) percentage of BrdU. (E) livers stained with Tunel. (F) percentage of Tunel. (Li, R., Lee et al. 2015)

as well as the regulation of pancreatic islet hormones synthesis and secretion (Panneerselvam, Kannan et al. 2019). In Ngn3-Btc and PD-L1 treated mice, there was a decrease in the CD3 T cells, that was because of the reduced CD4 T helper cells (Figure 95A-B) (Li, R., Lee et al. 2015). This decrease was a result of lower rate of proliferation and higher rate of apoptosis in CD3 cells, as indicated by BrdU and Tunel levels, respectively (Figure 95C-F) (Li, R., Lee et al. 2015). Furthermore, there was a significant decrease of expression interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) in CD4 cells in mice treated with Ngn3-Btc and PD-L1 (Figure 96) (Li, R., Lee et al. tolerance test, correlated with the non-diabetic normal levels and were significantly lower than the Ngn3-Btc treatment alone (Figure 94F-I) (Li, R., Lee et al. 2015). In addition, this gene therapy led to the formation of periportal neo-islets that highly expressed the four major islet hormones, glucagon, insulin, somatostatin and pancreatic polypeptide, with these results being persistent over time (Li, R., Lee et al. 2015). Ngn3-Btc and PD-L1 neo-islets, also, had a significant increase of the expression of the islet transcription factors, Pdx1, Pax6 and Nkx6.1 in comparison to Ngn3-Btc neo-islets (Li, R., Lee et al. 2015). Pax6 has a central role in the pancreatic development and differentiation,

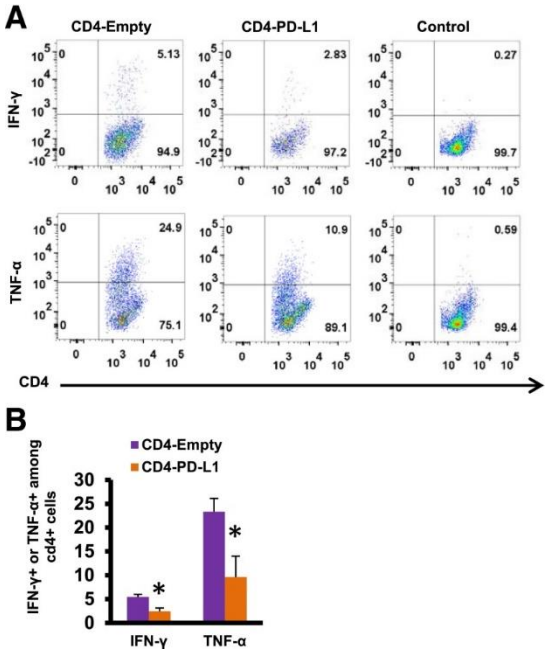


Figure 96 IFN-γ and TNF-α proteins were assessed in isolated CD4+ T cells purified from the spleen of nondiabetic NOD mice. (Li, R., Lee et al. 2015)

2015). Many cytokines, including those that are implicated in pathogenesis of diabetes in NOD mice, are downregulated in the treated mice with PD-L1 in comparison to Ngn3-Btc only therapy, indicating the constriction of autoimmune response in the pancreas that is offered by PD-L1 (Li, R., Lee et al. 2015). The immunosuppressive expertise that is given from PD-L1 expression, was specialized only in the pancreas, as skin allografts were rejected from the treated mice (Figure 97) (Li, R., Lee et al. 2015). Splenocytes from the treated mice with PD-L1 were transferred to NOD-SCID mice, causing them diabetes earlier than the mice treated without PD-L1, while when euglycemic mice treated with PD-L1 were infected with splenocytes from newly diabetic NOD mice seemed to remain diabetes free, showcasing the protection that was gained from this gene therapy (Figure 97) (Li, R., Lee et al. 2015).

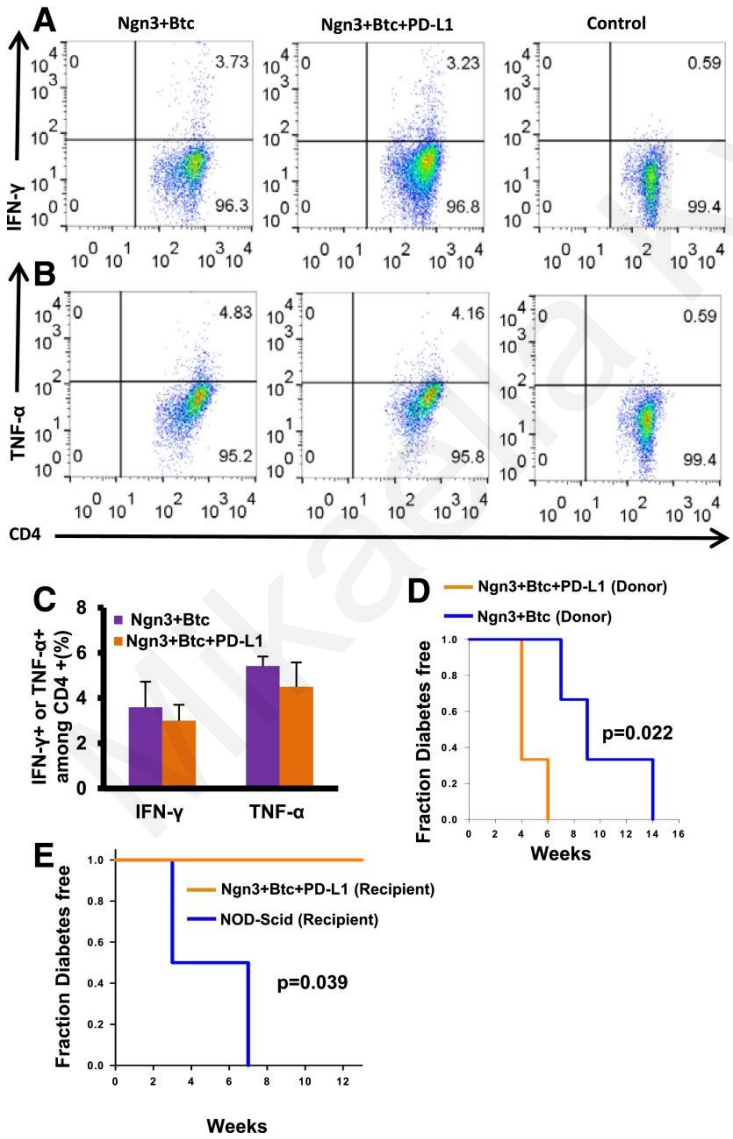


Figure 97 No peripheral immunosuppression occurred in the mice treated with Ngn3-Btc and PD-L1. (A–C) IFN-γ– and TNF-α–positive CD4+ T cells isolated from spleens of treated mice. (D) Diabetes induction in NOD-Scid mice after adoptive transfer of splenocytes from Ngn3-Btc+PD-L1 or controls. (E) Diabetes induction in Ngn3-Btc+PD-L1 or control mice after adoptive transfer of splenocytes from newly diabetic NOD mice. (Li, R., Lee et al. 2015)

In 2014 Lee, Ryu et al. induced Nk6-1 and Ngn3 to enteroendocrine K cells with the expectation to differentiate them into pancreatic β-cells. At the beginning when they compared mRNA expression of K cells with islets, insulin, Nkx6-1 and Ngn3 were absent in K cells, while Pax4 mRNA was absent in the islets (Lee, Ryu et al. 2014). Therefore, the scientists transfected rat Nkx6-1 into K cells and rat Nkx6-1 mRNA was expressed in those cells and islets, but not in K cells (Figure 98A)(Lee, Ryu et al. 2014). Insulin 1 was expressed only a little in the Nkx6-1 K cells (Figure 98C) (Lee, Ryu et al. 2014). As a second step, the scientists introduced Ngn3, as well, with adenoviral vector and resulting to full expression of insulin 1 but not insulin 2 (Figure 98F) (Lee, Ryu et al. 2014). When the Nkx6-1 K cells that were treated with adenoviral vector, were cultured in a medium for the decrease of cell death, the cells formed clusters of spheroid cells that were composed of mostly living cells (Figure 99A) (Lee, Ryu et al. 2014). At 3 days approximately 40% of the same cells were producing insulin and at 6 days approximately 55% were insulin-producing cells, while the Nkx6-1 K cells were at 20% and 10% respectively (Figure 99B, C) (Lee, Ryu et al. 2014). The expression of glucagon, somatostatin and pancreatic polypeptide was stable between the K cells and the treated K cells (Figure 99E) (Lee, Ryu et al. 2014). When the treated

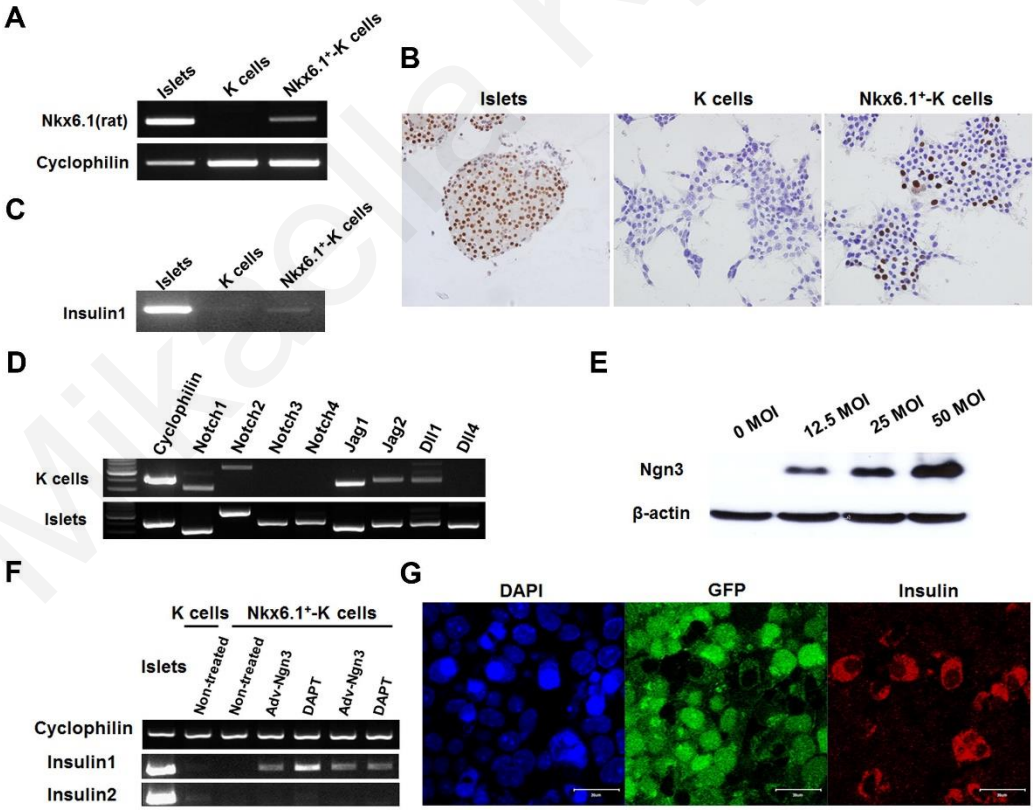


Figure 98 Reprogramming of Nkx6.1+-K cells to β-cells. (A, C, D, F) RT-PCR. (B) Immunostaining, NKX6.1 (brown). (E) Western blot analysis. (G) Confocal microscopy. (Lee, Ryu et al. 2014)

cells were incubated with glucose, they secreted insulin, however, the secretion of insulin was not increased after a higher amount of glucose incubation (Lee, Ryu et al. 2014).

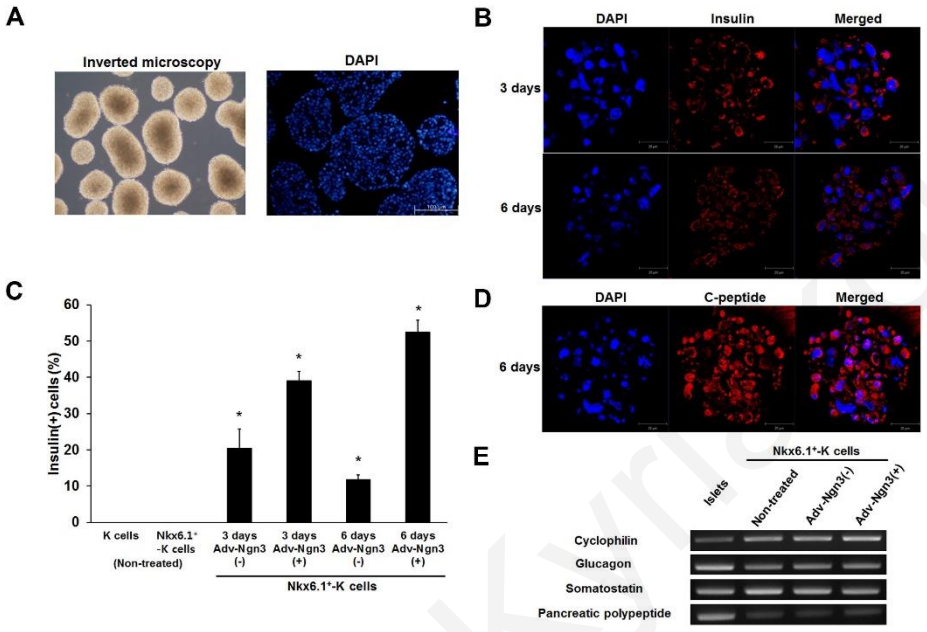


Figure 99 Reprogramming of Nkx6.1+-K cells to β-cells after Adv-Ngn3 infection and reaggregation in suspension culture. Inverted microscopy (A). Confocal microscopy (B, D). The percentage of insulin-positive cells was calculated (C). RT-PCR (E). (Lee, Ryu et al.)

DISCUSSION

T1D is a worldwide spread disease, by which many human beings are suffering from it. The main goal of any therapy for T1D is for the patient to have near normal blood glucose levels at all times. Gene therapy intends to target certain genes or proteins in order to achieve near normal blood glucose levels in an efficient, safe and specific way.

In this thesis, essential genes and proteins that can be overexpressed for a therapeutic result in T1D were examined, exploring their benefits as well as their limitations. Gene therapy is put in use for the establishment of this goal, as the expression of genes and proteins are impossible to be inflected by any other medical techniques. In many studies that were explored, gene therapy was used for immunological interventions, the prevention of autoimmune destruction of β-cells and to reduce the reliance of insulin by T1D patients.

Overexpression of certain genes and proteins caused the animal models to return to euglycemia after diabetes occurrence or delayed the onset of the disease. On some occasions, neo-islets were formed and on other occasions, gene or protein therapy preserved the pancreatic β-cell mass. In some studies insulin was often secreted in near normal levels, whereas in other studies, several pathways or hormones regulated the normal blood glucose levels.

Therapy with IGF1, via AAV, in the pancreas of NOD mice seemed to suppress the development of diabetes and having a protecting effect to the mice, via the abolishment of autoimmune β-cell destruction and hence insulin production. Additionally, topical IGF1 delivery caused a normal energy metabolism. Moreover, topical delivery of IGF1 prevented the progressive loss of corneal nerve in NOD mice, by employing AMPK and AKT pathways.

On another note, Reg3g delivered by plasmid, was able to reduce insulitis and increase the regeneration of β-cells. Moreover, Reg3g gene therapy seemed to inhibit the β-cell apoptosis that was induce by Th1 cytokines, via downregulating their production. When Reg3g was delivered with a lentivirus vector, it mitigated the results of STZ- induced diabetes in mice. The β cells were proliferating and protected from the apoptosis, with the first causing the secretion of insulin, because of the elevated levels of AAT-1 serum.

Moreover, co-expression of NK1, a fragment of HGF and IL4 cytokine via a dsAAV8 vector induced high proliferation rates of β-cells contributing to larger islets, augmented, lengthened glycemic homeostasis, and decreased appearance of insulitis. In a small percentage of the STZ diabetic mice, this treatment was able to reverse diabetes. The delivery of HGF with a plasmid vector to STZ diabetic mice, caused the amelioration of diabetes, by producing higher levels of circulating insulin and lower levels of blood glucose. This therapy was able to promote β-cell proliferation and diminish β-cell apoptosis, thus preserving β-cell mass. Delivering HGF via adenovirus and CA, a cytomegalovirus immediate-early enhancer and a modified chicken β-actin promoter, made possible transfecting the gene with a lower dose of adenovirus and thus inhibiting liver toxicity and other pathological problems. Additionally, the protection of β-cells offered by this therapy persisted for longer period, than when the HGF was delivered with a cytomegalovirus promoter. In another research, the scientists intended to deliver HGF as an immunosuppressive gene therapy strategy, to replace immunosuppressive drugs in an effort to protect islet allografts in diabetic rats. Indeed, HGF was able to induce prolonged improvement in blood glucose levels when used in conjunction with islet allografts. A research employed a RGD peptide into an Adenoviral vector to deliver XIAP and HGF into transduced islets. Their results were similar with the previous research. This therapy was able to inhibit the β-cell apoptosis and promoted the revascularization of the transplanted islets.

G6Pase gene was transduced in STZ-diabetic rats increasing to near normal levels the plasma insulin and decreasing the blood glucose levels significantly in fasting conditions, without causing them to go in a hypoglycemic state after a prolonged fasting. However, when the treated rats were fed, a moderate but corrected hyperglycemia state occurred. That can be attributed to the low dose of delivered G6Pase, however a higher dose could cause hepatotoxicity to the rats.

β-cell specific expression of Klotho gene constricted T cell infiltration, as well as the β-cell apoptosis caused by autoimmunity in NOD diabetic mice. Nevertheless, Klotho overexpression seemed to be unable to lower the blood glucose in near normal levels. Another study indicated that Klotho overexpression increased the β-cell mass and β-cell replication, as well as augmented insulin expression. Lastly, Klotho seemed to attenuate insulitis in the NOD diabetic mice.

Delivery of Ngn3 with AAV8 was unable to reverse hyperglycemia effectively and caused hepatotoxicity to the treated STZ diabetic mice. However, when a plasmid expressing Ngn3 and AdVhFIX made the mice euglycemic and they responded great to glucose tolerance tests. In

another study, where Ngn3 was delivered with a first-generation transcription factor the STZ diabetic mice returned to euglycemia, while their serum insulin levels were reduced, indication of a decreased glucose metabolism. As mentioned before AAV delivery of Ngn3 is unable to restore glycemic homeostasis, so the scientists used, in parallel, vaccine adjuvants that temporarily reduced blood glucose levels. Evidence of possible treatment method. In the same study, pre-treatment with Ngn3 was able to rescue mice from developing hyperglycemia in a time-dependent manner.

Btc therapy is not commonly used alone, many studies use Btc in conjunction with Ngn3, as Btc showed in several studies that it could not induce euglycemic state on its own. However, one study showcased that Btc therapy inhibited islet apoptosis and induced higher proliferation of β-cells, lowering blood glucose temporarily, via insulin secretion. Btc therapy, also, caused the formation of new blood vessels in allografts of diabetic mice.

When scientists delivered Pdx1 via AAV vector to STZ diabetic mice, the mice remained hyperglycemic. However, adding the AdVhFIX reversed diabeted in the same mice without signs of liver toxicity. In a research Pdx1 was delivered to CAD-NOD mice, the mice developed normal glucose clearance response to glucose tolerance test. Nonetheless, insulin production was relatively low indicating that glucose homeostasis was due to other factors. The scientists showcased that Pdx1 therapy results were connected to a shift of Th1 to Th2 cytokine. Lastly, the treated mice's splenocytes did not have the capability to treat other mice's splenocytes. Another, research with Pdx1 overexpression, but to the hAMSCs, demonstrated their ability to differentiate into islet-like cells. These cells expressed pancreatic marker genes including insulin when exposed to a high glucose medium.

Leptin therapy with an AAV vector intracerebroventricularly, into STZ diabetic mice, depleted hyperglycemia, hyperphagia and weight loss, while it did not increase insulin production. One injection of leptin was able to sustain the results that were observed for a long time. Hyperleptinemia reversed partially hyperglycemia and fully reversed hyperketonemia in $Lepr^{flox/flox}$ mice. Additionally, leptin therapy increased insulin sensitivity in STZ-diabetic mice. When a leptin osmotic pump was implanted in STZ diabetic mice, their blood glucose levels were decreased, while their insulin levels remained downregulated. Leptin treated mice were unable to tolerate prolonged fasting because of energy substrate limitations, low plasma glycerol, NEFA and TAG levels. High leptin expression and glycerol depletion are needed for hypoglycemia in STZ

diabetic mice to occur. In Akita rats leptin overexpression reversed hyperglycemia, but low plasma insulin levels persisted. Ketone bodies were decreased and the life span of the rats was expanded compared to the non-treated rats. Another study demonstrated that leptin acts in a dose-dependent way to induce euglycemia, and leptin and pancreatic islet co-therapy was explored. Low dose of leptin and islets induced glucose euglycemia, normal insulin production and inhibited dyslipidemia. A study combined PEGylated leptin antagonist with insulin therapy. Leptin is an insulin antagonist and thus, the leptin antagonist increased the body weight and adiposity, as well as the plasma insulin and, lastly a mild nonsignificant glucose tolerance deterioration.

A study, that delivered Ngn3 and Btc discovered that the insulin production, which is induced by the therapy, emerged in two waves. The first wave of insulin secretion was due to hepatocytes and the second by oval cells that differentiated into insulin β-cells. A second study that delivered Ngn3 in conjunction with Btc, managed to induce hepatic periportal neo islets with, both, the morphology, and the function of β-cells. Specifically, this co-therapy lowered blood glucose levels and increased insulin secretion, as well as reversed the dyslipidemia that occurred due to the diabetes induced by STZ. Another study that explored the effect of Ngn3-Btc therapy discovered the production of neo-islets, however these newly induced islets were targeted through autoimmunity destruction. To overcome this obstacle, scientists overexpressed SOCS1, a cytokine signaling suppressor, resulting in 50% protection from the autoimmune destruction of neo-islets, while it did not protect them from insulitis. However, the immunosuppression protection that the therapy offered, was specific. A similar study, but with PD-L1 expression, instead of SOCS1, engineered neo-islets that resisted T-cell destruction, causing tolerance to them. This therapy provided topical immunosuppression and induced glucose homeostasis, while the neo-islets expressed islet hormones, without any toxic results in the liver. A different combinational therapy study showcased the partial reprogramming of K cells to β-cells, via Ngn3 and Nk6.1 overexpression. The reprogrammed K cells expressed a small amount of insulin 1 mRNA.

In conclusion, gene therapy has been explored as a potential treatment for T1D. However, the studies made are not sufficiently exploring the pathological results of the therapies throughout the life span of several animal models. Not only the positive effects, but the negative effects of the therapies should be investigated in the models, in order to be able to proceed in clinical trials. Additionally, several combinational therapies should be explored even further, as well as how they interact with each other and what complications arise through them. Gene therapy is a very

promising therapy to treat or even reverse diabetes permanently, without the need of people affected with T1D to use chronic drugs and to suffer from their adverse effects.

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