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# A FISHING EXPEDITION FOR ANTIDIABETIC DRUGS: IDENTIFYING NEW AVENUES TO STIMULATE BETA-CELL REGENERATION

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# A fishing expedition for antidiabetic drugs: Identifying new avenues to stimulate beta-cell regeneration
## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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*To those who never had the chance*

*When I was struggling in making a decision, I remember that my brain was resisting my reasoning a great deal: "What you're aiming for, (my brain) was telling my heart, is futile; the world as you desire it to be, without anyone being hungry or cold, a world that is fair for all people, does not exist and it will never exist". But my heart echoed from deep within and replied to my brain: "Indeed, I know such world does not exist, but it will, because I simply desire to. In my every heartbeat I want and long for such world. Yes, I believe in a world that does not exist; but by believing in such world, I create it; something that we never truly desire enough, that is what we name impossible to happen.*

*Report to Greco, Nikos Kazantzakis*

# ABSTRACT

All types of diabetes are characterized by loss of the insulin-producing β-cells that reside in the pancreas. While current treatments can manage the disease, there is still no cure available. Understanding and promoting endogenous β-cell regeneration is an attractive approach that can lead to an eventual cure for diabetes. This thesis aims to discover new molecular pathways that can promote β-cell regeneration by using mainly the zebrafish model system, which can spontaneously regenerate its β-cells following injury.

In **Papers I&II**, we performed a transcriptomics profiling of the microenvironment of the zebrafish regenerating islet using microarray and RNA-Seq technologies respectively and we coupled this profile with in vivo genetic screens. We aimed to identify upregulated genes that code for secreted proteins that when overexpressed in the same zebrafish model could stimulate β-cell regeneration at supraphysiological levels. In **Paper I**, we found that by overexpressing *igfbp1a*, we promoted transdifferentiation of pancreatic α-cells to β-cells. This phenotype was conserved in mouse and human islet cultures suggesting that the pathway could translate to mammals. In **Paper II**, we showed that *folr1* overexpression stimulated β-cell regeneration in the zebrafish through differentiation of duct-residing pancreatic progenitors. The observed phenotype was mediated through membrane-bound *folr1* and treatment with folinic acid, a ligand of *folr1*, increased β-cell formation in zebrafish and pig islet cultures. Overall, these two in vivo genetic screens uncovered two important pathways stimulating β-cell regeneration in a variety of experimental models.

In **Paper III**, we aimed to identify the target of CID661578 a potent small molecule that potentiates β-cell regeneration in zebrafish. Experimentally, we combined a modified yeast-2-chemical screen together with in vivo zebrafish genetic models and uncovered MNK2 as the molecular target of CID661578. Mechanistically, CID661578 boosted protein translation by binding and removing MNK2 from the translation initiation complex as shown by in vitro biochemical and in vivo zebrafish experiments. In summary, this work ascribes a new role for the translation initiation complex in β-cell regeneration

In **Paper IV**, we characterized the liver to pancreas interaction following β-cell injury with the aim to identify secreted proteins/metabolites from the liver that stimulate β-cell regeneration. By performing a genetic screen in zebrafish larvae, we revealed a role for molybdenum metabolism in stimulating β-cell regeneration.

Overall, the work performed in this thesis identified four new molecular pathways that could stimulate β-cell regeneration in an array of experimental models that could be further advanced as potential diabetes therapies.

# POPULAR SCIENCE SUMMARY OF THE THESIS

One of the great challenges of modern medicine is the development of regenerative therapies to tackle diseases that are caused by damaged tissues. Insulin is a hormone secreted from a specialized clump of cells sitting in the pancreas named β-cells. Insulin is important for our survival as it helps by efficiently managing the energy we get following our meals. People suffering from diabetes usually have a much-reduced number of these β-cells and therefore cannot control their energy needs efficiently, a condition that will eventually lead to death. Therefore, a promising strategy to cure diabetes is to increase the number of β-cells through a regenerative medicine approach.

In this thesis, we used a small tropical fish, called zebrafish, that has a surprisingly similar pancreas and β-cells to humans. In the laboratory, we can make the zebrafish diabetic by killing almost all its β-cells similar to what happens to humans suffering from the disease. However, zebrafish is unique in the sense that after the injury happens, they can immediately make new β-cells and cure diabetes, something that we humans cannot do. In this work, we aimed to identify how the diabetic zebrafish can regenerate their β-cells and transfer this knowledge in order to cure diabetes in humans.

In the studies I&II, we tried to find genes important for making the zebrafish β-cells regenerate. To do that we isolated cells of the pancreas, before and after making the zebrafish diabetic and compared the two conditions to see which genes are changing immediately after the injury. We found that when we gave the zebrafish large amounts of the proteins that derive from two of these genes, named *igfbp1a* and *folr1*, they regenerated their β-cells even faster than the zebrafish with normal amounts of these proteins. In addition, we saw that these proteins could create new β-cells in other models like mice and human cultured cells (*igfbp1*) as well as pigs (*folr1*). In study III, we aimed to find the exact mechanism a chemical called CID661578 works. This experimental drug is one of the best chemicals that we can find to promote a strong increase in β-cell numbers in the diabetic zebrafish compared to the untreated fish, but nobody knew what this chemical target in the body. In this work, we have identified that this experimental drug targets a protein named MNK2, which is implicated in diabetes for the first time. We further showed that this experimental drug increased β-cell generation in pigs and was functional in human cells suggesting that it could be used as a potential therapy for diabetes. Lastly, in study IV we asked how other tissues and more specifically the liver can interact with the pancreas to promote β-cell regeneration. To do that, we extracted the liver from healthy and diabetic zebrafish and looked at genes that make proteins with increased levels following injury and might make a product that can enter the bloodstream. We found that a gene involved in the molybdenum (a metal) processing in the liver could further stimulate β-cell regeneration in the diabetic zebrafish. Overall, this work has contributed to four new ways that could be potentially used to regenerate β-cells in the human pancreas and tested as cures for diabetes.

# LIST OF SCIENTIFIC PAPERS

I. Jing Lu,* Ka-Cheuk Liu*, Nadja Schulz*, **Christos Karampelias**, Jérémie Charbord, Agneta Hilding, Linn Rautio, Philippe Bertolino, Claes-Göran Östenson, Kerstin Brismar, Olov Andersson.
IGFBP1 increases β-cell regeneration by promoting α-to β-cell transdifferentiation.
*The EMBO journal, 2016, 35, 2026-2044.*

II. **Christos Karampelias**, Habib Rezanejad, Mandy Rosko, Likun Duan, Jing Lu, Laura Pazzagli, Philippe Bertolino, Carolyn E. Cesta, Xiaojing Liu, Gregory S. Korbutt, Olov Andersson.
Reinforcing one-carbon metabolism via folic acid/Folr1 promotes β-cell differentiation.
*Nature Communications, 2021, 12, 3362*

III. **Christos Karampelias**, Charlotte L. Mattsson, Habib Rezanejad, Jiarui R. Mi, Xiaojing Liu, Jason W. Locasale, Gregory S. Korbutt, Olov Andersson.
MNK2 deficiency potentiates β-cell regeneration via hypertranslation.
*(Manuscript)*

IV. **Christos Karampelias**, Etty Bachar-Wikström, Olov Andersson.
Characterization of liver-to-pancreas crosstalk following β-cell loss reveals a role for the molybdenum cofactor in β-cell regeneration. *(Manuscript)*

(*These authors contributed equally to this work)

**Publications not included in the thesis**:

I. Abdelfattah El Ouaamari, Ercument Dirice, Nicholas Gedeon, Jiang Hu, Jian-Ying Zhou, Jun Shirakawa, Lifei Hou, Jessica Goodman, **Christos Karampelias**, Guifeng Qiang, Jeremie Boucher, Rachael Martinez, Marina A. Gritsenko, Dario F. De Jesus, Sevim Kahraman, Shweta Bhatt, Richard D. Smith, Hans- Dietmar Beer, Prapaporn Jungtrakoon, Yanping Gong, Allison B. Goldfine, Chong Wee Liew, Alessandro Doria, Olov Andersson, Wei-Jun Qian, Eileen Remold-O'Donnell, and Rohit N. Kulkarni.
SerpinB1 promotes pancreatic β-cell proliferation.
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II. Laura Pazzagli, **Christos Karampelias**, Randi Selmer, Olov Andersson, Carolyn E. Cesta.
Investigating the association between prenatal exposure to folic acid and risk of neonatal diabetes/hyperglycemia and type 1 diabetes: A Norwegian register-based study.
*Pediatric Diabetes*. 2021; 1- 5.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-IT | 5-Iodotubercidin |
| ASCL1b | Achaete-scute family BHLH transcription factor 1b |
| ARX | Aristaless related homeobox |
| ALDH1 | Aldehyde dehydrogenase 1 |
| CDK4 | Cyclin dependent kinase 4 |
| CK7 | Cytokeratin 7 |
| DHFR | Dihydrofolate reductase |
| DNMT1 | DNA methyltransferase 1 |
| DPP4 | Dipeptidyl peptidase-4 |
| DYRK1A | Dual specificity tyrosine phosphorylation regulated kinase 1A |
| EIF4E | Eukaryotic translation initiation factor 4E |
| EIF4G | Eukaryotic translation initiation factor 4G |
| ESC | Embryonic stem cell |
| FOXO1 | Forkhead box O1 |
| FOLR1 | Folate receptor 1 |
| GABA | Gamma aminobutyric acid |
| GAD | Glutamic acid decarboxylase |
| GIP | Glucose-dependent insulinotropic polypeptide |
| GLP-1 | Glucagon-like peptide 1 secretion |
| GWAS | Genome-wide association studies |
| HES1 | Hes family BHLH transcription factor 1 |
| HHEX | Hematopoietically expressed homeobox |
| HNF1β | Hepatocyte nuclear factor 1 homeobox B |
| IA2A | Insulinoma-associated protein 2 autoantibody |
| INS | Insulin |
| LIF | Leukemia inhibitory factor |
| MAFA | MAF BZIP transcription factor A |
| MAFB | MAF BZIP transcription factor B |
| MNK1 | MAP kinase-interacting serine/threonine-protein kinase 1 |
| MNK2 | MAP kinase-interacting serine/threonine-protein kinase 2 |

| | |
|---|---|
| MOCS2 | Molybdenum cofactor synthesis 2 |
| MODY | Maturity Onset Diabetes of the Young |
| MTZ | Metronidazole |
| NEUROD1 | Neuronal differentiation 1 |
| NEUROG3 | Neurogenin 3 |
| NKX6.1 | NK6 homeobox 1 |
| nPod | Network for pancreatic organ donors |
| NTR | Nitroreductase |
| OPP | O-propargyl-puromycin |
| PAX4 | Paired box 4 |
| PAX6 | Paired box 6 |
| PDX1 | Pancreatic and duodenal homeobox 1 |
| PPAR-γ | Peroxisome proliferator-activated receptor gamma |
| PTF1a | Pancreas associated transcription factor 1a |
| PROCR | Protein C receptor |
| SERPINB1 | Serine peptidase inhibitor, clade b, member 1 |
| SGLT2 | Sodium/glucose cotransporter 2 |
| SOX9 | SRY-box transcription factor 9 |
| TGFb | Transforming growth factor b |
| WISP1 | WNT1-inducible signaling pathway protein 1 |
| YAP | Yes-associated protein 1 |
| ZnT8A | Zinc transporter 8 autoantibodies |

# 1 INTRODUCTION

## 1.1 DIABETES

The term Diabetes Mellitus characterizes a heterogeneous group of diseases that share the common phenotype of dysregulated glucose metabolism. It is estimated that 415 million people were diagnosed with diabetes in 2015, a number that can rise to 642 million by 2040. Around 5 million deaths were attributed to diabetes in 2015 and the global cost for diabetes was estimated to be 673 billion US dollars (1). Similar estimates are reported from multiple sources (2–4). It is evident from these reports that diabetes poses a significant challenge to healthcare systems worldwide and the trend points to an even greater increase of people suffering from the disease in years to come.

Diabetes pathophysiology is tightly linked to the pancreas. Pancreas is an endoderm-derived organ that has been traditionally separated in an exocrine and an endocrine compartment. The major cell types of the exocrine compartment are the acinar and ductal cells that have a critical role in digestion. The endocrine pancreas consists of the hormone-secreting populations that cluster in structures termed islets and consist of: glucagon producing α-cells, insulin producing β-cells, pancreatic polypeptide producing γ-cells, somatostatin producing δ-cells and ghrelin producing ε-cells. β-cells secrete insulin in response to elevated glucose levels after a meal that signals to tissues important in metabolic regulation including the liver, muscle and adipose tissues. Insulin triggers a signaling cascade in the receiving tissues that promotes glucose uptake to be used in downstream metabolic processes or stored as potential energy source (5).

**Diabetes classification**

Diabetes can be characterized as an umbrella term encompassing a wide range of diseases. Scientists have tried to classify subtypes of diabetes mainly based on the cause of the dysregulated glucose metabolism. MODY is the monogenic inherited form of diabetes that represents 1-2% of the total diabetic population. MODY arises from mutations in genes important for β-cell function and in extreme cases can manifest with dorsal pancreatic agenesis as is the case in MODY4 patients that have a homozygous mutation in the PDX1 protein (6,7).

Type 1 diabetes affects approximately 10% of all diabetics. Patients with type 1 diabetes suffer from an almost complete loss of the β-cell population due to an autoimmune attack. Specifically, the immune system recognizes certain β-cell autoantigens including among others GAD, IA2A, INS and ZnT8A and eventually triggers a -what is considered so far-CD8+ mediated destruction of β-cells (8,9). Both genetic and environmental factors contribute to disease onset. It is important to note that more than half of the genetic loci implicated with type 1 diabetes as described by GWAS studies are responsible for immunoregulation. For example, the human leukocyte antigen (HLA) class I region has been shown to be responsible for half the genetic risk of developing type 1 diabetes (10,11). In

addition, recent evidence demonstrates that β-cells upregulate the expression of HLA class II genes to act as antigen presenting cells (12). The environmental factors that can trigger the onset of the autoimmune attack remain to be found with certain viral infections been shown so far as one possible trigger that can contribute to disease onset (9). Technical advances in recent years have given a clearer view of disease. Two studies using mass cytometry compared pancreata from control and type 1 diabetes donors at different stages of the disease. The ability to simultaneously assess the expression levels of more than 30 important pancreatic and immune cell markers revealed an abundance of immune cell infiltration in the exocrine part of the pancreas. Islet morphology was also affected in type 1 diabetes and there was evidence suggestive of cell fate changes in the endocrine compartment (13,14). Finally, a large-scale GWAS study coupled with the powerful single-nucleus assay for transposase-accessible chromatin sequencing (snATAC-Seq) methodology to sample the epigenome of type 1 diabetics also showed a strong involvement for the exocrine cells in disease onset (15). Overall, these new tools have shown a previously underappreciated role for the exocrine pancreas in type 1 diabetes.

Type 2 diabetes is considered the prevalent type of the disease affecting more than 85% of the diabetic population. Interestingly, there have been recent efforts that further classify type 2 diabetes in several subgroups based on various key clinical parameters of the disease that could lead in improved clinical management (16,17). In general, type 2 diabetes is characterized by the inability of the peripheral tissues to respond to insulin. Specifically, β-cells secrete insulin in response to the high glucose levels following a meal. Insulin binds to its receptor, for instance in the hepatocytes of the liver, to trigger the insulin signaling cascade. This results in increased glucose uptake, shut down of glucose production and increased glycolysis and lipid production (5). In type 2 diabetes insulin cannot regulate these processes and that leads to insulin resistance, glucose intolerance and eventually the development of the disease (18). Whether β-cell dysfunction or insulin resistance comes first is a topic widely debated in the type 2 diabetes field (19,20). Nonetheless, while type 2 diabetes is mainly a result of a different cause, there has been a documented decline in β-cell mass in advanced stages of the disease that contributes to the rapid deterioration of patients (21–24). The decline in β-cell numbers has been attributed so far to the immense metabolic stress that the β-cells have to cope with (25). Overall, it is clear from the literature that a decrease in β-cell numbers is a common feature in all types of diabetes (Figure 1) and potential curative therapies should be focused in replenishing the β-cell population.
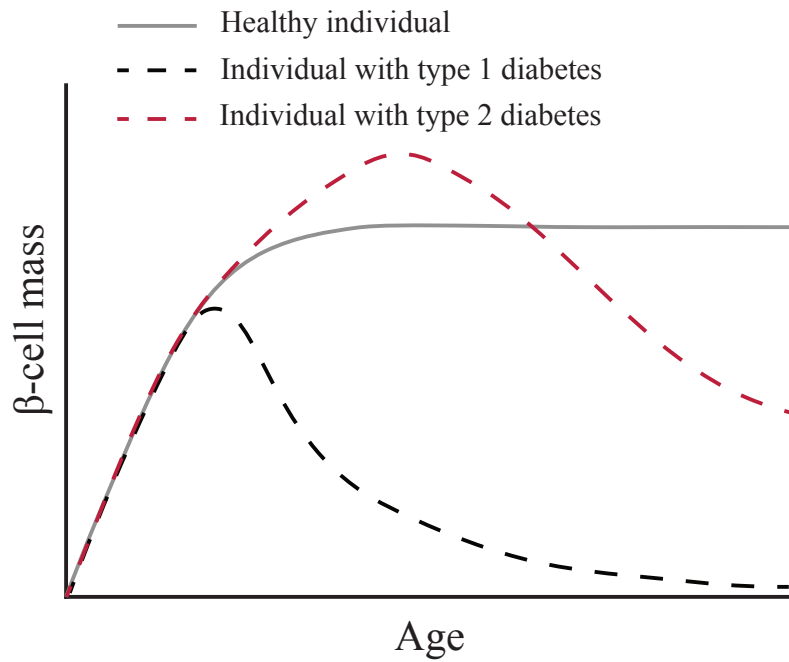
*Figure 1: Schematic showing the β-cell mass changes for healthy individuals as well as individuals with type 1 and type 2 diabetes.*

# 2  CURRENT THERAPEUTIC APPROACHES FOR DIABETES

## 2.1  INSULIN

Currently, there is no known cure for diabetes. All treatments aim to manage the disease and advances in the field have been significant over the past several years. This thesis is written during the 100-year mark from the discovery of insulin (1921-2021). Therefore, it is necessary to start this section by acknowledging that the single most important treatment for diabetes that exists today is insulin supplementation. Insulin was isolated in 1921 by seminal work performed by Banting and Best in pancreatectomized dogs (26). More importantly, the authors refused to take a patent on insulin so that it will be freely available for everyone to use at affordable prices saving millions of lives. To this day, insulin and its modified analogues are the golden standard when it comes to treatment of patients with diabetes and it is the only treatment that can keep people with type 1 diabetes alive.

There are several commercially available insulin analogues to clinicians that can help tailor-make the treatment to individual patients' needs. Insulin modifications both to the amino acid synthesis as well as the composition of the injected solution have generated three types of insulin analogues: 1- rapid, 2-regular and 3-long-lasting insulin analogues (27,28). Rapid and regular acting insulin analogs can manage glycaemia in a more physiological way but can cause hypoglycemia, especially during the night. Long lasting insulin analogues have the advantage of causing fewer hypoglycemic episodes, however they do not have the pick of

insulin concentration that would mimic the physiological response after a meal (27). As is the case with most treatments, administration of insulin and its analogues has still not achieved to phenocopy the physiological response of humans following a meal, with the episodes of hypoglycemia being the most common side-effects. Moreover, insulin is known to act as a mitogen stimulating proliferation of cells (29). Therefore, it has to be noted here that there are epidemiological studies suggesting that exogenous insulin administration in diabetics might promote cancer formation (29,30). However, this claim has been disputed by Giovanucci et al as tough to conclude, given the limitations of the current study designs and diabetes being a risk factor for cancer development (31). Overall, insulin analogues and the continuously evolving methods to deliver them in humans is still today the best solution for managing diabetes.

## 2.2  CELLULAR-BASED THERAPIES

### Islet transplantation

Insulin supplementation is of particular importance to patients with type 1 diabetes that lack insulin-secreting β-cells. One logical approach as a cure of this disease is to replenish the β-cell population. One way to replenish the β-cell population in patients is through islet transplantation from cadaveric donors. A landmark study by Shapiro and colleagues in 2000 established an improved protocol for islet transplantation, which is now commonly referred to as the Edmonton protocol (32). The clinical trial using this protocol demonstrated for the first time that type 1 diabetics (58% of the participants), can survive without insulin injections for one year following transplantation of purified islets from cadaveric donors. Further, even when the grafts stop secreting insulin and patients returned to exogenous insulin administration, the remaining transplanted β-cells were enough to reduce episodes of hypoglycemia (33). A recent phase 3 clinical trial corroborated these results (34). As is the case with any transplantation procedure, the shortage of donors as well as the immunosuppressant regimen used are the major shortcomings of the Edmonton protocol (35). However, there is great hope with the recent advancements in the field of stem cell differentiation towards β-cells that an abundant source of β-cells is achievable.

### Stem-cell based therapies for diabetes

hESCs retain the ability to differentiate into all tissues and cells of the body. Additionally, the novelty of induced pluripotency (iPSC) from differentiated human tissues has generated hope in the field of regenerative medicine for new therapies to a number of diseases characterized by cell loss (36). As mentioned, a shortage of islet donors is the limiting factor for large-scale cellular transplantation to tackle diabetes. However, differentiation of hESCs or donor-derived iPSCs to β-cells holds great promise as an unlimited supply of in vitro generated β-cells. Pioneer work by three different research groups have generated what is considered today established protocols of hESC differentiation to β-cells (37–40). These initial protocols followed the developmental stages of β-cells as shown in animal model systems (see below for an overview of the process). In the first optimization efforts of the protocols, the

efficiency of generating β-cells was low and these β-cells lack critical characteristics of β-cell physiology, for instance robust response to a glucose challenge (37,39). However, when these β-like cells were transplanted in mice they matured to fully functional β-cells showing that the potential for this approach to be successful is high (37,38).

Since the establishment of the first protocols, there have been incremental advances in the stem cell field that generally revolve around three approaches. First, translate the in vivo understanding of molecular signals governing β-cell differentiation into the existing protocols. For example, modulating the Yap signaling pathway that has been shown to be important for β-cell differentiation in mice can increase the maturity of β-cells generated in vitro (41–43). A second approach tries to isolate purified populations of each stage of the differentiation protocol to achieve higher differentiation efficiency to β-cells. As an example, sorting for glycoprotein 2 (GP2$^+$) pancreatic progenitors can yield a higher percentage of differentiated β-cells (44–46). Third, recent evidence points to cell aggregation and cell-cell contact as a crucial parameter for differentiation of pancreatic progenitors in vitro (47,48). This breakthrough will pave the way for different materials to be explored as determinants of β-cell differentiation. It has to be noted that although efforts to efficiently differentiate iPSCs to β-cells did not have a high success rate, one of the latest protocols that includes modulation of cellular adhesion signaling pathways managed to achieve this (48). Importantly, clinical trials have been initiated (clinical trial IDs: NCT03163511 and NCT02239354) to test the already available protocols in patients demonstrating that this approach is coming closer to be used in clinical practice.

## 2.3   PHARMACOLOGICAL APPROACHES

Various drugs have been developed targeting different levels of glucose metabolism in critical tissues like the intestine and the liver. Contrary to type 1 diabetes that insulin supplementation is necessary for survival, these drugs have shown success in managing type 2 diabetes progression. This section aims to briefly review, some of the most important drug classes targeting glucose metabolism.

**Metformin**

Metformin is a synthetic derivative of a natural product from the plant Galega Officinalis and is the most prescribed drug for patients with type 2 diabetes. Its wide use in clinics can be attributed to its potent effect in lowering glucose levels combined with minimal and tolerable side-effects (49). The paradox with metformin is that its exact mechanism of action is still not clear, something surprising given its wide use in diabetes management. Initial experimental evidence in animal models suggested that the main beneficial effects of metformin was through suppressing glucose production from the liver (50,51). A liver specific knockout mice of the metformin transporter could abrogate the effects of metformin in lowering glucose levels (52). Further, mitochondrial targeting of glucose production via metformin treatment in hepatocytes has been proposed to involve inhibition of the activity of the

mitochondrial glycerophosphate dehydrogenase (53). However, recent findings have challenged this mechanism of action (54). Nevertheless, it seems that there is an agreement among researchers that at least part of the glucose lowering effects of metformin are exerted through the master regulator of metabolism, adenosine monophosphate (AMP)-activated protein kinase (49,55). Additionally, development of a slow-release analogue of metformin suggested that the main glucose lowering effects of the drug might not involve the liver to the extent previously thought. Instead, the intestine has been proposed as a major site for metformin effect by activating glucose utilization by colonic enterocytes and an interesting observation of elevated GLP-1 (56). Nonetheless, the exact mechanism of action of the most prescribed antidiabetic drug remains elusive.

**Incretin-based therapies**

Perhaps one of the most intriguing clinically approved therapies for diabetes are incretin-based therapies. While the commercial drugs are relatively new, the concept termed as "the incretin effect" has been described since the first half of the 20th century. Briefly, the incretin effect refers to the observation that an oral route of glucose delivery stimulates a higher level of insulin secretion compared to an intravenous glucose injection (57). This field of study began with the discovery of GIP and GLP-1, as two hormones secreted from gut endocrine cells that stimulate insulin secretion in the pancreas (58–60). The incretin-based therapies for diabetes aim to potentiate the incretin effect and are developed as two independent lines of therapeutics: peptide-based analogues of GLP-1 that act as GLP-1 receptor agonists and DPP4 chemical inhibitors that block degradation of the incretins and as a result prolong their actions. The first GLP-1 receptor agonist approved for the clinics, was isolated from the lizard's Heloderma suspectum venom and was named exendin-4 (60). Using exendin-4 as the basis for drug development, there are a handful of clinically approved GLP-1 based agonists that are effective in treating type 2 diabetes. Importantly, one of them, liraglutide, has been approved by the FDA as a drug against obesity, another metabolic disorder linked to diabetes (60,61). Shortly after the approval of the first peptide analog of GLP-1 as an antidiabetic treatment, the first DPP4 inhibitor, sitagliptin, got approval for use in the clinics in 2006. 15 years of clinical evidence suggests that the peptide-based GLP-1 agonists have a more potent antidiabetic effect than the DPP4 inhibitors. On top of the insulin secretion stimulation, GLP-1 receptor agonists have been shown to have additional beneficial metabolic effects such as lower glucagon secretion and promote satiety (60,62). Overall, incretin-based therapies have a potent and well-tolerated clinical profile that makes them an emerging option in management of type 2 diabetes.

**Sulfonylureas and meglitinides**

Contrary to metformin, sulfonylureas and meglitinides have a defined mechanism of action. Upon binding to Kir6.2 ATP-sensitive potassium channel, this class of drugs blocks potassium efflux and depolarizes the plasma membrane that leads to an increased calcium influx. Calcium influx stimulates insulin granules exocytosis and therefore insulin secretion. The drawback of a persistent use of sulfonylureas is an eventual desensitization of the β-cells.

The difference between sulfonylureas and meglitinides is that meglitinide-based therapies exert their action in faster way than sulfonylureas (63).

**Thiazolidinediones**

Thiazolidinediones are PPAR-γ agonists. PPAR-γ is an important metabolic mediator of metabolism and it is expressed in crucial metabolic tissues including the adipose and the liver. Drugs of this class have been discontinued and re-approved as controversy arose regarding adverse cardiovascular effects, which now have been dismissed (63).

**SGLT2 inhibitors**

SGLT2 inhibitors target the kidney which is a major tissue responsible for reabsorption of glucose from circulation. SGLT2 transporter has a low affinity for glucose, however its capacity to reabsorb glucose aimed for excretion is high. Therefore, SGLT2 inhibition is a logical target that can potentiate glucose excretion from the body and as a consequence lower circulating glucose levels (63,64). Apart from type 2 diabetics, SGLT2 inhibitors have been tested in patients suffering from type 1 diabetes as well, however adverse effects were observed (65,66).

Conclusively, all the above approved treatments for management of type 1 and type 2 diabetes have greatly improved the survival and quality of life of the patients. Yet, none of the above has managed to efficiently eliminate hypoglycemic events that can be as dangerous as hyperglycemia (67) and control insulin secretion from β-cells in a physiological manner for a prolonged period of time. It is therefore evident the need to identify drugs and pathways that can replenish the endogenous β-cell population as one of the most promising approaches that can cure the disease.

# 3   HOW TO MAKE A β-CELL?

The endpoint of regenerative medicine is to create fully functional, mature cell types that can take over and perform the physiological function of the lost tissue. This applies to the field of β-cell regeneration as well. However, in order to promote the generation of β-cells, first we need to understand how mature β-cells are formed. In this section, a summary of β-cell development is described which however is not meant to be exhaustive as this is not the main topic of this thesis.

**Overview of pancreatic induction and β-cell differentiation**

Induction of the pancreatic fate and β-cell specification is mainly studied in the mouse model and this section refers to mouse β-cell development unless otherwise stated. The overall process of β-cell specification follows a well-established trajectory. Following gastrulation and the specification of the three germ layers (endoderm, mesoderm, ectoderm), the prospective pancreatic field is specified in the foregut endoderm. Two invaginations of the foregut endoderm, termed pancreatic buds, emerge at around embryonic day E9.5. This first specified pancreatic field is marked by the expression of the PDX1 and PTF1a transcription

factors. These are characteristic transcription factors that define the first multipotent pancreatic progenitors of the mouse. From E9 to E12.5 the epithelium within the two pancreatic buds undergoes dramatic morphogenetic changes. Initially, the PDX1$^+$ multipotent pancreatic progenitors proliferate to create a stratified epithelium followed by formation of microlumens. At around E11 the two buds and the microlumens start to fuse and an elaborate epithelial plexus is generated. The pancreatic plexus is remodeled into a highly complex ductal epithelium that has traditionally separated into the tip and trunk domains that give rise to acinar and duct/endocrine cells respectively. Starting at E12.5, a developmental stage known as secondary transition begins that refers to a massive differentiation of the multipotent pancreatic progenitors to all lineages of the pancreas. Following birth, endocrine cells continue to proliferate for the first weeks followed by maturation to fully functional β-cell populations (68–71).

## 3.1 PANCREATIC INDUCTION

Molecular signals from surrounding developing tissues and structures are responsible for the induction of the prospective PDX1$^+$ pancreatic field. Major developmental signaling pathways have been shown to play a role in the initial pancreatic field induction including activin-βB, fibroblast growth factor (FGF), bone morphogenetic factors (BMPs) and the Notch signaling pathways (69). These signals are secreted by surrounding developing tissues and structures such as the mesenchyme, notochord and the lateral plate mesoderm. It is important to note that the different pancreatic buds are induced independently as they are exposed to signals from different tissues. All the signaling cascades in the developing endoderm lead to the specification of the multipotent pancreatic progenitors. There are two well-established facts regarding the pancreatic progenitor population. First, there is a defined core transcriptional network of transcription factors important for the establishment of the pancreatic progenitors that includes the transcription factors: PDX1, PTF1A, SOX9, NKX6.1, HNF1β and HES1 (72–78). For example, PDX1 knockout mice present with pancreatic agenesis while a knockout of HES1, which is a Notch-induced transcriptional repressor, causes pancreatic hypoplasia due to diminished proliferation (72,75,76). Observations from the *Hes1* knockout mice together with data from knockouts of various components of the notch signaling pathway has shed light to the second established fact that notch signaling is critical for maintaining the multipotency of the pancreatic progenitors at all embryonic stages. Importantly, genetic manipulations of the notch signaling pathway has caused a number of phenotypes from formation of ectopic pancreatic tissues in other endodermal organs to precocious differentiation of the pancreatic progenitors to endocrine cell fates (75,79–86).

## 3.2 SECONDARY TRANSITION AND ENDOCRINOGENESIS

Notch signaling plays an important role during the secondary transition of the pancreas in segregating the tip and trunk domains of the developing plexus (84). The exact mechanism of how this process happens is not still clear, however expression studies have demonstrated that notch signaling is active in the trunk domain of the plexus while the proacinar tip domain

downregulates notch signaling. Increased notch signaling in the trunk domain maintains the expression of NKX6-1 that in turn represses expression of the PTF1A transcription factor (80,87). The reverse relationship of these two transcription factors is present in the tip domain where high PTF1A expression suppressing the expression of NKX6-1 (88). This mutually inhibitory behavior of the two transcription factors leads to the establishment of the tip-trunk morphology of the epithelial plexus from E12.5 and onwards.

## 3.3   ENDOCRINE CELL FORMATION

Lineage-tracing studies using various Cre and inducible Cre[ER] transgenic mice under the control of trunk specific markers like SOX9 and HNF1β have shown that endocrine cell lineages are derived from this structure of the epithelial plexus (68). The major transcription factor involved in the formation of endocrine cells during the secondary transition in mouse is NEUROG3. NEUROG3-knockout mice lack endocrine cells formation and lineage tracing efforts using a knock-in NEUROG3:Cre[ER] mouse proposed that all endocrine lineages are derived from NEUROG3^{+} cells (89–91). Subsequent studies have shed light on the regulation of NEUROG3 expression by notch signaling as well as by post-translational modifications (mainly phosphorylation of certain amino acids) (79,92–95). The exact mechanism of how NEUROG3^{+} cells delaminate form the epithelial plexus to differentiate and form the islets is still not clear. Initially, it was thought that the trunk bipotent progenitors went through an epithelial-to-mesenchyme transition (EMT) and single NEUROG3^{+} cells could differentiate to one of the endocrine lineages (68,96–98). However, recent evidence convincingly show that most likely these endocrine progenitor cells do not go an EMT transition but rather bud from the epithelium and cluster at the same time to form what the authors of this work has termed as peninsulas (99).

NEUROG3 marks the initiation of the endocrine cell differentiation program. Subsequently, the newly-formed endocrine cell types are characterized by a specific gene regulatory network. Immediately under the control of NEUROG3 induction are the transcription factors ARX and PAX4 which are mutually inhibitory and restrict cells to the glucagon-secreting α-cells and β/δ-cell fate respectively (100,101). This initial signaling cascades lead to the establishment of the different endocrine lineages characterized by high ARX, PAX6 and MAFB expression in α-cells, PDX1, NKX6.1 and NEUROD1 in β-cells and HHEX in δ-cells (69,70). MAFB and MAFA transcription factors are crucial for the functional maturation of α- and β-cells respectively (69,102).

Finally, following birth there is a transient wave of β-cell proliferation that is considered to be driven by CDK4 mediated cell-cycle regulation and is associated with β-cell immaturity (103,104). The ability of β-cells to proliferate diminishes dramatically after the first weeks of life and the signaling molecules driving this early proliferation phase are under scrutiny for β-cell regenerative purposes (105,106). The majority of proliferating β-cells in early life seem to correlate with an immature state. This was shown by distinct transcriptomic transitions by two recent exceptional studies of Zeng et al and Puri et al (107,108). Following this

proliferation wave, β-cells acquire their functional maturation state. Nutritional state has been shown to be important for the maturation process and a significant role has been assigned to the mTOR signaling cascade for this nutrient-induced maturation (109–112). Lastly, detailed transcriptomics characterization of the mouse pancreas development at single-cell level have broadly confirmed the findings obtained from mouse genetics. These single-cell RNA-SEQ studies have identified in finer detail the transition between the different progenitor populations and these findings remain to be confirmed in vivo using lineage-tracing of these proposed new subpopulations (113–115).

## 3.4 HUMAN β-CELL DEVELOPMENT

Most of the data regarding human development come from studying donor pancreata at different developmental stages. The overall process follows the developmental trajectory of the mouse β-cell development with the endoderm adjacent to the notochord receiving instructive signals to differentiate to PDX1$^+$ multipotent pancreatic progenitors. A primary and secondary transition in endocrinogenesis has not been observed in human samples. NEUROG3 expression in the human pancreas reaches high levels around the end of the first trimester (116,117). A recent transcriptomics analysis defined the pancreatic populations using bulk RNA-SEQ methodology and showed that the NEUROG3$^+$ population is indeed characterized by the expression of transcription factors like ARX and FEV similar to the mouse endocrine progenitor population (118). However, there are reports from hESC differentiation protocols and from individuals with NEUROG3 mutations that β-cells are still able to form, showing that there might be additional transcription factors that can compensate for a loss of NEUROG3, contrary to the mouse model (119,120). Finally, endocrine cells arise and expand postnatally similar to mouse β-cells. As it is evident our understanding of β-cell development in humans is lacking. Nevertheless, with the development of the new transcriptomics and epigenomics tools on the single-cell level, a more detailed view of the process will soon be realized. For example, two recent studies using chromatin-immunoprecipitation sequencing and single-cell RNA-SEQ approaches in isolated human pancreatic progenitors showed a conservation between mouse and humans regarding the expression of genes involved in critical signaling pathways for progenitor differentiation (121,122).

## 3.5 β-CELL DEVELOPMENT IN ZEBRAFISH

Zebrafish which is the focus of this thesis have a fully functional pancreas with all the major cell types performing identical functions to their mammalian counterparts. By day 3 of development, the zebrafish pancreas contains a single primary islet located in the head of the pancreas (Figure 2). This single islet makes it an excellent model to study endocrine cell formation and regeneration. Similar to mouse pancreatic development, the zebrafish pancreas is a result of a fusion between a dorsal and a ventral pancreatic bud (123). The ease of imaging in the developing zebrafish embryo has revealed that the two pancreatic buds have distinct contributions at least in early β-cell development. The dorsal bud gives rise to an early primary islet in the head region of the developing zebrafish pancreas, making it an ideal

model for studying β-cell development and regeneration. The ventral bud then contributes to the majority of the pancreas tissue thereafter (124–126). When it comes to the pancreatic specification from the endoderm, the only distinction identified so far compared to the mammalian process is the need for active sonic hedgehog (Shh) signaling in zebrafish, while in mice Shh signaling is inhibitory for the formation of the early pancreatic progenitors (70,127). The Shh signaling acts in a non-cell autonomous manner in endodermal cells adjacent to the prospective pancreatic progenitors and it is necessary β-cell differentiation (128). Early pancreatic progenitors are characterized by the expression of Pdx1 and Ptf1a as in the mouse model (125,129,130). Notch signaling has a clear contribution to the development of the endocrine lineages in zebrafish, with notch inhibition via pharmacological or genetic approaches leading to precocious endocrine cell formation (129,131,132).
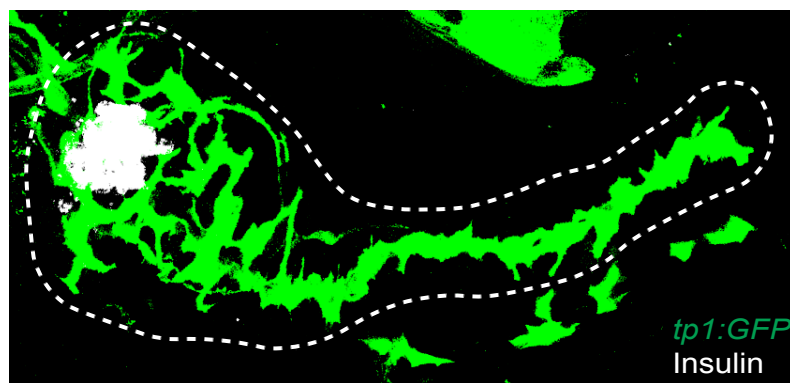


*Figure 2: Maximun projection of 5 dpf zebrafish pancreas. Tg(tp1:GFP) is used to visualize the ductal tree and insulin staining shows the primary islet location at the head of the pancreas. White dashed line outlines the whole pancreas.*

Contrary to mammals, Neurog3 is not expressed in the zebrafish pancreas and is not required for pancreatic endocrinogenesis. Instead work by Flasse et al convincingly showed that Ascl1b and Neurod1 are the transcription factors responsible for endocrinogenesis in the zebrafish pancreas (133). Importantly, Neurod1 levels seem to be important for the differentiation towards the α- and β-cell fates similar to Neurog3 expression in mice (134). It is still unclear if there is an exact developmental process similar to the secondary transition. Yet, pancreatic progenitors residing in the duct epithelial cells of the zebrafish have been traced to differentiate to β-cells and show similar expression to mammalian progenitor with high levels of Nkx6.1 and Sox9b (135–138). Recent efforts to functionally characterize maturation of zebrafish β-cells rely on calcium imaging, an important component of the insulin secretion machinery. Insulin immunostaining also is used as a tool to assess functional maturation of β-cells however a reliable ELISA assay is lacking to definitively show glucose dependent insulin secretion in this model. On the other hand, the calcium-efflux imaging experiments have shown that zebrafish β-cells respond rapidly to glucose injections, suggesting via an indirect manner that they have the machinery necessary to perform glucose-stimulated insulin secretion as early as 4 dpf (days post fertilization) (139–141). Overall,

these studies demonstrate the conservation of β-cell developmental mechanisms from zebrafish to mouse and human.

# 4  β-CELL REGENERATION

In the first part of the introduction, it was established that loss of β-cells is an important element in diabetes manifestation and progression. As described, available therapies for patients with diabetes can manage the disease but no cure is available. hESCs-derived β-cells hold currently the biggest promise for a next-in-line therapy and the increased understanding of human β-cell development will certainly lead to improved protocols of β-cell differentiation. Nevertheless, stimulating regeneration of the endogenous β-cell population is the most physiologically relevant approach that can approximate a cure for the disease. Therefore, it is of paramount importance to understand β-cell regeneration in animal models and translate these findings into potential therapeutics for clinical use.

## 4.1  MODELS OF β-CELL REGENERATION

**Physical injuries**

Pancreatectomy refers to the surgical removal of a portion of the pancreas and it is the first method of pancreas injury attempted in mammals. While the organ does not fully regenerate in mice, there are reports of a certain extent of β-cell regeneration in mice that mainly involve proliferation of preexisting β-cells (142). Pancreatic duct ligation (PDL) is the second widely used surgical procedure to damage the pancreas in mammalian models. During this process the main pancreatic duct is ligated leading to atrophy and enzymatic distraction of the distal site of the ligation leaving the rest of the pancreas healthy to be used as a control. PDL injury seems to reawaken a progenitor pool within the ductal cell epithelium to differentiate to β-cells in mice (143).

**Genetic models of diabetes**

The non-obese diabetic (NOD) mice have been used to mimic type 1 diabetes in mammals. These mice are characterized by pancreatic inflammation, termed pancreatitis, that lead to elevated leukocyte infiltration of the pancreas, destruction of β-cells as well as detectable levels of autoantibodies found in type 1 diabetic patients (144). A second genetic mouse model of β-cell destruction is based on mutation of the transcription initiation factor 1A (TIF-Ia). This model shows a profound induction of β-cell apoptosis in mice followed by β-cell regeneration, however it has not been widely used by researchers in the field (145).

**Chemically induced β-cell damage**

Streptozotocin to a large extent and alloxan to a lesser extent have been the golden standard of pancreatic injury models in rodents. Both alloxan and streptozotocin is taken up specifically by the GLUT2 transporter in the β-cells. Streptozotocin causes DNA damage

followed by apoptosis. Different dose regiments of streptozotocin have been used to mimic different extents of β-cell damage (146). A recent study suggested that several low doses of STZ injections in mice cause β-cell dedifferentiation suggesting that injury model can be potentially used in studies of β-cell dedifferentiation as well (147). Moreover, single cell RNA-SEQ of mice injected with a single high dose of STZ provided a detailed transcriptomic characterization of the islet cell population in this injury model (148). In zebrafish, streptozotocin treatment does not seem to have the same extent of β-cell damage as in rodents (149–151). Alloxan generates reactive oxygen species in rodent β-cells and that is considered as the primary mechanism of β-cell death (146).

**Combination of genetic and chemically-induced models**

In mice the diphteria toxin-based models has been widely used as a method to ablate almost 100% of β-cells. In this model, the diphtheria toxin receptor (DTR) is expressed under the control of the insulin promoter. Consequently, addition of diphtheria toxin selectively ablates 99% of the β-cells. Using this method it was first discovered that other endocrine cells can transdifferentiate to β-cells (see following chapter) (152). The combination of genetic and chemical approaches to mimic β-cell loss are more popular in the zebrafish model. In fact, the majority of the studies in the field of zebrafish β-cell regeneration take advantage of the nitroreductase (NTR)-metronidazole (MTZ) approach (153,154). The NTR-MTZ is also the main injury model used in this thesis to study β-cell regeneration. In this model, a transgenic zebrafish expresses the bacterial enzyme NTR under the control of the insulin promoter. When a prodrug called MTZ is added to the fish water, NTR converts MTZ to a toxic byproduct and therefore β-cells are specifically and efficiently ablated. This model leads to an almost complete ablation of β-cells. The standardized assay used in this thesis is based on the NTR-MTZ model. *Tg(ins:FP-NTR)* zebrafish larvae are allowed to grow until 3 dpf and then MTZ is added to the water for 24 hours to efficiently ablate the β-cells. Following β-cell ablation, a 2-day regeneration window is given for the zebrafish larvae where the genetic and chemical pertubations are tested for their phenotype on β-cell regeneration at 6 dpf (Figure 3). On the contrary, the DTR based system was also adopted in the zebrafish system but it is not widely used as it causes high levels and unspecific toxicity (155). In addition, a different system based on human CASPASE8 induction using a chemical dimerizer was described as an alternative to the NTR-MTZ system. Using the CASPASE8-based system both β-cell and acinar cell regeneration has been observed in zebrafish (156,157).
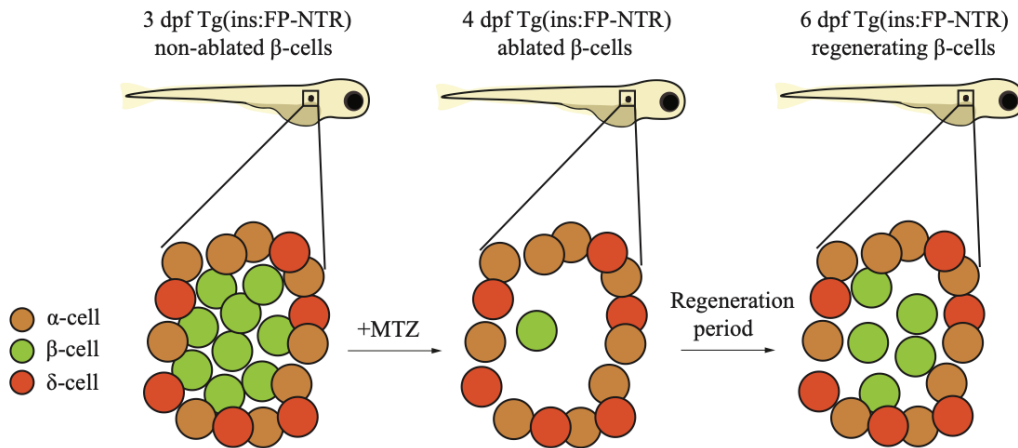
*Figure 3: Schematic describing the NTR-MTZ zebrafish injury model that is used in this work to model diabetes.*

## 4.2 CELLULAR MECHANISMS OF β-CELL REGENERATION

### 4.2.1 Proliferation

Proliferation of preexisting β-cells has been argued as the main mechanism of β-cell regeneration in mouse models of pancreatectomy (158,159). This has been expanded recently to include other injury models of the mouse pancreas including streptozotocin and PDL using a newly developed lineage tracing system of β-cells (160). In the majority of these studies, the pancreatic injury does not lead to 100% destruction of β-cells and it has been speculated that the extent of the injury might stimulate different mechanisms of regeneration (142). On the contrary, proliferation of the few surviving β-cell is not a major contributor in the zebrafish models of injury, probably due to the high percentage of β-cell injury achieved (161). While proliferation has been shown to be a major mechanism of β-cell regeneration in mammals, the basal levels of β-cell proliferation in zebrafish, mouse and humans are very low (124).

A potent and specific stimulator of human β-cell proliferation has so far been elusive. The best and most reproducible hit from various screens so far is the chemical compound harmine. Harmine was discovered in a chemical screen for inducers of MYC transcriptional activation in HepG2 hepatocyte cells. Harmine could stimulate murine β-cell proliferation following pancreatectomy as well as human β-cell proliferation (162). Importantly it was shown that the mechanism of action was through inhibition of DYRK1A kinase but unfortunately the proliferative effect was not specific to β-cells as ductal and other endocrine cell proliferation was observed (162,163). Based on these findings there were attempts to generate more specific and potent DYRK1A inhibitors and the results of these efforts remain to be seen (164). Moreover, a third research group synthesized and identified a compound that they named GNF4877 that promotes β-cell proliferation in the diptheria toxin-induced β-

cell mouse injury model. Importantly, the induced proliferation was dependent on DYRK1A signaling inhibition similar to the previous studies (165). Collectively, this results demonstrate a major involvement of the DYRK1A inhibition in mouse and human β-cell proliferation.

Apart from harmine, adenosine signaling and TGFb inhibition seem to be heavily involved in β-cell proliferation following β-cell injury. An unbiased screen in zebrafish showed that NECA, an adenosine agonist, stimulates β-cell proliferation in vivo with three of the other top hits of the screen also targeting the adenosine pathway (161). Independently, another group showed that adenosine kinase inhibition stimulates β-cell proliferation in murine and pig islet cultures (166). Important observations led to TGFb signaling via SMADs as important regulators of β-cell proliferation. It was shown that older mice have very limited capacity to proliferate because the trithorax group of epigenetic regulators activated INK4 that acts as a cell cycle inhibitor (105,167–169). Further evidence from transcriptomics analysis of insulinoma tumours, rare β-cell malignancies with high proliferation rates, revealed that SMAD-mediated signaling was one of the most prominent drivers of the uncontrolled proliferation phenotype (170). Inhibition of TGFb signaling indeed stimulated β-cell proliferation in both mice and human cells transplanted in immunocompromised mice (171,172). Strikingly, a combination of harmine and TGFb inhibitors increased human β-cell proliferation up to averages of 5%-8% which is the highest reported currently in the literature (173).

Circulating peptides are thought of as attractive alternatives to traditional chemical approaches of creating new drugs, the reason being they have the advantage of circulating in the bloodstream and therefore be readily available in all tissues as well as have higher specificity for their target in vivo compared to small molecules. A number of circulating peptides have been shown to stimulate β-cell proliferation. In an induced model of mouse insulin resistance, El-Ouaamari and colleagues discovered that a circulating factor from the mouse liver mediates the compensatory  β-cell proliferation (174). Follow-up work revealed that Serpinb1 was the circulating protein secreted from the liver that stimulated β-cell proliferation in zebrafish, mouse and human β-cells under various injury models (175). Moreover, already commercially available GLP-1 analogues promote the proliferation of murine β-cells in the streptozotocin mouse injury model, but the effect seem to decline with age (167). Similar observations are made in human islet grafts transplanted in mice, while a synergy between GLP-1 agonists and DYRK1A inhibition has been reported in vitro (176,177). This age-dependent decline in proliferative capacity upon GLP-1 agonists is observed with two additional signaling pathways, namely PDGF receptor signaling and WISP1 related signaling. Although is not clear if the circulating PDGF can induce β-cell proliferation, overexpressing WISP1 was shown to induce β-cell proliferation and rescue mice from streptozotocin injury (106,178).

The vast majority of the aforementioned studies and signaling pathways have been identified either by assessing known signaling pathways that affect cellular proliferation or by in vitro

screens. Zebrafish larvae is a great model to perform larger scale chemical screens to identify novel pathways to stimulate β-cell proliferation. Two such screens have been performed so far both taking advantage the fluorescent ubiquitylation-based cell cycle indicator (FUCCI) system (179). In a zebrafish adaptation of the FUCCI system, a fluorescently-labelled zCdt1 protein that marks the G1 phase and fluorescently-labelled zGeminin that marks the S/G2/M phases of the cell cycle are expressed under the control of the insulin promoter. Using this system, Tsuji et al performed a chemical screen and found that serotonin, retinoic acid and glucocorticoids can stimulate β-cell proliferation in vivo (180). In particular, the serotonin signaling pathway is a known contributor to the pregnancy-induced β-cell expansion in mice (181). A more automated version of the zebrafish FUCCI system is termed LUCCI and was developed by Charbord et al (182). In this genetic system, the fluorescent label fused with the zgeminin is replaced with the nanoluciferase gene. This enabled a higher-throughput of the screen in vivo and identified a SIK kinase inhibitor as a potent stimulator of β-cell proliferation in zebrafish and mice (182). Additional work identified distinct roles for different members of the SIK protein family in mice and human cells (183). These studies demonstrate the power of zebrafish to perform medium-large scale chemical screens for inducers of β-cell proliferation.

As mentioned, despite the discovery of all the different signaling pathways regulating β-cell proliferation in zebrafish, mice and humans, the induced proliferation rate achieved is low. An array of new methodologies has shown an unprecedented level of β-cell heterogeneity that might explain this phenomenon. Using in vivo lineage-tracing, Flattop (Fltp) a WNT signaling pathway reporter, was shown to mark a non-proliferative subpopulation of mouse β-cells (184). Further, a LIF[+] marked subpopulation of β-cells was demonstrated to have an enhanced capacity to proliferate in humans (185). This opens up possibilities for future studies to take advantage of this heterogeneity to develop more targeted treatments for β-cell proliferation.

Overall, β-cell proliferation following injury is the most well-studied mechanism of β-cell regeneration. Although, the scientific community still has not found a combination that can stimulate human β-cells to proliferate to levels that can treat the disease, advancements in the field are rapid. Nonetheless, stimulating β-cell proliferation is possible when there are some β-cells left following injury. In case of type 1 diabetes, there are hardly any β-cells remaining and it will be a challenge for even the most potent drugs to fully regenerate the β-cells. Other mechanisms of β-cell regeneration have been proposed when β-cell injury is nearly 100% and will be discussed.

### 4.2.2 Transdifferentiation

One of the first examples of one mature cell type directly reprogrammed to another was shown in the neuronal field when fibroblasts were converted to neurons upon genetic manipulation (186). Examples like this, sparked hope that other cell types could be transdifferentiated directly to β-cells as a way to stimulate β-cell regeneration. The first approach was pioneered by Zhou and colleagues demonstrating that overexpressing the key

developmental transcription factors NEUROG3, PDX1 and MAFA in pancreatic acinar cells could transdifferentiate them to insulin producing β-cells (187). These newly-formed β-cells were shown to revert diabetes in mouse however one of the follow-up studies was retracted from the scientific literature bringing up questions regarding the validity of the data (188). A handful of studies followed-up on this discovery and showed that a similar genetic approach could stimulate β-cell formation from intestinal, stomach, liver and other endocrine cells. Different genetic approaches were used in the different systems. First, overexpressing the same transcription factors mentioned above, second an induced FOXO1 inhibition and third missexpressing/deleting key transcription factors of endocrine cells (189–192). Moreover, as demonstrated by the Collombat group using elegant mouse genetics overexpressing of PAX4 transcription factors in α- or δ-cells can stimulate β-cell regeneration after streptozotocin treatment in mice (193,194). Further evidence showed that forced expression of just PDX1 and MAFA in α-cells could rescue diabetes in the NOD model by transdifferentiation of α-cells to β-cells and can reprogram human α-cells in vitro as well (195). Lastly, genetic manipulation of ARX and DNMT1 in α-cells could transdifferentiate these cells to β-cells in mice and human cultures from type 1 diabetics (196).

All these studies demonstrate that upon genetic manipulation different cell types can transdifferentiate to β-cells, with acinar and α-cells being in the center of this approach. However, the question remained: is transdifferentiation of a mature cell-type to β-cells something that can naturally occur in injury conditions? A seminal study by Thorel et al provided clear evidence that upon complete ablation of β-cells, there is a small percentage of α-cells transdifferentiating to β-cells in mice. The model of injury used in this study was the DTR based genetic-chemical ablation approach (152). This study and specifically this mouse model formed the basis for subsequent results showing that the somatostatin-secreting δ-cells could transdifferentiate to β-cells upon β-cell injury. This transdifferentiation event was age-dependent with older mice losing the capacity to rejuvenate the β-cells through δ-cell regeneration (197). Importantly, human α-cells transduced with Pdx1 and MafA in vitro transdifferentiated to β-cells and could rescue the development of diabetes after transplantation in the DTR mouse injury model (198). Similarly to the mouse DTR system, the zebrafish MTZ-NTR system causes a near-complete ablation of β-cells and α- to β-cell conversion was initially thought to be the predominant mechanism of β-cell regeneration in this model (199). Interestingly, the first study by Ye et al implicated proglucagon signaling into α- to β-cell conversion data that have been corroborated independently in mice (199,200). However, development of better tools for lineage-tracing the α-cell population in zebrafish using the *arx* promoter showed a minimal contribution of this cell type towards β-cell regeneration (201). No other cell conversion to β-cells has been reported so far in this model and no chemical or genetic screen has been performed for inducers of cell transdifferentiation to β-cells. The only chemical inducer of α- to β-cell conversion identified so far is GABA and its downstream signaling pathway. In one of these studies, artemether treatment which was shown to activate GABA-related signaling, could improve β-cell regeneration in the caspase-induced zebrafish model, but no lineage-tracing was performed to

identify the origin of these cells (157,202). Controversially, the mouse data of these two papers are currently challenged by other groups claiming that they do not observe the expected phenotype following GABA treatment and instead they provide evidence for a dedifferentiated phenotype upon chemical exposure (203). Whether or not these results stand the test of rigorous reproducibility remains to be seen.

Lastly, efforts are made to understand the level of plasticity of mature cell types in the mouse and human pancreas. Using ATAC-SEQ, Ackermann et al discovered a previously unappreciated plasticity of human α-cells on the epigenomic level and in genomic loci of important β-cell regulators hinting their ability to transdifferentiate (204). Additionally, Van der Meulen et al proposed that there is a rare population of immature β-cells that derive from α-cells and present a neogenic pool at the mouse islet periphery. However, whether these cells can respond to injury and replenish the β-cell population is unknown (205). Intriguingly, a Procr$^+$ islet cell population was also shown to contribute to islet regeneration however this expression data does not seem to be reproduced by another single-cell RNA-SEQ study (114,206). In sum, direct cellular conversion to β-cells is a promising strategy to replenish the β-cell mass, yet our understanding and manipulation of the relevant pathways are still in its infancy.

### 4.2.3 β-cell neogenesis

The existence of adult stem-like progenitors is well-established in certain tissues like the intestine and the hematopoietic systems. These adult stem cell have the ability to regenerate certain cell types following injury (207,208). There is the belief that the pancreas has such progenitor cells that can be stimulated upon β-cell injury and differentiate to β-cells. More than 20 years of research into the identification of such progenitors has given no clear answer and is perhaps the most controversial topic in the field of β-cell regeneration.

The potential progenitor cells of the pancreas are thought to reside in/along the pancreatic ducts. These cells have similarities in gene expression profile with the developing epithelial cells of the trunk domain that constitute the progenitors of the duct/endocrine cells later in life. As was the case with the other two cellular mechanisms of β-cell regeneration, the majority of the data regarding β-cell neogenesis derive from mice. As it was stated previously, pancreatectomy and streptozotocin induced β-cell injury causes the β-cells to proliferate and that seems to be the predominant mode of regeneration in these models (158–160). However, a study in 2008 suggested that ductal cells can reexpress Neurog3 upon PDL injury, differentiate to β-cells and subsequently proliferate (209). This was the first evidence of the elusive progenitor in the ductal cell compartment; however, this study lacked a definitive lineage-tracing experiment to conclude its existence. Besides, in the DTR-induced ablation of acinar and endocrine tissues there was evidence for ductal-to-β-cell differentiation, showing that this phenotype might be conserved across models (210). A number of lineage tracing studies followed the initial observation in an effort to trace the ductal cells and assess their ability to differentiate to β-cells. Lineage-tracing with Hnf1b, Hes1 and Sox9 all markers of the ductal cells failed to show any evidence of ductal cell

contribution to β-cell differentiation following injury (77,81,211). However, traced cells in all these transgenic mice showed contribution of the embryonic epithelial ductal cells to all adult endocrine cell types. The only positive evidence towards the existence of a ductal pancreatic progenitor was that Sox9$^+$ ductal cells could initiate expression of NEUROG3 but failed to fully differentiate to β-cells (211). It has to be noted here that none of these genetic tracing models are ideal as they mark at best 70% of the mature ductal cells and as low as 40% in the case of the Hnf1b:Cre$^{ER}$ transgenic mouse line. Yet, a very recent study utilized a different ROSA26-RFP reporter and the Hnf1b:Cre$^{ER}$ driver and achieved an approximate 95% of ductal labeling, suggesting that this new genetic tool can be a better alternative to trace the ductal cells in mice (212). Moreover, temporal non-cre mediated tracing using florescent reporters also confirmed that pancreatic duct ligation reawakens the NEUROG3 expression in pancreatic ducts and surviving endocrine cells but no evidence of β-cell neogenesis was observed (213). However, a very recent study using a NEUROG3:Cre$^{ER}$ tracing mouse line showed that these duct-residing cells could differentiate to β-cells in young mice (214).

On the contrary, a number of studies using different set of markers for lineage-tracing the ductal cells have suggested the existence of such progenitors. Carbonic anhydrase driven Cre expression that labels around 50% of the adult mouse duct showed that regenerating β-cells following PDL can be traced at an early age (215). Even in the case of transdifferentiation upon PAX4 overexpression, it has been postulated that ductal cells give rise to α-cells before the α- to β-cell conversion, but no lineage-tracing of the duct was performed in these studies (194). Moreover, cells with high ALDH1 activity termed centroacinar cells have been shown to be the facultative progenitors of the pancreatic ducts (216). It was recently proposed that ALDH1B1 marks a progenitor population of the adult pancreas however no injury model was performed in this study to assess if these cells can be induced to differentiate to β-cells (217). Further, it has been postulated that the location of the ductal cells compared to the islets might indicate the progenitor pool as intraislet ductal can be induced to differentiate to β-cells in young mice (218). Finally, two independent studies have revealed roles for f-box and WD repeat domain-containing 7 (FBW7) and a fast-mimicking diet in β-cell neogenesis in mice (219,220).

There is strong evidence that ductal cells of zebrafish can differentiate to β-cells both during and upon injury in the NTR-MTZ model, both in larva as well as in adult stages. Importantly, ductal cell neogenesis is thought to contribute up to 70% of the regenerating β-cells in adult zebrafish (135,136). The most commonly used tool to lineage-trace the ductal cell utilizes the notch signaling pathway reporter and can label up to 75% of the intrapancreatic duct (131,221). As one can deduce, notch signaling is an important determinant of β-cell neogenesis and upon treatment with notch inhibitors there is a pronounced endocrine cell differentiation (129,131). The extrapancreatic duct has been hypothesized to contribute to β-cell regeneration but no lineage-tracing tool has been developed to confirm this hypothesis. Chemical screen for inducers of β-cell neogenesis both with and without β-cell ablation have identified crucial roles for retinoic acid, NfkB and cyclin-dependent kinase 5 (Cdk5) mediated signaling pathways as potential therapeutic targets (222–225). Further, overnutrition

and a high calory diet that used to model type 2 diabetes in zebrafish also showed evidence for progenitor differentiation to β-cells, suggesting that this mechanism can be further explored in mammalian type 2 diabetes models (155,226,227). Moreover, the zebrafish is a great platform to identify markers of this population that we can then use to definitively identify if such population exist in mammals. A summary of the signaling pathways identified so far to be involved in β-cell regeneration is shown in table 1.

| Signaling pathways, secreted proteins and small molecules involved in β-cell regeneration | | |
|---|---|---|
| *β-cell proliferation* | *Transdifferentiation to the β-cell fate* | *β-cell neogenesis* |
| DYRK1A inhibition (Harmine/5-IT/GNF4877 treatment) | Combinations of NEUROG3, MAFA and PDX1 overexpression in various non-β-cell populations | Inhibition of the Notch signaling pathway |
| Adenosine signaling pathway | PAX4 missexpression in α- and δ-cells | Retinoic acid signaling pathway |
| Inhibition of TGFb signaling pathway | GABA signaling pathway (?) | CDK5 inhibition |
| Serpinb1 overexpression | Simultaneous DNMT1 and ARX loss in α-cells | FBW7 loss |
| GLP-1 agonists | | |
| PDGFR signaling pathway | | |
| WISP1 overexpression | | |
| Serotonin signaling pathway | | |
| SIK signaling pathway | | |

*Table 1: Table summarizing the major signaling pathways, small molecules and secreted proteins identified so far to be involved in β-cell regeneration from model organism studies.*

## 4.3 HUMAN β-CELL REGENERATION

The ultimate goal in the field of β-cell regeneration is to translate findings from preclinical models into therapies for diabetes. However, is there any evidence for β-cell regeneration in humans? Of course, we need to mention that the data we have regarding pancreatic development and regeneration are derived from the invaluable contribution of human pancreatic donors. Initiatives like the nPod network, the Alberta Diabetes Institute Islet Core and the Exeter Archival Diabetes biobank have revolutionized human diabetes research providing donor samples in a systematic and organized way (228,229).

β-cell expansion in humans have been documented in the cases of obesity-induced insulin resistance and pregnancy (another situation of insulin resistance). The exact pathways involved in this compensatory β-cell expansion is not yet detailed but studies in rodents point to β-cell proliferation as the likely mechanism of the expansion (230). Nevertheless, it needs to be mentioned that the dramatic increase in mouse β-cell expansion in models of obesity, insulin resistance or pregnancy is not directly translating to the human observational data. Importantly, no changes in β-cell proliferation or apoptosis is observed in human sections contrary to the mouse models where β-cell proliferation is the predominant means of expansion. Instead, it has been reported that small islet clusters and scattered insulin cells within the human ducts are observed in insulin resistant individuals (231–233).

In a seminal study of individuals with long-standing type 1 diabetes by Keenan and colleagues, it was revealed that a few β-cells survive even in extreme cases of diabetes. Interestingly, some of the insulin positive cells were found in the ductal cell compartment of the pancreas adding evidence to the duct-progenitor pool hypothesis in humans (234). It also has to be mentioned that insulin scattered cells within ducts have been observed in pancreatic sections from healthy individuals (235,236). Expanding on this study, work from other groups have corroborated the evidence that insulin positive cells are detected in the pancreas of patients with type 1 diabetes and that some of these individuals can secrete very low but detectable amounts of c-peptide. Two of these studies reported an extremely low amount of β-cells associated with the ducts while the most recent effort using a large number of donor pancreata from the nPod network showed indeed insulin$^+$ cells around the ductal compartment (237–241). These data suggests that human ducts might be more plastic compared to the mouse counterparts and a progenitor pool might be present after all.

# 5  AIMS

The overall aim of the present thesis is to uncover new molecular pathways that stimulate pancreatic β-cell regeneration.

Specific aims:

**Papers I&II**: To perform transcriptomic analyses of regenerating zebrafish islets and screen for putatively secreted proteins that can stimulate β-cell regeneration

**Paper III**: To identify the molecular target of the small molecule CID661578, the most potent hit of an in vivo chemical screen for stimulators of β-cell regeneration

**Paper IV**: To explore the liver-to-pancreas crosstalk during β-cell regeneration

# 6 RESULTS AND DISCUSSION

## 6.1 PAPER I

As was established in the introduction of this thesis, regenerating the β-cell population is an attractive approach to generate new therapeutics for diabetes. In order to develop new drugs to stimulate β-cell regeneration in humans, we first need to understand how this process occurs in vivo. The zebrafish model has a tremendous capacity to regenerate its β-cell population following an almost complete ablation. In Papers I&II, we aimed to understand the changes occurring in the islet microenvironment following β-cell ablation to identify pathways important for β-cell regeneration in the zebrafish model and translate the findings to mammals.

To this end, we used the zebrafish NTR-MTZ model to ablate β-cells. We performed a microarray analysis of isolated islets from 4 dpf zebrafish larvae with and without β-cell ablation. We sampled the islets directly after β-cell ablation as we hypothesized that we could identify pathways immediately upregulated upon injury. We focused our analysis on upregulated genes coding for potentially secreted proteins, as we reasoned they would make ideal drug candidates given their ability to circulate in the bloodstream (Figure 4).
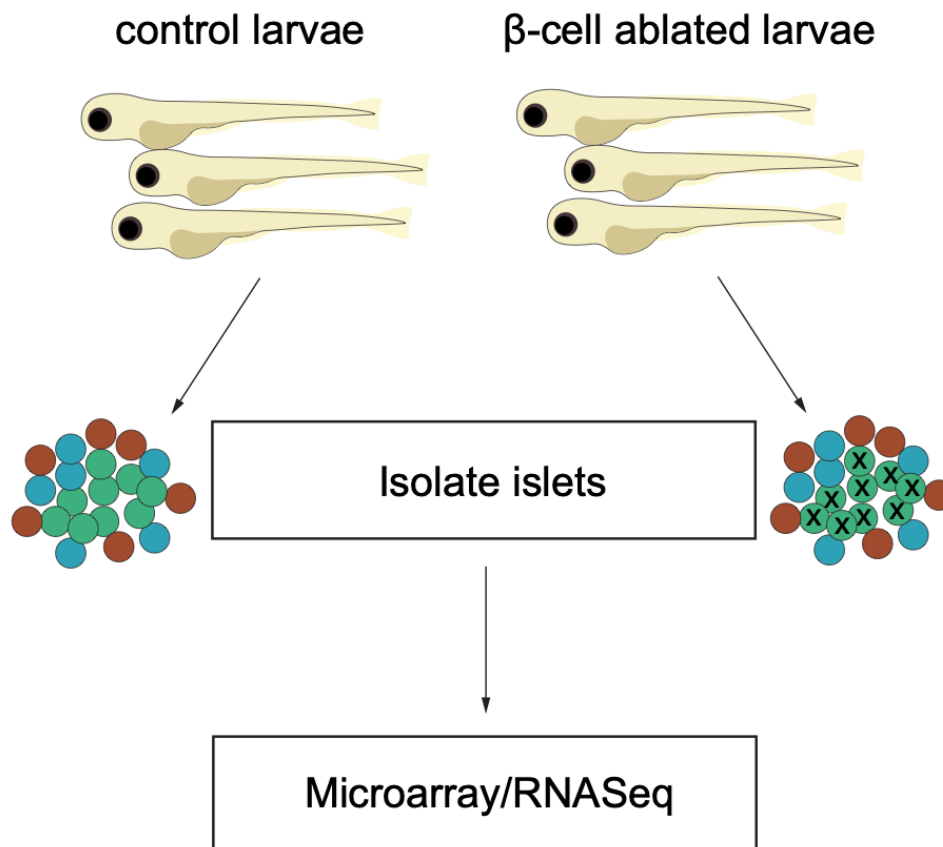
*Figure 4: Schematic showing the experimental design of the genetic screen for inducers of β-cell regeneration. Briefly, primary islets were isolated from zebrafish larvae directly after β-cell ablation. RNA was extracted, and Microarray/RNA-Seq was performed for Papers I&II respectively. Adapted from Paper II.*

In Paper I we showed that overexpressing the circulating protein Igfbp1a in our β-cell ablation model stimulated β-cell regeneration at supraphysiological levels. Igfbp1a-induced β-cell formation was specific in injury conditions and the newly-formed β-cells were functional as shown by the co-expression with Pcsk1 and lower glucose levels. The cellular source of the new β-cells in this system were the α-cells of the pancreas suggesting that Igfbp1a promoted α- to β-cell transdifferentiation. We also observed an increase in β-cell proliferation suggesting that both regenerative mechanisms are important for the observed phenotype. Lastly, treatment with Igfbp1 increased the number of insulin- and glucagon-bihormonal cells in mouse and human islets, suggesting that at least the transdifferentiation phenotype can be translated to mammalian systems.

Transdifferentiation of α- to β-cell has been shown to be an alternative that can partially restore glucose levels in mice (152,198,242). Our work, identified a secreted protein Igfbp1 as stimulator of this process. Mice lacking Igf1 in the pancreas also manifest with an enlarged islet after streptozotocin challenge (243). Coupled with the increased appearance of

34

bihormonal glucagon/insulin cells in isolated mouse and human islets, this data suggests that the functionality of Igfbp1 can be conserved to mammalian systems.

## 6.2 PAPER II

Paper II builds on Paper's I approach (Figure 4) to explore the possibility of additional upregulated and potentially secreted proteins that could stimulate β-cell regeneration. In Paper II we used RNA-Seq to sample the transcriptome of the regenerating islets as we reasoned that we could identify more upregulated genes with the use of a more advanced transcriptomics method (Figure 4). Indeed, using RNA-Seq we identified an additional 16 upregulated genes coding for potentially secreted proteins that were not present in the microarray dataset. We also identified *igfbp1a* and *lxn* to be upregulated in the RNA-Seq dataset showing that there is an overlap between the two approaches.

Subsequently, we cloned the 16 genes under the control of the ubiquitous *actb2* promoter and overexpressed them in our regeneration assay. In this paper, we show that overexpressing *folr1* increased β-cell regeneration in the zebrafish larvae to similar levels as *igfbp1a* that was used in this study as a positive control. Using temporal lineage-tracing of the intrapancreatic duct together with genetic approaches to restrict the overexpression of *folr1* to different cellular compartments of the zebrafish pancreas, we showed that the newly-formed β-cells were derived from a ductal source.

Expression of *FOLR1* in two independent human single-cell RNA-Seq studies showed a restricted expression in a subset of ductal cells (244,245). Ductal cells have been predicted to be the progenitor cells of the pancreas and the expression of FOLR1 coupled to the in vivo data showed that it could mark a potential progenitor population. Interestingly, *FOLR1* expression was correlated with *CEACAM6* in one of the human single-cell RNA-Seq datasets. CEACAM6 has been also predicted to mark a progenitor population in human adult pancreas (246). Further, FOLR1 was shown as one of the top markers of pancreatic progenitors hESC differentiation to β-cells (44). Taken together, these results together with our observations suggests a potential use of FOLR1 as a functional marker of ductal cells with progenitor potential in humans.

Folr1 belongs to a class of receptors responsible for the uptake of folic acid derivatives into the cell (247). Once folic acid is taken up by the cells, it is converted to tetrahydrofolate which is the main carbon donor. The two metabolic cycles involved in these cellular processes are named folate cycle, which refers to the cycling of the folate intermediates, and the one-carbon metabolism, which is the series of metabolic reactions that transfer the carbon to different molecules for biosynthetic processes. Numerous biosynthetic processes are dependent on one-carbon metabolism involving methylation reaction, nucleotide biosynthesis and polyamine metabolism (248,249). In this work, we show that the genetically-induced β-cell regeneration through *folr1* overexpression can be recapitulated by chemical treatments with folate intermediates. Treatment with methotrexate that blocks tetrahydrofolate generation in the cells, blocks the increase of β-cell regeneration upon *folr1* overexpression.

Additionally, treatment with folinic acid promotes β-cell regeneration in larvae and juvenile zebrafish.

To identify the potential mechanism of one-carbon involvement in β-cell regeneration, we performed an untargeted metabolomics characterization of zebrafish larvae with/without β-cell ablation as well as with/without folinic acid treatment. First, enrichment analysis of the significantly changed metabolites showed that folinic acid treatment indeed changes one-carbon metabolism and folate cycle. Second, we identified pathways relating to lipid, carnitine and nucleotide metabolism as the most significantly affected metabolic pathways following folinic acid treatment. Third, we show that serine metabolism is important in β-cell regeneration without folinic acid treatment, establishing a new pathway to be explored in β-cell regeneration. Serine metabolism is directly linked to the mitochondrial one-carbon metabolism demonstrating a link between folinic acid treatment and serine metabolism in zebrafish. Interestingly, a substantial amount of carnitine-linked lipids was differentially regulated between the baseline regeneration process (i.e comparing metabolites with/without β-cell ablation) and the β-cell regeneration following folinic acid treatment. Recent evidence suggests that one-carbon metabolism and serine pathways can converge on the lipid biosynthetic pathway and our data corroborates these results (250,251). Lastly, we believe that the comparison of the metabolites differentially regulated upon β-cell ablation is a useful resource for researchers studying β-cell regeneration and it will point to novel pathways involved in the process.

Finally, in Paper II we show that folinic acid and tetrahydrofolic acid treatments can stimulate β-cell generation in neoneatal pig islet cultures. These cultures are enriched in intraislet ductal cells and both our compounds increased the number of Ins$^+$CK7$^+$ colabelled cells. Only tetrahydrofolic acid increased the number of β-cells in these cultures showing that treatment with folinic acid may need a modified treatment period to fully differentiate these cells (Figure 5). These data are in line with research showing that the intraislet ductal population contributes to β-cell formation in young mice, suggesting that perhaps this population is more prone to differentiate (218).
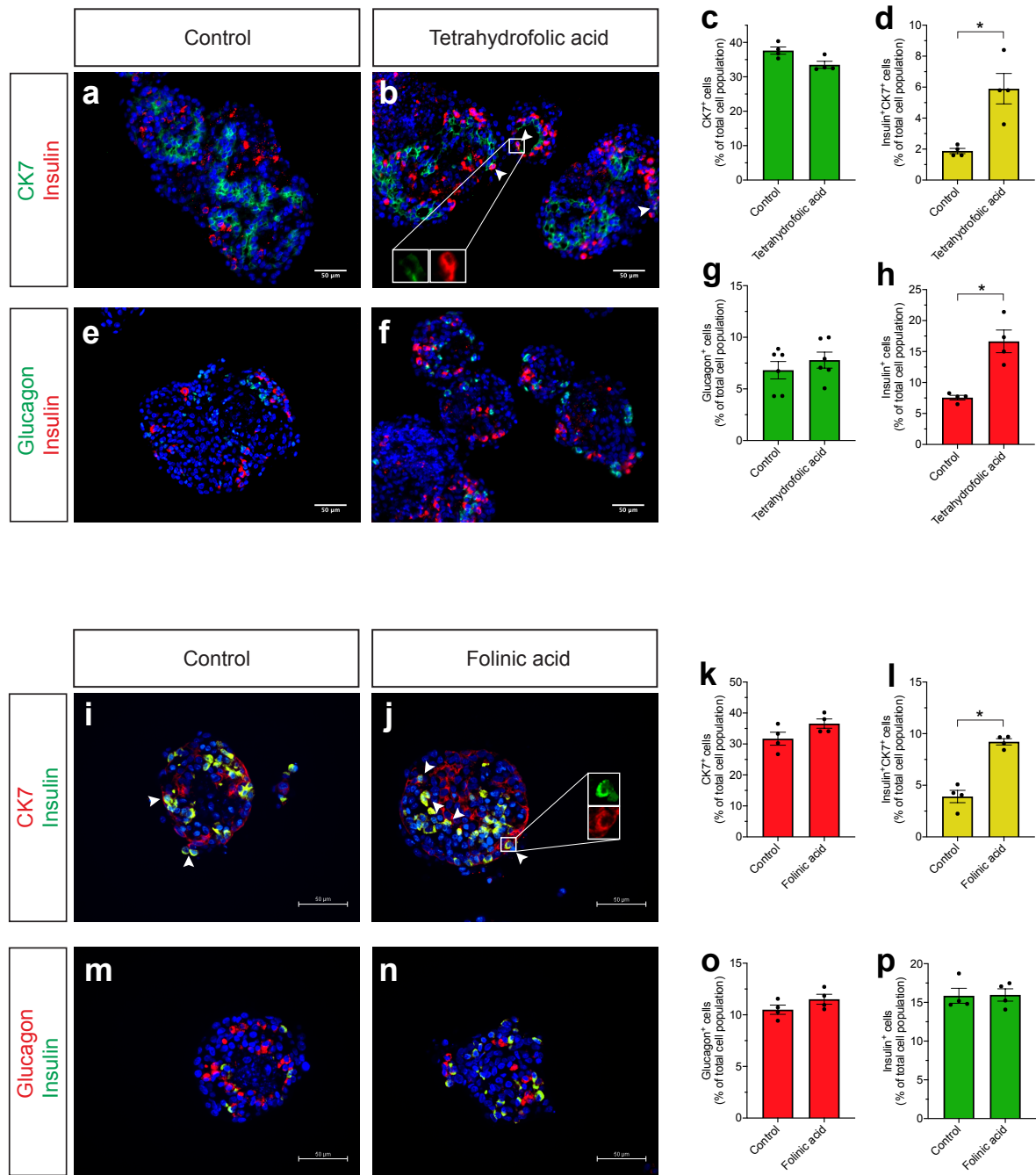
*Figure 5: Folate intermediates stimulate β-cell formation in neonatal pig islets. **a-d**, THF stimulates β-cell formation from ductal cells in neonatal pig islets. Images of control (**a**) and THF-treated (**b**) neonatal pig islets immunostained for insulin and CK7, a duct-specific marker in pigs. Quantification of the percentage of CK7+ and CK7+Insulin+ cells is shown in (**c**) and (**d**), respectively. Arrowheads point to CK7+Insulin+ cells. n=4. (**d**) *P=0.0286. **e-h**, THF increased β-cell but not α-cell formation in the in vitro neonatal pig islet culture. Control (**e**) and THF-treated (**f**) neonatal pig islets immunostained for insulin and glucagon. Quantification of the percentage of β-cells and α-cells is shown in (**g**) and (**h**), respectively. n=4-6; (**h**) *P=0.0286. **i-l**, Folinic acid stimulates β-cell formation from ductal cells in neonatal pig islets. Images of control (**i**) and folinic acid-treated (**j**) neonatal pig islets immunostained for insulin and CK7. Quantification of the percentage of CK7+ and CK7+Insulin+ cells is shown in (**k**) and (**l**), respectively. Arrowheads point to CK7+Insulin+ cells. n=4. (**l**) *P=0.0286. **m-p**, Folinic acid did not increase the percentage of α- or β-cells in the in vitro neonatal pig islet culture. Control (**m**) and folinic acid-treated (**n**) neonatal pig islets immunostained for insulin and glucagon.*

*Quantification of the percentage of α- and β-cells is shown in (o) and (p), respectively. n=4. The scale bar is 50 μm for all pictures in the figure. All statistical analyses were performed with a Mann-Whitney test.*

Overall, in Paper II we reveal a role for *folr1* and one-carbon metabolism to stimulate β-cell generation in zebrafish and pig. Our metabolomics analysis suggested that this effect is most likely relating to changes in lipid, serine and nucleotide metabolic pathways. Moreover, our expression data suggests that FOLR1 could mark a potential progenitor population in human pancreas that could be potentially induced to differentiate to β-cells. Our results further support the existence of duct-residing pancreatic progenitors in zebrafish in relation to published studies (135,136) and suggests that this population might be present in young pigs, and adding a new pathway to be explored for its potential translational potential. Finally, given the safety profile of folic acid use in clinics, epidemiological studies exploring an association between folic acid supplementation and diabetes manifestation are currently in preparation as a follow-up on this work.

## 6.3 PAPER III

Most chemical screens in zebrafish are performed with FDA-approved drugs as these chemicals can already be used in clinical practice. However, screening for small molecules with no known mechanism of action can lead to the discovery of novel pathways to tackle disease. In the largest to-date in vivo chemical screen for stimulators of β-cell regeneration, Andersson et al discovered five potent small molecule inducers of β-cell regeneration. Four of these small molecules targeted the adenosine pathway while the fifth, CID661578, had no known mechanism of action (161). The aim of Paper III is to identify the molecular target of CID661578 and explore its mechanism of action.
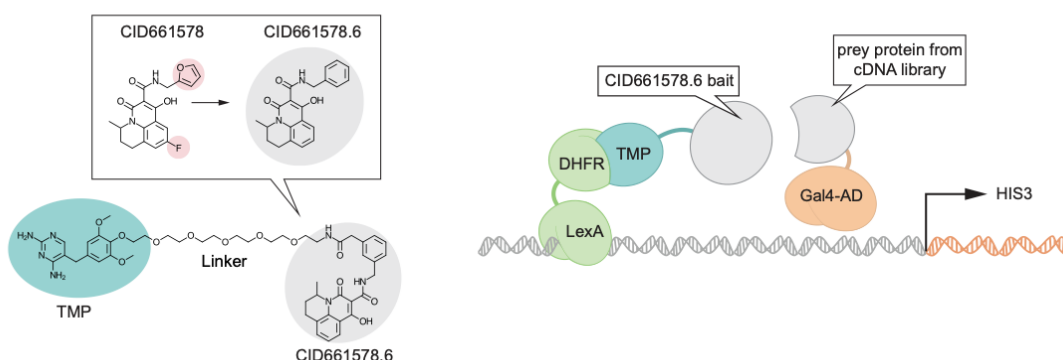


*Figure 6: Schematic showing YchemH screen identifying the molecular target of CID661578. A modified, linker-containing CID661578 molecule was added to yeast cultures that independently expressed two sets of cDNA libraries (the first one from zebrafish embryos and the second from human islet preparations) to screen for potential protein interactors. Adapted from manuscript III.*

To identify the molecular target of CID661578, we utilized a modified Yeast-two-hybrid system, termed yeast-chemical-hybrid (YChemH) screen. Briefly, this system takes advantage of the yeast strain that express the GAL4 activation domain fused to a cDNA library and the DNA binding domain linked to the DHFR protein. An interaction between CID661578 and the two components of the yeast-two-hybrid system is necessary for the yeast to transcribe the necessary proteins to synthesize histidine. Therefore, the yeast is placed in histidine-free media and when yeast survival is observed that marks a potential interaction between the CID661578 and the prey. The original molecule CID661578 was chemically modified to include a linker and two yeast libraries containing cDNA from zebrafish embryos and human islets were screened to identify the protein binding to the small molecule (Figure 6). By using this method, we identified that CID661578 binds to both the zebrafish and human MNK2. Moreover, we validated the interaction between Mnk2b (the zebrafish homologue of MNK2) and CID661578 in vivo by generating a mutant Mnk2b zebrafish, morpholino knockdown of Mnk2b and overexpressing Mnk2b in the ductal cells of the zebrafish. Additionally, a structurally similar inhibitor of MNK2, cercosporamide (Figure 7), increased β-cell regeneration in zebrafish larvae and no additive effect was observed after co-treatment with both chemicals. Further, knockdown/knockout of Mnk2b induces β-cell

regeneration in zebrafish larvae while overexpressing either the zebrafish or the human *MKNK2* abolishes the effect of CID661578 on β-cell regeneration.
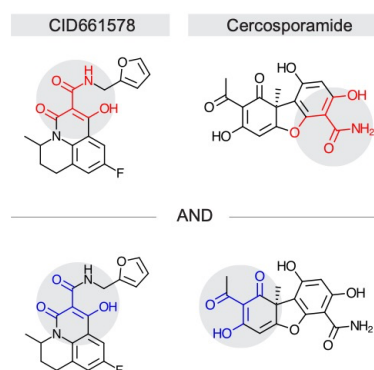


*Figure 7: Comparison of the structures of CID661578 and cercosporamide (a known inhibitor of MKNK2). Cercosporamide shares two similar structural groups with CID661578, colored in red and blue.*

Lineage-tracing showed that the newly-formed β-cells in zebrafish were derived from the notch-responsive ductal progenitors. Similar to Paper II, treatment with CID66178 and the known inhibitor of MNK2, cercosporamide, stimulated differentiation of pig ductal cells to β-cells in vitro. Staining of human pancreas sections showed that MNK2 is expressed in endocrine cells but also in/along the ductal cell compartment.  Interestingly, we observed a sparse population of duct and duct-associated cells to express MNK2, suggesting perhaps that inhibition of MNK2 of these cells might differentiate them to endocrine progenies. This is the second pathway identified in this work suggesting that ductal cell can differentiate to β-cells in zebrafish and pigs. Overall, combined the data from both Paper II and Paper III one could argue that the pig system might be a better alternative to the mouse model for studying the ductal population as a source for β-cell differentiation given the controversy surrounding the topic (77,81,158–160,211,213,218–220). Indeed, mouse evidence suggest the existence of an intraislet endocrine progenitor population later in life while the differentiation potential of the mouse duct is limited to prenatal and perinatal developmental stages (205,206,214,215,218).

MNK2 is part of the translation initiation complex, binding to the scaffold EIF4G, and has a known role to phosphorylate EIF4E together with the paralogue MNK1. MNKs are the only known kinases that phosphorylate EIF4E but the exact role of this phosphorylation has not yet been determined (252–255). Surprisingly, when we aimed to identify the kinetics of MNK2 inhibition upon CID661578 treatment, we found that CID661578 did not inhibit phosphorylation of EIF4E and a large-scale in vitro screen showed that CID661578 does not significantly affect the kinase activity of any of the 140 human kinases screened. Then we performed immunoprecipitation experiments of the translation initiation complex and observed that CID661578 inhibits the binding of MNK2 to the EIF4G scaffold and that increases the interaction between EIF4E-EIF4G on the 5'cap of mRNAs.

To reveal the molecular mechanism of action of CID661578, we performed untargeted metabolomics analysis of zebrafish larvae treated with CID661578. The results confirmed the potent glucose-lowering effect of the drug that we observed using a well-established in vitro

glucose measurement assay. Moreover, the levels of most of the amino acids were altered upon treatment, reinforcing the idea that protein translation is affected. Next, we used OPP incorporation and showed that CID661578 increases protein synthesis in vivo and blocking the interaction between EIF4E-EIF4G could abolish the effect of the drug confirming our in vitro observations. MNK2 has also been recently implicated in metabolic disorders. Cercosporamide treatment has been shown to reduce glucose levels in mice, while MNK2 was recently shown to affect adipocyte metabolism in mice (256–258).

In summary, in Paper III we identified MNK2 as the molecular target of CID661578, one of the most potent hits from an in vivo chemical screen for inducers of β-cell regeneration. We showed that the source of the new β-cells were the duct-residing progenitors in zebrafish and pig models highlighting the translation potential of our work. Mechanistically, CID66178 blocked the binding of EIF4G-MNK2, changed the binding dynamics of the translation initiation complex on the 5' cap and increased protein synthesis. Our results are the first to demonstrate that protein synthesis dynamics affect cell fate decisions in the pancreas. Nonetheless, it has been shown in other systems of adult stem cell niches that increasing protein synthesis can stimulate differentiation of the progenitor cells (259–262). Therefore, our results are in agreement with the existing literature and expands it to the pancreas field.

## 6.4 PAPER IV

The advantage of looking for pathways stimulating β-cell regeneration in vivo is that the regeneration occurs when all relevant surrounding tissues are present. A handful of studies have explored the possibility of secreted proteins from other tissues to induce β-cell regeneration in the pancreas. For example, Serpinb1 and Adiponectin have been shown to stimulate β-cell regeneration while secreted from the liver and adipose tissues respectively (175,263). Particularly the liver has been explored as a tissue that can secrete signals that stimulate β-cell proliferation in mouse insulin resistance (174,175). Further, blocking glucagon signaling in hepatocytes stimulates pancreatic α-cell hyperplasia via circulating amino acids (264,265). The aim of Paper IV is to study the crosstalk between hepatocytes and β-cell regeneration and similar to Papers I&II identify a secreted factor that can be used as potential treatment for diabetes.

Studies have shown that upon overexpression of PDX1 liver hepatocytes and ductal cells can transdifferentiate to β-cells (192,266–268). In the first part of Paper IV, we explored the possibility of developing liver cells to transdifferentiate naturally to β-cells in our zebrafish NTR-MTZ β-cell ablation model. We did not observe any spontaneous transdifferentiation of hepatocytes to β-cells following β-cell injury. Then we damaged the hepatocytes of the zebrafish larvae by treatment with acetaminophen, an established model of hepatocyte toxicity (269). Although we did not observe any changes in β-cell development upon hepatocyte damage, we noticed a delayed liver development upon β-cell ablation. This data showed that β-cells/insulin is important for proper hepatocyte development in the developing zebrafish larva.
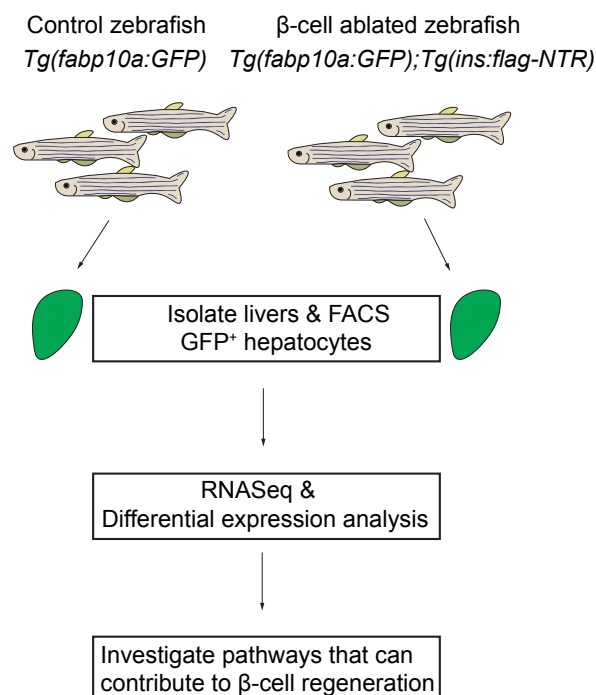
*Figure 8: Schematic showing the experimental design of the genetic screen for hepatocyte-specific inducers of β-cell regeneration. Briefly, hepatocytes were FACS from 2-month zebrafish with/without β-cell ablation. RNA-Seq was used to characterize the transcriptome of the isolated. Adapted from Manuscript IV.*

Then, we aimed to identify if there are any secreted proteins/metabolites from the hepatocyte that can induce β-cell regeneration. To this end, we characterized the transcriptome of hepatocytes following β-cell ablation in 2-month old zebrafish (Figure 8). Our transcriptomics analysis showed that glycolysis and lipid-related metabolic processes were the ones mostly affected early after β-cell ablation. Focusing our analysis on the upregulated genes, we identified only one significantly upregulated gene that we have not previously phenotyped, *sdf2l1*, that code for a potentially secreted protein but overexpression of this gene in the zebrafish larvae had no effect on β-cell regeneration. It is worth mentioning that both *serpinb1* and *igfbp1a* were upregulated in our transcriptomics data but the upregulation did not reach significant levels. Subsequently, we focused on the significantly upregulated enzymes as we hypothesized that they might be involved in a metabolic pathway that produces a secreted metabolite. For this reason, we overexpressed all the significant upregulated enzymes in the hepatocytes and ablated the β-cells followed by β-cell quantification. Only overexpression of the small isoform of the *mocs2* enzyme had a small yet significant increase in β-cell regeneration. Experiments using a stable line overexpressing *mocs2* showed that the increased β-cell regeneration phenotype was present in around 70% of the experiments suggesting that there are additional changes that need to work in concert with the *mocs2* overexpression for the full phenotype to be present, or that the overexpression model is not robust.

Moreover, treatment with a molybdenum salt (sodium molybdate) stimulated β-cell regeneration in zebrafish larvae. Sodium molybdate treatment has been shown to reduce glucose levels in a diverse set of model organisms including flies, mice and rats (270–272). Our results, expand on this observation and suggested that the molybdenum metabolism can be used to increase the β-cell number in zebrafish larvae.

Overall, Paper IV shows that there is no spontaneous hepatocyte-to-β-cell conversion in our regeneration model. Our genetic screen failed to identify a hepatocyte-derived secreted protein that can potentiate β-cell regeneration in zebrafish but found that the *mocs2* enzyme can be involved in the process. Mocs2 is part of the molybdenum biosynthetic pathway and is responsible for the generation of the metabolite molybdopterin that is an intermediate of the molybdenum cofactor metabolite (273). Interestingly, this pathway, through targeting gephyrin, was speculated to have a role in the α- to β-cell transdifferentiation observed in mice (157). Further, data from the mouse phenotype database show that the heterozygote *Mocs2* mice mutant have elevated glucose. Unfortunately, the *Mocs2* knockout mice die a few days after birth making it difficult to assess the full effect of the phenotype (274). However, this data presents a promising starting point to exploit molybdenum metabolism in β-cell regeneration.

# 7 CONCLUSIONS AND FUTURE PERSPECTIVES

Pancreatic β-cell regeneration has emerged as a promising therapeutic approach for patients suffering from diabetes due to extensive β-cell loss. The work performed during this thesis aimed to identify previously unknown molecular pathways important for β-cell regeneration in animal model systems and can potentially translate to new therapies. As part of this work, and using a zebrafish model of β-cell injury, we identified four new avenues to stimulate β-cell regeneration including: Igfbp1 overexpression, reinforcing one-carbon metabolism, stimulating protein translation through a novel interactor of MNK2 and manipulating molybdenum metabolism.

This work was performed using mainly one zebrafish model of β-cell injury. The pathways identified were also assessed for their potency to affect the normal course of β-cell and other endocrine cell development, but the phenotype appeared restricted to conditions of β-cell injury. Further, most of these pathways increased β-cell formation in mammalian models of β-cell biology. Yet, it would be interesting to assess the potency of these pathways to stimulate β-cell regeneration in additional models of β-cell injury, like the PDL procedure and in zebrafish and mouse model of compensatory β-cell hyperplasia as is the case of high-fat diet and pregnancy (151,227,230).

Our work provides additional evidence for the existence of facultative progenitors in the ductal tree of zebrafish that can be induced to differentiate to β-cells. Further, the neonatal pig islet culture model shows that it can also be exploited for studies of ductal cell as source for β-cell formation in mammals. The ongoing debate over the existence of these progenitors mainly revolves around the mouse model which is considered as the golden standard in the field to model pancreatic diseases. Yet, the data from all the different mouse models of β-cell injury show a somewhat clear picture. Ductal cells of the mouse pancreas can produce hormone producing cells during early mouse development (approximately up until 1 month of age) and afterwards they lose their ability to differentiate to hormone producing cells. However, few studies have identified pathways and chemicals to enhance or perhaps prolong this process. It would be interesting to assess the potency of one-carbon metabolism and CID661578 to reawaken the differentiation of such ductal progenitors in mouse models of β-cell injury. Further, drawing conclusions from the increasing number of transcriptomics studies of the human pancreas, in addition to the work performed in this thesis, it seems that human ducts have a higher degree of heterogeneity and spontaneous endocrine cells co-expressing or appearing next to human ductal cells. Based on these results one could speculate that perhaps human ductal cells are more prone to differentiate to β-cells compared to the mouse model.

Additionally, treatment with CID661578 promotes β-cell regeneration by increasing protein translation in the ductal cells. Future work should identify the exact mRNAs molecules that are hypertranslated and are causative for the observed phenotype. Our results implicate for

the first time the protein translation initiation as a target for regenerative medicine and it would be interesting if this effect is translatable to other organs and tissues.

A potential regenerative therapy for diabetes not only needs to be effective but also safe to use in the clinics. In the case of Igfbp1 overexpression perhaps the increase β-cell regeneration can be counterbalanced by the systemic effects that an IGF signaling inhibitor can have on tissues like the liver and muscle. However, folic acid treatment is a safe supplement that is being taken by women prior and during pregnancy. It would be intriguing to assess the potency of folic acid in a randomized control trial study in people with type 1 diabetes and if there is a treatment window that can increase β-cell neogenesis and decrease the need for insulin supplementation.

Lastly, it needs to be mentioned that the results of this thesis can be attributed to extensive collaborative efforts between research groups that tried to translate findings to mammalian systems and identify in detail the exact molecular mechanisms of how these four pathways stimulate β-cell regeneration. So a final conclusion of this work, is that one person and even one research team alone can advance scientific discoveries to a certain extent, yet it is only through collaborative efforts from multiple individuals and teams we can achieve the desired result, which is advancement of knowledge and improvement of human health.

# 8 ACKNOWLEDGEMENTS

*Ithaca gave you this wonderful journey.*
*Without Ithaca you wouldn't have set out.*
*She has nothing left to give you now.*
*And if you find her poor, Ithaca won't have fooled you.*
*(Ithaca, C.P Cavafy)*
Read the whole poem, it is good for your health.

This long, wonderful journey finally ended. And it would not have been possible to conclude if it was not for the constant support of so many people through all these years. A big thank you to all who dragged my ass all the way to the end.

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