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FISHING FOR SMALL MOLECULES TO TREAT DIABETES, FROM A BETA CELL PERSPECTIVE

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Cover illustration: Immunofluorescence images taken from eight postnatal day 0 moue islets after whole mount staining. Single focal plane confocal images are shown for each islet. Green represents insulin, red glucagon, white somatostatin, blue DAPI.

Fishing for small molecules to treat diabetes, from a beta cell perspective

Thesis for Doctoral Degree (Ph.D.)

By

Lipeng Ren

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To my grandpa

献给我的爷爷

Popular science summary of the thesis

With the help of insulin, blood glucose can be used in cells to produce or storage the energy one needs in daily life. Blood glucose rises after a meal, β cells in islets of pancreas sense the change and release insulin to the blood to increase the usage of glucose in cells. However, people with diabetes have impaired or decreased number of β cells thus cannot produce enough insulin to balance blood glucose. To discover drugs that can increase the number or function of insulin-producing β cells, we use diabetic zebrafish as our experimental model to perform drug screening.

Paper I: Self-replication rarely happens in β cells of adult humans. In a screen using a specific zebrafish model in which self-replication can be monitored by bioluminescence, one chemical called HG-9-91-01 was identified, as it increased β cell replication and decreased glucose levels in diabetic zebrafish. HG-9-91-01 was further found to promote replication of mouse and human β cells. Mechanistic studies revealed that HG-9-91-01 induced β cell replication though a transient unfolded protein response (UPR), a biological process which helps cells to cope with misfolded protein.

Paper II: Beside self-replication, other types of cells in pancreas have the potential to be converted to insulin-producing β cells. To find drugs that could promote the conversion and expand β cell population, we performed screening in diabetic zebrafish, in which β cells were killed, and the conversion can be monitored and assessed by bioluminescence. 4 chemicals were identified in the screen. We found the chemicals increased number of newly formed β cells, and reduced glucose levels in the diabetic zebrafish. However, in the lineage-tracing experiments where other types of cells were marked with florescent protein and kept track of to see if they express insulin and become β cells after drug treatment, only one chemical A-674563 induced a mild conversion of glucagon-expressing α cells to β cells. The other chemicals failed to promote the conversion of somatostatin-expressing δ cells or elastase-expressing acinar cells to β cells. Overall, we found conversion of other types of cells to β cells was rare and is difficult to stimulate with chemicals.

Paper III: Instead of increasing the number of β cells, another chemical called Adjudin, identified in the screen described in Paper II, was found to promote the function of β cells. Adjudin increased the function of β cells in zebrafish, enhanced the function of islets isolated from new-born mice, and improved the function of

impaired islets from a type 2 diabetes mouse model. Apart from the effect on β cells, we found Adjudin stimulated glucose uptake in zebrafish liver and human liver cells. In type 2 diabetic mice that received Adjudin treatment, we found a decrease in their blood glucose. Overall, Adjudin could be a potential drug for diabetes therapy.

Abstract

Maintenance of glucose homeostasis necessitates a precise control of insulin secretion. Pancreatic β cells sense fluctuation of blood glucose and secret insulin in response. However, functional β cell mass is decreased in diabetes. Here, to recover functional β cell mass, we use zebrafish as a model to perform in vivo drug discovery.

In paper I, to discover drugs that could expand β cell mass through proliferation, we performed an *in vivo* screen in zebrafish based on a luminescence ubiquitination-based cell cycle indicator (LUCCI), identified a small molecule called HG-9-91-01, an inhibitor of salt-inducible kinases (SIKs), as a mitogen of mouse and human β cells. Mechanistic studies found HG-9-91-01 induced a transient upregulation of ATF6-dependent unfolded protein response (UPR), which together with other downstream effectors including CRTC1, CRTC2, mTOR and IRE1 led to a mitogenic response in β cells.

In paper II, to discover drugs that could increase β cell mass from other origins, we performed *in vivo* screening in zebrafish for stimulators that convert glucagon-expressing α cells, somatostatin-expressing δ cells, and elastase-expressing acinar cells to β cells. 4 hits were identified in the screens and shown to promote β cell regeneration as well as reduce glucose in zebrafish with β cell ablation. However, only one hit called A-674563 induced a modest increase in reprogramming of α cells to β cells in lineage tracing experiments, whereas the other hits failed to promote reprogramming. Spontaneous conversion of α- or δ-cells to β cells was rare, and no conversion of acinar cells to β cells was observed during β cell regeneration. Together, reprogramming of other pancreatic cells to β cells is rare and difficult to stimulate by small molecules in zebrafish.

In paper III, a small molecule called Adjudin, identified in paper II, was discovered to promote β cell function in zebrafish. In translational studies using *in vitro* cultured mouse islets, we found that Adjudin promoted functional maturation of isolated islets from postnatal day 0 (P0) mice, as they gained capability of glucose responsive insulin secretion; Adjudin also improved recovery of islets from a type 2 diabetic mouse model. Moreover, Adjudin stimulated hepatic glucose uptake, an effect we further found largely independent of insulin in zebrafish and validated in primary human hepatocyte (PHH) formed spheroids with insulin resistance. Next, we examined Adjudin in a type 2 diabetic mouse model (*db/db* mice), observed

improved glucose homeostasis in the mice that received Adjudin treatment. Together, Adjudin may serve as a potential therapeutic drug for diabetes.

To summarize, using *in vivo* drug screening in zebrafish, we identified small molecules that could either expand β cell mass or promote β cell function, such findings may pave the way for future research and development of a novel treatment for diabetes.

List of scientific papers

I. In vivo screen identifies a SIK inhibitor that induces β cell proliferation through a transient UPR.

   Jérémie Charbord, **Lipeng Ren***, Rohit B. Sharma*, Anna Johansson, Rasmus Ågren, Lianhe Chu, Dominika Tworus, Nadja Schulz, Pierre Charbord, Andrew F. Stewart, Peng Wang, Laura C. Alonso & Olov Andersson.

   Nature metabolism. 2021 May;3(5):682-700.

II. In vivo screening for small molecules promoting cellular reprogramming to insulin-producing β-cells.

   Lipeng Ren*, Jérémie Charbord*, Lianhe Chu, Nicole Schmitner, Jiarui Mi, Ka-Cheuk Liu, Dominika Tworus, Olov Andersson.

   Manuscript.

III. Adjudin improves beta cell maturation, hepatic glucose uptake and glucose homeostasis.

   Lipeng Ren, Jérémie Charbord, Lianhe Chu, Aurino M Kemas, Maria Bertuzzi, Jiarui Mi, Chen Xing, Volker M Lauschke, Olov Andersson.

   Diabetologia. 2023

   *These authors had equal contribution to the work.

Scientific paper not included in the thesis

I. Inhibition of mammalian mtDNA transcription paradoxically activates liver fatty acid oxidation to reverse diet-induced hepatosteatosis and obesity.

   Shan Jiang, Taolin Yuan, Laura S Kremer, Florian A Rosenberger, Fynn M Hansen, Melissa Borg, Diana Rubalcava-Gracia, Mara Mennuni, Roberta Filograna, David Alsina, Jelena Misic, Camilla Koolmeister, **Lipeng Ren**, Olov Andersson, Anke Unger, Tim Bergbrede, Raffaella Di Lucrezia, Rolf Wilbom, Juleen R Zierath, Anna Krook, Patrick Giavalisco, Matthias Mann, Nils-Göran Larsson.

   bioRxiv. 2023.09.22.558955

Contents

List of abbreviations

AKT	Protein kinase B
AMPK	AMP-activated protein kinase
ATP	Adenosine 5´-triphosphate
CD	Cluster of differentiation
DNMT	DNA methyltransferase
DYRK	Dual-specificity tyrosine phosphorylation-regulated kinase
ERR	Estrogen-related receptor
FOXO1	Forkhead box O1
GCK	Glucokinase
GLP-1	Glucagon-like peptide-1
GTT	Glucose tolerance test
GSIS	Glucose stimulated insulin secretion
hGSC	Human gastric stem cell
HK1	Hexokinase I
hiPSC	Human induced pluripotent stem cell
hPSC	Human pluripotent stem cell
LDHA	Lactate dehydrogenase
LUCCI	Luminescence ubiquitination-based cell cycle indicator
MAFA	v-Maf musculoaponeurotic fibrosarcoma oncogene homologue A
NEUROD1	Neurogenic differentiation factor 1
NGN3	Neurogenin 3
NKX6.1	NK6 transcription factor-related, locus 1
P	Postnatal day
PDX1	Pancreatic and duodenal homeobox factor 1
PHH	Primary human hepatocytes
PP1	Protein phosphatase 1
PPARγ	Peroxisome proliferator-activated receptor gamma

Robo	Roundabout
ROCK	Rho-associated protein kinase
SC-β cell	Stem cell-derived β-like cell
SC-islet	Stem cell-derived islet
SIK	Salt-inducible kinase
TGF	Transforming growth factor
UPR	Unfolded protein response

1 Introduction

1.1 Diabetes

With an estimated number of 536.6 million people living with diabetes worldwide in 2021 (1), it has become one of the global health emergencies. Diabetes is a chronic disease characterized by high blood glucose. Long-term raised blood glucose can lead to damage to multiple organs and increase the risk of mortality (2). There are mainly two types of diabetes, namely type 1 diabetes and type 2 diabetes.

1.1.1 Type 1 diabetes

Type 1 diabetes is caused by autoimmune system mediated selective destruction of β cells, consequently there are few β cells remaining and reduced insulin release (3). The disease most often develops in children and juveniles, newly diagnosed individuals usually carry autoantibodies to insulin (IAA), glutamic acid decarboxylase (GAD), zinc transporter 8 (ZNT8) and/or insulinoma-associated autoantigen 2 (IA2) (4). Studies of postmortem pancreases of recent onset type 1 diabetes individuals showed that cluster of differentiation (CD) 8 $CD8^+$ T cells, $CD4^+$ T cells, $CD3^+$ T cells, $CD68^+$ macrophages, and $CD20^+$ B lymphocytes are present in the islets (5, 6). Mechanisms behind type 1 diabetes is heterogeneous, both genetic and environmental factors could affect susceptibility to the disease. HLA (human leukocyte antigen) locus is a genomic region most associated with the disease. Nutrition, viral infections and microbiome are potential environmental factors involved in the development of the disease (7, 8). Regarding β cells, it was found that β cell dysfunction and loss of β cell mass precedes the onset of type 1 diabetes (9), individuals with recent onset type 1 diabetes were reported to have approximately 10% of normal β cell mass in autopsy studies (10). If individuals with high risk for type 1 diabetes can be identified by biomarkers at early stage, retainment of β cell function/mass might be beneficial to prevent or delay the disease progression. Nevertheless, most individuals retain some β cells even after long-term duration of type 1 diabetes (11, 12). Even though it is difficult to know whether these cells are newly formed β cells or β cells that escaped autoimmune

destruction, the finding suggests that other than reducing autoimmune mediated destruction, stimulating β cell regeneration represents a promising approach for treatment of type 1 diabetes.

1.1.2 Type 2 diabetes

With insulin resistance in liver, muscle and/or adipose tissue as well as β cell dysfunction being its main features, type 2 diabetes accounts for over 90% of all diabetes cases (13). The long-term hyperglycemia caused by insulin resistance and β cell dysfunction can lead to microvascular and macrovascular complications like neuropathy, retinopathy and peripheral vascular diseases, eventually resulting in multiorgan dysfunction and damage (13). Type 2 diabetes is a multifactorial disease with both genetic and environmental factors involved in its development. Hundreds of genetic variants have been identified in genome-wide association studies (GWAS) to be associated with increased risk of type 2 diabetes (14, 15). Insulin resistance has a strong association with obesity and physical inactivity. With insulin resistance, glucose homeostasis is disrupted by increased output and decreased uptake of glucose from peripheral tissues. The mechanisms of insulin resistance include impaired insulin signaling, mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, and inflammation (16, 17). β cell failure is another essential factor for pathogenesis of type 2 diabetes (18). Under chronic hyperglycemia and relative insulin deficiency, β cells secret insulin continuously to meet physical needs. They get exhausted, lose their function and even identity over time, as a result, a 39% reduction in β cell mass was found in individuals with type 2 diabetes on average (19). Loss of β cells could be due to increased apoptosis (20-22) and/or dedifferentiation of β cells (23-25). Therefore, the challenge of restoring normoglycemia in type 2 diabetes may be achieved through reducing insulin resistance and simultaneously restoring functional β cell mass.

1.2 β cells

The crucial physical role of β cells can be explained by the life-threatening feature of untreated type 1 diabetes. The estimated β cell mass in healthy adults is 0.25–2 grams (19, 26). Despite the relatively small mass, they exert profound effects on the body. A better understanding of their developmental and functional maturation processes will be beneficial for developing treatment for diabetes.

1.2.1 Development

β cells are the only type of cells which can produce and release insulin, they are clustered together with glucagon-producing α cells, somatostatin-producing δ cells, pancreatic polypeptide (Ppy)-producing γ cells, and ghrelin-producing ε cells to compose islets, the endocrine of pancreas. Islets are scattered through pancreas and function as a glucose homeostatic regulator.

Initial specification of pancreas happens when pancreatic and duodenal homeobox factor 1 (PDX1) expressing dorsal and ventral buds arise from the foregut of the endoderm. The buds branch and develop on opposing sides of foregut independently, and eventually get fused into a single interconnected organ as a result of foregut rotation. Meanwhile, duct of the ventral bud gives rise to the main duct of the pancreas whereas the duct of dorsal buds becomes the accessory duct. The period after fusion is characterized by formation of differentiated cells, including endocrine, exocrine and ductal cells (27, 28).

Glucagon-expressing cells are the first endocrine cells showed up in mice during development, followed by insulin and other hormone expressing cells, whereas the first appeared endocrine cells in human pancreas are insulin-expressing cells (29). Besides cells that express a single hormone, bihormonal cells, for example cells co-expressing both insulin and glucagon, can be found in early developmental pancreas in both mice and human (30, 31). Number of bihormonal cells declines over time but the fate of these bihormonal cells remains unclear (32-34). Newly differentiated endocrine cells aggregate to form islets and continue to differentiate until mature.

It is estimated that healthy adult has around 3.2 million islets, which composes 1–2% of pancreas (35). β cells are the predominant cell type in adult islets, constituting around 60% of islet cells, followed by α cells (around 30%), δ cells (around 10%), γ cells (<5%) and ε cells (<1%) (36, 37). Human islets have intermingled β cells and α cells, while zebrafish and mouse islets usually have β cells in its core with α cells at the periphery. Moreover, islets of zebrafish, mouse and human are innervated, highly vascularized, and very heterogeneous in terms of shape, size, cell type composition, innervation, blood supply and function (38-41).

1.2.2 Functional maturation

Functional maturation describes the process of acquiring the capability of glucose responsive insulin secretion in β cells. Being the only type of cells producing and releasing insulin, precisely controlling of insulin secretion in β cells is a necessity for maintenance of glucose homeostasis. A better understanding of the mechanisms behind this process favors the functional recovery of dysfunctional β cells in diabetes, and generation of better functioning stem cell-derived β-like cells (SC-β cells).

Glucose stimulated insulin secretion (GSIS) is the best functional characteristic of β cells. In this process, highly vascularized environment in islets enables β cells to efficiently sense elevated glucose, glut transporters facilitate transportation of glucose into β cells. Once inside the cells, glucose is first phosphorylated by glucokinase, goes through glycolytic reactions and yields pyruvate. Then pyruvate, after being transported to mitochondria, enters the tricarboxylic acid (TCA) cycle, leads to oxidative phosphorylation to generate ATP. The resulting increased ATP to ADP ratio causes closure of membrane ATP-sensitive potassium channel, leading to membrane depolarization and opening of voltage-gated calcium channels. Calcium influx to cytoplasm triggers exocytosis of insulin (42). Mature β cells exert robust insulin secretion in response to elevated glucose.

Another aspect of functional maturation is to reduce basal insulin secretion. One of the most characteristic features of immature β cells is that they display low glucose threshold and high basal insulin secretion, meaning that high levels of insulin being secreted even when glucose is low (43, 44).

Metabolic changes in β cells during development is important for their maturation. Transition of dietary patterns in mice, from a fat-rich milk diet to a carbohydrate-rich chow diet, is coupled with β cell maturation (45-48). Islets from weaned mice displayed lower basal insulin secretion and better glucose responsive insulin secretion compared with that of suckling mice, further studies revealed that islets of weaned mice had suppressed oxygen consumption and ATP production in low glucose, and enhanced oxidative phosphorylation in high glucose (46). Furthermore, diet transition during weaning induced a signaling-pathway switch from the nutrient sensor to the energy sensor, namely from target of rapamycin (mTORC1) to 5′ adenosine monophosphate-activated protein kinase (AMPK), led to enhanced mitochondrial biogenesis and a switch to oxidative metabolism in β cells, resulting in functional maturation of β cells (47). Weaning induced changes in islet specific microRNAs that are associated with β cell maturation, modulating expression of those microRNAs in postnatal islets changed metabolic enzymes and led to acquisition of glucose responsive insulin secretion (45). Along the same line, estrogen-related receptor γ (ERRγ) has been shown to drive metabolic maturation of β cells. Increased expression of ERRγ in islets during development is coupled with increased oxidative phosphorylation, electron transport chain and ATP production. Islets from β cell specific *Errγ* knockout mice displayed reduced or abrogated insulin secretion in response to glucose. Transient *Errγ* knockout islets failed to increase oxygen consumption rate in response to high glucose (49). Moreover, overexpression of *Errγ* in SC-β cells drove their metabolic maturation and enhanced their insulin secretion in response to glucose (49). Metabolic switch from predominantly glycolysis to oxidative phosphorylation in β cells involves DNA methylation mediated inhibition of disallowed genes as well, *de novo* DNA methyltransferase 3 alpha (DNMT3A) is exclusively expressed in islet β cells, by

binding to the promoters of genes encoding Hexokinase 1 (HK1), which has a higher affinity for glucose compared to glucokinase (GCK), and lactate dehydrogenase A (LDHA), which turns pyruvate to lactate in glycolysis, DNMT3A repressed both genes expression. Specific deletion of *Dnmt3a* in β cells led to upregulated expression of *Hk1* and *Ldha*, increased lactate production and basal insulin secretion in islets (50).

Transcription factors of β cells are important for maturation. Many transcription factors have been identified to determine the β cell lineage (28, 51, 52) or regulate adult β cell function (53), but only a handful were shown to promote β cell maturation. Among them, MAFA (v-maf musculoponeurotic fibrosarcoma oncogene homologue A), a basic leucine zipper which activates insulin transcription, is critical for the establishment of β cell functionality. It is exclusively expressed in β cells from embryonic day 13.5 onward in mouse (54), in human its expression increases with age (55). Study in a rat insulinoma cell line INS-1 cells has shown that MAFA can positively regulate mRNA expression of *Glut2*, *Pcsk1*, *Gck*, *Glp1r*, *Pdx1*, *Nkx6.1* and *Pcx* (56). Islets of *Mafa* deficient mice showed decreased mRNA expression of *Glut2*, *Neurod1* and *Pdx1* (57). *Mafa* specific deletion in pancreas led to a reduction in mRNA expression of *Glut2*, *G6pc2*, *Slc30a8*, *Sytl4*, *Stxbp1* and *Atp2a2* in mouse islets (58). MAFA together with NEUROD1 and HNF1β activates transcription of *Glut2* through the proximal promoter region and 3′ downstream distal enhancer of the gene (59). Furthermore, overexpression of *Mafa* in postnatal day 2 (P2) immature rat islets increased mRNA expression of *Gck*, *Glp1r*, *Neurod1*, and *Nkx6.1*, had no effect on cellular insulin content, most importantly resulted in acquisition of GSIS (60). Later MAFA was shown to drive maturation of human fetal islets and SC-β cells (61). Mechanistic study revealed that expression of MAFA during β cell development was regulated by thyroid hormone, the activated receptor of which bound to promoter of *Mafa* and enhanced its expression, resulted in functional maturation of β cells (62, 63). Similar to MAFA, thyroid hormone has been shown to promote functional maturation of rat islets, human fetal islets, and SC-β cells (61, 62), it is

now used for production of SC-β cells *in vitro* (64, 65). In zebrafish thyroid hormone was also shown to promote pancreatic islet maturation (66). PDX1 is known to regulate insulin expression, overexpression of *Pdx1* in P2 rat islets increased cellular insulin content but failed to improve GSIS (60). As discussed before, *Errγ* overexpression promoted maturation of human SC-β cells (49). A few transcription factors were shown to maintain functional maturity of β cells. Mouse islets with specific deletion of *Neurod1* in adult β cells showed a profile similar to immature islets, as those islets had increased expression of *Npy* (67), *Ldha* and glycolytic genes, exhibited high basal insulin secretion, high basal oxygen consumption and no response to glucose challenge (68). Similarly, loss of *SIX3* in human adult islets (69) and *Rfx6* in mouse adult islets (70) resulted in upregulation of disallowed genes and impaired insulin secretion.

Functional maturation of β cells involves changes in regulation of insulin vesicles. Membrane depolarization induced influx of calcium ion is the triggering signal for insulin exocytosis. High basal insulin secretion was found to be associated with high Ca^{2+} sensitivity to insulin vesicles in immature β cells. Synaptotagmin 4 (Syt4) is a component of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex which regulates Ca^{2+}-triggered vesicle exocytosis. Syt4 increased gradually during β cell development and was shown to play a role in reducing Ca^{2+} sensitivity of insulin vesicles. Deletion of *Syt4* in mouse did not change expression of β cells markers like GLUT2, PDX1, MAFA, NKX6.1, but led to high basal insulin secretion in P14 islets, conditional overexpression of *Syt4* in β cells decreased basal insulin secretion in islets of P4 and P7 mice without changing β cell markers like MAFA, NKX6.1, and PDX1, suggesting it is important for β cell functional maturation (71, 72). Moreover, inactivation of calcineurin/NFAT (Nuclear Factor of Activated T cells) signaling disrupted biogenesis and maturation of insulin vesicles in mouse β cells (73).

Circadian Clock was shown to have important roles in β cell maturation. The gradually increasing expression of islet circadian clock transcription factor BMAL1 (basic helix-loop-helix ARNT like 1) was observed in rat and human islets during

development (74). Specific deletion of *Bmal1* in mouse β cells at an early stage led to impaired GSIS in islets, although had no impact on expression of maturation markers and disallowed genes (74). In another study, pancreas explants from a circadian reporter mouse (*Bmal1-Luc*) showed robust circadian rhythms, selective *Bmal1* deletion in pancreas resulted in defective glucose responsive insulin secretion and severe glucose intolerance *in vivo*, islets isolated from the mutant mice had normal islet insulin content but defective GSIS (75). Moreover, circadian entrainment by daily feeding/fasting the stem cell-derived islets (SC-islets) was shown to reduce their basal insulin secretion and increase GSIS (76).

Islet architecture is important for functional maturity (77). Studies showed that Roundabout (Robo) receptors in β cells were required for a mature islet architecture in mouse (78, 79), *Robo* deficient β cells had normal expression of β cell functional markers and GSIS, however, islets with *Robo* deficiency in β cells showed decreased synchroneity of Ca^{2+} oscillations *in vivo* (80).

In mouse, GSIS is developed in β cells 1–2 weeks postpartum (43). More specifically, it was reported that most genes related to maturation reach highest expression by P4, and genes associated with calcium-mediated processes are likely to be the key molecular mechanism limiting further β cell maturation, since they increase as β cell develops (81). Maturation of human β cells takes more time (55, 82), it was suggested that human β cells are functionally mature by the age of one year (83).

Of note, the maturing process of β cells is coupled with a decrease in their proliferation capacity. β cells are highly proliferative, functionally immature in the neonatal stage, and functionally mature, barely proliferative in the adult (84-86). Forcing adult β cells to re-enter cell cycle resulted in an immature phenotype in mouse islets (87). Compromising adult β cell function by reducing insulin expression led to a proliferative status of mouse β cells (88).

1.3 Expand functional β cell mass in diabetes

1.3.1 β cell proliferation

Proliferation of human β cells reaches its highest rate (approximately 2% of proliferating β cells) at the first year of childhood, declines rapidly afterwards and becomes low or undetectable in adult (89). In mouse, β cells have their highest proliferation rate at P4–P7 (90). One of the efforts in the field is to identify stimulus that could expand β cell mass endogenously through proliferation. The reason why β cells resist proliferation remains elusive, but studies suggest constrained regulatory molecules of cell cycle in cytoplasm, loss of mitogenic molecules, and changes in epigenetic regulation may be part of the obstacles (89, 91). Severe hyerglycemia may block the capacity of proliferation in β cells, a recent study found short-term management of glycaemia released β cell proliferation capacity in mouse (92). Zebrafish is an ideal animal model for *in vivo* drug discovery, and several screens and studies used zebrafish for examining β cell proliferation. A chemical screen using a transgenic zebrafish model with which β cell can be conditionally ablated and regenerated β cells can be examined by a fluorescent reporter found that NECA, an adenosine agonist, can promote proliferation of β cells in zebrafish and mouse (93), adenosine kinase inhibition was further shown to selectively stimulate β cell proliferation in mouse, rat and porcine, but had no effect on human β cell proliferation unfortunately (94). Like adenosine kinase inhibitors, plenty of stimulus have been identified as drivers of β cell proliferation in animal models but failed to stimulate proliferation of human β cells, the reason might be due to the structure and molecular differences in islets between rodent and human (89, 95-97). Notably, some of them has been shown to enhance human β cell proliferation. Based on the method of FUCCI (fluorescence ubiquitination-based cell cycle indicator) (98), another zebrafish model LUCCI (luminescence ubiquitination-base cell cycle indicator), in which the S/G2/M marker geminin was fused with luciferase and expressed under control of the insulin promoter, was developed to enable efficient assessment of β cell proliferation based on production of bioluminescence. *In vivo* screening with the

model discovered an inhibitor of salt-inducible kinases HG-9-91-01. It induced β cell proliferation in zebrafish and mouse consistently, and had a modest dose-depedent effect on human β cells. Further analysis revealed that proliferation induced by HG-9-91-01 was mediated by a transient activation of the unfolded protein response (UPR) (99). Liver insulin receptor knockout (LIRKO) mouse showed increased β cell proliferation, and mechanistic studies identified a liver secreted proteinase inhibitor SerpinB1 as a driver of β cell proliferation in zebrafish, mouse and human (100). With the model of c-Myc promoter driving expression of luciferase in HepG2 liver cells, *in vitro* human cell line based high throughput screen identified Harmine, an inhibitor of dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), that promoted significant proliferation of human β cells (101). Inhibition of DYRK1A disrupted the repressive DREAM (Dp/Retinoblastoma(Rb)-like/E2F/MuvB) complex and converted which to a pro-proliferative form, resulting in quiescent β cells reentering the cell cyle (102). Following Harmine, two DYRK1A inhibitors namely GNF4877 and 5-iodotubericidin (5-IT) were found to be more potent in promoting β cell proliferation (103). Compared to Harmine alone, a more potent effect was also observed when combined with transforming growth factor β (TGFβ) superfamily inhibitors (104), glucagon-like peptide-1 (GLP1) receptor agonists (105).

1.3.2 Differentiation of stem-cells to β cells

Based on developmental knowledge gained from zebrafish, mouse and human, through stepwise imitating and modulating important signaling pathways involved in development of pancreas and islets with growth factors and chemicals, human pluripotent stem cells (hPSCs) were differentiated to β-like cells *in vitro* in 2014 (64, 65). This opens a new door for diabetes therapy as hPSCs derived β-like cells reduced hyperglycemia in a diabetic mouse model weeks after transplantation. Albeit human stem cell-derived β cells (SC-β cells) expressed β cell transcription factors and displayed glucose responsive insulin secretion in static GSIS, they were shown to resemble immature human β cells (106). Unlike human mature β cells, SC-β cells generated from initial protocols expressed lower levels of key β

cell transcription factors, contained less insulin per cell, secreted less insulin in response to high glucose, and showed dysregulated insulin secretion in dynamic GSIS (64, 65). Thus, one of the main efforts in the field is to improve the functionality of SC-β cells. Approaches like modulating protocols (e.g use of WNT4 (107), latrunculin A (108)) to improve differentiation efficiency, sorting to enrich SC-islet cells (109), implicating 3D culture for SC-islets (40), and circadian entrainment (76) were shown to improve their functionality (110, 111). Despite still being less functional compared to primary human islets, SC-islets generated from improved protocols showed low basal insulin secretion and biphasic insulin secretion in dynamic GSIS (108, 112, 113). Another effort in SC-islets is to reduce the generation of off-target cells like enterochromaffin cells, which secret serotonin and normally reside in intestine (114, 115).

Drug screen in zebrafish has identified hits that could increase differentiation efficiency of stem cells to β cells, as retinoic acid was indeed first identified to promote endoderm differentiation to pancreatic fate in zebrafish (116). Moreover, an inhibitor of Cdk5 roscovitine was shown to promote β cell differentiation both in *in vivo* models including zebrafish and mouse pancreas ductal ligation model, and in *in vitro* inducible differentiation models using mouse embryonic pancreatic explants and human induced pluripotent stem cells (hiPSCs) (117). A zebrafish genetic screen discovered that overexpression of folate receptor 1 stimulated differentiation of β cells in zebrafish, and follow-up experiments using neonatal pig islet aggregates showed that folinic acid promoted β formation likely from ductal progenitors (118). Moreover, screens using SC-islet platforms discovered chemicals that promote an endodermal lineage including IDE1 and IDE2 (TGFβ activator) (119), stauprimide (nucleoside diphosphate kinase B (NME2) inhibitor) (120), Fasudil and RKI-1447 (rho-associated protein kinase (ROCK) inhibitor) (121), a potential protein kinase C (PCK) activator indolactam V (ILV) that promotes generation of pancreatic progenitors from endoderm (122), and a ROCK inhibitor H1152 that improves the functional maturity of SC-β cells (115, 123).

Endogenous differentiation of progenitor cells to β cells would have been ideal to expand functional β cell mass, however, the existence of pancreatic progenitors in adult is highly controversial (124-127). It is generally accepted that pancreatic progenitors, if existing, barely contribute to β cell mass in the adult, at least in mouse.

1.3.3 Conversion of non-β cells to β cells

Conversion of non-β cells to β cells is a fascinating way to expand β cell mass. Using a mouse model in which genetic ablation inducing extreme/near total β cell loss, somatostatin-expressing δ cells (when ablating β cells before puberty) (128), or glucagon-expressing α cells (when ablating β cells after puberty) (129), were shown to turn into β cells spontaneously. Later in the same mouse model, Ppy-expressing γ cells were found to also upregulate insulin (130). α cells are the most abundant cells in islets following β cells. Conversion of α-to-β cells is the most studied process, even though the conversion occurred only in 1–2% of α cells after near total β cell loss in mouse (129, 131). A unique type of immature β cells at periphery of islets were shown to be intermediate cells derived from α cells (132). Studies in mouse demonstrated that overexpression of *Pax4* or *Pdx1*, or deletion of *Arx* in α cells led to their conversion to β cells (133-135), deletion of *Dnmt1* and *Arx* resulted in more α cells transdifferentiating to β cells compared to deletion of *Arx* alone (136), inhibition of insulin signaling and inactivation of Smoothened-mediated signaling promoted the conversion of α cells to β cells (134). In human, α cells were reprogrammed to β-like cells by ectopic expression of *MAFA* and *PDX1* (137, 138), approximately 40% of human α cells can be reprogrammed to insulin-producing cells *in vitro* with this method (131, 138). Despite still maintaining expression of some α cell markers, human α cell derived β-like cells showed glucose responsive insulin secretion *in vitro*, reversed mouse diabetes after being transplanted to *in vivo*, and had reduced immunogenicity (138). Small molecules, like gamma-aminobutyric acid (GABA) and artemisinins, were showed to drive conversion of α-to-β cells in mouse and have potential to change the identity of human α cells *in vitro* (139, 140), but the results were not reproducable and thus

remain controversial (141, 142). A recent study using an efficient genetic tracing system mediated by a dual recombinase showed conversion of non-β cells to β cells only occurred in mice with extreme β cell loss, no conversion of non-β cells to β cells was observed in adult pancreas under physiological conditions (125).

It was shown that ectopic expression of the transcription factors *Mafa*, *Ngn3* and *Pdx1* in mouse acinar cells resulted in their adoption of a β cell profile, these cells expressed functional markers of β cells, shared similar ultrastructure to endogenous β cells, and suppressed hyperglycemia in diabetic mouse (143, 144). Furthermore, human exocrine pancreatic cells were reprogrammed to insulin-producing cells through ectopic expression of *NGN3*, *PAX4*, *PDX1* and *MAFA*, exocrine cell-derived β-like cells had glucose response and normalized hyperglycemia in diabetic mice after transplantation (145). Exogenously transducing *NGN3* to human pancreatic ductal cells was shown to induce their transcriptional expression of endocrine genes (146).

Mouse gastrointestinal cells with misexpression of *Mafa*, *Ngn3* and *Pdx1* were converted to β-like cells (147, 148). Later, a stepwise protocol was developed to induce human adult gastric stem cells (hGSCs) to β-like cells through sequential activation of the three transcription factors, hGSCs-derived organoids had biphasic insulin secretion in response to glucose, normalized hyperglycemia in diabetic mice after transplantation (149). Moreover, the protocol displayed high reprogramming efficiency, with around 70% of hGSCs being converted to insulin-producing cells *in vitro* (149).

Other than that, human insulin-producing cells can be generated from hepatocytes through ectopic expression of PDX1 (150, 151), skin fibroblasts through chemical induced transdifferentiation (152), and gallbladder cells through ectopic expression of *NEUROG3*, *PAX6*, *PDX1* and *MAFA* (131, 153). β-like cells derived from those cells were shown to have glucose responsive insulin secretion *in vitro*, but only hepatocytes and skin fibroblasts derived cells ameliorated hyperglycemia *in vivo* in a diabetic mouse model after cell transplantation (151, 152).

Consistence with the mouse study, zebrafish α cells can be spontaneously converted to β cells after near total β cell loss (154). Genetic screening in zebrafish found that Igfbp1 promoted β cell regeneration through conversion of α-to-β cells by inhibiting Igf signaling. Moreover, treatment with IGFBP1 recombinant protein resulted in increased number of bihormonal (insulin^{+}glucagon^{+}) cells in both mouse and human islets (155). Recent studies indicated that somatostatin 1.1 (Sst1.1) positive cells are a prominent source for β cell regeneration in zebrafish as nearly all regenerated β cells had Sst1.1 expression (156, 157), but further validation needs to be done with proper lineage tracing experiments. Zebrafish ghrelin-expressing ε cells were also found to contribute to β cell regeneration after extreme β cells loss (158), albeit to a lesser extent. Zebrafish acinar cells can be induced to express insulin by inhibition of Ptf1a (159), but these cells were not fully converted to β cells.

1.3.4 Recovery of dysfunctional β cells

β cells become dysfunctional and lose their identity under chronic stressed conditions. Studies from mouse suggested that dedifferentiation occurred in both type 1 and type 2 diabetes (25, 160, 161). In human, a study estimated that approximately 31.9% of β-cells were dedifferentiated in type 2 diabetes compared to 8.7% in controls based on immunohistochemical analysis, concluding that dedifferentiation could be the key mechanism for β cell loss in human type 2 diabetes (23). Evidence of dedifferentiation of β cells in human type 2 diabetes includes losing expression of MAFA, NKX6.1 and PDX1 (162, 163), displaying blunting insulin secretion in response to glucose (164, 165), upregulation of the β cell dysfunctional marker ALDH1A3 (aldehyde dehydrogenase 1 family member A3) (23, 166), and adoption of markers of other endocrine cells (23). Molecular mechanisms like endoplasmic reticulum (ER) stress, oxidative stress, hypoxia and inflammation were implicated in β cell dedifferentiation (167).

Management of hyperglycemia with insulin benefits the recovery of dedifferentiated β cells in diabetes. It was reported that insulin therapy redifferentiated dedifferentiated β cells in a diabetic mouse model (168). Results

from human clinical trials showed that intensive insulin therapy significantly improved β cell function in newly diagnosed type 2 diabetes patients (169). A hypoxia-inducible factor-1α (HIF-1α) inhibitor PX-478 was shown to increase expression of β cell functional markers, decrease expression of β cell dysfunctional markers, improve β cell function, lower hyperglycemia in diabetic mouse models, and improve function of high glucose induced dysfunctional human islet organoids (170). Chemical screens have been employed to discover compounds that facilitate the recovery of β cells. Screening based on quantitative PCR (qPCR) in human ductal carcinoma cell line PANC-1 identified BRD7552 as a *PDX1* inducer, an effect that was validated in primary human islets (171). Based on a dual fluorescent reporter with which *Ins1* and *Pdx1* promoter activity can be accessed by live imaging, screen in MIN6 β cells found a small molecule called carbamazepine that can increase expression of *Ins1*, *Ins2*, and *Pdx1* in primary mouse islets likely through inhibition of Nav1.7 sodium channel (172). Treatment with carbamazepine in non-obese diabetic (NOD) mice resulted in reduced insulitis, improved glucose tolerance, and decreased incidence of type 1 diabetes (173). Urocortin 3 (UCN3) is a well-known maturation marker of β cells (43, 174). Using β cell UCN3 expression as a readout, activin receptor-like kinase 5 (ALK5) inhibitor II was discovered in a screen performed on primary mouse islets. ALK5 inhibitor II can restore maturation in dedifferentiated β cells and maintain β cell identity under cytokine stress (175). Screening on a transgenic zebrafish line where the *pdx1* promoter drives expression of luciferase identified HC toxin, a histone deacetylase (HDAC) inhibitor, as a stimulator of *pdx1*. HC toxin was shown to promote expression of β cell functional markers (i.e. *INS, PDX1*, *NEUROD1*, *PCSK1*, and *NKX2.2*) in hiPSCs-derived β-like cells, enhance glucose responsive insulin secretion in primary mouse and human islets (176). A study found that a small molecule mediated forkhead box O1 (FOXO1) inhibition induced dedifferentiation of MIN6 β cells. Screening on the dedifferentiation model identified a small molecule named loperamide, which prevented dedifferentiation of MIN6 cells induced by FOXO1 inhibitor, increased insulin expression and promoted GSIS in islets from diabetic human (177). We discovered a small molecule called Adjudin

from a zebrafish *in vivo* screen, showed that Adjudin decreased proliferation and promoted functional maturation of P0 mouse islets. Moreover, Adjudin increased expression of β cell functional markers, decreased expression of disallowed genes, increased insulin secretion in islets from a type 2 diabetic mouse model, thus shows potential to recover the function of islets from type 2 diabetes (178).

Despite the field favors the theory of β cell dedifferentiation, findings from another human type 2 diabetes study argued against it, in which only a quantitatively small portion of dedifferentiated β cells was observed (179).

1.4 Liver in glucose homeostasis

Liver plays a crucial role in glucose homeostasis. Approximately one third of glucose is disposed by liver postprandially (180, 181), and 90% of endogenous glucose is produced by liver during fasting periods (182).

After meals, glucose is absorbed by the intestine and reaches the liver via the portal vein. Through the glucose transporter, predominantly Glut2, glucose enters hepatocytes. The process of glucose uptake in hepatocytes is not regulated by insulin. Inside hepatocytes, glucose is primarily used to synthesize glycogen. Excess glucose goes through *de novo* lipogenesis and becomes fatty acids. Only some glucose undergoes oxidation to carbon dioxide in hepatocytes, which differs from muscles, where glucose is primarily consumed in oxidation. Within hepatocytes, glucose is phosphorylated to glucose 6-phosphate by glucokinase. Glucose 6-phosphate can be metabolized to uridine 5′ diphosphate (UDP)-glucose for glycogen synthesis. During carbohydrate overfeeding, excess glucose 6-phosphate can be metabolized to acetyl-CoA, which is a precursor for fatty acid synthesis (183).

During fasting, glucose is produced in hepatocytes through glycogenolysis and gluconeogenesis. Glycogenolysis involves the enzymatic debranching and breakdown of glycogen to glucose 1-phosphate, which can be further converted into glucose. Gluconeogenesis uses precursors like lactate, fructose, glycerol, and

alanine to synthesize glucose. In short-term fasting, glycogenolysis is the predominant contributor to blood glucose, gluconeogenesis gradually takes over and predominate the glucose production in prolonged fasting following the decrease and depletion of glycogen (183).

Insulin is a powerful hormone in regulation of glucose metabolism. Insulin promotes glycogen synthesis through AKT (protein kinase B) mediated inactivation of glycogen synthase kinase 3 (GSK-3), a kinase phosphorylating thus decreasing activity of glycogen synthase, and activation of protein phosphatase 1 (PP1), a kinase dephosphorylating thus increasing activity of glycogen synthase. Insulin inhibits glycogenolysis through PP1 mediated inactivation of glycogen phosphorylase. Insulin inhibits gluconeogenesis through inactivation of FOXO1, which results in transcriptional downregulation of gluconeogenic genes (184, 185). Besides directly regulating enzyme activity by phosphorylation and dephosphorylation in liver, insulin can indirectly inhibit gluconeogenesis through inhibiting secretion of glucagon in pancreatic α cells, and lipolysis in adipose tissue, the latter leads to less lipolytic products like fatty acids and glycerol delivered to liver for gluconeogenesis (184, 185).

In type 2 diabetes, glucose regulation in liver is severely compromised under insulin resistance. Increased diacylglycerol activates protein kinase Cε (PKCε), the resulting inhibitory effect on kinase activity of the insulin receptor decreases glycogen synthesis stimulated by insulin (186). High level of glucagon promotes gluconeogenesis and glycogenolysis. The decreased inhibitory effect on lipolysis by insulin in adipocytes increases delivery of free fatty acids and glycerol to liver, resulting in more gluconeogenic precursors and acetyl-CoA mediated activation of pyruvate carboxylase, which collectively lead to more gluconeogenesis (185, 187, 188).

1.5 Skeletal muscle and adipose tissue in glucose homeostasis

Skeletal muscle disposes 60-80% of ingested glucose (189-191). Glucose is primarily used in oxidation to supply energy for muscle contraction, excess of which is stored as glycogen. Glucose transport in muscle primarily relies on Glut4, a glucose transporter normally localized to intracellular vesicles, translocates to the cell membrane when responding to stimuli to facilitate glucose transport. Insulin serves as a well-known stimulator that facilitates Glut4 translocation through phosphorylation cascades (192). Exercise stimulated Glut4 translocation occurs in an insulin-independent manner, mechanistic studies suggest involved signaling pathways includes AMPK, calcium, nitric oxide and reactive oxygen species (193).

Adipose tissue disposes less that 20% of glucose postprandially (194). Insulin stimulates glucose uptake in adipocytes via translocation of Glut4. Unlike liver and muscle where glucose is stored as glycogen predominantly, less than 5% of the taken-up glucose ends up as glycogen in adipocytes, with at most 50% being used for triglyceride synthesis (195), and a considerable amount being metabolized to and secreted as lactate (196).

In type 2 diabetes, dysregulated insulin signaling disrupts glucose uptake and storage in muscle and adipocytes, increased lipolysis in adipocytes results in accumulation of ectopic lipid in other tissues, which together worsen the disease.

1.6 Pharmaceutical treatment of diabetes

For both types of diabetes especially type 2 diabetes, healthy lifestyle behaviors are beneficial for glycemic control. Metabolic surgery (bariatric surgery) appears to be an effective treatment for type 2 diabetes (197, 198). However, in most cases, pharmaceutical intervention is essential.

1.6.1 Insulin therapy

With β cells being destroyed by immune system, consequent insulin deficiency once made type 1 diabetes a fatal disease. The discovery and deployment of insulin in the clinic turned the disease into a manageable condition (199). In healthy individuals, basal level of insulin is needed to sustain basal metabolism, the rise of glucose upon a meal requires a robust insulin secretion. Correspondingly, through increasing or decreasing complexity of the hexamers-to-monomers process, long-acting and rapid-acting insulin have been developed over the years based on human regular insulin and possesses the same insulin structure as produced by β cells, but when injected subcutaneously often shows a delay of action (200). When glucose is lowered by insulin in healthy individuals, the feedback signals suppress insulin secretion in β cells to prevent a continuous glucose decrease. However, lack of such suppression regulation makes hypoglycemia a most common and sometimes dangerous adverse effect for insulin therapy (201). Other adverse effects include insulin-induced body weight gain and lipohypertrophy in injection sites of skin (202).

Besides type 1 diabetes, insulin is recommended for glycemic control in type 2 diabetes when in presence with other metabolic disorders or glycemic goals are not achieved by other glucose lowering agents (203, 204).

Among the efforts to improve insulin therapy including developing even faster- or longer-acting insulin, oral insulin, better inhaled insulin and closed-loop insulin delivery (199), the concept and development of glucose responsive insulin is most intriguing, the principle of which is that insulin is modified to be inactive initially, its activation occurs when binding to carbohydrates (205).

1.6.2 Sodium-glucose cotransporter-2 inhibitors

Sodium-glucose cotransporter-2 (SGLT2) is specifically expressed in kidney, it mediates approximately 90% of glucose reabsorption in human. By inhibiting SGLT2, the sodium-glucose cotransporter-2 inhibitors (SGLT2i) drug enhances glucose excretion in kidney (glycosuria) thus decrease systemic glucose (206,

207). Moreover, studies in clinical trials showed that SGLT2i therapy has protective effects on cardiovascular and renal function (207, 208). SGLT2i medicines are approved by both the US Food and Drug Administration (FDA) and the European Medicine Agency (EMA) for glycemic control in type 2 diabetes.

The common adverse effect associated with the use of SGLT2i is increased risk of mild genital infections (207, 208).

1.6.3 Incretin mimetics and Dipeptidyl peptidase-4 inhibitors

Incretin hormones are secreted by enteroendocrine cells after meals, they curb glucose increase by stimulating insulin secretion. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are the two main incretin hormones. Besides augmenting insulin secretion, GLP-1 suppresses glucagon secretion, reduces appetite and decelerates gastric emptying (209, 210), GIP promotes satiety, increases insulin sensitivity in adipose tissue, however, stimulates glucagon secretion (209, 210).

Drugs in this class include GLP-1 receptor agonists, and more recently developed GIP and GLP-1 receptor co-agonists (e.g. the recently FDA approved tirzepatide). Those drugs not only have high efficacy to lower glucose, but also reduce body weight and decrease, or have potentials to decrease, cardiovascular risk (208, 210). Gastrointestinal effects are the most common side effect of the drug class (208).

Dipeptidyl peptidase-4 inhibitors (DPP-4i) are a class of medicines that increase incretin levels through inhibition of enzymes inactivating incretin hormones (208, 209).

1.6.4 Metformin

Metformin belongs to the biguanide class. With high efficacy in lowering glucose and a high safety profile, it is the first line medicine and has been used for over 60 years for glycemic control in type 2 diabetes (211). Even though the exact mechanism behind metformin induced glucose lowering effect is still unclear,

mechanistic investigations indicate that several actions may contribute, including inhibition of gluconeogenesis in hepatocytes, increase of insulin sensitivity in skeletal muscle, decrease of inflammation in metabolic organs, and modulation of microbiome and function of gastrointestinal tract (211). Long-term use of metformin may result in low serum vitamin B12 (212).

1.6.5 Sulfonylureas

Sulfonylureas binds to sulfonylurea receptor 1 subunit on the ATP sensitive potassium channel, leading to membrane depolarization and insulin secretion in β cells. Because the stimulation of insulin secretion is regardless of glycemic level, the risk of sulfonylureas treatment is hypoglycemia (213) and with time β cell exhaustion (214).

1.6.6 Thiazolidinediones

Thiazolidinediones are insulin sensitizers acting as agonists of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) and have high efficacy in lowering glucose. Even though PPARγ is also expressed in liver and skeletal muscle, its expression in adipose tissue is predominant. Activation of PPARγ increases insulin sensitivity through actions like promoting lipid storage in adipocytes, lowering free fatty acid in serum, and increasing secretion of adipokines (215, 216). The adverse effects associated with use of thiazolidinediones include body weight gain, congestive heart failure, fluid retention, and bone fracture (216).

1.6.7 Pramlintide

Pramlintide is an analogue of a peptide hormone called amylin, which is co-secreted with insulin from β cells. Pramlintide lowers glucose through inhibiting glucagon secretion, decelerating gastric emptying and enhancing satiety. It is an FDA approved drug for adjunctive treatment for type 1 diabetes (202).

1.6.8 Anti-CD3 antibody

Anti-CD3 monoclonal antibody (teplizumab) is the first FDA approved drug that delays the onset of type 1 diabetes (217). It is a humanized antibody adapted from mouse. The antibody inhibits T cell activation by blocking the chain of CD3 on T cell receptor and preventing antigen recognition, resulting in preserved β cell function and retained insulin production (218, 219). A study has shown that anti-CD3 antibody can delay the onset of the disease by approximately 2 years (220). Adverse effects of the therapy include rash, decreased white blood cells and headache.

1.7 β cell replacement therapy for diabetes

β cells continuously sense fluctuation of blood glucose and release insulin accordingly in response. The tight and precise control of insulin secretion in β cells makes β cell replacement therapy a promising approach for diabetes treatment, especially for type 1 diabetes (221).

Whole pancreas transplantation and pancreatic islet transplantation have been used in the clinic for diabetes treatment, and shown to be able to effectively normalize glycemic levels, prevent hypoglycemia and reduce the complications of diabetes (222). Downside of the therapy is the need of systemic immunosuppression to prevent transplantation caused allogeneic rejection. The widespread use of pancreas or islet transplantation is limited because of the lack of human pancreas or islets.

Meanwhile, the technique to produce human SC-islets *in vitro* is fast-developing, can provide adequate amount of cells for transplantation. Despite that SC-islets are not as functional as human primary islets, SC-islets are currently under clinical trials and shows positive preliminary results in glycemic control in type 1 diabetes patients (114). Like pancreas/islets transplantation, SC-islets transplantation faces the problem of allogeneic rejection, current prevention strategies include encapsulating SC-islets, using immunosuppressants, and genetic modification of SC-islets to escape immune attack (114).

Harmine is under phase I clinical trial (NCT05526430). It is among the most potent drugs that promote human β cell proliferation. Even though the results of the clinical trial are not yet available, concerns have been raised about its use in type 1 diabetes: it does not specifically targe β cells (i.e. induces proliferation of α cells and δ cells (97)); with current harmine stimulated proliferation rate in β cells, it may take 1-3 years to reach a meaningful β cell mass to achieve insulin-independence (221), although this might change with simultaneous delivery of GLP-1 receptor agonists.

2 Research aims

Deficiency of insulin-producing β cells is a common feature for both type 1 and late-stage type 2 diabetes. Insulin therapy is a good way to preserve normoglycemia in diabetes, a better alternative could be to recover functional β cell mass endogenously so that glycemic fluctuations can be monitored and responded by β cells in real-time.

Zebrafish have conserved islet structure and are easy to manipulate. To discover drugs and pathways that increase functional β cell mass, zebrafish is used as a model for our *in vivo* drug discovery.

The overall aim of the project is to screen, identify and characterize chemicals that could expand functional β cell mass thus provide a possible cure for diabetes.

Specific aims:

Paper I: In vivo screen in zebrafish to identify and characterize chemicals stimulating proliferation of β cells.

Paper II: In vivo screen in zebrafish to identify and characterize chemicals potentiating reprogramming of non-β cells to β cells in the pancreas.

Paper III: To explore and functionally characterize effects of a small molecule called Adjudin on pancreatic islets and liver, and its role in diabetes.

3 Results, discussion and future perspectives

3.1 Paper I

Title: In vivo screen identifies a SIK inhibitor that induces β cell proliferation through a transient UPR.

In an effort to screen for small molecules that promote β cell proliferation, we developed a LUCCI zebrafish model based on the S/G2/M marker geminin. The model enables us to assess β cell proliferation in an efficient and quantitative manner. Through monitoring geminin levels in β cells by bioluminescence, both cell cycle blockers and activators were identified, indicating increases in geminin may occur by activation of the cell cycle, as well as accumulation of geminin may occur when blocking the cell cycle in a phase where geminin is expressesd. Among the activators, HG-9-91-01 (hereafter named HG) was identified as the most potent one. In translational studyies, we examined the effect of HG in mouse and human primary islets, found a consistent increase of proliferation in β cells of both species.

The SIK protein family is a known target of HG. Knock-down of *sik1*, *sik2a* or *sik3* increased LUCCI signal in zebrafish, and overexpression of *Sik1* blocked HG induced β proliferation in mouse islets. Next, we performed single-cell RNAseq on HG-treated islets to delineate the mechanism, and discovered β cells with HG treatment had a transient UPR before entering cell cycle. The transient UPR was validated in mouse islets in both *in vivo* and *in vitro* experiments and shown to be necessary for HG-mediated proliferation based on a ATF6 knock-down experiment in mouse islets. To investigate how the SIK family regulates UPR, we performed various experiments and demonstrated that: 1) overexpression of *Sik1* in MIN6 cells blocked HG induced UPR; 2) there was no direct interaction between SIK1 and ATF6; 3) HG-mediated upregulation of ATF6 was likely regulated by targets of SIKs, the cAMP-regulated transcriptional coactivators CRTC1 and CRTC2. Furthermore, we found chemical inhibition of mTOR (target of ATF6) and IRE1 (another UPR pathway) partially blocked HG-mediated proliferation, whereas

chemical activation of ATF6 was not enough to stimulate β cell proliferation. Together, HG inhibits the SIK family, especially SIK1, which downstream effectors including CRTC, ATF6, mTOR and IRE1 collectively induce β cell proliferation.

HG could be used in cell replacement therapy by expanding β cell number *in vitro* before cell transplantation. Although we demonstrated SIK1 as the main effector of HG, a recent study reported that activation of SIK2 stimulated β cell proliferation (223), therefore a better characterization of HG and its targets is needed as well as whether the different SIKs have opposing roles in β cell proliferation. Nevertheless, several factors may limit further development of HG in diabetes therapy: HG stimulated δ cell proliferation in zebrafish and mouse islets, suggesting the proliferative effects are not β cell specific, even though it was more potent in β cells; HG induced proliferation resulted in compromised β cell function in mouse islets; since the SIK family functions as a suppressor of gluconeogenesis in liver (224), inhibition of SIK with HG may have opposite effects on glycemic control.

Regarding to β cell proliferation in diabetes therapy, more studies to decipher why β cells are resistant to proliferate would guide more specific drug discovery methods, thus likely lead to more potent drugs. Another obstacle needed to be overcome is to develop β cell targeted mitogens that stimulate proliferation specifically in β cells. Moreover, one thing to keep in mind when aiming for β cell mitogens in the clinical setting is that an increase in β cell proliferation is usually coupled with compromised β cell function.

3.2 Paper II

Title: In vivo screening for small molecules promoting cellular reprogramming to insulin-producing β-cells.

Based on luciferase reporters built on Cre-lox system and the β cell ablation model, we examined reprogramming of *gcga*-expressing α cells, *sst2*-expressing δ cells and *ela3l*-expressing acinar cells to β cells in zebrafish, respectively, after extreme β cell loss. Consistent with what has been shown in the mouse (128, 129),

we observed spontaneous conversion of α cells and δ cells to β cells after β cell ablation, even though the conversion was low. We did not observe spontaneous conversion of acinar cells to β cells in our study. To discover chemicals that promote reprogramming to β cells, we performed chemical screens in zebrafish. A total number of 4794 chemicals was screened in each type of reprogramming.

In the screen for reprogramming of α cells to β cells, we identified an Akt inhibitor A-674563 (hereafter called A67) and a matrix metalloproteinase inhibitor NSC-405020 (hereafter called NSC) as potential hits. Both compounds promoted β cell regeneration, reduced glucose in zebrafish with β cell ablation. However, only A67 induced a modest conversion in subsequent lineage tracing experiment. The discovery of A67 goes in line with previous findings, where inhibition of insulin signaling stimulates reprogramming of α cells to β cells (134, 155).

In the screen for reprogramming of δ cells to β cells, we identified a CDK1/2 Inhibitor BMS-265246 (hereafter called BMS) and a PDGFR inhibitor CP-673451 as potential hits. Both compounds promoted β cell regeneration, reduced glucose in zebrafish with β cell ablation, but showed no effects in reprogramming of δ cells to β cells in subsequent lineage tracing experiments.

In the screen for reprogramming of acinar cells to β cells, we identified the same hit from δ-to-β cell screen BMS and another chemical named triciribine as potential hits. BMS failed to induce reprogramming in subsequent lineage tracing experiments. Triciribine was later found to be a false positive autoluminescent hit.

To explore the origin of regenerated β cells induced by the hits, we tested whether *krt4*-expressing ductal cells (225) contributed to the induced β cell regeneration. Lineage tracing experiments showed that the 4 hits failed to increase β cells derived from the *krt4* lineage. Therefore, more studies need to be done to find out the cellular origins of the induced β cells.

These results indicate that it is difficult to stimulate reprogramming to β cells in the pancreas *in vivo* with chemicals, at least with a single chemical and a relatively

short-term treatment. Screening with Cre-lox system in early developmental stage of zebrafish can lead to meaningful findings, but possible leakage of Cre and high plasticity of islet cells at this stage may result in misexpression of Cre in unexpected cells, which can make the experimental results hard to interpret. *In vivo* drug screening has a big advantage in drug discovery, but it comes with a downside: when a chemical leads to multiple effects, it is often difficult to distinguish whether one specific effect is a direct or indirect effect. A better understanding of this may lead to different avenues of follow-up studies. Together, using small molecules to stimulate *in vivo* reprogramming of none-β cells to β cells for diabetes therapy might be a long-term challenge.

3.3 Paper III

Title: Adjudin improves beta cell maturation, hepatic glucose uptake and glucose homeostasis.

Adjudin was identified from the α-to-β cell screen described in Paper II, but was less potent in the screen compared to the other two hits A67 and NSC. In the follow-up studies, we found that Adjudin failed to stimulate reprogramming of α cells to β cells in lineage tracing experiments and failed to promote β cell regeneration in zebrafish. However, we observed increased insulin promoter activity and mRNA expression, as well as decreased glucose levels in zebrafish with β cell ablation after Adjudin treatment. Those results led us to examine its effect on β cell function. We performed live imaging to analyze intracellular calcium signals in β cells using a zebrafish line *Tg(ins:GCaMP6s)*, and found that Adjudin enhanced calcium response to glucose in regenerated β cells, suggesting Adjudin treatment led to a better β cell function *in vivo*.

To translate the findings and examine whether islets are a direct target of Adjudin, we tested Adjudin on isolated islets from mice of different stages. We found, instead of affecting function of adult islets, Adjudin treatment led to functional improvements in P0 islets, including increased insulin secretion in high glucose media, reduced basal insulin secretion, upregulated transcriptional expression of

mature β cell markers. Consistent with what has been shown before (84-86), we found Adjudin-induced functional improvements in P0 islets were coupled with a decrease in β cell proliferation. In islets from type 2 diabetic *db/db* mice, Adjudin increased insulin secretion in high glucose media, upregulated transcriptional expression of mature β cell markers, and downregulated transcriptional expression of disallowed genes, suggesting Adjudin improves the recovery of dysfunctional islets.

Observations in zebrafish without β cell ablation drove us to another direction. In those fish, Adjudin treatment resulted in unchanged β cell function but reduced glucose. Moreover, with Adjudin treatment we observed glucose lowering effects even in an insulin deficient zebrafish model. By employing fluorescent glucose analogue 2-NBDG in zebrafish, we identified the liver as another target of Adjudin. Adjudin enhanced liver glucose uptake in different diabetic zebrafish models including fish with β cell ablation, fish pretreated with glucose, and fish with insulin deficiency. More importantly, we found that Adjudin promoted glucose uptake in primary human hepatocyte (PHH) formed spheroids with insulin resistance. These results indicate that: liver is another target that contributes to Adjudin-mediated glycaemic control; Adjudin induced glucose lowering effects is at least partially insulin-independent.

Based on the findings in β cells and liver, we examined the therapeutic potential of Adjudin in *db/db* mice. Adjudin treatment decreased nonfasting blood glucose, improved glucose tolerance, and mice with the treatment showed signs of improved β cell recovery. However, we did not observe increased blood insulin in glucose tolerance test (GTT). Instead, Adjudin-treated mice reduced glucose faster with relatively less insulin during GTT. The results suggest that other target(s) (e.g. liver) may play a more important role in glycemic control *in vivo*.

Several future avenues are warranted to explore: Adjudin may improve the function of SC-islets for cell replacement therapy. The insulin-independent glucose lowering effect may represent a model to study insulin-independent

glucose uptake. Since type 1 diabetes patients have insulin deficiency, Adjudin (if approved) may be used for type 1 diabetes treatment. Limitations of the study: the molecular mechanisms of Adjudin's action on islets and liver are not clear; Adjudin's effects on human islets especially islets from human type 2 diabetes are not examined; Adjudin's effects on muscle and adipose tissue in mouse are not examined. Another factor that may limit its future use in diabetes therapy is that Adjudin is a potential male contraceptive drug, although the contraceptive effects were shown to be reversible (226).

4 Concluding remarks

The constituent papers in this thesis cover 3 aspects of expanding functional β cell mass in a drug discovery context.

Paper I focuses on β cell proliferation, in this study we developed a new screening model (LUCCI) for β cell proliferation, performed *in vivo* screens in zebrafish, identified a small molecule (HG-9-91-01) as an inducer of β cell proliferation in zebrafish, mouse and human, and discovered an important role of SIK (especially SIK1) and UPR in β cell proliferation. Nevertheless, several obstacles still remain, as the effect on human β cell proliferation is generally low and it is hard to stimulate specifically β cell proliferation, areas that future research may expand on.

Paper II focuses on reprogramming of non-β cells to β cells in the pancreas, in this study we performed *in vivo* screens in zebrafish, identified potential hits as stimulators of reprogramming. The hit A-674563 promoted modest reprogramming of α cells to β cells. Other hits promoted β cell regeneration without stimulating expected reprogramming. Thus, it is possible that cellular origins other than the ones we examined are more prone to enable reprogramming, or that reprogramming events are more common in other injury models or species.

Paper III focuses on β cell function, in this study we discovered that a hit Adjudin from Paper II enhanced β cell function in zebrafish, as well as in immature and dysfunctional mouse islets. Moreover, Adjudin promoted glucose uptake in zebrafish and human hepatocyte formed spheroids, and improved glucose homeostasis in a type 2 diabetic mouse model. Small molecues like Adjudin, which simultaneously exert multiple benificial effects on diabetes, might fill a gap among the arsenal of drugs targeting diabetes.

Together, the studies presented in this thesis represent a significant advancement in *in vivo* drug discovery, enhance our understanding of β cell biology, and hold the potential to improve heath of human with diabetes.

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