Alpha cells transdifferentiation in the pathophysiology and potential treatment of diabetes

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(I confirm that the word count of this thesis is less than 100,000 words)

Dedication

I dedicate my thesis to my beloved family, teachers and friends

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GENERAL SUMMARY

Diabetes mellitus (DM) is frequently associated with the loss of beta cells and gain of alpha cells. Therefore, here we aimed to target the plasticity of alpha cells to regenerate beta cells. For this purpose, we tested a range of therapeutic agents using *in vitro* approach (BRIN-BD11 cells and alpha-TC 1.9 cells) and *in vivo* models (Swiss TO NIH mice and $Glu^{CreERT2}$; ROSA26-eYFP transgenic mice).

Here we found beta cell regenerative effects of taurine using both *in vitro* cell culture and *in vivo* Swiss TO NIH diabetic mice models. Other studies using cultured alpha-TC 1.9 cells, we found plasticity-altering effects of artemether, GABA, taurine, sitagliptin and exendin-4 with respect to alpha to beta transdifferentiation.

Finally, using $Glu^{CreERT2}$;ROSA26-eYFP mice we employed alpha cell lineage tracing approach that was easy to identify transdifferentiation of alpha cells. Here, we developed multiple low dose streptozotocin-induced diabetic mice model that showed persistent hyperglycaemia with severe impact on body parameters and islets morphology. Importantly, STZ mediated loss of beta cells triggered natural alpha to beta transdifferentiation. This was further associated with decreased glucagon and increased local GLP-1 expression by alpha cells. Interestingly, taurine, liraglutide, sitagliptin, insulin, GABA and analogues of GIP and Oxm significantly enhanced alpha to beta cell transdifferentiation. Moreover, except insulin all these agents as well as rosiglitazone, metformin and xenin also promoted beta cell proliferation. While, taurine, liraglutide, sitagliptin, dapagliflozin, insulin, nicotinamide, analogues of GIP and Oxm protected beta cells from apoptotic death. In contrast, tolbutamide promoted beta cell apoptosis. Surprisingly, sitagliptin enhanced alpha cell proliferation while it was prevented by insulin, GABA, analogues of GIP, xenin and Oxm. In addition, metformin, analogous of GIP and Oxm increased alpha cell apoptosis.

Overall, the present thesis highlights the possible role of GLP-1, GIP, glucagon, insulin, and GABA signaling mechanism in activating the transdifferentiation of alpha to beta cells. Therefore, we suggest that alpha cells might provide a source of beta cell regeneration and that therapeutic intervention with selected drugs may provide a means to treat diabetes in man.

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AUC	Area under the curve
α	Alpha
BSA	Bovine serum albumin
β	Beta
Ca^{2+}	Calcium
cAMP	Cyclic-AMP (3, 5'-cyclic monophosphate)
CPM	Counts per minute
dH_2O	Distilled water
DM	Diabetes mellitus
DPP-4	Dipeptidyl peptidase-4
ELISA	Enzyme linked immunosorbent assay
FBS	Foetal bovine serum
Fig.	Figure
g	Gram(s)
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide-1
GLUT	Glucose transporter
h	Hour(s)
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethyl-piperazine-N'-2-thane-sulphonic acid
i.p.	Intraperitoneal
IU	International units
Kg	Kilogram(s)
KCl	Potassium chloride
KRBB	Krebs ringer bicarbonate buffer
l	Litre(s)
M	Molar
mg	Milligram
min	Minute(s)

ml	Millilitre(s)
mm	Millimetre(s)
mmol	Millimole(s)
µ	Micro
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
n	Number of observations
ng	Nanogram(s)
nmol	Nanomole(s)
nM	Nanomolar
Oxm	Oxyntomodulin
P	Probability
PBS	Phosphate buffered saline
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rpm	Revolutions per minute
sec	Second(s)
S.E.M.	Standard error of the mean
STZ	Streptozotocin
t	Time
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
U	Unit(s)
µg	Microgram(s)
µl	Microlitre(s)
v/v	Volume per volume
w/v	Weight per volume

DECLARATION

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Chapter 1

General Introduction

1.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a group of metabolic disorders characterised by elevation of blood glucose typically beyond its normal range (Fan et al., 2017). The possible reasons include impaired insulin secretion and insulin sensitivity (Unnikrishnan et al., 2017; Fan et al., 2017). If diabetes is untreated for a long time, then several organs including eyes, heart, kidneys, etc. undergo long-term damage or failure in their function (Fan et al., 2017). Its symptoms include increased appetite, polyuria, polydipsia, loss of weight, impairment of eyesight due to retinopathy (Fan et al., 2017). The ketoacidosis is frequently associated with chronic hyperglycaemia, which may offer some life-threatening consequences (Fan et al., 2017). It is a fatal disease increasing globally at an alarming rate (Olokoba et al., 2012). Indeed, it is estimated in 2011 that about 366 million people suffered from diabetes while this population would rise to 552 million people by the year 2030 (Olokoba et al., 2012; Fan et al., 2017; Unnikrishnan et al., 2017). In 2011, it was predicted that more than 4.6 million people died with diabetes (Olokoba et al., 2012). The prevalence of diabetes varies as per geography, race, environment, diet and lifestyle (Olokoba et al., 2012). It is assumed that in the next two decades, the prevalence of diabetes will increase at its alarming rate (Olokoba et al., 2012).

In 1936, the vast population of diabetes was categorized into two broad hyperglycaemic pathological conditions (Olokoba et al., 2012). The first category is referred to as type 1 DM that includes an absolute absence of insulin (Olokoba et al., 2012). Type 1DM individuals are diagnosed by recognizing some genetic factors and by serological signs of autoimmune events ongoing in the pancreatic islets (Olokoba et al., 2012). While, another type 2 DM is developed as a result of metabolic syndrome and is described in 1988 as a much more prevalent category (Olokoba et al., 2012). In this type of diabetes, the severity of hyperglycaemia offers pathophysiological alterations in several target tissues (Olokoba et al., 2012). Type 2 DM can exist for a long time with no symptoms and therefore, it is sometimes referred to as a silent killer disease (Olokoba et al., 2012). However, it can be diagnosed by measuring fasting blood glucose or performing an oral glucose tolerance test (Olokoba et al., 2012).

1.2 PANCREAS

1.2.1 Islet architecture

Pancreatic islets are comprised of a diverse population of endocrine cells assembled to form islands or islets of Langerhans. It is estimated that 0.5 to 2.0 cm^3 volume of the human pancreas consists of around 3.2 or more million islets (Silva et al., 2018). Islet composition differs from species to species. Human islets comprise a random distribution of about 60% of beta cells, about 30% of alpha cells, and up to 10% another delta, PP- and epsilon cells. While rodent species show a somewhat different cellular composition and distribution with central beta cells surrounded by other cells. These endocrine cells produce and secrete hormones such as insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), PYY (γ-cells) and ghrelin (ε-cells) (Silva et al., 2018). Recent studies showed that 3-D mapping of islet architecture. According to their study, a healthy human pancreas consists of about 3.2 million islets, which show diameters of 108 μm and a volume of 690 pL. Most of them (60%) show islet area in the range of 1000-10,000 μm^2 (Silva et al., 2018).

1.2.2 Insulin secretion and its action

Following the ingestion of a meal, glucose enters in to the bloodstream, which conveys it to the pancreas. In the pancreas, glucose is transported into beta cells through Glut2 transporters. If blood glucose is high, then a large amount of glucose is delivered into beta cells and vice versa. The transported glucose is then oxidized to CO_2 and H_2O through series of glycolysis and oxidation pathways. The end result generates ATP. The resultant increased ATP:ADP ratio causes the closing of ATP-dependent SUR1/Kir6.2 potassium channels. This inhibits the outflow of potassium ions (K^+), resulting in intracellular accumulation. The net intracellular charge becomes less negative, which leads to membrane depolarization. The effect of depolarisation causes the opening of voltage-gated calcium ion (Ca^{2+}) channels required for the entry of calcium ions inside the beta cells. In addition, intracellular granules simultaneously release calcium ions to raise intracellular calcium. In addition, phospholipase C also triggers the intracellular release of calcium ions through the regulation of G-Protein coupled receptors signaling. The net effect of increased intracellular calcium concentration causes the release of insulin from stored granule packets by exocytosis.

This is the general glucose-dependent mechanism of insulin secretion from beta cells. Other mechanisms involve stimulation of insulin secretion by the amino acids such as arginine and leucine, sulphonylureas, CCK, and incretin hormones such as GLP-1 or GIP.

For the action of insulin, it's binding to insulin receptors is needed. This receptor is present on the cell surface in the form of a homodimer. Insulin interacts with the exterior faced alpha part of homodimer and triggers enzyme activity of another interior faced-beta part of a homodimer. This signal leads to the activation of endogenous protein insulin receptor substrates (IRS). The activation of the IRS triggers intracellular signaling cascade (PI3K/Akt/mTOR signaling pathway) for the insertion of Glut4 transporter into the membrane. Therefore, ultimately glucose is allowed to enter into cells. The insulin effect is prominent in many muscle cells, adipose tissues, and liver cells. Insulin also increases glycogen synthesis and fatty acid synthesis.

1.3 PATHOPHYSIOLOGY OF TYPE 1 AND TYPE 2 DIABETES

1.3.1 Autoimmune destruction of beta cells in type 1 diabetes

Type 1 diabetes involves autoimmune-mediated eradication of pancreatic beta cells. The immune systems are build-up for the recognition of foreign invaders and their precise elimination to protect the body. However, certain unexpected defects in the immune system lead to the removal of the body's beta cells and the resultant condition is categorized as type-1 diabetes mellitus (T1DM). As such, T1DM involves more than 50 different genetic alterations. Out of them, major genetic defects have been found in major histocompatibility complex (MHC) region, which is frequently referred to as human leukocyte antigen (HLA) occurs at Chr 6, which accounts for 40-50% of T1D susceptibility. In addition, the variable numbers of tandem repeats in insulin promoter (Ins-VNTR, IDDM 2) loci and the cytotoxic T lymphocyte-associated antigen-4 gene (CTLA-4) loci contribute about 15% of defective condition (Paschou et al., 2018). Certain immunological factors such as immunotolerance, cellular immunity and humoral immunity may also increase the risk of T1DM. An immune tolerance involves an increase in potentially cytotoxic immune cells. Such dangerous cells are expected to be clear through the elimination process called central

tolerance. However, a mutation in AIRE gene can lead to defective central tolerance mechanisms and thereby generate T1DM (Paschou et al., 2018). Cellular immunity describes the elimination of beta cells through apoptotic pathways, mainly by activation of caspase activity. While some assumptions suggest the involvement of necrosis and necroptosis. It is also believed that certain proinflammatory cytokines such as IL-1, TNF-α and INF-γ is can be activated by the autoreactive T-lymphocyte within pancreatic vicinity. The humoral immunity describes the involvement of autoantibodies against beta cells specific molecules such as GAD65, tyrosyl phosphatase (IA-2), insulin (IAA) and zinc transporter (ZnT8). Overall, such a defective immune system in T1DM leads to the loss of beta cells (Paschou et al., 2018).

1.3.2 Type 2 diabetes is associated with beta cell dysfunction

1.3.2.1 Insulin resistance

Insulin resistance is a metabolic condition in which cells fail to respond to insulin. This causes abnormality in the delivery of glucose, leading to the accumulation of glucose in the bloodstream. Many cells, including muscle and adipose tissue, are affected by this abnormal condition. Indeed, insulin resistance causes the failure of the liver to respond to insulin, which results in unrestrained endogenous glucose production. Insulin resistance results in the synthesis of new fatty tissues and increases weight gain. Therefore, insulin resistance is common in obese, hyperglycaemic people.

The action of insulin uses PI3K/Akt/mTOR signaling pathway to activate insulin receptors. However, the block of this pathway by certain factors leads to loss of sensitivity of the receptor. In recent studies, the biphasic threshold phenomenon of insulin response is suggested. According to this hypothesis, insulin response operates in a biphasic manner under different physiological conditions. In the case of pregnancy, insulin resistance is believed to be useful for a mother to divert glucose to much-needed brain nourishment of both mother and fetus by reducing insulin sensitivity in less important sites. This may involve blocking the communication between insulin receptor substrate (IRS) and PI3K through the action of placental growth factors. In contrast, during obesity, the prolonged secretion of insulin creates

desensitization of insulin receptors. Moreover, increased endogenous fatty acids or cholesterol also interferes in the binding of insulin to its receptor.

In the case of prolonged hypersecretion of insulin, beta cells increase the synthesis and release of insulin in line with increased metabolic demand. This leads to a condition called hyperinsulinemia, which is needed to compensate for increased blood sugar. However, this compensatory phase is responsive as it manages to lower blood glucose to its normal range. However, failure in the compensatory phase may lead to a rise in fasting or postprandial sugar levels. Finally, the combination of failed compensatory phase and prolonged insulin resistance culminates in type 2 diabetes. In addition, beta cells lose their ability to synthesize and secrete insulin normally.

1.3.2.2 Glucotoxicity

Loss of beta cells is believed to be a common incidence in type 1 and severe type 2 diabetes. Several reports have stated that prolonged hyperglycaemia weakens the glucose-dependent insulin synthesis and secretion. Glucose desensitization, β-cell exhaustion, and glucotoxicity are major characteristic features of prolonged hyperglycaemia. In the case of glucose desensitization, an increased level of glucose leads to compromise of beta cell's exocytosis machinery. However, it is a reversible phenomenon and is reversed after insulin release is inhibited. β cell exhaustion involves the rapid depletion of secretory granules following chronic exposure to secretagogues. Glucotoxicity also triggers the loss of beta cells via the apoptotic route (Poitout et al., 2002). The mechanisms of glucotoxicity involving changes in gene expression induce an adverse effect on insulin synthesis. This involves the impairment of two main beta cell transcription factors such as Pdx1 and inducer of rat insulin promoter element 3b1. Previous evidence suggested that the level of insulin gene repressor CCAAT/enhancer-binding protein β and proto-oncogene c-myc is amplified. The presence of proto-oncogene c-myc results in the loss of formation of β-cells exposed to hyperglycaemia (Poitout et al., 2002). Chronic hyperglycaemia is frequently associated with biochemical changes such as the generation of oxidative stress. Previous *in vitro* studies have been suggested that reactive oxygen generation following prolonged hyperglycaemic condition adversely affected insulin gene transcription while it was reversed by the use of the antioxidants amino-guanidine and *N*-acetyl-cysteine (NAC) (Poitout et al., 2002).

1.3.2.3 Lipotoxicity

Similar to glucotoxicity effects, lipotoxicity is another condition that causes beta cell damage. Unlike normal condition in which fatty acids are fuels to beta cells, chronic exposure of elevated fatty acids leads to lipotoxicity. Such chronic exposure of beta cells to lipids causes a rise in insulin secretion but hinders glucose-dependent insulin release. Moreover, lipid deposition suppresses insulin synthesis in the presence of hyperglycaemia by downregulating Pdx1 expression. Eventually, elevated fatty acids lead to beta cell death via a programmed cell death event (Poitout et al., 2002). There is a debate that lipotoxicity is associated with enhanced fatty acid oxidation, which leads to a reduction in glucose breakdown or involvement of esterification of fatty acids. Some reports suggest that certain intermediate precursors of fatty acid esterification pathway exert the adverse effect of hyperlipidemia. Nonetheless, it reflects the profound alteration in fatty acid metabolism rather than glucose metabolism after exposure to prolonged elevated fatty acids. The metabolic evidence for this premise was tested and reviewed in detail by previous studies. Accordingly, the concurrent occurrence of both hyper-glucose and-lipids leads to the generation of beta-cell toxic metabolite long-chain fatty acyl CoAs. Correspondingly, several other intermediate metabolites of downstream of lipid oxidation pathways have been believed to exert a deleterious effect on ATP-sensitive potassium channel, PKC, uncoupling protein-2 (UCP-2), and synthesis and secretion of insulin (Poitout et al., 2002).

1.4 PREVENTION OF TYPE 2 DIABETES

1.4.1 Pharmacological anti-diabetic drugs

It is well known that loss of weight and an active lifestyle are the key elements for the prevention of type 2 diabetes. A low-calorie diet and regular exercise are useful in managing obesity-induced diabetes. At present, some currently available antidiabetic drugs are helpful in managing type 1 or type 2 diabetes mellitus (Unnikrishnan et al., 2017; Fan et al., 2017).

1.4.2 Biguanides

The widely used biguanide agent is metformin that lowers endogenous glucose production as well as glucose absorption by intestine and enhances insulin sensitivity, fatty acid catabolism, glucose uptake. Metformin can activate AMP-activated protein kinase that is essential in hepatic gluconeogenesis. It offers less risk of hypoglycemia in comparison to other insulin secretory drugs (Olokoba et al., 2012).

1.4.3 Sulphonylureas

Tolbutamide, glyburide, glipizide are some examples of this class. Tolbutamide secretes insulin blocking the potassium channel on the membrane of beta cells. However, it offers hypoglycaemic complications, weight gain, allergies (Olokoba et al., 2012).

1.4.4 Meglitinides

Repaglinide and Nateglinide also stimulate insulin secretions by interacting with ATP-dependent K-channel on beta cells. They are effective in controlling postprandial blood glucose control. Their effect lasts long for a short duration and therefore offers less risk compared to sulphonylureas (Olokoba et al., 2012).

1.4.5 Thiazolidinediones (TZD)

Rosiglitazone is the example of TZD, which serves as an insulin sensitizer, and interacts with nuclear peroxisomes proliferator-activated gamma. The activation of this transcription factor leads to the activation of specific genes in fatty cells and enhances the uptake of glucose and fatty acids. It offers complications such as heart failure, stroke and bone fractures (Olokoba et al., 2012).

1.4.6 Alpha-glucosidase inhibitors

Acarbose, voglibose and miglitol are some examples of this class, which slow down the digestion of carbohydrates by inhibiting degradation enzyme alpha-glucosidase (Olokoba et al., 2012).

1.4.7 Incretins and DPPIV inhibitors

Intestinal hormones GLP-1 and GIP secreted by L-cells or K-cells, respectively, are effective in secreting insulin and regulation of beta cell growth. However, they are

degraded rapidly by the action of dipeptidyl peptidase-4 (DPP-IV). Therefore, enzyme resistant peptide analogues such as exendin-4 and liraglutide (GLP-1) or DPP-IV inhibitor sitagliptin are used to treat diabetes (Prately et al., 2007; Moffett et al., 2014; Vasu et al., 2014a).

1.4.8 Insulin

In severe cases of diabetes, an exogenous injection of insulin is given to the diabetic individual. People with type 1 diabetes are being treated with exogenous insulin. Several short-, long-, and intermediate-acting insulin analogues have been developed. Lispro, glargine and lente are some examples of insulin analogues (Burge et al., 1997).

1.4.9 Need for research in the context of beta cell regeneration

Recently it has been noticed that beta cells loss in type 1 DM and type 2 DM is congruous with an expansion of alpha cells. The possible assumptions for such changes could be a loss of alpha cells growth regulatory molecules such as insulin or GABA that led to alpha cell hyperplasia (type 1 DM & type 2 DM) (Feng et al., 2017) or to survive in hyperglycaemic toxic environment; beta cells may commit dedifferentiation, which ultimately turns their phenotypic transition into alpha cells (Type 2 DM) (Talchai et al., 2012). There is an urgent need for research to regenerate beta cells as a treatment for type 1 or type 2 diabetes because of the several reasons such as limitation of currently available drugs, failure of compensation by natural regeneration mechanism such as beta cells proliferation in a toxic metabolic condition, barriers of islet transplantations, less knowledge regarding beta cell regeneration phenomenon.

1.5 ISLET PLASTICITY

1.5.1 Alpha cell transdifferentiation as a source of beta cell regeneration

The pancreas organ develops from endodermal lineage consists of progenitor cells. These cells give rise to fully functional, committed cells such as alpha, beta, delta, etc. Different mechanisms such as replications, proliferation and programmed cell death regulate the growth of alpha and beta cells. However, the cellular loss is frequently

observed in certain extreme conditions such as hyperglycaemic stress. To compensate such loss, specialised cells offer genomic plasticity, which makes them adapt the required function (Collombat et al., 2010; Talchai et al., 2012; Spijker et al., 2013). Each cell possesses its own set of transcription factors, which are essential for maintaining their identity (Habener et al., 2013). These transcription factors decide activation and repression of specified genes and therefore the main function of the cells such as insulin or glucagon synthesis (Habener et al., 2013) Figure 1.5 explains alpha and beta cell specified markers. Therefore, if alpha cells need to produce insulin, they are required to express beta cell-specific identity factors primarily Pax4, Pdx1 and Maf A (Lu et al., 2014). Simultaneously they are bound to repress their own identity markers such as Arx, Pax6, MafB, Men1 and Dnmt (Habener et al., 2013). This would therefore results in a flip from glucagon producing alpha to insulin-producing alpha cells. This process is called transdifferentiation and the resultant alpha cells either are called transdifferentiated alpha cells or newly formed beta cells (Collombat et al., 2010; Talchai et al., 2012; Habener et al., 2013; Spijker et al., 2013).

Alpha cells consist of about 6 regulatory elements such as G1 to G5 and cAMP-responsive element (CRE) that regulate glucagon gene expression (Figure 1.1). Aristaless related homeobox (Arx) is the major identity marker of alpha cells, which controls the glucagon expression. The paired box protein (Pax-6) is essential to regulate the function of many other factors like MafB, Beta2 and PC2. G1 to G4 elements are required to upregulate glucagon synthesis. The Pax6 and MafB interactions stimulate these sites, while Pdx1, Pax4 and Nkx6.1 repress (Figure 1.1) (Gosmain et al., 2013). During organ development, the relative expression of Arx and Pax4 is a decisive phenomenon to generate alpha or beta cell fates. These transcription factors could antagonize each other by interacting via their domains (Gosmain et al., 2013).

1.5.2 Alpha cell transdifferentiation using genetic manipulation

Previous studies have found the possible role of transcription factors in fate changing or transdifferentiation of alpha and beta cells **(**Habener et al., 2012; Puri et al., 2015; Van et al., 2015). Some misexpressions of transcription factors in alpha or beta cells revealed the cellular plasticity **(**Habener et al., 2012; Puri et al., 2015). Some studies have been demonstrated that alpha cell-specific overexpression of Pax4 and Arx

suppression is responsible for their transdifferentiation into beta cells (Collombat et al. 2005, 2009; Courtney et al., 2013). An author Wilcox et al. (2013) showed that the deletion of Arx developed bihormonal cells expressing glucagon as well as insulin, while the effect was absent upon its temporary repression. In line with this, Courtney *et al.* (2013) also showed the loss of Arx led to the transition of alpha to beta cells. Pancreatic and duodenal homeobox 1 (Pdx1) is another essential marker for beta cell development. Previous studies have been demonstrated that beta cell-specific depletion causes loss of beta cell characteristics, while alpha cell-specific overexpression led to their transdifferentiation into beta cell. Similarly, reports on beta cell marker forkhead box protein O1 (FoxO1) showed the fate adaptation of either alpha cell or Ngn^{3+} progenitor cell type after deletion of FoxO1 (Lu et al., 2014). DNA methyltransferase1 (Dnmt1) reduction is associated with suppression of glucagon gene and function of alpha cells. Overall, these attempts highlight the importance of transcription factors in the manipulation of the identity and function of alpha or beta cells. Therefore, the relative expression of transcription factors allows the transdifferentiation of alpha to beta cells (Habener et al., 2013; Lu et al., 2014).

1.5.3 Alpha cell transdifferentiation after beta cells injury or loss

Recent studies demonstrated the evidence of alpha cell transdifferentiation after extreme (>90%) beta cell destruction using alloxan- or streptozotocin-induced diabetic rodent models (Thorel et al., 2010). Importantly, it has also been observed that selective removal of alpha cells disappeared the presence of bi-hormonal cells when beta cells challenged with extreme stress (Habener et al., 2013; Lu et al., 2014).

1.5.4 Alpha cell transdifferentiation using small therapeutic molecule

Due to the plasticity of alpha cells, the specific transcription factors could be the druggable targets to repress or activate specific gene functions. This strategy could be beneficial for the regeneration of beta cells through transdifferentiation of alpha into beta cells (Wagner et al., 2010; Xu et al., 2016). In this attempt, Fomina-Yadlin et al. (2010) tested about 30,710 small compounds and found the potential drug BRD7389. Similarly, GW8510 (Choudhary et al., 2014) was found with such transdifferentiating potential. These drugs were screened using ChemBank computation simulation methods. BRD7389 is considered to interact and inhibit RSK protein family within

alpha cells, which initiates transdifferentiation (Fomina-Yadlin et al., 2010). GW8510 found to downregulate the phosphorylation of Brsk1 and Camkk2 (Choudhary et al., 2014) or regulate p53 in a JNK- and p38-dependent activation of insulin (Fomina-Yadlin et al., 2012).

In line with this, Ben-Othman et al. (2017) suggested the alpha cell transdifferentiation potential of GABA, which is classically considered as growth, and hormone regulator of alpha cells. Interestingly, prolonged treatment with GABA restored beta cells in streptozotocin-ablated beta cell mass. Importantly, the authors went on to show that the loss of beta cells was compensated by surrounding alpha cells. In turn, the ductal cells turned into alpha cells to compensate the resultant alpha cell loss (Ben-Othman et al., 2017). In this way, GABA could restore islet size even after two cycles of streptozotocin mediated beta cell loss.

Similarly, the testing of 280 small molecules by Li et al. (2017) demonstrated that the anti-malarian drug artemether holds the alpha cell transdifferentiation potential. This drug is believed to use GABA receptor machinery of alpha cells to repress Arx and activate Pax4 genes, which are essential alterations for alpha to beta cell transdifferentiation (Figure 1.3). In particular, the author highlighted that the artemether targets gephyrin cluster, a stabilizer of $GABA_A$ receptors that leads to receptor activation followed by stimulation of chloride ions influx. This favors to the certain unknown signals of Arx inactivation. In addition, Arx is then translocated from nuclear site to cytoplasm, which gives a space for activation of Pax4 transcription factor that ultimately inhibits glucagon and upregulates insulin gene within the alpha cell domain (Li et al., 2017). In contrast, later the studies on artemether and GABA showed that lack of alpha cell transdifferentiating effects of these molecules (Ackermann et al., 2018; Van et al., 2018, Shin et al., 2019). According to Van et al. (2018), the artemether not only represses alpha cells' identity but also affects the identity of beta cells. Nevertheless, other studies using various drugs showed events of alpha cell transdifferentiation that includes activin A (activin signaling; Brown et al., 2016), incretin hormones **(**Lee et al., 2018; Zhang et al., 2019), IGFBP1 (inhibiting IGF signaling; Lu et al., 2016), caerulein (Piran et al., 2014), antagonistic glucagon receptor antibody (Wei et al., 2019), vimentin (Cheng et al., 2015).

However, there are few drawbacks of these studies such as the use of immortalized islet cell lines, lack of alpha cell lineage models, use of non-diabetic models in some cases, effects on body parameters. Nevertheless, the findings are inconsistent and not enough to clear the understanding of alpha cell transdifferentiation (Xu et al., 2016). Likewise, the low ability of beta cell regeneration and the failure in the reproduction of similar effects highlights the need for further research (Wagner et al., 2010; Xu et al., 2016).

1.6 RESEARCH FOCUS IN PRESENT THESES

1.6.1 Selection of therapeutically potential agents

In this context, we aimed in this thesis to determine the potential therapeutic molecules and using them to promote the alpha cell transdifferentiation into beta cells. For this purpose, we selected interesting molecules that have already shown some beneficial effects on the islet or glycemic improvement. Moreover, we considered their possible role in relation to alpha cell regulation.

Insulin and GABA are classical secretory products of beta cells (Ben-Othman et al., 2017). The regulate glucagon secretion as well as the population of alpha cells through directly interacting insulin or GABA receptors present on the alpha cell surface (Ben-Othman et al., 2017). In addition, taurine has been widely studied in a neurological context where it is considered as an agonist of $GABA_A$ receptors (Caletti et al., 2015). Therefore, loss of beta cells should be lowering or absolutely lacking the concentration of these secretory molecules. Hence, it is interesting to study the role of their signaling routes in transdifferentiation.

Further, after extreme beta cell injury, alpha cells found to secrete GLP-1, as their genome is mainly equipped for glucagon synthesis (Vasu et al., 2014a; Moffett et al., 2015b). In accordance, recently glucagon family enteroendocrine peptides produced by intestinal L and K cells have been receiving much attention due to their beneficial effects on the islet cells (Moffett et al., 2013; Hasib et al., 2018). It is well known that the proglucagon gene is processed by PC2 in alpha cells to produce glucagon, while PC1/3 processing in intestinal L-cells results in GLP-1, GLP-2, and oxyntomodulin

(Pocai, 2012) (Processing pattern is shown in Figure 1.4). Surprisingly, ectopic expression of PC1/3 has recently been observed in alpha cells, which results in the production of GLP-1, GLP-2, and oxyntomodulin (Dhanvantari et al., 1996; Wideman et al., 2007; Vasu et al., 2014a; Moffett et al., 2014/2015b). It is noteworthy that, although the source of GLP-1, oxyntomodulin, and glucagon is a unique gene, they possess a huge difference in their structure and physiological function (Soni et al., 2016). While, in enteroendocrine K cells, the precursor of GIP is processed by PC1/3 or PC2 to produce bioactive GIP, more interestingly; it is also believed that GIP is produced by PC2 enzyme also in pancreatic alpha cells (Gutierrez-Aguilar et al., 2011; Vasu et al., 2014a; Moffett et al., 2015b). Similarly, xenin peptide is mainly released by K cells; however, it is unexpectedly detected in alpha cells (Khan et al., 2017). The primary function of all these enteroendocrine peptides is to regulate glucose homeostasis, satiety, gastric and pancreatic hormone release by affecting various islet target sites (Hasib et al., 2018; Craig et al., 2018). The peptides derived from L- and K- cells have previously been shown to exert insulinotropic and protective effects on beta cells (Vasu et al., 2014a; Moffett et al., 2015b). However, little is known about their effects on alpha cells. Such intestinal peptides are predominantly attractive therapies for influencing the alpha cell transdifferentiation since they belong to a glucagon-peptide family and unexpectedly produced by alpha cells under some circumstances. Previously GLP-1 is reported to exert transdifferentiation effect in alpha cells **(**Bulotta et al., 2002; Lee et al., 2018; Zhang et al., 2019). However, its closely related peptide family such as oxyntomodulin, GIP and xenin have not been studied with respect to alpha cell transdifferentiation. Therefore, it is interesting to ascertain the role of these peptides on the alpha cell transdifferentiation. In the present study, we tested analogs of oxyntomodulin, GIP and xenin i.e. (D-Ala^{2})GIP, xenin-25[Lys^{13}PAL] and (D-Ser^{2})-Oxm[Lys^{38}PAL]. It is known that both alpha and beta cells offer the binding sites for oxyntomodulin, GIP, and xenin (Pocai, 2012; Moffett et al., 2015b; Craig et al., 2018).

In addition, despite showing anti-diabetic potential, incretin hormones are limited in their action due to their rapid clearance by endogenous DPP-IV enzyme. Their role in beta cell regeneration and protection is not clear (Habener et al., 2013). Therefore, we selected liraglutide and sitagliptin to overcome these degradation barriers. Further, alpha cells are equipped with SGLT-2 transporter 1 and 2. Previously SGLT-2

inhibitor showed a beneficial effect on islet morphology. Another molecule nicotinamide has widely been studied for inducing differentiation in cultured pluripotent cells. This highlights the potential of nicotinamide to manipulate the cellular transcription factors. It is also interesting to investigate whether conventional anti-diabetic drugs such as rosiglitazone, tolbutamide that are known to manage glycaemia play any role in alpha cell transdifferentiation. Overall, by considering their previous beneficial effects in managing diabetes and their potential targets available on alpha cells, we in the thesis aimed to evaluate their actions in transdifferentiation of alpha to beta cells.

1.6.2 Selection of experimental cell line model

For Chapter 3 study, we employed a unique cell line referred as BRIN-BD11 cells, which are clonal hybrid cell lines produced by a combination of NEDH rat pancreatic β- cells with RINm5F cells (McClenaghan et al., 1996). The BRIN-BD11 cells mimic the phenotype of normal pancreatic β- cell coupled with immortality in tissue culture (McClenaghan et al, 1996). BRIN-BD11 are glucose-responsive insulin-secreting cell lines that are used to study physiology and pathophysiology of the pancreatic β-cells (McClenaghan et al, 1996). While, for Chapter 4 study, we employed Alpha TC1.9, which are clones of alpha TC1 cell line (Hamaguchi et al., 1990; Powers et al., 1990). Alpha TC1.9 are primarily generated from alpha cell tumor having a transgenic expression of the SV40 large T-antigen oncogene coupled into the rat preproglucagon promoter (Hamaguchi et al., 1990; Powers et al., 1990). They resemble to normal pancreatic α- cells and produce large amounts of glucagon mRNA and peptide (Powers et al., 1990). The detailed experimental procedure using BRIN-BD11 cells and Alpha TC 1.9 cells were explained in Chapter 2.

1.6.3 Alpha cell labeled $Glu^{CreERT2}$; ROSA26-eYFP transgenic mice

In the present thesis, we aimed to study alpha cells behaviour in response to diabetes and treatment drugs. For Chapter 5-9 studies, we employed novel transgenic mice. Several lines of studies has previously been shown that transdifferentiation of alpha to beta cells is a transitional phenomenon, which involves simultaneous inhibition of alpha cell characteristics and expression of beta cell phenotype (Quoix et al., 2007; Thorel et al, 2010; Lee et al., 2019). This transition may therefore become difficult to

detect by the conventional immunostaining method as it particularly recognises only glucagon (Thorel et al, 2010; Lee et al., 2019). According to previous studies, glucagon is nearly undetectable in the later transdifferentiated beta cell (Thorel et al, 2010; Lee et al., 2019). Therefore, we need a unique approach to permanent labelling of the alpha cells. To overcome this barrier, here we used a new transgenic mouse model, referred to as $Glu^{CreERT2}$; ROSA26-eYFP mice, that expresses the enhanced yellow fluorescent protein (eYFP) precisely in alpha cells (Quoix et al., 2007; Shiota et al., 2017; Parker et al., 2018; Campbell et al., 2019).

Full details of the generation and characterisation of $Glu^{CreERT2}$;ROSA26-eYFP mice provided by Professor Frank Reimann (University of Cambridge) are mentioned in Appendix B as well as described elsewhere (Campbell et al, 2019). In brief, GLU-CreERT2-09 mice were generated. *Cre*-recombinase an estrogen receptor 2 mediated inducible enzyme coded by *iCreERT2* sequence was expressed under proglucagon promoter. Tamoxifen is an estrogen receptor 2 activator, therefore it acts as an inducer of *iCreERT2*. As such *iCreERT2* sequence isolated from a pBS plasmid encoding ERT2-iCre-ERT2 sequence and placed in between the proglucagon start codon in exon 2 and stop codon in exon 6 in the murine based BAC RP23-343C17. Positive recombinants were isolated using appropriate antibiotic selection and characterised by PCR and restriction analysis. Using the microinjection technique, the resultant DNA was introduced into the ova of C57B6/CBA F1 parents and embryos were transferred into pseudo pregnant females (Central Biomedical Services at Cambridge University). Transgene copy number was determined by RT-PCR. While, Another strain Rosa26-EYFP mice (derived from B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J from Jacksons) contains loxP-STOP-loxP-eYFP sequence. For successful expression of enhanced yellow fluorescent protein (eYFP) in alpha cells, the STOP sequence needs to be excised. This phenomenon is achieved by crossing Rosa26-EYFP mice with GLU-CreERT2-09 mice. Finally, double positive off-springs need tamoxifen injection for proper labelling of alpha cells with YFP fluorescent. Mice were bred in-house at Coleraine using breeding pairs derived from the colony originally maintained at University of Cambridge, UK. The presence of Cre-ERT2 and ROSA26eYFP transgenes were assessed by PCR genotyping as previously described (Campbell et al, 2019). The primers used for genotyping of $Glu^{CreERT2}$;ROSA26-eYFP mice are listed in Table 2.6 of Chapter 2. In this study, $Glu^{CreERT2}$;ROSA26-eYFP mice were

intraperitoneally injected with tamoxifen (7 mg/mouse) to induce yellow fluorescent protein. Later, mice were administered using multiple low dose streptozotocin (50 mg/kg) to develop diabetes and considered as diabetic when blood glucose level exceeded above the normal blood range. To achieve severe beta cell damage, around 30 mmol/L blood glucose level was considered as a good sign and therefore reflects as a suitable diabetic model for studying beta cells loss and regeneration phenomenon.

1.7 HYPOTHESIS

- Beta cell loss occurs in diabetes and is associated with the expansion of alpha cells
- It is possible to activate the expression of beta cell markers within alpha cells using therapeutic agents (Figure 1.5)
- Stimulation of such alpha cell transdifferentiation may be beneficial for the treatment of diabetes in man.

1.8 AIM/ OBJECTIVES

Using:

- $Glu^{CreERT2}$; ROSA26eYFP transgenic mice and Swiss-TO NIH mice.
- Mice with diabetes induced by multiple low dose STZ, and non-diabetic controls
- Rodent BRIN-BD11 and Alpha-TC 1.9 cell lines

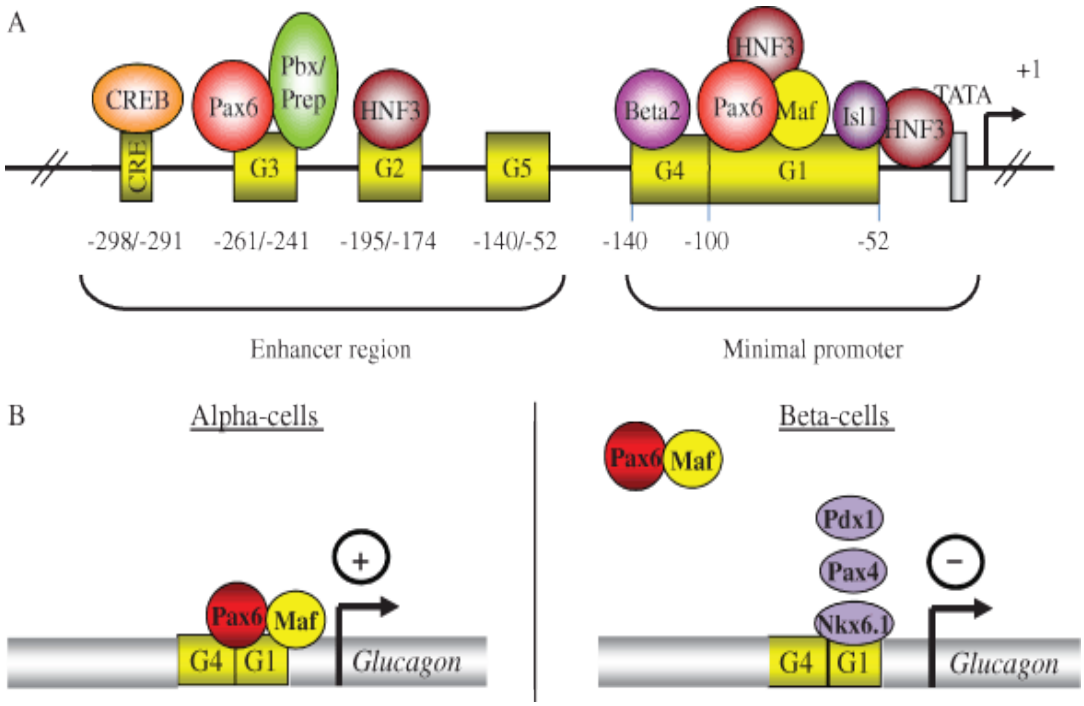
Histological & functional studies used to evaluate:

- Diabetes associated alterations in islet alpha cells (proliferation and apoptosis)

- Transformation associated with co-expression of insulin within YFP labelled alpha cells.

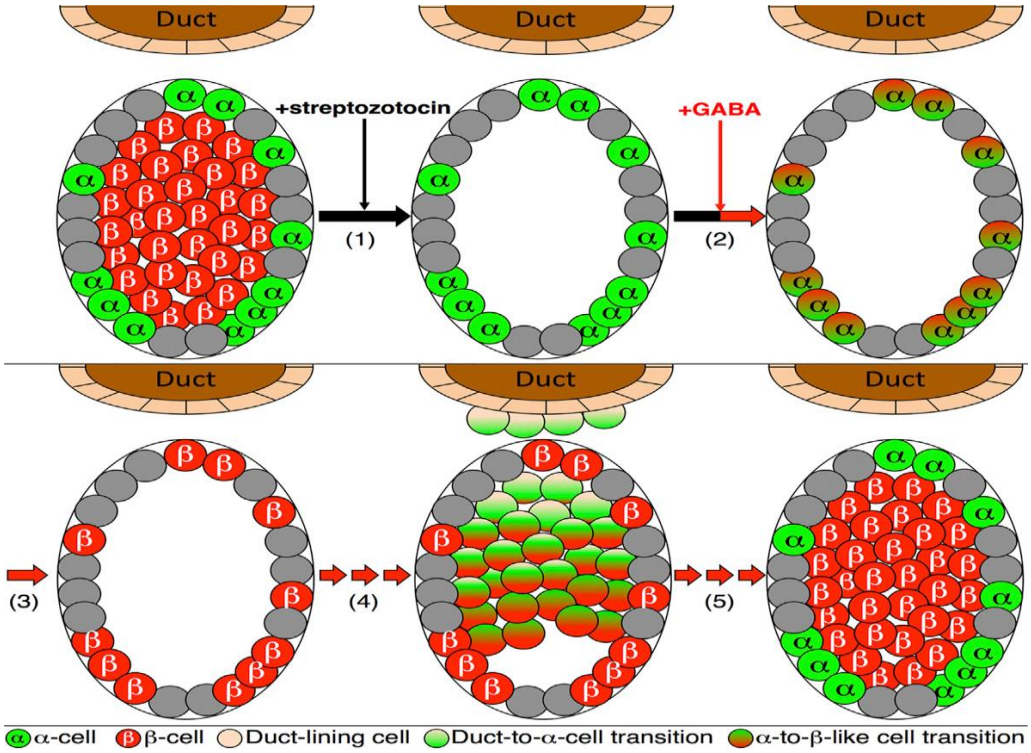
- Actions of therapeutic agents (GLP-1 mimetics, DPP-IV inhibitors, SGLT2 inhibitors, TZD, metformin, GABA, artemether, taurine, antidiabetic peptides, sulphonylureas, insulin, taurine, artemether) on islet morphology, reprogramming of alpha cells to functional beta cells and blood glucose homeostasis.

Figure 1.1 Schematic diagram of the differential regulation of glucagon gene expression within alpha and beta cells (Gosmain et al., 2013)



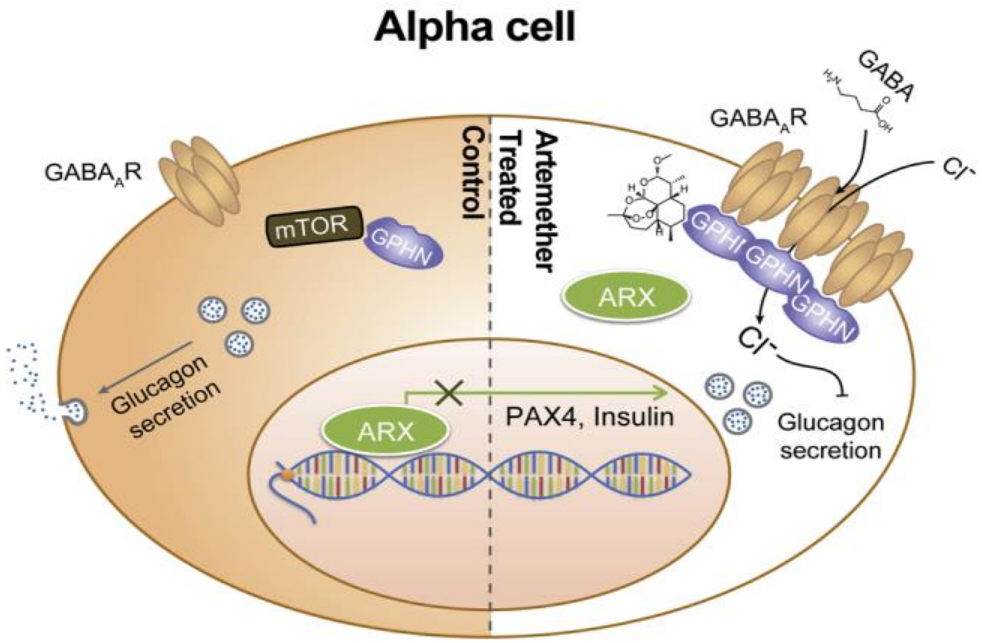
Schematic representation of alpha cell-specific glucagon promoter. (A) Structure of the rat glucagon gene promoter. It is formed by an enhancer region and a minimal promoter region that guides different types of transcription factors involved in glucagon gene expression. (B) Hypothetical representation of α- and β- cell-specific presence of transcription factors that either enhance or inhibits glucagon gene, respectively.

Figure 1.2 Schematic diagram of treatment with GABA during the beta cell loss and induction of alpha cell to beta cell transdifferentiation (Ben-Othman et al., 2017)



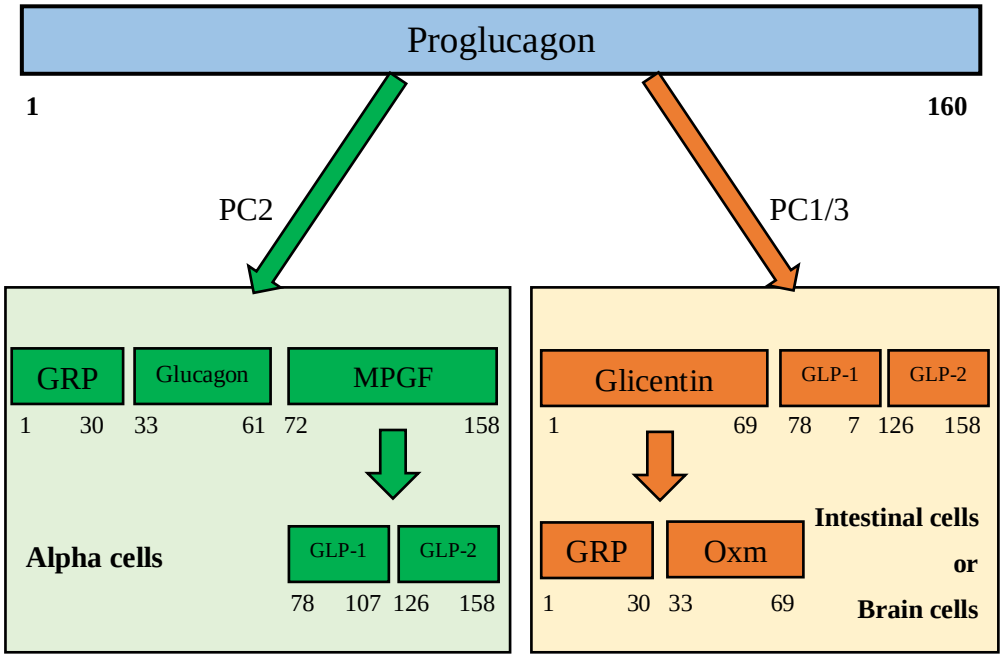
The schematic diagram shows GABA mediated alpha to beta cell transdifferentiation. The STZ mediated loss of beta cells was repaired by the administration of GABA by promoting alpha to beta cell transdifferentiation. Thus, the gradual conversion of alpha to beta cells eventually restored a significant population of central beta cells. In turn, the scarcity of alpha cells was balanced by the continuous transition of duct lining cells to alpha cells.

Figure 1.3 Schematic diagram of the artemether regulation of GABA receptor machinery to induce alpha cell to beta cell transdifferentiation (Li et al., 2017)



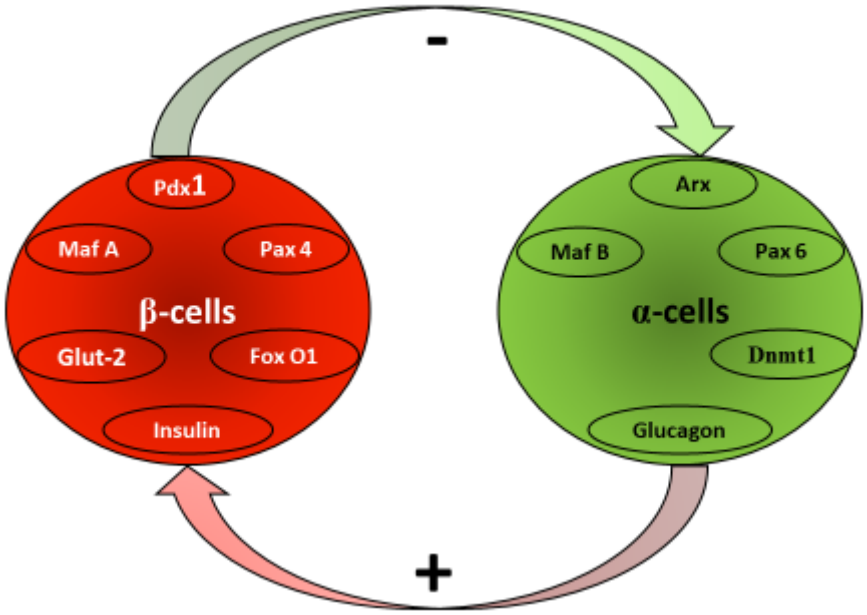
Representative image shows the transdifferentiation signaling mechanism within alpha cells. Artemether utilizes the stabilization of gephyrin, thereby mediated $GABA_A$ receptor signaling to repress Arx function. This, in turn, inhibits glucagon expression and expresses Pax 4 function to enhance insulin expression in alpha cells.

Figure 1.4 Schematic diagram of the post-translational processing pattern of proglucagon gene (Pocai, 2012)



The schematic representation shows the processing pattern of proglucagon found in different tissues. Proglucagon (160 a.a.) is a common precursor gene found in pancreatic alpha, intestinal L and brain cells. However, it is differentially processed in all these cells due to the action of different processing enzymes, namely PC2 and PC1/3. In alpha cells, PC2 generates GRPP, glucagon, and precursor MPGF for subsequent processing into GLP-1 & GLP-2. While in gut & brain cells, PC1/3 generates GLP-1, GLP-2 and precursor glicentin, which is subsequently, derives GRPP & oxyntomodulin (oxm). The number represents amino acid position in the peptide sequence (Pocai et al., 2012).

Figure 1.5 Schematic diagram of the alpha and beta cell-specific markers (+/- indicates treatments required to restore beta cell mass) (Van et al., 2015)



Schematic representation shows beta- and alpha- cell specific identity markers. Beta cells mainly possess insulin, Pax 4, Pdx1, Maf A, Glut 2 and FoxO1. While alpha cells contain glucagon, Arx, Maf B and Dnmt1. During diabetes, beta cells tend to dedifferentiate into alpha cells. Therefore, the therapeutic intervention that can lower the beta cells dedifferentiation and promote alpha to beta cell transdifferentiation could be a potential treatment for diabetes.

Chapter 2

General Materials and Methods

2.1 ACUTE ANIMAL STUDIES

2.1.1 Animals

All experiments were carried out under the UK Animals (Scientific Procedures) Act 1986 & EU Directive 2010/63EU and approved by the University of Ulster Animal Welfare and Ethical Review Body (AWERB). Animals were maintained in an environmentally controlled laboratory at 22 ± 2^0C with a 12 h dark and light cycle and given *ad libitum* access to standard rodent diet (10% fat, 30% protein and 60% carbohydrate; Trouw Nutrition, Northwich, UK) and drinking water.

2.1.2 Taurine experiments using Swiss TO NIH mice (Chapter 3)

Acute *in vivo* effects of taurine was studied using Swiss TO NIH male mice (10-12 weeks old) received from Envigo RMS (UK) Ltd, Oxford, UK. Mice were kept on observation with standard diet and normal drinking water at day -2. Mice were randomized to four groups. One group was injected with saline (0.09% NaCl; i.p.; day 1 to day 14). The second group was injected with multiple low dose streptozotocin to induce hyperglycaemia (50 mg/kg; i.p.; day 5 to day 9). The third group was supplemented with taurine (2% in drinking water; day 1 to day 14). The fourth group was injected with multiple low dose streptozotocin (50 mg/kg; i.p.; day 5 to day 9) as well as supplemented taurine (2% in drinking water; day 1 to day 14) and to study preventive effect of taurine on beta cell damage induced by streptozotocin. The study design is represented in Figure 2.4.

2.1.3 Animal experiments using $Glu^{CreERT2}$; ROSA26-eYPF mice (Chapter 5-9)

The studies were conducted in 15 week old $Glu^{CreERT2}$; ROSA26-eYPF transgenic mice maintained on C57BL/6 background (Figure 2.3). Mice were bred in-house at Coleraine using breeding pairs derived from the colony originally maintained at University of Cambridge, UK. The breeding pairs of the $Glu^{CreERT2}$; ROSA26-eYFP transgenic mice were donated by Professor Frank Reimann (University of Cambridge). Full details of the generation and characterisation of $Glu^{CreERT2}$;ROSA26-

eYFP mice are mentioned in Appendix B as well as described elsewhere (Campbell et al, 2019; Richards et al. 2014). The presence of Cre and ROSA26^{eYFP} transgenes were assessed by PCR genotyping as previously described (Quoix et al. 2007; Campbell et al, 2019). The primers used for this genotyping study are listed in Table 2.6. In this study, all Glu^{CreERT2};ROSA26-eYFP mice were intraperitoneal injected with 100 µl of tamoxifen at concentration of 7 mg/mouse (Stock was prepared by dissolving tamoxifen at 25 mg/ml in filtered corn oil). Following tamoxifen administration, multiple low dose streptozotocin (50 mg/kg body weight, i.p.; n=6) in 0.1 M sodium citrate buffer (pH 4.5) or saline vehicle (0.9% w/v NaCl, i.p.; n=6) was injected daily over a period of 5 days to induce insulin-deficient diabetes (Vasu et al., 2014a). Groups of mice (n=5 to 7) then received once or twice daily administration of treatment for 10 days. The entire treatment pattern for Chapter 5 to Chapter 9 are illustrated in Figure 2.6 to Figure 2.10. The body weight, cumulative food and fluid intake as well as circulating glucose levels were assessed regularly. At the end of the treatment period, non-fasting plasma insulin and glucagon concentrations were determined. At termination, pancreatic tissues were excised, divided as per head and tail, and processed for determination of pancreatic hormone content following tissue lysis using hand held homogeniser with acid-ethanol solution [1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% (v/v) H_20].

2.1.4 Biochemical analyses (Chapter 3, 5-9)

Blood samples were collected from the tail of animal into ice-chilled heparin coated microcentrifuge tubes. Blood glucose was measured using a portable a Bayer Ascencia Counter blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK). For plasma insulin and glucagon, blood was collected to chilled fluoride/heparin coated micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged using a Beckman micro-centrifuge (Beckman Instruments, Galway, Ireland) for 10 min at 12,000 rpm. Plasma was extracted and stored at -20^{0}C, until required. Insulin and glucagon concentrations were subsequently assessed by an in-house insulin radioimmunoassay (Flatt & Bailey 1981) or commercially available glucagon ELISA kit (EZGLU-30K, Merck Millipore), respectively. Pancreatic glucagon content was

measured as per manufacture's instruction using commercially available glucagon RIA kit (GL-32K Merck Millipore).

2.1.5 Pancreatic immunohistochemistry (IHC) (Chapter 3, 5-9)

Pancreatic tissues fixation was carried out using 4% PFA. Fixed tissues were processed for antibody staining as described from our laboratory (Vasu et al., 2014a). Processed tissues were embedded in paraffin wax and sectioned (7 µm). Sections were blocked using 2% BSA and then incubated with respective primary antibodies overnight at 4^0C, and then appropriate fluorescent secondary antibodies (Table 2.1). To stain nuclei, a final incubation was carried out at 37^0C with 300 nM DAPI (Sigma-Aldrich, D9542). In addition, co-staining of mouse anti-insulin (1:1000; Abcam, ab6995) or guinea pig anti-glucagon (PCA2/4, 1:200; raised in-house) with rabbit anti-Ki-67 (1:200; Abcam ab15580) or TUNEL reaction mixture (Roche Diagnostics Ltd, UK) was used to assess beta-cell proliferation and apoptosis, respectively. To investigate beta cell lineage, co-staining of insulin or glucagon, as above, with rabbit anti-GFP (1:1000; Abcam, ab5450) was employed. Imaging was carried out using an Olympus fluorescent microscope (Olympus system microscope, model BX51) fitted with DAPI (350 nm) FITC (488 nm) and TRITC (594 nm) filters and a DP70 camera adapter system. Cell^F imaging and ImageJ software were used to assess islet area, beta-cell area, alpha-cell area. ImageJ software was employed to evaluate beta- and alpha-cell proliferation and apoptosis, as well as GFP co-expression with either insulin or glucagon positive cells. All counts were determined in a blinded manner with ≥70 to 100 islets analysed per treatment group.

2.1.6 Iodination of insulin for RIA (Chapter 3, 5-9)

According to optimized protocol at Diabetes Research Lab (UUC), bovine insulin was iodinated. Using dichloromethane solvent, iodogen (1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenylglycoluril) was dissolved to prepare solution of concentration of 100 µg/ml. An aliquot of iodogen solution (100 µl) was transferred into a series of clean eppendorfs followed formation of uniform layer of iodogen at the bottom by drying

iodogen drop in fume hood. This iodogen coating served as a catalyst for the iodination reaction. The bovine insulin stock (1 mg/ml) was prepared using 10 mM HCl, followed by 1:8 dilution at 125 µg/ml using 500 mM phosphate buffer (pH 7.4). Later, bovine insulin (20 µl) and sodium iodide (5 µl) ($Na^{125}I$ 100 mCi/ml stock, Perkin Elmer, Cambridge, UK) were mixed together and transferred into iodogen coated tube. The tube was incubated on ice-chilled box for 15 min with gentle tapping at bottom for proper agitation at every 2-3 min. Finally, the reaction was terminated by removing reaction mixture to fresh Eppendorf. The reaction tube was rinsed with 500µl of 50mM sodium phosphate buffer and mixed with fresh reaction mixture tube.

For the purification of ^{125}I-insulin, the reaction mixture was run on a RP-HPLC machine at a flow rate of 1.0 ml/min using a Vydac C- 8 (250 x 4.6 mm) analytical column, while 0.12% (v/v) TFA/water used as a solvent. Over 60 min, acetonitrile concertation was raised from 0 to 56%, while 70% for 5 min. Using a fraction collector (Frac-100, LKB), fractions were acquired per min over 67 min run of HPLC (Figure 2.1). To measure label counts on a gamma counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter, from each fraction 5 µl label was added to LP-3 tubes. The fractions with maximum counts were chosen and mixed with 1 ml of 40 mM sodium phosphate buffer (pH 7.4) containing 1 g/100 ml BSA and 0.02 g/100 ml thimerosal. Finally, antibody binding ability of fractions was confirmed using various antibody dilutions (1:25,000-1:45,000) followed by mixing of fractions showing same good biding capacity. The fractions were stored at 4^0C until further use in RIA.

2.1.7 Modified dextran-coated charcoal insulin RIA (Chapter 3, 5-9)

As described by Flatt and Bailey (1981), a modified dextran-coated charcoal radioimmunoassay is a robust method for detecting concentration of insulin in cells, tissues or plasma. In this method, rat insulin standard, ^{125}I-labelled bovine insulin, and using guinea pig anti-porcine antibody were employed to determining the concentration of unknown insulin in samples. Stock RIA buffer of pH 7.4 was a mixture of disodium hydrogen orthophosphate (Base; 40 mM) constituted with 0.3% (w/v) NaCl and 0.02% (w/v) thimerosal), and sodium dihydrogen orthophosphate (Acid; 40 mM). For standard preparation, a serial dilution (0.039 to 20 ng/ml) of rat

insulin standard 40 ng/tube (100 µl) was carried out using working RIA buffer. For antibody preparation, dilution (1:25000 to 1:45000) of guinea pig anti-porcine antibody was carried out to acquire 40% binding. In LP-3 tubes, 200 µl of each unknown samples and standards were transferred in duplicates and triplicates respectively followed by addition of 100 µl of diluted antibody. A control tube (antibody plus assay buffer), a tube for non-specific binding (only assay buffer) and a tube for total counts (no reagents) were prepared. The ^{125}I-labeled tracer solution was prepared in working RIA buffer to obtain 10,000 counts/min/100 µl of solution. Addition of 100 µl of tracer to each tube was carried out followed by incubation for 48 h at 4^0C. Following 48 h incubation, for distinct separation of bound and unbound ^{125}I-labelled insulin, we used 5% charcoal solution coated with dextran T-70 were prepared and diluted (1:5) using stock RIA buffer. The addition of 1 ml of activated charcoal to each tube (except the total tubes) was carried out followed by incubation at 4^0C for 20 min. After incubation, tubes were centrifuged 2500 rpm for 20 min at 4^0C in a Sorvall centrifuge followed by removal of supernatant. Finally, radiation of the residual unbound (free) ^{125}I-labelled insulin bound to charcoal precipitate was measured with a gamma counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter). Counts attached to antibody (total counts minus counts bound to charcoal) were inversely proportional to the quantity of insulin exist in standards or unknown samples. As it is shown in Figure 2.2, the insulin present in the unknown samples was measured using a rat insulin standard curve.

2.1.8 Measurement of pancreatic glucagon content (Chapter 5-9)

Pancreatic tissue from Glu^{CreERT2};ROSA26e-YFP mice treated with saline, streptozotocin and treatments mentioned in Chapters 5-9 were extracted using acid-ethanol method. Pancreatic glucagon content was measured using Glucagon RIA kit (250-tubes GL-32K, Millipore, MA, USA) according to manufacturer's instruction. The concentration of glucagon in the sample was estimated using standard curve of known glucagon standards (concentration ranging from 15.625 pg/ml– 125 pg/ml). 125I-Glucagon tracer was used as label (specific activity 603 µCi/µg; 111 kBq).

2.1.9 Measurement of cellular and plasma glucagon content (Chapter 4, 5-9)

Plasma glucagon assay was performed according to manufacturer's protocol. About 150 µl of plasma samples from $Glu^{CreERT2}$;ROSA26e-YFP mice studies (Chapter 5-9) and cellular extracts from alpha TC 1.9 cells (Chapter 4) were aliquoted into fresh eppendorf tubes. Then acetonitrile (225 µl) was added to sample tube and mixed vigorously for 5 sec followed by incubation at R.T. for 30 min. After incubation, tubes were centrifuged at 17,000 x g for 5 min. Later, the supernatant (300 µl) was transferred to new tube and dried using Speed Vac concentrator at 80^0C for 2 h and stored at 4^0C until ready for assay use. Glucagon ELISA assay 96-well plate was washed and decanted three times with diluted wash buffer (300 µl) prior to use. The assay buffer were added to blank (30 µl) remaining wells (20 µl) respectively. The glucagon standards (10 µl) were serially added to respective wells. From each QC1 and QC2 sample 10 µl volume was also added. The unknown samples (10 µl) were added to rest of wells followed by addition of antibody of (20 µl) mixture to each well. Plates were covered with sealer and incubated for 48 h at 4^0C. After incubation, reaction solution were removed and wells were washed with and decanted three times with diluted wash buffer (300 µl) prior to use. Then enzyme solution (100 µl) was added to each well. Plates were covered with sealer and incubated for 30 min at R.T. on plate shaker. Then reaction mixture was decanted and wells were washed with and decanted six times with diluted wash buffer (300 µl) prior to use. Then substrate solution (100 µl) was added to each well followed by gentle shaking for ~1 min. Finally, relative light units were measured immediately at ~425 nm using luminometer plate reader. Unknown plasma glucagon concentration was determined using standard glucagon curve.

2.1.10 Measurement of pancreatic GLP-1 content (Chapter 6)

Pancreatic tissue from $Glu^{CreERT2}$;ROSA26e-YFP mice treated with saline or multiple low dose of streptozotocin (Chapter 6) were extracted using acid-ethanol method. Pancreatic GLP-1 content was measured using GLP-1 ELISA kit Active (EGLP-35K, Millipore, MA, USA) according to manufacturer's instruction. The amount of GLP-1

in the sample was estimated using reference curve of known GLP-1 standards (concentration ranging from 2 pM/ml– 100 pM/ml)

2.2 *IN VITRO* STUDIES

2.2.1 Culture of BRIN-BD11 cells (Chapter 3)

The BRIN-BD11 rat clonal β-cell line exhibits glucose-dependent insulin secretion in response to various modulators and its establishment was described previously. BRIN-BD11 cells were grown at 37^0C in an atmosphere of 5% CO_2 and 95% air in tissue culture flasks (Orange Scientific, Braine-l'Alleud, Belgium) containing RPMI-1640 tissue culture medium supplemented with 11.1 mM glucose, 10% (v/v) foetal calf serum, antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin). Cells seeding carried out in 24-multiwell plates and incubated overnight at 37^0C to anchor cells at bottom of the flask.

2.2.2 Culture of alpha TC 1.9 cells (Chapter 4)

Alpha TC.19 mouse clonal cells were grown at 37^0C in an atmosphere of 5% CO_2 and 95% air in DMEM tissue culture medium containing 2 mM L-glutamine, sodium pyruvate, high glucose supplemented with 10% (v/v) fetal calf serum, antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin) and 1.1 mM glucose as earlier described. Cells were seeded in 12-well plate and 6-well plate to perform immunocytochemical staining and PCR experiments respectively. The cells were allowed to attach for overnight at 37^0C. After attachment, the culture medium was replaced with a range of agents (Table 2.5) and incubated for 72 h at 37^0C. Schematic outline of experiment using α-TC 1.9 cells has shown in Figure 2.5.

2.2.3 Immunocytochemical staining (ICC) (Chapter 3, 4)

A glass coverslip of size 16 mm was sterilized using absolute ethanol. After air-drying in laminar airflow cabinet, coverslips were put in each of 12-well plate. BRIN-BD11 cells (Chapter 3) or alpha-TC 1.9 cells (Chapter 4) were cultured on the coverslip with a density of 20,000 cells per well (n=3). The RPMI (BRIN BD11 cells) or DMEM (alpha-TC 1.9 cells) media supplemented with treatments was added to each well (Table 2.4 and Table 2.5) and incubated for 37^0C for 12 h or 72 h respectively. After incubation, cells were washed using PBS and fixed in 4% PFA for 30 min at room temperature. For antigen retrieval, pre-warmed-citrate buffer was added to each well and incubated for 20 min at 90^0C. A blocking buffer containing 2% BSA was added to each well to avoid non-specific binding of antibodies. Cells were incubated overnight with primary antibody (Table 2.1). After washing with PBS buffer, cells were incubated with secondary antibody for 1hr at 37^0C (Table 2.1). For staining nucleus, DAPI was added to cells and incubated for 15 min at 37^0C. Finally, cells were mounted with mounting medium and placed on a glass slide. The immunofluorescence was determined using a fluorescent microscope (Olympus System Microscope BX51, Olympus instruments, UK) equipped with FITC filter (488 nm) and TRITC filter (594 nm). Immunofluorescence images were captured using a DP70 camera adapter system and analysis was carried out using Cell^F imaging software (Olympus System Microscope BX51, Olympus instruments, UK).

2.2.4 Cell viability assessment using MTT assay (Chapter 4)

To study gene expression, alpha TC1.9 cells (Chapter 4) were cultured in 24-well plates with a density of 150,000 cells per well and allowed to anchor for 4-6 h. After cellular attachment, DMEM media was supplemented with various treatments (Table Table 2.5) and incubated at 37^0C for 72 h respectively. After the incubation, cells were washed with HBSS solution. Each well was added with MTT solution (300 µl per well; 0.5 mg/ml; Thiazolyl blue tetrazolium bromide; Sigma) and incubated for 1 h at 37^0C. For this, MTT stock (25 ml) was prepared by dissolving MTT (12.5 mg) into KRBB buffer (2.5 ml) without glucose and then diluted to KRBB with glucose (22.5 ml). After incubation DMSO (250 µL=l) was added to each well and sample (100 µl) was transferred to fresh 96-well transparent plate. The fluorescent intensity was measured at 570 nm using fluorimeter equipped with Softmax software.

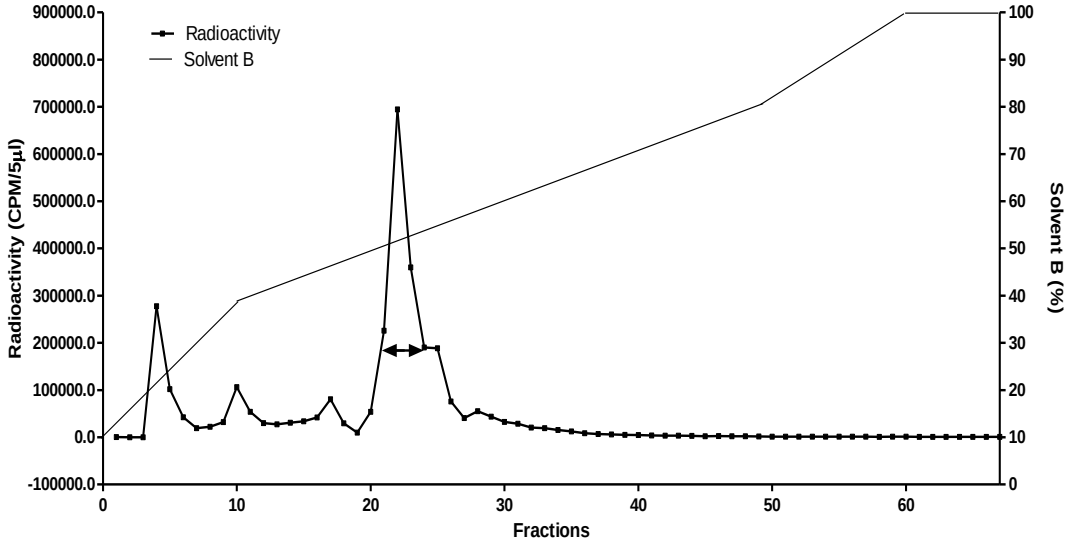
2.2.5 Polymerase chain reaction (PCR) (Chapter 3, 4)

To study gene expression, BRIN-BD11 cells (Chapter 3) or alpha TC1.9 cells (Chapter 4) were cultured in 6-well plates with a density of 10^6 cells per well and allowed to anchor for 4-6 h. After cellular attachment, RPMI (BRIN BD11 cells) or DMEM (alpha-TC 1.9 cells) media was supplemented with various treatments (Table 2.4 and Table 2.5) and incubated at 37^0C for 12 h or 72 h respectively. After the incubation, cells were washed with HBSS solution and lysed using Trizol. Lysed cells were centrifuged at 12,000 rpm for 10 min at 4^0C. The supernatant was collected and stored at -20^0C. Using chloroform-isopropanol, m-RNA was extracted and quantified at 260 nm absorbance using Nanodrop (Labtech Internation). According to the manufacturer's instructions, superscript II reverse transcriptase-RNase H kit (Invitrogen, UK) was used to prepare cDNA at 42^0C for 50 min. The reaction mixture (20 µl) contains 3 µg of total RNA, oligodT (0.5 mg), dNTP (10 mM) and Superscript II reverse transcriptase (200 U). RT-PCR was used to perform amplification of c-DNA and quantifying gene expression of various genes shown in Table 2.2 (Chapter 3) and Table 2.3 (Chapter 4). The reaction mixture was prepared using SYBR green (12.5 µl; LightCycler 480 SYBR Green I Master), primers (1 µl; forward and reverse, Table 2.2 & Table 2.3, Invitrogen, UK), respective cDNA (1 µl) and nuclease-free water (9.5 µl). The c-DNA was denaturated initially at 95^0C for 5 min and finally at 95^0C for 30 s. It was annealed and extended at 58^0C for 30 s and at 72^0C for 30 s respectively for 34 cycles. At 60^0C - 90^0C temperature, the melting curve was obtained. MiniOpticon two-colour real-time PCR detection system (BioRad, UK) was used to obtain real-time result. Data evaluation was performed using the ΔΔCt method in comparison with ACTB expression. Primers used for *in vitro* PCR studies using rodent BRIN-BD11 cells (Chapter 3) and mouse alpha TC1.9 cells (Chapter 4) are listed in Table 2.2 and 2.3 respectively.

2.3 STATISTICAL ANALYSIS

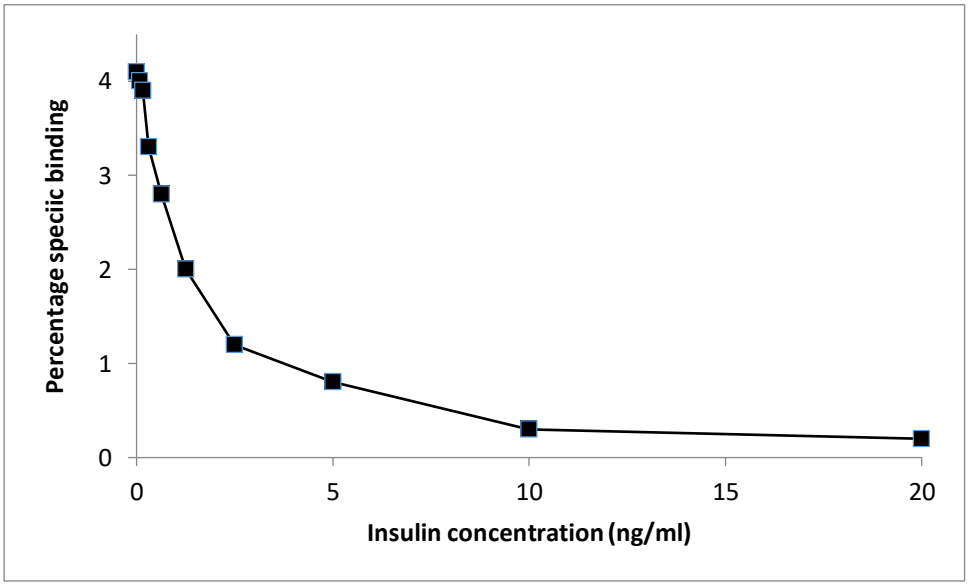
Statistical analyses were performed using GraphPad PRISM software (Version 5.0). Values are expressed as mean ± S.E.M. Comparative analyses between groups were carried out using a One-way ANOVA with Bonferroni's post hoc test or student's unpaired t-test, as appropriate. The difference between groups was considered significant if $P<0.05$.

Figure 2.1 Fractioning of iodinated bovine insulin using HPLC



Using reverse-phase HPLC, iodinated ^{125}I-bovine insulin was fractionated. To measure label counts on a gamma counter (Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter, 5 µl of each fraction was added to LP-3 tubes. The fractions with maximum counts were chosen between 20 to 26 min.

Figure 2.2 Typical standard curve of rat insulin standards for insulin immunoassay



Standard curve of rat insulin standard ranging concentration from 0.039 ng/ml to 20 ng/ml.

Figure 2.3 Representative image of Glu^{CreERT2}; ROSA26e-YFP mice (non-diabetic)

Representative image shows two months old Glu^{CreERT2}; ROSA26e-YFP mice bred in-house at Coleraine using breeding pairs derived from the colony originally maintained at University of Cambridge, UK.

Figure 2.4: Schematic diagram of the therapeutic agents and design of animal study using Swiss-TO NIH mice (Chapter 3)

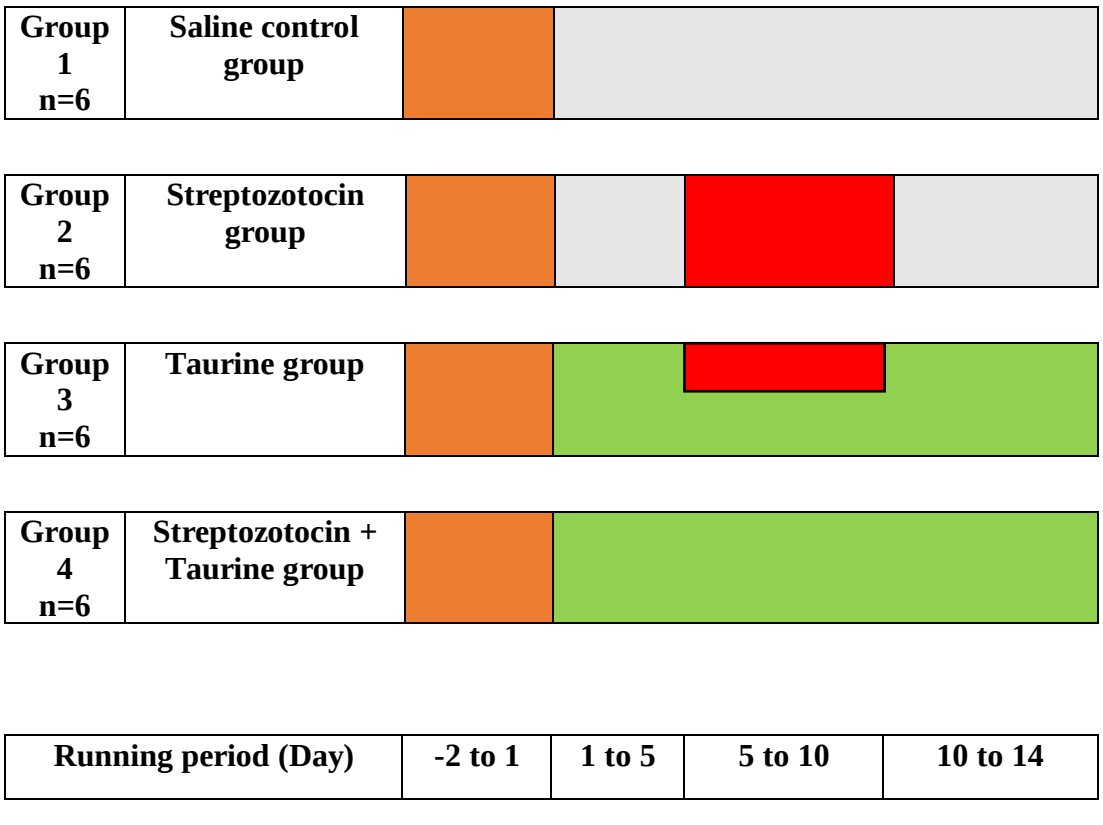


Figure 2.5: Schematic diagram of the therapeutic agents and design of cell culture study using alpha TC 1.9 cells (Chapter 4)

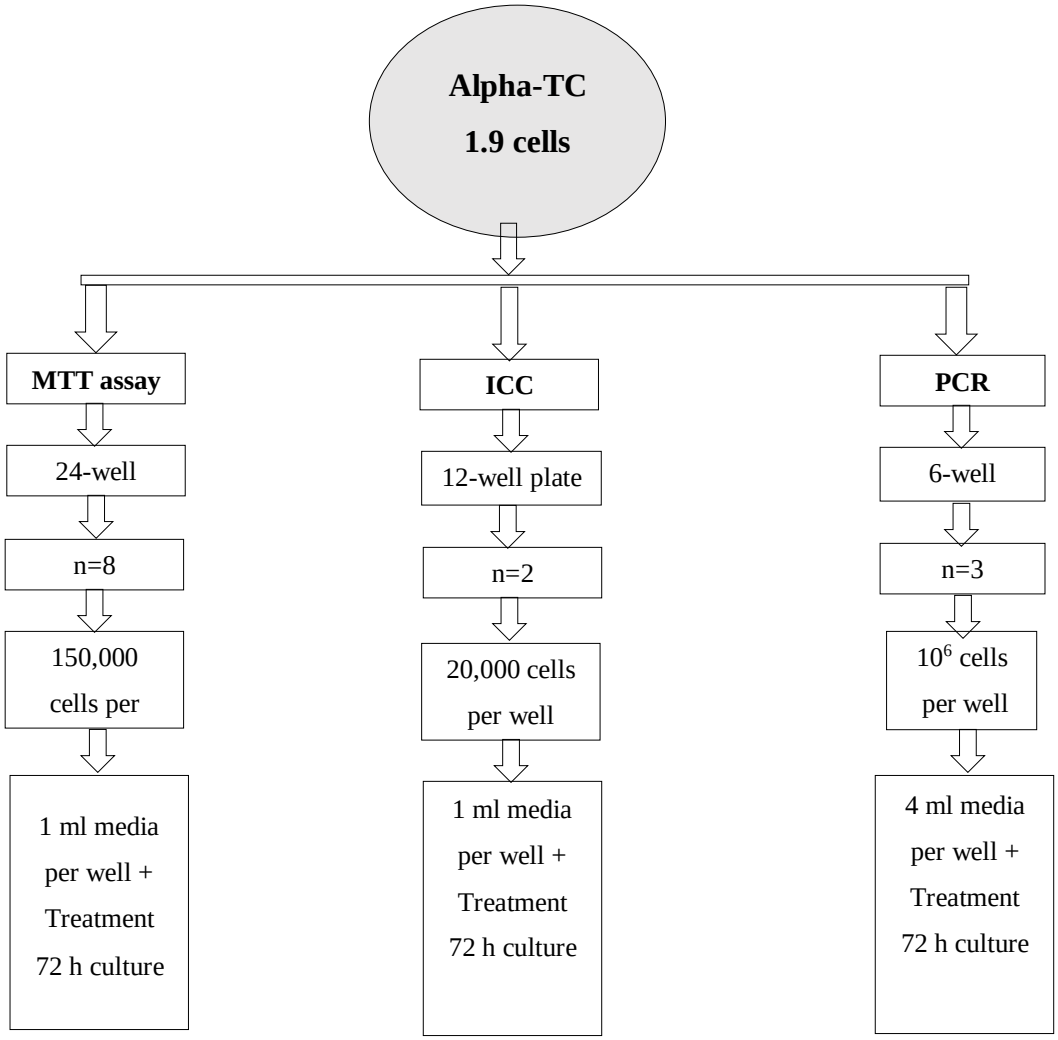


Figure 2.6: Schematic diagram of the therapeutic agents and design of animal study using Glu^{CreERT2}; ROSA26e-YFP mice (Chapter 5)

Group 1	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Saline control		
Group 2	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin control		
Group 3	n = 5	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Taurine (5 % in drinking water)
Group 4	n = 5	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Artemether (100 mg/kg; oral; once a daily)
Running period (Day)		-16	-15 to -14	-13 to -9	-8 to 0	1-10

Tamoxifen administration period

Observation period

Saline administration period

Multiple low dose of streptozotocin administration period (50 mg/kg/day; ip)

Treatment administration period

Figure 2.7: Schematic diagram of the therapeutic agents and design of animal study using Glu^{CreERT2}; ROSA26e-YFP mice (Chapter 6)

Group 1	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Saline control		
Group 2	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin control		
Group 3	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Liraglutide (25 nmol/kg, i.p, twice a daily)
Group 4	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Sitagliptin (50 mg/kg, oral, once a daily)
Group 5	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Dapagliflozin (1 mg/kg, oral, once a daily)

Running period (Day)	-16	-15 to -14	-13 to -9	-8 to 0	1-10

Tamoxifen administration period

Observation period

Saline administration period

Multiple low dose of streptozotocin administration period (50 mg/kg/day; ip)

Treatment administration period

Figure 2.8: Schematic diagram of the therapeutic agents and design of animal study using Glu^{CreERT2}; ROSA26e-YFP mice (Chapter 7)

Group 1	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Saline control		
Group 2	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin control		
Group 3	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Insulin (1 U/kg; i.p.; thrice a daily)
Group 4	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	GABA (10 mg/kg; i.p.; once a daily)
Group 5	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Nicotinamide (150 mg/kg, ip; once a daily)

Running period (Day)	-12	-11 to -10	-9 to -5	-4 to 0	1 to 10

Tamoxifen administration period

Observation period

Saline administration period

Multiple low dose of streptozotocin administration period (50 mg/kg/day; ip)

Treatment administration period

Figure 2.9: Schematic diagram of the therapeutic agents and design of animal study using Glu^{CreERT2}; ROSA26e-YFP mice (Chapter 8)

Group 1	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Saline control		
Group 2	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin control		
Group 3	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Rosiglitazone (10 mg/kg; oral, once a daily)
Group 4	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Tolbutamide (20 mg/kg; oral, once a daily)
Group 5	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Metformin (100 mg/kg; oral, once a daily)

Running period (Day)	-10	-9	-8 to -4	-3 to 0	1 to 10

Tamoxifen administration period

Observation period

Saline administration period

Multiple low dose of streptozotocin administration period (50 mg/kg/day; ip)

Treatment administration period

Figure 2.10: Schematic diagram of the therapeutic agents and design of animal study using Glu^{CreERT2}; ROSA26e-YFP mice (Chapter 9)

Group 1	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Saline control		
Group 2	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin control		
Group 3	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	D-Ser 2 Oxyntomodulin GluPal (50 nmol/kg; i.p; twice a day)
Group 4	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	D-Ala2 GIP (50 nmol/kg; i.p; twice a day)
Group 5	n = 6	Tamoxifen Injection (7 mg/mouse)	Observation	Streptozotocin	Hyperglycaemia observation	Xenin-25 GluPal (50 nmol/kg; i.p; twice a day)

Running period (Day)	-10	-9	-8 to -4	-3 to 0	1 to 10

Tamoxifen administration period

Observation period

Saline administration period

Multiple low dose of streptozotocin administration period (50 mg/kg/day; ip)

Treatment administration period

Table 2.1: List of the antibodies and their dilutions for IHC and ICC studies in islets or cells (Chapter 3-9)

Primary antibody					
Target	**Host**	**Clonality**	**Immunogen**	**Dilution**	**Source**
Insulin	Mouse	Monoclonal	Human insulin	1/1000	Abcam, ab6995
Insulin	Guinea pig	Polyclonal	Human insulin	1/200	Abcam, ab7842
Glucagon	Guinea pig	Polyclonal	Human insulin	1/200	Raised in-house PCA2/4
Ki67	Rabbit	Polyclonal	Synthetic peptide corresponding to Human Ki67	1/200	Abcam, ab15580
GLP-1	Mouse	Monoclonal	Human	1/200	Santacruz 65388
Arx	Rabbit	Polyclonal	Recombinant fragment corresponding to Human Arx aa 381-460	1/200	Abcam, ab235060
Pdx1	Guinea pig	Polyclonal	Recombinant fusion protein containing N-terminal sequence from mouse PDX1	1/200	Abcam, ab 47308
GFP	Rabbit	Polyclonal	Recombinant full length protein corresponding to GFP	1/1000	Abcam, ab 6556

PC1/3	Mouse	Monoclonal	Human	1/100	Abcam, ab55543
Pax6	Rabbit	Polyclonal	Synthetic peptide corresponding to Mouse PAX6 aa 267-285	1/200	Abcam, ab5790
Secondary antibody					
Target	**Host**	**Reactivity**	**Immunogen**	**Dilution**	**Source**
IgG	Goat	Mouse	Gamma Immunoglobins	1/400	Alexa Fluor 488, Invitrogen, UK
IgG	Goat	Mouse	Gamma Immunoglobins	1/400	Alexa Fluor 594, Invitrogen, UK
IgG	Goat	Guinea pig	Gamma Immunoglobins	1/400	Alexa Fluor 488, Invitrogen, UK
IgG	Goat	Guinea pig	Gamma Immunoglobins	1/400	Alexa Fluor 594, Invitrogen, UK
IgG	Goat	Rabbit	Recombinant full-length protein	1/400	Alexa Fluor 488, Invitrogen, UK
IgG	Donkey	Rabbit	Gamma Immunoglobins	1/1000	Alexa Fluor 594, Invitrogen, UK

Table 2.2: List of the primers and their sequence for gene expression studies (Rat primers; Chapter 3)

Sr.no	Gene	Primer title	Primer sequence
Proliferation genes			
1	Norvegicus neurogenin 3	NEUROG 3 F	GCAGAGCAGATAAAGCGTGC
		NEUROG 3 R	TCGCCTGGAGTAAATTGCGT
2	Integrin β1	ITGB F	CTCAATGATGCGTGCGGAAG
		ITGB R	AAGGCGTTGGGAGTTACAGG
Apoptosis genes			
3	B-cell CLL/lymphoma 2	BCL-2 F	TGTGGAGAGCGTCAACCGGGAG
		BCL-2 R	ATCAAACAGAGGCCGCATGCTG
4	BCL2-associated X protein	BAX F	TGGACTTCCTCCGGGAGCGG
		BAX R	CTGGGGGCCTCAGCCCATCT
5	Nuclear factor of kappa light polypeptide gene enhancer in β - cells 1	NFKB1 F	CCTGGATGACTCTTGGGAAA
		NFKB1 R	TCAGCCAGCTGTTTCATGTC
Anti-oxidant defence genes			
6	Superoxide dismutase 1 (soluble)	SOD1 F	ACGGGGTGCTGGTTTGCGTC
		SOD1 R	TTCAGCACGCACACGGCCTT
7	Superoxide dismutase 2, mitochondrial	SOD2 F	TCCCAAGGGAAACACTCGGCTTT
		SOD2 R	AAACCACTGGGTGACATCTACCAGA
8	Catalase	CAT F	CGTGCTGAATGAGGAACAGA

		CAT R	AGTCAGGGTGGACCTCAGTG
9	Glutathione peroxidase 1	GPX-1 F	TCCCTGCGGGGCAAGGTACTAC
		GPX-1 R	TTCGTTCTTGGCGTTCTCCTGATG
Transdifferentiation genes			
10	Glucagon	GCG F	CTCAGCTCAGTCCCACAAGG
		GCG R	AGCTGCCTTGTACCAGCATT
11	Paired box 6	PAX6 F	ACCTCCTCGTACTCCTGCAT
		PAX6 R	CCCATGGGCTGACTGTTCAT
12	Aristaless related homeobox	ARX F	CTCTTCCGTTGGCTGTCCAT
		ARX R	GGAGGAAGAGAAGGTTGGGC
13	Insulin	INS F	TACCAGCATCTGCTCCCTCT
		INS R	TGCTGGTTCAAGGGCTTTAT
14	Pancreatic & duodenal homeobox 1	Pdx1 F	GAACGCTGGAACAGGGAAGT
		Pdx1 R	CCCCAGTCTCGGTTCCATTC
15	Glucose transporter 2	GLUT 2 F	CATTCGGAACAGGACCTGGAT
		GLUT 2 R	AGGTGCATTGATCACACCGA
16	β-actin	ACTB F	CCACCATGTACCCAGGCATT
		ACTB R	CGGACTCATCGTACTCCTGC

Table 2.3: List of the primers and their sequence for gene expression studies (Mouse primers; Chapter 4)

Sr.no	Transdifferentiation genes	Primer title	Primer sequence
1	Glucagon	GCG F	TTGAGAGGCATGCTGAAGGG
		GCG R	TCTCGTCAGAGAAGGAGCCA
2	Insulin	INS F	TCAACATGGCCCTGTGGAT
		INS R	AAAGGTGCTGCTTGAAAAAGC
3	Arx	ARX F	TTTTCTAGGAGCAGCGGTGT
		ARX R	GACAGATTGACAGGCGGACA
4	PC1/3	PC1/3 F	TCTGGTTGTCTGGACCTCTGAGT
		PC1/3 R	CATCAAGCCTGCCCCATTCTTT
5	Pax6	Pax6 F	AGGTCAGGCTTCGCTAATGG
		Pax6 R	AGAGTTTTCTCCACGGACGC
6	β-actin	ACTB F	AGGAGTACGATGAGTCCGGC
		ACTB R	GCAGCTCAGTAACAGTCCGC

Table 2.4: List of the therapeutic agents used for cell culture studies (Chapter 3)

Sr.no.	Agents	Concentrations used
1	RPMI media control	-
2	Streptozotocin	10 mM
3	Taurine	20 mM
4	Streptozotocin + Taurine	10 mM + 20 mM

Table 2.5: List of the therapeutic agents used for cell culture studies (Chapter 4)

Sr.no.	Agents	Concentrations used
1	DMEM media (control)	-
2	Artemether	10 µM
3	Taurine	20 mM
4	GABA	10 mM
5	Exendin	10^{-9} M
6	Sitagliptin	25 µM

Table 2.6: List of the primers and their sequence for PCR genotyping of $Glu^{CreERT2}$;ROSA26-eYFP transgenic mice (Mouse primers; Chapter 5-9)

Sr.no	Genotyping primers	Primer title	Primer sequence
1	Housekeeping control Beta catenin (220BP)	Forward	AAGGTAGAGTGATGAAAGTTGTT
		Reverse	CACCATGTCCTCTGTCTATTC
2	Cre lines iCre002/003 fragment (537BP)	Forward	GACAGGCAGGCCTTCTCTGAA
		Reverse	CTTCTCCACACCAGCTGTGGA
3	GLUCre-ERT2 (759BP)	Forward	CCACCTTCTAGAATGTGCCTG
		Reverse	CATCTGCATGCAAAGCAATATAGC
4	EYFP (442BP)	Forward	GACGTAAACGGCCACAAGTT
		Reverse	GGATCTTGAAGTTCGCCTTG

Chapter 3

Beta-Cells Protective and Regenerative Ability of Taurine in Streptozotocin Induced Diabetes

3.1 SUMMARY

Taurine is a plentiful free semi-essential amino acid found in many parts of the mammalian body. Earlier taurine has been shown its beneficial effects on the management of obesity and hyperglycaemia. However, its exact effects on islet morphology and beta cell regeneration are yet unclear. Therefore, in the present chapter, we aim to determine the therapeutic action of taurine using BRIN-BD11 clonal beta cells and Swiss TO NIH diabetic mice.

The 2% taurine supplementation in the drinking water of diabetic mice showed less effect on body parameters including blood glucose but showed slightly beneficial effects on pancreatic insulin. The islet morphology was disrupted by streptozotocin but improved with taurine treatment. This was associated with increased beta cell area and declined alpha cell area. The involvement of taurine in the enhancement of beta cell proliferation was confirmed by hormone as well as gene expression *in vitro* studies. Interestingly, similar effects were observed in taurine supplemented non-diabetic controls. Moreover, the same criteria used to study the role of taurine in STZ induced beta cell apoptosis and oxidative stress. Interestingly, taurine offered protection to the beta cells. Furthermore, STZ was induced dedifferentiation of beta cells while taurine prevented this detrimental transition. Indeed, taurine encouraged the beta cells to maintain their identity by promoting the gene of insulin and other beta cell marker.

Due to a lack of cell-lineage tracing mechanism in the current mice model, it is not clear whether the regenerated beta cells were from the sole supply of pre-existing beta cells or the contribution of alpha cells as well. Nevertheless, our findings from the current chapter suggest that taurine may be beneficial in the promotion of beta cell regeneration. In conclusion, taurine showed various morphological and gene expressing actions in the protection and restoration of beta cells.

3.2 INTRODUCTION

Taurine (2-aminoethylsulphonic acid) is a semi-essential, non-proteinic sulphur containing amino acid (Bustamante et al., 2001). In *Latin*, a bull is referred to as *taurus* so the compound derived from ox-bile was named taurine in 1827 by German researchers Tiedemann and Gmelin (Tiedemann et al., 2001). Due to its multi-beneficial properties, many scientists have referred taurine as a wonder molecule. Taurine plays a vital role in many fundamental biological processes and exerts a variety of physiological actions (Huxtable, 1992). Taurine is involved in the production of bile acid, antioxidation (Huxtable, 1992), osmolarity (Schaffer et al., 2000), membrane stabilization (Kulakowski et al., 1984), neurotransmission (Caletti et al., 2015), anti-inflammation and regulation of calcium homeostasis (Satoh, 1998; Palmi et al., 1999; Foos et al., 2002; Park et al., 2004; Lim et al., 2004).

Taurine is detected in animal brain and liver, synthesized by cysteine sulfinic acid pathway (Jacobsen et al., 1968; Huxtable, 1992). Taurine exerts its effect through GABA or glycine receptors and is transported by TauT transporter. Taurine is structurally similar to GABA and its analogues are homotaurine and hypotaurine (Gossai et al., 2009a, 2009b). They have been shown to exhibit several therapeutic benefits (Gossai et al., 2009a/2009b; Caletti et al., 2015). The capacity to synthesize taurine in animal cells is very low so mammals rely on supply of taurine in the diet. However, taurine concentration is also influenced by disease and age of the individual. Taurine intake is estimated to be approximately 40-400 mg per day. It has been found that taurine level is decreased in diabetes characterised due to low intestinal absorption rates and a high renal excretion compared to normal individuals. Normal taurine level in plasma is important for managing diabetes and its complications (Huxtable, 1992).

Recently, several animal studies have been shown that diabetes can be managed by maintaining appropriate taurine concentration (Brøns et al., 2004; Patel et al., 2015). Taurine supplementation has been found to ameliorate hyperglycaemia and improved glucose intolerance (Carneiro et al., 2009). In addition, some data showed taurine administration to improve hyperinsulinemia, insulin resistance and impaired insulin sensitivity in type 2 individuals (Patel et al., 2015). Taurine may protect beta cells via mitochondrial anti-oxidation or anti-inflammation (Lee et al., 2011). Taurine has also been shown to exert anti-obesity action by lowering cholesterol and enhancing

lipolysis in adipocytes (Santos-Silva et al., 2015). Moreover, taurine supplementation resulted in improve a diabetic-related complications such as retinopathy, neuropathy and nephropathy (Agca et al., 2014; Caletti et al., 2012/2015). In this study, we have studied the acute protective effect of taurine on streptozotocin-induced beta cell damage in NIH Swiss TO mice. Diabetes related changes were evaluated by histological and functional studies. Further, the *in vitro* effect of taurine was studied using cultured BRIN-BD11 cells.

3.3 MATERIALS AND METHODS

Materials and methods for this study have been discussed in Chapter 2.

3.4 RESULTS

3.4.1 *In vivo* effects of taurine on the body parameters

Body weight and cumulative food intake were found to be unchanged in taurine supplemented normal mice compared to saline treated controls. However, diabetic mice with or without taurine supplementation exhibited reduced both body weight (Figure 3.1A, B) and cumulative food intake (Figure 3.1C) compared to controls. Cumulative fluid intake was increased with all treatments but not significantly compared to saline treated controls (Figure 3.2A). Blood glucose (Figure 3.2B, 3.2C) and plasma insulin (Figure 3.2D) were found to be unchanged in non-diabetic mice supplemented with taurine. However, diabetic mice with or without taurine supplementation displayed significantly increased blood glucose levels ($p < 0.001$; Figure 3.2B & 3.2C) and reduced plasma insulin in compare to saline control ($p<0.001$; Figure 3.2D). Pancreatic insulin content was found to be significantly reduced in diabetic mice, but increased in taurine supplemented mice in comparison with non-diabetic mice (Figure 3.2E).

3.4.2 *In vivo* effects of taurine on islet morphology

Representative images (Figure 3.3A) are included showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). Islet area (Figure 3.3C), beta cell (Figure 3.4A) and alpha cell areas (Figure 3.4B) were similar in saline-treated and taurine supplemented non-diabetic mice. However in diabetic animals, streptozotocin significantly reduced islet area ($p<0.01$; Figure 3.3C) as well as beta cell area ($p<0.001$; Figure 3.4A). In addition, streptozotocin significantly augmented alpha cell area as compared to non-diabetic mice ($p<0.001$; Figure 3.4B). It was found that, as compared to diabetic control mice, taurine supplemented diabetic mice exhibited significantly elevated both islet area ($p<0.05$; Figure 3.3C) and beta cell area ($p<0.01$; Figure 3.4A). The alpha cell area ($p<0.05$; Figure 3.4B) as compared to saline control, was found to be significantly elevated in diabetic mice administered with taurine, while it was found be significantly reduced in comparison with streptozotocin-treated diabetic control. ($p<0.05$; Figure 3.4B). Taurine supplementation in non-diabetic mice also significantly ($p<0.05$ & $p<0.05$; Figure 3.3B) increased the number of islets per mm2 of the pancreas compared to both non-diabetic and diabetic controls. Streptozotocin reduced the number of islets per mm2 in the pancreas of diabetic mice (Figure 3.3B). While taurine supplemented diabetic mice showed no significant difference in a number of islets per mm^2 compared to non-diabetic controls, it markedly increased the count compared to diabetic mice (Figure 3.3B). Streptozotocin-treated diabetic mice exhibited a significant increase in small size islets ($p<0.01$; Figure 3.3D) with a significant decline in medium size islets ($p<0.01$; Figure 3.3D). A drastic loss in large size islets was observed after streptozotocin treatment (Figure 3.3D). When compared to saline controls, non-diabetic mice given taurine had significantly increased medium size islets with a reduction in large size islets ($p<0.05$; Figure 3.3D). Taurine increased proportion of small size islets ($p<0.05$ to $p<0.01$) with a significant decline in large size islets compared to non-diabetic controls with or without taurine supplementation ($p<0.5$ to $p<0.01$; Figure 3.3D). Taurine supplementation in diabetic mice also increased number of small size islets significantly compared to non-diabetic control mice. However, taurine supplementation in diabetic mice was found to result in significantly decreased number of smaller size islets and increased amount of medium size islets ($p<0.01$; Figure 3.3D) compared to diabetic controls. Taurine supplementation also dramatically increased large size islets distribution as compared to diabetic controls ($p<0.001$; Figure 3.3D).

3.4.3 *In vivo* effects of taurine on apoptosis of islet beta cells

Representative images show immunostaining for TUNEL (green) and insulin (red) are presented in Figure 3.5A. Streptozotocin treated diabetic mice displayed significantly increased beta cell apoptosis frequency compared to non-diabetic controls ($p<0.01$; Figure 3.5B). Taurine supplementation of these mice had no effect on beta cell apoptosis, while; administration of taurine to diabetic mice resulted in a significant rise in apoptosis frequency compared to saline controls ($p<0.05$; Figure 3.5B). Interestingly, taurine reduced beta cell apoptosis significantly as compared to streptozotocin treated control mice. ($p<0.05$; Figure 5B).

3.4.4 *In vitro* effects of taurine on apoptosis and antioxidation in BRIN-BD11 cells

In order to observe apoptosis *in vivo* and *in vitro*, TUNEL assay was performed to detect a programmed death event triggered in the beta cell. Representative images (Figure 3.6A) show immunostaining for TUNEL (green) /insulin (red). It was found that STZ significantly increased beta cell apoptosis rate ($p<0.001$; Figure 3.6B). Interestingly, taurine supplementation significantly decreased beta cell apoptosis at 12hr exposure ($p<0.01$; Figure 3.6B). In addition, STZ significantly reduced Bcl-2 expression ($p<0.05$; Figure 3.7) while taurine supplementation alone and together with streptozotocin slightly decreased Bcl-2 gene expression (Figure 3.7). As expected, Bax was significantly upregulated by streptozotocin ($p<0.05$; Figure 3.7) while it was found to be significantly downregulated after chronic exposure to taurine. ($p<0.05$; Figure 3.7). However, NF- κ B was upregulated with streptozotocin ($p<0.05$; Figure 3.7), while it was slightly downregulated with taurine. This suggests that taurine reduced apoptosis. To assess the effects of taurine on oxidative stress triggered in beta cells, anti-oxidant gene expression was evaluated. Intriguingly, it was found that taurine supplementation alone and together with STZ significantly increased SOD1 ($p<0.05$; Figure 3.8). Although, the expression of SOD2 and GPX-1 was less affected; taurine plus streptozotocin slightly down-regulated CAT (Figure 3.8).

3.4.5 *In vivo* effects of taurine on the proliferation of beta cells

Representative images (Figure 3.9A) show immunostaining for Ki67 (green) /insulin (red). Taurine supplementation in normal mice resulted in a significant ($p<0.01$) rise in beta cell proliferation compared to saline controls and STZ diabetic. Streptozotocin treated diabetic mice showed a slight rise in beta cell proliferation compared to saline treated controls. Taurine supplementation in diabetic mice caused a significant increase in beta cell proliferation compared to all other groups ($p<0.05$ to $p<0.001$; Figure 3.9B).

3.4.6 *In vitro* effects of taurine on beta proliferation in BRIN-BD11 cells

In order to investigate the effect of taurine on beta cell function**,** hormone secretion and expression, BRIN-BD11 cells were incubated with taurine alone or with STZ for 12 hr at 37^0C. To evaluate the effect of taurine on the proliferation of beta cells, Ki67 staining was performed. Representative images (Figure 3.10A) show immunostaining for Ki67 (green) /insulin (red). Interestingly, *in vitro* analyses revealed a subpopulation of beta cells treated with STZ followed by taurine with increased Ki67 staining (Figure 3.10B; $p<0.05$). This suggests that taurine promotes the proliferation of beta cells. Furthermore, PCR analysis illustrated that the chronic exposure to streptozotocin did not affect Ngn3+ gene expression, however, taurine alone and in addition to streptozotocin slightly downregulated Ngn3+ gene in 12 hr cultured BRIN-BD11 beta cells (Figure 3.11). In contrast, β1integrin gene was significantly downregulated by streptozotocin ($p<0.05$), while, taurine alone and in presence of streptozotocin significantly upregulated β1 integrin gene ($p<0.05$; Figure 3.11).

3.4.7 *In vitro* effects of taurine on cell-dedifferentiation in BRIN-BD11 cells

The effect of taurine on transdifferentiation, glucagon, and insulin expression was examined in cultured BRIN-BD11 cells exposed to the different conditions. Representative images (Figure 3.12A) show immunostaining for glucagon (green) /insulin (red). Cells showing immunoreactivity for both glucagon and insulin were increased in number by STZ treated cells ($p<0.05$) and reduced by taurine addition (Figure 3.12A). In addition, the effect of taurine on transdifferentiating genes of cultured BRIN-BD11 cells was assessed by examining gene expression (Figure 3.13). It was found that STZ significantly decreased Pdx1 ($p<0.05$) and insulin ($p<0.05$)

whereas exposure to taurine significantly enhanced both Pdx1 ($p<0.05$) and insulin expression ($p<0.05$). Moreover, taurine showed little or no effect on alpha cell specific genes (Figure 3.13). Thus, taurine might promote beta cell identity by enhancing expression of beta cell specific genes.

3.5 DISCUSSION

In the current study, an animal model with dead and damaged beta cells was generated by streptozotocin exposure, treatment with taurine, protected their beta cells from cytotoxic attack and increased pancreatic beta cell mass by promoting proliferation and inhibiting apoptosis. Pancreatic insulin content was enhanced and expansion of alpha cell area was supressed.

3.5.1 *In vivo* effect of taurine on body parameters

The function of beta cells is modulated by various nutrients, hormones, cell-cell interactions and neural signaling in the pancreas (Vasu et al., 2014a, 2014b)**.** The hyperglycaemia resulted in increased insulin demand, glucotoxicity, inflammation, and oxidative stress. This results in a detrimental effect on insulin synthesis and secretion by pancreatic beta cells (Moffett et al., 2014)**.** Streptozotocin is a cytotoxic inducer of diabetes in mammals. It is a structural analog of glucose and enters into beta cells via GLUT2 transporter. Due to this streptozotocin is considered selectively toxic to pancreatic beta cells. Streptozotocin acts as a suppressor of glucose catabolism and inhibits synthesis and secretion of insulin. Streptozotocin causes DNA fragmentation via DNA methylation, nitric oxide generation, free radical production in oxidative stress, NAD+ depletion and PARP activation. Pancreatic beta cells exposed to streptozotocin therefore commit apoptotic cell death. An elevated blood glucose and a drastic reduction in plasma and pancreatic insulin content represent the sign of streptozotocin-induced beta cell destructions (Yin et al., 2006).

3.5.2 Effects of taurine on islet morphology (*in vivo*) and transdifferentiation related changes in beta cells (*in vitro*)

Earlier studies on streptozotocin-induced beta cell destruction reported that streptozotocin promoted a remarkable loss of beta cells with subsequent gain of alpha

cells. These alterations resulted in a detrimental effect on area, size and number area of islets (Yin et al., 2006; Moffett et al., 2014; Vasu et al., 2014a/2015). Islets with small size and irregular shape were observed after streptozotocin-induced destruction of beta cells. Taurine supplementation stimulated beta cell area in association with augmentation of total islet area in diabetic animals. This appeared to be consistent with the increased medium and large size islets as well as increased number of overall islets. Streptozotocin mediated massive destruction of beta cells resulted in a substantial rise in alpha cells, suggesting the lack of paracrine action of beta cells in the normalization of islet architecture (Yin et al., 2006). In contrast, taurine reduced alpha cell mass after exposure to streptozotocin. This suggests that taurine might be suppressing alpha cell mass similar to GABA or insulin or by promoting transdifferentiation of alpha to beta cells. Further lineage studies will be needed to investigate effect of taurine on transdifferentiation. Taurine acts as an agonist of the $GABA_A$ receptor in the brain (Gossai et al., 2009a/b). Some studies have demonstrated that GABA can transdifferentiate pancreatic alpha cells into beta cells (Ben-Othman et al., 2017; Li et al., 2017). Future work should be focussed on the investigating effect of taurine on the transdifferentiating alpha cell to beta cells using alpha cell lineage tracing strains. In addition, our data demonstrated an effect of taurine on transdifferentiating genes of cultured BRIN-BD11 cells. It was found that taurine promoted beta cell specific genes including (Pdx1 and Insulin) while it showed little or no effect on alpha cell specific genes. The result is consistent with demonstration of immunoreactivity for beta cell and alpha cell markers. Therefore, we have demonstrated at least to some extent, compensations in the area and number of islets, beta cell area and islet size distribution of diabetic mice result from the proliferation of beta cells induced by taurine supplementation.

3.5.3 Beta cell-growth promoting effects of taurine

The role of amino acids in regulating beta cell mass has already been elucidated (Boujendar et al., 2002). Using *in vitro* and *in vivo* studies, we emphasized a regenerative ability of taurine on beta cells. This is a crucial outcome because islet cells have been considered to possess a low ability for regeneration. The origin of new beta cells during injury is still controversial (Boujendar et al., 2002). Although some reports attributed the source of beta cells after injury solely to the replication of pre-existing beta cells, others proclaimed that non-beta cells may also transdifferentiate

into new beta cells (Yang et al., 2011; Talchai et al., 2012; Weir et al., 2013; Brereton et al., 2014). To address this possibility, we aimed to find whether duplication of beta cells contributed to the restoration of beta cells after taurine supplementation. Some studies have also attributed a role to protein Ngn3+ in duplication of beta cells and explained that a latent downregulation of Ngn3+ genes is crucial for beta cells to activate mitogenesis and proliferation (Rukstalis et al., 2009). Another study on proliferation inferred that β1intregrin proteins are major checkpoints of cell division and activate different signaling cascades for the proliferation of beta cells (Li et al., 2005). Previously, Nakatsuru et al, (2018) also suggested role of taurine in proliferation of beta cell area. Therefore, our findings clearly suggest that taurine supplementation promotes the proliferation of beta cells *in vitro*.

3.5.4 Beta cell-protective effects of taurine

Apoptosis, along with proliferation, is observed immediately after the beta-cell injury. An enhanced rate of apoptosis could be responsible for beta cell loss (Boujendar et al., 2002). Consistent with this postulate, our study found that insulin content was drastically reduced in both *in vivo* and *in vitro* in STZ treated beta cells. This therefore highlights the possible role of apoptosis in beta cell depletion. Although TUNEL labeling is suitable for detecting DNA fragmentation, the method does not accurately reflect the possibility of apoptotic gene induction after beta cell injury, therefore, we evaluated the apoptotic gene expression *in vitro* (Boujendar et al., 2002). It is known that the anti-apoptotic protein Bcl-2 prevents cell death by maintaining the structural integrity of the mitochondrial membrane. It is also involved in inactivation of other pro-apoptotic proteins such as Bax, BAK, BID, and caspase-9 (Hardwick et al., 2013). In contrast, Bax protein promotes apoptosis by forming pores in the mitochondrial membrane (Hardwick et al., 2013). Similarly, NF- κ B also promotes apoptosis in response to outer cell injury signals by inducing expression of other pro-apoptotic proteins such as FasL, death receptor 4 and Fas/CD95. Indeed, overwhelming evidence has revealed the contribution of NF- κ B to apoptosis. Increasing lines of evidence also suggest the possible involvement of NF- κ B in preventing cell death (Lee et al., 2007). Therefore, our evidences clearly suggest that taurine may prevent beta cell apoptosis following the streptozotocin exposure both *in vivo* and *in vitro.*

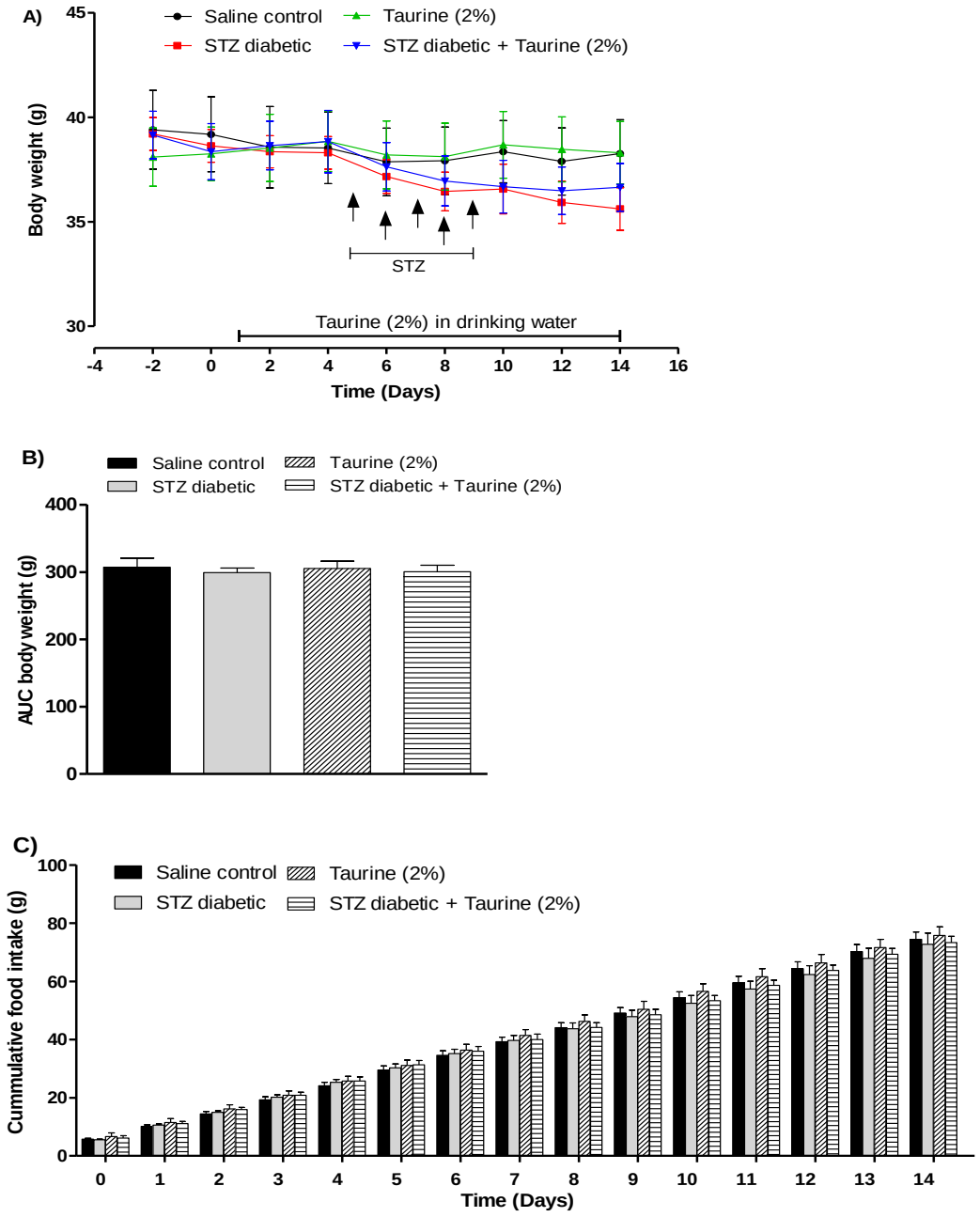
3.5.5 Taurine provides anti- oxidant defence to beta cells

Oxidative stress, in addition to apoptosis, is responsible for streptozotocin-mediated beta cell death (Vasu et al., 2013, 2014b, 2015, 2016)**.** During oxidative stress, the generation of harmful superoxide ions is injurious to cell components such as membrane, organelle, and genetic material (Vasu et al., 2014b)**.** However, anti-oxidant enzymes such as superoxide dismutase (SOD1 or SOD2) can protect cell integrity by promoting the breakdown of lethal superoxide ions into less harmful molecules like ethanol or O2 (Vasu et al., 2016)**.** In addition, glutathione peroxidase (GPX-1) breakdowns to the less harmful entities (Vasu et al., 2015)**.** However, increased catalase (CAT) is consistent with an increased rate of oxidative stress (Vasu et al., 2014b)**.** Thus, our results suggest that taurine may play an important role in protecting beta cells from oxidative stress generated by streptozotocin attack.

3.5.6 Concluding remark

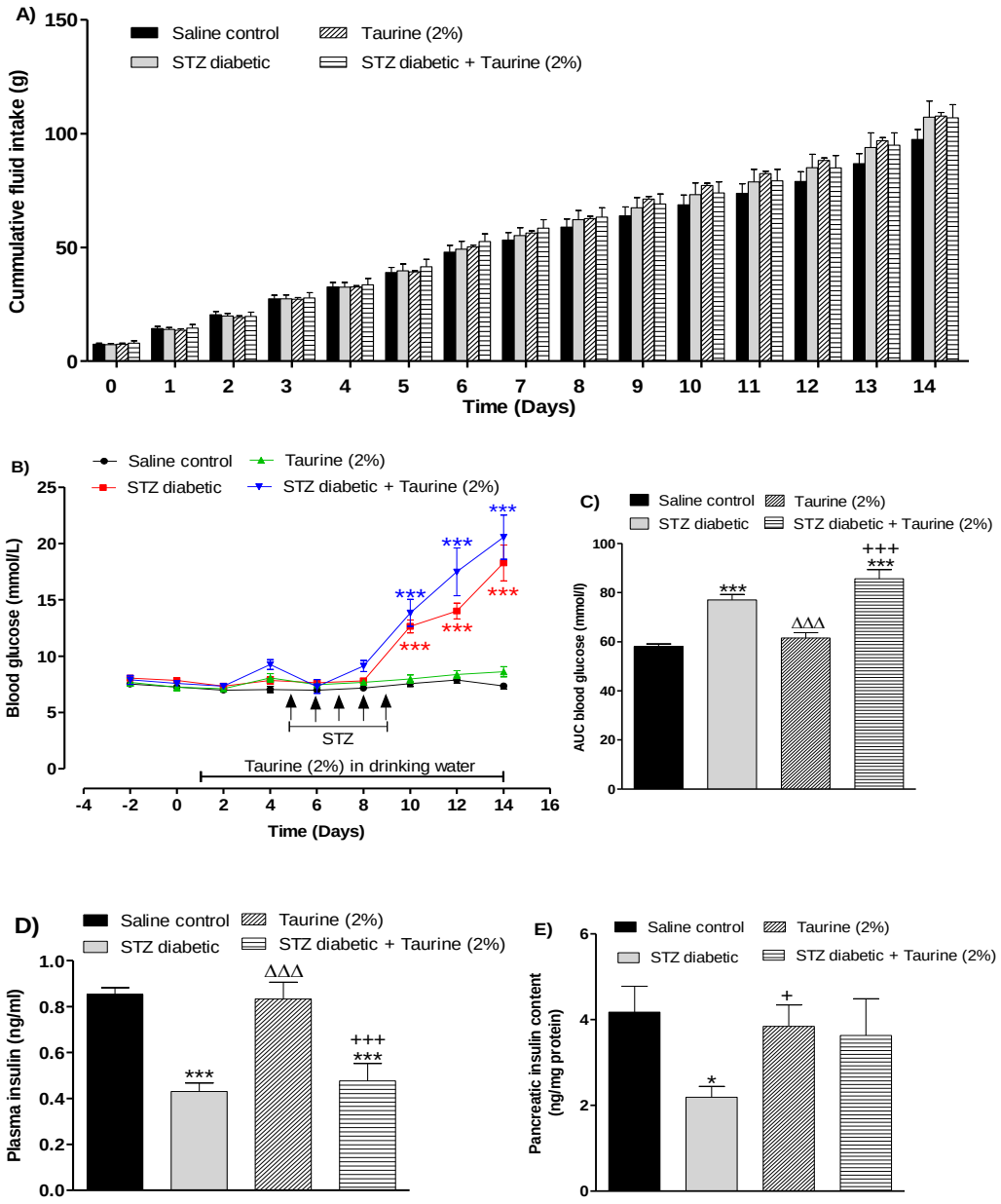
These combined observations suggest that taurine supplementation in streptozotocin-induced diabetes may protect beta cells by promoting proliferation and inhibiting apoptosis along with suppression of alpha cell mass. Therefore, taurine may be therapeutically useful in protecting and regenerating beta cells (by pre-existing beta cells) and improving insulin release to treat type 1 and type 2 diabetes. Further studies involving alpha cell lineage tracing are required to investigate the possible role of taurine in beta cells generation from non-beta cell (α-cell) source.

Figure 3.1 Effects of taurine on (A,B) body weight and (C) food intake in normal and streptozotocin diabetic mice



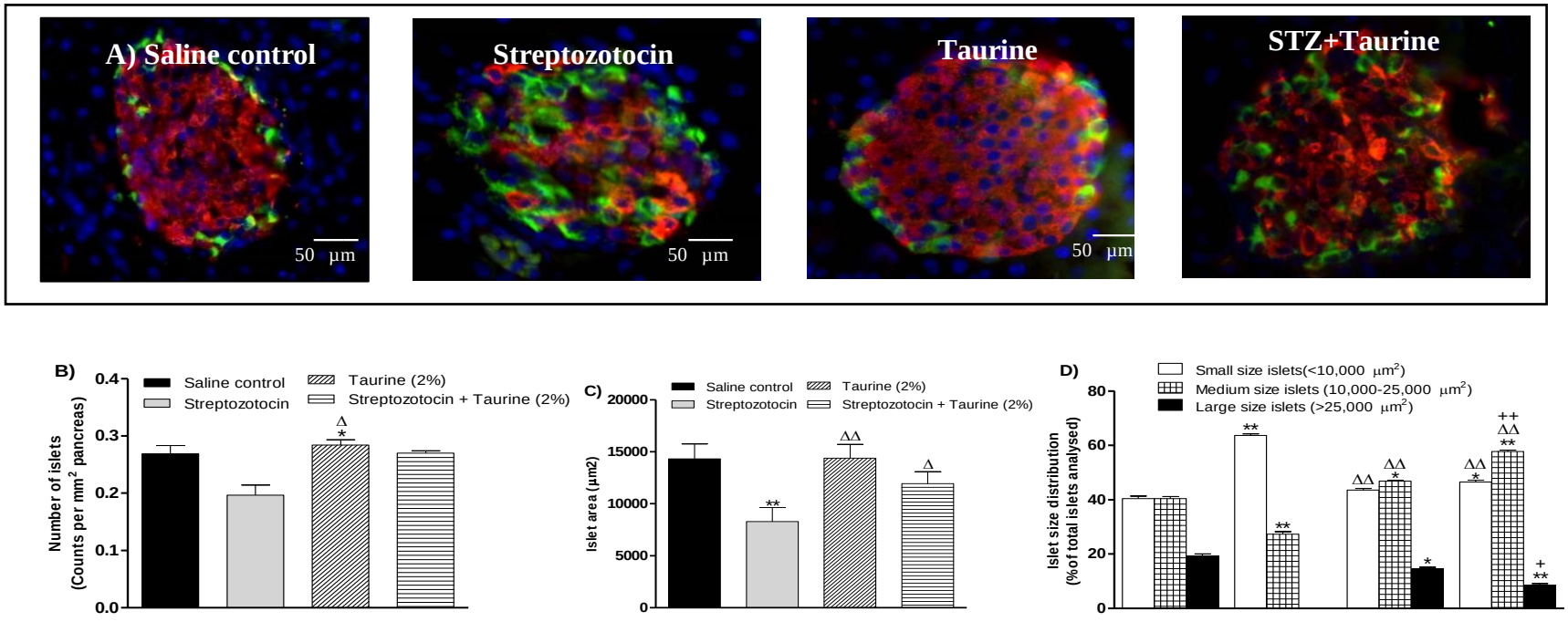
(A,B) Body weight and (C) food intake were measured at the end of the study in Swiss TO mice. Taurine was included at 2% drinking water of normal and streptozotocin diabetic mice. Controls received normal tap water. Values represent means ± SEM for 6 mice.

Figure 3.2 Effects of taurine on (A) fluid intake, (B,C) blood glucose, (D) plasma insulin and (E) pancreatic insulin intake in normal and streptozotocin diabetic mice



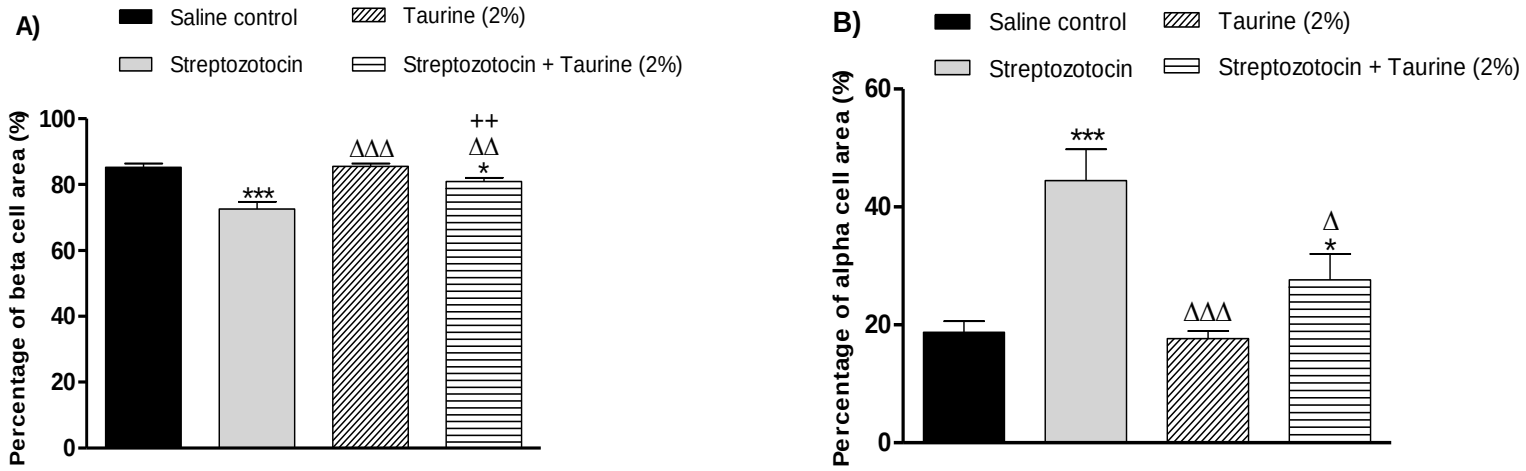
(A,B) Body weight and (C) food intake were measured at the end of the study Swiss TO mice. Taurine was included at 2% drinking water of normal and streptozotocin diabetic mice. Controls received normal tap water. Values represent means ± SEM for 6 mice. *p<0.05 and ***p<0.001 compared to saline control group. ΔΔΔp<0.001 compared to streptozotocin treated group. +p<0.05 and +++p<0.001 compared to taurine control group.

Figure 3.3 Effects of taurine on (B) islet numbers, (C) islet area and (D) islet size-distribution in normal and streptozotocin diabetic mice



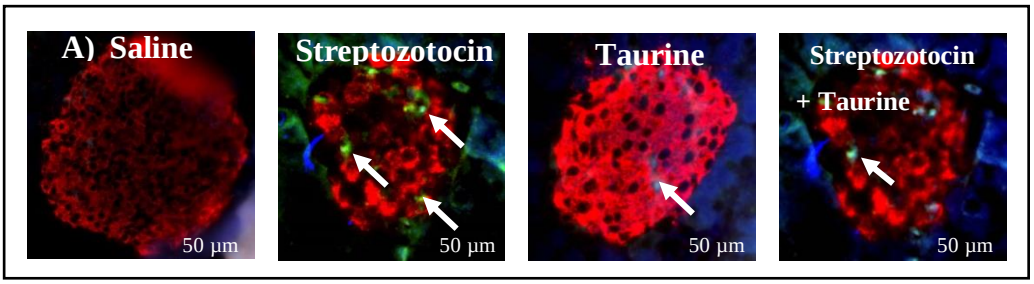
Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). (B) Islet number, (C) Islet area and (D) islet size distribution were analysed by using Cell^F and image J at the end of the study of Swiss-TO mice. Taurine was included at 2% drinking water of normal and streptozotocin diabetic mice. Values represent means ± SEM for 6 mice (>70 islets per group). **p<0.05 and **p<0.01 compared to saline control group. Δp<0.05 and ΔΔp<0.01 compared to streptozotocin treated group. ++p<0.01 compared to taurine treated group. Scale bars: 50 µm.

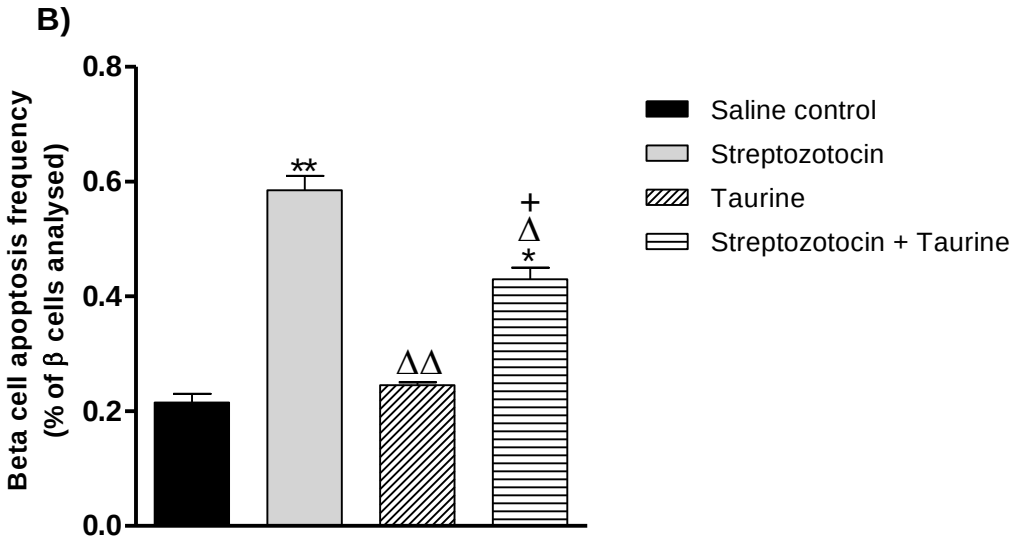
Figure 3.4 Effects of taurine on (A) beta cell area and (B) alpha cell area in normal and streptozotocin diabetic mice



(A) Percentage of beta cell area and (B) percentage of alpha cell area measured by using cell^F and image J at the end of the study of Swiss-TO mice. Taurine was included at 2% drinking water of normal and streptozotocin diabetic mice. Values represent means ± SEM for 6 mice (>70 islets per group). *p<0.05 and ***p<0.001 compared to saline control group. Δp<0.05, ΔΔp<0.01 and ΔΔΔp<0.001 compared to streptozotocin treated group. ++p<0.01 compared to taurine treated group.

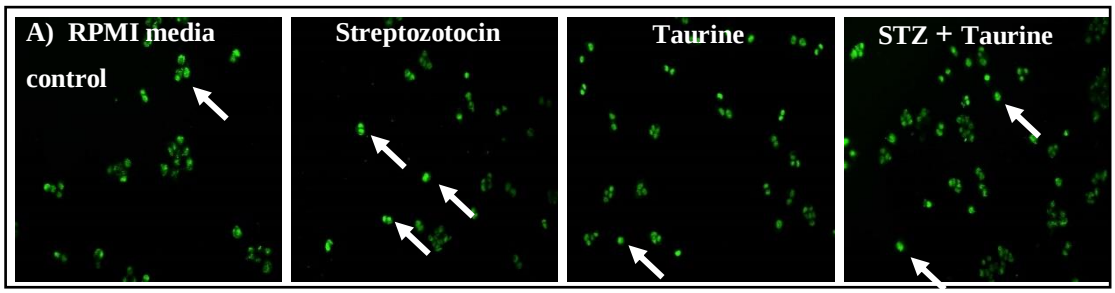
Figure 3.5 Effects of taurine on (A,B) apoptosis of beta cells in normal and streptozotocin diabetic mice



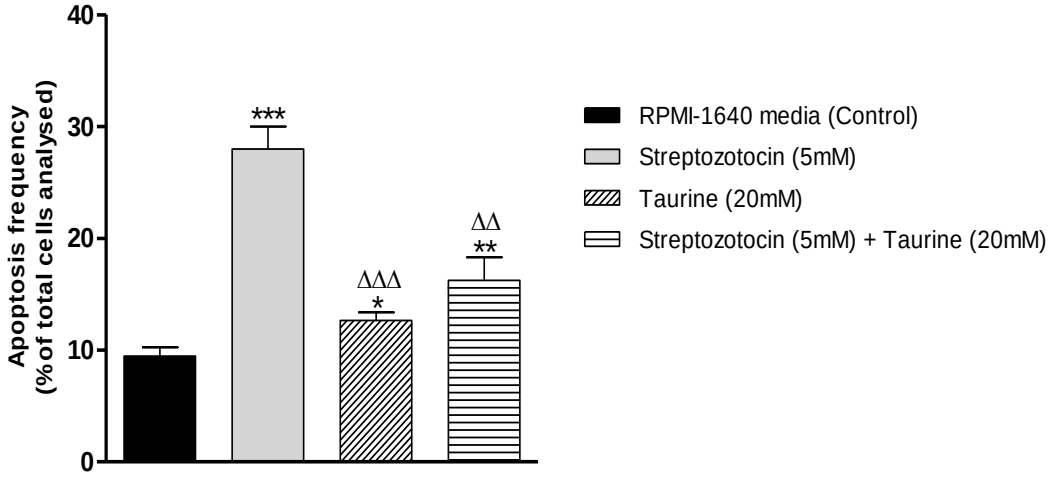


Representative images (A) showing immunostaining for TUNEL(green) and Insulin (red). (B) Percentage of beta cell apoptosis measured by using Cell^F and image J at the end of study of Swiss-TO mice. Taurine was included at 2% drinking water of normal and streptozotocin diabetic mice. Values represent means ± SEM for 6 mice (100-120 islets per group). *$p<0.05$ and **$p<0.01$ compared to saline control group. Δ$p<0.05$ and ΔΔ$p<0.01$ compared to streptozotocin treated group. +$p<0.05$ compared to taurine group. Scale bars: 50 µm.

Figure 3.6 Effects of chronic (12 h) exposure to taurine (20 mM) in presence and absence of streptozotocin (5 mM) on (A, B) apoptosis of BRIN-BD11 cells

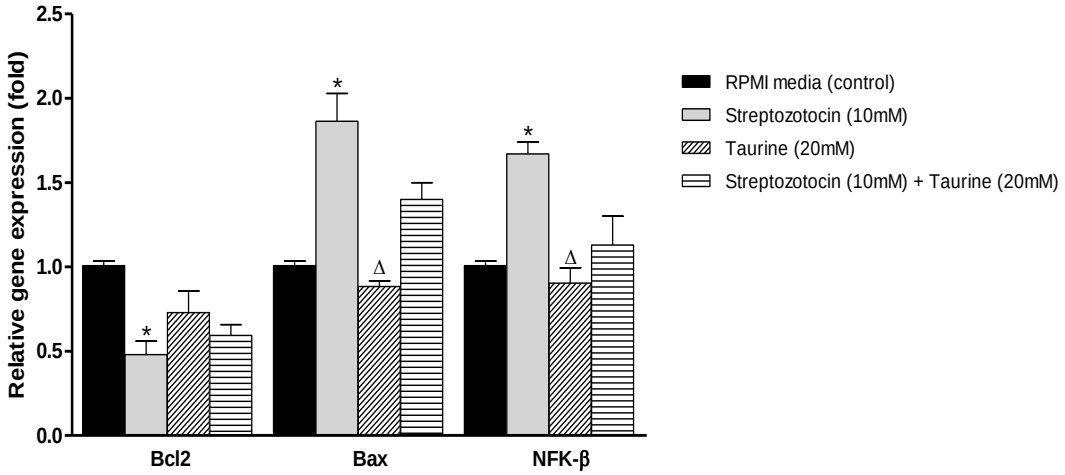


B)



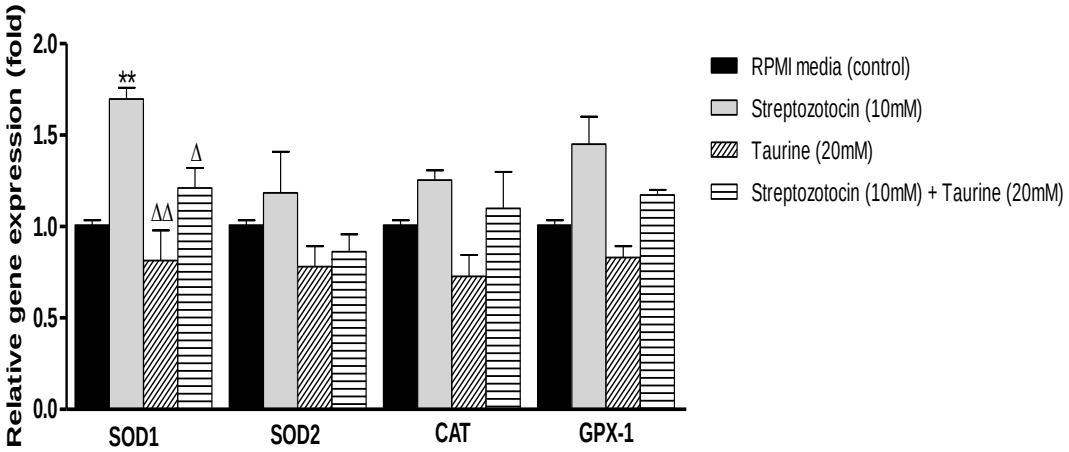
Representative images (A) showing immunostaining for TUNEL (green). Effects of chronic (12 h) exposure to taurine (20 mM) in presence and absence of streptozotocin (5 mM) on (B) apoptosis of beta cells expressed as apoptosis frequency (% of total cells analysed). Values represent means ± SEM for n=4. *p<0.05, **p<0.01 and ***p<0.001 compared to RPMI media control group. ΔΔp<0.01 and ΔΔΔp<0.001 compared to streptozotocin treated group. Scale bars: 50 µm.

Figure 3.7 Effects of taurine on apoptotic gene expression in BRIN-BD11 cells in presence and absence of streptozotocin



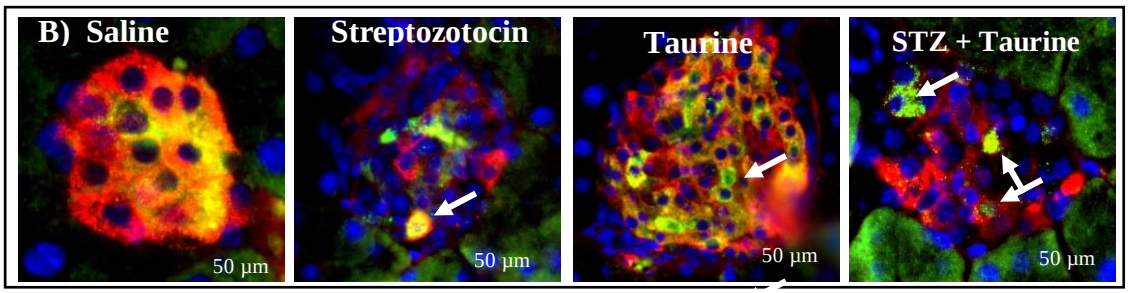
BRIN-BD11 cells were cultured in RPMI-1640 media supplemented with treatments for 12 h. Relative gene expression of Bcl2, Bax and NFK-β were measured using RT-PCR. Gene expression was normalized to β-actin expression and relative gene expression (fold).Values are mean ± SEM (n=3). *$p<0.05$ compared to saline control group. Δ$p<0.05$ compared to streptozotocin treated group.

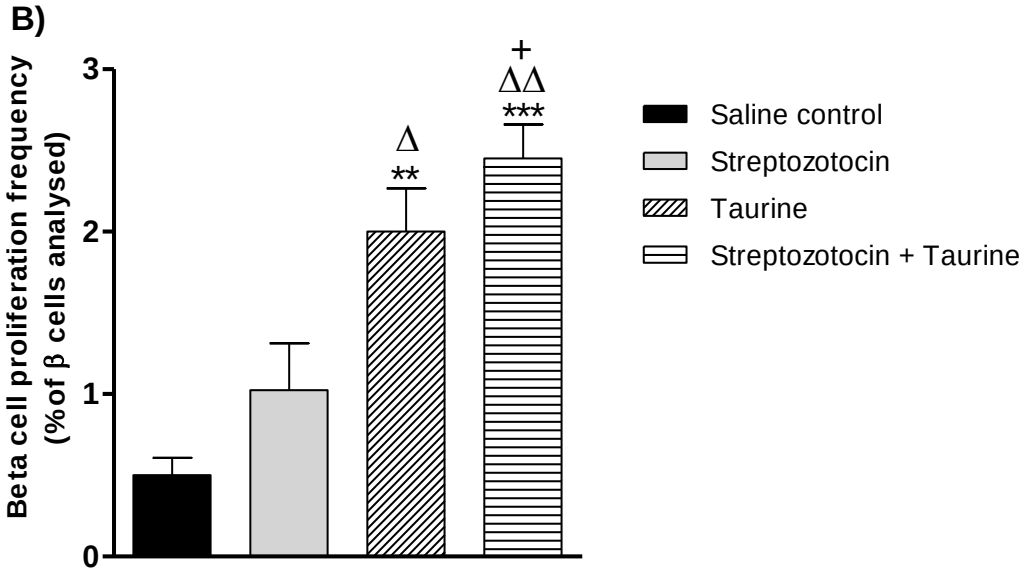
Figure 3.8 Effects of taurine on anti-oxidant defence gene expression in BRIN-BD11 cells in presence and absence of streptozotocin



BRIN-BD11 cells were cultured in RPMI-1640 media supplemented with treatments for 12 h. Relative gene expression of SOD1, SOD2, CAT and GPX-1 were measured using RT-PCR. Gene expression was normalized to β-actin expression and relative gene expression (fold).Values are mean ± SEM (n=3). **$p<0.01$ compared to saline control group. Δ$p<0.05$, ΔΔ$p<0.01$ compared to streptozotocin treated group.

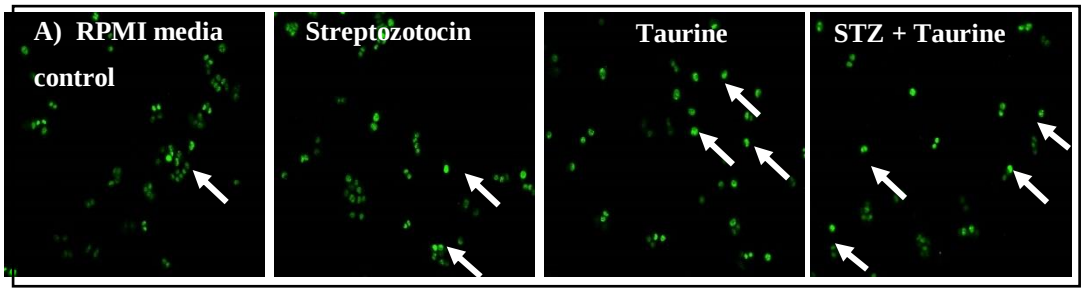
Figure 3.9 Effects of taurine on (A, B) proliferation of beta cells in normal and streptozotocin diabetic mice



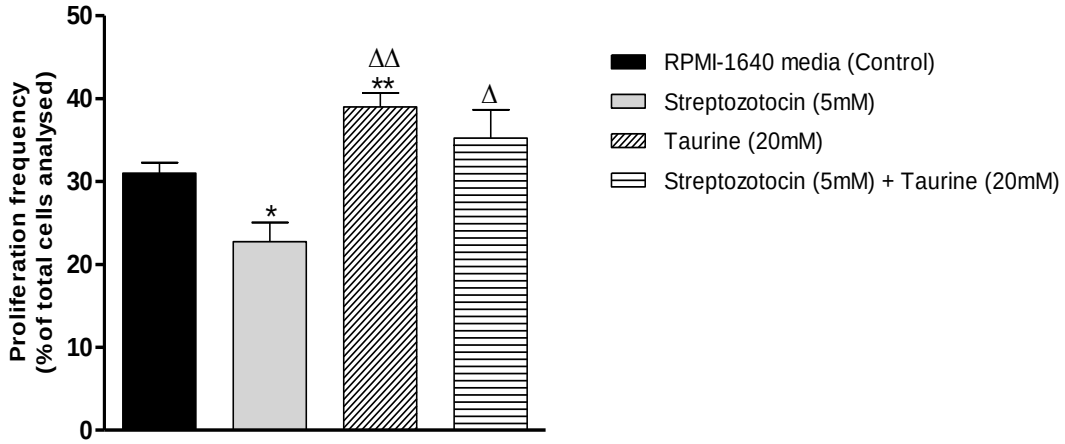


Representative images (A) showing immunostaining for DAPI (blue), Ki67(green) and Insulin (red). (B) Percentage of beta cell proliferation measured by using Cell^F and imageJ at the end of study of Swiss-TO mice. Taurine was included at 2% drinking water of normal and streptozotocin diabetic mice. Values represent means ± SEM for 6 mice (100-120 islets per group). **p < 0.01 and ***p < 0.001 compared to saline control group. ΔΔp<0.01 compared to streptozotocin treated group. +p<0.05 compared to taurine control. Scale bars: 50 µm.

Figure 3.10 Effects of chronic (12 h) exposure to taurine (20 mM) in presence and absence of streptozotocin (5 mM) on (A, B) proliferation of BRIN-BD11 cells

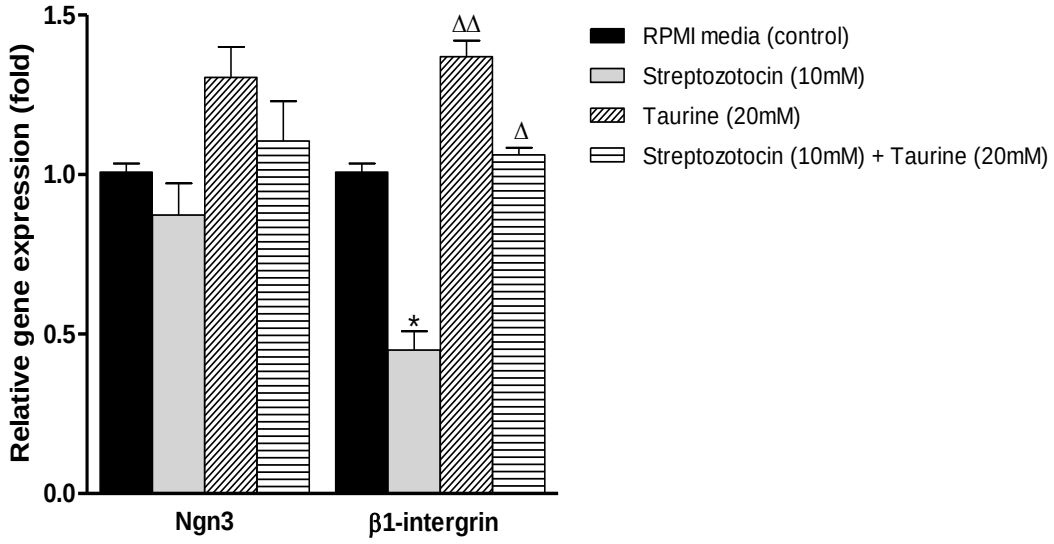


B)



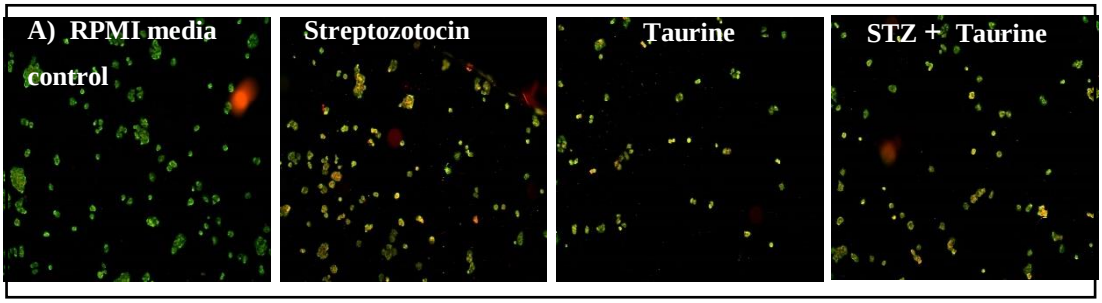
Representative images (A) showing immunostaining for Ki67 (green). Effect of chronic (12 h) exposure to taurine (20 mM) in presence and absence of streptozotocin (5 mM) on (B) proliferation of beta cells expressed as proliferation frequency (% of total cells analysed). Values represent means ± SEM for n=4. *p< 0.05 and **p< 0.01 compared to RPMI media control group. Δp<0.05 and ΔΔp<0.01 compared to streptozotocin treated group. Scale bars: 50 µm.

Figure 3.11 Effects of taurine on proliferating gene expression in BRIN-BD11 cells in presence and absence of streptozotocin

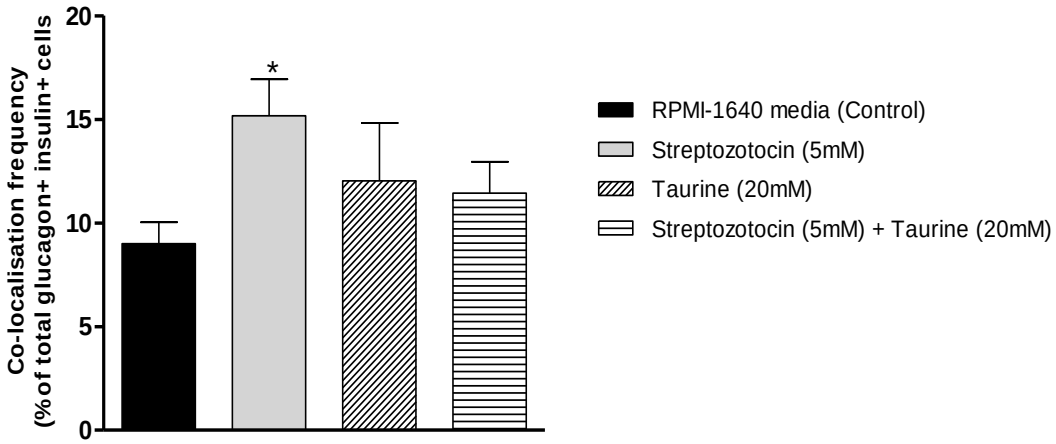


BRIN-BD11 cells were cultured in RPMI-1640 media supplemented with treatments for 12 h. Relative gene expression of ngn3 and β-1 integrin were measured using RT-PCR. Gene expression was normalized to β-actin expression and relative gene expression (fold).Values are mean ± SEM (n=3). *$p<0.05$ compared to saline control group. Δ$p<0.05$, ΔΔ$p<0.01$ compared to streptozotocin treated group.

Figure 3.12 Effects of chronic (12 h) exposure to taurine (20 mM) in presence and absence of streptozotocin (5 mM) on (A,B) dedifferentiation of BRIN-BD11 cells

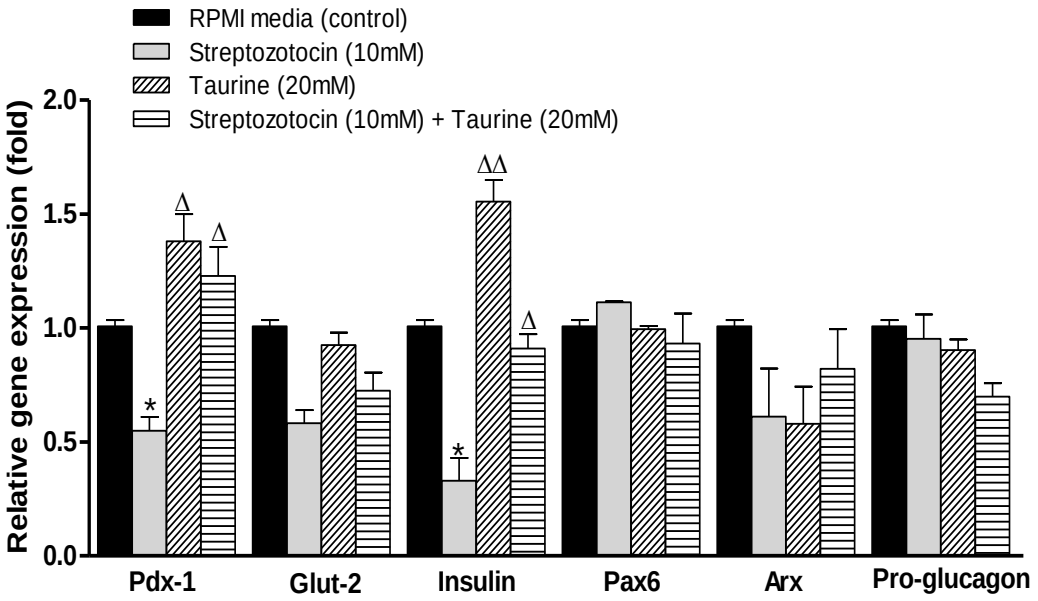


B)



Representative images (A) showing immunostaining for insulin (green) & glucagon (red). Effects of chronic (12 h) exposure to taurine (20 mM) in presence and absence of streptozotocin (5 mM) on (B) dedifferentiation of beta cells expressed as co-localization frequency (% of glucagon+/insulin+ cells analysed). Values represent means ± SEM for n=4. *p<0.05 compared to RPMI media control group. Scale bars: 50 µm.

Figure 3.13 Effects of taurine on trans-differentiating gene expression in BRIN-BD11 cells in presence and absence of streptozotocin



BRIN-BD11 cells were cultured in RPMI-1640 media supplemented with treatments for 12 h. Relative gene expression of Pdx1, Glut2, Insulin, Arx, Pax6 and Pro-glucagon were measured using RT-PCR. Gene expression was normalized to β-actin expression and relative gene expression (fold).Values are mean ± SEM (n=3). *$p<0.05$ compared to saline control group. Δ$p<0.05$ and ΔΔ$p<0.01$ compared to streptozotocin treated group.

Chapter 4

Effects of therapeutic agents on transdifferentiation and related hormone production and gene expression in alpha-TC 1.9 cells

4.1 SUMMARY

A developing approach to form new beta cells is through the transformation of glucagon producing-alpha cells into insulin-producing beta cells. Nevertheless, the finding of the target sites that can be managed by chemical transdifferentiation needs more insight into adaptive changes of early state and the terminating transformed state. Therefore, to understand this mechanism at the molecular and genetic levels, here we sought to determine the effects of different therapeutic agents on mouse α-TC 1.9 clonal pancreatic alpha cell line.

Here, we were cultured α-TC 1.9 cells at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. We measured the fluorescent intensity of each expressed hormone and quantified using gene expression analysis. Importantly we found that GABA, taurine, sitagliptin and exendin-4 were showed promoting glucagon expression in alpha cells. This could suggest that treatments either proliferating alpha cells enhancing the glucagon gene, which might be essential for the transdifferentiation mechanism. Moreover, artemether, sitagliptin and exendin-4 decreased Arx gene. Interestingly, only exendin-4 showed facilitated insulin expression in cultured alpha cells. However, other beta cell marker Pax4 was found to be in the suppressed state in all treatment groups. GABA and exendin-4 showed increased GLP-1 expression. Surprisingly, we found artemether significantly reduced the cell viability of alpha cells.

Thus, transdifferentiation is considered as a chronological phenomenon and needs a strong message from beta-cell injury to activate this mechanism; we, therefore, suggest from this chapter that normal alpha cell lines are at least responding to these tested molecules. Future studies with mimicking the diabetic environment as well as long-term exposure of these drugs would clear the detail outcomes. Previous studies have shown the link between glucagon/GLP-1 signaling and alpha transdifferentiation into beta cells. Our results suggest that the glucagon expression is increased by all the treatments except the artemether. Thus, we can speculate that glucagon/GLP-1 signaling might be an important part of alpha to beta transition.

4.2 INTRODUCTION

Type-1 and type-2 diabetes mellitus are frequently described to a lack of beta cells, which causes severe hyperglycaemia (Moffett et al., 2015a; Vasu et al., 2015, 2016). In such cases, the regeneration of beta cells could be a better therapeutic alternative than existing therapies to compensate for insulin deficiency (Cheng et al., 2015). Previous studies on modulation of islet plasticity have demonstrated that non-beta cells such as alpha cells hold promising regenerative ability to contribute new beta cells mass (Thorel et al., 2010; Cheng et al., 2015; Stanojevic et al., 2015). More interestingly, alpha cells either possess innate ability to protect and nourish beta cells by secreting ectopic GLP-1 or by adapting beta cells phenotypes, often observed during state of islet insult (Vasu et al., 2014a; Puri et al., 2015). Such extraordinary fate changing behaviour of alpha cells offers an instinctive therapeutic route to produce insulin during the diabetic state. However, the detailed mechanism of alpha cells fate change into beta cells is yet unclear (Gromada et al., 2007; Stanojevic et al., 2015).

Previous studies on gene manipulation have already revealed that the transdifferentiation of alpha into beta cells may result either from predominant expression of beta cell-specific markers such as Pax4 (Collombat et al., 2009), Pdx1 (Yang et al., 2011), Neurog3 (Zhu et al., 2017) and MafA (Matsuoka et al., 2017); or relative inhibition of alpha cell identity genes such as Arx (Wilcox et al., 2013), Men1 (Lu et al., 2010), and Dnmt1 (Chakravarthy et al., 2017). Further, despite showing some controversial evidence certain drugs such as GABA (Ben-Othman et al., 2017; Ackermann et al., 2018), artemisinin (Li et al., 2017), BRD7389 (Dina et al., 2010), activin (Brown et al., 2016), IGFBP1 (Lu et al., 2016), and GLP-1 (Lee et al., 2018) have been found to potentiate alpha cell transdifferentiation. Although GABA and artemether have been shown to promote alpha to beta conversion, recent reports have failed to replicate their roles in alpha cell transdifferentiation (Ackermann et al., 2018; Van et al., 2018). Therefore, it is interesting to further confirm their role in this process.

Because activation of a single gene is enough to induce alpha cell transition into beta cells (Collombat et al., 2009; Dina et al., 2010; Yang et al., 2011), here we aim to investigate whether exogenous therapeutic agents can exert similar effects. The

ectopic expression of beta cells specific markers exclusively in alpha TC 1.9 cells would therefore reflect their transition into beta cells (Dina et al., 2010). In this context, here we cultured a well-established pancreatic alpha cells line, namely mouse α-TC 1.9 cells to detect expression of beta cells specific characteristics upon chronic exposure to exogenous chemical inducers. Recent research has demonstrated that in insulin-deficient mouse models, pancreatic alpha cells were found to adapt beta cells markers following extreme loss existing beta cells (Thorel et al., 2010; Cheng et al., 2015). Therefore, it is interesting to examine the effects of artemether, GABA, taurine, sitagliptin and exendin-4 on cultured α-TC 1.9 cells with respect to adaptation of beta cells characteristics.

4.3 MATERIALS AND METHODS

Materials and methods for this study has been discussed in Chapter 2.

4.4 RESULTS

4.4.1 Chronic (72 h) *in vitro* effect of various therapeutic agents on immunostaining for hormonal expression in relation to alpha cells transdifferentiation into beta cells

4.4.1.1 Glucagon/Arx double staining

Representative images (Figure 4.1A) show immunostaining for glucagon (green) and Arx (red). Here, α-TC 1.9 cells were cultured in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively, at 37^0C for 72 h. As shown in Figure 4.1 B, glucagon colour intensity was unaltered by artemether, GABA and taurine, but, it was significantly ($p<0.05$ to $p<0.001$) enhanced by both sitagliptin and exnedin-4 when compared to control media. Similar trend was observed on Arx colour intensity with a slight increase observed with sitagliptin and exendin-4 in comparison with control media (Figure 4.1C).

4.4.1.2 Glucagon/insulin double staining

Representative images (Figure 4.2 A) show immunostaining for glucagon (green) and insulin (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. As it is shown in Figure 4.2B, glucagon colour intensity was unaltered by artemether, GABA and taurine while it was significantly ($p<0.05$) enhanced by both sitagliptin and exendin-4 treated cells when compared control media. Further, although insulin colour intensity was unaltered by all groups, exndin-4 showed significantly ($p<0.01$) higher insulin intensity compared to DMEM control (Figure 4.2C).

4.4.1.3 Glucagon/GLP-1 double staining

Representative images (Figure 4.3A) show immunostaining for glucagon (green) and GLP-1 (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. Here we found that glucagon colour intensity was enhanced ($p<0.05$ to $p< 0.001$) by all treatments (Figure 4.3B). While GLP-1 colour intensity was unaltered by taurine and sitagliptin, it was significantly ($p<0.05$ to $p<0.01$) augmented by artemether, GABA and Exendin-4 (Figure 4.3C).

4.4.1.4 Pdx-1/Pax-6 double staining

Representative images (Figure 4.4A) show immunostaining for Pdx-1 (green) and Pax-6 (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. Unexpectedly, none of the treatment regimens altered the colour intensity of both Pdx-1 (Figure 4.4B) and Pax-6 (Figure 4.4C) when compared to media control.

4.4.1.5 PC1/3/insulin double staining

Representative images (Figure 4.5A) show immunostaining for PC1/3 (green) and insulin (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. Unexpectedly, none of the treatment regimens had altered the colour intensity of either PC1/3 (Figure 4.5B) or insulin (Figure 4.5C) when compared to media control. However, a slight increase in trend was observed in GABA, taurine and exendin-4 treated cells compared to control (Figure 4.5C).

4.4.2 Chronic (72hr) *in vitro* effect of various therapeutic agents on the gene expression of alpha and beta cells specific marker proteins in relation to alpha cells transdifferentiation into beta cells

4.4.2.1 Glucagon gene expression

Effects of treatments were examined on glucagon gene expression in α-TC 1.9 cells. As shown in Figure 4.6, glucagon gene expression was significantly ($p<0.05$) reduced by artemether and GABA, but it was unaltered by all other treatments. However, a decreasing trend was observed with taurine, sitagliptin and exendin-4 compared to control (Figure 4.6).

4.4.2.2 Insulin gene expression

Effects of treatments on insulin gene expression in α-TC 1.9 cells are shown in Figure 4.7. Insulin gene expression was significantly ($p<0.01$) reduced by artemether while it was unaltered by all other treatments. Although not significant, a slight increase in trend was observed with exendin-4 (Figure 4.7).

4.4.2.3 Pax-4 gene expression

Effects of treatments on Pax-4 gene expression were examined in α-TC 1.9 cells. As shown in Figure 4.8, Pax4 gene expression was significantly ($p<0.05$ to $p<0.01$)

reduced by all the treatments ($p<0.05$ to $p<0.01$). However, detectable level of expression fold was observed with GABA, taurine and exendin-4 (Figure 4.8).

4.4.2.4 Arx gene expression

Effects of treatments were examined on Arx gene expression in α-TC 1.9 cells are shown in Figure 4.9. Arx gene expression was significantly ($p<0.05$ to $p<0.01$) reduced by artemether, sitagliptin and exendin-4 while unaltered by GABA and taurine (Figure 4.9).

4.4.2.5 PC1/3 gene expression

Effects of treatments on PC1/3 gene expression were examined in α-TC 1.9 cells. As shown in Figure 4.10, PC1/3 gene expression was not significantly altered by artemether, taurine and sitagliptin. Interestingly, it was significantly ($p<0.05$) enhanced by GABA and exendin-4 when compared to media control (Figure 4.10).

4.4.3 Effects of 72 h culture with different therapeutic agents on the cellular glucagon content in α-TC 1.9 cells

Effects of treatments were examined on cellular glucagon content of α-TC 1.9 cells are shown in Figure 4.11. Cellular glucagon content was significantly ($p<0.05$ to $p<0.01$) increased by taurine, GABA, sitagliptin and exendin-4 when compared to cells cultured in control media. Artemether did not affect glucagon concentration compared to cells cultured in control media (Figure 4.11).

4.4.4 Chronic (72 h) *in vitro* effect of various therapeutic agents on cell viability of alpha cells

Effects of treatments were examined on cell viability α-TC 1.9 cells measured by MTT assay. It was found that artemether had significant ($p<0.001$) cell toxicity compared

to control. No other treatments affected the cell viability in comparison to control (Figure 4.12).

4.5 DISCUSSION

Cellular transdifferentiation, is a process that recruits fully mature cells into other phenotypically and functionally different cells. It is considered as more convenient and feasible path to generate insulin-producing cells over the use of pluripotent stem cells followed by their conversion into desired cell type (Gottesfeld et al., 2000; Xu et al., 2016). Accordingly, small molecule-driven fate changing approach offers a potential treatment for diabetes that results from a loss of beta cell mass (Fomina-Yadlin et al., 2010; Xu et al., 2016). In present study, we mainly focussed on chemically-induced alpha cell transdifferentiation into beta cells, which is similar to beta cell regeneration by genetic manipulation as mentioned in earlier reports (Collombat et al., 2009; Lu et al., 2010; Yang et al., 2011; Wilcox et al., 2013). Several recent studies have reported *in vitro* alpha cell transdifferentiation using a range of small molecules including artemether **(**Li et al., 2017), BRD7389 (Fomina-Yadlin et al., 2010), GW8510 **(**Choudhary et al., 2014) and exendin-4 (Lee et al., 2018). Intriguingly, earlier Li et al. (2017) used a fluorescent intensity as an analysis method to confirm the relative protein expression in cultured alpha TC1.9 cells. In contrast, other studies (Fomina-Yadlin et al., 2010; Choudhary et al., 2014; Lee et al., 2018) employed gene analysis methods to confirm protein expression. In present study, we used both experimental analysis methods. In particular, we tested the fate-changing role of artemether, GABA, taurine, sitagliptin and exendin-4 in 72 h cultures of glucagon producing alpha-TC 1.9 cells.

4.5.1 Effects of test compounds on alpha cells identity markers (Glucagon, Arx and Pax6)

We found an increased glucagon immunostaining and cellular glucagon content in alpha cells with sitagliptin and exendin-4 treatments. Artemether and GABA reduced glucagon gene expression. However, GABA and taurine- treated cells showed a trend towards increasing glucagon immunostaining and perhaps increased cellular glucagon content. Thus, we speculate that the observed enhanced expression of glucagon could

be a sign of alpha cell proliferation and pro-glucagon gene promotion as reported in earlier studies by Lee et al. (2018). In addition, it is also noteworthy that glucagon itself may possess some important role in alpha cells transdifferentiation (Ye et al., 2015). Although in present study, the staining intensity and cellular glucagon content were found be significantly enhanced with GABA, taurine, sitagliptin and exendin-4, it is valid to speculate that interference of pro-glucagon peptide with glucagon antibody could also contribute to observed effects. This notion has also been supported by Nie et al (2000).

The expression of alpha cell identity factor Arx was decreased with sitagliptin and exendin-4. The presence of Pax6 is an essential transcription factor for glucagon gene expression (Sander et al., 1997). However, in present study, none of the treatments significantly altered on Pax6, and further studies investigating the actions of these tested drugs on Pax6 expression are merited. Overall, we suggest that glucagon activation might be a hallmark event in the progressive transdifferentiation of alpha to beta cells.

4.5.2 Effects of test compounds on local production of GLP-1 in alpha cells (GLP-1 and PC1/3)

We found the visible level of local GLP- 1 or PC1/3 expression. In line with this, Ye et al., (2015) demonstrated the presence of GLP-1 & glucagon receptors throughout islets claiming that glucagon/GLP-1 signaling could suppress Arx transcription factor in alpha cells. The glucagon signalling also maintained alpha cell growth through controlling proliferation, neogenesis and transdifferentiation events (Ye et al. 2015, 2016). Glucagon gene activation is also associated with the production of glucagon related peptides including GLP-1 (Vasu et al., 2014a; Moffett et al., 2014; Ye et al. 2015). The possible involvement of an increase in the expression of these other pro-glucagon gene products in relation to alpha to beta cell transdifferentiation has been widely reported in recent years (Whalley et al., 2011; Ye et al. 2015; Lee et al., 2018).

4.5.3 Effects of test compounds on beta cells identity markers (Insulin, Pdx-1 and Pax4)

Expression of beta cell identity factor Pdx1 in glucagon producing alpha cells can convert them into insulin producing beta cells (Yang et al., 2011). Nevertheless, in

current study, none of the treatments significantly affected Pdx1 expression. In line with this, another beta cell identity factor Pax4 was reduced by all the test compounds. Notably, alpha cells are considered as lacking Pax-4 and Pdx-1. However, a detectable level of Pax-4 was observed with exendin-4, GABA and taurine. In contrast, previous studies showed ectopic expression Pdx1 or Pax4 in transdifferentiated alpha cells (Collombat et al., 2009; Thorel et al., 2010; Ben-Othman et al., 2017; Lee et al., 2018). Thus, further studies to explore the effects of long-term exposure of the tested drugs on the expression status of Pdx1 and Pax4 are required.

Interestingly, both the insulin gene and insulin hormone expression were detected in alpha cells after exposure of exendin-4. Our findings are consistent with previous reports on incretin peptides used in the context of alpha cells transdifferentiation (Lee et al. 2018; Zang et al., 2019). It is also noteworthy that according to previous reports alpha cell transdifferentiation is a chronological phenomenon (Thorel et al., 2010; Lee et al. 2018; Zang et al., 2019). Moreover, some evidence suggests that the early deactivation of Arx can induce Pax4 to induce insulin gene expression, while glucagon can be deactivated later (Wilcox et al., 2013). Therefore, the long-term actions of tested compounds are validated to assess chronological activation or inhibition of alpha to beta identity markers.

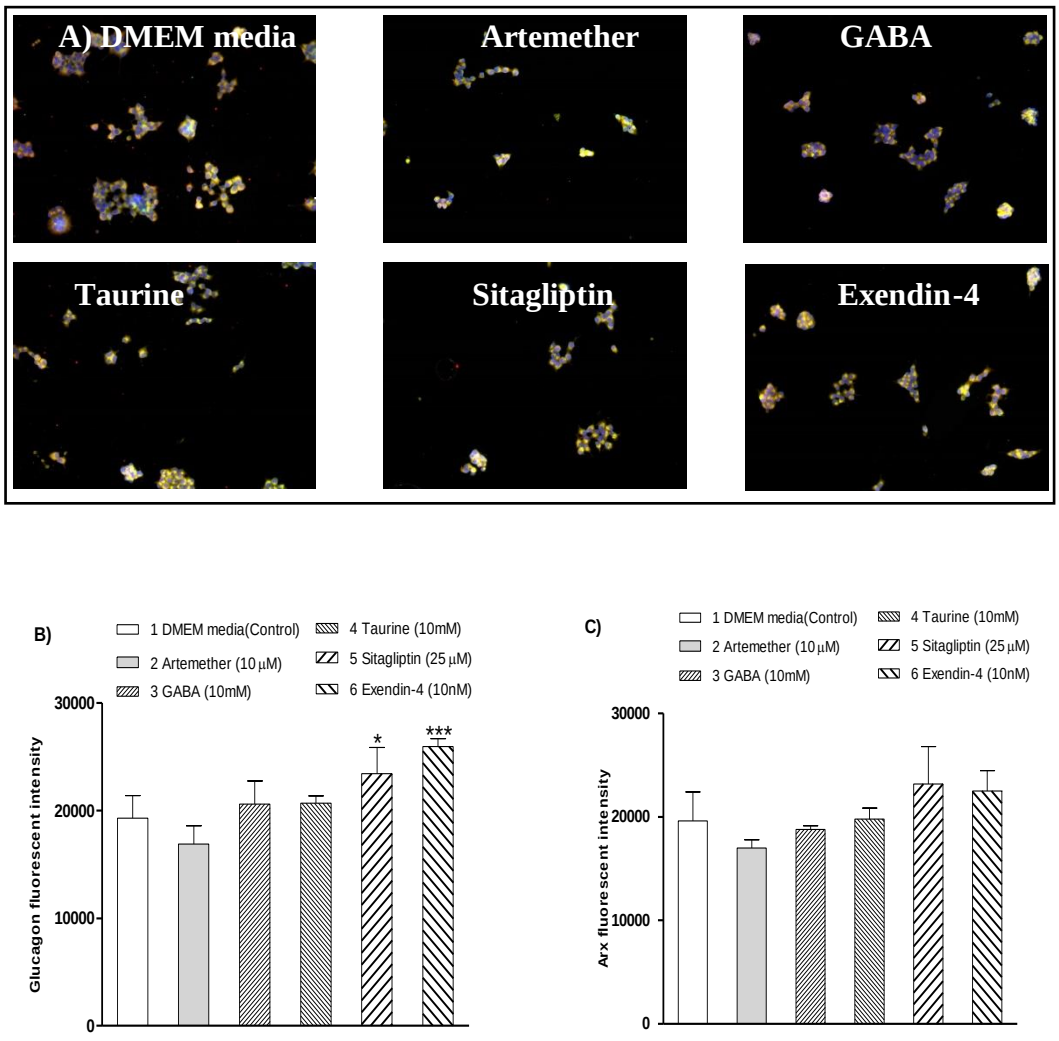
4.5.4 Effects of test compounds on the viability of cells

Importantly, GABA, taurine, sitagliptin and exendin-4 showed no toxic effect on the viability of alpha cells at the concentrations tested. Interestingly, artemether induced a reducing trend in intensity, gene expression and cellular concentration of glucagon. Although Li et al. (2017) showed effect of artemether favours reduction of glucagon and Arx, this was contradicted in a later study by Van et al, (2018). This demonstrated that artemether reduced identity markers of all the islet cells including beta cells and alpha cells. In line with this, we observed that the artemether reduced gene expression of Arx, glucagon, insulin and Pax4. Therefore, our findings strengthen the outcomes and conclusions of Van et al, (2018), suggesting that their findings are secondary to a toxic effect of artemether. However, further studies using different doses are required to explore the toxic effects of artemether on alpha cells.

4.5.5 Concluding remarks:

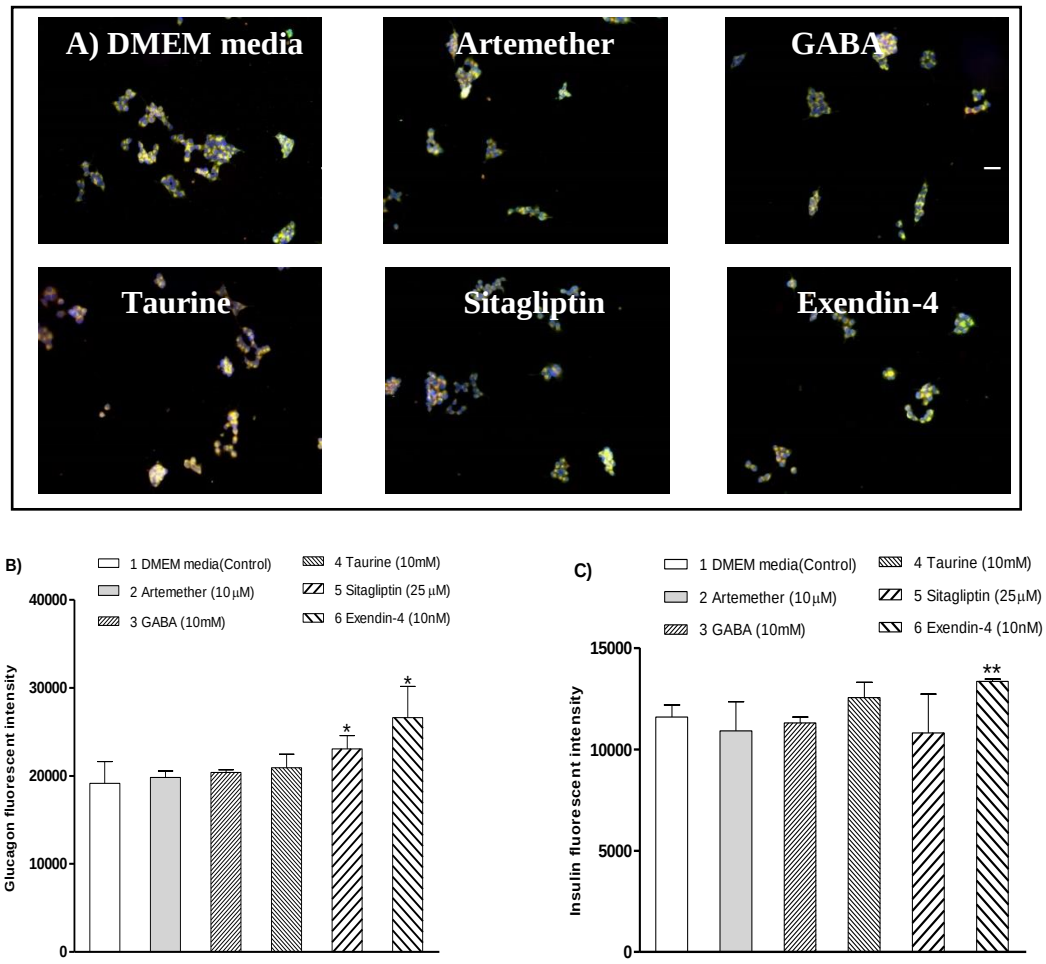
Lee et al. (2018) observed presence of immature bihormonal cells and fully transdifferentiated alpha to beta cells after exendin-4 for 2 and 7 days respectively. This highlights again a chronology of transdifferentiation events. In present study, only exendin-4 was able to induce insulin expression over a 3 day period. Sitagliptin showed negligible detection of insulin expression. We could speculate that sitagliptin might be protecting local GLP-1 and that GLP- 1 may enhancing insulin activation. Therefore, we suggest that increased GLP-1 signaling using exogenous GLP-1 receptor analogues might play a role in alpha cell transdifferentiation. GABA, taurine and sitagliptin showed increased glucagon expression therefore we speculate that alpha cells may be preparing for either proliferation or immatutre transdifferentiation. Nevertheless, further studies employing a longer period of exposure and with different concentrations of artemether, GABA, taurine and sitagliptin would be required to the effects of transdifferentiation of alpha TC cells in the present system. However, no cell line can replicate the properties of primary alpha cells within islets, suggesting that *in vitro* culture is a convenient but poor substitute for direct *in vivo* measurements.

Figure 4.1 Chronic (72 h) *in vitro* effect of various therapeutic agents on immunostaining for glucagon and Arx protein expression in relation to alpha cells transdifferentiation into beta cells.



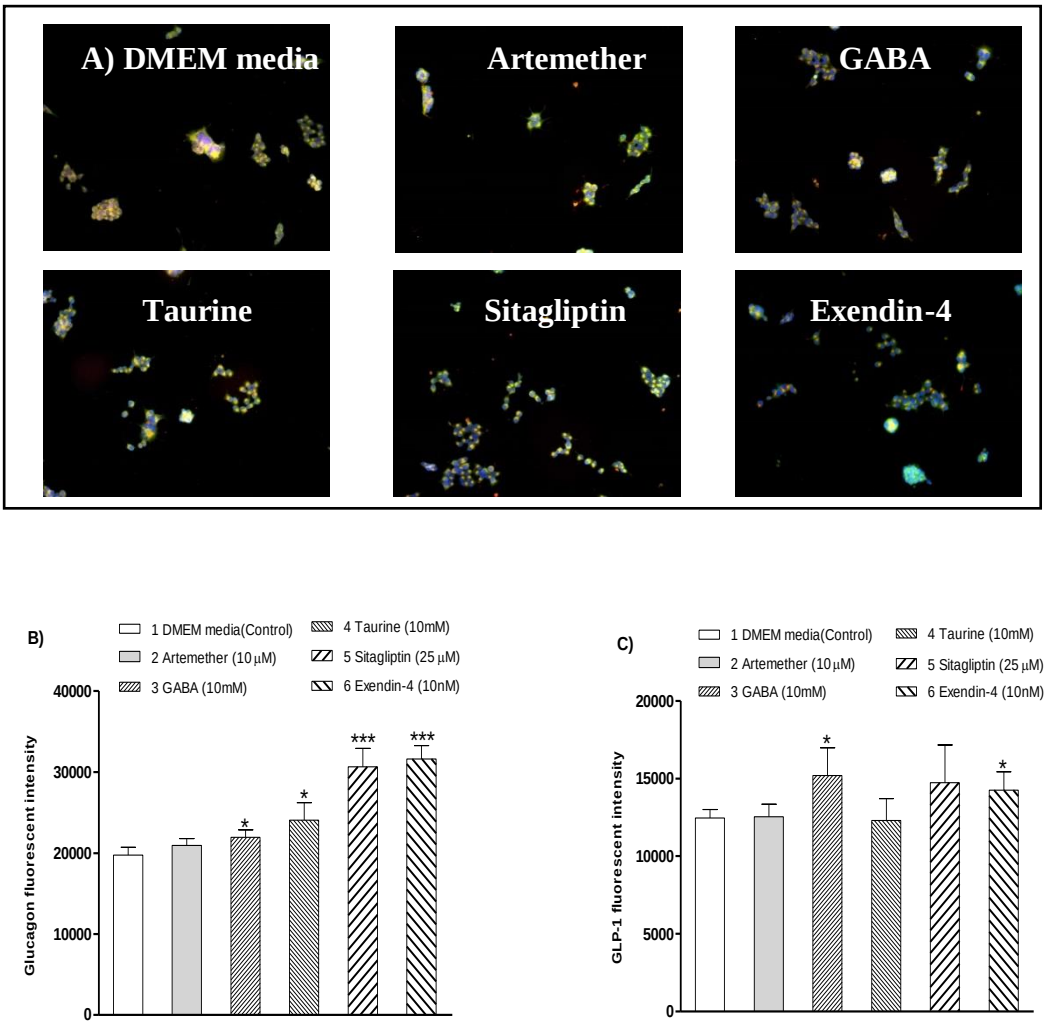
Effects of treatments on glucagon and Arx protein immunoreactivity in α-TC 1.9 cells. Representative images (A) showing immunostaining for glucagon (green) and Arx (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. The immunoreactivity for each protein was measured using fluorescent microscope. The fluorescent intensity for each protein was obtained under FITC filter (Glucagon: Green channel; 488 nm) or TRITC filter (Arx: Red channel; 594 nm), and expressed as fluorescent intensity of glucagon or Arx, respectively. Values represent means ± SEM for n=4; ~ 20,000 cells were analysed for each condition. *p<0.05 and ***p<0.001 compared to DMEM media. Scale bars: 50 μm.

Figure 4.2 Chronic (72 h) *in vitro* effect of various therapeutic agents on immunostaining for glucagon and insulin protein expression in relation to alpha cells transdifferentiation into beta cells



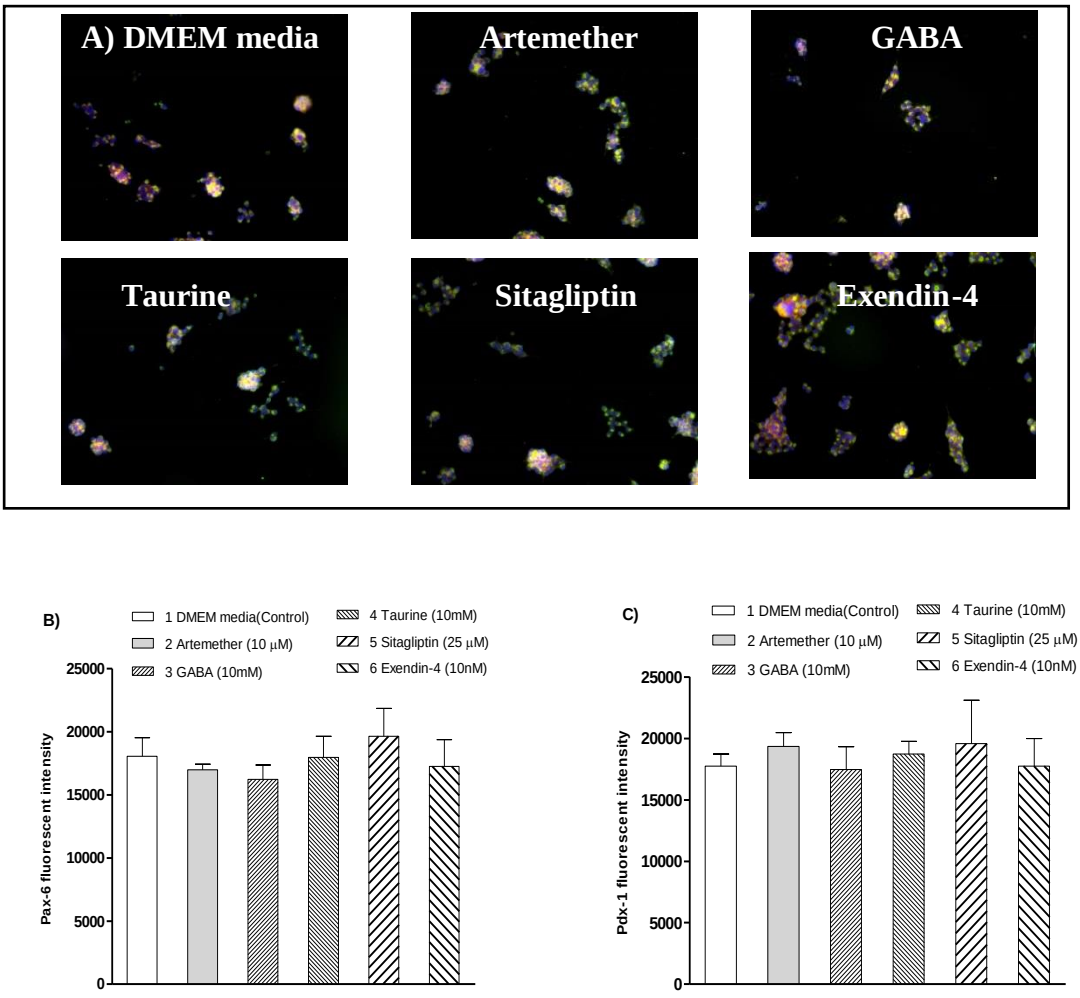
Effect of treatments on glucagon and insulin protein immunoreactivity in α-TC 1.9 cells. Representative images (A) showing immunostaining for glucagon (green) and insulin (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. The immunoreactivity for each protein was measured using fluorescent microscope. The fluorescent intensity for each protein was obtained under FITC filter (Glucagon: Green channel; 488 nm) or TRITC filter (Insulin: Red channel; 594 nm), and expressed as fluorescent intensity of glucagon or insulin, respectively. Values represent means ± SEM for n=4; ~ 20,000 cells were analysed for each condition. *p<0.05 and ** p<0.01 compared to DMEM media. Scale bars: 50 μm.

Figure 4.3 Chronic (72 h) *in vitro* effect of various therapeutic agents on immunostaining for glucagon and GLP-1 protein expression in relation to alpha cells transdifferentiation into beta cells.



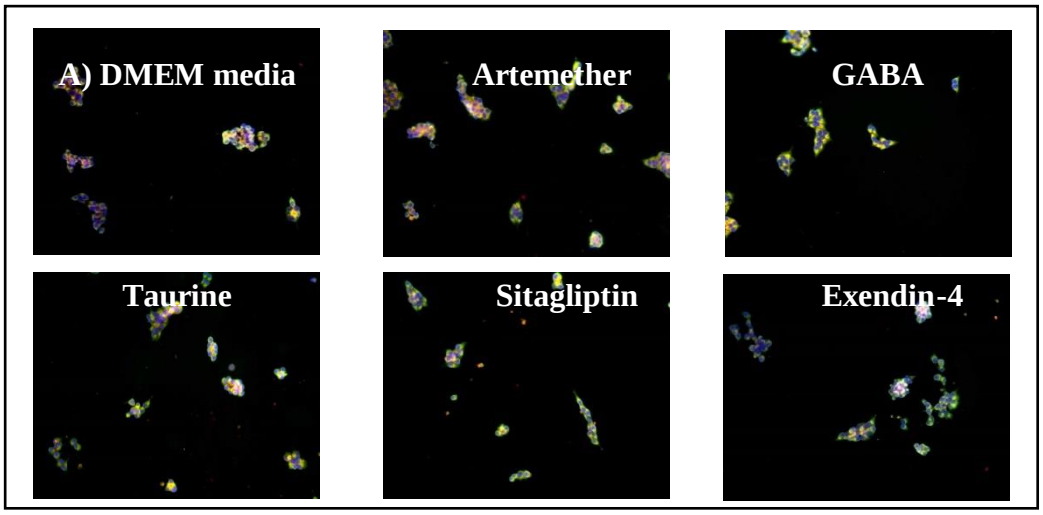
Effect of treatments on glucagon and GLP-1 protein immunoreactivity in α-TC 1.9 cells. Representative images (A) showing immunostaining for glucagon (green) and GLP-1 (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. The immunoreactivity for each protein was measured using fluorescent microscope. The fluorescent intensity for each protein was obtained under FITC filter (Glucagon: Green channel; 488 nm) or TRITC filter (GLP-1: Red channel; 594 nm), and expressed as fluorescent intensity of glucagon or GLP-1, respectively. Values represent means ± SEM for n=4; ~ 20,000 cells were analysed for each condition. *p<0.05 and ***p<0.001 compared to DMEM media. Scale bars: 50 µm.

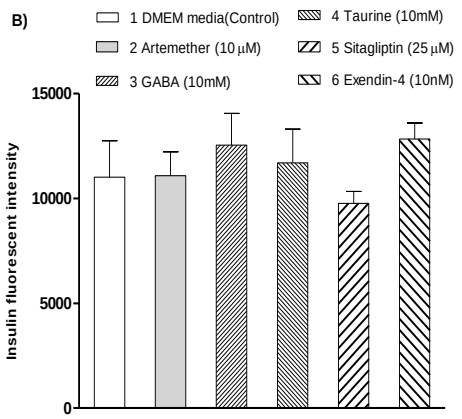
Figure 4.4 Chronic (72 h) *in vitro* effect of various therapeutic agents on immunostaining for Pax-6 and Pdx-1 protein expression in relation to alpha cells transdifferentiation into beta cells.

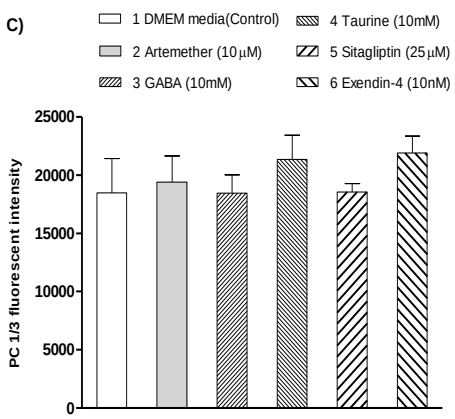


Effect of treatments on Pax 6 and Pdx-1 protein immunoreactivity in α-TC 1.9 cells. Representative images (A) showing immunostaining for Pax-6 (green) and Pdx-1 (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. The immunoreactivity for each protein was measured using fluorescent microscope. The fluorescent intensity for each protein was obtained under FITC filter (Pax-6: Green channel; 488 nm) or TRITC filter (Pdx-1:Red channel; 594 nm), and expressed as fluorescent intensity of Pax-6 or Pdx-1, respectively. Values represent means ± SEM for n=4; ~ 20,000 cells were analysed for each condition. Scale bars: 50 µm.

Figure 4.5 Chronic (72 h) *in vitro* effect of various therapeutic agents on immunostaining for insulin and PC1/3 protein expression in relation to alpha cells transdifferentiation into beta cells.

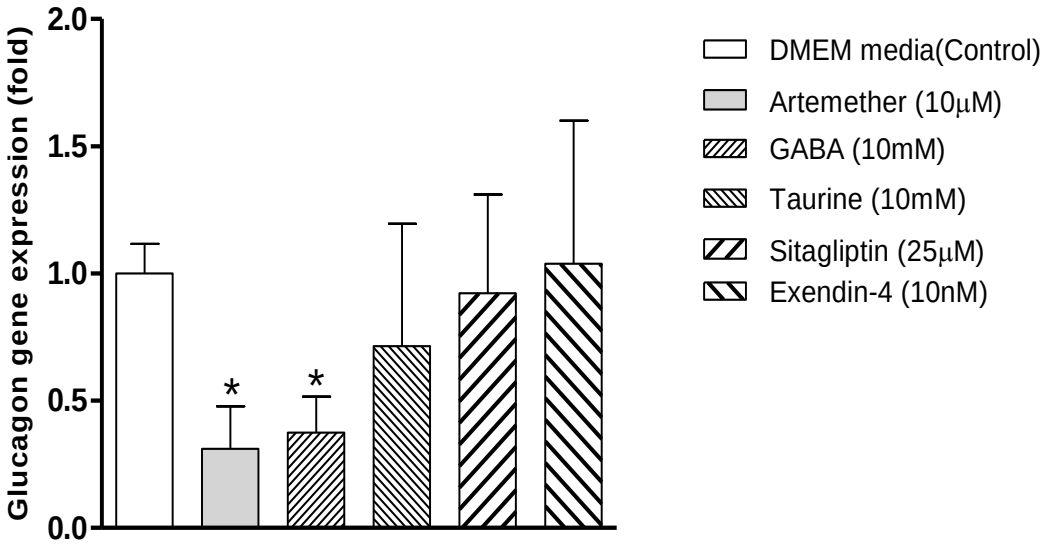






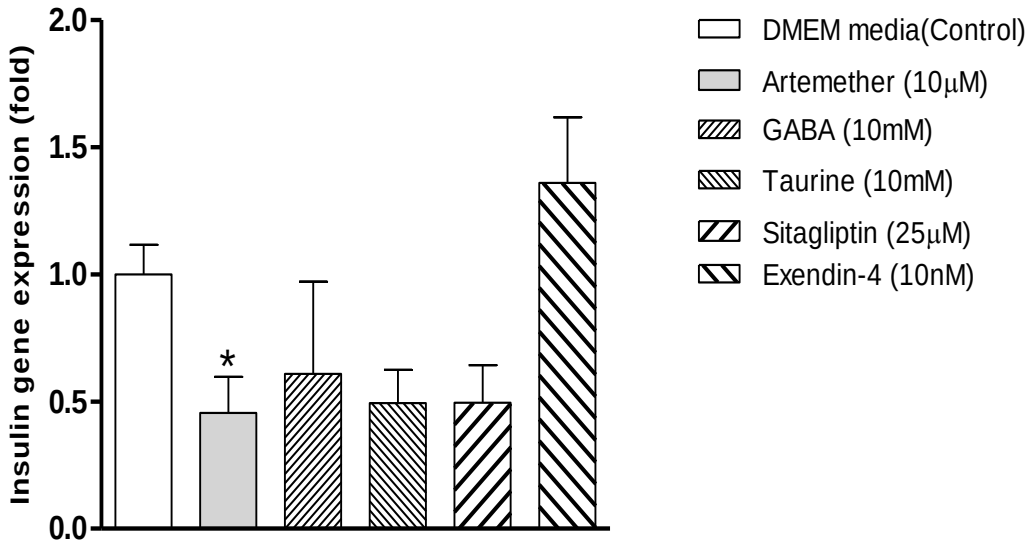
Effect of treatments on insulin and PC1/3 protein immunoreactivity in α-TC 1.9 cells. Representative images (A) showing immunostaining for insulin (green) and PC1/3 (red). Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. The immunoreactivity for each protein was measured using fluorescent microscope. The fluorescent intensity for each protein was obtained under FITC filter (Insulin: Green channel; 488 nm) or TRITC filter (PC1/3: Red channel; 594 nm), and expressed as fluorescent intensity of insulin or PC1/3, respectively. Values represent means ± SEM for n=4; ~ 20,000 cells were analysed for each condition. Scale bars: 50 μm.

Figure 4.6 Chronic (72 h) *in vitro* effect of various therapeutic agents on the alpha cell-specific glucagon gene expression in the context of alpha cells transdifferentiation into beta cells.



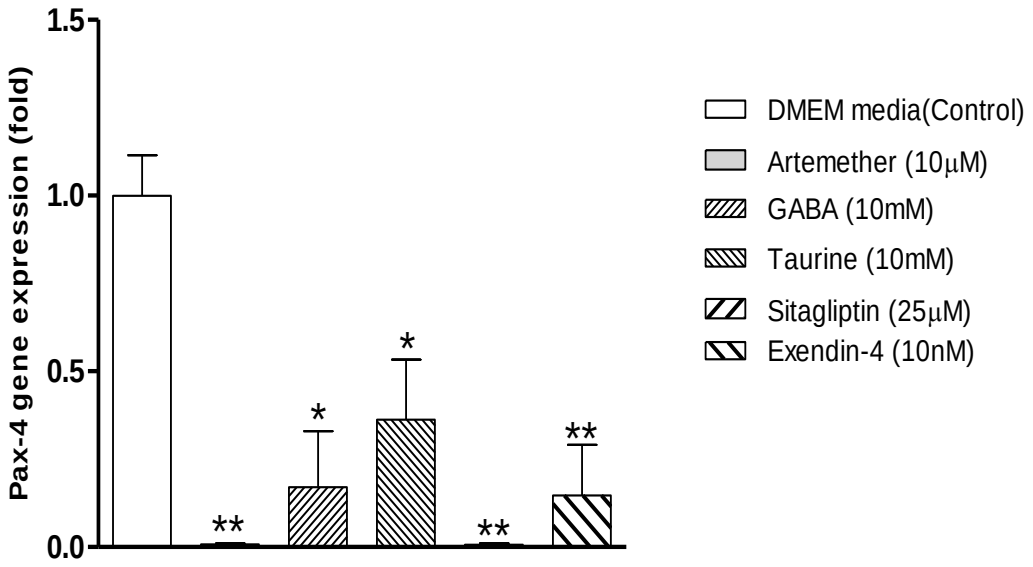
Effect of treatments on glucagon gene expression in α-TC 1.9 cells. Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. In the end, cells were lysed using Trizol reagent, mRNA was extracted from the lysed cells, and cDNA was prepared. The relative mRNA expression of the glucagon gene was measured using RT-PCR. The gene expression was normalized to β-actin reference gene and expressed as relative gene expression (fold).Values are mean ± SEM (n=3). *$p<0.05$ compared to DMEM media.

Figure 4.7 Chronic (72 h) *in vitro* effect of various therapeutic agents on the beta cell-specific insulin gene expression in the context of alpha cells transdifferentiation into beta cells.



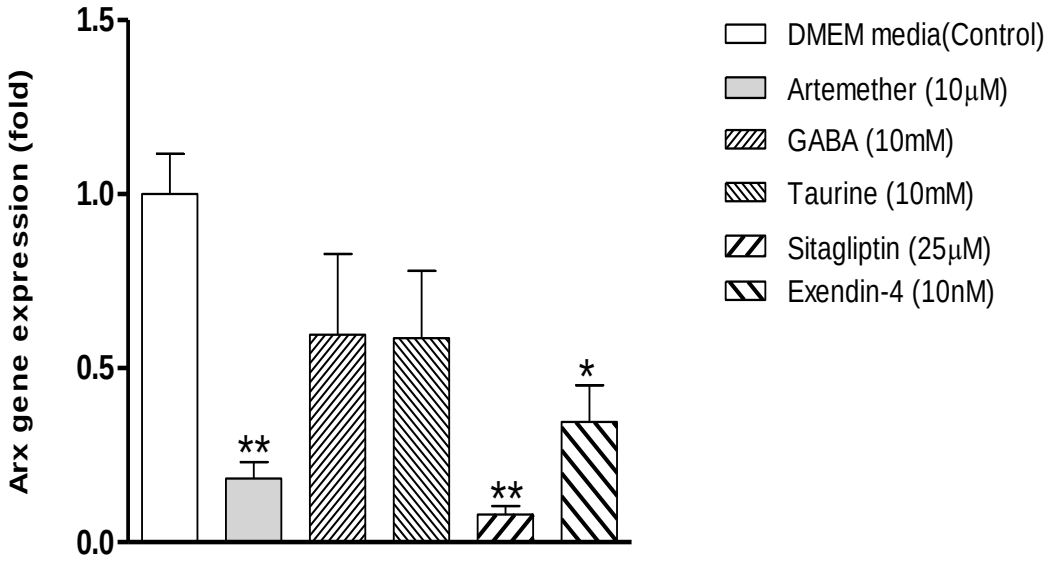
Effect of treatments on insulin gene expression in α-TC 1.9 cells. Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. In the end, cells were lysed using Trizol reagent followed by mRNA was extracted from the lysed cells, and cDNA was prepared. The relative mRNA expression of the insulin gene was measured using RT-PCR. The gene expression was normalized to β-actin reference gene and expressed as relative gene expression (fold).Values are mean ± SEM (n=3). *$p<0.05$ compared to DMEM media.

Figure 4.8 Chronic (72 h) *in vitro* effect of various therapeutic agents on the beta cell-specific Pax-4 gene expression in the context of alpha cells transdifferentiation into beta cells.



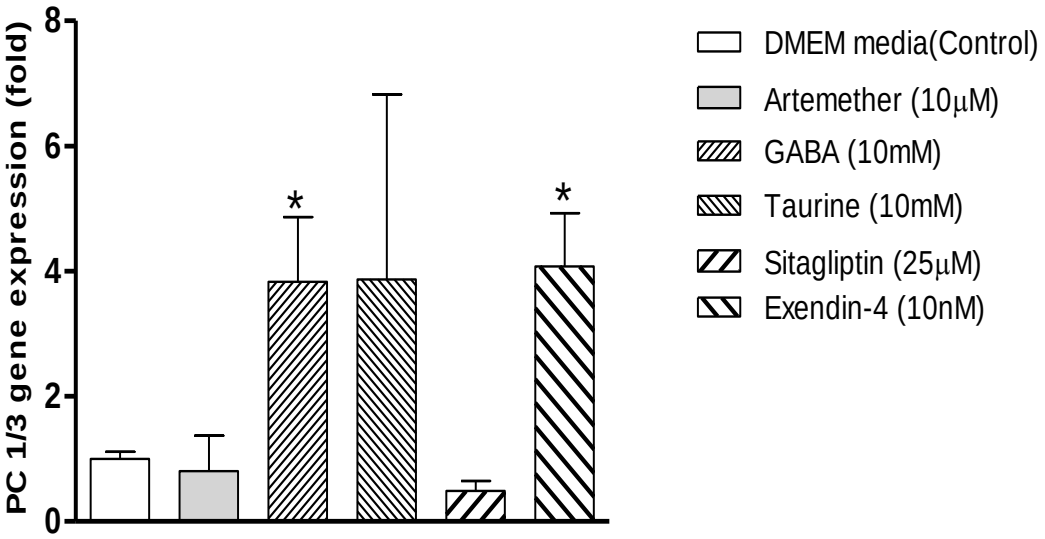
Effect of treatments on Pax-4 gene expression in α-TC 1.9 cells. Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. In the end, cells were lysed using Trizol reagent followed by mRNA was extracted from the lysed cells, and cDNA was prepared. The relative mRNA expression of the Pax-4 gene was measured using RT-PCR. The gene expression was normalized to β-actin reference gene and expressed as relative gene expression (fold).Values are mean ± SEM (n=3). *$p<0.05$ and **$p<0.01$ compared to DMEM media.

Figure 4.9 Chronic (72 h) *in vitro* effect of various therapeutic agents on the alpha cell-specific Arx gene expression in the context of alpha cells transdifferentiation into beta cells.



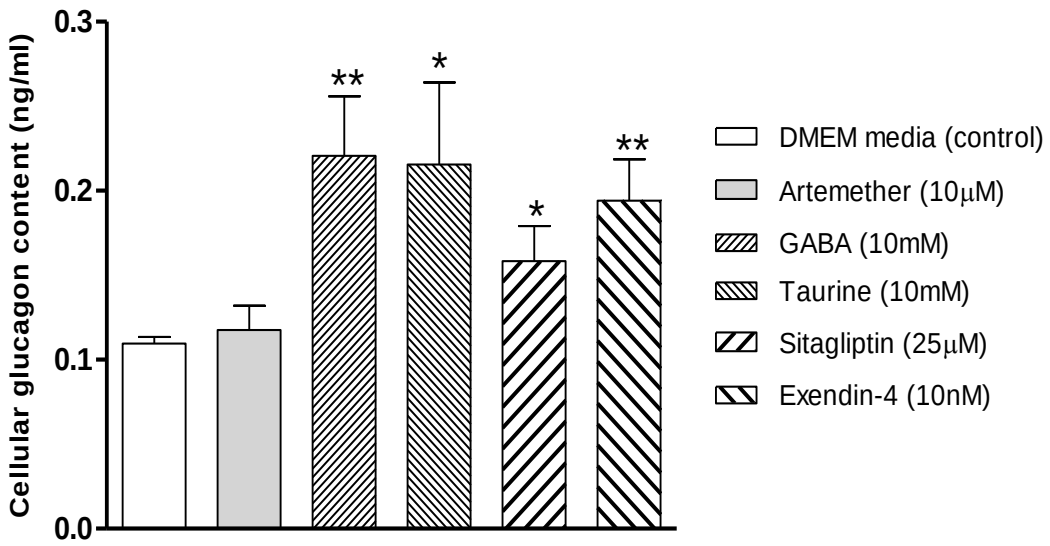
Effect of treatments on Arx gene expression in α-TC 1.9 cells. Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. In the end, cells were lysed using Trizol reagent followed by mRNA was extracted from the lysed cells, and cDNA was prepared. The relative mRNA expression of the Arx gene was measured using RT-PCR. The gene expression was normalized to β-actin reference gene and expressed as relative gene expression (fold).Values are mean ± SEM (n=3). *$p<0.05$ and **$p<0.01$ compared to DMEM media.

Figure 4.10 Chronic (72 h) *in vitro* effect of various therapeutic agents on the alpha cell-specific PC1/3 gene expression in the context of alpha cells transdifferentiation into beta cells.



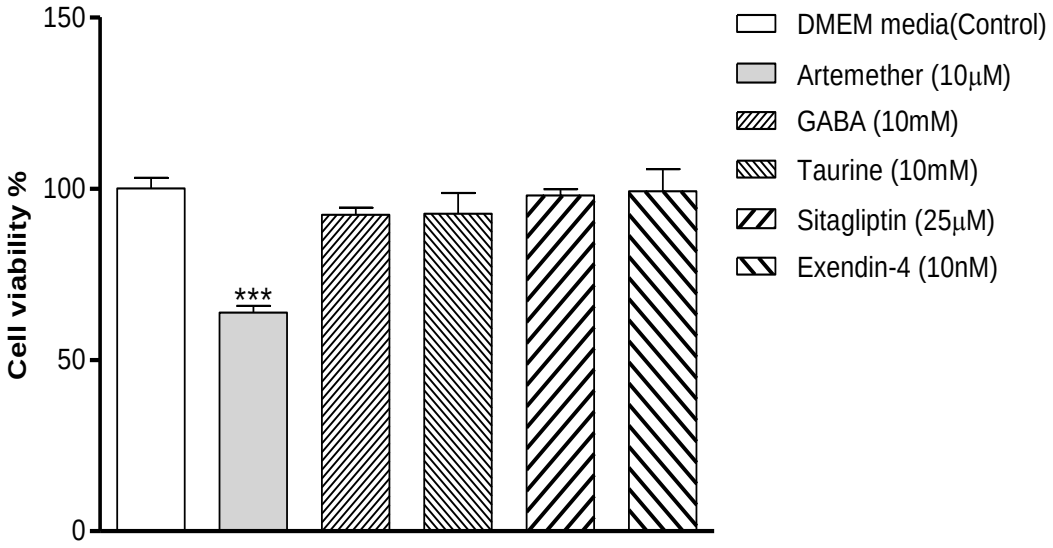
Effect of treatments on PC1/3 gene expression in α-TC 1.9 cells. Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. In the end, cells were lysed using Trizol reagent followed by mRNA was extracted from the lysed cells, and cDNA was prepared. The relative mRNA expression of the PC1/3 gene was measured using RT-PCR. The gene expression was normalized to β-actin reference gene and expressed as relative gene expression (fold). *$p<0.05$ compared to DMEM media (control). Values are mean ± SEM (n=3).

Figure 4.11 Effect of different therapeutic agents on the cellular glucagon content from α-TC 1.9 cells



Effect of treatments on cellular glucagon content from α-TC 1.9 cells. Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. In the end, cell viability was assessed using chemiluminescent glucagon ELISA, and expressed as cellular glucagon content (ng/ml).Values are mean ± SEM (n=8). *$p<0.05$ and **$p<0.01$ compared to DMEM media.

Figure 4.12 Effect of different therapeutic agents on the viability of α-TC 1.9 cells, as assessed by MTT assay



Here, α-TC 1.9 cells were cultured at 37^0C for 72 h in DMEM media supplemented with artemether, GABA, taurine, sitagliptin, and exendin-4 respectively. In the end, cell viability was assessed using MTT assay and expressed as percentage. Values are mean ± SEM (n=8). ***$p<0.001$ compared to DMEM media.

Chapter 5

Effects of taurine and artemether on islet morphology and alpha-cell transdifferentiation in insulin-deficient diabetic $Glu^{CreERT2}$; ROSA26-eYFP mice

5.1 SUMMARY

Taurine is a conditional amino acid, has widely been known as ameliorating neuropathy and hyperglycaemia, but little is known about its actions on beta cells regeneration. Moreover, artemether an anti-malarian drug has recently been identified as regenerating beta cells from transdifferentiation of alpha cells. However, to gain more knowledge about these two small molecules in the context of alpha to beta cell transition, here we used alpha cell lineage strain $Glu^{CreERT2}$; ROSA26-eYFP mice.

The 10 days supplementation of taurine (5% in drinking water) and artemether (100 mg/kg) did not show beneficial changes in body parameters but showed decreased plasma glucagon level. Interestingly taurine significantly enhanced pancreatic beta cell proliferation and protected from apoptotic loss. Alpha cell lineage study showed taurine showed suppression of glucagon expression in GFP labelled alpha cells ($Glucagon^{negative}/GFP^{positive}$ cells). Moreover, it also showed an increased number of bihormonal cells that carries both glucagon and insulin hormones ($Glucagon^{positive}/Insulin^{positive}$ cells). Finally, all the streptozotocin treated islets showed increased transdifferentiated alpha to beta cells ($GFP^{positive}/Insulin^{positive}$ cells), interestingly further significant augmentation observed with taurine exposure. According to one study, an artemether showed transdifferentiating potential, but here we found no effect on transdifferentiation. Moreover, artemether was also found to be neutral on islet morphology.

Here we showed that taurine could promote the growth of survived beta cells and prevent their further loss. However, it showed no effect on alpha cell proliferation or apoptosis yet it showed a reduction in the alpha cell area. Therefore, we suggest that taurine helps to regenerate beta cells from both the origins such as alpha cells as well as pre-existing beta cells. On contrast, 10 days treatment with the artemether failed to induce alpha cell transdifferentiation. However, earlier studies showed its potential to induce alpha to beta conversion, we suggest that acute dosing of artemether could not induce alpha cell transdifferentiation.

5.2 INTRODUCTION

Pancreatic beta-cell loss has been frequently observed in both type 1 and type 2 diabetes (Rhodes et al., 2005; Tomita, 2017). Due to depletion of insulin hyperglycaemia develops. During embryonic development, progenitor cells develop into fully differentiated cells (Van et al., 2015). Therefore, the islets are comprised of 85-90 % of beta cells, ~10-15% alpha cells, and least delta or PP cells. Only beta cells are genetically specialized for insulin production and secretion. Mature beta cells possess active gene *INS* to produce insulin. This gene is regulated by transcription factors, namely Pdx1, Pax4, NKx6.2, Foxo1, and MafA. In contrast, alpha cells are specialized to produce and secrete glucagon. Mature alpha cells possess active gene *GCG* which is controlled by Arx, Pax6, MafB, and Dnmt1 (Van et al., 2015). However, the phenotype of pancreatic islet cells is not rigid and they possess genetic plasticity (Lu et al., 2014). Recent studies have identified that such mature cells have a transdifferentiation potential which is induced after the loss of neighbouring cells. It has been observed that the reprogramming of alpha cells into insulin-producing cells can occur after the loss of beta-cell mass (Collombat et al., 2009; Bramswig et al., 2013; Choudhary et al., 2014; Lee et al., 2018).

In recent studies, induction of Pax-4-loss-of function in mice favoured expansion of alpha cell mass at the expense of beta cells. Conversely, Arx deficient mice showed loss of alpha cell identity accompanied by a rise in beta cells. Similarly, ectopic expression of Pdx1 in progenitor cells favoured beta cells at the expense of alpha cells. By using cell lineage tracing, studies have shown that near-complete ablation of beta cells triggered transdifferentiation of alpha cells into beta cells (Lu et al., 2014). Another study using pancreatic duct ligation and alloxan treated mice showed that beta cells originated from alpha cells. Initially, Maf B transcription factor was expressed in these newly formed beta cells, but later Maf A was appeared, suggesting that alpha cells gradually adopted the identity of beta cells. Moreover, Men1 disruption specifically in alpha cells caused alpha cells to display characteristics of beta cells (Lu et al., 2014).

Taking this into consideration, it is important to investigate the reprogramming potential of modulating alpha cells to generate of beta cells. It might be possible to reprogram alpha cells without genetic modifications (Xu et al., 2016; Ackermann et

al., 2018). Small molecules are low mass chemical entities that demonstrate novel benefits and potential in determining cell fates. Small molecules exert a rapid biological action. They may reversibly interact with the target protein. The structural stability and lower cost of small molecules make them more applicable than synthetic peptides. Their action could be enhanced by modifying structure or dose. Small molecules can effectively regulate cellular pathways and gene expression (Xu et al., 2016; Ackermann et al., 2018).

The conversion of the alpha cells using drug based-genetic modulation to generate beta cells is as yet controversial. Earlier studies reported that screening of 30,710 small molecules using mouse alpha cell line, identified BRD 7389 and GW8510 with epigenetic potential. BRD 7389 and GW8510 molecules inhibited of RSK kinase and upregulated p53 transcription that induces insulin gene in alpha cells, respectively (Dina et al., 2010; Fomina-Yadlin et al., 2012; Choudhary et al., 2014). Another study using alpha cell and zebrafish islets, screened about 280 clinically approved small molecules to identify potential to manipulate alpha cells. Interestingly, they found that GABA and anti-malarian drug artemisinin downregulated ARX gene express in alpha cells via $GABA_A$ receptor thereby, promotes conversion to beta cells (Ben-Othman et al., 2017; Li et al., 2017). In addition, another study using streptozotocin-induced diabetes in C57BL/6J mice showed that combined treatment with GABA and sitagliptin promoted beta cell regeneration (Liu et al., 2017). In contrast, more recent studies reported that GABA and malarian drug artesunate failed to transform alpha cells into beta cells (Ackermann et al., 2018; Van et al., 2018). The distinct mechanism of alpha cell reprogramming using small drugs is, however, not yet clear, but we assume that remodeling alpha cells with small drugs might potentially promote the regeneration of beta cells.

It remains unclear whether new beta cells originates from pre-existing beta cells or alpha cells that can be promoted by transdifferentiation using small molecules (Lee et al, 2018; Thorel et al, 2010). To discriminate the two scenarios, we utilized $Glu^{CreERT2}$; ROSA26-eYFP mice that allowed specific detection of alpha cells (Quoix et al., 2007). Multiple-dose of streptozotocin model was used to induce beta-cell damage (Vasu et al., 2014a; Moffett et al., 2014). The effect of small molecules on the regenerative capacity of alpha cells was evaluated.

Taurine is sulfur-containing intracellular amino acid (Bustamante et al., 2001). An earlier report showed that taurine can promote insulin secretion and reduce hyperglycaemia (Carneiro et al., 2009; Ribeiro et al., 2009; Batista et al., 2012). Although the main source of taurine is the diet, it was believed to be present in pancreatic alpha cells (Santora et al., 2011). In nerve cells, taurine exerts a similar effect as GABA neurotransmitter via $GABA_A$ or glycine receptors (Horikoshi et al., 1988; Furukawa et al., 2014). However, both receptors are also found on alpha cells (Li et al., 2013); it is interesting to investigate the effect of taurine on the regenerative capacity of alpha cells and recently artemether showed alpha to beta transdifferentiation. Artemether, similar to GABA has been shown to bind gephyrin to suppress Arx and thereby promote alpha to beta reprogramming (Ben-Othman et al., 2017; Li et al., 2017). However, this hypothesis was contradicted by another study showing that artemether is not reprogramming alpha cells (Ackermann et al., 2018; Van et al., 2018). Therefore, it is interesting to confirm the role of artemether on transdifferentiation of alpha cells.

5.3 MATERIALS AND METHODS

Materials and methods for this study have been discussed in Chapter 2.

5.4 RESULTS

5.4.1 Effect of taurine and artemether on body parameters

Streptozotocin- treated $Glu^{CreERT2}$; ROSA26 e-YFP mice alone and in combination with taurine or artemether showed significant ($p<0.05$ to $p<0.001$) reduction in body weight (Figure 5.1A). This was consistent with AUC values shown in Figure 5.1B. In addition, diabetic mice displayed a significant increase in food intake compared with control mice (Figure 5.1C). Taurine had no significant effect on food intake (Figure 5.1C). As would be expected, streptozotocin significantly ($p<0.01$ to $p<0.001$) evoked high blood glucose when compared with the saline group (Figure 5.2A). Both taurine and artemether were unaltered increased blood glucose (Figure 5.2A). This

was corroborated by unchanged overall AUC measures (Figure 5.2B). In addition, fluid intake was also significantly ($p<0.01$ to $p<0.001$) increased in all diabetic groups, except those treated with taurine at some observational points, compared to saline mice (Figure 5.3C). In harmony, plasma insulin was significantly ($p<0.01$ to $p<0.001$) decreased, and it did not alter after administration of the treatments (Figure 5.3A). Similarly pancreatic insulin content was significantly ($p<0.05$ to $p<0.01$) lowered in all diabetic groups compared to saline control (Figure 5.3A, B). The plasma glucagon concentration was significantly ($p<0.05$ to $p<0.01$) decreased while pancreatic glucagon content was unaltered in all diabetic groups compared to saline controls (Figure 5.3C, D).

5.4.2 Effects of taurine and artemether on islet morphology

Figure 5.4A shows the representative islets from $Glu^{CreERT2}$; ROSA26-eYFP mice illustrating an immunoreactivity for insulin and glucagon. As expected, streptozotocin-treated mice islets treated with saline or combination of treatments showed a significant ($p<0.1$ to $p<0.001$) decrease in islet area, plus the number of islets when compared to saline control (Figure 5.4B, C). The tail pancreas of all groups appeared to consist of a greater number of islets compared to head part of the islet (Figure 5.4B). Islet size distribution was negatively affected by streptozotocin alone and in combination with artemether. The percentage of small size islets increased and large size islets disappeared compared with respective saline groups. However, large-sized islets were found to be slightly recovered in taurine-treated mice (Figure 5.4D). After streptozotocin administration and injection of treatments, a dramatic ($p<0.01$ to $p<0.001$) loss of islet beta cells was observed as compared to the saline group. Interestingly, taurine significantly ($p<0.01$) augmented beta cell area (Figure 5.5A). Along with the loss of beta cells, alpha cell area was significantly increased with all treatments when compared to saline islets. Taurine reduced alpha cell area ($p<0.01$) compared to STZ diabetic mice (Figure 5.5B)

5.4.3 Effects of taurine and artemether on proliferation and apoptosis of beta and alpha cells

Representative islets are shown in Figure 5.6A and 5.7A, displaying immunoreactivity for Ki67/insulin and TUNEL/insulin. Interestingly taurine significantly ($p<0.01$) augmented beta-cell proliferation when compared with both saline mice and STZ mice (Figure 5.6B). As expected, beta-cell apoptosis was significantly ($p<0.05$ to $p<0.01$) higher in streptozotocin treated mice irrespective of treatment (Figure 5.7B). Intriguingly, it was further suppressed remarkably ($p<0.05$) by taurine treatment compared with respective diabetic control (Figure 5.7B).

Representative islets, depicted in Figures 5.8A and 5.9A highlight staining for Ki67/glucagon and TUNEL/glucagon, respectively. As compared to the saline group, alpha cell proliferation was significantly ($p<0.05$ to $p<0.01$) higher than in all other treatment groups (Figure 5.8B). Importantly, there was a tendency of reduction in alpha cell proliferation after taurine supplementation relative to STZ treated animals (Figure 5.8B). Finally, alpha cell apoptosis remained unaltered in all treatment groups compared with respective non-diabetic control (Figure 5.9B).

5.4.4 Effects of taurine and artemether on alpha cell lineage tracing

Here, Glucagon+/GFP+ colocalized cells & Glucagon+/GFP- cells represent undifferentiated alpha cells, and Glucagon-/GFP+ cells represent reprogrammed alpha cells. Figure 5.10A shows representative islets for GFP and glucagon staining. It was reported that about 50-76 % of alpha cells were found to be coexpressed GFP along with glucagon (Figure 5.10B). This is congruous with about 24-50% the expression of only glucagon positive cells observed in these mice (Figure 5.10C). Intriguingly, only GFP positive cells were significantly ($p<0.05$ to $p<0.001$) increased with almost all streptozotocin treated groups when compared with saline-treated mice (Figure 5.10D). In addition, this expression was further significantly ($p<0.05$) enhanced after taurine supplementation in comparison with STZ-treated diabetic animals (Figure 5.10D).

5.4.5 Effect of taurine and artemether on generation of bihormonal cells

Figure 5.11A shows the representative islets for containing insulin and glucagon staining. Interestingly, all treatment groups had significantly ($p<0.05$ to $p<0.01$) increased bihormonal cells coexpressing insulin/glucagon compared with that of the control group, Importantly, taurine treated mice showed a trend of increasing bihormonal cells compared with STZ mice (Figure 5.11B).

5.4.6 Effect of taurine and artemether on alpha to beta transdifferentiation

To address the possibility that alpha cells represent a source of beta cell regeneration, we analysed fluorescently labeled alpha cells (GFP+ cells) coexpressing insulin. Representative images depicting GFP/insulin containing islets mice are displayed in Figure 5.12A. Interestingly, beta-cell ablation by streptozotocin alone or in combination with treatments significantly increased the percentage of GFP+/insulin+ co-localised cells compared to the saline group. Furthermore, taurine was found to significantly ($p<0.05$ to $p<0.01$) enhance this transdifferentiation in both the head and tail regions of the pancreas when compared to STZ group (Figure 5.12B).

5.5 DISCUSSION

Breakthroughs in cell differentiation offered new insights for understanding cellular events occurring during diabetes. It is believed that fully differentiated mature cells such as pancreatic alpha cells retain some level of plasticity. As such, the genomic flexibility of alpha cells makes them guardians of beta cells during the islet architectural crisis. Here we demonstrate distinct evidence for alpha to β cells transdifferentiation occurring naturally in multiple low dose streptozotocin-induced beta-cell loss, and astonishingly this was further augmented by treatment with taurine, but not by artemether. In addition, taurine augmented beta-cell proliferation and suppressed beta-cell apoptosis. However, artemether was found to be ineffective in affecting beta cell regeneration. Therefore, we suggest that taurine might play an important role in establishing beta-cell mass by promoting alpha to beta-cell transition and partially encouraging proliferation or survival of pre-existing beta cells.

5.5.1 Effects of treatments on body parameters

Streptozotocin-induced severe diabetes together with increased food intake and body weight loss. Taurine supplementation had a little effect on body weight but the hyperphagia was greatly suppressed. Thus, taurine but not artemether might have a tendency to prevent hyperphagia in uncontrolled diabetes. As expected, streptozotocin selectively demolished beta cells thereby resulting in loss of insulin-dependent glucose hyperglycaemia. Indeed, both taurine and artemether had no beneficial effect on blood glucose, plasma insulin and pancreatic insulin content in STZ diabetic mice. During uncontrolled diabetes, gluco-lipo toxicity affects adversely its beta-cell secretory response (Vasu et al., 2014a; Moffett et al., 2015a). Earlier reports have suggested that long-term supplementation of taurine has hypoglycaemic action (Carneiro et al., 2009; Ribeiro et al., 2009; Batista et al., 2012). Some evidence inferred that artemether abolishes glucose-induced insulin secretion in beta cells (Van et al., 2018). However, further studies of its chronic action of taurine and artemether are required to evaluate beneficial effects on the prevention of weight loss and hyperglycaemia.

5.5.2 Effects of treatments on islet morphology

Streptozotocin-targeted removal of beta cells critically affects the islet architecture, and therefore, it is often associated with the hyperplasia of alpha cells. As such, we observed an uncontrolled loss of beta cells did exhibit major alterations in islet structure. Therefore, the small size and irregularly shaped islets appeared in the vicinity. Interestingly, taurine supplementation in diabetic animals augmented beta-cell area. This was also reflected in the enriched distribution of medium and large size islets with a greater number of overall islets. It is supposed that the loss of communication by the majority of beta cells generally triggers alpha cell hyperplasia (Vasu et al., 2014a). Similar observations were evident in our streptozotocin-treated mice islets. However, taurine suppressed alpha cell expansion to some extent. Notwithstanding the importance of this, the exact mechanism of transdifferentiation is yet unknown. Therefore, it seems reasonable to infer from an observed reduction in alpha cell mass that taurine either may stimulate alpha cell conversion to beta cells or inhibit alpha cell mass in the same way as insulin and GABA.

5.5.3 Effects of treatments on alpha cell transdifferentiation

Importantly, in the present study after extreme beta cell loss glucagon+/insulin+ bihormonal cells emerged in all diabetic groups irrespective of treatment. Taurine showed a tendency to increase in arising bihormonal cells. In line with this, taurine also promoted GFP+/Insulin+ cells clearly suggesting alpha to beta transdifferentiation. Earlier studies on GABA showed the possible involvement of GABA receptors in this transition process. **(**Ben-Othman et al., 2017)

Moreover, taurine has been widely studied with respect to neurological GABA receptor activation. Therefore, it is interesting to elucidate further its long-term effects of taurine on alpha cell transdifferentiation. Earlier studies showed that artemether follows GABA signaling mechanism to transdifferentiate alpha cells. In contrast, later reports contradicted its role and stated that artemether has no action on alpha cell transdifferentiation. Our findings are consistent with later reports, suggesting that artemether might not effective in alpha cell programming. However, we used acute dosing of artemether; therefore, further long-term use of artemether is merited.

5.5.4 Alpha cell hyperplasia and glucagon expression

By using streptozotocin mediated beta-cell loss, we found that that glucagon secretion was significantly decreased. Earlier studies have revealed that hyperglycaemia promotes PC1/3, but not PC2 gene regulation. Some studies have mentioned that a subset of alpha cell mass release primarily GLP-1, while other alpha cells provide glucagon secretion (Whalley et al., 2011). Additionally, it was recently shown that the upregulation of glucagon may play an important role in alpha to beta transdifferentiation. However, further research is crucial to elucidate how several peptides derived from the processing of pre-proglucagon serve to regenerate lost islet cell function after injury to the beta cells (Ye et al., 2015). Taken together, these data reveal a crucial action of taurine, but not artemether on reduction glucagon secretion in addition to streptozotocin, suggesting it might stimulate other peptides of the pre-proglucagon gene. In support of this, previous evidence suggesting the role of taurine in the stimulation of somatostatin, a known inhibitor of glucagon secretion. Further

study is required to evaluate the action of taurine suppression of glucagon release during a lack of insulin state (Santos-Silva et al., 2015).

5.5.5 Limitations

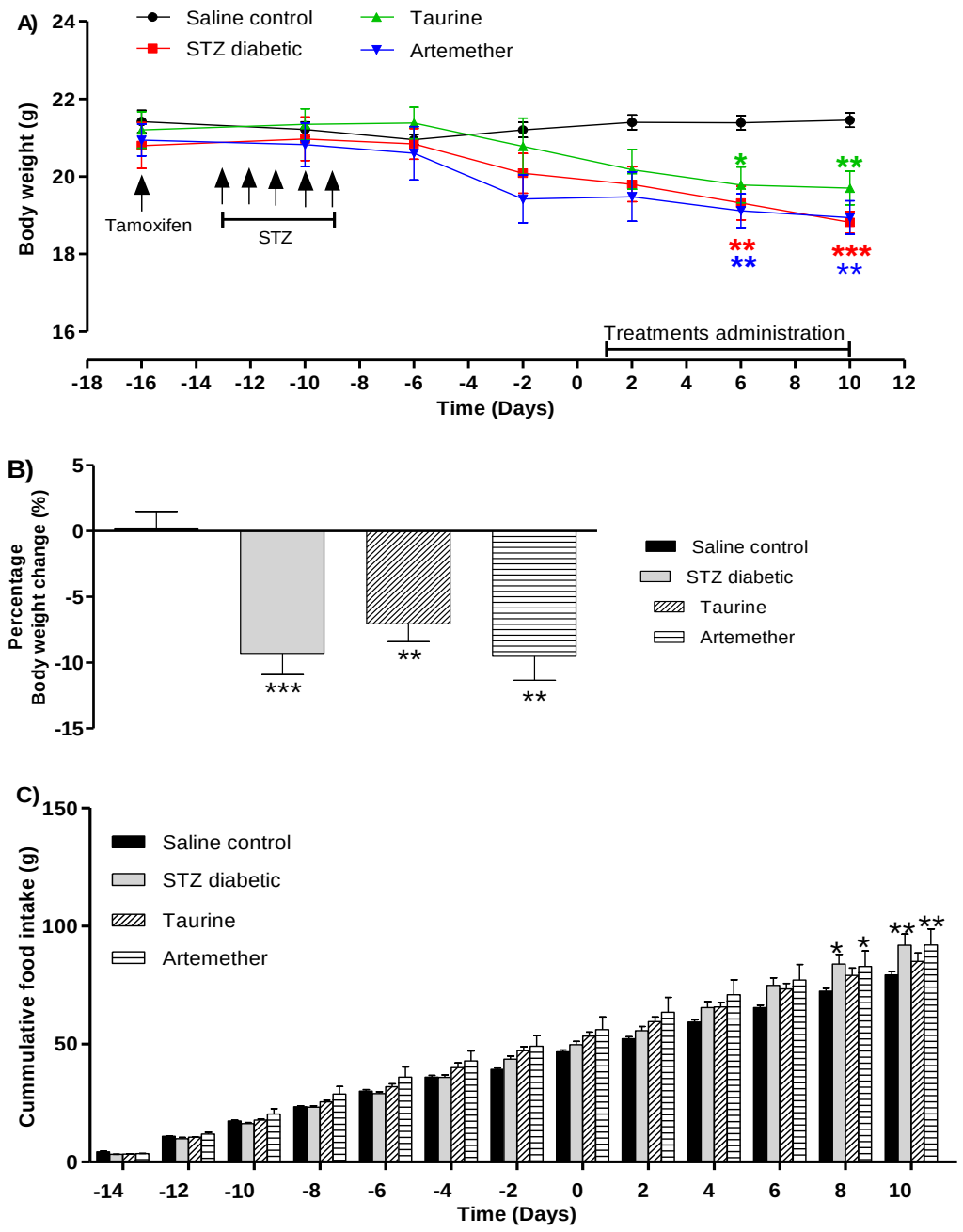
In the current animal model, GFP-labelled alpha cells without or with insulin co-expression were found to appear after streptozotocin exposure. This suggests that substantial beta-cell injury is required to stimulate alpha to beta cell regeneration (Thorel et al., 2010). However, artemether was found to exert no effect on transdifferentiation. Interestingly, taurine significantly promoted alpha to beta transdifferentiation. Owing to the limitations of our current tools, we were unable to discriminate whether insulin and GFP co-expressing cells were positive or negative for glucagon. In addition, diverse expression of GFP in alpha cells causes stronger fluorescent levels in a few cells, while its expression was undetectable in others. We were not able to elucidate, whether the absence of fluorescence is caused by a limitation of detection tool or correlates with the actual lack of GFP expression. Similarly, previous studies were explained using the same strain (~76% alpha-cell tagged) and Cre/loxP approach (Crabtree et al., 2003; Quoix et al., 2007). However, it is not a major limitation because GFP tagged a large population of alpha cells (50%-73.5%). Although few cells were not labelled with GFP, it could be claimed that glucagon expression reflects normal alpha cells instead of GFP labelled cells. Overall, these combined observations suggest that $Glu^{CreERT2}$; ROSA26-eYFP mice are a more reliable model for specifically highlighting alpha cell transdifferentiation (Quoix et al., 2007).

5.5.6 Concluding remarks

Thus, we suggest for the first time that taurine may promote the natural transdifferentiation of alpha cells. In support of this, a recent report on GABA and artemether was already suggested a positive effect on transdifferentiation. However, other reports have been denied the involvement of artemether in alpha cell reprogramming. Our results for artemether are consistent with this supposition. The previous study involved long-term administration of artemether, whereas we have observed the more acute effect of artemether. Therefore, further studies explaining the

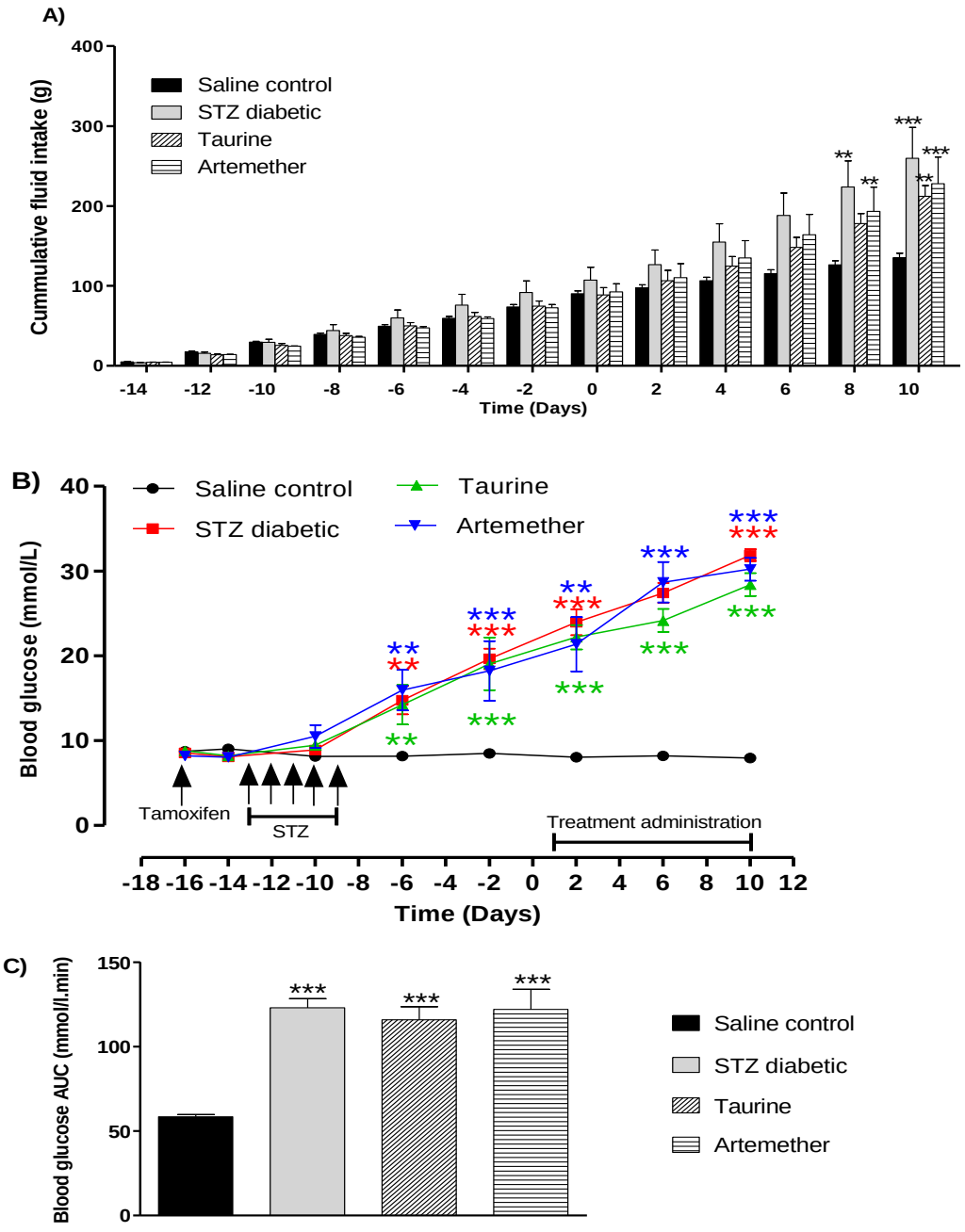
long-term effects of artemether on alpha cell reprogramming is essential. Owing to the agonist of the $GABA_A$ receptor, taurine might play a similar role to GABA. Previous reports have been revealed that taurine is present in alpha cells. Therefore, further studies on the role of taurine on pathways involved in transdifferentiation of alpha cells to beta cells are required.

Figure 5.1 Effects of taurine & artemether on (A, B) body weight and (C) food intake in normal and streptozotocin diabetic mice



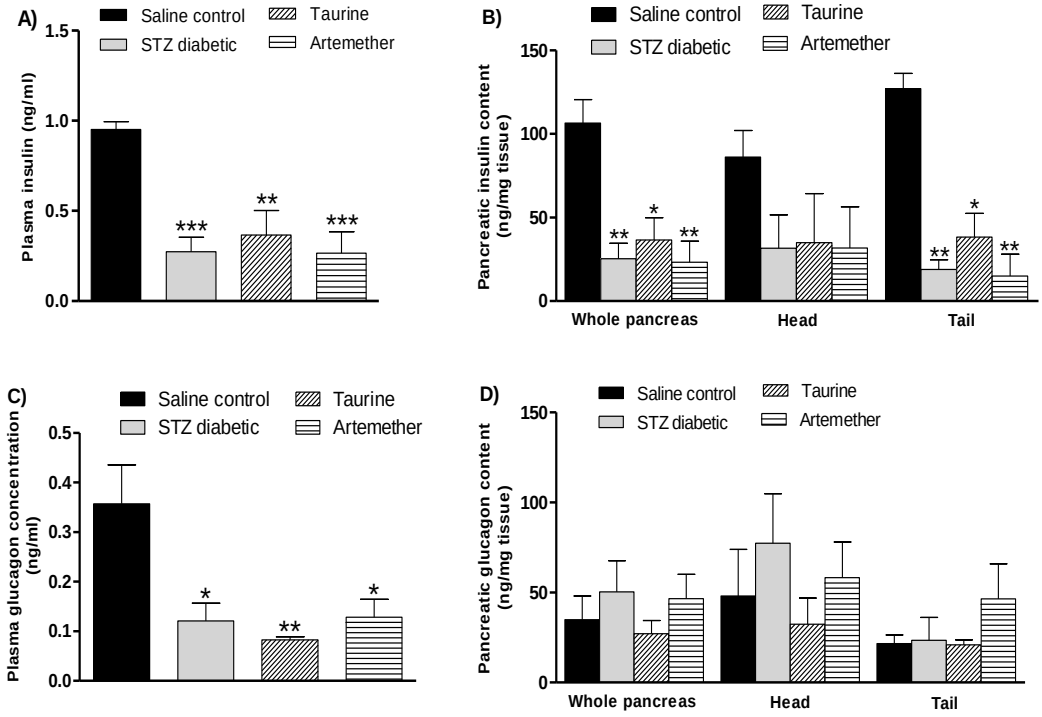
(A,B) Body weight and (C) food intake were measured throughout of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice. *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group.

Figure 5.2 Effects of taurine & artemether on (A) fluid intake and (B, C) blood glucose in normal and streptozotocin diabetic mice



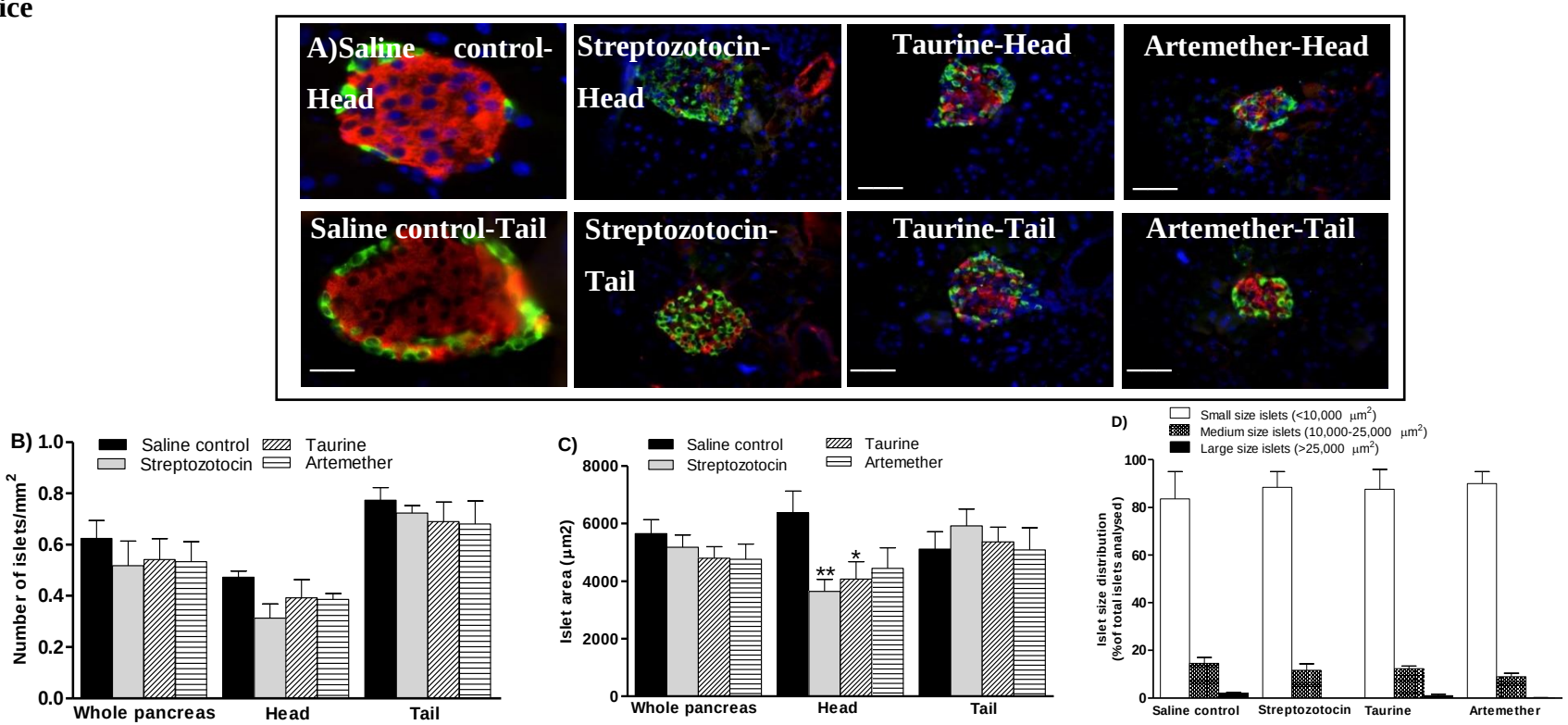
(A,B) Blood glucose and (C) fluid intake were measured throughout the study in $Glu^{CreERT2}$;ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice. **p < 0.01 and ***p <0.001 compared to saline control group.

Figure 5.3 Effects of taurine & artemether on plasma and pancreatic (A, B) insulin or (C, D) glucagon concentration in normal and streptozotocin diabetic mice



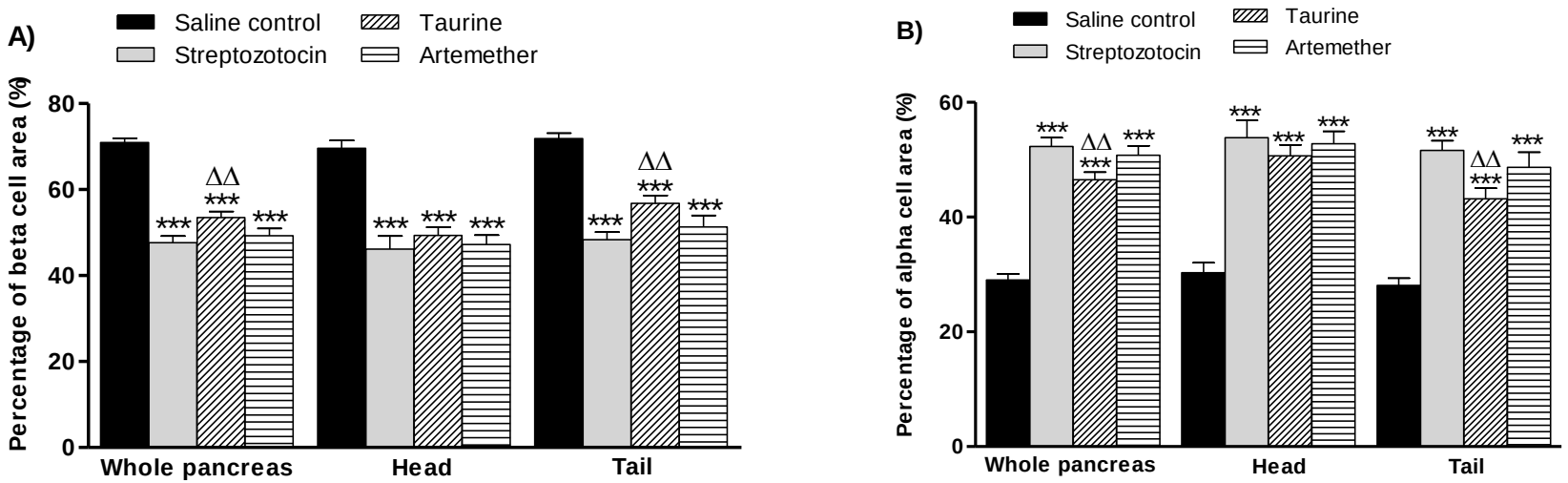
Plasma and pancreatic (A,B) insulin and (C,D) glucagon were measured at the end of the study in $Glu^{CreERT2}$;ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control group.

Figure 5.4 Effects of taurine and artemether on islet (A) number, (B) area and (C) size-distribution in normal and streptozotocin diabetic mice



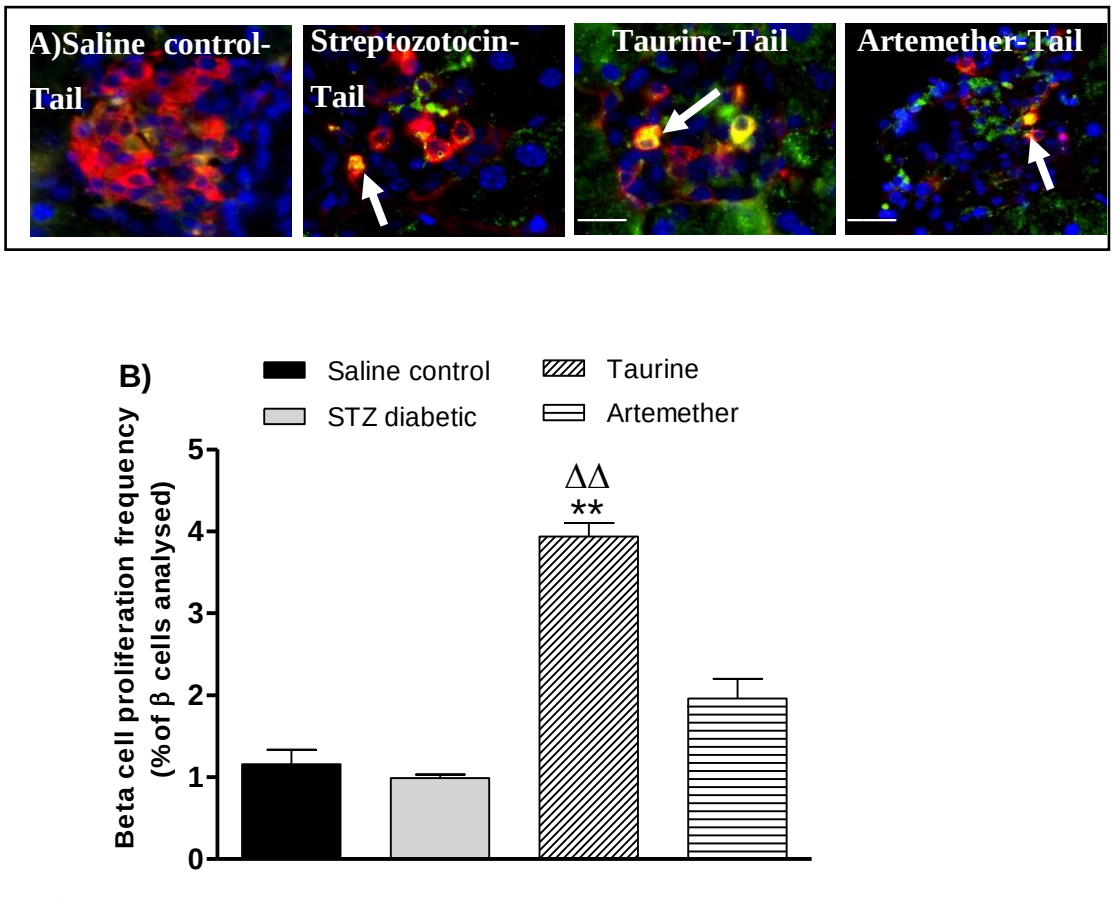
Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). Islet (B) number, (C) area and (D) size-distribution were analysed using Cell^{F} and ImageJ at the end of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice (100-150 islets per group). **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 compared to streptozotocin treated group. Scale bars: 50µm.

Figure 5.5 Effects of taurine and artemether on (A) beta cell area and (B) alpha cell area in normal and streptozotocin diabetic mice



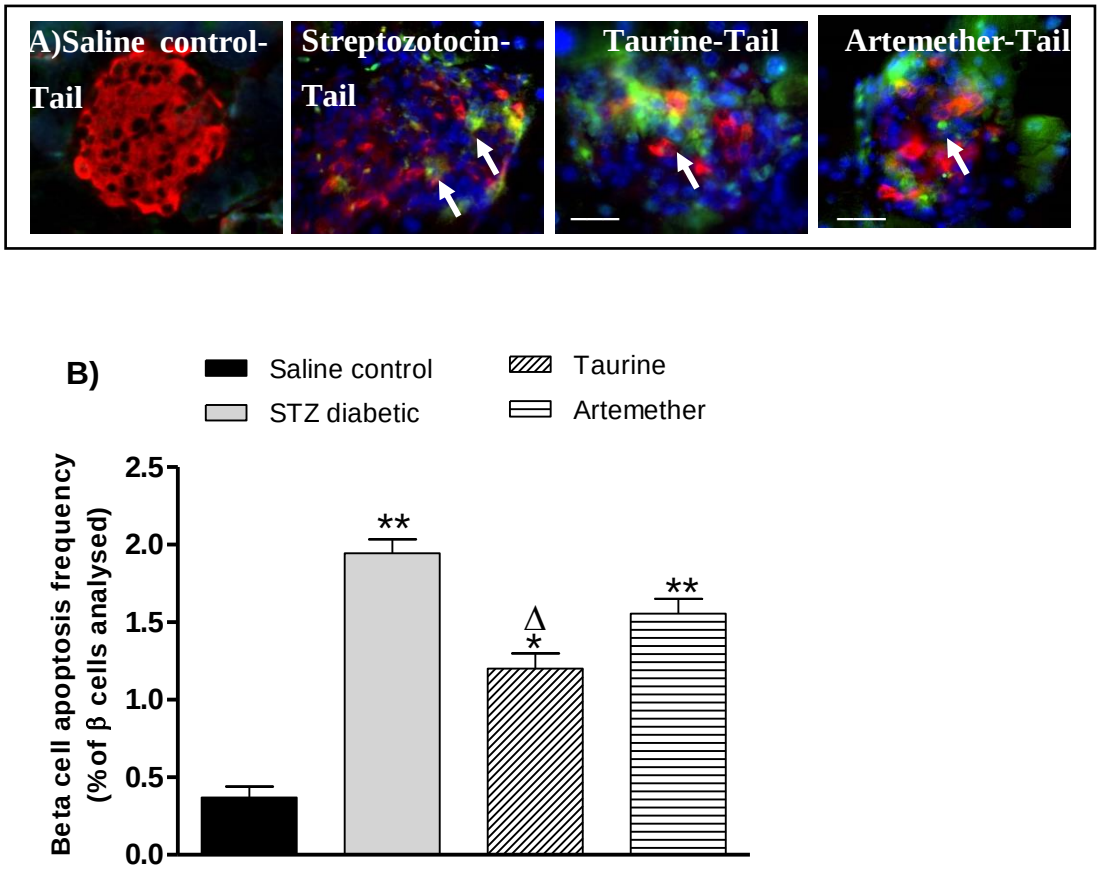
(A) Beta cell area, (B) alpha cell area were analysed using Cell^{F} and ImageJ at the end of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice (100-150 islets per group). **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. $\Delta p < 0.05$ and $\Delta\Delta p < 0.01$ compared to streptozotocin treated group.

Figure 5.6 Effects of taurine and artemether on (A, B) beta cell proliferation in normal and streptozotocin diabetic mice



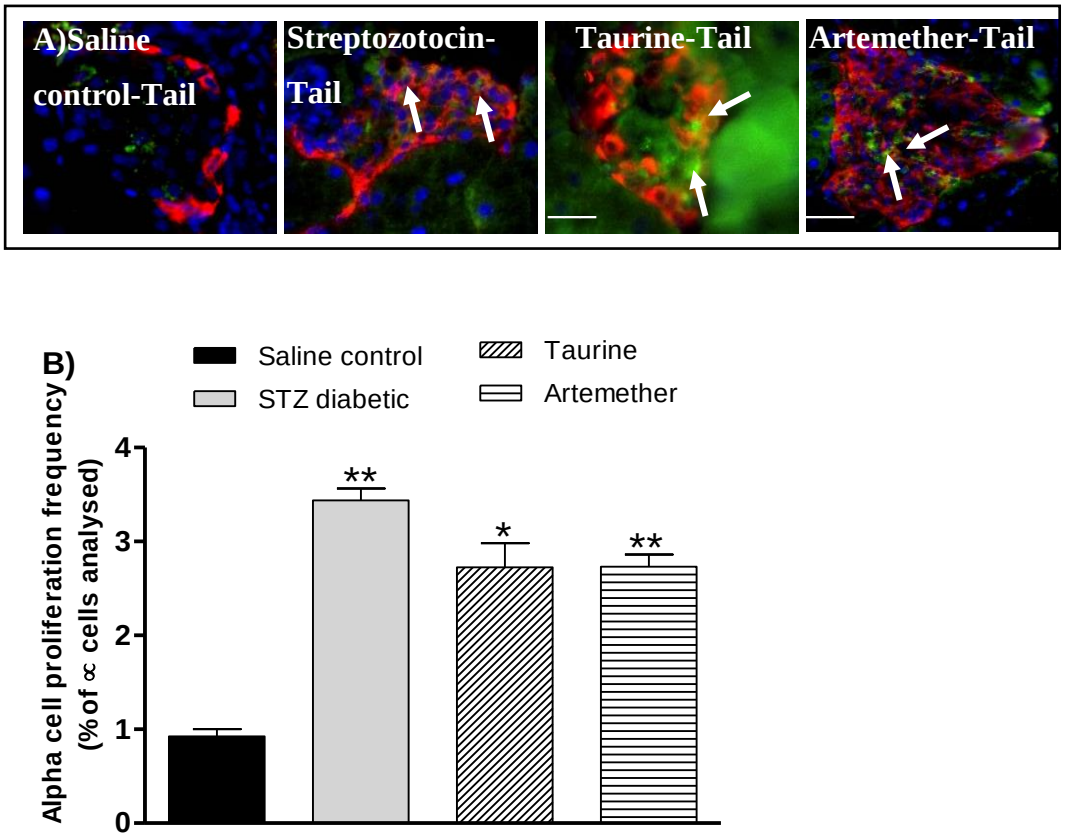
Representative images (A) showing immunostaining for DAPI (blue), Ki67 (green) and Insulin (red). (B) Beta cell proliferation were analysed using Cell^{F} and ImageJ at the end of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice (>60 tail islets per group). **p<0.01 compared to saline control group. ΔΔp<0.01 compared to streptozotocin treated group. Scale bars: 50µm.

Figure 5.7 Effects of taurine, artemether on (A, B) beta cell apoptosis in normal and streptozotocin diabetic mice



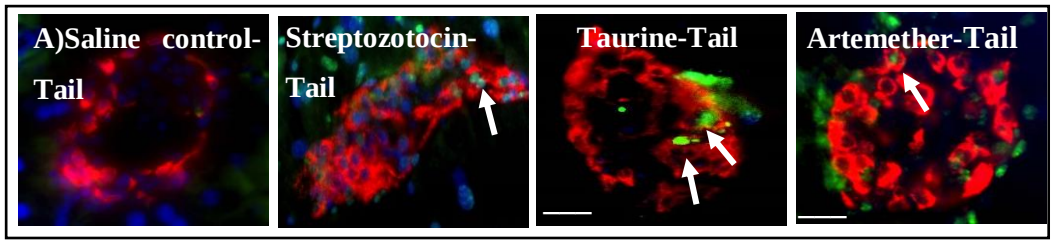
Representative images (A) showing immunostaining for DAPI (blue), TUNEL (green) and Insulin (red). (B) Beta cell apoptosis were analysed using Cell^{F} and ImageJ at the end of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice (>60 tail islets per group). *$p < 0.05$ and **$p < 0.01$ compared to saline control group. Δ$p < 0.05$ compared to streptozotocin treated group. Scale bars: 50µm.

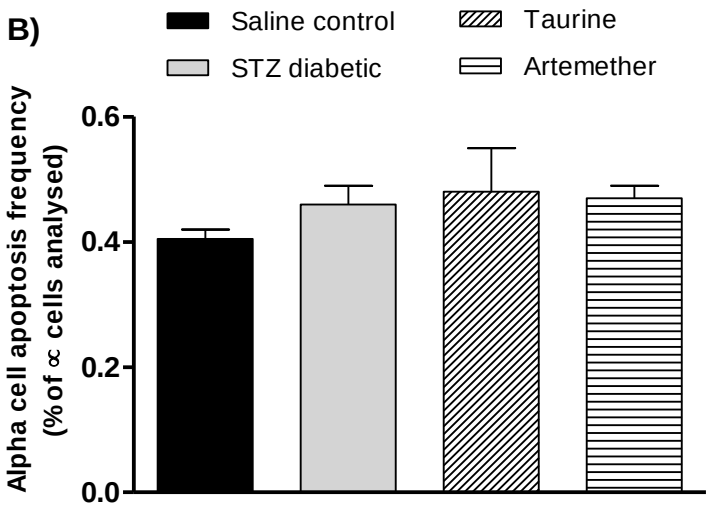
Figure 5.8 Effects of taurine and artemether on (A, B) alpha cell proliferation in normal and streptozotocin diabetic mice



Representative images (A) showing immunostaining for DAPI (blue), Ki67 (green) and Glucagon (red). (A) Alpha cell proliferation were analysed using Cell^{F} and ImageJ at the end of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 6 mice (>60 tail islets per group). *p < 0.05 and **p < 0.01 compared to saline control group. Δp < 0.05 compared to streptozotocin treated group. Scale bars: 50µm.

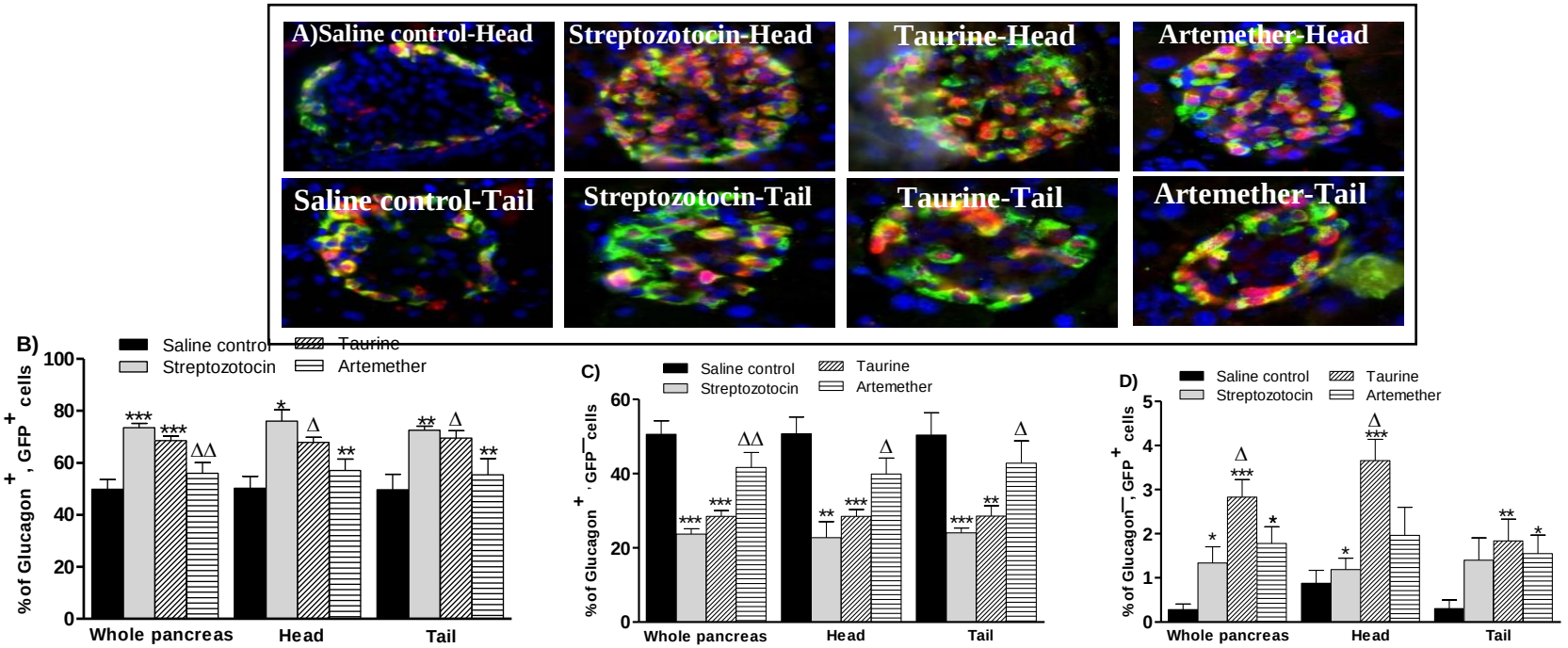
Figure 5.9 Effects of taurine, artemether on (A, B) alpha cell apoptosis in normal and streptozotocin diabetic mice





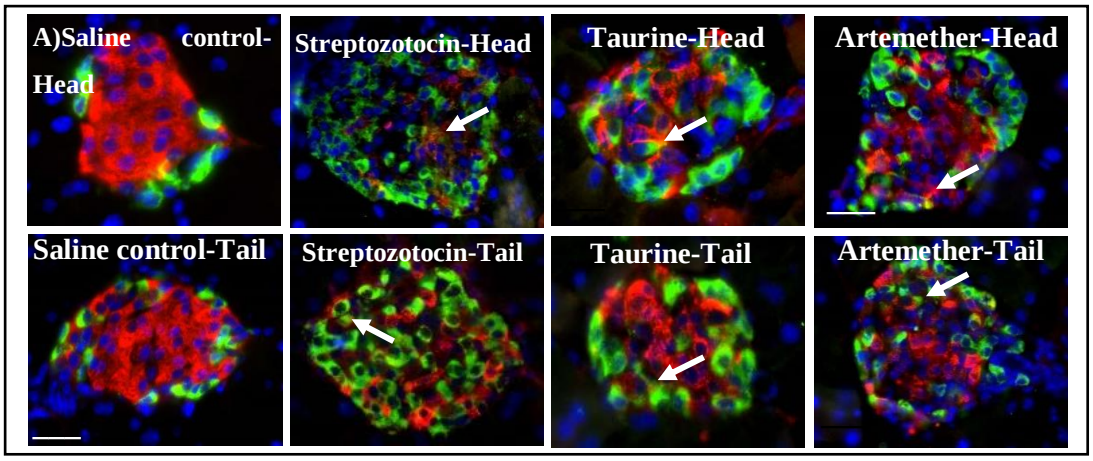
Representative images (A) showing immunostaining for DAPI (blue), TUNEL (green) and Glucagon (red). (A) Alpha cell apoptosis were analysed using Cell^{F} and ImageJ at the end of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice (>60 tail islets per group). Scale bars: 50µm.

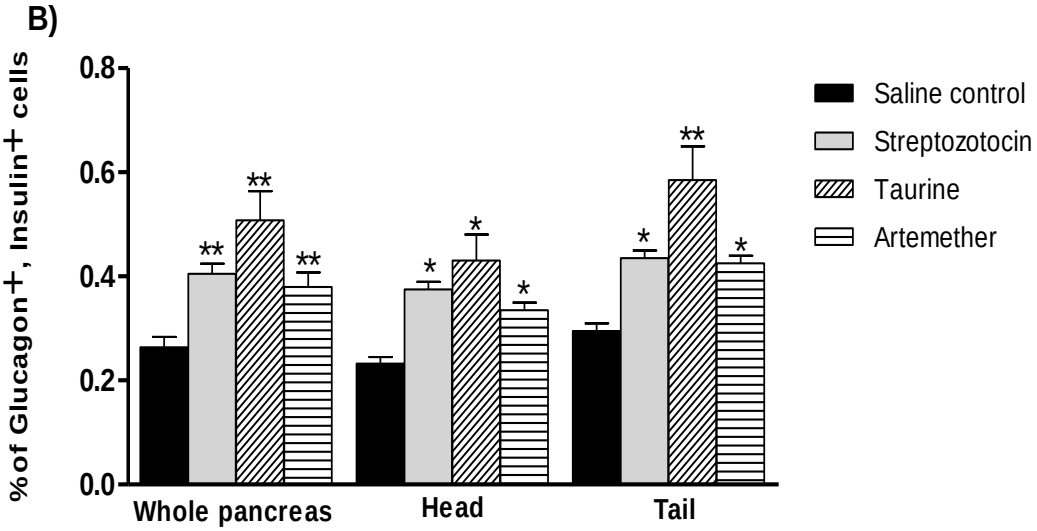
Figure 5.10 Effects of taurine and artemether on alpha cells lineage (A) $Gln^{postive}/GFP^{positive}$, (B) $Gln^{postive}/GFP^{negative}$ and (C) $Gln^{negative}/GFP^{positive}$ in normal and streptozotocin diabetic mice



Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and GFP (red). (B) Percentage of glucagon positive & GFP positive cells, (C) Glucagon positive & GFP negative cells and (D) percentage of glucagon negative & GFP positive cells were analysed using $Cell^F$ and ImageJ at the end of the study in $Glu^{CreERT2}$;ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 6 mice (>70 islets per group).). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. Δ$p < 0.05$ and ΔΔ$p < 0.01$ compared to streptozotocin treated group. Scale bars: 50µm.

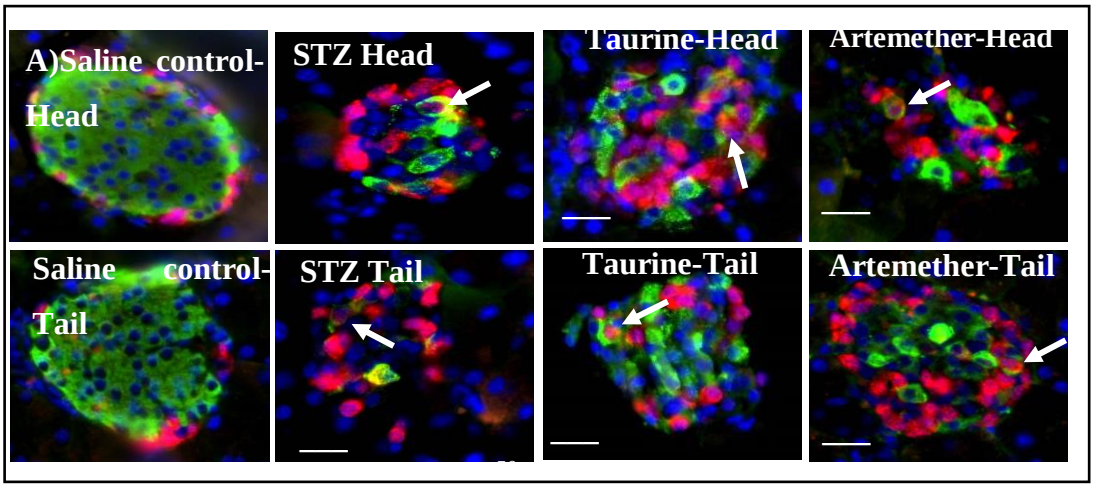
Figure 5.11 Effects of taurine & artemether on generation of bi-hormonal cells (A,B) Gln^{postive}/Insulin^{positive} in normal and streptozotocin diabetic mice

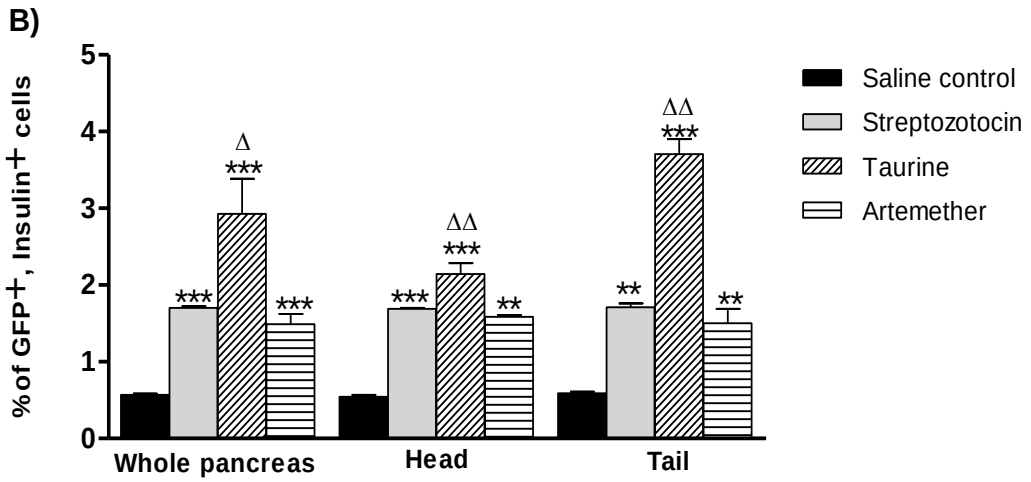




Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). (B) Glucagon^{positive}/Insulin^{positive} cells were analysed using Cell^{F} and ImageJ at the end of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 5 or 6 mice (100-150 islets per group). *p<0.05 and **p<0.01 compared to saline control group. Scale bars: 50µm.

Figure 5.12 Effects of taurine & artemether on alpha cells transdifferentiation into beta cells (A,B) GFP^{postive}/Insulin^{positive} in normal and streptozotocin diabetic mice





Representative images (A) showing immunostaining for DAPI (blue), insulin (green) and GFP (red). (B) GFP^{positive}/Insulin^{positive} cells were analysed using Cell^{F} and ImageJ at the end of the study in Glu^{CreERT2};ROSA26-eYFP. Taurine was included at 5% drinking water of streptozotocin diabetic mice. Controls received normal tap water and injected with saline. Oral dose of 100 mg/kg of artemether was administered every day. Values represent means ± SEM for 6 mice (100-150 islets per group). **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 and ΔΔp < 0.01 compared to streptozotocin treated group. Scale bars: 50µm.

Chapter 6

Effects of liraglutide, sitagliptin and dapagliflozin on islet morphology and alpha-cell transdifferentiation in insulin-deficient diabetic $Glu^{CreERT2}$; ROSA26-eYFP mice

6.1 SUMMARY

Intestinal peptide GLP-1 has been well known for its beneficial actions on pancreatic and extrapancreatic sites. Interestingly, due to sharing a common precursor in intestinal L and pancreatic alpha cells, GLP-1 can be expressed in both the cells for different purposes. As such, incretin effects on increasing insulin secretion and encouraging beta cell growth are well known. It is therefore interesting to know their purpose to produce locally during beta cell stress. Hence, here we sought to determine the role of liraglutide, sitagliptin and SGLT-2 inhibitor dapagliflozin using $Glu^{CreERT2}$; ROSA26-eYFP mice.

A 10 days administration of liraglutide (25 nmol/kg) for twice a daily; sitagliptin (50 mg/kg), and dapagliflozin (1 mg/kg) for once a daily showed a decreasing trend in food and fluid intake but no effect observed on blood glucose, body weight, and plasma insulin. There was a slight increasing trend in pancreatic insulin with liraglutide and sitagliptin compared STZ diabetic. However, plasma glucagon was decreased with liraglutide and sitagliptin, while a decreasing trend was observed in pancreatic glucagon by all the treatments. Although all treatments showed less effect on islets parameters, a significant increase was observed in beta cell area. This was associated with the promotion of beta cell growth by liraglutide and sitagliptin while protection by all the treatments. While dapagliflozin prevented beta cell loss by inhibiting apoptosis, importantly, sitagliptin found to promote proliferation of alpha cells. The effect of streptozotocin on GLP-1 expression by alpha cells was measured by immunostaining and pancreatic GLP-1 measurement. As such, STZ induced GLP-1 expression compared to saline controls. Finally, using alpha cell lineage we found liraglutide and sitagliptin increased a significant amount of transdifferentiation.

Thus, our data emphasize the role of GLP-1 signaling in the conversion of the alpha cell into beta cells. We suggest that incretin peptides may interact directly or indirectly on alpha cells, while dapagliflozin mainly protects beta cells.

6.2 INTRODUCTION

Severe diabetes is often associated with the loss of functional beta cells; thereby, blood glucose is elevated in an uncontrolled fashion (Moffett et al., 2015a; Vasu et al., 2015/2016). During the pancreatic organ development, cellular fates are pre-determined that are highly specific for various functions (Puri et al., 2015; Van et al., 2015). As such, different types of committed cells work together to ensure the proper functioning of the pancreas. However, recently, it has been noticed that during an extreme beta cell-stress, other local cells such as alpha **(**Thorel et al., 2010; Lee et al., 2018), delta (Chera et al., 2014), and exocrine (Solar et al., 2009) cells may sense the urgent need and exhibit the cell fate switching mechanism (Puri et al., 2015). This is an interesting phenomenon, which is offered by pancreas due to exclusive genomic plasticity, and therefore possess a robust potential to regenerate beta-cell mass to maintain the blood glucose level in diabetes (Puri et al., 2015). In this context, alpha cells are considered major contributors to supply new beta cells (Thorel et al., 2010; Cheng et al., 2015; Stanojevic et al., 2015).

Earlier gene modulation studies **(**Collombat et al., 2009; Lu et al., 2010; Yang et al., 2011; Wilcox et al., 2013) revealed the role of transcription factors in determining the alpha cell fate, and their rhythmic manipulation can induce beta cells characteristics within alpha cells. However, recent advances in regenerative medicines demonstrate the therapeutically based induction of alpha transdifferentiation (Lu et al., 2016; Ben-Othman et al., 2017; Li et al., 2017; Ackermann et al., 2018, Lee et al., 2018). However, the precise mechanism underlying alpha to beta transition is yet unclear (Gromada et al., 2007; Stanojevic et al., 2015). Therefore, the pharmacological based approach is necessary to understand the promotion of alpha to beta cell transdifferentiation.

More interestingly, recent research has been reported that alpha cells can store and release GLP-1 after beta cell loss. (Vasu et al., 2014a; Moffett et al., 2014/2015b). Unlike glucagon synthesis by PC2 processing, GLP-1 is PC1/3 processed translational product of pre-proglucagon gene (Dhanvantari et al., 1996; Wideman et al., 2007; Moffett et al., 2014/2015b). It is a major hormone of intestinal L-cells, yet ectopically produced by alpha cells. It is believed that alpha cells retain beta cell guardian properties during extreme conditions. Previous reports have suggested the role of

GLP-1 produced by both L-cells and alpha cells to protect beta cells (Vasu et al., 2014a). Encouragingly recent reports found that GLP-1 actively participates in the alpha cells transdifferentiation into beta cells (Bulotta et al., 2002; Lee et al., 2018; Zhang et al., 2019). Notwithstanding, it is noteworthy that endogenous GLP-1 has certain biological limitations such as rapid degradation by dipeptidyl IV (DPP-IV). Thus, these limitations of GLP-1 impede the therapeutic application of its native forms (Moffett et al., 2015a, 2015b). Indeed, other alternatives exhibiting protracted pharmacodynamics profile of GLP-1 have merit.

In this context, here we examined DPP-IV resistant GLP-1 analogue and DPP-IV inhibitor in relation to alpha cell transdifferentiation. As such, liraglutide and sitagliptin were found previously to be highly effective DPP-IV resistant GLP-1 analogue and DPP-IV inhibitors respectively (Green et al., 2006; Bailey et al., 2010; Moffett et al., 2015a; Millar et al., 2017). Furthermore, to test the role of glucose transporter in alpha cells, we examined the role of dapagliflozin, which serves as SGLT-2 inhibitor (Millar et al., 2017; Asahara et al., 2019), in the context of beta cells regeneration from reprogrammed alpha cell. SGLT-2 transporter is a glucose transporter that facilitates glucose flow using sodium gradient. They are mostly found in the kidney where they to reduces the renal glucose load (Suga et al., 2019). Thus, the present study was undertaken using $Glu^{CreERT2}$; ROSA26e-YFP mice treated with multiple low dose STZ to determine whether liraglutide, sitagliptin, and dapagliflozin recruits alpha cells or remaining beta cells to compensate beta cell loss.

6.3 MATERIALS AND METHODS

Materials and methods for this study have been discussed in Chapter 2.

6.4 RESULTS

6.4.1 Effects of liraglutide, sitagliptin, and dapagliflozin on body parameters

All mice treated with STZ without or with drug treatment showed significant ($p<0.05$ to $p<0.001$) decrease in body weight (Figure 6.1A). The area under curve measures

were significantly decreased for all treatments compared with STZ-treated mice, as shown in Figure 6.1B.

STZ increased food intake ($p<0.01$) at some observation points compared with saline controls (Figure 6.1C). After administration of STZ alone or in combination with sitagliptin, fluid intake was significantly ($p<0.05$ to $p<0.01$) raised and unaltered by liraglutide and dapagliflozin in comparison with saline-treated animals (Figure 6.2A). As expected, all STZ treated diabetic mice exhibited marked ($p<0.001$) elevation of blood glucose as compared to control mice. None of the treatment regimens exhibited any beneficial hypoglycemia action (Figure 6.2B). This was corroborated by a marked ($p<0.001$) rise in AUC compared with saline (Figure 6.2C).

Plasma insulin ($p<0.05$ to $p<0.001$) and pancreatic insulin ($p<0.05$ to $p<0.01$) were markedly decreased in all diabetic animals compared to saline controls (Figure 6.3A, B). However, liraglutide and sitagliptin groups showed a trend to increase pancreatic insulin content compared to STZ controls (Figure 6.3B). Streptozotocin treated mice exhibited a tendency towards decreased plasma (Figure 6.3C) and pancreatic glucagon concentrations (Figure 6.3D). A marked ($p<0.05$ to $p<0.01$) reduction in plasma glucagon was noted in liraglutide and sitagliptin groups (Figure 6.3C).

6.4.2 Effects of liraglutide, sitagliptin, and dapagliflozin on islet morphology

Figure 6.4A shows representative images of staining for insulin and glucagon in the islets of $Glu^{CreERT2}$; ROSA26-eYFP mice. The overall number of islets per mm^2 pancreas was decreased by STZ with significance ($p<0.05$) observed in head pancreas, compared with saline islets (Figure 6.4B). Importantly, the tail pancreas of all groups had a greater number of islets compared with head region (Figure 6.4B). Liraglutide significantly ($p<0.05$) increased islets in the tail region as compared to STZ controls (Figure 6.4B). As shown in Figure 6.4C, all diabetic treatment groups exhibited significantly ($p<0.05$ to $p<0.001$) decreased the total islet area. Unexpectedly, none of the treatment regimens improved islet area when compared with diabetic controls (Figure 6.4C). However, all diabetic groups had a significantly ($p<0.05$ to $p<0.01$) reduced percentage of large islets compared to saline controls (Figure 6.4D).

Compared with the control group, all streptozotocin treated groups showed significant ($p<0.001$) reduction in beta-cell area (Figure 6.5A). In contrast, all treatment regimens exhibited significantly ($p<0.05$ to $p<0.001$) improved percentage beta-cell area as compared to STZ alone (Figure 6.5A).

In harmony, the alpha cell area was significantly ($p<0.001$) expanded after streptozotocin administration alone or in combination with drugs (Figure 6.5B). Dispute this alpha cell area was significantly ($p<0.01$ to $p<0.001$) reduced by all treatments compared to diabetic controls (Figure 6.5B).

6.4.3 Effects of liraglutide, sitagliptin, and dapagliflozin on proliferation and apoptosis of beta and alpha cells

Figure 6.6A and 6.7A display representative staining images for Ki67/insulin and TUNEL/insulin, respectively. Importantly, liraglutide and sitagliptin significantly ($p<0.05$ to $p<0.01$) increased beta-cell proliferation frequency as compared to both saline control and STZ treated group (Figure 6.6B). In contrast, all STZ treated groups showed marked ($p<0.001$) increase of beta-cell apoptosis frequency in comparison with saline-treated mice (Figure 6.7B). However, as compared to STZ alone, all other treatment significantly ($p<0.05$ to $p< 0.01$) reduced the frequency of beta-cell apoptosis (Figure 6.7B).

Figure 6.8A and 6.9A depicts representative staining images for Ki67/glucagon and TUNEL/glucagon, respectively. Notably, a significant ($p<0.01$ to $p<0.001$) rise in alpha cell proliferation was noted in all diabetic mice when compared to saline control (Figure 6.8B). Importantly, sitagliptin significantly ($p<0.05$) increased alpha cell proliferation in comparison with STZ group (Figure 6.8B). Finally, none of the diabetic groups showed any change in alpha cell apoptosis frequency (Figure 6.9B).

6.4.4 Effects of liraglutide, sitagliptin, and dapagliflozin on alpha cell lineage tracing

Representative images illustrating immunostaining for glucagon and GFP are presented in Figure 6.10A. Here, Glucagon+/GFP+ colocalized cells &

Glucagon+/GFP- cells represent undifferentiated alpha cells, and Glucagon-/GFP+ cells represent reprogrammed alpha cells. About 51 to 74% of cells were GFP labeled alpha cells (Figure 6.10B). Accordingly, 35 to 48% of cells were noted to glucagon positive alpha cells (Figure 6.10C). Importantly, only GFP positive cells were significantly ($p<0.05$ to $p<0.001$) increased in all diabetic mice compared to saline control (Figure 6.10D). Thus it was further significantly ($p<0.05$ to $p<0.01$) promoted by liraglutide and sitagliptin with respect to STZ treated group (Figure 6.10D).

6.4.5 Effects of liraglutide, sitagliptin, and dapagliflozin on generation of bihormonal cells

Figure 6.11A shows representative islets exhibiting immunoreactivity for glucagon and insulin coexpression. All treatments significantly enhanced ($p<0.05$ to $p<0.01$) number of bihormonal cells compared to saline controls (Figure 6.11B). Liraglutide and sitagliptin significantly ($p<0.05$ to $p<0.01$) augmented glucagon+/insulin+ co-expressing cells compared to STZ group (Figure 6.11B)

6.4.6 Effects of streptozotocin on ectopic expression of GLP-1 by alpha cells

Figure 6.12A shows representative islets exhibiting immunoreactivity for GFP and GLP-1 coexpression. Interestingly, streptozotocin-induced diabetes dramatically ($p<0.001$) increased GLP-1 production by GFP-labelled alpha cells as compared to saline control. (Figure 6.12B). Saline controls showed a negligible expression of GLP-1 (Figure 6.12B). Consistent with this, pancreatic GLP-1 content was aslo significantly ($p<0.001$) increased in STZ-diabetic control as compared to saline controls (Figure 6.12C).

6.4.7 Effects of liraglutide, sitagliptin, and dapagliflozin on alpha to beta transdifferentiation

Representative islets showing immunoreactivity for GFP and insulin are depicted in Figure 6.13A. Intriguingly, STZ induced destruction of beta cells significantly ($p<0.01$ to $p<0.001$) enhanced GFP+ alpha cells coexpressing insulin compared with saline

controls. This was further augmented significantly ($p<0.05$ to $p<0.001$) by all treatments (Figure 6.13B).

6.5 DISCUSSION

Here, we demonstrate that the pancreatic islets possess the plasticity to originate beta cells from reprogramming alpha cells during pathophysiological conditions where the uttermost beta cells injury occurred. Intriguingly, we observed the natural alpha to beta cell transdifferentiation phenomenon using novel $Glu^{CreERT2}$; ROSA26-eYFP mice following multiple low dose of streptozotocin. More interestingly, we found that this transition process was further augmented by liraglutide and sitagliptin but not by dapagliflozin. Importantly, all treatments encouraged existing beta cells through either enhanced proliferation or suppressed apoptosis. Therefore, we suggest that liraglutide and sitagliptin may contribute to generating beta cells partially from both pre-existing beta cells and guardian alpha cells, while dapagliflozin simply protects pre-existing beta cells.

6.5.1 Effects of treatments on islet morphology

Similar to earlier observations, the present study showed adverse effects of multiple low doses of STZ on islet morphology (Vasu et al., 2014a). Contrary to this, liraglutide and sitagliptin but not by dapagliflozin showed beneficial effects on islet morphology. In line with this, liraglutide and sitagliptin were able to increase a distinct beta cell mass and reduced the alpha cells area. These effects are consistent with the action of GLP-1 as elucidated in previous reports (Vasu et al., 2013/2014a; Moffett et al., 2015b)

To ascertain whether the increased beta cell mass is derived from survival beta cells, we assessed proliferation and apoptosis of beta cells. As expected, we found that beta cell proliferation was unaltered whilst beta cell apoptosis was greatly increased in STZ-treated islets (Vasu et al., 2014a). In contrast, liraglutide and sitagliptin enhanced beta-cell proliferation while all treatments reduced beta cell death. Thus, it could be deduced that liraglutide and sitagliptin regulate both the growth and protection of beta cells while only later effect is found with dapagliflozin. This notion is supported by

previous studies, which strengthen our view on the survival of existing beta cells. (Green et al., 2006; Vasu et al., 2014a; Irwin et al., 2015a; Merovci et al., 2015; Jurczak et al., 2018; Asahara et al., 2019)

6.5.2 Evidence of glucagon+/insulin+ bihormonal cells in diabetic mice

Earlier studies have suggested that alpha cells start producing insulin without affecting glucagon expression, thereby representing two hormone storing cells (Thorel et al., 2010; Brown et al., 2016; Lee et al., 2018). Eventually, these bihormonal cells become deficient in glucagon expression leading to fully mature beta cells (Thorel et al., 2010). Notably, in the present study, a detectable number of bihormonal cells was evident after streptozotocin-induced stress and this was further enhanced by liraglutide and sitagliptin but not dapagliflozin. From these observations, we speculate that alpha cells gained beta cell identity through temporarily expressing both cell phenotypes.

6.5.3 GFP-tagged alpha cells ectopically expressing insulin

In the present study, we demonstrate whether insulin is co-expressed in GFP-labelled alpha cells following beta cells stress and further manipulation by treatment regimens. Interestingly, we observed that STZ mediated severe beta cell injury resulted in detectable insulin-expression by GFP-labelled alpha cells. Liraglutide and sitagliptin were found to further significantly increased insulin+ and GFP+ co-expressing cells. However, the action of dapagliflozin was found to similar to the STZ group suggesting that dapagliflozin neither stimulated nor inhibited the transdifferentiation process. Finally, we observed that the presence of glucagon+/insulin+ cells, glucagon-/GFP+ or insulin+/GFP+ cells were increased in liraglutide and sitagliptin groups. Therefore, we suggest that liraglutide and sitagliptin may play an important role in the acceleration of alpha to beta cell transdifferentiation.

6.5.4 Changing pattern of glucagon gene processing after beta-cell injury

The observed hyperplasia of alpha cells might be expected to cause a rise in plasma glucagon concentrations (Thorel et al., 2010; Menge et al., 2011; Vasu et al., 2014a). However, we observed a decreased level of both plasma and pancreatic concentration glucagon concentrations. Alpha cell hyperplasia was determined based on glucagon or GFP immuno-stained cells. Expression of glucagon appeared to be brighter in alpha cells, which contrasts with lowered circulating glucagon concentrations. One might

speculate that pro-glucagon precursor protein could also show cross-reactivity with its glucagon antibody expressed (Nie et al., 2000). Thus, proglucagon precursor might increase its expression of other glucagon related peptides such as GLP-1. Consistence with this, diabetic mice showed significantly increased alpha cell area with stimulation of proliferation by sitagliptin. Some evidence points to the possible role of such locally produced GLP-1 in the proliferation of alpha cells (Lee et al., 2018). Interestingly we found that ectopic expression of GLP-1 was upregulated by more than 90% in the alpha cell population of STZ diabetic animals, whereas it was almost undetectable in normal mice islets.

It is not clear however how injury to beta cells locally induces GLP-1 production in alpha cells, and how such locally produced GLP-1 may induce the expression of the insulin gene in alpha cells (Moffett et al., 2014; Vasu et al., 2014a; Lee et al., 2018). One possible explanation might be that beta-cell injury signals interact with TGR5, GPR119, GPR 120 or GRP 40 receptors on alpha cells to induce PC1/3 and thereby GLP-1 production (Whalley et al., 2011). As such, local GLP-1 may be either directly or indirectly involved in the regeneration of beta cells (Lee et al., 2018). This might be involved activating some unknown direct pathways in alpha cells that led to the expression of insulin in alpha cells. Alternatively, this might be mediated through indirect routes such as by increasing insulin (Nakashima et al., 2018) or somatostatin (Ørgaard et al., 2017) release from remaining beta or delta cells to suppress glucagon secretion. Overall, consistent with previous reports on GLP-1 (Lee et al., 2018; Zhang et al., 2019), here we confirmed that liraglutide (GLP-1 analog) and sitagliptin (DPP-IV inhibitor to avoid local GLP-1 degradation) might possess the potential to restore functional beta cells. Previous reports reported GLP-1 mediated alpha cell transdifferentiation highlighted the possible involvement of PI3K/AKT/FOXO1 pathway (Zhang et al., 2019) or fibroblast growth factor 21 (FGF21) (Lee et al., 2018). Therefore, further research is needed to elucidate the exact role of GLP-1 in the context of alpha cell transdifferentiation.

The presence of SGLT-2 on the plasma membrane of alpha cells is yet controversial. (Solini et al., 2017; Suga et al., 2019)**.** Consistent with our findings, earlier studies showed that DAPA inhibits SGLT-2 transporters to increase glucagon secretion (Bonner et al., 2015; Pedersen et al., 2016; Suga et al., 2019). In contrast,

canagliflozin, other inhibitors of SGLT-1 has been reported to suppress glucagon secretion (Suga et al., 2019). Therefore, it is interesting to explore further the role of SGLT-1 inhibition with respect to alpha cell transdifferentiation. From this, we can speculate that inhibition of SGLT-2 lowers glucose uptake by alpha cell, which mimics the hypoglycaemic state (Pedersen et al., 2016; Suga et al., 2019) and inhibits glucagon secretion (Suga et al., 2019). Taken together, the reduced glucagon concentrations in the present study might suggest that increased glucose uptake is the adaptation criteria used by alpha cells following beta cell injury. In contrast, previous studies reported that prolonged hyperglycaemia might impair normal glucagon secretory behaviour of alpha cells (Thorel et al., 2010; Menge et al., 2011; Vasu et al., 2014a). Thus, testing the role of other glucose transporters in alpha cells would be a part of further investigation, in the context of transdifferentiation.

6.5.5 Expression pattern of fluorescent protein in alpha cells

The present study has certain limitations. The saline-treated $Glu^{CreERT2}$; ROSA26-eYFP mice used as a control showed approximately 55% of alpha cell tagging, while all other diabetic mice with or without treatments showed nearly 76% alpha cell labelling. This difference between optimization and experimental observation might be due to the variable responses of individual animals to exogenous tamoxifen bioavailability. Taken these together, we speculate that the presence of glucagon positive/GFP negative cells resulted from a lack of EYFP expression in alpha cells rather than dedifferentiation of other non-alpha cells such as beta cells into glucagon-positive cells. However, it is not a significant limitation since a substantial number of alpha cells in the experimental mice were tagged with its fluorescent protein. A similar expression pattern was observed in previous studies (Quoix et al., 2007; Parker et al., 2018). Therefore, we suggest that $Glu^{CreERT2}$; ROSA26-eYFP mice were a convenient and reliable model for studying the role of alpha cells in pathophysiology.

6.5.6 Effect of treatments on body parameters

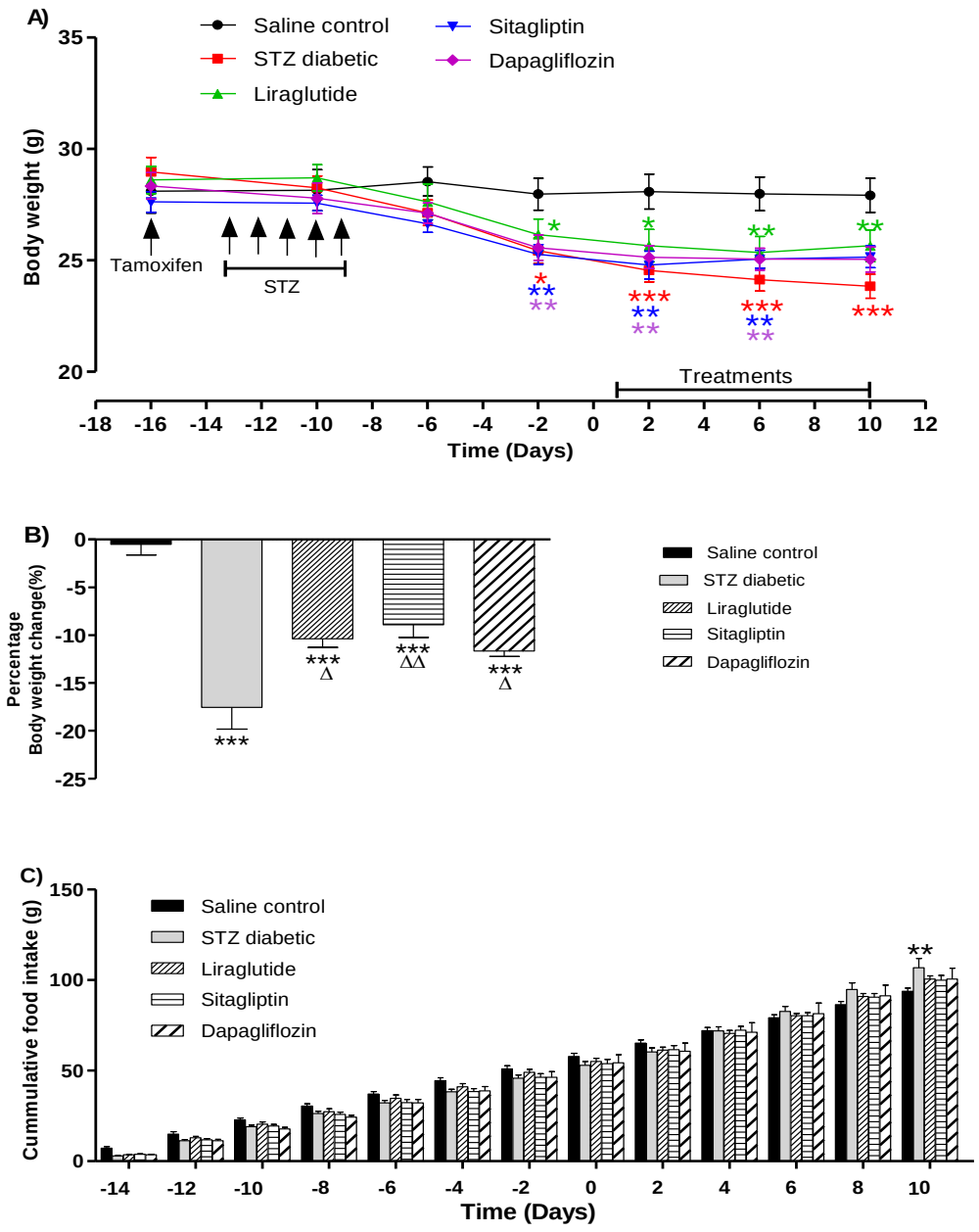
An absolute diabetic condition developed with streptozotocin in $Glu^{CreERT2}$; ROSA26-eYFP mice was associated with severe loss of body weight and high demand for food and water. Furthermore, a marked increase in blood glucose and reduction of plasma

and pancreatic insulin were observed in the diabetic mice. These observations are consistent with previous STZ induced diabetic models (Vasu et al., 2014a). Importantly, none of the treatments could prevent these uncontrolled diabetic symptoms over the acute period of administration. Thus, we speculate that alpha to beta transdifferentiation occurred during the extreme diabetic condition and was not a single consequence of changes in blood glucose control. Previously all treatments at higher doses showed beneficial effects on body weight and hyperglycaemia in other rodent models of diabetes (Bailey et al., 2010; Vasu et al., 2013; Merovci et al., 2015; Millar et al., 2017; Wang et al., 2017; Kanno et al., 2019; Asahara et al., 2019). However, in the future, it would be interesting to assess the impact of improved glycaemia on this treatment-mediated alpha to beta cell transition.

6.5.7 Concluding remarks

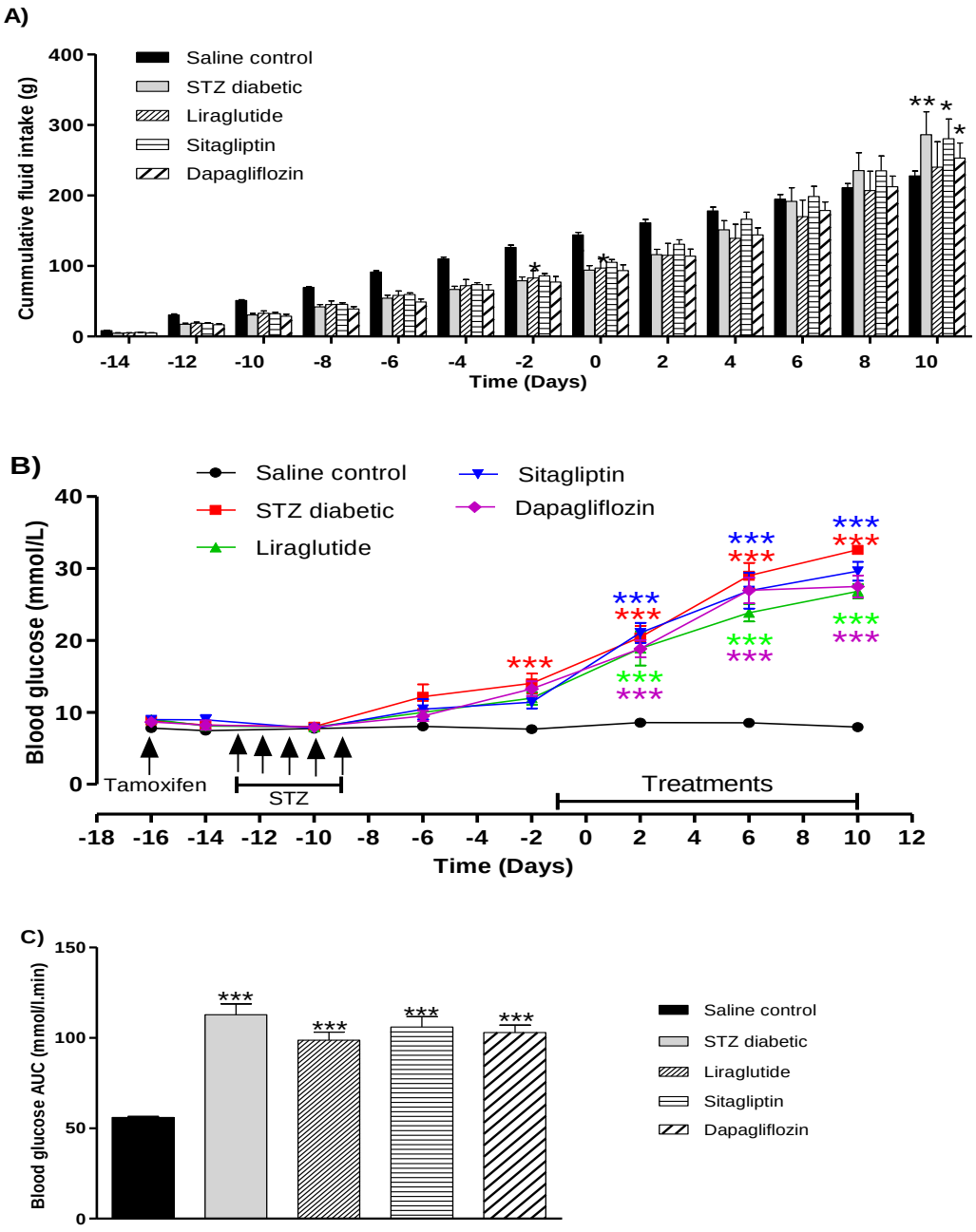
In conclusion, our findings clearly suggest that GLP-1 signaling might play an important in alpha to beta transdifferentiation. However, further studies of its chronic use of liraglutide and sitagliptin are merited. Furthermore, a lack of effect of dapagliflozin provides a clue that SGLT-2 is not important for alpha cell glucose uptake and alpha cell transdifferentiation. Although in the present study, dapagliflozin was unable to alter this transdifferentiation at a given dose, it is vital to explore its chronic action using another dose concentration.

Figure 6.1 Effects of liraglutide, sitagliptin and dapagliflozin on (A, B) body weight and (C) food intake in normal and streptozotocin diabetic mice



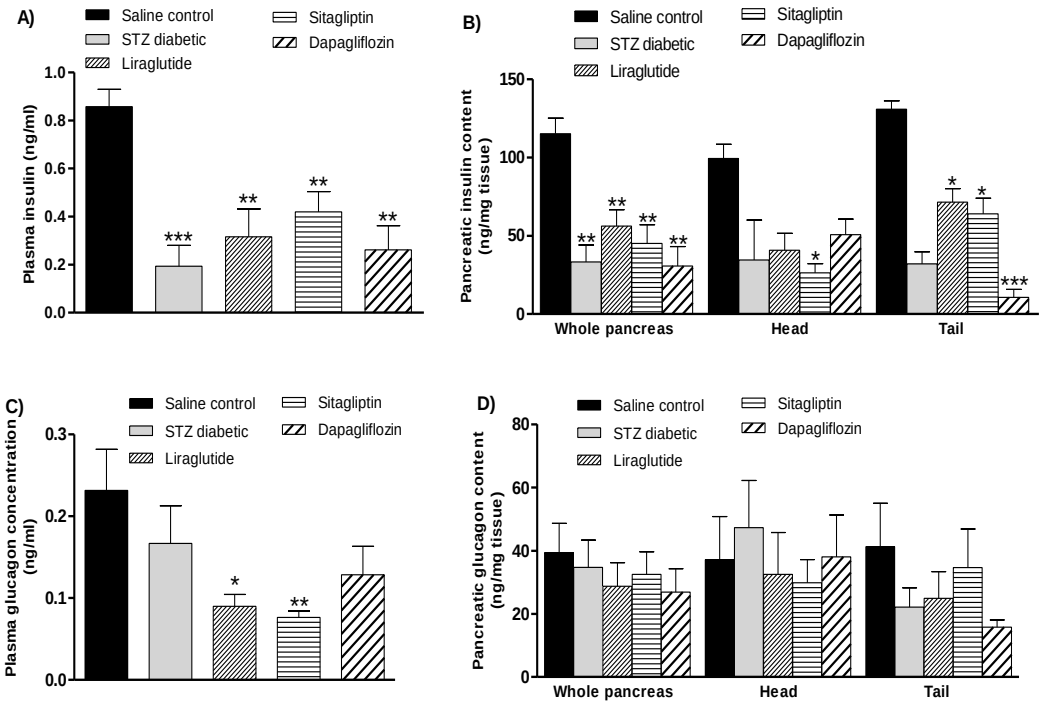
(A,B) Body weight and (C) food intake were measured throughout the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic $Glu^{CreERT2}$;ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to saline control group. Δp<0.05 and ΔΔp<0.01 compared to streptozotocin treated group.

Figure 6.2 Effects of liraglutide, sitagliptin and dapagliflozin on (A) fluid intake and (B, C) blood glucose in normal and streptozotocin diabetic mice



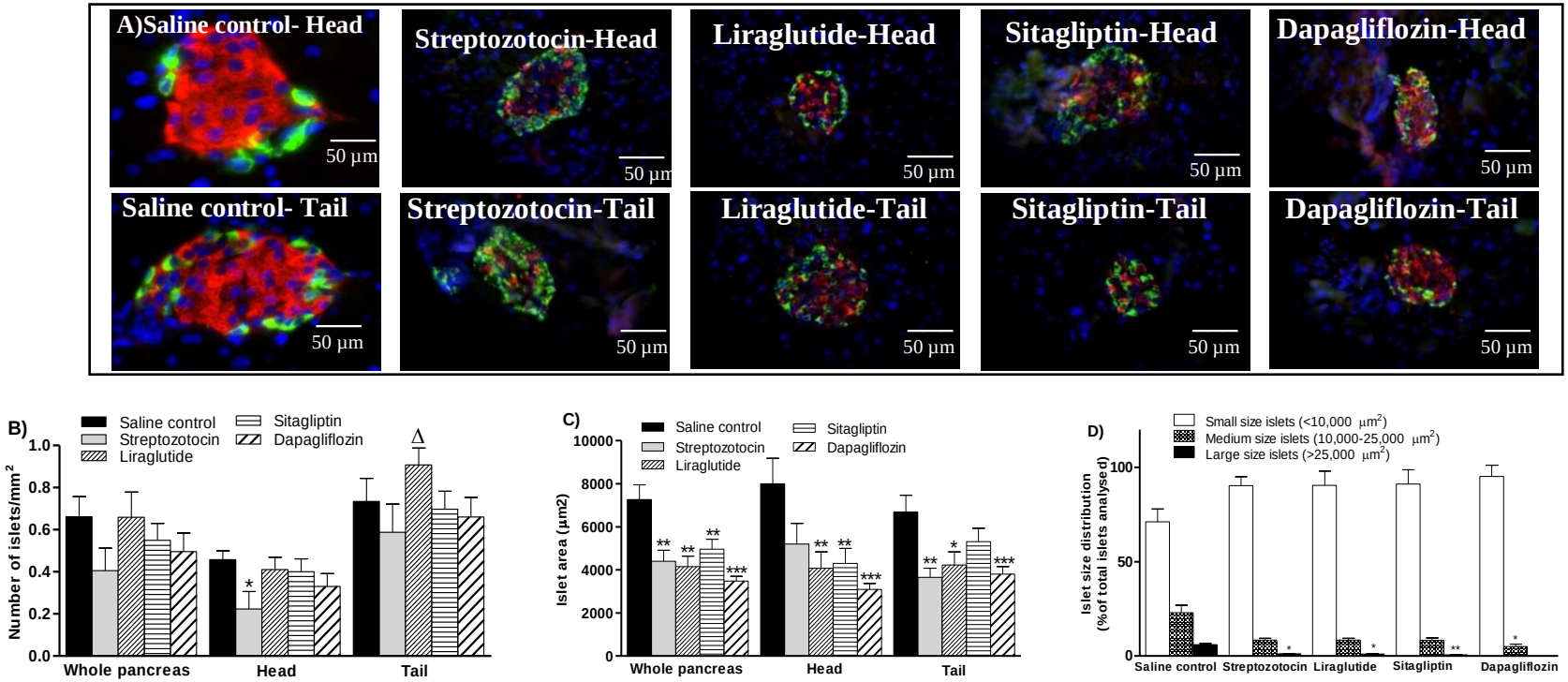
(A,B) Blood glucose and (C) fluid intake were measured throughout the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to saline control group.

Figure 6.3 Effects of liraglutide, sitagliptin and dapagliflozin on plasma and pancreatic (A, B) insulin or (C, D) glucagon concentration in normal and streptozotocin diabetic mice



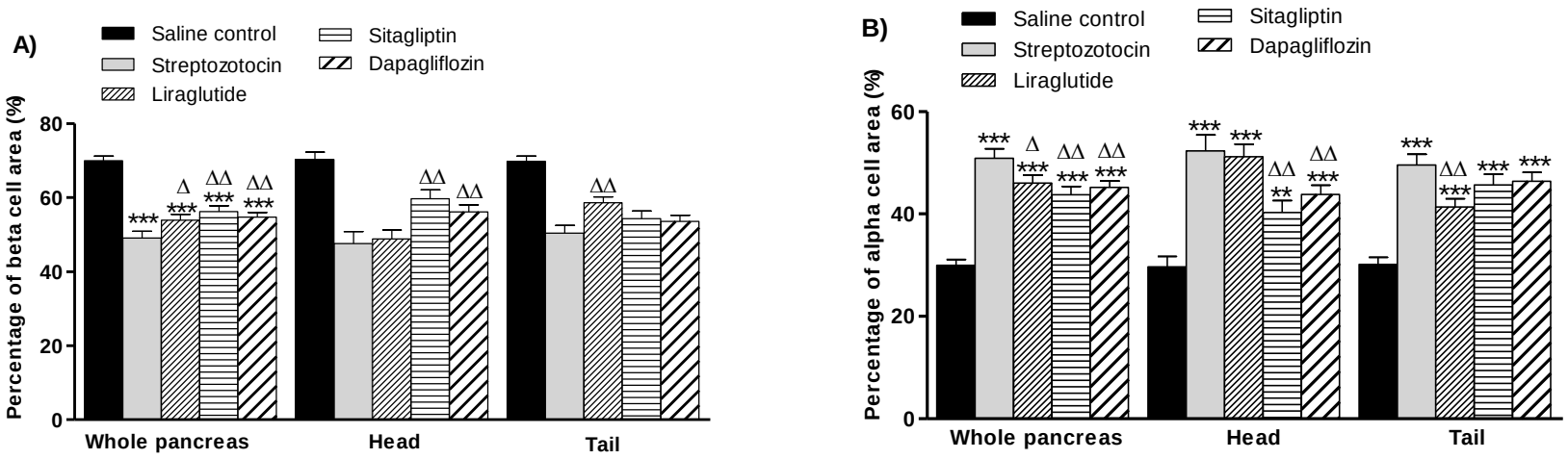
Plasma and pancreatic (A,B) insulin and (C,D) glucagon were measured at the end of the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic $Glu^{CreERT2}$;ROSA26-eYFP. Controls received twice-daily injection of saline vehicle.Values represent means ± SEM for 6 mice. *p<0.05, **p<0.01 and ***p <0.001 compared to saline control group.

Figure 6.4 Effects of liraglutide, sitagliptin and dapagliflozin on islet (A) number, (B) area and (C) size-distribution in normal and streptozotocin diabetic mice



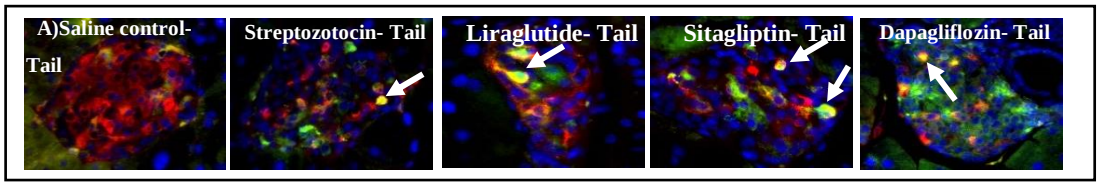
Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). Islet (B) number, (C) area and (D) size-distribution were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice (100-150 islets per group). *p<0.05 and **p < 0.01 compared to saline control group. Δp<0.05 and ΔΔp<0.01 compared to streptozotocin treated group. Scale bars: 50 µm.

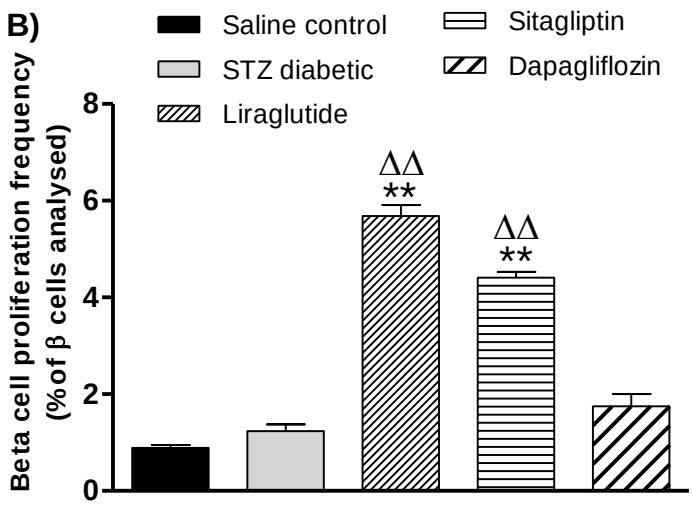
Figure 6.5 Effects of liraglutide, sitagliptin and dapagliflozin on (A) beta cell area and (B) alpha cell area in normal and streptozotocin diabetic mice



(A) Beta cell area, (B) alpha cell area were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice (100-150 islets per group). ***p <0.001 compared to saline control group. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ and $\Delta\Delta\Delta p < 0.001$ compared to streptozotocin treated group.

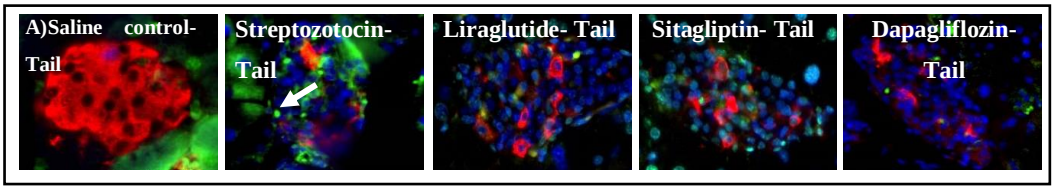
Figure 6.6 Effects of liraglutide, sitagliptin and dapagliflozin on (A, B) beta cell proliferation in normal and streptozotocin diabetic mice

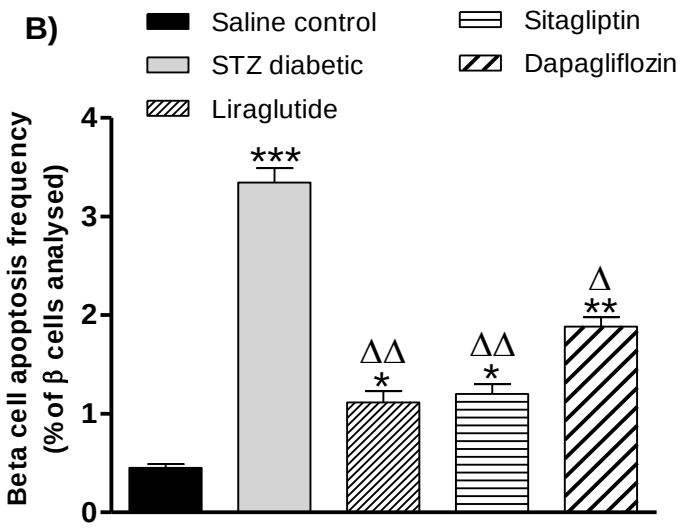




Representative images (A) showing immunostaining for DAPI (blue), Ki67 (green) and Insulin (red). (B) Beta cell proliferation were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice (>60 tail islets per group). **$p < 0.01$ compared to saline control group. ΔΔ$p < 0.01$ compared to streptozotocin treated group. Scale bars: 50 µm.

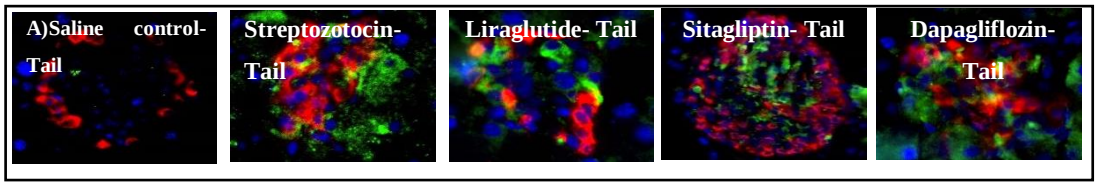
Figure 6.7 Effects of liraglutide, sitagliptin and dapagliflozin on (A, B) beta cell apoptosis in normal and streptozotocin diabetic mice

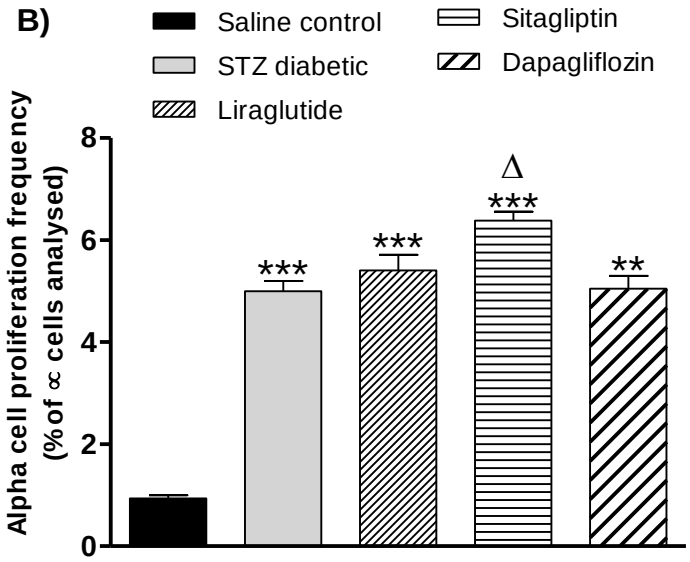




Representative images (A) showing immunostaining for DAPI (blue), TUNEL (green) and Insulin (red). (B) Beta cell apoptosis were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle.Values represent means ± SEM for 6 mice (>60 tail islets per group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. Δ$p < 0.05$ and ΔΔ$p < 0.01$ compared to streptozotocin treated group. Scale bars: 50 µm.

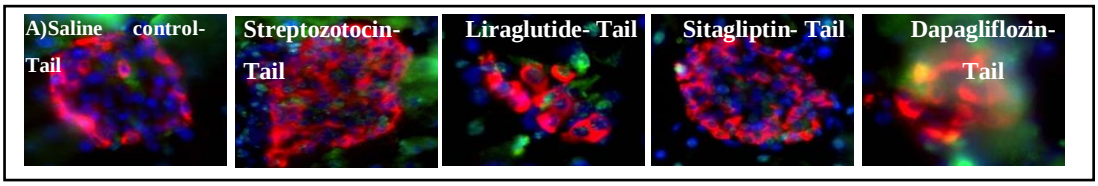
Figure 6.8 Effects of liraglutide, sitagliptin and dapagliflozin on (A, B) alpha cell proliferation in normal and streptozotocin diabetic mice

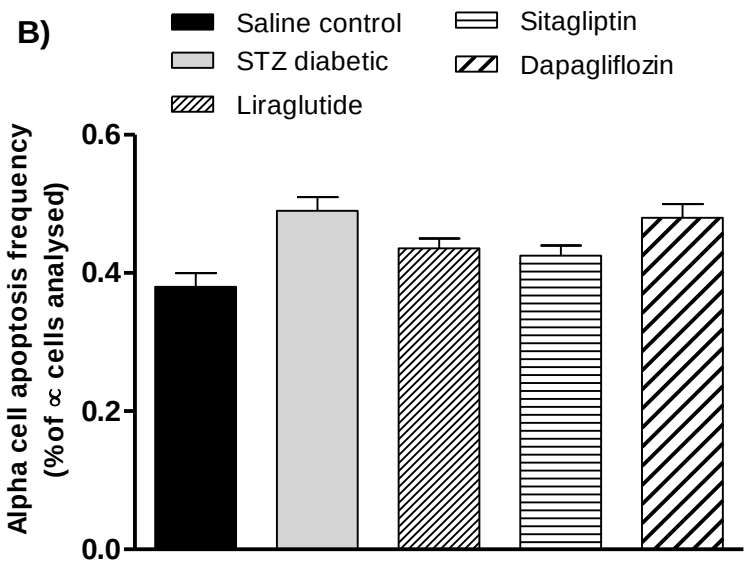




Representative images (A) showing immunostaining for DAPI (blue), Ki67 (green) and Glucagon (red). (A) Alpha cell proliferation were analysed using Cell^{F} and ImageJ at the end of the study Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice (>60 tail islets per group). **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 compared to streptozotocin treated group. Scale bars: 50 µm.

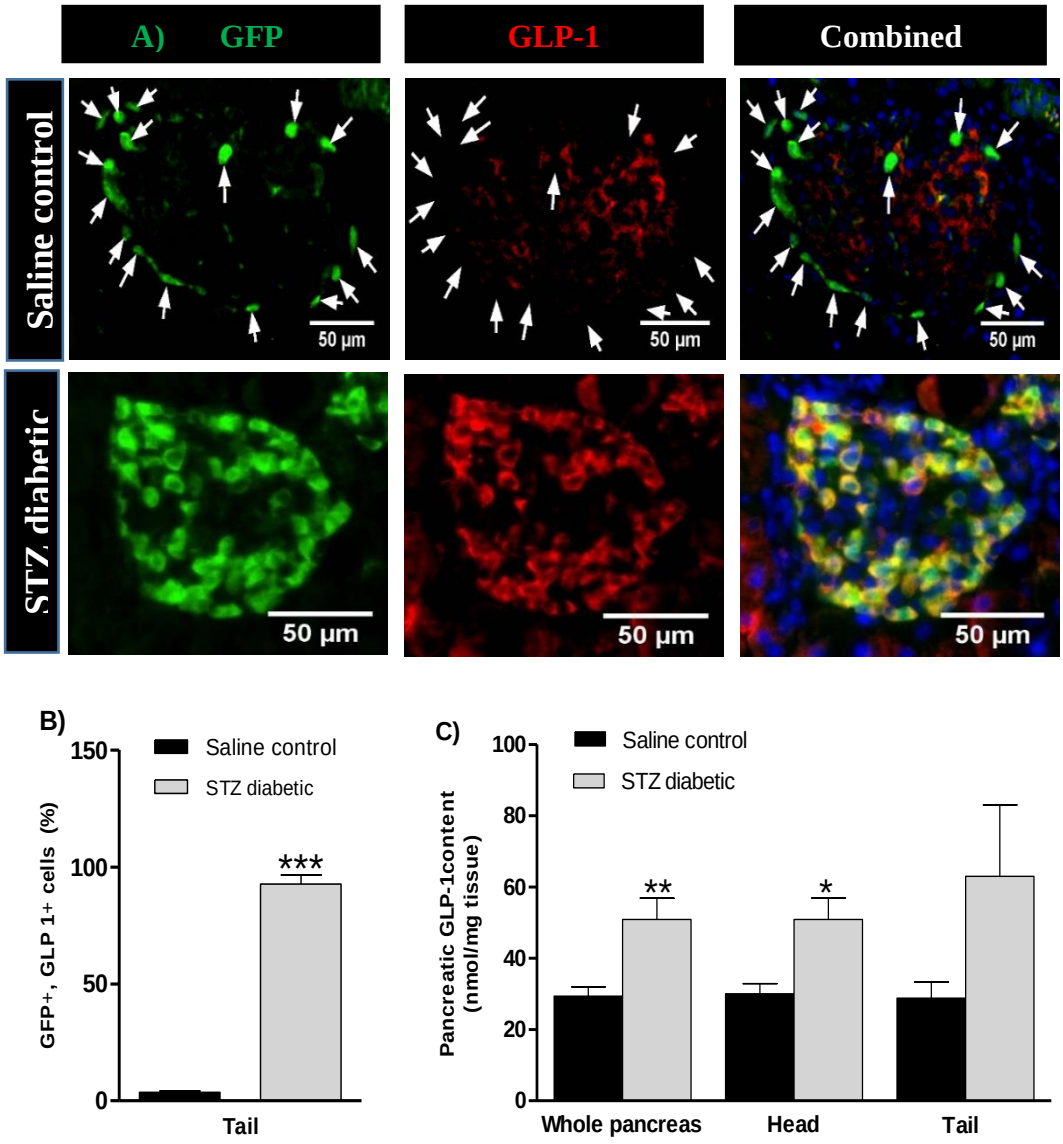
Figure 6.9 Effects of liraglutide, sitagliptin and dapagliflozin on (A, B) alpha cell apoptosis in normal and streptozotocin diabetic mice





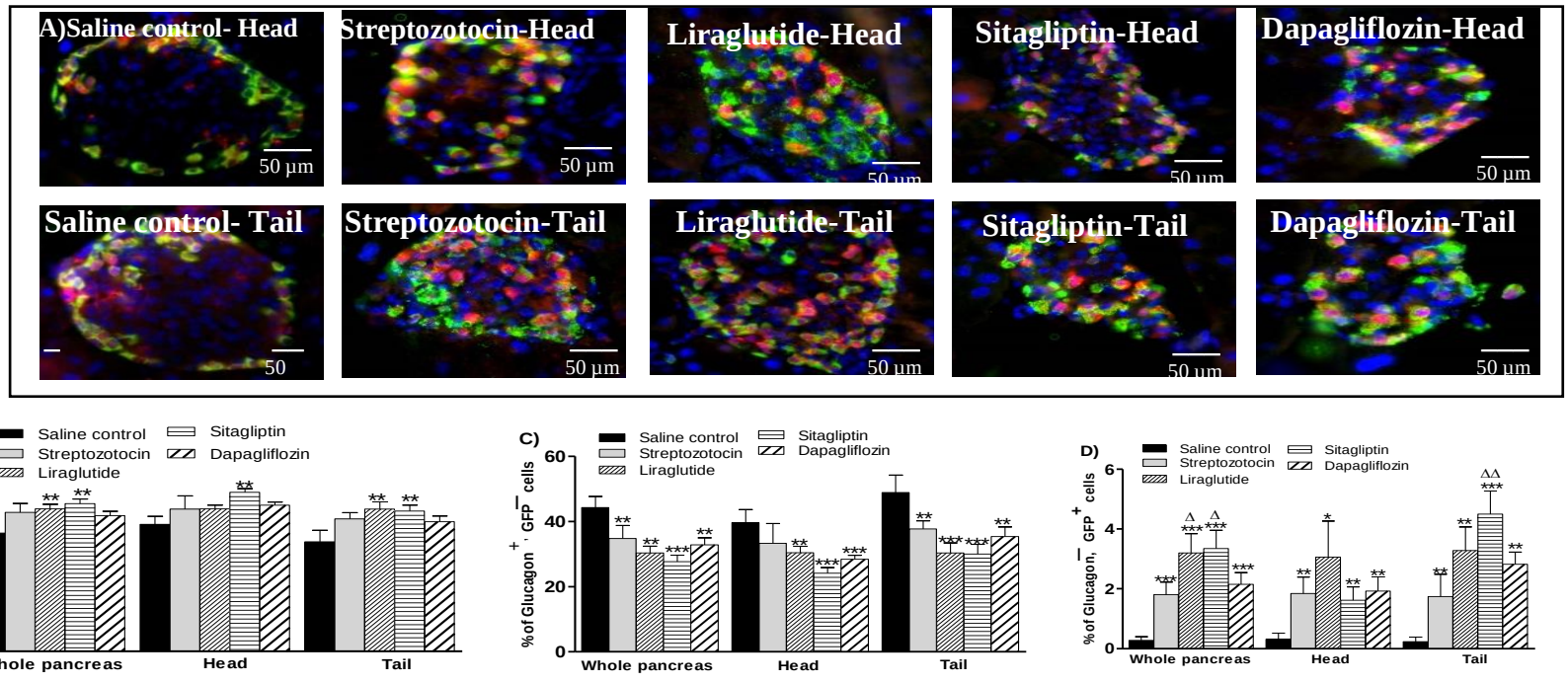
Representative images (A) showing immunostaining for DAPI (blue), TUNEL (green) and Glucagon (red). (A) Alpha cell apoptosis were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice (>60 tail islets per group). Scale bars: 50 µm.

Figure 6.10 Effects of liraglutide, sitagliptin and dapagliflozin on (A, B) GLP-1 expression by GFP^{postive}/GLP-1^{positive} cells and (C) pancreatic GLP-1 content in normal and streptozotocin diabetic mice



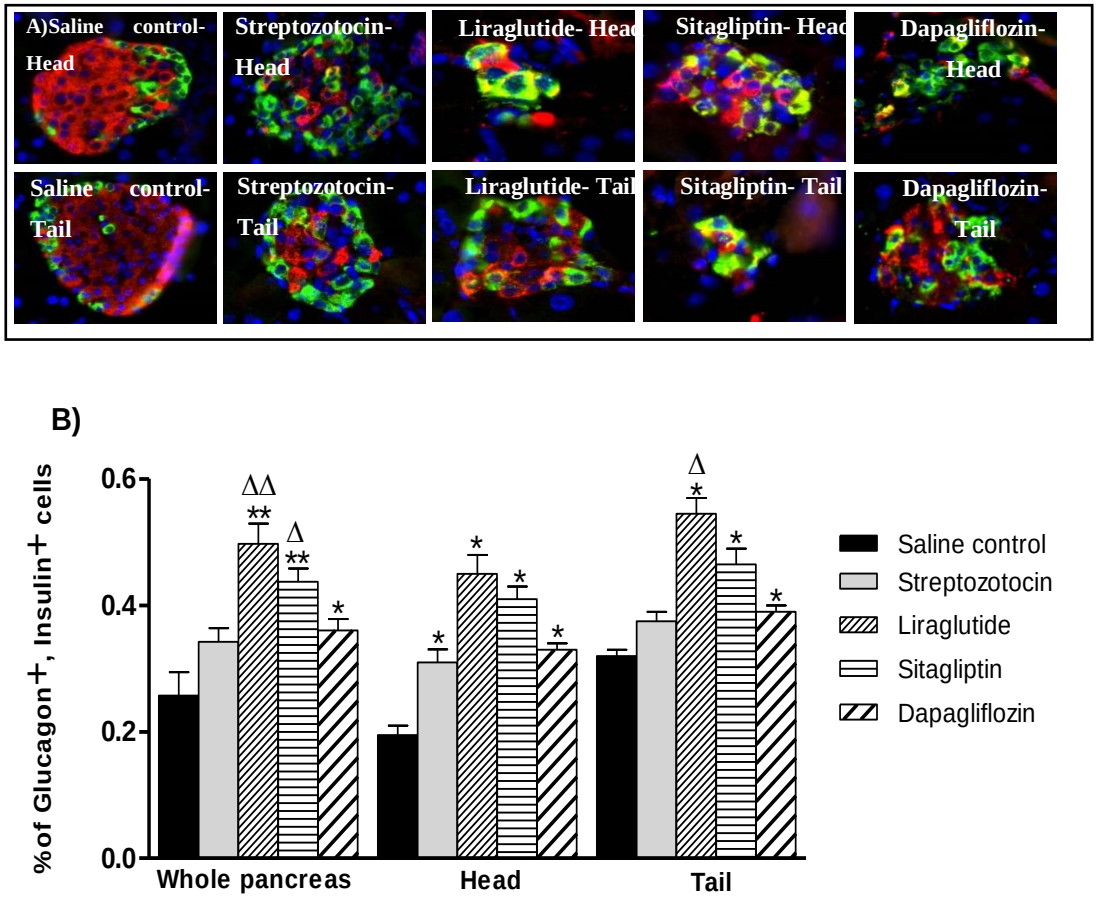
Representative images (A) showing immunostaining for DAPI (blue), GFP (green) and GLP-1 (red). Effects of streptozotocin on immuno-expression of GLP-1 by GFP labelled alpha cells expressed as percentage of GFP positive & GLP-1 positive α-cells (B) and pancreatic GLP-1 content measured using GLP-1 ELISA (C) in streptozotocin treated Glu^{CreERT2};ROSA26-eYFP male mice. Arrows indicates GLP-1^{negative}/GFP ^{positive} α-cells from saline control group. Values represent means ± SEM for 6 mice (~100 islets per group). *$p < 0.05$ and **$p < 0.01$ compared to saline control group. Scale bars: 50 µm.

Figure 6.11 Effects of liraglutide, sitagliptin and dapagliflozin on alpha cells lineage (A) Gln^{postive}/GFP^{positive}, (B) Gln^{postive}/GFP^{negative} and (C) Gln^{negative}/GFP^{positive} in normal and streptozotocin diabetic mice



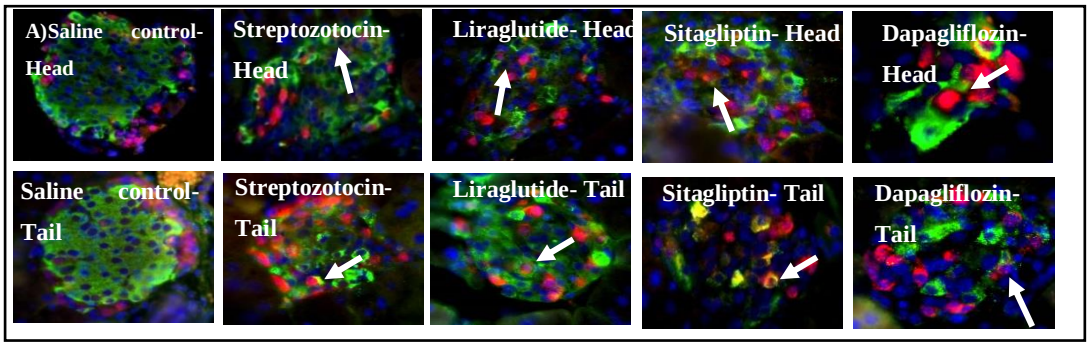
Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and GFP (red). (B) Percentage of glucagon positive & GFP positive cells (C) percentage of glucagon positive & GFP negative cells, and (D) Percentage of glucagon negative & GFP positive cells were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice (100-150 islets per group). **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 and ΔΔp < 0.01 compared to streptozotocin treated group. Scale bars: 50 µm.

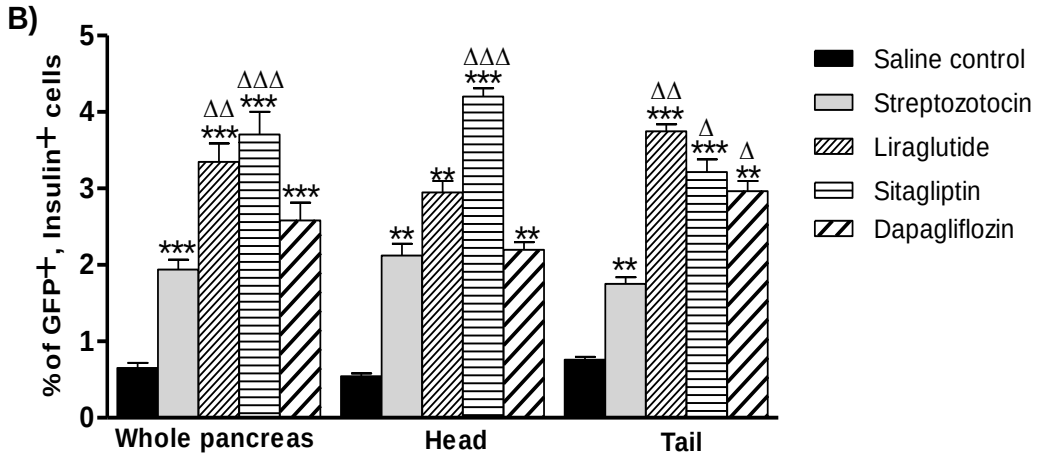
Figure 6.12 Effects of liraglutide, sitagliptin and dapagliflozin on generation of bi-hormonal cells (A, B) Gln^{postive}/Insulin^{positive} in normal and streptozotocin diabetic mice



Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). (B) Glucagon^{positive}/Insulin^{positive} cells were analysed using Cell^{F} and ImageJ at the end of the study Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice (100-150 islets per group). *p < 0.05 and **p <0.01 compared to saline control group. Δp < 0.05 and ΔΔp < 0.01 compared to streptozotocin treated group. Scale bars: 50 µm.

Figure 6.13 Effects of liraglutide, sitagliptin and dapagliflozin on alpha cells transdifferentiation into beta cell (A, B) GFP^{postive}/Insulin^{positive} in normal and streptozotocin diabetic mice





Representative images (A) showing immunostaining for DAPI (blue), insulin (green) and GFP (red). (B) GFP^{positive}/Insulin^{positive} cells were analysed using Cell^{F} and ImageJ at the end of the study Twice a daily i.p. dose of 25 ng/kg of liraglutide and a single daily oral dose of 25 ng/kg of each sitagliptin and dapagliflozin was administered for 10 days in diabetic Glu^{CreERT2};ROSA26-eYFP. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 6 mice (100-150 islets per group). **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05, ΔΔp < 0.01 and ΔΔΔp <0.001 compared to streptozotocin treated group. Scale bars: 50 µm.

Chapter 7

Effects of insulin, GABA and nicotinamide on islet morphology and alpha-cell transdifferentiation in insulin-deficient diabetic $Glu^{CreERT2}$; ROSA26-eYFP mice

7.1 SUMMARY

The damage to beta cells causes a loss in the counter-regulatory mechanism of alpha cells. Usually, the growth and function of alpha cells are largely regulated by insulin and GABA. However, after loss paracrine action with beta cells, it is interesting to understand how alpha cells respond to insulin and GABA signaling especially in any demand to their transdifferentiation into beta cells. Moreover, nicotinamide is an inducer of differentiation machinery, which previously facilitated stem cells into beta cells. Therefore, in this chapter, we explored the alpha cell transdifferentiation potential of insulin, GABA, and nicotinamide.

The $Glu^{CreERT2}$;ROSA26-eYFP mice with diabetes induced by multiple low dose streptozotocin was administered a day thrice with insulin (1U/kg) and once with GABA (10 mg/kg) or nicotinamide (150 mg/kg) for 10 days of the period. Although none of the treatment shows beneficial effects on preventing hyperglycaemia and other body parameters, insulin and GABA improved islet morphology by increasing beta cell area with decreasing alpha cell area. GABA significantly enhanced pancreatic insulin content, while plasma insulin was unaltered by all diabetic treated groups. Plasma glucagon had a declining trend in all diabetic groups but not in nicotinamide. Insulin and nicotinamide prevented beta cells from apoptotic death. Moreover, insulin and GABA inhibited alpha cell expansion; in addition, GABA promoted beta cell proliferation as well. Interestingly, insulin and GABA significantly stimulated alpha to beta cell transdifferentiation.

Therefore, from this chapter, we assume that insulin and GABA re-establishes the paracrine mechanism to some extent to control alpha cell expansion. In addition, they divert alpha cells to beta cells through transdifferentiation. Apart from this, GABA encouraged existing beta cells while insulin or nicotinamide protected them from further injury. Overall, we suggest that the abundant number of insulin and GABA receptors on the alpha cells might be the interesting therapeutic targets to guide an alpha cell to generate new beta cells.

7.2 INTRODUCTION

Diabetes mellitus is a disease of uncontrolled hyperglycaemia, which results from the gradual loss secretory function or mass of the beta cells (Moffett et al., 2015a; Vasu et al., 2015, 2016). Due to severe glycemic challenges, the beta cells lose their innate ability of neogenesis or proliferation (Vasu et al., 2014a, 2014b). However, recent observations have highlighted the importance of islet plasticity to compensate for the cellular loss (Puri et al., 2015). Earlier it was believed that every cell has a committed function, but recent advances revealed that cells are not rigid but rather possess fate-changing characteristics (Habener et al., 2012; Van et al., 2015; Puri et al., 2015). In this context, alpha cells represent a cellular pool capable of supplying significant numbers of new beta cells (Thorel et al., 2010; Cheng et al., 2015; Stanojevic et al., 2015). Recent studies demonstrate that manipulation of islet plasticity at genomic level represents a possible approach to generate beta cells via conversion of alpha cells **(**Collombat et al., 2009; Lu et al., 2010; Yang et al., 2011; Wilcox et al., 2013). Although certain genes such as Pax4 **(**Collombat et al., 2009), Pdx1(Yang et al., 2011) or Arx **(**Wilcox et al., 2013) have been identified as key regulators in disrupting alpha cell identity while promoting beta cell phenotype, the exact mechanism underlying transdifferentiation process remains principally unknown (Gromada et al., 2007; Stanojevic et al., 2015). Moreover, in the last two decades, several pharmacological based approaches have been reported to achieve this but its actual mechanism of alpha transdifferentiation remains elusive **(**Brown et al., 2016; Lu et al., 2016; Ben-Othman et al., 2017; Li et al., 2017; Lee et al., 2018).

It is noteworthy that beta cells secrete insulin in conjunction with GABA and zinc, all of which are believed to affect glucagon synthesis and secretion from adjacent alpha cells (Bansal et al., 2008). As well, insulin and GABA receptors are present on alpha cells (Bansal et al., 2008/2011). Usually, these receptors modulate alpha cell function to work in harmony with beta cells (Bansal et al., 2008). However, it is interesting to elaborate the behaviour of these receptors to cope with the changed surroundings after the loss of paracrine connection with adjacent beta cells. Previous studies demonstrate a role of GABA in the protection and regeneration of beta cells (Soltani et al., 2011; Bansal et al., 2011). Notwithstanding this, Ben-Othman et al. (2017) reported GABA mediated alpha to beta cell transdifferentiation using lineage tracing. These authors

demonstrated that GABA signaling on alpha cells suppressed Arx expression, which thereby activated insulin expression in alpha cells. Moreover, the authors went on to show that duct cells contributed to the emerging alpha cells, which subsequently converted into new beta cells. In contrast, another cell lineage study using GABA treatment found no evidence of alpha cell transdifferentiation (Ackermann et al., 2018). Similar observations were noted in another non-lineage study using GABA treatment in the rhesus monkey (Shin et al., 2019). In line with this, controversial evidence has been reported with GABA agonist artemether (Li et al., 2017; Van et al., 2018). However, these studies (Ackermann et al., 2018; Van et al., 2018; Shin et al., 2019) displayed certain experimental discrepancies and limitations compared to Ben-Othman et al (2017) study. Therefore, further insight into GABA mediated alpha cell transdifferentiation is required.

Another quest is to ascertain the role of insulin in alpha cell transdifferentiation. It is well known that lack of insulin-producing beta cells triggers alpha cell transdifferentiation (Liang et al., 2011; Cheng et al., 2015). It makes sense that the absence of insulin signaling might activate insulin expression from non-beta cells such as alpha cells, while the presence of insulin could inhibit transdifferentiation (Ye et al., 2016; Lee et al., 2018). Contrary to this hypothesis, it is noteworthy that alpha cell transdifferentiation occurred even in its presence of exogenous insulin, which was administered to prolong the survival of mice with severe diabetes (Thorel et al., 2010; Wang et al., 2014; Cheng et al., 2015). If the concept of insulin lacking triggering conversion of alpha to beta cells is correct, then the glucagon secreting cultured alpha TC cell line which is devoid of surrounding beta cells and therefore deprived of insulin would be expected to transdifferentiate into beta cells. However, this is not reported in the literature. Therefore, it is interesting to further elucidate the role of insulin signaling during extreme beta cell ablation.

Another molecule, nicotinamide has been widely studied in relation to insulin synthesis from sources of non-beta cells such as mesenchymal stem cells (Yang et al., 2015), bone marrow cells (Dong-Qi et al., 2004), other endocrine cells (Vaca et al., 2003) or progenitors cells (Mngomezulu et al., 2000). Interestingly, there is no report on the action of nicotinamide on alpha cells transdifferentiation, but it is known to protect and preserve beta cell mass when administered with streptozotocin.

In the present study, we tested the effect of insulin, GABA and nicotinamide on islet morphology and alpha cells transdifferentiation using $Glu^{CreERT2}$; ROSA26-eYFP mice. Moreover, we also aimed to ascertain the role of these treatments in the regeneration of beta cells from pre-existing beta cells.

7.3 MATERIALS AND METHODS

Materials and methods for this study have been discussed in Chapter 2.

7.4 RESULTS

7.4.1 Effects of insulin, GABA and nicotinamide on body parameters of $Glu^{CreERT2}$; ROSA26-eYFP mice

Streptozotocin-borne severe diabetes led to significant ($p<0.05$ to $p<0.01$) weight loss compared to $Glu^{CreERT2}$; ROSA26-eYFP mice controls (Figure 7.1A). Exogenous insulin slightly improved weight change but none of the treatments achieved significance (Figure 7.1A). This was further corroborated by AUC data showing no significant difference among all the groups compared to saline control (Figure 7.1B). Food and fluid consumption was significantly ($p<0.05$ to $p<0.001$) increased in all treatment groups in comparison to saline-treated mice, with the slightly declining trend observed with insulin and GABA at some observations points (Figure 7.1C and 7.2A). Unpredictably, significantly ($p<0.001$) higher levels of blood glucose were evident among all diabetic groups when compared with normal mice (Figure 7.1B). Importantly, none of the treatment regimens showed a glucose-lowering effect as compared to STZ-administered groups (Figure 7.1B). In marked contrast, blood glucose after 1hr of insulin treatment showed distinct reductions as compared to saline controls (Figure 7.2B). The AUC values were significantly ($p<0.01$ to $p<0.001$) increased in all diabetic groups, compared with normal mice (Figure 7.2C). However, AUC values recorded after 1 hr of insulin administration showed significantly ($p<0.01$) lower values compared to the control STZ group (Figure 7.2C). STZ-triggered diabetes was with significantly ($p<0.01$ to $p<0.001$) reduced plasma and

pancreatic insulin concentrations as compared to saline controls (Figure 7.3A, B). Importantly, its GABA treated group showed a significant increase ($p<0.05$) in pancreatic insulin content (Figure 7.3B). Unexpectedly, STZ-treated diabetic group controls or those treated with insulin, GABA or nicotinamide showed similar plasma glucagon concentrations when compared to normal mice (Figure 7.3C). Similarly, pancreatic glucagon content was unaltered in all diabetic groups compared to saline control (Figure 7.3D).

7.4.2 Effects of insulin, GABA and nicotinamide on islet morphology

Figure 7.4A illustrates representative images indicating staining for insulin (red) and glucagon (green) from the islets of $Glu^{CreERT2}$;ROSA26-eYFP mice.

As shown in Figure 7.4B, C; the total number of islets was moderately decreased in all groups compared to controls (Figure 7.4B). Whilst, total islet area was significantly ($p<0.05$ to $p<0.001$) reduced in all STZ mice compared to saline mice (Figure 7.4C). Intriguingly, insulin and GABA significantly ($p<0.05$) increased islet area in comparison with STZ treated controls (Figure 7.4C). Notably, large size islets were found to absent in streptozotocin treated controls and those receiving nicotinamide (Figure 7.4D). In accordance, small size islets were slightly increased as compared to controls (Figure 7.4D). As would be expected, beta cell area was significantly ($p<0.01$ to $p<0.001$) decreased in all groups together with a significant ($p<0.001$) expansion of alpha cell area, compared to saline-treated groups (Figure 7.5A, B).

Interestingly, insulin and GABA treatments significantly ($p<0.05$) increased beta cell area in comparison with STZ group (Figure 7.5A). Concomitantly, alpha cells area was significantly ($p<0.001$) increased within diabetic groups when compared with saline controls (Figure 7.5B). Insulin and GABA significantly ($p<0.05$) reduced alpha cell area compared with STZ group (Figure 7.5B).

7.4.3 Effect of insulin, GABA and nicotinamide on beta cell proliferation and apoptosis

Figure 7.6A shows representative images indicating staining for Ki67 (green) and insulin (red) from the islets of $Glu^{CreERT2}$; ROSA26-eYFP mice. GABA showed significant ($p<0.01$) enhancement of beta cell proliferation as compared to both saline and STZ groups (Figure 7.6B), while it was unaltered by insulin and nicotinamide. Representative images indicating staining for TUNEL (green) and insulin (red) from the islets of $Glu^{CreERT2}$; ROSA26-eYFP mice are illustrated in Figure 7.7A. As would be expected, all diabetic mice showed significantly ($p<0.05$ to $p<0.01$) increased beta cell apoptotic death (Figure 7.7B). In contrast, insulin and nicotinamide significantly ($p<0.05$) reduced beta cell death, while it was unchanged after GABA treatment (Figure 7.7B).

7.4.4 Effect of insulin, GABA and nicotinamide on alpha cell proliferation and apoptosis

Figure 7.8A shows representative images indicating staining for Ki67 (green) and glucagon (red) from the islets of $Glu^{CreERT2}$; ROSA26-eYFP mice. Notably, when compared to saline controls, significant ($p<0.01$ to $p<0.001$) alpha cell proliferation was evident in all treatment groups (Figure 7.8B). It was markedly ($p<0.05$) reduced by insulin and GABA in comparison with STZ group (Figure 7.8B). Representative images indicating staining for TUNEL (green) and glucagon (red) from the islets of $Glu^{CreERT2}$; ROSA26-eYFP mice are illustrated in Figure 7.9A. Alpha cell apoptosis was not changed in of no treatment groups when compared to saline normal controls and the STZ group (Figure 7.9B). Insulin showed a tendency to reduce alpha cell apoptosis rate in comparison with STZ group (Figure 7.9B).

7.4.5 Effect of insulin, GABA and nicotinamide on alpha cell lineage tracing

Figure 7.10A illustrates representative images indicating staining for glucagon (green) and GFP (red). Here, Glucagon+/GFP+ colocalized cells & Glucagon+/GFP- cells represent undifferentiated alpha cells, and Glucagon-/GFP+ cells represent reprogrammed alpha cells. As shown in Figure 7.10B, up to 65% of glucagon positive alpha cells were irreversibly tagged with GFP. While 38-55 % of the cells did not

express GFP (Figure 7.10D). Furthermore, glucagon negative/GFP positive cells were significantly ($p<0.05$ to $p<0.001$) increased in all treated groups when compared to saline controls (Figure 7.10C). Interestingly, insulin significantly ($p<0.05$) enhanced such glucagon lacking GFP localise cells as compared to STZ treated groups (Figure 7.10C).

7.4.6 Effect of insulin, GABA and nicotinamide on the generation of bihormonal cells

Figure 7.11A illustrates representative images indicating staining for insulin+ (red) and glucagon+ (green) bihormonal cells from the islets of $Glu^{CreERT2}$; ROSA26-eYFP mice. The emergence of bihormonal cells was significantly ($p<0.01$) increased in both insulin and GABA treated groups, while STZ and nicotinamide unaltered this population when compared with saline controls (Figure 7.11B). Moreover, insulin and GABA significantly increased bihormonal cells significantly ($p<0.05$) as compared to STZ groups (Figure 7.11B).

7.4.7 Effect of insulin, GABA and nicotinamide on reprogramming of alpha cells into beta cell

Figure 7.12A shows representative images indicating staining for insulin+ (green) and GFP+ (red) co-expressing cells from the islets of $Glu^{CreERT2}$; ROSA26-eYFP mice. Importantly, streptozotocin-induced significant ($p<0.001$) co-expression of insulin in GFP+ alpha cells (Figure 7.12B). Interestingly, these insulin co-expressed cells were further enhanced in number ($p<0.05$ to $p<0.001$) by insulin and GABA (Figure 7.12B). However, nicotinamide did not affect its number of such cells (Figure 7.12B).

7.5 DISCUSSION

It has recently been suggested that the formation of new beta cells after severe beta cell injury primarily depends on the transition of other non-beta cells or proliferation of residual beta cells (Thorel et al., 2010; Lee et al., 2018). However, the major events underlying the contribution of alpha cells to restore beta cell pool has not been clearly

understood (Stanojevic et al., 2015). In this study, we demonstrate whether alpha cells or other remaining beta cells are involved in the production of insulin, and examine the possible effect of insulin, GABA, and nicotinamide on this process using $Glu^{CreERT2}$ ROSA26 e-YFP mice. Also, we addressed the contribution of proliferation and apoptosis of both beta and alpha cells to altered the islet architecture following diabetes induced by streptozotocin in $Glu^{CreERT2}$; ROSA26-eYFP mice.

7.5.1 Effects of treatments on islet morphology

Previous reports provided evidence for severe impairment in islet symmetry after streptozotocin attack on beta cells in diabetic animals (Vasu et al., 2014a). In the present study, it is reflected by shrunken both islet size and the number of total islets. Moreover, anomalous islet size distribution resulted in the loss of large-sized islets. In contrast, insulin and GABA showed a beneficial compensatory effect on the islet area. Furthermore, the number of islets was also enhanced with insulin and GABA, while size distribution tended to be rehabilitated. The dramatic distortion in islet structure in diabetic mice resulted in a significant reduction in the beta-cell area. This loss of beta cells caused an increase in the alpha cell area. However, insulin and GABA slightly restored beta cell mass. Previous studies have shown the beneficial effects of insulin and GABA on beta cell expansion (Wang et al., 2014; Purwana et al., 2014; Cheng et al., 2015). Therefore, we can speculate that beta cells might be regenerated at the expense of alpha cells.

7.5.2 Alpha cells acquire bihormonal characteristics

Several lines of evidence have been revealed that alpha cells may express two hormones before transforming into functional beta cells (Piran et al., 2014; Brown et al., 2016). To ascertain this possibility, we used the double staining immunoreactivity for glucagon and insulin. Interestingly, we found that the presence of bihormonal cells in all treated groups. Indeed, the bihormonal cells were significantly increased by insulin and GABA. In addition, double staining for glucagon with GFP revealed that the presence of GFP+/Glucagon- cells were increased after insulin and GABA treatment. These observations suggest that glucagon expression is suppressed in alpha cells, which might be important for the transformation event.

7.5.3 Regeneration of beta cells through reprogramming of alpha cells

Reestablishment of beta cell mass is a novel concept to help manage diabetes, which can be achieved by an ample supply of mature beta cells derived from either pancreatic islet tissue transplantation or reprogramming of other endocrine cells (Puri et al., 2015). Recent investigations have revealed that beta cell injury in mouse models may trigger endocrine cell reprogramming into beta cells (Thorel et al., 2010; Ben-Othman et al., 2017; Lee et al.,2018). Due to plasticity, islet alpha cells are believed to be the best suitable source to regenerate new beta cells (Puri et al., 2015). Therefore, in an attempt to ascertain the possible involvement of alpha cells into the regeneration of beta cells, we used $Glu^{CreERT2}$; ROSA26-eYFP mice to provide ease detection of alpha cell lineage. Accordingly, we examined whether GFP-tagged alpha cells to start producing insulin in response to extreme islet injury triggered by STZ. Importantly, we found the increased population of GFP labelled- alpha cells carrying insulin. More importantly, such insulin+/GFP+ cells were further augmented in the presence of insulin and GABA treatment regimens. In contrast, nicotinamide did not influence such co-expression. Therefore, we suggest that insulin and GABA may possess certain beneficial effects on alpha to beta transition.

These findings are consistent with previous studies on GABA by Ben-Othman et al (2017), where authors showed a chronic effect of GABA on alpha cell transdifferentiation. Further, they went on to explain the possible involvement of $GABA_A$ receptor in repressing Arx and inducing Pdx1 in alpha cells to emerge beta cells. On the contrary, some studies have contradicted the role of GABA in transdifferentiation (Ackermann et al., 2018; Shin et al., 2019). However, these studies did not use diabetic models. In the present study, we used lineage tracing in diabetic mice together with a higher dose of GABA compared to use by Ben-Othman et al (2017). Using this dose, we explore the long-term effects of GABA on alpha cell transdifferentiation. Similarly, insulin effects are controversial with respect to alpha cell transdifferentiation. It makes sense that the loss of beta cells develops insulin deficiency, which may trigger alpha cell conversion to beta cells. In the present study, we found insulin enhanced alpha cell transdifferentiation. This finding is in line with previous studies **(**Thorel et al., 2010; Wang et al., 2014; Cheng et al., 2015), which also showed alpha cell transdifferentiation after exogenous insulin was administered to help the survival of mice from a diabetic attack. From this, we can not be ruled out that insulin exerts its effects either directly through insulin receptors present on alpha

cells or by indirectly managing glycaemia. This would be an area for future research. Furthermore, earlier studies showed nicotinamide exert beneficial effects on inducing beta cell markers *in vitro* cultured stem cells (Otonkoski et al., 1993; Kunisada et al., 2012). Therefore, it is interesting to explore the long-term effect of nicotinamide in this context.

7.5.4 Alpha cells tagging in $Glu^{CreERT2}$; ROSA26-eYFP mice

In the present study, the fluorescent protein EYFP was expressed exclusively in alpha cells in response to a single dose of tamoxifen. We found that a substantial population of alpha cells were fluorescently labeled with GFP in all mice studied. Importantly, the EYFP expression trend was similar in all groups, including saline control. Therefore, the presence of glucagon positive/GFP- cells may reflect the failure of GFP expression in some alpha cells rather than an ectopic expression of glucagon in non-alpha cells. This notion was further supported by the presence of a minor number of bihormonal cells. Nevertheless, this limitation can be overcome in the future by selecting multiple doses of tamoxifen. Overall, $Glu^{CreERT2}$; ROSA26-eYFP mice model is suitable for studying alpha to beta cells transdifferentiation in curing pathophysiology.

7.5.5 Alpha cells hyperplasia and glucagon expression

Pancreatic tissue islets possess a repair mechanism to help avoid absolute loss of hormone-producing function. In particular, islets are composed of primarily beta cells, and their loss has frequently been observed in the presence of uncontrolled expansion of alpha cell mass (Whalley et al., 2011). In harmony with this, we observed hyperplasia of alpha cells following the elimination of beta cells using STZ. Surprisingly, plasma glucagon concentrations were to be decreased in diabetic animals. As suggested in chapter 6, these findings indirectly suggest that glucagon gene might play some unique role in the conversion of alpha to beta cells. In accordance, previous studies (Whalley et al., 2011; Moffett et al., 2015b; Ye et al., 2015; Lee et al., 2018) point to the synthesis of glucagon gene product other than glucagon, since PC1/3 expression has been detected in alpha cells. Therefore, further studies are needed to elucidate the importance of glucagon gene products with respect to alpha cell transdifferentiation.

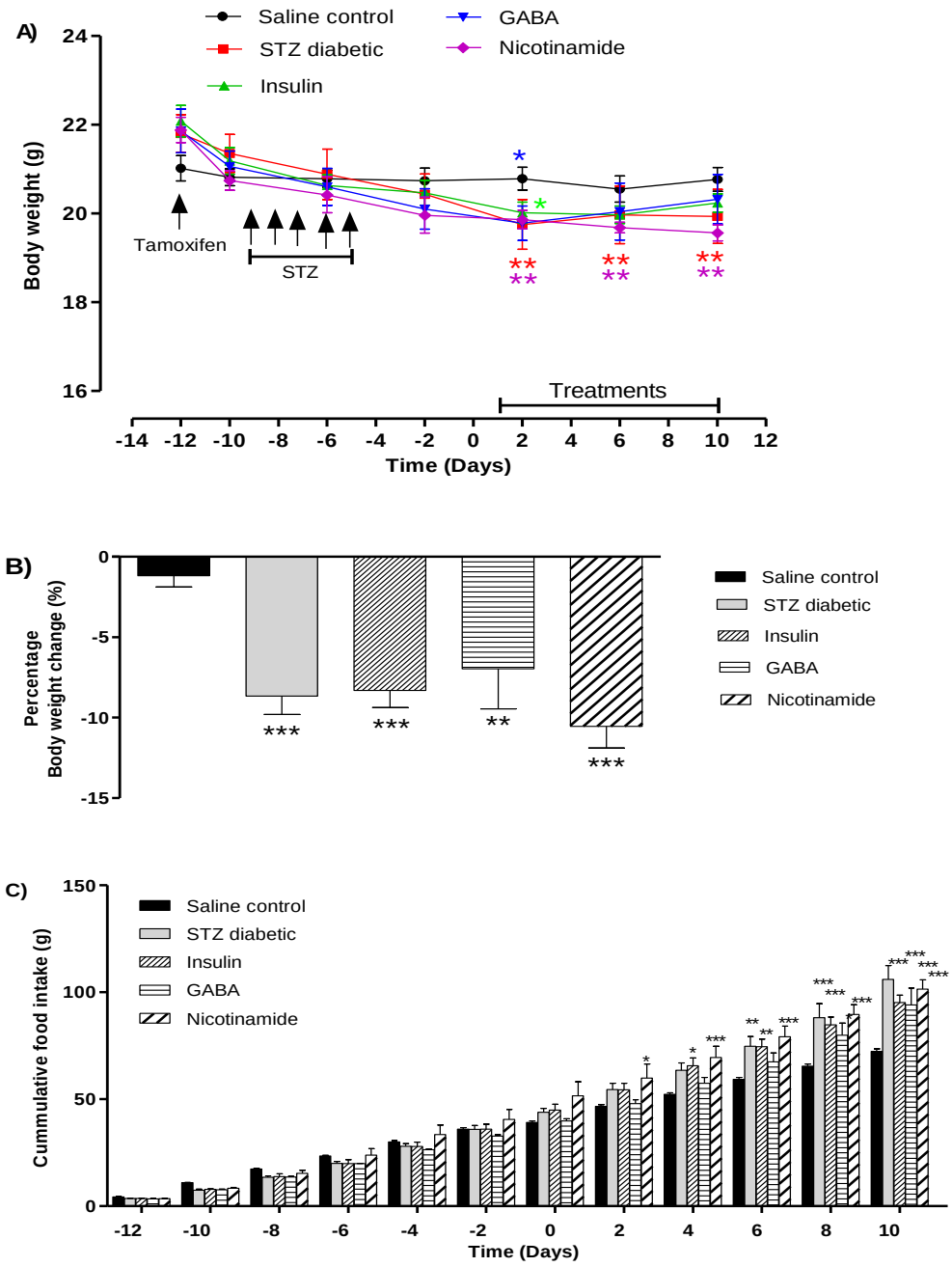
7.5.6 Effects of treatments on Body parameters

As would be expected, streptozotocin caused severe induction of diabetes in $Glu^{CreERT2}$; ROSA26-eYFP mice. The diabetic animals consumed more food and water than normal mice reflecting hyperphagia and polydipsia (Havel et al., 2000). However, the amount of food consumption was not sufficient to counter the uncontrolled loss of body weight. STZ-induced a massive loss of beta cells, insulin deprived resulted in uttermost hyperglycaemia. Although treatments had no direct beneficial effect on lowering blood glucose, a slight increase in total pancreatic insulin content were observed in both the insulin and GABA groups. One possible explanation is that the pancreas may still possess regenerative ability; however, the secretory function of regenerated beta cells might be adversely affected by extreme hyperglycaemic shock. Besides, regenerated beta cells might be in the juvenile stage of development. Furthermore, alpha cell transdifferentiation has been observed in extreme diabetic conditions (Thorel et al., 2010; Ben-Othman et al., 2017). Therefore, we suggest that future research should elucidate the role of the long-term glucose-lowering effects of the treatments in alpha cell transdifferentiation.

7.5.7 Concluding remarks

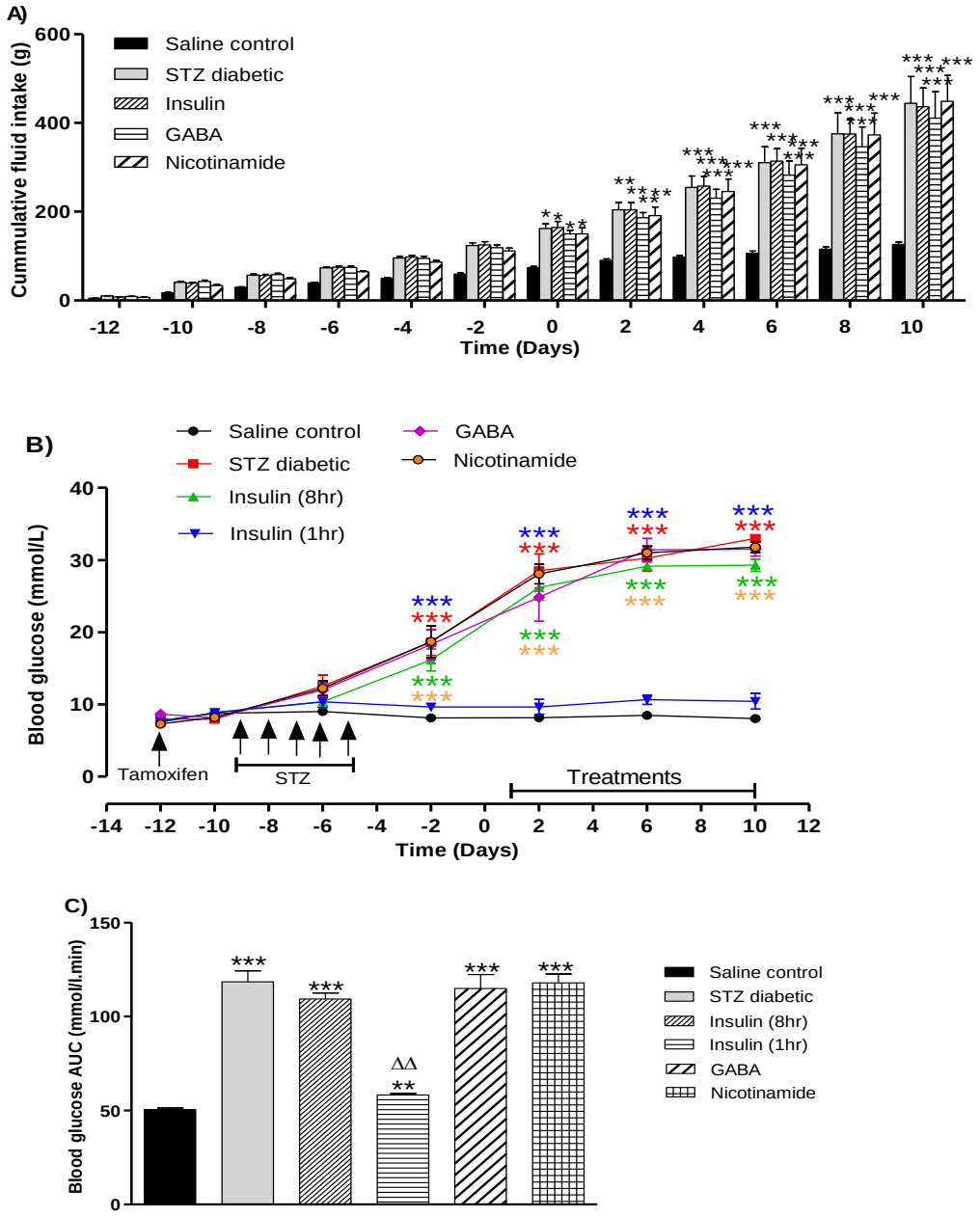
In conclusion, our study using $Glu^{CreERT2}$ ROSA e-YFP mice revealed that insulin and GABA signalling might induce alpha cell transdifferentiation. Although nicotinamide at preferred dose has not been shown to affect transdifferentiation over the acute period, beneficial effects were observed on beta cell protection with respect to reduction in the apoptotic rate. Therefore, doses of nicotinamide would be helpful to fully evaluate its role in alpha cell reprogramming. Further, understanding of the role of insulin receptor and GABA signaling within alpha cells to enable their transition to insulin-producing cells mass provides a robust diabetes therapy to enhance the compensation of beta-cell loss.

Figure 7.1 Effects of insulin, GABA and nicotinamide on (A, B) body weight and (C) food intake in normal and streptozotocin diabetic mice



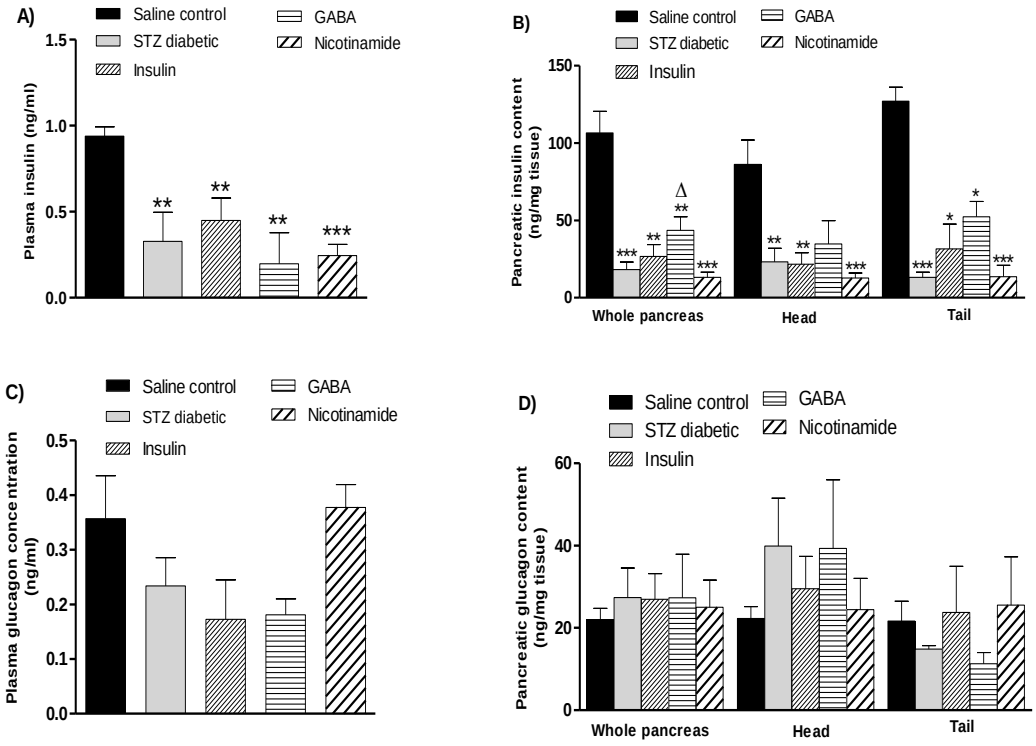
(A,B) Body weight and (C) food intake were measured throughout the study. Twice a daily i.p. injection of 1U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-6 mice. *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group.

Figure 7.2 Effects of insulin, GABA and nicotinamide on (A) fluid intake and (B, C) blood glucose in normal and streptozotocin diabetic mice



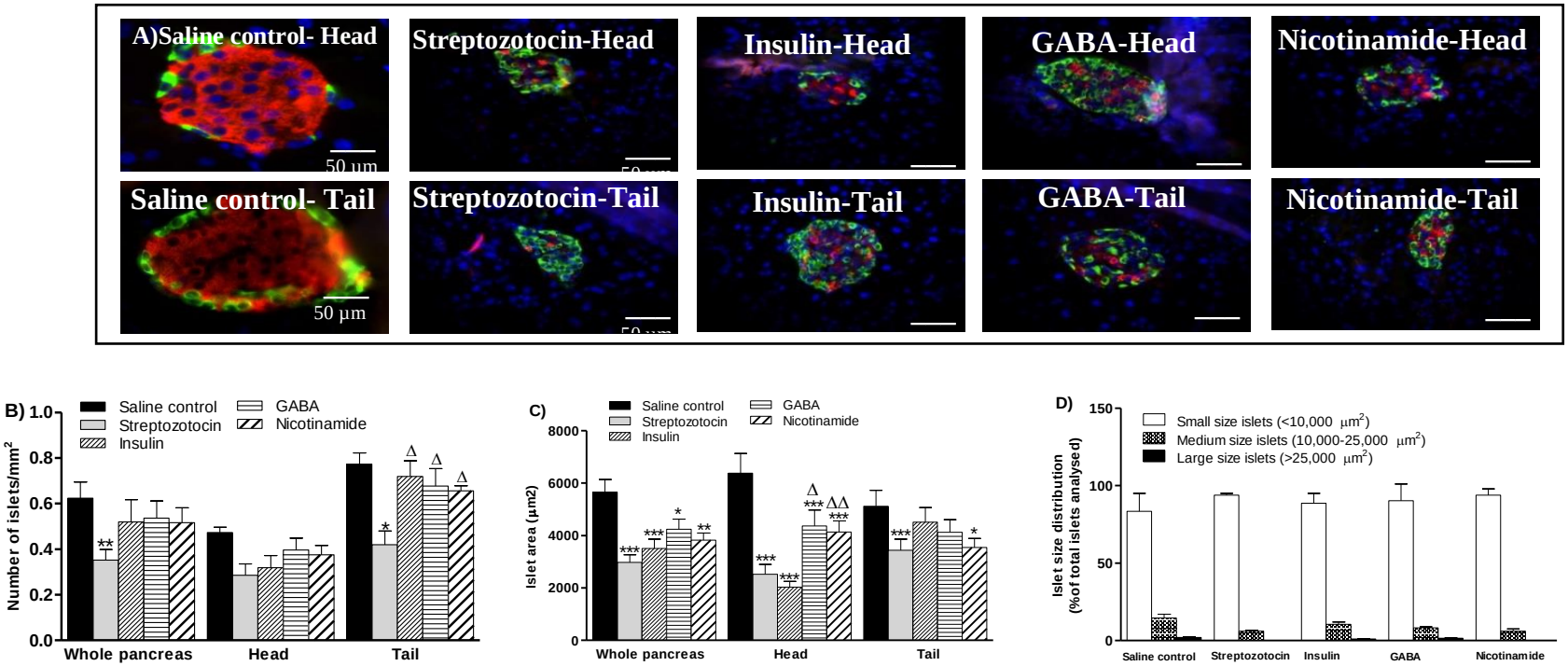
(A,B) Blood glucose and (C) fluid intake were measured throughout the study. Twice a daily i.p. injection of 1U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150mg/kg of nicotinamide was received for 10 days by diabetic $Glu^{CreERT2}$;ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice. *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group. ΔΔp < 0.01 compared to streptozotocin treated group.

Figure 7.3 Effects of insulin, GABA and nicotinamide on plasma and pancreatic (A, B) insulin or (C, D) glucagon concentration in normal and streptozotocin diabetic mice



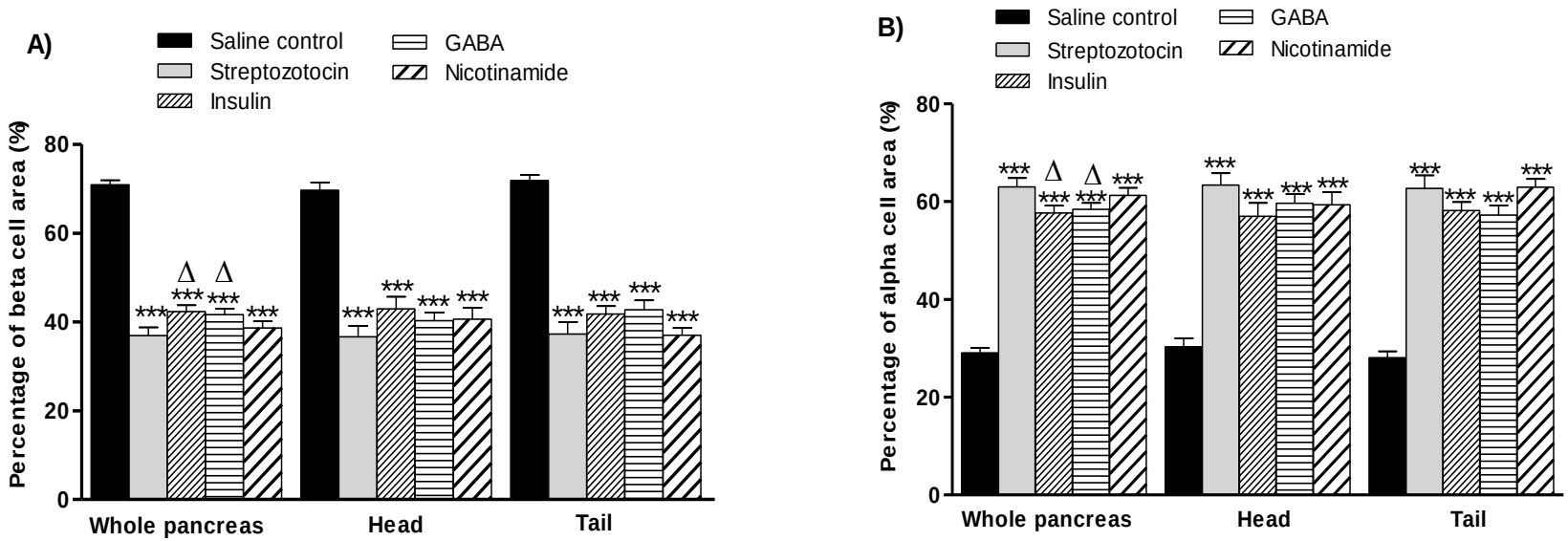
Plasma and pancreatic (A,B) insulin and (C,D) glucagon were measured at the end of the study. Twice a daily i.p. injection of 1U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150 mg of nicotinamide was received for 10 days by diabetic $Glu^{CreERT2}$;ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice. **$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. $\Delta p < 0.05$ compared to streptozotocin treated group.

Figure 7.4 Effects of insulin, GABA and nicotinamide on islet (A) number, (B) area and (C) size-distribution in normal and streptozotocin diabetic mice



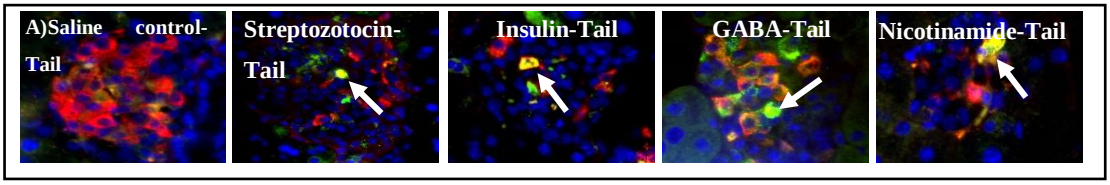
Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). Islet (B) number, (C) area and (D) size-distribution were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. injection of 1U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (100-150 islets per group). *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 compared to streptozotocin treated group. Scale bars: 50 µm.

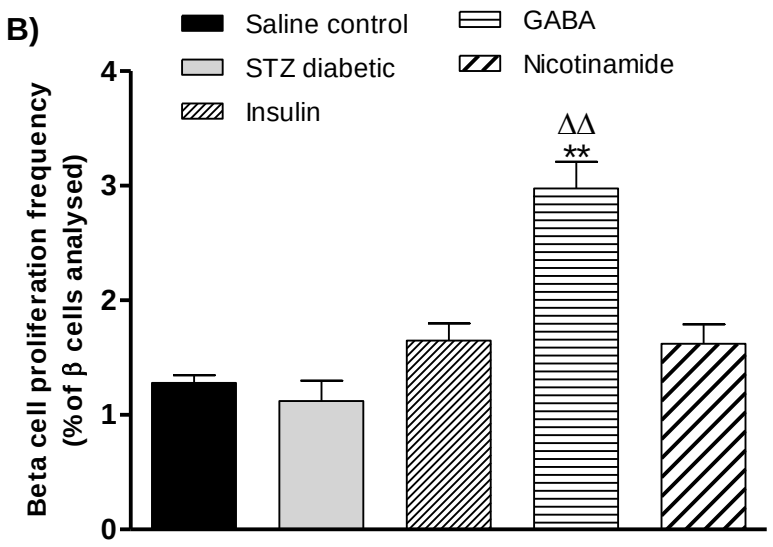
Figure 7.5 Effects of insulin, GABA and nicotinamide on (A) beta cell area and (B) alpha cell area in normal and streptozotocin diabetic mice



(A) Beta cell area, (B) alpha cell area were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. injection of 1U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (100-150 islets per group). **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. $\Delta p < 0.05$ compared to streptozotocin treated group.

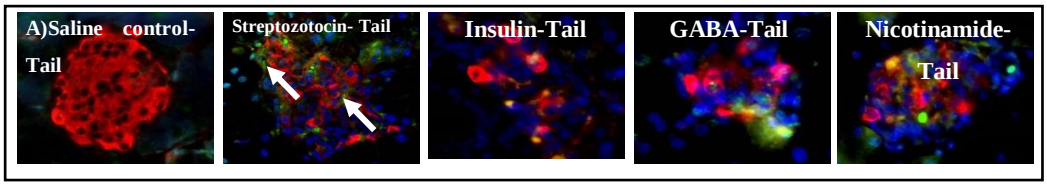
Figure 7.6 Effects of insulin, GABA and nicotinamide on (A, B) beta cell proliferation in normal and streptozotocin diabetic mice

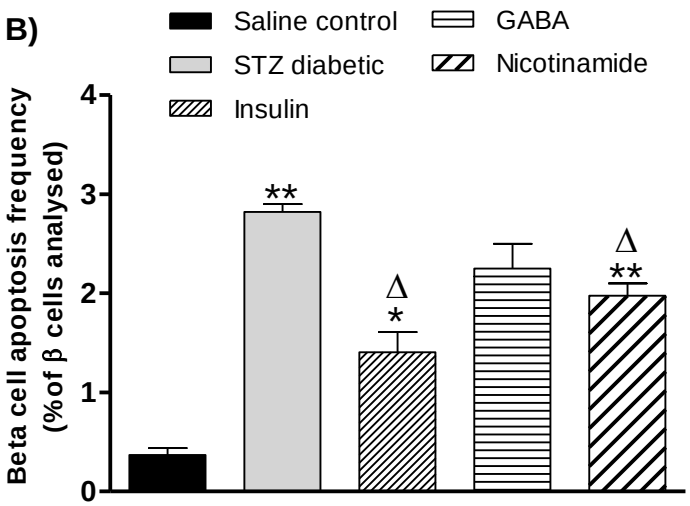




Representative images (A) showing immunostaining for DAPI (blue), Ki67 (green) and Insulin (red). (B) Beta cell proliferation were analysed using Cell^{F} and ImageJ at the end of the study.Twice a daily i.p. injection of 1U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150 mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (>60 tail islets per group). **p < 0.01 compared to saline control group. ΔΔp < 0.01 compared to streptozotocin treated group. Scale bars: 50 µm.

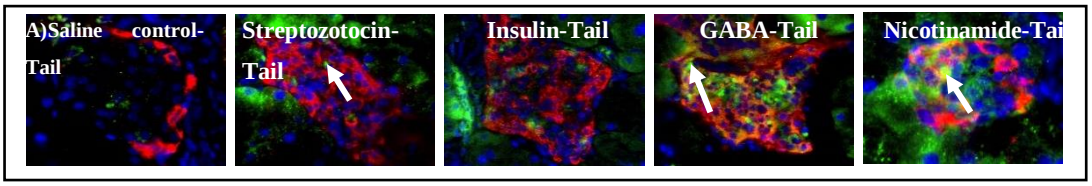
Figure 7.7 Effects of insulin, GABA and nicotinamide on (A, B) beta cell apoptosis in normal and streptozotocin diabetic mice

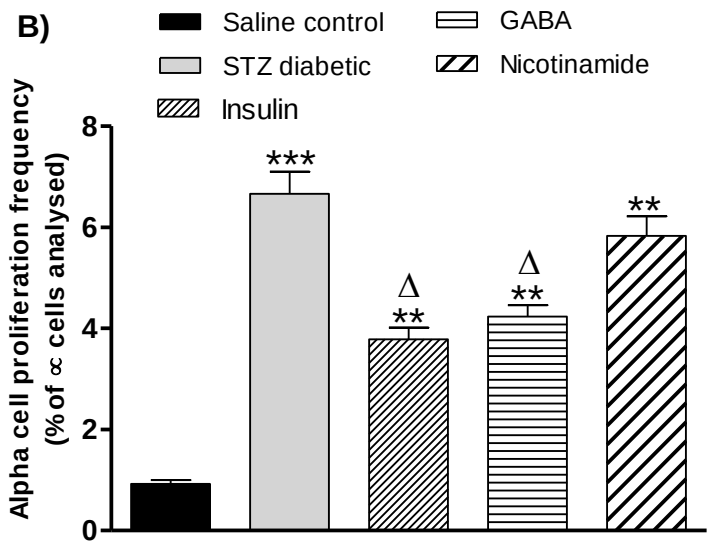




Representative images (A) showing immunostaining for DAPI (blue), TUNEL (green) and Insulin (red). (B) Beta cell apoptosis were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. injection of 1 U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150 mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (>60 tail islets per group). *$p < 0.05$ and **$p < 0.01$ compared to saline control group. $\Delta p < 0.05$ compared to streptozotocin treated group. Scale bars: 50 µm.

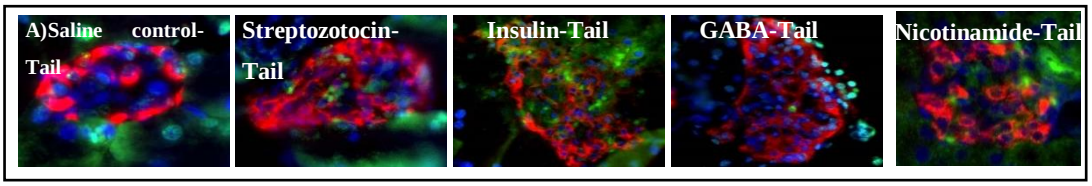
Figure 7.8 Effects of insulin, GABA and nicotinamide on (A, B) alpha cell proliferation in normal and streptozotocin diabetic mice

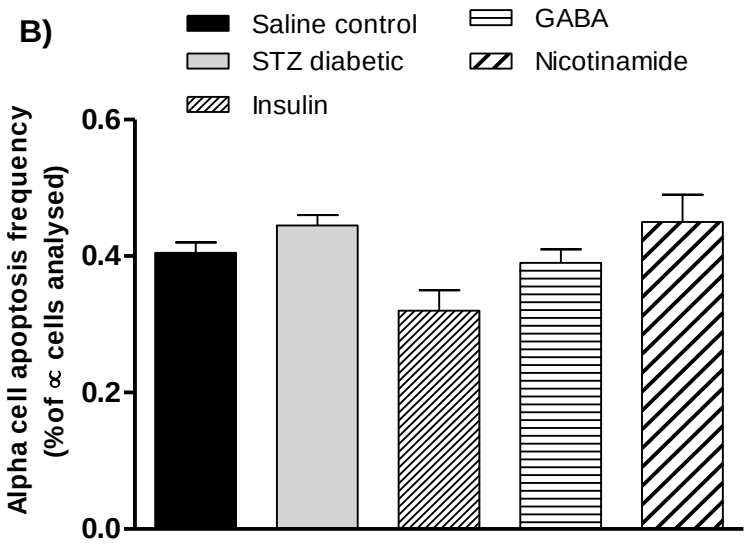




Representative images (A) showing immunostaining for DAPI (blue), Ki67 (green) and Glucagon (red). (A) Alpha cell proliferation were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. injection of 1 U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (>60 tail islets per group). **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 compared to streptozotocin treated group. Scale bars: 50 µm.

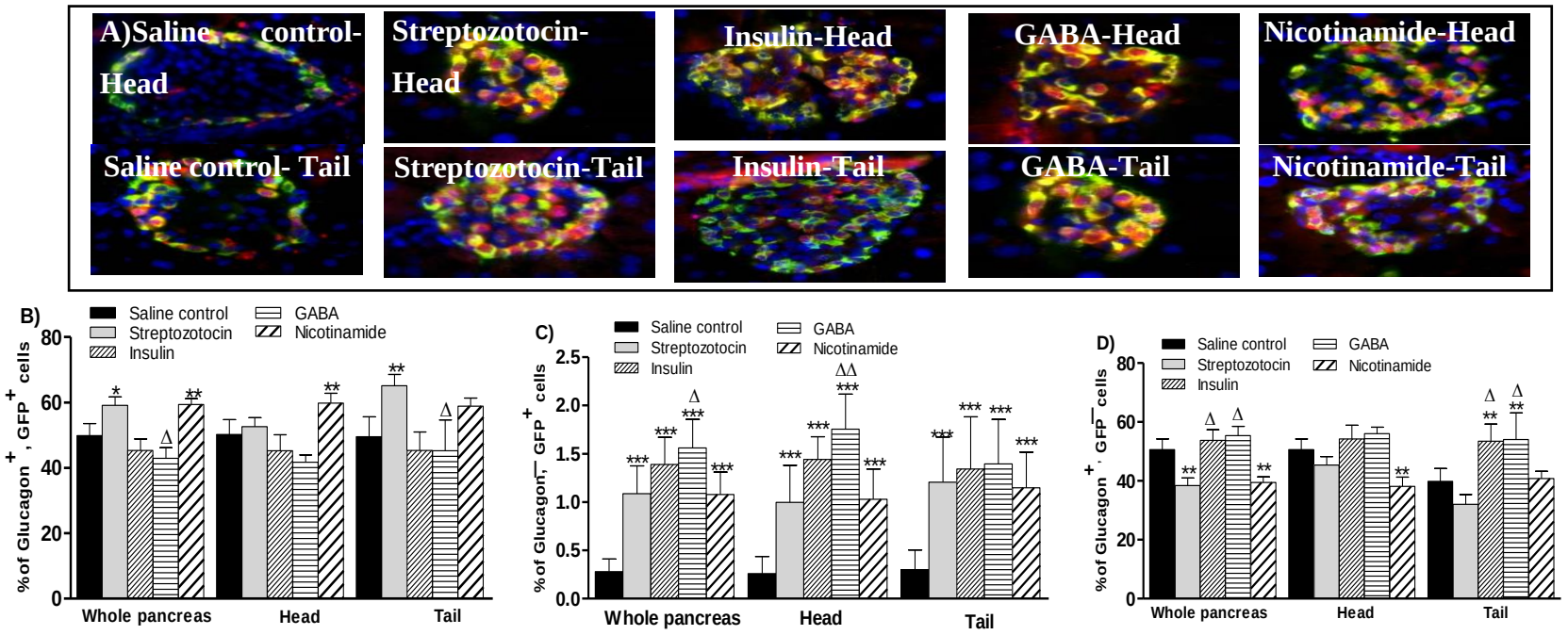
Figure 7.9 Effects of insulin, GABA and nicotinamide on (A, B) alpha cell apoptosis in normal and streptozotocin diabetic mice





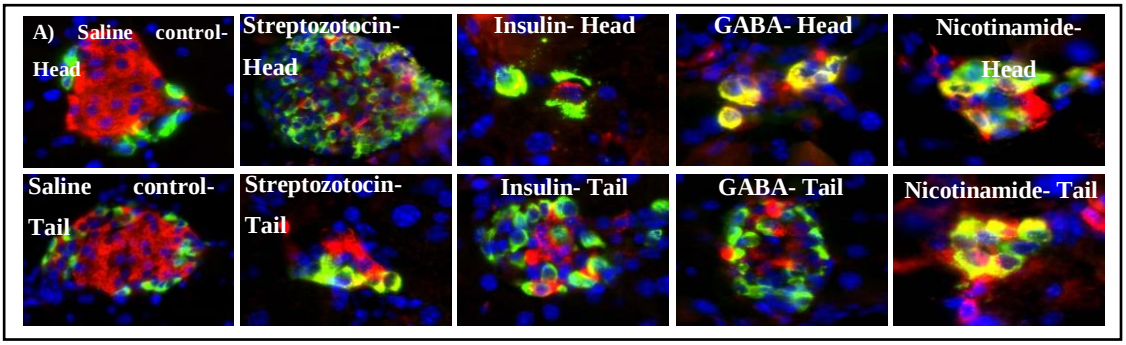
Representative images (A) showing immunostaining for DAPI (blue), TUNEL (green) and Glucagon (red). (A) Alpha cell apoptosis were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. injection of 1 U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150 mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (>60 tail islets per group). Scale bars: 50 µm.

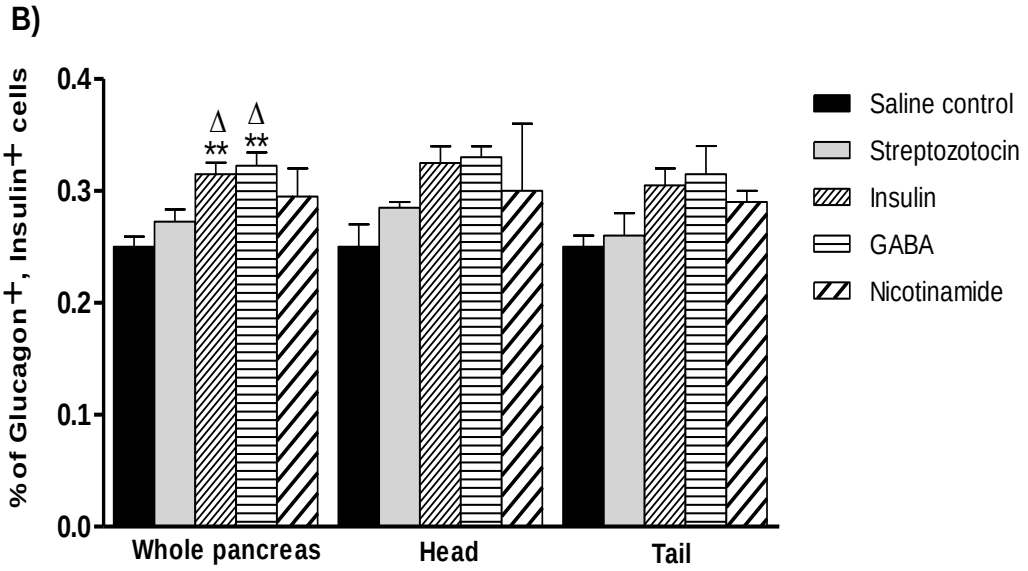
Figure 7.10 Effects of insulin, GABA and nicotinamide on alpha cells lineage (A) $Gln^{postive}/GFP^{positive}$, (B) $Gln^{postive}/GFP^{negative}$ and (C) $Gln^{negative}/GFP^{positive}$ in normal and streptozotocin diabetic mice



Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and GFP (red). **(B)** Percentage of glucagon positive & GFP positive cells, (C) percentage of glucagon negative & GFP positive cells and (D) Glucagon positive & GFP negative cells were analysed using $Cell^F$ and ImageJ at the end of the study. Twice a daily i.p. injection of 1 U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150 mg of nicotinamide was received for 10 days by diabetic $Glu^{CreERT2}$;ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (100-150 islets per group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. Δ$p < 0.05$ and ΔΔ$p < 0.01$ compared to streptozotocin treated group. Scale bars: 50 µm.

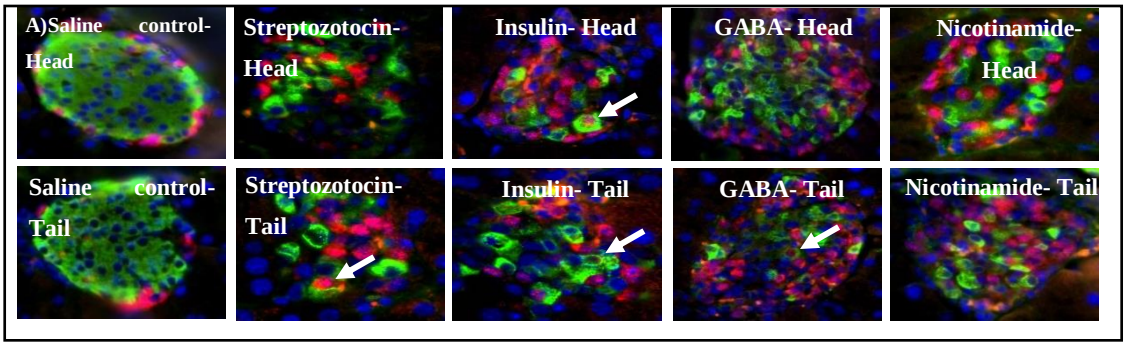
Figure 7.11 Effects of insulin, GABA and nicotinamide on generation of bi-hormonal cells (A, B) Gln^{postive}/Insulin^{positive} in normal and streptozotocin diabetic mice

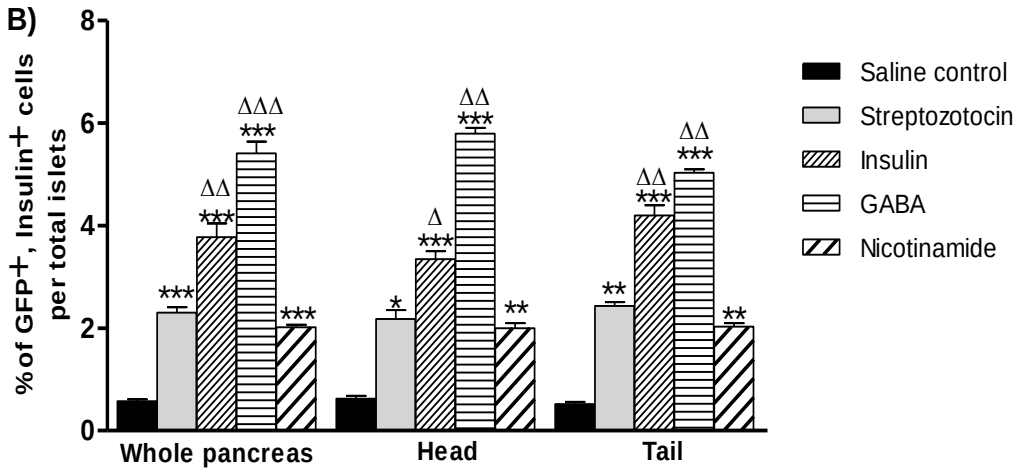




Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). (B) Glucagon^{positive}/Insulin^{positive} cells were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. injection of 1 U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150 mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (100-150 islets per group). $^{**}p < 0.01$ compared to saline control group. $\Delta p < 0.05$ compared to streptozotocin treated group. Scale bars: 50 µm.

Figure 7.12 Effects of insulin, GABA and nicotinamide on alpha cells transdifferentiation into beta cell (A, B) GFP^{postive}/Insulin^{positive} in normal and streptozotocin diabetic mice





Representative images (A) showing immunostaining for DAPI (blue), insulin (green) and GFP (red). (B) GFP^{positive}/Insulin^{positive} cells were analysed using Cell^{F} and ImageJ at the end of the study. Twice a daily i.p. injection of 1 U//kg of insulin and a single daily i.p. injection of 10 mg/kg of GABA or 150mg of nicotinamide was received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Controls received twice-daily injection of saline vehicle. Values represent means ± SEM for 5-7 mice (100-150 islets per group). *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05, ΔΔp < 0.01 and ΔΔΔp < 0.001 compared to streptozotocin treated group. Scale bars: 50 µm.

Chapter 8

Effects of rosiglitazone, tolbutamide and metformin on islet morphology and alpha-cell transdifferentiation in insulin-deficient diabetic $Glu^{CreERT2}$; ROSA26-eYFP mice

8.1 SUMMARY

Diabetes is a multi-disorder disease, which includes hyperglycaemia, impaired insulin secretin, or even loss of beta cells. Several treatments are available to reduce its complications. Rosiglitazone, tolbutamide, and metformin are few of them. Despite their extensive therapeutic use, very little is studied about their role in the islet repairs especially beta cell regeneration. Therefore, the present chapter aims to explore their roles in islet morphology and transdifferentiation of alpha to beta cells.

As expected, streptozotocin-induced severe hyperglycaemia in $Glu^{CreERT2}$;ROSA26-eYFP mice together with the loss of weight, polyphagia and polydipsia. However, once a daily injection of rosiglitazone (10 mg/kg bw), tolbutamide (20mg/kg bw) and metformin (100mg/kg bw) preventive effects on the alterations in food and fluid intake over the 10 days of the period. All the treatments showed declining plasma glucagon observed with a decrease in the pancreatic glucagon by rosiglitazone. Streptozotocin showed disrupted islet architecture with a decreased number of islets. Intriguingly, metformin improved islet structure by promoting area and proliferation of beta cell area with a reduction in the alpha cell area. Similarly, rosiglitazone increased beta cell proliferation. In contrast, tolbutamide did not protect beta cells instead promoted apoptosis. All the treatments showed an increasing trend in alpha to beta cell transdifferentiation compared to saline control; however, it was unchanged compared to diabetic control. Only metformin showed a slightly higher but still not significant transdifferentiated alpha compared to STZ diabetic. However, metformin increased the population of bihormonal cells compared to STZ.

Nevertheless, these conventional anti-diabetic drugs in combination with benefits on pancreatic islets, might be affected to their target sites a well, such as liver, insulin secretion or PPARγ. These effects slightly reflected in improvements in food and fluid intake. Importantly none of the treatments showed significant enhancement in alpha to beta transdifferentiation. Therefore, we suggest that PPARγ, SUR and AMPK signaling may not be effective in alpha cell transdifferentiation. However, transdifferentiation induced after beta cell loss was not inhibited by any of the treatments; rather, metformin showed an increasing trend. Therefore, further long-term studies using these regimens are merited.

8.2 INTRODUCTION

Absolute lack of pancreatic beta cells is a condition found in type 1 or severe type 2 diabetes (Moffett et al., 2015a; Vasu et al., 2015/2016). Reestablishment of insulin-producing pool in a severe state of diabetes is, therefore, a major medical challenge (Cheng et al., 2015).

Reformation of islet cells after a serious injury is a focus for rigorous attempts in the pathophysiology of diabetes and regenerative medicine (Cheng et al., 2015). Pancreatic islets cells form a paradigm for understanding the fate changing and protective behaviour of closely organised cells to harmonise pancreatic function during the critical period (Habener et al., 2012; Van et al., 2015; Puri et al., 2015). In this context, beta cell injury or loss has been observed to induce restoration demand from neighbouring cells notably alpha cells (Thorel et al., 2010; Cheng et al., 2015; Stanojevic et al., 2015). Interestingly, alpha cells not only release glucagon but they are also capable of reprogramming themselves to protect and restore beta cell identity (Vasu et al., 2014a; Puri et al., 2015). Although the exact mechanism is not fully understood, the intensive efforts have been made to convert alpha cells into beta (Gromada et al., 2007; Stanojevic et al., 2015).

In particular, injury to beta cells activates transdifferentiation mechanism among the adjacent alpha cells to start producing insulin for a compensatory purpose (Thorel et al., 2010; Liang et al., 2011; Cheng et al., 2015). Such inherent ability of alpha cells to display beta cell characteristics may hold a therapeutic potential to treat type 1 or type 2 diabetes (Thorel et al., 2010; Liang et al., 2011; Lee et al., 2018).

As such, previous studies have demonstrated that manipulation of transcription factors of alpha cells or beta cells may help to produce insulin from alpha cells **(**Collombat et al., 2009; Lu et al., 2010; Yang et al., 2011; Wilcox et al., 2013; Zhu et al., 2017; Matsuoka et al., 2017; Chakravarthy et al., 2017). Further, several chemical-based approaches have been gaining more attention due to their ease and robust impact on alpha cell transdifferentiation (Dina et al., 2010; Brown et al., 2016; Lu et al., 2016; Li et al., 2017; Ben-Othman et al., 2017; Ackermann et al., 2018; Lee et al., 2018).

Rosiglitazone is an insulin-dependent modulator of PPARγ that regulates functions of a variety of tissues such as adipose tissues and hepatic tissues (Boughton et al., 2017).

Accordingly, it reduces hepatic glucose production and lowers blood glucose by increasing glucose uptake and storage by adipose and skeletal tissues (Kavak et al., 2002). In contrast, tolbutamide belonging to the class of sulphonylureas has the ability to modulate of ATP-sensitive K^+ (K_{ATP}) channels, present on pancreatic beta cells to facilitate the increase in insulin release (Maedler et al., 2005). Another popular anti-diabetic drug metformin mainly regulates AMPK pathway to inhibit the endogenous hepatic glucose production (Lundquist et al., 2016). Although, all of these anti-diabetic drugs have been widely studied for their effective glucose-lowering actions to manage type 2 diabetes complications, very little is known about their direct effects on islet cell functions.

In the current study, we studied the effect of these treatment drugs on beta cell regeneration from alpha or other sources in transgenic $Glu^{CRE.ERT2}$; ROSA26-eYFP mice. Our findings support the notion that alpha cells dedifferentiate after STZ induced injury and subsequently convert into beta cells, and that this process is unlettered by rosiglitazone, tolbutamide and metformin. However, all treatments contribute to the preservation of pre-existing beta cell mass either by promoting proliferation or by inhibiting apoptosis of beta cells.

8.3 MATERIAL AND METHODS

Materials and methods for this study have been discussed in Chapter 2.

8.4 RESULTS

8.4.1 Effects of rosiglitazone, tolbutamide and metformin on body parameters of $Glu^{CreERT2}$; ROSA26-eYFP mice

In the present study, severe diabetes was induced in $Glu^{CreERT2}$; ROSA26-eYFP mice using multiple low dose of streptozotocin. As expected, streptozotocin adversely ($p<0.01$ to $p<0.01$) affected body weight of treated mice compared to normal controls, and never returned to normal throughout the experimental period (Figure 8.1A). This was further corroborated using AUC values of percentage of body weight change

(Figure 8.1B). Similar trend ($p<0.01$ to $p<0.01$) was observed in mice treated with each of the three drug regimens compared to normal mice, while no improvements in body weight were observed compared to STZ group (Figure 8.1A, B). A significant ($p<0.05$ to $p<0.001$) increase in food intake was observed in all STZ groups compared to control mice. However, it was significantly ($p<0.001$) reduced in mice treated with rosiglitazone or metformin in comparison to STZ treated mice (Figure 8.2A).

The severe ($p<0.001$) hyperglycaemia induced by STZ was unaltered by the doses of rosiglitazone, tolbutamide or metformin used (Figure 8.2B). This was further evident from AUC values for blood glucose as shown in Figure 8.2C. In line with this, plasma insulin and pancreatic insulin were significantly ($p<0.01$ to $p<0.001$) reduced in all diabetic mice compared to saline mice (Figure 8.3A, B). Pancreatic insulin content was slightly increased in tolbutamide group as compared to STZ group (Figure 8.3B). Plasma glucagon was found to be reduced in all diabetic groups, with a significant ($p<0.05$) effect observed in metformin (Figure 8.3C). A similar trend was observed in pancreatic glucagon concentration plus a significant decrease with rosiglitazone compared to STZ (Figure 8.3D).

8.4.2 Effects of rosiglitazone, tolbutamide and metformin on islet morphology

Figure 8.4A shows representative images of staining for insulin and glucagon in the islets of $Glu^{CreERT2}$; ROSA26-eYFP mice. As in previous chapters, streptozotocin changed islet architecture, decreased the total number of islets ($p<0.05$) reduction was noted in STZ group (Figure 8.4A, B). The tail of pancreas showed greater number of islets (Figure 8.4B). Islet area was found to be slightly reduced in all treatment groups with a significant ($p<0.01$) reduction in STZ islets compared to saline-treated groups (Figure 8.4C). The large-sized islets were completely absent in STZ, rosiglitazone and metformin groups with a slight corresponding increase in small size islets (Figure 8.4D). Compared to saline-treated mice, beta cell area was significantly ($p<0.001$) reduced in all treatment groups, while it was significantly ($p<0.05$) greater in metformin when compared to STZ group (Figure 8.5A). In contrast, the alpha cell area was significantly ($p<0.01$ to $p<0.001$) greater in all groups when compared to normal

mice (Figure 8.5B). It was significantly ($p<0.05$) reduced by metformin compared to STZ group (Figure 8.5B).

8.4.3 Effect of rosiglitazone, tolbutamide and metformin on beta cell proliferation and apoptosis

Representative islets showing immunoreactivity for beta cell proliferation as Ki67/Insulin (Figure 8.6A) and beta cell apoptosis as TUNEL/insulin are depicted in Figure 8.7A. Beta cell proliferation was significantly ($p<0.05$ to $p<0.01$) higher in rosiglitazone and metformin-treated groups as compared to both nondiabetic and diabetic controls (Figure 8.6B). This effect was absent in tolbutamide and STZ compared to saline group (Figure 8.6B). Further, compared to saline controls beta cell apoptosis was significantly ($p<0.05$ to $p<0.001$) higher in all treated groups while it was increased ($p<0.05$) by tolbutamide but unaltered by rosiglitazone and metformin compared to STZ group (Figure 8.7B).

8.4.4 Effect of rosiglitazone, tolbutamide and metformin on alpha cell proliferation and apoptosis

Representative islets showing immunoreactivity for alpha cell proliferation as Ki67/glucagon (Figure 8.8A) and alpha cell apoptosis as TUNEL/glucagon are depicted in Figure 8.9A. Alpha cell proliferation was significantly ($p<0.05$ to $p<0.001$) elevated in all diabetic animals compared to normal mice (Figure 8.8B). None of the treatments reduced alpha cell proliferation compared to STZ group (Figure 8.8B). While alpha cell apoptosis was observed only in metformin-administered mice when compared saline controls (Figure 8.9B).

8.4.5 Effects of rosiglitazone, tolbutamide and metformin on alpha cell lineage tracing

Representative islets showing immunoreactivity for glucagon and GFP are shown in Figure 8.10A. Here, Glucagon+/GFP+ colocalized cells & Glucagon+/GFP- cells represent undifferentiated alpha cells, and Glucagon-/GFP+ cells represent reprogrammed alpha cells. As shown in Figure 8.10B about 62% of alpha cells expressed GFP fluorescent. While ~50% of alpha cells were unlabelled with GFP fluorescent (Figure 8.10C). Glucagon negative/GFP positive cells were significantly ($p<0.05$ to $p<0.001$) augmented in all treated groups when compared to normal mice (Figure 8.10D). More intriguingly, in its pancreatic tail tolbutamide and metformin showed significantly promoted ($p<0.01$ & $p<0.05$ resp.) promotion of only GFP positive alpha cells as compared to STZ groups (Figure 8.10D).

8.4.6 Effects of rosiglitazone, tolbutamide and metformin on generation of bihormonal cells

Representative islets showing immunoreactivity for glucagon and insulin are depicted in Figure 8.11A. Diabetic groups alone and in combination with rosiglitazone and tolbutamide showed a significant ($p<0.05$ to $p<0.01$) increase in insulin+/glucagon+ colocalized cells compared to saline controls. This was further enhanced ($p<0.01$) in the metformin group compared to STZ groups. Importantly, tolbutamide did not alter the number of bihormonal cells (Figure 8.11B).

8.4.7 Effects of rosiglitazone, tolbutamide and metformin on reprogramming of alpha cells into beta cell

Representative islets showing immunoreactivity for GFP and insulin are depicted in Figure 8.12A. Compared to saline groups all treatment groups were significantly ($p<0.001$) increased GFP+/insulin+ colocalized cells, while further increasing trend was observed in rosiglitazone and metformin when compared to STZ group (Figure 8.12B).

8.5 DISCUSSION

Recent studies have showed the instinctive ability of pancreas to reform new beta cells at the expense of alpha cells during extreme conditions (Thorel et al., 2010). In this regard, small molecules have been shown to possess the ability to activate or suppress specific genes through modulation of transcription factors (Fomina-Yadlin et al., 2010). However, the exact mechanism of the transdifferentiation process is not clearly understood (Cheng et al., 2015). In line with this view, here we used small molecules such as rosiglitazone, tolbutamide and metformin in relation to their possible effects on alpha cell transdifferentiation into beta cells. Further, we elucidated the role of these molecules with respect to the contribution of pre-existing beta cells to newly formed beta cells.

8.5.1 Effects of treatments on body parameters

Previous reports demonstrate that streptozotocin induces abnormal hyperglycaemia due to a dramatic loss of insulin (Havel et al., 2000; Vasu et al., 2014a). We observed that streptozotocin treated mice developed an uncontrolled high glucose level. This persistent hyperglycemia was associated with a sudden loss in weight of diabetic animals. We observed no changes in body weight after tested regimens. However, the accompanying hyperphagia was significantly decreased by rosiglitazone and metformin. In addition, metformin also decreased fluid intake at some observation points. Accordingly, rosiglitazone and metformin appear to exert some beneficial effects on diabetes symptoms. In control, no treatment regimens modified abnormalities of blood glucose, plasma insulin or pancreatic insulin. However, there was an increasing trend in plasma and pancreatic insulin with tolbutamide and metformin. Thus, we suggest that a higher dose would be needed to control severe hyperglycaemia over the 10 days of administration. Nevertheless, the absence of blood glucose effect means that any changes observed in islet morphology are due to its days terminates and not a mere consequence of decreased blood glucose. Plasma glucagon was found to be decreased in all STZ treated groups and metformin reduced it further. The possible explanation for this might be a diversion of glucagon gene processing might be diverting towards GLP-1which is known to protect beta cells (Vasu et al., 2014a; Moffett et al., 2015b; Lee et al., 2018).

8.5.2 Effects of treatments on islet morphology

Our diabetic model exhibited extreme beta cells loss and destroyed islet symmetry following STZ exposure. This is mirrored by a reduction of islet size together with total islet number. Furthermore, the distribution of large size islets was also adversely affected. None of the treatments showed a beneficial effect on the size, number and distribution of islets, but they enhanced beta cell area and reduced alpha cell mass. Similar adverse effects were noted in a previous study (Vasu et al., 2014a). Further proliferation studies revealed the involvement of rosiglitazone and metformin in promoting the replication of pre-existing beta cells.

8.5.3 Effects of treatments on alpha cell transdifferentiation

In order to know more about the possible role of alpha cell transdifferentiation, we employed an alpha cell lineage staining approach. We found that the transition of alpha cells into beta cells following STZ treatment. An increasing trend in alpha cell transdifferentiation was observed after rosiglitazone and metformin. In contrast, tolbutamide had no effect on this transition. Previous studies have reported the emergence of bihormonal cells, which are believed to be a transitional state of alpha cell reprogramming to beta cells (Zhang et al., 2019). Here we found that after the administration of STZ, bihormonal cells arose in all groups with significant enhancement in the metformin group. Cells expressing these two hormones were evident with rosiglitazone and tolbutamide treatment. Therefore, we speculate that metformin might possess transdifferentiation ability. Metformin inhibits hepatic gluconeogenesis (Irwin et al, 2010). While previous reports showed that blocking of hepatic glucagon receptors induces alpha cell hyperplasia. The present diabetic model showed alpha cell hyperplasia together with glucagon suppression. An earlier report indicated a link between liver and alpha cell transdifferentiation (Ye et al., 2015). However, further studies are needed to understand the details of the link between hepatic glucagon signaling and alpha cell transdifferentiation. Moreover, further chronic action of metformin remains to be established.

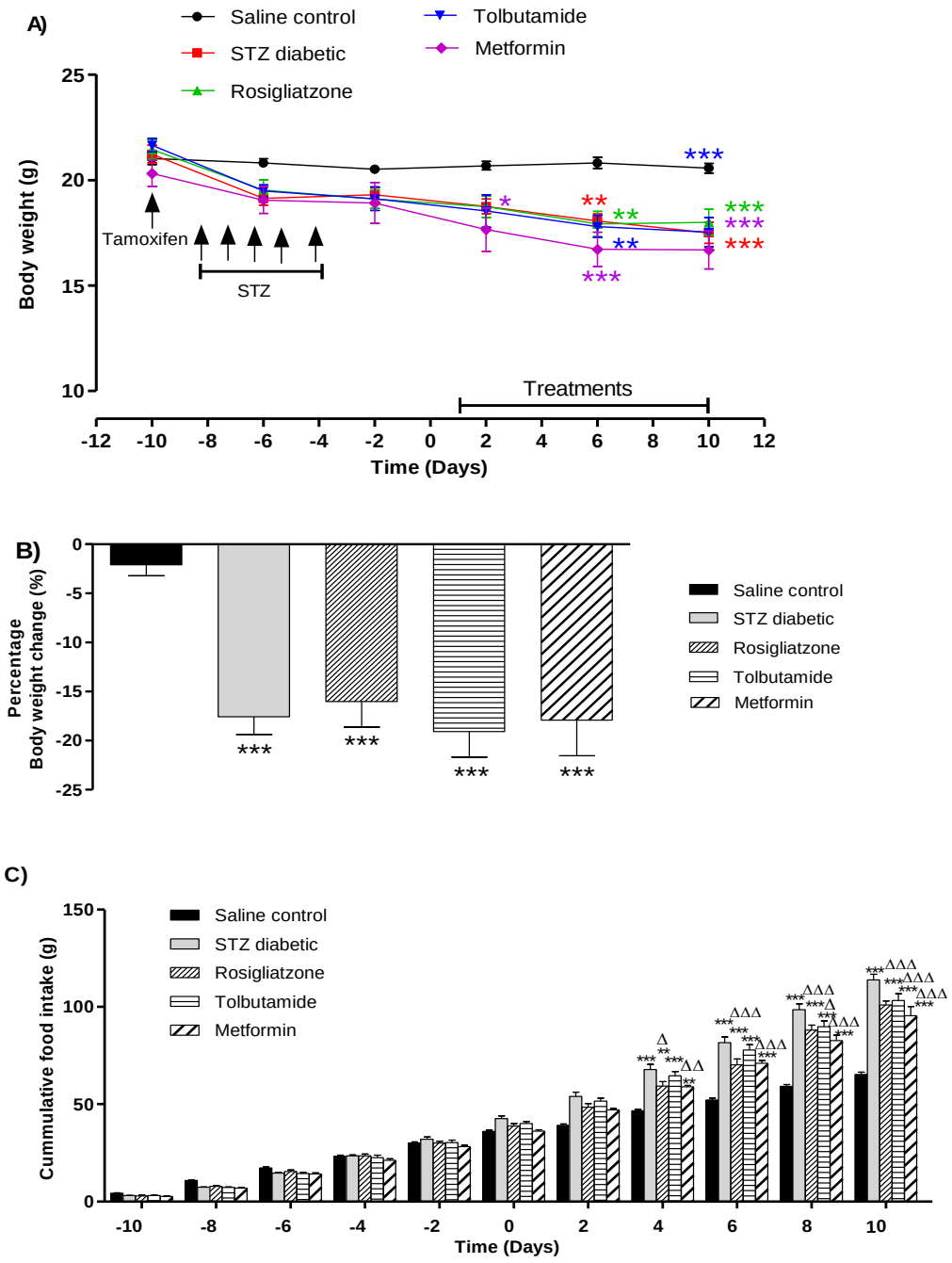
8.5.4 Fluorescent labelling of alpha cells

The expression of GFP in glucagon positive alpha cells explains the labelling of alpha cells in GluCre ROSA mice (Quoix et al., 2007). Here we observed that a substantial population of alpha cells irreversibly labeled with a fluorescent protein. Indeed, diabetic mice showed an increased number of labeled alpha cells compared to the saline group, which supports the notion that alpha cells emerged after beta cells loss because of the replication of pre-existing alpha cells. In line with this, tolbutamide enhanced glucagon lacking GFP cells. We also found that alpha cell proliferation was persistent in all treated diabetic groups. Only metformin promoted in alpha cell apoptosis. Notably, rosiglitazone showed a slight decline in alpha cell proliferation. These findings are consistent with the reduced alpha cell area by all these treatment regimens.

8.5.5 Concluding remarks

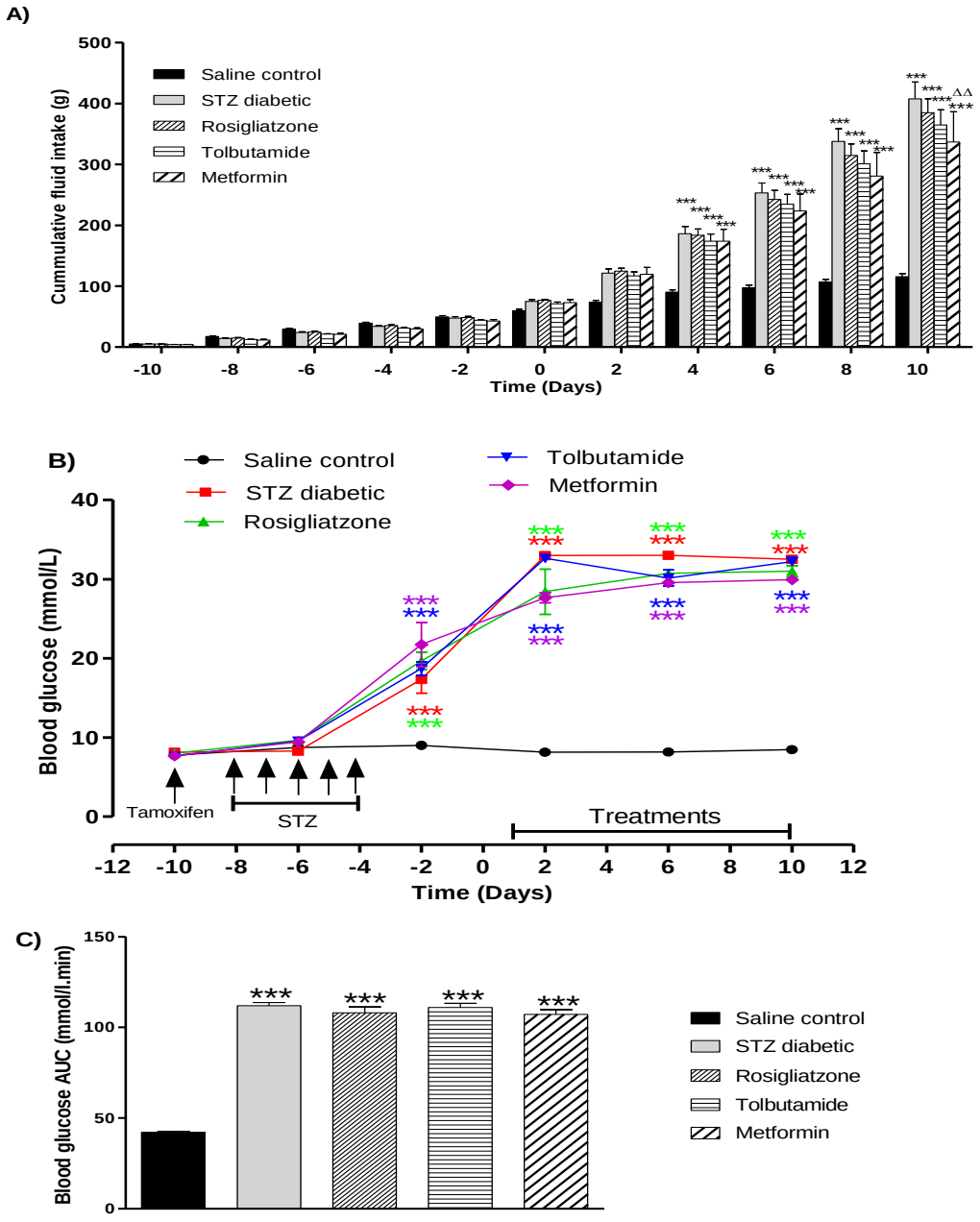
Although long-term studies with other doses are required, these findings support the notion that the potential tendency of metformin toward promoting alpha cell transdifferentiation. Nevertheless, rosiglitazone partially promoted the replication of residual beta cells. In the future, the detailed understanding regarding alpha cell transdifferentiation using all these treatment regimens may well open up new avenues to promote regenerative therapy in managing diabetic complications.

Figure 8.1 Effects of rosiglitazone, tolbutamide and metformin on (A, B) body weight and (C) food intake in normal and streptozotocin diabetic mice



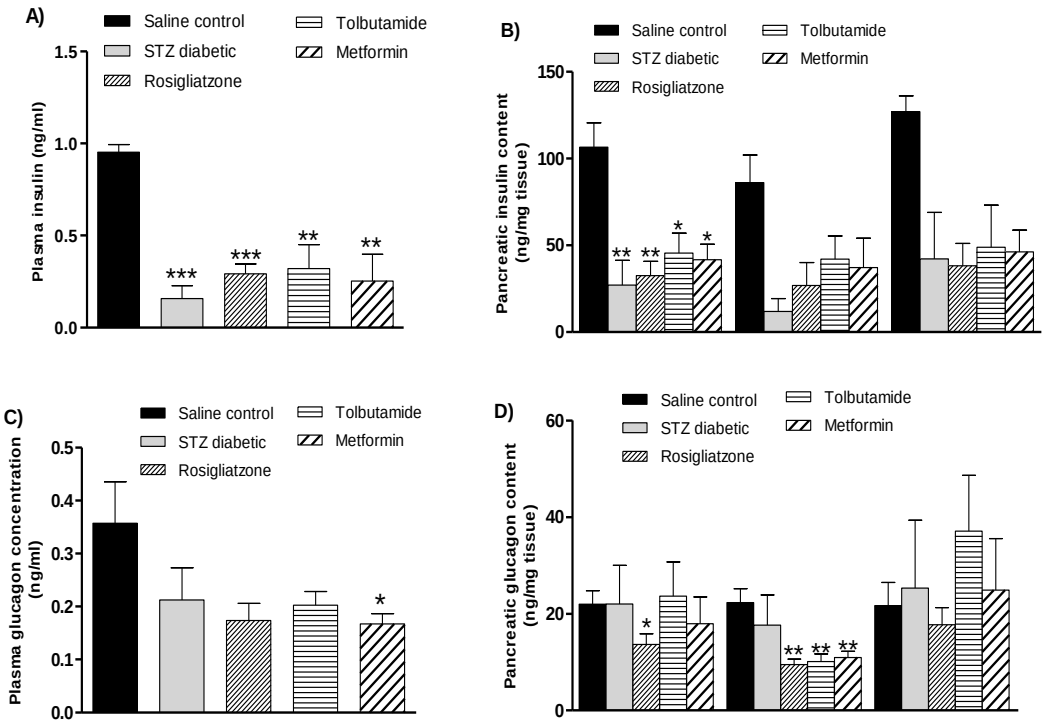
(A,B) Body weight and (C) food intake were measured throughout the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic $Glu^{CreERT2}$;ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice. *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 and ΔΔΔp < 0.001 compared to streptozotocin treated group.

Figure 8.2 Effects of rosiglitazone, tolbutamide and metformin on (A) fluid intake and (B, C) blood glucose in normal and streptozotocin diabetic mice



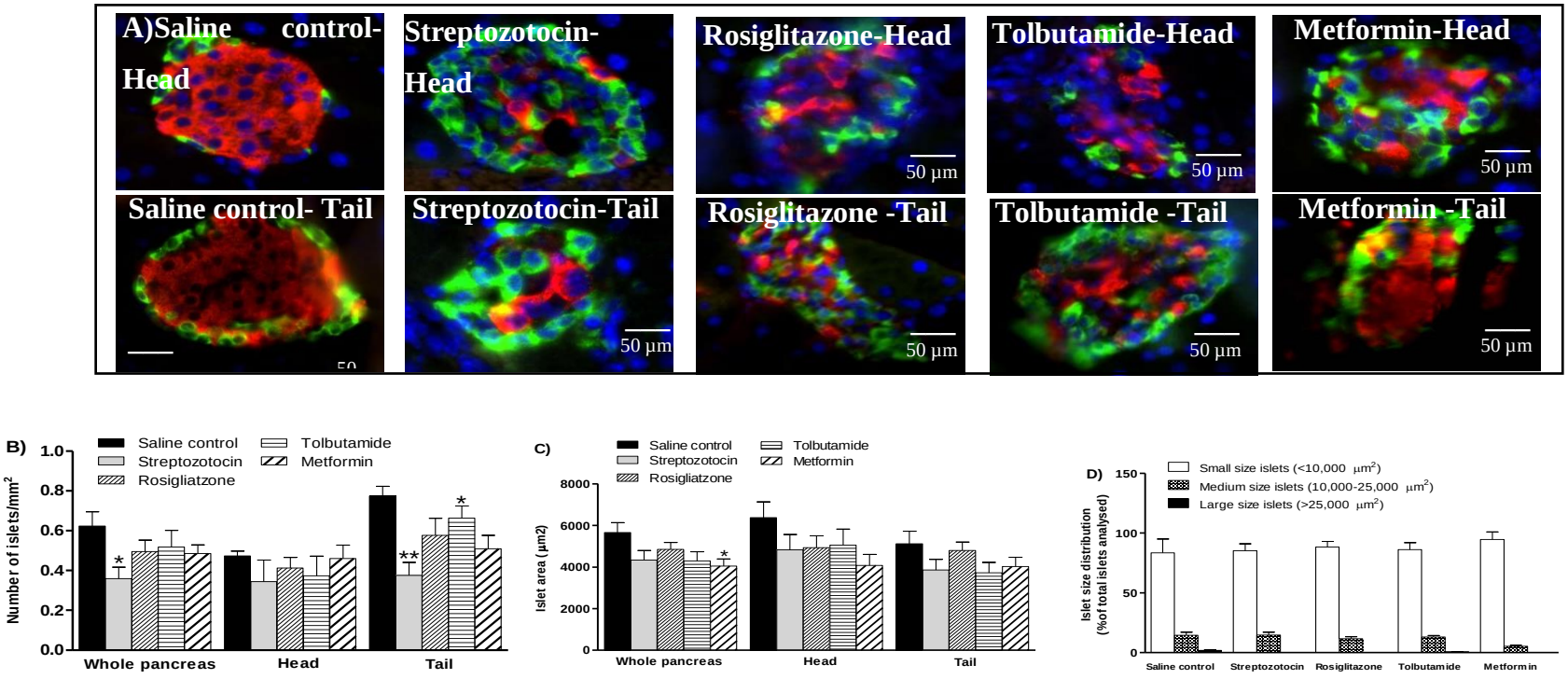
(A,B) Blood glucose and (C) fluid intake were measured throughout the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic $Glu^{CreERT2}$;ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice. ***p <0.001 compared to saline control group. ΔΔp < 0.01 compared to streptozotocin treated group.

Figure 8.3 Effects of rosiglitazone, tolbutamide and metformin on plasma and pancreatic (A, B) insulin or (C, D) glucagon concentration in normal and streptozotocin diabetic mice



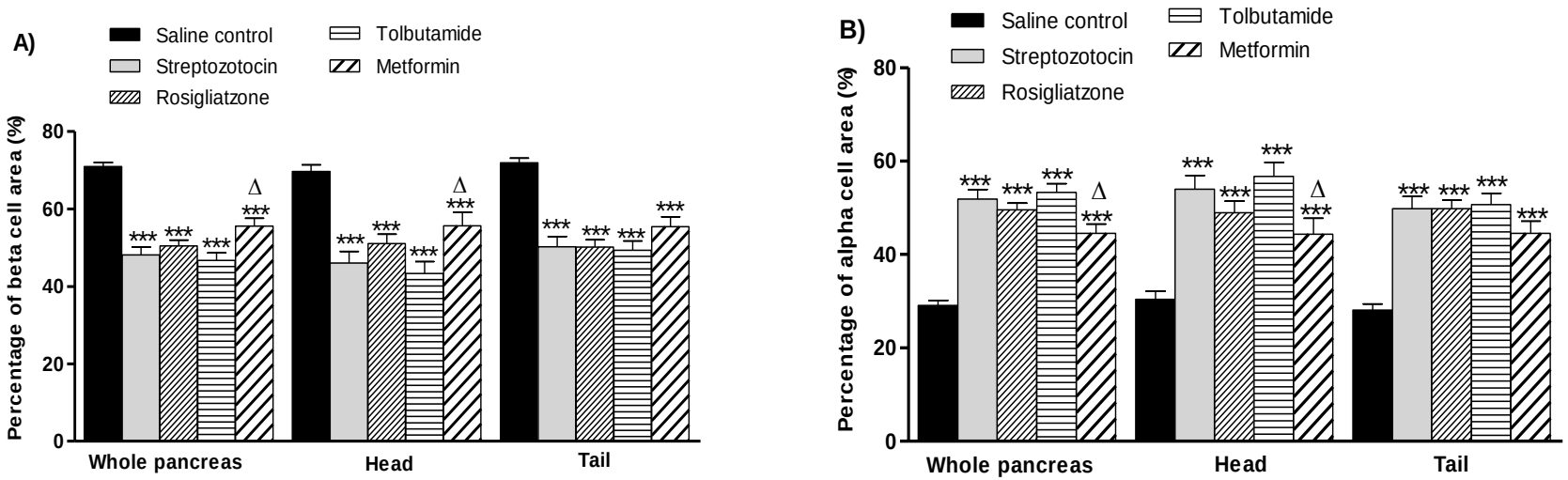
Plasma and pancreatic (A,B) insulin and (C,D) glucagon were measured at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic $Glu^{CreERT2}$;ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control group.

Figure 8.4 Effects of rosiglitazone, tolbutamide and metformin on islet (A) number, (B) area and (C) size-distribution in normal and streptozotocin diabetic mice



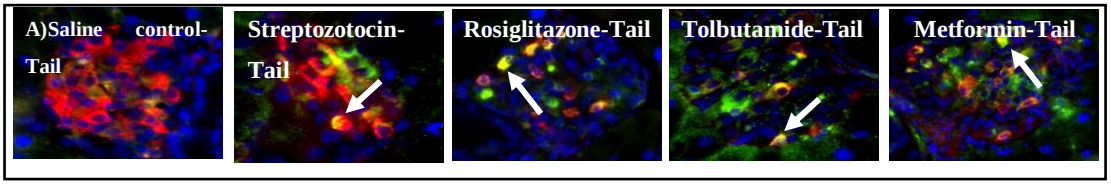
Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). Islet (B) number, (C) area and (D) size-distribution were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (100-150 islets per group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. $\Delta\Delta p < 0.01$ compared to streptozotocin treated group. Scale bars: 50 µm.

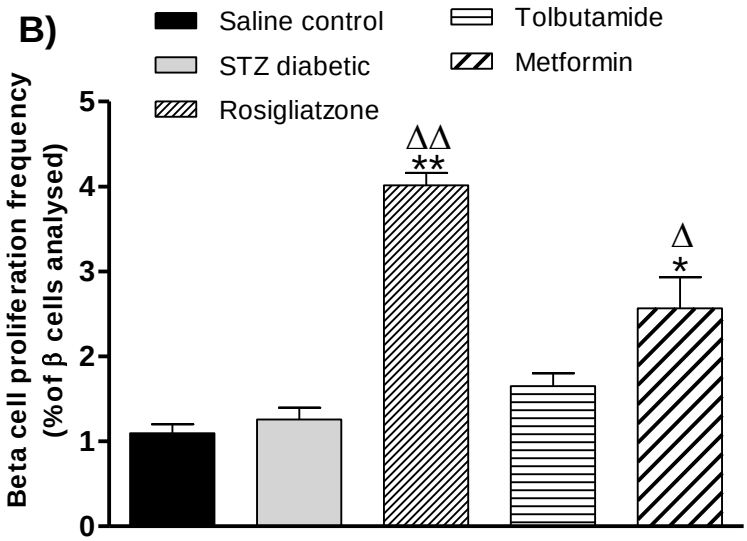
Figure 8.5 Effects of rosiglitazone, tolbutamide and metformin on (A) beta cell area and (B) alpha cell area in normal and streptozotocin diabetic mice



(A) Beta cell area, (B) alpha cell area were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (100-150 islets per group). **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ and $\Delta\Delta\Delta p < 0.001$ compared to streptozotocin treated group.

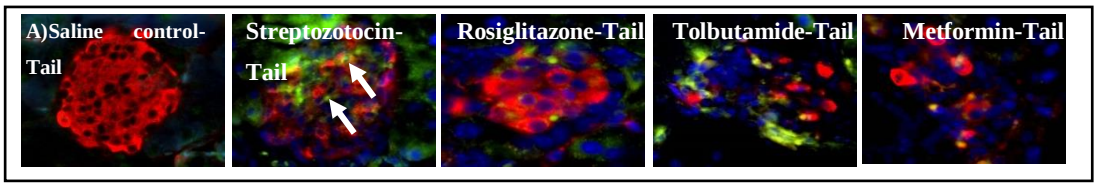
Figure 8.6 Effects of rosiglitazone, tolbutamide and metformin on (A, B) beta cell proliferation in normal and streptozotocin diabetic mice

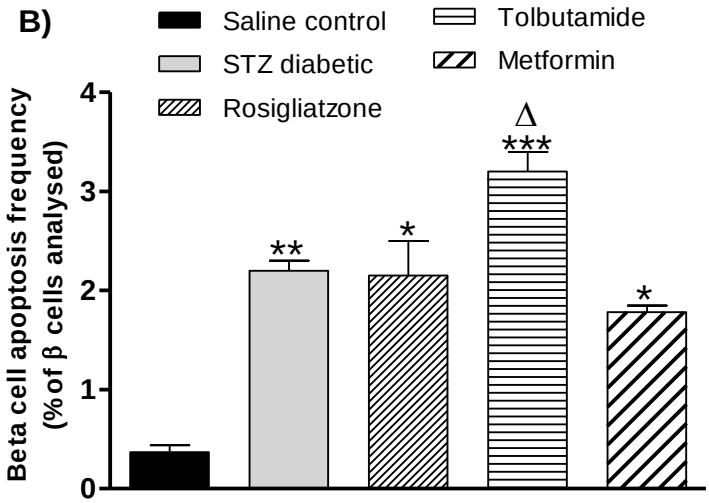




Representative images (A) showing immunostaining for DAPI (blue), Ki67 (green) and Insulin (red). (B) Beta cell proliferation were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (>60 tail islets per group). *$p < 0.05$ and **$p < 0.01$ compared to saline control group. $\Delta p < 0.05$ and $\Delta\Delta p < 0.01$ compared to streptozotocin treated group. Scale bars: 50 µm.

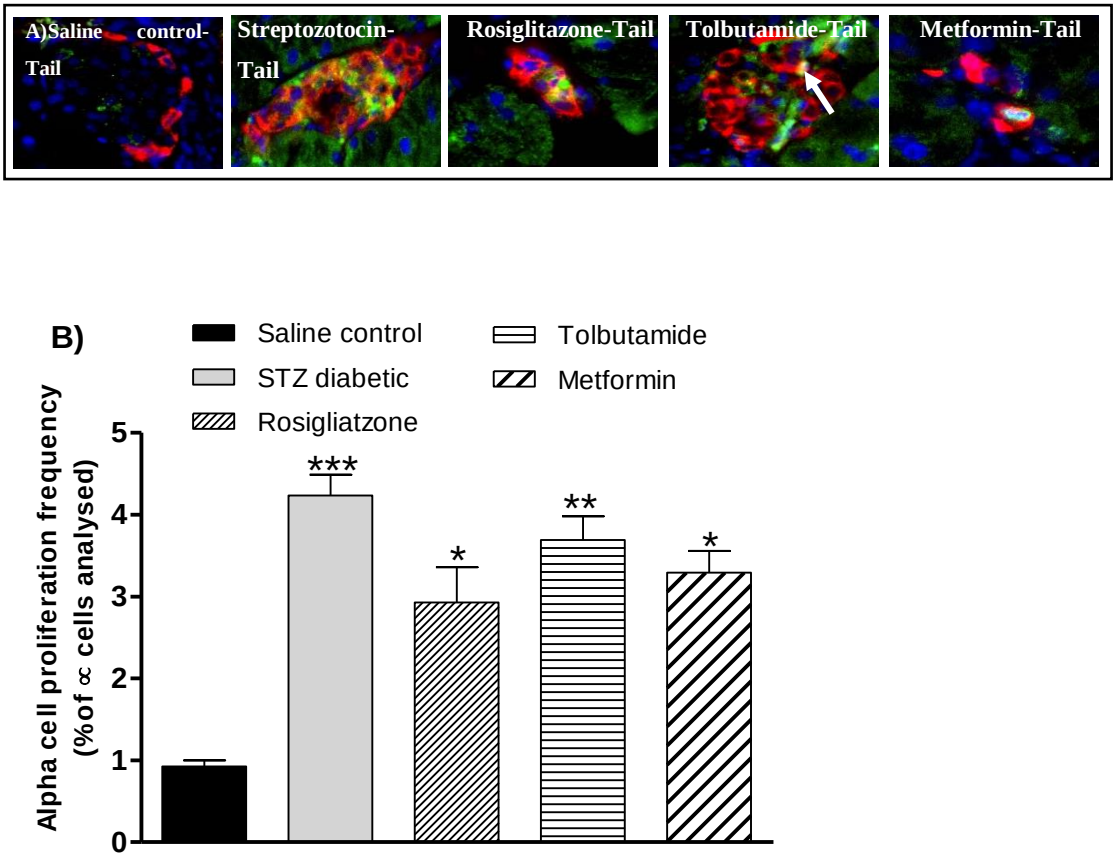
Figure 8.7 Effects of rosiglitazone, tolbutamide and metformin on (A, B) beta cell apoptosis in normal and streptozotocin diabetic mice





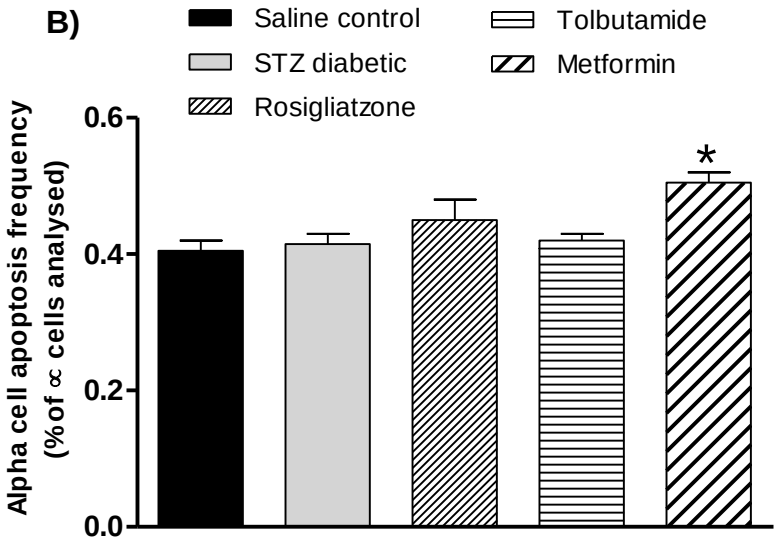
Representative images (A) showing immunostaining for DAPI (blue), TUNEL (green) and Insulin (red). (B) Beta cell apoptosis were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (>60 tail islets per group). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. Δ$p < 0.05$ compared to streptozotocin treated group. Scale bars: 50 µm.

Figure 8.8 Effects of rosiglitazone, tolbutamide and metformin on (A, B) alpha cell proliferation in normal and streptozotocin diabetic mice



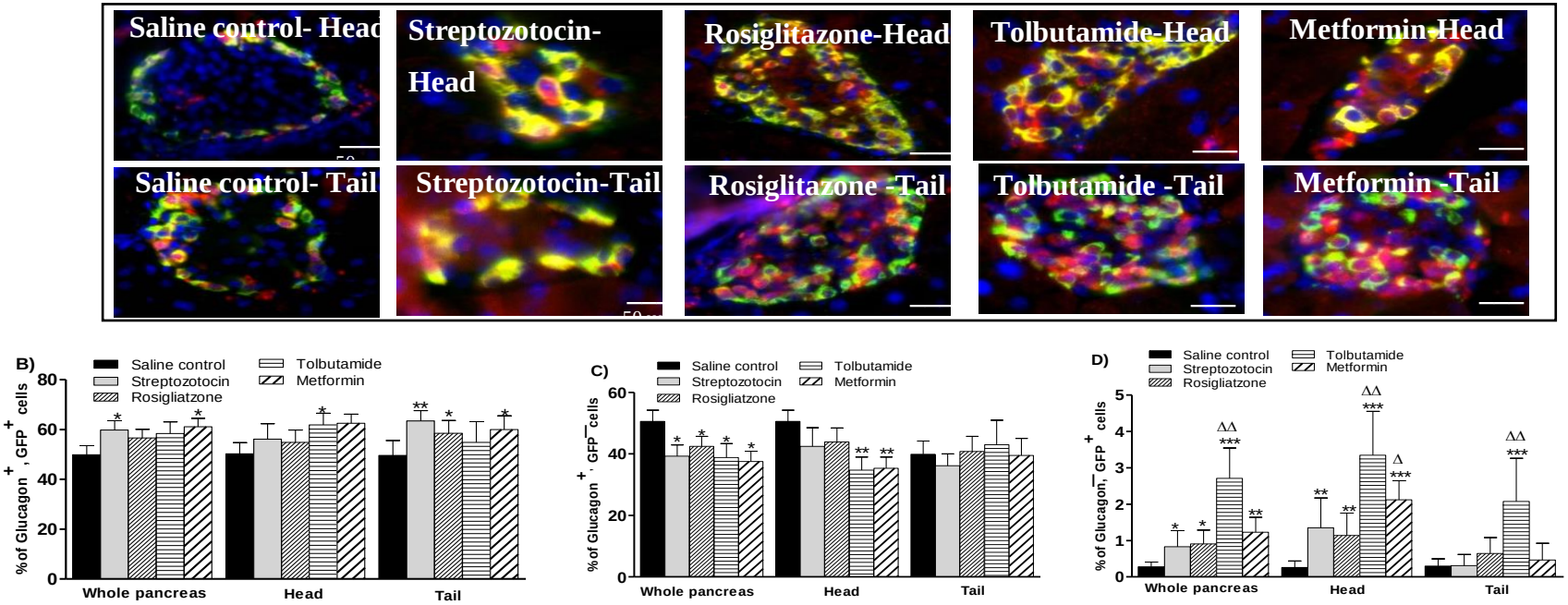
Representative images (A) showing immunostaining for DAPI (blue), Ki67 (green) and Glucagon (red). (A) Alpha cell proliferation were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (>60 tail islets per group). *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group. Scale bars: 50 µm.

Figure 8.9 Effects of rosiglitazone, tolbutamide and metformin on (A, B) alpha cell apoptosis in normal and streptozotocin diabetic mice



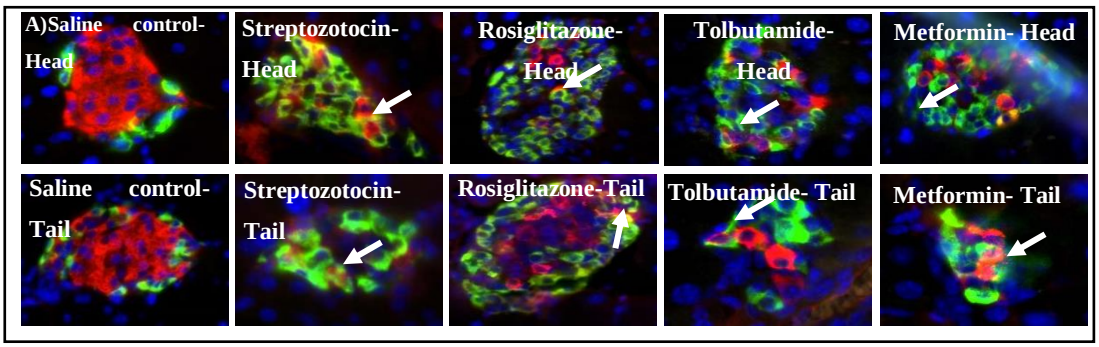
Representative images (A) showing immunostaining for DAPI (blue), TUNEL (green) and Glucagon (red). (A) Alpha cell apoptosis were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (>60 tail islets per group). *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group. Scale bars: 50 µm.

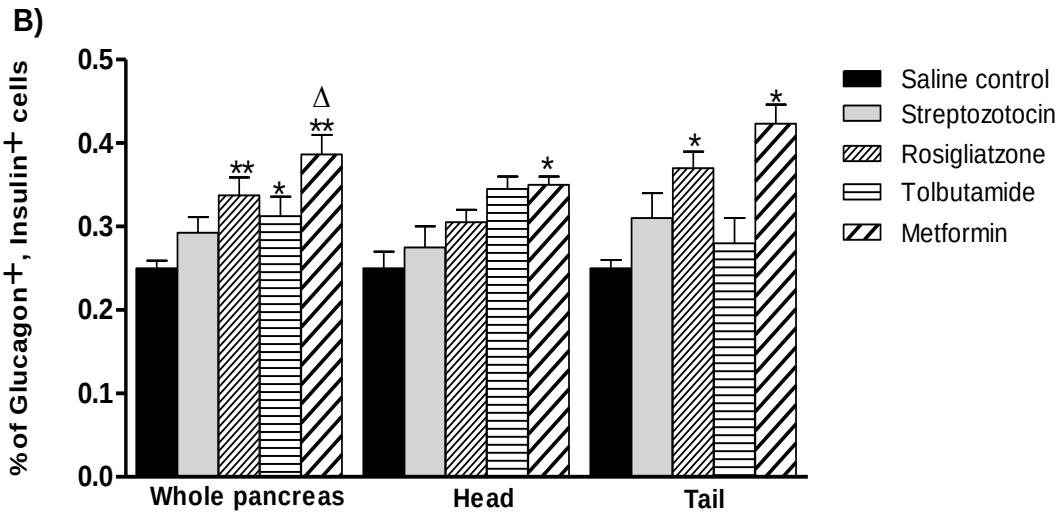
Figure 8.10 Effects of rosiglitazone, tolbutamide and metformin on alpha cells lineage (A) Gln^{postive}/GFP^{positive}, (B) Gln^{postive}/GFP^{negative} and (C) Gln^{negative}/GFP^{positive} in normal and streptozotocin diabetic mice



Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and GFP (red). (B) Percentage of glucagon positive & GFP positive cells (C) Glucagon positive & GFP negative cells (D)percentage of glucagon negative & GFP positive cells and were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (~70 islets per group). *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 and ΔΔp < 0.01 compared to streptozotocin treated group. Scale bars: 50 µm.

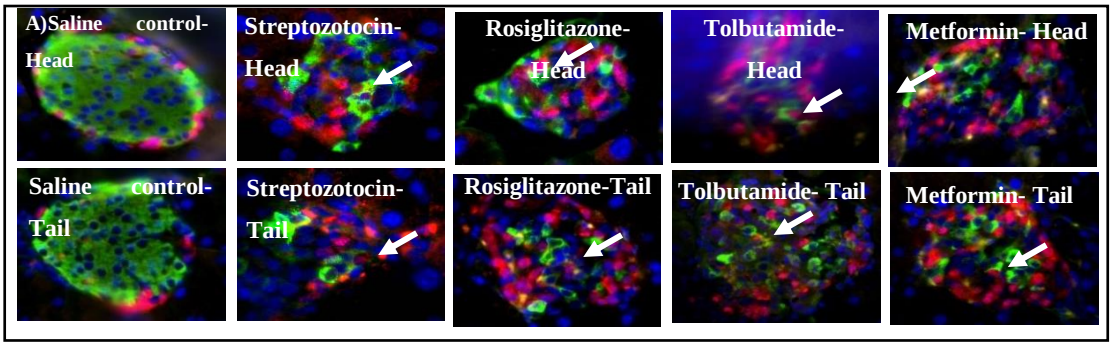
Figure 8.11 Effects of rosiglitazone, tolbutamide and metformin on generation of bi-hormonal cells (A, B) Gln^{postive}/Insulin^{positive} in normal and streptozotocin diabetic mice

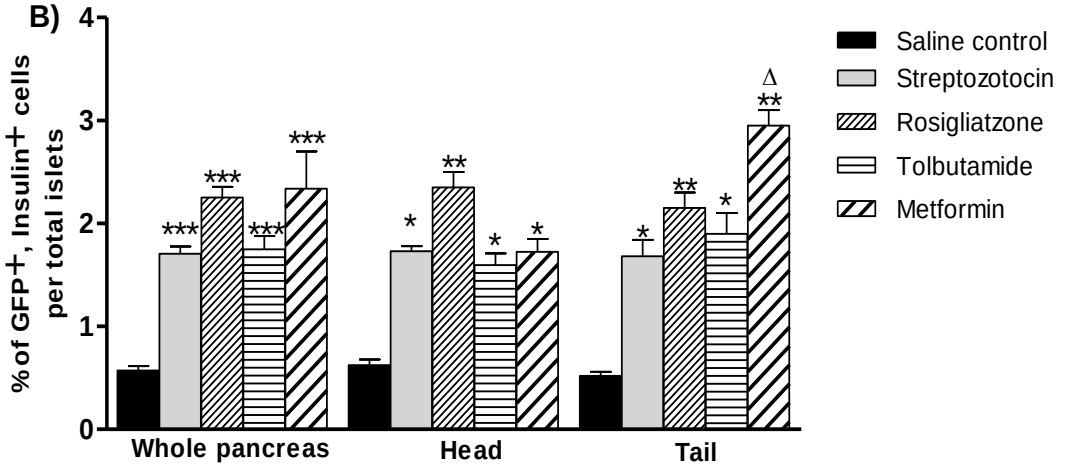




Representative images (A) showing immunostaining for DAPI (blue), glucagon (green) and insulin (red). (B) Glucagon^{positive}/Insulin^{positive} cells were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (100-150 islets per group). *$p < 0.05$ and **$p < 0.01$ compared to saline control group. $\Delta p < 0.05$ compared to streptozotocin treated group. Scale bars: 50 µm.

Figure 8.12 Effects of rosiglitazone, tolbutamide and metformin on alpha cells transdifferentiation into beta cell (A, B) GFP^{postive}/Insulin^{positive} in normal and streptozotocin diabetic mice





Representative images (A) showing immunostaining for DAPI (blue), insulin (green) and GFP (red). (B) GFP^{positive}/Insulin^{positive} cells were analysed using Cell^{F} and ImageJ at the end of the study. A daily single oral dose of saline vehicle, rosiglitazone (10 mg/kg), tolbutamide (20 mg/kg) and metformin (100 mg/kg) were received for 10 days by diabetic Glu^{CreERT2};ROSA26-eYFP mice. Values represent means ± SEM for 5 or 6 mice (100-150 islets per group). *p < 0.05, **p < 0.01 and ***p <0.001 compared to saline control group. Δp < 0.05 compared to streptozotocin treated group. Scale bars: 50 µm.

Chapter 9

Effects of long-acting GIP, xenin and oxyntomodulin peptide analogues on islet morphology and alpha-cell transdifferentiation in insulin-deficient diabetic $Glu^{CreERT2}$; ROSA26-eYFP mice

(This chapter has been published)

Sarnobat D, Moffett RC, Gault VA, Tanday N, Reimann F, Gribble FM, Flatt PR & Irwin N (2019) Effects of long-acting GIP, xenin and oxyntomodulin peptide analogues on alpha-cell transdifferentiation in insulin-deficient diabetic $Glu^{CreERT2}$; ROSA26-eYFP mice. *Peptides* 170205.

9.1 SUMMARY

Enzyme-resistant long-acting forms of the gut-derived peptide hormones, glucose-dependent insulinotropic polypeptide (GIP), xenin and oxyntomodulin (Oxm) have been generated, and exert beneficial effects on diabetes control and pancreatic islet architecture. The current study has employed alpha-cell lineage tracing in $Glu^{CreERT2}$;ROSA26-eYFP transgenic mice to investigate the extent to which these positive pancreatic effects are associated with alpha- to beta-cell transdifferentiation. Twice-daily administration of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] for 10 days to streptozotocin (STZ)-induced diabetic mice did not affect body weight, food intake or blood glucose levels, but (D-Ser^2)-Oxm[Lys^{38}PAL] reduced ($P<0.05$ to $P<0.001$) fluid intake and circulating glucagon. (D-Ala^2)GIP and (D-Ser^2)-Oxm[Lys^{38}PAL] also augmented ($P<0.05$ and $P<0.01$, respectively) pancreatic insulin content. Detrimental changes of pancreatic morphology induced by STZ in $Glu^{CreERT2}$;ROSA26-eYFP mice were partially reversed by all treatment interventions. This was associated with reduced ($P<0.05$) apoptosis and increased ($P<0.05$ to $P<0.01$) proliferation of beta-cells, alongside opposing effects on alpha-cells, with (D-Ala^2)GIP and (D-Ser^2)-Oxm[Lys^{38}PAL] being particularly effective in this regard. Alpha-cell lineage tracing revealed that induction of diabetes was accompanied by increased ($P<0.01$) transdifferentiation of glucagon positive alpha-cells to insulin positive beta-cells. This islet cell transitioning process was augmented ($P<0.01$ and $P<0.001$, respectively) by (D-Ala^2)GIP and (D-Ser^2)-Oxm[Lys^{38}PAL]. (D-Ser^2)-Oxm[Lys^{38}PAL] also significantly ($P<0.05$) promoted loss of alpha-cell identity in favour of other endocrine islet cells. These data highlight intra-islet benefits of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] in diabetes with beta-cell loss induced by STZ. The effects appear to be independent of glycaemic change, and associated with alpha- to beta-cell transdifferentiation for the GIP and Oxm analogues.

9.2 INTRODUCTION

The most common forms of diabetes, type 1 and type 2 diabetes mellitus (T1DM and T2DM), are associated with loss or dysfunction of insulin-producing pancreatic beta-cells (Moffett et al., 2013, 2015a; Vasu et al., 2014a, 2015). In addition, hyperglucagonaemia and alpha-cell expansion are also characteristic of diabetes (Lund et al., 2016), together resulting in absolute hyperglycaemia. Thus, an interplay between alpha- and beta-cell mass appears fundamental in the progression of diabetes. Regeneration of beta-cells from various intrinsic sources has been investigated as a means to manage diabetes, including pancreatic endocrine (Vasu et al., 2016; Lee et al., 2018) and exocrine tissue (Lysy et al., 2013), as well as liver (Yanagimachi et al., 2016), stomach (Ariyachet et al., 2016) and stem cells (Borowiak et al., 2018). Among these methods, local pancreatic alpha-cells are established as natural, physiologically relevant, contributors to the recruitment of new beta-cells (Thorel et al., 2010; Stanojevic et al., 2015).

In this regard, it has been demonstrated that pancreatic beta-cell injury can promote transition of glucose-elevating alpha-cells to glucose-lowering beta-cells (Thorel et al., 2010; Liang et al., 2011; Cheng et al., 2015). Thus, despite their conflicting roles in the control of blood glucose, alpha- and beta-cells share essentially similar transcriptomes (Van et al., 2015). The exact mechanisms underlying this transdifferentiation process remain unclear (Stanojevic et al., 2015), and attempts to pharmacologically induce alpha- to beta-cell transdifferentiation have yielded some inconsistent findings to date (Fomina-Yadlin et al., 2010; Brown et al., 2016; Lu et al., 2016; Ben-Othman et al., 2017; Ackermann et al., 2018; Li et al., 2018). Therefore, further investigation of alternative methods to augment pancreatic endocrine cell transdifferentiation pathways is required.

In relation to this, the prohormone convertase (PC) enzymes that process the glucagon gene within alpha-cells are more promiscuous than first imagined. PC1/3 was initially believed to exclusively process the glucagon gene within intestinal L-cells to yield the incretin hormone glucagon-like peptide-1(GLP-1), as well as the related gene products glucagon-like peptide-2 (GLP-2) and oxyntomodulin (Oxm) (Pocai, 2012; Vasu et al., 2014a). In contrast, pancreatic PC2 directed the post-translational production of glucagon by pancreatic alpha-cells (Moffett et al., 2014). However, PC1/3 is expressed

in alpha-cells, particularly in response to islet stress (Moffett et al., 2014; Vasu et al., 2014a; Pathak et al., 2015). Moreover, GLP-1 has notable islet benefits including promotion of beta-cell growth and survival (Vasu et al., 2014a), and has recently been suggested to positively affect alpha- to beta-cell transitioning (Lee et al., 2018). Similar to GLP-1, its sister incretin hormone glucose-dependent insulinotropic polypeptide (GIP), classically believed to be generated solely from enteroendocrine K-cells, has been shown to be synthesised and released locally from alpha-cells (Gutierrez-Aguilar et al., 2011; Moffett et al., 2015b). In addition, a second K-cell derived peptide product, known as xenin-25, has also been evidenced within the endocrine pancreas as well as intestinal K-cells (Khan et al., 2017). Both GIP and xenin induce benefits on beta-cell proliferation and protection against apoptosis (Vasu et al., 2014a; Craig et al., 2018), with xenin also suggested to potentiate the bioactivity of GIP (Taylor et al., 2010; Martin et al., 2012, 2014, 2016). However, the potential positive and therapeutically relevant, impact of non-classic islet hormones such as GIP and xenin, as well as the related glucagon gene product Oxm, on alpha-cell transdifferentiation have not been elucidated to date.

In the present study, we have utilised well-characterised long-acting, enzyme resistant peptide forms of GIP, xenin and Oxm, namely (D-Ala^{2})GIP, xenin-25[Lys^{13}PAL] and (D-Ser^{2})-Oxm[Lys^{38}PAL] (Martin et al., 2013; Lynch et al., 2014; Gault et al., 2015) to investigate the lineage fate of individual alpha-cells in Glu^{CreERT2};ROSA26-eYFP transgenic mice with streptozotocin (STZ)-induced beta-cell loss. Notably, sub-chronic administration of all three analogues has previously been shown to result in numerous metabolic advantages in diabetic rodents, in addition to positive effects on pancreatic islet architecture (Martin et al., 2012; Irwin et al., 2015b; Millar et al., 2016). Here we investigate whether these pancreatic islet benefits are linked to favourable effects on cell growth and survival, or transdifferentiation of alpha- to beta-cells.

9.3 MATERIALS AND METHODS

Materials and methods for this study have been discussed in Chapter 2.

9.4 RESULTS

9.4.1 Effects of peptide treatments on body weight, cumulative food and fluid intake, non-fasting glucose, insulin and glucagon

Body weight was significantly ($p<0.05$ to $p<0.001$) reduced in STZ diabetic mice when compared to lean controls (Figure 9.1A). Twice daily administration of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] did not alter body weight (Figure 9.1A). Food intake initially declined in diabetic mice, but was elevated ($p<0.05$ to $p<0.001$) from day 6 onwards, and treatment interventions did not dramatically alter this (Figure 9.1B). A similar profile was observed in terms of fluid intake, but (D-Ser^2)-Oxm[Lys^{38}PAL] did significantly ($p<0.05$ to $p<0.001$) decrease fluid intake on days 8 and 10 (Figure 9.2B). None of the treatment regimens altered the high levels ($p<0.001$) of circulating glucose in STZ diabetic mice (Figure 9.2A). In addition, plasma insulin concentrations were marginally increased by all treatments on day 10, but remained significantly reduced ($p<0.001$) when compared to lean controls (Figure 9.3A). As expected, pancreatic insulin concentrations were dramatically reduced ($p<0.001$) in STZ diabetic mice (Figure 9.3B). However, twice daily treatment with either (D-Ala^2)GIP or (D-Ser^2)-Oxm[Lys^{38}PAL] significantly increased ($p<0.05$ and $p<0.01$, respectively) pancreatic insulin, but the content was still reduced ($p<0.001$) compared to lean controls (Figure 9.3B). Plasma glucagon was not altered in STZ diabetic mice, but twice daily (D-Ser^2)-Oxm[Lys^{38}PAL] administration significantly ($p<0.05$) reduced circulating glucagon compared to STZ control mice (Figure 9.3C). In comparison to saline control, pancreatic glucagon content was unchanged in all treatments (Figure 9.3D). As such, a slight decreasing trend was observed in (D-Ala^2)GIP or (D-Ser^2)-Oxm[Lys^{38}PAL] treated groups. However, it was significantly ($p<0.05$) reduced by xenin-25[Lys^{13}PAL] compared to both saline controls and STZ diabetic control (Figure 9.3D).

9.4.2 Effects of peptide treatments on islet morphology

Representative images of pancreatic tissue form each treatment group stained fluorescently for insulin, glucagon and DAPI are shown in Figure 9.4A. The STZ diabetic mice had significantly reduced ($p<0.05$ to $p<0.001$) pancreatic islet area and

number, as well as beta-cell area, with increased ($p<0.001$) alpha-cell area (Figure 9.4A-E). All treatments returned islet area and number to levels not significantly different from lean control mice (Figure 9.4B&C). Beta-cell area was significantly elevated ($p<0.05$) by both (D-Ala^2)GIP and xenin-25[Lys^{13}PAL] treatment (Figure 9.4D), and all treatments decreased ($p<0.05$) alpha-cell area (Figure 9.4E).

9.4.3 Effects of peptide treatments on pancreatic alpha- and beta-cell proliferation and apoptosis

Representative images of islets co-stained with TUNEL reagent and insulin (Figure 9.5A) or glucagon (Figure 9.6A) from each treatment group are also shown. Beta-cell apoptosis was substantially increased ($p<0.001$) in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice (Figure 9.5B). All treatments partially reversed this effect, with (D-Ala^2)GIP and (D-Ser^2)-Oxm[Lys^{38}PAL] significantly ($p<0.05$) reducing beta-cell apoptotic rate compared to diabetic control mice (Figure 9.5B). Alpha-cell apoptosis was not altered in STZ mice, but (D-Ala^2)GIP and (D-Ser^2)-Oxm[Lys^{38}PAL] increased ($p<0.05$) apoptotic rate of alpha-cells when compared to lean control mice (Figure 9.6B). In terms of alpha- and beta-cell proliferation, all three treatments substantially ($p<0.05$ to $p<0.01$) increased beta-cell proliferative rate when compared to both diabetic and lean control mice (Figure 9.7B). Alpha-cell proliferation was augmented ($p<0.01$) in STZ diabetic mice, with all three treatment regimens significantly reducing ($p<0.05$) this effect (Figure 9.8B). Representative images of islets co-stained with Ki-67 and insulin (Figure 9.7A) or glucagon (Figure 9.8A) are shown.

9.4.4 Effects of peptide treatments on islet cell lineage

Representative images of islets co-stained with GFP and insulin are shown in Figure 9.10A. Alpha-cell lineage was investigated in Glu^{CreERT2};ROSA26-eYFP mice by co-staining islet sections with glucagon and GFP, as depicted in Figure 9.10A-D. STZ-induced diabetes was associated with increased ($p<0.05$) numbers of glucagon positive/GFP positive (Figure 9.10B) and decreased ($p<0.05$) glucagon positive/GFP negative (Figure 9.10C) cells. The treatment regimens did not affect the expression of

these glucagon positive cells (Figure 9.10B,C). However, glucagon negative/ GFP positive islet cell numbers were increased ($p<0.01$) in STZ diabetic mice, with treatment of (D-Ser^2)-Oxm[Lys^{38}PAL] further increasing ($p<0.05$) the number of these cells (Figure 9.10D). Finally, numbers of GFP positive/insulin positive cells were also significantly ($p<0.01$) elevated in diabetic mice, and further amplified ($p<0.01$ and $p<0.001$, respectively) by treatment with (D-Ala^2)GIP and (D-Ser^2)-Oxm[Lys^{38}PAL], respectively (Figure 9.10C).

9.5 DISCUSSION

Previous work reveals that sustained activation of GIP, xenin or Oxm cell signalling pathways induces metabolic benefits in diabetes, linked in part to expansion of beta-cell mass (Martin et al., 2012; Irwin et al., 2015b; Millar et al., 2016). The exact mechanism behind this beneficial endocrine pancreatic effect is not fully understood, despite knowledge that GIP, xenin and GLP-1 receptor activation exert beta-cell pro-survival effects (Moffett et al., 2014; Vasu et al., 2014a; Craig et al., 2018), with Oxm known to function as a GLP-1 receptor ligand (Irwin et al., 2015a, 2015b). In this regard, recent studies have shown that transition of adult, glucagon expressing, alpha-cells to functional beta-cells, by a process termed transdifferentiation (Thorel et al., 2010; Cheng et al., 2015), represents an important means of regenerating beta-cells in diabetes. Therefore, in the present study, we have employed STZ-diabetic Glu^{CreERT2};ROSA26-eYFP transgenic mice to investigate the impact of long-acting forms of GIP, xenin and Oxm, namely (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL], on glucose homeostasis, islet morphology and pancreatic alpha-cell lineage. Unlike single large doses of STZ, the multiple low dose model employed here exhibits insulin deficiency and diabetes due to lymphocyte infiltration of islets and cytokine-induced beta-cell death (King et al., 2012).

9.5.1 Effects of peptide treatments on morphology, proliferation and apoptosis of islets cells

In keeping with previous observations (Yanagimachi et al., 2016), (D-Ala^2)GIP augmented beta-cell mass and reduced the alpha-cell expansion associated with STZ administration. This was in part due to increased beta-cell proliferation and reduced

beta-cell apoptosis, as documented previously with activation of beta-cell GIP receptors (Taylor et al., 2010; Moffett et al., 2014). Interestingly, alpha-cell apoptosis was enhanced, and proliferation decreased, in (D-Ala^{2})GIP and xenin-25[Lys^{13}PAL] treated mice, which would also contribute to the observed pancreatic islet architecture changes in these mice. This may be somewhat unexpected given that both GIP and xenin are both known to increase glucagon secretion which presumably reflects changes in the intracellular signalling pathways leading to exocytosis (Cassidy et al., 2008; Craig et al., 2019). In addition, differences in alpha- and beta-cell apoptosis rates in this mouse model is likely related to the direct effects of STZ on beta-cell health, as a result of the beta-cell GLUT2 specificity of this alkylating antineoplastic agent (King et al., 2012). The other treatment modalities evoked essentially similar effects on both beta- and alpha-cell growth and survival, with all three treatments reducing alpha-cell area and increasing beta-cell mass, although the latter just failed to reach significance in the case of (D-Ser^{2})-Oxm[Lys^{38}PAL]. In addition, only administration of the GIP and Oxm analogues significantly augmented overall pancreatic insulin concentrations, in keeping the beta-cell regenerative effects of these two peptides.

9.5.2 GLP-1 and GIP signaling with respect to alpha cell transdifferentiation

Given that the STZ-induced diabetic Glu^{CreERT2};ROSA26-eYFP mice presented with reduced pancreatic beta-cell area coupled with an increase in alpha-cell area, it would seem counterintuitive that activation of glucagon receptor signalling by Oxm could exert beneficial effects. However, in agreement with the current findings, sustained treatment with (D-Ser^{2})-Oxm[Lys^{38}PAL] has been shown to evoke improvements of pancreatic islet morphology in a T1DM mouse model generated through single high dose STZ administration (Pathak et al., 2015; Irwin et al., 2015b). Moreover, (D-Ser^{2})-Oxm[Lys^{38}PAL] was the only treatment that reduced characteristic STZ-induced increases of fluid intake and circulating glucagon concentrations in the current setting. Thus, the positive impact of GLP-1 receptor activation in T1DM (Irwin et al., 2015b), coupled with glucagon receptor activation by (D-Ser^{2})-Oxm[Lys^{38}PAL], may prove surprisingly beneficial in T1DM.

Interestingly, induction of diabetes in Glu^{CreERT2};ROSA26-eYFP mice was also associated with increased transition of glucagon-positive alpha-cells to insulin positive beta-cells, as has been observed previously (Thorel et al., 2010S; tanojevic et al.,

2015). More prominently, this transdifferentiation process was further enhanced by (D-Ala^{2})GIP and (D-Ser^{2})-Oxm[Lys^{38}PAL] treatment, but not by xenin-25[Lys^{13}PAL]. Thus, the benefits of xenin-25[Lys^{13}PAL] on pancreatic morphology may be independent of alpha- to beta-cell transdifferentiation, although further study would be required to confirm this. Given that local islet secretion of GIP is elevated in STZ-induced diabetes (Vasu et al., 2014a), it might also suggest that the xenin analogue was unable to augment GIP bioactivity at the level of the endocrine pancreas, in contrast to the well described GIP potentiating effects of xenin (Gault et al., 2015; Hasib et al., 2017). However, unlike (D-Ala^{2})GIP, native GIP has an extremely short biological half-life (Martin et al., 2013), which may also be important in this regard.

Further to the above islet changes, the number of alpha-cells losing their identity was increased in the diabetic mouse model, in accordance with upregulated alpha- to beta-cell transdifferentiation. (D-Ala^{2})GIP, and particularly (D-Ser^{2})-Oxm[Lys^{38}PAL], again augmented this islet cell transitioning effect. Thus, induction of diabetes by STZ appears to be linked to positive effects on islet cell transdifferentiation, likely as an adaptive measure to help re-establish normal metabolism, which were further enhanced by the treatment interventions. For (D-Ser^{2})-Oxm[Lys^{38}PAL], it is difficult to fully appreciate the relative impact of GLP-1 and glucagon receptor signalling in the islet cell transdifferentiation benefits, especially since Oxm is a recognised GLP-1 receptor biased agonist (Pocai, 2012), but yet only a small subset of alpha-cells express the GLP-1 receptor (Richards et al., 2014). Thus, in the current setting, glucagon receptor signalling may be equally, or more important than GLP-1, for (D-Ser^{2})-Oxm[Lys^{38}PAL] induced pancreatic effects, or these effects might involve alpha-delta cell cross-talk, as the latter also express GLP-1 receptors (Richards et al., 2014). Intriguingly, similar to GIP (Vasu et al., 2014a), glucagon gene upregulation was observed in alpha-cells following the loss of beta-cell paracrine interactions (Stanojevic et al., 2015). This would lead to increased processing and secretion of gene products such as Oxm (Ye et al., 2015), that may play an important role in ultimately defining islet cell identity in diabetes. Whether related molecular events also occur with the islet xenin gene in response to diabetes and beta-cell loss, still needs to be investigated.

9.5.3 Effects of peptide treatments on body parameters

None of the treatments was able to rescue body weight loss or suppress progression to overt hyperglycaemia in STZ-diabetic mice at the doses employed. This could be important given that improved glycaemic status is known to independently prevent loss of beta-cell identity (Wang et al., 2014). Thus, the observed adaptations of pancreatic morphology seem likely to be due to direct actions of the peptides on islet cells and not a secondary consequence of changes in blood glucose In this regard, it would have been intriguing to consider the impact of simple insulin supplementation, and related improved glycaemic status, on beta-cell identity in the current setting. Long-acting GIP and Oxm analogues have previously been demonstrated to suppress blood glucose levels in STZ diabetic rodents (Irwin et al., 2015b; Pathak et al., 2015; Yanagimachi et al., 2016), and differences from the present study likely relate to variations in the mouse strains used, severity of diabetes induced, the peptides and dosing regimens and length of experimentation. Thus, a more prolonged treatment regimen may have resulted in improved glucose handling, which could have also beneficially influenced islet cell transdifferentiation. In addition, interference of GIP receptor signalling by long-term administration of the partial GIP receptor antagonist, (Pro^3)GIP (Pathak et al., 2015), did exacerbate hyperglycaemia and further impair glucose tolerance in STZ diabetic mice (McClean et al., 2007). Unfortunately, related antagonists of xenin and Oxm signalling pathways are not available to investigate this effect further.

9.5.4 Limitations

Notwithstanding our clear-cut findings, and use of state-of-the-art cell lineage tracing technologies, the present study does have some minor limitations. As such, GFP expression was only detectable in around 50-60% of glucagon positive alpha-cells in normal mice. This was increased to approximately 70% in the diabetic rodents, which likely represents alpha-cell expansion induced by STZ administration (Liang et al., 2011; Vasu et al., 2014a). It is likely that refining the method of promoter induction would significantly increase the percentage of GFP positive alpha-cells. Despite this, earlier studies in $Glu^{CreERT2}$;ROSA26-eYFP mice confirm that activation of the Cre recombinase enzyme specifically within alpha-cells does not affect normal islet cell transdifferentiation processes (Thorel et al., 2010), indicating that our findings on alpha- to beta-cell transitioning are reliable. Although an alpha- to beta-cell

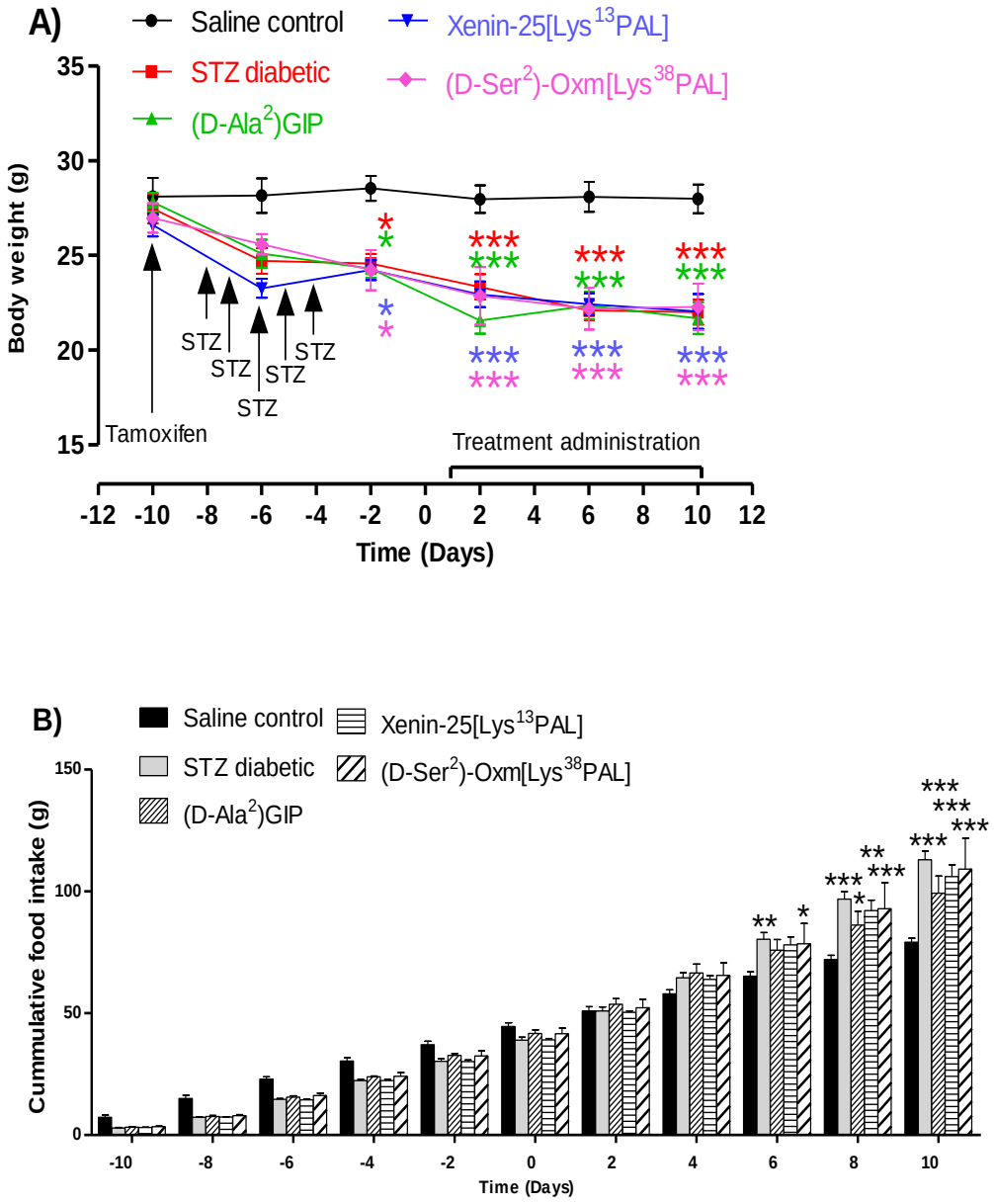
transdifferentiation seems the most likely explanation of our findings, we cannot exclude that transient activation of CreERT2 resulted in EYFP-reporter activation in STZ-resistant beta-cells, as we previously observed some recombination events in aged islets of tamoxifen naïve mice, even though 99% of EYFP-positive cells in that study also expressed proglucagon (Campbell et al., 2019). This suggests that within the limitations of immunohistochemical co-localisation, only alpha-cells ever activate the Cre-reporter (Campbell et al., 2019). It is thus theoretically possible that our results arose from transient proglucagon-promoter activity in STZ-treated beta-cells, escaping apoptosis through transient dedifferentiation to a proglucagon (co-) expressing cell type, and subsequently losing proglucagon expression again. However, importantly for this study, the respective treatment interventions would still have resulted in increased rescue of alpha-cell mass through this alternative, in our view less likely, mechanism. Similarly, we are unable to determine the relative impact of enhanced beta-cell proliferation, reduced beta-cell apoptosis and positive alpha- to beta-cell transdifferentiation to the overall beneficial beta-cell regenerative effects evoked by each of the treatments. Further to this, owing to methodological restrictions, we are not able to examine triple staining of GFP, glucagon and insulin, which could have provided some further useful information in relation to islet cell lineage status. Moreover, use of a mouse model where diabetes induction is principally autoimmune related, such as humanised non-obese diabetic (NOD) SCID mice, may help to ascertain full translational potential of the GIP, xenin and Oxm compounds. However, from a therapeutic viewpoint, the extent of restoration of islet and beta-cell area induced by each treatment regimen is highly encouraging. Furthermore, direct comparison against a clinically approved antidiabetic drug, such as a GLP-1 mimetic (Irwin et al., 2015a), or use of single-cell RNA sequencing to help uncover regulatory relationships between relevant genes and any potential cellular heterogeneity (Wang et al., 2019), would also have been interesting.

9.5.5 Concluding remarks

In conclusion, long-term administration of (D-Ala^{2})GIP, xenin-25[Lys^{13}PAL] or (D-Ser^{2})-Oxm[Lys^{38}PAL] improved pancreatic islet morphology in STZ-diabetic mice through positive effects on alpha- and beta-cell growth and survival. In addition, the GIP and Oxm analogues augmented transdifferentiation of mature alpha-cells to insulin positive beta-cells in this mouse model of insulin deficient diabetes.

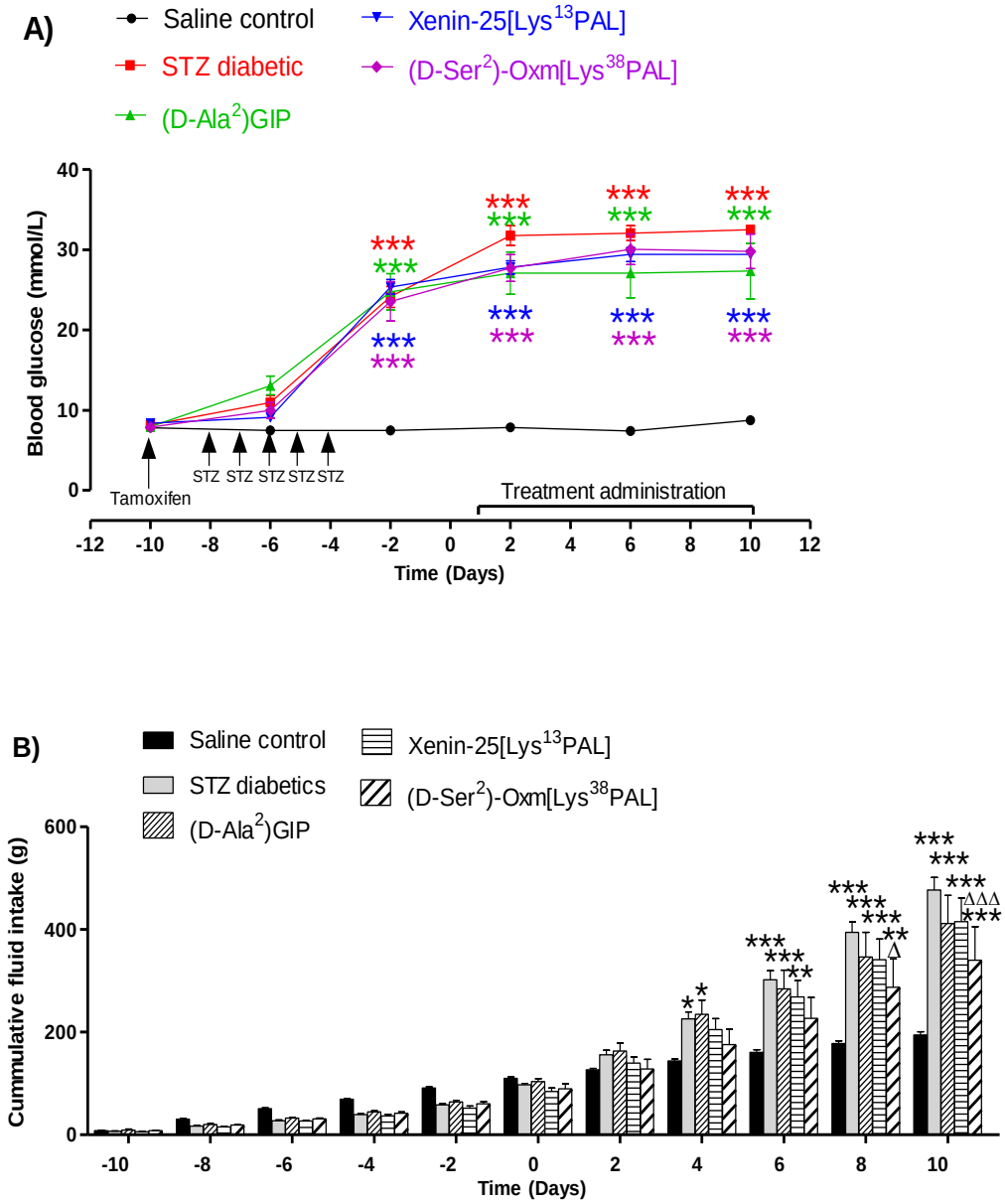
Understanding the cellular mechanisms underlying these benefits would also be of considerable interest and merits further detailed study. Taken together, these data emphasise the benefits of endogenous and enzyme resistant forms of GIP, xenin and Oxm in diabetes, and highlight their complementary mechanisms to potentially augment or regenerate beta-cell mass.

Figure 9.1 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on body weight and food intake in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice



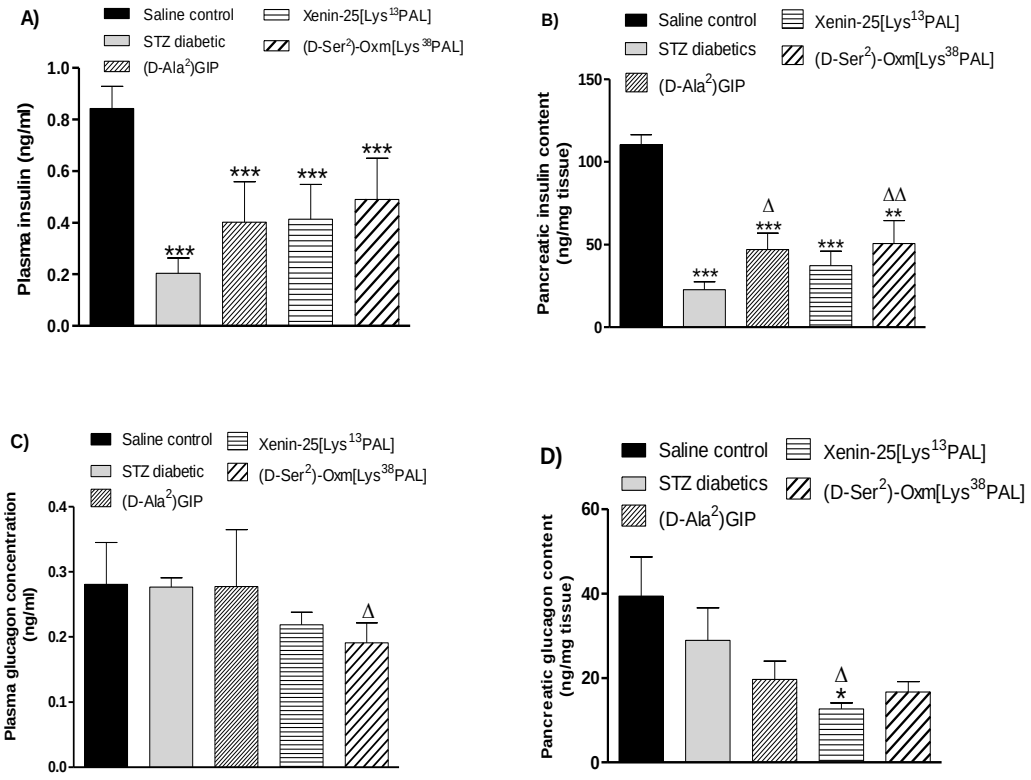
(A) Body weight and (B) food intake were measured prior to, and 10 days during, twice daily treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw) in STZ Glu^{CreERT2};ROSA26-eYFP mice. Values represent mean ± SEM for 6 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to lean controls.

Figure 9.2 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on blood glucose and fluid intake in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice



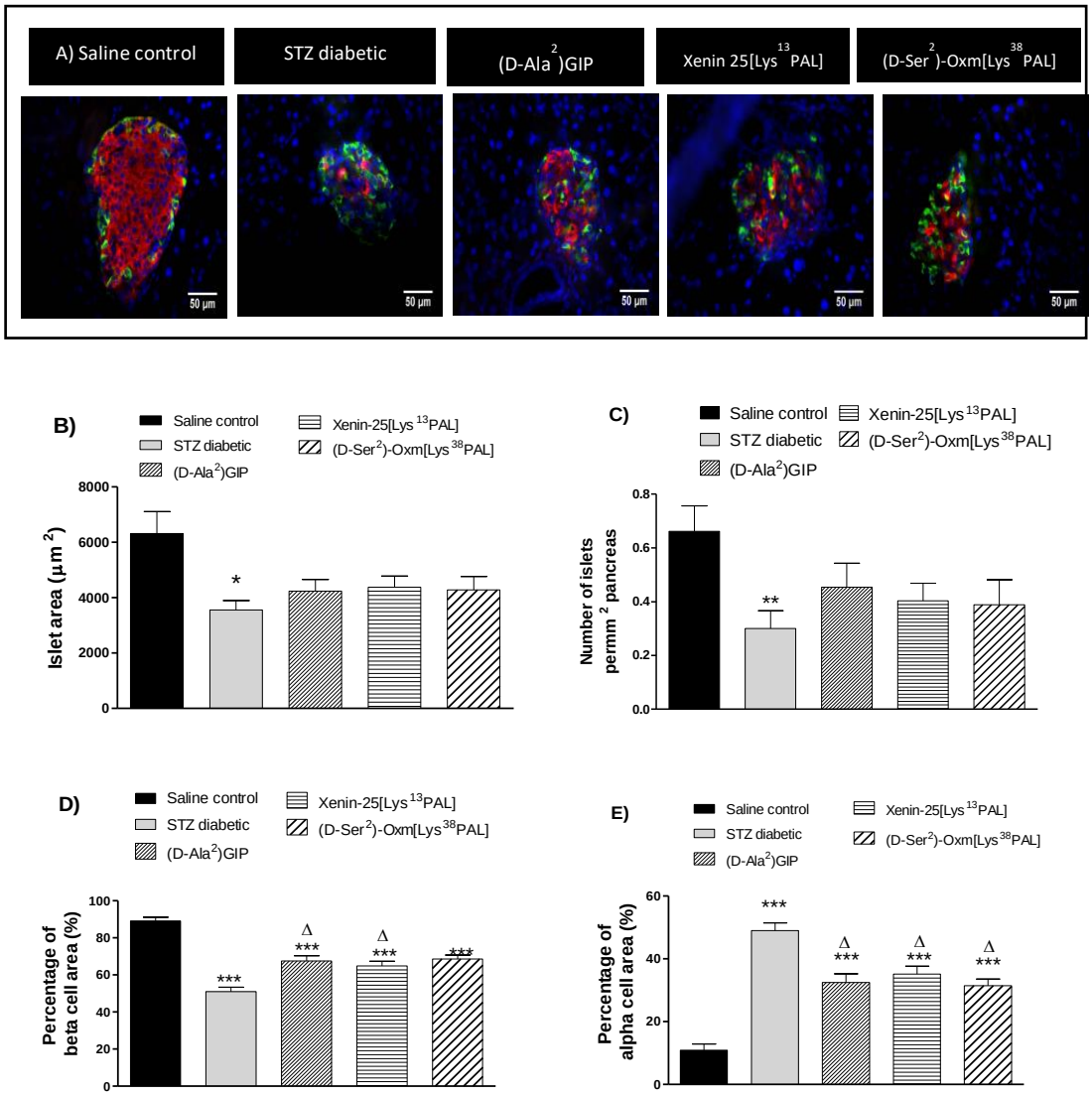
(A) Blood glucose and (B) fluid intake were measured prior to, and 10 days during, twice daily treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw) in STZ Glu^{CreERT2};ROSA26-eYFP mice. Values represent mean ± SEM for 6 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to lean controls. Δp<0.05 and ΔΔΔp<0.001 compared to STZ diabetic controls.

Figure 9.3 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on plasma and pancreatic insulin or glucagon in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice



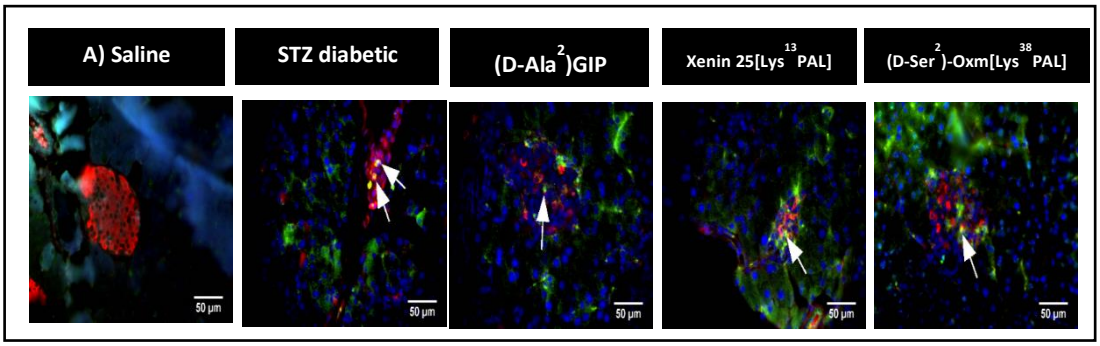
(A) Plasma insulin, (B) pancreatic insulin content, (C) plasma glucagon and (D) pancreatic glucagon content were assessed at the end of the treatment period, twice daily treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw) in STZ Glu^{CreERT2};ROSA26-eYFP mice. Values represent mean ± SEM for 6 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to lean controls. Δp<0.05 and ΔΔ p<0.01 compared to STZ diabetic controls.

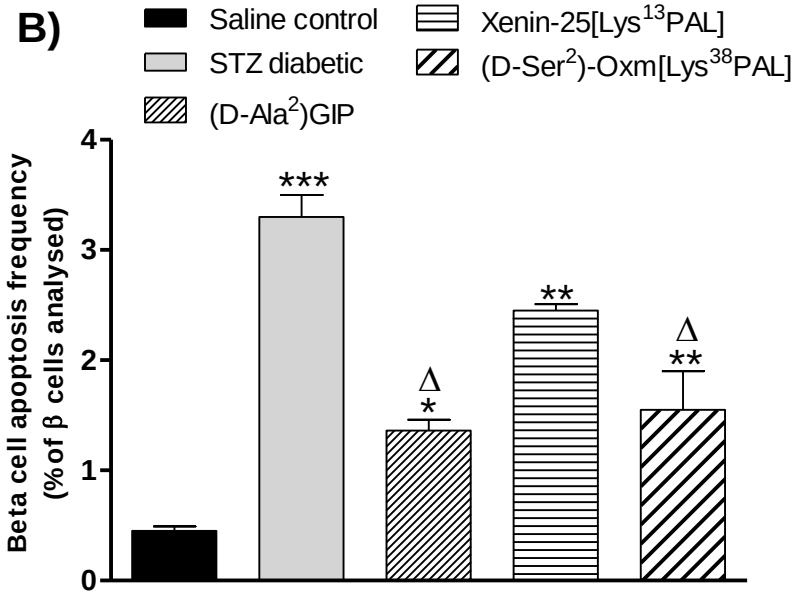
Figure 9.4 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on islet morphology in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice



(A-E) Parameters were assessed in STZ-diabetic Glu^{CreERT2};ROSA26-eYFP mice after 10 days twice daily treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw). (A) Representative images (40X) of islets showing insulin (red), glucagon (green) and DAPI (blue) immunoreactivity from each group of mice; scale bar 50 µm. (B) Islet area, (C) islet number, (D) percentage of beta cell area and (E) percentage of alpha cell area were measured using Cell^F image analysis software. Values represent mean ± SEM for 6 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to lean controls. Δp<0.05 compared to STZ diabetic controls.

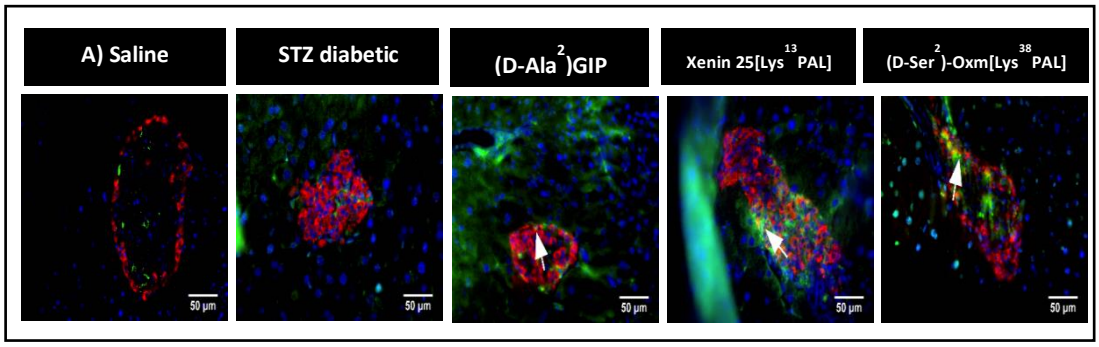
Figure 9.5 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on beta cell apoptosis in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice

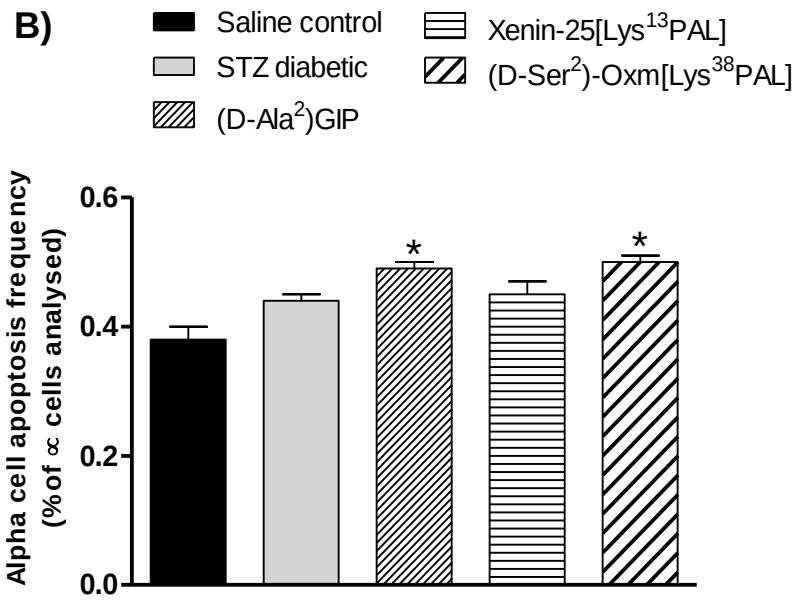




(A,B) Parameters were assessed in STZ-diabetic Glu^{CreERT2};ROSA26-eYFP mice after 10 days treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw). (A) Representative images (40X) of islets showing insulin (red), TUNEL (green) and DAPI (blue) immunoreactivity from each group of mice; scale bar 50 µm. Arrows indicate apoptotic cells. (B) Pancreatic beta cell apoptosis was measured using TUNEL staining and quantified with ImageJ software. Values are mean ± SEM for 6 mice. ***$p<0.001$ compared to lean controls. Δ$p<0.05$ compared to STZ diabetic controls.

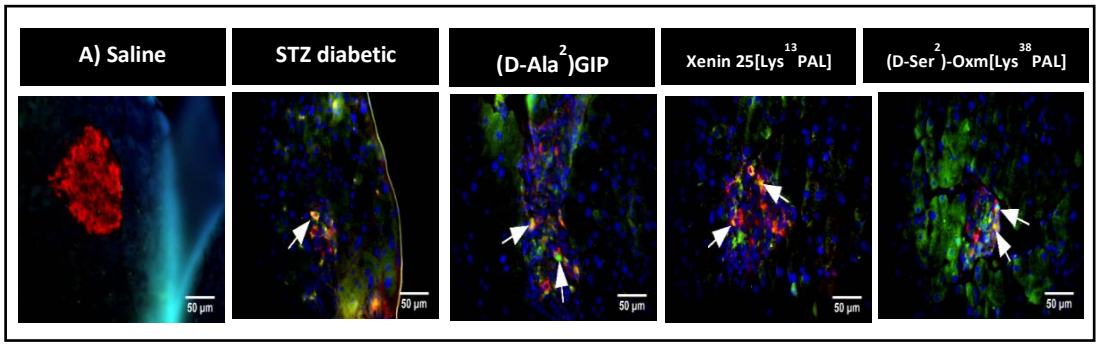
Figure 9.6 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on alpha cell apoptosis in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice

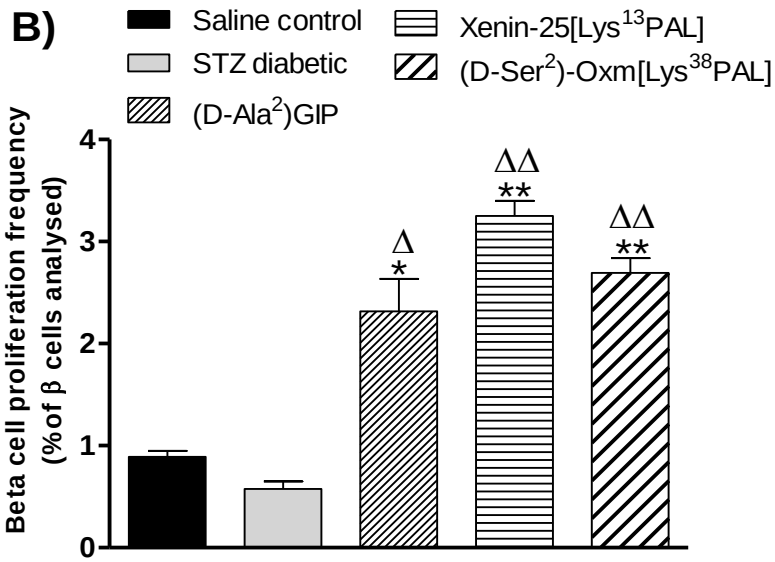




(A,B) Parameters were assessed in STZ-diabetic Glu^{CreERT2};ROSA26-eYFP mice after 10 days treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw). Representative images (40X) of islets showing glucagon (red), TUNEL (green) and DAPI (blue) immunoreactivity from each group of mice; scale bar 50 µm. Arrows indicate apoptotic cells. (B) Pancreatic alpha cell apoptosis was measured using TUNEL staining and quantified with ImageJ software. Values are mean ± SEM for 6 mice. *$p<0.05$ compared to lean controls.

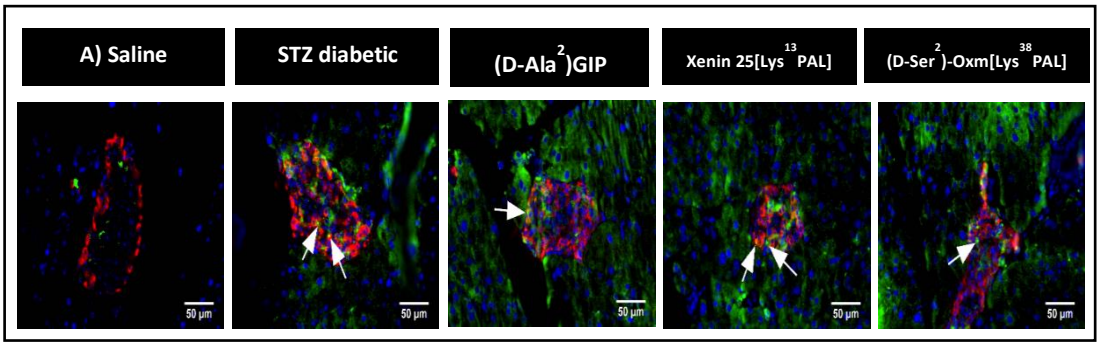
Figure 9.7 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on beta cell proliferation in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice

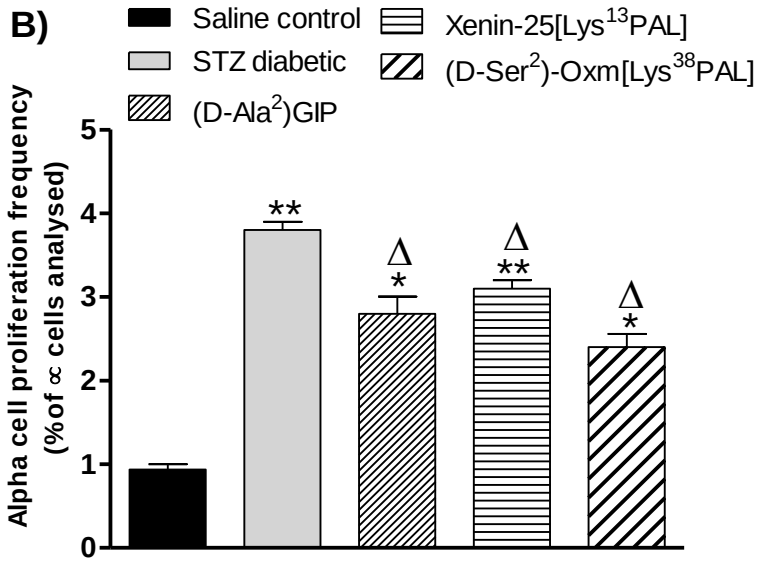




(A,B) Parameters were assessed in STZ-diabetic Glu^{CreERT2};ROSA26-eYFP mice after 10 days treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw). Representative images (40X) of islets showing insulin (red), Ki67 (green) and DAPI (blue) immunoreactivity from each group of mice; scale bar 50 µm. Arrows indicate proliferating cells. (B) Pancreatic beta cell proliferation was measured using Ki67 staining and quantified with ImageJ software. Values are mean ± SEM for 6 mice. *$p<0.05$ and **$p<0.01$ compared to lean controls. Δ$p<0.05$, ΔΔ$p<0.01$ compared to STZ diabetic controls.

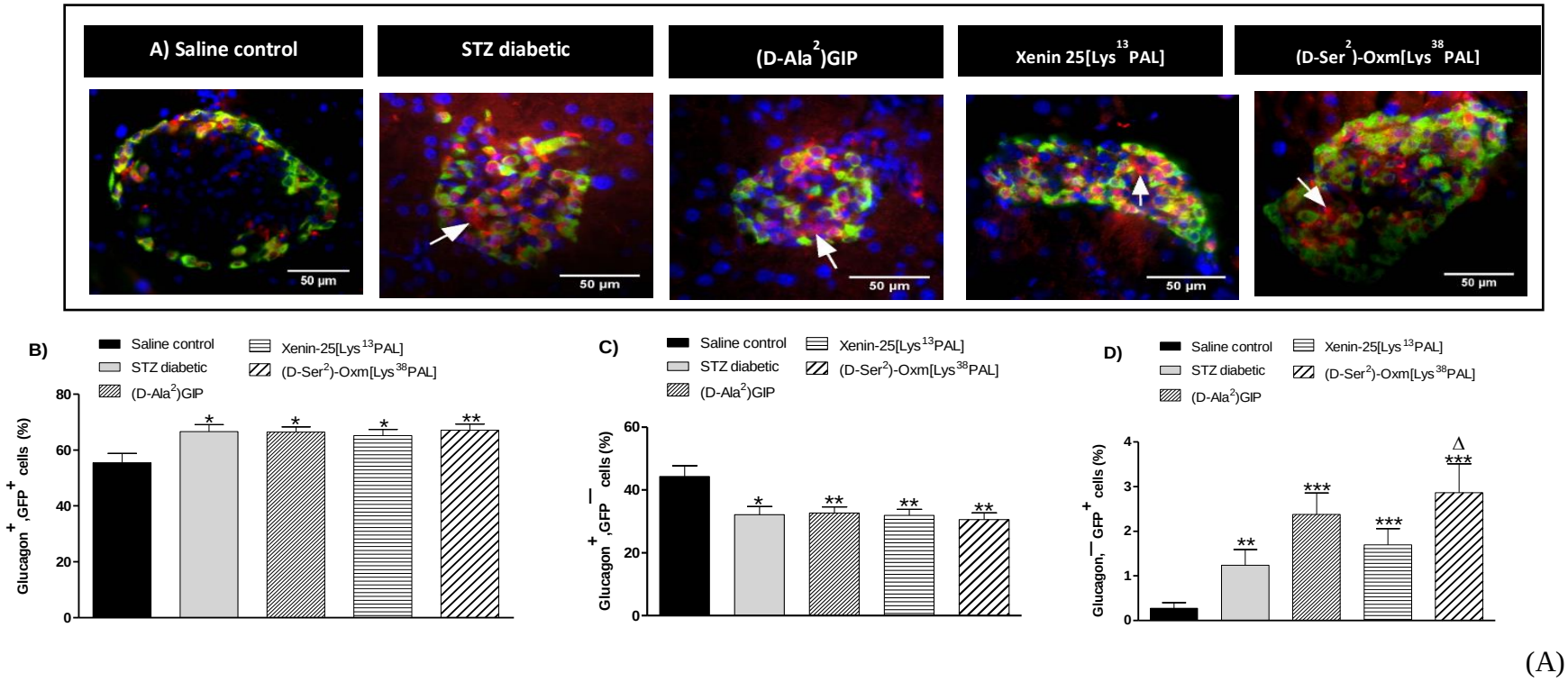
Figure 9.8 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on alpha cell proliferation in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice





(A,B) Parameters were assessed in STZ-diabetic Glu^{CreERT2};ROSA26-eYFP mice after 10 days treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw). Representative images (40X) of islets showing glucagon (red), Ki67 (green) and DAPI (blue) immunoreactivity from each group of mice; scale bar 50 µm. Arrows indicate proliferating cells. (B) Pancreatic beta cell proliferation was measured using Ki67 staining and quantified with ImageJ software. Values are mean ± SEM for 6 mice. *$p<0.05$ and **$p<0.01$ compared to lean controls. Δ$p<0.05$ compared to STZ diabetic controls.

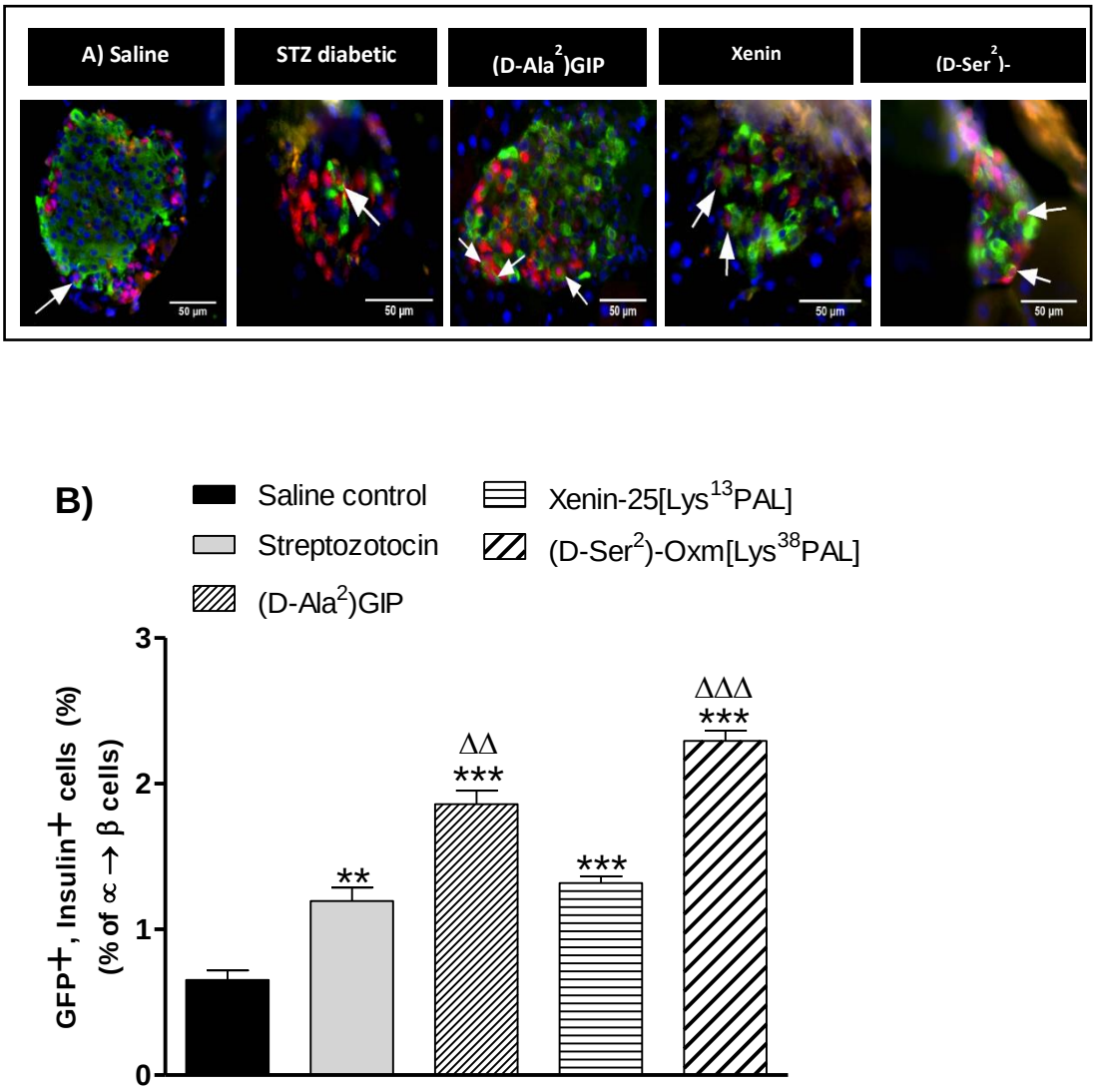
Figure 9.9 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on pancreatic alpha-cell lineage in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice



(A)

Representative images (40X) of islets showing glucagon (green) and GFP (red) immunoreactivity from each group of mice; scale bar 50 µm. (B-D) Parameters were assessed in STZ-diabetic Glu^{CreERT2};ROSA26-eYFP mice after 10 days treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw). (B) Glucagon^{+ve}/GFP^{+ve}, (C) glucagon^{+ve}/GFP^{-ve} and (D) glucagon^{-ve}/GFP^{+ve} islet cells. Arrows indicate glucagon^{-ve}/ GFP^{+ve} islet cells. Values are mean ± SEM for 6 mice. *p<0.05, **p<0.01 and ***p<0.001 compared to lean controls. Δp<0.05 compared to STZ diabetic controls.

Figure 9.10 Effects of (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] and (D-Ser^2)-Oxm[Lys^{38}PAL] on alpha-cell transdifferentiation in STZ diabetic Glu^{CreERT2};ROSA26-eYFP mice



(A) Representative images (40X) of islets showing insulin (green), GFP (red) and DAPI (blue) immunoreactivity from each group of mice; scale bar 50 µm. (B) GFP^{+ve}/insulin^{+ve} islet cells were counted in STZ-diabetic Glu^{CreERT2};ROSA26-eYFP mice after 10 days treatment with saline vehicle, (D-Ala^2)GIP, xenin-25[Lys^{13}PAL] or (D-Ser^2)-Oxm[Lys^{38}PAL] (each at 50 nmol/kg bw). Arrows indicate double stained cells. Values are mean ± SEM for 6 mice. **p<0.01 and ***p<0.001 compared to lean controls. ΔΔp<0.01 and ΔΔΔp<0.001 compared to STZ diabetic controls.

Chapter 10

General Discussion

10.1 Alpha cells as potential treatment for diabetes

Today, the incidence of diabetes mellitus, a disease of persistent high blood glucose levels, is rising at a worrying rate globally (Unnikrishnan et al., 2017). Diabetes is characterised by a sudden (Type- 1DM) or progressive (Type-2 DM) loss of beta cells. In former case, the cause is a genetic defect in beta cells, which make them foreign entities for body's own immune system. While in the later class, it may be a result of the progressive increase in the burden of insulin demand along with surrounding toxic environment (Fan et al., 2017). However, type-2 DM is a complex disease state with insulin resistance being an important contributor, which also impacts on beta cell dysfunction (Fan et al., 2017). Obesity is the main cause of insulin resistance and type-2 DM (Kahn et al., 2006) but not all obese people progress towards diabetes (Kahn et al., 2006). Several currently available drugs can reverse diabetes to certain extent by activating beta cell function mainly through enhancing insulin secretion (Irwin et al., 2015a). On the other hand, diabetes may progress even more after management with non-insulin treatments such as sulphonylureas (Moreno-Fernandez et al., 2019). Therefore, insulin is recommended in severe cases to avoid further complications (Kahanovitz et al., 2017).

Several lines of evidence support the notion that beta cells loss is also associated with type-2 diabetes (Thorel et al., 2010; Vasu et al., 2014a). Indeed, it is noteworthy that the type-2DM is a progressive disease in which beta cells commit programmed-death by means of failure in compensation mechanism (Vasu et al., 2014a). Glucotoxicity is considered as a main reason for beta cell apoptosis (Robertson et al., 2003; Boughton et al., 2017). It can cause irreversible damage in beta cell function and stress the cells towards apoptosis (Robertson et al., 1992; Gleason et al., 2000; Boughton et al., 2017). Lipotoxicity has been found to impair insulin gene expression, which eventually can lead to beta cell apoptosis (Cnop et al., 2001; Poitout et al., 2010). Several forms of metabolic damage can generate oxidative and endoplasmic reticulum stress that increases vulnerability of beta cells (Tiedge et al., 1997; Robertson, 2006). In addition to these factors, inflammation and amyloid deposition can also lead to damaged beta cells (Boni-Schnetzler et al., 2008; Richardson et al., 2009; Jurgens et al., 2011).

Pancreatic islet cells possess low replication capacity (Boujendar et al., 2002). Thereby during diabetes and the associated apoptotic environment, the weak proliferation ability of beta cells is either lost or further hampered (Boujendar et al., 2002; Vasu et al., 2014a)**.** Several studies have showed that reduction of beta cell mass is associated with increased apoptosis rate together with reduced proliferation of beta cells (Moffett et al., 2014; Vasu et al., 2014a)**.** In contrast, Type -1DM involves T-immune cells mediated beta cell destruction (Burrack et al., 2017). In such cases, exogenous insulin is the only survival remedy for people with T1DM. However, it is not clear that insulin is a target antigen for T-cells. It seems to be an exogenous insulin exerts its glucose lowering effects irrespective of T-cell-mediated elimination. Thereby, it is important to note that beta cells themselves or relevant identity markers could be a possible target for immune system probably due to genetic defect (Burrack et al., 2017). Therefore, the scarcity of insulin needs to be addressed in order to assure the survival and well-being of people with diabetes. To overcome this barrier, several attempts have been made to transplant whole pancreas or isolated islets from multiple donors (Matsumoto, 2010). However, it this offers major obstacles such as difficulties in donor supply, islet isolation, preservation of beta cell function and immune rejection (Matsumoto, 2010). Therefore, there is an urgent need to compensate scarcity of insulin by providing alternative approaches for provision of new beta cells.

Recent studies have shown that several non-beta cell sources such as islet alpha or delta cells, exocrine cells, liver, stomach skin and pluripotent stem cells offer the regenerative ability for lost insulin production (Solar et al., 2009; Chera et al., 2014; Lee et al., 2018). In particular, alpha cells are believed to be natural and abundant regenerative source (Thorel et al., 2010). Earlier, alpha cells have been known to regulate and protect adjacent beta cells in islets. Accordingly, previous studies demonstrated that alpha cells could produce GLP-1, which is classically secreted by intestinal L-cells to promote growth and function of surrounding beta cells (Moffett et al., 2014; Vasu et al., 2014a). Interestingly, several gene manipulation studies on alpha cells have demonstrated that the forceful repression of their own markers or induction of beta cell specific transcription factors can lead to expression of the insulin gene **(**Collombat et al., 2009; Yang et al., 2011). While, some opposite attempts have also been taken to convert beta cells back to alpha cells **(**Lu et al., 2010; Wilcox et al., 2013). Overall, these attempts have been clearly confirmed that the function and role

of alpha cells are not rigid as assumed earlier; rather they possess fragile genome, which can be exploited for the therapeutic purpose. In addition, recently studies by Thorel et al (2010) reported that the alloxan-mediated extreme beta cell loss activated transdifferentiation of alpha cells into beta cells.

Therefore, in the present thesis, we used diabetic rodent models to evaluate alterations in islet morphology, contribution of alpha cell transdifferentiation and potential treatments. We employed multiple low doses of streptozotocin to generate mice with severe diabetes. The resultant persistent hyperglycaemia is considered as a sign of extreme injury to the beta cells. Earlier reports on streptozotocin demonstrated the alteration in the normal body parameters, and a distortion of islet architecture together with increased beta cell apoptosis. Streptozotocin is believed to induce apoptotic pathways and oxidative stress exclusively in beta cells (Vasu et al., 2014a). Therefore, the islets, which are mainly, composed of beta cells, undergoes a sudden loss in number and total islet area together with the reduction of plasma and pancreatic insulin levels. Interestingly, over the period, STZ- generated empty areas of beta cells within islets which were largely balanced by immediate expansion of alpha cell mass. Our observations from Chapter 3 and Chapter 5 to Chapter 9 are consistent with such events. Therefore, we confirmed that this diabetic model is a good choice for the studying diabetes related changes in relation to testing our hypothesis of alpha cell transdifferentiation.

To explore the therapeutic potential of well-known small molecule taurine with respect to beta cell re-establishment during diabetes, we employed BRIN-BD11 cell lines (*in vitro*) and Swiss-TO mice (*in vivo),* as documented in Chapter 3. Interestingly we found that taurine promoted proliferation and reduced apoptosis of beta cells. These findings clearly suggest that taurine plays important role in the growth of beta cells through either encouragement or protection of beta cells. This was further confirmed by studying the growth-related gene- and protein- expression. Consistent with this line of thought, earlier studies have been reported that anti-oxidant and anitapototoic properties of taurine in protecting beta cells from IL and NO stress (Merezak et al.,2001; Arany et al., 2004).

Importantly, alpha cells were found to be increased as a result of loss of beta cells. As such, taurine prevented this expansion. In Chapter 3, we were not able to confirm

whether these reduced alpha cells committed apoptosis or transdifferentiated into beta cells, as we found taurine promoted the population of beta cells. One of the limitations of the methodology employed in Chapter 3 study was the inability to track alpha cell lineage changes in the mouse strain employed. Therefore, we sought to use transgenic $Glu^{CreERT2}$; ROSA26eYFP mice that could easily detect the alpha cells and offer us its functional property in the context of transdifferentiation.

To study the *in vitro* effects of taurine on transdifferentiation we utilised two different cultured cell lines, namely BRIN-BD11 cells and alpha TC-1.9 cells. As such, we found no significant alterations in the expression of beta and alpha identity markers using 12 h cultured insulin-producing BRIN-BD11 cells. Thus, here we confirmed that taurine did not induce any alpha cell markers in beta cells. Moreover, taurine promotes of beta growth through stimulating beta cells identity markers such as Pdx-1 and insulin. However, in contrast to this, we observed no effect of taurine on Pdx1 or insulin expression in 72 h cultured alpha TC-1.9 cells. Besides, taurine showed increased expression of glucagon hormone in alpha TC-1.9 cells. This result is consistent with previous studies that utilised alpha TC-1.6 cells (Bessho et al., 2014). It has been suggested that taurine might be potentiating K_{ATP} and Ca^{2+} channels on alpha cells to enhance glucagon accumulation. Earlier studies on glucagon suggested its possible role in alpha cells transdifferentiation (Ye et al., 2015). Notably, these types of studies on cultured cell lines provide a relatively weak picture compared with observations on primary alpha cells within islets exposed to diabetic milieau.

Indeed, in contrast to *in vitro* effects of taurine on alpha TC 1.9 cells, we found in Chapter 5 that taurine promoted the transdifferentiation of alpha cells into beta cells in a transgenic $Glu^{CreERT2}$; ROSA26eYFP mice. Moreover, plasma glucagon concentrations were found to be lowered in taurine treated diabetic mice as compared to saline controls. This effect was seemed to be secondary to alterations occurred after extreme beta cell injury upon administration of multiple low dose of streptozotocin. A similar effect was observed with incretin peptides, sitagliptin and GABA. Thus, we can speculate that transdifferentiation occurs as a chronological process in diabetes as reported by several other studies (Thorel et al., 2010; Whalley et al., 2011; Ye et al., 2015; Lee et al., 2018). It appears that glucagon might be increasing at the beginning of transdifferentiation. Apart from this action, the beta protective effects of taurine

were also observed in $Glu^{CreERT2}$; ROSA26eYFP mice which clearly suggests that taurine has a role in maintaining beta cell mass by proliferation of pre-existing beta cells and hampering the cell death. These observations were similar with the findings observed in Chapter 3. Moreover, earlier studies support our observations of a beta cell protective effect of taurine (Lee et al., 2011; Santos-Silva et al., 2015).

We also studied the anti-malarian small molecule artemether with respect to alpha to beta cell transdifferentiation. Recent research by Li et al. (2017), showed the involvement of artemether in $GABA_A$ receptor-dependent activation of insulin gene. This study also demonstrated that artemether supresses Arx marker of alpha cells and activates Pax4 marker of beta cells. Surprisingly, we found no alpha to beta cell transdifferentiation either in the *in vivo* or *in vitro* studies with artemether. Accordingly, artemether significantly decreased the alpha cell viability with the same dose that was used by Li et al. (2017). In parallel with this, it also reduced Arx gene expression. Moreover, the drug negatively affected the cell viability. Although Li et al. (2017) demonstrated the possible involvement of artemether in alpha cell transdifferentiation, later reports, in addition to ours, failed to reproduce the similar findings (Ackermann et al., 2018; Van et al., 2018). As such, Li et al (2017) suggested that reduction of Arx and glucagon observed after artemether treatment was due to suppression of Arx and expression of insulin. We found the artemether reduced Arx but no effect on glucagon. While, Van et al, (2018) reported that artemether not only reduced alpha cell identity markers but also induced similar effects on beta cell identity factors. Taken together, it is valid to suggest that such reduction in alpha cells identity factors may be result of alpha cell apoptosis due to lethal effects of artemether rather than progression to beta cell phenotype. However, further investigation is would be useful to confirm the exact action of artemether on alpha cell transdifferentiation.

The incretin hormones GLP-1 and GIP, naturally produced by enteroendocrine cells of the intestine are now believed to produced also in pancreatic alpha cells (Moffett et al., 2014; Vasu et al., 2014a). The main function of these hormones is to carry intestinal food signals to pancreas and regulate secretion of insulin and glucagon as well as normal growth of beta cells and alpha cells (Moffett et al., 2014). Much has been evaluated regarding their beneficial effects on islet morphology (Moffett et al., 2014; Vasu et al., 2014a). The detailed study on benefits of incretin hormone

signalling on the islet morphology and glycaemia of diabetic animals was explained by Moffett et al (2014, 2015a, 2015b). The author also demonstrated the ectopic expression of GLP-1 by alpha cells during extreme diabetic condition. Accordingly, an injury to beta cells triggers such local production of GLP-1 in alpha cells. In line with this, more recent studies have been demonstrated the effect of GLP-1 and exendin-4 on alpha to beta cell transdifferentiation (Bulotta et al., 2002; Lee et al., 2018; Zhang et al., 2019). However, natural GLP-1 is degraded within a short period of time and therefore an enzyme resistant or enzyme inhibiting approach is needed to overcome this barrier (Vasu et al., 2014a).

With this consideration, here in Chapter 6, we employed the GLP-1 analogue liraglutide and a DPP-IV inhibitor sitagliptin to potentiate the GLP-1 signalling with more intensity. Both agents have become established and well utilised treatments for diabetes in man. Interestingly, we found that liraglutide and sitagliptin significantly enhanced alpha to beta cell transdifferentiation. This was congruous with the presence of bi-hormonal cells. Therefore, it clearly suggests that GLP-1 signalling might play an important role in alpha to beta cell transdifferentiation. Previous studies by Whalley et al (2011) demonstrated that beta cell dysfunction was observed with 2 h exposure of STZ with isolated islets. This was associated with deprived glucose-induced insulin secretion, yet unchanged glucagon release by surrounding alpha cells. This suggests that alpha cells were normal in their functioning. Later, islets were exposed for 2 days with higher concentrations of streptozotocin, resulting in extreme stress to beta cells which ultimately upregulated GLP-1 with no alteration glucagon level. This clearly suggested that alpha cells produced GLP-1 for compensating extreme condition. Similarly, we observed increased GLP-1 expression by alpha cells while either no change or reduction in glucagon concentration. Similar findings were also observed by Lee et al (2018).

Recent evidence postulates that peptide products derived from the processing of the glucagon gene may play some beneficial role in alpha cell transdifferentiation (Ye et al., 2015; Stanojevic et al., 2015). Therefore, in Chapter 9, we tested analogues of oxyntomodulin, GIP and xenin. Consistent with previous studies on such analogues, we have shown beneficial effects on islet morphology (Irwin et al., 2015; Millar et al., 2016; Hasib et al., 2017). Therefore, we suggest that these peptides might improve

islet architecture by promoting beta cell mass and reducing alpha cell mass. These findings prompted us to quest for whether recapitulation of beta cell mass is a result of the contribution of alpha cells or survived beta cells. Intriguingly D-Ser2 oxyntomodulin GluPal and D-ala2 GIP have been found to increase the number of reprogrammed alpha cells. However unexpectedly, xenin-25 GluPal was unable to alter the additional transdifferentiation impact of STZ, which is perhaps surprising because xenin is known to mediate its effect partially through GIP machinery. For instance, we hypothesize that direct modulation of GIP signaling by D-ala2 GIP could be more effective rather than xenin-25 GluPal's involvement through its indirect potentiation. Moreover, D-Ala2 GIP is more stable and resistant to enzyme degradation compared to locally produced GIP (Martin et al., 2013). However, we envisage that further long-term effect of xenin-25 GluPal would elaborate its distinct role in transdifferentiation.

It is known that both alpha and beta cells offer the binding sites for oxyntomodulin, GIP, and xenin (Pocai, 2012; Moffett et al., 2015b; Craig et al., 2018). Further, it is noteworthy that both oxyntomodulin and xenin have no known specific receptors, however they are believed to modulate the receptors specific for their closely related sister peptides, yet their mode of action could widely differ from the independent effect of such peptides (Pocai, 2012; Pathak et al., 2015; Craig et al., 2018). In particular, oxyntomodulin is known to exert its effects through modulating both GLP-1 and glucagon receptors which are both expressed by alpha and beta cells (Pocai, 2012; Moffett et al., 2015b). While xenin has been reported to enhance GIP signaling through the use of neurotensin subtypes receptors present on the islet cells (Irwin et al., 2015a; Craig et al., 2018). Besides, GIP has its specific receptors on beta and alpha cells (Irwin et al., 2015a; Moffett et al., 2015b). Taken together, we suggest that GLP-1/glucagon or GIP signalling might play a beneficial role in the transdifferentiation of alpha cells.

It is assumed that an extreme loss of beta cells may result in activation of a beta cell compensation mechanism and alpha cells transdifferentiation (Thorel et al., 2010). Loss of beta cells results into loss of paracrine connections with alpha cells. It is also associated with loss of beta cells secretory products including insulin, GABA and Zn^{2+} (Kawamori et al., 2009). If the lack of beta cells markers could trigger alpha to beta

cell conversion then the question is why do isolated cultured pure alpha cell lines not convert into beta cells, because they are completely devoid of surrounding paracrine beta cells. In addition, injuries to beta cells do not necessarily lead to absolute eradication of beta cells; rather some beta cells survive through extreme stress insult (Thorel et al., 2010). Moreover, earlier studies showed that reprogrammed alpha cells could compensate a vast number of beta cells (Thorel et al. 2010; Ben-Othman et al., 2017; Lee et al; 2018). So why does insulin secreted by initial transdifferentiated alpha cells not hinder further transdifferentiation? In contrast, several studies have shown that alpha cells contributed to beta cell mass even after long-term administration of exogenous insulin employed for the survival of diabetic animals (Thorel et al., 2010; Wang et al., 2014; Cheng et al., 2015). A similar effect was explained with treatment GABA (Ben-Othman et al., 2017). However, some contradictory evidence reported on insulin and GABA signalling which failed to demonstrate alpha to beta transdifferentiation (Ye et al., 2016; Ackermann et al., 2018; Lee et al., 2018; Shin et al., 2019). However, these studies have some limitations including use of non-diabetic mice, low doses, short treatment period and lack of alpha cell lineage method.

Accordingly, in Chapter 7, we tested insulin, GABA and nicotinamide with respect to alpha transdifferentiation. Interestingly we found that both insulin and GABA significantly increased alpha to beta cells transdifferentiation. This suggests that insulin and GABA signaling either directly or indirectly modulates alpha cell transdifferentiation pathways. Earlier studies by Ben-Othman et al., 2017, suggested that GABA regulated transdifferentiation pathways through activating $GABA_A$ receptors on alpha cells. Earlier in Chapter 6 and 9, we showed that the incretin peptides GLP-1 and GIP may stimulate alpha transdifferentiation. These peptides are also known to stimulate insulin secretion from beta cells (Irwin et al., 2015a). This highlights role of insulin signalling in transdifferentiation. Whether, GLP-1 acts through direct signalling receptor or via recruiting any other intermediates such as insulin or somatostatin or any other unknown signals is yet to be discovered.

Unfortunately, we found alpha cells transdifferentiation was unaffected by other treatments including dapagliflozin, nicotinamide, rosiglitazone, tolbutamide and metformin. However, we suggest that future long-term studies with different doses

might reveal beneficial effects of these small molecules on alpha cells transdifferentiation. Another query concerns the possible role of changes in the level of glycaemia induced by the various treatments on islet morphology and individual cell phenotype. This might clearly be an important factor in the action of insulin but it is notable that the islets effects of other drugs were observed without significant alleviation of the hyperglycaemia. This presumably reflects the doses chosen but most notably the severe damage induced by multiple dose STZ administration. In general, the comparative *in vitro* and *in vivo* effects of different treatments with respect to alpha to beta cells are listed in Table 10.1 and 10.2 respectively.

10.2 Limitations

Although much has been achieved in the present research, there are some limitations with respect to studies of alpha cells lineage in $Glu^{CreERT2}$; ROSA26eYFP mice. As the presence of fluorescent protein in alpha cells was heterogeneous, its relative expression among alpha cells was variable in different islets. Importantly saline treated islets showed lesser expression of YFP compared to all other diabetic or treatment islets. The reason for this might be a low population of alpha cells evident in saline treated normal islets. In addition, the alpha cell hyperplasia observed in STZ diabetic islets might reflect the high rate of alpha replication that could have led to loss of Cre-induced labelled alpha cells over the time following the initial dose of tamoxifen. Such observations were previously noted in several other papers (Quoix et al., 2007; Shiota et al., 2017; Parker et al., 2018). Nevertheless, we found that $Glu^{CreERT2}$; ROSA26eYFP mice was a reliable and convenient model to study of alpha cell transdifferentiation (Quoix et al., 2007; Parker et al., 2010; Campbell et al., 2019).

10.3 Concluding remark future prospective

In conclusion, alpha cell expansion was evident immediately after STZ induced-extreme loss of beta cells; while pre-existing beta cells were reduced dramatically. This was consistent with emergence of bihormonal cells containing both insulin and glucagon plus the subsequent transdifferentiation to alpha cells. Interestingly we observed that taurine, GLP-1 analogues, DPP-IV inhibitors, analogues of gut-derived peptides, insulin and GABA might have a potential to regenerate beta cells from

transdifferentiating alpha cells and/or pre-survived beta cells. In contrast, artemether and nicotinamide did not improve islet morphology. Although xenin-25 GluPal did not promote the regeneration of beta cells from alpha cells origin, it has promoted pre-existing beta cells to contribute to new beta cell mass. Similarly, dapagliflozin, tolbutamide and metformin showed little beneficial effects on islet morphology. Although our acute period study could not show beneficial effects on blood glucose regulation, previous evidence revealed that alpha cell transdifferentiation in diabetic mice accompanied improved glycemic control over a long period 10 months by Thorel et al (2010) or 2.7 months by Ben-Othman et al (2017). Therefore, further research will be necessary to evaluate the longer-term effects and durability of the actions of all the tested compounds on beta cell regeneration, transdifferentiation of alpha cells and the maintenance of normal islet architecture. Furthermore, although we confirmed the transdifferentiation of alpha cells, the full beta-like functionality such as glucose sensing and insulin secretory mechanism would be required to vindicate their functional stability and thus further insight into this adaptation is merited.

These thesis outcomes provide an interesting understanding of the mechanism of the alpha cells transdifferentiation. Transdifferentiated alpha cells may be a novel strategy to manage type 1 diabetes as well as type 2 diabetes that suffers from beta cell loss. In addition, it is interesting that alpha cells could survive in type 1 patients irrespective of immune attack on islets. Therefore, transdifferentiated alpha cells after start expressing insulin could be unrecognised from immune target. Future research will gain knowledge into this area. Although transdifferentiation of alpha cells seems to be challenging process, there is well-established limb regeneration process that works naturally in many lizard (Alibardi , 2014). The similarity of transdifferentiation with limb regeneration is that both process begins with extreme injury. Previous reports suggested that inured cell sends either apoptotic signals, or some immune signals (inflammation) to activate transdifferentiation specific pathways like Wnt-signaling in neighbouring cells. This triggers differentiation process in the cells. It is noteworthy that GLP-1 has been found to mediate such Wnt signaling (Yu-Ting et al., 2012). Future research is needed to explore this mechanism. Although limb-regeneration is very a slow process and takes from few months to years for the regeneration of entire organ, it is important to understand more about this process to gain clear understanding of alpha cells transdifferentiation.

Taken all together, therefore here we suggest that alpha cells may offer a better source of beta cell regeneration for treatment of diabetes.

Table 10.1: Summary of comparative *in vitro* effects of different treatments on alpha TC 1.9 cell with respect to control (Chapter 4)

Parameters / Treatments	Hormone expression fluorescent intensity					Gene expression					Cellular glucagon	Cell viability
	Glucagon	Arx	Insulin	GLP-1	Pax-6/ Pdx-1/ PC-1/3	Glucagon	Insulin	Pax 4	Arx	PC1/3		
Artemether	Less affected	Less affected	Less affected	Less affected	Less affected	Decreased ($p<0.05$)	Decreased ($p<0.05$)	Decreased ($p<0.01$)	Decreased ($p<0.01$)	Less affected	Less affected	Decreased ($p<0.001$)
GABA	Less affected	Less affected	Less affected	Increased ($p<0.05$)	Less affected	Decreased ($p<0.05$)	Less affected	Decreased ($p<0.05$)	Less affected	Increased ($p<0.05$)	Increased ($p<0.01$)	Unaffected
Taurine	Less affected	Less affected	Less affected	Less affected	Less affected	Less affected	Less affected	Decreased ($p<0.05$)	Less affected	Less affected	Increased ($p<0.05$)	Unaffected
Sitagliptin	Increased ($p<0.05$)	Less affected	Less affected	Less affected	Less affected	Less affected	Less affected	Decreased ($p<0.01$)	Decreased ($p<0.01$)	Less affected	Increased ($p<0.05$)	Unaffected
Exendin-4	Increased ($p<0.01$)	Less affected	Increased ($p<0.01$)	Increased ($p<0.05$)	Less affected	Less affected	Slightly increased	Decreased ($p<0.01$)	Decreased ($p<0.05$)	Increased ($p<0.05$)	Increased ($p<0.01$)	Unaffected

Table 10.2: Summary of comparative *in vivo* effects of different treatments on beta cell regeneration with respect to diabetic group (Chapter 3 and Chapter 5-9)

Parameters / Treatments	Body parameters	Islet morphology	Beta cell apoptosis	Alpha cell apoptosis	Beta cell proliferation	Alpha cell proliferation	Bihormonal cell generation	Alpha to beta cell transdifferentiation
Chapter 3								
STZ + Taurine group	Less affected	Slightly Improved	Decreased ($p<0.05$)	-	Increased ($p<0.01$)	-	-	-
Chapter 5-9								
Taurine	Less affected	Slightly Improved	Decreased ($p<0.05$)	Less affected	Increased ($p<0.01$)	Slightly decreased	Slightly increased	Promoted ($p<0.05$)
Artemether	Less affected	Slightly Improved	Less affected	Less affected	Less affected	Slightly decreased	Less affected	Less affected
Liraglutide	Less affected	Slightly Improved	Decreased ($p<0.01$)	Less affected	Increased ($p<0.01$)	Less affected	Increased ($p<0.01$)	Promoted ($p<0.01$)
Sitagliptin	Less affected	Slightly Improved	Decreased ($p<0.01$)	Less affected	Increased ($p<0.01$)	Increased ($p<0.05$)	Increased ($p<0.05$)	Promoted ($p<0.001$)
Dapagliflozin	Less affected	Slightly Improved	Decreased ($p<0.05$)	Less affected	Less affected	Less affected	Less affected	Less affected

Insulin	Less affected	Slightly Improved	Decreased ($p<0.05$)	Slightly decreased	Less affected	Decreased ($p<0.05$)	Increased ($p<0.05$)	Promoted ($p<0.01$)
GABA	Less affected	Slightly Improved	Less affected	Less affected	Increased ($p<0.01$)	Decreased ($p<0.05$)	Increased ($p<0.05$)	Promoted ($p<0.001$)
Nicotinamide	Less affected	Less affected	Decreased ($p<0.05$)	Less affected	Less affected	Less affected	Less affected	Less affected
Rosiglitazone	Less affected	Less affected	Less affected	Less affected	Increased ($p<0.01$)	Slightly decreased	Slightly increased	Less affected
Tolbutamide	Less affected	Less affected	Increased ($p<0.05$)	Less affected	Less affected	Less affected	Slightly increased	Less affected
Metformin	Less affected	Slightly Improved	Less affected	Increased ($p<0.01$)	Increased ($p<0.05$)	Slightly decreased	Increased ($p<0.01$)	Less affected
(D-Ala2)GIP	Less affected	Slightly Improved	Decreased ($p<0.05$)	Increased ($p<0.05$)	Increased ($p<0.05$)	Decreased ($p<0.05$)	Unaffected	Promoted ($p<0.01$)
Xenin-25 [Lys13PAL]	Less affected	Slightly Improved	Less affected	Less affected	Increased ($p<0.01$)	Decreased ($p<0.05$)	Less affected	Less affected
(D-Ser$_2$)-Oxm [Lys$_{38}$ PAL]	Less affected	Less affected	Decreased ($p<0.05$)	Increased	Increased ($p<0.01$)	Decreased ($p<0.05$)	Less affected	Promoted ($p<0.001$)

Chapter 11

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APPENDICES

A. MATERIALS:

Sr.no.	Materials	Suppliers
1	Sodium hydroxide (NaOH)	BDH Chemicals Ltd
2	Calcium chloride dihydrate (CaCl2.2H2O)	BDH Chemicals Ltd
3	D-glucose	BDH Chemicals Ltd
4	Disodium hydrogen orthophosphate (Na2HPO4)	BDH Chemicals Ltd
5	Dichloromethane (CH2Cl2)	BDH Chemicals Ltd
6	Dimethyl sulphoxide (DMSO)	BDH Chemicals Ltd
7	Potassium chloride (KCl)	BDH Chemicals Ltd
8	Sodium chloride (NaCl)	BDH Chemicals Ltd
9	Hydrochloric acid (HCl)	BDH Chemicals Ltd
10	Sodium bicarbonate (NaHCO3)	BDH Chemicals Ltd
11	Magnesium sulphate (MgSO4)	BDH Chemicals Ltd
12	Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (Poole, UK)
13	Bovine serum albumin (BSA)	Sigma-Aldrich (Poole, UK)
14	Dextran T-70	Sigma-Aldrich (Poole, UK)
15	Bovine insulin	Sigma-Aldrich (Poole, UK)
16	Collagenase-V, Clostridium histolyticum	Sigma-Aldrich (Poole, UK)
17	Probenecid	Sigma-Aldrich (Poole, UK)
18	Ethylene glycol-bis-N, N, N’, N’-tetraacetic acid (EGTA)	Sigma-Aldrich (Poole, UK)
19	Thimerosal	Sigma-Aldrich (Poole, UK)

20	Trypan blue stain	Sigma-Aldrich (Poole, UK)
21	Activated charcoal	Sigma-Aldrich (Poole, UK)
22	HPLC grade acetonitrile	Sigma-Aldrich (Poole, UK)
23	HPLC grade ethanol	Sigma-Aldrich (Poole, UK)
24	Roswell Park Memorial Institute medium (RPMI 1640) tissue culture medium	Gibco Life Technologies Ltd (Paisley, Strathclyde, UK)
25	Hanks Buffered Saline Solution (HBSS)	Gibco Life Technologies Ltd (Paisley, Strathclyde, UK)
26	Trypsin/ Disodium ethylenediaminetetraacetate (EDTA)	Gibco Life Technologies Ltd (Paisley, Strathclyde, UK)
27	Foetal bovine serum (FBS)	Gibco Life Technologies Ltd (Paisley, Strathclyde, UK)
28	Penicillin and streptomycin	Gibco Life Technologies Ltd (Paisley, Strathclyde, UK)
29	Radiolabelled sodium iodide (Na125I)	Perkin Elmer (UK)
30	Enzyme-linked immunosorbent assay (ELISA) kits for Glucagon	Millipore (Millipore, Watford, UK).
31	Enzyme-linked immunosorbent assay (ELISA) kits for GLP-1	Millipore (Millipore, Watford, UK).
32	Glucagon RIA kit	Millipore (Millipore, Watford, UK).
33	(D-Ala^{2})GIP	SynPeptide (China)
34	Xenin-25[Lys^{13}PAL]	SynPeptide (China)
35	(D-Ser^{2})-Oxm[Lys^{38}PAL]	SynPeptide (China)
36	Exendin-4	SynPeptide (China)

37	Liraglutide	SynPeptide (China)
38	Sitagliptin	APExBIO (Huoston)
39	Dapagliflozin	AdooQ (US)
40	Taurine	Sigma-Aldrich (Poole, UK)
41	Nicotinamide	Sigma-Aldrich (Poole, UK)
42	Streptozotocin	Sigma-Aldrich (Poole, UK)
43	Artemether	Tokyo chemical industry (TCI) UK
44	GABA	Tokyo chemical industry (TCI) UK
45	Rosiglitazone	Tokyo chemical industry (TCI) UK
46	Metformin	Tokyo chemical industry (TCI) UK
47	Tolbutamide	Sigma-Aldrich (Poole, UK)
48	Isoflurane	Zoetis (UK)

Purified water (18.2 MΩ-cm purity) used in the experiments was obtained from an Elga PURELAB Ultra system (Elga, Celbridge, Ireland).

B. SUPPLEMENTARY MATERIAL

The detailed information on the generation of $Glu^{CreERT2}$; ROSA26-eYFP provided by Cambridge collaborator Professor Frank Reimann:

B.1 Creation of proglucagon-promoter driven Cre-recombinase expressing $Glu^{CreERT2}$;ROSA26-eYFP transgenic mice

To express the tamoxifen inducible *Cre*-recombinase *iCreERT2* under the control of the proglucagon promoter we replaced the sequence between the proglucagon start codon in exon 2 and stop codon in exon 6 in the murine based BAC RP23-343C17 (Children's Hospital Oakland Research Institute, Oakland, CA, USA) by the *iCreERT2* sequence (isolated from a pBS plasmid encoding ERT2-iCre-ERT2 sequence (Casanova et al, 2002) a kind gift by Rolf Sprengel, Max Planck Institute for Medical Research, Heidelberg, Germany) using Red/ET recombination technology (Genebridges, Heidelberg, Germany). Briefly, *iCreERT2* sequence was amplified by PCR adding proglucagon gene specific 3' and 5' sequences (see oligonucleotides tabulated below) and homologous recombination was achieved upon co-transforming an rpsLneo-modified BAC (Reimann et al, 2008) containing E.coli DH10B clone with the PCR product and the plasmid pSC101-BAD-gbaA, which provides the recombination enzymes (Genebridges). Positive recombinants were isolated using appropriate antibiotic selection and characterised by PCR and restriction analysis. Identity and correct positioning of the introduced *iCre* sequence was confirmed by direct sequencing using primers given in supplementary Table1. BAC-DNA for microinjection was purified using the large-construct Maxi-Prep kit (Qiagen) and dissolved at ~ 1-2 ng/µl in injection buffer containing (mmol/l): 10 Tris-HCl pH 7.5, 0.1 EDTA, 100 NaCl, 0.03 spermine, 0.07 spermidine. Pronuclear injection into ova

derived from C57B6/CBA F1 parents and reimplantation of embryos into pseudopregnant females was performed by the Central Biomedical Services at Cambridge University. DNA of pups was isolated from ear clips by proteinase K digestion and screened for the transgene by PCR using the following primer pairs: Ert2001/mGLP005, iCre002/003 (and RM41/42, which amplifies *β-catenin* sequence used as a DNA quality control). Transgene copy number was estimated by RT-PCR comparing CT numbers for a transgene specific probe (iCre-004, 005 and -probe) and Kir6.2 (Kcnj11-forw, -rev and -probe). Initially two transgenic strains were established (estimated transgene copy number): GLU-CreERT2-09 (1), Glu-CreERT2-017 (1). Initial characterization did not reveal any differences and a more in depth characterization was performed on GLU-CreERT2-09 (see below), which is the strain used in this study and available on reasonable request.

Supplementary Table 1:

Name	Sequence
iCre002	GAC AGG CAG GCC TTC TCT GAA
iCre003	CTT CTC CAC ACC AGC TGT GGA
iCre004	GCC GAA ATT GCC AGA ATC AG
iCre005	CAA TGT GGA TCA GCA TTC TCC
iCre-probe	6-FAM-TGA AGG ACA TCT CCC GCA CCG-TAMRA
mGLP005	CAT CTG CAT GCA AAG CAA TAT AGC
GLU008	AAT TGA GCT CAT TTG GAC TGC C
RM41	AAG GTA GAG TGA TGA AAG TTG TT
RM42	CAC CAT GTC CTC TGT CTA TTC
mKcnj11-fw	CCC GCT TCG TGT CCA AGA
mKcnj11-rev	CAG CGT GGT GAA CAC ATC CT
mKcnj11-probe	6-FAM-CAA CGT CGC CCA CAA GAA CAT TCG A-BHQ-1
mGLUCre003	TGC TCC CCC ATC ACC CCC TAC CCA CCC CCA TTC TGT GTT CCA TCA GGC AGA AAA AAA ATC CAC CAT GTC CAA CCT GCT GAC TGT GCA C
mGLUCre004	TAC ATC CCA AGT GAC TGG CAC GAG ATG TTG TGA AGA TGG TTG TGA ATG GTG AAA TAC CTA AGC TGT GGC AGG GAA ACC C
ERT2-001	CCA CCT TCT AGA ATG TGC CTG
ERT2-002	GTG GTT CCT GTC CAA GAG CA

Oligonucleotides used to create and verify GLU-CreERT2 mice

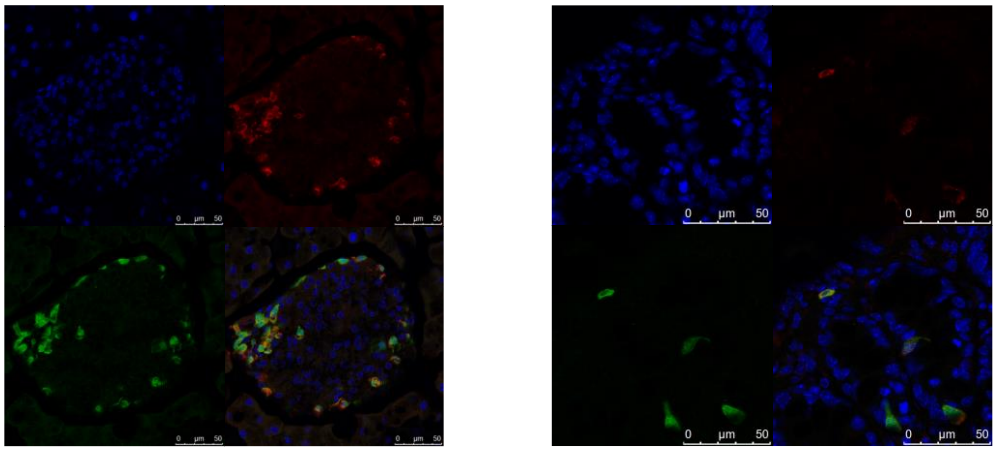
B.2 Characterisation of GLU-CreERT2-009:

GLU-CreERT2-09 mice were crossed with Rosa26-EYFP mice (derived from B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J from Jacksons). Double positive off-springs were subcutaneously injected with 400 µl tamoxifen dissolved at 20 mg/ml in corn oil. Pilot experiments indicated that this dosing regiment results in maximal induction 7 days after injection. Mice were sacrificed 7 days after the injection and pancreas and intestinal sections (duodenum, colon) were isolated into ice-cold L15 medium, intestinal contents were removed and tissues fixed in 4% paraformaldehyde in PBS over night at 4ºC. Tissues were dehydrated successively in 15% (5-6 hrs) and 30% sucrose (over night) in PBS and mounted in Optimal cutting temperature (OCT) compound, with the intestinal tissues rolled up in a "swiss-roll" with the epithelium facing inwards and stored at -80ºC. Tissue slices (~8 µm) were produced with a Cryostat, mounted on poly-lysine coated microscope slides and air dried. Slides were washed and blocked with 1% BSA and 5% donkey serum in PBS and then incubated with primary antibodies in this solution with the addition of 0.05% Tween20 at room temperature over night. Secondary antibody (1:300) and Hoechst (Sigma 14533 dissolved 5mg/ml in dH_2O; 1:1300) incubation was performed in the same medium for 1 h after at least 4 washed in antibody free medium, also performed after secondary antibody incubation and slices were finally mounted in ProLong Gold (Life Technologies). Antibodies used: Primary antibodies: Proglucagon antibody (FL-180, Sc-13091, raised in rabbit, Santa Cruz; 1:200), GFP antibody (Ab 5450, raised in goat, Abcam, 1:1,000). Secondary antibodies: Donkey anti-goat Alexa Fluor 488 (A11055, Life Technologies, 1:300); Donkey anti-rabbit Alexa Fluor 546 (A10040, Life Technologies; 1:300).

Images were taken with a confocal microscope (LeicaSP8) and (co-)localization of GFP- and Gcg-staining, selecting cells on the basis of one or the other, was performed non-blinded. GLU-CreERT2 mice showed good selectivity upon induction (>90% of EYFP labeled cells in the pancreas and colon also stained for proglucagon) with slightly lower ~80% selectivity in the duodenum (Suppl. Figure 1, Suppl. Table 2). The penetrance also reached >90% in the pancreas, but in the large (colon) and small (duodenum) intestine this was notably lower, with only ~70% and 40% of proglucagon positive cells showing successful reporter recombination (Suppl. Table 2). We also assessed the leakiness of the inducible transgene in the absence of tamoxifen induction. As we previously reported labelling of intestinal cells not actively expressing proglucagon in our non-inducible GLU-iCre mouse model (Zac-Varghese et al, 2014), we included deliberately older mice in this analysis. In the intestine EYFP positive cells were only seen very sporadically in the absence of tamoxifen induction; the majority of spontaneously labelled cells was also proglucagon positive (Suppl. Table 3). This applied also to the pancreas, however, higher % age of pancreatic α-cells were labelled even in the absence of induction with clear variability between animals. In summary, the GLU-iCreERT2 model created here compares favorably to our previously described GLU-iCre model, which with age accumulated proglucagon-negative cells in the intestine, possibly through lineage tracing (Zac-Varghese et al, 2014) and is comparable to GLU-CreERT2 models created by others (Ackermann et al, 2017; Shiota et al, 2017).

Supplementary Figure 1:

a) b)



Example images after tamoxifen induction

GLU-CreERT2 x Rosa26EYFP mice were treated 7 days s/c with tamoxifen as described above and pancreas (a) and large intestine (b) were stained for GFP (green) and proglucagon (red). Blue = DAPI stain to identify cellular nuclei. Confocal images with scale bars as indicated.

Supplementary Table 2:

IHC Results - Line 9 (induced)									
	PANCREAS			COLON			DUODENUM		
ID	**3199**	**3625**	**4198**	**3199**	**3625**	**4198**	**3199**	**3625**	**4198**
(age at harvest in weeks)	10.5	11.5	10.5	10.5	11.5	10.5	10.5	11.5	10.5
No of GFP+ve cells	399	474	301	179	232	97	67	62	45
No of GFP+ve cells that are GCG+ve	399	463	299	179	203	88	52	46	36
%GFP+ve cells that are GCG+ve	**100.0**	**97.7**	**99.3**	**100.0**	**87.5**	**90.7**	**77.6**	**74.2**	**80.0**
	Mean	**99**		**Mean**	**93**		**Mean**	**77**	
No of GCG+ve cells	418	571	310	252	300	122	100	144	86
No of GCG+ve cells that are GFP+ve	399	463	299	179	203	88	52	46	36
%GCG+ve cells that are GFP+ve	**95.5**	**81.1**	**96.5**	**71.0**	**67.7**	**72.1**	**52.0**	**31.9**	**41.9**
	Mean	**91**		**Mean**	**70**		**Mean**	**42**	

Immunohistochemical characterization of GLU-CreERT2-009 mice after tamoxifen induction.

Supplementary Table 3:

IHC Results - Line 9 (uninduced)									
	PANCREAS			COLON			DUODENUM		
ID	4305	4306	8156	4305	4306	8156	4906	4907	8156
(age at harvest in weeks)	46	46	14	46	46	14	41	41	14
No of GFP+ve cells	34	164	10	4	1	6	0	1	1
No of GFP+ve cells that are GCG+ve	33	164	10	4	1	3	0	1	0
%GFP+ve cells that are GCG+ve	**97.1**	**100**	**100**	**100**	**100**	**50**	**100**	**100**	**0**
	Mean	**99**		**Mean**	**83**		**Mean**	67	
No of GCG+ve cells	205	415	104	100	101	82	87	66	91
No of GCG+ve cells that are GFP+ve	33	164	10	4	1	3	0	1	0
%GCG+ve cells that are GFP+ve	**16.1**	**39.5**	**9.6**	**4.0**	**1.0**	**3.7**	**0**	**1.5**	**0.0**
	Mean	**22**		**Mean**	**3**		**Mean**	**1**	

Immunohistochemical characterization of GLU-CreERT2-009 mice without tamoxifen induction.

B.3 Supplementary References:

1. Casanova, E. *et al* (2002) ER-based double iCre fusion protein allows partial recombination in forebrain. *Genesis* **34** 208-214.

2. Reimann, F. *et al* (2008) Glucose Sensing in L Cells: A Primary Cell Study. *Cell Metabolism* **8** 532-539.

3. Zac-Varghese, S. *et al* (2014) The Peutz-Jeghers kinase LKB1 suppresses polyp growth from intestinal cells of a proglucagon-expressing lineage in mice. *Dis Model Mech* **7** 1275-1286.

4. Ackermann AM, Zhang J, Heller A, Briker A & Kaestner KH (2017) High-fidelity Glucagon-CreER mouse line generated by CRISPR-Cas9 assisted gene targeting. *Mol Metab* **6** 236-244.

5. Shiota, C. *et al* (2017) $Gcg^{CreERT2}$ knockin mice as a tool for genetic manipulation in pancreatic alpha cells. *Diabetologia* **60** 2399-2408.

6. Campbell JR, Martchenko A, Sweeney ME, Maalouf M, Psichas A, Gribble FM, Reimann F, Brubaker PL (2019) Essential role of munc18-1 in the regulation of glucagon-like peptide-1 secretion. *Oxford Academic (Currently under review).*