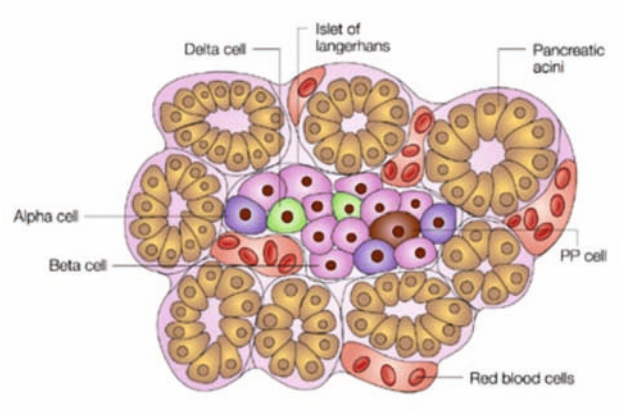
Dipeptidyl Peptidase IV inhibition activates CREB and improves islet vascularization through the VEGF-A/VEGFR-2 pathway

BALAJI SAMIKANNU



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der Justus-Liebig-Universität Giessen

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Dedicated to my beloved father and my family ....

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Abbreviations

Glp-1- Glucagon like Peptide-1

EGF- Epidermal Growth Factor

NOD- Non obese Diabetic

GIP- Glucose- dependent insulinotropic peptide

cAMP- Cyclic adenosine monophosphate

CREB- cAMP Response element binding

CBP- CREB- Binding Protein

PDX-1- pancreatic and duodenal homeobox gene-1

PKA- Protein Kinase A

INS-1 – Insulinoma cell line

IFN-γ- Interferon gamma

GLP-1R- Glucagon Like Peptide Receptor-1

GPCR- G-protein coupled Receptor

GHRH- Growth hormone releasing hormone

DPP-IV- Dipeptidyl Peptidase IV

CD26- Cluster of Differentiation 26

IDDM- Insulin dependent diabetes mellitus

VEGF- Vascular Endothelial Growth factor

REG- Regenerating Gene

PDGF- Platelet-derived growth factor

FGF- Fibroblast growth factor

HIF-1 α- Hypoxia Inducible Factor-1 α

VEGFR2- Vascular Endothelial Growth Factor receptor 2

PGC-1 α -Peroxisome proliferator-activated receptor gamma coactivator-1 alpha

BrdU- Bromo-de-oxyuridine

IPGTT- Intraperitoneal Glucose tolerance test

ELISA- Enzyme Linked Immunosorbant Assay

PBS- Phosphate Buffered Saline

RIP- Rat Insulin Promoter

FCS- Fetal Calf Serum

Summary

Substitution of pancreatic islets is a potential therapy to treat diabetes and it depends on reconstitution of islet's capillary network. In this study, we addressed the question whether stabilization of Glucagon-Like-Peptide-1 (GLP-1) by inhibiting Dipeptidyl Peptidase-IV (DPP-IV) increases β-cell mass by modulating vascularization.

Mouse or porcine donor islets were implanted under kidney capsule of diabetic mice treated with DPP-IV inhibitor, sitagliptin. Grafts were analyzed for insulin production, β-cell proliferation and vascularization. In addition, the effect of sitagliptin on sprouting and Vascular Endothelial Growth Factor (VEGF)-A expression was examined *ex vivo*. The cAMP response element-binding (CREB) and VEGF-A/ Vascular Endothelial Growth Factor Receptor (VEGFR)-2 signaling pathway leading to islet vascularization was explored.

Sitagliptin increased mean insulin content of islet grafts and area of insulin-positive tissue as well as β-cell proliferation. Interestingly, sitagliptin treatment also markedly increased endothelial cell proliferation, microvessel density and blood flow. Finally, GLP-1 (7-36) stimulated sprouting and VEGF expression, which was significantly enhanced by sitagliptin- mediated inhibition of DPP-IV. Our *in vivo* data demonstrate that sitagliptin treatment phosphorylated CREB and induced islet vascularization through VEGF-A/VEGFR-2 signaling pathway. This study paves a new pathway for improvement of islet transplantation in treating diabetes mellitus.

Zusammenfassung

Der Ersatz von Langerhansschen Inselzellen stellt eine Therapiemöglichkeit des Diabetes Mellitus dar und hängt von der Wiederherstellung der Kapillarnetze der Inseln ab. In dieser Arbeit wird geprüft, ob eine Stabilisierung des Glucagon-Like-Peptide-1 (GLP-1) durch Inhibition der Dipeptidylpeptidase-IV (DPP-IV) eine Vergrößerung der Betazellmasse durch Veränderung der Vaskularisierung hervorruft.

Von Mäusen oder Schweinen stammende Inseln wurden unter die Nierenkapsel von diabetischen Mäusen transplantiert. Diese Mäuse wurden mit dem DPP-IV-Inhibitor Sitagliptin behandelt. Die Transplantate wurden auf ihre Insulinproduktion, Betazellproliferation und Vaskularisierung untersucht. Zusätzlich analysiert wurden der Effekt des Sitagliptins auf die Angiogenese sowie die Expression von Vascular Endothelial Growth Factor (VEGF)-A. Die cAMP response element-binding (CREB) und VEGF-A/Vascular Endothelial Growth Factor Receptor (VEGFR)-2-Signalwege waren darüber hinaus Objekt der Forschung.

Sitagliptin führte zu einer Erhöhung des mittleren Insulingehalts der Transplantate, zu einer flächenmäßigen Vergrößerung des Insulin-positiven Gewebes und zu Betazellproliferation. Bemerkenswerterweise erhöhten sich durch die Behandlung mit Sitagliptin auch die Proliferation der Endothelzellen, die Kapillardichte und der Blutfluss. Schließlich wirkte GLP-1 stimulierend auf Angiogenese und VEGF-Expression, welche aufgrund der Inhibition von DPP IV durch GLP-1 signifikant verbessert wurden.

Unsere in vivo gesammelten Daten demonstrieren, dass eine Behandlung mit Sitagliptin CREB phosporyliert und eine vermehrte Vaskularisierung von Langerhansschen Inseln durch den VEGF-A/VEGFR-2-Signalweg induziert. Diese Arbeit ebnet einen neuen Weg zu einer Verbesserung der Inseltransplantation in der Behandlung des Diabetes Mellitus.

1. Introduction

1.1 Pancreas

1.1.1Pancreas anatomy

The pancreas serves two major functions: (i) the production of digestive enzymes, which are secreted by exocrine acinar cells and routed to the intestine by a branched ductal network; and (ii) the regulation of blood sugar, which is achieved by endocrine cells of the islets of Langerhans (1). The pancreas is often described as two organs in one, due to the distinct function and organization of its endocrine and exocrine components. In higher vertebrates, it might more properly be thought of as four organs, as it comprises anatomically distinct dorsal and ventral lobes. Referred to in humans as the tail and head, respectively, these two pancreatic lobes arise as thickenings along the dorsal and ventral surfaces of the posterior foregut, near the prospective hepatic endoderm (1).

The bulk of the mature pancreas is comprised of acinar cells, connected to the intestine via a highly branched ductal tree, while islets are primarily scattered through the central regions of the organ. Several separate endocrine cell types comprise the islet: β -cells are the most prominent (50-80% of the total, depending on species) (2; 3) and tend to segregate to the islet core, with other cell types arranged closer to the mantle.

1.1.2 Beta cell Biology

The islets of Langerhans, discovered by a medical student 121 years ago, occupy a pivotal position in the endocrine control of fuel metabolism. The islets of Langerhans, taken together, can be thought of as a single organ occupying - 1% of the pancreas. Islets have a central core of insulin-containing β-cells and a surrounding mantle of glucagon-containing α-cells, somatostatin containing δ-cells, and pancreatic polypeptide-containing PP cells (4). The largest portion of the pancreas, the dorsal lobe, is derived from the embryonic dorsal anlage, but a smaller portion, the ventral lobe making up the inferior aspect of the head, is derived

from the ventral anlage. The islets of the dorsal lobe have mantles, which contain mostly α-cells and δ-cells, whereas the islets of the ventral lobe have mantles that consist mostly of PP and δ-cells. Islets are innervated by sympathetic nerves, parasympathetic nerves, and the poorly understood peptidergic nerves (5).

Islets are richly vascularized by direct arteriolar blood flow. Studies with microspheres have shown that islets, which make up only 1% of the mass of the pancreas, receive at least 10 % of the blood flow (6). Small arterioles enter the β-cell-containing core of the islet through discontinuities or pores of the mantle, and then break into a glomerular-like network of fenestrated capillaries which coalesce into collecting venules either during or after leaving the islet (7).

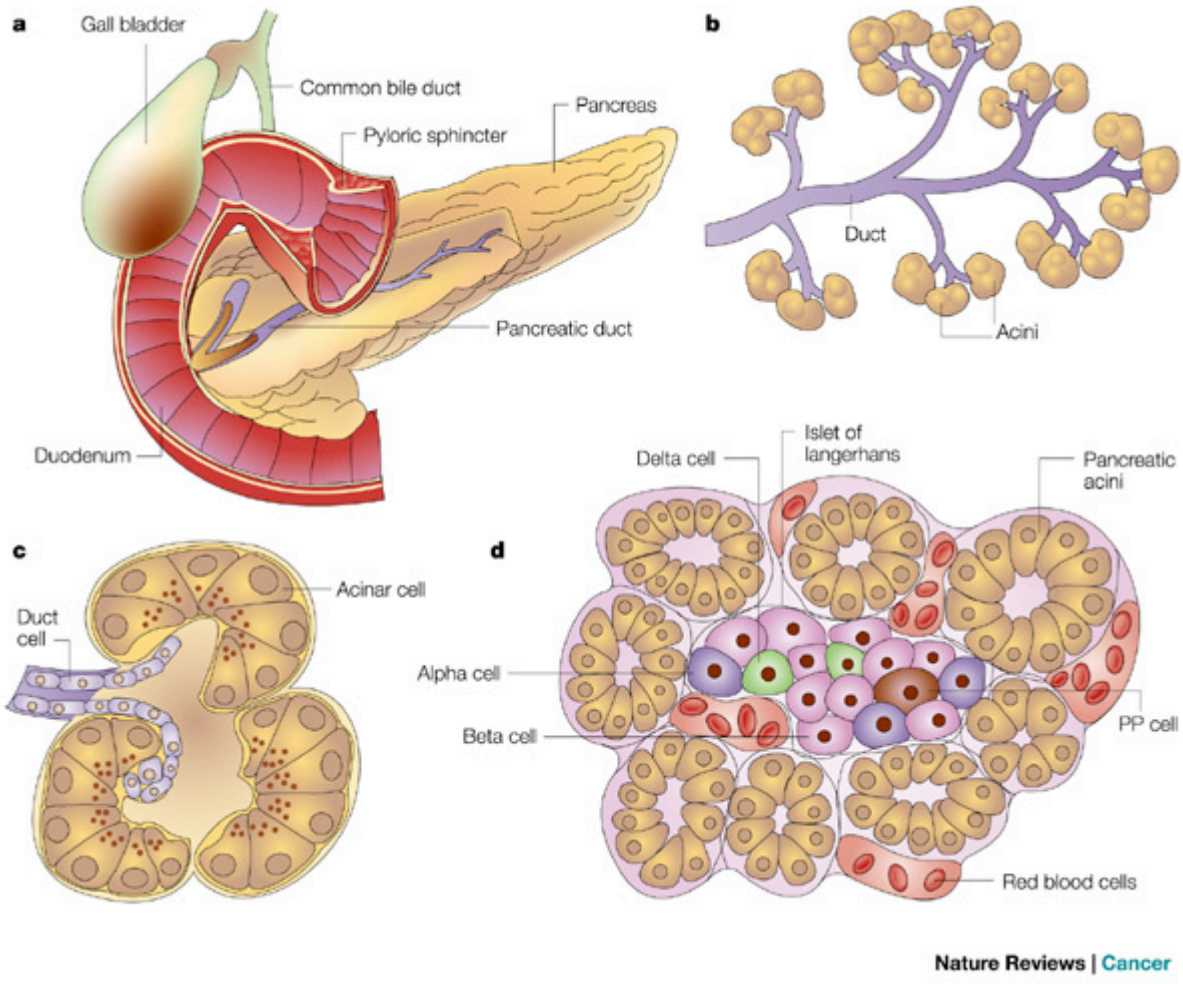

Fig 1-1 Anatomy of Pancreas a. Gross anatomy of the pancreas; **b**. The exocrine pancreas; **c**. A single acinus; **d**. A pancreatic islet embedded in exocrine tissue.

The microvasculature of the core has a unique relationship with β-cells, which are now known to be polarized- with one domain facing an arterial capillary and another facing a venous capillary (8). The β-cells resembles other epithelial cells, having a basolateral portion and an apical portion; the latter contains relatively more insulin secretory granules. In addition, there are canaliculi, which are located between the lateral surfaces of β-cells, and span the distance from the arterial to the venous domains. These canaliculi contain large numbers of microvilli, which have been found to be enriched in the recently described β-cell glucose transporter (9). The presence of these elaborate structures suggests that interstitial fluid flows through these canaliculi in an arterial to venous direction and that glucose uptake of β-cells is carried out primarily by the transporters on these microvilli. Thus, these canaliculi may serve as the initial interface for glucose sensing by the β-cells. Through these glucose transporters extracellular glucose rapidly equilibrates with cytosolic glucose and is phosphorylated, with glucokinase serving a key rate-limiting function; glucose metabolism is tightly linked to the rate of insulin secretion (10).

1.2 Pancreas Regeneration

Regeneration of the endocrine pancreas has been studied in different experimental models that differ in the extent and selectivity of the tissue injury that is inflicted. The two major types of injury are caused by toxic drugs and surgery, respectively (11).

1.2.1 Toxic Injury

Selective destruction of beta cells can be obtained by injecting streptozotocin or alloxan. Streptozotocin is a DNA-alkylating agent and alloxan a generator of oxygen free radicals, both causing extensive DNA damage (12) . The capacity to regenerate following a toxic insult like streptozotocin rapidly declines during the first 5 days of life in rats (13). This indicates that beta-cell regeneration beyond the critical perinatal time window is inefficient, possibly because there is no more neogenesis operating from precursor cells. Interestingly, when the

hormone Glp-1 is administered to streptozotocin-treated newborn rats, beta-cell neogenesis is stimulated, and this results in improved glucose homeostasis persisting at adult age (14). In a recently described experimental model of β-cell destruction by alloxan, the combination of two factors, gastrin and EGF, were found to restore glycemic control in mice (15). In this model of beta-cell regeneration, a beta-cell growth rate of 30% per day was observed, leading to a beta-cell population doubling time of only 3 days. Although there was no complete regeneration of the original β-cell number after subtotal destruction, regenerative growth induced by the gastrin and EGF treatment led to the restoration of 30–40% of the normal beta-cell mass within 7 days. The treatment had no effect on beta-cell replication, cell size, or apoptosis, and therefore, the regenerative effect could be attributed to neogenesis from precursor cells. Indeed, a pulse chase labeling with the thymidine analog bromo de-oxyuridine confirmed an influx of labeled cells from a replicating insulin-negative pool into the regenerating islets. The proliferating precursor cells express ductal-type cytokeratin markers (15). In NOD mice, beta cells are destroyed by a spontaneous autoimmune reaction leading to a type 1-like diabetes condition. Immune suppression in combination with Glp-1 (analog) treatment could restore normoglycemia and improve islet histology (16).

1.2.2 Surgical Injury

Partial pancreatectomy represents another model of tissue injury wherein regeneration has been studied in rodents. Surgical removal of part of the pancreas is followed only by a limited regenerative growth, and there is never a complete restoration of the original pancreatic volume. Furthermore, the regenerative response is proportional to the amount of pancreas removed. If half the pancreatic volume is left after hemipancreatectomy, this residual pancreas will grow only 20%. In the case of one-third of the volume being left after two-thirds pancreatectomy, the residual pancreas grows only 30%. When only one-tenth of the volume is left after subtotal pancreatectomy, the residual pancreatic volume grows 80% (17). Glp-1 receptor knockout mice showed worse glucose intolerance after partial pancreatectomy

compared with wild-type mice, and this correlated with a significant defect in β-cell mass regeneration (18). An experimental model that leads to robust hyperplasia of β-cells is partial duct ligation. This consists in the closure, or ligation, with surgical thread of part of the main duct of the pancreas. As a consequence of this obstruction, exocrine secretory products will leak into the interstitial space and lead to tissue damage and inflammation. The part of the pancreas that lies downstream of the ligation (~50% of the pancreatic volume) is not affected histologically and continues to function normally. During the first week post ligation, a pronounced β-cell hyperplasia occurs in the ligated part, although the animals remain normoglycemic. β-cell replication is only slightly elevated, which is strongly suggesting that the increased β-cell number results from self-renewal by the β-cells. This is also indirectly indicated by the observation of intermediate phenotypes between ductal and endocrine cells (19).

1.3 Incretin Hormones

Incretin hormones are released from enteroendocrine cells from the gut and are secreted into the blood stream when food containing glucose, fat or protein hydrolysate enters the duodenum. Glucose-dependent insulinotropic peptide (GIP, also referred to as gastric inhibitory polypeptide) and glucagon-like peptide 1 (GLP-1) are the main incretin hormones (20; 21). GLP-1 is produced in the L-cells, which are located in high concentration in the distal intestine, whereas GIP arises in the K-cells, which are found predominantly in the more proximal intestine (22).

1.3.1 Glucagon Like Peptide-1 (GLP-1)

The 30-amino acid peptide hormone, glucagon-like peptide 1, is a product of the glucagon gene. This gene is expressed, not only in the A cells of the pancreas, but also in the endocrine L cells of the gut mucosa (23; 24). The L cells are at least as numerous as are A cells in the pancreas. However, the outcome of glucagon gene expression in the gut differs completely

from that of the pancreas. The primary translation product, proglucagon, a peptide of 160 amino acids, contains, apart from the glucagon sequence, two glucagon-like sequences designated GLP-1 and GLP-2. They are glucagon-like because, with respect to amino acid sequence, they are about 50% homologous to glucagon. When the prohormone is processed in the pancreas, the glucagon sequence is cleaved out, whereas the part containing the GLP is secreted as a single, large peptide (25; 26). In the gut, the glucagon sequence remains in a larger, presumably inactive, peptide designated glicentin, while the two glucagon-like peptides are cleaved out and secreted separately, in parallel with glicentin (25). Glicentin and the two glucagon like peptides are secreted in parallel, so results from investigations of enteroglucagon secretion might still, to some extent, be applicable to GLP physiology. Glicentin might be processed further, leading to the formation of an inactive N-terminal fragment and oxyntomodulin, the latter of which consists of the entire glucagon sequence and the C-terminal extension also found in glicentin. This molecule possesses some of the biological actions of glucagon (27), but is generally less potent and usually present in concentrations that are lower than those required for biological effects (28).

GLP-1 is normally secreted in response to meal ingestion (29), with lipids and carbohydrates most active in stimulating secretion (30). The L cell is an open type endocrine cell with apical microvilli reaching the gut lumen, suggesting that the luminal concentration of lipids and carbohydrates might regulate L cell secretion directly. Meal responses occur rapidly (between 5 and 30 min) after initiation of the meal, seemingly in contradiction to the predominantly distal location of the L cells within the gut. This has led to speculations that neural or endocrine mechanisms could play a role in initiating GLP-1 secretion. However, in support of a major role for luminal factors with respect to the meal response, luminal perfusions with lipids or carbohydrates have been demonstrated to elicit GLP-1 secretion (30). In addition, the meal response strongly correlated with the gastric emptying rate (31), indicating that it is the

rate of exposure of the gut mucosa to nutrients that determines the magnitude of the GLP-1 response (32).

1.3.2 Actions of GLP-1

GLP-1 is a potent direct stimulator of insulin and somatostatin secretion from β- and δ-cells, respectively, and suppresses glucagon secretion from α-cells, either directly or indirectly, by the paracrine suppressive actions of insulin (33). The actions of GLP-1 to stimulate insulin secretion from β-cells are directly dependent on the glucose concentrations. The effectiveness of GLP-1 as an insulin secretagogue increases as the glucose levels rise and is attenuated as glucose levels fall (34) . This important property of GLP-1, and the other incretins such as GIP, to auto regulate the potencies of their actions on augmenting insulin secretion in step with ambient glucose concentrations provides a means to protect against hypoglycemia. A cellular mechanism in β-cells to explain this interdependence between glucose and GLP-1 actions is described below and involves a synergetic cross-talk between the glycolysis (glucose metabolism) and cyclic adenosine monophosphate (cAMP) signaling pathways. This mutual interdependence between glucose metabolism and GLP-1 actions on β-cells is referred to as the glucose competence concept, that is, glucose is required for β-cells to respond to GLP-1, and GLP-1 (or other incretins) is required to render β-cells competent to respond to glucose (35). In addition to stimulating glucose-dependent insulin secretion, GLP-1 stimulates transcription of the insulin gene, proinsulin mRNA levels, insulin biosynthesis, and accumulation of cellular stores of insulin (36). GLP-1 stimulates the formation of cAMP and the cAMP signaling pathway stimulates transcription of the insulin gene by activating the DNA-binding transcription factor CREB (37), which binds to a key cAMP response element (CRE) located in the promoter of the insulin gene and thereby enhances the efficiency of transcription of the gene. The intracellular signaling cascade that mediates cAMP-induced effects on insulin gene transcription remains unclearly defined (38). Nuclear activity in response to GLP-1–mediated increases in cAMP includes recruitment of the CREB-binding

protein (CBP) that couples the protein–DNA complex. This complex consists of CREB bound to the CRE and CBP bound to CREB, resulting in enhancement of insulin gene transcription. Discrete from its effects on insulin gene expression via cAMP/CREB, GLP-1 also stimulates the recruitment of PDX-1 from the cytosol to the nucleus, leading to enhanced DNA binding and transcriptional activity of the insulin gene. Whereas translocation of PDX-1 was shown to be dependent on cAMP/protein kinase A (PKA) (39), the PDX-1 DNA binding activity was determined to be phosphatidylinositol (PI) 3-kinase dependent (40).

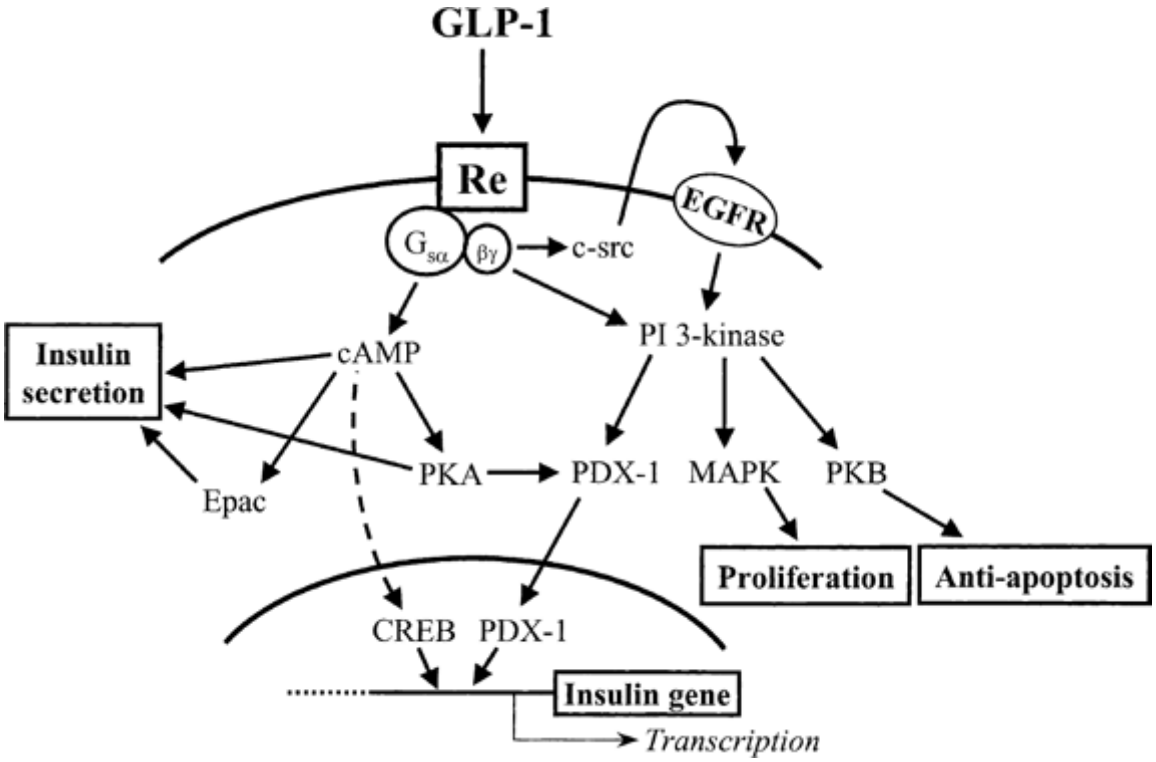

Fig1.2. Functional responses to glucagon-like peptide-1 (GLP-1) receptor signaling in β-cells.

1.3.3 GLP-1 and β cell proliferation

GLP-1 increases the expression level of the β-cell-specific transcription factor pancreatic and duodenal homeobox gene-1 (PDX-1) (36), which is implicated in the regulation of the expression of insulin, GLUT2, and glucokinase genes and in β-cell differentiation (41-43). In addition, the glucoincretin increases in vitro β-cell proliferation nonadditively with glucose via a phosphatidylinositol (PI) 3-kinase/protein kinase C ζ signaling pathway in β (INS-1)

cells (40; 44), as well as the islet mass in mouse pancreas in vivo (45). Finally, GLP-1 induces several immediate early response genes and proto-oncogenes in INS cells that are implicated in cell growth/apoptosis control, such as c-*fos*, c-*jun*, junD, and nur77 (46; 47). GLP-1 (10 nM) was shown to induce an increase in DNA synthesis as measured by tritiated thymidine incorporation in the INS832/13 insulinoma cell line and in rat pancreatic islets, following a 24-hr incubation (40). Exendin-4, a long-acting GLP-I agonist, stimulates both the differentiation of β-cells from ductal progenitor cells (neogenesis) and proliferation of β-cells when administered to rats in a partial pancreatectomy rat model of type 2 diabetes (48).

1.3.4 GLP-1 and β cell neogenesis

The de novo formation of islet cells in the adult pancreas and in particular β cell neogenesis is a controversial process with evidence indicating that it can occur, especially in the rodent and some recent evidence from transgenic mice showing otherwise (49). Many adult in vivo models have been used to demonstrate the potential for the ductular network to give rise to new β cells, including interferon gamma (IFN-γ) over expression, plastic wrapping of the pancreatic duct (in order to induce mild pancreatitis) and administration of gastrin and EGF (50).The ductal origin of β-cells from the insitu progenitors during acute injury had recently been controversial as cell lineage tracing studies concluded that the pancreatic ductal epithelium does not contribute to β-cell growth during acute regeneration(51)

1.3.5 Glucagon Like Peptide-1 Receptor

GLP-1R is a specific 7-transmembrane receptor guanine nucleotide-binding protein (G-protein) coupled receptor (GPCR). It was first cloned from rat pancreatic islets (52) and later from a human pancreatic insulinoma (53; 54) and a gut tumor cell line (55).The rat and human GLP-1R exhibit a 95% amino acid homology and are 90% identical (52; 54), differing at 42 amino acid positions (56). The human GLP-1R gene is located on the long arm of

chromosome 6p21 (57). GLP- 1R is a 64-kDa protein (58). GLP-1R is a member of the class B family consisting of many classical hormone receptors (59). Within class B, the receptors for the peptide hormones form a subclass of the glucagon receptor family, which also include receptors for glucagon, GLP-2, GIP, growth hormone releasing hormone (GHRH), and secretin (60-62).

1.3.6 GLP-1 inhibitor-Dipeptidyl Peptidase IV (DPP -IV)

DPP IV, a 110 KD cell surface glycoprotein, is a widely distributed serine protease that cleaves two amino acids from small peptides containing alanine or proline in the second position of the N-terminus of the peptide (63) .This enzyme, also known as the T-cell antigen CD26, is found in many locations including intestinal and renal brush-border membranes, on hepatocytes and capillary endothelial cells and in a soluble form in plasma (64). Although its specificity suggests a role of the metabolism of many endogenous peptides, it seems as though DPP IV activity is especially critical for inactivation of GLP-1. The *in vivo* N-terminal truncation of both endogenous and exogenous GLP-1 was likely to have a physiological role in mediating the rapid degradation of native GLP-1 (65; 66). The peptide has an apparent plasma half-life of only 1–2 minutes, with a metabolic clearance rate of 5–10 L/min, exceeding the cardiac output by a factor of 2 to 3 (67). In patients with type 2 diabetes, studies suggest a correlation between glycemic control and plasma DPP IV activity, but this does not seem to be correlated to the amount of intact biologically active hormone in plasma (68). Targeted disruption of the CD26 gene had increased levels of intact endogenous GLP-1 supporting the importance of DPP IV in GLP-1 metabolism (69). DPP IV inhibition was able to completely prevent the N-terminal degradation of native GLP-1 *in vivo* and this was also associated with enhancement of its insulinotropic effects (70; 71).

1.3.7 Dipeptidyl Peptidase Inhibitor

Dipeptidyl peptidase-IV inhibitors, that can block the DPP-IV enzyme, can increase the endogenous GLP-1 level and thus enhances the incretin action. DPP-IV inhibitors are novel oral glucose-lowering agents, which may be used as monotherapy or in combination with other antidiabetic compounds, metformin, thiazolidinediones or even sulfonylureas. Sitagliptin, vildagliptin and saxagliptin are already on the market, either as single agents or in fixed-dose combined formulations with metformin. Other compounds, such as alogliptin and linagliptin, are in a late phase of development (72). The proliferative and anti-apoptotic actions of GLP-1 on islet β-cells have been mirrored by studies using DPP-IV inhibitors in diabetic rodents. High-fat–fed mice treated with low-dose streptozotocin followed by 2–3 months of treatment with des-fluoro-sitagliptin showed improvement in fasting and postprandial hyperglycemia, increased pancreatic insulin content, and increased numbers of insulin-positive β-cells in association with normalization of β -cell mass and restoration toward normal of the β-cell: β-cell ratio (73).

1.4 Diabetes mellitus

Diabetes mellitus is a major health problem affecting more than 4% of the global population. There are two forms of the disease; type 1 diabetes results from autoimmune destruction of the β- cells of the islets of Langerhans and type 2 diabetes occurs as a result of a combination of reduced insulin sensitivity and impaired function of the insulin-secreting β-cells. The age of onset of type 2 diabetes is normally much later than for type 1, which occurs in childhood (74).

1.4.1 Type 1 Diabetes

Type 1 diabetes (Insulin dependent diabetes mellitus (IDDM) or Juvenile onset diabetes), is an autoimmune disease, where the islets are destroyed by immune system. The pathogenesis of the disease is attributed to both genetic and environmental trigger. In Europe the incidence of Type 1 diabetes is highest in the youngest age group. The rate of increase in incidence was

6.3%, 3.1% and 2.4% for children aged 0-5, 5-9 and10-14 years, respectively and the rapid increase in youngest age group is of concern for the policy makers (75).

1.4.2 Type 2 Diabetes

Type 2 diabetes is characterised by disorders of insulin resistance and insulin secretion. It is the most challenging health problems in the 21st century both in developed and developing countries, is among the leading causes of death and diabetic macro- and microvascular complications, resulting in increased disability and enormous health care costs. In the European Region, an average total diabetes prevalence of 7.8 % in the adult population (20 - 79 years) or 48.4 million persons has been estimated in 2003 (76).

1.4.3 Islet transplantation as a cure for Type 1 Diabetes

The patients suffering from Type 1 diabetes require exogenous insulin for the normalization of blood glucose level but the normalization of metabolic control cannot be achieved. Recent clinical trials and experimental models suggest that islet transplantation would be of potential use for type 1 diabetes. Owing to the introduction of better technology for the separation of islets and recent advances in immunosuppressive strategies, islet transplantation results have markedly improved. But major challenges remain to be addressed for the lifelong use of immunosuppressive drugs, which limits the indication for islet transplantation (77).

1.4.4 Islet transplantation and regeneration

Regeneration of islets was made possible by auto transplanting islets from the pancreatectomised mice and rats. These islet grafts had an increased blood flow due to improved vascular engraftment, which also increased the capacity of insulin secretion and larger endocrine volume. This was attributed to the release of survival signals such as VEGF, GLP-1 and REG (78). Islet transplantation after 70% partial pancreatectomy enhanced the β-cell mass and insulin content in the remnant pancreas resulting from increased β -cell proliferation/neogenesis and also due to prevention of β-cell apoptosis (79).

1.5 Islet vasculature

Islets contains a glomerulus-like micro vessel arrangement that is specific to islets alone and not observed in other tissues (80). Pancreatic islets are highly vascularized by a dense network of capillaries and are approximately five times denser in islets than in the surrounding exocrine tissue. Islets receive 7-10% of the total pancreatic blood flow, although they account of 1% of the total pancreatic mass (78; 81).

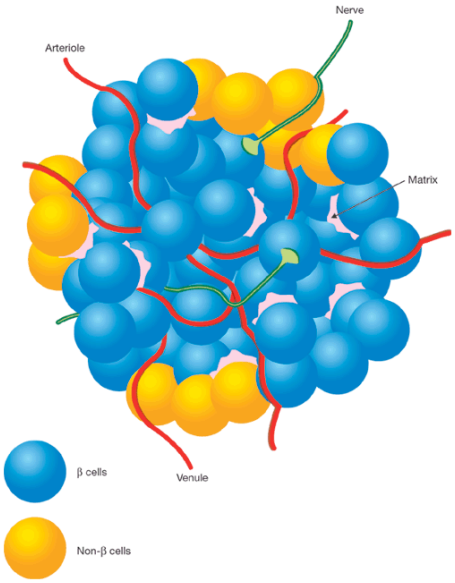

Fig 1-3. Three-dimensional architecture of an islet. Islets are richly irrigated by a well developed microvasculature and endocrine cells are in contact with extracellular matrix (presumably deposited, at least in part, by neighboring endothelial cells) as well as being selectively innervated. Adapted from Halban PA, 2004 (82)

The blood flow mechanism in islets is an inner-outer pattern, whereby the capillaries perfuse β-cells before other islet cell types (83). β-cells are closely associated with islet endothelial cells and are no more than one cell distant from the blood stream (84).

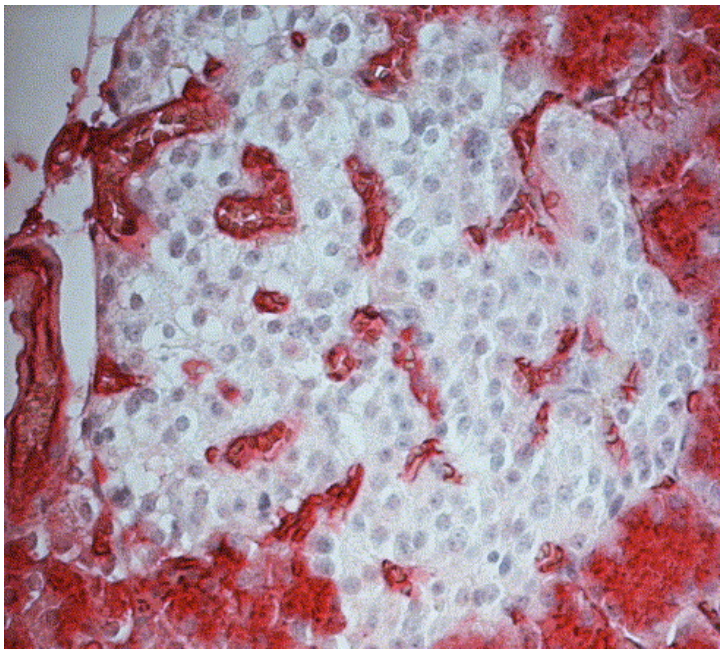

Fig1-4. Micrograph showing the rich vascularity of a human pancreatic islet. The micro vascular endothelium is visualized by staining with the lectin Bandeiraea simplicifolia (red). Adapted from Olsson, R, 2006 (84)

1.5.1 The Pancreatic islet endothelial cell

The endothelial cells and hematopoietic cells seem to derive from the same precursor cell, the haemangioblast. The islet endothelium functions as both a cellular barrier as well as allows rapid passage of proteins such as the endocrine hormones into the blood stream (84). The islet endothelial fenestrae are sites through which proteins can quickly permeate, which crucially depends on the VEGF-A secretion from the neighbouring β-cells (85). Several of the most important vasoactive substances such as nitric oxide (NO), angiotensin II and endothelin are produced or processed by the islet endothelial cells themselves (86). Several studies indicate that islet endothelial cells produce a number of factors involved in the regulation of angiogenesis, including both potent proangiogenic factors such as VEGF and angiostatic factors such as endostatin, pigment epithelial-derived factor and α_1-antitrypsin (87; 88).

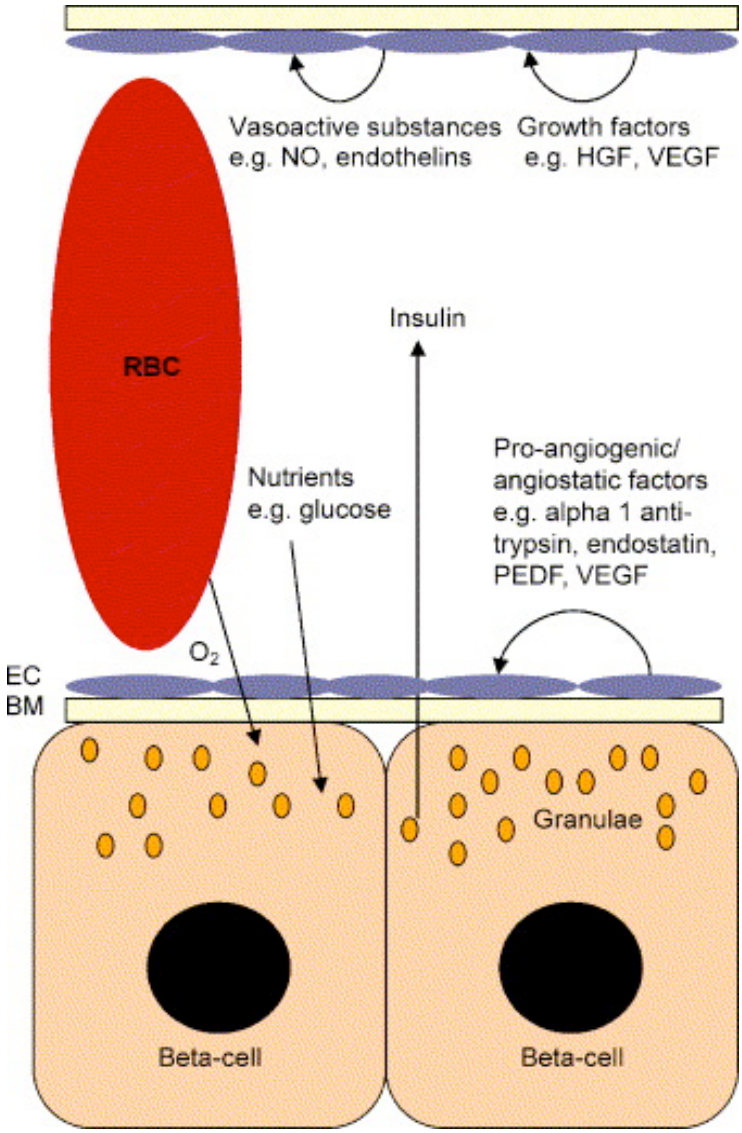

Fig 1-5. Schematic picture outlining some major functions of the islet endothelial cell. RBC, red blood cell; NO, nitric oxide; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; PEDF, pigment endothelial derived growth factor; EC, endothelial cell; BM, basal membrane. Adapted from Olsson R, 2006 (84).

1.5.2 Revascularization of Islet graft

Islet isolation often severe the microvasculature and damages the endothelial cells. Therefore the transplanted islets must re-establish a functional vascular network, by a process believed to involve angiogenesis and vasculogenesis (89). Revascularization of transplanted islets should be rapid and adequate for its survival and function or else it will be deprived of oxygen and nutrients, resulting in the islet cell death and early graft failure (90). On the contrary these

transplanted islets are less vascularized immediately after transplantation, and even several weeks thereby maintaining hypoxic conditions than the native islets (91). Until recently endothelial cells from the transplant recipient were thought to be the major contributor of angiogenesis but recent finding by using lac Z or GFP- tagged donor endothelial cells, suggest that the donor endothelial cells also play an important role in islet vascularization (92-94).

1.5.3 Proangiogenic factors in the revascularization of islet graft

Although there are several proangiogenic factors involved in the revascularization of islet graft, factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), Angiopoietin-2 and Platelet-derived growth factor-B(PDGF-B) promote the vascularization of islet grafts (95; 96). VEGF is also expressed in the pancreatic islets, but its expression in transplanted islets is significantly reduced 2–3 days post transplantation (94).

1.5.4 Angiogenesis of islet engraftment

Vasculogenesis and angiogenesis are the two mechanisms for the formation of new blood vessels. Vasculogenesis is the formation of new blood vessels from angioblasts that occurs in embryo where as angiogenesis is the formation of new blood vessels from pre-existing blood vessels which occurs in adult. Angiogenesis is further divided into vascular sprouting and intussusception. Vascular sprouting is the elongation/outgrowth of a pre-existing vessel and intussusception is the formation of new blood vessels by the ingrowths' of transcapillary tissue pillars into existing blood vessels, which increases the vascular density (97). Isolated pancreatic islets are avascular and they depend on the neo-vasculation of microvasculature to re-establish nutritional blood supply. This condition leads to prolonged hypoxia as long as the islets are dependent on the diffusion of oxygen and nutrients from surrounding tissues. In the islet transplantation model, the islets are exposed to hypoxia immediately after transplantation in the portal as well as under the kidney capsule (98; 99).

1.5.5 The HIF-1α system

The HIF system is activated in hypoxic cells to induce angiogenesis. HIF-1 is an αβ heterodimeric transcription factor of which the HIF-1β subunit is constitutively produced whereas the HIF-1α subunit is produced upon induction by hypoxia. The HIF-1α is hydroxylated and thus the transcription activity is inhibited, meaning that the HIF-1 heterodimer is formed during hypoxic conditions. But the cellular threshold for the activation of HIF-1 system seems to differ between tissues. Following the formation of HIF-1αβ heterodimer, it interacts with the hypoxic response elements to induce the transcription of pro-angiogenic factors such as VEGF, VEGFR2 and NOS. Furthermore, it also induces the expression of several pro and anti-angiogenic factors (100). Angiogenesis is induced when tissue produces more pro-angiogenic factors than anti-angiogenic factors (97).

1.5.6 HIF-1α independent regulation of VEGF and Angiogenesis

PGC-1 is a nuclear protein of 90 kDa containing SR domains and an RNA-binding motif characteristic of certain splicing factors (101). PGC-1α is a powerful modulator of oxidative metabolism whereby it regulates the oxidative metabolism, mitochondrial biogenesis and respiration. PGC-1α along with the ERR-α, is a mediator of signalling in response to deprivation of nutrients and oxygen, and that it powerfully regulates *VEGF* and other angiogenic factors to elicit neovascularization *in vivo* (102).

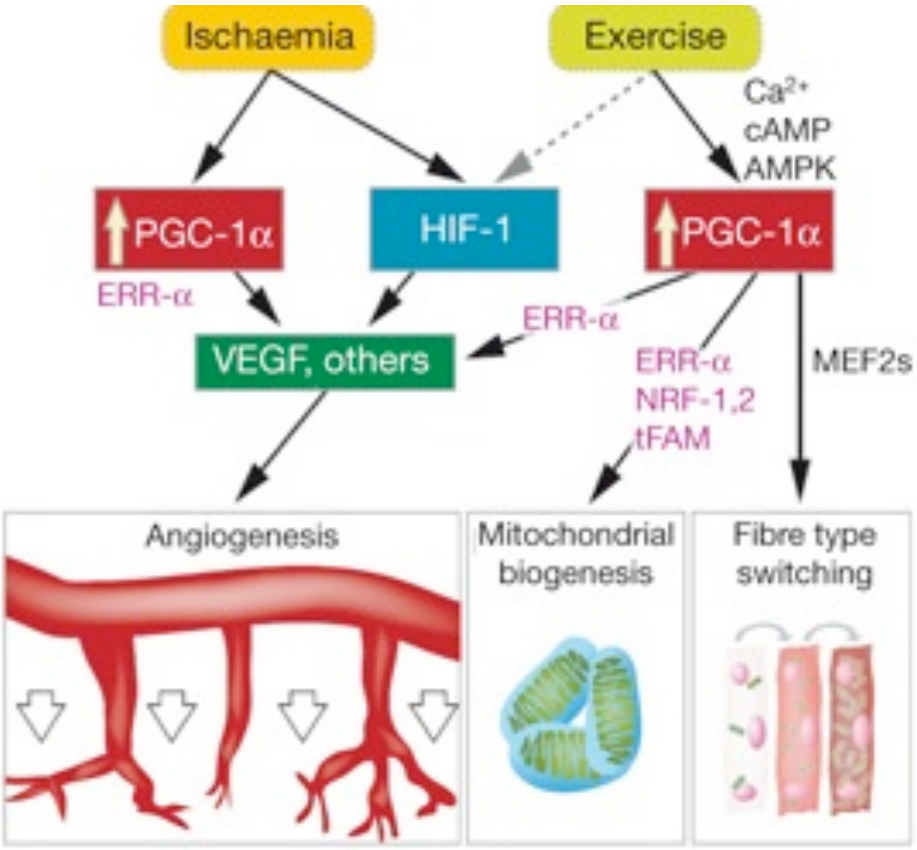

Fig 1-6. Model for the role of PGC-1α in the regulation of angiogenesis during exercise and in response to ischemia. Adapted from Arany Z, 2008 (102).

1.5.7 Role of CREB in angiogenesis

CREB is a cellular transcription factor and plays an important role in endothelial cell function and angiogenesis by mediating cellular responses to VEGF(103)

1.4.5.8 Role of mTOR and P70S6Kinase in vasculogenesis

Activated mTOR regulates the rate of protein synthesis for select mRNAs by activating the translational proteins S6Kinase and 4E-binding protein 1. It also regulates the cell growth and angiogenesis(104) and induces VEGF, platelet-derived growth factor-β and transforming growth factor-α(105).

2. Aim of the study

The general aim of this thesis is to study the role of Dipeptidyl peptidase inhibition on β-cell regeneration and revascularization in pancreatectomy and islet transplantation. In particular, we sought to study the role of endogenous incretin hormone, GLP-1 on β-cell proliferation and revascularization during pancreratectomy and islet transplantation under kidney capsule. Thus the specific aim of this thesis includes.

1. To evaluate the role of DPP-IV inhibition on β-cell proliferation.
2. To study the revascularization potential of the islet graft upon DPP-IV inhibition.
3. To study the pathway involved in the role of regeneration and revascularization.

3.Materials and Methods

3.1 Materials

3.1.1 Chemicals

Chemicals	Company
3,3'-diaminobenzidine tetra Hcl	Sigma
Avertine	Sigma
Biotinylated Bandereira Simplicifolia	Sigma
Bovine Fibrinogen	Sigma
Bovine Serum Albumin (BSA)	Sigma
Bromodeoxyuridine	Sigma
Collagenase	Sigma
Crystal Violet	Sigma
Dithiothreitol (DTT)	Invitrogen
DNAse I	Qiagen
Donkey serum	Jackson ImmunoResearch
Ethanol	Merck
Ethylenediaminetetraacetic acid (EDTA)	Fluka
Fetal Calf Serum (FCS)	Biowest

Ficoll	PAA
Formaldehyde	Roth
Fuchsin	Sigma
Gentamycin	Invitrogen
Glo-Reagent	Promega
Glucose	Sigma
Glutamine	Invitrogen
Hank’s Buffered Salt Solution (HBSS)	Invitrogen
Hepes Buffer	Sigma
Hoechst 33342	Sigma
Isoflurane	Baxter
Isopropanol	Merck
Kayser’s Glycerine Gelatine	Sigma
Levamisole	Sigma
MCDB131 Medium	Invitrogen
Mouse Alkaline Phosphatase	DAKO
Neuraminidase Type X	Sigma
Oligo (dT)20	Invitrogen
Paraformaldehyde	Merck

Penicillin/Streptomycin	Invitrogen
Peroxidase Anti Mouse IgG2	DAKO
Phosphate Buffered Saline (PBS)	B Braun
Picric Acid	Sigma
Prolong Gold	Invitrogen
RNAse-Free Water	Invitrogen
Sitagliptin	Merck
Sodium Hydroxide	Merck
StreptABComplex	DAKO
Streptozotocin (STZ)	Sigma
Sucrose	Merck
SYBR Green	Invitrogen
Thrombin	Invitrogen
Tissue-Tek OCT Compound	Sakura
Trasylol	Bayer
Tris-Hcl	Sigma
Trizol	Invitrogen
Trypan Blue	Sigma

3.1.2 Antibodies

Primary Antibody	Dilution	Company
Guinea Pig Anti-Insulin, PolyClonal	1:100 (IHC, IF)	DAKO
Mouse Anti-BrdU, Monoclonal	1:100 (IHC)	DAKO
Rabbit CREB (48H2), Monoclonal	1:100 (IF)	Cell Signal
Rabbit p70S6 Kinase (49D7), Monoclonal	1:100 (IF)	Cell Signal
Rabbit pCREB (Ser133) (87G3), Monoclonal	1:100 (IF)	Cell Signal
Rabbit pmTOR (Ser 2448)(D9C2), Monoclonal	1:100 (IF)	Cell Signal
Rabbit VEGF (A-20), Polyclonal	1:100 (IF)	Santa Cruz
Rabbit VEGF R2 (55B11), Monoclonal	1:100 (IF)	Cell Signal
Rat Anti-Mouse CD11B, Monoclonal	1:100 (IF)	Immunotools

Secondary Antibody	Dilution	Company
FITC-APure Donkey Anti Rat	1:500 (IF)	Jackson ImmunoResearch
FITC-APure Donkey Anti.Guinea Pig	1:500 (IF)	Jackson ImmunoResearch
Rhod Red-X-APure Donkey Anti-Guinea Pig	1:500 (IF)	Jackson ImmunoResearch
Rhod Red-X-APure Donkey Anti-Rabbit	1:500 (IF)	Jackson ImmunoResearch

3.1.3 Kits

Kits	Company
BCA Protein Assay Kit	Thermo Scientifc Pierce
Human Insulin ELISA Kit	DRG Instruments
Mouse Insulin ELISA Kit	DRG Instruments
Mouse VEGF Quantikine® ELISA Kit	R&D Systems
Porcine Insulin ELISA Kit	DRG Instruments
RNeasy Micro Kit	Qiagen
RNeasy Mini Kit	Qiagen
SuperScript® III Reverse Transcriptase	Invitrogen

3.1.4 Human Primer sequences for realtime PCR

Genes	Forward Primer	Reverse Primer
ARNT	GCTGGGAGATCAGAGCAACAGCTACAA	TGTTTCTTTCCAGAGGGACTG
GAPDH	TGATGACATCAAGAAGGTGG	TTTCTTACTCCTTGGAGGCC
HIF-1α	TTCACCTGAGCCTAATAGTCC	CAAGTCTAAATCTGTGTCCTG
PGC-1α	AGTACAACAATGAGCCTTCAA	CATCAAATGAGGGCAATC
VEGF-A	CCTCCGAAACCATGAACTTT	TTCTTTGGTCTGCATTCACATT
VEGF-R2	CCAGTCAGAGACCACGTTT	AGTCTTTGCCATCCTGCTGA

3.1.5 Instruments

Instrument	Company
Cell Processor (COBE 2991)	COBE
Cryostat Maschine (Leica CM1850)	Leica
ELISA Plate Reader	Brethold Technologies-Mithra LB940
Fluorescent Microscope	Leica Microsystems
Gel Doc	Vilber Lourmat
Hamilton Syringe	Hamilton
Laser-Dopler Flow Cytometer	Perimed
NanoDrop 1000 Spectrophotometer	Thermo Scientific
OneTouch® Ultra®2 Glucometer	LifeScan
StepOne Plus™ Real-Time PCR System	Applied Biosystems

3.1.6 Softwares

Softwares		Company
Adobe Illustrator	CS4	Adobe
Adobe Photoshop	CS4	Adobe
EndNote	X4	Thomson Reuters
GraphPad Prism	5.0	GraphPad Software

Image J 1.45	National Institutes of Health
Leica Application Suite	Leica
Motic Image Plus 2.0	Motic

3.1.7 Animals

Male C57Bl/6 mice and inbred athymic NMRI nu/nu mice (12 weeks old) were purchased from Charles River (Sulzfeld, Germany). Animal research was approved by Regional Commission Giessen, Germany under the code number GI20/11-Nr.15/2006. Animal husbandry was performed according to the German Animal Welfare Law as published in the latest version under http://bundesrecht.juris.de/tierschg.

3.2 Methods

3.2.1 Experimental Design

Parital Pancreatectomy (60%) and Islet transplantation model was used to study the effect of DPP-IV inhibition on β-cell regeneration.

3.2.1.1 Partial Pancreatectomy

Partial Pancreatectomy (60%) was done in 12 weeks old male C57Bl/6 mouse. The animals were anaesthetized with avertine and maintained on isoflurane. Animals were shaved off the hair in the abdominal region. A midline insicion was made and the pancreas was identified as a pinkish organ along with the spleen by slowly pulling out the intestine with a moisturized guaze.Now the pancreas was exposed along with the spleen. The blood vessels were tied with an absosrbable suture one in the region of spleen and the other at the head region of the pancreas where it joins with the duodenum. Now the spleenic porton of the pancreas was removed with a pair of scissors leaving behind the hepatic or intestinal region of

the pancreas. Now the abdomen was sutured with an absorbable suture and the skin was clamped with surgical staples. Sham operation was also done for control animals by performing the surgery without removing the pancreas

3.2.1.2 Experimental Groups:

The partially pancreatectomised and sham operated mice were randomly assigned into two different groups.

Px+NC - Partial Pancreatectomy (60%) and Normal chow fed animals

Px+SG - Partial pancreatecttomy (60%) and 5.5 % sitagliptin mixed diet.

The animals were kept at ad libidum feed and water and maintained in 12:12 Light : Dark cycle until the intended period of time

3.2.1.3 Blood Glucose measurement

Blood glucose was measured using One Touch® Ultra®2 (LifeScan) on every alternate days from the tail vein.

3.2.1.4 Bromo-de-oxyuridine (BrdU) injection

Bromodesoxyuridine (BrdU; 100 mg/kg body weight) was injected intravenously for three days to study the endogenous β-cell proliferation.

3.2.1.5 Intraperitoneal Glucose Tolerance Test (IPGTT)

Intraperitoneal Glucose Tolerance Test was performed at the end of the experiment. After measuring the blood glucose at 0 min, glucose solution (20%) was immediately injected intraperitoneally to each mouse and the thereafter the blood glucose was measured at 30, 60,90 and 120 minutes post glucose injection.

3.2.1.6 Pancreatic Insulin content measuremnet

After the intended days of treatment the remaining pancreas was removed from 5 animals in each group and the pancreas was homogenised with IRI buffer and the insulin extracted with acid-ethanol method overnight.

3.2.1.7 Insulin ELISA

Insulin content of the pancreas was estimated using a mouse Insulin ELISA kit (DRG, Germany).For the measurement of the insulin content the manufacture's protocol was followed.

3.2.1.8 Immunohistochemistry-Light Microscopy

After the intended days of treatment the remaining pancreas was removed from 5 animals from each group and fixed with zamboni's fixative.After 30 minutes of fixation, the pancreas was washed 3x with PBS and kept in PBS solution overnight at 4°C.The next day it is kept in sucrose solution overnight at 4°C.Then it is embedded in a cryoblock using OCT medium and frozen.The cryosections were made and stained for Guinea Pig Anti-Insulin (Polyclonal,Dako) and anti-BrdU antibodies to measure the area of islet and the rate of proliferation.

3.2.2 Islet transplantation

The experimental design was investigated in three islet transplantation models with mice as recipients.

3.2.2.1 Mouse islet transplantation

Sitagliptin (5.5% w/w) was administered to mice together with their chow due to short

duration of action in rodents (Altromin, Lage, Germany) while control mice received standard lab chow without sitagliptin. Blood glucose was monitored regularly with a handheld glucometer. We used a murine model of minimal islet mass transplantation. A suboptimal number of islets isolated from syngenic donor mice (50-70 per graft) were transplanted under the capsule of the upper pole of the kidney in C57Bl6 mice made diabetic with streptozotocin (180 mg/kg). Diabetes was defined as non-fasting blood glucose >350 mg/dl measured at two successive days. Mice were anaesthetized with 2.5% avertine (200 µL/100 g, Sigma), and the kidney was accessed by an incision on the left flank. A small nick was made in the kidney capsule with the bevel of a 20-gauge needle over the inferior renal pole. The sterile polyethylene tubing containing islets was then connected to a 0.5 mL Hamilton syringe and advanced under the capsule through the nick toward the superior pole of the kidney where the islets were deposited. The wound was closed in two layers with absorbable sutures. Three days after explantation of the graft, the blood glucose levels were analysed again to verify the recurrence of diabetes. Kidneys containing islet grafts were removed from the recipient mouse for further processing after 10 weeks.

3.2.2.2 Human islet transplantation

In the second model of islet transplantation 2,000 islet equivalents of human islets were transplanted beneath the kidney capsule of diabetic nu/nu NMRI mice. Human pancreatic islets were obtained from a single multiorgan donor utilizing a continuous digestion-filtration device modified as described before (106). After 10 weeks of treatment with sitagliptin the human islet grafts were retrieved for insulin extraction. At the end of the experiment bromodesoxyuridine (BrdU @ 100mg/kg body weight) was injected i.v. for three days to study β-cell proliferation in islet grafts.

3.2.2.3 Porcine islet transplantation

Porcine islets were isolated using previously described techniques of collagenase digestion and Ficoll purification (107; 108) at the Islet Isolation Facility of Third Medical Department, Uni-Giessen. About 2,000 islet equivalents of porcine islets were transplanted beneath the kidney capsule of diabetic nu/nu NMRI mice.

3.2.3 Insulin content of islet grafts

For insulin measurement, the transplants were homogenized mechanically and dissolved in acid ethanol, as described before (109). The supernatant was collected for species-specific insulin ELISA after centrifugation at 3,000 rpm for 10 minutes (DRG Instruments GmbH, Marburg, Germany).

3.2.4 Islet area measurement

Kidneys were retrieved 10 weeks after human islet transplantation, snap-frozen in O.C.T. medium, serial sections were made at 7µm thick. Every 20^{th} section was stained for insulin to identify the β-cells. The sections were fixed in Zamboni's fixative for 10 minutes and washed thrice for 5 minutes. After blocking in PBS containing 1% BSA and 2% donkey serum, the cryosections were probed with antibodies insulin (Polyclonal Guinea pig anti-insulin (1:200), Cat.No:A0564, Dako, Germany) and Monoclonal Antibody M1/70.15 to CD11b (Mouse) (1:200,Cat-No:22159111, Immunotools, Germany) at 4°C overnight. Secondary Rhodamine Red-coupled anti-guinea pig and FITC-coupled anti-mouse (1:500, Jackson ImmunoResearch, USA) antibodies were applied on the next day. The cover slips were mounted with Prolong Gold (Invitrogen), visualized and photographed using a Leica DMLB microscope (Leica, Germany) equipped with Leica DFC420C CCD and processed in Leica Application Suite. Insulin immunostained sections were scanned using Motic image analysis software 2.0 system

and analyzed. Total β-cell area was determined in 5-10 sections from the control and the sitagliptin-fed group.

3.2.5 Proliferation of β-cells

Staining for bromo de-oxyuridine (BrdU) incorporation was performed as described before (109). Briefly, sections were incubated with a mixture of nuclease and mouse alkaline phosphatase-conjugated anti-BrdU monoclonal antibody (dilution 1:200,Cat. no. 1758756, Boehringer Mannheim) for 1 h at room temperature, washed for 15 min with Tris-HCl, and incubated with a peroxidase antimouse IgG2 for 30 min and stained with 3,3'-diaminobenzidine tetra hydrochloride using peroxidase. After BrdU labelling the sections were washed and then stained for insulin with guinea pig anti-insulin polyclonal antibody (dilution 1:200, incubation for 1 h at room temperature) and anti-guinea pig alkaline phosphatase secondary antibody (Dako). To calculate the proliferation rate of β- cells, cells stained for both insulin and BrdU were counted using light microscopy. The number of total β-cells inspected for proliferation in a single graft was 1000-1200. Results were expressed as the percentage of BrdU positive β-cells.

3.2.6 Blood flow and microvascular Density

The blood perfusion of the islet graft and the adjacent renal cortex was measured by laser-Doppler flow cytometry (PF 4001-2, Perimed, Stockholm, Sweden) with a needle probe (411 mm tip; outside diameter, 0.45 mm; Perimed). The flow probe was positioned perpendicular to the immobilized tissue surface by the use of a micromanipulator, and care was taken not to cause any compression of the tissue.

Microvessels in histologic sections were identified by staining with biotinylated Bandereira simplicifolia (BS-1, Cat.No: L 5391 Sigma) as previously described (110). Briefly, slides were pretreated with neuraminidase type X (Cat.No:N2133, Sigma) and incubated with

biotinylated BS-1 at 4°C overnight. After three washings with PBS, they were incubated with StreptABComplex (Dako) for 30 minutes at room temperature. Subsequently, fuchsin and 1 mM levamisole was added. Vascular area cross sections within grafts were calculated from lectin-positive fields in each section with a light microscope (200x) combined with an image analysis system (Motic, Wetzlar, Germany). Vascular density was expressed as percentage of vascular area to total area examined.

3.2.7 Mouse islet isolation and culture

Pancreatic islets were isolated from C57Bl6 or RIP-VEGF mice, a transgenic mouse model with human vascular endothelial growth factor (VEGF) production in β-cells under the control of the rat insulin promoter (RIP), at the age of 9-12 weeks (109). Briefly, pancreata were distended by collagenase solution (2.5 mg/ml), incubated in a water bath for 12 min at 37°C and shaken by hand for 3 min under visual inspection. After washing twice in ice cold HBSS and centrifugation (900rpm, 3min) the remaining pellet was resuspended in Parkers FCS medium (TCM199 medium supplemented with 5% FCS, HEPES buffer, penicillin/streptomycin and gentamycin). Pure islets were handpicked under a stereomicroscope, which were incubated overnight (37°C, humidified air) to recover from the isolation stress.

3.2.8 Assessment of VEGF secretion and DPP-IV activity

VEGF concentrations of cell culture supernatants were measured by ELISA (R&D Systems). DPP-4 activity in plasma samples or pancreatic islet was determined using the Glo-reagent for biological fluids (Promega).

3.2.9 Ex vivo Islet Sprouting

Bovine fibrinogen, supplemented with trasylol (prevents hyperfibrinolysis) was diluted in PBS Dulbecco's to a final concentration of 1.7mg/ml. To the wells of a 24-well plate 300 µl

of this solution was added. After removing the exocrine tissue from the 24h pre-cultured islets and washing in PBS, around 20-30 islets were embedded into each of the prepared gels. All test substances were mixed into the liquid gels prior to polymerization with 5µl of thrombin (1000 U/ml). After clotting at room temperature gels were equilibrated with MCDB131 medium (Invitrogen) containing 5% glutamine in an incubator with 5% CO_2. The fibrin clots were soaked with 300µl Parkers FCS medium and cultured for 48h. After 48 h cultures, gels were fixed with 3% formaldehyde for 6 - 12 h and stained with crystal violet. Washed gels were transferred onto slides and fixed with Kayser's glycerine gelatine. Islet vitality was scrutinized at two time points, firstly directly prior to the angiogenesis assay using the trypan blue staining and secondly by determination of islet morphology in the fixed gels. Vital islets did not disperse and had smooth shape without cellular protrusions. Photos were taken and sprout length was determined using Motic software (Wetzlar, Germany). Following the isolation procedure every individual experiment was carried out with an appropriate control condition.

3.2.10 Quantitative Realtime PCR in human islet transplant

3.2.10.1 RNA isolation

Human islet transplants were carefully dissected from nu/nu NMRI kidney capsule one day or 14 days following islet transplantation. Total RNA was immediately extracted with TRI reagent (Invitrogen) and the RNA pellet obtained after isopropanol and ethanol precipitation was dried and resuspended in 50 µl of RNase-free water. To prevent contamination of RNA by RNAses all standard precaution was taken.

3.2.10.2 Measurement of RNA Concentration

RNA concentration was measured using NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington). The ratio of sample absorbance at 260 and 280 nm (260/280) was measured and

it should be approximately 2.0 for pure RNA. Sample concentration was given in ng/µl based on its absorbance at 260 nm.

3.2.10.3 DNAse Treatment

All RNA samples were treated with DNAse to eliminate any genomic DNA contamination. The following reagents were mixed in a microfuge tube and incubated at 37ºC for 15 min. To inactivate DNAse I, 1 µl of 25mM EDTA was added and incubated at 65^{o}C for 15 min. The reaction was collected by a brief centrifuge and stored at -80 ^{o}C until used for reverse transcription.

RNA	1 µg
10X Reaction Buffer	1 µl
DNase I (1 U/µl)	1 µl
RNAse/DNAse free water	upto 10 µl

3.2.10.4 cDNA synthesis

The sample containing 11 µl of the DNAse treated RNA was used for cDNA synthesis.The RNA was reverse transcribed into cDNA using reverse transcriptase and Oligo(dT) primers.The RNA sample was mixed with 10mM 10 mM dNTPs (final concentration), 0.5µ g Oligo(dT)20 and H2O to 7µl. This mixture was heated at 65°C for 5 min, and quickly chilled on ice. Then, 4 µl of 5 x firststrand buffer and 2 µl of 0.1 M DTT were added to the sample and incubated at 42°C for 2 min.Finally, 1 µl of SuperScript II RT (200 U) was added to the mixture (total volume 25µl), followed by incubating at 42°C for 50 min and heating at 70°C for 15 min. A sample of 1µg total RNA was used for cDNA synthesis from Oligo (dT) 20 primers using the Superscript ^{TM} II first strand synthesis system (Invitrogen).

3.2.10.5 Real -Time PCR analysis

Real time PCR or qPCR is used to detect the amplification of a specific target sequence from cDNA using gene-specific primers.

The reaction mixture consists of the following reagents.

SYBR Green Master Mix (2x)	5µl
cDNA template	0.5µl
Primers (F+R) 20pmol/µl	0.5 µl
RNAse/DNAse free H_2O	upto 10µl

Real time- PCR was carried out StepOne Plus real-time PCR cycler (Applied Biosystems) using the following programs:

Steps	Temp.	Time	Number of Cycles
Enzyme activation	95°C	15 min	1 cycle
Denaturation	95°C	15 sec	40 cycles
Annealing	60°C	30 sec	
Extention	72°C	30 sec	

After amplification of the products a melting curve analysis was performed to analyze the specificity of the products using the following steps.

Steps	Temp	Time	No.of Cycles
Denaturation	95°C	30 sec	1 cycle
Starting Temp.	60°C	30 sec	1 cycle
Melting step	60°C	10 sec	80 cycles

The threshold value ct for each individual PCR product was calculated by the instruments' software and CT values obtained for the target gene were normalized by subtracting the CT values obtained with the reference gene GAPDH. All data are presented as the ratio of the target gene/GAPDH. The relative abundance of the different genes was calculated by the comparative ΔΔCT method (111).

3.2.11 Immunohistochemistry-Fluorescent Microscopy

3.2.2.11.1 Measurement of VEGF, VEGFR-2, CREB, pCREB, pMTOR and P70S6K expression

Sections were made from the retrieved graft and fixed with Zamboni's fixative for 10 min and washed thrice for 5 min in PBS. After blocking in PBS containing 1% BSA and 2% donkey serum, the cryosections were probed with antibodies against insulin (polyclonal guinea pig anti-insulin antibody, Dako, Germany), Rabbit Polyclonal VEGF (Santa Cruz), VEGFR2, CREB and pCREB (Cell signaling) and Rabbit monoclonal pMTOR and P70S6K antibody (Cell signaling). Primary antibodies were visualized with FITC- coupled anti-Guinea pig and rhodamine-coupled anti-rabbit antibodies (Jackson Immuno Research, USA). Sections were mounted with Prolong Gold (Invitrogen), visualized and photographed using Leica DMLB microscope (Leica, Germany). The images were analyzed using Image J software for VEGF and VEGFR-2 area and for number of CREB and pCREB positive β-cell nuclei.

3.2.12 Statistical analysis

Statistical analysis was performed by Prism 5.0 (GraphPad Software, San Diego California, USA) using one-way analysis of variance and Bonferroni post-hoc tests. For two-group tests the student *t*-test was applied. Data from the transplantation experiment were plotted as survival curves with 'cure of diabetes' as non-recurrent event and analyzed by the Mantel-Haenszel log rank test.

4. Results

4.1 Sitagliptin increases proliferation of β-cells after pancreatectomy

In this study we used pancreatectomy model and fed the mice with sitagliptin to assess the impact of pancreatectomy and sitagliptin on β-cell proliferation. We found that sitagliptin treatment increased the number of BrdU positive cells, indicating the proliferation potential of β-cells after pancreatectomy and sitagliptin treatment. The number of BrdU positive cells increased significantly ($p<0.05$) in the sitagliptin treatment compared to the control group (**Figure 1 A, B and C**).

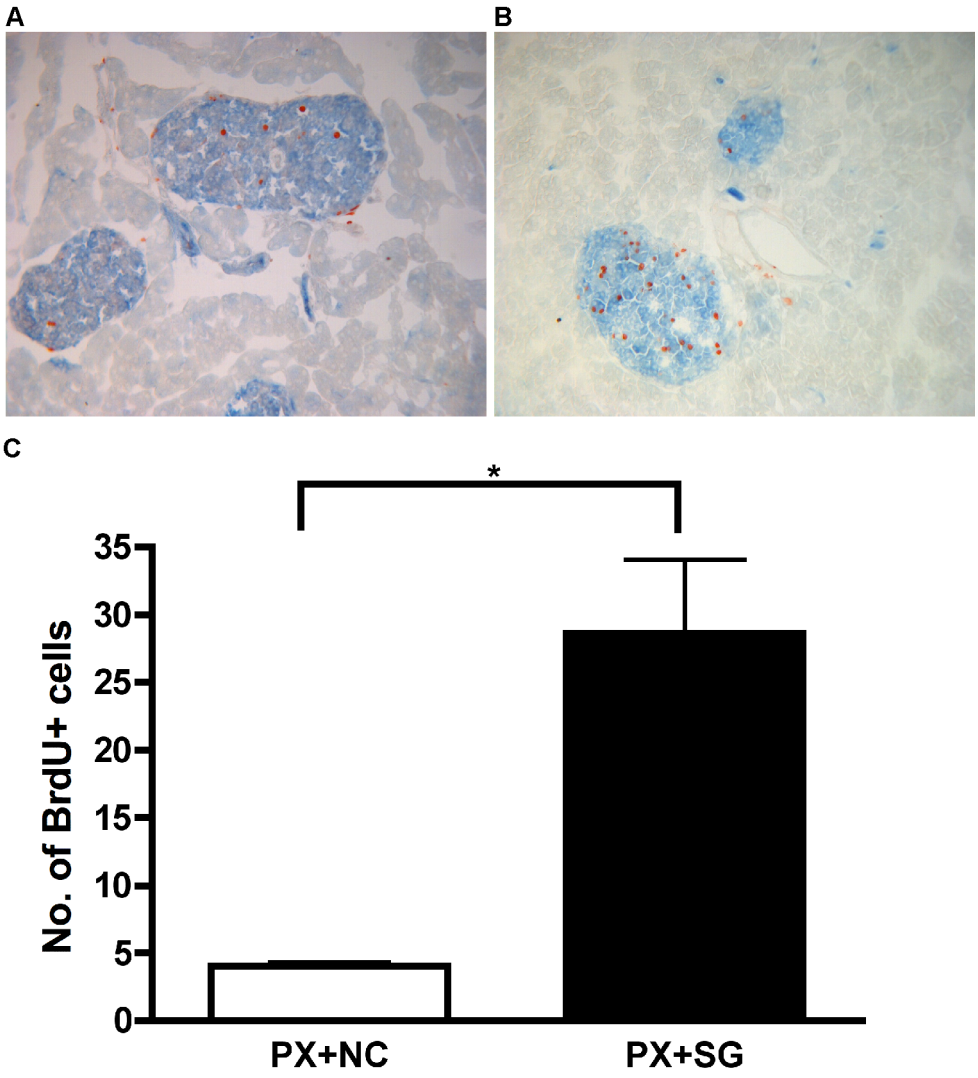

Figure 4-1. β-cell proliferation after pancreatectomy and sitagliptin treatment. **(A-Control, B-Sitagliptin).** Light microscopic picture showing the proliferation of β-cell as measured by BrdU staining. **(C)**. Quantitative measurement of β-cell proliferation.

4.2 Sitagliptin increases Insulin area after pancreatectomy

Next we assessed the insulin area after pancreatectomy and sitagliptin treatment and found that the insulin area was significantly ($p<0.05$) increased in the sitagliptin treated than the control group (**Figure 2**)

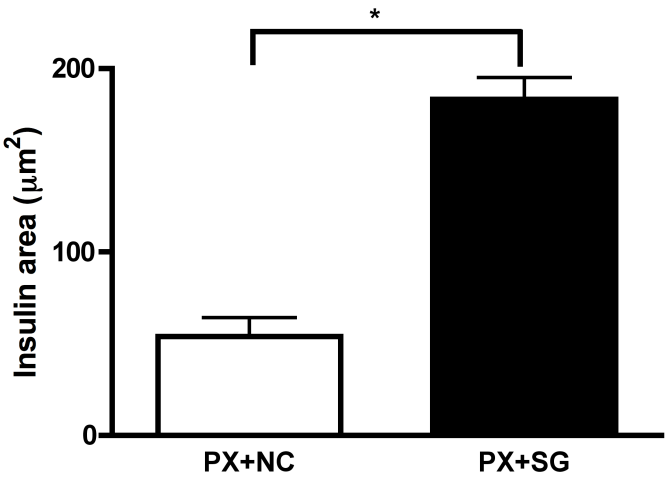

Figure 4- 2. Quantitative Insulin area after pancreatectomy and sitagliptin treatment

4.3 Sitagliptin improves glucose tolerance in pancreatectomized mice

Intraperitoneal glucose tolerance test was performed in both control and sitagliptin group after pancreatectomy and found that sitagliptin treatment improved the glucose tolerance significantly ($p<0.05$) than the control group **(Figure 3)**

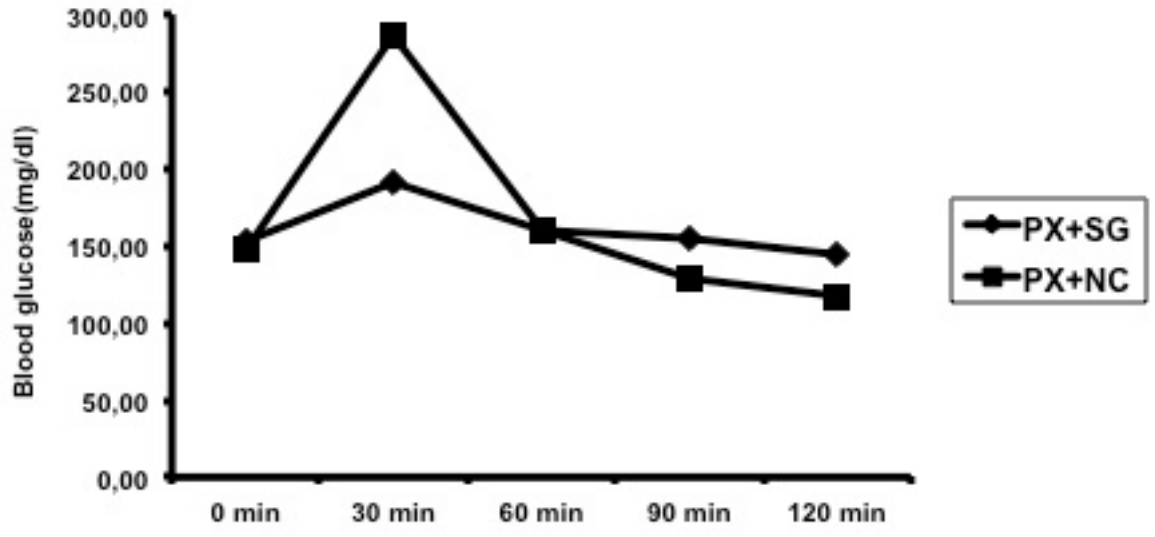

Figure 4-3: Intraperitoneal glucose tolerance test.

4.4 DPPIV concentration and activity reduced by sitagliptin in both plasma and pancreas

Since the sitagliptin treatment increased both the proliferation and insulin area of β-cells, we determined whether this might be due to actual inhibition of DPP-IV by sitagliptin and found that sitagliptin treatment significantly ($p<0.05$) decreased both the concentration and activity in pancreas and plasma (**Figure4A, B, C and D**).

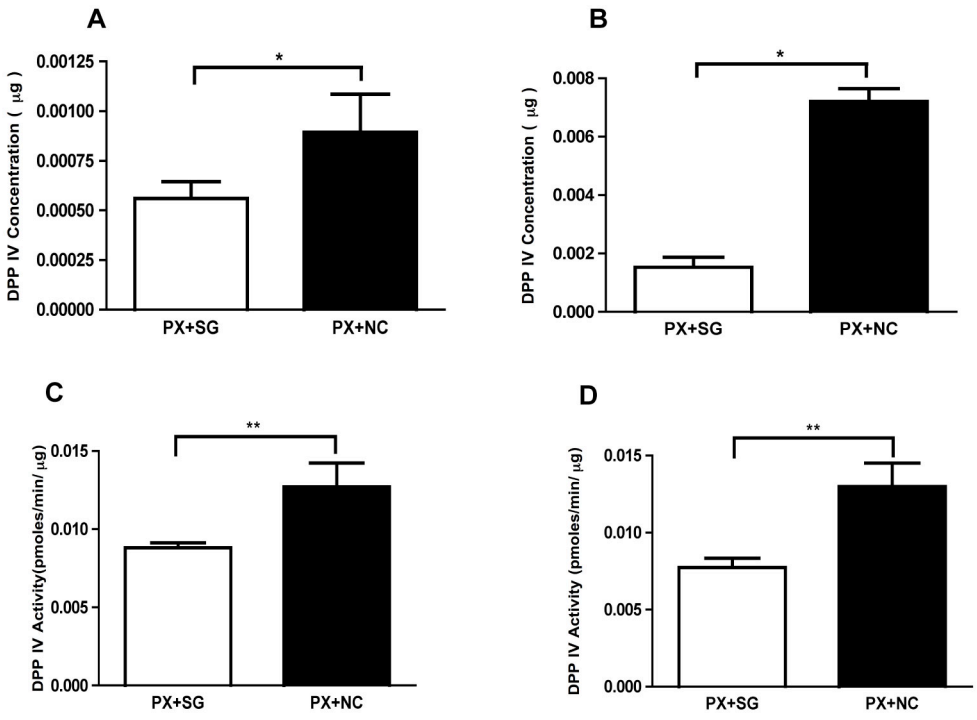

Figure 4-4. DPPIV concentration and activity in pancreas and plasma. (**A, C**). DPPIV concentration and activity was measured using Glo-reagent in pancreas. (**B, D**). DPPIV concentration and activity in plasma. PX+SG- pancreatectomy and sitagliptin; PX+NC- Pancreatectomy and Normal Chow.

4.5 Sitagliptin accelerates restoration of normal blood glucose levels

To determine the effect of sitagliptin on the cure of diabetes after pancreatic islet transplantation blood glucose levels were measured over a period of ten weeks. Cure of diabetes was defined as sustained reduction of blood glucose < 200 mg/dl.

Diabetic C57Bl6 mice were hyperglycemic with a mean blood glucose level of 350 ± 80 mg/dl. In previous studies it was shown that progression of severity of diabetes induced by streptozotocin (STZ) in rats was reduced by DPP-IV inhibitor(112). Subsequent to transplantation with a suboptimal number of pancreatic islets blood glucose levels gradually decreased. Three and ten weeks following transplantation 46% and 92% of the control group had returned to non-fasting blood glucose concentrations of less than 200 mg/dl, respectively. In contrast, 100% of sitagliptin-treated mice reached non-fasting blood

glucose concentrations already after 3 weeks (**Figure 5A, B**). Thus, sitagliptin treatment had a beneficial effect on transplantation-mediated restoration of normal blood glucose levels in diabetic mice.

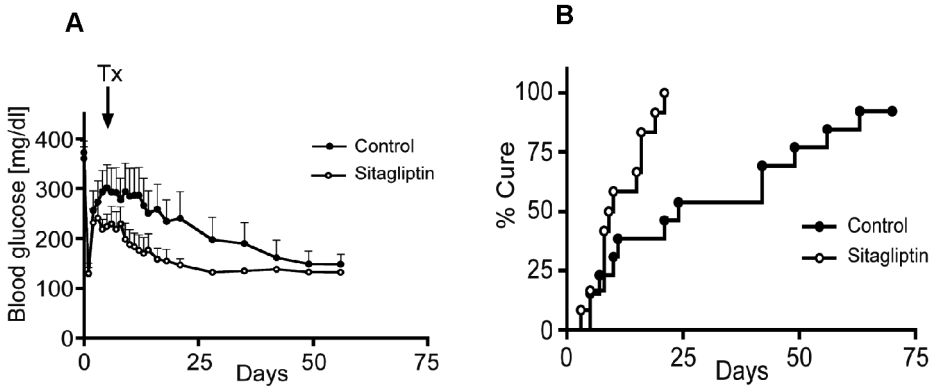

Figure 4-5: Sitagliptin accelerates restoration of normal blood glucose levels. **(A)** C57Bl6 mice were treated with a single dose of streptozotocin (180 mg/kg) and placed on either a normal chow diet or diet containing 5.5% sitagliptin following syngenic islet transplantation (day 0). Blood glucose levels (mg/dl) were determined in the control and sitagliptin treated mice. Tx = transplantation. Control = chow fed mice. Sitagliptin = Recipient mice fed with 5.5% sitagliptin for 10 weeks. **(B)** Survival curves demonstrating cure from diabetes (< 200 mg/dl) by transplantation up to 70 days after transplantation ($p < 0.0072$ *vs.* control). n=12. Data represent mean ± SEM.

4.6 Improved insulin production through enhanced β -cell proliferation

To determine the underlying cause of accelerated restoration of blood glucose levels we assessed the effect of sitagliptin on insulin production. Grafts and pancreases from recipient mice were retrieved and insulin content was determined. The mean insulin content of both mouse (**Figure 6A**) and human (**Figure 6B**) islet grafts was significantly higher in the sitagliptin-treated compared to the control group (mouse: 48 ± 10 *vs.* 35 ± 11 µg insulin/g kidney, $p < 0.03$; human: 106 ± 6 *vs.* 58 ± 6 µg insulin/g kidney, $p < 0.01$).

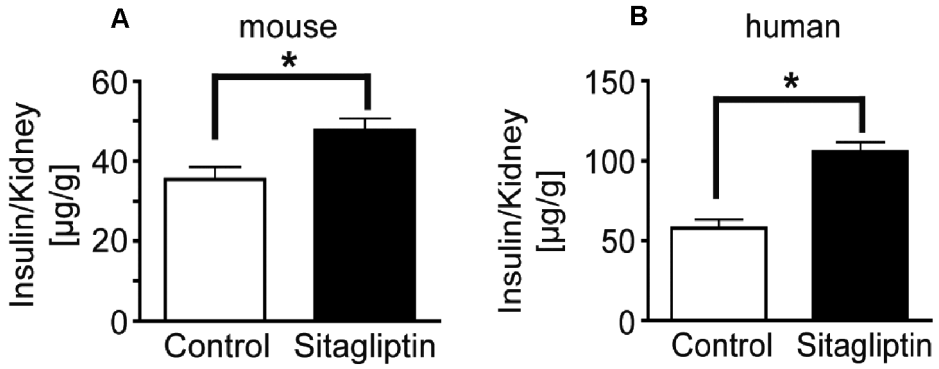

Figure 4-6: Sitagliptin improves insulin production. (A) Pancreatic insulin content of mouse islet grafts transplanted to the kidney capsule given as µg insulin per g kidney. Control = normal chow, Sitagliptin = 5.5 % sitagliptin included into the rodent chow ($p < 0.05$; n=6). **(B)** Insulin content of transplanted human islets 10 weeks after transplantation of 2,000 islet equivalents each and feeding the recipient NMRI nu/nu mice either with normal chow or chow enriched with 5.5% sitagliptin ($p < 0.01$). Data represent mean ± SEM.

These results were confirmed by immunocytochemistry of sections of transplants and pancreases utilizing insulin antibodies. Insulin-positive areas were markedly greater in graft sections of sitagliptin-treated mice (mouse: 1629 ± 126 µm2 *vs.* 1151 ± 145 µm2, $p < 0.005$; human: 3113 ± 690 µm2 *vs.* 1812 ± 10 µm2, $p < 0.01$) (**Figure 7A and B**). Staining with CD11b antibodies revealed no difference of immune cell infiltration (**Figure 7C**).

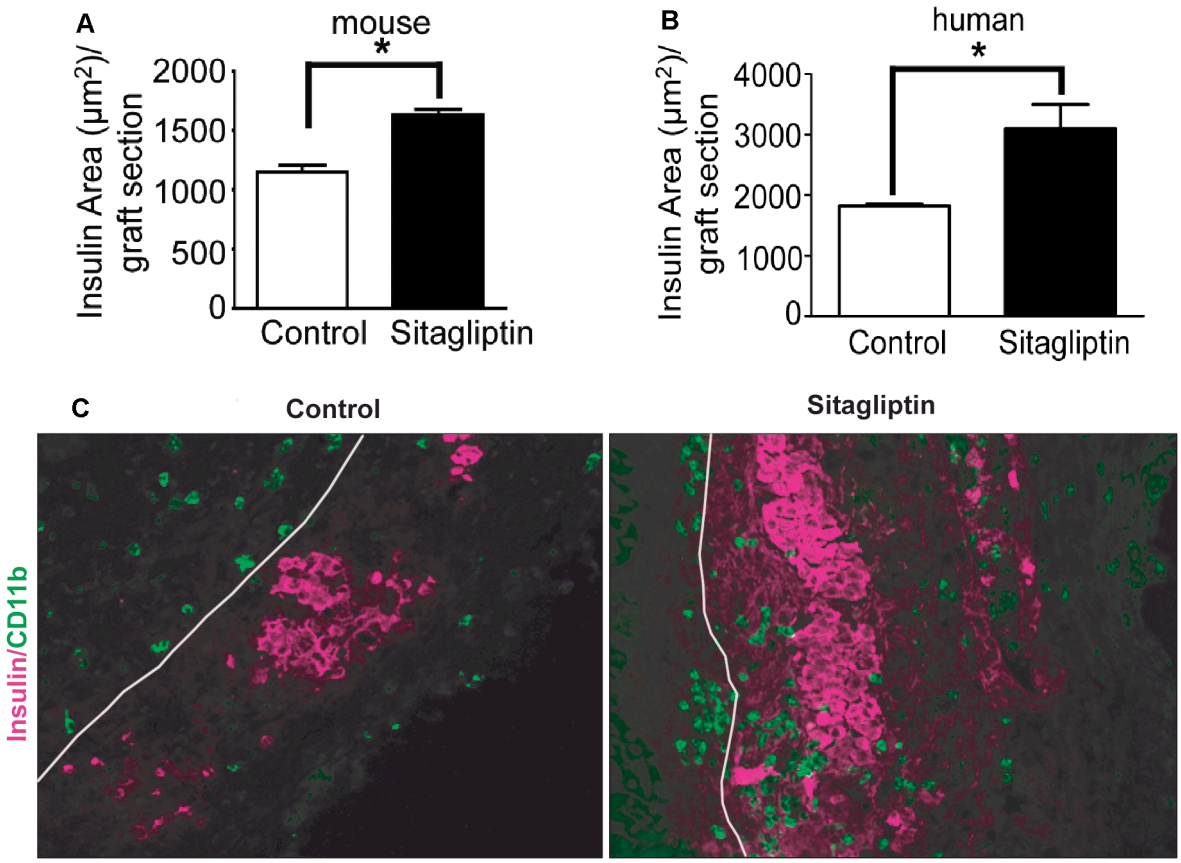

Figure 4-7: Sitagliptin increases grafted insulin area. (A, B). Insulin area (μm^2) of sections of mouse (A) and human (B) islet grafts transplanted to the kidney capsule. Insulin positive graft area for human islets was 3113 ± 690 μm^2 in sitagliptin-treated mice and 1812 ± 10 μm^2 in control mice (n=6, 10 sections per graft, $p < 0.01$). Data represent mean ± SEM. (C) Effect of DDP-IV inhibition by 5.5% sitagliptin on insulin area and immune cell migration to human islets transplanted to kidney capsule of NMRI nu/nu mice. Grafted kidneys were recovered as described 10 weeks after transplantation. Representative cryo sections were stained for insulin (magenta) and CD11b (green)-positive immune cells (neutrophils, macrophages). The white line demarcates the graft and kindey cortex. Area stained positive for insulin was larger in the sitagliptin group compared to control. No difference of immune cell infiltration was observed.

In sections of host pancreases insulin could not be detected indicating complete abrogation of endogenous insulin production by streptozotocin (data not shown). Larger amounts of insulin can be due to increased insulin biosynthesis per cell or greater number of actively producing β-cells or both.An earlier study reported that sitagliptin treatment in partially pancreatectomized mice increased β-cell mass in remnant pancreas(113). To determine changes in the biosynthesis of insulin we measured the expression level of PGC-1α and PDX-1 in islet grafts. Altered expression of these genes in pancreatic islets is accompanied by changes of cyclic AMP levels and glucose stimulated insulin secretion. [19][20] Interestingly, the expression of both genes was upregulated (**Figure 8A, B**).

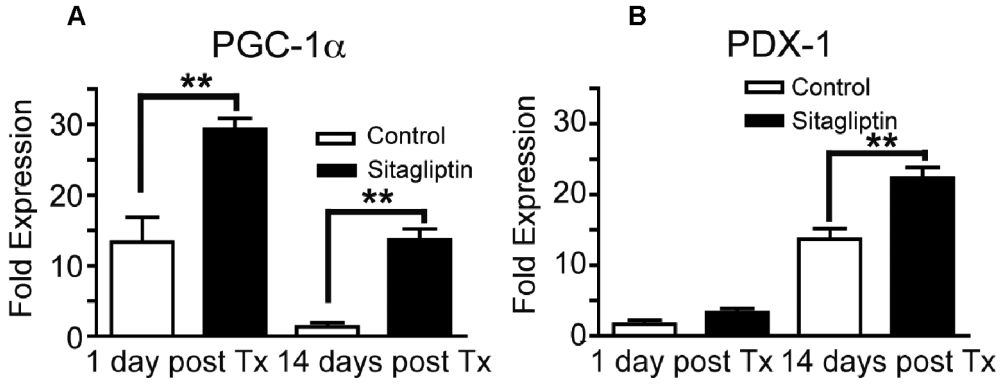

Figure 4-8: Sitagliptin promotes upregulation of PGC-1α and PDX-1. (**A,B**) Human islet grafts were retrieved from NMRI-nu/nu mice fed with either 5.5% sitagliptin or normal chow one day or fourteen days after transplantation (Tx). After RNA isolation and cDNA synthesis the relative expression of the indicated genes was quantified by real time PCR in triplicates. The effects of different stimulations on individual gene expression were calculated and normalized to endogenous reference gene GAPDH using comparative ΔΔCT method (* $p < 0.05$, ** $p < 0.001$).

This, however, could also be explained by an increased number of β-cells. Moreover, the areas of insulin positive tissue suggest an increased number of β-cells. This might be due to an enhanced proliferation rate or an antiapoptotic effect of sitagliptin. The anti-apoptotic gene Bcl-2 was increased by sitagliptin (**Figure 9A**). However, we did not detect a significant difference in the expression of the pro-apoptotic gene Bax (**Figure 9B**). Thus, we determined whether β-cell proliferation was affected by sitagliptin. Sitagliptin treatment significantly increased the number of BrdU-positive β-cells from 4.5 ± 0.5 per μm2 to 7.5 ± 2.6 per μm2 ($p < 0.01$; **Figure 9C**). These data suggest that the observed increased production of insulin after sitagliptin treatment is mainly due to enhanced β-cell proliferation.

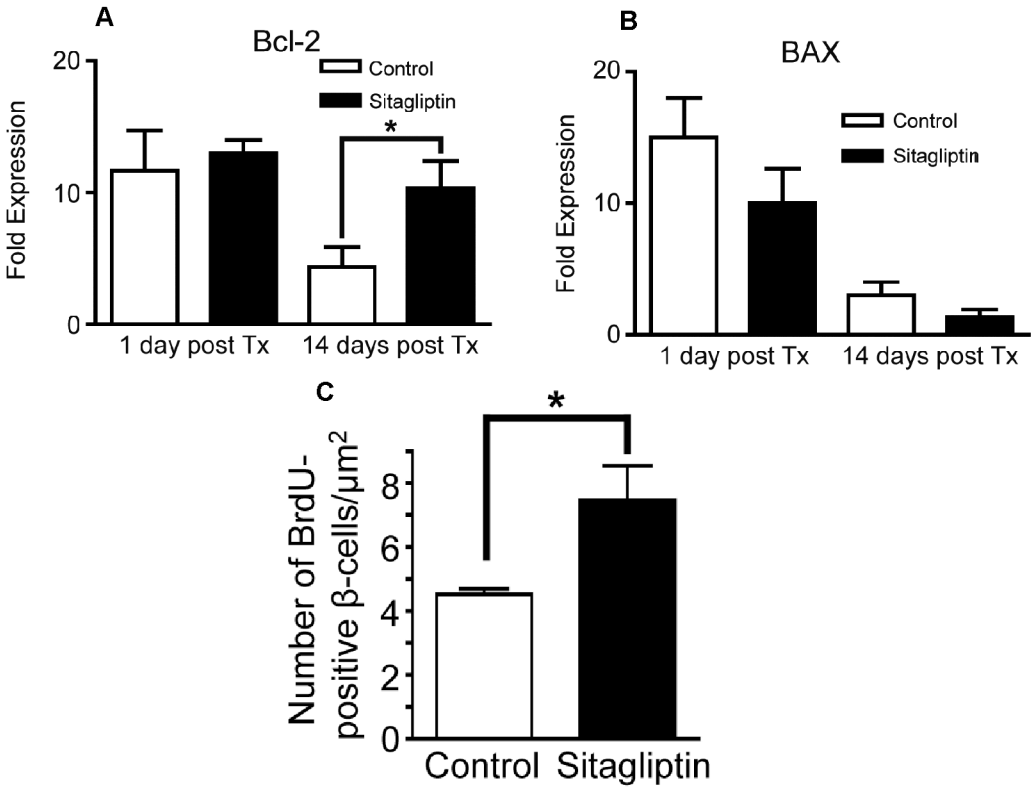

Figure 4-9:Sitagliptin increases anti-apoptotic gene Bcl-2 expression and promotes β-cell proliferation. **(A).** The anti-apoptotic gene Bcl-2 was increased by sitagliptin. **(B).** No difference in the expression of the pro-apoptotic gene Bax. **(C).** Quantitative analysis of β-cells positive for bromodesoxyuridine (BrdU) in islet grafts transplanted to the kidney

capsule of mice on normal chow or chow containing 5.5% sitagliptin ($p < 0.01$; n=6; 10 sections per graft). Data represent mean ± SEM.

4.7 Sitagliptin improves vascularization

Previously, it has been demonstrated that the capillary network of transplanted pancreatic islets has to be reconstituted(109) and vascularization of them occurred within 10-14 days(114). To examine the effect of sitagliptin on re-vascularization islet transplants were retrieved after 10 weeks. Vascular density within the graft was calculated by morphometry after endothelial cell staining (**Figure 10A**). Sitagliptin treatment significantly increased the area occupied by endothelial cells from 53 ± 8 µm2 to 164 ± 15 µm2 ($p < 0.001$; **Figure 10B**) as well as the number of proliferating endothelial cells within and around the graft from 11 ± 1 per µm2 to 31 ± 5 per µm2 ($p < 0.002$; **Figure 10C**). Collectively, these data suggest that DPP-IV inhibition by sitagliptin increases revascularization.

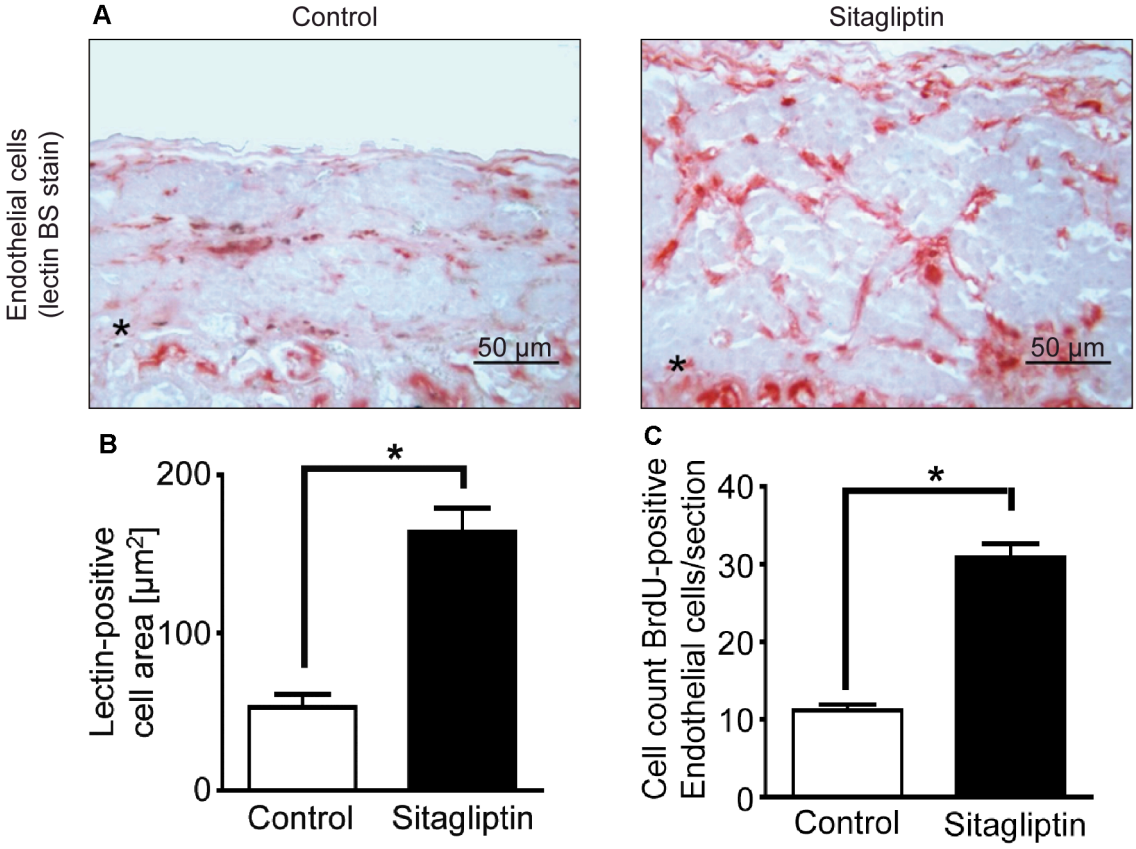

Figure 4-10: Sitagliptin enhances vascularization. (**A**) Representative picture of immunostaining of islet graft with lectin BS-1 (red) at the kidney capsule of control and sitagliptin-treated islet graft. Both graft area and lectin BS-1-positive areas are larger compared to control. The asterisk * marks the border between islet graft and kidney. (**B**) Quantitative analysis of area (μm^2) stained positive for lectin BS-1 in islet grafts supplanted to the kidney capsule of C57Bl56 mice fed either with control chow or chow enriched with 5.5% sitagliptin (n=6; 10 sections per graft, $p < 0.001$). (**C**) Quantitative analysis of bromodesoxyuridine (BrdU)/lectin BS-1 double-positive cells in islet grafts transplanted to the kidney capsule of mice either on normal chow or chow containing 5.5% sitagliptin (n=6; 10 sections per graft, $p < 0.001$).

4.8 DPP-IV inhibition increases functional blood flow to transplanted islets

To determine if re-vascularization also resulted in an improved blood flow we applied laser-Doppler flowmetry. A needle probe was used to record signals up to 200 µm under the kidney capsule. Control samples showed a negative difference between the kidney's and the graft's regular blood flow of -34.5 % (95% confidence interval -41; -28). Importantly, the difference in blood flow in sitagliptin-treated mice was significantly lower -9.7 % (95% confidence interval -16; -2; $p < 0.01$) (**Figure 11**).

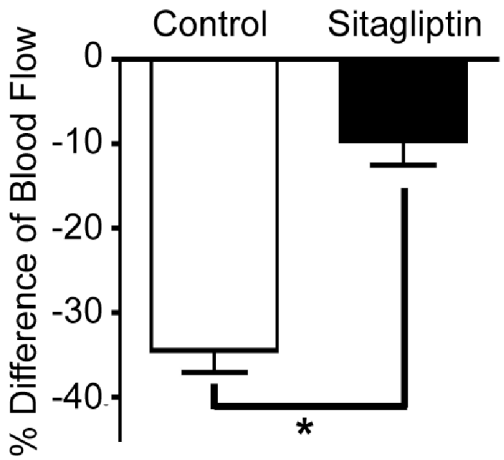

Figure 4-11: DPP-IV inhibition increases functional blood flow to transplanted islets. Blood flow measured by Doppler ultrasound probe simultaneously on the kidney capsule surface and at the site of the grafted islets. The difference (%) of kidney and graft blood flow is plotted on the ordinate ($p < 0.01$; n= 6). Data represent mean ± SEM.

Taken together, these data demonstrate that sitagliptin enhances functional vascularization of transplanted pancreatic islets. Interestingly, gene expression analysis suggests that the VEGF signaling pathway is modulated by sitagliptin treatment (HIF-1α, ARNT, VEGF-A, VEGF-R2) (**Figure 12A to D**).

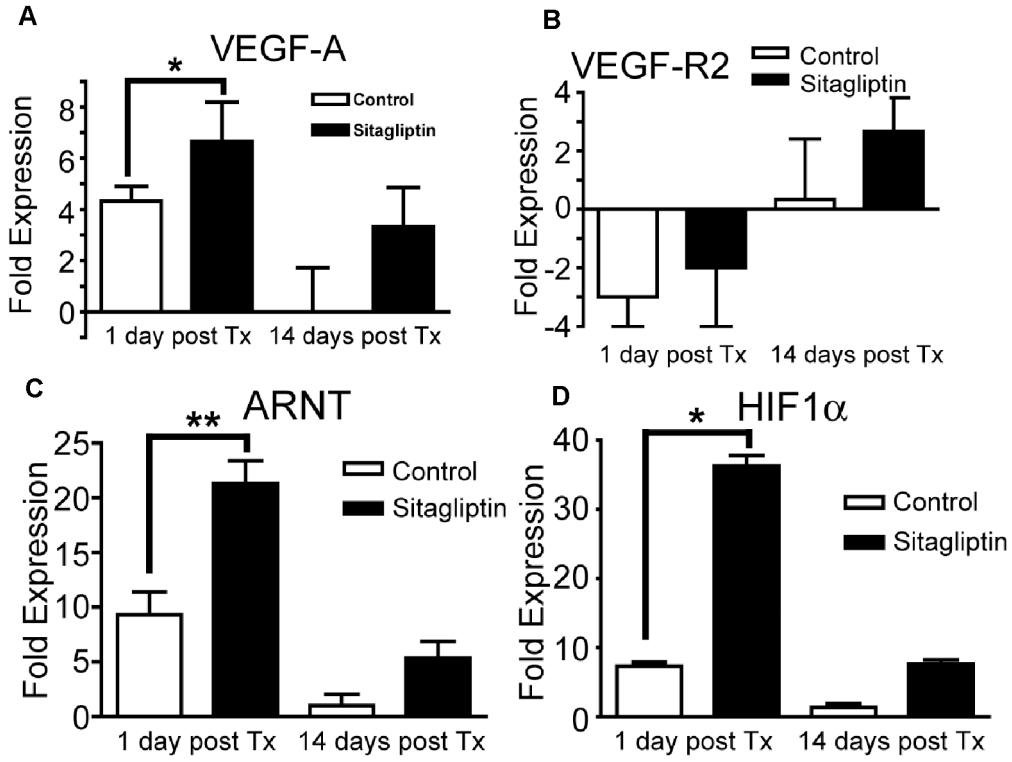

Figure 4-12: VEGF signaling pathway is modulated by sitagliptin treatment. (**A to D**) Human islet grafts were retrieved from NMRI-nu/nu mice fed with either 5.5% sitagliptin or normal chow one day or fourteen days after transplantation (Tx). After RNA isolation and cDNA synthesis the relative expression of the indicated genes was quantified by real time PCR in triplicates. The effects of different stimulations on individual gene expression

were calculated and normalized to endogenous reference gene GAPDH using comparative ΔΔCT method (* $p<0.05$, ** $p<0.001$).

4.9 Sitagliptin increases VEGF secretion in vitro

Local blood perfusion is important for regular function and β-cell survival in islet grafts(115; 116). Since sitagliptin improved re-vascularization, we wondered whether it actually increases the VEGF secretion *in vitro* from islets. For this purpose we treated isolated islets with Glp-1 (7-36) and analyzed the cell culture supernatants for VEGF secretion. Treatment with Glp-1 (7-36) but not Glp-1 (9-36) resulted in a significant increase of VEGF release from normal islets (**Figure 13**). Sitagliptin further enhanced the effect of Glp-1 (7-36) resulting in a 4-fold increase of VEGF. The use of RIP-VEGF islets demonstrated that VEGF was released from the pancreatic β-cells (**Figure 13**).

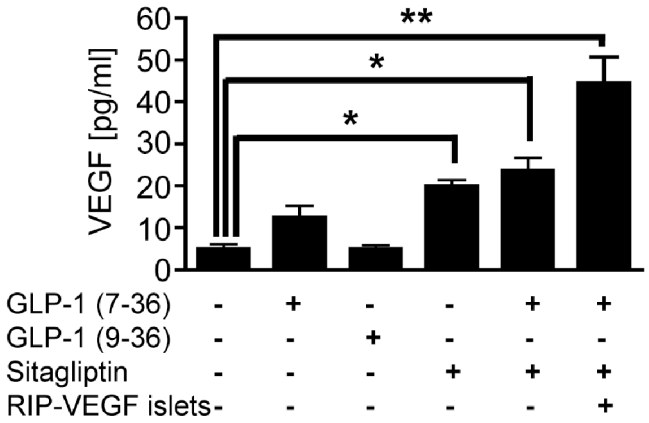

Figure 4-13: DPP-IV/Glp-1 regulates secretion of VEGF by β-cells. Mouse islets were cultured free floating in Parkers FCS medium in the presence of Glp-1 (7-36), Glp-1 (9-36), or sitagliptin. RIP-VEGF islets = islets isolated from mice synthesizing human VEGFA165 under the control of the rat insulin promoter (RIP). After 24 h of incubation the content of VEGF in the supernatant was determined. Glp-1 (7-36) resulted in higher VEGF content of the supernatant than without the presence of Glp-1 (7-36). Islets

cultivated in the presence of Glp-1 (7-36) and 5 μM sitagliptin released even more VEGF (normal islets * $p < 0.05$ and RIP-VEGF islets ** $p < 0.01$) compared to islets incubated with Glp-1 (7-36) alone. Data represent mean ± SEM.

These islets were derived from a mouse strain producing VEGF under the control of rat insulin promoter as described before(109). The result suggests that sitagliptin induces release of VEGF from islets. Next we investigated whether the release of VEGF increases any growth or proliferation of islet endothelial cells *in vitro*.

4.10 Sitagliptin enhances islet endothelial cell sprouting

An earlier study demonstrated that VEGF-A controls angiogenic sprouting in the early postnatal retina(117). To analyze whether the release of VEGF increases sprouting of islet endothelial cells, we isolated islets and studied the effect of sitagliptin on islet endothelial cell sprouting into fibrin gels. Interestingly, DPP-IV activity was nearly 30-fold higher in islets compared to mouse plasma (6126 ± 320 μmol/min/mg protein *vs.* 215 ± 18 μmol/min/mg, $p < 0.0001$). The addition of bFGF induced low levels of sprouting, which was greatly enhanced by VEGF. The addition of Glp-1 (7-36) but not addition of inactive Glp-1 (9-36) enhanced the effect of bFGF. Sitagliptin is an inhibitor of DPP-IV that cleaves Glp-1 (7-36) to Glp-1 (9-36). As DPP-IV activity was abundant in our assay we tested whether addition of sitagliptin to bFGF + Glp-1 (7-36) can further enhance sprouting. This combination resulted in an even greater sprouting activity as bFGF + VEGF (**Figure 14 A,B)**. Taken together these data suggest that sitagliptin enhances VEGF release and thereby increases islet endothelial cell proliferation necessary for functional viability of transplanted islets by modulating DPP-IV/Glp-1.

Next we worked on the mechanism that promoted VEGF secretion and maintained functional viability of transplanted islets *in vivo.*

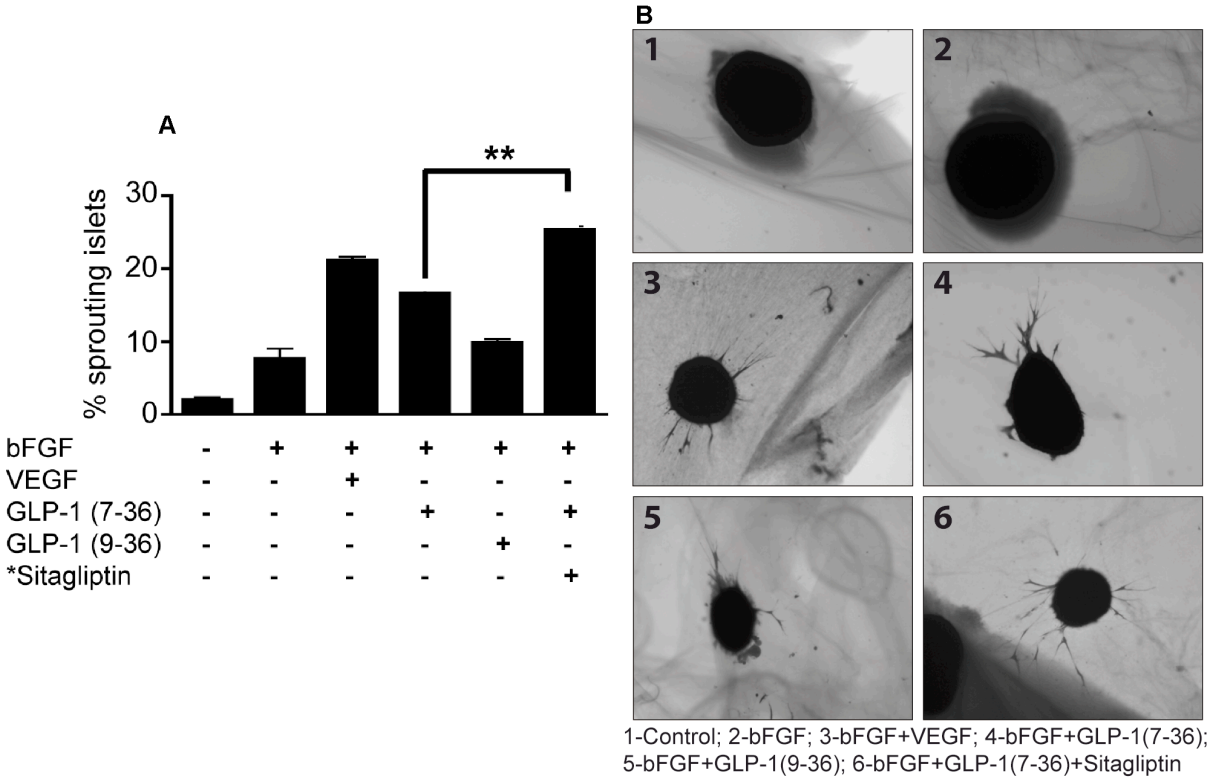

Figure 4-14: Sitagliptin enhances islet endothelial cell sprouting. Analysis of the effect of different factors regarding their capacity to induce islet sprouting. bFGF = 5 ng/ml human basicfibroblast growth factor, VEGF = 5 ng/ml human vascular endothelial growth factor 165, Glp-1 = 50 nM Glucagon-like peptide 1; either the 7-36 peptide or 50 nM 9-36 fragment. Glp-1 (7-36) is cleaved by dipeptidyl peptidase IV (DPP-IV) resulting in Glp-1 (9-36), sitagliptin = 5 μM sitagliptin. All data represent mean ± SEM and significance was tested using ANOVA with a Bonferroni post hoc test where ** represents $p < 0.01$ for the percentage of sprouting islets in the presence of bFGF and Glp-1 (7-36) versus bFGF, Glp-1(7-36) and DPP-IV inhibitor sitagliptin demonstrating an additional effect on endothelial sprouting by DPP-IV inhibition. (**B**) Representative pictures of sprout-like structures emerging from islets into the fibrin gel due to endothelial proliferation of resident islet endothelial cells.

4.11 Sitagliptin improves secretion of VEGF in transplanted islets

Since sitagliptin treatment improved transplantation outcome and moreover enhanced VEGF secretion, thereby increasing the sprouting capacity of isolated islets, we examined VEGF secretion from transplanted islets, which will lead to proliferation of host vascular endothelial cells(118). For this purpose grafts were retrieved and analyzed for the presence of VEGF protein by immunohistochemistry using anti-VEGF antibody. Sitagliptin treatment significantly ($p < 0.05$) increased the total VEGF expression compared to control (**Figure 15**). VEGF is essential for vasculogenesis- de novo formation of blood vessels(118) and our data indicate that sitagliptin treatment increases the secretion of VEGF improving vasculogenesis.

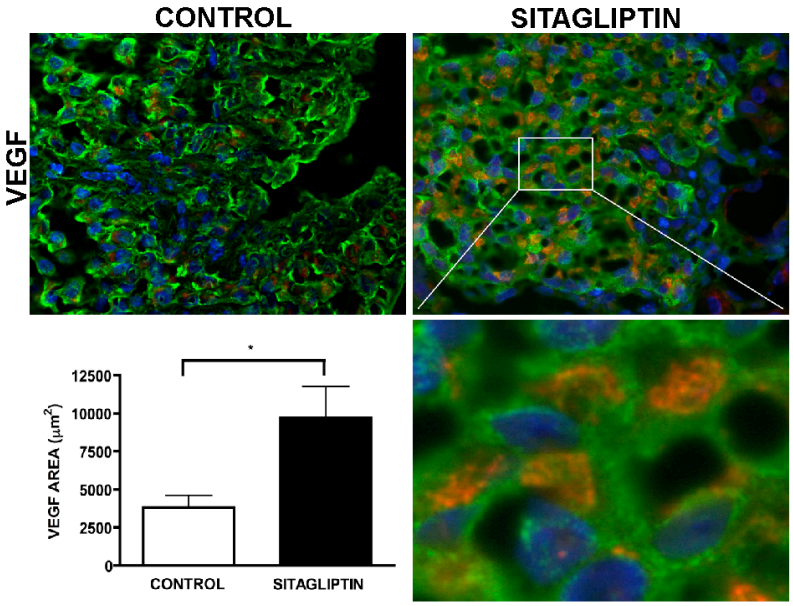

Figure 4-15: Sitagliptin treatment increases VEGF Expression. Sections were treated with Insulin and VEGF antibodies at 4°C overnight and then treated with secondary antibodies. The image was captured using Leica fluorescent microscope and analyzed using image J software. VEGF staining was more pronounced in the sitagliptin treated group compared to the control. The area showing VEGF staining was significantly ($p < 0.05$) increased in the sitagliptin group as represented by their respective bar graphs. n = 4; Data represents mean ± SEM.

4.12 Sitagliptin sensitizes VEGFR2 in transplanted islets

VEGF exerts its proangiogenic action by binding to VEGFR-2(119). To assess this, graft sections were analyzed for VEGFR2 expression using anti-VEGFR-2 antibody. We found that sitagliptin treatment significantly ($p < 0.05$) increased VEGFR-2 expression within the transplanted islets (**Figure 16**). This result indicates that the proangiogenic action of VEGF was further promoted by sensitization of VEGFR-2.

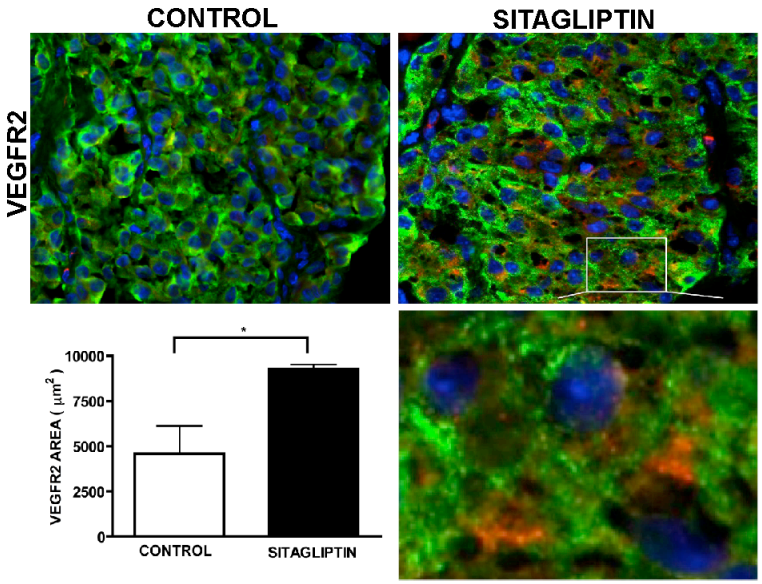

Figure 4-16: Proangiogenic action of VEGF is augmented by VEGFR-2 sensitization. Sections were treated with Insulin and VEGFR-2 antibodies at 4°C overnight and then treated with secondary antibodies. The image was captured using Leica fluorescent microscope and analyzed using image J software. VEGFR-2 staining was more pronounced in the sitagliptin treated group compared to the control. The area showing VEGFR-2 staining was significantly ($p < 0.05$) increased in the sitagliptin group as represented by their respective bar graphs. n = 4; Data represents mean ± SEM.

4.13 Sitagliptin activates CREB pathway

An earlier study reported that Glp-1 receptor agonists activate CREB and Insulin Receptor Substrate (IRS)-2, thereby promoting β-cell growth and survival(120). Similarly, a recent

report suggested that the protective role of VEGF-A on neurons and vascular endothelial cells is by CREB phosphorylation through VEGFR-2/ERK signaling(121). This prompted us to explore whether sitagliptin increases islet vascularization via CREB phosphorylation and VEFG/VEGFR-2 signaling. We retrieved kidney grafts and analyzed them for the activation of CREB by immunocytochemistry. Sitagliptin treatment enhanced CREB expression and phosphorylation compared to control. The number of nuclei with translocated CREB protein was significantly ($p < 0.01$) increased in the sitagliptin-treated group (**Figure 17 A, B**). These results indicate that DPP-IV inhibition induces vasculogenesis of transplanted islets by VEGF secretion through phosphorylation of CREB in transplanted β-cells.

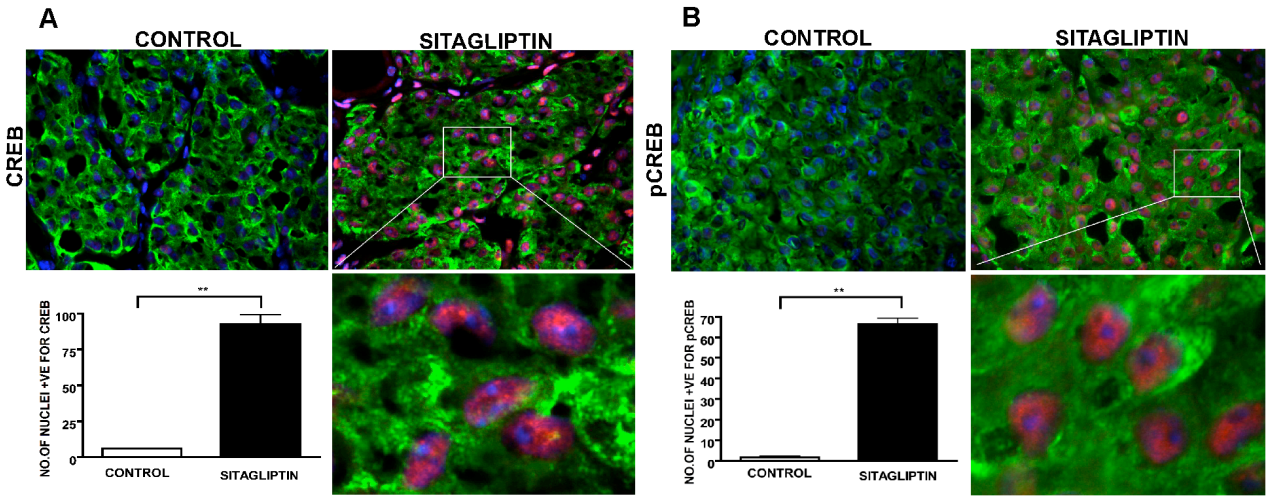

Figure 4-17: Sitagliptin phosphorylates CREB and aids in revascularization of islet grafts. Sections were treated with Insulin, CREB and pCREB antibodies at 4°C overnight and then treated with secondary antibodies. The image was captured using Leica fluorescent microscope and analysed using image J software. CREB **(A)** and pCREB **(B)** staining was more pronounced in the sitagliptin treated group compared to the control. The number of nuclei stained positive for CREB **(A)** and pCREB **(B)** was significantly ($p < 0.01$) increased in the sitagliptin group as represented by their respective bar graphs. n = 4; Data represents mean ± SEM.

4.14 Sitagliptin enhances mTOR and P70S6K expression

Since DPP-IV inhibition activated CREB in transplanted islets, we next studied the mTOR/P70S6K pathway that will lead to the cell growth and proliferation(122). For this we retrieved kidney grafts and analyzed them for the expression of pMTOR and P70S6K. Sitagliptin treatment increased the expression of pMTOR and P70S6K as evidenced by immunohistochemistry (**Figure 18A, B**).

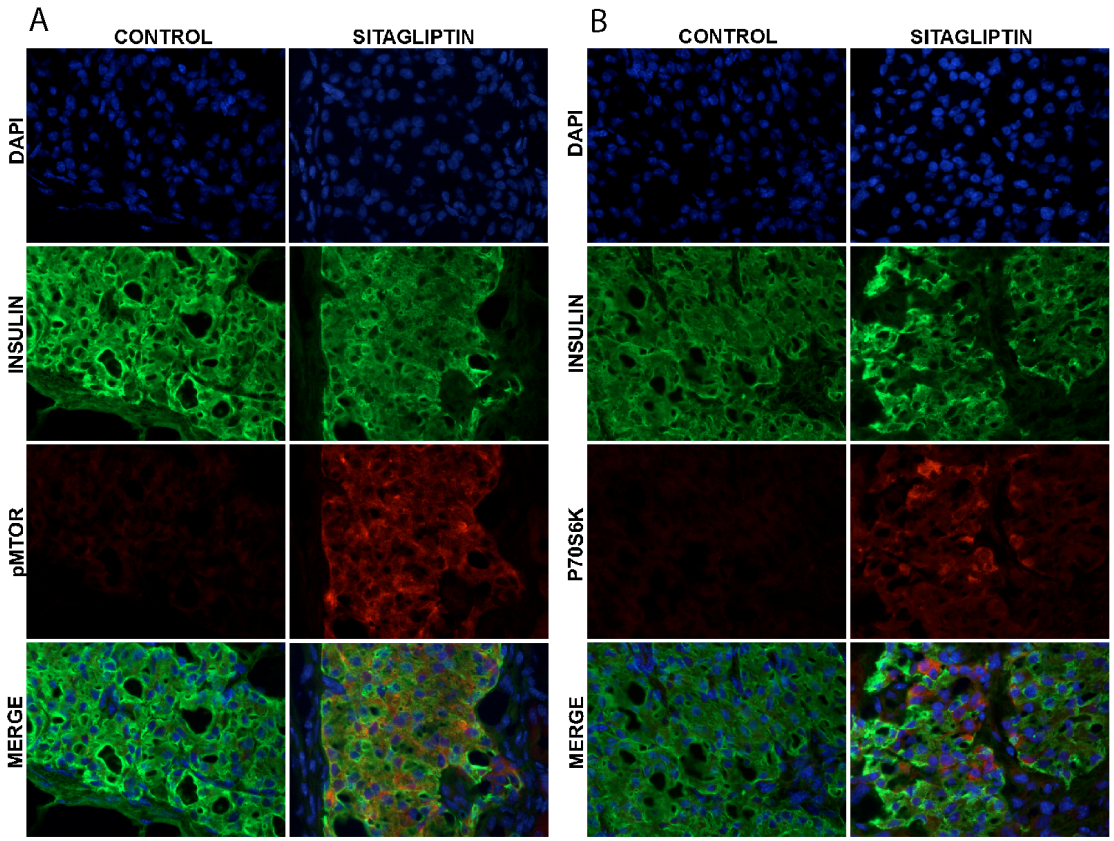

Figure 4-18: Sitagliptin phosphorylates mTOR and activates P70S6K. Sections were treated with Insulin, pMTOR **(A)** and P70S6K **(B)** antibodies at 4°C overnight and then treated with secondary antibodies. The image was captured using Leica fluorescent microscope.

5.Discussion

Sitagliptin is considered to enhance active GLP-1 levels by suppressing proteolytic DPP-IV activity resulting in increased β -cell survival in rodent models of diabetes and islet transplantation(112; 123-125). On the other hand, it has been demonstrated that the efficiency of pancreatic islet transplantation depends on re-vascularization. Therefore, we have investigated mechanisms underlying the effect of sitagliptin on partial pancreatectomy and pancreatic islet transplantation with focus on angiogenesis. Our data suggest that sitagliptin accelerates normalization of the blood glucose level in a diabetes model by stabilizing GLP-1, which in turn results in increased insulin production due to an increase in β-cell mass, VEGF expression and vascularization.

5.1 Sitagliptin promotes β-cell proliferation and insulin area in partial pancreatectomy

Partial pancreatectomy is the most commonly used model for the study of β-cell regeneration(126). Experimental evidence indicate that the β-cells were derived from preexisting islets rather than islet neogenesis(126). Our data suggest that the number of BrdU and insulin positive cells was significantly increased with sitagliptin treatment in partial pancreatectomised mice. Moreover, the insulin area was also significantly increased after sitagliptin treatment. There are controversies regarding the cellular origin of β-cell replication. In particular, the ductal progenitor hypothesis remains debated vigorously(51). Lineage tracing studies indicate that specialized progenitors do not contribute to adult β-cells, not even during acute β-cell regeneration after injury, but β-cells are the products of uniform self- renewal(127). The cell lineage tracing experiments show compelling evidence that pancreatic ductal epithelium does not contribute to β-cell growth during acute regeneration but may have originated from a source other than ductal epithelium(128). Our data suggest that sitagliptin improves the diabetic outcome by inhibiting DPP-IV activity and concentration. It also increased the number of BrdU positive β-cells and ductal cells and

insulin area in pancreatic sections. However, we cannot decide from our experiments whether ductal cells or β-cells are the main source of proliferation and enlarged insulin area.

5.2 The DPP-IV-Glp-1-VEGF axis

Pancreatic islets are highly vascularized and formation of their microvessels depends on the expression of VEGF in β-cells(129). Endothelial cells and microvessels are needed for coordinated insulin secretion of hundreds of β-cells within pancreatic islet(124; 125). Our results show that sitagliptin treatment increased the number of proliferating endothelial cells and also the area occupied by endothelial cells, indicating that actual vascularization takes place after islet transplantation. Studies in mutant mice with pancreas specific expression of VEGF in pancreatic islets have supported the idea of an association between the vascular system and β-cell mass. There is experimental evidence not only in pancreatic islet transplantation(129; 130) but also for streptozotocin-induced diabetes(92) and pregnancy(131). While endogenous VEGF released from grafted pancreatic islets was beneficial for transplantation outcome, prolonged pharmaceutical administration resulted in decreased islet functionality(132) and microvascular leakage(133). However, no link between GLP-1 receptor activation and VEGF release has been described so far.

Our data show that DPP-IV was highly active in islets isolated with collagenase. Inhibition of DPP-IV by sitagliptin accelerated normalization of the blood glucose level in a diabetes model by stabilizing GLP-1. This phenomenon correlated with enhanced insulin production due to an increase in β-cell mass. Interestingly, sitagliptin treatment also resulted in an increased expression of VEGF, improved vascularization and functional blood flow to the graft. This correlation *in vivo* raises the question whether vascularization is a direct or indirect effect of sitagliptin treatment. Sitagliptin treatment inhibits DPP-IV, which is known to cleave the hormone Glp-1 (7-36) into its inactive form Glp-1 (9-36). Glp-1 is secreted from the gut and has several functions including the regulation of insulin production(134). Our finding that VEGF secretion and endothelial cell sprouting is enhanced by inhibition of DPP-

IV in the presence of Glp-1 (7-36), but not in the presence of the cleavage product Glp-1 (9-36) indicates that the DPP-IV regulates VEGF expression via stabilization of Glp-1. Our data furthermore suggest that this axis controls both local and systemic availability of VEGF necessary for vascularization.

Our *in vitro* data demonstrated that Glp-1 alone significantly increased VEGF release from pancreatic islets. It is possible that *in vivo* DPP-IV inhibition activated additional pro-angiogenic pathways. There is not only cell membrane abundant peptidase but also a catalytically active soluble form. Basically, the enzyme would cleave dipeptides from all proteins with proline in the penultimate position, however *in vivo* proteolysis could be affected by modulating factors, for example glycation of the catalytic site due to hyperglycemia in models of diabetes(135). DPP-IV is believed to primarily modulate the availability of GLP-1, but other peptides cleaved by DPP-IV, such as GLP-2 or vasoactive peptide(136) could be involved. Chemical inhibition of DPP-IV was reported to reduce attraction of leukocytes in a T-lymphocyte induced immune response(137). However, recruitment of CD11b+ immune cells into the transplantation site was not different between treated and control in our model.

5.3 Potential intracellular links between GLP-1 receptor activation and VEGF synthesis in the β-cell

Islet cells are avascular after islet isolation and reduced islet vascularity immediately after islet transplantation contributes to the early loss of islets and its function(93).The classical activation of VEGF transcription is regulated by HIF-1α when oxygen concentrations decrease. VEGF synthesis can also be regulated by genetic rather than hypoxia-induced HIF-1α activation(138). In our study we observed increased gene expression of HIF-1α and ARNT. It was reported that the CREB binding protein (CBP), which in turn was activated by GLP-1 via the cAMP and Protein Kinase A pathway(139) had a stabilizing effect on the formation of the complex between HIF-1α and its DNA-binding partner, ARNT(140). Our

findings also showed an increased expression of PGC-1α mRNA levels, which is up regulated in proliferating islets(139) and increases VEGF synthesis through Estrogen-related Receptor-α.(102). Thus based on our data we suggest that the main mechanism of VEGF expression is GLP-1 stabilization by inhibition of DPP-IV through sitagliptin under conditions of pancreatic islet transplantation. The observed increase in proliferative activity of both β-cells and endothelial cells by DPP IV inhibition is attributed to the increase in the VEGF induced cell proliferation mediated by GLP-1.

5.4 Glp-1 receptor activation, CREB phosphorylation, mTOR activation and VEGF synthesis in β-cells by DPP-IV inhibition

The vascularization of islets could be increased by several interventions. One approach could be by increasing the proangiogenic factors responsible for formation, proliferation and maturation of islet endothelial cells into fully functional islet vasculature(89). This approach was demonstrated to increase functional islet mass by improving the angiogenesis of isolated porcine and murine islets(109). In our study we chose to inhibit DPP-IV so that the available Glp-1 could be used for activation of VEGF, thereby inducing vascularization of transplanted islets. VEGF is required for vasculogenesis(118) and our data demonstrate that sitagliptin treatment increased VEGF expression in transplanted islets. Moreover, experimental evidence suggests that the observed vascular endothelial cell response of VEGF was mediated by VEGFR-2(118). Our data shows that sitagliptin treatment enhanced the expression of VEGFR-2 in transplanted islets further supporting vascularization.

Interestingly, Glp-1R agonists activate CREB and Insulin Receptor Substrate (IRS)-2, promoting β-cell growth and survival(120). Also a recent finding suggests that mTOR is linked to the incretin signaling to HIF induction, promoting pancreatic islet viability(141). Similarly the protective role of VEGF-A on neurons and vascular endothelial cells is by phosphorylation of CREB through VEGFR-2/ERK signaling(121). Our data show that

sitagliptin treatment induced phosphorylation of CREB resulting in its activation of the VEGF-A/VEGFR-2 signaling pathway.

Thus we conclude that the main mechanism of VEGF expression and islet vascularization is Glp-1 stabilization by DPP-IV inhibition through sitagliptin followed by Glp-1R activation leading to phosphorylation of CREB and activation of mTOR, which in turn modulates VEGF-A/VEGFR-2 signaling thereby increasing the vascularization of transplanted islets. The observed increase in proliferative activity of both β-cells and endothelial cells by DPP-IV inhibition is attributed to activation of P70S6K mediated by increased availability of Glp-1(**Figure 19**).

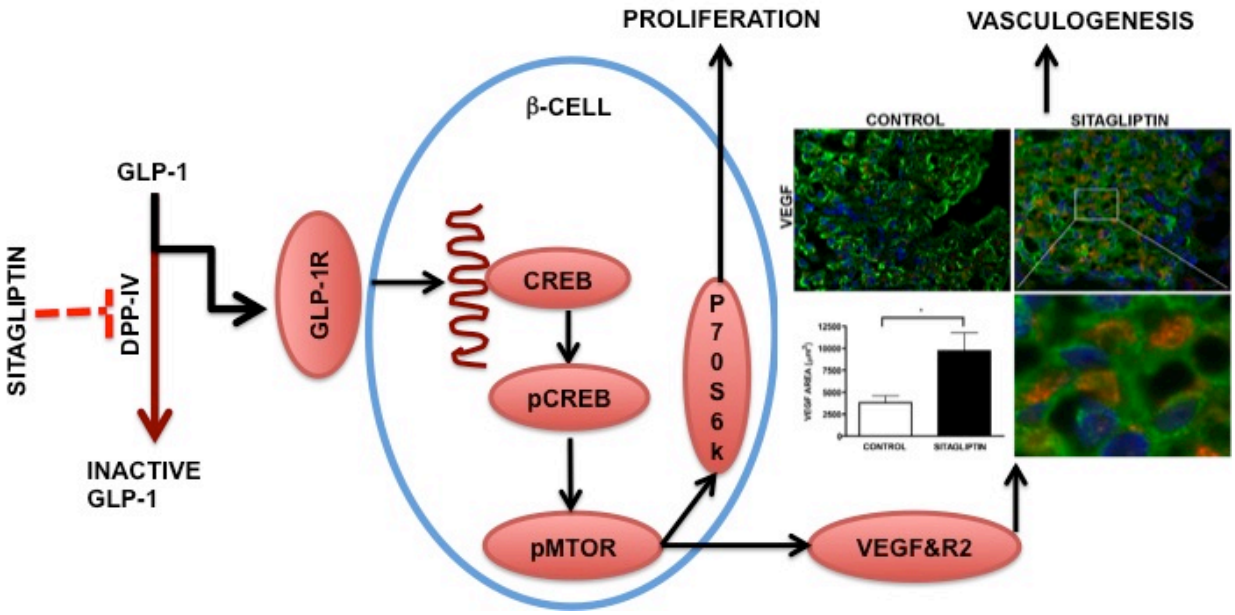

Figure 5-19: Model of CREB, mTOR and P70S6K activation and the VEGF-A/VEGFR-2 pathway regulating secretion of VEGF by DPP-IV inhibition in β-cells.

Taken together, we have discovered a new pathway by which sitagliptin improves vascularization of transplanted mouse, porcine and human islets. From a therapeutic perspective this mechanism could open new venues to prevent the decline of insulin secreting capacity in in pancreatic islet transplantation.

6. Reference

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7. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Place and Date

BALAJI SAMIKANNU

8. Appendix

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