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Effects of a carbohydrate restricted diet on the metabolic state and progressive pancreatic beta-cell loss in transgenic mice expressing a dominant negative GIP receptor

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Für meine Eltern

1. Introduction

Diabetes mellitus is a major health concern worldwide. Today about 7% of all germans suffer from diabetes and its accompanying diseases.

The number of diabetics in Germany has increased continually during the last four decades and experts estimate that the number will further rise to about 10 million until 2010 (Diabetes-Union 2004). Exhaustive scientific research in this area is necessary to uncover mechanisms of the disease and to generate new therapeutic strategies.

GLP-1 (glucagon-like-peptide 1) and also GIP (gastric inhibitory polypeptide or glucose-dependent insulinotropic polypeptide) belong to the incretin hormones which enhance the release of insulin after meal ingestion. Both GIP and GLP-1 were shown to act antiapoptotic and mitogenic on pancreatic beta-cells, both in vitro and in vivo. Transgenic mice expressing a dominant negative GIP receptor ($GIPR^{dn}$) under the control of the rat insulin 2 gene promoter have recently been shown to develop early onset diabetes mellitus and a severe reduction in pancreatic beta-cell mass, resembling a malformation of the endocrine pancreas (Herbach et al. 2005). These data confirm the importance of an intact GIP/GIPR-axis in glucose homeostasis and postnatal development of the endocrine pancreas.

Since nutrition is an important issue in diabetic patients and also one of several environmental factors causing diabetes, the advantage of a carbohydrate restricted nutrition for diabetics and for persons who are at high risk of developing diabetes is discussed frequently. The diabetic phenotype of $GIPR^{dn}$ transgenic mice can be ameliorated using a carbohydrate restricted diet, leading to an increased life-span as compared to transgenic mice fed a conventional diet (Herbach 2002). Therefore, at least in this mouse model of diabetes mellitus, the beneficial effect of a carbohydrate restricted diet seems proven.

Recent studies in healthy mice suggested that the number of islets is fixed during adult life and that islet neogenesis does not play a role in the expansion of beta-cell mass (Dor et al. 2004). It is rather believed that both islet and beta-cell hypertrophy occur in situations of increased insulin demand and diabetes mellitus (Weir et al. 2001). $GIPR^{dn}$ transgenic mice have been shown to exhibit a severely reduced pancreatic beta-cell mass and reduced islet neogenesis already at 10 days of age (Herbach et al. 2005). In order to get more insight into the morphological changes of

the endocrine pancreas of diabetic GIPR^{dn} transgenic mice, the islet and beta-cell numbers as well as the mean volumes of islets and beta-cells were determined, using state of the art quantitative-stereological methods. Non-transgenic healthy siblings served as controls and to determine the physiological postweaning development of islet and beta-cell numbers and mean volumes in a time course, where the beta-cell mass is known to double in rodents (Finegood et al. 1995).

A recent review suggested prevention of glucolipotoxicity by reducing glycemia, which is known to have beneficial effects on diabetes-associated beta-cell apoptosis, as one approach for diabetes therapy (Ahrén 2005). The aim of this study was therefore to analyze the effects of a carbohydrate restricted diet on glucose control and beta-cell function of GIPR^{dn} transgenic mice as compared to transgenic mice fed a breeding diet. Morphologically, in particular the effects of feeding the carbohydrate restricted diet on beta-cell numbers and frequency of apoptosis were determined under physiological conditions and in diabetic GIPR^{dn} transgenic mice as compared to wild-type and transgenic mice fed a conventional breeding diet.

For further characterization and better understanding of the animal model, the expression pattern of the transgenic GIP receptor in comparison to the endogenous murine GIP receptor was examined.

2. Literature review

2.1 Diabetes mellitus

2.1.1 Definition and description of diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action, or both.

Clinical symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening consequences of diabetes are hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome.

Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputation, and Charcot joints; as well as autonomic neuropathy causing gastrointestinal, genitourinary, cardiovascular symptoms and sexual dysfunction. Patients with diabetes show an increased incidence of atherosclerotic cardiovascular, peripheral vascular, and cerebrovascular disease (The Expert Committee on the diagnosis and classification of diabetes mellitus 2002).

2.1.2 Classification of human diabetes mellitus

The classification of human diabetes mellitus is updated to the latest scientific results. The terms insulin-dependent and non-insulin-dependent diabetes mellitus were eliminated for they classified the patients based on treatment rather than etiology.

The term malnutrition-related diabetes mellitus was also eliminated because it does not exist any evidence that diabetes can be directly caused by protein deficiency (The Expert Committee on the diagnosis and classification of diabetes mellitus 2002). The actual classification of the different types of diabetes mellitus is listed below.

I. Type 1 diabetes
   a) immune mediated
   b) idiopathic

II. Type 2 diabetes

III. Other specific types

   a) Genetic defects of β-cell function, for example
   1. MODY 1 – MODY 3

   b) Genetic defects in insulin action, for example
   1. Type A insulin resistance
   2. Leprechaunism

   c) Diseases of the exocrine pancreas, for example
   1. Pancreatitis
   2. Neoplasia

   d) Endocrinopathies, for example
   1. Cushing´s Syndrome
   2. Hyperthyroidism

   e) Drug- or chemical-induced, for example
   1. Glucocorticoids
   2. β-adrenergic agonists

   f) Infections, for example
   1. Congenital rubella
   2. Cytomegalovirus

   g) Uncommon forms of immune-mediated diabetes, for example
   1. “Stiff-man” syndrome
   2. Anti-insulin receptor antibodies

   h) Other genetic syndromes sometimes associated with diabetes, for example
   1. Down´s syndrome
   2. Klinefelter´s syndrome

IV. Gestational diabetes mellitus

2.1.3 Pathophysiology of type 1 diabetes mellitus

Type 1 diabetes mellitus is characterized by an autoimmune destruction of pancreatic beta-cells and a susceptibility for ketoacidosis. If there is no evidence for autoimmune mediated destruction of beta-cells, the cases are classified as type 1 idiopathic.

The autoimmune destruction of beta-cells has multiple genetic predispositions and is also related to environmental factors (The Expert Committee on the diagnosis and classification of diabetes mellitus 2002).

About 17 different loci of inherited susceptibility for type 1 diabetes are known. The major genetic determinant lies within the major histocompatibility complex. More than 90% of patients who develop type 1 diabetes have abnormalities in this locus (Devendra et al. 2004).

Several environmental factors that may lead to autoimmunity have been investigated. Different virus infections have been associated with the generation of autoantibodies against beta-cells, e.g. Enterovirus and Rubella. Common childhood vaccinations showed an induced autoimmunity in one study. This was not confirmed by other studies (Devendra et al. 2004). More recent studies reported a possible association of early feeding with gluten or cereals with risk of type 1 diabetes mellitus. The mechanism how ingestion of gluten or cereals may cause autoimmunity is not clear but it has been suggested that an aberrant immune response to cereal antigens in an immature gut immune system may be involved in susceptible individuals (Norris et al. 2003; Ziegler et al. 2003).

The rate of beta-cell destruction is variable in type 1 diabetes mellitus. In infants and children it is mainly rapid whereas in adults it is mainly slow. Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age. Patients often become dependent on insulin for survival (The Expert Committee on the diagnosis and classification of diabetes mellitus 2002).

2.1.4 Pathophysiology of type 2 diabetes mellitus

Type 2 diabetes mellitus is characterized by insulin resistance and beta-cell dysfunction resulting in a relative insulin deficiency. Insulin sensitivity of different tissues is influenced by many factors such as genetics, age, acute exercise, nutrition and obesity (DeFronzo 1979; Barnett et al. 1981; Bogardus et al. 1985; Chen et al.

1987; Prigeon et al. 1995). First it was thought that insulin resistance is the primary abnormality causing beta-cell dysfunction and exhaustion due to a prolonged, increased secretory demand of insulin (DeFronzo and Ferrannini 1991; Leahy et al. 1992). Later it became clear that reductions in both insulin sensitivity and beta-cell function are present early in the course of the development of type 2 diabetes (Kahn 2003). Beta cell dysfunction is characterized by a decrease of insulin secretion in response to intravenous glucose (Perley and Kipnis 1967), a lack of the first phase insulin secretion and a diminished second phase (Donath and Halban 2004), alterations in the pulsatile pattern of insulin secretion (O´Rahilly et al. 1988) and an inefficient processing of proinsulin to insulin (Kahn and Halban 1997; Sempoux et al. 2001).

The increased insulin resistance leads to an increased demand of insulin. The pancreatic beta-cells are not able to compensate for this enhanced demand, resulting in a relative insulin deficiency and hyperglycemia. The reasons for the insufficient secretion of insulin remain unclear and are therefore discussed controversially in literature. A loss of beta-cell mass has been suggested by many authors while others did not support this view (see chapters 2.4 and 2.5).

Type 2 diabetes mellitus is usually diagnosed years after the onset of pathological processes in the pancreatic beta-cells. Hyperglycemia develops only gradually and is at earlier stages often not severe enough for the patient to notice any of the classic symptoms of diabetes mellitus (The Expert Committee on the diagnosis and classification of diabetes mellitus 2002). The reason for this slow onset of the disease is that pancreatic beta-cells can temporarily adapt to the increased demand of insulin. The deterioration of beta-cell function is thought to occur in four different phases ranging from successful adaptation to the increased demand via mild and severe decompensation to decompensation with structural damage. Many different factors are thought to play a role in the damage of beta-cells, such as islet amyloid, glucotoxicity and lipotoxictity (Weir et al. 2001).

2.2 Nutrition and diabetes mellitus

Dietary measures are an important part in diabetes therapy and possibly also a means of prevention for persons who have a high risk for developing diabetes.

Some of the decisive factors for nutrition of diabetic patients are outlined here.

2.2.1 The glycemic index (GI)

The glycemic index was introduced as a means to compare carbohydrate-containing foods due to their glycemic responses. The GI is expressed as a percentage of the AUC (area under curve) glucose, following ingestion of a standard amount of food in comparison to the glycemic excursion, following an equivalent amount of a carbohydrate standard (glucose or white bread) (Jenkins et al. 1981).

The GI of foods is determined by the nature of its carbohydrates, its composition and the food processing (e.g. cooking) (Jenkins et al. 1981; Brand et al. 1985).

Diets low in GI were soon found to have a positive effect on glucose metabolism. In healthy adults, a low GI diet reduced serum fructosamine concentrations, 12-hour blood glucose profile, total serum cholesterol and 24-hour urinary C-peptide excretion. (Jenkins et al. 1987). In type 2 diabetic patients, a reduction in plasma glucose, serum cholesterol, and LDL cholesterol was observed. Insulin sensitivity and the capacity for fibrinolysis were enhanced by low GI diets (Järvi et al. 1999).

A study in type 1 diabetic patients revealed that a low GI diet improves the daily blood glucose profile, reduces HbA1c levels and the number of hypoglycemic events. No effect on fasting plasma triglycerides, cholesterol, and HDL cholesterol levels was observed. The diet used in this study also contained a high amount of dietary soluble fiber, probably adding to the beneficial effect of the low GI (Giacco et al. 2000).

Low GI diets not only affect the actual meal positively but also improve glucose tolerance to the following meal. The reason for this effect is unknown (Jenkins et al. 1980; Wolever et al. 1988).

Two pathways have been proposed through which low GI diets may exert their effect: First, low GI foods induce lower blood glucose concentrations and therefore lower insulin secretion than do high GI foods with the same amount of carbohydrates (Willett et al. 2002). Second, foods with a high glycemic index led to an increased insulin resistance in rats (Byrnes et al. 1995). Furthermore, low GI foods generally have a higher content of resistant starch and dietary fiber, leading to a delayed glucose absorption (Järvi et al. 1999; Mann 2001).

Prospective studies indicate that low GI diets may also reduce the risk of type 2 diabetes mellitus (Salmeron et al. 1997; Schulze et al. 2004).

Thinking of the diminished first-phase insulin response in type 2 diabetes mellitus, the advantage of a delayed glucose absorption is obvious.

2.2.2 Dietary fiber

Since a low GI diet is often associated with a high content of dietary fiber, many studies concentrated on the effects of high-fiber diets. Dietary fiber is defined as the sum of polysaccharides and lignin that are resistant to enzymes produced in the human gastro-intestinal tract, and therefore pass the small intestine without being hydrolyzed or absorbed (Asp et al. 1981).

Much of the interest in dietary fiber and diabetes has focused on the effects of soluble fiber, such as pectin, and guar-gum (Asp et al. 1981).

In type 2 diabetic patients, a diet containing high amounts of soluble fiber reduced basal and postprandial hyperglycemia, urinary glucose excretion, and mean serum total- and LDL-cholesterol (Aro et al. 1981; Ray et al. 1983).

A later study in which diets with an average fiber content (24g/day total, 8g soluble) and a high fiber content (50g/day total, 25g soluble) were compared, confirmed an improved glycemic control after intake of the high-fiber diet (Chandalia et al. 2000). However, the positive effect can not be attributed to the soluble fibers alone.

A study that investigated the effect of whole grains revealed an improve in insulin sensitivity in overweight hyperinsulinemic adults. Most of the fiber in the whole-grain foods was insoluble, suggesting that soluble fiber may not be necessary to improve insulin sensitivity (Pereira et al. 2002).

Soluble fibers reduce GIP (glucose-dependent insulinotropic polypeptide) and enhance GLP-1 (glucagon-like-peptide 1) responses after ingestion. This is probably caused by a lower amount of absorbable carbohydrates (Morgan et al. 1979). Furthermore, some soluble fibers are able to slow absorption of food by creating a gel-like substance (Anderson 1986).

In spite of the positive effects, the intake of soluble fiber was found to be unrelated to the risk of diabetes in older women (Meyer et al. 2000).

In type 1 diabetes mellitus, both soluble and insoluble fibers yielded a significant inverse association with HbA1c levels. In multivariate stepwise regression, only the soluble fiber fraction was found to significantly predict HbA1c levels (Buyken et al. 1998).

Ingestion of insoluble fiber was shown to cause an accelerated insulin response, associated with an earlier response of biologically active GIP in healthy subjects. GLP-1 levels remained unaffected (Weickert et al. 2005).

In type 1 diabetes, the prevalence of severe ketoacidosis fell significantly with higher intakes of total fiber. In stepwise logistic regression, only the insoluble fiber fraction was found to significantly predict the risk of severe ketoacidosis. However, a higher fiber intake does not seem to provide much additional protection for patients in whom severe ketoacidosis is a less common complication (Buyken et al. 1998).

In prospective analysis, the intake of insoluble fiber was inversely associated with risk of type 2 diabetes mellitus (Meyer et al. 2000; Montonen et al. 2003).

In contrast to these results is the study of Juntunen et al. (2002) who suggest that the positive effects of high-fiber diets do not depend on the fiber content, but on the structural properties of the food.

They compare two types of rye bread, one with a high content of whole kernels and one with a high content of soluble fiber (i.e. oat β-glucan). Both breads led to a reduction in the insulin response after ingestion when compared to a control bread, as well as to a lowered GIP and GLP-1 response.

The authors therefore propose that the structural and compositional properties of fiber play more of a role in the regulation of the insulin response than the amount of fiber. This is in agreement with the study of Järvi et al. (Järvi et al. 1999) who found that consumption of a diet with a low GI and a preserved food structure improved glucose and insulin responses.

Pereira et al. (2002) compared the effects of diets high in either whole grain products or refined grain products and found that insulin sensitivity was increased after intake of the whole-grain foods.

In a 10 year follow up study, the inverse association between the intakes of total grain and whole grains and the incidence of type 2 diabetes mellitus was observed. High fiber intake was associated with a reduced risk of type 2 diabetes mellitus (Montonen et al. 2003). Additionally, prospective cohort studies have shown an inverse association between dietary fiber consumption and risk of type 2 diabetes mellitus. Cereal fiber appeared to contribute most to the positive effect (Salmeron et al. 1997; Meyer et al. 2000; Montonen et al. 2003; Schulze et al. 2004).

In summary, it is very difficult to examine the effects of either soluble or insoluble fiber independently from each other and from the structure of food that also seems to play an important role in the positive effect of high fiber diets on human diabetic patients. Probably both fiber types contribute to the improvement of glucose homeostasis via their different mode of operation.

Nevertheless, it is obvious that glucose homeostasis can be improved by dietary measures.

2.3 Enteroinsular axis and incretin hormones

2.3.1 Definition of the enteroinsular axis

The connection between the gut and the pancreatic islets was named “enteroinsular axis” by Unger and Eisentraut in 1969.

Later on Creutzfeldt (1979) defined that this enteroinsular axis consists of nutrient, neural and hormonal signs from the gut directed at the pancreatic islets (Creutzfeldt 1979). Furthermore he determined the criteria an incretin hormone has to fulfill: first it must be released by nutrients, particularly carbohydrates and second it must stimulate insulin secretion at physiological levels in the presence of elevated blood glucose levels.

Today, two hormones have been discovered that fulfill these criteria: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like-peptide-1 (GLP-1).

2.3.2 Incretin hormones

2.3.2.1 GIP

The human GIP gene is located on the long arm of chromosome 17 and consists of six exons and five introns (Fehmann et al. 1995). A large precursor peptide of 153 amino acids is transcribed, translated and then processed by trypsin-like cleavage mainly to the biologically active form GIP1-42 (Tseng et al. 1992).

The amino acid sequence of human GIP shows a high degree of sequence homology to porcine, bovine and rat GIP.

GIP is produced in the K-cells, distributed in the small intestine, mainly in the duodenum and upper jejunum in humans. In rats, K-cells can also be found in the ileum and immunoreactive GIP is also found in the rat submandibular salivary gland, although GIP protein levels were only one-eighth of those found in the duodenum (0.28 ± 0.05 vs. 2.22 ± 0.26 ng per mg of tissue) (Tseng et al. 1992).

GIP is released into the blood after nutrient intake. Most of all, long-chain fatty acids and carbohydrates are secretagogues for GIP.

Its insulinotropic activity is strictly glucose-dependent, requiring a threshold value of approximately 6mmol/l, thereby forming a safeguard against hypoglycemia (Creutzfeldt and Nauck 1992).

In the pancreatic beta-cell, GIP exerts its insulinotropic effect by stimulating insulin secretion, proinsulin gene transcription and insulin biosynthesis (Fehmann et al. 1995). In studies using GIP antagonists, GIP-induced insulin secretion is inhibited by up to 86% in cell culture (Gault et al. 2003). Another study examined the effects of a GIP receptor antagonist administered to rats and found that the postprandial insulin release was decreased by 72% (Tseng et al. 1996 a).

A mitogenic and antiapoptotic effect of GIP on beta-cells was shown in vitro and in vivo (Trümper et al. 2001; Trümper et al. 2002; Hansotia and Drucker 2004 a). GIP is able to enhance glucagon secretion but only at blood glucose levels of 5 mmol/l or lower, thus not at physiological concentrations. It was shown that GIP augments somatostatin secretion in the perfused rat pancreas (Fehmann, 1995).

GIP stimulates lipoprotein lipase activity and fatty acid synthesis in adipose tissue. Under diminished insulin action this effect is even more pronounced (Zhou et al. 2005). Furthermore, it inhibits glucagon-induced lipolysis and increases insulin-binding affinity in adipocytes (Yip et al. 1998).

GIP reduces glucagon-stimulated hepatic glucose production, an effect that is augmented in the presence of insulin (Fehmann et al. 1995).

A role of GIP-dependent cortisol secretion is discussed in patients with nutritional Cushing´s syndrome (Groussin et al. 2002; Swords et al. 2005).

After it was discovered that GIP inhibits gastric acid secretion in man only at supraphysiological concentrations, the original name of GIP, gastric inhibitory polypeptide, was switched to the now common name glucose-dependent insulinotropic polypeptide (Fehmann et al. 1995).

The enzyme dipeptidyl peptidase IV (DPPIV) is responsible for the N-terminal degradation of GIP in vivo, yielding GIP (3-42) as the primary metabolite (Deacon et al. 2000).

DPPIV is a cell-surface protease that can be found in the kidney, intestinal brush-border membranes, hepatocytes, endothelial cells and in a soluble form in plasma (Deacon 2004). Degradation of GIP occurs rapidly, resulting in a half-life of approximately 7 min (Deacon et al. 2000).

2.3.2.2 GLP-1

Glucagon-like peptide 1 (GLP-1) is processed from the proglucagon gene in intestinal L-cells. In humans, pigs and rats, L-cells are located in the ileum, colon and rectum (Creutzfeldt and Nauck 1992; Fehmann et al. 1995).

The proglucagon gene is composed of six exons and five introns, located on the long arm of chromosome two (Fehmann et al. 1995). Four isoforms of GLP-1 are processed from the gene with only GLP-1 (7-37) and GLP-1 (7-36) amide showing an insulinotropic effect (Habener 1993).

The amino acid sequence of GLP-1 is identical in all mammals (Fehmann et al. 1995).

Since L-cells are predominantly positioned in the distal parts of the gut, GLP-1 has been questioned as incretin hormone (Fehmann et al. 1995). However, GLP-1 release is prompt after oral ingestion of a mixed meal. Therefore nutrients, the autonomic nervous system and humoral mediators have been discussed to control GLP-1 release (Fehmann et al. 1995). In rats it has been proven that infusion of exogenous GIP in physiological doses stimulates secretion of intestinal proglucagon-derived peptides. This could not be confirmed in human studies (Roberge and Brubaker 1993).

The insulinotropic action of GLP-1 is tightly regulated by blood glucose levels, requiring a glucose threshold of approximately 108 mg/dl (Creutzfeldt and Nauck 1992). If blood glucose levels fall below 90 mg/dl, GLP-1 action is attenuated, below 50 mg/dl, GLP-1 effects are completely abrogated (Habener 1993). GLP-1 has a mitogenic effect on beta-cells. It was shown to increase islet cell proliferation and islet mass when administered to Umea+/? normoglycemic mice (Edvell and Lindström 1999). In rats, GLP-1 was found to increase beta-cell replication, neogenesis of beta-cells and beta-cell mass (Xu et al. 1999). Furthermore, treatment of ZDF (zucker diabetic fatty) rats with GLP-1 led to a decreased number of apoptotic beta-cells (Farilla et al. 2002).

Somatostatin secretion from delta-cells is stimulated by GLP-1, whereas glucagon secretion from alpha-cells is inhibited. Gastric motility and acid secretion are lowered by GLP-1. GLP-1 augments lipolysis and lipogenesis in adipose tissue (Habener 1993). A role of GLP-1 in central neurotransmission has been discussed (Fehmann et al. 1995).

GLP-1 is even more sensible to degradation by DPPIV than GIP, resulting in a half-life of 1-1.5 min (Deacon et al. 2000).

2.3.3 Incretin hormone receptors

2.3.3.1 Structure and expression of incretin hormone receptors

The GIP and GLP-1 receptor (GIPR and GLP-1R) both belong to the class two of G-protein coupled receptors along with the secretin, glucagon, GLP-2 and growth-hormone releasing hormone receptors.

The receptors are named after their principal and only physiological relevant ligand, e.g. GIP receptor, secretin receptor. None of the related peptide ligands show a significant biological meaningful cross-reaction with other receptors than their own.

Structurally, the G-protein coupled receptors form a seven transmembrane-spanning protein, containing a disulfide bond that links the first and second extracellular loop, a signal peptide and a large N-terminal portion important for ligand binding (Mayo et al. 2003).

The human GLP-1R gene is located on chromosome six. 13 exons can be found in the GLP-1 gene of humans, mice and rats. It is translated into a 463 amino acid sequence (Fehmann et al. 1995). 90% sequence homology has been found among the human and the rat GLP-1R (Brubaker and Drucker 2002).

The human GIPR gene has been mapped to chromosome 19 with 13 exons identified. The length of the amino acid sequence is 455 in rats, 462 in hamsters and 466 in humans (Brubaker and Drucker 2002; Mayo et al. 2003). The murine GIP receptor is located on chromosome 7 and consists of 460 amino acids (http://www.signaling-gateway.org).

Both receptors are widely expressed throughout the organism. The GLP-1R mRNA has been isolated from pancreatic islets, brain (Fehmann et al. 1995), stomach, intestine, hypothalamus, heart, lung, liver and kidney in rats (Kieffer and Habener 1999). In dogs, the GLP-1R was also detected in adipose tissue and skeletal muscles (Mayo et al. 2003).

GIPR expression was found in rat pancreatic beta-cells, stomach, duodenum, proximal small intestine, adrenal gland, pituitary and brain (telencephalon, diencepahlon, brainstem and cerebellum) by RT-PCR (Usdin et al. 1993). By means of in situ hybridization, the receptor was discovered in heart, lung and endothelium of

major blood vessels in rats (Usdin et al. 1993). Furthermore, the GIPR is expressed on rat adipocytes (Yip et al. 1998) and osteoblasts in mice (Xie et al. 2005).
Functional GIP receptors are present in hyperplastic adrenal cells of Cushing´s disease but not in healthy humans. This is in contrast to rats, where the receptor is widely expressed in the adrenal gland physiologically (Groussin et al. 2002; Swords et al. 2005).

2.3.3.2 Signal transduction

Both, the GIP and the GLP-1 receptor are coupled to a heterotrimeric G protein through which they predominantly stimulate cAMP production upon ligand binding (Tseng and Zhang 1998). Different domains in the third intracellular loop are responsible for the specific coupling of the G protein (Mayo et al. 2003). cAMP potentiates the glucose-induced closure of ATP-sensitive potassium channels (K_{ATP}), resulting in cellular depolarization, activation of voltage-dependent Ca^{2+} channels (VDCC), and influx of Ca^{2+}. The rise of intracellular Ca^{2+} levels then triggers exocytosis of insulin (Habener 1993; Lu et al. 1993; Kieffer and Habener 1999).
Additionally, it has been proven that GIP stimulates activation of mitogen-activated protein kinase (Kubota et al. 1997), phospholipase A2 (Ehses et al. 2001) as well as the phosphatidylinositol 3-kinase/protein kinase B pathway (Trümper et al. 2001).

2.3.3.3 Homologous desensitization of the GIP receptor

G-protein coupled receptors undergo rapid homologous desensitization.
Desensitization of G-protein coupled receptors can be mediated by four different mechanisms:

- receptor internalization (Böhm et al. 1997)
- down-regulation after long-term continuous exposure to agonist (Böhm et al. 1997)
- uncoupling from G-protein by receptor phoshporylation mediated by GRKs (G protein-coupled receptor kinases) (Böhm et al. 1997; Tseng and Zhang 2000)
- interaction of RGS (regulators of G protein signalling) with G protein subunits (Tseng and Zhang 1998)

Different studies show that all four mechanisms may play a role in the desensitization of the GIPR. Receptor internalization is a short-term regulatory process occurring within minutes in response to GIP (Hinke et al. 2000).
At high glucose levels, GIP becomes ineffective. Down regulation of GIP mRNA levels with a statistically significant difference can be measured after only six hours exposure of INS(823/13) cells to high glucose concentrations (Lynn et al. 2003).
The role of GRKs in GIPR desensitization was shown in a study of Tseng et al. (2000). Overexpression of GRK2 or β-arrestin-1 in βTC3 cells clearly reduced the insulinotropic effect of GIP (Tseng and Zhang 2000). Additionally, RGS (regulators of G protein signalling) have been shown to play an important role in the desensitization of the GIP receptor. In this process, RGS proteins act as guanosine triphosphatase-activating proteins to decrease the half-life of the activated Gα subunit. Overexpression of the RGS2 protein in L293 cells transfected with GIP receptor cDNA resulted in an attenuation of GIP-stimulated cAMP production (Tseng and Zhang 1998).

2.3.4 The role of incretins in diabetes mellitus

The pathophysiology of type 2 diabetes mellitus in humans involves a reduced incretin effect (Creutzfeldt and Nauck 1992; Nauck et al. 2004).
First it was thought that only the almost absent insulinotropic effect of GIP was the cause for the impaired incretin effect. Exogenous human GIP barely stimulates insulin secretion in type 2 diabetic patients even at supraphysiological concentrations, whereas the insulinotropic action of GLP-1 is preserved (Creutzfeldt and Nauck 1992; Nauck et al. 1993).
Later, GLP-1 secretion was found to be reduced in type 2 diabetic patients compared to healthy humans. Healthy subjects showed a significantly higher and more prolonged GLP-1 (intact) response after meal ingestion, with a maximum around 90-120 minutes, whereas levels of intact GLP-1 in patients were down to preprandial levels at 75-120 minutes. Since 24-hour GLP-1 secretion is normal in first-degree relatives of diabetic patients, the deficiency in GLP-1 secretion is probably a consequence of the diabetic condition rather than the cause (Vilsboll et al. 2001).
Different mechanisms of the reduced GIP effect have been discussed.

Tseng et al. (1996) examined streptozotocin diabetic rats displaying almost four-fold elevated fasting serum glucose levels and decreased fasting serum insulin levels when compared to healthy rats. The serum GIP concentration was nine-fold increased and duodenal GIP mRNA was 80% higher in diabetic vs. control rats. Diabetic rats also showed a reduced insulinotropic effect of GIP, whereas the GLP-1 effect was undisturbed. These findings led to the assumption that the GIPR undergoes homologous desensitization as a consequence to the high levels of GIP (Tseng et al. 1996).

However, serum GIP levels in type 2 diabetic humans are reported controversely. In different studies, GIP levels were found to be either increased, normal or even decreased when compared to healthy subjects (Creutzfeldt and Nauck 1992).

Holst et al. (1997) proposed that beta-cells in type 2 diabetic patients do not express the GIPR or express a defective GIPR. However, no mutations of the GIPR gene, associated with type 2 diabetes mellitus were found (Holst et al. 1997).

A significant decrease (75 ± 5%) of GIPR mRNA in the pancreatic islets along with a marked decrease of GIP receptor protein levels were found in VDF (Vancouver diabetic fatty) rats (Lynn et al. 2001). Additionally, no cAMP response to GIP was observable in isolated islets of VDF rats. In contrast to streptozotocin diabetic rats, no elevation in serum GIP levels was detected in VDF rats. Consequently, the loss of GIPR was not caused by down-regulation after prolonged stimulation by the ligand. Instead, the downregulation of the GIPR expression could have been caused by the high levels of blood glucose (Lynn et al. 2003) (See chapter 2.3.3.3).

However, the theory of a diminished expression of the GIPR on beta-cells or an expression of a defective GIPR in diabetic humans was neglected later, since recent studies reported a relatively well-preserved insulinotropic effect of GIP when administered as a bolus injection (Nauck et al. 2004).

In several studies, the early phase (0-20min) of insulin secretion in diabetic patients after meal ingestion was markedly decreased and delayed but both incretins could augment this early response when administered externally. In contrast, GIP had very little effect on the late phase insulin response (20-120min), while GLP-1 was still capable to induce normal beta-cell response to glucose in diabetic patients (Vilsboll et al. 2002; Vilsboll et al. 2003). However, insulin secretion in type 2 diabetic patients can still be stimulated by GIP if the peptide is administered as a bolus (Meier et al. 2004). After the insulinotropic effect of GIP in physiological doses was found to be

reduced by 50% in glucose tolerant first-degree relatives of type 2 diabetic patients compared to healthy subjects (Meier et al. 2001) it was argued that a GIP postreceptor defect of the intracellular machinery, that could be genetically determined, would be the most reasonable explanation for the reduced GIP effect (Vilsboll et al. 2002; Nauck 2004 a).

Vilsboll et al. (2003) showed that diabetic patients with completely different etiology, eg. maturity-onset diabetes of the young (MODY 3), latent autoimmune diabetes in adults (LADA) and type 1 diabetes mellitus, were similarly incapable of responding to GIP while a significantly higher response to GLP-1 was seen. Therefore, the assumed GIP postreceptor defect is probably secondary to diabetes rather than genetically determined (Vilsboll et al. 2003).

The variety of the results that led to different explanations indicates that further investigations are needed to shed light on the mechanisms involved in the role of incretin hormones in the pathophysiology of diabetes mellitus.

2.3.5 Animal models for incretin research

2.3.5.1 GIPR^{dn} transgenic mice

Transgenic mice expressing a dominant negative GIP receptor were created by Volz in 1997 (Volz 1997). The aim was to generate a mutated human GIP receptor that shows unchanged binding affinity for GIP but is unable to induce signal transduction. The mutated GIP receptor was then expressed in pancreatic beta-cells of mice under the control of the rat insulin gene 2 promoter. GIPR^{dn} transgenic mice were found to develop a severe diabetic phenotype (Volz 1997; Herbach 2002; Herbach et al. 2005).

This new animal model of diabetes mellitus allows to examine the effects of the expression of a defective GIP receptor on glucose homeostasis and the endocrine pancreas. Different targeted mutations were introduced into different copies of the cDNA of the human GIP receptor:

a) deletion of 24 base pairs, position 955-978
b) point mutation Ala -> Glu, position 1018-1020
c) both mutations at once

The three mutations were cloned into the area of the cDNA that codes for the third intracellular loop of the GIP receptor. This sequence of G-protein coupled seven-transmembrane receptors is essential for signal transduction (Lefkowitz and Caron 1988)

The cDNA fragments were cloned into the shuttle expression vector pTEJ-8. In this vector, the cDNA is under control of the human ubiquitin promoter which guarantees a ubiquitous strong expression.

First, functional analyses were performed in vitro with CHL-cells expressing the native human GIP receptor and with cells expressing the three mutated receptors.

Examination of the binding affinity of the three mutated receptors showed that the receptors with the deletion of 24 bp and double mutation exhibited nearly the same affinity as the wildtype receptor while the receptor with the point mutation had a much lower affinity for GIP:

- K_D= 0,22 x 10^{-9} M wildtype receptor
- K_D= 0,35 x 10^{-9} M receptor with double mutation
- K_D= 0,44 x 10^{-9} M receptor with deletion of base pairs
- K_D= 0,79 x 10^{-7} M receptor with point mutation (Volz 1997)

Therefore, the receptor with the single point mutation was excluded from further investigations.

In CHL-cells expressing the receptor with the deletion of 24 bp, a low increase of cAMP levels was measurable after stimulation with GIP (40% above basal values). Cells expressing the receptor with both mutations showed no increase in cAMP levels.

Therefore, this receptor fulfilled all demands of a dominant negative receptor and was chosen for generating transgenic mice.

The cDNA construct with the mutated GIPR was then isolated from the pTEJ-8-vector and cloned into the pGEM-7-vector. This vector possessed the necessary *Xba*I and *Hind*III sites for cloning the fragment into the RIP-1 vector. This vector has been successfully used to achieve beta-cell specific expression of cDNA constructs in mice (Hanahan 1985). Regulatory sequences are the rat insulin gene promoter 2 and the termination sequence of the SV40 t-antigen.

Prokaryotic sequences were eliminated by restriction with the enzymes *Sal*I and *Ssp*I. The resulting fragments were separated using an ethidiumbromide-free gel.

After further purification and dialysis, the RIP GIPR^{dn} cDNA construct was used for microinjection.

After cyle synchronisation and superovulation female mice were mated with male mice. The fertilized zygotes were isolated and the construct was transferred to the male pronucleus by microinjection. Afterwards the zygotes were positioned into the tube of pseudo-pregnant female mice. 57 offspring were born and twelve of them were identified GIPR^{dn} transgenic mice by PCR.

Transgenic mice expressing the dominant negative GIP receptor were recently characterized clinically and pathomorphologically (Herbach 2002; Herbach et al. 2005).

GIPR^{dn} transgenic mice were found to develop severe diabetes mellitus. The onset of diabetes mellitus in these mice was found to occur between 14 and 21 days of age, probably due to the beginning intake of rodent chow. Fasting and postprandial blood glucose levels were significantly elevated in transgenic mice in comparison to healthy control mice, while serum insulin concentrations were significantly lower (18.8-fold in male transgenic mice and 6.1-fold in female transgenic mice), demonstrating an absolute insulin deficiency.

Transgenic mice showed severe hyperphagia, polydipsia and polyuria when compared to control mice.

The composition of pancreatic islets was disturbed accompanied by a changed distribution of the endocrine cells in islets. A shift towards endocrine non-beta-cells was determined in transgenic mice. The total volume of islets, beta-cells and isolated beta-cells were dramatically reduced in transgenic mice, while the total volume of alpha-cells was increased.

In addition, GIPR^{dn} transgenic mice showed a drastically reduced life-span (male mice: 360 ± 17 days vs. 192 ± 24 in transgenic mice; female mice: 346 ± 28 vs. 239 ± 16 in transgenic mice)(Herbach 2002).

2.3.5.2 GIP receptor knockout (GIPR $^{-/-}$) mice

The function of the GIP receptor was knocked out by replacement of the exons four and five that code for the N-terminal extracellular region using the PGK-neo cassette, thereby creating GIPR$^{-/-}$ mice (Miyawaki et al. 1999).

$GIPR^{-/-}$ mice show no abnormalities in general behaviour or feeding. Plasma glucose, HbA1c, plasma triglycerides, total cholesterol and free fatty acid levels in plasma did not differ from wildtype mice.

During an intraperitoneal glucose tolerance test (IPGTT), no difference in glucose tolerance was observed in knockout mice vs. wild-type mice. No differences in either fasting plasma glucose levels or fasting insulin levels, were observed between control and $GIPR^{-/-}$ mice. However, peak levels of blood glucose were delayed and maximum levels of blood glucose were significantly higher in knockout mice compared to wild-type mice during OGTT. Additionally, insulin levels were significantly lower in knockout mice vs. wild-type mice 15 minutes after glucose challenge (641 ± 54 pg/ml vs. 1101 ± 68 pg/ml).

Thus, $GIPR^{-/-}$ mice display glucose intolerance after oral glucose administration and impaired glucose induced insulin secretion, the later being typical for type 2 diabetes mellitus (Miyawaki et al. 1999).

In another study, Miyawaki et al. (2002) examined the influence of a high fat diet on $GIPR^{-/-}$ and $GIPR^{+/+}$ mice. After 50 weeks, wild-type mice had gained 35% body weight compared to wild-type mice on a control diet. In knockout mice no weight gain was detected.

The excessive fat intake leads to a hypersecretion of GIP in wild-type mice that promotes nutrient uptake and triglyceride accumulation. To further investigate the effect of GIP on obesity development, $GIPR^{-/-}$ mice were crossbred with genetically obese ob/ob (Lep^{ob}/Lep^{ob}) mice to generate double-homozygous ($GIPR^{-/-}$, Lep^{ob}/Lep^{ob}) mice. Obese Lep^{ob}/Lep^{ob} mice exhibit a marked increase in adipose tissue. The body weight of the double-homozygous mice was reduced by 23% compared with the Lep^{ob}/Lep^{ob} mice, confirming the role of the GIPR in obesity development. In the absence of GIP signalling fat can not be stored in adipocytes efficiently and instead is used as the primary energy source (Miyawaki et al. 2002).

A later study addressed the question whether adaptative mechanisms for the missing GIP effect exists in $GIPR^{-/-}$ mice (Pamir et al. 2003).

In vitro perfusion of pancreata of knockout and wild-type mice with GIP resulted in no insulin response, thereby confirming the abolished GIP signalling. Glucose and arginin provoked a comparable insulin response in GIPR knockout and wild-type pancreata.

However, GLP-1 infusion caused a higher peak and a sustained insulin release in pancreata of $GIPR^{-/-}$ mice compared to glucose and arginin.
Basal levels of cAMP in isolated islets of $GIPR^{-/-}$ mice were increased. GLP-1 stimulated cAMP levels were also significantly elevated, suggesting a higher sensitivity of the beta-cells to GLP-1.
These findings show that the functional knockout of the GIP receptor leads to a compensatory increase in the coupling efficiency of the GLP-1R.
Those compensatory changes may lay at a postreceptor level in the beta-cell since GLP-1 levels were not elevated in $GIPR^{-/-}$ versus $GIPR^{+/+}$ mice (Pamir et al. 2003).
GIP is thought to have an anabolic effect on bone metabolism, therefore bone formation was studied in $GIPR^{-/-}$ mice. $GIPR^{-/-}$ mice were found to exhibit less bone formation, smaller bone size, lower bone mass, and alterations in bone microarchitecture and biomechanical properties (Xie et al. 2005).

2.3.5.3 GLP-1 receptor knockout ($GLP\text{-}1R^{-/-}$) mice

$GLP\text{-}1R^{-/-}$ mice were created by replacing two exons encoding the first and third transmembrane domains and intervening peptide sequence with a PGK-neo cassette.
$GLP\text{-}1R^{-/-}$ mice showed normal feeding behaviour and weight gain. In contrast to $GIPR^{-/-}$ mice, $GLP\text{-}1R^{-/-}$ mice did not only exhibit abnormal glucose tolerance and diminished insulin secretion after oral glucose challenge, but they also showed fasting hyperglycemia and increased levels of blood glucose after intraperitoneal glucose injection compared to wild-type mice (Scrocchi et al. 1996).
Histological evaluation of the pancreatic islets revealed normal beta-cell mass but the main contribution to the total beta-cell volume in $GLP\text{-}1R^{-/-}$ mice came from medium-sized islets. This was in contrast to wild-type mice in which the large islets contributed most to the beta cell mass. Furthermore, $GLP\text{-}1R^{-/-}$ mice displayed more alpha-cells located in the center of the islets than wild-type mice, whereas the number of peripherally located alpha-cells was similar (Ling et al. 2001).
In vitro studies were performed on isolated islets of $GLP\text{-}1R^{-/-}$ and $GLP\text{-}1R^{+/+}$ mice. The estimated amount of insulin mRNA appeared comparable in wild-type and knockout mice as was the total pancreatic insulin content. Glucose-induced insulin release was shown to be well preserved in islets of knockout mice (Flamez et al.

1998). Therefore, the question arose whether a compensating mechanism for the loss of GLP-1R signalling exists, especially since GIP plasma levels were increased in GLP-1R$^{-/-}$ mice (Preitner et al. 2004). Therefore, pancreas perifusion in the presence of glucose (16,6mmol/l) with and without GIP (1nmol/l) was performed and resulted in a 80% greater mean integrated insulin response in GLP-1R$^{-/-}$ mice vs. wild-type mice. The insulin response to glucagon was not different between wild-type and knockout islets.

Proinsulin mRNA and pancreatic insulin content were found to be reduced in GLP-1R knockout mice, while pancreatic glucagon content was comparable to control mice. Pancreatic somatostatin content was also increased in GLP-1R$^{-/-}$ mice (Pederson et al. 1998).

In summary, GIP secretion and action was enhanced in GLP-1R$^{-/-}$ mice, resulting in a partial compensation of GLP-1 effects. However, GIP compensation was not sufficient to restore glucose-stimulated insulin secretion to normal levels in vitro.

2.3.5.4 Double Incretin Receptor Knockout Mice

Double incretin receptor knockout (DIRKO) mice were created by crossing GIPR$^{-/-}$ mice and GLP-1R$^{-/-}$ mice.

DIRKO mice were viable and fertile, exhibiting normal behaviour. Food intake over a 24h period revealed small but significant increases at some time points in DIRKO mice versus wild-type mice. Fasting blood glucose levels were normal in knockout mice. Female DIRKO mice showed a tendency towards lower fasting insulin levels compared to female controls (Hansotia et al. 2004).

During oral glucose tolerance test the AUC glucose in female DIRKO mice was approximately twice that of wild-type females and about 40-50% higher than in single incretin receptor knockout mice. In male mice, the same pattern was found although the elevations of blood glucose were less distinct. Plasma insulin levels measured during the OGTT were significantly lower in DIRKO mice than in wild-type mice.

During an intraperitoneal glucose tolerance test, male DIRKO mice showed normal basal blood glucose levels and also normal glucose tolerance after intraperitoneal glucose injection. Female knockout mice had normal basal blood glucose values but the AUC glucose was comparable to that after OGTT.

Examination of the insulin response during IPGTT in female mice unmasked that the first-phase insulin secretion was totally absent (Preitner et al. 2004).
The disturbed glucose tolerance was not a result of a diminished sensitivity to insulin, since glycemic response to exogenous insulin was comparable in wild-type and DIRKO mice (Hansotia et al. 2004).
The blunted first-phase insulin secretion was confirmed in pancreas perfusion experiments. Surprisingly, this defect occurs despite normal pancreatic insulin contents in DIRKO mice.
In DIRKO mice, the kinetics of the insulin response were investigated by islet perifusion and were comparable to wild-type mice, nevertheless, the amplitude and the total amount of secreted insulin was reduced (Preitner et al. 2004).
Taken together, the inactivation of both incretin receptors results in impaired glucose tolerance but not in a diabetic phenotype. This leads to the conclusion that as yet unidentified compensatory mechanisms exist.

2.3.5.5 Mt-Exendin-4 transgenic mice

Exendin-4 is a potent agonist for the mammalian GLP-1 receptor that was originally discovered in the venom of a lizard (Heloderma suspectum). Exendin-4 showed a much longer in vivo half-life with a prolonged duration of action as compared to GLP-1 and it is not subject to degradation by DPPIV (dipeptidyl peptidase IV).
Exendin-4 was expressed in transgenic mice under the control of the mouse metallothionein I promoter. Its activity can be induced by administration of zinc.
Mt-Exendin-4 transgenic mice were viable, fertile and developed normally. Fasting blood glucose levels were normal under basal and induced transgene expression.
Blood glucose excursion and glucose-stimulated insulin release was comparable in control and Mt-Exendin-4 transgenic mice under basal transgene expression following oral or intraperitoneal glucose challenge. When transgene expression was induced, a significant reduction in glycemic excursion following oral and intraperitoneal glucose loading, associated with a significant increase in insulin levels was observable. Basal levels of transgene expression had no effect on short-term or long-term food intake but short-term water intake was significantly reduced. Short-term food intake was only reduced significantly after induction of transgene expression.

Islet histology and numbers were comparable in control versus transgenic mice. Although exendin-4 is thought to stimulate beta-cell replication and neogenesis (Zhou et al. 1999; Stoffers et al. 2000), no sign for islet neogenesis was detected in Mt-Exendin-4 transgenic mice (Baggio et al. 2000).

In spite of ongoing exposure to exendin-4, no evidence of significant impairment of GLP-1R dependent actions could be found.

Since G-protein coupled receptors desensitize quickly during exposure to their agonist, the effects of a long-acting agonist were examined more closely. Exposure to exendin-4 led to a greater degree of GLP-1 receptor desensitization as compared to similar incubations with GLP-1 in vitro. However, wild-type and Mt-Exendin-4 transgenic mice did not display differences in glycemic response after one week of exendin-4 administration. These findings argue against the development of a significant GLP-1 receptor desensitization, coupled to control of glucose homeostasis in vivo (Baggio et al. 2004).

2.3.5.6 CD26$^{-/-}$ mice and F344/DuCrj rats

CD26 or DPPIV is a serine protease, responsible for degradation of GIP and GLP-1 as described above (see chapter 2.3.2).

CD26$^{-/-}$Mice

The gene coding for CD26 was inactivated by homologous recombination in embryonic stem cells.

CD26$^{-/-}$ mice were fertile and healthy. Male mice exhibited a slightly decreased body weight compared to controls. Fasting levels of glucose, insulin and GLP-1 were similar in wildtype and CD26$^{-/-}$ mice. During an OGTT, blood glucose levels in CD26$^{-/-}$ mice were significantly lower after 15, 30 and 60 minutes vs. in control mice. At 120 and 180 minutes, blood glucose concentrations were comparable again, underlining the glucose-dependency of incretin action.

Glucose-stimulated insulin levels were significantly elevated in CD26$^{-/-}$ mice versus wild-type mice (Marguet et al. 2000).

F344/DuCrj Rats

In the F344/DuCrj rat strain, a point mutation was discovered in the DPPIV gene, that leads to an amino acid substitution in the active-site sequence of DPPIV. As a result, DPPIV activity is abolished in plasma and tissue of the rats.

During OGTT, significantly lower blood glucose levels were observed in F344/DUCrj rats vs. control rats 30 and 60 minutes after glucose challenge. At 120 minutes, blood glucose levels were similar in F344/DuCrj and control rats.

Intraduodenal glucose administration yielded similar results as the OGTT. Additionally, insulin secretion was enhanced at 15 and 30 minutes. Measurement of GLP-1 concentrations revealed not only significantly higher basal values in F344/DuCrj rats but also significantly higher levels after glucose challenge.

Therefore, the improved glucose tolerance may be caused by elevated GLP-1 and insulin levels (Nagakura et al. 2001).

Both animal models demonstrate the possibilities of DPPIV inhibition in improving glucose tolerance which could be of decisive meaning in diabetes therapy. Moreover, both models offer themselves for studying effects of chronic inhibition of DPPIV.

2.4 Apoptosis

2.4.1 Definition of apoptosis

Apoptosis or programmed cell death is a physiological component of the development and health of multicellular organisms. The term apoptosis is of Greek origin meaning “to fall away from” in analogy to leaves falling off trees (Reed 2000).

During embryogenesis, apoptosis is needed, for example, to remove interdigital webs, to perforate the vaginal canal, and to reduce excessive cells in the nervous and hematopoietic systems (Wang 2001).

In adults, apoptosis is the key to destroy unwanted cells at the right time. If this balance between cell death and cell proliferation is disturbed, a variety of diseases can occur.

In many forms of cancer, antiapoptotic proteins are mutated or antiapoptotic factors are upregulated, leading to an inability of the organism to react to DNA damage, mutations or chemotherapeutical agents, and thereby to an accumulation of damaged cells. Apoptosis is also necessary to eliminate autoreactive T-lymphocytes.

If those cells persist, due to a disturbance in cell death, an autoimmune lymphoproliferative syndrome (ALPS) can result (Fesik 2000). In Alzheimer´s disease, neurons commit suicide prematurely because the accumulation of amyloid-ß peptides has a neurotoxic effect. This usually results in the common memory loss (Danial and Korsmeyer 2004).

2.4.2 Apoptosis compared to necrosis

Necrosis is another mechanism of cell death that usually occurs after severe cell damage, and differs from apoptosis in several characteristics.

Necrosis is uncontrolled cell death that commonly involves a group of neighboring cells, while apoptosis is strictly regulated and only affects single cells. During necrosis, cells begin to swell, and finally disrupt, resulting in leakage of cell contents which leads to inflammation of surrounding tissue. Apoptotic cells shrink, thereby losing contact to their adjacent cells, the chromatin condenses, DNA is fragmented, and finally the cell breaks into compact membrane-enclosed structures, the so called apoptotic bodies, that are engulfed by macrophages, leaving no inflammation (Scaglia et al. 1995; Chandra et al. 2001).

2.4.3 Mechanisms of apoptosis

2.4.3.1 Death signals

Apoptosis is induced by a variety of stimuli that either come from outside the cell and are transmitted by ligand binding of the death receptors on the cell surface, or from inside the cell which are mediated by the mitochondria. Though there are many different stimuli, e.g. DNA damage, growth factor withdrawal, toxins, radiation, they activate a common cell death machinery, leading to the characteristic features of apoptotic cell death (Fesik 2000).

2.4.3.2 Caspases

Caspases, short for cysteinyl aspartate-specific proteases, are enzymes activated in most cases of apoptotic cell death. They specifically cleave their substrates after certain accessible aspartatic acid residues.

Substrates include many different kinds of molecules, e.g. signal transduction proteins, cytosceletal and nuclear matrix proteins as well as DNA repair proteins (Reed 2000).

So far, 12 mammalian caspases have been discovered, numbered in the chronological order of their identification, and divided into three different groups according to their function. The initiator caspases-2, -8, -9, -10, and -12 cleave and thereby activate the executioner caspases-3, -6 and -7. Caspases-1, -4, -5 and -11 are mainly involved in the proteolytic maturation of proinflammatory cytokines (Philchenkov 2004).

Caspases are synthesized in the cell as inactive zymogens, the procaspases. At their N-terminus, they carry a prodomain followed by a large and a small subunit. The prodomains of the initiator caspases are long carrying modular regions, essential for the interaction with adaptor proteins. These modules contain death effector domains (DED), and caspase recruitment domains (CARD). A linker region connects the catalytical subunits. Proteolytical processing results in the separation of the large and the small subunit. A heterotetramer, the active enzyme, is being built, consisting of two small and two large subunits (Philchenkov 2004).

2.4.3.3 Death receptor pathway

External signals are transmitted by activation of death receptors. These death receptors are integral membrane proteins, exposed at the cell surface, and are activated by binding specific ligands. The most common representatives of death receptors are Fas (CD95), ligand FasL (Fas Ligand), TNFR1 (tumor necrosis factor receptor 1), ligand TNF (tumor necrosis factor) and receptors DR4 and DR5 (death receptors 4 and 5) with their ligand TRAIL (TNF-related apoptosis-inducing ligand) (Fesik 2000). Upon binding of a ligand, the activated receptor recruits an adaptor protein to its cytoplasmatic tail via the death domain. At its free end, the protein carries a DED to bind initiator procaspase-8. The complex built of the receptor, the connected adaptor protein, and the caspase is called DISC, death inducing signaling complex (Reed 2000; Philchenkov 2004).

The activated caspase-8 then proteolytically cleaves executioner procaspase-3, thereby moving further downstream in the apoptotic cascade. (Fig. 2.1)

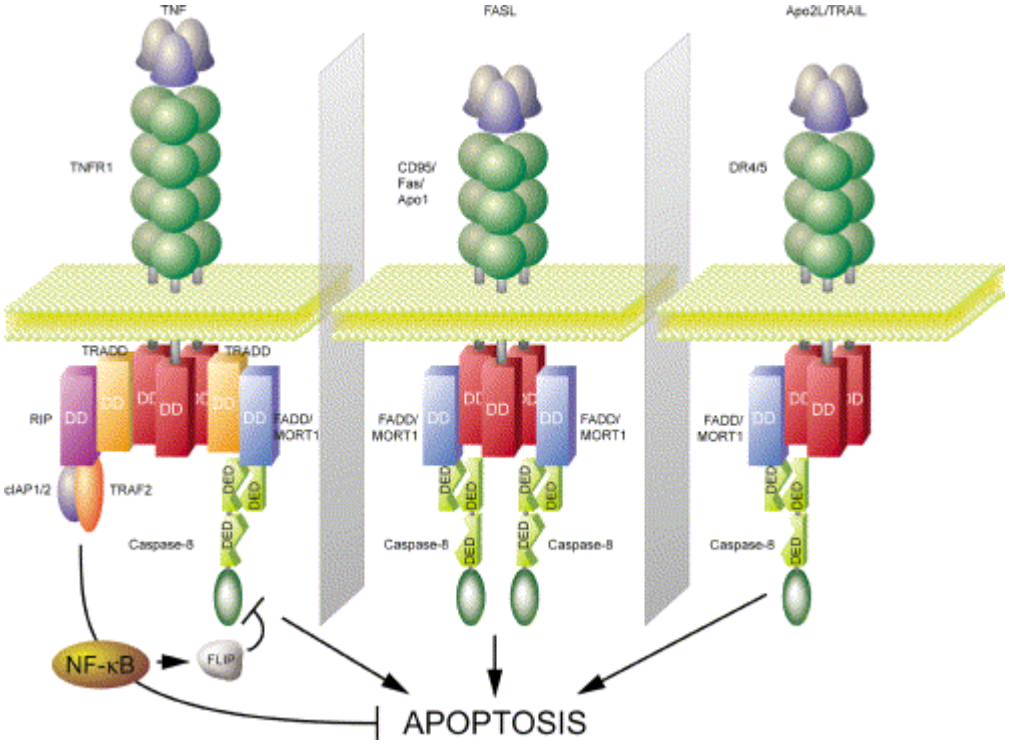

Figure 2.1 Extrinsic death receptor pathway

The distinct composition of the Death-Inducing-Signaling Complex (DISC) downstream of the various death receptors TNFR1, CD95, and DR4/5 is illustrated (Danial and Korsmeyer 2004).

2.4.3.4 Mitochondrial pathway

A number of mitochondrial proteins, which usually reside in the intermembrane space of mitochondria, are able to set off apoptosis when released to the cytosol after an apoptotic stimulus.

One of those proteins, and the most important one, is cytochrome c. After its release into the cytosol, it binds to Apaf-1. Thereupon, the affinity of Apaf-1 for ATP/dATP is increased about 10-fold (Wang 2001). Binding of ATP/dATP leads to a conformational change, and the so called apoptosome is formed, a multimeric Apaf-1 and cytochrome c complex. Due to this conformational change, the CARD (caspase recruitment domain) of Apaf-1 becomes exposed, and binds multiple procaspase-9 molecules.

Only when bound to the apoptosome, procaspase-9 molecules can activate themselves, and then process downstream executioner caspases, such as caspase-3 (Wang 2001). (Fig 2.2)

An important crosslink between the mitochondrial and death receptor pathway exists, which will be described in chapter 2.3.3.1.

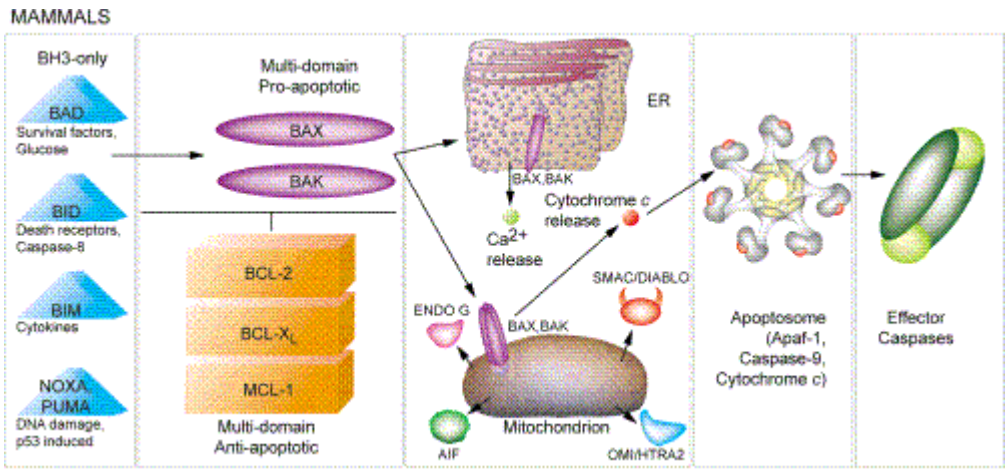

Figure 2.2 Mitochondrial pathway

Forming of the apoptosome and activation of executioner caspases. (Danial and Korsmeyer 2004)

2.4.4 Regulatory mechanisms

2.4.4.1 Bcl-2 family

The Bcl-2 proteins are important regulators of cell death, and execute their effect via the mitochondria. Bcl-2 proteins are divided into three groups, according to the Bcl-2 homology domains they have in common.

Bcl-2 itself and four relatives hold all four of these domains, and promote cell survival. Bax, Bak and Bok share three Bcl-2 domains with the antiapoptotic Bcl-2 group, but support cell death. Also proapoptotic are the BH3-only proteins, such as Bim, Bad, and Bid, which only have the BH3-domain in common with the Bcl-2 group (Fesik 2000; Adams 2003).

Whether a cell lives or dies, mainly depends on the ratio of anti- to proapoptotic molecules.

BH3-only proteins are activated either by an intrinsic apoptotic stimulus or by cleavage by caspase-8. They can inactivate antiapoptotic Bcl-2 proteins, as well as activate Bax and Bak (Adams 2003). Bax and Bak are thought to oligomerize and form pores in the mitochondrial membrane to release cytochrome c and other cytotoxins into the cytosol (Muchmore et al. 1996). The turning-on of the BH3-only protein by caspase-8, displays an important crosslink between the death receptor and mitochondrial pathway. If the activation of executioner caspases by the death

receptor pathway does not suffice to overcome prosurvival proteins, this crosslink can amplify the signal for cell death.

2.4.4.2 IAPs and Smac/Diablo

IAPs, inhibitors of apoptosis, were initially detected in baculoviruses, where they inhibited death of host cells (Crook et al. 1993). All IAPs share one to three BIR domains (baculovirus IAP repeat) which have a characteristic signature sequence.
IAPs bind specifically to effector caspases-3 and -7, and also initiator caspase-9, rendering their action impossible by blocking the substrate´s access to the caspase (Danial and Korsmeyer 2004).
Antagonist of the IAPs is Smac/Diablo, a cytotoxin that is released from the mitochondria upon an apoptotic signal. The first four amino acids of Smac bind to the BIR domain of IAPs which then release the bound caspase.
The ability of Smac/Diablo to counter the inhibition of IAP, if the damage to mitochondria is severe and Smac is released at high levels, provides a powerful way to ensure rapid execution of apoptosis. If the damage is not severe enough, a high concentration of IAPs will save a viable cell from death (Wang 2001).

2.4.5 Role of apoptosis in the endocrine pancreas

2.4.5.1 Physiological role of apoptosis

Apoptosis is a rapid process that usually takes no longer than three hours in the endocrine pancreas. Morphological evidence of this event is even more shortlived, detectable for only one hour. Therefore, apoptosis is rarely seen in slowly renewed tissue, such as pancreatic beta-cells (Scaglia et al. 1995). Investigations of apoptosis of the endocrine pancreas through the lifetime of rats, investigated at 12 different time points, revealed a biphasic curve with peaks at day five and day 545 (Hanke 2000).
The peak of apoptosis of the endocrine pancreas at day five could not be confirmed by other investigators. Instead, they found a peak at days 13-17 (Scaglia et al. 1997) or day 14 (Petrik et al. 1998). At the same time, also extensive replication of beta-cells takes place, suggesting a remodeling of the endocrine pancreas as has been

found in other organs, such as the kidney or central nervous system (Scaglia et al. 1997).

Petrik et al. (1998) found that the apoptotic wave in the endocrine pancreas of neonatal rats coincides with a diminished expression of IGF-II, whereas IGF-I was not detectable yet. Concomitant with the low levels of IGF-II, a higher expression of iNOS, inducible nitric oxide synthase, appeared in beta-cells. The temporal linkage of these two events allows the presumption that IGF-II protects beta-cells from apoptosis, and when IGF-II levels do not suffice, nitric oxygen (NO) driven by a high expression of iNOS can lead to apoptosis. NO is also thought to be an important effector molecule in the autoimmune destruction of beta-cells in type 1 diabetes mellitus (Mandrup-Poulsen 2001).

A high rate of apoptosis was also found in the beta-cells of rats after giving birth. Three to four days after giving birth, the incidence of apoptosis rises, thereby leading to a faster involution of the post partum endocrine pancreas (Finegood et al. 1995).

2.4.5.2 Role of apoptosis in type 1 diabetes mellitus

Type 1 diabetes mellitus is characterized by an autoimmune destruction of beta-cells, leading to a dramatically reduced functional beta-cell mass. At the time of diagnosis, beta-cell mass is already reduced by 70-80% (Cnop et al. 2005). Several studies in animal models of type 1 diabetes mellitus have shown that apoptosis rather than necrosis is the process that underlies beta-cell death (Kurrer et al. 1997; O´Brien et al. 1997).

Any kind of destruction of beta-cells, during which antigen is shed, could cause presentation of this antigen and activation of beta-cell specific T-cells. The cause for this initial destruction is still unknown. Many initiating factors have been discussed, such as virus infections (Hyöty and Taylor 2002), toxins and abnormal physiological tissue turnover (Liadis et al. 2005).

Even the neonatal wave of apoptosis, leading to a remodeling of the endocrine pancreas of rodents, has been suggested to invoke inflammatory processes and trigger autoimmune diabetes (Trudeau et al. 2000).

The autoimmune assault of pancreatic beta-cells leads to an inflammatory reaction of the pancreatic beta-cells called “insulitis”. Death of the beta-cells is probably caused by either direct contact with macrophages and T-cells and/or exposure to soluble

mediators secreted by these cells, such as cytokines, nitric oxide, and oxygen free radicals (Cnop et al. 2005).

During autoimmune destruction, macrophages are thought to occur first in islets, followed by $CD8^+$ and $CD4^+$ cells. This infiltration is accompanied by a high IL-1β and TNF-α expression in the immune cells and iNOS expression in both the immune cells and the beta-cells. The role of the proinflammatory cytokines in beta-cell destruction was proven by in vitro studies in human islets (Delaney et al. 1997).

Cytokines probably induce the expression of stress response genes. Thereafter, the protein kinases c-Jun, p38 mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK) are activated, which influence transcription factors regulating iNOS production.

Because of their high rate of protein synthesis, beta-cells are particularly susceptible to ER stress. Cytokines are also thought to be able to trigger ER stress and the release of death signals from the mitochondria. Finally, cytokines directly induce pro-apoptotic genes, such as Bid, Bak and caspase-3 (Cnop et al. 2005).

2.4.5.3 Role of apoptosis in type 2 diabetes mellitus

While the role of apoptosis in type 1 diabetes mellitus seems to be guaranteed, it is still contentious in type 2 diabetes.

In rodent models of type 2 diabetes, a dramatic decrease of beta-cell mass is often reported (Pick et al. 1998; Jörns et al. 2002). Studies in humans regarding this issue are controverse. Butler et al. (2003) revealed a 41% deficit in volume density of beta-cells in the pancreas in lean subjects with type 2 diabetes mellitus, and a 63% decrease in obese type 2 diabetic subjects (Butler et al. 2003). However, in a study by Guiot et al. (2001) the beta-cell mass was not reduced in diabetic patients (Guiot et al. 2001). A study in Japanese type 2 diabetic patients also found a significant reduction of the volume density of beta cells in the islets and the total beta-cell mass (Sakuraba et al. 2002).

ZDF (zucker diabetic fatty) rats, an animal model of type 2 diabetes mellitus that has many similarities to human type 2 diabetes, were found to be not able to compensate beta-cell mass in the face of insulin resistance, as compared to non-diabetic ZF rats. Despite a high replication rate of beta-cells, ZDF rats showed a decrease in beta-cell

mass when compared to the obese control rats. This decrease was supposed to result from a higher rate of apoptosis (Pick et al. 1998).
Similarly, Butler et al. (2003) discovered a decreased volume density of beta-cells in the pancreas in lean and obese humans with type 2 diabetes mellitus, compared to nondiabetic age- and weight-matched subjects. They also found a higher rate of apoptosis in these patients. In contrast to rats, beta-cell replication was low, not only in diabetic patients, but also in nondiabetic counterparts. They came to the conclusion that beta-cell replication is not important in humans. Instead, new islet formation from exocrine ducts was observed, and found to be intact in patients with type 2 diabetes mellitus (Butler et al. 2003).
Many factors for this apoptosis-mediated decrease in beta-cell mass have been discussed, first and foremost amyloid and glucotoxicity.

Amyloid

Deposits of amyloid are thought to play a major role in the progressive loss of beta-cell mass due to beta-cell apoptosis in type 2 diabetic patients.
Islet amyloid is derived from the islet amyloid polypeptide (IAPP) that is coexpressed and co-secreted with insulin. IAPP expression is increased with insulin resistance. Human IAPP, but not murine IAPP, spontaneously forms fibrils in an aqueous environment (Ritzel and Butler 2003). Amyloid was shown to cause beta-cell apoptosis in vitro, possibly by causing leaks in cell membranes (Janson et al. 1999). Moreover it was discovered that amyloid increases vulnerability of beta-cells for apoptosis after replication in vitro. Even replication alone caused a higher incidence of apoptosis in beta-cells in vitro (Ritzel and Butler 2003). Additionally, amyloid upregulates the proapoptotic genes *c-fos*, *fosB*, *c-jun* and *junB* (Tucker et al. 1998) and increases expression of the proapoptotic factors p53 and p21 (Zhang et al. 1999).
However, amyloid was not detectable in every type 2 diabetic patient, and was found only in 10% of patients with impaired fasting glucose, who already had a 40% loss of beta-cell mass (Butler et al. 2003). In the study of Japanese type 2 diabetic patients, amyloid was found in about 15% of the islets of diabetic patients, but was very rare in healthy subjects (0~2%) (Sakuraba et al. 2002). A possible explanation was given in the study of Janson et al. (1999). They found out, that small IAPP oligomers that are not detectable by light microscopy are toxic, thereby causing loss of beta-cells,

whereas the large extracellular amyloid deposits visible by light microscopy are inert (Janson et al. 1999).

Glucotoxicity

High levels of glucose have a toxic effect on human beta-cells in vitro. High glucose levels induce the production and release of cytokines, especially IL-1β, in the pancreatic beta-cell which results in an upregulation of the Fas receptor. Finally, caspase 8 and 3 are activated. In addition, FLIP, short for FLICE (caspase 8)-inhibitory protein, was found to be decreased in human islets of type 2 diabetic patients (FLICE stands for FADD-like ICE-inhibitory protein; FADD is an adaptor protein; ICE stands for Interleukin 1β converting enzyme). FLIP, in a certain critical amount, leads to cell proliferation via Fas signaling. If FLIP levels are decreased, Fas-mediated signaling induces apoptosis (Maedler et al. 2002; Maedler et al. 2002 a). However, in a recent study, the involvement of IL-1ß in apoptosis of human beta-cells could not be proven. In this study, IL-1ß neither induced iNOS expression nor upregulated Fas expression (Welsh et al. 2005).

Chronic hyperglycemia was found to reduce insulin gene expression by downregulation of two major transcription factors of the insulin gene, pancreatic-duodenal homeobox-1 (Pdx-1) and the activator of the rat insulin promoter element 3b1 (Poitout and Robertson 2002).

Pro-apoptotic signals, induced by high levels of glucose, show some similarities to those induced by cytokines. For example, high glucose levels may also cause ER (endoplasmatic reticulum) stress and generation of reactive oxygen species (Donath and Halban 2004).

Chronic hyperglycemia could also lead to long-term increases in cytosolic Ca^{2+} that could in turn act pro-apoptotic (Grill and Bjorklund 2001).

These various results suggest that glucotoxicity exerts its effect by different mechanisms. Therefore, the insufficient insulin competence in type 2 diabetes could not only result from a diminished insulin production, but also from a reduced beta-cell mass due to destruction of beta-cells by apoptosis.

2.4.6 Beta-cell death in animal models of type 2 diabetes mellitus

In Psammomys obesus (sand rat), type 2 diabetes mellitus is induced nutritionally by feeding a high-energy diet. The total beta-cell volume in the pancreas was reduced by more than 70%, accompanied by a 90% reduction of total pancreas insulin

content. Ultrastructurally, different signs of necrosis were found in pancreatic beta-cells, including the rupture of cell membranes and a massive vacuolization of the cytoplasm. Signs of apoptosis were not observed (Jörns et al. 2002).
In another study, signs of apoptosis as mechanism for beta-cell death were found. Necrotic events were also evident but only at late stages of the disease (Donath et al. 1999).
Leibowitz et al. (2001) showed that the Pdx-1 gene is not expressed in postnatal pancreatic islets of Psammomys obesus. Other transcription factors seem to be sufficient to sustain basal expression of the insulin gene. Pdx1 is probably not necessary to maintain normoglycemia if the animals are fed a low calorie diet. This specific genetic feature is probably responsible for the failure of beta-cells to cope with an increased demand for insulin (Leibowitz et al. 2001).
Another animal model exhibiting an increased rate of beta-cell apoptosis is the Akita mouse. Akita mice harbour a spontaneous mutation in the *Ins2* gene that leads to the production of a mutant form of proinsulin 2 and causes early-onset diabetes with a decreased beta-cell mass. The loss of beta-cells starts at birth and progressively moves on. It is supposed to result from immense ER (endoplasmatic reticulum) stress induced by the retention of the mutant proinsulin 2 in the ER (Wang et al. 1999; Oyadomari et al. 2002).
Recently, a human IAPP transgenic rat (the HIP rat) has been obtained that spontaneously develops diabetes, characterized by islet amyloid formation and decreased beta-cell mass. The expression of human IAPP leads to an increased rate of beta-cell apoptosis in these rats (Butler et al. 2004).
Several lines of transgenic mice expressing human IAPP also have been established. They only deposit human IAPP in their islets when the metabolic environment is altered, e.g. when they are crossbred with mouse models of obesity or fed a high-fat diet (Masiello 2006).

2.5 Renewal of the beta-cell mass

2.5.1 Introduction

Since all kinds of diabetes mellitus are associated with a decreased mass of functional beta-cells, much effort is made to understand how beta-cell mass can regenerate. Pancreatic beta-cells are a slowly renewing tissue with a production of

new beta-cells by division of about 3% in 24 hours in the rat. This figure declines rapidly with increasing age when insulin resistance usually becomes more pronounced (Swenne 1982). The replication rate of human beta-cells is even lower than that in rodents (Bonner-Weir and Weir 2005). However, many animal models emphasize that regenerative capacity of beta-cells exists.

2.5.2 Animal models for the study of beta-cell renewal

2.5.2.1 IGF-II transgenic mice

A massive increase in beta-cell mass was observed in transgenic mice overexpressing insulin-like growth factor-II. Concomitant, an abundant immunoreactivity of the beta-cell transcription factor Pdx-1 and a decrease in the apoptosis rate were found (Petrik et al. 1999).

2.5.2.2 GLP-1 and Exendin-4 infusion of diabetic rodents

GLP-1 and its longer acting agonist exendin-4 stimulates the expression of Pdx-1, resulting in an increase in islet size in db/db mice (Stoffers et al. 2000) and in pancreatectomized Sprague-Dawley rats (Xu et al. 1999). GLP-1 infusion of ZDF rats for two days led to an increase in proliferating cells, an expansion of beta-cell mass and a decrease in apoptosis (Farilla et al. 2002). However, overexpression of exendin-4 and the knockout of the GLP-1R in genetically engeneered mice did not alter beta cell mass.

2.5.2.3 Glucose administration

Glucose administration to streptozotocin diabetic rats provoked a massive increase in beta-cell mass after only 24 hours (Bernard et al. 1998; Bernard et al. 1999). However, this impressive recovery did not result in any functional improvement of the beta-cells (Bernard et al. 1998). Another study showed that chronic hyperglycemia even leads to a progressive loss of beta-cell differentiation in 90% pancreatectomized rats (Jonas et al. 1999). These results demonstrate that the effect of glucose on beta-cells is depending on the glucose concentration and duration of infusion.

It is also important to stress that glucose affects survival of human and rodent islet cells differently. Graded increases in glucose from 100mg/dl to 200mg/dl and above induce apoptosis of human beta cells in vitro in a dose-dependent fashion. This is in contrast to rodent islets where glucose levels up to 200mg/dl decrease apoptosis (Maedler et al. 2001).

2.5.2.4 Pancreatectomy

90% pancreatectomy in rats results in massive proliferation and differentiation of ductal epithelium into exocrine and endocrine pancreas (Bonner-Weir et al. 1993). This process is accompanied by an increase in Pdx-1 protein levels (Sharma et al. 1999).

These studies suggest that beta-cell mass does not only increase by replication but also by neogenesis of beta-cells.

Neogenesis of pancreatic beta-cells is a topic that is discussed controversially in literature. Different theories exist as to where putative stem cells that are able to create new beta-cells might be located.

2.5.3 Locations of putative stem cells/progenitor cells

Pancreatic duct cells as progenitor cells

Bonner-Weir et al. (1993, 2005) support the view that mature duct cells are the main progenitor source for pancreatic islet cells. In a 90% pancreatectomized rat, they observed two pathways of pancreas regeneration: 1, replication of pre-existing differentiated exocrine and endocrine cells and 2, proliferation of duct cells that form focal areas of regeneration that give rise to new endocrine and exocrine cells. The duct cells are supposed to dedifferentiate transiently, replicate and then differentiate into the various cell types of the pancreas (Bonner-Weir et al. 1993). This theory is supported by the discovery that Pdx-1 protein levels are enhanced during the period of differentiation (Sharma et al. 1999).

Not long ago, putative multipotent pancreatic progenitor cells were isolated by flow cytometry. They were identified by expression of c-Met, the hepatocyte growth factor receptor, that is essential for pancreatic development. These putative progenitors formed colonies in vitro and expressed islet and acinar characteristics as well as the

duct marker cytokeratin 19 and nestin, a possible marker of neural stem cells. About 1% of duct cells were found to be c-Met-positive (Suzuki et al. 2004).
Another possibility would be that duct cells directly transdifferentiate into beta-cells. Bernard et al. (1999) found insulin-positive duct cells and islets invaded by cells originating from ducts (Bernard et al. 1999).

Other pancreatic cells as presumptive beta-cell precursors

Neogenesis of beta-cells was detected in other kinds of pancreatic cells, too. Guz et al. (2001) examined beta-cell regeneration in mice with streptozotocin-induced diabetes. They found presumptive beta-cell precursors during development to reappear during regeneration. These beta-cell precursors first express Pdx-1 and somatostatin and then Pdx-1, somatostatin (SOM) and insulin (IN). Additionally, GLUT-2 expressing cells were discovered which also occur during development. It was proposed that delta- and alpha-cells revert to an immature phenotype and then express Pdx-1/SOM/IN and GLUT-2 respectively (Guz et al. 2001).
Lipsett and Finegood (2002) observed beta-cell neogenesis in response to two-four days of glucose-infusion of rats. After glucose infusion, many small duct-like structures appeared in a focal pattern (Bonner-Weir et al. 1993; Lipsett and Finegood 2002). However, no increase in duct cell proliferation before or during the time of so called focal area formation was observed. Instead, the authors came to the conclusion that the focal areas arise through dedifferentiation of acinar tissue (Lipsett and Finegood 2002).
A major advance was made when Seaberg at al. (2004) isolated multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. These precursors form floating colonies in vitro and express markers, characteristic of pancreatic and neural precursors, e.g. Pdx-1 and nestin. The pancreas-derived multipotent precursors (PMPs) were able to generate β_3-tubulin positive neurons, GFAP positive astrocytes, 04 positive oligodendrocytes, pancreatic alpha-cells, delta-cells and insulin positive and C-peptide positive pancreatic beta-like cells.
The colonies are functional and release insulin in a glucose-dependent fashion. However, the PMPs have a limited capacity for self-renewal and lack the stem cell markers Oct4 and Nanog (Seaberg et al. 2004).

Putative pancreatic stem cells outside the pancreas

Putative pancreatic stem cells were discovered in the bone marrow of adult mice. Using the CRE-LoxP system, transgenic mice were generated expressing green fluorescent proteins in bone marrow cells, if the insulin gene was transcribed. The bone marrow cells expressing the green fluorescent protein were isolated from male mice and transplanted into lethally irradiated female recipient mice. The bone marrow cells that inserted into the pancreatic islets of the recipient mice exhibited markers and physiological behavior characteristic of pancreatic beta- cells. They expressed insulin, GLUT-2 and typical transcription factors. The differentiation into beta-like cells may occur directly or via an intermediary pool of one or more multipotent cell phenotypes (Ianus et al. 2003).

2.5.4 Other mechanisms of islet-cell regeneration

Dor et al. (2004) examined the postnatal development of the endocrine pancreas, using genetic lineage tracing. They developed an inducible pulse and chase system, labeling the beta-cells in mice. Cells generated after the pulse are labeled if they derive from existing beta-cells. No new islets were found to be formed during adult life, regardless of the size of the islets. Additionally, the percentage of labeled beta-cells remained stable in chased mice, showing no dilution of the labeled population which would be the consequence if beta-cells are created by another source. Even after a 70% pancreatectomy, no new islets were discovered although BrdU incorporation in pancreatic cells increased. Therefore, the authors concluded that the number of islets during adult life is fixed and that new beta-cells that form subsequently derive from pre-existing beta-cells (Dor et al. 2004).

Supporting this view, a study of Georgia and Bhushan (2004) concentrated on the exact mechanisms of replication of beta-cells. They revealed that cyclin D2 plays an essential role in the regulation of beta-cell replication. Cyclins are key components of the cell cycle machinery.

Cyclin $D2^{-/-}$ mice showed a striking reduction in total beta-cell mass, BrdU incorporation and islet size when compared to control mice (Georgia and Bhushan 2004).

The diversity of the results of these studies and the lack of sturdy proofs for one of the introduced theories clearly show the need for further investigations.

3. Animals, materials and methods

3.1 Transgenic mice

Transgenic mice were generated as described by Volz (1997) and characterized by Herbach (Herbach 2002; Herbach et al. 2005). Hemizygous female transgenic mice and age-matched littermate controls, bred on a CD1 genetic background, were investigated in this study. Animals were kept on a 12 hour light, 12 hour dark cycle at 22°C +/- 2°C. Mice were weaned at the age of 21 days, tail tip biopsies were taken for PCR analysis and each animal was marked by numeric ear perforation. Food and water was provided *ad libitum*, except where noted. Half of the animals was fed a rodent breeding chow, the other half was fed a carbohydrate restricted diet (both diets from Altromin, Lage, Germany, see Table 3.2). Before animals received the carbohydrate restricted diet, the breeding chow and carbohydrate restricted diet were mixed 1:1 from day 21 until day 28 in order to prevent a glucose malnutrition which young animals are very sensible to and to let the gastrointestinal tract get accustomed to the high fiber content of the carbohydrate restricted diet.
For the age and number of animals investigated in each test, see Table 3.1.

Analyses/parameters	45 - 50 days				90 days				175 - 180 days			
	wild-type		*transgenic*		*wild-type*		*transgenic*		*wild-type*		*transgenic*	
	BD	CRD	BD	CRD	BD	CRD	BD	CRD	BD	CRD	BD	CRD
Expression analyses of endogenous and transgenic GIPR					2		2					
Blood glucose fasted + postprandial	6	8	6	8	4	4	8	6	6	4	6	8
Oral glucose tolerance test	5	6	5	4					6	6	7	7
Insulin sensitivity test	6	6	6	5								
Serum GIP values	4	4	5	5	4	4	5	5	4	4	5	5
Serum insulin values	6	8	5	8	4	4	7	6	7	4	5	8
Body weight	6	6	5	6	4	4	5	5	4	4	5	5
Total volumes and volume densities of islets, beta-cells and isolated beta-cells	6	6	5	6	4	4	5	5	4	4	5	5
Replication of islet cells	4	4	4	6	4	4	5	5	4	4	5	5
TUNEL assay	5	4	5	6	4	4	5	5	4	4	5	5
Number, numerical density and mean volume of beta-cells and islets	5	5	5	5					4	4	4	4

Table 3.1 Numbers of investigated animals

	rodent breeding chow Altromin C1313	carbohydrate restricted diet Altromin C1009
Protein (%)	23.6	17.0
Fat (%)	4.9	7.0
Fiber (%)	3.7	34.0
Ashes (%)	6.4	8.0
Disaccharides (%)	5.9	1.3
Energy (kcal/kg)	2,825.6	1,343.6

Table 3.2 Composition of the different diets

3.2 Materials

3.2.1 Antibodies

Primary antibodies and dilution:

Monoclonal Rat anti-Bromodeoxyuridine (BrdU) 1:50	Oxford Biotechnology, Oxfordshire, Great Britain
Polyclonal Guinea Pig anti-Insulin 1:2000	DAKO, Hamburg, Germany

Secondary antibodies and dilution:

Pig anti-Rabbit Ig, biotinylated 1:50	DAKO, Hamburg, Germany
Rabbit anti-Rat IgPO 1:50	DAKO, Hamburg, Germany
Rabbit anti-Guinea Pig IgPO 1:50	DAKO, Hamburg, Germany

3.2.2 Chemicals

Agarose	Roth, Karlsruhe, Germany
Bacto-Agar	Difco Laboratories, Detroit, USA
Benzoylperoxid	Merck, Darmstadt, Germany
Boric acid	Roth, Karlsruhe, Germany
Bromophenol blue	Merck, Darmstadt, Germany
Chloroform	Riedel-de Haen, Deisenhofen, Germany
DAB (3,3 Diaminobenzidinetetrahydrochloride-dihydrate)	Kem-En-Tec Diagnostic Kopenhagen, Denmark
DEPC (Diethylpyrocarbonate)	Sigma, Deisenhofen, Germany
Dimethylanilin	Merck, Darmstadt, Germany

DTT (1,4-Dithio-DL-threitol)	Roth, Karlsruhe, Germany
EDTA	Sigma, Deisenhofen, Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidiumbromide (solution 1%)	Merck, Darmstadt, Germany
Ethyleneglycol monobutylether	Merck, Darmstadt, Germany
Glacial acetic acid	Roth, Karlsruhe, Germany
Glycerol	Merck, Darmstadt, Germany
Hydrochloric acid (25%)	Merck, Darmstadt, Germany
Hydrogen peroxide (30%)	neoLab, Heidelberg, Germany
2-Hydroxymethylmethacrylate	Riedel-de Haen, Deisenhofen, Germany
Magnesium chloride hexahydrate	Merck, Darmstadt, Germany
Mayer´s Hemalaun	Applichem, Darmstadt, Germany
Methylmethacrylate	Riedel-de Haen, Deisenhofen, Germany
Paraffine	Engelbrecht, Edermuende, Germany
Polyethyleneglycol 400	Merck, Darmstadt, Germany
Potassium chloride	Merck, Darmstadt, Germany
2-Propanol	Roth, Karlsruhe, Germany
SDS (Sodiumdodecylsulfate) Ultra Pure	Roth, Karlsruhe, Germany
Sodiumchloride	Applichem, Darmstadt, Germany
Spermidine	Sigma, Deisenhofen, Germany
Target Retrieval Solution	DAKO, Hamburg, Germany
Tris (Tris-(hydroxymethyl)-aminomethane)	Roth, Karlsruhe, Germany
Xylene cyanol FT	Sigma, Deisenhofen, Germany
Xylol	W.Graen GmbH & Co., Munich, Germany

3.2.3 Enzymes and other reagents

BrdU (Bromodeoxyuridine)	Roche, Mannheim, Germany
Calibration solution	Hitado Diagnostic Systems, Moehnesee, Germany
DNase I (10U/µl)	Roche, Mannheim, Germany
DNTP	Eppendorf, Hamburg, Germany
Glucose	Sigma-Aldrich, Steinheim, Germany
Hemolyzing solution	Hitado Diagnostic systems,

	Moehnesee, Germany
Insulin (Huminsulin 40)	Lilly, Giessen, Germany
M-MLV-RT (200U/µl)	Invitrogen, Karlsruhe, Germany
Normal pig serum	DAKO, Hamburg, Germany
Normal rabbit serum	MPI Biomedicals, Eschwege, Germany
Proteinase K (for immunohistochemistry)	DAKO, Hamburg, Germany
Proteinase K (for biochemical analyses)	Boehringer, Ingelheim, Germany
Random primer hexamers (600µg/ml)	Roche, Mannheim, Germany
RNAlater®	Ambion (Europe) Ltd., Huntingdon, Great Britain
RNaseOUT™ (Rnase-Inhibitor) (40 U/µl)	Invitrogen, Karlsruhe, Germany
StreptABComplex/HRP	DAKO, Hamburg, Germany
Trizol®	Invitrogen, Karlsruhe, Germany

3.2.4 Kits

Apoptag™ Apoptosis detection kit	MP Biomedicals, Illkirch, France
Rat Insulin RIA Kit	Linco Research, Missouri, USA
Rat/Mouse GIP Elisa Kit	Linco Research, Missouri, USA
RNeasy Mini Kit	Qiagen, Hilden, Germany
Taq DNA Polymerase Kit	Qiagen, Hilden, Germany

3.2.5 Molecular weight standards for DNA and RNA

Gene Ruler™ (100bp-DNA-Ladder)	MBI Fermentas, St. Leon Roth, Germany
Gene Ruler™ (1kb-DNA-Ladder)	MBI Fermentas, St. Leon Roth, Germany
PUC-Mix-Marker 8	MBI Fermentas, St. Leon Roth, Germany

3.2.6 Equipment

Shandon Citadel™ 1000	Thermo Electron Corporation, Germany
Eagle Eye II	Stratagene, Heidelberg, Germany
γ - Counter LB2111	Berthold, Bad Wildbad, Germany

Heating block	Biometra, Goettingen, Germany
Histomaster 2050/DI	Bavimed, Birkenau, Germany
Incubator	Mettler, Giessen, Germany
Photomacroscope Wild M 400	Wild, Heerbrugg, Switzerland
Spectra Max 250 (ELISA-Reader)	Molecular Devices, Sunnyvale, USA
Spectrophotometer	Beckman, Palo Alto, USA
Super GL_{easy}	Dr. Mueller Geraetebau GmbH, Freital, Germany
TBS 88 Paraffin Embedding System	medite Medizintechnik, Burgdorf, Germany
Tissue homogenizer (ART-Mirco D8)	ART Labortechnik, Müllheim, Germany
Video camera DFC 320	Leica, Heerbrugg, Switzerland
Videoplan® Image analysis system	Zeiss-Kontron, Eching, Germany
Centrifuges:	
Laboratory Centrifuge 1K15	Sigma, Deisenhofen, Germany
Table Centrifuge with Cooling (5417R)	Eppendorf, Hamburg, Germany
Microtomes:	
HM 315 microtome	Microm GmbH, Walldorf, Germany
HM 360 microtome	Microm GmbH, Walldorf, Germany
Thermocycler:	
Biometra® Uno II Thermocycler	Biometra, Goettingen, Germany
Gene Amp® PCR System 9700	Perkin Elmer, Cologne, Germany
Mastercycler® gradient	Eppendorf, Hamburg, Germany

3.2.7 Composition of buffers

3.2.7.1 Buffer for molecular biological methods

3.2.7.1.1 Cutting buffer

2.5 ml 1 M Tris-HCl (pH 7.5)
5.0 ml 0.5 M EDTA (pH 8.0)
1.0 ml 5 M NaCl
250µl 1 M DTT
127µl Spermidine (500mg/ml)
ad 50ml Aqua bidest.
Storage at 4°C.

3.2.7.1.2 DEPC-H_2O (0.1 %)

1ml DEPC were dissolved in 1000ml Aqua bidest. over night on a magnetic stirrer. Afterwards it was autoclaved three times. Storage at room temperature.

3.2.7.1.3 10x Dnase I-buffer

250 mM Tris-HCl (pH 8.3)
375 mM KCl
15 mM $MgCl_2$
filtered sterile, autoclaved and aliquoted, stored at –20°C.

3.2.7.1.4 Proteinase K solution

20mg/ml were dissolved in Aqua bidest. and aliquoted, stored at –20°C.

3.2.7.1.5 TE-buffer

10 mM Tris-HCl (pH 8.0)
1 mM EDTA

3.2.7.2 Buffer for Agarose gel electrophoresis

3.2.7.2.1 50x TAE-buffer

242 g Tris-base
57.1 ml Glacial Acetic Acid
100 ml EDTA (0.5M, pH 8.0)
ad 1000ml Aqua bidest.

3.2.7.2.2 1xTAE-buffer

10 ml 50x TAE-buffer
ad 500 ml Aqua bidest.

3.2.7.2.3 10x TBE-buffer

108 g Tris-base
55g boric acid
40 ml 0.5 M EDTA (pH 8.0)
ad 1000ml Aqua bidest.
Autoclaved

3.2.7.2.4 1x TBE-buffer

100 ml 10x TBE

ad 1000ml Aqua bidest.

3.2.7.3 Buffers for embedding and immunohistological procedures

3.2.7.3.1 Solution A

338 mg benzoylperoxide (25% water)

20 ml methylmethacrylate

60 ml hydroxymethylmethacrylate

16 ml ethyleneglycol monobutylether

2 ml polyethylene glycol 400

3.2.7.3.2 TBS (Tris-Buffer-Saline) stock solution (ph 7.6/0.05M)

60.5 g Tris-base dissolved in 700ml Aqua dest.

Add 2n HCl until pH 7.6 is reached

Ad 1000ml Aqua dest.

Add 90 g sodium chloride and adjust again to pH 7.6

3.2.7.3.3 TBS

100 ml TBS stock solution

900 ml Aqua dest.

3.3 Identification of transgenic mice by PCR

3.3.1 Primers

For the detection of transgenic mice, oligonucleotide primers with the following sequence were used:

- 5´-ACA GNN TCT NAG GGG CAG ACG NCG GG-3´ sense (Tra1)
- 5´-CCA GCA GNT NTA CAT ATC GAA GG-3´ antisense (Tra3)

(Synthese, Ludwig-Maximilians-University, Munich, Germany)

These primers bind to the human cDNA of the mutated GIP receptor and also to the endogenous murine GIP receptor. The primers were chosen from areas where the known DNA sequence of the human, rat, mouse and hamster GIP receptor is highly conserved. Wherever the sequence varies in these animals, oligonucleotide

synthesis was performed to allow all nucleotides (“N” in primer sequence) to integrate (Volz 1997).
The mutated human GIP receptor and the endogenous murine receptor can be distinguished in the PCR by their number of base pairs. The PCR product of the murine GIP receptor contains about 500 base pairs, whereas the PCR product of the mutated human GIP receptors consists of about 140 base pairs.

3.3.2 DNA isolation

At weaning of the mice, tail tip biopsies were taken and stored at –20°C until assayed. For DNA extraction, the tail tip of approximately 0.4 cm length was incubated in 400µl Mastermix over night in a heating block at 55°C.
Mastermix:
375 µl Cutting buffer
20 µl 20% SDS
5 µl Proteinase K (20mg/ml)

Thereafter, undigested components were separated by centrifugation for two minutes at 15,000 rpm. The supernatant was poured into another Eppendorf cup, and 400µl isopropanol were added to precipitate DNA. The DNA pellet was washed twice with 900µl 70% EtOH, the liquid phase was discarded and the DNA pellet was dried at room temperature. DNA was suspended in 100 - 200µl 1*TE buffer, according to the size of the pellet when dried. To make sure that the DNA was dissolved completely it was stored at 4°C for at least 24h before proceeding with the PCR.

3.3.3 PCR

1µl of the suspended DNA was mixed with 19µl of the Master Mix in PCR-analysis cups (Eppendorf, Germany). DNA and components of the Mastermix were kept on ice during the procedure. The Taq DNA polymerase was stored at –20°C until it was added to the Mastermix.

Mastermix:

Aqua bidest.	3.65µl
Q-Solution	4.00µl
10x buffer	2.00µl
MgCl2	1.25µl
DNTPs	4.00µl
sense primer	2.00µl
antisense primer	2.00µl
Taq Polymerase	0.10µl
Template	1.00µl

PCR conditions :

Primers	Tra1 / Tra3		
denaturation	94° C	4 min	
denaturation	94° C	1 min	
annealing	60° C	1 min	39x
extension	72° C	2 min	
final extension	72° C	10 min	

Until further use, the PCR samples were stored at either 4°C (short-term) or at –20°C (long-term).

DNA of a transgenic mouse was used as positive control, DNA of a wild-type mouse was used as negative control, H_2O served as quality control.

3.3.4 Gel electrophoresis

DNA fragments were separated by size via electrophoresis in a TAE agarose gel. 9µl/l ethidiumbromide (0.1%) was added to the gel solution. The TAE running buffer also contained 9µl/l ethidiumbromide (0.1%). Ethidiumbromide binds double stranded DNA by interpolation between the base pairs and fluorescence may be seen when irradiated in the UV part of the spectrum.

DNA samples were coloured with 4µl of 6* loading dye. In the first well of each row 6µl PUC-Mix-Marker was placed for determination of the length of the amplified DNA fragments and the remaining wells were filled with 24µl of the samples.
Afterwards, electrophoresis was run for 45 minutes at 90 volt.
To visualize the bands, the gel was put on an ultraviolett transilluminator and a digital picture was taken to document the result (Figure 3.1).

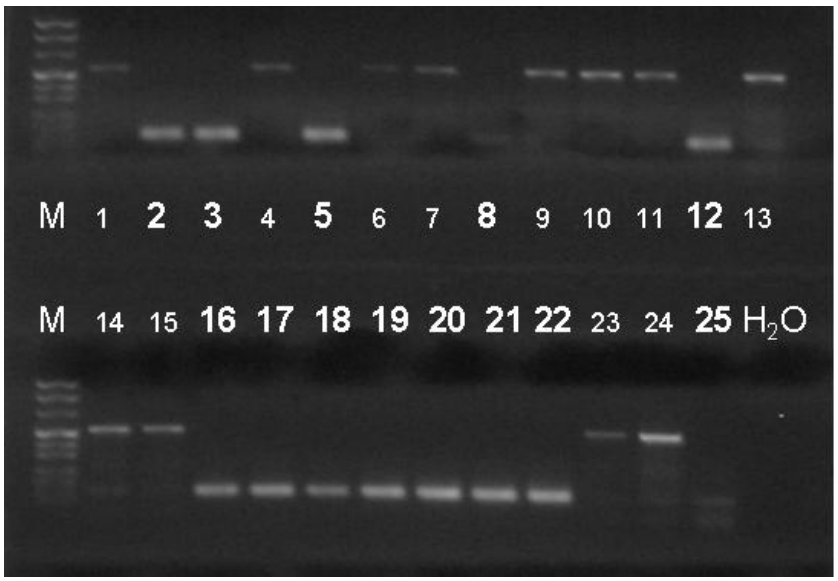

Figure 3.1 shows a PCR result of GIPR^{dn} transgenic and control mice. At the beginning of each row, the PUC Mix Marker 8 (M) is shown. Probes from control animals show a DNA fragment of 500 base pairs (endogenous GIP receptor). Transgenic animals exhibit a DNA-fragment of 140 base pairs (mutated GIP receptor, GIPR^{dn}). The identification number of animals is shown between the two rows, transgenic animals are written in bold, large numbers.

3.4 Analyses of gene/transgene expression on RNA level

Mice were sacrificed by cervical dislocation at the age of 90 days. Organ samples and fat were collected immediately and either frozen on dry ice until storage at -80°C or transferred to a plastic falcon containing RNAlater.
While working with RNA, these procedures were followed closely to prevent a degradation of RNA by RNases:

- gloves were worn at all times and changed regularly
- all solutions and plastic cups were autoclaved, glass was sterilized at 180°C for 12 hours

- gel chambers and gel combs were cleaned off proteins in 1% SDS solution over night, then washed with hot water and aqua bidest.
- all reaction steps were carried out on ice

3.4.1 Isolation of RNA

1ml TRIZOL® was filled in 5ml plastic falcons. 30 – 50mg of the samples were transferred to the falcon and immediately homogenized with a tissue homogenizer (Art-Mirco D 8) at 23,500 rpm for 30 - 60 seconds. The homogenizer was cleaned between the samples with aqua bidest., 0.2M NaOH and then again with aqua bidest. RNA was isolated according to the producer´s manual, either with Trizol® or with the RNeasy Mini Kit.

The RNA pellet was solubilised in 20 - 200µl 0.1% DEPC-H_2O depending on its size.

Following the isolation, the concentration of RNA was measured with a spectrophotometer at 260 nm and 280 nm wave length.

The quality of RNA was controlled by gel electrophoresis in an 1% TBE gel and was considered to be good when the 18s and 28s bands showed up clearly.

3.4.2 Dnase I-digest and reverse transcription for RT-PCR

5µg of the isolated RNA were incubated with RNase-free DNase I at 37°C for 30min to eliminate DNA contaminations in the sample. Reagents were added as follows:

x µl total RNA (5µg)

- ad 17µl DEPC-H_2O
- 2µl 10x DNase I digestion buffer
- 1µl DNase I

The enzyme was inactivated afterwards at 75°C for 10min.

After elimination of DNA contaminations, the concentration of RNA was measured again.

400ng DNase I-digested RNA were used for reverse transcription with M-MLV reverse transcriptase. Reagents were applied as follows:

x µl RNA, Dnase I-digested (400ng)

- ad 10µl PCR-H_2O
- 1µl random primer hexamers
- 1µl dNTP Mix 10mM → 5min at 65°C → chill on ice
- 2µl 5x first-strand buffer
- 2µl 0.1M DTT
- 1µl RNase OUT → 10min at 25°C → 2min at 37°C
- 2µl 5x first-strand buffer
- 1µl M-MLV RT (200U/µl) → 50min at 37°C

After inactivation of the enzyme at 70°C for 15min, the cDNA was chilled on ice, centrifuged shortly and then stored at -20°C until further use.

3.4.3 RT-PCR

2µl of the cDNA solution were used for RT-PCR. The Mastermix for the PCR was prepared in the following way:

Aqua bidest.	7.65µl
Q-Solution	4.00µl
10x buffer	2.00µl
MgCl2	1.25µl
dNTPs	1.00µl
Sense primer	1.00µl
Antisense primer	1.00µl
Taq Polymerase	0.10µl
Template	2.00µl

For detection of the expression of the endogenous murine GIP receptor (mGIPR), transgenic dominant negative human GIP receptor, and beta-actin as housekeeping gene the following primers were generated (5´-> 3´):

Murine GIP receptor: mGIPR 3 (sense) + mGIPR 6 (antisense)

mGIPR 3 (se) AGCCATCCTCACCCGAGATCAG

mGIPR 6 (as) TCCCACGATCACCAGCAGATG

Transgenic dominant negative GIP receptor ($GIPR^{dn}$): GIPRdn1 (sense) + GIPRdn (antisense)

GIPRdn1 (se)	TTTTTATCCGCATTCTTACACGG	deletion-specific
GIPRdn (as)	ACCTCGTGGACACCCAGCAG	

beta-actin: beta-actin 1 + beta-actin 2

beta-actin 1	GGCATCGTGATGGACTCCG
beta-actin 2	GCTGGAAGGTGGACAGTGAG

RT-PCR conditions:

Primer	mGIPR / $GIPR^{dn}$			beta-actin		
denaturation	94° C	5´		94°C	5´	
denaturation	94° C	45´´		94°C	1´	
annealing	62° C	45´´	36x	60°C	1´	36x
extension	72° C	1´15´´		72°C	1´30´´	
final extension	72° C	10´		72°C	10´	

Gel electrophoresis was performed as described in chapter 3.4.3. Instead of TAE, TBE was used as buffer.

Bands were visualized and documentated by a Stratagene Eagle Eye II.

3.5 Blood glucose

Blood glucose was measured in all mice at weaning, using the Super GL_{easy} System. Furthermore, 15-hour fasting blood glucose levels and postprandial blood glucose levels after 1.5 hours refeeding were determined in each animal before euthanisation, using the Super GL_{easy} System.

10µl blood were drawn into a capillary and put into an Eppendorf cup containing 500µl hemolysing solution. The Super GL_{easy} System was calibrated with a control solution of known concentration and then the samples were measured.

3.6 Oral glucose tolerance test (OGTT)

An oral glucose tolerance test (OGTT) was performed at the age of 50 days in five control and six transgenic mice fed with breeding chow and in seven control and five transgenic mice fed the carbohydrate restricted diet. Another OGTT was carried out

at the age of 175 days in six control and seven transgenic mice fed with breeding chow and in six control and seven transgenic mice fed a carbohydrate restricted diet.
Animals were fasted for 15 hours and a basal blood sample was taken from the nicked tail tip for determination of basal blood glucose (t = 0 minutes). Mice were then gently fixed and 11.1µl/g body weight of an 1M glucose solution were administered via gavage tube. Further blood samples were collected at 10, 20, 30, 60, 90 and 120 minutes for determination of blood glucose. Blood glucose levels were determined with the Super GL_{easy} System as described above.

3.7 Insulin sensitivity test

At the age of 50 days, an insulin sensitivity test was carried out in 24 mice of which six control and six transgenic mice were fed with breeding chow and six control and five transgenic mice were fed the carbohydrate restricted diet.
Mice were randomly fed. At the beginning, a basal blood sample was taken from the nicked tail tip to determine blood glucose (t= 0 minutes). Thereafter, mice were gently fixed and 1U/kg body weight insulin was injected intraperitoneally. Further blood samples were obtained at 10, 20, 30 and 60 minutes for determination of blood glucose. Blood glucose levels were measured with the Super GL_{easy} System as described before.

3.8 Serum insulin levels and serum GIP levels

Blood was collected from the retroorbital plexus under ether anesthesia of fed 45- (n= 28), 90- (n= 16) and 180-day-old mice (n= 24). All mice were starved over night (15 hours) and fed for 1.5 hours prior to sampling.
Serum was separated by centrifugation (10 minutes, 10,000xg) and stored at -80°C until assayed.
Serum insulin of mice of all age groups was measured using the sensitive Rat Insulin RIA kit (Linco Research, Missouri, USA), which shows 100% cross reactivity with murine insulin according to the manufacturer´s manual.
Serum GIP levels were determined using a Rat/Mouse GIP Elisa kit (Linco Research, Missouri, USA).

Both the RIA and the ELISA were carried out according to the producer´s manual.

3.9 Body weight

All mice were weighed at weaning, and prior to sacrifice. Body weight was determined to the nearest 0.1g.

3.10 Daily food and water intake

Daily food and water intake was surveyed for 5 days in a 24-hour interval at the age of 120 days. Seven wild-type and seven $GIPR^{dn}$ transgenic mice fed the rodent breeding chow and six wild-type and seven transgenic mice fed the carbohydrated restricted diet were included in this test. Each mouse was kept in a separate cage for this time and the bottom of the cages was covered with paper towels in order to recognize fallen food particles more easily. Food and water intake were determined at the same time every day (09:30 am).

3.11 Pancreas preparation

Mice were starved over night for 15h. Then blood was collected from the tail vein to determine fasting blood glucose levels and 1ml/100g body weight of a 10mM BrdU solution in aqua dest. were injected intraperitoneally. Mice were fed for 1.5h before sacrifice by exsanguination under ether anesthesia and blood was collected from the retroorbital plexus for determination of serum GIP and insulin levels as described above. The pancreas was removed immediately, carefully separated from adjacent tissues, weighed to nearest mg, placed in a tissue capsule on a piece of foam-rubber sponge to avoid distortion and fixed in 10% neutral buffered formalin.
After fixation at room temperature for 24 hours, the pancreas was embedded in Agar, sectioned perpendicular to this longitudinal axis into parallel slices of approximately 1mm thickness, with the first cut positioned randomly within an interval of 1mm length at the splenic end of the pancreas (Wanke et al. 1994). Slices were placed in tissue capsules on a piece of foam-rubber sponge with the right cut surface facing

downward. Then embedding in paraffin (every first slice) and plastic (GMA/MMA; every second slice) was proceeded.

3.12 Histology

3.12.1 Embedding procedures

Pancreatic tissue was embedded in paraffin and plastic.

Embedding procedure:

Paraffin	
H_2O	1 hour
Ethyl alcohol 70%	2x 2 hours
Ethyl alcohol 96%	2x 2 hours
Ethyl alcohol 100%	2x 1.5 hours
Xylol	3x 1 hour
Paraffin 65°C	3 hours
Paraffin 65°C	6 hours

Plastic	
Rinsing solution	3 hours
Ethyl alcohol 30%	2x 1 hour
Ethyl alcohol 50%	2x 1 hour
Ethyl alcohol 70%	2x 1 hour
Ethyl alcohol 96%	2 hours
Ethyl alcohol 96%	2x 3 hours

After dehydration, samples chosen for embedding in plastic were incubated at 4°C for 4 hours in a GMA/MMA 1:1 solution on a shaker, thereafter samples were incubated in solution A at 4°C for 2 hours also on a shaker. 60µl dimethylanilin was added to solution A to start polymerization. Then plastic cups were filled with the same solution A and samples were placed in the cups with the right cut surface facing downward. The plastic cups were kept in a water bath at 4°C over night to allow polymerisation.

3.12.2 HE staining of plastic sections

Sections were cut on a rotary microtome, dried on a heating plate and stored in an incubator at 64°C over night before staining with hematoxylin-eosin.

Table 3.2 Hematoxylin-Eosin

Reagents	Time
Mayer´s Hemalaun	20min
running tap water	10min
HCl-ethyl alcohol	10sec

running tap water	10min
heating plate	5min
Eosin-Phloxin	20min
Aqua dest.	2x 2min
heating plate	5min
Xylol	2min
Covering with Coverquick®	

3.12.3 TUNEL assay and immunohistochemistry on paraffin sections

Approximately 5µm thick paraffin sections for histology were cut with a HM 315 microtome and mounted on Starfrost® adhesive glass slides. Before starting immunohistochemistry, the slides were stored in an incubator at 45°C for at least one day. Apoptotic cells were stained with the TUNEL assay according to the manufacturer´s manual, the indirect immunoperoxidase method was applied to stain for replicating cells (BrdU-containing cells) and insulin positive cells.
Immunohistochemical staining techniques are shown in tables 3.3 – 3.5. Temperatures are room temperature if not stated otherwise.

Table 3.3 TUNEL assay

Reagent	Time
Xylol	15min
Ethyl alcohol 100%	2x 5min
Ethyl alcohol 96%	3min
Ethyl alcohol 70%	3min
Aqua dest.	2min
TBS	5min
Proteinase K	5min
Aqua dest.	2x 2min
3% H_2O_2 in TBS	5min
TBS	2x 5min
Equilibration buffer	2min
Tdt Enzyme 37° 38.5µl reaction buffer + 16.5µl enzyme	1h
Stop buffer 1ml Stop buffer + 34ml Aqua dest.	shake 15sec incubate 10min
TBS	3x 1min
Anti-digoxigenin-peroxidase	30min
TBS	3x 3min

DAB	10min
Aqua dest.	2x 1min 1x 5min
Hematoxylin	2min
Running tap water	2min
Ascending ethyl alcohol series	2min
Xylol	2min
Covering with Coverquick®	

Table 3.4 BrdU Immunohistochemistry

Reagent	Time
Xylol	30min
Ethyl alcohol 100%	2min
Ethyl alcohol 96%	2min
Ethyl alcohol 70%	2min
Aqua dest.	2min
Target retrieval solution 1:10 95°	20min
Cool at room temperature	20min
TBS	2x 2min
Normal rabbit serum 1:5	30min
Rat anti-human BrdU 1:50	1h
TBS	2x 2min
3% H_2O_2 in Aqua dest.	10min
TBS	3x 2min
Rabbit anti-Rat IgPO 1:50	30min
TBS	3x 2min
DAB	8min
Aqua dest.	1min
Hematoxylin	2min
Running tap water	2min
Ascending ethyl alcohol series	2min
Xylol	2min
Covering with Coverquick®	

Table 3.5 Insulin Immunohistochemistry

Reagent	Time
Xylol	20min
Ethyl alcohol 100%	2min
Ethyl alcohol 96%	2min
Ethyl alcohol 70%	2min

Aqua dest.	2min
1% H_2O_2 in Aqua dest.	15min
TBS	10min
Normal Rabbit Serum 1:10	30min
Guinea Pig anti-Insulin 1:2,000	2h
TBS	10min
Rabbit anti-guinea pig IgPO 1:50	1h
TBS	10min
DAB	5min
Running tap water	2min
Hematoxylin	1min
Running tap water	2min
Ascending ethyl alcohol series	2min
Xylol	2min
Covering with Coverquick®	

3.12.4 Immunohistochemistry on plastic sections

Approximately 2.0 µm thick sections for histology were cut on a rotary microtome, stretched in a warm water bath and dried on a heating plate at 64°C. The indirect immunoperoxidase method was applied to stain for insulin positive cells, as described in the table 3.6. Since a complete cross reaction between guinea pig and rabbit immunglobulins has been described in the manufacturer´s manual, biotinylated pig anti-rabbit Ig were used as secondary antibody. Temperatures are room temperature if not stated otherwise.

Table 3.6 Insulin Immunohistochemistry

Reagents	Time
TBS	5min
0,1% HCl	10min
Heating plate 64°	2min
Target retrieval solution 1:10 95°	10min
Heating plate 64°	2min
TBS	5min
1% H_2O_2 in Aqua dest.	15min
TBS	10min
Normal pig serum 1:10	30min
Guinea pig anti-Insulin 1:50 37°	2h
TBS	10min

biot.pig anti-rabbit Ig 1:50 37°	2h
TBS	10min
Streptavidin-Biotin Complex 1:100	30min
TBS	10min
DAB	10min
Running tap water	5min
Hematoxylin	15min
Running tap water	5min
Xylol	2min
Covering with Coverquick®	

3.13 Morphometric analyses of the pancreas

3.13.1 Determination of the pancreas volume

Photographs of paraffin sections immunostained for insulin, showing the complete cut surface of all pancreas slices were taken at a final magnification of 16x, using a Wild M 400 photomacroscope (Wild, Switzerland). At the beginning of each set, an object micrometer (Zeiss, Germany) was photographed under the same conditions for calibration. Morphometric evaluation was carried out on a Videoplan® image analysis system (Zeiss-Kontron, Germany), attached to a microscope by a colour video camera. The cross-sectional area of the pancreas was determined planimetrically by circling the contours on printouts. The volume of pancreas, $V°_{(Pan)}$, before embedding was calculated from the pancreas weight divided by the specific weight of mouse pancreas (1.08 mg/mm^3) (Wanke et al. 1994).

The volume of the pancreas without the volume of residual adjacent tissues was calculated according to the formula:

$$V_{(Pan)} = \frac{\Sigma A_{(Pan)}}{\Sigma A_{(Pan)} + \Sigma A_{(non\ Pan)}} \times V°_{(Pan)}$$

3.13.2 Determination of total volumes and volume densities of islets in the pancreas, beta-cells in islets and isolated beta-cells in the pancreas

Measurements of areas of the islet profiles, islet beta-cells and isolated beta-cells were carried out on the same sections immunostained for insulin. Isolated beta-cells are referred to as an indicator for islet neogenesis (Bonner-Weir et al. 1993). Isolated beta-cell areas were defined as small clusters of beta-cells, containing four nuclear

profiles or less. Images were displayed on a colour monitor at an 850x final magnification, and the profiles of islets, beta-cells or isolated beta-cells were measured planimetrically by circling their contours with a cursor on the digitizing tablet of the image analysis system.
Parameters were calculated as follows:

Volume density of islets in the pancreas:

$$Vv_{(Islets/Pan)} = \frac{\Sigma A_{(Islets)}}{\Sigma A_{(Pan)}}$$

Total islet volume:

$$V_{(Islets,Pan)} = Vv_{(Islets/Pan)} \times V_{(Pan)}$$

Volume density of beta-cells in the islets:

$$Vv_{(Beta\text{-}cells/Islets)} = \frac{\Sigma A_{(Beta\text{-}cells)}}{\Sigma A_{(Islets)}}$$

Total volume of beta-cells in the islets:

$$V_{(Beta\text{-}cells,Islets)} = Vv_{(Beta\text{-}cells/Islets)} \times V_{(Islets,Pan)}$$

Volume density of isolated beta-cells in the pancreas:

$$Vv_{(isol.beta\text{-}cells/Pan)} = \frac{\Sigma A_{(isol.\ beta\text{-}cells)}}{\Sigma A_{(Pan)}}$$

Total volume of isolated beta-cells in the pancreas:

$$V_{(isol.beta\text{-}cells,Pan)} = Vv_{(isol.beta\text{-}cells/Pan)} \times V_{(Pan)}$$

3.13.3 Determination of islet cell replication and apoptosis

Profiles of BrdU and TUNEL positive nuclei of cells of the endocrine pancreas were counted using paraffin sections stained for BrdU and TUNEL, respectively.
With a random start within a section of the first slice of pancreas, the whole slide was surveyed by meandering, neglecting every second stripe. Stained nucleus profiles and not stained nucleus profiles of islet cells were counted in every second stripe and the areas of the corresponding islets were measured planimetrically. The number of nucleus profiles per area was calculated by dividing the number of counted nuclear profiles by the measured area of islet profiles and then the nuclear profile number

was extrapolated to the total islet area by multiplying the number of profiles per area and the total islet area. The total islet area was determined as described in 3.13.2. The number of BrdU and TUNEL positive nuclear profiles are stated as number of positive cells per 100,000 nuclei.

3.13.4 Determination of number and size of islets and beta cells

For the determination of the total islet number and total beta-cell number, plastic sections of pancreata of both feeding groups at the age of 45 (n= 5 per group) and 180 days (n= 4 per group) were analysed.

Counting was performed by applying the disector principle. The disector is a three-dimensional stereologic probe, which allows unbiased assumption-free counting and sizing of particles (Sterio 1984).

The physical disector, which consists of a pair of physical section planes separated by a known distance was used to estimate the numerical density of pancreatic islets in the pancreas and beta-cells in the islets. The distance between both sections (disector height) should be approximately 30% of the diameter of the objects to be counted (Howard and Reed 2005). All islets or beta-cells sampled in the primary section, which were not present in the reference section, were counted (Q^-). The process of counting Q^- was then repeated by interchanging the roles of the primary and reference section, thereby increasing the efficiency by a factor of two.

To determine the disector height, the nominal thickness of the plastic sections was controlled, using a resectioning technique. Five sections, which were not used for sampling, were selected from different section series, embedded in agar, re-embedded in GMA/MMA, and stained with hematoxylin-eosin. The thickness of the re-embedded sections was then obtained by two-point-distance measurement on a Videoplan® image analysis system. The measured mean thickness of the sections was 2.11 ± 0.42 µm.

Calculation of the numerical density, the total number and mean volume of islets and beta-cells is influenced by tissue shrinkage due to the embedding procedure. Since tissue shrinkage was found to be lower in plastic embedded tissue than in other embedding materials (Gardella et al. 2003) and since the comparison of different groups was of main interest, tissue shrinkage was neglected in this study.

Tissue shrinkage of GMA/MMA embedded kidney tissue was determined to constitute 25% of the original volume (Wanke 1996). In the liver, tissue shrinkage due to GMA embedding resulted in a 35% loss of volume (Gerrits et al. 1987).

3.13.4.1 Determination of number and size of islets in the pancreas

Prior to counting islets, the mean diameter of islet profiles was determined from 12 pancreas sections of six mice (three wild-type and three transgenic). The mean minimum diameter turned out to be 94 µm, therefore, a disector height of 30 µm was chosen.

Sections for 12 disectors per wild-type mouse and for 16 disectors in transgenic mice were cut on a rotary microtome, dried on a heating plate, incubated at 65°C over night and then stained with hematoxylin-eosin.

Islets were counted under the microscope at a final magnification of 100x. Clusters of beta-cells were only counted as islets when more than four nuclear profiles were visible.

On the average 29 (range: 23–40) islets were counted in 45 day old control mice, 39 (range: 30-49) islets were counted in 180 day old control mice, 18 (range: 12-22) islets were counted in 45 day old $GIPR^{dn}$ transgenic mice, and 27 (range: 19-37) islets were counted in 180 day old transgenic mice.

The area of the pancreas cut surface was used as disector area and was determined by point counting morphometry.

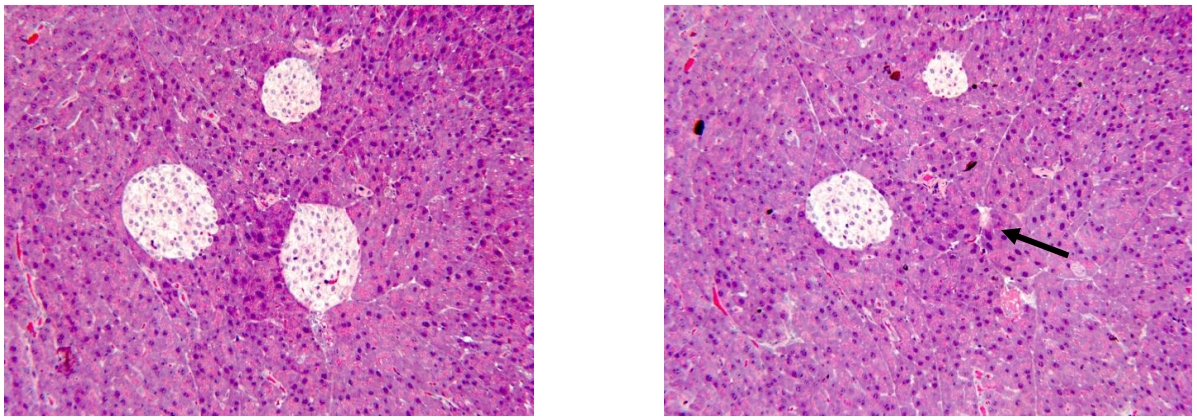

Figure 3.1 Disector for counting islets

Three islets are visible in the primary section. In the reference section one of the islets has vanished. Therefore it is counted. Plastic sections, hematoxylin-eosin staining. Original magnification 250x.

The numerical density of islets in the pancreas, $N_{VS(Islets/Pan)}$, was calculated by dividing the total number of islets counted in all disectors (Q^-) by the cumulative volume of the disectors (cross-sectional area of pancreas x disector height). The total number of pancreatic islets, $N_{(Islets,Pan)}$, was calculated as the product of the

numerical density of islets in the pancreas ($Nv_{s(Islets/Pan)}$) and the total pancreas volume $V_{(Pan)}$.

The mean islet volume ($v_{(Islets)}$) was obtained by dividing the volume density ($Vv_{(Islets/Pan)}$) by the numerical density of the islets in the pancreas ($Nv_{s(Islets/Pan)}$).

3.13.4.2 Determination of number and size of beta cells in the islets

For selection of the appropriate disector height for counting beta-cells, the approximate diameter of beta-cell nuclei was determined. For this purpose, the diameter of beta-cell nucleus profiles that appeared to be cut centrally was determined by planimetrically circling its cut surface on immunohistologically stained sections on a Videoplan® image analysis system. The mean diameter of the nuclei of beta-cells was 5.7µm. Based on these results, a disector height of ~2.0 µm (2.1 µm) was chosen according to Howard and Reed (2005). Three series of five sections of each animal were sectioned. From the stack of serially cut sections, one section was drawn at random by means of a random number (rn) between two and five (the baseline section was not used for sampling). The second section was sampled by means of rn±1.

The selected sections were stained immunohistochemically for insulin. With a random start within the first slice of pancreas the whole primary section was surveyed and islets in every second stripe were sampled until a total number of five islets in wild-type mice and seven islets in transgenic mice was reached. The number of disectors, five in wild-type mice and seven in transgenic mice, were determined in a pilot study. Light microscopic photographs of the selected islets and the corresponding islets in the reference section were taken with a colour video camera, using a 40x objective and printed at a final magnification of 1530x.

A plastic transparency with an unbiased counting frame was applied to the print-outs and the number of beta-cells was counted according to the unbiased counting rule (Sterio 1984). In case of small islets which were smaller than the counting frame the whole islet area was used as dissector area and a plastic transparency with equally spaced test points (n= 35) was used in order to estimate the area of the islet by point counting morphometry.

The upper and right frame line were considered as inclusion lines and nuclei within the frame or touching one of those lines were counted. The lower and left frame line were considered as exclusion lines, i.e. cell nuclei touching one of these lines were

not counted. On the average 58 (range: 48 – 72) beta-cell nuclei were counted in control mice and 38 (range: 11 – 50) in GIPR^{dn} transgenic mice.

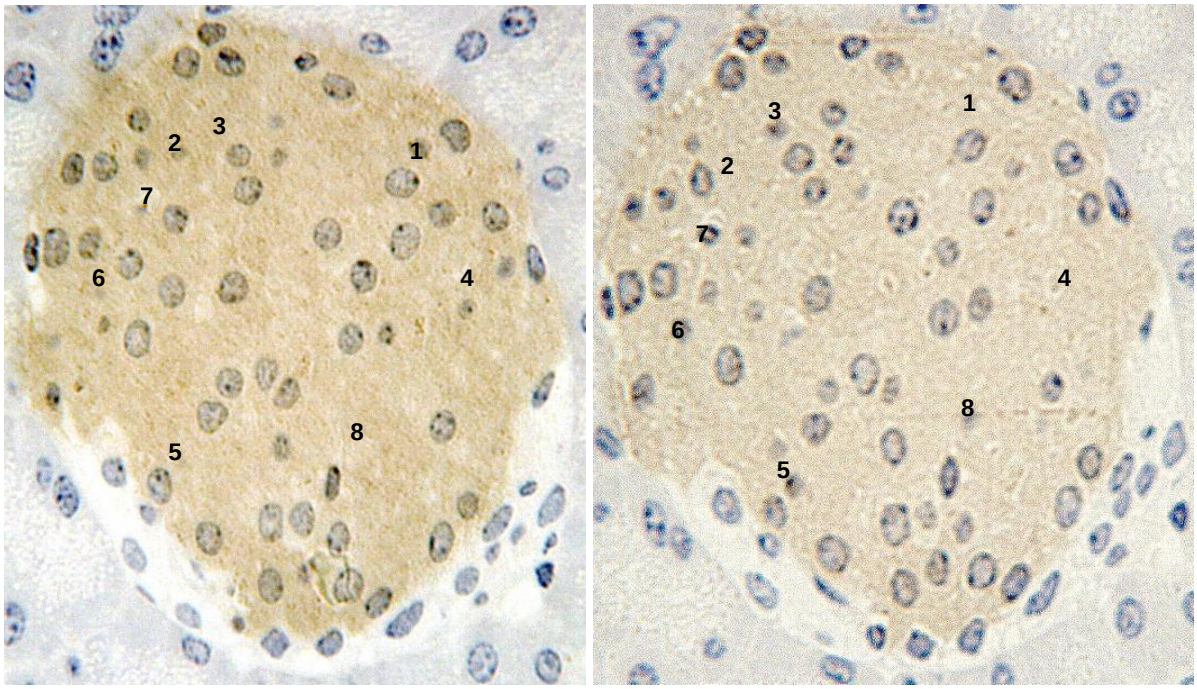

Figure 3.2 Disector for counting beta-cells

A corresponding primary and reference section of a wild-type mouse are shown. Nuclei that were counted are numbered. Nuclei were counted with the double dissector principle by interchanging the roles of the primary and reference section. The total islet profile was used as disector area, i.e. no subsampling with an unbiased counting frame was done in this example. Original magnification of 1530x.

The numerical density of beta cells in islets ($Nv_{s(Beta\text{-}cells/Islets)}$) was calculated by dividing the total number of nuclei counted in all disectors (Q^-) by the cumulative volume of the disectors (area of the unbiased counting frame x disector height x number of disectors). To obtain the area of the counting frame taking the magnification factor into account, its length and width were measured, divided by the magnification factor 1530x, and then multiplied.

The total number of beta-cells in the islets ($N_{(Beta\text{-}cells,Islets)}$) was estimated by multiplying the numerical density of beta-cells in the islets ($Nv_{s(Beta\text{-}cells/Islets)}$) and the total beta cell volume ($V_{(Beta\text{-}cells,\ Islets)}$).

The mean beta-cell volume ($v_{(Beta\text{-}cells)}$) was obtained by dividing the volume density ($Vv_{(Beta\text{-}cells/Islets)}$) by the numerical density of the beta-cells in the Islets ($Nv_{s(Beta\text{-}cells/Islets)}$).

3.14 Statistical analysis

The effects of genetic group, diet and age on the different parameters were investigated by univariate variance analysis. Comparisons between the different groups were carried out by two-tailed Student's *t*-test (SPSS program package; SPSS, Inc., Chicago, IL, USA). P values < 0.05 were considered significant. Data are presented as means and standard error of means (SEM) throughout the study.

4. Results

4.1. Gene/transgene expression on RNA level

The expression of the murine endogenous GIP receptor, and the dominant negative GIP receptor was investigated in a wide organ spectrum. Tissue samples were taken from M. quadriceps, heart, cerebrum, adipose tissue, pancreas, liver, stomach, duodenum, ileum, colon, spleen, kidney and lung. For number of investigated animals see table 3.1.

4.1.1 Expression pattern of the endogenous GIP receptor in wild-type mice

Expression of the murine endogenous GIP receptor was found in M. quadriceps, heart, cerebrum, adipose tissue, pancreas, stomach, duodenum, ileum, colon, spleen (very weak band) and kidney. No expression was found in the liver and in the lung (Fig 4.1).

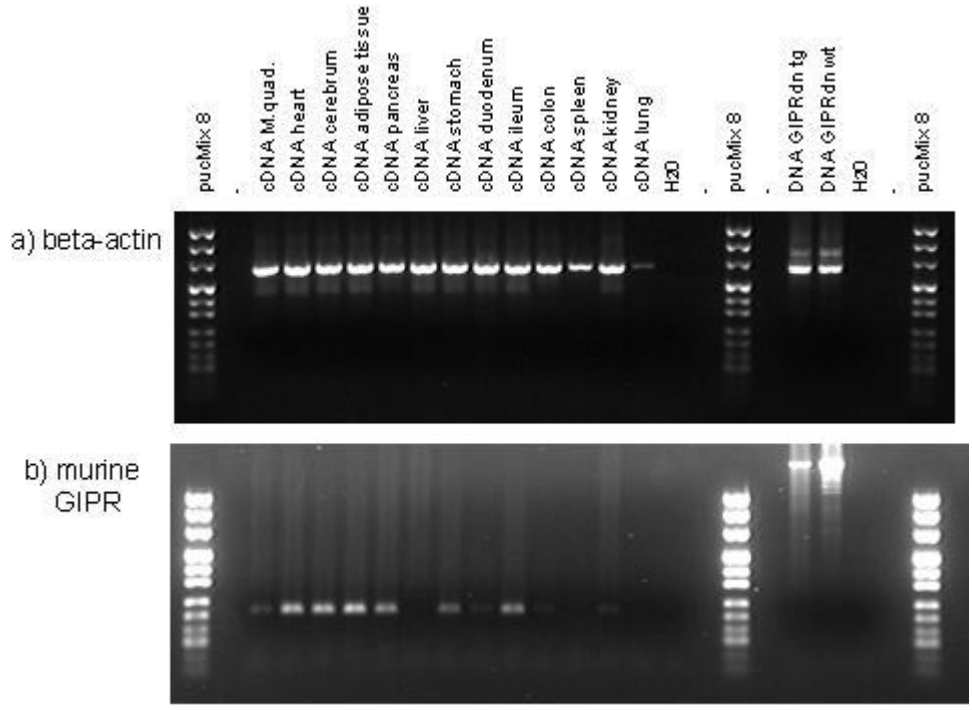

Figure 4.1 Expression pattern of the endogenous GIP receptor in a wild-type mouse

a) Expression of the beta-actin gene. Expression is found in all organs.

b) Expression of the endogenous murine GIP receptor. Expression is found in the muscle, heart, cerebrum, adipose tissue, pancreas, stomach, duodenum, ileum, colon, spleen and kidney.

4.1.2 Expression pattern of the endogenous and transgenic GIPR in GIPR^{dn} transgenic mice

The transgenic GIP receptor was found to be expressed in the M. quadriceps, heart, adipose tissue, pancreas, stomach, duodenum, ileum, colon, spleen, kidney and lung. No expression of the transgenic GIP receptor was detected in the cerebrum and in the liver.

Expression of the murine endogenous GIP receptor was observed in the M. quadriceps, heart, cerebrum, adipose tissue, pancreas, stomach, duodenum, ileum, colon, spleen, kidney and lung. No expression was detected in the liver (Figure 4.2).

The RT-PCR for the transgenic GIP receptor was run in wild-type mice to control the specifity of the primers and no expression was found. (data not shown)

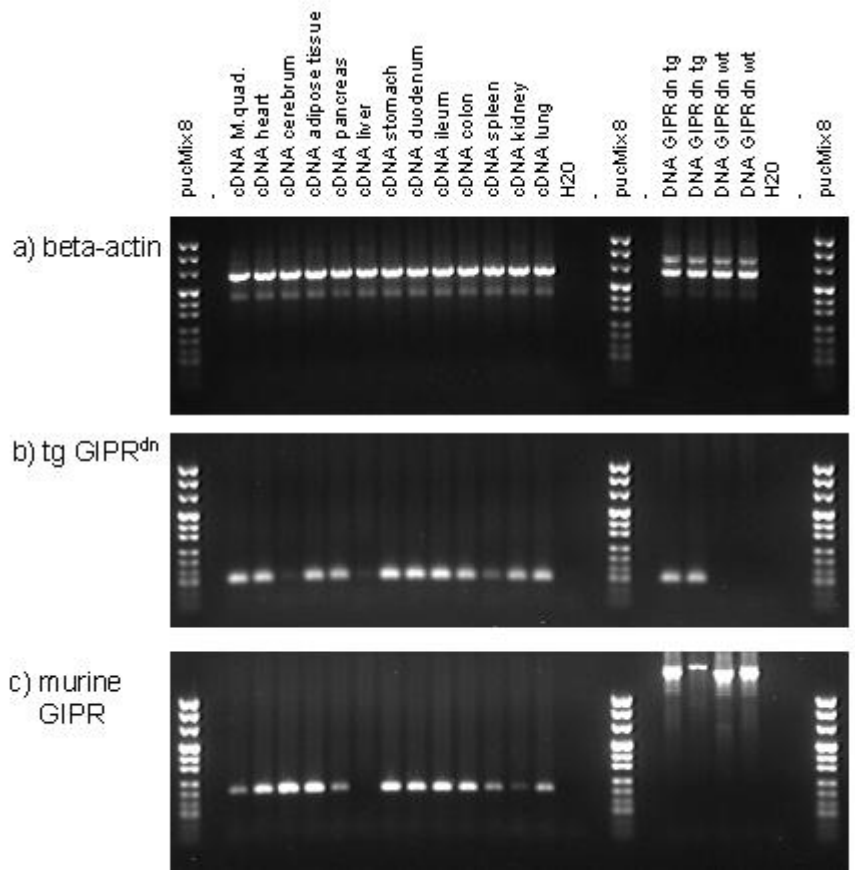

Figure 4.2 Expression pattern of the endogenous and transgenic GIPR in a GIPR^{dn} transgenic mouse

a) Expression of the beta-actin gene. Expression is found in all organs.

b) Expression of the transgenic GIP receptor in M. quadriceps, heart, adipose tissue, pancreas, stomach, duodenum, ileum, colon, spleen, kidney.

c) Expression of the endogenous murine GIP receptor in M. quadriceps, heart, adipose tissue, pancreas, stomach, duodenum, ileum, colon, spleen, kidney, lung.

4.2 Blood glucose

4.2.1 Blood glucose levels at weaning

Mice were weaned at the age of 21 days and a blood sample was taken from the tail vene of randomly fed animals, when tail tip biopsies were performed. Female wild-type mice showed mean blood glucose levels of 130.7 ± 3.4 mg/dl. $GIPR^{dn}$ transgenic mice exhibited significantly elevated blood glucose levels with mean levels of 343.8 ± 17.2 mg/dl. Data are means ± SEM, (n= 30 per group).

4.2.2 Fasting blood glucose

$GIPR^{dn}$ transgenic mice fed the breeding diet (BD) showed at least 2.9-fold higher (2.9 – 5.1-fold) fasting blood glucose levels than diet-matched wild-type mice, irrespective of the age at sampling. Transgenic mice fed the carbohydrate restricted diet (CRD) exhibited significantly increased (1.6 – 2.4-fold) fasting blood glucose levels when compared to diet-matched wild-type mice mice regardless of the age at sampling.

Fasting blood glucose levels of $GIPR^{dn}$ transgenic mice fed the carbohydrate restricted diet were lower than those of transgenic mice fed the breeding diet but this difference did not reach statistical significance. However, analysis of variance revealed a significant effect of diet and genetic group on fasting blood glucose concentrations. The diet had a significant effect only among transgenic mice.

At the age of 45 days, wild-type mice fed the carbohydrate restricted diet showed significantly elevated (1.3-fold) blood glucose levels when compared to age- matched wild-type mice fed the breeding diet.

No significant difference in fasting blood glucose levels were found with the progression of age, irrespective of the diet fed or genetic group (Fig 4.3).

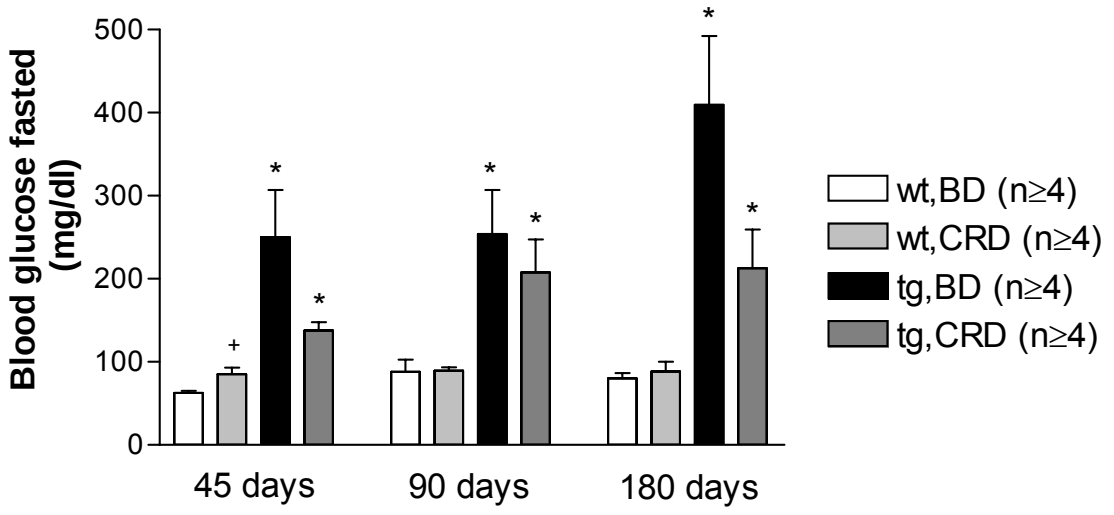

Figure 4.3 Fasting blood glucose levels

GIPR^{dn} transgenic mice (tg) of both feeding groups exhibit higher fasting blood glucose levels than age- and diet-matched wild-type mice (wt) at all ages investigated. At 45 days, wild-type mice fed the carbohydrate restricted diet (CRD) show higher fasting blood glucose levels than wild-type mice fed the breeding diet (BD). Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD; + $p<0.05$ wt, CRD vs. wt, BD.

4.2.3 Postprandial blood glucose

GIPR^{dn} transgenic mice fed the breeding diet showed 3.3-fold to 5.2-fold higher postprandial blood glucose levels than age- and diet-matched wild-type mice regardless of the age investigated.

GIPR^{dn} transgenic mice fed the carbohydrate restricted diet exhibited at least 1.6-fold (1.6-fold – 2.6-fold) higher postprandial blood glucose levels than age- and diet-matched wild-type mice at all ages investigated.

Transgenic mice fed the carbohydrate restricted diet showed 57% lower postprandial blood glucose levels than age-matched transgenic mice fed the breeding diet at all ages investigated ($p<0.05$).

At the age of 180 days, wild-type mice fed the carbohydrate restricted diet exhibited 23% lower postprandial blood glucose levels than age-matched wild-type mice fed the breeding diet ($p<0.05$).

Postprandial blood glucose levels of GIPR^{dn} transgenic mice of both feeding groups were significantly higher at the age of 90 days vs. 45 days (≥ 1.6-fold) (Fig 4.4).

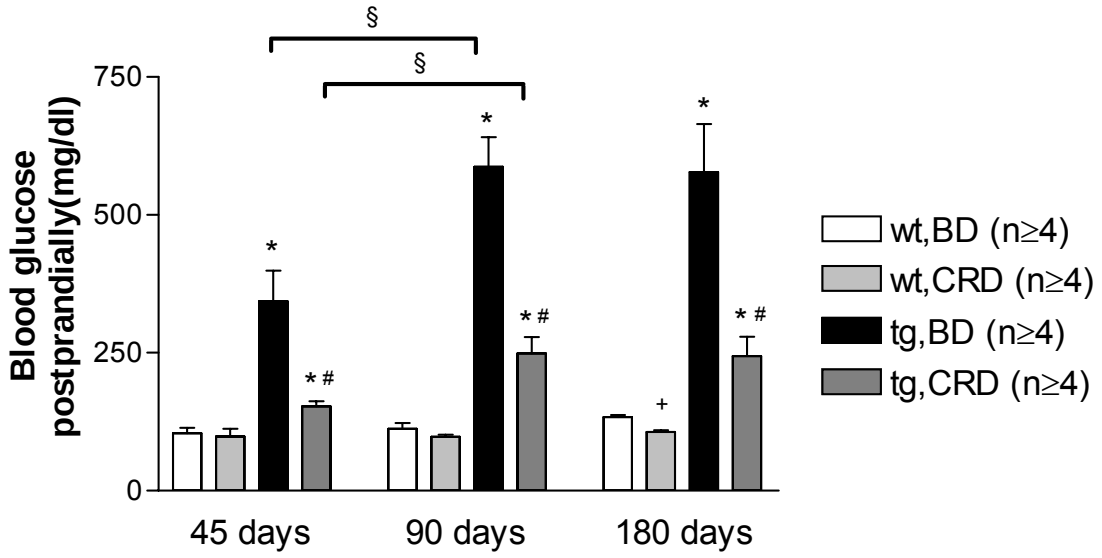

Figure 4.4 Postprandial blood glucose levels

GIPR^{dn} transgenic mice (tg) of both feeding groups exhibit higher postprandial blood glucose levels than age- and diet-matched wild-type mice (wt) at all ages investigated. Postprandial blood glucose levels of transgenic mice fed the carbohydrate restricted diet are lower than those of transgenic mice fed the breeding diet, regardless of age. At 180 days of age, wild-type mice fed the carbohydrate restricted diet (CRD) show significantly lower postprandial blood glucose levels than age-matched wild-type mice fed the breeding diet (BD). Postprandial blood glucose levels of GIPR^{dn} transgenic mice were significantly higher at 90d vs. 45d. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD; + $p<0.05$ wt, CRD vs. wt, BD; § $p<0.05$ 45d vs. indicated time point.

4.3 Oral glucose tolerance test (OGTT)

Oral glucose tolerance tests were performed at the age of 50 days and 175 days. For numbers of investigated animals see table 3.1.

4.3.1 OGGT at the age of 50 days

Basal blood glucose levels of GIPR^{dn} transgenic mice fed the breeding diet were significantly elevated when compared to diet-matched wild-type mice (142.0 ± 17.6 vs. 80.2 ± 5.5 mg/dl; n.s.). Transgenic mice fed the carbohydrate restricted diet showed higher basal blood glucose levels than diet-matched wild-type mice (187.2 ± 34.3 vs. 89.7 ± 8.3 mg/dl). GIPR^{dn} transgenic mice of both feeding groups showed significantly increased blood glucose levels at 10, 20, 30, 60, 90 and 120 minutes after glucose load as compared to diet-matched wild-type mice. There were no

significant differences in blood glucose levels comparing wild-type mice or GIPR^{dn} transgenic mice of the different feeding groups at the time points investigated after glucose challenge (Fig 4.5).

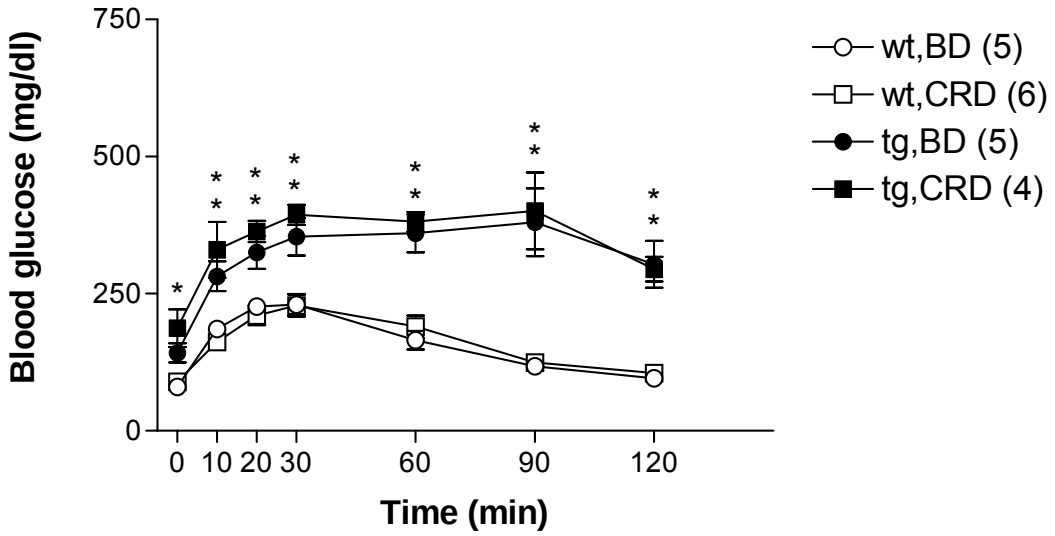

Figure 4.5 Oral glucose tolerance test at the age of 50 days

GIPR^{dn} transgenic (tg) mice fed the breeding diet (BD) show significantly elevated basal blood glucose levels as compared to respective wild-type mice (wt). Transgenic mice fed the breeding diet and transgenic mice fed the carbohydrate restricted diet (CRD) show significantly higher blood glucose levels at 10, 20, 30, 60, 90 and 120 min after oral glucose challenge than diet-matched wild-type mice. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt.

Calculation of the AUC glucose during OGTT revealed an at least 2.2-fold higher AUC glucose of GIPR^{dn} transgenic mice as compared to diet-matched wild-type mice ($p<0.05$) (Fig 4.6).

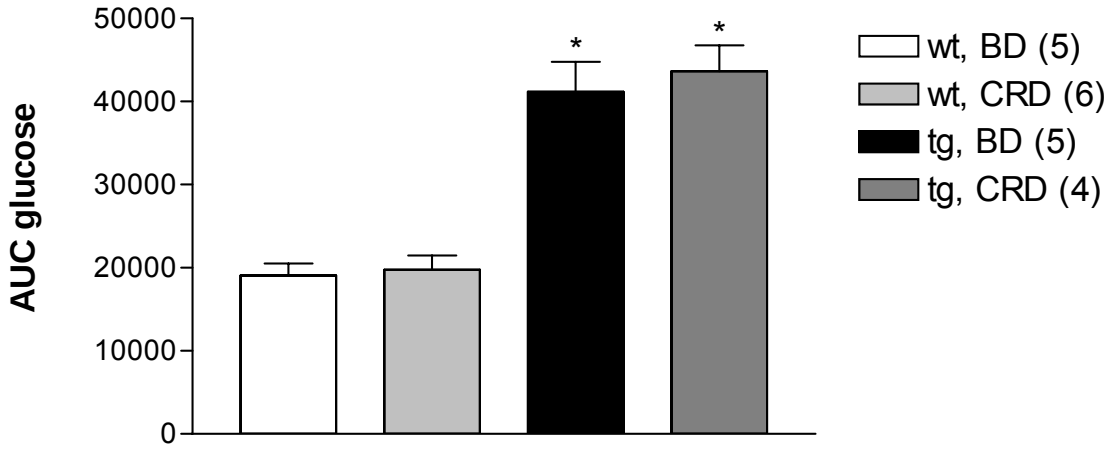

Figure 4.6 AUC glucose of OGTT at the age of 50 days

GIPR^{dn} transgenic mice (tg) show significantly higher AUC glucose values than diet-matched wild-type mice (wt). BD, breeding diet; CRD, carbohydrate restricted diet. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt.

4.3.2 OGTT at the age of 175 days

Comparing transgenic and wild-type mice of both feeding groups, GIPR^{dn} transgenic animals exhibited significantly elevated blood glucose levels at all time points investigated.

Basal blood glucose levels of GIPR^{dn} transgenic mice fed the carbohydrate restricted diet were 50% lower than those of transgenic mice fed the breeding diet but the difference only reached borderline significance ($p=0.055$).

Transgenic mice fed the carbohydrate restricted diet showed significantly lower blood glucose levels at 10 and 20 minutes after oral glucose challenge in comparison to transgenic mice fed the breeding diet.

Wild-type mice fed the carbohydrate restricted diet exhibited higher blood glucose levels at 30, 60, 90 and 120 minutes after oral glucose load as compared to wild-type mice fed the breeding diet.

The results clearly show that transgenic animals were glucose intolerant. GIPR^{dn} transgenic mice fed the carbohydrate restricted diet were more competent to compete the glucose challenge than transgenic mice fed the breeding diet (Fig 4.7).

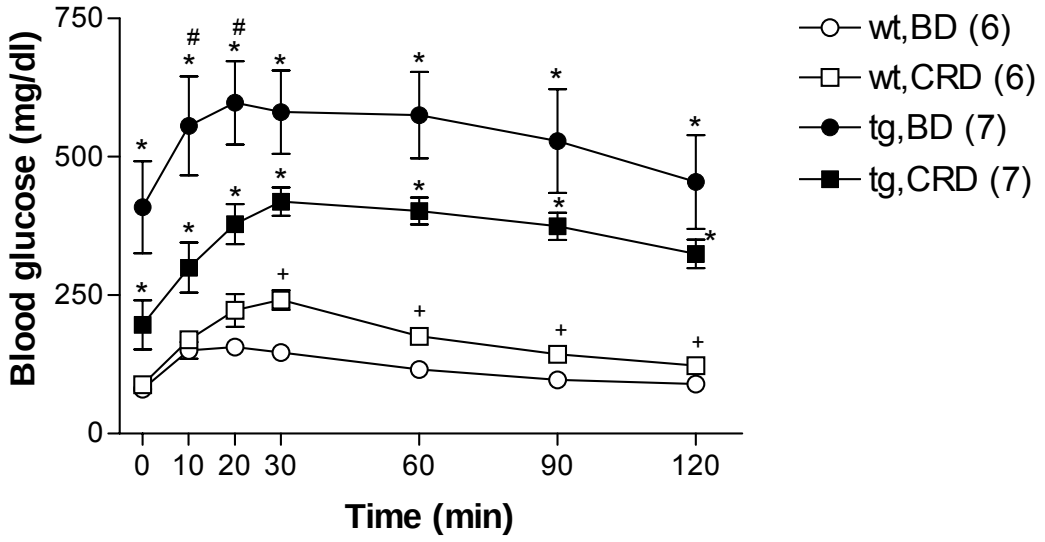

Figure 4.7 Oral glucose tolerance test at the age of 175 days

GIPR^{dn} transgenic (tg) mice fed the breeding diet (BD) and tg mice fed the carbohydrate restricted diet (CRD) show significantly elevated blood glucose levels at all time points investigated when compared to diet-matched wild-type (wt) mice. Transgenic mice fed the carbohydrate restricted diet exhibit significantly lower blood glucose levels at 10 and 20 min after oral glucose load when compared to transgenic mice fed the breeding diet. Wild-type mice fed the carbohydrate restricted diet show significantly higher blood glucose levels at 30, 60,90 and 120 min after glucose challenge as compared to wild-type mice fed the breeding diet. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD; + $p<0.05$ wt, CRD vs. wt, BD.

GIPR^{dn} transgenic mice fed the breeding diet exhibited a 4.6-fold higher AUC glucose as compared to diet-matched wild-type mice ($p<0.05$). Transgenic mice fed the carbohydrate restricted diet showed a 2.2-fold higher AUC glucose in comparison to diet-matched wild-type mice and a 32% reduced AUC glucose vs. transgenic mice fed the breeding diet ($p<0.05$).

Wild-type mice fed the carbohydrate restricted diet exhibited a 1.5-fold higher AUC glucose than wild-type mice fed the breeding diet ($p<0.05$) (Fig 4.8).

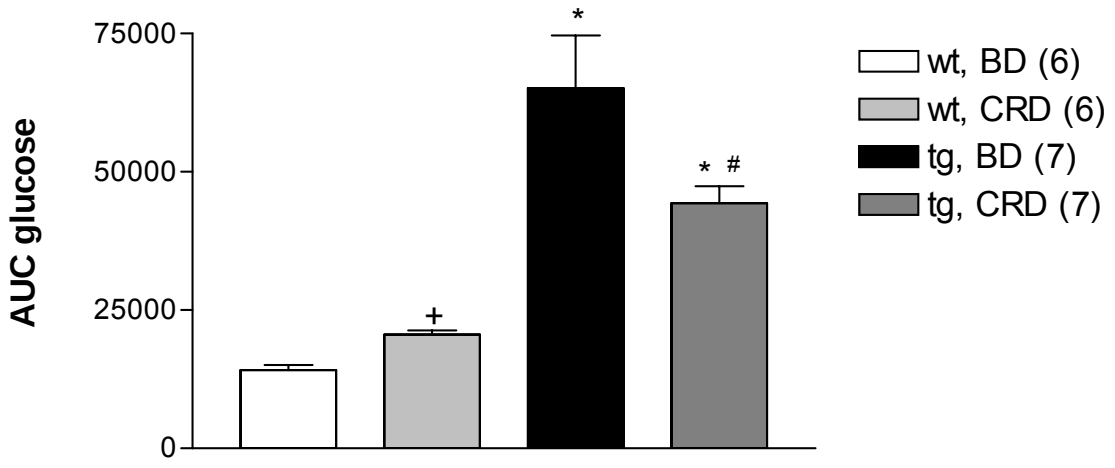

Figure 4.8 AUC glucose of the OGTT at the age of 175 days

GIPR^{dn} transgenic mice (tg) fed the breeding diet (BD) show a significantly higher AUC glucose than diet-matched wild-type mice (wt) and transgenic mice fed the carbohydrate restricted diet (CRD). Transgenic mice fed the carbohydrate restricted diet exhibit a significantly elevated AUC glucose as compared to diet-matched wild-type mice. Wild-type mice fed the carbohydrate restricted diet show significantly a higher AUC glucose than wild-type mice fed the breeding diet. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD; + $p<0.05$ wt, CRD vs. wt, BD.

4.4 Insulin sensitivity test

An insulin sensitivity test was carried out at the age of 50 days to analyze glucose disposal in response to exogenous insulin.

Results are expressed as percentage of decrease from basal values.

Blood glucose levels of randomly fed mice before insulin administration were 133.7 ± 8.2 mg/dl and 128.3 ± 7.3 mg/dl in control mice fed the breeding diet and carbohydrate restricted diet, respectively, and 489.8 ± 17.1 mg/dl and 350.0 ± 16.6 mg/dl ($p<0.05$) in GIPR^{dn} transgenic mice fed the breeding diet and carbohydrate restricted diet, respectively.

Blood glucose levels of transgenic mice fed the breeding diet did not decrease as much as blood glucose levels of diet-matched wild-type mice 10 and 20 minutes after insulin injection ($p<0.05$).

There was no significant difference in insulin sensitivity between transgenic mice of both feeding groups and between transgenic and wild-type mice fed the carbohydrate restricted diet.

Wild-type mice fed the breeding diet exhibited a marked decrease in blood glucose levels 10 minutes after insulin administration. In wild-type mice fed the carbohydrate restricted diet, this decrease was less pronounced ($p<0.05$). However, at 20 and 30 minutes after insulin injection, the blood glucose decrease of wild-type mice fed the different diets were nearly identical. At 60 minutes though, wild-type mice fed the breeding diet showed a further decrease in blood glucose levels, while blood glucose levels in wild-type mice fed the carbohydrate restritced diet remained constant ($p<0.05$).

Transgenic animals fed the breeding diet clearly show a less marked reaction to insulin administration in comparison to diet-matched wild-type mice, especially in the first 20 minutes though they still respond to insulin administration (Fig 4.9).

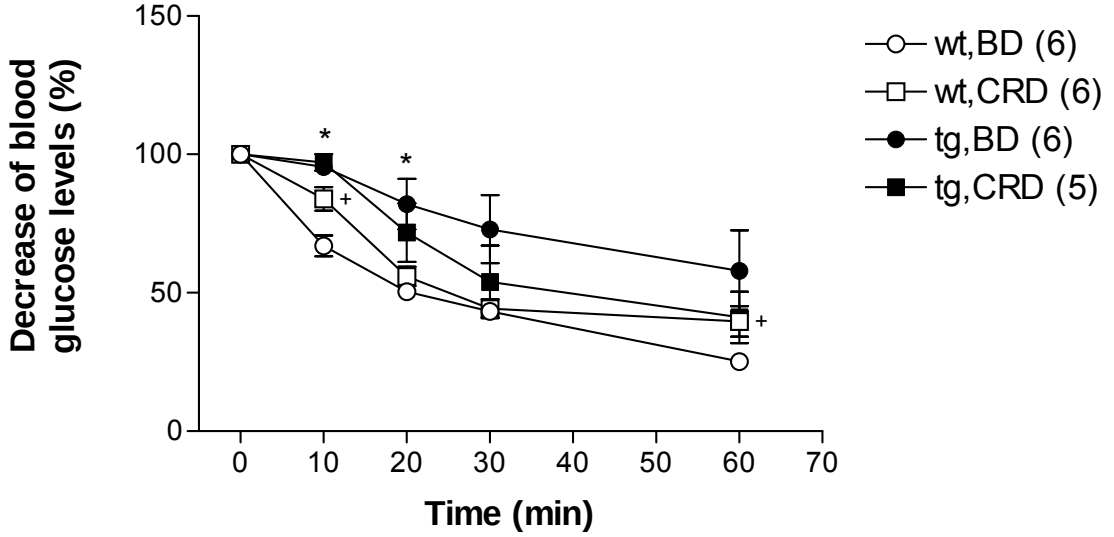

Figure 4.9 Insulin sensitivity test at the age of 50 days

GIPR^{dn} transgenic (tg) mice fed the breeding diet (BD) show a significantly lower decrease of blood glucose levels at 10 and 20 min as compared to diet-matched wild-type (wt) mice. Wild-type mice fed the carbohydrate restricted diet show a significantly lower reduction of blood glucose levels at 10 and 60 min when compared to wild-type mice fed the breeding diet. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD; + $p<0.05$ wt, CRD vs. wt, BD.

The calculation of the AUC glucose of the insulin sensitivity test revealed a 1.6-fold higher AUC glucose of transgenic mice fed the breeding diet as compared to diet-matched wild-type mice ($p<0.05$).

No significant differences comparing transgenic and wild-type mice fed the breeding diet, and transgenic mice and wild-type mice of both feeding groups could be detected (Fig 4.10).

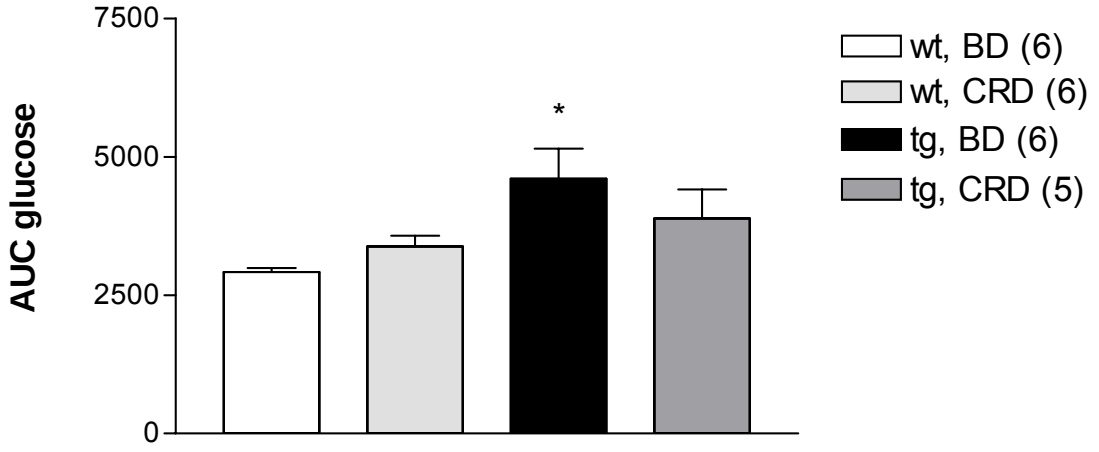

Figure 4.10 AUC glucose of the insulin sensitivity test

GIPR^{dn} transgenic mice (tg) fed the breeding diet (BD) show a significantly higher AUC glucose than diet-matched wild-type mice (wt). CRD, carbohydrate restricted diet. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt.

4.5 Serum levels of insulin and GIP

4.5.1 Serum levels of insulin

Postprandial insulin levels were determined in order to determine meal-induced insulin secretion.

Analysis of variance revealed a significant effect of the genetic group on serum insulin levels.

At the age of 45 days, wild-type mice fed the carbohydrate restricted diet exhibited significantly lower postprandial serum insulin levels than wild-type mice fed the breeding diet. Postprandial serum insulin levels of wild-type and transgenic mice fed the carbohydrate restricted diet were significantly higher at 90 days of age as compared to 45 days of age. There were no significant differences in serum insulin levels between GIPR^{dn} transgenic mice of both feeding groups at any age investigated. However, mean serum insulin levels were at least 50% reduced (50% – 66%) in transgenic mice fed the breeding diet vs. age- and diet-matched wild-type mice and at least 17% (17% – 41%) decreased in transgenic mice fed the carbohydrate restricted diet in comparison to age- and diet-matched wild-type mice (Fig 4.11).

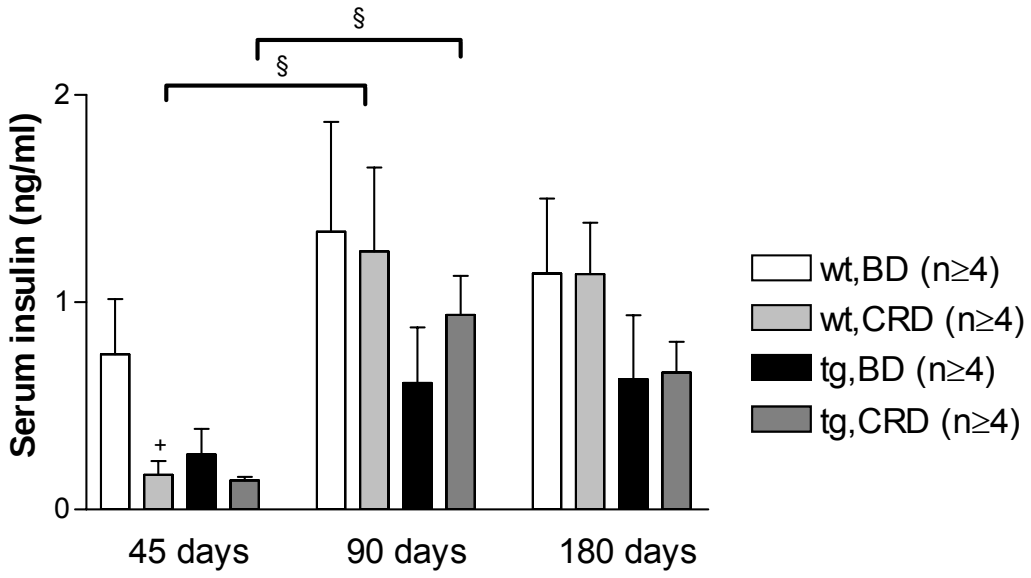

Figure 4.11 Postprandial serum insulin levels

At the age of 45d, wild-type (wt) mice fed the carbohydrate restricted diet (CRD) exhibit significantly lower serum insulin levels than wild-type mice fed the breeding diet (BD). Serum insulin levels of wild-type and transgenic (tg) mice fed the carbohydrate restricted were significantly higher at 90d vs. 45d of age. Data are means ± SEM; (n) number of animals investigated. + $p<0.05$ wt, CRD vs. wt, BD; § $p<0.05$ 45d vs. indicated time point.

4.5.2 Serum levels of GIP

Serum GIP levels were examined 1.5 hours after refeeding following a 15-hour fasting period.

Analysis of variance revealed a significant effect of diet and age in serum GIP concentrations.

There were no significant differences of GIP serum levels between wild-type and transgenic animals of one feeding group or between wild-type and $GIPR^{dn}$ transgenic animals of the different feeding groups at any age investigated. However, serum GIP levels showed a tendency to be reduced in all carbohydrate restricted diet-fed transgenic mice vs. breeding-diet fed transgenic mice and in carbohydrate restricted diet-fed wild-type mice vs. breeding diet-fed wild-type mice at 45 and 180 days of age.

At 90 days of age, $GIPR^{dn}$ transgenic mice fed the breeding diet showed 1.7-fold higher serum GIP levels than diet-matched wild-type mice but the difference did not reach statistical significance.

At the age of 90 days, serum GIP levels were 3.6-fold ($p<0.05$) higher in wild-type mice and 5.5-fold ($p<0.05$) in transgenic mice fed the carbohydrate restricted diet as compared to respective 45-day-old mice (Fig 4.12).

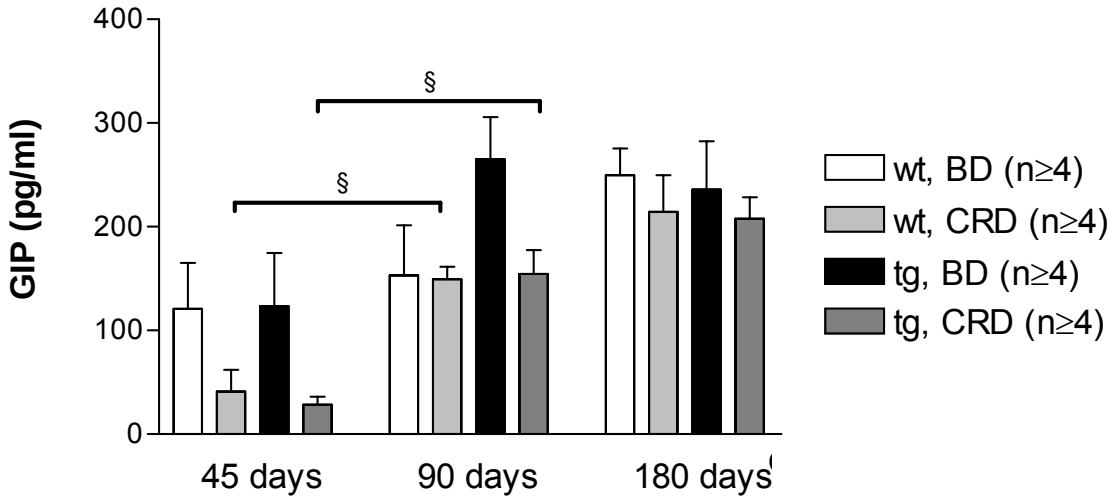

Figure 4.12 Serum GIP levels

No significant differences of serum GIP levels between wild-type (wt) and $GIPR^{dn}$ transgenic (tg) mice were evident, irrespective of the diet fed. Differences of serum GIP levels between wild-type animals of both feeding groups and transgenic mice of both feeding groups (BD, breeding diet; CRD carbohydrate restricted diet) did not reach statistical significance. Serum GIP levels of wild-type and transgenic mice fed the carbohydrate restricted diet were significantly higher at 90d vs. 45d of age. Data are means ± SEM; (n) number of animals investigated. § $p<0.05$ 45d vs. indicated time point.

4.6 Daily food and water intake

To determine the daily food and water intake, each mouse was kept in a separate cage for five days and food and water intake was measured every day at the same time. Mice were 120 days old at the beginning of the experiment.

4.6.1 Food intake

$GIPR^{dn}$ transgenic mice fed the breeding diet exhibited a significantly increased food intake (59%) per day when compared with diet-matched wild-type mice.

No difference was found in food intake comparing transgenic and wild-type mice fed the carbohydrate restricted diet.

There was no significant difference in food intake between $GIPR^{dn}$ transgenic mice of both feeding groups.

Wild-type mice fed the carbohydrate restricted diet showed a significantly higher (25%) food intake per gram body weight than wild-type mice fed the breeding diet. (Fig 4.13)

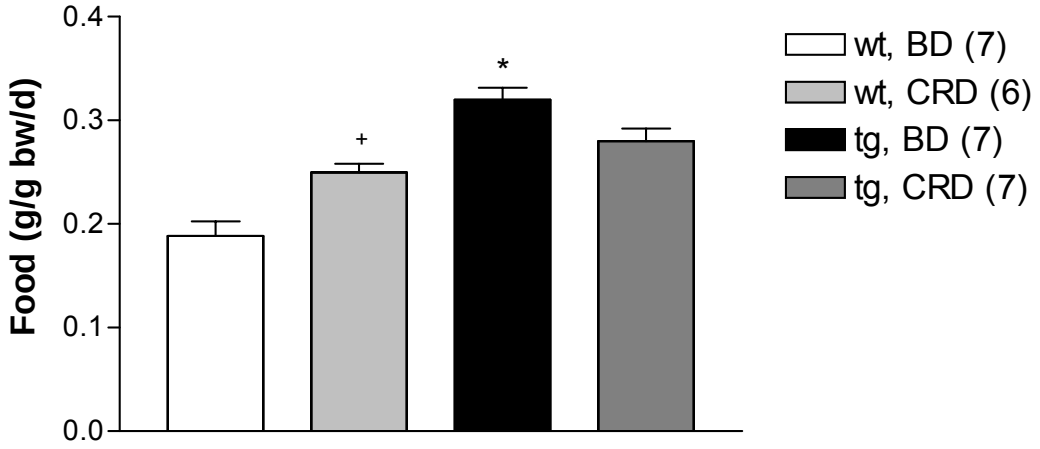

Figure 4.13 Mean daily food intake

Transgenic (tg) mice fed the breeding diet (BD) show a higher food intake per gram bodyweight than diet-matched wild-type mice (wt). Wild-type mice fed the carbohydrate restricted diet (CRD) consume more food per gram body weight than wild-type mice fed the breeding diet. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; + $p<0.05$ wt, CRD vs. wt, BD.

4.6.2 Water intake

Water intake of $GIPR^{dn}$ transgenic mice fed the breeding diet was 6.4-fold higher than that of wild-type mice fed the breeding diet ($p<0.05$). $GIPR^{dn}$ transgenic mice fed the carbohydrate restricted diet exhibited a significantly larger water intake than diet-matched controls and a significantly lower water intake than transgenic mice fed the carbohydrate-restricted diet (66% lower).

Furthermore, wild-type mice fed the carbohydrate restricted diet exhibited a significantly higher water consume than wild-type mice fed the breeding diet (Fig 4.14).

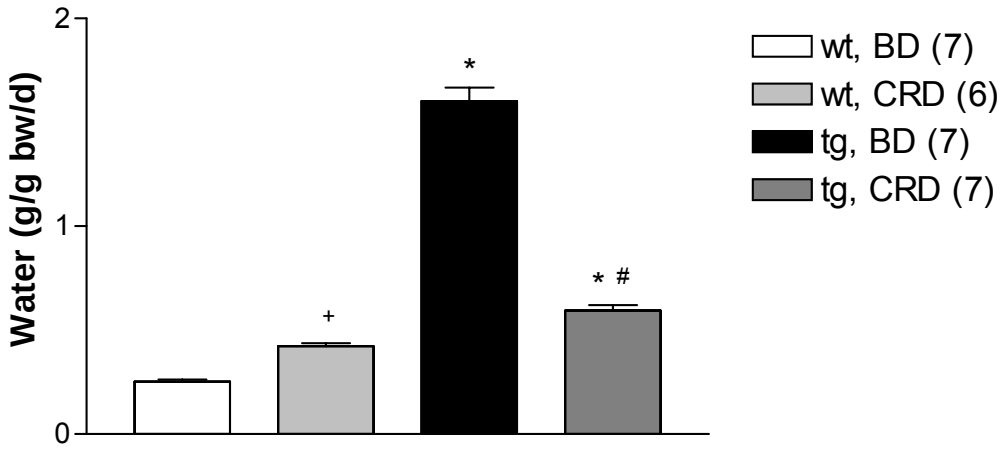

Figure 4.14 Mean daily water intake

Water intake is significantly higher in GIPR^{dn} transgenic (tg) mice fed the breeding diet (BD) than in wild-type (wt) mice fed the breeding diet and in transgenic mice fed the carbohydrate-restricted diet (CRD). Transgenic mice fed the carbohydrate restricted diet show a significantly higher water intake than diet-matched controls. Wild-type mice fed the carbohydrate restricted diet exhibit a significantly larger water consume than wild-type mice fed the breeding diet. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD; + $p<0.05$ wt, CRD vs. wt, BD.

4.7 Body weight

At weaning, the body weight of wild-type mice was significantly higher than body weight of transgenic mice (12.8 ± 0.5 g vs. 10.9 ± 0.3 g; n=30/group).

Postprandial body weight was investigated in mice after refeeding for 1.5 hours following a 15-hour fasting period.

At the age of 45 and 180 days, GIPR^{dn} transgenic mice fed the carbohydrate restricted diet weighed 23% ($p<0.05$) and 20% ($p<0.05$) less than diet-matched transgenic mice. Wild-type mice fed the carbohydrate restricted diet weighed significantly less than wild-type mice fed the breeding diet at the age of 45 and 90 days (44% and 20%, respectively).

GIPR^{dn} transgenic mice fed the breeding diet showed a 1.2-fold higher body weight at the age of 180 days as compared to 45 days of age ($p<0.05$).

Body weight of GIPR^{dn} transgenic mice fed the carbohydrate restricted diet was 1.3-fold higher at the age of 90/180 days vs. 45 days of age (Fig 4.15).

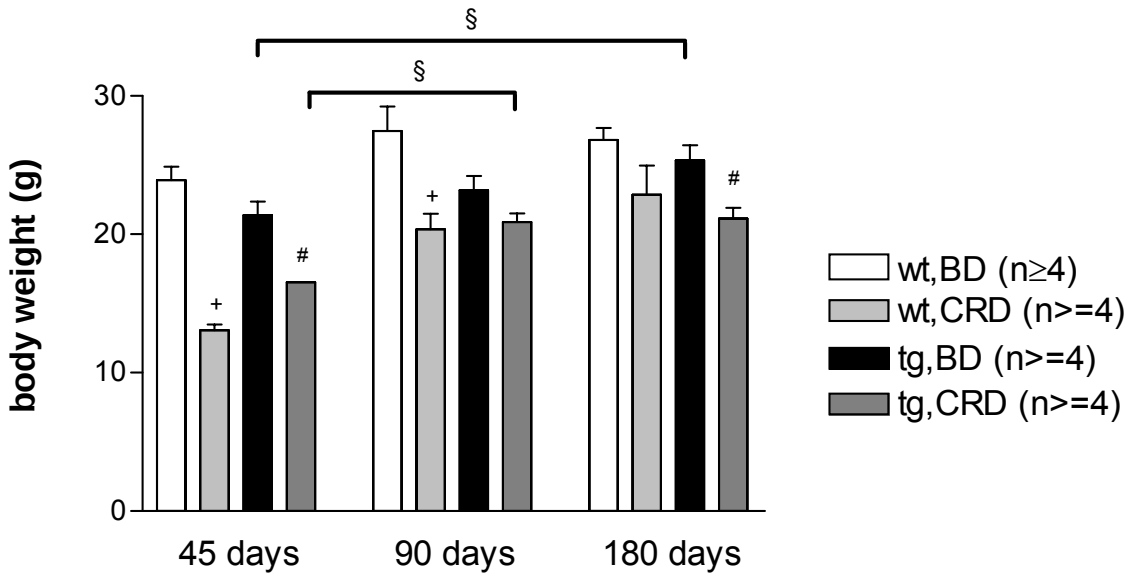

Figure 4.15 Body weight

GIPR^{dn} transgenic (tg) mice fed the carbohydrate restricted diet (CRD) weigh significantly less than transgenic mice fed the breeding diet (BD) at the age of 45 and 180 days. Wild-type (wt) mice fed the carbohydrate restricted diet weigh significantly less than wild-type mice fed the breeding diet at the age of 45 and 90d. Body weight of transgenic mice fed the breeding diet is significantly higher at 180d vs. 45d of age. Transgenic mice fed the carbohydrate restricted diet show a higher body weight at 90d of age as compared to 45d. Data are means ± SEM; (n) number of animals investigated. + $p<0.05$ wt, CRD vs. wt, BD; # $p<0.05$ tg, CRD vs. tg, BD; § $p<0.05$ 45d vs. indicated time point.

4.8 Morphometric data of the pancreas

4.8.1 Pancreas volume

At the age of 45 days, the pancreas volume, $V_{(Pan)}$, was significantly lower in transgenic and wild-type mice fed the carbohydrate restricted diet when compared to transgenic and wildtype mice fed the breeding diet, respectively.

Wild-type mice fed the carbohydrate restricted diet exhibited a significantly higher $V_{(Pan)}$ at 90 days of age compared to 45 day-old counterparts (1.5-fold). Transgenic mice fed the carbohydrate restricted diet showed a significantly higher $V_{(Pan)}$ at the age of 90 days (1.7-fold) in comparison to 45 day old animals.

There were no significant differences in the $V_{(Pan)}$ comparing transgenic and wild-type mice of either feeding group at any age (Fig 4.16).

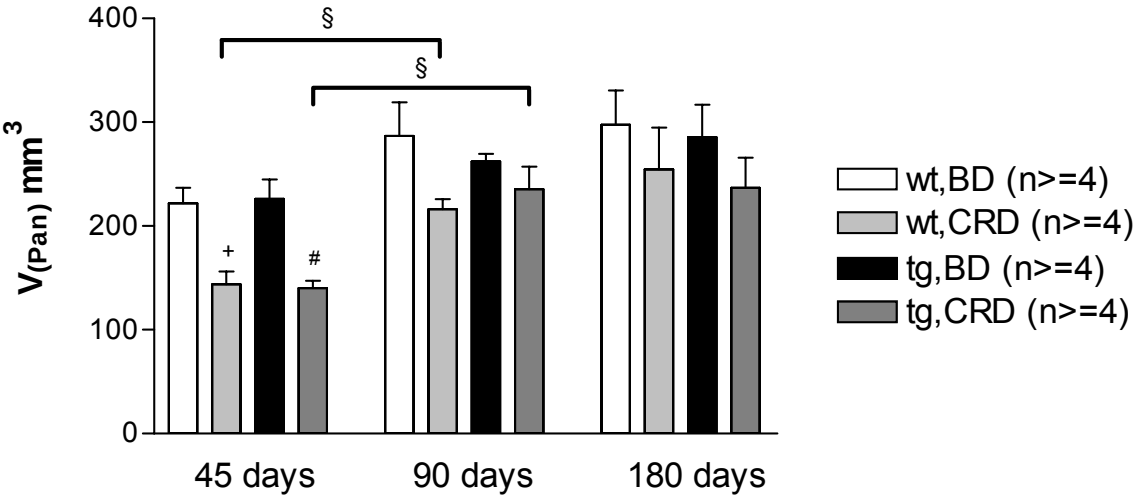

Figure 4.16 Total pancreas volume, $V_{(Pan)}$

At the age of 45d, wild-type (wt) and transgenic (tg) mice fed the carbohydrate restricted diet (CRD) exhibit a significant lower V(Pan) than wild-type and transgenic mice fed the breeding diet (BD). Wild-type mice fed the carbohydrate restricted diet show a significantly higher V(Pan) at the age of 90d as compared to 45d. Transgenic mice fed the carbohydrate resitricted diet exhibit a significantly higher V(Pan) at 90d than at 45d. Data are means ± SEM; (n) number of animals investigated. + $p<0.05$ wt, CRD vs. wt, BD; # $p<0.05$ tg, CRD vs. tg, BD; § $p<0.05$ 45d vs. indicated time point.

4.8.2 Volume density of islets in the pancreas

$GIPR^{dn}$ transgenic mice fed the breeding diet exhibited a 38% - 85% lower volume density of islets in the pancreas, $Vv_{(Islets/Pan)}$, than age- and diet-matched wild-type mice at all ages investigated ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet showed a 56% decreased $Vv_{(Islets/Pan)}$ as compared to age- and diet-matched wild-type mice only at 45 days of age ($p<0.05$).

No difference in the $Vv_{(Islets/Pan)}$ could be discovered comparing transgenic mice fed different diets.

At 90 days of age, wild-type mice fed the carbohydrate restricted diet showed a significantly lower $Vv_{(Islets/Pan)}$ than age matched wild-type mice fed the breeding diet (50%).

Wild-type mice fed the breeding diet showed a significantly higher (1.5-fold) $Vv_{(Islets/Pan)}$ at the age of 90 days than at 45 days. In contrast, transgenic mice fed the breeding diet exhibited a significantly reduced $Vv_{(Islets/Pan)}$ at 90 days of age vs. 45 days (Fig 4.17).

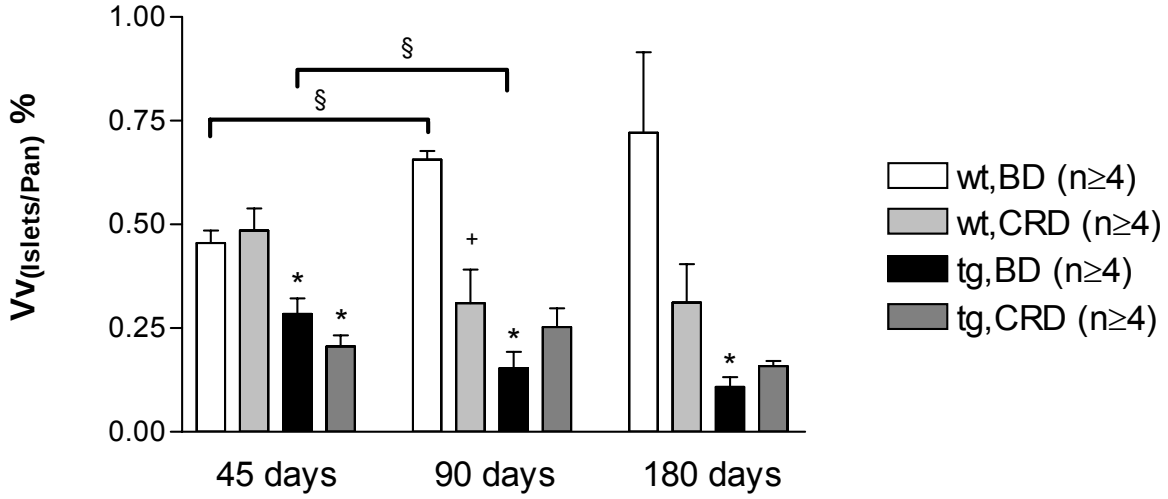

Figure 4.17 Volume density of islets in the pancreas, $Vv_{(Islets/Pan)}$

$GIPR^{dn}$ transgenic (tg) mice fed the breeding diet (BD) show a significantly lower Vv(Islets/Pan) than corresponding wild-type (wt) mice at all ages investigated. Transgenic (tg) mice fed the carbohydrate restricted diet (CRD) exhibit a significantly lower Vv(Islets/Pan) than respective wild-type (wt) mice at 45d. Wild-type mice fed the carbohydrate restricted diet show a reduced Vv(Islets/Pan) vs. wild-type mice fed the breeding diet at the age of 90d ($p<0.05$). Wild-type mice fed the breeding diet exhibit a higher Vv(Islets/Pan) at the age of 90d vs. 45d ($p<0.05$). $GIPR^{dn}$ transgenic mice show a significantly lower Vv(Islets/Pan) at 90d vs. 45d. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; + $p<0.05$ wt, CRD vs. wt, BD; § $p<0.05$ 45d vs. indicated time point.

4.8.3 Total islet volume

The total islet volume, $V_{(Islet,Pan)}$, of $GIPR^{dn}$ transgenic mice fed the breeding diet was 38% to 85% reduced as compared to age- and diet-matched wild-type mice, irrespective of the age at sampling ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet showed a significantly lower (62%) $V_{(Islet,Pan)}$ at the age of 45 days in comparison to age- and diet-matched wild-type mice.

At the age of 45 days, transgenic mice fed the carbohydrate restricted diet showed a significantly reduced $V_{(Islet,Pan)}$ in comparison to transgenic mice fed the breeding diet (56%).

Wild-type mice fed the carbohydrate restricted diet exhibited a largely reduced $V_{(Islet,Pan)}$ as compared to age-matched wild-type mice fed the breeding diet at 90 and 180 days of age.

GIPR^{dn} transgenic mice fed the breeding diet showed a significantly reduced $V_{(Islet,Pan)}$ at 180 days vs. 45 days (55%). The $V_{(Islet,Pan)}$ of transgenic mice fed the carbohydrate restricted diet was significantly higher at 90 days vs. 45 days (2.0-fold). The $V_{(Islet,Pan)}$ of wild-type mice fed the breeding diet was significantly higher at 90 days vs. 45 days (1.8-fold) (Fig 4.18).

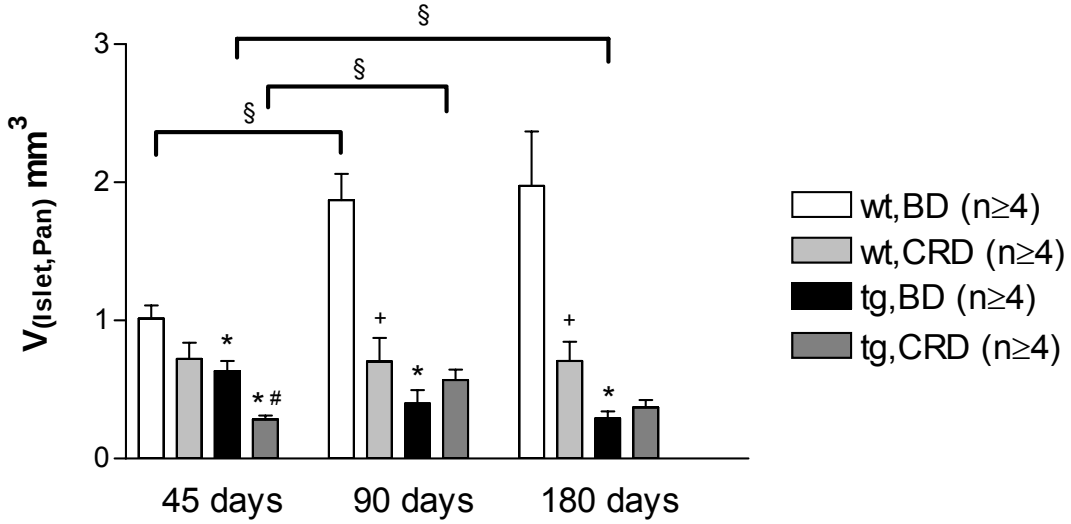

Figure 4.18 Total islet volume, $V_{(Islet,Pan)}$

Transgenic mice (tg) fed the breeding diet (BD) show a significantly reduced V(Islet,Pan) in comparison to age- and diet-matched wild-type mice (wt), regardless of the age investigated. At the age of 45 days, transgenic mice fed the carbohydrate restricted diet show a decreased V(Islet,Pan) as compared to transgenic mice fed the breeding diet ($p>0.05$). Wild-type mice fed the carbohydrate restricted (CRD) diet exhibit a significantly lower V(Islet,Pan) than wild-type mice fed the breeding diet at 90 and 180d. The V(Islet,Pan) of 90-day-old wild-type mice fed the breeding diet and of 90-day-old transgenic mice fed the carbohydrate restricted diet is higher than that of 45-day-old counterparts ($p<0.05$). Transgenic mice fed the breeding diet show a significantly lower V(Islet,Pan) at the age of 180d than at 45d. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; + $p<0.05$ wt, CRD vs. wt, BD; # $p<0.05$ tg, CRD vs. tg, BD; § $p<0.05$ 45d vs indicated time point.

4.8.4 Volume density of beta-cells in islets

GIPR^{dn} transgenic mice fed the breeding diet showed a 66% reduced $Vv_{(Beta-cells/Islets)}$ at the age of 45 days, an 88% reduction at 90 days, and a 86% reduction at 180 days as compared to age- and diet-matched wild-type mice ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet exhibited a 44% reduction of $Vv_{(Beta-cells/Islets)}$ at the age of 45 days, 70% reduction at 90 days, and a 63%

reduction at the age of 180 days in comparison to age- and diet-matched wild-type mice ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet exhibited an 1.6-fold – 2.5-fold higher $Vv_{(Beta\text{-}cells/Islets)}$ than transgenic mice fed the breeding diet ($p<0.05$).

In $GIPR^{dn}$ transgenic mice fed the breeding diet, the $Vv_{(Beta\text{-}cells/Islets)}$ was at least 57% lower at 90 and 180 days of age vs. 45 days ($p<0.05$). Transgenic mice fed the carbohydrate restricted diet showed a significantly reduced $Vv_{(Beta\text{-}cells/Islets)}$ at 90 days (~50%) of age in comparison to 45 days (Fig 4.19).

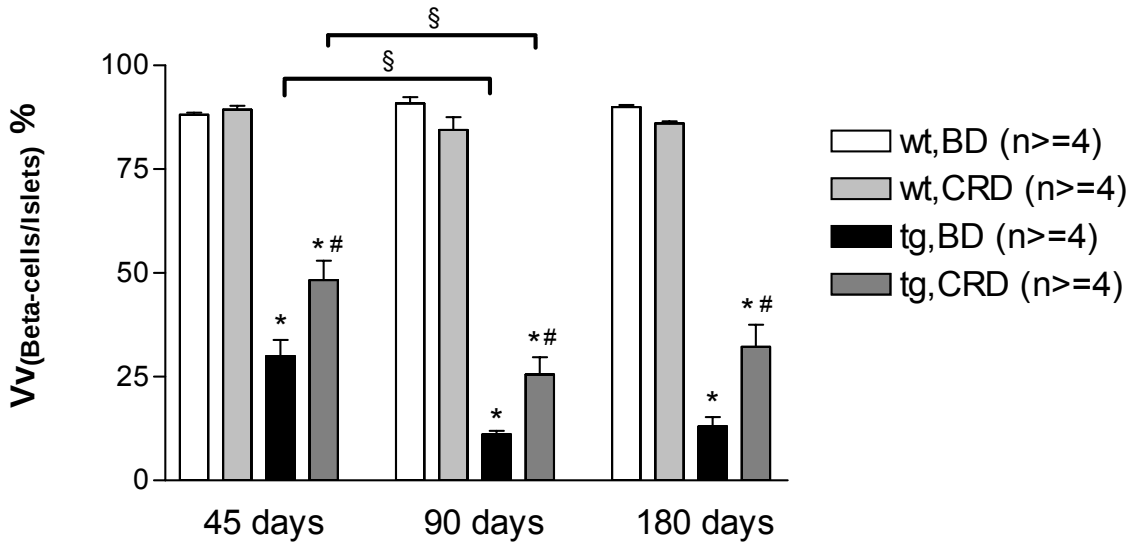

Figure 4.19 Volume density of beta-cells in islets, $Vv_{(Beta\text{-}cells/Islets)}$

Transgenic (tg) mice of both feeding groups show a significantly reduced Vv(Beta-cells(Islets) in comparison to age- and diet-matched wild-type mice (wt), irrespective of the age at sampling. Transgenic mice fed the carbohydrate restricted diet (CRD) exhibit a higher Vv(Beta-cells/Islets) than transgenic mice fed the breeding diet (BD), regardless of the age investigated ($p<0.05$). Transgenic mice of both feeding groups show a lower Vv(Beta-cells/Islets) at 90 days vs. 45d ($p<0.05$). Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD; § $p<0.05$ 45d vs.indicated time point.

4.8.5 Total beta-cell volume

Analysis of variance revealed a significant influence of diet, genetic group and the interaction diet * genetic group, as well as the interaction diet * age * genetic group on $V_{(Beta\text{-}cells,Islets)}$.

Transgenic mice fed the breeding diet showed an at least 82% reduced $V_{(Beta\text{-}cells,Islets)}$ in comparison to age- and diet-matched wild-type mice at all ages investigated ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet exhibited a significantly reduced $V_{(Beta\text{-}cells,Islets)}$ as compared to diet-matched wild-type mice at 45 and 180 days of age (62% and 79%, respectively).

$GIPR^{dn}$ transgenic mice fed the carbohydrate restricted diet exhibited a 29% lower $V_{(Beta\text{-}cells,Islets)}$ than transgenic mice fed the breeding diet at 45 days of age but the difference did not reach statistical significance. At the age of 90 days, $GIPR^{dn}$ transgenic mice fed the carbohydrate restricted diet showed a 3.3-fold higher ($p<0.05$), and at the age of 180 days a 3.3-fold higher (borderline significance, $p=0.08$) $V_{(Beta\text{-}cells,Islets)}$ than transgenic mice fed the breeding diet.

Wild-type mice fed the carbohydrate restricted diet showed a 64% lower $V_{(Beta\text{-}cells,Islets)}$ in comparison to wild-type mice fed the breeding diet at 90 and 180 days of age ($p<0.05$).

Wild-type mice fed the breeding diet exhibited a 1.9-fold higher $V_{(Beta\text{-}cells,Islets)}$ at the age of 90 days vs. 45-day-old counterparts ($p>0.05$), while diet-matched transgenic mice showed a 76% lower $V_{(Beta\text{-}cells,Islets)}$ at 90 days of age as compared to respective 45-day-old mice ($p<0.05$). In both wild-type and transgenic mice fed the carbohydrate restricted diet, $V_{(Beta\text{-}cells,Islets)}$ did not change with increasing age (Fig 4.20).

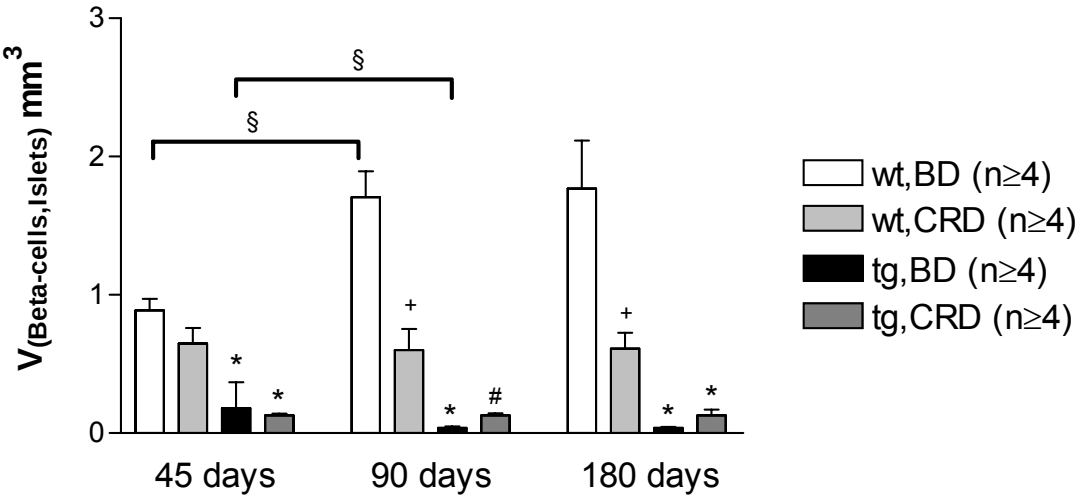

Figure 4.20 Total beta-cell volume, $V_{(Beta\text{-}cells,Islets)}$

$GIPR^{dn}$ transgenic (tg) mice fed the breeding diet (BD) and transgenic mice fed the carbohydrate restricted diet (CRD) exhibit a lower V(Beta-cells,Islets) than respective wild-type (wt) mice at any age investigated. At 90 days of age, transgenic mice fed the carbohydrate restricted diet show a significantly higher V(Beta-cells,Islets) as compared to age-matched transgenic mice fed the breeding diet. Wild-type mice fed the carbohydrate restricted diet exhibit a significantly lower V(Beta-cells,Islets), than wild-type mice fed the breeding diet at the age of 90 and 180 days. Wild-type mice fed the breeding diet show a higher V(Beta-cells,Islets) at 90d vs. 45d ($p<0.05$), while diet-matched transgenic mice show a lower V(Beta-cells,Islets) at 90 and 180d vs. 45d ($p<0.05$). Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; + $p<0.05$ wt, CRD vs. wt, BD; # $p<0.05$ tg, CRD vs. tg, BD; § $p<0.05$ 45d vs. indicated time point.

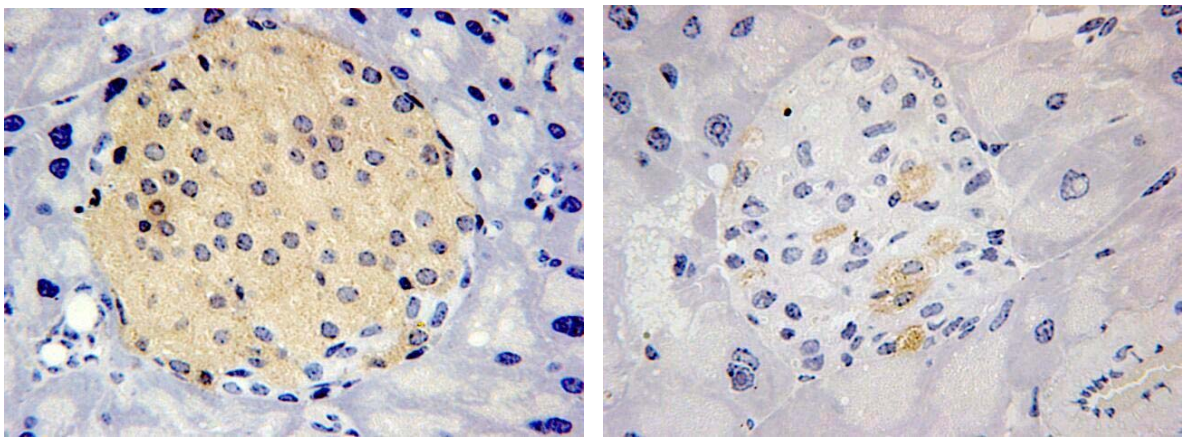

Figure 4.21 Islet of a 180-day-old wild-type mouse (left) and of a 180-day-old transgenic mouse (right), showing the typical staining pattern for insulin in wild-type mouse islets and the reduced immunostaining for insulin in the islet of a $GIPR^{dn}$ transgenic mouse

Immunohistochemical staining for insulin; plastic sections, original magnification 400x.

4.8.6 Volume density of isolated beta-cells in the pancreas

At 45 and 180 days of age, the volume density of isolated beta-cells in the pancreas was at least 72% lower in transgenic mice fed the breeding diet than in diet-matched wild-type mice ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet exhibited a significantly reduced (70%) $Vv_{(isol.beta\text{-}cells/Pan)}$ in comparison to age- and diet-matched wild-type mice at the age of 45 and 90 days.

At 180 days of age, transgenic mice fed the carbohydrate restricted diet showed a 2.5-fold higher $Vv_{(isol.beta\text{-}cells/Pan)}$ as compared to transgenic mice fed the breeding diet ($p<0.05$).

Comparing wild-type animals, wild-type mice fed the carbohydrate restricted diet had a 33% lower $Vv_{(isol.beta\text{-}cells/Pan)}$ than wild-type mice fed the breeding diet ($p<0.05$) at the age of 45 days.

Wild-type mice fed the breeding diet exhibited 50% less $Vv_{(isol.beta\text{-}cells/Pan)}$ at the age of 180 days vs. 45 days ($p<0.05$). Transgenic mice fed the breeding diet showed a 60% lower $Vv_{(isol.beta\text{-}cells/Pan)}$ at 90 days in comparison to 45 days ($p<0.05$) (Fig 4.22).

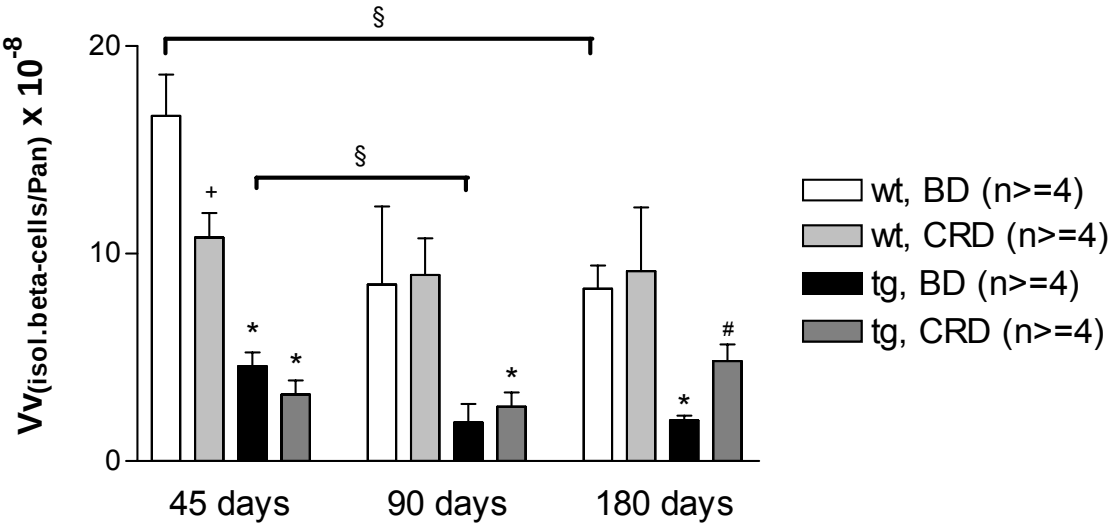

Figure 4.22 Volume density of isolated beta-cells in the pancreas, $Vv_{(isol.beta\text{-}cells/Pan)}$

At 45 and 180 days of age, Vv(isol.beta-cells/Pan) is significantly reduced in transgenic (tg) mice fed the breeding diet as compared to respective wild-type (wt) mice. At 45 and 90 days of age, transgenic mice fed the carbohydrate restricted diet (CRD) show a significantly lower Vv(isol.beta-cells/Pan) than respective wild-type mice. At 180 days, transgenic mice fed the breeding diet (BD) exhibit a significantly lower Vv(isol. beta-cells/Pan) than transgenic mice fed the carbohydrate restricted diet. Wild-type mice fed the carbohydrate restricted diet (CRD) exhibit a significantly lower Vv(isol.beta-cells/Pan) as compared to wild-type mice fed the breeding diet (BD) at 45d. Wild-type mice fed the breeding diet exhibit a lower Vv(isol.beta-cells/Pan) at 180d vs. 45d ($p<0.05$). Transgenic mice fed the breeding diet show a reduced Vv(isol.beta-cells/Pan) at 90 vs. 45d ($p<0.05$). Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; + $p<0.05$ wt, CRD vs. wt, BD; # $p<0.05$ tg, CRD vs. tg, BD; § $p<0.05$ 45d vs.indicated time point.

4.8.7 Total volume of isolated beta-cells in the pancreas

$GIPR^{dn}$ transgenic mice fed the breeding diet showed a severely reduced $V_{(isol.beta\text{-}cells,Pan)}$ as compared to age- and diet-matched wild-type mice at all ages investigated ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet also exhibited a largely decreased $V_{(isol.beta\text{-}cells,Pan)}$ when compared to age- and diet-matched wild-type mice at 45 and 90 days of age ($p<0.05$); and at 180 days (n.s.).

At 45 days of age, wild-type mice fed the carbohydrate restricted diet showed a significantly lower (58%) $V_{(isol.beta\text{-}cells,Pan)}$ than age-matched wild-type mice fed the breeding diet. The same tendency was observed comparing $GIPR^{dn}$ transgenic mice fed the different diets.

Wild-type mice fed the breeding diet exhibited a 33% reduced $V_{(isol.beta\text{-}cells,Pan)}$ at the age of 180 days vs. 45 days ($p<0.05$) (Fig 4.23).

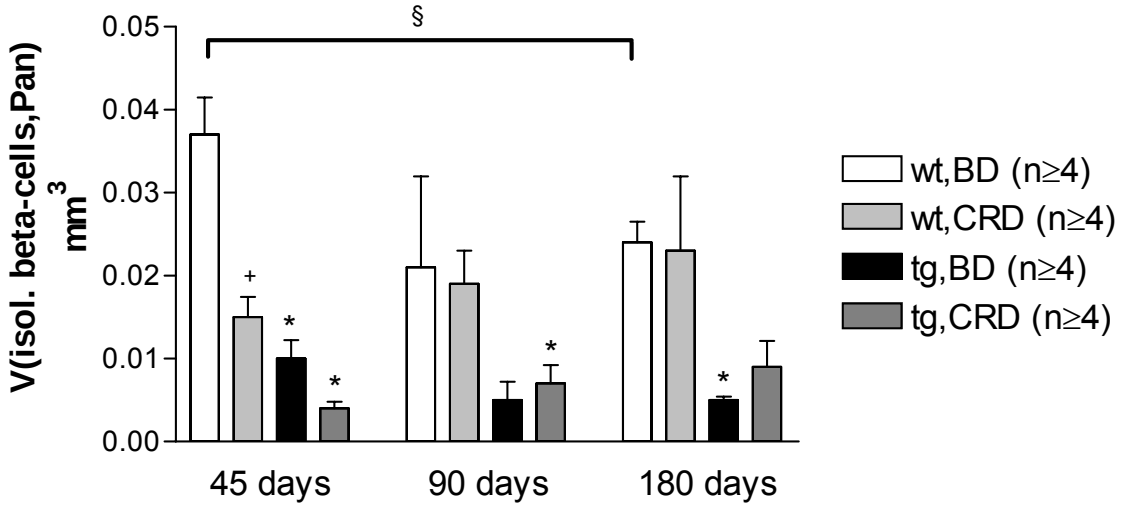

Figure 4.23 Total volume of isolated beta-cells in the pancreas, $V_{(isol.beta\text{-}cells,Pan)}$

Transgenic (tg) mice fed the breeding diet (BD) show a significantly lower V(isol.beta-cell,Pan) than age- and diet-matched wild-type (wt) mice at the age of 45 and 180d. Transgenic mice fed the carbohydrate restricted diet (CRD) exhibit a significantly reduced V(isol.beta-cells,Pan) as compared to age- and diet-matched wild-type mice at 45 and 90d of age. Wild-type mice fed the carbohydrate restricted diet show a significantly lower V(isol.beta-cells,Pan) than age-matched wild-type mice fed the breeding diet at 45d of age. Wild-type mice fed the breeding diet exhibit a lower V(isol.beta-cells,Pan) at 180d vs. 45d of age. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; + $p<0.05$ wt, CRD vs. wt, BD; § $p<0.05$ 45d vs. indicated time point.

4.8.8 Replication of islet cells

BrdU positive islet cells were counted in immunohistologically stained sections in order to estimate the cell replication rate.

Analysis of variance revealed a significant influence of age on islet cell replication.

At 45 days of age, the amount of BrdU positive islet cells was ~3.4-fold higher in $GIPR^{dn}$ transgenic mice fed the breeding diet and also ~1.6-fold higher in transgenic mice fed the carbohydrate restricted diet in comparison to diet-matched wild-type mice (n.s.).

At 90 days of age, wild-type mice fed the breeding diet still exhibited BrdU-positive cells, wheras almost no replicating cells were found in transgenic mice fed the breeding diet and in wild-type and transgenic mice fed the carbohydrate restricted diet. At 180 days of age, almost no BrdU-positive cells were found irrespective of the genetic group (Table 4.1).

	BrdU positive cells/100,000 cells		
Group	45 days	90 days	180days
BD wt	210 ± 80 (4)	199 ± 96 (4)	$0.5x10^{-3}$ ± $0.5x10^{-3}$ (4)
CRD wt	235 ± 192 (4)	$0.8x10^{-3}$ ± $0.5x10^{-3}$ (4)	n.d. (4)
BD tg	702 ± 263 (4)	n.d. (5)	$8.0x10^{-4}$ ± $8.9x10^{-4}$ (5)
CRD tg	382 ± 207 (6)	$2.0x10^{-3}$ ± $1.3x10^{-3}$ (5)	$4.0x10^{-3}$ ± $4.5x10^{-3}$ (4)

Table 4.1 BrdU positive cells per 100,000 cells

Data are means ± SEM. Breeding diet (BD), carbohydrate restricted diet (CRD), wild-type animals (wt), $GIPR^{dn}$ transgenic animals (tg). n.d. not detectable. (n) number of animals investigated.

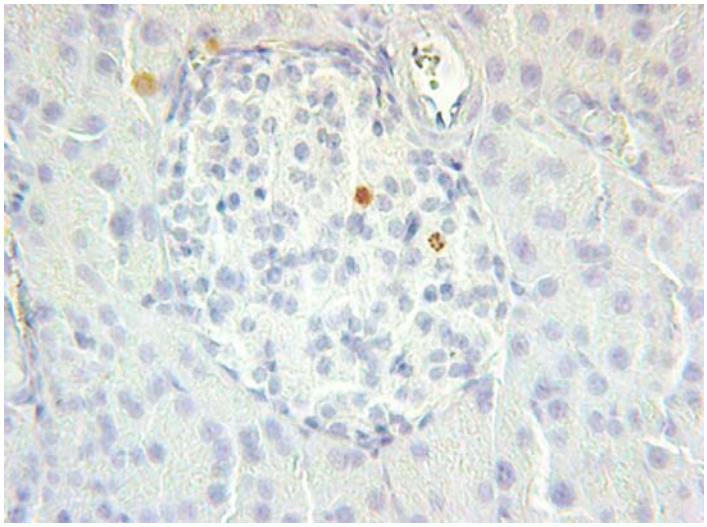

Figure 4.24 BrdU positive nuclear profiles in an islet of a 45 day old wild-type mouse

Immunohistochemical staining for BrdU, magnification 400x.

4.8.9 Apoptosis of islet cells

TUNEL positive islet cells were counted in paraffine sections in order to estimate the rate of apoptotic cell death.

Analysis of variance revealed a significant effect of the genetic group on the rate of apoptotic islet cell death.

At 45 days of age, three-times more apoptotic cells were counted in $GIPR^{dn}$ transgenic mice fed the breeding diet as compared to diet-matched wild-type mice (n.s.). At the age of 90 days, the numerical frequency of apoptotic cells was higher in

transgenic mice in comparison to wild-type mice, irrespective of the diet fed (n.s.). (Table 4.2)

	TUNEL positive cells/100,000 cells		
Group	45 days	90 days	180days
BD wt	68 ± 53 (5)	n.d. (4)	n.d. (4)
CRD wt	n.d. (4)	n.d. (4)	n.d. (4)
BD tg	186 ± 82 (5)	222 ± 136 (5)	n.d. (5)
CRD tg	n.d. (6)	240 ± 115 (5)	168 ± 111 (5)

Table 4.2 TUNEL positive cells per 100,000 cells

Data are means ± SEM. Breeding diet (BD), carbohydrate restricted diet (CRD), wild-type animals (wt), transgenic animals (tg). n.d. not detectable. (n) number of animals investigated.

4.8.10 Numerical density of islets in the pancreas

At the age of 45 and 180 days, $Nv_{s(Islets/Pan)}$ was significantly lower (55% and 58%) in $GIPR^{dn}$ transgenic mice fed the breeding diet in comparison to diet-matched wild-type mice.

Transgenic mice fed the carbohydrate restricted diet showed a 38% reduction of $Nv_{s(Islets/Pan)}$ at the age of 45 days and a 44% reduction at the age of 180 days as compared to diet-matched wild-type mice ($p<0.05$).

$Nv_{s(Islets/Pan)}$ was significantly higher (1.8-fold) in transgenic mice fed the carbohydrate restricted diet than in transgenic mice fed the breeding diet at 45 and 180 days of age.

There was no significant difference comparing wild-type mice of the different diet groups (Fig 4.25).

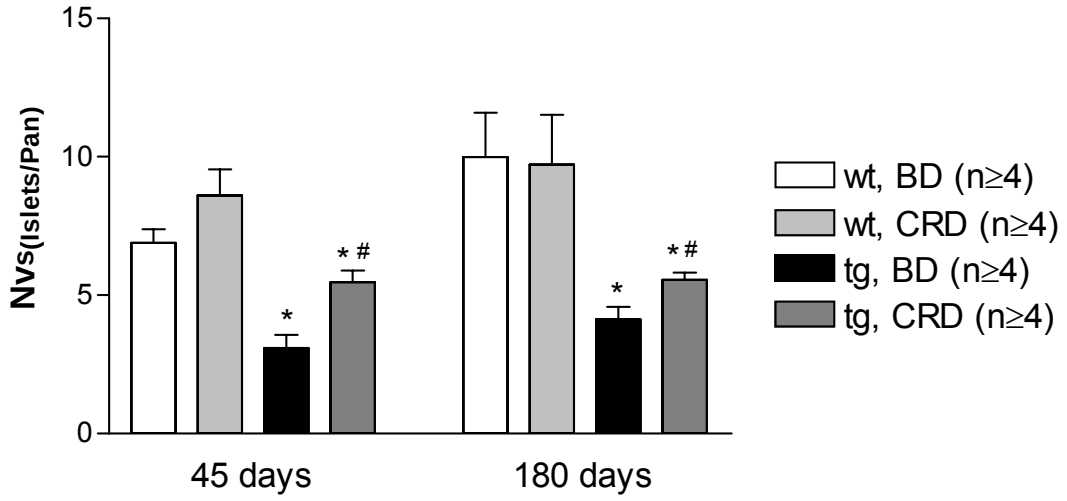

Figure 4.25 Numerical density of islets in the pancreas, $Nvs_{(Islets/Pan)}$

At 45 and 180 days of age, transgenic (tg) mice fed the breeding diet (BD) show a significantly smaller Nvs(Islet/Pan) than diet-matched wild-type mice (wt) and transgenic mice fed the carbohydrate restricted diet (CRD). Transgenic mice fed the carbohydrate restricted diet exhibit a significantly smaller Nvs(Islets/Pan) than diet-matched controls. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD.

4.8.11 Total number of islets

Analysis of variance revealed a significant influence of age, genetic group and the interaction diet * genetic group, as well as the interaction age * genetic group on the total number of beta-cells.

At the age of 45 days, $N_{(Islets,Pan)}$ was severely reduced (58%) in $GIPR^{dn}$ transgenic mice fed the breeding diet and in transgenic mice fed the carbohydrate restricted diet (41%) as compared to diet-matched wild-type mice ($p<0.05$). No differences were evident comparing wild-type and transgenic mice of the different diet groups.

At the age of 180 days, the reduction of the total number of islets in $GIPR^{dn}$ transgenic mice fed the breeding diet was even more pronounced (64%) in comparison to diet-matched wild-type mice.

$N_{(Islets,Pan)}$ was reduced by 41% in transgenic mice fed the carbohydrate restricted diet in comparison to diet-matched wild-type mice (n.s.).

No difference was evident comparing wild-type mice or transgenic mice of the different feeding groups at 180 days of age.

Wild-type and transgenic mice fed the breeding diet showed a significantly higher $N_{(Islets,Pan)}$ at the age of 180 days vs. 45 days (1.8-fold and 1.6-fold, respectively).

Wild-type and GIPR^{dn} transgenic mice fed the carbohydrate restricted diet exhibited a higher $N_{(Islets,Pan)}$ at the age of 180 days as compared to 45 days (1.8-fold and 1.7-fold, respectively; n.s.) (Fig 4.26).

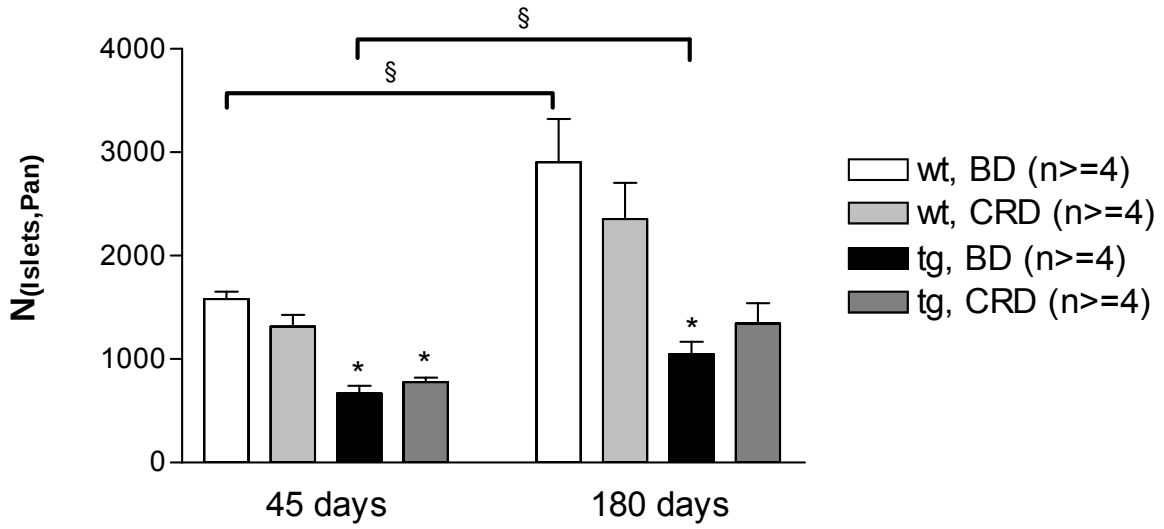

Figure 4.26 Total number of islets, $N_{(Islets,Pan)}$

Transgenic (tg) mice fed the breeding diet (BD) exhibit a significantly lower N(Islets,Pan) as compared to diet-matched wild-type (wt) mice at 45 and 180 days of age. GIPR^{dn} transgenic mice fed the carbohydrate restricted diet (CRD) show a reduced N(Islets,Pan) as compared to diet-matched controls at the age of 45 days ($p<0.05$). Wild-type and transgenic mice fed the breeding diet show a higher N(Islets,Pan) at the age of 180 days vs. 45 days ($p<0.05$). Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; § $p<0.05$ 45d vs. 180d.

4.8.12 Mean islet volume

At 45 days of age, the mean islet volume, $v_{(Islets)}$, was severely reduced (44%) in transgenic mice fed the carbohydrate restricted diet as compared to diet-matched wild-type mice ($p<0.05$).

The mean islet volume of 45-day-old transgenic mice fed the carbohydrate restricted diet was strikingly smaller (66%) than that of transgenic mice fed the breeding diet ($p<0.05$).

GIPR^{dn} transgenic mice fed the breeding diet showed a 55% reduced $v_{(Islets)}$ as compared to diet-matched wild-type mice at the age of 180 days (borderline significance, $p=0.06$).

Transgenic mice fed the breeding diet exhibited a 70% reduced $v_{(Islets)}$ at the age of 180 days in comparison to 45 days ($p<0.05$). Wild-type mice fed the carbohydrate

restricted diet showed a significantly smaller $v_{(Islets)}$ at the age of 180 days vs. 45 days (52%) (Fig 4.27).

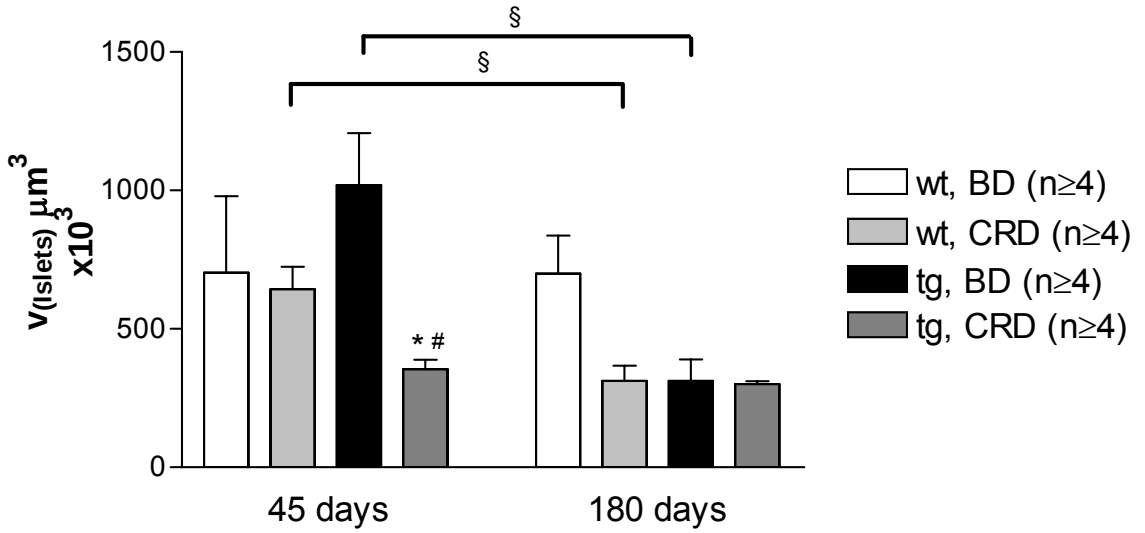

Figure 4.27 Mean islet volume, $v_{(Islets)}$

At 45 days of age, the v(Islets) in GIPR^{dn} transgenic mice (tg) fed the carbohydrate restricted diet (CRD) is significantly smaller than in diet-matched wild-type mice (wt). GIPR^{dn} transgenic mice fed the carbohydrate restricted diet show a significantly smaller v(Islets) than transgenic mice fed the breeding diet at the age of 45d. v(Islets) is significantly reduced at the age of 180d vs 45d in wild-type mice fed the carbohydrate restricted diet and in transgenic mice fed the breeding diet. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; # $p<0.05$ tg, CRD vs. tg, BD; § $p<0.05$ 45d vs. 180d.

4.8.13 Numerical density of beta-cells in islets

The numerical density of beta-cells in islets, $Nv_{s(Beta\text{-}cells/Islets)}$, was significantly decreased in GIPR^{dn} transgenic mice fed the breeding diet as compared to age- and diet-matched wild-type mice (47%) and vs. GIPR^{dn} transgenic mice fed the carbohydrate restricted diet (33%) at the age of 45 days ($p<0.05$). 45-day-old transgenic mice fed the carbohydrate restricted diet showed a 29% reduced $Nv_{s(Beta\text{-}cells/Islets)}$ in comparison to age- and diet-matched wild-type mice ($p<0.05$).

At 180 days of age, transgenic mice fed the carbohydrate restricted diet exhibited a 29% reduced $Nv_{s(Beta\text{-}cells/Islets)}$ as compared to diet-matched wild-type mice ($p<0.05$). The same tendency was observed in transgenic mice fed the breeding diet vs. diet-matched wild-type mice but this difference did not reach statistical significance.

$Nvs_{(Beta\text{-}cells/Islets)}$ of 180-day-old wild-type mice fed the carbohydrate restricted diet was significantly lower as compared to 45-day-old diet-matched wild-type mice (Fig 4.28).

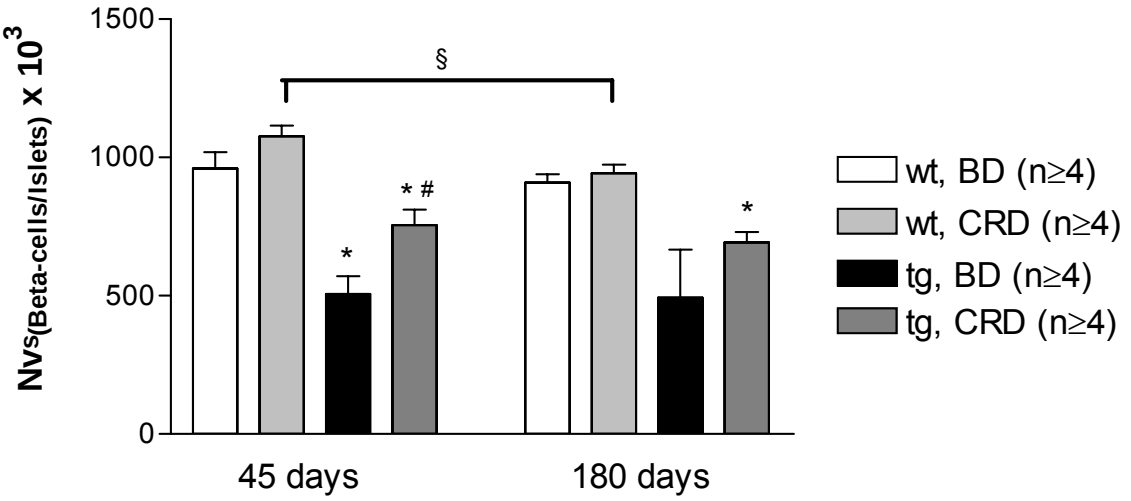

Figure 4.28 Numerical density of beta-cells in islets, $Nvs_{(Beta\text{-}cells/Islets)}$

At 45 days of age, $GIPR^{dn}$ transgenic (tg) mice fed the breeding diet (BD) exhibit a significantly lower Nvs(beta-cells/Islets) than diet-matched wild-type mice (wt) and than transgenic mice fed the carbohydrate restricted diet (CRD). $GIPR^{dn}$ transgenic mice fed the carbohydrate restricted diet show a significantly reduced Nvs(beta-cells/Islets) in comparison to diet-matched wild-type mice at 45 and 180 days of age. At 180 days of age, wild-type mice fed the carbohydrate restricted diet show a significantly reduced Nvs(Beta-cells/Islets) as compared to 45 days of age. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; + $p<0.05$ wt, CRD vs. wt, BD; § $p<0.05$ 45d vs. 180d.

4.8.14 Total number of beta-cells

Analysis of variance revealed a significant influence of diet, genetic group and the interaction diet * genetic group, the interaction diet * age, as well as the interaction diet * genetic group * age on the total number of beta-cells.

$GIPR^{dn}$ transgenic mice of both diet groups showed a striking reduction of at least 87% of $N_{(Beta\text{-}cells,Islets)}$ as compared to diet-matched wild-type mice at the age of 45 days ($p<0.05$).

At the age of 180 days, $N_{(Beta\text{-}cells,Islets)}$ of $GIPR^{dn}$ transgenic mice fed the breeding diet was reduced by 98% in comparison to diet-matched wild-type mice ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet exhibited an 83% reduced $N_{(Beta\text{-}cells,Islets)}$ in comparison to diet-matched wild-type mice at 180 days of age ($p<0.05$).

Transgenic mice fed the carbohydrate restricted diet exhibited a 5.0-fold higher $N_{(Beta\text{-}cells,Islets)}$ than transgenic mice fed the breeding diet at the age of 180 days (n.s.).

Wild-type mice fed the carbohydrate restricted diet exhibited a significantly reduced $N_{(Beta\text{-}cells,Islets)}$ as compared to wild-type mice fed the breeding diet at 180 days of age (64%).

$N_{(Beta\text{-}cells,Islets)}$ of wild-type mice fed the breeding diet was 1.7-fold higher at the age of 180 days than at 45 days of age ($p<0.05$), whereas in $GIPR^{dn}$ transgenic mice fed the breeding diet $N_{(Beta\text{-}cells,Islets)}$ was 79% lower at 180 days of age vs. 45 days (n.s.) (Fig 4.29).

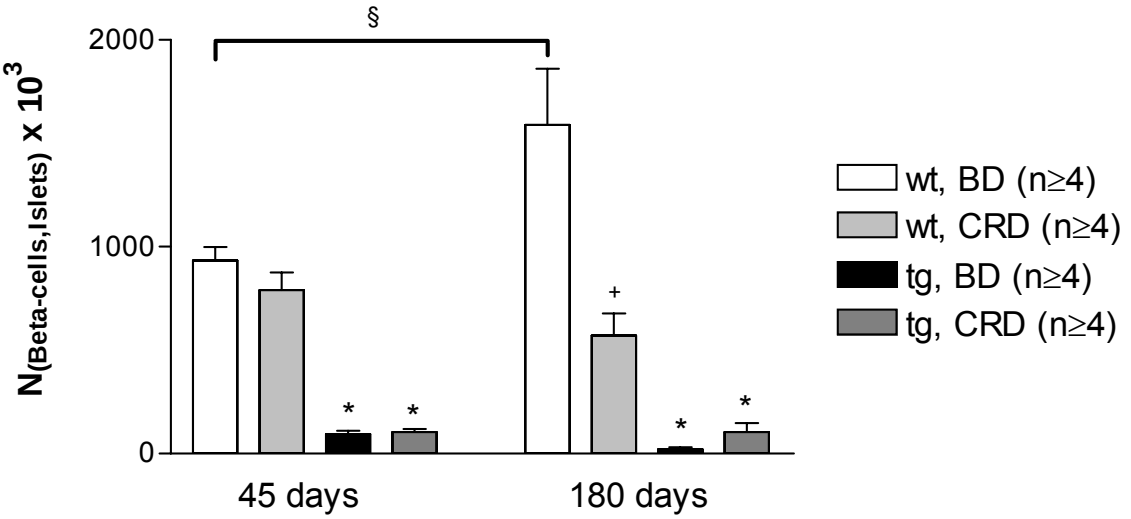

Figure 4.29 Total number of beta-cells, $N_{(Beta\text{-}cells,Islets)}$

$GIPR^{dn}$ transgenic mice (tg) fed the breeding diet (BD) and also $GIPR^{dn}$ transgenic mice fed the carbohydrate restricted diet (CRD) show a significantly smaller N(Beta-cells,Islets) at 45 and 180 days than respective wild-type (wt) mice. At 180 days of age, wild-type mice fed the carbohydrate restricted diet exhibit significantly less beta-cells than wild-type mice fed the breeding diet. N(Beta-cells,Islets) of wild-type mice fed the breeding diet was significantly higher at the age of 180d vs. 45d. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt; + $p<0.05$ wt, CRD vs. wt, BD; § $p<0.05$ 45d vs. 180d.

4.8.15 Mean beta-cell volume

At 45 days of age, $GIPR^{dn}$ transgenic mice fed the breeding diet showed a 33% reduced mean beta-cell volume as compared to diet-matched wild-type mice ($p<0.05$). At 180 days of age, $v_{(Beta\text{-}cell)}$ was even more markedly reduced (66%; $p<0.05$). Transgenic mice fed the carbohydrate restricted diet exhibited a 46%

reduced mean beta-cell volume at the age of 180 days vs. diet-matched wild-type mice ($p<0.05$) (Fig 4.30).

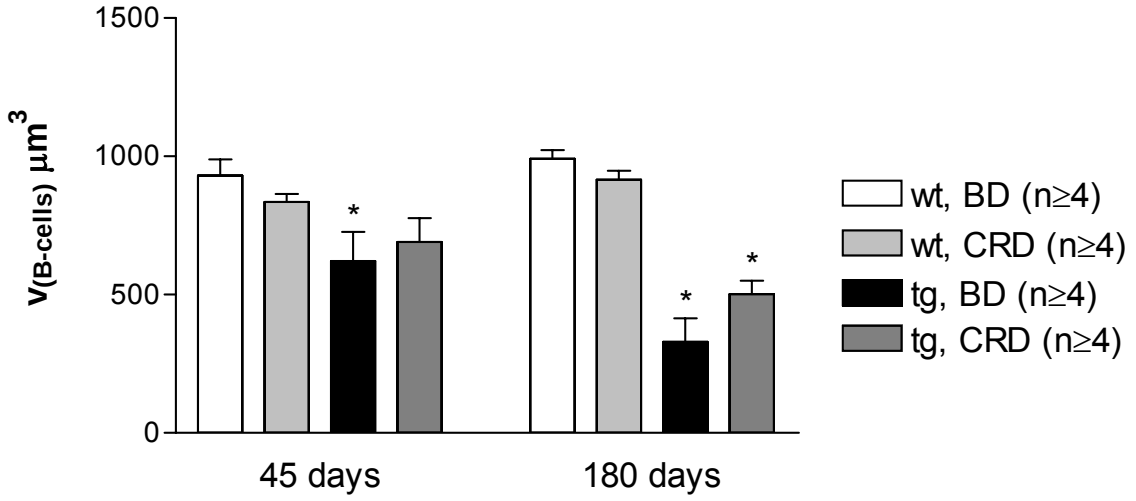

Figure 4.30 Mean beta-cell volume, v(Beta-cell)

$GIPR^{dn}$ transgenic (tg) mice fed the breeding diet (BD) exhibit a significantly reduced v(Beta-cell) as compared to diet-matched wild-type mice (wt) at the age of 45 and 180 days. At 180 days of age, $GIPR^{dn}$ transgenic mice fed the carbohydrate restricted diet (CRD) show a significantly reduced v(beta-cells) vs. diet-matched wild-type mice. Data are means ± SEM; (n) number of animals investigated. * $p<0.05$ tg vs. diet-matched wt.

5. Discussion

GIPR^{dn} transgenic mice have recently been shown to develop early onset diabetes mellitus and exhibit a severe reduction of pancreatic beta-cell mass (Herbach et al. 2005).

The aim of this study was to investigate the effects of a carbohydrate restricted diet on the metabolic status and the progressive beta-cell loss of GIPR^{dn} transgenic mice. Female mice were used because the diabetic condition of female GIPR^{dn} transgenic mice was better controlled by dietary measures than that of male GIPR^{dn} transgenic mice (Herbach et al.,unpublished data). Nontransgenic female littermates fed the same diet and transgenic and nontransgenic mice fed a breeding diet served as controls. Various clinical parameters were determined and quantitative stereological analyses of the endocrine pancreas were performed. In addition, the expression of the endogenous, murine and the transgenic GIP receptor was analyzed.

5.1 Expression analyses of endogenous and transgenic GIPR

In order to further characterize GIPR^{dn} transgenic mice, the expression of the murine, endogenous and the human, transgenic GIP receptor was investigated.

The murine, endogenous GIP receptor RNA was expressed widely throughout the organism of GIPR^{dn} transgenic and wild-type mice. The expression pattern was similar to that found in rats. Usdin et al. (1993) found GIP receptor mRNA by RT-PCR in the stomach, duodenum, proximal small intestine, adipose tissue, adrenal gland, pituitary and in the telencephalon, diencephalon, brainstem and cerebellum. They did not find expression of the receptor in the spleen and liver. In GIPR^{dn} transgenic mice, expression of the murine, endogenous GIP receptor RNA was found in the lung while it was not detectable there in wild-type mice. However, the not detectable expression in the lung of wild-type mice could be due to the low RNA-content of the lung sample.

The expression of the GIP receptor RNA does not necessarily lead to the processing of the mRNA to the protein. The expression of the murine GIP receptor in the pancreas was analysed on protein level, using immunohistochemistry. The used antibody (rabbit α-human GIPR) did not stain positive in mouse samples, either due to lack of cross-reaction with the murine GIPR or because of protein levels below detection limit.

RNA of the transgenic GIP receptor was found in a wide variety of organs in transgenic mice. Therefore, the rat insulin 2 gene promoter used to create $GIPR^{dn}$ transgenic mice, did not lead to a beta-cell specific expression of the transgene on RNA level. This finding is consistent with observations from other groups (Vasavada et al. 1996; Drayton et al. 2003; Iwakura et al. 2005): Vasavada et al. (1996) found expression of their RIP2-controlled transgene (PTHrP = Parathyroid Hormone related Protein) in the pancreas, liver, stomach, heart, brain, kidney and skin by RNase protection analysis. However, the level of expression was low in other organs compared to the pancreas. Drayton et al. (2003) found their RIP2-controlled transgene (LTβ = lymphotoxin β) to be expressed in the pancreas and in the kidney, as shown by in situ hybridization. Iwakura et al. (2005) showed expression of ghrelin-mRNA in RIP2-ghrelin transgenic mice in the pancreas and in the brain. However, no ghrelin-like immunoreactivity was detected in the brain by immunohistochemical analysis. Likewise, Hanahan (1985) analysed the expression of the RIP2-controlled transgene on protein level and found an expression only in the pancreas (Hanahan 1985).

In summary, the expression of the $GIPR^{dn}$ was shown in a wide organ spectrum. Since the expression on protein level could not be confirmed to date, the physiological impact of the ubiquitous expression remains speculative.

5.2 Blood glucose, serum parameters, OGTT and IST

At weaning, $GIPR^{dn}$ transgenic mice already exhibited significantly higher blood glucose levels than control mice. This finding confirms the results of Herbach et al. (2005) who found the onset of diabetes mellitus in $GIPR^{dn}$ transgenic mice to occur with the beginning intake of rodent chow, between 14 and 21 days of age. With the beginning intake of rodent chow, the participation of the entero-insular axis in glucose homeostasis becomes more important (Morgan 1996), and thereby the defect in the entero-insular axis of $GIPR^{dn}$ transgenic mice becomes clinically relevant.

Fasting blood glucose levels were significantly elevated in $GIPR^{dn}$ transgenic mice at all ages investigated, regardless of the diet fed. However, transgenic mice fed the carbohydrate restricted diet showed lower fasting blood glucose levels than transgenic mice fed the breeding diet. A significant impact of the diet fed on fasting blood glucose levels was found by variance analysis. Since GIP is not known to play

a role in the regulation of fasting blood glucose levels in mice (Baggio et al. 2000 a), the severely disturbed function of beta-cells and the diminished total beta-cell mass of GIPR^{dn} transgenic mice are thought to cause elevated fasting blood glucose levels (Herbach et al. 2005).
Postprandial blood glucose levels were also found to be significantly higher in all GIPR^{dn} transgenic mice examined as compared to wild-type mice. GIPR^{dn} transgenic mice fed the carbohydrate restricted diet exhibited significantly lower postprandial blood glucose levels than transgenic mice fed the breeding diet. This finding may be a result of the lower disaccharide content of the carbohydrate restricted chow. Additionally, the high content of fiber slows the absorption of nutrients in the gut, leading to a better metabolic control of GIPR^{dn} transgenic mice fed the carbohydrate restricted diet (Järvi et al. 1999).

The oral glucose tolerance tests confirmed the diabetic phenotype of GIPR^{dn} transgenic mice, regardless of nutrition and underlined the beneficial effect of the carbohydrate restricted diet on glucose tolerance in 175-days-old transgenic mice fed the carbohydrate restricted diet and the breeding diet. A higher glucose-induced insulin secretion and the higher beta-cell mass of transgenic mice fed the carbohydrate restricted diet could explain the improved glucose tolerance.
At the age of 50 days, wild-type animals of both feeding groups showed the same glucose curve during OGTT. At the age of 175 days, wild-type mice fed the carbohydrate restricted diet exhibited significantly higher blood glucose levels after 30 minutes and a significantly higher AUC glucose, thereby displaying mild glucose intolerance as compared to wild-type mice fed the breeding diet. The slight glucose intolerance could be due to a reduced glucose-induced insulin secretion in wild-type mice fed the carbohydrate restricted diet, since low GI (glycemic index) diets are known to reduce insulin secretion (Willett et al. 2002). Since the total beta-cell volume and the number of beta-cells of wild-type mice fed the carbohydrate restricted diet were significantly lower than those of wild-type mice fed the breeding diet (see below), lowered glucose-induced insulin secretion may result from a decreased beta-cell mass.

An insulin sensitivity test was performed to further characterize the diabetic phenotype of GIPR^{dn} transgenic mice, and to assess the effect of a carbohydrate

restricted diet on insulin sensitivity. Wild-type mice fed the carbohydrate restricted diet exhibited a delayed response 10 minutes after insulin administration in comparison to wild-type mice fed the breeding diet. Furthermore, blood glucose levels of wild-type mice fed the carbohydrate restricted diet rose after 30 minutes, while blood glucose levels in wild-type mice fed the breeding diet further decreased. The increase in blood glucose levels of wild-type mice fed the carbohydrate restricted diet could be due to counterregulatory mechanisms leading to gluconeogenesis, e.g. secretion of glucagon. However, no significant difference could be found in the AUC glucose during the insulin sensitivity test between wild-type mice fed the carbohydrate restricted diet and wild-type mice fed the breeding diet. The decrease in blood glucose levels ten minutes after insulin injection was also less pronounced in transgenic mice fed the carbohydrate restricted diet vs. diet-matched wild-type mice. Therefore, the improved insulin sensitivity of human diabetic patients on a high fiber diet that is described in literature could not be confirmed in $GIPR^{dn}$ transgenic mice fed the carbohydrate restricted diet (Pereira et al. 2002). However, in the study of Pereira et al. (2002), overweight hyperinsulinemic adult human patients were examined. $GIPR^{dn}$ transgenic and wild-type mice are neither overweight nor hyperinsulinemic which could be an explanation why an improvement in insulin sensitivity as a result of feeding a carbohydrate restricted diet was not observed in this study.

Transgenic mice fed the breeding diet showed a less pronounced decrease of blood glucose levels after the first 20 minutes of the insulin sensitivity test and a significantly higher AUC glucose than wild-type mice fed the breeding diet, indicating insulin resistance. However, increased glucagon secretion and thereby enhanced gluconeogenesis were observed in $GIPR^{dn}$ transgenic mice during glucose tolerance tests, which could be responsible for the delayed reaction to the exogenous insulin of $GIPR^{dn}$ transgenic mice (Herbach et al. 2005). Pamir et al. (2003) analyzed the insulin sensitivity of $GIPR^{-/-}$ mice, by injecting 10U/kg insulin. They did not find a difference in blood glucose lowering action over one hour between GIPR knockout and control mice. However, the high dose of insulin (10-fold higher than the dose used in this study) might have been able to overcome an existing insulin resistance. Since GIP usually increases insulin-binding affinity in adipocytes, the loss of GIP action is thought to lead to insulin resistance in adipose tissue (Yip et al. 1998).

Transgenic mice showed approximately 50% lower postprandial serum insulin levels vs. wild-type mice statistically proven by ANOVA. In a recent study, postprandial serum insulin levels were found to be significantly reduced in both male and female $GIPR^{dn}$ transgenic mice fed a conventional diet. In addition, early phase glucose-induced insulin secretion was absent in transgenic mice even when GIP or GLP-1 were co-administered during glucose tolerance tests (Herbach et al. 2005). These findings show an absolute insulin deficiency in $GIPR^{dn}$ transgenic mice.
In $GLP\text{-}1R^{-/-}$ and $GIPR^{-/-}$ mice, fasting insulin levels were reported to be similar to wild-type mice. After an oral glucose challenge, serum insulin levels of GIPR and GLP-1R knockout mice were significantly reduced vs. control mice in the early phase after glucose challenge. In DIRKO (double incretin receptor knockout) mice, the reduction in serum insulin levels 15 minutes after glucose challenge was even more pronounced than in single receptor knockout mice. One hour after oral glucose challenge, serum insulin levels were not different between DIRKO mice and corresponding controls (Scrocchi et al. 1996; Miyawaki et al. 1999).
In 45-day-old wild-type mice fed the carbohydrate restricted diet serum insulin was lower than in age-matched wild-type mice fed the breeding diet, thereby confirming the insulin-lowering effect of a low GI diet (Willett et al. 2002).

Serum GIP levels did not differ between wild-type and transgenic mice when no DPPIV-inhibitor was added to the serum. Serum of wild-type and $GIPR^{dn}$ transgenic mice where a DPPIV-inhibitor was added showed significantly higher GIP levels in female transgenic mice vs. wild-type mice fed a conventional diet (Herbach et al., unpublished data). These results underline the importance to add a DPPIV-inhibitor, though the manufacturer´s manual claims that it is not necessary.
In several human studies, GIP levels were reported to be higher in patients with type 2 diabetes than in healthy control subjects (Nyholm et al. 1999; Nauck et al. 2004). Higher GIP levels in diabetic humans probably occur to compensate for the reported reduced insulinotropic activity of GIP in diabetic patients (Nauck et al. 1993; Meier et al. 2001). In $GIPR^{dn}$ transgenic mice, GLP-1 levels were reported to be increased (Herbach et al. 2005). In $GIPR^{-/-}$ mice, GLP-1 levels were unaltered but GLP-1 induced insulin responses were significantly greater in knockout vs. wild-type mice (Pamir et al. 2003). In $GLP\text{-}1R^{-/-}$ mice, GIP levels were increased vs. wild-type mice (Pederson et al. 1998). These results demonstrate the plasticity of the entero-insular

axis, suggesting that in the mouse the lack of action of one incretin is partly compensated by upregulation of the other incretin hormone, thereby preventing mostly from the development of diabetes mellitus. In GIPR^{dn} transgenic mice, the secretion of both incretins is increased. However, neither GIP nor GLP-1 exert an insulinotropic effect during GTT (glucose tolerance test), leading to a disturbed glucose homeostasis (Herbach et al. 2005). In DIRKO mice, mean fasted GIP levels were similar in wild-type and knockout mice. After oral glucose challenge, the levels of circulating GIP were significantly higher in wild-type compared with DIRKO mice. In contrast, circulating levels of GLP-1 were lower in DIRKO mice in both the fasted state and following glucose challenge (Hansotia et al. 2004). These results demonstrate similar changes in DIRKO mice and human type 2 diabetic patients, who also exhibited a reduced GIP-effect and reduced GLP-1 secretion.

In summary, GIPR^{dn} transgenic mice exhibited increased fasting and postprandial blood glucose levels, a disturbed glucose tolerance and lower serum insulin levels than age- and diet-matched wild-type mice. Transgenic mice fed the carbohydrate restricted diet showed a better controlled glucose homeostasis, as evidenced by lower fasting and postprandial blood glucose levels and an improved glucose tolerance at the age of 175 days, in comparison to transgenic mice fed the breeding diet.

5.3 Food and water intake

The diabetic state of GIPR^{dn} transgenic mice fed the breeding diet was accompanied by severe hyperphagia and polydipsia. These are common findings in diabetic patients (Report of the Expert Committee on the diagnosis and classification of diabetes mellitus 2002). Transgenic mice fed the carbohydrate restricted diet showed only marginally increased food and water intakes as compared to diet-matched wild-type mice. Wild-type mice fed the carbohydrate restricted diet showed a significantly higher food intake than wild-type mice fed the breeding diet. This result can be explained by the low energy content of the carbohydrate restricted diet. The water intake of wild-type mice fed the carbohydrate restricted diet was also significantly higher than that of wild-type mice fed the breeding diet. The high amount of fiber in the diet is probably the cause for the higher demand of water. These results are in line with the results of Herbach (2002).

GIPR^{dn} transgenic mice fed the carbohydrate restricted diet showed a significantly lower water intake than transgenic mice fed the breeding diet. The reason for this difference is probably the significantly elevated blood glucose levels in transgenic mice fed the breeding diet that lead to a higher urine glucose excretion which in turn results in a greater renal loss of water (Herbach 2002).

In summary, the clinical investigations of the present study confirmed that GIPR^{dn} transgenic mice show a severe diabetic phenotype which was further characterized by the investigations of this study.
The feeding of a carbohydrate restricted diet to GIPR^{dn} transgenic mice led to a better metabolic control, as evidenced by lower fasting and postprandial blood glucose levels and a significantly improved glucose tolerance at 175 days of age.

5.4 Morphometric investigations of the endocrine pancreas

The expression of a dominant negative GIP receptor was found to be associated with a severe reduction of the total volumes of islets and beta-cells, and the volume density of beta-cells in islets in GIPR^{dn} transgenic mice as compared to control mice, regardless of nutrition and the age at sampling.
The volume density of beta-cells in islets was found to be higher in GIPR^{dn} transgenic mice fed the carbohydrate restricted diet than in transgenic mice fed the breeding diet at all ages investigated. This results leads to the conclusion that the unique islet composition of rodent islets was better preserved in GIPR^{dn} transgenic mice fed the carbohydrate restricted diet. The clustering of beta-cells in rodent islets is thought to be connected with the oscillations in membrane potential and Ca^{2+} levels in response to high glucose concentrations. This oscillatory response may constitute the molecular basis for the typical pulsatility in insulin release (Cabrera et al. 2006). Therefore, the better preserved volume fraction of beta-cells in the islets of GIPR^{dn} transgenic mice fed the carbohydrate restricted diet could result in a better function of beta-cells and the observed improved glucose homeostasis in comparison to GIPR^{dn} transgenic mice fed the breeding diet.
At 90 days of age, the total volume of beta-cells was significantly higher in GIPR^{dn} transgenic mice fed the carbohydrate restricted diet vs. transgenic mice fed the breeding diet; at 180 days of age, the total volume of beta-cells was increased to the

same extent in transgenic mice fed the carbohydrate restricted diet (n.s.) A reduced total volume of beta-cells is a common finding in rodent models of diabetes, e.g. in Psammomys obesus where beta-cell mass is reduced up to 70% (Donath et al. 1999) and in the Akita mouse (Masiello 2006). In human type 2 diabetic patients it is still discussed whether beta-cell mass is decreased, or not. Several studies reported a decreased total volume or volume density of beta-cells in the pancreas of diabetic patients (Sakuraba et al. 2002; Butler et al. 2003), whereas others did not observe a reduction in beta-cell mass (Guiot et al. 2001). GLP-1R$^{-/-}$ mice exhibited comparable mean beta-cell volumes and beta-cell numbers to wild-type mice. However, female GLP-1R$^{-/-}$ mice showed a markedly increased alpha- to beta-cell volume ratio and an increase of alpha-cell number (Ling et al. 2001). In GIPR$^{-/-}$ mice, beta-cell area as a percentage of total pancreatic area was significantly increased in knockout vs. wild-type mice. The staining intensity for insulin was reduced in GIPR$^{-/-}$ islets, though, and the pancreatic insulin content was significantly lower (Pamir et al. 2003).

The total islet volume was 2.0-fold higher at the age of 90 days vs. 45 days of age in wild-type mice fed the breeding diet. The same was true for the total volume of beta-cells resulting in a constant volume density of beta cells and non-beta cells in the islets. Therefore, the cellular composition of the islets did not change.

In wild-type mice fed the carbohydrate restricted diet, the total volumes of islets and beta-cells remained stable, resulting in a significantly lower total islet volume and total beta-cell volume at 90 and 180 days of age vs. wild-type mice fed the breeding diet. These findings could be due to a lower rate of islet-/beta-cell replication and neogenesis and/or a higher rate of apoptosis of islet-/beta-cells than in wild-type mice fed the breeding diet. Given that glucose is the most important stimulant for beta-cell replication and neogenesis, a lowered rate of replication and neogenesis is more probable. Different studies showed that long-term glucose infusion leads to an increase in beta-cell mass, resulting from both beta-cell hyperplasia and hypertrophy (Bonner-Weir et al. 1989; Bernard et al. 1999; Lipsett and Finegood 2002). The islet cell replication rate of wild-type mice fed the carbohydrate restricted diet was found to be very low at the age of 90 days, where the replication rate was still high in wild-type mice fed the breeding diet. This finding supports the view, that high islet cell replication led to the increase in the total islet and beta-cell volume of wild-type mice fed the breeding diet.

In GIPR^{dn} transgenic mice fed the breeding diet, the total volumes of islets and beta-cells were reduced at the age of 90 and 180 days as compared to 45 days of age. The volume density of beta-cells in the islets was severely reduced at 90 and 180 days of age in comparison to 45 days of age, resulting in a shift towards non-beta-cells in transgenic mice fed the breeding diet.
In contrast, in GIPR^{dn} transgenic mice fed the carbohydrate restricted diet, the total islet volume was significantly higher at 90 days of age vs. 45 days of age, while the total beta-cell volume remained constant, resulting in a lower volume density of beta-cells in the islets at 90 days of age vs. 45 days of age. Therefore, a shift towards endocrine non-beta-cells was also evident in GIPR^{dn} transgenic mice fed the carbohydrate restricted diet. The shift towards non-beta-cells in GIPR^{dn} transgenic mice is in line with the results of recent studies in human diabetic patients (Yoon et al. 2003) and GIPR^{dn} transgenic mice (Herbach et al. 2005). The decrease in beta-cell mass in transgenic mice fed the breeding diet might be a result of the prevailing high blood glucose levels, leading to glucotoxicity-induced beta-cell death and a progressive loss of beta-cell mass. Since glucose homeostasis was found to be improved in GIPR^{dn} transgenic mice fed the carbohydrate restricted diet, glucotoxicity is likely to be reduced, leading to preserved beta-cell mass with the progression of age and a 3.3-fold higher total volume of beta-cells as compared to transgenic mice fed the breeding diet.

The total volume of isolated beta-cells, used as an indicator for islet neogenesis (Bonner-Weir et al. 1993), was significantly reduced at the age of 45 days in GIPR^{dn} transgenic mice, irrespective of the diet fed. This result implies that islet neogenesis was disturbed in transgenic vs. wild-type mice at a time when replication and neogenesis were high in wild-type mice. Islet neogenesis was shown to be largely reduced in GIPR^{dn} transgenic mice already at 10 days of age, before overt diabetes mellitus developed (Herbach et al. 2005). Dysregulation of the balance between neogenesis and apoptosis in the phase of remodeling of the endocrine pancreas is thought detrimental for the maintenance of beta-cell mass (Trudeau et al. 2000). This concept is supported by studies in GIPR^{dn} transgenic mice and other experimental models for diabetes mellitus (Petrik et al. 1999 a; Bonner-Weir 2000).

The total volume of isolated beta-cells was reduced in wild-type mice fed the carbohydrate restricted diet when compared to wild-type mice fed the breeding diet, leading to the conclusion that wild-type mice fed the carbohydrate restricted diet show reduced islet neogenesis resulting in the lower total islet volume. An explanation for the reduced rate of replication/neogenesis of wild-type mice fed the carbohydrate restricted diet could be the low concentration of the most important mitogenic stimulans, glucose, in the carbohydrate restricted diet.

In order to show the mechanisms of changes in beta-cell mass, islet-cell replication and apoptosis were examined. Islet-cell replication showed a tendency to be increased in transgenic vs. wild-type mice fed the carbohydrate restricted diet and was more than doubled in transgenic mice fed the breeding diet vs. respective controls (n.s.).Since beta-cell mass does not increase in transgenic mice, the higher replication most likely accounts for non-beta-cells (Herbach et al. 2005). Since the endocrine pancreas is a slowly turning tissue with a replication rate of about 3% per day in healthy rodents (Finegood et al. 1995), it is very difficult to quantify cell replication. Increased rates of apoptosis and replication were mainly found in rodents in the first 2-3 weeks of life when a remodeling of the endocrine pancreas takes place (Scaglia et al. 1997). High replication rates of islet cells were also reported between one and two, and between three and four months of age in rodents, the time course when total beta-cell mass doubled in healthy rodents. Thereafter, replication of islet cells was rather slow and the rate of apoptosis reached that of replication (2-3% per day), leading to a stable beta-cell mass (Finegood et al. 1995).
Under physiological conditions, apoptosis is a very rare and rapid process in the endocrine pancreas of adult rodents, that takes not more than three hours and its morphological evidence is even more short lived (about 1 hour) (Scaglia et al. 1995). In this study, a higher rate of apoptosis was found in $GIPR^{dn}$ transgenic mice of both feeding groups at the age of 90 days. At 180 days of age, apoptotic islet cells were only found in transgenic mice fed the carbohydrate restricted diet.
Basal rates of apoptosis in the endocrine pancreas of rats are reported to be ~1.5% per day (Scaglia et al. 1997). In a recent study, the whole pancreas of Akita and wild-type mice was exhaustively sectioned in order to determine the number of apoptotic cells per islet. Even using this time consuming and expensive method, no difference in apoptotic frequency was found comparing Akita and wild-type mice, despite Akita

mice displaying severe ER-stress and progressive loss of beta-cell mass (Izumi et al. 2003).Therefore, it was not unexpected to find low and non-significant rates of apoptosis in this study.

In addition to the reduced islet mass, $GIPR^{dn}$ transgenic mice exhibited a significantly lower total number of islets than control mice. The total number of islets was higher in transgenic and wild-type mice at the age of 180 days as compared to 45 days of age, irrespective of the diet fed. Therefore, islet neogenesis in $GIPR^{dn}$ transgenic mice is not completely abolished. The results of the study of Dor et al. (2004), claiming that the number of islets is fixed during adult life could therefore not be confirmed. In other studies, islets were described as a dynamic tissue that expands continually through adulthood. The islet size was reported to increase due to addition of new islet cells and an increase in individual cell volume (hypertrophy) in healthy mice (Montanya et al. 2000; Bonner-Weir 2000 a). In contrast, the present study shows that neither the mean islet volume, nor the mean beta-cell volume increases with age, irrespective of the genetic group and diet fed. Therefore, the higher total volumes of islets and beta-cells found at the age of 180 days in wild-type mice fed the breeding diet solely resulted from neogenesis of islets and beta-cell hyperplasia, and not from islet and beta-cell hypertrophy.

The total number of beta-cells was strikingly reduced in $GIPR^{dn}$ transgenic mice at both ages investigated. This can result from a lowered rate of replication and neogenesis, and/or an elevated rate of apoptosis of beta-cells of transgenic mice. Since GIP action is abolished by the mutated GIP receptor, it is not able to unfold its mitogenic effect. Furthermore, GLP-1 was shown to have no effect on the insulin secretion in $GIPR^{dn}$ transgenic mice (Herbach et al. 2005) so it might be possible that its mitogenic effect is similarly disturbed. Additionally, the permanently elevated blood glucose levels could trigger beta-cell apoptosis by glucotoxicity. Glucose has been reported to have an ambiguous effect on beta-cell mass. Glucose in limited concentrations administered for a short period of time leads to an increase in beta-cell mass (as described above) while excessive amounts of glucose over a prolonged period exert negative effects on beta-cell function and also may negatively affect beta-cell mass by inducing apoptosis (Kaiser et al. 2003). Both, reduced neogenesis

and increased apoptosis presumably contribute to the reduced beta-cell number of $GIPR^{dn}$ transgenic mice.

At 180 days of age, the total number of beta-cells is reduced in wild-type mice fed the carbohydrate restricted diet when compared to wild-type mice fed the breeding diet but the mean volume of beta-cells was unchanged. Therefore, the difference in beta-cell mass of wild-type fed the carbohydrate restricted diet vs. wild-type mice fed the breeding diet resulted from the stagnating beta-cell mass in wild-type mice fed the carbohydrate restricted diet due to a lower replication/neogenesis rate.

Beta-cell hypertrophy is reported in studies using glucose infusions to stimulate expansion of beta-cell mass of nondiabetic and mildly diabetic (due to streptozotocin administration), rats (Bernard et al. 1998). Beta-cell hypertrophy may also contribute to the increase in beta-cell mass as a means of adaptation to an increased insulin demand due to insulin resistance in diabetic subjects (Weir et al. 2001; Weir and Bonner-Weir 2004). In contrast, $GIPR^{dn}$ transgenic mice did not only show beta-cell hypoplasia but also beta-cell hypotrophy. In $GIPR^{dn}$ transgenic mice, this adaptation mechanism was not triggered, apparently, since transgenic mice fed the breeding diet exhibited the reduction in beta-cell size already at an age of 45 days. Similarly, no compensatory beta-cell hypertrophy was observed in type 2 diabetic patients despite chronic exposure to high glucose levels (Klöppel et al. 1985).

In summary, feeding of a carbohydrate restricted diet improved the glycemic control of $GIPR^{dn}$ transgenic mice. Feeding of this diet also exerted a significant effect on pancreatic beta-cell mass and the total number of beta-cells. The improved glucose homeostasis, however, did not restore beta-cell mass or function, but prevented from the progressive decline in both beta-cell mass and number, observed in $GIPR^{dn}$ transgenic mice fed the breeding diet. A complete restoration of beta-cell mass by dietary intervention can not be expected since $GIPR^{dn}$ transgenic mice already exhibit a reduced beta-cell mass at 10 days of age, due to a disruption of the postnatal development of the endocrine pancreas (Herbach et al. 2005).

Surprisingly, the feeding of the carbohydrate restricted diet led to an impaired glucose tolerance in wild-type mice fed the carbohydrate restricted diet as compared to wild-type mice fed the breeding diet. This may be explained by a reduced total volume of beta-cells, reduced replication of beta-cells and a lower number of beta-

cells in wild-type mice fed the carbohydrate restricted diet resulting in a lower functional beta-cell mass.

The results of the clinical and morphological investigations of transgenic mice lead to the conclusion that a carbohydrate restricted/high fiber diet could be of deciding advantage in human type 2 diabetic patients. The improved glucose homeostasis could prolong the stadium of stable adaptation by reducing loss of beta-cell mass due to glucotoxicity, thereby delaying the need for insulin therapy. A recent study underlined the need for therapy that targets specific mechanisms, e.g. inhibition of apoptosis by preventing glucolipotoxicity (Ahrén 2005). Delaying insulin therapy by any means would be an important advantage, since insulin therapy is often a psychologic problem for diabetic patients and also involves the risk of hypoglycaemic episodes.

The present study shows that $GIPR^{dn}$ transgenic mice are an excellent model to study the effects of dietary intervention on glucose homeostasis and to investigate how the diet partly compensates for the disturbed development of the endocrine pancreas.

6. Summary

Effects of a carbohydrate restricted diet on the metabolic state and progressive pancreatic beta-cell loss in transgenic mice expressing a dominant negative GIP receptor ($GIPR^{dn}$)

In the last years, gastrointestinal hormones like glucose-dependent insulinotropic polypeptide (GIP) have been proven to play a role in the pathogenesis of diabetes mellitus in humans and animal models of diabetes mellitus.

Furthermore, the influence of dietary measures on the diabetic status in type 2 diabetic patients is a topic frequently discussed in the literature. The aim of this study was to investigate the effects of a carbohydrate restricted diet on the metabolic state and the progressive loss of beta-cells in transgenic mice expressing a dominant negative GIP receptor under the control of the rat insulin gene promoter. Results were compared to diet-matched wild-type littermates and to transgenic mice fed a standard breeding diet. For further characterization and better understanding of the animal model, the expression pattern of the murine endogenous and the transgenic GIP receptor was examined on RNA level. Clinical parameters were analyzed, including fasting and postprandial blood glucose levels, serum insulin and serum GIP concentrations, oral glucose tolerance tests and insulin sensitivity tests. In addition, daily food and water intake was investigated. Quantitative morphological investigations were carried out on the endocrine pancreas using unbiased stereological methods. Evaluated parameters included the total volumes of islets and beta-cells, volume densities of islets in the pancreas and beta-cells in the islets, total numbers of islets and beta-cells, mean volume of islets and beta-cells. In addition, apoptotic and replicating islet-cells were quantified.

Examination of the expression pattern of the transgenic GIP receptor and the endogenous murine GIP receptor showed that both, the endogenous and the transgenic GIP receptor are expressed in a wide variety of organs, such as pancreas, brain and adipose tissue.

Clinical investigations showed a severe diabetic phenotype of $GIPR^{dn}$ transgenic mice which could be ameliorated by feeding the carbohydrate restricted diet. Quantitative-stereological analyses of the pancreas revealed a striking reduction of the total volumes of pancreatic islets, beta-cells in the islets and isolated beta-cells in

the pancreas of transgenic mice, irrespective of the diet fed. Total numbers of islets and beta-cells were also largely reduced in transgenic vs. wild-type mice. Analyses of variance revealed a significant influence of diet, genetic group and the interaction of diet * genetic group, as well as the interaction diet *genetic group * age on the total number of beta-cells. A higher rate of apoptosis of islet cells was found in 90-day-old transgenic mice vs. age-matched wild-type mice, which could contribute to the reduced beta-cell mass in transgenic mice.

In summary, transgenic mice expressing a dominant negative GIP receptor under the control of the rat insulin gene promoter develop a severe diabetic phenotype and striking changes in quantitative parameters of the endocrine pancreas. In $GIPR^{dn}$ transgenic mice, feeding of a carbohydrate restricted diet resulted in an improved glucose homeostasis, as evidenced by lower fasting and postprandial blood glucose levels and an improved glucose tolerance, as well as a higher volume density of beta-cells in islets, a higher mean islet volume at the age of 45 days, and a stable beta-cell mass during the investigated period. The findings of the present study provide evidence for a beneficial effect of a carbohydrate restricted diet on progressive beta-cell loss in $GIPR^{dn}$ transgenic mice. Therefore, $GIPR^{dn}$ transgenic mice are a valuable model to study the effects of dietary interventions on glucose homeostasis and the progressive loss of beta-cells.

Zusammenfassung

Einfluss einer kohlenhydratarmen Diät auf den metabolischen Status und den progressiven Verlust an pankreatischen Betazellen bei transgenen Mäusen, die einen dominant negativen GIP Rezeptor ($GIPR^{dn}$) exprimieren

In den letzten Jahren wurde gezeigt, dass gastrointestinale Hormone wie glucose-dependent insulinotropic polypeptide (GIP), eine Rolle in der Pathogenese des Diabetes mellitus bei Menschen und bei diabetischen Tiermodellen spielen. Außerdem wird der Einfluss von dietätischen Maßnahmen auf den diabetischen Status bei Typ 2 Diabetikern in der Literatur zunehmend diskutiert. Ziel dieser Studie war es, den Einfluss einer kohlenhydratarmen Diät auf den metabolischen Status und die Entwicklung des endokrinen Pankreas bei transgenen Mäusen, die einen dominant negativen GIP Rezeptor unter der Kontrolle des Ratteninsulingenpromoters

exprimieren, zu untersuchen. Die Ergebnisse wurden mit denen von gleichartig gefütterten Wildtyp Geschwistertieren und denen von transgenen Mäusen, die ein Standard-Zuchtfutter erhielten, verglichen. Um das Tiermodell weiter zu charakterisieren, wurde das Gewebemuster der Expression des endogenen, murinen GIP Rezeptors und des transgenen GIP Rezeptors auf RNA Ebene untersucht. Es erfolgte eine Analyse Diabetes-relevanter klinischer Parameter, wie basale- und postprandiale Blutglukosespiegel, Serum-Insulin- und Serum-GIP-Konzentrationen, sowie orale Glukosetoleranztests und Insulinsensitivitätstests. Außerdem wurde der tägliche Futter- und Wasserverbrauch untersucht. Die Erfassung histopathomorphologischer Veränderungen des endokrinen Pankreas erfolgte quantitativ-stereologisch an drei Altersgruppen. Die Untersuchungen beinhalteten die Bestimmung des Gesamtinsel- und –betazellvolumens, der Volumendichte der Inseln im Pankreas und der Betazellen in den Inseln, der Gesamtinsel- und -betazellzahl, des mittleren Volumens der Inseln und Betazellen. Ferner wurden apoptotische und replizierende Zellen der Pankreasinseln quantitativ bestimmt.

Die Untersuchung des Gewebemusters der Expression des transgenen und des endogenen, murinen GIP Rezeptors ergab eine Expression in zahlreichen Organen, u.a. im Pankreas, Gehirn und Fettgewebe.

Die klinischen Untersuchungen zeigten, dass $GIPR^{dn}$ transgene Mäuse beider Fütterungsgruppen einen schweren diabetischen Phänotyp aufweisen, der durch die Fütterung der kohlenhydratarmen Diät verbessert werden konnte.

Durch die quantitativ-stereologische Untersuchung des Pankreas wurde eine hochgradige Reduktion des Gesamt-Inselvolumes, Betazell-Volumens und des Gesamtvolumens der isolierten Betazellen bei $GIPR^{dn}$ transgenen Mäusen festgestellt, unabhängig von der verabreichten Diät. Die Gesamtzahl der Inseln und der Betazellen, die mittels modellfreier stereologischer Methoden ermittelt wurde, war bei transgenen Mäusen ebenfalls deutlich verringert ($p<0.05$). Die Varianzanalyse zeigte einen signifikanten Einfluss der Diät, der Transgenität und der Interaktion Diät * Transgenität, als auch der Interaktion von Diät * Transgenität * Alter auf die Anzahl der Betazellen. Bei 90 Tage alten transgenen Mäusen wurde eine höhere Apoptoserate als bei gleichaltrigen Wildtyptieren gefunden, die zu der verminderten Betazellmasse bei transgenen Mäusen beitragen könnte.

Die Ergebnisse zeigen, dass trangene Mäuse, die einen dominant negativen GIP Rezeptor unter der Kontrolle des Ratteninsulingenpromoters exprimieren, einen hochgradigen diabetischen Phänotyp und prägnante quantitative, morphologische Veränderungen des endokrinen Pankreas entwickeln. Das Füttern der kohlenhydratarmen Diät führte bei $GIPR^{dn}$ transgenen Mäusen sowohl zu einer verbesserten Glukosehomeostase, wie anhand der Nüchtern- und postprandialen Blutglukosespiegel und der verbesserten Glukosetoleranz gezeigt werden konnte, als auch zu einer höheren Volumendichte der Betazellen in den Inseln, einem höheren mittleren Inselvolumen im Alter von 45 Tagen und einer stabilen Betazellmasse im Untersuchungszeitraum.

Die Resultate der vorliegenden Studie weisen eine günstige Beeinflussung des progredienten Diabetes-assoziierten Betazellverlustes durch die kohlenhydratarme Diät bei $GIPR^{dn}$ transgenen Mäusen nach. Deswegen sind $GIPR^{dn}$ transgene Mäuse ein wertvolles Tiermodel, um den Einfluss von dietätischen Massnahmen auf die Glukosehomeostase und die postnatale Entwicklung des endokrinen Pankreas zu untersuchen.

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