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# Physiological and morphological characterization of transgenic pigs expressing a dominant-negative glucose-dependent insulinotropic polypeptide receptor ($GIPR^{dn}$) – a large animal model for diabetes research

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# Table of contents

# 1 Introduction

Diabetes mellitus is a major public health problem characterized by high blood glucose levels due to defects in insulin secretion and/or insulin action (ADA 2010). There are two major types of diabetes. Type 1 diabetes mellitus, previously called insulin dependent diabetes mellitus (IDDM) or juvenile-onset diabetes develops due to immunogenic destruction of pancreatic β-cells resulting in a deficient insulin production. Around 90% of diabetes affected people suffer from type 2 diabetes mellitus, previously called non insulin dependent diabetes mellitus (NIDDM) or adult-onset diabetes. Defects on insulin secretion, insulin resistance and a reduction of β-cell mass characterize this diabetes type (ADA 2010; Butler et al. 2003; Sakuraba et al. 2002). The world prevalence of diabetes among adults (aged 20 to 79 years) is 6.4% in the year 2010, i.e. 285 million people are affected (Shaw et al. 2010). In 2030, an increase to 7.7% and 439 million adults, respectively, is expected (Shaw et al. 2010). Furthermore, diabetes appeared to be a considerable cause of premature mortility as 6.8% of global mortility are due to diabetes (Roglic et al. 2010). Therefore diabetes is not only a problem of the individual suffering from severe secondary lesions but also of the society due to high expenses. Health expenditure for diabetes only for the European region is expected to correspond to 105.5 billion USD in the year 2010 and 124.6 billion USD in the year 2030 (IDF 2009). North America represents the region with the highest costs as 214.2 billion USD are budgeted for 2010 and 288.7 billion USD for 2030 (IDF 2009). Changed living circumstances related to overly nutrition and lack of physical activity promote the development of diabetes (Leahy 2005). The number of adults with diabetes will increase among others in developing countries indicating a growing burden of diabetes in the world (Shaw et al. 2010). However, the reasons for diabetes are still not completely clarified, whereby a great effort on research is going on. Animal models are needed to get insight in different aspects of this disease with techniques that are not applicable in

humans whereas one single animal model is not sufficient to cover all aspects of this multifactorial disease (Larsen 2009).

One aspect of diabetes is linked to the incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) which potentiate insulin secretion after nutrient ingestion mediating the so-called “incretin effect” (Elrick et al. 1964; McIntyre et al. 1964). Patients with type 2 diabetes mellitus exhibited an impaired incretin effect whereby the insulinotropic action of GIP is reduced to almost absent while this of GLP-1 is preserved (Nauck et al. 1993b).

Young adult $GIPR^{dn}$ transgenic pigs, generated by lentiviral gene transfer, showed parallels to type 2 diabetes patients as they exhibited a significantly reduced β-cell mass and disturbed glucose tolerance compared to controls (Renner 2008). The aim of this work was to further characterize $GIPR^{dn}$ transgenic pigs physiologically and morphologically at different age stages to find out more about the role of the GIP/GIPR axis in the pathogenesis of type 2 diabetes mellitus.

# 2 Review of the literature

## 2.1 The incretin hormone system

At the beginning of the 20th century a substance called “secretin” released from the duodenum was found to be responsible for the stimulation of pancreatic cells provoking a secretion of the pancreatic juice (Bayliss et al. 1902). The term “incretin” was coined by La Barre in 1932 to denote this substance as a glucose-lowering and intestinal-derived factor (La Barre 1932). When in 1960 the radioimmunoassay was developed, leading to the possibility to determine serum insulin levels, an important progress on the incretin concept was made. By radioimmunoassay proof was furnished, that an oral glucose load mediated a higher insulin secretion compared to an intravenous glucose load (Elrick et al. 1964; McIntyre et al. 1964). The first incretin hormone was identified at the end of the 1960`s (Brown et al. 1969). Based on its ability to inhibit gastric acid secretion in dogs, it was named gastric inhibitory polypeptide (GIP) (Brown et al. 1971; Brown 1971; Brown et al. 1975). However, further investigations were performed and GIP was also characterized as insulin-stimulating factor (Dupre et al. 1973). As this characteristic was not only detectable after pharmacological doses like inhibition of gastric acid secretion but also at physiological levels, GIP was renamed glucose-dependent insulinotropic polypeptide, thus maintaining the original acronym. Cloning and sequencing of mammalian proglucagon genes and complementary DNAs revealed a second incretin hormone, glucagon-like peptide-1 (GLP-1), with potent insulinotropic properties (Schmidt et al. 1985). These two hormones, GIP and GLP-1, work in an additive manner and mediate the so-called incretin effect which describes the phenomenon, that glucose given orally causes a higher insulin response compared to an isoglycemic intravenous glucose infusion (Nauck et al. 1993a).

## 2.2 Glucose-dependent insulinotropic polypeptide (GIP)

### 2.2.1 Synthesis, secretion and degradation of GIP

The bioactive 42-amino acid peptide form of GIP derives from a proGIP protein precursor (Takeda et al. 1987). Secretin, glucagon, growth-hormone releasing factor and vasoactive intestinal peptide are structurally related hormones to GIP (Sherwood et al. 2000). Moody et al. showed that the sequence or porcine GIP differs from that of human GIP only by two single base changes at the DNA level, demonstrating a high sequence identity between the two species (Moody et al. 1984). Synthesis and secretion of GIP take place in the K-cells, which were mainly identified in the duodenum and the proximal jejunum (Buchan et al. 1978). Throughout the entire small intestine also a small number of cells producing both GIP and GLP-1 were detected revealing colocalization of both hormones (Mortensen et al. 2003). The enzyme dipeptidyl peptidase-4 (DPP-4) is responsible for the degradation of GIP. In humans, the half-life of biologically active GIP was determined being between 5 and 7 minutes using a N-terminal directed radioimmunoassay to distinguish between the biological active form GIP-(1-42) and the predominantly existing non-insulinotropic metabolite GIP-(3-42) (Deacon et al. 2000).

### 2.2.2 GIP receptor and signal transduction

Nutrient ingestion stimulates GIP secretion from the intestinal K-cells, whereas fat is the most potent GIP stimulator in humans in contrast to carbohydrates in pigs (Baggio et al. 2007; Rijkelijkhuizen et al. 2009). After binding to its specific GIP receptor (GIPR), GIP can execute its insulinotropic ability. The GIPR is a member of the seven transmembrane-spanning, heterotrimetric G-protein-coupled receptor superfamily (Usdin et al. 1993). The N-terminal extracellular domain of the GIPR and the first extracellular loop act as regions of the highest binding affinity for GIP, whereas the first transmembrane domain is essential for receptor activation (Gelling et al. 1997). The carboxyl-terminal tail (CT) seemed not to play an important role in intracellular signal transduction, but receptor expression depends on a minimum chain length of approximately 405 amino

acids and specific serine residues in the CT are required for plasma membrane insertion (Wheeler et al. 1999). Glucose represents the primary stimulation factor for insulin secretion (Malaisse et al. 1979) . When glucose is metabolized in the β-cell the ATP/ADP ratio increases resulting in closure of ATP sensitive $K^+$ channels, membrane depolarization, activation of voltage gated $Ca^{2+}$ channels followed by an increase in intracellular $Ca^{2+}$ levels and insulin-granule exocytosis (Ashcroft et al. 2004). GIP mediates increases in intracellular $Ca^{2+}$ levels and additionally shows direct effects on insulin exocytosis mechanisms (Ding et al. 1997; Holst et al. 2004; MacDonald et al. 2002; Wahl et al. 1992). Binding of GIP to the GIPR leads to G-Protein coupling at the third intracellular loop of the GIPR followed by increases in cAMP levels (Cypess et al. 1999; Hallbrink et al. 2001; Harmar 2001; Salapatek et al. 1999; Takhar et al. 1996; Wheeler et al. 1993). Elevated cAMP levels induce both protein kinase A (PKA)-dependent (Ding et al. 1997) and -independent pathways (Seino et al. 2005). PKA stimulates several intracellular events like rising of intracellular $Ca^{2+}$ levels, which cause, in the case of pancreatic β-cells, enhanced exocytosis of insulin-containing granules (Ding et al. 1997). Mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) and phospholipase $A_2$ ($PLA_2$) pathways contribute to direct PKA-independent effects of GIP on insulin secretion (Ehses et al. 2001; Kubota et al. 1997; Straub et al. 1996).

### 2.2.3 Biological actions of GIP

Originally, GIP was identified based on its ability to inhibit gastric acid secretion in dogs (Brown et al. 1975). Over the years the insulinotropic action of GIP and the other metabolic effects were considered important, although the effects of GIP are not restricted to the endocrine pancreas. GIP is also involved in a wide range of physiological actions in the adipose tissue, the bone metabolism and the nervous system (Baggio et al. 2007). Several studies in GIPR knockout ($GIPR^{-/-}$) mice (Miyawaki et al. 1999), double incretin receptor knockout (DIRKO) mice (Hansotia et al. 2004) and $GIPR^{dn}$ mice (Herbach et al. 2005) accounted for the elucidation of the various physiological roles of GIP. In the

following the biological actions of GIP in the endocrine pancreas and extrapancreatic tissues are introduced.

#### 2.2.3.1 The endocrine pancreas

After food intake GIP is secreted from the intestinal K-cells and binds to its specific receptor initiating a potentiation of insulin secretion (Dupre et al. 1973). Hypo-, eu- and hyperglycemic clamp studies in combination with GIP infusion confirmed the hypothesis that the insulinotropic effect of GIP is glucose-dependent (Elahi et al. 1979; Kreymann et al. 1987; Nauck et al. 1993a). GIP seemed not to be effective under fasting conditions in humans indicating that there is a glucose threshold for its insulinotropic action (Elahi et al. 1979). While GLP-1 was detected to be involved in the regulation of fasting glycemia (Baggio et al. 2000), GIP appeared to act only as a potentiator of glucose-induced insulin secretion (Lewis et al. 2000). Nevertheless, there are also studies suggesting GIP being capable to influence insulin secretion under fasting conditions in humans (Amland et al. 1985a). Furthermore, the overall incretin effect mediated to 50-70% by GIP was detected by immunoneutralization studies with intravenous administration of GIPR antagonists or antibodies for the GIPR (Gault et al. 2002b; Lewis et al. 2000; Tseng et al. 1996; Tseng et al. 1999). The pro-proliferative and anti-apoptotic effects of GIP play an important role in the remodeling of the pancreas (see 2.2.3.2). Moreover, GIP stimulates insulin gene transcription and protein synthesis in the β-cell (Fehmann et al. 1995; Wang et al. 1996) as well as it mediates an up-regulation of glucose-sensing elements (Wang et al. 1996). Not only insulin but also the other islet hormones glucagon, somatostatin and pancreatic polypeptide are regulated by GIP (Adrian et al. 1978; Amland et al. 1985b; Ipp et al. 1977). The influence of GIP on glucagon secretion is discussed controversially as on the one hand GIP was incapable to effect glucagon secretion in normal humans (Elahi et al. 1979; Nauck et al. 1993b) and on the other hand a glucagon-enhancing action of GIP under fasting conditions was detected (Meier et al. 2003). Whether the contribution of GIP on the regulation of somatostatin and pancreatic polypeptide in humans is important remains unclear (McIntosh et al. 2009).

#### 2.2.3.2 Pro-proliferative and anti-apoptotic effects of GIP on β-cells

As in type 2 diabetes patients β-cell mass is reduced by at least 65 % (Butler et al. 2003) and the insulinotropic effect of GIP is impaired, the influence of GIP on proliferation and differentiation of β-cells is an interesting aspect for diabetes research. The GIPR is highly expressed on β-cells (Moens et al. 1996) and there are several indications that GIP acts as a growth and metabolic factor for β-cells. Mitogenic signaling modules, like mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K), are activated by GIP, indicating that this incretin hormone is a pro-proliferative factor (Trumper et al. 2002). Several signal cascade studies were performed to investigate the influence of GIP on β-cell fate. Using the well-differentiated β-cell line, INS-1, growth factor properties of GIP were examined (Trumper et al. 2001). Detection of 5-bromo-deoxyuridine (BrdU) incorporation in GIP and glucose stimulated INS-1 cells using an ELISA revealed increases in the rate of DNA synthesis in INS-1 cells compared to cells stimulated with glucose only confirming mitogenic effects of GIP. Furthermore, activation of the major mitogenic signaling modules PKA/CREB, p44/p42 MAPK and PI3K/PKB by GIP were detected (Ehses et al. 2002). MAPK signaling modules are defined as cellular regulators of processes like differentiation and proliferation. The ERK1/2 is belonging to these signaling modules. GIP was shown to regulate all known kinases of the ERK1/2 module using two different β-cell lines for this experiment, the GIP transfected CHO-K1 cell line and the INS-1 cell line (Ehses et al. 2002). The identification of the cAMP-PKA-Rap1-ERK pathway demonstrated an important influence of GIP on cellular proliferation/differentiation and/or gene transcription events in pancreatic β-cells (Ehses et al. 2002). In another study (D-Ala$^2$)-GIP, a potent DPP-4 resistant GIP agonist, was utilized to examine effects on proliferation and apoptosis (Maida et al. 2009). Treatment of C57BL/6 mice with (D-Ala$^2$)-GIP before and concomitant with streptozotocin (STZ) administration tended to promote β-cell survival. Reduction of islet apoptosis was distinctly visible. Therefore it was concluded that GIPR activation is able to couple to proliferative and anti-apoptotic pathways in murine β-cells (Maida et al. 2009). A detailed analysis of the DPP-4 resistant GIP analog D-GIP$_{1-30}$ revealed

markedly beneficial effects on β-cells (Widenmaier et al. 2010). The experiments to examine the effects of D-$GIP_{1-30}$ were carried out in *in vitro* studies with INS-1 cells and in *in vivo* studies using different rodent models including STZ treated rats, Vancouver diabetic fatty (VDF) rats and obese Zucker diabetic fatty (ZDF) rats. The onset of cell death in Staurosporine (STS; an activator of the mitochondria-derived apoptotic pathways) treated INS-1 cells was significantly suppressed by D-$GIP_{1-30}$ compared to the natural occurring form of GIP, $GIP_{1-42}$. All *in vivo* studies showed improvement of glucose tolerance and insulin response when the rodents were treated with D-$GIP_{1-30}$. Histological analyses revealed a significantly larger β-cell area due to reduced apoptosis and maintenance of structural islet integrity (Widenmaier et al. 2010). Additionally, other anti-apoptotic actions of GIP were found. In one study caspase-3 activity was demonstrated to be reversed by GIP in INS-1 cells by inhibition of the cell death-associated p38 MAPK pathway after treatment with wortmannin, an inhibitor of the PI3K/PKB signal cascade (Ehses et al. 2003). GIP appeared to be able to regulate both cell growth and cell death via dynamic control of the p38 MAPK module. Another experiment showed that GIP decreases nuclear Foxo1 interaction with the Foxo1 response element in INS-1 β-cells, a process resulting in downregulation of the bax gene during glucolipotoxicity-induced apoptosis (Kim et al. 2005). These results could also be confirmed by *in vivo* experiments in VDF rats. Likewise, it was shown that the anti-apoptotic effects of GIP in β-cells are partially mediated through transcriptional regulation of Bcl-2, an apoptosis regulating gene product that facilitates cell survival (Kim et al. 2008). Widenmeier et al. examined how GIP is involved in the interactions between cytoplasmic Bcl-2 family members and the mitochondria in INS-1 cells during apoptosis. After treating the cells with STS, an activator of the mitochondria-derived apoptotic pathways, a significantly reduced stimulation of these pathways was observed following GIP application. Pro-survival responses to GIP during STS treatment could be explained by GIP provided β-cell survival via Akt-dependent suppression of p38 MAPK and JNK (Widenmaier et al. 2009).

However, studies of pro-proliferative and anti-apoptotic effects of GIP *in vivo* are very limited. Findings of increased relative β-cell volume in $GIPR^{-/-}$ mice (Pamir et al. 2003) and no apparent differences in islet size and number of DIRKO mice (Hansotia et al. 2004) are inconsistent with previous results in β-cell lines or rodent models treated with an GIP analog. Thus further *in vivo* investigations are needed to define the role of GIP for β-cell fate.

#### 2.2.3.3 Adipose tissue

Expression of functional GIPRs on rat adipocytes and 3T3-L1 cells (preadipocyte cell line) (Yip et al. 1998) as well as the occurrence of GIPR mRNA in adipose tissue (Usdin et al. 1993) suggested a function of GIP in fat metabolism. Also, fat ingestion is a potent stimulator of GIP secretion in humans (Falko et al. 1975), wherefore the anabolic effects of GIP related to fat metabolism were examined. Dose-dependent enhancement of lipoprotein lipase activity by GIP (Eckel et al. 1979) as well as prevention of obesity due to inhibited GIPR signaling indicate that GIP is a potent factor for lipogenesis (Isken et al. 2008; Miyawaki et al. 2002). Interestingly, lipolytic effects of GIP were discovered, too (McIntosh et al. 1999; Yip et al. 1998). A different role of GIP under fasting conditions and in the presence of circulating nutrients might explain these contrary actions (McIntosh et al. 2009).

#### 2.2.3.4 Bone

The presence of GIPR mRNA and protein in normal bone and osteoclast-like cell lines (SaSo2 and MG63) offered an indication of the regulatory effects of GIP on bone metabolism (Bollag et al. 2000; Bollag et al. 2001; Zhong et al. 2007). Stimulation of osteoblast-like cells (SaOS2) with GIP revealed anabolic effects of presumptive osteoblasts (Bollag et al. 2000). Zhong et al. analyzed the effect of GIP on osteoclast function and detected its role as a mediator in postprandial suppression of skeletal resorption (Zhong et al. 2007). *In vivo* studies confirmed the physiological relevance of GIP related to bone metabolism. Altered bone microarchitecture as well as reduced bone mass and size in $GIPR^{-/-}$ mice were detected (Tsukiyama et al. 2006; Xie et al. 2005)

Correspondingly, repressive effects on bone resorption and stimulative effects on bone formation were shown in GIP transgenic mice (Xie et al. 2007).

#### 2.2.3.5 Nervous system

In the central nervous system GIPR expression was substantiated in the cerebral cortex, hippocampus and olfactory bulb (Usdin et al. 1993). Interestingly, GIP mRNA could not be detected in the brain by in situ hybridization and PCR indicating the presence of a novel peptide in the brain (Usdin et al. 1993). Subsequent studies revealed stimulating effects of GIP on proliferation of hippocampal progenitor cells (Nyberg et al. 2005). Furthermore, $GIPR^{-/-}$ mice exhibited lower numbers of newborn cells in the dentate gyrus regions of the hippocampus (Nyberg et al. 2005) indicating a regulatory function of GIP in the central nervous system. Expression analyses of GIPR and GIP in the peripheral nervous system and the spinal cord revealed a widespread neuronal GIP expression (Buhren et al. 2009). An impaired peripheral axonal regeneration in $GIPR^{-/-}$ mice pointed towards beneficial effects of GIP/GIPR signaling with respect to nerve regeneration (Buhren et al. 2009).

## 2.3 Glucagon-like peptide-1 (GLP-1)

### 2.3.1 Secretion, synthesis and degradation

Mammalian proglucagon genes encode not only glucagon but also two other peptides named glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2), based on 50% sequence homology to glucagon (Bell et al. 1983). An insulinotropic action was detected for GLP-1 but not for GLP-2 (Schmidt et al. 1985). GLP-1 exists in two biological active forms, GLP-1 (7-36) and (7-37), whereby the (7-36)-amide is the mainly circulating form (Orskov et al. 1994). GLP-1 is a tissue-specific posttranslational proteolytic product of the proglucagon gene that is derived from the intestinal endocrine L-cells in the distal ileum and colon (Eissele et al. 1992; Mojsov et al. 1986). Various stimulators like nutrients, neural and endocrine factors mediate GLP-1 secretion. Carbohydrates and fat are the mainly stimulators for GLP-1 release

(Brubaker 2006). The half-life of GLP-1 is less than 2 minutes due to a fast degradation by the enzyme DPP-4 (Deacon et al. 1995).

### 2.3.2 GLP-1 receptor and signal transduction

Effects mediated by GLP-1 occur after binding to its specific receptor, the GLP-1 receptor (GLP-1R). This receptor is like the GIPR a member of the seven transmembrane-spanning, heterotrimeric G-protein-coupled receptor family (Mayo et al. 2003). GLP-1R expression was identified in several organs including the β-cells in the islets of Langerhans within the pancreas (Tornehave et al. 2008) as well as lung, heart, kidney, stomach, intestine, pituitary, skin, nodose ganglion neurons of the vagus nerve and several regions of the CNS (Baggio et al. 2007). After binding to the N-terminal extracellular region efficient coupling to G-proteins is mediated by distinct domains in the third intracellular loop. Increases of intracellular $Ca^{2+}$, activation of adenylate cyclase and phospholipase C and activation of PKA, PKC, PI3K, Epac2 and MAPK signal transduction pathways relay further signal transduction (Drucker et al. 1987; Holz et al. 1995; Thorens 1992; Wheeler et al. 1993).

### 2.3.3 Biological actions of GLP-1

Insulin secretion as well as insulin transcription and biosynthesis are stimulated via GLP-1 binding to its specific receptor (Baggio et al. 2007). An inhibitory effect of GLP-1 on glucagon secretion was observed (Creutzfeldt et al. 1996). Pro-proliferative and anti-apoptotic effects of GLP-1 on β-cells were demonstrated in several studies (Farilla et al. 2002; Farilla et al. 2003; Li et al. 2003; Maida et al. 2009), whereas GLP-1 revealed stronger influence on β-cell survival than GIP (Maida et al. 2009). Moreover, functions in other tissues were discovered. GLP-1 enhances cardiac function and mediates cardioprotection as well as neuroprotection and glucose uptake and storage in muscles (Drucker 2006; Nauck 2009). Reduction of glucose production in the liver, and gastric emptying as well as the effect of an appetite regulator could be added to the functions of GLP-1 (Nauck 2009).

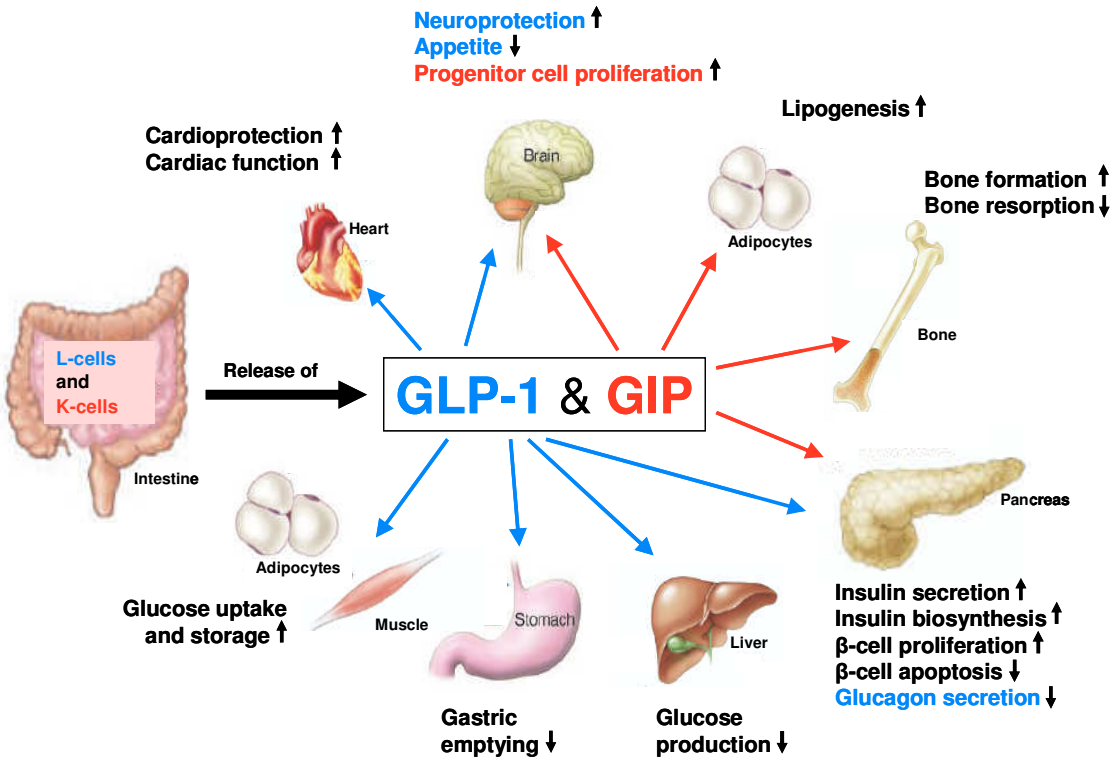


**Figure 2.1 Biological actions of GIP and GLP-1 (figure modified from Baggio et al. 2007 and Drucker 2007)**

## 2.4 The contribution of GIP and GLP-1 to type 2 diabetes mellitus

It has been shown that an oral glucose load resulted in higher insulin levels and thereby faster decline of blood glucose levels compared to an isoglycemic intravenous glucose load (Elrick et al. 1964; McIntyre et al. 1964). The incretin hormones GIP and GLP-1 were found to be responsible for this process. Thus the term “incretin effect” was coined for this observation. Approximately 60% of the total insulin secretion after food intake is mediated by the incretin hormones (Nauck et al. 1986a). Patients with type 2 diabetes mellitus exhibited no significant differences in C-peptide levels after oral and intravenous glucose administration, indicating a reduced incretin effect (Nauck et al. 1986b). Further investigations showed differences in secretion and insulinotropic action of GIP and GLP-1 in type 2 diabetic patients compared to healthy control subjects. Studies on GIP secretion in type 2 diabetic patients demonstrated normal secretion in most cases, but hypersecretion in some individuals (Ross et al.

1977; Vilsboll et al. 2001). While former studies showed a reduced GLP-1 secretion (Toft-Nielsen et al. 2001; Vilsboll et al. 2001), more recent studies disproved this finding showing normosecretion of GLP-1 in diabetic subjects (Vollmer et al. 2008). Studies on the insulinotropic action of both incretins revealed that the insulinotropic action of GIP was markedly impaired, while this of GLP-1 was preserved in type 2 diabetic patients (Nauck et al. 1993b). In the past it was hypothesized that due to changes in the level or defects in GIPR expression, the insulinotropic action of GIP is impaired in type 2 diabetic patients (Holst et al. 1997). Findings of a blunted insulin response to GIP in first-degree relatives of type 2 diabetes patients promoted the assumption of a defect GIPR in these subjects (Meier et al. 2001). Lynn et al. showed reduced GIPR mRNA expression in the pancreatic islets of ZDF rats (Lynn et al. 2001). Hyperglycemic partially pancreatectomized rats exhibited downregulated GLP-1 and GIPR expression providing a possible explanation for the impaired incretin effect in type 2 diabetes mellitus affected peoples (Xu et al. 2007). A meta-analysis of nine genome wide association studies revealed that GIPR variants influence glucose and insulin responses to an oral glucose challenge supporting the hypothesis that changes in GIPR play a role in the pathogenesis of type 2 diabetes mellitus (Nauck et al. 2004a; Saxena et al. 2010).

## 2.5 Incretins in diabetes research

To examine the role of the incretin hormones in general and their role in diabetic metabolism, several genetically modified rodent models have been established. In the following section these animal models are reviewed as well as further investigations on prolonging the action of GIP and/or GLP-1 as a therapeutic approach are summarized.

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

In 1999 Miyawaki et al. generated mice with a targeted mutation of the *GIPR* gene in order to investigate effects of the GIP/GIPR axis on glucose metabolism. The complete loss of GIPR function was controlled by stimulating isolated pancreatic islets of GIPR$^{-/-}$ mice with GIP. The isolated islets of

homozygous GIPR$^{-/-}$ mice showed no insulinotropic action following stimulation with glucose and GIP compared to controls (Miyawaki et al. 1999; Pamir et al. 2003). Histological abnormalities were found neither in the pancreas nor in the gastrointestinal tract or the adrenal gland (Miyawaki et al. 1999). In contrast, another study revealed an increased β-cell area (referred to total pancreatic area) of 45% and reduced staining intensity for insulin in immunohistochemically stained pancreatic sections of GIPR$^{-/-}$ mice (Pamir et al. 2003). Fasting glucose levels and blood glucose levels after intraperitoneal glucose injection were unaltered compared to controls, while blood glucose and plasma insulin levels after an oral glucose tolerance test were significantly elevated and reduced, respectively. (Miyawaki et al. 1999). The disruption of the GIP/GIPR axis led to reduced insulin gene transcription and protein biosynthesis and increased islet sensitivity to GLP-1 (Pamir et al. 2003).

Furthermore, GIPR$^{-/-}$ mice were protected from obesity compared to wild-type mice when fed a high-fat diet from 7 to 50 weeks of age and did not develop insulin resistance like obese control animals (Miyawaki et al. 2002). Also, diet-induced obesity was attenuated in GIPR$^{-/-}$ mice crossbred with a genetically obese mouse model (Lep$^{ob}$/Lep$^{ob}$ mice) compared to single-homozygous Lep$^{ob}$/Lep$^{ob}$ mice confirming that the protecting effects of GIP even occur in the absence of leptin, an adipokine that stimulates fat oxidation in peripheral tissues for body weight control (Miyawaki et al. 2002). Adiponectin presents another major adipokine. Three weeks of high-fat (HF) diet feeding in GIPR$^{-/-}$ mice caused significantly increased fat oxidation in skeletal muscles and significantly elevated adiponectin levels and GIPR$^{-/-}$ mice did not put on body weight compared to wild-type mice fed the HF diet (Naitoh et al. 2008). In contrast, GIPR$^{-/-}$ mice exhibited no differences in plasma adiponectin levels after 20 weeks of HF diet compared to GIPR$^{-/-}$ mice fed a control diet (Hansotia et al. 2007) concluding that GIPR signal interruption modulates adiponectin levels in the early stage of obesity induced by a HF diet (Naitoh et al. 2008). Recently, GIPR$^{-/-}$ mice were used to study whether the effects of GIP and estrogen on body weight and fat mass could be linked (Isken et al. 2008). Ovariectomized (OVX) GIPR$^{-/-}$ mice and wild-type mice were compared with respect to body

composition, energy metabolism and hypothalamic neurocircuitry. OVX GIPR$^{-/-}$ were protected from obesity going along with reduced cumulative food intake while OVX wild-type mice exhibited a significant higher body weight gain compared to OVX GIPR$^{-/-}$ and sham OVX mice. Interestingly, previous studies detected no influence of GIP on food intake behavior (Miyawaki et al. 2002) suggesting that the GIP/GIPR axis might be relevant to mediate feeding under changed physiological conditions like estrogen decline (Isken et al. 2008). Furthermore, GIP appeared to have age-associated effects on body composition. GIPR$^{-/-}$ mice showed improved insulin sensitivity and physical activity concomitantly to reduced fat mass compared to wild-type mice (Yamada et al. 2007). Low and high glycemic index (GI) diets were fed to GIPR$^{-/-}$ mice to evaluate the influence of GIP and age to body composition (Isken et al. 2009). Male wild-type (C57BL/6J) and GIPR$^{-/-}$ mice were fed the high-carbohydrate diet which differed only in its GI over 20 to 26 weeks starting at the age of 16 weeks (young-adult) and 44 weeks (aged). GIPR$^{-/-}$ genotype could not be linked to differences in body weight gain, body fat and hepatic triacylglycerol content. An increased cumulative energy intake was only observed in aged wild-type mice on high-GI diet while GIPR$^{-/-}$ mice were protected from this effect. GIPR deficiency improved insulin sensitivity in aged mice fed a high-GI diet. In agreement to this observation aged GIPR$^{-/-}$ mice on high-GI diet exhibited improved carbohydrate oxidation. Furthermore, an increased locomotor activity during the dark phase was referred to the aged GIPR$^{-/-}$ genotype in combination with the high-GI diet. These observations suggested that a blockage of GIPR signaling might be advantageous for aged humans consuming high-GI diets (Isken et al. 2009).

Effects of GIP on bone remodeling were examined using GIPR$^{-/-}$ mice. Lower bone mass and alterations in bone architecture in the knockout mice compared to wild-type animals indicated an anabolic effect of GIP on bone mass and quality (Xie et al. 2005). In this context, mice with a disruptive GIPR signaling were examined to evaluate the role of GIP on calcium deposition in the bone (Tsukiyama et al. 2006). After nutrient ingestion GIPR$^{-/-}$ showed significantly

elevated plasma calcium levels compared to controls concluding effects of GIP on the efficient storage of ingested calcium (Tsukiyama et al. 2006).

### 2.5.2 GLP-1 receptor knockout mice (GLP-1R$^{-/-}$)

GLP-1R$^{-/-}$ mice were generated to examine the physiological importance of GLP-1 for regulation of satiety and blood glucose (Scrocchi et al. 1996). GLP-1R$^{-/-}$ mice exhibited only mild fasting hyperglycemia but impaired glucose tolerance and reduced insulin secretion after an oral glucose challenge (Hansotia et al. 2004; Preitner et al. 2004; Scrocchi et al. 1996). In contrast to GIPR$^{-/-}$ mice, abnormal glycemic excursions were visible during an intraperitoneal glucose tolerance test (Scrocchi et al. 1996) as well as significantly reduced peak insulin levels compared to wild-type mice (Preitner et al. 2004). Defective glucose-induced insulin secretion and reduced *proinsulin* gene expression indicated multiple β-cell abnormalities (Pederson et al. 1998). However, total β-cell volume was unaltered in GLP-1R$^{-/-}$ mice while the distribution of the endocrine fraction was markedly changed. α-Cells were located more centrally and more medium-sized islets were detected in GLP-1R$^{-/-}$ mice indicating an influence of GLP-1 on islet topography (Ling et al. 2001). Body weight and food intake did not differ from controls, although GLP-1 is evidentially a potent inhibitor of food intake (Scrocchi et al. 1996). Enhanced glucose-stimulated GIP levels in GLP-1R$^{-/-}$ mice and increased GIP stimulated insulin release from the perfused pancreas or isolated islets of GLP-1R$^{-/-}$ mice revealed compensatory mechanisms in the enteroinsular axis (Flamez et al. 1999; Pederson et al. 1998). Additionally, the importance of endogenous GLP-1 for the control of bone metabolism was examined using GLP-1R$^{-/-}$ mice (Yamada et al. 2008). A diminished cortical bone mass and reduce bone stability in the tibia of GLP-1R$^{-/-}$ mice was detected. Increases of osteoclast numbers and bone resorption were stimulated indirectly through a modulation of the calcitonin expression by GLP-1. Thus, an essential role of GLP-1 for the regulation of bone resorption was substantiated (Yamada et al. 2008). Hyperinsulinemia and exercise are metabolic perturbations regulating the glucose flux insulin-dependently and insulin–independently, respectively. The

different glucoregulatory abilities of GLP-1 were examined using the GLP-1R$^{-/-}$ mouse model (Ayala et al. 2009). A hyperinsulinemic-euglycemic clamp experiment suggested an essential role for GLP-1 during the postabsorptive phase regulating glucose homeostasis insulin-dependently. However, GLP-1R$^{-/-}$ mice became hyperglycemic during exercise due to a failing suppression of hepatic endogenous glucose production and hepatic glycogen accumulation, concluding that GLP-1 regulates muscle glucose uptake and hepatic glucose flux also independent of its role as an incretin (Ayala et al. 2009).

### 2.5.3 Double incretin receptor knockout mice (DIRKO)

Mice lacking both GIPR and GLP-1R allowed to study physiological consequences of a complete loss of GIP and GLP-1 action on glucose homeostasis (Hansotia et al. 2004). Glucose tolerance following an oral glucose challenge was significantly more impaired compared to single incretin knockout (SIRKO) mice. Also, insulin levels were decreased after an oral glucose challenge and glycemic excursion following intraperitoneal glucose administration was abnormal while fasting glucose levels were not significantly elevated in DIRKO mice compared to controls. Histological analyses revealed no difference in the number and size of DIRKO versus wild-type islets. The administration of DPP-4 inhibitors confirmed GIPR and GLP-1R as principal targets for glucose-lowering actions of the inhibitors. Despite the absence of both incretin receptors DIRKO mice developed only a mild phenotype of glucose intolerance and reduced insulin secretion (Hansotia et al. 2004; Preitner et al. 2004).

Diet studies in DIRKO mice elucidated the influence of the lack of incretin signaling on insulin action (Ayala et al. 2008). DIRKO mice and control animals were fed a regular chow or high-fat (HF) diet for 12 weeks starting at 3 weeks of age. DIRKO mice exhibited decreased body weight gain and reduced muscle mass after being fed the chow diet. While control animals fed a HF diet showed increased body weight gain, this diet did not affect the weight gain of DIRKO mice. Furthermore, DIRKO mice showed increased energy expenditure and activity. Insulin action of both groups was evaluated by a hyperinsulinemic

euglycemic clamp study discovering enhanced whole-body insulin action in DIRKO mice and protection from HF diet induced insulin resistance. Also, glucose uptake in cardiac and muscle tissues was enhanced in DIRKO mice. These observations pointed towards the importance of incretins in regulating glucose homeostastis beyond the pancreas (Ayala et al. 2008).

### 2.5.4 Mice expressing a dominant-negative GIPR (GIPR$^{dn}$ transgenic mice)

GIPR$^{dn}$ transgenic mice represent another mouse model to examine the role of the GIP/GIPR axis. In contrast to models with a complete loss of GIP action, the reduction of GIPR signaling in GIPR$^{dn}$ transgenic mice led to a severe diabetic phenotype just before weaning (Herbach et al. 2005). Severe glucosuria, elevated serum glucose concentrations, reduced insulin levels and increased glucagon levels characterized the diabetic phenotype of GIPR$^{dn}$ transgenic mice. Additionally, marked changes in islet morphology were detected. Islet profiles were much smaller in size and number and revealed an altered composition. Total β-cell volume as well as total volume of isolated β-cells (a marker for islet neogenesis) were markedly reduced while the total volume of non-β-cells was increased indicating GIP as an important factor for postnatal islet and β-cell development (Herbach et al. 2005).

As special diets are discussed to improve glucose metabolism in diabetic patients, GIPR$^{dn}$ transgenic mice were fed a high-fiber/low-disaccharide diet to mimic new diet set ups (Herbach et al. 2008). GIPR$^{dn}$ transgenic mice fed a high-fiber diet showed reduced non-fasting blood glucose levels and a tendency towards declined fasting blood glucose levels compared to transgenic mice fed a regular diet. Also, β-cell function and insulin sensitivity were significantly improved. High-fiber diet positively affected survival rates of GIPR$^{dn}$ transgenic mice. Notably, improved β-cell function and glucose tolerance indicated a beneficial effect of high-fiber/low-disaccharide diet in GIPR$^{dn}$ transgenic mice (Herbach et al. 2008).

Nephropathy is one of the most common epiphenomena of diabetes. Age-related kidney lesions associated with a diabetic phenotype were examined in

GIPR$^{dn}$ transgenic mice at the age of 3, 8, 20 and 28 weeks (Herbach et al. 2009). Early renal changes like podocyte hypertrophy, reduced numerical volume density of podocytes in glomeruli and homogenous thickening of the glomerular basement membrane were detected by qualitative and quantitative morphological analyses. GIPR$^{dn}$ transgenic mice showed further renal and glomerular hypertrophy as well as mesangial expansion and matrix accumulation. At 28 weeks of age advanced glomerulosclerosis going along with tubointerstital lesions and proteinuria were observed. Hyperglycemia and renal alterations correlated obviously in GIPR$^{dn}$ transgenic mice indicating these mice as an appropriate model to study mechanisms involved in the onset and progression of diabetic nephropathy (Herbach et al. 2009).

### 2.5.5 GIP transgenic mice

The role of GIP as an anabolic factor for bone metabolism was already analyzed in GIPR$^{-/-}$ mice (Xie et al. 2005). Generating mice overexpressing GIP allowed to confirm previous results of GIPR$^{-/-}$ mice and to further define the impact of GIP on the skeleton. Data on bone density and histomorphometry were collected as well as bone markers for formation and resorption were determined concluding that an increased GIP signaling induces gain in bone mass (Xie et al. 2007) and prevents age-induced bone loss (Ding et al. 2008).
As GIP synthesis was detected in the hippocampal dentate gyrus GIP transgenic mice were utilized to study the effects of GIP on behavior (Ding et al. 2006). A number of behavioral tests were performed and GIP appeared to modulate the regulation of locomotor activity and exploration (Ding et al. 2006).

### 2.5.6 Prolonging the action of GIP, GLP-1 or both

The half-live of native GLP-1 and GIP is very short due to their fast degradation by the enzyme DPP-4 (Deacon et al. 1995; Deacon et al. 2000) Therefore strategies to prolong incretin hormone action were developed in order to use their beneficial effects on β-cell function for therapeutic purposes. In general, there are three different strategies to prolong incretin action:

1. N-terminal modification of GIP/GLP-1 to convey DPP-4 resistance
2. C-terminal modification of GIP/GLP to circumvent renal filtration
3. DPP-4 inhibition

To establish DPP-4 resistant analogs amino acid substitutions were induced. Although the most modifications including amino acid substitution generate DPP-4 resistance, the biological activity and receptor stimulation can differ markedly (Green et al. 2007). Several GIP analogs with modifications at $Tyr^1$ and $Ala^2$ were developed (Gault et al. 2002a; Gault et al. 2003a; Hinke et al. 2002; Irwin et al. 2009b; O'Harte et al. 1999; O'Harte et al. 2002). Comparing the activity of the analogs with the native GIP hormone, $Tyr^1$-modified forms were completely DPP-4 resistant and showed enhanced biological activity while $Ala^2$-modified analogs were only partially DPP-4 resistant and less efficient (Irwin et al. 2009a). Amino acid substitution within the GLP-1 sequence at $Ala^8$ seemed to be superior to modifications at $His^7$ (Green et al. 2006; Green et al. 2007; O'Harte et al. 2000). Weak agonists or even receptor antagonists were established by amino acid substitutions at $Glu^9$ for GLP-1 and $Glu^3$ for GIP, respectively (Gault et al. 2007; Green et al. 2004; O'Harte et al. 2007). Even if DPP-4 resistance is an important approach to prolong the action of the incretins, renal filtration is not prohibited by N-terminal modifications meaning that the effective period is limited to four hours due to rapid kidney elimination (Green et al. 2007).

C-terminal modification of GIP and GLP-1 demonstrated a functional strategy to circumvent renal filtration. Fatty acid acylation and PEGylation (attachment of polyethylene glycol chains) are used to promote binding to plasma proteins to delay renal elemination. Several C-terminal modifications for both GIP (Gault et al. 2008; Irwin et al. 2005a; Irwin et al. 2005b; Irwin et al. 2006; Kerr et al. 2009) and GLP-1 (Chou et al. 1997; Lee et al. 2006; Rolin et al. 2002) were generated and tested in preclinical studies. Dual modification by N-terminal acylation and C-terminal PEGylation of GIP generated a DPP-4 resistant long-acting GIP analog with a comparable efficiency to native GIP (Salhanick et al. 2005). Accordingly, Liraglutide represents a long-acting GLP-1 analog generated by

dual modification which has already been tested in type 2 diabetes patients (Nauck et al. 2006; Vilsboll et al. 2007).

Degradation of GIP and GLP-1 by DPP-4 leads to formation of metabolites without insulinotropic potential. Thus, a blockage of the enzyme DPP-4 provides an attractive method to prolong the action of GIP and GLP-1. To test the efficiency of various DPP-4 inhibitors several preclinical studies with animal models for type 2 diabetes mellitus were carried out (Ahren et al. 2000; Balkan et al. 1999; Deacon et al. 2002; Duez et al. 2009; Liu et al. 2009). Studies in humans could confirm the beneficial effects of DPP-4 inhibitors on glucose metabolism (Ahren et al. 2002). Vildagliptin, Sitagliptin and Saxagliptin are only three examples of the numerous existing DPP-4 inhibitors (Ahren 2007a, 2007b, 2009).

Exendin-4 represents a naturally existing GLP-1 mimetic. It was isolated from the salivary gland of the Gila Monster lizard *Heloderma suspectum* demonstrating approximately 53% sequence homology to GLP-1 (Eng et al. 1992; Goke et al. 1993). In addition, it is not a substrate for DPP-4 making it interesting for therapeutic use (Keating 2005; Nielsen et al. 2003). A synthetic derived form of exendin-4, the so called exenatide (Byetta®), was the first incretin-based pharmaceutical reaching the market (Green et al. 2007). Recently, long-term effects of elevated steady-state exendin-4 levels were examined using gene therapy. A helper-dependent adenovirus (HDAd) served as a vector for expressing the exendin-4 peptide (Samson et al. 2008). After 8 weeks of high-fat diet feeding mice were treated with the HDAd. Glucose homeostasis was positively influenced, while insulin levels did not increase. Additional changes on insulin sensitivity, adipokine levels, hepatic steatosis and energy expenditure gave impetus towards further investigations (Samson et al. 2008).

## 2.6 The pig as an animal model in research

Although rodent models are predominantly used in research, pigs provide great advantages for the translation of data to humans, due to many physiological and anatomical similarities. Skin and subcutaneous tissues only differ slightly

from that of humans, allowing comparison of kinetics and dynamics after injection of drugs (Meyer 1996). Sparse body hair, similar dermal-epidermal thickness ratio as well as similar size, orientation and distribution of blood vessels are only some analogies (Meyer 1996; Sullivan et al. 2001). Sullivan et al. compared studies about wound healing in various animal models leading to the conclusion that the pig correlates well to human (Sullivan et al. 2001). Investigations of intestinal absorption and nutrient requirements are also interesting for many fields of research. Therefore it is advantageous that pigs are omnivorous and the digestive effectiveness is comparable to humans (Miller et al. 1987). Constant training of pigs allows to perform most experimental procedures in conscious and unstressed animals (Larsen et al. 2004). Another advantage of pigs is the possibility to obtain larger amounts of blood or tissue samples compared to rodents. Placement of central venous catheters either temporary or permanent (Moritz et al. 1989; Smith et al. 1991) allows circumvention of repeated stressful blood sampling.

There are different opportunities to generate an adequate model for human diseases. Induction of random gene mutations by chemicals, treatment with drugs for targeted reduction of organ functions and generation of genetically modified animals can be discriminated. Also, spontaneous onset of diseases in pigs similar to humans is used as a basis for comparison.

### 2.6.1 Genetically modified pigs for translational research

The translation of scientific outcomes into clinical applicable strategies plays an important role in today's research and coined the term "translational medicine" (Wehling 2008). Appropriate animal models are needed to elucidate pathogenesis and pharmacological strategies in human diseases. Genetic modification is a useful method to generate animal models for a specific disease. Different methods available for genetic modification in pigs may lead to overexpression, impairment or loss of function of a gene allowing to evaluate the effects on the organism. In the following some highlights of genetically modified pigs for common diseases are presented.

#### 2.6.1.1 Cardiovascular disease

Heart diseases are a worldwide common problem. Nitric oxide plays an important role in multiple pathways among other things in cardiac development, angiogenesis and wound healing. The endothelial nitric oxide synthase (eNOS) was identified regulating vascular function, vascular structure and blood pressure. To receive information on its function transferable to humans transgenic pigs overexpressing eNOS were generated by somatic cell nuclear transfer (SCNT). Yucatan minipigs served as models for this study and appeared to be useful to clarify the role of eNOS for atherosclerosis, hypertension and wound healing (Hao et al. 2006).

#### 2.6.1.2 Cerebral diseases

Huntington disease (HD) is caused by a dominant mutation in a gene called *Huntingtin*. HD presents an autosomal inheritable progressive neuro-degenerative disorder, whereby both homozygous as well as heterozygous phenotypes lead to an onset of this disease commonly in the middle age. Involuntary, jerky movements as well as cognitive and psychiatric symptoms are typical disturbances of the affected peoples. As the endogenous mouse HD gene appeared to be not as pathogenic as in humans, pigs provided an attractive alternative. Characterization of the porcine HD gene revealed more similarity to human HD genes than those of rodents indicating pigs as a valuable model for physiological and pharmacotherapeutical studies of Huntington disease (Matsuyama et al. 2000).

Another neurodegenerative process in the brain is responsible for the onset of Alzheimer disease (AD). Although AD is delineated as multifactorial disturbance, AD onset is also elicited by an autosomal dominant mutation disorder. Mutations of the *amyloid precursor protein* (*APP*) gene and the *presinilin* genes (*PSEN*) resulted in higher production levels of the APP, whereby increases in APP expression cause neuritic plaques and formation of neurofibillary tangles. Discrepancies in disease development of mice compared to humans postulated a more homologous model. Göttingen minipigs expressing a specific splice variant of human APP, that carries an AD-causing

dominant mutation (Swedish mutation) were generated by so called handmade cloning (Kragh et al. 2009).

#### 2.6.1.3 Ophthalmic disease

In 1997 Petters et al. designed a pig model for retinitis pigmentosa (RP) representing pioneers in generation of transgenic pigs. RP is an incurable degenerative disease. Although various mutations in RP patients were identified, a common pattern of loosing eyesight was recognized. An early development of night vision loss caused by rod photoreceptor degeneration associated with a progressive loss of cone photoreceptors finally leads to blindness. As the mutation *Pro374Leu* is characterized causing severe rod degeneration it was chosen for generating a transgenic pig model. Pronuclear microinjection served as method for producing transgenic pigs. Pigs exhibited a rapid severe loss of rod photoreceptors in combination with moderate early cone photoreceptor degeneration deteriorating over time (Petters et al. 1997).

#### 2.6.1.4 Motor neuron disease

Spinal muscle atrophy (SMA) occurs in three different severity grades and is the most common inherited motor neuron disease. Degeneration of anterior horn cells of the spinal cord resulting in muscle atrophy distinguishes this autosomal recessive disorder. The loss of function of survival motor neuron-1 (SMN-1) was identified to be responsible for SMA onset. Identification and cloning of the porcine *SMN-1* gene was carried out. Notably, the absence of the SMN-1 protein in primary fibroblasts of a SMA type 1 patient was compensated by transfection with porcine SMN-1. Human and porcine SMN are consistent in sequence, localization and expression indicating that a transgenic swine model is feasible for further investigations of SMA (Lorson et al. 2008).

#### 2.6.1.5 Cystic fibrosis

Cystic fibrosis (CF) is a common autosomal recessive disease, which affects wide parts of the organism including intestine, lung, liver, gallbladder and male genital tract. The mutated *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene was discovered to be the trigger for degenerations. To obtain a

pig model for cystic fibrosis, the *CFTR* gene was disrupted by homologous recombination in porcine fibroblasts and transferred by somatic cell nuclear transfer (SCNT). Meconium ileus and pancreatic destruction occurred first in $CFTR^{-/-}$ piglets similar to human neonates as well as additional alterations of gallbladder and bile ducts. Lung and vas deferens of the testis exhibited no abnormalities similar to humans at a comparable age (Rogers et al. 2008).

#### 2.6.1.6 Diabetes

Genetic mutation of the *hepatocyte nuclear factor (HNF)-1α* was discovered to be responsible for the development of type 3 maturity-onset diabetes of the young (MODY3), an autosomal dominant inherited disease. Umeyama et al. generated transgenic pigs expressing the human *HNF-1α* mutant gene imitating pathophysiological characteristics of MODY3 in humans. Intracytoplasmic sperm injection (ICSI) combined with somatic cell nuclear transfer (SCNT) served as method for transgenic pig production. Piglets exhibited markedly elevated non-fasting blood glucose levels and a disturbed oral glucose tolerance. Altered morphology of glomerular structures and of islets of Langerhans in the pancreas were demonstrated in immunohistochemically stained sections. However, high mortality rate and early death during the neonatal period of the transgenic-cloned pigs require further studies to improve postnatal survival (Umeyama et al. 2009).

### 2.6.2 Pigs as models in type 2 diabetes mellitus research

Currently, the use of pigs in biomedical research is relatively small compared to the fraction of rodentiae (mice, rats) and lagomorphae (rabbits) (BMELV 2009). However, similarities to humans related to cardiovascular system and pancreas make them an excellent tool for diabetes research. Size, shape and position of the pig pancreas are very similar to that of humans as well as blood supply of endocrine and exocrine tissues (Murakami et al. 1997). Although the islet structure of young pigs is more diffuse than in adult humans, changes in islet structure with increasing age make them more comparable to adult humans (Jay et al. 1999; Ulrichs et al. 1995; van Deijnen et al. 1992; Wieczorek et al. 1998). α-Cells were predominantly detected in the dorsal pancreas both in the

core and in the periphery of the islets while pancreatic polypeptide (pp) containing cells were found almost exclusively in the ventral pancreas. This cell dispersion is true for both humans and pigs (Jay et al. 1999; Orci et al. 1979; Rahier et al. 1981; Stefan et al. 1983; Wieczorek et al. 1998). β-Cells represent the major part of endocrine cells in the ventral and dorsal pancreas of the pig whereas in humans pp-cells were shown to be the major endocrine fraction in the ventral pancreas (Jay et al. 1999; Orci et al. 1979; Rahier et al. 1981; Stefan et al. 1983; Wieczorek et al. 1998). Nevertheless, the β-cell content of the porcine endocrine tissue ranges between 60 to 80%, therefore resembling the human β-cell content (Larsen et al. 2004). Another similarity between humans and pigs is the main expression of islet amyloid polypeptide (IAPP) in β-cells (Lukinius et al. 1996). However, a dissimilarity between the human and pig IAPP sequence is present and only humans are prone to the formation of pancreatic amyloid (Larsen et al. 2004). Porcine insulin and GIP sequence are very similar to this of humans allowing their use in treatment and studies (Bromberg et al. 2006; Jornvall et al. 1981; Moody et al. 1984).

Some pig strains commonly used as models for type 2 diabetes mellitus are listed below. Minipigs are often preferred due to smaller size and less expenses in housing.

#### 2.6.2.1 Yucatan Minipigs

Two lines of Yucatan minipigs with either impaired glucose tolerance (so-called “low K”) or enhanced glucose tolerance (so-called “high K”) were produced by selective breeding (Phillips et al. 1979; Phillips et al. 1982). Minipigs with an impaired glucose tolerance showed reduced serum insulin levels, although storage and synthesis of insulin were intact. An altered pancreatic receptor response seemed to be responsible for glucose intolerance. However, fhe F7 generation of the low-K line exhibited no more glucose intolerance and therefore these pigs are currently not available anymore.

Furthermore, chemicals were used to generate diabetic Yucatan miniature pigs. Treatment with alloxan led to impaired glucose tolerance caused by partial β-cell damage, whereby feeding of a high-fat/high-cholesterol diet was used to provoke dyslipidemia. Increased atherosclerosis was discovered in diabetic

dyslipidemic minipigs. Atorvastatin, a potent drug for the treatment of hypercholesterinemia protected diabetic minipigs from atherosclerosis (Dixon et al. 2002).

Alloxan induced diabetic Yucatan minipigs were furthermore used to examine microangiopathies associated with diabetes like diabetic retinopathy. As in former studies dyslipidemia induced by a high-fat diet caused macrovascular disorders in Yucatan minipigs, it should be clarified whether microvascular changes are also associated with diabetes. Retinal capillary basement membrane thickening was detected making this model interesting for early intervention and treatment studies for diabetic retinopathy (Hainsworth et al. 2002).

Overfeeding of Yucatan minipigs led to obesity and was associated with lower insulin sensitivity compared to control-fed pigs (Sebert et al. 2005). Administration of a high-calorie diet before sexual maturation should give information about cellular and metabolic modifications and about IGF-1 levels and insulin sensitivity related to childhood obesity (Sebert et al. 2005).

#### 2.6.2.2 Sinclair minipigs

This pig strain served as a model for diabetes associated with dyslipidemia. Alloxan treatment was utilized to destroy insulin producing β-cells implicating diabetes. In addition to alloxan application feeding of a high-fat and high-cholesterol diet caused dyslipidemia in these diabetic minipigs and allowed to study peripheral vascular and coronary artery diseases. Diabetic minipigs exhibited enhanced contractile tension oscillations in coronary arteries and impaired endothelium-dependent relaxation compared to controls. Fatty streak formation in diabetic minipigs was markedly increased (Dixon et al. 1999).

#### 2.6.2.3 Göttingen minipigs

The metabolic syndrome describes risk factors associated with the development of type 2 diabetes. Decreased insulin sensitivity, enhanced insulin secretion as well as hypertension, dyslipidemia and microvascular complications characterize the metabolic syndrome, a typical prediabetic period. Female Göttingen minipigs fed a high-fat/high-energy diet exhibited some of the

metabolic disturbances observed in obese patients like elevated triglyceride levels and significantly higher area under the insulin curve after an intravenous glucose tolerance test (Johansen et al. 2001). Male Göttingen minipigs fed with a high-fat/high-energy diet for 3 months also developed slight hyperglycemia and compensatory hyperinsulinemia. Experiments related to insulin resistance in type 2 diabetic patients could be performed in this animal model (Larsen et al. 2002b).

As insulin secretion and action are impaired in type 2 diabetic patients and pulsatile insulin secretion was found to be important for insulin effects in the liver, muscle and adipose tissue, Göttingen minipigs were also used to study pulsatile insulin action to gain insight into the pathogenesis of type 2 diabetes mellitus. Larsen et al. evaluated the minipigs being a good model for further studies related to insulin pulsatility due to their similarities of insulin kinetics to humans and advantages in sampling compared to other species (Larsen et al. 2002a). After the treatment with nicotinamide (NIA) in combination with streptozotocin (STZ) Göttingen minipigs developed mild diabetes characterized by a reduction of β-cell mass as observed in type 2 diabetic patients. Insulin pulsatility studies were carried out to obtain information, whether disturbed pulsatile insulin secretion in type 2 diabetes patients is linked to reduced β-cell mass. Diabetic Göttingen minipigs exhibited normal insulin pulsatility concluding other mechanisms to be responsible causing the impaired insulin pulsatility seen in human type 2 diabetes patients (Larsen et al. 2003a).

Obese and obese-STZ treated Göttingen minipigs were used to investigate whether impaired insulin pulsatility as an early marker of β-cell dysfunction is only detectable when obesity and reduced β-cell mass are present concomitantly. Glucose-entrained insulin secretion was significantly reduced in obese-STZ minipigs while obese minipigs without STZ treatment showed no marked differences compared to controls. Accordingly, the coexistence of obesity and reduced β-cell mass in the Göttingen minipig caused further deterioration in β-cell function demonstrated by changes in insulin pulsatility. Due to this fact it was suggested that abnormalities in insulin pulsatility are a sensitive marker for β-cell dysfunction (Larsen et al. 2005).

The NIA-STZ treated Göttingen minipig presented also a feasible model for therapy studies. Effects of dipepitdyl peptidase-4 (DPP-4) inhibitors that protect the incretins GLP-1 and GIP from fast degradation by the enzyme DPP-4, and NN221, a long-acting GLP-1 derivate, on glucose metabolism of these pigs were evaluated (Larsen et al. 2003b; Ribel et al. 2002).

#### 2.6.2.4 Yorkshire strains

Yorkshire pigs are normal sized domestic pigs commonly used for atherosclerosis research. It was discovered, that Yorkshire pigs with induced diabetes by STZ treatment, fed a special diet high in fat and cholesterol exhibited accelerated atherosclerosis (Gerrity et al. 2001). Increases in triglyceride levels, blood glucose as well as accumulation and proliferation of arterial smooth muscle cells emerged in this animal model. Further investigations of this pig model were performed studying the mechanisms of smooth muscle cell proliferation. Insulin-like growth factor-1 (IGF-1) levels were increased and growth-enhancing effects of IGF-1 on smooth muscle cells were potentiated by long-chain fatty acids. Thus, enhanced IGF-1 and triglyceride levels in type 2 diabetic patients appeared to be responsible for the accelerated SMC proliferation and lesion progression in the vascular system (Askari et al. 2002).

Furthermore, wound healing investigations were carried out in Yucatan pigs as diabetic patients often suffer from wound healing problems. The diabetic state was reached by treatment with STZ. Several 1.5 x 1.5 squares were outlined on the dorsal skin to create wounds. Adhesive polyurethane chambers were placed around the wounds and served for standardized conditions during wound healing in a liquid environment. Diabetic pigs exhibited delayed reepithelialization and reduced IGF-1 levels in the wounds. This model is feasible for investigations of pathophysiological wound healing procedures and experiments of new therapies (Velander et al. 2008).

Recently, the correlation between type 2 diabetic patients and obesity traits in Yorkshire pigs was investigated. Pig genes associated with fat deposition were analyzed. Mapping of genes associated with human type 2 diabetes mellitus revealed that only the *TCF7L2* was related with five fat traits of the pig.

Functional gene candidate analyses are needed to find out more about the function of these genes and to establish the pig as a useful model for obesity and type 2 diabetes mellitus (Du et al. 2009).

#### 2.6.2.5 Chinese Guizhou minipig

High-fat diet is known to induce insulin resistance and increases in triglyceride levels in pigs. Atherosclerotic fatty streaks were observed in rats and rabbits after being fed a high-sucrose diet. A high-fat/high-sucrose diet was fed to Chinese Guizhou minipigs to provoke both altered glucose and lipid metabolism as well as abnormalities in the vascular system. The diabetic phenotype of the pigs was characterized by reduced insulin sensitivity, glucose intolerance and a decrease of pancreatic β-cells. Furthermore, pigs showed an altered lipid metabolism and aortic lipid accumulations. Establishment of this new animal model might offer the possibility to study whether dietary and diabetogenic factors influence the onset of atherosclerosis (Xi et al. 2004).

In the pathophysiology of human atherosclerosis inflammatory factors like TNF-α, ILK-1β and ILK-6 are increased. STZ treatment induced diabetes in Chinese Guizhou minipigs. Fasting blood glucose levels were controlled regularly to initiate insulin therapy at glucose levels over 10 mmol/L. Measurement of inflammatory factors TNF-α, ILK-1β and ILK-6 in the aortic intima by Western blot and RT-PCR showed distinct increases indicating diabetes as a potent risk factor for atherosclerosis (Lu et al. 2007).

## 2.7 GIPR$^{dn}$ transgenic pigs

Although a great research effort is carried out on diabetes, the pathogenesis of type 2 diabetes is still not completely clarified. Pigs utilized in type 2 diabetes research up to now primarily served as models for cardiovascular diseases and dyslipidemia, phenomena associated with diabetes. As spontaneous onset of diabetes has not yet been reported in pigs, chemical induction of diabetes served as the method of choice, whereby physiological processes during development of type 2 diabetes mellitus are disregarded. Pigs expressing a dominant-negative GIPR imitate the situation of an impaired insulinotropic effect

of GIP observed not only in type 2 diabetic patients but also in ~50% of their first degree relatives (Nauck et al. 2004b). This first genetically modified pig model for type 2 diabetes mellitus provides the possibility to investigate the effects of an impaired insulinotropic action of GIP and to define its role in the pathogenesis of type 2 diabetes mellitus. Generation and initial physiological and morphological characterization of GIPR$^{dn}$ transgenic pigs was carried out previously (Renner 2008). In the following generation of and previous results on GIPR$^{dn}$ transgenic pigs are summarized.

### 2.7.1 Generation of GIPR$^{dn}$ transgenic pigs

The GIPR is a seven transmembrane domain heterotrimeric G-protein coupled receptor including three extracellular loops, three intracellular loops, an amino-terminal extracellular domain as well as an intracellular carboxyl terminus. The GIPR$^{dn}$ differs from the endogenous GIPR by an eight amino-acid deletion (amino acid position 319-326/nucleotide position 955-978) and two additional point mutations (amino acid position 340/nucleotide position 1018-1020) causing and amino acid exchange from alanine to glutamate (Ala → Glu) in the third intracellular loop which is known to be essential for signal transduction (Cypess et al. 1999; Hallbrink et al. 2001; Harmar 2001; Salapatek et al. 1999; Takhar et al. 1996). The binding affinity of the ligand GIP to the GIPR$^{dn}$ is almost equal compared to the endogenous GIPR. However, binding of GIP to the GIPR$^{dn}$ does not lead to any further signal transduction (Herbach et al. 2005; Volz 1997).

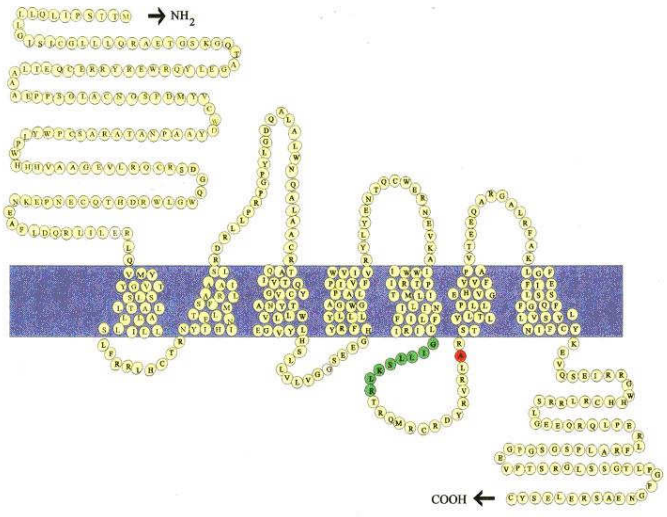


**Figure 2.2 Amino acid sequence of the human GIPR$^{dn}$ with marked mutations**

Targeted mutations of the GIPR$^{dn}$ are indicated in green (eight amino acid deletion) and red (amino acid shift (Ala → Glu)) (Renner 2008).

GIPR$^{dn}$ transgenic pigs were generated by lentiviral gene transfer. The lentiviral vector consisted of the cDNA of the human GIPR$^{dn}$ under the control of the rat insulin 2 (Ins 2) gene promoter (RIPII). Lentiviral vectors were injected into the perivitelline space of pig zygotes. Subsequently, the embryos were transferred laparoscopically to cycle synchronized recipient gilts. Two founder boars were selected to establish two transgenic lines by mating them to non-transgenic females. Genotyping of founder boars and their offspring was carried out by PCR and Southern blot analyses. GIPR$^{dn}$ mRNA expression was evaluated in isolated islets of Langerhans by RT-PCR. All transgenic animals showed GIPR$^{dn}$ expression in the islets of Langerhans, while in the islets of non-transgenic pigs no expression was detected.

### 2.7.2 Physiological characterization

To investigate the influence of GIPR$^{dn}$ expression on glucose metabolism blood glucose as well as serum fructosamine levels were evaluated. Additionally, oral and intravenous glucose tolerance tests were performed in GIPR$^{dn}$ transgenic pigs and controls. GIPR$^{dn}$ transgenic pigs exhibited no changes in fasting blood glucose levels from the age of 35 days up to 210 days. Non-fasted piglets

before weaning also showed normal blood glucose levels compared to controls. Furthermore, serum fructosamine levels, an intermediate-term marker of glucose tolerance, did not differ between GIPR$^{dn}$ transgenic pigs and controls at any time point.

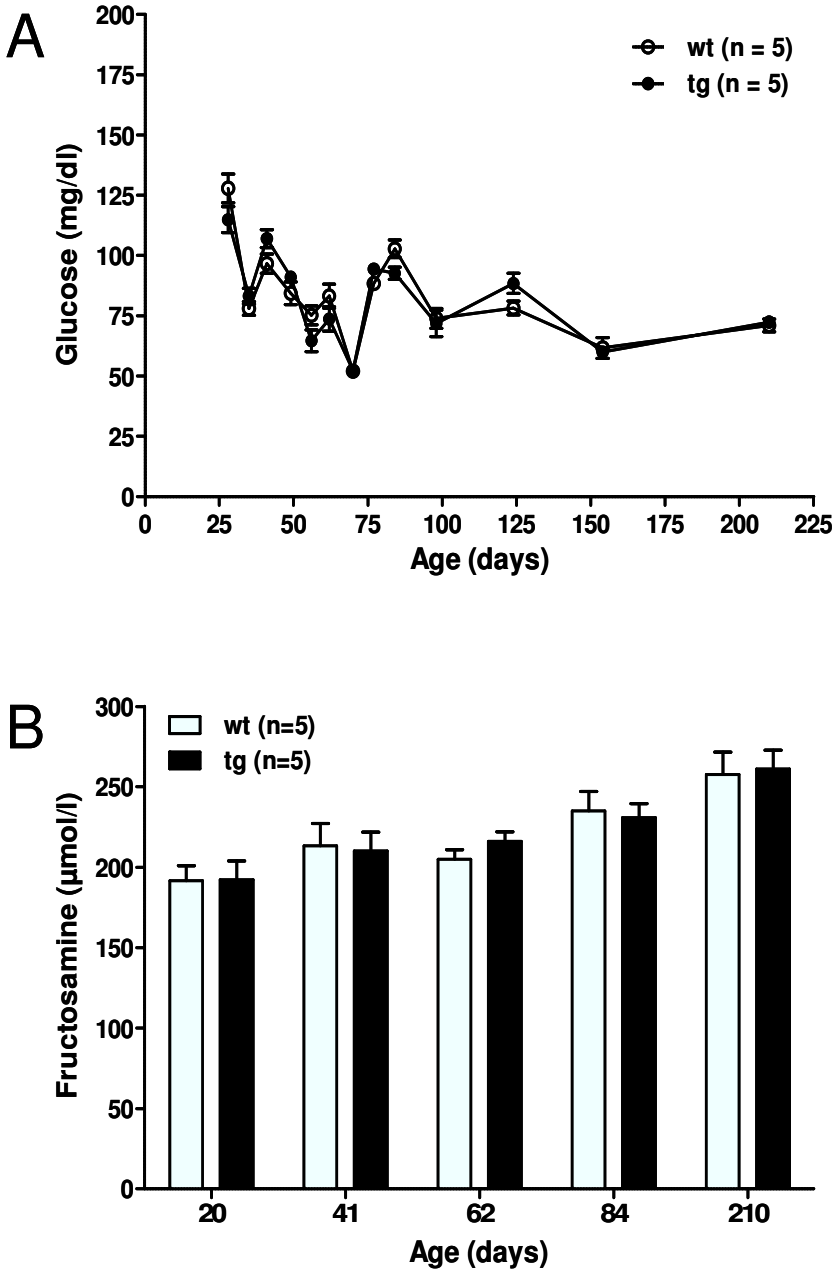


**Figure 2.3 Normal blood glucose and serum fructosamine levels in GIPR$^{dn}$ transgenic pigs (tg) compared to controls (wt)**

**(A)** Blood glucose levels; **(B)** serum fructosamine levels; n: number of animals investigated. Data are means ± SEM (Renner 2008).

An oral glucose tolerance test was performed in 5-month-old GIPR$^{dn}$ transgenic pigs and controls. Serum glucose levels of GIPR$^{dn}$ transgenic pigs were markedly elevated and the AUC glucose was significantly larger by 26% compared to their littermate controls. Furthermore, insulin levels were decreased and the AUC insulin demonstrated a significant reduction of 49% in transgenic pigs compared to control animals.

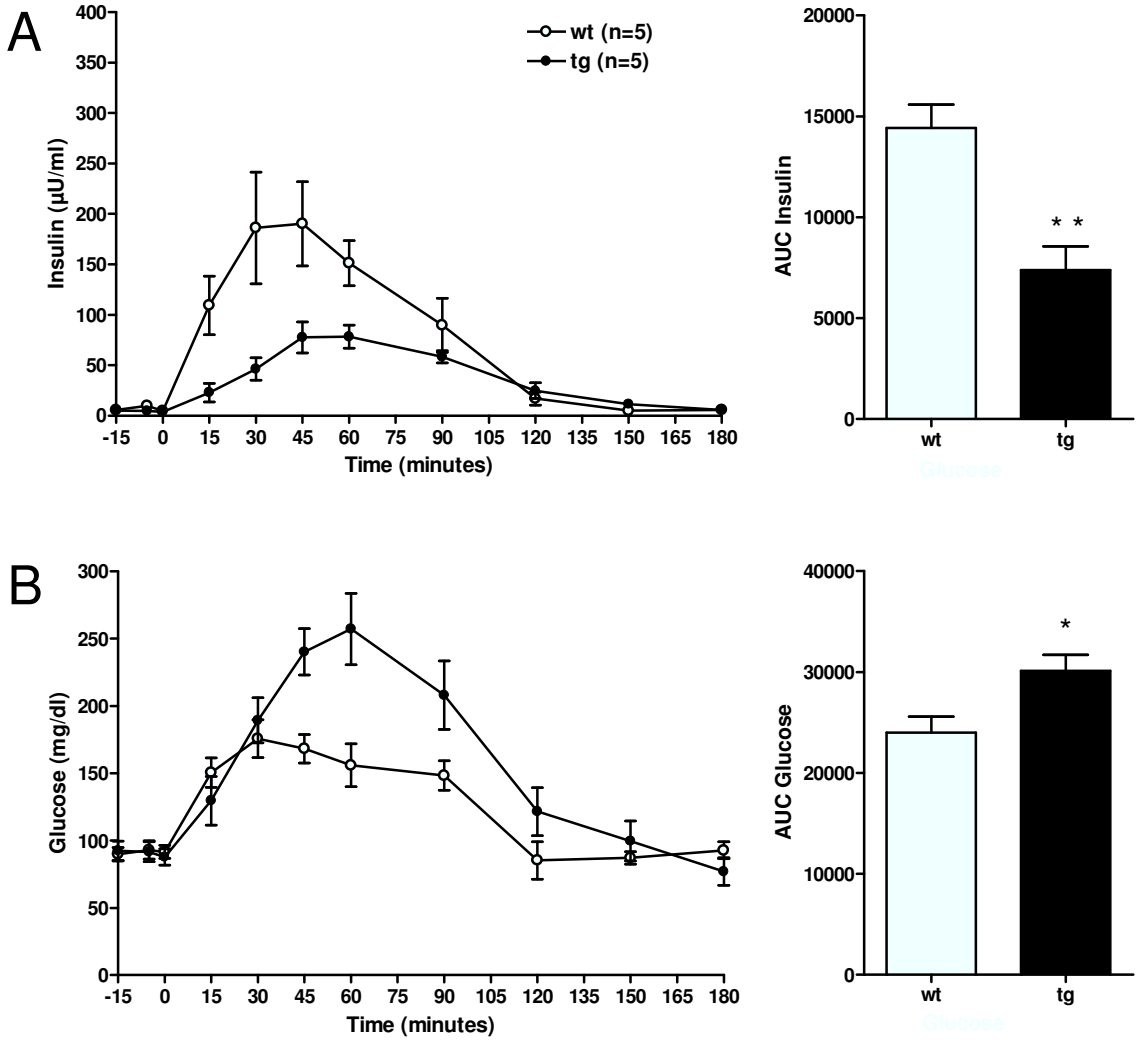


**Figure 2.4 Disturbed oral glucose tolerance and reduced insulin secretion in 5-month-old GIPR$^{dn}$ transgenic pigs (tg) compared to controls (wt).**

**(A)** Serum insulin levels and area under the insulin curve (AUC insulin); **(B)** serum glucose levels and AUC glucose; n: number of animals investigated. Data are means ± SEM; *: $p<0.05$, **: $p<0.01$ vs. control (Renner 2008).

The same collective of animals used for the OGTT was utilized to perform an intravenous glucose tolerance test at the age of 11 months. Significantly elevated blood glucose levels with a markedly increased AUC glucose by 10% as well as markedly reduced insulin levels and a significantly diminished AUC insulin by 52% furnished proof for a disturbed intravenous glucose tolerance going along with decreased insulin secretion in 11-month-old GIPR$^{dn}$ transgenic pigs compared to controls.

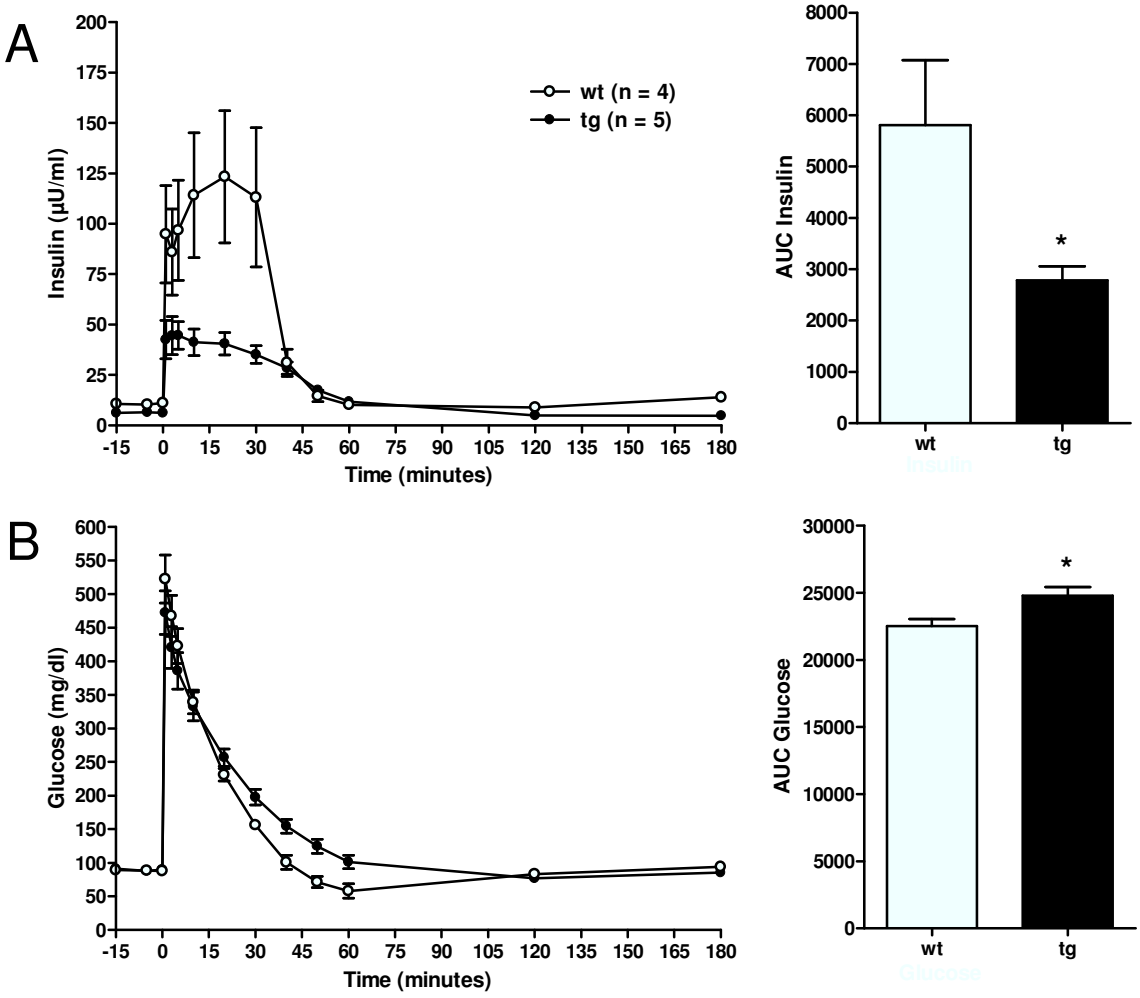


**Figure 2.5 Disturbed intravenous glucose tolerance and reduced insulin secretion in 11-month-old GIPR$^{dn}$ transgenic pigs (tg) compared to controls (wt).**

**(A)** Serum insulin levels and area under the insulin curve (AUC insulin); **(B)** serum glucose levels and AUC glucose; n: number of animals investigated. Data are means ± SEM; *: $p<0.05$ vs. control (Renner 2008).

### 2.7.3 Morphological characterization

In order to clarify the underlying reasons for disturbed oral and intravenous glucose tolerance and reduced insulin secretion quantitative stereological analyses of the pancreas and quantitative islet isolation were performed in 12 to 13-month-old GIPR$^{dn}$ transgenic pigs and control animals. While the left pancreatic lobe was used for islet isolation, the remnant pancreas served for quantitative stereological analyses.

The total number of islet equivalents (islets with a diameter of ~150 µm) was significantly reduced in GIPR$^{dn}$ transgenic pigs compared to littermate controls ($p<0.05$). Islet purity and vitality were identical in both groups.

**Table 2.1 Islet isolation results using the left pancreatic lobes of non-transgenic (wt) and GIPR$^{dn}$ transgenic pigs (tg) (n=3 in each group)**

| Type of Pig | Total IEQ Purified | Total IEQ/ g Organ | Islet Purity (%)* | Islet Vitality (%)* |
|---|---|---|---|---|
| wt #1 | 72,673 | 889.71 | 95 | 95 |
| wt #2 | 90,260 | 1519.53 | 85 | 80 |
| wt #3 | 71,658 | 1119.66 | 85 | 80 |
| **mean ± SEM** | **78,197 ± 6,038** | **1176.23 ± 184** | **88.3 ± 3.33** | **85 ± 5.0** |
| tg #1 | 4,053 | 50.66 | 85 | 80 |
| tg #2 | 6,026 | 81.76 | 85 | 80 |
| tg #3 | 6,240 | 89.14 | 85 | 80 |
| **mean ± SEM** | **5.439 ± 696** | **73.85 ± 11.79** | **85** | **80** |

IEQ: islet equivalent (islet of 150 µm in size); * estimation by two independent individuals after microscopic inspection (Renner 2008).

Quantitative stereological analyses revealed that the total volume of β-cells ($V_{(\beta\text{-cell, Pan})}$) and the volume density of β-cells ($Vv_{(\beta\text{-cell/Pan})}$) in the pancreas were significantly reduced in GIPR$^{dn}$ transgenic pigs compared to controls by 71% (Figure 2.6 A/B). The total volume of β-cells in the islets ($V_{(\beta\text{-cell, Islet})}$)

showed a marked reduction of 75%, whereas volume density of β-cells in the islets ($Vv_{(\beta\text{-cell/Islet})}$) was diminished by 11% (Figure 2.6 C/D). A clear difference between transgenic and wild-type animals was also observed in total islet volume ($Vv_{(Islet,\ Pan)}$) and volume density of islets ($Vv_{(Islet/Pan)}$) in the pancreas (Figure 2.6 E/F). A reduction of 72% for both parameters was determined. Total volume of isolated β-cells and volume density of isolated β-cells in the pancreas as marker for islet neogenesis did not differ between the two groups indicating other mechanisms (proliferation, apoptosis) being responsible for the reduction.

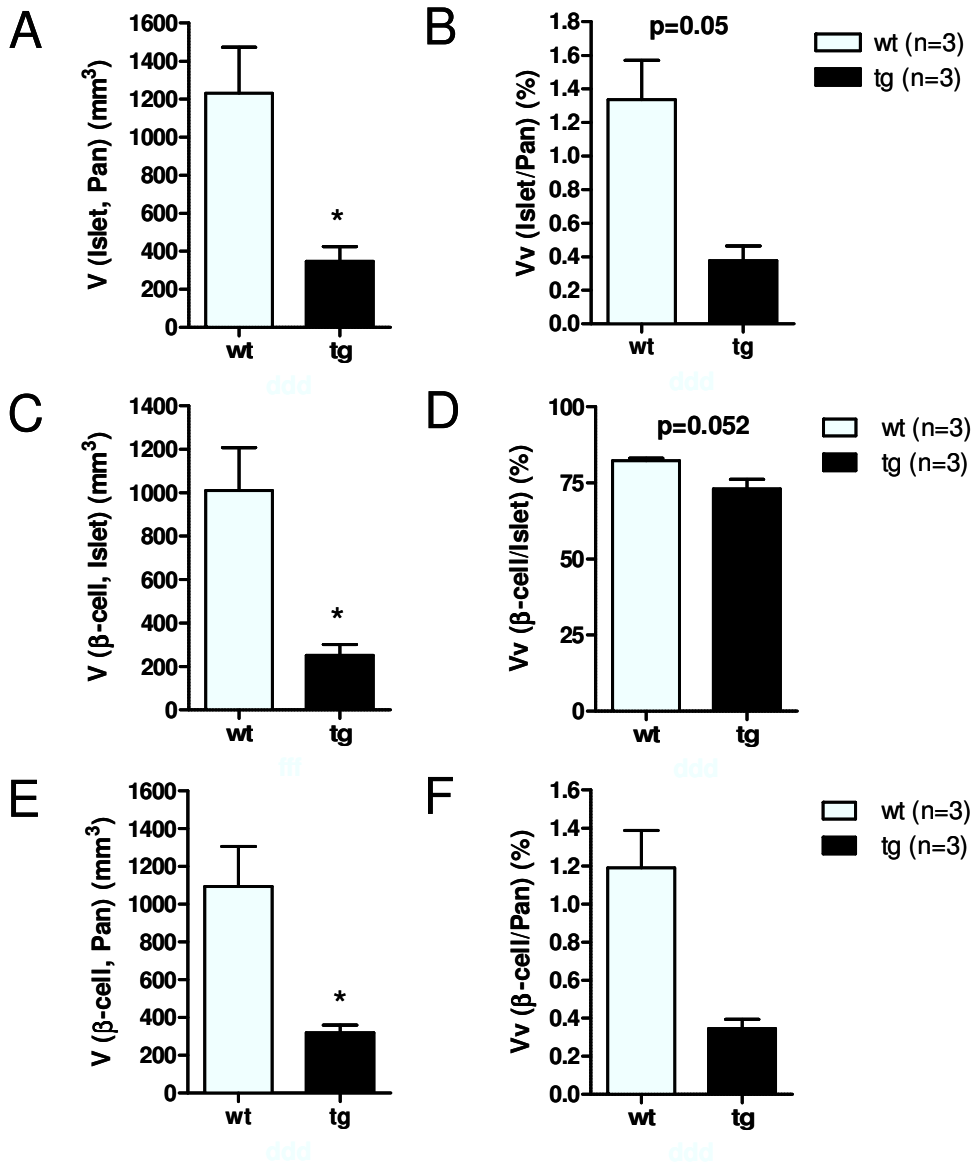


**Figure 2.6 Reduced total islet and β-cell volume in GIPR$^{dn}$ transgenic pigs (tg) compared to controls (wt).**

**(A/B)** Total volume ($V_{(islet, Pan)}$) and volume density ($Vv_{(islet/Pan)}$) of islets in the pancreas; **(C/D)** total volume ($V_{(\beta\text{-cell, islet})}$) and volume density ($Vv_{(\beta\text{-cell/islet})}$) of β-cells in the islets; **(E/F)** total volume ($V_{(\beta\text{-cell, Pan})}$) and volume density ($Vv_{(\beta\text{-cell/Pan})}$) of β-cells in the pancreas; data are means ± SEM; n: number of animals investigated; *:$p<0.05$ vs. control (Renner 2008).

# 3 Animals, Materials and Methods

## 3.1 Pigs

Pigs investigated in this study were hemizygous male and female transgenic and non-transgenic littermate or age-matched control animals. Stables were planar fixed and covered with straw. Once daily, animals were fed a commercial diet. Water was offered ad libitum. During all study procedures, animals were housed in single pens under controlled conditions. To prepare the pigs for the metabolic studies all experimental procedures were trained carefully prior to the tests. All animal experiments were carried out according to the German Animal Protection Law (209.1/211-2531-54/02).

**Table 3.1 Diet composition**

| | **Deuka primo care** (piglets up to 25 kg) | **Deuka porfina U** (growing and adult pigs) |
|---|---|---|
| MJ ME/kg | 14.0 | 12.6 |
| Crude protein % | 16.0 | 17.0 |
| Crude fat % | 4.3 | 3.0 |
| Crude fiber % | 5.0 | 6.5 |
| Crude ash % | 4.6 | 5.5 |
| Lysin % | 1.35 | 0.9 |
| Calcium % | 0.65 | 0.85 |
| Phosphorus % | 0.55 | 0.55 |
| Sodium % | 0.2 | 0.25 |

ME: metabolizable energy

## 3.2 Materials

### 3.2.1 Apparatuses

| | |
|---|---|
| Accu-jet® pro pipette controller | Brand, Wertheim |
| Agarose gel electrophoresis chamber | MWG-Biotech, Ebersberg |
| Analytical balance | Sartorius, Göttingen |
| AU 400 autoanalyzer | Olympus, Hamburg |
| Benchtop 96 tube working rack | Stratagene, La Jolla, USA |
| Gel documentation system | Bio Rad, Munich |

| | |
|---|---|
| GeneQuant *pro* | Amersham Bioscience, Freiburg |
| Glass case for glass rack | Roth, Karlsruhe |
| Heating plate with magnetic stirrer | IKA process equipment, Staufen |
| HM 315 microtome | Microm, Walldorf |
| Hybridization oven | H. Saur, Reutlingen |
| LB 2111 γ-counter | Berthold, Bad Wildbad |
| Leitz Wetzlar Microscope | Leica Microsystems, Wetzlar |
| Microwave | Siemens, Munich |
| MS1 Minishaker | IKA process equipment, Staufen |
| Multipette® plus | Eppendorf, Hamburg |
| pH-meter | WTW, Weilheim |
| Phosphorimager Storm 860 | GE Healthcare, Munich |
| Power Pac 300 | Bio Rad, Munich |
| Precision® Xceed™ Glucometer | Abbott, Wiesbaden |
| Scintillation counter LS6500 | Beckman, Palo Alto, USA |
| Staining box according to Schiefferdecker | Roth, Karlsruhe |
| Storage phosphor screen | Bio Rad, Munich |
| Thermomixer 5436 | Eppendorf, Hamburg |
| UV-Crosslinker | Biometra, Göttingen |
| Videoplan® image analysis system | Zeiss-Kontron, Eching |

*Thermocycler:*

| | |
|---|---|
| Biometra Uno Thermoblock | Biometra, Göttingen |
| Biometra TProfessional | Biometra, Göttingen |
| Mastercycler® gradient | Eppendorf, Hamburg |

*Centrifuges:*

| | |
|---|---|
| Heraeus Megafuge 1.0R | Heraeus, Munich |
| Table centrifuge with cooling (5417R) | Eppendorf, Hamburg |

### 3.2.2 Consumables

| | |
|---|---|
| Cavafix® Certo® central venous catheter | B. Braun, Melsungen |
| Centrifuge tubes (15 ml, 50 ml) | Falcon®, Becton Dickinson, Heidelberg |

| | |
|---|---|
| Cover slips 24x40 mm | Engelbrecht, Edermünde |
| Disposable plastic pipettes | Falcon®, Becton Dickinson, Heidlberg |
| Disposable syringes (2, 5, 10, 20 ml) | Codan Medical ApS, Roedby, Denmark |
| Disposable tubes for γ-counter | Sarstedt, Nümbrecht |
| Hybond-N+ Nylon membrane | GE Healthcare, Munich |
| Microscope slides Star Frost® | Engelbrecht, Edermünde |
| MicroSpin™ S-300 HR Columns | GE Healthcare, Munich |
| Microwave Tender Cooker 2.5 Quart | Nordic Ware, Minneapolis,USA |
| Millex™-GP syringe driven filter unit® (0.22 µm) | Millipore, Billerica, USA |
| Monovette® blood collection system (Serum, EDTA) | Sarstedt, Nümbrecht |
| OP-Cover (60 x 90 cm) | A. Albrecht, Aulendorf |
| Parafilm® M | American Can Company, Greenwich, USA |
| PCR reaction tubes (0.2 ml) | Braun, Wertheim |
| Perfusor® cable (50 cm) | B. Braun, Melsungen |
| Precision Xtra™ Plus blood glucose stripes | Abbott, Wiesbaden |
| Safe-Lock reaction tubes (1.5 ml, 2 ml) | Eppendorf, Hamburg |
| Skin adhesive spray | A. Albrecht, Aulendorf |
| Sterican® cannulas (18 G, 20 G) | B. Braun, Melsungen |
| 3-way-Stopcock | Fresenius Kabi, Bad Homburg |
| Uni-Link embedding cassettes | Engelbrecht, Edermünde |
| Vasco® OP Protect gloves | B. Braun, Melsungen |
| Vascocan® indwelling venous catheter | B. Braun, Melsungen |
| Vicryl (2-0) suture material | Ethicon, Norderstedt |

### 3.2.3 Chemicals

Comment: unless otherwise noted, all chemicals were used in p.a. quality

| | |
|---|---|
| Acetic acid (glacial acetic acid) | Roth, Karlsruhe |
| Agarose | Invitrogen, Karlsruhe |

| | |
|---|---|
| BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium) | Vector Laboratories, Burlingame, USA |
| Bromophenolblue | Serva, Heidelberg |
| Chloroform | Merck, Darmstadt |
| Citric acid | Merck, Darmstadt |
| D-(+)-glucose | Sigma-Aldrich, Deisenhofen |
| 3,3´ diaminobenzidine tetrahydrochloride | KemEnTec, Copenhagen, Denmark |
| Disodiumhydrogenphosphate | Merck, Darmstadt |
| EDTA | Sigma-Aldrich, Deisenhofen |
| Eosin | Roth, Karlsruhe |
| Ethanol | Roth, Karlsruhe |
| Ethidiumbromide (1 mg/ml) | Merck, Darmstadt |
| Formaldehyde solution, 37% | Sigma-Aldrich, Deisenhofen |
| Fuchsin + Substrate-Chromogen | Dako Cytomation, Hamburg |
| Glucose 50% solution | B. Braun, Melsungen |
| Glycerine | Roth, Karlsruhe |
| Hydrochloric acid (1 N) | Merck, Darmstadt |
| Hydrochloric acid (25%) | Merck, Darmstadt |
| Hydrogen peroxide (35%) | Roth, Karlsruhe |
| Isoamylalcohol (3-methyl-1-butanol) | Roth, Karlsruhe |
| Kalium chloride | Merck, Darmstadt |
| Kaliumdihydrogenphosphate | Merck, Darmstadt |
| Magnesium chloride (25 mM) | Qiagen, Hilden |
| Mayer`s Hemalaun | Applichem, Germany |
| 0.9% NaCl solution | B. Braun, Melsungen |
| Nuclear Fast Red | Roth, Karlsruhe |
| Phenol | Roth, Karlsruhe |
| 2-Propanol | Merck, Darmstadt |
| Sodiumacetate-trihydrate | Merck, Darmstadt |
| Sodium chloride | Merck, Darmstadt |
| Sodiumdodecylsulphate | Merck, Darmstadt |
| Sodium hydroxide (2 N) | Roth, Karlsruhe |
| Spermidine | Sigma, Deisenhofen |

| | |
|---|---|
| Tris-(hydroxymethyl)-aminomethane | Roth, Karlsruhe |
| Vectashield Mounting Medium with DAPI | Vector Laboratories, Burlingame, USA |
| Xylene | SAV LP, Flintsbach a. Inn |

### 3.2.4 Antibodies, drugs, enzymes and other reagents

#### 3.2.4.1 Antibodies

##### 3.2.4.1.1 Primary antibodies

| | |
|---|---|
| Monoclonal mouse anti-human Ki67 | Dako Cytomation, Hamburg |
| Polyclonal guinea pig anti-porcine insulin | Dako Cytomation, Hamburg |
| Polyclonal rabbit anti-human cleaved caspase-3 | Cell Signaling, Danvers, USA |
| Polyclonal rabbit anti-human GIPR | Acris, Herford |
| Polyclonal rabbit anti-human GLP-1R | Acris, Herford |
| Polyclonal rabbit anti-human glucagon | Dako Cytomation, Hamburg |
| Polyclonal rabbit anti-human somatostatin | Dako Cytomation, Hamburg |
| Polyclonal rabbit anti-human pancreatic polypeptide | Dako Cytomation, Hamburg |

##### 3.2.4.1.2 Secondary antibodies

| | |
|---|---|
| AP-conjugated donkey anti-rabbit IgG | Dianova, Hamburg |
| AP-conjugated goat anti-guinea pig IgG | Southern Biotech, Birmingham, USA |
| Biotinylated goat anti-rabbit IgG | Vector Laboratories, Burlingame, USA |
| Biotinylated goat anti-mouse IgG | Dako Cytomation, Hamburg |
| Cy 3-conjugated donkey anti-guinea pig IgG | Jackson Immunoresearch Laboratories, Baltimore, USA |
| FITC-conjugated donkey anti-mouse IgG | Jackson Immunoresearch Laboratories, Baltimore, USA |
| HRP-conjugated donkey anti-guinea pig IgG | Dianova, Hamburg |

| | |
|---|---|
| HRP-conjugated pig anti-rabbit IgG | Dako Cytomation, Hamburg |
| HRP-conjugated rabbit anti-guinea pig IgG | Dako Cytomation, Hamburg |

#### 3.2.4.2 Drugs

| | |
|---|---|
| Azaperon (Stresnil®) | Janssen Pharmaceutica, Beerse, Belgium |
| Cefquinom (Cobactan®) | Intervet, Unterschleißheim |
| Heparin-Sodium (25.000 IE/5 ml) | B. Braun, Melsungen |
| Ketamine hydrochloride (Ursotamin®) | Serumwerk Bernburg, Bernburg |
| Metamizol-Sodium (Vetalgin®) | Intervet, Unterschleißheim |
| Meloxicam (Metacam®) | Boehringer Ingelheim, Ingelheim |
| Xylazine (Xylazin 2%) | WDT, Garbsen |

#### 3.2.4.3 Enzymes

| | |
|---|---|
| Herculase® enhanced DNA Polymerase | Stratagene, La Jolla, USA |
| Proteinase K (20 mg/ml) | Roche, Mannheim |
| Restriction enzymes and -buffers | MBI Fermentas, St. Leon Roth |
| Ribonuclease A (RNase-A) (0.2 U/µl) | Roche, Mannheim |
| Taq Polymerase (5 U/ml) | Agrobiogen, Hilgerstshausen |

#### 3.2.4.4 Other reagents

| | |
|---|---|
| α-[32P]-dCTP | GE Healthcare, Munich |
| Blood & Cell Culture DNA Midi Kit | Qiagen, Hilden |
| dNTPs (dATP, dCTP, dGTP, dTTP) | MBI Fermentas, St. Leon Roth |
| 5 x first strand buffer | Invitrogen, Karlsruhe |
| Gel blotting paper (GB002) (Whatman-paper) | Schleicher & Schüll, Dassel |
| Goat serum | MP Biomedicals, Illkirch, France |
| Horse serum | MP Biomedicals, Illkirch, France |
| Jetquick Gel extraction Spin Kit | Genomed, Löhne |
| 10 x PCR buffer | Agrobiogen, Hilgertshausen |
| Porcine Insulin RIA Kit | Millipore, Billerica, USA |
| Porcine serum | MP Biomedicals, Illkirch, France |
| Porcine serum albumin | Sigma-Aldrich, Taufkirchen |
| Rabbit serum | MP Biomedicals, Illkirch, France |

| | |
|---|---|
| Rapid-Hyb buffer | GE Healthcare, Munich |
| Roti® Histokitt II | Roth, Karlsruhe |
| Synthetic porcine GIP | Bachem, Weil am Rhein |
| Synthetic Exendin-4 | Bachem, Weil am Rhein |
| Vectastain Elite ABC Kit | Vector Laboratories, Burlingam, USA |
| Vet-Sept® solution (10%) | A. Albrecht, Aulendorf |

### 3.2.5 Buffers, media and solutions

Comment: unless otherwise noted, in a Millipore machine (EASYpure® II, pure Aqua, Schnaitsee) de-ionized water was used as solvent which is termed aqua bidest.

#### 3.2.5.1 Chloroform-isoamylalcohol (CiA)

| | |
|---|---|
| 96 ml | chloroform |
| 4 ml | isoamylalcohol |

Storage at 4°C protected from light

#### 3.2.5.2 Citrate buffer (pH 6.0)

| | |
|---|---|
| 4.2 g | citrate monohydrate |
| ad 2000 ml | aqua bidest. |

#### 3.2.5.3 Citrate buffer for cleaved caspase-3 IHC (pH 6.0)

Stock solution A: 19.2 g citric acid in 1000 ml aqua bidest.
Stock solution B: 29.4 g trisodium citrate-dihydrate in 1000 ml aqua bidest.
working solution: 27 ml solution A + 123 ml solution B + 1.5 ml Tween 20 ad 1500 ml aqua bidest.

#### 3.2.5.4 Phenol-chloroform-isoamylalcohol (PCiA)

| | |
|---|---|
| 25 ml | phenol |
| 25 ml | CiA |

Storage at 4°C protected from light

#### 3.2.5.5 dNTP-mix

| | |
|---|---|
| 100 µl | of each dNTP (2M) |
| ad 4.6 ml | aqua bidest. |

Aliquoted; storage at -20°C

#### 3.2.5.6 PBS buffer

| | |
|---|---|
| 137 mM | NaCl |
| 10 mM | $Na_2HPO_4$ |
| 2.7 mM | KCl |
| 2 mM | $KH_2PO_4$ |

#### 3.2.5.7 Proteinase-K solution

| | |
|---|---|
| 20 mg | Proteinase K |
| 1 ml | Aqua bidest. |

Aliquoted; storage at -20°C

#### 3.2.5.8 TBS buffer (10x) (pH7.6)

| | |
|---|---|
| 90 g | NaCl |
| 60.5 g | Tris |
| ad 1000 ml | aqua bidest. |

#### 3.2.5.9 TE buffer

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

#### 3.2.5.10 Buffers for agarose gels

##### 3.2.5.10.1 TAE buffer (50x)

| | |
|---|---|
| 242 g | Tris (2 M) |
| 57.1 ml | glacial acetic acid |
| 100 ml | EDTA 0.5 M (pH 8.0) |
| ad 1000 ml | aqua bidest. |

#### 3.2.5.10.2 TAE running buffer (1x)

| | |
|---|---|
| 20 ml | 50 x TAE buffer |
| ad 1000 ml | aqua bidest. |

#### 3.2.5.10.3 Loading buffer for DNA (6x)

| | |
|---|---|
| 3 ml | glycerine |
| 7 ml | aqua bidest. |
| 1 point of a spatula | bromophenolblue |

Aliquoted; storage at 4°C

### 3.2.5.11 Solutions for Southern blot

#### 3.2.5.11.1 Denaturation solution

| | |
|---|---|
| 0.5 M | NaOH |
| 1.5 M | NaCl |

#### 3.2.5.11.2 Neutralization solution

| | |
|---|---|
| 1 M | NaCl |
| 0.5 M | Tris/HCl (pH 8.0) |

#### 3.2.5.11.3 SSC buffer (20x) (pH 7.0)

| | |
|---|---|
| 3 M | NaCl |
| 0.3 M | Sodiumacetate-trihydrate |

#### 3.2.5.11.4 Washing solution I

| | |
|---|---|
| 2 x | SSC |
| 0.1% | SDS |

#### 3.2.5.11.5 Washing solution II

| | |
|---|---|
| 1 x | SSC |
| 0.1% | SDS |

## 3.2.6 Oligonucleotides

| | |
|---|---|
| RIP2 (sense): | 5'-TAGTCGACCCCCAACCACTCCAAGTGGAG-3' |
| RIP2 (antisense): | 5'-CAGCCCTAACTCTAGACTCGAGGGATCCTA-3' |

Actin (sense): 5`-GCCAACCGTGAGAAGATGAC-3`
Actin (antisense): 5`-GGTCTCGAACATGATCTGG-3`

### 3.2.7 DNA molecular weight markers

| | |
|---|---|
| Gene Ruler$^{TM}$ (1 kb DNA Ladder) | MBI Fermentas, St. Leon Roth |
| Lambda DNA/EcoRI + HindIII-Marker | MBI Fermentas, St. Leon Roth |
| pUC Mix Marker 8 | MBI Fermentas, St. Leon Roth |

## 3.3 Methods

### 3.3.1 Identification of GIPR$^{dn}$ transgenic pigs

The identification of GIPR$^{dn}$ transgenic pigs was accomplished by PCR as well as by Southern blot analysis. Southern blot analysis furnished proof not only for discrimination of transgenic pigs from wild-type animals, but also for determination of the number of integration sites of the transgene.

#### 3.3.1.1 Polymerase chain reaction (PCR)

##### 3.3.1.1.1 DNA isolation from ear punches

Ear punches were obtained from 2-day-old piglets and stored at –20°C until further processing. Tissue digestion from ear samples (0.5 cm$^2$) was performed using 30 µl Proteinase K (20 mg/ml) and 400 µl lysis buffer as follows:

Composition of 5 ml lysis buffer:

| | |
|---|---|
| 500 µl | 160 mM saccharose |
| 800 µl | 80 mM EDTA pH 8.0 |
| 500 µl | 100 mM Tris/HCl pH 8.0 |
| 125 µl | 0.5% SDS |
| 3.175 ml | aqua bidest. |

Tissue samples and Proteinase K were added to the lysis buffer, mixed well and incubated overnight at 60°C. The next day, undigested components were separated by centrifugation (5 min, 13000 x g) and the supernatant was transferred into a new sterile reaction tube. Sodium chloride (400 µl, 4.5 M) and phenol-chloroform-isoamylalcohol (PCiA; 300 µl) were added and samples were

pivoted about 10 minutes for a thorough through-mixing of the components. To achieve phase separation samples were centrifuged (5 min, 13000 x g). The lower two phases containing lipids, RNA, carbohydrates and proteins were left in the reaction tube while the upper aqueous phase containing DNA was pipetted into a new sterile reaction tube. Then samples were washed with PCiA, pivoted and centrifuged at least three times until no more residua were visible between the lower and the upper phase. Afterwards the addition of 550 µl (0.7 x volume of the aqueous phase containing DNA) isopropanol induced DNA precipitation. The precipitated DNA could be transferred into a reaction tube containing 70% ethanol. This step was repeated once and DNA was kept in ethanol for several hours. Then ethanol was discarded and DNA was air-dried at room temperature for 6 minutes and resuspended with 55 µl Tris/HCl solution (10 mM). To ensure complete DNA resolution, samples were stored at 4°C overnight. The next day, DNA concentration was measured at 260 nm using a GeneQuant *pro* spectrophotometer (Amersham Bioscience, Freiburg). For PCR use, DNA concentration was adjusted to 100 ng/µl.

#### 3.3.1.1.2 PCR conditions

Transgene-specific primers were used to identify $GIPR^{dn}$ transgenic pigs:

RIP2 (sense): 5'-TAGTCGACCCCCAACCACTCCAAGTGGAG-3'

RIP2 (antisense): 5'-CAGCCCTAACTCTAGACTCGAGGGATCCTA-3'

To verify integrity of the genomic DNA a so-called house-keeping gene was amplified additionally. In this case β-actin was used which is expressed independently of cell type, cell cycle or external influences.

The following β-actin specific primers were used:

β-actin (sense): 5`-GCCAACCGTGAGAAGATGAC-3`

β-actin (antisense): 5`-GGTCTCGAACATGATCTGG-3`

The PCR reactions with a total volume of 25 µl were prepared on ice in 0.2 ml reaction tubes as follows:

**Table 3.2 Reaction batch for RIP2-hGIPR$^{dn}$/β-actin PCR**

| RIP2-hGIPR$^{dn}$/β-actin | |
|---|---|
| 10 x PCR buffer | 2.5 µl |
| $MgCl_2$ (25 mM) | 1.5 µl |
| dNTPs (2 mM) | 2.5 µl |
| Sense Primer (25 µM) | 0.5 µl |
| Antisense Primer (25 µM) | 0.5 µl |
| Taq DNA Polymerase (5 U/µl) | 0.2 µl |
| Aqua bidest. | 16.3 µl |
| Template (100 ng/µl) | 1 µl |
| Total volume | 25 µl |

PCR conditions are listed below:

**Table 3.3 Reaction conditions RIP2-hGIPR$^{dn}$/β-actin PCR**

| RIP2-hGIPR$^{dn}$/β-actin | | | |
|---|---|---|---|
| Denaturation | 95°C | 2 min | |
| Denaturation | 95°C | 30 s | 31 x |
| Annealing | 60°C | 30 s | |
| Elongation | 72°C | 1 min | |
| Final elongation | 72°C | 10 min | |

#### 3.3.1.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis served as a method for separating DNA strands according to their size. Thereto a 2% TAE-agarose solution was prepared. After boiling up the mixture in a microwave, ethidiumbromide (0 5 µg/ml) was added. Due to intercalation of the dye ethidiumbromide between the bases of nucleic acids DNA can be visualized and documented under UV-light. When the gel was cured running buffer was dumped into the chamber. The PCR products and a DNA molecular weight standard were loaded into the gel slots after addition of application of a 6 x loading dye to monitor the electrophoresis progress.

#### 3.3.1.2 Southern Blot

##### 3.3.1.2.1 Genomic DNA islolation from EDTA blood

For Southern blot analysis, genomic DNA extracted from EDTA blood (5 ml) using the Blood and Cell Culture DNA Midi kit® (Qiagen, Hilden, Germany) according to the manufacturer`s instructions was applied.

##### 3.3.1.2.2 Restriction digest

Genomic DNA strands were cut into smaller fragments using a restriction enzyme. Besides of the DNA (8 µg) the reaction batch with a total volume of 30 µl contained 30 U of the restriction enzyme *ApaI*, its appropriate buffer as well as Spermidine (0.1 M). After an overnight digestion at 37°C another 10 U of the restriction enzyme were added to each sample followed by an incubation step of one hour. Prior to the use of the samples for gel electrophoresis 6 x loading dye was pipetted to each sample for a better monitoring of the gel electrophoresis process.

##### 3.3.1.2.3 Gel electrophoresis and transfer of genomic DNA

DNA fragments were loaded into the gel slots of a 0.9% TAE agarose gel to separate them by size. A 1 kb ladder served as DNA molecular weight standard. When the samples, visualized by the bromophenol blue containing 6 x loading dye, reached the end of the gel, the gel electrophoresis could be stopped. The electrophoresis result was documented under UV-light using a gel documentation system. Prior to the blotting process, the gel was pivoted in 0.25 M hydrochloric acid for about 20 minutes depending on the color shift of the blue bands to yellow. After washing the gel for several minutes with aqua bidest. it was gently shook in denaturation solution until the color of the bands turned back to blue (around 45 minutes). Subsequently, the DNA was blotted onto a positive loaded nitrocellulose membrane by capillary transfer. Thereto the gel was placed upside-down on a plastic surface. Two layers of Whatman paper followed by multiple layers of absorbent paper were placed on the gel and weighed down by a weight of ca. 800 g to improve capillary transfer. The transfer was carried out overnight for 18-24 hours. The next day, the membrane was pivoted in neutralization solution for 5 minutes followed by 15 minutes of

air-drying. Cross linking of the transferred DNA with the membrane was accomplished under UV-light (120 J/cm$^2$) before the membrane was stored at room temperature until further processing.

#### 3.3.1.2.4 Probe establishment

The primers RIP2-sense and RIP2-antisense (see 3.3.1.1.2) were used to create a 720-bp probe. Plasmid DNA containing the RIP2-hGIPR$^{dn}$ construct in a concentration of 7 ng/µl served as template for the PCR.

**Table 3.4 Reaction batch probe Southern blot PCR**

| | Probe Southern blot |
|---|---|
| 10 x Herculase buffer | 10 µl |
| dNTPs (10 mM) | 2 µl |
| Sense primer (100 µM) | 0.5 µl |
| Antisense primer (100 µM) | 0.5 µl |
| Aqua bidest. | 83.5 µl |
| Herculase Taq Polymerase | 0.5 µl |
| Template | 3 µl |
| Total volume | 100 µl |

**Table 3.5 PCR conditions probe Southern blot PCR**

| | Probe Southern blot | | |
|---|---|---|---|
| Denaturation | 94°C | 4 min | |
| Denaturation | 94°C | 1 min | 34 x |
| Annealing | 62°C | 1 min | |
| Elongation | 72°C | 3.03 min | |
| Final elongation | 72°C | 10 min | |

The amplified PCR product was separated in a 1% TAE-agarose gel after addition of 20 µl 6x loading dye. Then, the designated band was excised using a scalpel blade and DNA was extracted from the gel using the Jetquick Gel Extraction Spin Kit according to the manufacturer's instructions. Subsequently, 2 µl of each extracted DNA sample as well as of the molecular weight standard Lambda/*Hind*III + *Eco*RI were loaded on a 1% TAE agarose gel. DNA concentration of the amplificates was estimated by comparison of the respective

band intensity with the intensity of the bands of the concentration standard Lambda/*Hind*III + *Eco*RI.

#### 3.3.1.2.5 Radioactive labeling of the probe

The Rediprime II Random Prime Labeling System® was used to label 50-70 ng of the probe radioactively with 50 $\mu$Ci α-[$^{32}$P]-dCTP (GE Healthcare) according to the manufacturer's instructions. Random priming is a method to label DNA. Oligonucleotides of accidential sequence are added to denatured single strand DNA for hybridization. The so called Klenow enzyme, a fragment of the DNA Polymerase I of E. coli, synthesizes the opposite strand using the oligonucleotides as primers. DNA is labeled by integration of radioactive nucleotides. A MicroSpin™ S-300 HR column was used for removing all not incorporated nucleotides. Nuclear radiation per minute (cpm: counts per minute) was measured by a scintillation counter using 5 µl of a 1:100 dilution of the labeled probe. To calculate the cpm value per µl of the labeled probe the following formula, where Cerenkov is a correction factor for the calculation without scintillation liquid, was utilized:

$$\text{cpm/µl} = \text{cpm} * 20\ (\text{dilution}) * 1.55\ (\text{Cerenkov})$$

#### 3.3.1.2.6 Hybridization, washing and signal detection

Prehybridization of the nylon membrane preceded the hybridization step. Therefore the nylon membrane was incubated in Rapid-Hyb buffer® for two hours at 65°C in a hybridization oven. For hybridization, the labeled probe was used in a concentration of $2 \times 10^6$ cpm per µl Rapid-Hyb buffer®. After denaturation of the adequate amount of probe for five minutes at 95°C and chilling on ice, the sample was added to the prehybridization solution and incubated overnight at 65°C. The next day, nonspecific bound radioactivity was removed by three washing steps: 1 x washing solution I for 20 minutes (RT); 2 x washing solution II for 20 minutes (65°C). Blots were exposed in a Phosphor-Imager cassette and visualized with a Phosphor-Imager (Storm 860).

### 3.3.2 Physiological characterization of GIPR$^{dn}$ transgenic pigs

#### 3.3.2.1 Surgical implantation of central venous catheters

For accomplishment of the glucose tolerance tests and GIP/Exendin-4 stimulation tests, two central venous catheters (Cavafix® Certo®, B.Braun) were surgically inserted into the external jugular vein under general anesthesia using a modified method of Moritz et al. (1989). After an intramuscular injection of 2 ml per 10 kg body weight (BW) Ketamin (Ursotamin®, Serumwerk Bernburg) and 0.5 ml per 10 kg BW Azaperon (Stresnil®, Janssen Pharmaceutica, Belgium) pigs could be prepared for surgery. The hair was extensively shaved around the neck and an indwelling catheter was placed into one of the ear veins. Surgical tolerance was reached and general anesthesia was maintained by an intravenous injection of Ketamin (Ursotamin®, Serumwerk Bernburg; dosage: 2 ml per 10 kg BW) and Xylazine (Xylazin®, 2%, WDT; dosage: 0.5 ml per 10 kg BW) as required. Metamizol (1 ml per 10 kg BW, Vetalgin®, Intervet) and Meloxicam (2 ml per 100 kg BW, Metacam®, Boehringer Ingelheim) were given intramuscularly for peri- and post-surgical analgesia. During surgery the inter digital reflex, nasal septum reflex and breathing pattern were controlled regularly. Following aseptic preparation of the surgical field a skin incision, five centimeters in length, was made followed by the exposure of the external jugular vein (Figure 3.1 A). Proximal and distal of the exposed vein a holding suture was placed to facilitate preparation of the intended site for vein cannulation (Figure 3.1 B). A venotomy was made and the catheters were inserted 10 to 15 cm into the vein depending on pig size (Figure 3.1 C). The objective was to position the catheters near the heart base (Figure 3.2). A proximal ligature was placed to stop blood reflux while a distal ligature was needed to fix the catheters (Figure 3.1 D). The incision was closed in two layers. External fixation of the catheters was carried out by a single suture to the skin (Figure 3.1 E). Further, the catheters were covered with sterile gauze and adhesive tape up to the withers level where they were coiled in a pouch to provide easy access (Figure 3.1F). An antibiotic (Cobactan® 2.5%, Intervet) was administered for three days (0.5 ml/10 kg BW) after catheter placement. The catheters were flushed with 250 IU Heparin/ml 0.9% isotonic NaCl solution (Heparin-Natrium, B. Braun, 0.9% NaCl, B. Braun) once daily. At the start of the

study period, all animals had fully recovered from the surgical procedure as evaluated by normal behavior and food intake.

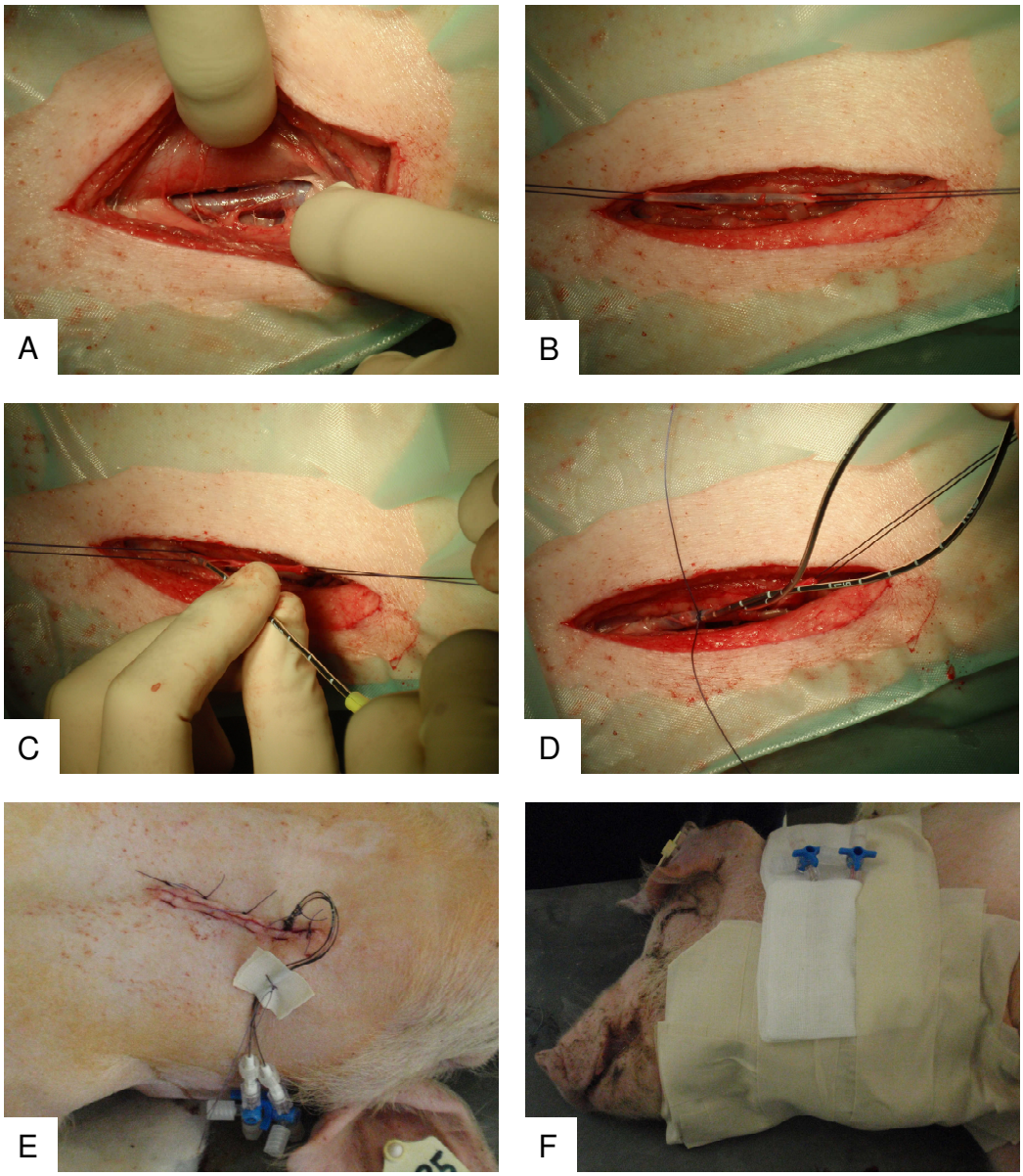


**Figure 3.1 Placement of two central venous catheters:**

**(A)** Exposition of the external jugular vein; **(B)** vein advanced by a proximal and distal holding suture; **(C)** placement of the first central venous catheter following venotomy; **(D)** placement of the second central venous catheter and distal fixation; **(E)** skin suture and external fixation of the central venous catheter; **(F)** pouch for easy access covered with adhesive tape.

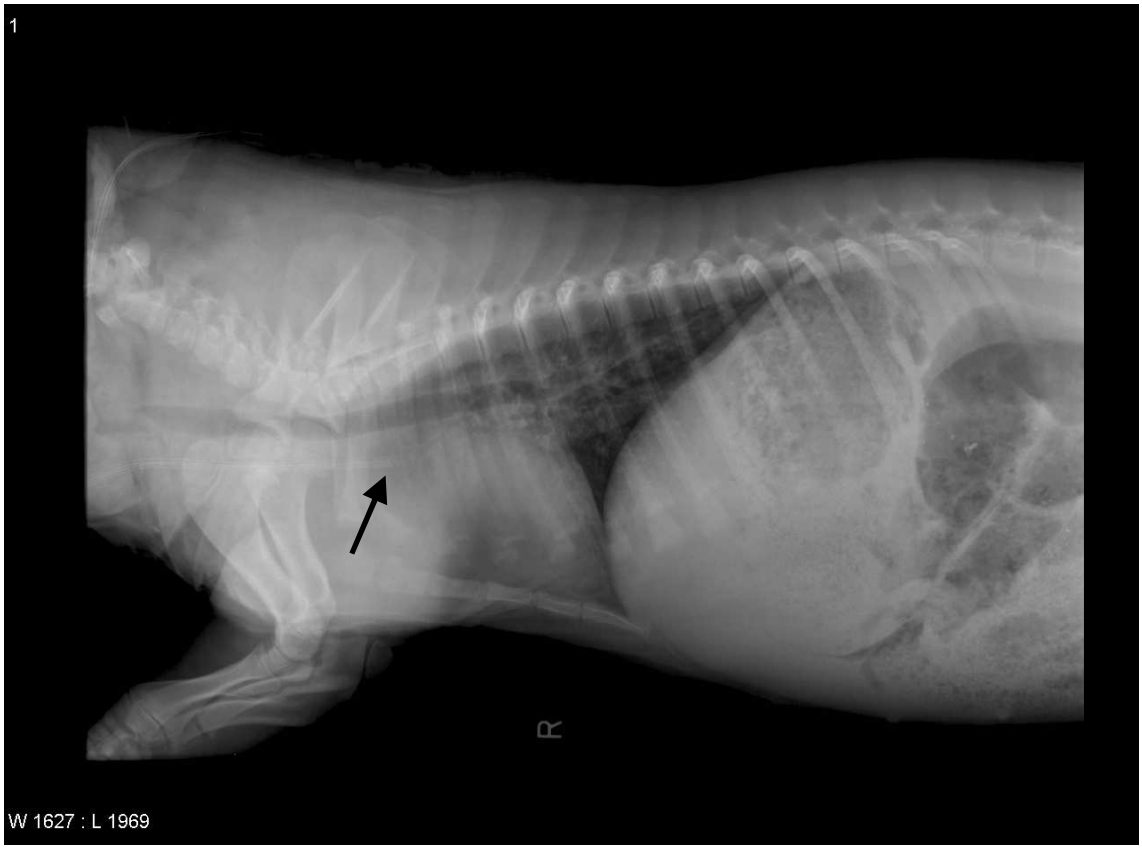


**Figure 3.2 Latero-lateral thoracic radiograph for determination of catheter placement (arrow marks the end of the catheter near the heart base)**

### 3.3.2.2 Oral glucose tolerance test (OGTT)

The OGTT was performed in 11-week-old non-restrained, freely moving animals. After an 18-h overnight fast, animals were fed 2 g glucose/kg body weight (BW) mixed with 50 g commercial pig fodder (Deuka primo care; Deuka, Düsseldorf, Germany). The meal was eaten from a bowl under supervision. Blood samples were obtained from the jugular vein catheter at -10, 0, 15, 30, 45, 60, 90 and 120 minutes relative to the glucose load. Serum glucose levels were determined using an AU 400 autoanalyzer (Olympus, Hamburg, Germany) while serum insulin levels were measured in duplicate using a porcine insulin radioimmunoassay (RIA) kit (Millipore, Billerica, USA) as described in 3.3.2.6.

### 3.3.2.3 Intavenous glucose tolerance test (IVGTT)

The IVGTT was performed in 11-week-old and 5-month-old pigs (22.5 ± 1.5 weeks). After an 18-h overnight fast, a bolus injection of concentrated 50% glucose solution (0.5 g glucose/kg BW) was administered through one marked central venous catheter. Blood was collected at -10, 0, 1, 3, 5, 10, 20, 30, 40, 60

and 90 minutes relative to the glucose load in 11-week-old pigs and at -10, 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50, 60 and 90 minutes relative to the glucose load in 5-month-old pigs. For blood sampling the second catheter was used to avoid a contamination with the applied glucose. Serum glucose and serum insulin levels were determined as described above (see 3.3.2.2).

#### 3.3.2.4 GIP/Exendin-4 concentration test

To determine the amount of GIP or Exendin-4 administrated in combination with glucose leading to a distinct higher insulin secretion compared to glucose administration only a GIP/Exendin-4 concentration test was performed in 11-week-old non-transgenic pigs. Three different concentrations of synthetic porcine GIP (40, 80 or 160 pmol/kg BW; Bachem, Weil am Rhein, Germany) and synthetic Exendin-4 (20, 40 and 60 pmol/kg BW; Bachem) were tested. Animals were studied on three occasions separated by at least 24 hours. After an 18-h fasting period a bolus of concentrated 50% glucose solution (0.5 g glucose/kg BW) was administered intravenously through one marked central venous catheter. Immediately after glucose administration, synthetic porcine GIP (40, 80 or 160 pmol/kg BW) or synthetic Exendin-4 (20, 40 and 60 pmol/kg BW) mixed with 1% of porcine serum albumin (Sigma-Aldrich, Taufkirchen, Germany) was applied intravenously. Blood samples were collected at -10, 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50 and 60 minutes relative to the glucose load through the second catheter. Serum glucose levels and serum insulin levels were measured as described above (see 3.3.2.2).

#### 3.3.2.5 GIP/Exendin-4 stimulation test

The GIP/Exendin-4 stimulation test was performed in 11-week-old $GIPR^{dn}$ transgenic and control animals. Following an 18-h fasting period, 0.5 g glucose/kg BW were administered intravenously as a bolus of concentrated 50% glucose solution through the central venous catheter. Immediately after glucose administration, 80 pmol/kg BW of synthetic porcine GIP (Bachem) or 40 pmol/kg BW of synthetic Exendin-4 (Bachem) mixed with 1% of porcine serum albumin (Sigma-Aldrich) were administered intravenously. Blood samples were collected at -10, 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50 and 60

minutes relative to the glucose load. Serum glucose levels and serum insulin levels were measured as described above (see 3.3.2.2).

#### 3.3.2.6 Determination of serum insulin levels by radioimmunoassay (RIA)

A porcine insulin RIA kit (Millipore, Billerica, USA) was used to analyze serum insulin levels during an OGTT, IVGTT and GIP/Exendin-4 concentration and stimulation test according to the manufacturer's instructions. In this RIA, a fixed concentration of labeled tracer antigen ($^{125}$I labeled insulin) was incubated with a constant dilution of anti-porcine insulin antiserum such that the concentration of antigen binding sites on the antibody was limited. The addition of unlabeled insulin in the form of a serum sample resulted in competition between labeled tracer and unlabeled insulin for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody decreased as the concentration of unlabeled antigen increased. This was measured after separating antibody-bound from free tracer and counting the antibody-bound fraction in a γ-counter. A calibration or standard curve was set up with increasing concentrations of standard unlabeled insulin and from this curve the amount of insulin in unknown samples was calculated. All samples were measured in duplicate. Only duplicates with a coefficient of variance (CV) less than 10% were accepted.

**Table 3.6 Assay flow chart of the RIA**

| | | |
|---|---|---|
| DAY 1 | Step 1 | Add assay buffer |
| | Step 2-3 | Add insulin standard/ QC or sample of unknown IC |
| | Step 4 | Add $^{125}$I-Insulin Tracer |
| | Step 5 | Add porcine insulin antibody |
| | Step 6 | Vortex, cover and incubate 20-24 h at 4°C |
| DAY 2 | Step 7 | Add precipitating reagent |
| | Step 8 | Vortex and incubate 20 min at 4°C |
| | Step 9-11 | Centrifuge at 4°C for 20 min, decant and count pellets using a γ-counter |

QC: quality control, IC: insulin concentration

### 3.3.3 Morphological characterization of GIPR$^{dn}$ transgenic pigs

#### 3.3.3.1 Pancreas preparation

Immediately after slaughter the whole pancreas was rapidly prepared. To avoid damage of pancreatic tissue due to autolysis the organ was immediately stored on ice for final dissection of fat, blood vessels and connective tissue. Then the pancreas was weighed. Prefixation in formalin preceded further manipulations of the organ to avoid damaging of the tissue. Subsequently, the pancreas was cut into 1 cm thick slices. To perform an area-weighted subsampling of pancreas tissue for quantitative stereological analyses slices were turned to their left side and covered by a 1 $cm^2$ point-counting grid. This procedure represented volume-weighted subsampling as pancreas slices were of the same thickness. All points hitting pancreas tissue were counted. One tenth of the total number of points hitting pancreas yielded the total sample number. A random number (X) between one and the quotient of total hitting points and total sample number (Y) were defined for determination of the sites of sample collection. Pieces with a volume of 0.5 $cm^3$ were taken at the sites X+Y; X+2*Y; X+3*Y … Selected samples were placed in an embedding cassette with the right cut surface facing downwards, fixed in 4% neutral buffered formalin at room temperature overnight, routinely processed and embedded in paraffin. A series of sections of approximately 4 µm thickness was cut with a HM 315 microtome (Microm) from half of the paraffin embedded samples. Sections were mounted on 3-aminopropyltriethoxy-silane-treated glass slides for immunohistochemistry. For determination of pancreas area, all insulin stained sections as well as a sheet of millimeter paper for calibration purposes were photocopied at a final magnification of 400% on a commercial photocopier showing the complete cut surface of all pancreas slices.

### 3.3.4 Quantitative stereological analyses

Morphometric evaluation was carried out on a Videoplan® image analysis system (Zeiss-Kontron, Eching, Germany) attached to a microscope by a color video camera. Photocopies of the cut surfaces of pancreas samples served for planimetric determination of the cross-sectional area of the pancreas. Planimetric measurements of α-, β-, δ- and pp-cell areas were carried out on

immunohistochemically stained sections by circling their outlines with a cursor on a digitizing tablet of the image analysis system. A color monitor displayed images of stained cells at an 850 x final magnification. Quantitative stereological analyses were performed in 11-week-old and 5-month-old animals using the whole pancreas and in young adult (1-1.4-year-old) animals using the pancreas without the left pancreatic lobe, which was used for islet isolation in former studies. The volume of the pancreas ($V_{(Pan)}$) was calculated by the quotient of the pancreas weight and the specific weight of the pig pancreas (1.07 g/cm$^3$). The specific weight was determined by the submersion method (Scherle 1970). Several morphometric parameters were defined. The volume densities of α-, β-, δ- and pp-cells in the islets ($Vv_{(\alpha\text{-cell/Islet})}$, $Vv_{(\beta\text{-cell/Islet})}$, $Vv_{(\delta\text{-cell/Islet})}$, $Vv_{(pp\text{-cell/Islet})}$) were obtained by dividing the total area of α-, β-, δ- and pp-cells in the islets by the total islet area ($A_{(Islet/Pan)}$). The total volumes of α-, β-, δ- and pp-cells in the islets ($V_{(\alpha\text{-cell, Islet})}$, $V_{(\beta\text{-cell, Islet})}$, $V_{(\delta\text{-cell, Islet})}$, $V_{(pp\text{-cell, Islet})}$) were determined as a product of the volume densities of α-, β-, δ- and pp-cells ($Vv_{(\alpha\text{-cell/Islet})}$, $Vv_{(\beta\text{-cell/Islet})}$, $Vv_{(\delta\text{-cell/Islet})}$, $Vv_{(pp\text{-cell/Islet})}$) and $V_{(Islet, Pan)}$. $V_{(Islet, Pan)}$ was calculated as the product of $Vv_{(Islet/Pan)}$ and $V_{(Pan)}$. Volume densities of the various endocrine cell types in the islets refer to the volume fraction of the particular endocrine cell type in relation to the cumulative volume of the various endocrine islet cells, thus excluding capillaries and other interstitial tissues in the islets. Further, the product of the volume density of β-cells in the pancreas (referring to β-cells in the islets and isolated β-cells in the pancreas; $Vv_{(\beta\text{-cell, Pan})}$) and $V_{(Pan)}$ resulted in the total volume of β-cells in the pancreas ($V_{(\beta\text{-cell, Pan})}$). To obtain the volume density of isolated β-cells (insulin positive single cells and small clusters of insulin positive cells not belonging to established islets) in the pancreas, the total profile areas of isolated β-cells were divided by $A_{(Pan)}$. The total volume of isolated β-cells in the pancreas ($V_{(iso\beta\text{-cell, Pan})}$), a parameter indicative of islet neogenesis, was determined as the product of $Vv_{(iso\beta\text{-cell, Pan})}$ and $V_{(Pan)}$.

**Table 3.7 Parameters determined by quantitative stereological analyses**

| | |
|---|---|
| $A_{(Pan)}$ | = determined planimetrically by circling cut surfaces of photocopied pancreas samples |
| $A_{(cell)}$ | = determined planimetrically by circling immunohistochemically stained cells |
| $V_{(Pan)}$ | = pancreas weight / specific weight of the pig pancreas (1.07 g/cm$^3$) |
| $Vv_{(cell/Pan)}$ | = $A_{(cell)}$ / $A_{(Pan)}$ |
| $V_{(cell, Pan)}$ | = $Vv_{(cell/Pan)}$ x $V_{(Pan)}$ |
| $Vv_{(cell/Islet)}$ | = $A_{(cell)}$ / $A_{(Islet)}$ |
| $V_{(cell, Islet)}$ | = $Vv_{(cell/Islet)}$ x $V_{(Islet, Pan)}$ |

A: area; Vv: volume density; V: total volume; cell: refers to either α-, β-, δ- or pp-cells

### 3.3.4.1 Immunohistochemical procedures

The indirect immunoperoxidase technique was used for the detection of insulin, glucagon, somatostatin and pancreatic polypeptide while the indirect alkaline phosphatase (AP) method was used for the detection of insulin and cleaved caspase-3. For immunostaining of GIPR and GLP-1R the horseradish peroxidase (HRP) -labeled streptavidin-biotin (LAB) method was used while the avidin-biotin-complex (ABC) technique was used for the detection of Ki67. For all immunohistochemical stainings, sections were deparaffinized in xylene for 15 minutes, rehydrated in a descending alcohol series and washed in distilled water. Endogenous peroxidase activity was blocked by incubation of the sections using 1% hydrogen peroxide in TBS buffer (pH 7.4) for 15 minutes. After each incubation step, sections were washed in TBS buffer for 10 minutes, except after non-immune serum incubation. Following visualization of the immunoreactivity and counterstaining, sections were washed in distilled water, dehydrated in an ascending alcohol series, cleared in xylene and mounted under coverslips using Roti® Histokitt II (Roth, Karlsruhe). Antibodies, pretreatment, chromogens and counterstainings used for the different immunohistochemistries are listed in Table 3.8. All immunohistochemistries were performed in 11-week-old, 5-month-old and 1-1.4-year-old GIPR$^{dn}$ transgenic pigs and control animals.

Immunohistochemistry for GIPR and GLP-1R was carried out by Prof. Werner Amselgruber and coworkers, Institute of Anatomy and Physiology, Universtiy of Stuttgart-Hohenheim, Germany.

#### 3.3.4.2 Hemalaun/Eosin staining

Hemalaun/Eosin staining was accomplished following immunohistochemistry for the proliferation marker Ki67(see Table 3.8) to allow identification of the islets of Langerhans within pancreas tissue. Sections were washed in tap water for five minutes after visualizing of the immunoreactivity for Ki67 using 3,3`-diaminobenzidine tetrahydrochloride dehydrate. For nuclear staining slides were incubated in Meyer`s Hemalaun solution for four minutes. Subsequently, sections were washed with tap water for five minutes and dipped four to five times in eosin solution. Sections were cleared in distilled water, dehydrated in an ascending alcohol series, cleared in xylene and mounted under coverslips using Roti® Histokitt II (Roth, Karlsruhe).

#### 3.3.4.3 Immunofluorescence

The indirect immunfluorescence method was used for a double immunohistochemical staining for the proliferation marker Ki67 and insulin. The sections were deparaffinized in xylene for 15 minutes, rehydrated in a descending alcohol series and washed in distilled water. Microwave heating for 20 minutes in citrate buffer (pH 6.0) was accomplished for antigen demasking. Following a cool-down period of 20 minutes endogenous peroxidase activity was blocked by incubation of the sections using 1% hydrogen peroxide in TBS buffer (pH 7.4) for 15 minutes. After washing the sections for 10 minutes in TBS they were incubated with normal donkey serum (dilution 1:10) for 30 minutes to reduce non-specific binding. The primary antibody for Ki67 (monoclonal mouse anti-human Ki67; dilution 1:8) and insulin (polyclonal guinea pig anti-porcine insulin; dilution 1:1000) were mixed with the appropriate amount of TBS buffer, applied on slides and incubated for 60 minutes. Then sections were washed again in TBS buffer for 10 minutes and thereafter incubated for one hour protected from light with a mixture of the secondary antibodies for Ki67 (FITC-conjugated donkey anti-mouse IgG; dilution 1:500) and insulin (Cy 3-conjugated donkey anti-guinea pig IgG; dilution 1:500) in TBS buffer containing 5% (vol/vol)

porcine serum. Then, sections were washed for 10 minutes in TBS for the last time, followed by counterstaining and mounted under coverslips using Vectashield Mounting Medium with DAPI for nuclear staining. Sections were stored at 4°C protected from light.

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**Table 3.8 Immunohistochemical procedures**

| Antigen | Pretreatment | Serum | Primary antibody | Dilution | Secondary antibody | Dilution | CM | Chromogen | Counterstaining |
|---|---|---|---|---|---|---|---|---|---|
| **GIPR** | X | Goat | Rabbit anti-human GIPR, polyclonal | 2 µg/ml | Goat anti-rabbit IgG, biotinylated | 1:400 | LAB | DAB | Hemalaun |
| **GLP-1R** | X | Goat | Rabbit anti-human GLP-1R, polyclonal | 2 µg/ml | Goat anti-rabbit IgG, biotinylated | 1:400 | LAB | DAB | Hemalaun |
| **Insulin** | X | Rabbit | Guinea pig anti-porcine insulin, polyclonal | 1:1000 | Rabbit anti-guinea pig IgG, HRP-conjugated | 1:50 | X | DAB | Hemalaun |
| **Glucagon** | 20 min MW (citrate buffer pH 6.0) | Pig | Rabbit anti-human glucagon, polyclonal | 1:50 | Pig anti-rabbit IgG, HRP-conjugated | 1:50 | X | DAB | Hemalaun |
| **Somatostatin** | 20 min MW (citrate buffer pH 6.0) | Pig | Rabbit anti-human somatostatin, polyclonal | 1:50 | Pig anti-rabbit IgG, HRP-conjugated | 1:50 | X | DAB | Hemalaun |
| **Pancreatic Polypeptide** | X | Pig | Rabbit anti-human pancreatic polypeptide, polyclonal | 1:100 | Pig anti-rabbit IgG, HRP-conjugated | 1:50 | X | DAB | Hemalaun |
| **Ki67 (+ Insulin)** | 20 min MW (citrate buffer pH 6.0) | Goat | Mouse anti-human Ki67, monoclonal | 1:8 | Goat anti-mouse IgG, biotinylated | 1:200 | ABC | DAB | Hemalaun/Eosin (Ki67) |
| | | | Guinea pig anti-porcine insulin, polyclonal | 1:1000 | Goat anti-guinea pig IgG, AP-conjugated | 1:100 | | BCIP/NBT | Hemalaun (Ki67 + Insulin) |
| **Cleaved Caspase-3 + Insulin** | 30 min steamer (citrate buffer pH 6.0) | Horse | Rabbit anti-human cleaved caspase-3, polyclonal | 1:200 | Donkey anti-rabbit IgG, AP-conjugated | 1:100 | X | BCIP/NBT | Nuclear Fast Red |
| | | | Guinea pig anti-porcine insulin, polyclonal | 1:1000 | Donkey anti-guinea pig IgG, HRP-conjugated | 1:200 | | DAB | |

CM: complex method; DAB: 3,3`diaminobenzidine tetrahydrochloride, BCIP/NBT: 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium; MW: microwave

#### 3.3.4.4 Proliferation rate of islet cells

Proliferating cells were detected by immunohistochemical staining for the proliferation marker Ki67 (see Table 3.8). Hemalaun/Eosin counterstaining allowed identification of the islets of Langerhans wihtin pancreatic tissue. Sections were screened for islet profiles. Immunostained-Ki67-positive (proliferating) as well as not immunostained islet cell nuclei were counted. Only nuclei with a clearly visible cell membrane were implied for the counting of not immunostained islet cell nuclear profiles. To screen for immunostained-Ki67-positive nuclei a 20 x objective lens was used while verification was carried out with a 40 x objective lens. A minimum of $10^4$ islet cells per animal was included in the quantification. Islet cell proliferation index was defined as the number of immunolabeled cell nuclei divided by the total number of cell nuclei counted, and expressed as the number of immunolabeled Ki67 positive cell nuclei per $10^5$ nuclear profiles.

#### 3.3.4.5 Proliferation/apoptosis rate of β-cells

Proliferation/apoptosis rates of β-cells were determined by double immunohistochemical staining for insulin and the proliferation marker Ki67 or the apoptosis marker cleaved caspase-3 (see Table 3.8). To verify the assumption that the number of nuclear profiles per β-cell area does not differ significantly from the number of nuclear profiles per islet area immunostained nuclear profiles of one section of 16 randomly selected animals of the three age groups were counted and compared to the corresponding results of the nuclear profile quantification using the H.E. stained sections. As no marked difference was detected, the counted number of nuclear profiles of this section was adjusted to the total β-cell area of all sections evaluated for the determination of β-cell proliferation/apoptosis rate. Subsequently all insulin positive cells within all immunohistochemically stained sections were surveyed for Ki67-positive/cleaved caspase-3-positive cell nuclei using a 20 x objective lens. Verification of Ki67-positive/cleaved caspase-3-positive cell nuclei was carried out with a 40 x objective lens. A minimum of $10^4$ β-cells per animal was included

in the quantification of β-cell proliferation and apoptosis. Islet cell proliferating/apoptotic index was determined as described in 3.3.4.4.

### 3.3.5 Statistics

All data are presented as means ± SEM. The results of glucose tolerance tests as well as incretin stimulation test were statistically evaluated by analysis of variance (Linear Mixed Models; SAS 8.2; PROC MIXED), taking the fixed effects of Group (wt, tg), Time (relative to glucose or hormone application) and the interaction Group*Time as well as the random effect of Animal into account. The same model was used to compare body weight gain of $GIPR^{dn}$ transgenic and control pigs. Pancreas weight and the results of quantitative stereological analyses were evaluated by the General Linear Models (GLM) procedure (SAS 8.2) taking the effects of Group (wt, tg), Age (11 wk, 5 mo, 1-1.4 yr) and the interaction Group*Age into account. Calculation of AUCs was performed using Graph Pad Prism 4 software. Statistical significance of differences between tg and wt pigs was tested using the Mann-Whitney-U test in combination with an exact test procedure (SPSS 16.0). P values less than 0.05 were considered significant.

# 4 Results

## 4.1 Identification of GIPR$^{dn}$ transgenic pigs by PCR and Southern blot analysis

GIPR$^{dn}$ transgenic pigs were genotyped by PCR using DNA of ear punches. A marked band was visible in GIPR$^{dn}$ transgenic pigs, while non-transgenic littermates showed no band. Genomic DNA of a wild-type pig and aqua bidest. were used as negative controls, while genomic DNA of a previously genotyped GIPR$^{dn}$ transgenic pig served as a positive control. Concurrently, a PCR using β-actin specific primers was performed in order to control DNA integrity. Two bands were visible if DNA was intact. The upper band showed the β-actin gene, while the lower one represented a pseudogene.

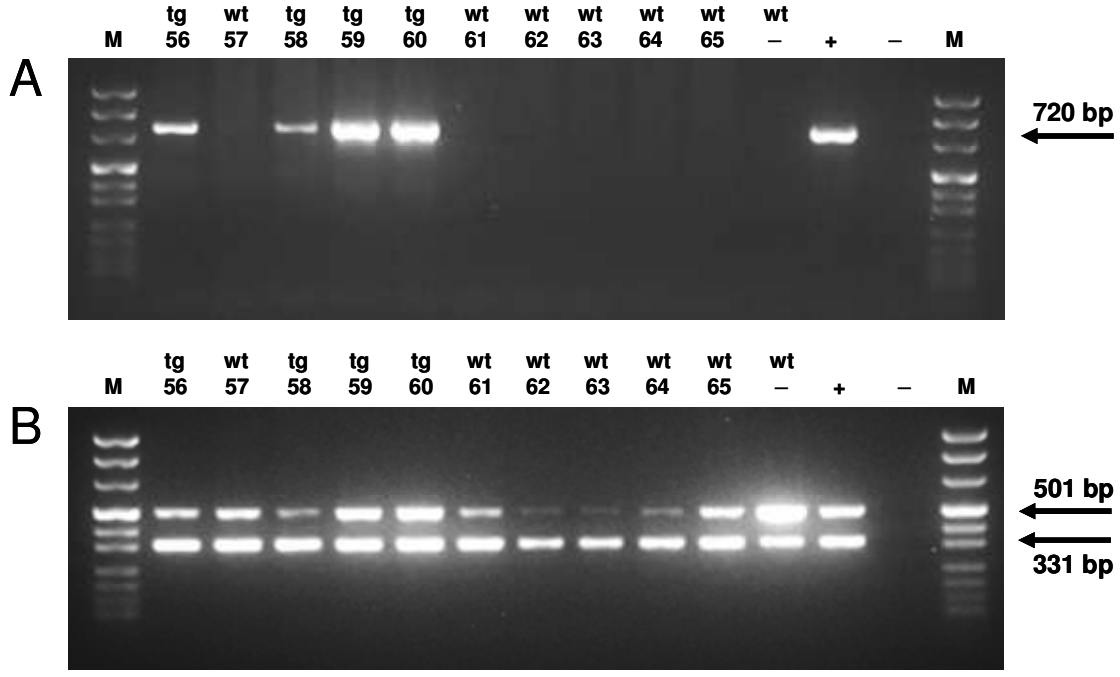


**Figure 4.1 Identification of GIPR$^{dn}$ transgenic pigs and littermate control animals by PCR analysis**

**(A)** Specific PCR analysis for the GIPR$^{dn}$ transgene; **(B)** β-actin PCR (control of DNA integrity); **(A/B)** tg: GIPR$^{dn}$ transgenic pig; wt: littermate non-transgenic pig; +: positive control (genomic DNA of a previously genotyped GIPR$^{dn}$ transgenic pig); -: negative control (genomic DNA of a wild-type pig (wt -) and aqua bidest.(-), respectively); M: pUC Mix Marker 8.

Southern blot analyses were performed to identify the number of integration sites of the GIPR$^{dn}$ construct in transgenic pigs. The founder generation of GIPR$^{dn}$ transgenic pigs was generated previously by lentiviral gene transfer. Two founder boars were selected to generate two transgenic lines. Both founders had two integration sites of the transgenic construct. Southern blot analyses of the F1-generation revealed transgenic pigs with one or two integrants proving germ line transmission and segregation according to Mendelian rules. To establish F2- and F3-generations of GIPR$^{dn}$ transgenic pigs that have been used in this study male offspring of both transgenic lines were mated to German Landrace-Swabian-Hall crossbred wildtype sows. Southern blot analyses of littermates were continued to control transmission of integrants.

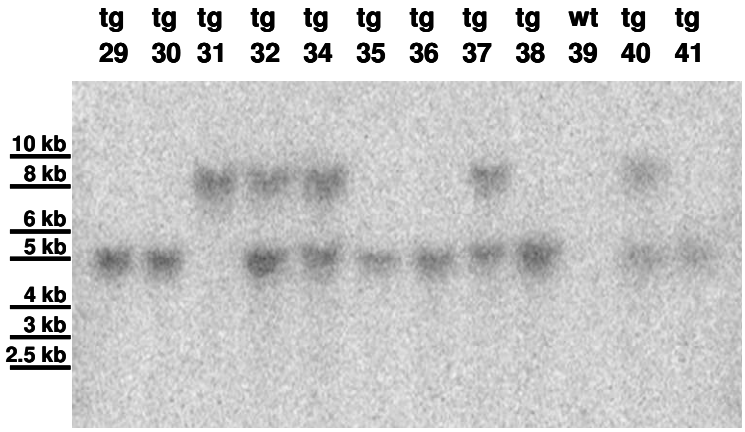


**Figure 4.2 Representative Southern blot analysis of one litter of GIPR$^{dn}$ transgenic pigs and non-transgenic littermates of the F3-generation**

Southern blot analysis of genomic DNA from EDTA blood of GIPR$^{dn}$ transgenic pigs (tg) and littermate control animal (wt); piglets show segregation of integration sites.

## 4.2 Normal body weight gain in GIPR$^{dn}$ transgenic pigs

GIPR$^{dn}$ transgenic pigs and age-matched controls were weighed regularly to clarify effects of GIPR$^{dn}$ expression on weight gain. The body weight of GIPR$^{dn}$ transgenic pigs did not differ from control animals at any time point investigated.

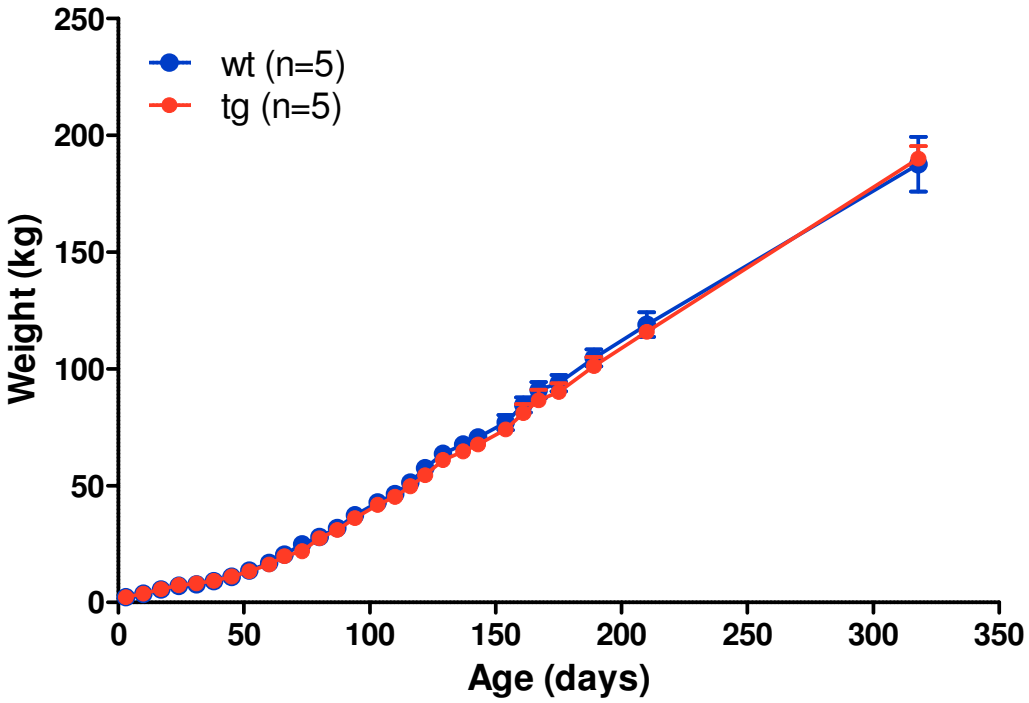


**Figure 4.3 Increase of body weight related to age**

Body weight gain of GIPR$^{dn}$ transgenic pigs (tg) compared to age-matched control pigs (wt). n: number of animals investigated. Data are means ± SEM (published in Renner et al. 2010).

## 4.3 Examination of GIPR$^{dn}$ specifity and in vivo function

To proof specific action of the GIPR$^{dn}$ in vivo, a stimulation test with glucose in combination with porcine GIP or the GLP-1R agonist Exendin-4 was performed. Previously, different GIP and Exendin-4 concentrations were tested to determine the amount of GIP/Exendin-4 needed to induce a markedly higher insulin secretion compared to the stimulation with only glucose (IVGTT).

### 4.3.1 GIP concentration test

Different GIP concentrations were tested in 11-week-old non-transgenic pigs (n=2). During the GIP concentration test a GIP bolus of 40, 80 or 160 pmol/kg BW was administered immediately after an intravenous glucose load (0.5 g/kg

BW). Prior, to receive reference values for serum glucose and insulin levels after an intravenous glucose load without bolus injection of GIP, an intravenous glucose tolerance test (IVGTT) was performed. Following a bolus injection of 40 pmol/kg BW GIP, insulin levels were almost equal compared to insulin levels after stimulation with glucose only, while the decrease of glucose levels was even slower. Bolus injection of 80 pmol/kg BW GIP caused a marked increase of insulin levels compared to an intravenous glucose load without GIP. Also, serum glucose levels were lowered nearly similar to values of the IVGTT. A 160 pmol/kg BW bolus injection of GIP had slightly less effect on insulin levels compared to the effects of an 80 pmol/kg BW bolus but markedly higher effects compared to a 40 pmol/kg BW GIP bolus. Serum glucose levels decreased most rapidly after administration of a 160 pmol/kg BW GIP bolus. As a GIP bolus of 80 pmol/kg BW resulted in markedly higher insulin levels compared to a bolus of only glucose this concentration was used for the GIP stimulation tests.

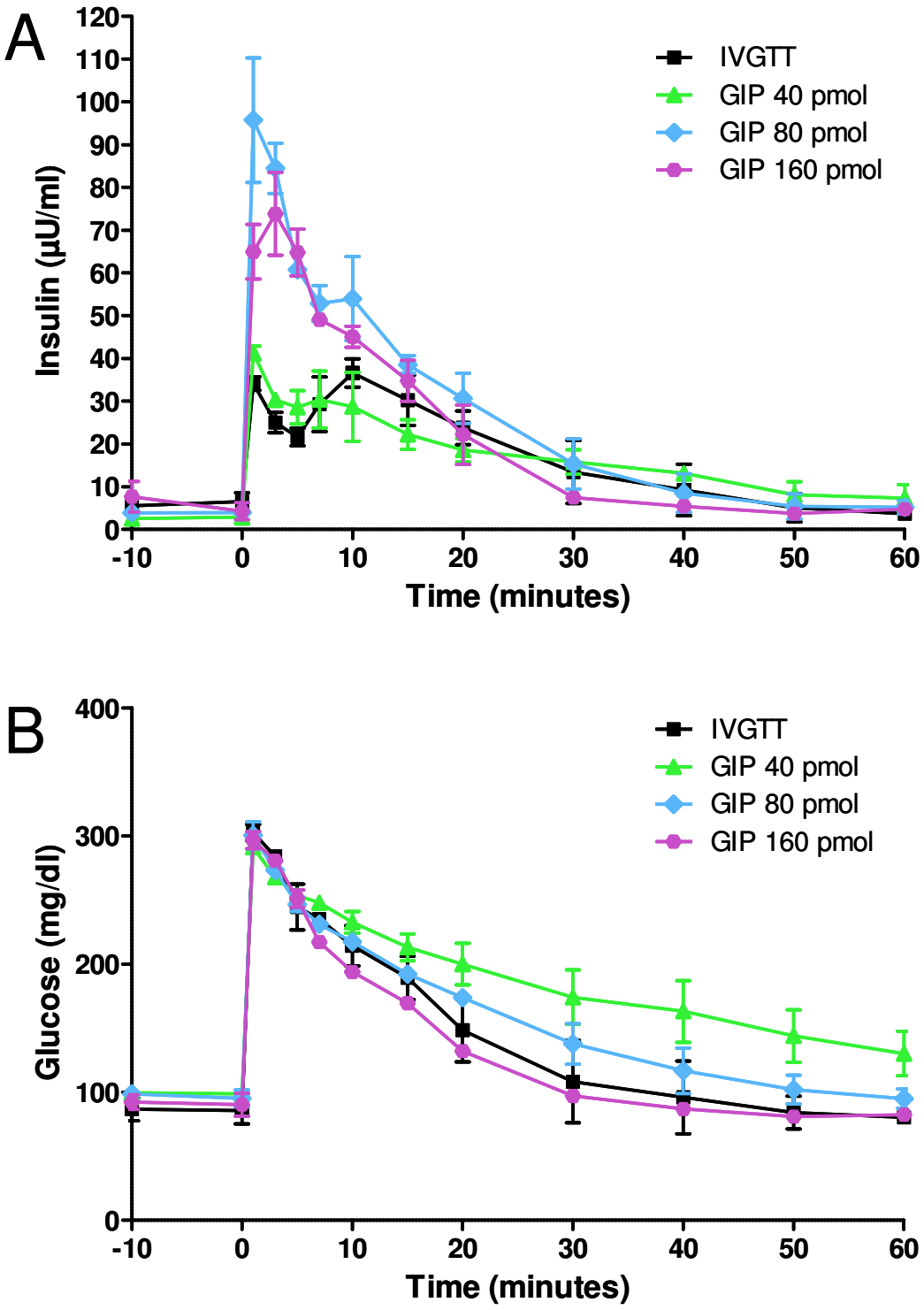


**Figure 4.4 GIP concentration test**

Intravenous glucose tolerance test (IVGTT) and IVGTT in combination with bolus application of different concentrations of synthetic porcine GIP in 11-week-old non-transgenic pigs. **(A)** serum insulin levels; **(B)** serum glucose levels. Data are means ± SEM.

### 4.3.2 Exendin-4 concentration test

Exendin-4 is a GLP-1 mimetic with equal binding affinity to the GLP-1 receptor as GLP-1 itself (Eng et al. 1992). GLP-1 has a short half-life (<2min) because of its fast degradation by the enzyme dipeptidyl peptidase IV (DPP-IV) (Deacon et al. 1995). In contrast to GLP-1, Exendin-4 is not degraded by DPP-IV involving a longer half-life, and thus provides the opportunity of a better monitoring of insulin level changes during a bolus challenge. The Exendin-4 concentration test was performed in 11-week-old non-transgenic pigs (n=2). Similar to the GIP concentration test an IVGTT was carried out to receive standard values. Exendin-4 was administrated as a bolus of 20, 40, and 60 pmol/kg BW. After a bolus injection of 20 pmol/kg BW no distinct changes in serum glucose and insulin levels were observed compared to glucose administration only, so that this concentration was excluded for further investigations. A clear increase in insulin levels was induced by Exendin-4 concentrations of 40 and 60 pmol/kg BW with insulin levels being higher following a 60 pmol/kg BW Exendin-4 bolus. Furthermore, both concentrations lowered serum glucose levels, whereas serum glucose levels after a 60 pmol/kg BW bolus injection decreased considerably below the levels of the IVGTT without Exendin-4 bolus injection while blood glucose levels following a 40 pmol/kg BW bolus were little below levels of the IVGTT. Due to the fact that the bolus administration of 40 pmol/kg BW of Exendin-4 resulted in a distinct elevation of insulin levels compared to the insulin levels during an IVGTT this concentration was selected for the Exendin-4 stimulation tests.

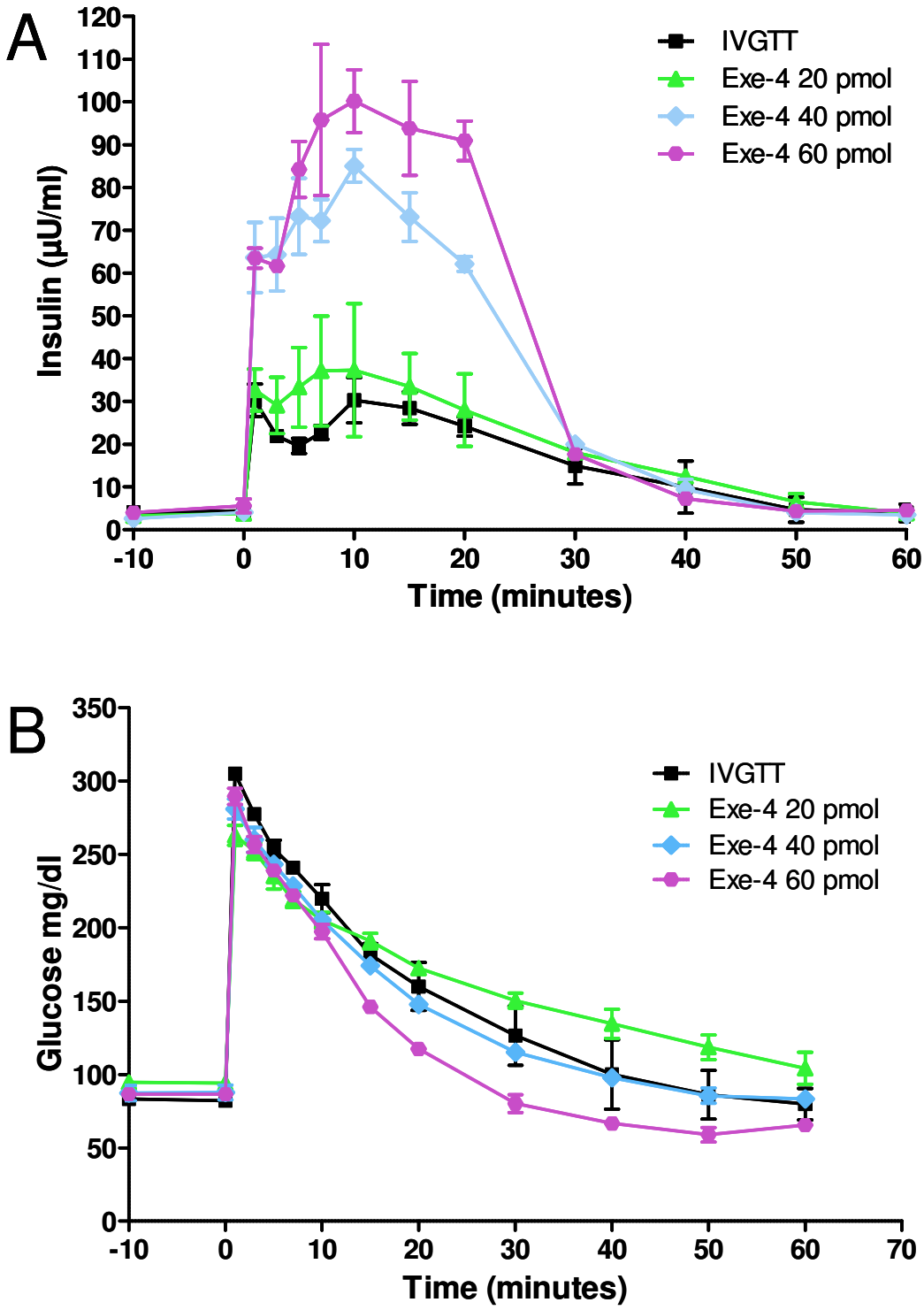


**Figure 4.5 Exendin-4 concentration test**

Intravenous glucose tolerance test (IVGTT) and IVGTT in combination with bolus application of different synthetic Exendin-4 concentrations in 11-week-old non-transgenic pigs. **(A)** serum insulin levels; **(B)** serum glucose levels. Data are means ± SEM.

### 4.3.3 Reduced insulinotropic effect of GIP in GIPR$^{dn}$ transgenic pigs

The GIPR$^{dn}$ is characterized by exhibiting equal binding affinity to GIP compared to the endogenous GIPR but prohibiting further signal transduction pathways (Herbach et al. 2005). In GIPR$^{dn}$ transgenic pigs both the endogenous GIPR and the GIPR$^{dn}$ are expressed. To evaluate whether expression of a GIPR$^{dn}$ impairs the insulinotropic action of GIP in vivo a stimulation test with exogenous administered GIP in combination with glucose was performed in 11-week-old GIPR$^{dn}$ transgenic pigs and control animals (n=4 per group). Additionally, an intravenous glucose tolerance test was carried out to receive baseline values. GIPR$^{dn}$ transgenic pigs exhibited an unaltered intravenous glucose tolerance and insulin levels compared to controls. The insulinotropic effect of GIP (80 pmol/kg BW) was significantly diminished ($p<0.01$) in GIPR$^{dn}$ transgenic vs. control pigs (Figure 4.6 A) leading to a moderately, although not significant, decelerated decrease of serum glucose levels (Figure 4.6 B). Detection of impaired insulinotropic action of GIP in GIPR$^{dn}$ transgenic pigs confirmed integrity and in vivo function of the GIPR$^{dn}$.

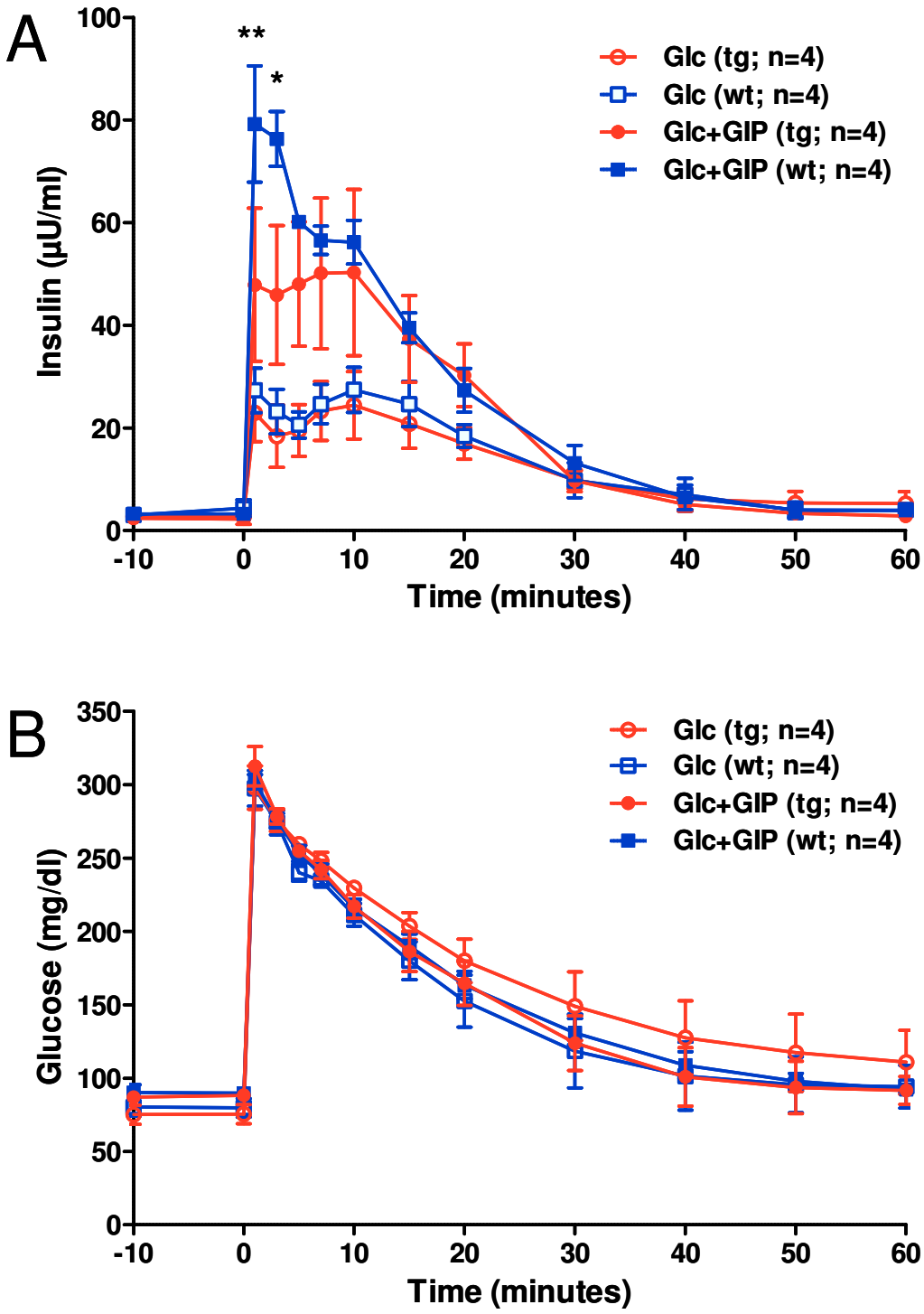


**Figure 4.6 Impaired insulinotropic action of GIP in GIPR$^{dn}$ transgenic pigs**

Intravenous glucose tolerance test (IVGTT) and IVGTT with application of 80 pmol/kg BW GIP in 11-week-old GIPR$^{dn}$ transgenic pigs (tg) and control animals (wt). **(A)** serum insulin levels; **(B)** serum glucose levels; Glc: glucose; n: number of animals investigated. Data are means ± SEM; *: $p<0.05$; **: $p<0.01$ vs. controls (published in Renner et al. 2010).

### 4.3.4 Enhanced insulinotropic effect of Exendin-4 in GIPR$^{dn}$ transgenic pigs

The Exendin-4 stimulation tests were accomplished in 11-week-old GIPR$^{dn}$ transgenic pigs and controls (n=4 per group) to evaluate the insulinotropic effect mediated by the GLP-1R and to exclude possible disruptive effects of the GIPR$^{dn}$ on GLP-1R function. Same binding affinity to the GLP-1 receptor but longer half-life of Exendin-4 vs. GLP-1 allowed a better monitoring of effects on glucose metabolism during a bolus challenge. Changes in insulin/glucose levels after an Exendin-4 and glucose bolus injection were compared to insulin/glucose levels during an intravenous glucose tolerance test without Exendin-4 application. The insulinotropic effect of Exendin-4 intravenously administered as a bolus was significantly enhanced ($p<0.01$) in GIPR$^{dn}$ transgenic pigs compared to controls (Figure 4.7 A). Insulin levels after an intravenous glucose load without Exendin-4 bolus application did not differ between both groups. Accordingly, the decline of serum glucose after a bolus injection of 40 pmol/kg BW Exendin-4 combined with glucose was significantly lower in GIPR$^{dn}$ transgenic pigs compared to the administration of glucose only ($p<0.05$), while control animals exhibited lower serum glucose levels following Exendin-4 stimulation compared to intravenous glucose application (Figure 4.7 B).

In summary, the results of both stimulation tests, GIP and Exendin-4, demonstrate that the GIPR$^{dn}$ specifically reduces insulinotropic action of GIP and does not impair the function of the related G-protein coupled GLP-1R.

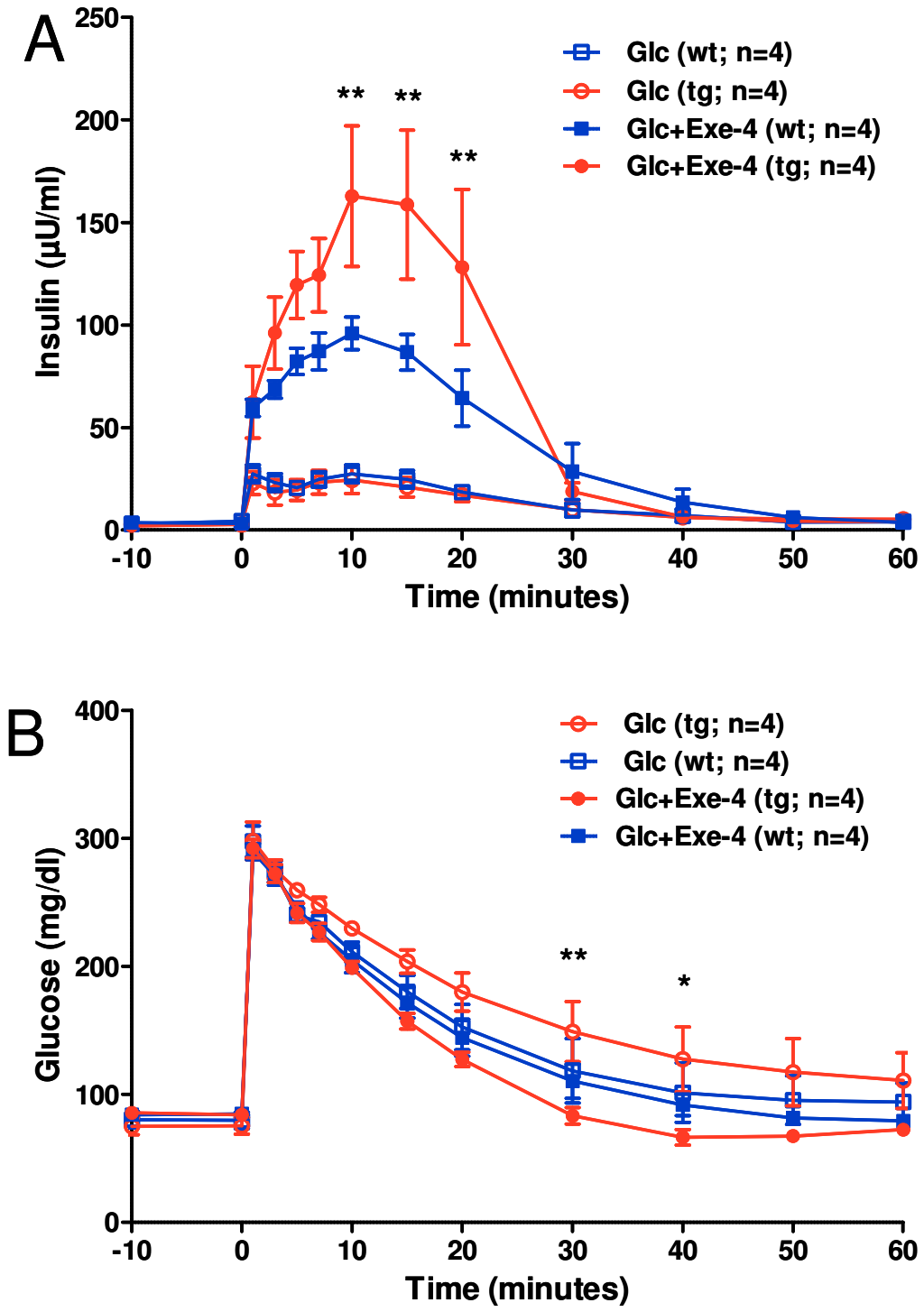


**Figure 4.7 Enhanced insulinotropic action of Exendin-4 in GIPR$^{dn}$ transgenic pigs**

Intravenous glucose tolerance (IVGTT) and IVGTT with application of Exendin-4 in 11-week-old GIPR$^{dn}$ transgenic pigs and control animals. **(A)** serum insulin levels; **(B)** serum glucose levels; Glc: glucose; n: number of animals investigated. Data are means ± SEM; *: $p<0.05$; **: $p<0.01$ vs. controls (published in Renner et al. 2010).

### 4.3.5 Unaltered immunohistochemical appearance of incretin receptors

Due to the results of the GIP/Exendin-4 stimulation tests, immunohistochemical stains for GIPR and GLP-1R were performed. Immunohistochemistry should clarify, if there were striking differences in GIPR/GLP-1R expression of $GIPR^{dn}$ transgenic pigs compared to control pigs. Immunohistochemical analysis of serial sections clearly showed, that both receptors (GIPR, GLP-1R) are selectively expressed in identical islet cells either of transgenic or wild type animals. Pancreatic exocrine cells, ductal cells, stroma cells and vascular cells were uniformly not stained with both antibodies used. Based on number and localization pattern the analysis of these serial sections indicated that the insulin producing β-cell is the labeled cell-type. At the cellular level both receptors were found to be homogenously distributed in the cytoplasm in a fine granular manner. Regional and cellular variations in the intensity of the immunoreaction could be found. Enhanced GLP-1R expression or reduced GIPR expression was not detected in 11-week-old $GIPR^{dn}$ transgenic pigs compared to controls. Hence, no correlation between the immunohistochemical signals of both receptors and the results from the GIP/Exendin-4 stimulation tests showing impaired insulinotropic action of GIP and enhanced insulinotropic action of Exendin-4, respectively, could be detected. Correspondingly, evaluation of the sections immunohistochemically stained for GIPR and GLP-1R in the other age-groups showed no apparent difference in the abundance and spatial distribution of both receptors comparing $GIPR^{dn}$ transgenic and control pigs (Figure 4.8 and Figure 4.9).

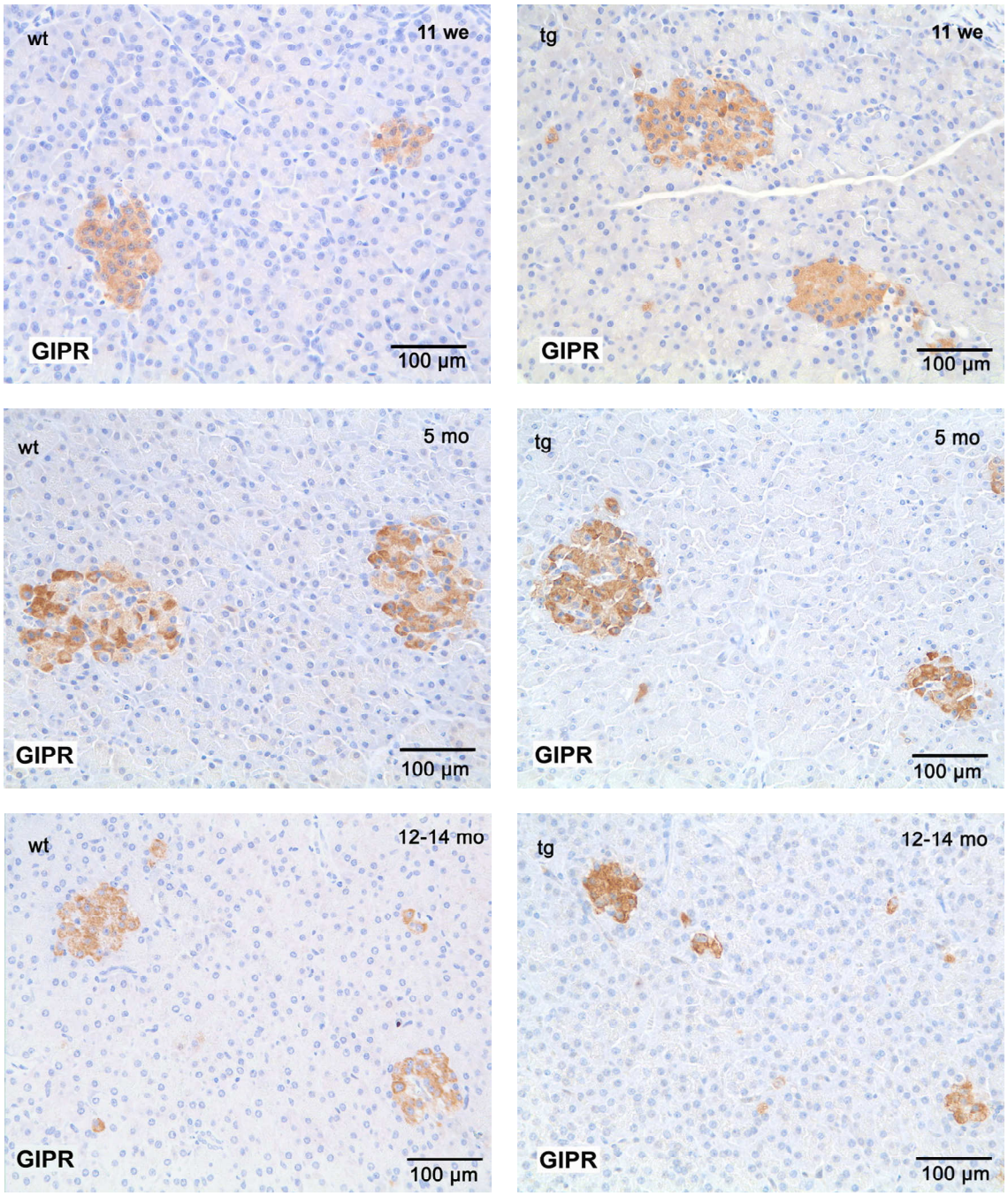


**Figure 4.8 Expression of the GIPR in GIPR$^{dn}$ transgenic pigs and controls**

Immunostaining for GIP receptor (GIPR); representative pancreas sections of a GIPR$^{dn}$ transgenic pigs (tg) and a non-transgenic control animal (wt) of all three age groups; we: weeks, mo: months; scale bar = 100 µm (published as supplementary material to Renner et al. 2010).

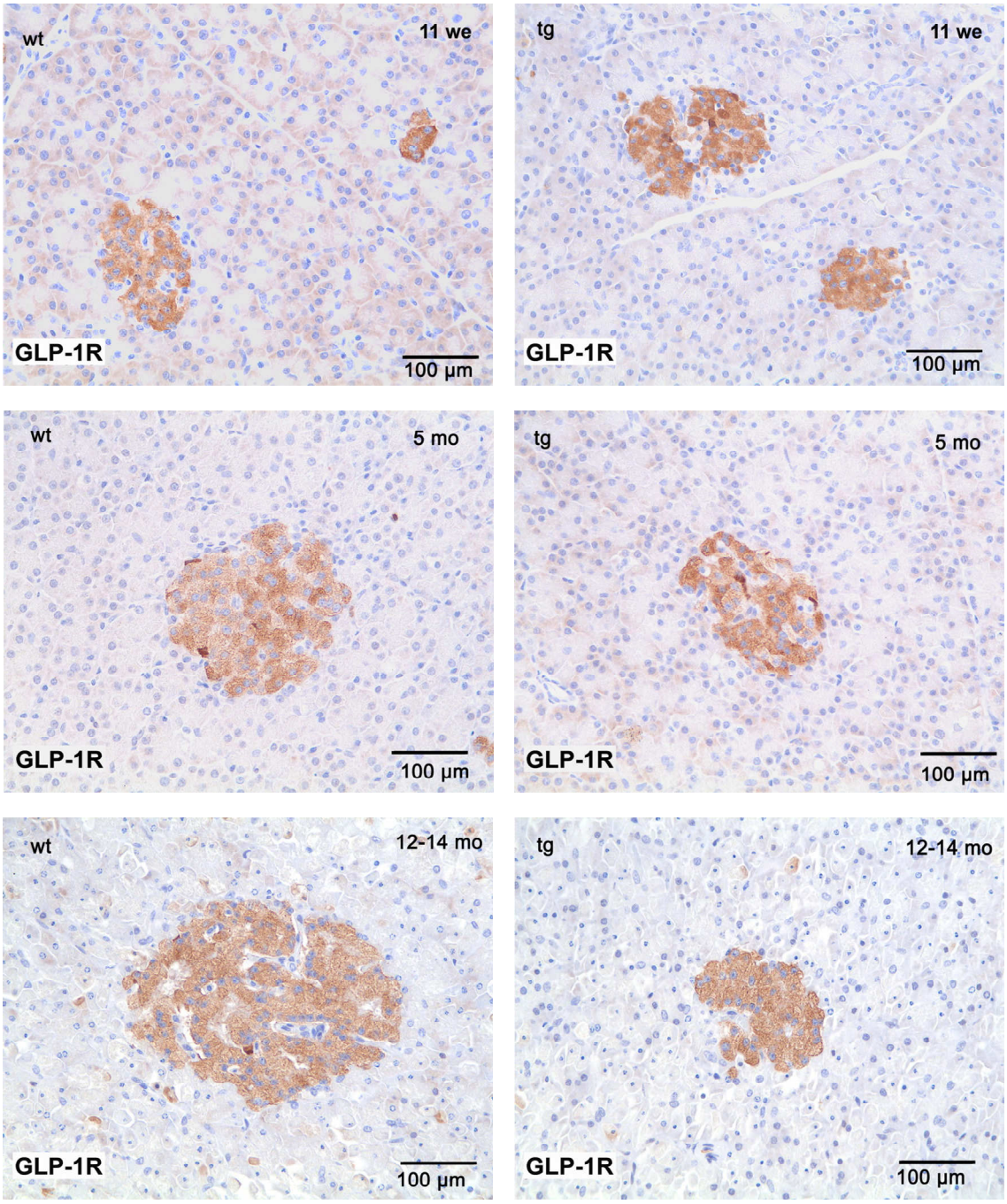


**Figure 4.9 Expression of the GLP-1R in GIPR$^{dn}$ transgenic pigs and controls**

Immunostaining for GLP-1 receptor (GLP-1R); representative pancreas sections of a GIPR$^{dn}$ transgenic pig (tg) and a non-transgenic control animal (wt) of all three age groups; we: weeks; mo: months; scale bar = 100 µm (published as supplementary material to Renner et al. 2010).

## 4.4 Results on glucose control of GIPR$^{dn}$ transgenic pigs

### 4.4.1 Disturbed oral glucose tolerance and delayed insulin secretion in 11-week-old GIPR$^{dn}$ transgenic pigs

The oral glucose tolerance test (OGTT) is used to obtain information on the body's ability in glucose metabolism and clearing excess of glucose from the bloodstream. The incretin hormones GIP and GLP-1 are released from the enteroendocrine cells into the bloodstream after ingestion and are estimated to account for up to 60% of the total insulin secretion (Nauck et al. 1986a). At 5 months of age GIPR$^{dn}$ transgenic pigs revealed a disturbed oral glucose tolerance going along with a significantly reduced insulin secretion as has been shown previously (Renner 2008). In order to evaluate the effects of GIPR$^{dn}$ expression on oral glucose tolerance and insulin secretion in younger animals, OGTTs were performed in 11-week-old GIPR$^{dn}$ transgenic (n=5) and control pigs (n=5). GIPR$^{dn}$ transgenic pigs exhibited elevated serum glucose levels ($p<0.05$) as well as a distinct delay in insulin secretion after glucose challenge (Figure 4.10). The area under the insulin curve (AUC insulin) during the first 45 minutes following glucose challenge was 31% ($p<0.05$) smaller in GIPR$^{dn}$ transgenic pigs than in age-matched controls. However, the total amount of insulin secreted during the experimental period (i.e. total AUC insulin until 120 minutes following glucose load) load was not different between the two groups (5155 ± 763 vs. 5698 ± 625; $p=0.351$). The AUC glucose was significantly elevated during the entire measuring period comparing GIPR$^{dn}$ transgenic and non-transgenic pigs ($p<0.05$). These findings indicate that expression of a GIPR$^{dn}$ in the pancreatic islets of transgenic pigs is sufficient to interfere with the incretin effect, but does initially not affect the total AUC insulin.

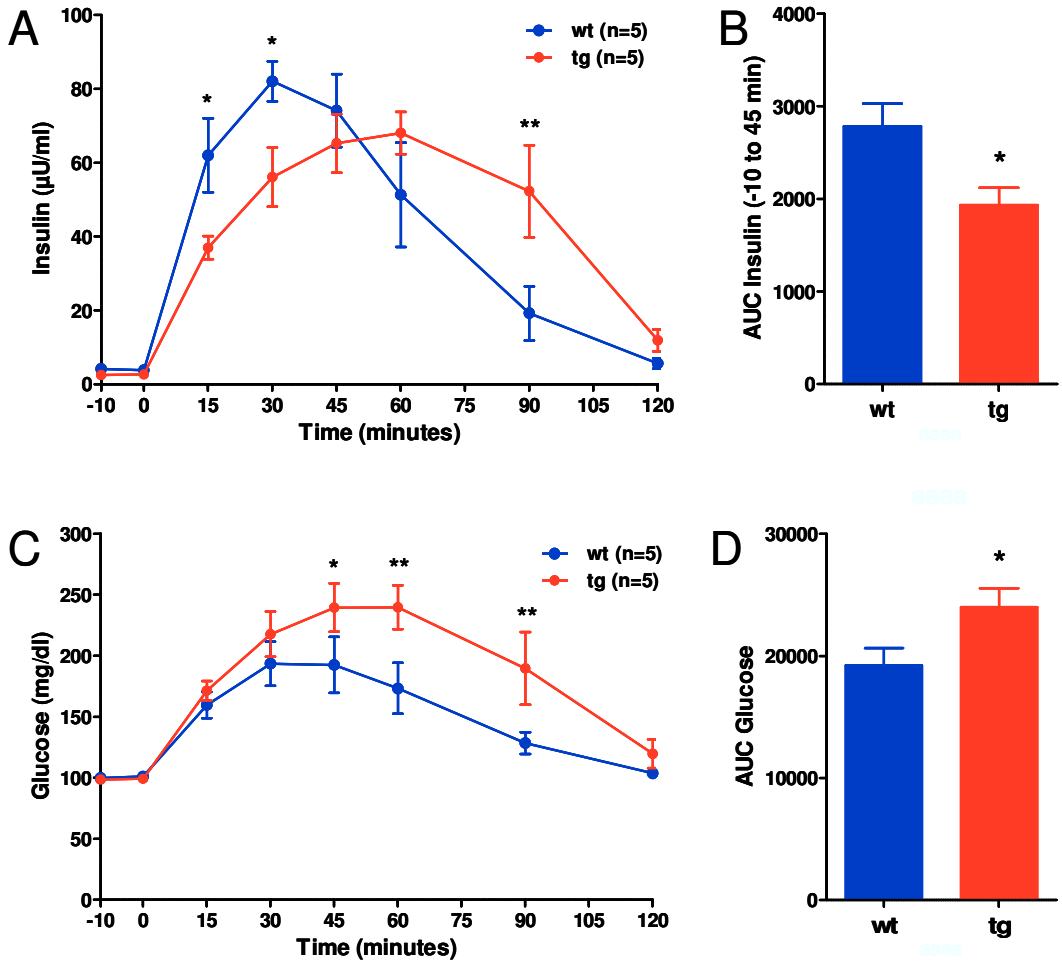


**Figure 4.10 Disturbed oral glucose tolerance in 11-week-old GIPR$^{dn}$ transgenic pigs**

Oral glucose tolerance and insulin secretion in 11-week-old GIPR$^{dn}$ transgenic pigs (tg) compared to non-transgenic pigs (wt). **(A)** serum insulin levels; **(B)** AUC insulin during the first 45 minutes; **(C)** serum glucose levels; **(D)** AUC glucose; **(A/C)** 0 minutes = point of glucose administration; n: number of animals investigated; **(B/D)** AUC: area under the curve. Data are means ± SEM; *: $p<0.05$; **: $p<0.01$ vs. controls (published in Renner et al. 2010).

### 4.4.2 Undisturbed intravenous glucose tolerance in 11-week-old and 5-month-old GIPR$^{dn}$ transgenic pigs

The intravenous glucose tolerance test (IVGTT) offers the possibility to evaluate glucose tolerance and insulin secretion by-passing the incretin effect. Additionally, the IVGTT is not subject to variations in glucose resorption rate like the oral glucose tolerance test. It has been shown previously that 11-month-old GIPR$^{dn}$ transgenic pigs have a disturbed intravenous glucose tolerance ($p<0.05$) as well as significantly diminished insulin secretion ($p<0.05$) (Renner 2008). To further characterize the GIPR$^{dn}$ transgenic pig model an IVGTT was performed in 11-week-old (n=5) and 5-month-old (22.5 ± 1.5 weeks; n=4) GIPR$^{dn}$ transgenic pigs and their age-matched controls (n=6/4). Eleven-week-old GIPR$^{dn}$ transgenic pigs exhibited no differences in serum glucose levels at each determined point of time following intravenous administration of 0.5 g/kg BW of concentrated 50% glucose solution compared to their controls ($p<0.633$) (Figure 4.11 C). Accordingly, the time course and amount of insulin secreted were not different between the two groups ($p<0.644$) (Figure 4.11 A). Determination of the area under the insulin curve (AUC insulin) revealed no alterations in GIPR$^{dn}$ transgenic pigs compared to controls ($p<0.644$) (Figure 4.10 B). Correspondingly, the area under the glucose curve (AUC glucose) did not differ between the two groups ($p<0.633$) (Figure 4.11 D). Serum glucose levels of 5-month-old GIPR$^{dn}$ transgenic pigs were only little elevated but significant differences could not be observed in comparison to glucose levels of control animals (Figure 4.12 C). Determination of the AUC glucose reflected this observation ($p<0.165$) (Figure 4.12 D). Although insulin secretion of 5-month-old GIPR$^{dn}$ transgenic pigs following an intravenous glucose load was lower compared to controls (Figure 4.12 A) no significance was reached. Likewise the AUC insulin showed no significant difference ($p<0.106$) comparing GIPR$^{dn}$ transgenic pigs and controls (Figure 4.12 B). However, a tendency towards reduced intravenous glucose tolerance and reduced insulin secretion in 5-month-old transgenic pigs was visible.

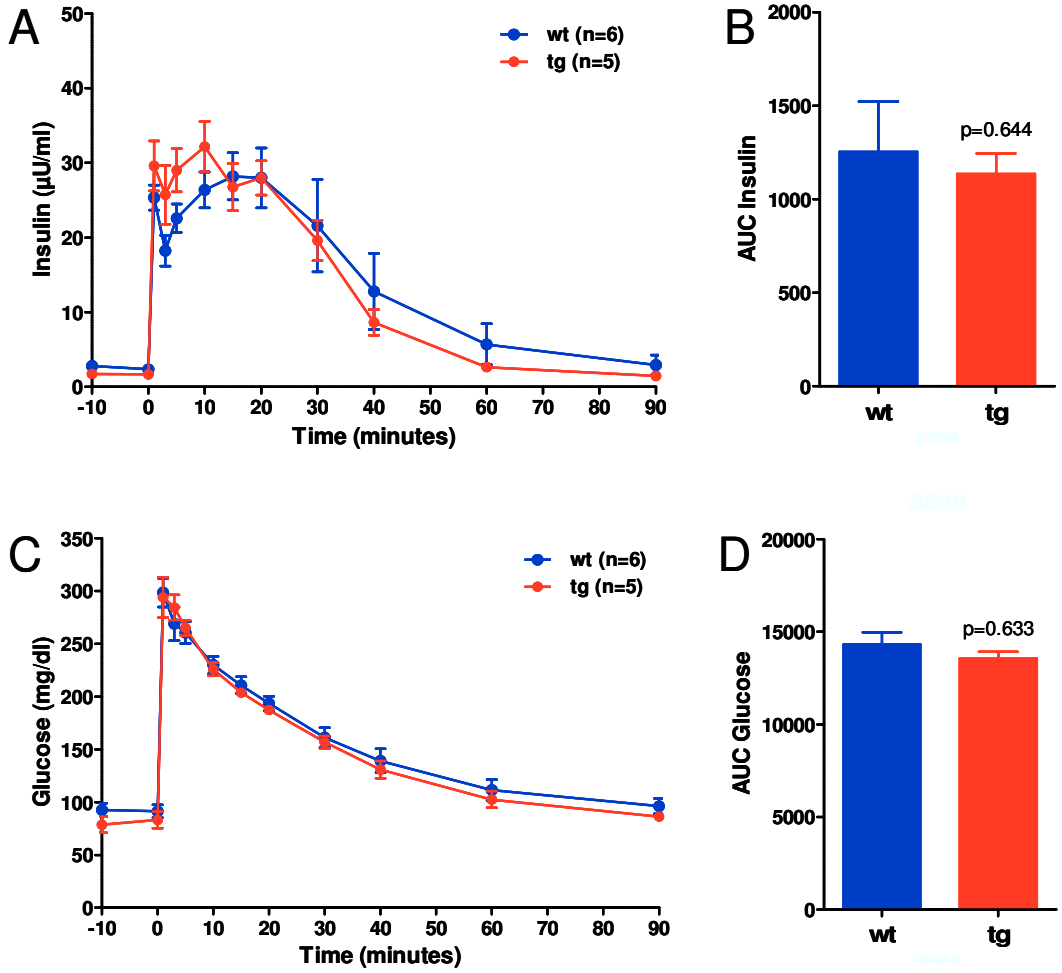


**Figure 4.11 Undisturbed intravenous glucose tolerance in 11-week-old GIPR$^{dn}$ transgenic pigs**

Intravenous glucose tolerance and insulin secretion in 11-week-old GIPR$^{dn}$ transgenic pigs (tg) compared to age-matched controls (wt). **(A)** serum insulin levels; **(B)** AUC insulin; **(C)** serum glucose levels; **(D)** AUC glucose; **(A/C)** 0 minutes = point of glucose administration; n: number of animals investigated; **(B/D)** AUC: area under the curve. Data are means ± SEM (published in Renner et al. 2010).

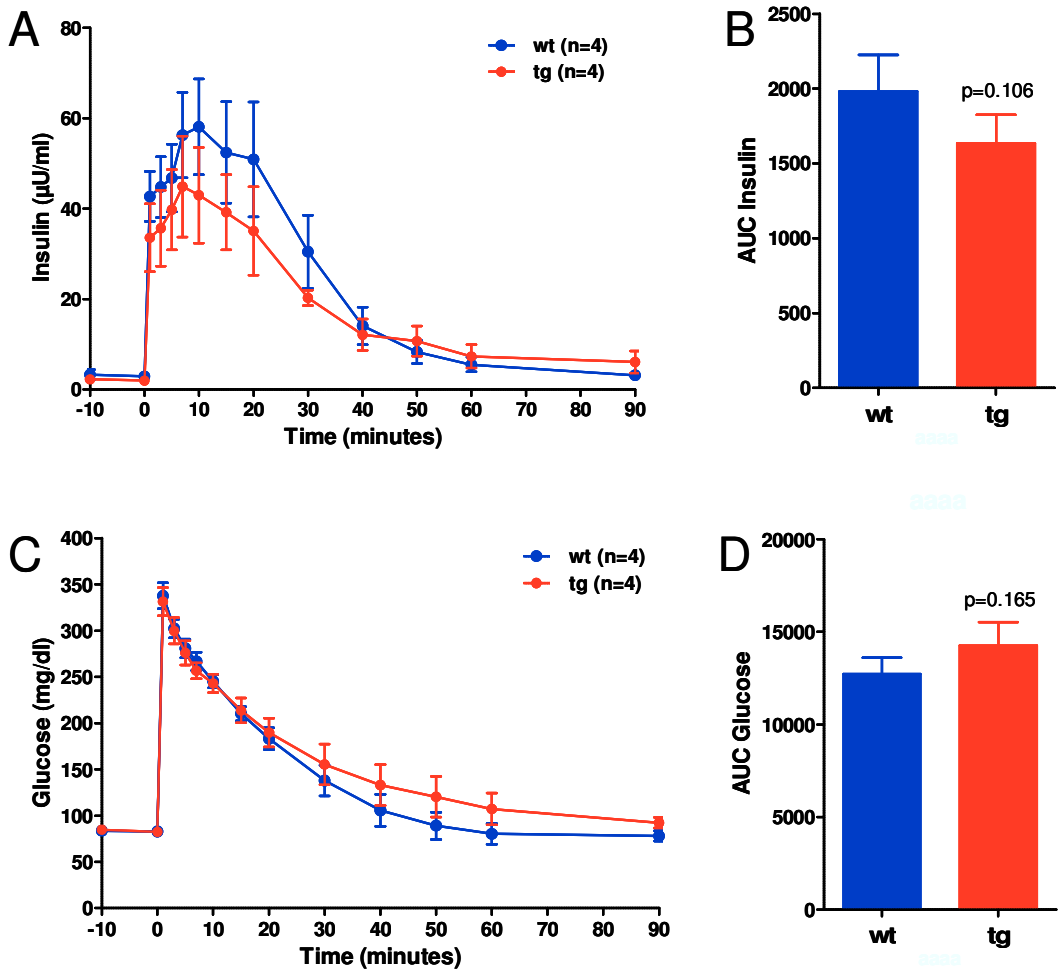


**Figure 4.12 Tendency towards reduced insulin secretion and disturbed IVGT in 5-month-old GIPR$^{dn}$ transgenic pigs**

Intravenous glucose tolerance test of 5-month-old GIPR$^{dn}$ transgenic pigs (tg) compared to age-matched controls (wt): **(A)** serum insulin levels; **(B)** AUC insulin; **(C)** serum glucose levels; **(D)** AUC glucose; **(A/C)** 0 minutes = point of glucose administration; n: number of animals investigated; **(B/D)** AUC: area under the curve. Data are means ± SEM (published in Renner et al. 2010).

## 4.5 Results of morphological analyses

### 4.5.1 Impaired age-related expansion of pancreatic β-cell mass in GIPR$^{dn}$ transgenic pigs

To clarify short-term as well as long-term effects of GIPR$^{dn}$ expression on the islets of Langerhans, quantitative stereological analyses of pancreata from 11-week-old, 5-month-old and 1-1.4-year-old GIPR$^{dn}$ transgenic pigs and controls were performed, whereby the parameters volume density of β-cells in the pancreas ($Vv_{(\beta\text{-cell/Pan})}$) and total β-cell volume ($V_{(\beta\text{-cell, Pan})}$) were determined. Additionally, volume density and total volume of isolated β-cells ($Vv_{(iso\beta\text{-cell/Pan})}$ and $V_{(iso\beta\text{-cell, Pan})}$) as a parameter for islet neogenesis were evaluated (Petrik et al. 1999; Xu et al. 1999). In 11-week-old GIPR$^{dn}$ transgenic pigs (n=5) neither the total volume of β-cells ($p<0.843$) nor the volume density of β-cells in the pancreas ($p<0.430$) differed from controls (n=5). At the age of 5 months the total β-cell volume ($V_{(\beta\text{-cell, Pan})}$) in the pancreas was diminished by 35% ($p<0.05$) in the group of GIPR$^{dn}$ transgenic pigs (n=4) compared to controls (n=4). Also, the volume density of β-cells in the pancreas ($Vv_{(\beta\text{-cell/Pan})}$) was reduced significantly ($p<0.05$) by 41%. Total β-cell volume as well as the volume density of β-cells in the pancreas of 1-1.4-year-old GIPR$^{dn}$ transgenic pigs and their controls (n=3 per group) has been previously examined by quantitative stereological analyses (Renner 2008). Here, total β-cell volume as well as the volume density of β-cells in the pancreas was found to be significantly reduced by 71% respectively ($p<0.05$). To raise the validity of these analyses two more animals per group were investigated. Including these additionally analyzed animals, GIPR$^{dn}$ transgenic pigs exhibited a significantly reduced total β-cell volume ($V_{(\beta\text{-cell, Pan})}$) by 56% ($p<0.01$) as well as a significantly diminished volume density of β-cells ($Vv_{(\beta\text{-cell/Pan})}$) in the pancreas by 61% ($p<0.01$) (Figure 4.13 A/B). Notably, total β-cell volume of non-transgenic pigs expanded markedly with increasing age (6-fold from 11 weeks to 5 months of age; 1.5-fold from 5 months to 1-1.4 years of age). In contrast, total β-cell volume of GIPR$^{dn}$ transgenic pigs rose between 11 weeks and 5 months of age (4-fold) but almost no further increase could be detected between 5-month-old and 1-1.4-year-old GIPR$^{dn}$ transgenic pigs (Figure 4.13 A). Volume density of β-cells in the pancreas increased from 11 weeks of

age up to 5 months of age by 1.5-fold in control animals while 5-month-old GIPR$^{dn}$ transgenic pigs showed no increase compared to 11-week-old transgenic pigs. 1-1.4-year-old GIPR$^{dn}$ transgenic pigs revealed a decreased volume density of β-cells in the pancreas (62%) compared to the other two age-groups while volume density of β-cells in the pancreas of 1-1.4-year-old control pigs was almost equal compared to 5-month-old-pigs (Figure 4.13 B). Volume density as well as the total volume of isolated β-cells (single insulin positive cells and small β-cell clusters) were not different between GIPR$^{dn}$ transgenic pigs and age-matched non-transgenic controls neither at 11 weeks of age (p=0.840/p=0.695) and 5 month of age (p=0.686/p=0.883) nor at 1-1.4 years of age (p=0.225/p=0.844). The volume density of isolated β-cells in the pancreas was highest in 11-week-old GIPR$^{dn}$ transgenic pigs and controls (tg=0.16%; wt=0.15%) compared to that of 5-month-old (tg=0.10%; wt=0.11) and 1-1.4-year-old (tg=0.07%; wt=0.08%) GIPR$^{dn}$ transgenic pigs and controls (Figure 4.13 D). The total volume of isolated β-cells was lowest in 11-week-old and highest in 5-month-old GIPR$^{dn}$ transgenic and control pigs (Figure 4.13 C). Correspondingly to quantitative-stereological analyses, qualitative histological assessment of pancreas sections revealed that pancreatic islet profiles of 5-month-old and 1-1.4-year-old GIPR$^{dn}$ transgenic pigs appeared smaller in size and reduced in number while there was no difference in 11-week-old transgenic pigs compared to controls. Representative pancreas sections from GIPR$^{dn}$ transgenic pigs and non-transgenic control animals are shown in Figure 4.14. In summary, these analyses revealed an impaired age-related expansion of β-cell mass leading to a significant reduction of β-cell mass 5-month-old and 1-1.4-year-old GIPR$^{dn}$ transgenic pigs.

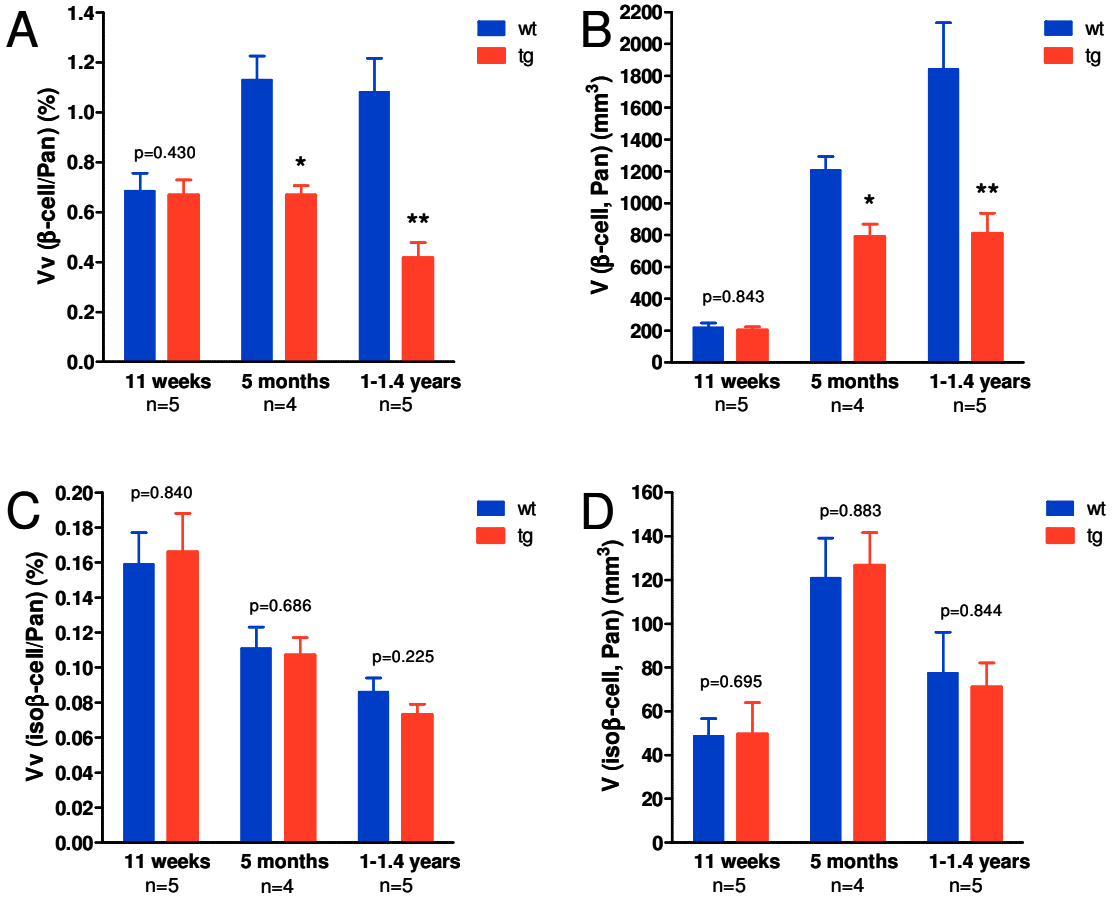


**Figure 4.13 Quantitative stereological analyses of GIPR$^{dn}$ transgenic pigs**

**(A/B)** Volume density (Vv $_{(\beta\text{-cell/Pan})}$) and total volume (V $_{(\beta\text{-cell, Pan})}$) of the β-cells in the pancreas; **(C/D)** volume density (Vv $_{(iso\beta\text{-cell/Pan})}$) and total volume (V $_{(iso\beta\text{-cell, Pan})}$) of isolated β-cells in the pancreas; tg: GIPR$^{dn}$ transgenic pigs; wt: non-transgenic control pigs; n: number of animals investigated per age group. Data are means ± SEM; *: $p<0.05$; **: $p<0.01$ (published in Renner et al. 2010).

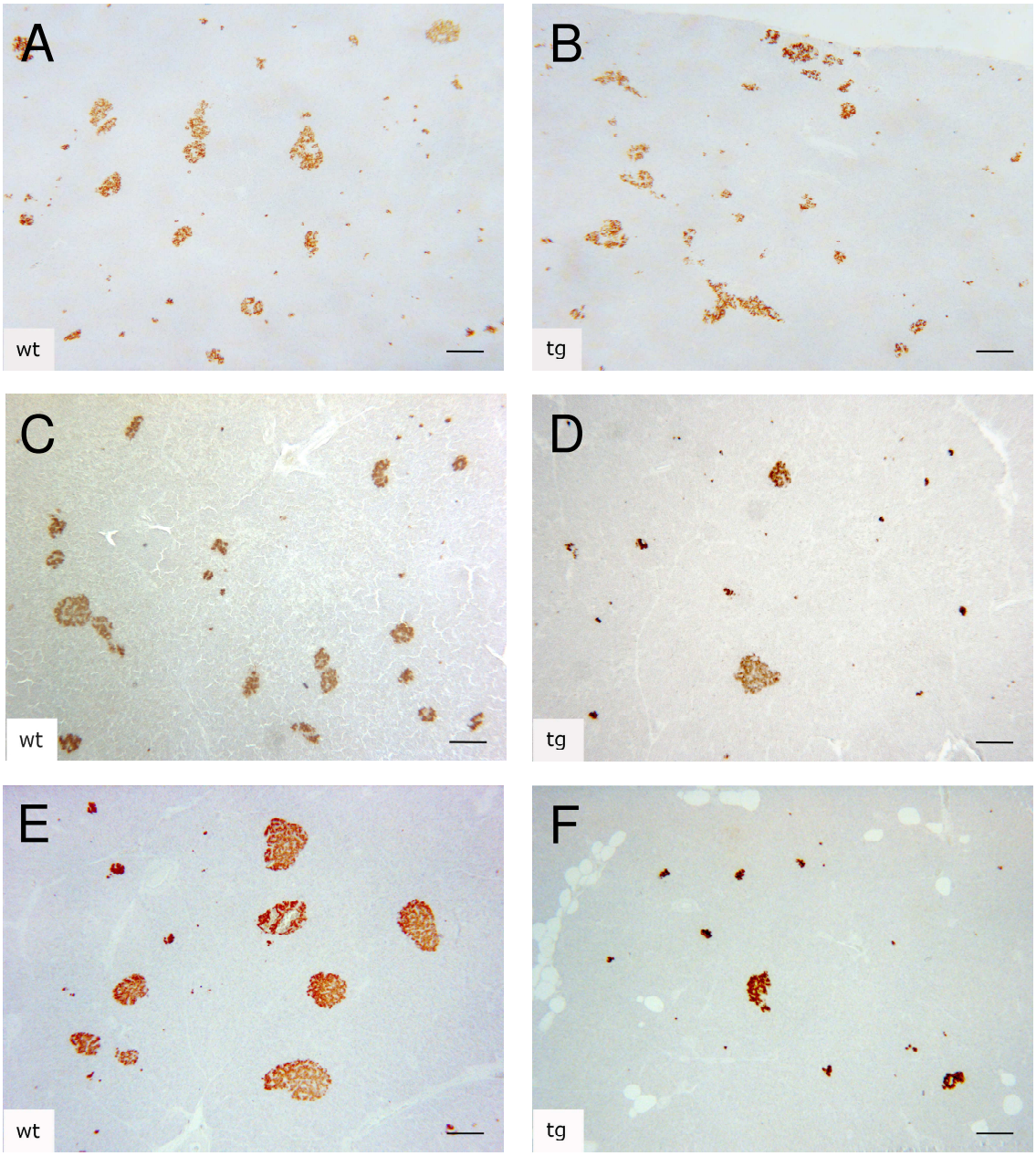


**Figure 4.14 Age-related reduction of β-cell mass in GIPR$^{dn}$ transgenic pigs**

Representative histological sections of pancreatic tissue immunohistochemically stained for insulin of a control (wt) and a GIPR$^{dn}$ transgenic pig (tg); **(A/B)** 11-week-old pigs; **(C/D)** 5-month-old pigs **(E/F)** 1-1.4-year-old pigs; scale bar = 200 µm (published in Renner et al. 2010).

### 4.5.2 Altered cellular composition of islets in GIPR$^{dn}$ transgenic pigs

Due to the knowledge of reduced total β-cell volume in GIPR$^{dn}$ transgenic pigs with increasing age, fractions of α-, δ- and pp-cells in the islets were investigated by quantitative stereological analyses. Additionally, the distribution of the different endocrine cells within the islets was evaluated qualitatively. Islets were defined as the cumulative volume of the various endocrine islet cells, thus excluding capillaries and other interstitial tissues in the islets. Immunohistochemistry was accomplished for each endocrine islet fraction. A similar age-dependent increase in total volumes of α-, δ- and pp-cells in established islets was seen in GIPR$^{dn}$ transgenic pigs and their age-matched controls. However, in comparison to controls, the increase of total β-cell volume of GIPR$^{dn}$ transgenic pigs was less pronounced from 11 weeks to 5 months of age. Importantly, there was no further augmentation of total β-cell volume in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs compared to 5 months of age, demonstrating that impaired GIPR function interferes with the physiological expansion of pancreatic β-cells. In addition, the fractional volume of β-cells in the islets ($V_{(\beta\text{-cell, Islet})}$) was decreased, while that of α- and δ-cells was increased in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs. However, the total volumes of these non-β-cell populations were not different from those of age-matched control pigs. Qualitative evaluation of the distribution of endocrine cells within the islets revealed insulin positive cells as the major fraction of the different endocrine cell types. Insulin positive cells were located in the central area of the islets. Glucagon positive cells (α-cells) represented the second most numerous endocrine fraction within the islet encircling them peripherally with some single cells being localized in the center of the islet. Commonly, somatostatin immunoreactive cells (δ-cells) were distributed in the periphery of the islet with a few isolated cells being spread over the islet. The appearance of pancreatic polypeptide positive cells (pp-cells) varied between the sections of each animal revealing no pp-cells in some sections up to even clusters of pp-cells in other sections. In general pp-cells were localized in the periphery of the islet or as single cells in the exocrine pancreas representing the least numerous fraction of the endocrine cell types. Representative immunohistochemical pictures are shown in Figure 4.15, Figure 4.16 and Figure 4.17.

**Table 4.1 Quantitative stereological analyses of the endocrine pancreas of GIPR$^{dn}$ transgenic pigs (tg) and wild-type control pigs (wt)**

| Parameter | Group | 11 wk (n = 5 wt, 5 tg) | | 5 mo (n=4 wt, 4 tg) | | 1-1.4 yr (n = 5 wt, 5 tg) | | Analysis of Variance | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | mean | SEM | mean | SEM | mean | SEM | Group | Age | Group*Age |
| Pancreas weight [g] | wt | 34.5 | 4.2 | 115.3 | 5.6 | 183.4 | 13.8 | n.s. | <0.0001 | n.s. |
| | tg | 32.7 | 3.1 | 125.7 | 6.1 | 206.1 | 4.9 | | | |
| $V_{V(\beta\text{-cell/Islet})}$ [%] | wt | 69.8 | 2.2 | 89.0 | 1.5 | 90.2 | 1.2 | 0.0066 | <0.0001 | 0.0024 |
| | tg | 70.8 | 1.3 | 87.0 | 1.2 | 76.4** | 3.2 | | | |
| $V_{V(\alpha\text{-cell/Islet})}$ [%] | wt | 14.1 | 1.2 | 5.0 | 0.8 | 5.0 | 0.7 | 0.0122 | <0.0001 | 0.0008 |
| | tg | 12.2 | 0.7 | 6.5 | 0.7 | 13.8** | 2.3 | | | |
| $V_{V(\delta\text{-cell/Islet})}$ [%] | wt | 13.5 | 2.8 | 4.3 | 0.8 | 2.2 | 0.6 | n.s. | <0.0001 | n.s. |
| | tg | 13.0 | 1.0 | 5.5 | 0.9 | 5.8* | 0.9 | | | |
| $V_{V(pp\text{-cell/Islet})}$ [%] | wt | 2.7 | 0.7 | 1.8 | 0.3 | 2.8 | 0.8 | n.s. | 0.0355 | n.s. |
| | tg | 3.9 | 0.5 | 1.3 | 0.3 | 3.8 | 1.1 | | | |
| $V_{(\beta\text{-cell, Islet})}$ [mm³] | wt | 168.7 | 29.5 | 1088.2 | 82.0 | 1694.6 | 251.7 | 0.0002 | <0.0001 | 0.0024 |
| | tg | 152.1 | 17.1 | 664.1* | 74.5 | 663.7** | 130.5 | | | |
| $V_{(\alpha\text{-cell, Islet})}$ [mm³] | wt | 32.6 | 8.7 | 58.4 | 6.3 | 95.7 | 17.4 | n.s. | <0.0001 | n.s. |
| | tg | 24.5 | 1.7 | 47.7 | 4.5 | 112.8 | 14.5 | | | |
| $V_{(\delta\text{-cell, Islet})}$ [mm³] | wt | 26.6 | 3.9 | 49.5 | 6.1 | 36.8 | 6.3 | n.s. | 0.0014 | n.s. |
| | tg | 25.9 | 1.8 | 39.6 | 3.5 | 47.5 | 5.2 | | | |
| $V_{(pp\text{-cell, Islet})}$ [mm³] | wt | 6.0 | 1.8 | 20.7 | 2.9 | 52.4 | 12.6 | n.s. | <0.0001 | n.s. |
| | tg | 8.2 | 1.7 | 9.3 | 2.1 | 30.3 | 7.9 | | | |

(published in Renner et al. 2010)

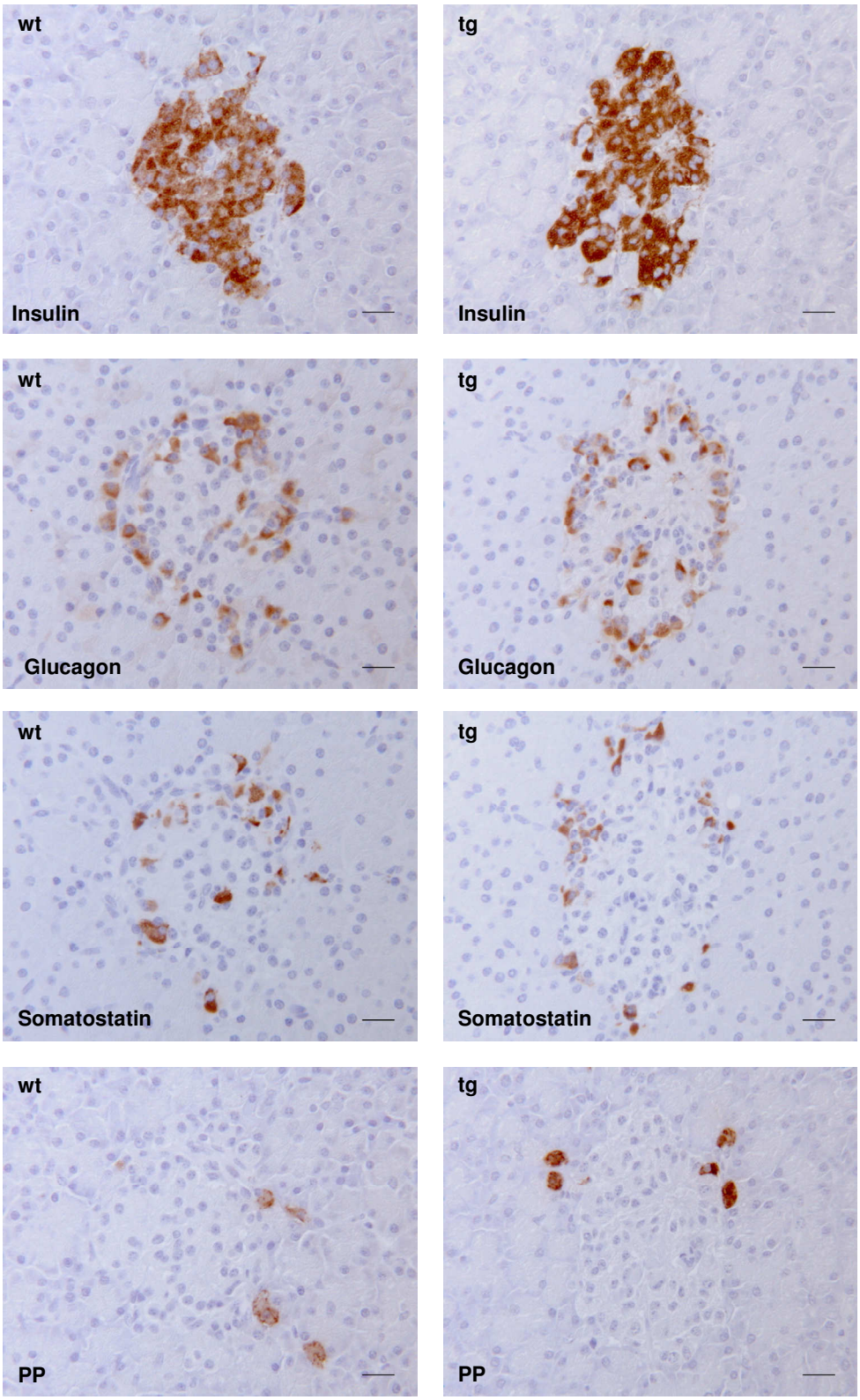


**Figure 4.15 Immunostaining of insulin, glucagon, somatostatin and pancreatic polypeptide (PP) containing cells**

Representative consecutive pancreas sections from an 11-week-old GIPR$^{dn}$ transgenic pig (tg) and a non-transgenic control pig (wt); scale bar = 20 µm (published as supplementary material to Renner et al. 2010).

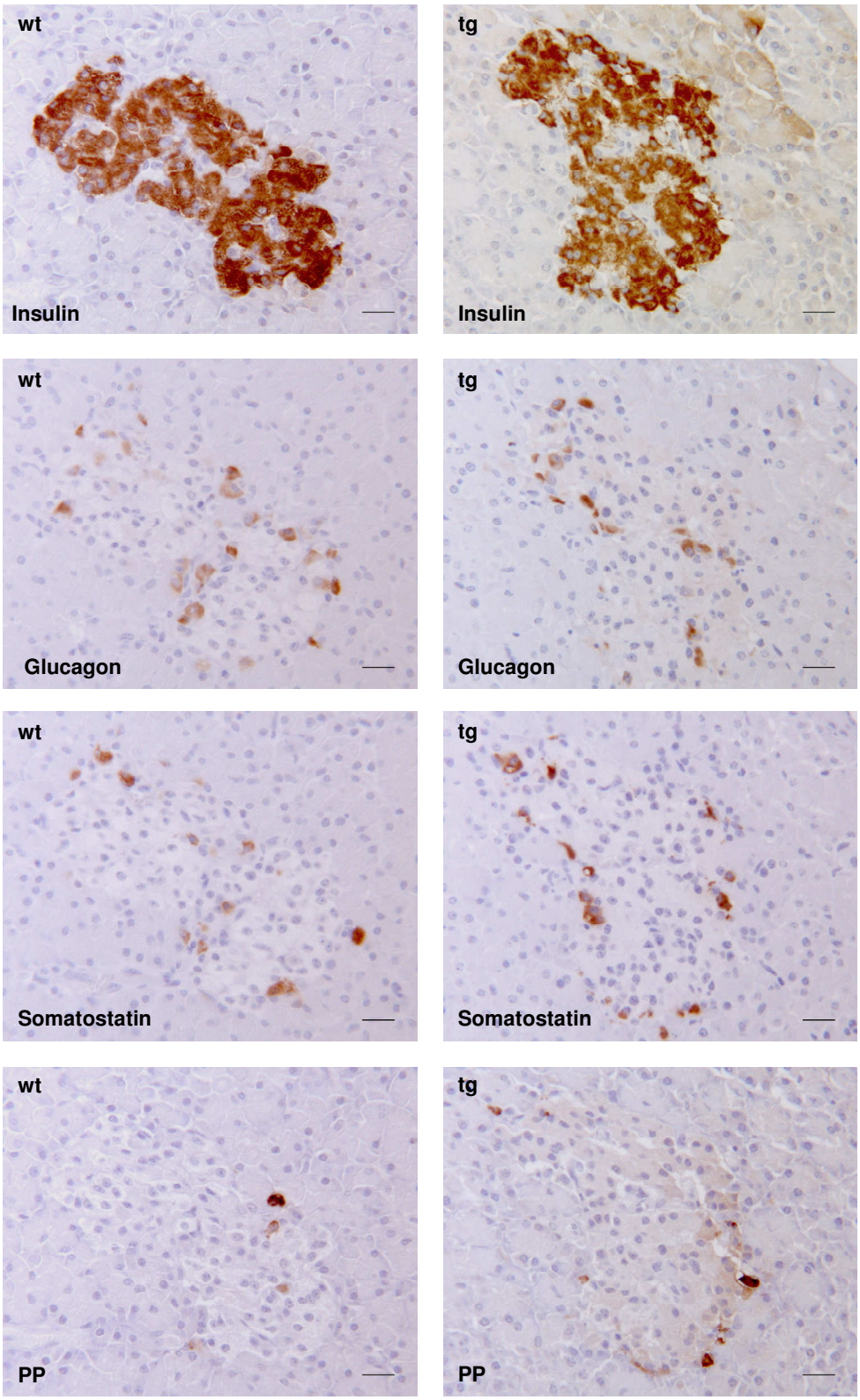


**Figure 4.16 Immunostaining of insulin, glucagon, somatostatin and pancreatic polypeptide (PP) containing cells**

Representative consecutive pancreas sections from a 5-month-old GIPR$^{dn}$ transgenic pig (tg) and a non-transgenic control pig (wt); scale bar = 20 µm (published as supplementary material to Renner et al. 2010).

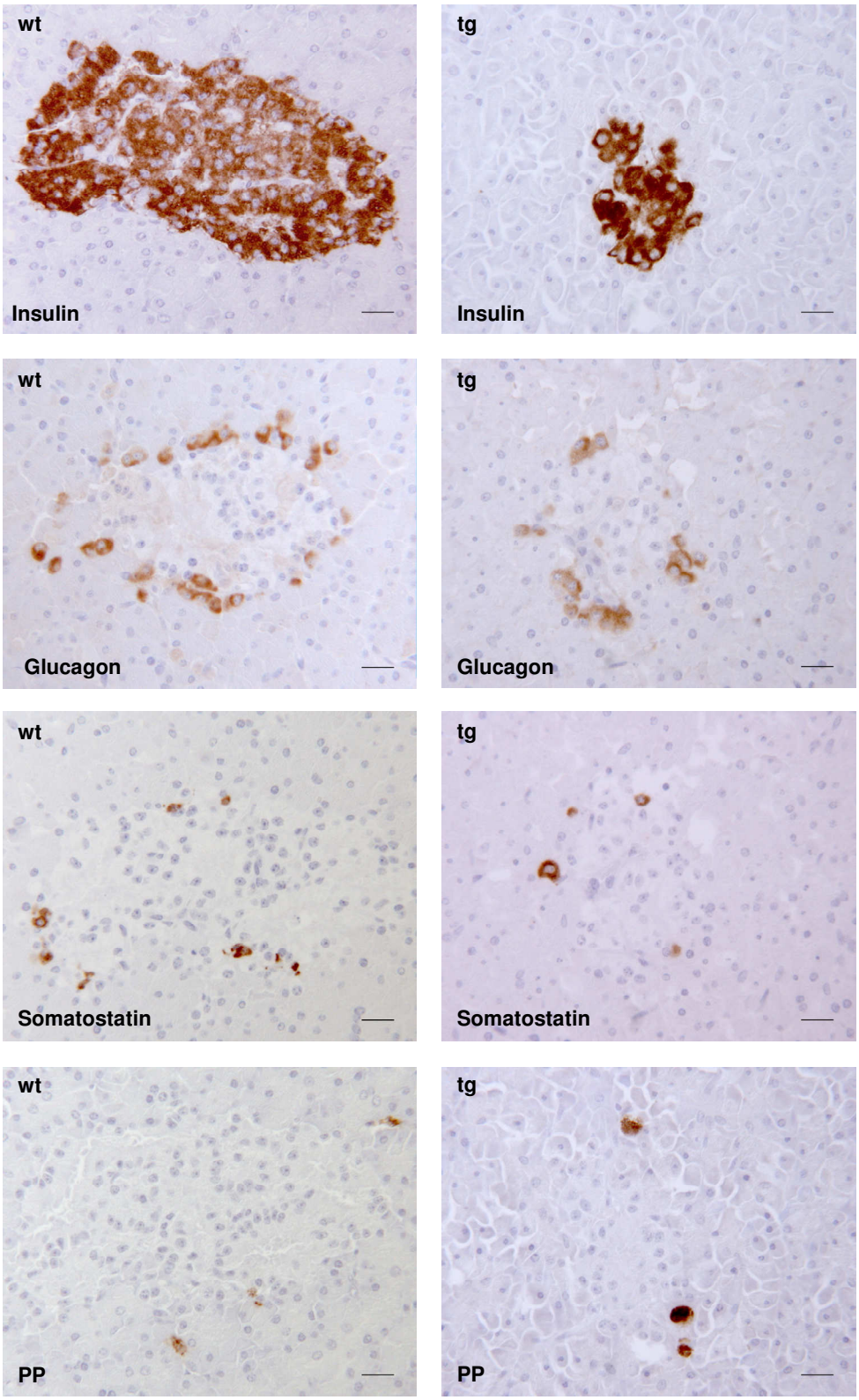


**Figure 4.17 Immunostaining of insulin, glucagon, somatostatin and pancreatic polypeptide (PP) containing cells**

Representative consecutive pancreas sections from an 1-1.4-year-old GIPR$^{dn}$ transgenic pig (tg) and a non-transgenic control pig (wt); scale bar = 20 µm (published as supplementary material to Renner et al. 2010).

### 4.5.3 Reduced proliferation rate of β-cells in GIPR$^{dn}$ transgenic pigs

In order to clarify the mechanism of impaired β-cell expansion in GIPR$^{dn}$ transgenic pigs, β-cell proliferation was determined by immunohistochemical staining for the proliferation marker Ki67. The Ki67 antigen is preferentially expressed during all active phases of the cell cycle ($G_1$, S, $G_2$ and M-phases), but it is absent in resting cells ($G_0$-phase) (Gerdes et al. 1984). The antigen is rapidly degraded as the cell enters the non-proliferative states (Scholzen et al. 2000). For double immunohistochemical staining of insulin and the proliferation marker Ki67 different variations of chromogens were tested: 3,3`diaminobenzidine (DAB) for insulin combined with Fuchsin for Ki67, Fuchsin for insulin combined with DAB for Ki67 and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) for insulin combined with DAB for Ki67. Furthermore, immunofluorescence was carried out using a FITC-conjugated secondary antibody for Ki67 and a Cy 3-conjugated secondary antibody for the detection of insulin containing cells. Combination of Fuchsin and DAB resulted in difficult identification of double positive cells for insulin and Ki67 independent which of both chromogens was chosen for the detection of Ki67 and insulin because of the high similarity between the red and the brown shade. Best results were obtained by staining insulin positive cells with BCIP/NBT and Ki67 positive nuclei with DAB. Staining by immunofluorescence revealed approximately equivalent results compared to combination of BCIP/NBT and DAB, but as technical efforts required for data analyses were considerably higher immunohistochemical staining was chosen. The proliferation rate of β-cells in double immunohistochemically stained sections was significantly reduced by 60% ($p<0.05$) in 11-week-old GIPR$^{dn}$ transgenic pigs (n=5 per group), while β-cell proliferation in 5-month-old (n=4 per group; $p<0.549$) and 1-1.4-year-old (n=5 per group; $p<0.352$) GIPR$^{dn}$ transgenic pigs was unaltered compared to age-matched controls. Comparing the age groups the total amount of proliferation rate of β-cells decreased with increasing age (Figure 4.19 A). In conclusion, analysis of β-cell proliferation rate points towards a diminished proliferation rate of β-cells as reason for reduced β-cell mass in GIPR$^{dn}$ transgenic pigs.

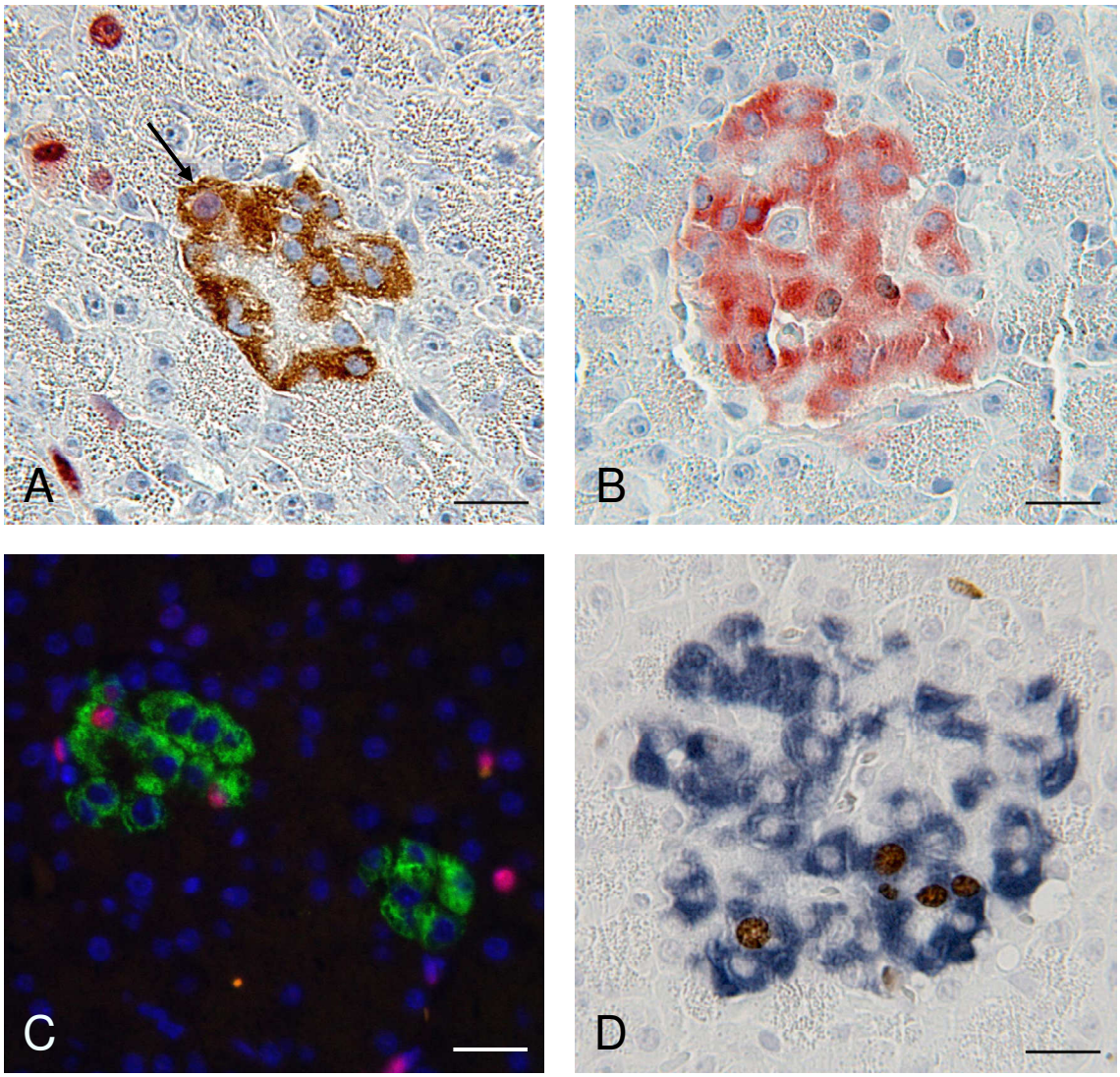


**Figure 4.18 Chromogen variations of double immunohistochemistry for insulin and the proliferation marker Ki67**

**(A)** DAB for insulin staining combined with Fuchsin for Ki67 staining; **(B)** Fuchsin for insulin staining combined with DAB for Ki67 staining; **(C)** Immunofluorescence (Insulin=green; Ki67=pink); **(D)** BCIP/NBT for insulin staining combined with DAB for Ki67 staining; pancreas sections from 11-week-old wild-type pigs served for this experiment; scale bar = 20 µm (Figure D published in Renner et al. 2010).

### 4.5.4 Reduced proliferation of islet cells in 11-week-old GIPR$^{dn}$ transgenic pigs

Single staining for the proliferation marker Ki67 in combination with a Hemalaun/Eosin counterstaining to localize islets of Langerhans allowed determination of proliferating islet cells. In 11-week-old GIPR$^{dn}$ transgenic pigs proliferation of islet cells was significantly reduced by 38% ($p<0.05$) compared to controls (n=5 per group). No differences in islet proliferation could be determined in GIPR$^{dn}$ transgenic pigs at the age of 5 months (n=4 per group; $p=0.549$) and at the age of 1-1.4 years (n=5 per group; $p=0.276$) compared to age-matched non-transgenic controls.

### 4.5.5 Tendency of more cleaved caspase-3 positive β-cells in 1-1.4-year-old GIPR$^{dn}$ transgenic pig

Changes in apoptosis rate were investigated by quantitative stereological analyses of immunohistochemically stained sections for cleaved caspase-3. Caspase-3 is one of the key factors of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins (Fernandes-Alnemri et al. 1994). Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated fragments. Cleavage of caspase-3 requires aspartic acid at the P1 position (Nicholson et al. 1995). The used cleaved caspase-3 (Asp175) antibody detects endogenous levels of the large fragment of activated caspase-3 resulting from cleavage adjacent to Asp175. Double immunohistochemistriy for cleaved caspase-3 and insulin was performed in all three age groups to evaluate a potential impact of GIPR$^{dn}$ expression on cell death in the β-cell compartment. Overall the proportion of cleaved caspase-3 positive cells was very low, with no significant difference between GIPR$^{dn}$ transgenic pigs and controls of all age classes. However, there was a trend ($p=0.075$) of more cleaved caspase-3 positive cells in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs as compared to age-matched controls.

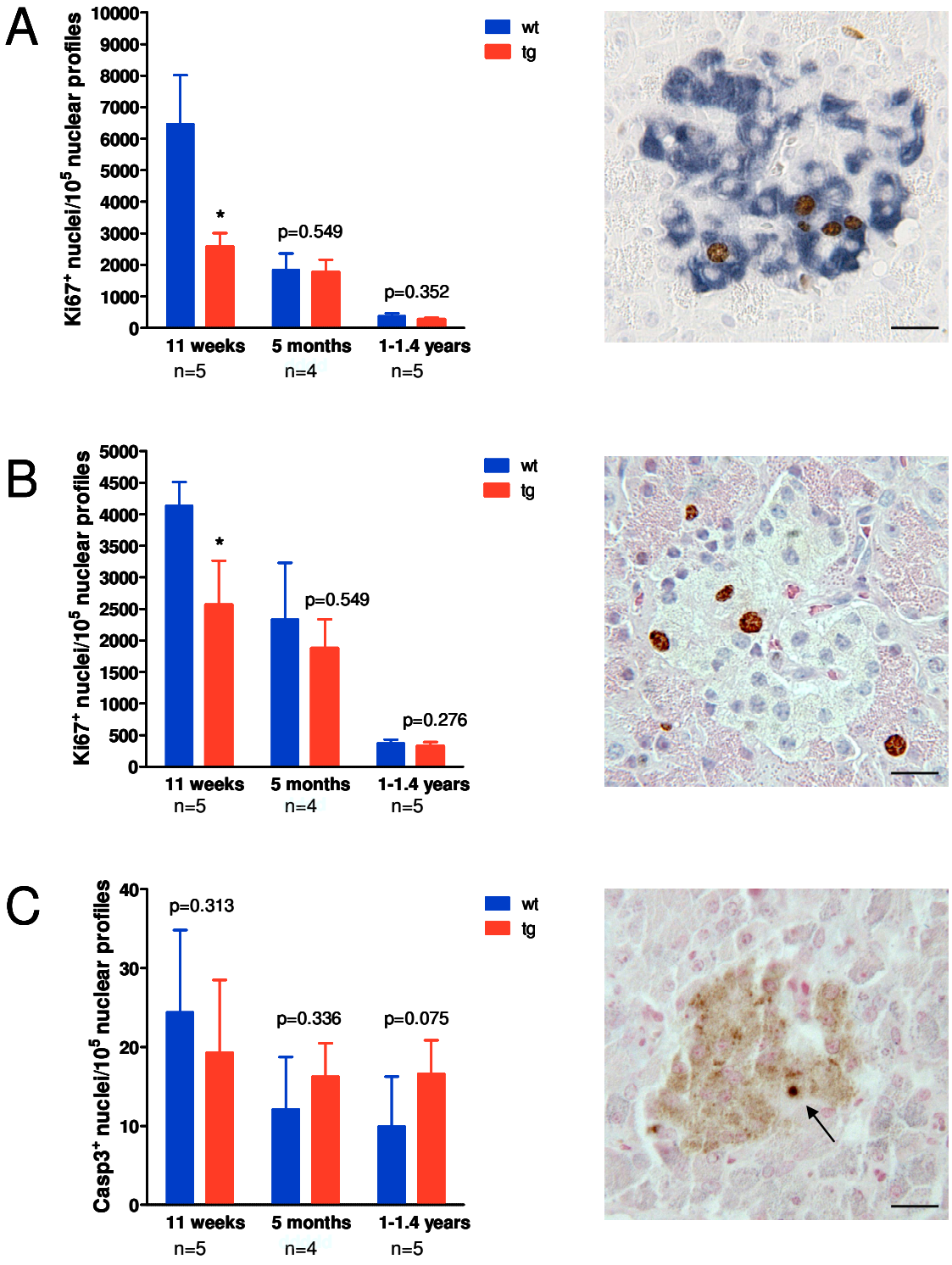


**Figure 4.19 Reduced proliferation rate of β-/islet cells in 11-week-old GIPR$^{dn}$ transgenic pigs and trend of elevated apoptosis rate of β-cells in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs**

**(A)** Proliferation rate of β-cells; representative pancreas section of double immunohistochemical staining for insulin and the proliferation marker Ki67;

**(B)** proliferation rate of islet cells; representative pancreas section of immunohistochemistry for the proliferation marker Ki67 in combination with Hemalaun/Eosin counterstaining for localization of the islets of Langerhans; **(C)** apoptosis rates of β-cells; representative pancreas section of double immunohistochemical staining for insulin and cleaved caspase-3; **(A/B/C)** tg: $GIPR^{dn}$ transgenic pigs; wt: non-transgenic control pigs; n: number of animals investigated per group; scale bar = 20 µm. Data are means ± SEM; *: $p<0.05$ (published in Renner et al. 2010).

# 5 Discussion

## 5.1 GIP/Exendin-4 concentration test

A dose-response study with three different concentrations of GIP (40, 80 and 160 pmol/kg BW) and Exendin-4 (20, 40 and 60 pmol/kg BW) was carried out to determine the amount of GIP/Exendin-4 needed to induce a markedly higher insulin secretion compared to the stimulation with only glucose. A bolus injection has the advantage to potentiate the insulin secretion more than a continuous intravenous infusion (Meier et al. 2004; Vilsboll et al. 2002) providing a better monitoring on glucose-lowering and insulin-enhancing effects of the different doses.

A dose of 40 pmol/kg BW GIP failed to distinctly enhance insulin levels compared to insulin levels following an intravenous glucose load. A distinct increase of insulin secretion was observed after a bolus injection of 80 and 160 pmol/kg BW GIP, although, unexpectedly, peak insulin levels after 80 pmol/kg BW GIP bolus injection were higher compared to a dose of 160 pmol/kg BW GIP. 160 pmol/kg BW GIP represent a very high dose compared to a GIP bolus of 80 pmol/kg BW GIP that was used in humans studies as a high dose (Meier et al. 2004) so that a receptor saturation at a concentration of 160 pmol/kg BW GIP is definitely conceivable. Furthermore, only two pigs were used to estimate the dose-dependent increase of insulin secretion wherefore one must also be aware of inaccuracy due to the small animal number. Glucose decrease after each GIP bolus concentration did not differ markedly from IVGTT without GIP bolus injection. The only slight glucose-lowering effects of GIP observed in this experiment might be due to the short half-life of GIP as GIP is rapidly degraded by the enzyme DPP-4 (Deacon et al. 2000). As the dose of 80 pmol/kg BW GIP showed a markedly increase in insulin response and the use of the two-fold amount of GIP provided no advantages, 80 pmol/kg BW was chosen for the GIP stimulation tests.

The Exendin-4 concentration test demonstrated clear dose-dependent increases of insulin secretion after bolus injection of 20, 40 and 60 pmo/kg BW

Exendin-4 compared to an intravenous glucose load without Exendin-4 bolus application. Correspondingly, serum glucose decreased more rapidly with rising Exendin-4 concentration. Although the insulin secretion after the administration of 60 pmol/kg BW Exendin-4 was higher than after 40 pmol/kg BW Exendin-4, 40 pmol/kg BW Exendin-4 was selected for the Exendin-4 stimulation tests as this concentration but not 20 pmol/kg BW resulted in a markedly elevated insulin secretion compared to an intravenous glucose load without Exendin-4 bolus. Additionally, the probability of the appearance of known side effects like vomiting and nausea were lower using 40 pmol/kg BW Exendin-4 as the appearance of side-effects is dose-dependent (Poon et al. 2005).

## 5.2 Examination of GIPR$^{dn}$ specifity

GIP/Exendin-4 stimulation tests were performed in 11-week-old GIPR$^{dn}$ transgenic pigs and control animals to clarify whether GIP signaling is reduced and GLP-1 signaling is sustained in transgenic pigs due to the expression of the GIPR$^{dn}$. GIPR$^{dn}$ transgenic pigs exhibited significantly diminished insulin levels indicating an impaired insulinotropic effect of GIP. Importantly, insulin levels after an intravenous glucose load without GIP revealed no differences between the two groups arguing against the suspicion that toxic effects due to GIPR$^{dn}$ expression are responsible for the impaired insulinotropic action of GIP in GIPR$^{dn}$ transgenic pigs. A residual function of GIPR signaling was expected because the GIPR$^{dn}$ is expressed in addition to the endogenous GIPR. Both receptors compete for GIP binding showing nearly the same binding affinity (Herbach et al. 2005; Volz 1997). In contrast, *in vitro* stimulation of isolated islets of GIPR$^{-/-}$ mice did not induce residual GIP function indicating a complete loss of GIPR signaling (Miyawaki et al. 1999). Furthermore, GIPR and GLP-1R belong to the same receptor family explaining that a supraphysiological dose of GIP might lead to an additional binding of GIP to the intact GLP-1R of GIPR$^{dn}$ transgenic pigs mediating a further increase in insulin secretion after intravenous administration of GIP and glucose (Brubaker et al. 2002; Wheeler et al. 1993).

Exendin-4 is a potent GLP-1R agonist used to demonstrate sustained GLP-1R activity in GIPR$^{dn}$ transgenic pigs. After stimulation with glucose and Exendin-4 11-week-old GIPR$^{dn}$ transgenic pigs exhibited significantly enhanced insulin secretion levels compared to controls. These results are consistent with findings in perfused pancreas and isolated islets of GIPR$^{-/-}$ mice and GLP-1R$^{-/-}$ mice where insulin responses to GLP-1 and GIP, respectively, were significantly greater in the knockout mice compared to controls (Pamir et al. 2003; Pederson et al. 1998). Hence, the enhanced insulin secretion after Exendin-4 bolus injection suggested the presence of compensatory mechanisms.

To clarify whether compensatory mechanisms comprise alterations in receptor expression immunohistochemical staining for GIPR and GLP-1R was performed. No apparent difference in the abundance and spatial distribution of both receptors were detected comparing GIPR$^{dn}$ transgenic pigs and controls. Notably, these results reflect only an impression but no quantification. The validity of quantification of immunohistology is disputable due to variables during fixation and processing and should be regarded as approximations only (Leong 2004). However, striking differences in the amount and intensity of staining can be detected by immunohistochemistry (Herbach et al. 2007).

## 5.3 Disturbed oral glucose tolerance in 11-week-old GIPR$^{dn}$ transgenic pigs

GIP and GLP-1 are responsible for up to 60% of the total amount of insulin secretion (related to C-peptide levels) after an oral glucose load whereby the degree of the incretin effect depends on the amount of glucose ingested (Nauck et al. 1986a; Nauck et al. 1986b). This observation demonstrates an important role of the incretins in glucose metabolism. Eleven-week-old GIPR$^{dn}$ transgenic pigs showed an impaired insulinotropic action of GIP due to a prohibited signal transduction after binding of GIP to the GIPR$^{dn}$ (Herbach et al. 2005; Volz 1997). A first functional consequence of the GIPR$^{dn}$ expression in the pancreas of 11-week-old GIPR$^{dn}$ transgenic pigs was a reduced oral glucose tolerance going along with significant higher serum glucose levels and delayed insulin secretion associated with a significant smaller area under the insulin curve

during the first 45 minutes compared to controls. The impaired insulinotropic action of GIP in GIPR$^{dn}$ transgenic pigs can explain the initially delayed insulin secretion. However, in 11-week-old GIPR$^{dn}$ transgenic pigs β-cell response to glucose is not diminished as the total amount of secreted insulin did not differ between transgenic pigs and controls. GIPR$^{dn}$ transgenic pigs showed an enhanced insulinotropic action of Exendin-4, a GLP-1 mimetic, in the stimulation test. An enhanced insulinotropic action of GLP-1 in GIPR$^{dn}$ transgenic pigs may compensate for the initially reduced insulin secretion and contribute in a balancing manner. In GIPR$^{-/-}$ and GLP-1R$^{-/-}$ mice such a compensatory action of the functional incretin hormone could also be observed (Flamez et al. 1999; Pamir et al. 2003; Pederson et al. 1998). No differences in the total β-cell volume and β-cell integrity of 11-week-old GIPR$^{dn}$ transgenic pigs were detected confirming that the disturbed oral glucose tolerance is the consequence of the impaired insulinotropic action of GIP. Normal fasting blood glucose and insulin levels, but altered glucose excursions that came along with significantly elevated blood glucose and reduced insulin levels after an OGTT were also observed in GIPR$^{-/-}$ mice (Miyawaki et al. 1999). Furthermore, early insulin secretion after oral glucose ingestion was impaired in GIPR$^{-/-}$ mice showing parallels to the findings in the GIPR$^{dn}$ transgenic pigs (Miyawaki et al. 1999). Insulin release after nutrient ingestion is biphasic and a defect within the first 30 minutes (as a marker for early insulin secretion) was detected to occur long before the development of diabetes (Del Prato 2003; Gerich 1997; Leahy 2005). Changes in the early insulin secretion result in an elevated postprandial rise of glucose levels (Leahy 2005) suggesting that the first phase of insulin secretion seems to be important to prime the tissues for the incoming nutrients. Moreover, GIP appeared to be the more potent incretin hormone contributing approximately 80% of the incretin induced insulin secretion (Gault et al. 2003b; Nauck et al. 1993a) wherefore an impaired action of GIP caused by GIPR$^{dn}$ expression in the islets of Langerhans can be responsible for a disturbed oral glucose tolerance as observed in 11-week-old GIPR$^{dn}$ transgenic pigs.

## 5.4 Undisturbed intravenous glucose tolerance in 11-week-old and 5-month-old GIPR$^{dn}$ transgenic pigs.

For further physiological characterization an intravenous glucose tolerance test was performed in 11-week-old and 5-month-old GIPR$^{dn}$ transgenic pigs. As previously described, 11-month-old GIPR$^{dn}$ transgenic pigs showed a disturbed intravenous glucose tolerance as well as a significantly diminished insulin secretion (Renner 2008). Eleven-week-old GIPR$^{dn}$ transgenic pigs exhibited an unaltered intravenous glucose tolerance and insulin secretion compared to controls. Five-month-old GIPR$^{dn}$ transgenic pigs also showed no significant differences in insulin secretion and serum glucose levels compared to non-transgenic pigs. However, insulin secretion tended to be lower and glucose decline was delayed in 5-month-old GIPR$^{dn}$ transgenic pigs. When glucose is administered intravenously the incretin effect is bypassed meaning that an impaired incretin effect alone cannot cause the development of a disturbed intravenous glucose tolerance in GIPR$^{dn}$ transgenic pigs. Quantitative stereological analyses revealed a significant reduction of the total β-cell volume in 5-month-old (35%) and 1-1.4-year-old (58%) GIPR$^{dn}$ transgenic pigs while total β-cell volume of 11-week-old GIPR$^{dn}$ transgenic pigs was unaltered compared to controls. These observations demonstrate that the progressive deterioration of intravenous glucose tolerance correlates with the progressive reduced expansion of β-cell mass in GIPR$^{dn}$ transgenic pigs.

In contrast, GIPR$^{-/-}$ mice showed no differences in blood glucose levels after an intraperitoneal glucose tolerance test that is comparable to an IVGTT (Miyawaki et al. 1999). However, it has to kept in mind that the incretin hormones can be released to some degree by enhanced glucose levels in the hepatoportal region following an intraperitoneal glucose load (Gallwitz et al. 1997).

## 5.5 Impaired age-related expansion of pancreatic β-cell mass in GIPR$^{dn}$ transgenic pigs

Determination of quantitative stereological parameters for β-cells were carried out using unbiased model-independent stereological methods (Gundersen et al.

1987; Gundersen et al. 1988; Kroustrup et al. 1983). In 5-month-old GIPR$^{dn}$ transgenic pigs the total β-cell volume and volume density of β-cells were significantly diminished compared to non-transgenic controls. Moreover, the growth of β-cell mass appeared to stagnate as no further increase of the total β-cell volume of 1-1.4-year-old GIPR$^{dn}$ transgenic pigs compared to the transgenic animals at 5 months of age was detected. Due to this fact this situation is described as an impaired expansion of β-cell mass. Total volume and volume density of isolated β-cells as a parameter for islet neogenesis (Petrik et al. 1999; Xu et al. 1999) were determined in all three age groups. It has been shown that besides the replication of already existing cells new β-cells or small new islets are formed by neogenesis from progenitor cells or by transdifferentiation from acinar cells (Bonner-Weir 2000; Montanya et al. 2009). GIPR$^{dn}$ transgenic pigs showed neither at the age of 11 weeks and 5 months nor at the age of 1-1.4 years differences in the total volume and volume density of isolated β-cells in the pancreas. Pigs at 5 months of age exhibited the highest total volume of isolated β-cell compared to the transgenic and non-transgenic pigs of the other two age groups. However, the total β-cell volume consists of all insulin positive cells including the single insulin positive cells and small clusters of insulin positive cells (isolated β-cells). With respect to this, the mean percentage of the total volume of isolated β-cells referring to the total β-cell volume is the highest in 11-week-old transgenic (tg) pigs and controls (wt) (tg=24.5%; wt=22.4%) and decreases with age (5 months: tg=16%; wt=10%; 1-1.4 years: tg=8.8%, wt=4.2%). These observations are in line with the age-related decrease of the volume density of the isolated β-cells in the pancreas. It has to be kept in mind to what extent isolated β-cells are a valid marker for islet neogenesis. The upper threshold to define insulin positive cells either as islets or as isolated β-cells, and therefore as markers for islet neogenesis, is not definitely determined whereas the interpretation of islet neogenesis data must be evaluated with caution. However, all analyses in GIPR$^{dn}$ transgenic pigs were carried out under the same conditions so that possible inaccuracies apply to all age groups. As the used parameters for the quantification of islet neogenesis in GIPR$^{dn}$ transgenic pigs did not differ from controls at any age

group other mechanisms like alterations in proliferation or apoptosis rate of β-cells might be responsible for the impaired expansion of β-cells in GIPR$^{dn}$ transgenic pigs (see below). Studies on the pancreatic development in rodents indicated that β-cell mass rather expands by replication than by neogenesis after the first weeks of life (Bouwens et al. 2005). Less information is available about the postnatal growth of the β-cell mass in pigs. Constant increases in β-cell mass after birth into adulthood associated by waves of apoptosis and mitosis were detected examining pancreata from pigs (Bock et al. 2003). Qualitative histological assessment of pancreas sections of all three age groups confirmed the results of the quantitative stereological analyses. Pancreatic islet profiles of 5-month-old and 1-1.4-year-old GIPR$^{dn}$ transgenic pigs appeared to be smaller in size and reduced in number indicating changes in β-cell remodeling due to GIPR$^{dn}$ expression.

## 5.6 Altered cellular composition of islets in GIPR$^{dn}$ transgenic pigs

Quantitative stereological analyses of the total β-cell volume and volume density revealed markedly reduced expansion of β-cells in GIPR$^{dn}$ transgenic pigs with increasing age. To control changes in the islet composition immunohistochemistry for α-, δ- and pp-cells were performed. The fraction of α- and δ- cells was increased in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs. Notably, only the volume densities of α- and δ- cells were altered in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs whereas the total volumes of the non-β-cells were unaltered compared to controls. This observation could be explained considering that concomitantly to the diminished β-cell fraction the total islet cell volume was reduced. Accordingly, studies in type 2 diabetic patients discovered changes in the cellular composition of the islets of Langerhans in the pancreas. Diabetic people exhibited an enhanced relative volume of α-cells going along with a reduced relative volume of β-cells (Deng et al. 2004; Yoon et al. 2003). GIPR$^{dn}$ transgenic mice showed a marked increase of the total volume of non-β-cells and a decrease of total β-cell volume compared to controls (Herbach et al. 2005). Qualitative histological assessment of randomly selected pancreas

sections of transgenic and control animals of each age group were performed to get more information about endocrine cell distribution within the islet. According to immunohistochemical studies of minipig pancreata insulin positive cells represented the major fraction of endocrine cells located in the central area of the islets while glucagon, somatostatin and pp-positive cells were primarily localized in the periphery of the islet of GIPR$^{dn}$ transgenic pigs (Wieczorek et al. 1998).

## 5.7 Reduced proliferation rate of β- and islet cells in GIPR$^{dn}$ transgenic pigs

The marked reduction of the total β-cell volume in 5-month-old and 1-1.4-year-old GIPR$^{dn}$ transgenic pigs but unaltered total volume of isolated β-cells as a marker for islet neogenesis required investigations related to proliferation and apoptosis (see 5.8) rates of β-cells. Immunohistochemical staining for the proliferation marker Ki67 revealed significantly reduced proliferation rates of β-cells as well as of islet cells in 11-week-old GIPR$^{dn}$ transgenic pigs compared to controls. Determination of proliferating islet cells included all endocrine cell types whereas proliferating endothelial as well as connective tissue cells were not counted. In GIPR$^{dn}$ transgenic pigs at 5 months of age and 1-1.4 years of age proliferation rate of both islet and β-cells reached no significant difference, but a tendency towards diminished proliferation was observed comparing 1-1.4-year-old transgenic animals and controls ($p<0.276$). The total β-cell volume in 11-week-old non-transgenic pigs showed a 6-fold increase up to the age of 5 months while the total β-cell volume from 5 months to 1-1.4 years of age not even doubled. According to these results, the proliferation rate of the young (11-week-old) non-transgenic pigs was 3-fold higher than in 5-month-old control pigs indicating that proliferation is important for β-cell mass expansion. The increase of the total β-cell volume is only modest (1.5-fold) from 5 months to 1-1.4 years of age going along with a markedly lower proliferation rate in these groups compared to the 11-week-old pigs which are in a developmental stage of massive β-cell expansion (Bock et al. 2003). Recently, experiments in mice

showed that β-cell proliferation and the ability of β-cells to regenerate decline with increasing age (Tschen et al. 2009).

Additionally, Hemalaun/Eosin (HE) staining in combination with immunodetection of Ki67 was carried out to obtain information about islet cell proliferation. The endocrine fraction in the pig pancreas is not clearly separated from the exocrine fraction by a reticular capsule as in rodents (Wieczorek et al. 1998) wherefore counterstaining with HE was utilized to make the islets of Langerhans visible. The course of islet cell proliferation showed the same age-related decrease pattern as was detected for β-cell proliferation. By absolute numbers, more proliferating cells occurred in 11-week-old non-transgenic pigs comparing the double immunohistochemistry (insulin Ki67) and immunohistochemistry for Ki67 combined with HE counterstaining. β-Cells represent the major fraction of endocrine cells in the islets of Langerhans with a content of 60-80% in pigs (Larsen et al. 2004). Even if in immunohistochemistry for Ki67 combined with HE counterstaining proliferating non-β-cells (α-, δ- and pp-cells) are included these cells represent only a minor fraction. Therefore it is possible that in double immunohistochemistry an almost similar number of proliferating cells is related to a lower number of nuclei as β-cell area is smaller than islet area. Another explanation for the higher proliferation rate of β-cells compared to islet cells could be that young (11 weeks) $GIPR^{dn}$ transgenic pigs showed primarily small islets and a lot of single β-cells compared to the other age groups. It has to kept in mind that small HE stained islets are more difficult to identify than single cells stained for insulin, possibly resulting in inaccuracies in determining proliferating islet cells due to overlooked cells. The reason carrying out two variants of analyzing the proliferation rate was that it might be possible that a proliferating β-cell dedifferentiates and transiently loses function as it replicates (Bonner-Weir 2000) and therefore might not be insulin positive during that period of time. As the results of both analyses were similar this assumption appeared not to play an important role for determination of the proliferation rate. Both analyses for the proliferation rate showed a significant reduction of proliferating β-/islet cells in 11-week-old $GIPR^{dn}$ transgenic pigs compared to controls and a tendency towards reduction in 1-1.4-year-old

GIPR$^{dn}$ transgenic pigs. These results are in line with previous *in vitro* studies showing that GIP has trophic effects on β-cells (Ehses et al. 2002; Trumper et al. 2001; Trumper et al. 2002). In contrast, GIPR$^{-/-}$ mice revealed a paradoxical increase in relative β-cell area (Pamir et al. 2003) providing no evidence that the trophic action of GIP is required for the maintenance of islet and β-cell integrity *in vivo* (Brubaker et al. 2004; Hansotia et al. 2004; Pamir et al. 2003). Both GIPR$^{-/-}$ and GLP-1R$^{-/-}$ mice exhibited compensatory mechanisms involving a higher action of the other incretin (Flamez et al. 1999; Pamir et al. 2003; Pederson et al. 1998). Furthermore, number and size of islets in DIRKO mice lacking functional expression of both the GIPR and the GLP-1R did not differ from controls assuming that unidentified compensatory mechanisms might balance the absence of both incretin receptors (Hansotia et al. 2004). In contrast, GIPR$^{dn}$ transgenic mice exhibited striking abnormalities in the endocrine pancreas concluding the importance of GIP signaling for postnatal islet and β-cell development as well as neogenesis of islets (Herbach et al. 2005). As a functional endogenous GIPR is present in transgenic mice the various compensatory mechanisms occurring in GIPR$^{-/-}$, GLP-1R$^{-/-}$ and DIRKO mice might not be triggered (Herbach et al. 2005). This conclusion might also apply to GIPR$^{dn}$ transgenic pigs.

There are different proliferation markers that can be used to determine the proliferation rate. Bromodeoxyuridine (BrdU), proliferating cell nuclear antigen (PCNA) and Ki67 are often utilized for this purpose. BrdU is a thymidine analogue incorporated during DNA synthesis. An uptake of BrdU labeling is restricted to cells in the S-phase of the cell cycle (Gratzner 1982). PCNA represents a subunit of the DNA-Polymerase-delta being essential for both DNA replication and DNA repair (Bravo et al. 1987; Prelich et al. 1987; Toschi et al. 1988). Even if its maximal expression occurs in the $G_1$ and S-phases PCNA is also present in the $G_0$-phase because of its long half life (8-20h) (Scott et al. 1991). In contrast, Ki67 is also visible in all phases of the cell cycle but is only rarely detectable in cells in the $G_0$-phase due to its short half life of about one hour (Bruno et al. 1992; Gerdes et al. 1984). Despite the accuracy of the BrdU method, this chemical must be administered to living animals including the

disadvantage to render previously collected pancreas samples useless. A study about the correlation of the labeling indices of the proliferation markers PCNA and Ki67 to the BrdU labeling index in rat renewing tissues supported immunohistochemistry for Ki67 as the preferable technique to assess proliferative activity compared to PCNA immunohistochemistry (Muskhelishvili et al. 2003). Due to this fact the proliferation marker Ki67 was selected being the best possible method for determination of the proliferation rate.

## 5.8 Tendency towards more cleaved caspase-3 positive β-cells in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs

GIPR$^{dn}$ transgenic pigs revealed significantly reduced total β-cell volume at 5 months and 1-1.4 years of age while total β-cell volume in 11-week-old transgenic pigs was unaltered compared to controls. The regulation of the β-cell mass is dynamic due to a balance between cell renewal (replication, neogenesis) and cell loss (necrosis, apoptosis) (Finegood et al. 1995). To find out whether this balance is altered in GIPR$^{dn}$ transgenic pigs investigations for proliferation (as described in 5.7) and apoptosis rates of β-cells were carried out. Apoptosis rates in GIPR$^{dn}$ transgenic pigs did not differ significantly from non-transgenic pigs at any age group using immunohistochemistry for cleaved caspase-3 and insulin. However, a tendency towards more cleaved caspase-3 positive cells in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs vs. controls was visible. An antiapoptotic effect has been proposed for GIP in several *in vitro* studies. Ehses et al. (2003) showed GIP as an inhibitor of cell death associated p38 MAPK pathway in β-INS-1 cells. Furthermore, it was demonstrated that GIP protects β-cells during glucolipotoxicity-induced apoptosis (Kim et al. 2005). Recently, it was discovered that GIP is involved in a pathway preventing the proapoptotic actions of p38 MAPK and JNK (Widenmaier et al. 2009). In type 2 diabetes patients apoptosis appeared to play an important role in β-cell reduction (Butler et al. 2003) and the insulinotropic action of the antiapoptotic factor GIP has been proven to be impaired both in humans affected by diabetes as well as in GIPR$^{dn}$ transgenic pigs.

Chronic elevation of blood glucose concentration is characteristic of the diabetes state and was discovered to derogate β-cell function and trigger β-cell apoptosis (Zhang et al. 2009), a phenomenon referred to as glucotoxicity (Kaiser et al. 2003). Hyperglycemia also accounts for oxidative stress that is potentiated by low expression and activity of anti-oxidative enzymes in the pancreas and documented to increase β-cell apoptosis (Del Guerra et al. 2005; Robertson et al. 2004). Likewise, endoplasmic reticulum stress activated by chronic hyperglycemia initiates apoptosis (Marchetti et al. 2007). Obesity, a main risk factor of diabetes, is accompanied with increased levels of leptin and free fatty acids exerting proapoptotic effects on β-cells due to lipotoxicity (Lupi et al. 2002). Furthermore, elevated islet amyloid content in the pancreas of diabetic patients was discovered to contribute to the loss of β-cells (Lorenzo et al. 1994) representing another factor for increased apoptotic processes. However, $GIPR^{dn}$ transgenic pigs did not develop diabetes up to an age of at least two years and showed normal body weight providing an explanation for the only slightly enhanced apoptosis rates of 1-1.4-year-old $GIPR^{dn}$ transgenic pigs compared to controls. Nevertheless, the tendency towards an increased apoptosis rate in 1-1.4-year-old $GIPR^{dn}$ transgenic pigs may have contributed to the reduced expansion of β-cell mass in $GIPR^{dn}$ transgenic pigs. It must also be mentioned that *in vivo* apoptotic cells are rapidly cleared by macrophages (O'Brien et al. 2002), making apoptosis a rapid process that can only be detected during a restricted time window (Donath et al. 2004) and might explain the small number of cleaved caspase-3 positive nuclei in the pancreatic sections.

Immunohistochemistry for cleaved caspase-3 is not the only method to detect apoptosis in tissue sections, wherefore the advantages of this technique compared to other apoptosis detection assays like the terminal transferase dUTP nick-end labeling (TUNEL) and the labeling of a cleavage product of cytokeratin 18 (CK18) are discussed. The TUNEL method is carried out as a standard method for the detection of apoptosis but one must be aware of the inaccuracy with this technique. Besides apoptotic nuclei also non-apoptotic nuclei are labeled (Kockx et al. 1998) as the TUNEL technique is not able to

distinguish between apoptosis, necrosis and autolysis (Grasl-Kraupp et al. 1995) indicating that the detection of DNA fragmentation is not specific for apoptosis. Furthermore, a well defined fixation and pretreatment of the samples is needed to obtain reliable results (Kockx et al. 1998; Negoescu et al. 1998). Caspases play an essential role in initiation, regulation and execution of downstream proteolytic events occurring during apoptosis whereby caspase-3 is on of the key factors (Fernandes-Alnemri et al. 1994; Slee et al. 2001). Detection of activated caspase-3 allows identification of apoptosis before the morphological features of apoptosis occur. However, apoptosis may also be triggered to caspase-independent pathways (Borner et al. 1999; Sperandio et al. 2000). Another possibility to detect apoptosis in tissue sections is the labeling of an apoptosis specific neo-epitope of CK18, a substrate of caspases 3, 6 and 7 cleaved during apoptosis (Caulin et al. 1997). The cleaved CK18 can be recognized by binding of the monoclonal antibody M30 to its specific epitope (Leers et al. 1999). Duan et al. (2003) compared the immunohistochemical stains for TUNEL, activated caspase-3 and cleaved CK18 to quantificate apoptosis in histological sections. Activated caspase-3 and cleaved CK18 labeling showed the best correlations to morphological analysis of apoptosis. Cleaved CK18 staining was associated with some background staining and therefore the signal to noise ratio with M30 was inferior to that obtained with activated caspase-3 immunohistochemistry (Duan et al. 2003). Hence, using cleaved caspase-3 labeling for determination of apoptotic β-cells in $GIPR^{dn}$ transgenic pigs and controls pointed towards being a reliable tool for the early identification and quantification of apoptotic cells in histological sections.

## 5.9 Concluding remarks

The analyses of the metabolic and morphological investigations revealed clear similarities of the $GIPR^{dn}$ transgenic pig to type 2 diabetes patients. First, the impaired insulinotropic action of GIP represents an important characteristic of both $GIPR^{dn}$ transgenic pigs and type 2 diabetes patients (Nauck et al. 1986a). Second, like diabetic humans $GIPR^{dn}$ transgenic pigs have a reduced β-cell mass. (Butler et al. 2003; Sakuraba et al. 2002). Third, deterioration of glucose

tolerance as observed in type 2 diabetes patients was also detected in GIPR$^{dn}$ transgenic pigs. However, GIPR$^{dn}$ transgenic pigs have normal fasting blood glucose and serum fructosamine levels indicating no diabetic phenotype until at least two years of age (Renner 2008). In contrast, GIPR$^{dn}$ transgenic mice developed already at three weeks of age a severe diabetic phenotype characterized by a marked hyperglycemia and striking abnormalities within the pancreas (Herbach et al. 2005). Different methods of transgenesis (lentiviral gene transfer vs. microinjection) as well as different copy numbers and/or integration sites might lead to different expression levels of the transgene involving different outcomes. Interestingly, a third line of GIPR$^{dn}$ transgenic mice showed similar physiological and morphological characteristics as the GIPR$^{dn}$ transgenic pig. The absence of chronic hyperglycemia is advantageous for evaluation of the GIP function *in vivo*. Therefore the results of the physiological and morphological analyses can be related to the impaired insulinotropic action of GIP disregarding effects due to hyperglycemia. The findings in GIPR$^{dn}$ transgenic pigs suggest that the function of GIP is not only restricted to that of an incretin but also plays an important role for the expansion of β-cell mass and that its partial loss cannot be fully compensated by hyperactivation of the GLP-1/GLP-1R system. Due to similarities to humans GIPR$^{dn}$ transgenic pigs offer a valuable tool to elucidate a possible role of GIP in the pathogenesis of type 2 diabetes mellitus.

# 6 Perspectives

$GIPR^{dn}$ transgenic pigs represent a valuable model to obtain more information about the function and effects of GIP within the glucose metabolism. Insulin resistance is a typical pathophysiological characteristic of type 2 diabetes mellitus (DeFronzo et al. 1991). Thus, it would be interesting to define the role of GIP with respect to insulin sensitivity. Hyperinsulinemic euglycemic glucose clamp techniques are used as a standard method to analyze insulin sensitivity. This test allows to gain more detailed information on the components of insulin sensitivity and insulin secretion overcoming the limitations of an OGTT due to variability of gastric emptying and glucose absorption from the gastrointestinal tract (Muniyappa et al. 2008; Trout et al. 2007). Striking differences in the proliferation rate of $GIPR^{dn}$ transgenic pigs were detected. Studying the proliferative signaling pathways of GIP might give new impetus how to stimulate β-cell proliferation or whether defects in proliferation signal cascades might contribute to the reduction of β-cell mass in type 2 diabetic patients. Holistic transcriptome and proteome analyses might be useful in this context. Obesity is known to be a major risk factor for the development of diabetes. $GIPR^{-/-}$ mice were resistant to obesity when fed a high-fat diet (Hansotia et al. 2007; Miyawaki et al. 2002). In this context, it would be interesting to perform diet studies using a high-fat diet to find out whether $GIPR^{dn}$ transgenic pigs are protected from obesity or whether it is possible to induce a diabetic phenotype feeding a high-fat/high-carbohydrate diet. Furthermore, $GIPR^{dn}$ transgenic pigs can be used for therapy studies, especially with respect to incretin-based therapies (GLP-1 receptor agonists like liraglutide or exenatide; DPP-4 inhibitors like vildagliptin or sitagliptin). Preclinical evidence suggested that GLP-1 receptor agonists may have stimulative effects on β-cell proliferation and neogenesis and prohibiting effects on apoptosis (Vilsboll 2009). However, there are still a lot of unresolved issues with respect to the effects of incretin-based therapies on β-cell mass. Therefore, $GIPR^{dn}$ transgenic pigs might be an excellent tool. Another use of $GIPR^{dn}$ transgenic pigs might be to test or develop

*in vivo* imaging techniques to assess the total β-cell mass (Moore 2009; Schneider 2008). In combination with therapy studies the effects of incretin-based therapies on β-cell mass could be determined in chronological sequence. Due to their similarity to humans new findings in the pigs might be translated to humans providing new advances in monitoring therapeutic trials.

In summary $GIPR^{dn}$ transgenic pigs seem to be a valuable animal model in various areas of diabetes research.

# 7 Summary


**Physiological and morphological characterization of transgenic pigs expressing a dominant-negative glucose-dependent insulinotropic polypeptide receptor ($GIPR^{dn}$) – a large animal model for diabetes research**

The incretin hormones GIP (glucose-dependent insulinotropic polypeptide) and glucagon-like peptide-1 (GLP-1) mediate the so-called incretin effect, which describes the phenomenon that glucose given orally causes a higher insulin response compared to an isoglycemic intravenous glucose load. The insulinotropic action of GIP is reduced to almost absent in type 2 diabetes patients, while the action of GLP-1 is vastly preserved. $GIPR^{dn}$ transgenic pigs were generated by lentiviral genetransfer to establish a large animal model to investigate the effects of an impaired insulinotropic action of GIP on glucose homeostasis. At the age of 5 months $GIPR^{dn}$ transgenic pigs showed a disturbed oral glucose tolerance going along with reduced insulin secretion. Eleven-month-old $GIPR^{dn}$ transgenic pigs exhibited an impaired intravenous glucose tolerance and reduced insulin secretion as well as a significantly reduced total β-cell volume compared to controls.

In this work different age classes (11 weeks, 5 months, 1-1.4 years) of $GIPR^{dn}$ transgenic were investigated to obtain detailed data about physiological and morphological characteristics. To proof specifity of the $GIPR^{dn}$ GIP/Exendin-4 stimulation tests were carried out in 11-week-old $GIPR^{dn}$ transgenic pigs. The insulinotropic action of intravenously injected porcine GIP was impaired, while this of Exendin-4, a GLP-1 mimetic, was enhanced in $GIPR^{dn}$ transgenic pigs compared to controls. Marked alterations in the expression profile of the GIPR and the GLP-1R were excluded as no apparent differences of immunohistochemically stained pancreas sections for GIPR and GLP-1R were detectable between $GIPR^{dn}$ transgenic pigs and controls at any age group. The effects of the impaired insulinotropic action of GIP on glucose metabolism were

investigated by oral and intravenous glucose tolerance tests. Eleven-week-old GIPR$^{dn}$ transgenic pigs exhibited significantly reduced oral glucose tolerance with a delay in insulin secretion compared to controls. The area under the insulin curve (AUC insulin) during the first 45 minutes following glucose load was 31% smaller in transgenic pigs compared to controls. The total amount of insulin secretion was not different between the two groups indicating that GIPR$^{dn}$ expression initially only interferes with the incretin effect. This was supported by the fact that intravenous glucose tolerance and insulin secretion in transgenic pigs were not different from controls. Five-month-old GIPR$^{dn}$ transgenic pigs exhibited a tendency towards reduced intravenous glucose tolerance and reduced insulin secretion in response to an intravenous glucose challenge.

To determine the reason for the alterations in glucose metabolism quantitative stereological analyses of the pancreas were performed. In 11-week-old pigs, transgenic and control groups showed similar β-cell mass. However, pancreatic β-cell mass was reduced by almost 40% in 5-month-old and by 60% in adult (1-1.4 years) GIPR$^{dn}$ transgenic pigs compared to controls. Furthermore, the cellular composition of the islets was analyzed by quantitative stereological investigations. The relative volumes of α- and δ-cells in the islets were increased in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs but the absolute volumes of these non-β-cell populations were not different from those of age-matched controls.

To investigate the reason for the reduced pancreatic β-cell mass in GIPR$^{dn}$ transgenic pigs, β-cell proliferation and apoptosis rate was determined performing a double-immunohistochemistry for insulin and the proliferation marker Ki67 and cleaved caspase-3, respectively. Eleven-week-old GIPR$^{dn}$ transgenic pigs showed significantly less Ki67 positive cell nuclei compared to controls, whereas proliferation rates in 5-month-old and 1-1.4-year-old GIPR$^{dn}$ transgenic pigs reached no statistical significance. No differences were shown in the apoptosis rates of GIPR$^{dn}$ transgenic pigs compared to controls at any age, although a trend of higher numbers of cleaved caspase-3 positive β-cells was visible in 1-1.4-year-old GIPR$^{dn}$ transgenic pigs.

In conclusion, GIPR$^{dn}$ transgenic pigs exhibit a comparable situation as in type 2 diabetes mellitus patients like impaired insulinotropic action of GIP, disturbed glucose tolerance and reduced β-cell mass. Moreover, the results of this work demonstrate an essential role of GIP for the physiological expansion of β-cell mass. In this context GIPR$^{dn}$ transgenic pigs represent a valuable model for further investigations on type 2 diabetes mellitus including diet studies and therapeutic trials.

# 8 Zusammenfassung


**Physiologische und morphologische Charakterisierung von transgenen Schweinen, die einen dominant-negativen Glukose-abhängigen insulinotropen Polypeptid Rezeptor (GIPR$^{dn}$ ) exprimieren – ein Großtiermodel für die Diabetesforschung**

Die Inkretinhormone glucose-dependent insulinotropic polypeptide (GIP) und glucagon-like peptide-1 (GLP-1) werden nach Nahrungsaufnahme aus den enteroendokrinen Zellen des Darms sezerniert und bewirken eine Steigerung der Insulinsekretion, den sogenannten Inkretineffekt. Bei Typ 2 Diabetes Patienten ist die insulinotrope Wirkung von GIP deutlich vermindert, während die insulinotrope Wirkung von GLP-1 weitgehend erhalten bleibt. Mittels lentiviralem Gentransfer wurden Schweine erstellt, die einen dominant-negativen GIP Rezeptor (GIPR$^{dn}$) exprimieren, um die Folgen einer verminderten insulinotropen Wirkung von GIP auf den Glukosemetabolismus näher zu erforschen. Im Alter von fünf Monaten zeigten GIPR$^{dn}$ transgene Schweine eine gestörte orale Glukosetoleranz, die mit einer reduzierten Insulinsekretion einherging. Bei 11 Monate alten GIPR$^{dn}$ transgenen Schweinen wurde eine gestörte intravenöse Glukosetoleranz und verminderte Insulinsekretion festgestellt sowie eine deutliche Reduktion des Gesamt-β-Zellvolumens.

Ziel dieser Arbeit war es GIPR$^{dn}$ transgene Schweine verschiedener Altersklassen (11 Wochen, 5 Monate, 1-1,4 Jahre) zu charakterisieren, um die Dynamik und den Verlauf der metabolischen und morphologischen Veränderungen zu erforschen. Zunächst wurde bei 11 Wochen alten GIPR$^{dn}$ transgenen Schweinen mittels GIP/Exendin-4 Stimulationstests die Spezifität des GIPR$^{dn}$ überprüft. Die insulinotrope Wirkung von intravenös verabreichten porcinem GIP war bei GIPR$^{dn}$ transgenen Schweinen signifikant vermindert, wohingegen die insulinotrope Wirkung von Exendin-4, einem GLP-1 Mimetikum, erhöht war. Deutliche Veränderungen in der Expressionshöhe des GIPR oder

GLP-1R konnten mittels immunhistochemischer Nachweise beider Rezeptoren in allen drei untersuchten Altersklassen ausgeschlossen werden. Die Auswirkungen der verminderten insulinotropen Wirkung von GIP auf den Glukosestoffwechsel wurden mitttels oraler und intravenöser Glukosetoleranztests charakterisiert. Elf Wochen alte $GIPR^{dn}$ transgene Schweine zeigten eine gestörte orale Glukosetoleranz sowie eine verzögerte Insulinsekretion. Im Zeitraum von null bis 45 Minuten nach Glukosegabe war die Fläche unter der Insulinkurve (AUC Inulin) bei den transgenen Tieren im Vergleich zu den Kontrolltieren 31% kleiner. Die gesamte Insulinsekretion (AUC Insulin bis 120 Minuten nach Glukosegabe) hingegen war nicht vermindert und auch die intravenöse Glukosetoleranz wies keine Veränderungen im Vergleich zu Kontrolltieren auf. Diese Ergebnisse deuten darauf hin, dass zunächst nur der Inkretineffekt durch die $GIPR^{dn}$ Expression beeinflusst wird. Bei fünf Monate alten Tieren war ebenfalls eine gestörte orale Glukosetoleranz zu erkennen. Die Insulinsekretion war nicht nur verzögert, sondern signifikant vermindert im Vergleich zur Kontrollgruppe. Neben den metabolischen Tests wurden quantitativ-stereologische Untersuchungen des Pankreas durchgeführt, um Auswirkungen der Expression des $GIPR^{dn}$ auf die Morphologie und/oder die Integrität der Langerhansschen Inseln detektieren zu können. Die Bestimmung des Gesamt-β-Zellvolumens bei elf Wochen alten Tieren ergab keinen Unterschied zwischen den beiden Gruppen. Fünf Monate alte $GIPR^{dn}$ transgene Schweine wiesen gegenüber den Kontrolltieren eine beinahe 40%ige Reduktion im Gesamt-β-Zellvolumen auf. Das Gesamt-β-Zellvolumen von 1-1,4 Jahre alten $GIPR^{dn}$ transgenen Tieren war um fast 60% vermindert. Die Reduktion des Gesamt-β-Zellvolumens von $GIPR^{dn}$ transgenen Tieren kann eine Erklärung für die Entwicklungen im Glukosestoffwechsel sein. Zusätzlich wurde die zelluläre Zusammensetzung der Inseln anhand quantitativ-stereologischer Untersuchungen der α-, δ- und PP-Zellen analysiert. Das relative Volumen der α- und δ-Zellen war bei 1-1,4 Jahre alten $GIPR^{dn}$ transgenen Schweinen erhöht, wohingegen das Gesamtvolumen dieser beiden endokrinen Fraktionen sich nicht von denen der Kontrollgruppe unterschied.

Untersuchungen der Proliferations- sowie der Apoptoserate von β-Zellen sollten Aufschluss über mögliche Ursachen für die Reduktion der β-Zellmasse ergeben. Die Auswertung der doppelimmunhistochemischen Färbung mit dem Proliferationsmarker Ki67 und Insulin ergab eine signifikant verminderte Proliferationsrate bei elf Wochen alten $GIPR^{dn}$ transgenen Schweinen im Vergleich zur Kontrollgruppe, wobei 5 Monate und 1-1,4 Jahre alte $GIPR^{dn}$ transgene Schweinen keinen signifikanten Unterschied zu ihren Kontrolltieren aufwiesen. Doppelimmunhistochemische Färbungen mit cleaved caspase-3 und Insulin dienten zur Bestimmung der Apoptoserate. Es konnten keine statistisch signifikanten Unterschiede der Apoptoserate zwischen transgenen Tieren und Kontrolltieren detektiert werden, obwohl 1-1,4 Jahre alte $GIPR^{dn}$ transgene Schweine einen Trend zu vermehrter Apoptose zeigten. Die verminderte Proliferation von β-Zellen deutet auf eine reduzierte Expansion von β-Zellen bei $GIPR^{dn}$ transgenen Schweinen hin.

$GIPR^{dn}$ transgene Schweine stellen ein interessantes Großtiermodell dar, da sie wichtige Parallelen zum Typ 2 Diabetes mellitus Patienten aufweisen: verminderte insulinotrope Wirkung von GIP, gestörte orale und intravenöse Glukosetoleranz sowie ein vermindertes Gesamt-β-Zellvolumen. Weiter lassen diese Daten eine bedeutende Rolle von GIP für die physiologische Expansion von β-Zellen erkennen. In diesem Zusammenhang wäre es interessant, das $GIPR^{dn}$ transgene Schweinemodell in Zukunft auch für Therapiestudien und Diätstudien heranzuziehen.

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# 9 Index of figures

# 10 Index of tables

# 11 Index of abbreviations

| | |
|---|---|
| AD | Alzheimer disease |
| ADP | adenosine diphosphate |
| APP | amyloid precursor protein |
| ATP | adenosine triphosphate |
| BCIP/NBT | 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium |
| BrdU | bromodeoxyuridine |
| BW | body weight |
| cAMP | cyclic adenosine monophosphate |
| CF | cystic fibrosis |
| CFTR | CF transmembrane conductance regulator |
| CK | Cytokeratin |
| cpm | counts per minute |
| CREB | cAMP response element-binding protein |
| CT | carboxyl terminal |
| CV | coefficient of variance |
| DAB | 3´3 diaminobenzodine tetrahydrochloride |
| DAPI | 4`,6-diamidino-2-phenylindole |
| DIRKO | double incretin receptor knockout |
| DNA | deoxyribonucleic acid |
| DPP-4 | dipeptidyl peptidase-4 |
| ds | double stranded |
| ELISA | enzyme linked immunosorbent assay |
| ERK | extracellular signal regulated kinase |
| FITC | fluorescein isothiocyanate |
| GI | glycemic index |
| GIP | glucose-dependent insulinotropic polypeptide |
| $GIPR^{dn}$ | dominant-negative glucose-dependent insulinotropic polypeptide receptor |

| | |
|---|---|
| GIPR | GIP receptor |
| $GIPR^{-/-}$ | GIPR knockout |
| GLP-1 | glucagon-like peptide-1 |
| GLP-1R | GLP-1 receptor |
| $GLP\text{-}1R^{-/-}$ | GLP-1R knockout |
| GLP-2 | glucagon-like peptide-2 |
| HD | Huntingon disease |
| HE | hematoxylin and eosin |
| HF | high-fat |
| HNF | hepatic nuclear factor |
| $H_2O_2$ | hydrogen peroxide |
| HP | horseradish peroxidase |
| IAPP | islet amyloid peptide |
| ICSI | intracytoplasmic sperm injection |
| IEQ | islet equivalents |
| IGF | insulin-like growth factor |
| ILK | interleukin |
| IPTT | intraperitoneal glucose tolerance test |
| IVGTT | intravenous glucose tolerance test |
| JNK | Jun N-terminal kinase |
| MAPK | mitogen-activated protein kinase |
| mg | milligram |
| ml | milliliter |
| mRNA | messenger RNA |
| NIA | nicotinamide |
| OGTT | oral glucose tolerance test |
| OVX | ovariectomized |
| PBS | phosphate buffered saline |
| PCiA | phenol-chloroform isoamylalcohol |
| PCNA | proliferating cell nuclear antigen |
| PCR | polymerase chain reaction |
| PI3K | phosphatidylinositol 3-kinase |

| | |
|---|---|
| PKA | protein kinase A |
| PKB | protein kinase B |
| PLA | pospholipase A |
| PSEN | presinilin genes |
| RIA | radioimmunoassay |
| RIP2 | rat insulin 2 promoter |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RP | retinitis pigmentosa |
| RT | room temperature |
| RT-PCR | reverse transcription PCR |
| SCNT | somatic cell nuclear transfer |
| SDS | sodium dodecyl sulphate |
| SIRKO | single incretin knockout |
| SMA | spinal muscle atrophy |
| SMN | survival motor neuron |
| STS | staurosporine |
| STZ | streptozotocin |
| TAE | tris-acetate buffer |
| TBS | tris-buffered saline |
| Tris | tris-(hydroxymethyl)-aminomethan |
| TNF α/β | tumor necrosis factor α/β |
| TUNEL | terminal transferase dUTP nick-end-labeling |
| UV | ultraviolet |
| VDF | Vancouver diabetic fatty |
| ZDF | Zucker diabetic fatty |

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