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Delta cell reprogramming during mouse pregnancy

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Vorsitzender der Promotionskomission

Success is the ability to go from one failure to another with no loss of enthusiasm.
– Winston Churchill

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List of abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ARX	aristaless related homeobox
BSA	bovine serum albumin
BrdU	5-bromo-2'-deoxyuridine
°C	degrees Celsius
CG	chorionic gonadotropin
Cre	Cre recombinase
DAPI	4´,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
E	embryonic day
E_2	estradiol
ECS	extracellular solution
EdU	5-Ethynyl-2´-deoxyuridine
ELISA	enzyme linked immunosorbent assay
EGF	epidermal growth factor
ESC	embryonic stem cell
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
G	gestational day
GABA	gamma-aminobutyric acid
Gcg	glucagon
GDM	gestational diabetes mellitus
GFP	green fluorescent protein
GH	growth hormone
GH-V	growth hormone variant
GLUT	glucose transporter
GR	glucagon receptor
GSDB	goat serum diluent buffer
hCG	human chorionic gonadotropin
hrs	hours
HTRF	homogeneous time resolved fluorescence
Ins	insulin
ip	intraperitoneal
iPSC	induced pluripotent stem cells
K_{ATP} channel	ATP sensitive potassium channel

KRBH	Krebs Ringer buffer HEPES
LPL	lipoprotein lipase
µM	micro molar
mM	milli molar
µL	micro liter
mL	milli liter
NeuroD1	neuronal differentiation 1
Ngn3	neurogenin 3
nm	nano meter
nM	nano molar
OCT	optimum cutting temperature
P_4	progesterone
PAX4	paired box gene
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDL	partial duct ligation
PL	placental lactogen
PRL	prolactin
Pdx1	pancreatic and duodenal homeobox 1
PFA	para-formaldehyde
PI	propidium iodide
PP	pancreatic ploypeptide
Ptf1a	pancreas specific transcription factor 1 a
qRT-PCR	quantitative real time polymerase chain reaction
ROI	region of interest
rpm	rounds per minute
sc	subcutaneous
Sst	somatostatin
Sstr	somatostatin receptor
T1D	type 1 diabetes mellitus
T2D	type 2 diabetes mellitus
TCA	tricarboxylic acid cycle
TAM	tamoxifen
TLB	tail lysis buffer
YFP	yellow fluorescent protein
x G	G-force

1 Introduction

1.1 The Pancreas

1.1.1 Anatomy

The pancreas is an elongated, glandular organ located in the posterior abdominal wall behind the stomach. Anatomically, the pancreas is divided in three regions, the pancreas head, the body and the tail (Parsons, et al. 1992). The majority of the pancreatic mass is concentrated in the head, which connects to the duodenum through the pancreatic duct. The body of the pancreas is thinned and stretches over the duodenojejunal intestine and extends to the narrow pancreas tail at the hilum of the spleen (Pandol 2010).

On the functional level, the pancreas is subdivided into two distinct cell lineages, exocrine and endocrine. The exocrine compartment consists of acinar cells and complex ductal networks and makes up approximately 98 % of the mature pancreatic organ. Acinar cells produce digestive enzymes in order to break down carbohydrates, fat and proteins in the duodenum. Moreover, epithelial cells lining at the ducts produce and secrete bicarbonate to neutralize the acid coming from the stomach. Together, this pancreatic juice is guided through the ductal network to be delivered into the duodenum. Secretion of these enzymes is regulated by hormones released by the stomach and duodenum in response to food intake (Means and Leach 2001). Interspersed within the exocrine parenchyma are small clusters of several hundreds to thousand endocrine cells, namely the islets of Langerhans. These micro-organs play a critical role in maintaining glucose homeostasis by secreting cell type specific hormones into the bloodstream (Sakula 1988). In order to deliver these hormones sufficiently into the circulation system, the pancreas facilitates a unique and complex vascular network. Pancreatic anatomic studies showed that the blood flow from the pancreas enters capillaries of the exocrine tissue surrounding the individual islets before entering the circulation system (Ballian and Brunicardi 2007). Venous drainage from the body and tail of the pancreas flows into the splenic vein, whereas the head region drains into the superior mesenteric vein (Bockman, et al. 1983).

1.1.2 Development

Not surprisingly, islet organogenesis has been shown to differ between human and mouse, however key developmental events seem to be roughly conserved (Nair and Hebrok 2015). Rodent pancreas development has been divided into three main transition phases (Fig. 1), each characterized by organ morphology and a controlled cascade of gene activation by the expression of key transcription factors (Rutter, et al. 1968; Kim, et al. 1997). The first transition phase, from embryonic day (E) 8.5 to 12.5, is characterized by the occurrence of morphological changes in the pancreatic epithelium and first specification of different pancreatic cell types takes place. Within the second transition phase, between E12.5 and E16.5, the majority of endocrine cell specification occurs. Finally, in a third transition phase from E16.5 to birth, differentiated endocrine cells migrate to form the islets of Langerhans (Rutter, et al. 1968; Pictet, et al. 1972; Herrera, et al. 1991; Pan and Wright 2011).

Morphologically, pancreas development begins with the evagination and thickening of a dorsal pancreatic bud from the embryonic foregut endoderm. This is followed by the emergence of the anlage of the ventral pancreatic bud and the common bile duct from the ventral foregut endoderm. The subsequent expansion and branching of both pancreatic buds is a tightly regulated process which relies on signaling from the notochord and active crosstalk between the pancreatic epithelium and the surrounding mesenchyme. During the second transition phase, rapid expansion, branching and fusion of the pancreatic dorsal and ventral buds occurs. As a result of gut tube rotation and stalk expansion, the two pancreatic buds eventually fuse into a single interconnected organ.(Wessells and Cohen 1967; Pictet, et al. 1972; Jorgensen, et al. 2007). This process includes the fusion of the pancreatic ducts, leaving a single common bile duct (duct of Wirsung) that runs the entire length of the pancreas. These morphological events coincide with the formation of the three main pancreatic cells types, endocrine islet cells and exocrine acinar and ductal cells (Pictet, et al. 1972; Schwitzgebel, et al. 2000). Lineage tracing experiments showed that all pancreatic cells derive from the same origin of multipotent pancreatic progenitor cells which have the capacity to differentiate into any of the three pancreatic lineages. These progenitor cells are characterized by the expression of *pancreatic and duodenal homeobox 1* (*Pdx1*) (Ohlsson, et al. 1993; Gu, et al. 2002). The initial expression of *Pdx1* starts already at E8.5 and defines the pancreatic cell fate before first morphological signs are visible. As organogenesis continues, *Pdx1* expression mainly persists in the pancreatic regions, but expands to a few epithelial cells of the later stomach and duodenum (Ohlsson, et al. 1993; Offield, et al. 1996). Yet, pancreatic cell fate specification is determined by the co-expression of *Pdx1* and

pancreas specific transcription factor 1a (*Ptf1a*). Studies showed that both transcription factors play a critical role for proper pancreas specification and were identified to contribute to all three pancreatic lineages. However, *Ptf1a* expression becomes restricted to exocrine acinar cell differentiation later during development (Krapp, et al. 1996; Hald, et al. 2008). The first sign for endocrine cell specification is the expression of the basic helix-loop-helix transcription factor *Neurogenin 3* (*Ngn3*) (Gu, et al. 2002). Transcription of *Ngn3* is observed in two distinct temporal waves from E8.5 until 15.5, starting with relatively low levels at the time of pancreatic budding, followed by a major peak at E14.5 and rapid decline thereafter (Schwitzgebel, et al. 2000). Interestingly, this biphasic expression pattern correlates with the first and second transition phase of pancreatic organogenesis and confirms the essential contribution of *Ngn3* expression in pancreatic endocrine lineage commitment. During the last decades studies have identified *Ngn3* as a master regulator of endocrine cell specification and its absence during development leads to a lack of endocrine cell formation, whereas the exocrine and ductal compartments develop normally (Gradwohl, et al. 2000; Schwitzgebel, et al. 2000; Gu, et al. 2002). Unlike multipotent pancreatic progenitor cell lineages, *Ngn3* expressing precursor cells have been shown to be less proliferative giving rise to only one single endocrine cell (Desgraz and Herrera 2009). The mechanisms inducing *Ngn3* expression are not completely clarified, but several studies revealed that Notch signaling functions as a negative regulator of *Ngn3*. Inhibition of Notch signaling on the other hand leads to overexpression of *Ngn3* and enhanced formation of beta cells (Apelqvist, et al. 1999; Murtaugh, et al. 2003; Nakhai, et al. 2008). Furthermore, it has been shown that *Pdx1* expressing progenitor cells that transiently induce *Ngn3* go through competence windows each allowing the generation of specific endocrine subtypes (Apelqvist, et al. 1999; Gradwohl, et al. 2000; Gu, et al. 2002; Jorgensen, et al. 2007). Thus, early *Ngn3* expression around E9.5 exclusively leads to the formation of alpha cells, whereas its expression around E11.5 suggests the predominant differentiation of beta and PP cell phenotypes. Finally, *Ngn3* activity from E14.5 onwards favors delta cell differentiation (Ohlsson, et al. 1993; Schwitzgebel, et al. 2000; Johansson, et al. 2007).

After endocrine cell fate is specified by the initiation of *Ngn3* expression, endocrine progenitor cells delaminate out of the epithelium and further differentiate towards distinct endocrine cell types. This process is influenced by expression of several downstream transcription factors of *Ngn3* signaling, including *aristaless related homeobox* (*Arx), paired box gene (Pax4), neuronal differentiation 1 (NeuroD1), homeobox protein Nkx2.2* and *Nkx6.1* (Bonal and Herrera 2008; Gouzi, et al. 2011). Numerous gene knockout studies have given insights into the different pathways controlling the selection of endocrine cell fates (Sander, et al. 2000; Collombat, et al. 2003; Matsuoka, et al. 2004; Collombat, et al. 2005). For

instance, *Arx* and *Pax4* expression have opposite roles in the process of islet cell fate differentiation. While *Pax4* expression leads to beta and delta lineage commitment, *Arx* promotes alpha and epsilon lineage specification (Collombat, et al. 2003) Furthermore, enhanced levels of *Pdx1*, *MafA* and *Nkx6.1* eventually lead to beta cell formation, whereas *Arx* and *MafB* expression is restricted to alpha cells (Kataoka, et al. 2002; Matsuoka, et al. 2004; Jensen, et al. 2005; Artner, et al. 2007). Accordingly, absence of these transcription factors leads to loss of alpha and beta cells resulting in a subsequent increase of delta cells (Sussel, et al. 1998; Collombat, et al. 2005).

Finally, within the last transition phase of pancreatic development the differentiated endocrine cells migrate away from the progenitor cell domain and form clusters within the pancreatic mesenchyme (Pictet, et al. 1972; Jorgensen, et al. 2007). By the end of gestation, these clusters of pancreatic endocrine cells have been fully generated and assembly into mature islets has been completed (Herrera, et al. 1991).

The development of human islets in comparison to mouse, shows some important differences including delayed appearance of key differentiation markers, a single transition phase of endocrine differentiation as well as dissimilarities in the timing of endocrine cell specification (Nair and Hebrok 2015). During the phase of pancreatic lineage separation, extended presence of *SOX9* is found in the human acinar cells and prolonged expression of *NKX6.1* in the endocrine lineage (Jennings, et al. 2013). Most contrary to mouse development is the appearance of only a single phased induction of *NGN3* expression leading to endocrine cell differentiation. However, timing of *NGN3* peak occurrence and expression in non-proliferative cells was consistent compared to mouse data (Salisbury, et al. 2014). Moreover, absence of *NKX2.2* expression was detected in human progenitor cells and did first appear after endocrine lineage commitment by *NGN3* expression (Jennings, et al. 2013). This might explain the differences in temporal differentiation of endocrine cell types compared to mouse. While mouse development revealed the formation of alpha cells prior to beta, PP and delta cells, first endocrine cells to arise in human are beta cells (Schwitzgebel, et al. 2000; Johansson, et al. 2007; McDonald, et al. 2012).

Summarily, human and mouse organogenesis display differences in presence and timing of developmental transcription factors, however key events in endocrine lineage commitment are comparable.

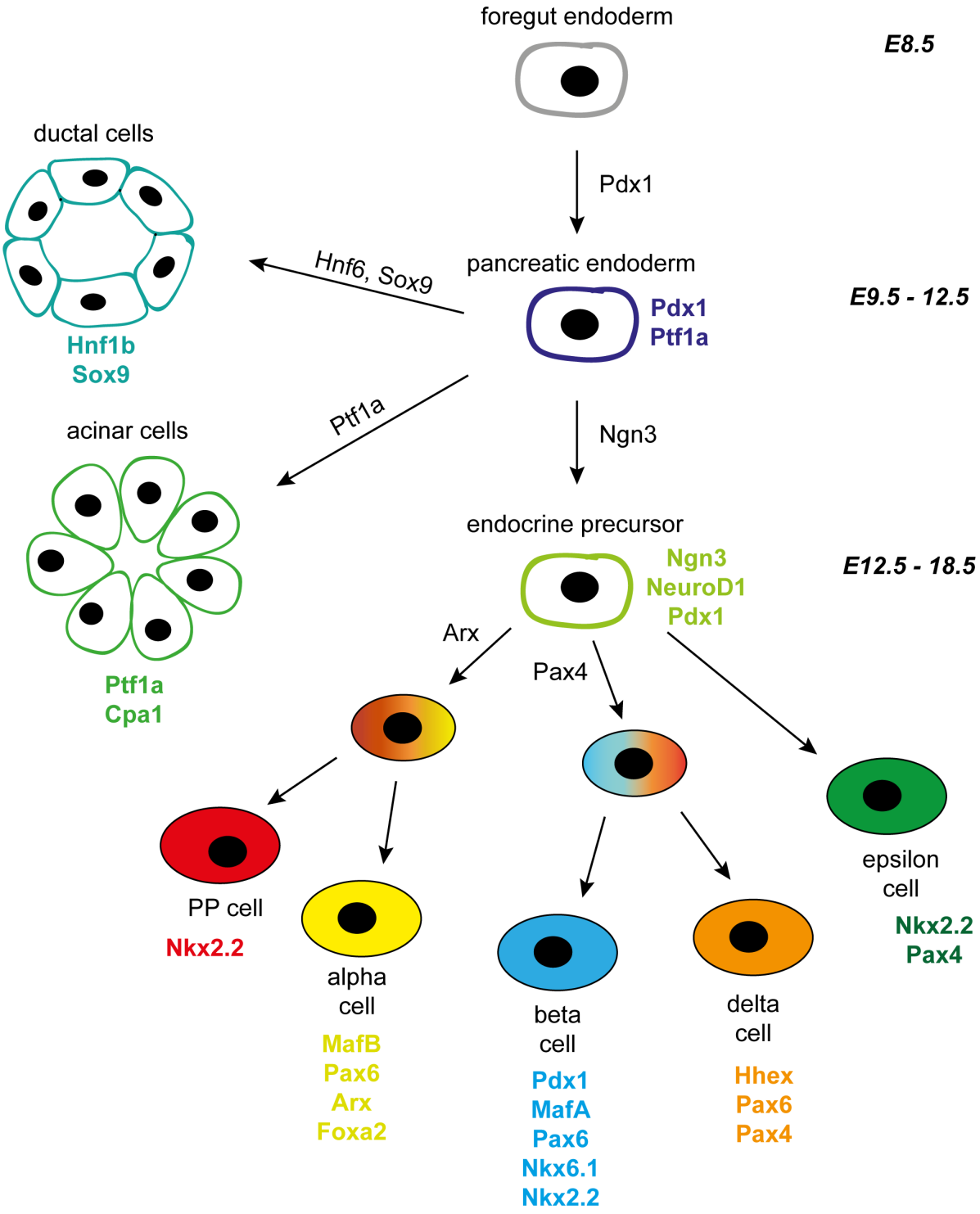

Figure 1: Scheme for rodent development of the three terminally differentiated cell types in the pancreas. According to Pan and Wright, 2011.

1.2 Islets of Langerhans

1.2.1 Islet architecture

The pancreatic islets were first described in 1869 by the german pathological anatomist Paul Langerhans. Although he provided a detailed description of these cell clusters their function remained unclear until 1893, when Édouard Lagusse, a french scientist, rediscovered the islet cells and postulated that they might serve an endocrine function (Sakula 1988). During the next century, numerous studies facilitated the discovery that the islets of Langerhans are complex micro-organs composed of five endocrine cell types, responsible for the maintenance of glucose homeostasis (Ceranowicz, et al. 2015).

The adult pancreas is composed of approximately 1-2 % endocrine cells and cluster size varies in a range from 40 up to 500 µm (Saito, et al. 1978; Bosco, et al. 2010). The islets of Langerhans are innervated by the autonomous nerve system (Smith and Porte 1976) and consist of multiple secretory endocrine cells, namely insulin secreting beta cells, glucagon producing alpha cells, somatostatin containing delta cells, the pancreatic polypeptides secreting (PP) cells and ghrelin containing epsilon cells (Brissova, et al. 2005). The most abundant cells within the islets are alpha and beta cells, but studies performed on the cytoarchitecture of pancreatic islets revealed that there are differences in islet composition among the different species (Brissova, et al. 2005; Cabrera, et al. 2006; Kim, et al. 2009; Bosco, et al. 2010). Islets of mice and other rodents contain approximately 75 % beta cells predominantly located in the central core of the islets, surrounded by a periphery forming mantle of alpha and delta cells. In contrast, human islets show a high heterogeneity in terms of cellular composition with beta cell fractions varying between 28 and 75 % (Brissova, et al. 2005). Moreover, pancreatic islets are highly vascularized by a dense network of capillaries (Zanone, et al. 2008) which also has been shown to differ substantially between human and rodent islets (Cabrera, et al. 2006; Brissova, et al. 2015; Cohrs, et al. 2017). Cohrs et al. provided evidence that human islets are less vascularized than mouse islets, as a result of reduced vessel diameter and density (Cohrs, et al. 2017). Moreover, their cellular arrangement differs compared to rodents, as the beta cells are intermingled by alpha and delta cells aligned along the blood vessels (Kim, et al. 2009; Bosco, et al. 2010; Cohrs, et al. 2017). The unique cellular composition of human pancreatic islets has been postulated to affect beta cell function, allowing the cells to respond to lower glucose concentration (3-5 mM) to which rodent islets are blind for (Henquin, et al. 2006; Dufrane, et al. 2007; Dai, et al. 2012).

1.2.2 Islet cell function

Beta cells represent the most abundant cell type in pancreatic islets and play a critical role in the maintenance of glucose homeostasis. The primary function of beta cells is to secrete insulin in response to increasing blood glucose levels. In order to precisely meet the metabolic demand, insulin biosynthesis and secretion has to be tightly controlled. Insulin is a peptide hormone first synthesized as a single polypeptide called preproinsulin. After cleavage, the precursor molecule proinsulin is packed in secretory vesicles and finally cleaved into insulin and C-peptide in equimolar amounts. Both are stored inside secretory granules waiting to be exocytosed from the cell upon intracellular signaling (Huang and Arvan 1995; Fu, et al. 2013). Unlike insulin, C-peptide is not metabolized by the liver and therefore represents a better measure of insulin secretion than insulin itself (Oram, et al. 2015). The rich vascularization within the islet and the close proximity of endocrine cells to this microvasculature, ensures rapid sensing of plasma glucose changes to allow prompt and appropriate secretory responses (Cabrera, et al. 2006; Cohrs, et al. 2017). Glucose is transported into the beta cells *via* specific transmembrane glucose transporters (GLUT1 in humans and GLUT2 in rodents). Within the cell, glucose is phosphorylated *via* glucokinase, and subsequently undergoes glycolysis to be metabolized to pyruvate which then enters the tricarboxylic acid cycle (TCA) in the mitochondria to produce adenosine triphosphate (ATP). The generation of ATP in the TCA increases the ATP/adenosine diphosphate (ADP) ratio, which serves as a second messenger to promote closure of ATP sensitive potassium channels (K_{ATP}-channels), leading to the depolarization of the plasma membrane. Consequently, opening of voltage gated calcium channels leads to Ca^{2+} influx which in turn initiates exocytosis of insulin granules (Wollheim, et al. 1987; Ashcroft, et al. 1994). This signaling cascade describes one of the two major signaling pathways to control insulin secretion and is called the triggering pathway. Alone, this pathway would not lead to sufficient insulin release, thus glucose also activates metabolic amplifying signals leading to an augmented triggering Ca^{2+} signal (Henquin 2000). However, these pathways are hierarchical, meaning the amplifying pathway depends on the initial triggering signal to affect the magnitude of insulin secretion (Kalwat and Cobb 2017).

Although glucose represents a primary stimulus for the secretion of insulin, it can also be regulated by several other nutrient factors. Beta cells can metabolize free fatty acids by beta-oxidation in the TCA, also leading to the production of ATP (Itoh, et al. 2003). Moreover, amino acids can be transported actively into the beta cells mediating membrane depolarization (Thams and Capito 1999).

The second main cell type within the islets of Langerhans is represented by the alpha cells, which make up approximately 19 % of the rodent and 35 % of the human endocrine cells (Brissova, et al. 2005). Their main function is to secrete glucagon in response to low glucose concentrations and thus maintain blood glucose levels within a glycemic range. Similar to beta cells, alpha cells store glucagon in secretory granules that are released by exocytosis upon intracellular Ca^{2+} influx (Brereton, et al. 2015). Glucagon secretion exerts systemic effects through the glucagon receptor (GR), especially in the liver, mobilizing glucose through gluconeogenesis, glycogenolysis and ketogenesis and therefore restoring blood glucose levels (Unson 2002). Cellular regulation of glucagon secretion is under debate, but paracrine signaling by released factors of neighboring beta and delta cells (Unger and Orci 2010) as well as neuronal regulation have been proposed mechanisms (Taborsky, et al. 1998).

Somatostatin (SST) producing delta cells represent with approximately 6 % (Brissova, et al. 2005) the third most abundant cell type of the pancreatic islet. This peptide hormone is also synthesized and secreted by neuroendocrine cells in other tissues including the hypothalamus, the central nervous system, peripheral neurons and the gastrointestinal tract (Arimura, et al. 1975; Hokfelt, et al. 1975). Basically, there are two bioactive forms of somatostatin. SST-14, consisting of 14 amino acids, which is the predominant form in the brain and released by the pancreatic delta cells, while SST-28 (28 amino acids) is a product of the intestinal cells (Francis, et al. 1990). Both forms bind to five specific membrane receptor subtypes (SSTR1-SSTR5), which are also expressed in alpha and beta cells (Kumar, et al. 1999). Although no absolute specificity of any receptor subtype was identified, the individual islet cell types have been demonstrated to prefer expression of certain receptors. Thus, in rodent islets beta cells predominantly contain SSTR5, whereas alpha cells mostly express SSTR2 (Ludvigsen, et al. 2004). In human islets on the other hand the SSTR2 has been shown to be predominantly expressed in both, alpha and beta cells (Kailey, et al. 2012). The wide distribution and short half life time of SST (less than 1 min) implies inhibitory action *via* paracrine signaling (Lewin 1992). However, knowledge about somatostatin secretion in the pancreas is limited, but similarities in the stimulus-response pathways of beta and delta cells have been suggested (Hauge-Evans, et al. 2012). Although it has been shown, that somatostatin exerts a paracrine inhibitory function on insulin and glucagon secretion (Orci and Unger 1975), its physiological relevance still remains unknown. Studies performed on Somatostatin knock out mice showed increased insulin and glucagon secretion upon glucose stimulation *in vivo* as well as *in vitro* (Hauge-Evans, et al. 2009). Excluding neuroendocrine cells as a source of SST release by islet isolation, this study demonstrated that the observed effect on hormone secretion is most likely attributed to the

absence of somatostatin secretion by the delta cells within the islets, confirming the importance of SST as a negative regulator of alpha and beta cell function.
In summary, the islets of Langerhans are well organized cellular networks and their characteristic architecture serves to facilitate strong interactions among the different cell types, which allows coordinated signaling for tight control of glucose homeostasis. Changes in cellular composition, dysfunction or loss of cells might affect the balance between hormone and counter regulatory hormone release and therefore lead to metabolic disorders like diabetes mellitus.

1.3 Diabetes Mellitus

1.3.1 Etiology of diabetes

Diabetes mellitus is a set of metabolic diseases with common characteristics like chronically hyperglycemia and glucose intolerance caused by insulin deficiency, defects in insulin secretion and action, or both. Chronic hyperglycemia is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and vascular system (Skyler 2012). Throughout the world an estimated 418 million people suffer from diabetes, which makes it necessary to develop novel therapeutic approaches to overcome this major health burden (IDF Diabetes Atlas, 2017).
Type 1 diabetes (T1D), one of the three main diabetes types, is an autoimmune disease characterized by the selective destruction of insulin-producing beta cells. The disease occurs as a consequence of genetic susceptibility and environmental factors, however the cause of this disorder is not clearly defined (Skyler and Ricordi 2011; Jerram and Leslie 2017). T1D is often first diagnosed when symptoms occur, although the pathogenic development of the disease usually begins years before that. Disease progression is initiated by islet cell autoimmunity characterized by elevated levels of pancreatic autoantibodies leading to a gradual decline of beta cell mass and function. Enhanced cell death during this prediabetic phase ultimately leads to a massive reduction in beta cell mass and subsequent onset of hyperglycemia and disease manifestation (von Herrath, et al. 2007; van Belle, et al. 2011).
The second main form, namely type 2 diabetes (T2D), accounts for the majority of diagnosed patients (95 %) (Wu, et al. 2017). T2D results from a combination of resistance to insulin action, inadequate insulin secretion, and excessive or inappropriate glucagon secretion (Skyler 2012). Although the mechanisms of disease progression are still incompletely understood, it is suggested that an inactive lifestyle, over-nutrition and genetic predisposition significantly contribute to the risk of T2D development (Kaneto, et al. 2013). T2D is a progressive disease initiated by a long prediabetic phase and associated with chronic

glucose intolerance and peripheral insulin resistance. Initially, this can be compensated by increased beta cell function and mass (Hanley, et al. 2010). However, prolonged insulin resistance and subsequently increasing beta cell work load results in cellular exhaustion and elevated cell death (Butler, et al. 2003). Finally, a gradual decline of beta cell mass and function leads to the development of T2D (Leahy, et al. 2010; Meier and Bonadonna 2013). Gestational diabetes mellitus (GDM) describes another form of diabetes characterized by glucose intolerance resulting in hyperglycemia first detected during pregnancy. With a prevalence of 2-6 % of all European pregnancies, it represents one of the most common pregnancy disorders (Buckley, et al. 2012). Pregnancy is accompanied with severe changes in glucose metabolism and therefore demands enormous plasticity of the endocrine pancreas (Soma-Pillay, et al. 2011). The development of insulin resistance serves as a physiological adaptation in order to decrease maternal glucose consumption and supply the developing fetus with sufficient nutrients. This leads to an increased demand for insulin and is compensated by increased beta cell function and islet mass expansion (Parsons, et al. 1992; Sorenson and Brelje 1997; Butler, et al. 2010; Rieck and Kaestner 2010). If this adaptation fails, gestational diabetes might develop (Sonagra, et al. 2014). During pregnancy, GDM is associated with fetal overgrowth leading to an increased risk of Caesarean section (Schmidt, et al. 2001). Even though gestational diabetes is a transient condition occurring during the last period of pregnancy, women have a higher risk of developing type 2 diabetes later on (Kim, et al. 2002). Moreover GDM has been demonstrated to facilitate long-term consequences for the offspring as well, including increased risk of diabetes, obesity, cardiovascular diseases and structural hypothalamic changes (Silverman, et al. 1991; Fraser and Lawlor 2014).

1.3.2 Therapeutic approaches

Unfortunately there is no cure for any of the disease types and existing treatment possibilities are still limited and accompanied by long-term side effects (Halban, et al. 2014). In some cases, T2D patients manage to achieve glycemic control by oral medications in combination with diet changes and exercise. However, the progressive nature of the disease leads more frequently to inevitable oral agent treatment in combination with basal insulin therapy as glycemic targets cannot be maintained at a certain point (Swinnen, et al. 2009). In contrast, patients suffering from type 1 diabetes are highly dependent on treatment with exogenous insulin to maintain normal blood glucose levels due to the absolute insulin deficiency. Nevertheless, this treatment cannot prevent long-term complications including the risk of hypoglycemic episodes, heart disease, nerve damage and moreover a decreased life expectancy. A promising treatment option for diabetic patients represents whole pancreas

transplantation, which is currently the only long-term treatment that restores glycemic control without exposing patients to the risk of severe hypoglycemia (Gruessner and Gruessner 2013). However, this procedure is an invasive surgery accompanied with life-long immunosuppression to prevent graft rejection and autoimmune islet destruction. Consequently, pancreas transplantation holds the risk of severe side effects and morbidity. The concept of allogenic islet transplantation represents a novel clinical approach by which healthy pancreatic islets are isolated from cadaveric organs and infused into the portal vein of the diabetic patient's liver. This treatment holds the promise of insulin independence without the invasiveness of whole organ pancreas transplantation (Samy, et al. 2014). Although, beta cell replacement therapy by either pancreas- or islet transplantation can reduce hypoglycemic episodes and improve life quality, the organ shortage and necessity of immunosuppressive medication are two major issues that prevent broad clinical application of the treatment (Bruni, et al. 2014). As an alternative approach pig to human islet xenotransplantation represents an encouraging strategy to overcome the shortcoming of organ donor shortage. However, this strategy is still experimental and needs further validation in order to assess the infectious risk of xenotransplantation across species barriers (Abrahante, et al. 2011) as well as solving the issue of T-cell mediated rejection (van der Windt, et al. 2012). These limitations, together with a shortage of donor organs has directed research to alternative beta cell sources for transplantation and led to identification of pluripotent human embryonic stem cells (ESCs) (Thomson, et al. 1998) and induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). Both are capable of developing into any cell type and could therefore replace the missing beta cells. However, utilization of these new sources still requires further optimization as there are problems associated with mutagenesis, tumorigenicity and immunogenicity (Pappas and Yang 2008; Gutierrez-Aranda, et al. 2010).

Therefore, another promising but yet unknown branch of research for diabetes therapy represents the usage of the body´s own regenerative capacity. Unfortunately, our understanding of mechanisms that control adaptive beta cell mass expansion and survival are still limited. Uncovering the plasticity of pancreatic endocrine mass under physiological conditions might unravel novel therapeutic approaches and represent a next fundamental step for future diabetes therapy.

1.4 Plasticity of the pancreatic endocrine mass

Throughout life, pancreatic beta cell mass is maintained by the equilibrium of cell replication, renewal and apoptosis (Bonner-Weir 2000). However, it has been demonstrated that beta cell mass slowly increases during lifetime in tight parallelism to the total body weight at any time (Montanya, et al. 2000). Nevertheless, beta cell mass is capable of dynamic adaptations to compensate changing metabolic conditions such as pregnancy, obesity or aging (Van Assche, et al. 1978; Defronzo 1979; Rieck and Kaestner 2010; Saisho, et al. 2013). For instance, during late pregnancy or obesity the increase in beta cell mass and the improvement of secretory activity serves as a physiological adaptation to meet the metabolic demand and to compensate insulin resistance. The lack of this adaptive process might lead to impaired insulin secretion and is in part related to the development of type 2 diabetes (Fonseca 2009). An autopsy study performed in 1985 revealed that not only beta cell mass is increased in nondiabetic obese patients, but also that type 2 diabetes is linked to a loss of pancreatic beta cell mass (Kloppel, et al. 1985). Subsequent studies suggested the decrease in mass is a result of combined stresses specifically directed at the beta cells (Halban, et al. 2014). Taken together, these findings suggest that the ability of beta cell mass expansion under increased metabolic conditions might be a key factor in preventing T2D.
Although it is known for decades, that changes in metabolic conditions are associated with a compensatory increase in beta cell mass and function (Green and Taylor 1972), the mechanisms involved are still not completely clarified. In general, three main mechanisms responsible for the formation of new beta cells have been suggested (Fig. 2): replication of existing beta cells, beta cell neogenesis *via* differentiation of new beta cells from endocrine progenitors or stem cells and transdifferentiation of non-beta cells to beta cells (Toselli, et al. 2014).

1.4.1 Replication of preexisting beta cells

Although multiple mechanisms have been implicated to be involved in adult beta cell mass maintenance and renewal, it has been demonstrated that replication of preexisting beta cells represents the major source for beta cell mass during adult life (Dor, et al. 2004). Moreover, beta cells have been shown to be very long lived and to maintain their capacity to replicate throughout lifespan (Teta, et al. 2005). In a study performed by Montanya and colleagues the rate of beta cell replication and apoptosis was determined throughout the first 20 month of life, in Lewis rats. While potent beta cell proliferation was observed during the initial 7 month of life, replication was progressively reduced thereafter. Moreover, it has been

shown that hypertrophy was responsible for beta cell mass increase in response to increased metabolic demands in the aged cohort, whereas levels of apoptosis remained stable from 1 to 20 month of life (Montanya, et al. 2000).

Similarly, islet cell proliferation in the human pancreas was shown to correlate with age (Meier, et al. 2008). While high frequencies of beta cell replication were observed within the first year of life, a rapid decline during early childhood marks proliferation in the adult human pancreas as an extremely rare event (Kassem, et al. 2000). However, postnatal expansion has been demonstrated to follow the same pattern as seen in mouse, namely by an increase in islet size rather than islet number (Georgia and Bhushan 2004).

Although turn-over rates have been shown to be rather low in the adult pancreas, beta cells are capable to compensate metabolic challenges by increased proliferation which has been suggested by various studies using genetic mouse models of obesity (Bock, et al. 2003; Georgia and Bhushan 2004; Hull, et al. 2005; Cox, et al. 2016). More precisely, Bock *et al.* could show a total islet volume increase of 3.6 fold in *ob/ob* mice, a mouse strain with a mutation in the leptin encoding gene resulting in profound obesity. Interestingly, total islet numbers did not differ in comparison to *ob/+* mice, suggesting islet cell hypertrophy and hyperplasia as main mechanism for beta cell mass expansion (Bock, et al. 2003). Similar results have been observed by Cox and colleagues using an inducible leptin receptor deficient mouse resulting in a threefold beta cell mass increase as a consequence of increased proliferation (Cox, et al. 2016). Furthermore, experimental models of pancreas injury confirmed that the initiation of proliferation represents the major mechanism of beta cell regeneration. Work performed by Nir and colleagues demonstrated that, after diphtheria toxin induced beta cell ablation with an efficiency of 70-80 %, the remaining pool of beta cells showed enhanced proliferation rates in the adult mouse and lead to recovery of glucose tolerance over time (Nir, et al. 2007). Moreover, lineage tracing experiments revealed that all beta cells contribute homogenously to islet growth, regeneration and maintenance (Brennand, et al. 2007).

A physiological model for a marked increase in pancreatic beta cell mass is represented by pregnancy, as increased insulin demand is compensated by beta cell mass expansion (Rieck and Kaestner 2010). Multiple studies performed in rats and mice demonstrated a massive beta cell growth primarily due to proliferation of preexisting beta cells (Parsons, et al. 1992; Sorenson and Brelje 1997; Beamish, et al. 2017). Also human pregnancy is associated with an increase in islet mass of up to 2 fold (Van Assche, et al. 1978). However, beta cell adaptation was mainly due to an increase in islet density represented by the appearance of increased numbers of small islets (Butler, et al. 2010). This suggests the influence of beta cell neogenesis rather than proliferation of existing beta cells during human pregnancy.

In summary, these and other studies have demonstrated that adult beta cell growth primarily occurs by self-duplication and that metabolic demand resembles the most powerful stimuli for beta cell mass expansion. Nevertheless, knowledge about contribution of endocrine non-beta cells or progenitor cells is lacking and might also play a crucial role in physiological adaptation of beta cell mass.

1.4.2 Beta cell neogenesis

Islet cell neogenesis describes the formation of new pancreatic endocrine cells and is known to be responsible for the initial formation of the endocrine pancreas during embryogenesis (Herrera, et al. 1991; Gu, et al. 2002). During pancreas development, multiple transcription factors are involved in the differentiation of pancreatic progenitor cells towards endocrine lineage commitment (Jensen, et al. 2005). It has been demonstrated, that the regenerative capacity of beta cells declines very rapidly after the first days of life and that neogenesis derived from precursor cells is not readily activated thereafter (Wang, et al. 1996). Yet, regeneration of beta cell mass might occur under certain physiological conditions. Thus, lineage tracing experiments by Abouna *et al*. have identified the contribution of non-beta cell progenitors to beta cell mass expansion during pregnancy. Moreover, it has been suggested that these precursor cells are associated with ductal epithelium (Abouna, et al. 2010). Remaining morphological evidence for beta cell regeneration derives from studies performed in experimental models of beta cell destruction or injury (Bouwens and Rooman 2005). Thus, Xu and colleagues observed the generation of new beta cells induced by partial duct ligation (PDL) in the pancreas of the adult mouse. Lineage tracing revealed the activation of highly proliferative, *Ngn3* expressing cells within the ductal lining. After isolation, these cells gave rise to functional beta cells *in vitro*. These findings suggest the contribution of multipotent progenitor cells in the ductal lining that activate *Ngn3* expression to induce beta cell regeneration upon injury by PDL (Xu, et al. 2008). A follow up study using selective *Ngn3* ablation, confirmed the key role of this transcription factor and its contribution in the neogenesis of beta cells in the injured pancreas of adult mice (Van de Casteele, et al. 2013). An alternative mechanism was described using an experimental model of beta cell destruction caused by alloxan in adult Bl/6 mice. Alloxan treatment mediated beta cell destruction and induced hyperglycemia after 1 day. Treatment with a combination of gastrin and *epidermal growth factor* (*EGF*) through an implanted pump, lead to enhanced beta cell growth and restoration of glycemia within 4 days. The authors observed the occurrence of newly formed islets without the influence of proliferation or hypertrophy, demonstrating the involvement of neogenesis from precursor cells. Moreover, these precursor cells are vastly

proliferative and express ductal cytokeratin, suggesting a transition of ductal cells towards endocrine beta cells (Rooman and Bouwens 2004)].

In summary, the mechanism of islet neogenesis from progenitor cells can lead to vigorous expansion and partial regeneration of beta cell mass, yet this pathway seems to be exclusive in embryonic development, postnatal growth and in response to severe pancreatic injury or beta cell destruction. Only little evidence supports the influence of islet cell neogenesis under physiological conditions and its quantitative contribution remains unknown. Nevertheless, the induction of endogenous beta cell neogenesis may provide a promising path to replenish beta cell mass in diabetes

1.4.3 Transdifferentiation of non-beta cells

Transdifferentiation is a process described by the conversion of a differentiated cell into another type of cell and depends on cellular reprogramming. A representative example for the occurrence of transdifferentiation in the pancreas is acinar to ductal metaplasia. This process is observed under severe stress conditions such as pancreatitis, and describes the differentiation of acinar cells into duct cells in order to replenish the damaged organ (Schmid 2002; Grippo and Sandgren 2012). Although acinar cells undergo morphologic and transcriptional conversion, they do not fully become ductal cells (Murtaugh and Keefe 2015; Beer, et al. 2016). These cells adopt in terms of gene expression pattern, however resemble embryonic progenitor cells properties (Chuvin, et al. 2017). Thereby, these cells remain highly proliferative and have the capacity to regenerate, whereas mature acinar and ductal cells are largely quiescent (Jensen, et al. 2005; Mills and Sansom 2015). Likewise, acinar cells have the ability to adopt an insulin-producing phenotype. This has been shown by *in vivo* reprogramming of acinar cells, using a combination of the three transcription factors *Ngn3, Pdx1* and *MafA*. The re-expression of this key developmental regulators results in the differentiation of insulin producing cells in a glucose sensitive manner to restore glucose homeostasis after destruction of the endogenous beta cells (Zhou, et al. 2008). Moreover, there is evidence for transdifferentiation within pancreatic islets after injury, shown by the potential of alpha cells to differentiate into beta cells. Thorel *et al.* used the glucagon-TetO system to label alpha cells prior to diphtheria toxin induced beta cell ablation. Lineage tracing experiments revealed that large fractions of regenerated beta cells derived from conversion of alpha cells (Thorel and Herrera 2010). A study performed by Collombat and colleagues confirmed alpha to beta cell conversion after overexpression of the *Pax4* gene in alpha cells. Conversion was observed under reactivation of the developmental transcription factor *Ngn3* (Collombat and Mansouri 2009). Moreover, the group of Collombat could demonstrate that the long-term administration of gamma-aminobutyric acid (GABA) leads to alpha to beta like

cell conversion *in vivo*. Subsequently, alpha cells are replaced by mobilization of duct-lining progenitor cells that reactivate the endocrine developmental program, adopt an alpha cell identity and migrate towards the islets (Ben-Othman, et al. 2017). Interestingly, Chera and colleagues could show that prior to puberty, pancreatic delta cells are capable to transdifferentiate into insulin producing beta cells, rather than alpha cells. This has been demonstrated by lineage tracing experiments and diphtheria toxin induced beta cell ablation two weeks after birth (Chera, et al. 2014).

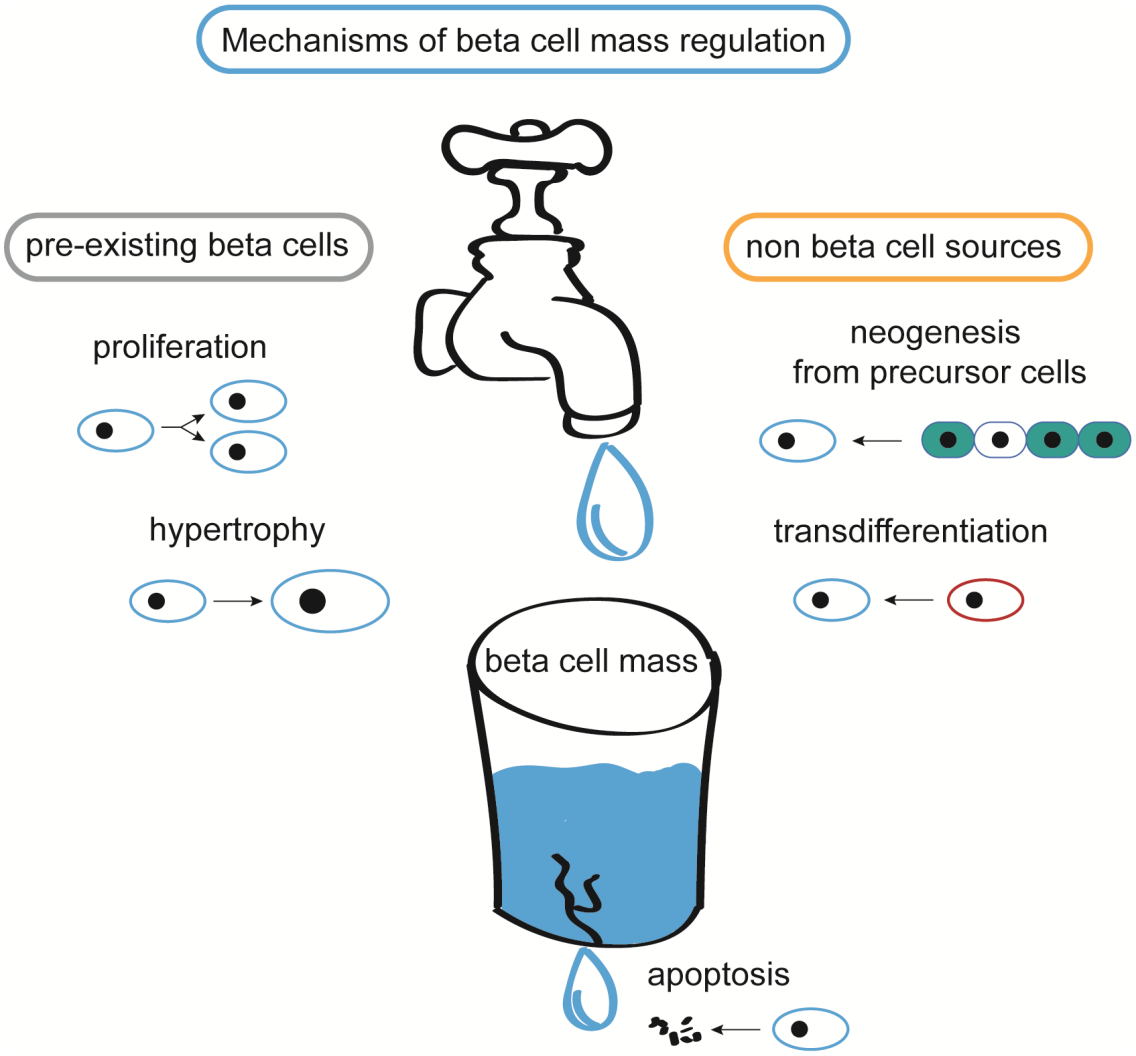

Figure 2: Mechanisms involved in the dynamic regulation of beta cell mass. Illustration of different mechanisms involved in beta cell mass regulation. Beta cell dependent mechanisms, such as replication of preexisting cells and hypertrophy are shown on the left side. Beta cells derived from non-beta cell sources, like neogenesis from ductal cells or transdifferentiation from other mature pancreatic cell types are shown on the right side. Reduction of beta cell mass can result from increased apoptotic cell death and is demonstrated at the bottom.

In summary, the pancreas is capable of adapting to metabolic challenges by different potential mechanisms that can modulate beta cell mass. However, knowledge about the induction of these processes was only provided using experimental model systems of pancreas injury and extreme metabolic conditions. Therefore, it remains unclear whether the induction of endocrine plasticity is a physiological or entirely artificial process of beta cell mass expansion and requires further investigation.

1.5 Pregnancy as a physiological model system for endocrine cell plasticity

1.5.1 The physiology of pregnancy

Pregnancy resembles a unique physiological event, characterized by complex metabolic adaptation processes under which influence pancreatic islets have to undergo major short-term structural and functional changes to adapt the increased demand for insulin (Parsons, et al. 1992). All endocrine and metabolic changes occurring within pregnancy represent an indispensable adaptation to meet the energy demand of the growing fetus and to prepare the maternal organism for labor and lactation. The adaptations during pregnancy also affect the maternal glucose and lipid metabolism (Grimes and Wild 2000). As the developing embryo uses glucose as a main source of energy, carbohydrates are redirected towards the fetus and the maternal energy production is switched towards lipid metabolism. This results in increased maternal levels of circulating free fatty acids, triglycerides, cholesterol and phospholipids (Catalano, et al. 1999). Transport of glucose represents a passive process *via* glucose transporters across the placenta and therefore glucose delivery depends on a concentration gradient between the maternal and fetal circulation system (Baumann, et al. 2002). In general, alterations in maternal metabolism can be divided in an anabolic and a catabolic phase. The anabolic phase is characterized by enhanced fat deposition in the maternal tissues and takes place during the first 2 trimesters of pregnancy. Multiple factors, including de novo lipogenesis (Palacin, et al. 1991) and enhanced lipoprotein lipase (LPL) activity (Alvarez, et al. 1996) have been reported to promote fat deposition. The switch to a catabolic metabolism occurs in the third trimester of pregnancy and leads to increased breakdown of these fat depots as a consequence of increased energy demand (Kurpinska, et al. 2015). As part of the systemic metabolic changes, the maternal body develops a physiological insulin resistance resulting in a 50-60 % decrease in insulin sensitivity with ongoing gestation (Catalano, et al. 1999). A condition of insulin resistance typically develops in the phase of pronounced fetal growth between the second and third trimester of gestation. On the other hand, beta cell function is increased leading to enhanced

insulin secretion already during the first trimester and steadily increases to a maximum in the third trimester (Sivan, et al. 1997). Although reduced insulin sensitivity does not reflect a pathological condition, similarities to impaired insulin action in T2D can be observed. It has been indicated, that the development of insulin resistance is associated with a decrease in the postreceptor insulin signaling cascade, primarily due to a decreased expression of the insulin receptor substrate 1 protein that drives tyrosine phosphorylation of the receptor (Friedman, et al. 1999). However, clinical studies have reported that maternal insulin sensitivity is completely restored approximately one year postpartum, starting to increase shortly after delivery (Sivan, et al. 1997; Berggren, et al. 2015). Studies using the euglycemic clamp technique confirmed comparable insulin sensitivity in comparison to non-pregnant women within three days after delivery. Moreover, enhanced insulin sensitivity was accompanied with an increase in insulin receptor concentration and protein expression, confirming the reversal of the underlying metabolic adaptation processes occurring during pregnancy (Kirwan, et al. 2004).

Studies in rodents have demonstrated that the changes in the metabolic state during pregnancy and the adaptions on beta cell function coincide with the rise of steroid hormones and placental lactogens (Fig. 3). These include progesterone, estrogens, chorionic gonadotropin, placental lactogen, prolactin and growth hormones (Brelje, 1993; Ernst, 2011 Galosy,1995; Georgia, 2010; Kim, 2010; Kosaka, 1988; Ryan, 1988; Sorenson, 1997; Soares, 2004). They are produced and secreted either by the pituitary gland, the corpus luteum, a temporary endocrine gland within the ovaries or later during pregnancy by the placenta. Studies in mice could demonstrate that hormonal secretion by the corpus luteum within the ovaries is essential to start and maintain pregnancy during the first and second trimester (Parkes 1928; Rubinstein and Forbes 1963). This is most likely linked to its secretion of the steroid hormone progesterone, which prepares the uterine lining for a potential pregnancy after ovulation. In case of fertilization, chorionic gonadotropin is secreted by cells of the blastocyst which signal the corpus luteum to further increase progesterone secretion and maintain pregnancy. If no fertilization takes place, the corpus luteum breaks down, resulting in decreasing progesterone levels and the endometrium is expelled (in humans) or lining degenerates back to normal size (in rodents) (Kumar and Magon 2012). Serum progesterone levels have been intensively studied in mice showing a sharp increase already during the first 2-3 days of pregnancy followed by a plateau until beginning of the third trimester. Within the last trimester of pregnancy, progesterone levels further increase and reach a peak at gestational day 16-18. The dramatic drop of serum progesterone levels at the end of gestation serves as a signal for the maternal body to induce labor (Murr, et al. 1974; Barkley, et al. 1979; Kosaka, et al. 1988). Besides its stimulatory effect on food intake

and fat deposition (Ladyman, et al. 2010), progesterone has been shown to induce glucose intolerance by decreased insulin binding and glucose transport (Nelson, et al. 1994). Similar to progesterone, also estrogen levels increase early during pregnancy although not as tremendous. Serum estrogen levels steadily increase with progress of pregnancy and decline shortly before delivery (Barkley, et al. 1979). Estrogen has been proven essential for embryo implantation (McCormack and Greenwald 1974), to maintain pregnancy (Albrecht, et al. 2000) and plays a fundamental role in the biosynthesis of progesterone (Albrecht and Pepe 1990). Moreover, it has been shown that the presence of estrogens amplifies the physiological effects of progesterone (Kastner, et al. 1990).

In the second and third trimester, the placenta takes over the production of hormones and releases a series of lactogens in addition to the described steroid hormones, resulting in a progressive augmentation of serum hormone levels with ongoing gestation. The most abundant lactogenic hormone secreted by the placenta is placental lactogen (PL). In humans it is secreted into the maternal and the fetal circulation system after the sixth week of pregnancy. In mice, placental lactogen-I (PL-I) is produced immediately after implantation and its expression increases until mid-gestation when it is replaced by PL-II until term. Both forms are synthesized by trophoblast giant cells (Galosy and Talamantes 1995). PL expression is associated with insulin resistance, increased insulin secretion and shown to promote lipolysis, which consequently inhibits gluconeogenesis (Brelje, et al. 1993; Kumar and Magon 2012).

During pregnancy there is also a progressive increase of maternal serum growth hormone (GH), which is produced by the pituitary gland during the first trimester. The expression of GH is replaced by a variant growth hormone (GH-V), which is synthesized by the placenta at about mid gestation. Levels of placental growth hormone increase gradually throughout pregnancy and have been indicated to reflect placental function and fetal growth (Lonberg, et al. 2003). Both, GH and PL have been observed to stimulate insulin-like growth factor (IGF) production to modulate the maternal metabolism and consequently contribute in redirecting carbohydrates towards the developing fetus (Handwerger and Freemark 2000).

Another relevant lactogenic hormone during gestation is prolactin (PRL), secreted by the pituitary gland. Prolactin levels increase dramatically when pregnancy gets to term as it regulates the onset of lactation and the synthesis of milk (Brelje, et al. 2004). Before, pituitary PRL secretion is inhibited by placental lactogens produced by the placenta (Galosy and Talamantes 1995). The effect of declining estrogen and progesterone levels in late pregnancy sharply increase PRL secretion (Grattan and Averill 1990; Bonafede, et al. 2011). These observations indicate that pregnancy induces a tightly regulated hormonal network with complex interactions among the different hormones. Finally, the secretion of lactogenic

hormones (PL, PRL and GH), has been shown to effect the regulation of islet mass increase and improves islet cell function during pregnancy (Parsons, et al. 1992; Brelje, et al. 1993; Brelje, et al. 2004; Georgia and Bhushan 2010; Ernst, et al. 2011).

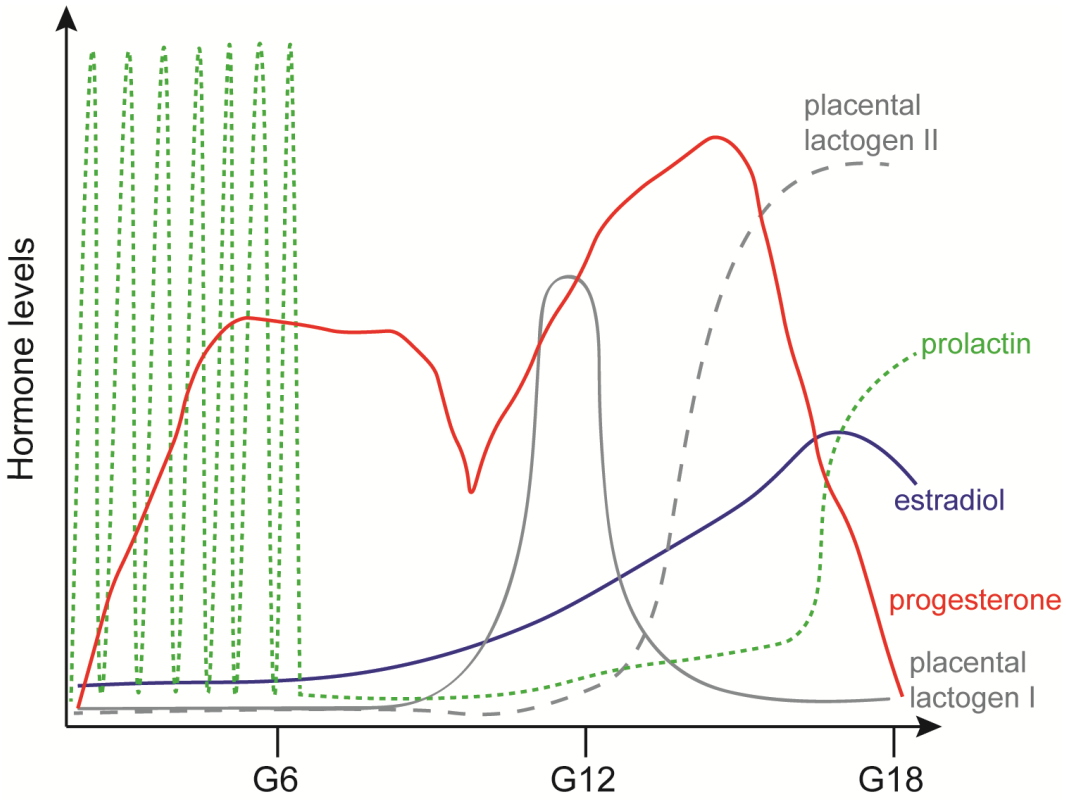

Figure 3: Changes in plasma hormone levels during mouse pregnancy. Serum levels of progesterone (red line) adapted from Murr, 1974; estradiol (blue line) adapted from Barkley, 1979; placental lactogen I and II (grey lines) and prolactin (green dashed line) adapted from Soares, 2004.

1.5.2 Adaptation of islets of Langerhans in response to pregnancy

The adaptation of islets of Langerhans is necessary to comply with the higher insulin demand caused by the increase in maternal insulin resistance and to counteract excessive carbohydrate supply towards the fetus (Zeng, et al. 2017). Failure of this adjustment is believed to be an underlying cause of gestational diabetes. The detailed mechanisms of morphological and functional beta cell compensation are not fully understood. However, several studies performed on isolated islets and perfused extracted pancreata from pregnant rodents have shown lowered glucose thresholds for insulin synthesis and increased glucose metabolism (Green and Taylor 1972; Bone and Taylor 1976; Parsons, et al. 1992). More precisely, Weinhaus and colleagues have observed an increase in glucose metabolism as result from elevated activity of glucokinase, hexokinase and glucose transporter 2 in isolated islets from pregnant rats (Weinhaus, et al. 1996). Moreover, the authors could observe the

same changes after PL and PRL treatment *in vitro*, suggesting that adaptive metabolic changes during pregnancy might be mediated by lactogen regulated events. Furthermore, elevated lactogen levels during late gestation lead to enhanced beta cell coupling *via* gap junctions and results in improved glucose induced insulin secretion (Sheridan, et al. 1988).

Morphological studies in rodents found a 1.6-4 fold increase in beta cell mass largely attributed to enhanced beta cell duplication and hypertrophy as predominant mechanism for beta cell mass expansion during pregnancy (Parsons, et al. 1992; Sorenson and Brelje 1997; Rieck and Kaestner 2010) BrdU incorporation in pregnant rats (Parsons, et al. 1992) and Ki67 labeling in mice (Rieck, et al. 2009) has demonstrated that the increase in beta cell proliferation is first observed within the second trimester, peaks around gestational day 14 and then gradually declines to control levels by the end of pregnancy. Moreover, insulin DNA contentincreased about 32 % and protein content was elevated about 62 % when measured in isolated islets from pregnant rats at term (Green and Taylor 1972). A recent study by Kim *et al.* provided evidence that the downstream of lactogenic signaling acts as a paracrine factor to initiate beta cell mass expansion during pregnancy. The authors found that activation of the prolactin receptor by lactogenic hormones increases transcription of the rate-limiting enzyme in serotonin synthesis. Consequently the *serotonin receptor gene Htr2b*, expressed on the beta cell surface, stimulates cell cycle progression and increases beta cell replication (Kim, et al. 2010). Furthermore, it has been shown that serum of pregnant women is capable to stimulate beta cell proliferation of neonatal rat islets *in vitro*. More precisely, proliferative activity was increased gradually with progression of gestation, while serum from non-pregnant women did not have an effect on cell replication. The mitotically active serum fraction contained placental lactogen, serum albumin and peptides involved in blood coagulation like fibrinogen and kininogen-1 (Nalla, et al. 2014).

The question whether other mechanisms than proliferation of preexisting beta-cells might also contribute to the enhanced beta cell mass, still remains unclear. Lineage tracing experiments using an inducible transgenic mouse strain to label endogenous beta cells prior to pregnancy indicated the role of non-beta progenitor cells due to a dilution of labeled beta cells during pregnancy (Abouna, et al. 2010). Likewise, Toselli and colleagues showed a drop from 97 % to 87 % beta cell fraction by mid-gestation using another beta cell lineage tracing mouse model (Toselli, et al. 2014). Moreover, *Ngn3* signaling was shown to be involved in the contribution of non-beta cells to beta cell mass increase during pregnancy. Further evidence for beta cell neogenesis has only been reported during human pregnancy. A morphological study, performed on pancreata obtained from autopsies of pregnant women, demonstrated an increased density of small islets during pregnancy and post-partum. Moreover, increased beta cell replication and apoptosis was not observed during pregnancy,

suggesting beta cell formation by other sources than replication of preexisting beta cells (Butler, et al. 2010). However, a study performed by van Assche and co-workers reported a 2-fold increase of islet mass attributed to increased number of beta cells and hyperplasia (Van Assche, et al. 1978). Thus, both human and rodent pregnancy is characterized by beta cell mass expansion to counteract insulin resistance and meet the growing metabolic demand. Yet, the underlying mechanisms are still not clarified and might differ among the species. Whereas beta cell proliferation and hypertrophy seem to be the most prominent mechanisms in rodent pregnancy, there is growing evidence that beta cell formation in humans arises in part from other sources than beta cell replication. However, the quantitative contribution can only account for a minor fraction to the total beta cell mass. Thus, there is need to clarify the role and extend of neogenesis in contributing to beta cell plasticity during pregnancy.

1.6 Aim

Although it is known for decades that pregnancy is associated with a compensatory increase in beta cell mass and function, the mechanisms involved are still not completely clarified. In general, the replication of existing beta cells, beta cell neogenesis *via* differentiation of new beta cells from endocrine progenitors or stem cells and transdifferentiation of non-beta cells have been suggested as main mechanisms responsible for the formation of new beta. However, knowledge about contribution of non-beta cell progenitors is lacking. Since the population of beta cells in the pancreas of diabetic patients is massively reduced, replication of surviving beta cells would not represent a reasonable therapeutic option. However, the induction of neogenesis or cellular reprogramming towards beta cell identity could provide a new beta cell population that can be further expanded by replication. Thus, understanding this intrinsic regenerative capacity of the endocrine pancreas might help to develop new treatments for diabetes therapy.

Therefore, the overall aim of this thesis is to study the mechanisms involved in the adaptation of beta cell mass and function during pregnancy, as a physiological model of islet plasticity. A first aim is to assess changes in pancreatic mass expansion at different time points during and after pregnancy and to investigate the role of proliferation. Although it has been demonstrated that proliferation of preexisting beta cells is the main mechanism for mass expansion during mouse pregnancy, the role of non-beta cell progenitors or reprogramming from differentiated non-beta cells is still lacking. Therefore, a second aim is to investigate the role of neogenesis or cellular reprogramming, indicated by the recurrence of progenitor gene expression of *Ngn3* in the compensatory response during early pregnancy. To fulfill this aim, *in vivo* experiments in transgenic reporter mice will be performed, combined with *in situ* analyses in tissue slices and *in vitro* studies on isolated islets.

2 Material and Methods

2.1 Mice

All mouse experiments were conducted in accordance with the German Animal Welfare Act, following the guidelines of the European Convention for the protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and approved by the Committee on the Ethics of Animal Experiments of the State Directory of Saxony (24-9168.11-1/2014-9; DD24-5131/339/21(TVV A 20/2015)). Mice were housed in a licensed animal facility on a 12h day-night cycle. The animals were maintained in groups of max. 5 animals per cage and had free access to water and chow (SNIFF rat/mouse maintenance 10 mm). All mouse experiments were conducted using one of the strains listed below (Table 1).

Table 1 : Overview of the applied mouse stains

Mouse strain	Abbreviation	Reference	Supplier
C57BL/6J	BL/6	-	Janvier Labs
Tg(Neurog3-cre/Esr1)Dam/J	Ngn3-CreER	Gu et al.,2002	The Jackson Laboratory
Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J	mTmG	Muzumdar et al., 2007	The Jackson Laboratory
B6.FVB(Cg)-Tg(Neurog3-cre)C1Able/J + Gt(ROSA)26Sortm1(EYFP)Cos	Ngn3-YFP	Mellitz et al., 2004	Gerard Gradwohl
Somatostatin-Cre; R26-YFP	Sst-Cre;YFP	Chera et al., 2014	F.M. Gribble Camebridge
B6(Cg)-Ins1tm1.1(cre)Thor/J	Ins1-Cre	Thorens et al., 2015	Bernard Thorens
Gt(ROSA)26Sortm1(CAG-tdTomato,-EGFP)Ees	nTnG	Prigge et al., 2013	The Jackson Laboratory

2.1.1 Genotyping of mice

To determine the genetic variants of the animals, genotyping was performed by polymerase chain reaction (PCR). Biopsies were conducted from the tail tip at weaning and genomic deoxyribonucleic acid (DNA) was extracted. Therefore, tail tips were transferred in

1.5 mL reaction tubes (VWR) and 25 µL tail lysis buffer (TLB, see Table 2) containing 1 mg/mL Proteinase K (Sigma Aldrich) was added. The reaction was incubated for 60 min at 55°C in a heating block, while shaking at 450 rpm (TMix, Analytik). Tissue was mashed thoroughly by using disposable spatula (Sarstedt) and further incubated for additional 60 min at 55°C. The suspension was heated for 10 min at 99°C and 175 µL ddH_2O was added and mixed properly. After 2 min centrifugation at 21100x g the supernatant was taken and analyzed by PCR. Reactions were performed with the respective primers for each mouse line (Table 3) according to the protocols given by the respective mouse supplier in a PCR cycler (Mastercycler, Eppendorf).

Table 2: Composition of tail lysis buffer

Reagent	Company	Final concentration
Tris HCl (pH 8.0)	Merck	50 mM/L
NaCl	Roth	100 mM/L
EDTA	SPO	100 mM/L
Tween 20	Merck	0.5 %
NP-40	Sigma	0.5 %

Table 3: Primers for Genotyping

Strain	Forward Primer (5´→ 3´)	Reverse Primer (5´→ 3´)
Ngn3-YFP (wildtype)	TCTCGCCTCTTCTGGCTTTC	CGGCAGATTTGAATGAGGGC
Ngn3-YFP (mutant)	AGGGCGAGGAGCTGTTCA	TGAAGTCGATGCCCTTCAG
Ngn3CreER	AACCTGGATAGTGAAACAGGGGC	TTCCATGGAGCGAACGACGAGACC
mTmG (wildtype)	CTCTGCTGCCTCCTGGCTTCT	CGAGGCGGATCACAAGCAATA
mTmG (mutant)	CTCTGCTGCCTCCTGGCTTCT	TCAATGGGCGGGGGTCGTT
Cre	CATTTTGGGCCAGCTAAACATT	CCCGGCAAAACAGGTAGTTA
YFP (wildtype)	CTGGCTTCTGAGGACCG	GACAACGCCCACACA
YFP (mutant)	AGGGCGAGGAGCTGTTCA	TGAAGTCGATGCCCTTCAG
nTnG (wildtype)	AAAGTCGCTCTGAGTTGTTAT	GGAGCGGGAGAAATGGATATG
nTnG (mutant)	AAAGTCGCTCTGAGTTGTTAT	CCAGGCGGGCCATTTACCGTAAG

2.1.2 Mating and pregnancy control

Female mice at the age of 8-12 weeks were mated with male mice of the corresponding strain and a vaginal plug check was performed every morning and afternoon. The day of plug detection was defined as gestational day 0 and female animals were separated immediately. In order to assess the progress of pregnancy, body weight of female mice was determined before mating and every 2-6 days during pregnancy. Non-fasting blood glucose levels were measured by a glucometer (AccuCheck Aviva, Roche) before mating and on the day of sacrifice, in a drop of blood from the tail tip. Animals were sacrificed by cervical dislocation and the accurate gestational stage was verified on the basis of embryo size.

2.2 Tamoxifen preparation and application

The Ngn3-CreER;mTmG reporter mouse strain was used for *in vivo* labeling of *Ngn3* positive cells during pregnancy. The *Ngn3* promoter driven expression of the inducible Cre/LoxP reporter system with the double-fluorescent mTmG indicator allows visualization of *Ngn3* positive cells during a desired timeframe in the adult pancreas when exposed to tamoxifen (TAM). The reporter mouse ubiquitously expresses membrane bound tdTomato switching to GFP after TAM administration in *Ngn3* expressing cells.
Tamoxifen (Sigma) powder was dissolved in 96 % ethanol to obtain a stock solution of 100 mg/mL. For subcutaneous (sc) injection, stock solution was further diluted in filter-sterilized corn oil (Sigma) to a final concentration of 20 mg/mL. Female animals received an absolute dose of 5 mg TAM in a total volume of 200 µL.

2.3 Experiments on isolated pancreatic islets

2.3.1 Pancreatic islet isolation

The islets of Langerhans were isolated from 8-12 week old female mice by enzymatic digestion. Collagenase type V from *Clostridium histolyticum* (Sigma) was dissolved in RPMI-1640 medium (Life Technologies) to a final concentration of 0.45 mg/mL and filtered through a 0.2 µm filter (Filtropur S 0.2, Startstedt AG). The collagenase solution was kept in 5 mL syringes with a 30-gauge cannula (BD Microlance) and stored on ice until injection.

Animals were sacrificed by cervical dislocation and the pancreas was exposed by abdominal incision. The major duodenal papilla was clamped underneath the ampulla of Vater and 3 mL of cold collagenase solution was injected into the common bile duct. The injected pancreas was removed and transferred in a 50 mL Falcon tube containing 2 mL of collagenase solution stored on ice. Digestion was performed in a water bath at 37°C for 8.5 min without and 1 min with gentle shaking by hand. The reaction was stopped by placing the tube on ice and addition of 45 mL ice-cold RPMI-1640 medium containing 10 % fetal bovine serum (FBS). After 2 min centrifugation at 900 rpm, supernatant was discarded and the pellet was resolved in RPMI medium with 10 % FBS. Subsequently, the solution was poured through a small sterile metal strainer (sieve size 422 µm, Sigma) placed on top of a sterile beaker. The solution was centrifuged for 2 min in a cooled centrifuge at 900 rpm and the supernatant was discarded. To purify islets a discontinuous Ficoll (Sigma) gradient 1.108, 1.096 and 1.037 g/mL was created on top of the pellet followed by centrifugation for 18 min at 1900 rpm with gentle start and stop. Purified islets were collected between the 1.096 and 1.037 g/mL layers and transferred into 50 mL tubes and filled up to 50 mL with RPMI medium containing 5 % FBS. The solution was centrifuged for 2 min at 1000 rpm and the pellet was collected from the bottom of the tube using a 10 mL pipette. For further washing, islets were transferred in a 50 mL falcon and 40 mL RPMI medium with 5 % FBS was added. After a 2 min centrifugation at 900 rpm, islets were placed into a 60 mm suspension culture dish (Corning) filled with RPMI medium with 5 % FBS. Finally, islets were handpicked, counted and rested overnight in an incubator at 37°C and 5 % CO_2.

2.3.2 Culture of pancreatic islets

For culture experiments, RPMI-1640 with 5.5 mM/L or 11.1 mM/L glucose, 1 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin was used. In order to simulate early pregnancy conditions, 100 ng/mL progesterone (P_4; Sigma), 150 ng/mL estradiol (E_2, Sigma) or both were added. Islets were kept in 24 well plates filled with 0.5 mL medium under a humidified atmosphere consisting of 95 % air and 5 % CO_2 at 37°C. Culture was performed for 5 days and media change was performed after 24 hrs.

2.3.3 Cell dispersion

Isolated islets were handpicked from the dish and transferred to a 15 mL falcon tube. Islets were washed twice with 10 mL sterile 1x phosphate buffered saline (PBS; Life technologies) and centrifuged at 300 rpm for 1 min at 4°C with soft start and stop. Islets were dissociated by adding 5 mL of a 1:1 mix accutase (Sigma) and dissociation solution (Sigma)

in a water bath at 37°C for 9-12 min, depending on islet size and number. To stop digestion, 5 mL of cold FBS were added and mixed thoroughly with a 1000 µL pipette (15x) to dissociate the cells. The solution was centrifuged at 1000 rpm for 2 min with fast start and stop and the cell pellet was resuspended in 1 mL of sterile PBS. For cell counting, a Neubauer cell chamber was used and 4 individual squares were counted to calculate the mean. The cells were placed on ice for transportation to the flow cytometer.

2.3.4 FACS sorting

Dissociated islet cells were sorted as bulk by flow cytometry. In order to sort only viable cells, 1 µL propidium iodide (PI; Life technologies) was added and mixed properly. Cells were sorted on a LSRII flow cytometer (Becton Dickson) according to their fluorescent signals and their relative cell size. Fluorescence activated cell sorting (FACS) experiments were performed together with Anne Gompf from the FACS facility (CRTD). For qRT-PCR, 300 YFP^+ and 300 YFP^- -cells were sorted individually into PCR tubes containing 2 µL of lysis buffer.

2.3.5 Quantification by qRT-PCR

Isolated islets from Sst-Cre;YFP virgin and pregnant female mice were dispersed directly after isolation procedure and sorted by flow cytometry. Bulk samples containing either 300 YFP^+ or 300 YFP^- cells were collected in PCR tubes with 2 µL lysis buffer and further processed by the sequencing facility for RNA extraction and cDNA synthesis. Due to the low amount of cells, cDNA was amplified using the Smart-seq2 method with 12 cycles. Yields of 0.8-2 ng/µL cDNA were collected.

For quantitative real time-PCR (qRT-PCR) using an AriaMx Real-Time PCR System (Agilent Technologies), cDNA was diluted 1:50 with distilled water. Reactions were performed in duplicates with SYBR®Green qPCR SuperMix (Promega) according to the protocol described in Table 4. The master mix for each well consisted of 2.5 µL diluted cDNA, 10 µL SYBR®Green and 5.5 µl ddH_2O. Appropriate primers (100 pM) were used in a 1:20 dilution and 2 µL of diluted primer pairs were added to the individual wells prior to the master mix. Primer sequences were optimized by melting curve analysis and are listed in Table 5. Technical replicates were performed for each probe and Ct values were obtained by automatic Ct analysis of the AriaMx Real-Time PCR System. For data quantification Ct values were averaged and determination of individual gene expression was performed as

relative quantification against two housekeeping genes by the $2^{-\Delta\Delta Ct}$ method (Livak, 2001). Values were normalized to the control probes set as 100 %.

Table 4: qRT-PCR protocol

	Temperature	Time	Repeats
hot start	95°C	10 min	1x
amplification			45x
denaturation	95°C	15 sec	
annealing	58°C	30 sec	
ligation	72°C	30 sec	
melting	58°C – 95°C	30 sec	Ramp 2x/°C

Table 5: Pimers for qRT-PCR

Gene	Forward (5' → 3')	Reverse (5' → 3')
Actb	GAGGTATCCTGACCCTGAAG	GGTCATCTTTTCACGGTTG
B2M	GCTATCCAGCGTACTCCAAA	TGCTGCTTACATGTCTCGAT
HPRT	TGGACAGGACTGAACGTCTT	TATGTCCCCTGTTGACTGGT
Gcg	AGGCTCACAAGGCAGAAAAA	CAATGTTGTTCCGGTTCCTC
Ins2	CAGCAAGCAGGAAGCCTATC	GCTCCAGTTGTGCCACTTGT
NeuroD1	AACAGGAAGTGGAAACATGACC	TCTTCCTCCTCCTCCTCTCC
Ngn3	AGTCGGGAGAACTAGGATGG	GGCAGTCACCCACTTCTG
Nkx2.2	CCTCCAATACTCCCTGCAC	GTAGGTCTGCGCTTTGGAG
Pdx1	AGTGGGCAGGAGGTGCTTA	ACGGTTTTGGAACCAGATTT
Sst	CACCGGGAAACAGGAACT	CAGCCTCATCTCGTCCTG

2.4 Immunohistochemistry

2.4.1 Preparation of pancreatic tissue sections

Tissue sections were prepared from pancreata received from female virgin mice or mice at different stages during or after pregnancy. Animals were sacrificed by cervical dislocation and the abdomen was opened to extract the pancreas. The organ was fixed for 2 hrs in 4 % para-Formaldehyde (PFA) in PBS at 4°C and subsequently transferred into 30 % sucrose solution in PBS overnight. Afterwards, the pancreas was embedded in Tissue-TEK® optimum cutting temperature (OCT) (A. Hartenstein) and snap frozen in 2-methylbutan (Roth) chilled liquid nitrogen. Frozen tissue sections of 10 µm thickness were prepared using a Cryostat (Nx70, Mnf. Thermo). The entire pancreas was sectioned in 6 consecutive stacks and sections were collected on clean, fat-free superfrost glass slides (StarFrost®, Engelbrecht GmnH) at -20°C. One entire set of 11-14 slides was used for mass analysis.

2.4.2 Preparation of pancreatic tissue slices

Pancreatic tissue slices were prepared from female virgin mice and pregnant mice at gestational day 6 (Marciniak, 2014). Briefly, 1.25 % low melting point agarose (Roth) was melted in extracellular solution (ECS) composed of 5 mM KCl, 140 mM NaCl, 2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 3 mM glucose and 10 mM HEPES using a microwave oven and was kept at 37°C in a water bath. Animals were euthanized by cervical dislocation and the abdominal cavity was opened using surgical scissors. Mice were placed under a stereomicroscope and the common bile duct was clamped off at the ampulla of Vater. 3 mL low melting point agarose solution was injected steadily using a 5-mL syringe and a 27-gauge needle. After injection, the hardened pancreas was resected and subsequently transferred into a 60-mm petri dish filled with ECS. Tissue was further processed by removing connective and adipose tissue and cut into smaller blocks of approximately 3-5 mm^3 size. Tissue pieces were transferred into a 35-mm petri dish and the dish was filled with agarose solution until tissue was fully submerged. After agarose was solidified, individual agarose blocks containing pancreatic tissue were excised and mounted on a probe plate. Slicing procedure was performed using a semi-automated Vibratome (Leica, VT 1200s) at an amplitude of 1.0 mm, speed of 0.2 mm/s and a step thickness of 150 µm. Slices were either kept in a 60-mm petri dish filled with Krebs-Ringer bicarbonate HEPES buffer (KRBH) (137 mM NaCl, 5.36 mM KCl, 0.34 mM Na_2HPO_4, 0.81 mM $MgSO_4$, 4.17 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.44 mM KH_2PO_4, 10 mM HEPES, 0.1 % BSA, 3 mM glucose, pH 7.3) for functional assays or fixed for 30 min in 4 % PFA in PBS at 4°C and washed in PBS at room temperature.

2.4.3 Staining

Immunohistochemistry on slices was accomplished in 24-well plates with a total volume of 300 µL / well. Using 2 slices per well, staining was performed in blocking solution containing 1 % goat serum, 0.6 % Triton-X 100, 900 mM NaCl and 40 mM sodium phosphate buffer in deionized water (goat serum diluent buffer (GSDB) 0.6 % Triton-X 100). Primary antibodies (Table 6) were diluted in GSDB 0.6 % Triton-X and incubated over night at 4°C, shaking. Afterwards, slices were washed 3 times in PBS for at least 10 min and secondary antibodies (Table 7) diluted in GSDB 0.6 % Triton-X was applied and incubated overnight at 4°C, shaking. Three additional washing steps were applied using PBS and slices were stored in fresh PBS until imaging.

Table 6: Primary antibodies and dilutions

Antigen	Conjugate	Company	Cat. no.	Dilution
GFP	rabbit	Life technologies	A-111222	1:1000
GFP	chicken	Abcam	AB13970	1:1000
Insulin	guinea-pig	DAKO	A0564	1:200
Ki-67	goat	Santa Cruz Biotech.	sc-7846	1:100
Somatostatin	goat	Santa Cruz Biotech.	sc-7819	1:200
Somatostatin	rat	Millipore	MAB354	1:100

Table 7: Secondary antibodies and dilutions

Antigen	Conjugate	Company	Cat. no.	Dilution
donkey anti-goat IgG	Alexa Fluor® 405	Abcam	AB175664	1:200
donkey anti-goat IgG	Alexa Fluor® 633	Invitrogen	A-21082	1:200
goat anti-guinea pig IgG	Alexa Fluor® 405	Abcam	AB175664	1:200
goat anti-guinea pig IgG	Alexa Fluor® 488	Invitrogen	A-11073	1:200
goat anti-guinea pig IgG	Alexa Fluor® 633	Invitrogen	A-21105	1:200
goat anti-rabbit IgG	Alexa Fluor® 488	Invitrogen	A-11008	1:200
goat anti-chicken IgG	Alexa Fluor® 488	Invitrogen	A-11039	1:200
goat anti-rat IgG	Alexa Fluor® 546	Invitrogen	A-11081	1:200
goat anti-rat IgG	Alexa Fluor® 633	Invitrogen	A-21094	1:200

Pancreatic cryosections were stained using GSDB solution containing 0.3 % Triton-X 100. Therefore, 4 slides of each mouse were adjusted to room temperature and washed with PBS for 5 min. Primary antibodies (Table 6) were diluted in GSDB 0.3 % Triton-X and 200 µL staining solution were applied on each slide and incubated overnight at 4°C in a humidified staining chamber. Slides were washed three times in PBS for at least 10 min and subsequently incubated for 2.5 hrs at RT with secondary antibodies (Table 7) diluted in GSDB 0.3 % Triton-X solution. After three additional washing steps in PBS, slides were covered in Mowiol (Carl Roth) and mounted with a glass coverslip (No.1, Engelbrecht GmbH). Slides were stored at 4°C until imaging.

Isolated islets were stained in 24-well plates containing a total volume of 250 µL/well. For optimal permeabilization, islets were kept in PBS with 1 % Triton X for 3 hrs at RT, shaking. Then islets were picked into fresh wells containing diluted primary antibodies (Table 6) in GSDB 1 % Triton-X solution and incubated over night at 4°C, shaking. Islets were washed for at least one hour in PBS and stained with conjugated secondary antibodies (Table 7) diluted in GSDB 1 % Triton-X overnight at 4°C shaking. Finally, islets were washed in PBS for 1 hour and embedded into a fibrinogen gel (3:1:1 HBSS, fibrinogen (10 mg/mL), thrombin (50 U/mL)) in a 35-mm dish and covered with PBS and stored at 4°C.

2.4.4 Data aquisition

Tissue slices were imaged in a 60-mm dish containing PBS and kept in place using a slice anchor (Warner instruments). Isolated islets were imaged in gels within a 30-mm dish containing PBS and cryosections were imaged with a mounted coverslip. Images from individual islets in slices, sections and isolated islets were acquired by confocal imaging (upright LSM780; Zeiss) using a C-Apochromat 20x/1.2 water corrected objective and pinhole was adjusted to one airy unit. Images were acquired individually in single track mode for each fluorophore. Fluorescence of GFP and Alexa Fluor 488 were excited at 488 nm laser wavelength and emission was detected from 490 to 560 nm, Tomato, Alexa Fluor 546 and 555 were excited at 561 nm and detected at 569 to 621 nm, Alexa Fluor 633 was excited at 633 nm and detected at 638 to 755 nm. DAPI was excited at a wavelength of 405 nm and emitted light was detected in a range from 410 to 510 nm. Images for the assessment of the fractional beta cell area were acquired using a slide scanner (Axio Scan.Z1; Carl Zeiss).

2.4.5 Data analysis

Data quantification was performed manually using Imaris (Bitplane AG, versions 8.2 and 8.3) and FIJI. Single planes of pancreatic cryosections were analysed in 2D for islet composition. Analysis was carried out by manual cell counting with FIJI using the cell counter plugin. For the assessment of pancreatic mass total pancreatic area and endocrine area were measured manually by FIJI. For every islet a single region of interest (ROI) was created and the area was measured. Tissue slices were analysed in 3D using Imaris. A 3D surface of the first 30 planes (45 µm) was assessed by the surface creation tool. Channels were masked and surface volume of the individual tracks was assessed by semiautomatic 3D volume reconstruction. Cell counting within the individual volumes was measured using the semiautomatic spot analysis tool. Double fluorescent cells were analysed using the spot function tool.

2.5 Tissue slice perifusion

Slices were pre-selected by identifying slices rich in islets using transmitted light. Selected slices were trimmed using forceps and scalpel to reduce the agarose surrounding the tissue. Slices were then rested for 90 min in KRBH buffer (3 mM glucose) containing 0.1 mg/mL Soybean Trypsin Inhibitor (Sigma) on a shaker at room temperature. After resting time, slices were transferred into closed perifusion chambers (Warner instruments) and

connected to a perifusion system (Biorep). A perifusion protocol (Table 8) with different glucose concentrations was applied with a flow rate of 100 µL/min and a sampling rate of 1 min. Prior to the actual stimulation protocol, a 90 min flushing step with 3 mM KRBH buffer was included, in order to wash out accumulated hormones and enzymes from the tissue. Perfusates were collected in 96-well plates and stored at -20°C. After perifusion, slices were collected in 500 µL acid ethanol (2 % HCl [37 %, 12 M] in absolute ethanol) and stored at -20°C. Finally, insulin secretion and content was assessed by measuring perfusates and slice lysates with an ultrasensitive insulin HTRF (Cisbio).

Table 8: Mouse tissue slice perifusion protocol

Step	Time [min]	Flow rate [µL/min]	Sampling rate [sec]	solution
1	90	100	180	3 mM KRBH
2	10	100	60	3 mM KRBH
3	40	100	60	16.7 mM KRBH
4	20	100	60	3 mM KRBH
5	10	100	60	16.7 mM KRBH + 60 mM KCl
6	10	100	60	3 mM KRBH

2.6 Hormonal profiling

2.6.1 Blood sampling and processing

Whole blood was taken from the retro-orbital plexus and collected in heparinized capillary tubes (Siemens Healthcare Diagnostics). Mice were anesthetized and 500-800 µl of blood was taken prior to euthanasia by cervical dislocation. Blood samples were immediately centrifuged at 2000x g for 20 min at 4°C and plasma was stored in aliquots of 10 µl at -80°C. Individual aliquots were thawed on ice prior to measurements. For the assessment of blood glucose levels blood was obtained by small incisions on the tail vein of the animal and concentrations were measured using a glucometer (Accu-Chek Aviva, Roche).

2.6.2 Glucose tolerance test

Intraperitoneal glucose tolerance tests were performed on mice fasted for 6 hrs. Animals were weight and blood glucose levels were assessed prior to the experiment. Subsequently, glucose (2 g/kg bodyweight) was injected intraperitoneally using a 1 mL syringe with a 23 gauge cannula. Blood glucose levels were determined at 15 min, 30 min, 60 min, 90 min and 120 min post injection. Additionally, 100 µL blood was taken from the tail vein at 0 and 30 min for the assessment of plasma insulin levels. Collected blood was

centrifuged at 2000x g for 20 min at 4°C and plasma collected in aliquots of 10 µl at -20°C. Plasma insulin levels were assessed in duplicates by applying a sample volume of 5 µL using a mouse ultrasensitive Insulin ELISA kit (Chrystal Chem) according to the manufacturer's instructions. Absorbance was measured at a wavelength of 405 nm and 630 nm using a plate reader (TECAN, infinite F200 Pro). Analysis was performed on averaged duplicates

2.6.3 Plasma hormone levels

Plasma hormone concentrations of several characteristic pregnancy hormones were determined from whole blood samples. Plasma collection and storage was accomplished as described above. Hormone levels were measured using the following ELISA kits in accordance to the manufacturer's instructions provided with each kit (Table 9).

Table 9: List of ELISA kits

ELISA	company	sample volume	dilution
mouse/rat progesterone	Alpco	5 µL	1:5
mouse/rat estradiol	Calbiotech	5 µL	1:5
mouse prolactin	RayBio	100 µL	1:5 1:50
mouse placental lactogen	Bluegene	50 µL	none
mouse chorionic gonadotropin 5	MyBioSource	50 µL	none

All measurements were performed in duplicates and measured using a plate reader (TECAN, infinite F200 Pro) at a wavelength of 450 nm.

2.7 Statistical analysis

Statistical analyses were performed using Prism 6 (Prism; GraphPad Software, San Diego, CA). Data is expressed as mean ± SEM. Statistics were analyzed using Prism 6. Data was compared by one- or two-way ANOVA or by an unpaired, two-tailed student's t-test. Multiple comparisons were adjusted by Šidák correction. Significant differences are indicated as: *p < 0.0 05, **p < 0.01, ***p < 0.005 or ****p < 0.001.

3 Results

3.1 Metabolic characterization of pregnancy

Pregnancy is accompanied with systemic metabolic changes of the maternal body in order to provide sufficient nutrients to the developing fetus. This includes profound changes in hormonal regulation and glucose metabolism. To investigate these physiological adaptations C57BL/6J mice (Bl/6) were examined to establish a detailed metabolic and hormonal profile before, during and after pregnancy. In total 5 time points were monitored, a pre gestational state (PreG) and 4 time points during gestation, one to cover changes within every trimester of pregnancy, namely gestational day (G) 6, 12 and 18. Selected tests were additionally performed at G14, the phase of maximal fetal growth. Furthermore, the time point 4 weeks after birth (28 post-partum) was assessed to study adaptations that might occur after pregnancy.

3.1.1 Weight gain and glucose homeostasis

The assessment of body weight gain during pregnancy showed a gradual increase after the first trimester from 20.5 ± 0.53 g in non-pregnant controls and 22.2 ± 0.71 g at G6 to 27.83 ± 0.73 g at G12, 31.04 ± 0.30 g at G14 and further to 35.39 ± 2.77 g at G18 (Fig. 4A). Body weight changes during gestation are mainly attributed to litter growth and size, which was comparable among all groups (data not shown). Moreover, pancreas weight was observed to increase similar to the total body weight until G14. Thereafter organ weight did not further increase although body weight did. However, this might be related to litter development as this phase represents the time of maximal growth. A significant increase of pancreas weight was first observed at G14 with 0.29 ± 0.02 g and at G18 with 0.30 ± 0.02 g in comparison to the non-pregnant control group with 0.22 ± 0.01 g (Fig. 4B).

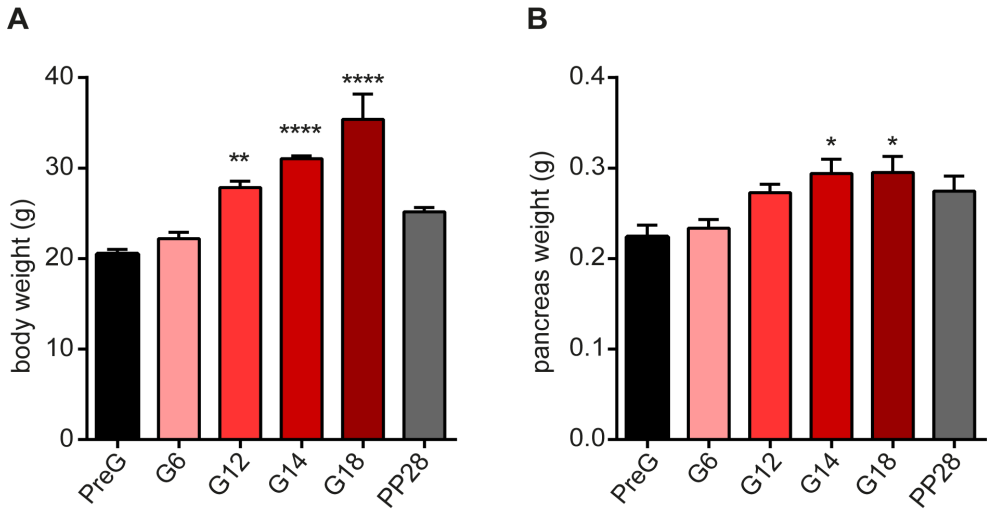

Figure 4: Body- and pancreas weight changes during mouse pregnancy. Body weight (A) and pancreas weight (B) gain during the different time course of pregnancy. Data are presented as mean ± SEM with n = 5-6 mice per group, analyzed by one-way ANOVA.

In order to characterize alterations in glucose metabolism throughout pregnancy, fasting blood glucose levels were measured after 6 hours starvation, followed by an intraperitoneal glucose tolerance test (ipGTT) prior to pregnancy (PreG) and at G6, G12, G18 and 4 weeks after delivery (PP28). To keep an appropriate distance between GTTs during pregnancy, tests were either performed at PreG, G6, G18 and PP28 or at PreG, G12 and PP28. Statistical analysis was performed on the combined dataset, however graphs have been divided for better visualization (Fig. 5B-C).

Fasting blood glucose levels did not demonstrate any significant changes during early and mid-gestation with glucose levels of 151.10 ± 5.48mg/dL at G6 and 150.00 ± 9.19 mg/dL at G12 in comparison to PreG with 140.64 ± 4.25 mg/dL. Interestingly, fasted blood glucose levels were significantly lower by the end of pregnancy at G18 with 121.30 ± 5.3 mg/dL and remained lower even 4 weeks after delivery 120.55 ± 4.27 mg/dL (Fig. 5A).

Glucose tolerance tests revealed that pregnant mice within the first two trimesters displayed significantly increased blood glucose levels 30 min after glucose challenge with values of 377.63 ± 8.61 mg/dL at G6 (Fig. 5B) and 393.00 ± 9.82 mg/dL at G12 (Fig. 5C) compared to the levels prior to pregnancy with 334.17 ± 13.56 mg/dL. At G18, mice displayed significantly improved glucose clearance after 90 min (Fig. 5B). Moreover, glucose tolerance was observed to be significantly improved after pregnancy at PP28 with blood glucose values of 290.22 ± 10.14 mg/dL after 30 min and 179.67 ± 6.54 mg/dL after 60 min glucose challenge (Fig. 5C).

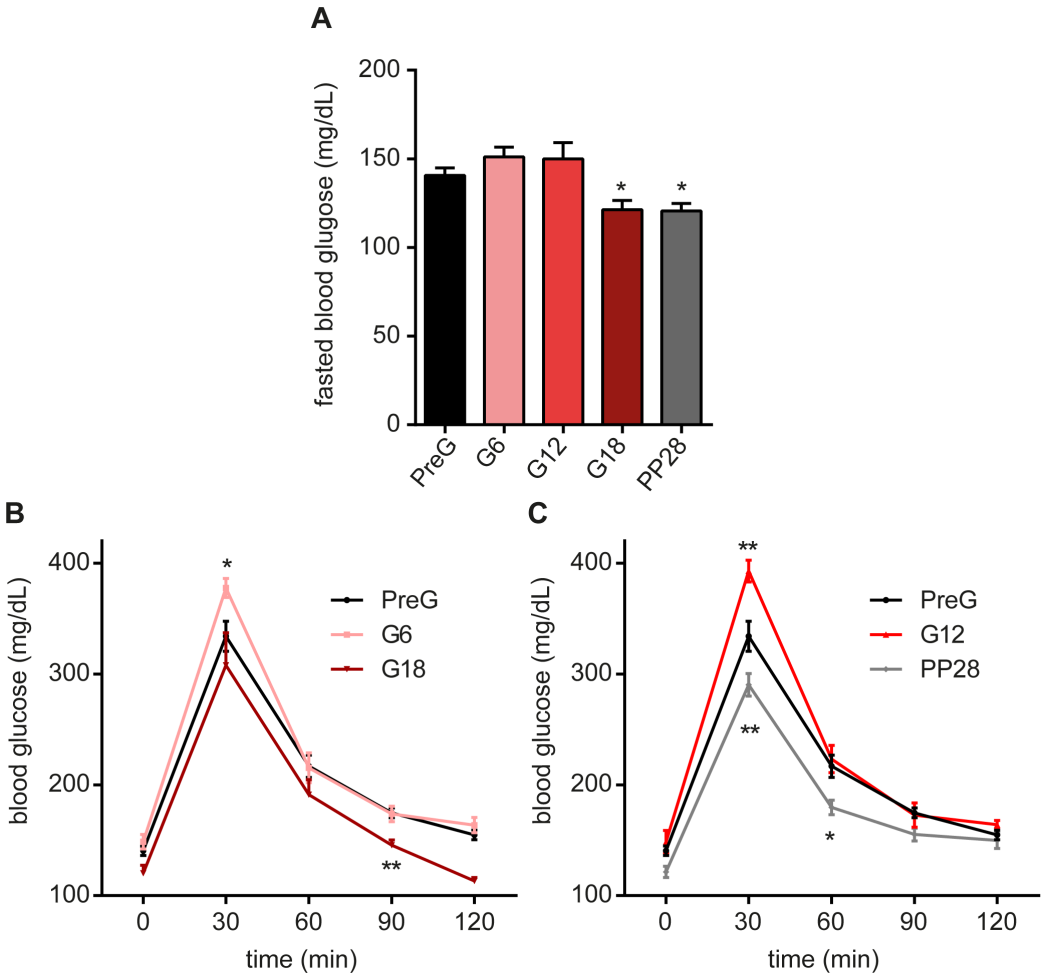

Figure 5: Glucose metabolism during and after mouse pregnancy. Fasting blood glucose levels after 6 hrs starvation (A). Blood glucose clearance over time at PreG, G6 and G18 (B) and at PreG, G12 and PP28 (C) Data are presented as mean ± SEM with n = 7-12 mice per group, analyzed by two-way ANOVA.

3.1.2 Hormonal profile during mouse pregnancy

Since changes in the hormonal profile are not only necessary to establish and maintain pregnancy but have been shown to contribute to important pancreatic endocrine adaptations, blood plasma samples were collected longitudinally prior to pregnancy and at G6, G12 and G18. The assessment of plasma concentrations of the steroid hormones progesterone (P_4) and estradiol (E_2) as well as of the lactogenic hormones prolactin (PRL) and placental lactogen (PLI and PLII) were measured by ELISA.

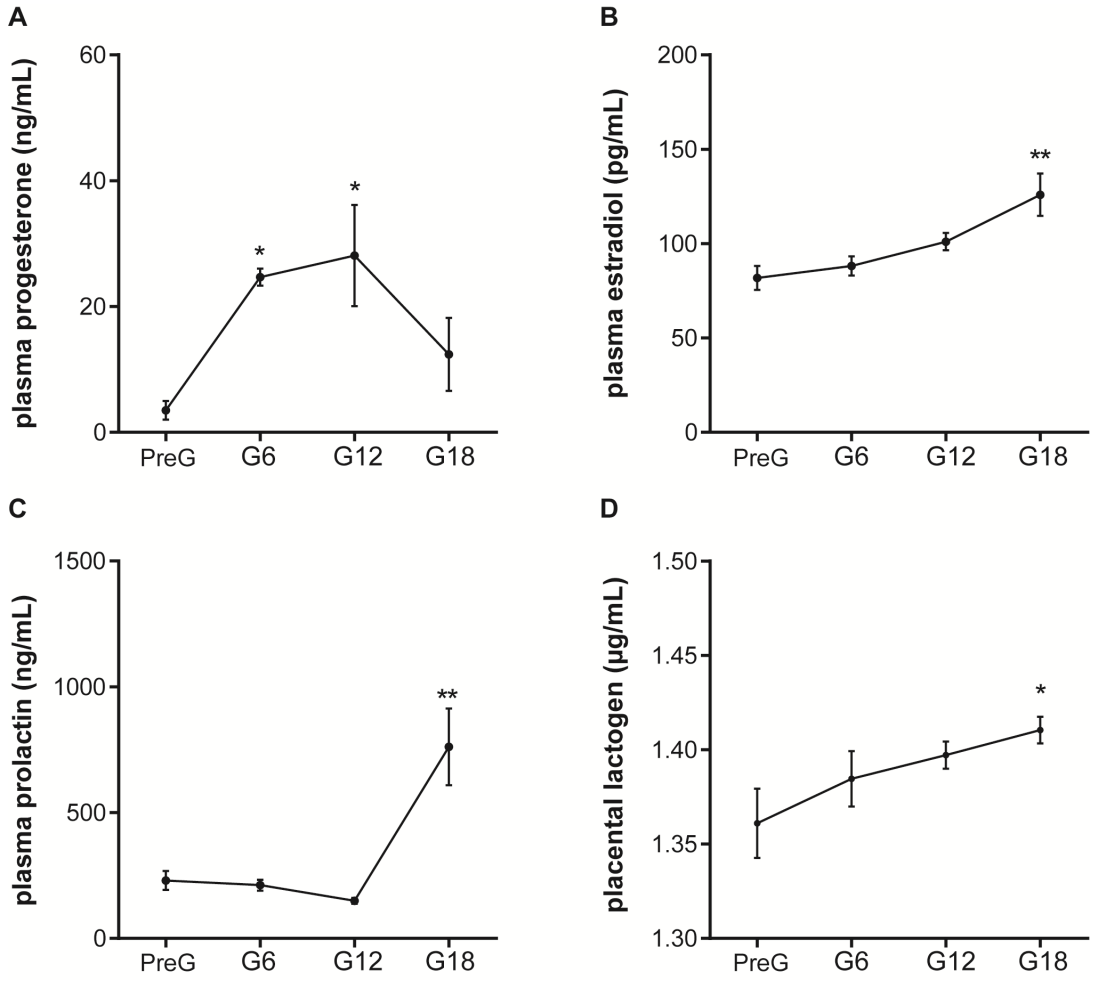

Figure 6: Hormonal changes during pregnancy. Plasma hormone levels of female BL/6 mice before and during pregnancy determined by ELISA. Plasma concentrations of progesterone (A), estradiol (B), prolactin (C) and placental lactogen (D). Data are presented as mean ± SEM with n = 5 mice per group, analyzed by one-way ANOVA.

Plasma P_4 levels (Fig. 6A) were observed lowest prior to pregnancy with 3.49 ± 1.50 ng/mL. Notably, early gestation was accompanied with a rapid increase in P_4 levels to 24.66 ± 1.35 ng/mL, resulting in a significant seven-fold augmentation already at G6. P_4 levels remained high during mid-gestation with 28.08 ± 8.05 ng/mL at G12 and decreased towards the end of pregnancy at G18 with plasma P_4 concentrations of 12.37 ± 5.80 ng/mL.
In contrast, plasma E_2 levels (Fig. 6B) were observed to increase steadily during gestation, resulting in significantly increased plasma level at G18 of 125.90 ± 11.19 pg/mL compared to 81.76 ± 6.37 pg/mL prior to pregnancy.
PRL concentrations (Fig. 6C) were observed to remain low throughout the first two trimesters of pregnancy with plasma levels comparable to the pre-gestational state (con 230.70 ± 37.7 ng/mL, G6 211.71 ± 21.66 ng/mL, G12 149.83 ± 12.10 ng/mL). However, by the end of pregnancy at G18, PRL concentration was increased significantly to a maximum of 761.63 ± 152.32 ng/mL.
Plasma concentrations of PL (Fig. 6D) displayed a similar activity pattern like E_2 concentrations with gradually increased values from 1.36 ± 0.02 µg/mL before gestation up to 1.41 ± 0.01 µg/mL at term.
While P_4 levels were elevated already very early during pregnancy and decreased prior to delivery, estradiol and lactogenic hormones rather increased with ongoing gestation, resulting in significantly increased concentration first observed by the end of pregnancy.

3.2 Islet mass adaptations during pregnancy

3.2.1 Assessment of endocrine mass

In order to study the expansion of endocrine cell mass during pregnancy, the entire pancreas of female mice at different stages of gestation and 4 weeks after delivery was sectioned and cryosections were imaged with a slide scanner. Pancreatic mass was assessed manually by measuring endocrine and exocrine area and total islet number was counted. Changes in islet mass were quantified by calculation of either endocrine weight, using the measured pancreas weight and the endocrine area, or by calculation of islet area as percentage of total pancreatic area (Fig. 7A-B).
A significant increase, both in mass and area, could be observed first at G14. Islet mass was found to increase 2 fold from 1.00 ± 0.08 mg in virgin female mice to 2.05 ± 0.24 mg at G14 and 2.00 ± 0.21 mg at G18. Interestingly, islet mass remained elevated also 4 weeks after delivery with 2.14 ± 0.29 mg and was therefore comparable mass at term (Fig. 7A-B).

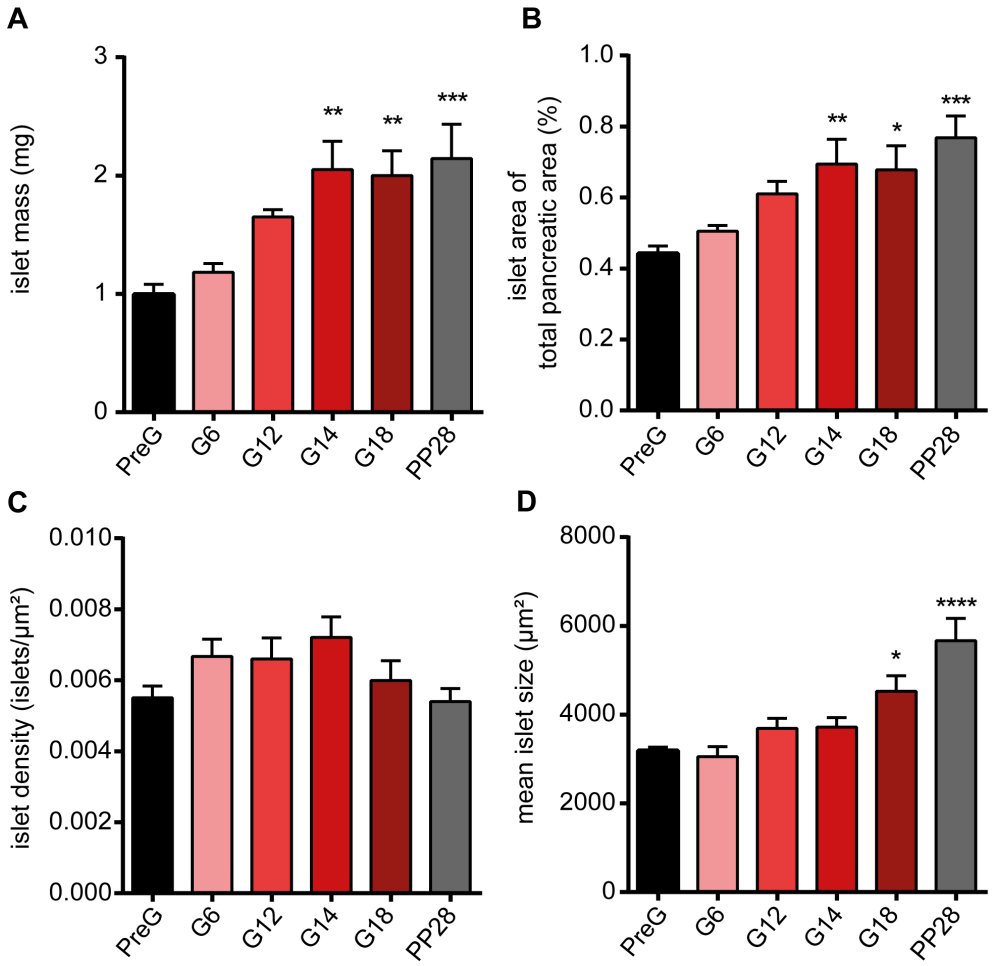

Figure 7: Islet mass and size adaptations during and after pregnancy. Islet mass increase at different stages of pregnancy calculated as mg (A) and percent (B) of total pancreatic mass/area. Islet density (C) calculated by the total amount of islets per µm² pancreas area. Mean islet size (D) measured by average islet area of all assessed islets. Data are presented as mean ± SEM with n = 5-6 mice per group, analyzed by one ANOVA.

Although the total amount of islets present within the pancreas during early and mid-gestation steadily increased from a mean of 817.40 ± 72.93 in control mice to 1361.20 ± 113.47 islets at G14 (data not shown), islet density did not differ significantly among the various stages of gestation or afterwards (Fig. 7C). This might be related to the increase in organ size, as islet density is calculated by the number of islets within the total assessed pancreatic area. However, mean islet size increased during pregnancy, with a significant elevation first observed at G18 of 4523.00 ± 353.32 µm² in comparison to 3185 ± 83.16 µm² in the control group. Interestingly, mean islet size was observed to increase after pregnancy even further to a peak area of 5667.60 ± 497.93 µm² (Fig. 7D).

In addition to islet mass expansion, individual islet size distribution was assessed throughout pregnancy. Therefore, islets were divided in four groups of different size (Fig. 8A-D).

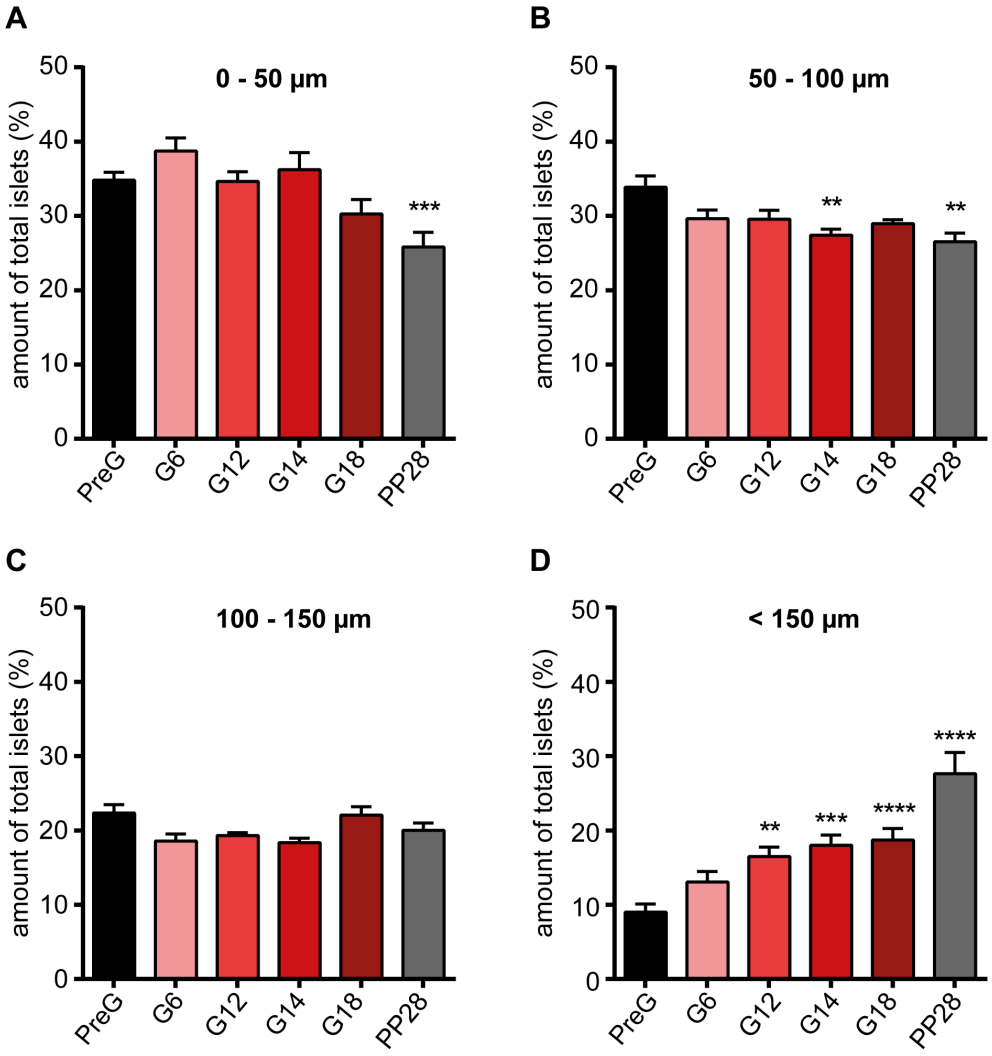

Figure 8: Islet size distribution during and after pregnancy. Islets with a diameter of 0-50 µm (A), 50-100 µm (B), 100-150 µm (C) and > 150 µm (D), calculated as percent of total counted islets. Data are presented as mean ± SEM with n = 5-6 mice per group. Data were analyzed by one-way ANOVA.

Contribution of small islets with a diameter up to 50 µm (Fig. 8A), did not change significantly throughout pregnancy, but was found to decrease significantly at PP28 (con 34.80 ± 1.08 %, PP28 25.79 ± 2.00 %). In pregnancy the frequency of middle sized islets between 50 and 100 µm (Fig. 8B) decreased from 33.85 ± 1.51 % in non-pregnant control to 27.40 ± 0.82 % at G14 and 26.52 ± 1.17 % at PP28. Islets with a diameter between 100 and 150 µm (Fig. 8C) were not found to differ in percentage of contributing endocrine mass during or after

pregnancy. Finally, percentage of islets bigger than 150 µm (Fig. 8D) increased gradually from 9.00 ± 1.09 % in virgin female mice to a significant increase of 16.48 ± 1.30 % at G12 and demonstrated an even more pronounced increase after delivery at PP28 with 27.66 ± 2.86 %. These results indicate a major contribution of proliferation to the observed islet mass increase during pregnancy.

3.2.2 Contribution of proliferation to endocrine mass expansion during pregnancy

To assess the contribution of islet cell proliferation at the different stages of gestation, sections were stained for the cellular marker Ki67 and the expression of insulin using immunohistochemistry. This approach allows to investigate the proliferative activity in general, but also to assess the fraction of proliferating beta cells throughout pregnancy. The ratio of endocrine cell division was calculated as percent of total islet cells and showed that 0.45 ± 0.02 % of islet cells underwent DNA synthesis under control conditions. This rate was significantly increased by more than 2-fold as early as G6 with 1.23 ± 0.15 % (Fig. 9A). Moreover, proliferation rates remained elevated with ongoing pregnancy at G12 with 1.07 ± 0.22 % and G14 with 1.27 ± 0.35 %. Endocrine cell division rates returned to pre-gestational levels already by the end of pregnancy at G18 with a percentage of 0.46 ± 0.10 % and seemed consistent even 4 weeks after delivery with 0.44 ± 0.11 % at PP28 (Fig. 9A). A similar profile could be observed for beta cell proliferation calculated as percentage of dividing beta cells within the beta cell fraction (Fig. 9B). Interestingly, throughout pregnancy the majority of dividing cells are represented by beta cells. However, 4 weeks after delivery significantly less beta cells underwent DNA synthesis in comparison to pregnant or non-pregnant animals (PP28 50.28 ± 5.46 %, Fig. 9C).

This data confirmed that proliferation seems to play a major role in the compensatory response during pregnancy, but due to the low proliferative activity it might not be the only mechanism involved in this process.

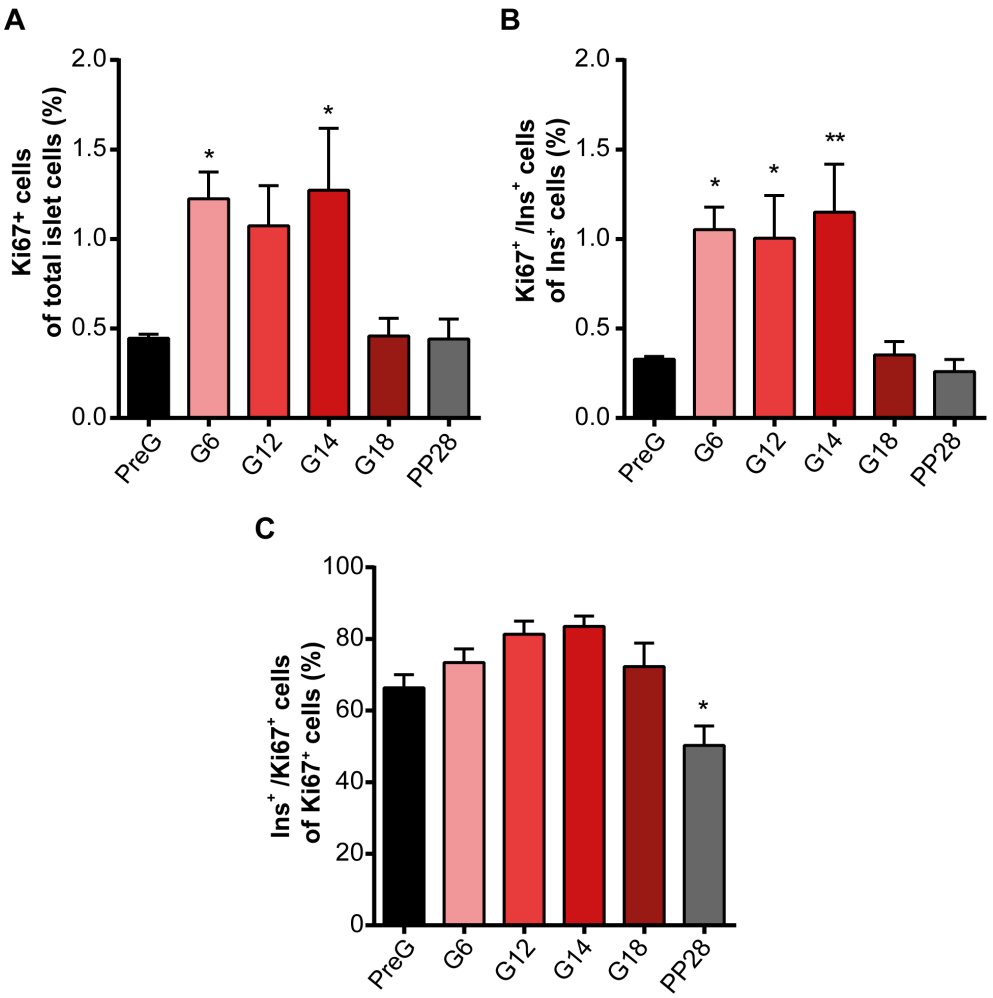

Figure 9: Islet cell proliferation during and after pregnancy. Percentage of Ki67 positive cells of total islet cells (A). Amount of Ki67 positive beta cells (B), calculated as percent of the beta cell fraction. Percent of proliferating insulin positive cells (C), calculated as percentage of all proliferating cells. Data are presented as mean ± SEM with n = 5-6 mice per group with >4000 cells/mouse. Data were analyzed by one-way ANOVA.

3.3 Influence of neogenesis on pregnancy

Besides proliferation of preexisting cells, islet cell mass increase can be achieved by other mechanisms like neogenesis, dedifferentiation or transdifferentiation. While islet cell neogenesis describes the formation of new pancreatic endocrine cells from pancreatic progenitor cells or stem cells which are localized within the exocrine compartments (Herrera, et al. 1991; Gu, et al. 2002), de- and transdifferentiation require the cellular transition of existing mature endocrine cells. This transition may occur directly from one pancreatic cell into another (transdifferentiation) or *via* an intermediary transition cell stage (dedifferentiation) followed by re-differentiation towards another cell type (Puri, et al. 2015). To investigate the contribution of these mechanisms to the increase in islet mass during pregnancy the recurrence of the progenitor gene *Ngn3* was assessed *in vivo.* As all pancreatic islet endocrine cells arise from *Ngn3* expressing precursor cells during development (Gradwohl, et al. 2000; Gu, et al. 2002; Gouzi, et al. 2011), a re-activation of this endocrine progenitor during pregnancy is therefore proposed to indicate adult beta cell neogenesis or dedifferentiation through a precursor cell state. For the purpose of *in vivo* labeling of *Ngn3* positive cells during pregnancy the double transgenic Ngn3-CreER;mTmG reporter mouse strain was used and pulse chase experiments by TAM administration were performed. The *Ngn3* promoter driven expression of the inducible Cre/LoxP reporter system with the double-fluorescent mTmG indicator allows the visualization of *Ngn3* positive cells during a desired timeframe in the adult pancreas, when exposed to tamoxifen (TAM). The reporter mouse ubiquitously expresses membrane bound tdTomato switching to GFP after tamoxifen induced Cre-mediated recombination in *Ngn3* expressing cells. Only those cells that express *Ngn3* in the presence of tamoxifen, as well as any subsequent daughter cells, will express GFP.

3.3.1 Ngn3 expression in the endocrine pancreas

As differentiation of exocrine acinar or ductal cells into endocrine cells has been observed to represent the major mechanism of endocrine beta cell neogenesis (Bonner-Weir, et al. 1993; Wang, et al. 1995; Jensen, et al. 2005), the occurrence of *Ngn3* expressing cells during pregnancy was first investigated in the exocrine part of the pancreas. Surprisingly, *Ngn3* expression was mainly observed in the endocrine pancreas, with only very few cells located within the exocrine tissue (Fig. 10).

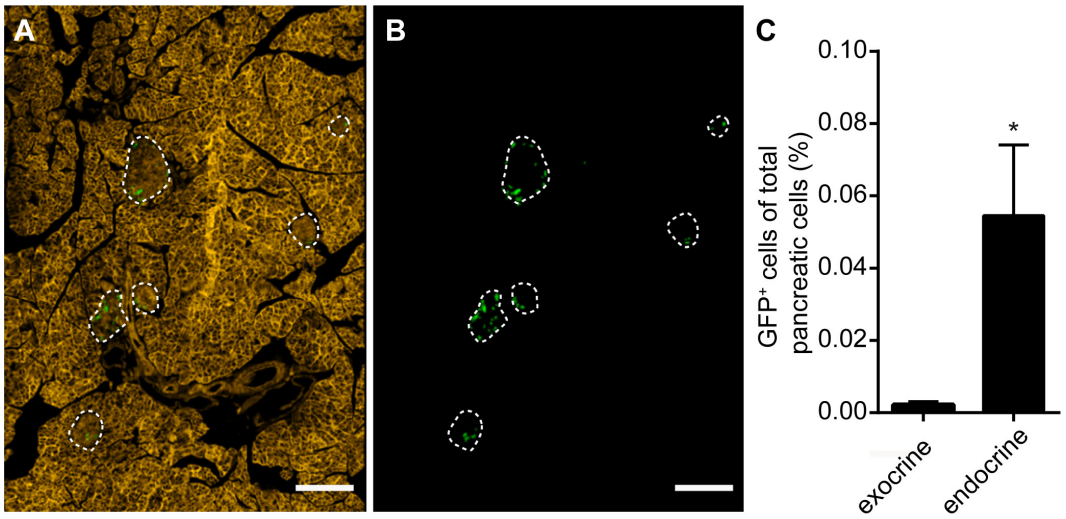

Figure 10: *Ngn3* expression during pregnancy is limited to the endocrine pancreas. Representative images of a pancreatic tissue section from Ngn3-CreER;mTmG mice after TAM administration for 2 days in a pregnant mouse at G4-6 showing recombined GFP positive cells (green) and tomato positive cells (orange) (A), and GFP expression only (B). Dotted lines indicate islets. Scale bar = 200 µm. Percentage of GFP expressing cells in non-pregnant controls within the exocrine and endocrine tissue (C), calculated as percent of all pancreatic cells. Data are presented as mean ± SEM with n = 5 mice with >100000 cell/mouse, analyzed by an unpaired, two-tailed t-test.

3.3.2 Increased Ngn3 promotor activity during early pregnancy

As *Ngn3* expression is limited to the endocrine pancreas, dynamics of intra-islet *Ngn3* promotor activity were further investigated by immunohistochemistry and manual cell counting analysis. For Cre-dependent recombination, mice were injected a single dose of TAM at different gestational days and sacrificed 2 days afterwards (Fig. 11A). Surprisingly, already under non-pregnant conditions *Ngn3* expression could be observed in 1.92 ± 0.34 % of all islet cells. Moreover, a significant increase of *Ngn3* promotor activity, visualized by GFP expression was shown only during the first trimester of pregnancy at G4-6 with 5.38 ± 0.67 %. Thereafter *Ngn3* promotor activity declined back to control levels (Fig. 11B-D).

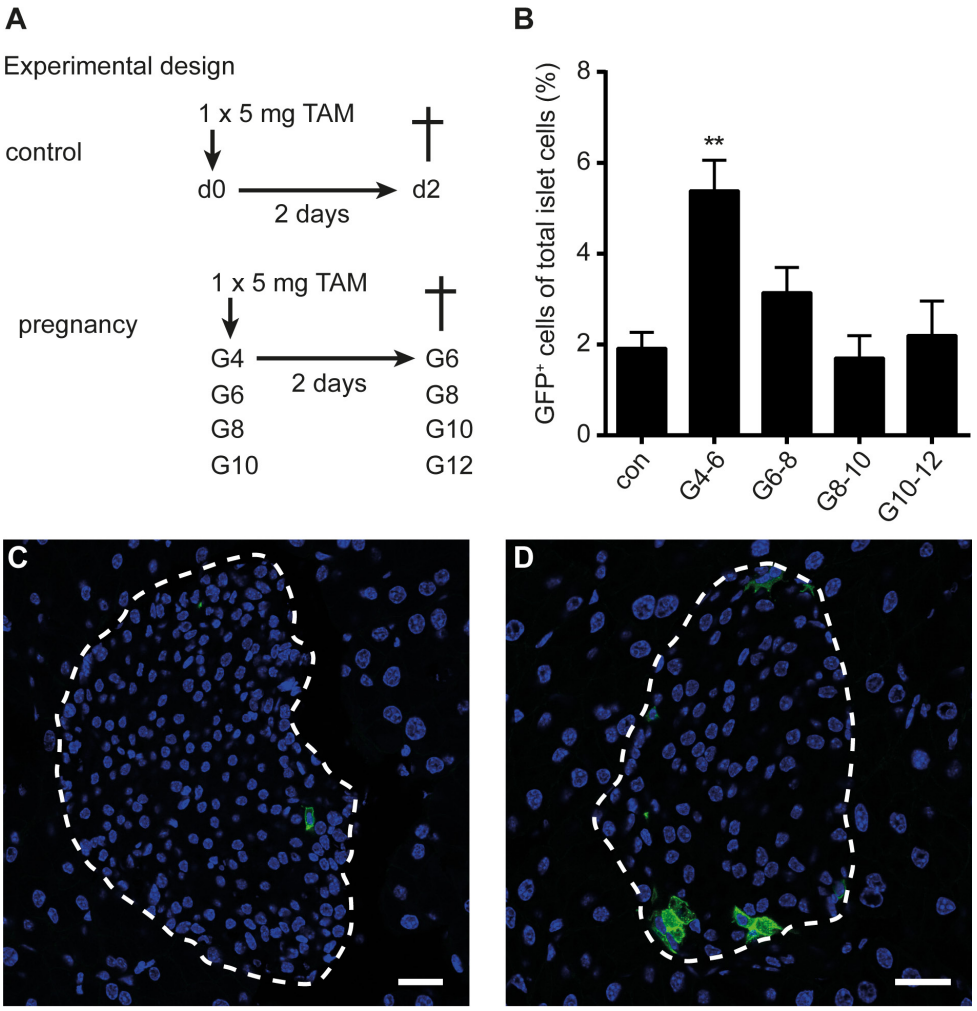

Figure 11: Recombination efficiency of *Ngn3* promotor activity during pregnancy. Experimental setup for TAM administration *in vivo* (A). Intra-islet *Ngn3* expression indicated by GFP expression, calculated as percentage of all islet cells (B). Representative images of islets from pancreatic tissue sections of Ngn3-CreER;mTmG mice after TAM administration of a virgin (A) and pregnant mouse at G4-6 (B). *Ngn3* indicated by GFP expression (green) and stained for DAPI (blue). Dotted line indicates an islet. Scale bar = 25 µm, n = 5-6 mice per group with >10000 cells/mouse. Data are presented as mean ± SEM, analyzed by one-way ANOVA.

To exclude proliferation as possible mechanism for the observed increase in *Ngn3* expression during early gestation, a lineage tracing experiment was performed by labeling preexisting *Ngn3* positive cells prior to pregnancy. Studies have shown that TAM administration may continue to label significant amounts of cells for up to 4 weeks after treatment (Reinert, et al. 2012). Thus, the labeling protocol was adjusted and included a 4 week waiting period after TAM administration to prevent further recombination thereafter

(Fig. 12A). *Ngn3* lineage tracing experiments revealed that the observed increase during pregnancy does not result from replication of preexisting *Ngn3* positive islet cells as the number of labeled cells in virgin and pregnant female mice did not differ significantly (1.40 ± 0.30 % control and 2.00 ± 0.70 % pregnant, Fig. 12B).

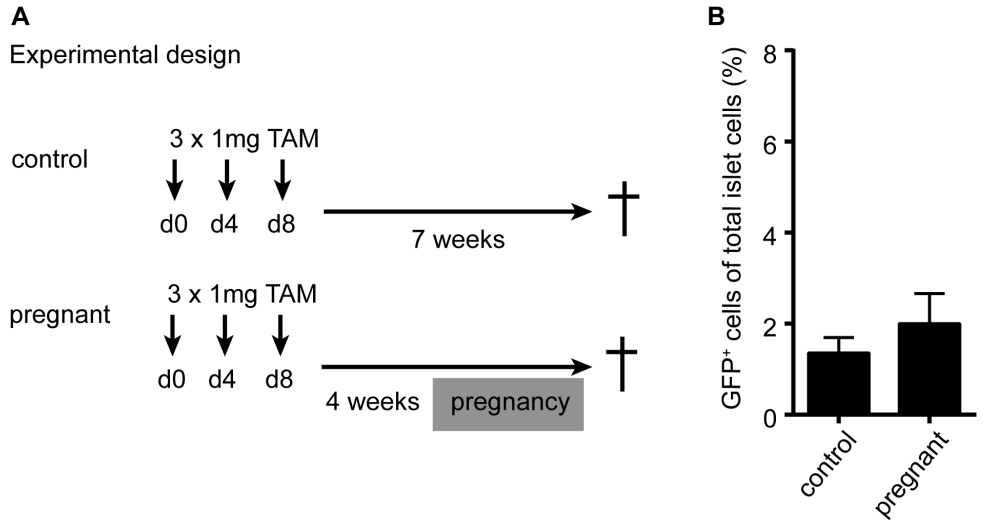

Figure 12: Lineage tracing of *Ngn3* positive cells. Scheme of TAM administration *in vivo* (A). Recombination frequency calculated as percent GFP positive cells of total analysed islet cells (B). Data are presented as mean ± SEM, n = 5-6 mice per group with >4000 cells/mouse, analyzed by an unpaired, two-tailed t-test.

The emergence of intra-islet *Ngn3* positive cells during early pregnancy was visualized by GFP expression using the TAM dependent induction of Cre activity in Ngn3-CreER;mTmG reporter mice as surrogate for *Ngn3* expression. To verify the accurate function of this reporter system the Ngn3-YFP reporter mouse strain was used in addition to analyse *Ngn3* expression during early pregnancy (Fig. 13A-C). Here, *Ngn3* promotor activity leads to transient expression of the yellow fluorescent protein (YFP) independent of Cre-recombination. The 3-dimensional analysis of islets in stained pancreatic tissue slices from Ngn3-YFP mice revealed that *Ngn3* is already expressed in non-pregnant control mice, similar to the results obtained from the Ngn3-CreER,mTmG mice. Moreover, mice at G6 of pregnancy showed a significantly increased amount of 7.66 ± 0.65 % YFP positive cells (Fig. 13C).

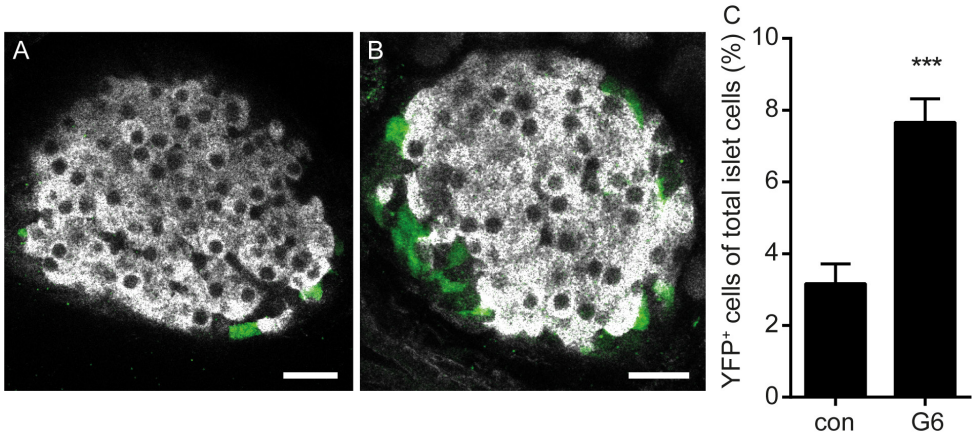

Figure 13: *Ngn3* expression during early pregnancy. Representative images of islets from pancreatic tissue slices of Ngn3-YFP mice of a virgin mouse (A) and a pregnant mouse at G6 (B). *Ngn3* indicated by YFP expression (green) and islet backscatter (grey). Scale bar = 25 µm. Recombination efficiency in islets (C), calculated as percentage of YFP positive cells in all islet cells, n = 5 mice per group with >5000 cells/mouse. Data is presented as mean ± SEM, analyzed by one-way repeated measure ANOVA and Sidak's multiple

In summary, both mouse strains confirmed a dynamic on- and offset of intra-islet *Ngn3* expression within a narrow time frame during early gestation as a result of *de novo* promotor activity.

3.3.3 Origin and fate of Ngn3-positive cells

In order to further characterize the emerging *Ngn3* positive cells, sections were examined for insulin, glucagon and somatostatin hormone expression by immunohistochemistry. Quantification of hormone expression in GFP positive cells revealed that under control conditions only 28.06 ± 1.27 % of the *Ngn3* expressing cells stained positive for insulin and 12.34 ± 1.64 % for glucagon, while the majority was positively labeled for somatostatin (67.66 ± 3.96 %) (Fig. 14A-C). Interestingly, at the peak incidence of *Ngn3* positive cells during pregnancy, G4-6, the somatostatin positive stained fraction significantly decreased (52.00 ± 5.50 %), whereas the insulin and glucagon fractions remained constant. Moreover, co-staining for all three endocrine hormones confirmed an increase in GFP positive, hormone negative ($Ins^-/Gcg^-/Sst^-$) cells from 3.50 ± 0.77 % in the control group to 19.87 ± 2.64 % at G4-6 (Fig. 14D-F). The observed loss of somatostatin expression and increase in hormone negative cells might indicate a loss of delta cell phenotype during pregnancy induced by *Ngn3* expression.

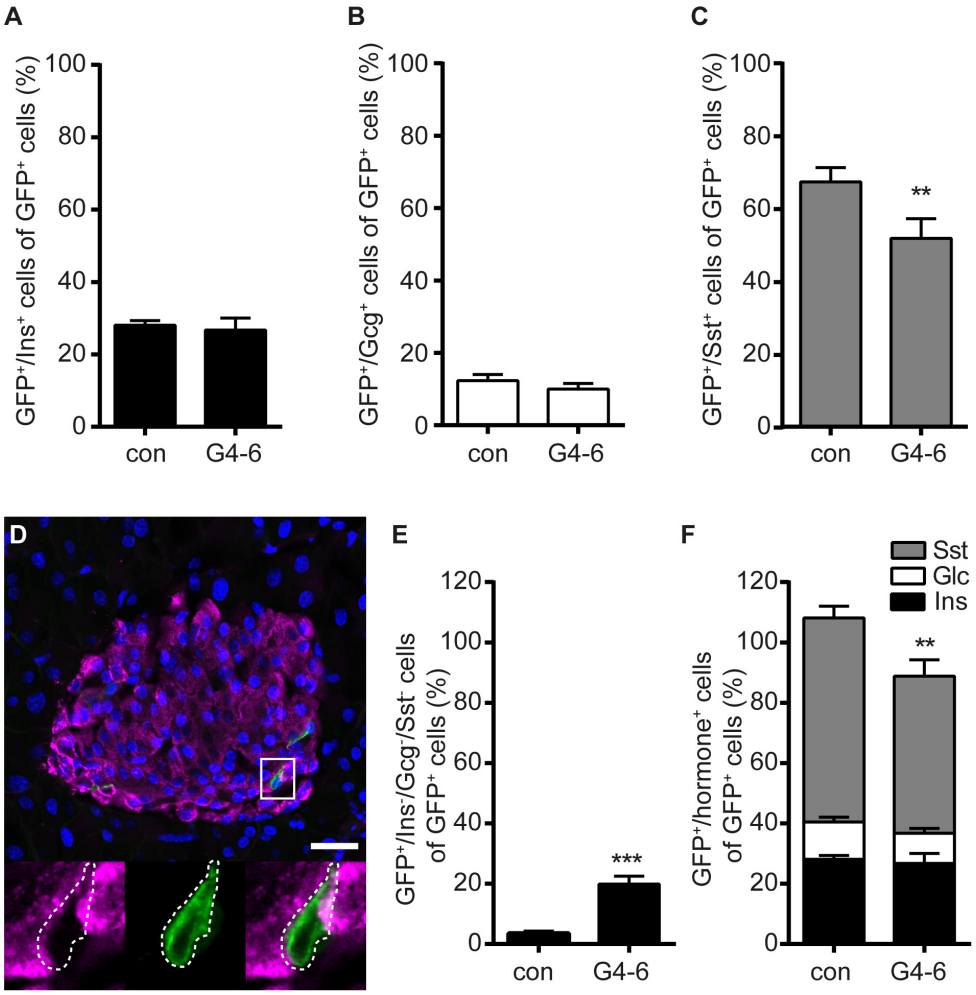

Figure 14: Hormone composition of emerging *Ngn3* positive cells. Composition of GFP positive cells, calculated as percent of GFP positive cells for insulin (A), glucagon (B) or somatostatin (C). Representative image of an islet from a TAM treated G4-6 pregnant mouse (D) with recombined GFP positive cells (green) stained for insulin, glucagon and somatostatin (magenta) and the nuclear marker DAPI (blue). Dotted line represents a GFP positive, triple hormone negative cell. Scale bar = 25 µm. Percent of GFP positive, triple hormone negative cells (E) calculated as fraction of all GFP positive cells. Cell type distribution (F) of insulin positive (black), glucagon positive (white) and somatostatin positive (grey) cells calculated as fraction of all GFP positive cells. Data are presented as mean ± SEM, n = 5-6 mice per group with >2500 cells/mouse, analyzed by an unpaired, two-tailed t-test.

3.3.4 Occurrence of islet cell dedifferentiation during pregnancy

To investigate if early pregnancy leads to *Ngn3* promotor activity and subsequent loss of hormone expression in delta cells, the Sst-Cre;YFP mouse line was used to harvest pancreatic organs at different states of gestation and thereafter. The Cre-mediated recombination under the somatostatin promotor results in irreversible expression of the fluorescent reporter YFP and therefore allows to lineage trace delta cells over time. Analysis of YFP positive cells was performed by immunofluorescence staining for somatostatin and insulin positive cells in pancreatic cryosections (Fig. 15 A-C).

Results showed that the fraction of delta cells (YFP^+/Sst^+) dropped significantly from 96.96 ± 1.00 % to 87.12 ± 1.83 % already within the first trimester of pregnancy at G6 (Fig. 15D). The percentage of this cell fraction remained significantly lower in comparison to virgin mice until mid-gestation. At the same time, an increase in YFP positive cells that did not express somatostatin nor insulin ($YFP^+/Ins^-/Sst^-$), could be observed from 1.54 ± 0.39 % under control conditions to 11.05 ± 1.36 % at G6 and 9.50 ± 1.63 % at G12 (Fig. 15E). This fraction of $YFP^+/Ins^-/Sst^-$ cells declined with proceeding pregnancy and was not significantly different to non-pregnant control levels at G18 or after pregnancy at PP14 and PP28. Moreover, a steady increase of YFP^+ cells expressing insulin (YFP^+/Ins^+) was observed throughout pregnancy with a significantly elevated value of 5.38 ± 0.74 % at G18 compared to 1.50 ± 0.75 % under control conditions (Fig. 15F).

This data suggests that a substantial number of somatostatin expressing delta cells lose their cell identity and become hormone negative at G6-12. With ongoing pregnancy these cells might partially convert into insulin producing beta cells.

Due to the increasing islet cell mass and elevated cell division rates during pregnancy, alterations in cell fractions can also be the result of diverse changes in cell mass and might not be related to cell conversions. For instance, the opposite development of YFP^+/Sst^+ and $YFP^+/Ins^-/Sst^-$ cell fractions could be caused by a selective enhancement of the $YFP^+/Ins^-/Sst^-$ cell mass. To evaluate the influence of cell mass changes on these findings, pancreatic mass during pregnancy was assessed by manual measurements of pancreatic endocrine and exocrine area of consecutive pancreas cryosections. Measurements of endocrine area and islet composition data were used to calculate the individual mass changes of the different YFP^+ cell types in order to visualize the absolute changes of each fraction (Fig. 15G-H).

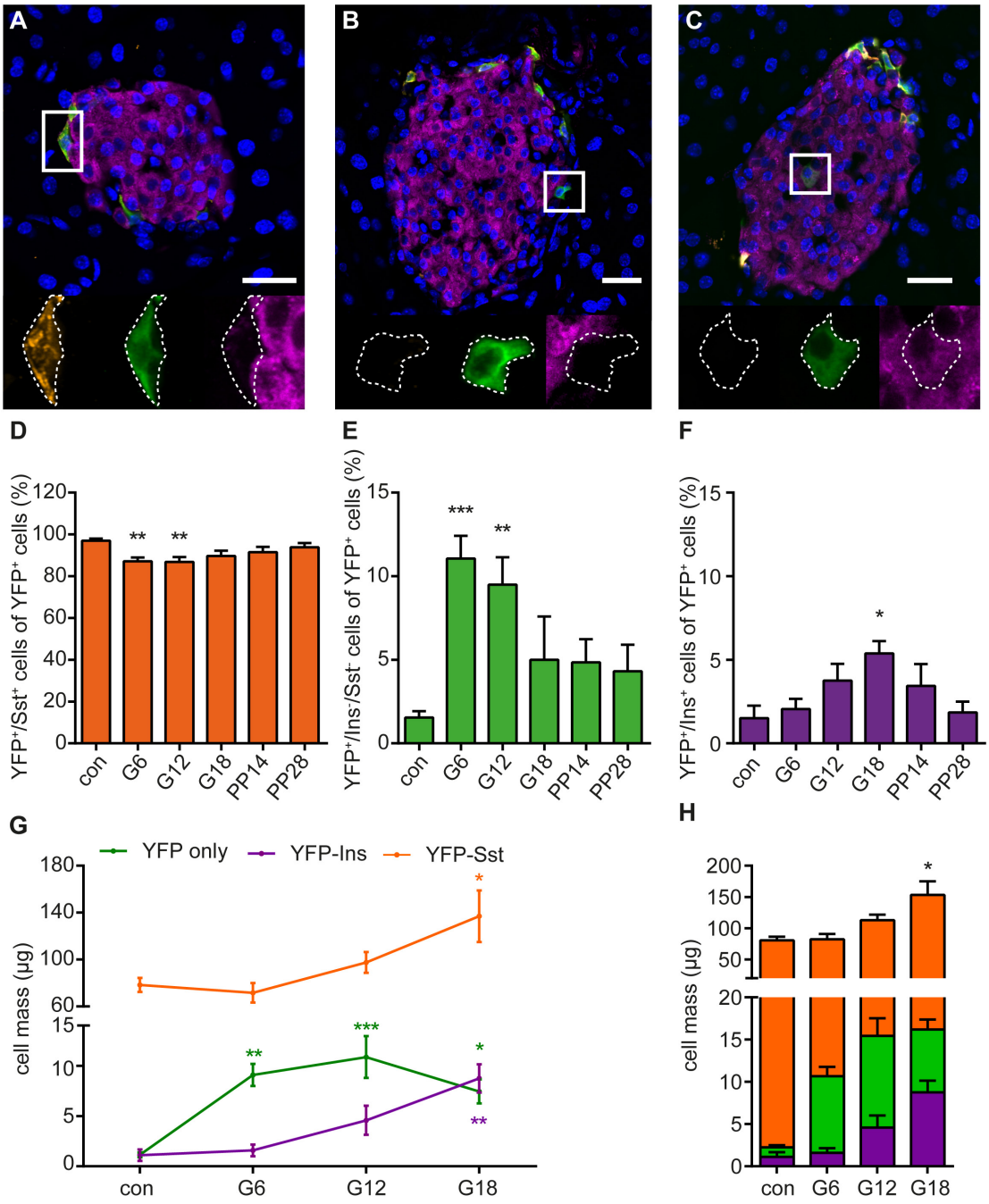

Figure 15: Lineage tracing of delta cells. Images of representative islets of a non-pregnant control mouse (A), a pregnant mouse at G6 (B) and a pregnant mouse at G18 (C) with recombined YFP$^+$ cells (green), stained for somatostatin (orange), insulin (magenta), and DAPI (blue). Square indicates the magnification of a single YFP$^+$/Sst$^+$ cell (A), YFP$^+$/Ins$^-$/Sst$^-$ cell (B) and YFP$^+$/Ins$^+$ cell (C). Scale bar = 25 µm. Composition of YFP positive cells shown as YFP$^+$/Sst$^+$ (D), YFP$^+$/Ins$^-$/Sst$^-$ (E) and YFP$^+$/Ins$^+$ (F) cells calculated as percent of all YFP positive cells. Combined analysis of endocrine mass and composition of the YFP$^+$ cell fractions during pregnancy (G-H). Cell mass changes of YFP$^+$/Sst$^+$ (orange), YFP$^+$/Ins$^-$/Sst$^-$ (green) and YFP$^+$/Ins$^+$ (purple) cells (G). Combined mass changes of all YFP$^+$ islet cells (H). Data are presented as mean ± SEM, n = 5-6 mice per group, analyzed by one-way repeated measure ANOVA.

Interestingly, YFP$^+$/Ins$^-$/Sst$^-$ cell mass was found to be significantly increased from 1.15 ± 0.23 µg in non-pregnant female mice to 9.09 ± 1.09 µg at G6 of pregnancy, while the YFP$^+$/Sst$^+$ cell mass did not show a significant drop in comparison to virgin mice (con 78.37 ± 5.90 µg, G6 71.65 ± 8.39 µg). The mass of the YFP$^+$/Ins$^-$/Sst$^-$ cell$^-$ fraction was further increased to 10.87 ± 2.08 µg at G12 and dropped to 7.45 ± 1.19 µg at G18 (Fig. 15G). YFP$^+$/Ins$^+$ cell mass was shown to gradually increase with a significant mass expansion not before G18 from 1.11 ± 0.56 µg to 8.74 ± 1.40 µg. The YFP$^+$/Sst$^+$ cell mass was shown to increase progressively from mid-gestation to term, with a significant increase at G18 from 78.37 ± 5.90 µg to 136.99 ± 21.98 µg, which might be related to a high proliferative rate during the third trimester.

In summary, the combined analysis of islet mass and islet cell composition at different phases of pregnancy revealed that fractional changes precede mass expansion and therefore rule out proliferation as major mechanism for the initial reprogramming of delta cells towards a hormone negative state.

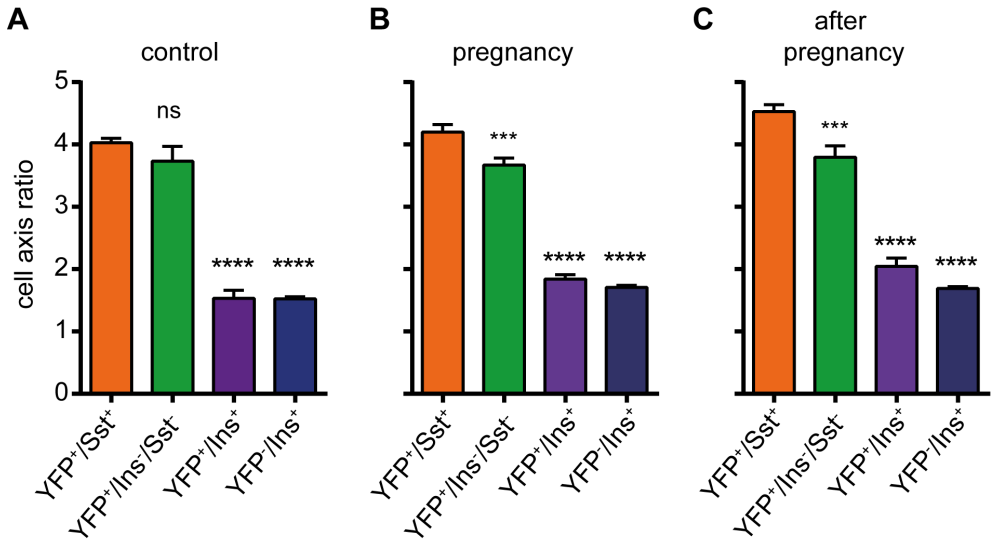

Figure 16: Cell shape of different islet cell types during pregnancy. Cell shape, calculated as ratio between width and length of cells under control conditions (A), during pregnancy (B) at G6, G12 and G18 and after pregnancy (C) at PP14 and PP28. Data are presented as mean ± SEM, n = 5-6 mice per group with >400 cells/mouse, analyzed by an unpaired, two-tailed t-test.

In addition to their different hormone expression, beta and delta cells vary significantly in their cell morphology. The cell shape of individual islet cells was assessed by manual measurements of longest *versus* shortest cell axis of the islet using FIJI. While delta cells (YFP$^+$/Sst$^+$) displayed an elongated cell shape with a cell axis ratio of 4.03 ± 0.07 under control conditions, beta cells (YFP$^-$/Ins$^+$) showed an almost round shape with a ratio of

1.52 ± 0.03. Interestingly, converted beta cells (YFP^+/Ins^+) showed an identical axis ratio like beta cells with 1.53 ± 0.13 (Fig. 16A-C). This specific cell shape did not change significantly during (G6, G12 and G18) or after pregnancy (PP14 and PP28). Moreover, cells that lost delta cell identity ($YFP^+/Ins^-/Sst^-$) showed a significantly smaller cell axis ratio in comparison to delta cells during pregnancy with 3.67 ± 0.11 and after pregnancy with 3.79 ± 0.18.

3.3.5 Detection of Ngn3 mRNA levels in vitro

To investigate whether explicitly delta cells activate *Ngn3* expression during early pregnancy, *Ngn3* mRNA expression in delta and non-delta cells was assessed by qRT-PCR. To do so, the Sst-Cre;YFP mouse line was used and islets were isolated islets from non-pregnant and pregnant mice at G6, dispersed into single cells and sorted for their YFP expression. *Ngn3* expression and its downstream target *NeuroD1* were clearly detectable in cells from virgin and G6 pregnant female mice. While YFP negative cells did not show any changes in *Ngn3* expression at G6 compared to virgin females, *Ngn3* expression in the delta cells significantly increased by 2.78 ± 0.41 fold during pregnancy (Fig. 17A-B). Likewise, *NeuroD1* expression was increased by 2.64 ± 0.93 fold only in the YFP positive cells during pregnancy, yet this augmentation was not statistically different (Fig. 17C-D).

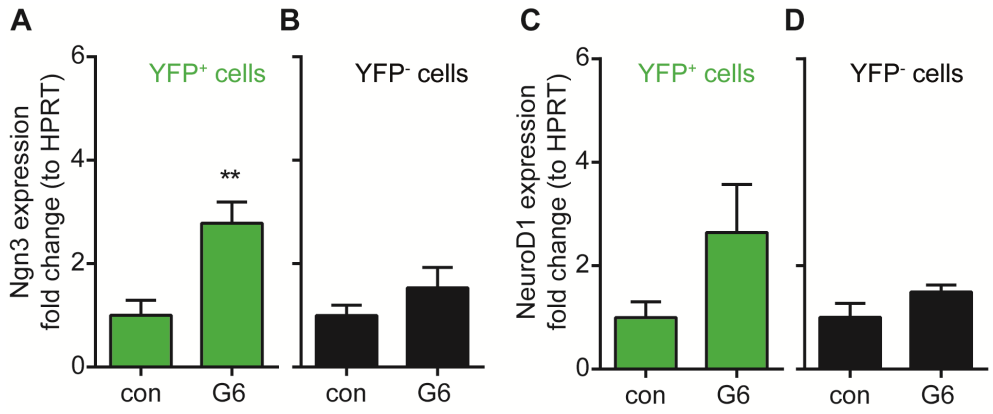

Figure 17: mRNA expression of *Ngn3* and *NeuroD1 in vitro*. Quantification of mRNA levels by qRT-PCR of FACS sorted cells. *Ngn3* expression calculated as fold change to the normalized mean control value of YFP positive cells (A) and YFP negative cells (B). Quantified mRNA levels of *NeuroD1* calculated as fold change to the normalized mean control value of YFP positive cells (C) and YFP negative cells (D). Data are presented as mean ± SEM, n = 5-6 mice per group with 300 cells/mouse, analyzed by an unpaired, two-tailed t-test.

Thus, emergence of *Ngn3* expression during pregnancy was shown to be significantly activated in delta cells and might lead to loss of somatostatin hormone expression.

3.3.6 Progesterone induces delta cell conversion in vitro

Increased *Ngn3* promotor activity and partial loss of delta cell phenotype occurred within a time window during early and mid-gestation. During this phase of pregnancy also metabolic and hormonal plasma concentrations were significantly altered. In particular the plasma steroid hormone P_4 levels were increased and glucose tolerance decreased. In order to elucidate whether there is a correlation between glucose homeostasis, hormonal changes and the loss of hormone expression in delta cells, *in vitro* studies with isolated islets of Sst-Cre;YFP female mice were performed.

Isolated islets were cultured for a prolonged period of 5 days in the presence of steroid hormones and elevated glucose concentrations. To elucidate the impact of every applied stimulus, islets were fixed after culture and stained by immunohistochemistry (Fig. 18A). This approach allows to perform lineage tracing of the delta cells and to quantify the specific effect of elevated glucose and/or increased hormone levels as a trigger to induce loss of somatostatin hormone expression in delta cells. Glucose concentrations were chosen according to fasting conditions by utilizing 5.5 mM glucose and elevated glucose concentrations, simulating the phase of insulin resistance occurring during pregnancy with 11.1 mM glucose concentration in the media.

Three dimensional analyses revealed no effect of elevated glucose concentrations per se on hormone expression levels in delta cells. However, slightly elevated fractions of YFP^+/Sst^- cells could be observed under basal conditions compared to the results obtained *in vivo* (6.43 ± 1.37 % *in vitro*; 1.54 ± 0.39 % *in vivo*). This might be an artifact of stress induced by the islet isolation procedure or *in vitro* culture itself. Interestingly, the presence of P_4 in the media resulted in a loss of somatostatin staining in YFP positive cells and thus, in an increase of YFP^+/Sst^- cells from 6.43 ± 1.37 % cultured in 5.5 mM glucose to 12.57 ± 1.36 % after culture in 5.5 mM glucose supplemented with P_4 (Fig. 18C). This effect was independent of the applied glucose concentration as elevated glucose of 11.1 mM did not reveal a significant increase compared to basal glucose (17.27 ± 1.85 %). The opposite development was observed in the YFP^+/Sst^+ cell fraction with a decrease from 93.57 ± 1.37 % cultured in 5.5 mM glucose to 87.43 ± 1.36 % after culture in 5.5 mM glucose and P_4 (Fig 18B). Remarkably, this effect was absent when islets were cultured in medium supplemented with a combination of P_4 and E_2 with values of 5.47 ± 1.03 %. Likewise, E_2 alone did not demonstrate any significant changes on YFP^+/Sst^- cell fraction (Fig. 18C). Significantly increased plasma E_2 levels were only observed during the last trimester of pregnancy when loss of somatostatin expression seemed to be completed. This effect of

combined hormone concentrations in the culture media might therefore suggest E_2 as an inhibitor of P_4 induced delta cell phenotype loss.

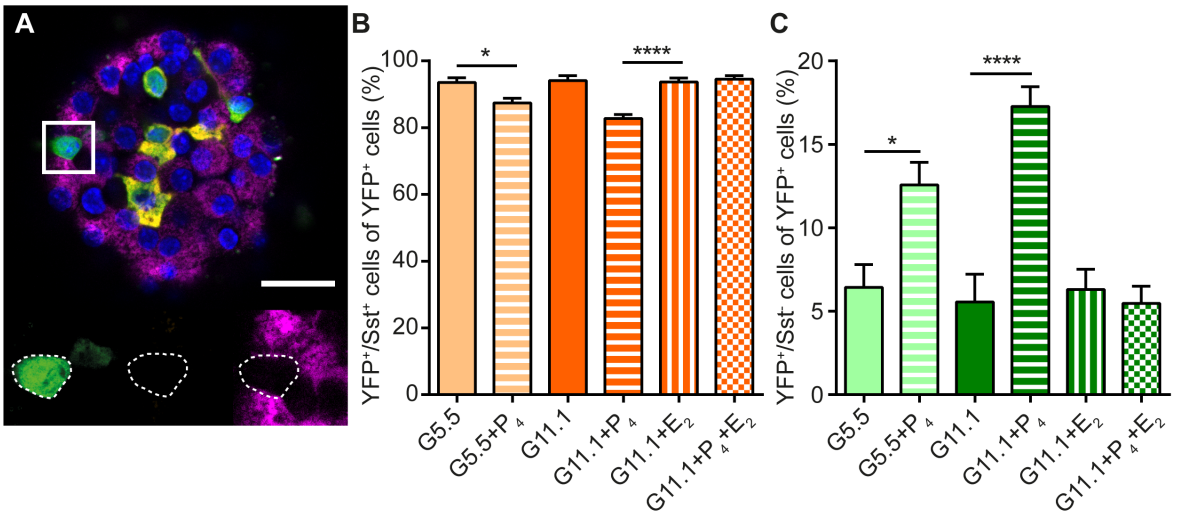

Figure 18: Effects of glucose concentration and presence of steroid hormones on isolated islets *in vitro*. Isolated islets from Sst-Cre;YFP mice were cultured for 5 days and stained by immunohistochemistry. Representative image of a stained islet for GFP (green), somatostatin (orange), insulin (magenta) and DAPI (blue). Scale bar = 25 µm (A). Percentage of YFP^+/Sst^+ cells (B) and YFP^+/Sst^- cells (C) after 5 day culture in 5.5 mM glucose (G5.5), 5.5 mM glucose supplemented with progesterone (G5.5+P_4), 11.1 mM glucose (G11.1), 11.1 mM glucose supplemented with progesterone (G11.1+P_4), 11.1 mM glucose supplemented with estradiol (G11.1+E_2) and 11.1 mM glucose supplemented with progesterone and estradiol (G11.1+P_4+E_2) (B). Data are presented as mean ± SEM, n = 5-6 mice per group with > 2000 cells/mouse, analyzed by one-way repeated measure ANOVA.

In summary, P_4 alone has been demonstrated to induce loss of hormone expression in delta cells *in vitro*, visualized by an increased fraction for YFP^+/Sst^- cells, whereas a combination of P_4 and E_2 demonstrated suppressive effects.

3.3.7 Delta cell dedifferentiation leads to increased beta cell function in vivo and in vitro

Early pregnancy initiated loss of delta cell identity resulting in significantly decreased amounts of somatostatin producing delta cells (Fig. 15D). This might impact insulin release as somatostatin produced by pancreatic delta cells is supposed to have an inhibitory function on beta cells (Hauge-Evans, et al. 2009). In order to elucidate the effects of early pregnancy on insulin secretion, pancreatic tissue slices were utilized to assess hormone secretion in response to different stimuli by perifusion (Fig. 19 A-C). For this purpose, tissue slices were generated from female virgin and pregnant Bl/6 mice at G8, corresponding to the phase of delta cell reprogramming between the first and second trimester of pregnancy. Slices were placed in closed perifusion chambers and connected to a perifusion machine.

Dynamic insulin secretion of perifused tissue slices revealed an increased glucose stimulated insulin secretion in pregnant mice at G8 compared to control mice, expressed as stimulation index over the mean basal insulin secretion (Fig. 19A). Quantification of stimulated insulin secretion, calculated by the area under the curve, showed a significant increase while total insulin content of perifused slices was comparable (Fig 19B-C). This data clearly showed increased glucose stimulated insulin secretion during mid-pregnancy at G8. However, this data could not prove whether the observed response is a result of diminished inhibition of beta cells *via* somatostatin secretion or linked to increased beta cell function. As *in vitro* culture of isolated islets in the presence of P_4 demonstrated an induction delta cell dedifferentiation, this stimulus was used to address whether delta cell dedifferentiation has an effect on islet cell function.

After a 5 day culture period, perifusion experiments were performed on isolated islets using the same protocol as before. Quantification of insulin kinetics in response to glucose demonstrated that prolonged culture with P_4 indeed alters glucose stimulated insulin secretion (Fig. 19D). The observed increase correlated well to the effect seen during pregnancy (Fig. 19A). Assessment of dynamic insulin secretion by the area under the curve confirmed increased beta cell function after P_4 culture (Fig. 19E). Notably, both conditions revealed a comparable total insulin content (Fig. 19F), indicating no changes in total beta cell mass after prolonged culture. Thus, elevated plasma hormone levels of P_4 during early pregnancy might be responsible for increased islet function *via* delta cell dedifferentiation.

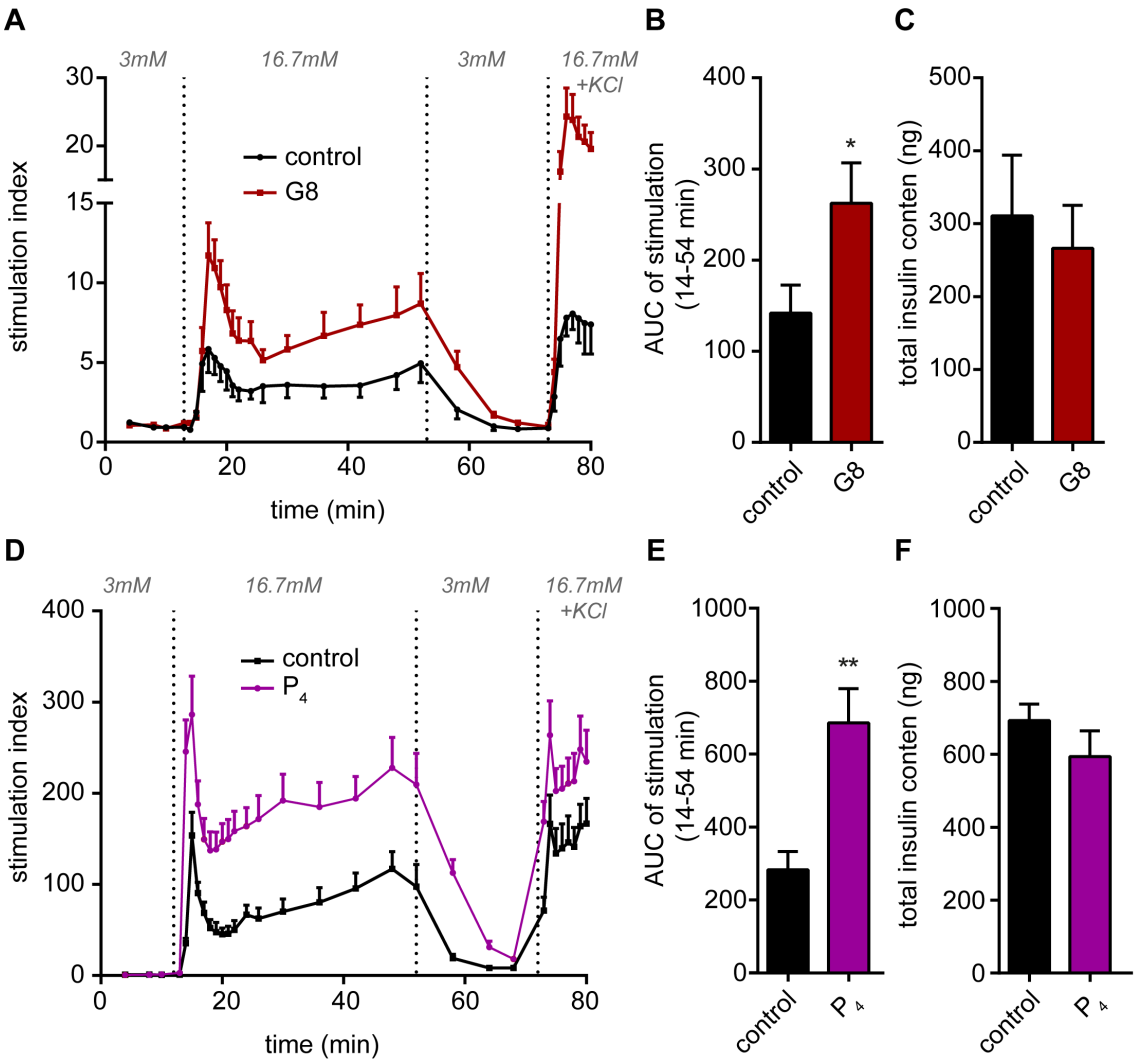

Figure 19: Dynamic insulin release during pregnancy and after culture *in vitro*. Insulin secretion from perifused pancreatic tissue slices of virgin and pregnant Bl/6 mice at G8 (A-C) and isolated islets cultured for 5 days with and without progesterone (D-F). Insulin response is calculated as stimulation index over the mean baseline response from tissue slices (A) and isolated islets (D). Quantification of stimulated insulin response, calculated as area under the curve in tissue slices from pregnant mice (B) and after *in vitro* culture (E). Total insulin content of perifused pancreatic tissue slices (C) and cultured islets (F). Data are presented as mean ± SEM, n = 5-6 mice per group with 4 slices or 30 islets respectively. Analysis was performed by one-way ANOVA.

4 Discussion

Pancreatic beta cells produce and secrete insulin, and are therefore responsible for lowering blood glucose concentrations. The amount of secreted insulin depends on the functional beta cell mass, determined by the absolute number and size of beta cells as well as their potential to secrete insulin. The population of beta cells is dynamic as they are capable to react to changes in metabolic demand over a short time period. Beta cell replenishment and death is regulated by cellular processes including replication of preexisting cells, hypertrophy, neogenesis and apoptosis. Throughout life these processes are balanced, resulting in a sufficient beta cell mass to maintain euglycemia within a narrow range. One of the most impressive physiological challenges for beta cell mass represents pregnancy, a unique metabolic condition demanding enormous structural and functional plasticity of the endocrine pancreas. Profound changes in hormonal regulation and metabolism favor glucose supply to the developing fetus, resulting in progression of maternal insulin resistance and subsequent compensation by beta cell mass and function (Green and Taylor 1972; Parsons, et al. 1992; Sorenson and Brelje 1997). The significance of this adaptation is illustrated by the progression of gestational diabetes, a condition that develops when beta cells fail to meet the elevated physiological demand (Barbour, et al. 2007) and can lead to deleterious short- and long-term effects on the fetus and the mother. While fetal overgrowth, underdevelopment of organs and premature birth are potential short-term risks for the developing fetus, predisposition to the development of metabolic syndrome and T2D, as well as cardiovascular diseases, represent the most significant long-term consequences for mother and child (Buckley, et al. 2012; Fraser and Lawlor 2014). However, only little is known about the contributing mechanisms of the compensatory response of beta cell mass during pregnancy that protect from the development of GDM. Although beta cell mass expansion has been mainly attributed to increased proliferation rates (Sorenson and Brelje 1997; Xue, et al. 2010; Beamish, et al. 2017), the influence of mechanisms including non-beta cell sources have not been fully clarified yet. Moreover, even less attention was attracted to responsible mechanisms in the early functional compensation. Therefore, this thesis aimed to assess metabolic adaptions occurring during and after pregnancy and to obtain more accurate data on the contribution of individual cellular processes underlying increased beta cells mass and function. These findings are essential for broader understanding of pancreas plasticity and to identify the underlying responsible signaling factors leading to beta cell mass adaptation. Moreover, deeper understanding of the intrinsic mechanisms involved in beta cell adaptations during pregnancy can provide novel targets

that could be used to enhance endogenous beta cell regeneration as potential future treatment options of diabetes.

4.1 Functional adaptations precede islet mass expansion during pregnancy

In this study, the physiological changes on maternal metabolism were comprehensively characterized across the different phases of rodent pregnancy and thereafter. Assessment of weight changes demonstrated significant and progressive weight gain from mid-gestation onwards, which in large parts results from size and growth of the litter. Notably, pancreatic weight increased similar to the total body weight during the first 14 days of pregnancy, indicating adaptations of this organ to the increased food intake and metabolic demand. Within the last trimester organ growth did not further increase and might indicate that compensatory mechanisms, at least in the exocrine pancreas that makes up 98 % of the entire mass, are mostly completed. Characterization of glucose homeostasis revealed that glucose intolerance developed already during early pregnancy and further progressed at mid-gestation, while pregnancy at term and even 4 weeks later revealed a better performance in glucose clearance with significantly lowered fasting blood glucose levels. Insulin sensitivity was measured under fasting conditions and after glucose challenge by insulin ELISA. However, these data were inconclusive which might be attributed to the prolonged sample storage, leading to partial degradation of the plasma insulin. These experiments will be repeated using C peptide as readout for beta cell function to circumvent the complications. Nevertheless, several studies already demonstrated significantly increased plasma insulin levels and suggest a significant rise in insulin secretion as pregnancy advances (Green and Taylor 1972; Bone and Taylor 1976; Parsons, et al. 1992; Catalano, et al. 1993; Sonagra, et al. 2014) Serum insulin levels were found significantly elevated by 75 % until mid-gestation and therefore demonstrate that the development of a transient insulin resistance is a hallmark of early to mid-pregnancy (Costrini and Kalkhoff 1971; Green and Taylor 1972). The increased demand for insulin during this stage is most likely compensated by enhanced beta cell function with negligible contribution of beta cells mass as a significant expansion could not be observed until the third trimester. Thus, beta cell function makes a major contribution during the first two trimesters of pregnancy whereas islet mass is of minor influence during that time and rather accounts for late adaptations.

The compensatory mass increase within the third trimester, also previously reported by others (Sorenson and Brelje 1997; Xue, et al. 2010), could be verified in this thesis, demonstrating a significant 2-fold increase by G18. Strikingly, no reduction of islet cell mass

could be observed up to 4 weeks post-partum and also mean islet size further increased. This data is contrary to literature, as it has been reported that beta cell volume returned to pre-gestational values shortly after birth (Rieck and Kaestner 2010). More precisely, regression of endocrine mass was shown to occur within ten days post-partum through decreased proliferation, elevated apoptosis and beta cell size reduction (Scaglia, et al. 1995; Beamish, et al. 2017). However, this effect could not be observed in this thesis.
Taken together, these results reveal that during rodent gestation a physiologic reduction of insulin sensitivity leads to distinct compensatory dynamics of beta cell function and mass.

4.2 Non-proliferative mechanisms contribute to the mass adaptations during pregnancy

Analyses of the proliferation rates by the cellular marker Ki67 demonstrated increased cell division rates with comparable levels throughout pregnancy, from G6 to G14. Notably, proliferative activity returned to pre-pregnancy levels shortly before parturition. Moreover, assessment of islet size distribution throughout pregnancy demonstrated a clear shift towards bigger islet diameter as pregnancy proceeds. These observations are in line with previous studies (Parsons, et al. 1992; Sorenson and Brelje 1997; Beamish, et al. 2017) showing elevated endocrine cell proliferation during pregnancy, however peak occurrence at about two-thirds of gestation could not be observed in this thesis. This might be related to the different experimental methods that were used, as labeling wit bromodeoxyuridine (BrdU) *in vivo* might result in a prolonged experimental windows and higher labeling efficiency compared to endpoint labeling with Ki67. Taken together, this data confirms previous reports and suggests proliferation as a major mechanism for endocrine mass expansion during pregnancy.
Several studies attributed the increased DNA synthesis to increased levels of hormone concentrations (Brelje, et al. 1993; Galosy and Talamantes 1995; Kim, et al. 2010). While progesterone has been shown to rise rather early during gestation (McCormack and Greenwald 1974; Murr, et al. 1974) with a suggested effect in beta cell function (Ashby, et al. 1978, 1981), lactogenic hormones were proposed to induce beta cell proliferation (Nielsen 1982; Brelje, et al. 1993; Sorenson, et al. 1993). Characterization of hormonal changes during pregnancy in this thesis confirmed that only the steroid hormone progesterone was significantly elevated during early and mid-gestation, while lactogenic hormones and estradiol increased gradually with significantly elevated plasma concentrations exclusively at term. These findings confirm the concept that placental lactogen and prolactin stimulate islet

proliferation at later stages of pregnancy resulting in endocrine mass expansion (Ryan and Enns 1988; Brelje, et al. 1993; Brelje, et al. 2004; Georgia and Bhushan 2010; Kim, et al. 2010). However, the observed proliferation rates in this thesis were rather low suggesting that other mechanisms cannot be excluded in the contribution of compensatory beta cell mass adaptation during pregnancy. Assessment of size distribution further support this hypothesis, as the fraction of small islets (0-50 µm diameter) remained constant during the course of gestation, indicating the influence of neogenesis. Furthermore, increasing size towards islets bigger than 150 µm might also be partially due to neogenesis of endocrine cells into preexisting islets.

In summary, the data shown in this thesis could confirm previous reports in terms of proliferation accounting predominantly for mass adaptations during mid- and late gestation, yet the influence of non-beta cell sources cannot be excluded.

4.3 Recapitulation of *Ngn3* promotor activity during early pregnancy

Since the source of beta cell mass expansion during gestation has not been entirely identified, this thesis aimed to determine whether other mechanisms, like neogenesis or dedifferentiation, under the re-activation of the developmental pathway involving *Ngn3* expression contribute.

Neogenesis of islet cells is defined as the new formation of endocrine cells from pancreatic progenitor or stem cells, frequently described to originate from the ductal compartment (Bonner-Weir, et al. 2004). This pathway has been shown to occur during embryonic development (Herrera, et al. 1991; Gu, et al. 2002), postnatal growth (Chintinne, et al. 2010) and in response to extreme artificial beta cell loss (Xu, et al. 2008; Van de Casteele, et al. 2013). Cellular reprogramming on the other hand defines the transition from a fully differentiated endocrine cell into another and can happen in two different ways, namely trans- and dedifferentiation. In case of transdifferentiation, a mature islet cell converts directly into another (Thorel and Herrera 2010). If the process occurs *via* an intermediate transition phase towards a multi-progenitor cell stage, it is called dedifferentiation and can be followed by a re-differentiation into a mature cell state (Puri, et al. 2015). Recent studies describe islet cell dedifferentiation as a mechanism of cell failure caused by cellular exhaustion and dysfunction associated with disease progression, e.g. T2D (Cinti, et al. 2016). This study however did not link dedifferentiation as a consequence of stress or exhaustion, but as a physiological adaptation.

It has been well established, that the transcription factor *Ngn3* plays an essential role in endocrine differentiation and that all islet cells derive from *Ngn3* positive precursors (Gradwohl, et al. 2000; Gu, et al. 2002; Gouzi, et al. 2011). Expression of *Ngn3* in the adult pancreas on the other hand is contrary. Its transient induction has been observed in hormone negative cells, leading towards differentiation of the endocrine lineage. These derive from cells located along the ductal lining (Xu, et al. 2008; Van de Casteele, et al. 2013) or acinar cells (Sostrup, et al. 2014) and initiation of *Ngn3* expression would subsequently lead to migration towards existing islets.

Contrary to literature, analysis on histological pancreas sections from adult mice demonstrated the emergence of *Ngn3* positive cells to be located within the islets themselves and not in the exocrine or ductal compartments. Yet, this is an endpoint approach, so *Ngn3* induction within the ductal or acinar cells followed by migration towards the islets of Langerhans cannot be fully excluded. However, the narrow time window and absence of elevated GFP expression in the exocrine cells during pregnancy makes this hypothesis highly unlikely.

Interestingly, low amounts of intra-islet *Ngn3* activity were observed in adult non-pregnant mice, indicating that *Ngn3* expression is indeed present in the adult pancreas. Nevertheless, pregnancy lead to a transient increase of *Ngn3* promotor activity, resulting in a considerable higher amount of GFP positive islet cells in the first trimester of pregnancy, dropping back to control levels at mid-term. Notably, G4 was the earliest tolerated time for TAM administration during pregnancy. Thus, it is possible, that induction of *Ngn3* activity might be initiated at an earlier time point. Moreover, the actual fraction of GFP positive cells in pregnant and control mice is likely to be higher, but limited due to the Cre-dependent recombination efficiency limited. Therefore, accurate function of *Ngn3* promotor activity was verified using a Cre-independent mouse line, the Ngn3-YFP. Remarkably, augmentation of *Ngn3* promotor activity correlated nicely under control conditions as well as during pregnancy at G6. As expected, Cre-independent YFP expression resulted in slightly higher *Ngn3* promotor activity under control and pregnancy. However, pregnant mice at G6 revealed a similar 2.5-fold increase compared to virgin control mice, demonstrating the faithful recapitulation of endogenous *Ngn3* activation during pregnancy.

These findings demonstrate that re-activation of the endocrine progenitor marker *Ngn3* also occurs under the physiological stress of pregnancy, which was contradictory discussed in literature (Teta, et al. 2007; Abouna, et al. 2010; Butler, et al. 2010; Xiao, et al. 2013). Further evidence of the recapitulation of the embryonic developmental pathway to regenerate functional beta cells has only been reported under artificial conditions of extreme

regenerative pressure, like beta cell ablation (Rooman and Bouwens 2004) or partial duct ligation (Xu, et al. 2008).

Nonetheless, it is well-known that islet cells exhibit a remarkable proliferative potential during pregnancy (Sorenson and Brelje 1997; Beamish, et al. 2017) and also this study has shown increased cell division rates at that time point of gestation. Thus, it might be possible, that the observed augmentation resulted from replication of preexisting cells, instead of newly onset *Ngn3* promotor activity. To rule out this hypothesis, lineage tracing experiments were performed on Ngn3-CreER;mTmG pregnant and virgin mice, by pre-labeling of *Ngn3* expressing cells. Results revealed no significant increase in GFP positive cell fractions, indicating *de novo Ngn3* promotor activity initiated by early pregnancy.

4.4 Onset of endogenous Ngn3 activity during early pregnancy is delta cell specific

Although, *Ngn3* activity was shown to be induced with the onset of pregnancy, the origin of these cells still remains unknown. Moreover, low GFP expression levels of 1.92 % were observed almost exclusively within the endocrine compartments under control conditions. This suggests the constant presence of the progenitor cell marker *Ngn3* in mature islet cells.

Studies have indicated the existence of precursor cell population located within the islet cells that also express *Ngn3* at both, embryonic and adult stages under normal physiological conditions (Jensen, et al. 2005). Additionally, these cells were reported to be hormone negative and fractional loss was observed with onset of pregnancy (Toselli, et al. 2014). Contrary to these findings, co-labeling with antibodies for three hormones (insulin, glucagon and somatostatin) revealed almost no hormone negative, GFP expressing cells under control conditions. These results indicate that *Ngn3* is expressed in a subpopulation of mature hormone expressing islet cells. Whether this cell population constantly expresses *Ngn3* or if it is transiently expressed among the islet cells remains unknown. Moreover, the time delay between recombination and fluorescent visualization did not allow precise identification of the exact origin of *Ngn3* activity, as it cannot be excluded that these cells derive from exocrine tissue and migrate into the islets after *Ngn3* expression. However, the consistency of the obtained data suggests the presence of *Ngn3* expression predominantly located within the somatostatin expressing delta cell population. A more beneficial approach would be the use of direct *Ngn3* staining at the individual stages of pregnancy. Unfortunately, intensive staining

trials during this thesis did not result in reliable *Ngn3* staining in the adult pancreas due to the lack of a sensitive anti-*Ngn3* antibody.

The expression of *Ngn3* in mature hormone expressing islet cells has already been demonstrated although only under conditions of regenerative stress and with much lower protein levels than during embryogenesis (Wang, et al. 2009). Yet, the authors propose that transient high levels of *Ngn3* allow the cell to gain autonomy along the endocrine differentiation pathway (Apelqvist, et al. 1999; Jensen, et al. 2005). Low expression levels on the other hand have been suggested to preserve the mature phenotype and maintain islet cell function (Wang, et al. 2009).

Likewise, this study demonstrated a transient increase of *Ngn3* expression initiated during early pregnancy by genetic lineage tracing as well as RNA expression levels. In order to address the cell fate of emerging *Ngn3* expressing cells during early pregnancy, a detailed characterization of endocrine phenotypes was performed by immunohistochemistry for insulin, glucagon and somatostatin hormone expression by manual, morphological analysis. Surprisingly, more than half of the *Ngn3* positive cells, indicated by GFP expression, were labeled for somatostatin under normal physiological conditions. Moreover, peak occurrence of *Ngn3* positive cells goes along with an increase in hormone negative cells in the GFP fraction, suggesting a dedifferentiation of mature islet cells towards a hormone negative cell state. Whether this resembles an endocrine progenitor cell state, an intermediate differentiation cell stage or just a lack of hormone secretion in a differentiated endocrine cell cannot be determined at this point. Yet, exclusively the amount of somatostatin expressing delta cells in the GFP fraction decreased during this time.

These results suggest that early pregnancy might rather lead to a dedifferentiation of delta cells by recapitulation of the endocrine developmental pathway through *Ngn3* induction, than islet neogenesis induced by the exocrine compartment. The surprising absence of *Ngn3* within the exocrine compartments further supports this hypothesis although it did not exclude islet neogenesis *via* a different mechanism.

4.5 Early pregnancy induces partial loss of delta cell identity

To confirm the occurrence of delta cell reprograming during pregnancy, the Sst-Cre;YFP mouse line was utilized. Lineage tracing was performed during pregnancy as well as 2 and 4 weeks post-partum by staining for somatostatin and insulin hormone expression. Interestingly, a considerable amount of somatostatin expressing delta cells seem to lose hormone expression within the first and second trimester of pregnancy, while a gradual

increase of YFP positive cells expressing insulin, showing a similar cell shape to beta cells was observed towards G18.

Taken together, these results indicate that the physiological changes during early pregnancy induce cellular reprogramming of somatostatin producing delta cells to insulin secreting beta cells. This process seems to involve re-expression of the developmental factor *Ngn3*, suggesting that a subpopulation of delta cells lose their cell fate, become hormone negative and re-differentiate into beta-cells.

The possibility of islet cell dedifferentiation from a mature hormone expressing endocrine cell type into beta-like cells has been described in recent studies (Collombat, et al. 2009; Chera, et al. 2014; Ben-Othman, et al. 2017). For instance, Collombat and colleagues demonstrated that long-term GABA administration induces continuous conversion of alpha to beta-like cells (Collombat, et al. 2009). Moreover, resulting alpha cell deficiency was compensated by constant alpha cell neogenesis through reactivation of the *Ngn3* controlled endocrine developmental program of duct-associated cells and subsequent migration towards the islets (Ben-Othman, et al. 2017). This study demonstrated the enormous plasticity of the pancreas, yet only under artificial conditions of extreme beta cell loss and the involvement of the exocrine compartment. Delta to beta cell conversion in particular has been described by Herrera and his group (Chera, et al. 2014). Importantly, conversion occurred in a defined sequence starting with dedifferentiation into hormone negative cells, replication followed by either re-differentiation back to delta cells or activation of *Ngn3* and subsequent differentiation into insulin producing beta cells. This process describes precisely the individual progressions of delta cell dedifferentiation also observed in this thesis. However, Chera et al. could only observe this conversion after total beta cell ablation and more importantly only in juvenile mice. Therefore, this thesis describes for the first time, the dedifferentiation of mature islet cells under the physiological conditions of mouse pregnancy. Furthermore, the physiological appearance of cellular reprogramming could also be demonstrated by changes in the specific cell shapes of the cells. Rodent delta cells are frequently described to exhibit a neuronal morphology, showing dendrite-like extensions that allow distinct interconnections and paracrine crosstalk within the islet (Grube and Bohn 1983; Baskin, et al. 1984; Gopel, et al. 2004). This elongated cell shape could be confirmed by measurements of x and y axis of individual YFP and somatostatin expressing cells. Moreover, a majority of the delta cell population was located at the periphery of the islet while beta cells with an almost round cell structure were predominantly found in the islet core. Interestingly, cell shape was demonstrated to differ significantly in cells undergoing conversion during pregnancy indicating their morphological adaptation.

However, all evidence leading to the hypothesis of delta cell dedifferentiation is based on the observation of changing fractions of hormone negative and insulin positive YFP expressing cells. Given that all of these cell types could be found already under non-pregnant conditions and considering elevated proliferation rates during pregnancy, this observation could be an artefact of different cell division rates of the individual cell types during the stages of pregnancy. In order to exclude this possibility, islet composition data was combined with pancreatic mass assessments in order to include the adaptive compensation of endocrine mass occurring during gestation. This analysis clearly demonstrated a significant increase in hormone negative YFP cells, while the total YFP mass remains unchanged at G6. Notably, somatostatin expressing YFP positive cell mass was observed to decrease slightly, yet statistical significance could not be observed. This might be linked to the low amount of delta cells in rodent islets and the high standard error at this time point. Although the mass of insulin expressing YFP positive cells was significantly elevated at G18, re-differentiation of hormone negative YFP cells towards insulin expressing YFP positive cells cannot be concluded, as total YFP positive cell mass was increased significantly at this point. Moreover, mass changes within the hormone negative YFP cells between the second and third trimester were not shown to be significantly decreased, meaning that proliferation of preexisting insulin expressing YFP positive cells might also be a possible mechanism for the elevated mass at term.

In summary, this data suggests loss of delta cell identity rather than proliferation of preexisting hormone negative cells as underlying mechanism during early pregnancy. After all, the re-differentiation of these cells towards a beta cell identity cannot be concluded for certain, as proliferation especially within the third trimester of pregnancy leads to increased YFP mass.

4.6 Progesterone initiates cellular reprogramming of delta cells

Lineage tracing data indicated that intra-islet cell conversion of delta to beta cells *via Ngn3* expression can be visualized under physiological conditions of rodent pregnancy. Still, the responsible signaling factors leading to the onset of this mechanism are not clarified.

Early pregnancy is characterized by profound alterations in glucose homeostasis and steroid hormone activity. A progressive insulin resistance commences already by the end of the first trimester resulting in a compensatory response by increased beta cell function and subsequent elevated plasma insulin levels (Costrini and Kalkhoff 1971; Green and Taylor 1972; Parsons, et al. 1992). Compensatory changes in beta cell mass controlled by hypertrophy and increased proliferation represent cellular processes that have been

observed predominantly from mid-pregnancy to term (Sorenson and Brelje 1997; Rieck and Kaestner 2010). Therefore, distinct functional adaptations precede mass at this point of pregnancy. Several studies during the last decades demonstrated correlations between changes in maternal islet growth and function with increased levels of circulating lactogenic hormones, like prolactin (PRL) and placental lactogen (PL) (Ryan and Enns 1988; Parsons, et al. 1992; Brelje, et al. 1993; Galosy and Talamantes 1995; Brelje, et al. 2004; Ladyman, et al. 2010). Notably, both PRL and PL, bind to the prolactin receptor, which is supposed to possess increased expression levels during pregnancy (Clarke and Linzer 1993; Moldrup, et al. 1993). Effects of these lactogenic hormones were suggested to increase insulin secretion and beta cell proliferation *in vitro* (Brelje, et al. 1993; Weinhaus, et al. 1996) and *in vivo* (Nielsen 1982; Vasavada, et al. 2000). In this thesis however, prominent alterations in lactogenic activity of prolactin and placental lactogen could not be observed before the third trimester of gestation in the utilized mouse model. Thus, lactogenic hormones might be responsible for adaptations at later stages of pregnancy, but did not induce cellular reprogramming by *Ngn3* activation. In contrast, changes in steroid hormone levels, especially progesterone are shown to rise very early during pregnancy (McCormack and Greenwald 1974; Murr, et al. 1974) and therefore correlate with the onset of islet cell conversion.

In order to investigate the particular effect of steroid hormones on delta cell conversion *in vitro* studies on isolated Sst-Cre;YFP mouse islets were performed and cell composition was assessed after prolonged culture. Strikingly, only the presence of progesterone lead to significantly increased amounts of converting delta cells, while no additional effect was observed with elevated glucose concentrations. Moreover, combined culture with estrogen and progesterone seemed to inhibit this effect. This results determined elevated progesterone levels as a potential signal to induce delta cell identity loss *in vitro*. Moreover, gradually rising levels of estrogen to significant values during the third trimester correlate with the decrease in hormone negative YFP expressing cells and the potential differentiation towards beta cells *in vivo*. Still, it is not entirely certain, whether progesterone induces the loss of the delta cell phenotype *via* the onset of *Ngn3* expression or by a different mechanism. Therefore, further culture experiments followed by quantitative real-time PCR are planned in order to assess mRNA expression levels of progenitor gene activity in order to correlate expression patterns with a loss in somatostatin gene translation.

4.7 Loss of delta cell identity leads to increased beta cell function

Since the completion of delta to beta cell conversion lasts over the entire duration of pregnancy and the total beta cell gain of this process is fairly low, this mechanism most likely serves a different purpose than beta cell mass compensation. Therefore, the initiation of cellular reprogramming itself might be an essential process in the early functional compensation during gestation.

The local secretion of Somatostatin produced by the pancreatic delta cells is supposed to act as an inhibitory regulator on glucose stimulated insulin secretion as well as arginine- induced glucagon secretion, which has been demonstrated *in vivo* and *in vitro* (Schuit, et al. 1989; Strowski, et al. 2000; Cejvan, et al. 2003). Studies using SST knock-out models demonstrated enhanced insulin and glucagon secretory responses further indicating its role in the negative regulation of alpha and beta cell function (Hauge-Evans, et al. 2009; Hauge-Evans, et al. 2012). Given the fact, that early pregnancy results in increased beta cell function, in the absence of mass adaptations, it can be hypothesized, that partial loss of delta cell identity serves as an indirect early compensatory mechanism to increase insulin secretion.

In order to test this hypothesis, pancreatic tissue slices were used to assess the dynamic insulin release in response to glucose of virgin and pregnant mice in the second trimester at G8. Insulin secretion kinetics clearly demonstrated an increased glucose stimulated insulin response in pregnant mice at G8 compared to non-pregnant age matched mice. Given that SST is a strong inhibitor of insulin secretion, it is surprising that low glucose concentrations did not reveal any elevated insulin response. However, it has been demonstrated that SST secretion is dose dependent and only released at glucose levels above 4 mM (Vieira, et al. 2007). Therefore, it has been proposed that SST is first released at glucose levels which are supposed to diminish glucagon secretion, thus preventing over-secretion of insulin and glucagon (Brereton, et al. 2015). Nevertheless, the here presented experiment only proves enhanced beta cell function during mid-pregnancy and is no evidence for diminished SST release due to a lower delta cell fraction. Thus, delta cell conversion was stimulated *in vitro* in the presence of progesterone and beta cell function was assessed by islet perifusion. Quantification of insulin kinetics demonstrated elevated insulin secretion when cultured with P_4. This effect nicely correlated with insulin responses visualized at G8 of pregnancy, supporting the hypothesis that delta cell dedifferentiation might serve as an early compensatory mechanism to increase beta cell function.

Subsequently, direct assessment of somatostatin secretion would be essential to illuminate the functional relevance of delta cell dedifferentiation during early pregnancy on increased beta cell function. However, this is a rather difficult approach due to the limited amount of delta cells and consequently low hormone release. So far, ELISA kits are not sensitive enough to measure somatostatin secretion using a reasonable amount of islets. To circumvent these limitations further investigations on delta cell function by measuring intracellular calcium dynamics are ongoing. These experiments might not only give further insights into delta cell signaling and alterations during pregnancy, but also unravel the functional similarities between beta cells and insulin expressing YFP positive cells.

4.8 Conclusion and perspectives

Pregnancy leads to increased maternal beta cell function and mass to compensate insulin resistance and increased metabolic demand. While mass expansion is mainly attributed to increased proliferative rates with peak occurrence in the third trimester of pregnancy, beta cell function is compensated already during early and mid-pregnancy resulting in elevated plasma insulin levels. This early functional compensation coincides with a transient increase of intra-islet *Ngn3* promotor activity predominantly in somatostatin producing delta cells. The subsequent partial loss of delta cell identity towards a hormone negative cell state is initiated by elevated progesterone levels. Estradiol on the other hand was identified as a repressor of this mechanism and might therefore lead to the offset of this process with preceding pregnancy when estradiol concentrations rise. Furthermore, delta cell dedifferentiation correlates with increased glucose stimulated insulin secretion at mid gestation. These findings indicate that delta cell dedifferentiation, initiated by *Ngn3* expression, might represent a compensatory mechanism to increase beta cell function during early pregnancy. These observations might uncover a novel role for delta cells and could be translatable to humans in order to provide new therapeutic opportunities to enhance beta cell function and foster the formation of beta cells.

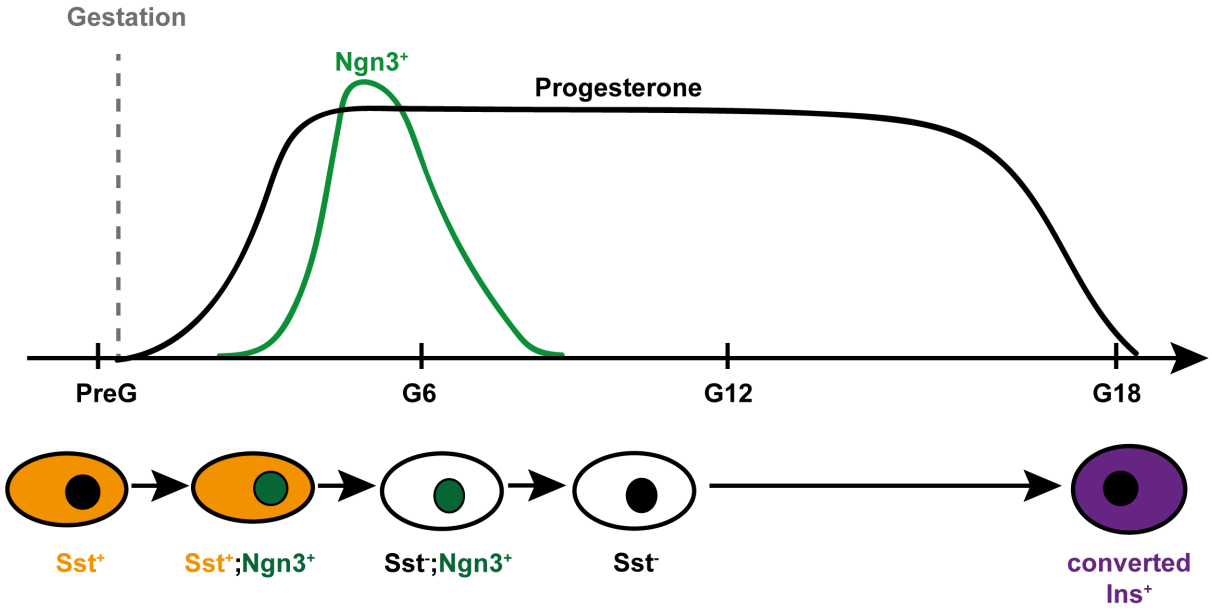

Figure 20: Sequence of delta cell dedifferentiation during pregnancy. Delta cells dedifferentiate in part and reprogram towards insulin producing cells during the course of pregnancy in Sst-Cre;YFP mice.

It is tempting to hypothesize that partial loss of delta cell identity leads to decreased amounts of secreted somatostatin and therefore less inhibitory action on insulin secretion to promote insulin secretion. However, the kinetics of somatostatin secretion need to be further investigated. One of the major limitations is the assessment of somatostatin secretion, due to the low amount of cells within rodent islets and the lack of sensitive readouts. Ongoing experiments measuring the intracellular calcium dynamics in delta cells might circumvent these limitations and give further insights into the role and function of delta cells during early pregnancy. Moreover, it remains uncertain whether reprogrammed delta cells further differentiate towards the beta cell state due to the dynamic adaptations in endocrine mass occurring within the last phase of pregnancy. Further studies addressing the proliferative capacity of these cell types over time would be necessary to draw more precise conclusions. Additionally, it will be very interesting whether this process is reversible after pregnancy and if so, to define the signals involved in this mechanism. Finally, it has to be investigated whether this mechanism also occurs during human pregnancy and if this is triggered by the same signaling cascade. If so, the findings in this thesis might have uncovered a novel role for delta cells and could provide new therapeutic opportunities to enhance beta cell function and foster the formation of beta cells.

5 Summary

Background

Diabetes mellitus is a set of metabolic diseases with common characteristics such as chronic hyperglycemia and glucose intolerance caused by insulin deficiency, defects in insulin secretion and action, or both. Up to now, there is no cure for this disease types as existing treatment possibilities are still limited and accompanied by long-term side effects. A promising approach in this regard would be the restoration of endogenous beta cell mass by induced regeneration. A requirement for the development of such an approach is to understand the regenerative capacities of endogenous beta cells and uncover the underlying signaling factors of this mechanism.

It is well established that functional beta cell mass is capable of dynamic adaptations to compensate changing metabolic conditions. Pregnancy represents a physiological setting for adaptive beta cell mass expansion and increased function as the maternal body undergoes enormous physiological adaptations in order to provide sufficient nutrients to the developing fetus. Although these compensatory changes are known for decades, the mechanisms involved are still not completely clarified.

Aim

The objective of this thesis was to study the mechanisms involved in the compensatory response of beta cell mass and function during pregnancy. Especially the role of non-beta cell sources, indicated by the re-activation of the developmental transcription factor *neurogenin 3* (*Ngn3)* was investigated.

Methods

Characterization of metabolic adaptations during pregnancy was assessed by glucose tolerance tests, plasma insulin levels and hormonal profiling. To address the individual adaptations occurring during pregnancy, a combined approach of *in vivo* experiments in transgenic reporter mice, *in situ* analysis in tissue slices and *in vitro* studies on isolated islets was utilized.

Pancreatic mass adaptations were quantified by point morphometry of stained cryosections from Bl/6 mice. Furthermore pancreatic tissue sections were stained from different reporter mouse lines at multiple stages during gestation and islet composition was assessed by immunohistochemistry and point counting analysis. Pancreatic tissue slices were utilized to investigate glucose stimulated insulin secretion during pregnancy and isolated islets were

cultured in the presence of pregnancy hormones to investigate their role in functional and morphological adaptions.

Results

Characterization of mouse pregnancy confirmed current knowledge from literature that pregnancy induces insulin resistance, increased insulin secretion and beta cell proliferation resulting in elevated endocrine mass. Mass expansion gradually increased from the second trimester leading to a two fold increase by the end of pregnancy and remained elevated even post-partum. Increased proliferative rates were visualized already in the first trimester and declined with pregnancy at term. Although, proliferation seemed to play a major role in the compensatory response during pregnancy, it might not be the only mechanism involved in this process. A subpopulation of islet cells was identified to initiate transient *Ngn3* promoter activity during the first trimester of pregnancy. A majority of these cells was characterized as somatostatin secreting delta cells. Interestingly, peak occurrence of *Ngn3* activity, lead to an increase in hormone negative, *Ngn3* expressing cells, indicating an endocrine progenitor cell state. Lineage tracing experiments revealed a fractional loss of delta cells and might be followed by differentiation towards a beta cell identity throughout the duration of pregnancy. Notably, the onset of *Ngn3* expression and consequent delta cell reprogramming correlated with changes in plasma steroid hormone levels, whereas delta cell dedifferentiation coincides with increased plasma insulin levels. *In vitro* culture of isolated islets demonstrated that indeed the presence of elevated progesterone concentrations in the media lead to a partial delta cell identity loss. Moreover, functional characterization revealed that progesterone also increased glucose stimulated insulin secretion after a 5 day culture period.

Conclusion

These findings provide evidence for a functional role of delta cells in the early compensatory adaptations during pregnancy. During early gestation a considerable amount of somatostatin expressing delta cells reprogram and might differentiate towards a beta cell identity. This process seemed to involve the re-expression of the developmental transcription factor *Ngn3* reinforced by plasma progesterone levels. Although the functional relevance of delta cell conversion needs further investigation, a major implication on early compensation to increase insulin secretion *via* less somatostatin mediated inhibition is suggested.

6 Zusammenfassung

Hintergrund

Diabetes Mellitus umfasst eine weit verbreitete Gruppe von Stoffwechselerkrankungen, die durch chronisch erhöhte Blutzuckerspiegel und Glukose Intoleranz gekennzeichnet sind. Den Krankheitsformen liegen unterschiedliche Mechanismen zugrunde, jedoch haben alle Formen einen Mangel an Insulin produzierenden Beta Zellen gemein. Bis heute gibt es keine Heilung und existierenden Behandlungsmöglichkeiten sind meist begleitet von Nebeneffekten. Ein vielversprechender Ansatz für die neuartige Diabetestherapie bietet die Wiederherstellung der körpereigenen Betazellmasse durch induzierte Regeneration. Als notwendige Voraussetzung für die Entwicklung einer Zellbasierten Therapie wird das umfassende Verständnis der körpereigenen Mechanismen zur Erneuerung dieser Zellen vorausgesetzt.
Es ist bekannt, dass Beta Zellen in ihrer Funktion anpassungsfähig sind und so kurzzeitig auf eine veränderte Stoffwechselsituation reagieren können. Schwangerschaft repräsentiert einen solchen physiologischen Zustand, da der mütterliche Körper sich enormen Anpassungen unterzieht um den Fetus optimal zu versorgen. Während der Schwangerschaft wächst die funktionell wirksame Betazellmasse um dem gesteigerten Insulin Bedarf gerecht zu werden. Obwohl diese Leistungssteigerung schon lang bekannt sind, konnten die zugrundeliegenden Mechanismen bisher nicht vollständig aufgeklärt werden.

Ziele

Ziel dieser Arbeit war die Charakterisierung der unterschiedlichen Mechanismen der Anpassung von Betazellmasse und Funktion während der Trächtigkeit von Mäusen. Hierbei sollte besonders die Rolle der Zellerneuerung unter Aktivierung embryonaler Signalwege eingehender untersucht werden.

Methoden

Die Erfassung der Stoffwechselveränderungen während der Trächtigkeit wurden durch intraperitoneale Glukosetoleranztests und Blutplasmawerte untersucht. Um die verschiedenen Anpassungsprozesse zu adressieren, wurde ein kombinierter Ansatz von *in vivo* Experimenten mittels Reportermausstämmen, sowie *in situ* Analyse an lebenden Gewebeschnitten und *in vitro* Studien mit isolierten, kultivierten Inseln durchgeführt.
Die Erhöhung der endokrinen Zellmasse wurde über die manuelle Auswertung gefärbter Längsschnitte der Bauspeicheldrüse quantifiziert. Zusätzlich wurde eine detaillierte Analyse der Zusammensetzung der Langerhans´scher Inseln vor, während und nach der Trächtigkeit

mittels unterschiedlichen Hormonfärbungen erstellt. Zur Ermittlung der endokrinen Funktion im trächtigen Tier wurden vitale Gewebeschnitte der Bauchspeicheldrüse verwendet. Zusätzlich wurden Inseln isoliert und mit verschiedenen Zusätzen kultiviert, um die Rolle einzelner Faktoren auf die Funktion und Morphologie der Inselzellen zu beurteilen.

Ergebnisse

Die Charakterisierung der Stoffwechselveränderungen während der Trächtigkeit in Mäusen konnte den aktuellen Wissensstand der Literatur bestätigen. So wurde eine gestörte Glukosetoleranz, erhöhte Insulin Ausschüttung und vermehrte Zellproliferation von Beta Zellen festgestellt. Auch die Gesamtmasse der endokrinen Zellen war zum Ende der Trächtigkeit signifikant erhöht. Eine Erniedrigung innerhalb der ersten vier Wochen nach Trächtigkeit konnte im Gegensatz zur Literatur nicht festgestellt werden. Obwohl die erhöhte Zellteilung während der Trächtigkeit zum Großteil für den Massenzuwachs verantwortlich ist, kann der Einfluss anderer Mechanismen, wie Neogenese oder Dedifferenzierung existierender Inselzellen nicht ausgeschlossen werden. So wurde eine Teilpopulation innerhalb den Langerhans´schen Inseln erkannt, die während des ersten Trimesters der Trächtigkeit den Transkriptionsfaktor *Neurogenin 3* (*Ngn3*) exprimiert. Die Mehrheit dieser Zellen wurde als Somatostatin sezernierenden Deltazellen identifiziert. Die höchste *Ngn3* Aktivität ging mit einer Erhöhung der Hormon negativen Zellen einher und legt die Vermutung eines Vorläuferzellstatus nahe. Rückverfolgung der Zellidentität mittels transgener Mäuse ergab eine prozentuale Erniedrigung der Deltazellen im ersten Trimester, gefolgt von der Differenzierung entgegen einer Betazellidentität zum Ende der Trächtigkeit. Weiterhin konnte gezeigt werden, dass der Anstieg an *Ngn3* Aktivität mit einem erhöhten Steroid und Insulin Hormonspiegel korreliert. Kulturexperimente mit isolierten Inseln konnten Progesteron als möglichen Auslöser für den partiellen Verlust an Deltazellidentität und gesteigerter Insulin Sekretion identifizieren.

Schlussfolgerung

Die Ergebnisse der vorliegenden Arbeit weisen möglicherweise auf eine neue funktionelle Rolle von Deltazellen während der Trächtigkeit hin. Im ersten Trimester führt eine Erniedrigung der Somatostatin produzierenden Zellmasse durch Aktivierung von *Ngn3* und zur Umwandlung in einen Hormon negativen Zellstatus und später zur Differenzierung von Beta Zellen. Als möglicher Auslöser für diesen Mechanismus wurde eine erhöhte Progesteron Konzentration im Blut identifiziert. Obwohl die funktionelle Relevanz dieses Prozesses nicht vollständig aufgeklärt wurde, wird die gesteigerte Betazellfunktion durch eine verringerte Somatostatin vermittelte Hemmung vermutet.

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8 List of figures

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10 Acknowledgements

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Anlage 1

Technische Universität Dresden
Medizinische Fakultät Carl Gustav Carus
Promotionsordnung vom 24. Juli 2011

Erklärungen zur Eröffnung des Promotionsverfahrens

1. Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

2. Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten: **entfällt**

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8. Ich habe die Zitierrichtlinien für Dissertationen an der medizinischen Fakultät der Technischen Universität Dresden zur Kenntnis genommen und befolgt.

Dresden, den _____

Julia Katharina Panzer

Anlage 2

Hiermit bestätige ich die Einhaltung der folgenden aktuellen gesetzlichen Vorgaben im Rahmen meiner Dissertation:

- o das zustimmende Votum der Ethikkommission bei Klinischen Studien, epidemiologischen Untersuchungen mit Personenbezug oder Sachverhalten, die das Medizinproduktegesetz betreffen

  Aktenzeichen der zuständigen Ethikkommission: **entfällt**

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  24-9168.24-1/2012-21
  24-9168.11-1/2014-9
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- o die Einhaltung von Datenschutzbestimmungen der Medizinischen Fakultät und des Universitätsklinikums Carl Gustav Carus.

Dresden, den

Julia Katharina Panzer