

Imaging the Pancreas

New Aspects on Lobular Development and Adult Constitution

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Front cover: OPT rendered image of a mouse embryonic day 15.5 gut segment epithelium including the stomach, the duodenum and the pancreas. The pancreatic lobes have been pseudocolored red, green and yellow, respectively.

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1. Abstract

The mouse pancreas is a mixed exocrine and endocrine gland consisting of three lobular compartments: the splenic, duodenal and gastric lobes. During embryogenesis, the pancreas forms from two progenitor populations located on the dorsal and ventral side of the primitive gut tube. These anlagen are brought in close proximity as the gut elongates and rotates, and fuse to form a single organ. The splenic and duodenal lobes develop from the dorsal and ventral anlagen, respectively.

In the adult pancreas, exocrine tissue secretes digestive enzymes into the gut lumen to support nutrient uptake. The endocrine *Islets of Langerhans* are scattered throughout the exocrine tissue and aid in regulation of energy homeostasis through the secretion of hormones. One of the key players in energy homeostasis is the pancreatic β -cell, which is the most abundant cell type of the islets. The β -cells regulate blood glucose levels through the action of insulin. Conditions where this regulation does not function properly are gathered under the common name of *Diabetes mellitus*.

Type 1 diabetes (T1D) is characterized by insulin deficiency due to autoimmune destruction of the β -cells. Using recently developed protocols for optical projection tomography (OPT) whole-organ imaging, we have revealed new spatial and quantitative aspects on β -cell mass dynamics and immune infiltration during the course of T1D development in the non-obese diabetic (NOD) mouse model. We show that although immune infiltration appears to occur asynchronously throughout the organ, smaller islets, mainly located in the periphery of the organ, preferentially lose their β -cells during early stages of disease progression. Larger islets appear more resistant to the autoimmune attack and our data indicate the existence of a compensatory proliferative capacity within these islets. We also report the appearance of structures resembling tertiary lymphoid organs (TLOs) in association with the remaining islets during later phases of T1D progression.

OPT has already proven to be a useful tool for assessments of β -cell mass in the adult mouse pancreas. However, as with other techniques, previous protocols have relied on a tedious degree of manual post-

acquisition editing. To further refine OPT-based assessment of pancreatic β -cell mass distribution in the murine pancreas, we implemented a computational statistical approach, Contrast-Limited Adaptive Histogram Normalisation (CLAHE), to the OPT projection data of pancreata from C57Bl/6 mice. This methodology provided increased islet detection sensitivity, improved islet morphology and diminished subjectivity in thresholding for reconstruction and quantification. Using this approach, we could report a substantially higher number of islets than previously described for this strain and provide evidence of significant differences in islet mass distribution between the pancreatic lobes. The gastric lobe stood out in particular and contained a 75% higher islet density as compared to the splenic lobe.

Although the development of the early pancreatic buds has been relatively well studied, later morphogenetic events are less clear and information regarding the formation of the gastric lobe has largely been missing. Using OPT we have generated a quantitative three-dimensional road map of pancreatic morphogenesis in the mouse. We show that the gastric lobe forms as a perpendicular outgrowth from the stem of the dorsal pancreas at around embryonic day (e) 13.5, which grows into a mesenchymal domain overlaying the pyloric sphincter and proximal part of the glandular stomach. By analyzing mutant mice with aberrant spleen development, we further demonstrate that proper formation of the gastric lobe is dependent on the initial formation of the closely positioned spleen, indicating a close interplay between pancreatic and splenic mesenchyme during development. Additionally, we show that the expression profile of markers for pancreatic multipotent progenitors within the pancreas is heterogenous with regards to lobular origin. Altogether, our studies regarding the morphogenesis and adult constitution of the mouse pancreas recognize lobular heterogeneities that add important information for future interpretations of this organ.

2. Papers included in this thesis

This thesis is based on the following papers that will be referred to in the text by their corresponding roman numerals (I-III).

- I. Alanentalo T, Hörnblad A, Mayans S, Karin Nilsson A, Sharpe J, Larefalk A, Ahlgren U, Holmberg D. Quantification and 3-D imaging of the insulinitis-induced destruction of β -cells in murine Type 1 Diabetes. *Diabetes*. 2010 Jul;59(7):1756-64.

- II. Hörnblad A*, Cheddad A*, Ahlgren U. An improved protocol for optical projection tomography imaging reveals lobular heterogeneities in pancreatic islet and β -cell mass distribution. *Islets*. 2011 Jul-Aug;3(4):204-8.

- III. Hörnblad A, Eriksson AU, Sock E, Hill RE, Ahlgren U. Impaired spleen formation perturbs morphogenesis of the gastric lobe of the pancreas. *PLoS One*. 2011;6(6):e21753.

*The two first authors contributed equally to this work.

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Papers not included in this thesis:

Growth-limiting role of endothelial cells in endoderm development. Sand FW, Hörnblad A, Johansson JK, Lorén C, Edsbacke J, Ståhlberg A, Magenheimer J, Ilovich O, Mishani E, Dor Y, Ahlgren U, Semb H. *Dev Biol*. 2011 Apr;352(2):267-77.

3. Abbreviations

AHE	Adaptive histogram equalization
BB	Bio breeding rat
BCM	β -cell mass
CLAHE	Contrast-limited adaptive histogram equalization
DL	Duodenal lobe of the pancreas
GADA	Glutamic acid decarboxylase antibodies
GL	Gastric lobe of the pancreas
GLUT2	Glucose transporter 2
IA-2A	IA-2 protein antibodies
IAA	Insulin autoantibodies
ICA	Islet cell antibodies
IR	Infrared
LADA	Latent autoimmune diabetes in adults
LSM	Laser scanning microscopy
MPC	Multipotent progenitor cells
MRI	Magnetic resonance imaging
NIR	Near infrared
NOD	Non obese diabetic mouse
NOD.H-2b	Mice congenic to NOD
OCT	Optical coherence tomography
OPT	Optical projection tomography
PET	Positron emission tomography
SL	Splenic lobe of the pancreas
SMP	Splanchnic mesodermal plate
SPECT	Single photon emission computed tomography
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TF	Transcription factor
TLO	Tertiary lymphoid organ
WHO	World health organization

4. Introduction

4.1 Function and structure of the mouse pancreas

The mouse pancreas is a mixed exocrine and endocrine gland consisting of three lobes, the splenic (SL), duodenal (DL) and the gastric lobes (GL) (Fig. 1a). The exocrine compartment constitutes more than 90% of the pancreatic tissue and produces digestive enzymes that are drained by the pancreatic ductal tree into the intestine where they aid in nutrient metabolism. The endocrine compartment is scattered throughout the larger exocrine parenchyma in micro-organs called the Islets of Langerhans. The major cell lineages of these islets include the glucagon-producing α -cells, insulin-producing β -cells, pancreatic polypeptide-producing γ -cells and somatostatin-producing δ -cells (Fig. 1b). The most abundant of

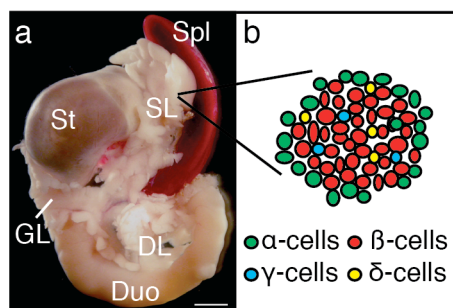


Figure 1. (a) Photomicrograph of a gut segment including the stomach, duodenum, spleen and pancreas from an adult wild-type mouse. (b) Schematic representation of an islet cross-section. Scalebar in (a) is 2mm. Abbreviations: St, stomach; Spl, spleen; SL, DL, GL, splenic, duodenal and gastric lobe, respectively.

these cells are the β -cells, which in the mouse are located in the core of the islets, and together with the α -cells are key players in the regulation of glucose homeostasis. β -cells secrete insulin into the blood stream as a response to the raised levels of blood glucose after a meal. Following insulin release, glucose and amino acid uptake and storage is stimulated in the main target tissues: the liver, muscles and adipose tissue. In contrast, glucagon is secreted by the α -cells upon low blood glucose and triggers the release of glucose from the liver into the blood as well as stimulation of fat catabolism. Somatostatin is an inhibitory hormone that in the pancreas suppresses the release of both insulin and glucagon and also modulates pancreatic exocrine secretion. Pancreatic polypeptide inhibits exocrine secretion, gall bladder motility, gastric emptying rate, and negatively regulates food intake. In addition to the above-mentioned hormones, various other peptides are produced in the endocrine pancreas, many of which are

thought to function in the body's regulation of energy homeostasis. Conditions where the intricate system for controlling blood glucose level does not function properly, either through improper insulin secretion or through failure to respond to insulin by the target tissues, is gathered under the common name of diabetes mellitus.

4.2. Diabetes mellitus

The first known record of diabetes dates back to a papyrus written by the Egyptian physician Hesy-Ra around 1500BC. The name *Diabetes* (meaning "siphon") dates back to ancient Greece and refers to the symptoms of frequent urination associated with diabetes. In the 17th century Thomas Willis added *mellitus* (meaning "honey-sweet") to the name due to the sweet taste of the urine of diabetic patients (Allan, 1953); this had been noted also in antiquity and tasting the urine was part of the regular examination. (For more detailed information of diabetes history: see (Eknoyan and Nagy, 2005; King and Rubin, 2003) and references therein)

Diabetes can roughly be divided into two different types; the less frequent but more severe type 1 diabetes (T1D), and the more frequent type 2 diabetes (T2D). According to the World Health Organization (WHO) 346 million people suffer from diabetes worldwide and the number is expected to increase substantially in the coming years. The Swedish Diabetes association estimates that the number of individuals suffering from diabetes in Sweden alone is about 350000, of which around 50000 suffer from T1D. Apart from the acute conditions (e. g. ketoacidosis and severe hypoglycaemia) resulting from a disturbed blood glucose regulation, diabetes is also accompanied by a number of secondary complications such as damage to nerves, the heart, blood vessels and the kidneys. The main cause for these complications is changes in the vascular tissues due to long term high concentration of glucose. Today diabetes and its associated complications constitute a mayor societal cost in many countries, and given the current increase in diabetes incidence, it is expected to increase dramatically in the future.

4.2.1. Type 1 Diabetes

T1D is a chronic immune-mediated disease characterized by the selective destruction of the insulin-producing β -cells. It is a multifactorial disease with both genetic and environmental

components. About 50% of the genetic component is thought to reside in the HLA locus encoding a large number of genes important for immune function (Daneman, 2006). The pathogenesis begins when environmental factors triggers the immune system in individuals with a genetic susceptibility for the disease. This causes abnormal lymphocyte infiltration or insulinitis of the pancreatic islets, and the immune cells subsequently kill the islets β -cells. Activated T-cells are thought to play the major role in the autoimmune destruction of β -cells, although the first detectable sign of autoimmunity is the appearance of β -cell specific auto-reactive antibodies produced by B-cells (Bluestone et al., 2010; Knip and Siljander, 2008). These antibodies, including islet cell antibodies (ICA), insulin autoantibodies (IAA), antibodies to glutamic acid decarboxylase (GADA) and antibodies to tyrosine phosphatase-related IA-2 protein (IA-2A), can to some extent be used to predict T1D susceptibility before clinical onset of the disease (Bingley et al., 1994; Knip and Siljander, 2008). The destruction of β -cells diminishes the secretory capacity for insulin and leads to clinical diabetes when the remaining β -cells cannot produce sufficient amounts of insulin to regulate blood glucose levels. Most T1D patients finally develop absolute insulin deficiency (Daneman, 2006). Although T1D in present times is not an inevitable death sentence as it was before the discovery of insulin by Banting and Mcleod in the 1920's, it still requires daily insulin treatment and in the long term inflicts substantial morbidity among diabetic patients.

4.2.2 Type 2 Diabetes

T2D is the current name of the different conditions previously gathered under the common name adult-onset diabetes. It results from a combination of insulin resistance, which is an inability of the target tissues to respond properly to insulin secretion, and improper insulin secretion. Several loci have been identified linking T2D to genetic susceptibility (Prokopenko et al., 2008; Scott et al., 2007; Sladek et al., 2007; Tsai et al., 2010) but it is also strongly associated with environmental factors such as a sedentary lifestyle, obesity and aging. The first phase in developing T2D is when peripheral target tissues fail to respond properly to insulin. This results in an increased insulin production by the β -cells and it has also been shown in rodents that β -cell mass is increased in obese individuals (Bonner-Weir, 2000). Onset of clinical T2D occurs in the second phase when the β -cells fail to maintain normoglycaemia due to impaired insulin secretion.

Morphometric analyses of diseased T2D patients have also shown that late stages of T2D are accompanied by a decrease up to 60% in β -cell mass (Butler et al., 2003).

As previously noted, the division of diabetes mellitus into two forms is coarse and other types of diabetes exist. One of them is latent autoimmune diabetes in adults (LADA), which has been considered a possible overlap between T1D and T2D (Boitard et al., 2005). It has also been shown that T1D and T2D frequently co-occur in the same families, suggesting common genetic susceptibility loci (Boitard et al., 2005; Tuomi, 2005). Moreover, in both forms the immune system is triggered; in T1D this is manifested as an autoimmune attack on the β -cells while T2D is accompanied by an auto inflammatory response (Donath and Shoelson, 2011). Most importantly, it is clear that β -cell function and changes in β -cell mass are key components in the aetiology of virtually any form of diabetes.

4.2.3 Mouse models of T1D – the NOD mouse

The availability of sophisticated genetic tools and the high degree of genetic homology between mice and humans makes the mouse one of the most widely used models in experimental diabetes research. There are several ways of experimentally mimicking T1D. Perhaps the earliest model to achieve a diabetes-like state in experimental animals was the partial or complete surgical removal of the pancreas. Since the β -cells are removed together with the rest of the pancreatic tissue, the animals are quickly rendered hyperglycaemic. It is also possible to induce hyperglycaemia through the administration of chemical compounds. Two of the most commonly used are streptozotocin and alloxan. Although acting through different pathways, both are glucose analogues that bind to the glucose transporter 2 (GLUT2) receptor and cause the selective destruction of β -cells (Lenzen, 2008). Both surgically and chemically induced models of diabetes have been widely used in islet transplantation experiments to facilitate the study of e.g. the outcome of anti-rejection therapies. They also constitute valuable tools for studying the direct and indirect physiological consequences of a hyperglycaemic state. However, these experimental mouse models of diabetes are not very well suited for the study of disease onset and aetiology, as the mechanism of β -cell destruction differs substantially from T1D in humans. In this respect, there are a number of animal models developing spontaneous diabetes that are

more similar to the human form. Among the most widely used are the Non Obese Diabetic (NOD) mouse and the Bio Breeding (BB) rat. The NOD mouse was isolated by Makino and colleagues around 30 years ago (Makino et al., 1980). The strain develops spontaneous diabetes with an incidence of 60-80% among females and 20-30% in males. The NOD pancreas starts to be infiltrated by immune cells at 3-4 weeks of age and by 10 weeks most mice display severe insulinitis. As in T1D patients, auto-reactive antibodies can be detected prior to clinical onset of diabetes, which occurs between 12 to 14 weeks of age in females and slightly later in males. It should be noted that both male and female mice develop early insulinitis although the process in males is slower (Young et al., 2009) and the subsequent number of diabetic males is lower. Sex hormones have been shown to play an important role for this difference and castrated NOD males display an increased incidence of T1D (Fitzpatrick et al., 1991) and administration of testosterone to NOD females delays T1D onset (Fox, 1992; Toyoda et al., 1996). Apart from the similarities in disease aetiology, it has also been demonstrated that NOD mice and human T1D share some susceptibility loci and molecular pathways that contribute to the disease (Wicker et al., 2005).

4.3 Pancreas development

4.3.1 Formation and specification of the gut tube

A highly complex process of morphogenetic movements drives the formation of the primitive gut tube from a flat, two-dimensional sheet of cells that constitute the endoderm in the embryonic day (e) 7.5 mouse embryo (Fig. 2). The sheet of endoderm that is located on the outside of the cup-shaped embryo starts to fold from the anterior and slightly later from the posterior. Between e8.5 and 9.5 the embryo turns from its inside out position (Fig. 2). This coincides with the lateral endoderm folding ventrally thereby closing the opening in the medial part of the gut tube. During these events, the primitive gut acquires regional identity across the anterior-posterior, dorsal-ventral and subsequently, radial axes. The regional identity is further refined to generate the primordium of the endodermally derived organs, including the pancreas. This is achieved through reciprocal signalling between the endoderm and the surrounding mesoderm and is manifested by differential expression of transcription factors (TF) in

the organ primordia (Spence et al., 2011; Wells and Melton, 1999; Zorn and Wells, 2009) For example, the region of lung formation is marked by the expression of *Nkx2.1*, *Sox2* marks the presumptive stomach, and the pancreatic anlagen is together with the duodenum, posterior stomach and bile duct marked by the expression of *Pdx1*.

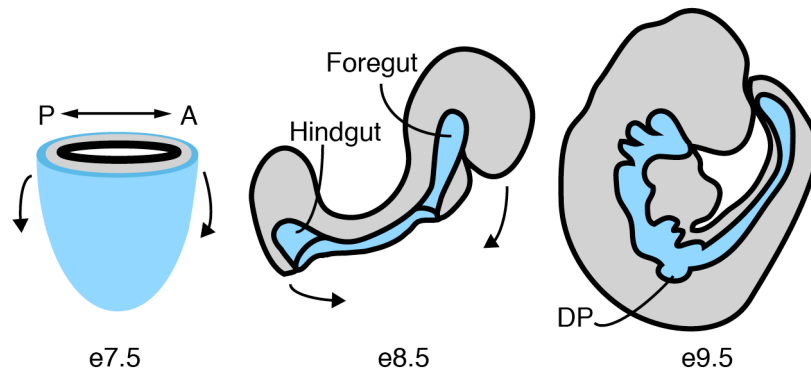


Figure 2. Schematic representation of primitive gut tube formation. The endoderm starts to fold from the anterior part of the embryo and slightly later from the posterior. As the embryo turns and the lateral endoderm folds ventrally, the complete primitive gut tube is formed. Abbreviations: A, anterior; P, posterior; DP, dorsal pancreatic bud.

4.3.2 Pancreas morphogenesis and differentiation

The first visible signs of pancreatic development occur around e9 when the dorsal endoderm starts to thicken and evaginate from a domain caudal to the presumptive stomach (Fig. 2 and Fig. 3)(for recent reviews on pancreas development, see (Gittes, 2009; Pan and Wright, 2011; Puri and Hebrok, 2010)). A ventral evagination develops slightly later on the opposite side of the primitive gut tube. As the pancreatic buds proliferate and expand into the surrounding mesenchyme, scattered microlumens appear stochastically throughout the pancreatic epithelium and by e12.5 they have coalesced into a complex continuous luminal network (Kesavan et al., 2009). As a consequence of the elongation and rotation of the gut, the two pancreatic buds fuse to form a single organ at e13.5 (Fig. 3). During subsequent development the luminal network matures into a proper tubular network (Kesavan et al., 2009) and there is a massive wave of differentiation termed the *secondary transition*. It has been suggested

that until this time point multipotent pancreatic progenitors (MPCs) located in the tips of the branching epithelium give rise to all three main pancreatic lineages; the exocrine, endocrine and ductal cells. However, starting from e14.5 the tip cells, which are delineated as $Pdx1^+/Cpa1^+/Amy^-$, start to express amylase and only give rise to exocrine progeny while endocrine cells differentiate from progenitor cells in the trunk region of the developing pancreas (Zhou et al., 2007). Another lineage tracing study showed that $Hnf1\beta^+$ cells give rise to all three cell lineages until this time point. During secondary transition the multipotent progenitor potential of these cells are restricted to endocrine and ductal progeny and subsequently they differentiate into ductal cells (Solar et al., 2009). The timing of exocrine differentiation is consistent between the two reports but it is unclear as to what extent these two cell populations overlap and if they are true markers for multipotency. A contradictory report suggests that all three main pancreatic lineages can be generated throughout gestation from multipotent progenitors expressing the TF

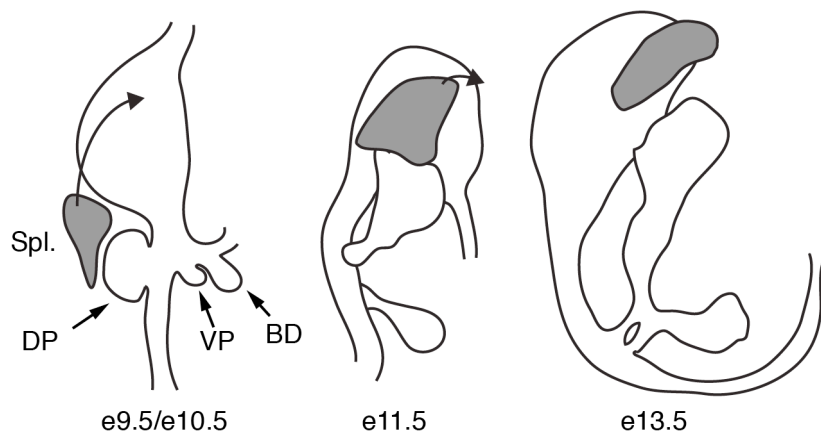


Figure 3. Schematic representation of spleno-pancreatic morphogenesis. The pancreas forms as a dorsal and ventral evagination from the primitive gut tube. These subsequently grow, branch and differentiate into the surrounding mesenchyme. As a consequence of the elongation and rotation of the gut, the two anlagen are brought into close proximity and fuse around e13.5. The spleen forms from a mesenchymal compartment in close proximity to the dorsal pancreatic bud at around e10.5. The presumptive spleen cells subsequently condense and migrate along the left side of the greater curvature of the stomach to finally adopt its adult shape and location. Abbreviation: Spl., spleen; DP, dorsal pancreas; VP, ventral pancreas; BD, bile duct.

Sox9 (Kopp et al., 2011a). An explanation for the discrepancy between these reports could be that the $Pdx^+/Cpa1^+/Amy^-$ and $Hnf1\beta^+$ cells are cells on the brink of differentiation that have been specified but not yet committed, whereas a subset of the $Sox9^+$ cells represents true MPCs. The pancreatic epithelium expands after secondary transition as exocrine cells continue to proliferate and new pancreatic branches are formed. The endocrine cells continue to differentiate and delaminate from the trunk region throughout gestation (Solar et al., 2009) but it is not until end-gestation and during the first weeks of postnatal life that they coalesce into proper islets. On the macroscopic level, the dorsal pancreatic primordium gives rise to the SL of the pancreas while the ventral primordium gives rise to the DL. The GL is conserved in a number of rodent species including hamster, rat and mouse, and have been suggested to correspond to the auricle or “ear” of the human pancreas, which is a protrusion along the gastroepiploic vessel present in around 50% of adults (Nagai, 2003). In mice, the GL have generally been considered a part of the dorsal pancreas, although it was only recently shown (Villasenor et al., 2010) and little is still known about the gross morphogenetic events and molecular mechanisms that lead to its formation.

4.3.3 Transcription factors and signalling pathways in pancreas development

Understanding of the molecular mechanisms governing pancreatic development and the formation of the pancreatic β -cell in particular is pivotal for the generation of stem cell based replacement therapies in diabetes. With this aim, numerous TFs and signalling pathways implicated in different steps of pancreatic development have been identified and the emerging picture is exceedingly complex. Several of the molecules involved have context-dependent multiple roles during development. One example is the TF *Pdx1* which is required for early pancreas formation (Jonsson et al., 1994), exocrine differentiation (Hale et al., 2005; Holland et al., 2002) and the maintenance of β -cell function (Ahlgren et al., 1998). In addition to the different TFs required for proper pancreas development, several other important genes and molecules have been identified such as components of the retinoic acid (Martin et al., 2005; Molotkov et al., 2005; Ostrom et al., 2008), FGF (Bhushan et al., 2001; Hart et al., 2003; Norgaard et al., 2003), Notch (Apelqvist et al., 1999; Hald et al., 2003; Murtaugh et al., 2003), and Wnt (Heiser et al., 2006; Murtaugh et al., 2005)

signalling pathways. As previously noted, the pancreas arises from two progenitor domains that display partially different gene expression profiles during early development and a number of knockout mice have been described that display distinctive phenotypes in the dorsal and the ventral buds. Among these are the null mice for the transcription factors *Isl1* (Ahlgren et al., 1997) and *Mnx1* (Harrison et al., 1999), the RA processing enzyme *RALDH2* (Martin et al., 2005), and the extracellular matrix molecule *Ncad* (Esni et al., 2001). All of these mutants exhibit an early truncation of the dorsal pancreas while the ventral bud forms normally. There are also a number of mutant mice displaying a more severe ventral phenotype, among them are null mice for the TFs *Gata4* and *Gata6* (Watt et al., 2007). Although many of these molecules have later functions during pancreas development, the discrepancies in early development between the dorsal and ventral buds indicate that multiple pathways might lead to the generation of the mature pancreatic cell types. These differences in gene expression/function could provide opportunities to refine our knowledge regarding the molecular processes required for the generation of the pancreatic cell lineages.

4.3.4 Tissue interactions during pancreas development

Proper pancreas patterning, induction and morphogenesis are dependent on tissue interactions between the endodermally derived pancreatic anlagen and the surrounding mesodermally derived mesenchyme. During very early stages of gut development, the pre-pancreatic endoderm is patterned by the adjacent germ layers (Wells and Melton, 2000). Subsequently, the notochord is thought to provide permissive signals for induction of the dorsal pancreatic program (Fig. 4) (Hebrok et al., 1998; Kim et al., 1997). As the dorsal pancreatic bud and notochord are intercalated by endothelial cells of the dorsal aorta (Fig. 4), signalling from these cells maintain the expression of *Pdx1* and induce *Ptf1a*, an important regulator of pancreatic differentiation (Yoshitomi and Zaret, 2004). Endothelial cells also promote the survival of a group of mesenchymal cells required for maintenance of *Ptf1a* expression (Jacquemin et al., 2006) and later exocrine differentiation (Ahlgren et al., 1997). Additionally, it has been suggested that endothelial cells induce endocrine differentiation (Lammert et al., 2001; Yoshitomi and Zaret, 2004) although in contrast to these findings recent reports conclude that endothelial cells restrain pancreatic differentiation, branching and growth (Magenheim

et al., 2011; Sand et al., 2011). The reason for these discrepancies still remains to be elucidated. It is possible that differences in the timing of expression onset and/or expression levels of transgenic constructs, and/or dual functions of blood vessels mediated by different molecular pathways, could explain these inconsistencies. The ventral pancreatic bud develops from cells in close proximity to the vitelline veins. However, as demonstrated by analyses of *Flik1*^{-/-} embryos, these vessels are dispensable for the induction of initial pancreatic gene expression in the ventral bud (Yoshitomi and Zaret, 2004). The ventral pancreatic precursors are part of a bipotential precursor domain that gives rise to both the liver and the ventral pancreas (Deutsch et al., 2001). In order to adopt the pancreatic program, it is essential that the ventral pancreatic progenitors escape FGF and BMP signalling from the cardiac mesoderm and septum transversum mesenchyme respectively (Deutsch et al., 2001; Rossi et al., 2001). By using a combination of transgenic tools and fetal surgical techniques, it has recently been shown that the pancreatic mesenchyme provide

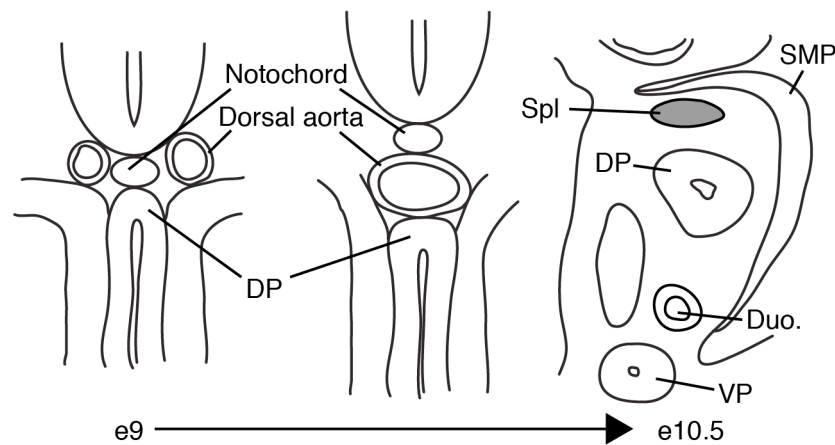


Figure 4. Tissue interactions during development of the dorsal pancreas. During early development, the notochord is located adjacent to the DP bud and is thought to provide permissive signals for induction of the pancreatic program. Later, the DP and notochord become intercalated by the dorsal aorta which provides additional cues for the maintenance of *Pdx1* expression and the survival of the DP mesenchyme. The leftward growth of the spleno-pancreatic region is driven by mesenchymal proliferation and is thought to be induced by signals from the SMP. The SMP has also been suggested as an inducer of spleen development. Abbreviations: DP, dorsal pancreatic bud; VP, ventral pancreatic bud; Spl., spleen; SMP, splanchnic mesodermal plate; Duo, duodenum.

crucial signals throughout development (Landsman et al., 2011) not only during early phases of pancreas morphogenesis. Altogether, this underscores the importance of dissecting the interplay between the pancreatic epithelium and adjacent tissues to understand the events leading to proper pancreatic morphogenesis and differentiation.

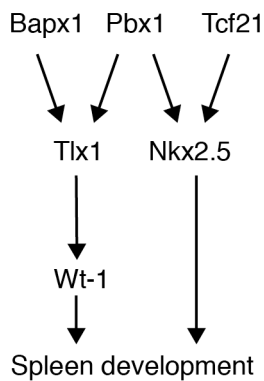
4.4. Spleen development and function

Located in the left side of the body cavity, between the diaphragm and the fundus of the stomach, the spleen is an organ that plays important roles in haematopoiesis and immunity. It serves as a filter of the blood and removes old/damaged red blood cells and blood-borne microorganisms (Brendolan et al., 2007; Mebius and Kraal, 2005). The spleen is positioned adjacent to the main lobe of the dorsal pancreas that consequently is referred to as the SL. During development, the spleen forms from an inducible cell population distinct from the stomach mesenchyme in close proximity to the dorsal pancreas. The first sign of spleen organogenesis is a mesenchymal condensation caudal to the presumptive stomach adjacent to the dorsal pancreatic epithelium at around e10.5 (Fig 3). This process is thought to be initiated in response to signals establishing left-right asymmetry in the embryo and spleen abnormalities are often considered indicative of heterotaxia syndromes (Burn and Hill, 2009). The splanchnic mesodermal plate (SMP), a transient organizer overlying the presumptive splenic mesenchyme, drives the left lateral growth of the spleno-pancreatic region and has been suggested to induce spleen development (Hecksher-Sorensen et al., 2004). In mice with a defective SMP, including the *Nkx3.2*^{-/-} and *Dh*^{+/-} mutants, normal spleen development is severely disturbed. As spleen morphogenesis proceeds, the splenic precursors dislocate from the pancreatic anlagen and migrate anteriorly along the left side of the greater curvature of the stomach to finally adopt the shape and position of the adult organ at around e14.5. Dislocation of the spleen from the dorsal pancreas requires the TF *Nkx3.2* (Asayesh et al., 2006) and the migratory process is guided by yet unidentified signals from the anterior part of the stomach (Burn et al., 2008). There is however few comprehensive studies that have attempted to analyse the morphogenesis of the entire spleno-pancreatic region during development in high resolution.

4.4.1 Genetic regulation of spleen development

Through analysis of mutant mice, a number of genes required for spleen development have been identified; some of them interact in a transcriptional hierarchy consisting of two parallel pathways (Fig. 5). One pathway includes *Nkx3.2* that regulates the expression of *Tlx1* (*Hox11*), which in turn acts upstream of *WT1*. The other pathway is constituted by *Tcf21* (*Pod1/Capsulin*) upstream of *Nkx2.5*. *Pbx1* is involved in both pathways and acts in concert with both *Bapx1* and *Tcf21* and regulates the expression of *Tlx1* and *Nkx2.5* (Brendolan et al., 2005; Brendolan et al., 2007). Additional mutants with abnormal spleen development are *Barx1* (Kim et al., 2007), *Nkx2.3* (Pabst et al., 1999), *Sox11* (Sock et al., 2004), *WTX* (Moisan et al., 2011), and the spontaneously derived *Dh* mutant (Green, 1967). Of these mutants, the most severe asplenic phenotype is displayed by the *Dh*^{+/-} mice, which completely fail to form a recognizable spleen primordium.

Figure 5. Schematic illustration of the transcriptional hierarchy controlling spleen development. *Bapx1* and *Tcf21* are key regulators in separate pathways during spleen development. *Pbx1* is a central co-regulator that regulates components in both pathways.



Adopted from Brendolan et al., 2005

Although asplenia or hypoplastic spleen has been reported for *Sox11* and *WTX* null mice, the nature of the splenic developmental defects in these mice has not yet been thoroughly assessed (Moisan et al., 2011; Sock et al., 2004).

4.4.2 Spleno-pancreatic interactions during development

The spleen and pancreas develops in very close proximity and analyses of *Nkx3.2*^{-/-}, *Dh*^{+/-} and *Ptf1a*^{-/-} mice have indicated that there is a close interplay between the spleen and the pancreas during development. In *Nkx3.2*^{-/-} and *Dh*^{+/-} mice the leftward growth of the entire spleno-pancreatic region is perturbed due to defects in the SMP (Hecksher-Sorensen, Watson et al. 2004). Furthermore, in *Nkx3.2* null mice the splenic mesenchyme fail to condense and dislocate from the pancreatic epithelium. As a consequence, continued signalling between the two tissues leads to the induction of ectopic cysts in the pancreatic epithelium. These cysts express markers of gut specific differentiation (Asayesh et al., 2006). In *Ptf1a*^{-/-} mice the exocrine

pancreas fail to develop and pancreatic endocrine cells are found in the spleen (Krapp et al., 1998).

4.5. Pancreas imaging

4.5.1 Challenges for pancreas imaging

An essential part of biological research is the visualisation of biological components and processes. Technical improvements aiding in visualisation are often accompanied by new discoveries. This holds true also for many aspects of pancreatic research. Currently, the rapid development of a range of biomedical imaging techniques can be expected to aid both in clinical settings as well as in preclinical studies of pancreas physiology, pancreas development and models of diabetes. Given the pivotal role of β -cell function and BCM in the different forms of diabetes, the most important aim for the development of imaging modalities in diabetes research is the possibility to accurately measure BCM and islet distribution non-invasively in humans. A functional method for scoring BCM in man would have important implications for the early diagnosis of T1D and also open up for the possibility to perform longitudinal studies of therapeutical regimes and the evaluation of transplantation protocols. To reach this aim a number of difficulties must be overcome: 1) the pancreas is an irregularly shaped organ located deep within the body in close proximity to several other major organs and blood vessels. This makes it difficult to access from the outside. 2) The islets constitute only a small fraction of the total pancreatic mass, they vary in size and are scattered in very high numbers throughout the much larger exocrine tissue. Thus, a non-invasive technique for spatial and quantitative assessments of human BCM must 1) include a non-toxic contrast agent that allows for detection through several cm of tissue and 2) have a very high resolution in order to visualize individual islets. Although immense efforts are aimed at scoring BCM in humans, many aspects of pancreas physiology can at present only be studied in animal models and therefore it is also of great interest to improve imaging techniques for this purpose. Examples hereof are gene ablation studies expected to affect pancreas development or physiology, lineage tracing, *ex vivo* studies of disease dynamics, novel protocols for islet transplantation and/or diabetes therapeutics.

4.5.2 Techniques for imaging and quantification of the endocrine pancreas

In recent years, a wide range of imaging techniques has been developed to meet the growing demands of biomedical research and to monitor molecular, cellular, and tissue interactions/dynamics in an unperturbed environment. With the advent of these techniques, new promising approaches have been applied to different aspects of pancreas biology and in particular to imaging of the endocrine pancreas. In the following paragraphs, a selection of technologies that have shown potential for islet imaging is presented. They have been divided into stereological, nuclear and optical imaging techniques based on their principle of detection.

4.5.2.1 Stereological techniques

Traditionally, *stereological* sampling techniques have been used to estimate BCM and islet number. Sections are sampled at different intervals throughout the tissue, followed by measurements of islet area and statistical analyses (Bock et al., 1999; Finegood et al., 1995; Hellerstrom and Hellman, 1963; Hellman et al., 1961). Comparisons are then made as means of islet area or other variables. This is a laborious and time-consuming method that can provide adequate numbers of relative islet area and number but cannot give extensive information of the overall spatial distribution of islets/BCM. Furthermore, they are based on the assumption that the BCM is homogeneously distributed within different regions of the pancreas. Mathematical models have been developed to estimate the pancreatic islet volume based on 2D sampling but they rely on additional assumptions regarding islet shape and have, even in an animal as small as the mouse, due to the anatomical properties of the pancreas been described as a “true stereological challenge” (Skau et al., 2001). A possible mean to obtain whole organ spatial and quantitative information of BCM and islet distribution is the digital reconstruction of sectioned specimen. For a tissue like the adult mouse pancreas, this would be an immense task to perform manually since it requires the sectioning of the entire specimen, staining of all sections, imaging of each section independently and finally reconstruction of the sections through computer processing. Perhaps not feasible to do manually, there are today automatic sectioning machines and imaging devices that could provide resources for this purpose. There are however no reports yet applying this type of technique on assessments of BCM

and islet number. The output data can be expected to have very high resolution and the approach therefore put high demands on computational resources both during reconstruction and subsequent image analyses. Important issues are also the risk of introducing artefacts both during the sectioning process and when aligning consecutive sections in the reconstruction.

4.5.2.2 Nuclear imaging techniques (MRI, PET and SPECT)

Magnetic Resonance Imaging (MRI) is used in a number of clinical settings to visualize anatomical structures. It is a non-invasive technique that takes advantage of the magnetic properties of hydrogen atoms (or other nuclei). As the specimen is placed in a strong magnetic field, the hydrogen atoms of the specimen align either parallel or anti-parallel to the magnetic field. When a second, oscillating, electromagnetic field is turned on, it imposes the hydrogen atoms to change position. When they fall back to the original state, radio waves are emitted that can be detected by the scanner. MRI does not suffer from limitations in imaging depth and it has a fairly high spatial resolution. In order to use MRI for BCM scoring, the main issue is the need for highly specific contrast agents that provide sufficient signal to compensate for the relatively low sensitivity of the technique. Promising attempts have been made in this direction in settings of islet transplantation (see (Ahlgren and Gotthardt, 2010) and references therein) although in order to monitor native β -cells *in vivo* there is an additional need for high binding specificity, which is not an absolute requirement when labelling islets *ex vivo* prior to transplantation. Nevertheless, MRI is perhaps the most promising technique for scoring of BCM in clinical settings and in longitudinal studies of experimental animal models provided that suitable β -cell-specific contrast agents can be developed.

Positron Emission Tomography (PET) and *Single Photon Emission Computed Tomography* (SPECT) are both techniques that rely on the detection of radioactive tracer molecules. The high detection sensitivity and poor spatial resolution of these techniques suggests that they will primarily become useful for quantitative BCM assessments. In comparison, SPECT is a cheaper and more widely available technique than PET. The radionuclides used in SPECT also often have longer half-lives, which enables easier labelling and use of tracer molecules. On the other hand, the shorter half-life of PET

radionuclides allows for increased detection sensitivity over a given period of time since they can be injected in higher activities without causing additional radiation damage to the subject (Rahmim and Zaidi, 2008). The major advantage of PET over SPECT is the extremely high detection sensitivity, which is around 2-3 times higher than in SPECT (Rahmim and Zaidi, 2008). In PET, radioactive labelling of the vesicular monoamine transporter 2 molecule have been used as a specific marker for β -cells in quantitative studies of BCM in both mice and men (Harris et al., 2008). A derivative of the GLP-1 analogue Exendin have shown promising results for BCM scoring using SPECT in rodents and is currently optimised for imaging in humans (Ahlgren and Gotthardt, 2010; Gotthardt et al., 2006). The overall availability of contrast agents for both techniques is however limited.

4.5.2.3 Optical imaging techniques (Confocal and epifluorescence microscopy, OCT and OPT)

Among the most widely used imaging techniques in experimental pancreatic research are the different forms of *laser scanning microscopy* (LSM). These include *confocal* LSM, *two-photon* LSM and *multi-photon* LSM. The basic concept of these techniques is that a focused laser beam scans the specimen at a focal plane within the tissue and a pinhole is used to reject light from out-of-focus areas. During the scan, information is only obtained from one laser focal spot at a time and to form a 2D image of the “tissue section”, the information from all focal spots needs to be assembled. By focusing the laser beam at different depths of the specimen, an image stack representing the full 3D volume can be created. LSM techniques have an excellent spatial resolution and there is an abundant pool of available contrast agents. As such, they are powerful techniques for imaging of small-scale specimen although light scattering and limitations in light penetration makes it difficult to image specimen larger than a few hundred micrometers. Because of the inaccessible location of the pancreas deep within the body, LSM have primarily been used as an *ex vivo* technique for studying different aspects of pancreas biology. However, in experimental setups, exteriorization of pancreatic tissue in anaesthetized mice has demonstrated the possibility to also perform *in vivo* imaging studies (Martinic and von Herrath, 2008; Nyman et al., 2008). Another approach using LSM

takes advantage of the transparency of the eye to study the physiology of transplanted islets *in vivo* in mice (Speier et al., 2008).

Another *ex vivo* setup used to estimate the islet distribution of the pancreas takes advantage of a normal *epifluorescent microscope* and detects emitted light from fluorescent proteins or fluorescently labelled probes (e.g. antibodies) (Kilimnik et al., 2009). As opposed to confocal microscopes, an *epifluorescent microscope* does not have a pinhole that excludes light from regions outside the focal plane. The collected images thus represent light emitted from the entire depth of the specimen. By using a motorized stage, images from all regions of the pancreas can be collected and tiled into one image depicting the entire pancreatic area. The β -cell area is then segmented from the image based on the islets fluorescence intensity. Parameters such as islet number, islet area, islet circularity and Feret's diameter can thus be calculated. However, the pancreas has to be flattened between a glass slide and a cover slip and subsequently cleared in glycerol to enable penetration of light through all regions of the gland. This procedure may adversely affect islet morphology and thereby the measured islet area. Moreover, there is an obvious risk that islets lying deeper within the tissue are masked by islets closer to the detector. Furthermore, the technique does not allow for mapping of BCM and islet distribution in three dimensions. In combination with mathematical modelling, this approach has been applied to assessments of BCM distribution in the developing and adult mouse pancreas (Jo et al., 2011; Kilimnik et al., 2009; Kim et al., 2009) and it has also been applied to sectioned samples from human T2D patients (Kilimnik, Zhao et al. 2011).

Optical Coherence Tomography (OCT) is a method that sends photons into a specimen and relies on the detection of the back-reflected light (Huang et al., 1991) to form an image. As different tissues have different back-scattering properties, OCT can image the intrinsic contrast of the tissue without the need for adding exogenous contrast-agents. In the context of pancreas imaging, islets show different back-scattering properties than the surrounding exocrine parenchyma and can thus be monitored via OCT (Villiger et al., 2009). However, since OCT detects the intrinsic contrast of the tissue, the detection is not limited exclusively to islets and additional structures with similar back-scattering properties such as blood

vessels might interfere with the islet data. A drawback is also the inability of OCT to detect fluorescent signals, which limits the possibility to detect gene and protein expression patterns. Initial attempts to image pancreatic islets *in vivo* have been promising although, as with LSM, the limitation in imaging depth requires externalization of the organ prior to scanning (Villiger et al., 2009) and excludes analyses of whole organs. The spatial resolution and imaging depth in this setup was 2-3 μm and 300 μm , respectively. This makes OCT a promising tool for longitudinal studies of at least regional islet morphology/distribution in experimental animals. It has also been suggested as a potential tool for cross-validation of probes developed for other imaging modalities such as MRI (Villiger et al., 2009).

Optical Projection Tomography (OPT) is a relatively new imaging technique that was initially developed as a tool for gene expression studies in embryonic specimens (Sharpe et al., 2002). It can

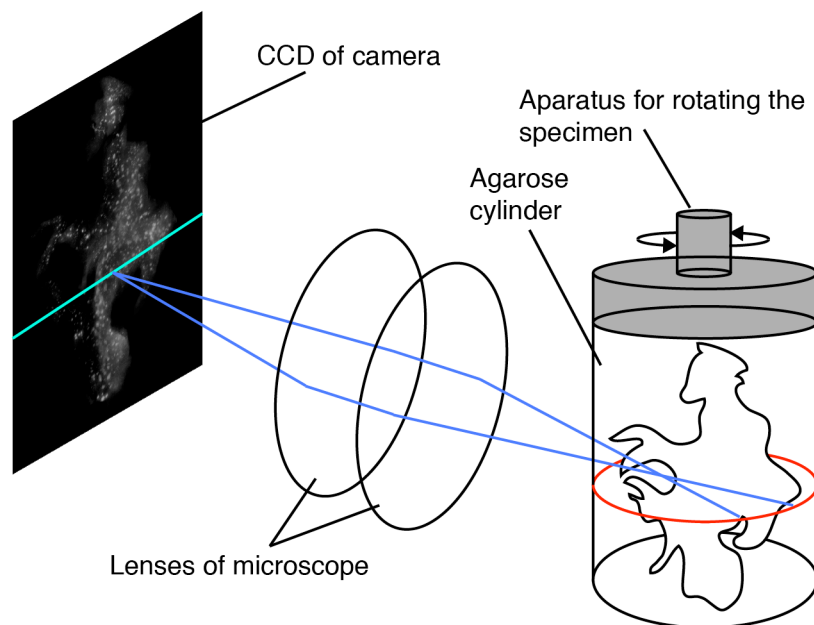


Figure 6. Schematic illustration of the OPT setup. A pancreas is mounted in a cylinder of agarose and rotated in the lightpath of a microscope. The light emitted from the specimen is focused onto the camera's CCD chip and images are collected throughout the full 360 degree rotation. (Image adapted from Sharpe et al., 2002)

essentially be described as the optical equivalent of X-ray CT. In a typical setup, a specimen is mounted in a cylinder of low melting point agarose gel and made semitransparent by immersion in a mixture of benzyl alcohol and benzyl benzoate. It is then placed in the light path of a microscope and rotated a full 360° while multiple images are collected on a CCD chip throughout the process (Fig. 6). By employing a back-projection algorithm on the projections, tomographic sections throughout the volume of the specimen are generated (Fig. 7). These can then be used to create interactive 3D models of for example gene or protein expression patterns without the need for dissecting away surrounding tissue. As the name implies, OPT is an optical imaging modality. However, as opposed to other optical techniques such as LSM and OCT it does not detect signal intensities at specific coordinates in 3D space. Instead the intensity value of each pixel in a projection image corresponds to the sum of intensities along a straight line through the specimen (Fig. 7).

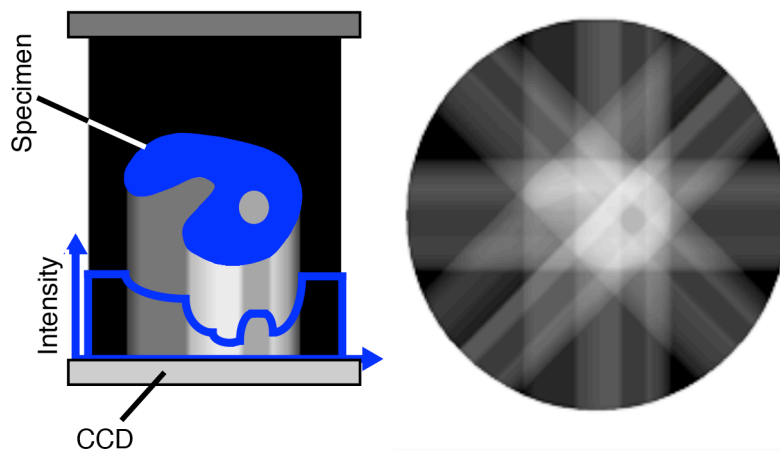


Figure 7. Illustration of the OPT principle. In OPT the recorded intensities of each pixel in a projection image represents the sum of intensities along a straight line through the specimen. Tomographic sections throughout the volume of the specimen are generated by combining the data from the projection images using a back-projection algorithm. Images kindly provided by J. Sharpe.

OPT has been successfully employed in the study of tissue interactions during pancreas development (Asayesh et al., 2006; Hecksher-Sorensen et al., 2004) and through recent improvements in

immunohistochemical and computational processing, it has also been adapted to imaging of the full BCM distribution in the intact adult mouse pancreas. The combination of the relatively high resolution (15-20 μm in an adult pancreas) and large imaging depth ($\sim 1\text{cm}$) makes it possible to visualise the BCM and islet distribution down to the level of individual islets (Alanentalo et al., 2007). As such, OPT provides an ideal platform for *ex vivo* imaging of BCM in the mouse. The abundant availability of highly specific contrast agents and the possibility to detect fluorescent markers also make it suitable for whole-organ analyses of more than one component of pancreas biology in the same specimen. Additionally, whole-organ OPT analyses can be combined with regional high-resolution LSM by collecting biopsies from region of interests after OPT-scanning (Alanentalo et al., 2008).

5.5.2.4 OPT – improvements and future potential in pancreas imaging

Since the relatively recent introduction of OPT, several optimizations and improvements of the technique have been described. Some of these include computational algorithms for improved resolution and reconstruction (Birk et al., 2011; Walls et al., 2007), determination of post-alignment values for reconstruction (Cheddad et al., 2011), noise filtering (Fumene Feruglio et al., 2010), and live imaging of mouse organ cultures (Boot et al., 2008). It is likely that this rapid development will continue in the coming years. One aspect of OPT that is under current development and will be of importance for pancreas studies is the potential for multichannel imaging. In commercial scanners, imaging is at present limited to three channels due to filter settings and camera sensitivity. One of these channels takes advantage of the endogenous tissue fluorescence and collects information of the overall anatomy of the specimen. Thus, it cannot be used for detection of specifically labelled structures. Furthermore, of the two remaining channels, only one (in the far red spectrum) provide sufficient signal-to-noise ratio to enable reliable quantitative measurements in large specimen. By changing the hardware settings to a camera with high sensitivity also in the near infrared (NIR) to infrared (IR) spectra, and adapt filter settings accordingly, it is possible to extend the number of specific quantifiable channels to at least three (Eriksson et al., manuscript in preparation). This would give the opportunity to, on the global scale, monitor and quantify

several cell types involved in pancreatic biology and relate them, in the same specimen, to fundamental pancreatic components such as the vasculature or the ductal tree. Obviously, this would have implications for several areas of pancreas research ranging from studies of β -cell regeneration to diabetes progression and many more. An increased multichannel capacity of OPT could also be used for the cross-evaluation of contrast-agents for non-invasive imaging techniques as long as the contrast-agent could be fluorescently labelled. Apart from increasing the number of detectable channels, extending the detection wavelengths into the NIR/IR spectra provides an additional advantage since it is in the range of optimal light penetrance in biological tissues. OPT thus have the potential to image even larger specimen than the mouse pancreas (Eriksson et al., manuscript in preparation). Important applications for these types of improvements in imaging capacity include whole-organ imaging of rat models for diabetes and quantitative and spatial analyses of islet transplantation experiments to the mouse liver. Taken together, although it is unlikely that OPT will constitute a platform for *in vivo* imaging of the pancreas, the high resolution, imaging depth and potential multichannel capacity makes it a useful tool that may contribute to reveal new information about different aspects of the normal and diseased pancreas.

5. Aims of this thesis

- To refine the current understanding regarding the quantitative and spatial dynamics of infiltrating lymphocytes and BCM during T1D progression in the NOD mouse.
- To further explore and develop the OPT methodology for whole-organ assessments of BCM and islet distribution in the mouse.
- To create a quantitative and spatial atlas of pancreatic morphogenesis in the mouse.
- To characterize the developing and adult gastric lobe of the pancreas and to elucidate the developmental requirements for its formation.

6. Results and Discussion

6.1 Paper I

6.1.1 OPT based assessment of BCM and islet number during T1D progression in the NOD mouse

Studies of BCM dynamics during T1D have traditionally relied on statistical interpolation of 2D data obtained from sectioned pancreata (Bock et al., 1999; Hellerstrom and Hellman, 1963; Hellman et al., 1961). Although sectioning provides high-resolution data of individual islets, the possibility to assess global distribution and overall BCM content during T1D disease progression is limited. Using a recently developed protocol for OPT based assessments of BCM distribution (Alanentalo et al., 2007), we have assessed the dynamics of β -cell destruction in the NOD and congenic NOD.H-2b mice at different time points between 3 and 16 weeks of age. Hereby, we provide the first comprehensive whole-organ spatial and quantitative description of BCM distribution during T1D aetiology in the NOD mouse.

6.1.2 Dynamics of β -cell destruction

In both NOD and NOD.H-2b, the adult islet number is established already at 3 weeks. In the NOD.H-2b mice the islet number were maintained throughout the assessed time points while the total pancreas volume and BCM increased. In the NOD mouse, similar kinetics occurred until 8 weeks of age when both BCM and islet number started to decrease. Initially, the decrease in islet number was more dramatic than the reduction in BCM. To investigate the reason for this discrepancy, we categorized the islets arbitrarily into three size categories. By cross-referencing the islet volumes to their spatial location we could reveal that the smaller islets, which are mainly located in the periphery of the organ, were preferentially lost during the early phases of BCM reduction (Fig. 8). There are several possible explanation for this phenomenon: 1) the physical properties of the islets: large islets have a lower surface-area-to-volume ratio than the small islets, thus a smaller percentage of the large islets are exposed to infiltrating immune cells; 2) compensatory proliferation occurs in the large islets in response to the infiltration process; 3) the infiltration process itself takes place in an ordered fashion from the periphery of

the organ towards the centre/local signals preferentially recruit infiltrating immune cells to small islet; 4) a combination of the above.

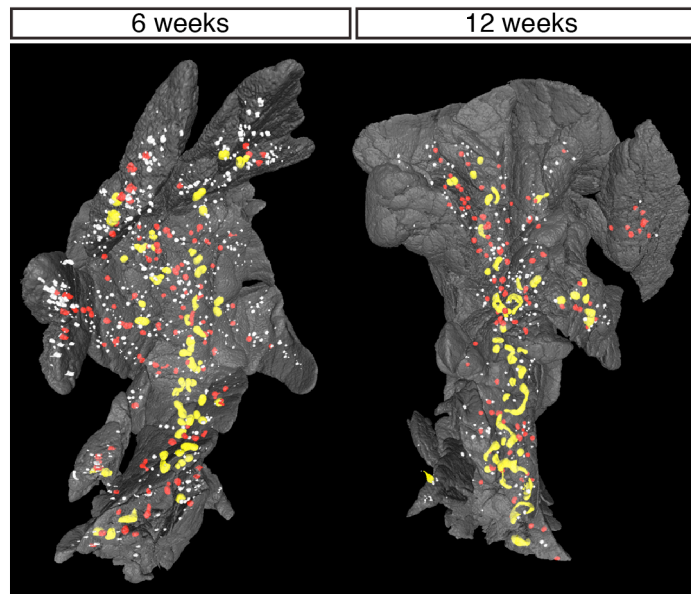


Figure 8. Comparison of BCM distribution in splenic NOD pancreata at 6 and 12 weeks. The images show OPT-rendered iso-surface reconstructions based on the signal from insulin antibodies and endogenous fluorescence. Islets are arbitrarily categorised into small (white), intermediate (red) and large (yellow). During early phases of disease, the islets belonging to the “small” category are preferentially lost.

6.1.3 Evidence for compensatory growth potential of large islets during later phases of disease progression in the NOD mice

In experimental models of diabetes/pancreas injury, β -cells have been suggested to regenerate through the duplication of mature β -cells (Dor et al., 2004; Nir et al., 2007; Teta et al., 2007), through neogenesis from specialized progenitors (Bonner-Weir et al., 1993; Xu et al., 2008) or through transdifferentiation from pre-existing cell types (Inada et al., 2008; Thorel et al., 2010; Wang et al., 1995). To elucidate whether there is a compensatory proliferative process paralleling the autoimmune destruction of β -cells, we compared the average volumes of the ten largest islets in NOD pancreata at 6 and 8 weeks with the volumes of the ten largest islets in the 12 and 16-week NOD mice in which the BCM had not collapsed. We could hereby

detect an increase in the average size of the largest islets prior to the decrease in BCM. This indicates that the remaining islets proliferate to meet the metabolic demand for insulin and compensate for the autoimmune destruction of β -cells. However, this does not exclude the possibility of other mechanisms for islet regeneration nor does it rule out a contribution from any of the other above-mentioned explanations for the preferential loss of small islets. Nevertheless, the indicated proliferative potential of the large islets is an observation that might offer an opportunity to target BCM expansion as a treatment in a prediabetic/diabetic stage.

6.1.4 Spatial distribution of insulinitis

The observed preferential loss of small islets in the periphery of the pancreas led us to ask if this was related to the spatial coordination of insulinitis progression within the organ or if it occurs due to inherent properties of the differentially sized islets. Pancreata were stained with antibodies against insulin and CD3, a marker for infiltrating T-cells, and the overall spatial distribution of infiltrating cell clusters in relation to the pancreatic islets was analyzed using OPT. A few foci of infiltrating CD3⁺ cells could be detected already at 3 weeks of age and the amount progressively increased during the course of evaluation. Surprisingly, the autoimmune infiltration did not appear in a coordinated fashion – at least during early stages of insulinitis. Instead, the infiltrating cells appeared randomly scattered throughout the pancreas and as the smaller islets disappeared, more CD3⁺ cells were accumulated around the remaining large islets. As immune infiltration advanced, these structures became more well-defined and by sectioning and subsequent immunohistochemical analyses of markers for B-cells (CD19) and high endothelial venules (MAdCAM-1) we could detect characteristics of so called tertiary lymphoid organs (TLOs). TLOs have been reported to form under various conditions of chronic inflammation (Aloisi and Pujol-Borrell, 2006) including in the NOD mouse (Lee et al., 2006). It has been speculated that when the immune system fail to deplete an antigen/infectious agent, TLOs forms at the site of infection/source of the antigen to increase the efficiency of the immune response (Aloisi and Pujol-Borrell, 2006). Local factors have been suggested to be important for the formation of TLOs since the most organized structures are found in highly infiltrated tissues (Aloisi and Pujol-Borrell, 2006). This also fits with our observation that infiltration appears to be asynchronous with

regards to spatial distribution during early phases of T1D in the NOD mouse. An alternative explanation is that the destruction of small islets occurs very rapidly; this would evade retention of infiltrating immune cells in the periphery of the pancreas. It is clear from our experimental data that infiltration of the larger islets begins before the dramatic drop in islet number around 8 weeks of age occurs, thus arguing against the possibility that it is the overall spatial coordination of the infiltration process that accounts for the preferential loss of smaller islets.

6.1.5 BCM and onset of diabetes in the NOD mouse

It is known that the time of onset for overt diabetes varies between individual NOD mice. In our data, this is reflected by a large variation in BCM between individuals within the 12 and 16-week cohorts of mice. Previous morphometric data in the NOD mouse have suggested that at onset of overt diabetes, the remaining BCM is at least ~30% of the controls (Sreenan et al., 1999). Our data indicates that overt diabetes does not occur until ~14% of the BCM remains. This is well in line with the numbers reported for human T1D where 5-20% of the islets remain at clinical onset of the disease (von Herrath and Homann, 2004). In the Sreenan study NOD/Scid mice were used as controls and together with the methodological differences this could explain the discrepancy in numbers between the two studies. It is striking that a relatively low percentage of the BCM manage to keep blood glucose levels at reasonable levels during a long period before T1D onset and underscores the advantages that an early diagnosis could give for treatment of the disease.

6.2 Paper II

6.2.1 A computational approach to improve OPT based assessments of the mouse pancreas

The pivotal role of β -cell function and BCM in the different forms of diabetes has made analyses of BCM and islet number indispensable for many areas of diabetes research. Previous studies have to a large extent relied on stereological sampling techniques for these assessments and it is only recently that technological advances has allowed for alternative approaches to analyse BCM and islet number. OPT is one of the aspiring techniques that has been successfully used

in studies of BCM distribution in the mouse (Alanentalo et al., 2007; Alanentalo et al., 2010; Sun et al., 2010a; Sun et al., 2010b). It allows for the extraction of BCM, islet number and islet co-ordinates throughout the volume of the intact mouse pancreas (Alanentalo et al., 2007). However, as with other techniques, previously described protocols for quantifications of BCM/islet distribution using OPT have relied on large amounts of manual post-acquisition editing. The calculation of BCM and islet number in these quantifications relies on an intensity threshold that is applied throughout the volume of a reconstructed 3D model. The spatial coordinates and volume of each object (islet) with an intensity above the applied threshold is then calculated and displayed in a list that can be cross-referenced back to the 3D model. The main difficulties associated with this protocol depend on the inherent differences in signal intensity between islets displaying strong and weak staining and the relation of these intensities to the unspecific endogenous background fluorescence from the surrounding tissue. If a low threshold is applied, the weak intensity islets are included but the strong islets tend to grow and become misshaped. Additionally, a low threshold makes it difficult to exclude unspecific background leading to an overestimation of the islet volume. The opposite is true for a high threshold; strong islets

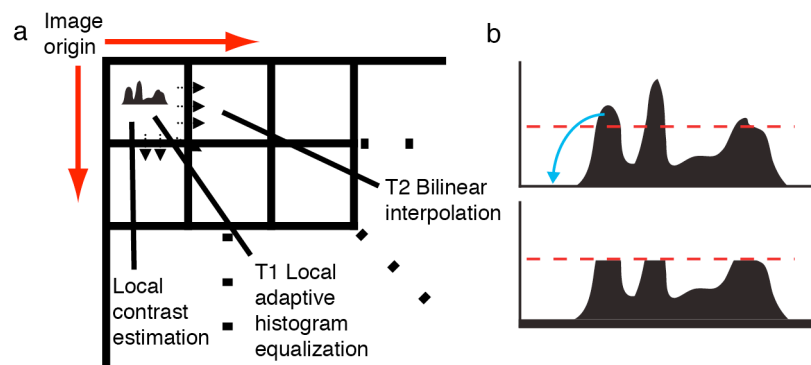


Figure 9. Illustration of the CLAHE procedure. (a) The image is divided into non-overlapping blocks; the contrast is estimated within each of the blocks and a clip limit is set to constrain the permissible contrast; artificial boundaries between blocks are eliminated using bilinear interpolation. (b) The clip limit distinguishes CLAHE from AHE and limits the amplification of contrast to avoid saturating pixels and increase noise intensity in homogenous areas. Values that exceed the clip limit are redistributed equally among all intensities in the histogram.

will maintain their proper size and morphology but at the cost of losing weaker islets. The latter scenario will result in a more accurate BCM measurement but with a less accurate islet number. To address these issues, we applied a computational statistical approach named *Contrast-Limited Adaptive Histogram Equalization* (CLAHE) (Zuiderveld, 1994) to projection images of adult mouse pancreata stained for insulin. CLAHE is an image analysis tool that facilitates the display of hidden features in an image by combining local contrast-enhancement with overall interpolation. CLAHE first equalizes the contrast within discrete areas (blocks) of an image (Fig. 9a). It differs from *Adaptive Histogram Equalization* (AHE) (Pizer et al., 1987) in that the histogram is clipped at a predefined value before computing the histogram equalization (Fig. 9b). This is done to limit the increase in contrast, thereby avoiding saturation of pixels and amplification of noise in areas with homogenous intensities. After local contrast enhancement, artificially boundaries between the blocks that are induced by the equalization process are removed by bilinear interpolation at the border regions. The result is an increased contrast in the overall image without sharp boundaries between regions.

6.2.2 CLAHE increases islet detection sensitivity in OPT based assessments of BCM distribution

By comparing reconstructions from identical data sets with and without CLAHE processing we could demonstrate that CLAHE significantly increases the sensitivity of OPT-based assessments of BCM and islet number. In our data, the number of islets detected was strikingly higher (~80%) than both previous OPT protocols and the numbers reported for the same strain with morphometric measurements (although the definition of an islet to be accounted for differs slightly between the methods). CLAHE did not only allow for detection of substantially more islets but it also helped preserve the shape and size of the strong intensity islets. In order to rule out that CLAHE induced artificial structures contributing to the calculated islet number and to validate the accuracy of the method, we performed two sets of experiments. First, we stained pancreata with secondary antibodies only and subjected these to CLAHE-processing and analyses. We could hereby not detect any islets or islet-like objects, thus validating the notion that CLAHE does not induce artificial islets in the 3D volume. Secondly, we identified regions containing ~50 islets (n=3) in the 3D rendering of a CLAHE-processed pancreas.

These regions were then isolated and subjected to sectioning and morphometric analyses. All islets detected by OPT could be accounted for in these analyses. This further supports the high islet number detected in our study. It is noteworthy that we also detected additional small islets consisting of only a few cells in these regions. A plausible explanation for this is the size limit of 10 voxels that we set in our protocol to avoid including artefacts caused by background noise. However, this is an observation that underscores the importance of cautiousness when comparing numbers obtained by different methodologies, and that a general awareness of the inherent limitations of different techniques used for BCM assessments is important. In addition to the increased islet detection sensitivity observed in CLAHE-processed data as compared to “normal” OPT, the increased ratio between specific signal and the surrounding unspecific background facilitates both the setting of dynamic range in the reconstruction process and the setting of threshold when performing segmentation. As a consequence of these improvements, CLAHE decreases manual intervention and overall subjectivity in islet segmentations as well as reduces the time needed for quantification of each specimen. We predict that this approach will be very useful not only for OPT-based assessments of BCM and islet distribution in the mouse. The technique should be possible to translate also to other organ systems and protein distribution patterns.

6.2.3 The pancreatic lobes display heterogeneous BCM and islet distribution

Although it is known that the pancreatic lobes develop in different milieus, there has been, to the best of our knowledge, no detailed study presented that address the lobular distribution of BCM and islet number in the mouse. We used OPT imaging in combination with the developed CLAHE approach to investigate this issue in eight-week healthy C57Bl/6 female mice (n=5). The pancreata were stained for insulin and the lobes were separated prior to scanning and computational processing. Aside from the strikingly high number of islets detected, this approach revealed significant differences in BCM and islet distribution between the pancreatic lobes. Noteworthy is the relative BCM (islet β -cell volume per lobular volume) that was ~40% higher in the DL as compared to the SL. The largest difference was however observed in the relative number of islets (islet number per lobular volume) in the three lobes. The DL displayed ~20% more

islets than the SL while the GL displayed strikingly ~75% more islets per lobe volume than the SL. Although the GL displayed a significantly higher relative islet number, these islets had a smaller average size than the islets in the other lobes and the total contribution of the GL to the BCM corresponded well with its contribution to the overall volume of the pancreas. Furthermore, our data is in agreement with the previous notion that the BCM constitute 1-2% of the total pancreatic volume. It is perhaps not very surprising that the most striking differences were displayed in the relative islet number. It is well known that the large islets in the mouse pancreas are located near the large blood vessels in the centre of the organ while small islets are located in the periphery. The size and number of islets likely reflects the microenvironments ability to give and receive signals as well as to provide sufficient nutrients for proper β -cell function. The GL is the smallest lobe and does not contain the largest blood vessels; the smaller size of the GL islets may thus be an issue of the overall lobular structure. Nevertheless, the heterogeneities in relative BCM and islet distribution reported in this study underscore the importance of taking lobular context into careful consideration when analysing BCM and/or islet distribution. This has implications for studies of mouse models of diabetes but also in the context of gene ablation studies aimed at understanding β -cell/islet development or in experimental models of β -cell regeneration. Furthermore, the lobular heterogeneities in islet distribution raises the question to whether β -cell mass is established in a coordinated fashion throughout the organ or if regional differences exists between the lobes.

6.3 Paper III

6.3.1 Spatial and quantitative dynamics of pancreas morphogenesis

Pioneering studies describing the morphogenesis of the rodent pancreas was performed more than 40 years ago (Pictet et al., 1972; Wessells and Cohen, 1967). With the advent of new imaging techniques, the opportunity to revisit and retrieve new knowledge of pancreas morphogenesis has been raised. OPT was initially developed as a tool for analysis of gene and protein expression in embryos (Sharpe et al., 2002) and it has previously been shown to facilitate imaging of the developing pancreas (Asayesh et al., 2006; Hecksher-

Sorensen et al., 2004; Sand et al., 2011). In this study we aimed to: 1) create a spatial and quantitative “atlas” of pancreatic morphogenesis in the mouse using OPT, and 2) investigate the morphological events associated with to GL formation. To address these issues, gut segments including the stomach, duodenum, pancreas and spleen from e10.5 to postnatal day 0 (P0) mice were subjected to OPT analyses. The pancreatic epithelium was visualized using antibodies against E-cadherin whereas the mesenchymal morphology was imaged based on the signal from endogenous tissue fluorescence. At each time point, mesenchymal and epithelial morphology was investigated and the volume of the pancreatic epithelium was measured. The hereby-created “road map” of pancreas morphogenesis demonstrates that the GL forms as a lateral branch from the stem of the dorsal pancreas at around e13.5 (Fig. 10). As development proceeds, the GL grows into a mesenchymal domain overlying the pyloric sphincter and the caudal-most part of the glandular stomach. Already at e15.5 the relative size between the pancreatic lobes has been established. From this time point the GL constitute ~10% of the pancreatic epithelium whereas the SL and the DL constitute ~55% and ~35% respectively. The similarity in the relative lobular size at later time points during pancreatic development suggests that pancreatic growth is homogenous between the lobes from e15.5 onwards. Moreover, the amount of mesenchyme



Figure 10. Epithelial morphogenesis of the mouse pancreas. OPT-generated iso-surface reconstructions based on antibodies against E-cadherin. The GL starts to form as a perpendicular outgrowth from the splenic lobe at e13.5. It subsequently grows over the pyloric sphincter and the proximal part of the glandular stomach. The specimens are displayed according to relative size. Scalebar: 1 mm. Abbreviations: SL, splenic lobe; GL, gastric lobe; DL, duodenal lobe.

surrounding the pancreatic epithelium is successively reduced towards the end of gestation. This is in line with both previous observations (Heller et al., 2002) and a recent publication where the mesenchymal to epithelial ratio was estimated by measuring their respective area at different time points on serial sections (Landsman et al., 2011). Noteworthy is also the fusion of the dorsal and ventral anlagen at \sim e13.5. It occurs at a position distal from the opening of the pancreatic ducts into the duodenum, at about the same time point as the first visible signs of GL formation. Taken together, these data show that the GL constitute a substantial portion of the pancreas that forms by perpendicular growth from the stem of the dorsal pancreatic anlagen into a lateral mesenchymal domain four days after the first visible signs of pancreas morphogenesis.

6.3.2 Splenogenesis promotes formation of the gastric pancreatic lobe

In an attempt to elucidate the basis for GL morphogenesis, we thoroughly investigated the morphological features of the mesenchyme surrounding the dorsal pancreatic bud prior to and during GL formation. We could hereby note that initial spleen formation between e10.5 and e13.5 coincided with the division of the dorsal pancreatic mesenchyme into a GL and a SL domain. The time of GL epithelial outgrowth corresponds to when spleen precursors condense and starts to dislocate from the dorsal pancreatic anlagen. Given these morphological features seen in wild type mice we hypothesized that in mice with aberrant spleen formation, the morphogenesis of the GL would be disrupted. In order to investigate this notion, we analysed the spleno-pancreatic morphology in mice deficient for the TFs *Nkx3.2* and *Sox11* and mice heterozygous for the *Dh* locus. These mice all display severe but various degrees of perturbed spleen morphogenesis. In both the *Dh*^{+/-} and *Nkx3.2*^{-/-} the GL epithelial domain were either completely absent or severely truncated at e14.5. Examination of these mutants at e12.5 indicate that the dorsal pancreatic mesenchyme does not split up into a GL and SL domain as a consequence of the inability to form a distinct splenic anlage. An aberrant gut looping in *Nkx3.2*^{-/-} mice could also contribute to the disturbed overall morphology of the pancreatic mesenchyme, which further complicates the phenotype of these mice. No thorough studies have been made concerning the asplenic phenotype of *Sox11*^{-/-} mice. However, our OPT analyses suggests that

Sox11^{-/-} mice do form an initial spleen anlage that is recognizable at e12.5 whereas at e14.5 shows various degree of perturbation. The corresponding GL morphology also ranges from an apparent truncation to essentially normal. These variations might be explained, at least in part, by the mixed background of the *Sox11*^{-/-} strain. Investigations of *Sox11*^{-/-} mice at later stages of development demonstrate that the dorsal pancreatic epithelium grows into the surrounding remnants of splenic mesenchyme. Thus, depending on the time point when spleen development fails, there is a varying degree of mesenchyme providing a template for the growth of the GL epithelium. In accordance with this notion, these mice also show a variable size and morphology of the GL in late gestation embryos. Taken together, the disturbed GL morphology observed in the analysed mutants and the analyses of spleno-pancreatic mesenchymal morphology in normal mice supports the hypothesis that initial spleen organogenesis is required for proper morphogenesis of the GL epithelium. We suggest that spleen organogenesis results in the subdivision of the dorsal pancreatic mesenchyme into a GL and a SL mesenchymal domain, which then provide templates for the growth of the GL and SL epithelium and as such impact the final gross morphology of the pancreas. We cannot exclude that the lack of specific gene function in the examined mutant mice directly contribute to disturbed GL development but it is clear that the most severe splenic phenotype also corresponds to the most severe perturbation in GL development. This strengthens the hypothesis that it is spleen organogenesis *per se* that mediates morphogenesis of the dorsal pancreatic mesenchyme and thus promotes the formation of the GL. It is well known that mesenchymal signals are important for the growth and differentiation of the pancreatic epithelium. Gene expression has been shown to be heterogenous even within regions of the pancreatic mesenchyme (Hecksher-Sorensen et al., 2004; Villasenor et al., 2010). In view of this knowledge, comparative studies of gene expression in the pancreatic mesenchymal compartments might provide information on whether unique signalling pathways are at play during the course of GL development. It could also aid in the identification of which signalling molecules are essential for growth and differentiation of the pancreatic epithelium in all of the lobes. In early pancreas development, it has been shown that the growth of the pancreatic epithelium can be supported by a variety of mesenchymal sources (Ahlgren et al., 1997; Golosow and Grobstein, 1962; Wessells and

Cohen, 1967) and although unique pathways might play a role during GL development, it is perhaps more likely that it is the spatial organization of the mesenchyme that shapes the overall morphology of the pancreas by providing a structural support into which the epithelium can grow. However, it is clear that the close interplay between the spleen and the dorsal pancreas during development should be taken into consideration when assessing knockout/transgenic mice with pancreatic phenotypes.

6.3.3 Gastric lobe formation appears independent on the L-R signalling pathway

Leftward growth of the spleno-pancreatic region is mediated by the SMP, a transient organizer overlaying the spleno-pancreatic mesenchyme at e10.5. The SMP consists of a mesodermally derived columnar cell layer under the direct influence of the left-right genetic cascade; it drives leftward growth of the spleno-pancreatic region mainly through proliferation but it is also a source of growth factors. One of these factors is FGF10, which plays an important role in early pancreas development (Bhushan et al., 2001) and has been suggested as a chemotactic factor for pancreatic growth (Hecksher-Sorensen et al., 2004). In *Dh*^{+/-} and *Nkx3.2*^{-/-} mice the SMP does not form or is severely truncated. To investigate the possibility that GL morphogenesis is dependent on a similar mechanism that directs early leftward growth, we investigated the SMP morphology in e10.5 *Sox11* embryos and screened the wild type GL mesenchyme between 12.5 and e14.5 for structures analogous to the SMP. In wild type embryos we could not detect any structure analogous to the columnar cells of the SMP and in *Sox11*^{-/-} mice the SMP formed normally. Furthermore, we could not detect increased proliferation in GL mesenchyme nor the expression of FGF10. These data indicate that GL formation is not dependent on a mechanism similar to that controlling the early leftward growth and that the SMP does not play a direct role for the formation of the GL other than as a possible inducer of splenic mesenchyme.

6.3.4 Cellular differentiation is delayed in the gastric pancreatic lobe

The outgrowth of the GL commences at e13.5 and by e14.5 it is a clearly discernible structure. This coincides with a transition of the pancreas from an immature state harbouring a large amount of MPCs

to a more differentiated state with a larger amount of progenitors specified for one of the three main pancreatic cell lineages. As previously mentioned, it has been suggested that the MPCs are located in the tips of the branching epithelial tree of the pancreas and that these cells express *Pdx1* and *Cpa1* while they are negative for the exocrine marker amylase (Zhou et al., 2007). At e13.5 these tip cells start to express amylase and only give rise to exocrine progeny. Given that the GL contains all major pancreatic cell lineages and that it forms at the same time as the tip cells switch from MPCs to exocrine progenitors, there are two possible sources of cells giving rise to the GL epithelium: 1) already specified exocrine and endocrine progenitors in the stem of the dorsal pancreas proliferate and grow into the GL mesenchymal domain or 2) the multipotent progenitor potential is prolonged in the GL as compared to the SL and MPCs in the tips gives rise to both endocrine and exocrine progeny during the initial phase of GL formation. In order to investigate these possibilities, we performed triple labelling experiments for *Pdx1*, *Cpa1* and Amylase between e12.5 and e15.5 on sections from the SL, DL and GL. Hereby we could detect the presence of $Pdx1^+$, $Cpa1^+$ and Amylase⁻ tip cells in the SL and DL until e13.5. At e14.5 the majority of the tip cells in these lobes were also Amylase⁺ whereas in the GL differentiated $Pdx1^+/Cpa1^+/Amylase^+$ were not abundant until e15.5. A number of recent reports have pointed out different markers for MPCs within the pancreas (Kopp et al., 2011b; Solar et al., 2009; Zhou et al., 2007). It still remains to be elucidated how these suggested markers for MPCs relate to each other and to a “true” multipotent progenitor. Nevertheless, the delay in GL differentiation as compared to the other lobes suggests that a thorough study of the spatial microenvironments of the pancreatic compartments could give clues to signals important for pancreatic cell lineage selection and differentiation. Although the prolonged presence of undifferentiated tip cells in the GL may only represent a general delay in differentiation, we cannot exclude that there is spatial differences in the mechanisms controlling pancreatic cell type differentiation. As noted above, there are known differences in gene expressions between the lobes both in the epithelium and in the mesenchyme (Ahlgren et al., 1997; Esni et al., 2001; Harrison et al., 1999; Hecksher-Sorensen et al., 2004; Martin et al., 2005; Villasenor et al., 2010; Watt et al., 2007). To what extent these differences influence developmental and functional aspects of pancreas biology are not fully understood. However, it is an

important issue that raises the question whether there are multiple/parallel pathways that can lead to the generation of mature pancreatic cell types or if only highly specific combinations of signalling molecules/TFs are needed for the differentiation of certain cell lineages (Fig. 11). Knowledge in this issue is of obvious interest for current day efforts to develop *in vitro*-protocols to generate β -cells for transplantation purposes. In this respect, the lobular heterogeneties recognized in the present study may provide an inherent system for the study of how pancreatic cellular differentiation is coordinated.

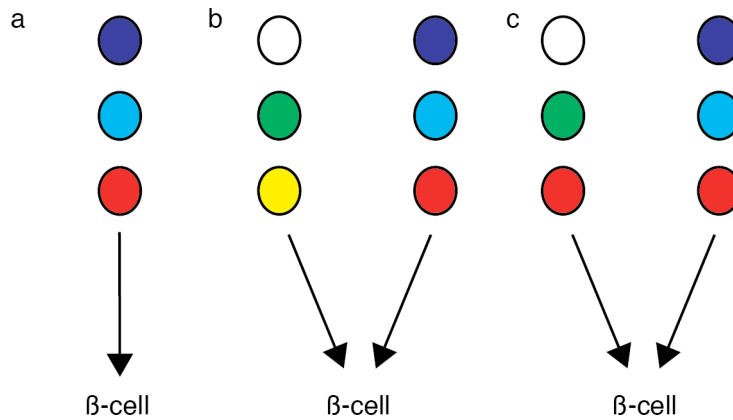
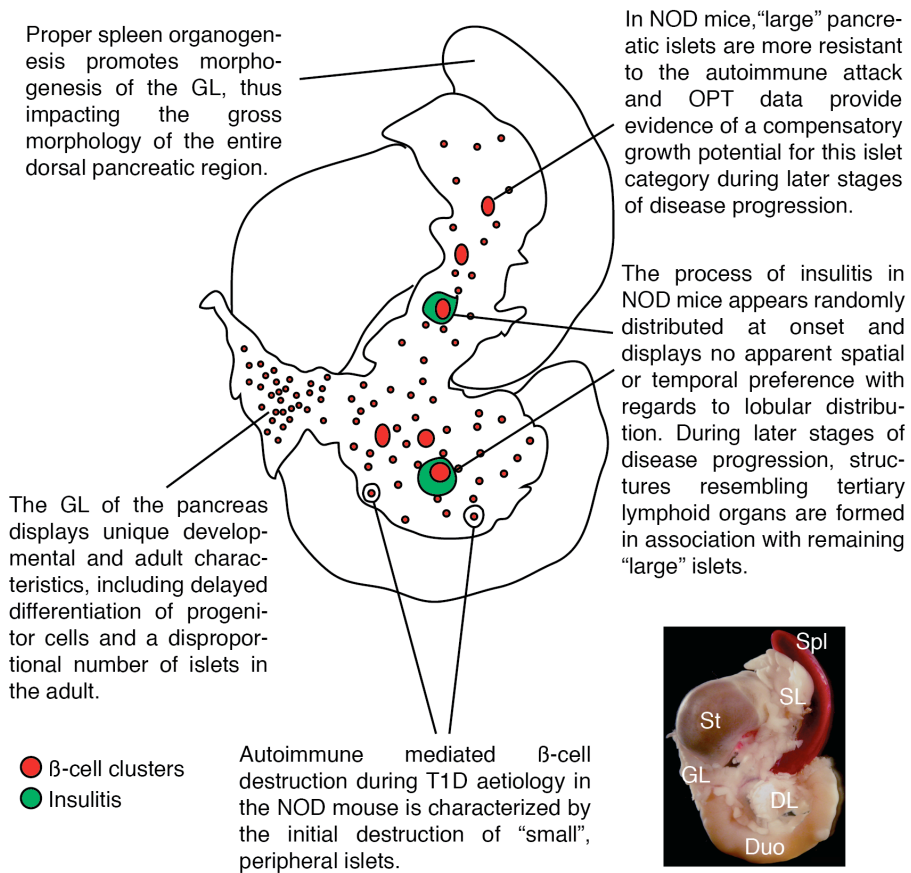


Figure 11. Possible scenarios for cellular differentiation in the pancreas. (a) Only a specific set of molecules can induce differentiation of a certain cell type. (b) Parallel pathways can achieve differentiation of a certain cell types. (c) A set of specific signalling molecules is absolutely required for the differentiation of a certain cell type but additional factors that can vary are also required.

7. Summary and Conclusions



CLAHE significantly contributes to increase detection sensitivity and to preserve islet morphology in OPT based assessments of BCM and islet distribution in the rodent pancreas. Hereby, this computational technique further strengthens the capability of OPT for various assessments of the normal pancreas and during diabetes.

Abbreviations: St, stomach; Spl, spleen; Duo, duodenum; SL, splenic lobe; DL, duodenal lobe; GL, gastric lobe

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