The role of N-cadherin and S1P/S1P1 in pancreas development

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Abstract

ABSTRACT

Organogenesis of epithelial organs requires interaction between epithelial and mesenchymal tissues. During pancreas development, mesenchyme-derived structures induce specification, growth and differentiation of pancreatic epithelial cells. For instance, notochord, endothelial cells, and pancreatic mesenchyme are essential for the development of the pancreas. N-cadherin deficient-embryos suffer from dorsal pancreas agenesis due to apoptosis of the mesenchyme surrounding the pancreatic epithelium. N-cadherin-deficient embryos expressing N-cadherin or E-cadherin ectopically in the heart rescue dorsal pancreas formation, indicating that the phenotype is secondary to cardio-vascular defects. These results suggest that circulating factors are important for dorsal pancreas development. Here we identify a sphingolipid, sphingosine-1-phosphate, to be important for dorsal pancreas formation. S1P acts on receptors (S1P1, S1P2, and S1P3) expressed in the pancreatic mesenchyme, inducing mesenchymal growth.

Studies on the S1P1 receptor knockout showed a reduced size of the dorsal pancreatic bud due to decreased proliferation of Pdx1+ cells. Other epithelial organs revealed growth defects. The lung epithelium had fewer branches and the stomach was smaller. This suggests that endothelial cells are essential for early development of the pancreatic epithelium.

Since N-cadherin deficient embryos die at embryonic day E10, due to the cardio-vascular phenotype, we used a conditional N-cadherin deficient-mouse which was intercrossed with a Pdx-1-Cre driven transgenic line to generate a pancreas specific knockout. Our results indicate that N-cadherin neither is important for pancreas morphogenesis nor endocrine specification. This could be due to variable recombination efficiency of the Pdx-1-Cre line during development. However, mutant β-cells have fewer mature insulin secreting granules in the N-cadherin deleted islets indicating that N-cadherin is required for β-cell granule turnover.

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Edsbagge J*, Johansson JK*, Esni F, Luo Y, Radice GL, Semb H.

Development. 2005 Mar:132(5):1085-92.

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II. **$S1P_1$ signaling control endoderm development.**

Fredrik Wolfhagen Sand, Christina Lorén*, Jenny K. Johansson*, Josefina Edsbagge, Anders Ståhlberg, Judith Magenheim, Ohad Ilovich, Eyal Mishani, Yuval Dor, Richard L. Proia, Ulf Ahlgren, and Henrik Semb.

Manuscript, *Contributed equally to this work

III. **N-cadherin is dispensable for pancreas development but required for β-cell granule turnover.**

Jenny K Johansson, Ulrikke Voss, Gokul Kesavan, Igor Kostetskii, Nils Wierup, Glenn L Radice, Henrik Semb.

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ABBREVIATIONS

ADP	Adenosine di phosphate
Arx	Aristaless-related homeobox gene
ATP	Adenosine tri phosphate
B-Geo	β-galactosidase-Neo^{R}fusion
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
Cdc	Cell division cycle
cKO	Conditional knockout
Cpa1	Carboxypeptidase A1
Cre	Cyclization recombination
DE-cadherin	Drosophila E-cadherin
Dlg	Disc large
DNA	Deoxyribonucleic acid
E	Embryonic day
EC	Extracellular cadherins
E-cadherin	Epithelial cadherin
ECM	Extra cellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
EP	EPLIN
FGF	Fibroblast growth factor

GLP1	Glucagon-like-peptide 1
GLUT2	Glucose transporter 2
HAV	Histidine, Alanine, Valine
HDL	High density lipoprotein
hESC	Human embryonic stem cells
Hlxb9	Homeo box HB9
Hnf	Hepatocyte nuclear factor
Ig	Immunoglobin
Ihh	Indian hedgehog
Isl1	Islet 1
Lgl	Lethal giant larvae
loxP	Locus of X-over P1
N-cadherin	Neural cadherin
Ngn3	Neurogenin-3
OPT	Optical Projection Tomography
Par	Partitioning defective
PALS1	Protein linked to protein associated with Lin-7
PATJ	Pals-associated tight junction protein
Pax	Paired box gene
P-cadherin	Placental cadherin
PDGF	Platelet-derived growth factor
Pdx1	Pancreatic and duodenal homeobox 1
Ptf1a	Pancreas specific transcription factor 1a
Rac1	RAS-related C3 botulinum substrate 1
R-cadherin	Retinal cadherin
RER	Rough endoplasmic reticulum

Rho-GTPase	Ras homology family of GTPase
RRP	Readily releasable pool
RT-PCR	Reverse transcription polymerase chain reaction
Shh	Sonic hedgehog
S1P	Sphingosine-1-phosphate
$S1P_{1-5}$	Sphingosine-1-phosphate receptor 1-5
Sox2	(Sex determing region Y)-box 2
Sox9	(Sex determing region Y)-box 9
Stat3	Signal transducer and activator of transcription-3
TGF	Transforming growth factor
TGN	Trans-golgi network
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

ABSTRACT

Organogenesis of epithelial organs requires interaction between epithelial and mesenchymal tissues. During pancreas development, mesenchyme-derived structures induce specification, growth and differentiation of pancreatic epithelial cells. For instance, notochord, endothelial cells, and pancreatic mesenchyme are essential for the development of the pancreas. *N-cadherin* deficient-embryos suffer from dorsal pancreas agenesis due to apoptosis of the mesenchyme surrounding the pancreatic epithelium. *N-cadherin*-deficient embryos expressing N-cadherin or E-cadherin ectopically in the heart rescue dorsal pancreas formation, indicating that the phenotype is secondary to cardio-vascular defects. These results suggest that circulating factors are important for dorsal pancreas development. Here we identify a sphingolipid, sphingosine-1-phosphate, to be important for dorsal pancreas formation. S1P acts on receptors ($S1P_1$, $S1P_2$, and $S1P_3$) expressed in the pancreatic mesenchyme, inducing mesenchymal growth.

Studies on the *$S1P_1$* receptor knockout showed a reduced size of the dorsal pancreatic bud due to decreased proliferation of $Pdx1^+$ cells. Other epithelial organs revealed growth defects. The lung epithelium had fewer branches and the stomach was smaller. This suggests that endothelial cells are essential for early development of the pancreatic epithelium.

Since N-cadherin deficient embryos die at embryonic day E10, due to the cardio-vascular phenotype, we used a conditional *N-cadherin* deficient-mouse which was intercrossed with a *Pdx-1-Cre* driven transgenic line to generate a pancreas specific knockout. Our results indicate that N-cadherin neither is important for pancreas morphogenesis nor endocrine specification. This could be due to variable recombination efficiency of the *Pdx-1-Cre* line during development. However, mutant β-cells have fewer mature insulin secreting granules in the *N-cadherin* deleted islets indicating that N-cadherin is required for β-cell granule turnover.

POPULÄRVETENSKAPLIG SAMMANFATTNING

β-celler producerar insulin som frisätts när blodsockret höjs, t.ex. efter en måltid. Patienter med diabetes saknar de insulinproducerande β-cellerna eller har nedsatt känslighet för insulin. För de patienter som saknar insulin kan det vara svårt att reglera blodsockernivåerna i blodet trots dagliga injektioner med insulin, varför transplanterbara insulinproducerande β-celler skulle kunna vara av stor nytta för dessa patienter. Hittills har försök gjorts med att injicera hormonproducerande celler från donatorer till patienter. Dessa patienter har klarat sig utan insulin i några år men har sedan behövt återgå till insulininjektioner. Ett annat problem med denna behandling är bristen på donatorer. För att komma runt detta görs försök med att framställa insulinproducerande β-celler från humana embryonala stamceller, dvs de stamceller som ger upphov till ett människo-foster. För att driva de humana embryonala stamcellerna till att bli insulinproducerande β-celler behövs forskning om hur dessa celler bildas normalt i bukspottkörtelns utveckling.

Tidigt i utvecklingen bildar bukspottkörteln två utbuktningar, så kallade buddar, från tarmen. Dessa två buddar växer sedan samman och bildar bukspottkörteln som består av exokrin och endokrin vävnad samt kanaler. Den exokrina vävnaden bildar de enzymer som används vid matspjälkningen. Dessa enzymer transporteras från de exokrina cellerna via kanalerna till tarmen. Den endokrina vävnaden bildar hormonproducerande celler. Dessa celler utsöndrar hormoner till blodet, t.ex. insulin.

I den här avhandlingen har vi fokuserat på blodkärl och proteinet N-cadherins inverkan på bukspottkörtelns utveckling. I det första arbetet har vi använt en musmodell där endast en av de två buddarna bildas. Denna musmodell saknar N-cadherin i embryots alla celler. Detta leder till att embryot dör tidigt under utveckling på grund av avsaknad av blodcirkulation. Forskare har visat att signalsubstanser ifrån blodcirkulationen är viktigt för bukspottkörtelns utveckling. Här har vi visat att plasma ifrån blodet är kapabel till att bilda den budd som saknas i musmodellen. Vi har även identifierat en lipid, sphingosine-

1-phosphate (S1P), som är nödvändig för bildandet av budden.

I det andra arbetet har vi använt oss av en genmodifierad mus som saknar receptorn $S1P_1$ och har en dramatisk kärldefekt. I denna modell bildas båda buddarna men de är mindre än buddarna hos kontrollmössen, även storleken på mage, lever och lungor är mindre.

Syftet med det tredje arbetet var att studera hur N-cadherin reglerar bukspottkörtelns utveckling. För att studera hur N-cadherin reglerar bukspottkörtelns utveckling använde vi oss av en musmodell som saknar N-cadherin endast i bukspottkörteln. Resultaten från denna studie i embryon visade att N-cadherin inte har en funktion i utvecklingen av bukspottkörteln eller i bildandet av de hormonproducerande cellerna. N-cadherin har dock visat sig ha en betydelse för utsöndrandet av insulin i vuxna möss. Detta beror på att mindre insulin bildas.

Sammanfattningsvis visar våra resultat att bukspottkörteln behöver signaler ifrån blodet, i detta fall sphingosine-1-phosphate, för att initiera bildandet av bukspottkörteln, samt att N-cadherin är viktigt för insulinfrisättningen.

INTRODUCTION

CELL ADHESION AND CADHERINS

CELL ADHESION

Cell adhesion mediates adhesive contacts between cells or helps cells to adhere to the extra cellular matrix (ECM) (Figure 1). Cell adhesion molecules (CAMs) prevent the tissue from dissociating. This was first studied in the 1950's, when Townes and Holfreter dissociated amphibian embryos into single cells and mixed them. Strikingly, upon reaggregation the cells sorted out to form their particular germ-layer [1]. From this experiment the researchers formed the idea of cell adhesion molecules expressed on the cell-surface. Today cell adhesion is implicated in signal transduction, morphogenesis, and in diseases such as cancer.

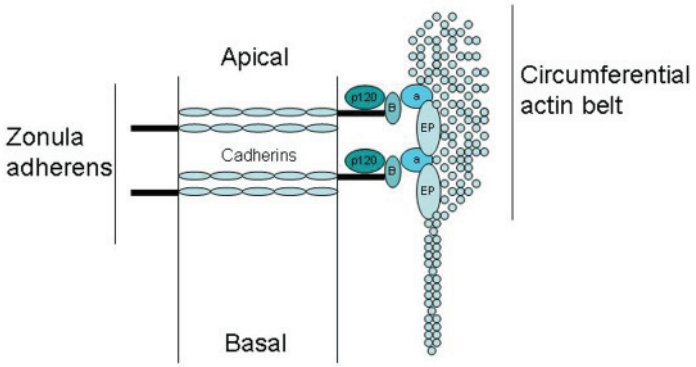

Figure 1. Adherens junction. Cadherins form homophilic interactions with their extracellular part. The intracellular part binds to a catenin complex. The catenin complex binds to EPLIN. EPLIN is required for linkage between cadherins and F-actin, and for maintaining the circumferential actin belt. Abbrevations: B, β-Catenin; a, α-Catenin; EP, EPLIN. The picture is modified from Nishimura et al 2009.

CELL ADHESION MOLECULES (CAMs)

CAMs are important during both the development of new tissue and the controlled growth and turnover of adult tissue. CAMs are cell surface glycoproteins that display two different kinds of adhesive mechanisms, Ca^{2+}-dependent and non-Ca^{2+}-dependent [2]. Based on their structures, CAMs are classified into

four major families; cadherins, selectins, integrins and the immunoglobin (Ig) superfamily [3]. Cadherins and selectins belong to the Ca^{2+}-dependent group, while integrins and immunoglobins belong to Ca^{2+}-independent group. Cadherins and immunoglobins promote cell-cell adhesion, whereas integrins mediate cell-matrix interactions [4, 5]. Selectins are involved in inflammatory responses.

THE CADHERIN SUPERFAMILY

The cadherin family of proteins can be divided into several groups. These subgroups include classical cadherins type I and type II, fat-like cadherins, seven-pass transmembrane cadherins, DCad102F-like cadherins, Desmosomal cadherins, and Protocadherins [6]. The classification of cadherins into different groups is based on the number of the extracellular cadherins repeats (EC). Each repeat consists of specific residues that bind calcium. Calcium provides structural rigidity of the extracellular domain, and is essential for the cadherins' adhesive function and protection against protease digestion (Figure 2) [7].

Classical cadherins are divided into type I and II based on the presence or absence of HAV (histidine, alanine, valine) tri-peptide within the most extracellular N-terminal repeat EC1. The EC1 domain promotes homophilic adhesive interactions of cadherins on opposing cells. The classical cadherins have five EC domains, a single-pass transmembrane part, and an intracellular part binding to the catenin complex [8].

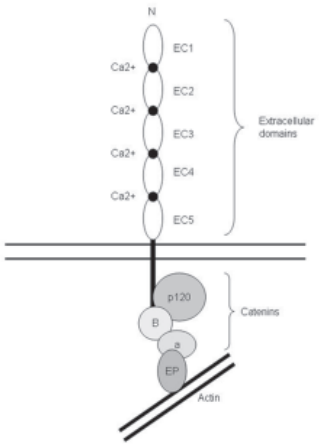

Figure 2. Structure of classical cadherins. Classical cadherins have an extracellular part containing five extracellular domains, a single transmembrane part, and an intracellular part. The intracellular part binds to the catenin complex and mediates the binding

between cadherins and F-actin. Abbreviations: N, N-terminal; EC, extracellular domain; B, β-Catenin; a, α-Catenin; EP, EPLIN. Picture modified from Ivanov et al 2001.

CADHERINS IN CELLULAR JUNCTIONS

Cells are connected with multiple types of junctions; tight junctions, adherens junctions, gap junctions, and desmosomes. The cell-cell junctions in epithelial cells contain tight and adherens junctions as well as desmosomes. These three junctional components are clustered together at the apical-lateral cell-cell contacts, forming the apical "junctional complex" [9]. The adherens junctions and desmosomes located in the junctional complex are termed zonal adherens (Figure 1).

The adhesive function of cadherins requires catenins (α-, β-, γ-catenin, and p120) that bind the cadherins to the actin fibers. The cytoplasmic part of classical cadherins binds to p120 [10] and β-catenin. P120 regulates cytoskeleton dynamics through direct modulation of the Rho GTPases [11] by inhibiting RhoA and activating Rac1 and Cdc42 [12-14]. β-catenin binds directly to α-catenin, which in turn, binds and bundles actin filaments and interact with other actin partners. Studies performed by Drees and colleagues and Yamada and colleagues showed that the cadherin-β-catenin-α-catenin complex associating with F-actin can not bind directly to F-actin. Only free α-catenin could link directly to F-actin. Later it has been shown that a molecule called EPLIN mediate the linkage between the cadherin-β-catenin-α-catenin complex and the F-actin [15, 16] (Figure1).

N-CADHERIN

N-cadherin was first identified as a cell adhesion molecule expressed in neural tissue [17], but was later found to be expressed in various non-neural tissues, such as the cardiac muscle [18], testis [19], kidney [20], and liver [21].

During embryogenesis, the expression pattern of N-cadherin changes as the cells undergo different morphological events such as gastrulation, neurolation, cardiogenesis, and somitogenesis [22, 23]. During gastrulation, when the cells delaminate from the primitive streak to form mesoderm, they switch from E-cadherin to N-cadherin expression. During neurolation the neural plate switches from E-cadherin to N-cadherin expression. After the neural tube closure, E-cadherin is lost and N-cadherin proteins are the major cadherin.

However, N-cadherin is not expressed in the neural crest cells where the neural tube closes [24]. In heart development, the mesoderm is divided into somatic mesoderm (dorsal) and the splanchnic mesoderm (ventral). N-cadherin expression is downregulated in the somatic mesoderm, but continues to be expressed in the splanchnic mesoderm [25]. When the somites are forming, N-cadherin is redistributed to the luminal side of the epithelium.

N-cadherin mutants die at E10. They express several developmental abnormalities such as malformed somites and yolk sac, undulated neural tube, dorsal pancreatic bud agenesis and severe cardiovascular defect, which is the cause of death [26, 27]. Due to the fact that N-cadherin is widely expressed in the embryo it is difficult to ascertain which phenotypes those are primary or secondary to the mutation. Therefore, the cardiac-mediated adhesion was restored by expressing either N-cadherin or E-cadherin in the heart of mutant embryos. These embryos survived one to two days longer than mutant *N-cadherin* embryos. N-cadherin and E-cadherin can restore cardiovascular phenotype and N-cadherin mediated cell-cell adhesion is required for cell survival [28].

As shown in rescue experiments, cadherins can compensate for each other. For instance, cadherin-11 is expressed in somites and may substitute for the loss of N-cadherin. There is also data showing that cadherin-11 can substitute for N-cadherin in skeletal muscle differentiation [26]. In embryonic stem cells E-cadherin and N-cadherin have been shown to compensate for each other [29].

N-cadherin is involved in many processes such as cell sorting, cell migration, and cell aggregation. In endothelial cells, N-cadherin regulates proliferation and motility by controlling VE-cadherin expression at the cell membrane [30]. In the cerebral cortex N-cadherin has been shown to be important for normal architecture of the neuroepithelial or radial glial cells and ablation of *N-cadherin* randomizes the internal structure of the cortex [31]. In endothelial cells, N-cadherin is required for pericyte recruitment [32].

EPITHELIAL ORGAN DEVELOPMENT

Endoderm has two functions; 1) to induce the formation of several mesodermal organs (notochord, heart, and blood vessels), and 2) to construct the lining of the digestive tube and respiratory tube. The digestive tube forms the pharynx, esophagus, stomach, small intestine and colon, while the respiratory tube forms the lungs (Figure 3). Caudal to the stomach liver, gallbladder, and pancreas are formed. The digestive and respiratory tubes are surrounded by mesenchyme that induces different endodermal structures along the anterior-posterior axis. Initially the expression of specific transcription factors is established to define the areas along the endodermal tube. These areas will become specific organs (e.g. Pdx1 expression) that define the future pancreatic and duodenal region. The endodermal epithelium then differentiates into the organ specific cell types. Concomitantly with specification and differentiation the tissues undergo morphogenesis. During this process the cells create cell-cell contacts and polarity, the tissues migrate and branches, and epithelial-mesenchymal interactions occurs. Coordination between these multiple processes results in the development of epithelial organs [33].

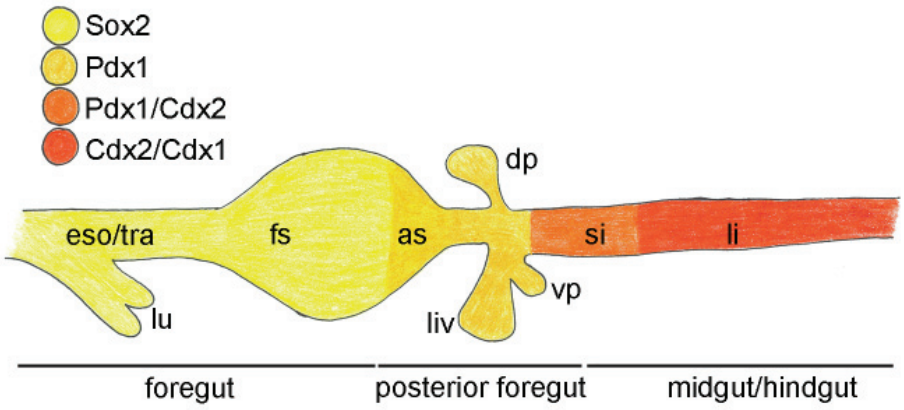

Figure 3. Anterior/posterior patterning of the digestive tract. Anterior foregut gives rise to esophagus, trachea, lung, and forestomach expressing Sox2. Posterior foregut gives rise to antral stomach, dorsal bud, ventral bud and liver expressing Pdx1. Midgut and hindgut develop small and large intestine expressing Cdx2 and Cdx1. Abbrevations: lu, lung; eso, esophagus; tra, trachea; fs, forestomach; as, antral stomach; dp, dorsal pancreas; liv, liver; vp, ventral pancreas; si, small intestine; li, large intestine. Picture modified from Guney et al 2009.

MORPHOGENESIS

Morphogenesis establishes appropriate environment for cells to interact and respond to signals. During epithelial organ development several cellular processes such as initiation and expansion of cell polarization, coordinated migration of cells towards particular chemo attractants, segregation of cells into specific groups, and cell survival occur. These morphogenetic processes can be triggered by signals like fibroblast growth factors (FGFs) secreted from the mesenchyme or by signaling pathways such as notch. For example, FGF has been shown to be important for lung development in mouse, while Notch has been proven to be required for tooth morphogenesis [34, 35].

CELLULAR POLARIZATION

Epithelial cells have three distinct surfaces, the apical surface facing the lumen, the lateral surface facing neighboring cells and the basal surface facing the ECM. Epithelial cells are polarized and their cell polarity is regulated by intrinsic mechanisms, such as coordinated targeting of vesicles with apical and basolateral proteins to distinct surfaces. There are three groups of proteins responsible for forming and maintaining baso-apical polarity: (1) the Par complex (Par proteins and atypical protein kinase C); (2) the Scribble (Scrib) complex (Scrib, Disc large (Dlg), and Lethal giant larvae (Lgl)); (3) the Crumbs (Crb) complex (Crb, PALS1, and PATJ) [36] (Figure 4). Extrinsic factors regulate polarity by forming junctional complexes to create barriers between apical-basal surfaces and cell contacts to the ECM. This influences the vesicular transport as well as transcription of genes necessary for establishing polarity.

Adherens junction proteins like cadherins are shown to play an important role during cell polarization. Disruption of adherens junctions can alter cell polarization. This was investigated by McNeill and colleagues by transfecting non-polar L-cells with E-cadherin. The cells expressing E-cadherin redistributed Na^+, K^+ ATPase from a diffuse pattern to the regions of cell-cell contacts. This indicates a direct role of cell adhesion molecules as inducers of cell polarity [37].

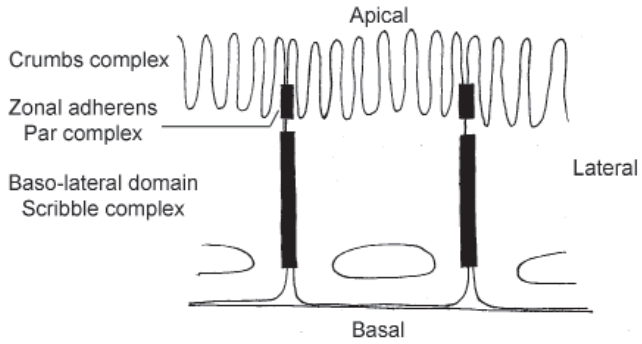

Figure 4. Cell polarization by polarity complexes. The apical side express Crumbs complex, while zonal adherens express Par complex and the baso-lateral domain express Scribble complex.

CELL MIGRATION

Cell migration is essential for normal development but also for responses to tissue damage and infections. Cell migration also occurs in diseases; cancer, atherosclerosis, and rheumatoid arthritis. Thus, preventing the migration of specific cell types could inhibit disease progression [38].

Migrating cells undergo changes in the cytoskeleton, cell-substrate adhesions and the extracellular matrix. Many cell types migrate as single cells, including leukocytes, and neuronal cells. Epithelial and endothelial cells migrate as sheets or group of cells. Cells moving as sheets or groups are connected via cell-cell adhesion to neighboring cells that generate strong forces, whereas single cells rely on cell-substrate interactions providing less force.

Migrating cells are guided via different chemo attractant ligands, most commonly chemokines or growth factors. For instance, angiogenic sprouting of blood vessels is dependent on vascular epidermal growth factor A (VEGF-A) for migration [39]. Cells migrate towards an area expressing higher levels of VEGF-A and will continue migrating until the sprouts migrate into a region of local hypoxia. Local hypoxia downregulate VEGF-A and cause the cells to stop migrating [40]. PDGFA is important for migration in mesodermal cells. Loss of PDGFA results in randomized migration [41]. Epithelial cells undergoing epithelial to mesenchymal transition (EMT), which is triggered by several signaling pathways; bone morphogenetic protein (BMP), FGF, and Wnt, is usually associated with the enhanced migratory behaviour. FGF induces EMT by down regulating E-cadherin and represses polarization via the transcription factor Snail.

Cadherins are not only important for EMT, but also to promote cell-cell adhesion. When cells migrate cadherin-mediated cell-cell interactions have to break and reform. DE-cadherin has been shown to be important in border cell migration in Drosophila. Cadherin 11 regulates fillipodia and lamellipodia formation via small RhoGTPases [42]. Lamellipodia formation is dependent on fibronectin-integrin interactions. The connection between the ECM and the integrins is also essential for cell-adhesion, development of directed protrusions, and cell polarity-processes highly relevant to migratory cells.

CELL SORTING

Cell sorting is a process where differentiated cells separate from one another and form clusters that constitute organized tissues. This process is mediated by classical cadherins. Cadherins are expressed to a variable degree in distinct tissues during development. For example, E-cadherin is expressed in all epithelial cells, N-cadherin in neural tissue and muscles, R-cadherin in forebrain and bone, and VE-cadherin in endothelial cells and so on. The specific expression pattern of cadherins combined with homophilic adhesive properties, may facilitate sorting of specific cell types in tissues. Experiments performed by mixing cadherin-negative L-cells transfected with E-cadherin and L-cells transfected with P-cadherin, showed that the cells were able to sort out and cluster [43]. Even mixtures of cells expressing the same cadherin but with different levels of adhesion sorted from one another [44].

CELL SURVIVAL

Cellular interactions with neighboring cells induce a variety of signaling events, including survival and differentiation. Studies in different genetic models where cadherins have been deleted reveal an essential role for these molecules in cell survival. For instance, studies on R-cadherin, N-cadherin, and E-cadherin show that cadherins are important for cell survival.

Ablation of *R-cadherin* in the ureteric bud epithelium show an altered morphology and branching behavior, including increased apoptosis [45]. It is not known if the decreased cell survival is directly due to the deletion of *R-cadherin* or if it is secondary due to the changes in morphology of the epithelium.

N-cadherin deficient embryos exhibit a decrease in cell survival. The deficient embryos have increased apoptosis within the collapsing neural folds and somites [26]. Since *N-cadherin* deficient embryos have a cardio-vascular phenotype, the researchers performed a rescue experiment expressing N-cadherin or E-cadherin in the heart. The embryos survived longer but were still smaller and exhibited increased apoptosis, demonstrating that N-cadherin is directly involved in cell survival [28, 46].

E-cadherin is also known to mediate cell survival. Arulanandam and colleagues have shown that E-cadherin promotes cell survival via *Rac1* and *Cdc42* by activating the signal transducer and activator of transcription-3 (Stat3). *Stat3* increases both the expression levels and activates *Rac1* and *Cdc42*. Morever, inhibitors of *Rac1* and *Cdc42* block E-cadherin-mediated *Stat3* activation, indicating that *Rac1* and *Cdc42*, are the mediators of the cadherin signal to *Stat3*.

ES-cells lacking E-cadherin undergo a dramatic induction of apoptosis. The survival signals may be mediated by an increase of *Rac1* and *Cdc42* activity, leading to *Stat3* stimulation. This could explain why Stat3 inhibition in cells expressing cell-cell adhesion can induce apoptosis [47].

DIFFERENTIATION

The generation of cellular diversity is called differentiation. Differentiation depends on many signaling pathways, e.g. Wnt, Hedgehog, and Notch. These signaling pathways activate various transcription factors, which in turn initiate cell differentiation, for example, suppression of Wnt signaling in the anterior endoderm is required for both liver and pancreas development [48]. The mesodermal tissues surrounding the posterior foregut provide secreted signals that promote liver and pancreas. In the ventral posterior foregut, BMP secreted by the septum transversum mesenchyme and FGF1 and FGF2 produced by the cardiac mesoderm promote liver development while concomitantly suppressing the pancreatic differentiation program [46, 49-54].

Initially, transcription factors pattern the endoderm along the anterior to posterior axis. For example, Sox2 is expressed in the anterior domain of the endoderm that will give rise to the esophagus and stomach; Pdx1 expression is found in the antral stomach, presumptive pancreas, common bile duct, and rostral duodenum [55]; Cdx2 is expressed in the entire postgastric epithelium

in the regions which will form intestine [56] (Figure 3). Initially, the presumptive pancreatic domain is marked by overlapping expression of Pdx1, Ptf1a, and Hlxb9/Hb9 [55, 57-59].

PANCREAS DEVELOPMENT

ANATOMY AND FUNCTION

The pancreas is an endoderm derived organ consisting of two major cell types: exocrine and endocrine cells (Figure 5). The exocrine part of the pancreas consists of acinar cells and ductal cells forming a highly branched duct system. The acinar cells produce digestive enzymes, such as proteases, lipases, and nucleases. These enzymes are secreted into the ductal system and transported to the intestine by the ampulla of Vater. The enzymes produced by the exocrine tissue are necessary for digesting food.

Approximately 98% of the pancreas consists of exocrine tissue; the other 2% are endocrine tissue [54]. The endocrine cells form clusters called islets of Langerhans. The islets are divided into five cell types; α-, β-, ε-, δ-, and pancreatic-polypeptide cells. These five cell types produce the hormones; glucagon, insulin, ghrelin, somatostatin, and pancreatic polypeptide respectively. The core of the islets contains mainly β-cells; which are surrounded by α-, ε-, δ-, and PP-cells. Insulin-producing β-cells make up for ~60-80% of the islet, while 15-20% are glucagon-producing α-cells; 5-10% are somatostatin-producing δ-cells, <2% are pancreatic-polypeptide-producing cells, and <1% is ghrelin-producing ε-cells [60].

Endocrine cells are important for controlling blood glucose homeostasis. After food intake, blood sugar levels increases, and insulin is released from β-cells. This is the signal for liver, muscle, and fat to store glucose and thereby control glucose homeostasis. In contrast, glucagon secretion is stimulated at low blood-sugar levels. The release of glucagon primes β-cells to secrete more insulin when the glucose levels rises. Somatostatin and pancreatic polypeptide apply inhibitory effects on both endocrine and exocrine secretion [60].

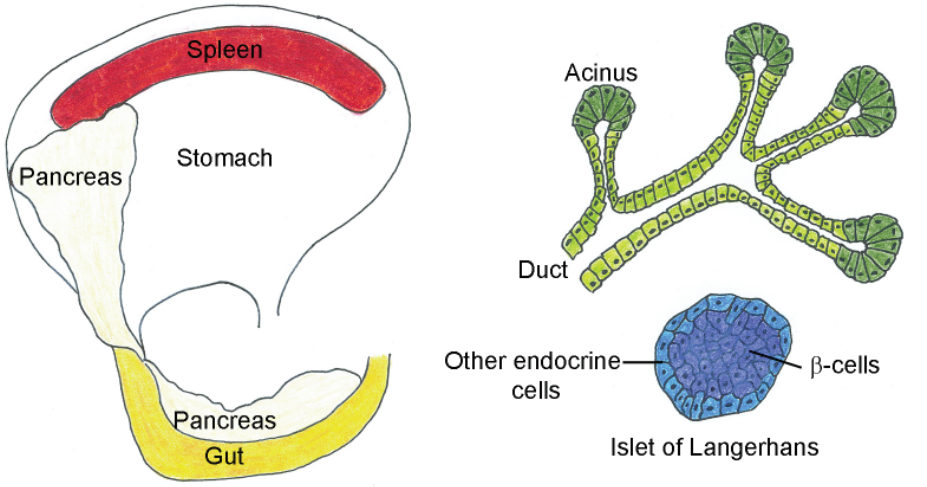

Figure 5. Anatomy of mouse pancreas. Pancreas develops posterior to the stomach and is connected to the gut via Ampulla of water. The pancreas consists of exocrine and endocrine tissues. The exocrine part is composed of acinar cells and ducts. The endocrine part forms islets of Langerhans.

INITIATION OF THE PANCREATIC PROGRAM

The pancreas develops from the posterior foregut endoderm expressing Pdx1/Ipf1. At E9.5 in the mouse, the dorsal and ventral buds of the pancreas begin to evaginate. All cell lineages in the pancreas originate from $Pdx1^{+}$ cells [61]. Deletion of *Pdx1* results in arrested pancreas development at E10.5. Before the dorsal pancreas starts budding the endoderm is in close proximity to the notochord (Figure 6). Factors from the notochord, such as activin-βB and FGF2, prevent the expression of the hedgehog genes. If the hedgehog genes, sonic hedgehog (Shh) and Indian hedgehog (Ihh) are not suppressed, the intestinal differentiation program will be initiated at the expense of dorsal pancreas development [62]. The ventral pancreas is not in contact with the notochord indicating that another pathway inhibits hedgehog genes in the ventral pancreas [63]. Patterning of the ventral bud relies on signals from the overlying cardiac mesenchyme and the lateral plate mesoderm.

When the dorsal pancreas starts to protrude, the notochord is no longer in close proximity to the dorsal pancreas. Instead the dorsal aortas fuse and become in direct contact with the dorsal pancreatic endoderm. Signals from the dorsal aorta have been shown to be important for differentiation and growth of the

pancreatic endoderm [64], partly by inducing expression of Ptf1a [65]. Ptf1a is expressed in the pancreas from E9.5 and Ptf1a^{+} cells give rise to all endoderm derived cell lineages of the pancreas. *Ptf1a* knockouts fail to form a ventral pancreas and formation of the dorsal pancreas does not proceed beyond the initial budding.

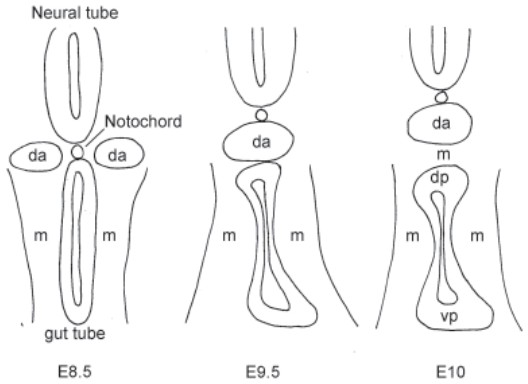

Figure 6. **Early pancreas development.** The developing gut endoderm is in contact with the notochord at E8.5. One day later the dorsal aortas have fused and are in close vicinity to the dorsal pancreatic endoderm. At E10 the mesenchyme is surrounding the dorsal pancreatic bud. Abbreviations: da, dorsal aorta; m, mesenchyme; dp, dorsal pancreas; vp, ventral pancreas. Picture modified from Slack, J 1995.

PANCREATIC MORPHOGENESIS

At E10 the pancreatic epithelium is surrounded by mesenchymal cells that control its growth and differentiation [66, 67]. Several mesenchymal factors are essential for proper pancreas growth. The transcription factor Isl1 is expressed throughout the dorsal mesenchyme during bud formation. In *Isl1* mutants, the mesenchyme is absent, and there is an associated failure of exocrine cell differentiation in the dorsal but not ventral pancreas [68]. There is also a complete loss of differentiated islet cells. By adding wild type mesenchyme to pancreas *Isl1-/-* epithelium, the exocrine phenotype could be rescued. This shows that *Isl1* in the mesenchyme is important for dorsal exocrine pancreas and that *Isl1* in the endoderm is required for the generation of all endocrine cells.

SPECIFICATION AND DIFFERENTIATION OF THE PANCREAS

Generation of a ductal tree reprints proliferation of the pancreatic epithelium and mesenchyme, branching morphogenesis, and fusion of the dorsal and ventral buds at E12.5. This branched epithelial organ contains the precursor cells for islets, acini, and ducts [69]. The tip cells in the ductal tree are marked by expression of Craboxypeptidase A1 (Cpa1), Pdx1, Ptf1a, and c-Myc [70]. At early stages, these cells contribute to all lineages by acting as multipotent progenitors. Later in development cells from the stalk in the ductal tree will give rise to endocrine- or ductal-cells, while the tip cells differentiate into acinar cells. The peak in exocrine and endocrine differentiation, begins at E13.5 in mouse, and is referred to as the secondary transition.

EXOCRINE LINEAGE

Differentiation of the epithelium into exocrine tissue is mesenchyme dependent. Cultures with pancreas epithelium lacking mesenchyme promote endocrine cell differentiation resulting in impaired exocrine differentiation [71-73]. Mouse pancreatic epithelium exposed to the ligands (activin and transforming growth factor-β1 (TGF-β1) for TGF-β signaling promotes development of endocrine cells, particular β-cells and PP-cells [74], and disrupts epithelial branching and acinar formation [75]. Furthermore, an antagonist of TGF-β signaling (follistatin) promotes exocrine differentiation [73].

DUCT LINEAGE

By E12.5 the specification of different lineages (exocrine, ductal, and endocrine) is initiated. The factors regulating ductal differentiation are not well understood [76]. However, several gene deletions have branching phenotypes. Inactivation of FGF10 or FGFR2IIIb leads to a reduced number of branches and decreased proliferation [77, 78]. The epidermal growth factor (EGF) family is also important for growth and branching [79]. Ablation of hepatocyte nuclear factor-6 (*Hnf-6*) demonstrate enlarged lumina and multiple cysts within the ducts as well as impaired formation of primary cilia and mislocation of the adherens junction proteins, including β-catenin [80]. Sox9 mutants also reveal cystic ducts and reduced exocrine differentiation [81]. Small Rho GTPases such as Cdc42 have also been demonstrated to be required for pancreatic duct differentiation. Ablation of *Cdc42* increased acinar differentiation at the expense of endocrine and duct lineage commitment [82].

ENDOCRINE LINEAGES

All endocrine cells are derived from Neurogenin-3^{+} cells (Ngn3). Ngn3^{+} cells arise from Sox9 progenitor cells within the duct epithelium. These cells differentiate, delaminate and migrate to form the islets of Langerhans (Figure 7). Mutant *Ngn3* lack all endocrine cells and die shortly after birth due to hyperglycemia [83, 84]. Forced expression of Ngn3 at different time point indicates that timing is important for epithelial cells to respond to Ngn3 and differentiate into distinct endocrine cell lineages [85]. Maturation and maintenance of endocrine cells is also dependent on *Ngn3* [86].

Downstream targets of *Ngn3* are the *Pax* and *Nkx* genes, *NeuroD*, *Isl1*, and *Arx*. Together these genes maintain the balance between the different endocrine cells [87]. Mice lacking expression of *Isl1*, *Pax6*, *NeuroD*, *Nkx 2.2*, or *Nkx 6.1* exhibit defects in endocrine formation, while *Pax4* and *Arx* mutants show a shift in endocrine lineages.

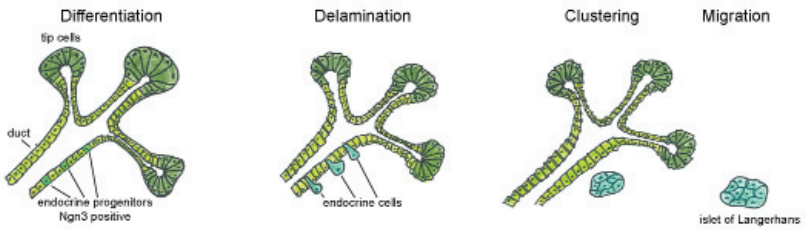

Figure 7. **Endocrine differentiation and morphogenesis.** The ductal epithelium consists of endocrine progenitor cells expressing Ngn3. The Ngn3^{+} cells delaminate from the ducts and cluster into islets of Langerhans. The islets migrate away from the ductal epithelium and are found within the exocrine tissue.

CELL ADHESION MOLECULES IN PANCREAS DEVELOPMENT

Several cell adhesion molecules are expressed during pancreas development. For instance, E-cadherin is expressed in all pancreatic epithelial cells when the pancreas starts to form and continues to be expressed in exocrine, ductal, and endocrine lineage throughout development and adulthood. To investigate if cadherins mediate organization of endocrine cells into islets of Langerhans, a dominant negative mutant of mouse *E-cadherin* was expressed in insulin-

producing cells, resulting in displacement of both E-cadherin and N-cadherin in the β-cells. As a result, the architecture of the islets was disorganized. β-cells were found as individual cells within the tissue, whereas α-cells aggregated into islets [88]. R-cadherin is also expressed in the majority of the cells in the pancreatic buds. In adults, R-cadherin is primarily expressed in the ductal system of the pancreas, on the apical side of the exocrine tissue, in intraductal endocrine cells, but low or no expression in endocrine cells [89, 90]. The *R-cadherin* mutant mice had no pancreatic phenotype (Dahl and Semb unpublished data). N-cadherin has been shown to be expressed in both dorsal and ventral bud at embryonic day E9.5 and being specifically expressed in the islets of adult pancreas [27, 91]. Ablation of *N-cadherin* resulted in a lack of the dorsal bud [27].

Another cell-cell adhesion molecule expressed in the pancreas is N-CAM. It is expressed both in pancreatic mesenchyme and epithelium. The expression becomes restricted to endocrine cells, and peripheral nerve endings and ganglia. *N-CAM* mutants develop a normal pancreas, consisting of all pancreatic cell types and islets of normal size and number, scattered within the exocrine tissue. However, lack of NCAM results in disrupted organization and morphology of the islets. α-cells were distributed centrally in the islets and not in the periphery. This suggests that N-CAM regulates cell type segregation within islets. Islet cell polarity was also affected in *N-CAM* mutant mice. E-cadherin and N-cadherin were distributed subcellularly, whereas R-cadherin distribution was unaffected [92].

BLOOD VESSELS IN PANCREAS DEVELOPMENT

During initiation of pancreas organogenesis the buds are in close vicinity to large vessels, the aorta dorsally and the vitelline veins ventrally. Several experiments have shown that endothelial signaling is important for pancreas development. Cleaver and colleagues removed the endothelial cell precursors of the dorsal aorta from frog embryos. Thus endocrine gene expression was inhibited, while the development of liver and neural tube proceeded normally [64, 93]. The result suggests that endothelial cells are required for endocrine development.

Recombination of prepatterned dorsal endoderm with dorsal aorta, notochord, and neural tube indicated that dorsal endoderm together with dorsal aorta can only initiate Pdx1 expression and insulin expression. Dorsal endoderm with

notochord and neural tube did not induce insulin. However, the notochord did induce Pdx1. This experiment showed that endothelial cells supply signals sufficient for insulin expression [64].

The third experiment showing that endothelial cells are important for insulin expression, was to analyze the role of VEGF-A. Overexpression of VEGF-A in the Pdx1-expressing domain resulted in increased vascularization. These endothelial cells instructed foregut cells to differentiate into pancreatic cells.

The results presented above all indicate that endothelial cells promote pancreas development. In vitro experiment on *Flk1*$^{-/-}$ embryos (VEGFR2) lacking endothelial cells induced Pdx1, indicating that aorta or endothelial cells are not required for initial pancreas development. The expression of Ptf1a was lost in the in the *Flk1*$^{-/-}$ embryos, indicating that endothelial cells are important for the appearance of the dorsal bud and to maintain Pdx1 expression [65].

SPHINGOSINE-1-PHOSPHATE

Sphingosine-1-phosphate (S1P) is a metabolite formed by the phosphorylation of sphingosine by sphingosine kinase [94, 95]. S1P can be recycled back to sphingosine by S1P phosphatase or be degraded by an S1P lyase [96, 97, 98]. S1P is produced and secreted by red blood cells, platelets, monocytes, mast cells, and possibly by endothelial cells [99-101]. Since S1P is highly soluble in water it is bound to high density lipoprotein (HDL) or to albumin in plasma [102].

S1P triggers several activities in cells including proliferation, migration, cytoskeletal changes, adhesion molecule expression, and anti-apoptotic effects [103-107]. Many of these activities are produced via the interaction of S1P with G protein-coupled receptors [108].

SPHINGOSINE-1-PHOSPHATE RECEPTOR-1

S1P bind to five different receptors, named $S1P_{1\text{-}5}$ [109]. The receptors bind to different G proteins. $S1P_1$ bind to $G_{i/0}$, $S1P_{2,3}$ bind to $G_{i/0}$, G_q, $G_{12/13}$, and $S1P_{4,5}$ bind to $G_{i/0}$, $G_{12/13}$ and thereby mediate different signaling pathways. Many cell types express several S1P receptors. For example, endothelial cells express $S1P_1$ and $S1P_3$, vascular smooth muscle cells express $S1P_2$ and $S1P_3$, and cardiac muscles express $S1P_1$, $S1P_2$, and $S1P_3$ [109].

$S1P_1$ is highly expressed in the cardiovascular system during mouse development [110] and in the adult mouse; in humans $S1P_1$ is expressed at significant levels in brain, heart, lung, spleen, liver and low expression was detected in kidney, muscle and thymus [111-113]. Mice lacking *$S1P_1$* die at E12.5-14.5 due to vascular maturation defects. These embryos develop a normal blood vessel network at E12.5, indicating that $S1P_1$ was not essential for endothelial cell differentiation, proliferation, migration and tube formation during vasculogenesis or for sprouting and branching of vessels during angiogenesis [110]. Instead, the association of vascular smooth muscle cells and pericytes was defected. The dorsal aorta was not covered by vascular smooth muscle cells. Cells were lining the ventral part of the aorta and not the dorsal. The defects in vascular smooth muscle cell coverage extended to arteries and capillaries in the brain and also to small vessels in the limbs. The lack of vascular smooth muscle cells resulted in a weakened vasculature with disrupted and leaky vessels. The defect seen in the *$S1P_1$* mutant might be caused by impaired vascular smooth muscle cell and pericyte differentiation, proliferation and/or migration. For example, fibroblast cells from the *$S1P_1$* mutant failed to migrate towards S1P in vitro [114].

INSULIN CELL MATURATION AND GLUCOSE HOMEOSTASIS

After digestion, a variety of nutritional factors in circulation, including amino acids, fatty acids and glucose initiate release of insulin. A serial of signaling cascades responsible for the suppression of hepatic glucose output, increased synthesis of glycogen and triglycerides, and stimulation of peripheral tissue uptake of glucose begin. In addition, hormones from the gut (incretins) are secreted, which increases insulin secretion from the pancreas in a glucose-dependent manner [115].

Insulin is synthesized as preproinsulin on the rough endoplasmic reticulum (RER). It is converted into proinsulin and transferred to the Trans-Golgi Network (TGN), where it is packed into immature secretory granules. These vesicles fuse directly to the plasma membrane or enter the recycling endosome system. The immature granules undergo several maturation processes, such as acidification, conversion of proinsulin into insulin and C-peptide, and loss of the coat protein clathrin [116, 117].

There are two pools of insulin secretory granules, the readily releasable pool (RRP) that is responsible for the initial phase of insulin secretion and a second reserve pool that is responsible for a more prolonged insulin secretion phase [118-121]. The RRP is pre-docked to the membrane and is released within 1-5 min. During this first phase approximately 15 granules per minute are released. The second phase continues for 5-60 min and releases 5 granules per minute [119]. A mouse β-cell contains more than 10 000 secretory granules [122]. Only 1-5% of the secretory granules belong to the RRP.

High glucose in the blood is sensed by glucose transporter 2 (GLUT2) in the plasma membrane. Via glycolysis the ratio of ATP/ADP increases and closes the ATP-sensitive K^+ channels. This leads to a depolarization that activates Ca^{2+} channels resulting in extracellular Ca^{2+} influx and fusion of insulin granules with the plasma membrane. At the same time GLP-1 binds to its receptor and activates cAMP that binds to EPAC. EPAC increases the numbers of insulin granules in the RRP at the plasma membrane (Figure 8). Together these processes results in insulin secretion [119].

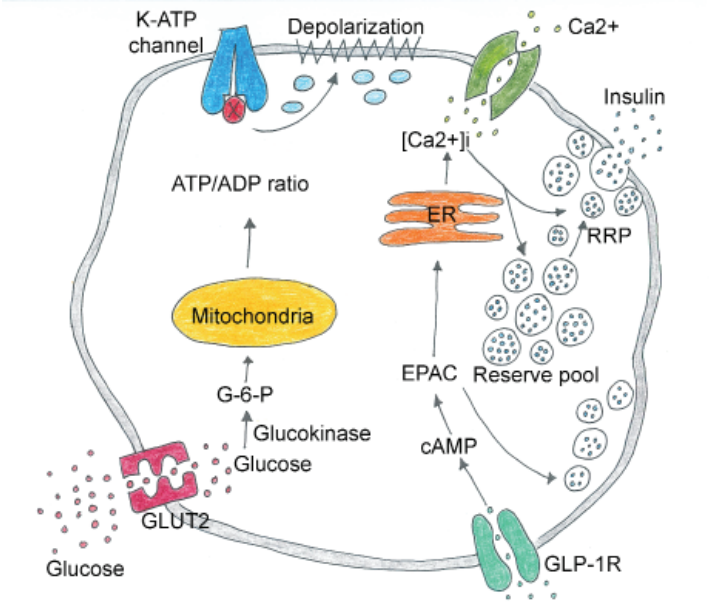

Figure 8. **Insulin signaling.** Glucose increase ATP/ADP ratio leading to closing of K^+ channels. Depolarization of the membrane, increased concentration of Ca^{2+} within the cell, and fusion of insulin granules with the plasma membrane increase cAMP. cAMP binds to EPAC to increase Ca^{2+} and thereby increasing the insulin granules within the readily releasable pool. Light blue dots represent insulin granules. Picture modified from Hou et al 2009.

DIABETES

The most common forms of diabetes are type 1 and 2, however there are several different types of diabetes. Type 1 diabetes is an autoimmune disease resulting in destruction of β-cells by the immune system. Type 2 diabetes is caused by insulin resistance. The pancreas in type 2 diabetic patient has an increased β-cell mass to compensate for the elevated need for insulin. Eventually this compensatory mechanism fails and the patient develops diabetes.

Diabetes is a disease that is associated with major complications, including retinopathy, neuropathy, kidney failure and cardiovascular defects. The diabetic retinopathy causes blindness, while diabetic neuropathy causes symptoms of tingling, pain, numbness, or weakness in the feet and hands. Kidney failure as well as cardiovascular defects is also common causes of death in diabetes patients (World Health Organization; www.who.int).

The most common treatment for patients with type 1 diabetes is daily injections of recombinant insulin. This is a fairly efficient treatment but excursions in blood glucose levels may still be difficult to control. Thus alternative treatment options are currently explored. Type 1 diabetic patients have been injected with donor islets. This procedure was able to restore euglycemia [123]. However, two major obstacles are associated with this procedure: only 10% of the patients were insulin free after 5 years of transplantation, and secondly there are not enough donors.

Thus alternative sources for β-cells are needed. One possibility would be to differentiate human Embryonic Stem Cells (hESC) into transplantable β-cells. hESC have been proven to be able to differentiate into insulin producing β-cells [124] and in vitro studies have also succeeded in differentiating hESC into insulin producing β-cells [125-127]. Efficient and reproducible differentiation protocols will be needed to develop fully functional, transplantable β-cells.

TECHNICAL CONSIDERATIONS

THE GENE-TRAP SYSTEM

Gene-trap mutagenesis is a technique that randomly generates loss-of-function mutations and reports the expression of many mouse genes. A Gene-trap vector contains a splice acceptor site immediately upstream of a promoterless reporter. Since the Gene-trap has no promoter of its own, it is driven by an endogenous promoter close to the insertion site. The Gene-trap also contains a β-galactosidase-Neo^Rfusion (β-Geo) cassette in order to work as a reporter. A disadvantage using a Gene-trap vector is that, because the insertion occurs in an intron, alternative splicing can sometimes take place. This might lead to lower levels of the wild-type transcripts, resulting in hypomorphic alleles [128].

THE *Cre/loxP*-SYSTEM

Traditional knockout mice may be hard to investigate since many mutants are lethal during embryogenesis. However, even if the mutants do survive until adulthood it might be hard to interpret the data. Since an observed phenotype might be due to a primary defect in another tissue. A solution to this problem is to use the *Cre-loxP* recombination system. The *Cre-loxP* system allows deletion of a gene and its expression in a particular tissue or cell type. *Cre* is a recombinase that recognizes 34 base-pair *loxP*-sequences and catalyses the recombination between two *loxP* sites by excising the intervening DNA [129]. A sequence that is flanked with two *loxP* sites is referred to as a floxed sequence. The floxed sequence will get excised from the genome in the presence of a *Cre*-recombinase.

RECOMBINATION VARIABILITY'S IN *Cre* TRANSGENES

A known problem with *Cre* transgenic mouse strains is the variability of recombination. This could be due to several factors, including integration site. Integration of a transgene into a site with an open chromatin structure is expected to increase availability for recombination events. Increased variability of transgenic expression is observed with increased number of backcrosses [130]. Different backgrounds of the responder mice may also affect variability in recombination efficiency.

AIM OF THE THESIS

The overall objective of this thesis was to understand the roles of S1P/$S1P_1$ and N-cadherin in pancreas development

The specific aims were:

Paper I. To address whether the pancreatic phenotype in *N-cadherin* deficient mice is due to a cell-autonomous function of N-cadherin within the mesenchyme or if it is secondary to other defects, e.g. cardiac and/or vascular function.

Paper II. To study the functional role of S1P signaling from endothelial cells in pancreas development using *$S1P_1$* deficient embryos.

Paper III. To investigate the consequences of *N-cadherin* ablation in the developing pancreas, specifically in morphogenesis and endocrine cell differentiation.

PAPERS IN SUMMARY

PAPER I

INTRODUCTION

N-cadherin is required for the formation of the dorsal pancreas, by mediating dorsal pancreatic mesenchymal cell survival [27]. *N-cadherin* deficient mice die of cell adhesion defects in the heart at E9.5. These mice also exhibit defects in the development of the nervous system, somites and yolk sac. Importantly, when cadherin function was restored in the heart of *N-cadherin* mutant mice, the embryos survived until E10.5-11 due to rescued heart and vascular function. The aim of this investigation was to address whether the pancreatic phenotype in *N-cadherin*-deficient mice reflects a cell-autonomous function of N-cadherin within the pancreatic mesenchyme or if it is secondary to other defects, e.g. cardiac and/or vascular function.

RESULTS

Cardiac-rescued *N-cadherin*-deficient mice develop a dorsal pancreas due to an intact circulatory system

Cardiac rescued *N-cadherin*-knockout mice were analyzed to assess if the lack of the dorsal pancreas in *N-cadherin*-deficient mice is due to a cell-autonomous function within the mesenchyme or if it secondarily caused by other defects (e.g. cardiac and/or vascular function). The most dramatic consequence of expressing N-cadherin within the heart in the *N-cadherin*-mutant mice was the restoration of an intact circulatory system. As a consequence of a functioning circulatory system the dorsal pancreas was formed. The lack of mesenchyme recruitment in the *N-cadherin*-deficient mice was not observed in the rescued *N-cadherin*-knockout mice. This indicates that N-cadherin does not play a cell-autonomous role in mesenchymal cell survival. To identify how the cardiac/vascular function affects pancreas development, explants from *N-cadherin*-deficient embryos were incubated with beads soaked in plasma. The dorsal pancreas was rescued. Endoderm with and without mesenchyme was also cultured with plasma to address whether plasma acts on mesenchyme or epithelium. Our results show that plasma acts primarily on mesenchyme.

S1P rescues the formation of the dorsal pancreas in *N-cadherin*-deficient pancreatic explants.

The dorsal aorta is in close vicinity to the pancreatic endoderm and the vascular smooth muscle cells of the dorsal aorta are derived from mesenchymal cells. It has been demonstrated that vascular maturation is deficient in mice lacking the S1P receptor $S1P_1$ due to deficient vascular smooth muscle cell recruitment. S1P is a lipid present in the circulation. To test whether the plasma-induced rescue of the dorsal pancreas could be mediated by S1P, we incubated explants with beads soaked in S1P. The explants developed a dorsal pancreas and S1P's effect was specifically seen on the mesenchyme.

S1P receptors in pancreas

S1P binds to receptors $S1P_{1-5}$ with high affinity. RT-PCR and in situ hybridization of these receptors show that $S1P_1$ is expressed in endothelial cells and $S1P_{2-3}$ are preferentially expressed in the mesenchyme, but that $S1P_{4-5}$ cannot be detected. S1P receptors are G-protein coupled and to block their activity we cultured explants with pertussis toxin that inactivates G_i. Pertussis toxin blocked the S1P-mediated rescue of the dorsal pancreas as well as early pancreas development in wt explants.

S1P regulates dorsal pancreatic mesenchymal cell proliferation

The mechanism by which S1P rescued the dorsal pancreas development may involve a direct effect on pancreatic mesenchyme. Mesenchymal development involves both cell migration and proliferation, and to investigate if S1P act on any of these processes, we cultured primary mesenchymal cells. BrdU-labeling experiments demonstrated that S1P increase the number of proliferating cells. Importantly, pertussis toxin inhibits this effect.

SUMMARY

We showed that restoring cardiac and circulatory function in *N-cadherin*-deficient mice by cardiac-specific expression of N-cadherin, rescues formation of the dorsal pancreas. This indicates that the phenotype observed is secondary to defects related to cardiac/vascular function. Based on this observation, we demonstrated that plasma and S1P-mediated G-protein-coupled signaling rescues formation of the dorsal pancreas in vitro.

PAPER II

INTRODUCTION

During early pancreatic development major blood vessels are in close contact with the pancreatic endoderm. The dorsal bud forms close to the dorsal aorta, while the ventral buds emerge close to the vitelline veins. Endothelial signals have been shown to be important for initiation of the dorsal bud. However, endothelial signals are not required for initiation of the ventral bud.

Later in development the blood vessels and the pancreatic endoderm get separated by invading mesenchyme. Blood vessels are embedded within the mesenchyme and endoderm-endothelial interactions continue. Previously, we have shown that a functional vascular system is required for initiating dorsal pancreas formation. Signaling cues from the circulation, in particular the bioactive sphingolipid metabolite S1P is essential. *S1P*$_1$ deficient embryos were analyzed to investigate the role of S1P signaling in endothelial cells during pancreas development.

RESULTS

Growth and branching morphogenesis of the pancreatic epithelium is compromised in *S1P*$_1$-deficient embryos

To measure the effects of specific ablation of S1P$_1$ in the pancreas, the pancreatic volume was scanned with Optical Projection Tomography (OPT). Analysis showed that the pancreatic buds from *S1P*$_1$-deficient embryos did not protrude as far as pancreatic buds from wild type embryos. The dorsal bud was significantly smaller in the *S1P*$_1$-deficient embryos. The ventral bud was also smaller but not significantly smaller. There were no differences in the structure of the tubular network in the distal parts of the dorsal and ventral buds. However, the proximal parts were less branched in the *S1P*$_1$-deficient embryos.

Other endoderm-derived organs, such as liver, ventricle, and lung, revealed a more general effect of *S1P* deletion on endodermal development, for example the lung had fewer branches, and liver and ventricle were smaller.

$S1P_1$ is required for proliferation of $Pdx1^+$ pancreatic progenitors

Possible explanations for smaller dorsal and ventral buds in the *$S1P_1$*-deficient embryos are less proliferating cells and/or more apoptosis. Wild type and mutant embryos were BrdU-pulse labelled and stained for Pdx1 and BrdU. There were significantly fewer proliferating $Pdx1^+$ cells in the *$S1P_1$*-deficient embryos compared to littermate controls. To investigate if there were more apoptotic cells, we stained for Caspase 3. No significant change was observed. We also looked at endocrine differentiation but there were no significant change between *$S1P_1$*-deficient and control embryos. Thus, $S1P_1$ is required for proliferation of multipotent $Pdx1^+$ pancreatic progenitor cells, but not for endocrine cell specification.

$S1P_1$ deficiency results in defective pancreatic morphogenesis in vitro

To exclude that the phenotype observed is caused by the cardiovascular defect, in vitro explants studies were performed. All control explants developed normally, while the *$S1P_1$*-deficient explants showed a rather diverse phenotype. The explants development spanned from severe growth defects to showing only size reduction and less branching.

Blood vessel ablation does not mimic the $S1P_1$ phenotype

Our results indicate that endothelial cells, via $S1P_1$, provide inductive cues that are necessary for pancreatic endoderm development. To test this, we ablated all blood vessels using quinolin-urea. After two days of incubation, starting at E11.5, there were no affect on the pancreatic endoderm, suggesting that expansion of progenitor cells beyond E11.5 does not require endothelial cells.

SUMMARY

Developing organism are all dependent on blood vessels providing oxygen and nutrients as well as inductive cues that control cell proliferation and cell specification. It is known that endothelial cells control dorsal pancreas outgrowth and early endocrine cell specification. However, it is not understood how endothelial cells control these processes. Here, we identify $S1P_1$ as a new signaling pathway that is necessary for development of foregut derived organs, such as lung, liver, stomach and pancreas. Ablation of *$S1P_1$* results in reduced size of the dorsal pancreatic bud due to decreased proliferation of $Pdx1^+$ cells.

PAPER III

INTRODUCTION

Previously we demonstrated that N-cadherin is expressed in the pancreatic epithelium at E9.5, but later becomes restricted to endocrine aggregates in mice. Furthermore, in the absence of N-cadherin the dorsal pancreatic bud fails to form. This is a secondary phenotype due to cardiac failure. The early lethality of *N-cadherin*-deficient embryos excludes a complete analysis of N-cadherin function in pancreas development. We generated a tissue specific knockout of *N-cadherin* in the early pancreatic epithelium to study the role of N-cadherin during pancreas formation and function.

RESULTS

N-cadherin expression during pancreas development

At E10.5-13.5, N-cadherin is expressed throughout the $Pdx1^+$ pancreatic epithelium. Later, at E14.5 N-cadherin is expressed at low levels in $Sox9^+$ cells, acinar cells and at high levels in mature endocrine cells. At E15.5, N-cadherin expression becomes restricted to a subpopulation of $Sox9^+$ cells, and never in $Ngn3^+$ cells. In addition $Isl1^+$ endocrine progenitors (hormone negative) exhibit a mosaic expression of N-cadherin and all hormone producing cells express N-cadherin. At this point acinar cells do not express N-cadherin. Throughout development N-cadherin is also expressed in neurons and blood vessels. From E18.5 and onwards N-cadherin is restricted to hormone producing cells, neurons and blood vessels.

Pdx1Cre-mediated ablation of *N-cadherin*

To study the function of N-cadherin during pancreas development *N-cadherin* was conditionally deleted in $Pdx1^+$ cells (cKO). To ensure that *N-cadherin* was ablated, N-cadherin expression was examined by immunofluorescence, and immunoblotting. At E13.5, the efficacy of *N-cadherin* ablation varied from <5% to almost complete ablation of *N-cadherin*. At E15.5, the expression varied between littermates, but N-cadherin expression was consistently maintained in more than 50% of the cells. From E18.5 and onwards, N-cadherin was no longer detectable in the pancreas of cKO individuals.

Pancreatic morphogenesis and endocrine specification is not affected in conditional *N-cadherin*-knockout mice.

To determine if N-cadherin is important for cell lineage specification, expression of specific markers for acinar, ductal, and endocrine cells was analyzed. No developmental defects were observed within the exocrine and endocrine compartment. To investigate if N-cadherin is important for initiation and/or maintenance of islet cell polarity, the distribution of characteristic epithelial junctional, apical and lateral markers was analyzed. However, the normal allocation of these cell polarity markers indicates that islet cell contacts and polarity is not altered. To understand if microtubule dynamics are altered in islets, α- and β-tubulin were analyzed. There was no difference observed between control and cKO. To investigate the role of N-cadherin in endocrine cell specification and islets formation we measured insulin area versus E-cadherin area. This experiment did not reveal any difference between control and cKO, suggesting that N-cadherin is not required for β-cell specification. To study if other hormone-producing cells were affected, the ratio of glucagon$^+$, PP$^+$, Somatostatin$^+$ cells versus insulin$^+$ cells, respectively, were estimated in adult mice. The ratio was not altered, suggesting that N-cadherin appears to be dispensable for endocrine development.

N-cadherin controls insulin granule turnover

Transmission electron microscopy studies of islets showed a significant overall reduction (27%) of insulin secreting granules in mutant islets. 16% of the β-cells contained very few insulin granules which are a 67% reduction. Even if the β-cells with very few insulin granules were not included in the analysis, the difference was statistical significant. In contrast there is no difference in immature granules in the cKO, suggesting that the decrease in mature granules is not due to a change in biogenesis of granules.

N-cadherin regulates insulin secretion

Insulin secretion was studied in response to low and high concentration of glucose. In response to low glucose insulin secretion was significantly reduced in cKO. At high glucose, insulin secretion was also reduced but the change was not significant.

SUMMARY

By ablating *N-cadherin* specifically during pancreas development we show that N-cadherin is not essential for pancreas organogenesis. However, the late onset and high variability of recombination, suggest that a potential requirement of N-cadherin during pancreas development prior to E15.5 cannot be ruled out. *N-cadherin*-deficient endocrine cells aggregate into islets with normal morphology, indicating that N-cadherin is not required for islets morphogenesis. However, ultrastructural analysis of adult islets revealed a reduction in number of mature insulin secretory granules in *N-cadherin*-deficient β-cells. In conclusion our findings suggest that N-cadherin is dispensable to pancreas morphogenesis and cell fate specification, but is required for insulin secretory granules turnover and insulin secretion.

CONCLUDING REMARKS

PAPER I

N-cadherin does not act cell-autonomously on the mesenchyme surrounding the dorsal pancreas. When expressing cadherins in the heart the mesenchyme was recruited and the dorsal pancreas started to form. This data show that the agenesis seen in *N-cadherin* deficient mice is secondarily due to cardiac-vascular defects. The rescue experiment led us to believe that circulation is important for dorsal pancreas development. Therefore we soaked beads in plasma from E15.5 embryos and cultured explants from *N-cadherin*-deficient mice for two days. The addition of plasma was able to rescue the dorsal pancreas. S1P, a lipid produced and secreted by red blood cells, platelets, monocytes, mast cells, and possibly by endothelial cells, was also able to rescue dorsal pancreas formation. S1P secreted from the dorsal aorta binds to its receptors in the mesenchyme ($S1P_{1-3}$) and induce mesenchymal proliferation, which induce dorsal pancreas formation.

PAPER II

$S1P_1$ knockout have defects in endodermal organ formation, such as pancreas, stomach, liver and lung. In the pancreas this is due to less proliferation of the endoderm and not by increased cell death. To evaluate if the phenotype is due to cardiovascular defects, blood vessels were ablated from E11.5 wild type explants. The $S1P_1$ phenotype was not observed, suggesting that $S1P_1$ only

has an effect early in development and not during secondary transition. In conclusion we identified a pathway, by which endothelial cells, via $S1P_1$, control early pancreas development.

PAPER III

Ablation of *N-cadherin* specifically during pancreas development demonstrates that N-cadherin is not essential for pancreas organogenesis. However, due to the late onset and high variability of recombination, a role of N-cadherin during early pancreatic development can not be ruled out.

Concomitant with islet formation the efficiency of recombination was high. Normal architecture was seen in the *N-cadherin* deficient islets indicating that N-cadherin is not required for islet morphogenesis. However, ultrastructural analysis of adult islets revealed a reduction in the number of mature insulin secretory granules in *N-cadherin*-deficient β -cells. Since the number of immature insulin secretory granules was unaffected we conclude that N-cadherin is not required for granule biogenesis. Consistent with the low number of mature insulin secretory granules, *N-cadherin*-deficient islets displayed less insulin secretion when stimulated with low glucose. We speculate that this is due to fewer granules docked to the membrane and/or fewer granules in the ready releasable pool. Our findings suggest that N-cadherin is dispensable for pancreas morphogenesis and cell fate specification, but that N-cadherin is required for insulin secretory granules turnover and insulin secretion.

FUTURE PERSPECTIVES

PAPER I AND II

Understanding how S1P regulates growth in endoderm derived organs would be of great importance. Most likely the mechanism involves endothelial cells that secrete molecule/molecules that is/are important for endodermal growth.

It could also be of interest to identify other compounds from plasma that can induce dorsal pancreas development. Preliminary results show that lysophosphatidic acid (LPA) is one potential compound.

PAPER III

To further analyze if N-cadherin plays a role in pancreas development, it is of importance to use a *Pdx1-Cre* transgenic mouse line with high recombination efficiency or other *Cre*-lines with an early onset of expression in the pancreas, such as *Ptf1a*. Thereby, the role of N-cadherin in morphogenesis can be studied in greater detail.

To analyze how N-cadherin is required for insulin granule turnover, different insulin secreting pathways need to be studied. It would be important to clarify if fewer granules are docked to the membrane or if there are fewer granules in the ready releasable pool. TEM studies could be used to address this issue. It could potentially be of interest to see if any of the components in the insulin secreting pathway is defected in the cKO. Insulin secretion studies on islet stimulated with different components involved in insulin signaling pathway would answer this. To analyze if there is less insulin content, perfusion studies on islets will be performed. By adding high percentage of triton, the membrane breaks and all insulin granules are secreted. Preliminary data show that the cKO has lower insulin content.

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