ENDOTHELIAL- EPITHELIAL INTERACTIONS Blood vessels friends or foes?

Wolfhagen Sand, Fredrik

2011

ENDOTHELIAL- EPITHELIAL INTERACTIONS

Blood vessels friends or foes?

Fredrik Wolfhagen Sand

Stem Cell Center

Department of Laboratory Medicine, Lund

Faculty of Medicine, Lund University

Sweden

With the approval of the Lund University Faculty of Medicine, this thesis will be defended on January 7th 2011 at 13.00 in the Belfragesalen, BMC D15, Lund

Supervisor: Professor Henrik Semb

Faculty Opponent: Professor Lena Claesson-Welsh.

Department of Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Sweden.

DOKUMENTDATABLAD enl SIS 61 41 21

Organization LUND UNIVERSITY Stem Cell and Pancreas Developmental Biology Stem Cell Center Department of Laboratory Medicine, Lund	Document name DOCTORAL DISSERTATION Date of issue 2011-01-07 Sponsoring organization
Author(s) Fredrik Wolfhagen Sand	

Title and subtitle ENDOTHELIAL- EPITHELIAL INTERACTIONS
Blood vessels friends or foes?

Abstract

Diabetes affects enormous amounts of patients. In type 1 diabetes the insulin producing β cells in the islet of Langerhans are destroyed by the immune system. The β cells, which are located in the pancreas, regulate blood glucose levels. The lack of insulin production requires administration of insulin. This is accomplished by insulin injections. Even though the treatment methods have been improved, we are still not able to artificially regulate the blood glucose levels as evenly as the β cells. The fluctuations of the blood glucose levels results in the long term in a number of complication, among the diabetic retinopathy. Diabetic retinopathy is the leading cause of blindness among the younger population.

Both to reduce the complications of diabetes and to find cures of the disease is important.

By using a model for diabetic retinopathy in mice we have been able to show that lack of the molecule N-CAM is protecting against the pathological neovascularization affecting diabetic patients.

The last 10 years have proven it possible to cure diabetes through transplantations of islets of Langerhans from cadaveric donors. The transplantations requires suppression of the immune response, this could lead to other complications. The amount of diabetic patients in need of transplantations far exceeds the availability of donors.

The use of stem cells to produce insulin producing β cells might be a possible way. To be able to direct the stem cells into β cells it is important to understand how these cells form during embryo development.

By the use of mice we have been able to study how blood vessels regulate the growth of the precursors of β cells. We have also be able to show that the formation of a tubular system in the pancreas is important for the regulation of which cell types that are to be formed.

Key words: N-CAM, diabetic retinopathy, S1P1, endoderm development, pancreas, blood vessels, Cdc42, tubulogenesis, diabetes

Classification system and/or index termes (if any):

Supplementary bibliographical information:		Language English
ISSN and key title: 1652-8220		ISBN 978-91-86671-48-8
Recipient's notes	Number of pages 196	Price
	Security classification	

Distribution by (name and address)

Signature _____ Date 2010-12-07

Endothelial- Epithelial interactions

Blood vessels friends or foes?

Fredrik Wolfhagen Sand

Stem Cell Center
Department of Laboratory Medicine, Lund
Faculty of Medicine, Lund University
Sweden

Lund University
Faculty of Medicine

About the cover: Numerous blood vessels (green) surround the dorsal pancreatic epithelium (red) in the $S1P_1$ mutant embryos.

ISBN 978-91-86671-48-8

ISSN 1652-8220

Lund University, Faculty of Medicine Doctoral Dissertation Series 2011:1

Printed by Media-Tryck, Lund, Sweden

Till min lilla familj

Table of content

List of papers

The thesis was based on the following papers, which will be referred to by their roman numerals.

I. J. Håkansson, A. Ståhlberg, F. Wolfhagen Sand, H. Gerhardt, and H. Semb. N-CAM Exhibits a Regulatory Function in Pathological Angiogenesis in Oxygen Induced Retinopathy. Under revision.

II. F. Wolfhagen Sand, A. Hörnblad, JK. Johansson, C. Lorén, J. Edsbagge, A. Ståhlberg, J. Magenheim, O. Ilovich, E. Mishani, Y. Dor, U. Ahlgren and H. Semb. Growth-limiting role of endothelial cells in endoderm development. Under revision.

III. G. Kesavan, F. Wolfhagen Sand, TU. Greiner, JK. Johansson, S. Kobberup, X. Wu , C. Brakebusch, and H. Semb. Cdc42-mediated tubulogenesis controls cell specification. Cell. 2009 Nov 13;139(4):791-801.

Abstract

Diabetes affects enormous amounts of patients. In type 1 diabetes the insulin producing β cells in the islet of Langerhans are destroyed by the immune system. The β cells, which are located in the pancreas, regulate blood glucose levels. The lack of insulin production requires administration of insulin. This is accomplished by insulin injections. Even though the treatment methods have been improved, we are still not able to artificially regulate the blood glucose levels as evenly as the β cells. The fluctuations of the blood glucose levels results in the long term in a number of complication, among the diabetic retinopathy. Diabetic retinopathy is the leading cause of blindness among the younger population.

Both to reduce the complications of diabetes and to find cures of the disease is important.

By using a model for diabetic retinopathy in mice we have been able to show that lack of the molecule N-CAM is protecting against the pathological neovascularization affecting diabetic patients.

The last 10 years have proven it possible to cure diabetes through transplantations of islets of Langerhans from cadaveric donors. The transplantations requires suppression of the immune response, this could lead to other complications. The amount of diabetic patients in need of transplantations far exceeds the availability of donors.

The use of stem cells to produce insulin producing β cells might be a possible way. To be able to direct the stem cells into β cells it is important to understand how these cells form during embryo development.

By the use of mice we have been able to study how blood vessels regulate the growth of the precursors of β cells. We have also be able to show that the formation of a tubular system in the pancreas is important for the regulation of which cell types that are to be formed.

Our results can be used to develop better methods for the production of β cells from stem cells.

Populärvetenskaplig sammanfattning

Diabetes är en av de vanligaste folksjukdomarna och tusentals diagnostiseras med sjukdomen varje år. Det finns två typer av diabetes, typ 1 och typ 2. I typ 1 förstörs de insulin producerande β-cellerna som finns i de Langerhanska öarna av kroppens immunförsvar. β-cellerna har till uppgift att reglera blodsockernivåerna. Avsaknaden av egen insulinproduktion leder till diabetes som behandlas med insulininjektioner. Trots kraftigt förbättrade behandlingsmetoder kan vi inte artificiellt reglera blodsocker nivårena lika jämnt som β-cellerna gör. Fluktuationerna av blodsockernivåerna leder på sikt till en mängd komplikationer, bland dem diabetisk retinopati. Diabetisk retinopati är den vanligaste orsaken till blindhet hos den yngre befolkningen.

Både att mildra komplikationerna av diabetes och att hitta behandlingsmetoder för att bota sjukdomen är viktig.

I en musmodell för diabetisk retinopati har vi kunnat visa att avsaknaden av molekylen N-CAM skyddar mot delar av den sjukliga nybildningen av blodkärl som sker hos diabetiker.

Under de senaste 10 åren har forskare visat att det är möjligt att tillfälligt bota diabetes genom att transplantera Langerhanska öar från avlidna donatorer till diabetiker. Transplantationen kräver att immunförsvaret hämmas, vilket kan leda till andra komplikationer. Antalet diabetiker i behov av transplantation är mångt mycket större än tillgången på donatorer.

Att använda stamceller för att ta fram insulinproducerande β-celler skulle kunna vara en möjlig väg. För att kunna få stamcellerna att bilda β-celler är det viktigt att förstå hur dessa celler bildas under fosterutvecklingen. Genom att använda en musmodell har vi kunnat studera hur blodkärl reglerar tillväxten av förstadier till β-cellerna. Vi har även kunnat visa att bildandet av rörsystemet i pankreas är viktigt för vilka celltyper som skall bildas i pankreas. Våra resultat kan användas för att ta fram bättre metoder för att bilda β-celler från stamceller.

Introduction

Developmental Biology-Why?

In a clinical perspective developmental biology can explain the underlying mechanism for several diseases, as diabetes and cancer. By understanding these mechanisms we can develop better therapies to treat or cure diseases.
By studying developmental biology we will understand that human kind is not the "crown of the creation", and hopefully this will teach us some humbleness towards the environment.
However, strictly scientifically it is important to describe, explain and understand the world around us, the more we know the more opportunities to do good we have.

In one of the first lectures I had on developmental biology the professor made a flower with developmental biology in the middle surrounded by other medical specialties like anatomy, cell biology, pathology and several others. He then stated that we would, or already had, probably seen other professors putting their own field in the center of the flower. However, who could argue with him when he put developmental biology in the center since it studies the formation of the human being, and without that the other specialities would not exist? I guess since that lecture I have always been interested in developmental biology. The mystery of the formation of an organism, from one large cell that fuses with one small and then give rise to the entire human being, will always fascinate me.

Background

Diabetes Mellitus

Diabetes Mellitus is a sever disease that resulted in the death of the patient before the insulin injections was introduced as a treatment. Diabetes can be divided into two main forms, type 1 and type 2. Type 1 diabetes is an autoimmune disease often with early onset, were the insulin producing β cells are destroyed. Type 2 is characterized by the resistance to insulin. Type 2 is caused both by genetic factors and to a large extent by the lifestyle of the patient [1, 2].

Type 1 diabetes is treated by insulin injections to control the blood sugar. Even though the modern therapies are highly efficient they are not capable of the fine tuning of the glucose levels as the β cells themselves are. The fluctuations of the blood glucose leads to a number of complications, like diabetic retinopathy, diabetic neuropathy, kidney failure and cardiovascular diseases. It has been shown that a better blood glucose control results in milder long term complications [3, 4].

Diabetic retinopathy is the dominating cause of blindness in the population under 60 years [5]. Diabetic retinopathy affects almost all Type 1 patients, diagnosed for more than 20 years and the majority of the Type 2 patients [6]. Hopefully these numbers will decrease as we see the effect of the modern, more effective, treatments of diabetes.

Diabetic retinopathy often develops without any early warning signs. In its first phase diabetic retinopathy is nonprolifatory but as the disease progresses it becomes proliferative. The new blood vessels that form can even penetrate the inner limiting membrane. In the worst scenario the blood vessel growth can cause the retina to detach. The damaged blood vessels of the retina also become leaky which could result

in reduced vision. In severe cases blood clots will result in black dots covering the field of vision (Figure 1).

Figure 1. Vision in diabetic retinopathy can be severely affected. Normal vision left and diabetic retinopathy vision right.

To avoid diabetic complications, which leads to reduced life quality and a reduced life span, Islets of Langerhans have been isolated from cadaveric donors and transplanted to diabetic patients [7].

These transplantations require immunosuppressive treatments but can restore normoglycemia without insulin injections.

The treatment has a number of obstacles, such as short supply of donor islets and poor graft survival and function. But it also is a proof of concept. Therefore other ways of generating functional and insulin producing β cells are currently investigated. Both induced pluripotent stem (iPS) cells and human embryonic stem (hES) cells are possible sources of unlimited β cells. But to be able to differentiate these stem cells, knowledge about the normal developmental program of the β cell is needed. Due to this, the field of pancreatic developmental biology has the past decade received enormous attention since it holds the blueprint for β cell formation. Various differentiation protocols have been tested but so far no one has been successful in generating glucose responsive insulin producing β cells in vitro.

However, co-transplantations of differentiated hES and dorsal pancreatic buds from mice has proven that the hES has the capacity to form insulin producing β cells [8].

Retina

The retina is the most accessible part of the CNS. In the retina the first processing of the visual information takes place. The retina is distinctly organized with the cell bodies arranged into three layers. The retinas borders are marked by the inner limiting membrane (ILM) closest to the vitreous body and the retina pigment epithelium closest to the choroid and the sclera. Underneath the ILM the axons from the retinal ganglion cells in the ganglion cell layer (GCL) is passing on its way to the optic nerve. In the GCL the astrocytes are also found. The astrocytes migrate into the retina just before birth [9]. The Müller cells, which are glia cells, have their cell bodies in the inner nuclear layer (INL) but their projections span the entire retina [10]. The microglia cells have a diverse origin and enter the retina in several waves. One of the functions of microglia is to act as macrophages of the retina [11].

Closest to the retina pigment epithelium is the light sensitive rods and cones located in the photoreceptor segments (PRS). The fact that the light has to pass through all the other layers (and thereby is scattered) to stimulate the photoreceptors has been used as an argument against intelligent design. The squids on the other hand have the photoreceptors closest to the light and in that way has a more superior organization of the eye compared to humans. The organization of the human retina is probably an evolutionary rest from when we were smaller animals, since in a small eye the inverted organization gives space saving advantages [12].

The blood vessels of the retina are organized into a superficial plexus in the GCL and a deeper plexus in the inner nuclear layer (INL) [9, 13]. The deeper layers of the retina are supplied by vessels outside the retina in the choroid. The blood vessels of

the retina enter through the optic nerve around birth in mice. Before this stage the retina has been supplied by the hyaloid vessels [14].

Neural cell adhesion molecule (N-CAM)

N-CAM is a cell-cell adhesion molecule and was the first to be identified [15]. Several splice variants are formed from the N-CAM gene [16]. All germ layers go through a period of N-CAM expression [17]. In the adult N-CAM is predominately expressed in the nervous system [18], the skeletal muscles [19] and the neuroendocrine organs, for example in the pancreatic islet of Langerhans [20]. In the retina N-CAM is highly expressed by both astrocytes and the Müller cells [21-23]. N-CAM knockouts have showed the importance of N-CAM for the development of the nervous system [24, 25]. The major phenotype of the N-CAM knockout is the defects of the olfactory bulb. In the islet of Langerhans N-CAM ablation resulted in disturbed distribution of the glucagon producing α cells and a more polarized appearance of the endocrine cells [26].

Through interplay with FGFR, N-CAM is involved in intra cellular signaling [27, 28] but apart from that the molecular understanding of N-CAM function is rather spare.

In cancer down regulation of N-CAM has been shown to correlate with poor prognosis [29]. Previous studies from our lab have revealed the importance of N-CAM as inhibitor of metastasis, trough effect of the pericyte endothelial interactions and regulation of the extra cellular matrix (ECM) [30, 31].

Blood vessels

The vascular system is the first functional organ of the human body. It supplies the body with oxygen and nutrients as well as removing waste products. The blood vessel hierarchy involves high pressure vessels originating from the heart, arteries and

arterioles, low pressure collecting vessels e.g. veins and venules and between these larger vessel types the capillaries. The different vessel types are to different degree covered by the mural cells, pericytes and vascular smooth muscle cells (vSMC) [32].
The stability of the blood vessels is depended on the mural cell coverage and when the pericytes and vSMC are detached from the blood vessels hemorrhages and leakiness appears [33, 34].
The blood vessels form through two different processes, termed vasculogenesis and angiogenesis. During vasculogenesis angioblasts (endothelial progenitors) cluster and form blood vessels[35]. This process was first believed to only occur during embryogenesis but it has been shown to take place during adulthood [36].
Angiogenesis on the other hand is when new blood vessels sprout from pre-existing vessels [37-39]. It is a dynamic process that involves proliferation, migration and differentiation of the endothelial cells. Angiogenesis requires a distinct interaction between the endothelial cells and the surrounding tissue; both other cell types and the extracellular matrix. Angiogenesis is fundamental to embryogenesis but is normally not occurring in the healthy adult, except for menstruating females. However in a number of pathological conditions angiogenesis plays a key role, such as in tumor development, wound healing, diabetic retinopathy, arteriosclerosis, psoriasis and arthritis [40].
VEGF-A is the most potent growth factor for the endothelial cells. VEGF-A expression is regulated by oxygen levels [41]. Hypoxia leads to the stabilization of hypoxia inducible factor 1α which is a transcription factor that promotes VEGF-A expression [42].
During angiogenesis the blood vessel sprouts send out filopodia just like the neural growth cone and the Drosophila tracheal sprouts [43, 44]. The filopodia are located on the tip cells of the vascular sprout. The filopodia are rich in VEGFR2 expression. The filopodia can therefore probe the surrounding to make sure that the sprout migrates in the right direction. The stalk cells response, to VEGF-A, are not migration but instead proliferation. VEGFR2 has been shown to be responsible for

both endothelial proliferation (stalk cells) and migration (tip cells) whereas the other receptor VEFGR1 function as a regulator of VEGFR2 signaling [45].

Upon hypoxia VEGF-A is not evenly distributed instead a gradient is formed [44]. The gradient is formed through the expression of five different splice variants of VEGF-A. The major ones are the VEGF-A_{120} which is a freely diffusible, the VEGF-A_{188} is attached to cells or extracellular matrix and VEGF-A_{164} has intermediate properties. The VEGF-A_{164} and VEGF-A_{188} have the ability to bind heparan sulfate proteoglycan (HSPG) and are therefore not freely diffusible [44, 46]. The migration of the tip cell is controlled by the VEGF-A gradient whereas the proliferation is regulated by the VEGF-A concentration.

The VEGF-A gradient formed due to the HSPG binding properties is broken down by matrix metalloproteinases during pathologies like diabetic retinopathy causing the ocular disease [47].

In the retina the astrocytes express VEGF-A that the blood vessels then use as a scaffold for their development. When a vascular plexus is formed it is important that the density of branch points is adequate. This is controlled in a competitive manner involving both the delta-like 4 (Dll4)-Notch 1 signaling pathway and the VEGF pathway [48].

To reduce tumor growth therapies targeting the tumor blood vessels has become a clinical reality in severe cases of cancer [49].

Endoderm development

Along the anterior-posterior axis the primitive gut tube is patterned due to interactions with the surrounding meso- and ectoderm [50]. This patterning determines where the gut derived organs should develop. The organs derived from the primitive gut tube include the pharynx, thyroid, parathyroid, esophagus, lungs, thymus, stomach, liver, pancreas, small and large intestine. The endoderm retains the plasticity even after initiation of organ formation. Recombination experiments were

the endodermal epithelium from one anlage is stripped from it surrounding mesenchyme and recombined with mesenchyme from another anlage shows that the mesenchyme has the potential to redirect organ specification and that the epithelium is still plastic [51].

The primitive gut tube itself develops into the esophagus, stomach, small and large intestine whereas the other organs, like the lung, liver and pancreas, bud off into the surrounding mesenchyme from the primitive gut tube (Figure 2).

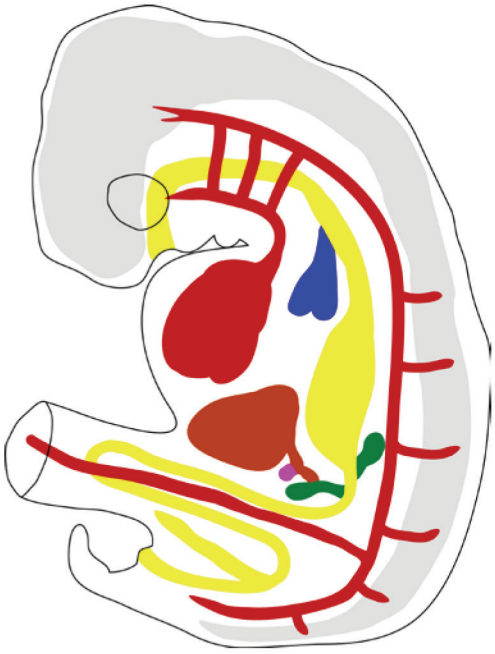

Figure 2. Schematic drawing of an embryo showing the neural tube in grey, the cardiovascular system in red, the primitive gut tube in yellow, the lung in blue, liver in brown, gallbladder in purple and the pancreas in green.

Pancreas development

Anatomy and function

The mature pancreas consists of both the exocrine-, which secretes digestive enzymes into the duodenum, and the endocrine compartment, which secretes hormones in to the blood stream.

The exocrine compartment contains both ductal cells, which secrete bicarbonate ions to neutralize the acid from the stomach, and the accini, which are the enzyme producing units.

The endocrine cells are arranged in the islet of Langerhans. The endocrine cell mass is small compared to the exocrine mass, less than 5 % [52, 53]. The majority of the endocrine cells are insulin producing β cells (60-80 %). Among the other endocrine cell types glucagon producing α cells are the most numerous with 15-20 % followed by the somatostatin producing δ cells with 5-10 % and pancreatic polypeptide producing PP-cells less than 2 %. The least numerous cell type is the ghrelin producing ε cell with less than 1 % of the endocrine mass [54]. The islet architecture is more defined in the mouse, with β cells in the core and the other endocrine cells in a mantle around, than in the human, were the endocrine cells are more mixed [55].

The islet of Langerhans are highly vascularized with special blood vessels containing small pores called fenestra, which allow hormone release from the endocrine cells to the blood stream [56].

Pancreatic morphogenesis

Pancreatic specification starts E8.5 when FGF2 and activin βB from the notochord represses Sonic hedgehog which allows Pdx-1 expression [57-59]. All epithelial cell types in the pancreas originate from Pdx-1^{+} progenitors [60]. In the *Pdx-1* deficient embryos the budding starts but is arrested and no pancreas is formed [61, 62].

Pancreatic development begins E9 with the budding of the epithelium in three locations, one dorsal and two ventral, in close connection with the endothelium [63]. The dorsal bud is in close contact with the newly fused dorsal aorta and the ventral buds forms in close contact with the septum transversum and the vitelline veins. One of the ventral buds regresses as its vitelline vein also regresses (Figure 3). The close interaction between the dorsal aorta and the dorsal pancreas is important for maintained expression of Pdx-1 and the initiation of the crucial pancreatic transcription factor Ptf1a, whereas the vitelline vein is not crucial for similar events in the ventral bud [64]. The remaining ventral and the dorsal bud later rotate and fuse to form the pancreas.

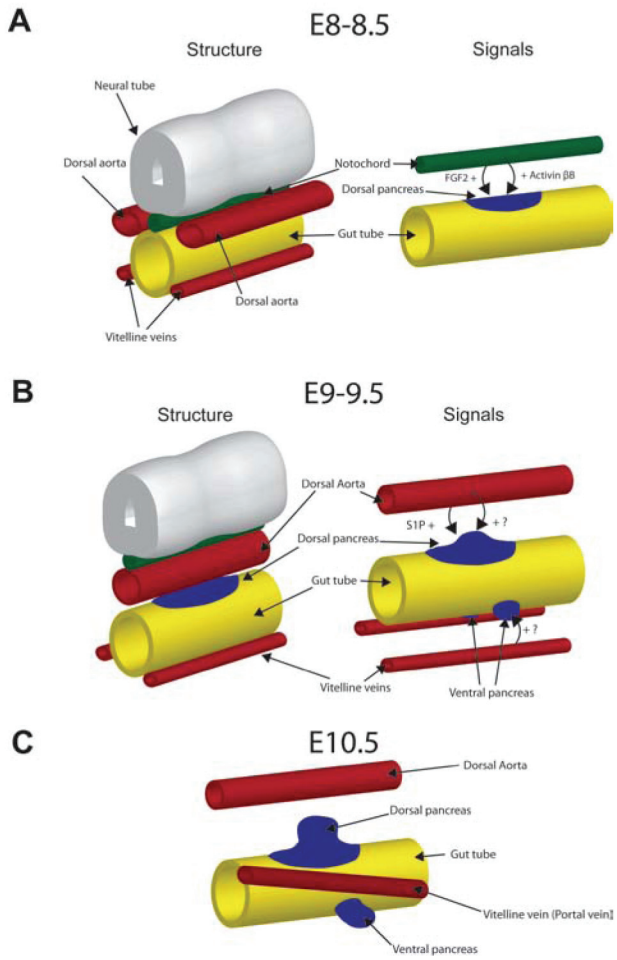

Figure 3. The pancreatic program is initiated at E8 when signals from the notochord inhibits Sonic hedgehog, which allows Pdx-1 expression (A). Later signals from the dorsal aorta promote growth of the dorsal pancreas (B). Due to the regression of one of the vitelline veins its corresponding ventral pancreatic bud also regresses (C). Courtesy Dr. Thomas Greiner.

During the first period around E10-11 all pancreatic epithelial cells express Pdx-1, Ptf1a, Cpa1, Sox9 and Nkx6.1 [65-67]. During later phases of development high expression of Pdx-1 and Nkx6.1 is kept by the β cells, whereas Sox9 becomes a ductal marker [68] and Ptf1a and Cpa1 is expressed by the exocrine cells [65].

After the initiation process the pancreatic buds are separated from the dorsal aorta and the vitelline vein respectively by the invasion of the splanchnic mesenchyme. However the endoderm-endothelial interactions are reestablished as the splanchnic mesenchyme is infiltrated by the blood vessels.

The communication between endoderm and mesenchyme is crucial for proper organ growth. In that perspective FGF10 signaling has got a lot of attention. FGF10 is expressed in the mesenchyme whereas the receptor, FGFR2b, is expressed on the epithelial cells. The mesenchymal cells express another isoform of the receptor named FGFR2c. FGFR2b and c binds different FGF's with different affinity, FGFR2b predominately binds FGF1, 3, 7 and 10 whereas FGFR2c predominately binds FGF1, 2, 4, 6 and 9 [69]. The FGF10-FGFR2b pathway has been shown to be fundamental for the expansion of the pancreatic progenitors. If this signaling pathway is also a determinant of the cells choice to expand or differentiate is not fully understood.

The mesenchyme also expresses other factors which have been proven to be important for pancreatic development. Loss of *N-Cadherin* results in dorsal bud agenesis due to loss of the surrounding mesenchyme [70]. Isl1, which is early expressed in the mesenchyme but later also in the endocrine cells, is fundamental for proper growth of the dorsal pancreas[71]. *Isl1* ablation result in dorsal pancreas agenesis. The mesenchymal cells also express Raldh, an enzyme important for the conversion of retinol (vitamin A) into retinoic acid. *Raldh* knockouts also fail to form the dorsal pancreatic bud [72].

Both endothelial cells and circulatory factors are important for the survival and proliferation of the mesenchyme [64, 73]. The mesenchymal cells express FGF10 which is important for the maintenance of Ptf1a [74]. The mesenchyme also plays a

role in cell fate specification. Explant investigations (cultures of embryonic organs in vitro) have shown that removal of the mesenchyme results in massive endocrine differentiation of the epithelium [75]. At the same time exocrine differentiation in these explants are compromised, suggesting that signals from the mesenchyme represses endocrine differentiation and that the mesenchyme at least has a permissive role in exocrine differentiation. FGF10 has been proposed to be this signal, but since FGF10 expression is not present when the majority of the endocrine and exocrine differentiation takes place, after E14.5, it is more likely that another molecule is responsible for this action.

Ductal compartment

Both the ventral and the dorsal bud originate from a ductal stalk connecting them to the duodenum. During the fusion of the two buds the duct trees, in most cases, fuses and give rise to the major pancreatic duct or duct of Wirsung. The major pancreatic duct is connected with the common bile duct in the ampulla of Vater. In some cases the dorsal duct tree does not fuses with the ventral duct tree but instead gives rise to the accessory pancreatic duct or Duct of Santorini, which then ends in the minor duodenal papilla.[55]

The pancreatic duct tree forms, except the stalk which is connected with the duodenum, through micro lumen formation around E10. These micro lumens expands and fuses to create the immature luminal network around E11-12 that is later remodeled into the mature ductal tree. During this process the epithelium is transformed from a multilayered epithelium into a single layered epithelium [76]. From the ductal compartment the endocrine cells are born and in the ductal ends the exocrine cells are formed.

Exocrine compartment

The exocrine part makes up more than 90 % of the pancreas, counting both exocrine and ductal compartment. The exocrine compartment consists of the acinus [55]. The

acinus matures from the tips of the ductal tree and keeps the expression of the Cpa1 and Ptf1a. The acinus drains into the ductal network. The acinar cells express both proteases and lipases that are secreted, through the pancreatic duct into the duodenum, upon food intake.

Endocrine compartment

During the formation of the ductal network the endocrine compartment is also evolving. All endocrine cells go through a short period of Ngn3 expression [77], before continuing development into; glucagon producing α cells, insulin producing β cells, pancreatic polypeptide producing PP cells, somatostatin producing δ cells or the rare ghrelin producing ε cells.

The different endocrine cell types are born in different proportions during development due to different competence windows of the epithelium [78]. Glucagon cells are born from the earlier $Ngn3^+$ progenitors, insulin and PP cells from slightly later $Ngn3^+$ progenitors and somatostatin cells from even later $Ngn3^+$ progenitor. These results do not support Ngn3 dosage dependence of differentiation. Other investigations have on the other hand shown that the dosage of Ngn3 affects the cell fate but not between different endocrine cell types, rather between endocrine and exocrine differentiation [79]. Low Ngn3 expression is also found in exocrine progenitors were as the traditional Ngn3 cells are expressing Ngn3 in high amounts.

Sphingosine-1-phosphate receptor 1 ($S1P_1$) and its ligand sphingosine-1-phosphate

The $S1P_1$ is a G-coupled receptor and its family consists of four other receptors $S1P_{2\text{-}5}$. The receptors were previously called Edg receptors since the first member Edg-1 ($S1P_1$) is an endothelial differentiation gene. The ligand S1P, which is a lipid derivate, produced by the phosphorylation of sphingosine by sphingosine kinase 1 (SphK1) and SphK2 [80]. S1P can be reverted to sphingosine by sphingosine phosphatase. S1P

is produced by platelets, erythrocytes, monocytes, mast cells and possibly also by endothelial cells [81-83]. The concentration of S1P in the surrounding tissue is low due to the degradation by S1P lyase [84]. In the blood S1P is bound to albumin and high density lipoprotein (HDL) [85].

Activation of $S1P_1$ by S1P is known to induce cell proliferation [86], migration [87] and morphogenesis [88].

In the pancreas three of the receptors are expressed, $S1P_{1-3}$ [73]. In situ histochemistry data suggests that $S1P_1$ is mainly expressed by the endothelial cells and $S1P_{2-3}$ are expressed by the mesenchyme in the pancreas.

Results from our lab have shown that the dorsal pancreas agenesis in the N-cadherin deficient embryos can be rescued in explants by the addition of S1P [73].

$S1P_1$ deficient mice are lethal around E14.5 due to cardiovascular defect [34]. The blood vessels become leaky and results in large hemorrhages due to the $S1P_1$ ablation. The leaky vessels are due to poor mural cell (pericytes and smooth muscle cells) coverage [34, 89].

Polarity and tube formation

Polarity or the definition of what is inside and what is outside is crucial for organs that are formed as pipes. Since the human body to a large extent consists of interacting pipes, consider the CNS, the cardiovascular system, the respiratory system and the gastrointestinal canal, polarity is fundamental to the entire organism.

The polarized cells have three defined locations, the apical, the basal and the lateral cell surface [90]. The apical surface defines the lumen; the basal surface faces the outside and the lateral surface faces the neighboring cells.

A tube can be formed in several different ways [91]. A flat sheet of polarized cells can fold and the two edges fuse to create the tube. This type of tube formation is called wrapping and can be seen in the formation of the neural tube [92].

A new tube can also form from an existing tube as a bud [90]. The bud grows out basally but remains connected with the apical lumen. This form of tube formation is commonly seen, for example in lung branching [90].

Cavitation is another form of tube formation where the central cells undergo cell death, maybe apoptosis, to create a cavity. This form of tube formation has been suggested for salivary gland [93, 94].

During cell hollowing a lumen is formed within a single cell, this can be observed in capillaries [95] and in the excretory cells of the Caenorhabditis elegans (C. elegans) [96].

In hollowing a multilayered epithelium is first formed. As a consequence of the establishment of polarity within the multilayered micro lumens are formed. These micro lumens fuse to form the mature lumen. This process can be seen in the organogenesis of the gut tube in C. elegans [97].

Cell division cycle 42 (Cdc42)

Cdc42 is a small Ras homology (Rho) GTPases. Cdc42 has been shown to be a key regulator of polarity and this function is conserved from yeast to mammals [98]. Rho GTPases cycles between their active GTP bound form and their inactive GDP bound form. Activation of the Rho GTPase is facilitated by guanine nucleotide exchange factors (GEFs). This process is energy dependent. The Rho GTPases are inactivated with the help of GTPase activating proteins (GAP). The GAPs activate the GTPase which then hydrolyses the GTP into a GDP. The switch from GTP to GDP then inactivates the signaling from the Rho GTPase. Rho GTPases can also be kept inactive by guanine dissociation inhibitors (GDIs).

Cdc42 is part of the partitioning defective (Par) complex, together with Par3, Par6 and atypical protein kinase C (aPKC). The par complex regulates polarity by participating in the formation of the apical domain; Cdc42 has been shown to control this process in an in vitro three dimensional (3D) model [99].

Rho GTPases has been shown to be involved in cytoskeleton regulation; specifically Cdc42 has been shown to control filopodia formation [100, 101].

The full knock out of Cdc42 is embryonic lethal and die at E7.5 [102]. Conditional Cdc42 knockouts have been generated in various organs for example; neural progenitor specific [103], skin specific [104] and liver specific [105]. In the neural progenitor specific conditional Cdc42 knockout apical progenitors are turned into basal progenitors. This results in a depletion of the self-renewing capacity of the cells. In the skin specific Cdc42 knockout differentiation of the progenitors into hair follicles were arrested. The liver specific Cdc42 knockout results in hepatomegaly (enlarged liver).

Methodological considerations

Oxygen Induced Retinopathy (OIR)

Diabetic retinopathy is hard to study in mice since they do not develop this vascular complication to elevated blood glucose levels to the same extent as humans. Instead the OIR model is used since it creates similar vascular complications in mice seen in diabetic patients [106]. OIR also closely resembles the retinal complications earlier seen in pretermed infants as a result of abrupt abortion of high oxygen treatment.

In OIR (Figure 4) the pups are left in normoxia between birth and P7 when they are placed in hyperoxia. The pups are kept in hyperoxia until P12. During this time the high oxygen levels represses VEGF-A expression causing the blood vessels to regress. The regression is most pronounced in the center of the retina and around the arteries and arterioles. The deeper plexa is also arrested. The return to normoxia results in a relative hypoxia in the avascular areas causing both revascularization and neovascularization. The new blood vessels do not only regrow into the avascular areas but grows perpendicular in both directions. The ILM is penetrated by the new perpendicular blood vessels causing epiretinal tufts.

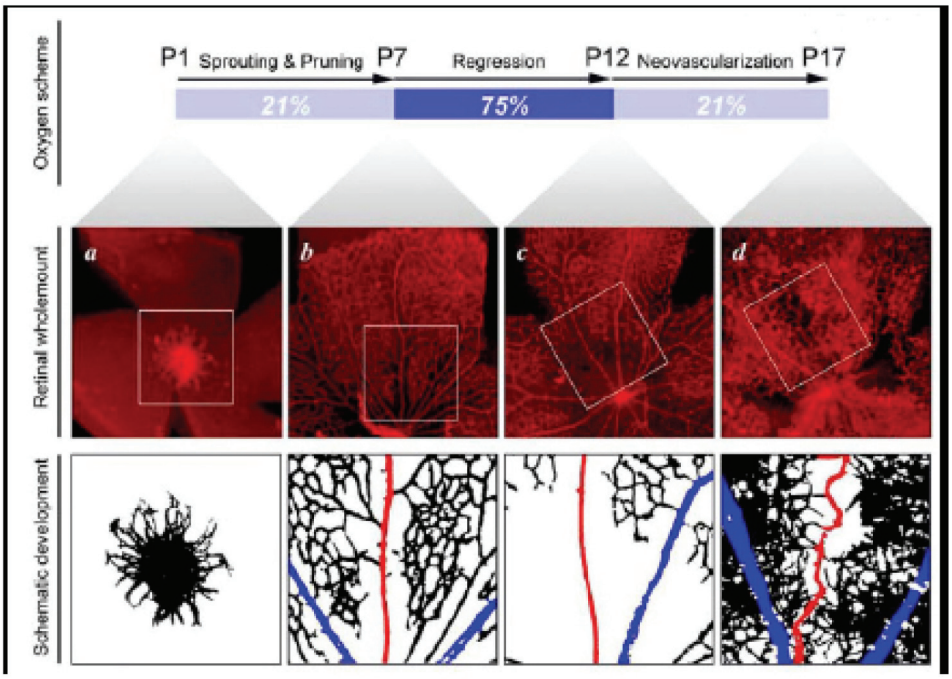

Figure 4. Schematics showing the vascular patterning in the OIR model. The top panel shows the oxygen-exposure scheme from P0 to P17. The middle panel (a-d) shows representative micrographs from P1 (a), P7 (b), P12 (c) and P17 (d). In the lower panel schematic drawings from the boxed region abover are illustrating capillaries (black), arteries (red) and veins (blue). Courtesy of Dr. Andrea Lundkvist.

Knockouts

To use mice as a model of human as we have done in all three papers might seem strange, but it is not. We have the same fundamental set up of organs, and the mouse is possible to genetic manipulate. It is important though to remember that the mouse is a model nothing more.

In paper I and II full knockouts are used. This technique was awarded the Nobel Prize in 2007. The first knockout mouse was preceded by the culture of mES [107]

cells, germ line transmission [108] and the ability to place genetic material at exact location using homologous recombination [109, 110]. By combining these techniques it became possible to target specific genes in ES cells [111, 112] and thereby producing knockouts. The ability to delete any given gene within the genome has taught us a lot about gene function in vivo.

The drawbacks with the full knockouts are that an observed effect within an organ might not be primary to the loss of function of the investigated gene, but might be secondary to defects in other organs. This is actually the case in paper II.

Knockouts are often produced in a mixed background of inbred mouse lines. To get rid of this mixed background the new mouse line is backcrossed to a mouse line of choice. By doing this for numerous generations eventually even the DNA sequence closest to the targeted area is exchanged. The genetic background has in some cases produced dramatic effect of the outcome of the gene deletion. The same gene deletion in two different mouse lines might cause very different phenotypes [113].

Time and/or tissue specific gene targeting

The fundament for creating time and/or tissue specific knockouts is the insertion of loxP sites around the gene of interest, and the ability to express the Cre recombinase [114]. The Cre recombinase recognizes the loxP sites and removes the DNA in between. If a full knockout is early lethal other approaches can be used to study the gene function. In paper III we have used a Cre ErT system [115, 116], were Cre ErT is expressed under the Pdx-1 promoter [117]. In the Cre ErT system Cre is not active until tamoxifen is administrated. This allows a time control of gene deletion. Moreover the Pdx-1 Cre Er system creates a mosaic situation were not all cells are targeted. This can, depending on the aim of the investigation be both a drawback and an advantage. To be able to address gene function in a single organ the regular Cre/Lox system is widely used. The Cre recombinase is expressed under a promoter of

choice and the LoxP sites flank the gene of interest. By choosing the promoter which should express Cre it is possible to create cell type specific knockout.
In some cases the effectiveness of the Cre recombinase excistion of the LoxP flanked site has been very ineffective [118]. It is therefore important to check the efficiency for instance using the floxed R26 mice [119]. Effective Cre recombinase will then result in expression of β galactosidase, which can be monitored.

Explants

To explant an organ/tissue means to isolate it from the organism and culture it in vitro. The organ/tissue is cultured intact e.g. the tissue is not dissociated to create a cell culture. To explant the primitive gut tube and its associated organs has been extensively used to understand the mechanisms behind organ formation.
In both paper II and III we have used explants cultures to address questions not possible to answer in vivo. Explant cultures can be carried out in different ways, we have used both filter cultures [120], where the explants is kept in the liquid-air inter phase and cultures on fibronectin coated cover slips [121].
On the cover slips the explants grows in a flat almost two dimensional fashion whereas on the filters the tissue, even though it flattens out, develops in a three dimensional way which more closely resembles the in vivo development.
In paper II the robust filter cultures were used whereas in paper III we used both systems. The cover slip system proved valuable in analysis of the tubular defects in paper II.

OPT and confocal microscopy

In all three papers we have used 3D visualization techniques to address morphological questions.

Confocal microscopy uses laser illumination and a pine hole to create high contrast images by avoiding out of focus light. Confocal imaging enables 3D reconstruction of the specimen and co-localization investigations.

OPT uses standard fluorescence or brightfield illumination and combine with tomography and rotation of the sample in 360°. The specimen is then reconstructed using algorithms [122]. The advantage of OPT is that it allows investigation of larger samples but the drawback is that the resolution is not as high as for confocal microscopy.

Aims of the thesis

The overall aim of the thesis was to study diabetes related blood vessel phenomena e.g. pathological angiogenesis in proliferative retinopathy and blood vessels role in early pancreatic development. Blood vessels as well as the pancreas are tubular organ, and an additional aim was to study tube establishment in pancreas development.

Specific aims were:

Paper I
To study the role of N-CAM in pathological angiogenesis of proliferative retinopathy.

Paper II
To investigate the effect of endothelial S1P/S1P$_1$ signaling on endoderm development.

Paper III
To describe tube formation during pancreas development and to address the role of tube establishment on cell fate specification.

Present investigations

Paper I: N-CAM Exhibits a Regulatory Function in Pathological Angiogenesis in Oxygen Induced Retinopathy

Introduction

Diabetic retinopathy is the leading cause of blindness among people younger than 60 years [5]. It is caused by damaged blood vessels as a consequence of the fluctuating blood glucose levels.

Both normal and pathological angiogenesis is driven by ischemia which elevates VEGF-A expression [123, 124]. A fundamental difference between normal retinal angiogenesis and the pathological counterpart is the formation of intravitreous epiretinal tufts. The mechanism behind the epiretinal tuft formation seen in diabetic retinopathy is largely unknown.

N-CAM is expressed throughout the retina and profound expression is found in Müller cells and astrocytes [21-23, 125]. In tumor angiogenesis *N-CAM* ablation results in decreased vascular stability [31]. The decreased vascular stability results in increased leakiness. These properties increase the risk of tumor metastasis.

To investigate whether N-CAM was also involved in pathological angiogenesis in proliferative retinopathy we used the OIR model [106].

Results

N-CAM deficient mice did not show any retinal vascular phenotype during normal development. *N-CAM* ablation had a protective role during proliferative retinopathy.

Without affecting the revascularization *N-CAM* ablation dramatically reduced the epiretinal tuft formation. The reduction of the tufts was due to decreased tuft endothelial cell proliferation. The protective role were gene-dose dependent, from wild type through heterozygote to homozygote. In wild type mice N-CAM did accumulate around the epiretinal tufts. The tufts are covered by pericytes, but since N-CAM accumulation are restricted to the borders of the tuft, astrocytes are the likely producers.

To address whether the reduced tuft formation could be explained by changed VEGF-A expression we measured VEGF-A mRNA levels. Opposite to what we expected we found elevated mRNA levels of VEGF-A. We also noted that there was no isoform shift of VEGF-A upon *N-CAM* ablation.

During tumor angiogenesis *N-CAM* ablation results in alterations of the ECM. To address if this was also the case in proliferative retinopathy we investigated the basement membrane proteins collagen IV, fibronectin and laminin α1. Despite *N-CAM* ablation we could not detect any changes of the ECM components.

Summary

Since N-CAM is accumulated around the wild type epiretinal tufts and *N-CAM* ablation results in reduced tuft proliferation it is likely that N-CAM is mechanistically involved in epiretinal tuft formation.

As VEGF-A expression levels were elevated N-CAM might regulate endothelial cell proliferation in an VEGF-A independent way. The elevated VEGF-A levels could also be a consequence of the reduced tuft formation. Normally the tufts, although dysfunctional, supplies the retina with higher oxygen levels reducing VEGF-A expression.

In conclusion we have identified N-CAM as a potential clinical target in diabetic retinopathy.

Paper II Growth-limiting role of endothelial cells in endoderm development

Introduction

During early steps of organogenesis of the endodermal derived organs lung, stomach, liver and pancreas, blood vessels are in close contact with the endoderm and its surrounding mesenchyme.

Previous results from our laboratory have shown the importance of a functional circulation for dorsal pancreatic development [73]. The bioactive sphingolipid metabolite S1P was able to rescue the dorsal bud in the circulation deficient *N-cadherin* mutants in vitro. Others have also shown that the endothelial cells are crucial for the pancreatic program to occur [63, 64, 74].

In the pancreas three receptors for S1P are expressed, $S1P_{1-3}$. The receptors have different expression pattern, and $S1P_1$ expression is concentrated to the blood vessels, whereas $S1P_{2-3}$ are expressed by the mesenchymal cells surrounding the pancreas.

To address if S1P-$S1P_1$ signaling within endothelial cells had a functional role in endoderm development $S1P_1$ deficient mice were analyzed.

Results

To investigate the effect of $S1P_1$ ablation on the endodermal derived organs such as lung, stomach, liver and pancreas, optical tomography was used. All investigated organs except the lung was statistically reduced in the $S1P_1$ mutants. The lung was also smaller however the reduction was not statistically significant.

To eliminate the possibility that the $S1P_1$ mutant's cardiovascular phenotype resulted in the observed endodermal phenotypes explant cultures of gut tube were performed. The littermate controls constantly developed normally whereas the $S1P_1$ deficient explants had perturbed growth. The in vitro phenotype of the $S1P_1$ mutants were rather diverse spanning from no growth to merely a reduction in size and branching of the epithelium.

A reduced size of an organ can either be due to reduced proliferation or increased cell death. To investigate these questions we quantified the proliferation index in the dorsal pancreas. The proliferation was decreased by 35 %. To address programmed cell death, apoptosis, we analyzed cleaved caspase-3. Very few positive cells were observed within the epithelium in both mutants and litter mate controls and therefore could this be excluded as an explanation for the reduced organ size.

We hypothesized that the defective blood vessels in the *S1P*$_1$ mutants would be possible to mimic in vitro by ablating the blood vessels totally. To our surprise the result was the totally opposite, we found an increase organ size and a hyperbranching of the epithelium.

The blood vessel ablation results instead indicated that blood vessels had a growth limiting effect during endoderm development. To address this we quantified the blood vessel density in the lung, stomach and pancreas. We could observe a 2-3 fold increase of the blood vessel density at E12.5. The autofluorescence of the liver made this investigation unsuccessful; instead we measured the relative expression levels of Pecam in the liver. We could show a relative increase of Pecam in the liver in the same magnitude as we had seen in the other organs.

Summary

Endothelial cells have previously been reported to induce pancreas budding [63] and the pancreatic program [64].

By analyzing the *S1P*$_1$ deficient embryos we could show that hypervascularization of the blood vessels have a growth limiting effect on several foregut derived organs. The blood vessel pattern at E12.5 in the investigate organs differ. In stomach and lung the blood vessels are located in the mesenchyme with limited direct contact with the epithelium, whereas in the liver and pancreas the blood vessels are located both in the mesenchyme and in direct contact with the epithelium. The difference in pattern of the blood vessels suggests that the inhibitory molecule from the blood vessels is either a diffusible molecule or a component of the ECM.

In conclusion our data suggests that the blood vessels counterbalance the stimulatory signals from the mesenchyme.

Paper III Cdc42-Mediated Tubulogenesis Controls Cell Specification

Introduction

Formation of polarity and subsequently the formation of tubes is essential in pancreatic organogenesis. The mechanisms behind these processes are poorly understood in vivo.

Tubulogenesis in the pancreas takes place at the same time as cell fate specification, if these two processes are separate or interdependent are unknown.

To address these questions we characterized the initial steps of tube formation in wild type embryos and analyzed *Cdc42* pancreatic mutants.

Results

Tube formation in the pancreas starts around E11. Before this stage the epithelium is multilayered and lack apical-basal polarity, except for the stunt connection with the duodenum. The tube formation is initiated by the appearance of microlumens indicated by the expression the apical marker mucin1. During the following days these microlumens expand and fuse to create a luminal network that is then remodeled into a mature single layered tubular system.

In the Cdc42 mutants the luminal structures were not formed. In the wild type epithelium targeting of apical components and tight junctional complex defines the apical-basal polarity in single cells. The neighbouring cells are induced by these first polarized cells to form common apical surfaces. In the *Cd42* deficient pancreas no

mutlicellular apical lumens are formed, instead auto- and intercellular lumens are formed.

To address if the lack of tubes had any effect of cell fate specification exocrine, duct and endocrine differentiation was investigated. *Cdc42* ablation in the pancreas results in increased exocrine differentiation on the expense of endocrine and duct differentiation.

The decreased endocrine and duct differentiation is a non-cell autonomous effect cased by the altered tissue architecture and the increase in epithelial exposure to ECM in the *Cdc42* mutants.

It has previously been shown that interaction between par3 and par6/aPKC is fundamental for the formation of apical polarity [126], the defects in tube formation seen in the *Cdc42* mutants might therefore be explained by disturbed interactions between par3 and par6/aPKC. Rho kinase (ROCK) is an inhibitor of the par3 interaction with par6/aPKC [127]. To address if ROCK inhibition could rescue tube formation in *Cdc42* mutants explants were incubated with the ROCK inhibitor Y27632. The inactivation of ROCK in the *Cdc42* deficient epithelium was sufficient to restore tube formation.

Summary

Proper tissue architecture in the pancreas is controlled by tube formation. Cell fate specification is tightly linked to the tissue architecture. By disrupting the tube formation the epithelium is exposed to elevated interactions with the mesenchyme. By regulating apical polarity Cdc42 is fundamental for pancreatic tube formation and thereby for cell fate specification.

Further perspectives

Paper I

It is intriguing that ablation of N-CAM can have both protective effects, as in the OIR model, and detrimental effects, as in the tumor models, in pathological angiogenesis.

To understand how N-CAM mechanistically regulates pathological angiogenesis would be of great importance. Since N-CAM is known to interact with FGFR signaling and proliferative retinopathy has been linked to proteolytic release of growth factors, a speculative explanation might be that N-CAM ablation results in reduced FGF signaling and thereby reduced tuft formation.

Paper II

To identify the growth limiting factor/factors from the blood vessels during endodermal development would be of great importance. Since neither the early stimulatory signals from the endothelial cells are known we can only speculate whether the signals are the same and have different effect during development or if it is different signals affecting the early endoderm and the later endoderm.

Previous results have highlighted the importance for circulatory factors in the early initiation of the pancreas [73]. A recent publication also linked embryonic blood flow to differentiation [128].

Taken together it is obvious that the vasculature does affect the endodermal development in different ways, to further characterizing these mechanisms will give us tools to develop better hES differentiation protocols.

Paper III

To fully understand the mesenchyme epithelial interactions governing cell fate specifications is of great importance to the pancreatic developmental field. In the present work we have identified the tube formation as a key determinant of these interactions. To understand these interactions on a molecular level would be valuable. By using *Cdc42* deficient mice we have shown that establishment of apical polarity is fundamental to tube formation. The normalization of the *Cdc42* deficient pancreas by the in vitro treatment with the ROCK inhibitor implies a function for Rho A in pancreatic tube formation.

The current failures to generate effective in vitro differentiation protocols for the generation of β cells might be due to the ignorance of the importance of proper microenvironment for cell fate specification.

Concluding remarks

During the last 10 years representatives from the hES cell field have promised treatments to various severe diseases, among them diabetes. Many of these promises have been unrealistic and harmed the credibility of the field.

Does this mean that hES cell based therapies are unrealistic? Far from, however the treatments are hardly going to be available in the near future. The back to basic strategy, even though not fully reaching the goal, used by the pharmaceutical company Novocell showed that applying knowledge from developmental biology is fruitful [129]. By applying knowledge from how the different steps in the development of the β cell is regulated they were able to differentiate hES cells into insulin producing cells. However the efficiency was rather low and the generated cells were not glucose responsive.

At the moment the puzzle of differentiating hES into functional cells contain many blank pieces, developmental biology can reveal their identity.

Personal remarks

I have had the privilege to be involved in three stunning scientific observations.
The first one already as a summer student in Gothenburg. My task was to perform in situ hybridizations of interesting genes in blood vessel biology. I performed the VEGF-A in situ on a retinal whole mount and my supervisor Dr. Holger Gerhardt stained it for the blood vessel marker isolectin. It was striking that the blood vessels sent out projections against the VEGF-A expressing astrocytes in front of the vascular plexa.

The second one was in the $S1P_1$ project. Our initial hypothesis was that the phenotypes observed were due to the defective blood vessels and that we would be able to phenocopy the $S1P_1$ ablation by ablating the blood vessels. To do this we got a blood vessel blocker through our collaborator Dr. Yuval Dor. After some initial testing with the blocker I decided to carry out a larger explant experiment.
The E11.5 explants were treated for several days with either the compound or vehicle. The explants were stained for Mucin1 and Pecam. In the blood vessel blocker treated explants Pecam stain was totally abolished. To our surprise the pancreas in the blood vessel ablated explants did not at all resemble the phenotype seen in the S1P1 mutant. Instead the pancreatic organ was enlarged and hyperbranched.

The third took place during the hectic revision of the Cdc42 paper. We had been asked by one of the reviewers to try to rescue the Cdc42 phenotype by inhibiting Rho kinase (ROCK) in vitro. When analyzing the outcome of the ROCK inhibition in the *Cdc42* mutants the first time we thought that the genotyping must be incorrect, the

epithelium was far too normal. But after several repetitions we could convince our self that ROCK inhibition and Cdc42 ablation neutralized each other.

The fantastic thing with events like these is their power to make it all worth it, all the failed experiments, the annoying mistakes as well as the stupid ones. I hope that I can add more of these positive events to my list in the future.

Acknowledgment

En avhandling är ett lagarbete. Laget består inte bara av kollegorna utan även av vänner och familj.

Jag vill passa på att tacka de människor som på ett eller annat sätt gjort denna avhandling möjlig.

Min handledare, doldisen, **Henrik** för ditt oerhörda engagemang för att producera förstklassig forskning.

Trots en lite tuffare tid till en början nere i Lund har du under (trots) min doktorandtid lyckats med nästan allting, anslag, Cell-pek och ett nytt center. Men kaffemaskinen Henrik den är en stor plump i protokollet, det blev ju lite bättre med den nya men här krävs nya tag för att nå samma nivå som Gbg.

Trots att du formellt inte har varit min bihandledare har du i praktiken fungerat som en sådan **Isabella**, det har jag verkligen uppskattat. Om du behöver några råd så vet du vart jag finns.

Christer för att jag fick möjligheten att börja i ditt labb, du är högst skyldig till att det blev utvecklingsbiologi för mig.

Holger G för att du lärde mig grunderna i forskningen, för ditt oerhörda engagemang, kunskap och omtanke.

Min mentor **Holger L** för trevliga samtal om både forskning och livet.

Mina kollegor som bidragit till att skapa en hjälpsam och ofta rå men hjärtlig atmosfär. **Jenny** labbets nav även nu när du bytt grupp. Du är den mest hjälpsamma människa jag träffat, visst du är hård som få och envisare än en åsna men ett större hjärta får man leta efter. **Thomas**, ditt engagemang och entusiasm saknas varje dag i labbet, dessutom hade ju ett år som detta varit extra roligt att ha dig i Skåne! **Gokul**, the guru of the lab. I know that you want to be my supervisor, but first you must learn to find the pancreas and to use the autoclave. **Martina** för alla fantastiska fester du fixast för oss och för att du höll Gokul i handen så han inte slog ihjäl sig när ni

åkte rollerblades. **Jackie**, labbets prinsessa, du får mig att skratta så ofta och ett skratt förlänger ju livet. Till min tidigare rumskamrat, **Karin** för alla roliga samtal under tiden som vi har opererat tillsammans. **Zarah**, jag lovar; snart har jag tid att hjälpa dig, men då får du stå ut med att jag retas! **Elvira**, labbets andra prinsessa, du har fortfarande en bit kvar till mästarinnans nivå, men du är på godväg. **Anders** för all hjälp med QPCR:en och för tävlingen mellan hES gruppen och utvecklingsbiologi gruppen, vi behöver ju inte nämna vem som vann. **Pia** för gemensamma ansträngningar med confocalen. **Marie** och **Camilla** för all hjälp med mössen. **Fina**, du har betytt väldigt mycket för blodkärlsprojektet. Att ha någon att ringa till när det strular som är så positiv är underbart. Dessutom var det fantastiskt kul att vara i Strasbourg med dig och Gabriella. Och **Gabriella** för att du på fullaste allvar kan lägga huvudet på sned och hävda att en tom frigolitlåda är för tung och för att du tog det för självklart att jag skulle släpa hem allt vinet till hotellet som du hade köpt till Mr P. **Michael** for showing interest in the injection experiments, we will make them work. **Oliver** you have the best thing in life ahead of you. **Karen** min nuvarande rumskamrat, för att du står ut med mig, det har inte de tidigare gjort. Fräulein **Yvonne** för att du "uppskattar" mina dåliga tyska. **Siqin** för att du får mig att skratta så ofta, en diskussion med dig är aldrig tråkig. **Xiaojie** min tidigare rumskamrat för att du har mycket skinn på näsan och ett stort hjärta. **Ingar** för fantastiska fredags-fikor. **Ingrid** för att du såg till att stämningen alltid var på topp. Novo-tjejerna **Katja, Jenny E, Nina,** och **Qianren** för att ni bidrar med extra kommersiell dramatik till labbet. **Jesper** för fantastisk danska och **Magda** för ordning. The motherhood kontoret med **Anti, Karro, Anna, Maria**[2] och **Hanna** för trevliga morgonpratstunder. **Jocke** för tålamod med retina-arbetet. **Sune** för alla diskussioner om allt från fotboll och barn till forskning. **Maria H** för att du bidrog med lugn och stabilitet till labbet.

Ulf för ditt oerhörda teknik- och biologiintresse. **Andreas** och **Christina** för ett fantastiskt jobb med endodermproverna.

Mikael, Johan, Alexander och **Tobbe** för alla trevliga kaffestunder.

Gamla **Medkem** för den bästa forskningsmiljö man kan tänka sig, jag saknar ölklubben, godisklubben och grillningarna på taket, men framförallt människorna.

Andrea för trevliga luncher och springturer både i Götet och i Lund.

Grapparna; **Bjarne** för bjarnegårdarna, **Rollo** för alarm, **Lamm** för en quickie, **Dr Ä** för helt sjuka vetenskapliga referenser, **LillePer** för dubbel botten och UV-bord,

Fricken för innovativa elstötar, **Långås** för power naps. Det måste vara dags för en ny resa snart!

Mattias, my brother in arms, nu blir det uppryckning med träningen för min del, är du på? **Jenny** för dyraste olivinköpet till den avslutande öl-fikan och för trevliga besök i Lund.

Kim för att du tillåter din dotter att lära sig skånska.

Denny, Rasmus, Jocke, Emil och **Rööken** för gemensamma intressen i forskning och Bonde-SM.

Mina vänner på **B11** som jag så ofta äter lunch med. **Eva** för alla diskussioner, som enligt min kära fru, ibland blir för hetsiga, men som jag alltid har uppskattat.

Alla mina vänner utanför labbet ingen nämnd ingen glömd.

Till P3's Christer-redaktion för verklig underhållning och kunskap.

Till min familj vill jag rikta ett extra stort tack. Till världens bästa svärföräldrar, **Lena** och **Lennart** för all hjälp. **Anna, Christian** och **Stina** för fantastiska resor.

Min underbara lillasyster **Anna**, min **mamma** och **Tomas** för all hjälp och omtanke. **Pappa** och **Tanja** för stöd och uppmuntran.

Sist och de som förtjänar det mest **Oscar**, för att du får mig att se vad som är viktigt, och **Elin**, för att du är världens roligaste, ni är mitt allt, snart är vi fyra.

References

1. Carey, V.J., et al., Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women. The Nurses' Health Study. Am J Epidemiol, 1997. **145**(7): p. 614-9.

2. Riserus, U., W.C. Willett, and F.B. Hu, Dietary fats and prevention of type 2 diabetes. Prog Lipid Res, 2009. **48**(1): p. 44-51.

3. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med, 1993. **329**(14): p. 977-86.

4. Nordwall, M., et al., Good glycemic control remains crucial in prevention of late diabetic complications--the Linkoping Diabetes Complications Study. Pediatr Diabetes, 2009. **10**(3): p. 168-76.

5. Kahn, H.A. and R. Hiller, Blindness caused by diabetic retinopathy. Am J Ophthalmol, 1974. **78**(1): p. 58-67.

6. Diabetic Retinopathy. Diabetes Care, 2002. **25**(suppl 1): p. s90-s93.

7. Shapiro, A.M., et al., Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med, 2000. **343**(4): p. 230-8.

8. Brolen, G.K., et al., Signals from the embryonic mouse pancreas induce differentiation of human embryonic stem cells into insulin-producing beta-cell-like cells. Diabetes, 2005. **54**(10): p. 2867-74.

9. Stone, J. and Z. Dreher, Relationship between astrocytes, ganglion cells and vasculature of the retina. J Comp Neurol, 1987. **255**(1): p. 35-49.

10. Cepko, C.L., et al., Cell fate determination in the vertebrate retina. Proc Natl Acad Sci U S A, 1996. **93**(2): p. 589-95.

11. Chen, L., P. Yang, and A. Kijlstra, Distribution, markers, and functions of retinal microglia. Ocular Immunology and Inflammation, 2002. **10**(1): p. 27-39.

12. Kroger, R.H. and O. Biehlmaier, Space-saving advantage of an inverted retina. Vision Res, 2009. **49**(18): p. 2318-21.

13. Fruttiger, M., et al., PDGF mediates a neuron-astrocyte interaction in the developing retina. Neuron, 1996. **17**(6): p. 1117-31.

14. De Schaepdrijver, L., et al., The hyaloid vascular system of the pig. A light and scanning electron microscopic study. Anat Embryol (Berl), 1989. **180**(6): p. 549-54.

15. Thiery, J.P., et al., Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. J Biol Chem, 1977. **252**(19): p. 6841-5.

16. Chuong, C.M. and G.M. Edelman, Alterations in neural cell adhesion molecules during development of different regions of the nervous system. J Neurosci, 1984. **4**(9): p. 2354-68.

17. Crossin, K.L., C.M. Chuong, and G.M. Edelman, Expression sequences of cell adhesion molecules. Proc Natl Acad Sci U S A, 1985. **82**(20): p. 6942-6.

18. Gower, H.J., et al., Alternative splicing generates a secreted form of N-CAM in muscle and brain. Cell, 1988. **55**(6): p. 955-64.

19. Dickson, G., et al., Human muscle neural cell adhesion molecule (N-CAM): identification of a muscle-specific sequence in the extracellular domain. Cell, 1987. **50**(7): p. 1119-30.

20. Rouiller, D.G., V. Cirulli, and P.A. Halban, Differences in aggregation properties and levels of the neural cell adhesion molecule (NCAM) between islet cell types. Exp Cell Res, 1990. **191**(2): p. 305-12.

21. Bartsch, U., F. Kirchhoff, and M. Schachner, Highly sialylated N-CAM is expressed in adult mouse optic nerve and retina. J Neurocytol, 1990. **19**(4): p. 550-65.

22. Sawaguchi, A., et al., Multistratified expression of polysialic acid and its relationship to VAChT-containing neurons in the inner plexiform layer of adult rat retina. J Histochem Cytochem, 1999. **47**(7): p. 919-28.

23. Wojciechowski, A.B., et al., Migratory capacity of the cell line RN33B and the host glial cell response after subretinal transplantation to normal adult rats. Glia, 2004. **47**(1): p. 58-67.

24. Cremer, H., et al., Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature, 1994. **367**(6462): p. 455-9.

25. Tomasiewicz, H., et al., Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. Neuron, 1993. **11**(6): p. 1163-74.

26. Esni, F., et al., Neural cell adhesion molecule (N-CAM) is required for cell type segregation and normal ultrastructure in pancreatic islets. J Cell Biol, 1999. **144**(2): p. 325-37.

27. Doherty, P. and F.S. Walsh, Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. Curr Opin Neurobiol, 1994. **4**(1): p. 49-55.

28. Williams, E.J., et al., Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron, 1994. **13**(3): p. 583-94.

29. Cavallaro, U. and G. Christofori, Cell adhesion in tumor invasion and metastasis: loss of the glue is not enough. Biochim Biophys Acta, 2001. **1552**(1): p. 39-45.

30. Hakansson, J., et al., Neural cell adhesion molecule-deficient beta-cell tumorigenesis results in diminished extracellular matrix molecule expression and tumour cell-matrix adhesion. Tumour Biol, 2005. **26**(2): p. 103-12.

31. Xian, X., et al., Pericytes limit tumor cell metastasis. J Clin Invest, 2006. **116**(3): p. 642-51.

32. Allt, G. and J.G. Lawrenson, Pericytes: cell biology and pathology. Cells Tissues Organs, 2001. **169**(1): p. 1-11.

33. Lindahl, P., et al., Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science, 1997. **277**(5323): p. 242-5.

34. Liu, Y., et al., Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J Clin Invest, 2000. **106**(8): p. 951-61.

35. Risau, W. and I. Flamme, Vasculogenesis. Annu Rev Cell Dev Biol, 1995. **11**: p. 73-91.

36. Asahara, T., et al., Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res, 1999. **85**(3): p. 221-8.

37. Risau, W., Mechanisms of angiogenesis. Nature, 1997. **386**(6626): p. 671-4.

38. Carmeliet, P. and R.K. Jain, Angiogenesis in cancer and other diseases. Nature, 2000. **407**(6801): p. 249-57.

39. Carmeliet, P., Mechanisms of angiogenesis and arteriogenesis. Nat Med, 2000. **6**(4): p. 389-95.

40. Presta, M., et al., Inflammatory cells and chemokines sustain FGF2-induced angiogenesis. Eur Cytokine Netw, 2009. **20**(2): p. 39-50.

41. Ikeda, E., et al., Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. J Biol Chem, 1995. **270**(34): p. 19761-6.

42. Fong, G.H., Regulation of angiogenesis by oxygen sensing mechanisms. J Mol Med, 2009. **87**(6): p. 549-60.

43. Gerhardt, H., et al., VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol, 2003. **161**(6): p. 1163-77.

44. Ruhrberg, C., et al., Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev, 2002. **16**(20): p. 2684-98.

45. Olsson, A.K., et al., VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol, 2006. **7**(5): p. 359-71.

46. Neufeld, G., et al., Vascular endothelial growth factor (VEGF) and its receptors. Faseb J, 1999. **13**(1): p. 9-22.

47. Lundkvist, A., et al., Growth factor gradients in vascular patterning. Novartis Found Symp, 2007. **283**: p. 194-201; discussion 201-6, 238-41.

48. Hellstrom, M., et al., Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature, 2007. **445**(7129): p. 776-80.

49. Ellis, L.M. and D.J. Hicklin, VEGF-targeted therapy: mechanisms of anti-tumour activity. Nat Rev Cancer, 2008. **8**(8): p. 579-91.

50. Grapin-Botton, A. and D.A. Melton, Endoderm development: from patterning to organogenesis. Trends Genet, 2000. **16**(3): p. 124-30.

51. Yasugi, S., Role of Epithelial-Mesenchymal Interactions in Differentiation of Epithelium of Vertebrate Digestive Organs. Development, Growth & Differentiation, 1993. **35**(1): p. 1-9.

52. Guney, M.A. and M. Gannon, Pancreas cell fate. Birth Defects Res C Embryo Today, 2009. **87**(3): p. 232-48.

53. Githens, S., The pancreatic duct cell: proliferative capabilities, specific characteristics, metaplasia, isolation, and culture. J Pediatr Gastroenterol Nutr, 1988. **7**(4): p. 486-506.

54. Edlund, H., Pancreatic organogenesis--developmental mechanisms and implications for therapy. Nat Rev Genet, 2002. **3**(7): p. 524-32.

55. Slack, J.M., Developmental biology of the pancreas. Development, 1995. **121**(6): p. 1569-80.

56. Konstantinova, I. and E. Lammert, Microvascular development: learning from pancreatic islets. Bioessays, 2004. **26**(10): p. 1069-75.

57. Kim, S.K., M. Hebrok, and D.A. Melton, Notochord to endoderm signaling is required for pancreas development. Development, 1997. **124**(21): p. 4243-52.

58. Hebrok, M., S.K. Kim, and D.A. Melton, Notochord repression of endodermal Sonic hedgehog permits pancreas development. Genes Dev, 1998. **12**(11): p. 1705-13.

59. Kim, S.K. and D.A. Melton, Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor. Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13036-41.

60. Gu, G., J. Dubauskaite, and D.A. Melton, Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development, 2002. **129**(10): p. 2447-57.

61. Ahlgren, U., J. Jonsson, and H. Edlund, The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. Development, 1996. **122**(5): p. 1409-16.

62. Offield, M.F., et al., PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development, 1996. **122**(3): p. 983-95.

63. Lammert, E., O. Cleaver, and D. Melton, Induction of pancreatic differentiation by signals from blood vessels. Science, 2001. **294**(5542): p. 564-7.

64. Yoshitomi, H. and K.S. Zaret, Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development, 2004. **131**(4): p. 807-17.

65. Murtaugh, L.C. and D. Kopinke, Pancreatic stem cells. 2008.

66. Sander, M., et al., Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. Development, 2000. **127**(24): p. 5533-40.

67. Lioubinski, O., et al., Expression of Sox transcription factors in the developing mouse pancreas. Dev Dyn, 2003. **227**(3): p. 402-8.

68. Seymour, P.A., et al., SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci U S A, 2007. **104**(6): p. 1865-70.

69. Pulkkinen, M.-A., et al., The IIIb isoform of fibroblast growth factor receptor 2 is required for proper growth and branching of pancreatic ductal epithelium but not for differentiation of exocrine or endocrine cells. Mechanisms of Development, 2003. **120**(2): p. 167-175.

70. Esni, F., et al., Dorsal pancreas agenesis in N-cadherin- deficient mice. Dev Biol, 2001. **238**(1): p. 202-12.

71. Ahlgren, U., et al., Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature, 1997. **385**(6613): p. 257-60.

72. Martin, M., et al., Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. Dev Biol, 2005. **284**(2): p. 399-411.

73. Edsbagge, J., et al., Vascular function and sphingosine-1-phosphate regulate development of the dorsal pancreatic mesenchyme. Development, 2005. **132**(5): p. 1085-92.

74. Jacquemin, P., et al., An endothelial-mesenchymal relay pathway regulates early phases of pancreas development. Dev Biol, 2006. **290**(1): p. 189-99.

75. Gittes, G.K., et al., Lineage-specific morphogenesis in the developing pancreas: role of mesenchymal factors. Development, 1996. **122**(2): p. 439-47.

76. Jensen, J., Gene regulatory factors in pancreatic development. Dev Dyn, 2004. **229**(1): p. 176-200.

77. Gradwohl, G., et al., neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A, 2000. **97**(4): p. 1607-11.

78. Johansson, K.A., et al., Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. Dev Cell, 2007. **12**(3): p. 457-65.

79. Wang, S., et al., Neurog3 gene dosage regulates allocation of endocrine and exocrine cell fates in the developing mouse pancreas. Dev Biol. **339**(1): p. 26-37.

80. Spiegel, S., D. English, and S. Milstien, Sphingosine 1-phosphate signaling: providing cells with a sense of direction. Trends Cell Biol, 2002. **12**(5): p. 236-42.

81. Pappu, R., et al., Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science, 2007. **316**(5822): p. 295-8.

82. Yatomi, Y., et al., Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood, 1995. **86**(1): p. 193-202.

83. Venkataraman, K., et al., Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circ Res, 2008. **102**(6): p. 669-76.

84. Schwab, S.R., et al., Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science, 2005. **309**(5741): p. 1735-9.

85. Murata, N., et al., Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. Biochem J, 2000. **352 Pt 3**: p. 809-15.

86. Zhang, H., et al., Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. J Cell Biol, 1991. **114**(1): p. 155-67.

87. Sadahira, Y., et al., Sphingosine 1-phosphate, a specific endogenous signaling molecule controlling cell motility and tumor cell invasiveness. Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9686-90.

88. Kupperman, E., et al., A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development. Nature, 2000. **406**(6792): p. 192-5.

89. Kono, M., et al., The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. J Biol Chem, 2004. **279**(28): p. 29367-73.

90. Hogan, B.L. and P.A. Kolodziej, Organogenesis: molecular mechanisms of tubulogenesis. Nat Rev Genet, 2002. **3**(7): p. 513-23.

91. Lubarsky, B. and M.A. Krasnow, Tube morphogenesis: making and shaping biological tubes. Cell, 2003. **112**(1): p. 19-28.

92. Colas, J.F. and G.C. Schoenwolf, Towards a cellular and molecular understanding of neurulation. Dev Dyn, 2001. **221**(2): p. 117-45.

93. Melnick, M. and T. Jaskoll, Mouse submandibular gland morphogenesis: a paradigm for embryonic signal processing. Crit Rev Oral Biol Med, 2000. **11**(2): p. 199-215.

94. Borghese, E., The development in vitro of the submandibular and sublingual glands of Mus musculus. J Anat, 1950. **84**(3): p. 287-302.

95. Wolff, J.R. and T. Bar, 'Seamless' endothelia in brain capillaries during development of the rat's cerebral cortex. Brain Res, 1972. **41**(1): p. 17-24.

96. Berry, K.L., et al., A C. elegans CLIC-like protein required for intracellular tube formation and maintenance. Science, 2003. **302**(5653): p. 2134-7.

97. Leung, B., G.J. Hermann, and J.R. Priess, Organogenesis of the Caenorhabditis elegans intestine. Dev Biol, 1999. **216**(1): p. 114-34.

98. Etienne-Manneville, S., Cdc42--the centre of polarity. J Cell Sci, 2004. **117**(Pt 8): p. 1291-300.

99. Martin-Belmonte, F., et al., PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. Cell, 2007. **128**(2): p. 383-97.

100. Miki, H., et al., Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. Nature, 1998. **391**(6662): p. 93-6.

101. Mattila, P.K. and P. Lappalainen, Filopodia: molecular architecture and cellular functions. Nat Rev Mol Cell Biol, 2008. **9**(6): p. 446-54.

102. Chen, F., et al., Cdc42 is required for PIP(2)-induced actin polymerization and early development but not for cell viability. Curr Biol, 2000. **10**(13): p. 758-65.

103. Cappello, S., et al., The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface. Nat Neurosci, 2006. **9**(9): p. 1099-107.

104. Wu, X., et al., Cdc42 controls progenitor cell differentiation and beta-catenin turnover in skin. Genes Dev, 2006. **20**(5): p. 571-85.

105. van Hengel, J., et al., Continuous cell injury promotes hepatic tumorigenesis in cdc42-deficient mouse liver. Gastroenterology, 2008. **134**(3): p. 781-92.

106. Smith, L.E., et al., Oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci, 1994. **35**(1): p. 101-11.

107. Evans, M.J. and M.H. Kaufman, Establishment in culture of pluripotential cells from mouse embryos. Nature, 1981. **292**(5819): p. 154-6.

108. Bradley, A., et al., Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature, 1984. **309**(5965): p. 255-6.

109. Smithies, O., et al., Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature, 1985. **317**(6034): p. 230-4.

110. Thomas, K.R., K.R. Folger, and M.R. Capecchi, High frequency targeting of genes to specific sites in the mammalian genome. Cell, 1986. **44**(3): p. 419-28.

111. Thomas, K.R. and M.R. Capecchi, Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell, 1987. **51**(3): p. 503-12.

112. Doetschman, T., et al., Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature, 1987. **330**(6148): p. 576-8.

113. Doetschman, T., Influence of genetic background on genetically engineered mouse phenotypes. Methods Mol Biol, 2009. **530**: p. 423-33.

114. Sauer, B. and N. Henderson, Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A, 1988. **85**(14): p. 5166-70.

115. Rossant, J. and A. McMahon, "Cre"-ating mouse mutants-a meeting review on conditional mouse genetics. Genes Dev, 1999. **13**(2): p. 142-5.

116. Hayashi, S. and A.P. McMahon, Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol, 2002. **244**(2): p. 305-18.

117. Zhang, H., et al., Efficient recombination in pancreatic islets by a tamoxifen-inducible Cre-recombinase. Genesis, 2005. **42**(3): p. 210-7.

118. Johansson, J.K., et al., N-cadherin is dispensable for pancreas development but required for beta-cell granule turnover. Genesis. **48**(6): p. 374-81.

119. Mao, X., Y. Fujiwara, and S.H. Orkin, Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci U S A, 1999. **96**(9): p. 5037-42.

120. Gittes, G.K. and P.E. Galante, A culture system for the study of pancreatic organogenesis J. Tissue Cult. Methods, 1993. **Volume 15**(Number 1 / March, 1993): p. 23-27.

121. Percival, A.C. and J.M. Slack, Analysis of pancreatic development using a cell lineage label. Exp Cell Res, 1999. **247**(1): p. 123-32.

122. Sharpe, J., et al., Optical projection tomography as a tool for 3D microscopy and gene expression studies. Science, 2002. **296**(5567): p. 541-5.

123. Stone, J., et al., Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. J Neurosci, 1995. **15**(7 Pt 1): p. 4738-47.

124. Stone, J., et al., Roles of vascular endothelial growth factor and astrocyte degeneration in the genesis of retinopathy of prematurity. Invest Ophthalmol Vis Sci, 1996. **37**(2): p. 290-9.

125. Bartsch, U., F. Kirchhoff, and M. Schachner, Immunohistological localization of the adhesion molecules L1, N-CAM, and MAG in the developing and adult optic nerve of mice. J Comp Neurol, 1989. **284**(3): p. 451-62.

126. Horikoshi, Y., et al., Interaction between PAR-3 and the aPKC-PAR-6 complex is indispensable for apical domain development of epithelial cells. J Cell Sci, 2009. **122**(Pt 10): p. 1595-606.

127. Nakayama, M., et al., Rho-kinase phosphorylates PAR-3 and disrupts PAR complex formation. Dev Cell, 2008. **14**(2): p. 205-15.

128. Shah, S.R., et al., Embryonic mouse blood flow and oxygen correlate with early pancreatic differentiation. Dev Biol.

129. D'Amour, K.A., et al., Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol, 2006. **24**(11): p. 1392-401.