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Maf and Mitf transcription factors regulate pancreatic endocrine cell differentiation and function

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PO Box 117
221 00 Lund
+46 46-222 00 00

Maf and Mitf transcription factors regulate pancreatic endocrine cell differentiation and function.

Magdalena Agata Mazur



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DOCTORAL DISSERTATION

With due permission of the Faculty of Medicine at Lund University, Sweden.

This thesis will be defended on September 5th at 9:00
in Segerfalksalen, BMC A10, Sölvegatan 19, Lund

Supervisor

Isabella Artner, PhD

Faculty opponent

Anne Grapin-Botton, PhD

Professor of Developmental Biology

The Danish Stem Cell Center (DanStem)

University of Copenhagen

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| <p>Diabetes - the imbalance in glucose homeostasis is partially caused by loss or dysfunction of pancreatic insulin producing β cells or development of insulin resistance. In Type 1 diabetes β cells are destroyed in the process of an autoimmune attack, whereas in Type 2 diabetes, islets produce insufficient amounts of insulin or the insulin cannot be used adequately. So far, the most promising therapy to treat Type 1 diabetes is pancreatic islet transplantation. However, this method cannot be used in a larger scale because of the severe scarcity of donors. Therefore, there is a great and urgent need to develop new methods and therapies. One of the potential sources of obtaining functional insulin cells are human embryonic stem cells (ESC) or inducible pluripotent stem cells (iPS). Hence, differentiating stem cells into functional β-cells requires a detailed understanding of pancreas development with the focus on molecular programs underlying β-cells formation and function. Pancreas development and differentiation of insulin and glucagon secreting cells is a complex process controlled by a variety of transcription factors. Two of these factors, MafA and MafB, have been shown to play a major role in the regulation of several genes critical to pancreas development and endocrine cell formation. MafB is essential for both α- and β-cell differentiation, as mice lacking MafB have fewer insulin+ and glucagon+ cells during pancreas development. In contrast, in mouse embryos lacking MafA, the development of β-cells is normal, but adult animals develop diabetes. In adult animals these two transcription factors have a distinct expression pattern, with MafB being expressed in α-cells, whereas MafA is exclusively found in β-cells. We have performed gene expression profiling of wild type and MafA/B mutant pancreata to identify genes important for β-cell maturation and function. In these microarray studies several known (insulin, glucagon, Glut2, PC2) but also novel genes were shown to be differentially expressed in MafB and MafA/MafB compound mutant embryos. Gene ontology analysis revealed that the differentially expressed genes were mainly associated with mature β-cell function. Our findings show that Neuronatin (Nnat), islet-specific zinc transporter (Slc30a8), islet-specific glucose-6-phosphatase catalytic subunit-2 protein (G6pc2) and Microphthalmia associated transcription factor (Mitf) are downregulated in embryonic and adult mutant pancreata. In contrast, the mRNA level of Retinol Binding Protein-4 (Rbp4) was upregulated in mutant tissue. Given the differences in spatio-temporal expression pattern of MafA and MafB in developing and adult pancreas, we propose that these two factors regulate islet β-cell formation and maturation in a unique and sequential manner.</p> <p>In MafA deficient models studied so far pancreas development is unaffected, most likely due to compensatory functions of MafB. Therefore, to be able to study the actual role of MafA in β-cell function we developed a β-cell-specific deletion of MafA. Our MafA^{Δcell} mutant animals lack expression of MafA and MafB in adult β-cells. In contrast to other MafA mutants, our animals have normal islet architecture, β-cells mass, β- to α-cell ratio, and MafB expression is restricted to α-cells. Thus we have created a system suitable for studying the true contribution of MafA to adult β-cell function.</p> <p>Our gene expression experiments have shown that in MafA and MafB mutant embryonic pancreata levels of Mitf expression are reduced comparing to levels found in wild type animals. We also show that Mitf loss of function mutation alters functionality of islet β-cell. In response to an intraperitoneal glucose challenge, but also during non-fasted conditions, Mitf mutant mice have lower blood glucose levels than wild type animals. Mutant islets secrete more insulin upon exposure to high glucose concentrations and Mitf mutant animals have higher circulating insulin levels in fasted conditions. Additionally, Mitf directly regulates the expression of genes regulating blood glucose levels and β-cell formation, which is significantly higher in Mitf mutant than in wild type animals. Thereby, we demonstrate that Mitf is an important factor in modulation of β-cell function.</p> | | | |
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Signature Magdalena Mazur Date 2013-07-01

I dedicate this thesis to my parents

“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”

Maria Curie-Skłodowska

Front cover: Adult mouse islets stained for Microphthalmia transcription factor, insulin, glucagon and DAPI

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List of papers included in the thesis

1. **MafA and MafB regulate genes critical to beta-cells in a unique temporal manner.**

Artner I, Hang Y, Mazur M, Yamamoto T, Guo M, Lindner J, Magnuson MA, Stein R.

Diabetes. 2010 Oct;59(10):2530-9

2. **Adult Beta Cell Function Depends on MafA Transcriptional Activity**

Elvira Ganic, Jesper K. Colberg, Magdalena A. Mazur, Isabella Artner
Manuscript

3. **Microphthalmia transcription factor (Mitf) regulates pancreatic β cell function.**

Mazur MA, Winkler M, Ganic E, Colberg JK, Johansson JK, Bennet H, Fex M, Nuber UA, Artner I.

Diabetes. 2013 Apr 22.

Paper I

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Paper III

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From Diabetes®, Vol. 62, 2013

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Abbreviations

Bcl2 - B-cell lymphoma 2
CREB - C-AMP response element-binding protein
DIG - digoxigenin
HLA - human leukocyte antigen
IAPP - islet amyloid polypeptide
IDX-1 – islet/duodenum homeobox -1
Ipfl – insulin promoter factor 1
IPGTT – intraperitoneal glucose tolerance test
Maf - v-maf musculoaponeurotic fibrosarcoma oncogene homolog
MHC – major histocompatibility complex
Mitf - Microphthalmia transcription factor
Mnx1 – motor neuron and pancreas homeobox 1
NK- natural killer cell
PC – prohormone convertase
RA – retinoic acid
RER - rough endoplasmatic reticulum
RIP - rat insulin promoter
RPE – retinal pigmented epithelium
RRP - readily releasable pool
STF-1 – somatostatin transcription factor
TGN - Trans Golgi Network

Abstract

Diabetes - the imbalance in glucose homeostasis is partially caused by loss or dysfunction of pancreatic insulin producing β -cells or development of insulin resistance. In Type 1 diabetes β -cells are destroyed in the process of an autoimmune attack, whereas in Type 2 diabetes, islets produce insufficient amounts of insulin or the insulin cannot be used adequately. So far, the most promising therapy to treat Type 1 diabetes is pancreatic islet transplantation. However, this method cannot be used in a larger scale because of the severe scarcity of donors. Therefore, there is a great and urgent need to develop new methods and therapies. One of the potential sources of obtaining functional insulin cells are human embryonic stem cells (ESC) or inducible pluripotent stem cells (iPS). Hence, differentiating stem cells into functional β -cells requires a detailed understanding of pancreas development with the focus on molecular programs underlying β -cells formation and function.

Pancreas development and differentiation of insulin and glucagon secreting cells is a complex process controlled by a variety of transcription factors. Two of these factors, MafA and MafB, have been shown to play a major role in the regulation of several genes critical to pancreas development and endocrine cell formation. MafB is essential for both α - and β -cell differentiation, as mice lacking MafB have fewer insulin⁺ and glucagon⁺ cells during pancreas development. In contrast, in mouse embryos lacking MafA, the development of beta cells is normal, but adult animals develop diabetes. In adult animals these two transcription factors have a distinct expression pattern, with MafB being expressed in α -cells, whereas MafA is exclusively found in β -cells.

We have performed gene expression profiling of wild type and MafA/B mutant pancreata to identify genes important for β -cell maturation and function. In these microarray studies several known (insulin, glucagon, Glut2, PC2) but also novel genes were shown to be differentially expressed in MafB and

MafA/MafB compound mutant embryos. Gene ontology analysis revealed that the differentially expressed genes were mainly associated with mature β -cell function. Our findings show that Neuronatin (Nnat), islet-specific zinc transporter (Slc30a8), islet-specific glucose-6-phosphatase catalytic subunit-2 protein (G6pc2) and Microphthalmia associated transcription factor (Mitf) are downregulated in embryonic and adult mutant pancreata. In contrast, the mRNA level of Retinol Binding Protein-4 (Rbp4) was upregulated in mutant tissue. Given the differences in spatio-temporal expression pattern of MafA and MafB in developing and adult pancreas, we propose that these two factors regulate islet β -cell formation and maturation in a unique and sequential manner.

In MafA deficient models studied so far pancreas development is unaffected, most likely due to compensatory functions of MafB. Therefore, to be able to study the actual role of MafA in β -cell function we developed a β -cell-specific deletion of MafA. Our MafA ^{$\Delta\beta$ cell} mutant animals lack expression of MafA and MafB in adult β -cells. In contrast to other MafA mutants, our animals have normal islet architecture, β -cells mass, β - to α -cell ratio, and MafB expression is restricted to α -cells. Thus we have created a system suitable for studying the true contribution of MafA to adult β -cell function.

Our gene expression experiments have shown that in MafA and MafB mutant embryonic pancreata levels of Mitf expression are reduced comparing to levels found in wild type animals. We also show that Mitf loss of function mutation alters functionality of islet β -cell. In response to an intraperitoneal glucose challenge, but also during non-fasted conditions, Mitf mutant mice have lower blood glucose levels than wild type animals. Mutant islets secrete more insulin upon exposure to high glucose concentrations and Mitf mutant animals have higher circulating insulin levels in fasted conditions. Additionally, Mitf directly regulates the expression of genes regulating blood glucose levels and β -cell formation, which is significantly higher in Mitf mutant than in wild type animals. Thereby, we demonstrate that Mitf is an important factor in modulation of β -cell function.

Streszczenie

Cukrzyca jest zespołem chorób metabolicznych w trakcie którego dochodzi do stanu podwyższonego stężenia glukozy we krwi, zwanej też hiperglikemią. Pacjenci objęci cukrzycą cierpią na zaburzenia związane z utrzymaniem prawidłowego poziomu cukru we krwi wynikające z utraty funkcjonalności przez komórki β , wydzielające hormon insulinę. Insulina pełni zasadniczą rolę w metabolizmie węglowodanów, sprzyja transporcie glukozy z krwi do tkanek, obniżając jednocześnie stężenie cukru we krwi. Zwyczajowo rozróżnia się dwa rodzaje cukrzycy: typ 1 i typ 2. Cukrzyca typu 1 (insulinozależna) wywołana jest zniszczeniem komórek beta trzustki, które w konsekwencji produkują zbyt małą ilość insuliny lub nie wytwarzają jej wcale. W przypadku cukrzycy typu 2 (insulinoniezależna) trzustka produkuje niewystarczającą ilość insuliny lub działa ona niewłaściwie (zjawisko insulinooporności). Przeszczep wysp trzustkowych wydaje się być jedną z najbardziej obiecujących form leczenia cukrzycy (typu 1), co udowodnił słynny już protokół z Edmonton. W związku z ogromnym zapotrzebowaniem i nie mogącą mu równocześnie sprostać małą ilością materiału dostępnego do przeszczepu (komórki wysp trzustkowych pobrane od zmarłych dawców) metoda ta nie jest stosowana na zbyt wysoką skalę. Istnieje zatem wciąż niezaspokojona potrzeba pozyskania nowych źródeł komórek produkujących insulinę. Jednym z potencjalnych źródeł funkcjonalnych komórek β są ludzkie macierzyste komórki embrionalne (ES embryonic stem cells) lub indukowane komórki macierzyste (iPS induced pluripotent stem cells), drogą inżynierii genetycznej. Niemniej jednak, rozwinięcie skutecznych metod walki z cukrzycą wymaga szczegółowego poznania skomplikowanych mechanizmów kontrolujących proces powstawania i funkcjonowania komórek beta.

Rozwój trzustki i powstawanie specjalnych struktur komórkowych wydzielających hormony jest kontrolowane przez wiele różnych białek, tzw. czynników transkrypcyjnych. Badania nad dwoma z nich MafA i MafB udowodniły ich ważną rolę w prawidłowym funkcjonowaniu trzustki poprzez kontrolę genów kluczowych dla rozwoju i funkcjonowaniu komórek α i β . MafB jest niezbędny w rozwoju zarówno komórek α i β , podczas gdy MafA obecny jest jedynie w komórkach β . Myszy z zaburzeniami w ekspresji białka MafB mają mniej komórek α i β . W przeciwieństwie, myszy z nokautem MafA mają normalny rozwój komórek α i β , lecz w okresie dorosłym występują u nich objawy cukrzycy. Obecność czynników MafA i MafB w trzustce ma dość unikatowy charakter, gdyż MafB jest obecny w rozwijających się komórkach produkujących insulinę oraz glukagon, w dorosłej trzustce obecny jest jedynie w komórkach α .

MafA jest produkowany wyłącznie przez komórki β .

W celu zbadanie ekspresji genów regulowanych przez MafA i MafB, przeprowadziliśmy analizę za pomocą mikromacierzy z użyciem trzustek z E18.5 zarodków mysich z nokautem czynników MafA/MafB. W rezultacie wyselekcjonowaliśmy kilka znanych już białek regulowanych przez te dwa czynniki (insulina, glukagon, Glut2, PC2), jak również kilka nowych. Analiza ontologiczna genów różniących się ekspresją w przypadku nokautu MafB wykazała, iż większość z tych genów jest ważna w funkcjonowaniu zróżnicowanych komórek produkujących insulinę. W uzyskanych przez nas wynikach zidentyfikowaliśmy Nnat (Neuronatin), Slc30a8 (it-specific zinc transporter), G6pc2 (islet-specific glucose-6-phosphatase catalytic subunit-2 protein) Mitf (Microphthalmia associated transcription factor) jako geny potencjalnie regulowane przez MafA i MafB. Poziom ekspresji tych genów był mniejszy u myszy MafA/MafB mutantów zarówno w trzustkach wyizolowanych z E18.5 płodów, jak i dorosłych trzustkach. W odróżnieniu od czterech wymienionych, poziom ekspresji czwartego z genów Rbp4 (Retinol Binding Protein-4) był podwyższony w trzustkach wyizolowanych z myszy mutantów białek MafA i MafB. Wziąwszy pod uwagę wyjątkowy charakter ekspresji czynników MafA i MafB w rozwijającej się i dorosłej trzustce oraz wyniki uzyskane z analizy mikromacierzy proponujemy, iż czynniki te w różny sposób regulują ekspresję genów ważnych w rozwoju.

Analiza wyników miromacierzy umożliwiła nam zidentyfikowanie kolejnego czynnika transkrypcyjnego Mitf (Microphthalmia transcription factor), którego poziom był zmniejszony w trzustkach wyizolowanych z E18.5 płodów mysich MafA/MafB mutantów. Nasze eksperymenty pokazały, iż Mitf jest obecny w rozwijającej się oraz dorosłej mysiej trzustce. Jak pokazały eksperymenty z myszami z upośledzoną funkcją Mitf, czynnik ten jest potrzebny do prawidłowego funkcjonowania trzustki, w szczególności komórek produkujących insulinę. Myszy z mutacją czynnika Mitf mają niższy poziom glukozy we krwi podczas testu obciążenia glukozą, jak również w warunkach na czczo. Wyspy trzustkowe pochodzące z myszy z mutacją genu Mitf wydzielają więcej insuliny podczas stymulacji roztworem glukozy o wysokim stężeniu w porównaniu do myszy kontrolnych. Dodatkowo, mutanty mają wyższy poziom insuliny na czczo. Ekspresja genów mających zasadniczy wpływ na poziom glukozy we krwi oraz rozwój trzustki była znacząco wyższa u myszy z mutacją czynnika Mitf. Czynnik Mitf jest bezpośrednim regulatorem ekspresji tych genów w komórkach β trzustki i dzięki temu może modelować funkcje komórek produkujących insulinę.

Populärvetenskaplig sammanfattning

Patienter med diabetes kan inte kontrollera blodsockernivåerna på grund av att de saknar insulinproducerande celler eller har icke fungerande insulinproducerande celler. Det finns olika typer av diabetes där typ 1 diabetes och typ 2 diabetes är de vanligaste formerna. Den mest framgångsrika behandling mot typ 1 diabetes är transplantation av insulinproducerande celler, så kallade öar, från donatorer. Dock finns det inte tillräckligt med donatorer. Ett annat alternativ är att framställa insulinproducerande celler från bland annat mänskliga embryonala stamceller. För att lyckas med detta krävs det att vi vet hur bukspottkörteln normalt bildas i däggdjur. Bukspottkörteln är det organ där de insulinproducerande cellerna finns.

Bukspottkörteln har bland annat till uppgift att producera hormoner (insulin och glukagon) som kontrollerar blodsockernivåerna. Denna process styrs av olika faktorer såsom MafA och MafB. MafB är viktigt för bildandet av både insulinproducerande och glukagonproducerande celler. Möss som saknar MafB har färre insulin- och glukagonproducerande celler men de insulin celler som finns kvar har MafA. Möss som saknar MafA är normala under fosterstadiet men utvecklar så småningom diabetes.

Jag har jämfört olika gener i vanliga möss och i möss som saknar Maf faktorer för att se vilka gener som är viktiga för insulincellers mognad och funktion. Vi såg att många kända faktorer var reglerade såsom insulin, glukagon, Glut2 och PC2 men också icke kända gener involverade i funktionen av insulinceller identifierades. I de möss som saknade Maf faktorer var följande gener nedreglerade; Neuronatin (Nnat), islet-specific zinc transporter (Slc30a8), islet-specific glucose-6-phosphatase catalytic subunit-2 protein (G6pc2) och microphthalmia associated transcription factor (Mitf) medans retinol binding protein-4 (Rbp4) var uppreglerad.

Jag har visat att det finns lägre nivåer av Mitf i bukspottkörteln från möss som saknar MafA och MafB. Jag har också visat att Mitf produceras i bukspottkörteln under fosterstadiet och i öarna i vanliga möss. Möss som saknar Mitf har icke fungerande insulinproducerande celler. Detta såg jag både när mössen injicerades med sockerlösning och när djuren fastades. När öarna ifrån dessa djur utsätts för socker utsöndrar de mer insulin och dessa möss har även högre nivåer av insulin i blodet när de är fastande. De faktorer som styr regleringen av blodsockernivåer i blodet och insulincellernas funktion finns i högre nivåer i möss som saknar Mitf än i vanliga möss. Jag har också visat att Mitf direkt reglerar dessa gener som styr både blodsocker nivåerna och insulincellernas funktion.

Introduction

Diabetes

Diabetes results from lack or dysfunction of pancreatic beta cells which produce and secrete the insulin hormone. In Type 1 diabetes, insulin producing beta cells are destroyed in the process of an autoimmune attack which results in loss of beta cell mass. In Type 2, beta cells become dysfunctional, islets produce insufficient amounts of insulin, or the subject develops insulin resistance and cannot use the insulin adequately. Diabetes represents a chronic metabolic disorder for which there is no permanent/final cure. To date, the most successful therapy to treat Type 1 diabetes is islet transplantation according to the Edmonton protocol [1]. However, this technique is severely limited by the number of cadaveric donors and the need for immunosuppressive treatment after the transplantation. Therefore, there is a great and urgent need to develop new methods and therapies in order to treat diabetes. One of the potential sources of obtaining functional insulin cells are human embryonic stem cells (ESC) and inducible pluripotent stem cells (iPS). Nevertheless, developing efficient and successful therapies requires a detailed understanding of pancreas development biology with the focus on molecular programs underlying β -cells formation and function.

Today's worldwide prevalence numbers predict nearly 300 million people with diabetes (8,3% of adults). Alarmingly, that number is likely to exceed 500 million in 2030 which corresponds to one in ten adult suffering from diabetes (<http://www.diabetesatlas.org/>). In Europe, around 7% of the entire population is estimated to have diabetes, of which 10% are diagnosed with Type 1. Remaining 90% of diabetic patients represent Type 2. Therefore, there is a high risk for Type 2 diabetes to become a global epidemic having a huge impact on health and economical status of the society.

Types of diabetes

The American Diabetes Association distinguishes between two main types of diabetes, Type 1 and Type 2, although diabetes may also manifest during pregnancy (gestational diabetes) and under other conditions such as drug or chemical toxicity and genetic disorders [2]. Diabetes results from a combination of mutations in specific genes and/or environmental factors. The aetiology behind

development of Type 1 diabetes is still not very well known, yet the autoimmune destruction of pancreatic beta cells is the resulting pathological finding. It is believed that variations in certain major histocompatibility complex loci (HLA) strongly contribute to the risk of Type 1 diabetes. However, this only accounts for ~50% of diabetic incidences suggesting that there are other genetic factors which are involved in Type 1 diabetes susceptibility. Type 1 diabetes comprises 10–15% of the diabetic cases in e.g. Australia [3], but this number can vary greatly and be up to 40% in other countries. (<http://www.jdrf.org.au/about-jdrf-australia/mediaroom>). In addition to genetic factors, the increasing number of Type 1 diabetic patients can result from both environmental triggers and insulin resistance [4] which are normally ascribed to Type 2 diabetes. Type 2 diabetes is the most common form of Diabetes Mellitus comprising ~85% of all diabetes cases globally. In Type 2 diabetes environmental factors (obesity, improper diet, lack of physical exercise) are much better understood than the underlying genetic factors [5], [6]. Hyperglycemia in Type 2 diabetes stems from either failure of beta cells to produce sufficient amounts of insulin or the peripheral insulin resistance in target tissues (skeletal muscle, liver, and adipose tissue). Interestingly, in many cases of insulin resistance, the decreased sensitivity to insulin is compensated by hypersecretion of insulin from the pancreatic islets [7]. This mechanism can act in advance to the drop in islet secretory function.

Diabetic complications and treatment

Up until recently, diabetes was a fatal disease that ended with diabetic ketoacidosis, in case of extremely high glucose levels (hyperglycemia) or coma resulting from low blood glucose (hypoglycemia). Discovery of insulin and its therapeutic role has dramatically changed this fatal condition into a chronic illness prolonging lives of many diabetic patients. Unfortunately, even though their life expectancy was significantly increased, they still face the long-term effects of increased blood glucose levels. Usually, complications associated with diabetes are divided into two groups: micro- and macrovascular describing damage to small or large (arteries) blood vessels respectively. Microvascular complications include retinopathy (eye disease), nephropathy (kidney disease) and neuropathy (neural damage). Most common macrovascular complications are coronary heart diseases manifesting as strokes.

Insulin is considered a key player in controlling hyperglycemia in Type 1 and some Type 2 diabetic patients. Subcutaneous injections are the most common form

of administering insulin. In spite of being the most predictable method, this delivery system is very invasive. An alternative strategy to wearisome insulin injections is pancreatic islet transplantation. The success of a cell replacement approach was greatly improved by a landmark study known as the Edmonton protocol [1]. In that study seven diabetic patients with transplanted islets remained insulin independent for a period up to one year. Since then much has been learned about many aspects of islet transplantations. Clinicians and researchers aim to optimize islet survival, function and engraftment after transplantation, and it is believed that clinical outcome of islet transplantation will continue to improve. However, scarcity of donors, gradual reduction in β -cell function and the necessity for immunosuppressive treatment limit this approach quite drastically. Hence, many research groups have focused on developing methods for generating alternative sources of new β cells. These might be obtained either after transdifferentiation of related cell types or directed differentiation from stem and progenitor cells [8].

Developmental biology of the pancreas

Anatomy and function of the pancreas

The pancreas is a glandular organ and in humans it is located across the back of the abdomen, behind the stomach. The terms head and tail are used to describe the two main regions of the organ, from proximal to distal (Figure 1A). Pancreatic organ consists of two distinct compartments: the exocrine (produces and secretes enzymes into the intestine) and endocrine (controls blood glucose levels by hormone secretion into the bloodstream). The exocrine pancreas is composed of acinar and ductal portions. Acinar cells secrete a variety of digestive enzymes (e.g. amylases, proteases, lipases, nucleases) that facilitate digestion of food and nutrient absorption (Figure 1B). These enzymes are transported by another component of the exocrine pancreas, the highly branched ductal system. The endocrine cell compartment makes up only a small percentage (1-2%) of the entire pancreatic organ and consists of five different cell types (α , β , Δ , PP and ϵ) (Figure 1C). Endocrine cells cluster into spheroidal structures, scattered throughout the exocrine tissue, called islets of Langerhans. Beta cells represent the majority of the endocrine compartment and compose 60-70% of the total islet mass. Remaining 15-20% are glucagon cells, 5-10% are somatostatin producing cells, less than 2%

are pancreatic polypeptide and only 1% are ghrelin cells. Interestingly, in the adult mouse pancreas ghrelin cells are extremely rare [9], [10].

In normal conditions, blood glucose levels are kept under tight control in such a manner that the insulin hormone is released from beta cells in response to elevated blood sugar levels after food intake. This is also a signal for glucose storage and its uptake by target tissues such as muscle, liver and adipose tissue. Concomitantly, insulin inhibits glucose production by the liver. Glucagon acts in opposite to insulin and its secretion is stimulated by low blood glucose levels. Glucagon promotes two processes involved in glucose formation glycogenolysis and gluconeogenesis.

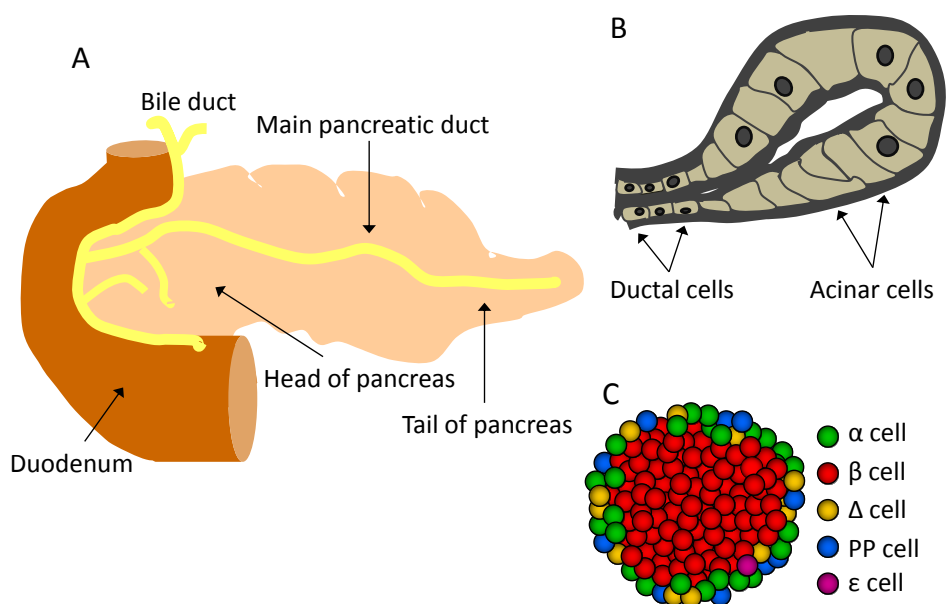


Figure 1. Structure of the adult human pancreas. The mature pancreas lies adjacent to the duodenum which is the first part of the small intestine (A). Histology of pancreatic acinar cells and ductal system (B). The endocrine compartment consisting of five different hormone expressing cell types (C).

Pancreas specification

Pancreas development similarly to many other organs involves a series of complex tissue interactions. The detailed description of this process is mostly based on *in vivo* and explants studies. The pancreas formation program is induced in the foregut/midgut junction of the endoderm by factors secreted from the mesoderm.

The pancreatic organ originates from a flat sheet of cells called definitive endoderm. The portion that will later give rise to the dorsal bud is a single layer of epithelial cells that are in close contact with the notochord (an axial mesoderm-derived structure). The dorsal endoderm stays adjacent to the notochord until the fusion of the two dorsal aortae (in mice E8.75-9.0). Lateral endoderm that is destined to become the ventral pancreas is adjacent to both splanchnic mesoderm and aortic endothelial cells. However, it is not in close contact with the notochord (Figure 2).

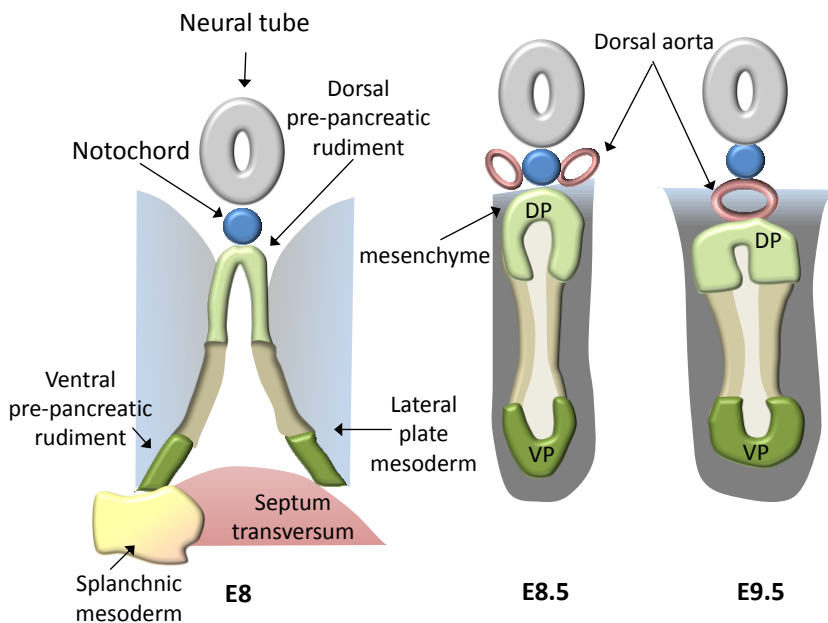


Figure 2. Early stages of pancreas development. Pancreas originates from two evaginations (ventral and dorsal) of the foregut endoderm. The ventral pre-pancreatic bud is surrounded by lateral plate mesoderm. There is also a thick layer of splanchnic mesoderm on the ventral side of the gut epithelium. The dorsal pre-pancreatic bud stays in close contact to the notochord until the fusion of the two dorsal aortae. Mesenchyme surrounding pancreatic buds (VP ventral bud, DP dorsal bud) provides signals for proliferation, branching and differentiation of the different pancreatic compartments.

The two pancreatic buds arise as thickenings of the dorsal and ventral posterior foregut from a specialized pre-patterned endodermal epithelium between E8.5-E9.5. The dorsal mesenchyme thickens and the neighboring endodermal region gives rise to the dorsal bud which forms at E9.5. The ventral bud lies in proximity to the liver diverticulum and it appears one day later. By E10.5 the two buds

undergo a series of morphological changes that results in the formation of a ductal tree. Mesoderm that lies adjacent to the buds sends signals that instruct pancreatic epithelial cells to proliferate, migrate and form evaginations that will branch out into the surrounding mesenchyme. Subsequently, differentiation and maturation of the pancreatic primordium occurs, followed by microlumen formation. Microlumen coalesce and form a highly branched ductal network. In the mouse, both acinar and ductal structures are easily visible as histologically distinct structures by ~E14.5. Along with the complex branching morphogenesis, endocrine cell precursors delaminate from the epithelium and cluster into islet structures within the mesenchyme. The cells that remain in the epithelium form a branched ductal network and acinar structures. The two pancreatic buds are brought together when the gut rotates clockwise placing the ventral bud beside its dorsal counterpart. Finally, pancreatic lobes fuse and form a mature organ prior to birth (Figure 3).

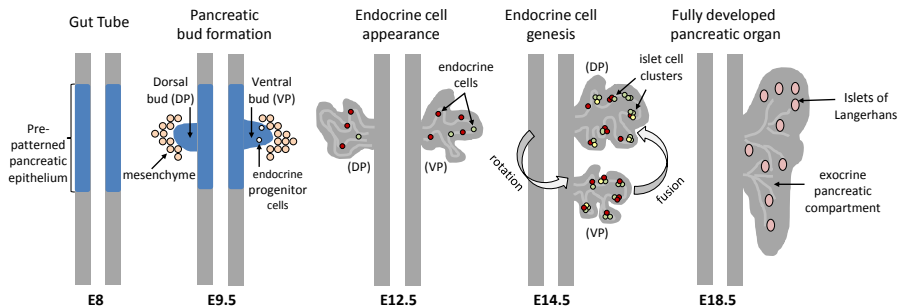


Figure 3. Overview of mouse pancreatic organogenesis showing different stages in the development of the mouse pancreas. At E8, pre-patterned regions of the primitive gut endoderm (foregut/midgut junction) form the two primitive evaginations. These outgrowths will later give rise to pancreatic buds by E9.5. First endocrine cells form along with the formation of the dorsal and ventral bud. Additionally, at E12.5 the pancreatic epithelium undergoes substantial proliferation and differentiation. Growing buds invade the surrounding mesenchyme and branching morphogenesis takes place. Subsequently, the ventral bud rotates clockwise and fuses with the dorsal bud. During the secondary transition, endocrine and acinar cell formation is at its peak. Finally, mature pancreas with clusters of endocrine cells (Islets of Langerhans), acinar and ductal structures forms prior to birth.

In the developing pancreas, endocrine cells can be detected relatively early and the first endocrine cell type to form is glucagon (E9.5). Insulin and ghrelin positive cells appear one day later at ~E10.5. It is hypothesized that these early glucagon and insulin expressing cells do not contribute to the adult endocrine pancreas and

they most likely die during the development [11]. Around the period of secondary transition (~E13.5) endocrine and acinar cell formation is at its peak and many mature insulin and glucagon expressing cells are generated. Later, at E14.5 first somatostatin positive cells can be found, whereas pancreatic polypeptide producing cells only appear shortly before birth at E18. Two to three weeks after birth the pancreas undergoes further remodeling and numerous new insulin producing β cells are formed at that time point [12], [13].

Distinct specification of dorsal and ventral pancreas anlagen

Growth of the two pancreas anlagen appears to be temporally independent. In regards to the cell types, composition and function of the both pancreatic buds are indistinguishable. However, different studies proved that the ventral and dorsal pancreas develop from the posterior foregut as two entirely distinct organs. In addition, their development results from distinct signaling patterns.

Dorsal pancreas specification

Induction of dorsal pancreas formation occurs when the dorsal pancreatic endoderm contacts the notochord which is a source of signals such as activin- β B (member of TGF- β family) and FGFs. Both activin and FGF were shown to activate Pdx1 expression in the dorsal endoderm by repressing Sonic hedgehog (Shh) transcription [14], [15]. Exclusion of the expression of the hedgehog gene family (Shh and Ihh) is an important element of the early pancreas specification since these factors promote intestinal instead of pancreatic fate [16], [17].

Retinoic acid (RA), which is produced by the Raldh2 enzyme, was shown to be crucial for the development of the dorsal bud. Raldh2-deficient mice lack the dorsal bud and have fewer mesenchymal cells. The expression of essential pancreatic markers e.g. Hb9, Isl1, Pdx1, Glut2 is also decreased in these mutants. However, RA is dispensable for the formation of the ventral pancreas and the liver [18], [19].

Expression of Hb9 (also called Hlxb9 or Mnx1) gene was shown to be essential for the dorsal pancreas evagination since Hb9 knockout animal models have dorsal pancreatic agenesis. The ventral pancreas develops but shows disturbances in β -cell differentiation and in islet architecture. In addition, maturation of the β cells is affected and glucose transporter Glut2 expression is lacking [20], [21].

Dorsal pancreas formation is also severely affected in embryos lacking the LIM homeodomain protein – Isl1. Mutant mice do not have dorsal mesenchyme over

the pancreatic bud and endocrine cells are missing. However, the development of the ventral pancreatic epithelium seems to be unaffected [22].

Ventral pancreas specification

During pancreas formation, the ventral portion of the endoderm is subjected to a quite different collection of signals and tissue interactions than the dorsal bud. During foregut morphogenesis the ventral foregut endoderm stays in vicinity of the cardiac mesoderm and septum transversum mesenchyme that derives from lateral plate mesoderm. These two tissues were shown to promote the choice of liver fate at the expense of the pancreatic [23], [24]. Both FGFs and BMP are thought to be the two candidates of the pro-liver instructive signals coming from surrounding mesenchyme [24]. FGF1 and FGF2 were shown to be specifically involved in patterning of the ventral foregut although FGF1/2 double knockout mice do not display liver or pancreas defects [25] probably due to the compensation from other FGF family members. Similarly to the FGF pathway, the BMP signaling is also needed for hepatocyte specification while it inhibits pancreas specification [24]. It is believed, that to overcome the effect of BMP and FGF, the ventral pancreas has to have a certain protective mechanism. Studies by Bort et al. have shown an important role for the homeobox gene Hex in that process. Its role is to control the proliferation of the ventral foregut endoderm prior to the specification into liver by allowing a subset of endoderm cells to grow beyond cardiogenic mesoderm. This is supported by the fact that ventral pancreas specification fails in Hex null mutant mouse embryos. However, the liver program is not affected [26].

Notch signaling is another pathway that may be involved in specification of the ventral foregut. Studies of the downstream Notch effector Hes1 showed that Hes1^{-/-} mutant mice have a hypoplastic pancreas with an increased number of glucagon cells which may be a result of premature differentiation of endocrine cells. In summary, the Notch signaling pathway during early pancreas development is responsible for keeping pancreatic progenitor cells in undifferentiated state. This enables proper growth and morphogenesis of the pancreas.

Transcriptional Regulation in Pancreatic Development

Transcription factors in the regulation of pancreas development and function

Intercellular signaling through different molecular pathways is crucial for proper endocrine and exocrine pancreas development. Pancreas formation and development have been studied in great details by many different research groups. Based on their findings, a large number of markers that elegantly define different stages of pancreas development have been identified. There are many transcription factors involved in the complex process of pancreas development which enables coordinated expression of different pancreatic cell-type specific genes. These factors act in a sequential cascade of inductive events which lead to cell fate decisions and eventually formation of a mature and functional pancreatic organ. Most of our knowledge about pancreas formation and function come from animal models in which specific genes were genetically modified (e.g. time and tissue dependent context).

Multipotent pancreatic progenitor markers

Pdx1 as a master regulator of pancreas development

Early expression of Pdx1 (also known as Ipfl, STF-1 or IDX-1) marks the territory of the future pancreas [27], [28]. Both pancreatic and duodenal progenitor cells express Pdx1. During early pancreas development Pdx1 expression initiates at E8.5 in the gut region when the foregut endoderm becomes committed to pancreatic precursor cells and the ventral pancreas starts to form. Approximately 12h later Pdx1 expression is also initiated in the dorsal pancreas (additionally in caudal stomach and proximal duodenum). Pdx1 expression is preceded by Hnf6 induction. Factors such as Hb9 and Isl1 trigger expression of Pdx1 in the dorsal and Hex1 in the ventral bud, respectively. In acinar and ductal cells, Pdx1 expression becomes downregulated at the end of embryonic development and by E16.5 its expression becomes restricted to endocrine lineage. From E19 Pdx1 is present only in insulin producing cells and ~20% of somatostatin cells, and very weakly in the exocrine compartment.

In mice, disruption of Pdx1 results in pancreatic agenesis with limited growth of the dorsal bud and fatal perinatal hyperglycemia [29]. In human patients homozygous mutations in the IPF1/PDX1 locus cause failure in pancreas

development and heterozygous mutations strongly predispose to maturity onset diabetes of the young (MODY4) and adult-onset Type 2 diabetes [30].

Pdx1 is also engaged in differentiation and maintenance of the β -cell phenotype. In β cells, Pdx1 activates insulin but also other genes that are important for glucose metabolism. These genes include e.g. glucose transporter (Glu2), glucokinase, and islet amyloid polypeptide (IAPP) [31–33]. Pdx1 directly binds to and activates the insulin gene promoter. Together with other transcriptional co-activators it forms a complex on the proximal insulin promoter [34].

Recently Pdx1 was shown to cause an alpha to beta cell conversion upon forced expression in Ngn3+ endocrine cells. The increase in the number of insulin producing cells at the expense of the glucagon cell lineage demonstrated by Yang et al. suggests that Pdx1 can specifically affect the early endocrine cell fate decision [35].

Ptf1a specifies early pancreatic progenitor cells

Ptf1a, also known as p48, is a helix-loop-helix transcription factor implicated in early specification of pancreatic progenitor cells. Its expression initiates in pancreatic progenitors shortly after budding of the two pancreatic anlagen. Ventral pancreas starts expressing Ptf1a at E8.5, whereas dorsal at E8.75 [36]. Initially Ptf1a is coexpressed with Pdx1 in pancreatic progenitors found in both pancreatic buds, although later (from E14) it becomes restricted to acinar cells [37]. Ptf1a mutant animals do not develop a ventral pancreas. Formation of the dorsal pancreas, which lacks ducts and acini, is greatly reduced. Endocrine cells, even though diminished in number, are present. This implies that Ptf1a is important for specification of the exocrine lineage, although it is dispensable for the formation of the endocrine cells [37], [38]. In addition, it was found that Ptf1a is not necessary for specification of Ngn3+ endocrine cells nor mature beta cells differentiation [39]. The Ptf1a protein is a part of a trimeric complex – PTF1 consisting of three distinct subunits (p75, p64 and p48). P75 subunit does not bind to the DNA, although it is responsible for targeting PTF1 to the nucleus. P64 and p48 contact the DNA as a heterodimer (no individual binding) recognizing two distinct DNA motifs. The PTF1 complex can directly bind to the promoters of exocrine specific genes such as elastase-1 and amylase [40], [41]

Hb9 controls formation of the dorsal pancreatic bud

Hb9 belongs to the bHLH transcription factor family. Hb9 is transiently expressed and it initially synthesized at E8 in the notochord and the dorsal and ventral gut endoderm where it forms a dorsal-ventral gradient at E9.5 [42]. Hb9 protein levels peak at E10.5. Later they gradually decline and in the adult pancreas, Hb9 is found in mature β cells. Hb9 expression in the dorsal bud precedes that of Pdx1. Experiments with Hb9 over-expression under the control of the Pdx1 promoter (extending Hb9 expression beyond E9–E10) led to impaired pancreas development. This suggests that early stages of pancreatic development require tight temporal regulation of Hb9 expression [43].

In mice with homozygous null mutation in Hb9 gene, the dorsal pancreatic bud fails to form. The ventral pancreas appears to be normal, although beta cell maturation is affected (lack of Pdx1, Nkx6.1 and Glut2 expression) and islet structure is disorganized [20], [21].

Sox9 is critical for maintaining the pool of pancreatic progenitors

Several members of the SRY-related (Sex-determining region Y–box, high mobility group box - Sox) genes have been reported to be expressed in the pancreas during development. Sox9 is present in both the fetal and adult human pancreas. Sox9 is initially detected in Pdx1+ cells of the pancreatic epithelium at E9-E9.5. Later during development Sox9 expression becomes restricted to ductal cells, but it is excluded from endocrine lineage committed progenitor cells or differentiated hormone expressing cells [44], [45]. In mice, pancreas-specific Sox9 ablation causes severe pancreatic hypoplasia of both pancreatic buds (decreased proliferation, increased apoptosis of progenitor cells).

A possible link between Sox9 and Notch signaling might be engaged in maintaining the pool of pancreatic progenitors [45]. Hes1 expression is severely downregulated in the absence of Sox9 activity. This suggests that Sox9 modulates Notch signaling by affecting Hes1 which is a known Notch effector. Furthermore, Sox9 was shown to bind and upregulate the Ngn3 gene which may enable its role as a mediator in the transition of pancreatic progenitor cells from Hes1+/Ngn3- to Hes1-/Ngn3+ [45], [46].

Pancreatic endocrine progenitor markers

Neurogenin3 specifies pancreatic endocrine cell lineage

Neurogenin3 (Ngn3 or Neurog3) belongs to the bHLH family of transcription factors and is critical for the specification of pancreatic endocrine cells. At E8.5 Ngn3 can be found in a few cells within the dorsal pre-patterned endoderm [47–49]. During pancreas development Ngn3 is expressed transiently in a biphasic manner with two peaks observed around E10 and E15, but it decreases by E17.5 [50]. Ngn3 expression highly correlates with the three distinct stages of endocrine cell development, the primary (E8.5-E12.5), secondary (E12.5-E16.5) and tertiary (E16.5-birth) transition (Figure 4). Ngn3 is only found in a small fraction of cells in developing pancreatic epithelium and its expression is a prerequisite in directing the differentiation of new endocrine cells in the islet. It was previously shown that Ngn3 is required for the development of all pancreatic endocrine cell lineages [47] and it is important for activation of endocrine lineage-committed transcription factors. This enables differentiation of endocrine progenitor cells towards mature hormone-producing cells [51]. Interestingly, once hormone expression is turned on, Ngn3 expression is switched off suggesting that Ngn3 is a marker of immature cells in the endocrine cell lineage [49].

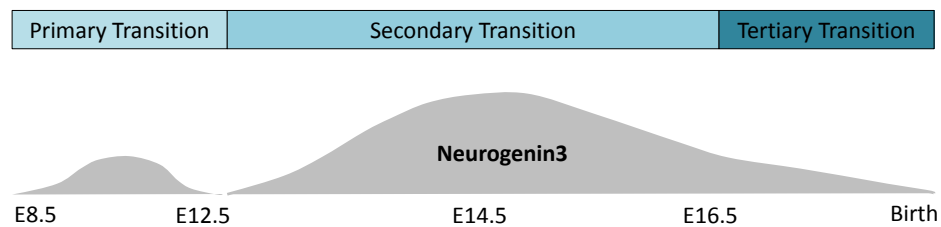


Figure 4. Schematic overview of the three transitional stages during pancreas endocrine cell development correlating with the temporal pattern of Neurogenin3 expression. First wave of Neurogenin3 expression occurs approximately at E8.5 – E11. As the pancreas reaches the secondary transition, the second wave of Ngn3 expression initiates ~E12.5 and peaks around E14.5-E15.5. Prior to birth Neurogenin3 expression declines to undetectable levels. Further remodeling and islet reorganization occurs during first few weeks after birth.

Ngn3^{-/-} animals have neither endocrine precursors nor islets of Langerhans. As a consequence, mutant mice develop diabetes and die soon after birth [47]. In contrast, studies using the Pdx1 promoter to drive ectopic Ngn3 expression showed precocious differentiation of pancreatic progenitor cells, mostly to α -cells [49], [52] However, recent studies, with tamoxifen-inducible transgenic mice

expressing Ngn3 under the Pdx1 promoter in the Ngn3 null background, showed the presence of competence windows, during which all different endocrine cell types can be generated. This indicates that the competence of Pdx1+ cells changes during the development and it depends on Ngn3 activation and that the glucagon cell fate is not necessarily the default pathway [53]. In 2010, elegant studies by Wang et al. demonstrated that Ngn3 gene dosage (Ngn3^{HIGH} stage) plays an essential role in commitment of pancreatic progenitors to endocrine cell lineage. Low and medium levels of Ngn3 expression may prevent cells from full differentiation and final hormone production. Consistent with this, cells with Ngn3^{LOW} expression may adopt acinar or ductal fate. This latter finding suggests that Ngn3^{LOW} cells may represent a plastic, although endocrine-biased cell population [54].

Isl1 controls development of islet cell lineage and dorsal pancreas mesenchyme

Isl1 is a primary LIM homeodomain transcription factor in developing and adult pancreas. Isl1^{-/-} mutant mice die during embryogenesis as a result of severe heart defects. Its expression was shown to be required for the formation of the dorsal pancreatic mesenchyme, although it is dispensable for the formation of the ventral mesenchyme and ventral exocrine pancreas [22]. A conditional deletion of Isl1 enabled studying its role in endocrine progenitors. Mice lacking Isl1 expression from pancreatic epithelium at E13.5, have drastically reduced numbers of hormone+ cells and the total endocrine cell mass is decreased. As a consequence, Isl1 mutant mice become diabetic which is at least partially attributed to defects in MafA expression, an important insulin regulator [55].

Endocrine lineage allocation factors

Endocrine lineage-specific markers control progression of pancreatic progenitor cells during their specification towards a unique endocrine cell type. Aberrations in the expression of these markers mostly affect allocation of different islet cell types (shift in ratio between the different cell types); whereas total numbers of endocrine cells remain unchanged.

Nkx family members regulate differentiation of pancreatic endocrine cells

In the pancreas, Nkx2.2 expression initiates at E8.75 in the Pdx1⁺ cells of the dorsal bud; in the ventral bud Nkx2.2 expression starts at ~E9.5. During the development (after E12.5) Nkx2.2 becomes restricted to Ngn3⁺ cells and later it can be found in α , β and PP cell population, however not in δ cells. Nkx2.2 mutant mice die shortly after birth due to severe hyperglycemia. Disrupted Nkx2.2 expression causes lack of insulin producing cells, diminished number of glucagon and PP cells. However, the presence of many islet hormone negative cells in mutant islets suggests that Nkx2.2 is required for the final differentiation of pancreatic endocrine progenitors [56]. Studies by Prado et al. showed that in Nkx2.2 null mutants these “arrested” cells express the hormone ghrelin [9]. In addition to its role as a transcriptional repressor, it can also act as an activator and both of these roles may contribute to specification of endocrine cell types in the pancreas [57], [58].

Nkx6.1 and Nkx6.2 also belong to the NK homeodomain family which plays central role in endocrine cell development. Both Nkx6.1 and Nkx6.2 are expressed early during the development and their expression is preceded by Pdx1. Nkx6.1 is first found at E10.5 in Ngn3⁺ cells of the pancreatic epithelium. Similarly to Nkx6.1, Nkx6.2 is also found in Pdx1⁺ but it becomes downregulated and by E15.5 it is present in a small population of α cells and exocrine cells. Its expression is absent from adult pancreas [59], [60]. Pdx1 deficiency causes loss of Nkx6.1 expression but no effect on Nkx6.2 was observed. This findings place Pdx1 upstream of Nkx6.1 but not Nkx6.2 [61]. Nkx6.1 mutant animals have smaller islets, due to reduction in beta cell number, although other endocrine cell types develop normally [59]. Interestingly, Nkx6.1 was shown to be required for beta cell formation only during the secondary transition since formation of insulin cells at E12.5 was unaffected in Nkx6.1 mutant animals. In contrast to Nkx6.1, Nkx6.2 appears to be dispensable for endocrine cell formation and Nkx6.2^{-/-} mice do not have obvious pancreatic phenotype [60]. Nevertheless, compound Nkx6.1/Nkx6.2 mutant embryos have a more pronounced decrease in beta and additional reduction in alpha cell number. This suggests that Nkx6.2 partially compensate for the loss of Nkx6.1 function [60].

Two members of the Pax family of transcription factors are important for endocrine cell fate and β cell development

Studies of Pax4 and Pax6 mutant animal models showed their importance for proper endocrine cell specification and function. Pax4 expression initiates ~E9.5 in differentiating endocrine cells of both ventral and dorsal pancreatic buds. During development its expression becomes restricted to the insulin expressing β cell population. Pax4 homozygous mice have a drastic decrease in β and δ cell populations which fail to develop. Mutant animals' growth is severely impaired; they also suffer from dehydration and as a consequence die within few days after birth. In contrast to insulin and somatostatin, the glucagon cell population is increased in Pax4^{-/-} mice [62]. Interestingly, Prado et al. showed that Pax4^{-/-} mice also have increased number of ghrelin⁺ cells, similarly to the Nkx2.2 knockout mice. This finding suggests a possible genetic link between these two transcription factors [9]. Pax4 was shown to be involved in direct inhibition of the alpha specific cell marker Arx [63]. In Arx mutant animals, a lineage switch with loss of alpha cells and increase in β and δ cell populations was observed, which suggest a strong complementation to the Pax4 mutant. These mutually repressive functions of both factors appear to be crucial for proper islet specification, especially α - and β -cell allocation, and are tightly connected to the levels of Arx and Pax4 transcripts. Recent study by Collombat et al. demonstrated that Pax4 misexpression driven by different cell-specific promoters forces both endocrine precursor cells and adult alpha cells to convert into beta cells [64].

During early pancreatic development Pax6 expression initiates at ~E9 and is present in cells in the region of the primitive gut that will later form the pancreas. Subsequently, Pax6 is found in progenitor cells in both pancreatic buds and some glucagon⁺ cells (from E10.5) and insulin⁺ (from E12.5). Later its expression is retained in all committed endocrine cells and differentiated hormone expressing cells, whereas it is not detected in the exocrine cells [65], [66]. Pax6 was demonstrated to play an important role in the development of eye, forebrain and pancreas. Mice with a point mutation in pax6 gene called Small eye Sey^{NEU/NEU} display reduced numbers of all endocrine cells, together with abnormal islet morphology, highlighting the importance of this transcription factor in pancreas development. Further studies identified Pax6 as an important activator of hormone gene transcription, since it directly binds to the promoter regions of the insulin, glucagon and somatostatin genes [65]. Homozygous Pax6 mutant mice do not form distinct islets, although remaining α -, β - and δ -cell populations are present.

The disorganized islets of Langerhans observed in these mutants may result from disrupted interaction between Pax6 and certain adhesion molecules or the absence of appropriate cell number required for normal islet morphogenesis [66]. Pax6 null mutant animals die soon after birth which precluded studies on the role of Pax6 postnatally. Therefore, a conditional inactivation of Pax6 gene approach was utilized, and Pax6 was specifically ablated from the endocrine cell population. Pax6 mutant animals display diabetic phenotype caused by hyperglycemia and hypoinsulinemia, followed by death usually 3-6 days after birth. These results further establish the Pax6 as key transcription factor in maintaining normal beta cell function [67]. In vitro experiments show that Pax6 regulates also several α -cell specific genes e.g. MafB, PC2 and NeuroD1/Beta2 [68]. In contrast to well established and known role of Pax6 during the development, its function in adult tissue maintenance is less clear. Recent studies using a conditional tamoxifen-inducible Cre transgene to inactivate Pax6 expression in adult mice showed that late loss of Pax6 expression leads to a drastic diabetic phenotype within few weeks after tamoxifen administration. In addition, expression of several beta cells genes (Pdx1, Glut2, PC1/3) was reduced [69]. This demonstrates a requirement for continuous Pax6 expression in adult islets to maintain proper glucose homeostasis and pancreatic β cell function.

Maturation factors

Maturation factors control late steps of islet cell development, but may also contribute to earlier stages of lineage specification and commitment. Often, loss of these factors does not cause dramatic effects in regards to endocrine cell formation. However, cell function is affected postnatally or in adult pancreas.

Maf transcription factors are important regulators of cellular differentiation

Members of Maf family of bZIP transcription factors were found to regulate gene expression and cell differentiation in many different tissues, also in the pancreas. Mafs are involved in a variety of biological processes, such as hematopoiesis, lens differentiation, oncogenesis, and segmentation of hindbrain. The first Maf protein, identified and described in 1989, was the v-maf oncogene. It was identified as the transforming gene in spontaneous musculoaponeurotic fibrosarcoma of the avian retrovirus A42 [70]. The Maf family of transcription factors consists of seven members that, based on their structures and function, are further divided into two

groups: small and large. Small Maf factors do not have a transactivation domain, in contrast to the large Mafs. There are four members of the large Maf proteins family: MafA, MafB, c-Maf and NRL. All members contain an N-terminal transactivation domain and a C-terminal basic leucine-zipper DNA-binding domain. The leucine zipper motif facilitates homo- and heterodimerization [71]. Maf proteins can heterodimerize with their family members or with other compatible bZIP proteins. Maf transcription factors recognize and bind to a long consensus DNA sequence called Maf recognition element (MARE [TGCTGAC(G)/TCAGCA]) reviewed in [72], [73]. Since all Maf factors recognize exactly the same consensus MARE element, they can compete with each other in regulation of MARE-dependent gene expression. Hence, in order to better understand the role that large Mafs play during the development and differentiation of the pancreas, it is important to define the spatio-temporal expression patterns of these regulatory proteins. Expression of the two Maf transcription factors, namely MafA and MafB appears to be vital for the β - and α -cell maturation in developing and postnatal pancreas.

MafA and MafB are the two principal Maf family members expressed in the pancreas that are vital for α and β cell maturation

MafA was initially identified as a β -cell specific factor that binds to the enhancer regions RIPE3b1/C1-A2 of the insulin promoter, in response to glucose [74–76]. MafA was shown to interact with two other transcriptional factors Pdx1 and NeuroD in activation of insulin gene transcription [77], [78]. Apart from MafA, two other members of large Maf family – MafB and cMaf were shown to bind to the insulin promoter. Similarly, MafA, MafB and cMaf can also regulate the glucagon promoter [79–81]. MafA is initially expressed in insulin producing cells that appear around the secondary transition and in adult it is also retained exclusively in beta cells [82], [83]. MafA was shown to be an important regulator of key β -cell genes and is considered to be a β -cell identity marker. Interestingly MafA inactivation does not seem to affect β -cell development. However, it affects the functionality of beta cells. The expression of several essential β -cell markers such as Insulin1, Insulin2, Pdx1, NeuroD and Glut2 is downregulated in MafA-/- mice. Moreover, mutant mice have abnormal islet structure with a change in β - to α -cell ratio. They are also glucose intolerant (defects in both glucose sensing and insulin secretion capacity) and develop diabetes 12 weeks after birth [84]. That

late phenotype suggests that MafA is crucial for maintenance of pancreatic islet architecture and function, although it is dispensable for the development of β cells.

MafB (also Kreisler - Krm11/MafB) was primarily described as the mutated locus in Kreisler (kr) mouse [85]. MafB is found in adult α cells, although during early pancreas development it is expressed in some Ngn3+ cells and also insulin and glucagon cells. Its expression initiates around E9.5-E10 and postnatally becomes restricted to glucagon+ cells [81], [86]. MafB deficient mice have 50% fewer insulin+ and glucagon+ cells during development, whereas the total number of endocrine cells is unchanged. This suggests an essential role for MafB in endocrine cell maturation and initiation of hormone expression. MafB was observed to directly bind to promoter regions of Pdx1, Nkx6.1, and Glut2 genes and their expression was selectively lost in insulin+ cells after E15.5 in MafB mutant islets [87]. MafB has been reported to have a crucial role in respiratory control which is attributed to specification of rhythmicogenic neurons. Mice deficient for MafB die within 2h after birth from renal failure and respiratory arrest caused by defects in preBötC neurons, which control breathing [88]. This precludes analysis of MafB's function in postnatal and adult islets mice. Therefore, until a conditional MafB ablation model is available, it is not yet possible to study the role of MafB in postnatal and adult pancreas.

Dynamic expression of Maf and MafB in developing and adult β cells

Recent studies of MafA and MafB transcription factors provide a model for a “molecular switch” in the expression profile in pancreatic β cells. It demonstrates that during development insulin+ cells initially express high levels of MafB and only a small fraction of β cells express MafA. However, with time MafB expression is efficiently replaced by MafA, which after birth is present in almost all insulin+ cells. In contrast to that, MafB expression is virtually absent from insulin+ cells a few weeks after birth and it is retained only in the glucagon cell population (Figure 5). In addition, the switch from MafB to MafA expression is associated with upregulation of Pdx1 expression [83], [89]. This appears to correlate with the findings identifying MafA as a direct transcriptional target of Pdx1 [90]. Additionally, studies suggesting the opposite relation, where Pdx1 itself is a MafA target highlight the importance of the crosstalk between these two transcription factors and its necessity for the proper β cell maturation and function.

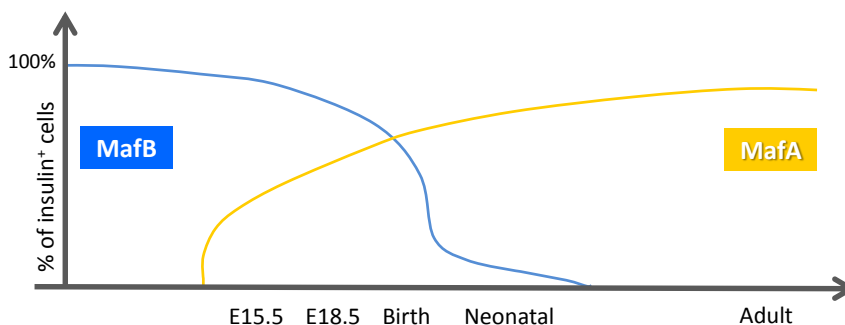


Figure 5. Dynamic expression of MafB and MafA in developing and adult β cells.

In summary, MafA and MafB are uniquely distributed in the pancreas, with MafA being exclusively present in beta cells while MafB is only found in alpha cells in the adult. These characteristics suggest that MafA and MafB have a distinct, non-overlapping role in regulating α - and β -cell gene expression in the adult pancreas.

Insulin – supreme factor in governing glucose homeostasis

Discovery of the insulin hormone

Insulin's name comes from the Latin *insula* which means "island". The hypothesis about a substance secreted from the pancreas, which controls blood glucose levels, comes from studies of pancreatectomized (surgical removal of all or fragments of pancreas) animals and dates back to 19th century. Since then, a clear connection between pancreas and diabetes was made, although the details remained ambiguous. In 1901, E.L. Opie established the link between the destruction of pancreatic islets and diabetes development. Insulin was isolated and purified in 1921 which initiated its therapeutic applications, first in experiments performed in dogs. Only one year later, the Canadian surgeon F. Banting and medical student Ch. Best, supervised by Professor J.J.R. McLeod, began their first human experiments which demonstrated the successful reversion of a diabetic phenotype in a 14 year-old patient. The "mysterious substance" obtained from the pancreas was given a name insulin and it has revolutionized the life of patients diagnosed with diabetes. The discovery of insulin was one of the most influential discoveries in medical history and in 1923 it was appreciated with the Nobel Prize which was awarded to F. Banting and J.J.R. McLeod.

Synthesis of insulin granules

β cells are the only endocrine cells in the pancreas that are able to synthesize the insulin hormone. The first step in insulin biogenesis is translation of insulin mRNA to a single chain precursor form preproinsulin. This process takes place on ribosomes of the rough endoplasmic reticulum (RER). Preproinsulin consists of a signal peptide, a B chain, an interconnecting - C peptide and a A chain. During insertion into the lumen, preproinsulin is converted to proinsulin upon removal of the N-terminal signal sequence [91]. The resulting proinsulin has three domains: an N-terminal B chain, a C-terminal A chain and a C peptide linking the latter two in the middle. Subsequently, proinsulin is transported by secretory vesicles from the RER to the Trans Golgi Network (TGN), where it forms immature insulin secretory vesicles with soluble zinc-containing hexamers reviewed in [92], [93]. Insulin is formed upon enzymatic reaction which cuts off the C peptide from proinsulin. During insulin granule maturation, the pH changes from 6.5 (in TGN) to 5-5.5 (in mature granules) facilitating the activity of the prohormone convertases such as PC1/3 and PC2, and carboxypeptidase E. The convertase enzymes are able to generate also other secretory products of β cells such as islet amyloid polypeptide/amylin and chromogranins [94]. Secretion of mature granules (exocytosis) results in release of equimolar ratios of both C peptide and insulin [91].

Insulin is a rather small protein and its molecular weight is about 6000 Daltons. Mature insulin is stored inside secretory vesicles as a solid hexamer bound by two zinc ions (Zn^{2+}). Thus, zinc appears to be important for insulin packaging, secretion, and signaling. In addition, zinc released together with insulin may control glucagon secretion from α cells [95], [96]. Zinc homeostasis is regulated by two main types of proteins: metallothioneins and zinc transporters. Metallothioneins are responsible for intracellular zinc storage and its trafficking to the secretory vesicles, whereas zinc transporters carry zinc across the biological membranes, reviewed in [97]. There are at least ten different members of the zinc transporter family described in mammals, and they can be found in the majority of mammalian tissues [98]. Slc30a8 (also ZnT8) has particularly high expression in pancreatic islets. Slc30a8 controls and facilitates zinc accumulation from the cytoplasm into insulin secretory vesicles [99]. Recently Slc30a8 was reported to be an autoantigen associated with the appearance of type 1 diabetes [100], [101]. Moreover, polymorphic variants in Slc30a8 are linked to the onset of type 2 diabetes [102], [103].

Mechanism of insulin secretion

Insulin stored in secretory vesicles is released from β cells in response to elevated glucose levels and this is referred to as the “first phase” of glucose-mediated insulin secretion. This appropriate stimulation causes insulin exocytosis and diffusion into islet capillaries. Glucose enters β cells via the high capacity, low affinity glucose transporter – Glut2 that is the only glucose transporter found in β cells [93], [104]. Glucose entry is sensed by glucokinase which subsequently phosphorylates glucose to glucose-6-phosphate. This represents the first step in glucose metabolism and generates ATP.

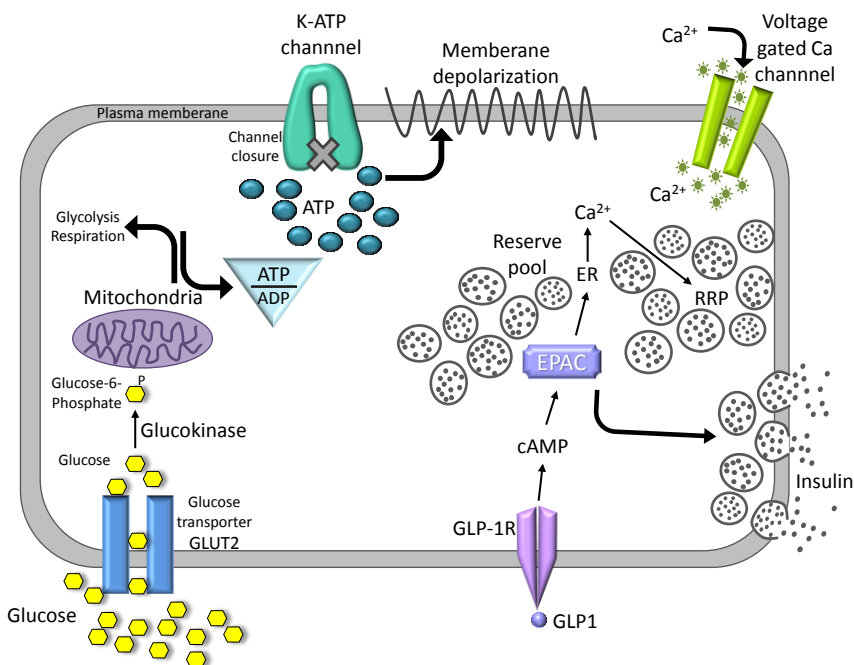


Figure 6. Insulin signaling and secretion. Glucose is transported into beta cells by the glucose transporter – Glut2. Following transport through the plasma membrane glucose is converted to glucose-6-P by the “glucose sensor” glucokinase. This generates high amounts of ATP and the resulting increased ATP to ADP ratio leads to the closure of K-ATP dependent (K_{ATP}) channels. The subsequent cellular depolarization activates voltage-dependent Ca^{2+} channels and triggers extracellular calcium influx into the cell. This activates mobilization of insulin secretory granules and their consequent fusion with the plasma membrane. GLP-1 incretin binds to the GLP-1R receptor on the plasma membrane and causes activation of adenylate cyclase which in turn increases intracellular cyclic AMP (cAMP) levels. Consequently, cAMP binds to EPAC which mediates the mobilization of Ca^{2+} ions from intracellular calcium depot, finally leading to insulin secretion.

The increased ATP to ADP ratio leads to the closure of K-ATP dependent (K_{ATP}) channels and is followed by plasma membrane depolarization. Depolarizing cell

membrane activates voltage-dependent Ca^{2+} channels and triggers extracellular calcium influx into the cell. This activates mobilization of insulin secretory granules and their consequent fusion with the plasma membrane. GLP-1 incretin binds to the GLP-1R receptor on the plasma membrane and causes activation of adenylyl cyclase which in turn increases intracellular cyclic AMP (cAMP) levels. Consequently, cAMP binds to EPAC (cAMP binding protein) that mediates the cAMP-dependent mobilization of Ca^{2+} ions from intracellular calcium depots. This increases the number of insulin granules in the readily releasable pool (RRP) that are pre-docked at the surface of the plasma membrane (Figure 6). RRP is responsible for the initial phase (I phase) of insulin secretion, whereas the reserve pool prolongs insulin secretion during the second phase (II phase) [105], [106]. The combination of the processes mentioned above triggers mobilization of insulin granules and eventually results in insulin secretion.

Microphthalmia transcription factor (Mitf)

Mitf is a transcription factor that belongs to the basic-helix-loop-helix leucine zipper (bHLH-Zip) protein family. Its expression has been associated with various cell types such as mast cells and osteoclasts [107], [108]. Mitf plays an important role during the development of pigment cells of different origin such as the neural crest-derived melanocytes and the retinal pigment epithelial (RPE) cells that are derived from neuroepithelium [109]. It is also recognized as the main regulator of melanogenesis since it activates the transcription of several pigment cell-specific genes both in the skin and retina [110], [111]. Mitf has also been linked to control genes involved in cell survival (Bcl2) [112] and cell cycle regulation (Cdk2, p16/Ink4A) [113], [114]. Mitf is able to bind to DNA and regulate its target gene expression either as a homo- or heterodimer. Studies by Hemesath et al. [115] showed that three other members (Tfe3, Tfeb, Tfec) of the MITF-TFE subfamily of bHLH proteins form stable heterodimers with Mitf and with each other. Interestingly, knockout studies of Tfe3, Tfeb, Tfec proved that the heterodimeric interactions between these members are not necessary for melanocyte development [116]. Mitf shares structural and biochemical features with other basic-helix-loop-helix proteins which are known to have a potent role as cell fate regulators. The MITF-TFE family proteins have three distinct regions: an identical basic region and a highly similar HLH and the leucine Zip domains. The basic region recognizes a canonical CANNTG DNA-binding sequence. Both the helix-

loop-helix and leucine Zip domains facilitate protein dimerization (homo- and/or heterodimerization), which is a prerequisite for binding to DNA [117].

Mitf isoform expression has spatio-temporal dependent context

The MITF-encoding locus on chromosome 3p is quite diverse and encompasses at least nine different promoters (Figure 7) known so far. The presence of multiple promoters creates the possibility to produce several different Mitf isoforms. These isoforms (MITF-MC, MITF-A, MITF-H, MITF-C, MITF-B, MITF-D, MITF-E, MITF-J, and MITF-M) differ in their N-terminal sequences, while the exon 2-9 containing the region of bHLH-Zip domain is identical (Figure 7).

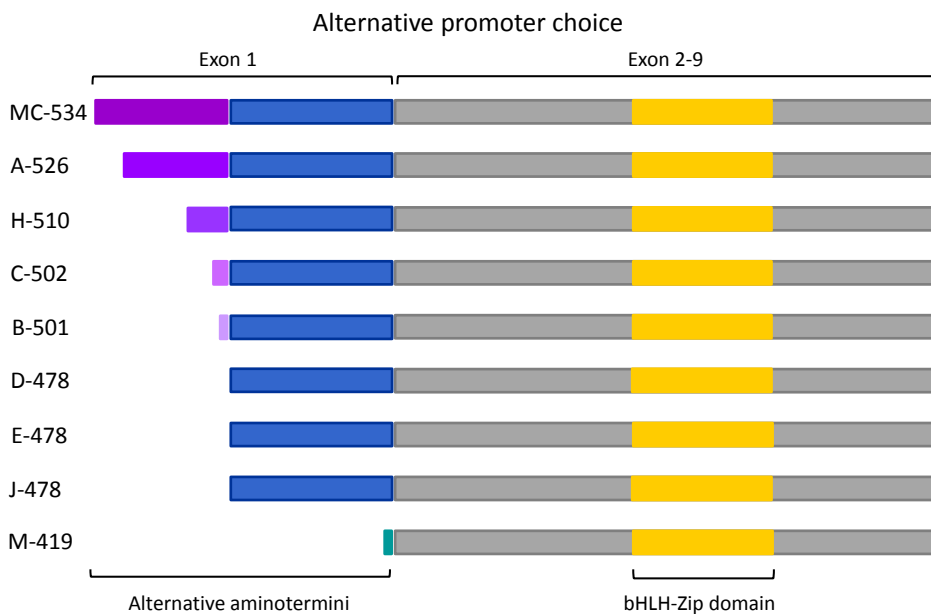


Figure 7. The structure of the murine *Mitf* gene and protein isoforms. Nine different promoters of the *Mitf* gene and their alternative N-terminal sequences based on differential promoter choice associated with nine distinct mRNA isoforms: MITF-MC, MITF-A, MITF-H, MITF-C, MITF-B, MITF-D, MITF-E, MITF-J, and MITF-M.

In addition, all *Mitf* isoforms produce alternative splice variants of exon 6a which results in incursion (+) or excursion (-) of 6 amino acids (ACIFPT) that are found proximal to the basic region of the protein (Figure 8). The presence of different exon 6a variants of MITF is not fully understood, but it might be related to different transcriptional activities and/or cell proliferation [118], [119]. Studies by

Bharti et al., showed that some Mitf isoforms are ubiquitously expressed, whereas others have more restricted expression pattern [120].

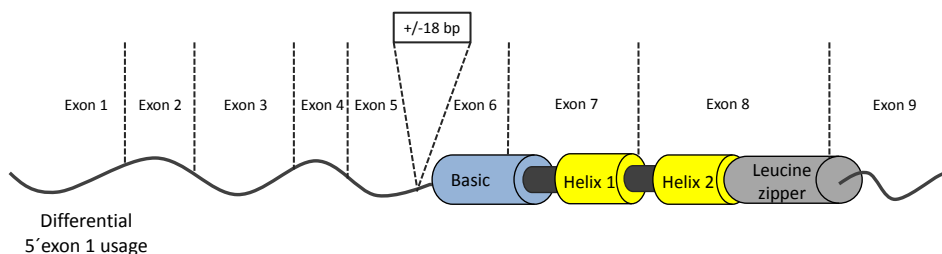


Figure 8. Schematic structure of the Mitf transcription factor showing the conserved basic helix-loop-helix-leucine-zipper motifs. Differential usage of exon 1 and distinct regions encoded by each of the 1-9 exons are shown. Inclusion or excusion of a 18bp region of exon 6a is depicted in a box.

Discovery of Mitf

The first mutation at *microphthalmia* locus was described by Paula Hertwig in 1942 based on the studies of the offspring from irradiated mice [121]. The animals were white with small eyes and subsequent studies showed that mutations in Mitf gene result in loss of neural-crest derived melanocytes affecting pigmentation, hearing, and defects in RPE differentiation [115], [122], [123]. Since then, the knowledge about the Mitf gene and its regulation has expanded, mostly based on the Mitf mouse mutant studies which have shown that Mitf is important for the physiology and pathology of many different organs and tissues, such as eye, ear, heart, immune system, bone, and skin [117]. These pleiotropic mutations are of particular interest for studying melanocyte biology, since Mitf has a pivotal role in melanocyte survival and pigmentation, and is often regarded as a master gene in melanogenesis (pigment cell formation). Many signaling molecules and/or transcription factors that are involved in melanocytes development affect either the expression of Mitf or its function. It was not until 1993 that the Mitf gene was cloned from mice bearing transgene insertions in the *microphthalmia* locus and shown to encode for a bHLP-Zip transcription factor [115], [124].

Neural crest and melanocyte development

Melanocyte precursors - melanoblasts originate from a unique embryonic structure in the vertebrate embryo called neural crest (NC) [125]. The neural crest forms from the dorsal neural tube or neural folds early during embryonic development. A transient population of neural crest derived pluripotent cells contributes to the

development of a variety of neuronal and non neuronal cell types which later compose different tissues and organs in the vertebrate body. Due to its great importance during the development it is sometimes referred to as a forth germ layer. During epithelio-mesenchymal transition cells become ready for migration within embryonic tissues. Upon delamination from the neural tube cells become more lineage restricted. Neural crest cells migrate from the neural tube throughout the embryo along several different pathways. During migration cells receive environmental signals that define their future cell fate choice and result in their homing to specific destinations. Eventually, they populate various places in the vertebrate embryo, aggregate and differentiate to numerous cell types and tissues. In mouse, melanocytes originate from non pigmented melanoblasts around embryonic day 10.5 (E10.5). Upon migration and proliferation the cells differentiate and begin to produce pigment at E16.5 [126].

Mitf - master gene in melanocyte development and function

Mitf mutant models are of particular interest for studying the mechanisms underlying melanocyte formation and consequently melanoma development, because lack of this gene causes abnormalities in neuroepithelial and neural crest-derived melanocytes. It is known to be crucial for pigment synthesis, since loss or reduction in Mitf expression in animals and humans results in pigmentation disturbances [125].

Mitf activates melanocytic pigmentation by regulating melanogenic enzymes such as tyrosinase (Tyr), tyrosinase related protein 1 (Tyrp-1) and melanin synthetic DOPAchrome tautomerase (Dct also under the name tyrosinase-related protein 2) [110]. Mitf regulates their expression through conserved DNA sequences, namely E-box or M-box. Mitf has the ability to discriminate between different E-box elements providing a mechanism which restricts the pool of genes that are likely to be regulated by Mitf. This mechanism improves the specificity by which Mitf is regulating the complex program of gene expression during melanocyte development [127].

In addition to genes that are essential for melanogenesis Mitf interacts and regulates the expression of many genes which are important for melanocyte survival or differentiation. These genes include B-cell lymphoma 2 (Bcl2), stem cell growth factor receptor (c-Kit), endothelin B receptor (Ednrb), its ligand endothelin 3 (Edn3) as well as several transcription factors such as paired box homeodomain transcription factor (Pax3) [128] and SRY related high mobility

group (HMG)-domain factor (Sox10) [129], [130].

Based on microarray studies Bcl2 was shown to be one of the Mitf-dependent KIT (proto oncogene c-Kit or tyrosine-protein kinase Kit) transcriptional target genes in primary melanocytes. MITF directly bound to the Bcl2 promoter in *in vivo* tests. Bcl2 is an antiapoptotic factor and Bcl2 homozygous knockout mice show sign of depigmentation caused by loss of melanocytes that die shortly after birth [131]. Studies by Opdecamp et al. showed that Mitf allows the entry of neural crest cells into the melanocytes migratory pathway and by influencing Kit plays a role in melanoblasts survival [132]. Kit is not only required for the onset of Mitf as shown by the studies using Kit null allele marked by LacZ expression, but also for the proper expression of Tyr which characterizes differentiation of melanoblasts to melanocytes [133].

Similarly to loss or defects in Kit expression, mutations in Ednrb gene also lead to defects in melanocytes [134], [135]. Ednrb encodes a G-coupled receptor for its ligand Edn3. Studies of Mitf expression in human melanocytes that were treated with Edn1/3 proposed a model where Edn-Mitf signaling pathway is regulated by several feedback loops (Figure 9).

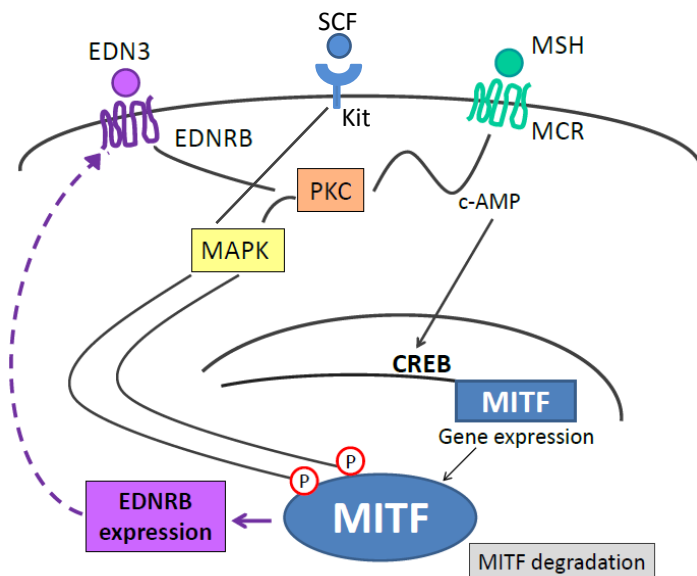


Figure 9. Endothelin, Kit and melanocortin, signaling pathways are involved in the modulation and the expression of the Mitf gene via MAPK and cAMP respectively.

Apart from Edn this pathway comprises many other signaling molecules such as Kit, MSH (melanocortin), c-AMP, PKC. Melanocytes stimulated with melanocortin increase c-AMP levels which lead to upregulation of MITF transcription via CREB (C-AMP response element-binding protein) in a melanocyte-restricted fashion [136]. In such manner the extracellular signals are linked to the expression of MITF and consequently the expression of many genes essential for melanocyte survival and development (Figure 9). Melanocytes stimulation via KIT results in a rapid MITF phosphorylation mediated by MAPK which subsequently recruits the p300 transcriptional activator [137], [138].

Mutations in EDNRB, EDN3 and SOX10 genes cause Waardenburg syndrome type IV (WS IV) and result in deafness and pigmentary abnormalities. It is conceivable that the genes implicated in WS etiology directly or indirectly regulate the expression of MITF. SOX10 was proven to strongly activate the MITF promoter in human melanocyte transfection studies [139]. Interestingly, SOX10 binding to the consensus regions on *Mitf* promoter can be prevented using Sox10 constructs with mutations that were identified in patients with WSIV [129], [139]. In cooperation with PAX3, SOX10 directly binds to the proximal region of the MITF promoter which has binding sites for both of these factors as shown in transfection assays [140]. Similarly to the SOX10 mutation, constructs with mutated PAX3 failed to activate MITF promoter [128].

In summary, there are many genes implicated in the regulation of *Mitf* gene. Concurrently *Mitf* regulates genes critical for the survival or maintenance of melanoblasts. The interactions of these different signaling molecules and transcription factors result in cell type specific but also isoform specific expression of MITF.

Mitf mutations and pathology

Mouse

Mitf was proven to be of great importance for pigment cell development and/or survival. The *Mitf* gene, with its at least nine different promoters known to date, is quite complex and there have been more than 20 different alleles described in *mitf* locus. These mutations are of spontaneous, chemical, radiation and insertional mutagenesis origin, comprising point mutations, insertions and deletions. Many of them lead to amino acid substitutions in critical molecular domains greatly

affecting the function of the *Mitf* gene [108]. There are both dominant and recessive types of *Mitf* alleles which affect melanocyte development to varying degrees. *Mitf* alleles can interact and complement each other in various ways. Most *Mitf* mutant mice are microphthalmic (Greek, *mikros* + *ophthalmos*, small eyes), hypopigmented with white spots or completely pigment deprived. Additional pleiotropic phenotypes are observed such as early onset deafness, reduced mast and natural killer (NK) cell numbers [141–143], decreased cardiac function [144]. Some *Mitf* mutants suffer from intrinsic osteopetrosis (defective secondary bone resorption) and failure of the incisors to appear [145].

Human

Mutations in the *MITF* gene cause Waardenburg syndrome type IIA in humans. Waardenburg syndrome (WS) is a group of inherited auditory-pigmentary disorders that causes loss of pigmentation and/or hearing [146], [147]. These symptoms result from abnormalities in proliferation or differentiation of neural crest (NC) derived melanocytes. Several genes such as *MITF* (WSII), *PAX3* (WSI and III), *EDN*, *EDNRB*, *SOX10* (WSIV) have been associated with different types of Waardenburg syndrome [139], [148]. The four different types of WS were categorized based on the presence or absence of additional symptoms [148]. The most common forms of Waardenburg syndrome are type I and II, while type III and IV are rare. Waardenburg syndrome is usually inherited in an autosomal dominant way. However, currently it is recognized as a genetically heterogeneous condition since not all forms are dominant and recessive also exist. In humans WS often manifests by a white forelock in the middle of the forehead, premature graying of the hair, white eyebrows and eyelashes. In addition, pigmentary disturbances of the iris of the eye resulting from the absence of functional melanocytes are also present. The heterochromia (different eye color) observed in subjects with WS can be complete (one normal and one hypoplastic iris) or segmental (partially hypoplastic iris) [149]. WS can also represent an inherited sensorineural deafness condition, caused by reduced or lack of the expression of one of the multiple transcription factors required during inner ear development both in the vestibular organ and cochlea.

Mitf and melanoma (skin cancer)

Melanoma is a melanocyte-derived highly metastatic tumor, which at its early stages is curable. Different studies linked increase in MITF expression to human cancers, mostly melanoma. However, the levels of Mitf expression differ greatly among melanoma cell lines and different cells in affected tissue. As already mentioned, Mitf regulates the expression of many genes critical for pigment production, but also genes that regulate proliferation, survival, invasiveness or resistance to apoptosis. The possibility of Mitf to affect cell shape and invasiveness plays an important role in understanding melanoma proliferation, metastasis as well as formation of mature melanocytes. It was shown that Mitf can regulate melanoma invasiveness, via the Diaph1 gene. Low levels of Mitf lead to a p27Kip1 dependent cell cycle arrest, changes in actin cytoskeleton which coincides with increased invasiveness through reduction in Diaph1 expression. In contrast to that, elevated Mitf levels cause increased proliferation but decreased invasiveness [150], [151]. This finding was consistent with previous observations suggesting that high levels of p27Kip1 are found to primary melanoma cells with metastatic potential [152].

Background on Mitf mutant mice used in Paper III

The Mitf^{ce} mutant allele was discovered in 1989 among breeding of DBA/2N mice. Based on genetic studies it was proposed that these animals represent a new mutation rather than genetic contamination. Phenotypical characterization of Mitf^{ce} mice showed pleiotropic effects ranging from white, pigment deprived coat color, microphthalmic eyes, defects in inner ear to reduced fertility. The Mitf allele was recognized as microphthalmia cloudy-eye (ce) [153]. The Mitf^{ce} allele was defined as a result of an autosomal recessive mutation which is characterized by a C to T substitution at bp 916 of the microphthalmia (mi) locus on mouse chromosome 6. This single point mutation causes the lack of the Mitf Zip domain due to the presence of a STOP codon between the HLH and Zip motif. This results in the synthesis of a truncated protein that is incapable of dimerizing and DNA binding [115].

Technical considerations

Cre-LoxP mediated recombination allows for conditional gene inactivation or activation

The Cre-loxP system is a sophisticated tool for tissue-specific gene inactivation. Cre-loxP stands for Cre - cyclization recombination and loxP – locus of X-over P1. The system is highly versatile and works in virtually any type of cell. This precise *in vivo* manipulation is of special interest when the conventional null mutation is lethal. The Cre gene encodes a DNA site-specific enzyme, the Cre recombinase, which is capable of recombining DNA once it recognizes specific DNA sequences. Cre recognizes a 34bp consensus sequence called loxP site. Specific recognition of those Cre recombination signal fragments enables deletion of genes that are flanked by two loxP sites oriented in the same direction (Figure 10A). If loxP sites are oriented in opposite directions the Cre recombinase mediates the inversion of the flanked fragment (Figure 10B).

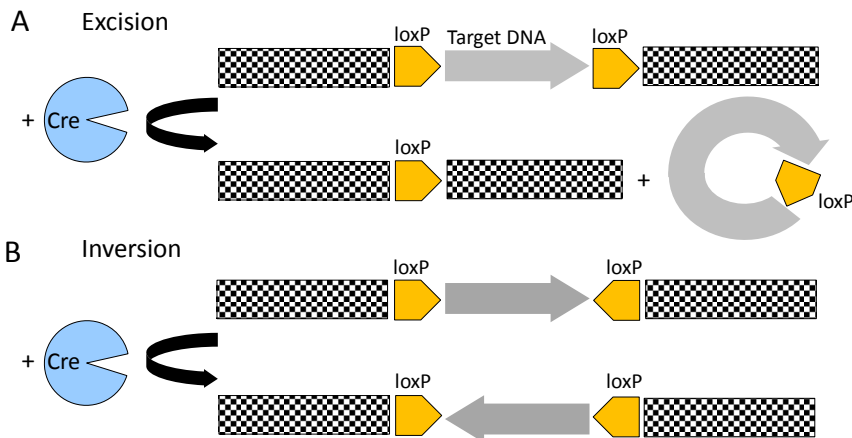


Figure 10. Two types of rearrangements resulting from the Cre/loxP system depending on the position and orientation of the loxP sites. The loxP sites work in pairs. When the Cre recombinase binds to the flanked by two loxP sites DNA fragment it cuts the loxP sites into two. After the excision of the target DNA, one loxP site is left, whereas the two flanking fragments of DNA are spliced together. Flanked DNA is excised and later degraded.

Cre is a 38 kDa protein originally found in the P1 bacteriophage and it belongs to the integrase family of site-specific recombinases. The P1 bacteriophage is a bacterial virus and contains DNA that is normally not found in plants or animals. Hence, it is highly unlikely to find the loxP sequence randomly in a genome. This feature makes Cre suitable for artificial insertion into animals or plants without the risk of excising other parts of organism genome. One other advantage of the Cre-loxP system is that, there is no need for additional co-factors facilitating the recombination process.

In order to conditionally delete genes two mouse lines are required. First transgenic line has a Cre recombinase driven by either a lineage/cell type specific or ubiquitous promoter. In the second transgenic line the gene of interest is “floxed” (flanked by loxP sites) in a direct orientation (Figure 10A). The Cre-loxP strain is made by crossing the two separate transgenic lines Cre and loxP. Upon crossing, recombination occurs only in the cells expressing Cre recombinase and consequently the gene of interest will be excised from the specific tissues. In contrast, the target gene remains active in all cells and tissues which do not express Cre.

Moreover, the Cre-loxP system can be combined with other regulatory elements, in order to express Cre only under certain conditions. This results in Cre enzyme being active e.g. in certain tissues or when animal diet is supplemented with chemicals such as tetracycline, doxycycline or tamoxifen. This improvement makes it possible to control gene expression in an inducible way. Using tamoxifen as an inducible factor requires designing a fusion protein, where Cre recombinase is fused to a hormone binding domain of the mutated estrogen receptor (ER) that cannot bind endogenous estrogen. In the presence of synthetic ligand (4-hydroxy tamoxifen - estrogen antagonist) the Cre fusion protein (Cre-ER^{Tamoxifen}) translocates to the nucleus where it executes its function. Tamoxifen can be administered at a desired time point during development, postnatally or in adult life.

The Cre-loxP system is an important tool for molecular biology controlling gene activity in spatio-temporal manner in almost any given tissue. However, Cre-loxP system is not ideal and its main drawback is the lack of perfectly tissue specific promoters. Also, establishing reliable and functional transgenic models requires significant amounts of time and money. Nevertheless, the Cre-loxP system has greatly advanced our understanding of mammalian gene function and provides a perfect tool for studies of human physiology and pathophysiology.

In situ hybridization

The *In situ* hybridization (ISH) technique, also known under hybridization histochemistry name, was first described in studies detecting DNA:RNA in 1969. Consequently, additional articles appeared where this technique was used to localize mRNA of several different proteins. The basic principle behind ISH is the restricted binding of labeled nucleotide probe to a complementary sequence of interest.

The detection is performed in morphologically preserved tissue sections (e.g. frozen or paraffin) and is highly sensitive, with the possibility to detect as few as 10-20 copies of mRNA per cell. Annealing of a labeled acid probe to its complementary target mRNA is followed by visualization of the location of the probe.

Advantages of *in situ* hybridization

- It is possible to fully use tissues that might be in small quantities (clinical biopsies, embryos etc.)
- Specific probes are generated quickly from known DNA fragments (in comparison to antibody production for immunochemical detection)
- The sequences of interest is directly detected in tissues or cells (in contrast to e.g. Northern blot technique)
- It provides both spatial and temporal resolution of gene expression
- It allows studies of genes interactions, gene relationships in specific diseases, genes as tissue markers and specific alleles activity

Choice of Probe

Selection of a probe is usually the first step of *in situ* hybridization. Probes are complementary sequences of labeled nucleotides and can be obtained by *in vitro* transcription of the gene of interest. Different probes vary greatly in size and they can be 100 bp or up to 1500 bp. There are four main types of ISH probes:

Double stranded DNA probes (dsDNA probes)

These probes are relatively easy to make (no subcloning is required). dsDNA probes can be joint with many different labels and the labeling techniques produce probes with high specific activity. However, due to the fact that these probes produce both strands, the DNA must be denatured prior to the hybridization step in

order for one strand to bind with the target mRNA. This creates a competition between the complementary strands for their target DNA/RNA and in consequence can negatively affect probe binding.

Single stranded DNA probes (ssDNA probes)

The use of ssDNA probes eliminates the risk for competition between the different DNA strands and no denaturation is required. These probes are usually 200-500bp in size. Creating this type of ISH probes is technically more complex than dsDNA and involves subcloning procedures

Single stranded complimentary RNA probes (ssRNA or riboprobes)

RNA probes have the advantage that RNA-RNA hybrids are significantly more stable than DNA-RNA complexes. This allows more stringent washes and enables post hybridization digestion with RNase in order to remove non bound RNA while sparing hybridized RNA. Post hybridization digestion with RNase reduces background staining increasing probe specificity. However, this type of probes generally gives higher degree of unspecific binding to different tissue components increasing background staining and lower probe penetration into the tissue. One more drawback of ssRNA probes is their sensitivity to RNases (RNA degrading enzymes).

Oligonucleotide probes

This type of probe can be directly produced synthetically from labeled nucleotides (deoxynucleotides) which is economical, although requires the knowledge about the desired nucleotide sequence. The generated probes are stable (resistant to RNases) with good tissue penetration properties (small size). One more advantage of this type of probes is that they are single stranded, hence there is no competition between probes

Probe labeling

In situ hybridization probes can have both radioactive and non-radioactive labels. Radioactively labeled probes are considered more sensitive than non-radioactive and they enable quantitative analysis of the target mRNA [154]. Radioactive probe labels contain nucleotides that have radioactive isotopes such as: H^3 , P^{32} , S^{35} , C^{14} , I^{125} .

Non-radioactive labels widely used with *in situ* hybridization include biotin, digoxin and digoxigenin (DIG), alkaline phosphatase and the fluorescent labels, fluorescein (FITC), Texas Red and rhodamine.

Signal detection

Signal from the radiolabeled probes can be detected using either photographic film or photographic emulsion.

Non-radioactively labeled probes can be hybridized to the target mRNA:

indirectly (digoxigenin detected by specific antibodies, biotin detected by avidin or streptavidin). This procedure requires that the probe contains a reporter molecule, introduced chemically or enzymatically, that can subsequently be detected by histochemistry.

Direct labeling (fluorophores directly coupled to the nucleotides) allows for immediate detection of the reporter molecule bound to the probe after the hybridization reaction.

In situ hybridization represents an important and powerful technique that enables localization of gene expression in tissues or single cells. It is an extremely sensitive method and allows for detection of very small amounts of mRNA, even from a single cell. In order to obtain valid data from *in situ* hybridization studies it is crucial to include proper controls, to show that the observed hybridization is due to binding of the real target and not just unspecific labeling. It is also important to carefully interpret the amount of detected mRNA to the protein that is generated, since the amount of target mRNA does not have to correlate with the same amount of translated protein. Therefore, combining immunohistochemistry and *in situ* hybridization can provide more evidence for the functional interpretations of the data.

Aims of the present investigation

The general aim of this thesis was to elucidate the role of MafA, MafB and Mitf transcription factors in the development and function of pancreatic β -cells. Understanding the role of specific transcription factors in controlling formation, maturation and function of the beta cells is of great importance for developing therapeutically useful insulin cells.

The specific aims were to:

- Paper I** To address the functional relationship between MafA and MafB transcription factors in the regulation of pancreatic β -cell formation and maturation by identifying novel MafA and MafB target genes.
- Paper II** To investigate the importance of MafA for β -cell function by analyzing the consequences of loss of MafA in mice, which lack expression of MafA specifically in adult β -cells.
- Paper III** To examine the role of Mitf in developing and adult pancreas based on studies of Mitf loss-of-function mice

Papers in summary

Paper I: MafA and MafB regulate genes critical to β -cells in a unique temporal manner

Introduction

Maf transcription factors are involved in a variety of biological processes. The majority of Maf target genes are important for development and differentiation. However, the total number of genes directly regulated by large Maf transcription factors is unknown. Based on previous *in vitro* and *in vivo* findings both MafA and MafB emerged as key regulators of pancreatic endocrine cell development and differentiation into functional hormone producing cells. This is due to their ability to regulate genes essential for endocrine cell function such as *insulin*, *glucagon*, *pdx1*, and *glut2*. MafA and MafB have a unique distribution pattern in the pancreas, with MafA being exclusively present in beta cells [76]. MafB is expressed in all developing insulin- and glucagon-producing cells, and in the adults becomes restricted to alpha cells. MafB deficient animals have a ~50% loss of insulin+ and glucagon+ cells while no difference was found between wild type and the mutant in total endocrine cell numbers [87]. These cells retained expression of many important alpha and beta cells regulatory proteins in both hormone+ and hormone- endocrine cells. MafA is important for maintaining β -cell identity, although MafA deficient mice do not have defects in developing pancreas. However, adult MafA mutant have abnormal islet structure and eventually develop diabetes [84].

In order to comprehensively identify genes controlled by MafA and MafB, and their involvement in beta cell formation and function we applied cDNA microarray analysis.

Results

In order to study the functional relationship between MafA and MafB, we performed gene-profiling studies on E18.5 pancreata isolated from MafA ^{Δ Panc} gene (targeting of the single coding exon of MafA), MafB^{-/-} and Maf ^{Δ Panc} MafB^{-/-} (the single-MafB exon coding sequence is replaced with a MafB-GFP allele by homologous recombination in R1 embryonic stem cells). On the basis of microarray several differentially regulated candidate genes have been selected. We

found multiple genes differentially regulated in MafB^{-/-} mutant, but not in MafA^{-/-} sample pancreata. Gene ontology analysis showed that the differentially expressed genes were associated with mature β -cell function e.g. ion binding and transduction and hormone secretion. Levels of the three candidate genes (ZnT8, G6pc2 and Nnat) were found to be decreased in MafA/MafB mutant pancreata, while the level of one (Rbp4) was found to be increased in mutant pancreata. We further analyzed the expression of those different potential MafA and MafB target genes in embryonic and adult mouse pancreata using *in situ* hybridization, immunofluorescence and Q-PCR (quantitative PCR) methods.

MafA and MafB are dynamically expressed in developing and adult pancreas. During early development (i.e. E14.5), MafB is found in almost all β cells while MafA is only present in less than half of the insulin⁺ cell population. The number of MafA⁺ insulin cells increases after the secondary transition, while MafB is still found in most β cells. In contrast, only few of the insulin cell population still expressed MafB at postnatal day 14 (P14) and MafB expression is virtually absent from insulin⁺ cells a few weeks after birth and it is retained only in the glucagon cell population. At this time point MafA was found in a large number (~80%) of β cells. After birth, expression of MafB becomes restricted to glucagon⁺ cells.

Slc30a8 (islet zinc transporter or ZnT8) expression is activated by MafB during embryogenesis and by MafA in islet β cells.

The zinc transporter Slc30a8 is localized in insulin secretory granules and is a major component for providing zinc to insulin maturation and/or storage processes in pancreatic beta cells. Zinc itself has a role in mediating the synthesis and action of insulin, in both physiological and in the pathological state (diabetes). To study the expression pattern in the developing pancreas (at E15.5 and E18.5) Slc30a8 mRNA expression was assessed by *in situ* hybridization analysis. In the wild type Slc30a8 was coexpressed with insulin⁺ and glucagon⁺ cells. However, in the MafB^{-/-} mutant pancreata its expression becomes restricted to insulin producing cells but is lost in glucagon⁺ cells. In the double mutant pancreata its expression is basically gone from both α and β cell types. This suggests that Slc30a8 is directly regulated by MafB during development. Slc30a8 protein was detected in islet β and α cells of 12-week old wild type islets by immunofluorescence. Slc30a8 expression was absent from the 3-month old islet insulin⁺ cells of MafA ^{Δ panc} mutants. The remaining Slc30a8 was found in glucagon⁺ cells. In addition, quantitative PCR analysis showed a 50% reduction of Slc30a8 expression in islets

of 12 week-old MafA^{Δpanc} mice.

This suggests that Slc30a8 expression is activated by MafB during embryogenesis and by MafA in islet β cells.

Nnat (Neuronatin) expression in β- cells is regulated only by MafB

Neuronatin was found to be involved in modulating ion channel activity in β cells. *In vitro* studies demonstrated that Nnat overexpression increased insulin secretion. In order to study cellular distribution of Nnat *in situ* hybridization was performed on wild type and mutant samples. Our analysis showed that at E15.5 and E18.5 Nnat mRNA was expressed in insulin+ cells but also in cells that did not express either insulin or glucagon. Nnat was mostly found in insulin+ cells by E18.5. Neuronatin expression was diminished in MafB and MafA/MafB mutant pancreata and in the mutant it was only detected in the remaining insulin+ cells. Nnat mRNA levels assessed in the Maf^{Δpanc} mutant adult islets were unchanged when compared to wild type, suggesting that MafA does not regulate Nnat expression either during development or in adult pancreas.

These results suggest Nnat expression in developing β-cells is only partially dependent on MafB

G6pc2 (islet-specific glucose-6-phosphatase catalytic subunit-2 protein) expression

G6pc2 is a major autoantigen in the onset of Type 1 diabetes. Our microarray data showed a significant downregulation of G6pc2 in embryonic MafB^{-/-} and Maf^{ΔPanc} MafB^{-/-} mutant pancreata and a drastic decrease in MafA^{ΔPanc} adult islets. The latter was confirmed with immunohistochemical analysis which demonstrated loss of G6pc2 from adult islets of MafA^{ΔPanc} pancreas. Altogether these results suggest that both MafB and MafA activate G6pc2 expression during development and in the adult pancreas.

Rbp4 (Retinol binding protein 4) expression is increased in E18.5 MafB mutant and MafA

Recently it has been suggested that some adipose tissue-secreted gene products may mediate many obesity-related diseases including diabetes. Rbp4 protein is believed to be one of them, since increased levels of Rbp4 cause systemic insulin resistance and downregulation of insulin serum levels [155]. Interestingly, in contrast to the previously described genes Rbp4 mRNA levels were found to be

upregulated in both MafB^{-/-} and MafA/MafB compound mutant pancreata. Cellular distribution of Rbp4 in the developing pancreas was studied by *in situ* hybridization. Rbp4 transcripts were found as early as E15.5 in the developing pancreas. In the wild type E15.5 pancreata Rbp4 is expressed in many insulin and glucagon+ cells. Later during development at E18.5 Rbp4 expression was decreased (compared to E15.5) and only detected in few wild type α , β and Δ cells. In MafB and MafA/MafB mutant pancreata Rbp4 levels increase at both developmental stages analysed. In addition the number of Rbp4+hormone- cells is drastically increased in MafB^{-/-} and MafA ^{Δ panc}MafB^{-/-} mutant pancreata, presumably representing the endocrine progenitor population that fails to express insulin and glucagon. Q-PCR analysis of adult MafA ^{Δ panc} mutant islets showed ~70% increase in Rbp4 mRNA levels. Rbp4 protein was present only in Δ cells in both wild type and MafA ^{Δ panc} mutant islets. Taken together, MafA and MafB factors negatively regulate production Rbp4 protein.

Summary discussion

In the present investigation we have performed gene expression profiling of wild type and Maf mutant pancreata to identify genes important for β -cell maturation and function. In these microarray studies several known (insulin, glucagon, GLUT2, PC2) but also novel genes were shown to be differentially expressed in MafB and MafA/MafB mutant embryos. Gene ontology analysis revealed that the differentially expressed genes were mainly associated with mature β -cell function. Our findings demonstrate that Nnat, Slc30a8 and G6pc2 are downregulated in embryonic and adult mutant pancreata. In contrast, the mRNA level of Rbp4 was upregulated in mutant tissue. Given the unique spatio-temporal expression pattern of MafA and MafB transcription factors in developing and adult mouse pancreas we propose, based on our results, that these two factors differentially regulate the expression of genes critical to β cells both during development and in the adult. Combining histological and gene expression analysis of differentially regulated genes demonstrated that MafA and MafB have a distinct, non-overlapping role in regulating α - and β -cell gene expression in the adult pancreas. MafB is more potent in regards to regulation of β -cell development, since most of the differentially expressed genes were common for both MafB^{-/-} and MafB^{-/-}MafA ^{Δ panc} mutant samples. MafB appears to partially compensate for the loss of – MafA ^{Δ panc} mice and MafB mRNA levels were found over 50% upregulated in adult MafA ^{Δ panc} samples, although it is not able to fully rescue the adult phenotype of the

MafA mutant mice. Consequently, our results show that in adult islets MafA controls expression of several genes that are primarily regulated by MafB (during development). This dynamic temporal pattern of expression with slightly different requirement for MafA and MafB expression at distinct stages in mouse β cells appears to be essential for differentiation of β -cells (MafB) and sustaining the role of genes crucial in mature insulin producing cells (MafA) respectively.

In summary, our results further establish an important role of Maf transcription factors in regulation of genes required for endocrine cell differentiation and function.

CONCLUSIONS

- In the pancreas MafA and MafB are expressed in a dynamic spatio-temporal pattern with MafA being exclusively present in beta cells.
- MafB is expressed in all developing insulin and glucagon producing cells, and in adult it is retained only in glucagon cells
- At E18.5 Neuronatin expression becomes restricted to mature β cells, and its expression is only partially dependent on MafB
- MafB primarily regulates Slc30a8 expression during pancreas development, while MafA is essential for its expression in adult β cells.
- MafA and MafB regulate G6pc2 expression.
- Rbp4 expression is increased in both embryonic and adult MafA/MafB knockout pancreata

Paper II: Adult β cell function depends on MafA transcriptional activity

Introduction

Unraveling the complex network of various transcription factors involved in regulation of the key pancreatic gene expression is a prerequisite in successful generation of therapeutically useful insulin producing cells. Previous studies have shown that Maf transcription factors are potent regulators of the insulin and glucagon hormones but also other genes essential to endocrine cell function such as Pdx1, and Glut2. MafA expression is only found in β cells both during the development and in adult islets. Pancreas development in MafA deficient mice

appears to be unaffected, most likely due to partial compensation by MafB. However, adult MafA mutant mice are diabetic with impaired islet morphology and reduced beta cell mass. In contrast to systemic deletion of MafA, β cell-specific deletion does not prolong MafB expression postnatally. Therefore, in the current study we took the advantage of the β cell specific deletion of MafA (MafA ^{$\Delta\beta$ cell}) using mice expressing Cre under the RIP - rat insulin promoter and crossed with MafA^{fl/fl} mice. This did not result in increased MafB expression and would truly reflect the exact role that MafA plays in β -cells.

Results

MafA ^{$\Delta\beta$ cell} mutant mice have unperturbed islet morphology

Immunohistochemical analysis of adult MafA ^{$\Delta\beta$ cell} showed normal islet organization with β -cells in the core and α -cells in the periphery, in contrast to MafA ^{Δ systemic} mutant mice, whose islets are disorganized. β -cell specific deletion of MafA does not affect the α - to β -cell ratio which is affected in MafA ^{Δ systemic} mutant (increased α - to β -cell ratio).

MafB expression is only found in α -cells in MafA ^{$\Delta\beta$ cell} mutant islet

Previously described MafA mutant models (MafA ^{Δ systemic} and MafA ^{Δ Panc}) have elevated levels of MafB expression [89]. In contrast to wild type, in these MafA mutant models MafB can be found in insulin+ cells, which most likely plays a compensatory role for the loss of MafA. This is a plausible explanation, since loss of MafA does not have a more drastic phenotype. Interestingly, we did not observe this compensatory effect of MafB in islets of 2 month-old MafA ^{$\Delta\beta$ cell} (lack of MafB expression in insulin+ cells, MafB mRNA levels are comparable to wild type).

MafA expression is lost from adult β -cells of MafA ^{$\Delta\beta$ cell} postnatally

Upon crossing of MafA^{fl/fl} mice with mice expressing Cre recombinase from the rat insulin promoter fragment we generated MafA ^{$\Delta\beta$ cell} animals. In these transgenic mice we observed significant reduction in MafA already at day P7. At that time point only 1/3 of the MafA ^{$\Delta\beta$ cell} mutant β cells expressed MafA

MafA ^{$\Delta\beta$ cell} mutant animals islet function is impaired

Even though islet architecture and α - to β -cell ratio remained unchanged in MafA ^{$\Delta\beta$ cell}, we decided to test the functionality of the mutant β -cells and challenged mice with glucose. Upon performing the IPGTT mutant mice, both males and females, display signs of glucose intolerance and clear glucose slower than wild type. Fasted blood glucose levels were similar to wild type. We then

analyzed mRNA expression levels of several key β -cell genes. Insulin 1 and insulin 2 were found to be downregulated in the $\text{MafA}^{\Delta\beta\text{cell}}$ although protein synthesis does not seem to be negatively affected. In addition, expression of the zinc transporter *Slc0a8* and islet-specific glucose transporter *Glut2* was abolished in $\text{MafA}^{\Delta\beta\text{cell}}$ mutants. *Pdx1* protein synthesis was not reduced in $\text{MafA}^{\Delta\beta\text{cell}}$ mutants, although mRNA levels were significantly downregulated in comparison to wild type. Altogether these results suggest that $\text{MafA}^{\Delta\beta\text{cell}}$ β -cell function is impaired.

Summary discussion

Using Cre-loxP mediated recombination of $\text{MafA}^{\text{fl/fl}}$ mice upon crossing with mice expressing Cre recombinase from the rat insulin promoter fragment; we created a model in which the $\text{MafA}^{\Delta\beta\text{cell}}$ mutant animals lack expression of *MafA* specifically in adult beta cells and no ectopic expression of *MafB* is detected. Even though the $\text{MafA}^{\Delta\beta\text{cell}}$ mutant islets lack *MafA* expression two weeks after birth, islets display normal organization, β -cell mass and no compensation from *MafB* was observed. Studies with other *MafA* deficient models proved that *MafB* can partially compensate for the loss of *MafA* in mutant β -cells, which would explain the relatively “mild” developmental phenotype of the mutant mice. However, *MafB* expression alone in $\text{MafA}^{\Delta\text{systemic}}$ mutant β cells is not able to rescue the adult phenotype. In contrast to that, $\text{MafA}^{\Delta\beta\text{cell}}$ animals, which do not have ectopic *MafB* expression in their β -cells, have normal islet organization, yet they are glucose intolerant as shown in intraperitoneal glucose tolerance. The loss in *Glut2* protein and mRNA in $\text{MafA}^{\Delta\beta\text{cell}}$ mutant mice is a direct result of *MafA* deletion.

CONCLUSIONS

- We have successfully created a suitable model for studying the true contribution of *MafA* to β -cell function.
- Intraperitoneal glucose tolerance tests show that $\text{MafA}^{\Delta\beta\text{cell}}$ mutant mice are glucose intolerant.
- A postnatal *MafA* deletion ($\text{MafA}^{\Delta\beta\text{cell}}$) reduces expression of β cell-specific transcription factors which leads to impaired β -cell function and glucose intolerance.

Paper III: Microphthalmia transcription factor regulates pancreatic β -cell function

Introduction

Our previous gene expression profiling studies showed that expression level of several known, but also some novel genes, was downregulated in MafA/B knockout pancreata. In this study, we present data on a potentially novel MafA/B target gene - Mitf (Microphthalmia transcription factor) expression level was downregulated ~50% in MafA/MafB compound mutant in comparison to wild type. Mitf is a bHLH transcription factor which function is primarily associated with regulation of melanocytic pigmentation, by regulating enzymes (TRP1, TRP2) that are essential for melanogenesis [110]. Given its important role in development and function of melanocytes but also other tissues and structures (e.g. in the eye and ear) we hypothesize that Mitf gene could have a role also during pancreas formation and function. Therefore, we used Mitf mutant mouse model in which the formation of functional protein is impaired due to a point mutation. This results in the synthesis of a truncated protein that is incapable of dimerizing and DNA binding [115].

Results

Mitf is expressed in all five hormone expressing cell types in the pancreas

Our immunohistochemical analysis showed that Mitf is expressed in the developing and adult pancreas. Mitf was initially observed at ~E18.5 in endocrine, exocrine and ductal compartment of the developing pancreas. However along the development Mitf is lost from exocrine and ductal tissue. Postnatally and in the adult pancreas Mitf is solely expressed in the endocrine cells.

Mitf^{ce/ce} mice have improved glucose tolerance and increased insulin secretion

Blood glucose measurements showed that Mitf mutant mice have significantly lower blood glucose levels both during fasted and random fed conditions. Intraperitoneal glucose tolerance test (IPGTT with overnight – 12h fasting) demonstrated that Mitf mutant mice have increased glucose clearance both at 12 weeks and 6 months of age. Additionally, Mitf mutant animals have significantly higher serum insulin levels in fasting conditions than wild-type animals. Next, we performed insulin secretion studies, which showed an enhanced insulin secretory

response in $Mitf^{ce/ce}$ islets in comparison to wild-type (treated with 16.7 mM glucose + 35 mM KCl).

$Mitf^{ce/ce}$ animals have unchanged number of endocrine cells

In order to investigate whether the elevated serum insulin levels observed in $Mitf^{ce/ce}$ mice result from an increase in β -cell mass, we performed quantitative immunohistochemical analysis. $Mitf^{ce/ce}$ mutant islets do not have any changes in the appearance of pancreatic islets. Similarly, an average β -cell area was unchanged in $Mitf^{ce/ce}$ mutant. Interestingly, Q-PCR measurement of pancreatic hormone levels showed insulin and PP mRNA levels significantly increased in $Mitf^{ce/ce}$ islets, while ghrelin transcription was decreased to only 20% of wild-type. Unchanged β -cell area (also number of other endocrine cell types) suggests that single β -cells produce more insulin. Electron microscope analysis demonstrated that $Mitf^{ce/ce}$ mutant β -cells granule morphology is comparable to the wild type, although there is a change in ratio of mature and immature insulin secretory granules.

$Mitf^{ce/ce}$ islets have increased expression of key β -cell genes

In order to investigate if the lack of functional $Mitf$ affects β -cell maturation and function, we immunolabeled pancreatic tissue samples for some of the known mature β -cell markers (Pax6, MafA, Pdx1, Nkx6.1, and Glut2). This examination did not show disturbances in the distribution and expression pattern of these genes in $Mitf$ mutant beta cells. However, we observed two-fold upregulation in Pax6 and Glut2 mRNA levels in $Mitf^{ce/ce}$ mutant β -cells.

$Mitf$ binds to and regulates Pax6 expression in β -TC6 cells

Previous studies demonstrated that $Mitf$, depending on the tissue and time context, can act as transcriptional activator or a repressor [110], [111], [156]. In the eye, $Mitf$ was shown to negatively affect Pax6 expression, while $Mitf$ and Pax6 together repress common target genes in the RPE. Our ChIP analysis performed on β -TC6 cells transfected with an expression vector containing FLAG-tagged $Mitf$ showed that $Mitf$ binds to two regions on Pax6 P0 promoter (Pax6^{EE} and Pax6^{PE}) which are important for Pax6 expression during development. Similarly $Mitf$ were obtained in α -TC6 cell line transfected with the same construct. Dual luciferase reporter assays show that pPax6P0-LUC activity is enhanced by co-transfection with Pax6 in HEK293 cells, but repressed by co-transfection with $Mitf$.

Summary discussion

Mitf transcription factor is primarily associated with differentiation of pigment producing cells and their function. Pancreatic endocrine cell differentiation depends on multiple transcription factors and our previous results demonstrated that MafA and MafB are essential activators of several key endocrine cell genes, especially in regards to β -cell differentiation and function.

Immunohistochemical analysis demonstrated that Mitf is initially expressed in pancreatic epithelium from E18.5 and postnatally becomes restricted to islet cells, whereas it is not found in exocrine and ductal compartments. Gene profiling studies of the Ngn3+ endocrine progenitors and their descendants showing that Mitf transcription increases after birth, while the highest expression is found in adult islets [157] confirm our results. This late expression pattern suggests that Mitf is required mostly postnatally and in adult. This coincides with major changes in pancreas morphology and function e.g. islet formation and establishing glucose responsiveness.

To determine the potential role of Mitf in pancreas development and function, we analyzed Mitf mutant mice that lack the Mitf Zip domain due to the presence of a STOP codon between the HLH and Zip domain fragment of Mitf gene. Hence, translated protein is truncated and incapable of dimerizing and DNA binding.

Histological analysis did not show changes in the expression pattern of important pancreatic β -cell markers, nor total islet β -cell area in Mitf^{ce/ce} mutant mice. However, isolated Mitf^{ce/ce} islets secrete more insulin in response to high glucose and KCl, suggesting increased secretory capacity. In addition, physiological tests (IPGTT) demonstrated enhanced glucose clearance in Mitf animals in comparison to wild type. Interestingly Mitf^{ce/ce} mutant mice have fasting hyperinsulinemia in the presence of slight hypoglycemia. Altogether, these results imply that loss of Mitf improves β -cell function.

Increased expression of Glut2, Pax4 and Pax6 mRNA could also contribute to the overall alterations in β -cell function. We demonstrate that enhanced Pax6 expression could play a crucial role in improvement of the Mitf^{ce/ce} β -cell activity, since Pax6 is an important regulator of several key β -cell genes like insulin, Glut2, PC1/3 and glucokinase.

Our ChIP and luciferase assay experiments show that Mitf binds to Pax4 and Pax6 regulatory elements most likely positively influencing β -cell function, by repressing the Pax6P0 regulatory region. This region is important for high Pax6

expression during pancreatogenesis and we conclude that Mitf is partially responsible for inactivation of Pax6 P0 promoter in developing pancreas.

We believe that potential interplay between Mitf and Pax6 transcription factors may also be involved in controlling pancreas development and endocrine cell function. These novel findings indicate a possible novel role of Mitf in the development of the pancreas, differentiation of pancreatic endocrine cells and function of the mature pancreatic organ.

CONCLUSIONS

- Mitf is specifically expressed in endocrine cells in the postnatal and adult pancreas.
- Mitf mutant mice have enhanced glucose tolerance and are protected from high blood glucose levels by elevated insulin secretion.
- Mitf is critical for proper hormone expression but not essential for endocrine cell specification and maintenance.
- Mitf is controlling β cell function by regulating β cell genes.
- Mitf binds to and represses Pax6 regulatory elements, suggesting that increased Pax6 mRNA levels in Mitf^{ce/ce} are a direct effect of the loss of Mitf.

Concluding remarks

During pancreas development many transcription factors are activated in order to form a mature and functional organ. Pancreatic progenitors are specified into different endocrine cell types and finally hormone producing cells based on the expression profile involving sequential cascades of inductive events. Therefore, transcription factors regulating this process need to be controlled in a tight spatio-temporal manner. Two members of the Maf family of transcription factors, MafA and MafB have emerged as crucial regulators of key pancreatic endocrine cell genes. Our results further emphasize the importance of these two factors in α - and β -cell development, maturation and function. In addition, the distinct spatio-temporal expression pattern of MafA and MafB is important for the proper regulation of β -cell specific genes both during development and in the adult. Moreover, we have recently developed a system to study the true contribution of MafA to β -cell function, by specifically deleting MafA from β -cells only. We demonstrate that the β -cell specific deletion of MafA does not prolong MafB expression in β -cells postnatally (in contrast to other Maf deficient models) and that the timing of MafA deletion is critical to β -cell function.

Based on the microarray results performed on E18.5 wild type and MafA/MafB knockout pancreata, we identified novel genes regulated by these two factors. Among differentially regulated genes in MafA/B mutant, expression of *Mitf* was downregulated in mutant embryonic pancreata. Further analysis showed that lack of functional *Mitf* specifically alters β -cell function. Interestingly, deficiency in *Mitf* expression enhances glucose tolerance and protects *Mitf* mutant mice from high blood glucose levels by elevated insulin secretion. This can, at least partially, be contributed to the *Mitf*'s ability to regulate β -cell specific genes. We also show that *Mitf* directly binds to and represses *Pax6* regulatory elements, suggesting that increased *Pax6* mRNA levels observed in *Mitf*^{ce/ce} result from the loss of *Mitf*.

Unraveling the spatio-temporal pattern of a gene expression is essential in understanding its biological role. Therefore, it will be of great importance to additionally apply both microarrays and various sequencing approaches to broaden our knowledge about the exact role of Microphthalmia transcription factor in pancreas development and function.

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