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ROLE OF EPIDERMAL AND FIBROBLAST GROWTH FACTORS IN PANCREATIC DEVELOPMENT

Mari-Anne Pulkkinen

Program for Developmental and Reproductive Biology
Biomedicum Helsinki

and

Transplantation Laboratory
Haartman Institute

and

Hospital for Children and Adolescents

University of Helsinki

Finland

ACADEMIC DISSERTATION

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in the Niilo Hallmann Auditorium of the Children´s Hospital,
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SUPERVISED BY

Docent Timo Otonkoski, M.D., Ph.D.
Program for Developmental and Reproductive Biology
Hospital for Children and Adolescents
University of Helsinki

REVIEWED BY

Docent Juha Partanen, Ph.D.
Institute of Biotechnology
University of Helsinki

and

Docent Jorma Toppari, M.D., Ph.D.
Departments of Pediatrics and Physiology
University of Turku

OFFICIAL OPPONENT

Professor Jens Høiriis Nielsen, Ph.D.
Department of Medical Biochemistry and Genetics
University of Copenhagen

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To my Mother Sirkka

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following publications, which are referred to in the text by their Roman numerals:

I M.-A. Huotari, J. Palgi and T. Otonkoski. Growth factor mediated proliferation and differentiation of insulin producing INS-1 and RINm5F cells: identification of betacellulin as a novel β-cell mitogen. Endocrinology 1998; 139:1494-1499

II P.J. Miettinen, M.-A. Huotari, T. Koivisto, J. Ustinov, J. Palgi, S. Rasilainen, E. Lehtonen, J. Keski-Oja and T. Otonkoski. Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors. Development 2000; 127:2617-2627

III M.-A. Huotari, P.J. Miettinen, J. Palgi, T. Koivisto, J. Ustinov, D. Harari, Y. Yarden and T. Otonkoski. ErbB signaling regulates lineage determination of developing pancreatic islet cells in embryonic organ culture. Endocrinology 2002; 143:4437-4446

IV M.-A. Pulkkinen, B. Spencer-Dene, C. Dickson and T. Otonkoski. The IIIb isoform of fibroblast growth factor receptor 2 is required for proper growth and branching of pancreatic ductal epithelium but not for differentiation of exocrine or endocrine cells. Mechanisms of Development 2003; 120:167-175

ABBREVIATIONS

AEC	3-amino-9-ethyl-carbazole
ANOVA	analysis of variance
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
BRN4	brain-4
CAM	cell adhesion molecule
CK	cytokeratin
CNS	central nervous system
DNA	deoxyribonucleic acid
e	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
ES	embryonic stem cell
FCS	fetal calf serum
FGF	fibroblast growth factor
Flk	fetal liver kinase (receptor for VEGF)
GH	growth hormone
GLP	glucagon-like peptide
GLUT-2	glucose transporter 2
IGF	insulin-like growth factor
HES	hairy and enhancer of split
HGF/SF	hepatocyte growth factor/scatter factor
HLGAG	heparan-like glycosaminoglycan
HNF	hepatocyte nuclear factor
Ig	immunoglobulin
MODY	maturity-onset diabetes of young
MMP	matrix metalloproteinase
NGF	nerve growth factor
NGN	neurogenin
NRG	neuregulin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDX1/IPF1	pancreatic duodenal homeobox 1/insulin promoter factor 1
PL	placental lactogen
PP	pancreatic polypeptide
PRL	prolactin
PTHrP	parathyroid hormone related protein
Reg	regenerating protein
RIP	rat insulin promoter
RNA	ribonuceic acid
RT	room temperature/reverse transcriptase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh	sonic hedgehog
TH	tyrosine hydroxylase
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

ABSTRACT

The pancreas develops from the foregut endoderm through the fusion of dorsal and ventral pancreatic buds. During development the pancreatic epithelium undergoes branching morphogenesis, in which endoderm-mesoderm interactions are needed for differentiation. Gene targeting experiments have shown the necessity of transcription factors for this process, but the extracellular regulatory mechanisms are not well understood.

The major aim of this study was to elucidate the role of EGFR-, erbB4-, and FGFR2b-mediated signaling in growth and differentiation of pancreatic endocrine progenitor cells.

First, effects of several growth factors were screened using INS-1 insulinoma cells. It was found that betacellulin, a member of the EGF-family, induced proliferation of these cells. The importance of EGFR-mediated signaling in pancreas development was further studied using mice that lack functional EGFR. In EGFR deficient mice both the proliferation and the number of beta cells was diminished. Also the migration of pancreatic islet cells from their embryonic ductal position was impaired due to diminished gelatinolytic activity. In the next set of studies expression of erbB4, another EGF-family receptor, and several EGF-family ligands were detected already at early phases of pancreas development. To further study the role of EGFR and erbB4 in pancreas development, several of their ligands were introduced to embryonic mouse pancreas cultures. In these experiments the EGFR ligands EGF, HB-EGF and betacellulin induced differentiation of beta cells, betacellulin being the most potent factor in this respect. NRG4, an erbB4 ligand, instead induced delta cell development. Both betacellulin and NRG4 were inhibitory to alpha cell formation, indicating that EGFR- and erbB4-mediated signaling favors the beta/delta cell lineages instead of the alpha cell lineage.

Finally, the role of FGFR2b in pancreas development was defined by analyzing the pancreatic phenotype of mice lacking functional FGFR2b. Pancreases of FGFR2b (-/-) mice were significantly diminished in size, and both the pancreatic ductal branching as well as duct cell proliferation was reduced. Differentiation of exocrine and endocrine cells on the other hand occurred normally. Addition of FGFR2b ligands (FGF7 and FGF10) into wild type pancreatic organ cultures stimulated duct cell proliferation. These findings suggest that FGFR2b-mediated signaling plays a major role in pancreatic ductal proliferation and branching.

In conclusion, EGFR, erbB4, FGFR2b, and their ligands appear as important regulators of pancreatic islet cell progenitors. Results obtained from these studies are directly linked to development of islet transplantation therapies. Knowledge of factors regulating beta cell differentiation, proliferation and survival is essential to be able to potently manipulate stem or progenitor cells towards terminally differentiated physiologically regulated transplantable beta cells.

INTRODUCTION

Type 1 (insulin-dependent) diabetes mellitus (IDDM) is an autoimmune disorder where destruction of insulin-producing pancreatic beta cells leads to insulin deficiency (Atkinson and Maclaren, 1994). There are currently approximately 70 000 type I diabetics in Finland (National Health Institute, 2000), and the incidence of the disease has increased steadily, being higher than in any other country (Karvonen et al., 2000). Current treatment of IDDM is based on insulin injection therapy that, however, does not provide continuous normoglycemia to the patients. As a consequence, long-term complications associated with the disease are a major reason for excess morbidity and mortality of IDDM patients.

Transplantation of isolated pancreatic islets, or beta cells, is considered as a promising approach to restore the required mass of functional beta cells in diabetic patients (Ricordi, 1996). Although encouraging long-term success has been reported from clinical islet allotransplantation (Shapiro et al., 2000), the major obstacle to a widespread clinical use of cell transplantation therapy in diabetes is the lack of donor tissue. Thus, new sources of transplantable insulin-producing cells are needed. Currently efforts are being made to generate insulin-producing cells from stem cells or tissue-specific progenitor cells (Bonner-Weir et al., 2000; Lumelsky et al., 2001).

Formation of insulin-producing beta cells can take place by two pathways: via neogenesis of beta cells from islet cell precursors or via replication of already existing beta cells. During fetal life majority of new beta cells develop from precursor stem cells, but newly developed beta cells also proliferate. In the adult pancreas the amount of both beta cell neogenesis and replication is more limited, but however, takes place (Bonner-Weir et al., 1993; Bouwens et al., 1997; Tyrberg et al., 1996). Understanding of the developmental biology and function of the pancreatic beta cell has progressed rapidly during recent years, mainly through the cloning and functional analysis of several tissue-specific transcription factors (Edlund, 1998; Sander and German, 1997). However, extracellular factors, i.e. growth factors, regulating beta cell neogenesis and replication during fetal as well as adult life remain in larger part unknown.

The following review of the literature first describes the ontogeny and transcriptional regulation of pancreas development. Thereafter the focus will be on the role of growth factors and extracellular matrix proteins in pancreas development, the major emphasis being on the role of epidermal growth factor and fibroblast growth factor families.

REVIEW OF THE LITERATURE

1. Overview of endocrine pancreas development

The mammalian pancreas is a mixed exocrine and endocrine organ that derives from the gut endoderm. Mature mammalian pancreas is comprised of the exocrine ductal and acinar compartment, which is responsible for the production and secretion of the digestive enzymes (amylase, lipase, carboxypeptidase etc.), and the endocrine compartment, which is responsible for the production of hormones controlling glucose homeostasis (insulin, glucagon, somatostatin and pancreatic polypeptide). The exocrine portion comprises approximately 99% of the adult mammalian pancreas. Endocrine cells, which comprise 1% of the total pancreatic mass, are located in the islets of Langerhans. Mature islets of Langerhans are discrete micro-organs scattered throughout the exocrine pancreas. They have a characteristic distribution of insulin-expressing cells in the central core of the islets and the peripheral location of non-insulin cells. The human pancreas contains about one million islets whilst in the mouse pancreas several hundred islets are detectable (Hughes, 1956; Slack, 1995).

1.1. Morphogenic events and cytodifferentiation in the developing mouse pancreas

Morphologically evident pancreas development begins by appearance of the dorsal pancreatic bud in the 3 mm long embryo, around the embryonic day 25 in human (Liu and Potter, 1962), and at embryonic day 9.5 (e9.5) in mouse (Slack, 1995).

Appearance of the pancreatic bud is preceded by formation of the three germ layers, endoderm, ectoderm and mesoderm by the end of gastrulation at e7.5 in the mouse. The endodermal germ layer will give rise to the digestive tract and the organs that branch from the main tube, including the pancreas. Before e8.5, the dorsal prepancreatic endoderm looses its contact with the notochord. The dorsal mesenchyme underlying the future pancreatic area condenses and the adjacent duodenal epithelium starts to evaginate forming the dorsal pancreatic bud at e9.5. Soon after (at e10.5) also the ventral bud will arise. As both the dorsal and ventral buds have formed, the pancreatic epithelial cells invade the surrounding mesenchyme. Mesenchymal signals stimulate proliferation, branching and growth of the developing pancreatic epithelium, finally leading to a highly branched differentiated organ where acinar and ductal structures are clearly distinguishable by e14.5. As duodenum and stomach start to rotate, the ventral and dorsal pancreatic buds finally come into contact and fuse around e17 to form a single organ (Figure 1) (Golosow and Grobstein, 1962; Slack, 1995; Wessells and Cohen, 1967).

Markers of endocrine cytodifferentiation can be detected at early phases of pancreas development. Expression of somatostatin mRNA is detected in the mouse gut endoderm already at e7.5 - 8.5. Glucagon and insulin mRNA expression can be detected at e8.5 - 9, and expression of PP mRNA soon after that. The appearance of scattered cells with immunoreactivity for insulin or glucagon can be seen at e9.5 but immunoreactivity for PP is found first at e18. Transcription of amylase and carboxypeptidase is detected at e10.5 - 12, and immunoreactivity for amylase can be seen at e14.5 (Gittes and Rutter, 1992; Herrera et al., 1991). The early endocrine cells are associated with the pancreatic ducts, and mature islets of Langerhans with central insulin-producing cells become detectable first at around e18 – 19 (Figure 1). Formation of mature islets requires sorting of the endocrine cells out of the ductal epithelium, migration of cells through the extracellular matrix, and cell reaggregation, processes where cell adhesion is of importance.

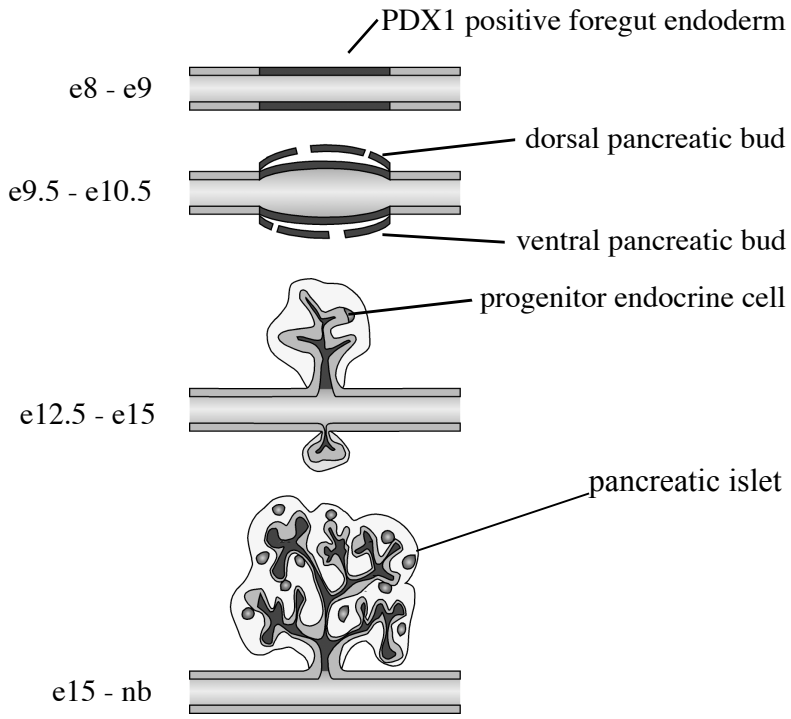

Figure 1. Progression of the mouse embryonic pancreas development (Modified from Edlund, 1998, Sander and German, 1997 and Kemp et al., 2003). Embryonic foregut endoderm designated to become pancreas starts to express transcription factor PDX1. Soon after both the dorsal and ventral pancreatic buds evaginate. Pancreatic epithelium branches, proliferates and invades the surrounding mesenchyme. Endocrine cells start to differentiate. Mature pancreatic islets form around birth.

It was previously suggested that pancreatic endocrine cells arise from common precursors co-expressing islet hormones (Teitelman et al., 1987). Common islet progenitor cells have also been shown to express peptide YY (Upchurch et al., 1994) or neuropeptide Y (Teitelman et al., 1993). It has also been shown that early embryonic endocrine cells in the duct wall transiently express tyrosine hydroxylase (TH) (Teitelman and Lee, 1987). More recently, it was proposed that cells co-expressing insulin and glucagon represent subpopulations of alpha cells that are capable for

transient insulin expression, and that both alpha and beta cells develop independently (Jensen et al., 2000a). This was verified by transgenic cell marking analysis by Herrera (Herrera, 2000). Additional markers for pancreatic endocrine multipotent precursors have been suggested. The most commonly accepted marker is cytokeratin (CK), which is specifically expressed by the pancreatic ductal epithelial cells (Bouwens et al., 1994). In addition, it has been suggested that nestin, which is a marker for neural stem cells, also marks pancreatic islet stem cells (Zulewski et al., 2001). It was later shown that nestin is expressed in the mesenchymal cells of the developing pancreas, but not in the pancreatic epithelial cells believed to represent the pancreatic progenitor cell pool (Selander and Edlund, 2002). Other novel pancreatic markers include ghrelin, which was initially isolated from the rat stomach as a ligand for the growth hormone secretagogue receptor (GHS-R). Ghrelin is expressed in the human pancreatic islets from early gestation to adulthood. Ghrelin positive islet cells do not express any of the known islet hormones (insulin, glucagon, somatostatin, or PP), and thus seem to constitute a new islet cell type (Wierup et al., 2002).

The dorsal and ventral pancreas initially develop separately and then fuse at the later phase of gestation to form a single organ (Liu and Potter, 1962). In adult mammals islet composition and function differ in dorsal and ventral part of the pancreas. In the dorsal islets number of glucagon producing alpha cells is significantly higher than in ventral islets, whereas majority of PP cells are located in the ventral islets. Beta cell number is similar both in the dorsal and ventral pancreatic lobes, however, glucose-stimulated insulin secretion and proinsulin biosynthesis are significantly higher in the dorsal islets (Baetens et al., 1979; Trimble et al., 1982).

2. Genetic regulation of pancreas development

Differentiation of early gut endoderm cells into fully mature endocrine and exocrine pancreatic cells is under strictly regulated genetic control. Already pioneer studies in the area of islet development stressed the importance of epithelial-mesenchymal interactions in the early pancreas development (Golosow and Grobstein, 1962; Pictet and Rutter, 1972; Wessells and Cohen, 1967). Recently, also the molecular mechanisms underlying pancreatic differentiation have started to become clarified.

2.1. Initiation of the pancreatic program

Early development events of ventral and dorsal pancreatic domains are independent. Signals from the notochord are important for development of the dorsal pancreas, whereas signals from the adjacent endothelial cells seem to be necessary for the initiation of both dorsal and ventral pancreatic development. The early endoderm is in tight contact with endothelium of the dorsal aorta and vitelline veins. Followingly, the dorsal and ventral pancreatic buds start to develop precisely

where endoderm previously contacted the endothelium. It has also been shown that isolated endoderm does not start expression of pancreatic markers unless it is cultured with endothelium. Also development of the liver has been shown to be dependent on close vicinity of the endothelium (Lammert et al., 2001; Lammert et al., 2003). The liver and the ventral pancreas are specified at the same time from the same group of ventral foregut cells that express the homeobox gene ***Prox1*** (Burke and Oliver, 2002). FGF signaling from the cardiac mesoderm induces local expression of sonic hedgehog (Shh) on ventral endoderm. Shh in turn is inhibitory to pancreas but permissive to liver development. Close vicinity of the cardiac mesoderm thus seems to be inhibitory for the initiation of ventral pancreas development. As ventral endodermal cells are cultured without cardiac mesoderm they start to express pancreatic marker PDX1 (see below) and fail to express liver markers (Deutsch et al., 2001). Dorsal prepancreatic endoderm is in tight contact with the adjacent notochord until the dorsal aorta fuse. This interaction of endoderm with notochord is necessary for proper dorsal pancreas formation. Notochord secretes inducing molecules (at least FGF2 and activin-βB) that permit initiation of the pancreatic program. Factors secreted by the notochord are responsible for inhibition of expression of hedgehog family signaling molecules in the adjacent gut endoderm. It has been shown that repression of both sonic hedgehog and indian hedgehog in the prepancreatic endoderm is necessary for the continuation of pancreatic differentiation. Both sonic and indian hedgehog are uniformly expressed in the gut endoderm anterior and posterior to the pancreas, however, they remain selectively excluded from the pancreatic buds throughout the development. Also later ectopic expression of Shh in the mouse pancreas prevents proper pancreatic morphogenesis (Apelqvist et al., 1997; diIorio et al., 2002; Hebrok et al., 1998; Hebrok et al., 2000; Kim et al., 1997).

2.2. Transcriptional regulation of pancreas development

A number of transcription factors have been implicated in pancreas development. These belong to the homeodomain family (Pdx1, Hb9, Pbx1, HNF6, Pax4, Pax6, Nkx2.2, Nkx6.1, Isl1, HNF1β, HNF4α, Brn4), the basic helix-loop-helix (bHLH) family (Ngn3, NeuroD, Hes1, p48) and the forkhead/winged helix family (Foxa2/HNF3β, Foxa1/HNF3α). Several homeodomain and bHLH transcription factors have a central role in controlling pancreatic endocrine differentiation, and are expressed already at early stages of pancreas development (Table 1 and Figure 2).

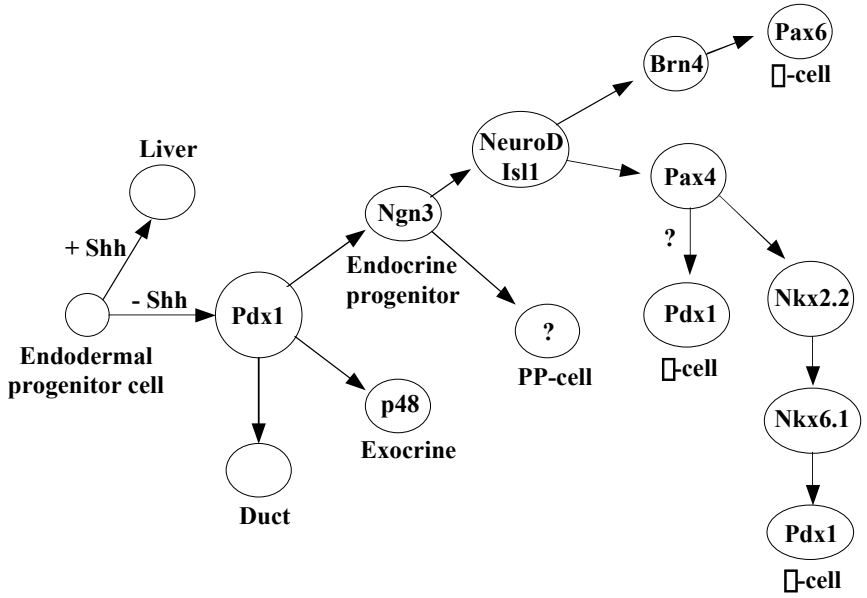

Figure 3. A simplified model for the hierarchy of transcription factor expression in the developing pancreas. The proposed position for each transcription factor is based on its timing of expression or timing of predominant functional role (modified from Edlund, 1998 and Kemp et al., 2003).

Pancreatic duodenal homeobox 1 (***Pdx1***) [human gene known as insulin promoter factor 1 (***Ipf1***)] is a homeodomain transcription factor with a key regulatory role both in pancreas development and in adult beta cell function. During mouse embryogenesis PDX1 is first detected at e8.5 in the ventral gut endoderm in cells later forming the ventral pancreatic bud. At e9.5 PDX1 is expressed both in the dorsal and ventral pancreatic buds, and during early pancreas development its expression is detected throughout the developing pancreatic epithelium. PDX1 expressing cells give rise to all pancreatic cell types: exocrine, endocrine and duct cells. Later in life PDX1 expression becomes restricted to beta and delta cells of the islets of Langerhans, and in dispersed endocrine cells of the duodenal wall (Guz et al., 1995; Jonsson et al., 1994). In the adult pancreas PDX1 acts as a key regulator of insulin gene expression (Ohlsson et al., 1993), and beta cell specific gene disruption of *Pdx1* results in reduced beta cell mass and diabetes (Ahlgren et al., 1998). Heterozygous mutations in human *Ipf1* gene have also been shown to cause MODY-type adult-onset diabetes (Stoffers et al., 1997a), and homozygous *Ipf1* mutations cause agenesis of the pancreas (Stoffers et al., 1997b). In mice carrying a null mutation of *Pdx1*, pancreatic development is arrested at very early stage.

However, the initial stages of pancreatic bud formation occur and early insulin- and glucagon-producing cells appear (Ahlgren et al., 1996; Jonsson et al., 1994). This points to the existence of additional upstream regulators of *Pdx1* (McKinnon and Docherty, 2001). In any case, the expression of PDX1 in gut endoderm is crucial for the pancreatic program to continue and all pancreatic tissues subsequently differentiate from PDX1-positive precursors.

Forkhead/winged helix transcription factor ***Foxa2*** (formerly known as ***Hnf3β***) has been shown to be a key regulator of early foregut development and also to act as an upstream regulator of *Pdx1* (Lee et al., 2002). FOXA2 is expressed in the early foregut endoderm, and also in the adult pancreatic islet and acinar tissue (Wu et al., 1997). *Pdx1* promoter region has binding sites for FOXA2, and it has been shown that FOXA2 acts as a transcriptional regulator of *Pdx1*. However, also other activators of the *Pdx1* gene must exist since beta cell specific deletion of *Foxa2* does not totally arrest PDX1 expression (Lee et al., 2002; Sund et al., 2001). **FOXA1** (formerly **HNF3α**) is initially expressed in the notochord and later on in all pancreatic islet cell types. In mice deficient for FOXA1 expression of proglucagon gene is reduced and mice die between P2 and P12 due to hypoglycemia (Kaestner et al. 1999).

Another homeobox gene having a key regulatory role in early pancreas development is ***Hlxb9*** (encoding **HB9**). HB9 is expressed at the early stages of mouse pancreatic development and later in life in the mature beta cells. HB9 expression is first seen at e8 in the notochord, dorsal gut endoderm and ventral prepancreatic endoderm. In the dorsal pancreatic bud, expression of HB9 precedes expression of PDX1, whereas in the ventral bud both transcription factors appear simultaneously. HB9 expression is detectable in both the dorsal and ventral pancreatic buds at e9.5. At e10.5, its expression becomes restricted to the dorsal pancreatic bud, and then ceases. At later stages when mature islets appear, HB9 can be detected in the mature beta cells. In mice deficient for *Hlxb9*, dorsal pancreatic bud fails to initiate its development, but ventral pancreas development starts normally. The ventral pancreatic epithelium generates all pancreatic cell types even in the absence of *Hlxb9*. However, the number of beta cells is decreased and islet cell organization perturbed. Persistent expression of *Hlxb9* under the *Pdx1* promoter leads to agenesis of both dorsal and ventral pancreas. This indicates that spatially and temporally regulated expression of *Hlxb9* is necessary for proper pancreas development (Harrison et al., 1999; Li et al., 1999; Li and Edlund, 2001).

PBX1 is expressed at e10.5 both in the mesenchymal cells adjacent to pancreatic buds and in the PDX1 positive pancreatic epithelial cells. At e14.5 PBX1 is expressed in the ductal epithelial cells, pancreatic mesenchymal cells, and in early exocrine cells. In the adult pancreas PBX1 expression is detected in ductal, endocrine and exocrine cells. In mice deficient for PBX1, pancreas is hypoplastic and both exocrine and endocrine differentiation is impaired. Thus, also PBX1 seems to be essential for normal pancreatic development (Kim et al., 2002).

Hepatocyte nuclear factor 6 (***Hnf6***) is a homeodomain transcription factor that is expressed in the epithelial cells of the pancreas starting at e9.5. Later, the expression of HNF6 is restricted to acinar and ductal cells. In mice deficient for HNF6, exocrine pancreas appears to develop normally but endocrine differentiation is impaired. Expression of the pro-endocrine gene *Ngn3* (see below) is almost abolished. It has also been shown that HNF6 binds to and stimulates the *Ngn3* promoter. HNF6 has previously been suggested to be a regulator of PDX1 and FOXA2. Their expression, however, is normal in HNF6 (-/-) pancreas (Jacquemin et al., 2000).

Hepatocyte nuclear factor 1β (**HNF1β**) and **HNF4α** have been shown to underlie two types of maturity-onset diabetes of the young (MODY) in human. MODY3 results from the mutations in *Hnf1β*, and MODY1 from the mutations in *Hnf4α*, which is a regulator of *Hnf1β*. In humans with heterozygous *Hnf1β* or *Hnf4α* mutation, insulin-secretion is impaired, leading to diabetes. In mice, carrying heterozygous mutations for *Hnf1β* or *Hnf4α*, pancreatic function is normal, however in HNF1β (-/-) mice a similar phenotype to human MODY3 exists. Expression of HNF1β is first detected at e10.5 in the dorsal pancreas of the developing mouse. From e15.5 onwards expression can be seen in all pancreatic cell types, both exocrine and endocrine (Ferrer, 2002; Nammo et al., 2002; Pontoglio et al., 1998; Shih et al., 2001).

Pancreatic endocrine cells develop from precursor cells expressing PDX1 and the bHLH-family transcription factor neurogenin3 (**NGN3**). All four islet cell types develop from NGN3 expressing cells that are found adjacent to ductal cells. However, coexpression of NGN3 with islet hormones (insulin, glucagon, somatostatin, PP) can not be detected. NGN3 expression is first observed at e9.5, and the number of NGN3 expressing cells increases until e15.5 - the time-point when islet cell differentiation peaks - and decreases thereafter. In the adult pancreas NGN3 positive cells are expressed within the endocrine islets and may represent pancreatic progenitor cells. In mice deficient for NGN3, all four islet cell types are missing at all stages of development. However, both exocrine and ductal tissues develop normally. Expression of NGN3 is strictly controlled. Its promoter has binding sites for HES1, which is a transcriptional repressor of bHLH genes and thus inhibits NGN3 expression. Also HNF6, FOXA2 and HNF1β bind to the *Ngn3* promoter, acting as its activators. NGN3 on the other hand acts as an upstream regulator for the transcription factors *Pax6*, *Pax4*, *NeuroD*, *Nkx6.1*, *Nkx2.2* and *Isl1* (Gradwohl et al., 2000; Gu et al., 2003; Gu et al., 2002; Lee et al., 2001).

NeuroD, a bHLH factor, is an important activator of insulin gene transcription, but is also required for generation of a normal mass of pancreatic beta and alpha cells. One of the earliest steps in the pancreatic endocrine differentiation is activation of NeuroD expression in cells coexpressing NGN3 and PDX1. NGN3 is an upstream activator of NeuroD, and though these two factors are expressed in different cells, it has been demonstrated that NeuroD-positive cells arise from cells expressing NGN3. NeuroD expression is detected at e9.5 in early glucagon expressing cells. Inactivation of *NeuroD* gene in mice leads to lethality 3-5 days postpartum due to severe hyperglycemia. In

newborn NeuroD (-/-) mice the number of beta cells is reduced by 75%, also numbers of alpha and delta cells are reduced (Jensen et al., 2000a; Naya et al., 1997).

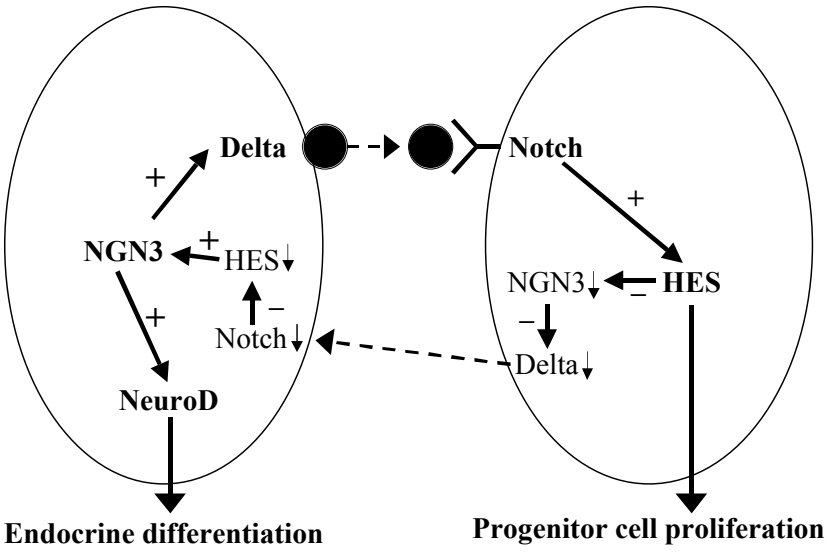

Figure 3. Lateral inhibition of neurogenin3 expression. NGN3 activation leads to endocrine differentiation of pancreatic progenitor cells and also to activation of Delta-Notch pathway. Activation of Notch-pathway causes activation of HES-type proteins that act as lateral inhibitors of NGN3 expression and thus allow expansion of pancreatic progenitor cell pool (Modified from Apelqvist et al., 1999, Jensen et al., 2000b).

bHLH proteins NGN3 and NeuroD are antagonized by the **Notch** pathway. Activation of Notch receptors leads to activation of Hairy and Enhancer-of-split (**HES**) -type proteins, which in turn act as transcriptional repressors of bHLH genes. Mice lacking Notch ligand Delta like-1 (Dll1) or the DNA-binding protein RBP-jk, which is an activator of HES1, have accelerated differentiation of pancreatic endocrine cells and subsequently severe pancreatic hypoplasia due to premature differentiation of pancreatic stem cells into endocrine cells. Also mice overexpressing NGN3 and mice lacking HES1 have a similar pancreatic phenotype. Lateral inhibition of NGN3 expression via Notch-pathway is necessary to allow the expansion of epithelial cells prior to differentiation (Figure 3). Premature overexpression of NGN3 will block the Notch-pathway, which then results into poorly branched ductal epithelium, blockage of exocrine development, and accelerated islet cell differentiation. The Notch signaling pathway is thus involved in the regulation of the balance between progenitor cell differentiation and proliferation during the pancreas development (Apelqvist et al., 1999; Jensen et al., 2000b; Lammert et al., 2000).

PTF1-p48 encodes a bHLH protein that is a 48 kD pancreas-specific subunit of the trimeric pancreas transcription factor PTF1. PTF1-p48 has been considered as the only known exocrine

pancreas-specific transcriptional regulator. Recently, however, PTF1-p48 was shown to regulate commitment of foregut cells, and majority of progenitors of islets, acini and ducts express PTF1-p48. PTF1-p48 seems to have an important role in determining whether prepancreatic cells continue pancreatic organogenesis or differentiate towards intestinal phenotypes. PTF1-p48 was previously considered as an exocrine transcription factor because mice homozygous for a null mutation of the p48 allele totally lack the exocrine pancreas. The ductal epithelium of the p48 (-/-) mice fails to grow and branch and although the endocrine pancreatic cells are formed, they disappear from their original location and migrate to the spleen later during development (Kawaguchi et al., 2002; Krapp et al., 1996; Krapp et al., 1998).

Islet-1 (***Isl1***) encodes a transcription factor belonging to the LIM homeodomain family. ISL1 expression is first detected at e9-9.5 in mesenchymal cells surrounding the dorsal pancreatic bud, and in glucagon positive cells. All developing pancreatic islet cells are positive for ISL1 expression and ISL1 is also expressed in all adult endocrine cells. In mice lacking ISL1, development of the dorsal pancreatic mesenchyme fails and subsequently there is also a failure in the development of the exocrine cells in the dorsal pancreas. In *Isl1* (-/-) mice there is also a complete loss of all islet cell types (Ahlgren et al., 1997).

Brain-4 (**BRN4**) is a member of pou-homeodomain proteins and is expressed in pancreatic epithelial progenitor cells that later differentiate into glucagon producing alpha cells. Expression of BRN4 precedes expression of ISL1 and expression of BRN4 appears to be the early sign of alpha cell development (Hussain et al., 2002).

Pax4 and ***Pax6*** are paired box genes belonging to the *Pax* gene family. PAX4 and PAX6 are closely related and have also been implicated in the pancreas development. PAX6 is expressed in a subset of cells in the developing pancreas already at e9. PAX4 is detected in the developing mouse pancreas at e9.5. During the secondary transition stage (at e14.5) the number of PAX4 expressing cells increases significantly. At this point some of the PAX4 positive cells coexpress insulin. In the adult pancreas PAX4 expression is no longer detectable. PAX6 instead is expressed in all endocrine cells of the developing pancreas as well as in all mature endocrine cells. Mice lacking a functional *Pax4* gene die within three days of birth. Macroscopically, their pancreas appears normal, but both the insulin-producing beta cells as well as the somatostatin-producing delta cells are absent. Instead, the number of glucagon-producing alpha cells is increased. Early pancreas development appears to be normal in *Pax4* (-/-) embryos, but their endocrine progenitor cells fail to mature into beta or delta cells. Also the markers of mature beta cells are absent. In mice lacking PAX6, mature beta, delta and PP-cells can be found, but their number is reduced and the islet formation is disrupted. Cells expressing glucagon are totally abolished. Exocrine differentiation, in contrast, appears to be normal. Mice lacking both PAX4 and PAX6 fail to develop any endocrine cells, suggesting that *Pax* genes are essential for endocrine cell differentiation during pancreatic development (Dohrmann et al., 2000; Sosa-Pineda et al., 1997; St.Onge et al., 1997).

Table 1. Major pancreatic transcription factors, their expression and functions

Transcription factor	Onset and site of pancreatic expression	Mouse & human mutations	References
Homeodomain factors			
Pdx1	e8.5, early pancreatic epithelium, beta and delta cells of mature pancreas	Pancreatic agenesis, heterozygous human mutation: MODY4	Guz et al., 1995; Jonsson et al., 1994; Ohlsson et al., 1993; Ahlgren et al. 1998; Stoffers et al. 1997a, b
Hb9	e8, early prepancreatic endoderm, mature beta cells	Dorsal pancreatic agenesis	Harrison et al., 1999; Li et al., 1999; Li and Edlund 2001
Pbx1	e10.5, ubiquitous	Hypoplastic pancreas	Kim et al., 2002
HNF6	e9.5, throughout the pancreatic buds	Impaired endocrine differentiation	Jacquemin et al., 2000
Pax4	e9.5, in developing endocrine cells, not found in mature pancreas	Deficiency of beta and delta cells, increase in alpha cells	Dohrmann et al., 2000; Sosa-Pineda et al., 1997
Pax6	e9, in endocrine cells	No alpha cells, decrease in other endocrine cell types	St-Onge et al., 1997
Nkx2.2	e9.5, throughout pancreatic bud, later in endocrine cells	Lack of beta cells, decrese in alpha and PP cells	Sussel et. al, 1998
Nkx6.1	e9.5, in beta cells	Decrease in beta cells	Sander et al., 2000
Isl1	e9, in all islet cells	No differentiated islet cells	Ahlgren et al., 1997
HNF1	e10.5, dorsal pancreas, mature beta cells	Heterozygous human mutation and homozygous mouse mutation: MODY3	Nammo et al., 2002; Pontoglio et al., 1998
HNF4	e5.5, in visceral endoderm, e9.5 in pancreatic primordium	Early embryonic lethal Heterozygous human mutaion: MODY1	Ferrer, 2002; Duncan et al., 1994
Brn4	e9, in alpha cell progenitors	No phenotype	Hussain et al., 2002
Basic helix-loop-helix factors			
Ngn3	e9.5, endocrine precursors	No endocrine cells	Gradwohl et al., 2000; Gu et al., 2002
NeuroD	e9.5, endocrine precursors and adult islet cells	Impaired islet development	Jensen et al., 2000a; Naya et al., 1997
Hes1	e9.5, pancreatic precursors	Premature endocrine differentiation	Jensen et al., 2000b; Apelqvist et al., 1999
Ptf1-p48	e10, exocrine, endocrine and ductal progenitors	No exocrine cells	Kawaguchi et al., 2002; Krapp et al., 1996; Krapp et al., 1998
Forkhead / winged helix factors			
Foxa2 / HNF3	e5.5-6.5, early foregut endoderm, adult acinar and islet tissue	Lack of foregut formation	Lee et al., 2002; Sund et al., 2001
Foxa1 / HNF3	e7.5, notochord, later in all islet cell types	Reduced proglucagon expression	Kaestner et al., 1999

Expression of NK homeodomain gene ***Nkx2.2*** is initially detected at e9.5 in the pancreatic epithelium, and during the following days NKX2.2 expression persists in most pancreatic cells. By e15.5 NKX2.2 expression becomes restricted to the endocrine cells, and in the adult pancreas its expression is detected in beta, alpha and PP-cells, but not in delta cells. **NKX6.1** is initially expressed in the developing pancreas at e10.5 in the majority of developing epithelial cells. By e15.5 NKX6.1 expression becomes restricted to the insulin-expressing cells. All NKX6.1 expressing cells coexpress NKX2.2. In mice deficient for NKX2.2 exocrine pancreas develops normally, but there is a reduction in islet-cell mass and islet morphology is disrupted. In NKX2.2 (-/-) mice insulin producing beta cells are absent, the number of glucagon-producing alpha cells is diminished and also the number of PP-cells is reduced. However, mutant pancreases contain a large population of islet cells that express some beta cell specific markers, but do not produce any pancreatic hormones. In mice lacking Nkx6.1 the gross pancreatic morphology appears normal, however, the pancreatic islets are smaller. Expression of glucagon, somatostatin and PP is normal but the number of insulin-producing cells is diminished. Expression of NKX2.2 is maintained in the *Nkx6.1* mutants, whereas expression of NKX6.1 is absent in the *Nkx2.2* mutants, suggesting that NKX6.1 acts downstream of NKX2.2 in beta cell development (Sander et al., 2000; Sussel et al., 1998).

3. Soluble regulatory molecules and microenvironment in pancreas development

Knowledge of ontogeny and transcriptional regulation of endocrine pancreas development has increased significantly during recent years. However, factors and mechanisms regulating the lineage differentiation of islet cells from multipotent precursors are much less well understood. Two types of co-operative signals are known to regulate cell proliferation, differentiation and survival: 1) signaling by soluble regulatory molecules (i.e. hormones and growth factors) and 2) cell-associated signals delivered by extracellular matrix (ECM) proteins and their receptors.

Endocrine differentiation of pancreatic islets has classically been thought to be induced by interaction of the mesenchymal cells with the adjacent epithelium (Hellerström and Swenne, 1985; Pictet and Rutter, 1972). More recently, it has been found that embryonic pancreatic epithelium forms islets even without contact to the fetal mesenchyme, suggesting that the endocrine pancreas is developing by default. However, signals derived from the mesenchyme and extracellular matrix are important for the proper morphology, growth and development of the entire organ, including ducts and acinar components of the pancreas (Gittes et al., 1996). Extracellular molecules also have an important regulatory role during the adulthood since it has been shown that neogenesis of islets from precursor cells occurs even in the adult pancreas. This has been clearly demonstrated in rodent models of pancreas regeneration (Vinik et al., 1996) and is suggested by autopsy observations in humans (Gepts, 1965).

3.1. Factors regulating beta cell replication and neogenesis

Beta cell mass can be stimulated to increase by normal physiological demands (e.g. obesity or pregnancy), by several pathological conditions (e.g. partial pancreatectomy or pancreatic islet inflammation), as well as in experimental conditions by nutrients (e.g. glucose and some amino acids) and growth factors (e.g. growth hormone and prolactin) (Bonner-Weir et al., 1983; Gu and Sarvetnick, 1993; Swenne et al., 1980). There are two pathways for increased beta cell mass: replication of pre-existing beta cells or neogenesis from stem cells. During fetal life, beta cells have a high capacity for replication, but majority of new beta cells is formed by neogensis from pancreatic ductal epithelial stem cells. Some of beta cells' proliferative potential is retained also in adulthood and beta cell neogenesis can be detected even in the adult pancreas. Glucose is an important stimulator of beta cell replication in both fetal and adult life (Hellerström and Swenne, 1985) but also several other factors are involved.

Lactogenic and somatotropic hormones can induce beta cell replication. This is best demonstrated by the fact that during pregnancy hyperplasia of pancreatic islets can be observed both in rodents and in humans. This is a direct effect of increased lactogenic or somatotropic activity in islets, and is mediated by placental lactogen (PL), prolactin (PRL) and growth hormone (GH), whose circulating levels are increased in pregnancy. Expression levels of receptors for prolactin and growth hormone, which also act as receptors for PL, are also elevated in pancreatic tissue from pregnant rats (Moldrup et al., 1993). PL has been implicated as the most potent inducer of enhanced islet cell mass during pregnancy. This effect is mediated via the PRL/GH receptor family, stimulating the Jak/Stat intracellular signaling pathway. Similarly, PRL, PL and GH induce both beta cell proliferation as well as insulin secretion *in vitro* (Brelje and Sorenson, 1991; Friedrichsen et al., 2001; Nielsen, 1982; Nielsen et al., 1999). Unlike in several other tissues, it appears that the effects of PRL, PL and GH in islets are IGF-I (insulin like growth factor-I) independent (Billestrup and Nielsen, 1991; De et al., 1995). *In vivo* effects of PRL on beta cells have been studied using mice overexpressing prolactin under the rat insulin II promoter (RIP). In these mice increased islet cell mass can be detected, mainly due to enhanced beta cell proliferation (Vasavada et al., 2000). Instead, in mice deficient for PRL receptor a clear reduction in beta cell mass is found. Also the islet size is reduced in PRLR (-/-) newborn mice as compared to wild type littermates. PRLR (-/-) mice are not diabetic, but their insulin secretory response to glucose is somewhat blunted (Freemark et al., 2002).

Insulin-like growth factors (IGFs) and insulin, which are considered as important regulators of organ growth in the developing fetus, are strong stimulators of beta cell replication and hypertrophy (Rabinovitch et al., 1982; Swenne et al., 1987). Insulin-like growth factors (IGF-I and IGF-II) share

a high degree of sequence homology with insulin and are produced by pancreatic islet cells both during development and tissue regeneration (Smith et al., 1991). Also receptors for IGFs are expressed by islet cells (Vanschravendijk et al., 1987). Both IGF-I and IGF-II can induce islet cell growth in the developing pancreas (Otonkoski et al., 1988; Rabinovitch et al., 1982). IGF-II seems to be stronger stimulator of beta cell growth than IGF-I and in mice overexpressing IGF-II the size of pancreatic islets is increased due to induced islet cell proliferation and diminished apoptosis (Petrik et al., 1998; Petrik et al., 1999). In mice lacking IGFR1 a reduction in the islet cell mass can be observed, similar phenotype is observed in mice heterozygous for IGFR1 and lacking insulin receptor substrate 2 (IGFR1+/-IRS2-/-) (Withers et al., 1999). In mice with beta cell specific deletion of IGFR1, islet cell mass is normal, but beta cells have reduced expression of Glut2 and glucokinase. This leads to defective glucose-stimulated insulin secretion and impaired glucose tolerance (Kulkarni et al., 2002), suggesting that IGFR1 is not essential for pancreatic islet development, but is involved in the regulation of beta cell function.

Hepatocyte growth factor/scatter factor (HGF/SF) is a mesenchyme-derived protein originally identified as a liver regeneration factor (Nakamura et al., 1989). C-met tyrosine kinase, the receptor for HGF, is expressed in epithelial cells of various developing organs and HGF is produced by distinct mesenchymal cells in close vicinity, suggesting that HGF and c-met mediate epithelial-mesenchymal interactions during development (Sonnenberg et al., 1993). Both HGF and c-met are expressed in the developing pancreas (Defrances et al., 1992). In developing human pancreas c-met expression is mainly associated with the developing beta cells and in the adult pancreas expression is restricted to the islet beta cells (Otonkoski et al., 1996). In NOD (non-obese diabetic) mice expression of c-met can be detected in ductal cells that are also positive for insulin (Bulotta et al., 2001). Exogenous HGF is mitogenic for the human fetal pancreatic beta cells *in vitro* (Otonkoski et al., 1994), this effect can be obtained also with medium secreted by fetal fibroblasts and can be blocked by HGF neutralizing antibody (Otonkoski et al., 1996). In one study HGF together with extracellular matrix was demonstrated to be mitogenic for adult human pancreatic beta cells *in vitro* (Hayek et al., 1995), in another study, however, it appeared that the increase was in the proliferation of duct cells (Lefebvre et al., 1998). Mice overexpressing HGF under the rat insulin II promoter (RIP-HGF) show increase in the islet cell mass as well as in the beta cell proliferation. Insulin content per beta cell is increased and insulin secretion in response to glucose is also higher in RIP-HGF mice. Increase in the beta cell number and insulin content is associated with mild hypoglycemia and hyperinsulinemia in these mice. In addition, islets isolated from RIP-HGF mice function more effectively after transplantation (Garcia-Ocana et al., 2000; Garcia-Ocana et al., 2001). In addition to several studies indicating HGF as a mitogenic factor for beta cells, it has been shown to induce transdifferentiation of exocrine cell line into insulin-producing cells (Mashima et al., 1996b).

Transforming growth factor β (TGF-β) family contains more than 60 family members that regulate cell proliferation, differentiation, morphogenesis and tissue remodeling starting from early embryogenesis. Members of the TGF-β family are subdivided into bone morphogenetic proteins (BMPs), TGF-βs, activins and inhibins, growth and differentiation factors (GDFs) and Müllerian inhibiting substance. These factors signal via two receptor serine/threonine kinase subclasses, type I (activin receptor-like kinase, ALK) and type II. Ligand binding to type II receptor leads to activation of the type I receptor kinase that subsequently phosphorylates cytoplasmic Smad proteins. Activated Smads enter the nucleus and act as transcriptional activators of target genes (Massagué J. and Chen, 2000).

TGF-β and activin are expressed in the developing pancreas (Crisera et al., 2000; Furukawa et al., 1995; Sanvito et al., 1994), as well as in the adult pancreatic tissue (Ogawa et al., 1993; Yamanaka et al., 1993). Exogenously added TGF-β inhibits pancreatic acinar development and promotes development of endocrine cells in embryonic mouse pancreas cultures (Sanvito et al., 1994). TGF-β also has a role in regulating islet morphogenesis in developing pancreas. It activates matrix metalloproteinase MMP-2 (see below), which in turn is necessary for islet morphogenesis (Miralles et al., 1998). Exogenous activin has been shown to inhibit epithelial branching morphogenesis of mouse embryonic pancreas rudiments in embryonic organ culture. This effect of activin is neutralized by follistatin (Ritvos et al., 1995), which is expressed in the pancreatic mesenchyme (Miralles et al., 1998), and is a known inhibitor of activins. Follistatin can also stimulate exocrine pancreas development and inhibit endocrine differentiation in embryonic rat pancreatic epithelial culture *in vitro* (Miralles et al., 1998) thus mimicking the effects of mesenchyme (Gittes et al., 1996). Activin A has also been shown to have a potential to convert pancreatic exocrine AR42J cell line into neuroendocrine direction (Ohnishi et al., 1995). As e15.5 mouse pancreatic cells are cultured in the presence of laminin-1 and bone morphogenetic proteins 4, -5 or -6, formation of cystic epithelial E-cadherin-positive cell clusters containing insulin-positive cells is promoted (Jiang et al., 2002).

Expression of type II activin receptors ActRIIA and ActRIIB has been demonstrated in the developing pancreas and also in the pancreatic islets of adult mice (Kim et al., 2000). Also type I activin receptors are expressed in the pancreas, most abundantly ALK7 (Watanabe et al., 1999), which also can induce phosphorylation of Smad2 in MIN6 mouse insulinoma cell line (Bondestam et al., 2001). Mice lacking both type II activin receptors (ActRIIA-/-B-/-) show defects in foregut patterning. In ActRIIA+/-B-/- embryos Shh is inappropriately expressed adjacent to pancreatic area thus disturbing pancreas development. Expression of Isl1 is also downregulated, and islets that are formed are reduced both in size and number. However, expression of exocrine markers is not affected. (Kim et al., 2000) In transgenic mice expressing mutant forms of activin receptors pancreatic islets are hypoplastic. Both in mice carrying a mutation of constitutively active activin receptor I (i.e. too high activin effect) and in mice carrying a serine/threonine kinase defective mutation in activin receptor II (i.e. no activin effect), the islet mass was diminished (Yamaoka et al.,

1998). Also in transgenic mice expressing truncated type II activin receptor proper formation of pancreatic islets is disturbed (Shiozaki et al., 1999).

Several additional factors have been implicated both in beta cell replication, differentiation as well as in the regulation of beta cell turnover or apoptosis.

Nicotinamide, which is an inhibitor of poly (ADP-ribose) polymerase, has been shown to induce beta cell differentiation in *in vitro* culture of both human (Otonkoski et al., 1993) and porcine (Otonkoski et al., 1999) fetal pancreatic islets. Nicotinamide has also been used for the induction of beta cell differentiation in cultured adult human pancreatic cells (Bonner-Weir et al., 2000) and to prevent diabetes in experimental animal models (Uchigata et al., 1983; Yamada et al., 1982). Though nicotinamide was not effective in preventing the onset of IDDM in first-degree relatives of type 1 diabetics (the ENDIT trial) (Schatz and Bingley, 2001), nicotinamide has so far proven to be the most potent *in vitro* inductor of beta cell differentiation. Sodium butyrate has also been shown to potently increase insulin content of fetal porcine pancreatic islets when it is used in combination with nicotinamide (Korsgren et al., 1993; Otonkoski et al., 1999).

Parathyroid hormone-related protein (PRTrP) and its receptors are expressed in the pancreatic islet cells (Drucker et al., 1989). In mice overexpressing PTHrP under the rat insulin II promoter islet cell hyperplasia, as a result of decreased beta cell apoptosis and increased beta cell neogensis, can be seen (Vasavada et al., 1996).

Reg (regenerating gene) has been isolated from regenerating islets of mice undergone 90 % partial pancreatectomy (Terazono et al., 1988). In non-regenerating pancreas Reg is expressed only in the exocrine tissue (Miyaura et al., 1991). Receptor for Reg is expressed both in the normal pancreatic islets as well as in regenerating islets (Kobayashi et al., 2000). Reg protein has been shown to stimulate beta cell growth both *in vitro* and *in vivo*(Watanabe et al., 1994b). In human fetal islet cell cultures, expression of Reg is increased during the increased beta cell replication, and decreased as beta cell differentiation is induced (Otonkoski et al., 1994). Another islet neogenesis-associated gene (INGAP) was isolated from cellophane wrapped hamster pancreases. INGAP (islet neogenesis associated protein) has 40 % identity to the rat Reg protein and like Reg, it is expressed only in the exocrine pancreas. Addition of INGAP to hamster pancreatic ductal epithelial cell culture or to rat pancreatic duct cell line induces DNA-synthesis (Rafaeloff et al., 1997).

Glucagon-like peptide-1 (GLP-1) is cleaved from proglucagon precursor and is secreted from intestinal enteroendocrine cells in response to oral glucose administration. GLP-1 has been shown to lower blood glucose both in NIDDM and IDDM patients. Binding of GLP-1 to its receptor on islet beta cells stimulates insulin secretion and increases insulin mRNA via insulin gene transcription. GLP-1 also inhibits glucagon secretion. Exendin-4, a GLP-1 analog with long half-life, has been shown to increase both beta cell replication and neogenesis in rats undergone partial

pancreatectomy (Xu et al., 1999), and to exert insulinotropic effects on pancreatic exocrine AR42J cell line (Zhou et al., 1999).

Tyrosine kinase receptor Flk-1 (receptor for VEGF) is expressed in cells lining rat fetal pancreatic ducts and in adult rat pancreatic ducts. Vascular endothelial growth factor (VEGF) instead, is produced by fetal and adult rat islets (Öberg et al., 1994; Rooman et al., 1997). Endothelial signals have been shown to be essential for induction of insulin expression in the foregut endoderm. Culture of prepancreatic (e8.5) endoderm with endothelial cells leads to pancreatic differentiation, whereas ablation of endothelial signals prevents pancreatic differentiation in endodermal culture. In mice expressing VEGF under the *Pdx1* promoter, pancreatic islet hyperplasia ensued, further indicating a role for endothelial factors in endocrine pancreas development (Lammert et al., 2001). Also *in vitro* addition of VEGF has islet cell promoting effects in fetal pancreas (Öberg-Welsh et al., 1997), whereas in adult rat pancreatic duct culture VEGF induces duct cell proliferation (Rooman et al., 1997).

Trk-A, a receptor for nerve growth factor (NGF), is expressed in fetal rat pancreatic ducts. In adult rat pancreas Trk-A is expressed in islet beta cells (Scharfmann et al., 1993), and also NGF is produced and secreted by adult rat beta cells (Rosenbaum et al., 1998). Inhibition of Trk-A activity in an *in vitro* culture system of fetal rat islets has been shown to inhibit normal islet morphogenesis (Kanakagantenbein et al., 1995). More recently a role has been demonstrated for NGF in the regulation of beta cell function and insulin secretion (Rosenbaum et al., 2001).

Another receptor tyrosine kinase, c-Kit, is present both in fetal and adult rat islets. In the adult pancreas Kit is expressed in some of the beta cells, whereas in the embryonic pancreas expression is seen in insulin positive and glucagon positive cells as well as in a specific epithelial cell population that stained negative for endocrine markers (Rachdi et al., 2001). Pancreatic expression of stem cell factor (SCF), the ligand for c-Kit, has not been demonstrated.

Platelet derived growth factor (PDGF) can induce proliferation of fetal rat islet cells in *in vitro* culture (Swenne et al., 1988). In addition, transfection of adult pacreatic beta cells with PDGF□-receptor also induces beta cell DNA synthesis in *in vitro* conditions (Welsh et al., 1990).

Retinoid receptors are expressed in the pancreatic exocrine compartment, and it has been shown that exogenous addition of either 9-cis retinoic acid or all-trans retinoic acid to early embryonic pancreas culture can direct pancreatic lineage selection between ductal and acinar phenotype (Kadison et al., 2001). Culturing of human fetal pancreas with retinoic acid has also been demonstrated to induce functional beta cell maturation (Tuch and Osgerby, 1990). Both retinoids as well as vitamin D analogs have been shown to inhibit growth of pancreatic cancer cells (Zugmaier et al., 1996). Vitamin D has also been demonstrated to inhibit RIN cell growth (Lee et al., 1994).

Gastrin receptors are expressed in the rat pancreatic ductal cells, and it has been shown that gastrin can induce pancreatic duct cell proliferation (Rooman et al., 2001). Gastrin has also been shown to stimulate beta cell neogenesis in duct-ligated rats, where transdifferentiation of acinar tissue towards ductal tissue occurs (Rooman et al., 2002).

3.2. Extracellular matrix, cell adhesion molecules and matrix metalloproteinases in pancreatic morphogenesis

Differentiation of the various pancreatic cell types occurs even before the organ gets its final morphogenetic form, characterized by well-formed islets of Langerhans scattered throughout the exocrine tissue. In the mature islets the endocrine cells are organized in a specific way: beta cells are in the central core of the islet surrounded by the non-beta cells. It is thought that these differentiated islet cells, first found close to the ductal epithelium, migrate out of their epithelial location and then reaggregate to form mature islets. Both cell surface adhesion molecules and molecules with proteolytic characteristics are considered to have a role in these processes.

Cell-cell and cell-matrix intercations have a role both during tissue morphogenesis as well as in the function of adult tissues. Several types of cell adhesion receptors (e.g. integrins, cadherins and members of the Ig superfamily) are involved in aggregation and organization of endocrine cells to form islets. Both calcium-dependent cadherin type cell adhesion molecules (CAMs) and calcium-independent CAMs (e.g. N-CAM) are expressed both during the pancreas development (Esni et al., 1999; Esni et al., 2001), and in the adult pancreatic islet cells (Langley et al., 1989). During pancreas development N-cadherin is initially expressed in pancreatic mesenchyme and endoderm and later in pancreatic islet cells. Mice deficient for N-cadherin suffered from dorsal pancreatic agenesis. However, N-cadherin (-/-) dorsal endoderm underwent evagination, branching and morphogenesis when cultured with wild type mesenchyme indicating that N-cadherin is essential for the survival of the dorsal pancreatic mesenchyme (Esni et al., 2001). Also R- and E-cadherin are expressed in the pancreas. It has been demonstrated that beta cell specific inhibition of their function perturbs clustering of endocrine cells into islets suggesting an important role in the regulation of adhesive properties of beta cells during the aggregation of endocrine cells into mature islets (Dahl et al., 1996). During the early stages of pancreas development, N-CAM is expressed both in the pancreatic mesenchyme and endoderm, later on its expression is restricted to endocrine islet cells and nerve fibers and ganglia. In pancreatic islets all endocrine cells show membranous N-CAM expression though this expression is much stronger in the peripheral non-beta cells than in beta cells. Inhibition of N-CAM function *in vitro* prevents the segregation of beta and non-beta cells and islets that are formed have a mixed organization of beta and non-beta cells. Furthermore, in mice deficient for N-CAM normal localization of endocrine cells within islets is lost. N-CAM thus seems to have a role in islet cell type segregation and organization (Cirulli et al., 1994; Esni et al., 1999).

The integrin family of adhesion receptors regulates interaction of cells with various extracellular matrix (ECM) components. Integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$, acting as receptors for fibronectin, vimentin and collagen IV, are expressed in the developing human pancreas in the ductal cells and clusters of cells branching from the ducts. Fibronectin and collagen IV are expressed in the basal membrane of pancreatic duct cells and vitronectin in the epithelial cells adjacent to pancreatic ducts. In differentiated islets of Langerhans the expression of both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ is downregulated. Inhibition of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ function in the fetal islet perturbs islet cell migration from ductal epithelium. This suggests that interaction of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ with their ligands has a specific role in the regulation of human islet morphogenesis during development (Cirulli et al., 2000).

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that are involved in tissue remodeling and extracellular matrix turnover during embryogenesis. MMPs are secreted as inactive pro-MMPs that are activated by proteolysis (Werb, 1997). MMPs are expressed in the pancreatic mesenchyme and could thus be involved in pancreatic epithelial invasion through the mesenchymal tissue (Reponen et al., 1992). Also rat pancreatic islets express several MMPs and their inhibitors (Barro et al., 1998). MMP-2 is activated from its inactive precursor form during the time when islet morphogenesis takes place. Inhibition MMP-2 activity leads to impairment of islet morphogenesis. TGF-β is a key activator of MMP-2 in the developing pancreas (Miralles et al., 1998). Also EGFR ligands have been shown to upregulate MMPs (Kondapaka et al., 1997), and in mice lacking EGFR secretion of MMPs is decreased in developing mandibles (Miettinen et al., 1999).

4. Epidermal growth factor (EGF) -family and erbB-receptors

4.1. Epidermal growth factor family and related receptors

All members of the epidermal growth factor (EGF) -family share the same basic structure: the EGF-like domain containing six characteristically spaced cysteine residues. This sequence folds into three disulfide-linked loops. This EGF unit is essential for ligand binding (Savage et al., 1972).

EGF-family growth factors are synthesized as membrane-anchored precursors that can be sequentially cleaved to release soluble, bioactive ligands. Epidermal growth factor (EGF) is the first identified member of the family. It was initially discovered by its potential to accelerate eye-opening and tooth eruption in newborn mice (Carpenter and Cohen, 1990). Other EGF -family growth factors are transforming growth factor -α (TGF-α) (Derynck, 1988), heparin binding EGF (HB-EGF) (Raab and Klagsbrun, 1997), amphiregulin (Shoyab and Plowman, 1989), epiregulin (Toyoda et al., 1997), betacellulin (Shing et al., 1993), and four neu differentiation factors (NDFs) also called neuregulins (NRGs) or heregulins (Carraway III et al., 1997; Holmes et al., 1992; Pinkas-Kramarski et al., 1994; Wen et al., 1992; Harari et al., 1999).

EGF-family growth factors activate a group of four receptor tyrosine kinases encoded by the erbB gene family (erbB1/EGF-receptor, erbB2/neu, erbB3 and erbB4) (Kraus et al., 1989; Plowman et al., 1993; Ullrich et al., 1984; Yamamoto et al., 1986). ErbB1/EGFR binds EGF-family ligands that share an EGF-like motif (EGF, TGF-α, amphiregulin, betacellulin, HB-EGF and epiregulin) (Barnard et al., 1994) but betacellulin, epiregulin and HB-EGF can also bind to erbB4. Binding to erbB4 leads to cellular responses distinct from those mediated by EGFR activation (Elenius et al., 1997; Komurasaki et al., 1997; Riese II et al., 1996). Neuregulins were first discovered from the search of a ligand for erbB2 (Holmes et al., 1992). NRGs however can not bind to erbB2 though they can phosphorylate it via heterodimerization with a distinct erbB receptor. It has since been shown that both erbB3 and erbB4 can function as receptors for NRG1 and NRG2 (Carraway III et al., 1997; Riese II et al., 1995), whereas NRG3 and NRG4 only bind erbB4 (Harari et al., 1999; Zhang et al., 1997) (Figure 4). Ligand for erbB2 has so far not been identified and it appears that erbB2 is a co-receptor for other erbBs.

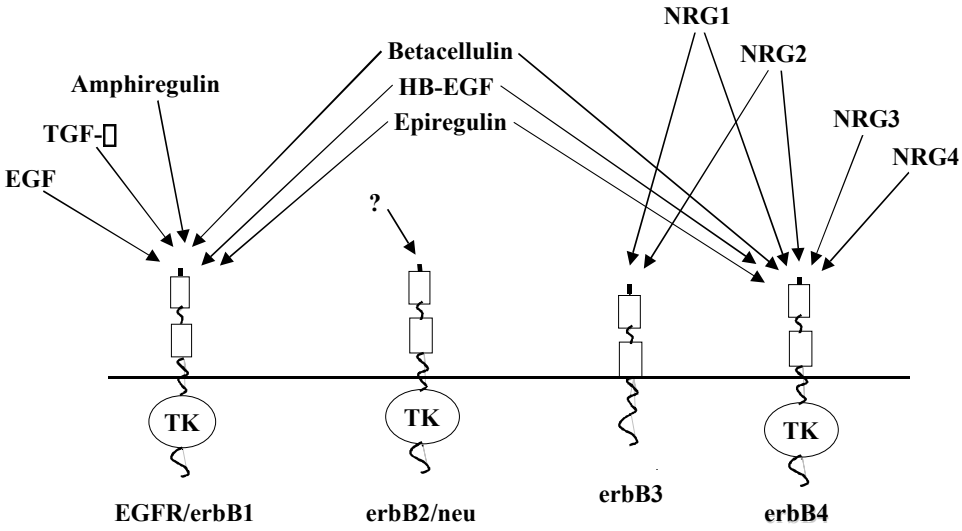

Figure 4. Ligand binding within EGF-family. ErbB-family of receptors consists of four members. EGFR/erbB1 binds all EGF-family ligands except neuregulins (NRGs). NRG1 and NRG2 bind to erbB3 and -4, NRG3 and NRG4 bind only to erbB4. Betacellulin, HB-EGF and epiregulin can bind both to EGFR and erbB4. Ligand for erbB2 has not been discovered. Ligand binding leads to phosphorylation of receptor tyrosine kinases. ErbB3 lacks the intrinsic tyrosine kinase activity.

Members of the erbB-family are structurally closely related. A single hydrophobic membrane anchor sequence separates the extracellular ligand-binding domain from the cytoplasmic domain that contains the highly conserved tyrosine kinase domain. Ligand binding to the extracellular domain induces formation of receptor homo- or heterodimers that subsequently trigger distinct downstream signaling cascades leading to cell differentiation or proliferation. Receptor dimerization is a prerequisite for subsequent receptor activation. In addition, dimerization increases the number of choices for the intracellular signaling pathways that are activated (Carraway III and Cantley, 1994; Riese II et al., 1995; Todderud and Carpenter, 1989). Receptor dimerization is hierarchial: erbB2 is a preferred heterodimerization partner for other erbBs (Graus-Porta et al., 1997) and involvement of erbB2 is essential in mediation of many cellular responses. For example no stimulation of tyrosine phosphorylation occurs in response to NRGs in cells that express erbB3 alone, but co-expression of erbB2 and -3 reconstitutes a high affinity receptor for NRG (Sliwkowski et al., 1994). ErbB2 prolongs and amplifies signal transduction by several EGF-like ligands by decelerating their rate of dissociation from the direct receptor.

Specificity of ligand-induced signal transduction is determined by the expression levels of specific erbB receptor combinations (Sundaresan et al., 1998) and by the unique downstream signals activated by each ligand (Sweeney et al., 2001). It has even been shown that different ligands for a single receptor dimer induce different patterns of phosphorylation of intracellular signaling molecules (Sweeney et al., 2000). Both EGFR and erbB4 can be translocated to the nucleus after binding of their ligand, and thus may directly function as transcription factors without activation of intracellular donwstream signaling-pathways (Lin et al., 2001; Ni et al., 2001). ErbB4 signaling diversity is even greater, since four structurally and functionally distinct erbB4 isoforms have been identified. These isoforms differ in their tissue distribution and in the intracellular signal transduction pathways they activate (Elenius et al., 1999; Junttila et al., 2000).

4.2. Expression pattern and function of erbB-receptors

EGF-receptor is expressed in most tissues of the body (Adamson et al., 1981) and its over-expression is related to epithelium-derived tumors. Other members of the erbB gene family have initially been described by their expression in tumor tissues and they posses significant roles in oncogenesis (Hynes and Stern, 1994). However, there is also wide distribution of these receptors in normal tissues. ErbB2 is essential in the regulation of normal cardiac and nervous system development and its expression in epithelial tissues and fibroblasts is broader than expression of other erbBs (Lee et al., 1995). ErbB3 expression is detectable in a variety of normal tissues of epithelial origin (Kraus et al., 1989). ErbB4 is expressed for example in heart, skeletal muscle, brain, kidney and pancreas (Plowman et al., 1993). In the mammary gland, all four erbB receptors are expressed in cell type- and developmental stage-specific patterns (Schroeder and Lee, 1998).

The importance of erbBs in developmental processes has been obtained from mice with null mutations for erbBs. Loss of functional EGFR leads to embryonic or perinatal lethality, and to abnormalities in EGF-responsive organs (skin, intestine), and in organs undergoing branching organogenesis during development (lung, kidney, mammary gland, pancreas and prostate). Interestingly, the severity of the EGFR deficiency was dependent on the genetic background. In CF-1 background homozygous EGFR mutation resulted in peri-implantation lethality, whereas mid-gestation lethality was seen in 129/Sv mice, and peri- or postnatal lethality in mice with CD-1 or C57BL/6J background (Miettinen et al., 1995; Miettinen et al., 1997; Siblia and Wagner, 1995; Threadgill et al., 1995). Also other members of the erbB gene family appear to be essential for normal organogenesis. Inactivation of erbB2 leads to embryonic death at e10.5 due to lack of cardiac myocyte differentiation (Lee et al., 1995). Also erbB3 (-/-) mice show cardiac abnormalities, and die at e13.5 due to defective heart valve formation. Another major defect in erbB3 (-/-) embryos is seen in the midbrain/hindbrain differentiation. (Erickson et al., 1997). ErbB4 is an essential *in vivo* regulator of both cardiac muscle differentiation and axon guidance in the central nervous system (CNS). Mice lacking erbB4 die during mid-embryogenesis due to abnormalities in the development of cardiac trabeculae. There are also striking changes in the innervation of hindbrain in the CNS (Gassmann et al., 1995).

Expression of erbB ligands is organ- and developmental stage-specific. While some erbB ligands (e.g. NRG1 and NRG2) are expressed in several tissues of the developing embryo (Carraway III et al., 1997; Meyer and Birchmeier, 1994), others (like NRG4) show very restricted expression patterns (Harari et al., 1999).

Mice with null mutation for NRG1 show similar phenotypic abnormalities to erbB2 and erbB4 deficient embryos and also die at e10.5 due to cardiac trabeculae malformation (Meyer and Birchmeier, 1995). Mice lacking TGF-α have abnormalities in their hair follicles and delayed eyelid development (Mann et al., 1993). Similar phenotype has been discovered in spontaneous mouse mutant *waved-1*, that express reduced amount of TGF-α (Luetteke et al., 1993). In mice deficient for EGF no overt phenotype can be observed. In mice deficient for amphiregulin ductal growth in the mammary gland is impaired, and in mice lacking EGF, TGF-α and amphiregulin lactogenesis is abrogated (Luetteke et al., 1999).

4.3. EGF-family and erbB-receptors in pancreas development and function

All four erbB receptors are expressed in the developing pancreas. EGFR is expressed throughout the fetal pancreas (Miettinen and Heikinheimo, 1992), expression of other erbB receptors is detected in primitive fetal ducts (Kritzik et al., 2000). ErbB receptor expression has also been detected in the newborn and adult pancreatic tissues (Kritzik et al., 2000; Press et al., 1990). Evidence for the role of erbB3 and especially EGFR in pancreas development has been obtained

from gene inactivation studies. ErbB3 (-/-) mice die at e13.5 therefore their pancreas development can not be studied properly. ErbB3 (-/-) embryos however have signs of attenuated pancreatic development and reduced endocrine differentiation (Erickson et al., 1997). EGFR (-/-) mice instead survive to term and their pancreatic phenotype was studied in the present work. There is no information about the role of erbB2 or erbB4 inactivation in the developing pancreas because embryos die already at e10.5, prior to pancreas organogenesis (Gassmann et al., 1995; Lee et al., 1995).

Several erbB ligands are also widely expressed in the developing pancreas, and their over-expression can affect pancreas growth and differentiation. In human fetal pancreas TGF-α is present both in exocrine and endocrine tissue. In islets it is coexpressed with insulin (Miettinen and Heikinheimo, 1992). In mouse pancreas TGF-α overexpression has been shown to result in hyperplasia of PDX1 positive ductular cells and progressive interstitial fibrosis. The metaplastic ductules that were formed, contained numerous insulin expressing cells indicating islet neogenesis, however, no increase in either acinar or islet cell mass could be detected (Jhappan et al., 1990; Song et al., 1999). Coexpression of TGF-α and gastrin instead significantly increases islet mass in mice expressing both transgenes (Wang et al., 1993). In addition, it has been shown that when islet neogenesis is induced by duct ligation, expression of both TGF-α and gastrin is upregulated (Wang et al., 1997). It has also been shown that both EGF and TGF-α are involved in the regulation of gastrin gene expression and are weak stimulants of gastrin release (Ford et al., 1997).

Also EGF is present both in the developing (Kajikawa et al., 1991) and adult (Krakowski et al., 1999a) pancreas, and it has been shown to induce pancreatic duct cell proliferation in several species. Systemic EGF treatment in pigs has been shown to induce hyperplasia of interlobular pancreatic ducts (Vinter-Jensen et al., 1997). As pancreatic rudiments from e12.5 mouse embryos were cultured in collagen gels in the presence of exogenously added EGF, increased formation of duct-like structures was detected (Sanvito et al., 1994). Culture of e13.5 rat isolated pancreatic epithelium with EGF also induced proliferation of epithelial cells (Cras-Meneur et al., 2001). In mice overexpressing EGF under the insulin promoter the islet size was increased and there was significant fibrosis around the islets (Krakowski et al., 1999a). In isolated adult rat pancreatic islets exogenously added EGF instead, has been shown to stimulate proinsulin biosynthesis and also DNA content of the islets (detected by ^{3}H-thymidine uptake) (Chattergee et al., 1986). In β-interferon transgenic mice there is inflammation-induced islet cell loss and concomitantly continual growth and differentiation in the pancreas. Islet cells in these mice have a unique capacity to regenerate from proliferating ducts. Transient upregulation of EGF, TGF-α and EGFR can be detected in these β-interferon transgenic mice in acinii undergoing differentiation into duct-like structures (Arnush et al., 1996). Transgenic overexpression of amphiregulin instead has been shown to induce cell proliferation in small pancreatic ducts (Wagner et al., 2002).

HB-EGF is a potent mitogen and chemotactic factor for fibroblasts, smooth muscle cells and keratinocytes (Elenius et al., 1997). During pancreatic development HB-EGF is abundantly expressed both in the endocrine pancreas and in the primitive duct cells from which the endocrine cells are derived. Expression of HB-EGF is not detected in the exocrine compartment. The expression pattern of HB-EGF in the fetal pancreas is similar to that of PDX1, suggesting that HB-EGF may function as a mediator of PDX1, and thus be involved in the development of the endocrine pancreas (Kaneto et al., 1997). NRG1 is also expressed in the ductal epithelial cells of the fetal pancreas (Kritzik et al., 2000) and NRG4, a novel member of the neuregulin family, is predominantly expressed in the pancreas (Harari et al., 1999).

Betacellulin was first identified in the conditioned medium of cell lines derived from mouse pancreatic beta cell tumors (TC-3 cells) (Shing et al., 1993). The mRNA for betacellulin is detected in thymus, lung, heart, liver, spleen, small intestine, pancreas, kidney, muscle, testis and uterus of the mouse. Expression of betacellulin is also detectable in pancreatic islets and ductal cells from normal adult mice. It is also present in several islet tumor cells as well as in the fetal mouse pancreas already at embryonic day 12 (Miyagawa et al., 1999; Shing et al., 1993). Betacellulin is equipotent with EGF in stimulating fibroblast proliferation (Watanabe et al., 1994a). This growth-promoting action of betacellulin is exerted through the EGF receptor (Watanabe et al., 1994a), and antibodies to the EGF receptor act as betacellulin antagonists (Modjtahedi and Dean, 1996). However, betacellulin not only stimulates tyrosine phosphorylation of EGFR, but it can also activate erbB4 (Riese II et al., 1996). Recent data also suggest the existence of a yet unknown receptor for betacellulin. In a pancreatic exocrine cell line, AR42J, apart from erbB1, betacellulin also binds to a protein different from the erbB proteins. This protein is tyrosine phosphorylated after binding betacellulin and its molecular weight is approximately 190 kDa. (Ishiyama et al., 1998). Betacellulin has been shown to exert mitogenic activities in many cell types. It stimulates proliferation of retinal pigment epithelial cells and vascular smooth muscle cells (Shing et al., 1993) as well as undifferentiated human fetal pancreatic epithelial cells (Demeterco et al., 2000). On the other hand, betacellulin also has differentiation inducing effects. Activin A and betacellulin can together convert exocrine AR42J cells into neuron-like cells expressing both insulin and pancreatic polypeptide (PP) (Mashima et al., 1996a) and in 90% pancreatectomized rats addition of betacellulin accelerates beta cell regeneration (Li et al., 2001).

5. Fibroblast growth factor (FGF) -family and FGF-receptors

5.1. Fibroblast growth factor (FGF) -family and related receptors

Fibroblast growth factors (FGFs) make up a family of at least 22 polypeptide growth factors in vertebrates. The 22 known FGFs have a molecular mass ranging from 17 to 34 kDa and share 13-71 % amino acid identity. Common feature of the FGF-family members is a strong affinity for heparin, and heparan-like glycosaminoglycans (HLGAGs), which are required for the binding of FGFs to their high affinity cell surface receptors. Most of the FGFs contain a signal sequence for secretion via classical secretory pathway, FGFs 1, 2, 9, 16 and 20 lack this leader sequence but FGFs 9, 16 and 20 are still secreted. FGFs are important regulators of embryonic development, but they also act in wound healing and are potent angiogenic factors. First members of the FGF-family (FGF1/acidic FGF and FGF2/basic FGF) were isolated from bovine pituitary extracts on the basis of their mitogenic activity on fibroblasts (Armelin, 1973; Gospodarowicz, 1974), also several other FGFs have fibroblast stimulating activities (Ornitz and Itoh, 2001; Powers et al., 2000).

FGFs signal through four cell-surface tyrosine kinase receptors (FGFR1, -2, -3 and -4) and induce receptor dimerization. This dimerization is facilitated by HLGAGs. FGF-receptors share 55 to 72 % homology at the protein level and are characterized by three extracellular immunoglobulin (Ig)-like domains, an acidic region between IgI and IgII, a transmembrane domain, and an intracellular tyrosine kinase domain. Both second and third Ig-loops are involved in ligand binding. Third of the extracellular Ig-like loops (IgIII) is spliced alternatively in FGFR1, -2 and -3 to form different receptor isoforms (Figure 5). Differential splicing may also result in the loss of IgI, which however, does not alter the binding capacity of the receptor. The FGFR4 gene has only one possible form. The IgIIIa receptor splice variant codes a truncated protein that does not contain the transmembrane part and thus can not act in signal transduction. IgIIIb and IgIIIc are expressed differentially and also have different ligand-binding specificities and thus mediate specific actions of different FGFs (Ornitz et al., 1996; Orr-Urtreger et al., 1993; Powers et al., 2000).

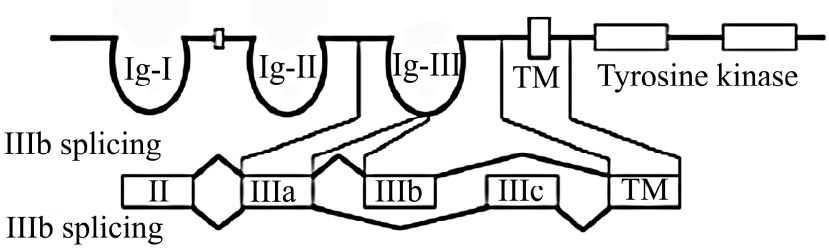

Figure 5. Alternative splicing of FGF-receptors (Modified from De Moerlooze et al., 2000)

5.2. Expression pattern and function of FGF-receptors

FGFR variants are expressed in different locations during the development and thus seem to have unique roles in signal transduction. Both FGFR1 and FGFR2 are detected in the primitive ectoderm of the postimplantation embryo. Later during development the expression patterns of FGFRs 1, 2, and 3 are distinct and expression of either IIIb or IIIc splice variant is dependent upon cell lineage so that IIIb exon is predominantly expressed in epithelial tissues whereas IIIc is located primarily in the mesenchyme. In addition to variable tissue distribution of different FGFRs, they also vary in their ligand specificity so that some ligands preferably bind to IIIb isoforms, whereas others show activity toward IIIc isoforms. FGF1 is the only ligand that binds to all FGF receptor splice variants as well as to FGFR4 (Ornitz et al., 1996; Orr-Urtreger et al., 1993; Powers et al., 2000).

FGFs and their receptors have a central role in development. FGF signaling is involved in cell division of the mouse embryo even prior to implantation (Chai et al., 1998). FGFs are also involved in the formation of the three germ layers during gastrulation. FGFs also have crucial roles for example in the development of the nervous system (Crossley et al., 1996), the lung (Sekine et al., 1999), and most prominently in the development of the limb (Martin, 1998). Mutations in FGFRs are associated with heritable autosomal dominant skeletal disorders in human (De Moerlooze and Dickson, 1997). In mouse, attempts to study specific organ development after inactivation of individual FGFs or both isoforms of a specific FGFR have often failed due to early embryonic lethality. Mice deficient for both isoforms of FGFR1 die during gastrulation and display severe growth retardation and defective mesoderm patterning (Deng et al., 1994; Yamaguchi et al., 1994). Embryos lacking IIIc isoform of the FGFR1 resemble embryos deficient for both isoforms of the FGFR. Mice lacking FGFR1b are viable and show no apparent phenotype, indicating that IIIc is the dominant FGFR1 isoform (Partanen et al., 1998). Mice lacking both isoforms of FGFR2 die at e4.5 - 5.5, soon after implantation (Arman et al., 1998). Also null mutations of FGF4 (Feldman et al., 1995) and FGF8 (Sun et al., 1999) lead to early embryonic lethality prior to organ induction. On the other hand, due to functional redundancy within FGF- and FGFR-families, mutations in a single FGF gene can also yield to little or no apparent phenotype. For example mice lacking FGFR4 (Weinstein et al., 1998) have no apparent embryonic phenotype. Loss of FGF5 and FGF7 results in abnormal hair growth (Guo et al., 1996; Hebert et al., 1994), whereas FGF3 deficient mice have perturbed ear development (Mansour, 1994). FGFR3 deficient mice have problems with long bone and inner ear development (Colvin et al., 1996).

The IIIb isoform of FGFR2 (FGFR2b) has been demonstrated as an important regulator of vertebrate organogenesis. Its role in mouse development has more specifically been studied using FGFR2b dominant-negative receptors (Celli et al., 1998; Peters et al., 1994), and by deleting the exon encoding FGFR2b (De Moerlooze et al., 2000). Inactivation of FGFR2b leads to death of fetuses at birth due to agenesis of lungs. Both mice carrying a dominant-negative mutation for

FGFR2b as well as FGFR2b deficient mice have a failure in the development of limbs, abnormally curled tail and open eyes. Anterior pituitary is absent and this is associated with craniofacial abnormalities, such as cleft palate, abnormal tooth development and reduced otic capsules. There are defects also in the development of the salivary gland, mammary gland (Mailleux et al., 2002), inner ear (Pirvola et al., 2000) and skin.

FGF10 is considered to be the major ligand for FGFR2b, and it is usually expressed in the mesenchyme surrounding the developing FGFR2b positive epithelia (Ohuchi et al., 2000). For example in the developing lung, FGF10 is expressed in the mesenchyme in close vicinity of developing lung buds. FGF10 was initially discovered because it is a homologue of FGF7 (Igarashi et al., 1998). However, whilst FGF7 only binds to and activates FGFR2b, FGF10 can in addition bind to FGFR1b (Figure 6) (Lu et al., 1999). The phenotype of mice lacking FGF10 seems to be quite similar to FGFR2b (-/-) phenotype. FGF10 (-/-) fetuses survive to birth, but then also die due to lack of lungs. Another characteristic feature in FGF10 mutants is the absence of limbs. In addition FGF10 (-/-) fetuses like FGFR2b mutants lack anterior pituitary and salivary glands. They also have defects in the development of teeth, inner ear, kidney, hair follicles, thymus, mammary gland and pancreas (Bhushan et al., 2001; Mailleux et al., 2002; Min et al., 1998; Ohuchi et al., 2000; Sekine et al., 1999). Unlike FGF10 mutants, mice deficient for FGF7 are viable, and only abnormalities detected in FGF7 mutants is their rough hair and slightly disturbed ureteric bud growth (Guo et al., 1996; Qiao et al., 1999).

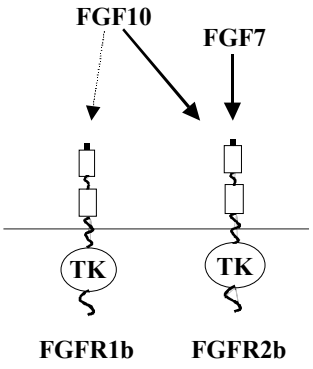

Figure 6. FGF7 binds only to FGFR2b whereas FGF10 can also bind to FGFR1b

5.3. FGFs in pancreas development and function

FGFs and their receptors have been implicated during embryonic pancreas development as well as in the function of adult beta cells. Some studies point out a role for FGFs in the regulation of early endoderm differentiation as well as in the initial stages of pancreas development. FGF4 is expressed in primitive-streak mesoderm and it has been shown to induce posterior endodermal markers (Wells and Melton, 2000). One of the signaling factors expressed in the notochord during pancreas specification is FGF2, which induces Pdx1 expression in endodermal *in vitro* culture (Hebrok et al., 1998). More recently, it was also demonstrated that FGFs secreted by early cardiac mesoderm induce hepatic markers while repressing pancreatic markers (Deutsch et al., 2001).

Expression of FGFR1, FGFR4, FGFR2b and some FGF ligands has previously been demonstrated in the developing rodent pancreas (Arany and Hill, 2000; Le Bras et al., 1998; Miralles et al., 1999). Expression of the IIIc isoform of FGFR1 has been shown both in epithelial and mesenchymal cells of fetal rat pancreas, whereas expression of FGFR1b is seen only in the epithelial fraction (Cras-Méneur and Scharfmann, 2002). Expansion of pancreatic epithelial endocrine precursor cells with relevant growth factors also induced expression of FGFR1b, indicating that it might be a marker for pancreatic progenitor cells (Cras-Méneur and Scharfmann, 2002). FGFR1 expression has also been shown in adult mouse beta cells as well as in the exocrine tissue (Hart et al., 2000). Also FGFR2 expression is detected in the developing rodent pancreas (Miralles et al., 1999) and in the adult mouse pancreatic beta cells (Hart et al., 2000). IIIb isoform of FGFR2 is expressed in the epithelial compartment of the developing mouse pancreas, whereas expression of FGFR2c is seen in the embryonic pancreatic mesenchyme (Elghazi et al., 2002). Expression of FGFR4 has been detected in the epithelial part of rat embryonic pancreas (Öberg-Welsh and Welsh, 1996; Elghazi et al., 2002; Le Bras et al., 1998), whereas in the adult rat pancreas its expression is undetectable (Le Bras et al., 1998). Recently, cDNA endoding novel protein that is highly homologous to known FGFRs was isolated. This novel protein was nominated fibroblast growth factor receptor 5, and its expression was abundant in the pancreas. FGFR5 was demonstrated to bind FGF2, but it lacks the tyrosine kinase domain (Sleeman et al., 2001; Kim et al., 2001).

Some FGF ligands are also expressed in the developing rodent pancreas. Expression of FGF10 is detected in the mesenchyme surrounding the epithelial pancreatic buds from e9.5 to e11.5. (Bhushan et al., 2001). FGF7 expression becomes detectable in late embryonic pancreas (Miralles et al., 1999). FGF1, FGF7 and FGF10 as well as FGF2, FGF4 and FGF5 have all been detected in adult mouse beta cells (Hart et al., 2000).

Exogenous FGF1 has been shown to induce epithelial growth and differentiation of exocrine cells in epithelial cultures from e11.5 rat pancreas (Miralles et al., 1999). Also FGF2 has been shown to induce proliferation of epithelial cells prepared from e13 rat pancreas (Le Bras et al., 1998). In e11.5 rat epithelial pancreatic culture FGF7 and FGF10 induce both epithelial growth as well as exocrine differentiation. An *in vitro* increase in the proliferation of pancreatic endocrine progenitor cells but a decrease in the number of differentiated endocrine cells by exogenous addition FGF7 has also been demonstrated (Elghazi et al., 2002). FGF7 also induces duct cell proliferation in the adult rat pancreas. (Krakowski et al., 1999b; Yi et al., 1994). Overexpression of FGF7 in beta cells resulted in disorganized islet growth and emergence of hepatocytes within islets (Krakowski et al., 1999a; Krakowski et al., 1999b). Transgenic expression of FGF8 under the glucagon promoter induced hepatocyte differentiation in the peripheral part of pancreatic islets. Overexpression of FGF10 in similar experiments induced appearance of both ductal and acinar cells within the islets and disruption of normal beta cell development (Yamaoka et al., 2002).

Further evidence for the role of FGF-mediated signaling in pancreas development and function has been obtained from gene inactivation studies. The pancreas of mice expressing a soluble dominant-

negative form of FGFR2b appeared hypoplastic (Celli et al., 1998). Furthermore, e11.5 rat pancreatic rudiments cultured with antisense oligonucleotides to FGFR2b resulted in a reduced DNA content and reduced number of amylase producing cells (Miralles et al., 1999). In mice expressing a dominant-negative FGFR2b construct under the *Pdx1* promoter, pancreas development and function, however, are normal. In mice deficient for FGF10 (the major ligand for FGFR2b) pancreas is hypoplastic and lacks branched structures. In FGF10 null mice severe reduction in the number and proliferation of PDX1-positive pancreatic epithelial progenitor cells is evident from e10.5 onwards. Subsequently, a marked reduction in the number of islet cells expressing insulin can be seen (Bhushan et al., 2001). In mice deficient for FGF7 (another ligand for FGFR2b), instead, no obvious pancreatic phenotype can be seen (Guo et al., 1996). This is in agreement with the observations that FGF7 in the developing pancreas is not detected until late in gestation (Miralles et al., 1999), whereas FGF10 is expressed from the initial stages of pancreas development (Bhushan et al., 2001). Mice overexpressing FGF10 under the *Pdx1* promoter have increased proliferation of pancreatic epithelium and subsequently hyperplasia of the pancreas (Hart et al., 2001). However, as is the case in FGF10 deficient mice (Bhushan et al., 2001), the numbers of differentiated endocrine cells are also reduced in the *Pdx1*-FGF10 mice (Hart et al., 2001).

The IIIc isoform of FGFR1 has been shown to be essential for proper beta cell function in the adult mouse. Mice expressing a dominant-negative FGFR1c construct under the *Pdx1* promoter become diabetic by the age of 10 weeks. There is an atypical islet organization and 25% decrease in beta cell number, but no increase in beta cell apoptosis. Expression of several functionally important beta cell genes, such as GLUT-2 and PC1/3, is also downregulated (Hart et al., 2000).

AIMS OF THE STUDY

1. To screen for possible beta cell growth and/or differentiation factors (I).
2. To study the role of epidermal growth factor (EGF) -family peptides and receptors in pancreas growth and development (II, III).
3. To study the role of fibroblast growth factor receptor 2IIIb and its ligands in pancreas growth and development (IV).

GENERAL METHODOLOGY

Detailed description of used methodology can be found in the original publications.

Materials (I-IV)

Recombinant human IGF-I, bFGF, PDGF AA, PDGF BB, EGF and natural human TGF were obtained from Upstate Biotechnology. Recombinant human IGF-II, VEGF, betacellulin, TGF-, HB-EGF, FGF7, FGF10 and anti-human betacellulin antibody were obtained from R&D systems. Recombinant human NGF-, all-*trans*-retinoic acid, nicotinamide, and sodium butyrate were obtained from Sigma. Recombinant human activin A and porcine PRL were obtained through the National Hormone and Pituitary Program (NIH). 1,25-dihydroxyvitamin D_3 was a product of Leo Pharmaceutical Products. Recombinant human GH (Genotropin) was from Pharmacia AB. Recombinant human HGF was a gift from Dr. Jeffrey Rubin (National Cancer Institute, Bethesda, USA). Recombinant human NDF was a gift from Dr. Walter Birchmaier (Max Delbrück Centrum, Berlin, Germany). Non-specific goat IgG was provided by Dr. Ilkka Seppälä (Haartman Institute, University of Helsinki, Finland). NRG4 was synthesized as described (Harari et al., 1999). NRG4 inhibitory antibody was generated by injecting New Zealand White rabbits five times with a purified, refolded synthetic peptide comprising the EGF-domain of NRG4 (Harari et al., 1999), with antigenicity boostered by standard Freund's adjuvant protocol. IgB4, a fusion protein between the extracellular ligand binding portion of erbB4 and the Fc portion of human immunoglobulin G was synthesized as described (Chen et al., 1996).

Cell culture studies (I)

INS-1 and RINm5F cell lines were provided by Dr. Claes Wollheim (University of Geneva, Switzerland). Cells were cultured in tissue culture flasks at 37°C under a humified condition of 95% air and 5% CO_2 in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 g/ml streptomycin and 10 % FCS. For INS-1 cell cultures 1 mM sodium butyrate and 50 M 2-merchaptoethanol was added to the medium. For assays the cells were detached by a brief incubation with trypsin/EDTA. For proliferation studies, the cells were plated in 96-well plates and allowed to attach for 24 hours. For the following 24 hours the cells were incubated in the medium containing only 0.5% FCS. For the final 24 hours incubation the studied growth factor was added to the medium. For the measurement of DNA synthesis 1 Ci/ml of ^{3}H-thymidine was added to the cells for the final 4 hours. The cells were then harvested and transferred on glass fiber filters and ^{3}H radioactivity was measured in a liquid scintillation counter. Alternatively, the cells were plated in 12-well plates and incubated for 4 or 7 days with complete

medium containing the studied growth factor. Cell numbers were then counted in a hemocytometer. For detection of insulin content, the cells were plated in 12-well plates and allowed to attach in complete medium for 24 hours and then the studied growth factor was added to the medium for 7 days. The cells were then detached and homogenized by sonication. Cellular insulin content was measured by radio-immuno-assay in dilutions of acid ethanol extracts. Cellular DNA content was measured fluorometrically (Hinegardner, 1971).

Generation and genotyping of the EGFR (-/-) mice (II, III)

EGFR was disrupted as described previously (Miettinen et al., 1995). Embryos and newborn pups used in this study derived from intercrosses between EGFR (+/-) mice. Plug-date was considered as e0.5. E12.5 - 15.5 embryos were genotyped by PCR and Southern blot analysis as described previously (Miettinen et al., 1995), while older EGFR (-/-) embryos could be recognized from their open-eye phenotype. For histological analysis newborn mice plus embryos at e12.5, 13.5, 14.5, 15.5 and 16.5 were collected. For pancreatic organ cultures e12.5 embryos were collected. For cell proliferation experiments, pregnant animals or pups were injected intraperitoneally with bromodeoxyuridine (BrdU; 100 μg/g animal mass; Sigma) 2 hours before killing the animals.

Generation and genotyping of the FGFR2b (-/-) mice (IV)

The targeted disruption of FGFR2b, and the genotyping of offsprings by PCR analysis of genomic yolk sac DNA has been described previously (De Moerlooze et al., 2000). Mutant embryos could be recognized from their limb-lacking phenotype. Mutant and wild type littermates were collected at e12.5, 13.5, 14.5, 15.5 and 18.5 for histological and in situ analysis, at e15.5 for RNA analysis, and at e12.5 for pancreatic organ cultures.

Blood glucose levels (II)

Random blood glucose recordings were obtained from newborn animals at the time they were killed using a portable glucose meter (MediSense Inc., Waltham, USA).

Pancreatic organ cultures (II, III, IV)

Pancreatic explant cultures from wild type, EGFR deficient and FGFR2b deficient e12.5 embryos were used to study the *in vitro* development of the pancreas. The duodenal loop along with the pancreatic rudiment and stomach were microdissected. The tissues were then cultured by a technique originally designed for embryonic kidney (Saxen and Lehtonen, 1987). Tissue explants consisting of dorsal and ventral pancreatic buds (mesenchyme plus epithelium), stomach and duodenal loop, were placed on Nucleopore filters (1.0 μm pore size, Costar) on metal grids and

cultured at the air-liquid interphase in serum-free I-MEM (Improved Eagle´s Minimum Essential Medium, Gibco) supplemented with transferrin (30 μg/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml) and either EGF (20 ng/ml), TGF-α (20 ng/ml), HB-EGF (20 ng/ml), betacellulin (20 ng/ml), NRG4 (1 ng/ml), anti-human betacellulin antibody (1 μg/ml), anti NRG-4 antibody (1:100), IgB4 (10 μg/ml), non-specific goat IgG (10 μg/ml), FGF7 (50 ng/ml), FGF10 (50 ng/ml), or cultured in plain medium (knock out embryos and control group). The media were changed every second day. After one or five days in culture, explants were fixed for 4 hours at room temperature (RT) in Bouin´s fixative. After rinsing with 50% alcohol, tissues were stored in 70% alcohol prior to dehydration and paraffin embedding.

Histological analysis (II, IV)

Samples were fixed either in 4% paraformaldehyde or Bouin's fixative and processed into serial paraffin sections using routine procedures. For general morphology, deparaffinized sections were stained with hematoxylin. The sections were then examined under light microscope, photographed or morphometrically analyzed directly under light microscope. E15.5 pancreases were used for the analysis of ductal branching. For this purpose whole pancreases were sectioned through and the number of ductal lumens was counted under the light microscope from every 20^{th} section (6-8 sections/each pancreas) using a 10 μm grid. Number of ductal openings/mm^2 was then calculated.

Immunohistochemistry (II, III, IV)

For quantitative morphometric analysis of pancreatic cell types, an entire tissue block was sectioned for immunohistochemical stainings. Every fifth or seventh consecutive section was stained with the same primary antibody. Deparaffinized, rehydrated sections were incubated for 2 hours at RT in 3% normal goat or rabbit serum (Zymed, South San Francisco, CA) in PBS (pH 7.4) or for 10 minutes in Ultra Vision blocking reagent (Lab Vision, Fremont, CA) to block nonspecific binding sites. Sections were incubated either overnight at +4 °C or for one hour at RT with primary antibody, diluted in PBS containing 3% normal serum (mouse anti-human E-cadherin, Transduction Laboratories; guinea pig anti-porcine insulin, rabbit anti-human glucagon, rabbit anti-human somatostatin, rabbit anti-human pancreatic polypeptide, DAKO; rabbit anti-human amylase, monoclonal anti-pan cytokeratin (mixture); Sigma). All antisera were pretested for optimal dilutions and staining conditions. After rinsing several times with PBS, sections were incubated with biotinylated-goat anti-rabbit (ready-to-use, Lab Vision) or biotinylated-goat anti-mouse (ready-to-use, Lab Vision) for 20 minutes at RT, rinsed and incubated with streptavidin peroxidase (ready-to-use, Lab Vision) for 10 minutes at RT. The sections were finally developed with AEC substrate (Lab Vision). Alternatively, after rinsing off the primary antibody the sections were incubated for 30 minutes with biotinylated-goat anti-rabbit IgG (biotinylated-rabbit anti-mouse IgG for CK)

(Zymed), diluted in PBS, rinsed and incubated with peroxidase conjugated streptavidin (ZYMED), diluted in PBS for 30 min at RT. The sections were developed with 3-amino-9-ethyl-carbazole (AEC) substrate and rinsed with distilled water. Light counter-staining was performed with hematoxylin. For simultaneous detection of insulin-, glucagon- and cytokeratin-positive cells or insulin- and E-cadherin-positive cells, fluorochrome (FITC, TRITC and AMCA)-conjugated secondary antibodies (Jackson Immunolaboratories) were used.

Cell proliferation was quantified by staining with Ki67 nuclear antigen or with anti-BrdU antibody (BrdU labeled pups and embryos). For double staining of hormone plus Ki67/BrdU, the staining protocol was continued from the one described above. The slides were treated with 10 mM citrate buffer (pH 6.0) for 25 minutes in a microwave oven (for Ki67 staining) or with 0.1% pepsin in 0.1 M HCl at RT for 30 minutes (for BrdU staining) to reveal antigenic sites and rinsed in PBS. Double staining was performed using the Vectastain ABC-kit (Vector, Burlingame, CA). The sections were incubated for 1 hour at RT in 3% goat or horse blocking serum in PBS (pH 7.4) and incubated with rabbit polyclonal Ki67 antibody (NovoCastra, Newcastle, UK), diluted 1:500 in PBS containing 3% goat serum or with mouse monoclonal anti-BrdU antibody diluted 1:100 in 3% horse serum. After rinsing with PBS, sections were incubated for 30 minutes with biotinylated antibody, rinsed, incubated for 30 min in Vectastain ABC-alkaline phosphatase reagent, rinsed, and developed with AP substrate (Vector Blue®). After color development, slides were rinsed in distilled water and mounted with Aquamount.

After the immunohistochemical stainings the numbers of positively stained cells was either counted manually under the light microscope or the slides were included in the computerized image analysis using KS400 Image Analysis software.

Detection of apoptosis (II)

For detection of apoptosis deparaffinized sections were stained using the terminal dideoxynucleotidetransferase (Tdt)-mediated ddUTP nick end labelling (TUNEL) procedure. The sections were permeabilized by microwave treatment in 10 mM citric acid, followed by preincubation in 5 mM $CoCl_2$ Tdt buffer for 10 minutes and the digoxigenin-conjugated ddUTP labeling of the nicked DNA ends by Tdt (5 mM $CoCl_2$, 5 mM Tdt-Buffer, 0.23 mM ddATP, 0.13 mM dig-ddUTP, 0.58 U/μl Tdt) at 37˚C for 60 minutes. To detect the labeled cells, the sections were treated with 2 % blocking reagent (Boehringer Mannheim) in 150 mM NaCl, 100 mM Tris-HCl buffer (pH 7.5) and treated with anti-digoxigenin Fab fragments (0.19 U/ml in blocking buffer) at 37˚C for 60 minutes. Finally, the reaction products were visualized by incubation with a peroxidase dye, NBT/BCIP, in 67 % DMSO, for up to 60 minutes. Nuclei were counter-stained with hematoxylin. Similar procedure without the Tdt-treatment was used as a negative control for every sample. Cell specificity of apoptosis was attained by insulin immunocytochemistry following TUNEL-staining.

To detect DNA fragmentation at tissue level DNA was purified from neonatal pancreases using a commercial kit (Apoptotic DNA ladder kit, Boehringer Mannheim). The purified DNA samples were nick-end labeled with digoxigenin-ddUTP. Gel electrophoresis was performed and the DNA blotted onto a Hybond N+ membrane. The membrane was then incubated with an anti-digoxigenin-alkaline phosphatase antibody and further with CSPD, a chemiluminescent substrate for alkaline phosphatase.

RNA extraction (I, II, III, IV) **and analysis** (I, II, IV)

Total RNA was prepared by guanidinium thiocyanate extraction followed by CsCl gradient centrifugation (Sambrook et al., 1989) or by using GenElute total RNA isolation kit (Sigma). mRNA was purified from total RNA preparations with oligo(T)-coated magnetic microbeads (Dynabeads, Dynal A.S.).

Total RNA (approximately 10 μg/lane) or mRNA (1.2 μg/lane) was fractionated on a 1.2 % formaline-agarose gel and transferred to a nylon membrane (Hybond-N, Amersham) by capillary blotting. The cDNA probes were ^{32}P-labeled by a random priming method (Prime-A-Gene Labeling System, Promega). Following probes were used: Mouse EGFR cDNA and rat erbB-2 cDNA corresponded to the extracellular domains of the receptors. Human erbB-3, erbB-4 cDNAs and mouse 72-kDa type IV collagenase (Reponen et al., 1992) cDNAs represented the whole coding areas of corresponding mRNAs. cDNA clones used for probe generation in work number IV were provided by following investigators: Dr. Helena Edlund, Umeå, Sweden (mouse Ngn-3); Dr. Ole Madsen, Gentofte, Denmark (rat Nkx6.1); Dr. Peter Wellauer, Lausanne, Switzerland (mouse p48). Hybridizations were done in buffer containing 1% SDS, 1M NaCl, and 8% dextran sulfate overnight at 65°C. The blots were washed at 65°C in 1 x SSC and finally in 0.5 x SSC. Hybridization signals were visualized using a Bio-imaging analyzer (Fuji Photo Film Co., Ltd). The hybridization signals were normalized against the housekeeping gene cyclophilin (Bergsma et al., 1991) or β-actin.

Reverse transcriptase (RT) PCR and primers (III)

Prior to reverse transcription RNA samples were DNase-I treated for 50 minutes at +37°C. Thereafter the samples were phenol/chloroform extracted and precipitated after addition of 1 volume of 7.5 M NH_4OAc and 2.5 volumes of ethanol. Total RNA was precipitated by centrifugation and washed once with 75% EtOH. After precipitation with ethanol the samples were dissolved in RNase-free water. Total RNA (2 μg) was then reverse transcribed at +37°C for 90 min. The reaction was inactivated at 95°C for 5 min. One μl of RT reaction mixture was used for PCR amplification. The PCR samples were heated to 95°C for 5 minutes to activate the polymerase and then cycled for 35 times: denaturation 45 sec at 94°C, annealing 45 sec at 58°C - 63°C, elongation

45 sec at 72°C on GenAmp PCR System 9600 apparatus. The PCR samples were electrophoresed through 1,5 % agarose gel and visualized with ethidium bromide staining.

Following primers were used:

Rat GAPDH (5´-GTC TTC ACC ACC ATG GAG AAG GCT´& 5´-TGT AGC CCA GGA TGC CCT TTA GTG´; EMBL/Genbank M17701, position 325-854, fragment size 530)

Mouse EGF (5´TTG AAA TGG CCA ATC TGG ATG G´ & 5´TGA CAC CAT GAT TTC AGC CAC T; EMBL/Genbank J00380, position 2368-2855, fragment size 488 bp)
Mouse TGF-α (5´GTT CTC AGG TCC AGC CAG TC & 5´GGT TCT CTC CTT CCA CCA GAT´; EMBL/Genbank U65016, position 2685-3225, fragment size 541 bp)
Mouse HB-EGF (5´TCT GGA GCG GCT TCG GAG AG´ & 5´CAC GCC CAA CTT CAT TTC TC´; 5´primer exon 2 EMBL/Genbank L36024 and 3´primer exon 5 EMBL/Genbank L36027 corresponding to human HB-EGF cDNA EMBL/Genbank M60278, position 336-873, fragment size 538 bp)
Mouse betacellulin (5´CAC AGC ACA GTT GAT GGA CC´ & 5´CCG TTA AGC AAT ATT GGT CTC 3´; EMBL/Genbank L08394, position 100-649, fragment size 550 bp)
Mouse NRG4 (5´CTC ACT CTT ACC ATC GCG GC´ & 5´CAG CCT TAT CTA TAC TGC TGA C´, position 328-710, fragment size 383 bp, (Harari et al., 1999))

In situ hybridization, whole mount in situ and probes (IV)
In situ hybridization was performed on deparaffinized tissue sections essentially as described previously (Kettunen et al., 1998). The sections were hybridized with 10^6 cpm of [^{35}S]-labeled antisense riboprobes, prepared using the following murine cDNA templates: Pdx1 p48, Ngn3, Pax6, FGFR2b, FGFR2c and rat Islet 1. Whole mount in situ hybridization was performed as described (Revest et al., 2001). Digoxigenin-labeled mouse Pdx1 cDNA was used for the hybridization. The cDNA clones used for the generation of probes were provided by following investigators: Dr. Päivi Kettunen, Helsinki, Finland (mouse FGFR2c); Dr. Helena Edlund, Umeå, Sweden (mouse Ngn-3); Dr. Ole Madsen, Gentofte, Denmark (rat Nkx6.1); Dr. Peter Wellauer, Lausanne, Switzerland (mouse p48); Dr. Chris Wright, Nashville, TN, USA (mouse Pdx1); Dr. Peter Gruss, Göttingen, Germany (mouse Pax6) and Dr. Samuel Pfaff, La Jolla, USA (rat Islet 1). FGFR2b cDNA was generated as described (De Moerlooze et al., 2000).

Gelatin zymography, zymographic and reverse zymographic assays (II)
Analyses for gelatinase activity (Chin and Werb, 1997) and caseinolysis-in-agarose assays (Saksela, 1981) were carried out as described. Tissues were dissected and digested with collagenase (4 mg/ml in Hanks buffer) in a shaking water bath (37°C for 5 minutes). Tissues were then cultured in RPMI 1640 supplemented with 10 % FCS, 10 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine for 3 days. Samples of conditioned medium were collected and diluted in non-reducing 2 x Laemmli sample buffer. Equal amounts of protein were subjected to SDS-PAGE

under non-reducing conditions. For gelatin zymography assays the gels were washed twice after electrophoresis with 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 2.5% Triton X-100 to remove SDS. This was followed by brief rinsing in washing buffer without Triton X-100 and then incubation in substrate buffer (50 mM Tris-HCl buffer (pH 7.6) containing 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 1 % Triton X-100 and 0.02 % NaN_3) for overnight at 37°C. The gels were then stained with Coomassie brilliant blue, destained with 10 % acetic acid and 10 % methanol, dried and photographed. Zymograms were then densitometrically scanned and the gelatinolytic bands were quantified by NIH Image-analysis software. Conditioned medium from human fibrosarcoma HT-1080 cells containing gelatinases A and B was used as mobility markers. For caseinolysis assays the gels were washed with 2.5 % triton-X in PBS and then placed on caseinolysis gels that contained 1 U plasminogen (Cromogenix), casein (1.5% milk powder) and 1.2% agarose (LITEX) in 0.1 M Tris-HCl buffer (pH 8.0) and incubated at 37°C until lysis zones were observed. The positions of plasminogen activators (tPA and uPA) in SDS gels were determined as lysis zones in the indicator zymography gels. To analyze the levels of plasminogen activator inhibitor-1 (PAI-1), the SDS was removed by washing with 2.5 % Triton X-100 in PBS, and human uPA (2 U/ml) was added for the last 30 minutes rinse. uPA induces plasminogen-mediated digestion of the casein, except at the zones containing PAIs, which can be seen as lysis-resistant bands on caseinolysis gels.

NRG4 antibody and betacellulin neutralizing antibody binding (III)

To test specificity of the NRG4 antibody, NRG4 and EGF (as negative control) were both radiolabeled with ^{125}I (2 x 10^6 cpm/µl) using Iodogen (Pierce), and cleaned on a G25 Sepharose column. 10 µl of NRG4 antisera from two independently injected rabbits were pre-adsorbed to protein-A sepharose beads in HNTG buffer at +4°C for 30 minutes. Beads were washed three times in HNTG buffer, blocked in HNTG + 0.1 % BSA for 30 minutes at +4°C, spinned, and incubated for a further 2 hours with 5 µl of radio-labeled ligand at +4°C. The same protocol was repeated for control antibodies and soluble Fc-conjugated erbB1. Beads were then washed four times with HNTG, before being boiled in reducing sample buffer, proteins resolved by SDS-PAGE and bound ligand detected by autoradiography. Ability of anti-human betacellulin antibody to inhibit betacellulin activity was studied using A431 cell line (ATCC, no: CRL-1555) (Giard et al., 1973). For this purpose cells were plated in 6-well plates and allowed to attach in D-MEM (Gibco) supplemented with 10 % FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine for 24 hours. Cells were then serum-starved for overnight, incubated for 5 minutes at 37°C with betacellulin, anti-human betacellulin or without additions (control group). Betacellulin was then added to cells treated with anti-human betacellulin antibody for another 5 minutes incubation. Cells were then lysed in ice cold lysis buffer (25 mM Hepes, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, leupeptin 20 µg/ml, aprotinin 20 µg/ml, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM Na-orthovanadate) and left on ice for 10 minutes. The whole cell extract

was then cleared by centrifugation, boiled in reducing gel sample buffer, and resolved by 10 % SDS-PAGE before transfer onto nitrocellulose filters. Filters were blocked for overnight at +4°C in TBST buffer (10 mM TrisHCl (pH 8), 150 mM NaCl, 0.1% Tween 20) containing 5 % milk. The filters were then blotted with anti EGFR [pY^{1086}] phosphospecific antibody in blocking buffer for 2 hours at RT, followed by conjugation with a secondary antibody linked to horseradish peroxidase in blocking buffer for 1 hour at RT. Subsequently proteins were detected using the ECL detection system.

Statistics

Results are presented as the mean ± SEM. Significance of observed differences between experimental groups were tested by one-way ANOVA followed by Fischer's PLSD test, or in the case of two groups by Student's unpaired t test. In case of skewed data non-parametric methods were used (Kruskal Wallis test and Mann Whitney U test). All statistical analyses were performed using Statview 5.0 software (SAS institute, Cary, NC).

RESULTS AND DISCUSSION

Growth factor effects on proliferation and differentiation of insulin producing cell lines (I)

The aim of the present study was to screen the effect of several peptide growth factors, hormones and pharmacological agents on proliferation and differentiation of insulin producing INS-1 cell line. INS-1 is a relatively differentiated rat insulin-producing cell line with substantially high level of insulin expression and with glucose responsive insulin secretion (Asfari et al., 1992). We hypothesized that the growth regulation of this cell line could thus resemble more closely the regulation of primary β-cells than in more undifferentiated, glucose unresponsive lines, such as RINm5F.

Table 2. Effect of studied growth factors on INS-1 proliferation and insulin content (+ stimulation; - suppression; 0 no effect; N.D. not done)

	Effect on proliferation	Effect on insulin content
GH	+	0
PRL	+	0
IGF-1	+	0
IGF-2	+	N.D.
VEGF	+	0
PDGF	0	N.D.
NGF	0	N.D.
TGF-β	0	N.D.
Activin A	-	+
bFGF	0	N.D.
HGF	0	N.D.
EGF	0	N.D.
TGF-α	0	N.D.
NRG1	0	N.D.
Betacellulin	+	0
Nicotinamide	-	+
Sodium butyrate	-	+
Nic. + Sod.but.	-	+
Vitamin D_3	-	+
Retinoic acid	-	0

In accordance with the hypothesis, several of the factors that were mitogenic to INS-1 cells had also previously been demonstrated as beta cell mitogens in studies done with primary cells (Brelje and Sorenson, 1991; Nielsen et al., 1992; Stout et al., 1997). From the factors that were tested only betacellulin emerged as a novel beta cell mitogen. Interestingly, other members of the EGF family tested (EGF, TGF-α) did not stimulate beta cell proliferation. Nicotinamide, sodium butyrate, vitamin D_3 analog and activin A inhibited INS-1 growth while increasing cellular insulin content. Effects of EGF-family members on proliferation of less differentiated RINm5F cell line (Dereli et al., 1988) was also studied. In contrast to the INS-1 line, betacellulin did not induce RINm5F cell proliferation, whereas both EGF and TGF-α had a small stimulatory effect.

The results indicated that betacellulin is mitogenic for differentiated beta cells. This is in agreement with studies showing that betacellulin is mitogenic for several cell types (Shing et al., 1993), including fetal pancreatic epithelial cells (Demeterco et al., 2000). However, the potential of betacellulin to induce proliferation of beta cells had not been demonstrated previously. Vitamin D, activin A, nicotinamide and sodium butyrate emerged as potential factors for beta cell differentiation. Effect of nicotinamide and sodium butyrate was additive, as has been shown to be the case in primary fetal porcine cell cultures as well (Korsgren et al., 1993; Otonkoski et al., 1999). Beta cell differentiation inducing effect of nicotinamide and activin A has also been demonstrated previously (Mashima et al., 1996a; Otonkoski et al., 1993). Overall, the results thus suggest that the INS-1 cell line is a useful model for studies of mechanisms regulating beta cell growth and differentiation.

Expression of erbB receptors and ligands in pancreas (I, II, III)

After identification of betacellulin, a member of the EGF-family, as a novel beta cell mitogen, our following aim was to study the expression profile of erbB receptors and ligands in the pancreas. Expression of EGFR has been demonstrated throughout the developing human pancreas (Miettinen and Heikinheimo, 1992), other erbBs are also expressed in the developing pancreas (Krahl et al., 2000). Also several of the erbB ligands are present during the pancreas development. TGF-α expression is abundant in the developing pancreas (Miettinen and Heikinheimo, 1992). Expression of both betacellulin and HB-EGF has been demonstrated in the ductal cells of the developing pancreas (Kaneto et al., 1997a; Miyagawa et al., 1999), and NRG4, a novel ligand of erbB4, is also abundantly expressed in the pancreas (Harari et al., 1999).

To study the expression of erbBs in insulinoma cell lines and in primary pancreatic tissue, mRNA was isolated and subjected to northern analysis. All known EGFR splice variants were expressed in both INS-1 and RINm5F cells, as well as in the adult rat pancreas. ErbB2 and erbB3 were expressed in the newborn mouse pancreas, in the adult rat pancreas, and in RINm5F cells, but not in INS-1 cells. Low level of erbB4 expression could be detected in the newborn mouse pancreas with

northern analysis, but not in INS-1 or RINm5F cells. As the erbB4 expression was further studied with immunohistochemical staining, it became evident that it is expressed in the ductal epithelial tissue of the developing pancreas, and in the glucagon producing alpha cells of the adult pancreas. A similar expression pattern was at the same time reported by others (Krahl et al., 2000).

Expression of erbB ligands was studied with RT-PCR and with northern analysis. A low level of endogenous betacellulin expression was detected in both RINm5F and INS-1 cell lines. Betacellulin was also expressed in the mouse pancreas throughout development. Also EGF, TGF-□, HB-EGF and NRG4 were present in the mouse pancreas at all studied stages (e12.5, newborn and adult).

In summary, expression of erbB receptors and many of their ligands was observed in the developing as well as in the adult pancreas, suggesting an important regulatory role for the EGF-family in pancreas development and function.

Functional EGFR is essential for normal pancreatic development (II)

Since EGFR is expressed throughout the developing pancreas and since the pancreas also develops through branching morphogenesis, we next wanted to study the role of EGFR in pancreas development *in vivo*. For this purpose mice lacking functional EGFR were used. Inactivation of EGFR leads to epithelial immaturity and to death within the first postnatal week of life. Epithelial development of EGFR (-/-) mice is impaired in several organs (e.g. skin, gastrointestinal tract), and organs developing via branching morphogenesis (e.g. lungs, mammary gland) are also affected (Miettinen et al., 1995; Miettinen et al., 1997; Siblia and Wagner, 1995; Threadgill et al., 1995). Analysis of EGFR (-/-) pancreases implicated impairment in islet cell migration and differentiation in the absence of EGFR. In EGFR (-/-) embryos, the appearance of insulin-producing beta cells was delayed, and also the number of glucagon-producing alpha cells was lowered. Though pancreases in the EGFR (-/-) embryos were significantly smaller than in the wild type littermates, the differences in blood glucose levels of the pups were insignificant, indicating that the early postnatal lethality was likely not a consequence of inadequate insulin supplies.

Islet morphogenesis is disturbed in EGFR (-/-) mice (II)

All four major islet cell types were found in the EGFR (-/-) pancreases. However, their organization as islets was disturbed. Whilst in the wild type pups the endocrine cells formed round pancreatic islets separated from the pancreatic ducts, in the EGFR (-/-) pups the endocrine cells tended to stay in streak-like structures associated with the ducts. In the newborn EGFR (-/-) pancreases over 60 %

of the beta cells were duct-associated, whereas in the wild type pancreases only approximately 35 % of the beta cells remained in contact with ducts.

To find out the reason for the impaired islet formation in EGFR (-/-) pancreases, the expression of both cell adhesion molecules, and ECM degrading proteases was studied. Cadherins are cell adhesion molecules expressed in the developing pancreas and implicated in the islet formation (Cirulli et al., 1994; Rouiller et al., 1991). Disruption of E-cadherin function leads to impaired formation of compact islets due to problems in cell-cell adhesion (Dahl et al., 1996). However, no difference was seen in the aggregation of individual islet cells to each other in mice lacking EGFR, and also the E-cadherin-like immunoreactivity remained normal, indicating that cell-cell adhesion within the islets was not affected in the absence of functional EGFR. Matrix metalloproteinases (MMPS) are involved in the remodeling of the extracellular matrix during the embryogenesis (Werb, 1997). MMPs are expressed in the pancreatic mesenchyme and it has been shown that activation of MMP-2 is required for proper islet morphogenesis (Miralles et al., 1998; Reponen et al., 1992). MMPs are upregulated by EGFR ligands (Kondapaka et al., 1997) and secretion of MMPs has been shown to be diminished in cultured mandibular processes from EGFR (-/-) embryos (Miettinen et al., 1999). Indeed, we found out that MMP-activity was also reduced in the EGFR (-/-) pancreases. Both the total gelatinolytic activity and the amount of active form of MMP-2 was reduced in late gestational EGFR (-/-) pancreases. Production of plasminogen activator inhibitor-1 (PAI-1), an activator of MMPs, was instead increased in the EGFR (-/-) pancreases. It thus appears that the reduced MMP-activity and the increased PAI-1 production are responsible for the impaired islet formation seen in the EGFR (-/-) pancreases.

Beta cell proliferation and number are reduced in EGFR (-/-) mice (II)

The overall size of the EGFR (-/-) pancreases was reduced as compared to the wild type. Also the development of beta cells was clearly delayed. Islet cell differentiation in the EGFR (-/-) embryos was further studied using a whole-mount organ culture system (see methods). After the culture period the EGFR (-/-) explants were clearly smaller and contained less ductal branches than the wild type explants (number of luminal cross-sections 514±190 versus 1675±358, $p<0.005$). Additionally, the proportion of insulin-producing beta cells was reduced significantly (27±6 % versus 48±8 %, $p<0.01$), whereas the proportion of alpha cells was increased. Proliferation of pancreatic beta cells was quantitated 2 hours after injection of the animals with BrdU. BrdU-labeling index of beta cells was reduced significantly both in the newborn (2.6±0.4 % versus 5.8±0.9%, $p<0.01$) and in the one week old (1.4±0.5 % versus 4.8±0.6 %, $p<0.01$) EGFR (-/-) pups. However, no difference could be detected in the number of apoptotic beta cells between the wild type and EGFR (-/-) pancreases. Based on these findings, it appears that the reduced number of beta cells in EGFR (-/-) mice is due to reduced proliferation and delayed differentiation.

ErbB mediated signaling favors development of beta and delta cells and inhibits development of alpha cells (III)

To further clarify the role of erbB-mediated signaling in pancreatic islet development, several erbB ligands were introduced to whole-mount pancreatic organ culture. Pancreatic explants were cultured for 5 days with EGF, HB-EGF, betacellulin, NRG4, or with plain medium. Addition of erbB ligands to explant culture did not change the ratio of exocrine to endocrine cells. However, several changes were observed within the endocrine cell population. Beta cell proportion was induced by EGF, HB-EGF, and most significantly, by betacellulin. Proportion and number of delta cells was increased by NRG4, a novel erbB4 ligand. Betacellulin was also able to induce delta cell number in the explant culture. Proportion of glucagon producing alpha cells instead was significantly reduced by addition of betacellulin, EGF, and NRG4. As a consequence an increase in beta to alpha cell ratio and delta to alpha cell ratio was detected.

Both EGFR and erbB4 are required for differentiation of islet cells (II, III)

We had already previously demonstrated that beta cell differentiation is diminished in the absence of functional EGFR (see above). We next wanted to block the signaling through erbB4 because it is known that both betacellulin and NRG4 bind to it (Harari et al., 1999; Riese II et al., 1996) and they were the most effective inducers of beta and delta cell differentiation in our explant model. ErbB4 was blocked indirectly, using soluble erbB4 receptor in the explant cultures. This soluble erbB4 binds its endogenous ligands as added to the culture system. Its addition to the explant culture for 5 days had no effect on beta to alpha cell ratio. The number of somatostatin producing delta cells instead was dramatically reduced.

We next wanted to see if betacellulin and NRG4 effects could be overcome by inhibiting their receptors. For this purpose betacellulin or NRG4 was added to the explant culture together with the soluble erbB4. The delta-cell inducing effect of NRG4 could be blocked with the soluble erbB4, whereas the beta-cell inducing effect of betacellulin could not be inhibited. EGFR is known to be the primary receptor for betacellulin (Riese II et al., 1996). We therefore next investigated whether the effect of betacellulin could be inhibited in the absence of EGFR. For this purpose betacellulin was added to explants of EGFR (-/-) pancreases. Indeed, in the EGFR (-/-) cultures, betacellulin failed to induce beta cell differentiation. These results demonstrate that betacellulin-induced differentiation of beta cells is not mediated via erbB4. Instead, it appears that EGFR is the primary receptor for betacellulin in the developing pancreas. Data obtained also further confirms that effects of NRG4 are mediated exclusively via erbB4.

We had previously shown that both betacellulin and NRG4 are expressed endogenously in e12.5 mouse pancreas (see above). Inhibitory antibodies to betacellulin and NRG4 were added to the explant culture to study the importance of endogenous betacellulin and NRG4 in islet cell differentiation. Neutralizing anti-BTC antibody did not induce any significant response in the islet cell differentiation in this model. NRG4 inhibitory antibody instead induced greater than 50 % reduction in the development of somatostatin-expressing delta cells. These results indicate that endogenous activity of betacellulin is not necessary for proper beta cell differentiation but could perhaps be overcome by other EGFR ligands. NRG4 instead appears to be an important regulator of delta cell differentiation.

Results obtained here demonstrate for the first time that EGF-family ligands can regulate the lineage determination of pancreatic endocrine precursor cells. Both betacellulin and EGF favor beta cell differentiation, NRG4 instead favors delta cell development. These results are in agreement with findings demonstrating that betacellulin promotes beta cell differentiation (Li et al., 2001; Mashima et al., 1996a). A functional role for NRG4 had not been demonstrated previously.

FGFR2b is required for proliferation and branching of the pancreatic ductal epithelium (IV)

Several FGFs and their receptors have been implicated in the pancreatic development (Le Bras et al., 1998; Miralles et al., 1999). Especially FGF10, a ligand for FGFR2b, has been shown to be important for growth and differentiation in the developing pancreas (Bhushan et al., 2001). Since FGFR2b and its ligands have been shown to have a regulatory role in the development of organs forming via branching morphogenesis (De Moerlooze et al., 2000; Sekine et al., 1999), and since we were able to show FGFR2b expression throughout the developing pancreatic epithelium, we next wanted to study the role of FGFR2b in the pancreas development. For this purpose mice lacking functional FGFR2b were used. FGFR2b (-/-) mice survive to term, but die at birth due to defective lung formation. FGFR2b (-/-) fetuses also lack limbs, eyelids, mammary glands and anterior pituitary. Development of the salivary gland and inner ear are also impaired (De Moerlooze et al., 2000; Mailleux et al., 2002; Pirvola et al., 2000).

FGFR2b (-/-) pancreases were greatly reduced in size, and both the proliferation and the number of CK positive ductal cells was diminished. At e12 - 13 there was over 50 % reduction in both the number of ductal cells (225±24 CK positive cells versus 566±94, $p<0.02$) and in their proliferation index (2.5±0.6 % versus 5.0±0.7 %, $p<0.04$). Also at e15.5 a similar reduction in the number of both CK positive cells (4373±430 versus 9602±2276, $p<0.08$) and in the number of luminal cross-sections (380±11 ductal lumens / mm^2 versus 626±32, $p<0.002$) was detected. These results clearly demonstrate that both pancreatic duct cell proliferation and epithelial branching are impaired in the absence of functional FGFR2b.

To further study the importance of FGFR2b-mediated signaling in the development of the pancreatic ductal tree, FGFR2b ligands were introduced to the pancreatic explant culture system. For this purpose wild type e12.5 pancreases were dissected as described (see methods) and cultured with FGF7 or FGF10 for 24 hours, and the proliferation index of CK positive cells was counted. The proportion of proliferating ductal cells was significantly increased after addition of either FGF7 (12.1±1.67 % versus 7.9±1.3 %, $p<0.05$) or FGF10 (11.9±1.96 % versus 7.9±1.3 %, $p<0.05$) as compared to the control. These results are in agreement with previous studies showing that in the absence of FGF10 pancreatic ductal branching is severely disturbed (Bhushan et al., 2001). Our results also confirm the previous finding showing that the increased amount of FGFR2b ligands induces pancreatic duct cell proliferation (Elghazi et al., 2002; Hart et al., 2001; Yi et al., 1994).

Pancreatic differentiation is not impaired in the absence of functional FGFR2b (IV)

Since FGFR2b (-/-) pancreases were clearly reduced in size and also had problems with ductal branching and proliferation, we next wanted to study their endocrine and exocrine differentiation. It had previously been suggested that FGFR2b is specifically required for the development of the exocrine pancreas (Miralles et al., 1999), and that its over-stimulation is inhibitory to endocrine pancreas differentiation (Elghazi et al., 2002). Previous studies have also demonstrated reduced expression of PDX1 and subsequent problems in the endocrine differentiation in mice carrying a null mutation for FGF10, a major ligand of FGFR2b (Bhushan et al., 2001). Based on these findings, we expected FGFR2b (-/-) mice to have problems in both endocrine and exocrine differentiation.

To study pancreatic differentiation, the level and expression pattern of several pancreatic transcription factor genes was studied by in situ hybridization and northern analysis. Expression of PDX1, p48, PAX6, ISL1 and NGN3 was detected in both mutant and wild type pancreases. The relative expression level of an endocrine transcription factor NGN3, an exocrine transcription factor p48, and a beta cell specific transcription factor NKX6.1 were all reduced by approximately 50 % in the FGFR2b (-/-) pancreases. This suggested that there is no major defect in the differentiation of endocrine or exocrine cell in the absence of FGFR2b. To confirm the results obtained from gene expression analyses, we next stained sections from e15.5 and e18.5 mutant pancreases for endocrine (insulin, glucagon, somatostatin and PP) and exocrine (amylase) markers. All these pancreatic proteins were present in FGFR2b (-/-) pancreases, further indicating that differentiation of pancreatic cell types does not require FGFR2b-mediated signaling. Finally, cell differentiation was studied using the explant culture of both FGFR2b deficient and wild type pancreases. After five days culture the total cell number in the FGFR2b (-/-) explants was significantly lower than in the wild type explants (2737±525 versus 4073±540, $p<0.05$). The number (992±182 versus 2381±236, $p<0.001$) and proportion (39±3.4 % versus 58 ± 3.7 %, $p<0.01$) of ductal cells was also clearly

reduced. The relative proportion of endocrine cells however, was not significantly reduced, though the beta cell number was lower than in wild type explants. The proportion of exocrine cells was even higher in FGFR2b (-/-) explants than wild type. Addition of FGF7 or FGF10 to the wild type explant cultures induced a small reduction in the number of endocrine cells. A similar effect of FGF7 has previously been demonstrated in rat embryonic pancreatic epithelial culture (Elghazi et al., 2002).

Taken together, the pancreatic phenotype of FGFR2b (-/-) mice indicates a crucial role for this receptor in the proliferation of pancreatic ductal epithelial cells, as well as in the proper branching of the pancreatic ductal tree. However, in contrast to previous findings (Miralles et al., 1999), we show that FGFR2b is not required for differentiation of the exocrine pancreas. Also endocrine differentiation can occur without functional FGFR2b, though proper amount of FGFR2b-mediated signaling appears to be necessary for balanced development of the islet cells.

Mice lacking functional FGF10, which is considered as a major ligand for FGFR2b, also develop a hypoplastic pancreas. Proliferation and branching of the pancreatic ductal epithelium is severely disturbed in these mice. However, in addition to the branching defect, also the expression of several pancreatic transcription factors is abolished and the endocrine differentiation is markedly reduced (see Table 3). It is thus likely that FGFR2b is not the only mediator of FGF10 effects, since the pancreatic phenotype of FGFR2b (-/-) mice is clearly less severe. FGFR1b, which also can bind FGF10 (Lu et al., 1999), is highly expressed in the developing pancreatic epithelium (Cras-Méneur and Scharfmann, 2002) and is thus the likely additional mediator of FGF10 effects (Figure 6).

Table 3. Comparison of FGFR2b (-/-) and FGF10 (-/-) pancreatic phenotypes

	FGFR2b (-/-)	FGF10 (-/-)
Pancreatic size	Reduced	Reduced
Ductal branching	Reduced	Reduced
Epithelial proliferation	Reduced	Reduced
Pdx1 expression	Normal	Reduced/Absent
Endocrine differentiation	Slightly reduced	Greatly reduced

SUMMARY AND CONCLUSIONS

Due to recent success in clinical islet transplantation as a treatment for type I diabetes (Shapiro et al., 2000), the search for new sources of transplantable insulin-producing cells has intensified. It has been suggested that development of insulin-producing cells can be induced from embryonic stem cells (Lumelsky et al., 2001; Soria et al., 2000), or from adult pancreatic ductal stem cells (Bonner-Weir et al., 2000; Ramiya et al., 2000). Knowledge of the factors controlling development of beta cell precursors and also the factors controlling differentiation of these precursors into mature beta cells is essential before engineering of stem cells for the production of beta cells will be possible. The present studies aimed to elucidate the role of some of the peptide growth factors involved in these processes.

On the basis of the results obtained from this thesis study the following conclusions can be drawn:

- Differentiated insulin-producing cell lines appear to be suitable models for screening of growth factors that regulate beta cell proliferation and differentiation. Betacellulin emerges as a potential novel growth factor for the differentiated beta cell.
- All erbB receptors as well as several of the EGF-family ligands are present in the developing pancreas. Signaling via EGF-receptor is essential for the migration of the developing pancreatic islet cells through the surrounding mesenchyme. Functional EGFR is also needed for the proper level of beta cell proliferation and differentiation. In the absence of EGFR signaling, the endocrine precursor cells prefer to differentiate toward the alpha cell phenotype, whereas increased amount of EGFR ligands EGF, HB-EGF and betacellulin shift the differentiation balance toward the beta cell phenotype, betacellulin being the strongest inducer in this respect. However, presence of betacellulin is not an absolute requirement for beta cell differentiation indicating that redundancy within the EGF-family is quite abundant. EGFR ligands are involved in the induction of beta cell differentiation from endocrine precursor cells.
- Although betacellulin has been shown to bind erbB4, the results obtained here indicate that its effects in the pancreas are mediated solely through EGFR. Further studies are needed to elucidate the pancreatic receptors and signaling pathways activated by betacellulin.
- NRG4 is an erbB4 ligand that is expressed predominantly in the pancreas. In the developing pancreas NRG4 supports differentiation of the somatostatin producing delta cells and shifts the differentiation balance of pancreatic endocrine precursors toward beta and delta cell lineages instead of the alpha cell lineage.

- ErbB4 is expressed abundantly in the epithelium of the developing pancreas. Its inactivation *in vitro* inhibits the development of delta cells. However, the effect of erbB4 inactivation in the developing pancreas *in vivo* has not been studied since embryos lacking functional erbB4 die already prior to pancreas organogenesis. Further studies are thus needed to determine the exact role of erbB4 in the developing pancreas.
- FGFR2b is expressed throughout the developing pancreas. Our results demonstrate that FGFR2b mediated signaling is essential for pancreatic ductal branching and proliferation. FGFR2b and its ligands seem to be important regulators of the pancreatic endocrine progenitor cell mass.
- Since EGFR, erbB4, FGFR2b and their ligands are important regulators of pancreatic endocrine progenitors, these factors could be used when producing insulin-producing cells from stem cells or tissue-specific progenitor cells.

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