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EPIDERMAL GROWTH FACTOR RECEPTOR IN PANCREATIC BETA CELL MASS REGULATION

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Academic Dissertation

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Immunofluorescence of a human pancreatic islet (left) and a mouse pancreatic islet (right): insulin (red), glucagon (green) and nuclei (blue).

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The arduous journey through the wilderness is made worthwhile by the moments, when the desert blooms, and for a fleeting moment one can believe in miracles.
Leena Palotie

(translated from "Erämaataipaleen tekevät vaivalloisen matkan arvoiseksi ne hetket, jolloin erämaa kukkii ja kiitävän hetken voi uskoa ihmeeseen." Leena Palotie)

TABLE OF CONTENTS

TABLE OF CONTENTS **4**

ABSTRACT **7**

LIST OF ORIGINAL PUBLICATIONS **9**

ABBREVIATIONS **10**

INTRODUCTION **12**

REVIEW OF THE LITERATURE **13**

1. Overview of the pancreas **13**

2. The formation and regulation of beta cell mass **13**

2.1 The embryonic formation of beta cell mass 13

2.2 Beta cell mass during neonatal period and adulthood 16

2.3 Gestational beta cell compensation 17

2.4 Obesity 20

2.5 Growth factors stimulating beta cell proliferation 21

2.6 Small molecules inducing beta cell proliferation 23

2.7 Signaling pathways activated by the mitogenic factors 24

2.8 Regulators of beta cell cycle progression 27

2.9 The role of survivin in beta cell proliferation 29

2.10 Pancreatic regeneration 29

2.11 Pancreatic plasticity 30

3. Diabetic beta cell loss **34**

3.1 Inflammatory cytokines in type 1 diabetes 34

3.2 Beta cell loss in type 2 diabetes 37

3.3 ER stress 38

3.4 Beta cell protection 39

4. Epidermal growth factor (EGF)- family and erbB-receptors **40**

4.1 ErbB ligands 41

4.2 ErbB receptors 41

4.3 ErbB receptors in cancer 44

4.4 ErbB receptor expression and signaling in pancreas 44

4.5 ErbB receptor ligands in pancreas 47

AIMS OF THE STUDY **50**

MATERIAL AND METHODS **51**

1. Animal work **51**
1.1 E1-DN mice (I, II) 51
1.2 INS-CA-EGFR mice (III) 51
1.3 Blood glucose and *in vivo* testing of glucose tolerance (I,III) 52
1.4 Islet isolation (I, II, III) 52
1.5 Partial duct ligation (I) 52
1.6 High-fat diet treatment (I) 52
1.7 Pregnancy experiment (I, II) 53
1.8 Streptozotocin experiments (III) 53
2. Human primary islet cell culture **53**
3. DNA extraction and genotyping (I-III) **54**
4. mRNA expression (I-III) **55**
5. Protein detection (I-III) **56**
5.1 Western blotting (III) 56
5.2 Immunohistochemistry (I-III) 57
5.3 Microscopy and scoring of results (I-III) 59
6. Optical projection tomography (OPT) (II-III) **60**
6.1 Organ preparation and OPT scanning 60
6.2 OPT sample reconstruction, visualization and quantification 60
7. Detection of proliferation and cell death (I-III) **60**
7.1 Proliferation (I-III) 60
7.2 Cell viability (III) 61
7.3 Apoptosis (I, III) 61
8. Statistical analysis (I-III) **62**
9. Ethical considerations (I-III) **62**
9.1 Animal experiments 62
9.2 Human islet experiments 62

RESULTS AND DISCUSSION **63**
1. EGFR expression in the mouse and the human pancreas **63**
2. EGFR in beta cell mass regulation (I-III) **64**
2.1 EGFR in beta cell mass regulation during normal physiology (III) 64
2.3 Beta cell mass regulation during obesity (I) 68
2.4 Beta cell mass regulation during pregnancy (I, II, unpublished data) 69
2.5 Beta cell mass regulation after PDL (I) 73
3. EGFR in beta cell protection **74**
3.1 Protection against diabetes, *in vivo* models (III) 74

3.2 Protection against cytokines, *in vitro* (III) 75
4. Limitation of the study...77

CONCLUSIONS AND FUTURE PERSPECTIVES 79

ACKNOWLEDGEMENTS 81

REFERENCES 84

ORIGINAL PUBLICATIONS 111

ABSTRACT

Pancreatic beta cells produce and secrete insulin into circulation in order to control blood glucose levels. The amount of beta cells is tightly controlled throughout life. If the number or function of beta cells is compromised, glucoregulation does not function properly, leading to dysglycemia. In the case of type 1 diabetes, beta cells are selectively targeted by an autoimmune attack, and die through apoptosis. Type 2 diabetes develops due to peripheral insulin resistance, combined with pancreatic beta cell failure. Individuals with type 2 diabetes show a reduced beta cell mass and increased beta cell apoptosis, leading to a relative lack of insulin. Treatments that replenish beta cell mass in diabetic patients could enable restoration of normal glycemic control and represent a potentially curative therapy. A better understanding of the factors that regulate or protect beta cell mass and function is important for the development of new treatments for all forms of diabetes.

The major aim of the present study was to elucidate the role of epidermal growth factor receptor (EGFR) in beta cell mass regulation and protection against diabetogenic insults. Two mouse models were generated for these studies: one with downregulated EGFR signaling in beta cells through expression of a dominant-negative EGFR mutation, and the other with increased EGFR signaling through expression of constitutively active EGFR in beta cells.

In the dominant-negative EGFR mouse model beta cell mass failed to expand during obesity and gestation, suggesting that EGFR signaling is crucial in beta cell mass regulation during these metabolic challenges. Subsequent mechanistic studies identified survivin as a candidate EGFR-regulated target mediating beta cell mass expansion during gestation. EGFR activation during the neonatal period led to increased beta cell proliferation and mass. In contrast, in adults EGFR activation had only a minor effect on beta cell proliferation and mass. Furthermore, EGFR activation protected beta cells against apoptosis *in vivo* during diabetogenic insults and *in vitro* when challenged with inflammatory cytokines. Mechanistic studies suggested that EGFR signaling protected isolated islets from cytokine-mediated beta cell death by repressing the proapoptotic protein Bim.

In conclusion, EGFR signaling appears to be important in beta cell mass expansion during metabolic challenges. The observation that EGFR stimulation protects against experimental

diabetes suggests that enhancing EGFR signaling could prevent or revert beta cell loss in diabetes.

LIST OF ORIGINAL PUBLICATIONS

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

I Hakonen E, Ustinov J, Mathijs I, Palgi J, Bouwens L, Miettinen PJ and Otonkoski T
Epidermal growth factor (EGF)-receptor signalling is needed for murine beta cell mass expansion in response to high-fat diet and pregnancy but not after pancreatic duct ligation. Diabetologia. 2011 Jul;54(7):1735–43.

II Hakonen E, Ustinov J, Palgi J, Miettinen PJ and Otonkoski T
EGFR signaling promotes β-cell proliferation and survivin expression during pregnancy. Plos One. 2014 9(4):e93651

III Hakonen E, Ustinov J, Eizirik D, Sariola H, Miettinen PJ and Otonkoski T
In vivo activation of the PI3K-Akt pathway in mouse beta cells by the *EGFR* mutation L858R protects against diabetes. Diabetologia. 2014 May;57(5):970-979

In addition some unpublished data are presented.

ABBREVIATIONS

AR	amphiregulin
Bad	BCL-2 associated agonist of cell death
Bim	BCL-2 like 11
Birc5	baculoviral IAP-repeat containing 5, also known as survivin
BrdU	bromodeoxyuridine
BTC	betacellulin
CA-EGFR	constitutively active EGFR
CDK	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
CNTF	ciliary neurotrophic factor
DNA	deoxyribonucleic acid
DPP-4	dipeptidyl peptidase-4
E1-DN	EGFR dominant negative
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPGN	epigen
EPR	epiregulin
ER	endoplasmic reticulum
Erk1/2	extracellular signal-regulated kinases 1 and 2
FFA	free fatty acid
FoxO1	forkhead box O1
FoxM1	forkhead box M1
GCK	Glucokinase
GD	Gestational day
GH	growth hormone
GIP	gastric inhibitory peptide, also known as glucose-dependent insulinotropic peptide
GLP	glucagon-like peptide
Glut2	glucose transporter 2
GSK3β	glycogen synthase kinase 3 beta
HB-EGF	heparin binding EGF
HGF	hepatocyte growth factor
Htr2B	serotonin receptor 2B

IFNγ	interferon gamma
IGF-1	insulin-like growth factor 1
IL-1β	interleukin 1 beta
iNOS	inducible nitric oxide synthase
INS-CA-EGFR	Mouse model with doxycycline-inducible expression of constitutively active EGFR in beta cells (INS-rtTA::TetOP-EGFR^{L858R})
IRS2	insulin receptor substrate 2
JAK2	janus kinase 2
KO	knockout
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
MEK1/2	mitogen-activated protein kinase 1/2
MLDS	multiple low dose streptozotocin
mTOR	mammalian target of rapamycin
Ngn3	neurogenin 3
NO	nitric oxide
NRG	neuregulin
OPT	optical projection tomography
PDGF	platelet derived growth factor
PDL	partial/pancreatic duct ligation
PI3K	phosphatidylinositol 3-kinase
PKCζ	protein kinase C zeta
PL	placental lactogen
PTEN	phosphatase and tensin homolog
Prl	prolactin
Prl-R	prolactin receptor
PUMA	Bcl-2 binding component 3
STAT	signal transducer and activation of transcription
STZ	streptozotocin
Tph	Tryptophan hydroxylase
T1D	type 1 diabetes mellitus
T2D	type 2 diabetes mellitus
TGF-α	transforming growth factor -α
TNF-α	tumor necrosis factor -α

INTRODUCTION

Pancreatic β-cells produce and secrete insulin into blood circulation in a tightly regulated manner. All forms of diabetes mellitus are characterized by the loss of functional beta cell mass, leading to insufficient insulin secretion and hyperglycemia. In the case of type 1 diabetes mellitus (T1D) beta cells are selectively targeted by an autoimmune attack and die through apoptosis, eventually leading to total insulin deficiency (Eizirik et al., 2009). There are currently approximately 40 million type 1 diabetics worldwide (Tuomilehto, 2013), and incidence is currently highest in Finland, with 64,3 cases per 100 000 persons in children younger than 15 years in 2011 (Harjutsalo et al., 2013). Current treatment of T1D is based on insulin injection therapy, which, while effective, does not provide the same degree of glycemic control as functional pancreatic beta cells, and does not fully prevent complications associated with the disease.

Modern lifestyle, with its abundant nutrient supply and reduced physical activity, has resulted in a dramatic increase in the incidence of type 2 diabetes mellitus (T2D). It is estimated that approximately 340 million people worldwide have T2D (Danaei et al., 2011). The underlying etiology consists of insulin resistance in muscles and liver, as well as pancreatic beta cell failure (Defronzo, 2009). Individuals with T2D show a reduced beta cell mass, and increased beta cell apoptosis (Butler et al., 2003). Furthermore, studies of obese people report an inverse relationship between blood glucose levels and beta cell volume (Ritzel et al., 2006), illustrating that dysregulation of the beta cell mass impairs insulin secretion capacity. Current therapies for T2D consist of metformin (inhibitor of hepatic gluconeogenesis) with or without insulin secretagogues (sulfonylureas, GLP-1 analogues and gliptins), insulin sensitizers (glitazones) or SGTL-2 inhibitors, or injectable insulin. These treatments rarely achieve the physiological glycemic control of a fully functional pancreas.

Treatments that replenish beta cell mass in diabetic patients could enable restoration of normal glycemic control and represent a potentially curative therapy. Better understanding of the factors that regulate or protect the mass and function of beta cells is thus important for the development of new treatments. EGFR is a tyrosine kinase receptor, that is abundantly present in pancreatic beta cells. It has mitogenic properties, thus making it a potentially crucial player in the regulation of beta cell mass. My thesis aims to elucidate the role of EGFR in beta cell mass regulation in normal physiological situations and during diabetogenic insults.

REVIEW OF THE LITERATURE

1. Overview of the pancreas

The pancreas is a mixed endocrine and exocrine organ that derives from the gut endoderm. The exocrine compartment, which consists of ductal and acinar cells, is responsible for the production and secretion of digestive enzymes (amylase, lipase, carboxypeptidase etc.). The endocrine compartment is responsible for the production of hormones controlling glucose homeostasis and comprises five different hormone-secreting cell types: glucagon-secreting alpha-cells, insulin-secreting beta cells, somatostatin-releasing delta-cells, ghrelin-producing epsilon-cells and pancreatic polypeptide-secreting PP-cells. The endocrine cells aggregate to form the islets of Langerhans, which consist of the endocrine cells listed above intermingled with blood vessels, neurons, and stroma. Endocrine cells comprise only one to two percent of the total pancreatic organ. The human pancreas contains about one million islets, while the mouse pancreas contains about one thousand islets (Alanentalo et al., 2006).

2. The formation and regulation of beta cell mass

2.1 The embryonic formation of beta cell mass

Morphologically evident development of the pancreas begins with the appearance of the dorsal pancreatic bud at the dorsal forgut endoderm, around the embryonic day 29 in humans (Jennings et al., 2013) and at embryonic day 9.5 in mice (Pan and Wright, 2011) (Figure 1). Shortly afterwards, at E10 in mouse embryo, the ventral pancreatic bud emerges to the ventral side of the forgut endoderm. The first wave of pancreatic development, referred to as the primary transition (E9.5-E12.5 in the mouse), includes active proliferation of the pancreatic progenitors to generate a stratified epithelium followed by pancreatic tubulogenesis (Figure 1). Individual cells acquire apicobasal polarity and then form rosettes around a nascent central lumen (Kesavan et al., 2009; Villasenor et al., 2010). Newly formed microlumens are initially unconnected but eventually fuse into a luminal plexus, which produces the tubular network (Kesavan et al., 2009; Villasenor et al., 2010). The first differentiated endocrine cells start to appear at this stage of development and all islet peptides can be detected (Jørgensen et al., 2007). Most of the early endocrine cells

synthesize glucagon; additionally, cells that produce both insulin and glucagon are present. Double-positive cells do not contribute to mature islets (Herrera, 2000), but some of the first-wave endocrine cells seem to become incorporated into mature islets (Gu et al., 2002). At E11.5, the gut tube begins to undergo its first coiling movements. That brings the dorsal and ventral buds closer together, and they then fuse at E12.5 (Jørgensen et al., 2007). By E12.5, the epithelium starts to undergo specification and patterning, segregating the epithelium into "tip" and "trunk" domains (Figure 1). The tip domains contain multipotential pancreatic cells that give rise both to endocrine and exocrine cells, while the adjacent trunk epithelial region consists of an endocrine-duct bipotential progenitor pool (Zhou et al., 2007). After the primary transition, tip progenitors lose their multipotency and become preacinar cells.

The second phase, referred to as the secondary transition, starts at E13.5 and consists of a massive differentiation wave. Acinar cells arise from the extending tip epithelium, while endocrine cells start to express neurogenin 3 (Ngn3) and delaminate from the trunk epithelium (Figure 1). $Ngn3^{+}$ endocrine precursors undergo dynamic changes in gene expression, resulting in activation of different transcription factors (e.g. Pax4, Arx, Rfx6, NeuroD, Pax6, Isl1) and hormones, forming all five endocrine cell types (Desgraz and Herrera, 2009; Gu et al., 2002). Transcription factors Pax4, Pdx1 and Nkx6.1 act as critical beta cell determinants (Collombat et al., 2003; Gannon et al., 2008; Henseleit et al., 2005; Sosa-Pineda et al., 1997; St-Onge et al., 1997), while Arx determines alpha cell identity (Collombat et al., 2003; 2005). At E16.5, endocrine cells start to coalesce and round up into mature islets. Trunk epithelial cells that do not activate Ngn3 eventually contribute to the ductal tree.

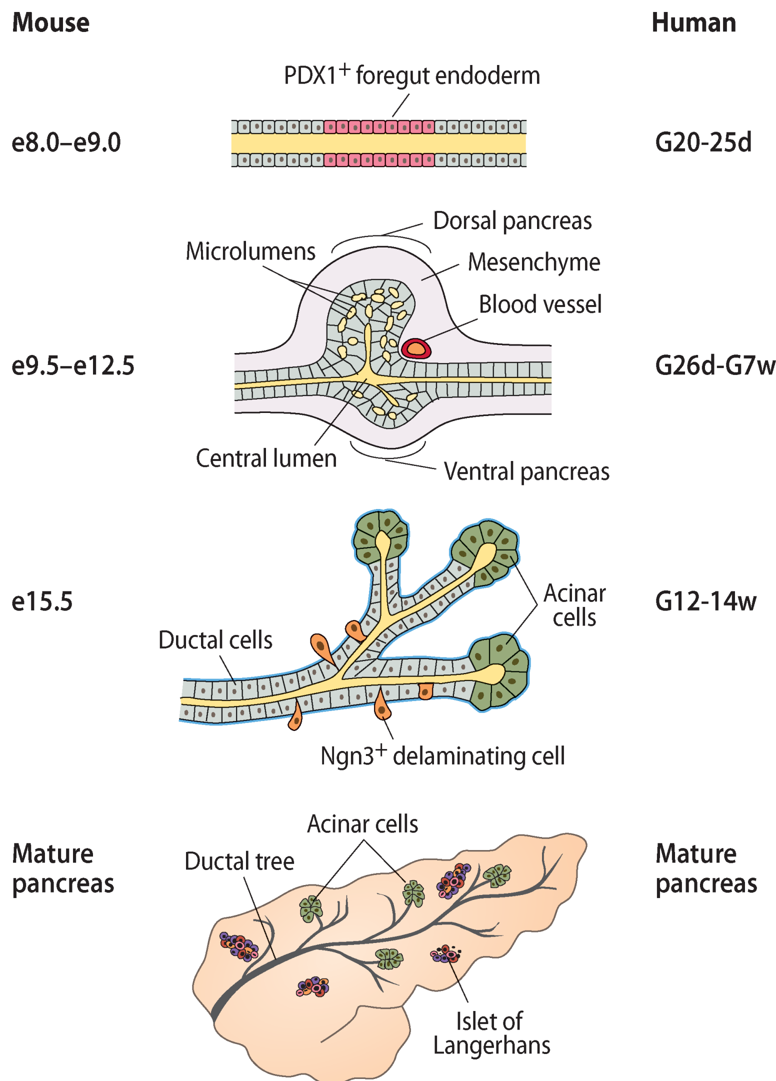

Figure 1 *Overview of pancreatic organogenesis. First a Pdx1⁺ region of gut tube epithelium becomes pancreas-specified. Soon after both the dorsal and ventral pancreatic buds evaginate into the surrounding mesenchyme. Shortly after, scattered microlumens arise between epithelial cells. The microlumens coalesce to form a primitive plexus, which progressively remodels to form single-layered epithelium consisting of branched ducts. Ngn3⁺ cells delaminate and migrate from the trunk epithelium and differentiate into hormone-producing cells. Acinar cells arise from the tip region and form exocrine secretory units at the end of the terminal ducts. The details in the figure are based on Pan and Wright, 2011; Shih et al., 2013.*

At birth, about 80% of the islet cell mass is generated by the proliferation and differentiation of endocrine progenitors, with the remaining 20% coming from proliferation of differentiated islet cells (Bouwens and Rooman, 2005).

2.2 Beta cell mass during neonatal period and adulthood

Both rodent and human fetal insulin-positive beta cells are immature, and have a reduced ability to synthesize and secrete insulin (Asplund et al., 1969; Bliss and Sharp, 1992; Jermendy et al., 2011; Otonkoski et al., 1988). Functional maturation starts at late gestation and continues during the first postnatal weeks. Especially after birth, the beta cells mature rapidly in order to maintain the glycemic level. Transcriptional regulators of beta-cell maturation include at least Pdx1, NeuroD and MafA, (Aguayo-Mazzucato et al., 2011).

In addition to maturation, during the neonatal period, beta cells replicate at a high rate in order to form the required beta cell mass, as has been shown in rodents (Finegood et al., 1995; Montanya et al., 2000). In general, during the first months of life, beta cell mass increases with body weight (Bonner-Weir, 2000; Montanya et al., 2000). In adulthood, when bodyweight remains stable, beta cells maintain a slowly renewing population, with steady low levels of proliferation (<0,5% divide per day) (Bouwens and Rooman, 2005; Teta et al., 2007).

Data from autopsy studies suggests that similar processes also occur in humans. There is a peak in beta cell proliferation during the neonatal period, and it fades after the age of two years (Gregg et al., 2012; Meier et al., 2008). With older age, beta cell proliferation further slows down, and beta cell mass may decline, both in rodents and humans (Gregg et al., 2012; Meier et al., 2008; Teta et al., 2005). The dynamics of beta cell mass at different age milestones is illustrated in Figure 2.

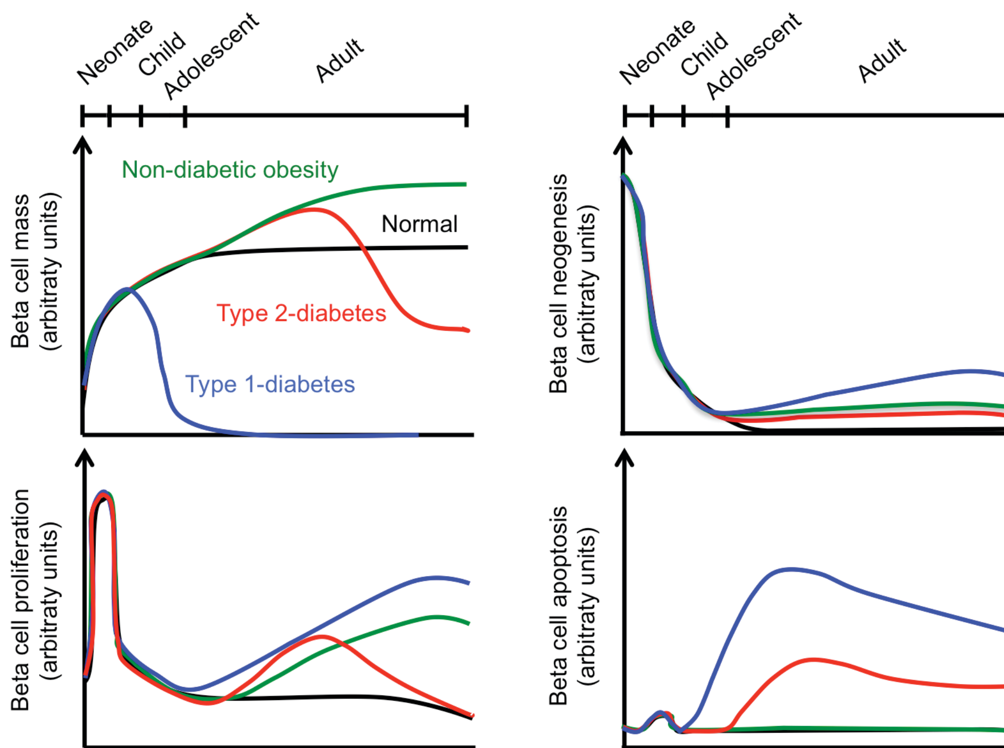

Figure 2 *Illustration of the current view regarding beta cell dynamics during human lifespan. Changes in beta cell mass are the sum of beta cell proliferation, neogenesis, and size, minus the degree of beta cell death. The details in the figure are based on the following references: Ackermann and Gannon, 2007 and Prentki and Nolan, 2006.*

However, because of physiological changes in insulin demand, due to conditions such as pregnancy, hyperglycemia, pancreatic injury, or peripheral insulin resistance, there is a compensatory increase in beta cells mass in both rodents and humans (Bernard et al., 1998; Butler et al., 2010; 2003; Karnik et al., 2007; Pan et al., 2013; Yi et al., 2013). Replication of pre-existing beta cells is the principal method for formation of new beta cells in adult rodents (Dor et al., 2004; Georgia and Bhushan, 2004). Additionally, beta cell neogenesis has been reported in models of pancreatic injury (Inada et al., 2008; Pan et al., 2013).

2.3 Gestational beta cell compensation

During pregnancy, the maternal beta cell mass expands to compensate for the increased insulin demand (Kim et al., 2010; Parsons et al., 1995). Pregnancy is probably the most robust physiological stimuli to induce beta cell mass expansion in the adult pancreas. In

pregnant rodents, beta cell proliferation increases dramatically, with a peak occurring after midgestation, and returns to postpartum levels after gestational day 18.5 (Parsons et al., 1992; Sorenson and Brelje, 1997). The main stimuli of beta cell proliferation during pregnancy are lactogens (placental lactogen and prolactin), although growth hormone (GH) has similar effects and is also elevated during pregnancy (Brelje et al., 2004; Huang et al., 2009). The peak in β-cell proliferation coincides with the peak of lactogen levels. Both placental lactogen (PL) and prolactin (Prl) bind to the same prolactin receptor (Prl-R). The most important mediator of lactogen signaling is the Janus kinase 2/signal transducer and the activator of transcription 5 (JAK2/STAT5) pathway (Brelje et al., 2002; Karnik et al., 2007). Upon ligand binding to Prl-R, JAK2 kinase is activated and STAT5 is phosphorylated. Phosphorylated STAT5 then dimerizes and translocates to the nucleus, where it regulates gene expression as a transcription factor (Karnik et al., 2007). In addition to the JAK2/STAT5 pathway, the Prl-R activates the phosphatidylinositol-3 kinase (PI3K)/Akt (also known as protein kinase B) pathway and the mitogen activated protein kinase (MAPK) pathway (Amaral et al., 2003; 2004).

Several studies have explored the gene expression changes that occur during pregnancy. Lactogens control β-cell mass expansion at multiple levels, including induction of the transcription factor *FoxM1* (Zhang et al., 2010), downregulation of *Menin* (Karnik et al., 2007), and upregulation of survivin (*Birc5*) (Rieck et al., 2009). Furthermore, Prl-R signaling leads to upregulation of serotonin-synthesizing enzymes *Tph1* and *Tph2* through the JAK2/STAT5 pathway (Schraenen et al., 2010). Consequently, the beta cells start to synthesize and secrete serotonin, which then acts in an autocrine/paracrine manner through Htr2B receptor to stimulate beta cell proliferation (Kim et al., 2010). Prl-R- and Htr2B-deficient mice fail to increase beta cell proliferation during pregnancy (Huang et al., 2009; Kim et al., 2010). Known mechanisms stimulating beta cell proliferation during pregnancy are illustrated in Figure 3.

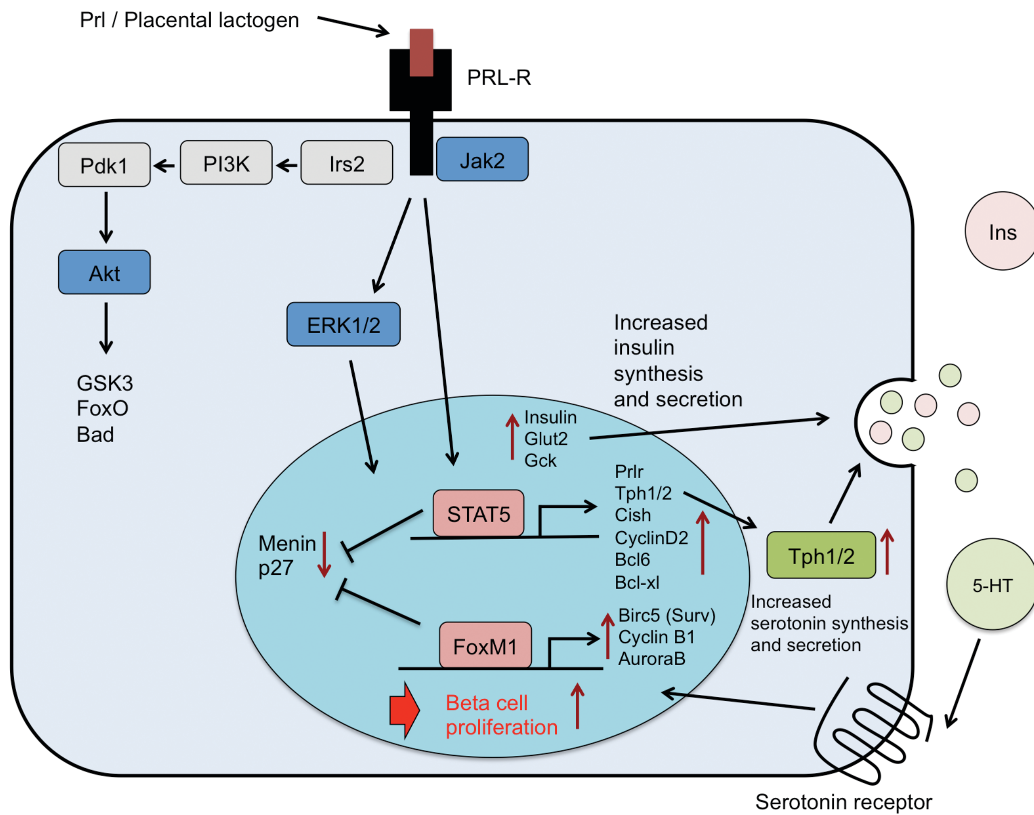

Figure 3 *Known mechanisms stimulating beta cell proliferation during pregnancy. Activation of PRLR upon binding of Prl/PL induces activation of JAK2/STAT5, PI3K and MAPK pathways. These lead to changes in gene expression, as illustrated. An upward-pointing arrow indicates that the expression is increased and a downward-pointing arrow indicates that expression is decreased. Increased expression of Thp1/2 leads to synthesis and secretion of serotonin, which then acts in a paracrine/autocrine manner to stimulate beta cell proliferation. The details in the figure are based on Rieck and Kaestner, 2010.*

Some studies report that, in addition to increased beta cell proliferation, the number of islets increases during pregnancy (Huang et al., 2009). Supporting this, a lineage-tracing experiment by Abouna et al. shows that some non-β-cells differentiate into insulin-producing cells during pregnancy, suggesting islet neogenesis (Abouna et al., 2010). Furthermore, autopsy studies of pregnant women have shown that there is an increase in the number of small pancreatic islets rather than increased β-cell proliferation (Butler et al., 2010).

After delivery, beta cell mass returns to normal levels within 10 days through increased beta cell apoptosis, decreased beta cell proliferation, and beta cell atrophy (Scaglia et al., 1995).

2.4 Obesity

Obesity is another well-studied physiological phenomenon that induces compensatory beta cell mass expansion. For example the db/db mice, which lack a functional leptin receptor, experience a twofold increase in beta cell mass by eight weeks of age (Wang and Brubaker, 2002). Ob/ob mice, which have a mutation in leptin itself, have a similar phenotype (Tomita et al., 1992). In a corresponding rat model, the Zucker fatty rat (fa/fa), which also has a mutation in the leptin receptor, there is increased beta cell mass and proliferation prior to the onset of T2D (Pick et al., 1998). In rodents, the compensatory beta cell mass expansion is probably based mostly on the proliferation of existing beta cells (Dor et al., 2004; Teta et al., 2007).

Similar compensatory events seem also to take place in humans. Obese non-diabetic humans have a higher beta cell mass than lean humans (Butler et al., 2003), and there is an inverse relationship between blood glucose levels and beta cell mass based on autopsy studies (data from T2D patients) (Ritzel et al., 2006). Additionally, insulin-resistant humans have higher beta cell mass than non-insulin-resistant humans, implying that the driving force for beta cell mass compensation could be insulin resistance (Mezza et al., 2013). Furthermore, transplantation of human islets to obsogenic environment in mice leads to doubling of the transplanted beta cell mass (Gargani et al., 2013). In humans, neogenesis has been suggested as the primary method of beta cell mass expansion (Butler et al., 2003), which is further supported by the discovery of increased numbers of CK19 and insulin double-positive cells, as well as insulin and glucagon double-positive cells, in insulin-resistant humans (Mezza et al., 2013).

The factors that induce the compensatory beta cell mass expansion during obesity are incompletely understood. Increased nutrient supply in the bloodstream is an important stimulus, especially glucose and free fatty acids (FFA) (Alonso et al., 2007; Bernard et al., 1998; Bonner-Weir et al., 1989; Hirose et al., 1996). Glucagon-like peptide (GLP)-1 production from the L-cells in the intestine might also potentiate beta cell mass expansion (van Citters et al., 2002). Additionally, there is convincing evidence that during insulin resistance, the liver secretes humoral factors that stimulate beta cell proliferation (Ouaamari et al., 2013); as well recently, betatrophin (also known as lipasin [Zhang, 2012], atypical angiopoetin-like 8 [Quagliarini et al., 2012] and re-feeding-induced fat and liver protein [Ren et al., 2012]) was shown to be such a factor (Yi et al., 2013). Overexpression of betatrophin

highly stimulates the proliferation of mouse beta cells *in vivo*, and it is secreted from the liver during insulin resistance. However, so far there is no evidence showing that betatrophin would increase the proliferation of human islets (Jiao et al., 2013).

2.5 Growth factors stimulating beta cell proliferation

Several studies show that systemic circulating factors can stimulate beta-cell proliferation (listed in Table 1). Glucose itself is a mitogen; the infusion of glucose in rodents causes an increase in beta cell proliferation (Alonso et al., 2007; Bernard et al., 1998; Bonner-Weir et al., 1989). Several hormones, including insulin (Paris et al., 2003), PL (Brelje et al., 1993; Vasavada et al., 2000), Prl (Brelje et al., 1993; Freemark et al., 2002), insulin-like growth factor (IGF)-1 (George et al., 2002; Hügl et al., 1998), GLP-1 (Xu et al., 1999), betacellulin (BTC) (Huotari et al., 1998; Oh et al., 2011), epidermal growth factor (EGF) (Huotari et al., 1998), gastric inhibitory peptide (GIP) (Renner et al., 2010), gastrin (Téllez et al., 2011), growth hormone (GH) (Brelje et al., 1993; Liu et al., 2004) and betatrophin (Yi et al., 2013) can stimulate rodent beta cell replication. Additionally, platelet-derived growth factor (PDGF) (Chen et al., 2011), hepatocyte growth factor (HGF) (Garcia-Ocana et al., 2000; Otonkoski et al., 1996), and serotonin (Kim et al., 2010) have been shown to stimulate beta cell proliferation. The major mitogenic signaling pathways that these factors activate are discussed in more detail below.

Table 1 *Summary of growth factors promoting beta cell proliferation and their in vivo and in vitro effects.*

Growth factors	*In vivo* effect	References	*In vitro* effect	References
Placental lactogen	RIP-mPL1: BCM and proliferation ↑	Vasavada et al 2000	Proliferation ↑	Brelje et al 1993
Prolactin	PRL-R$^{-/-}$: BCM ↓	Freemark et al 2002	Proliferation ↑	Brelje et al 1993
GH	GH$^{-/-}$: BCM and proliferation ↓	Liu et al 2004	Proliferation ↑	Brelje et al 1993
GLP-1	Exendin-4 treatment in vivo: BCM and proliferation ↑ GLP1-R$^{-/-}$: BCM No change	Xu et al 1999 Ling et al 2001 Scrocchi et al 1996	Exendin-4: Proliferation ↑	Song et al 2008
Betatrophin	Liver expression: BCM and proliferation ↑	Yi et al 2013	ND	
Gastrin	Gastrin treatment after PPx: BCM and proliferation ↑	Tellez et al 2011	Together with EGF increases BCM, differentiation? Proliferation ND	Suarez-Pinzon et al 2005a
EGF	EGF$^{-/-}$: BCM no change	Luetteke et al 1999	Proliferation of embryonic pancreatic epithelium ↑ Proliferation ↑	Cras-Meneur et al 2001 Huotari et al 1998
BTC	BTC$^{-/-}$: BCM no change	Jackson et al 2003	Proliferation ↑	Huotari et al 1998
HGF	RIP-HGF: BCM and proliferation ↑	Garcia-Ocana et al 2000	Proliferation ↑	Otonkoski et al 1996
IGF-1	RIP-IGF1: After MLDS BCM and proliferation ↑	George et al 2002	Proliferation ↑	Hugl et al 1998
PDGF	RIP-Cre x PDGFRa $^{fl/fl}$: Reduced BCM and mass	Chen et al 2011	PDGF-AA: Proliferation ↑	Chen et al 2011
Serotonin	Htr2B$^{-/-}$: Reduced BCM and proliferation during gestation	Kim et al 2010	Increased proliferation of β-cells	Kim et al 2010

BCM, beta cell mass; GH, growth hormone; GLP-1, glucagon-like peptide; EGF, epidermal growth factor; BTC, betacellulin; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; ND, not done; PPx, partial pancreatoctomy; MLDS, multiple low dose streptozotocin; PDGFRa, platelet-derived growth factor receptor a; Htr2B, serotonin receptor 2B.

GLP-1 receptor agonists (e.g. exendin-4 and liraglutide) have been shown to induce beta cell proliferation in rodents (Drucker and Nauck, 2006; Song et al., 2008; Xu et al., 1999). Surprisingly the GLP-1R knockout (KO) mice have only modestly impaired glucose homeostasis and normal beta cell mass (Ling et al., 2001; Scrocchi et al., 1996). Nevertheless, the GLP-1 analogues do promote beta cell proliferation in rodents (Drucker and Nauck, 2006; Song et al., 2008; Xu et al., 1999), potentiate glucose-stimulated insulin secretion (MacDonald et al., 2002), reduce beta cell apoptosis (Buteau et al., 2004; Farilla et al., 2002) and lower body weight by decreasing food intake.

GLP-1 receptor agonists and inhibitors of dipeptidyl peptidase-4 (DPP-4) also known as gliptins, which limit GLP-1 degradation, are now therapeutically used in T2D patients. Clinical trials with exenadine and liraglutide show reduction in fasting and postprandial glucose, associated with weight loss (Drucker, 2006). Gliptins have similar but milder effects on glycaemia (Drucker, 2006). Whether these drugs also induce beta cell proliferation in humans is unclear. *In vitro* studies with human islets have reported a modest increase in beta cell proliferation induced by liraglutide (from 0,017% to 0,082%) (Rutti et al., 2012). Furthermore, human islet-grafts transplanted into mice treated with alogliptin showed increased beta cell proliferation (Jurczyk et al., 2013). The first autopsy study showed that incretin therapy could increase beta-cell mass, but, alarmingly, the study subjects also had increased levels of exocrine dysplasia and alpha-cell hyperplasia (Butler et al., 2013). However, it has been argued that the diabetic controls were substantially mismatched in regard to age and duration of diabetes in this first autopsy study (Drucker, 2013). Additionally, there has been discussion and concern about the possibility of increased risk of pancreatitis and thyroid cancer in patients using GLP-1 agonists (Bjerre Knudsen et al., 2010; Drucker, 2013; Garber et al., 2009; Singh et al., 2013).

2.6 Small molecules inducing beta cell proliferation

High-throughput screens have aimed to find small molecules that induce beta cell proliferation. These screens have identified the adenosine pathway as a potent pathway to stimulate beta cell proliferation. Both the adenosine agonists and adenosine kinase inhibitors, which prevent the degradation of endogenous adenosine, have been shown to induce beta cell proliferation in rodents (Andersson et al., 2012; Annes et al., 2012).

Glucokinase (GCK) activators are also capable of inducing beta cell proliferation (Grimsby et al., 2003; Nakamura et al., 2009). Increased GCK activity has also been shown to stimulate human beta cell proliferation in patients with glucokinase-activating mutations (Kassem et al., 2010). Some glucokinase activators are currently in clinical trials for their antidiabetic potential (Zhang et al., 2013). In the first prolonged clinical trial, however, with the GCK activator MK-0941 was associated with increased risk of hypoglycemia and did not lead to sustained improvement of glycemic control (Meininger et al., 2011).

2.7 Signaling pathways activated by the mitogenic factors

There are several signaling pathways that promote beta cell proliferation in the rodent beta cell. Important mitogenic pathways include the PI3K/Akt, the PI3K/protein kinase C zeta (PKCζ), the MAPK, and the JAK/STAT pathways (Elghazi and Bernal-Mizrachi, 2009; Font de Mora et al., 2003; Karnik et al., 2007; Velázquez-Garcia et al., 2011). Multiple other signaling pathways also link growth factors to beta cell replication. Furthermore, all pathways crosstalk and intersect with other mitogenic pathways, making the actual situation more complex. An overview of these pathways is presented below and in Figure 4.

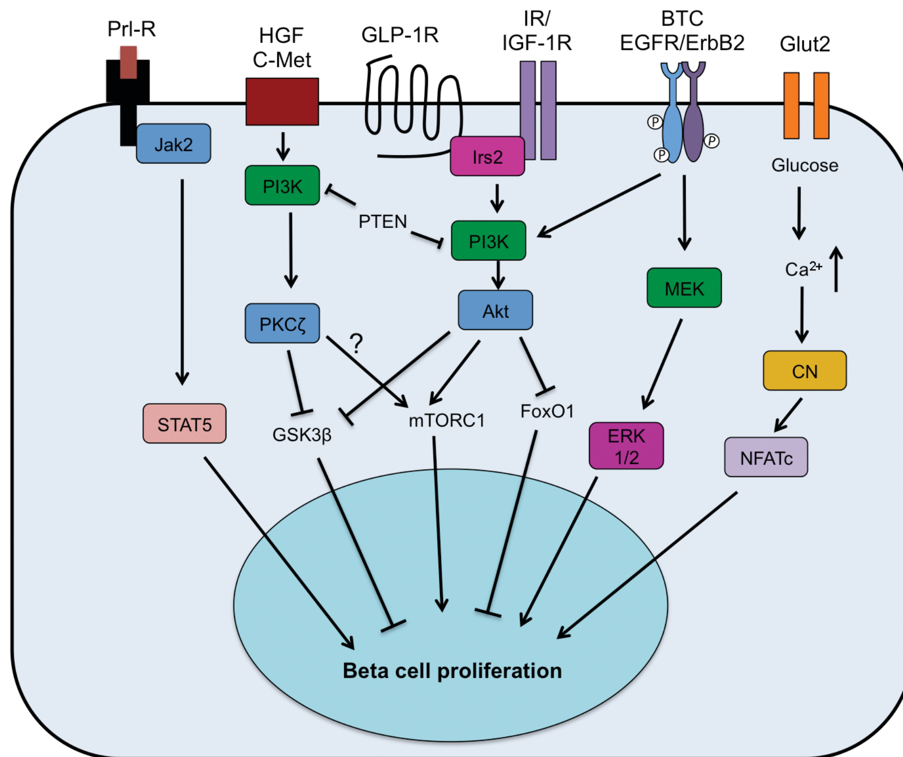

Figure 4 *Simplified overview of some of the multiple signaling pathways participating in beta cell replication. Also presented are some important hormones/growth factors/nutrients that activate these pathways. For abbreviations see pages 10-11.*

The PI3K pathway can be activated by many growth factors, including insulin, IGF-1, GLP-1, and HGF. Insulin, IGF-1, and GLP-1 receptor activation transmits signals by phosphorylating the insulin receptor substrate 2 (IRS2), which leads to activation of PI3K (Elghazi and Bernal-Mizrachi, 2009; Park et al., 2006). PI3K activation leads to phosphorylation of Akt, which in turn phosphorylates and inactivates GSK3beta and FoxO1. Additionally, Akt leads to activation of mammalian target of rapamycin (mTOR), which plays an important role in the regulation of beta cell mass. mTOR is part of two complexes, mTORC1 and mTORC2 (Blandino-Rosano et al., 2012). mTORC1 seems to play the more important role for beta cell proliferation. Inhibition of mTORC1 by rapamycin blocks beta cell proliferation induced by activation of Akt or by pregnancy (Balcazar et al., 2009; Zahr et al., 2008).

PI3K also activates the PKCζ, which can stimulate the proliferation of beta cells (Velázquez-Garcia et al., 2011). HGF, parathyroid hormone-related protein, and GLP-1 are all known to lead to PKCζ activation (Buteau et al., 2001; Vasavada et al., 2007b). PKCζ phosphorylates

and activates mTOR, leading to increased expression of cyclins (Velázquez-Garcia et al., 2011).

Additionally, the MAPK pathway is an important signaling cascade in beta cell proliferation. Many growth factor receptors, including platelet-derived growth factor receptor (Chen et al., 2011), insulin receptor (Siddle, 2011), and EGFR (Cras-Meneur et al., 2001), mediate their mitogenic signals via the MAPK pathway. MAPK pathway activation is initiated when the phosphorylated receptor activates Ras (a GTPase) to swap its GDP to GTP. Activated Ras can activate Raf, which then activates mitogen-activated protein kinase (MEK) leading to phosphorylation of extracellular signal-regulated kinase (Erk) 1/2. Erk1/2 phosphorylates multiple proteins, including transcription factors, leading to changes in transcription. The importance of this pathway in beta cells has been demonstrated by targeted disruption of Ras-guanine exchange factor GRF1, which leads to a reduction of beta cell proliferation and mass (Font de Mora et al., 2003).

The Janus kinase-signal transducer JAK2-STAT5 pathway stimulates beta cell proliferation during gestational beta cell compensation. Pregnancy-associated lactogens bind to the prolactin receptor and activate STAT5, leading to its nuclear entry (Amaral et al., 2003; Karnik et al., 2007). Phospho-STAT5 regulates the transcription of many cell cycle-related genes, including cyclin D2.

Glucose itself is a potent beta cell mitogen. This is probably mediated via increased cytosolic calcium concentration and the auto/paracrine effects of insulin secretion. Increased calcium concentration activates the calcineurin phosphatase, leading to dephosphorylation of the nuclear factor of activated T-cells (NFAT) transcription complexes (Goodyer et al., 2012). Consequently NFAT translocates to the nucleus, where it regulates expression of cell cycle related genes. Calcineurin inhibitors such as cyclosporine and tacrolimus, which are used as immunosuppressive agents in organ transplantation, have diabetogenic effects due to reduced insulin secretion (Kesiraju et al., 2014).

Finally, branched chain amino acids (leucine, isoleucine, and valine) have been shown to induce beta cell proliferation (Swenne, 1985), and this effect is probably mediated through the mTOR pathway (Xu et al., 1998).

2.8 Regulators of beta cell cycle progression

The actual cell-cycle progression is controlled by the expression and function of three major classes of proteins: cyclins, cyclin dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs). Most of our knowledge about these factors comes from rodent studies, but the cell cycle machinery of quiescent human beta cells differs significantly from that of rodent beta cells (Fiaschi-Taesch et al., 2013a).

In rodent beta cells, cyclins D1, D2, and D3 are all expressed, but in human beta cells only cyclins D1 and D3 are detectable (Fiaschi-Taesch et al., 2013a). Cyclin D1 is not required for beta cell development as cyclin D1 KO mice are viable and have normal islet number and size (Georgia and Bhushan, 2004; Kushner et al., 2005), yet ectopic expression of cyclin D1 in human and rodent beta cells increases beta cell proliferation (Cozar-Castellano et al., 2004; Zhang et al., 2005). Cyclin D2 is expressed at higher levels than D1, and is crucial for normal postnatal beta cell mass expansion in mice (Georgia and Bhushan, 2004; Kushner et al., 2005). Since human islets contain very little or no cyclin D2, this suggests either that cyclin D2 is not needed for human beta cells or that its absence is the reason why human beta cells do not proliferate (Fiaschi-Taesch et al., 2013a). Cyclin E is also expressed in the pancreatic islets of both mouse and human beta cells; however the role of this cyclin has not been described (Cozar-Castellano et al., 2006a; Fiaschi-Taesch et al., 2013a). CDK4 and CDK2 are expressed in the murine islets, while no CDK6 expression is detectable. In contrast, human beta cells also express CDK6 in addition to CDK2 and CDK4, these however are located in the cytoplasm instead of nucleus (Fiaschi-Taesch et al., 2013b).

CKI proteins inhibit beta cell proliferation. Of these Cip/Kip and INK4 (inhibitors of CDK4) members are expressed in the islets. All four INK4 proteins are expressed in beta cells: $p18^{Ink4c}$, $p15^{Ink4b}$, $p16^{Ink4a}$, and $p19^{Ink4d}$. Of these, the first two are important inhibitors of murine beta cell proliferation. For example, $p18^{Ink4c}$ KO mice have increased beta cell mass (Pei et al., 2004), and $p15^{Ink4b}$ /$p18^{Ink4c}$ double-KO mice develop pancreatic endocrine tumors (Latres et al., 2000). $p16^{Ink4a}$ plays an important role in inhibiting islet cell proliferation in an age-dependent way (Krishnamurthy et al., 2006). The expression of $p16^{Ink4a}$ increases with age, and $p16^{Ink4a}$-deficient old mice have increased beta cell proliferation. The expression of $p16^{Ink4a}$ increases as Ezh2 expression declines with age (Chen et al., 2009). The role of $p19^{Ink4d}$ has not been described.

All three members of the Cip/Kip protein family are expressed in the islets $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. $p27^{Kip1}$ is highly expressed in the murine islets, and represses beta cell proliferation (Cozar-Castellano et al., 2006b; Karnik et al., 2005; Uchida et al., 2005). Menin regulates the expression of $p27^{Kip1}$ by histone methylation. Furthermore, patients with Men1 mutations frequently develop insulinomas and nonfunctioning pancreatic neuroendocrine tumors (Gonçalves et al., 2014). In human beta cells, p57 and p21 are the only cell cycle molecules that are located in the nucleus, aside from pRb (Fiaschi-Taesch et al., 2013a). The involvement of p57 in suppression of human beta cell proliferation is further supported by focal hyperinsulism, a condition in which, due to loss of the maternal allele in chromosome 11, the expression of p57 is decreased and this is associated with increased beta cell proliferation (Kassem et al., 2001). Furthermore, knockdown of $p57^{Kip2}$ expression in human islets promotes beta cell replication when these islets are transplanted into mice (Avrahami et al., 2014).

In addition to the previously mentioned cyclins, various transcription factors participate in the regulation of beta cell proliferation. At G1, the cyclin-Cdk complexes activate E2F family transcription factors, which regulate expression of genes that promote S-phase initiation, including other cyclins and proteins involved in DNA synthesis and chromosome duplication. All seven E2F factors are expressed in the islets, and E2F1, -4, and -6 are the most abundant (Cozar-Castellano et al., 2006b; Fajas et al., 2004; Iglesias et al., 2004). The pocket protein family members retinoblastoma protein (Rb), p107, and p130 regulate the activity of E2F proteins. Cyclin-Cdk complexes phosphorylate pocket proteins, reducing their binding to E2F. However, the deletion of Rb in beta cells had only a minor effect on beta cell proliferation (Vasavada et al., 2007a). Additionally, the deletion of p107 or p130 alone did not affect beta cell replication or function (Harb et al., 2009). Functional redundancy has been reported between the Rb-related members of the pocket protein family, and, supporting this, the combined loss of pRb and p130 in beta cells results in increased beta cell proliferation and apoptosis (Harb et al., 2009). Finally, p53 represses beta cell proliferation *in vivo*, and Rb and p53 double-KO mice frequently develop insulinomas and other islet tumors (Harvey et al., 1995; Williams et al., 1994). The lack of p53 alone, however, does not lead to insulinoma formation, even though it leads to formation of numerous other tumors (Donehower et al., 1992), demonstrating the complexity of the interplay between the various cell cycle molecules in beta cells.

2.9 The role of survivin in beta cell proliferation

Survivin is a member of the chromosomal passenger protein complex, and has been shown to play an important role in the regulation of beta cell proliferation. Survivin is transiently expressed in mouse pancreatic islets during embryonic and perinatal periods, but disappears from the adult pancreas (Jiang et al., 2008; Wu et al., 2009). However, it is upregulated during pancreatic regeneration and pregnancy (Rieck et al., 2009; Wu et al., 2012). Mice with beta cell-specific survivin deletion develop insulin-deficient diabetes after birth due to a failure of beta cell mass expansion (Jiang et al., 2008; Wu et al., 2009). In addition to being a mitotic regulator, survivin also has antiapoptotic properties. Overexpression of survivin under the insulin promoter protected beta cells from staurosporine-induced cell death *in vitro,* and improved islet graft survival (Dohi et al., 2006). Interestingly, EGFR signaling has been linked to survivin in many cancer studies (Kanwar et al., 2011), and in beta cells, the EGFR-MAPK pathway has been shown to regulate survivin stability (Wang et al., 2010), thus it is a potentially important EGFR regulated molecule in beta cells.

2.10 Pancreatic regeneration

The pancreas of rodents is able to regenerate, at least to some extent, if the majority of beta cells is lost due to partial pancreatectomy, inflammation or toxins (Bonner-Weir et al., 1983; Nir et al., 2007; Van De Casteele et al., 2013; Wang et al., 1995; Xu et al., 2008). After a partial pancreatectomy, there is active regeneration in the remaining part of the organ (Bonner-Weir et al., 1983; Hayashi et al., 2003) which is further increased with gastrin treatment (Téllez et al., 2011). Pancreatic/partial duct ligation (PDL) is a classic model of pancreatic regeneration. In PDL, the pancreatic duct is ligated, which causes degeneration of acinar cells in the ligated part of the pancreas and leads to doubling of beta cell mass via the formation of $Ngn3^{+}$ embryonic-like progenitor cells in the ducts (Van De Casteele et al., 2013; Wang et al., 1995; Xu et al., 2008). However, there are conflicting reports that find no increase in beta cell mass after PDL (Kopp et al., 2011; Rankin et al., 2013). A third example of pancreatic regeneration is toxin-induced diabetes. If nearly all beta cells are lost, the pancreas is able to regenerate new beta cells (Nir et al., 2007; Thorel et al., 2010). The source of new beta cells during pancreatic regeneration in all of these models has long been debated, and is discussed in more detail below.

2.11 Pancreatic plasticity

One possible method to generate new beta cells *in vivo*, in addition to the proliferation of pre-existing beta cells, is transdifferentiation from other cell types within the pancreas. This has led to a search for strategies to transdifferentiate pancreatic progenitor or adult cells into insulin-producing cells. These studies have revealed plasticity of adult cells present in the pancreatic epithelium (e.g. acinar, duct and alpha-cells) to beta cells. Possible sources of new beta cells are illustrated in Figure 5.

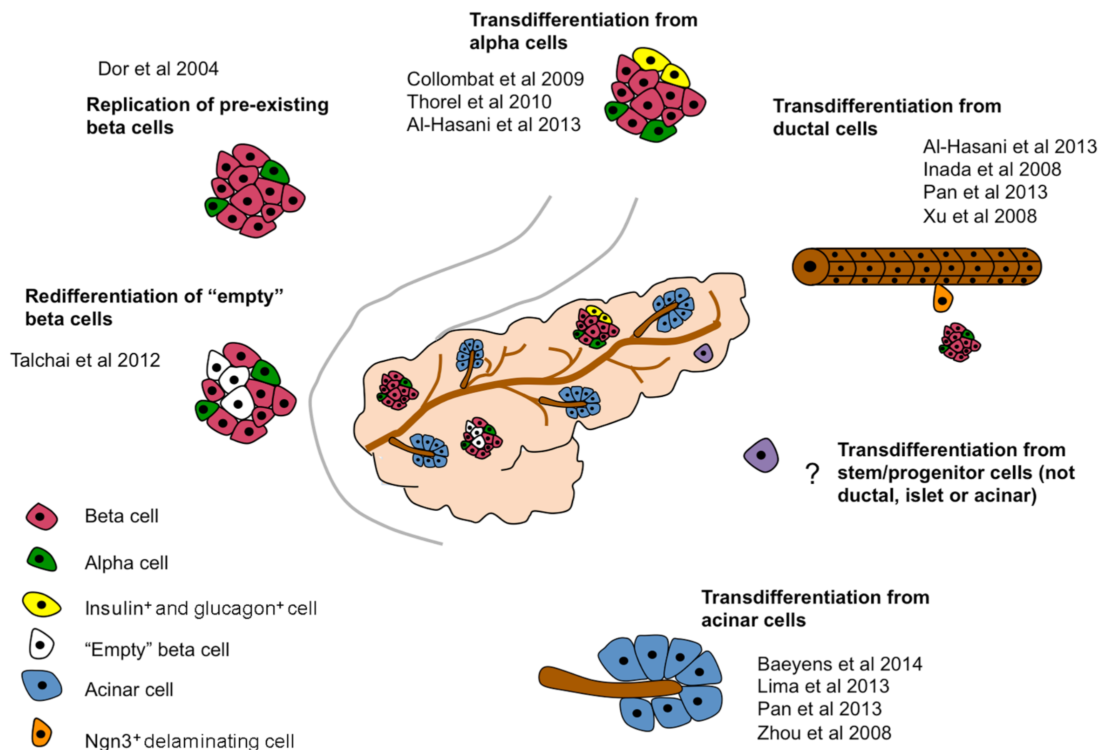

***Figure 5** Possible sources of new insulin-producing cells from the adult pancreas. Summary of the several processes that have been described and proposed. Adapted from Bonner-Weir and Weir, 2005.*

Duct cells

The capacity of duct cells to give rise to new beta cells in the adult mouse is controversial, since contradictory results have been reported. Some Cre-LoxP based lineage-tracing studies found no endocrine cells marked for the duct lineage after birth or injury (Furuyama et al., 2011; Kopp et al., 2011; Solar et al., 2009), while other studies did (Inada et al., 2008; Pan et al., 2013). There are several explanations for the controversial lineage tracing findings (Lysy

et al., 2013). These include at least the heterogeneity between the ductal cells and the long duration of action of tamoxifen, which activates the estrogen receptor-responsive transgenes (Reinert et al., 2012).

The existence of beta cell progenitors in the duct epithelium is supported in the study by Xu et al. (Xu et al., 2008), in which they show that PDL induced injury can activate beta cell progenitors in the ductal lining of adult mouse pancreas. Three days after PDL some of the cytokeratin positive ductal cells started to express Ngn3, and seven days after PDL, Ngn3 lineage-traced cells were double-positive with islet hormones, implying endocrine differentiation of the $Ngn3^+$ positive cells. When these $Ngn3^+$ cells were sorted out seven days after PDL, they could differentiate into glucose-responsive beta-like cells in embryonic pancreas explant cultures *in vitro*.

Furthermore, a recent study from Collombat's group shows that duct cells can transdifferentiate into alpha cells and consequently into beta cells (Al-Hasani et al., 2013). In this study, Pax4 was overexpressed in alpha cells, turning them into beta cells. The glucagon shortage recruited duct-lining precursor cells that transiently express Ngn3 and successively adopt a $glucagon^+$ and a beta-like cell identity. This could repeatedly regenerate the whole beta cell mass, and reverse several rounds of toxin-induced diabetes.

In the human pancreas, the frequency of insulin-positive cells within the duct is about 0,5% in healthy, normal-weight individuals (Reers et al., 2009) and it increases up to 1,5% during beta cell compensation, e.g. during insulin resistance (Mezza et al., 2013) and pregnancy (Butler et al., 2010), suggestive of duct-to-beta cell transdifferentiation.

Acinar cells

Acinar cells can be reprogrammed to beta cells *in vivo* by an adenoviral-mediated over-expression of key beta cell transcription factors Pdx1, Ngn3, and MafA (Zhou et al., 2008). One month after adenoviral injection, more than 20% of the acinar cells overexpressing these transcription factors, had transdifferentiated to insulin-positive beta-like cells. In diabetic streptozotocin-treated animals, this led to a significant reduction of hyperglycemia. Another study, in which the same transcription factors were ectopically expressed in a doxycycline-dependent manner, failed to reproduce the transdifferentiation of exocrine

cells, but reported transdifferentation of intestinal cells to insulin-producing cells (Chen et al., 2014).

Without genetic modification, many lineage-tracing studies have been negative for transdifferentiation of acinar cells into beta cells *in vivo*. A lineage-tracing study with elastase I promoter showed no transdifferentiation from acinar cells into insulin-positive beta cells during injury provoked by a 70% pancreatectomy, PDL, or caerulin-induced pancreatitis (Desai et al., 2007). However, two recent studies have shown that acinar cells can be reprogrammed into insulin-producing cells *in vivo* (Baeyens et al., 2014; Pan et al., 2013). The first study by Pan et al showed that Ptf1a-labeled acinar cells contributed to beta cell regeneration following PDL. After PDL, some of the acinar cells acquired the molecular features of multipotent progenitor cells, and gave rise to both acinar and ductal cells that occasionally started to express Ngn3 and became hormone-producing cells, similar to the process observed during embryonic development. In the study by Baeyens et al. (Baeyens et al., 2014), the transient administration of EGF and ciliary neurotrophic factor (CNTF) to adult mice with chronic alloxan-induced hyperglycemia led to the transdifferentiation of acinar cells into insulin-producing cells. This transdifferentiation was dependent on STAT3 signaling and was mediated through transient Ngn3 expression in acinar cells (Baeyens et al., 2014).

The *in vitro* transdifferentiation of acinar cells into beta-like-cells has been reported with both mouse and human cells (Lima et al., 2013; Minami et al., 2005). In a study by Minami et al (Minami et al., 2005) mouse acinar cells were cultured in suspension culture with EGF and nicotinamide. While the frequency of insulin-positive cells was only 0,01% in the initial preparation, it increased to approximately five percent after five days in these culture conditions. Human exocrine cells can be transdifferentiated into insulin-producing cells *in vitro* upon transduction of adenoviruses containing Pdx1, Ngn3, MafA, and Pax4 (Lima et al., 2013). The reprogramming can be further enhanced by suppressing epithelial-mesenchymal transition via inhibitors of Rho-associated kinase and transforming growth factor-β1 (Lima et al., 2013).

Centroacinar cells

Centroacinar cells are cells residing in the pancreatic epithelium at the junction of the terminal duct and acini. The function of these cells is largely unknown. They proliferate following partial pancreatectomy and are thus suggested to play an important role in

pancreatic regeneration following injury (Hayashi et al., 2003). When they are isolated, they form *in vitro* self-renewing "pancreatospheres" that contain capacity to contribute to both endocrine and exocrine lineages (Rovira et al., 2010). Whether differentiation of endocrine cells from centroacinar cells occurs *in vivo* is unknown.

Alpha cells

Pax4 overexpression in the glucagon-positive alpha cells has been shown to turn them into functional beta cells (Collombat et al., 2009). Furthermore, the following shortage of glucagon is reversed by mobilizing duct-lining precursors, which transiently express Ngn3 and start to express glucagon before transforming into insulin-positive beta-like cells (Al-Hasani et al., 2013). Alpha cells can also convert into insulin-producing cells spontaneously after an extreme beta cell loss (Thorel et al., 2010). However, the efficiency of this process seems to be much lower than what can be detected after forced overexpression of Pax4 in alpha cells. Whether alpha-to-beta cell transdifferentiation occurs in humans is unclear. *In vitro* it has been shown that human beta cells can undergo a conversion into glucagon-producing alpha cells (Spijker et al., 2013). Furthermore, treatment of human islets with methyltransferase inhibitor induces bihormonal cells, suggestive of alpha-to-beta cell transdifferentiation *in vitro* (Bramswig et al., 2013).

Dedifferentiation

In addition to transdifferentiation, cell plasticity has been shown to occur also in the other direction, i.e. beta cells can dedifferentiate into "empty" chromogranin A^{+}/hormone^{-} cells (Sherry et al., 2006; Talchai et al., 2012). FoxO1 appears to be important in maintaining the mature beta cell phenotype: in mice with beta cell specific ablation of FoxO1, the beta cells lose their expression of key beta cell genes such as insulin, glucokinase, and Glut2. Furthermore, during persistent hyperglycemia, e.g. in *db/db* mice, FoxO1 expression decreases, leading to a loss of insulin and mature beta cell markers (Talchai et al., 2012). Beta cell dedifferentiation has also been reported in NOD mice: early in the autoimmune process some beta cells lose insulin expression and escape from autoimmune destruction (Sherry et al., 2006). It is unclear whether beta cell dedifferentiation also occurs in humans, and, if so, whether it has a role in the pathogenesis of diabetes.

3. Diabetic beta cell loss

3.1 Inflammatory cytokines in type 1 diabetes

In T1D, beta cells are selectively targeted by autoimmune attack, and they die through apoptosis. Factors triggering the autoimmune process are unknown, but both environmental and genetic factors play a role (Concannon et al., 2009). Among the environmental factors infectious diseases (mostly enteroviruses), gut microbiome, early exposure to cow's milk, and low vitamin D levels have been especially implicated (Atkinson, 2012). When the autoimmune process is initiated, macrophages and T-lymphocytes are important players in beta cell destruction. Macrophages can induce target cell apoptosis via the synthesis of proinflammatory cytokines interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNFα), as well as via nitric oxide (NO) and other free radicals (Eizirik and Mandrup-Poulsen, 2001; Kolb-Bachofen and Kolb, 1989). Activated T-cells produce apoptosis-inducing cytokines, such as TNFα, lymphotoxin, and interferon gamma (IFNγ). The CD8+ cytotoxic T lymphocytes probably play the most important role of T-lymphocytes. CD8+ T lymphocytes are the predominant infiltrating cells in the pancreases of T1D patients (Bottazzo et al., 1985), and NOD mice that lack functional CD8+ T cells do not develop insulitis or diabetes (Serreze et al., 1994; Sumida et al., 1994). In addition to secreting cytokines, T-cells also express molecules that can directly induce target-cell apoptosis, such as the Fas ligand and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

The proinflammatory cytokines IL-1β, TNF-α and IFN-γ are direct mediators of beta cell death, and all activate the intrinsic (mitochondrial) pathway of apoptosis (Eizirik and Mandrup-Poulsen, 2001; Gurzov and Eizirik, 2011; Thomas et al., 2009). While these factors separately do not have a major cytotoxic effect, their combinations do (Arnush et al., 1998; Mandrup-Poulsen, 1996; Thomas et al., 2002). *In vitro* exposure of human or rodent islets to IL-1β and IFNγ induces expression changes in approximately 700 genes as shown by microarray analysis (Cardozo et al., 2001a; 2001b; Kutlu et al., 2003; Rasschaert et al., 2003). The transcription factors NF-κB and STAT1 are central mediators of these changes (Cardozo et al., 2001a; Rasschaert et al., 2003). IL-1β induces activation of NF-κB in rodent and human cells (Eizirik and Mandrup-Poulsen, 2001). NF-κB regulates the expression of inducible nitric oxide synthase (iNOS) resulting in the production of NO in beta cells (Darville and Eizirik, 1998; Kutlu et al., 2003). Free radical NO is toxic to rodent beta cells and its production leads to apoptosis. Human islets are more resistant to NO and the apoptosis after cytokine

exposure is independent of NO generation (Eizirik and Mandrup-Poulsen, 2001; Rabinovitch et al., 1994). IFNγ activates STAT1, and its signaling leads to expression of proapoptotic genes such as Bim (Barthson et al., 2011). In addition to inducing changes in gene expression, cytokines can lead to alternative splicing of target genes (Nogueira et al., 2013; Ortis et al., 2010). Important gene expression changes are summarized in Figure 6.

NO formation leads to endoplasmic reticulum (ER) stress by decreasing the expression of SERCA (sarco/endoplasmic reticulum $Ca2^{+}$ ATPase), depleting ER $Ca2^{+}$ stores. Because $Ca2^{+}$ is required for the protein binding and chaperoning ability of ER chaperones, severe ER $Ca2^{+}$ depletion will impair the quantity of ER protein folding and lead to unfolded protein response (Cardozo et al., 2005). Prolonged and severe ER stress leads to beta cell apoptosis (Eizirik and Cnop, 2010) (see 3.3).

Classically, it is thought that the first symptoms of T1D occur only once over 80-90% of the beta cells have been destroyed (Eisenbarth, 1986). However, some studies suggest that 40-50% of beta cells might be present at the onset of hyperglycemia (Akirav et al., 2008). Nevertheless, there is a marked gap between the onset of autoimmunity and the onset of diabetes.

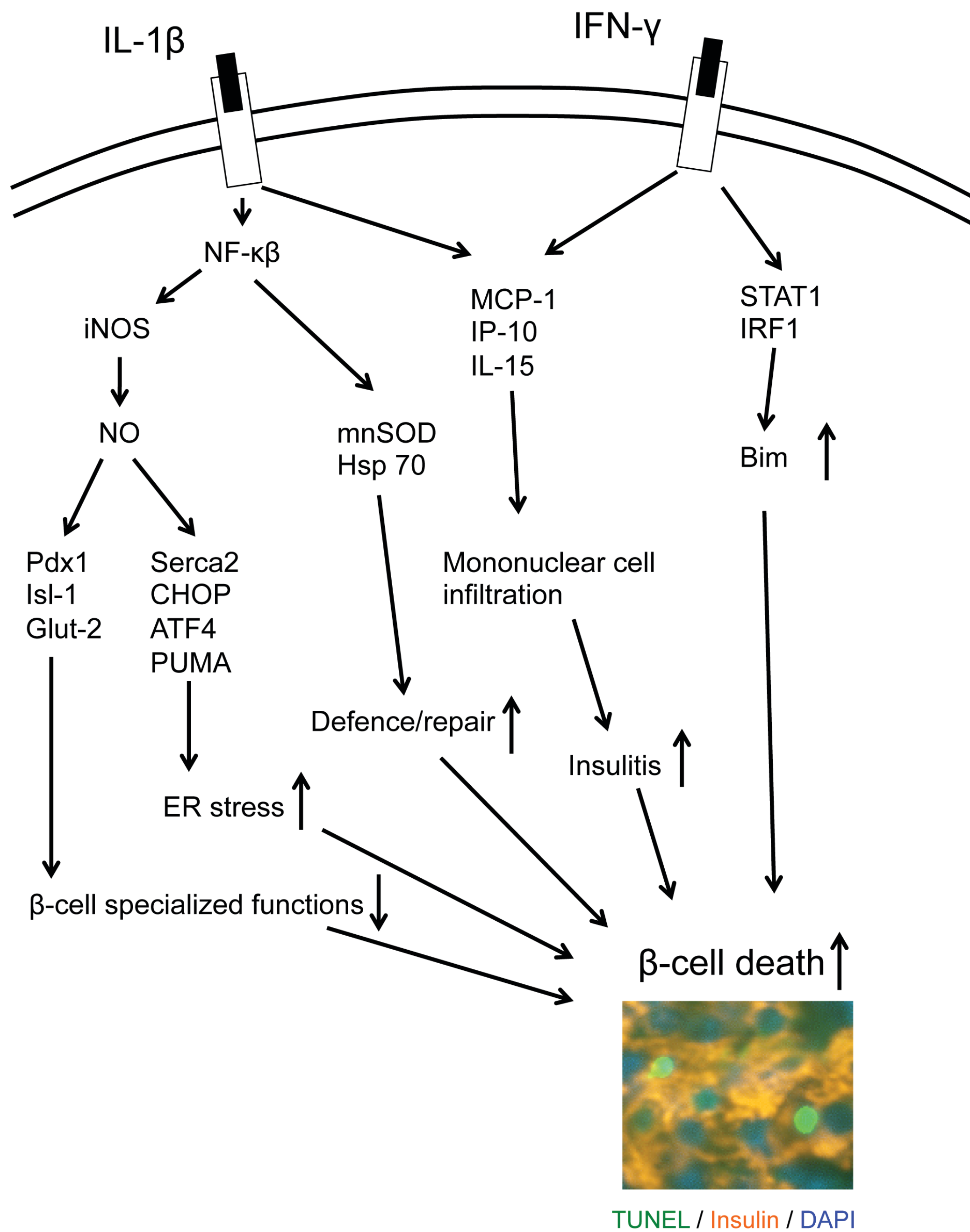

Figure 6 *A simplified model of the most important known gene networks participating in cytokine-induced beta cell death. The most important transcription factors in this process are NF-κB, which is activated by IL-1β, and STAT1, which is activated by IFN-γ. The details in the figure are based on the reference Cnop et al., 2005.*

3.2 Beta cell loss in type 2 diabetes

The factors affecting the predisposition to T2D are not well understood. Only every third obese individual develops T2D. Genetic factors definitely play a major role, but environmental factors also contribute to the pathogenesis of T2D pathogenesis (Velloso et al., 2013). T2D-related polymorphism has been detected within numerous genes, and these genes relate to different tissues and functions, among them adipocyte, hepatic and skeletal muscle metabolism, insulin action and pancreatic islet function (Ahlqvist et al., 2011). Intrauterine environmental factors also affect the risk of developing T2D: low birth weight and intrauterine growth retardation are associated with increased risk of T2D (Hales and Barker, 2001), as is maternal hyperglycemia in diabetic pregnancy (Dabelea et al., 2000). The effect of intrauterine environmental factors on T2D predisposition is likely due to epigenetic modifications (Simmons, 2007). However, obesity is by far the most important predisposing factor to T2D, and the consumption of energy-dense and fat-rich diets is the main environmental factor related to obesity (Prentki and Nolan, 2006; Schulz et al., 2002).

Both functional defects and a decreased beta cell mass are considered to contribute to the beta cell failure in T2D. The loss of beta cell mass might be induced by insufficient beta cell replication, dedifferentiation, or beta cell apoptosis. Probably all of these factors contribute to the loss of beta cell mass detected in T2D patients. There is evidence of beta cell apoptosis in T2D from both mouse models and human autopsy samples. Furthermore, both obese and lean T2D patients have apoptotic beta cells (Butler et al., 2003). The primary cause of this apoptosis is not clear; hyperglycemia, saturated fatty acids, islet amyloid polypeptide and IL-1β have all been implicated.

Short-term hyperglycemia (30 mmol/l glucose for 2 hours) induces ER stress, as shown by upregulation of unfolded protein response genes (Elouil et al., 2007), and prolonged hyperglycemia (five days with 16.5 mM glucose) results in the induction of proapoptotic genes and subsequently increased apoptosis (Federici et al., 2001). Additionally, it has been suggested that hyperglycemia induces the production of IL-1β by beta cells and intra-islet macrophages, causing subsequent NF-κB activation and apoptosis (Maedler et al., 2002).

Saturated fatty acids, such as palmitate, induce apoptosis and activation of the ER stress pathway (Cnop et al., 2001; Kharroubi et al., 2004). It is conceivable that a high FFA load that exceeds the beta cells' esterification capacity impairs ER functions and triggers an ER stress

response. ER stress leads to the activation of PERK and IRE1a as well as ATF6 and Bip. In addition, ATF4 and CHOP get activated, which probably contributes to FFA-induced apoptosis (Kharroubi et al., 2004).

Finally, deposits of amyloid may play a role in beta cell apoptosis. These deposits are characteristic of many T2D patients (Westermark and Wilander, 1978). Furthermore, overexpression of islet amyloid polypeptide, a precursor of amylin, leads to apoptosis in mouse beta cells (Huang et al., 2007).

3.3 ER stress

Beta cells are vulnerable to ER stress, since they produce and secrete large amounts of insulin. Insulin biosynthesis accounts for up to 50% of the total protein production in beta cells (Schuit et al., 1988). After stimulation with high glucose, the insulin synthesis is increased more than tenfold (Schuit et al., 1988). Hyperglycemia, FFAs, and cytokines can all trigger the unfolded protein response via different mechanisms. Irreversible ER stress leads to beta cell apoptosis. ER stress is probably one of several factors contributing to beta cell apoptosis and loss in both T1D and T2D (Eizirik et al., 2008).

The unfolded protein response is controlled by the ER sensors IRE1, PERK, and ATF6. These proteins become active when unfolded proteins accumulate in the lumen, and BiP dissociates them to assist in protein folding. These sensors translate information into signals that modulate expression of genes and proteins. Activated IRE1 cleaves 26 nucleotides from the mRNA encoding XBP1, generating spliced XBP1 (sXBP1). sXBP1 acts as a potent transcriptional transactivator of genes that reduce ER stress, including chaperones. AFT6 activation leads to its translocation into the nucleus, where it binds to the ER stress element in genes encoding ER chaperone proteins, increasing protein-folding activity in the ER. PERK phosphorylates eIF2alpha, decreasing the protein synthesis and thus the functional demand of the ER. Mutations in PERK are associated with Wollcott-Rallison syndrome, which causes permanent neonatal diabetes (Delépine et al., 2000). Activation of PERK due to ER stress induces a general decrease in translation but some selected proteins, such as ATF4, are translated more efficiently. AFT4 regulates expression of many genes including CHOP. Prolonged and excessive ER stress may trigger apoptosis through c-Jun N-terminal kinase (JNK), CHOP, and Atf3, and inhibition of Bcl-2 and/or activation of the proapoptotic members of the Bcl-2 family (Puthalakath et al., 2007; Zinszner et al., 1998).

IL-1β and IFNγ induce ER stress by NO formation and Ca^{2+} depletion, as described above. This leads to activation of IRE1a, to consequent XBP1 splicing, and to eIF2a/ATF4/CHOP/Bim activation, but not ATF6 activation. Since ATF6 is not activated, BiP expression is not increased. This deprives the beta cells of an important compensatory mechanism for cell survival during ER stress. Supporting this, overexpression of BiP leads to decreased CHOP induction, and to partial protection against IL-1β- and IFNγ-induced apoptosis (Wang et al., 2007).

3.4 Beta cell protection

Akt is a serine/threonine-specific protein kinase that is activated by PI3K and plays a key role in multiple cellular processes. Akt signaling is one of the most important pathways in regulating beta cell survival, and thus the several growth factors that activate Akt, including IGF-1 and GLP-1, protect beta cells against apoptosis (Bernal-Mizrachi et al., 2001; Elghazi and Bernal-Mizrachi, 2009; Tuttle et al., 2001). The mechanisms by which Akt protects beta cells are incompletely understood. Phosphorylation of FoxO1 can have protective effects under acute stress (Martinez et al., 2008). The phosphorylation and inactivation of the Bcl-2 family protein Bad probably has a protective role (Elghazi and Bernal-Mizrachi, 2009). Additionally, the inhibition of GSK3beta activity has been shown to promote beta cell survival (Mussmann et al., 2007). PTEN is a negative regulator of Akt signaling, and thus deletion of PTEN in beta cells results in increased activation of Akt and also in protection against beta cell apoptosis (Nguyen et al., 2006; Stiles et al., 2006; Zeng et al., 2013).

GLP-1 analogue treatment of Zucker diabetic fatty rats or db/db mice reduces beta cell apoptosis (Farilla et al., 2002; Wang and Brubaker, 2002). Similarly, treatment of human islets with GLP-1 *in vitro* inhibits apoptosis (Buteau et al., 2004; Ferdaoussi et al., 2008). The molecular mechanism by which GLP-1 agonists protect against beta cell death involves many pathways. Induction of antiapoptotic proteins such as Bcl-2 and Bcl-xl, and activation of PI3K are all involved. In addition, IL-1β-mediated death is inhibited by inactivating the c-Jun N-terminal kinase (JNK) (Ferdaoussi et al., 2008; Li et al., 2005a). Pdx-1 is also important in this protective mechanism. Mice with a beta-cell-specific inactivation of the Pdx1 gene exhibit a complete loss of the anti-apoptotic effects of exendin-4 (Li et al., 2005b). Furthermore, the activation of GLP-1R induces the antiapoptotic JunB and the ER chaperone Bip (Cunha et al., 2009).

Several transgenic mouse models have been created to study the mechanism of beta cell death in response to a diabetogenic environment. For example deletion of connexins in beta cells predisposes them to cytotoxic death (Klee et al., 2011), overexpression of Pax4 decreases streptozotocin-induced beta cell death via downregulation of Glut2 expression (Hu He et al., 2011), and overexpression of NF-κB inhibitor in beta cells prevents MLDS-induced diabetes (Eldor et al., 2006).

In addition to proteins, chemical chaperones can prevent beta cell apoptosis. Chemical chaperones stabilize proteins and improve their folding and export from the ER, thus reducing ER stress (Welch and Brown, 1996). For example, treatment of ob/ob (leptin deficient) mice with the chemical chaperone 4-phenyl butyric acid and taurine-conjugated ursodeoxycholic acid (TUDCA) improves glycemia after 10 days (Ozcan et al., 2006). Importantly, there is also evidence that ER stress contributes to the beta cell death detected in T1D, and supporting this treatment of NOD mice with TUDCA results in decreased diabetes incidence (Engin et al., 2013).

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

In 1962 Stanley Cohen isolated from the salivary gland a protein that induced eyelid opening and tooth eruption in newborn mice (Cohen, 1962). Further experiments showed that this protein could stimulate the proliferation of epithelial cells, and it was named Epidermal Growth Factor (EGF) (Cohen, 1965). A decade later, the EGF receptor (EGFR) was identified as a 170 kD membrane protein that increased the incorporation of phosphorus into EGFR in response to EGF treatment (Carpenter et al., 1978); shortly after, EGFR was recognized as a receptor tyrosine kinase. It activates signaling pathways that have essential roles in embryonic development, as well as in the adult. Finally, EGFR is overexpressed in several epithelial tumors, and currently there are several anti-EGFR based therapies used in oncology.

EGFR is a member of the ErbB tyrosine kinase family, which consists of EGFR (ErbB1/HER1), ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4 (Kraus et al., 1989; Plowman et al., 1993;

Ullrich et al., 1984; Yamamoto et al., 1986); see Figure 7. EGFR family receptors are ubiquitously expressed in various cell types.

4.1 ErbB ligands

ErbB receptor activation is tightly regulated by the availability of ligands. There are 11 known ligands, which are divided into three groups (Jones et al., 1999) (Figure 7). The first includes EGF (Cohen, 1965), transforming growth factor alpha (TGF-α) (Derynck et al., 1984), amphiregulin (AR) (Shoyab et al., 1989) and epigen (EPGN) (Kochupurakkal et al., 2005), all of which bind specifically to EGFR. The second group includes betacellulin (BTC) (Shing et al., 1993), heparin-binding EGF (HB-EGF) (Higashiyama et al., 1991), and epiregulin (EPR) (Toyoda et al., 1995), which bind to both EGFR and ErbB4. The third group is composed of neuregulins or heregulins (NRG1-4), and this group is further subdivided based on these ligands' ability to bind to ErbB3 and ErbB4 (NRG1 and 2) (Holmes et al., 1992; Wen et al., 1992), or only to ErbB4 (NRG3 and 4) (Carraway et al., 1997; Harari et al., 1999).

4.2 ErbB receptors

All ErbB family members contain an extracellular ligand-binding domain, a single membrane-spanning region, a juxtamembrane nuclear localization signal and a cytoplasmic tyrosine kinase domain. Ligand binding to the ErbB receptor induces conformational changes that lead to either homo- or heterodimerization of the receptor. This interaction activates the receptor tyrosine kinase, which causes autophosphorylation of the cytoplasmic tails of each dimer pair. It was long thought that ErbB3 did not have intrinsic kinase activity (Guy et al., 1994). However, more recently it has been discovered that ErbB3 harbors a low level of kinase activity (a thousandfold less than EGFR) (Shi et al., 2010). ErbB2 does not have any known ligands (Klapper et al., 1999), but downstream signaling of activation of ErbB2 is readily achieved through heterodimerization. ErbB2 is actually the preferred heterodimerization partner for other ErbBs (Graus-Porta et al., 1997). Furthermore, substantial overexpression of ErbB2 appears to force spontaneous homodimer formation in the absence of ligands (Alvarado et al., 2009; Tebbutt et al., 2013).

In addition to direct ligand binding, the ErbB receptors can be transactivated by other receptors. What is interesting in this context is that in pancreatic beta cells, the GLP-1 receptor has been shown to transactivate EGFR through HB-EGF cleavage (Buteau et al.,

2003). EGFR-mediated PI3K kinase activation is important at least for the proliferative and antiapoptotic effect of GLP-1 (Buteau et al., 2003; 2006). Other G-protein-coupled receptors can also induce rapid stimulation of metalloproteinases that cleave an EGF-like ligand precursor e.g. pro-HB-EGF, which then binds to EGFR and activates downstream signaling pathways (Prenzel et al., 1999). Furthermore, the binding of GH or Prl to their receptors activates the JAK2, which phosphorylates the cytoplasmic domain of ErbB1 or ErbB2, in addition to activating STAT5, as previously discussed (Yamauchi et al., 1997; 2000). Other stimuli including hyperglycemia (Han et al., 2011), bacterial products (Yan et al., 2011), and TNF (Yamaoka et al., 2008) have also been shown to induce EGFR transactivation.

EGFR activation stimulates many complex signaling pathways that are tightly regulated by the presence and identity of the ligand, the heterodimer composition, and the availability of the phosphotyrosine-binding proteins. The two primary signaling pathways activated by the EGFR are the MAPK and PI3K pathways. Additionally, EGFR can activate the phospholipase C-protein kinase C (PLCγ-PKC) and STAT pathways (Marmor et al., 2004); see Figure 7.

During pancreatic development, EGFR-induced activation of MAPK pathway is important for proliferation of pancreatic epithelial cells (Cras-Meneur et al., 2001). This pathway is also important for adult human pancreatic duct-cell proliferation (Rescan et al., 2005). Beta cells contain several other growth factor receptors that also induce activation of the MAPK pathway, such as the PDGF-receptor and FGF-receptors (Chen et al., 2011; Le Bras et al., 1998; Powers et al., 2000).

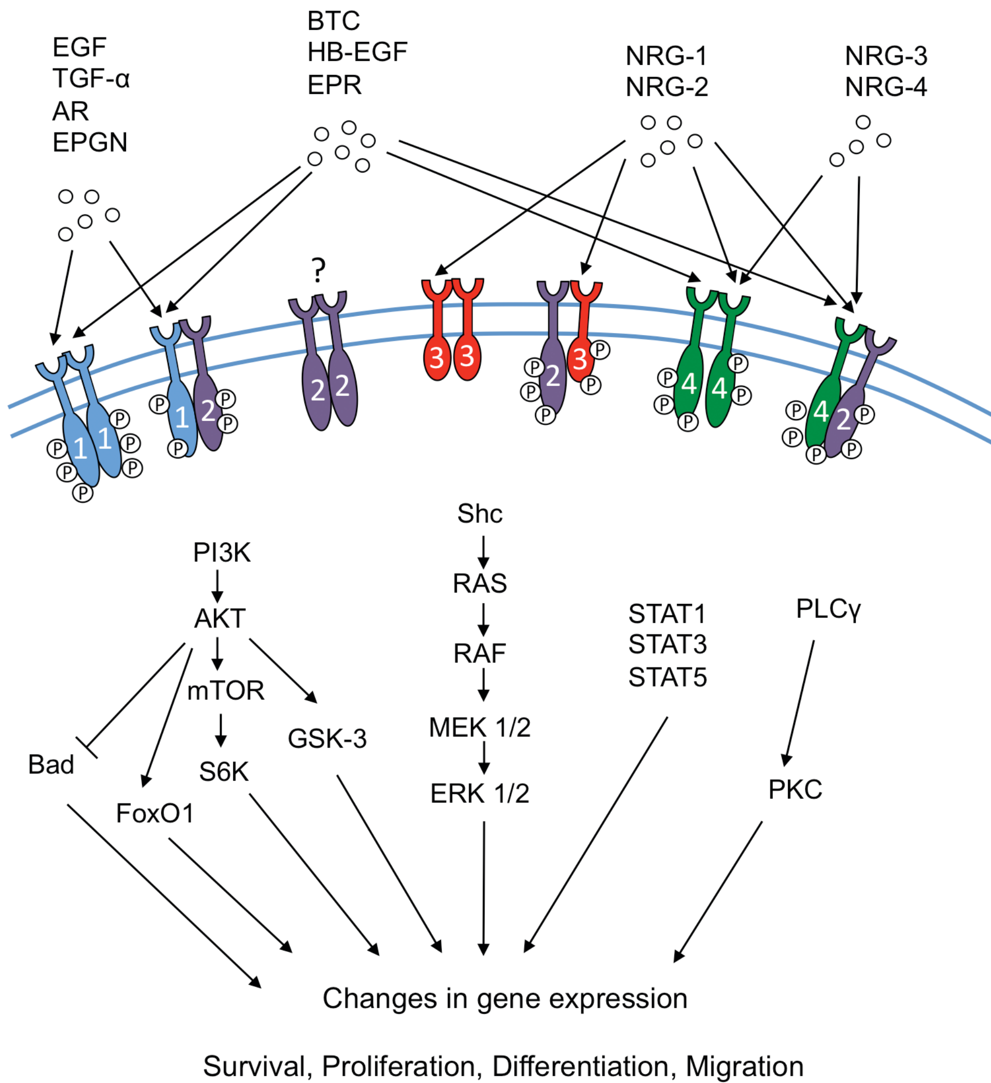

***Figure 7** Schematic illustration of the ErbB ligands, possible combinations of ErbB receptor dimers, and activated signaling pathways. Binding of a ligand induces formation of homo- or heterodimers with other ErbB receptors. The activated receptor activates downstream signaling pathways, namely the PI3K-Akt, the MAPK, the STAT, and the PKC pathways, which ultimately lead to changes in gene expression. For abbreviations, see pages 10-11.*

The other major signaling pathway that EGFR activates in the beta cell is PI3K. Activated EGFR phosphorylates PI3K, which leads to activation of PDK1, and thus phosphorylation of Akt. Akt affects several downstream signaling molecules, e.g. mTOR, GSK-3beta, FoxO proteins, and Bad, affecting either transcription or survival (Elghazi et al., 2007).

4.3 ErbB receptors in cancer

The ErbB network dysregulation is implicated in multiple human cancers. ErbB1 and ErbB2 in particular are constitutively activated as a result of autocrine ligand production, receptor overexpression, or activating mutations (Marmor et al., 2004) and this has clinical relevance in the treatment of lung, breast, and pancreatic carcinoma and head and neck tumors (Tebbutt et al., 2013).

The most common mutation of EGFR is a deletion of the extracellular region resulting in constitutive activation of the receptor (Pedersen et al., 2001). Another common mutation is a point mutation in exon 19, leading to substitution of arginine for leucine (L858R) and a constitutively activated receptor (Choi et al., 2007).

EGFR inhibitors are currently used in the treatment of advanced cancer of the colon, rectum, pancreas, lungs and upper airways (Yarden and Pines, 2012). EGFR can be inhibited by the monoclonal antibodies cetuximab (Erbitux) and panitumab (Vectibix), which bind to the extracellular part of the receptor, preventing binding of the endogenous ligands, and can also be inhibited by the small-molecule TKIs e.g. erlotinib (Tarceva) and gefitinib (Iressa), which inhibit phosphorylation of the intracellular tyrosine kinase by blocking the ATP-binding site (Yarden and Pines, 2012). When considering the multiple tissues and processes in which the EGFR participates, it is somewhat surprising that the most common side effect of these drugs is folliculitis (Bachet et al., 2012).

4.4 ErbB receptor expression and signaling in pancreas

During development, EGFR and its ligands are broadly expressed in the pancreas (Huotari et al., 2002; Miettinen and Heikinheimo, 1992). In the adult mouse pancreas, EGFR is mostly expressed in the islets of Langerhans and ductal epithelium (Miettinen et al., 2006; 2008; Miyagawa et al., 1999). In humans, EGFR is also abundantly present in the islets (Danzer et al., 2012; Hanley et al., 2011). The EGFR -/- mice have immature development in several epithelial organs; for instance, they have thin skin, fewer hair follicles, and no hair outgrowth (Miettinen et al., 1995). The pancreatic organ size is smaller, and the islets are located adjacent to the ducts, showing a migration defect (Miettinen et al., 2000). This might be due to reduced matrix metalloproteinase activity, which is needed to degrade the basal lamina (Miettinen et al., 2000). However, gene ablation studies of MMPs in the developing pancreas showed no effect on islet formation (Perez et al., 2005). Another possibility for

how EGFR regulates islet migration from the ductal epithelium is that EGFR stimulates Rac-1 signaling, which regulates E-cadherin-mediated cell-cell adhesion and actin rearrangements to facilitate islet cell movement (Greiner et al., 2009). Deletion of Rac1, however, had no obvious effect on pancreas morphogenesis either (Heid et al., 2011). EGFR signaling might also affect differentiation, since in the EGFR KO mice, the formation of insulin-positive cells *in vitro* embryonic cultures was reduced by 50% (Miettinen et al., 2000).

The function of EGFR signaling in the postnatal period has been studied with an EGFR dominant-negative mouse (Miettinen et al., 2006). These mice express a dominant-negative EGFR under the Pdx1 promoter, leading to approximately 40% reduction in EGFR and downstream signaling pathway activation upon EGF stimulation. These mice show a postnatal beta cell proliferation defect, resulting in an 80% reduction in beta cell mass in homozygous animals, and they become hyperglycemic by two weeks of age. Since the islets from EGFR dominant-negative mice did not show any defect in beta cell development or in insulin secretion; the diabetic phenotype was thus due to a reduced beta cell proliferation and mass.

EGFR is also important for the effect of GLP-1 in beta cells; it has been shown that the transactivation of EGFR by GLP-1R is required for the mitogenic effect of incretins (Buteau et al., 2003). It has been proposed that GLP-1R activates c-Src, which induces proteolytic cleavage of the endogenous EGFR ligands from transmembrane precursors. GLP-1 analogues are currently used therapeutically in the treatment of T2D to increase insulin secretion and to reduce appetite as explained in 2.5.

The dimerization partner of EGFR impacts the downstream signaling pathways that are activated, and, theoretically, the receptor combinations that form in the pancreas are dependent on the available receptor partners. All ErbB receptors are expressed in the mouse pancreas during development and in the adult islets (ErbB4 mostly in the alpha cells) (Huotari et al., 2002; Kritzik et al., 2000; Oh et al., 2011); however, only heterodimers with EGFR and ErbB2, in addition to EGFR homodimers, have been shown to be functionally important (Huotari et al., 2002; Oh et al., 2011). BTC-induced proliferative effect is dependent on the activation of both ErbB2 and EGFR (Oh et al., 2011). The exact physiological role of ErbB2 in the pancreas is not known, since the ErbB2−/− mice die before

pancreatic organogenesis at E10.5 (Erickson et al., 1997; Lee et al., 1995), and there are no reports of pancreas-specific *ErbB2* gene ablation.

Although the ErbB expression pattern is clear in the murine pancreas, it is more obscure in the human adult pancreas. All ErbBs have been detected in human islets at transcript level (Kutlu et al., 2009), but only EGFR and ERBB3 at protein level (Danzer et al., 2012; Hanley et al., 2011; Miyagawa et al., 1999). However, all ERBBs have been shown to be present in human ductal cells (Rescan et al., 2005).

It is unclear whether and how ErbB3 influences beta cell development and function. ErbB3$^{-/-}$ mice had smaller pancreases, thinner surrounding mesenchyme, and fewer islet hormone positive cells at E13.5, which might be due to a pancreatic developmental defect or due to defects in the surrounding mesenchyme (Erickson et al., 1997). However, these mice die at E13.5 (Erickson et al., 1997), and there are no reports of pancreas-specific *ErbB3* ablation. SNPs within an exon of *ERBB3* gene are associated with T1D (Keene et al., 2012). Nevertheless, since these SNPs do not alter the aminoacid sequence of ERBB3, the conceivable functional effect is mediated via altered mRNA expression, splicing, or stability.

ErbB4 protein has been detected in mouse pancreas at E12.5, and in adult mice it is expressed in the ducts and alpha cells (Huotari et al., 2002; Kritzik et al., 2000). Blocking of ErbB4 signaling in pancreatic explant cultures reduced the number of forming delta cells (Huotari et al., 2002). ErbB4−/− mice die at E10.5 due to cardiac and neural defects (Gassmann et al., 1995). Pancreas-specific gene ablation studies are needed to shed light on the role of ErbB4 in pancreatic development. A summary of genetically modified ErbB receptor and ligand mouse models is presented in Table 2.

Table 2 Summary of genetically modified ErbB receptor and ligand mouse models and their pancreatic phenotype.

Target gene	Pancreatic phenotype	References
Egfr$^{-/-}$	Streak like islets, poor branching	Miettinen et al 2000
ErbB2$^{-/-}$	Embryonically lethal at E10.5	Erickson et al 1997 Lee et al 1995
ErbB3$^{-/-}$	Embryonically lethal at E13.5	Erickson et al 1997
ErbB4$^{-/-}$	Embryonically lethal at E10.5	Gassmann et al 1995
Btc$^{-/-}$	None	Jackson et al 2003
TGFα$^{-/-}$	None	Luetteke et al 1993
Ereg$^{-/-}$	None	Lee et al 2004
Epigen$^{-/-}$	None	Dahlhoff et al 2013
Egf$^{-/-}$ **and** ***AR***$^{-/-}$	None	Luetteke et al 1999
Egf$^{-/-}$ **and** ***AR***$^{-/-}$ **and** ***TNFα***$^{-/-}$	Not reported	Troyer et al 2001
Insulin-Egf	Increased islet size, disorganized, insulin-positive cells in the ducts	Krakowski et al 1999
Pdx1-HB-Egf	Disorganization, fibrosis, metaplastic ductal epithelium	Means et al 2003
Elastase-AR	Ductal cell proliferation	Wagner et al 2002
β-actin-BTC	Increased insulin secretion but BCM normal	Dahlhoff et al 2009

4.5 ErbB receptor ligands in pancreas

EGF, TGF-alpha, BTC and NRG4 are among the ErbB ligands that are expressed in the pancreas (Huotari et al., 2002; Korc et al., 1992; Miyagawa et al., 1999). There is extensive functional overlay among different EGFR ligands. Thus, the knockouts of EGF, BTC, AR, EPR, EPGN, or TGF-alpha have no pancreatic phenotype, see Table 2 (Dahlhoff et al., 2013; Jackson et al., 2003; Lee et al., 2004; Luetteke et al., 1999; 1993; Troyer et al., 2001).

During development, EGF appears to stimulate the proliferation of the pancreatic epithelium, but not the differentiation; conversely, the inhibition of MAPK signaling activates endocrine differentiation (Cras-Meneur et al., 2001). In addition, the systemic administration of EGF in pigs resulted in the induction of ductal proliferation with fewer insulin-positive cells (Vinter-Jensen et al., 1997). Furthermore, overexpression of HB-EGF under the Pdx1 promoter results in disorganization of both exocrine and endocrine

compartments, with ductal metaplasia and fibrosis (Means et al., 2003). Nevertheless, targeted overexpression of EGF under the insulin promoter in mice increases islet size, also showing increased beta cell proliferation (Krakowski et al., 1999). On the other hand, overexpression of BTC improves glycemia without affecting beta cell mass (Dahlhoff et al., 2009), while overexpression of AR in acinar cells results in ductal cell proliferation without affecting beta cells (Wagner et al., 2002).

EGF has been shown to induce beta cell regeneration in several murine diabetes models, usually together with gastrin or leukemia inhibitory factor (LIF) or CNTF. In adult diabetic animals, co-treatment with EGF and gastrin induces pancreatic regeneration in diabetic NOD mice (Suarez-Pinzon et al., 2005b). This might result from the effects of EGF and gastrin on beta cells, immune cells, or both. Additionally EGF, together with gastrin, increases β-cell mass after streptozotocin-induced (in rats) or alloxan-induced (in mice) diabetes (Rooman and Bouwens, 2004; Solar et al., 2009). The origin of the new beta cells in this model is controversial. Lineage-tracing studies have shown that they do not originate from ductal cells (Solar et al., 2009). Furthermore, co-treatment with EGF and gastrin seemed also to increase human beta cell mass *in vitro* and *in vivo* when islets were transplanted into mice (Suarez-Pinzon et al., 2005a). Additionally, *in vitro* EGF and LIF have been shown to induce transdifferentiation of murine acinar cells into endocrine cells *in vitro*, and transplantation of these newly formed beta cells restored normoglycemia in alloxan-treated mice (Baeyens et al., 2005) This transdifferentiation seems also occur *in vivo* after alloxan-induced diabetes followed by EGF and CNTF treatment (Baeyens et al., 2014). The origin of new beta cells in this context appears to be Ptf1a-positive exocrine cells (Baeyens et al., 2014). This effect is mediated through the JAK/STAT3-induced Ngn3 expression (Baeyens et al., 2006; 2014). In another study, it was shown that EGF induces transdifferentiation of acinar cells into insulin-producing cells *in vitro* together with nicotinamide (Minami et al., 2005).

BTC was first found from the MIN6 β-cell line as a growth-promoting factor (Shing et al., 1993). It is expressed in many tissues, particularly in the pancreas, liver, kidney and small intestine. In the pancreas, its primary expression sites appear to be α-cells and ducts (Miyagawa et al., 1999). BTC seems to be a potent beta cell mitogen, with a proliferative effect comparable to GH and Prl in INS-1 cells *in vitro* (Huotari et al., 1998). Furthermore, adenoviral overexpression of BTC in pancreatic ductal cells leads to increased beta cell proliferation *in vivo* (Tokui et al., 2006). BTC also stimulates the proliferation of human fetal

islet-like cell clusters (Demeterco et al., 2000). BTC induces the differentiation of β-cells in embryonic mouse pancreatic explants (Huotari et al., 2002). This effect was dependent on EGFR and not affected by the blocking of the ErbB4 receptor. The ubiquitous overexpression of BTC improved glucose metabolism in mice (Dahlhoff et al., 2009). Furthermore, the administration of a recombinant protein or adenoviral vector expressing BTC into STZ-induced diabetic mice increases beta cell proliferation and restores normoglycemia (Shin et al., 2008) and this effect is mediated through EGFR and ErbB2 (Oh et al., 2011).

TGF-α is expressed in duct cells, acinar cells, and islets of the human fetal and adult pancreas (Huotari et al., 2002; Korc et al., 1992; Miettinen and Heikinheimo, 1992). Systemic overexpression of TGF-α leads to progressive pancreatic interstitial fibrosis and ductular metaplasia (Jhappan et al., 1990). TGF-α overexpression induced metaplastic ducts can differentiate into islets by co-overexpression of gastrin under the insulin promoter (Wang et al., 1993).

Epiregulin has been shown to increase beta cell proliferation of both INS-1 cells and RINm5F cells *in vitro* (Kuntz et al., 2005). Epiregulin is expressed at a low level in human ductal and acinar cells, and its expression is upregulated in pancreatic cancer (Zhu et al., 2000).

Finally, the ErbB4 ligand NRG4 is expressed in mouse pancreas as early as E13 (Harari et al., 1999; Huotari et al., 2002). In E12.5 mouse pancreatic explants, NRG-4 stimulates the differentiation of somatostatin-producing δ-cells, while at the same time suppressing α-cell development.

AIMS OF THE STUDY

This study was designed to elucidate the role of EGFR signaling in beta cell mass dynamics in normal physiological conditions as well as during diabetogenic insults. The specific aims were:

1. To study the role of EGFR signaling in beta cell mass regulation in physiological situations of beta cell mass increase (pregnancy and metabolic syndrome) and during pancreatic regeneration (partial/pancreatic duct ligation)

2. To study the possible molecular mechanisms by which EGFR regulates beta cell proliferation during pregnancy

3. To study the effect of EGFR activation on beta cell proliferation at different ages *in vivo*

4. To study the protective effect of EGFR activation in beta cells against experimentally induced diabetes

MATERIAL AND METHODS

1. Animal work

1.1 E1-DN mice (I, II)

The EGFR dominant-negative (E1-DN) transgene consisted of the mouse *Pdx1* promoter, followed by a rabbit beta-globin second intron, the human kinase-deficient *EGFR* cDNA CD533 (Contessa et al., 1999), and sequences coding for a myc tag and a growth hormone polyA signal. This transgene was used to generate a single transgenic line (Miettinen et al., 2006) (Figure 9). Expression of the kinase-deficient EGFR CD533 leads to a dominant negative phenotype (Kashles et al., 1991). In addition to blocking the activity of EGFR, it can also inhibit the tyrosine kinase activity of other ErbB receptors by heterodimerization (Redemann et al., 1992; Spivak-Kroizman et al., 1992). The role of EGFR signaling in beta cell mass expansion during obesity, pregnancy, and pancreatic regeneration was studied with these mice. The E1-DN was generated in FVB background. For the experiments, the offspring of E1-DN male mice mated with wild-type FVB female mice were used, to exclude the possible intrauterine effects of maternal hyperglycemia. Transgene-negative littermates and wild-type FVB mice were used as controls. Cohort sizes were three to 13 mice depending on the experiment.

1.2 INS-CA-EGFR mice (III)

The Ins-rtTA::TetOP-EGFRL858R (INS-CA-EGFR) mice were generated by crossbreeding Ins-rtTA mice provided by Dr Y. Dor, Hebrew University-Hadassah Medical School (Nir et al., 2007), with TetOP-EGFRL858R mice obtained from Dr H. Varmus, National Cancer Institute (Politi et al., 2006). The TetOP-EGFR construct consists of a tetracyclin operator followed by a human EGFR cDNA with a point mutation in exon 21 (c.2573 T>G), leading to substitution of lysine to arginine at the position 858 (L858R, also known as L834R, which corresponds to an EGFR residue numbering without a 24-residue membrane targeting signal sequence). L858R is an activating mutation leading to a constitutively active form of the EGFR (CA-EGFR). The resulting background for INS-CA-EGFR mice was a mix of C57Bl, CBA, and FVB mouse strains. The INS-rtTA mice were originally generated into a C57Bl background and bred with FVB mice in our facility resulting in a mix of strains C57bl and FVB.

Doxycycline was administered to the drinking water (0,5 mg/ml doxycycline and 3% v/w sucrose; for controls only 3% v/w sucrose) or through the diet (T.2018.12 with 625 mg/kg of doxycycline, Tekland, Harlan) for indicated time periods. Cohort sizes were four to 11 mice depending on the experiment.

1.3 Blood glucose and *in vivo* testing of glucose tolerance (I,III)

Blood glucose values were measured from mice at various ages from the whole blood using a OneTouch Ultra glucometer (Lifescan).

For the intraperitoneal glucose tolerance test, mice were fasted overnight or for 5h (in cases of pregnant mice). Glucose (1 mg/g body weight) was injected intraperitoneally and blood samples were collected at 0, 30, 60 and 120 min time points. Insulin was analyzed from plasma with ELISA (Chrystal Chem).

1.4 Islet isolation (I, II, III)

Islets from one-to-six-month-old mice were isolated by first mechanically dispersing the tissue, followed by standard collagenase digestion (Collagenase P; Roche Diagnostics), sedimentation and subsequent handpicking under a stereomicroscope.

1.5 Partial duct ligation (I)

In the partial/pancreatic duct ligation (PDL) experiment the pancreatic duct of anesthetized nine-week-old mice was ligated in Brussels (in Professor Luc Bouwens's laboratory in Vrije Universiteit), as described in rats by Wang et al (Wang et al., 1995) with some minor modifications. The mice were culled seven days after the operation, and the head and tail parts of the pancreas were collected separately for beta cell mass and proliferation analysis.

1.6 High-fat diet treatment (I)

To study the role of EGFR during obesity-induced beta cell mass expansion, E1-DN heterozygous and wild-type mice were fed a high-fat diet (TD 06414, Harlan Laboratories, Indianapolis, IN, USA) or standard chow (Altromin 1324; Altromin, Lage, Germany) for eight

weeks. The high-fat diet consisted of 35% of fat (vs four percent in the standard chow) and 60% of the diet's kilocalories were derived from fat (vs 11% in the standard chow) After eight weeks, glucose tolerance, beta cell mass and proliferation were analyzed.

1.7 Pregnancy experiment (I, II)

To study the role of EGFR during gestational beta cell mass expansion 12-week-old E1-DN heterozygous or wild-type female mice were mated with male FVB mice. Mating was confirmed by the presence of a vaginal plug the next morning, designated as day 0.5 of gestation. An intraperitoneal glucose tolerance test was performed on gestational day 13.5 (GD13.5), and the mice were culled and the pancreases collected on day 13.5, 14.5, and 18.5 of gestation for measurement of beta cell mass.

1.8 Streptozotocin experiments (III)

Streptozotocin (STZ) induced hyperglycemia was used to study the protective effect of EGFR activation. STZ was prepared in 0.1 M sodium citrate at pH 4.5. For the single-dose STZ treatment, three-month-old mice were fasted before injection for 5h, followed by intraperitoneal injection of STZ (200 mg/kg body weight). STZ is specifically incorporated into beta cells, as STZ is only transported by glucose transporter 2 (GLUT2), present mostly in beta cells (Wang and Gleichmann, 1998). STZ induces beta cell demise mostly via DNA alkylation, subsequent DNA strand breaks, and consequent nicotinamide adenine dinucleotide (NAD^+) depletion (Yamamoto et al., 1981).

In the multiple low-dose STZ (MLDS) experiment, mice of the same age were injected intraperitoneally with STZ (50 mg/kg body weight) for five consecutive days without fasting. Day 0 was designated as the first day of injection of STZ. Multiple low-dose streptozotocin causes an initial mild toxic insult, and then a progressive immune-mediated β-cell apoptosis (Kantwerk-Funke et al., 1991).

2. Human primary islet cell culture

Human islets were received from Uppsala, Sweden through the European Consortium for Islet Transplantation. Islets were isolated using standard methods (Ricordi et al., 1988). Details of the organ donors are shown in Table 3. Islet purity was checked by dithizone

staining. Upon arrival, the islets were cultured overnight in non-adherent culture dishes (Corning Incorporated, NY, USA) in Ham's F10, supplemented with 0.5% BSA. The next day the islets were changed to RPMI media, supplemented with penicillin (100 IU/ml), streptomycin (100 IU/ml), and 10% (vol/vol) fetal calf serum either with or without human placental lactogen 500 ng/ml (Affiland) and with or without gefitinib 10 µM (Sellect chemicals, Houston, TX, USA) for 96h in non-adherent culture dishes. Bromodeoxyuridine (BrdU) 30 µg/ml (Sigma-Aldrich) was added for the last 48h.

Formalin-fixed islets were washed in PBS and spun down at high speed in fluid agar. Agar-containing cell pellets were embedded in paraffin, processed with routine methods, and sectioned. Proliferation was analysed as BrdU/C-peptide or BrdU/ChromograninA double-positive cells relative to all C-peptide- or ChromograninA-positive cells. All C-peptide- or ChromograninA-positive cells found in the section were included in the analysis (altogether, 503-5731 cells per sample).

Table 3 *Characteristics of human organ donors and purity of the islets obtained (percentage of islets in the received cell material). N/A, not available.*

Age of the donor	Gender	HbA1c (%)	Purity
68	Female	6,0	54%
57	Female	5,5	85%
72	Female	5,7	87%
69	Female	N/A	35%

3. DNA extraction and genotyping (I-III)

Genomic DNA was extracted from tail or ear samples. Tissue pieces were digested overnight with proteinase K in lysis buffer followed by isopropanol precipitation. The resulting pellet was dissolved in TE (1 mM EDTA in 10 mM Tris-HCl buffer, pH 8.0). Primers used for genotyping are listed in Table 4. PCR reaction was performed according to the following PCR protocol: denaturation for 2 min at 95°C, followed by 36 cycles of 35 sec at 95°C, 35 sec at 59°C and 35 sec at 72°C, followed by a 7 min extension at 72°C.

Table 4 List of primers used for genotyping.

Primer	Forward sequence	Reverse sequence
E1-DN	AAGAGCGAGTTGGCACTCAG	TACAAGCTGTGGTCCGCTAT
INS-rtTA	TAGATGTGCTTTACTAAGTCATCGCG	GAGATCGAGCAGGCCCTCGATGGTAG
TetOP-EGFR-L858R	GCTGCAGGAGAGGGAGCTTG	AAACGGTCACCCCGTAGCTC

4. mRNA expression (I-III)

Total RNA from cells and tissues was isolated using the NucleoSpin RNAII kit (Macherey-Nagel) without on-column DNase treatment. DNase treatment was done separately followed by RNA purification with NucleoSpin RNA CleanUp or NucleoSpin RNA XS kit according to manufacturer's instructions (Macherey-Nagel). RNA quality was controlled with NanoDrop 1000 spectrophotometer (Thermo Scientific). cDNA was synthesized from approximately 50 ng of islet RNA or 2 µg of total pancreatic lysate. Real-time PCR was done with SYBR Green JumpStart Taq ReadyMix for Quantitative PCR (Sigma-Aldrich) with Corbett Rotor-Gene 6000 instrument (Qiagen). The reactions were prepared with a Corbett CAS-1200 liquid handling system (Qiagen). All reactions were performed in duplicates on at least three biological replicates. The median C_T values were used for ΔΔCT analysis (Livak and Schmittgen, 2001) with the housekeeping gene Cyclophilin G as endogenous control. An exogenous positive control was used as a calibrator for all the real-time PCRs. Primer sequences are presented in Table 5.

Table 5 List of primers used for RT-PCR.

Primer	Forward sequence	Reverse sequence
CyclophilinG	CAATGGCCAACAGAGGGAAG	CCAAAAACAACATGATGCCCA
STAT1	AAAGTCATGGCTGCCGAGAA	TACTTCCCAAAGGCGTGGTC
iNOS	AAGATGGCCTGGAGGAATGC	TGCTGTGCTACAGTTCCGAG
IP-10	AGTGCTGCCGTCATTTTCTG	TCCCTATGGCCCTCATTCTCA
PUMA (Bcl-2-binding component 3)	ACCACCATCTCAGGAAAGGC	CAAAGTGAAGGCGCACTGG
BIM (Bcl2-like 11)	GATGGCAAGCCCTCTCACTT	AAGCAAACAGTCGTTGGCAC
DP-5	GCTACCGGAGCAGTGTCATT	CCGTGCACCTAACCAACTGA
Bcl-2	GGGATGACTTCTCTCGTCGC	TCACCCCATCCCTGAAGAGT
Glut2	AACCGGGATGATTGGCATGT	CACGTAACTCATCCAGGCGA
Birc5/Survivin	GACTGCAAAGACTACCCGTCA	GATGTGGCATGTCACTCAGG
Tph1	TCAAAAACTGGCAACGTGCTAC	GGCATGTCCAGAAAGTGCATG
Tph2	GTCAATTACCCGTCCCTTCTC	TCAATACTTCTGGTGTCTTTCAG
EGFR (mouse)	ATGACGCATTCCTCCCTGTA	TGATAATGCAGGTCTCTTCCA
EGFR (human)	GCGTGGACAAGTGCAACCTT	CTGAGGCAGGCACTCTGGG
Htr2B	GGAGATATTTGTGTGGATAGG	TCCCGAAATGTCTTATTGAAGAG

5. Protein detection (I-III)

5.1 Western blotting (III)

Equal amounts of protein were resolved by Any kD SDS page gel (BioRad). Immunoblot was performed with primary antibodies listed in Table 6. Primary antibodies were detected with horseradish peroxidase conjugated secondary antibody (Cell Signaling technology) and chemiluminescence (GE Healthcare). Protein loading was normalized for islet-actin or β–tubulin signal through densitometric signal quantitation with ImageJ software (NIH).

5.2 Immunohistochemistry (I-III)

Tissues were fixed with four percent PFA, embedded in paraffin and sectioned. Deparaffinised rehydrated sections were treated with 1 mmol/l EDTA buffer (pH 8) or citrate buffer (pH 6.0) in a microwave oven to reveal antigen sites. The sections were incubated for 10 min in three percent H_2O_2 (J.T.Baker, Avantor Performance Materials) followed by 10 min incubation in Ultra V Block (Thermo Scientific) to block non-specific binding sites prior to incubation overnight at 4°C with a primary antibody. For primary antibodies see Table 6. Secondary antibody incubations were carried out the next day for 30 min in RT. For light microscopy staining, the sections were incubated for 30 min at room temperature with a biotinylated secondary antibody as indicated in Table 7 (Zymed Laboratories), rinsed, and incubated with peroxidase-conjugated streptavidin (Zymed Laboratories). The sections were finally developed with 3-amino-9-ethyl-carbazole substrate (Thermo Scientific). For fluorescence microscopy, appropriate Alexa secondary antibodies (Invitrogen) were used as indicated in Table 7. Nuclear staining was performed with DAPI (Vectashield with DAPI, Vector Laboratories).

Table 6 *List of primary antibodies.*

Peptide/protein target	Manufacturer, catalog #	Species raised in	Dilution for IHC	Dilution for WB
Insulin	Dako Cytomation, A0564	Guinea pig; polyclonal	1:1000	
Ki67	Novocastra; NCL-Ki67p	Rabbit; polyclonal	1:1000	
BrdU	Dako Cytomation, M0744	Mouse; monoclonal	1:100	
Survivin	Cell Signaling Technology, #2808	Rabbit; monoclonal	1:100	
C-peptide	Cell Signaling Technology, #4593	Rabbit; polyclonal	1:100	
ChromograninA	Dako Cytomation, A0430	Rabbit; polyclonal	1:1000	
EGFR (human)	NeoMarkers, Ab10	Mouse; monoclonal	1:1000	
EGFR (human)	Novocastra; NCL-EGFR-384	Mouse; monoclonal	1:100	
EGFR (human)	Cell Signaling Technology # 4267	Rabbit; monoclonal	1:100	1:1000
EGFR (human and mouse)	Rockland; 100-401-149	Rabbit; polyclonal	1:1000	1:2000
EGFR-L858R	Cell Signaling Technology # 3197	Rabbit; monoclonal	1:100	1:1000
Phosphor-EGFR Y1068	Abcam; ab40815	Rabbit; monoclonal		1:5000
Phospho-Akt	Cell Signaling Technology # 4060	Rabbit; monoclonal		1:2000
Total Akt	Santa Cruz Biotechology; sc-8312	Rabbit; polyclonal		1:500
Phospho-Erk	Cell Signaling Technology #	Rabbit; monoclonal		1:2000
Bim	Cell Signaling Technology #2819	Rabbit; polyclonal		1:1000
Beta tubulin	Cell Signaling Technology #2146	Rabbit; polyclonal		1:1000
Actin	Santa Cruz Biotechology; sc-1616 HRP	Goat; Polyclonal		1:1000

Table 7 List of secondary antibodies.

Species targeted against	Manufacturer, catalog #	Species raised in	Dilution used	Fluorochrome
Anti-Rabbit	Life Technologies; A21206	Dnk	1:500	488
Anti-Rabbit	Life Technologies; A21207	Dnk	1:500	594
Anti-Guinea pig	Life Technologies; A11076	Goat	1:500	594
Anti-Mouse	Life Technologies; A11029	Goat	1:500	488
Biotinylated anti-Rabbit	Zymed; 81-6140	Goat	1:200	-
Biotinylated anti-Mouse	Zymed; 64-6440	Rabbit	1:200	-
HRP-conjugated anti-Rabbit	Cell Signaling Technology #7074	Goat	1:2000	-

5.3 Microscopy and scoring of results (I-III)

Fluorescence images were captured with a Zeiss AxioImager 2 microscope, and light microscopy images with an Olympus BX51 microscope.

To analyze β-cell mass, pancreases were sectioned, and five sections were analyzed at 100-150 µm intervals. These sections were stained for insulin, counterstained with haematoxylin, and morphometrically analyzed directly under light microscopy using the Image-Pro Analyzer 6.0 software (Media Cybernetics). The β-cell mass was calculated by multiplying the relative cross-sectional area of the insulin-positive area per total pancreatic tissue area by the weight of the pancreas.

In the CA-EGFR experiment malignant transformation capacity was assessed. After nine months of doxycycline treatment the pancreases were evaluated for possible premalignant or malignant transformations. This evaluation was done both macroscopically and

histologically. Five hematoxylin-eosin stainings per pancreas were evaluated at 100-150 µm intervals, for pancreatic dysplasia and malignancy by a pathologist blinded to the sample identity.

6. Optical projection tomography (OPT) (II-III)

6.1 Organ preparation and OPT scanning

The pancreases were processed as published by Alanentalo et al (Alanentalo et al., 2006). The antibodies were guinea pig anti-swine insulin (Dako Cytomation) and goat anti-guinea pig IgG (Alexa, A11076, Invitrogen). OPT scanning was done using the Bioptonics 3001 OPT scanner (Bioptonics), with exciter D560/40nm and emitter 610nmLP or exciter 425/40nm and emitter 475nmLP when visualizing Alexa 594 and 488 respectively. The scanning settings (identical for all specimens) were: rotation degree 0.9 µm; pixel size 9 µm for P1 pancreases, 17 µm for P30 pancreases; resolution 1024 x 1024 pixels; exposure time was set manually depending on the intensity of the fluorescence.

6.2 OPT sample reconstruction, visualization and quantification

Reconstructions were made with NRecon v1.6.3.3 (SkyScan). Isosurface reconstructions were generated using Imaris v7.6.3 (Bitplane). Islet volumes were segmented using the “background subtraction (local contrast)” thresholding option, and the intensity threshold was set manually for each pancreas.

7. Detection of proliferation and cell death (I-III)

7.1 Proliferation (I-III)

To measure proliferation, sections were stained for Ki67 by immunohistochemistry. Ki67 is an antigen present during all active phases of the cell cycle, and is commonly used to mark proliferating cells. Results were analyzed as percentage of Ki67 and insulin double-positive cells relative to total number of insulin-positive cells.

For measurement of DNA synthesis (an indicator of beta cell proliferation *in vivo)*, BrdU was added to the drinking water (1 mg/ml) or media (30 µg /ml) for the desired time period. This enabled the detection of long-term proliferation. BrdU incorporation was studied with immunohistochemistry, and proliferation was quantified by analyzing BrdU and insulin or C-peptide double-positive cell relative to the total number of insulin or C-peptide positive cells.

7.2 Cell viability (III)

The viability of pancreatic islet cells was assessed with a LIVE/DEAD Viability/Cytotoxity Kit (Invitrogen) after a 48h exposure to cytokines. This analysis is based on calcein dye that is retained in the live cells producing a green fluorescence and ethidium homodimer-1 that enters only damaged membranes and undergoes a 40-fold enhancement in fluorescence upon binding to nucleic acids producing a red fluorescence. The percentage of dead islet cells was estimated by three individual observers, two of them unaware of the sample identity and the average of these values was used for the final analysis.

7.3 Apoptosis (I, III)

Cleaved caspase 3 (I)

Caspase 3 is a critical executioner of apoptosis in beta cells (Gurzov and Eizirik, 2011). Activation of caspase 3 requires its proteolytic cleavage to the active form, which is done by the initiator caspase 9 (Gurzov and Eizirik, 2011) and an antibody specific to the cleaved form can therefore be used to detect apoptosis. Apoptotic beta cells were detected with insulin and cleaved caspase 3 double immunohistochemistry, and quantified as the percentage of caspase 3 and insulin double-positive cells relative to all insulin-positive cells.

TUNEL (III)

In situ terminal transferase-mediated dUTP nick end-labeling (TUNEL) was one of the methods used to detect apoptosis. This method was used to analyze mice treated with multiple low-dose streptozotocin for beta cell apoptosis. Citrate buffer microwave treatment was used for antigen retrieval, and the sections were incubated with TUNEL staining mixture according to the manufacturer’s protocol (Roche), followed by insulin staining. Results were analyzed as percentage of TUNEL and insulin double-positive cells relative to total number of insulin-positive cells.

8. Statistical analysis (I-III)

In all the studies, the data are presented as the mean ±SEM. Student's unpaired t-test was used to compare differences between two groups (I, II). Differences between more than two groups were calculated using one-way ANOVA with Bonferroni (III) or Tukey post hoc test (I, II). In study III the normality of the sample distribution was evaluated and data that were normally distributed according to the Kolmogorov-Smirnov and Shapiro-Wilk tests were analysed by Student's unpaired t-test. The non-parametric Mann-Whitney U-test was used to assess differences between non-Gaussian distributions. The log-rank (Mantel-Cox) test was used for survival analysis (III). $\chi 2$ test was used for the MLDS-induced lymphocyte infiltration (III). Analyses were performed using Graph Pad Prism 6 or SPSS PASW Statistics 22 software.

9. Ethical considerations (I-III)

9.1 Animal experiments

All experimental procedures were approved by the National Animal Experiment Board of Finland (permit number ESAVI-2010-09238/Ym-23). The mice were housed on a 12-h light-dark cycle and fed ad libitum without restriction to physical activity. The wellbeing of mice was checked daily. If any signs of discomfort or impending death emerged (weight loss >20%, immobility), the mice were culled immediately.

9.2 Human islet experiments

Human islets were received from Uppsala, Sweden through the European Consortium for Islet Transplantation. The ethical board of the Children's Hospital, University of Helsinki and the ethical board of the University of Uppsala approved all the procedures. Islets were obtained with consent from the legal next-of-kin. Received islets were leftovers from clinical islet transplantation. All samples were processed anonymously, used only for the intended purposes and discarded afterwards.

RESULTS AND DISCUSSION

1. EGFR expression in the mouse and the human pancreas

The expression pattern of EGFR was analyzed in the mouse and the human pancreas. During development, pancreatic epithelium demonstrated strong EGFR immunoreactivity in the human pancreas (Figure 8). Mesenchymal cells surrounding and intermingled with the pancreatic epithelium were negative for EGFR. In the newborn and adult mice, EGFR-like immunoreactivity was detected in pancreatic islets and in some ductal cells, but not in acinar cells. In adult human pancreatic samples, EGFR immunoreactivity was weaker, but detectable in endocrine cells.

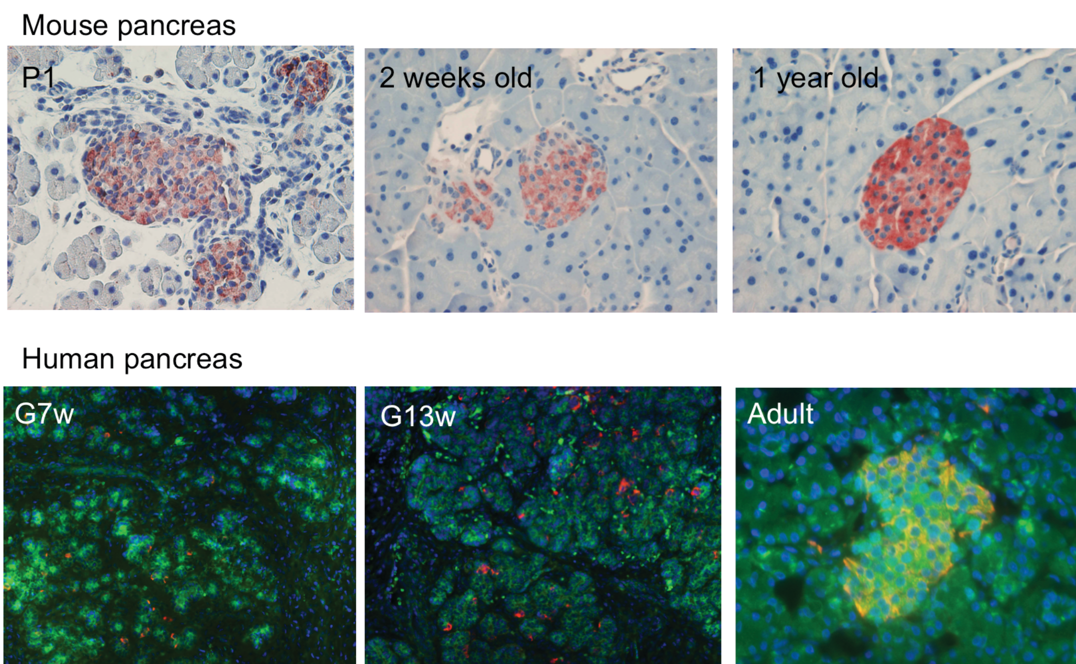

Figure 8 *Upper panel: EGFR (red) immunohistochemistry from, from newborn (P1), 2-weeks-old, and 1-year-old mouse pancreas.*

Lower panel: EGFR (green) and insulin (red) immunofluorescence from 7- and 13-weeks-old fetal pancreas as well as adult (20-years-old female) human pancreas. Double-positive cells shown in yellow.

Others have detected a similar expression pattern for EGFR in the mouse and the human pancreas (Hanley et al., 2011; Kritzik et al., 2000). Furthermore, cell surface proteomics analysis has revealed that EGFR is amongst the 18 most highly expressed cell-surface kinase receptors in the mouse beta cell (Stützer et al., 2012).

2. EGFR in beta cell mass regulation (I-III)

To investigate EGFR signaling *in vivo* in beta cell mass regulation, two mouse models were used: one that has defective EGFR signaling in beta cells (E1-DN), and another one with constitutively active EGFR under tetracyclin regulation (INS-CA-EGFR) (Figure 9). In the EGFR dominant-negative mouse model, the effect of the transgene on EGFR and downstream signaling pathway activation was reduced by approximately 40% (both Akt and Erk phosphorylation). In the constitutively active model, the transgene expression led to approximately a threefold increase in EGFR phosphorylation, and this L858R mutated receptor preferentially activated the Akt instead of Erk pathway in isolated islets. These mice were exposed to different physiological and diabetogenic conditions, as described below, and their beta cell mass and proliferation were analyzed (see Figure 9).

2.1 EGFR in beta cell mass regulation during normal physiology (III)

Previously it was shown that EGFR signaling is essential for normal beta cell mass formation. $EGFR^{-/-}$ mice have impaired islet migration and reduced beta cell proliferation at birth (Miettinen et al., 2000). Furthermore, E1-DN homozygous mice have 80% reduced beta cell mass at two weeks of age (Miettinen et al., 2006). This is mostly due to insufficient beta cell proliferation during the first two postnatal weeks (Miettinen et al., 2006).

However, it remained to be determined what would happen if EGFR were activated specifically in the beta cells during pancreatic development or in the adult. We generated mice with tetracyclin-inducible expression of a constitutively active EGFR in pancreatic beta cells. These mice were generated by crossbreeding Ins-rtTA (Nir et al., 2007) mice with TetOP-$EGFR^{L858R}$ mice (Politi et al., 2006). Upon doxycycline administration, EGFR-L858R was expressed specifically in the insulin-positive and not in the glucagon-positive cells. The L858R mutation causes ligand-independent tyrosine phosphorylation of the receptor (Choi et al., 2007), and facilitates its dimerization (Shan et al., 2012). Expression of L858R in beta cells led

to a threefold increase in EGFR phosphorylation and a twofold increase in Akt phosphorylation in pancreatic islets. However, no increase was detected at Erk phosphorylation. This is in line with previous reports showing that the EGFR L858R mutation preferentially leads to the activation of the PI3K-Akt pathway (Sordella et al., 2004).

To investigate the effect of EGFR activation at different ages, the mice were given doxycycline to activate the transgene at different stages and for different time periods. First, the mice were given doxycycline during embryonic development from E12.5 (when the first insulin-positive cells are present) to postnatal day 1 (P1). EGFR^{L858R} expression during embryogenesis (E12.5-P1) increased insulin-positive volume in the newborn mice. The proliferation rate of beta cells in the doxycycline-treated newborn mice was 40% higher than in controls, which may explain the observed difference in the insulin-positive volume.

In contrast, activation of the transgene in the young and adult murine pancreas had only a negligible effect on beta cell proliferation or mass. One-month-old INS-CA-EGFR mice that received doxycycline for one month had no change in beta cell mass or proliferation. When three-month-old mice were given doxycycline for three months, no significant increase in beta cell mass and proliferation was noted.

In conclusion, these results show that EGFR activation boosts beta cell mass in the neonatal period, but activation of the transgene in the adult murine pancreas has only a minor effect on beta cell proliferation and mass. These results are in line with the results from reverse experiments with the dominant-negative mouse model, in which most of the negative effects on beta cell proliferation took place during the neonatal period, with no difference in beta cell proliferation in adult mice (Miettinen et al., 2006).

Why does the activation of the same receptor lead to different outcomes, depending on the age of the animal? This is not a new phenomenon, as many developing events are temporo-spatially regulated. It is also possible that a single signal is not enough to trigger beta cell proliferation; during the neonatal period many growth factors are elevated in the bloodstream, probably making the beta cells more prone to multiple mitogenic signals. Furthermore, aged beta cells have been shown to proliferate poorly in many studies (Meier et al., 2008; Teta et al., 2005). This might be due to depletion of cell cycle activators or excessive activation of cell cycle inhibitors. At the least, expression of the cell cycle inhibitor

$p16^{INK4a}$ increases with age (Chen et al., 2009; 2011; Krishnamurthy et al., 2006). However, with an appropriate stimulation, aged beta cells can be induced to re-enter the cell cycle, as has been shown with PDGF overexpression (Chen et al., 2011) and phosphatase and tensin homolog (PTEN) deletion, which is a negative regulator of Akt (Yang et al., 2014). Injection of tamoxifen to Pdx1-CreER x $PTEN^{flox/flox}$ mice also resulted in increased beta cell proliferation at the age of 12 months. PTEN-inactivating mutations and deletions have resulted in pancreatic endocrine tumor formation as well (Jiao et al., 2011). These studies suggest that the Akt activity is under a strict negative regulation in pancreatic beta cells.

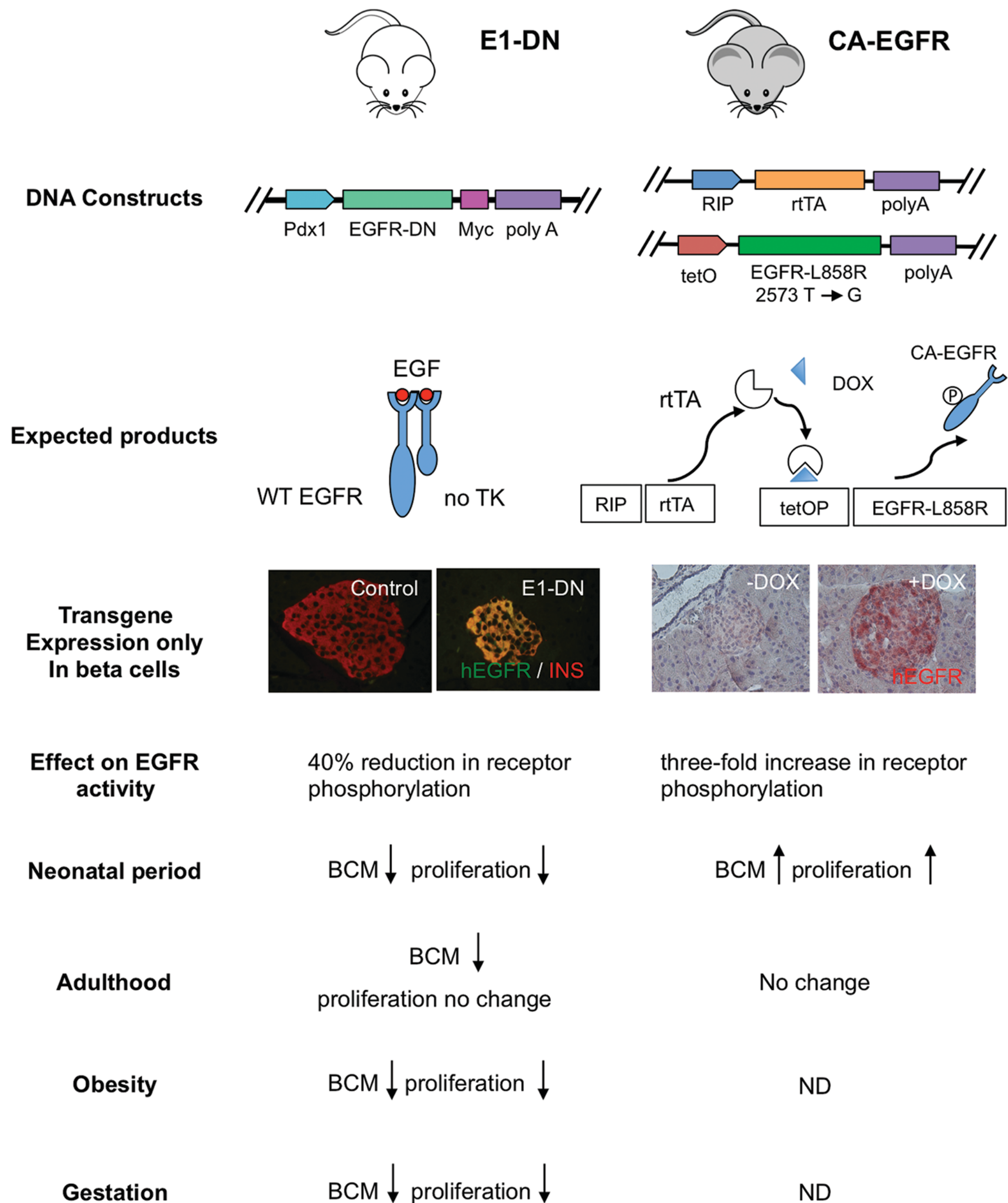

Figure 9 *Summary of results from mouse models used in these studies. E1-DN mice express a dominant-negative EGFR in beta cells, resulting in approximately 40% reduction in EGFR phosphorylation after EGF stimulation. CA-EGFR mice express doxycycline dependently, a constitutively active form of EGFR resulting in approximately a threefold increase in the receptor activation. An upward-pointing arrow indicates that BCM or proliferation is increased. A downward-pointing arrow indicates that BCM or proliferation is decreased. TK, tyrosine kinase; rtTA, reverse tetracyclin transactivator; WT, wild type; BCM, beta cell mass; ND, not done.*

2.3 Beta cell mass regulation during obesity (I)

During obesity, beta cell mass expands to compensate for increased insulin demand. The mechanisms by which the beta cell mass increases during obesity are unclear. To explore whether EGFR signaling is essential to this process, the E1-DN mice and wild-type mice were fed either a high-fat or standard diet for eight weeks. After eight weeks, the high-fat fed E1-DN mice were diabetic, and while the beta cell mass of wild-type mice had increased by 70%, the E1-DN mice failed to show any signs of beta cell mass increase, suggesting that EGFR signaling is crucial to this process.

Recently it was shown that glucose and intralipid infusion induces beta cell mass expansion via a pathway that includes EGFR signaling, mTOR activation, and FoxM1-mediated cell proliferation (Zarrouki et al., 2013). The increase in beta cell mass under these conditions was dependent on EGFR signaling, since the EGFR tyrosine kinase inhibitor AG1478 completely blocked FoxM1 upregulation and the compensatory beta cell mass expansion. Additionally, FoxM1 expression increases during high-fat feeding (Zarrouki et al., 2013), and is upregulated in nondiabetic ob/ob mice (Davis et al., 2010). In future experiments it will be interesting to test the possibility that the E1-DN mice failed to increase beta cell mass due to a defective FoxM1 upregulation.

Another possible mechanism for the EGFR involvement in the high-fat diet-induced beta cell mass increase is through the regulation of survivin (*Birc5*) expression. Transcriptomics analysis has shown that survivin is upregulated during nondiabetic obesity in mouse islets (Keller et al., 2008). Since we have shown that survivin expression is under EGFR regulation during pregnancy, it will be interesting to evaluate whether the same mechanism also applies to beta cell mass increase during obesity.

A third possible mechanism for EGFR involvement is via the GLP-1 system. It has been proposed that GLP-1 production from the L-cells in the intestine stimulates beta cell mass expansion during a high-fat diet (van Citters et al., 2002). Since EGFR transactivation by the GLP-1 receptor is needed for the mitogenic effect of incretins (Buteau et al., 2003), it might be that EGFR signaling is needed in beta cells during high-fat feeding to mediate GLP-1 effects.

2.4 Beta cell mass regulation during pregnancy (I, II, unpublished data)

During pregnancy, the maternal beta cell mass expands to compensate for the increased insulin demand (Kim et al., 2010; Parsons et al., 1992). To investigate whether EGFR signaling is needed in this process, we mated E1-DN and wild-type mice. In this model, similarly to the obesity model, the E1-DN mice completely failed to show signs of beta cell compensation. The beta cell proliferation and mass of E1-DN mice did not increase, leading to impaired glucose tolerance contrary to the wild-type mice that showed marked beta cell proliferation and increased beta cell mass. These results suggest that unaffected EGFR signaling is crucial for the beta cell mass expansion during gestation.

In order to investigate the mechanisms by which EGFR participates in these events, we analyzed the expression of genes known to be involved in the gestational beta cell proliferation from wild-type and E1-DN mouse islets. Survivin is one of the genes upregulated during pregnancy (Rieck et al., 2009). It was originally discovered as an inhibitor of apoptosis. Later on it was discovered that survivin is also involved in cell division: it forms complexes with chromosomal passenger proteins including aurora B kinase, INCENP, and Borealin (Kanwar et al., 2011). We discovered that survivin was expressed at a lower level in the E1-DN mice during gestation when compared to the wild-type mice. All the other genes studied (*Tph1*, *Tph2*, *FoxM1*, *Prlr* and *Egfr*) were expressed at a similar level in both genotypes. Our results are the first to show that survivin expression is under EGFR regulation in pancreatic beta cells during gestation.

There are several possible mechanisms by which EGFR can activate survivin expression, summarized in Figure 10. Prl/PL binding to PRL-R activates JAK2, which likely transactivates the EGFR. After EGFR activation, the signal transduction could theoretically be mediated through PI3K-mTOR-FoxM1, shown to be the signaling pathway during beta cell mass compensation in the intralipid and glucose-infusion model (Zarrouki et al., 2013), as survivin is a known target of FoxM1 (Wang et al., 2005). Another option is that survivin transcription is regulated through the route EGFR-PI3K-HIF1alpha-survivin, a signaling pathway present in cancer cells (Peng et al., 2006). The third option is that the effect goes through the EGFR-Erk pathway, which has been shown to regulate survivin protein stability in beta cells (Wang et al., 2010). To test this, we stimulated isolated islets with PL and with or without the EGFR inhibitor gefitinib, the MEK inhibitor PD0325901, or the mTOR inhibitor rapamycin. PL-

induced survivin upregulation was blocked by the EGFR inhibitor gefitinib, as well as by the MEK inhibitor and by rapamycin. Thus, it seems that PL-induced survivin expression is regulated by EGFR activation, and the mechanisms include MAPK and mTOR pathways. Since survivin forms complexes with chromosomal passenger proteins to regulate cell division (Kanwar et al., 2011) and its absence in beta cells reduces beta cell proliferation (Jiang et al., 2008), it seems likely that the inability of E1-DN islets to upregulate survivin properly, affects the replication potential of the beta cells and thus leads to insufficient proliferation during gestation.

Since the dominant-negative EGFR also downregulates signaling through the other ErbB receptors (Spivak-Kroizman et al., 1992), future studies are needed to elucidate the possible contribution of ErbB2-4 receptors in beta cell mass expansion during metabolic challenges.

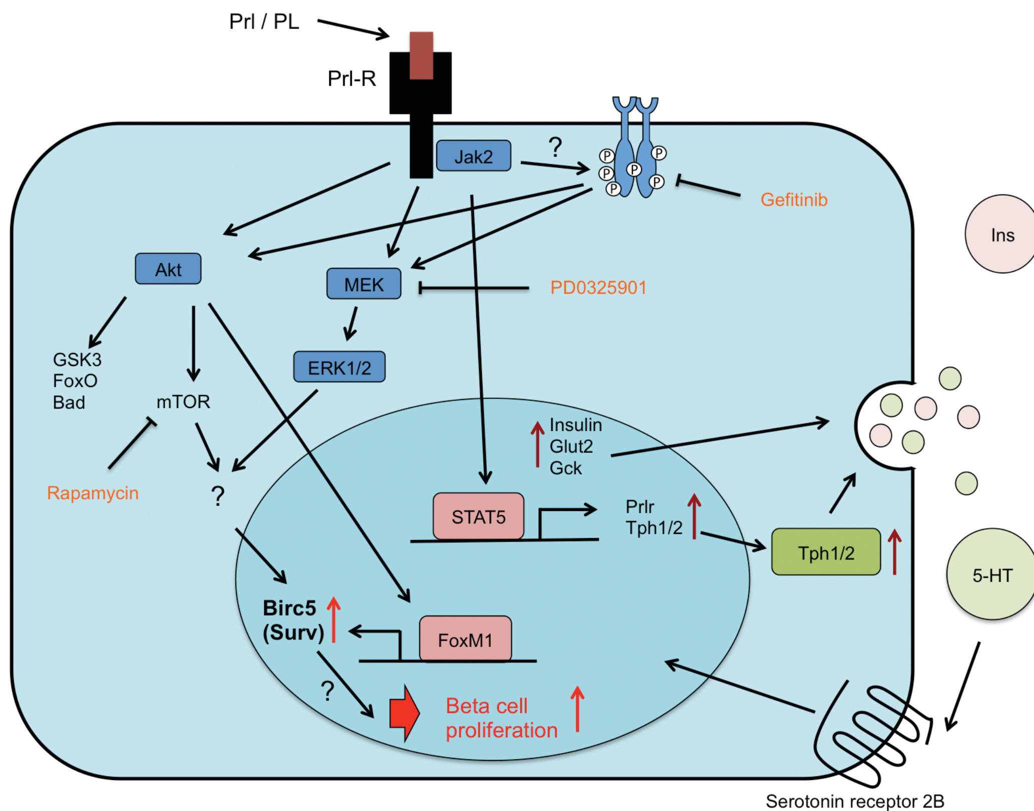

Figure 10 *Illustration of the proposed model of how EGFR participates in beta cell proliferation during pregnancy. Binding of a ligand to Prl-R leads to JAK2-STAT5 pathway activation and subsequent upregulation of many cell cycle related genes, as well as Tph1/2, which leads to production of serotonin (5-HT). Prl-R activation also leads to activation of the EGFR, probably through Jak2. EGFR activation is needed for Prl/PL-induced upregulation of survivin. Inhibitors used are shown in orange: gefitinib inhibits the tyrosine kinase activity of EGFR, rapamycin is an mTOR inhibitor and PD0325901 is a MEK inhibitor. For abbreviations see pages 10-11.*

In humans, the adaptive changes in β-cell mass during pregnancy are less clearly correlated with increased beta cell proliferation. It has been shown that the beta cell mass increases during gestation, but the increase is achieved by increased numbers of new small islets rather than by increased proliferation (Butler et al., 2010). Alternatively, it was recently suggested that the lack of proliferating beta cells in human autopsy or cadaveric samples might be an artifact of the postmortem state (Caballero et al., 2013). Nevertheless, when human islets are stimulated *in vitro* with prolactin or placental lactogen, the β-cell proliferation has been shown to increase modestly, but at a lower level than in rodents and there is marked subject-to-subject variation (Brelje et al., 1993).

In an attempt to validate the relevance of our findings to human β-cells, we also studied PL-induced proliferation in human islets obtained from four female organ donors (ages 57-72 years) (unpublished data). Isolated islets were cultured with PL 500 ng/ml with or without EGFR inhibitor gefitinib (10 μM) for 96h, and the proliferating β-cells were recorded by BrdU/C-peptide double-positive cells. Overall, the β-cell proliferation was very low (baseline 0.2%). Islets from three out of four donors responded to PL stimulation by increasing proliferation, the best response being a 3.5-fold increase in β-cell cell labeling index (from 0.2% to 0.7%; see Figure 11). However, β-cells from one donor were completely unresponsive to PL stimulation. All donors were postmenopausal which may have affected β-cell proliferation and could explain the unresponsiveness to PL stimulation. Single nucleotide polymorphisms in the 5' UTR and promoter region of *PRLR* have been associated with gestational diabetes (Le et al., 2013); since we did not analyze these SNPs from our organ donors, it is also possible that some of the donors had SNPs affecting *PRLR* expression and translation. Nevertheless, the PL treatment increased human β-cell proliferation by 2.5-fold, and this effect was blocked by the EGFR inhibitor gefitinib, implying that EGFR signaling might also participate in the PL-induced proliferation of human β-cells.

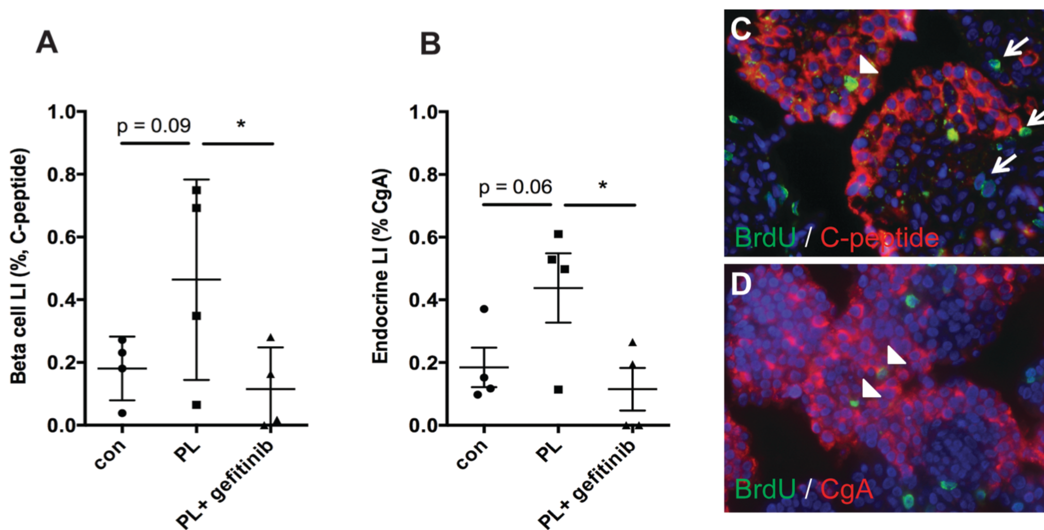

Figure 11 *EGFR signaling is needed for PL-induced beta cell proliferation in human islets. Isolated human islets were cultured with PL 500 ng/ml with or without EGFR inhibitor gefitinib 10 µM for 96h. BrdU was added to the media for the last 48h. A: Quantification of proliferating β-cells analyzed by BrdU and C-peptide double-positive cells relative to the total number of C-peptide-positive cells. B: Quantification of proliferating endocrine cells analyzed by BrdU and ChromograninA (CgA) double-positive cells relative to the total number of CgA-positive cells; n=4 in each group. C: Representative C-peptide and BrdU double immunofluorescence from PL-stimulated human islets (arrowhead: a double-positive cell and arrows: C-peptide negative proliferating cells) and D: Representative CgA and BrdU double immunofluorescence from PL-stimulated human islets (arrowheads: double-positive cells). Bars represent the mean ±SEM for each group. * p < 0.05.*

2.5 Beta cell mass regulation after PDL (I)

In addition to obesity and pregnancy, we wanted to study the involvement of EGFR signaling during pancreatic regeneration. For this, we chose the pancreatic duct ligation (PDL) model, which is considered to involve neogenesis from Ptf1a-positive acinar cells (Pan et al., 2013), as well as carbonic anhydrase-positive ductal cells (Inada et al., 2008) and proliferation of pre-existing beta cells (Xu et al., 2008). After PDL, the relative growth of the beta cell surface area was equal in the E1-DN and the wild-type mice. Furthermore, the increase in beta cell

proliferation was equal in the two genotypes, implying that EGFR signaling is not important in beta cell proliferation after PDL.

Since our transgene expression was under the Pdx1 promoter, EGFR signaling was unaffected in the exocrine cells (Miettinen et al., 2006), leaving open the question of whether EGFR signaling is essential in neogenesis after PDL. Additionally, it has been reported that acinar cells transdifferentiate into beta cells with EGF and CNTF stimulation after alloxan-induced diabetes (Baeyens et al., 2014). It is thus possible that EGFR signaling also plays a role in the acinar-to-endocrine transdifferentiation after PDL. However, beta cell proliferation after PDL increased at a similar level in the E1-DN and the wild-type mice, suggesting that in this model, the factors inducing beta cell proliferation are different from those operating in obesity and pregnancy.

3. EGFR in beta cell protection

3.1 Protection against diabetes, *in vivo* models (III)

Activation of the PI3K pathway protects beta cells against various pro-apoptotic stimuli (Bernal-Mizrachi et al., 2001; Elghazi and Bernal-Mizrachi, 2009; Tuttle et al., 2001). Since EGFR also activates the PI3K pathway, we hypothesized that $EGFR^{L858R}$ might protect beta cells against apoptosis. In order to study this, mice with CA-EGFR expression were exposed to agents that induce beta cell stress and death. The first diabetogenic challenge was done with a high-dose STZ, after which the wild-type mice developed overt hyperglycemia, while the CA-EGFR mice developed less-severe hyperglycemia and had a slightly higher survival rate. Furthermore, beta cell mass and insulin levels were higher in the CA-EGFR mice 40 days after STZ injection, showing that expression of CA-EGFR had either prevented beta cell loss or promoted regeneration. Since the effect on glycemia was immediate and we did not detect any difference in the beta cell proliferation rate, the more likely explanation is that CA-EGFR protected beta cells from apoptosis.

Next, we exposed the mice to multiple low-dose streptozotocin treatment (MLDS), which causes an initial mild toxic insult and then progressive immune-mediated beta cell apoptosis (Kantwerk-Funke et al., 1991). CA-EGFR protected these mice against hyperglycemia in this model as well. Importantly, in the MDLS model we also detected a reduction in the apoptosis rate, showing that EGFR activation does protect beta cells from apoptosis.

Additionally, CA-EGFR reduced the islet immune-cell invasion after MLDS. This might be secondary to the reduced beta cell death, and thus to the decreased priming of the immune system against beta cell antigens.

STZ is a toxic glucose analogue that accumulates in beta cells through GLUT2 (Lenzen, 2008; Wang and Gleichmann, 1998). In some other transgenic mouse models, particularly in the Pax4 overexpression mouse model, protection against STZ has been suggested to be mediated at least partially through a reduced level of Glut2 expression (Hu He et al., 2011). To exclude the protective effect of CA-EGFR via a reduced STZ entry to the cells, GLUT2 levels were studied; no differences between the mouse groups were detected, ruling out this pitfall.

3.2 Protection against cytokines, *in vitro* (III)

In type 1 diabetes, the proinflammatory cytokines IL-1β, IFN-γ and TNF-α contribute to beta cell apoptosis and the development of insulitis (Eizirik and Mandrup-Poulsen, 2001; Eizirik et al., 2009; Thomas et al., 2009). To investigate whether CA-EGFR directly protected beta cells against cytokine-induced beta cell death, isolated islets were exposed to cytokines and cell viability analyzed. CA-EGFR had a significant protective effect in this context as well.

To elucidate the molecular mechanisms by which EGFR signaling protects against beta cell death, we exposed islets from CA-EGFR and wild-type mice to a cytokine cocktail and analyzed the expression levels of selected genes. The cytokine-induced beta cell death is mediated via activation of the intrinsic pathway of cell death, with an important role for DP5, PUMA, and BIM (Barthson et al., 2011; Gurzov and Eizirik, 2011). In our model, CA-EGFR significantly suppressed *Bim* but did not modify *Dp5* or *Puma* expression. It was previously shown that silencing of *Bim* in beta cells protects against cytokine-induced cell death (Barthson et al., 2011). Furthermore, *PTPN2* (Santin et al., 2011) and *GLIS3* (Nogueira et al., 2013), two candidate genes for type 1 diabetes, contribute to beta cell apoptosis via BIM modulation. *Bim* expression is regulated by multiple pathways in neurons and fibroblasts, including the PI3K-Akt-FoxO and the Erk1/2 MAPK pathways (Ley et al., 2005; Qi et al., 2006), and the relationship between EGFR and Bim has been previously reported in lung adenocarcinomas with L858R mutation. In cancer cells with EGFR L858R mutation, EGFR inhibition leads to *Bim* up-regulation, implying that $EGFR^{L858R}$ suppresses *Bim* (Costa et al., 2007; Gong et al., 2007). Furthermore, pharmacological inhibition of the PI3K pathway

causes inactivation of Akt, allowing dephosphorylation and nuclear entry of FOXO-3A, which induces *Bim* mRNA expression (Dijkers et al., 2000; Ley et al., 2005). Thus, our results, showing that EGFR-induced Akt activation leads to suppressed Bim expression, are in line with these previous studies. However, we are the first to show that EGFR signaling affects Bim expression and protein levels in pancreatic beta cells.

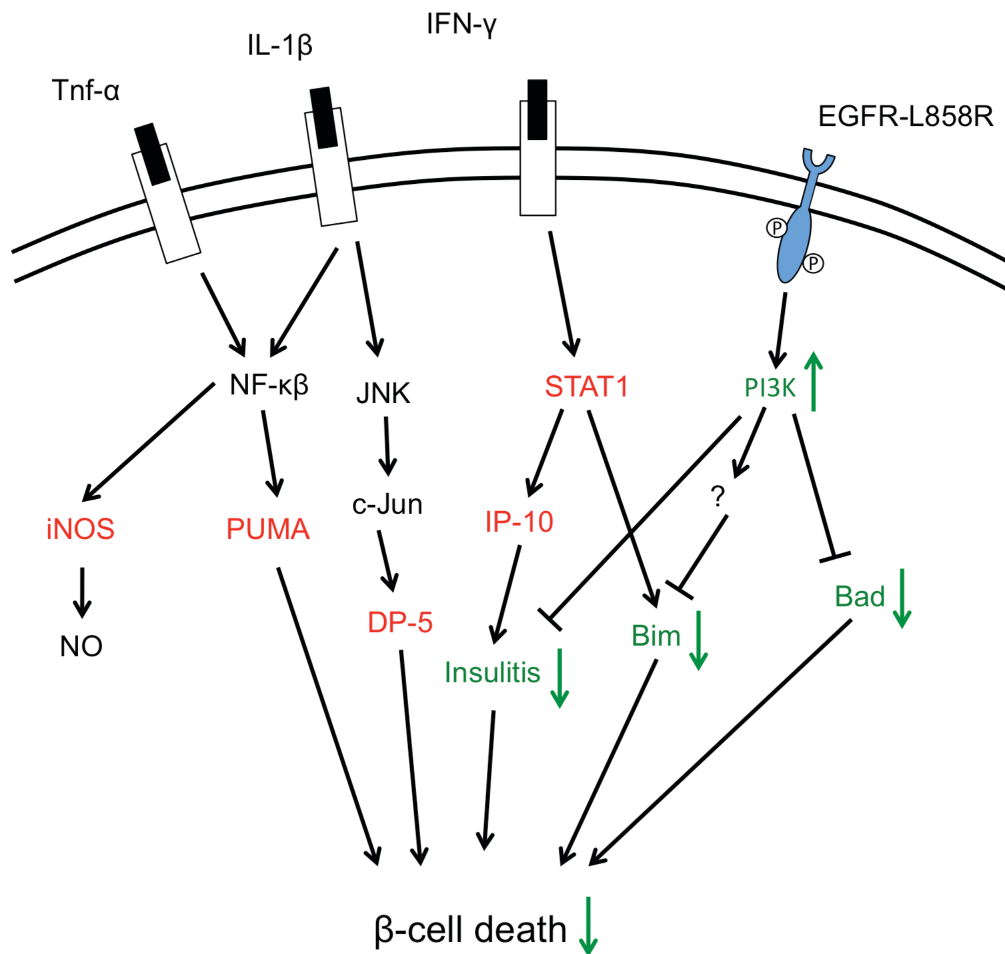

Figure 12 *Illustration of the proposed model of how EGFR activation protects beta cells against apoptosis. Genes/proteins that were altered in CA-EGFR mice either by expression or phosphorylation during cytokine exposure are presented in green. Expression of genes that were studied but did not show any changes are in red and proteins/genes that were not studied are in black. EGFR*^{L858R} *activates the PI3K, which leads to phosphorylation and inactivation of Bad and downregulation of Bim. Additionally, EGFR*^{L858R} *signaling reduced insulitis. Together these factors led to beta cell death reduction in different models of experimental diabetes.*

L858R mutation facilitates EGFR dimerization (Shan et al., 2012), and thus also increases signaling through heterodimers with other ErbB receptors. What is interesting in this context is the finding that SNPs in the *ERBB3* gene are associated with T1D (Keene et al., 2012). More studies are needed to elucidate the contribution of other ErbB kinases to beta cell proliferation and protection detected in this study.

4. Limitation of the study

A major problem with using transgenic mouse models is how to translate these findings to humans, and particularly to clinical medicine. There are several differences between mice and men, e.g. factors that promote beta cell proliferation in rodents have attenuated or no effect with human beta cells (Parnaud et al., 2008).

In addition, the transgenic approach itself has some weaknesses. First, all of the mouse models used in these experiments are generated by pronuclear injection. Due to the random nature of transgene integration, position-site-dependent effects may alter the transgene expression and also alter the expression of other genes at the site of insertion (Gama Sosa et al., 2010; Magnuson and Osipovich, 2013). Second, the promoters used in these constructs are not physiological, which might lead to extremely high levels of the transgene product. Especially in the case of insulin promoter, the transgene expression might vary according to the metabolic state, meaning that during STZ-induced hyperglycemia, insulin biosynthesis is highly stimulated in the surviving beta cells, potentially resulting in a higher transgene expression than under normal physiological conditions. Third, both the insulin and Pdx1 promoters have been shown to have ectopic expression in the hypothalamus, which might affect energy intake and thus metabolism (Magnuson and Osipovich, 2013; Wicksteed et al., 2010).

In general, the selection of promoters affects our results. If the expression of the dominant-negative EGFR had been targeted at acinar cells, the results with the partial duct ligation experiment could have been different. Similarly, if we had expressed the $EGFR^{L858R}$ in acinar cells, it would have been interesting to evaluate whether this could lead to acinar-to-endocrine transdifferentiation. Furthermore, it would have been interesting to express $EGFR^{L858R}$ in multipotent pancreatic progenitors to evaluate the effect of EGFR activation in pancreatic development.

The major limitation in the human islet work is that the islets were from postmenopausal females. The age of the islet donors may affect the responsiveness of beta cells to lactogenic stimulation. Additionally, by the time we received the islets, they had already been artificially cultured for several days, which may also affect the proliferative capacity.

CONCLUSIONS AND FUTURE PERSPECTIVES

Knowledge about the factors that regulate and protect beta cell mass has increased dramatically over the last several years. Since both type 1 and type 2 diabetes are characterized by a reduction in functional beta cell mass, there is great interest in identifying methods to preserve or regenerate beta cells for therapy. One of the potential targets is EGFR, a tyrosine kinase receptor abundantly present in the pancreatic islets. BTC and EGF, ligands of the EGFR, have been shown to be among the top 20 most potent inducers of beta cell proliferation. Furthermore, it has been shown that EGFR is necessary for neonatal beta cell proliferation. In this thesis I aimed to elucidate the role of EGFR in beta cell mass regulation during situations in which beta cell mass either increases to compensate for an increased insulin demand or decreases due to diabetogenic conditions. Mouse models with beta cell-specific EGFR downregulation and activation were used, as were human islets.

The results show that EGFR signaling is crucial for beta cell mass expansion during some metabolic challenges. Even a partial attenuation of EGFR signaling in beta cells interfered with the normal compensatory increase in beta cell proliferation and beta cell mass during obesity and pregnancy. The mechanistic studies suggested that EGFR signaling during gestation is needed for the upregulation of survivin.

In our studies, the effect of EGFR inhibition or activation on beta cell mass in normal physiological situations was negligible. This might also explain why there are no diabetogenic side effects with the EGFR inhibitors used for the treatment of advanced cancers. The most common adverse effect of these drugs is folliculitis (Bachet et al., 2012). However, if indications of EGFR inhibitors in clinical setting enlarge to also encompass other diseases, and younger individuals, the possible diabetogenic effect should be carefully evaluated.

EGFR activation protected against experimental diabetes *in vivo*. Furthermore, EGFR protected against cell death induced by diabetogenic cytokines *in vitro*. Subsequent mechanistic studies showed that EGFR signaling repressed the proapoptotic protein Bim

during cytokine exposure, and this likely contributed to the reduced cell death in islets with constitutively active EGFR.

Translating these findings to human diabetes is challenging. First, results obtained with the mouse beta cells are not necessarily similar to those that would be obtained with human beta cells. In terms of diabetes treatment, it is very important to evaluate the role of EGFR in human beta cells. If EGFR activation proves stimulating to the proliferation of human beta cells, therapeutic applications will require development of tissue-specific carrier proteins due to the broad expression pattern and oncogenic nature of EGFR.

Each advance raises additional questions. Future studies will reveal the molecular mechanism through which EGFR signaling regulates beta cell mass increase during nondiabetic obesity. Part of this data was based on the signaling stimulated by the $EGFR^{L858R}$ and it remains to be elucidated, whether similar beta cell protective effects are achievable with activation of the wild-type receptor. Finally, we showed that $EGFR^{L858R}$ results in Bim repression, but the exact molecular mechanism of how Bim expression is regulated remains open.

In summary, these studies show that proper EGFR signaling is crucial for beta cell mass expansion during metabolic challenges, and that EGFR stimulation protects beta cells against apoptosis. These results suggest that activating EGFR signaling could prevent or revert beta cell loss in diabetes.

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