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Analysis of a new transgenic mouse model with β-cell-specific overexpression of human betacellulin

Work performed under supervision of
Dr. Marlon R. Schneider

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Untersuchung eines neuen transgenen Mausmodels mit β-Zell-spezifischer Überexpression des humanen Betacellulins

Arbeit angefertigt unter der Leitung von
Dr. Marlon R. Schneider

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Dedicated to my family

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ABBREVIATIONS

µg	microgram
µl	microliter
µM	micromol
Ala31	alanine 31
AREG	amphiregulin
Asp32	aspartic acid
AUC	area under the curve
bGH	bovine growth hormone
bp	base pairs
BTC	betacellulin
C.P.	*caudate putamen*
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
co	control
Cre	causes recombination
Cy3	cyanine 3
Cy5	cyanine 5
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPGN	epigen
ErbB	epidermal growth factor receptor
EREG	epiregulin
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GH	growth hormone

$GIPR^{dn}$	dominant negative glucose-dependent insulinotropic polypeptide receptor
GLK	glucokinase
HB-EGF	heparin binding-epidermal growth factor
hBTC	human betacellulin
HEK 293	human embryonal kidney 293 cells
HER	human epidermal growth factor receptor
hGH	human growth hormone
HIP	human insulin promoter
HNF-1 α	hepatocyte nuclear factor-1 α
HRP	horseradish peroxidase
IGF-I	insulin-like growth factor I
IL-18	interleukin-18
INS	insulin gene
Ins2	insulin 2 gene
L112	leucine 112r
L2	line 2
L3	line 3
MAPK	mitogen-activated protein kinase
MIN-6	mouse insulinoma cell line-6
MODY	maturity onset diabetes in the young
mRNA	messenger ribonucleic acid
NOD	non-obese diabetic mice
nm	nanometer
ob/ob	obese diabetic mice
pA	polyadenylation
PACAP	pituitary adenylate cyclase-activating polypeptide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-L1	programmed death-1 ligand
Pdx1	pancreatic duodenal homeobox factor-1
PI3K	phosphoinositide 3-kinase
PIP	porcine insulin promoter

PVDF	polyvinylidienfluorid
rAd-BTC	recombinant adenovirus-BTC
Ras	rat sarcoma
rhBTC	recombinant human betcellulin
RIP	rat insulin promoter
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Shc	src homology 2 containing protein
STAT3	signal transducer and activator of transcription 3
STAT5	signal transducer and activator of transcription 5
STZ	streptozotocin
TATA box	Goldberg-Hogness box
TBS	tris buffered saline
tg	transgenic
TGFA	transforming growth factor - alpha
TRITC	tetramethylrhodamine-5-(and 6)-isothiocyanate
UTR	untranslated region
Y111	tyrosine 111

1 INTRODUCTION AND OBJECTIVES

Since 1982 genetically modified mice have been widely employed in the biological and medical research in order to investigate different human and animal diseases and also to test possible therapies. Recently, transgenic animal models have also been generated to develop new treatment options in diabetes mellitus. The transgenic technology has been used for targeting molecules like hormones (Cayrol *et al.* 2006), oncogenes (Pelengaris *et al.* 2002), growth factors (Garcia-Ocana *et al.* 2001) or transcription factors (Ahlgren *et al.* 1998) in the pancreatic islets. Thereby, the rat (Hanahan 1985; Vasavada *et al.* 1996), mouse (Hara *et al.* 2003) and human (Hotta *et al.* 1998; Krakowski *et al.* 1999) insulin promoters have been employed to control the overexpression of the transgene in the pancreatic β-cells. However, a big drawback of the most frequently recommended rat insulin promoter is the detected expression of the transgene not only in β-cells but also in several areas of the brain (Gannon *et al.* 2000; Martin *et al.* 2003).

The use of mice as animal models is still useful for studies investigating basic problems of β-cell physiology. However, researches regarding, for instance, islet xenotransplantation approaches require large animal models. Due to similarities to humans in the blood glucose level regulation and in the general physiology, pigs are excellent candidates for such studies. Furthermore, results of different studies support the possibility to obtain pancreatic islets from newborn and adult pigs for xenotransplantation (MacKenzie *et al.* 2003; Cardona *et al.* 2006; Hering *et al.* 2006). Therefore, there is a considerable interest in the development of tools for the genetic modifications of porcine pancreatic islets for the delivery of gene products direct to β-cells.

The aim of the present study was to create an expression cassette for the β-cell-specific overexpression of transgenes under the control of the porcine insulin promoter.
The functionality of the construct was verified by expressing human betacellulin, a ligand of the EGFR family, known for its ability to stimulate β-cell differentiation in vitro and in transgenic mouse model.

2 LITERATURE REVIEW

2.1 The Epidermal Growth Factor Receptor system

2.1.1 Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors comprises the following members: ErbB1/EGFR/HER1, ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4 (Figure A).

Studies on the structure of EGF receptors revealed the presence of several common regions, including an extracellular ligand-binding region, a single membrane-spanning region and a cytoplasmic part with a tyrosine kinase-containing domain (Holbro & Hynes 2004).

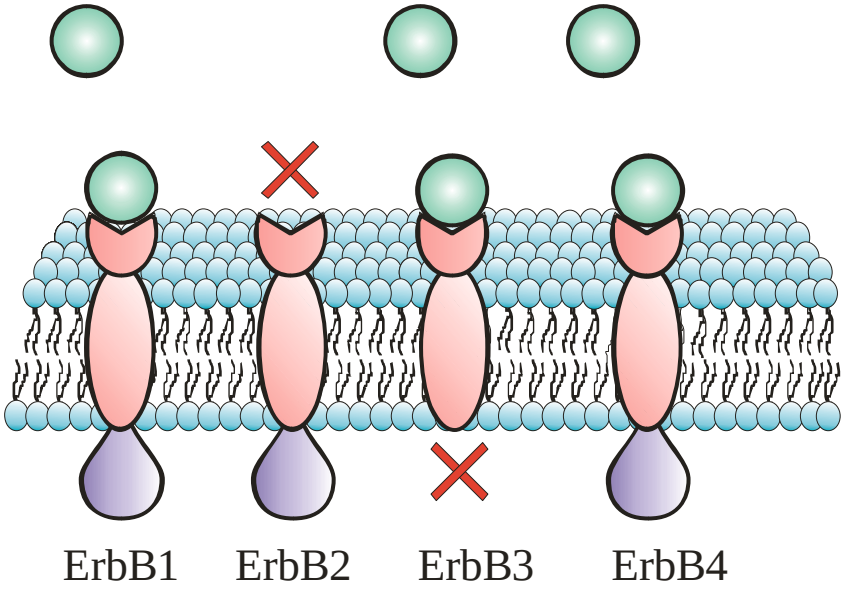

Figure A Epidermal Growth Factor Receptor and the related ErbB2-4 receptors.

The EGFR and many of its ligands are broadly expressed in the gastrointestinal tract, including the pancreas (Miettinen & Heikinheimo 1992). In the adult pancreas, the EGF receptor is predominantly expressed in the islets of Langerhans and ductal epithelial cells (Ueda *et al.* 2004). The expression of ErbB2 in the developing mouse pancreas has been detected in the ducts (Kritzik *et al.* 2000), as well as in the proliferating human adult duct cells (Rescan *et al.* 2005). ErbB3 is expressed in the developing mouse pancreatic ducts (Kritzik *et al.* 2000) and mesenchyme surrounding the pancreas (Erickson *et al.* 1997). The ErbB4 protein has been detected in the mouse pancreas as early as embryonic (E) day 12.5 (Huotari *et al.* 2002) and it is expressed in the fetal mouse pancreatic ducts (Kritzik *et al.* 2000). In the proliferating human adult pancreatic duct cells ErbB4 is expressed only weakly (Rescan *et al.* 2005).
Moreover, the presence of the ErbB receptors has been shown also in other tissues like peripheral nervous system (Woldeyesus *et al.* 1999), heart (Gassmann *et al.* 1995; Lee *et al.* 1995), and several epithelial organs (Miettinen *et al.* 1995; Sibilia *et al.* 1998).

The activation of ErbB receptors initiates a rich network of signaling pathways, culminating in variety of responses ranging from cell division to death. The phosphorylated residues are recognized as docking sites by different signal molecules, which activation promotes intracellular signaling cascades and control variety of genetic processes. Additionally, the activation of ErbBs occurs not only through ligand binging, but also via heterologous signals, like hormones, neurotransmitters, lymphokines and stress inducers (Carpenter 1999). However, two of the ErbB receptors make an exception to this rule. The “ligandless” ErbB2 receptor requires heterodimerisation with another ErbB receptor to be activated (Klapper *et al.* 1999). Also the ErbB3 receptor needs a dimerisation with a second ErbB receptor to become phosphorylated and to initiate signalling pathways (Guy *et al.* 1994). Therefore, neither ErbB2 nor ErbB3 in isolation can perform its function as membrane receptor. The most important downstream signaling pathways of ErbB receptors (Figure B) are the Ras- and Shc-activated MAPK networks, as well as phosphoinositide 3-kinase (PI3K)-activated pathway (Yarden & Sliwkowski 2001).

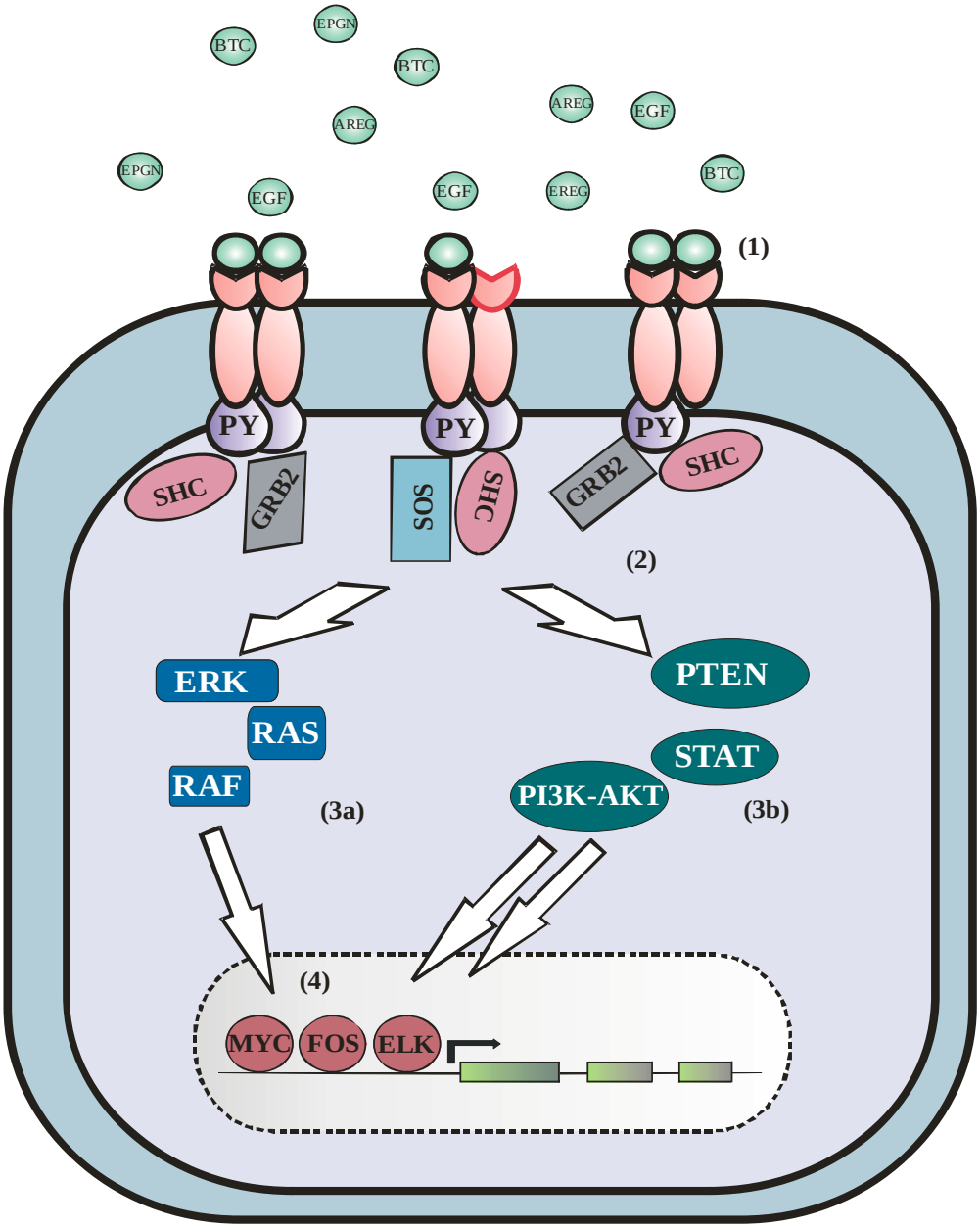

Figure B The EGFR system: Binding of a ligand (1) permits the receptor dimerisation and activation (2) of downstream signaling pathways, (3a and 3b) changing the activity of multiple nuclear transcription factors and cellular transcriptional program (4).

The biological role of the ErbB receptors and their ligands has been often studied by using genetically modified mouse models. The epidermal growth factor receptor (EGFR)-deficient mice exhibited a significant reduction of the pancreas weight and disturbed pancreas morphogenesis (Miettinen *et al.* 2000). These alterations have been suggested to result from the impaired branching of the ductal tree. Although EGFR activation was reduced by only 40%, the transgenic mice exhibited a significant

decreased β-cell mass and became diabetic within two weeks of birth. To study the role of the EGF receptor in adult pancreas, a mouse model expressing a kinase-deficient and dominant-negative EGFR under the control of *Pdx1* (pancreatic duodenal homeobox factor-1) promoter was generated (Miettinen *et al.* 2006). These mice showed an impaired postnatal β-cell growth, resulting in diabetes (Miettinen *et al.* 2006).

Moreover, the other ErbB receptors are probably also required for an appropriate function of the pancreas. Knockout mouse models for ErbB2-, ErbB3- and ErbB4 exhibited early prenatal mortality, mostly before pancreatic organogenesis could take place (Gassmann *et al.* 1995; Lee *et al.* 1995; Erickson *et al.* 1997).

ErbB receptors have been shown to play an important role in the development and functionality of many other organs. For instance, EGFR knockout mice revealed the necessity of this receptor for the proper craniofacial development (Miettinen *et al.* 1995). In this study, the heterozygous $Egfr^{+/-}$ mice exhibited a normal weight and reproductive ability, while $Egfr^{-/-}$ animals survived only a few days after birth because of lethal defects in the respiratory system and epithelial immaturity in several organs. Additionally, absence of a functioning EGFR in these mice led to abnormalities in the epidermis and hair coat. The skin was thinner and almost transparent, with only few hair follicles (Miettinen *et al.* 1995). Other studies on mice lacking the *Egfr* gene also demonstrated alterations in skin, kidney, brain, liver and gastrointestinal tract (Miettinen *et al.* 1995; Sibilia & Wagner 1995; Threadgill *et al.* 1995).

Numerous studies demonstrated that alterations of ErbB receptor activity were associated with the appearance of severe cancer types including carcinomas of the breast (Slamon *et al.* 1987; Slamon *et al.* 1989; Barnes *et al.* 1992; Prenzel *et al.* 1999; Gschwind *et al.* 2002), colon and rectum (Shirai *et al.* 1995; Yang *et al.* 1997), endometrium (Berchuck *et al.* 1991), lung (Brandt-Rauf *et al.* 1994), ovary (Tanner *et al.* 1996) and prostate gland (Zhau *et al.* 1992; Fox *et al.* 1994). Abnormalities in the ErbB pathway system are often the reason for initiation and progression of cancer. Overproduction of ligands, overexpression of receptors or constitutive activation of receptors can drive the cell to uncontrolled proliferation. One of the best characterized ErbB ligands connected with human cancers is TGFA (transforming growth factor-

alpha). Overexpression of TGFA and EGFR, particularly in lung, ovary and colon tumors, predicts poor recovery chances (Sandgren *et al.* 1990; Salomon *et al.* 1995). Overexpression of ErbB2 is often associated with the invasive ductal breast cancers (Slamon *et al.* 1987; Slamon *et al.* 1989), however alterations in ErbB2 alone are insufficient for breast cancer progression (Ross & Fletcher 1999). Importantly, the heterodimers ErbB2/ErbB3 represent an extremely high mitogenic complex (Pinkas-Kramarski *et al.* 1998). Nevertheless, it has been shown that down-regulation of ErbB2 follows decrease of ErbB3 expression as well (Lane *et al.* 2000; Neve *et al.* 2000; Basso *et al.* 2002; Motoyama *et al.* 2002). This observation suggests a close connection between these two partners in the initiation of signaling pathways and cell proliferation.

2.1.2 Ligands of the Epidermal Growth Factor Receptor family

There are seven known EGFR ligands: epidermal growth factor (EGF), transforming growth factor-alpha (TGFA), amphiregulin (AREG), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EREG) and epigen (EPGN) (Harris *et al.* 2003; Schneider & Wolf 2009). The genes encoding four of these ligands, EREG, EPGN, AREG and BTC are loated on the same chromosome in mice and humans (chromosomes 5 and 4, respectively) (Pathak *et al.* 1995). This tight linkage suggests that they may have arisen through a tandem gene duplication event. In general, the overall organization of the genes encoding members of the EGF family is highly conserved. These genes consist of 6 exons, where the first one encodes the 5'UTR and signal peptide. Exon 2 encodes the N-terminal precursor. The third exon encodes the mature EGF with the two first disulfide loops and the fourth accounts for expression of the third loop of the EGF-like domain. Exon 5 encodes the cytoplasmic region and exon 6 the 3'UTR (Harris *et al.* 2003).

The ligands of the EGFR family are synthesized as type I transmembrane proteins consisting of an N-terminal part, EGF motif, a short juxtamembrane stalk and a carboxy-terminal domain. The EGF motif contains 40 amino acids with six cysteines arranged as three disulfide bridges and forming three loops (Harris *et al.* 2003; Schneider & Wolf 2009;). An amino acid between the second and the third loop is called "hinge residue". The probable function of this structure is to simplify the movement of these two structures (van Zoelen *et al.* 2000).

Cleavage of the EGFR ligands by surface proteases allows releasing the mature ligands from the cell membrane and their activity as growth factors (Massague & Pandiella 1993).

2.1.2.1 Amphiregulin (AREG)

AREG was identified in the human breast adenocarcinoma cell line, MCF-7 (Shoyab *et al.* 1988). This growth factor is expressed in many tissues, including placenta, ovary, testis, heart, pancreas, spleen, kidney, lung, ovary, colon and breast (Plowman *et al.* 1990). Under normal conditions, amphiregulin is not expressed in the liver. Nevertheless some studies demonstrated an upregulated AREG gene expression after liver injury or partial hepatectomy (Berasain *et al.* 2005; Castillo *et al.* 2006; Fausto *et al.* 2006). Amphiregulin activation appears to have an important functional role in the development and maturation of different organs, including mammary gland (Kenney *et al.* 1996; Sternlicht *et al.* 2005), bones (Qin *et al.* 2005) or placenta (Fukami *et al.* 2009). Due to its ability to inhibit the growth of many human tumors and to stimulate the proliferation of other cells, like fibroblasts, keratinocytes or epithelial cells, AREG is known as a bifunctional growth factor (Plowman *et al.* 1990; Cook *et al.* 1991; Normanno *et al.* 1994; Chu *et al.* 2005).

Reduced expression of AREG was detected in prolactin receptor knockout mice (Ormandy *et al.* 2003). Also, female AREG-deficient mice exhibited a defective mammary gland development and inadequate quality or quantity of milk (Luetteke *et al.* 1999). Together, these findings indicate that amphiregulin might be regulated by prolactin during the pregnancy and is necessary for an appropriate growth of mammary tissue.

Amphiregulin seems to have an effect on other organs as well. For instance, overexpression of AREG in mouse pancreas caused an increased proliferation of pancreatic duct cells (Wagner *et al.* 2002). Results of such investigations suggested that amphiregulin induces a mitogenic response in pancreatic duct cells through activation of Ras, CDK2 or CDK4 (Wagner *et al.* 2002). Moreover, studies on human pancreatic cancer cell lines indicated an elevated expression of amphiregulin in the cytoplasm of these cells being a signal of a more advanced clinical stage of the disease. These

findings suggested the contribution of AREG to aberrant activation of EGF receptor in pancreatic tumor cells (Ebert *et al.* 1994).

2.1.2.2 Epidermal Growth Factor (EGF)

EGF was originally detected in the mouse submaxillary gland (Cohen 1962). This ligand is known for its ability to stimulate precocious tooth eruption and eyelid opening in newborn mice (Cohen 1962), skin development in fetal lambs (Wynn *et al.* 1995) and lung maturation in fetal rabbits (Catterton *et al.* 1979). Moreover, EGF has been shown to have several functions in the bone cells such as stimulation of the osteoblast proliferation (Ng *et al.* 1983) or inhibition of collagen production (Hata *et al.* 1984).

Transgenic mice overexpressing EGF under the control of the human insulin promoter exhibited significant morphological changes within their pancreata (Krakowski *et al.* 1999). The pancreatic islets of these animals were larger than the islets of their non-transgenic littermates. Furthermore, a significant fibrosis around the islets was observed in the pancreas of transgenic animals. Treatment with EGF has been shown to stimulate the human adult pancreatic duct cells to expand and transdifferentiate (Rescan *et al.* 2005). Adult human duct cells employed for this study exhibited an increased activation of ErbB1 and ErbB2 receptors, followed by elevated proliferation of a particular fraction of the pancreatic duct cells. The authors (Rescan *et al.* 2005) suggested a possible role of EGF in converting pancreatic duct cells into insulin producing cells in diabetic patients. Moreover, EGF has been postulated to stimulate β-cell differentiation and islet neogenesis regulation during the pancreas development (Yasuda *et al.* 2007). EGF has been also shown to have a positive effect on glucose metabolism in diabetic animals (Suarez-Pinzon & Rabinovitch 2008; Lim *et al.* 2009) increasing the insulin secretion and reducing the glucose levels. However, some other studies did not support this theory, postulating that EGF was not able to modulate the plasma insulin content after glucose administration (Jansen *et al.* 2006).

2.1.2.3 Epigen (EPGN)

Epigen, a novel member of the EGFR family, was first described in mouse keratinocytes (Strachan *et al.* 2001). EPGN has a restricted tissue distribution; it is present in heart, liver and testis (Strachan *et al.* 2001). Moreover, EPGN is expressed in the inner and outer root sheath of hair follicles in newborn mouse skin (Kochupurakkal *et al.* 2005). So far, the function of epigen in the pancreas has not been investigated.

In the epithelial cells, epigen stimulates the phosphorylation of the EGFR and MAPK kinase proteins (Strachan *et al.* 2001). Although the binding affinity of EPGN to receptor is 100-fold lower than receptor binding affinity of EGF, its mitogenic potency is significantly higher (Kochupurakkal *et al.* 2005). These authors suggested that the low affinity of the ligand to the receptor may elicit such a potent response because of ineffective internalization and recycling of the ligand-receptor complex.

Recently, a transgenic mouse model with ubiquitously expression of epigen has been generated by Dahlhoff and colleagues (Dahlhoff *et al.* 2009). The transgenic mice were characterized by visibly reduced body size and impaired development of the first coat. Histological examination revealed extraordinarily enlarged sebaceous glands in the skin of EPGN-transgenic mice, suggesting a function of the EGFR pathways in the proliferation and/or differentiation of sebocytes.

2.1.2.4 Epiregulin (EREG)

Epiregulin was identified as a growth inhibitory factor from the conditioned medium of the fibroblast tumor cell line NIH-3T3 (Toyoda *et al.* 1995). This protein consists of 46 amino-acid residues and exhibits a dual biological activity, resembling in this regard AREG. On the one hand, it stimulates the proliferation of fibroblasts, hepatocytes, smooth muscle cells and keratinocytes (Shirakata *et al.* 2000; Takahashi *et al.* 2003). On the other hand, it inhibits the growth of several tumor-derived epithelial cell lines (Lee *et al.* 2004). EREG was found to be an autocrine growth factor in relatively low levels in the human keratinocytes and in tissue-resident macrophages (Shirasawa *et al.* 2004). Mice lacking epiregulin showed chronic dermatitis in the ear, face and neck area (Shirasawa *et al.* 2004). The authors demonstrated that epiregulin is a crucial molecule

for the regulation of IL-18 mRNA expression in keratinocytes. In contrast, Lee et al. reported that an independent line of EREG knockout mice did not display any abnormal phenotype (Lee *et al.* 2004) postulating that loss of epiregulin could be compensated by other EGFR ligands. Epiregulin as appears also to be an important factor in the female reproduction (Park *et al.* 2004) and liver regeneration (Toyoda *et al.* 1995). Investigations using insulinoma cell lines suggested that epiregulin may be a growth and insulinotropic factor in pancreatic β-cells (Kuntz *et al.* 2005). Treatment of rat insulinoma cell lines with epiregulin resulted in increased proliferation of these cells and elevated insulin production (Kuntz *et al.* 2005).
Interestingly, overexpression of epiregulin is often correlated with pancreas and prostate cancer development (Torring *et al.* 2000; Zhu *et al.* 2000).

2.1.2.5 Heparin-binding EGF-like growth factor (HBEGF)

HB-EGF was first described as a heparin binding mitogen in fibroblasts and smooth muscle cells (Besner *et al.* 1990; Higashiyama *et al.* 1991). HB-EGF is also present in the wound fluid, skin, lung and heart (Vaughan *et al.* 1992a; Vaughan *et al.* 1992b). The presence of HB-EGF have been confirmed both in the normal islets of Langerhans (Kaneto *et al.* 1997) and in the pancreatic cancer cells (Kobrin *et al.* 1994). Moreover, presence of HB-EGF in the duct cells of fetal and neonatal rat pancreas suggests a possible involvement of this protein in pancreas development (Kaneto *et al.* 1997).

Regenerative and therapeutic effects of HB-EGF on pancreatic β-cells have been postulated by Kozawa *et al.* using a diabetic mouse model (Kozawa *et al.* 2005). Results of this study demonstrated that treatment of a diabetic mouse with HB-EGF stimulated proliferation and differentiation of pre-existing β-cells leading to an increase in β-cell mass and improvement of glucose metabolism. However, overexpression of this growth factor in pancreas has been shown to promote pancreas cancer (Ito *et al.* 2001; Hurtado *et al.* 2007), fibrosis and epithelial metaplasia (Means *et al.* 2003).

HB-EGF was detected also in the epithelial cells of hair follicle (Rittie *et al.* 2007). Furthermore, its expression in the advancing epithelial margin and islands of regenerating epithelium within burn wounds suggests a role for this protein in wound healing (Marikovsky *et al.* 1993). Deletion of the HB-EGF gene resulted in early

postnatal mortality because of defected cardiac chamber dilation and heart valve malformation (Yamazaki *et al.* 2003), hypetrophic cardiomiocytes and hypoplastic lung (Iwamoto *et al.* 1999; Jackson *et al.* 2003). It is the only EGFR ligand whose deficiency causes early postnatal mortality. Loss of this ligand has been connected with epidermal hyperplasia and impaired differentiation of keratinocytes. For instance, keratinocyte-specific HB-EGF-deficient mice were characterized by decreased wound closure ability (Shirakata *et al.* 2005).

2.1.2.6 Transforming-Growth-Factor-α (TGFA)

TGFA was initially identified in medium conditioned by retrovirus transformed rodent cells (Ozanne *et al.* 1980; Twardzik *et al.* 1982; Marquardt *et al.* 1983). This growth factor is also expressed in epidermis and hair follicle. Moreover, TGFA mRNA has been detected in the preimplantation mouse embryos (Rappolee *et al.* 1988), in the anterior pituitary (Samsoondar *et al.* 1986), skin keratinocytes (Coffey, Jr. *et al.* 1987) and brain (Wilcox & Derynck 1988). Also, presence of the TGFA in the fetal and neonatal upper digestive tract and pancreas suggests a possible functional role of this protein in the growth and development of these organs (Hormi *et al.* 1995).

Overexpression of TGFA under control of the elastase promoter in transgenic mice has been shown to cause serious alterations in the growth and differentiation of several adult organs (Sandgren *et al.* 1990). In these animals the relative weight of the coagulation gland, colon, small intestine, liver and pancreas was increased by up to 3-fold. However, the average body weight of transgenic mice was significantly decreased. Despite these changes, there was no evidence for endocrine pancreatic dysfunction. Wagner *et al.* reported that ubiquitous TGFA overexpression in mouse pancreas caused the development of cancer similar to the human disease (Wagner *et al.* 2002). Mitogenic properties of TGFA have been shown in different studies using a transgenic mouse model with overexpression of this growth factor. For instance, Jhappan *et al.* demonstrated liver neoplasia and abnormal development of pancreas in TGFA transgenic mice (Jhappan *et al.* 1990). Also, Sandgren *et al.* presented the upregulated TGFA as an impotrant factor enhancing growth and development of pancreas and liver cancers in the transgenic mouse model (Sandgren *et al.* 1993).

Deficits in the expression of TGFA lead to phenotype forms including wavy hairs, curly whiskers and altered hair follicle structure (Luetteke *et al.* 1993; Mann *et al.* 1993). TGFA is implicated in wound healing (Schultz *et al.* 1987), cell migration (Barrandon & Green 1987) and bone resorption (Stern *et al.* 1985). In addition, it has been demonstrated that also uncleaved, membrane spanning form of this ligand can initiate signal transduction through EGFR on the surface of the contiguous cells *in vitro* (Brachmann *et al.* 1989; Wong *et al.* 1989).

2.1.3 Betacellulin

2.1.3.1 Structure of betacellulin

Betacellulin is a 32-kilodalton polypeptide growth factor, originally isolated from the conditioned medium of a mouse pancreatic tumor β-cell line, βTC3 (Shing *et al.* 1993). BTC is a glycoprotein composed of 80 residues, synthesized as a membrane-spanning precursor with 177 amino acids (Figure C). In this form pre-BTC is composed of an extracellular part, a transmembrane part and a cytoplasmic part. The extracellular part contains a signal peptide for the localization to the secretory pathway and EGF-like domain including six cysteine residues forming three characteristic disulfide bonds. In EGFR ligands, these intermolecular loops are necessary for recognizing and binding of the ligand to the receptor (Carpenter & Cohen 1990).

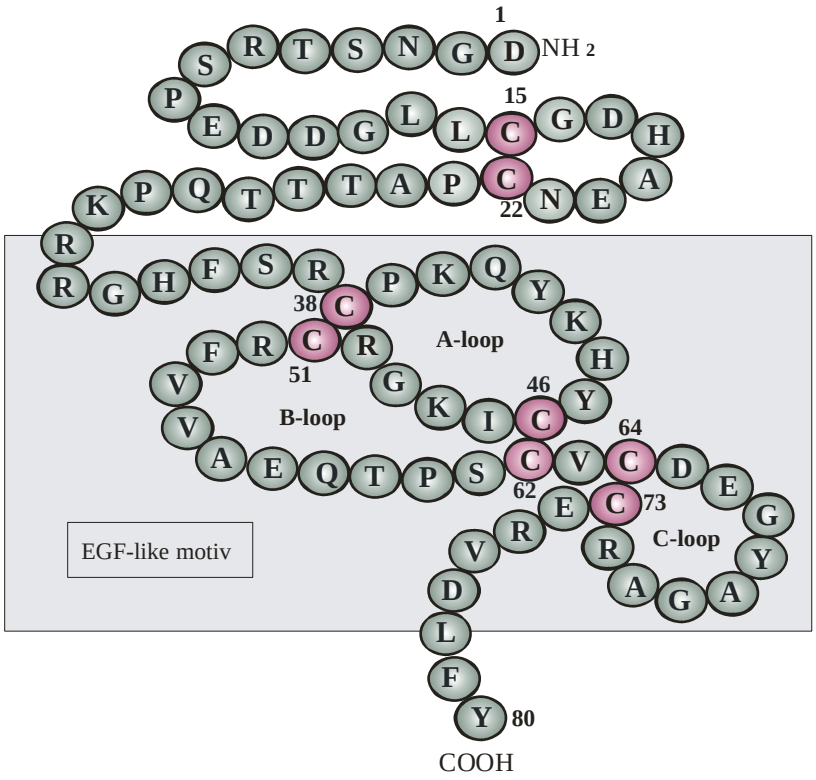

Figure C Structure of a mature human betacellulin. A, B, C disulfide loops within the EGF-like domain.

A single copy gene for mouse BTC maps to chromosome 5 whereas the human betacellulin gene is localized on the chromosome 4 (Pathak *et al.* 1995). The human and bovine precursor BTC share 88% sequence identity, bovine and mouse precursors are 79% identical, while identity between sequences of human and mouse pre-BTC reaches 74% (Dunbar & Goddard 2000). The mature form of human betacellulin is released after proteolytical cleavage of the precursor protein between Ala31 and Asp32, as well as between Y111 and L112 (Shing *et al.* 1993; Dunbar *et al.* 1999). This growth factor exhibits the strongest affinity to ErbB1 receptor, but it can also bind to receptor dimers containing ErbB4. Moreover, the ability of betacellulin to recognize and activate the highly oncogenic receptor dimer ErbB2/ErbB3 has been demonstrated (Alimandi *et al.* 1997).

2.1.3.2 Expression of betacellulin

Betacellulin is predominantly expressed in pancreas, liver, kidney and small intestine, but also in heart, lung, testis, ovary and colon. BTC is also present in various body fluids, including milk (Dunbar *et al.* 1999), suggesting that its absorption trough the small intestine influences the neonatal development and that BTC might be an important factor for growth and development of various neonatal organs including the pancreas. Investigations on fetal and neonatal pancreas demonstrated betacellulin to be localized to the primitive duct cells in fetal pancreas and to some islet cell populations associated with insulin producing cells (Miyagawa *et al.* 1999). This growth factor was able to stimulate the proliferation of fetal pancreatic epithelial cells and to promote growth, differentiation and survival of these cells (Seno *et al.* 1996; Miyagawa *et al.* 1999).

Betacellulin has been also found in tumor cells, such as mouse sarcoma 180 and fibrosarcoma BPV-11 cell lines, also in human breast adenocarcinoma MCF-7 cells (Sasada *et al.* 1993).

2.1.3.3 Functions of betacellulin in the endocrine pancreas

Several *in vitro* and *in vivo* studies showed a positive impact of BTC on β-cell differentiation and insulin production (Watada *et al.* 1996; Huotari *et al.* 1998; Mashima *et al.* 1999; Yasuda *et al.* 2007). Human embryonic stem cells have a very wide replication capacity; therefore they could be an efficient and renewable source of insulin-producing cells (Demeterco *et al.* 2000). The treatment of human ES cells with betacellulin and nicotinamide has been demonstrated to sustain the expression of PDX1, a transcription factor necessary for pancreatic development and β-cell maturation, and to induce β-cell differentiation (Cho *et al.* 2008). Results of several other studies also suggest that BTC may modify the activity of several transcriptions factors, known for their negative impact on insulin production, and support the activity of the PDX1 gene promoter (Watada *et al.* 1996). One possible explanation of this phenomenon could be that BTC causes the demethylation of chromosomal DNA to release the insulin gene promoter from inactivation (Watada *et al.* 1996).

Other *in vitro* researches showed that the activity of BTC could be intensified in the presence of activin A, a member of transforming growth factors-β family of cytokines (Mashima *et al.* 1996; Demeterco *et al.* 2000). Activin A induces endocrine differentiation, whereas BTC shows a mitogenic impact on undifferentiated pancreatic epithelial cells (Demeterco *et al.* 2000). This growth factor has also the ability to support betacellulin in converting multipotent pancreatic AR42J cells into pancreatic neuroendocrine cells (Mashima *et al.* 1996). Moreover, studies using a rat fetal pancreas cell line indicated the involvement of betacellulin and activin A in the inhibition of β-cell division and increase of β-cell volume during pancreas development (Demeterco *et al.* 2000; Yasuda *et al.* 2007).

Furthermore, application of betacellulin inhibited the expression of amylase and glucagon, and elevated the expression of insulin and ghrelin in an *in vitro* model of murine embryonic pancreas (Thowfeequ *et al.* 2007). However, there is a single study not supporting the concept that betacellulin has a significant effect in the improvement of glucose metabolism (Sjoholm & Kindmark 1999).

Beta-cell regeneration occurs through both increasing the replication of pre-existing β-cells and neogenesis from the precursor cells located in the pancreatic duct (Bonner-Weir *et al.* 1981; Dutrillaux *et al.* 1982). There are many studies showing improvement of glucose tolerance after treatment with BTC in animal models of diabetes (Yamamoto *et al.* 2000; Li *et al.* 2001). Hyperglycemia can be caused by administration of β-cell toxins such as streptozotozin or alloxan and by pancreatectomy. BTC was shown to promote regeneration and neoformation of β-cells mainly from somatostatin- and PDX1- positive cells (Li *et al.* 2008). Yamamoto and colleagues postulated that rhBTC (recombinant human BTC) was able to ameliorate glucose tolerance in the alloxan treated diabetic mouse by promoting β-cell differentiation from ductal and acinar cells. Indeed, injections of rhBTC significantly improved the glucose tolerance and contributed to a significant increase in body weight (Yamamoto *et al.* 2000). Additionally, Shin *et al.* showed a significantly higher level of serum insulin in diabetic mice up to two weeks after rAd-BTC (recombinant adenovirus BTC) treatment, indicating a positive impact of betacellulin on β-cell regeneration (Shin *et al.* 2008). Also, it is noteworthy that the delivery of a combination of Neurod and BTC genes to the liver induced islet neogenesis and reversed diabetes in STZ-treated diabetic mice (Kojima *et al.* 2003). In another diabetic mouse model, mice previously treated with STZ, presented a significantly increase of β-cell number and insulin positive islets after BTC application (Li *et al.* 2003). Here, daily administration of betacellulin significantly reduced the plasma glucose levels and elevated the plasma insulin concentrations (Li *et al.* 2003). Glucose tolerance tests confirmed the improvement of glucose homeostasis in these animals and additional morphometric analysis showed an increased β-cell mass without increasing β-cell size.

Potential functions of betacellulin have been demonstrated also in many other organs. For instance, BTC was shown to stimulate the proliferation of both normal mammary cells and breast tumor cells (Beerli & Hynes 1996; Daly *et al.* 1999) and to induce the terminal phase of mammary epithelial differentiation and secretion (Alimandi *et al.* 1997; Pinkas-Kramarski *et al.* 1998). Transgenic mice ubiquitously overexpressing BTC were characterized by high early postnatal mortality, caused by several defects in lung, and reduced body weight (Schneider *et al.* 2005). Additionally, pathological alterations like cataract and bone malformations were observed. The study of Dahlhoff *et al.* described several changes in the intestine of the BTC-transgenic mice. The small

intestine of transgenic mice was significantly briefer but simultaneously increased in weight. Moreover, BTC has been shown to stimulate the cell proliferation in intestinal crypts and to support the survival of nascent adenomas in the *Apc*$^{+/Min}$ mice (Dahlhoff *et al.* 2008).

Similar to many other ligands of the EGFR family, betacellulin is also involved in reproduction. Thus, a function for BTC as a mediator of luteinizing hormone, prostaglandin and progesterone receptors has been reported (Park *et al.* 2004; Ashkenazi *et al.* 2005; Shimada *et al.* 2006). In addition, the studies of Gratao and colleagues on the transgenic mice overexpressing betacellulin did not demonstrate any alterations in the uterus and ovaries development but exhibited defects in sexual maturity and blastocyst implantation (Gratao *et al.* 2008).

2.2 Animal models of diabetes

2.2.1 Pharmacological and surgical induction of diabetes in different animal species

There are two types of diabetes mellitus: type 1 and type 2. Diabetes mellitus type 1 is an autoimmune disease that results in destruction of insulin producing β-cells in the pancreas. Because of lack of insulin, fasting blood glucose in type 1 diabetic patients increases enormous (Eisenbarth 2003). Diabetes mellitus type 2 is predicted on alterations and dysfunction of insulin receptors leading to insulin resistance or reduced insulin sensitivity (Porte, Jr. 1991).

The most common species used as animal models of diabetes are mice, rats, rabbits and recently pigs. These animals are often selected because of easy breeding requirements, short generation intervals, large number of progeny and the similarity of their morphology and pathology to humans (Huge *et al.* 1995). Other species like nonhuman primates, cats or dogs also exhibit many advantages as animal models of human diseases, but because of restricted availability and ethical considerations, they are rather seldom used.

There are many possibilities to induce diabetes in the healthy organism. The most commonly used method is application of β-cell toxins such as streptozotocin and alloxan (Portha *et al.* 1974; Waguri *et al.* 1997; Li *et al.* 2003; Pomaro *et al.* 2005). These substances can be administrated intraperitoneally, intravenously, subcutaneously or orally, depending on the species and aim of investigation. Perfusion of alloxan causes destruction of β-cells only in the particular segment of the pancreas, while β-cells in the non-perfused segment remain intact. Therefore, this diabetes model allows to clarify, for instance, the process of β-cell regeneration from the ductal pancreatic epithelium (Yamamoto *et al.* 2000). Streptozotocin, in contrast, destroys all β-cells in the pancreas of the treated animal (Li *et al.* 2003).

Another method to generate an animal model of diabetes is pancreatectomy, the surgical removal of a part or of the whole pancreas (Stump *et al.* 1988; Li *et al.* 2001; Kobayashi *et al.* 2004). In this way type 1 diabetes can be induced. However, this technique is applied mostly in the large animal models, particularly in the swine.

Because of the similarity of human type 2 diabetes to cat type 2 diabetes mellitus, cats have often been used to investigate the insulin resistance (Henson & O'Brien 2006; Hoenig 2006). Results of such studies showed that a high supply of glucose had the highest impact on β-cell dysfunction. So, it is possible to induce diabetes mellitus using only the phenomenon of glucotoxicity on the pancreatic cells (Zini *et al.* 2009).

2.2.1.1 Mouse

The most common and applied method to induce diabetes in the mouse is infusion of alloxan or streptozotocin (Boquist 1977; Boquist & Lorentzon 1980; Leiter *et al.* 1983; Zhao *et al.* 2005). In addition, the Cre-*loxP*-mediated gene recombination has been applied in order to investigate the role of insulin signaling (Bruning *et al.* 1998; Michael *et al.* 2000). Dysfunction of major insulin receptors in the target tissues, skeletal muscle, resulted in hypertriglyceridemia, however, the glucose metabolism was not impaired (Bruning *et al.* 1998; Michael *et al.* 2000). Furthermore, obese and leptin-deficient (ob/ob) mice are widely employed as mouse models of type 2 diabetes mellitus (Coleman 1982; Drel *et al.* 2006). The lack of leptin, a hormone necessary for the appetite regulation, causes uncontrolled food intake and massive obesity in ob/ob mice.

Consequently, these mutant mice display several symptoms of type 2 diabetes mellitus (Coleman 1982; Drel *et al.* 2006).

Since 1980, non-obese diabetic mice (NOD) have been employed as a useful animal model of type 1 diabetes mellitus. These mice are distinguished by high incidence of spontaneous diabetes, resulting from insulitis, what means infiltration and destruction of pancreatic cells by the own immune system, particularly by $CD4^+$ and $CD8^+$ T cells (Anderson & Bluestone 2005).

A monogenic diabetic mouse model, called Akita mouse, has been established by Yoshioka and colleagues (Yoshioka *et al.* 1997). This diabetic mouse model, characterized by early onset, an autosomal dominant mode of inheritance and primary dysfunction of β-cells, resembles human maturity onset diabetes of the young (MODY) and was employed in different studies to investigante the background of this disease (Wang *et al.* 1999; Izumi *et al.* 2003). Hyperglycemia in the Akita mice is caused by mutation in the insulin 2 gene leading to disruption of an intramolecular disulfide bond (Wang *et al.* 1999). Importantly, Akita mice do not exhibit either obesity or insulitis.

Another diabetic mutant mouse model has been generated using N-ethyl-N-nitrosourea (ENU) (Herbach *et al.* 2007). The Munich *Ins2*^{C95S}mutant mice exhibited a thymine to adenine transversion in the insulin 2 gene at nucleotide position 1903 in exon 3. Heterozygous mutant mice demonstrated a significant lower serum insulin level, pancreatic insulin content and homeostasis model assasment (HOME), as compared with their wild-tipe littermates.

2.2.1.2 Rat

Similar to mice, diabetic rats can be generated by administration of streptozotocin and alloxan or through a pancreatectomy. It has been reported that injection of STZ in the neonatal rats caused destruction of the most islets and provided an useful model for diabetes mellitus (Cantenys *et al.* 1981; Dutrillaux *et al.* 1982; Wang *et al.* 1994). Streptozotocin has also been employed as a diabetes-inducing agent in order to investigate the structure and functional alteration of the insulin receptor (Kergoat *et al.* 1988). In this study, rats with non-insulin-dependent diabetes were employed to

demonstrate the lack of significant changes in the insulin receptor autophosphorylation between diabetic and healthy animals.

Pancreatectomy is a method of induction of experimental diabetes in the rat as well (Liu *et al.* 2000; Delghingaro-Augusto *et al.* 2009). Additionally, Goto-Kakizaki rats are also useful as animal models of diabetes. Due to defects in the glucose- stimulated insulin secretion, Goto-Kakizaki rats spontaneously develop peripheral insulin resistance, hyperinsulinemia, and hyperglycemia, observable already 4 weeks after birth. Therefore, this model provides a valuable tool for dissecting the pathogenesis of the insulin resistance (Chen & Ostenson 2005; Kindel *et al.* 2009; Matsumoto *et al.* 2009).

2.2.1.3 Rabbit

Rabbits are also often used as experimental animals for diabetes mellitus. In the most studies, diabetes has been induced by administration of the β-cell toxins STZ and alloxan (Ragazzi *et al.* 2002; Choi *et al.* 2003; Pomaro *et al.* 2005; Eller *et al.* 2006; Breen *et al.* 2008; Habibuddin *et al.* 2008). Nevertheless, surgical techniques in the creation of diabetes in rabbits are sometimes employed (Lasserre *et al.* 2000; Catala *et al.* 2001).
The experimental rabbits manifested disturbances in the glucose metabolism and thereby they could be employed to test new therapy methods of disorders connected with diabetes mellitus, like arrhythmias (Zhang *et al.* 2007), impaired wound healing (Breen *et al.* 2008) or nephropathies (Mumtaz *et al.* 2004; Mir & Darzi 2009).

2.2.1.4 Pig

While the rodent models are appropriated for answering basic questions of β-cell pathophysiology, translational studies, particularly regarding islet transplantations, would greatly benefit from large animal models. Pigs seem to be the best candidates for this role: they are readily available and produce a large amount of progeny. Also, similarities to humans in nutrition, pancreas development and morphology, and metabolism make the swine an interesting and useful model for the study of diabetes (Huge *et al.* 1995).

Induction of insulin-dependent diabetes in pigs has been achieved by use of streptozotocin and alloxan (Marshall 1979; Gabel *et al.* 1985; Grussner *et al.* 1993; Dixon *et al.* 1999; Larsen *et al.* 2002; Larsen *et al.* 2006; Velander *et al.* 2008). The intravenous administration of 200 mg/kg STZ in Yucatan mini-pigs induced diabetes with high mortality as a consequence of massive β-cell destruction (Phillips *et al.* 1980). A mild diabetes mellitus in Göttingen mini-pigs has been reported to originate by injection of 80 mg/kg STZ (Kjems *et al.* 2001).

In humans a 20-30% reduction of β-cell mass leads to an increase of the fasting glucose level and abnormalities in the glucose metabolism (Kendall *et al.* 1990). Similar changes are able to be induced in animals using pancreatectomy. Therefore pigs serve as an especially appropriate species for such studies. Nevertheless, to induce significant hyperglycemia and alterations comparable to humans, it is necessary to remove 80% of the porcine pancreas, while a 40% pancreatectomy results only in mild changes (Lohr *et al.* 1989). Several studies described a swine model of diabetes created by a complete pancreatectomy (Stump *et al.* 1988; Kobayashi *et al.* 2004). The treated animals exhibited abnormalities like an increase of the fasting glucose level, decrease of insulin secretion, athrophy of hepatocytes and decreased glycogen storage in the liver. These findings allow the use of total pancreatectomized pigs to investigate the complications of diabetes.

An important aspect supporting the employment of pigs as experimental animals is the possibility to obtain insulin-producing tissue from these animals for transplantation purposes. Allotransplantations, moving the pancreatic islets from one organism to another of the same species, became a useful therapy method against type 1 diabetes mellitus. Unfortunately, in humans the discrepancy between the number of recipients and the number of donors of pancreatic islets restricts the usage of this therapy in clinical practice on a large scale (Shapiro *et al.* 2000; Shapiro *et al.* 2006). Therefore, pigs might represent a possible source of insulin-producing tissue for transplantations into humans (Prabhakaran & Hering 2008). So far, experiments of transplantation of pancreatic islets from pigs into rats (Rogers *et al.* 2006), mice (Wu *et al.* 2000), dogs (Abalovich *et al.* 2009) or non-human primates(Hering *et al.* 2006; Dufrane & Gianello 2008) have been performed. The results of these studies indicate an improved glucose

metabolism in the recipient organisms; however, rejection of the xenografts still remains the major problem of this therapy.

2.2.1.5 Non-human primates

Reflecting their close phylogenetic relationship with humans (Lu *et al.* 2008), metabolic and hormonal alterations in monkeys seem to be similar to those in humans. Therefore, primates would be an excellent animal model of different pathological abnormalities. Diabetes mellitus can be initiated in monkeys also by administration of streptozotocin or alloxan (Gibbs *et al.* 1966; Dufrane *et al.* 2006; Zou *et al.* 2006), and through a partial and total pancreatectomy as well (Qiao *et al.* 2009). Several studies performed on monkeys pointed to an analog glucose metabolism of these animals to humans. Thus, 50 mg/kg streptozotocin appeared to be the optimal dose to increase the fasting and non-fasting blood glucose up to 6-fold and to generate a stable non-human primate model with all typical patterns of type 1 diabetes mellitus (Dufrane *et al.* 2006; Rood *et al.* 2006).

2.2.1.6 Dog and cat

Companion animals such as dogs and cats share the same living environment as humans and are exposed to similar illness agents (Kooistra *et al.* 2009). Feline diabetes mellitus closely resembles human type 2 diabetes mellitus (Henson & O'Brien 2006; Kooistra *et al.* 2009), whereas canine diabetes exhibits similarities to human type 1 diabetes mellitus (Catchpole *et al.* 2008). Clinical studies have shown that both living style and genetic predispositions are important factors causing diabetes mellitus in cats. Moreover, analog to humans, age and obesity were found to be the most important risk factors for diabetes in cats (Panciera *et al.* 1990).

Diabetes in dogs and cats can also be induced by injection of streptozotocin or alloxan (Nelson *et al.* 1990; Anderson *et al.* 1993) and through pancreatectomy as well (Gupta *et al.* 2002). In the canine diabetes model, the portal insulin levels have been shown to determine the suppression of the hepatic glucose production and inhibition of the precursors for gluconeogenesis (Fisher *et al.* 1996; Giacca *et al.* 1999; Gupta *et al.*

2002). Moreover, a canine model of diabetes has been employed for auto- and xeno-transplantation experiments (Stagner *et al.* 2007).

2.2.2 Genetically modified mice in diabetic research

2.2.2.1 Knockout mouse models

Another possibility to investigate the influence of different molecules on β-cells is transgenic technology, the mouse being the most frequently employed specie. Especially useful are modifications within insulin (Esposito *et al.* 2003; Yang & Chen 2009) and leptin receptors (Chen *et al.* 1996) or a total knockout of genes encoding these receptors (Accili *et al.* 1996; Joshi *et al.* 1996; Jackerott *et al.* 2002). Targeted disruption of the insulin receptor leads to the development of several metabolic disorders including diabetes mellitus (Taylor *et al.* 1991; Esposito *et al.* 2003). Mice homozygous for a null allele of the insulin receptor gene are born apparently without any developmental alterations. However, shortly after birth, the homozygous knockouts exhibit a massive hyperglycaemia and hyperketonaemia leading to death due to ketoacidosis within 72 hours (Accili *et al.* 1996). Moreover, a total absence of the insulin receptor in the homozygous animals resulted in other metabolic disorders like skeletal muscle hypotrophy, increase of the plasma triglyceride level and fatty infiltration of the liver (Joshi *et al.* 1996). Nevertheless, heterozygous mice, lacking only one allele of the insulin receptor gene did not develop any metabolic alterations, as indicated in the glucose tolerance tests (Joshi *et al.* 1996).

A proper regulation of the β-cell function depends on cross-talk between leptin and insulin signaling pathways (Morioka *et al.* 2007). Depending on the employed transgenic strategy for the creation of a leptin receptor knockout mouse model, the influence on glucose metabolism can be different. For instance, knockout mice lacking a functional leptin receptor within pancreatic β-cells and hypothalamus developed obesity, fasting hypereinsulinemia, impaired glucose-stimulated insulin release and glucose intolerance (Covey *et al.* 2006). Mice lacking the leptin receptor only within pancreatic β-cells did not manifest any alterations in insulin sensitivity but exhibited improved glucose tolerance due to enhanced insulin secretion which was consistent with a lack of tonic inhibitory action of leptin on β cell secretion (Morioka *et al.* 2007).

One of the most important transcription factors for development of the pancreas and other foregut structures is pancreatic duodenal homeobox factor-1 (Pdx-1) (Offield *et al.* 1996). Knockout mice lacking the Pdx-1 gene exhibit pancreatic agenesis and congenital diabetes (Stoffers *et al.* 1997; Edlund 2001; Johnson *et al.* 2003). Transgenic mice with a haploid deficiency of Pdx-1 demonstrated an impaired glucose metabolism, reduced insulin release and β-cell proliferation (Johnson *et al.* 2003), confirming the function of Pdx-1 as a mediator in the glucose-stimulated insulin gene transcription (Marshak *et al.* 1996).

Also the loss of another transcription factor, hepatocyte nuclear factor-1α (HNF-1α), leads to body weight reduction and to defective insulin secretory response to glucose and arginine in a transgenic mouse model (Fajans *et al.* 2001). Disruption of this gene in mice caused a phenotype similar to one of the six known genetic forms of maturity onset diabetes in the young (MODY), namely MODY-3. Due to such investigations, the involvement of HNF-1α in the regulation of β-cell differentiation and β-cell mass has been uncovered.

Signal transducer and activator of transcription 3 (STAT3) deficient mice have been generated in order to investigate the function of this ubiquitous transcription factor on the postnatal glucose homeostasis (Cui *et al.* 2004). This study revealed the development of diabetes mellitus, including body weight reduction, hyperglycaemia and hyperinsulinemia, in mice lacking STAT3 within hypothalamus and pancreatic β-cells (Cui *et al.* 2004).

Studies investigating mouse models with a dysfunction of either cyclin D or partner cyclin-dependent protein kinase (Cdk) demonstrated an important role of these molecules for a proper β-cells proliferation and insulin synthesis (Rane & Reddy 2000; Georgia & Bhushan 2004). Cyclin $D2^{-/-}$ mice exhibited insulin-deficient diabetes caused by reduced postnatal α- and β-cell replication. $Cdk\text{-}4^{-/-}$ mice showed abnormalities similar to cyclin D2-deficient mice; in addition, the body weight of the knockout animals was 40% lower than wild-type controls (Martin *et al.* 2003).

Glucokinase (GLK) is a member of the hexokinase family and responsible for the phosphorylation of glucose to glucose-6-phosphate. This serves as a glucose-sensing mechanism for regulation of the insulin secretion in the pancreatic β-cells (Meglasson & Matschinsky 1986). Knockout mouse models confirmed a crucial role of glucokinase in the glucose metabolism. GLK-deficient mice generated by Grupe and colleagues showed massive hyperglycaemia and hyperacidemia, and died during the first five days after birth (Grupe *et al.* 1995). Nevertheless, mice with one functioning GLK allele developed only mild diabetes, comparable to MODY in humans (Grupe *et al.* 1995).

2.2.2.2 Transgenic mouse models

Transgenesis became a widespread strategy to create appropriate animal models for investigations on diabetes mellitus and for developing possible therapy methods. An important aspect in the generation of transgenic mouse lines is selection of an adequate promoter to control the expression of the transgene.
The most commonly used promoter in diabetes research is the insulin promoter. In different studies rat, mouse, porcine or human insulin promoters have been applied to induce diabetes in the transgenic mouse models (Inada *et al.* 2004; Anneren *et al.* 2007; Hara *et al.* 2007; Watanabe *et al.* 2007).

The employment of transgenic mice overexpressing hormones (Davani *et al.* 2004), tyrosine kinases (Anneren *et al.* 2007), transcription factors (Jackerott *et al.* 2006) or enzymes (Kebede *et al.* 2008) supported investigations concerning the function of these molecules in the development of diabetes. For instance, expression of a dominant negative glucose-dependent insulinotropic polypeptide receptor ($GIPR^{dn}$) under the control of the rat proinsulin gene promoter induced diabetes mellitus in transgenic mice (Herbach *et al.* 2005). Also Kebede and colleagues showed that overexpression of fructose-1,6-bisphosphatase, a gluconeogenic enzyme upregulated in the pancreatic islets, under the rat insulin promoter contributed to insulin secretory dysfunction, increase of the serum glucose level and finally to the development of type 2 diabetes mellitus (Kebede *et al.* 2008).

A proper function of signal transducers and activators of transcription (STAT) proteins has been shown to be crucial for an adequate prenatal development (Cui *et al.* 2004; Yashpal *et al.* 2004). The rat insulin promoter has been employed to control the transcription of a dominant-negative and a constitutive active mutant form of the STAT5 gene (Jackerott *et al.* 2006). Due to these investigations, a role for STAT5 in glucose homeostasis has been uncovered.

Furthermore, the employment of a rat insulin promoter confirmed the diabetogenic role of glucocorticoids (Davani *et al.* 2004). Mice overexpressing the rat glucocorticoid receptor under the control of the rat insulin promoter showed an inhibited insulin secretion, hyperglycaemia and impaired glucose tolerance (Davani *et al.* 2004).

However, the rat insulin promoter is not the only one used in the diabetes research. Hara *et al*. employed a mouse insulin promoter causing the overexpression of the hepatocyte nuclear factor-6 and detected a significant loss of β-cell mass in transgenic mice (Hara *et al.* 2007). A diabetic mouse model has also been generated using a mutant form of the hepatocyte nuclear factor-1α regulated by the porcine insulin promoter (Watanabe *et al.* 2007). Results of this study indicated that expression of the transgene driven by the porcine insulin promoter induced a decrease in body weight, disturbed islet neogenesis and impaired insulin secretion in transgenic mice (Watanabe *et al.* 2007).

Insulin promoters have also often been applied to regulate the expression of molecules known to ameliorate the diabetic phenotype in mice. Miyawaki and colleagues demonstrated that overexpression of a cyclin-dependent kinase 4 in β-cells under the control of the human insulin promoter (HIP) led to glycaemic normalization and to improved plasma lipid concentration in transgenic, obese and insulin resistant db/db mice (Miyawaki *et al.* 2008). Moreover, a human insulin promoter has been useful for investigations on potentially protective impact of a programmed death-1 ligand (PD-L1) against autoimmune diabetes (Wang *et al.* 2008). In this study a human insulin promoter regulated the overexpression of PD-L1 in the pancreatic β-cells in the non-obese diabetic (NOD) mice. The proliferation rate of diabetogenic T-cells in the transgenic NOD mice was significantly reduced. Furthermore, a diabetic phenotype appeared in the transgenic animals significantly slower than in the control NOD mice.

A significant improvement of the glucose metabolism has been observed in transgenic mice expressing the hepatocyte growth factor in β-cells under the control of the rat insulin promoter (Garcia-Ocana *et al.* 2001). These animals exhibited an increased islet size and islet number, as well as insulin-mediated hypoglycaemia, suggesting a positive impact of this growth factor on the endocrine pancreas (Garcia-Ocana *et al.* 2001).

Although the insulin promoter is the most frequently used in the research on glucose metabolism, there are also some studies employing a promoter sequence of the mouse metallothinein-I gene (Portanova *et al.* 1990; Robertson *et al.* 2008). Overexpression of both the insulin-like growth factor I (IGF-I) (Robertson *et al.* 2008) and the human growth factor (hGH) (Portanova *et al.* 1990) in transgenic animals resulted in body weight reduction, significant hypoglycaemia and elevated glucose metabolism. However, the widespread expression of the transgene in these mouse models can be a complicating factor for the interpretation of the results.

A new mouse model, introduced in the following study, provides a possibility to achive β-cell-specific expression of different gene products and to investigate potential effects of these products in new therapies against diabetes mellitus.

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Specific transgene expression in mouse pancreatic β-cells under the control of the porcine insulin promoter

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Running head:
Transgene expression under the porcine insulin promoter

Abstract

The availability of regulatory sequences directing tissue-specific expression of transgenes in genetically modified mice and large animals is a prerequisite for the development of adequate models for human diseases. The rat insulin 2 gene (*Ins2*) promoter, widely used to achieve transgene expression in pancreatic β-cells of mice, also directs expression to extrapancreatic tissues and performs poorly in isolated pancreatic islets of human, mouse, and pig. To evaluate whether the full 5' untranslated region (UTR) of the porcine insulin gene (*INS*) confers robust and specific expression in β-cells we generated an expression cassette containing 1500 bp of the porcine *INS* 5' UTR and the 3' UTR of the bovine growth hormone gene (*GH*). The cassette was designed to allow easy exchange of the sequences to be expressed and easy removal of the vector backbone from the expression cassette. To evaluate the properties of the cassette, we initially inserted a cDNA encoding human betacellulin, a growth factor known to affect structural and functional parameters of β-cells. After confirming the functionality and specificity of the construct in vitro, transgenic mouse lines were generated by pronuclear DNA microinjection. Using RT-PCR, immunohistochemistry and immunofluorescence, we show that transgenic mice expressed human betacellulin exclusively in β-cells. Confirming the proposed insulinotropic effect of betacellulin, transgenic mice showed improved glucose tolerance. We conclude that the newly designed expression cassette containing 1.500 bp of the porcine insulin promoter 5' UTR confers robust and specific transgene expression to β-cells *in vitro* and in transgenic mice.

Keywords: porcine insulin promoter, transgenic mice, betacellulin, EGFR, glucose tolerance test.

1 Introduction

Genetically modified mice greatly contributed to advance our understanding of the physiology and pathology of the endocrine pancreas. A key component of this technology is the targeted expression of gene products in specific cell types of the pancreatic islets. The rat (Hanahan 1985; Dahl *et al.* 1996; Vasavada *et al.* 1996), mouse (Hara *et al.* 2003), and human (Hotta *et al.* 1998; Krakowski *et al.* 1999) insulin promoters have been frequently used to direct expression of oncogenes, hormones, growth factors, transcription factors, reporter genes, and more recently of the enzyme Cre recombinase (Ahlgren *et al.* 1998; Postic *et al.* 1999; Gannon *et al.* 2000; Dor *et al.* 2004) specifically to pancreatic β-cells in transgenic mice. A major caveat of the rat insulin promoter, however, is the reported ectopic expression in certain areas of the brain, potentially resulting in phenotypes in both β-cells and neural cells (Gannon *et al.* 2000; Martin *et al.* 2003).

While the mouse model is appropriate for answering basic questions of β-cell pathophysiology, translational studies, particularly regarding islet transplantation, would greatly benefit from large animal models. Pigs seem to be the best candidates for this purpose: they are readily available, produce a large progeny, and regulate blood glucose levels similarly to humans. In addition, porcine neonatal islet cell clusters (MacKenzie *et al.* 2003; Cardona *et al.* 2006) and islets from adult pigs (Hering *et al.* 2006) are an interesting source of insulin-producing tissue for transplantation purposes. In this regard, there is an urgent need of genetic tools for the tissue-specific delivery of gene products to porcine pancreatic β-cells. Unfortunately, the rat insulin promoter was shown to perform very poorly in isolated islets from pigs, humans, and mice (Londrigan *et al.* 2007). The porcine insulin gene (*INS*) contains three exons and two introns, and highly conserved cis-acting elements were identified in the 5' flanking region (Han & Tuch 2001). Recently, a fragment of the porcine *INS* promoter containing approximately 680 bp of the 5' untranslated region (UTR) was active in a cell type-specific manner in vitro, but failed to confer expression in transgenic mice (Watanabe *et al.* 2007). Addition of a cytomegalovirus enhancer resulted in transgene expression both in vivo and in vitro, but at the expense of cell type specificity (Watanabe *et al.* 2007).

Here, we report that an expression cassette containing 1500 bp of the 5' UTR from the porcine *INS* gene (including the first exon and the first intron) and the 3' UTR of the bovine growth hormone gene (*GH*) is efficient to drive transgene expression in a robust and specific manner to β-cells in vitro and in transgenic mouse models. To test the functionality of the cassette we have chosen the cDNA encoding human betacellulin, a ligand of the EGFR (Schneider & Wolf 2009) known to stimulate β-cell differentiation in vitro and to improve glucose tolerance in diabetic models and in transgenic mice.

2 Material and Methods

2.1 Construction of the transgene

A ~1500-bp fragment of the 5' flanking region of the porcine insulin gene (*INS*) was amplified by polymerase chain reaction (Expand High Fidelity^{PLUS} PCR System, Roche Diagnostics, Mannheim, Germany) using the sense primer (*Sal*I restriction site underlined) 5'-TGT ACT GTC GAC GAG TTC AGC TGA GCT GGC TC-3' and the antisense primer (*Hind*III restriction site underlined) 5'-CGC TAG AAG CTT TGG GGG ACG GGC GGC GTT-3' (**see Figure 1A**). Template was a plasmid pGEM+INS (Flaswinkel et al., unpublished) containing the whole porcine *INS* gene. The human (h) betacellulin (*BTC*) cDNA was amplified with the same PCR system employing the pTB 1560 vector (a gift of Dr. Y. Shing, Children's Hospital, Boston, MA) as a template and the primers (*Hind*III restriction site underlined) 5'-ACT AAG CTT CAC CCC CCG CCA TGG ACC GGG CCG CCC GGT-3', sense, and (*Eco*RV restriction site underlined, HA-tag in bold) 5'-TAG GAT ATC TTA **AGC GTA GTC TGG GAC GTC GTA TGG GTA** AGC AAT ATT TGT CTC TTC AAT ATC-3', antisense.

The PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) and Sp6/T7-sequenced to confirm amplification fidelity. The porcine *INS* promoter (PIP) was then subcloned into the *Sal*I and *Hind*III sites of a pBluescript vector (Stratagene, La Jolla, CA) containing the bovine (b) *GH* 3' untranslated region and polyadenylation signal (pA) within its *Xba*I and *Not*I sites (**Figure 1B**). Next, the *BTC* cDNA was cloned as a *Hind*III/*Eco*RV fragment between PIP and bGHpA (**Figure 1B**). Correct positioning of each element was confirmed by restriction enzyme digests and sequencing from the PIP through the hBTC cDNA into the bGHpA using the primer 5'-CATCTCGGCAGGAGGACGT-3' (**Figure 1A**).

2.2 Cell transfection

All cell culture reagents were purchased from PAA (Pasching, Austria). Immortalized mouse hepatocytes (a gift from Dr. D. Accili, Columbia University, NY), human embryonal kidney 293 cells (HEK 293), and the MIN-6 mouse pancreatic β-cell line (a gift from Dr. J. Miyazaki, Osaka University) were cultured in low glucose (1 g/L) DMEM medium with 10% fetal bovine serum. $2x10^6$ cells were transfected either with the PIP-hBTC construct or with the pGMAX-GFP vector using the Amaxa Nucleofector

system (Lonza, Cologne, Germany). After transfection cells were cultured in high glucose medium (4.5 g/L) for 48 hours and harvested for protein isolation.

2.3 Western blot analyses

Cells were homogenized in lysis buffer (Cell Signaling, Danvers, MA, USA) and 30 µg total protein were separated by SDS-PAGE and transferred to PVDF membranes by electroblotting. Loading of equal amounts of protein for each sample was verified with Ponceau staining. A rabbit antibody that reacts specifically with human BTC (R&D, Systems, Wiesbaden, Germany) and a secondary HRP-conjugated goat anti-rabbit-antibody (Cell Signaling) were employed. Bound antibodies were detected using an enhanced chemiluminescence detection reagent (ECL Advance Western Blotting detection kit, GE Healthcare) and appropriate x-ray films (GE Healthcare).

2.4 Generation of transgenic mice

The PIP-hBTC expression cassette was released from the vector backbone by *Sal*I/*Not*I double digestion, purified by agarose gel electrophoresis, diluted to 2 ng/µl in injection buffer (Nagy *et al.* 2003) and employed for pronuclear microinjection into fertilized oocytes from the inbred strain FVB/N. The injected zygotes were transplanted into the oviducts of pseudopregnant females and potential founder animals were screened by PCR. The animals had free access to a standard rodent diet (V1534, Ssniff, Soest, Germany) and water. All experiments were approved by the author's institutional committee on animal care and carried out in accordance with the German Animal Protection Law with permission from the responsible veterinary authority.

2.5 Immunohistochemistry and immunofluorescence

Animals were anesthetized and killed by cervical dislocation. Pancreata and other organs were removed immediately after the animal's death, carefully trimmed free of adjacent tissues, weighed, fixed in 4% paraformaldehyde (in PBS, pH 7.4) and embedded in paraffin. For immunohistochemical localization of BTC, the streptavidin-biotin method was applied. Five µm thick sections were cut, deparaffinized and heated for 20 min in a microwave in 10 mM sodium citrate buffer for antigen retrieval. The primary antibody (same as for Western blot, dilution 1:300) was incubated for 2 hours at room temperature, followed by a biotin-conjugated rabbit anti-goat secondary antibody (Dako, Hamburg, Germany). Diaminobenzidine (Sigma, Taufkirchen,

Germany) served as chromogen. Finally, the sections were counterstained with hematoxylin. Colocalization of BTC with insulin and glucagon was studied by multicolor immunofluorescence: Five µm paraffin sections pretreated as described above were incubated for 1 hour at room temperature with the primary antibodies diluted in Tris-buffered saline (TBS, pH 7.6). After three washes with TBS, sections were incubated for 1 hour with fluorescence-labeled secondary antibodies. We used the previously described goat anti-human BTC antibody (R&D, Systems, Wiesbaden, Germany), rabbit anti-glucagon and guinea pig anti-insulin antibodies (Dako, Hamburg, Germany). The secondary antibodies all rose in donkey and coupled to FITC, Cy3, or Cy5 were from Dianova (Hamburg, Germany). After the last washing step, slides were mounted with Vectashield antifade solution (Vector Laboratories, Grünberg, Germany) containing DAPI as a nuclear counterstain. Confocal optical sections (pixel size 50 x 50 nm, pinhole size 1 Airy unit corresponding to an optical thickness of 0.7 - 1 µm) were recorded using a confocal laser scanning microscope (LSM 510 Meta, Zeiss, Oberkochen, Germany) with a 40 × PlanNeofluar oil immersion objective (NA 1.3). For excitation of DAPI, FITC, TRITC and Cy5 laser lines of 364, 488, 543 and 633 nm were used. Fluorescence signals were detected through the following emission filters: BP 385 - 470 nm for DAPI, BP 505 - 530 nm for FITC, BP 560 - 615 for Cy3 and LP 650 for Cy5. A polychromatic multichannel detector (Meta detector, Zeiss) was used to discriminate autofluorescence from immunofluorescence signals.

2.6 Reverse transcriptase PCR

Isolation of total RNA, first strand cDNA synthesis, and semiquantitative RT-PCR were performed as described earlier (Schneider *et al.* 2001). The primers previously described for amplifying the human BTC cDNA were employed for the detection of transgene-specific BTC mRNA expression. The integrity of the template cDNA was confirmed by amplifying a sequence of the β-actin gene (sense, 5'-GGCATCGTGATGGACTCC-3'; antisense, 5'-GTCGGAAGGTGGACAGGG-3').

2.7 Glucose metabolism studies

After fasting for 14 hours, blood was drawn from the retro-orbital sinus of ether anesthetized mice for determination of blood glucose. For glucose tolerance test, fasted mice (16 hours) were injected intraperitoneally with glucose (1.5 g/kg body weight). Blood samples were obtained by puncture of the tail vein immediately before glucose

administration and at the indicated time points after injection. A glucometer (Precision, Abbott Diabetes Care, Wiesbaden, Germany) was used to determine glucose levels.

2.8 Statistical analyses

The results of glucose tolerance tests were statistically evaluated by analysis of variance (Linear Mixed Models; SAS 8.2; PROC MIXED), taking the fixed effects of Group (transgenic vs. control), Time (relative to glucose application) and the interaction Group*Time as well as the random effect of Animal into account (Verbeke & Molenberghs 2001).

3 Results

3.1 Evaluation of promoter activity in vitro

To evaluate the overall functionality and specificity of the construct, cell lines representing different cell types were transfected either with the PIP-hBTC or with the pMAX-GFP vector. Transfection efficiency, evaluated 24 hours after the transfection by monitoring GFP fluorescence, was similar for all lines (~50% green cells, data not shown). After 48 hours, cells transfected with PIP-hBTC were harvested for protein isolation and subjected to Western blot analysis employing an antibody that recognizes specifically human BTC. As shown in **Figure 1C**, human BTC was clearly detected in protein extracts from mouse MIN-6 β-cells, but not in extracts from mouse hepatocytes or human embryonic kidney (HEK 293) cells. These in vitro findings indicate that the PIP-hBTC construct is functional and suggest that the employed porcine insulin promoter is selectively activated in β-cells.

3.2 Generation of PIP-hBTC transgenic mice

After microinjection of the PIP-hBTC construct, forty mice were born and three of them were identified as being transgenic by PCR and Southern blot analyses (data not shown). One transgenic animal (#11) consistently failed to generate transgenic progeny and was therefore sacrificed. The remaining two founders (#16 and #38) transmitted the transgene to their descendents in a mendelian fashion, originating two PIP-hBTC transgenic lines (L2 and L3, respectively).

3.3 Specific expression of the PIP-hBTC transgene in insulin-positive cells of the endocrine pancreas

To evaluate expression of the construct in different organs, RT-PCR analysis was performed for RNA samples from lung, liver, small intestine, kidney, muscle, and pancreatic islets. As shown in **Figure 2A**, transgene expression was detected exclusively in the pancreatic islets in L2 transgenic mice. The same result was obtained for L3 mice (data not shown). Immunohistochemistry against hBTC also failed to detect positive cells in the above mentioned and additional organs such as the heart, stomach and brain (data not shown). To evaluate whether the used porcine insulin promoter directs expression to certain regions of the ventral brain, as described for the rat insulin

promoter (Gannon *et al.* 2000a), we additionally studied the pons, caudate putamen, and the hypothalamus. In none of these brain regions we detected transgene transcripts by RT-PCR (**Figure 2B**) or hBTC by immunohistochemistry (data not shown). In the pancreas of L2 and L3 transgenic mice, hBTC-positive cells were readily detected within the islets, but not in the exocrine compartment (**Figure 2C**). The number of cells expressing the transgene was considerably higher in the islets of L3 transgenic mice as compared to founder #11 and L2 animals (**Figure 2D**). To further evaluate whether the employed PIP sequence specifically directs expression of the transgene to insulin-producing cells, we examined colocalization of hBTC with insulin and glucagon by multicolor immunofluorescence and confocal laser scanning microscopy. In both transgenic PIP-hBTC and non-transgenic control mice, insulin-specific immunofluorescence was evident in the cytoplasm of the majority of islets cells, while a smaller proportion of the cells were negative for insulin and positive for glucagon (**Figure 2E**). Importantly, in transgenic animals, hBTC staining was only observed in insulin-positive cells, indicating that the PIP sequence employed is adequate for targeting exclusively pancreatic β-cells (**Figure 2E**). Evaluation of pancreatic islets from transgenic fetuses sacrificed on day 17.5 of pregnancy revealed that the β-cell specificity of the employed promoter is already present at this early stage of islet formation (**Figure 2F**).

3.4 PIP-hBTC transgenic mice show no overt phenotypic abnormalities

Transgenic mice from both lines developed normally, were fertile, and did not show any obvious abnormalities. Histological analysis of the pancreas did not reveal any pathological alteration in PIP-hBTC transgenic mice, in agreement with our previous results in mice with overexpression of BTC in both the exocrine and the endocrine compartment (Dahlhoff *et al.* 2009c). Body and organ weights were evaluated in transgenic males and females and gender-matched littermates at the age of 5 months (n=4-6/group). No differences were observed in total body weight, and in absolute or relative organ weights, irrespective of the line (data not shown).

3.5 PIP-hBTC transgenic mice show improved glucose tolerance

Intraperitoneal glucose tolerance tests showed that the blood glucose concentrations rose similarly in control and in PIP-hBTC transgenic mice from line 2 and 3 after glucose injection (**Figure 3A**). However, glucose clearance was improved in transgenic mice from both lines in the second half of the test period (**Figure 3A**). When the results were expressed as area under the glucose curve, a significant difference was observed between L2 and L3 transgenic mice and their control littermates, respectively (**Figure 3B**).

4 Discussion

Here we show that an expression cassette containing 1500 bp of the 5' UTR from the porcine insulin gene (including the first exon and the first intron) and the 3' UTR of the bovine *GH* gene is efficient for driving transgene expression in a robust and specific manner to β-cells *in vitro* and in transgenic mice.

To test the functionality of the cassette we have chosen a cDNA encoding the EGFR ligand betacellulin (Schneider & Wolf 2009). Betacellulin is known to stimulate β-cell differentiation in vitro and to improve glucose tolerance in diabetic models and in transgenic mice (reviewed in Dahlhoff *et al.* 2009) - although a few studies failed to demonstrate such an effect (Sjoholm & Kindmark 1999). Recently, we demonstrated an improved glucose tolerance in mice with ubiquitous overexpression of betacellulin (Schneider *et al.* 2005; Dahlhoff *et al.* 2009). This finding needed cautious interpretation, since betacellulin levels were also increased in other tissues. In the present study we demonstrate an improved glucose tolerance in an animal model with β-cell-specific overexpression of betacellulin. Also in agreement with our previous results is the lack of pathological alterations in PIP-hBTC transgenic mice. This is in contrast to the multiple pathological effects including islet disaggregation, fibrosis, and even ductal metaplasia observed in transgenic mice with β-cell-specific overexpression of the EGFR ligands EGF (Krakowski *et al.* 1999) and heparin-binding EGF (Means *et al.* 2003). This observation indicates that BTC has unique actions on pancreatic islets as compared to other EGFR ligands. The transgenic mouse lines described here are valuable for detailed studies of glucose metabolism and β-cell regeneration following induction of diabetes e.g. after injection of streptozotocin.

The new expression cassette described here represents a significant improvement over available expression systems in different ways. First, our cassette potentially allows β-cell-specific expression of gene products in transgenic mice without the unwanted expression in other tissues such as the central nervous system. Nevertheless, the level and specificity of transgene expression in new lines are always difficult to predict due to position effects and require careful characterization (Martin & Whitelaw 1996). Second,

the ease of exchanging the sequences to be expressed in the cassette and the availability of restriction sites for "rare cutter" enzymes for removing the relevant sequences from the vector backbone render this construct a handy tool for the generation of transgenic mice for β-cell specific overexpression of a variety of proteins. Third, it is plausible to predict that the transgene cassette will also perform well in pigs, promoting a widespread use of this species in diabetes research.

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Figures

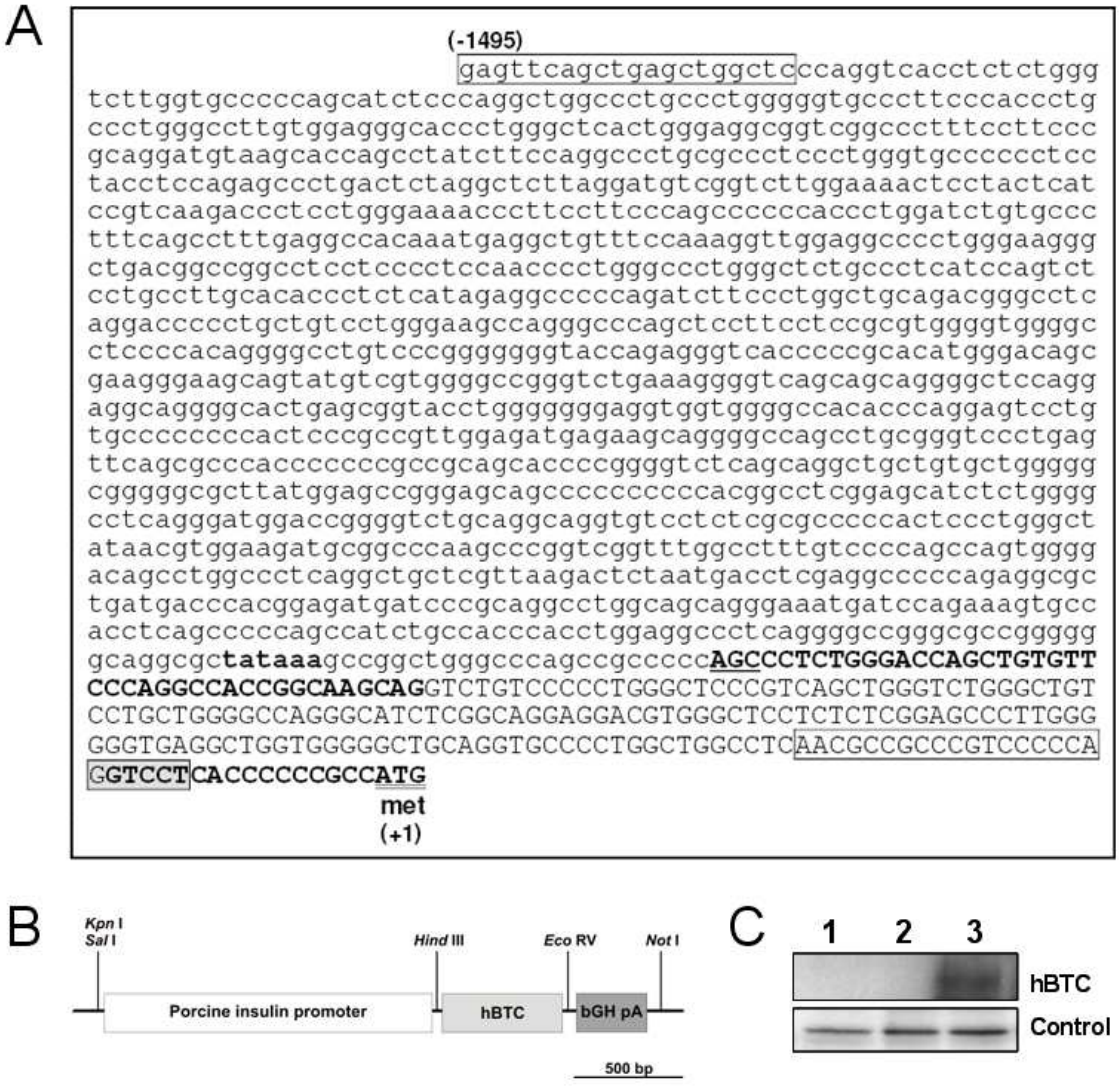

Figure 1 Generation of the PIP-hBTC construct. **A**: nucleotide sequence of the porcine insulin gene sequence employed in this study. The 5' flanking region is shown in lower case (the consensus TATA box is shown in bold). The transcription initiation site is underlined, the transcribed sequence is shown in upper case (cDNA sequence in bold). The translation initiation site is double-underlined and the sequence of the primers used to amplify the promoter is boxed. The sequence changed to a *Hind*III site (AAGCTT) in the second primer is highlighted in grey. **B**: schematic representation of the construct employed to generate transgenic mice including the position of relevant restriction sites. **C**: Western blot analysis of hepatocytes (1), kidney cells (2) and MIN-6 β-cells (3) transfected with the PIP-hBTC construct showing expression of human BTC exclusively in the β-cells. Staining of an unspecific band is shown as a loading control.

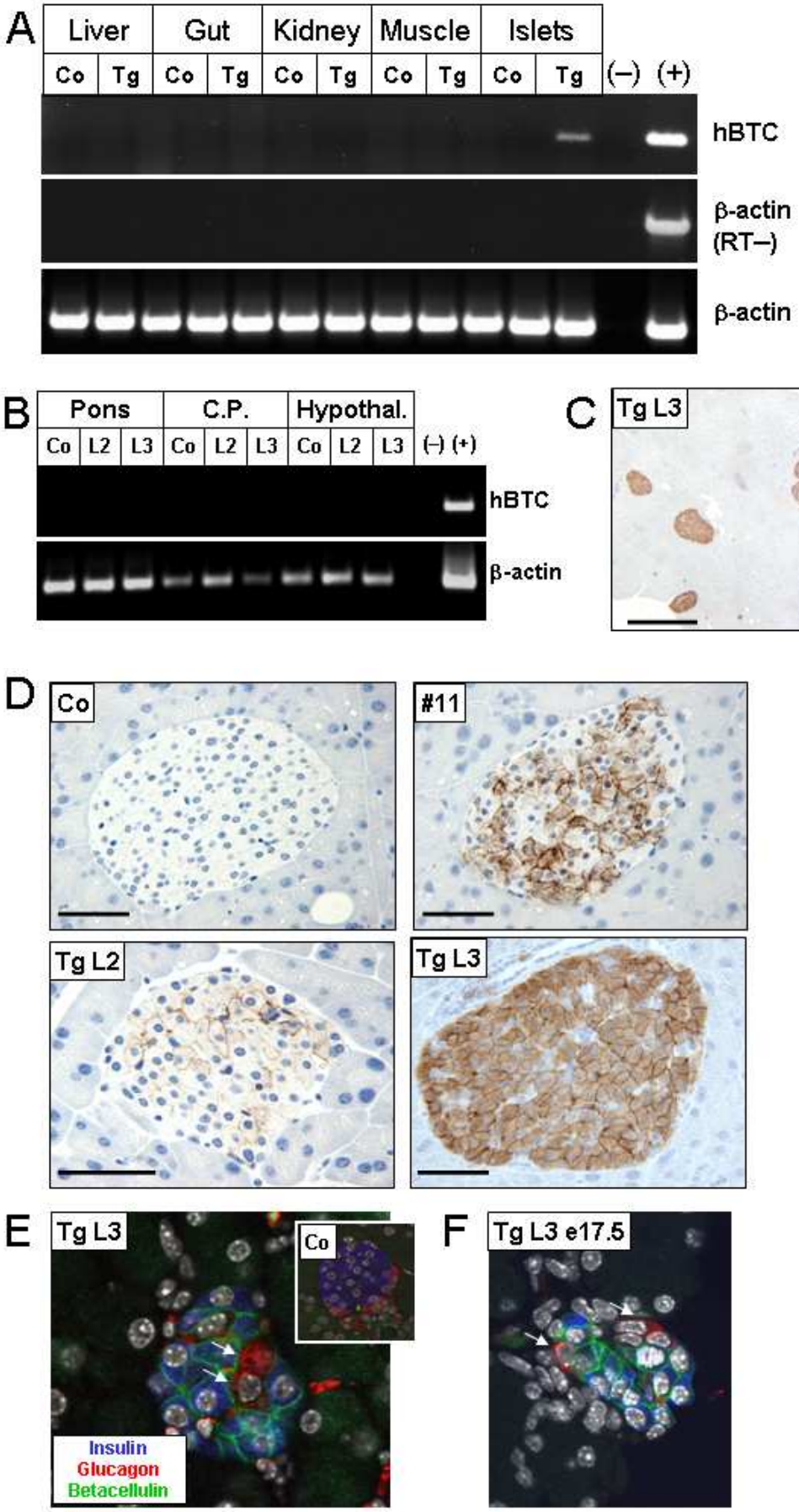
A
Liver
Gut
Kidney
Muscle
Islets
Co
Tg
(–)
(+)
hBTC
β-actin
(RT–)
β-actin
B
Pons
C.P.
Hypothal.
Co
L2
L3
(–)
(+)
hBTC
β-actin
C
Tg L3
D
Co
#11
Tg L2
Tg L3
E
Tg L3
Co
Insulin
Glucagon
Betacellulin
F
Tg L3 e17.5

Figure 2 Tissue and cell-specific expression of the PIP-hBTC construct in transgenic mice. **A, B**: RT-PCR analysis showing that transgene transcription is restricted to the pancreatic islets. Data in A are from L2 animals. C.P.: caudate putamen. **C**: Immunohistochemical staining of hBTC showing exclusive transgene protein expression within the pancreatic islets of L3 transgenic mice. **D**: Immunohistochemical detection of hBTC in the islets of transgenic mice as compared to non-transgenic control mice at higher magnification. Scale bars represent 500 µm in C and 50 µm in D. **E, F**: Simultaneous visualization of insulin, glucagon, and hBTC by multicolor immunofluorescence and confocal laser scanning microscopy in the islets of an adult PIP-hBTC (E), an adult non-transgenic control mouse (insert in E), and a PIP-hBTC fetus at day 17.5 of pregnancy (F).

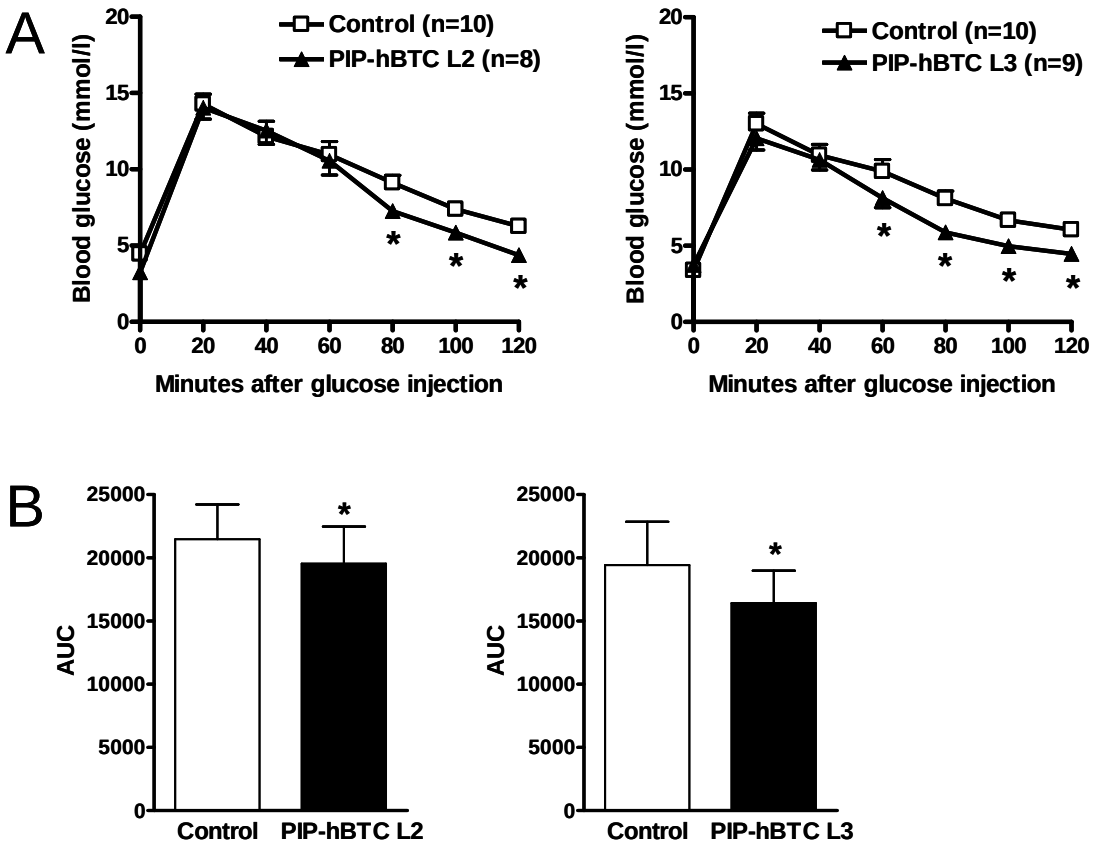

Figure 3 Glucose metabolism in BTC-tg mice. Intraperitoneal glucose tolerance test (A) revealed a significant reduction of the area under the curve (AUC, B) in PIP-hBTC transgenic mice from L2 and L3 as compared to control littermates. Values represent means ± SEM of two independent experiments for L2 and L3. *: $P < 0.05$.

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4 DISCUSSION

In the present study transgenic mice overexpressing human betacellulin under the control of the porcine insulin promoter were generated in order to investigate the ability of a new cassette to direct expression of the transgene specifically to the pancreatic β-cells. Both *in vitro* and *in vivo* analyses performed in this study showed that the expression cassette containing 1500 base pairs of the 5' UTR (untranslated region) from the porcine insulin gene and the 3' UTR of the bovine *GH* gene efficiently drives the transgene expression in a robust and specific manner to β-cells. Other advantages of this new construct are the ease of exchanging the sequences to be expressed in the cassette and the availability of restriction sites for "rare cutter" enzymes for removing the relevant sequences from the vector backbone. It renders this construct a handy tool for the generation of transgenic mice with β-cell specific overexpression of a variety of proteins.

The employment of insulin promoters in transgenic technology became a useful strategy to create animal models exhibiting not only diabetic symptoms but also an improved glucose metabolism. Transgenic animals overexpressing, for example, hepatocyte growth factor (Garcia-Ocana *et al.* 2001), pituitary adenylate cyclase-activating polypeptide (PACAP) (Yamamoto *et al.* 2003) or hepatocyte nuclear factor-1 α (Watanabe *et al.* 2007) under the control of the rat, human or porcine insulin promoter permitted to investigate the potentially positive function of these proteins in the insulin secretion and blood glucose reduction. Moreover, the usage of experimental diabetic mice with an transgenic overexpression of hyperglycaemia-avoiding molecules driven by insulin promoters (Miyawaki *et al.* 2008; Wang *et al.* 2008) demonstrated a possibly therapeutic approach to prevent the progression of diabetes mellitus in patients.

Depending on the employed promoter, the targeted gene can be expressed in many different organs (Sandgren *et al.* 1990) or in one or more particular tissues (Yamamoto *et al.* 2003; Burkhardt *et al.* 2006). Also position effects can be an obstacle in the creation of a transgenic animal lines with a tissue specific expression of the transgene (Festenstein *et al.* 1996). The frequently used rat insulin promoter has been shown to

induce the transcription of the transgene not only in the pancreas but also in some parts of the brain, including hypothalamus (Gannon *et al.* 2000; Covey *et al.* 2006). In the present study the porcine insulin promoter was employed to elicit a β-cell specific expression of the transgene with the long-term goal of generating BTC transgenic pigs.

Importantly, both PIP-hBTC transgenic mouse lines 2 and 3 used in the presenting study expressed the hBTC-mRNA solely within the islets of Langerhans. Reverse transcription PCR and immunhistochemical staining of different organs from the wild-type and transgenic mice against hBTC supported the conclusion of islet-specificity of the expression. Investigations using a multicolour immunofluorescence confirmed the β-cell specificity of the expression. Moreover, additional analyses of several brain areas of the transgenic animals confirmed the islet-specificity.

The potential of the PIP promoter to direct the β-cell specific expression of the transgene may stimulate the creation of large transgenic animal models overexpressing anti-diabetic agents solely in the pancreatic islets. Such an approach could be particularly worthwhile in the generation of transgenic pigs as donors for transplantations of the pancreatic islets into humans. Furthermore, results of the previous investigations (Yamamoto *et al.* 2000; Dahlhoff *et al.* 2009) and of the present study as well confirm betacellulin as an effective factor improving the glucose homeostasis and preventing diabetes mellitus.

Molecular analysis of the porcine insulin gene and promoter (German *et al.* 1995; Ohneda *et al.* 2000; Melloul *et al.* 2002) provide a background for understanding the strict β-cell-associated insulin transcription. It has been demonstrated that regulation of the insulin gene occurs by various *cis*-acting elements localized in the 5'-flanking region of the insulin promoter (German *et al.* 1995; Ohneda *et al.* 2000). These elements are potentially able to induce the β-cell specific transcription of any gene placed downstream. This activity of the porcine insulin promoter has been also employed to regulate the expression of genes of interest *in vitro* and *in vivo* (Watanabe *et al.* 2007). However, so far there are hardly publications describing animal models with a porcine insulin promoter to direct the expression of a transgene.

During mouse embryogenesis, the insulin-containing cells appear in the dorsal pancreatic bud at embryonic day (E) 11.5 and one or two days later in the ventral pancreatic bud (Herrera *et al.* 1991). At E 18.5 the typical islets are already formed with the centrally located β-cells surrounded by other endocrine cells (Herrera *et al.* 1991). The immunohistochemical analyses of pancreatic tissue of the PIP-hBTC transgenic and wild type mice from E 17.5 detected expression of the transgene in the originating pancreatic islets, demonstrating activity of the porcine insulin promoter already during the embryonic development. Nevertheless, expression of the transgene under control of the mouse PDX1 promoter in the embryonic pancreas was detectable already at E 12.0 (Dichmann *et al.* 2003).

A positive effect of betacellulin on the stimulation of β-cell differentiation (Mashima *et al.* 1996; Thowfeequ *et al.* 2007) and increase of insulin production (Mashima *et al.* 1996; Huotari *et al.* 1998;) was previously showed via *in vitro* investigations. Importantly, PIP-hBTC transgenic mice from lines 2 and 3 generated in the present study exhibited an improved glucose tolerance. These results confirm the results of other studies involving the overexpression of this growth factor in a mouse model (Schneider *et al.* 2005; Dahlhoff *et al.* 2009), suggesting a positive effect of this protein also in the glucose homeostasis. However, it must be considered that in the above mentioned studies betacellulin levels were elevated not only within the pancreas but also in many other tissues as well (Schneider *et al.* 2005; Dahlhoff *et al.* 2009).

Furthermore, treatment of the alloxan- and streptozotocin-diabetic rodents with recombinant betacellulin, appeared to have a significant impact on the improvement of glucose metabolism (Yamamoto *et al.* 2000; Li *et al.* 2003). Nevertheless, there is at least one study showing that betacellulin had no significant impact on insulin secretion and blood glucose reduction (Sjoholm & Kindmark 1999).

Overexpression of different growth factors or their receptors is often associated with pathological alterations. Human BTC transgenic mice from L2 and L3 did not show any alterations in the body weight. Also, general conditions of these animals were unimpaired. In contrast, studies employing mouse models with an ubiquitously overexpression of BTC demonstrated an early postnatal mortality rate and reduced body weight gain (Schneider *et al.* 2005). A retarded growth has been also reported in mice

overexpressing other EGFR ligands such as TGFA (Sandgren *et al.* 1993) or EGF (Chan & Wong 2000; Wong *et al.* 2000). Moreover, in contrast to betacellulin, the elevated expression of epithelial growth factors like TGFA (Sandgren *et al.* 1990) and HB-EGF (Means *et al.* 2003) has been shown to cause epithelial metaplasia, pancreatic hyperplasia and fibrosis in transgenic mice (Sandgren *et al.* 1990; Means *et al.* 2003). Furthermore, even though betacellulin is known for its mitogenic activity (Shing *et al.* 1993; Kim *et al.* 2003), histological analysis of different tissues from PIP-hBTC mice did not detect any alterations pointing to tumor development. The life-span of the transgenic animals remained unimpaired.

5 SUMMARY

Analysis of a new transgenic mouse model with β-cell specific overexpression of human betacellulin

In the present study, a new transgenic mouse model was generated in order to evaluate whether the full 5' untranslated region (UTR) of the porcine insulin gene was able to confer expression of a transgene robustly and specifically in the pancreatic β-cells.

An expression cassette containing 1500 base pairs of the 5' UTR porcine insulin gene promoter and 3' UTR of the bovine growth hormone gene was designed to permit easy exchange of the sequences to be expressed. The functionality of the cassette was verified by employing a cDNA encoding human betacellulin, a growth factor known to affect structural and functional parameters of β-cells.

The functionality of the PIP-hBTC construct was initially evaluated by conducting *in vitro* experiments. Transfection of different cell types with the PIP-hBTC vector, followed by Western blot analysis of the isolated proteins, showed the presence of human betacellulin solely in the mouse MIN-6 β-cell line.

Next, two transgenic mouse lines (L2 and L3) were generated by pronuclear DNA microinjection. For evaluation of expression specificity, tissue samples of the liver, gut, kidney, muscle and pancreatic islets were removed from the transgenic and control mice, and analysed using reverse transcription PCR and immunhistochemistry. These investigations demonstrated the presence of human betacellulin only within the endocrine pancreas of the transgenic animals with concomitant absence of transgene expression in any other organ or in tissues of control littermates.

Considering the reported ectopic activity of the rat insulin promoter in some brain areas of transgenic mice, the potential action of the porcine insulin promoter in different parts of the brain was analysed using reverse transcription PCR and immunhistochemistry. Transcripts for human betacellulin were never detected in any of the examined regions of the ventral brain.

Employment of multicolour immunofluorescence and confocal laser scanning microscopy confirmed expression of human betacellulin solely by insulin producing cells of the transgenic mice, confirming the β-cell specificity of the expression.

PIP-hBTC transgenic mice from both lines developed normally, were fertile and did not exhibit any abnormalities. Also, histological analysis of the pancreas and other organs removed from older individuals did not reveal any pathological alterations.

Confirming the known insulinotropic effects of betacellulin, transgenic mice demonstrated an improved glucose metabolism: during intraperitoneal glucose tolerance test, PIP-hBTC mice cleared glucose significantly faster as compared with their control littermates.

Taking together, the newly designed expression cassette, containing 1500 base pairs of the 5'UTR of the porcine insulin promoter, drove the expression of the transgene robustly and specifically to β-cells, *in vitro* and in transgenic mice. It also represents a significant improvement over available expression systems due to easy exchange of the sequences to be expressed. Moreover, the possibility of eliciting a β-cell-specific expression of gene products in transgenic large animal models without unwanted expression in other tissues could become useful for future studies in diabetes research.

6 ZUSAMMENFASSUNG

Untersuchung eines neuen transgenen Mausmodells mit β-Zell-spezifischer Überexpression des humanen Betacellulins

Ziel dieser Studie war die Erstellung eines transgenen Mausmodells zur Überprüfung der Fähigkeit des 5’ untranslatierten Bereich (UTR) des Schweineinsulin-Genes, eine stabile und spezifische Expression des Transgens in pankreatischen β-Zellen hervorzurufen.

Das Expressionskonstrukt besteht aus 1500 Basenpaaren des 5’ UTR des Schweineinsulin-Promotors (Porcine Insulin Promoter, PIP) und dem 3’ UTR des Rinderwachstumshormon-Genes (bovine Growth Hormone, bGH). Der Aufbau des Konstruktes ermöglicht eine einfache Verwendung für Sequenzen unterschiedlicher Transgene. Die Funktionalität der Kassette konnte durch den Einsatz der für das humane Betacellulin (hBTC) kodierende cDNA nachgewiesen werden. Betacellulin ist ein Wachstumsfaktor der die funktionellen und strukturellen Parameter der β-Zellen beeinflussen kann.

Die spezifische Expression des Konstruktes wurde zunächst mit Hilfe von *in vitro* Experimenten überprüft. Verschiedene Zelltypen wurden mit dem PIP-hBTC Vektor transfiziert. Der Expressionsnachweis von BTC in den Zelllysaten erfolgte mittels Western-Blot-Analysen. Das hBTC-Protein konnte ausschließlich in der MIN6-Insulinoma Zelllinie gefunden werden.

Anschließend wurden zwei transgene Mauslinien (L2 und L3) durch DNA-Mikroinjektionen in die Vorkerne befruchteter Eizellen erstellt. Um die Expressionsspezifität *in vivo* beurteilen zu können, wurden Gewebeproben aus Leber, Darm, Niere, Muskel und isolierten pankreatischen Inseln von transgenen Mäusen und Kontrolltieren (Wurfgeschwister) entnommen und sowohl durch die Reverse Transkriptase-Polymerase-Kettenreaktion (RT-PCR) als auch durch immunhistochemische Verfahren untersucht. Das humane BTC konnte hierbei nur innerhalb des endokrinen Pankreas der transgenen Mäuse nachgewiesen werden.

Da es Untersuchungen gibt, in denen eine zusätzliche Aktivität des Ratteninsulin-Promotors in einigen Gehirnbereichen transgener Tiere festgestellt worden sind, wurde

eine potentielle Aktivität des Schweineinsulin-Promotors im Gehirn der hBTC-transgenen Mäuse überprüft. Die Ergebnisse der RT-PCR und der Immunhistochemie haben keinerlei Expression des hBTC in den Gehirnen der transgenen Tiere gezeigt.

Zur Überprüfung der β-Zell-Expressionsspezifität wurde eine konfokale Mehrfarb-Immunfluoreszenzmikroskopie durchgeführt. Hierbei wurde das humane Betacellulin ausschließlich in den Insulin-produzierenden Zellen nachgewiesen.

Obwohl mehrere Studien über mitogene Eigenschaften des humanen BTC berichten, konnten wir keine Entwicklungsstörungen und Fertilitätsprobleme in PIP-hBTC-Mäusen beobachten. Außerdem ergaben die histologischen Analysen der Pankreatiden und anderen Organen der älteren Tiere keine pathologischen Veränderungen.

Die Blutglukose-senkende Wirkung von Betacellulin wurde auch in dieser Studie bestätigt: In intraperitonealen Glukose-Toleranz-Tests zeigten die PIP-hBTC-transgenen Tiere eine signifikant schnellere Senkung des Blutglukosespiegels im Vergleich zu ihren Kontrollgeschwistern.

Zusammenfassend kann gesagt werden, dass das für diese Arbeit generierte Konstrukt mit seinen 1500 Basenpaaren des 5'UTR des Schweineinsulin-Promotors zu einer stabilen und spezifischen Expression eines Transgens ausschließlich in den β-Zellen des Pankreas führt. Ein weiterer Vorteil dieses Konstrukts ist der einfache Austausch der zur Expression vorgesehenen Sequenz. Darüber hinaus, bietet eine β-Zell-spezifische Überexpression (ohne ungewollte Expression in anderen Organen) eines gewünschten Genprodukts die Möglichkeit in Großtiermodellen beispielsweise Studien über neue Therapiemethoden, wie Xenotransplantationen, gegen Diabetes Mellitus durchzuführen.

7 BIBLIOGRAPHY

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