In vitro development of islets from human adult pancreatic tissues

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ACADEMIC DISSERTATION

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CONTENTS

ABBREVIATIONS

ANOVA	analysis of variance
BM	bone marrow
BrdU	bromodeoxyuridine
CAM	cell adhesion molecule
CHIB	cultivated human islet bud
CK	cytokeratin
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
ESC	embryonic stem cell
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GLP-1	glucagon-like peptide-1
GLUT-2	glucose transporter 2
gw	gestational week
HGF	hepatocyte growth factor
HNF	hepatocyte nuclear factor
IFN-γ	interferon-gamma
Ig	immunoglobulin
Ihh	Indian hedgehog
INGAP	islet neogenesis-associated proteins
ip	intraperitoneally
MACS	magnetic cell sorting
MEM	minimal essential medium
MMF	mycophenolate mofetil
MODY	maturity-onset diabetes of young
N-CAM	neural cell adhesion molecule
ngn3	neurogenin 3
NIC	nicotinamide
NOD	non-obese diabetic
PBS	phosphate buffered saline
PC1/3	prohormone convertase 1/3
Pdx1/ Ipf1	pancreatic duodenal homeobox 1/ insulin promoter factor 1
PFA	paraformaldehyde
PP	pancreatic polypeptide
Reg	regenerating genes
Shh	Sonic hedgehog
SHC	small hepatocytes
TGF	transforming growth factor
TRITC	tetramethylrhodamine isothiocyanate

LIST OF ORIGINAL PUBLICATIONS

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

I. **Gao R**, Ustinov J, Pulkkinen MA, Lundin K, Korsgren O, Otonkoski T. Characterization of endocrine progenitor cells and critical factors for their differentiation in human adult pancreatic cell culture. Diabetes 2003; 52: 2007-2015.

II. **Gao R,** Ustinov J, Korsgren O, Otonkoski T. *In vitro* neogenesis of human islets reflects the plasticity of differentiated human pancreatic cells. Diabetologia 2005; 48: 2296-2304.

III. **Gao R,** Ustinov J, Korsgren O, Mikkola M, Lundin K, Otonkoski T. Maturation of *in vitro*-generated human islets after transplantation in nude mice. Molecular and Cellular Endocrinology 2007; 264: 28-34.

IV. **Gao R,** Ustinov J, Korsgren O, Otonkoski T. Effects of immunosuppressive drugs on *in vitro* neogenesis of human islets: mycophenolate mofetil inhibits the proliferation of ductal cells. American Journal of Transplantation, *in press*

ABSTRACT

Transplantation of isolated islets from cadaver pancreas is a promising possibility for the optimal treatment of type 1 diabetes. The long-term function of human pancreatic islet grafts may depend on the neogenesis of beta cells from epithelial precursors within the grafted tissue. Islet neogenesis can be induced in adult mouse and human pancreatic ductal cell cultures. Using the *in vitro* model for human islet neogenesis, we investigated the characteristics of potential pancreatic precursor cells, the optimal conditions for the proliferation, differentiation, and maturation of the cultivated human islets buds (CHIBs), and finally the effects of immunosuppressive drugs on islet neogenesis.

In this study, we first reproduced the culture of *in vitro* generation of endocrine cells from human adult pancreatic tissue. By tracing the bromodeoxyuridine-labeled cells in differentiated islet buds, we found that the pancreatic progenitor cells represented a subpopulation of cytokeratin 19 (CK19)-positive ductal cells but not nestin-positive cells. Serum-free culture was found to be an absolute requirement for the endocrine differentiation to occur. Also, overlay of the cells with Matrigel was essential, whereas nicotinamide had a potentiating effect.

One problem in most of the experiments performed with human pancreatic cells is the presence of preexisting islet cells at the onset of culture. We thus examined their involvement in islet cell neogenesis. The dedifferentiation of preexisting islet cells was identified in monolayer culture. About 6-10% of endocrine cells acquired a transitional phenotype by coexpressing the ductal marker CK19. Significant cell proliferation was only observed in CK19-positive cells, but not in chromogranin A-positive endocrine cells. Consequently, it is possible that the dedifferentiated islet cells started to proliferate after losing their endocrine phenotype, and might have acted as precursors of newly differentiated islet cells during the differentiation phase.

The *in vitro*-derived human islets were morphologically and functionally immature when compared with normal islets. Their insulin mRNA levels were only 4-5% of that found in fresh human islets, and glucose-stimulated insulin release of CHIBs was 3 times lower than that of islets. Moreover, some immature endocrine cells in the CHIBs coexpressed insulin and glucagon, and clusterin was distributed in all four types of endocrine cells. After

transplantation in nude mice, CHIBs became mature with one type of hormone per endocrine cell, and clusterin expression became restricted in alpha cells. In addition, we also found that in both fresh islet and CHIB transplants many cells coexpressed endocrine markers and ductal marker CK19 as a sign of ductal to endocrine cell transition.

Finally the study of the effects of immunosuppressive drugs on precursor cell proliferation and differentiation has shown that mycophenolate mofetil (MMF) severely hampered duct-cell proliferation, and significantly reduced the total DNA content indicating its antiproliferative effect on the precursors. Tacrolimus mainly affected differentiated beta cells by decreasing the insulin content per DNA as well as the proportion of insulin-positive cells. Sirolimus and daclizumab did not show any individual or synergistic side effects suggesting that sirolimus and daclizumab are amenable for use in clinical islet transplantation.

In summary, we confirm the capacity of endocrine differentiation from progenitors present in the adult human pancreas. The plasticity of differentiated cell types of human pancreas may be a potential mechanism of human pancreas regeneration. Ductal cell differentiation into endocrine cells in transplanted islets may be an important factor in sustaining the long-term function of islet transplants. The immunosuppressive protocol appears to be an important determinant of long-term clinical islet graft function.

REVIEW OF THE LITERATURE

1. Histology of pancreas and islets of Langerhans

Pancreas is an endoderm-derived organ consisting of a large exocrine (acini and ducts, 99%) and a small endocrine (islets of Langerhans, 1%) compartment. The exocrine pancreas is a lobulated compound acinar gland. The acinar cells produce and secrete a variety of digestive enzymes, including proteases, amylases, lipases and nucleases. Another component of the exocrine pancreas is the highly branched ductal epithelium, which transports the digestive enzymes and bicarbonate ions to the intestine (Githens, 1994). The endocrine cells, which secrete hormones into the bloodstream, are mainly grouped into the islets of Langerhans embedded in the exocrine tissue (Figure 1).

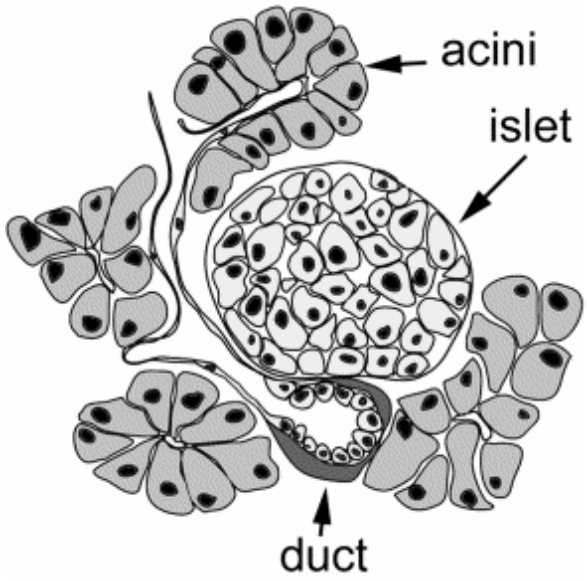

Figure 1. Adult mouse pancreas structure. The mammalian pancreas has three main tissue types: exocrine acini, ducts, and endocrine islets. Three acini are shown draining into a small intercalated duct, an islet is shown embedded in the exocrine tissue and in close association with the duct system. Adapted from Gu et al., 2003.

Islets are made up of four principal types of endocrine cells, organized in a stereotypical topological order in mice with insulin-producing beta cells (60-80%) in the core, glucagon-

producing alpha cells (15-20%), somatostatin-producing delta cells (5-10%), and pancreatic polypeptide-producing PP cells (<2%) at the periphery of the islets (Orci and Unger, 1975; Edlund, 2002). However, in humans the organization of different endocrine cells is more complex (Brissova et al., 2005). Compared with exocrine tissue and other organs, islets are highly vascularized. The endocrine cells are closely surrounded by a dense capillary network, which is about 5 times denser than the capillary network of the exocrine tissue (Konstantinova and Lammert, 2004). Insulin is the most important hormone of the islets, which is stored in secretory granules of beta cells and released for the maintenance of the blood glucose homeostasis. Glucagon functions together with insulin to regulate glucose metabolism. Somatostatin and pancreatic polypeptide regulate the secretion of other hormones and exocrine enzymes (Adrian et al., 1978; Roncoroni et al., 1983). All the types of islet cells, in addition to their specific hormones, also express a number of markers of neuroendocrine cells, such as synaptophysin and chromogranin A. The human pancreas, an organ of 70-150 grams in weight and 15-25 cm in length, is divided into head, body and tail to designate regions from proximal to distal. The head part contains more PP-rich islets while the tail part contains more glucagon-positive cells. There are about one million islets in the human pancreas while the number of islets in the mouse pancreas is only several hundred.

2. The development of pancreas

During embryonic development, both endocrine and exocrine cells develop from common stem cells within the early gut endoderm. The pancreas arises from the endoderm as a dorsal and a ventral bud which fuse together to form the single organ. The early pancreatic bud shows uniform expression of the pancreatic duodenal homeobox 1 (Pdx1) gene (Guz et al., 1995). In the mouse developing pancreas, the endocrine cells start to differentiate before the exocrine cells, and in the mouse the earliest hormone-expressing cells appear at embryonic day 9.5 (E9.5). These early hormone-expressing cells are largely glucagon-expressing although some cells also express insulin. Coexpression of different hormones by the same cells is often observed at early stages (Teitelman et al., 1993; Slack, 1995). The exocrine cells, which form branched ducts and acini, appear at E14.5 in the mouse (Edlund, 1998; Kim and Hebrok, 2001). At this stage, the endocrine cells migrate to the mesenchyme and aggregate to form clusters of islets in close proximity to the ductal epithelia. The formation and maturation of the islets continues until neonatal life. The events underlying the early period of pancreas development have not yet been fully elucidated in humans (Polak et al., 2000). The hormone expression during human pancreas development, evident as rare

epithelial cells immunoreactive for insulin, was first detected at 52 days post-conception while the expression of glucagon and somatostatin appeared one week later at gestational week (gw) 8.5 (Piper et al., 2004). Different from the development of rodent pancreas in which islets are formed properly only after birth, human islets form relatively early during fetal development (Polak et al., 2000; Piper et al., 2004). In humans, the formation of islets begins at gw 12, and during gw 13-16 small aggregates of endocrine cells grow out from pancreatic ducts and become vascularized. Eventually, from gw 17 islets lose their contact with ducts and form the characteristic architecture. Apart from islets, human fetal pancreas also contains a large number of small beta-cell clusters. These "extra-insular" beta-cell units are still present in the adult pancreas and are found to be associated with ductules (Bouwens and Pipeleers, 1998). Endocrine cells coexpressing multiple hormones also appear during early development of human pancreas (De Krijger et al., 1992). The turnover of double hormone to single hormone expression in endocrine cells is regarded as a sign of maturation (Polak et al., 2000).

Hormone coexpressing cells have originally been thought as common endocrine precursors (Teitelman and Lee, 1987; Teitelman et al., 1993; Alpert et al., 1988; Upchurch et al., 1994). For instance, cells coexpressing both glucagon and insulin were thought to give rise to fully differentiated alpha and beta cells. However, by transgenic lineage tracing it has been found that both alpha and beta cells develop independently although they probably arise from a common progenitor, which transiently expresses the transcription factor neurogenin 3 (ngn3) (Jensen et al., 2000a; Herrera, 2000; Gu et al., 2002).

2.1. Transcriptional regulation in pancreas development

Knowledge about the role of transcription factors in pancreatic development has rapidly advanced in recent years. The initiation and maintenance of distinct gene expression programs is required to establish the distinct cell types from uncommitted precursor to mature and terminally differentiated cells. Recent genetic studies have identified a network of transcription factors, which are implicated in regulating the development of pancreas at different stages (Figure 2). Several transcription factors play key roles in the initiation of gut endoderm, formation of pancreas and subsequent differentiation of endocrine and exocrine cells (Wilson et al., 2003; Habener et al., 2005).

Endodermal and pancreatic determination

A variety of hepatocyte nuclear factors (**HNFs**), originally identified as regulators of liver-specific gene transcription, have been found to be involved also in the development of the pancreas. HNF1b, Foxa1 (HNF3α), and Foxa2 (HNF3β) are suggested to initiate the early expression of Pdx1 (Wilson et al., 2003). Mice null for Foxa2 or HNF1b die before pancreas formation (Ang and Rossant, 1994; Weinstein et al., 1994; Coffinier et al., 1999), but embryoid bodies lacking Foxa2 fail to activate the expression of Pdx1, suggesting that Foxa2 may act as an upstream regulator of Pdx1 in the prepancreatic endoderm and pancreatic buds (Gerrish et al., 2000).

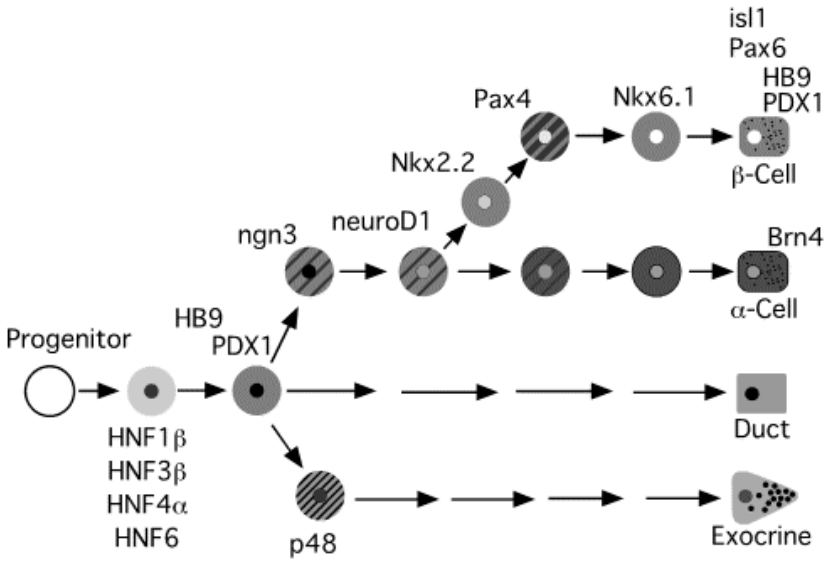

Figure 2. A model for the roles of a number of transcription factors in endocrine and exocrine differentiation in the developing pancreas. The proposed position for each transcription factor is based on its timing of expression, timing of predominant functional role, or both. Adapted from Wilson et al., 2003.

The early prepancreatic endoderm selectively expresses two homeodomain transcription factors, HB9 and Pdx1. **HB9** is necessary for pancreatic dorsal bud induction. Expression of HB9 precedes Pdx1 in the dorsal prepancreatic endoderm, and commences simultaneously with Pdx1 in the ventral prepancreatic endoderm (Li et al., 1999). HB9 is down-regulated when the pancreatic buds form but reappears in the differentiated beta cells. In embryonic

mice homozygous for targeted mutations in the *Hlxb9* gene that encodes HB9, the dorsal prepancreatic endoderm fails to express Pdx1 and formation of the dorsal pancreatic bud never initiates (Harrison et al., 1999). This indicates that HB9 functions upstream of Pdx1 in the dorsal pancreatic bud (Wilson et al., 2003).

Pdx1, a member of the ParaHox group of homeodomain transcription factors, is also known as insulin promoter factor 1 (Ipf1) in humans (Ohlsson et al., 1993), somatostatin transactivating factor 1 (STF1) (Leonard et al., 1993) and islet-duodenal-homeodomain protein 1 (IDX1) (Miller et al., 1994). Pdx1 is required for pancreas development both in mice and humans (Jonsson et al., 1994; Offield et al., 1996; Stoffers et al., 1997b). Pdx1-expressing cells, as common pancreatic precursors, can give rise to all pancreatic cell types: endocrine, exocrine and ductal cells. In mice, Pdx1 expression starts at E8.5 in the dorsal gut endoderm, preceding insulin and glucagon expression. At E9.5 it appears in all cells of the dorsal and ventral pancreatic buds. Subsequently, Pdx1 expression becomes more and more restricted to islet cells (Brooke et al., 1998; Coulier et al., 2000). In postnatal animals, Pdx1 is expressed mainly in pancreatic beta cells, and recent studies suggest it is required for functions in the mature beta cells. Knockout mice with homozygous mutation of the *pdx1* gene selectively lack a pancreas (Jonsson et al., 1994). In a patient with pancreatic agenesis the lack of a pancreas is associated with a single nucleotide deletion in the *Ipf1* gene (Stoffers et al., 1997b). Mice that are heterozygous for *pdx1* develop normally but have impaired glucose tolerance. These mice have more non-beta cells than wild-type mice suggesting that a deficiency in the *pdx-1* gene may skew the islet cell lineages towards developing into non-beta cells (Dutta et al., 1998). However, in transgenic mice a beta-cell specific deficiency of *pdx1* causes reduced beta-cell mass and diabetes (Ahlgren et al., 1998). Heterozygous mutation of the *Ipf1* gene in humans has also been reported to contribute to MODY-type diabetes (Stoffers et al., 1997a).

Endocrine differentiation

Genetic evidence in mice supports a role for **HNF6** as an upstream activator of ngn3 expression. HNF6 is initially expressed throughout the pancreatic buds, but after birth disappears in the mature islets (Rausa et al., 1997; Watada, 2004). Mice lacking HNF6 have severely reduced ngn3 expression in the developing pancreas, and a severely reduced number of endocrine cells with no organized islets at birth (Jacquemin et al., 2000).

Neurogenin 3 (**ngn3**) is a member of a family of basic helix-loop-helix transcription factors that is generally involved in the determination of neural precursor cells in the neuroectoderm. Ngn3 is expressed in discrete regions of the nervous system and in individual cells or small cell clusters within or adjacent to epithelium of the embryonic pancreas. During mouse pancreas development, ngn3 expression has been observed as early as E9.5. Their number increases and peaks at E15.5 just before the formation of recognizable islets at E17, and decreases thereafter until undetectable at birth or in the adult pancreas. **Hes-1**, the downstream target of Notch signaling in the pancreas, inhibits the expression of ngn3 (Lee et al., 2001). Direct cell lineage tracing reveals that ngn3-positive cells are islet progenitors and give rise to all endocrine islet cells during the mouse embryogenesis and in adults (Gu et al., 2002). In ngn3 knockout mice exocrine tissue and pancreatic ducts are nearly normal but endocrine cells fail to form, and these mice die postnatally from diabetes (Gradwohl et al., 2000).

NeuroD/BETA2 is expressed slightly later than ngn3 during pancreatic development and persists in the mature islets, and ngn3 can bind and activate the neuroD gene promoter (Huang et al., 2000). It has been demonstrated that neuroD is critical for the normal development of pancreas and glucose homeostasis. Like ngn3, ectopic expression of neuroD induces premature endocrine differentiation in pancreas, indicating that it is a pro-endocrine gene (Schwitzgebel et al., 2000). Mice carrying a targeted disruption of the neuroD gene develop severe, early-onset diabetes due to an arrest of beta-cell population as well as other islet cell types and eventually die around birth (Naya et al., 1997).

Exocrine differentiation

While cells expressing ngn3 are defined to become endocrine cell, other epithelial cells can become part of exocrine pancreas. The basic helix-loop-helix factor **p48** is required to drive these precursor cells to the exocrine fate. Mice lacking p48 fail to develop any exocrine pancreas with the absence of both acini and the ductal trees although islets form within mesenchyme and migrate to the spleen (Krapp et al., 1998). However, a study, based on lineage tracing of p48 gene *Ptf1a* expression, has revealed that *Ptf1a* is also required to commit foregut endoderm cells to the pancreatic lineages including all acinar cells and most ductal and islet cells (Kawaguchi et al., 2002). No factors that may control the expression of p48 have yet been found (Wilson et al., 2003).

Endocrine cell specification

Ngn3-expressing cells are progenitors for all four endocrine cell subtypes. However, ectopic expression of ngn3 in the early pancreatic bud drives most of the epithelial cells to endocrine alpha-cell fate (Schwitzgebel et al., 2000). This alpha-cell fate seems to the default direction for ngn3-positive progenitors, therefore additional signals are required to deviate these cells to alternate fates such as beta cells. A number of transcription factors that express selectively in endocrine lineage in the developing pancreas and that could play a role in endocrine cell subtype determination have been identified. However, no data have convincingly demonstrated that any of these factors are either necessary or sufficient to control endocrine cell fates (Wilson et al., 2003).

Two members of the Pax family, **Pax4 and Pax6**, play important roles in the differentiation of specific endocrine cell lineages during mouse pancreas development. Pax4 expression is first detected in E10 embryos in only a few cells of the pancreatic bud, then the Pax4-positive cells increase significantly just before the peak of endocrine cell genesis at E15. Little or no Pax4 can be detected in the mature pancreas (Smith et al., 1999; Dohrmann et al., 2000). Pax4 is required for the normal development of beta and delta cells. Mice lacking the Pax4 gene do not generate beta and delta cells and develop diabetes at birth (Sosa-Pineda et al., 1997). Pax6 is first expressed in a few cells of the pancreatic bud at E9.5, and then maintained in all endocrine cells throughout pancreas development as well as in all mature endocrine cells. In mice lacking Pax6, alpha cells are absent, but reduced numbers of beta, delta, and PP cells can be found (St-Onge et al., 1997).

Nkx2.2 and Nkx6.1 both belong to the NK homeodomain family of transcription factors. Nkx2.2 is expressed broadly in the pancreatic bud until E13 when it becomes localized to the ngn3-expressing progenitor cells. Later at E15.5, Nkx2.2 can be detected in all endocrine cell types. As development proceeds, Nkx2.2 becomes restricted to beta cells, most alpha cells and PP cells but is excluded from mature somatostatin-producing delta cells. Mice lacking Nkx2.2 have a complete absence of insulin-producing beta cells, and a reduction in alpha and PP cells (Sussel et al., 1998). Nkx6.1 is initially (E9.0-9.5) expressed predominantly in the dorsal pancreatic epithelium in a subset of Pdx1-positive cells. Later in development, Nkx6.1 becomes restricted to the differentiated beta cells. Knockout data reveal a severe reduction in insulin-producing cells, but normal numbers of other islet cell types, suggesting a key role of Nkx6.1 in beta-cell differentiation (Sander et al., 2000). The absence of Nkx6.1 expression in

the islets of mice lacking Nkx2.2 suggests Nkx2.2 lies upstream of Nkx6.1 in the beta-cell differentiation pathway.

2.2. Epitheliomesenchymal interactions and intercellular signals in pancreas development

Pancreas development requires tropic stimuli, "mesenchymal factors", from the associated mesenchyme (Golosow and Grobstein, 1962). It has long been thought that mesenchyme is necessary for all components of pancreatic development (Wessells and Cohen, 1967; Fell and Grobstein, 1968). In recent years, however, several *in vitro* studies in rodents have found mature islets formed from embryonic epithelium in the absence of mesenchyme whereas the mesenchyme was required for proper differentiation of the primitive epithelium into exocrine pancreas (Gittes et al., 1996; Miralles et al., 1998b). Gittes et al. (1996) have shown that, when pancreatic epithelial rudiments were transplanted under the mouse kidney capsule with their surrounding mesenchyme, they developed well into mature pancreatic tissue including acini, ducts and islets. In contrast, the isolated epithelia without mesenchyme under the same experimental condition formed only pure islets. Both the inductive effect of the mesenchyme on the proper development of the exocrine tissue and its repressive effect on the development of the endocrine cells might be mediated by soluble factors. Follistatin could represent one of these mesenchymal factors that mimic the pancreatic mesenchyme to exert either inductive effects on exocrine development or inhibitory effects on endocrine development (Miralles et al., 1998b).

In vitro experiments with embryonic mouse tissues have demonstrated that vascular endothelial cells provide inductive signals for islet cell development (Lammert et al., 2001). In a later study, Nikolova et al. (2006) found that laminins, among other vascular basement membrane proteins, as endothelial signals, promote insulin gene expression and beta-cell proliferation.

Many of the signaling pathways likely to govern the cell interactions in the developing pancreas have been identified. These include the transforming growth factor-β (TGF-β), Notch, Hedgehog, fibroblast growth factor (FGF), and epidermal growth factor (EGF) pathways (Figure 3) (Kim and Hebrok, 2001).

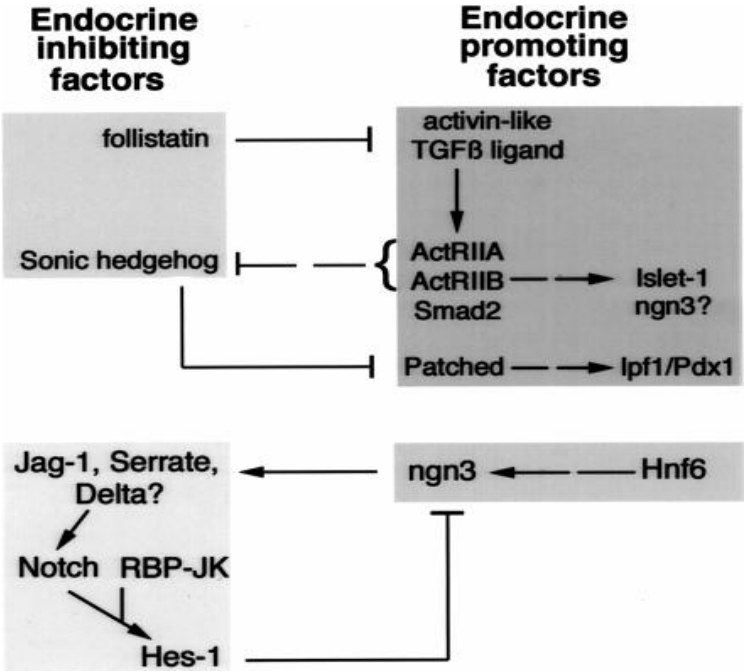

Figure 3. Signaling molecules that promote or inhibit endocrine cell differentiation in pancreas development. Established interactions between molecules indicated by solid lines with arrows (down-arrow) denoting stimulation and blunt lines (perp) denoting inhibition of activity. Indirect interactions indicated by dashed lines, with arrowheads denoting stimulation and blunt lines repression of target gene expression. Adapted from Kim and Hebrok, 2001.

TGF-β signaling is a major regulator of pancreatic endocrine and exocrine cell fates. Two major TGF-β ligands activin and TGF-β1 and their respective receptors are expressed in embryonic pancreas epithelium and mesenchyme. *In vitro* exposure of embryonic mouse pancreas to activin or TGF-β1 promotes the development of endocrine cells, particularly beta and PP cells (Sanvito et al., 1994), and disrupts epithelial branching and acinar formation (Ritvos et al., 1995). In developing pancreas, TGF-β is also involved in regulating islet morphogenesis through activating matrix metalloproteinase MMP-2 (Miralles et al., 1998a). Studies on activin receptor function *in vivo* support these earlier *in vitro* results. Type II activin receptors ActRIIA and ActRIIB are broadly expressed in the pancreatic epithelium of the midgestation mouse embryo, and later in adult islets (Kim and Hebrok, 2001). Type II activin receptor-negative transgenic mice show islet hypoplasia (Yamaoka et al., 1998), and studies on null mutations in type II activin receptors ActRIIA and ActRIIB indicate islet

differentiation is likely regulated by activin receptor-mediated TGF-β signaling (Kim et al., 2000).

The initiation of the pancreatic program requires the signals specifying the pancreas region within the developing gut endoderm. One important component of this early specification involves the exclusion of the hedgehog gene family, *Sonic hedgehog* (*Shh*) and *Indian hedgehog* (*Ihh*). Before pancreatic morphogenesis, repression of *Sonic hedgehog* in posterior foregut endoderm by factors secreted by the notochord like activin and FGF2 prevents intestinal differentiation and promotes the pancreatic development (Hebrok et al., 1998). In addition to this role, **Hedgehog signaling** pathway also regulates cell fates of embryonic pancreas. In *Ihh*$^{-/-}$-deficient embryos, the relative number of insulin- and glucagon-positive cells is marginally increased compared with wild-type mice (Hebrok et al., 2000). Endocrine cell overgrowth is more severe in embryos deficient for *Shh*. Ectopical expression of *Shh* in the pancreatic region results in severe hypoplasia of dorsal pancreas in transgenic mice (Apelqvist et al., 1997). These results suggest that Hedgehog signaling is necessary for limiting the differentiation of pancreatic endocrine cells.

Studies on **Notch signaling** in pancreas have shown the importance of Notch-mediated control of pancreatic fate determinations via control of ngn3. Similar to the generation of neurons during neurogenesis, pancreatic endocrine cells are also specified by lateral specification mediated by the Notch signaling pathway. Notch signaling involves cells expressing high quantities of the ligands like Delta, Serrate, or Jagged to activate Notch receptors on adjacent cells. In concert with the DNA-binding protein RBP-Jk, Notch receptors promote transcription of Hes genes, which subsequently repress expression of neurogenin and other target genes (Edlund, 2001; Kim and Hebrok, 2001). Inactivation of Delta-like gene 1 (Dll1) or RBP-Jk leads to accelerated differentiation of pancreatic endocrine cells paralleled by a depletion of the pool of precursor cells (Apelqvist et al., 1999). Hes1-deficient mice have precocious development of endocrine cells and exocrine cell defects (Jensen et al., 2000b). In contrast, activated expression of Notch1 in developing pancreas could maintain the undifferentiated state of pancreatic precursors and prevent the differentiation of both exocrine and endocrine lineages (Hald et al., 2003; Murtaugh et al., 2003). Taken together, Notch signaling appears to control the choice between differentiated endocrine and progenitor cell fates in the developing pancreas, and a block in the activation of Notch receptors causes high ngn3 expression and in turn promotes the endocrine fate.

Fibroblast growth factors (FGFs) and their receptors (FGFRs 1-4) have been identified as mediators of epithelial-mesenchymal interactions in different organs. FGFs like FGF1, FGF7 and FGF10, and receptors like FGFR1-IIIb and FGFR2-IIIb are expressed throughout pancreatic development. During pancreas specification, FGF2 expressed in the notochord can induce Pdx1 expression in endodermal organ culture (Hebrok et al., 1998). Later in the developing pancreas, **FGF signaling** has been reported to play important roles in the proliferation of epithelial progenitor cells (Bhushan et al., 2001; Hart et al., 2003) and the development of exocrine tissue (Miralles et al., 1999). *In vitro* studies have shown that FGFs can induce proliferation of epithelial cells and differentiation of exocrine cells in rat embryo cultures (Miralles et al., 1999; Le Bras et al., 1998; Elghazi et al., 2002). FGF7 also induces duct cell proliferation in the adult pancreas of rats (Yi et al., 1994; Krakowski et al., 1999). Overexpression of FGF7 in beta cells causes disruption of islet growth and emergence of hepatocytes within islets (Krakowski et al., 1999). Overexpression of FGF10 also results in disruption of normal beta-cell development and appearance of both ductal and acinar cells within the islets (Yamaoka et al., 2002). In FGF10 null mutants, a marked decrease in the proliferation of Pdx1-positive progenitor cells is found, and the subsequent growth, differentiation and branching morphogenesis of the pancreatic epithelium are arrested (Bhushan et al., 2001). Inhibition of FGFR2 signaling results in a significant reduction of acinar cells and loss of islets (Celli et al., 1998; De Moerlooze et al., 2000). Another study on FGFR2b knockout mice has shown that FGFR2b is necessary for the proliferation and branching of pancreatic ducts but not for the differentiation of endocrine or exocrine cells (Pulkkinen et al., 2003). FGF ligands and receptors are also expressed in the adult mouse pancreas and localized to the beta cells. Transgenic mice expressing soluble dominant-negative forms of FGF receptors have shown that, besides the decrease in beta-cell number, the expression of several functional components in beta cells such as GLUT-2 and PC1/3 was downregulated demonstrating the importance of FGF signaling for beta-cell function (Hart et al., 2000).

It has been proposed that EGF family and their receptors are implicated in the control of cell proliferation and differentiation in a large number of organs including pancreas. EGF-family growth factors activate a group of four receptor tyrosine kinases encoded by the erbB gene family (erbB1/EGFR, erbB2, erbB3 and erbB4) (Kraus et al., 1989; Plowman et al., 1993; Ullrich et al., 1984; Yamamoto et al., 1986). All four erbB receptors and several erbB ligands

like EGF, TGF-α, and betacellulin are expressed in the developing as well as adult pancreas (Miettinen and Heikinheimo, 1992; Kritzik et al., 2000; Press et al., 1990; Krakowski et al., 1999; Miyagawa et al., 1999). **EGF signaling** has important roles during pancreatic development and in adults. Overexpression of TGF-α in mouse pancreas results in hyperplasia of Pdx1-positive ductular cells and progressive interstitial fibrosis with no increase in either acinar or islet cell mass (Jhappan et al., 1990; Song et al., 1999). However, coexpression of TGF-α and gastrin significantly increases islet mass in mice expressing these two transgenes (Wang et al., 1993). Also both TGF-α and gastrin expressions are upregulated in islet neogenesis induced by duct ligation (Wang et al., 1997). Overexpression of EGF under the insulin promoter causes increased islet size and fibrosis around the islets (Krakowski et al., 1999). Exogenous addition of EGF in rat pancreas embryo culture leads to increased proliferation of undifferentiated epithelial precursor cells and increased formation of duct-like structures (Cras-Meneur et al., 2001). Betacellulin, a novel member of the EGF family, not only activates tyrosine phosphorylation of EGFR, but also erbB4 (Riese et al., 1996). Betacellulin can stimulate the proliferation of undifferentiated human fetal pancreatic cells (Demeterco et al., 2000). On the other hand, it also can induce beta-cell differentiation or act as a beta-cell mitogen (Huotari et al., 1998). Betacellulin together with activin A convert exocrine AR42J cells to insulin-expressing cells (Mashima et al., 1996). In 90% pancreatectomized rats, addition of betacellulin accelerates beta-cell regeneration (Li et al., 2001). A recent study has reported that erbB1/EGFR and erbB4 signaling influences the lineage determination of the endocrine cells (Huotari et al., 2002). The development of beta cells is boosted by betacellulin while the erbB4 ligand neuregulin-4 appears to favor the development of delta cells. Instead, both these two factors and EGF appear to reduce the proportion of alpha cells. Transgenic mice deficient in EGFR show impaired islet morphogenesis and delayed beta-cell differentiation (Miettinen et al., 2000).

It has been well established that basement membrane at the interface between epithelium and mesenchyme plays a key role in epithelial-mesenchymal interactions in numerous developing organ systems (Yurchenco et al., 2004). The role of extracellular matrix (ECM) in the growth and differentiation of the pancreas has attracted increasing attention in recent years. The formation of ductal structures from isolated epithelia grown in the mouse basement-membrane-rich Matrigel suggests that basement membrane and its components are important for ductal cell differentiation and three-dimensional ductal morphogenesis (Gittes et al., 1996). Laminins seem to be the key molecules in many of cell-matrix interactions. Laminin-1

(α1β1γ1), a glycoprotein of some epithelial basement membranes, has been reported to induce pancreatic ductal morphogenesis possibly through an interaction with an α6-containing integrin. However, the ductal cell differentiation occurs independent of mouse laminin-1 signaling (Crisera et al., 2000). In this study, the authors also found that those epithelial cells that maintain intimate contact with Matrigel-containing mouse laminin-1 appear to differentiate into exocrine structures whereas the cells that are spared direct contact with it appear to organize into discrete clusters of islets. This notion seems to argue against a role for laminin-1 in pancreatic endocrine development as laminin-1 could specifically promote the differentiation of beta cells from mouse fetal precursor cells (Jiang et al., 1999). Islet cell-ECM interactions together with cell-cell interactions also coordinate migration and adhesion of putative endocrine precursors and subsequent islet cell aggregation and segregation (Cirulli et al., 2000).

Previous evidence indicates that cell adhesion molecules (CAMs) may have some functions in cell type differentiation and tissue pattern determination during development (Edelman, 1986; Takeichi, 1991). During pancreas development, several types of CAMs, such as Ca^{2+}-dependent E-cadherin and Ca^{2+}-independent neural CAM (N-CAM), play important roles in the aggregation of endocrine cells into three-dimensional islets. Inhibition of cadherin function through ectopic expression of a dominant negative E-cadherin protein interferes with embryonic clustering of beta cells (Dahl et al., 1996). Islet architecture in mice lacking N-CAM is abnormal with alpha cells found throughout the islet core (Esni et al., 1999). It has been suggested that E-cadherin provides the cohesive force to cells within aggregates irrespective of their types while different expressions of N-CAM and possibly other CAMs may contribute to the development of the characteristic architecture of islets with beta cells located in the center and alpha cells at the periphery (Rouiller et al., 1991; Cirulli et al., 1994).

3. Beta-cell renewal in adults

Two mechanisms of beta-cell formation, replication of a differentiated beta cell or neogenesis from ductal precursor cells, are maintained postnatally and even in the adult. The capacity of postnatal beta cells to grow or regenerate via replication or neogenesis seems to be low. However, several experimental models have shown that under certain conditions, the growth and neogenesis of beta cells can be reactivated (Peters et al., 2000; Bouwens and Rooman, 2005) .

3.1. Islet regeneration in experimental animal models

Duct ligation

It has long been known that duct ligation may trigger islet neogenesis (Hultquist et al., 1979). However, this process was studied in detail by Wang et al. (1995). Destruction of tail part of the pancreas in adult rats by duct ligation resulted in pronounced changes including replacement of exocrine acini by ductual complexes and significant growth of islet cells. By bromodeoxyuridine (BrdU) labeling, it was also shown that double increase of beta-cell population within the first week after ligation results from the differentiation of non-endocrine ductal precursors but not from the replication of differentiated beta cells (Wang et al., 1995). Thus, in this model, the proliferation and differentiation of exocrine duct cells represents the major mechanism of endocrine beta-cell neogenesis. In another experiment, rat pancreatic tissue was observed one day after duct ligation. The finding of intermediate cells which contain both insulin and amylase granules indicated neoformation of beta cells through transdifferentiation of acinar cells (Bertelli and Bendayan, 1997).

Partial pancreatectomy

Substantial regeneration of both endocrine and exocrine pancreas occurs after a 90% partial pancreatectomy in young adult rats. In this model two pathways are supposed to be involved in pancreatic regeneration: replication of preexisting, differentiated endocrine and exocrine cells; proliferation and subsequent differentiation of ductal epithelium to form new pancreatic lobules including new acini and islets that are indistinguishable from the preexisting ones. The second pathway is considered of particular importance since it provides evidence of the neogenesis of the islets from precursor cells in the adult pancreas (Bonner-Weir et al., 1993). The upregulation of clusterin, originally reported as a cell-aggregating factor (Blaschuk et al., 1983), has also been found in the pancreatic regenerating tissues of pancreatectomized rats, suggesting that clusterin may act as a regulatory factor involved in the neogenesis of the pancreas (Min et al., 2003).

IFN-γ or TGF-α/gastrin transgenic mice

Islet neogenesis has also been provoked in a model of transgenic mice in which interferon-gamma (IFN-γ) is driven off an insulin promoter (Gu and Sarvetnick, 1993). The expression of this cytokine in islets leads to local inflammation and tissue destruction, which consequently results in duct hyperplasia and budding of new islets from hyperplastic ductules. In transgenic mice which overexpress two growth factors, gastrin and TGF-α, islet neogenesis

can be reactivated in the ductular epithelium of the pancreas. Overexpression of a TGF-α transgene only causes metaplastic ductules containing numerous insulin-expressing cells that resemble protodifferentiated precursors of the fetal pancreas. However, these insulin-expressing duct cells are not fully differentiated islets. The differentiation can be promoted by the ectopic expression of the gastrin transgene (Wang et al., 1993).

Cellophane wrapping

A model of partial pancreatic duct obstruction by cellophane wrapping in adult hamsters provides further evidence of the development from duct to islet cells (Rosenberg et al., 1983). After 8 weeks with the head of the pancreas wrapped in cellophane, there was a 2.5-fold increase in islet cell mass. Cellophane wrapping in the Vervet monkey pancreas also resulted in a noticeable increase in endocrine cell volume as well as ductal cell proliferation (Wolfe-Coote et al., 1998).

Alloxan perfusion

Selective beta-cell damage can be obtained by administration of alloxan or streptozotocin. A model of transient diabetes has been established in mice by perfusing the body and tail of the pancreas with alloxan (Waguri et al., 1997). Both proliferation and differentiation took place in the pancreas. In the beta-cell-depleted pancreas, neogenesis of beta cells from the precursors located within the ductular epithelium was observed, while in the non-alloxan-perfused part of the pancreas beta-cell regeneration was based mainly on the proliferation of preexisting beta cells (Peters et al., 2000).

Neonatal streptozotocin model

Beta-cell growth has also been studied in newborn rats treated with streptozotocin (Bonner-Weir et al., 1981; Cantenys et al., 1981; Dutrillaux et al., 1982; Wang et al., 1996). Streptozotocin induces subtotal beta-cell damage with concomitant hyperglycaemia followed by rapid beta-cell regeneration, which is mainly thought to be generated by differentiation from duct epithelial cells. However, the beta-cell function is gradually diminished resulting in the reappearance of diabetes. It has been shown that the extent of beta-cell regeneration depends on the timing of beta-cell damage and that this potential declines rapidly during the first days of life (Wang et al., 1996).

3.2. Islet regeneration-associated factors

Glucagon-like peptide-1 (**GLP-1**) is generated from the proglucagon precursor by prohormone convertase cleavage, and is secreted from intestinal L-cells into the circulation. GLP-1 not only stimulates glucose-dependent insulin secretion, but also expands islet mass by promoting beta-cell proliferation and islet neogenesis. **Exendin-4**, a structural analog of GLP-1, has often been used instead of GLP-1 due to its longer circulating half-life. GLP-1 or exendin-4 has been shown to induce cellular transdifferentiation from exocrine to endocrine cells in several exocrine cell lines (Zhou et al., 1999; Hui et al., 2001). In addition, GLP-1 agonists can promote the differentiation and maturation of fetal islet precursor cells (Hardikar et al., 2002; Movassat et al., 2002). The importance of GLP-1 agonists for stimulating beta-cell proliferation and islet neogenesis has also been demonstrated in normal as well as diabetic rodents (Edvell and Lindstrom, 1999; Xu et al., 1999; Tourrel et al., 2001; Stoffers et al., 2000).

Hepatocyte growth factor (**HGF**) is a mesenchyme-derived factor, originally identified as a circulating molecule implicated in liver regeneration after hepatic injury. HGF and its receptor c-met have been observed in the pancreatic islets of several species. Several *in vitro* studies have demonstrated that exogenous HGF is a mitogen and an insulinotropic agent for the human fetal and adult pancreatic beta cells (Otonkoski et al., 1994a; 1996; Hayek et al., 1995). Local overexpression of HGF under the rat insulin II promoter (RIP-HGF) could result in an increase both in islet size and beta-cell number. RIP-HGF mice also display lower blood glucose concentrations than their normal littermates, and inappropriate hyperinsulinemia (Garcia-Ocana et al., 2000). An *in vivo* study has shown that ip administration of HGF ameliorates hyperglycemia in diabetic mice after transplantation of marginal quantities of pancreatic islets (Nakano et al., 2000). Recently, conditional ablation of c-met in beta cells was shown to result in reduced islet size, decreased insulin levels, and impaired glucose tolerance, suggesting an important role of HGF/c-met signaling in islet growth, function, and glucose homeostasis (Dai et al., 2005).

A differentiatial cDNA screen on regenerative pancreas of mice after 90% partial pancreatectomy led to the cloning of the Regenerating genes (**Reg**) (Terazono et al., 1988; Unno et al., 1992). Reg protein has been shown to promote beta-cell growth both *in vivo* and *in vitro* (Watanabe et al., 1994). In human fetal islet cell culture, expression of Reg is increased during the increased beta-cell replication but decreased when the differentiation of

beta cells is induced (Otonkoski et al., 1994b). In cellophane-wrapped pancreas of hamsters, islet neogenesis-associated proteins (**INGAP**) has been identified, which is expressed during islet neogenesis and thought to be capable of initiating duct cell proliferation, a crucial process in pancreatic neogenesis (Rafaeloff et al., 1997).

Gastrin and its receptors are reported to be transiently expressed in the fetal pancreas in a period of pronounced islet neogenesis, *in vitro* transdifferentiated duct-like cells, and ductal complexes of rat pancreas after duct ligation. Gastrin infusion in duct-ligated rats not only increases the proliferation of ductal cells in the regenerative foci, but also stimulates beta-cell neogenesis by increasing beta-cell mass from transdifferentiated exocrine pancreas, indicating gastrin can extend the process of neogenesis already induced by duct ligation (Rooman et al., 2001; 2002). The combination of EGF and gastrin can induce islet regeneration in alloxan-treated mice (Rooman and Bouwens, 2004). The role of gastrin in islet neogenesis has also been demonstrated in double-transgenic mice overexpressing gastrin and TGF-α, in which ectopic expression of gastrin promotes the beta-cell differentiation in TGF-α induced metaplastic ductules (Wang et al., 1993).

Nicotinamide (**NIC**) was originally used to prevent the development of diabetes in experimental animals (Uchigata et al., 1983; Yamada et al., 1982). Clinical trials suggest that NIC administration may preserve beta-cell function and delay or prevent the development of type I diabetes if started early enough in the course of the disease (Elliott and Chase, 1991), but recent studies showed that NIC fails to prevent or delay the disease onset (Schatz and Bingley, 2001; Cabrera-Rode et al., 2006). Most importantly, NIC has been proven as a potent inducer of endocrine differentiation in both human and porcine fetal pancreatic cells (Otonkoski et al., 1993; 1997).

4. Diabetes mellitus and cell-replacement therapy

There are two main types of diabetes mellitus: type 1 (insulin-dependent) diabetes and type 2 (insulin-independent) diabetes. While type 2 diabetes is a more complex and heterogeneous range of conditions usually involving a degree of insulin non-responsiveness in the target tissues, type 1 diabetes is a single cell disorder, which results from specific autoimmune-mediated destruction of the insulin-producing beta cells within the pancreatic islets of Langerhans. Since the discovery of insulin more than 80 years ago, replacement therapy by injected insulin has been the only option for patients with type 1 diabetes. Although insulin

therapy has developed enormously, in many cases the patients still can not achieve normal control of blood glucose levels and there is also a consequent risk of long-term complications. The biological alternative, the re-introduction of beta cells into the organism, seems more attractive because no mechanical device could ever mimic the fine-tuned control of nutrient-induced insulin release by a normal beta cell (Otonkoski et al., 2005; Santana et al., 2006).

4.1. Islet transplantation and immunosuppression

The beta-cell replacement therapy has been established by clinical islet transplantation trials. Injection of islets isolated from cadaveric organ donor pancreata into the portal vein of a type 1 diabetic patient can result in independence of injected insulin (Shapiro et al., 2000; Ryan et al., 2001). Introduction of glucocorticoid-free immunosuppressive protocols has been one of the main reasons for the improved success of clinical islet transplantation in type 1 diabetes. The Edmonton protocol has recently been confirmed in an international multi-center trial, which demonstrated that 44% patients attained insulin independence with good glycemic control one year after the islet transplantation (Shapiro et al., 2006). Although immunosuppressive medication is still mandatory to prevent islet graft rejection, the beta-cell toxicity of these drugs may be an important issue for long-term outcome. It appears that many of the drugs commonly used, including sirolimus, tacrolimus and mycophenolate, do have negative effects on beta-cell function and viability *in vitro* (Paty et al., 2002). Thus it is possible that the immunosuppressive regimen itself may prevent the success in some cases of islet transplantation. Furthermore, this transplantation procedure most often needs more than one donor pancreas for each patient. In order for beta-cell replacement therapy to really have a major clinical impact, new sources of transplantable insulin-producing cells need to be developed.

4.2. Alternative sources of beta cells

4.2.1. Pancreatic stem cells

It seems to be a more logical step to make a beta cell from a pancreatic duct cell than from other possible sources. A specific cell type, characterized by the expression of two transcription factors Pdx1 and ngn3, represents the precursor of all islet cell types (Jensen et al., 2000a; Wilson et al., 2003). The pancreatic islet stem cells have been fairly well characterized in the developing pancreas. However, much more controversy remains concerning the possible existence and nature of stem or precursor cells in the adult pancreas. Numerous studies in rodents have shown that even the adult pancreas has a considerable

capacity to regenerate after subtotal pancreatectomy or injury from ductal occlusion. A general view has been that the islet precursor cells reside within the pancreatic ductal epithelium, and that neogenesis would be an important component in islet regeneration, in addition to replication of endocrine cells (Bonner-Weir et al., 1993; Vinik et al., 1996). More primitive types of islet-associated stem cells have also been identified, particularly those characterized by the expression of the neural precursor marker nestin (Zulewski et al., 2001). These concepts were seriously challenged by a transgenic cell lineage tracing analysis in the mouse, which clearly showed that in the postnatal life, all new beta cells were derived from preexisting insulin-expressing cells, with no contribution from an insulin-negative cell pool (Dor et al., 2004). Thus, beta-cell proliferation is suggested to be the dominant source of new beta cells during adult life. It is possible that adult pancreatic stem cells do exist, but their contribution to beta-cell mass is small (Dor, 2006).

Beta-cell generation from pancreatic ductal cells

While there still remains controversy about the location and identity of potential islet stem/progenitor cells, several studies clearly suggest that new beta cells could be generated *in vitro* from cultured pancreatic duct cells. Ramiya et al. (2000) first described the *in vitro* generation of new islets from pancreatic ductal epithelial cells isolated from adult prediabetic NOD (non-obese diabetic) mice. These cells formed well-organized islet-like structures characterized by the expression of insulin, glucagon and other islet-associated markers. Implantation of these *in vitro*-derived islets could reverse the hyperglycemia of diabetic mice.

Human pancreatic duct cells have also been successfully grown and induced to differentiate *in vitro* (Bonner-Weir et al., 2000). This method is based on the culture of mixed cells derived from left-over fractions after human islet isolation. The cells are first expanded into monolayer culture, then induced to differentiate by applying serum-free medium supplemented with nicotinamide and a thin layer of commercial extracellular matrix (Matrigel) to induce the formation of three-dimensional cysts from which the islet cells (CHIBs) bud out. The level of insulin gene expression in the CHIBs is only about 5 % of that seen in freshly isolated pure islets, but they are able to release insulin in response to glucose. More recently, it has also been shown that beta-cell differentiation can be induced from human pancreatic ductal cells when cotransplanted with human fetal pancreatic tissues (Hao et al., 2006).

Studies in rodents have shown that the combination of EGF and gastrin stimulated the proliferation of pancreatic ductal cells and their differentiation into islets (Wang et al., 1993; Rooman et al., 2002). This same growth factor combination also induced a clear increase in the number of beta cells when applied on cultured human adult islet cells (Suarez-Pinzon et al., 2005). Analysis of cell populations and their proliferation in these experiments suggested that the increase in beta-cell number resulted from the differentiation of islet-associated duct cells rather than replication of preexisting beta cells. An endocrine differentiation program can also be activated in human pancreatic duct cells through the forced expression of ngn3 in these cells (Heremans et al., 2002).

Islet-associated tissue stem cells

Beta-cell neogenesis can also be induced from the nestin-positive cells of islets. Nestin, an intermediate filament protein, often considered as a neural stem cell marker, is expressed in adult pancreatic stellate cells and vascular endothelium (Klein et al., 2003; Lardon et al., 2002). However, based on the studies of Zulewski et al. (2001), nestin-positive cells can be grown out of purified islets and passaged repeatedly in media favoring neural stem cell proliferation. Upon confluence, these multipotential cells differentiated into cells expressing pancreatic exocrine and endocrine genes as well as some hepatic markers. The authors concluded that nestin-positive islet-derived progenitor cells may participate in the neogenesis of islet endocrine cells. Clonal nestin-positive cells isolated from adult mouse pancreas have also been shown to give rise to neural and pancreatic lineages (Seaberg et al., 2004). Controversial to these studies, a detailed analysis of early human pancreatic development showed that endocrine precursors do not express nestin (Piper et al., 2002). Two transgenic lineage tracing experiments in mice also conclusively showed that pancreatic islet cells are not derived from nestin-expressing precursors (Treutelaar et al., 2003; Delacour et al., 2004).

Plasticity of pancreatic cell types

Adult acinar cells of rodents show remarkable capacity to transdifferentiate into ductal epithelial cells *in vivo* after pancreatic duct occlusion as well as *in vitro* (Rooman et al., 2000; Lardon et al., 2004). These transdifferentiated cells may also give rise to new islet tissue both *in vivo* (Rooman et al., 2002) and *in vitro* after stimulation with specific growth factors (Baeyens et al., 2005). Also islet cells may dedifferentiate into a more primitive proliferative epithelial phenotype. This has been shown in cultured human islets which could be expanded for several passages *in vitro* as epithelial cells expressing Pdx-1/Ipf-1 but not insulin (Beattie

et al., 1999). Gershengorn et al. (2004) showed that human islet cells may even undergo epithelial-mesenchymal transition into fibroblastoid cells which can, after a period of substantial expansion, redifferentiate into aggregates of epithelial cells with a low level expression of islet endocrine genes (Figure 4). Similar results was shown by Ouziel-Yahalom et al. (2006) demonstrating that adult human islet cells can be significantly expanded *in vitro*, and redifferentiate to normal beta cells in betacellulin-containing differentiation medium. However, these two studies are severely challenged by a recent lineage-tracing study, which conclusively shows that the fibroblast-like cells described in above two studies are not derived from a beta-cell or endoderm pancreatic origin. By contrast, these fibroblast-like cells appear to represent mesenchymal stem cells similar to those isolated from bone marrow (Chase et al., 2007).

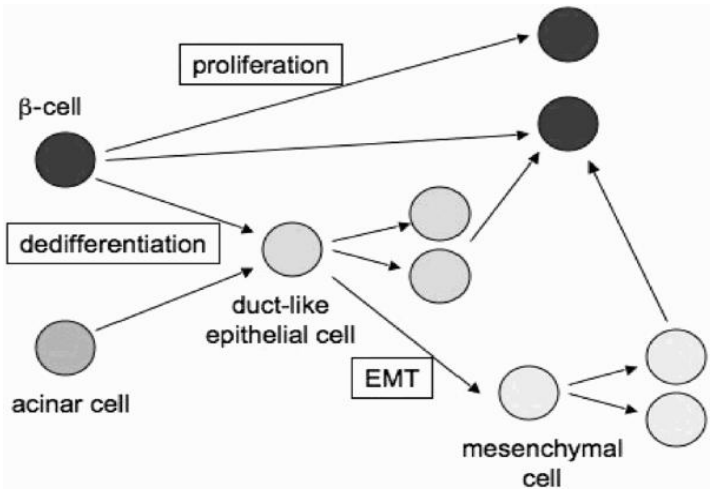

Figure 4. Potential pathways of beta-cell expansion. Direct proliferation is relatively active in many rodents, but human beta cells have an extremely low capacity to replicate. Instead, it appears that human beta cells can expand through a phase of transient dedifferentiation into a more primitive duct-like epithelial cell, or even through epithelial-mesenchymal transition (EMT) into a mesenchyme-like islet precursor. Adapted from Otonkoski et al., 2005.

4.2.2. Other types of somatic stem cells

Progenitor/stem cells from other tissues may also serve as suitable starting material for the generation of beta cells. Many studies have shown that stem cells derived from bone marrow (BM) have the potential to differentiate towards many directions such as muscle, bone and

cartilage. In particular, the multipotent stem cells in BM can differentiate even towards ectodermal or endodermal cells (Jiang et al., 2002). Although the true multipotency of BM-derived stem cells is quite controversial, several studies have suggested that BM stem cells can contribute to the pancreatic islet cell population in various models of islet regeneration (Ianus et al., 2003; Hess et al., 2003). It has also been shown that BM-derived stem cells can differentiate *in vitro* into insulin-producing cells with many characteristics of true beta cells (Tang et al., 2004).

Since pancreas and liver arise from common endodermal progenitors and share the expression of many phenotype-maintaining transcription factors, liver stem cells could be taken as another attractive source for the generation of new beta cells. Convincing evidence has been presented demonstrating the transdifferentiation of immortalized rat hepatocytes into functional insulin-producing cells after transduction with a superactive form of the Pdx-1 gene (Cao et al., 2004). After a period of maturation induced by hyperglycemia, the phenotype of these cells closely resembled that of true beta cells, and transplantation of the cells in diabetic NOD-Scid mice could reverse their hyperglycemia. Pdx-1 overexpression in cell lines derived from human fetal hepatocytes has yielded similarly promising results (Zalzman et al., 2003). In another study, adult rat hepatic oval "stem" cells, which are capable of differentiation to hepatocytes and bile duct epithelium, can transdifferentiate into pancreatic endocrine hormone-producing cells when cultured in a high-glucose environment (Yang et al., 2002). Small hepatocytes (SHC) identified in the adult liver as hepatic progenitor cells were also shown to possess the plasticity to differentiate into insulin-producing cells, and further Pdx1-transfected SHC cells could release significantly increased amounts of insulin (Nakajima-Nagata et al., 2004).

The central nervous system may offer an alternative source of cells from which to generate pancreatic beta cells. Neural progenitor/stem cells from various brain regions can be isolated and expanded *in vitro* for a long period. Mature beta cells and nerve cells share many common features despite originating from different embryonic germ layers. Recently the *in vitro* differentiation of rat neural stem cells into insulin-expressing cells has been reported (Burns et al., 2005). The differentiated cells expressed transcriptional and functional elements associated with a mature beta-cell phenotype, and showed functional responses typical of pancreatic beta cells.

4.2.3. Embryonic stem cells

Since the establishment of human embryonic stem cell (hESC) lines in 1998 (Thomson et al., 1998), much attention has been directed on ES cells as a renewable source for cell replacement therapies since they can give rise to cells from all three embryonic germ layers *in vitro*, including pancreatic beta cells. The first mouse embryonic stem cell (mESC) lines were established already in 1981 (Evans and Kaufman, 1981; Martin, 1981). So far, most of the studies have focused on the differentiation of insulin-producing cells from mouse ES cells.

The study by Soria et al. (2000) generated insulin-producing cells from mES cells using a cell-trapping system which transfected with a human insulin promoter to selectively induce insulin-producing cells. When transplanted into diabetic mice, these cells were able to restore normoglycemia. However, only 1% of the cells were insulin-positive and the reproducibility of the protocol was poor. This strategy has later been enhanced by using the Nkx6.1 promoter in the gene trap (Leon-Quinto et al., 2004).

A report of generation of insulin-producing three-dimensional structures from mouse ES cells through a five-step protocol (Lumelsky et al., 2001) initiated much interest in this area. It was modified from protocols originally designed to select nestin-expressing cells in the differentiation process of neural stem cells. This study was followed by some modifications in several groups (Hori et al., 2002; Blyszczuk et al., 2003; Miyazaki et al., 2004; Moritoh et al., 2003). These modifications resulted in insulin-positive cell clusters with more beta-cell specific characteristics, including the ability to rescue diabetic mice upon transplantation (Hori et al., 2002; Blyszczuk et al., 2003). It was, however, later shown that many of the insulin-positive cells generated using this protocol were not synthesizing insulin de novo, but that the insulin was concentrated from the culture medium and released by apoptotic cells (Rajagopal et al., 2003; Hansson et al., 2004). Furthermore, it was shown that the generated insulin-expressing cells had neuronal features. It has been proposed that the potential of ES cells to generate cells of the endoderm lineage and consequently pancreatic precursors is lost when the cells are predetermined to an ectodermal fate by selectively enriching the cultures with cells expressing nestin (Sipione et al., 2004; Blyszczuk et al., 2004).

Two groups have shown that cells of all islet lineages, including insulin-producing cells, do develop during the spontaneous differentiation of mES cells as embryoid bodies (Shiroi et al., 2002; Kahan et al., 2003). However, the number of insulin-positive cells in these cultures

remained very low (<0.1%). Several other protocols to generate functional insulin-producing cells from mouse ES cells have also been developed during recent years. Blyzczyk et al. (2004) showed that by omitting the nestin selection step and instead allowing the cells to spontaneously differentiate into "multi-lineage progenitor cells", islet-like clusters with embryonic beta-cell characteristics could be generated. These clusters released insulin in a glucose-dependent manner, but were not able to restore normoglycemia in diabetic mice. The performance of the cells was further enhanced by over expression of Pax4, a transcription factor of importance for later stages of beta-cell differentiation, and this time even the cell transplantation experiments were successful. Islet-like cell clusters containing cells capable of insulin synthesis and secretion have also been generated by various growth factor combinations (Kim et al., 2003; Micallef et al., 2005; Shi et al., 2005).

It is far from certain that the principles established in mES cells will be useful in hES cells. Insulin-producing clusters were also generated from hES cells by the nestin-selection protocol (Lumelsky et al., 2001) with minor modifications, which increased the insulin content of the clusters by 30-fold compared to monolayer cells (Segev et al., 2004). However, much controversy remains regarding whether the insulin-producing cells generated *in vitro* from ES cells are true beta cells. Recently it was also suggested that the ES cell-derived insulin-expressing cells might be of extra-embryonic endoderm origin, displaying some endodermal and beta-cell characteristics (Milne et al., 2005). The same conclusion was drawn from a comparison of insulin-producing cells generated from wild type versus HNF6 knock out mouse ES cells, suggesting that the insulin-producing cells seen in spontaneously differentiating embryoid bodies are of visceral endodermal lineage, not beta cells (Houard et al., 2003). Therefore, the production of definitive endoderm is an essential step to generate final therapeutically useful pancreatic endocrine cells. Two recent reports have shown that definitive endoderm and subsequent pancreatic endocrine cells can be produced from hES cells (D'Amour et al., 2005; 2006). In the presence of high concentrations of activin A and low serum, hES cells were differentiated into definitive endoderm cells occupying up to 80% of the cultures. Further, the same group developed a five-stage protocol that directed hES cells through a series of endoderm intermediates to hormone-expressing endocrine cells. The insulin content of newly formed endocrine cells approached that of adult human islets, however, C-peptide release was only minimally responsive to glucose.

AIMS OF THE STUDY

1. To characterize the cell types representing the endocrine precursors in cultured adult human pancreatic tissue and to identify the essential determinants for their differentiation (I).
2. To further analyze the involvement of preexisting human endocrine cells in the *in vitro* islet neogenesis and to purify a pool of endocrine precursor cells (II).
3. To study the maturation and differentiation of CHIBs and fresh isolated islets after transplantation under the kidney capsule of nude mice (III).
4. To examine the effects of immunosuppressive drugs on islet development in our culture model (IV).

MATERIAL AND METHODS

1. Tissue preparation and *in vitro* cultures (I-IV)

Human islets were isolated from cadaveric donors based on previously described methods (Ricordi et al., 1988) in Uppsala, Sweden. After enzymatic digestion and Ficoll gradient purification, the dissociated cell aggregates were collected in 15 fractions. A pool of fractions rich in ductal fragments (30-60%) and poor in islets (3-20%) was shipped on ice to Biomedicum Helsinki for subsequent cultures. Totally, pancreatic tissues from 70 donors were used in these studies. All procedures were approved by institutional ethical committees in Sweden and Finland.

The fresh pancreatic cell clusters were first expanded as monolayer cultures in 6-well plates with CMRL 1066 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (PromoCell, Heidelberg, Germany). After 5-7 day expansion, the media were changed into serum-free DMEM/F12 (National Public Health Institute, Helsinki, Finland) supplemented with ITS (5 mg/l insulin + 5 mg/l transferrin + 5 μg/l sodium selenite, Sigma, St. Louis, MO), 2 g/l bovine serum albumin, 8 mmol/l glucose, 10 mmol/l nicotinamide (Sigma). The cells were then overlaid with Matrigel (BD Biosciences, Bedford, MA), a basement membrane preparation from Engelbreth-Holm-Swarm mouse tumor cells according to the manufacturer's instructions with the exception of dilution (1:10) and overnight gelling time at 37°C. Within 2-3 weeks after gel overlay, the cells were collected for further analyses.

2. Immunosuppressive drug tests (IV)

The drugs and their concentrations were selected based on plasma drug level in protocols currently used in clinical islet transplantation. Drug tests were performed in parallel culture wells either for the first three days of monolayer culture to observe the effect on ductal cell proliferation or through both proliferation and differentiation phases (4-5 weeks) with one or more of the following agents: 10 ng/ml sirolimus (Wyeth Europa Ltd, Berkshire, UK), 5 ng/ml tacrolimus (Fujisama GmbH, Munich, Germany), 20 μg/ml mycophenolate mofetil (MMF, Roche, Basel, Switzerland), 10 μg/ml daclizumab (Roche).

3. Methods

3.1. Cell processing (I-IV)

For preparing cytocentrifuge slides from the cultured cells, the cell clusters were dissociated with 0.05% trypsin and 0.02% EDTA, washed in PBS, and spun to microscope slides by centrifugation at 700 rpm for 8 min. The intact monolayer cultures were prepared for staining directly in eight-chamber slides. Eight-chamber and cytospin slides were fixed in 4% paraformaldehyde (PFA) for 15 min and rinsed in PBS. Alternatively, harvested cells were fixed in 4% PFA for 1 h. After rinsed with PBS, the cells were suspended in 2% agarose-PBS solution, and centrifuged to form compact pellets, which were further embedded in paraffin for sectioning.

3.2. Immunochemical and immunofluorescent stainings (I-IV)

Immunostaining was carried out using the primary antibodies listed in the table. Non-specific binding was blocked by preincubation in 3% normal serum according to the type of the secondary antibody, followed by incubation of primary antibodies for 1 hour at room temperature or overnight at 4°C. Microwave treatment was necessary to retrieve the antigenicity for CK19, nestin, BrdU, Ki67 etc., whereas 0.1% pepsin-0.1 mol/l HCL was optimal for hormone retrieving. Biotinylated goat anti-rabbit and biotinylated rabbit anti-mouse IgGs (ZYMED, San Francisco, CA) were used as secondary antibodies. Peroxidase conjugated streptavidin (ZYMED) was used by developing the substrate of 3-amino-9-ethylcarbazole. Light counterstaining was performed with hematoxylin. The Vectastain ABC-kit (Vector, Burlingame, CA) was used for dual-color staining. For double immunofluorescence staining, the conjugated secondary antibodies, FITC-conjugated donkey anti-rabbit, FITC-conjugated donkey anti-guinea pig, FITC-conjugated donkey anti-mouse, TRITC-conjugated donkey anti-rabbit, TRITC-conjugated donkey anti-guinea pig, and TRITC-conjugated donkey anti-mouse IgGs were used (Jackson ImmunoResearch, West Grove, PA).

3.3. BrdU labeling (I-II)

For determining cell proliferation during monolayer expansion, the cultured cells were labeled with 10 µg/ml bromodeoxyuridine (BrdU, ZYMED) for 24 h at various time points before fixation in 4% PFA. A pulse-chase protocol, in which cells were first labeled with BrdU for 24 h and then incubated in the absence of BrdU until final processing, was used to trace the fates of proliferating cells during later differentiation.

Table. Primary antibodies used in immunochemical and immunofluorescent stainings.

Antibodies	source	used in
guinea pig anti-insulin	DAKO	I-IV
rabbit anti-glucagon	DAKO	I-IV
rabbit anti-somatostatin	DAKO	II, III
rabbit anti-pancreatic polypeptide	DAKO	II, III
rabbit anti-chromogranin A	DAKO	I-III
mouse anti-synaptophysin	Boehringer Mannheim	II
rabbit anti-α-amylase	DAKO	I
mouse anti-cytokeratin 19	DAKO	I-IV
rabbit anti-N-CAM	Dr. V Cirulli, The Whittier Institute for Diabetes, La Jolla, CA, USA	II
mouse anti-clusterin	Santa Cruz	III
mouse anti-vimentin	DAKO	I, II
rabbit anti-nestin	Dr. Urban Lendahl, Karolinska Institute, Stockholm, Sweden	I
rabbit anti-Pdx-1	Dr. Chris Wright, Vanderbilt University, Nashville, TN, USA	I
rabbit anti-Nkx6.1	Dr. Ole Madsen, Hagedorn Research Institute, Gentofte, Denmark	I
mouse anti-BrdU	DAKO	I, II
rabbit anti-Ki67	Novocastra	III, IV

3.4. Insulin and DNA content (I-II, IV)

For the measurement of insulin and DNA contents, the collected cells from different experimental conditions were washed 3-4 times in PBS, resuspended in 300 µl distilled cold water, and homogenized by sonication on ice. An aliquot of the homogenates in duplicate was analyzed fluorometrically or enzymatically for the DNA content, and another extracted with acid ethanol overnight and measured for insulin content using a solid-phase RIA kit (DPC, Los Angeles, CA).

3.5. Insulin release (I)

Dynamic insulin release from differentiated CHIBs was studied with a perifusion system as described previously (Otonkoski et al., 1999). Briefly, batches of selected pure human islets or harvested cysts/CHIBs were loaded in separate perifusion chambers and exposed to Krebs-Ringer bicarbonate buffer supplemented with 20 mmol/l HEPES and 0.2% BSA at a flow rate of 0.25 ml/min. After a 60-min stabilizing period in 1.67 mmol/l glucose, the cells were stimulated with 16.7 mmol/l glucose and 10 mmol/l theophylline. Fractions were collected every 4 min and analyzed for their insulin contents.

3.6. RNA extraction and Northern blotting (I)

Total RNA from pure human islets and harvested CHIBs was extracted using the Gen Elute Mammalian Total RNA Kit (Sigma). Total RNA (10 µg/lane for CHIBs and 2.5 µg/lane for islets) was fractionated on a 1.2% formalin-agarose gel and transferred to a nylon membrane by capillary blotting. The cDNA probe was ^{32}P-labeled by a random priming method. Hybridizations were done in buffer containing 1% SDS, 1 mol/l NaCl, and 8% dextran sulfate overnight at 65°C. The blots were washed at 65°C in 1×SSC and finally in 0.5×SSC. The hybridization signals were visualized using a Bio-imaging analyzer and normalized by the housekeeping gene cyclophilin (Pulkkinen et al., 2003).

3.7. Transplantation of CHIBs and fresh islets (I, III)

Six- to 8-week-old male athymic nude Balb/c (nu/nu) mice were purchased from Harlan. The mice were housed in isolators (Scantainer) and had water and food ad libitum. Animals were anesthetized with 0.1 mg/kg fentanyl citrate, fluanisone 3.3 mg/kg and diatsepam 5 mg/kg intraperitoneally. Approximately 5 µl of packed CHIBs harvested from 4-week cultures were injected with a microinjector under left kidney capsule. The animals were killed 2 weeks or 3 months after transplantation, the left kidney was removed, and the graft site was dissected and

fixed for 6 h in Bouin's fixative or overnight in 4% PFA. Human fresh islet transplantation was performed by the same method in Uppsala. The islet grafts were removed and processed 2 weeks or 3 months after transplantation and sent to our laboratory for immunohistochemical analysis.

3.8. Cell separation (II)

The initial cell clusters were dissociated with 0.16 mg/ml trypsin and 0.1 mmol/l EDTA and filtered through a 30-µm nylon mesh to remove residual cell clumps. The single cell suspension was incubated for 15 min at 6 °C with microbeads conjugated to a monoclonal antibody against N-CAM diluted 1:5. Cell separation was carried out on a MiniMACS magnetic cell separation system according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The N-CAM-negative cells were purified by passage through two consecutive MS columns while N-CAM-positive cells were retained in the columns.

4. Statistics (I, IV)

The differences of insulin and DNA contents, proportions of endocrine cells, and CHIB numbers at various experimental conditions were analyzed with Student's *t* test within two groups and one-way ANOVA and the Fischer's PLSD test at 95% significance level for multiple comparisons (Statview 4.1, Abacus, Berkeley, CA).

RESULTS AND DISCUSSION

1. Cell expansion and differentiation (I)

The only previous study to demonstrate the development of islet buds from adult human pancreatic tissue was published by Bonner-Weir et al. (2000). In our study, we reproduced this culture method and confirmed the capacity of endocrine differentiation from progenitors present in the adult human pancreas. The whole culture period included two phases: expansion for 7-10 days and subsequent differentiation for 2-3 weeks. After 2-day attachment, the cells started to grow and formed a monolayer. Within 7–10 days of monolayer expansion, two major cellular phenotypes were observed: cuboid epithelial cells and spindle-like cells. Two peaks of proliferation appeared separately at days 3–4 for ductal cells and days 7–8 for nestin-expressing cells. To determine which cell type represented the endocrine precursor, we labeled the monolayer cells with BrdU separately at the waves of CK19-positive cell proliferation or nestin-positive cell proliferation and chased their differentiation fates in the CHIBs. After 4-week culture, we found that BrdU-labeled endocrine cells appeared only in CHIBs from cultures that had been labeled early at the time of active CK19-positive cell proliferation. BrdU labeling at the time of nestin-positive cell growth did not result in chromogranin A/BrdU double-positive cells. Compared with the same aliquot of cells at monolayer culture, final harvested cellular insulin content and insulin-to-DNA ratio increased by eight- and five-fold, respectively. Immunocytochemistry analysis and measurement of insulin content demonstrated that insulin-producing cells along with CK19-positive ductal cells were enriched in the CHIBs. We also compared insulin and glucagon mRNA levels from two separate CHIB preparations with those of freshly isolated islets. The normalized insulin mRNA levels in CHIBs were only 4–5% of that found in islets, whereas glucagon mRNA levels were similar in CHIBs and islets. These results are consistent with an immature stage of islet differentiation, and they also confirm that the insulin immunostaining in CHIBs is due to insulin synthesis and not just uptake from the insulin-containing medium, as has recently been shown to be the case in embryonic stem cell differentiation experiments (Rajagopal et al., 2003).

2. Critical factors for *in vitro* endocrine differentiation (I)

On the basis of the experimental conditions used in the previously described successful protocol (Bonner-Weir et al., 2000), we investigated in more detail the critical role of its individual components. It is obvious that the differentiation process involved combined

functions of extracellular matrix and soluble factors. Our results demonstrated that serum-free medium was absolutely necessary for the development of islet cells, and serum inhibited the formation of CHIBs. This is consistent with previous observations showing that serum suppresses islet differentiation (Otonkoski et al., 1999). Extracellular matrix has been shown to play a crucial role in cell differentiation through rearrangement of the cytoskeletal network. In our study, the Matrigel overlay procedure was found to be necessary for the formation of three-dimensional structure, and without this manipulation, insulin/DNA ratio was 50% lower than the standard condition. Consistent with previous reports (Otonkoski et al., 1993; 1999), the addition of NIC promoted the development of endocrine cells. However, NIC was not an absolutely required factor because its omission decreased the numbers of CHIBs and their insulin content per DNA by only 40%. FGF-7 is known to be a potent mitogen for a variety of epithelial cell types and to support the growth of embryonic pancreatic epithelium while repressing endocrine development (Bottaro et al., 1990; Elghazi et al., 2002). As FGF-7 was included in the published protocol, we also included it in the serum-free medium during the differentiation phase. However, we could not identify any major effects on the growth and/or differentiation of the pancreatic cells, and omission of FGF-7 did not affect the results. Thus, in further optimization of the method, it seems more logical to apply this type of growth factors for the initial expansion of the progenitor cells rather than in the differentiation phase.

3. Insulin release in CHIBs compared with fresh isolated islets (I)

Perifusion studies were performed to study the CHIBs' responsiveness to the stimulation of glucose and theophylline. For each perifusion experiment, 100 CHIBs were selected after 5 weeks of culture. Thirty pure islets were hand-picked from freshly isolated human islet fractions to be used as a positive control. The cells were challenged sequentially with 16.7 mmol/l glucose and 16.7 mmol/l glucose plus 10 mmol/l theophylline. Both islets and CHIBs responded in a biphasic manner to glucose, and theophylline potentiated the response equally in both types of cells. Compared with basal insulin secretion at 1.67 mmol/l glucose, the first-phase insulin response was 4-fold in the CHIBs and 12-fold in the islets. The basal rate of insulin release, as related to the DNA content, was similar in both cell types.

4. Dedifferentiation of preexisting islet cells (II)

In the second study of *in vitro* development of human islet cells, we focused on the role of the preexisting differentiated islet cells, which were present at the onset of culture. Our results showed that a significant proportion of islet cells started to express CK19 soon after plating,

thus attaining a transitional phenotype. These transitional cells were relatively small with a weaker staining intensity than non-transitional endocrine or ductal cells. They were frequently identified during the expansion phase but never in the fresh fractions at the onset or in the differentiated CHIBs. More than 10% of all endocrine cells co-expressed chromogranin A and CK19 after the first 24 h of culture. During the following 4 days, this proportion gradually diminished after the initial peak. Further double immunostaining of insulin, glucagon, somatostatin and pancreatic polypeptide together with CK19 showed that transition occurred in all four endocrine cell types. Although it is theoretically possible that these transitional cells could represent preexisting ductal cells that start to express endocrine proteins, this is unlikely because these cells are encountered at an early stage of culture, characterized by decreasing numbers of endocrine cells, and a decrease in the cellular insulin content by 50%. The transitional cells were only observed for a limited time, suggesting that they stopped expressing markers of fully differentiated islet cells (such as chromogranin A). Significant epithelial cell proliferation was only observed in CK19-positive cells, but not in chromogranin A-positive cells. Consequently, it is possible that the dedifferentiated islet cells started to proliferate after losing their endocrine phenotype, and may have acted as precursors of newly differentiated islet cells during the differentiation phase. Recently, the process of dedifferentiation and redifferentiation in cultured human islets cells has also been reported by Gershengorn et al. (2004). However, in contrast to the islet–ductal transition found in our study, they showed a reversible epithelial-to-mesenchymal transition. The transitional vimentin-positive cells were positive for proinsulin mRNA until passage 10, suggesting that dedifferentiated beta cells were proliferative. However, the redifferentiated epithelial cells had a very low insulin expression level of less than 0.02% of that seen in human islets. In comparison, the CHIBs from our culture were much more differentiated, with 5% of the insulin level found in pure human islets. However, it is clear that the expansion of the dedifferentiated epithelial cells in our study was very limited in comparison with the long-term proliferation reported for the mesenchymal cells.

5. Elimination of preexisting islet cells by cell sorting (II)

Many studies published so far on human islet cell neogenesis have been hampered by contamination of preexisting islet cells in the starting material. This is a difficult problem because it appears to be impossible to identify any fractions from dispersed human pancreatic cells processed for islet isolation that would be completely devoid of islet endocrine cells. We developed a method for the elimination of these endocrine cells based on their expression of

N-CAM (Cirulli et al., 1994). Magnetic microbeads coated with N-CAM antibody effectively bound to the islet cells, allowing us to generate 99.7% endocrine-cell-free human pancreatic cells. When plated in culture, most of these cells expressed CK19 and proliferated. However, the differentiation phase was dramatically altered. The formation of three-dimensional cystic structures with small islet buds, which we have regularly seen in 50 consecutive experiments, did not occur when the Matrigel overlay and serum-free culture medium were applied for the expanded N-CAM-negative cell population. This was not the result of poor viability of the cells, because their rate of proliferation was not clearly different from that of the unseparated cell population. We performed the MACS experiments in order to deplete the preexisting islets. However, our results showed that a cell population coexpressing N-CAM and CK19 was also depleted from the N-CAM-negative fraction. These cells were also detected by double immunocytochemistry in the uncultured fresh material. It is possible that the N-CAM/CK19 double-positive cells could represent a pool of islet cell progenitors or partially dedifferentiated islet cells. This interesting pool of cells needs to be characterized in more detail in further studies.

6. The immaturity of CHIBs and their maturation after transplantation (III)

We have previously shown that insulin mRNA levels in CHIBs were only 4-5% of that found in fresh human islets and glucose-stimulated insulin release of CHIBs was 3 times lower than that of islets, which suggested that the neogenic islets were functionally immature as compared with normal islets. Therefore, I examined their morphological signs of immaturity in comparison with human fetal and adult islets, and maturation process after transplantation in nude mice. In the human fetal pancreas many double insulin/glucagon-positive and a few insulin/somatostatin-positive cells were present, and clusterin expression was not only located in glucagon- and PP-positive cells but also in some insulin- and somatostatin-positive cells. In contrast, in the adult pancreas, only single hormone-expressing mature cell types were seen, and clusterin expression was limited to all glucagon- and a subpopulation of PP-positive cells. Similar to fetal endocrine cells, many newly formed endocrine cells in CHIBs were found to co-express insulin and glucagon. It has been demonstrated that the double hormone-bearing cells indicate newly differentiated endocrine cells on their path to the final single hormone phenotype of mature state both in the human fetal pancreas and in experimental islet regeneration (De Krijger et al., 1992; Gu et al., 1994). In early human fetal pancreas, clusterin appeared in scattered beta cells but not in beta cells of well-organized islets perhaps suggesting its role in islet aggregation. The wider expression of clusterin in CHIB endocrine

cells may also indicate its involvement in the 3-dimensional islet organization during neogenesis, which is very similar to the clusterin expression during the development of rat pancreas (Min et al., 1998). Overall, the findings suggest that endocrine differentiation from cultured human adult pancreatic tissue mimics embryonic pancreas development.

Previous studies have shown that human fetal islet cells are able to mature functionally and morphologically when grafted under the kidney capsule of normoglycemic or diabetic nude mice, indicating that the kidney capsule is likely to provide a suitable environment for the maturation of the immature islet-like clusters generated from our cultures (Tuch, 1991; Hayek and Beattie, 1997; Castaing et al., 2001). CHIB grafts were examined for the expression of clusterin and the three major types of endocrine hormones (insulin, glucagon and somatostatin) at an early (2 wk) and late (3 mo) time point after engraftment. Obvious signs of maturation were detected in the transplanted CHIBs between 2 weeks and 3 months post-transplantation. Both insulin/glucagon, insulin/CK-19 and insulin/clusterin double-positive cells disappeared during this time. This is in line with previous studies showing functional and morphological maturation of human fetal pancreatic islet cells grafted to kidney or pancreas in normoglycemic or diabetic nude mice (Tuch, 1991; Hayek and Beattie, 1997; Castaing et al., 2001).

7. Regeneration and replication of transplanted human islets and CHIBs (III)

Human islet regeneration may be important for the outcome of clinical islet transplantation. Thus, I studied the replication of grafted beta cells and looked for signs of islet cell replication or neogenesis. Similar to previous reports (Tyrberg et al., 1996; 2001; Bogdani et al., 2003), we also found very low beta-cell replication in transplanted human islets suggesting that proliferation of preexisting beta cells is not able to support the long-term function of human islet grafts.

No proliferating beta cells were detected in 2-wk CHIB grafts. In contrast, proliferating CK19-positive ductal cells were frequently detected in both 2-wk islet and CHIB grafts. Transitional cells coexpressing the neuroendocrine pan-islet-cell marker chromogranin A and CK19 were detected in all 2-wk islet grafts as well as 2-wk CHIB grafts. Insulin and CK19 coexpression was also seen but less frequently than chromogranin A/CK19 double-positive cells. However, colocalization of chromogranin A and CK19 in 3-mo islet grafts or CHIB grafts disappeared. In fresh islets and CHIBs, no transitional cells coexpressing endocrine and

ductal markers were seen. The finding of frequent chromogranin A/CK-19 double-positive cells in 2-wk fresh islet and CHIB grafts would suggest that endocrine differentiation occurs after transplantation. Although it is possible that the appearance of chromogranin A/CK19 double-positive cells could reflect beta-cell dedifferentiation toward ductal cells, the scattering of these cells within duct structures but not among islets would be more consistent with islet neogenesis from transplanted ductal cells. We can, however, not exclude the possibility of dedifferentiation, particularly since this was found to take place *in vitro* during monolayer culture of islets. The idea of islet neogenesis from ductal cells in transplanted human islets is consistent with the positive correlation of transplanted ductal cells on the long-term function of clinical islet grafts (Street et al., 2004) and *in vivo* endocrine differentiation of CK19-positive ductal cells under the stimulation of human fetal pancreatic factors (Hao et al., 2006).

8. Effects of immunosuppressive drugs on *in vitro* neogenesis of human islets (IV)

Although immunosuppressive medication is still mandatory to prevent islet graft rejection, the beta-cell toxicity of these drugs may be an important issue for long-term outcome. It appears that many of the drugs commonly used, including sirolimus, tacrolimus and MMF, do have negative effects on beta-cell function and viability *in vitro* (Paty et al., 2002). In this part, we tested the effects of immunosuppressive drugs currently used in clinical islet transplantation on the proliferation and differentiation of ductal cells. After three day incubation with different drugs, MMF and its combination with sirolimus or tacrolimus induced a 90% reduction in the cell proliferation in comparison with the control condition. After 4-5-week exposure to various drugs, only MMF reduced the total DNA content at the end of the experiment. Tacrolimus and sirolimus did not affect the DNA content, and in combination with MMF they did not provide additional effects. Tacrolimus was the only drug which, alone or in combinations, induced a significant 50% decrease in the insulin/DNA ratio of the cultured cells and decreased the percentage of insulin-positive beta cells. These findings are consistent with previous reports of negative effects of tacrolimus on islet cell function and survival (Paty et al., 2002; Hui et al., 2005). Also in the current experiments, these effects are most likely caused by direct effects on the differentiated beta cells, for example through inhibition of insulin gene transcription (Oetjen et al., 2003). It is possible that some of the effects of tacrolimus could also be due to altered differentiation of beta cells from duct-like cells. However, the lack of any inhibitory effects by tacrolimus on the proliferation of ductal cells or their aggregation during the differentiation phase suggests that the major mechanism

is targeted on the differentiated endocrine cells. MMF has also been shown to have an inhibitory effect on insulin secretion of cultured human and rat islets (Paty et al., 2002; Hui et al., 2005). However, in our experiments, the major effect of MMF was very clearly targeted on the proliferation of islet precursor cells. The antiproliferative effect of MMF on the pancreatic CK19-positive ductal cells in our cultures is consistent with the study that MMF inhibits the proliferation of human intrahepatic biliary CK19-positive epithelial cells *in vitro* (Liu et al., 2005). Furthermore, MMF may have affected islet neogenesis in our study through inhibition of cell adhesion molecule N-CAM expression accompanied by a loss of cell-cell contacts. Sirolimus has been reported to reduce the viability of rat and human islets at supra-therapeutic concentrations (Bell et al., 2003). In contrast, several other studies have demonstrated little or no adverse effects of sirolimus on islet function (Fabian et al., 1993; Kneteman et al., 1996). Our results indicated that sirolimus, within the therapeutic range of plasma drug concentrations, neither affected proliferation of ductal cells nor islet neogenesis. We did not test daclizumab separately. However, it is clear that when combined with sirolimus and tacrolimus as in the clinically used protocols, daclizumab had no additional toxic effects on proliferation or differentiation.

SUMMARY AND CONCLUSIONS

The pancreatic islet stem cells have been fairly well characterized in the developing pancreas. However, much more controversy remains concerning the possible existence and nature of stem or precursor cells in the adult pancreas. A general view has been that the islet precursor cells reside within the pancreatic ductal epithelium, and that neogenesis would be an important component in islet regeneration, in addition to replication of endocrine cells (Bonner-Weir et al., 1993; Vinik et al., 1996). Two studies have shown the *in vitro* generation of endocrine cells from adult pancreatic ductal cell culture (Ramiya et al., 2000; Bonner-Weir et al., 2000). The present studies are mainly focused on detailed analyses of the characteristics of endocrine progenitor cells, the plasticity of preexisting islet cells, and the maturation of *in vitro*-generated CHIBs. In addition, the effects of immunosuppressive drugs, currently used in clinical islet transplantation, on *in vitro* neogenesis of human islets have been evaluated. Based on the results obtained, we can draw several major conclusions as follows:

1. The capacity of endocrine differentiation from progenitors present in the adult human pancreas has been confirmed in our cultures, and the pancreatic progenitor cells represent a subpopulation of ductal epithelial cells but not nestin-positive cells. In our culture model, the optimal condition for the development of neoislets is serum-free medium plus overlaid Matrigel.
2. *In vitro* islet neogenesis at least partly represents the dedifferentiation of preexisting non-proliferative islet cells into a proliferative duct-cell-like phenotype with further redifferentiation under appropriate conditions. The plasticity of differentiated human pancreatic cell types may be an important mechanism of human pancreas regeneration.
3. *In vitro*-generated human islets are immature both morphologically and functionally. These islet cells can become mature after transplantation into nude mice. Ductal cell differentiation into endocrine cells in transplanted islets may be an important factor in sustaining the long-term function of islet transplants.
4. Our findings of the effects of immunosuppressive drugs on islet neogenesis suggest that sirolimus and daclizumab are amenable for use in clinical islet transplantation. The immunosuppressive protocol may be an important determinant of long-term clinical islet graft function.

Our present results provides good evidence that islet neogenesis can be induced from cultured adult human pancreatic ductal cells. However, since acinar cells have the capacity to

transdifferentiate into ductal cells, and also we found in our culture that human islet cells can dedifferentiate into duct-like phenotype, further studies are needed to identify the nature of endocrine progenitors. Whether a pool of endocrine progenitor cells is within original ductal cells, or comes from transdifferentiated acinar cells and dedifferentiated islet cells, remains to be clarified.

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