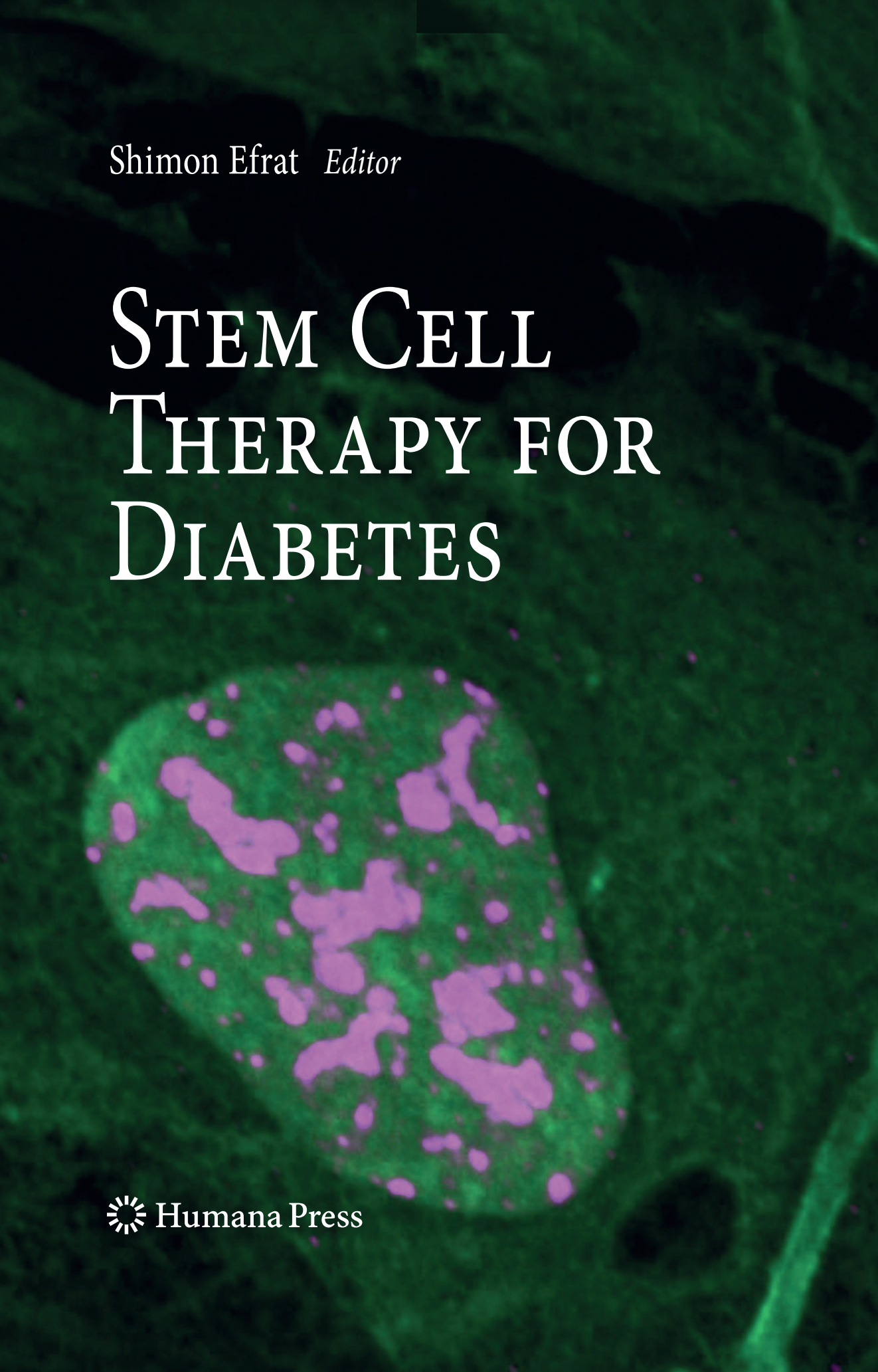
Shimon Efrat *Editor*

# Stem Cell Therapy for Diabetes



Humana Press

# Stem Cell Biology and Regenerative Medicine

**Series Editor**
Kursad Turksen, Ph.D.
krusksen@ohri.ca

For other titles published in this series, go to
http://www.springer.com/series/7896

Shimon Efrat
Editor

# Stem Cell Therapy for Diabetes

Humana Press

*Editor*
Shimon Efrat
Department of Human Molecular
Genetics & Biochemistry
Tel Aviv University
Sackler school of Medicine
69978 Tel Aviv
Israel

ISBN 978-1-60761-365-7 e-ISBN 978-1-60761-366-4
DOI 10.1007/978-1-60761-366-4

Library of Congress Control Number: 2009939144



Printed on acid-free paper

springer.com

# Preface

Regenerative medicine is an old human dream, and for the first time in human history its realization is within reach. Diabetes ranks high on the priority list of diseases that can benefit from regenerative medicine interventions. β-cell function is lost in both type 1 and type 2 diabetes. In type 1 β-cell loss results from autoimmune destruction. In type 2 the exact mechanisms of β-cell functional deterioration remain poorly understood, but they likely involve exposure to agents such as islet amyloid polypeptide and free fatty acids, coupled with cell "exhaustion" owing to increased demands for insulin and insufficient β-cell renewal. The incidence of both types of diabetes is on the rise, and the supply of human donor pancreatic tissue for β-cell replacement falls far short of the demand.

Stem cells hold a promise for providing an abundant source of cells for cell therapy for diabetes. The generation of human embryonic stem cell lines created expectations for an imminent unlimited supply of all cell types needed in regenerative medicine. A decade later, harnessing the potential of embryonic stem cells remains an attractive prospect, but the initial optimism was replaced by a more realistic appreciation of the difficulties involved in realizing this potential. As a result, the alternative source of tissue stem cells has also become a topic of intense investigation. Tissue stem cells possess a more limited proliferation capacity and offer fewer differentiation choices compared with embryonic stem cells, but it may be easier to realize their therapeutic potential.

This book reviews the three main approaches for the generation of sufficient numbers of insulin-producing cells for restoration of an adequate β-cell mass: β-cell expansion, stem cell differentiation, and nuclear reprogramming. The first section, *Beta-Cell Expansion and Regeneration*, opens with a description of our current knowledge of β-cell development, which can be utilized in the stimulation of β-cell renewal by replication or neogenesis. This is followed by a review of the updated status of β-cell replacement through pancreas and islet transplantation, which forms the clinical framework in which surrogate β cells can be evaluated as they become available. The next three chapters assess the prospects of generating β cells from preexisting β cells or their normal progenitors. Assuming that residual β cells exist in patients with type 1 diabetes leads to the possibility that their renewal can be stimulated in vivo. Alternatively, donor islet expansion in vitro may serve as a source

for allogeneic β-cell transplantation. These prospects rely on a detailed understanding of the regulation of β-cell replication and differentiation under normal and pathological conditions.

The second section, *Beta Cells from Non-Beta Cells*, considers alternative cell sources for deriving insulin-producing cells and opens with an overview of the intricate makeup of normal β cells. Although insulin administration cannot avoid diabetic complications, it represents a safe treatment, thereby posing a high bar for the quality and safety of surrogate β cells. Thus, β-cell function must be understood in detail to allow its mimicking to a close approximation in surrogate β cells, primarily with respect to accurate release of insulin in response to physiological signals. The following four chapters evaluate the potential of embryonic and tissue stem/progenitor cells, as well as mature cells from pancreatic and nonpancreatic tissues, to be differentiated or reprogrammed into β-like cells. This might be achieved using soluble factors to effect changes in gene expression in target cells, or, alternatively, by transfer of genes encoding transcription factors capable of inducing such changes. Once sufficient numbers of differentiated cells are generated, they will likely have to be assembled into a miniorgan structure to be fully functional and protected from immune rejection following transplantation.

The third section of the book, *Tissue Engineering and Immune Protection*, discusses cell interaction with matrix scaffolds, compares the merits of employing autologous or banked allogeneic cell sources for generation of surrogate β cells, and evaluates ways for protecting both endogenously generated and transplanted cells from recurring autoimmunity and graft rejection. Among possible approaches, cell encapsulation may help solve both the structural and immunological issues; however, it faces a number of difficult technical problems that have to be tackled before clinical application can be considered.

I hope that this book will be of interest to investigators, clinicians, and students interested both in stem cell application in regenerative medicine and cell therapy of diabetes. These are rapidly evolving research areas, but the contributions collected herein from leading experts in both fields capture the state of the art. They represent essential reading for those interested in tracking the progress in application of one of the most exciting new developments in biomedicine toward a cure for diabetes.

Tel Aviv, Israel Shimon Efrat, Ph.D.

# Contents

**Part I Beta-Cell Expansion and Regeneration**

**1 Pancreas and Islet Development** . . . . . 3
George K. Gittes, Krishna Prasadan, and Sidhartha Tulachan

**2 Islet and Pancreas Transplantation** . . . . . 41
Davide Mineo, Gaetano Ciancio, George W. Burke, Rodolfo Alejandro, and Camillo Ricordi

**3 Cell Cycle Regulation in Human Pancreatic Beta Cells** . . . . . 85
Nathalie Fiaschi-Taesch, George Harb, Esra Karsiloglu, Karen K. Takane, and Andrew F. Stewart

**4 Islet Regeneration** . . . . . 105
Xiaobo Xu, Joke D'Hoker, Nico De Leu, Xiangwei Xiao, Yves Heremans, Mark Van De Casteele, and Harry Heimberg

**5 Beta-Cell Expansion in Vitro** . . . . . 123
Shimon Efrat

**Part II Beta Cells from Non-beta Cells**

**6 What Does It Take to Make a Beta Cell?** . . . . . 137
Gordon C. Weir and Susan Bonner-Weir

**7 Generation of Beta Cells from Acinar Cells** . . . . . 153
Luc Baeyens, Ilse Rooman, and Luc Bouwens

**8 Generation of Beta Cells from Pancreatic Duct Cells and/or Stem Cells** . . . . . 167
Susan Bonner-Weir and Arun Sharma

**9 Adult Cell Reprogramming: Using Nonpancreatic Cell Sources to Generate Surrogate Beta Cells for Treatment of Diabetes** . . . . . 183
Irit Meivar-Levy, Vered Aviv, and Sarah Ferber

**10 Embryonic Stem Cells as a Potential Cure for Diabetes** . . . . . 203
Michael A. Bukys and Jan Jensen

**Part III Tissue Engineering and Immune Protection**

**11 Functional Tissue Reconstruction with the Use of Biologic Scaffolds** . . . . . 223
Stephen F. Badylak, Jennifer B. Ogilvie, and Thomas W. Gilbert

**12 Immunoisolation in Cell Transplantation** . . . . . 241
Riccardo Calafiore and Giuseppe Basta

**13 Prevention of Islet Graft Rejection and Recipient Tolerization** . . 263
Eitan M. Akirav and Kevan C. Herold

**Index** . . . . . 281

# Contributors

**Eitan M Akirav** Section of Human Translational Immunology, Yale University School of Medicine, New Haven, CT 06520 USA, eitan.akirav@yale.edu

**Rodolfo Alejandro** Diabetes Research Institute; Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, L. Miller School of Medicine, University of Miami, FL, USA, ralejand@med.miami.edu

**Vered Aviv** Endocrine Institute, Sheba Medical Center, Tel-Hashomer 52621; Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel-Aviv University, Tel Aviv 69978, Israel, vrdaviv@gmail.com

**Stephen F. Badylak** Departments of Surgery and Bioengineering, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA badylaks@upmc.edu

**Luc Baeyens** Cell Differentiation Unit, Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, lbaeyens@vub.ac.be

**Giuseppe Basta** Department of Internal Medicine, University of Perugia, 06126 Perugia, Italy, giuseppe.basta@unipg.it

**Susan Bonner-Weir** Research Division, Section on Islet Transplantation and Cell Biology, Department of Medicine, Harvard Medical School, Harvard Stem Cell Institute, Joslin Diabetes Center, Boston, MA 02215, USA, susan.bonner-weir@joslin.harvard.edu

**Luc Bouwens** Cell Differentiation Unit, Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, lucbo@vub.ac.be

**Michael A. Bukys** Department of Stem Cell Biology and Regenerative Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA, bukysm@ccf.org

**George W Burke** Diabetes Research Institute; DeWitt Daughtry Family Department of Surgery; Division of Transplantation, Lillian Jean Kaplan Renal and

Pancreas Transplant Center, University of Miami, FL, USA, gburke@med.miami.edu

**Riccardo Calafiore** Department of Internal Medicine, University of Perugia, 06126 Perugia, Italy, islet@unipg.it

**Gaetano Ciancio** DeWitt Daughtry Family Department of Surgery; Division of Transplantation, Lillian Jean Kaplan Renal and Pancreas Transplant Center, University of Miami, FL, USA, gciancio@med.miami.edu

**Joke D'Hoker** Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, joke.dhoker@vub.ac.be

**Nico De Leu** Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, nico.de.leu@vub.ac.be

**Shimon Efrat** Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel, sefrat@post.tau.ac.il

**Sarah Ferber** Endocrine Institute, Sheba Medical Center, Tel-Hashomer 52621; Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel-Aviv University, Tel Aviv 69978, Israel, sarahferber@gmail.com

**Nathalie Fiaschi-Taesch** Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA, nmt2@pitt.edu

**Thomas W Gilbert** Departments of Surgery and Bioengineering, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA, gilberttw@upmc.edu

**George K. Gittes** Division of Pediatric Surgery, Children's Hospital of Pittsburgh, Pittsburgh, PA 15213, USA, gittesgk@chp.edu

**George Harb** Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA, geh19@pitt.edu

**Harry Heimberg** Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, harry.heimberg@vub.ac.be

**Yves Heremans** Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, yves.heremans@vub.ac.be

**Kevan C Herold** Section of Human Translational Immunology, Yale University School of Medicine, New Haven, CT 06520 USA, kevan.herold@yale.edu

**Jan Jensen** Department of Stem Cell Biology and Regenerative Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA, jensenj2@ccf.org

**Esra Karsiloglu** Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA, karslioglue@upmc.edu

**Irit Meivar-Levy** Endocrine Institute, Sheba Medical Center, Tel-Hashomer, 52621 Israel, irit.meivar-levy@sheba.health.gov.il

**Davide Mineo** Diabetes Research Institute, University of Miami, FL, USA; Department of Internal Medicine and University Policlinic, Tor Vergata University of Rome, Italy, dmineo@med.miami.edu

**Jennifer B Ogilvie** Departments of Surgery and Bioengineering, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA, ogilviejb@upmc.edu

**Krishna Prasadan** Division of Pediatric Surgery, Children's Hospital of Pittsburgh, Pittsburgh, PA 15213, USA, krishna.prasadan@chp.edu

**Camillo Ricordi** Diabetes Research Institute; DeWitt Daughtry Family Department of Surgery, University of Miami, FL, USA, ricordi@miami.edu

**Ilse Rooman** Cell Differentiation Unit, Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, irooman@vub.ac.be

**Arun Sharma** Department of Medicine, Harvard Medical School, Harvard Stem Cell Institute, Joslin Diabetes Center, Boston, MA 02215, USA, arun.sharma@joslin.harvard.edu

**Andrew F. Stewart** Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA, stewarta@pitt.edu

**Karen K. Takane** Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA, takane@pitt.edu

**Sidhartha Tulachan** Division of Pediatric Surgery, Children's Hospital of Pittsburgh, Pittsburgh, PA 15213, USA, sidhartha.tulachan@chp.edu

**Mark Van De Casteele** Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, mvdcaste@vub.ac.be

**Gordon C. Weir** Research Division, Section on Islet Transplantation and Cell Biology, Department of Medicine, Harvard Medical School, Harvard Stem Cell Institute, Joslin Diabetes Center, Boston, MA 02215, USA, Gordon.weir@joslin.harvard.edu

**Xiangwei Xiao** Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, xiangwei.xiao@vub.ac.be

**Xiaobo Xu** Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium, xiaobo.xu@vub.ac.be

# Part I
# Beta-Cell Expansion and Regeneration

# Chapter 1
# Pancreas and Islet Development

**George K. Gittes, Krishna Prasadan, and Sidhartha Tulachan**

**Abstract** The development of the pancreas and the pancreatic islets has been an area of particular scientific interest over the last several years as our attention has turned toward the possible engineering of progenitor cells and stem cells into pancreatic β cells and pancreatic islets. Pancreatic development is a highly complex process in which two morphologically distinct tissue types must derive from one simple epithelium. Although the parent endoderm from which the exocrine tissue (including acinar cells, centroacinar cells, and ducts) and the endocrine islets are derived appears to be homogeneous, it is clear that there are selected cells within the early endoderm that are destined to become either endocrine or nonendocrine lineages. The identification of these cells and the processes that determine whether or not they will become islets is of paramount importance to the engineering of stem cells into β cells. Moreover, there is a repertoire of events that allows these endocrine progenitor cells to disconnect from the epithelial lining during development. In this chapter we discuss the various key elements of basic pancreatic development. Specifically, we focus on the intercellular factors, such as growth factors, that may influence these developmental processes, as well as the important known intracellular transcription factors, which have been shown to establish a developmental hierarchy that determines lineage selection and cell fate.

## 1.1 Basic Pancreas Embryology and Development of Pancreatic Endocrine Cells

The first morphologic evidence of the pancreas is a condensation of mesenchyme overlying the dorsal aspect of the endodermal gut tube in the foregut, just distal to the stomach, on the 26th day of gestation in humans and at approximately 9.5 days gestation in mice (approximately the 25th somite stage). Some 2–4 h later

G.K. Gittes (✉)
Division of Pediatric Surgery, Children's Hospital of Pittsburgh, Pittsburgh, PA 15213, USA
e-mail: gittesgk@chp.edu

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_1,

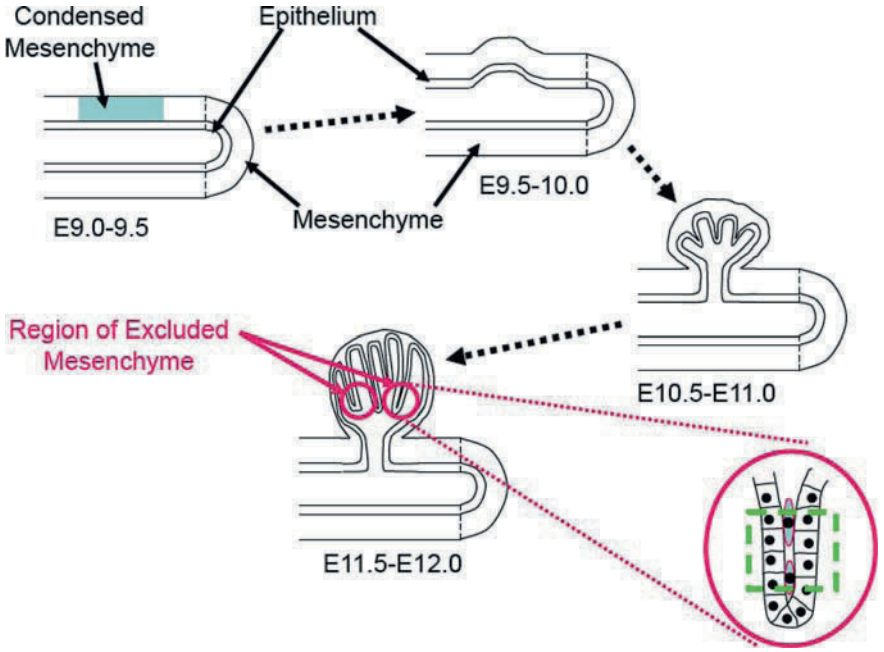



**Fig. 1.1 Schematic representation of epithelial–mesenchymal interactions**. Mesenchyme condensation at E9–E9.5 leads within the next 12–24 h to epithelium evagination. This is followed shortly by the onset of branching morphogenesis, which results in exclusion of most of the mesenchyme from within the clefts of the branched epithelium (see inset). This relative exclusion of mesenchyme may predispose to endocrine differentiation, since absence of contact with mesenchyme is thought to lead to endocrine differentiation (putative endocrine progenitor region shown in the green dotted-line box in the inset)

there is evagination of the dorsal epithelium; then about 12 h later in the mouse and 6 days later in humans, the ventral bud begins to arise from the caudal aspect of the hepatic–biliary bud evagination. Ventral bud evagination occurs through a process that morphologically resembles that of the dorsal bud, but is regulated by a markedly different set of molecules. The pancreatic buds undergo a unique pattern of branching morphogenesis. Unlike the more typical right-angle outgrowth of branches seen in, for example, lung and kidney, the pancreas undergoes a more arboreal pattern with acute-angle branching. Thus, owing to the resulting proximity of adjacent branches to one another, this branching tends to exclude intervening mesenchyme (Fig. 1.1). This exclusion may in turn influence the amount of epithelial–mesenchymal contact, and hence lineage selection.

Owing to gut rotation, together with elongation of the dorsal and ventral stalks, the two buds come into contact with one another within the forming C-loop of the duodenal anlage. This contact and subsequent fusion of the buds occur around E12–E13 in the mouse and on day 37–42 in humans. Coalescence of the two buds leads to formation of the future duct of Wirsung, whereas the future duct of Santorini

(smaller and accessory) originates from the proximal portion of the dorsal bud epithelium. Around E13–E14 in the mouse, dramatic changes occur in the cellular architecture of the pancreas, such as major amplification of endocrine cell numbers, particularly β cells (termed the secondary transition), and rapid branching morphogenesis with acinar cell differentiation.

Glucagon-containing α cells are the first endocrine cell types seen in the mouse, at E9 (Pictet et al., 1972), whereas significant numbers of insulin-containing β cells are not typically seen until the secondary transition period. Hormone-positive cells in the epithelium typically lose connection with the epithelial lumen (possibly through a change in cell division polarity) from perpendicular to parallel to the basement membrane (Pictet, 1972). It has been suggested that this loss of epithelial connection parallels the epithelium-to-mesenchyme transformation that occurs in other tissues. Over the next several days the delaminating endocrine cells accumulate along the ducts and blood vessels in a linear pattern, often referred to as the "cord region" of the E14–E18 mouse pancreas. These "cordlike" endocrine cell collections coalesce over the next few days into aggregates that represent the first islets of Langerhans. In adults, the islets of Langerhans constitute approximately 1–2% of the mass of the pancreas and consist of β cells that produce insulin and amylin, α cells producing glucagon, δ cells producing somatostatin, PP cells producing pancreatic polypeptide, and ϵ cells producing ghrelin.

## 1.2 Early Tissue Interactions

Pancreas development has classically been described as an epithelial–mesenchymal interaction, but earlier key tissue interactions occur before the appearance of the pancreatic mesenchyme.

### *1.2.1 Notochord*

Once the gut tube is established, the development of the dorsal pancreas is controlled by the overlying notochord. In mice, the notochord is in contact with the dorsal prepancreatic endoderm from the time of notochord formation up until E8 (somite 13), at which time the paired dorsal aortas fuse in the midline to intervene between the notochord and the dorsal foregut (Fig. 1.2). Kim et al. showed that removal of the notochord from early chicken embryos prevented proper dorsal pancreas formation and inhibited expression of pancreas-specific genes (Pictet et al., 1972; Kim et al., 1997). In a subsequent study Hebrok et al. followed up on an incidental observation that sonic hedgehog (SHH), which they had used as a marker of notochord, was specifically absent in the prepancreatic endoderm (Hebrok et al., 1998). Elegant grafting experiments showed that notochord proximity to the endoderm could suppress SHH expression. Similarly, deleting notochord in explanted cultures resulted in SHH expression ectopically in the pancreatic region, with failure of pancreatic development. With a candidate approach of known notochord-produced

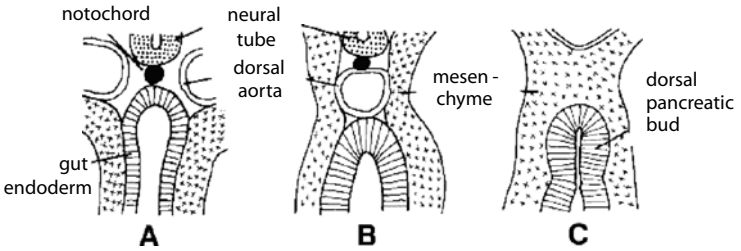



**Fig. 1.2 Early tissue interactions in pancreas development**. (**a**) At E8 a close contact exists between the dorsal aspect of the gut endoderm and the notochord. (**b**) The two paired dorsal aortas fuse in the midline by E8.5–E9.0 to intervene between the gut epithelium and notochord. **(c)** The mesenchyme proliferates to create a distance between the dorsal epithelium and the dorsal aorta by E9.0–E9.5 (reproduced with permission from Slack, 1995)

morphogens, either activin βB or fibroblast growth factor 2 (FGF2) at physiological concentrations could replace the notochord effect. Based on the observation that exogenous SHH could override the pancreas-inducing effect of activin βB and that the notochord makes SHH, it seems that SHH itself must be a key antipancreatic factor, rather than merely being a marker of nonpancreatic endoderm. In contrast to the dorsal pancreas, the ventral pancreas derives from ventral endoderm, which has no contact with the notochord, under the control of signals from the overlying cardiogenic mesenchyme.

### *1.2.2 Endothelium*

Since fusion of the paired aortas in the midline leads to loss of contact with the notochord, Lammert et al. hypothesized that the aortic endothelium may acquire an inductive role in pancreatic development (Lammert et al., 2001). They showed that the dorsal aorta could induce the formation of pancreatic budlike structures and that insulin-positive cells were specifically found in proximity to endothelium, a finding reminiscent of in-vivo normal development. This instructive role for blood vessels is suggested for the ventral pancreas as well, since the ventral endoderm in the prepancreatic region is in proximity to vitelline veins. Yoshitomi et al., however, suggested that endothelial induction of the ventral pancreas is less clear-cut and that induction may involve additional mechanisms (Yoshitomi and Zaret, 2004).

### *1.2.3 Mesenchyme*

Subsequent to contact with the dorsal aorta, there is a proliferation of pancreatic mesenchyme that envelops the pancreatic epithelium, thus separating the pancreatic epithelium from the dorsal aorta (Fig. 1.2). This early enveloping mesenchyme is thought to harbor key permissive and instructive signals for the generation

of differentiated pancreatic cell types and for proper pancreatic morphogenesis. Early studies from the 1960s and 1970s showed that mesenchyme from many different organs, even from a chicken embryo extract, was able to stimulate proliferation and cytodifferentiation of undifferentiated pancreatic epithelium. This finding led Rutter and colleagues to undertake an extensive search for a presumed "mesenchymal factor." Heterologous tissue recombination experiments showed that mesenchyme could induce endocrine cells to form from embryonic foregut epithelial cells (Kramer et al., 1987) or heterotopically from allantoic cells (Stein and Andrew, 1989). Mesenchyme was found to be critical for acinar development, and in the presence of basement membrane ducts develop in lieu of mesenchyme (Gittes et al., 1996). Furthermore, the absence or depletion of mesenchyme revealed that there was a "default" differentiation of pancreatic epithelium toward islets (Gittes et al., 1996; Miralles et al., 1998b). Further studies revealed that the age, location, proximity, and contact of the epithelial cells with mesenchyme induced epithelial differentiation. Younger mesenchyme grown with an older epithelium induced a greater number of α cells and fewer acinar cells. On the other hand, older mesenchyme induced the same epithelium to undergo greater acinar differentiation, specifically in the region of the contact, but induced a more mature insulin-positive endocrine phenotype when not in contact with the epithelium (Rose et al., 1999; Li et al., 2004). These data suggest that there are proexocrine factors in mesenchyme that are cell-contact-dependent and that additional diffusible proendocrine/proinsulin factors, which are secreted by the mesenchyme, are also present.

Raphael Scharfmann's group recently showed that proteoglycans (glypicans and syndecan) and the proteoglycan-producing enzyme heparan sulfate α-sulfotransferase are localized to the epithelial–mesenchymal interface and can induce exocrine differentiation. Other studies from the same laboratory have further delved into the multiple effects of mesenchyme on lineage selection. It appears that contact of mesenchyme with epithelium may enhance the expression of hairy enhancer of split 1 (HES1) through NOTCH signaling, thereby inhibiting expression of neurogenin-3 (NGN3), a key determinant of pancreatic endocrine lineage selection, growth, and differentiation (see below), and suppressing endocrine differentiation (Duvillié et al., 2006).

A new and interesting role for pancreatic mesenchyme has recently been suggested by studies of BAPX1, a member of the NKX family of transcription factors expressed in pancreatic mesenchyme. *bapx1*-null mutant mice had failure of separation of pancreatic mesenchyme and spleen, leading to formation of gutlike evaginations from the prepancreatic foregut. These results support an instructive role for pancreatic mesenchyme in diverting foregut epithelium away from the intestinal lineage, possibly mediated by PTF1a, a transcription factor for early specification of pancreatic progenitor cells (Asayesh et al., 2006).

Many studies have identified specific molecules in mesenchyme that have inductive influences on pancreatic epithelium. It is now widely accepted that pancreatic mesenchymal–epithelial interactions, much like in most other developing epithelial–mesenchymal organs, are mediated through numerous growth factors.

## 1.3 Soluble Factors and Signaling Pathways Regulating Pancreas Development

### *1.3.1 Fibroblast Growth Factors (FGFs)*

FGFs are well known to mediate multiple developmental processes, are expressed in many epithelial–mesenchymal interface regions, and particularly play an important role in regulating branching morphogenesis (Hogan, 1999). They are a large family of ligands (greater than 20) that signal through four different tyrosine kinase FGF receptors (FGFR1–4).

Pancreatic mesenchymal FGF signaling to the epithelium specifically favors duct and acinar differentiation (Dichmann et al., 2003; Hart et al., 2003; Norgaard et al., 2003). Scharfmann's group showed that FGF ligands 1, 7, and 10 were expressed in the pancreatic mesenchyme, whereas FGF receptor 2B (FGFR2B), a specific receptor isoform that binds all three of those FGF ligands, was expressed in pancreatic epithelium. FGF ligand signaling to FGFR2B induces pancreatic epithelial proliferation, both in vitro and in vivo, but at the apparent expense of cellular differentiation (Celli et al., 1998; Le Bras et al., 1998a, b; Elghazi et al., 2002). Similarly, FGF7 and FGF10 signaling has been implicated in mesenchyme-to-epithelium signaling in the developing human pancreas (Ye et al., 2005). Further, Miralles et al. showed that NOTCH signaling was a critical mediator of FGF10-induced embryonic pancreas epithelium proliferation and suppression of differentiation (Miralles et al., 2006). More recent studies have suggested that FGF signaling may be a key factor in specifying the pancreatic mesenchyme itself (Manfroid et al., 2007). Thus, FGF signaling clearly plays a pivotal role in regulating many aspects of pancreatic development.

### *1.3.2 Transforming Growth Factor β (TGF-β)*

The TGF-β superfamily is a large family of factors with roles in nearly every biological process known, particularly developmental processes. The superfamily consists of four major subfamilies: (1) TGF-β isoforms proper (including TGF-β 1, 2, and 3 in mammals); (2) activins; (3) bone morphogenic proteins (BMPs); and (4) other types, including, for example, MIS and growth differentiation factors (GDFs). All of these molecules signal through a large family of typically heterodimeric receptors to activate SMADs and other intracellular pathways to initiate cell-specific responses.

#### 1.3.2.1 TGF-β Isoforms

The TGF-β isoforms are present in the embryonic pancreas as early as E12.5. The three ligands are coexpressed in the epithelium initially and during gestation become progressively focused to acinar cells (Crisera et al., 1999, 2000). Key receptors for these ligands, including TGF-β receptor type I (TβRI/Alk5) and type II (TβRII) show a similar expression pattern, suggesting that together they mediate TGF-β isoform signaling in the developing pancreas (Tulachan et al., 2007). These receptors

are localized to the epithelium and mesenchyme early (E12.5), but at later gestation (E18.5) are found specifically in the pancreatic ducts.

The exact role of TGF-β isoforms in pancreas development is controversial. As exogenously added TGF-β induced enhanced endocrine differentiation, Sanvito et al. suggested a proendocrine role (Sanvito et al., 1994). However, the observed effects may have been due to acinar autolysis during prolonged organ explant cultures with relative endocrine protection. Miralles et al. showed that TGF-β isoforms were important regulators of matrix metalloproteases, which control migration of endocrine progenitors to form the islet architecture (Miralles et al., 1998a). Since both *tgf-β1/2/3* triple-null mutant mice and *tβrII*-null mutant mice are early embryonic lethal, a dominant-negative form of TβRII has been used to study inhibition of TGF-β isoform signaling. Expression of the dominant-negative form of the TβRII receptor in embryonic pancreas results in enhanced proliferation and accumulation of periductal endocrine cells at mid-to-late gestation (Tulachan et al., 2007). These data, together with the ontogeny data mentioned above, suggest that TGF-β signaling to ductal progenitors normally serves to restrict the recruitment of ductal or periductal cells into the endocrine lineage.

#### 1.3.2.2 Activins and BMPs

Two other key subfamilies within the TGF-β superfamily are activins and BMPs and these two share many binding partners, receptors, and inhibitors. It has been found that activins are expressed in early gut endoderm (Manova et al., 1995; Verschueren et al., 1995) and in the early pancreatic rudiment.

Activin A and B are expressed in the developing pancreatic endocrine cells, particularly in glucagon-positive cells (Furukawa et al., 1995; Maldonado et al., 2000). Exogenous activin in pancreas explant cultures inhibited branching morphogenesis, and follistatin, a known inhibitor of activin present in mesenchyme, was able to replace the proexocrine/antiendocrine effects of mesenchyme (Miralles et al., 1998b). Identifying these potential proendocrine effects of activin ligands on pancreas development led to an interesting series of experiments in cultured AR42J cells (pancreatic tumor cells with progenitor qualities). Exogenous activin induced a neuroendocrine phenotype in these cells, with 25% becoming positive for pancreatic polypeptide, though none was positive for insulin or glucagon (Ohnishi et al., 1995). Many of the treated cells underwent apoptosis, so the investigators then added a growth inducer [betacellulin, a member of the epidermal growth factor (EGF) family, or hepatocyte growth factor (HGF)]. Surprisingly, these growth inducers led to 10% of the cells becoming insulin-positive (Ohnishi et al., 1995; Mashima et al., 1996a, b). Further investigations into the mechanism by which activin may induce the formation of insulin-positive cells led to the finding that activin can specifically decrease expression of both ARX (a transcription factor critical for α-cell differentiation) and preproglucagon in AR42J cells, in αTC cells (a mouse tumor cell line derived from glucagonoma) and in human islets (Mamin and Philippe, 2007). This effect may be mediated directly through induced expression of NGN3 (Zhang et al., 2001).

Loss-of-function studies using a dominant-negative activin receptor II showed islet hypoplasia (Yamaoka et al., 1998; Shiozaki et al., 1999). Surprisingly, a similar islet hypoplasia phenotype was also seen with constitutively active activin receptor II. These results suggest that a specific window of activin receptor II signal dosing is necessary for proper islet development.

Kim et al. analyzed *activin-receptor type IIA* and/or *activin receptor IIB*-null mutant mice (Kim et al., 2000). Particularly in the presence of an additional *activin receptor IIA* heterozygous mutation, *activin receptor IIB*-null mutants were born with a small annular pancreas, similar to mice with altered Indian hedgehog signaling (Hebrok et al., 1998). As discussed earlier, notochord-derived activin inhibits SHH expression in the prepancreatic endoderm, and inappropriate SHH expression was seen in these *activin receptor type II* mutants. Further studies of the *activin receptor IIB* homozygous null mutants with the additional *activin receptor IIA* heterozygous mutation revealed mainly a reduction in endocrine cells with islet hypoplasia. Thus, regardless of the ligand that may be involved, the activin receptor type II family seems to be important for pancreatic morphogenesis and specifically endocrine and islet development.

Despite the extensive work implicating TGF-β superfamily signaling in pancreatic development, little is known about BMP-specific pathways. Exogenous BMP4, 5, and 6 are all able to induce dispersed E15.5 mouse pancreatic cells to form insulin-positive epithelial colonies when grown in the presence of laminin (Jiang et al., 2001). BMP ligands are known to be expressed in the developing pancreas (Hogan, 1996; Dichmann et al., 2003; Jiang and Harrison, 2005; Goulley et al., 2007). In AR42J cells, BMP signaling was found to be necessary for cell proliferation (Hua et al., 2006) as well as glucagon-like peptide 1 (GLP1)-induced insulin-positive differentiation (Yew et al., 2005). However, a transgenic mouse model expressing BMP6 under the *Pdx1* promoter developed complete pancreatic agenesis, a phenotype we attribute to an epiphenomenon of overinduction of intestinal smooth muscle in the duodenal anlage, which could have disrupted pancreatic development.

SMADs are the downstream intracellular mediators of most known TGF-β signaling. SMAD molecules are present in neonatal islets (Brorson et al., 2001), are necessary for insulin-positive differentiation of AR42J cells (Zhang et al., 1999; Yew et al., 2005) and are needed for proper regulation of the endocrine progenitor cell compartment in vivo (Harmon et al., 2004; Goto et al., 2007). There is an assumed role of SMADs in pancreatic development and differentiation because 50% of pancreatic cancers have the *SMAD4* mutation (Hahn et al., 1996). However, work with *smad4* transgenic mouse models has not uncovered a role for SMAD4 in pancreatic development (Bardeesy et al., 2006; Simeone et al., 2006).

Transgenic *Smad6* overexpression (which inhibits expression of SMAD1, 5, and 8, the canonical downstream mediators of BMP signaling) did not have a developmental phenotype. However, transgenic *Smad7* overexpression (which inhibits expression of BMP-mediating SMADs 1, 5, and 8, as well as TGF-β isoform/activin-mediating SMADs 2 and 3) led to a dramatic (85–90%) reduction in the number of β cells present at birth (Smart et al., 2006). These results suggest

that SMAD2 and 3 (which are specifically lost with SMAD6 expression, but not with SMAD7 expression) may play a specific role in β-cell differentiation, and the specific loss of β cells and not α cells in *Smad7* overexpressing mice suggests a possible role for SMADs in regulating the balance between formation of β cells versus α cells.

#### 1.3.2.3 Growth Differentiation Factor 11 (GDF11)

GDF11 was identified as a possible ligand mediating the activin receptor IIA and IIB signaling that appears to favor β-cell differentiation. Two separate studies showed slightly different results with *gdf11*-null mutant mice (Harmon et al., 2004; Dichmann et al., 2006). Harmon et al. showed that the null-mutant mice developed more NGN3-positive endocrine progenitor cells, less mature insulin-positive β cells, and more glucagon-positive α cells, whereas Dichmann et al. found an overall 43% reduction in the size of the pancreas due entirely to a loss of acinar cells. Additional experiments were performed to test the dependence of NGN3-positive cell formation on GDF11 and activin receptor IIA and IIB signaling on the formation of mature endocrine cells. Goto et al. further found that activin receptor IIB signaling through SMAD2 was important in promoting endocrine development (Goto et al., 2007).

### *1.3.3 NOTCH Signaling*

NOTCH is a cell-membrane-bound receptor that serves to maintain cells in an undifferentiated state when bound by NOTCH ligands such as JAGGED, SERRATE, or DELTA-like. In *Drosophila*, cells with less NOTCH activation adopt a neuronal fate, whereas adjacent cells with more NOTCH activation adopt an epidermal fate in a process called "lateral inhibition." However, no clear lateral inhibition corollary has been established in pancreatic development. A landmark paper from the Edlund laboratory demonstrated that NOTCH signaling was a key mediator of fate decision in pancreatic development (Apelqvist et al., 1999). Null mutant mice for *delta1*, encoding a NOTCH ligand present in the developing pancreas, and for *rbpjk*, encoding a transcription factor target of NOTCH signaling, both exhibited an accelerated and excessive commitment of the early embryonic pancreatic epithelium to the endocrine lineage, suggesting that NOTCH signaling was necessary to prevent endocrine differentiation of these progenitor cells.

Further studies have confirmed a key role for other members of the NOTCH-signaling pathway. Early NOTCH signaling favors nonendocrine lineages over endocrine lineages and is critically mediated by HES1 and NGN3. HES1 is a transcription factor upregulated by NOTCH and responsible for NGN3 suppression. The *hes1*-null mutant mice have severe pancreatic hypoplasia (Jensen et al., 2000b), owing not to apoptosis but rather to an inappropriate early commitment of precursor cells to becoming endocrine cells. Acinar cell differentiation is also regulated by NOTCH signaling (Hald et al., 2003). Transgenic expression of a constitutively active intracellular domain of NOTCH1 led to a diminution in mature

endocrine and acinar cells, suggesting that active NOTCH signaling may select for a progenitor epithelial cell population (Murtaugh et al., 2003). The mechanism by which NOTCH maintains the proliferation of a pancreatic stem/progenitor pool may involve mesenchymal FGF signaling (Norgaard et al., 2003; Miralles et al., 2006). The control point for NOTCH receptor function may be enzymes that regulate sugar residues on the NOTCH receptor. For example, in zebrafish, manic fringe is an enzyme that can alter NOTCH receptor function and thereby drive premature NGN3 expression and endocrine differentiation (Xu et al., 2006). Further zebrafish analysis showed that NOTCH signaling can affect later cell lineage selection within the endocrine compartment. For example, mutations in *DeltaA* (encoding a NOTCH ligand) showed a shift of endocrine lineage selection away from α cells and toward β cells (Zecchin et al., 2007).

Lastly, NOTCH appears to play a potentially positive role in duct formation, suggested by the fact that *Notch* mutants lack cells positive for duct markers (Lorent et al., 2004; Yee et al., 2005).

### *1.3.4 Hedgehog Signaling*

The hedgehog signaling pathway regulates differentiation in many developing tissues. The three hedgehog ligands, sonic (SHH), desert (DHH), and Indian (IHH), all bind to the receptor patched (PTC), thus relieving PTC-induced repression of membrane-bound smoothened, which then in turn regulates the GLI family of transcription factors. In the early embryo SHH is expressed in essentially the entire gut epithelium except for the pancreatic domain of the foregut. Adjacent notochord has a SHH-suppressive effect on the endoderm in the region of the pancreas (Hebrok et al., 1998). Cyclopamine, a steroid alkaloid that inhibits SHH signaling at the receptor level, could induce heterotopic pancreas development, presumably by expanding the pancreatic field in the gut, but only into areas that are already PDX1-positive (stomach and duodenum). SHH inhibition by activin secreted from the notochord controls formation of the dorsal pancreatic field in the endoderm, whereas in the ventral pancreatic anlage SHH is inhibited by FGF secreted from cardiogenic mesenchyme (Deutsch et al., 2001). IHH, DHH, and the receptor PTC are expressed in the foregut and pancreas (Hebrok et al., 2000; Thomas et al., 2000), and *ihh*-null mutants are born with a small pancreas (Hebrok et al., 2000).

The complexity of hedgehog signaling and pancreatic development is underscored by the paradoxical fact that in zebrafish hedgehog signaling is actually necessary for the formation of pancreatic endocrine cells (Roy et al., 2001; diIorio et al., 2002, 2007).

### *1.3.5 Retinoids*

Several studies have investigated the role of endogenous or exogenous retinoid signaling in the developing pancreas. Retinoid binding proteins and retinoic acid receptors have been found in both developing pancreatic islets and insulinoma

cell lines (Chertow et al., 1979, 1983; Kato et al., 1985; Kobayashi et al., 2002; Tulachan et al., 2003; Martin et al., 2005; Stafford et al., 2006). Similarly, exogenous retinoids can enhance the proportion of insulin-positive cells in isolated chick embryo endoderm (compared with the proportion of glucagon-positive cells) and can induce the dorsal lip cells of *Xenopus* gastrula to form the pancreas, including endocrine and acinar elements (Moriya et al., 2000a, b). In the embryonic mouse pancreas, retinoids induce endocrine and ductal differentiation (Tulachan et al., 2003; Shen et al., 2007) and influence the later differentiation between ductal and acinar/exocrine (Kobayashi et al., 2002). RALDH2, the enzyme that produces retinoic acid, is present in the developing pancreas, specifically in the mesenchyme (Tulachan et al., 2003; Martin et al., 2005; Molotkov et al., 2005; Stafford et al., 2006). Mesodermal retinoic acid appears to signal to the endoderm to induce pancreatic differentiation (Martin et al., 2005; Stafford et al., 2006). Conversely, exogenous retinoic acid can expand the pancreatic field within the endoderm (Stafford and Prince, 2002; Chen et al., 2004; Stafford et al., 2004, 2006).

The strain of *raldh2*-null mutant mice lack a dorsal pancreas (Martin et al., 2005; Molotkov et al., 2005), and cells in the normal dorsal pancreatic bud (including insulin- and glucagon-positive cells) were shown to have activation of retinoic acid responsive pathways. The role of RALDH2 in ventral pancreas development is less clear, and the normal absence of RALDH2 in the developing pancreas after E12.5 also suggests a diminished role for retinoids in later stages of pancreatic development.

### *1.3.6 Epidermal Growth Factor (EGF) Family*

The EGF family of growth factors consists of at least 30 member ligands, which signal through at least four ERBB tyrosine kinase receptors. The overall complexity of EGF-family signaling makes it difficult to understand its role in pancreatic development. Many EGF ligands are expressed in the embryonic pancreas (Huotari et al., 2002). HB-EGF, a membrane-bound ligand, is expressed in early embryonic pancreatic ducts and later in neonatal islets (Kaneto et al., 1997). It predominantly colocalizes with PDX1, and its promoter elements are bound and activated by PDX1.

Many studies have used exogenous EGF ligands to manipulate pancreatic development and induce β-cell formation. When betacellulin (an EGF family member) and activin A were added to AR42J cells, 100% of the cells became insulin-positive (Mashima et al., 1996a). Similarly, betacellulin treatment of E11.5 mouse pancreas cultures induced insulin-positive cell differentiation, with expansion of the number of PDX1-positive epithelial cells and an increase in the number of insulin-positive cells at the expense of acinar cells (Thowfeequ et al., 2007). However, any true endogenous role for betacellulin remains unknown since betacellulin-null mutant mice develop with a normal pancreas (Jackson et al., 2003).

Among the four EGF receptors, ERBB1 has been the one most studied in connection with pancreatic development. ERBB1 is expressed throughout the embryonic

mouse pancreas, and null mutant mice have diminished endocrine cells, with an overall smaller pancreas, perhaps owing to decreased branching morphogenesis (Miettinen et al., 2000). More recently, a transgenic mouse with pancreas-specific (*Pdx1* promoter) expression of a dominant-negative form of the ERBB1 receptor was able to survive beyond the neonatal period and showed loss of postnatal β-cell proliferation, supporting a role for EGF receptors in postnatal as well as prenatal β-cell growth (Miettinen et al., 2000). Further analysis of ERBB2–4 function awaits conditional mutants since all three null mutants are early embryonic lethal (Gassmann et al., 1995; Lee et al., 1995; Erickson et al., 1997).

### *1.3.7 Hepatocyte Growth Factor (HGF)*

Much evidence has accumulated to suggest that HGF is a proendocrine mesenchymal growth factor. HGF and its receptor c-MET are expressed in embryonic mouse pancreatic mesenchyme and epithelium, respectively (Sonnenberg et al., 1993). Similarly, human pancreatic fetal mesenchymal cells express high levels of HGF. The conditioned medium from these cells was able to induce β-cell proliferation and the formation of islet-like cell clusters.

Transgenic expression of HGF under the rat insulin promoter leads to an increased number of islets with enhanced insulin content (Garcia-Ocana et al., 2000), and conditional deletion of c-MET in insulin-expressing cells led to reduced numbers of islet cells (Dai et al., 2005; Roccisana et al., 2005). The importance of HGF signaling to c-MET in the regulation of pancreatic endocrine differentiation was also supported by experiments in cell lines. HGF prevented dexamethasone-induced acinar differentiation of AR42J cells and, when combined with activin A, induced insulin-positive differentiation of AR42J cells (Mashima et al., 1996b). Similarly, using a pancreatic cell line resembling ducts (ARIP cells), HGF alone stimulated insulin-positive differentiation. Interestingly, these cells recapitulated the canonical pancreatic endocrine developmental pathway, with early (6-h onset) expression of NGN3 and later (24 h) expression of NEUROD (Anastasi et al., 2005).

### *1.3.8 WNT Signaling*

A highly complex family of signaling molecules, WNTs and their associated signaling pathway molecules have been shown to play a role in multiple aspects of pancreatic development. The WNT ligands typically signal through the transmembrane receptors frizzled (FZD), together with the coreceptor lipoprotein-related peptide 5/6 (LRP5/6), to stabilize a key intracellular factor, β-catenin.

A role for WNT signaling was recently established in pancreatic specification within the foregut of *Xenopus* (McLin et al., 2007). Absence of WNT8 in the mesoderm prevented foregut determination within the endoderm. Similarly, ectopic WNT signaling prevented the normal formation of foregut from the anterior endoderm, resulting in the absence of liver and pancreas. This WNT-induced foregut inhibition

appears to be mediated by β-catenin-induced VENT2 expression. VENT2 is a homeodomain-containing transcription factor that represses *HHex*, a key-patterning gene for foregut development. The role of WNT/β-catenin signaling in pancreatic development is complex and dependent on the time and place of WNT signaling. A detailed analysis of expression patterns of WNT pathway components revealed that several WNT ligands and FZD receptors, as well as LRP5/6 and secreted frizzled-related peptides (sFRP), are expressed in the developing pancreas (Heller et al., 2002). Consistent with the role of WNTs in foregut and pancreas specification, *Pdx1-Wnt1* and *Pdx1-Wnt5a* transgenic mice had pancreatic agenesis and severe pancreatic hypoplasia, respectively, confirming a specific role for WNTs in suppressing pancreatic development.

The complexity of *Pdx1* promoter-driven studies of WNT signaling is best illustrated by studies from the Hebrok laboratory, in which an early expressing *Pdx1-Cre*, a late-expressing *Pdx1-Cre*, and a tamoxifen-regulatable *Pdx1-Cre/ER* were all used to conditionally express a constitutively active stabilized β-catenin. With either the early expressing *Pdx1-Cre* or tamoxifen treatment of the *Pdx-Cre/ER* embryos at E11.5 there was near-total pancreatic hypoplasia owing to overactive canonical WNT signaling (Heiser et al., 2006). These results suggest that some WNT signaling (perhaps canonical) may affect exocrine pancreas development, whereas other WNTs (perhaps noncanonical, cadherin-mediated) may affect endocrine pancreas growth and development.

The complex and varied nature of the different reports is reflective of the extreme complexity of WNT signaling, and further studies will likely continue to delineate the exact mechanisms involved.

### *1.3.9 Blood Vessel- and Endothelial-Derived Factors*

Recently there has been increased interest in the role of endothelial cells and possibly blood flow in pancreas development, especially in regard to endocrine cells. Teleologically, since normal endocrine cell function is critically dependent on an intimate relationship with capillaries in order to allow homeostatic sensing, it seems logical that there would be a carefully orchestrated, interdependent development of endothelial and endocrine cells. Lammert et al. demonstrated a critical role for aortic endothelial cells in the induction of PDX1 expression in the adjacent endoderm, as well as in subsequent evagination followed by insulin expression (Lammert et al., 2001). Removal of the dorsal aorta from *Xenopus* embryos led to the absence of pancreatic endocrine development. Similarly, overexpression of vascular endothelial growth factor A (VEGF-A) under a *Pdx1* promoter led to more vessels and more islets (in exchange for much less acinar tissue) and ectopic insulin-positive cells in the stomach.

Interestingly, the developing endocrine cells do not form their own basement membrane and thus are dependent on endothelial cells to make the basement membrane for them (Nikolova et al., 2006). Furthermore, endocrine cells are stimulated by basement membrane-derived laminin, bound to β1-integrins on the endocrine

cells, to proliferate and to increase insulin synthesis. Thus, throughout development there is a complex and ever-changing relationship among foregut endoderm, endocrine cells, endothelium, mesenchyme, and blood flow.

### *1.3.10 Glucagon-Family (and Other Peptide Hormones) Signaling*

The glucagon family of peptide hormones includes proglucagon-derived peptides such as glucagon itself, glucagon-like peptide-1 (GLP1), GLP2, etc., as well as glucose-dependent insulinotropic peptide (GIP), secretin, vasoactive intestinal peptide (VIP), and others. Although the role of these peptides has generally been well studied in endocrine physiology, roles in pancreatic development are only recently becoming apparent.

Several lines of evidence suggest that glucagon signaling is necessary for the early differentiation of insulin-expressing cells. First, in-vitro studies of cultured pancreas show that glucagon is necessary for early formation of insulin-positive cells (E11–E13), but not later in the E15 pancreas (Prasadan et al., 2002). Glucagon is specifically generated from proglucagon by the action of prohormone convertase 2 (PC2), and *pcsk2*-null mutant animals, which lack glucagon, showed a similar loss of early formation of insulin-expressing cells, but with retention of the secondary transition (Vincent et al., 2003). Glucagon-receptor-null mutant mice were found to have a similar absence of early phase insulin-expressing cells (Vuguin et al., 2006).

A possible role for GLP1 in β-cell development has been suggested because of the known function of GLP1 in promoting insulin synthesis and secretion in β cells, as well as promoting β-cell growth (Buteau et al., 1999; Stoffers et al., 2000; Buteau et al., 2001). Moreover, the GLP1 analogue exendin-4 can convert AR42J cells and ARIP cells into insulin-expressing cells (Zhou et al., 1999; Hui et al., 2001; Yew et al., 2004). Mature α cells have PC2 and do not make GLP1, but Wilson et al. found that immature glucagon-positive cells in the embryonic pancreas have PC1/3, and therefore presumably make GLP1 (Wilson et al., 2002). Suzuki et al. showed that a relatively unusual form of GLP1 (1–37), which unlike other GLP1 forms is present in α cells, could stimulate formation of insulin-glucagon double-positive cells in the epithelium of the embryonic pancreas or in ducts (Suzuki et al., 2003). These early insulin–glucagon double-positive cells may represent the first wave of endocrine cells in the early pancreas.

GIP and its receptor have also been implicated in β-cell development (Huypens et al., 2000). GIP regulates key pancreatic endocrine-determining transcription factors, including GATA4, ISL1, and PDX1 (Jepeal et al., 2005), and a GIP analogue is able to enhance insulin-positive differentiation in embryonic stem cells (Marenah et al., 2006).

Apart from the glucagon family, other peptide hormones, such as the pancreatic polypeptide family of peptides (PP, PYY, and NPY), have been studied in pancreatic development, but mainly as possible markers of progenitor cells. No specific signaling role in development has been found for these molecules, other than localization

in the vicinity of a potential early endocrine progenitor (Jackerott and Larsson, 1997).

### *1.3.11 Extracellular Matrix and Cell Adhesion Molecules*

Beyond the clearly established role of mesenchyme, the extracellular matrix molecules, especially the basement membrane, play many important roles in pancreatic development. The embryonic pancreatic epithelium is contained within a continuous sheath of basement membrane that creates the epithelial–mesenchymal interface (Hisaoka et al., 1993). There are microscopic breaks in this sheath in the region in which early endocrine cells are forming. Matrigel, which consists mainly of laminin-1, was found to induce duct formation in isolated E11 mouse pancreatic epithelium (Gittes et al., 1996). Laminin-1 through interactions with epithelial α6-containing integrin, mediates pancreatic duct formation (Crisera et al., 2000). Li et al. showed that laminin-1 mediates pro-exocrine induction by the mesenchyme (Li et al., 2004). Laminin-1 has also been shown to have a pro-β-cell role slightly later in gestation, specifically, as a tissue culture substrate which enhanced β-cell differentiation in dispersed E13 pancreatic epithelial cells, through binding to α-dystroglycan (Jiang et al., 1999, 2001).

In addition to pancreatic epithelial cell interactions with the extracellular matrix and mesenchyme, cell–cell interactions are also very important. Cadherins are calcium-dependent cell-membrane-bound molecules that mediate cell–cell adhesion and the sorting of different cell populations. E-cadherin and R-cadherin expression is localized to the ducts, and then downregulated as cells move out of the ducts and start forming islets (Sjodin et al., 1995; Dahl et al., 1996). N-cadherin shows a different pattern than R- or E-cadherin, localizing to mesenchyme but not epithelium in the E9.5 pancreas. After E9.5, N-cadherin becomes localized to the endoderm, and by E12.5 is only seen in the islets (Esni et al., 2001). Other cell adhesion molecules, such as N-CAM and Ep-CAM, have also been implicated in pancreatic development and differentiation (Cirulli et al., 1994, 1998).

### *1.3.12 Other Extracellular Molecules*

Numerous other extracellular molecules with potentially important influences on pancreatic development have been studied, but in less detail. Scharfmann's group has studied the role of VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) in embryonic pancreas to show that these molecules can enhance amylase-positive and insulin-positive cell growth. Similarly, they have found that calsenilin, a neuronal presenilin regulator, is present in all endocrine cells, and its inhibition in zebrafish led to a marked decrease in the number of endocrine cells, as well as to failure of islet cell aggregation. Recently, the Scharfmann group has surprisingly found that glucose is necessary for endocrine but not exocrine pancreas development in vitro. In the absence of glucose (except possibly for some glucose in the

added serum), exocrine pancreas developed normally, but endocrine progenitors were unable to progress past the NGN3-positive stage (Guillemain et al., 2007).

The Breant group has recently demonstrated an interesting role for glucocorticoid receptor signaling in regulating β-cell mass. Initially, these researchers showed that late-gestational malnutrition of pregnant rats led to decreased fetal β-cell mass owing to a decreased numbers of islets (Garofano et al., 1997), which seemed to correlate with poor proliferation and greater senescence of β cells in the adult mice (Garofano et al., 1998, 1999; Garofano et al., 2000). These studies suggested that type II diabetes may stem from in-utero and perinatal insults. This in-utero effect may well be due to enhanced glucocorticoid levels, which in turn can decrease β-cell mass and islet numbers (Blondeau et al., 2001).

## 1.4 Transcription Factors Regulating Pancreas Development

Studies of pancreas development over the last 15 years have been dominated by efforts to elucidate the roles of transcription factors and their hierarchies, often using genetically modified animal models. Some of the key transcription factors that have been identified are PDX1, PBX1, PTF1a, PAX6, PAX4, NGN3, NEUROD, and NKX family proteins. Here we describe the role and expression pattern of these and other transcription factors during pancreas development (Table 1.1, Figs. 1.3 and 1.4).

**Table 1.1** Transcription factor expression and function in pancreatic development

| Factor | Expression pattern | Function |
|---|---|---|
| PDX1 | E8–E9: Foregut epithelium<br>E9–E10: Prepancreatic and pancreatic epithelium<br>Midgestation: Nonendocrine cells (low level of expression)<br>Mid-to-late gestation: β cells (high level of expression) | Probably contributes to pancreas specification of the endoderm<br>Directs pancreatic budding<br>Necessary for early development of all lineages, including acinar and ongoing endocrine development.<br>Expression in early endocrine cells favors β cells over α cells<br>Important for proper glucose sensing in β cells, in cooperation with PBX1 |
| PTF1A | E9: Pancreas-specified endoderm within the PDX1 domain<br>E9–E14: Progenitors of all pancreatic lineages, except for a few α cells<br>After E14: Acinar-committed cells | Confers the pancreatic field within the PDX1 domain of the endoderm<br>Regulates NOTCH signaling at RBPJ level<br>Necessary for acinar development<br>Part of acinar enzyme transcriptional regulatory complex |
| NGN3 | Committed endocrine progenitor cells with limited proliferative potential | Induces commitment of epithelial progenitors to become endocrine cells, with different fates selected depending on when in gestation NGN3 is expressed |

**Table 1.1** (continued)

| Factor | Expression pattern | Function |
|---|---|---|
| PAX6 | E10: Some early endocrine progenitors<br>All endocrine progenitors, except for β/δ-lineage committed cells | Regulates endocrine hormone transcription<br>Enhances endocrine cell numbers, especially α cells<br>Suppresses ghrelin synthesis |
| PAX4 | E10: Marks some early endocrine progenitor cells<br>Endocrine cells that have committed to the β/δ-lineage | Increases numbers of β/δ-lineage cells owing to ARX suppression<br>Increases numbers of mature β cells<br>Suppresses ghrelin and PAX6-mediated glucagon production |
| ARX | Endocrine progenitor cells downstream of NGN3 expression | Diverts endocrine cells away from the β/δ-lineage, toward the α/PP-lineage |
| PBX1 | E10: Early epithelium and mesenchyme<br>All developing endocrine cells | Fosters endocrine development<br>Fosters mesenchymal induction of exocrine development<br>Enhances glucose sensing in mature β cells in cooperation with PDX1 |
| NKX2.2 | E9.5: All epithelial cells<br>E10.5: 50% of epithelial cells, but not specifically endocrine-committed cells | Enhances formation of β cells and, to a lesser extent, α cells<br>Suppresses ϵ-cell formation<br>Turns on MAFA in β-cell progenitors<br>Enhances duct formation in zebrafish |
| NKX6.1 and 6.2 | E9.5: All epithelial cells<br>E11–E13: NKX6.1 marks all PDX1$^+$ cells, but NKX6.2 shuts off<br>NKX6.1 marks post-NGN3 expression endocrine progenitors<br>NKX6.2 marks some glucagon and acinar cells by E15 | Either NKX6.1 or NKX6.2 is required for early PDX1$^+$ cells to become β cells<br>NKX6.1 is necessary for β cell expansion in the secondary transition and for β-cell maturation<br>Either NKX6.1 or NKX6.2 is required for proper α-cell development |
| MAFB | Endocrine committed cells after NGN3 expression, before β-cell expansion | Expands β-cell and α-cell progenitor pools |
| MAFA | Maturing β cells after the secondary transition | Regulates insulin gene transcription |
| HNF1β | E8: Foregut epithelium<br>E9–E10: Pancreatic epithelium<br>Ongoing scattered expression in the uncommitted epithelium | Turns on HNF6<br>Necessary for dorsal and ventral bud evagination<br>Mediates HNF6-induced production of early, proliferative endocrine progenitors |
| HNF6 | Early endoderm just downstream of HNF1β<br>E9–E10: Pancreatic epithelium<br>Ongoing scattered expression in the uncommitted epithelium | Regulates PDX1 expression and pancreatic field size<br>Activates HNF1β in the commitment of epithelial progenitor cells to the endocrine lineage |
| HNF3β | All early gut endoderm<br>E9–E10: Pancreatic epithelium | Enhances endocrine cell maturation, including specifically PDX1 expression in mature β cells and glucagon expression in α cells |

**Table 1.1** (continued)

| Factor | Expression pattern | Function |
|---|---|---|
| SOX9 | E9–E10: Early pancreatic epithelium<br>Later marks uncommitted epithelial cells (progenitors of all lineages) | Regulates HNF6- and HNF1β-induced commitment of epithelial progenitors to the endocrine lineage (see above)<br>Maintains epithelial cells in a progenitor state, likely through NOTCH signaling |

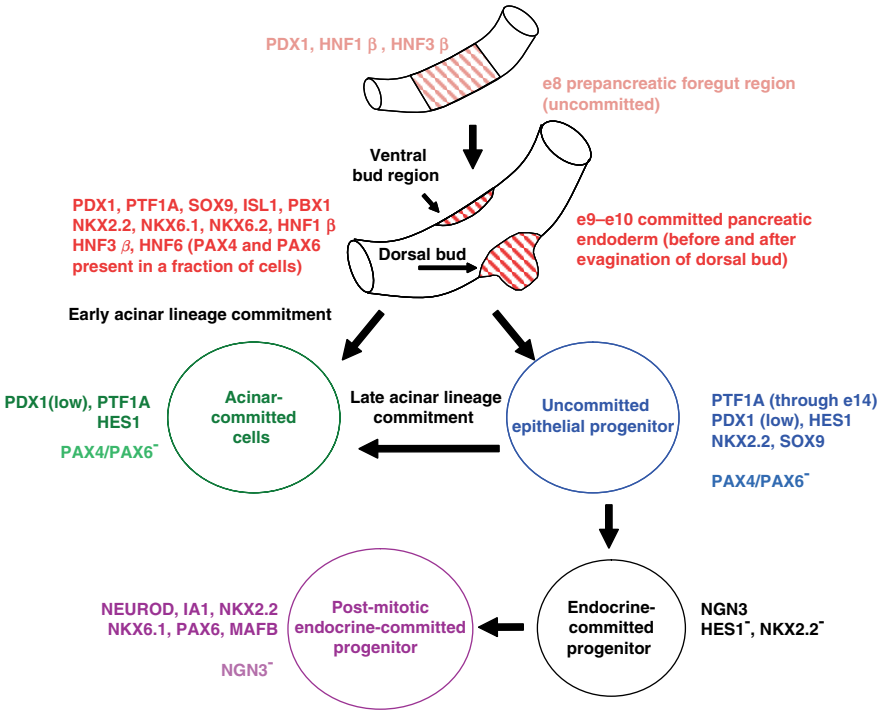



**Fig. 1.3 Schematic representation of early pancreatic lineages and their transcription factors**. Different stages of pancreas development are depicted from the earliest time of pancreas field specification to commitment to acinar or endocrine lineages (later stages are shown in Fig. 1.4)

## *1.4.1 PDX1*

Pancreatic duodenal homeobox 1 (PDX1), also called STF1, IDX1, and IPF1, and its *Xenopus* ortholog x1Hbox8, was originally identified based on its ability to bind the insulin and somatostatin genes (Leonard et al., 1993; Ohlsson et al., 1993; Miller et al., 1994). PDX1 is first expressed at E8.5 (10 somites) in the prepancreatic region of the mouse foregut (Guz et al., 1995; Jonsson et al., 1995; Offield et al., 1996),

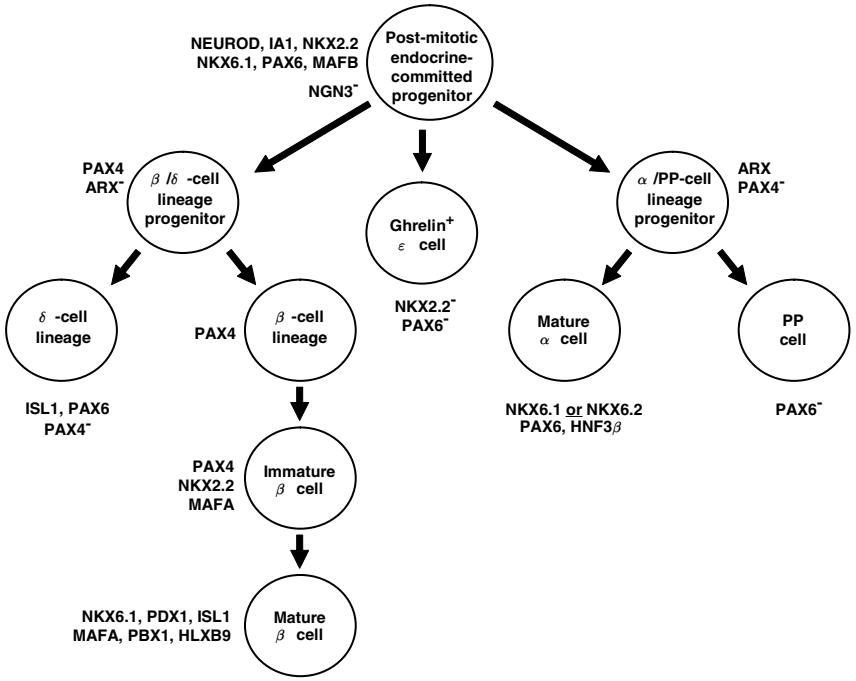



**Fig. 1.4 Schematic representation of transcription factor expression during endocrine cell differentiation after NGN3 is turned off** (last stage from Fig. 1.3)

which correlates with the earliest point in time at which foregut explants can form pancreas ex vivo. It appears that *Pdx1* gene regulatory elements in areas I–III of the *Pdx1* upstream sequence confer the expression of PDX1 in the early pancreas and duodenum (Stoffers et al., 1999; Gannon et al., 2001; Wiebe et al., 2007). Though initially limited to uncommitted epithelial cells, the developmental role for PDX1 is still significant throughout pancreatic development (Guz et al., 1995; Jonsson et al., 1995; Wu et al., 1997; Jensen et al., 2000b; Gu et al., 2002). The *pdx1*-null mutation in mice and humans causes pancreatic agenesis (Jonsson et al., 1994; Stoffers et al., 1997), with only a few insulin- and glucagon-expressing cells present in a primitive dorsal bud (Ahlgren et al., 1996). When PDX1 expression was blocked using a tetracycline-regulatable transgenic knock-in system at E12, a severe blunting of pancreatic development resulted, with only small ductal structures. However, when PDX1 expression subsequently stopped, at E14, the result was complete absence of both acini and β cells (Holland et al., 2002; Hale et al., 2005). A further refinement of the role of PDX1 in pancreas development was elucidated using mice bearing a hypomorphic *Pdx1* allele (Δ/Δ), which resulted in delayed and diminished PDX1 expression (Fujitani et al., 2006). Expressing this hypomorphic *Pdx1* allele in a *pdx1*-null mutant background allowed for stepwise PDX1 genetic "dosing," with partial rescue of pancreas phenotype at the highest subnormal "dose" of PDX1. The

Δ/+ genotype resulted in replacement of β cells with α cells and PP cells, with the α cells located throughout the islet, suggesting that enhanced α-cell growth in *Pdx1*-deficient pancreas is due to the loss of a normal β-cell inhibition of α-cell development (Gannon et al., 2008).

Beyond its developmental role, PDX1 is a key glucose-responsive regulator of insulin synthesis in β cells (MacFarlane et al., 1994; Marshak et al., 1996). The regulatory elements in areas I and II of the *Pdx1* upstream sequence, in cooperation with area IV, together confer β-cell specificity for PDX1 expression (Samaras et al., 2002; Gerrish et al., 2004; Van Velkinburgh et al., 2005; Wiebe et al., 2007).

### *1.4.2 PBX1*

PBX1 is a member of the TALE homeodomain transcription factor family (Dutta et al., 2001). In the early embryonic pancreas PBX1 is expressed in both epithelium and mesenchyme and subsequently becomes localized to ducts and islet cells. The *pbx1*-null mutant mice lack endocrine cells owing specifically to the absence of PBX1 in the epithelium, but then also lack exocrine cells as a result of the specific loss of a PBX1-induced pro-exocrine mesenchymal factor (Kim et al., 2002).

PBX1 and PDX1, together with one of various third partners, form a transcriptional regulatory complex that is important in pancreatic differentiation (Peers et al., 1995; Swift et al., 1998). The PDX1:PBX1 heterodimer has a 20-fold greater affinity for the insulin gene than the PDX1 monomer (Peers et al., 1995). Mice expressing only a PDX1 mutant that cannot interact with PBX1 could still specify pancreatic cells and develop all pancreatic cell types, but these committed cells were then unable to amplify further. This PBX1 requirement for proper PDX1 function is also demonstrated in chick foregut endoderm, where ectopic PDX1 expression (without concomitant PBX1 expression) induced pancreas-like evaginations, with suppression of intestinal patterning factors, but with no further pancreatic growth and differentiation (Grapin-Botton et al., 2001).

### *1.4.3 PTF1A*

Pancreas-specific transcription factor 1a (PTF1A or p48) is a basic helix-loop-helix (bHLH) protein that is part of a large heterotrimeric transcriptional regulator that regulates acinar enzyme gene expression (Cockell et al., 1989; Petrucco et al., 1990). PTF1A also has an important role in early specification of pancreatic progenitor cells. Essentially all acinar cells, 95% of ductal cells, 75% of α cells, and 100% of non-α endocrine cells are derived from PTF1A-positive progenitor cells. PTF1A is first expressed at E9.5 in duodenal cells destined to give rise to dorsal and ventral pancreas (Krapp et al., 1998; Burlison et al., 2008). From E9.5 to E12.5, PTF1A and PDX1 are coexpressed in pancreatic progenitor cells (Kawaguchi et al., 2002; Lin et al., 2004).

The *ptf1a*-null mutants failed to develop an exocrine pancreas in mice and zebrafish (Lin et al., 2004; Afelik et al., 2006). In mutant mice the endocrine cells develop from an aborted dorsal pancreatic bud and a minuscule ventral bud, and then migrate out through the mesenchyme to populate the spleen (Krap et al., 1998; Kawaguchi et al., 2002; Lin et al., 2004). Humans with a *PTF1A* nonfunctioning mutation are born without a pancreas, and have neonatal diabetes (Sellick et al., 2004). In addition to these known functions, PTF1A has a highly orchestrated and complex set of interactions with NOTCH downstream intercellular mediators (RBP-J's) to regulate target patterning genes and acinar-specific genes.

### *1.4.4 NGN3*

NGN3 is a bHLH transcription factor downstream of NOTCH-mediated intracellular signaling (Apelqvist et al., 1999; Jensen et al., 2000a; Lee et al., 2001). NGN3 is first expressed at E9, and peaks at E15.5, but by E17.5 is nearly gone from the pancreas (Gradwohl et al., 2000; Jensen et al., 2000a; Gu et al., 2002). NGN3-positive cells are mitotic, but quickly give rise to postmitotic cells expressing NEUROD, NKX6.1, and PAX6 (Jensen et al., 2000a), with concomitant down-regulation of NGN3, owing in part to autorepression (Gu et al., 2004; Smith et al., 2004).

All pancreatic endocrine cells derive from NGN3-positive cells (Gu et al., 2002), and thus NGN3 is a good marker for endocrine progenitor cells. However, early overexpression of NGN3 induces premature commitment to an endocrine lineage with exit from the cell cycle, resulting in only small clusters of glucagon-positive cells (Apelqvist et al., 1999; Schwitzgebel et al., 2000; Johansson et al., 2007). Nevertheless, NGN3 appears to have the ability to initiate the full pancreatic endocrine program when expressed at the proper time. With a transgenic "add-back" system, NGN3 expression at E11 to E12 in an *ngn3*-null mutant background induced the formation of many PP- and insulin-expressing cells (Johansson et al., 2007). A similar "rescue" of endocrine pancreas was found when NGN3-positive cells isolated from either an E13.5 embryonic pancreas or from a duct-ligated adult pancreas were grafted into an *ngn3*-null mutant foregut in vitro (Xu et al., 2008) (see Chap. 4). These data provide strong evidence for NGN3 as a marker of embryonic and adult-derived pancreatic islet stem cells.

Potential downstream mechanisms by which NGN3 may activate the endocrine program include binding and activating E-boxes of the NEUROD regulatory sequence (Huang et al., 2000). The *ngn3*-null mutant mice lack NEUROD (Gradwohl et al., 2000), and transgenic overexpression of NEUROD induces a phenotype similar to NGN3 overexpression in mice or in ductal cell lines (Schwitzgebel et al., 2000; Heremans et al., 2002; Gasa et al., 2008). Other suggested NGN3 targets include Iroquois-type homeobox proteins (IRX1 and 2) expressed in early pancreatic endoderm and in α cells (Petri et al., 2006), NEUROD2, which is present in embryonic pancreas and in α-cell lines (Gasa et al., 2008), and Insulin-associated 1 (IA1), a zinc-finger protein, (Breslin et al., 2003; Mellitzer et al., 2006).

### 1.4.5 NEUROD

Potential downstream mechanisms by which NGN3 may activate the endocrine program are of obvious interest. One immediate downstream target of NGN3 is *neuroD* (Gradwohl et al., 2000; Huang et al., 2000; Jensen et al., 2000a, b; Gu et al., 2002; Gasa et al., 2008), which binds and activates E-boxes of the *neuroD* regulatory sequence as an NGN3-E47 heterodimer (Huang et al., 2000). The close relationship between NGN3 and NEUROD expression is highlighted by the fact that NEUROD overexpression, in either transgenic mice or ductal cell lines, induces a phenotypic change similar to that seen with NGN3 overexpression (Schwitzgebel et al., 2000; Heremans et al., 2002; Gasa et al., 2008). NEUROD expression is dependent on NGN3 since *ngn3*-null mutant mice lack NEUROD (Gradwohl et al., 2000). The onset of NEUROD expression in endocrine cells of the embryonic pancreas represents a very important transition from proliferative NGN3-positive cells to postmitotic cells (Jensen et al., 2000a; Gu et al., 2002). Unlike *ngn3*-null mutant mice, however, *neuroD*-null mutant mice are still able to form all pancreatic endocrine cell types, but the number of cells is drastically reduced owing to late-gestation apoptosis, depending on the mouse strain (Naya et al., 1997; Huang et al., 2002). A role for NEUROD in suppressing non-β-cell lineages has been suggested by the fact that addition of a *neuroD*-null mutation to *nkx2.2*-null mutant mice (lacking β- , α- , and PP-cells, see below) rescues α and PP cells (Chao et al., 2007).

### 1.4.6 PAX6

PAX6, a member of the PAX family of transcription factors, contains a paired-box DNA-binding domain and a homeodomain (Turque et al., 1994). It is first expressed at E9.0 within an endocrine-committed population of PDX1-positive cells and subsequently in NGN3- , NEUROD- , and ISL1-expressing epithelial cells. Thus, PAX6 expression is retained in cells committed to the endocrine lineage (Sander et al., 1997; St-Onge et al., 1997; Heller et al., 2004). Though PAX6-positive cells give rise to endocrine cells, and PAX6 binding sites have been found on the promoter region of the preproglucagon, insulin and somatostatin genes (Sander et al., 1997), PAX6 does not appear to be critical for hormone gene expression. The *pax6*-null mutant mice, or a dysfunctional small-eye mutant of the *Pax6* gene, were both able to form endocrine cells, though at a reduced rate and especially with very few or no glucagon cells (Sander et al., 1997; St-Onge et al., 1997). The E19 pancreas of *pax6*-null mutant mice contained numerous ghrelin-positive ϵ cells, suggesting that PAX6 may normally serve to downregulate ghrelin expression (Heller et al., 2005).

### 1.4.7 PAX4 and ARX

PAX4, another member of the PAX family of transcription factors, is a ß-cell progenitor marker, which is required for β-cell development and differentiation (Dahl et al., 1997). Overexpression of a constitutively active NOTCH mediator in the PAX4-positive subset of $NGN3^{+}$ lineage-committed cells led to a surprising

metaplasia of the cells into ducts, suggesting that PAX4-positive cells may still represent an important multipotent progenitor cell (Greenwood et al., 2007). PAX4 is first expressed in the prepancreatic duodenum and then is present in the evaginating dorsal pancreas at E9.5 (Sosa-Pineda et al., 1997). PAX4 expression peaks at E13–E15 (Wang et al., 2004), coincident with the burst of new insulin-positive cells in the secondary transition. However, PAX4 seems necessary only for formation of mature ß cells and not for their function, as its expression disappears in mature ß cells (Smith et al., 1999). The *pax4*-null mutant mice fail to develop ß and δ cells (Sosa-Pineda et al., 1997; Wang et al., 2004), whereas the number of glucagon- and ghrelin-positive cells increased significantly, suggesting that PAX4 may normally act as a transcriptional repressor of glucagon and ghrelin expression (Wang et al., 2008). Evidence indicates that PAX4 directly inhibits expression of ARX, a homeobox-containing gene that enhances glucagon-positive cell differentiation (Collombat et al., 2005).

The *arx*-null mutant mice provide further evidence for opposing roles for PAX4 and ARX in α/PP-cell versus ß/δ-cell development. These mice have no α cells, and the α-cell precursors seem to be shunted toward the ß-cell and δ-cell lineage owing to unopposed PAX4 expression (Collombat et al., 2005). Conversely, overexpression of ARX in PDX1-positive progenitor cells diverted most ß-cell and δ-cell precursors toward α cells and PP cells with no change in the total number of endocrine cells (Collombat et al., 2007).

### *1.4.8 NKX2.2*

NKX2.2 is a homeodomain protein containing an engrailed-homologous repressor domain (Pedersen et al., 2005). NKX2.2 is first expressed in the developing pancreas at E9.5 (Sussel et al., 1998), and by E10.5 half of the pancreatic epithelial cells are NKX2.2-positive and coexpress PDX1 (Chiang and Melton, 2003). At later stages, NKX2.2 expression focuses on NGN3-positive cells and persists in most endocrine cells, except for the δ cells (Sussel et al., 1998). This expression of NKX2.2 during different phases of pancreatic development appears to be under the control of three different first exons, each having different regulatory elements (Watada et al., 2003).

The most notable phenotype of *nkx2.2*-null mutation in mice is the absence of detectable ß cells, an 80% reduction in α cells, a "modest" reduction in PP cells, and no effect on δ cells (Sussel et al., 1998). However, there was a large increase in the number of ghrelin-positive ϵ cells, suggesting that NKX2.2 can normally induce insulin-positive differentiation and repress ϵ-cell formation (Sussel et al., 1998). Other studies showed that NKX2.2 can bind and activate the *MafA* (Raum et al., 2006) and insulin genes (Cissell et al., 2003), being part of the mechanism by which NKX2.2 controls ß-cell formation.

### *1.4.9 NKX6.1 and NKX6.2*

The homeobox genes *Nkx6.1* and *Nkx.6.2* play a central role in pancreatic ß-cell development and function (Oster et al., 1998a, b; Sander et al., 2000; Henseleit et al.,

2005). Mature β cells express NKX6.1, suggesting an ongoing role in mature ß-cell function (Sander et al., 2000), whereas NKX6.2 expression is restricted to embryonic glucagon-positive and amylase-positive cells and is turned off after E15.5. In *nkx6.1*-null mutant mice the insulin-positive cells failed to expand after E13, resulting in an 85% reduction in ß cells (Sander et al., 2000). The *nkx6.1/nkx6.2* double-null mutant mice had a 92% reduction in ß cells, suggesting an important role for NKX6.1 in the generation of ß cells, specifically at the secondary transition, with some ability of NKX6.2 to compensate for this loss (Sander et al., 2000; Henseleit et al., 2005). Transgenic expression of either NKX6.1 or NKX6.2 under the *Pdx1* promoter, but not under the *Ngn3* promoter, could rescue the ß-cell loss in *nkx6.1*-null mutant mice. This suggests that NKX6.2 can replace NKX6.1 function if expressed in the proper cell and that early NKX6.1 expression in a PDX1-positive domain, before NGN3 expression, is necessary for ß-cell formation (Nelson et al., 2007).

### *1.4.10 MAFA and MAFB*

MAFA and MAFB are members of a large family of basic leucine-zipper transcription factors that are active in many developmental processes. MAFA is not necessary for ß-cell formation, but is required for ß-cell function as a critical regulator of the insulin gene (Olbrot et al., 2002). MAFA is first expressed in insulin-positive cells during the secondary transition (Matsuoka et al., 2004). It appears that the MAFA-positive–insulin-positive cells may derive from MAFB-positive–insulin-positive progenitor cells (Artner et al., 2006). MAFB is turned off in insulin-positive cells as they transition from immature to mature ß cells (Nishimura et al., 2006). This transition from MAFB to MAFA depends on MAFB function, since MAFB binds and activates the *MafA* gene (Artner et al., 2007). The *mafB*-null mutants have delayed development of early insulin-positive and glucagon-positive cells and a 50% reduction in both cell types, with an abundance of hormone-negative cells that appear to be of the endocrine lineage, suggesting that MAFB is a key regulator of α- and ß-cell maturation (Artner et al., 2007).

### *1.4.11 HNF Cascade*

HNF6 or ONECUT1 is a cut homeodomain protein expressed in the endoderm as early as E8 in the region of the foregut-midgut junction (Lemaigre et al., 1996; Poll et al., 2006). HNF6 expression in the early endoderm is controlled by vHNF1/HNF1β, which binds to *Hnf6* regulatory elements (Haumaitre et al., 2005; Poll et al., 2006). The *vhnf1*-null mutation in mice and *vHNF1/HNF1β* mutations in humans and zebrafish all lead to ventral pancreatic agenesis and an atrophic dorsal pancreas (Barbacci et al., 2004; Haumaitre et al., 2005). HNF6 regulates gut formation through HNF3β/FOXA2 expression and activates the *Pdx1* gene (Wu et al.,

1997). Thus, *hnf6*-mutant mice are born with a hypoplastic pancreas owing to a severely reduced PDX1-positive field of the pancreas-specific foregut endoderm (Jacquemin et al., 2000, 2003). After early prepancreatic endodermal expression, HNF cascade genes are expressed briefly in E9–E10 epithelia (Jacquemin et al., 2000, 2003) and turn off late in gestation (Gannon et al., 2001).

### *1.4.12 SOX9*

SOX9 is expressed in progenitor cells that can give rise to all pancreatic cell types (Akiyama et al., 2005) and may serve as a key mediator of the commitment of NGN3-positive endocrine progenitors. SOX9 can bind and upregulate the *Ngn3* gene and is transiently coexpressed with NGN3 (Lynn et al., 2007b; Seymour et al., 2007). Furthermore, SOX9 interacts with two HNF proteins, vHNF1/HNF1β and HNF6, which suggests a role for SOX9 in mediating HNF control over NGN3-positive cell populations (Lynn et al., 2007b; Seymour et al., 2007).

### *1.4.13 MYT1, GATA Factors, HB9, SOX4, ISL1, HEX, PROX1, and BRAIN4*

The *Myt1* gene can generate two different zinc-finger transcription factors that interact with NGN3 in the developing pancreas (Gu et al., 2004; Wang et al., 2007). Transgenic expression of a dominant-negative form of MYT1a in the NGN3 domain blocked α- and ß-cell development by 30–40%, whereas *myt1*-null mutant mice developed abnormal multihormone-expressing pancreatic endocrine cells.

The homeodomain protein HLXB9, or HB9, expressed in the pancreatic domain of the foregut, is important for dorsal pancreatic development and formation of all β cells (Li and Edlund, 2001). The *hb9*-null mutant mice develop specifically without a dorsal pancreas, and the remaining pancreas has a 65% reduction in ß cells. HB9 expression in the epithelium is downregulated after E12, and transgenic overexpression of HB9 using a *Pdx1-HB9* construct led to global pancreatic hypoplasia and pancreatic intestinalization (Li and Edlund, 2001).

SOX4 is expressed first in early pancreatic buds and later in the islets. The *sox4*-null mutation is embryonically lethal at E14.5, and in vitro explant cultures of mutant pancreas have reduced endocrine cell differentiation (Wilson et al., 2005).

The LIM homeodomain protein ISL1 is expressed in both early pancreatic epithelium and the dorsal mesenchyme in a pattern similar to that of PBX1 (Ahlgren et al., 1997). ISL1-positive cells are postmitotic and likely downstream of NEUROD-positive and upstream of PAX6-positive cells (Jensen et al., 2000b). The *isl1*-null mutation is lethal, and the embryos die at E9.5. The *isl1*-null mutant embryos lack dorsal mesenchyme and fail to form a dorsal pancreas. In-vitro experiments in which wild-type mesenchyme is added can rescue exocrine–acinar pancreatic development but not endocrine development, which suggests that ISL1 expression specifically in

the epithelium is necessary for the development of all endocrine cells, whereas ISL1 in the mesenchyme is necessary for production of an exocrine–acinar-inductive factor (Ahlgren et al., 1997).

GATA4 and GATA6 are zinc-finger transcription factors expressed in the developing pancreas (Ketola et al., 2004; Ritz-Laser et al., 2005; Decker et al., 2006). The *gata6*- and *gata4*-null mutants die early in development. A pancreas-specific null mutation of *gata6* produced severe pancreatic agenesis, whereas pancreas-specific *gata4*-mutant mice were normal (Decker et al., 2006).

HEX is a hox-related homeodomain protein present in both the early foregut and the pancreatic epithelium from E13 to E16 (Bort et al., 2004). It is also expressed in the ventral foregut endoderm, where it induces proliferation and development of the ventral pancreas (Deutsch et al., 2001).

PROX1 is a homeodomain protein that marks pancreatic and liver progenitor cells in the endoderm (Burke and Oliver, 2002). PROX1 expression continues in the developing pancreas and by E15 focuses on NGN3-positive cells, endocrine cells, and ducts. The *prox*1-null mutant mice die at E15 with a small pancreas and with loss of secondary transition endocrine cells owing to premature cell cycle exit (Wang et al., 2005).

The Pou-domain protein BRAIN4 binds and activates the glucagon gene (Hussain et al., 1997). BRAIN4 is restricted to α-cell progenitors (Heller et al., 2004) and persists in mature α cells and in a few PP cells of the late-gestational embryonic pancreas. BRAIN4 is not necessary for α-cell formation; however, transgenic expression of BRAIN4 under a *Pdx1* promoter can induce glucagon expression in ß cells (Heller et al., 2004).

## 1.5 MicroRNAs

MicroRNAs (miRNAs) are small 20- to 22-base RNA molecules derived from larger primary RNA transcripts through intranuclear (Drosha enzyme) and cytosolic (Dicer enzyme) processing. The miRNAs can regulate gene expression at the post-transcriptional mRNA level through either translational inhibition or mRNA degradation and play a role in normal ß-cell function (Poy et al., 2004). In zebrafish, inhibition of a specific miRNA (miR375) disrupted normal islet formation (Kloosterman et al., 2007). Pancreas-specific deletion of Dicer resulted in failure to produce mature miRNA, causing global disruption of pancreatic architecture, with ductal ectasia and loss of ß cells, reminiscent of the *hnf6*-null mutant phenotype (Lynn et al., 2007a).

A role for miRNAs in pancreatic regeneration through post-transcriptional regulation of *Ngn3* has been identified. NGN3 was not found in regenerating islets after pancreatectomy (Lee et al., 2006). This was attributed to suppression by miRNAs, since a 200-fold increase in the level of *Ngn3* mRNA was seen in these pancreatectomy specimens, but without detectable NGN3 protein (Joglekar et al., 2007). Other RNA-binding and inhibiting molecules include Vg1RBP, an RNA-binding protein that binds to an untranslated region of a newly-identified shirin gene

(Spagnoli and Brivanlou, 2006). *Xenopus* morphants for Vg1RBP had no insulin or PDX1 expression, and ectopic expression of this RNA-binding protein led to ectopic pancreas.

## 1.6 Summary

The formation of the pancreas and the pancreatic islets represents an extremely complicated process that is the subject of continuing intensive investigation, particularly toward the goal of engineering new sources of β cells. New and emerging scientific fields and technologies will surely deepen our understanding of these processes and help us to better assimilate the extensive information into a cohesive depiction of how the pancreas forms.

## References

Afelik S, Chen Y, Pieler T. (2006) Combined ectopic expression of Pdx1 and Ptf1a/p48 results in the stable conversion of posterior endoderm into endocrine and exocrine pancreatic tissue. Genes Dev. 20:1441–1446.

Ahlgren U, Jonsson J, Edlund H. (1996) The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. Development. 122:1409–1416.

Ahlgren U, Pfaff SL, Jessell TM, et al. (1997) Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature. 385:257–260.

Akiyama H, Kim JE, Nakashima K, et al. (2005) Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. Proc Natl Acad Sci USA. 102:14665–14670.

Anastasi E, Santangelo C, Bulotta A, et al. (2005) The acquisition of an insulin-secreting phenotype by HGF-treated rat pancreatic ductal cells (ARIP) is associated with the development of susceptibility to cytokine-induced apoptosis. J Mol Endocrinol. 34:367–376.

Apelqvist A, Li H, Sommer L, Beatus P, et al. (1999) Notch signaling controls pancreatic cell differentiation. Nature. 400:877–881.

Asayesh A, Sharpe J, Watson RP, et al. (2006) Spleen versus pancreas: strict control of organ interrelationship revealed by analyses of Bapx1-/- mice. Genes Dev. 20:2208–2213.

Artner I, Blanchi B, Raum JC, et al. (2007) MafB is required for islet β cell maturation. Proc Natl Acad Sci USA. 104:3853–3858.

Artner I, Le Lay J, Hang Y, et al. (2006) MafB: an activator of the glucagon gene expressed in developing islet alpha- and β-cells. Diabetes. 55:297–304.

Barbacci E, Chalkiadaki A, Masdeu C, et al. (2004) HNF1β/TCF2 mutations impair transactivation potential through altered co-regulator recruitment. Hum Mol Genet. 13:3139–3149.

Bardeesy N, Cheng KH, Berger JH, et al. (2006) Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev. 20:3130–3146.

Blondeau B, Lesage J, Czernichow P, et al. (2001) Glucocorticoids impair fetal β-cell development in rats. Am J Physiol Endocrinol Metab. 281:E592–E599.

Bort R, Martinez-Barbera JP, Beddington RS, et al. (2004) Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. Development. 131:797–806.

Breslin MB, Zhu M, Lan MS. (2003) NeuroD1/E47 regulates the E-box element of a novel zinc finger transcription factor, IA-1, in developing nervous system. J Biol Chem. 278:38991–38997.

Brorson M, Hougaard DM, Nielsen JH, et al. (2001) Expression of SMAD signal transduction molecules in the pancreas. Histochem Cell Biol. 116:263–267.

Burke Z, Oliver G. (2002) Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. Mech Dev. 118:147–155.

Burlison JS, Long Q, Fujitani Y, et al. (2008) Pdx-1 and Ptf1a concurrently determine fate specification of pancreatic multipotent progenitor cells. Dev Biol. 316:74–86.

Buteau J, Foisy S, Rhodes CJ, et al. (2001) Protein kinase Czeta activation mediates glucagon-like peptide-1-induced pancreatic β-cell proliferation. Diabetes. 50:2237–2243.

Buteau J, Roduit R, Susini S, et al. (1999) Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in β (INS-1)-cells. Diabetologia. 42: 856–864.

Celli G, LaRochelle WJ, Mackem S, et al. (1998) Soluble dominant-negative receptor uncovers essential roles for fibroblast growth factors in multi-organ induction and patterning. EMBO J. 17:1642–1655.

Chao CS, Loomis ZL, Lee JE, et al. (2007) Genetic identification of a novel NeuroD1 function in the early differentiation of islet alpha, PP and epsilon cells. Dev Biol. 312:523–532.

Chen Y, Pan FC, Brandes N, et al. (2004) Retinoic acid signaling is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in *Xenopus*. Dev Biol. 271:144–160.

Chertow BS, Baranetsky NG, Sivitz WI, et al. (1983) Cellular mechanisms of insulin release. Effects of retinoids on rat islet cell-to-cell adhesion, reaggregation, and insulin release. Diabetes. 32:568–574.

Chertow BS, Buschmann RJ, Kaplan RL. (1979) Cellular mechanisms of insulin release. Effects of retinol on insulin release and islet ultrastructure. Diabetes. 28:754–761.

Chiang MK, Melton DA. (2003) Single-cell transcript analysis of pancreas development. Dev Cell. 4:383–393.

Cirulli V, Baetens D, Rutishauser U, et al. (1994) Expression of neural cell adhesion molecule (N-CAM) in rat islets and its role in islet cell type segregation. J Cell Sci. 107:1429–1436.

Cirulli V, Crisa L, Beattie GM, et al. (1998) KSA antigen Ep-CAM mediates cell-cell adhesion of pancreatic epithelial cells: morphoregulatory roles in pancreatic islet development. J Cell Biol. 140:1519–1534.

Cissell MA, Zhao L, Sussel L, et al. (2003) Transcription factor occupancy of the insulin gene in vivo. Evidence for direct regulation by Nkx2.2. J Biol Chem. 278:751–756.

Cockell M, Stevenson BJ, Strubin M, et al. (1989) Identification of a cell-specific DNA-binding activity that interacts with a transcriptional activator of genes expressed in the acinar pancreas. Mol Cell Biol. 9:2464–2476.

Collombat P, Hecksher-Sørensen J, Broccoli V, et al. (2005) The simultaneous loss of Arx and Pax4 genes promotes a somatostatin-producing cell fate specification at the expense of the alpha- and β-cell lineages in the mouse endocrine pancreas. Development. 132:2969–2980.

Collombat P, Hecksher-Sørensen J, Krull J, et al. (2007) Embryonic endocrine pancreas and mature β cells acquire alpha and PP cell phenotypes upon Arx misexpression. J Clin Invest. 117:961–970.

Crisera CA, Maldonado TS, Kadison AS, et al. (2000) Transforming growth factor-β 1 in the developing mouse pancreas: a potential regulator of exocrine differentiation. Differentiation. 65:255–259.

Crisera CA, Rose MI, Connelly PR, et al. (1999) The ontogeny of TGF-β1, -β2, -β3, and TGF-β receptor-II expression in the pancreas: implications for regulation of growth and differentiation. J Pediatr Surg. 34:689–694.

Dahl E, Koseki H, Balling R. (1997) Pax genes and organogenesis. Bioessays. 19:755–765.

Dahl U, Sjødin A, Semb H. (1996) Cadherins regulate aggregation of pancreatic β-cells in vivo. Development. 122:2895–2902.

Dai C, Huh CG, Thorgeirsson SS, et al. (2005) Beta-cell-specific ablation of the hepatocyte growth factor receptor results in reduced islet size, impaired insulin secretion, and glucose intolerance. Am J Pathol. 167:429–436.

Decker K, Goldman DC, Grasch CL, et al. (2006) Gata6 is an important regulator of mouse pancreas development. Dev Biol. 298:415–429.
Deutsch G, Jung J, Zheng M, et al. (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development. 128:871–881.
Dichmann DS, Miller CP, Jensen J, et al. (2003) Expression and misexpression of members of the FGF and TGFβ families of growth factors in the developing mouse pancreas. Dev Dyn. 226:663–674.
Dichmann DS, Yassin H, Serup P, et al. (2006) Analysis of pancreatic endocrine development in GDF11-deficient mice. Dev Dyn. 235:3016–3025.
diIorio P, Alexa K, Choe SK, et al. (2007) TALE-family homeodomain proteins regulate endodermal sonic hedgehog expression and pattern the anterior endoderm. Dev Biol. 304:221–231.
diIorio PJ, Moss JB, Sbrogna JL, et al. (2002) Sonic hedgehog is required early in pancreatic islet development. Dev Biol. 244:75–84.
Dutta S, Gannon M, Peers B, et al. (2001) PDX:PBX complexes are required for normal proliferation of pancreatic cells during development. Proc Natl Acad Sci USA. 98: 1065–1070.
Duvillié B, Attali M, Bounacer A, et al. (2006) The mesenchyme controls the timing of pancreatic β-cell differentiation. Diabetes. 55:582–589.
Elghazi L, Cras-Méneur C, Czernichow P, et al. (2002) Role for FGFR2IIIb-mediated signals in controlling pancreatic endocrine progenitor cell proliferation. Proc Natl Acad Sci USA. 99:3884–3889.
Erickson SL, O'Shea KS, Ghaboosi N, et al. (1997) ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. Development. 124:4999–5011.
Esni F, Johansson BR, Radice GL, et al. (2001) Dorsal pancreas agenesis in N-cadherin- deficient mice. Dev Biol. 238:202–212.
Fujitani Y, Fujitani S, Boyer DF, et al. (2006) Targeted deletion of a cis-regulatory region reveals differential gene dosage requirements for Pdx1 in foregut organ differentiation and pancreas formation. Genes Dev. 20:253–266.
Furukawa M, Eto Y, Kojima I, et al. (1995) Expression of immunoreactive activin A in fetal rat pancreas. Endocr J. 42:63–68.
Gannon M, Ables ET, Crawford L, et al. (2008) pdx-1 function is specifically required in embryonic β cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis. Dev Biol. 314:406–417.
Gannon M, Gamer LW, Wright CV. (2001) Regulatory regions driving developmental and tissue-specific expression of the essential pancreatic gene pdx1. Dev Biol. 238:185–201.
Garcia-Ocaña A, Takane KK, Syed MA, et al. (2000) Hepatocyte growth factor overexpression in the islet of transgenic mice increases β cell proliferation, enhances islet mass, and induces mild hypoglycemia. J Biol Chem. 275:1226–1232.
Garofano A, Czernichow P, Bréant B. (1997) In utero undernutrition impairs rat β-cell development. Diabetologia. 40:1231–1234.
Garofano A, Czernichow P, Bréant B. (1998) Beta-cell mass and proliferation following late fetal and early postnatal malnutrition in the rat. Diabetologia. 41:1114–1120.
Garofano A, Czernichow P, Bréant B. (1999) Effect of ageing on β-cell mass and function in rats malnourished during the perinatal period. Diabetologia. 42:711–718.
Garofano A, Czernichow P, Bréant B. (2000) Impaired β-cell regeneration in perinatally malnourished rats: a study with STZ. FASEB J. 14:2611–2617.
Gasa R, Mrejen C, Lynn FC, et al. (2008) Induction of pancreatic islet cell differentiation by the neurogenin-neuroD cascade. Differentiation. 76:381–391.
Gassmann M, Casagranda F, Orioli D, et al. (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature. 378:390–394.
Gerrish K, Van Velkinburgh JC, Stein R. (2004) Conserved transcriptional regulatory domains of the pdx-1 gene. Mol Endocrinol. 18:533–548.

Gittes GK, Galante PE, Hanahan D, et al. (1996) Lineage-specific morphogenesis in the developing pancreas: role of mesenchymal factors. Development. 122:439–447.
Goto Y, Nomura M, Tanaka K, et al. (2007) Genetic interactions between activin type IIB receptor and Smad2 genes in asymmetrical patterning of the thoracic organs and the development of pancreas islets. Dev Dyn. 236:2865–2874.
Goulley J, Dahl U, Baeza N, et al. (2007) BMP4-BMPR1A signaling in β cells is required for and augments glucose-stimulated insulin secretion. Cell Metab. 5:207–219.
Gradwohl G, Dierich A, LeMeur M, et al. (2000) Neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci USA. 97:1607–1611.
Grapin-Botton A, Majithia AR, Melton DA. (2001) Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. Genes Dev. 15: 444–454.
Greenwood AL, Li S, Jones K, et al. (2007) Notch signaling reveals developmental plasticity of Pax4(+) pancreatic endocrine progenitors and shunts them to a duct fate. Mech Dev. 124:97–107.
Gu G, Dubauskaite J, Melton DA. (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development. 129:2447–2457.
Gu G, Wells JM, Dombkowski D, et al. (2004) Global expression analysis of gene regulatory pathways during endocrine pancreatic development. Development. 131:165–179.
Guillemain G, Filhoulaud G, Da Silva-Xavier G, et al. (2007) Glucose is necessary for embryonic pancreatic endocrine cell differentiation. J Biol Chem. 282:15228–15237.
Guz Y, Montminy MR, Stein R, et al. (1995) Expression of murine STF-1, a putative insulin gene transcription factor, in β cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. Development. 121:11–18.
Hahn SA, Schutte M, Hoque AT, et al. (1996) DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science. 271:350–353.
Hald J, Hjorth JP, German MS, et al. (2003) Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuates endocrine development. Dev Biol. 260:426–437.
Hale MA, Kagami H, Shi L, et al. (2005) The homeodomain protein PDX1 is required at mid-pancreatic development for the formation of the exocrine pancreas. Dev Biol. 286:225–237.
Harmon EB, Apelqvist AA, Smart NG, et al. (2004) GDF11 modulates NGN3+ islet progenitor cell number and promotes β-cell differentiation in pancreas development. Development. 131:6163–6174.
Hart A, Papadopoulou S, Edlund H. (2003) Fgf10 maintains Notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells. Dev Dyn. 228:185–193.
Haumaitre C, Barbacci E, Jenny M, et al. (2005) Lack of TCF2/vHNF1 in mice leads to pancreas agenesis. Proc Natl Acad Sci USA. 102:1490–1495.
Hebrok M, Kim SK, Melton DA. (1998) Notochord repression of endodermal Sonic hedgehog permits pancreas development. Genes Dev. 12:1705–1713.
Hebrok M, Kim SK, St Jacques B, et al. (2000) Regulation of pancreas development by hedgehog signaling. Development. 127:4905–4913.
Heiser PW, Lau J, Taketo MM, et al. (2006) Stabilization of β-catenin impacts pancreas growth. Development. 133:2023–2032.
Heller RS, Dichmann DS, Jensen J, et al. (2002) Expression patterns of Wnts, Frizzleds, sFRPs, and misexpression in transgenic mice suggesting a role for Wnts in pancreas and foregut pattern formation. Dev Dyn. 225:260–270.
Heller RS, Jenny M, Collombat P, et al. (2005) Genetic determinants of pancreatic epsilon-cell development. Dev Biol. 286:217–224.
Heller RS, Stoffers DA, Liu A, et al. (2004) The role of Brn4/Pou3f4 and Pax6 in forming the pancreatic glucagon cell identity. Dev Biol. 268:123–134.
Henseleit KD, Nelson SB, Kuhlbrodt K, et al. (2005) NKX6 transcription factor activity is required for alpha- and beta-cell development in the pancreas. Development. 132:3139–3149.

Heremans Y, Van De Casteele M, in't Veld P, et al. (2002) Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3. J Cell Biol. 159:303–312.

Hisaoka M, Haratake J, Hashimoto H. (1993) Pancreatic morphogenesis and extracellular matrix organization during rat development. Differentiation. 53:163–172.

Hogan BL. (1996) Bone morphogenetic proteins in development. Curr Opin Genet Dev. 6:432–438.

Hogan BL. (1999) Morphogenesis. Cell. 96:225–233.

Holland AM, Hale MA, et al. (2002) Experimental control of pancreatic development and maintenance. Proc Natl Acad Sci U S A. 99(19):12236–12241.

Hua H, Zhang YQ, Dabernat S, et al. (2006) BMP4 regulates pancreatic progenitor cell expansion through Id2. J Biol Chem. 281:13574–13580.

Huang HP, Chu K, Nemoz-Gaillard E, et al. (2002) Neogenesis of β-cells in adult BETA2/NeuroD-deficient mice. Mol Endocrinol. 16:541–551.

Huang HP, Liu M, El-Hodiri HM, et al. (2000) Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol Cell Biol. 20:3292–3307.

Hui H, Wright C, Perfetti R. (2001) Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells. Diabetes. 50:785–796.

Huotari MA, Miettinen PJ, Palgi J, et al. (2002) ErbB signaling regulates lineage determination of developing pancreatic islet cells in embryonic organ culture. Endocrinology. 143:4437–4446.

Hussain MA, Lee J, Miller CP, et al. (1997) POU domain transcription factor Brain 4 confers pancreatic alpha-cell-specific expression of the proglucagon gene through interaction with a novel proximal promoter G1 element. Mol Cell Biol. 17:7186–7194.

Huypens P, Ling Z, Pipeleers D, et al. (2000) Glucagon receptors on human islet cells contribute to glucose competence of insulin release. Diabetologia. 43:1012–1019.

Jackerott M, Larsson LI. (1997) Immunocytochemical localization of the NPY/PYY Y1 receptor in the developing pancreas. Endocrinology. 138:5013–5018.

Jackson LF, Qiu TH, Sunnarborg SW, et al. (2003) Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. EMBO J. 22:2704–2716.

Jacquemin P, Durviaux SM, Jensen J, et al. (2000) Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. Mol Cell Biol. 20:4445–4454.

Jacquemin P, Lemaigre FP, Rousseau GG. (2003) The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. Dev Biol. 258:105–116.

Jensen J, Heller RS, Funder-Nielsen T, et al. (2000a) Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes. 49:163–176.

Jensen J, Pedersen EE, Galante P, et al. (2000b) Control of endodermal endocrine development by Hes-1. Nat Genet. 24:36–44.

Jepeal LI, Fujitani Y, Boylan MO, et al. (2005) Cell-specific expression of glucose-dependent-insulinotropic polypeptide is regulated by the transcription factor PDX-1. Endocrinology. 146:383–391.

Jiang FX, Cram DS, DeAizpurua HJ, et al. (1999) Laminin-1 promotes differentiation of fetal mouse pancreatic β-cells. Diabetes. 48:722–730.

Jiang FX, Georges-Labouesse E, Harrison LC. (2001) Regulation of laminin 1-induced pancreatic β-cell differentiation by alpha6 integrin and alpha-dystroglycan. Mol Med. 7:107–114.

Jiang FX, Harrison LC. (2005) Convergence of bone morphogenetic protein and laminin-1 signaling pathways promotes proliferation and colony formation by fetal mouse pancreatic cells. Exp Cell Res. 308:114–122.

Joglekar MV, Parekh VS, Mehta S, et al. (2007) MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3. Dev Biol. 311:603–612.

Johansson KA, Dursun U, Jordan N, et al. (2007) Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. Dev Cell. 12:457–465.

Jonsson J, Ahlgren U, Edlund T, et al. (1995) IPF1, a homeodomain protein with a dual function in pancreas development. Int J Dev Biol. 39:789–798.

Jonsson J, Carlsson L, Edlund T, et al. (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. Nature. 371:606–609.

Kaneto H, Miyagawa J, Kajimoto Y, et al. (1997) Expression of heparin-binding epidermal growth factor-like growth factor during pancreas development. A potential role of PDX-1 in transcriptional activation. J Biol Chem. 272:29137–29143.

Kato M, Kato K, Blaner WS, et al. (1985) Plasma and cellular retinoid-binding proteins and transthyretin (prealbumin) are all localized in the islets of Langerhans in the rat. Proc Natl Acad Sci USA. 82:2488–2492.

Kawaguchi Y, Cooper B, Gannon M, et al. (2002) The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet. 32:128–134.

Ketola I, Otonkoski T, Pulkkinen MA, et al. (2004) Transcription factor GATA-6 is expressed in the endocrine and GATA-4 in the exocrine pancreas. Mol Cell Endocrinol. 226:51–57.

Kim SK, Hebrok M, Li E, et al. (2000) Activin receptor patterning of foregut organogenesis. Genes Dev. 14:1866–1871.

Kim SK, Hebrok M, Melton DA. (1997) Notochord to endoderm signaling is required for pancreas development. Development. 124:4243–4252.

Kim SK, Selleri L, Lee JS, et al. (2002) Pbx1 inactivation disrupts pancreas development and in Ipf1-deficient mice promotes diabetes mellitus. Nat Genet. 30:430–435.

Kloosterman WP, Lagendijk AK, Ketting RF, et al. (2007) Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. PLoS Biol. 5:e203.

Kobayashi H, Spilde TL, Bhatia AM, et al. (2002) Retinoid signaling controls mouse pancreatic exocrine lineage selection through epithelial-mesenchymal interactions. Gastroenterology. 123:1331–1340.

Kramer B, Andrew A, Rawdon BB, et al. (1987) The effect of pancreatic mesenchyme on the differentiation of endocrine cells from gastric endoderm. Development. 100:661–671.

Krapp A, Knöfler M, Ledermann B, et al. (1998) The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes Dev. 12:3752–3763.

Lammert E, Cleaver O, Melton D. (2001) Induction of pancreatic differentiation by signals from blood vessels. Science. 294:564–567.

Le Bras S, Czernichow P, Scharfmann R. (1998a) A search for tyrosine kinase receptors expressed in the rat embryonic pancreas. Diabetologia. 41:1474–1481.

Le Bras S, Miralles F, Basmaciogullari A, et al. (1998b) Fibroblast growth factor 2 promotes pancreatic epithelial cell proliferation via functional fibroblast growth factor receptors during embryonic life. Diabetes. 47:1236–1242.

Lee CS, De León DD, Kaestner KH, et al. (2006) Regeneration of pancreatic islets after partial pancreatectomy in mice does not involve the reactivation of neurogenin-3. Diabetes. 55:269–272.

Lee KF, Simon H, Chen H, et al. (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature. 378:394–398.

Lee JC, Smith SB, Watada H, et al. (2001) Regulation of the pancreatic pro-endocrine gene neurogenin3. Diabetes. 50:928–936.

Lemaigre FP, Durviaux SM, Truong O, et al. (1996) Hepatocyte nuclear factor 6, a transcription factor that contains a novel type of homeodomain and a single cut domain. Proc Natl Acad Sci USA. 93:9460–9464.

Leonard J, Peers B, Johnson T, et al. (1993) Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells. Mol Endocrinol. 7:1275–1283.

Li H, Edlund H. (2001) Persistent expression of Hlxb9 in the pancreatic epithelium impairs pancreatic development. Dev Biol. 240:247–253.
Li Z, Manna P, Kobayashi H, et al. (2004) Multifaceted pancreatic mesenchymal control of epithelial lineage selection. Dev Biol. 269:252–263.
Lin JW, Biankin AV, Horb ME, et al. (2004) Differential requirement for ptf1a in endocrine and exocrine lineages of developing zebrafish pancreas. Dev Biol. 274:491–503.
Lorent K, Yeo SY, Oda T, et al. (2004) Inhibition of Jagged-mediated Notch signaling disrupts zebrafish biliary development and generates multi-organ defects compatible with an Alagille syndrome phenocopy. Development. 131:5753–5766.
Lynn FC, Skewes-Cox P, Kosaka Y, et al. (2007a) MicroRNA expression is required for pancreatic islet cell genesis in the mouse. Diabetes. 56:2938–2945.
Lynn FC, Smith SB, Wilson ME, et al. (2007b) Sox9 coordinates a transcriptional network in pancreatic progenitor cells. Proc Natl Acad Sci USA. 104:10500–10505.
MacFarlane WM, Read ML, Gilligan M, et al. (1994) Glucose modulates the binding activity of the β-cell transcription factor IUF1 in a phosphorylation-dependent manner. Biochem J. 303:625–631.
Maldonado TS, Kadison AS, Crisera CA, et al. (2000) Ontogeny of activin B and follistatin in developing embryonic mouse pancreas: implications for lineage selection. J Gastrointest Surg. 4:269–275.
Mamin A, Philippe J. (2007) Activin A decreases glucagon and arx gene expression in alpha-cell lines. Mol Endocrinol. 21:259–273.
Manfroid I, Delporte F, Baudhuin A, et al. (2007) Reciprocal endoderm-mesoderm interactions mediated by fgf24 and fgf10 govern pancreas development. Development. 134:4011–4021.
Manova K, De Leon V, Angeles M, et al. (1995) mRNAs for activin receptors II and IIB are expressed in mouse oocytes and in the epiblast of pregastrula and gastrula stage mouse embryos. Mech Dev. 49:3–11.
Marenah L, McCluskey JT, Abdel-Wahab YH, et al. (2006) A stable analogue of glucose-dependent insulinotropic polypeptide, GIP(LysPAL16), enhances functional differentiation of mouse embryonic stem cells into cells expressing islet-specific genes and hormones. Biol Chem. 387:941–947.
Marshak S, Totary H, Cerasi E, et al. (1996) Purification of the β-cell glucose-sensitive factor that transactivates the insulin gene differentially in normal and transformed islet cells. Proc Natl Acad Sci USA. 93:15057–15062.
Martín M, Gallego-Llamas J, Ribes V, et al. (2005) Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. Dev Biol. 284:399–411.
Mashima H, Ohnishi H, Wakabayashi K, et al. (1996a) Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. J Clin Invest. 97:1647–1654.
Mashima H, Shibata H, Mine T, et al. (1996b) Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. Endocrinology. 137:3969–3976.
Matsuoka TA, Artner I, Henderson E, et al. (2004) The MafA transcription factor appears to be responsible for tissue-specific expression of insulin. Proc Natl Acad Sci USA. 101:2930–2933.
McLin VA, Rankin SA, Zorn AM. (2007) Repression of Wnt/β-catenin signaling in the anterior endoderm is essential for liver and pancreas development. Development. 134:2207–2217.
Mellitzer G, Bonné S, Luco RF, et al. (2006) IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas. Embo J. 25:1344–1352.
Miettinen PJ, Huotari M, Koivisto T, et al. (2000) Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors. Development. 127:2617–2627.
Miller CP, McGehee RE Jr, Habener JF. (1994) IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. EMBO J. 13:1145–1156.
Miralles F, Battelino T, Czernichow P, et al. (1998a) TGF-β plays a key role in morphogenesis of the pancreatic islets of Langerhans by controlling the activity of the matrix metalloproteinase MMP-2. J Cell Biol. 143:827–836.

Miralles F, Czernichow P, Scharfmann R. (1998b) Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. Development. 125:1017–1024.

Miralles F, Lamotte L, Couton D, et al. (2006) Interplay between FGF10 and Notch signaling is required for the self-renewal of pancreatic progenitors. Int J Dev Biol. 50:17–26.

Molotkov A, Molotkova N, Duester G. (2005) Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. Dev Dyn. 232: 950–957.

Moriya N, Komazaki S, Asashima M. (2000a) In vitro organogenesis of pancreas in *Xenopus laevis* dorsal lips treated with retinoic acid. Dev Growth Differ. 42:175–185.

Moriya N, Komazaki S, Takahashi S, et al. (2000b) In vitro pancreas formation from *Xenopus* ectoderm treated with activin and retinoic acid. Dev Growth Differ. 42:593–602.

Murtaugh LC, Stanger BZ, Kwan KM, et al. (2003) Notch signaling controls multiple steps of pancreatic differentiation. Proc Natl Acad Sci USA. 100:14920–14925.

Naya FJ, Huang HP, Qiu Y, et al. (1997) Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev. 11:2323–2334.

Nelson SB, Schaffer AE, Sander M. (2007) The transcription factors Nkx6.1 and Nkx6.2 possess equivalent activities in promoting β-cell fate specification in Pdx1+ pancreatic progenitor cells. Development. 134:2491–2500.

Nikolova G, Jabs N, Konstantinova I, et al. (2006) The vascular basement membrane: a niche for insulin gene expression and beta cell proliferation. Dev Cell. 10:397–405.

Nishimura W, Kondo T, Salameh T, et al. (2006) A switch from MafB to MafA expression accompanies differentiation to pancreatic β-cells. Dev Biol. 293:526–539.

Norgaard GA, Jensen JN, Jensen J. (2003) FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development. Dev Biol. 264:323–338.

Offield MF, Jetton TL, Labosky PA, et al. (1996) PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development. 122:983–995.

Ohlsson H, Karlsson K, Edlund T. (1993) IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J. 12:4251–4259.

Ohnishi H, Ohgushi N, Tanaka S, et al. (1995) Conversion of amylase-secreting rat pancreatic AR42J cells to neuronlike cells by activin A. J Clin Invest. 95:2304–2314.

Olbrot M, Rud J, Moss LG, et al. (2002) Identification of β-cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. Proc Natl Acad Sci USA. 99:6737–6742.

Oster A, Jensen J, Edlund H, et al. (1998a) Homeobox gene product Nkx 6.1 immunoreactivity in nuclei of endocrine cells of rat and mouse stomach. J Histochem Cytochem. 46:717–721.

Oster A, Jensen J, Serup P, et al (1998b) Rat endocrine pancreatic development in relation to two homeobox gene products (Pdx-1 and Nkx 6.1). J Histochem Cytochem. 46:707–715.

Pedersen JK, Nelson SB, Jorgensen MC, et al. (2005) Endodermal expression of Nkx6 genes depends differentially on Pdx1. Dev Biol. 288:487–501.

Peers B, Sharma S, Johnson T, et al. (1995) The pancreatic islet factor STF-1 binds cooperatively with Pbx to a regulatory element in the somatostatin promoter: importance of the FPWMK motif and of the homeodomain. Mol Cell Biol. 15:7091–7097.

Petri A, Ahnfelt-Rønne J, Frederiksen KS, et al. (2006) The effect of neurogenin3 deficiency on pancreatic gene expression in embryonic mice. J Mol Endocrinol. 37:301–316.

Petrucco S, Wellauer PK, Hagenbüchle O. (1990) The DNA-binding activity of transcription factor PTF1 parallels the synthesis of pancreas-specific mRNAs during mouse development. Mol Cell Biol. 10:254–264.

Pictet RL, Clark WR, Williams RH, et al. (1972) An ultrastructural analysis of the developing embryonic pancreas. Dev Biol. 29:436–467.

Pictet R, Rutter W. (1972) Development of the embryonic endocrine pancreas. In: Steiner DF, Frenkel M eds. Handbook of Physiology. American Physiology Society; Washington DC:pp 25–66.

Poll AV, Pierreux CE, Lokmane L, et al. (2006) A vHNF1/TCF2-HNF6 cascade regulates the transcription factor network that controls generation of pancreatic precursor cells. Diabetes. 55:61–69.

Poy MN, Eliasson L, Krutzfeldt J, et al. (2004) A pancreatic islet-specific microRNA regulates insulin secretion. Nature. 432:226–230.

Prasadan K, Daume E, Preuett B, et al. (2002) Glucagon is required for early insulin-positive differentiation in the developing mouse pancreas. Diabetes. 51:3229–3236.

Raum JC, Gerrish K, Artner I, et al. (2006) FoxA2, Nkx2.2, and PDX-1 regulate islet β-cell-specific mafA expression through conserved sequences located between base pairs –8118 and –7750 upstream from the transcription start site. Mol Cell Biol. 26:5735–5743.

Ritz-Laser B, Mamin A, Brun T, et al. (2005) The zinc finger-containing transcription factor Gata-4 is expressed in the developing endocrine pancreas and activates glucagon gene expression. Mol Endocrinol. 19:759–770.

Roccisana J, Reddy V, Vasavada RC, et al. (2005) Targeted inactivation of hepatocyte growth factor receptor c-met in β-cells leads to defective insulin secretion and GLUT-2 downregulation without alteration of β-cell mass. Diabetes. 54:2090–2102.

Rose MI, Crisera CA, Colen KL, et al. (1999) Epithelio-mesenchymal interactions in the developing mouse pancreas: morphogenesis of the adult architecture. J Pediatr Surg. 34:774–780.

Roy S, Qiao T, Wolff C, et al. (2001) Hedgehog signaling pathway is essential for pancreas specification in the zebrafish embryo. Curr Biol. 11:1358–1363.

Samaras SE, Cissell MA, Gerrish K, et al. (2002) Conserved sequences in a tissue-specific regulatory region of the pdx-1 gene mediate transcription in pancreatic β cells: role for hepatocyte nuclear factor 3 β and Pax6. Mol Cell Biol. 22:4702–4713.

Sander M, Neubüser A, Kalamaras J, et al. (1997) Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. Genes Dev. 11:1662–1673.

Sander M, Sussel L, Conners J, et al. (2000) Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of β-cell formation in the pancreas. Development. 127:5533–5540.

Sanvito F, Herrera PL, Huarte J, et al. (1994) TGF-β 1 influences the relative development of the exocrine and endocrine pancreas in vitro. Development. 120:3451–3462.

Schwitzgebel VM, Scheel DW, Conners JR, et al. (2000) Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development. 127:3533–3542.

Sellick GS, Barker KT, Stolte-Dijkstra I, et al. (2004) Mutations in PTF1A cause pancreatic and cerebellar agenesis. Nat Genet. 36:1301–1305.

Seymour PA, Freude KK, Tran MN, et al. (2007) SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci USA. 104:1865–1870.

Shen CN, Marguerie A, Chien CY, et al. (2007) All-trans retinoic acid suppresses exocrine differentiation and branching morphogenesis in the embryonic pancreas. Differentiation. 75:62–74.

Shiozaki S, Tajima T, Zhang YQ, et al. (1999) Impaired differentiation of endocrine and exocrine cells of the pancreas in transgenic mouse expressing the truncated type II activin receptor. Biochim Biophys Acta. 1450:1–11.

Simeone DM, Zhang L, Treutelaar MK, et al. (2006) Islet hypertrophy following pancreatic disruption of Smad4 signaling. Am J Physiol Endocrinol Metab. 291:E1305–E1316.

Sjödin A, Dahl U, Semb H. (1995) Mouse R-cadherin: expression during the organogenesis of pancreas and gastrointestinal tract. Exp Cell Res. 221:413–425.

Slack JM. (1995) Developmental biology of the pancreas. Development. 121:1569–1580.

Smart NG, Apelqvist AA, Gu X, et al. (2006) Conditional expression of Smad7 in pancreatic β cells disrupts TGF-β signaling and induces reversible diabetes mellitus. PLoS Biol. 4:e39.

Smith SB, Ee HC, Conners JR, et al. (1999) Paired-homeodomain transcription factor PAX4 acts as a transcriptional repressor in early pancreatic development. Mol Cell Biol. 19:8272–8280.

Smith SB, Watada H, German MS. (2004) Neurogenin3 activates the islet differentiation program while repressing its own expression. Mol Endocrinol. 18:142–149.

Sonnenberg E, Meyer D, Weidner KM, et al. (1993) Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. J Cell Biol. 123:223–235.

Sosa-Pineda B, Chowdhury K, Torres M, et al. (1997) The Pax4 gene is essential for differentiation of insulin-producing β cells in the mammalian pancreas. Nature. 386:399–402.

Spagnoli FM, Brivanlou AH. (2006) The RNA-binding protein, Vg1RBP, is required for pancreatic fate specification. Dev Biol. 292:442–456.

Stafford D, Hornbruch A, Mueller PR, et al. (2004) A conserved role for retinoid signaling in vertebrate pancreas development. Dev Genes Evol. 214:432–441.

Stafford D, Prince VE. (2002) Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. Curr Biol. 12:1215–1220.

Stafford D, White RJ, Kinkel MD, et al. (2006) Retinoids signal directly to zebrafish endoderm to specify insulin-expressing β-cells. Development. 133:949–956.

Stein B, Andrew A. (1989) Differentiation of endocrine cells in chick allantoic epithelium combined with pancreatic mesenchyme. Cell Differ Dev. 26:173–180.

Stoffers DA, Heller RS, Miller CP, et al. (1999) Developmental expression of the homeodomain protein IDX-1 in mice transgenic for an IDX-1 promoter/lacZ transcriptional reporter. Endocrinology. 140:5374–5381.

Stoffers DA, Kieffer TJ, Hussain MA, et al. (2000) Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. Diabetes. 49:741–748.

Stoffers DA, Zinkin NT, Stanojevic V, et al. (1997) Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet. 15:106–110.

St-Onge L, Sosa-Pineda B, Chowdhury K, et al. (1997) Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. Nature. 387:406–409.

Sussel L, Kalamaras J, Hartigan-O'Connor DJ, et al. (1998) Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic β cells. Development. 125:2213–2221.

Suzuki A, Nakauchi H, Taniguchi H. (2003) Glucagon-like peptide 1 (1-37) converts intestinal epithelial cells into insulin-producing cells. Proc Natl Acad Sci USA. 100:5034–5039.

Swift GH, Liu Y, Rose SD, et al. (1998) An endocrine-exocrine switch in the activity of the pancreatic homeodomain protein PDX1 through formation of a trimeric complex with PBX1b and MRG1 (MEIS2). Mol Cell Biol. 18:5109–5120.

Thomas MK, Rastalsky N, Lee JH, et al. (2000) Hedgehog signaling regulation of insulin production by pancreatic β-cells. Diabetes. 49:2039–2047.

Thowfeequ S, Ralphs KL, Yu WY, et al. (2007) Betacellulin inhibits amylase and glucagon production and promotes β cell differentiation in mouse embryonic pancreas. Diabetologia. 50:1688–1697.

Tulachan SS, Doi R, Kawaguchi Y, et al. (2003) All-trans retinoic acid induces differentiation of ducts and endocrine cells by mesenchymal/epithelial interactions in embryonic pancreas. Diabetes. 52:76–84.

Tulachan SS, Doi R, Kawaguchi Y, et al. (2007) TGF-β isoform signaling regulates secondary transition and mesenchymal-induced endocrine development in the embryonic mouse pancreas. Dev Biol. 305:508–521.

Turque N, Plaza S, Radvanyi F, et al. (1994) Pax-QNR/Pax-6, a paired box- and homeobox-containing gene expressed in neurons, is also expressed in pancreatic endocrine cells. Mol Endocrinol. 8:929–938.

Van Velkinburgh JC, Samaras SE, Gerrish K, et al. (2005) Interactions between areas I and II direct pdx-1 expression specifically to islet cell types of the mature and developing pancreas. J Biol Chem. 280:38438–38444.

Verschueren K, Dewulf N, Goumans MJ, et al. (1995) Expression of type I and type IB receptors for activin in midgestation mouse embryos suggests distinct functions in organogenesis. Mech Dev. 52:109–123.

Vincent M, Guz Y, Rozenberg M, et al. (2003) Abrogation of protein convertase 2 activity results in delayed islet cell differentiation and maturation, increased alpha-cell proliferation, and islet neogenesis. Endocrinology. 144:4061–4069.

Vuguin PM, Kedees MH, Cui L, et al. (2006) Ablation of the glucagon receptor gene increases fetal lethality and produces alterations in islet development and maturation. Endocrinology. 147:3995–4006.

Wang Q, Elghazi L, Martin S, et al. (2008) Ghrelin is a novel target of Pax4 in endocrine progenitors of the pancreas and duodenum. Dev Dyn. 237:51–61.

Wang J, Elghazi L, Parker SE, et al. (2004) The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic β-cell differentiation. Dev Biol. 266:178–189.

Wang J, Kilic G, Aydin M, et al. (2005) Prox1 activity controls pancreas morphogenesis and participates in the production of "secondary transition" pancreatic endocrine cells. Dev Biol. 286:182–194.

Wang S, Zhang J, Zhao A, et al. (2007) Loss of Myt1 function partially compromises endocrine islet cell differentiation and pancreatic physiological function in the mouse. Mech Dev. 124:898–910.

Watada H, Scheel DW, Leung J, et al. (2003) Distinct gene expression programs function in progenitor and mature islet cells. J Biol Chem. 278:17130–17140.

Wiebe PO, Kormish JD, Roper VT, et al. (2007) Ptf1a binds to and activates area III, a highly conserved region of the Pdx1 promoter that mediates early pancreas-wide Pdx1 expression. Mol Cell Biol. 27:4093–4104.

Wilson ME, Kalamaras JA, German MS. (2002) Expression pattern of IAPP and prohormone convertase 1/3 reveals a distinctive set of endocrine cells in the embryonic pancreas. Mech Dev. 115:171–176.

Wilson ME, Yang KY, Kalousova A, et al. (2005) The HMG box transcription factor Sox4 contributes to the development of the endocrine pancreas. Diabetes. 54:3402–3409.

Wu KL, Gannon M, Peshavaria M, et al. (1997) Hepatocyte nuclear factor 3β is involved in pancreatic β-cell-specific transcription of the pdx-1 gene. Mol Cell Biol. 17:6002–6013.

Xu X, D'Hoker J, Stangé G, et al. (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell. 132:197–207.

Xu Y, Wang S, Zhang J, et al. (2006) The fringe molecules induce endocrine differentiation in embryonic endoderm by activating cMyt1/cMyt3. Dev Biol. 297:340–349.

Yamaoka T, Idehara C, Yano M, et al. (1998) Hypoplasia of pancreatic islets in transgenic mice expressing activin receptor mutants. J Clin Invest. 102:294–301.

Ye F, Duvillié B, Scharfmann R. (2005) Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells. Diabetologia. 48:277–281.

Yee NS, Lorent K, Pack M. (2005) Exocrine pancreas development in zebrafish. Dev Biol. 284:84–101.

Yew KH, Hembree M, Prasadan K, et al. (2005) Cross-talk between bone morphogenetic protein and transforming growth factor-β signaling is essential for exendin-4-induced insulin-positive differentiation of AR42J cells. J Biol Chem. 280:32209–32217.

Yew KH, Prasadan KL, Preuett BL, et al. (2004) Interplay of glucagon-like peptide-1 and transforming growth factor-β signaling in insulin-positive differentiation of AR42J cells. Diabetes. 53:2824–2835.

Yoshitomi H, Zaret KS. (2004) Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development. 131:807–817.

Zecchin E, Filippi A, Biemar F, et al. (2007) Distinct delta and jagged genes control sequential segregation of pancreatic cell types from precursor pools in zebrafish. Dev Biol. 301: 192–204.

Zhang YQ, Kanzaki M, Furukawa M, et al. (1999) Involvement of Smad proteins in the differentiation of pancreatic AR42J cells induced by activin A. Diabetologia. 42: 719–727.

Zhang YQ, Mashima H, Kojima I. (2001) Changes in the expression of transcription factors in pancreatic AR42J cells during differentiation into insulin-producing cells. Diabetes. 50 (Suppl 1):S10-S14.

Zhou J, Wang X, Pineyro MA, et al. (1999) Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. Diabetes. 48:2358–2366.

# Chapter 2
# Islet and Pancreas Transplantation

**Davide Mineo, Gaetano Ciancio, George W. Burke, Rodolfo Alejandro, and Camillo Ricordi**

**Abstract** Islet allotransplantation for patients with brittle type 1 diabetes mellitus (T1DM) is a minimally invasive and relatively safe procedure that can induce sustained, normalized glucose control and restore C-peptide secretion, with reduction of hypoglycemic episodes, stabilization or delay of chronic complications, and better quality of life. Current immunosuppressive protocols have significantly improved short-term outcomes, whereas long-term results are still inadequate (from 80% to 10% insulin-independence from 1 to 5 years post-transplant). Principal limitations include: imperfections in the islet isolation process, auto- and alloimmunity, allosensitization, immunosuppression-related toxicity, and unsuitability of the intrahepatic implantation site. More efficient isolation methods, safer and more efficient immunosuppressive agents in tolerogenic strategies, and alternative transplant site(s) may resolve these limitations in the near future. Simultaneous pancreas–kidney (SPK) transplantation is the optimal treatment for patients with T1DM with end-stage renal disease. Restoration of normoglycemia after pancreas transplant, as well as of renal function after kidney transplant, results in significant improvement of neuropathy, retinopathy, and nephropathy. Novel immunosuppressive therapies, improvements in surgical techniques, and better understanding of postoperative recipient care have improved results of SPK transplants consistently over the past decade. Future directions include optimization of immunosuppression, allowing freedom from insulin injection therapy while maintaining normoglycemia, and avoidance of chronic transplant glomerulopathy, with durable normalization of kidney function, thus improving quality of life as well as extending patient survival.

## 2.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is a cell-specific autoimmune disease triggered by environmental factors (e.g., viral infections, toxins, diet nutrients or antigens) in genetically predisposed individuals [e.g., human leukocyte antigen (HLA)

D. Mineo (✉)
Diabetes Research Institute, University of Miami, Miami, FL, USA
e-mail: dmineo@med.miami.edu

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_2,

class II DR/DQ, insulin-VNTR, and CTLA4 genes], primarily children and young adults. This chronic process leads to selective destruction of the insulin-producing β cells within the pancreatic islets. The resultant complete deficit of insulin, the main hormone regulating glucose as well as lipid and protein metabolism, causes hyperglycemia, which leads to acute (ketoacidosis) and chronic (retinopathy, nephropathy, neuropathy) complications, hypercoagulability, dyslipidemia, and accelerated atherosclerosis, with poorer quality of life, increased cardiovascular disease, and reduced life expectancy (The Diabetes Control and Complications Trial Research Group 1993; Zimmet et al., 2001; Leroith et al., 2003).

T1DM represents 5–10% of all cases of DM. It is estimated that in 2010 the worldwide prevalence of T1DM will be 0.1–0.5% of the general population, more than 6 million patients (1 out of 100–300 newborns), and its incidence will be 30–50 new patients per each 100,000 individuals, with a 3% increase yearly, mainly in developing nations acquiring a western lifestyle and diet. In addition to racial and regional differences involving the genetic background and environmental triggers, possible reasons for such an increase in T1DM frequency are the rise in childhood obesity and increasing sedentary lifestyle, which cause metabolic stress by development of insulin resistance and inflammatory injury to β cells with functional exhaustion, thus accelerating the onset and progression of the disease (accelerator theory) (The Diabetes Control and Complications Trial Research Group, 1993; Zimmet et al., 2001; Leroith et al., 2003; Yoon and Jun, 2005; Daneman, 2006; Wilkin, 2008).

T1DM-related micro- and macro-vasculopathy are the main causes of blindness, chronic end-stage renal disease requiring dialysis, and peripheral limb amputations and deformities, together with associated disabilities, comorbidities, and death. Their impact involves some 10% of total health-care expense in Western countries, with over $100 billion spent every year in United States and over $200 billion worldwide. Daily exogenous insulin is the treatment of choice in association with tailored diet and physical exercise programs. Novel insulin formulations (e.g., glargine and lispro analogues) together with infusion-pump and glucose-sensor technologies have significantly improved metabolic control, with lower rates of side effects, prevention or reduction of chronic complications, and better quality of life (The Diabetes Control and Complications Trial Research Group 1993; Zimmet et al., 2001; Leroith et al., 2003; Daneman, 2006).

## 2.2 Pancreatic Islet Allotransplantation

Intensive insulin treatment in T1DM has been associated with increasing severe hypoglycemic episodes, which can associate with cardiovascular accidents and deterioration of glucose control. Up to 10–20% of long-standing T1DM patients cannot achieve stable metabolic control or avoid life-threatening hypoglycemia and progressive complications, owing primarily to diabetic neuropathy with hypoglycemia unawareness and a concomitant alteration of the contraregulatory mechanisms.

Attempting tight glycemic control is of major importance in view of the high mortality rate among such subjects while they wait for over 4 years for a pancreas transplant. In this subgroup of T1DM patients, β-cell replacement therapy by allogeneic pancreatic islet transplantation (IT) might be an attractive, less invasive, and safer option than pancreas transplantation. Despite the fact that it improves glucose control, chronic complications, and quality of life, and provides longer graft survival and function, pancreas transplantation has an higher risk of perioperative morbidity and mortality (The Diabetes Control and Complications Trial Research Group 1993, 1997; Zimmet et al., 2001; Gruessner et al., 2004; Gruessner and Sutherland, 2005; Ryan et al., 2006; Lipshultz and Wilkinson, 2007; Gerstein et al., 2008; McCrimmon, 2008).

The pancreatic islets of Langerhans, which contain the insulin-producing β cells, are functionally complex endocrine structures that detect minimal changes in blood levels of glucose and other metabolites and maintain metabolic homeostasis through a fine real-time secretion of specific hormones. IT is an alternative therapy that can restore physiological glucose sensing and insulin delivery in patients with unstable T1DM (Cabrera et al., 2006; Leibiger and Berggren, 2008).

Clinical indications for IT include T1DM patients with basal or stimulated C-peptide of less than 0.3 ng/ml and imminent or current end-stage renal disease who will receive a kidney transplant, namely simultaneous islet–kidney (SIK) transplants from the same donor, or who already had a kidney transplant and will receive an islet-after-kidney (IAK) transplant from a different donor. IT alone (ITA) is a valid option for T1DM patients with normal or minimally altered renal function and frequent acute and severe metabolic complications requiring urgent medical care (e.g., life-threatening hypoglycemic episodes, severe hyperglycemia, or recurrent ketoacidosis); and/or with incapacitating physical and emotional problems with insulin therapy; and/or with failure of insulin management to prevent chronic complications (Ryan et al., 2006; Marzorati et al., 2007).

The main goal of IT is to achieve stable, normalized glycemic control and absence of severe hypoglycemic episodes, thus improving quality of life, preventing long-term diabetic complications, and reducing procedure- and immunosuppression-related side effects. Insulin independence, although desirable, is not necessarily the primary goal of IT, although a significant reduction in insulin requirements and the restoration of C-peptide secretion are desirable and have some beneficial effects (Ryan et al., 2006; Leitao et al., 2008a).

## *2.2.1 Islet Transplantation Procedures*

### 2.2.1.1 Recipient and Donor Selection

Selection of IT recipients is based primarily on the following criteria: patients who have had T1DM for at least 5 years, are 18–65 years of age, with a body mass index (BMI) less than 26 kg/m$^2$, and have one or more of the following conditions: severe, incapacitating hypoglycemic episodes with lack of awareness (based on Clarke or

Hypo scores); poor, labile glucose control [according to mean amplitude glucose excursion (MAGE) or lability index], with hemoglobin-A1c (HbA1c) greater than 8.0% despite intensive insulin therapy and care; and progressive diabetic complications. Exclusion criteria include: nephropathy [creatinine > 1.6 mg/dl, estimated glomerular filtration rate (eGFR) < 80 ml/min, and albuminuria >300 mg/24 h], unstable diabetic retinopathy or neuropathy, or any condition limiting islet engraftment and survival or immunosuppression (e.g., hepatitis) (Ryan et al., 2004, 2006; Marzorati et al., 2007).

Criteria for selection of multiorgan, brain-deceased, and heart-beating donors include: subjects of 25–45 years of age, with BMI greater than 25 kg/m$^2$, and negative record or evidence of DM or other severe or chronic illness, transmissible infective agent or disease, under toxic substance or drug abuse. Several donor characteristics may positively influence the outcomes of the isolation process and the islet yield: age 16–40, BMI > 27, male gender; traumatic death; normoglycemia while hospitalized; use of steroids and vasopressors, especially pitressin, with hemodynamic stability; shorter duration of cardiac arrest and hypotension; and a larger organ size with surface integrity and no edema (Lakey et al., 1996; Nano et al., 2005; Ryan et al., 2006; Marzorati et al., 2007; Ponte et al., 2007; Hanley et al., 2008).

Donor–recipient ABO compatibility is required, together with negative lymphocyte cross-match and panel reactive antibody (PRA) of less than 20%. In SIK, HLA-matching is quite strict in order to guarantee kidney graft survival, whereas in ITA and IAK histocompatibility is not required. This strategy, although limiting the recurrence of autoimmunity, which relies on intrinsic β-cell antigenicity, increases the risk of HLA-dependent allorejection (Roep et al., 1999; Bosi et al., 2001).

#### 2.2.1.2 Pancreas Procurement, Islet Isolation, and Transplantation

The pancreatic islets of Langerhans are tight mixed clusters of different endocrine cells scattered throughout the pancreas, each type secreting a specific hormone: α cells (glucagon), β cells (insulin and amylin), δ cells (somatostatin), and PP cells (pancreatic polypeptide). It is estimated that the number of islets in a normal human pancreas is about 1 million, but significant variations can occur depending on donor age, sex, or weight and organ size and integrity (Ricordi, 1992; Leroith et al., 2003; Cabrera et al., 2006; Leibiger and Berggren, 2008).

The islet isolation process is designed to obtain an adequate yield of integral and functional islets from donor pancreata. Pancreas procurement and preservation are key steps for a successful isolation, requiring a short (<10 min) warm ischemia time (interval between uncontrolled non-heart-beating up to resumption of heart activity), organ recovery by an expert surgical team (preferably from the same IT program), pancreas storage in standard iced-chilled preservation solution, and short (<12 h) cold ischemia time (interval between pancreas harvesting and the islet isolation) (Ricordi, 1992; Lakey et al., 1996; Lee et al., 2004; Ponte et al., 2007; Porrett et al., 2007; Hanley et al., 2008).

Despite an increase in organ donations, rates of pancreas recovery remain unsatisfactory and much lower than those of other solid organs. Indeed, from more than

8000 multiorgan donors available in the United States in 2006, only 2000 pancreata were recovered, and only 1440 were used for transplantation, with the remaining not being retrieved because of poor organ and donor quality (63%, mainly owing to altered exocrine and/or endocrine function), placement-related issues and time constraints (9%), or other undefined causes (28%). Furthermore, IT centers receive a pancreas only after it has not been accepted for whole organ transplantation at the local, regional, or national level, often when the cold ischemia time has exceeded the ideal. A recent pancreas allocation scheme attempts to minimize this time, placing organs from donors over 50 years or BMI of more than 30 kg/m$^2$ directly for IT, but it may include older subjects with reduced islet function and mass or borderline diabetics with higher islet mass but lower insulin secretion capacity. A poor utilization of potential "islet donor pancreata" has also been reported. In fact, in the United States in the period 2000–2004, from the overall pool of pancreata available, only 22.3% were used for whole organ transplantation ("optimal" glands); of the remaining ones, 48.5% were considered "suitable islet donors" (11% "optimal" and 89% "standard"), but only 2.1% of them (only 8.7% of the "optimal" donors) were used for IT. There is a wide margin for improvement in pancreas allocation and utilization, including the use of "optimal" donors and a fair noncompetitive organ distribution between IT and pancreas transplantation programs, which might fulfill the demand of the small T1DM population requiring β-cell replacement (Lakey et al., 1996; Deng et al., 2004; Ihm et al., 2006; Porrett et al., 2007; Stegall et al., 2007; Hanley et al., 2008).

A semiautomated method of mechanically enhanced enzymatic pancreas dissociation in a digestion-dissociation chamber (Ricordi chamber), with different blends of lytic enzymes (e.g., collagenases and proteases), is used to release the islets from the surrounding interstitial-connective and exocrine tissues. A semiautomated purification technique in a computerized centrifuge system (COBE 2991), with various density gradient solutions (e.g., glucose-based), is performed thereafter to separate the endocrine from the exocrine cells (Fig. 2.1). Finally, a small volume (<5 ml) of highly purified islet product is recovered and undergoes a 2-day culture for cell recovery from the traumatic isolation process. The cell culture medium is enriched with trophic and antioxidant substances (e.g., insulin, nicotinamide, L-glutathione) to prevent oxidative stress and apoptosis, preserving β-cell function and survival (Ricordi et al., 1988; Ricordi, 1992; Ichii et al., 2006).

This interval also allows for assessment of islet survival, content, and function prior to transplantation, thus determining product clinical suitability by FDA-approved tests. Islet counting is performed at optical microscope from final product samples using diphenylthiocarbazone (DTZ) staining (selectively binding to zinc–insulin granules with red coloring). The islet mass is calculated using an algorithm whereby islets are scored according to their diameter and counted as the number of islet equivalents (IEQ) based on a standard islet size of 150 μm. Product purity is evaluated as a percentage of DTZ-stained endocrine cells compared to unstained exocrine cells. Islet viability is assessed by fluorescent inclusion–exclusion dyes selectively binding to viable or necrotic cells. Islet function is determined in vitro by measuring glucose-mediated insulin release in static incubation

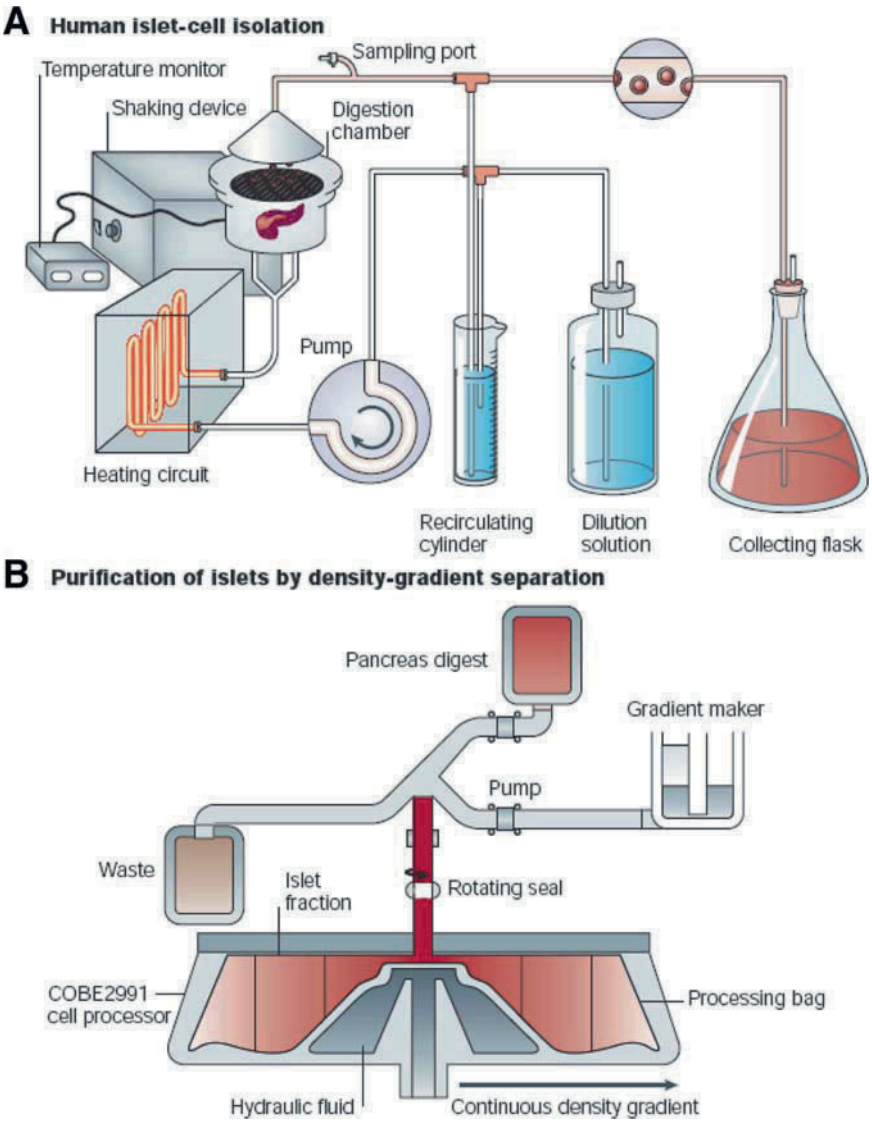



**Fig. 2.1 Schematic representation of the human islet isolation (a) and purification (b) process.** Reproduced with permission from Ricordi and Strom, 2004

(low- then high-glucose challenge) and expressed as a stimulation index (SI, ratio of stimulated-to-basal insulin release). A decision for transplant is made when sufficient islets are recovered (minimum 350,000 IEQ, or 5000 IEQ/kg of recipient body weight) and specific product release criteria are met: endocrine tissue > 30%; islet viability > 70%; SI value > 1; negative Gram stain; endotoxins levels < 5 EU/kg (Ricordi, 1992).

Despite significant progress, even in the most experienced centers fewer than 50% of the pancreata processed with the intent to transplant provide a sufficient number of islets; moreover, more than 50% of the pancreatic islet content is lost in the process, as a result of donor brain-death related events, suboptimal organ preservation, deficient isolation process, and inadequate β-cell cytoprotection. Overall, these conditions limit the chances of a satisfactory islet yield from a single pancreas, so that frequently more than one donor is required to collect the number of islets needed to normalize glucose control or achieve insulin independence (Nano et al., 2005; Pileggi et al., 2006, Ponte et al., 2007).

IT occurs via microembolization into the hepatic portal venous system, with islet entrapping in the peripheral branches, at the presinusoid level due to size restriction, followed by their engraftment and revascularization from the hepatic vasculature, with immediate function and sustained survival. The transplant is performed by gravity infusion from a closed-bag system containing the heparinized islet product in the main portal vein through percutaneous transhepatic catheterization, under fluoroscopic and ultrasound guidance, using local anesthesia and conscious sedation, with close monitoring of portal pressure. This minimally invasive interventional radiologic procedure lasts approximately 1 h and patients are discharged from the hospital within 24–48 h, once clinically stable and if no complications arise. In SIK, or if there are contraindications to this approach (e.g., risk of hemorrhage, anatomical anomalies), cannulation of a tributary of the portal vein, such as the mesenteric or umbilical vein, is performed by laparotomy or laparoscopy (Baidal et al., 2003; Pileggi et al., 2006; Goss et al., 2003).

## *2.2.2 Clinical Protocols*

### 2.2.2.1 Historical Protocols

Following the first case of a functioning allogeneic IT reported in 1980, several trials in patients with T1DM were performed in late 1980s, mainly as SIK and IAK or in combination with other solid organ transplants. Variable numbers of pancreatic islets, purified from cadaver single-donors, were injected into the liver during the main organ transplant or through a transient intraportal catheter as a post-transplant percutaneous procedure. The immunosuppressive regimens were those traditionally used in solid organ transplants, combining corticosteroids (prednisolone or methylprednisolone), purine antagonist azathioprine or calcineurin inhibitor (CNI) cyclosporine A, with lymphodepleting polyclonal antibodies added at induction in a few trials [e.g., diverse animal-derived antithymocyte globulin (ATG)] (Largiadr et al., 1980; Mintz et al., 1988).

The first promising results in IT were reported in the context of multiorgan transplants in the early 1990s using the new CNI tacrolimus, with greater immunosuppressive effect and fewer side effects than cyclosporine A, as a maintenance drug. Later on, mycophenolate mofetil (MMF), a prodrug of mycophenolate acid (MPA), a purine synthesis inhibitor, was launched as a maintenance drug with equal

immunosuppressive efficacy but lower nephrotoxicity than CNI. At the same time, more efficient induction strategies were tested, and the two monoclonal antibodies daclizumab and basiliximab, targeting the IL2 receptor/CD25 on T-lymphocytes with functional and proliferative inhibition, were used with significant reduction of acute rejection episodes. In contrast, muromonab-OKT3, targeting the T-cell surface marker CD3 with profound lymphodepletion, was tested but soon abandoned owing to severe cytokine release. In a few trials, bone marrow cells (BMCs) or hematopoietic stem cells (HSCs) from the same single-donors were coinfused, using lymphodepleting nonmyeloablative conditioning, in the attempt to induce recipient hematopoietic chimerism and islet graft tolerance, but islet graft survival was not maintained after discontinuation of immunosuppressive drugs (Tzakis et al., 1990; Ricordi et al., 1992; Gores et al., 1993; Alejandro et al., 1997; Secchi et al., 1997; Oberholzer et al., 2000; Pileggi et al., 2004).

The overall results of this first decade of IT trials were encouraging but not satisfactory, and limited islet graft survival, high rates of primary nonfunction, only transient insulin independence, and relevant immunosuppressive side effects were often observed. Indeed, post-transplant reduction of insulin requirements and improvement in glycemic control rarely lasted long term, with only 10% of islet recipients maintaining insulin independence at 1 year (Bretzel et al., 1999).

A main obstacle in achieving consistent positive results was the diabetogenic effect of corticosteroids and CNIs on β-cell function and survival, as well as on the development of peripheral insulin resistance. Post-transplantation DM occurs in more than 50% of solid organ transplant recipients, including pancreas, with incidence increasing with dose and duration of immunosuppressive therapy. Moreover, drug-dependent increment of lipids is associated with increased allograft loss and toxicity. Glucolipotoxicity may cause β-cell dysfunction and loss (Subramanian and Trence, 2007; Vantyghem et al., 2007; Poitout and Robertson, 2008).

Corticosteroids (dose > 5 mg/day) can induce hyperglycemia by decreasing insulin-mediated glucose uptake in peripheral tissues, with insulin resistance, and by inhibiting insulin production and secretion, with β-cell dysfunction and possibly apoptosis. Increased hepatic gluconeogenesis, reduced glycogen synthesis, and lipolysis also occur. Hyperlipidemia is due to increased VLDL synthesis and downregulation of LDL receptor and lipoprotein lipase activity, resulting in increased LDL cholesterol and triglycerides and reduced HDL cholesterol. Both metabolic alterations may result in overall increased cardiovascular risk after transplant (Poitout and Robertson, 2008).

CNIs frequently cause hyperglycemia and hyperlipidemia. High-dose tacrolimus (trough levels > 6 ng/ml) is more diabetogenic but less deleterious for lipids than cyclosporine A (trough levels > 300 ng/ml). Hyperglycemia is consequent to decreased insulin synthesis and secretion. Morphological anomalies are present, including reduced β-cell density, loss of secretory granules, cytoplasmatic swelling and vacuolization, and possibly apoptosis. Such alterations seem to be dose-dependent and reversible by drug discontinuation, with no chronic cumulative toxicity on β cells. Effects on insulin sensitivity are still being debated, with some animal and clinical studies reporting increased hyperinsulinemia and insulin

resistance. Dyslipidemia, with increased LDL cholesterol and impaired VLDL and LDL clearance, also occurs. Increased LDL oxidation and lipoprotein levels with accelerated atherosclerosis, as well as increased vascular tone and resistance with hypertension, contribute to a greater cardiovascular risk (Vantyghem et al., 2007).

#### 2.2.2.2 Current Protocols

In late 1990s, new immunosuppressants, such as mTOR inhibitors, sirolimus and everolimus, and novel anti-inflammatory agents, such as TNFα blockers infliximab (chimeric monoclonal antibody) and etanercept (recombinant fusion protein), allowed avoidance of corticosteroids and reduction of tacrolimus dosage in specifically designed ITA protocols (Table 2.1) (Mineo et al., 2008c).

In 2000, the Edmonton group reported remarkable results from a steroid-free protocol including daclizumab at induction and high-dose sirolimus (trough levels 12–15 ng/ml during the first trimester and then 10–12 ng/ml) plus low-dose tacrolimus (trough levels 3–6 ng/ml) at maintenance. After 1 year, virtually all recipients were insulin-free, with normalized HbA1c and absence of severe hypoglycemia. Insulin independence was obtained infusing more than 10,000 IEQ/kg or more than 700,000 IEQ total (full islet mass), from two or more fresh islet transplant infusions (Shapiro et al., 2000; Ryan et al., 2002).

Subsequently, the Miami group successfully introduced a 2-day culture stage in supplemented medium prior to transplant, to allow β-cell recovery from the isolation process, thus increasing islet viability while preserving islet mass. This time period permits the administration of induction strategies that can prevent acute rejection episodes and improve long-term outcomes. It also allows the shipment of islet products to remote facilities for transplantation. The same group also attempted to achieve recipient hematopoietic chimerism and islet graft tolerance infusing HSCs from the same single-donor, without any conditioning, but neither recipient chimerism nor islet graft function persisted after discontinuation of immunosuppression 1 year after transplantation (Froud et al., 2005; Mineo et al., 2008a).

Later on, the Minneapolis group showed that a more potent lymphodepletion at induction, using rabbit ATG (rATG) or a modified humanized OKT3 (hOKT3γ1 ala-ala), together with an IT-specific anti-inflammatory strategy using etanercept, achieved insulin independence from a single-donor with less than 10,000 IEQ/kg (marginal islet mass). Sirolimus and low-dose tacrolimus or MMF were used at maintenance (Hering et al., 2004, 2005).

Since the year 2000 many groups have adopted similar immunosuppressive strategies in IT, for a total of more than 700 transplants in about 400 recipients at some 50 centers worldwide, according to data from the Clinical Islet Transplant Registry (CITR), with comparable results in terms of prolonged improvement of glucose metabolism and rate of insulin independence at 1 year, steady at about 70–80% among the most experienced groups (Shapiro et al., 2006, Alejandro et al., 2008).

**Table 2.1** Main clinical islet allotransplantation trials after the year 2000 (adapted from Marzorati et al., 2007)[a]

| Author | Transplant | T1DM | Number of Pts | IEQ/kg | Induction | Mantainance | Graft function | Graft duration (C-pept) |
|---|---|---|---|---|---|---|---|---|
| Shapiro et al., 2000 | ITA | Yes | 7 | 11,547 | Dac | Sir, Tac | 100% ins ind | >12 months 67% |
| Hirshberg et al., 2003 | ITA | Yes | 6 | > 10,000 | Dac | Sir, Tac | 50% ins ind | >22 months 83% |
| Hering et al., 2004 | ITA | Yes | 6 | > 10,300 | OKT3γ1(Ala-Ala) | Sir, Tac | 67% ins ind | >12 months 83% |
| Frank et al. 2004 | ITA | Yes | 9 | 15,475 | Dac | Sir, Tac | 100% ins ind | >26 months 57% |
| | IAK | | 4 | | | | | >26 months 20% |
| Goss et al., 2004 | ITA | Yes | 10 | > 10,000 | Dac | Sir, Tac | 50% ins ind | >18 months 90% |
| Lehmann et al., 2004 | SIK | Yes | 9 | 16,172 | Dac | Sir, Tac | 84% ins ind | >12 months 89% |
| Hering et al., 2005 | ITA | Yes | 8 | 7,271 | ATG, Dac, Eta | Sir, Tac later MMF | 100% ins ind | >12 months 62% |
| Froud et al., 2005 | ITA | Yes | 16 | 13,552 | Dac, Inf | Sir, Tac | 100% ins ind | >26 months 80% |
| Kempf et al., 2005 | ITA, SIK, IAK | Yes | 22 | > 10,000 | Dac; Bas | Sir,Tac; Eve, CyA | 83% ins ind | >12 months 100% |
| Ryan et al., 2005 | ITA | Yes | 65 | 11,910 | Dac, Inf (10); Alem (9) | Sir, Tac | 100% ins ind | >60 months 80% |
| Warnock et al., 2005 | ITA | Yes | 10 | 13,806 | ATG, then Dac | Sir, Tac, or MMF (2) | 100% ins ind | 6–21 months 100% |
| Toso et al., 2006 | IAK | Yes | 8 | 12,530 | Dac | Sir, Tac | 71% ins ind | >12 months |
| O'Connell et al., 2006 | ITA | Yes | 6 | 17,958 | Dac | Sir, Tac | 50% ins ind | >18 months 83% |
| Shapiro et al., 2006 | ITA | Yes | 23 | 13,473 | Dac | Sir, Tac | 58% ins ind | >24 months 70% |
| Ghofaili et al., 2007 | ITA | Yes | 11 | 14,312 | Dac | Tac, MMFor Sir (1); Exen | 73% ins ind | 4–30 months 100% |
| Badet et al., 2007 | ITA | Yes | 10 | 11,089 | Dac | Sir, Tac | 80% ins ind | >24 months 80% |
| Maffi et al., 2007 | ITA | Yes | 19 | 11,477 | Dac | Sir, Tac or MMF (6), CyA (1) | 65% ins ind | >24 moths 33% |
| Gillard et al., 2008 | ITA | Yes | 5 | 4,700 | ATG | Sir | Reduced ins req | >30 months 40% |
| | | | 5 | 6,400 | | Sir, Tac | 60% ins ind | >24 months 60% |
| Gerber et al., 2008 | SIK | Yes | 13 | 345,000 (tot) | Dac | Sir, Tac | 31% ins ind | >48 months 40% |

**Table 2.1** (continued)

| Author | Transplant | T1DM | Number of Pts | IEQ/kg | Induction | Mantainance | Graft function | Graft duration (C-pept) |
|---|---|---|---|---|---|---|---|---|
| Cure et al., 2008c | IAK | Yes | 7 | 14,779 | Dac, Inf or Eta | Sir, Tac or MMF (2); Aza (1); CyA (2); Pdn (3) | 30% ins ind | >36 months 86% |
| Gangemi et al., 2008 | ITA | Yes | 4 | 24,385 | Dac | Sir, Tac | 100% ins ind | >30 months 50% |
| | | | 6 | 11,483 | Dac, Eta | Sir, Tac; Exen# | | >21 months 80% |
| Mineo et al. 2008 | IT+HSC | Yes | 6 | 8,611 | Dac, Inf | Sir, Tac | Reduced ins req | >15 months 67% |
| Tan et al., 2008 | IAK | Yes | 7 | 11,820 | Alem | Sir, Tac | 60% ins ind | >24 months |
| Bellin et al., 2008 | ITA | Yes | 6 | 11,872 | ATG, Eta | CyA and Eve later MMF | 70% ins ind | >36 months |
| Froud et al. 2008a | ITA | Yes | 3 | 450,000 (tot) | Alem, Eta | Sir and Tac or MMF | 100% ins ind (2) | >24 months 100% |

[a]IEQ/kg = islet equivalent per kilogram; ins ind = insulin independence; ins req = insulin requirement; MMF = mycophenolate mofetil; Pts = Patients; T1DM = type 1 diabetes mellitus; tot = total IEQ. Transplant**:** IAK = islet after kidney; IT + HSC = hematopoietic stem cells-islet transplant; ITA = islet transplantation alone; SIK = simultaneous islet and kidney transplantation. Induction**:** Alem = alemtuzumab; ATG = antithymocyte globulin; Bas = basiliximab; Dac = daclizumab; Eta = etanercept; FATG = fresenius antithymocyte globuline; Inf = infliximab; OKT3$\gamma$1(Ala-Ala) = humanized anti-CD3 monoclonal antibody. Mantainance**:** Aza = azathioprine; CyA = cyclosporine A; Eve = everolimus; Exen = exenatide; MMF = mycophenolate mofetil; Pdn = prednisolone; Sir = sirolimus; Tac = tacrolimus.The number of recipents included for a specific variable is shown in parentesis.

New immunosuppressive or immunomodulatory drugs are being tested to reduce side effects while attempting to use single-donor islet infusion, in order to avoid recipient allosensitization and overcome organ shortage. Preliminary promising results show significant improvements in short-term islet function and survival. The groups in Miami and Edmonton are using alemtuzumab, an anti-CD52 lymphodepleting monoclonal antibody, as an induction agent, while including MMF at maintenance, rather than tacrolimus or sirolimus. Similarly, the group in San Francisco is using rATG and etanercept at induction, with sirolimus plus efalizumab, an anti-LFA1/CD11a leukocyte antiadhesion monoclonal antibody, for maintenance (Froud et al. 2008; Posselt et al., 2008a; Shapiro et al., 2008).

Recently, in order to improve islet function, and possibly survival, as well as to prevent long-term graft exhaustion, the glucagon-like peptide-1 (GLP1) synthetic analogue exenatide, administered subcutaneously at meals, has been given from the time of the first islet infusion (University of Illinois group) or after islet graft dysfunction (Miami and Vancouver groups). The Miami group also reported patients receiving islet retransplants who had been under chronic exenatide treatment prior to the supplemental infusion. Overall, exenatide therapy seems to improve islet engraftment as well as islet graft function and survival, normalizing glucose control and favoring insulin independence (Ghofaili et al., 2007; Faradji et al., 2008; Froud et al., 2008; Gangemi et al., 2008; Faradji et al., 2009).

The Edmonton protocol has also been tested in several IAK and SIK trials. In both settings, resulting rates of insulin independence were not always comparable with ITA, varying from 30 to 70% at 1-year post-transplant, but similar stable, normalized glucose control and sustained C-peptide secretion were achieved, also significantly improving function and longevity of kidney grafts without either increasing the risk of kidney rejection or inducing premature decline in its function. Recently, a report from one SIK trial showed successful islet engraftment and function using alemtuzumab and an Edmonton regimen, with 60% insulin independence at 1 year and 100% kidney graft survival for more than 2 years (Toso et al., 2006; Cure et al., 2008a; Gerber et al., 2008; Tan et al., 2008).

## *2.2.3 Results*

### 2.2.3.1 Clinical Outcomes

Long-term results from different groups have shown that the rate of insulin independence using the Edmonton protocol declines post-transplant to 50% at 2 years, 30% at 3 years, and 10% at 5 years, although 70–80% of recipients have detectable C-peptide (>0.5 ng/ml), with 50–60% reduction in insulin requirement and normalized HbA1c (<6.5%). This progressive islet allograft loss and exhaustion seem mainly due to auto- and allorejection, immunosuppressant-related islet graft toxicity and implantation-site related unsuitability. Recently, the group in Minneapolis reported the achievement of 60% insulin independence for more than 3 years post-transplant, using rATG and etanercept as induction together with mTOR inhibitors plus CNIs (later changed for MMF) at maintenance (Ryan et al., 2005; Bellin et al., 2008).

Significant metabolic improvements are achieved and maintained after IT, even with only partial islet graft function, including stability of glucose control with normalized HbA1c and corrected substrata metabolism, amelioration of insulin sensitivity with reduced insulin requirements, absence of severe hypoglycemia with restored awareness, and improved quality of life. In particular, both the first-phase insulin secretion after an intravenous glucose tolerance test (IVGTT) and the area-under-the-curve (AUC) of C-peptide secretion after an oral mixed-meal tolerance test (MMTT) appear to be restored, with normalization of glucose levels and reduction of glucose excursion at the subcutaneous glucose monitoring system (CGMS) (Figs. 2.2 and 2.3). Notably, glucagon response to hypo- and hyperglycemia appears partially restored, with recovery of sympathoadrenal response and reduced hepatic glucose output, respectively, thus contributing to improved metabolic control after transplant (Luzi et al., 2001; Paty et al., 2002; Rickels et al., 2005a; Meier et al., 2006; Poggioli et al., 2006; Rickels et al., 2006a; Rickels et al., 2007; Gorn et al., 2008; Leitao et al., 2008b; Poggioli et al., 2008; Tharavanij et al., 2008).

Beneficial effects of IT are also evident for long-term diabetic complications, with stabilization or reduced progression of retinopathy and even improvement of neuropathy, with reduced nerve expression of receptor of advanced glycated end-product (RAGE) and increased nerve conduction. The effects on renal function are discordant, with some reports showing a decline in renal function after a long period subsequent to transplantation, whereas others show stability. Acceleration of the diabetic nephropathy as well as renal toxicity per se have been ascribed to immunosuppressive therapy. Prompt implementation of antihypertensive nephroprotective therapies and appropriate recipient selection, especially in ITA, including T1DM patients with virtually normal renal function and presumably slow progression of the diabetic nephropathy, are recommended for limiting post-transplant renal side effects. Results primarily from IAK recipients indicate that IT can induce improvements in cardiovascular and endothelial function (e.g., improved diastolic function, increased nitric oxide production), atherothrombotic profile (e.g., reduced lipid oxidation, delayed intimal media thickening), with fewer cardiovascular events and better survival in IT recipients (90 vs. 50% at 7 years). Overall, together with the improvement in glycemic control, IT seems to be protective for kidney graft function and to increase its longevity. The prolonged C-peptide secretion may contribute to such beneficial effects by reducing nerve dysfunction and increasing blood flow in cardiac and renal districts, with myocardial and glomerular vasodilatation, improving cardiovascular and kidney function, and slowing the progression of diabetic macro- and microangiopathy (Johansson et al., 2000; Wahren et al., 2000; Hansen et al., 2002; Fiorina et al., 2003a, b; Fiorina et al., 2005a, b; Lee et al., 2005; Venturini et al., 2006; Ryan et al., 2006; Del Carro et al., 2007; Fung et al., 2007; Maffi et al., 2007; Senior et al., 2007; Thompson et al., 2008; Warnock et al., 2008, Leitao et al., 2009).

At islet graft dysfunction, long- and short-acting insulin analogues (e.g., glargine and lispro), and/or the incretin-mimetic exenatide, are gradually started. The latter seems to have direct effects on β cells (increased glucose-dependent insulin secretion, restored first-phase secretion, better insulin processing, and higher amylin

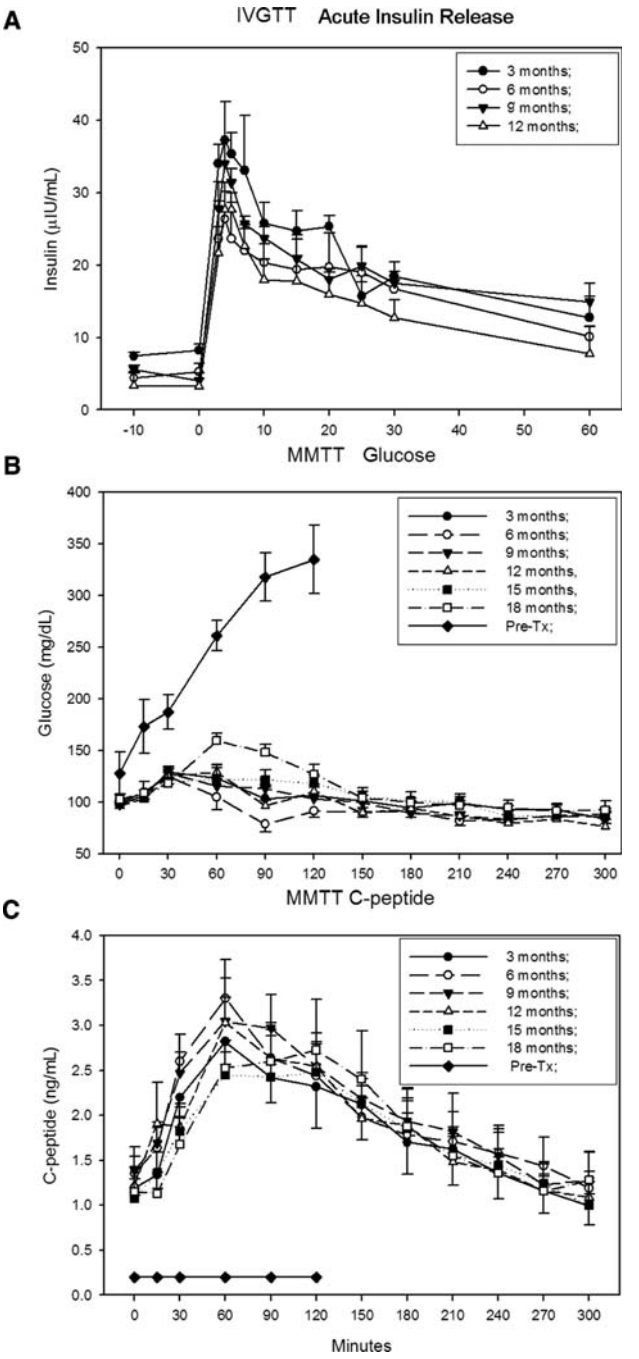



**Fig. 2.2 Intravenous glucose (IVGTT) (a) and mixed-meal (MMTT) (b and c) tolerance tests, pre- and post-islet allotransplantation.** Reproduced with permission from Faradji et al., 2008

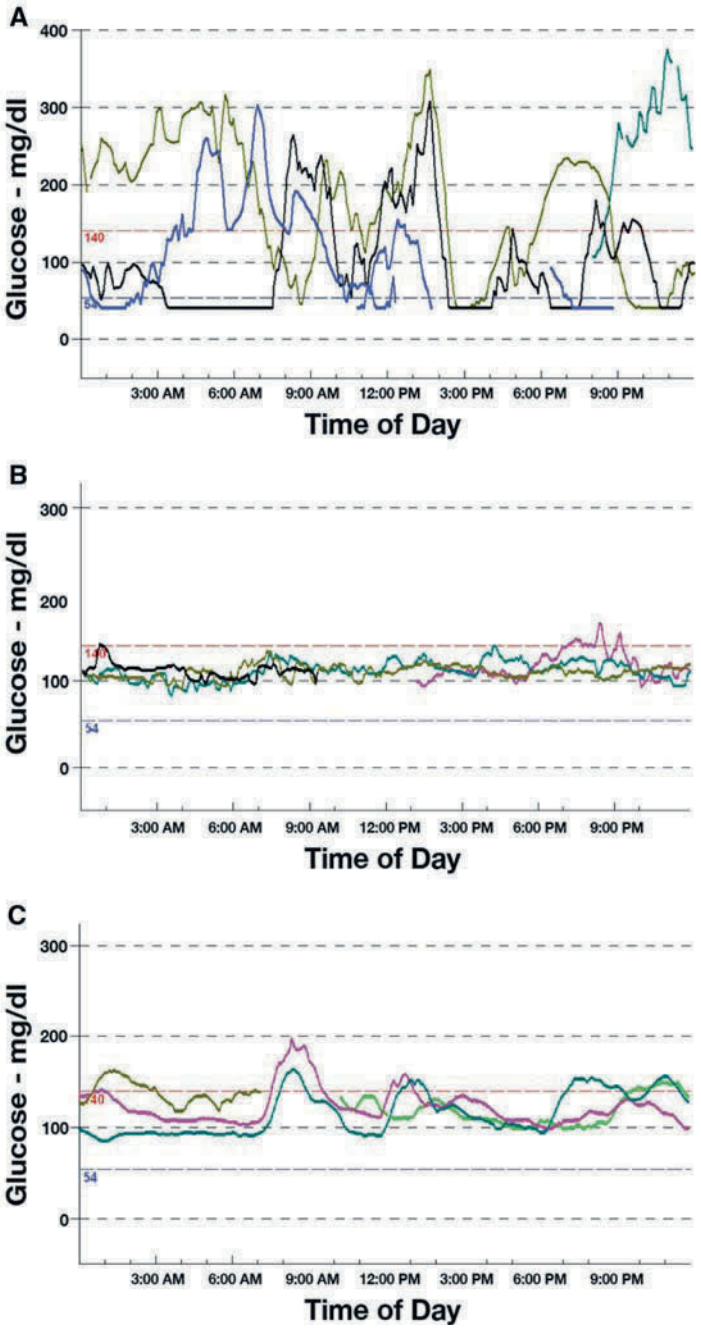



**Fig. 2.3 Continuous glucose monitoring system (CGMS) profiles pre- (a) and post-islet (b) allotransplantation, and at islet graft dysfunction (c).** Different lines represent different days of glucose monitoring. Reproduced with permission from Gorn et al., 2008

synthesis) and indirect effects on glucose metabolism (reduced glucagon secretion, lower hepatic gluconeogenesis, reduced gastric empting, and delayed glucose absorption). Whether reduction of apoptosis or regeneration of β cells can occur, as observed in experimental models, is not yet clear. Exenatide may also aid in protecting β cells from immunosuppression-related toxicity. Several side effects (e.g., vomiting, nausea), the risk of pancreatitis, and the possible worsening of preexisting diabetic gastroparesis may limit its use (D'Amico et al., 2005; Ranta et al., 2006; Cure et al., 2008b; Ranganath, 2008).

#### 2.2.3.2 Islet Graft Monitoring

The clinical management of islet transplant recipients relies on the combination of several immune responses and metabolic parameters together with blood trough levels of immunosuppressants and recipient clinical status, including immunosuppressive-related side effects and toxicity symptoms.

The immune alloresponse is monitored principally by mixed lymphocyte alloreaction (MLR) and panel reactive alloantibody (PRA) assays for cellular and humoral reactivity, respectively. Evaluation of cytotoxic gene expression levels (e.g., granzyme B) or ATP production in in-vitro stimulated $CD4^+$ T-lymphocytes may represent helpful tools for confirming the clinical picture and the islet graft course, together with cytokine measurement and characterization or other soluble markers. Recurrent autoimmunity can be detected by reappearance of T1DM-specific autoantibodies (e.g., anti-GAD65, anti-IA2, and anti-insulin) and seems to be associated with lower insulin-independence rates and shorter islet graft survival. Histological signs of selective destruction of β-cell allograft as well as autoreactive cytotoxic and memory T cells against specific β-cell epitopes have been also described (Stegall et al., 1996; Bosi et al., 2001; Han et al., 2004; Pinkse et al., 2005; Huurman et al., 2008; Huurman et al., 2009; Mineo et al., 2008b; Monti et al., 2008, Saini et al., 2008).

Monitoring islet graft function for detection or prediction of β-cell dysfunction or failure is based on insulin requirements and blood HbA1c, glucose, C-peptide, and insulin levels measured in the fasting state or after stimulation testing (e.g., intravenous arginine tolerance test, IVGTT, and MMTT). Several indices of islet graft function are derived from these measurements (e.g., acute insulin or C-peptide release, fasting C-peptide/glucose ratio, 90-min glucose). Composite indices are also calculated based on insulin requirements, HbA1c, and the number of infused IEQ, such as the beta score. The use of CGMS or of the MAGE index derived from daily glucose measurements with finger-sticks can help detect early graft dysfunction. Unfortunately, none of these indices is completely reliable or standardized, resulting in detection of metabolic alterations when it is too late to intervene with modifications of the immunosuppressive therapy for rescuing the islet graft (Teuscher et al., 1998; Geiger et al., 2005; Rickels et al., 2005b; Faradji et al., 2007b; Rickels et al., 2007b; Gorn et al., 2008; Baidal et al., 2009).

To date, limited imaging methods are clinically available for visualizing or monitoring the islet graft in vivo. Luciferase-transduced bioluminescence optical

imaging, despite high sensitivity, has limited depth penetration and is not applicable to human studies. High-sensitivity (e.g., 3-tesla) magnetic resonance imaging of islets labeled with different tracers (e.g., superparamagnetic iron nanoparticles) is being tested in animal settings with promising results for clinical application. Positron emission tomography with 18-fluorodeoxy-D-glucose has been used recently in human setting to assess intrahepatic islet engraftment and survival in the immediate postinfusion period. Percutaneous hepatic biopsy is not routinely used owing to procedure-related risks (e.g., bleeding) and lack of certainty of retrieval of islet graft tissue (Eich et al., 2007; Medarova and Moore, 2008).

## *2.2.4 Complications and Limitations*

### 2.2.4.1 Recipient- and Graft-Related Complications

Acute complications during the islet infusion procedure are rare (<2–6%), and include: intraabdominal bleeding, pleural or abdominal effusions, peripheral portal vein branches thrombosis, and transient transaminitis. Novel radiological techniques, intracatheter-tract coagulants, and recipient peritransplant antithrombotic prophylaxis have reduced their incidence. Intrahepatic focal steatosis and amyloid deposits may follow IT, but their effect on islet graft function and survival is still unclear (Bhargava et al., 2004; Froud et al., 2004; Barshes et al., 2005; Hafiz et al., 2005; Westermark et al., 2008).

The extended period of the islet allograft survival in recent protocols has involved long-term immunosuppression-related side effects in virtually all recipients, primarily common or opportunistic infections (mainly skin, respiratory, and urinary tracts), and direct immunosuppressive toxicity (Table 2.2). Several serious adverse events have been observed that required hospitalization and specific therapy (e.g., profound neutropenia, pneumonia, ovarian cysts), but only one death could be attributed to immunosuppression (viral meningitis). Extremely rare are viral reactivations (e.g., EBV, CMV) or de novo malignancies, with only 13 neoplasms reported (two papillary thyroid carcinomas, six squamous and two basal-cell skin carcinomas, one ovarian and one breast cancer, one pulmonary nodule) in approximately 400 IT recipients according to data of CITR (Cure et al., 2004; Hafiz et al., 2004; 2005; Faradji et al., 2007a, Alejandro et al., 2008; Cure et al., 2008c).

Sirolimus has opposing effects on insulin secretion and action, which appear to be cell- , species- , and dose-dependent, and act by inhibition of insulin-receptor signal transduction and of the kinases regulating the β-cell cycle. Beta-cell dysfunction and reduction of insulin secretion seem to occur only at doses higher than those used in the clinical setting, whereas increased basal and glucose-stimulated insulin levels with reduced apoptosis have been seen at therapeutic concentrations. In skeletal muscle and adipose cells, long-term exposure seems to reduce insulin-dependent glucose uptake and insulin sensitivity, whereas in the short term opposite effects have been observed. Reversible, dose-dependent dyslipidemia also occurs (Subramanian and Trence, 2007; Vantyghem et al., 2007).

**Table 2.2** Immunosuppression-related side effects in islet allotransplantation (adapted from Leitao et al., 2008a) [a]

| | Side-effect | Agent | Treatment | Commentary |
|---|---|---|---|---|
| Gastrointestinal | Oral ulcers (>70%) | Sirolimus | Topical symptomatic | – |
| | Diarrhea (>60%) | Sirolimus<br>MMF | Switch to MMF<br>Switch to enteric-coated MS | Exclude infections (*Clostridium difficile*/ CMV) |
| | Nausea/vomiting (˜50%) | Sirolimus/tacrolimus | Symptomatic | Exclude drug toxicity |
| Hematological | Anemia (>90%) | Sirolimus/ tacrolimus/ MMF or iron deficiency | Iron supplement, recombinant eritropoetin | |
| | Mild leucopenia (˜100%) | Sirolimus/MMF | – | Normalizing within 3 months |
| | Neutropenia <500/μl (>20%) | Sirolimus/MMF/ Cotrimazol/valganciclovir/ CMV infection | GCSF if <500/μl or <1000/μl with fever | <500/μl with fever (rare, 1/26 recipients) requires hospitalization and broad-spectrum antibiotic therapy |
| | Severe lymphopenia (>10%) | Alemtuzumab and thymoglobulin | Frequent monitoring for infections | Desirable effect, can last up to one year |
| | Mild thrombocytopenia (>60%) | Sirolimus | – | Spontaneous remission. ITP in alemtuzumab users - no cases in islet transplant recipients |
| Dermatological | Acne (>50%) | Sirolimus | Topical treatment | – |
| | Folliculitis (>20%) | Sirolimus | Topical treatment | – |
| | Eczema (>10%) | Tacrolimus | Topical treatment | Can be severe, requiring drug discontinuation |
| Metabolic | Dyslipidemia (>50%) | Sirolimus | Statins | Increase in LDL-cholesterol |
| | Impaired insulin secretion (variable) | Tacrolimus/sirolimus | – | See immunosuppressive beta cell toxicity |
| | Hypophosphatemia (>90%) Hypomagnesemia (>60%) | Sirolimus/tacrolimus/MMF | Oral replacement. | Mild |

**Table 2.2** (continued)

| | Side-effect | Agent | Treatment | Commentary |
|---|---|---|---|---|
| Gonadal | Ovarian cysts (>60%)<br>Altered menses (>60%) | Sirolimus | Hormonal<br>Surgery in selected cases | Oligo- or amenorrhea. Gonadothropins dysregulation. No polycystic pattern at ultrasound |
| Cardiovascular | Transient hypotension (rare) | Alemtuzumab | Symptomatic | – |
| | Increase in blood pressure (>30%) | Tacrolimus/sirolimus | Initiation/increase in antihypertensive medication | – |
| | Peripheral edema (>50%) | Sirolimus | Diuretics | – |
| Renal | Proteinuria/albuminuria, decrease in glomerular filtration rate (variable) | Tacrolimus/sirolimus | ACEi/ARB, statins | Stable if adequate treatment of diabetic nephropathy classical risk factors (i.e., LDL-cholesterol and blood pressure) is provided |
| Neurological | Tremors, paresthesias, headache, mild depression (>10%) | Tacrolimus | Switch to MMF | Can be severe: disabling pain syndrome and leukoencephalopathy |

[a] ACEi = angiotensin-converting-enzyme inhibitors; ARB = angiotensinogen receptors blockers; CMV = cytomegalovirus; GCSF = granulocyte colony-stimulating factor; ITP = immune thrombocytopenic purpura; LDL = low-density lipoprotein; MS = mycophenolate sodium.

MPA may also have a detrimental effect on β cells, by reducing insulin secretion and inducing apoptosis, as well as on peripheral insulin sensitivity, with most of such data coming from experimental settings, whereas lipid metabolism is not affected. The enteric-coated formulation mycophenolate sodium has recently shown better gastrointestinal tolerability and absorption than MMF and is increasingly used to avoid toxicity from other immunosuppressive drugs (Havrdova et al., 2005; Gao et al., 2007; Subramanian and Trence, 2007; Park et al., 2009).

All the immunosuppressive agents can interfere with islet engraftment and β-cell self-renewal. Indeed, sirolimus has antiproliferative and antiangiogenic effects on duct and islet cells that may impair β-cell engraftment and neovascularization as well as viability and regeneration. Tacrolimus and MPA also have negative effects on duct and islet cell proliferation and differentiation, preventing β-cell neogenesis or replication. No negative effects of everolimus, a newly introduced mTOR inhibitor, on glucose metabolism, have yet been reported, although it can induce dyslipidemia (Bussiere et al., 2006; Cantaluppi et al., 2006a, b; Marcelli-Tourvieille et al., 2007; Nir et al., 2007; Zahr et al., 2007).

Renal toxicity is still a major side effect of immunosuppressive therapy. Tacrolimus may cause acute vasomotor vasculopathy with tubular necrosis and/or chronic fibrotic vasculopathy with glomerulosclerosis and interstitial fibrosis. Moreover, sirolimus may induce acute renal dysfunction and/or chronic proteinuria by increasing glomerular permeability and injury or by suppressing the compensatory renal cell proliferation and repair capacity. Their combined use in IT can have synergic negative effects on renal function per se or may cause the progression of diabetic nephropathy, especially in the presence of pretransplant abnormalities (e.g., microalbuminuria, reduced eGFR), whereas the alternative use of MPA-based regimens could prevent renal injury (Rangan, 2006; Williams and Haragsim, 2006).

Supportive therapy is normally used to counteract systemic immunosuppressant-related side effects, such as angiotensin converting enzyme inhibitors (ACEi) or angiotensinogen receptor blockers (ARB), statins or ezetimibe, together with bone marrow stimulants (e.g., granulocyte-colony stimulating factor, erythropoietin), anti-infective prophylaxes, and dietary supplements (e.g., iron). In most cases prompt treatment of complications minimizes recipient morbidity without any sequela (Hafiz et al., 2005; Faradji et al. 2007).

#### 2.2.4.2 Transplant-Related Limitations

Similarly to the pretransplantation period, many factors can contribute to a significant post-transplantation islet loss especially during the early postinfusion phase, reducing the effective number of functioning islets available during the follow-up period. Because of that often a second or third donor islet infusion is required to achieve insulin independence and durable normalization of glucose control in the recipients.

In particular, during islet infusion, an intravascular instant blood-mediated inflammatory reaction (IBMIR) seems responsible for destroying 50–70% of the infused β cells. An upregulation of tissue factor and other molecules on islet

cell surface after the isolation process is capable of triggering innate immunity via activation of coagulation, complement, inflammation, and natural antibodies, destroying the islets. Peritransplant anticoagulant prophylaxis with heparin can counteract this reaction (Moberg et al., 2002; Johansson et al., 2005; Eich et al., 2007).

A progressive intrahepatic islet graft dysfunction and loss also occurs owing to: poor revascularization, chronic hypoxia, absent reinnervations, proinflammatory milieu, drug toxicity, glucolipotoxicity, fat and amyloid deposition, islet functional overload, premature apoptosis, and lack of regeneration. Several ongoing experiments are aimed at identifying alternative and less hostile implantation sites for islet allograft, with the omental pouch, the thymus, or the bone marrow being the most attractive. Recently, islet autotransplantation in the forearm muscle of a child with genetically determined pancreatitis has shown a prolonged (over 2 years) restoration of insulin secretion and normalized glucose control, while requiring minimal exogenous insulin therapy (Desai et al., 2003; Bhargava et al., 2004; Pileggi et al., 2006; Huang et al., 2008; Merani et al., 2008; Rafael et al., 2008; Westermark et al., 2008; Lau and Carlsson, 2009).

Finally, a major concern of IT is the recipient-wide allosensitization from the multiple HLA-mismatched donor infusions performed to achieve insulin independence, hypothetically jeopardizing the chances of receiving future organ transplants (e.g., kidney or pancreas). Pretransplant PRA levels higher than 15–20% and donor-specific antibodies (DSA) seem associated with reduced islet graft survival. Post-transplant positive PRA levels and de novo DSA may occur after drug dose reduction for persistent or serious side effects (e.g., infections) but their impact on islet graft loss is still unclear. Allosensitization seems absent or minimal under the recommended trough levels of immunosuppression, which also seem able to contain low PRA levels (<5–15%), but it occurs constantly when immunosuppression is discontinued, such as after islet graft failure. High PRA levels (>50%) with DSA and cross-reacting non-DSA may persist for a long time. A slower immunosuppressive tapering could minimize or prevent sudden and massive antigen exposition from residual islet graft (Mohanakumar et al., 2006; Rickels et al., 2006b; Campbell et al., 2007a, b; Cardani et al., 2007).

#### 2.2.4.3 Current Challenges and Future Perspectives

Several technical and clinical limitations still persist in IT (Fig. 2.4). Various cytoprotective strategies and agents are currently under investigation to improve organ preservation and islet yield and survival through the isolation process, such as perfluorocarbons in a two-layer method, new lytic enzyme blends or purification methods, and JNK or caspase inhibitors (Kin et al., 2006; Barbaro et al., 2007; Emamaullee et al., 2007; Sabek et al., 2008; Varona-Santos et al., 2008).

New immunological and possibly tolerogenic strategies, including more selective lymphodepleting drugs, costimulatory blockade, and anti-inflammatory agents, are being tested for increasing islet allograft longevity, preventing allorejection and

| ① | ② | ③ | ④ | ⑤ | ⑥ |
|---|---|---|---|---|---|
| Donor/Organ | Pancreas recovery | Islet Processing | Intra - hepatic Site | Drug Toxicity | Immunity |
| Age/BMI<br>Organ fibrosis<br>Fat Infiltration<br>Brain Death<br>Intensive Care | Recovery Technique<br>Warm/Cold Ischemia<br>Preservation Solution<br>Organ Shipment | Enzyme<br>Purification<br>Culture<br>Cell-Viability<br>Cell-Potency | Hypoxia<br>Gluco-toxicity<br>Apoptosis | Beta-Cell Toxicity<br>Lipo-Toxicity<br>Anti-Proliferation<br>Anti-Angiogenesis | IBMIR (acute)<br>Allo-Rejection<br>Auto-Immunity<br>Allo-Sensitization |

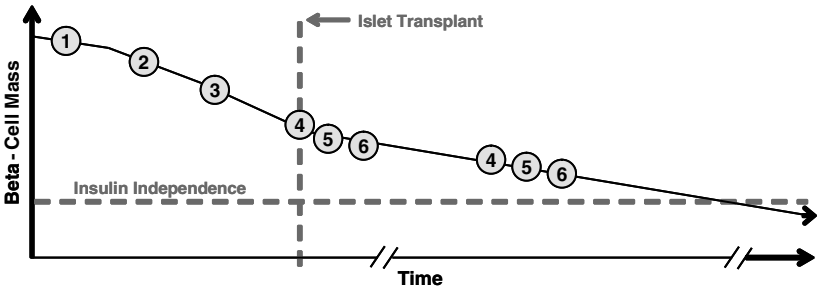



**Fig. 2.4 Main challenges in clinical islet allotransplantation.** Reproduced with permission from Mineo et al. 2008c

recurrent autoimmunity, and reducing recipient side effects and islet graft toxicity (Vincenti and Kirk, 2008). The Clinical Islet Transplant Consortium, including centers in North America and Europe, is starting different phase II–III trials using novel agents (e.g., anti-CD20/B-cell-depleting rituximab, anti-CD80/86 costimulatory blockade belatacept, immunomodulatory deoxyspergualin, anti-inflammatory agent lisofylline, and IBMIR-blocker low-molecular-weight dextran) to improve outcomes. Standardized procedures are also used, with the goal of obtaining approval for IT as a standard health-care procedure, thus allowing for insurance reimbursement. Indeed, costs of IT are very high, approximately $250,000 in the first 2 years post-transplantation, and only a few countries (e.g., Canada) have included this procedure as an optional treatment for selected patients with T1DM.

### *2.2.5 Conclusions*

IT as treatment for brittle T1DM has recently achieved successful graft function, with long-term metabolic improvements and minimal procedure-related complications. Unfortunately, islet recovery from isolation and post-transplant graft durability with the current methods and protocols are still unsatisfactory. Several limitations remain, including auto- and alloimmunity, allosensitization, immunosuppressive-related toxicity, and implantation-site unsuitability. In the near future, improvements in both the isolation process and islet cytoprotection, as well as new, less toxic immunological agents together with tolerogenic protocols, and alternative implantation sites, may overcome such challenges (Ricordi, 2003; Ricordi and Strom, 2004; Shapiro, 2008).

## 2.3 Simultaneous Pancreas–Kidney Transplantation

Simultaneous pancreas–kidney transplantation (SPK) is considered the best treatment option for patients with T1DM and end-stage renal disease (ESRD). The pancreas transplant can restore euglycemia, providing long-term insulin independence; increase patient survival; stabilize or improve diabetic retinopathy and neuropathy; and, in combination with the kidney transplant, eliminate the need for long-term dialysis (Gruessner and Sutherland, 2005; Leichtman et al., 2008).

More potent immunosuppression agents, improvements in surgical techniques, and better understanding of postoperative complications have led to consistent improvement in SPK transplantation results over the past decade. Drainage of the exocrine pancreas and duodenal segment into the bladder (Fig. 2.5) is used largely in respect to enteric drainage for safety reasons and for identifying changes in transplant function by monitoring urine amylase. Ten-year survival rates for patients and pancreas are 84% and 76%, respectively, among the best long-term survival reported in patients with T1DM/ESRD (Burke and Ciancio, 1997; Burke et al., 1998a; Burke et al., 2001; Gruessner and Sutherland, 2005; Leichtman et al., 2008).

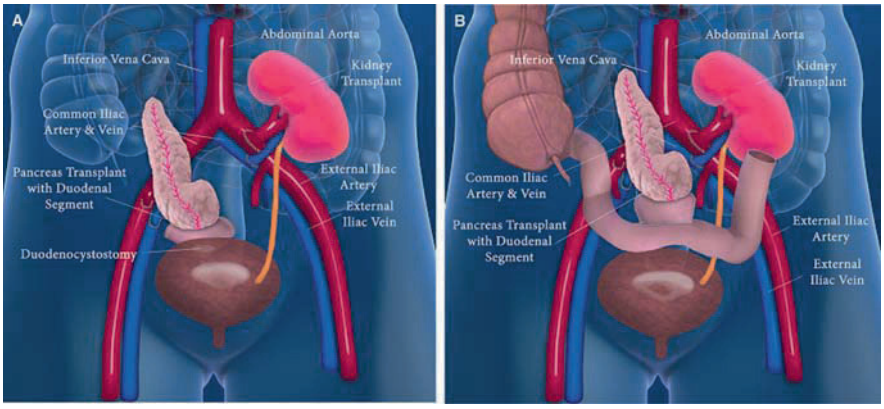



**Fig. 2.5 Schematic representation of pancreas-kidney transplantation.** (**a**) Bladder-drained transplant. (**b**) One option for enteric drainage of pancreas graft in pancreas–kidney transplantation

### *2.3.1 Clinical Protocols*

#### 2.3.1.1 Maintenance Immunosuppression

The incidence of acute rejection (AR) in SPK transplantation has been decreasing over the past decade as a result of advances in immunosuppression. The most common agents for maintenance immunosuppression in SPK transplantation presently are tacrolimus and MMF; other drugs such as cyclosporine A, sirolimus, and azathioprine are also used in different combinations. Corticosteroids are still administered, but there is a trend toward steroid-free immunosuppression protocols with the goal of reducing the consequent adverse effects (Burke et al., 1998b; Ciancio

et al., 2000a; Burke et al., 2004b; Gruessner and Sutherland, 2005; Cantarovich and Vistoli, 2009; Mineo et al., 2008c; Singh and Stratta, 2008).

#### 2.3.1.2 Induction Therapy

The recent therapeutic protocols in kidney and kidney–pancreas transplantation attempt to reduce the incidence and severity of AR as well as prevent long-term chronic (vascular) allograft dysfunction (CAD). The methodologies include reduction of CNIs and of their short- and long-term nephrotoxicity, reduction or avoidance of corticosteroids, use of adjunctive maintenance antiproliferative agents (e.g., mTOR inhibitors), and utilization of new agents, such as nonlymphodepleting monoclonal antibodies (daclizumab or basiliximab), or lymphodepleting monoclonal (alemtuzumab) and polyclonal (e.g., rATG) antibodies. The percentage of patients treated with induction therapy has been increasing and was more than 75% in the most recently reported data from the International Pancreas Transplant Registry (IPTR) 2004 (Gruessner and Sutherland, 2005).

Daclizumab

A series of studies has been published analyzing the safety and efficacy of daclizumab as induction therapy in SPK transplant recipients (Bruce et al. 2000; Burke et al., 2001; Lo et al., 2001a, b; Stratta et al., 2001; Burke et al., 2002a, b; Stratta et al., 2002). The results of a multicenter survey using daclizumab as induction therapy showed a low incidence of AR when used in combination with tacrolimus, MMF, and corticosteroids in SPK transplant recipients (Bruce et al., 2001). The survey reported experience with 71 SPK transplant recipients receiving 4–5 daclizumab doses ($n = 45$) or 1–3 doses ($n = 26$). There were no differences in patient and kidney graft survival rates, 98 vs. 96% and 92 vs. 92%, respectively. However, there was a trend toward improved pancreas graft survival rates in the group receiving 4–5 doses, compared with 1–3 doses (96 vs. 85%, $p = 0.07$). Although more patients receiving 1–3 doses had rejection (54%) than patients receiving 4–5 doses (24%), there was no dose–response relationship between the total number of doses or the adjusted total milligram/kilogram dose and time to rejection. All patients with functioning grafts had good renal and pancreatic allograft function at 6 and 12 months. The overall incidence of major infection was 27%, and there were no differences in the incidence of infection between the two groups. No major adverse events were attributed to daclizumab use. In conclusion, excellent short-term outcomes were noted in this retrospective, multicenter survey of initial experience with daclizumab induction in combination with tacrolimus, MMF, and corticosteroids in SPK transplant recipients.

The safety and efficacy of two dosing regimens of daclizumab as an adjunctive immunosuppressive agent versus no antibody induction in SPK transplant recipients receiving tacrolimus and MMF as primary immunosuppression were investigated in a multicenter, open label, comparative trial (Stratta et al., 2002). SPK transplant recipients were randomized to one of three groups: daclizumab 1 mg/kg every

14 days for five doses (Group I), daclizumab 2 mg/kg every 14 days for two doses (Group II), and no antibody induction (Group III). A total of 166 patients were randomized into the three groups [Group I ($n = 70$), Group II ($n = 74$), Group III ($n = 22$)]. At a minimum follow-up of 3 months, patient, kidney and pancreas graft survival rates were similar among the three groups. However, the rates of acute renal allograft rejection were 18% for Group I, 8% for Group II, and 36% for Group III ($p < 0.005$). The probabilities of either kidney or pancreas allograft rejection were 22% for Group I, 8% for Group II, and 38% for Group III. At 3 months, the actuarial event-free survival (no AR, allograft loss, or death) rates were 67%, 81%, and 50% in Group I, II, and III, respectively. Although the follow-up was short, this study emphasized the important role of induction antibodies in reducing AR.

### Daclizumab in Combination with rATG

The use of new immunosuppressive agents continues to be associated with reduced rates of AR episodes in SPK transplant recipients (Burke et al., 2002a, b). Forty-two SPK transplant recipients were included in a prospective, randomized trial in which they received rATG and daclizumab, tacrolimus, and corticosteroids as baseline immunosuppression. They were then randomized to receive either MMF or sirolimus in addition to baseline immunosuppression. Twenty-two patients received MMF and 20 received sirolimus. There were three episodes of AR (7.1%). These were in the MMF group, all in patients who were off either MMF (wound infection, pneumonia) or corticosteroids. Each of these episodes was corticosteroid-resistant, but responsive to antibody therapy (OKT3 or rATG). Actuarial patient, kidney, and pancreas allograft survivals were 100%, 100%, and 95% in the sirolimus group and 100%, 100%, and 100% in the MMF group (Burke et al., 2002b).

A similar study (Gallon et al., 2007) reported the effect of two tacrolimus-based maintenance regimens on long-term renal allograft function in SPK transplant recipients [tacrolimus/MMF ($n = 22$) vs. tacrolimus/sirolimus ($n = 20$)] (Schaapherder et al., 1993). All patients received rATG as induction therapy. The difference from the previous study (Burke et al., 2002b) was that both regimens included prednisone-free maintenance. Patient and pancreas graft survival rates at 6 years were the same, but kidney allograft survival was higher in the tacrolimus/MMF group (90.7% vs. 70.7%, $p = 0.09$). The incidence of AR and rate of decline in eGFR were similar in both groups (Gallon et al., 2007).

### Alemtuzumab

A nonrandomized study of 75 pancreas–kidney and solitary pancreas recipients who received alemtuzumab (four doses for induction and twelve doses within the first year) and MMF ($\geq$2 gr/day) for induction and maintenance therapy was reported (Gruessner et al., 2005). Thirty milligrams of alemtuzumab was given intravenously intraoperatively for induction as well as for maintenance dosing, the latter doses administered when the absolute lymphocyte count increased to 200/mm$^3$ or more; the maximum number of alemtuzumab doses was limited to ten within the first

year. In a 6-month follow-up the results were compared with an historical group of 266 consecutive pancreas recipients using rATG induction and tacrolimus maintenance. Patient survival at 6 months for SPK transplant recipients was 90%; for pancreas-after-kidney (PAK) recipients 91%; and for pancreas transplant alone (PTA) recipients 97% ($p \geq 0.4$).

The patient survival rates were not different between the control group and the three study groups ($p \geq 0.06$). Pancreas graft survival at 6 months in the study group for SPK transplant recipients (vs. historical control) was 81% (vs. 79%; $p \geq 0.66$); for PAK recipients 91% (vs. 85%; $p \geq 0.59$); and for PTA recipients 71% (vs. 84%; $p \geq 0.07$). Kidney graft survival in the historical control versus the study group at 6 months for SPK transplant recipients was 81% vs. 85%; ($p \geq 0.2$). The incidence of a first (reversible) rejection episode at 6 months in the study versus the control group for SPK transplant recipients was 41% (vs. 9%; $p \geq 0.0003$); for PAK recipients 14% (vs. 10%; $p \geq 0.89$); and for PTA recipients 19% (vs. 26%; $p \geq 0.36$). In all three recipient categories the median "modification of renal disease" level at 6 months was higher and the median serum creatinine concentration was lower in the study versus control groups, but the differences did not reach statistical significance. The conclusion was that the combination of alemtuzumab and MMF was associated with an acceptable rejection rate (albeit higher than expected for SPK transplants), and good (graft and native) kidney function; it eliminated undesired CNI- and corticosteroid-related side effects, but a long-term follow-up is warranted.

More recently a single-center nonrandomized retrospective sequential study was reported (Kaufman et al., 2006) comparing the effect of alemtuzumab ($n = 50$) and rATG ($n = 38$) as an induction immunosuppression for recipients of SPK transplant given a prednisone-free maintenance regimen in combination with tacrolimus/sirolimus-based maintenance therapy. The overall 1-year patient and graft survival rates were similar for the two treatment groups. The 1-year actual patient survival rates for recipients who received alemtuzumab and rATG were 96% and 100%, respectively ($p =$ ns); the 1-year actual death-censored kidney graft survival rates were 95% and 97.4%, respectively ($p =$ ns); the 1-year actual death-censored pancreas graft survival rates were 92% and 100%, respectively ($p =$ ns); the 12-month actual rejection rates were 6.1% and 2.6%, respectively ($p =$ ns). At 12 months, the serum creatinine values for the alemtuzumab and rATG group were $1.45 \pm 0.36$ and $1.29 \pm 0.43$, respectively ($p =$ ns). Viral infectious complications were statistically significantly lower in the alemtuzumab group. Despite the study limitation, both alemtuzumab and rATG induction were effective in facilitating a prednisone-free maintenance protocol in SPK transplant recipients.

The use of alemtuzumab as induction therapy in SPK transplant recipients has increased substantially. Lately, the impact of steroid-free maintenance immunosuppression in pancreas transplantation using alemtuzumab as induction therapy has been evaluated in a single-center study (Muthusamy et al., 2008), where 102 pancreas transplantations were performed in 100 patients with tacrolimus and MMF, with no maintenance corticosteroids. With a median follow-up of 17 months, patient, pancreas and kidney graft survival (actuarial) was 97%, 89%, and 94%, respectively. Overall incidence of rejection was 25%. The incidence of CMV

and BKV infections was 6.8% and 3.8%, respectively. This experience suggested that alemtuzumab is safe and effective. Furthermore, steroid-free maintenance was achieved in 83% of the patients with a 25% incidence of rejection.

A cautious tone should be used in the context of corticosteroid-free immunosuppression, since a recent report from the Minnesota group showed that occurrence of AR has a far greater impact on kidney graft survival (15 years actuarial) than the development of new onset DM (NODM) (Matas et al., 2008). This may dampen some of the enthusiasm for steroid-free protocols in which the high rate of AR may well translate into worse long-term graft (and hence patient) survival.

In another study (Clatworthy et al., 2007) alemtuzumab was given subcutaneously in 21 SPK transplant recipients. The rate of AR was 14% at 1 year. This route of administration was recommended because lymphocyte depletion was comparable to that seen in patients receiving intravenous alemtuzumab. Recently, alemtuzumab was compared with rATG (Farney et al. 2008) and basiliximab induction therapy (Magliocca et al., 2008). The use of alemtuzumab for induction therapy after SPK transplants was found to be as safe and effective as rATG and basiliximab. Furthermore, the outcome was not inferior to that of the other two induction therapies. It is important to note that there was a higher incidence of CMV infections in the alemtuzumab group and since then a single dose (rather than two) has been used.

## *2.3.2 Results*

### 2.3.2.1 Patient and Graft Survival

Long- and short-term patient survival rates have improved steadily over the years. Patient survival rates at 1 year have been higher than 90% since the earliest eras, and are now more than 95% for SPK transplantations performed in 2002/2003. Overall, 5-year survival rates have also improved and are higher than 80%. Survival rates at 10 years are 69% for SPK transplantation. One-year pancreas graft survival rates are 85%, and 1-year kidney graft survival rates are 92%. The 5-year pancreas graft survival reached 69%, and the 5-year kidney graft survival was 77% for the 1998/1999 period. The 10-year pancreas and kidney graft survival rates for the 1992/1993 were 46% and 45%, respectively (Gruessner and Sutherland, 2005). These numbers are similar to those in recent reports (Leichtman et al., 2008). At the University of Miami 10-year survival rates for patients, pancreas, and kidney are 8%, 76%, and 51%, respectively (Burke et al., 2001).

### 2.3.2.2 Diabetic Nephropathy

The effects of pancreas transplantation on diabetic nephropathy are among the most studied benefits of pancreas transplantation. A pivotal study demonstrated that pancreas transplantation can reverse preexisting histological lesions of diabetic nephropathy in the native kidneys, but reversal requires more than 5 years of normoglycemia (Fioretto et al., 1998). Another study reported on 32 T1DM patients

that were evaluated before and 1 year after successful PTA and compared with 30 matched nontransplanted T1DM patients. Evidence for improvement of renal function after pancreas transplantation was found, documented by the reduction of urinary excretion of protein with stable creatinine concentration and clearances (Coppelli et al., 2005).

#### 2.3.2.3 Diabetic Retinopathy

Retinopathy is the most common microvascular complication of DM. The majority of our patients who undergo SPK transplantation have already developed some degree of retinopathy and most of them have received laser therapy (LT). Patients with advanced retinopathy are less likely to benefit from a SPK transplant.

A prospective study evaluated 33 PTA recipients and 35 patients with T1DM who did not receive PTA. At baseline, 9% of PTA and 6% of non-PTA patients had no diabetic retinopathy, 24% and 29% had nonproliferative diabetic retinopathy (NPDR), and 67% and 66% had laser-treated and/or proliferative retinopathy (LT/PDR), respectively. No new case of diabetic retinopathy (DR) occurred in either group during at least 1 year of follow-up. In the NPDR PTA group, 50% of patients improved by one grading and 50% showed no change. In the LT/PDR, stabilization was observed in 86% of cases but worsening of retinopathy occurred in 14% of patients. In the NPDR non-PTA group, DR improved in 20% of patients, remained unchanged in 10%, and worsened in the remaining 70%. In the LT/PDR non-PTA group, retinopathy did not change in 43% and deteriorated in 57% of patients. Overall, the percentage of patients with improved or stabilized DR was significantly higher in the PTA group (Giannarelli et al., 2006). Another report concluded that advanced DR is present in a high proportion of SPK transplant recipients as a consequence of the duration of T1DM (mean of 24.6 years) and the presence of ESRD. More than 90% of patients have stable DR following transplantation (Pearce et al., 2000).

#### 2.3.2.4 Diabetic Neuropathy

Polyneuropathy is a very common (almost 100%) complication of both T1DM and ESRD, and advanced motor, sensory, and autonomic neuropathies are very frequent in patients undergoing SPK transplantation. Improvement or stabilization of gastric function was observed in 12 out of 23 (52%) SPK transplant recipients versus 5 out of 12 (41.7%) T1DM recipients who underwent kidney transplant alone (KTA) (Hathaway et al., 1994). SPK transplant recipients also demonstrated significant improvement in postural adjustment ratio (Navarro et al., 1998). Sensory and motor neuropathies, as measured by nerve conduction studies, have also shown improvement in SPK transplant recipients (Muller-Felber et al. 1993; Navarro et al., 1998).

#### 2.3.2.5 Quality of Life

Patients who received SPK transplants consistently reported an improvement in their quality of life (Sureshkumar et al., 2005). SPK transplantation had a significant positive effect on DM-related quality of life even though SPK transplantation is a complex surgical procedure.

SPK transplantation has been viewed as a higher-cost and higher-risk surgical procedure than kidney transplant, and it is unclear if SPK transplantation offers better health and quality of life outcomes than insulin therapy plus KTA. A study found that both SPK and KTA recipients report better health and quality of life, but SPK transplant recipients also report greater improvement in physical health and in areas that are DM-specific than those of KTA (Gross et al., 2002).

### *2.3.3 Complications and Limitations*

Surgical complications are more common after pancreas transplantation, compared to kidney transplantation. Nonimmunological complications of pancreas transplantation (including thrombosis and graft pancreatitis) account for graft losses in 5–10% of cases. These usually occur within 6 months of transplantation and are as important an etiology of pancreas graft loss in SPK transplantation as AR (Ciancio et al. 1996b; Gruessner et al., 1996; Gruessner and Sutherland, 2001; Gruessner and Sutherland, 2005).

#### 2.3.3.1 Hypercoagulation in SPK

T1DM has been shown to result in hypercoagulation, as assessed by numerous studies involving different components of the clotting cascade. In addition, hyperlipidemia, commonly associated with DM, further contributes to such hypercoagulation. Subsequent to SPK transplantation, with restoration of euglycemia, the lipid profile usually normalizes (Burke et al., 1998) or is treated, when necessary, with lipid-lowering medications. Although the uremic effect on platelets could offset the hypercoagulation associated with DM, our experience suggests that it does not.

In our program, a thromboelastogram (TEG) performed at the time of transplantation surgery has confirmed this hypercoagulable state in a remarkably consistent pattern (Burke et al., 2004). Generally, rheologic assessment, including the combination of shortened prothrombin time (PT), partial thromboplastin time (PTT), and elevated platelet count (>400,000/$mm^3$), fibrinogen (>400 mg/dl), and hematocrit (>40%), along with hyperlipidemia, are all features associated with hypercoagulability and conceptually integrated into the TEG. As each of these factors can vary over time, performing TEG at the time of surgery is the most helpful test in determining the degree of hypercoagulability at transplant.

The pancreas transplant portion of SPK transplant has historically been more prone to thrombosis than other solid organ transplants. This has been ascribed to

several factors, including: the degree of organ injury (i.e., the cumulative effect of preterminal donor injury with hypotension and hypoperfusion) and ischemia–reperfusion damage (Coppelli et al., 2005), which affect all solid organ transplants; technical issues (e.g., the size of the vessels or the method of vascular reconstruction) (Troppmann et al., 1996) [however, others have described low rates of pancreas transplant thrombosis with similar vascular techniques (Sollinger, 1996)]; and the use of desmopressin in the therapy of diabetes insipidus in the donor. This was associated with impaired microcirculation and subsequent pancreas transplant thrombosis (Burke et al., 2004).

When viewed in the context of Virchow's triad, thrombosis can in fact be predicted. Virchow's triad incorporates hypercoagulability, endothelial damage, and venous stasis as the criteria for venous thrombosis, and all three criteria are met in solid organ pancreas transplant. The hypercoagulable state is clearly defined in these patients with T1DM and ESRD by the TEG (Burke et al. 2004). The endothelial damage is associated with all solid organ transplants undergoing a period of cold and warm ischemia, with subsequent reperfusion injury after release of the vascular clamps. Such events result in the well-described ischemia–reperfusion injury with endothelial damage from numerous mediators, including cytokines, $O_2$ radicals, and nitric oxide. The venous stasis occurs when the spleen is removed from the tail of the pancreas and the major source of blood flow through the splenic vein is lost. The splenic vein remains with its high capacitance, but only with the limited flow from the arterial side of the pancreas. The superior mesenteric vein similarly no longer receives venous return from the small bowel, and is limited to small pancreatic venous radicals to maintain flow. Furthermore, immunosuppression itself, mostly CNIs (tacrolimus, cyclosporine A), can induce endothelial damage and hypercoagulability by enhancing secretion of procoagulant factors, for example, endothelin (Burke et al., 1999). Thus, pancreas transplant fulfills Virchow's triad for propensity to venous thrombosis.

The TEG-demonstrated hypercoagulability of patients with DM and ESRD has led us to use heparin intraoperatively when the degree of hypercoagulability is matched with the degree of operative field hemostasis. The PT loss rate of 1% from thrombosis shows that this has been an effective strategy, while reoperating from bleeding is also low (2%) (Burke et al. 2004). Anticoagulation may also confer protection to the distal extremity, which suffers an ischemia–reperfusion injury itself (typically subclinical) after cross-clamping the iliac artery and vein while transplantation is being performed. Since pancreas transplantation can be performed without anticoagulation with a similarly low rate of thrombosis, the demonstration of the hypercoagulable state in T1DM with ESRD by TEG may be more important as a risk factor for atherosclerosis (i.e., an issue for long-term patient survival). When seen in the context of other risk factors for atherosclerosis (e.g., hypertension, obesity, DM, insulin resistance, dyslipidemia, all components of the metabolic syndrome), the greatest benefit of the demonstration of the hypercoagulable state may lie in its subsequent therapy. Appropriate anticoagulation with aspirin therapy or other medications, in addition to the correction of DM, hypertension, renal failure, and dyslipidemia related to SPK transplantation, may result in reduced atherosclerosis.

Although SPK transplantation prolongs patient survival (Burke et al., 2001), recognition and treatment of the hypercoagulable state, along with new approaches to inflammation and atherosclerosis, may allow further improvement.

#### 2.3.3.2 Bladder-Drained Pancreas Transplant

Bladder-drained pancreas transplants (Fig. 2.5) are associated with multiple urological (Ciancio et al., 1995; Ciancio et al., 1996b) and metabolic complications, requiring enteric conversion in 14–50% of most reported series, although only in 8% of our 390 consecutive SPK transplants.

Hematuria occurs frequently but generally resolves early after transplantation with conservative measures. Late-occurring hematuria may be caused by formation of a bladder stone on the staple or suture line. Approximately 5% of patients will require interventions such as Foley catheter placement, irrigation, and cystoscopy for evacuation of clots. Urinary tract infections are common; they occur in as many as half of all cases. Although the urinary pH is generally alkaline and maintains pancreatic proenzymes in an inactivated state, a urinary tract infection can reduce the pH enough to activate these digestive enzymes. Enterokinase present in the brush border of the duodenal mucosa may activate the proenzyme trypsinogen and thereby initiate the pancreatic enzyme activation cascade. Other proteases, such as plasmin, thrombin, and fibrolysin, as well as bacterial enzymes, may also activate trypsinogen to trypsin. The severe burning from urethritis is attributed to autodigestion by the activated pancreatic enzymes trypsinogen and chymotrypsinogen. If untreated, these symptoms may progress to urethral disruption and later stricture. Treatment of urethral complications requires both enteric conversion and urological expertise (Ciancio et al. 1996a). Fortunately, this complication has become less common with the use of small duodenal segments.

Metabolic acidosis is caused by the excretion into the bladder of large quantities of alkaline pancreatic secretions. Most patients take supplemental sodium bicarbonate orally to minimize the degree of acidosis. With time, most of these patients are able to decrease their oral sodium bicarbonate intake.

Fluid management can become problematic for these patients because of the potential for relatively large volume losses. Patients are at risk for dehydration, which can be exacerbated by poor intake as a result of gastric-motility problems commonly associated with T1DM. The symptoms from dehydration can be further compounded when patients have preexisting orthostatic hypotension related to diabetic autonomic neuropathy. Fluid balance can be improved in some patients by the administration of fludrocortisone acetate. Out of our 390 patients who underwent SPK transplantation with bladder drainage, 20% were readmitted within the first year for correction of acidosis and/or dehydration. Their serum creatinine concentrations usually returned to baseline after the administration of intravenous fluids with bicarbonate. Occasionally, patients experience a persistent rise in creatinine associated with episodes of dehydration and require conversion to enteric drainage of the pancreatic secretions.

Urine leaks owing to breakdown of the duodenal segment can occur years after transplantation, but are usually encountered within the first 2–3 postoperative months. The causes of early urine leaks are most often technical in nature and generally require surgical correction with prolonged Foley catheter drainage. Late-occurring leaks can be caused by high pressure in the duodenum during urination. The onset of abdominal pain with elevated serum amylase, which can mimic reflux pancreatitis or AR, is a typical presentation. Imaging studies utilizing a cystogram or CT scanning may be necessary to confirm the diagnosis. Operative intervention may be required, including reanastomosis to the bladder or to bowel.

Despite these complications, bladder drainage of the pancreas graft has many advantages. Early and late complications may cause morbidity; however, these are rarely lethal because enteroenterostomy and, hence, potential intraperitoneal enteric spillage can be avoided.

Another advantage of bladder drainage is the ability to monitor the patient for graft rejection. A decrease of more than 50% in urine amylase activity after pancreas transplantation signals possible AR. The decrease in urinary amylase may be the only clinical indication of a problem, with no change in the serum concentration of creatinine or glucose or the activity of serum amylase or lipase. A biopsy of the pancreas should be performed to confirm the diagnosis of rejection.

After the administration of rejection therapy with corticosteroids or antilymphocyte preparations, the need for repeat pancreatic biopsy can be determined by measuring urine amylase activity. If low urine amylase activity persists after rejection therapy, pancreas biopsy is indicated. In contrast, if urine amylase activity is restored to baseline and the blood-glucose concentration remains high after therapy, the causative factor is steroid therapy with insulin resistance, rather than rejection, and pancreatic biopsy is not necessary.

#### 2.3.3.3 Enteric-Drained Pancreas Transplant

When pancreas transplantation was first performed in the early 1970s, the results of enteric drainage (ED) were poor. The small-bowel drainage procedure fell into disfavor because of anastomotic leaks with abscess formation. Resultant sepsis caused high rates of morbidity and mortality. Recently, more centers are experiencing success with ED (Reddy et al., 1999; Stratta et al., 2000; Monroy-Cuadros et al., 2006; Lipshultz and Wilkinson, 2007) because of improvement in donor management, optimized surgical techniques during organ procurement, better preservation solutions, improvement of the implantation procedure, and new immunosuppressive drugs (Ciancio et al., 1997; 1998; Ciancio et al., 1999; Ciancio et al., 2000a; Gruessner and Sutherland, 2005). Enteric drainage techniques (Fig. 2.5) vary in bowel arrangement, level of anastomosis, site of the recipient small bowel, and choice of either stapled or hand-sewn anastomosis (Di Carlo et al., 1998).

Of the pancreas transplantations performed in 2002/2003, 82% in the SPK, 72% in the PAK, and 57% in the PTA categories were ED. Of the few ED transplants carried out before 1996/1997, most were done with a Roux-en-Y limb of the recipient bowel, but in the 2002/2003 ED pancreas transplantations only 29% used a Roux-en-Y limb (Gruessner and Sutherland, 2005).

The most serious complication of enteric-drained pancreas transplantation is a leak from the anastomotic site. This serious problem occurs 1–6 months after transplantation and results in fever, abdominal discomfort, and leukocytosis. CT scans are helpful in diagnosing the problem. The mandatory treatment is surgical exploration and repair of the enteric leak. Gastrointestinal bleeding may occur at the duodenal–enteric suture line as a result of perioperative anticoagulation and inadequate homeostasis. When conservative management is not sufficient, reoperation is necessary (Reddy et al., 1999; Stratta et al., 2000).

Enteric drainage has some advantages over bladder drainage. First, because exocrine pancreas secretions are enterically directed, metabolic acidosis and dehydration do not occur and bicarbonate supplementation is not needed. Second, this procedure is not associated with urological complications, such as urinary infections, hematuria, bladder stones, and urinary leaks. Third, fewer laboratory tests are required because there is no reason to monitor urinary activity. However, rejection episodes may progress undiagnosed before treatment is started, and this delay increases the possibility of allograft loss.

### *2.3.4 Conclusions*

The optimal treatment for T1DM in the context of ESRD, where the primary goal is to restore normal glucose metabolism and then kidney function, is achieved by whole pancreas and kidney allograft transplantation. The administration of lymphodepleting agents continues to increase, whereas use of IL2 receptor antagonists is declining. The main goal of induction therapy is to provide a strong and long-term immunosuppressive effect for protocols that include steroid avoidance, CNIs minimization, or even monotherapy maintenance. The current trend is to reduce or minimize the number of immunosuppressive drugs in order to prevent or avoid side effects and adverse events. The challenge is to find the balance between benefit (protection from AR and long-term graft function) and risk (side effects, infection, cancers).

**Acknowledgments** We thank Mr. John Wilkes for assistance in reviewing and editing the manuscript.

For further information including transplant data and annual reports

U.S. Department of Health and Human Services (http://www.hhs.gov); Organ Procurement and Transplantation Network (http://www.optn.org); Scientific Registry of Transplant Recipients (http://www.ustransplant.org); Health Resources and Services Administration (http://www.hrsa.gov); Collaborative Islet Transplant Registry (http://www.citregistry.org); and Clinical Islet Transplant consortium (www.citisletstudy.org).

## References

Alejandro R, Barton FB, Hering BJ, et al. (2008) Collaborative Islet Transplant Registry Investigators. 2008 Update from the Collaborative Islet Transplant Registry (CITR). Transplantation. 86:1783–1788.

Alejandro R, Lehmann R, Ricordi C, et al. (1997) Long-term function (6 years) of islet allografts in type 1 diabetes. Diabetes. 46:1983–1989.

Badet L, Benhamou PY, Wojtusciszyn A, et al. (2007) Expectations and strategies regarding islet transplantation: metabolic data from the GRAGIL 2 trial. Transplantation. 84:89–96.
Baidal DA, Faradji RN, Messinger S, et al. (2009) Early metabolic markers of islet allograft dysfunction. Transplantation. 87:689–697.
Baidal DA, Froud T, Ferreira JV, et al. (2003) The bag method for islet cell infusion. Cell Transplant. 12:809–813.
Barbaro B, Salehi P, Wang Y, et al. (2007) Improved human pancreatic islet purification with the refined UIC-UB density gradient. Transplantation. 84:1200–1203.
Barshes NR, Lee TC, Goodpastor SE, et al. (2005) Transaminitis after pancreatic islet translantation. J Am Coll Surg. 200:353–361.
Bellin MD, Kandaswamy R, Parkey J, et al. (2008) Prolonged insulin independence after islet allotransplants in recipients with type 1 diabetes. Am J Transplant. 8:2463–2470.
Bhargava R, Senior PA, Ackerman TE, et al. (2004) Prevalence of hepatic steatosis after islet transplantation and its relation to graft function. Diabetes. 53:1311–1317.
Bosi E, Braghi S, Maffi P, et al. (2001) Autoantibody response to islet transplantation in type 1 diabetes. Diabetes. 50:2464–2471.
Bretzel RG, Brandhorst D, Brandhorst H, et al. (1999) Improved survival of intraportal pancreatic islet cell allografts in patients with type-1 diabetes mellitus by refined peritransplant management. J Mol Med. 77:140–143.
Bruce DS, Sollinger HW, Humar A, et al. (2001) Multicenter survey of daclizumab induction in simultaneous kidney-pancreas transplant recipients. Transplantation. 72:1637–1643.
Burke GW, Ciancio G. (1997) Critical care issues in the renal and pancreatic allograft recipient. In: Civetta JM, Taylor RW, Kirby RR (eds). Critical Care, 3rd edn. Lippincott; Philadelphia.
Burke GW, Ciancio G, Alejandro R, et al. (1998a) Cholesterol control: long-term benefit of pancreas-kidney transplantation with FK506 immunosuppression. Transplant Proc. 30: 513–514.
Burke GW, Ciancio G, Alejandro R, et al. (1998b) The use of tacrolimus and mycophenolate mofetil (MMF) for pancreas/kidney transplantation with or without OKT3 induction. Transplant Proc. 30:1544–1545.
Burke GW, Ciancio G, Cirocco R, et al. (1999) Microangiopathy in kidney and simultaneous pancreas/kidney recipients treated with tacrolimus: evidence of endothelin and cytokine involvement. Transplantation. 68:1336–1342.
Burke GW, Ciancio G, Figueiro J, et al. (2002a) Can acute rejection be prevented in SPK transplantation. Transplant Proc. 34:1913–1914.
Burke GW, Ciancio G, Figueiro J, et al. (2002b) Steroid-resistant acute rejection following SPK: importance of maintaining therapeutic dosing in a triple-drug regimen. Transplant Proc. 34:1918–1919.
Burke GW, Ciancio G, Figueiro J, et al. (2004a) Hypercoagulable state associated with kidney-pancreas transplantation. Thromboelastogram-directed anti-coagulation and implications for future therapy. Clin Transplant. 18:423–428.
Burke GW, Ciancio G, Olson L, et al. (2001) Ten-year survival after simultaneous pancreas/kidney transplantation with bladder drainage and tacrolimus-based immunosuppression. Transplant Proc. 33:1681–1683.
Burke GW, Ciancio G, Sollinger HW. (2004b) Advances in pancreas transplantation. Transplantation. 77:S62–S67.
Bussiere CT, Lakey JR, Shapiro AM, et al. (2006) The impact of the mTOR inhibitor sirolimus on the proliferation and function of pancreatic islets and ductal cells. Diabetologia. 49: 2341–2349.
Cabrera O, Berman DM, Kenyon NS, et al. (2006) The unique cyto-architecture of human pancreatic islets has implications for islet cell function. Proc Natl Acad Sci USA. 103:2334–2339.
Campbell PM, Salam A, Ryan EA, et al. (2007a) Pretransplant HLA antibodies are associated with reduced graft survival after clinical islet transplantation. Am J Transplant. 7:1242–1248.

Campbell PM, Senior PA, Salam A, et al. (2007b) High risk of sensitization after failed islet transplantation. Am J Transplant. 7:2311–2317.
Cantaluppi V, Biancone L, Romanazzi GM, et al. (2006a) Antiangiogenic and immunomodulatory effects of rapamycin on islet endothelium: relevance for islet transplantation. Am J Transplant. 6:2601–2611.
Cantaluppi V, Biancone L, Romanazzi GM, et al. (2006b) The impact of the mTOR inhibitor sirolimus on the proliferation and function of pancreatic islets and ductal cells. Diabetologia. 49:2341–2349.
Cantarovich D, Vistoli F. (2009) Minimization protocols in pancreas transplantation. Transpl Int. 22:61–68.
Cardani R, Pileggi A, Ricordi C, et al. (2007) Allosensitization of islet allograft recipients. Transplantation. 84:1413–1427.
Ciancio G, Burke GW, Miller J. (2000a) Current treatment practices in immunosuppression. Expert Opin Pharmacother. 1:1307–1330.
Ciancio G, Burke GW, Nery J, et al. (1995) Urologic complications following simultaneous pancreas-kidney transplantation. Transplant Proc. 27:3125–3126.
Ciancio G, Burke GW, Nery JR, et al. (1996a) Urethritis/dysuria after simultaneous pancreas-kidney transplantation. Clin Transplantation. 10:67–70.
Ciancio G, Burke GW, Roth D, et al. (1997) Tacrolimus. Curr Opin Transplant. 2:62–67.
Ciancio G, Burke GW, Roth D, et al. (1998) Tacrolimus and Mycophenolate Mofetil as primary immunosuppression for renal allograft recipients. In: Racusen LC, Solez K, Burdick J (eds). Kidney Transplant Rejection: Diagnosis and Treatment. Marcel Dekker; New York.
Ciancio G, Burke GW, Roth D, et al. (1999) Tacrolimus and mycophenolate mofetil regimens in transplantation: benefits and pitfalls. Biodrugs. 11:395–407.
Ciancio G, Burke GW, Viciana AL, et al. (1996b) Destructive allograft fungal arteritis following simultaneous pancreas-kidney transplantation. Transplantation. 61:1172–1175.
Ciancio G, Lo Monte A, Buscemi G, et al. (2000b) Use of tacrolimus and mycophenolate mofetil as induction and maintenance in simultaneous kidney pancreas transplantation. Transplant Int. 13:S191–S194.
Clatworthy MR, Sivaprakasan R, Butler AJ, et al. (2007) Subcutaneous administration of alemtuzumab in simultaneous pancreas-kidney transplantation. Transplantation. 84:1563–1567.
Coppelli A, Giannarelli R, Vistoli F, et al. (2005) The beneficial effects of pancreas transplant alone on diabetic nephropathy. Diabetes Care. 28:1366–1370.
Cure P, Froud T, Leito CB, et al. (2008c) Late Epstein Barr virus reactivation in islet after kidney transplantation. Transplantation. 86:1324–1325.
Cure P, Pileggi A, Alejandro R. (2008b) Exenatide and rare adverse events. N Engl J Med. 358:1969–1970.
Cure P, Pileggi A, Froud T, et al. (2004) Alterations of the female reproductive system in recipients of islet grafts. Transplantation. 78:1576–1581.
Cure P, Pileggi A, Froud T, et al. (2008a) Improved metabolic control and quality of life in seven patients with type 1 diabetes following islet-after-kidney transplantation. Transplantation. 85:801–812.
Daneman D. (2006) Type 1 diabetes. Lancet. 367:847–858.
Del Carro U, Fiorina P, Amadio S, et al. (2007) Evaluation of polyneuropathy markers in type 1 diabetic kidney transplant patients and effects of islet transplantation: neurophysiological and skin biopsy longitudinal analysis. Diabetes Care. 30:3063–3069.
Deng S, Vatamaniuk M, Huang X, et al. (2004) Structural and functional abnormalities in the islets isolated from type 2 diabetic subjects. Diabetes. 53:624–632.
Desai NM, Goss JA, Deng S, et al. (2003) Elevated portal vein drug levels of sirolimus and tacrolimus in islet transplant recipients: local immunosuppression or islet toxicity? Transplantation. 76:1623–1625.
Di Carlo V, Castoldi R, Cristallo M, et al. (1998) Techniques of pancreas transplantation through the world: An IPITA Center Survey. Transplant Proc. 30:231–241.

D'Amico E, Hui H, Khoury N, et al. (2005) Pancreatic β-cells expressing GLP-1 are resistant to the toxic effects of immunosuppressive drugs. J Mol Endocrinol. 34:377–390.

Eich T, Eriksson O, Lundgren T. (2007) Nordic Network for Clinical Islet Transplantation. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. N Engl J Med. 356:2754–2755.

Emamaullee JA, Stanton L, Schur C, et al. (2007) Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long-term function in islet transplantation. Diabetes. 56:1289–1298.

Faradji RN, Cure P, Ricordi C, et al. (2007a) Care of the islet transplant recipient: immunosuppressive management and complications. (Chapter 3) In: Shapiro AMJ, Shaw J (eds). Islet Transplantation and β cell Replacement Therapy. Informa Healthcare, 147–148, London, UK.

Faradji RN, Froud T, Messinger S, et al. (2009) Long-term metabolic and hormonal effects of exenatide on islet transplant recipients with allograft dysfunction. Cell Transplant. (in press).

Faradji RN, Monroy K, Messinger S, et al. (2007b) Simple measures to monitor β-cell mass and assess islet graft dysfunction. Am J Transplant. 7:303–308.

Faradji RN, Tharavanij T, Messinger S, et al. (2008) Long-term insulin independence and improvement in insulin secretion after supplemental islet infusion under exenatide and etanercept. Transplantation. 86:1658–1665.

Farney A, Sundeberg A, Moore P, et al. (2008) A randomized trial of alemtuzumab vs. antithymocyte globulin induction in renal and pancreas transplantation. Clin Transplant. 22:41–49.

Fioretto P, Steffes MW, Sutherland DE, et al. (1998) Reversal of lesions of diabetic nephropathy after pancreas transplantation. N Engl J Med. 339:69–75.

Fiorina P, Folli F, Bertuzzi F, et al. (2003a) Long-term beneficial effect of islet transplantation on diabetic macro-/micro-angiopathy in type 1 diabetic kidney-transplanted patients. Diabetes Care. 26:1129–1136.

Fiorina P, Folli F, Maffi P, et al. (2003b) Islet transplantation improves vascular diabetic complications in patients with diabetes who underwent kidney transplantation: a comparison between kidney-pancreas and kidney-alone transplantation. Transplantation. 75:1296–1301.

Fiorina P, Gremizzi C, Maffi P, et al. (2005a) Islet transplantation is associated with an improvement of cardiovascular function in type 1 diabetic kidney transplant patients. Diabetes Care. 28:1358–1365.

Fiorina P, Venturini M, Folli F, et al. (2005b) Natural history of kidney graft survival, hypertrophy, and vascular function in end-stage renal disease of type 1 diabetic kidney-transplanted patients beneficial impact of pancreas and successful islet cotransplantation. Diabetes Care. 28:1303–1310.

Frank A, Deng S, Huang X, et al. (2004) Transplantation for type I diabetes: comparison of vascularized whole-organ pancreas with isolated pancreatic islets. Ann Surg. 240:631–640.

Froud T, Baidal DA, Faradji R, et al. (2008a) Islet transplantation with Alemtuzumab induction and calcineurin-free maintenance immunosuppression results in improved short and long-term outcomes. Transplantation. 86:1695–1701.

Froud T, Faradji RN, Pileggi A, et al. (2008b) The use of exenatide in islet transplantation recipients with chronic allograft dysfunction: safety, efficacy and metabolic effects. Transplantation. 86:36–45.

Froud T, Ricordi C, Baidal DA, et al. (2005) Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience. Am J Transplant. 5:2037–2046.

Froud T, Yrizarry JM, Alejandro R, et al. (2004) Use of D-STAT to prevent bleeding following percutaneous transhepatic intraportal islet transplantation. Cell Transplant. 13:55–59.

Fung MA, Warnock GL, Ao Z, et al. (2007) The effect of medical therapy and islet cell transplantation on diabetic nephropathy: an interim report. Transplantation. 84:17–22.

Gallon LG, Winoto J, Chhabra D, et al. (2007) Long-term renal transplantation function in recipient of simultaneous kidney and pancreas transplant maintained with two prednisone-free maintenance immunosuppressive combinations: Tacrolimus/Mycophenolate Mofetil versus Tacrolimus/Sirolimus. Transplantation. 83:1324–1329.

Gangemi A, Salehi P, Hatipoglu B, et al. (2008) Islet transplantation for brittle type 1 diabetesthe UIC Protocol. Am J Transplant. 8:1250–1261.

Gao R, Ustinov J, Korsgren O, et al. (2007) Effects of immunosuppressive drugs on in vitro neogenesis of human islets: mycophenolate mofetil inhibits the proliferation of ductal cells. Am J Transplant. 7:1021–1026.

Geiger MC, Ferreira JV, Hafiz MM, et al. (2005) Evaluation of metabolic control using a continuous subcutaneous glucose monitoring system in patients with type 1 diabetes mellitus who achieved insulin independence after islet cell transplantation. Cell Transplant. 14: 77–84.

Gerber PA, Pavlicek V, Demartines N, et al. (2008) Simultaneous islet-kidney vs pancreas-kidney transplantation in type 1 diabetes mellitus: a 5 year single centre follow-up. Diabetologia. 51:110–119.

Gerstein HC, Miller ME, Byington RP, et al. (2008) Action to control cardiovascular risk in diabetes study group: effects of intensive glucose lowering in type 2 diabetes. N Engl J Med. 358:2545–2559.

Ghofaili KA, Fung M, Ao Z, et al. (2007) Effect of exenatide on β cell function after islet transplantation in type 1 diabetes. Transplantation. 83:24–28.

Giannarelli R, Copelli A, Sartini MS, et al. (2006) Pancreas transplant alone has beneficial effects on retinopathy in type 1 diabetic patients. Diabetologia. 49:2977–2982.

Gillard P, Ling Z, Mathieu C, et al. (2008) Comparison of sirolimus alone with sirolimus plus tacrolimus in type 1 diabetic recipients of cultured islet cell grafts. Transplantation. 85: 256–263.

Gores PF, Najarian JS, Stephanian E, et al. (1993) Insulin independence in type I diabetes after transplantation of unpurified islets from single donor with 15-deoxyspergualin. Lancet. 341: 19–21.

Gorn L, Faradji RN, Messinger S, et al. (2008) Impact of islet transplantation on glycemic control as evidenced by a continuous glucose monitoring system. J Diab Sci Tech. 2:221–228.

Goss JA, Goodpastor SE, Brunicardi FC, et al. (2004) Development of a human pancreatic islet-transplant program through a collaborative relationship with a remote islet-isolation center. Transplantation. 77:462–466.

Goss JA, Soltes G, Goodpastor SE, et al. (2003) Pancreatic islet transplantation: the radiographic approach. Transplantation. 76:199–203.

Gross CR, Limwattananon C, Matthees B, et al. (2002) Impact of transplantation on quality of life in patients with diabetes and renal dysfunction. Transplantation. 70:1736–1746.

Gruessner RW, Burke GW, Stratta R, et al. (1996) Multicenter analysis of the first experience with FK506 for induction and rescue therapy after pancreas transplantation. Transplantation. 61:261–273.

Gruessner RW, Kandaswamy R, Humar A, et al. (2005) Calcineurin inhibitor and steroid-free immunosuppression in pancreas-kidney and solitary pancreas transplantation. Transplantation. 79:1184–1189.

Gruessner AC, Sutherland DE. (2001) Report from the International Pancreas Transplant Registry 2000. Transplant Proc. 33:1643–1646.

Gruessner AC, Sutherland DE. (2005) Pancreas transplant outcomes for United States (US) and non-US cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) as of June 2004. Clin Transplant. 19:433–455.

Gruessner RW, Sutherland DE, Gruessner AC. (2004) Mortality assessment for pancreas transplants. Am J Transplant. 4:2018–2026.

Hafiz MM, Faradji RN, Froud T, et al. (2005) Immunosuppression and procedure-related complications in 26 patients with type 1 diabetes mellitus receiving allogeneic islet cell transplantation. Transplantation. 80:1718–1728.

Hafiz MM, Poggioli R, Caulfield A, et al. (2004) Cytomegalovirus prevalence and transmission after islet allograft transplant in patients with type 1 diabetes mellitus. Am J Transplant. 4:1697–1702.

Han D, Xu X, Baidal D, et al. (2004) Assessment of cytotoxic lymphocyte gene expression in the peripheral blood of human islet allograft recipients. Elevation precedes clinical evidence of rejection. Diabetes. 53:2281–2290.

Hanley SC, Paraskevas S, Rosenberg L. (2008) Donor and isolation variables predicting human islet isolation success. Transplantation. 85:950–955.

Hansen A, Johansson BL, Wahren J, et al. (2002) C-peptide exerts beneficial effects on myocardial blood flow and function in patients with type 1 diabetes. Diabetes. 51:3077–3082.

Hathaway DK, Abell T, Cardoso S, et al. (1994) Improvement in autonomic and gastric function following pancreas-kidney versus kidney-alone transplantation and the correlation with quality of life. Transplantation. 27:816–822.

Havrdova T, Saudek F, Boucek P, et al. (2005) Metabolic effect of sirolimus versus mycophenolate mofetil on pancreatic graft function in the early posttransplant period. Transplant Proc. 37:3544–3545.

Hering BJ, Kandaswamy R, Ansite JD, et al. (2005) Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. J Am Med Assoc. 293:830–835.

Hering BJ, Kandaswamy R, Harmon JV, et al. (2004) Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. Am J Transplant. 4: 390–401.

Hirshberg B, Rother KI, Digon BJ 3rd , et al. (2003) Benefits and risks of solitary islet transplantation for type 1 diabetes using steroid-sparing immunosuppression: the National Institutes of Health experience. Diabetes Care. 26:3288–3295.

Huang X, Moore DJ, Ketchum RJ, et al. (2008) Resolving the conundrum of islet transplantation by linking metabolic dysregulation, inflammation, and immune regulation. Endocr Rev. 29: 603–630.

Huurman VA, Hilbrands R, Pinkse GG, et al. (2008) Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. PLoS ONE. 3:e2435.

Huurman VA, Velthuis JH, Hilbrands R, et al. (2009) Allograft-specific cytokine profiles associate with clinical outcome after islet cell transplantation. Am J Transplant. 9:382–388.

Ichii H, Wang X, Messinger S, et al. (2006) Improved human islet isolation using nicotinamide. Am J Transplant. 6:2060–2068.

Ihm SH, Matsumoto I, Sawada T, et al. (2006) Effect of donor age on function of isolated human islets. Diabetes. 55:1361–1368.

Johansson BL, Borg K, Fernqvist-Forbes E, et al. (2000) Beneficial effects of C-peptide on incipient nephropathy and neuropathy in patients with Type 1 diabetes mellitus. Diabet Med. 17:181–189.

Johansson H, Lukinius A, Moberg L, et al. (2005) Tissue factor produced by the endocrine cells of the islets of Langerhans is associated with a negative outcome of clinical islet transplantation. Diabetes. 54:1755–1762.

Kaufman DB, Leventhal JR, Gallon LG, et al. (2006) Alemtuzumab induction and prednisone-free maintenance immunotherapy in simultaneous pancreas-kidney transplantation comparison with rabbit antithymocyte globulin induction—long term results. Am J Transplant. 6:331–339.

Kempf MC, Andres A, Morel P, et al. (2005) Logistics and transplant coordination activity in the GRAGIL Swiss-French multicenter network of islet transplantation. Transplantation. 79: 1200–1205.

Kin T, Mirbolooki M, Salehi P, et al. (2006) Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using preoxygenated perfluorocarbon. Transplantation. 82:1286–1290.

Lakey JR, Warnock GL, Rajotte RV, et al. (1996) Variables in organ donors that affect the recovery of human islets of Langerhans. Transplantation. 61:1047–1053.

Largiadr F, Kolb E, Binswanger U. (1980) A long-term functioning human pancreatic islet allotransplant. Transplantation. 29:76–77.

Lau J, Carlsson PO. (2009) Low revascularization of human islets when experimentally transplanted into the liver. Transplantation. 87:322–325.

Lee TC, Barshes NR, Brunicardi FC, et al. (2004) Procurement of the human pancreas for pancreatic islet transplantation. Transplantation. 78:481–483.
Lee TC, Barshes NR, O'Mahony CA, et al. (2005) The effect of pancreatic islet transplantation on progression of diabetic retinopathy and neuropathy. Transplant Proc. 37:2263–2265.
Lehmann R, Weber M, Berthold P, et al. (2004) Successful simultaneous islet-kidney transplantation using a steroid-free immunosuppression: two-year follow-up. Am J Transplant. 4:1117–1123.
Leibiger IB, Berggren PO. (2008) Insulin signaling in the pancreatic β-cell. Annu Rev Nutr. 28:233–251.
Leichtman AB, Cohen D, Keith D, et al. (2008) Kidney and pancreas transplantation in the United States, 1997–2006: The HRSA breakthrough collaboratives and the 58 DSA challenge. Am J Transplant. 8:946–957.
Leitao CB, Cure P, Messinger S, et al. (2009) Stable renal function after islet transplantation: importance of patient selection and aggressive clinical management. Transplantation. 87: 681–688.
Leitao CB, Cure P, Tharavanij T, et al. (2008a) Current challenges in islet transplantation. Curr Diab Rep. 8:324–331.
Leitao CB, Tharavanij T, Cure P, et al. (2008b) Restoration of hypoglycemia awareness after islet transplantation. Diabetes Care. 31:2113–2115.
Leroith D, Taylor SI, Olefky JM (eds). (2003) Diabetes Mellitus. A Fundamental and Clinical Text, 3rd edn. Lippincott, Williams Wilkins; Philadelphia.
Lipshultz GS, Wilkinson AH. (2007) Pancreas-kidney and pancreas transplantation for the treatment of diabetes mellitus. Endocrinol Metab Clin North Am. 36:1015–1038.
Lo A, Stratta RJ, Alloway RR, et al. (2001a) Initial clinical experience with interleukin-2 receptor antagonist induction in combination with tacrolimus, mycophenolate mofetil and steroids in simultaneous kidney-pancreas transplantation. Transplant Int. 14:396–404.
Lo A, Stratta RJ, Alloway RR, et al. (2001b) Limited benefits of induction with monoclonal antibody to interleukin-2 receptor in combination with tacrolimus, mycophenolate mofetil, and steroids in simultaneous kidney-pancreas transplantation. Transplant Proc. 33: 1701–1703.
Luzi L, Perseghin G, Brendel MD, et al. (2001) Metabolic effects of restoring partial β-cell function after islet allotransplantation in type 1 diabetic patients. Diabetes. 50:277–282.
Maffi P, Bertuzzi F, De Taddeo F, et al. (2007) Kidney function after islet transplant alone in type 1 diabetes: impact of immunosuppressive therapy on progression of diabetic nephropathy. Diabetes Care. 30:1150–1155.
Magliocca JF, Odorico JS, Pirsch JD, et al. (2008) Comparison of alemtuzumab with basiliximab induction in simultaneous pancreas-kidney transplantation. Am J Transplant. 8: 1702–1710.
Marcelli-Tourvieille S, Hubert T, Moerman E, et al. (2007) In vivo and in vitro effect of sirolimus on insulin secretion. Transplantation. 83:532–538.
Marzorati S, Pileggi A, Ricordi C. (2007) Allogeneic islet transplantation. Expert Opin Biol Ther. 7:1627–1645.
Matas AJ, Gillingham KJ, Humar A, et al. (2008) Posttransplant diabetes mellitus and acute rejection: impact on kidney transplant outcome. Transplant Int. 85:338–343.
McCrimmon R. (2008) The mechanisms that underlie glucose sensing during hypoglycaemia in diabetes. Diabet Med. 25:513–522.
Medarova Z, Moore A. (2008) Non-invasive detection of transplanted pancreatic islets. Diabetes Obes Metab. 10(Suppl 4):88–97.
Meier JJ, Hong-McAtee I, Galasso R, et al. (2006) Intrahepatic transplanted islets in humans secrete insulin in a coordinate pulsatile manner directly into the liver. Diabetes. 55: 2324–2332.
Merani S, Toso C, Emamaullee J, et al. (2008) Optimal implantation site for pancreatic islet transplantation. Br J Surg. 95:1449–1461.

Mineo D, Cure P, Baidal DA, et al. (2008b) Defining the immune response levels in islet allotransplantation by the immune cell function assay Immuknow-Cylex. Transplantation. 86(Suppl 2449).

Mineo D, Ricordi C, Xu X, et al. (2008a) Combined islet and hematopoietic stem cell allotransplantation: a clinical pilot trial to induce chimerism and graft tolerance. Am J Transplant. 8:1262–1274.

Mineo D, Sageshima J, Burke GW, et al. (2008c) Minimization and withdrawal of steroids in pancreas and islet transplantation. Transplant Int. 22:20–37.

Mintz DH, Alejandro R, Miller J. (1988) Human islet transplantation; the University of Miami experience. In: van Schilfgaarde R, Hardy MA (eds). Transplantation of the Endocrine Pancreas in Diabetes Mellitus. Elsevier Science; Philadelphia, PA.

Moberg L, Johansson H, Lukinius A, et al. (2002) Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. Lancet. 360:2039–2045.

Mohanakumar T, Narayanan K, Desai N, et al. (2006) A significant role for histocompatibility in human islet transplantation. Transplantation. 82:180–187.

Monroy-Cuadros M, Salazar A, Yilmaz S, et al. (2006) Bladder vs enteric drainage in simultaneous pancreas-kidney transplantation. Nephrol Dial Transplant. 21:483–487.

Monti P, Scirpoli M, Maffi P, et al. (2008) Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. J Clin Invest. 118:1806–1814.

Muthusamy ASR, Vaidya AC, Sinha S, et al. (2008) Alemtuzumab induction and steroid-free maintenance immunosuppression in pancreas transplantation. Am J Transplant. 8:2126–2131.

Müller-Felber W, Landgraf R, Scheuer R, et al. (1993) Diabetic neuropathy 3 years after successful pancreas and kidney transplantation. Diabetes. 42:1482–1486.

Nano R, Clissi B, Melzi R, et al. (2005) Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. Diabetologia. 48:906–912.

Navarro X, Sutherland DE, Kennedy WR. (1998) Long-term effects of pancreas transplantation on diabetic neuropathy. Ann Neurol. 42:727–736.

Nir T, Melton DA, Dor Y. (2007) Recovery from diabetes in mice by β cell regeneration. J Clin Invest. 117:2553–2561.

Oberholzer J, Triponez F, Mage R, et al. (2000) Human islet transplantation: lessons from 13 autologous and 13 allogeneic transplantations. Transplantation. 69:1115–1123.

O'Connell PJ, Hawthorne WJ, Holmes-Walker DJ, et al. (2006) Clinical islet transplantation in type 1 diabetes mellitus: results of Australia's first trial. Med J Aust. 184:221–225.

Park YJ, Ahn HJ, Chang HK, et al. (2009) The RhoGDI-alpha/JNK signaling pathway plays a significant role in mycophenolic acid-induced apoptosis in an insulin-secreting cell line. Cell Signal. 21:356–364.

Paty BW, Ryan EA, Shapiro AM, et al. (2002) Intrahepatic islet transplantation in type 1 diabetic patients does not restore hypoglycemic hormonal counterregulation or symptom recognition after insulin independence. Diabetes. 51:3428–3434.

Pearce IA, Ilango B, Sells RA, et al. (2000) Stabilisation of diabetic retinopathy following simultaneous pancreas and kidney transplant. Br J Ophthalmol. 84:736–740.

Pileggi A, Cobianchi L, Inverardi L, et al. (2006) Overcoming the challenges now limiting islet transplantation: a sequential, integrated approach. Ann NY Acad Sci. 1079:383–398.

Pileggi A, Ricordi C, Kenyon NS, et al. (2004) Twenty years of clinical islet transplantation at the Diabetes Research Institute-University of Miami. Clin Transpl:177–204.

Pinkse GG, Tysma OH, Bergen CA, et al. (2005) Autoreactive CD8 T cells associated with β cell destruction in type 1 diabetes. Proc Natl Acad Sci USA. 102:18425–18430.

Poggioli R, Enfield G, Messinger S, et al. (2008) Nutritional status and behavior in subjects with type 1 diabetes, before and after islet transplantation. Transplantation. 85:501–506.

Poggioli R, Faradji RN, Ponte G, et al. (2006) Quality of life after islet transplantation. Am J Transplant. 6:371–378.

Poitout V, Robertson RP. (2008) Glucolipotoxicity: fuel excess and β-cell dysfunction. Endocr Rev. 29:351–366.
Ponte GM, Pileggi A, Messinger S, et al. (2007) Toward maximizing the success rates of human islet isolation: influence of donor and isolation factors. Cell Transplant. 16:595–607.
Porrett PM, Yeh H, Frank A, et al. (2007) Availability of suitable islet donors in the United States. Transplantation. 84:280–282.
Posselt A, Szot G, Frassetto L, et al. (2008) Islet allograft survival in type 1 diabetics using a calcineurin-free based on Efalizumab and Sirolimus. Am J Transplant. 8(Suppl 2):S235.
Rafael E, Tibell A, Rydn M, et al. (2008) Intramuscular autotransplantation of pancreatic islets in a 7-year-old child: a 2-year follow-up. Am J Transplant. 8:458–462.
Rangan GK. (2006) Sirolimus-associated proteinuria and renal dysfunction. Drug Saf. 29: 1153–1161.
Ranganath LR. (2008) The entero-insular axis: implications for human metabolism. Clin Chem Lab Med. 46:43–56.
Ranta F, Avram D, Berchtold S, et al. (2006) Dexamethasone induces cell death in insulin-secreting cells, an effect reversed by exendin-4. Diabetes. 55:1380–1390.
Reddy KS, Stratta RJ, Shokouh-Amiri MH, et al. (1999) Surgical complications after pancreas transplantation with portal-enteric drainage. J Am Col Surg. 189:305–313.
Rickels MR, Kearns J, Markmann E, et al. (2006b) HLA sensitization in islet transplantation. Clin Transpl. 77:413–420.
Rickels MR, Naji A, Teff KL. (2006a) Insulin sensitivity, glucose effectiveness, and free fatty acid dynamics after human islet transplantation for type 1 diabetes. J Clin Endocrinol Metab. 91:2138–2144.
Rickels MR, Naji A, Teff KL. (2007b) Acute insulin responses to glucose and arginine as predictors of β-cell secretory capacity in human islet transplantation. Transplantation. 84: 1357–1360.
Rickels MR, Schutta MH, Markmann JF, et al. (2005b) {β}-Cell function following human islet transplantation for type 1 diabetes. Diabetes. 54:100–106.
Rickels MR, Schutta MH, Mueller R, et al. (2005a) Islet cell hormonal responses to hypoglycemia after human islet transplantation for type 1 diabetes. Diabetes. 54:3205–3211.
Rickels MR, Schutta MH, Mueller R, et al. (2007a) Glycemic thresholds for activation of counterregulatory hormone and symptom responses in islet transplant recipients. J Clin Endocrinol Metab. 92:873–879.
Ricordi C. (1992) Pancreatic islet cell transplantation 1892–1992. In: Ricordi C (ed). One Century of Transplantation for Diabetes. Landes; Austin.
Ricordi C. (2003) Islet transplantation: a brave new world. Diabetes. 52:1595–1603.
Ricordi C, Lacy PE, Finke EH, et al. (1988) Automated method for isolation of human pancreatic islets. Diabetes. 37:413–420.
Ricordi C, Strom TB. (2004) Clinical islet transplantation: advances and immunological challenges. Nat Rev Immunol. 4:259–268.
Ricordi C, Tzakis AG, Carroll PB, et al. (1992) Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation. 53:407–414.
Roep BO, Stobbe I, Duinkerken G, et al. (1999) Auto- and alloimmune reactivity to human islet allografts transplanted into type 1 diabetic patients. Diabetes. 48:484–490.
Ryan EA, Bigam D, Shapiro AM. (2006) Current indications for pancreas or islet transplant. Diabetes Obes Metab. 8:1–7.
Ryan EA, Lakey JR, Paty BW, et al. (2002) Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes. 15:2148–2157.
Ryan EA, Paty BW, Senior PA, et al. (2005) Five-year follow-up after clinical islet transplantation. Diabetes. 54:2060–2069.
Ryan EA, Shandro T, Green K, et al. (2004) Assessment of the severity of hypoglycemia and glycemic lability in type 1 diabetic subjects undergoing islet transplantation. Diabetes. 53: 955–962.

Sabek OM, Cowan P, Fraga DW, et al. (2008) The effect of isolation methods and the use of different enzymes on islet yield and in vivo function. Cell Transplant. 17:785–792.

Saini D, Ramachandran S, Nataraju A, et al. (2008) Activated effector and memory T cells contribute to circulating sCD30: potential marker for islet allograft rejection. Am J Transplant. 8:1798–1808.

Schaapherder AFM, van Oosterhout ECCA, Bode PJ, et al. (1993) Pancreatic graft survival after arterial thrombosis in simultaneous renal-pancreatic transplantation. Clin Transplant. 7:37–42.

Secchi A, Socci C, Maffi P, et al. (1997) Islet transplantation in IDDM patients. Diabetologia. 40:225–231.

Senior PA, Zeman M, Paty BW, et al. (2007) Changes in renal function after clinical islet transplantation: four-year observational study. Am J Transplant. 7:91–98.

Shapiro AMJ. (2008) Islet transplantation: the imperative need for continued clinical trials. Nat Clin Pract Nephrol. 4:662–663.

Shapiro AMJ, Koh A, Kin T, et al. (2008) Outcomes following Alemtuzumab induction in clinical islet transplantation. Am J Transplant. 8(Suppl 2):S213.

Shapiro AMJ, Lakey JR, Ryan EA, et al. (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med. 343:230–238.

Shapiro AMJ, Ricordi C, Hering BJ, et al. (2006) International trial of the Edmonton protocol for islet transplantation. N Engl J Med. 355:1318–1330.

Singh RP, Stratta RJ. (2008) Advances in immunosuppression for pancreas transplantation. Curr Opin Organ Transplant. 13:79–84.

Sollinger HW. (1996) Pancreatic transplantation and vascular graft thrombosis (Editorial). J Am Coll Surg. 182:362–363.

Stegall MD, Dean PG, Sung R, et al. (2007) The rationale for the new deceased donor pancreas allocation schema. Transplantation. 83:1156–1161.

Stegall MD, Lafferty KJ, Kam I, et al. (1996) Evidence of recurrent autoimmunity in human allogeneic islet transplantation. Transplantation. 61:1272–1274.

Stratta RJ, Alloway RR, Hodge E, et al. (2002) A multicenter, open-label, comparative trial of two daclizumab dosing strategies versus no antibody induction in combination with tacrolimus, mycophenolate mofetil, and steroids for the prevention of acute rejection in simultaneous kidney-pancreas transplant recipients: 6-month interim analysis. Transplant Proc. 34:1903–1905.

Stratta RJ, Alloway RR, Lo A, et al. (2001) A multicenter trial of two daclizumab dosing strategies versus no antibody induction in simultaneous kidney-pancreas transplantation: interim analysis. Transplant Proc. 33:1692–1693.

Stratta RJ, Gaber AO, Shokouh-Amiri MH, et al. (2000) A prospective comparison of systemic-bladder versus portal-enteric drainage in vascularized pancreas transplantation. Surgery. 127:217–226.

Subramanian S, Trence DL. (2007) Immunosuppressive agents: effects on glucose and lipid metabolism. Endocrinol Metab Clin North Am. 36:891–905.

Sureshkumar KK, Patel BM, Markatos A, et al. (2005) Quality of life after organ transplantation in type 1 diabetics with end-stage renal disease. Clin Transplant. 20:19–25.

Tan J, Yang S, Cai J, et al. (2008) Simultaneous islet and kidney transplantation in seven patients with type 1 diabetes and end-stage renal disease using a glucocorticoid-free immunosuppressive regimen with alemtuzumab induction. Diabetes. 57:2666–2671.

Teuscher AU, Kendall DM, Smets YF, et al. (1998) Successful islet autotransplantation in humans: functional insulin secretory reserve as an estimate of surviving islet cell mass. Diabetes. 47:324–330.

Tharavanij T, Betancourt A, Messinger S, et al. (2008) Improved long-term health-related quality of life after islet transplantation. Transplantation. 86:1161–1167.

The Diabetes Control and Complications Trial Research Group. (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med. 329:977–986.

The Diabetes Control and Complications Trial Research Group. (1997) Hypoglycemia in the diabetes control and complications trial. Diabetes. 46:271–286.
Thompson DM, Begg IS, Harris C, et al. (2008) Reduced progression of diabetic retinopathy after islet cell transplantation compared with intensive medical therapy. Transplantation. 85: 1400–1405.
Toso C, Baertschiger R, Morel P, et al. (2006) Sequential kidney/islet transplantation: efficacy and safety assessment of a steroid-free immunosuppression protocol. Am J Transplant. 6: 1049–1058.
Troppmann C, Gruessner AC, Benedetti E, et al. (1996) Vascular graft thrombosis after pancreatic transplantation: univariate and multivariate operative and operative risk factor analysis. J Am Coll Surg. 182:285–316.
Tzakis AG, Ricordi C, Alejandro R, et al. (1990) Pancreatic islet transplantation after upper abdominal exenteration and liver replacement. Lancet. 336:402–405.
Vantyghem MC, Marcelli-Tourvielle S, Pattou F, et al. (2007) Effects of non-steroid immunosuppressive drugs on insulin secretion in transplantation. Ann Endocrinol. 68:21–27.
Varona-Santos JL, Pileggi A, Molano RD, et al. (2008) c-Jun N-terminal kinase 1 is deleterious to the function and survival of murine pancreatic islets. Diabetologia. 51:2271–2280.
Venturini M, Fiorina P, Maffi P, et al. (2006) Early increase of retinal arterial and venous blood flow velocities at color Doppler imaging in brittle type 1 diabetes after islet transplant alone. Transplantation. 81:1274–1277.
Vincenti F, Kirk AD. (2008) What's next in the pipeline. Am J Transplant. 8:1972–1981.
Wahren J, Ekberg K, Johansson J, et al. (2000) Role of C-peptide in human physiology. Am J Physiol Endocrinol Metab. 278:759–768.
Warnock GL, Meloche RM, Thompson D, et al. (2005) Improved human pancreatic islet isolation for a prospective cohort study of islet transplantation vs best medical therapy in type 1 diabetes mellitus. Arch Surg. 140:735–744.
Warnock GL, Thompson DM, Meloche RM, et al. (2008) A multi-year analysis of islet transplantation compared with intensive medical therapy on progression of complications in type 1 diabetes. Transplantation. 86:1762–1766.
Westermark GT, Westermark P, Berne C, et al. (2008) Nordic Network for Clinical Islet Transplantation. Widespread amyloid deposition in transplanted human pancreatic islets. N Engl J Med. 359:977–979.
Wilkin TJ. (2008) Diabetes 1 and 2, or one and the same? Progress with the accelerator hypothesis. Pediatr Diabetes. 9:23–32.
Williams D, Haragsim L. (2006) Calcineurin nephrotoxicity. Adv Chronic Kidney Dis. 13:47–55.
Yoon JW, Jun HS. (2005) Autoimmune destruction of pancreatic β cells. Am J Ther. 12:580–591.
Zahr E, Molano RD, Pileggi A, et al. (2007) Rapamycin impairs in vivo proliferation of islet β-cells. Transplantation. 84:1576–1583.
Zimmet P, Alberti KG, Shaw J. (2001) Global and societal implications of the diabetes epidemic. Nature. 414:782–787.

# Chapter 3
# Cell Cycle Regulation in Human Pancreatic Beta Cells

**Nathalie Fiaschi-Taesch, George Harb, Esra Karsiloglu, Karen K. Takane, and Andrew F. Stewart**

**Abstract** For decades, it had been assumed that pancreatic β cells were terminally differentiated and thus unable to replicate, and that β-cell replication did not exist in any quantitatively meaningful way. This view has changed dramatically in the past decade, with abundant data demonstrating that fetal, neonatal, and adult rodent β cells replicate at physiologically important rates. These new data have resulted in a plethora of new reports exploring the nutrient, growth factor, and signaling cascades that lie upstream and regulate the cell cycle machinery that controls rodent β-cell replication. Moreover, myriad reports of murine genetic models of cell cycle molecule knockout or overexpression have appeared and have documented unequivocally the importance and therapeutic relevance of cell cycle regulatory mechanisms in murine β cells. These events contrast with the pace of development of new knowledge regarding human β-cell replication. It seems clear that unlike in rodents, spontaneous replication of adult human β cells is uncommon. Further, standard manipulations, nutrients, and growth factors that induce rodent β-cell replication fail to do so in human β cells. In this chapter we focus on the molecular control of cell cycle progression in human β cells, illustrate the differences between human and rodent β-cell cycle regulatory control, and provide examples of approaches to inducing human β-cell replication.

## 3.1 Introduction

Several observations in the past decade have underscored the critical importance and potential therapeutic relevance of enhancing β-cell replication. One important advance was the Edmonton report in 2000, which demonstrated that β-cell replacement with human cadaveric islets in patients with diabetes can be efficacious in restoring glycemic control (Shapiro et al., 2000; Ryan et al., 2005). However, it also demonstrated that the widespread application of β-cell replacement therapy is

N. Fiaschi-Taesch (✉)
Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA
e-mail: nmt2@pitt.edu

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_3,

limited by a number of factors, including an inability to generate sufficient numbers of human β cells. This report highlighted the need for expanding human β-cell mass in vitro and in vivo.

A second important pair of observations was the documentation by Butler and others in 2003–2005 (Butler et al., 2003; Yoon et al., 2003; Meier et al., 2005) that showed: (a) that human β cells remain present and are continuously trying to regenerate in subjects with type 1 diabetes, and (b) that autopsy of patients with type 2 diabetes displays marked reductions in β-cell mass. Thus, both type 1 and type 2 diabetes can be considered β-cell-deficiency diseases.

A third surprising and important observation arose from the genome-wide association studies in 2007–2008 (reviewed in Florez, 2008; Lindgren and McCarthy, 2008), which employed an unbiased population genetic approach for identifying genetic loci associated with type 2 diabetes. To the surprise of many, most of the genetic loci associated with type 2 diabetes represented genes likely involved with β-cell function, such as a zinc transporter, the potassium inward rectifier, the Wolfram syndrome gene, and certain cell cycle genes.

A fourth area of progress in recent years has been in stem cell differentiation into β cells. Kroon et al. and others (Kroon et al., 2008; Zaret and Grompe, 2008) have demonstrated that it is possible to generate human β cells in at least small numbers from human embryonic stem cells. More recently, investigators have begun to develop techniques for inducing pluripotent stem cells from adult somatic cells, such as dermal fibroblasts, by reprogramming them to become stem-cell-like, and then coaxing them along the β-cell differentiation program (Park et al., 2008; Tateishi et al., 2008).

Collectively, these observations have focused attention on developing techniques for inducing human β-cell expansion, regeneration, and replication. This is a challenging goal, given the accepted view that adult pancreatic β cells do not replicate under normal circumstances. This perception changed abruptly in 2004 when Dor et al. demonstrated that normal adult mouse β cells did replicate and that replication was one of the means, if not the principal one, by which adult β-cell mass was maintained in mice (Dor et al., 2004). This important paper was accompanied by a flurry of others demonstrating that knockout or overexpression of molecules known to control the G1/S transition in cell cycle progression in other cell types affected β-cell replication in mice, with corresponding changes in β-cell mass, β-cell function, and glycemic homeostasis (Hanahan, 1985; Efrat et al., 1988; Pestell et al., 1999; Rane et al., 1999; Tsutsui et al., 1999; Franklin et al., 2000; Milo-Landesman et al., 2001; Georgia and Bhushan, 2004; Karnick et al., 2005; Kushner et al., 2005; Uchida et al., 2005; Zhang et al., 2005; Cozar-Castellano et al., 2006a, b; Cozar-Castellano et al., 2006c; Heit et al., 2006; Krishnamurthy et al., 2006; Harb et al., 2009). Thus, in 2009, most diabetes researchers would agree that rodent β cells can and do replicate and that this process can be experimentally altered in rodents by manipulating many of the molecules that control the G1/S transition.

In this chapter, we discuss the fundamental mechanisms that control adult β-cell replication. In particular, we focus on the G1/S checkpoint in the cell cycle, which represents a critical checkpoint in most cell types and a central target for

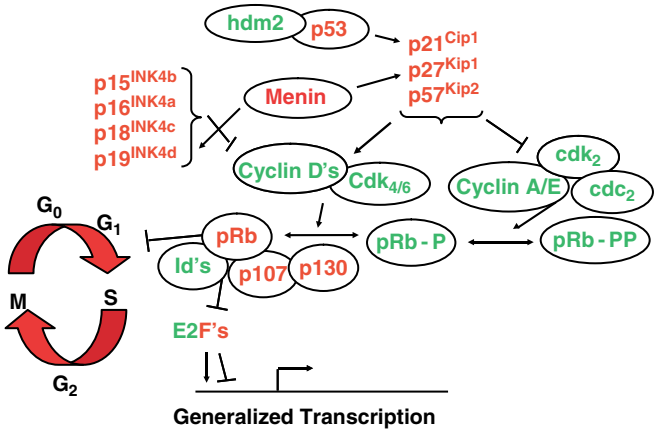



**Fig. 3.1 Components of the G1/S checkpoint proteome in human islets.** The scheme is based on immunoblots for each protein performed on extracts of isolated human islets from multiple donors. Molecules shown in green are proteins that stimulate cell cycle progression and those in red are cell cycle inhibitors. This is a useful model for studying the mechanisms of cell cycle control in human β cells, but it also has shortcomings. For example, since it is based on analyses of whole human islets, which contain many cell types apart from β cells, it may not accurately reflect events in β cells. In addition, the hierarchical organization and interaction is deduced from other cell types and species and is likely far more complex, as well as tissue-specific. Reproduced with permission from Fiaschi-Taesch et al., 2009

growth factors and nutrients acting to prevent or to stimulate cell cycle progression (Fig. 3.1). This pathway is also referred to as the "pRb pathway," since the final common molecule at the end of the pathway is the retinoblastoma protein, pRb. This pathway has been intensely studied in *Drosophila*, *C. elegans*, *Saccharomyces*, and mice, particularly in murine developmental models. The G1/S transition has also been studied extensively in human cancer, as some 90% of human cancers include abnormalities in pRb or upstream molecules that regulate pRb function. At the time of our review in 2006 (Cozar-Castellano et al., 2006a) we focused on the mechanisms that control the G1/S checkpoint in mouse β cells and pointed out that surprisingly little was known about the control of cell cycle progression in human β cells. Recently, we have updated this G1/S regulation story as it applies to the murine β cell (Harb et al., 2009). Several excellent recent reviews on cell cycle control in murine β cells are available (Pestell et al., 1999; Heit et al., 2006). In this chapter we focus on events that regulate the G1/S transition in human β cells and on attempts to use this knowledge in a therapeutic manner to expand human β-cell mass and augment human β-cell function.

## 3.2 Differences between Human and Rodent Beta Cells

It is important to note that although the basic biology of rodent and human β cells may be similar, human islets differ in many important ways from their rodent

counterparts. This suggests the possibility that molecular events controlling G1/S transition in mouse β cells may not apply to human β cells, and vice versa. Examples of human–rodent β-cell differences follow: (1) The fundamental architecture of human islets is different from that of rodents, with β cells forming a core in rodent islets, surrounded by a mantle of glucagon- , somatostatin- , PP- , and ghrelin-producing cells, whereas in human islets these several cell types are admixed and there is no mantle (Cozar-Castellano et al., 2004; Brissova et al., 2005; Cabrera et al., 2006). (2) GLUT2 is the principal glucose transporter in mouse and rat β cells, but is barely expressed in human β cells, where GLUT1 likely subserves the glucose transport function (De Vos et al., 1995; Schuit, 1997), which is why rodent β cells are much more sensitive to streptozotocin than human β cells. (3) The transcription factor neurogenin-3 is essential for mouse β-cell development, and its loss in mice leads to neonatal diabetes resulting from failure of β-cell development (Gradwohl et al., 2000). In contrast, humans with inactivating mutations in neurogenin-3 develop intestinal disease but are able to develop functional β cells (Wang et al., 2006). (4) Heterozygous loss of the transcription factors HNF4α, HNF1α, and HNF1ß results in maturity-onset diabetes of the young (MODY) syndromes in humans (MODY1, 3, and 5, respectively), but has no appreciable phenotype in mice (Stoffel and Duncan, 1997; Pontoglio et al., 1998; Yorifuji et al., 2004). (5) It has been clear for decades that among rodents subjected to partial pancreatectomy, up to 90% are able to regenerate their β cells as well as their exocrine pancreas. Recent studies by Meier et al. and others demonstrate that this is not true for humans (Kumar et al., 2008; Menge et al., 2008). (6) Islet amyloid polypeptide (IAPP), a protein secreted from β cells, self-assembles and denatures in human but not rodent islets (Matveyenko and Butler, 2006). (7) Cell cycle control is not the same in rodent and human islets: cyclin-dependent kinase-3 (CDK3) is absent in rodent islets (Malumbres and Barbacid, 2005) but present in humans; CDK6 is absent (or expressed at very low levels) in mouse islets, but is robustly expressed in human islets (Fiaschi-Taesch et al., 2009); and cyclin $D_2$ is essential for β-cell development and function in the mouse (Georgia and Bhushan, 2004; Kushner et al., 2005), but is present at very low levels in human β cells (Lavine et al., 2008; Fiaschi-Taesch et al., 2009). (8) Finally, although there are many rodent (mouse, rat, and hamster) insulinoma cell lines (Hohmeier and Newgard, 2004; Cozar-Castellano et al., 2008), continuously growing, insulin-producing human β-cell lines were difficult to generate. This suggests fundamental differences in oncogenic mechanisms as they relate to rodent versus human β cells.

Thus, with the realization that stimulation of human β-cell replication might be important in treating types 1 and 2 diabetes, the increasingly lucid picture of the molecular mechanisms for control of rodent β-cell replication, and the increasing evidence that human β cells are broadly similar to—but also very different from—rodent β cells, we elected to focus our attention on understanding and manipulating the molecular control mechanisms of G1/S progression in human β cells (Fiaschi-Taesch et al., 2009).

## 3.3 Embryonic and Neonatal Human Beta Cells Can Replicate

It has been clear for years that fetal and neonatal human β cells normally can and do replicate. For example, in 2000, using Ki67 staining, Kassem and Glaser showed that embryonic and neonatal human β cells could replicate (Kassem et al., 2000), mirroring events in embryonic and neonatal mouse β cells. More recently, Meier and Butler reaffirmed these observations in human neonates (Meier et al., 2008). Over the years Hayek and his collaborators have also demonstrated that embryonic and neonatal human β cells can replicate both in vitro and when transplanted into mouse models (Hayek et al., 1995; Hayek and Beattie, 1997). Thus, there seems to be no argument regarding the ability of human embryonic and neonatal β cells to replicate. However, the logistical barriers to using embryonic and neonatal human β cells as a starting material for widespread human β-cell replacement therapy are obvious. It would be preferable to be able to use adult human cadaveric β cells as a starting material for widespread β-cell experimentation and expansion.

## 3.4 Evidence for Limited Replication of Adult Human Beta Cells

In contrast to embryonic and neonatal human β cells, the record is equally clear in demonstrating that adult human β cells do not normally replicate. For example, the same Kassem and Glaser study cited above (Kassem et al., 2000) demonstrates that β-cell replication in humans comes to a halt at around 6 months of age, findings mirrored by Meier and Butler (Meier et al., 2008). More extensive studies in humans with types 1 and 2 diabetes and their healthy controls have also demonstrated that adult human β cells do not replicate with any frequency (Butler et al., 2003; Yoon et al., 2003; Meier et al., 2005).

What about maneuvers that might activate human β-cell replication? As noted above, whereas partial or subtotal pancreatectomy is a standard research manipulation for inducing β-cell replication in rodents, recent studies by Meier et al. demonstrate that this does not occur in humans (Menge et al., 2008). They studied human patients who had undergone a subtotal pancreatectomy for removal of a tumor or because of pancreatitis and were subjected to computerized tomography (CT) postoperatively. These subjects demonstrated no change, that is, no regeneration, in total pancreatic mass postoperatively. Several of these patients required a second surgical procedure on their pancreatic remnant, which provided an opportunity to examine the original remnant for evidence of β-cell regeneration or β-cell replication. None was found. Thus, although β-cell regeneration and replication are fundamental characteristics of the rodent pancreas, they apparently do not apply to the human endocrine or exocrine pancreas.

These findings have been supported by reports of long-term follow-up of healthy human pancreatic donors (Kumar et al., 2008). In these studies, a healthy person donated approximately half of his or her pancreas to a relative for a pancreas transplant. When the healthy donors were studied years later, the majority had developed either glucose intolerance or frank diabetes. Thus, whereas a hemipancreatectomy has little or no long-term consequences in rodents, in humans it results in irreversible β-cell impairment, without apparent regeneration.

Weir et al. and Tyrberg et al. studied human islets transplanted into immunodeficient rodent recipients (Davalli et al., 1995; Tyrberg et al., 1996). In both studies, human β-cell replication, as assessed by tritiated thymidine labeling or BrdU incorporation, was extremely low (<1%). Most studies using adult human islets in vitro support this observation. For example, in a recent report by Parnaud and Halban, β cells isolated from adult human islets did not replicate in appreciable quantities (Parnaud et al., 2008). We have reported similar findings (Cozar-Castellano et al., 2004; 2008). However, recent lineage-tracing evidence from the Efrat group suggests that dedifferentiated cells derived from adult human β cells do replicate in vitro (Russ et al., 2008; Bar et al., 2008; see Chapter 5).

Growth factors have been widely employed in an attempt to induce β-cell replication in rodents, and in many instances remarkable proliferation rates were induced. Examples of rodent β-cell mitogenic growth factors include prolactin, placental lactogen, hepatocyte growth factor, parathyroid hormone-related protein, insulin, insulin-like growth factor-1, betacellulin, epidermal growth factor, trefoil factor-3, tumor-derived growth factor-alpha, glucagon-like peptide 1, exendin-4, gastrin, and others (Kulkarni et al., 1999; Garcia-Ocaña et al., 2001; De Leon et al., 2003; Suarez-Pinzon et al., 2005; Fueger et al., 2008). In most instances, when these agents were explored in adult human islets, β-cell replication was either not induced at all or if induced, only minor increments were observed. This is exemplified by the Parnaud–Halban report described above (Parnaud et al., 2008). Similar results have been reported for nutrients, such as glucose and free fatty acids, which induce rodent β-cell replication (Alonso et al., 2007) but apparently do not induce adult human β-cell replication.

Finally, several classes of signaling molecules, downstream of the growth factors enumerated above, have been implicated in driving β-cell replication in mice. Examples include members of the PI3 kinase-AKT cascade, the MAP kinase cascade, the WNT–β-catenin cascade, intracellular calcium signaling pathways, and others. However, in each case studied in human β cells, constitutive activation of members of these cascades produced little or no β-cell replication (Rao et al., 2005; Vasavada et al., 2007). Even when human β-cell replication rates doubled or tripled the basal rate was so low that a threefold increment still resulted in fewer than 1% of the β cells replicating.

From the forgoing it seems clear that although embryonic or neonatal human β cells can and do replicate, adult human β cells differ from their rodent or embryonic/neonatal counterparts in that they are particularly resistant to efforts to drive replication: they do not replicate, or do so only modestly under normal circumstances.

## 3.5 Attempts to Engineer Beta-Cell Replication

Landmark studies by Efrat and Hanahan in the 1980s demonstrated that the G1/S transition could be manipulated to result in β-cell replication. These investigators demonstrated that targeted expression of the SV40 large T-antigen (which disrupts pRb–E2F interactions and p53 function) in β cells of transgenic mice led to marked cell proliferation (Hanahan, 1985; Efrat et al., 1988; Milo-Landesman et al., 2001). Using this approach, researchers developed permanently replicating murine β-cell lines such as the ßTC3 and MIN6 cells (Efrat et al., 1988; Milo-Landesman et al., 2001; Hohmeier and Newgard, 2004). In a therapeutic paradigm, Efrat demonstrated that β-cell proliferation was reversible with removal of T-antigen, which was achieved by placing the T-antigen gene under the control of the tetracycline repressor. Murine β cells harboring this genetic system were able to control hyperglycemia when they were transplanted into diabetic mice after treatment with tetracycline analogues to regulate β-cell expansion (Milo-Landesman et al., 2001).

Levine and collaborators have explored complementary approaches, using T-antigen and the RAS signaling cascade and later telomerase (TERT), in attempts to immortalize human β cells and generate β-cell lines (Wang et al., 1997; Dufayet de la Tour et al., 2001;). In these studies, human β cells induced to proliferate with the *Ras* oncogene were able to proliferate, but lost their ability to replicate with time and also dedifferentiated, losing the ability to secrete insulin. In an effort to induce sustained human β-cell replication, the Levine group developed human β-cell lines expressing both RAS and TERT. This resulted in continuously growing cell lines derived from human β cells, but markers of β-cell differentiation were lost.

Building on this experience, Narushima et al. reported in 2005 that TERT and T-antigen could be employed together to drive human β-cell proliferation, and that this resulted in prolonged and marked expansion of β-cell-derived cells (Narushima et al., 2005). However, these expanded cells lost their differentiated β-cell characteristics. In a clever molecular strategy, TERT and T-antigen were added to the cells using retroviruses in which the TERT and T-antigen coding regions were flanked by loxP sites, so they could be excised and deleted from these cells following expansion using an adenovirus expressing Cre recombinase. The removal of TERT and T-antigen resulted in a marked deceleration of replication and redifferentiated into functional β cells. These cells expressed expected β-cell markers, contained dense-core secretory granules, and were able to reverse hyperglycemia when transplanted into mouse models. Although these results are promising, they have not been replicated by others, nor has the NAKT15 cell line been made available to other investigators. Thus, whether or not this approach holds promise remains to be seen.

## 3.6 Components of the Human Islet G1/S Transition Proteome

Following the rapid advances in understanding the murine cell cycle and their obvious implications for driving rodent β-cell replication, and keeping the increasingly apparent differences between rodent and human β cells in mind, we elected to survey

the proteome components of the G1/S checkpoint in human adult cadaveric islets, with the hope of observing differences that might be instructive or informative for developing methods to induce adult human β-cell replication and expansion.

These studies have been reported recently (Fiaschi-Taesch et al., 2009). They demonstrated that human islets, like murine islets, contain all 12 of the regulatory components of the G1/S transition depicted in Fig. 3.1, namely all possible cell cycle inhibitors. Thus, all three pocket proteins (pRb, p107, and p130), all four INK4s (p15, p16, p18, and p19), all three CIP/KIPs (p21, p27, and p57), and the cell cycle inhibitors menin and p53 are present in human islets. This is quite unusual, because although many cell types contain some of the cell cycle inhibitors, few express all of them. Of course, detailed immunohistochemistry is needed to document which of these are present in β cells, as opposed to non-β cells, in the islet, but if one wanted to design a cell type that could not or would not replicate, this is one way in which that goal might be accomplished. No wonder adult human β cells do not replicate!

With regard to molecules that drive cell cycle progression, most were present. One surprise was that cyclin $D_2$, known to be essential to mouse β-cell development and function (Georgia and Bhushan, 2004; Kushner et al., 2005), was present in low abundance, if at all, in human islets (Fiaschi-Taesch et al., 2009). Indeed, a recent report from Lavine independently indicates that cyclin $D_2$ is absent in human β cells, a clear difference from rodent islets (Lavine et al., 2008). Another example of a human–rodent difference resides with E2F2, which is present in murine islets (Cozar-Castellano et al., 2008) and has been shown to be essential for β-cell development and accrual (Iglesias et al., 2004). In contrast, human β cells appear to lack E2F2 (Fiaschi-Taesch et al., 2009). Both the D-cyclin differences and the E2F differences merit future study.

We elected to focus on one additional difference. Rodent islets contain one canonical D-cyclin partner, CDK4 (Martin et al., 2003; Cozar-Castellano et al., 2006c; 2008). Global loss of CDK4 in mice results in a very restricted phenotype that includes failure of β-cell replication with resultant β-cell hypoplasia (Rane et al., 1999; Tsutsui et al., 1999). This appears to reflect the absence in mouse β cells of CDK6, the CDK4 homologue that might have been able to compensate for the lost CDK4 function. Neither we nor the Barbacid group have been able to detect CDK6 in murine islets at significant levels (Martin et al., 2003; Cozar-Castellano et al., 2006c; 2008). On the other hand, we readily detected abundant CDK6 in human islets and in human β cells, respectively, by immunoblotting and immunohistochemistry (Fiaschi-Taesch et al., 2009). Although human islets also express CDK4, it appears less abundant than CDK6 in β cells. Thus, human and murine β cells are the obverse of one another with respect to CDK6 and CDK4 expression.

This surprising observation led us to ask whether CDK6 either alone or in combination with cyclin $D_1$ would be able to drive human β-cell replication. Moreover, as we had previously demonstrated that CDK4 in combination with cyclin $D_1$ (but not alone) was able to drive human β-cell replication (Cozar-Castellano et al., 2004), we compared the ability of both of these CDKs, alone or in combination with cyclin $D_1$, to phosphorylate pRb and to stimulate human β-cell replication. The CDKs and cyclin $D_1$ were delivered using replication-defective adenovirus vectors. The results

demonstrated that CDK6 alone, but not CDK4 alone, was able to stimulate both pRb phosphorylation and replication in human β cells, the latter increasing 10-fold from 0.3 to 3% (Cozar-Castellano et al., 2004; Fiaschi-Taesch et al., 2009). Cyclin $D_1$ alone also led to pRb phosphorylation and stimulated human β-cell replication, about 12-fold. However, the most robust effects were seen with the combination of CDK6 and cyclin $D_1$, which led to the greatest pRb phosphorylation and a 40-fold increase in human β-cell replication (Fiaschi-Taesch et al., 2009).

As noted above, increments in β-cell replication rate are commonly associated with decreases in β-cell differentiation. In order to assess the differentiation state of the human islets, we assessed glucose-stimulated insulin secretion (GSIS) in islets cotransduced with CDK6 and cyclin $D_1$. GSIS was normal in human islets transduced with CDK6 and cyclin $D_1$ alone and in cells cotransduced with both (Fiaschi-Taesch et al., 2009). There was even a suggestion that CDK6 transduction alone enhanced GSIS in human islets. Thus, CDK6 plus cyclin $D_1$ robustly stimulates human β-cell replication, while allowing β cells to retain a differentiated phenotype.

These results were obtained using in-vitro approaches, thus raising a question as to whether similar effects could be achieved in vivo. To address this issue, we employed a marginal mass human islet transplant model, in which human islets were transplanted into nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice rendered diabetic by administration of streptozotocin (Fig. 3.2a). Sham-operated mice remained diabetic, with blood glucose in the range of 400–500 mg/dl for the 6 weeks of the study. As a positive control, 4000 human islet equivalents (IEQ) normalized fasting blood glucose (50 mg/dl) and postprandial glucose (180 mg/dl). As a marginal mass negative control, transplantation of 1500 IEQ resulted in only a minor decline in random blood glucose from 500 to 450 mg/dl, and fasting glucose from 350 to 220 mg/dl. In contrast, overexpression of CDK6 plus cyclin $D_1$ resulted in a dramatic enhancement in β-cell engraftment and function: 1500 human IEQ transduced with CDK6 together with cyclin $D_1$ achieved a similar effect as 4000 positive-control human islets, leading to random blood glucose values of 160–180 mg/dl, and fasting blood glucose values of 50–60 mg/dl (Fiaschi-Taesch et al., 2009). Thus, the CDK–cyclin-transduced islets engrafted efficiently, manifested a differentiated phenotype, and could be argued to function three times more effectively than normal human islets.

What about human β-cell proliferation in vivo? To ask this question, we harvested grafted kidneys from the transplanted mice and assessed β-cell replication rates using BrdU incorporation in vivo (Fig. 3.2b,c). Replication rates in control β-cell grafts were in the 0.2% range, whereas in the CDK–cyclin-transduced grafts they were 25-fold higher, in the 5% range (Fig. 3.2d) (Fiaschi-Taesch et al., 2009). These results indicate that human β-cell replication is maintained in vivo and that replicating β cells remain functional. Perhaps of equal importance, the model of human islet transplantation into immunodeficient diabetic mice allows direct observation of dynamic changes in human β-cell replication (i.e., BrdU labeling in response to manipulations that augment replication). We believe this is an important methodological advance.

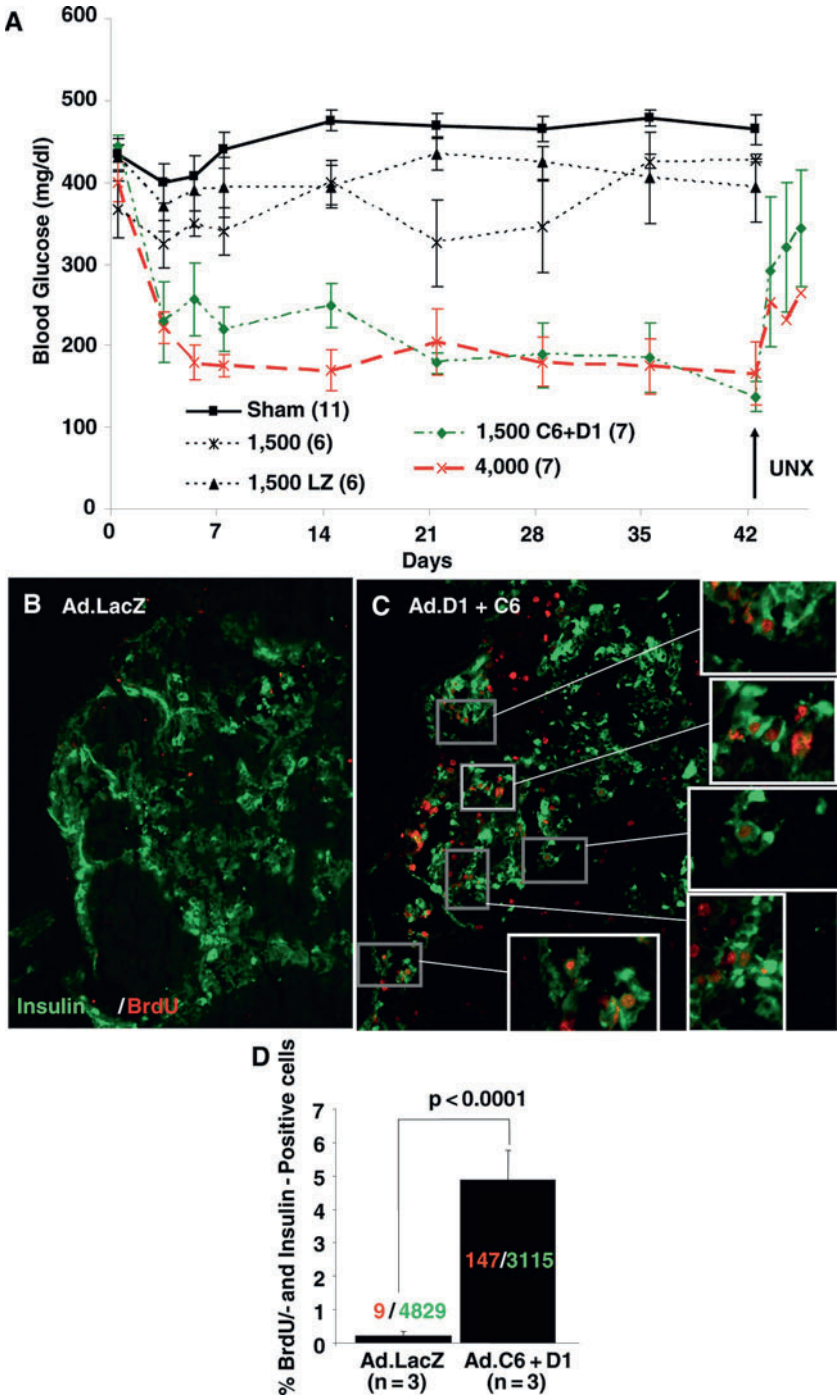



**Fig. 3.2** (continued)

## 3.7 Interpretation of Human Beta-Cell Cycle Activation by CDK6 and Cyclin $D_1$

These studies represent an advance but are still rudimentary. They are an advance because the human islet G1/S proteome had not been previously catalogued comprehensively and because the results indicate that: (1) human β cells can be induced to proliferate at striking and previously unanticipated rates; (2) human β-cell replication can be induced and studied using in-vivo models; and (3) two cell cycle regulators can induce human β-cell replication, while retaining differentiated function.

On the other hand, these studies are rudimentary in several respects. First, the in-vivo studies lasted only 6 weeks. Will β-cell function decline at a later point in time or, conversely, will prolonged β-cell proliferation lead to unwanted expansion of β-cell mass and function, resulting in severe hypoglycemia? Since D-cyclins and CDKs can be oncogenic, will uncontrolled proliferation and transformation occur in these grafts?

The studies are rudimentary in another sense: They show that the cell cycle can be effectively manipulated using two cell cycle molecules. However, there are more than 30 G1/S regulatory molecules in human islets (Fiaschi-Taesch et al., 2009): Are CDK6 and cyclin $D_1$ the best targets? Might cyclin A and E and their CDK1/CDK2 partners be superior candidates? Newgard et al., Attie et al., Hussain et al., and our own group have suggested that these G1/S molecules can also be employed to drive β-cell replication (Cozar-Castellano et al., 2008; Fueger et al., 2008; Song et al., 2008; Lavine et al., 2008). Moreover, these studies were performed using whole islets and do not specifically address which G1/S molecules are present in human β cells, as compared to other endocrine cell types and nonendocrine cells within the islets. This issue will require extensive confirmation using immunohistology and other approaches.

There are mechanistic questions as well. We have shown that CDK6, which can drive human β-cell replication and phosphorylation of pRb, appears to be localized

**Fig. 3.2** (continued) **The combination of CDK6 and cyclin $D_1$ delivered to human islets accelerates proliferation and enhances islet graft function. (a)** Blood glucose levels in streptozotocin-diabetic NOD-SCID mice transplanted with CDK6- and cyclin $D_1$-transduced human islets. The number of mice in each group is shown in parentheses. CDK6 plus cyclin $D_1$ transduction reduces the therapeutic mass of human islet transplant approximately threefold. The accelerated β-cell proliferation does not reduce, but rather enhances, human β-cell function in vivo. UNX, unilateral nephrectomy. **(b,c)** Effects of control virus (**b**) or CDK6 plus cyclin $D_1$ (**c**) on β-cell proliferation as assessed by BrdU (*red*) incorporation into insulin-positive cells (*green*) in human islet grafts. This is perhaps the first example of robust human β-cell replication induced in vivo. (**d**) Quantitation of β-cell proliferation rate in control and CDK–cyclin-treated human islet grafts. Proliferation rate of control human β cells is very low, as is widely reported, but proliferation rate in CDK–cyclin-treated islets is approximately 25-fold higher. Adapted with permission from Fiaschi-Taesch et al., 2009

in human β cells exclusively in the cytoplasm (Fiaschi-Taesch et al., 2009). If so, how is CDK6 able to phosphorylate pRb, a nuclear protein? Is it possible that CDK6 remains in the cytosol and recruits or retains CIP/KIPs in the cytosolic compartment, allowing unrestricted activation of CDK1, CDK2, and their cyclin A/E partners?

Another question concerns the G1/S inhibitors. In mice, disruption of p27 plus p18, or menin alone, or p16 alone all lead to expansion of β-cell mass and function (Franklin et al., 2000; Karnick et al., 2005; Krishnamurthy et al., 2006). Is it possible to knock down pocket proteins, KIP/CIPs, INK4s, and/or menin in human β cells? If so, would that result in rapid replication of human β cells? These are all important questions that have to be addressed.

## 3.8 Cell Cycle Inhibitors in Human Beta Cells

Much has been learned from mouse models regarding cell cycle inhibitors. Whereas loss of p27 in mice has little discernible effect on β cells under basal conditions (Uchida et al., 2005), when combined with a high-fat diet or leptin receptor deficiency it leads to marked β-cell replication and expansion (Uchida et al., 2005). Interestingly, p27 loss combined with p18 loss in mice also results in β-cell replication (Franklin et al., 2000). More recently, it has been shown that the menin protein is part of a transcriptional complex that upregulates the tumor suppressors INK4s, p18, and p27 (Karnick et al., 2005). Germline loss of both alleles of menin in mice results in embryonic lethality. However, loss of a single allele, later accompanied by somatic loss of the second allele in β cells, results in downregulation of p18 and p27, accompanied by β-cell proliferation, expansion of β-cell mass, and insulin-mediated hypoglycemia (Karnick et al., 2005). These events would appear to apply to human β cells as well, since humans with biallelic loss of menin in β cells develop insulinomas as part of the multiple endocrine neoplasia (MEN) type 1 syndrome (Stratakis and Marx, 2005).

Several groups have reported that loss of p27 alone results in a MEN-like syndrome in humans (Pellegrata et al., 2006; Georgitsi et al., 2007; Agarwal et al., 2009), suggesting that p27 is particularly important in repressing cell cycle progression in human endocrine cells. In addition, it has been reported that rare individuals among a large series of patients with an apparent MEN1 syndrome who do not display menin mutations have pathologic polymorphisms in p15, p18, p21, and p27, suggesting that these cell cycle inhibitors play important restraining roles in human endocrine cell replication (Agarwal et al., 2009).

INK4a/p16 has also attracted attention as a cell cycle inhibitor in mice, in which loss of its gene permits β-cell replication, as well as in humans, in which the relevant gene locus has appeared in genome-wide studies as being associated with type 2 diabetes. The studies of Sharpless et al. (Krishnamurthy et al., 2006) make it clear that p16 is a suppressor of β-cell replication that increases in activity with age and likely mediates the age-related decline in β-cell replication rates. Indeed, disruption of the p16 gene removes the age-related decline in β-cell replication in mice, and expedites the recovery of β-cell mass in older mice treated with streptozotocin. As

suggested in the following section, this makes p16 regulation an attractive target for pathophysiologic, mechanistic, and therapeutic studies in humans.

Thus, exploration of the individual and combined roles of cell cycle inhibitors as potential targets in inducing human β-cell replication and expanding β-cell mass is certainly warranted.

## 3.9 Epigenetic Changes in Beta-Cell Cycle Control

Regulation of the G1/S transition likely involves epigenetic changes. For example, pRb represses cell cycle progression not only by inhibiting E2F-induced transcriptional activation of cell cycle controlling genes (Pestell et al., 1999; Cozar-Castellano et al., 2006a; Heit et al., 2006; Harb et al., 2009), but also by attracting histone deacetylases (HDACs) to these same loci, in effect closing exposed promoter regions and thereby preventing cell cycle activation (Pestell et al., 1999; Cozar-Castellano et al., 2006a; Heit et al., 2006; Harb et al., 2009). As more is learned about cell cycle regulation, it becomes clearer that epigenetic control is at least as important as traditional transcriptional control in activating cell cycle progression. As another example, menin, which is known to participate in the transcriptional activation of p18 and p27, also serves as part of the trithorax group of transcriptional activators that act as histone methyltransferases (Hughes et al., 2004; Karnick et al., 2005). This field is in its infancy as it relates to the β cell, but it is an area that merits active investigation. Preliminary reports suggest that epigenetic mechanisms are accessible to manipulation for expanding human β-cell mass through proliferation (Hughes et al., 2004; Karnick et al., 2005).

## 3.10 Future Therapeutic Directions in Human Beta-Cell Cycle Control

What are the ultimate goals of inducing human β-cell replication? One aim would be to enhance the replication, survival, and function of human cadaveric islet β cells, so that fewer islets would be required for successful islet transplantation. At present it takes islets from two to four cadaver pancreata to generate sufficient numbers of human islets to reverse diabetes in a single recipient (Shapiro et al., 2000; Ryan et al., 2005). If the CDK6–cyclin $D_1$ strategy could be deployed in humans as discussed above (Fig. 3.2a) and a threefold or more increment in engraftment efficiency and function could be achieved, then perhaps a single pancreas could provide sufficient islets for transplantation into several patients. This would, of course, be a useful advance.

As no functional human β-cell lines exist (Hohmeier and Newgard, 2004), a second goal would be to generate continuously growing human β-cell lines, which could be stored frozen and used to replace β cells in patients with type 1 and type 2 diabetes, just as keratinocyte grafts are used in burn patients and frozen

corneas are used for corneal transplants. Unraveling the secrets of human β-cell cycle control might permit this type of expansion. Such a prospect raises obvious concerns about unregulated cell proliferation, but one can envision ways to reduce them (cell implantation in cell-impermeable chambers, inclusion of suicide genes, etc.). Parenthetically, these concerns also apply to cells derived from embryonic stem cells and other stem cell approaches.

Another goal is to use cell cycle regulatory approaches to enhance the numbers of β cells derived from embryonic stem cells (Kroon et al., 2008; Zaret and Grompe, 2008) or from induced pluripotent stem (iPS) cells (Park et al., 2008; Tateishi et al., 2008). For example, if embryonic stem cell technology can generate a few million β cells, perhaps the techniques described herein could be used to turn these few million into a few billion β cells.

Still another approach might be to develop "personal β cells" using iPS technology (Park et al., 2008; Tateishi et al., 2008). In this scenario, one might obtain skin biopsy-derived fibroblasts from a type 1 or type 2 diabetic patient, reprogram them into stem cells, differentiate them into a small number of β cells as has been described (Park et al., 2008; Tateishi et al., 2008), and then use the methods discussed herein to expand this pool. Since these β cells are derived from a single donor, they could be replaced without immunosuppression in a type 2 diabetic patient or with immunosuppression in a type 1 diabetic patient.

A final goal might be to acquire a few residual adult β cells from a type 1 or type 2 diabetic patient by fine-needle biopsy or another technique and employ the approaches described above for ex-vivo growth of these few β cells into large numbers of patient-specific β cells.

Clearly, none of the approaches described above is ready for clinical application; however, consideration of these scenarios may help our thinking on the best ways to utilize the knowledge gained from the human β-cell G1/S proteome analyses. One obvious bioengineering question relates to the optimal control of CDK and cyclin expression. To date, we have employed viral constructs with the CMV promoter, a ubiquitous and constitutive promoter. Would a regulated promoter or a β-cell-specific promoter be preferable, or is expression of CDK6–cyclin $D_1$ in non-β cells beneficial, as it would stimulate proliferation of β-cell precursors? These are questions that should be addressed.

Another key question relates to the optimal employment of viral vectors. Is an adenovirus an appropriate vector for driving human β-cell replication? Some would argue that it is a poor vector because its persistence is brief. This perception comes from human studies with cystic fibrosis and homozygous hypercholesterolemia, where adenoviral delivery of CFTR and LDL-receptor, respectively, failed because adenovirus-transduced cells persisted for only a few weeks (Flotte, 2007; Kaiser, 2007). On the other hand, we have shown that in immunocompromised models an adenovirus persists and expresses cargo cDNAs for at least a few months (Fiaschi-Taesch et al., 2008; Rao et al., 2005), which may be all that is needed for expanding the β-cell mass. Indeed, given the concerns mentioned above regarding unregulated cell proliferation, as well as specific concerns raised by retroviral or lentiviral integrating vectors (Cavazzana et al., 2004; Hacein-Bey-Abina et al., 2008; Kaiser,

2003), it could be argued that transient, nonintegrating viruses such as an adenovirus may be optimal for diabetes cell replacement therapy. Patients with type 1 diabetes receiving allogeneic human cadaveric islets or other cell replacement therapies would have to be immunosuppressed to prevent allograft rejection. Thus, "rejection" of adenovirus-transduced islets will likely not occur in the absence of a fully effective immune system, leading to long-term persistence, as we have demonstrated in immunodeficient mice. Further, optimal engraftment may require transgene expression for only a few weeks, making long-term transduction unnecessary. In these settings, an adenovirus may be preferred over a lentivirus.

Lentiviruses have the advantage of stable expression, but carry the risk of insertional mutagenesis (Kaiser, 2003; Cavazzana et al., 2004; Hacein-Bey-Abina et al., 2008), so many investigators, patients, and regulatory agencies are reluctant to consider their use. On the other hand, more is being learned about optimal design and use of retroviral vectors to avoid insertional mutagenesis (Kaiser, 2003; Cavazzana et al., 2004; Hacein-Bey-Abina et al., 2008), which may enable their safe use in the future.

Are there ways to avoid gene therapy altogether? One approach might be to deliver proteins using peptide targeting domains (PTDs) or TAT sequences, polybasic residues (arginines, lysines, and histidines) added to the N-terminus or C-terminus of a protein that can allow its entry into cells with fair efficiency, with a half-life of hours to a day (Klein et al., 2004; Gump and Dowdy, 2007). This method avoids concerns associated with the use of viral vectors and gene therapy. On the other hand, it has several drawbacks: sustained therapy is not possible without repeated treatment (a disadvantage for long-term in-vitro therapy and the development of cell lines); there is no way to target specific cells (e.g., β cells, as opposed to endothelial or other islet cell types); and it cannot be used effectively in vivo.

Yet another approach might be to identify key cell cycle activators or inhibitors in human islets and employ high-throughput screening for small molecules that might manipulate their activity. For example, now that we know that exogenous CDK6 and cyclin $D_1$ are effective in expanding human β cells, it makes sense to screen small-molecule libraries for agents that activate endogenous CDK6 and cyclin $D_1$.

Further, with increasing knowledge concerning the control of replication of human β cells it may become possible to generate a complete map of the pathways that activate cell cycle progression in these cells. For example, although it seems clear that CDK4 is downstream of PI3 kinase and AKT in murine β cells (Fatrai et al., 2006), not much is known about the control of CDK6 expression in human β cells. Thus, if a specific signaling pathway upstream of CDK6 is identified, it will become an attractive target for a high-throughput screening for small molecules that might manipulate the activity of this pathway. Analogously, whereas it is now clear that many cell cycle inhibitors are active in human β cells and that knockdown of cell cycle inhibitors in the mouse stimulates (or is at least permissive for) β-cell replication, little is known about regulation of cell cycle inhibitors in human β cells. Conceivably, cell cycle inhibitors may also be targets for small-molecule regulation.

## 3.11 Conclusions

In closing, let us note that these are exciting but early times in the induction of human β-cell replication. Ten years ago, most experts would have said that adult human β cells cannot divide and are terminally differentiated. This view has changed dramatically with the observations discussed herein: adult human β cells can clearly be induced to divide. The areas described above represent the next frontiers for achieving clinically significant human β-cell expansion through the coopting of normal cell cycle control mechanisms.

**Acknowledgments** The authors wish to thank the Juvenile Diabetes Research Foundation, the American Diabetes Association and the National Institutes of Health (NIDDK R-01 55023) for their support of this work. We also thank the Don and Arleen Wagner Family Foundation and Pam and Scott Kroh for their support of this work.

## References

Agarwal SK, Mateo CM, Marx SJ. (2009) Rare germline mutations in cyclin-dependent kinse inhibitor genes in MEN-1 and related states. J Clin Endocrinol Metab (e-pub ahead of print).

Alonso LC, Yokoe T, Zhang P, et al. (2007) Glucose infusion in mice: a new model to induce β-cell replication. Diabetes. 56:1792–1801.

Bar Y, Russ HA, Knoller S, et al. (2008) HES1 is involved in adaptation of adult human β cells to proliferation in vitro. Diabetes. 57:2413–2420.

Brissova M, Fowler MJ, Nicholson WE, et al. (2005) Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. J Histochem Cytochem. 53:1087–1097.

Butler AE, Janson J, Bonner-Weir S, et al. (2003) Beta cell deficit and increased β cell apoptosis in humans with diabetes. Diabetes. 52:102–110.

Cabrera O, Berman D, Kenyon NS. (2006) The unique architecture of the human pancreatic islet has implications for islet cell function. Proc Nat Acad Sci USA. 103:2334–2339.

Cavazzana-Calvo M, Thrasher A, Mavillo F. (2004) The future of gene therapy. Nature. 427: 779–781.

Cozar-Castellano I, Fiaschi-Taesch N, Bigatel TA, et al. (2006a) Molecular control of cell cycle progression in the pancreatic beta cell. Endocr Rev. 27:356–370.

Cozar-Castellano I, Harb G, Selk K, et al. (2008) Lessons from the first comprehensive characterization of cell cycle control in rodent insulinoma cell lines. Diabetes. 57:3056–3068.

Cozar-Castellano I, Haught M, Stewart AF. (2006b) The cell cycle inhibitory protein, $p21^{cip}$, is Not essential for maintaining beta cell cycle arrest or beta cell function in Vivo. Diabetes. 55: 3271–3278.

Cozar-Castellano I, Takane KK, Bottino R, et al. (2004) Induction of beta cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenoviral delivery of cyclin-dependent Kinase-4 and Cyclin $D_1$. Diabetes. 53:149–159.

Cozar-Castellano I, Weinstock M, Haught M, et al. (2006c) Comprehensive characterization of the $G_1$/S proteome in the islets of mice transgenic for hepatocyte growth factor, placental lactogen, or both: unique involvement of $p21^{cip}$. Diabetes. 55:70–77.

Davalli AM, Ogawa Y, Ricordi C, et al. (1995) A selective decrease in β cell mass of human islets transplanted into diabetic nude mice. Transplantation. 59:817–820.

De Leon DD, Deng S, Madani R. (2003) Role of endogenous glucagon-like peptide-1 in islet regeneration after partial pancreatectomy. Diabetes. 52:365–371.

De Vos A, Heimberg H, Qartier E, et al. (1995) Human and rat β cells differ in glucose transporter but not glucokinase gene expression. J Clin Invest. 96:2489–2495.

Dor Y, Brown J, Martinez OI, Melton DA. (2004) Adult pancreatic ß-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 429:41–46.

Dufayet de la Tour D, Halvorsen T, Demeterco C, et al. (2001) Beta cell differentiation from a human pancreatic cell line in Vitro and in vivo. Mol Endocrinol. 15:476–483.

Efrat S, Linde S, Kofod H, et al. (1988) Beta-cell derived from transgenic mice expressing a hybrid insulin gene-oncogene. Proc Nat Acad Sci USA. 85:9037–9041.

Fatrai S, Elghazi L, Balcazar N, et al. (2006) Akt Induces β-cell proliferation by regulating cyclin D1, Cyclin D2, and p21 levels and cyclin-dependent kinase-4 activity. Diabetes. 55:318–325.

Fiaschi-Taesch NM, Berman-Weinberg D, Sicari BM, et al. (2008) Hepatocyte growth factor (HGF) enhances engraftment and function of non-human primate islets. Diabetes. 57: 2745–2754.

Fiaschi-Taesch NM, Bigatel TA, Sicari BM, et al. (2009) A survey of the human pancreatic beta cell G1/S proteome reveals a potential therapeutic role for Cdk-6 and cyclin $D_1$ in enhancing human beta cell replication and function in Vivo. Diabetes (e-pub ahead of print).

Florez JC. (2008) Newly identified loci highlight β cell dysfunction as a key cause of type 2 diabetes: where are the insulin resistance genes?. Diabetologia. 51:1100–1110.

Flotte TR. (2007) Gene therapy: the first two decades and the current state of the art. J Cell Physiol. 213:301–305.

Franklin DS, Godfrey VL, O'Brien DA, et al. (2000) Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity. Mol Cell Biol. 20:6147–6158.

Fueger PT, Schisler J, Lu D, et al. (2008) Trefoil factor-3 stimulates human and rodent pancreatic β cell replication with retention of function. Mol Endocrinol. 22:1251–1259.

Garcia-Ocaña A, Vasavada RC, Takane KK, et al. (2001) Using Beta Cell Growth Factors to Enhance Human Pancreatic Islet Transplantation. J. Clin. Endocrinol. Metab. 86:984–988.

Georgia S, Bhushan A. (2004) ß cell replication is the primary mechanism for maintaining postnatal β cell mass. J Clin Invest. 114:963–968.

Georgitsi M, Raitila A, Karhu A, et al. (2007) Germline CDKN1B/$p27^{kip1}$ mutation in multiple endocrine neoplasia. J Clin Endocrinol Metab. 92:3321–3325.

Gradwohl G, Dierich A, LeMeur M, et al. (2000) Neurogenin-3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Nat Acad Sci USA. 97:1607–1611.

Gump JM, Dowdy SF. (2007) TAT protein transduction: the molecular mechanism and therapeutic prospects. Trends in Molec Med. 13:443–448.

Hacein-Bey-Abina S, Garrigue A, Wang GP, et al. (2008) Insertional oncogenesis in four patients after retrovirus-mediated gene therapy of SCID-X1. J Clin Invest. 118:3132–3142.

Hanahan D. (1985) Heritable formation of pancreatic ß-cell tumors in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature. 315:115–122.

Harb G, Cozar-Castellano I, Fiaschi-Taesch NM, et al. (2009) Cell cycle control in the pancreatic beta cell. In: Kandeel F (ed.) *Islet Transplantation: Biology, Immunology and Clinical Applications*. Springer; New York (in press).

Hayek A, Beattie GM. (1997) Experimental transplantation of human fetal and adult pancreatic islets. J Clin Endocrinol Metab. 82:2471–2475.

Hayek A, Beattie GM, Cirulli V. (1995) Growth factor/matrix-induced proliferation of human adult ß-cells. Diabetes. 44:1458–1460.

Heit JJ, Karnik SK, Kim SK. (2006) Intrinsic regulators of pancreatic β-cell proliferation. Ann Rev Cell Dev Biol. 22:311–338.

Hohmeier H, Newgard CB. (2004) Cell lines derived from pancreatic islets. Mol Cell Endo. 228:121–128.

Hughes CM, Rosenblatt-Rozen O, Milne TA, et al. (2004) Menin associates with a trithorax family histone methyltransferase complex and with the Hoxc8 locus. Molecular Cell. 13:587–597.

Iglesias A, Murga M, Laresgoiti U, et al. (2004) Diabetes and exocrine pancreatic insufficiency in E2F1/E2F2 double mutant mice. J Clin Invest. 113:1398–1407.

Kaiser J. (2003) Seeking the cause of induced leukemias in X-SCID trial. Science. 299:495.

Kaiser J. (2007) Gene transfer an unlikely contributor to patient's death. Science. 318:1535.
Karnick SK, Hughes CM, Gu X, et al. (2005) Menin regulates pancreatic islet growth by promoting histone methylation and expression of genes encoding p27kip1 and p18Ink4c. Proc Nat Acad Sci USA. 102:14659–14664.
Kassem SA, Ariel I, Thornton PS, et al. (2000) Beta cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. Diabetes. 49:1325–1333.
Klein D, Ribeiro MM, Mendoza V, et al. (2004) Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Comm. 323:473–478.
Krishnamurthy J, Ramsey MR, Ligon KL. (2006) p16ink4a induces an age-dependent decline in islet regenerative potential. Nature. 443:453–457.
Kroon E, Martinson LA, Kadoya K, et al. (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nature Biotechnol. 26:443–452.
Kulkarni RN, Brüning JC, Winnay JN, et al. (1999) Tissue-specific knockout of the insulin receptor on β cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell. 96:329–339.
Kumar AF, Greussner RWG, Seaquist ER, et al. (2008) Risk of glucose intolerance and diabetes in hemipancreatectomized donors selected for normal preoperative glucose metabolism. Diabetes Care. 31:1639–1643.
Kushner J, Ciemerych MA, Sicinska E, et al. (2005) Cyclins D2 and D1 are essential for postnatal pancreatic β cell growth. Mol Cell Biol. 25:3752–3762.
Lavine JA, Raess PW, Davis DB, et al. (2008) Overexpression of pre-pro-cholecystokinin stimulates β cell proliferation in mouse and human islets with retention of islet function. Mol Endocrinol. 22:2716–2728.
Lindgren CM, McCarthy MI. (2008) Mechanisms of disease: genetic insights into the etiology of type 2 diabetes and obesity. Nat Clin Pract Endocrinol Metab. 4:156–163.
Malumbres M, Barbacid M. (2005) Mammalian cyclin-dependent kinases. Trends Biol Sci. 30:630–641.
Martin J, Hunt SL, Dubus P, et al. (2003) Genetic rescue of cdk-4-null mice restores pancreatic β cell proliferation but not homeostatic cell number. Oncogene. 22:5261–5269.
Matveyenko AV, Butler PC. (2006) Beta cell deficit due to increased apoptosis in the human islet amyloid polypeptide transgenic (HIP) rat recapitulates the metabolic defects present in type 2 diabetes. Diabetes. 55:2106–2114.
Meier JJ, Bushan A, Butler AE, et al. (2005) Sustained β cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration?. Diabetologia. 48:2221–2228.
Meier JJ, Butler AE, Saisho Y, et al. (2008) Beta cell replication is the primary mechanism subserving the postnatal expansion of β cell mass in humans. Diabetes. 57:1584–1594.
Menge BA, Tannapfel A, Belyaev O, et al. (2008) Partial pancreatectomy in adult humans does not provoke β cell regeneration. Diabetes. 57:142–149.
Milo-Landesman D, Surana M, Berkovich I, et al. (2001) Correction of hyperglycemia in diabetic mice transplanted with reversibly immortalized pancreatic β cells controlled by the tet-on regulatory system. Cell Transplant. 10:645–650.
Narushima M, Kobayashi N, Okitsu T, et al. (2005) A human ß-cell line for transplantation therapy to control type 1 diabetes. Nature Biotechnol. 23:1274–1282.
Park I-H, Aroroa N, Huo H, et al. (2008) Disease-specific pluripotent stem cells. Cell. 134: 877–886.
Parnaud G, Bosco D, Berney T, et al. (2008) Proliferation of sorted human and rat β cells. Diabetologia. 51:91–100.
Pellegrata NS, Quintillana-Martinez L, Siggelkow H, et al. (2006) Germline mutation in p27kip1 causes a multiple endocrine neoplasia syndrome in rats and humans. Proc Nat Acad Sci USA. 103:15558–15563.
Pestell RG, Albanese C, Reutens AT, et al. (1999) The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. Endocr Rev. 20: 501–534.

Pontoglio M, Sreenan S, Roe M, et al. (1998) Defective insulin secretion in hepatocyte nuclear factor 1 α-deficient mice. J Clin Invest. 101:2215–2222.
Rane S, Dubus P, Mettus RV, et al. (1999) Loss of expression of cdk4 causes insulin-deficient diabetes and cdk4 activation results in β cell hyperplasia. Nature Genet. 22:44–52.
Rao P, Roccisana J, Takane KK, et al. (2005) Gene transfer of constitutively active Akt markedly improves human islet transplant outcomes in diabetic SCID mice. Diabetes. 54:1664–1675.
Russ HA, Bar Y, Ravassard P, et al. (2008) In vitro proliferation of cells derived from adult human β cells revealed by cell-lineage tracing. Diabetes. 57:1575–1583.
Ryan EA, Paty BW, Senior PA, et al. (2005) Five year follow-up after clinical islet transplantation. Diabetes. 54:2060–2069.
Schuit FC. (1997) Is glut-2 required for glucose sensing?. Diabetologia. 40:104–111.
Shapiro AMJ, Lakey JRT, Ryan EA, et al. (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. New Engl J Med. 343:230–238.
Song WJ, Schreiber WE, Zhong E, et al. (2008) Exendin-4 stimulation of cyclin A2 in β-cell proliferation. Diabetes. 57:2371–2381.
Stoffel M, Duncan SA. (1997) The maturity-onset diabetes of the young (MODY1) transcription factor HNF1α regulates expression of genes required for glucose transport and metabolism. Proc Nat Acad Sci USA. 94:13209–13214.
Stratakis CA, Marx SJ. (2005) Multiple endocrine neoplasias in the era of translational medicine. Horm Metab Res. 37:343–346.
Suarez-Pinzon WL, Yan Y, Power R, et al. (2005) Combination therapy with epidermal growth factor and gastrin increases β cell mass and reverses hyperglycemia in diabetic NOD mice. Diabetes. 54:2596–2601.
Tateishi K, He J, Taranova O, et al. (2008) Generation of insulin-secreting islet-like clusters from human skin fibroblasts. J Biol Chem. 283:31601–31607.
Tsutsui T, Hesabi B, Moons D, et al. (1999) Targeted disruption of cdk4 delays cell cycle entry with enhanced p27-kip activity. Mol Cell Biol. 19:7011–7019.
Tyrberg B, Eizirik DL, Hellerstrom C, et al. (1996) Human pancreatic β cell deoxyribonucleic acid synthesis in islet grafts decreases with increasing organ donor age but increases in response to glucose stimulation in vitro. Endocrinology. 137:5694–5699.
Uchida T, Nakamura T, Hashimoto N, et al. (2005) Deletion of cdkn1b ameliorates hyperglycemia by maintaining compensatory hyperinsulinemia in diabetic mice. Nature Med. 11:175–182.
Vasavada RC, Wang L, Fujinaka Y, et al. (2007) Protein kinase C-ζ activation markedly enhances ß-cell proliferation: an essential role in growth factor-mediated ß-cell mitogenesis. Diabetes. 56:2732–2743.
Wang S, Beattie GM, Mally MI, et al. (1997) Isolation and characterization of a cell line from the epithelial cells of the human fetal pancreas. Cell Transplant. 6:59–67.
Wang J, Cortina G, Wu V, et al. (2006) Mutant neurogenin-3 in congenital malabsorptive diarrhea. N Engl J Med. 355:270–280.
Yoon KH, Ko H, Cho JH. (2003) Selective ß cell loss and α cell expansion in patients with type 2 diabetes mellitus in Korea. J Clin Endocrinol Metab. 88:2300–2308.
Yorifuji T, Kurokawa K, Mamada M, et al. (2004) Neonatal diabetes mellitus and neonatal polycystic dysplastic kidneys: phenotypically discordant recurrence of a mutation in the hepatocyte nuclear factor 1β gene due to germline mosaicism. J Clin Endocrinol Metab. 89:2905–2908.
Zaret KS, Grompe M. (2008) Generation and regeneration of cells of the liver and pancreas. Science. 322:1490–1494.
Zhang X, Gaspard JP, Mizukami Y, et al. (2005) Overexpression of cyclin D1 in pancreatic β cells in vivo results in islet hyperplasia without hypoglycemia. Diabetes. 54:712–719.

# Chapter 4
# Islet Regeneration

**Xiaobo Xu, Joke D'Hoker, Nico De Leu, Xiangwei Xiao, Yves Heremans, Mark Van De Casteele, and Harry Heimberg**

**Abstract** Although the mechanisms controlling endocrine progenitor-cell differentiation during pancreas development have been described in considerable detail, our knowledge of islet-cell renewal in the postnatal pancreas was based until recently on indirect evidence and remained quite descriptive. This has changed with recent genetic lineage-tracing studies, which have provided evidence for a central role of β-cell replication in islet turnover in the adult pancreas, both under normal physiological conditions and following moderate injury. In contrast, recent work from our group demonstrates that severe tissue injury activates multipotent islet-cell progenitors, which can differentiate in vitro into all types of islet cells. In this chapter we provide an overview of experimental models available for studying β-cell renewal and the major mechanisms underlying this process.

## 4.1 Introduction

Restoration of the β-cell mass in type 1 diabetes or increase of the number of β-cells in type 2 diabetes would be an obvious solution for the inadequate release of endogenous insulin. Islet transplantation has proven to be successful in clinical trials (Robertson, 2004), but its widespread clinical application is severely limited by a shortage of organ donors and the deleterious side effects of immunosuppression. The need for life-long immunosuppression following transplantation might possibly be avoided by cell encapsulation, graft immunomodulation, or recipient tolerization (covered elsewhere in this book). Availability of donor tissue can be improved by several alternative approaches for generation of islet β cells. These include differentiation of embryonic stem cells into functional β cells through a series of in-vitro manipulations, followed by a maturation period in vivo (Kroon et al., 2008); stimulation of β-cell proliferation in vitro (Ouziel-Yahalom et al., 2006); and in-vitro transdifferentiation from isolated acinar (Baeyens et al., 2005) or duct cells (Bonner-Weir et al., 2000). Moreover, endogenous β cells and/or their progenitor/stem cells

H. Heimberg (✉)
Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium
e-mail: harry.heimberg@vub.ac.be

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_4,

are obvious targets for in-vivo generation of new β cells by self-renewal (Dor et al., 2004) or differentiation (Xu et al., 2008). Finally, new β cells were reported to be generated in vivo by transdifferentiation from pancreatic duct cells (Inada et al., 2008) or from bone-marrow-derived cells (Ianus et al., 2003), although the latter remains controversial. This chapter focuses on in-vivo islet regeneration, both by self-renewal of residual β cells and by differentiation of endogenous islet-cell progenitors (referred to as "neogenesis"). Owing to its noninvasive character and the employment of autologous cells, which avoid the need for immunosuppression to prevent allograft rejection, in-vivo regeneration of islets may represent an ideal therapy for diabetes, in combination with the possibility of reprogramming the autoimmune system (Waldmann et al., 2008).

During embryogenesis, β cells are generated by differentiation of stem and progenitor cells. A major absolute increase in β-cell mass parallels the gradual increase in body weight during the perinatal period, with up to 10% of β cells replicating per day (Bernard-Kargar and Ktorza, 2001). In older rodents, β-cell proliferation rate drops to less than 1% per day (Teta et al., 2005). Under normal physiological conditions, the β-cell mass is maintained by a balance between low rates of replication (Dor et al., 2004) and apoptosis (Finegood et al., 1995). Owing to this slow turnover, adult β cells have always been considered terminally differentiated, with little or no self-renewal capacity, similar to neurons and cardiomyocytes. However, this view has been challenged recently by studies that underscore the importance of new β-cell formation in the adult pancreas, raising questions about the mechanisms responsible for this renewal. Elucidating these mechanisms may reveal the full potential of regenerative medicine for the treatment of diabetes.

## 4.2 Measuring Islet Regeneration

The β-cell mass is determined by the balance between cell formation and death, both of which are regulated by alteration of (patho)physiological conditions, including change of body weight, pregnancy, β-cell damage, and insulin resistance. Currently the common way to estimate β-cell mass involves quantifying the total surface area of insulin-positive cells in pancreas sections. Owing to the variation in β-cell density throughout the pancreas, this method requires evaluation of a large number of tissue sections representing the entire organ. Restoration of β-cell function, as measured by glucose clamp assays or blood glucose levels, provides an even more significant measure, which evaluates the effect of experimental manipulations on regeneration of a functional β-cell mass in diabetic animal models.

## 4.3 Experimental Models for Inducing Beta-Cell Regeneration

Several animal models of tissue damage have been developed over the past decades, which allow studying regeneration of the β-cell mass.

### 4.3.1 Beta-Cell Ablation by Toxins

Rodent β cells are selectively sensitive to the diabetogenic drugs alloxan (ALX) and streptozotocin (STZ), which are glucose analogues that enter the cells through glucose transporter type 2 (GLUT2). Expression of this transporter, which is high in rodent, but not in human β cells, forms the basis for the cell type- and species-specificity of toxicity (Schnedl et al., 1994; De Vos et al., 1995). Both toxins act by generation of reactive oxygen species (ROS) (Szkudelski, 2001; Lenzen, 2008). ALX generates ROS in a cyclic reaction with its reduction product dialuric acid. Glucokinase, an important element of the β-cell glucose-sensing system, is a direct target of ALX (Meglasson et al., 1986). STZ, a nitrosourea, alkylates DNA and indirectly generates superoxide radicals via induction of poly ADP-ribosylation. The degree of β-cell destruction resulting from these toxins depends on the dose and frequency of administration. In neonatal rodents, intravenous administration of STZ induces proliferation of residual β cells, but this effect is insufficient for restoration of long-term glucose tolerance (Bonner-Weir et al., 1981) unless the animals are treated with the hormone glucagon-like peptide 1 (GLP1) (Tourrel et al., 2001).

An antimitotic drug, conophylline, promotes endocrine differentiation in cultured E14.5 pancreas rudiments and augments the β-cell mass in STZ-treated neonatal rats. This effect is associated with an increased number of islet-like cell clusters and expression of the developmental transcription factor pancreatic duodenal homeobox 1 (PDX1) in duct cells (Ogata et al., 2004). Rapid regeneration of β cells in neonatal STZ-treated rats is associated with hyperplasia of α cells that synthesize and secrete increased levels of GLP1, which probably targets immature β cells coexpressing proglucagon (Thyssen et al., 2006). Increased numbers of cells coexpressing somatostatin and insulin, or somatostatin and PDX1, are also seen in neonatal STZ-treated rats following treatment with betacellulin and activin A (Li et al., 2004). Importantly, the β-cell capacity for regeneration following toxin-induced insult declines rapidly during the first days of life (Wang et al., 1996).

In adult rodents, spontaneous restoration of the β-cell mass following toxin-induced β-cell ablation occurs only when exposure to the toxin is limited to a portion of the pancreas using selective perfusion: in the unexposed part, preexisting β cells proliferate, whereas in the exposed part, neogenesis from duct cells was suggested (Waguri et al., 1997). Similar to STZ-treated neonatal rats, a population of somatostatin$^+$/PDX1$^+$ cells was observed in islets of adult mice injected with STZ. These double-positive cells may be similar to islet precursor cells found in embryonic pancreas (Fernandes et al., 1997). Exogenous insulin therapy promoted restoration of the β-cell mass to levels of up to 40% of normal values. This was associated with presence of two putative precursor cell types, one expressing GLUT2 and the other insulin and somatostatin (Guz et al., 2001). Interestingly, in adult mice that received STZ together with the GLP1 analogue exendin-4, expression of PDX1 increased and the *Ngn3* gene, encoding the master switch transcription factor neurogenin-3, became activated in islet-like cell clusters (Kodama et al., 2005). Most remarkably, the combination of gastrin and epidermal growth factor (EGF) rapidly normalized hyperglycemia in ALX-treated diabetic mice (Rooman

and Bouwens, 2004). Under these conditions, the β-cell mass doubled within 3 days, without a change in β-cell size, replication rate, or apoptosis. Within one week, 30–40% of the normal β-cell mass was restored. New β cells were generated from an insulin$^-$ cell pool, as shown by pulse-chase labeling with the thymidine analogue bromodeoxyuridine (BrdU). Moreover, 65% of insulin$^+$ cells at day 4 of treatment expressed duct-cell cytokeratins (Rooman and Bouwens, 2004), suggesting a ductal origin. Gastrin and EGF are thus primary candidate factors for regulation of β-cell neogenesis. Since β cells do not express cholecystokinin-B (CCKB), the high-affinity receptor for gastrin, gastrin-responsive cells are likely to be different from β cells (Rooman et al., 2001). Another member of the EGF family, betacellulin, improved glucose tolerance in alloxan-treated diabetic mice by increasing β-cell volume, at least partly through accelerated neogenesis from duct cells (Yamamoto et al., 2000).

### *4.3.2 Surgical Methods for Inducing Pancreas Injury*

Partial pancreatectomy (PPx) is the surgical removal of part of the pancreas, both exocrine and endocrine. When only 50–70% of the pancreas is removed, mice remain normoglycemic. Following 50%-PPx the β-cell mass in the remaining half of the pancreas fully recovers within 4 weeks. Beta-cell renewal does not depend on activation of NGN3 (Lee et al., 2006), a marker of activated β-cell progenitors in embryonic mouse pancreas (Gu et al., 2002); rather, it is due primarily to enhanced proliferation of preexisting β cells (Dor et al., 2004). Mechanisms proposed to regulate this compensatory growth include signaling through the insulin receptor substrate 2 (IRS2)/AKT/FOXO1 pathway (Peshavaria et al., 2006) and the GLP1 receptor pathway (De Leon et al., 2003). Immediately following surgery, multiple small cell clusters containing a few β cells appear, followed by an increased number of larger islets, in which proliferation involves the transcription factor FOXM (Ackermann et al., 2008). STZ-treated diabetic mice with fasting plasma glucose of more than 200 mg/dl display rapid normalization of glycemia following 50%-PPx, most likely by proliferation of residual β cells (Hardikar et al., 1999).

After 90%-PPx diabetes develops within 3 weeks, and the β-cell mass regenerates thereafter to more than 40% of normal (Bonner-Weir et al., 1983). This surgery is quite difficult to perform in mice, so most of the available data were obtained from rat studies. Discordance between exocrine and endocrine growth was found at 3 weeks following surgery: although the mitotic index of β cells in 90%-PPx rats was doubled, compared to sham-operated animals, proliferation of exocrine cells returned to normal levels (Brockenbrough et al., 1988). The appearance of BrdU-labeled PDX1$^+$ duct cells, which disappeared when BrdU-positive endocrine and exocrine cells appeared, suggests that the newly formed β cells were derived from duct cells (Bonner-Weir et al., 1993; Sharma et al., 1999). Addition of the long-acting GLP1 analogue exendin-4 during 10 days following 90–95%-PPx attenuated the development of diabetes by stimulating neogenesis and replication of β cells

(Xu et al., 1999). When STZ-treated diabetic rats grafted with syngeneic islets to normalize blood glucose underwent 90%-PPx, focal areas of regenerating pancreas appeared (Finegood et al., 1999). In this setting, betacellulin further improved glucose metabolism by promoting β-cell regeneration (Li et al., 2001). These findings suggest that the precursor population for both endocrine and exocrine tissue is not susceptible to damage by STZ. Interestingly, PPx results in a similar regeneration in humans as well. Children under 2 years of age diagnosed with nesidioblastosis (also known as persistent hyperinsulinemic hypoglycemia of infancy) who underwent 90–95%-PPx showed complete pancreatic regeneration in two-thirds of all examined cases, as evidenced by ultrasound measurements (Berrocal et al., 2005).

Partial duct ligation (PDL) is performed by irreversibly clamping off the main duct that drains digestive enzymes from acinar cells in the pancreas tail, resulting in acute pancreatitis from leakage of digestive enzymes into the interstitial space. The severely injured tissue subsequently remodels (Xu et al., 2008; see the description below and Fig. 4.1). The nonligated part (head) of the pancreas remains unaffected and can be used as an internal control. It has been suggested that, in response to the injury, some acinar cells transdifferentiate into duct cells (Bouwens, 1998), whereas most acinar cells undergo p53-dependent apoptosis, thereby stimulating proliferation of duct cells (Scoggins et al., 2000). Upon proliferation, the duct cells form tubular structures, and the islet-cell mass in rat (Hultquist and Joensson, 1965; Wang et al., 1995) and mouse pancreas (Xu et al., 2008) is greatly expanded (80% increase in β-cell mass within 1 week following PDL). Emergence of cellular phenotypes that are intermediary between duct or acinar and endocrine cells suggests that neogenesis may play an instrumental role in the observed increase in β-cell mass (Bouwens and Rooman, 2005). Blood glucose levels and body weight of experimental animals remain similar to sham-treated controls throughout the entire follow-up period. PDL

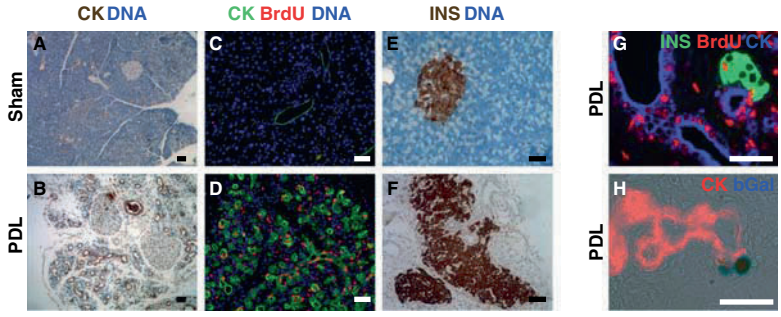



**Fig. 4.1 Partial duct ligation (PDL) induces profound morphological changes and endocrine neogenesis in the ligated tail of mouse pancreas. (a–f)** Immunostaining for cytokeratin (CK), BrdU, and insulin (INS) in PDL and sham-operated pancreas shows formation of ductal tubular structures and large, irregular islet structures on day 14 following ligation. (**g**) Immunostaining for INS, BrdU, and CK shows a β-cell cluster in close proximity to a replicating duct structure 7 days after PDL. (**h**) Histochemical staining for bGal in ligated *Ngn3-lacZ* pancreas (day 7) shows reactivated *Ngn3* gene expression near a duct structure. Bar = 100 μm

induces the expression of several growth factors and cytokines, including gastrin, transforming growth factor alpha (TGFα), interleukin (IL)-1 alpha (IL1α), IL1β, IL6, IL10, tumor necrosis factor alpha (TNFα), Fas ligand, and leukemia inhibitory factor (LIF) (Wang et al., 1997; Yasuda et al., 1999; De Breuck et al., 2006). An additional increase in the expansion rate of the β-cell mass of rats with ligated pancreatic duct can be achieved by gastrin administration (Rooman et al., 2002). The key role of gastrin in controlling β-cell neogenesis is demonstrated by inhibition of PDL-induced increase in β-cell mass using a gastrin antagonist, which blocks the CCKB receptor (Bouwens and Rooman, 2005).

Another method for (partially) obstructing the duct is cellophane wrapping (Rosenberg et al., 1983; Rosenberg, 1998). Two weeks following surgery, this method induces the appearance of small aggregates of proliferating endocrine cells, apparently budding off from pancreatic ducts and resulting in a doubling of β-cell mass within 6 weeks (Rosenberg et al., 1983). The effective factor was identified in a protein mixture extracted from wrapped pancreas and termed islet neogenesis-associated protein (INGAP) (Rafaeloff et al., 1997). INGAP is a REG3-family member and confers resistance to STZ-induced hyperglycemia (Rosenberg et al., 2004; Taylor-Fishwick et al., 2006).

### *4.3.3 Genetic Ablation of Beta Cells*

Regenerative processes can also be triggered by genetic ablation of β cells. The ablation, which is achieved by β-cell-specific expression of toxins in transgenic mice, allows study of the dynamics of β-cell regeneration in a diabetic environment without confounding factors, such as global pancreas injury, recurring autoimmune destruction of newly-generated β cells, and nonspecific cellular toxicity of chemical toxins. Moreover, these models can be combined with genetic lineage tracing, which provides a powerful tool for unraveling the mechanisms of islet regeneration.

Constitutive expression of a dominant-negative form of the KIR6.2 subunit of the ATP-sensitive potassium channel ($K^+_{ATP}$) under control of the human insulin promoter in adult β cells led to increased β-cell apoptosis, possibly by inducing chronic membrane depolarization and high $Ca^{2+}$ levels, leading to severe hyperglycemia at 4 weeks of age (Miki et al., 1997). Surprisingly, after 6 months of age, hyperglycemia spontaneously recovered to normal levels (Oyama et al., 2006). The continued expression of the dominant-negative KIR6.2 subunit in the older transgenic mice excluded the possibility that loss of the transgene was responsible for the recovery. Instead, the appearance of new β cells adjacent to proliferating intraislet duct-like cells (cells labeled with the lectin DBA, an indicator of uncommitted pancreatic epithelial–ductal cells, of which a subset was PDX1- and/or insulin-positive), most likely played a major role in the regenerative process.

Reversible repression of PDX1 expression in adult β cells impairs expression of insulin and GLUT2, reduces islet area without evidence for increased β-cell

apoptosis, and leads to diabetes within 2 weeks (Holland et al., 2005). PDX1 repression was associated with increased cell proliferation, predominantly in the exocrine pancreas but also within islets, and with upregulation of genes implicated in β-cell regeneration, including several REG-family members. Following reexpression of PDX1, normoglycemia was restored within 4 weeks. During this period, $PDX1^{high}$/$insulin^{+}$ and $PDX1^{low}$/$insulin^{-}$ cells were observed, embedded in or closely associated with ducts.

In the PANIC-ATTAC model (pancreatic islet β-cell apoptosis through targeted activation of caspase 8), β-cell death is induced through treatment with a chemical dimerizer (Wang et al., 2008). These mice show extensive β-cell regeneration and full normalization of glucose levels 2 months following termination of treatment. Recovery rate can be enhanced by various pharmacological interventions, including agents acting on the GLP1 axis and agonists of peroxisome proliferator-activated receptor gamma (PPARγ). During recovery, insulin-expressing cells appear in the ducts, and increased numbers of $GLUT2^{+}$/$insulin^{-}$/$PDX1^{-}$ cells are detected in the islets, which may serve as β-cell precursors.

Another recently-developed ablation model uses tamoxifen-inducible *c-Myc* expression in β cells, leading to massive β-cell apoptosis (after a short burst of proliferation) and diabetes (Cano et al., 2008). Shutoff of *c-Myc* expression leads to a gradual regeneration of β cells over the course of 3 months and glycemia reversion close to normal values. This process is seemingly mediated by β-cell replication, rather than neogenesis from pancreatic ducts. However, this study may be confounded by leakiness of *c-Myc* expression, which may cause the β-cell replication observed during the recovery period, thereby obviating the need for neogenesis.

An elegant model for β-cell-specific ablation is based on doxycycline-inducible and β-cell-specific expression of diphtheria toxin, resulting in apoptotic death of up to 80% of β cells and severe diabetes (Nir et al., 2007). Withdrawal of doxycycline after 1 week of treatment resulted in a spontaneous regeneration of the β-cell mass, leading to recovery from diabetes within 2 months. A similar spontaneous regenerative response was seen after hyperglycemia that lasted for more than 4 months. Genetic lineage-tracing analysis suggested that enhanced proliferation of surviving β cells played a major role in regeneration. However, as noted by the authors, a minor contribution of non-β cells to β-cell regeneration could not be excluded. Furthermore, it remains possible that in other settings, for example those involving near-total ablation of β cells, other cells are being recruited to the β-cell lineage.

Assessment of the relative contribution of pancreatic and extrapancreatic non-β cells to islet regeneration in the ablation models will depend on the development of additional tracing models using other cell-specific promoters. Furthermore, ablation models can be crossed with mouse strains that express a fluorescent marker under control of promoters from genes encoding transcription factors known to play roles in islet development or putative progenitor cell markers, thereby permitting the purification of β-cell progenitors in the course of β-cell regeneration. Taken together, the observations made using genetic β-cell ablation systems indicate that although β cells regenerate relatively slowly, their regenerative capacity can potentially be used therapeutically in diabetic patients.

## 4.4 Islet Regeneration: Proliferation and/or (Trans)Differentiation?

### *4.4.1 Proliferation of Preexisting Beta Cells*

In the developing pancreas, β cells differentiate from a population of multipotent NGN3-positive progenitor cells (Gu et al., 2002). Newly formed β cells are initially mitotically inactive, but near the end of fetal development enter a phase of intensive proliferation, which continues through early postnatal life. Thereafter, a much slower rate of β-cell proliferation persists throughout adulthood (Finegood et al., 1995; Teta et al., 2007) and progressively declines with age (Swenne, 1983; Krishnamurthy et al., 2006; Maedler et al., 2006). The length of the β-cell replication cycle is estimated at ±15 h (Swenne, 1982). Replication of a mature β-cell is followed by a refractory period, during which it does not initiate a new round of replication (Teta et al., 2007). Thus, overall β-cell turnover in the adult is rather slow, representing the net result of low levels of both β-cell replication and apoptosis and a relatively long half-life of β cells (Bonner-Weir, 2001; Teta et al., 2005). Measurements of thymidine analogue incorporation in adult mice suggest that β-cell half-life is 2 months or more; however, in aged mice it appears to increase to more than 1 year (Rankin and Kushner, 2009). Despite the slow turnover of β cells under normal physiological conditions, the β-cell mass can expand rapidly through proliferation in an adaptive response to conditions involving an increased demand for insulin, such as insulin resistance (Bruning et al., 1997; Kulkarni et al., 2004), obesity (Kloppel et al., 1985; Butler et al., 2003), and pregnancy (Sorenson and Brelje, 1997). Beta-cell proliferation can also be activated in response to pancreatic injury. Lineage tracing of mouse β cells using the insulin promoter and knockout of genes encoding key cell-cycle regulators, such as cyclin $D_2$, indicate that replication of preexisting β cells is involved in the postnatal generation of new β cells, as well as in the regeneration of the adult β-cell mass following 70%-PPx or β-cell ablation (Dor et al., 2004; Georgia and Bhushan, 2004; Nir et al., 2007). PPx reduces the refractory period in mouse β-cell replication, compared to normal animals. Consecutive labeling of dividing cells with different thymidine analogues in neonatal, pregnant, and PPx mice detected only rare β cells labeled with more than one analogue, suggesting that β-cell precursors or stem cells contribute to postnatal β-cell (re)generation only to a limited extent (Teta et al., 2007). Clonal analysis and pulse-labeling of β cells demonstrate that, at least in healthy adult mice, all β cells possess equal proliferation potential (Brennand et al., 2007).

Further support for the notion that β-cell mass expansion depends to a large extent on proliferation of preexisting β cells is provided by the fact that a majority of the known regulators of β-cell mass affect β-cell proliferation. Cell cycle regulators, such as cyclin-dependent kinase-4 (Rane et al., 1999), cyclin $D_1$ and $D_2$ (Cozar-Castellano et al., 2004; Georgia and Bhushan, 2004; Kushner et al., 2005; Zhang et al., 2005); cyclin–kinase inhibitors of the CIP/KIP and INK families (Rane et al., 1999; Uchida et al., 2005; Georgia and Bhushan, 2006; Krishnamurthy et al.,

2006); tumor suppressors (pRb and p53) (Harvey et al., 1995; Cozar-Castellano et al., 2004; Vasavada et al., 2007); and the transcription factors E2F1 (Fajas et al., 2004; Iglesias et al., 2004), c-MYC (Laybutt et al., 2002; Pelengaris et al., 2002), and HNF1/4 alpha (Gupta et al., 2007; Maestro et al., 2007) regulate both β-cell proliferation and mass. Similarly, growth factors, such as placental lactogens (Vasavada et al., 2000; Cozar-Castellano et al., 2006), growth hormone (Liu et al., 2004), EGF, keratinocyte growth factor (Krakowski et al., 1999), insulin, and insulin-like growth factor I (George et al., 2002; Otani et al., 2004), also induce an increase in β-cell mass via β-cell replication. The transcription factor forkhead box M1 (FoxM1) (Zhang et al., 2006) and the coactivator/histone-3 lysine-4 methytransferase menin, encoded by *Men1* (Karnik et al., 2005; Karnik et al., 2007) also control β-cell replication. Given the limited capacity of β cells to undergo multiple rounds of replication (Teta et al., 2007), identifying the key (epi)genetic elements involved in this limitation is crucial for stimulating β-cell replication in therapeutic strategies in vivo. Understanding which cell type(s) drive(s) adult β-cell replication and which β cells have the highest replication capacity may also facilitate β-cell expansion in vitro.

### *4.4.2 Differentiation of Pancreatic Progenitor Cells*

Hormone-negative, nestin-positive cells present in islets, termed islet-derived progenitor cells (NIPCs), were shown to be capable of differentiation into endocrine and exocrine pancreas, as well as liver (Zulewski et al., 2001). However, subsequent studies demonstrated that nestin was not an appropriate marker of islet progenitor cells; rather, it was shown to be expressed in the mesenchyme of the developing mouse pancreas (Selander and Edlund, 2002) and in the endothelium of adult rat and human pancreas (Lardon et al., 2002; Klein et al., 2003).

A large number of reports have provided indirect evidence for the existence of extraislet pancreatic stem/progenitor cells, mainly within or near the ductal lining. Many of these have already been referred to above (Wang et al., 1995; Fernandes et al., 1997; Waguri et al., 1997; Rosenberg, 1998; Finegood et al., 1999; Sharma et al., 1999; Xu et al., 1999; Yamamoto et al., 2000; Guz et al., 2001; Bonner-Weir et al., 2004; Ogata et al., 2004; Rooman and Bouwens, 2004; Bouwens and Rooman, 2005; Holland et al., 2005; Oyama et al., 2006; Wang et al., 2008). Apart from these descriptive in-vivo data, putative progenitor cells have been isolated and differentiated in vitro. Stem/progenitor cells with the capacity for clonal expansion and differentiation toward multiple pancreatic lineages can be isolated by fluorescence-activated cell sorting, based on negative selection for hematopoietic/vascular antigens (CD45, TER119, c-KIT, and FLK1) and the presence of the hepatocyte growth factor receptor c-MET. When grafted into the pancreas of 4-week-old recipients, further differentiation toward exocrine and endocrine pancreas cell types was noted (Suzuki et al., 2004). Moreover, adult multipotent precursors can be isolated from both islet- and duct-cell preparations in a colony-formation

assay in a serum-free medium (Seaberg et al., 2004). Remarkably, these cells coexpress neural and pancreatic precursor markers and can differentiate into distinct populations of neuronal and glial cells, pancreatic endocrine, and exocrine cells, as well as stellate cells. Since these pancreas-derived cells do not express markers of embryonic stem cells, mesoderm, or neural crest cells, the existence of an intrinsic pancreatic precursor cell population was postulated. Finally, the existence of endocrine stem/progenitor cells within the epithelial compartment of the adult human pancreas was suggested by experiments in which nonendocrine pancreas epithelial cells (NEPECs) were genetically labeled, selected for drug resistance to eliminate contaminating mesenchymal cells, and cotransplanted with fetal pancreas tissue under the kidney capsule of immunodeficient mice (Hao et al., 2006). These conditions resulted in the appearance of β cells. In the absence of evidence for β-cell replication or cell fusion, these experiments led to the conclusion that the β cells originated from stem/progenitor cells.

In the above-mentioned studies the initial cell populations were rather heterogeneous and poorly defined. In contrast, a homogeneous cell population can be isolated following PDL from the pancreas of adult mice expressing a green fluorescent protein (GFP) reporter gene under the control of the *Ngn3* promoter (Xu et al., 2008) (Fig. 4.2). NGN3 expression is induced following PDL in a subset of pancreatic cells, most of which are located in the duct epithelium. *Ngn3* gene knockout prevents the PDL-induced increase in β-cell mass, demonstrating the importance of *Ngn3* gene expression in this process. Remarkably, the ultrastructure of the adult $NGN3^+$ cells is similar to that of bona fide embryonic pancreas endocrine progenitors. When grafted into embryonic pancreas explanted from *Ngn3*-null mice, adult $NGN3^+$ cells differentiate into the different islet cell types, providing direct evidence for the existence of islet-cell progenitors in the adult mouse pancreas (Xu et al., 2008) (Fig. 4.2). Although a large fraction of the adult $NGN3^+$ cells was found among or near duct cells, confirming their origin from duct cells awaits cell lineage-tracing studies.

The ductal origin of adult β cells was recently addressed in transgenic mice driving tamoxifen-regulated Cre recombinase expression under control of the duct-specific human carbonic anhydrase II promoter (Inada et al., 2008; see Chapter 8). When crossed with a loxP-STOP-loxP-lacZ reporter strain and treated with tamoxifen, faithful expression of β-galactosidase was observed in duct cells and ganglia. Following PDL, duct-cell-derived β-galactosidase appeared in endocrine and newly-formed acinar cells, identifying duct cells as facultative progenitors for both cell types in the injured pancreas. We did not observe formation of new acinar cells in the PDL pancreas, possibly owing to subtle differences in surgical techniques. Although these studies provide evidence for the existence of islet-cell progenitors among adult mouse duct cells, it is not known whether all duct cells, or only a distinct subset of them, are capable of differentiation into β cells. These two possibilities would define β-cell regeneration in this system as either duct-cell transdifferentiation or progenitor cell differentiation, respectively.

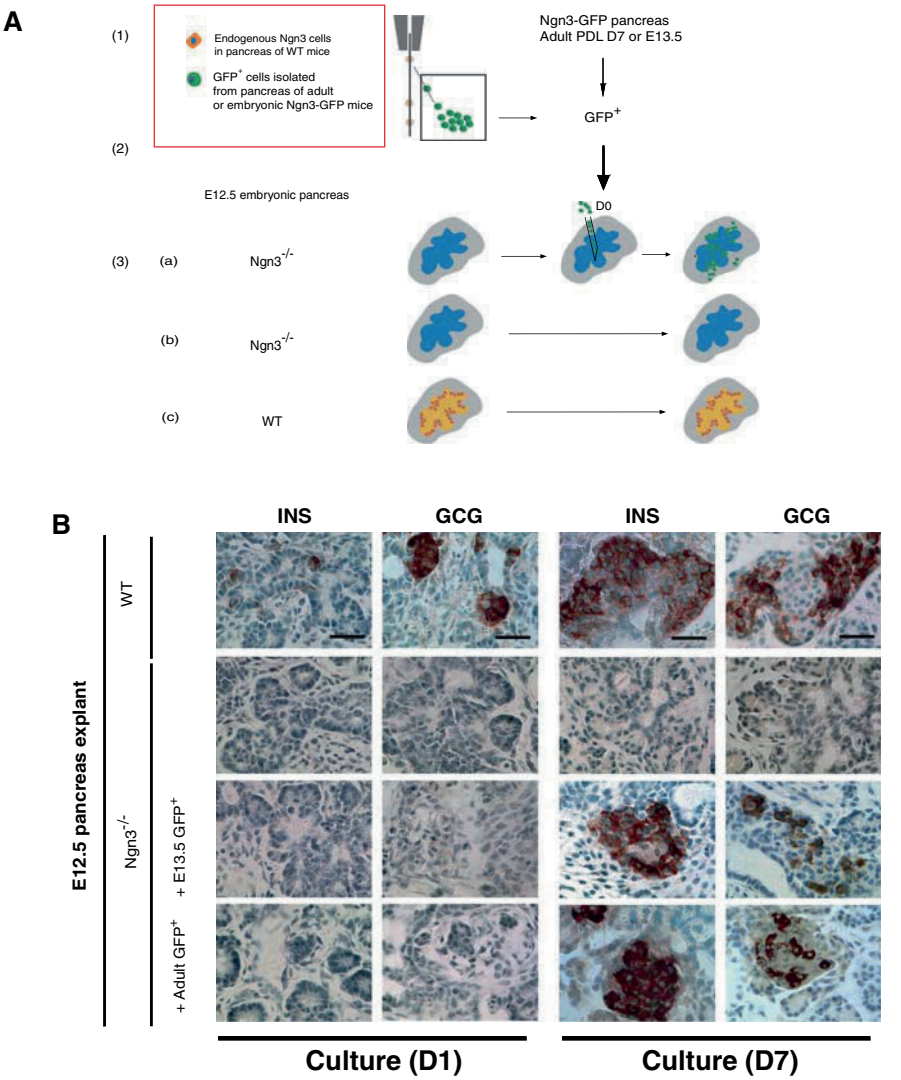



**Fig. 4.2 NGN3$^+$ cells from adult pancreas differentiate in vitro into insulin-expressing cells.** **(a)** Schematic overview of the experiment: GFP$^+$ cells were isolated by flow cytometry from adult (PDL day 7) or embryonic (E13.5) pancreas of *Ngn3-GFP* mice. Embryonic pancreas was explanted from homozygous *Ngn3*-null mice or wild types (WT) at E12.5. One day later (day 0), 500 GFP$^+$ cells were microinjected into the embryonic pancreas and cultured for 1 or 7 days. (**b**) After 1 day in culture, WT embryonic explants expressed insulin and glucagon, whereas *Ngn3* $^{-/-}$ embryonic explants did not, even when injected with GFP$^+$ cells from E13.5 or adult PDL pancreas. After 1 week of culture, WT explants expressed insulin and glucagon, whereas *Ngn3* $^{-/-}$ explants did not. However, when engrafted with GFP$^+$ cells from E13.5 or adult PDL pancreas, the islet hormones were detected in *Ngn3* $^{-/-}$ explants. Bar = 100 μm (adapted from Xu et al., 2008)

### *4.4.3 Transdifferentiation from Pancreatic Exocrine Cells*

Rodent acinar cells can transdifferentiate in vitro into all cell types of the endocrine pancreas (Baeyens et al., 2005; Minami et al., 2005; Okuno et al., 2007; see Chapter 7). In explants of adult pancreas from transgenic mice expressing an inducible Cre gene under control of the elastase promoter, acinar-to-duct cell transdifferentiation was supported by lineage tracing (Means et al., 2005). However, using the same mouse strain, acinar-to-β cell transdifferentiation was not observed in established models of β-cell regeneration (70%-PPx, PDL, caerulein-induced pancreatitis, and treatment with exendin-4) (Desai et al., 2007). Nevertheless, expression of the transcription factors NGN3, PDX1, and MAFA in mouse acinar cells in vivo using adenovirus vectors triggered acinar-to-β-cell transdifferentiation in a subset of cells through nuclear reprogramming (Zhou et al., 2008).

## 4.5 Effects of Cell Microenvironment: The Niche that Allows Beta-Cell Mass Expansion

A stem-cell niche is the specialized microenvironment where stem cells reside (Xie and Spradling, 2000). In response to injury, these resident stem/progenitor cells are activated to proliferate and differentiate to promote tissue regeneration. Unfortunately, the pancreatic stem/progenitor cell niche remains ill-defined, and much additional research is needed to identify its precise location and mechanism of action. Nevertheless, it has been suggested that factors released from endothelial cells promote β-cell replication/regeneration (Hess et al., 2003; Johansson et al., 2006), and recently the concept of a pancreatic vascular niche has been proposed (Nikolova et al., 2006). Endothelial cells and foregut endoderm crosstalk during embryonic development. Pancreatic bud formation is initiated in close contact with endothelial cells of the dorsal aorta and the vitelline veins, both of which supply the primordial foregut endoderm with instructive signals necessary for pancreas formation (Cleaver and Melton, 2003). In turn, the early developing pancreatic endoderm secretes differentiation- and proliferation-inducing signals that instruct nearby endothelial cells to form blood vessels. This reciprocal signaling (Lammert et al., 2003) continues during adulthood, since β cells depend on the presence of vascular-derived basement membrane components, such as laminins, for their proliferation and function. In turn, β cells attract endothelial cells by secretion of vascular endothelial growth factor-A to form a capillary network with a vascular membrane in β-cell proximity. This membrane provides cues for β-cell proliferation and insulin expression and can therefore be defined as a crucial component of the pancreatic niche (Nikolova et al., 2006). At present, it is unknown whether activation of islet-cell progenitor differentiation in the adult pancreas depends on similarly specified niche components. Clearly, a better understanding of the putative pancreatic niche would greatly benefit β-cell regeneration strategies.

## References

Ackermann Misfeldt A, Costa RH, Gannon M. (2008) Beta-cell proliferation, but not neogenesis, following 60% partial pancreatectomy is impaired in the absence of FoxM1. Diabetes. 57:3069–3077.

Baeyens L, De Breuck S, Lardon J, et al. (2005) In vitro generation of insulin-producing β cells from adult exocrine pancreatic cells. Diabetologia. 48:49–57.

Bernard-Kargar C, Ktorza A. (2001) Endocrine pancreas plasticity under physiological and pathological conditions. Diabetes. 50:S30–S35.

Berrocal T, Luque AA, Pinilla I, et al. (2005) Pancreatic regeneration after near-total pancreatectomy in children with nesidioblastosis. Pediatr Radiol. 35:1066–1070.

Bonner-Weir S. (2001) β-cell turnover: its assessment and implications. Diabetes. 50(Suppl 1):S20–S24.

Bonner-Weir S, Trent DF, Honey RN, et al. (1981) Responses of neonatal rat islets to streptozotocin: limited B-cell regeneration and hyperglycemia. Diabetes. 30:64–69.

Bonner-Weir S, Trent DF, Weir GC. (1983) Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. J Clin Invest. 71:1544–1553.

Bonner-Weir S, Baxter LA, Schuppin GT, et al. (1993) A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes. 42:1715–1720.

Bonner-Weir S, Taneja M, Weir GC, et al. (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci USA. 97:7999–8004.

Bonner-Weir S, Toschi E, Inada A, et al. (2004) The pancreatic ductal epithelium serves as a potential pool of progenitor cells. Pediatr Diabetes. 5(Suppl 2):16–22.

Bouwens L. (1998) Transdifferentiation versus stem cell hypothesis for the regeneration of islet β-cells in the pancreas. Microsc Res Tech. 43:332–336.

Bouwens L, Rooman I. (2005) Regulation of pancreatic β-cell mass. Physiol Rev. 85:1255–1270.

Brennand K, Huangfu D, Melton D. (2007) All β cells contribute equally to islet growth and maintenance. PLoS Biol. 5:e163.

Brockenbrough JS, Weir GC, Bonner-Weir S. (1988) Discordance of exocrine and endocrine growth after 90% pancreatectomy in rats. Diabetes. 37:232–236.

Bruning JC, Winnay J, Bonner-Weir S, et al. (1997) Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. Cell. 88:561–572.

Butler AE, Janson J, Bonner-Weir S, et al. (2003) Beta-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. Diabetes. 52:102–110.

Cano DA, Rulifson IC, Heiser PW, et al. (2008) Regulated β-cell regeneration in the adult mouse pancreas. Diabetes. 57:958–966.

Cleaver O, Melton DA. (2003) Endothelial signaling during development. Nat Med. 9: 661–668.

Cozar-Castellano I, Takane KK, Bottino R, et al. (2004) Induction of β-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1. Diabetes. 53:149–159.

Cozar-Castellano I, Weinstock M, Haught M, et al. (2006) Evaluation of β-cell replication in mice transgenic for hepatocyte growth factor and placental lactogen: comprehensive characterization of the G1/S regulatory proteins reveals unique involvement of p21cip. Diabetes. 55:70–77.

De Breuck S, Baeyens L, Bouwens L. (2006) Expression and function of leukaemia inhibitory factor and its receptor in normal and regenerating rat pancreas. Diabetologia. 49:108–116.

De Leon DD, Deng SP, Madani R, et al. (2003) Role of endogenous glucagon-like peptide-1 in islet regeneration after partial pancreatectomy. Diabetes. 52:365–371.

De Vos A, Heimberg H, Quartier E, et al. (1995) Human and rat β cells differ in glucose transporter but not in glucokinase gene expression. J Clin Invest. 96:2489–2495.

Desai BM, Oliver-Krasinski J, De Leon DD, et al. (2007) Preexisting pancreatic acinar cells contribute to acinar cell, but not islet β cell, regeneration. J Clin Invest. 117:971–977.

Dor Y, Brown J, Martinez OI, et al. (2004) Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 429:41–46.
Fajas L, Annicotte JS, Miard S, et al. (2004) Impaired pancreatic growth, β cell mass, and β cell function in E2F1 (–/–)mice. J Clin Invest. 113:1288–1295.
Fernandes A, King LC, Guz Y, et al. (1997) Differentiation of new insulin-producing cells is induced by injury in adult pancreatic islets. Endocrinology. 138:1750–1762.
Finegood DT, Scaglia L, Bonner-Weir S. (1995) Dynamics of β-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. Diabetes. 44:249–256.
Finegood DT, Weir GC, Bonner-Weir S. (1999) Prior streptozotocin treatment does not inhibit pancreas regeneration after 90% pancreatectomy in rats. Am J Physiol. 276:E822–E827.
George M, Ayuso E, Casellas A, et al. (2002) Beta cell expression of IGF-I leads to recovery from type 1 diabetes. J Clin Invest. 109:1153–1163.
Georgia S, Bhushan A. (2004) Beta cell replication is the primary mechanism for maintaining postnatal β cell mass. J Clin Invest. 114:963–968.
Georgia S, Bhushan A. (2006) p27 Regulates the transition of β-cells from quiescence to proliferation. Diabetes. 55:2950–2956.
Gu G, Dubauskaite J, Melton DA. (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development. 129:2447–2457.
Gupta RK, Gao N, Gorski RK, et al. (2007) Expansion of adult β-cell mass in response to increased metabolic demand is dependent on HNF-4alpha. Genes Dev. 21:756–769.
Guz Y, Nasir I, Teitelman G. (2001) Regeneration of pancreatic β cells from intra-islet precursor cells in an experimental model of diabetes. Endocrinology. 142:4956–4968.
Hao E, Tyrberg B, Itkin-Ansari P, et al. (2006) Beta-cell differentiation from nonendocrine epithelial cells of the adult human pancreas. Nat Med. 12:310–316.
Hardikar AA, Karandikar MS, Bhonde RR. (1999) Effect of partial pancreatectomy on diabetic status in BALB/c mice. J Endocrinol. 162:189–195.
Harvey M, Vogel H, Lee EY, et al. (1995) Mice deficient in both p53 and Rb develop tumors primarily of endocrine origin. Cancer Res. 55:1146–1151.
Hess D, Li L, Martin M, et al. (2003) Bone marrow derived stem cells initiate pancreatic regeneration. Nat Biotechnol. 21:763–770.
Holland AM, Gonez LJ, Naselli G, et al. (2005) Conditional expression demonstrates the role of the homeodomain transcription factor Pdx1 in maintenance and regeneration of β-cells in the adult pancreas. Diabetes. 54:2586–2595.
Hultquist GT, Joensson LE. (1965) Ligation of the pancreatic duct in rats. Acta Soc Med Ups. 70:82–88.
Ianus A, Holz GG, Theise ND, et al. (2003) In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest. 111: 843–850.
Iglesias A, Murga M, Laresgoiti U, et al. (2004) Diabetes and exocrine pancreatic insufficiency in E2F1/E2F2 double-mutant mice. J Clin Invest. 113:1398–1407.
Inada A, Nienaber C, Katsuta H, et al. (2008) Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci USA. [Epub ahead of print].
Johansson M, Mattsson G, Andersson A, et al. (2006) Islet endothelial cells and pancreatic β-cell proliferation: studies in vitro and during pregnancy in adult rats. Endocrinology. 147: 2315–2324.
Karnik SK, Chen H, McLean GW, et al. (2007) Menin controls growth of pancreatic β-cells in pregnant mice and promotes gestational diabetes mellitus. Science. 318:806–809.
Karnik SK, Hughes CM, Gu X, et al. (2005) Menin regulates pancreatic islet growth by promoting histone methylation and expression of genes encoding p27Kip1 and p18INK4c. Proc Natl Acad Sci USA. 102:14659–14664.
Klein T, Ling Z, Heimberg H, et al. (2003) Nestin is expressed in vascular endothelial cells in the adult human pancreas. J Histochem Cytochem. 51:697–706.

Kloppel G, Lohr M, Habich K, et al. (1985) Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. Surv Synth Pathol Res. 4:110–125.
Kodama S, Toyonaga T, Kondo T, et al. (2005) Enhanced expression of PDX-1 and Ngn3 by exendin-4 during β cell regeneration in STZ-treated mice. Biochem Biophys Res Commun. 327:1170–1178.
Krakowski ML, Kritzik MR, Jones EM, et al. (1999) Transgenic expression of epidermal growth factor and keratinocyte growth factor in β-cells results in substantial morphological changes. J Endocrinol. 162:167–175.
Krishnamurthy J, Ramsey MR, Ligon KL, et al. (2006) p16INK4a induces an age-dependent decline in islet regenerative potential. Nature. 443:453–457.
Kroon E, Martinson LA, Kadoya K, et al. (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol. 26:443–452.
Kulkarni RN, Jhala US, Winnay JN, et al. (2004) PDX-1 haploinsufficiency limits the compensatory islet hyperplasia that accurs in response to insulin resistance. J Clin Invest. 114:828–836
Rankin MM, Kushner JA. (2009) Adaptive beta-cell proliferation is severely restricted with advanced age. Diabetes. 58:1365–1372.
Kushner JA, Ciemerych MA, Sicinska E, et al. (2005) Cyclins D2 and D1 are essential for postnatal pancreatic β-cell growth. Mol Cell Biol. 25:3752–3762.
Lammert E, Cleaver O, Melton D. (2003) Role of endothelial cells in early pancreas and liver development. Mech Dev. 120:59–64.
Lardon J, Rooman I, Bouwens L. (2002) Nestin expression in pancreatic stellate cells and angiogenic endothelial cells. Histochem Cell Biol. 117:535–540.
Laybutt DR, Weir GC, Kaneto H, et al. (2002) Overexpression of c-Myc in β-cells of transgenic mice causes proliferation and apoptosis, downregulation of insulin gene expression, and diabetes. Diabetes. 51:1793–1804.
Lee CS, De Leon DD, Kaestner KH, et al. (2006) Regeneration of pancreatic islets after partial pancreatectomy in mice does not involve the reactivation of neurogenin-3. Diabetes. 55: 269–272.
Lenzen S. (2008) The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia. 51:216–226.
Li L, Seno M, Yamada H, et al. (2001) Promotion of β-cell regeneration by betacellulin in ninety percent-pancreatectomized rats. Endocrinology. 142:5379–5385.
Li L, Yi ZH, Seno M, et al. (2004) Activin A and betacellulin. Effect on regeneration of pancreatic β-cells in neonatal streptozotocin-treated rats. Diabetes. 53:608–615.
Liu JL, Coschigano KT, Robertson K, et al. (2004) Disruption of growth hormone receptor gene causes diminished pancreatic islet size and increased insulin sensitivity in mice. Am J Physiol Endocrinol Metab. 287:E405–E413.
Maedler K, Schumann DM, Schulthess F, et al. (2006) Aging correlates with decreased β-cell proliferative capacity and enhanced sensitivity to apoptosis: a potential role for Fas and pancreatic duodenal homeobox-1. Diabetes. 55:2455–2462.
Maestro MA, Cardalda C, Boj SF, et al. (2007) Distinct roles of HNF1β, HNF1alpha, and HNF4alpha in regulating pancreas development, β-cell function and growth. Endocr Dev. 12:33–45.
Means AL, Meszoely IM, Suzuki K, et al. (2005) Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates. Development. 132:3767–3776.
Meglasson MD, Burch PT, Berner DK, et al. (1986) Identification of glucokinase as an alloxan-sensitive glucose sensor of the pancreatic β-cell. Diabetes. 35:1163–1173.
Miki T, Tashiro F, Iwanaga T, et al. (1997) Abnormalities of pancreatic islets by targeted expression of a dominant-negative KATP channel. Proc Natl Acad Sci USA. 94: 11969–11973.

Minami K, Okuno M, Miyawaki K, et al. (2005) Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. Proc Natl Acad Sci USA. 102:15116–15121.

Nikolova G, Jabs N, Konstantinova I, et al. (2006) The vascular basement membrane: a niche for insulin gene expression and beta cell proliferation. Dev Cell. 10:397–405.

Nir T, Melton DA, Dor Y. (2007) Recovery from diabetes in mice by β cell regeneration. J Clin Invest. 117:2553–2561.

Ogata T, Li L, Yamada S, et al. (2004) Promotion of β-cell differentiation by conophylline in fetal and neonatal rat pancreas. Diabetes. 53:2596–2602.

Okuno M, Minami K, Okumachi A, et al. (2007) Generation of insulin-secreting cells from pancreatic acinar cells of animal models of type 1 diabetes. Am J Physiol Endocrinol Metab. 292:E158–E165.

Otani K, Kulkarni RN, Baldwin AC, et al. (2004) Reduced β-cell mass and altered glucose sensing impair insulin-secretory function in βIRKO mice. Am J Physiol Endocrinol Metab. 286: E41–E49.

Ouziel-Yahalom L, Zalzman M, Anker-Kitai L, et al. (2006) Expansion and redifferentiation of adult human pancreatic islet cells. Biochem Biophys Res Commun. 341:291–298.

Oyama K, Minami K, Ishizaki K, et al. (2006) Spontaneous recovery from hyperglycemia by regeneration of pancreatic β-cells in Kir6.2G132S transgenic mice. Diabetes. 55:1930–1938.

Pelengaris S, Khan M, Evan GI. (2002) Suppression of Myc-induced apoptosis in β cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. Cell. 109: 321–334.

Peshavaria M, Larmie BL, Lausier J, et al. (2006) Regulation of pancreatic β-cell regeneration in the normoglycemic 60% partial-pancreatectomy mouse. Diabetes. 55:3289–3298.

Rafaeloff R, Pittenger GL, Barlow SW, et al. (1997) Cloning and sequencing of the pancreatic islet neogenesis associated protein (INGAP) gene and its expression in islet neogenesis in hamsters. J Clin Invest. 99:2100–2109.

Rane SG, Dubus P, Mettus RV, et al. (1999) Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-islet cell hyperplasia. Nat Genet. 22:44–52.

Robertson RP. (2004) Islet transplantation as a treatment for diabetes—a work in progress. N Engl J Med. 350:694–705.

Rooman I, Bouwens L. (2004) Combined gastrin and epidermal growth factor treatment induces islet regeneration and restores normoglycaemia in C57Bl6/J mice treated with alloxan. Diabetologia. 47:259–265.

Rooman I, Lardon J, Flamez D, et al. (2001) Mitogenic effect of gastrin and expression of gastrin receptors in duct-like cells of rat pancreas. Gastroenterology. 121:940–949.

Rooman I, Lardon J, Bouwens L. (2002) Gastrin stimulates β-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. Diabetes. 51: 686–690.

Rosenberg L. (1998) Induction of islet cell neogenesis in the adult pancreas: the partial duct obstruction model. Microsc Res Tech. 43:337–346.

Rosenberg L, Brown RA, Duguid WP. (1983) A new approach to the induction of duct epithelial hyperplasia and nesidioblastosis by cellophane wrapping of the hamster pancreas. J Surg Res. 35:63–72.

Rosenberg L, Lipsett M, Yoon JW, et al. (2004) A pentadecapeptide fragment of islet neogenesis-associated protein increases β-cell mass and reverses diabetes in C57BL/6 J mice. Ann Surg. 240:875–884.

Schnedl WJ, Ferber S, Johnson JH, et al. (1994) STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes. 43:1326–1333.

Scoggins CR, Meszoely IM, Wada M, et al. (2000) p53-dependent acinar cell apoptosis triggers epithelial proliferation in duct-ligated murine pancreas. Am J Physiol Gastrointest Liver Physiol. 279:G827–G836.

Seaberg RM, Smukler SR, Kieffer TJ, et al. (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol. 22:1115–1124.
Selander L, Edlund H. (2002) Nestin is expressed in mesenchymal and not epithelial cells of the developing mouse pancreas. Mech Dev. 113:189–192.
Sharma A, Zangen DH, Reitz P, et al. (1999) The homeodomain protein IDX-1 increases after an early burst of proliferation during pancreatic regeneration. Diabetes. 48:507–513.
Sorenson RL, Brelje TC. (1997) Adaptation of islets of Langerhans to pregnancy: β-cell growth, enhanced insulin secretion and the role of lactogenic hormones. Horm Metab Res Hormon- und Stoffwechselforschung. 29:301–307.
Suzuki A, Nakauchi H, Taniguchi H. (2004) Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting. Diabetes. 53:2143–2152.
Swenne I. (1982) The role of glucose in the in vitro regulation of cell cycle kinetics and proliferation of fetal pancreatic B-cells. Diabetes. 31:754–760.
Swenne I. (1983) Effects of aging on the regenerative capacity of the pancreatic B-cell of the rat. Diabetes. 32:14–19.
Szkudelski T. (2001) The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res. 50:537–546.
Taylor-Fishwick DA, Bowman A, Hamblet N, et al. (2006) Islet neogenesis associated protein transgenic mice are resistant to hyperglycemia induced by streptozotocin. J Endocrinol. 190:729–737.
Teta M, Long SY, Wartschow LM, et al. (2005) Very slow turnover of β-cells in aged adult mice. Diabetes. 54:2557–2567.
Teta M, Rankin MM, Long SY, et al. (2007) Growth and regeneration of adult β cells does not involve specialized progenitors. Dev Cell. 12:817–826.
Thyssen S, Arany E, Hill DJ. (2006) Ontogeny of regeneration of β-cells in the neonatal rat after treatment with streptozotocin. Endocrinology. 147:2346–2356.
Tourrel C, Bailbe D, Meile MJ, et al. (2001) Glucagon-like peptide-1 and exendin-4 stimulate β-cell neogenesis in streptozotocin-treated newborn rats resulting in persistently improved glucose homeostasis at adult age. Diabetes. 50:1562–1570.
Uchida T, Nakamura T, Hashimoto N, et al. (2005) Deletion of Cdkn1b ameliorates hyperglycemia by maintaining compensatory hyperinsulinemia in diabetic mice. Nat Med. 11:175–182.
Vasavada RC, Garcia-Ocana A, Zawalich WS, et al. (2000) Targeted expression of placental lactogen in the β cells of transgenic mice results in β cell proliferation, islet mass augmentation, and hypoglycemia. J Biol Chem. 275:15399–15406.
Vasavada RC, Cozar-Castellano I, Sipula D, et al. (2007) Tissue-specific deletion of the retinoblastoma protein in the pancreatic β-cell has limited effects on β-cell replication, mass, and function. Diabetes. 56:57–64.
Waguri M, Miyagawa J, Yamamoto K, et al. (1997) Morphological analysis of neo-islet formation associated with β-cell differentiation. Studies on a new diabetic mouse model induced by selective alloxan perfusion. Diabetes. 46:840–840.
Waldmann H, Adams E, Cobold S. (2008) Reprogramming the immune system: co-receptor blockade as a paradigm for harnessing tolerance mechanisms. Immunol Rev. 223: 361–370.
Wang RN, Kloppel G, Bouwens L. (1995) Duct- to islet-cell differentiation and islet growth in the pancreas of duct-ligated adult rats. Diabetologia. 38:1405–1411.
Wang RN, Bouwens L, Kloppel G. (1996) Beta-cell growth in adolescent and adult rats treated with streptozotocin during the neonatal period. Diabetologia. 39:548–557.
Wang RN, Rehfeld JF, Nielsen FC, et al. (1997) Expression of gastrin and transforming growth factor-alpha during duct to islet cell differentiation in the pancreas of duct-ligated adult rats. Diabetologia. 40:887–893.
Wang ZV, Mu J, Schraw TD, et al. (2008) PANIC-ATTAC: a mouse model for inducible and reversible β-cell ablation. Diabetes. 57:2137–2148.

Xie T, Spradling AC. (2000) A niche maintaining germ line stem cells in the Drosophila ovary. Science. 290:328–330.

Xu G, Stoffers DA, Habener JF, et al. (1999) Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes. 48:2270–2276.

Xu X, D'Hoker J, Stange G, et al. (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell. 132:197–207.

Yamamoto K, Miyagawa J, Waguri M, et al. (2000) Recombinant human betacellulin promotes the neogenesis of β-cells and ameliorates glucose intolerance in mice with diabetes induced by selective alloxan perfusion. Diabetes. 49:2021–2027.

Yasuda H, Kataoka K, Ichimura H, et al. (1999) Cytokine expression and induction of acinar cell apoptosis after pancreatic duct ligation in mice. J Interferon Cytokine Res. 19:637–644.

Zhang H, Ackermann AM, Gusarova GA, et al. (2006) The FoxM1 transcription factor is required to maintain pancreatic β-cell mass. Mol Endocrinol. 20:1853–1866.

Zhang X, Gaspard JP, Mizukami Y, et al. (2005) Overexpression of cyclin D1 in pancreatic β-cells in vivo results in islet hyperplasia without hypoglycemia. Diabetes. 54:712–719.

Zhou Q, Brown J, Kanarek A, et al. (2008) In vivo reprogramming of adult pancreatic exocrine cells to β-cells. Nature. 455:627–632.

Zulewski H, Abraham EJ, Gerlach MJ, et al. (2001) Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes. 50:521–533.

# Chapter 5
# Beta-Cell Expansion in Vitro

**Shimon Efrat**

**Abstract** Ex-vivo development of human insulin-producing cells is considered a promising approach for the generation of an abundant supply of cells for β-cell replacement therapy for diabetes, which is currently limited by the shortage of islet donors. The most obvious cell source for these approaches is the islets themselves. However, over the past two decades investigators have been frustrated by their failure to significantly expand functional β cells from cultured adult human islets. As a result, the research emphasis has turned to exploring the potential of embryonic and tissue stem cells to differentiate into β-like cells, as detailed in other chapters in this book. On the other hand, work in recent years has cautiously renewed hopes for the feasibility of ex-vivo expansion of adult human β cells.

## 5.1 Beta-Cell Replication in Vivo

Most of our knowledge of regulation of the β-cell mass in vivo is derived from rodents. The increase in β-cell mass in neonatal mice is largely due to β-cell replication (Georgia and Bhushan, 2004). Lineage-tracing studies demonstrated that β-cell replication is also the major mechanism for normal β-cell turnover in adult mice (Dor et al., 2004; Teta et al., 2007), as well as for islet regeneration following tissue damage (Nir et al., 2007; Cano et al., 2008). Evidence suggests that the replicative capacity is not restricted to a specialized subpopulation; rather, all mouse β cells are equally capable of replication (Brennand et al., 2007). Subsequent to tissue damage, other mechanisms, in addition to β-cell replication, may play a role in islet regeneration, depending on the severity of the damage. Xu et al. have recently shown that progenitor cells located in pancreatic ducts contribute to islet recovery following severe pancreatic injury in adult mice (Xu et al., 2008; see Chapter 4). Lineage tracing of mouse duct cells in pancreas injury studies support these findings (Inada et al., 2008; see Chapter 8). Beta-cell renewal persists, and even intensifies, in the

S. Efrat (✉)
Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv, 69978, Israel
e-mail: sefrat@post.tau.ac.il

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_5,

face of autoimmune β-cell destruction in nonobese diabetic mice, although the net result is an absolute decrease in β-cell mass (Akirav et al., 2008).

Studying β-cell replication in humans is, of course, much harder. Nevertheless, human pancreas autopsy studies indirectly support the ability of mature human β cells to proliferate in vivo, both in normal tissue maintenance (Meier et al., 2005) and in response to increasing demands for insulin, such as in pregnancy and obesity. Beta-cell damage in type 1 or type 2 diabetes also seems to stimulate β-cell replication (Butler et al., 2003). A recent study in children (2 weeks to 21 years of age) that combined data from abdominal tomography and pancreas autopsy reported that the increase in β-cell mass in infancy is associated with β-cell replication (Meier et al., 2008). Excessive β-cell replication is also responsible for a rare disease termed hyperinsulinism of infancy (Kassem et al., 2000).

## 5.2 Beta-Cell Replication in Vitro

Removal of differentiated cells from their tissue context into the artificial environment in tissue culture induces multiple changes in their gene expression pattern. There are few examples of primary, untransformed, differentiated adult human cells that can be expanded in tissue culture without loss of phenotype. Peripheral blood leukocytes are a notable exception, probably because their in-vivo environment does not involve permanent attachment. Most cells, however, inhabit a complex niche in their normal tissue location, in which they interact with neighboring cells, extracellular matrix (ECM), soluble factors, blood capillaries, and nerve cells. These interactions play key roles in regulating cell phenotype and replication. In vitro most of these interactions are disrupted, leading to extensive changes in gene expression and cellular phenotype.

Intact human islets can be kept in suspension cultures for months without a significant decline in insulin production and secretion (Nielsen et al., 1979). However, under these conditions islet cells do not replicate. When allowed to attach to the culture dish surface, cells begin to migrate out of the islet structure and dedifferentiate within a period of several weeks. During this time, limited β-cell proliferation can be stimulated by various agents. Growth factors (e.g., growth hormone, placental lactogen, prolactin, and glucagon-like peptide-1) and metabolites (particularly glucose) were shown to stimulate a small number of population doublings of rodent insulin-positive cells before cell phenotype was lost (see Nielsen et al., 2001 for review). Attempts at culturing adult human islet cells resulted in a similar loss of β-cell markers in the proliferating cells following a small number of cell-population doublings (Hayek et al., 1995; Beattie et al., 1997; Beattie et al., 1999). In addition, these cultures underwent senescence following more than 15 population doublings. In an effort to mimic the normal cell environment in vivo, cell aggregation and ECM were employed, but preservation of β-cell function remained limited (Beattie et al., 2002). Thus, assessment of β-cell proliferation in vitro beyond the initial culture period is difficult, since the β-cell phenotype is lost. This loss may reflect

β-cell dedifferentiation or β-cell death, accompanied by expansion of cells from a non-β-cell origin. Recent genetic lineage-tracing studies in cells cultured from transgenic mouse islets allowed tracking of mouse β-cell fate in vitro for the first time (Atouf et al., 2007; Chase et al., 2007; Morton et al., 2007; Weinberg et al., 2007). These studies established that mouse β cells dedifferentiated and survived for a few weeks. Dedifferentiation was much faster (days) when the islets were dissociated into single cells, compared with culture of intact islets (weeks) (Weinberg et al., 2007). However, no significant β-cell proliferation could be detected in these studies. Following several weeks in culture, the cell population was taken over by cells derived from a non-β-cell origin. Thus, it was concluded that mouse β cells could not proliferate in vitro under the culture conditions employed.

Gershengorn et al. reported a protocol for expansion of adult human islet cells up to $10^{12}$-fold (Gershengorn et al., 2004). In this procedure intact islets were cultured, and cells migrating out of the islets were propagated in a simple serum-containing medium. The cell doubling time was about 60 h, and the cell morphology resembled that of fibroblasts. The cells were devoid of β-cell markers and instead expressed a number of mesenchymal markers, such as vimentin. These researchers postulated that β cells underwent epithelial-mesenchymal transition (EMT) upon entrance into the cell cycle; however, there was no direct evidence for the origin of the expanded cells from β cells.

In contrast to this expansion protocol, which cultured intact islets, our group used a protocol involving dissociation of the primary islets into single cells (Ouziel-Yahalom et al., 2006). This resulted in a rapid cell dedifferentiation within the first week in culture, followed by induction of replication in most cells. These cells differed in some of their properties from those expanded by Gershengorn et al.: Their doubling time was longer, about 7 days, and their overall expansion rate was lower, only $<10^5$-fold (about 16 population doublings). Although we could detect costaining for insulin and cell replication markers in a minority of cells, there was no direct evidence for the origin of most replicating cells from β cells. Moreover, activation of vimentin expression in cells expressing β-cell markers, such as insulin, supported the occurrence of EMT in these cultures.

Subsequent work from the Gershengorn group abandoned the EMT hypothesis and suggested instead that the expanded cells, termed human islet progenitor cells (hIPC), were derived from mesenchymal stem cells (MSC) normally present in the islets. hIPCs expanded in vitro were shown to express MSC markers and differentiate in vitro into mesodermal cell types, such as adipocytes and osteocytes (Davani et al., 2007). The presence of MSC in human islet preparations was supported by the work of another research group (Gallo et al., 2007). However, the presence of MSC in islets in vivo has not been confirmed, and their occurrence in islet cultures may result from contaminating duct tissue (Seeberger et al., 2006).

Gao et al. presented evidence for dedifferentiation of cultured human islet cells into CK19-positive cells with a duct-cell-like phenotype (Gao et al., 2005). These cells could be expanded in vitro; however, in the absence of a rigorous lineage-tracing study, it was hard to confirm their origin from β cells. It is possible that these cells were derived from duct cells contaminating the islet preparations, which have

been shown to possess a limited proliferation capacity in vitro (Bonner-Weir et al., 2000; Suarez-Pinzon et al., 2005; Yatoh et al., 2007).

Thus, although different groups were able to significantly expand isolated human islet cells in culture, the relatively low purity of these islet preparations and the rapid loss of β-cell phenotype made it difficult to evaluate what fraction of the expanded cells, if any, was derived from β cells.

## 5.3 Lineage-Tracing of Cultured Human Beta Cells

To monitor the fate of cultured human β cells following dedifferentiation directly, we developed a lineage-tracing approach based on a Cre-loxP-mediated DNA recombination system delivered by lentivirus vectors (Russ et al., 2008). Beta cells dissociated from isolated human islets were specifically labeled with green fluorescent protein (GFP) expressed under the cytomegalovirus promoter. In this system GFP expression was blocked by a loxP-flanked DNA fragment (Fig. 5.1). Removal of the block using Cre recombinase expressed under control of the insulin promoter activated GFP expression only in β cells. Using this method we were able to label

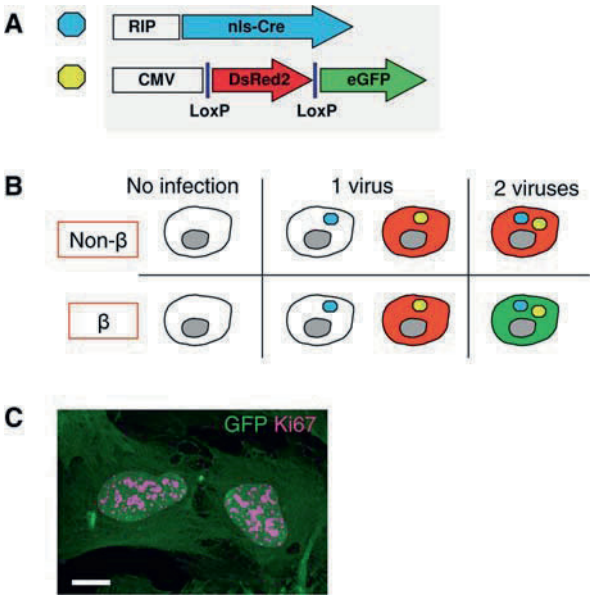



**Fig. 5.1 In-vitro proliferation of cells derived from human β cells. (a)** Isolated islets were dissociated and infected with an insulin promoter–Cre recombinase lentivirus and a reporter lentivirus containing a GFP gene downstream of a loxP-flanked stop fragment. (**b**) Cell-specific removal of the stop fragment in β cells is expected to activate GFP expression. (**c**) Dividing label-positive cells stained for Ki67 are shown at passage 10 (following about 70 days in culture). Bar = 10 μm. **Panel A** is reproduced with permission from Russ et al., 2008.

over 50% of the insulin-positive cells present in the original islet cell preparation and found evidence for massive proliferation of cells derived from them, in contrast to the reports on transgenic mouse β cells (Atouf et al., 2007; Chase et al., 2007; Morton et al., 2007; Weinberg et al., 2007). Label-positive, insulin-negative cells derived from β cells of 15 human donors aged 17–60 were shown to proliferate for a maximum of 16 population doublings. We did not observe age-related differences in the proliferation capacity of the cells within this age range. The approximate doubling times of the labeled cells derived from β cells and the GFP-negative cells present in the same culture were quite similar, about 7 days, as evidenced by the fact that the fraction of GFP-labeled cells remained stable throughout the culture period, about 20%. Given the labeling efficiency, this figure indicates that some 40% of the cultured islet cells were derived from β cells.

The proliferation of cells derived from β cells depended on soluble factor(s) secreted by the non-β cells present in the islet cell culture, as judged by the finding that FACS-sorted GFP-positive cells proliferated poorly unless their culture medium was supplemented with medium conditioned by GFP-negative cells (Russ et al., 2008). The latter finding is supported by the work of Parnaud et al., which showed that β cells purified from isolated human islets by labeling with Newport Green failed to proliferate, in a striking difference with the massive replication capacity of similarly purified adult rat β cells cultured under the same conditions (Parnaud et al., 2008). It should be noted, however, that Parnaud et al. sorted primary insulin-positive cells, whereas we sorted dedifferentiated β cells following proliferation in cell culture, which makes it difficult to compare the two studies.

Analysis of mouse islet cells by our virus lineage-tracing method revealed a much lower proliferation of cells derived from mouse β cells, compared with human β cells, under similar culture conditions (Russ et al., 2008). These findings confirmed the results obtained with transgenic mouse islets and suggested that the culture conditions were more favorable for human than for mouse β-cell expansion.

Taken together, these studies demonstrate a remarkable species difference with respect to β-cell proliferation in vitro: Whereas rat β cells replicate in the absence of support from other pancreatic cells (Parnaud et al., 2008), human dedifferentiated β cells seem to require soluble factor(s) released by non-β cells (Russ et al., 2008) and mouse dedifferentiated β cells cannot replicate even in the mixed culture (Atouf et al., 2007; Chase et al., 2007; Morton et al., 2007; Weinberg et al., 2007; Russ et al., 2008). We do not know the cause of these differences; however, these findings prescribe caution in extrapolating conclusions obtained from studies of rodent β cells to human β cells.

## 5.4 Redifferentiation of Cells Expanded from Human Beta Cells

Our findings demonstrate that cells derived from adult human β cells can be expanded in tissue culture in sufficient numbers to provide all the cell needs for human β-cell replacement at the current availability of human islet donors, provided

that their lost phenotype can be restored. Despite studies on cell cycle regulation in β cells (see Cozar-Castellano et al., 2006; Heit et al., 2006 for reviews, and Chapter 3), our understanding of the relationship between β-cell replication and expression of differentiated functions remains limited. It is unknown whether differentiated β cells in human pancreas in vivo must undergo temporary dedifferentiation before entering the cell cycle. Nevertheless, it appears that induction of significant replication in vitro requires cell delamination out of the normal epithelial structure, a process that results in dedifferentiation. Thus, even if β-cell replication in vivo does not involve dedifferentiation, the latter seems inevitable for significant in-vitro proliferation. If the β-cell phenotype cannot be preserved during proliferation in culture, ways must be found to induce ex-vivo redifferentiation of the expanded cells or restore their function in vivo following transplantation.

We hypothesized that the dedifferentiated cells retain an epigenetic memory of their β-cell phenotype, which may allow their redifferentiation using relatively simple manipulations, either in vitro or in vivo. Interestingly, in MSC expanded from adult human islets the insulin gene was found in an open chromatin structure, although it was not expressed (Mutskov et al., 2007). In an analogous manner, chromosomal regions important for β-cell function may maintain an open conformation in the dedifferentiated cells derived from β cells, but their transcription rates may be low owing to reduced expression of key transcription factors. Upregulation of these factors by overexpression, or indirectly by other treatments, may restore the normal pattern of β-cell gene expression in the expanded cells. It is also possible that the epigenetic memory is gradually eroded during proliferation in culture, thereby limiting the redifferentiation capacity to cells in early passages.

Since dedifferentiation was associated with entry into the cell cycle, it is tempting to postulate that inhibition of cell replication may induce redifferentiation. Serum-free medium, which is depleted of most growth factors and inhibits cell replication, was shown by two research groups to induce limited insulin expression in cultures of human islet cells devoid of insulin (Gershengorn et al., 2004; Lechner et al., 2006). However, since the precise origin of the expanded cells in these cultures was not determined, it is difficult to discern whether this phenomenon represents redifferentiation of dedifferentiated β cells or de novo differentiation of cells from another source. Moreover, these findings with serum-free medium were not reproduced by other studies (Ouziel-Yahalom et al., 2006; Kayali et al., 2007).

We reported that treatment with the EGF-family member betacellulin restored varying degrees of insulin production and secretion in expanded islet cells, which had lost their insulin content, to levels ranging from normal insulin content in cells from some donors to none in others (Ouziel-Yahalom et al., 2006). We could not identify an obvious cause for these variations, such as donor age, sex, or health status, or the quality of islet isolation or shipment. Our preliminary results showed that the expanded cells were capable of differentiation in vivo into insulin-producing cells (unpublished results). As with the experiments using serum-free medium, those with betacellulin suffered from the inability to distinguish between β-cell redifferentiation and de novo differentiation, and they should be repeated with lineage-traced cells to definitively determine the source of the insulin-expressing cells that emerge following treatment.

hIPCs were also shown to form insulin-producing cells when transplanted under the renal capsule of immunodeficient mice—more efficiently than in vitro (Davani et al., 2007). As in the other experiments with these cells, the relevance of these findings to evaluating β-cell redifferentiation approaches is unclear, since the cellular origin of the hIPCs has not been determined.

## 5.5 Signaling Pathways Involved in Ex-Vivo Human Beta-Cell Dedifferentiation and Replication

An attractive approach for identifying molecular targets for redifferentiation of cells expanded from adult human β cells involves elucidation of changes in gene expression that occur during adaptation of these cells to growth in culture. Of particular interest are changes in signal transduction pathways, as these may affect expression of multiple genes downstream. In a recent study we found that human β-cell dedifferentiation and entrance into the cell cycle in vitro correlated with activation of the NOTCH pathway and downregulation of the cell cycle inhibitor p57 (Bar et al., 2008). Using lineage-labeled cells, we showed that the NOTCH intracellular domain (NICD) and its downstream target HES1 appeared in the nuclei of cells derived from β cells that lost insulin expression. In the developing pancreas the NOTCH pathway regulates important cell-fate decisions, including the switch from proliferation to differentiation. Forced expression of NOTCH inhibits pancreas cell differentiation (Hald et al., 2003; Murtaugh et al., 2003), whereas mice with null mutations in genes encoding NOTCH pathway components exhibit accelerated differentiation of endocrine pancreas (Apelqvist et al., 1999; Jensen et al., 2000). The NOTCH pathway is not normally expressed in the adult pancreas; however, it is activated under conditions associated with cell dedifferentiation and replication, such as regeneration following experimental pancreatitis (Jensen et al., 2005), pancreatic neoplasia (Miyamoto et al., 2003), metaplasia of cultured pancreatic exocrine cells (Rooman et al., 2006; see Chapter 7), and in rat β cells exposed to cytokines (Darville and Eizirik, 2006).

Inhibition of HES1 upregulation using small hairpin RNA (shRNA) resulted in higher levels of p57 in β cells, compared with cells treated with a nontarget shRNA, and diminished β-cell proliferation (Bar et al., 2008). Moreover, inhibition of HES1 upregulation reduced β-cell dedifferentiation, as manifested in higher levels of insulin and the β-cell transcription factors PDX1 and NEUROD1, although it did not totally prevent cell dedifferentiation. These findings suggest that a partial cell dedifferentiation is independent of HES1 activity and cell replication; however, induction of advanced dedifferentiation and cell replication requires HES1 upregulation (Fig. 5.2). This interpretation is supported by the finding that the bulk of decrease in insulin mRNA in cultured human β cells occurs during the first week in culture, preceding the peak in HES1 mRNA levels. It is therefore possible that loss of most of the insulin content is a precondition for β-cell entrance into the cell cycle in vitro. The findings emphasize the role of components of the NOTCH pathway in the transition of quiescent β cells into a dedifferentiated, proliferative

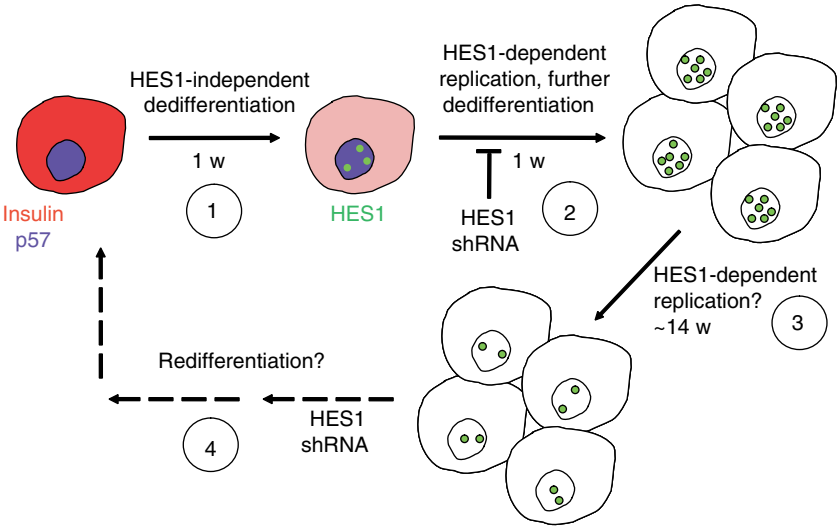



**Fig. 5.2 Proposed model for β-cell dedifferentiation, replication, and redifferentiation. (1)** During the initial days in culture insulin expression declines, whereas HES1 expression is induced. **(2)** HES1 induction blocks p57 expression, induces β-cell replication, and causes further dedifferentiation. HES1 shRNA prevents these events. **(3)** HES1 levels decline; cell replication continues for about 14 additional weeks until cells senesce. **(4)** HES1 shRNA, along with other effectors, may be useful in induction of cell redifferentiation following expansion (Reproduced with permission from Efrat S (2008) Rev Diabet Stud 5:110–116.)

state in vitro and demonstrate a negative correlation between replication and maintenance of differentiated function in cultured β cells. These findings suggest that significant β-cell expansion inevitably involves dedifferentiation and will require the development of ways to achieve cell redifferentiation following expansion. Components of the NOTCH pathway may represent molecular targets for induction of redifferentiation in the expanded cells.

In a recent publication Ikonomou et al. reported the involvement of activated β-catenin signaling in hIPC proliferation in vitro (Ikonomou et al., 2008). However, since the source of these cells has not been correlated to β cells, it is difficult to propose a role for β-catenin in replication of dedifferentiated β cells based on these results. Changes in this pathway, as well as in other candidate pathways, should be analyzed in lineage-traced cultured human β cells to identify additional potential targets for manipulation of expanded dedifferentiated β cells in order to restore their normal phenotype.

## 5.6 Future Prospects

Although cells derived from adult human islet β cells can be significantly expanded in culture, an efficient method for their in-vitro redifferentiation has not yet been

identified. Nevertheless, preliminary transplantation studies do suggest the potential of these cells to redifferentiate into insulin-producing cells in vivo and reverse hyperglycemia. Thus, the search is on for an efficient in-vitro redifferentiation protocol. Alternatively, if it is eventually concluded that full redifferentiation of these cells can only be achieved in vivo, their therapeutic potential will have to be carefully evaluated against the risks of transplanting undifferentiated cells. This risk is expected to be far smaller than that involved in transplantation of cells derived from embryonic stem cells, as the residual replicative potential of cells derived from human islets is very limited.

**Acknowledgments** Work in my laboratory is supported by the Juvenile Diabetes Research Foundation, Israel Science Foundation, and the European Union Beta Cell Therapy Consortium.

## References

Akirav E, Kushner JA, Herold KC. (2008) Beta-cell mass and type 1 diabetes: going, going, gone? Diabetes. 57:2883–2888.

Apelqvist A, Li H, Sommer L, et al. (1999) Notch signalling controls pancreatic cell differentiation. Nature. 400:877–881.

Atouf F, Park CH, Pechhold K, et al. (2007) No evidence for mouse pancreatic β-cell epithelial-mesenchymal transition in vitro. Diabetes. 56:699–702.

Bar Y, Russ HA, Knoller S, et al. (2008) HES1 is involved in adaptation of adult human β cells to proliferation in vitro. Diabetes. 57:2413–2420.

Beattie GM, Cirulli V, Lopez AD, et al. (1997) Ex vivo expansion of human pancreatic endocrine cells. J Clin Endocrinol Metab. 82:1852–1856.

Beattie GM, Itkin-Ansari P, Cirulli V, et al. (1999) Sustained proliferation of PDX-1+ cells derived from human islets. Diabetes. 48:1013–1019.

Beattie GM, Montgomery AM, Lopez AD, et al. (2002) A novel approach to increase human islet cell mass while preserving β-cell function. Diabetes. 51:3435–3439.

Bonner-Weir S, Taneja M, Weir GC, et al. (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci USA. 97:7999–8004.

Brennand K, Huangfu D, Melton D. (2007) All β cells contribute equally to islet growth and maintenance. PLoS Biol. 5:1520–1529.

Butler AE, Janson J, Bonner-Weir S, et al. (2003) Beta cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. Diabetes. 52:102–110.

Cano DA, Rulifson IC, Heiser PW, et al. (2008) Regulated β-cell regeneration in the adult mouse pancreas. Diabetes. 57:958–966.

Chase LG, Ulloa-Montoya F, Kidder BL, et al. (2007) Islet-derived fibroblast-like cells are not derived via epithelial-mesenchymal transition from Pdx-1 or insulin-positive cells. Diabetes. 56:3–7.

Cozar-Castellano I, Fiaschi-Taesch N, Bigatel TA, et al. (2006) Molecular control of cell cycle progression in the pancreatic β-cell. Endocr Rev. 27:356–370.

Darville MI, Eizirik DL. (2006) Notch signaling: a mediator of β-cell de-differentiation in diabetes? Biochem Biophys Res Commun. 339:1063–1068.

Davani B, Ikonomou L, Raaka BM, et al. (2007) Human islet-derived precursor cells are mesenchymal stromal cells that differentiate and mature to hormone-expressing cells in vivo. Stem Cells. 25:3215–3222.

Dor Y, Brown J, Martinez OI, et al. (2004) Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 429:41–46.

Gallo R, Gambelli F, Gava B, et al. (2007) Generation and expansion of multipotent mesenchymal progenitor cells from cultured human pancreatic islets. Cell Death Differ. 14:1860–1871.

Gao R, Ustinov J, Korsgren O, et al. (2005) In vitro neogenesis of human islets reflects the plasticity of differentiated human pancreatic cells. Diabetologia. 48:2296–2304.
Georgia S, Bhushan A. (2004) Beta cell replication is the primary mechanism for maintaining postnatal β cell mass. J Clin Invest. 114:963–968.
Gershengorn MC, Hardikar AA, Wei C, et al. (2004) Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. Science. 306:2261–2264.
Hald J, Hjorth JPTT, German MS, et al. (2003) Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuates endocrine development. Dev Biol. 260:426–437.
Hayek A, Beattie GM, Cirulli V, et al. (1995) Growth factor/matrix-induced proliferation of human adult β-cells. Diabetes. 44:1458–1460.
Heit JJ, Karnik SK, Kim SK. (2006) Intrinsic regulators of pancreatic β-cell proliferation. Annu Rev Cell Dev Biol. 22:311–338.
Ikonomou L, Geras-Raaka E, Raaka BM, et al. (2008) Beta-catenin signalling in mesenchymal islet-derived precursor cells. Cell Prolif. 41:474–491.
Inada A, Nienaber C, Katsuta H, et al. (2008) Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci USA[Epub ahead of print].
Jensen JN, Cameron E, Garay MV, et al. (2005) Recapitulation of elements of embryonic development in adult mouse pancreatic regeneration. Gastroenterology. 128:728–741.
Jensen J, Pedersen EE, Galante P, et al. (2000) Control of endodermal endocrine development by Hes-1. Nat Genet. 24:36–44.
Kassem SA, Ariel I, Thornton PS, et al. (2000) Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy. Diabetes. 49:1325–1333.
Kayali AG, Flores LE, Lopez AD, et al. (2007) Limited capacity of human adult islets expanded in vitro to redifferentiate into insulin-producing β-cells. Diabetes. 56:703–708.
Lechner A, Nolan AL, Blacken RA, et al. (2006) Redifferentiation of insulin-secreting cells after in vitro expansion of adult human pancreatic islet tissue. Biochem Biophys Res Commun. 327:581–588.
Meier JJ, Bhushan A, Butler AE, et al. (2005) Sustained β-cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia. 48: 2221–2228.
Meier JJ, Butler AE, Saisho Y, et al. (2008) Beta-cell replication is the primary mechanism subserving the postnatal expansion of β-cell mass in humans. Diabetes. 57:1584–1594.
Miyamoto Y, Maitra A, Ghosh B, et al. (2003) Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell. 3:565–576.
Morton RA, Geras-Raaka E, Wilson LM, et al. (2007) Endocrine precursor cells from mouse islets are not generated by epithelial-to-mesenchymal transition of mature β cells. Mol Cell Endocrinol. 270:87–93.
Murtaugh LC, Stanger BZ, Kwan KM, et al. (2003) Notch signaling controls multiple steps of pancreatic differentiation. Proc Natl Acad Sci USA. 100:14920–14925.
Mutskov V, Raaka BM, Felsenfeld G, et al. (2007) The human insulin gene displays transcriptionally active epigenetic marks in islet-derived mesenchymal precursor cells in the absence of insulin expression. Stem Cells. 25:3223–3233.
Nielsen JH, Brunstedt J, Andersson A, et al. (1979) Preservation of β cell function in adult human pancreatic islets for several months in vitro. Diabetologia. 16:97–100.
Nielsen JH, Galsgaard ED, Møldrup A, et al. (2001) Regulation of β-cell mass by hormones and growth factors. Diabetes. 50(Suppl 1):S25–S29.
Nir T, Melton DA, Dor Y. (2007) Recovery from diabetes in mice by β cell regeneration. J Clin Invest. 117:2553–2561.
Ouziel-Yahalom L, Zalzman M, Anker-Kitai L, et al. (2006) Expansion and redifferentiation of adult human pancreatic islet cells. Biochem Biophys Res Commun. 341:291–298.
Parnaud G, Bosco D, Berney T, et al. (2008) Proliferation of sorted human and rat β cells. Diabetologia. 51:91–100.

Rooman I, De Medts N, Baeyens L, et al. (2006) Expression of the Notch signaling pathway and effect on exocrine cell proliferation in adult rat pancreas. Am J Pathol. 169:1206–1214.
Russ HA, Bar Y, Ravassard P, et al. (2008) In vitro proliferation of cells derived from adult human β cells revealed by cell-lineage tracing. Diabetes. 57:1575–1583.
Seeberger KL, Dufour JM, Shapiro AM, et al. (2006) Expansion of mesenchymal stem cells from human pancreatic ductal epithelium. Lab Invest. 86:141–153.
Suarez-Pinzon WL, Lakey JR, Brand SJ, et al. (2005) Combination therapy with epidermal growth factor and gastrin induces neogenesis of human islet β-cells from pancreatic duct cells and an increase in functional β-cell mass. J Clin Endocrinol Metab. 90:3401–3409.
Teta M, Rankin MM, Long SY, et al. (2007) Growth and regeneration of adult β cells does not involve specialized progenitors. Dev Cell. 12:817–826.
Weinberg N, Ouziel-Yahalom L, Knoller S, et al. (2007) Lineage tracing evidence for in vitro dedifferentiation but rare proliferation of mouse pancreatic β-cells. Diabetes. 56:1299–1304.
Xu X, D'Hoker J, Stangé G, et al. (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell. 132:197–207.
Yatoh S, Dodge R, Akashi T, et al. (2007) Differentiation of affinity-purified human pancreatic duct cells to β-cells. Diabetes. 56:1802–1809.

# Part II
# Beta Cells from Non-beta Cells

# Chapter 6
# What Does It Take to Make a Beta Cell?

**Gordon C. Weir and Susan Bonner-Weir**

**Abstract** As diabetes with its devastating complications results from β-cell deficiency, there is a compelling need to know more about the culprit. Whereas type 1 diabetes has a near complete loss of β cells owing to autoimmune destruction, type 2 diabetes is associated with a 40–60% reduction in β-cell mass. Insulin resistance, brought on by our Western lifestyle coupled with genetic factors, is clearly of major importance, but diabetes only develops when β-cell deficiency with its associated dysfunctional insulin secretion occurs. Replenishment of β cells by transplantation or by stimulating regeneration of endogenous islets would eliminate the diabetic state. Improving the dysfunctional insulin secretion of diabetes could also provide help. This chapter is written with an eye toward how β cells, or some kind of β-cell surrogate, might function when transplanted. It is important to understand β cells in their normal pancreatic environment to fully appreciate the compromises that will necessarily accompany β-cell replacement therapy.

## 6.1 The Advantageous Anatomic Location of Beta Cells

Beta cells are located within islets, which are clusters of cells consisting in humans of about 70% β cells and 25% non-β islet cells; most of the latter are α cells producing glucagon, smaller numbers of δ cells producing somatostatin, and PP cells producing pancreatic polypeptide. The dorsal lobe of the pancreas, which is derived from the dorsal embryonic anlagen, comprises the tail and upper portion of the head of the pancreas and accounts for more than 80% of its weight (Stefan et al., 1982). The lower part of the head of the pancreas is the ventral lobe, which is derived from the ventral embryonic anlagen. The non-β cells in islets of the dorsal lobe are mostly α cells, whereas in the ventral lobe they are mainly PP cells. There is some evidence

G.C. Weir (✉)
Research Division, Section on Islet Transplantation and Cell Biology, Department of Medicine, Harvard Medical School, Harvard Stem Cell Institute, Joslin Diabetes Center, Boston, MA 02215, USA
e-mail: gordon.weir@joslin.harvard.edu

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_6,

that dorsal lobe islets may function better than ventral lobe islets when transplanted (Trimble et al., 1982).

Secretion of insulin and glucagon into the portal vein, with the liver as a downstream target, creates an efficiency for metabolism in general and for glycemic control specifically. Insulin and glucagon are released in an oscillatory pattern of secretion, with a periodicity of 4–5 min (Porksen et al., 1997). The coordination of these oscillations is probably controlled by oscillations of glycolysis (Tornheim, 1997) and an intrinsic neural network within the pancreas (Stagner et al., 1980). Insulin is secreted in periodic bursts that are impressive in their magnitude, such that the insulin concentration in the portal vein may be tenfold higher than peripheral levels and vary two- to fourfold within minutes (Song et al. 2000). Although not well understood, this bursting pattern of secretion is likely to be important for insulin action in the liver and may have secondary effects beyond the liver, perhaps in muscle and adipose tissue.

Islets are scattered throughout the pancreas. A human pancreas typically contains 1 million islet equivalents (IE), an IE being defined as a hypothetical islet with a diameter of 150 μm. However, there can be considerable variation among individuals, which is due in large part to differences in insulin sensitivity and body size. Thus, variation between 500,000 and 2,000,000 IE can be expected (Ritzel et al. 2006). Human islets are typically between 75 and 200 μm in diameter; islets larger than 400 μm are uncommon. We have estimated an IE to contain on average 1565 cells, of which 1140 are β cells; thus adult humans have about 500 million to 2 billion β cells.

## 6.2 Islet Blood Flow and the Relationship between Islet Cell Types

Islets have a highly developed microvasculature; although they occupy only about 2% of the pancreatic volume, they receive 10% of the pancreatic blood flow (Lifson et al., 1980). Islets contain a glomerular network of capillaries with fenestrated endothelium (Bonner-Weir and Orci, 1982). These very permeable capillaries may facilitate the rapid release of large amounts of insulin into the portal vein. The relationship between islet cell types and the microvasculature has been best studied in rodents, in which islets have a central core of β cells surrounded by a mantle of non-β cells. Small arterioles penetrate through porelike openings in the discontinuous islet mantle cell layer into the islet core. Within the core a glomerular-like network of capillaries is formed, which then exit through the mantle as capillaries or venules (Bonner-Weir and Orci, 1982). Moreover, β cells have a polarized structure, in which one domain is exposed to arterial inflow and another domain, from which insulin is released, faces venous outflow (Bonner-Weir, 1988) (Fig. 6.1). This polarity can be more evident when β cells are seen to form a columnar epithelium.

In this network, known as the islet-acinar portal blood flow (Weir and Bonner-Weir, 1990), blood flows via small capillaries from the β cells in the islet core to the islet mantle cells and then to the acinar cells. As a result, glucagon- and

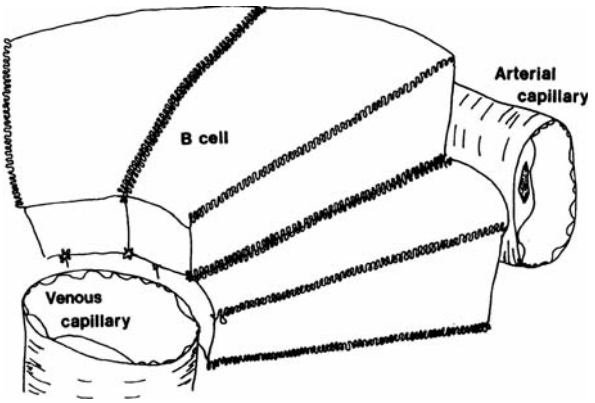



**Fig. 6.1 Polarization of β cells.** Beta cells have two capillary faces, one arterial and the other venous. The lateral interfaces of β cells are smooth surfaces. However, where three or more β cells meet, a canalicular system is found that extends from one capillary face to the other. The canaliculi contain microvilli that are enriched in glucose transporters. This specialized arrangement suggests that interstitial fluid may flow through these canaliculi in an arterial-to-venous direction (Weir and Bonner-Weir, 1990)

somatostatin-containing islet cells and acinar cells are exposed to very high concentrations of insulin and other β-cell secretory products (Fig. 6.2), a fact that likely has important physiological ramifications. There is good evidence that suppression of glucagon secretion by glucose is due in large part to the local inhibitory effects

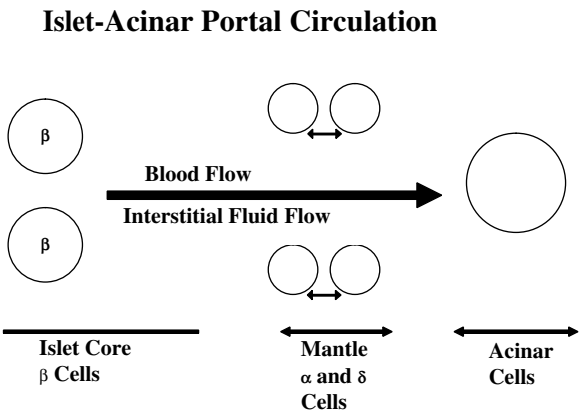



**Fig. 6.2 Islet-acinar portal circulation.** Blood and interstitial fluid in islets flow downstream from the β-cell-containing core to the islet mantle, which contains glucagon-secreting α cells and somatostatin-secreting δ cells, and then on to the acinar cells. Therefore the mantle and acinar cells are bathed in high concentrations of insulin and other β-cell secretory products. There are likely paracrine interactions between α and δ cells. Beta cells adjacent to mantle cells may be influenced by local glucagon or somatostatin secretion, but β cells in the core probably never see high concentrations of these hormones

of glucose-stimulated insulin secretion (GSIS). This was best shown when glucose suppression of glucagon secretion was abolished by passive immunization with insulin antibodies that were infused into the pancreas arteries (Maruyama et al., 1984; Stagner and Samols, 1992). The mechanisms of glucagon suppression are likely to be more complex. In particular, there may be other important mediators of glucagon suppression within islets (Gromada et al., 2007), and the sympathetic nervous system can exert a suppressive effect (Taborsky et al., 2002). Other evidence supporting the importance of the islet-acinar portal circulation comes from the finding that acinar cell secretion can be reduced by removal of local insulin secretion with passive immunization (Trimble et al., 1985).

Human islets have a somewhat more complex anatomy than rodent islets, but the similarities are impressive. Some recent studies have suggested that non-β cells are randomly distributed throughout the center of islets. This conclusion is incorrect; the distribution is not random but rather highly organized. Human islets often consist of several subunits, each of which resembles a rodent islet with a core of β cells and a surrounding mantle of non-β cells, as is shown is Fig. 6.3. The microvasculature of human islets has not yet been elucidated, but it may be similar to that of rodents. This supposition is based upon the finding that infusion of insulin antiserum into

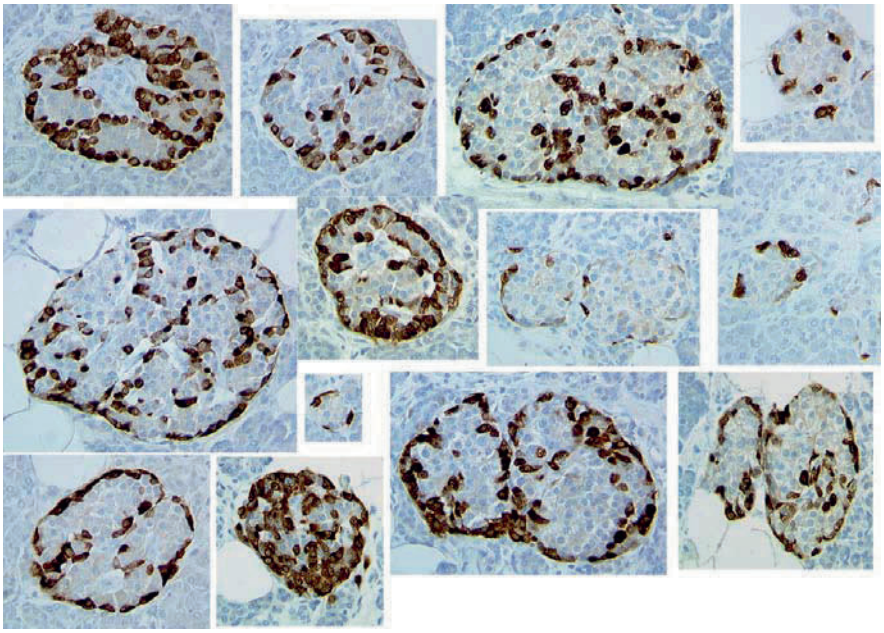


**Fig. 6.3 Glucagon immunostaining of human islets.** This montage shows the variability of human islets. In many islets, particularly the larger ones, there are subunits that consist of β cells surrounded by a mantle of glucagon-containing cells. Thus, the anatomical core–mantle relationship between β and α cells found in rodent islets is maintained in human islets, but as multiple adjacent subunits. Smaller islets are more likely to have the simpler rodent configuration

human pancreas arteries can also abolish glucose suppression of glucagon secretion (Stagner and Samols, 1992). This anatomical and functional arrangement raises the question as to how aggregates of β cells would function were they not accompanied by non-β islet cells. This would be an issue for transplantation if stem cell biology could provide only β cells for transplantation. Owing to the direction of islet blood flow and presumably interstitial fluid flow from core to mantle, it seems likely that the function of β cells is not significantly influenced by mantle cells. Thus, one could predict that transplantation of pure β cells would result in normoglycemia (for a more detailed discussion of this issue see below).

## 6.3 Why Are Islets Distributed throughout the Pancreas in Mammals?

In nonmammalian species, groups of islet cells may be found that are not contained within the exocrine pancreas, with a notable example being the large Brockman body of fish. It was suggested by Henderson that islets might have a beneficial effect upon the exocrine pancreas by enhancing food digestion and absorption, which could have provided an evolutionary advantage (Henderson, 1969). Insulin is an anabolic hormone, which might promote acinar cell survival and function. In agreement with this concept, in type 1 diabetes pancreatic weight is lower (Gepts, 1965) and the output of exocrine enzymes is less than normal (Trimble et al., 1985). Such a beneficial effect might also be exerted during embryological development, in that local secretion of insulin might enhance the development of acinar cells through the islet-acinar portal circulation. It would be interesting to know more about the embryological development of the glomerular-like vasculature in islets, particularly because endothelial cells have been found to be important for β-cell development (Cleaver and Melton, 2003).

## 6.4 A Dominant Role for Beta Cells in Maintaining Blood Glucose Homeostasis

The importance of this single cell type is clear because its absence results in death. There are no meaningful backup mechanisms. The function of β cells depends upon plasma glucose levels, which are typically maintained within a narrow range of 4–7 mM. Many factors influence β-cell function, but the dominance of glucose is clear. When β cells are subjected to chronic hypoglycemia, they undergo involution, probably by apoptosis and β-cell atrophy, as has been shown in rodents with transplantable insulinomas (Chen et al., 1992). Conversely, when β cells are subjected to even mild chronic hyperglycemia, as in the state of impaired glucose tolerance, the β-cell phenotype is markedly altered (Brunzell et al., 1976; Laybutt et al., 2003). The most notable finding is a profound loss of early (first phase) GSIS, while responses to some other secretagogues, such as isoproterenol, are preserved (Robertson and Porte, 1973).

## 6.5 Phenotype of the Typical Beta Cell

Beta-cell heterogeneity is discussed later, but let us now focus on a typical β cell. Beta cells have a diameter of about 10 μm and contain large amounts of insulin. Each β cell contains about 30 pg of insulin, which means that an IE has about 45 ng of insulin. Insulin, which accounts for about 10% of β-cell protein, is stored in secretory granules, which have a dense core of crystallized insulin surrounded by a light halo. The precursor proinsulin is efficiently processed to insulin, such that the granules contain only about 1–2% proinsulin, with the rest consisting of equimolar amounts of insulin and C-peptide. The control of insulin biosynthesis, which must be replenished after meals, is exerted mainly by translation. Transcription is fully turned on in most situations. Insulin mRNA has a long half-life of about 36 h and its levels are quite stable (Iype et al., 2005). Beta cells contain about 10,000 granules, of which some 1000 are docked and primed (Straub and Sharp, 2004; Rutter and Hill, 2006). In response to a glucose challenge, insulin is released with a first-phase burst, which lasts for less than 5 min, followed by a second phase of secretion. Insulin released during the first phase is thought to come from primed granules. The insulin stores far exceed the needs; it can be estimated that less than 20% of stored insulin is released during a meal. The storage of insulin is a complex process in which granules are constantly turned over as old granules are degraded by crinophagy or autophagy (Halban and Wollheim, 1980; Jung et al., 2008). Interestingly, there is preferential secretion of newly synthesized insulin (Halban, 1982).

Not only do glucose levels precisely regulate insulin secretion, but they are also the dominant control mechanism for proinsulin biosynthesis and β-cell replication (Weir and Bonner-Weir, 2007). This regulation is exerted by changes in glucose metabolism that are tightly linked to the extracellular glucose concentration. Glucose levels inside β cells are the same as those outside, thanks to facilitated glucose transporters; in rodents this is GLUT2, but in humans the dominance of GLUT2 is less clear (De Vos et al., 1995). The rate of glucose metabolism is controlled by the rate-limiting step of glycolysis, which is glucose phosphorylation by glucokinase. Glucokinase has a $K_m$ of about 8 mM, which is ideal for phosphorylating physiological levels of glucose (Matschinsky, 1996). The best-understood GSIS pathway is exerted by mitochondrial metabolism, which results in an increased ratio of ATP to ADP that closes an ATP-dependent potassium channel ($K^+_{ATP}$) (Henquin, 2000). This results in membrane depolarization, which opens voltage-dependent calcium channels, leading to a great increase in intracellular calcium that triggers exocytosis. This pathway is called the $K^+_{ATP}$-dependent or triggering pathway, but there is a separate $K^+_{ATP}$-independent or augmentation pathway that also relies on metabolism (Henquin, 2000). The mechanisms responsible for this latter pathway are unknown and represent one of most important unsolved problems in β-cell biology.

Insulin secretion is influenced by many other factors. The incretin hormones glucagon-like peptide-1 (GLP1) and gastrointestinal insulinotropic peptide (GIP) are clearly important. The parasympathetic nervous system is responsible for the cephalic phase of insulin secretion with meals (Teff et al., 1991). Adrenergic

stimulation with locally released norepinephrine suppressing insulin secretion is likely important for preventing hypoglycemia during exercise. An increase in amino acid levels with meals is thought to enhance insulin secretion. Free fatty acids appear to be important for maintaining some insulin secretion when glucose levels are low (Stein et al., 1996). There are no doubt other influences that remain to be defined.

## 6.6 Beta-Cell Turnover and Heterogeneity

Human β cells have a very slow turnover, such that loss of cells from a low rate of apoptosis is replenished by the generation of new β cells by replication of existing β cells and neogenesis (Butler et al., 2003; Bonner-Weir et al., 2004; see Chapters 4 and 8). It seems clear that some β cells can live for years; thus one aspect of β-cell heterogeneity is age. New β cells derived from duct precursors may be functionally different from new β cells generated by replication. Cells in a state of postmitotic senescence or in a proapoptotic phase may have other differences. The local environment of β cells could also create differences. Such environmental differences might include residence in dorsal versus ventral pancreatic lobes and adjacency to blood vessels or nerves or to α or δ cells, such that a small population of β cells could be influenced by the paracrine secretion of glucagon or somatostatin. Beta-cell heterogeneity in GSIS has been shown in dispersed islet cells studied with a variety of techniques (Van Schravendijik et al., 1992). Some of this heterogeneity may be an in-vitro artifact that is not seen in cells within the pancreas (Bennett et al., 1996). The coordination of insulin secretion within islets is influenced by electrical signals and small molecules exchanged via gap junctions (Serre-Beinier et al., 2000). This coordination appears to create synchrony of secretion, which could make heterogeneity less evident.

## 6.7 Insulin Secretion in Normal Physiological Conditions

As we move further into transplantation of isolated islets and hopefully surrogate β cells derived from other sources, such as stem cells, it is important to understand how the performance of grafted cells might compare to that of normal pancreatic islets. The ability of islets to control blood glucose levels and disposition of nutrients in target tissues is very efficient. The reciprocal relationships between insulin and glucagon regulate glycogen metabolism and gluconeogenesis and therefore control glucose uptake and output by the liver. This system is so efficient that a large carbohydrate meal is often associated with a glucose rise of less than 1 mM. The timing and magnitude of insulin release is driven by coordination of the cephalic signals via parasympathetic nerves, incretin hormones, and a rise in the plasma concentrations of glucose and perhaps amino acids. The first and second phases of GSIS, which are clearly seen in intravenous glucose challenge, likely also occur following meals but are obscured by the mixture of signals.

Efficient and rapid suppression of insulin secretion is also critically important. Although glucagon and epinephrine are considered the main defenders against hypoglycemia, suppression of insulin secretion by falling glucose levels is probably more important (Cryer, 2008). Beta cells stop secreting insulin within a minute when blood glucose levels fall below 3 mM. This efficient shutdown of insulin secretion by low glucose levels is important for prevention of postprandial hypoglycemia and hypoglycemia during exercise, when muscle uptake of glucose increases. These remarkable capabilities of pancreatic β cells, which secrete insulin into the portal vein, are important to keep in mind when thinking about what must be accomplished by transplanted β cells grafted into a liver or subcutaneous site.

## 6.8 Does the Normal Relationship among Beta Cells, Non-beta Cells, and Blood Vessels Have to Be Reestablished in a Graft Site?

The ideal treatment for the β-cell deficiency of diabetes would be to generate new islets in the pancreas, which could theoretically be done by stimulating islet neogenesis from duct cells (Suarez-Pinzon et al., 2005). These new islets could be expected to be perfectly normal. If there were a treatment that stimulated β-cell replication in existing islets, it seems likely that their function would be normal, although there are some interesting questions as to whether the ratio of α cells to β cells and their interactions would remain normal.

A very different challenge is faced with the transplantation of islet cells or some other kind of insulin-producing cells. The question becomes a practical issue because it is possible that stem cell biology will create β cells without non-β cells. Even if non-β cells are generated, it will be a major challenge to create grafts that have a normal islet structure, for the reasons described above. However, because islet grafts containing few non-β cells are successful in curing diabetes in rodents and because of what is known about the cellular interactions of normal islets, it seems highly likely that non-β cells are not needed.

It is reassuring that dispersed islet cells, which have been purified with flow cytometry to be enriched in β cells, function well when transplanted into diabetic mice (Keymeulen et al., 1997; King et al., 2007). These dispersed cells were 95% β cells and were reaggregated before being transplanted. The grafts contained small numbers of non-β cells, such that the normal relationship between β cells and non-β cells was not reestablished. To make matters worse, the normal rich glomerular-like vasculature was not reestablished. When islets are transplanted, most of their revascularization occurs by angiogenesis from the recipient; however, vasculature of grafts was found to be very sparse, compared to that of normal islets in the pancreas (Mattsson et al., 2003). This presumably is the major reason for the relative hypoxia of islet grafts in both liver and kidney. These grafts have oxygen tensions of about 5 mm Hg, compared to 40 mm Hg found in pancreatic islets (Carlsson et al., 2001). Attempts have been made to promote vascularization of transplanted islets by various strategies, including overexpression of vascular endothelial growth

factor (VEGF), but these have been unsuccessful (Brissova and Powers, 2008). Other strategies are being tried, but it seems likely that the normal islet vasculature, which is established as islets develop either embryologically or from postnatal neogenesis, would be very difficult to recapitulate in a transplant situation.

In summary, it is clear from rodent studies that grafts containing transplanted islets have a microanatomy that is very different from that of normal islets in the pancreas. The relationships between β and non-β cells are completely disrupted, and vasculature is poorly developed (Mattsson et al., 2003; King et al., 2007). The same distorted anatomy must also occur when subjects with type 1 diabetes receive islet transplants. There are also concerns about innervation. We know that some innervation takes place in transplanted islets (Korsgren et al., 1993), but its physiological relevance is not known. The good news is that islet transplants work well in human autograft situations and in experimental animals, and even in human subjects with islet allografts, in spite of the toxicity of immunosuppressive drugs and continuing immune attack.

## 6.9 How Large Should Islets Be for Optimal Transplantation?

Differences in islet size within the pancreas seem unlikely to be very important for function. There is no reason to think that a β cell in a small islet secretes insulin differently than one in a large islet. Differences have been found when large and small islets are compared in vitro (Colella et al., 1985), but these differences are likely due to in-vitro phenomena. Islets in culture are deprived of their rich vasculature and are therefore dependent upon oxygen diffusion from the medium. Thus, small islets will be well oxygenated, but islets over 150 μm in diameter will have some hypoxia of centrally-located β cells, and islets over 200 μm will have frank central necrosis (Dionne et al., 1993). This difference is expected to have an impact on secretory function, which is a major issue for islet transplantation because all isolated islets used for transplantation are subject to this problem. The situation becomes even worse when islets are cultured at high density. Furthermore, islets in a fresh transplant site, such as in the portal vein tributaries of the liver, are typically clumped together, so that even small islets in the center of a clump are at risk for necrosis. Nonetheless, there is evidence that small islets are more efficacious than large ones when transplanted (Lehmann et al., 2007).

There are related issues when islets are encapsulated prior to transplantation to protect them from immune destruction (see Chapter 12). Relatively small aggregates of islet cells in the range of 40–50 μm in diameter should have advantages over larger ones, whether enclosed in gel beads, conformal coatings, or planar devices. Modeling of oxygen consumption shows that packing density can be markedly improved, which could be especially important for reducing the large surface area that might be required for planar devices (Lewis, 2008). Another benefit is the potential minimization of hypoxia-induced release of chemokines, such as tissue factor or monocyte chemotactic protein-1 (MCP1), which could contribute to a destructive immune reaction.

With all of these considerations taken into account, future islet transplantation may employ aggregates of islet cells of a well-defined size. This may be accomplished by cell processing facilities, once it becomes possible to generate islet cells from stem/progenitor cells. Such aggregates may also be generated from fetal or neonatal porcine islet cells, but adult porcine islet size will be more difficult to regulate because of a strong tendency to undergo apoptosis. We are confident that transplanted aggregates of optimal size will be able to reverse the diabetic state and have an acceptable response time for release or shutoff of insulin secretion as required by normal physiology. However, as is the case of transplantation of whole islets, these grafts will have a very different microanatomy than islets in the pancreas. Unless new science finds a way to reorganize the topography of these clusters to create normal vasculature, innervation, and contacts between β and non-β cells, the grafts will predictably be poorly vascularized aggregates of cells with little resemblance to normal islets.

## 6.10 How Good Must a Beta Cell Be to Succeed When Transplanted?

It has often been suggested that surrogate β cells that differ from normal β cells may provide acceptable transplantation results. The easiest and most compelling argument we can make is that β cells have to be perfectly normal or they will not be able to maintain truly normal glucose levels in response to challenges such as meals, exercise, and fasting. However, there could be value in cells that have defective GSIS but can respond to other agents. We know that β cells in subjects with impaired glucose tolerance (IGT) who are not able to respond to an acute glucose challenge can respond to amino acids, parasympathetic stimulation, and GLP1 (Weir and Bonner-Weir, 2004). Thus, if cells with these characteristics were transplanted they might produce IGT in recipients, which is much better than frank diabetes. It has become apparent that the transcription factor MAFA is important for the final maturation of β cells (Nishimura et al., 2006). Moreover, its expression is deficient in the diabetic state and in neonatal β cells, which also have impaired GSIS (Aguayo-Mazzucato C, Sharma A, Weir GC, Bonner-Weir S and Weir GC, unpublished data). Thus, it can be argued that some type of less-than-perfect β cell might have some value for the treatment of both type 1 and type 2 diabetes.

A similar problem may be encountered with the use of subpar or even normal islet cells contained in immunoprotective capsules because the dynamics of insulin release may be delayed or prolonged. A normal β-cell can release insulin very quickly via its fenestrated vasculature, but insulin secreted from within a capsule must diffuse through fluid and a barrier before reaching small vessels. This means that there will be some delay with mealtime release of insulin, which will probably result in postprandial hyperglycemia. Possibly even more worrisome is the likely difficulty of promptly shutting off insulin release, which could create problems with hypoglycemia 3–4 h after meals or with exercise.

The possibility of using insulin-producing cell lines has been raised. One approach has been to use an oncogene, such as T antigen, to create a cell line that would allow expansion of β cells, whereupon the oncogene could be turned off when differentiated β cells are required (Efrat et al., 1995). There are obvious safety concerns associated with this approach, but this possibility will continue to be explored. The notion of using cells that continue to have cell line characteristics seems fraught with problems. Not only will β-cell turnover not be regulated with the same precision as in normal β cells, but the rapid growth characteristics cannot be compatible with the unique differentiation that is responsible for normal insulin secretion.

Engineering of liver cells to allow glucose stimulation of insulin production has been attempted. One approach employed a recombinant adeno-associated virus expressing a single-chain insulin analogue (SIA), which possessed insulin biological activity without enzymatic conversion, under the control of hepatocyte-specific L-type pyruvate kinase promoter (Lee et al., 2000). This creative gene therapy strategy did lead to more insulin output as plasma glucose levels increased, but the kinetics of release and the precision of the quantity of insulin released did not even come close to matching the physiological needs of people with either type 1 or 2 diabetes (Halban et al., 2001; Halban, 2004). Results obtained with engineered insulin-producing cells in mouse recipients can be misleading because mice have excellent counterregulation mechanisms, which can prevent hypoglycemia from being appreciated. As pointed out earlier, β-cell surrogates must be able to quickly and precisely shut off insulin secretion as glucose levels fall. With transplants of such engineered cells into mice, it is important that the mice be subjected to a prolonged fast to see if severe hypoglycemia develops.

Transdifferentiation of cells derived from endoderm toward a β-cell phenotype has been a goal of a number of investigators. Some success was demonstrated with fetal human liver cells, which were transduced with human telomerase and *Pdx1* genes. These cells contained an impressively large amount of stored insulin, secreted insulin in a regulated manner, and reversed diabetes when transplanted into immunodeficient diabetic mice (Zalzman et al., 2003). More recently, there has been apparent success in the transdifferentiation of pancreatic exocrine cells in mice to β cells by introducing three transcription factor genes (*Ngn3*, *Pdx1*, and *MafA*) with adenoviral vectors (Zhou et al., 2008). These transdifferentated cells have many characteristics of normal β cells, but more study is needed to fully understand their potential (see Chapter 9).

## 6.11 Testing to Determine How Close Insulin-Producing Cells Are to Normal Beta Cells

Many laboratories are trying to make β cells that can be used for β-cell replacement (Bonner-Weir and Weir, 2005). As alluded to above, these efforts include differentiation of human embryonic stem cells or iPS cells (Kroon et al., 2008), generation of β cells from pancreatic duct cells (Bonner-Weir et al., 2000), expansion of existing

β cells with subsequent redifferentiation (Ouziel-Yahalom et al., 2006), transdifferentiation of acinar cells (Zhou et al., 2008) or liver cells (Ferber et al., 2000), and a variety of other bioengineering approaches described in the chapters of this book. All the approaches have resulted in cells that make insulin, but the key issue is how close they are functionally to normal β cells. Although we argued earlier that cells falling a little short of normal β cells could possibly have some clinical value, it is clear that our goal should be to produce the real thing in large numbers. Several papers have proposed a variety of criteria that should be fulfilled (Halban, 2004), and the Juvenile Diabetes Research Foundation (JDRF) has supported laboratories to make such determinations available to the community of scientists. Criteria might include the following:

1. *Storage of insulin.* A β cell should contain about 30 pg of insulin.
2. *Conversion of proinsulin to insulin.* The ratio of stored insulin to proinsulin should be close to 99:1.
3. *Characteristic secretory vesicles.* When evaluated by electron microscopy, β cells should have vesicles with dense cores containing insulin crystals. In humans these can have a square or rectangular shape, but in rodents the shape is usually round. The dense core is surrounded by a light-colored halo that contains cleaved C-peptide.
4. *Glucose-stimulated insulin secretion* (GSIS). This can be assayed in static incubation or perfusion of cells or cell clusters. Some 10,000 β cells (about 10 islets) will secrete about 10 ng of insulin over a period of 30 min in response to a glucose concentration of 16.7 mM. This means that one cell will secrete about 1 pg. With stimulation of cyclic AMP elicited by agents such as isobutylmethylxanthine (IBMX) or exendin-4, they will secrete two to three times as much at the same glucose concentration. It is best to quantify secretion as a function of DNA; β cells and most other cell types contain 6–7 pg of DNA. Expressing secretion as a function of insulin content can be misleading if the cells have become deregulated, as will happen when tissue culture media contain high glucose concentrations.
5. *Basal insulin secretion.* Measurements of basal insulin secretion are problematic. Basal insulin secretion in the presence of a low glucose concentration, such as 2.8 mM, can vary considerably, depending upon how the experiment is performed (Weir et al., 1986). Assays are more reliable with perifused cells than in static incubation. Owing to these issues with basal secretion, fold stimulation determinations can be misleading.
6. *Cell function in vivo*. Transplanted β cells should reverse streptozotocin (STZ)-induced diabetes in immunocompromised mice. Aggregates of cells are usually placed under the kidney capsule. Cure is often considered to be a fed glucose value below 11 mM. It should be remembered that fed glucose levels in mice can often be 10–11 mM, whereas in humans 5–7 mM is the norm. Human β cells have a lower set point for GSIS than mouse β cells. Therefore, a sufficient number of human β cells in mice can drive blood glucose toward normal human levels, as demonstrated in a recent study employing β cells derived from human

embryonic stem cells (Kroon et al., 2008). It is worth remembering that human insulin has a lower biological activity in mice than mouse insulin. Therefore, 1000 IE or more of human islets are needed to cure STZ-diabetes in a mouse, whereas 250 or fewer mouse islets will be successful.

7. *Expression of key proteins.* To identify different islet cell types within a population of cells, staining for insulin or C-peptide is the standard for β cells. To conclusively characterize β cells, staining for other proteins that are relatively β-cell-specific, such as IAPP, PDX1, NKX6.1, MAFA, PC1/3, and PC2, is helpful.

## 6.12 Summary

Pancreatic β cells have a highly-specialized phenotype, which makes possible the precise and effective insulin secretion that maintains blood glucose levels within a very narrow range. Some of this function is dependent upon the unique islet anatomy in the pancreas. For β-cell replacement, transplanted cells should have a β-cell phenotype that is as normal as possible, so as to optimize their function in the abnormal environment of a transplant site.

## References

Bennett BD, Jetton TL, Ying G, et al. (1996) Quantitative subcellular imaging of glucose metabolism within intact pancreatic islets. J Biol Chem. 27:3647–3651.

Bonner-Weir S. (1988) Morphological evidence for pancreatic polarity of β-cell within the islets of Langerhans. Diabetes. 37:616–621.

Bonner-Weir S, Orci L. (1982) New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes. 31:883–939.

Bonner-Weir S, Taneja M, Weir GC, et al. (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci USA. 97:7999–8004.

Bonner-Weir S, Toschi E, Inada A, et al. (2004) The pancreatic ductal epithelium serves as a potential pool of progenitor cells. Pediatr Diabetes. 5(Suppl 2):16–22.

Bonner-Weir S, Weir GC. (2005) New sources of pancreatic beta-cells. Nat Biotechnol. 23: 857–861.

Brissova M, Powers AC. (2008) Revascularization of transplanted islets: can it be improved? Diabetes. 57:2269–2271.

Brunzell JD, Robertson RP, Lerner RL, et al. (1976) Relationships between fasting plasma glucose levels and insulin secretion during intravenous glucose tolerance tests. J Clin Endocrinol Metab. 42:222–229.

Butler AE, Janson J, Bonner-Weir S, et al. (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes. 52:102–110.

Carlsson PO, Palm F, Andersson A, et al. (2001) Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes. 50:489–495.

Chen L, Appel MC, Alam T, et al. (1992) Factors regulating islet regeneration in the post-insulinoma NEDH rat. Adv Exp Med Biol. 321:71–84.

Cleaver O, Melton DA. (2003) Endothelial signaling during development. Nat Med. 9:661–668.

Colella RM, Bonner-Weir S, Braunstein LP, et al. (1985) Pancreatic islets of variable size—insulin secretion and glucose utilization. Life Sci. 37:1059–1065.

Cryer PE. (2008) The barrier of hypoglycemia in diabetes. Diabetes. 57:3169–3176.
De Vos A, Heimberg H, Quartier E, et al. (1995) Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. J Clin Invest. 96:2489–2495.
Dionne KE, Colton CK, Yarmush ML. (1993) Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes. 42:12–21.
Efrat S, Fusco-DeMane D, Lemberg H, et al. (1995) Conditional transformation of a pancreatic β-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. Proc Natl Acad Sci USA. 92:3576–3580.
Ferber S, Halkin A, Cohen H, et al. (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med. 6:505–506.
Gepts W. (1965) Pathological anatomy of the pancreas in juvenile diabetes. Diabetes. 14: 619–633.
Gromada J, Franklin I, Wollheim CB. (2007) Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains. Endocr Rev. 28:84–116.
Halban PA. (1982) Differential rates of release of newly synthesized and of stored insulin from pancreatic islets. Endocrinology. 110:1183–1188.
Halban PA. (2004) Towards a perfect beta cell replacement. Semin Cell Dev Biol. 15:325–326.
Halban PA, Kahn SE, Lernmark A, et al. (2001) Gene and cell-replacement therapy in the treatment of type 1 diabetes: how high must the standards be set? Diabetes. 50:2181–2191.
Halban PA, Wollheim CB. (1980) Intracellular degradation of insulin stores by rat pancreatic islets in vitro: an alternative pathway for homeostases of pancreatic insulin content. J Biol Chem. 255:6003–6006.
Henderson JR. (1969) Why are the islets of Langerhans? Lancet. ii:469–470.
Henquin JC. (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes. 49:1751–1760.
Iype T, Francis J, Garmey JC, et al. (2005) Mechanism of insulin gene regulation by the pancreatic transcription factor Pdx-1: application of pre-mRNA analysis and chromatin immunoprecipitation to assess formation of functional transcriptional complexes. J Biol Chem. 280:16798–16807.
Jung HS, Chung KW, Won Kim J, et al. (2008) Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. Cell Metab. 8:318–324.
Keymeulen B, Anselmo J, Pipeleers D. (1997) Length of metabolic normalization after rat islet cell transplantation depends on endocrine cell composition of graft and on donor age. Diabetologia. 40:1152–1158.
King AJ, Fernandes JR, Hollister-Lock J, et al. (2007) Normal relationship of beta- and non-beta-cells not needed for successful islet transplantation. Diabetes. 56:2312–2318.
Korsgren O, Jansson L, Andersson A, et al. (1993) Reinnervation of transplanted pancreatic islets. A comparison among islets implanted into the kidney, spleen, and liver. Transplantation. 56:138–143.
Kroon E, Martinson LA, Kadoya K, et al. (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol. 26:443–452.
Laybutt DR, Glandt M, Xu G, et al. (2003) Critical reduction in beta-cell mass results in two distinct outcomes over time. Adaptation with impaired glucose tolerance or decompensated diabetes. J Biol Chem. 278:2997–3005.
Lee HC, Kim SJ, Kim KS, et al. (2000) Remission in models of type 1 diabetes by gene therapy using a single-chain insulin analogue. Nature. 408:483–488.
Lehmann R, Zuellig RA, Kugelmeier P, et al. (2007) Superiority of small islets in human islet transplantation. Diabetes. 56:594–603.
Lewis AS (2008) Elminating oxygen supply limitations for transplanted microencapsulated islets in the treatment of type 1 diabetes. PhD Thesis. Massachusetts Institute of Technology, Cambridge, MA.

Lifson N, Kramlinger KG, Mayrand RR, et al. (1980) Blood flow to the rabbit pancreas with special reference to the islets of Langerhans. Gastroenterology. 79:466–473.
Maruyama H, Hisatomi A, Orci L, et al. (1984) Insulin within islets is a physiologic glucagon release inhibitor. J Clin Invest. 74:2296–2299.
Matschinsky FM. (1996) A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes. 45:223–241.
Mattsson G, Jansson L, Nordin A, et al. (2003) Impaired revascularization of transplanted mouse pancreatic islets is chronic and glucose-independent. Transplantation. 75:736–739.
Nishimura W, Kondo T, Salameh T, et al. (2006) A switch from MafB to MafA expression accompanies differentiation to pancreatic beta-cells. Dev Biol. 293:526–539.
Ouziel-Yahalom L, Zalzman M, Anker-Kitai L, et al. (2006) Expansion and redifferentiation of adult human pancreatic islet cells. Biochem Biophys Res Commun. 341:291–298.
Porksen N, Nyholm B, Veldhuis JD, et al. (1997) In humans at least 75% of insulin secretion arises from punctuated insulin secretory bursts. Am J Physiol. 273:E908–E914.
Ritzel RA, Butler AE, Rizza RA, et al. (2006) Relationship between beta-cell mass and fasting blood glucose concentration in humans. Diabetes Care. 29:717–718.
Robertson RP, Porte JD. (1973) The glucose receptor. A defective mechanism in diabetes mellitus distinct from the beta adrenergic diabetes. J Clin Invest. 52:870–876.
Rutter GA, Hill EV. (2006) Insulin vesicle release: walk, kiss, pause. . .then run. Physiology (Bethesda). 21:189–196.
Serre-Beinier V, Le Gurun S, Belluardo N, et al. (2000) Cx36 preferentially connects beta-cells within pancreatic islets. Diabetes. 49:727–734.
Song SH, McIntyre SS, Shah H, et al. (2000) Direct measurement of pulsatile insulin secretion from the portal vein in human subjects. J Clin Endocrinol Metab. 85:4491–4499.
Stagner JI, Samols E. (1992) The vascular order of islet cellular perfusion in the human pancreas. Diabetes. 41:93–97.
Stagner JI, Samols E, Weir GC. (1980) Sustained oscillations of insulin, glucagon, and somatostatin from the isolated canine pancreas during exposure to a constant glucose concentration. J Clin Invest. 65:939–942.
Stefan Y, Orci L, Malaisse-Lagae F, et al. (1982) Quantitation of endocrine cell content in the pancreas of nondiabetic and diabetic humans. Diabetes. 31:694–700.
Stein DT, Esser V, Stevenson BE, et al. (1996) Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. J Clin Invest. 97:2728–2735.
Straub SG, Sharp GW. (2004) Hypothesis: one rate-limiting step controls the magnitude of both phases of glucose-stimulated insulin secretion. Am J Physiol Cell Physiol. 287:C565–C571.
Suarez-Pinzon WL, Lakey JR, Brand SJ, et al. (2005) Combination therapy with epidermal growth factor and gastrin induces neogenesis of human islet beta-cells from pancreatic duct cells and an increase in functional beta-cell mass. J Clin Endocrinol Metab. 90:3401–3409.
Taborsky GJ Jr, Ahren B, Mundinger TO, et al. (2002) Autonomic mechanism and defects in the glucagon response to insulin-induced hypoglycaemia. Diabetes Nutr Metab. 15: 318–323.
Teff KL, Mattes RD, Engelman K. (1991) Cephalic phase insulin release in normal weight males: verification and reliability. Am J Physiol. 261:E430–E436.
Tornheim K. (1997) Are metabolic oscillations responsible for normal oscillatory insulin secretion? Diabetes. 46:1375–1380.
Trimble ER, et al. (1982) Functional differences between rat islets of ventral and dorsal pancreatic origin. J Clin Invest. 69:405–413.
Trimble ER, Bruzzone R, Gjinovci A, et al. (1985) Activity of the insulo-acinar axis in the isolated perfused rat pancreas. Endocrinology. 117:1246–1252.
Van Schravendijik CFH, Kiekens R, Pipeleers DG. (1992) Pancreatic beta cell heterogeneity in glucose-induced insulin secretion. J Biol Chem. 267:21344–21348.
Weir GC, Bonner-Weir S. (1990) Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. J Clin Invest. 85:983–987.

Weir GC, Bonner-Weir S. (2004) Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes. 53(Suppl 3):S16–S21.
Weir GC, Bonner-Weir S. (2007) A dominant role for glucose in beta cell compensation of insulin resistance. J Clin Invest. 117:81–83.
Weir GC, Leahy JL, Barras E, et al. (1986) Characteristics of insulin and glucagon release from the perfused pancreas, intact isolated islets, and dispersed islet cells. Horm Res. 24:62–72.
Zalzman M, Gupta S, Giri RK, et al. (2003) Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells. Proc Natl Acad Sci USA. 100:7253–7258.
Zhou Q, Brown J, Kanarek A, et al. (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature. 455:627–632.

# Chapter 7
# Generation of Beta Cells from Acinar Cells

**Luc Baeyens, Ilse Rooman, and Luc Bouwens**

**Abstract** It is becoming increasingly clear that differentiated adult somatic cells retain the capacity to be reprogrammed into other cell types. In the case of the pancreas, a switch from an acinar to a β-cell phenotype in vitro can be induced by soluble agents, such as growth factors and cytokines. We found that the combination of epidermal growth factor and leukemia inhibitory factor stimulated the transdifferentiation of rat acinar cells into β cells in culture. The transdifferentiation, or cellular reprogramming, appears to recapitulate embryonic events, such as expression of the transcription factor NGN3, which is characteristic of pancreas proendocrine progenitor cells. The NOTCH-signaling pathway, whose activity is normally restricted to embryonic pancreas development, is also reactivated during transdifferentiation. Inhibition of this pathway in the same experimental model leads to further stimulation of β-cell neogenesis from adult acinar cells. Engraftment of the acinar-derived β cells results in correction of glycemia in alloxan-diabetic mice. The phenotype of the transdifferentiated cells is stable in vivo, resulting in normal and safe function following transplantation. This approach opens ways for β-cell replacement therapy by transplantation or regeneration.

## 7.1 Introduction

Recent work by Takahashi et al. on reprogramming of adult fibroblasts to pluripotent embryonic stem cells (Takahashi et al., 2007) has sparked an increased interest in the capacity of adult somatic cells to be reprogrammed into a different phenotype. Transdifferentiation, the reprogramming of "terminally" differentiated cells into another differentiated phenotype, has been studied for many years in different types of tissues (Brockes and Kumar, 2002; Thowfeequ et al., 2007; Tsonis et al., 2004). This process is also referred to as metaplasia, cell plasticity, or lineage switching. It is important to distinguish cell fate switching as a consequence

L. Baeyens (✉)
Cell Differentiation Unit, Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, Brussels 1090, Belgium
e-mail: lbaeyens@vub.ac.be

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_7,

of nuclear transfer to a new cytoplasm or as a result of gene transfer from cell fate changes induced by cell environment. In this chapter we focus on the latter type of transdifferentiation.

Transdifferentiation usually involves a dedifferentiation intermediate stage. Cells in this stage may enter the cell cycle and either proliferate before activating the new differentiation program or proceed directly into differentiation without replication.

A decade ago we proposed transdifferentiation as an alternative to stem cells for β-cell neogenesis (Bouwens, 1998). Although most studies suggest that in the adult pancreas new β cells are generated from duct cells (Bonner-Weir and Weir, 2005), the idea that islet cells could also originate from pancreatic acinar cells is quite old. The French histopathologist E. Laguesse, who was the first to introduce the term "islets of Langerhans" for the pancreatic endocrine microorgans, suggested this possibility (Laguesse, 1896). Later histopathological studies confirmed the existence of transitional or intermediate forms between acinar and islet cells under particular experimental or pathological conditions and in many animal species.

So-called "mixed cells" or "acinar-islet cells," which contain both exocrine and endocrine granules, have been reported by many electron microscopists (Leduc and Jones, 1968; Setalo et al., 1972). Another piece of evidence supporting the occurrence of acinar-to-β-cell transdifferentiation was provided by immunohistochemical studies describing cells that coexpress the acinar enzyme amylase and insulin following experimental pancreatic injury in rats (Bertelli and Bendayan, 1997; Lardon et al., 2004a). One of these procedures, pancreatic duct ligation, consists of a surgical intervention in which the ducts draining the tail part of the pancreas are obstructed by a silk thread, provoking a pancreatitis-like phenomenon in the splenic part of the organ. However, a recent genetic lineage-tracing study using the elastase promoter, which allows permanent marking of cells of acinar origin, failed to demonstrate an acinar origin of β cells in mice that were subjected to three different procedures of pancreatic injury, including duct ligation (Desai et al., 2007). This study employed the elastase-CreERT transgene, which drives tamoxifen-inducible acinar-cell-specific DNA recombination in a reporter transgene.

In contrast, in another recent genetic lineage-tracing study, in-vivo virus-mediated transfer of three genes encoding transcription factors that play key roles in normal β-cell development, NGN3, PDX1, and MAFA, was found to robustly reprogram acinar cells into β-like cells (Zhou et al., 2008). The resulting cells expressed genes essential for β-cell function and were able to ameliorate hyperglycemia in experimental mouse models of diabetes. Although it is uncertain whether these cells can sustain a precise glycemic control as well as normal β cells, these findings hold promise for future treatment of diabetes mellitus. The newly-formed β cells remain scattered within the pancreas and do not form new islet structures, a feature that may affect their survival and functionality. However, this may be less problematic in humans, since scattered β cells are found within the normal human pancreas (Bouwens and Pipeleers, 1998). Another challenge to overcome in this approach is the elimination of adverse effects associated with intraparenchymal injection and virus infection (German, 2008).

Genetic lineage tracing is likely to remain a powerful tool in ascertaining the origin of new β cells from acinar cells in future in-vivo experimental models. There is also an obvious need for defining the extracellular signals involved in normal differentiation of acinar cells, which may lead to new approaches for their reprogramming to β cells.

## 7.2 In-Vitro Dedifferentiation of Acinar Cells

Another approach for studying transdifferentiation or reprogramming of acinar cells is attempting its induction ex vivo, where the microenvironmental conditions can be thoroughly manipulated by controlling the composition of the culture medium. The rationale for evaluating acinar cells as a potential source for β cells is twofold. First, acinar cells are the most abundant cell type in the adult pancreas and therefore represent the most attractive alternative endogenous source for deriving β cells without the need for cell proliferation. Second, the acinar cell lineage is the most closely related developmentally to the pancreatic endocrine lineage. Indeed, acinar and islet cells share a common progenitor, which segregates early in pancreas embryogenesis from the duct lineage progenitor (Gu et al., 2002) (Fig. 7.1). The dosage of the transcription factor p48/PTF1A was recently found to determine the balance between acinar- and islet-cell differentiation in the developing pancreas (Dong et al., 2008).

Thus, from a developmental point of view acinar cells may be more attractive candidates for endocrine progenitors than duct cells, although the latter have been studied much more extensively in this respect. Moreover, adult acinar tissue displays a remarkable plasticity in vitro. When acinar cells are isolated from adult pancreas, they undergo a spontaneous dedifferentiation into ductlike cells. This has been shown in different species (Arias and Bendayan, 1993; Vila et al., 1994; Rooman et al., 2000) and has also been confirmed by genetic lineage tracing in Elastase-CreERT mice (Means et al., 2005). We showed that during this dedifferentiation rat acinar cells lose their exocrine characteristics, such as expression of digestive proenzymes, within the first days in culture. They also downregulate the acinar transcription factors p48/PTF1A and MIST1. On the other hand, they activate expression of genes characteristic of embryonic-fetal pancreatic progenitor cells, such as the gene encoding the transcription factor PDX1 and genes expressed in both ductal cells and embryonic-fetal pancreatic epithelium, such as the one encoding cytokeratin-20 (Rooman et al., 2000). Another embryonic/ductal activated gene encodes for the transcription factor SOX9, which is also considered a pancreatic progenitor marker. The cells also activate expression of receptors for growth factors such as gastrin (Rooman et al., 2001), vascular endothelial growth factor (VEGF) (Rooman et al., 1997), and leukemia inhibitory factor (LIF) (De Breuck et al., 2006), which may play roles in pancreas development. These factors were found to affect proliferation or differentiation of the dedifferentiated cells in culture. These cells also express receptors for netrin, a secreted factor that plays a role in guiding migration of pancreatic progenitor cells during development (De Breuck et al., 2003).

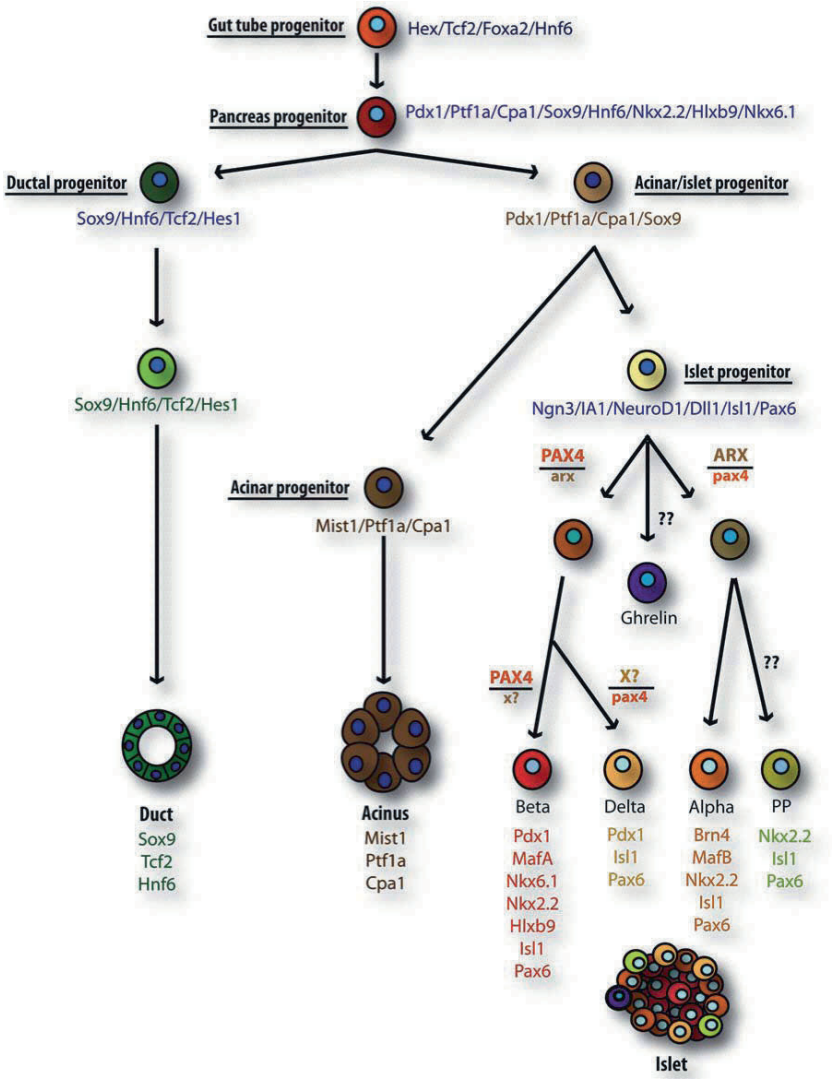



**Fig. 7.1 Embryonic development of the pancreas**. Schematic illustration of pancreatic lineages during rodent embryonic development showing the relationships between duct, acinar, and endocrine cells and their progenitors. Each differentiation stage or cell type is characterized by expression of a particular set of transcription factors

Another interesting receptor that is reexpressed by dedifferentiated acinar cells is NOTCH, along with its downstream target HES1 (Miyamoto et al., 2003; Rooman et al., 2006). Interfering with the NOTCH-signaling pathway in mouse acinar cell cultures had an inhibitory effect on the dedifferentiation process (Miyamoto et al., 2003). During early pancreas development NOTCH signaling maintains the progenitor population via HES1, which blocks expression of the cell cycle inhibitor p57

(Apelqvist et al., 1999; Murtaugh et al., 2003; Georgia et al., 2006) and prevents differentiation into specific lineages. Acinar cell dedifferentiation can be modulated by histone deacetylase inhibitors, such as sodium butyrate and trichostatin-A, and by the ADP-ribosylase inhibitor nicotinamide (Rooman et al., 2000), indicating that chromatin remodeling is involved in the observed changes in gene expression.

The reactivation of embryonic transcription factors, receptors, and signaling pathways supports the hypothesis that acinar cells dedifferentiate into an "uncommitted" progenitor state. This state may be induced by the absence of differentiation maintenance signals, which are normally present in the adult pancreatic environment and are lost during cell isolation. Alternatively, tissue dissociation or tissue injury may activate processes that induce these changes in gene expression. It was recently reported that the metalloproteinase MMP7 is involved in the activation of the NOTCH pathway during acinar dedifferentiation (Sawey et al., 2007). Minami et al. recently reported on the role of cell-to-cell contacts during the in-vitro dedifferentiation of acinar cells (Minami et al., 2008). It is known that alterations in tissue architecture and cellular communications can fundamentally change the differentiation state of cells, and that the response to reprogramming stimuli can differ significantly when cells are taken out of their normal environment. Minami et al. showed that the loss of E-cadherin-mediated cell-to-cell contact was an important step during acinar cell dedifferentiation into a progenitor-like state and that its recovery played an important role during in-vitro transdifferentiation of acinar cells into endocrine cells. PI3-kinase plays an essential role in transducing the E-cadherin signal.

In addition to losing their functional characteristics, dedifferentiated acinar cells become responsive to agents known to control embryonic development. In this progenitor-like state treatment with such agents allows reprogramming of these cells into various phenotypes, including β cells (see below). On the other hand, in the presence of the synthetic glucocorticoid dexamethasone a hepatocyte-like phenotype can be induced directly from acinar cells without the need for prior dedifferentiation (Lardon et al., 2004) (Fig. 7.2). This transdifferentiation is reminiscent of the capacity of embryonic pancreas progenitors following primary transition to become reprogrammed into liver cells by dexamethasone (Shen et al., 2003).

Acinar cells can also transdifferentiate into ductal cells in vivo (Lardon and Bouwens, 2005). An acinar-to-adipocyte transdifferentiation in vivo has also been reported (Bonal et al., 2008). What may be the biological role of this plasticity? One possibility is that in cases of pancreatic injury, such as in ductal obstruction or some forms of pancreatitis, acinar cells dedifferentiate or die to prevent damage caused by activation of exocrine enzymes. In acute pancreatitis, the transient dedifferentiation of acinar cells is well-documented, with temporary acquirement of fetal-ductal characteristics (Jensen et al., 2005). The reactivation of the NOTCH pathway in this model is essential for restoration of the acinar differentiation (Siveke et al., 2008). Another interesting possibility is that this acinar cell plasticity is an evolutionary relic. It was recently shown that when *Xenopus* tadpoles undergo metamorphosis, their exocrine pancreas remodels by massive dedifferentiation of exocrine acinar cells into a progenitor cell phenotype. The acinar cells lose their zymogen granules

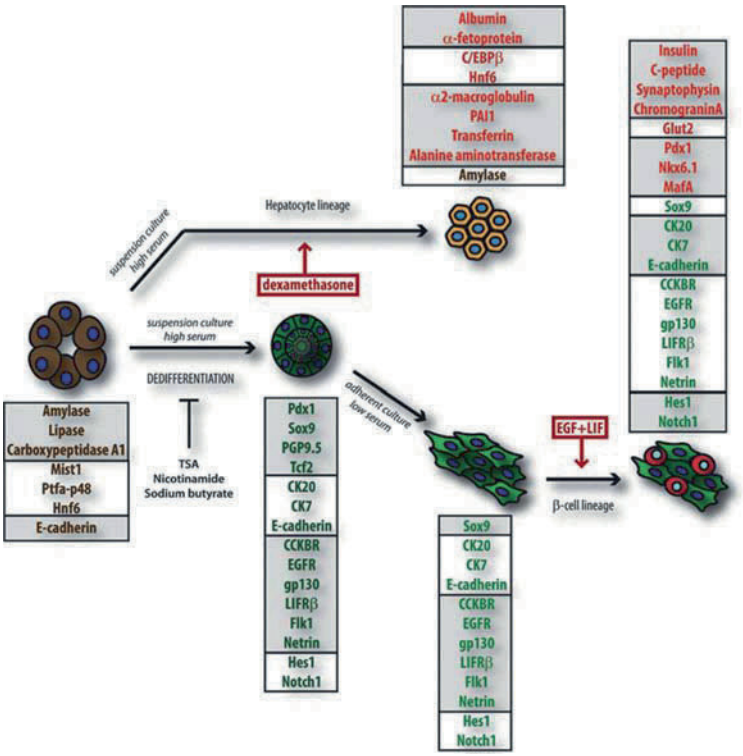



**Fig. 7.2 Acinar cell transdifferentiation**. Schematic illustration of acinar cell reprogramming in vitro induced by soluble factors. Proteins characteristic of acinar cells are indicated in brown, those expressed in dedifferentiated acinar cells are shown in green, and those activated in reprogrammed hepatocyte-like cells (induced by dexamethasone) or β-like cells (induced by EGF+LIF) are shown in red.

and activate *Pdx1*, *Notch1*, and *Hes1* gene expression, after which they redifferentiate into acinar and duct cells. In this way, the ductal system, which is absent in the tadpole, is reformed in the adult amphibian pancreas (Mukhi et al., 2008). These changes in gene expression and differentiation are similar to those observed in rodent acinar cells during dedifferentiation (see above).

## 7.3 In-Vitro Transdifferentiation of Acinar Cells into Beta Cells

Our group was the first to report that transdifferentiation of primary rat acinar cells into endocrine β-like cells can be induced ex vivo with soluble factors, epidermal growth factor (EGF), and LIF (Baeyens et al., 2005). A second group, led by S. Seino, reported on the induced transdifferentiation of murine acinar cells

into β-like cells in the presence of EGF and another differentiation-inducing agent, nicotinamide (Minami et al., 2005). Whereas the latter group used genetic lineage tracing to demonstrate the acinar origin of the newly generated murine β cells, we developed a lectin-tracer-based method to confirm the acinar origin of such cells in other species, such as rats (Baeyens L et al., unpublished results). Lectin tracing was performed by intraparenchymal injection of a fluorescent wheat germ agglutinin (WGA) before pancreas dissociation and cell isolation. In this way, WGA labels only the acinar cells and none of the other cell types, such as duct cells, centroacinar cells, islet cells, blood vessels, or mesenchymal cells. The label is maintained in the cytoplasm and is stable for more than 10 days. Following in-vitro transdifferentiation, newly formed β cells contained this acinar tracer, which demonstrated their acinar origin. It has also been shown that β cells can be generated from acinar tissue collected from mouse models of type 1 diabetes, demonstrating that metabolic disorders do not interfere with the capacity of acinar cells to transdifferentiate into β cells (Okuno et al., 2007).

Transdifferentiation was also reported for the acinar-derived rat tumor cell line AR42J. In the presence of hepatocyte growth factor, glucagon-like peptide-1, or the combination of activin A and betacellulin, these cells could be converted into insulin-producing cells (Mashima et al., 1996; Mashima et al., 1996a; Zhou et al., 1999). Interestingly, the embryonic proendocrine transcription factors NGN3 and PAX4 were induced in these cells (Zhang et al., 2001; Kanno et al., 2006). This cell line was also shown to transdifferentiate into hepatocyte-like cells in the presence of dexamethasone (Tosh et al., 2002) or following expression of the liver-specific transcription factor C/EBPβ α (Shen et al., 2000).

## 7.4 Mechanism of Acinar-to-Beta-Cell Transdifferentiation

We investigated the molecular mechanism involved in transdifferentiation of acinar cells into β cells induced by EGF and LIF. LIF exhibits a wide range of biological activities, including induction of proliferation and differentiation of different cell types (Kurzrock et al., 1991). It is also known to maintain pluripotency of murine embryonic stem cells. During neurogenesis, LIF regulates the differentiation of neural precursors into neurons or glial cells (Viti et al., 2003). In the latter study, it was also shown that EGF increases the competence of LIF as an inducer of astrocyte differentiation. Furthermore, LIF is considered a key signal for injury-induced nerve regeneration in the adult (Niwa et al., 1998; Chambers and Smith, 2004). We demonstrated an increased expression of LIF and its receptor in injured pancreas tissue (De Breuck et al., 2006). In light of the many similarities between neurogenesis and pancreatic islet formation, it seems likely that signals regulating differentiation in the neural system may exhibit a similar effect in the pancreas.

In our in-vitro transdifferentiation model, a strong inhibition of β-cell neogenesis can be observed after blocking EGF or LIF signal transduction, namely by inhibiting the EGF receptor or the JAK2 or STAT3 mediators of cytokine receptor signaling.

The strongest effect was achieved by preventing STAT3 activation in this model. The latter observation is in accordance with the effects described during neuronal development, in which EGF signaling was shown to amplify the responsiveness of LIF-mediated signaling at the level of STAT3 activation (Baeyens et al., 2006).

In EGF/LIF-treated rat acinar cells (Baeyens et al., 2006), as well as in EGF/nicotinamide-treated mouse acinar cells (Minami et al., 2005), reexpression of NGN3 was noted during transdifferentiation (Fig. 7.2). This transcription factor is not expressed postnatally, but is known to be crucial for the development of endocrine cells in the embryonic pancreas during secondary transition (Gradwohl et al., 2000; Schwitzgebel et al., 2000; Gu et al., 2002). We found a transient upregulation of *Ngn3* mRNA and protein expression immediately preceding, and partially overlapping, the expression of insulin (Baeyens et al., 2006). The upregulation of NGN3 and the ensuing expression of insulin and other β-cell markers could be significantly inhibited by specific chemical inhibitors of JAK2 and STAT3 signaling (Baeyens et al., 2006). RNA interference with specific siRNA to silence *Ngn3* expression led to a strong inhibition of β-cell neogenesis (Baeyens et al., 2006). These findings demonstrate that NGN3 expression is just as necessary for endocrine differentiation in adult pancreatic cell reprogramming as it is for islet development. It is noteworthy that following EGF/LIF-induced differentiation most NGN3-expressing cells gave rise to insulin-positive cells and that only very few cells expressing glucagon or other islet hormones appeared in the culture. This might be explained by the upregulation of specific transcription factors downstream of NGN3.

The signaling cascade leading to final endocrine cell differentiation is tightly controlled by the balance among the different cell-specifying transcription factors. If the relative abundance of one set of transcription factors outweighs the others, the equilibrium within the endocrine progenitor pool shifts toward one particular cell type. An example is given by the opposite actions of PAX4 and ARX (Fig. 7.1). Although originating from the same NGN3-positive progenitor, an excess of PAX4 will push the cells toward the β/δ cell lineage. On the other hand, if ARX outweighs PAX4, the progenitor cell will progress into the α/PP cell lineage. Beta-cell neogenesis induced by EGF and LIF is characterized not only by a reexpression of NGN3, but also by the specific upregulation of its downstream target PAX4 (unpublished observations). If we take into account that no changes were observed in ARX expression, this may help explain why this treatment generates predominantly β cells.

Immediately preceding NGN3 expression in EGF/LIF-treated acinar cells a transient increase in *Hnf6* transcript and protein levels was noted. HNF6 is another transcription factor associated with pancreas progenitor cells during embryonic development and is known to transactivate the *Ngn3* promoter prior to initiation of endocrine differentiation (Jacquemin, et al., 2000). The appearance of HNF6 is accompanied by a striking downregulation of the NOTCH signaling factor HES1 and a rapid increase in HES6. Although not demonstrated in the pancreas, HES6 is a known inhibitor of HES1 during neuronal development (Bae et al., 2000). HES1 expression in embryonic pancreas cells is induced by neighboring cells expressing NOTCH ligands, such as DELTA or JAGGED. In cells expressing the NOTCH

receptor HES1 represses *Ngn3* and thus prevents endocrine differentiation. Instead, these cells remain in the progenitor pool and may differentiate to exocrine cells at a later point in time when NOTCH and HES1 are downregulated. This process, which is also known as lateral inhibition, is responsible for restricting the number of pancreatic epithelial cells that differentiate into endocrine cells (Apelqvist et al., 1999; Gu et al., 2002; Murtaugh et al., 2003). Therefore, the NOTCH–HES1 pathway can be considered an endocrine–exocrine gatekeeper. This gatekeeper function also operates during adult acinar cell transdifferentiation. Indeed, in acinar cell cultures treated with EGF and LIF, only 10% of the cells can be reprogrammed into β cells and exhibit NOTCH–HES1 signaling activity (Baeyens L et al., unpublished results). Hyperactivation of NOTCH signaling by an excess of ligands enhances antiendocrine signaling and renders the dedifferentiated acinar cells insensitive to the growth factor treatment. In contrast, specific inhibition of active NOTCH signaling by RNA interference releases the cells from this inhibition and amplifies the potential of the cells to respond to proendocrine stimuli. This effect in manifested in a pronounced increase in the number of cells adopting a β-cell phenotype. The same effect is obtained by treatment with a soluble form of the extracellular domain of NOTCH1 in the culture medium. By competitively inhibiting the interaction of NOTCH ligands with their receptor on acinar cells, this treatment increases the fraction of insulin-positive cells from 10% to about 33%. Thus, at least one-third of acinar cells can be reprogrammed into β cells by culturing them in the presence of EGF, LIF, and soluble NOTCH1. In combination with RNA interference an even higher efficiency can be obtained. Thus, transdifferentiation from acinar cells represents a very robust method for β-cell neogenesis.

Taken together, after a first step of partial dedifferentiation in vitro, adult acinar cells become responsive to agonists of the JAK2–STAT3 signaling pathway, such as EGF and LIF. This pathway may activate HNF6 and, subsequently, NGN3 expression, resulting in a cascade of transcription factors that determine the β-cell phenotype (Fig. 7.2). The endocrine reprogramming is controlled by the NOTCH1–HES1 gatekeeper system.

## 7.5 Phenotype and Function of Beta Cells Generated from Acinar Cells

In-vitro analyses revealed that the β-like cells obtained from transdifferentiated acinar cells expressed insulin, C-peptide, and PDX1 proteins at levels similar to those observed in normal islet β cells (Baeyens L et al., unpublished results). The insulin content of the β-like cells was similar to that of islets, but glucose-induced insulin secretion was 50% lower. When examined by immunocytochemistry for other β-cell markers, such as the glucose transporter GLUT2, the secretory granule components chromogranin-A and islet amyloid polypeptide, and the transcription factor MAFA, the newly-generated β cells appeared immature compared with islet β cells (Baeyens L et al., unpublished results). Engraftment of these cells under the kidney capsule of

diabetic animals led to maturation of the grafted insulin-positive cells within 1 week. They acquired normal levels of the β-cell markers noted above, suggesting further maturation in vivo. Transplantation of only $10^5$ insulin-positive cells obtained from acinar cell cultures was sufficient to normalize glycemia in severely hyperglycemic recipients (Baeyens L et al., unpublished results). This number of cells is approximately the same as the number required for normalizing glycemia with islet β cells. This finding indicates that as yet unidentified factors in vivo induce further maturation of acinar-derived β cells to the normal functional state and raises hopes for using these cells in cell replacement therapy for diabetes.

## 7.6 Translation to the Clinic

Treatment of type 1 diabetes by islet transplantation is hampered by, among other problems, the shortage of organ donors. This scarcity is aggravated by the fact that more than one organ is needed to obtain sufficient islets for one recipient. The failure of many grafts to maintain normoglycemia for more than a year post-transplantation may be explained by a suboptimal number of transplanted cells. Therefore, developing alternative sources of transplantable β-like cells is important for efficient application of this therapy. At present acinar cells, the most abundant cell type in the pancreas, are discarded during human islet isolation, but they represent such a source.

Thus far, transdifferentiation of acinar cells to β cells has been studied only in rats and mice. Preliminary attempts to reproduce our work on rodent cells in human acinar cells have not yet been successful (unpublished observations). It is possible, but unlikely, that human acinar cells do not possess a transdifferentiation capacity. More likely, however, is the possibility that species-related differences exist at the level of signaling factors or pathways involved in the regulation of transdifferentiation/reprogramming events. Species-related differences are known to exist even between evolutionarily close species, such as mouse and rat. Indeed, the protocol that we employed successfully with rat acinar cells does not seem to work with mouse acinar cells. The protocol that was reported by Minami and colleagues (Minami et al., 2005) to work with mouse cells does not work with rat cells (unpublished observations). Therefore, before this approach can be developed into a cell replacement therapy, protocols and agents that can effect human acinar cell reprogramming must be identified. Previous studies have already shown that human acinar cells possess some differentiation plasticity, as judged by their ability to undergo a phenotypical switch to duct-like cells (Hall and Lemoine, 1992). However, others have reported that in cultures of human exocrine pancreas tissue acinar cells selectively die by apoptosis, leaving cultures enriched in duct cells (Street et al., 2004; Klein et al., 2008). Thus, finding appropriate culture conditions in which viable human acinar cells can be maintained is a first step in the translation of this research.

## 7.7 Future Work

The abundance of acinar tissue that can be obtained from donor pancreata makes efforts toward neogenesis of β cells from acinar cells highly worthwhile. In-vitro studies with rat acinar cells unequivocally demonstrated the occurrence of acinar-to-β-cell transdifferentiation. It is possible that such transdifferentiation occurs in other experimental models but, if so, its contribution has been overlooked. Many reports, including by our own group, have described β-cell neogenesis from duct cells. In these studies the conclusion regarding the ductal origin of β cells was based primarily on the appearance of insulin-positive cells within the ductal epithelium surrounding a lumen. A commonly used marker for duct cells is a specific pattern of cytokeratin expression, but this pattern is also acquired by dedifferentiated acinar cells (Rooman et al., 2000). A similar problem faced investigators attempting to identify the cell type from which pancreatic adenocarcinoma originates. Recent genetic lineage-tracing studies have proposed that the cancer cells may originate from acinar cells that undergo dedifferentiation following a chronic injury, rather than from duct cells (Guerra et al., 2007; De La O et al., 2008; Habbe et al., 2008). Along the same line of thought, the role of centroacinar cells in different models of β-cell neogenesis has to be addressed. It is thought that these cells are not labeled in lineage-tracing studies employing promoters of genes encoding acinar enzymes, but this possibility has not been rigorously evaluated. In a recent study by Guerra et al. (Guerra et al., 2007), Cre recombinase driven by the elastase-promoter labeled centroacinar cells. Thus, an adequate lineage-tracing method for labeling both duct cells and centroacinar cells has to be developed and tested side by side with the acinar cell lineage tracing in the available experimental models of β-cell neogenesis. Such studies may provide further support for a physiological role of acinar-to-β-cell differentiation.

We still lack understanding of the molecular mechanisms that underlie the dedifferentiation phase of the transdifferentiation process. Dedifferentiation is assumed to be a prerequisite for the reprogramming of acinar cells into β cells. In our in-vitro rat acinar cell transdifferentiation model, induction of dedifferentiation upon cell isolation and culture is obvious, as judged by downregulation of several acinar cell markers. This aspect has not been addressed in detail in the in-vivo mouse acinar cell transdifferentiation model using viral transduction of transcription factors (Zhou et al., 2008). The molecular signals and mechanisms involved in dedifferentiation should therefore be further studied. Elucidating them may allow manipulation of this process in vivo without gene transfer. Dedifferentiation can also predispose the cells to neoplasia, thus providing further impetus for fully understanding its role and mechanisms.

## References

Apelqvist A, Li H, Sommer L, et al. (1999) Notch signalling controls pancreatic cell differentiation. Nature. 400:877–881.

Arias AE, Bendayan M. (1993) Differentiation of pancreatic acinar cells into duct-like cells in vitro. Lab Invest. 69:518–530.
Bae S, Bessho Y, Hojo M, et al. (2000) The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Development. 127:2933–2943.
Baeyens L, Bonne S, German MS, et al. (2006) Ngn3 expression during postnatal in vitro β cell neogenesis induced by the JAK/STAT pathway. Cell Death Differ. 13:1892–1899.
Baeyens L, De Breuck S, Lardon J, et al. (2005) In vitro generation of insulin-producing β cells from adult exocrine pancreatic cells. Diabetologia. 48:49–57.
Bertelli E, Bendayan M. (1997) Intermediate endocrine-acinar pancreatic cells in duct ligation conditions. Am J Physiol. 273:C1641–C1649.
Bonal C, Thorel F, Ait-Lounis A, et al. (2008) Pancreatic inactivation of c-Myc decreases acinar mass and transdifferentiates acinar cells into adipocytes in mice. Gastroenterology [Epub ahead of print].
Bonner-Weir S, Weir GC. (2005) New sources of pancreatic β-cells. Nat Biotechnol. 23:857–861.
Bouwens L. (1998) Transdifferentiation versus stem cell hypothesis for the regeneration of islet β-cells in the pancreas. Microsc Res Tech. 43:332–336.
Bouwens L, Pipeleers DG. (1998) Extra-insular β cells associated with ductules are frequent in adult human pancreas. Diabetologia. 41:629–633.
Brockes JP, Kumar A. (2002) Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nat Rev Mol Cell Biol. 3:566–574.
Chambers I, Smith A. (2004) Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene. 23:7150–7160.
De Breuck S, Baeyens L, Bouwens L. (2006) Expression and function of leukaemia inhibitory factor and its receptor in normal and regenerating rat pancreas. Diabetologia. 49:108–116.
De Breuck S, Lardon J, Rooman I, et al. (2003) Netrin-1 expression in fetal and regenerating rat pancreas and its effect on the migration of human pancreatic duct and porcine islet precursor cells. Diabetologia. 46:926–933.
De La OJP, Emerson LL, Goodman JL, et al. (2008) Notch and Kras reprogram pancreatic acinar cells to ductal intraepithelial neoplasia. Proc Natl Acad Sci USA [Epub ahead of print].
Desai BM, Oliver-Krasinski J, De Leon DD, et al. (2007) Preexisting pancreatic acinar cells contribute to acinar cell, but not islet β cell, regeneration. J Clin Invest. 117:971–977.
Dong PD, Provost E, Leach SD, et al. (2008) Graded levels of Ptf1a differentially regulate endocrine and exocrine fates in the developing pancreas. Genes Dev. 22:1445–1450.
Georgia S, Soliz R, Li M, et al. (2006) p57 and Hes1 coordinate cell cycle exit with self-renewal of pancreatic progenitors. Dev Biol. 298:22–31.
German MS. (2008) New β-cells from old acini. Nat Biotechnol. 26:1092–1093.
Gradwohl G, Dierich A, LeMeur M, et al. (2000) Neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci USA. 97:1607–1611.
Gu G, Dubauskaite J, Melton DA. (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development. 129:2447–2457.
Guerra C, Schuhmacher AJ, Canamero M, et al. (2007) Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. Cancer Cell. 11:291–302.
Habbe N, Shi G, Meguid RA, et al. (2008) Spontaneous induction of murine pancreatic intraepithelial neoplasia (mPanIN) by acinar cell targeting of oncogenic Kras in adult mice. Proc Natl Acad Sci USA [Epub ahead of print].
Hall PA, Lemoine NR. (1992) Rapid acinar to ductal transdifferentiation in cultured human exocrine pancreas. J Pathol. 166:97–103.
Jacquemin P, Durviaux SM, Jensen J, et al. (2000) Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. Mol Cell Biol. 20:4445–4454.
Jensen JN, Cameron E, Garay MVR, et al. (2005) Recapitulation of elements of embryonic development in adult mouse pancreatic regeneration. Gastroenterology. 128:728–741.

Kanno R, Ogihara T, Igarashi Y, et al. (2006) Activin A-induced expression of PAX4 in AR42J-B13 cells involves the increase in transactivation of E47/E12. Biochim Biophys Acta. 1759:44–50.
Klein T, Heremans Y, Heimberg H, et al. (2008) Investigation and characterization of the duct cell-enriching process during serum-free suspension and monolayer culture using the human exocrine pancreas fraction. Pancreas [Epub ahead of print].
Kurzrock R, Estrov Z, Wetzler M, et al. (1991) LIF: not just a leukemia inhibitory factor. Endocr Rev. 12:208–217.
Laguesse E. (1896) Recherches sur l'histogenie du pancreas chez le mouton. J Anat Physiol. 32:209–255.
Lardon J, Bouwens L. (2005) Metaplasia in the pancreas. Differentiation. 73:278–286.
Lardon J, De BS, Rooman I, et al. (2004) Plasticity in the adult rat pancreas: transdifferentiation of exocrine to hepatocyte-like cells in primary culture. Hepatology. 39:1499–1507.
Lardon J, Huyens N, Rooman I, et al. (2004a) Exocrine cell transdifferentiation in dexamethasone-treated rat pancreas. Virchows Arch. 444:61–65.
Leduc EH, Jones EE. (1968) Acinar-islet cell transformation in mouse pancreas. J Ultrastruct Res. 24:165–169.
Mashima H, Ohnishi H, Wakabayashi K, et al. (1996) Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. J Clin Invest. 97:1647–1654.
Mashima H, Shibata H, Mine T, et al. (1996a) Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. Endocrinology. 137:3969–3976.
Means AL, Meszoely IM, Suzuki K, et al. (2005) Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates. Development. 132:3767–3776.
Minami K, Okano H, Okumachi A, et al. (2008) Role of cadherin-mediated cell-cell adhesion in pancreatic exocrine-to-endocrine transdifferentiation. J Biol Chem. 283:13753–13761.
Minami K, Okuno M, Miyawaki K, et al. (2005) Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. Proc Natl Acad Sci USA. 102:15116–15121.
Miyamoto Y, Maitra A, Ghosh B, et al. . (2003) Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell. 3:565–576.
Mukhi S, Mao J, Brown DD. (2008) Remodeling the exocrine pancreas at metamorphosis in Xenopus laevis. Proc Natl Acad Sci USA. 105:8962–8967.
Murtaugh LC, Stanger BZ, Kwan KM, et al. (2003) Notch signaling controls multiple steps of pancreatic differentiation. Proc Natl Acad Sci USA. 100:14920–14925.
Niwa H, Burdon T, Chambers I, et al. (1998) Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev. 12:2048–2060.
Okuno M, Minami K, Okumachi A, et al. (2007) Generation of insulin-secreting cells from pancreatic acinar cells of animal models of type 1 diabetes. Am J Physiol Endocrinol Metab. 292:E158–E165.
Rooman I, De Medts N, Baeyens L, et al. (2006) Expression of the Notch signaling pathway and effect on exocrine cell proliferation in adult rat pancreas. Am J Pathol. 169:1206–1214.
Rooman I, Heremans Y, Heimberg H, et al. (2000) Modulation of rat pancreatic acinoductal transdifferentiation and expression of PDX-1 in vitro. Diabetologia. 43:907–914.
Rooman I, Lardon J, Flamez D, et al. (2001) Mitogenic effect of gastrin and expression of gastrin receptors in duct-like cells of rat pancreas. Gastroenterology. 121:940–949.
Rooman I, Schuit F, Bouwens L. (1997) Effect of vascular endothelial growth factor on growth and differentiation of pancreatic ductal epithelium. Lab Invest. 76:225–232.
Sawey ET, Johnson JA, Crawford HC. (2007) Matrix metalloproteinase 7 controls pancreatic acinar cell transdifferentiation by activating the Notch signaling pathway. Proc Natl Acad Sci USA. 104:19327–19332.
Schwitzgebel VM, Scheel DW, Conners JR, et al. (2000) Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development. 127:3533–3542.

Setalo G, Blatniczky L, Vigh S. (1972) Development and growth of the islets of Langerhans through acino-insular transformation in regenerating rat pancreas. Acta Biol Acad Sci Hung. 23:309–325.

Shen CN, Seckl JR, Slack JM, et al. (2003) Glucocorticoids suppress β cell development and induce hepatic metaplasia in embryonic pancreas. Biochem J. 375:41–50.

Shen CN, Slack JM, Tosh D. (2000) Molecular basis of transdifferentiation of pancreas to liver. Nat Cell Biol. 2:879–887.

Siveke JT, Lubeseder-Martellato C, Lee M, et al. (2008) Notch signaling is required for exocrine regeneration after acute pancreatitis. Gastroenterology. 134:544–555.

Street CN, Lakey JR, Rajotte RV, et al. (2004) Enriched human pancreatic ductal cultures obtained from selective death of acinar cells express pancreatic and duodenal homeobox gene-1 age-dependently. Rev Diabet Stud. 1:66–79.

Takahashi K, Tanabe K, Ohnuki M, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 131:861–872.

Thowfeequ S, Myatt EJ, Tosh D. (2007) Transdifferentiation in developmental biology, disease, and in therapy. Dev Dyn. 236:3208–3217.

Tosh D, Shen CN, Slack JM. (2002) Differentiated properties of hepatocytes induced from pancreatic cells. Hepatology. 36:534–543.

Tsonis PA, Madhavan M, Tancous EE, et al. (2004) A newt's eye view of lens regeneration. Int J Dev Biol. 48:975–980.

Vila MR, Lloreta J, Real FX. (1994) Normal human pancreas cultures display functional ductal characteristics. Lab Invest. 71:423–431.

Viti J, Feathers A, Phillips J, et al. (2003) Epidermal growth factor receptors control competence to interpret leukemia inhibitory factor as an astrocyte inducer in developing cortex. J Neurosci. 23:3385–3393.

Zhang YQ, Mashima H, Kojima I. (2001) Changes in the expression of transcription factors in pancreatic AR42J cells during differentiation into insulin-producing cells. Diabetes. 50(Suppl 1):S10–S14.

Zhou Q, Brown J, Kanarek A, et al. (2008) In vivo reprogramming of adult pancreatic exocrine cells to β-cells. Nature. 455:627–632.

Zhou J, Wang X, Pineyro MA, et al. (1999) Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. Diabetes. 48:2358–2366.

# Chapter 8
# Generation of Beta Cells from Pancreatic Duct Cells and/or Stem Cells

**Susan Bonner-Weir and Arun Sharma**

**Abstract** Since diabetes is caused by the loss of the insulin-producing β cells, its reversal by replacement of these cells by transplantation or by replenishment from endogenous sources seems straightforward. In the pancreas, two mechanisms for β-cell growth are *replication* of preexisting β cells and *neogenesis* or the differentiation of new β cells from progenitor/stem cells that were not β cells. Replication and neogenesis are not mutually exclusive, and there is no biological reason for there to be only one mechanism for replenishment of the islet cells. This chapter focuses on the renewed interest in the identification, expansion, and differentiation of adult pancreatic progenitor/stem cells that can lead to more β cells, either in vivo or in vitro.

## 8.1 Introduction

A goal of regenerative medicine is the therapeutic use of stem cells to treat disease. Since diabetes is caused by the loss of the insulin-producing β cells, its reversal by replacement of these cells by transplantation or by replenishment from endogenous sources would seem to be straightforward. However, such therapy is hindered by the limited islet tissue currently available for transplantation from cadaveric pancreases. Although there has been progress in deriving insulin-producing cells from embryonic stem (ES) cells (Jiang et al. 2007; Kroon et al. 2008) and potentially from induced pluripotent stem (iPS) cells, there is increased interest in using endogenous sources for β-cell replenishment. For this reason, the regenerative process in the pancreas is of particular interest. Whereas tissue-specific stem cells are well documented in skin, intestine, and testes, pancreatic stem cells have been elusive at best. In the liver, even though oval cells are tissue-specific stem cells, regeneration occurs mainly through replication of existing differentiated hepatocytes (Fausto and Campbell 2003). Similarly in the pancreas, two mechanisms for β-cell growth are

S. Bonner-Weir (✉)
Department of Medicine, Harvard Medical School, Harvard Stem Cell Institute, Joslin Diabetes Center, Boston, MA 02215, USA
e-mail: susan.bonner-weir@joslin.harvard.edu

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_8,

*replication* of preexisting β cells and *neogenesis* or the differentiation of new β cells from progenitor/stem cells that were not β cells. Replication and neogenesis are not mutually exclusive, and there is no biological reason for there to be only one mechanism for replenishment of the islet cells. It is clear that in the pancreas replication of β cells is an important mechanism for renewal and growth, but there are compelling data indicating that after-birth progenitors, or even possibly stem cells, have a role in the renewal and growth of islets.

The continued and substantial growth (20-fold) of islet tissue after birth in rodents and humans (with additional rapid compensatory growth in response to increased demand), while there is a very low replication of existing β cells, suggests a contribution from adult progenitor/stem cells (Bonner-Weir 2000). In humans, compensatory growth of β-cell mass with obesity shown in a small careful study in 1985 (Kloppel et al. 1985) was confirmed in larger studies (Butler et al. 2003; Yoon et al. 2003). In rodents there is clear evidence of pancreatic regeneration after some types of injuries (Brockenbrough et al. 1988; Jensen et al. 2005; Desai et al. 2007) with replication as the major mechanism for expanding the β-cell mass during pregnancy, in obesity/insulin resistance, or in normal adult growth (Parsons et al. 1995; Bruning et al. 1997; Bock et al. 2003; Dor et al. 2004). However, in humans the role of replication in adult compensation is less clear (Meier et al. 2008); neogenesis may be a more important component in expanding the β-cell mass than replication of preexisting β cells. In humans telomere shortening is limiting to replication, and the rate of β-cell replication appears to be very low (Tyrberg et al. 2001). Furthermore, enlarged islets that would be the result of continued replication of preexisting β cells are rarely seen in a human pancreas. There is a large body of correlative evidence of islet neogenesis (Bonner-Weir et al. 1993; Gu and Sarvetnick 1993; Wang et al. 1993; Xu et al. 1999; Li et al. 2003), but until recently there has been a dearth of rigorous lineage-tracing studies. Moreover, adult stem cells that may or may not be contained within the ductal structures may also contribute to this replacement, but their contribution has been unclear. This chapter focuses on the renewed interest in the identification, expansion, and differentiation of adult pancreatic progenitor/stem cells that can lead to more β cells either in vivo or in vitro.

## 8.2 Definition of Terms

In the area of stem cell/progenitors there have been many claims and interpretations based on fuzzy definitions. So for clarity, we first provide some working definitions:

- *Adult stem cell:* Adult stem cells are undifferentiated cells found throughout the body after birth that multiply by cell division to replace dying cells and regenerate damaged tissue. Although these cells are considered to self-renew indefinitely and generate all the cell types of that particular organ, the attributes are usually not well shown.
- *Facultative stem cell:* The term facultative or functional stem cell has been used to indicate a differentiated epithelial cell that can be called upon to replicate

repeatedly and serve a stem cell role, but it usually does not. When cultured on collagen gel in defined media with growth factors known to stimulate their replication, primary hepatocytes behave like facultative stem cells with clonal expansion (sixfold increase/14 days) and by dedifferentiation/regression attain a more primitive phenotype (Block et al. 1996). When overlaid with Matrigel these expanded cells expressed liver-specific genes. Hepatocytes are the usual source for liver regeneration and can be considered the functional stem cell of the liver; it is only when this regeneration pathway is blocked that liver stem cell or oval cell differentiation is evoked (Michalopoulos 2007). Similarly, adult corneal epithelial cells, considered a transiently-amplified population with irreversible commitment, have been shown to be reprogrammed to different epidermal phenotypes depending on the dermis on which they are placed (Ferraris et al. 2000). This term has also been applied to the pancreas (Bonner-Weir and Sharma 2002; Dor and Melton 2008).

- *Progenitor cell:* Progenitor cell, often used synonymously with precursor cell, indicates a cell that gives rise to another more differentiated cell type. These cells have limited self-renewal capacity and can give rise to several cell types.
- *Transdifferentiation:* As defined by Tosh and Slack, this term represents the direct conversion of one mature cell phenotype to another (Li et al. 2005), a process that is not the same in concept as *dedifferentiation* of a fully differentiated cell to a more progenitor-like phenotype before redifferentiation to perhaps a different differentiated phenotype.

## 8.3 Are There Pancreatic Stem Cells?

Adult pancreatic stem cells may contribute to the replenishment of β cells, but their existence remains controversial. There have been a number of approaches to isolate and identify pancreatic stem cells with varying degrees of credibility.

Oval cells, thought to be adult stem cells in the liver, have been identified as periductal swarms of small cells in the atrophied pancreas of rats maintained on copper-deficient diets (Rao et al. 1989; Reddy et al. 1991). With repletion of copper, the atrophied pancreas refills with hepatocytes rather than with acinar cells, leading to the idea that there were hepatocyte stem cells within the pancreas. Indeed, when these pancreatic oval cells were transplanted into the liver, they expressed a number of hepatocyte genes, and when transplanted into mice deficient in fumarylacetoacetate hydrolase they repopulated the liver and in some cases corrected liver function (Grompe 2003; Wang et al. 2003). Within the pancreas, as we have previously reported, basal cells that are small and ovoid with fairly undifferentiated cytoplasm are rarely seen (about one in 100–200 cells) in the common or main pancreatic ducts of normal adult rats (Bonner-Weir and Sharma 2002). However, there are no data on the capacity of any of these oval cells to form pancreatic cell types.

In 2000 Ramiya et al. reported the generation of islet cells from pluripotent stem cells from pancreatic ducts isolated from adult nonobese diabetic (NOD) mice after

long-term culture (Ramiya et al. 2000). Interestingly, when transplanted into diabetic mice these cells improved the glycemic levels, but these results have not been duplicated by other researchers.

More recently mesenchymal stem cells (MSCs) isolated from human pancreas were reported as expressing transcription factors of pancreatic endocrine development during expansion. After 15 days of culture in differentiation media containing activin A and hepatocyte growth factor (HGF), these cells expressed insulin, glucagon, and glucokinase mRNA (Baertschiger et al. 2008). However, the islet-specific transcripts were shown with 40–45 cycles of amplification and so may only indicate a low level of leaky expression from open chromatin. Although MSCs are likely present within the pancreas, they are unlikely to contribute directly to epithelial cell renewal.

Another approach has been the isolation and clonal expansion of single cells after the exclusion of hematopoietic and vascular endothelial cell markers (CD45, TER119, c-KIT, FLK1); two reports (Seaberg et al. 2004; Suzuki et al. 2004) identified such putative pancreatic stem cells from the mouse pancreas and showed low-level expression of insulin and other pancreatic marker transcripts. In the paper by Suzuki et al., cells isolated from neonatal pancreas using cell surface markers, in particular the HGF receptor c-MET, produced expanded colonies expressing islet and acinar characteristics, the duct marker cytokeratin 19 (CK19), nestin, and markers of hepatocytes and gastrointestinal cells. In the study by Seaberg et al., rare single cells (one cell out of 3000–9000) obtained from either adult mouse islets or ducts could form colonies of 2000–10,000 cells expressing markers of both neurons and pancreatic cells, including nestin, neurogenin-3 (NGN3), and PDX1. These pancreas-derived cells had limited capacity for self-renewal, lacked the stem cell markers OCT4 and NANOG, and were of neither mesodermal nor neural crest origin. After further differentiation, single clones expressed specific proteins for neurons and pancreas (4–6% of the cells), including markers of β- (insulin and GLUT2), α- (glucagon), δ- (somatostatin), and acinar (amylase) cells. Numerous islet transcripts (insulin II, nestin, *Ngn3*, *NeuroD1*, and *Pax6*) were detected by RT-PCR, but many of these are also expressed in neural tissue. However, insulin I is the β-cell-specific gene in rodents, whereas insulin II is expressed in embryonic neural tissues. Surprisingly, neither the endoderm markers GATA4 and HNF3β nor cytokeratins (ductal cell markers) were detected. Moreover, these rare cells have not yet been localized within the pancreas and have not yet been shown to become functional islets.

Another approach used transgenic mice expressing a mouse telomerase reverse transcriptase (mTERT)-green fluorescent protein (GFP) fusion protein as a stem cell marker (Breault et al. 2008). After 3 days of treatment with exendin-4, a glucagon-like peptide 1 (GLP1)-receptor agonist that has been shown to stimulate neogenesis and replication of β cells (Xu et al. 1999), expression of both GFP and endogenous mTERT mRNA was increased (Carlone et al., abstract for the ISSCR annual meeting 2007). In addition, $GFP^+$ cells were found by immunostaining and flow cytometry in the *Dolichos biflorus* agglutinin lectin $(DBA)^+$ (ductal) cells but not within the islets.

Although these data are suggestive, the contribution of these putative pancreatic stem cells and their identity have not yet been determined.

## 8.4 In-Vitro Evidence of Pancreatic Progenitors in Ducts

Studies in vitro have provided considerable evidence for the induction of islet phenotype/genes from nonislet pancreatic cells, particularly ductal cells. A number of pancreatic ductal cell lines were induced in vitro to express islet hormones and other markers in response to GLP1/exendin-4 (Zhou et al. 1999; Bulotta et al. 2002; Zhou et al. 2002), activin A and either HGF or betacellulin (Mashima et al. 1996a; Mashima et al. 1996b), addition of PDX1 protein (Noguchi et al. 2003), and serum-free media and aggregation (Hardikar et al. 2003).

### *8.4.1 Progenitors in Human Pancreatic Duct-Enriched, Islet-Depleted Tissue*

Such tissue, remaining after islet isolation, has been used extensively. In 2000 we were able to generate in vitro islet-like structures from such islet-depleted tissue that were glucose-responsive and had mature islet phenotypes (Bonner-Weir et al. 2000), and our data were confirmed and extended by Otonkoski's group (Gao et al. 2003). The evidence indicates that these new islets were generated from ductal epithelial cells. However, Gao et al. reported that when they removed NCAM-positive cells, which they assumed to be the β cells, as in rodents, from the starting material they could no longer generate insulin-positive cells in vitro (Gao et al. 2005). However, we found that NCAM is also expressed in much of the human ductal tree, so more than just β cells were removed in that study. Using another approach, Zhao et al. reported that they were able to generate new insulin-producing cells in vitro from human pancreatic tissue after removing insulin-producing cells by in-vitro streptozotocin (STZ) treatment (Zhao et al. 2005); however, the lack of STZ cytotoxicity for human β cells casts doubt on the complete removal of insulin-positive cells at the start. Hao et al. labeled human pancreatic cells with a lentivirus after depleting dithizone-stained clusters containing β cells and showed that cotransplantation of these cells with human fetal pancreas resulted in islets differentiated from lentiviral-labeled adult tissue (Hao et al. 2006). In all of these studies, the possible expansion of a few contaminating islet cells (particularly β cells) lingers and hinders conclusive interpretations.

To show more conclusively that the progenitors were solely in the ductal population, we developed a strategy to purify human ductal cells using immunomagnetic beads and antibody against the cell surface antigen CA19-9 (Yatoh et al. 2007). The preparations remaining after human islet isolation are mainly the terminal end of the ductal tree: the acini, the small intercalated and terminal ductules, as well as the centroacinar cells. Immunostaining indicated that these ductal cells were the population in which most cells also expressed the transcription factor SOX9, suggested

as necessary for maintenance of the pancreatic progenitor pool (Seymour et al. 2007). Tissue from 17 pancreases was dispersed and either purified for duct cells by CA19-9 antibody or left unpurified but dispersed and termed "crude" ducts. FACS analysis determined that the viable CA19-9-positive fraction was almost 100% pure ductal epithelial cells, almost entirely $CK19^+$ cells with no $insulin^+$ cells (and no detectable insulin mRNA by qRT-PCR), whereas the unpurified, dispersed cells (crude duct) were 56% $CK19^+$ and 0.4% $insulin^+$ of the total number of cells (0.7% of $CK19^+$ cells). Cells were expanded as monolayers, aggregated under serum-free conditions, and transplanted into normoglycemic NOD-severe combined immunodeficient (SCID) mice. In crude duct grafts, the fraction of $insulin^+$ cells increased to 6.1% of $CK19^+$ cells, whereas aggregation of purified ducts (containing no $insulin^+$ cells) induced $insulin^+$ cells (0.1% of $CK19^+$ cells) that further increased to 1.1% of the $CK19^+$ cells in grafts. Insulin mRNA levels mirrored these changes.

In the latter grafts, all $insulin^+$ cells were in duct-like structures, whereas in crude duct grafts, 85% were found in such structures. In control experiments with dispersed islets mixed with the purified and crude duct cultures before aggregation and transplant, most of the $insulin^+$ cells were excluded from the ductal structures. Some $insulin^+$ cells coexpressed duct markers (CK19 and CA19-9) and HSP27, a marker of nonislet cells, suggesting the transition from duct. These data support the conclusion that purified duct cells from adult human pancreas can differentiate into insulin-producing cells. The low frequency of this differentiation may be due to our inadequate conditions or to the possibility that only a subpopulation of cells has this potential.

One of the more successful modifications in culture conditions has been the addition of epidermal growth factor (EGF) and gastrin. Using human pancreatic preparations that included islets, ducts, and acinar cells, Suarez-Pinzon et al. reported a doubling of β cells after 4 weeks of treatment with a combination of EGF and gastrin (Suarez-Pinzon et al. 2005). Parallel increases in the number of cells expressing the duct marker CK19 and the transcription factor PDX1 led the authors to conclude that the increase in the number of β cells was due to activation of neogenesis from the pancreatic ducts. Although these studies support the idea that duct cells can serve as progenitor cells, conclusive evidence for this supposition requires lineage tracing.

### *8.4.2 Progenitors in Mouse Pancreas Ductal Cells*

Mice expressing a reporter transgene have been used to provide in-vitro evidence of pancreatic progenitors within the ducts. We tried two different approaches using adult mouse insulin promoter (MIP)-GFP mice to facilitate the initial deletion of preexisting β cells and, after culture, detection of newly formed β cells. In the first (Kikugawa and Bonner-Weir, unpublished data), using collagenase, we isolated the pancreatic portion of the common bile duct (referred to hereafter as common pancreatic duct) from 2–3-month-old mice; we minced it, expanded it in RPMI 1640 + 10% FBS for 10 days, and then changed to a serum-free differentiation medium

(DMEM/F12 + 1 g/L ITS supplement, 2 g/L BSA, and 10 mM nicotinamide) for 11 days, with different combinations of growth factors. Insulin I and II mRNAs were found with 35 cycles of RT-PCR after treatment with either keratinocyte growth factor (KGF) or exendin-4, but only insulin II mRNA was detected after treatment with betacellulin and activin A. In the second approach (Kikugawa et al. 2009) whole pancreas was minced and digested with collagenase, the islets were isolated, and the islet-depleted tissue cultured for 3 days in RPMI + 10% FBS + 20 ng/ml EGF. Using the COPAS large-bore FACS sorter, we deleted aggregates with $GFP^+$ cells (GFP is a long-lived protein and so should have remained in the β cells following 3 days in culture even if they became degranulated). FACS analysis of dispersed aliquots of the remaining aggregates confirmed that there were no remaining $GFP^+$ cells. Attached GFP-depleted aggregates were cultured in differentiation media (with EGF and exendin-4); after 3 weeks of visual observation, aggregates were dispersed, and FACS analysis showed that 1.8% of the cells were $GFP^+$. Our mouse data support the concept that within the adult exocrine (acinar and ductal) pancreas there are cells that can give rise to $insulin^+$ cells in vitro.

Other researchers have prospectively isolated putative pancreatic progenitors using cell surface markers. Kim's group screened cells from fetal E15.5 mouse pancreas with a panel of 30 stem cell markers (Sugiyama et al. 2007). They found enrichment of $NGN3^+$ cells with CD133 (prominin), CD49f (integrin alpha 6) low, and CD24 (a sialoglycoprotein anchored by a GPI link to the cell surface) when each of these three cell surface markers was used singly to sort cells and even more enrichment when the three were combined, suggesting that these cell surface molecules can be used to isolate an embryonic islet progenitor cell population. The cell population that was nonhematopoietic ($CD133^+/CD49f^{low}$ cells) comprised 13% of the recovered cells, with 8% of them immunostainable for NGN3. Interestingly, these cells were shown to survive and differentiate with some expression of islet markers when cultured on mouse embryonic fibroblasts or on PA6 stromal cells. PA6 cells have been shown to induce neural differentiation in mouse ES cells via a stromal cell-derived inducing activity (SDIA).

Almost simultaneously, Taniguchi's group isolated purported progenitor cells from neonatal and adult mice as a subset of $CD133^+/CD34^-/CD45^-/TER119^-$ ductal cells (Oshima et al. 2007). The sorted nonhematopoietic $CD133^+$ cells accounted for 2.6 ± 3% of the total pancreatic cells in neonates and 1.2 ± 0.3% in adults; essentially all expressed cytokeratin 7 (CK7). Moreover, they were $CD49f^{low}$, CD29 (integrin $\beta1)^+$, c-$KIT^{-/low}$, $THY1^-$, and $SCA1^-$. Of these cells 3–4% expanded clonally. After 14 days in culture the cells from neonates expressed various islet, acinar, and ductal markers by RT-PCR (35 cycles) and immunohistochemistry, but those from adults only expressed ductal markers CK7 and carbonic anhydrase II. More recently, another group isolated and cultured $CD24^-$ pancreatic duct cells from 1-month-old mice after finding that only the interlobar and intralobar ducts, and not the main ducts nor the intercalated (smallest) ducts, stained for CD24 (Wang et al. 2008). Under their conditions some 3% of these $CD24^-$ cells were able to form colonies, which were then about 95% $CK19^+$. With further culture

under differentiation conditions, they found induction of insulin II mRNA and some insulin immunostaining.

All of these studies using various cell surface antigens to sort different populations of cells from murine pancreas found a population of cells that had at least some capacity for clonal expansion and that could express different markers of differentiated pancreatic cells. Further studies are needed to identify these cells and their location within the pancreas more exactly, as are transplantation experiments to show whether they can become functional islet cells.

## 8.5 In-Vivo Evidence of Postnatal Pancreatic Progenitors

It is generally accepted that sometime after E15.5, there are distinct differentiated ducts in the developing mouse pancreas that are different from the embryonic pancreatic progenitors that form the tubular structures often referred to as embryonic ducts. Carbonic anhydrase II, used as a marker for mature ducts (Hale et al. 2005), is only expressed in mouse ducts from E18 onward (Inada et al. 2006). After birth the duct can be thought of as a tree with the trunk being the common bile duct as it courses through the pancreas, the two main ducts as the first branches, with progressively smaller interlobar and intralobar ducts branching to small intercalated and then terminal ductules. The ductal epithelium is continuous with the acini, with centroacinar cells forming the transitional cell type between duct and acinar cells. There are differences in cell type (columnar to squamous) and immunoreactivity to antibodies along the ductal tree, but in any segment of the tree the cells appear to be fairly homogeneous. The appearance of hormone-expressing cells budding from the pancreatic ducts of different segments has suggested that progenitor/stem cells will be found in the ducts. Although there are numerous references to new islet formation and increased neogenesis, we summarize only a few of the more striking models.

### *8.5.1 Insulin Promoter-Interferon γ Transgenic Mice*

Transgenic mice overexpressing interferon γ under the regulation of the rat insulin promoter provide a useful model of enhanced neogenesis (Sarvetnick and Gu 1992). In these mice there is a continual destruction of islets with a continued proliferation of ductal epithelium and formation of new islets. Initially it was thought that the insulitis seen in this model might have triggered the neogenesis, but similar duct proliferation and islet neogenesis were observed with this transgene on an immunocompromised background. Many of the transcription factors seen in pancreas development are expressed in these proliferating ducts, suggesting that the regeneration follows the pattern of gene expression of pancreatic development (Kritzik et al. 1999; Kritzik et al. 2000). There was enhanced PDX1 protein expression in some of the ducts. As new islets are clearly derived from the ducts,

the authors suggested the presence of a "well-defined and functional stem cell population" in the adult pancreatic ducts (Sarvetnick and Gu 1992).

### *8.5.2 Metallothionein-TGFα Transgenic Mice*

In a second transgenic mouse model, overexpression of TGFα in the exocrine tissue led to sustained proliferation of the ductal epithelium (Jhappan et al. 1990; Wang et al. 1993). In the resulting metaplastic ducts 6% of the cells had insulin immunostaining. More recent examination of these mice indicated enhanced PDX1 expression in the metaplastic ducts (Song et al. 1999), a finding that is consistent with reexpression of PDX1 protein following replication, as shown in regeneration following partial pancreatectomy (Sharma et al. 1999). Furthermore, focal expression of PAX6 was found, suggesting the initiation of islet neogenesis (Song et al. 1999).

### *8.5.3 Partial (90%) Pancreatectomized Rats*

In the 90% pancreatectomy (Px) rat model a well-defined remnant of 10% of pancreas weight and islet mass left in a young adult rat regenerates to 27% of the weight of the sham-operated pancreas with 45% of the sham islet mass by 4 weeks after surgery (Bonner-Weir et al. 1983; Bonner-Weir et al. 1993: Xu et al. 1999). The regeneration that occurs in this model is by both replication of preexisting differentiated acinar and β cells and neogenesis, resulting in the rapid formation of whole new lobes (Bonner-Weir et al. 1993). After surgery, ductal cells rapidly replicate and dedifferentiate, with a marked increase in PDX1 protein, a transcription factor known to be important for both pancreas development and β-cell function (Sharma et al. 1999). By 72 h after surgery, small clumps of branching ductal structures project from the common pancreatic duct; in histological sections these clumps are seen as well-defined regions of proliferating ductules. Although little to no PDX1 protein was detected in the epithelium of the quiescent common pancreatic duct from unoperated or sham Px animals 24 h post-Px, most of the ductal epithelial cells in Px animals were $BrdU^{+}PDX1^{-}$ or $BrdU^{+}$ $PDX1^{+}$. By 3 days after Px, few cells of the common pancreatic duct epithelium were $BrdU^{+}$ but most cells still expressed PDX1 protein. By 7 days after Px, most of these regions had differentiated into new pancreatic lobes with a normal composition of exocrine and endocrine cells. Our findings from this model led to the hypothesis that differentiated (mature) pancreatic ductal cells can function as progenitors for new islets after birth, responding to stimulus by replication and regression to a less differentiated phenotype and then forming new acini and new islets (Sharma et al. 1999; Bonner-Weir et al. 2004).

Recently this model, with a less severe resection (50–70%), was extrapolated to mice (Dor et al. 2004; Lee et al. 2006; Peshavaria et al. 2006; Teta et al. 2007; Desai et al. 2007; Ackermann Misfeldt et al. 2008). Whereas two of these studies

concluded that replication was the only β-cell regeneration mechanism following mouse Px (Dor et al. 2004; Teta et al. 2007), at least two others (Peshavaria et al. 2006; Ackermann Misfeldt et al. 2008) reported islet neogenesis. Conclusions have been drawn about the need for severity of injury to evoke neogenesis in the mouse, but this has not been clearly shown.

### *8.5.4 Ductal Ligation*

The partial ductal ligation model has the advantage of having regeneration of both acini and islets localized distal to the ligation, with little to none in the nonligated portion (proximal to the ligation), allowing a comparison of labeling in regenerated and nonregenerated tissue within the same pancreas (Edstrom 1971; Hultquist et al. 1979; Wang et al. 1995; Rooman et al. 2002; Lardon et al. 2004). From studies of this model in adult rats it was clear that there was replication of ductal cells, increased hormone-positive cells budding from ducts, and increased β-cell mass. Furthermore, after duct ligation, stem cell markers such as c-KIT and nestin, the transcription factors PDX1 and NKX2.2, and netrin-1 were seen in the duct cells, with c-KIT and nestin double-positive cells showing high proliferative activity (Peters et al. 2005).

Extrapolation of this well-established rat model to the adult mouse to take advantage of expression of reporter transgenes has recently produced important data that address questions about the origin of the islet cells in the adult. A previous controversy as to whether the replicating ducts originated from transdifferentiated acinar cells or preexisting duct tissue was recently settled by the study of Desai et al. with the Cre-loxP system of lineage tracing using an acinar-specific elastase-1 promoter; they found no marked islets after ductal ligation and concluded that acinar cells did not contribute to new islet formation after pancreatic ligation (Desai et al. 2007).

Using this same model with various NGN3 reporter mice, Heimberg's group elegantly showed that NGN3, the transcription factor marker of the endocrine pancreatic progenitor, was induced in cells in or adjacent to the pancreatic ducts (Xu et al. 2008). By isolating these cells with flow cytometry and subsequently transplanting them into explants of fetal pancreas from *Ngn3*-null mice, they showed that these cells gave rise to β cells and other islet cells. Thus, they demonstrated convincingly that there are multipotent progenitor cells within the adult mouse pancreas that can be activated to increase the islet mass.

Similarly, to test the hypothesis that duct cells are progenitors that can give rise to β cells, we took the direct approach of genetically marking mouse ductal cells using duct-specific carbonic anhydrase II (CAII)-promoted Cre expression for lineage tracing (Inada et al. 2008). Inducible CAII-Cre:Rosa26R (R26R) mice were labeled by treatment with a tamoxifen pellet for 3 weeks from 4 to 7 weeks of age, ductal ligations were performed at 8 weeks, and the animals sacrificed

at 10 weeks. In the nonligated portions of pancreas from the tamoxifen-treated 10-week-old double-transgenic mice, the frequency of positive islets tended to be increased but was not statistically different from that in the controls. In contrast, in the distal, regenerated pancreas 42% of the islets were marked, compared to 12% in the nonligated pancreas of the same animals. These data provide direct evidence that CAII-positive cells act as endocrine progenitors and provide a shared lineage of ductal, acinar, and islet cells after birth.

## 8.6 How Large Is the Contribution of Neogenesis to Islet Mass?

The significance of the contribution of islets from neogenesis to islet mass after birth has been questioned (Dor et al. 2004; Teta et al. 2007), since the proliferation of β cells peaks around birth and decreases thereafter, reaching adult levels in rodents only about the time of weaning. Even in a human autopsy study (Meier et al. 2008) β-cell replication was found to be higher during the first five postnatal years. Yet, from birth to 4 weeks of age, the rodent pancreas size increases about 15-fold (Dore et al. 1981; Scaglia et al. 1997). Even with the significant β-cell replication during this period, an estimated 30% of the new β cells seen at day 31 could not be accounted for by replication of preexisting β cells (Bonner-Weir et al. 2004).

We used another variation of the duct-specific promoter CAII-Cre transgenic mouse to estimate the extent of new islet formation during the neonatal period. Pancreases of double heterozygous CAII-Cre:R26R transgenic mice were analyzed for loxP-inducible β-galactosidase expression on the day of birth and at 4 weeks (Inada et al. 2008). Since CAII is seen in ducts only after E18.5, at a time when a large founder population of pancreatic islets and acini has already been specified, only a fraction of the islets and acini is expected to be duct-derived after birth. The proportion of β-galactosidase-positive islets out of total islets showed a substantial increase from day 0 to 4 weeks of age: at birth few, if any, marked islets were seen, but at 4 weeks of age 37.8% of the islets (ranging from 30% to 64% in individual mice) were marked. At 4 weeks (just past weaning) many ducts and patches of acinar cells (some in lobular pattern) also expressed β-galactosidase. Whereas some newly-differentiated β cells may have coalesced with previously-formed islets instead of forming new islets, many of the labeled islets were in or adjacent to labeled acini. Moreover, when the labeled β cells were quantitated, 23.6 ± 2.2% expressed β-galactosidase, indicating that they originated from CAII-expressing duct cells. These data suggest that during the neonatal period the contribution of islet and acinar cells newly differentiated from ductal progenitors to islet mass is substantial.

There are implications of such a large population of islets being formed postnatally even in mice, which have greater replicative capacity than humans. An impairment of this neonatal neogenesis could have significant effects on the β-cell mass, which in rodents increases dramatically after weaning (3–4 weeks), even

though the β-cell replication rate approaches that of adults (Scaglia et al. 1997). Such a scenario occurs in the GK rat, a model of type 2 diabetes, in which there is early growth retardation, including impaired neogenesis, but no clear intrinsic secretory defect; the secretory defect is secondary to the reduced β-cell mass and was seen after weaning (Portha 2005).

## 8.7 Is There a Population of Multipotent Progenitor/Stem Cells that Are Activated as Needed?

Our lineage-tracing experiments show that pancreatic cells that express CAII are the origins of new islets and acini during normal growth and in response to injury. Even so, it is still unclear if there is a population within the CAII-expressing duct cells, maybe even a stem cell/oval cell population, that has this potential and has to be activated, as was suggested by Xu et al. (Xu et al. 2008). Our results (Sharma et al. 1999) from the regenerating rat pancreas in which all duct cells transiently express PDX1 protein, a marker of the embryonic pancreatic progenitors, and lose expression of some of their genes/proteins, suggest that most, if not all, the duct cells may have the potential for being pancreatic progenitors. Our working hypothesis has been that differentiated (mature) pancreatic ductal cells can function as progenitors for new islets after birth in response to a stimulus that triggers their replication and regression to a less differentiated phenotype, a phenotype equivalent to that of embryonic pancreatic progenitors (Sharma et al 1999; Bonner-Weir et al 2004). Further work is needed to resolve this question.

**Acknowledgments** The authors thank those scientists who have worked with us through the years for their discussions and contributions. The research was supported by grants from NIH and JDRF.

## References

Ackermann Misfeldt A, Costa RH, Gannon M (2008) Beta-cell proliferation, but not neogenesis, following 60% partial pancreatectomy is impaired in the absence of foxm1. Diabetes 57: 3069–3077

Baertschiger RM, Bosco D, Morel P et al (2008) Mesenchymal stem cells derived from human exocrine pancreas express transcription factors implicated in beta-cell development. Pancreas 37:75–84

Block GD, Locker J, Bowen WC et al (1996) Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGFβ in a chemically defined (HGM) medium. J Cell Biol 132:1133–1149

Bock T, Pakkenberg B, Buschard K (2003) Increased islet volume but unchanged islet number in ob/ob mice. Diabetes 52:1716–1722

Bonner-Weir S (2000) Perspective: postnatal pancreatic beta cell growth. Endocrinology 141:1926–1929

Bonner-Weir S and Sharma A (2002) Pancreatic stem cells. J Pathol 197:519–526

Bonner-Weir S, Trent DF, Weir GC (1983) Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. J Clin Invest 71:1544–1553

Bonner-Weir S, Baxter LA, Schuppin GT et al (1993) A second pathway for regeneration of the adult exocrine and endocrine pancreas: a possible recapitulation of embryonic development. Diabetes 42:1715–1720

Bonner-Weir S, Taneja M, Weir GC et al (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci USA 97:7999–8004

Bonner-Weir S, Toschi E, Inada A et al (2004) The pancreatic ductal epithelium serves as a potential pool of progenitor cells. Pediatric Diabetes 5:15–22

Breault DT, Min IM, Carlone DL et al (2008) Generation of mTERT-GFP mice as a model to identify and study tissue progenitor cells. Proc Natl Acad Sci USA 105:10420–10425

Brockenbrough JS, Weir GC, Bonner-Weir S (1988) Discordance of exocrine and endocrine growth after 90% pancreatectomy in rats. Diabetes 37:232–236

Bruning JC, Winnay J, Bonner-Weir S et al (1997) Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. Cell 88:561–572

Bulotta A, Hui H, Anastasi E et al (2002) Cultured pancreatic ductal cells undergo cell cycle re-distribution and beta-cell-like differentiation in response to glucagon-like peptide-1. J Mol Endocrinol 29:347–360

Butler AE, Janson J, Bonner-Weir S et al (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110

Desai BM, Oliver-Krasinski J, De Leon DD et al (2007) Preexisting pancreatic acinar cells contribute to acinar cell, but not islet beta cell, regeneration. J Clin Invest 117:971–977

Dor Y and Melton DA (2008) Facultative endocrine progenitor cells in the adult pancreas. Cell 132:183–184

Dor Y, Brown J, Martinez OI et al (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429:41–46

Dore BA, Grogan WM, Madge GE et al (1981) Biphasic development of the postnatal mouse pancreas. Biol Neonate 40:209–217

Edstrom C (1971) Further qauntitative structural studies of the pancreatic islet parenchyma in rats with duct ligation. Acta Soc Med Uppsal 76:127–138

Fausto N and Campbell JS (2003) The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 120:117–130

Ferraris C, Chevalier G, Favier B et al (2000) Adult corneal epithelium basal cells possess the capacity to activate epidermal, pilosebaceous and sweat gland genetic programs in response to embryonic dermal stimuli. Development 127:5487–5495

Gao R, Ustinov J, Pulkkinen MA et al (2003) Characterization of endocrine progenitor cells and critical factors for their differentiation in human adult pancreatic cell culture. Diabetes 52:2007–2015

Gao R, Ustinov J, Korsgren O et al (2005) In vitro neogenesis of human islets reflects the plasticity of differentiated human pancreatic cells. Diabetologia 48:2296–2304

Grompe M (2003) Pancreatic-hepatic switches in vivo. Mech Dev 120:99–106

Gu D and Sarvetnick N (1993) Epithelial cell proliferation and islet neogenesis in IFN-g transgenic mice. Development 118:33–46

Hale MA, Kagami H, Shi L et al (2005) The homeodomain protein PDX1 is required at mid-pancreatic development for the formation of the exocrine pancreas. Dev Biol 286: 225–237

Hao E, Tyrberg B, Itkin-Ansari P et al (2006) Beta-cell differentiation from nonendocrine epithelial cells of the adult human pancreas. Nat Med 12:310–316

Hardikar AA, Marcus-Samuels B, Geras-Raaka E et al (2003) Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates. Proc Natl Acad Sci USA 100:7117–7122

Hultquist GT, Karlsson U, Hallner AC (1979) The regenerative capacity of the pancreas in duct-ligated rats. Exp Pathol 17:44–52

Inada A, Nienaber C, Fonseca S et al (2006) Timing and expression pattern of carbonic anhydrase II in pancreas. Dev Dyn 235:1571–1577

Inada A, Nienaber C, Katsuta H et al (2008) Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci USA 105:19915–19919

Jensen JN, Cameron E, Garay MV et al (2005) Recapitulation of elements of embryonic development in adult mouse pancreatic regeneration. Gastroenterology 128:728–741

Jhappan C, Stahle C, Harkins RN et al (1990) TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61: 1137–1146

Jiang J, Au M, Lu K et al (2007) Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells 25:1940–1953

Kikugawa RKH, Akashi T, Yatoh S et al (2009) Differentiation of COPAS-sorted non-endocrine pancreatic cells into insulin-positive cells in the mouse. Diabetologia 52: in press

Kloppel G, Löhr M, Habich K et al (1985) Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. Surv Synth Pathol Res 4:110–125

Kritzik MR, Jones E, Chen Z et al (1999) *Pdx1* and *Msx-2* expression in the regenerating and developing pancreas. J Endocrinol 163:523–530

Kritzik MR, Krahl T, Good A et al (2000) Transcription factor expression during pancreatic islet regeneration. Mol Cell Endocrinol 164:99–107

Kroon E, Martinson LA, Kadoya K et al (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells *in vivo*. Nat Biotechnol 26:443–452

Lardon J, Huyens N, Rooman I et al (2004) Exocrine cell transdifferentiation in dexamethasone-treated rat pancreas. Virchows Arch 444:61–65

Lee CS, De León DD, Kaestner KH et al (2006) Regeneration of pancreatic islets after partial pancreatectomy in mice does not involve the reactivation of neurogenin-3. Diabetes 55: 269–272

Li L, Seno M, Yamada H et al (2003) Betacellulin improves glucose metabolism by promoting conversion of intraislet precursor cells to beta-cells in streptozotocin-treated mice. Am J Physiol Endocrinol Metab 285:E577–583

Li WC, Horb ME, Tosh D et al (2005) *In vitro* transdifferentiation of hepatoma cells into functional pancreatic cells. Mech Dev 122:835–847

Mashima H, Ohnishi H, Wakabayashi K et al (1996a) Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. J Clin Invest 97:1647–1654

Mashima H, Shibata H, Mine T et al (1996b) Formation of insulin producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. Endocrinol 137:3969–3976

Meier JJ, Butler AE, Saisho Y et al (2008) Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 57: 1584–1594

Michalopoulos GK (2007) Liver regeneration. J Cell Physiol 213:286–300

Noguchi H, Kaneto H, Weir GC et al (2003) PDX1 protein containing its own antennapedia-like protein transduction domain can transduce pancreatic duct and islet cells. Diabetes 52: 1732–1737

Oshima Y, Suzuki A, Kawashimo K et al (2007) Isolation of mouse pancreatic ductal progenitor cells expressing CD133 and c-met by flow cytometric cell sorting. Gastroenterology 132: 720–732

Parsons JA, Bartke A, Sorenson RL (1995) Number and size of islets of Langerhans in pregnant human growth hormone-expressing transgenic, and pituitary dwarf mice: effect of lactogenic hormones. Endocrinol 136:2013–2021

Peshavaria M, Larmie BL, Lausier J et al (2006) Regulation of pancreatic beta-cell regeneration in the normoglycemic 60% partial-pancreatectomy mouse. Diabetes 55:3289–3298

Peters K, Panienka R, Li J et al (2005) Expression of stem cell markers and transcription factors during the remodeling of the rat pancreas after duct ligation. Virchows Arch 446:56–63

Portha B (2005) Programmed disorders of beta-cell development and function as one cause for type 2 diabetes? The GK rat paradigm. Diabetes Metab Res Rev 21: 495–504

Ramiya VK, Maraist M, Arfors KE et al (2000) Reversal of insulin dependent diabetes using islets generated *in vitro* from pancreatic stem cells. Nat Med 6:278–282

Rao MS, Dwivedi RS, Yeldandi AV et al (1989) Role of periductal and ductular epithelial cells of the adult rat pancreas in pancreatic hepatocyte lineage. Am J Physiol 134: 1069–1086

Reddy JK, Rao MS, Yeldandi AV et al (1991) Pancreatic hepatocytes: an *in vivo* model for cell lineage in pancreas of adult rat. Digest Dis Science 36:502–509

Rooman I, Lardon J, Bouwens L (2002) Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. Diabetes 51: 686–690

Sarvetnick NE and Gu D (1992) Regeneration of pancreatic endocrine cells in interferon-gamma transgenic mice. Adv Exp Med Biol 321:85–89

Scaglia L, Cahill CJ, Finegood DT et al (1997) Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. Endocrinol 138:1736–1741

Seaberg RM, Smukler SR, Kieffer TJ et al (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22:1115–1124

Seymour PA, Freude KK, Tran MN et al (2007) Sox9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci USA 104:1865–1870

Sharma A, Zangen DH, Reitz P et al (1999) The homeodomain protein IDX-1 increases after an early burst of proliferation during pancreatic regeneration. Diabetes 48:507–513

Song SY, Gannon M, Washington MK et al (1999) Expansion of PDX1-expressing pancreatic epithelium and islet neogenesis in transgenic mice overexpressing transforming growth factor a. Gastroenterology 117:1416–1426

Suarez-Pinzon WL, Lakey JR, Brand SJ et al (2005) Combination therapy with epidermal growth factor and gastrin induces neogenesis of human islet β-cells from pancreatic duct cells and an increase in functional β-cell mass. J Clin Endocrinol Metab 90:3401–3409

Sugiyama T, Rodriguez RT, McLean GW et al (2007) Conserved markers of fetal pancreatic epithelium permit prospective isolation of islet progenitor cells by FACs. Proc Natl Acad Sci USA 104:175–180

Suzuki A, Nakauchi H, Taniguchi H (2004) Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting. Diabetes 53:2143–2152

Teta M, Rankin MM, Long SY et al (2007) Growth and regeneration of adult beta cells does not involve specialized progenitors. Dev Cell 12:817–826

Tyrberg B, Ustinov J, Otonkoski T et al (2001) Stimulated endocrine cell proliferation and differentiation in transplanted human pancreatic islets: effects of the *ob* gene and compensatory growth of the implantation organ. Diabetes 50:301–307

Wang TC, Bonner-Weir S, Oates PS et al (1993) Pancreatic gastrin stimulates islet differentiation of transforming growth factor alpha-induced ductular precursor cells. J Clin Invest 92: 1349–1356

Wang RN Klöppel G, Bouwens L (1995) Duct- to islet-cell differentiation and islet growth in the pancreas of duct-ligated adult rats. Diabetologia 38:1405–1411

Wang X, Foster M, Al-Dhalimy M et al (2003) The origin and liver repopulating capacity of murine oval cells. Proc Natl Acad Sci USA 100(Suppl 1):11881–11888

Wang CY, Gou SM, Liu T et al (2008) Differentiation of CD24- pancreatic ductal cell-derived cells into insulin-secreting cells. Dev Growth Differ 50:633–643

Xu G, Stoffers DA, Habener JF et al (1999) Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes 48:2270–2276

Xu X, D'Hoker J, Stangé G et al (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 132:197–207

Yatoh S, Dodge R, Akashi T et al (2007) Differentiation of affinity-purified human pancreatic duct cells to β-cells. Diabetes 56:1802–1809

Yoon KH, Ko SH, Cho JH et al (2003) Selective β-cell loss and α-cell expansion in patients with type 2 diabetes mellitus in Korea. J Clin Endoc Metab 88:2300–2308.

Zhao M, Amiel SA, Christie MR et al (2005) Insulin-producing cells derived from human pancreatic non-endocrine cell cultures reverse streptozotocin-induced hyperglycaemia in mice. Diabetologia 48:2051–2061

Zhou J, Wang X, Pineyro MA et al (1999) Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. Diabetes 48:2358–2366

Zhou J, Pineyro MA, Wang X et al (2002) Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX1 and HNF3bα transcription factors. J Cell Physiol 192:304–314

# Chapter 9
# Adult Cell Reprogramming: Using Nonpancreatic Cell Sources to Generate Surrogate Beta Cells for Treatment of Diabetes

**Irit Meivar-Levy, Vered Aviv, and Sarah Ferber**

**Abstract** Regenerative medicine is designed to produce new cells for repair or replacement of diseased and damaged tissues. Embryonic and adult stem cells have been suggested as attractive sources for generation of new differentiated cells. The leading dogma has maintained that once animal cells are committed to a specific lineage, they become "terminally differentiated" and can no longer change their fate. However, in recent years increasing evidence has demonstrated the remarkable ability of some differentiated cells to convert into a different cell type via a process termed developmental redirection or nuclear reprogramming. For example, abundant human cell types, such as dermal fibroblasts and adipocytes, could potentially be harvested and converted into other, medically important cell types, such as neurons, cardiomyocytes, or pancreatic β cells. In this chapter we review the potential use of adult tissue, specifically liver and bone marrow, to provide a source of tissue for generating functional insulin-producing cells. This approach might generate custom-made autologous surrogate β cells for treatment of diabetes and possibly circumvent both the shortage of cadaveric human donor tissue and the need for life-long immunosuppression.

## 9.1 Adult Cell Reprogramming

Nuclear reprogramming of differentiated cells in mammals offers an attractive approach for generating surrogate cells for treatment of degenerative diseases such as diabetes (Ferber 2000; Slack and Tosh 2001; Meivar-Levy and Ferber 2006; Eberhard and Tosh 2008; Zhou and Melton 2008). Until recently it had been thought that differentiated cells could only be generated from embryonic or adult stem cells. However, a growing body of evidence suggests that differentiated cells can be converted into a completely different phenotype in a process termed transdifferentiation (Eberhard and Tosh 2008). Transdifferentiation takes place in nature in a few specific cases: for example, in salamanders and chickens when the eye lens is

I. Meivar-Levy (✉)
Endocrine Institute, Sheba Medical Center, Tel-Hashomer 52621, Israel
e-mail: irit.meivar-levy@sheba.health.gov.il

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_9,

removed, cells of the iris turn into lens cells (Ito et al. 1999; Makarev et al. 2007; Tsonis 2007). Although this natural ability is relatively rare, transdifferentiation of mature cells can be forced using transcription factors or extracellular soluble factors, which induce profound changes in gene expression, leading to a different cellular phenotype.

The possibility of redirecting cell differentiation by overexpression of master regulator genes was demonstrated 20 years ago by Weintraub et al. using *MyoD*, a gene encoding a muscle-specific transcription factor (Weintraub et al. 1989). Overexpression of this gene was sufficient for switching a number of nonmuscle cell types into muscle-like cells. Activation of the muscle phenotype in nonmuscle cells was further augmented by inducing epigenetic modifications with the hypomethylating agent 5-azacytidine (Boukamp et al. 1992).

Switches between cell types have also been successfully achieved in cells from several other tissues, particularly in cells derived from the hematopoietic lineage. Overexpression of key transcription factors in these cells activated and/or repressed the expression of numerous genes that together resulted in changes in cell fate (Heavey et al. 2003; Newsome et al. 2003). In these cases the transdifferentiation process involved a transient dedifferentiation step (Heavey et al. 2003). In the reprogramming process of hematopoietic cells and in *MyoD*-induced reprogramming, cell proliferation was found to be crucial for the establishment of the new cell phenotype (Weintraub et al. 1989; Heavey et al. 2003). However, other lineage switches do not appear to require cell division, as in developmental redirection of liver into pancreas or of pancreatic exocrine cells into β cells (Meivar-Levy et al. 2007; Zhou et al. 2008).

Differentiated cell phenotypes are maintained by expression of combinations of transcription factors and by soluble factors present in the cellular niche. In some cases, a single transcription factor, which acts as a master regulator, can force a particular path of development in a dominant way in the presence of other factors, as in the above-mentioned examples of the MYOD family of basic helix-loop-helix (bHLH) factors, which can force differentiation into muscle cells in a variety of cell types in culture (Weintraub et al. 1989), and the eyeless/PAX6 factor, which can force eye development in many of the *Drosophila* imaginal discs (Tarpin et al. 2002).

The hierarchy of transcription factors active in pancreatic organogenesis, and the role of many soluble factors in promoting β-cell maturation, have been studied intensely. The rationale that underlies the concept of inducing a pancreatic phenotype in adult cells, such as liver cells, is that the sequential or combinatorial ectopic expression of these factors may be capable of inducing a similar developmental redirection along the pancreatic lineage in adult tissues as well. It is assumed that maximal transdifferentiation will be achieved when the optimal combination of pancreatic growth and transcription factors endows a sufficient number of target cells with mature β-cell characteristics and function.

We devote most of the chapter to the liver as a potential target for transdifferentiation into β cells because this tissue has attracted much interest in the last decade, as reflected in numerous publications, whereas the capacity of other cells has been less explored.

## 9.2 Conversion of Liver Cells into Pancreas Cells

### *9.2.1 The Rationale for Using Liver as a Source for Pancreatic Tissue*

The liver is the largest organ in the human body, with a high level of functional redundancy (Desmet 2001). Unlike β cells, liver regenerates efficiently, mainly by the proliferation of mature hepatocytes (Thorgeirsson 1996). Human hepatocytes can be propagated in vitro for months, and the number of cells can be expanded substantially. Moreover, since liver has an important role in neutralizing toxins and, compared with pancreatic β cells, hepatocytes have much higher levels of catalase and dismutase enzymes (Desmet 2001), surrogate β cells derived from liver may resist cellular assaults that kill normal β cells.

The liver and the pancreas are developmentally related, as both are derived from appendages of the upper primitive foregut endoderm. It has been suggested that following the separation of the liver and pancreas during organogenesis in the primitive ventral endoderm, multipotent cells that are capable of giving rise to both hepatic and pancreatic lineages are maintained in both tissues (Deutsch et al. 2001). The two mature tissues share many characteristics, including responsiveness to glucose and expression of a large group of specific transcription factors (Otsuka et al. 2003). Conversion of pancreatic acinar cells into hepatocytes in both rodents and humans has been reported under experimental, pathological, and malignant conditions (Rao and Reddy 1995; Shen et al. 2000; Zhou et al. 2008).

A comparison of liver and pancreas development in other species further emphasizes the close relationship between these two organs. In lower organisms, such as worms and eels, there is no spatial separation between the two organs (Kito et al. 1982; Yang et al. 1999), and the "hepatopancreas" functions as both liver and pancreas (Deutsch et al. 2001).

### *9.2.2 Reprogramming Liver Cells Using Ectopic Expression of Pancreatic Transcription Factors*

Numerous pancreatic transcription and developmental factors have been demonstrated to participate in endowing liver cells with pancreatic characteristics. The capacity of activating pancreatic gene expression in liver cells was first demonstrated in mice by systemic administration of a recombinant adenovirus carrying the *PDX1* gene (Ferber et al. 2000). Studies performed by multiple groups indicated that PDX1 plays a central role in regulating pancreas development and β-cell function (Miller et al. 1994; Waeber et al. 1996; Watada et al. 1996b; Kaneto et al. 2008). Heterozygosity in human *PDX1* mutations causes maturity-onset diabetes of the young and probably type 2 diabetes (Jonsson et al. 1994; Offield et al. 1996; Stoffers et al. 1997). PDX1 regulates expression of several islet cell-specific genes (Waeber et al. 1996; Watada et al. 1996a; Watada et al. 1996b) and participates

in mediating glucose regulation of insulin gene expression (Melloul et al. 2002; Andrali et al. 2008). The pattern of PDX1 expression during embryogenesis and its ability to stimulate insulin gene transcription suggest that it functions in both the regionalization of the primitive gut endoderm and the maturation and function of the pancreatic β cells (St-Onge et al. 1999; Edlund 2002; Kaneto et al. 2008). Moreover, ectopic expression of PDX1 in pancreatic non-β-cell types, such as duct/epithelial cells or exocrine cells, is obligatory for their differentiation into insulin-secreting cells (Baeyens et al. 2005; Minami et al. 2005; Bonner-Weir et al. 2008; Zhou et al. 2008).

#### 9.2.2.1 In-Vitro Reprogramming of Embryonic or Fetal Liver Cells

It is reasonable to assume that immature liver cells may be more susceptible to acquiring pancreatic characteristics owing to their multipotency, compared with adult cells. The bile-duct-derived progenitors termed oval cells have been characterized in rodents, but their human equivalents have not yet been found. Liver oval cells, considered to be the hepatic stem cells, have been shown to possess several developmental options, including hepatocytes, biliary epithelium, intestinal epithelium, and pancreatic acinar epithelium. These cells express high levels of surface THY1.1, cytokeratin-19, OC.2, and OV6, as well as cytoplasmic α-fetoprotein and γ-glutamyltranspeptidase. Yang and colleagues demonstrated that exposure of oval cells to high levels of glucose and nicotinamide in vitro and removal of leukemia inhibitory factor (which is known to inhibit stem cell differentiation) causes their transdifferentiation into pancreatic endocrine hormone-producing cells (Yang et al. 2002). These cells form three-dimensional islet-like clusters and express several pancreatic transcription factors, including PDX1, as well as islet-specific hormones. In several studies hepatocyte progenitor cells isolated from rodents were induced to differentiate into insulin-producing cells (Nakajima-Nagata et al. 2004; Jin et al. 2007; Kawasaki et al. 2008). Small hepatocytes (SHCs; Nakajima-Nagata et al. 2004; Kawasaki et al. 2008) expressing the stem cell marker nestin were induced to transdifferentiate into insulin-producing cells using a multistep protocol (Kawasaki et al. 2008). The cells were cultured in the presence of growth and differentiation factors, followed by delivery of the *PDX1* gene with an adenovirus. These cells expressed a number of β-cell transcripts and secreted insulin in response to insulin secretagogues.

Adult mouse liver epithelial progenitor cells (LEPCs) acquired the phenotype of pancreatic endocrine progenitor cells upon ectopic expression of PDX1, but the cells were not functional in vitro (Jin et al. 2007). LEPCs expressing PDX1 proliferated vigorously and expressed many transcription factors involved in β-cell development, including NGN3, NEUROD1, NKX2.2, NKX6.1, PAX4, PAX6, IsL1, and MAFA, as well as endogenous PDX1, but did not secrete insulin. When cultured in high-glucose and low-serum medium supplemented with cytokines the cells stopped proliferating and gave rise to functional β cells, without expression of exocrine or other islet-cell markers. When transplanted into diabetic severe-combined immunodeficient (SCID) mice, PDX1-expressing LEPCs ameliorated hyperglycemia by

secreting insulin in a glucose-regulated manner (Jin et al. 2007). The induction of a functional β-cell phenotype was also documented in fetal human liver cells. These cells, which were first immortalized by retroviral introduction of the gene encoding the catalytic subunit of human telomerase, were induced to differentiate into insulin-producing cells by a lentiviral vector containing *PDX1* and a neomycin-resistance gene (Zalzman et al. 2003). Upon treatment with soluble factors, such as activin A, serum withdrawal, and supplementation with insulin, transferrin, and selenium (ITS), the cells exhibited improved β-cell-like function, as manifested by an insulin content of up to 60% of that of normal human β cells (Zalzman et al. 2005). Processed insulin was secreted in a glucose-regulated manner, and the cells ameliorated hyperglycemia in nonobese diabetic (NOD)-SCID mice for long periods of time (Zalzman, et al. 2005).

Taken together, these data suggest that PDX1 triggers only a limited degree of differentiation toward the β-cell phenotype in immature liver cells in vitro. This is not surprising, given that most adult cells exhibit a lower differentiation level during cell proliferation. Supplementing the culture with defined soluble factors, arrest of cell proliferation by serum deprivation, and growth in a three-dimensional configuration may reduce cell proliferation through proper cell contact and promote maturation of the β-cell phenotype. Indeed, in fetal human liver cells expressing PDX1, switch to culture in serum-free medium, which increased their differentiation, was associated with a fourfold increase in doubling time (Zalzman et al. 2005).

#### 9.2.2.2 In-Vitro Reprogramming of Adult Rodent and Human Liver Cells

Primary liver cells isolated from adult rodents, as well as rodent hepatocyte cell lines, were used to study the effect of PDX1 in induction of liver-to-pancreas transdifferentiation (Cao et al. 2004; Li et al. 2005; Lu et al. 2005; Fodor et al. 2007). In all the reported studies, *PDX1*-transduced hepatocytes expressed β-cell-specific markers. However the β-cell-like function of these transdifferentiated liver cells was unclear. Fodor et al. reported that primary cultures of mature rat hepatocytes infected with a lentivirus containing human *PDX1* expressed insulin and secreted it in a glucose-regulated manner. Furthermore, transplantation of transduced hepatocytes under the kidney capsule in hyperglycemic NOD-SCID mice reversed diabetes, as the blood glucose level significantly decreased and the mice gained weight (Fodor et al. 2007). However, stably-transfected cells of the rat hepatic cell line WB-1 that overexpressed an activated form of PDX1 (PDX1-VP16; see Section 2.5.1 below) required further stimulation by hyperglycemia in vivo or long-term culture in high-glucose medium to become fully functional insulin-secreting cells (Cao et al. 2004).

The ability to induce functional redirection of mature human liver cells has a substantial therapeutic potential, since it may allow autologous cell replacement therapy for diabetics. Therefore, several studies analyzed the possibility of activating the β-cell phenotype in adult human liver cells. A pioneering study demonstrated the ability to manipulate primary adult human liver cells in culture (Sapir et al.

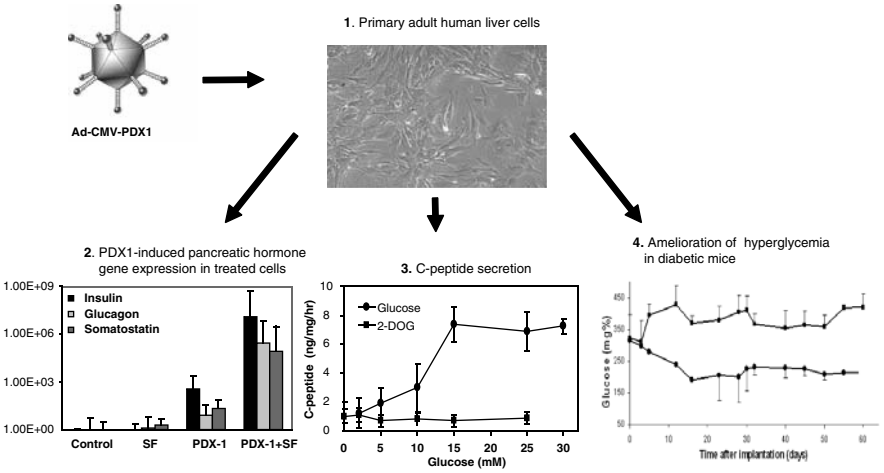



**Fig. 9.1 PDX1-induced reprogramming of adult human liver cells in vitro.** Adult human liver cells, shown in (**1**) in a phase-contrast image, were transduced with a *PDX1* virus and treated with soluble factors (SF) nicotinamide and EGF. The cells underwent a comprehensive developmental shift into functional insulin-producing cells, as judged by the expression of insulin mRNA (**2**), and glucose-regulated insulin secretion (**3**). The treated cells ameliorated hyperglycemia when transplanted under the renal capsule of diabetic, immunodeficient mice (**4**) (squares, PDX1-expressing liver cells; circles, untreated cells) (Reproduced with permission from Sapir et al. 2005)

2005) (Fig. 9.1). Cells isolated from adult human liver were propagated in vitro for multiple passages, and upon *PDX1* transduction and treatment with soluble factors [epidermal growth factor (EGF) and nicotinamide] up to 50% of PDX1-expressing cells activated insulin expression. Insulin produced in PDX1-expressing adult human liver cells was processed, stored in secretory granules, and secreted in response to elevated glucose concentrations. The β-cell-like function of these cells was demonstrated by their capacity to correct hyperglycemia in NOD-SCID mice. Human C-peptide secretion and amelioration of hyperglycemia persisted for the duration of the experiment (60 days). Activation of expression of islet transcription factors, including the endogenous human PDX1, was consistent with the functional properties of PDX1-expressing adult human liver cells (Sapir et al. 2005). These data demonstrate the ability to activate the β-cell phenotype and function in vitro not only in immature liver cells but also in adult, fully-differentiated liver cells, as summarized in Table 9.1.

### 9.2.2.3 In-Vivo Reprogramming of Adult *Xenopus* and Rodent Liver Cells

Studies performed in mice and in *Xenopus* suggest that the β-cell phenotype can also be activated by ectopic expression of transcription factors in vivo in mature, differentiated liver cells (Ber et al. 2003; Horb et al. 2003; Kojima et al. 2003; Koizumi et al. 2004; Imai et al. 2005; Kaneto et al. 2005a; Kaneto et al. 2005b) (Table 9.2).

**Table 9.1** Activation of the β-cell phenotype in differentiated liver cells by developmental redirection in vitro

| Cell source | Pancreatic transcription factor used | Induced phenotype | References |
|---|---|---|---|
| Rat WB-1 cells | PDX1-VP16<br>PAX4 | Partial pancreas-specific gene expression<br>Maturation upon transplantation in vivo | Cao et al. (2004)<br>Tang et al. (2006a)<br>Tang et al. (2006b) |
| Primary culture of rat hepatocytes | PDX1<br>PDX1-VP16 | Pancreas-specific gene expression<br>Insulin production and secretion<br>Amelioration of hyperglycemia upon transplantation in vivo | Fodor et al. (2007)<br>Yamada et al. (2006) |
| Human hepatoma cells, HepG2 | PDX1-VP16 | Pancreas-specific gene expression<br>Insulin production and secretion | Li et al. (2005) |
| Primary culture of adult human liver cells | PDX1 + Nicotinamide + EGF | Pancreas-specific gene expression<br>Insulin production and secretion<br>Amelioration of hyperglycemia upon transplantation in vivo | Sapir et al. (2005) |

**Table 9.2** Activation of the β-cell phenotype in liver cells by developmental redirection in vivo

| Model | Pancreatic transcription factor used | Induced phenotype | References |
|---|---|---|---|
| *Xenopus* tadpoles | PDX1-VP16 | Pancreas-specific gene expression | Horb et al. (2003) |
| STZ-diabetic mice | PDX1 | Pancreas-specific gene expression<br>Insulin production and secretion<br>Amelioration of hyperglycemia | Ferber et al. (2000)<br>Ber et al. (2003) |
| CAD-NOD diabetic mice | PDX1 | Pancreas-specific gene expression<br>Insulin production and secretion<br>Amelioration of hyperglycemia<br>Immune modulation | Shternhall-Ron et al. (2007) |
| STZ-diabetic mice + 40% hepatectomy | PDX1 | Pancreas-specific gene expression<br>Insulin production and secretion<br>Amelioration of hyperglycemia | Koizumi et al. (2004) |
| STZ-diabetic mice | PDX1-VP16 | Insulin production and secretion<br>Amelioration of hyperglycemia | Imai et al. (2005) |
| STZ-diabetic mice | PDX1-VP16<br>MAFA<br>NEUROD1<br>NGN3 | Insulin production and secretion<br>Amelioration of hyperglycemia | Kaneto et al. (2005a)<br>Kaneto et al. (2005b)<br>Wang et al. (2007) |
| STZ-diabetic mice | NEUROD1 + betacellulin | Pancreas-specific gene expression<br>Insulin production and secretion<br>Amelioration of hyperglycemia | Kojima et al. (2003) |

Transient ectopic expression of PDX1 in liver following systemic administration of first-generation E1-deleted recombinant adenovirus (FGAD) induced expression of a large repertoire of pancreatic genes. Surprisingly, ectopic expression of PDX1 led to long-lasting production and secretion of processed, biologically-active

insulin, despite the short-term expression of the *PDX1* transgene in liver, owing to the lack of integration of the adenovirus vector (Ferber et al. 2000; Ber et al. 2003) (Fig. 9.2). This can be explained by the finding that PDX1 induced its own expression (autoinduction). Insulin- and glucagon-producing cells were located primarily in the proximity of hepatic central veins, possibly allowing direct hormone release into the blood stream and an endocrine mode of action of the secreted hormones. This localization is not expected to affect normal hepatic function, which would have been caused by paracrine effects of locally-produced insulin at superphysiological concentrations (Ber et al. 2003).

Hepatic insulin production triggered by PDX1 expression was functional, not only restoring euglycemia in streptozotocin (STZ)-induced diabetic mice (Ferber et al. 2000), but also preventing STZ-induced hyperglycemia even 8 months after the initial infection with the *PDX1* virus (Ber et al. 2003). These data demonstrate the phenotypic stability of the modified cells, as well as the fact that liver insulin-producing cells are resistant to the β-cell-specific toxin STZ, possibly owing to high levels of catalase and dismutase activity that prevents accumulation of free

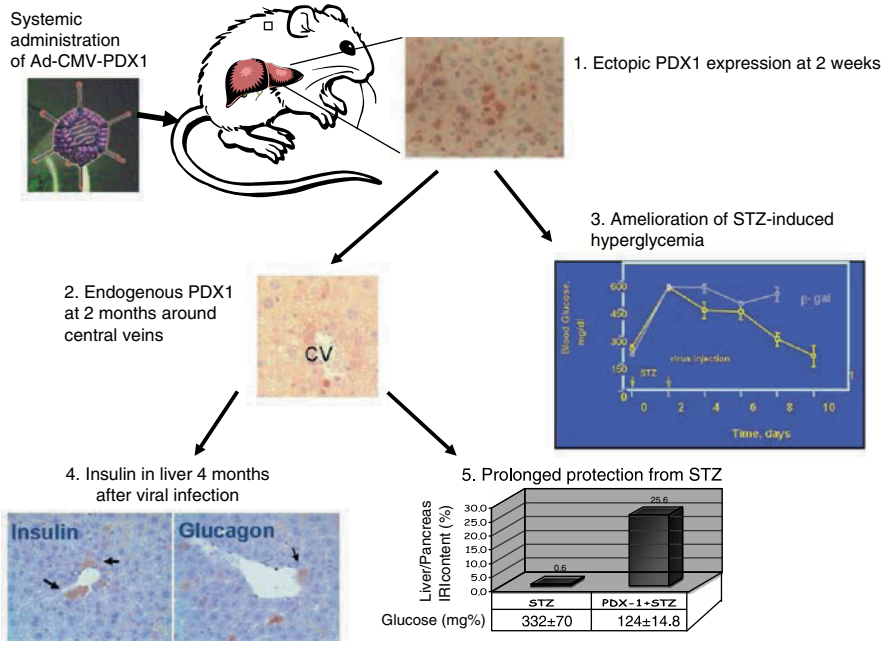



**Fig. 9.2 PDX1-induced reprogramming of liver cells in adult mice in vivo.** The short-term effects of ectopic PDX1 expression in liver (**1**) resulted in long-term PDX1 expression in liver cells located mainly in the proximity of central veins (**2**). Insulin secreted from liver cells of mice infected with the *PDX1* virus ameliorated hyperglycemia (**3**). Transient PDX1 expression induced long-term transdifferentiation, as manifested by insulin- and glucagon-positive cells 4 months after treatment (**4**). Hepatic insulin production 8 months after treatment was 40-fold higher, compared to control mice, and prevented STZ-induced hyperglycemia (**5**) (Reproduced with permission from Ferber et al. 2000 and Ber et al. 2003)

radicals generated by STZ metabolism in the cells (Schnedl et al. 1994; Tabiin et al. 2001). Moreover, surrogate β cells derived from liver cells may be resistant to cellular assaults directed against β cells. In fact, a recent study demonstrated that the insulin-producing cells in liver were protected from autoimmune attacks that destroyed β cells (Shternhall-Ron et al. 2007). This study utilized the model of cyclophosphamide-accelerated diabetes in NOD mice (CAD-NOD). Some 43% of the overtly diabetic CAD-NOD mice infected with the *PDX1* virus became normoglycemic and maintained a stable body weight. Although pancreatic islets of these mice showed lymphocyte infiltration, insulin-positive cells in their livers did not exhibit any signs of inflammation (Shternhall-Ron et al. 2007).

### *9.2.3 Liver-to-Pancreas Transdifferentiation Involves Hepatic Dedifferentiation*

Transdifferentiation refers to the conversion of a differentiated cell into a cell with another differentiated phenotype (Slack and Tosh 2001). This implies shutoff of the expression of genes responsible for the original cell phenotype, as well as activation of genes associated with the newly-acquired phenotype. Several studies reported a downregulation in expression of hepatocyte-specific genes, such as albumin, during the activation of the pancreatic phenotype in liver cells (Horb et al. 2003; Li et al. 2005). Other studies revealed that NEUROD1 activated the pancreatic phenotype in liver cells in vivo without turning off the host cell repertoire of genes (Kojima et al. 2003). However, the efficiency of the reprogramming process in vitro using the same transcription factor increased when liver cells underwent a prior dedifferentiation process (Yatoh et al. 2006).

To evaluate the full extent of changes in gene expression associated with liver-to-pancreas developmental redirection induced by ectopic expression of PDX1, cDNA microarray analyses were performed (Meivar-Levy and Ferber, unpublished data). The microarray studies revealed great changes in gene expression (Fig. 9.3). Although activating hundreds of genes, many of them pancreatic, PDX1 also suppressed the expression of hundreds of other genes, many of them liver-specific genes, such as albumin, glutamate synthetase, and glucose-6-phosphatase. PDX1 expression also activated expression of α-fetoprotein, a marker of immature hepatocytes. This occurred without induction of abnormal cell proliferation, both in vitro and in vivo (Meivar-Levy et al. 2007). The effects of PDX1 on hepatic gene expression were accompanied by suppression of the hepatic transcription factor C/EBPβ, which in turn could be responsible for the decline in other hepatic gene expression. Moreover, induction of hepatic dedifferentiation was necessary but insufficient for efficient activation of the pancreatic phenotype in PDX1-expressing liver cells (Meivar-Levy et al. 2007).

These profound changes in gene expression likely reflect epigenetic modifications. A strong indication for the occurrence of epigenetic modifications is the long-lasting activation of the pancreatic phenotype, despite diminished transgene

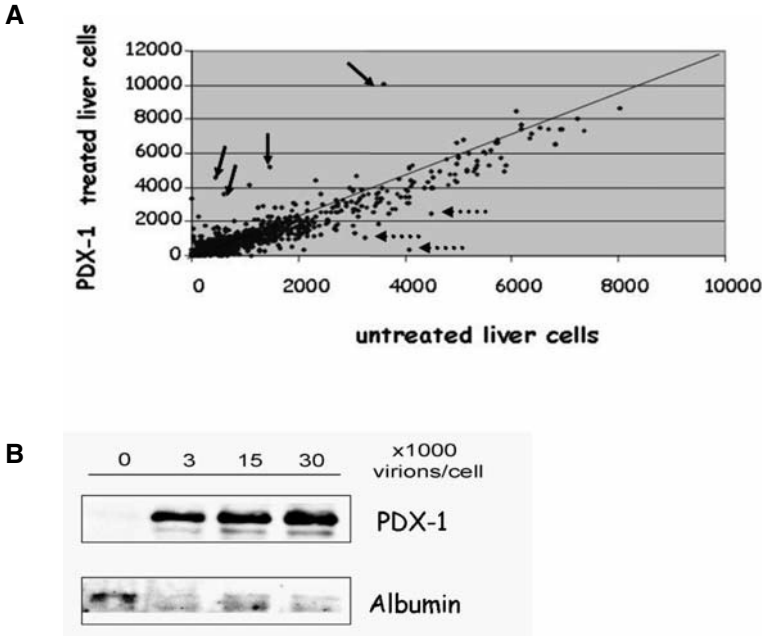



**Fig. 9.3 PDX1 induces profound changes in gene expression in adult human liver cells in vitro.** (**a**) cDNA microarray analyses demonstrate that ectopic expression of PDX1 in adult human liver cells induces profound changes in expression of hundreds of genes. (**b**) In addition to induction of hundreds of pancreatic genes, ectopic expression of PDX1 in adult liver cells repressed hepatic markers such as albumin

expression. This indicates that the transdifferentiation change is stable and probably depends on epigenetic events, which change the pattern of gene expression in the reprogrammed cells. We still have to prove unequivocally that the newly-acquired phenotype is transmitted to daughter cells during proliferation both in vivo and in vitro.

### *9.2.4 Which Is the Vector of Choice for Inducing Liver Transdifferentiation into Beta Cells?*

The ability to use a transient trigger for inducing stable transdifferentiation, as demonstrated above with the adenovirus-*PDX1* vector, represents an advantage in future therapy of diabetic patients, as it does not require insertion of genetic information into the host genome and therefore is considered safer, compared with integrating vectors. Adenovirus vectors remain episomal and are diluted upon cell replication, thereby limiting the time of transgene expression (Becker et al. 1994; Young et al. 2006). This transient infection works because PDX1 produced by the

transgene activates expression of the endogenous *PDX1* gene. Indeed, the majority of studies exploring this process used recombinant adenoviruses to deliver the transgene (Young et al. 2006).

When the transgene cannot activate its endogenous counterpart, it must be integrated into the cell genome. Currently, only a few vectors have been able to mediate long-term transgene expression in postmitotic cells with good safety records (Walther and Stein 2000; Lundstrom 2003; Young et al. 2006). The most commonly-used vehicles for gene delivery into mammalian cells exploit the high infectivity of DNA- or RNA-based viruses engineered to express the gene of interest and are devoid of replication capacity, such as retroviral, lentiviral, or adeno-associated virus (AAV) vectors. Although these vectors are stably integrated into the host genome, their integration raises the risk of insertional mutagenesis following vector delivery.

It has recently been suggested that PDX1 protein transduction may be sufficient for activating the pancreatic phenotype in liver cells in vivo (Koya et al. 2008). Recombinant PDX1 protein injected into STZ-diabetic mice promoted both β-cell regeneration and liver cell reprogramming, leading to restoration of normoglycemia. Amelioration of hyperglycemia was associated with an increase in islet cell number and induction of insulin production in liver cells. Reprogramming of adult cells using protein delivery represents a substantial safety advantage over viral vectors as a tool for future use in regenerative medicine.

### *9.2.5 Optimizing Transcription Factor-Induced Transdifferentiation of Liver into Beta Cells*

PDX1-induced transdifferentiation of liver toward the β-cell phenotype both in vivo and in vitro is partial: the number of insulin-producing cells is low, and the amount of mature insulin produced and secreted by the transdifferentiated cells is lower than that of pancreatic β cells. Numerous studies have tried to optimize the process using different approaches.

#### 9.2.5.1 PDX1 Fusion with VP16

PDX1 has been shown to activate target genes in association with several cofactors, such as PBX (Dutta et al. 2001). The expression of these cofactors is limited in liver and so may interfere with the ability of PDX1 to function efficiently. To overcome the need for cofactors, an active form of PDX1 was created by fusion of the activation domain of the herpes simplex virus transcription factor VP16 to the C-terminus of PDX1 (Tumbar et al. 1999; Hall and Struhl 2002). This fused protein, termed PDX1-VP16, can activate target genes independently of coactivators. Moreover, VP16 might also affect chromatin condensation and remodeling of PDX1 target genes in the host genome (Tumbar et al. 1999; Memedula and Belmont 2003). Multiple studies have examined the potential of VP16 to promote PDX1-induced

liver-to-pancreas transdifferentiation in vivo and in vitro (Horb et al. 2003; Imai et al. 2005; Kaneto et al. 2005b; Li et al. 2005; Tang et al. 2006a; Tang et al. 2006b; Yamada et al. 2006). In an early study, Horb et al. expressed the frog homologue of PDX1 as a fusion protein with the transcriptional activation domain of VP16 (Horb et al. 2003). PDX1 activation in transgenic tadpoles allowed the conversion of most of the liver cells into exocrine and endocrine pancreas cells. The massive transformation of the tadpole's liver into pancreas suggests that activation of PDX1 by VP16 may overcome some of the barriers of hepatocyte predisposition to transdifferentiation, possibly including the absence of some transcription factors, as well as certain aspects of chromatin structure. Activation of both endocrine and exocrine pancreas genes by ectopic expression of PDX1-VP16 was also reported in human HepG2 (hepatoma) cells (Li et al. 2005). Upon treatment, these cells expressed insulin, glucagon, and somatostatin, as well as the pancreatic exocrine marker amylase.

PDX1-VP16 was found to be more efficient than PDX1 in inducing the pancreatic phenotype and function in liver in vivo (Cao et al. 2004; Imai et al. 2005; Kaneto et al. 2005b; Yamada et al. 2006). Yamada et al. reported a significant increase in the number of insulin-producing cells in vitro detected upon treatment with PDX1-VP16 (<1% with PDX1, as opposed to 5–15% with PDX1-VP16) (Yamada et al. 2006). Similarly, it was shown that the effect of PDX1-VP16 in vivo was higher than that of PDX1. PDX1-VP16 lowered blood glucose levels faster, and the glucose levels throughout the experiment were significantly lower than those in PDX1-treated mice (Imai et al. 2005; Kaneto et al. 2005b).

#### 9.2.5.2 Using Combinations of Pancreatic Transcription Factors

PDX1 plays a central role in pancreas development, and most studies utilized it as an inducer of transdifferentiation. Nevertheless, several studies have examined the ability of other pancreatic transcription factors, such as NEUROD1 and NGN3, to induce this process (Kojima et al. 2003; Yatoh et al. 2006; Song et al. 2007; Wang et al. 2007). NEUROD1 and NGN3 are members of the bHLH transcription factor family and both are involved in pancreas development (Bernardo et al. 2008; Pearl and Horb 2008). Kaneto et al. demonstrated that both of these factors were incapable of individually inducing transdifferentiation of liver cells into β-like cells, but they both significantly promoted the effect of PDX1-VP16 in this process. The combined treatment of PDX1 and NEUROD1 or NGN3 markedly increased insulin promoter activity in vitro, as well as insulin expression in mouse liver and amelioration of hyperglycemia in vivo (Kaneto et al. 2005b).

Although a *NEUROD1* adenovirus did not activate the pancreatic phenotype in mouse liver (Kaneto et al. 2005b), the same transgene did activate pancreatic gene expression in liver when delivered by a helper-dependent adenovirus vector (Kojima et al. 2003). NEUROD1 expression in combination with betacellulin

treatment further promoted the transdifferentiation process and corrected STZ-induced diabetes in mice (Kojima et al. 2003). Interestingly, ectopic NEUROD1 expression in liver induced expression of both downstream and upstream pancreatic transcription factors, including PDX1 (Kojima et al. 2003). However, whereas in mouse PDX1-treated liver cells glucagon and insulin were produced in different cells (Ber et al. 2003), the pancreatic hormones were coproduced within the same cell in mouse liver expressing NEUROD1 (Kojima et al. 2003). Moreover, the hormone-producing cells in mouse liver expressing NEUROD1 were located primarily in the liver capsule (Kojima et al. 2003), whereas in PDX1-treated liver both insulin- and glucagon-expressing cells were located around central veins (Ber et al. 2003; Imai et al. 2005). It is still unclear whether the differences in outcome between the two models are due to differences in the activity of the two transcription factors, changes in the infection capacity of the viral vectors, or the different populations of progenitor cells in liver transduced by each vector.

Additional studies focused on the ectopic expression of the pancreatic transcription factor MAFA. This basic leucine-zipper transcription factor is a potent transactivator of the insulin gene and is expressed later in pancreas development, compared with PDX1, NGN3, and NEUROD1, as well as in mature β cells. MAFA increased insulin promoter activity in HepG2 cells, but failed to induce insulin production in liver in vivo (Kaneto et al. 2005a). However, MAFA expression, together with PDX1 and NEUROD1, substantially increased insulin production and secretion, compared to PDX1 treatment alone, and ameliorated hyperglycemia in a diabetic mouse model (Kaneto, et al. 2005a; Kaneto, et al. 2005b).

#### 9.2.5.3 Using Soluble Factors to Promote Transcription-Factor-Induced Liver-to-Pancreas Transdifferentiation

The effect of activin A, betacellulin, nicotinamide, EGF, hepatocyte growth factor (HGF), and other soluble factors on the transdifferentiation process has been examined both in vivo and in vitro (Kojima et al. 2003; Koizumi et al. 2004; Sapir et al. 2005; Zalzman et al. 2005).

In a model of primary culture of adult human liver cells, EGF and nicotinamide were documented to promote transdifferentiation of PDX1-expressing cells into endocrine pancreas cells. Betacellulin, a member of the EGF family of growth factors (Demeterco et al. 2000), has been implemented in promoting NEUROD1-induced liver-to-pancreas transdifferentiation in mice (Kojima et al. 2003). Combined ectopic expression of NEUROD1 with betacellulin treatment induced high levels of insulin production and ameliorated hyperglycemia in STZ-diabetic mice. Hepatic regeneration following 70% hepatectomy was also demonstrated to improve the effect of PDX1 by accelerating the transdifferentiation process toward the pancreatic phenotype (Miyatsuka et al. 2003). The improved efficiency could be due to the increased number of proliferating, and possibly multipotent, cells in the regenerating organ. The invasiveness of the proposed approach

might be circumvented by treating the mice with growth factors, such as HGF or interleukin-6, as their levels increase during liver regeneration, which may promote the PDX1-induced process.

## 9.3 Conversion of Bone-Marrow-Derived Mesenchymal Stem Cells into Pancreas Cells

Bone marrow (BM) is a source of both hematopoietic stem cells and mesenchymal stem cells (MSC). MSC reside in the stromal fraction of the BM, which provides the cellular microenvironment that supports hematopoeisis. MSC are multipotent and under appropriate experimental conditions can differentiate into lineages of mesenchymal tissues, such as bone, cartilage, fat, tendon, and muscle (Krause et al. 2001; Jiang et al. 2002). They are also thought to be able to differentiate into endodermal and ectodermal cells, such as vascular endothelial cells, neurons, lung cells, and hepatocytes, although this remains controversial (Fausto 2004; Ong et al. 2006). Based on their ability to adhere to plastic supports, MSC can be isolated from BM and expanded in culture. These cells were suggested as an attractive source for the generation of surrogate β cells (Chen et al. 2004; Moriscot et al. 2005)

A number of studies have analyzed whether BM-derived MSC can differentiate into insulin-producing cells and restore normal β-cell function both in vivo and in vitro (Ianus et al. 2003; Tang et al. 2004). In both cases it was suggested that BM-derived MSC differentiated along the insulin-producing cell lineage and ameliorated hyperglycemia in STZ-induced diabetic mice. However, other studies have failed to show a similar differentiation process (Choi et al. 2003; Kojima et al. 2004; Lechner et al. 2004) and have suggested that the donor BM-derived cells may have promoted endogenous β-cell regeneration (Hess et al. 2003; Mathews et al. 2004). Although some insulin-positive BM-derived cells were present in sections of STZ-damaged pancreatic tissue, their relative scarcity and the lack of PDX1 expression suggested that the BM-derived cells did not directly differentiate into insulin-producing cells. However, since recruitment of MSC to the damaged pancreas is rather inefficient in restoring β-cell function, other studies have taken a genetic manipulation approach for the generation of insulin-producing cells from MSC (Karnieli et al. 2007; Li et al. 2007). Expression of PDX1 in BM-derived MSC using lentivirus or adenovirus vectors resulted in activation of an islet-cell phenotype in these cells. PDX1 expression activated the expression of multiple islet genes, including insulin. A significant insulin content (1% of the content of normal human islets), as well as glucose-stimulated insulin release, were demonstrated in vitro (Karnieli et al. 2007), and insulin secretion was stimulated by incubating the cells in the presence of GLP1 (Li et al. 2007). Cell transplantation into STZ-diabetic immunodeficient mice resulted in further differentiation; euglycemia was obtained within 2 weeks and maintained for a prolonged period of time (Karnieli et al. 2007). Glucose tolerance tests showed a response to glucose load with a clearance rate that was parallel

to that of healthy mice. These data suggest that BM-derived MSC may serve as a cell source for generating functional insulin-producing cells for the treatment of diabetes.

## 9.4 From the Bench to Bedside: Challenges in Using Adult Cell Reprogramming in Regenerative Medicine

Adult cell reprogramming involves a number of problems that must be solved before it can be employed therapeutically:

1. The amount of insulin produced in reprogrammed adult cells is less than that of pancreatic β cells; therefore ways have to be developed for increasing insulin production in transdifferentiated liver and MSC.
2. The β-cell niche in vivo is known to play a crucial role in the final stages of β-cell differentiation. Therefore forced transdifferentiation in vitro or in a non-pancreatic adult organ may be incomplete without signals provided by this niche. Ways must be found to replace these signals to improve the phenotype of the transdifferentiated cells.
3. We do not yet know whether the reprogrammed cells proliferate and if the newly acquired phenotype can be transmitted to daughter cells.
4. Liver cell predisposition to transdifferentiation is still an enigma. It is possible that more than one cell population in the liver (and in extraendodermal tissues) could serve as a pancreatic progenitor. Moreover, the process could be driven by stochastic incidences related to chromatin compaction and promoter accessibility. Indeed, chemical compounds that modulate chromatin structure and DNA methylation, such as 5-azacytidine, may potentiate the transcription-factor-induced process.
5. It is not clear whether combined ectopic expression of several pancreatic transcription factors is more efficient in inducing reprogramming toward the pancreatic lineage, but it seems that PDX1 may prove indispensable in this process.
6. It is believed that the reprogramming process occurs mainly between developmentally-related tissues, such as liver and pancreas. It has yet to be determined whether a similar process also occurs between developmentally-unrelated tissues.

Notwithstanding these issues, adult cell reprogramming may allow the generation of autologous tissues in need, thus overcoming both the limited supply of transplantable tissues from cadaver donors and the requirement for life-long immunosuppression for preventing allograft rejection. In the case of cell replacement therapy for diabetes, the diabetic patient himself may be the donor of his own therapeutic tissue (Fig. 9.4).

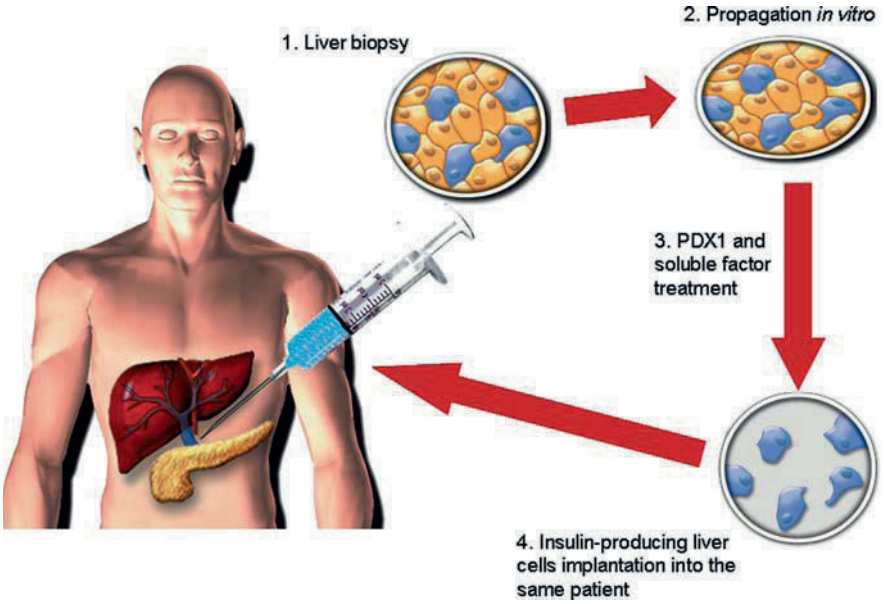



**Fig. 9.4 Schematic representation of a possible therapeutic approach involving implantation of autologous liver cells transdifferentiated in vitro**. Adult human liver cells can be obtained by liver biopsy from a patient with diabetes (**1**); expanded in vitro (**2**); treated by expression of PDX1 and incubation with growth and differentiation factors, which convert them into functional insulin-producing surrogate β cells (**3**); and transplanted into the same patient (**4**)

## References

Andrali SS, Sampley ML, Vanderford NL et al. (2008) Glucose regulation of insulin gene expression in pancreatic beta-cells. Biochem J 415:1–10

Baeyens L, De Breuck S, Lardon J et al. (2005) In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. Diabetologia 48:49–57

Becker TC, Noel RJ, Coats WS et al. (1994) Use of recombinant adenovirus for metabolic engineering of mammalian cells. Methods Cell Biol 43:161–189

Ber I, Shternhall K, Perl S et al. (2003) Functional, persistent, and extended liver to pancreas transdifferentiation. J Biol Chem 278:31950–31957

Bernardo AS, Hay CW, Docherty K (2008) Pancreatic transcription factors and their role in the birth, life and survival of the pancreatic beta cell. Mol Cell Endocrinol 294:1–9

Bonner-Weir S, Inada A, Yatoh S et al. (2008) Transdifferentiation of pancreatic ductal cells to endocrine beta-cells. Biochem Soc Trans 36:353–356

Boukamp P, Chen J, Gonzales F et al. (1992) Progressive stages of "transdifferentiation" from epidermal to mesenchymal phenotype induced by MyoD1 transfection, 5-aza-2′-deoxycytidine treatment, and selection for reduced cell attachment in the human keratinocyte line HaCaT. J Cell Biol 116:1257–1271

Cao LZ, Tang DQ, Horb ME et al. (2004) High glucose is necessary for complete maturation of Pdx1-VP16-expressing hepatic cells into functional insulin-producing cells. Diabetes 53: 3168–3178

Chen LB, Jiang XB, Yang L (2004) Differentiation of rat marrow mesenchymal stem cells into pancreatic islet beta-cells. World J Gastroenterol 10:3016–3020

Choi JB, Uchino H, Azuma K et al. (2003) Little evidence of transdifferentiation of bone marrow-derived cells into pancreatic beta cells. Diabetologia 46:1366–13674

Demeterco C, Beattie GM, Dib SA et al. (2000) A role for activin A and betacellulin in human fetal pancreatic cell differentiation and growth. J Clin Endocrinol Metab 85:3892–3897

Desmet VJ (2001) Organization principles. In: Arias IM, Boyer JL, Chisari FV et al. (eds) The Liver: Biology and Pathobiology, Lippincott, Williams and Wilkins

Deutsch G, Jung J, Zheng M et al. (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128:871–881

Dutta S, Gannon M, Peer B et al. (2001) PDX:PBX complexes are required for normal proliferation of pancreatic cells during development. Proc Natl Acad Sci USA 98:1065–1070

Eberhard D, Tosh D (2008) Transdifferentiation and metaplasia as a paradigm for understanding development and disease. Cell Mol Life Sci 65:33–40

Edlund H (2002) Pancreatic organogenesis—developmental mechanisms and implications for therapy. Nat Rev Genet 3:524–532

Fausto N (2004) Hepatic differentiation potential of commercially available human mesenchymal stem cells. Tissue Eng 12:3477–3485

Ferber S (2000) Can we create new organs from our own tissues? Isr Med Assoc J 2:32–36

Ferber S, Halkin A, Cohen H et al. (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 6:568–572

Fodor A, Harel C, Fodor L et al. (2007) Adult rat liver cells transdifferentiated with lentiviral IPF1 vectors reverse diabetes in mice: an ex vivo gene therapy approach. Diabetologia 50:121–130

Hall DB, Struhl K (2002) The VP16 activation domain interacts with multiple transcriptional components as determined by protein-protein cross-linking in vivo. J Biol Chem 277:46043–46050

Heavey B, Charalambous C, Cobaleda C et al. (2003) Myeloid lineage switch of Pax5 mutant but not wild-type B cell progenitors by C/EBPalpha and GATA factors. EMBO J 22:3887–3897

Hess D, Li L, Martin M et al. (2003) Bone marrow-derived stem cells initiate pancreatic regeneration. Nat Biotechnol 21:63–70

Horb ME, Shen CN, Tosh D et al. (2003) Experimental conversion of liver to pancreas. Curr Biol 13:105–115

Ianus A, Holz GG, Theise ND et al. (2003) In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 111:843–850

Imai J, Katagiri H, Yamada T et al. (2005) Constitutively active PDX1 induced efficient insulin production in adult murine liver. Biochem Biophys Res Commun 326:402–409

Ito M, Hayashi T, Kuroiwa A et al. (1999) Lens formation by pigmented epithelial cell reaggregate from dorsal iris implanted into limb blastema in the adult newt. Dev Growth Differ 41: 429–440

Jiang Y, Jahagirdar BN, Reinhardt RL et al. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49

Jin CX, Li WL, Xu F et al. (2007) Conversion of immortal liver progenitor cells into pancreatic endocrine progenitor cells by persistent expression of PDX1. J Cell Biochem 104:224–236

Jonsson J, Carlsson L, Edlund T et al. (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371:606–609

Kaneto H, Matsuoka TA, Nakatani Y et al. (2005a) A crucial role of MafA as a novel therapeutic target for diabetes. J Biol Chem 280:15047–15052

Kaneto H, Nakatani Y, Miyatsuka T et al .(2005b) PDX1/VP16 fusion protein, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. Diabetes 54:1009–1022

Kaneto H, Matsuoka TA, Miyatsuka T et al. (2008) PDX1 functions as a master factor in the pancreas. Front Biosci 13:6406–6420

Karnieli O, Izhar-Prato Y, Bulvik S et al. (2007) Generation of insulin-producing cells from human bone marrow mesenchymal stem cells by genetic manipulation. Stem Cells 25: 2837–2844

Kawasaki H, Mizuguchi T, Kikkawa Y et al. (2008) In vitro transformation of adult rat hepatic progenitor cells into pancreatic endocrine hormone-producing cells. J Hepatobiliary Pancreat Surg 15:310–317

Kito H, Ose Y, Mizuhira V et al. (1982) Separation and purification of (Cd, Cu, Zn)-metallothionein in carp hepato-pancreas. Comp Biochem Physiol C 73:121–127

Koizumi M, Doi R, Toyoda E et al. (2004) Hepatic regeneration and enforced PDX1 expression accelerate transdifferentiation in liver. Surgery 136:449–457

Kojima H, Fujimiya M, Matsumura K et al. (2003) NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 9:596–603

Kojima H, Fujimiya M, Matsumura K et al. (2004) Extrapancreatic insulin-producing cells in multiple organs in diabetes. Proc Natl Acad Sci USA 101:2458–2463

Koya V, Lu S, Sun YP et al. (2008) Reversal of streptozotocin-induced diabetes in mice by cellular transduction with recombinant pancreatic transcription factor pancreatic duodenal homeobox-1: a novel protein transduction domain-based therapy. Diabetes 57:757–769

Krause DS, Theise ND, Collector MI et al. (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377

Lechner A, Yang YG, Blacken RA et al. (2004) No evidence for significant transdifferentiation of bone marrow into pancreatic beta-cells in vivo. Diabetes 53:616–623

Li WC, Horb ME, Tosh D et al. (2005) In vitro transdifferentiation of hepatoma cells into functional pancreatic cells. Mech Dev 122:835–847

Li Y, Zhang R, Qiao H et al. (2007) Generation of insulin-producing cells from PDX1 gene-modified human mesenchymal stem cells. J Cell Physiol 211:36–44

Lu S, Wang WP, Wang XF et al. (2005) Heterogeneity in predisposition of hepatic cells to be induced into pancreatic endocrine cells by PDX1. World J Gastroenterol 11: 2277–2282

Lundstrom K (2003) Latest development in viral vectors for gene therapy. Trends Biotechnol 21:117–122

Makarev E, Call MK, Grogg MW et al. (2007) Gene expression signatures in the newt irises during lens regeneration. FEBS Lett 581:1865–1870

Mathews V, Hanson PT, Ford E et al. (2004) Recruitment of bone marrow-derived endothelial cells to sites of pancreatic beta-cell injury. Diabetes 53:91–98

Meivar-Levy I, and Ferber S (2006) Regenerative medicine: using liver to generate pancreas for treating diabetes. Isr Med Assoc J 8:430–434

Meivar-Levy I, Sapir T, Gefen-Halevi S et al. (2007) Pancreatic and duodenal homeobox gene 1 induces hepatic dedifferentiation by suppressing the expression of CCAAT/enhancer-binding protein beta. Hepatology 46:898–905

Melloul D, Marshak S, Cerasi E (2002) Regulation of insulin gene transcription. Diabetologia 45:309–326

Memedula S, Belmont AS (2003) Sequential recruitment of HAT and SWI/SNF components to condensed chromatin by VP16. Curr Biol 13:241–246

Miller CP, McGehee RE Jr, Habener JF (1994) IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. Embo J 13:1145–1156

Minami K, Okuno M, Miyawaki K et al. (2005) Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. Proc Natl Acad Sci USA 102:15116–15121

Miyatsuka T, Kaneto H, Kajimoto Y et al. (2003) Ectopically expressed PDX1 in liver initiates endocrine and exocrine pancreas differentiation but causes dysmorphogenesis. Biochem Biophys Res Commun 310:1017–1025

Moriscot C, de Fraipont F, Richard MJ et al. (2005) Human bone marrow mesenchymal stem cells can express insulin and key transcription factors of the endocrine pancreas developmental pathway upon genetic and/or microenvironmental manipulation in vitro. Stem Cells 23: 594–603

Nakajima-Nagata N, Sakurai T, Mitaka T et al. (2004) In vitro induction of adult hepatic progenitor cells into insulin-producing cells. Biochem Biophys Res Commun 318:625–630
Newsome PN, Johannessen I, Boyle S et al. (2003) Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. Gastroenterology 124:1891–1900
Offield MF, Jetton TL, Labosky PA et al. (1996) PDX1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development 122:983–985
Ong SY, Dai H, Leong KW (2006) Hepatic differentiation potential of commercially available human mesenchymal stem cells. Tissue Eng 12:3477–3485
Otsuka M, Hoshida Y, Kato N et al. (2003) Liver chip and gene shaving. J Gastroenterol 38:89–92
Pearl EJ, Horb ME (2008) Promoting ectopic pancreatic fates: pancreas development and future diabetes therapies. Clin Genet 74:316–324
Rao MS, Reddy JK (1995) Hepatic transdifferentiation in the pancreas. Semin Cell Biol 6: 151–156
Sapir T, Shternhall K, Meivar-Levy I et al. (2005) From the cover: cell-replacement therapy for diabetes: generating functional insulin-producing tissue from adult human liver cells. Proc Natl Acad Sci USA 102:7964–7969
Schnedl WJ, Ferber S, Johnson JH et al. (1994) STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes 43:1326–1333
Shen CN, Slack JM, Tosh D (2000) Molecular basis of transdifferentiation of pancreas to liver. Nat Cell Biol 2:879–888
Shternhall-Ron K, Quintana FJ, Perl S et al. (2007) Ectopic PDX1 expression in liver ameliorates type 1 diabetes. J Autoimmun 28:134–142
Slack JM, Tosh D (2001) Transdifferentiation and metaplasia—switching cell types. Curr Opin Genet Dev 11:581–586
Song YD, Lee EJ, Yashar P et al. (2007) Islet cell differentiation in liver by combinatorial expression of transcription factors neurogenin-3, BETA2, and RIPE3b1. Biochem Biophys Res Commun 354:334–339
St-Onge L, Wehr R, Gruss P (1999) Pancreas development and diabetes. Curr Opin Genet Dev 9:295–300
Stoffers DA, Ferrer J, Clarke WL et al. (1997) Early onset type-II diabetes mellitus (MODY 4) linked to IPF1. Nat Genet 17:138–139
Tabiin MT, Tuch BE, Bai L et al. (2001) Susceptibility of insulin-secreting hepatocytes to the toxicity of pro-inflammatory cytokines. J Autoimmun 17:229–242
Tang DQ, Cao LZ, Burkhardt BR et al. (2004) In vivo and in vitro characterization of insulin-producing cells obtained from murine bone marrow. Diabetes 53:1721–1732
Tang DQ, Cao LZ, Chou W et al. (2006a) Role of Pax4 in Pdx1-VP16-mediated liver-to-endocrine pancreas transdifferentiation. Lab Invest. 86:829–841
Tang DQ, Lu S, Sun YP et al. (2006b) Reprogramming liver-stem WB cells into functional insulin-producing cells by persistent expression of Pdx1- and Pdx1-VP16 mediated by lentiviral vectors. Lab Invest 86:83–93
Tarpin M, Gehring WJ, Bierne J (2002) Conversion of a postocellar into an ocellar region as a transdetermination event occurring in adult ribbonworms. Mech Dev 118:39–44
Thorgeirsson SS (1996) Hepatic stem cells in liver regeneration. Faseb J 10:1249–1256
Tsonis PA (2007) Regeneration via transdifferentiation: the lens and hair cells. Hear Res 227:28–31
Tumbar T, Sudlow G, and Belmont AS (1999) Large-scale chromatin unfolding and remodeling induced by VP16 acidic activation domain. J Cell Biol 145:1341–1354
Waeber G, Thompson N, Nicod P et al. (1996) Transcriptional activation of the GLUT2 gene by the IPF-1/STF-1/IDX-1 homeobox factor. Mol Endocrinol 10:1327–1334
Walther W, Stein U (2000) Viral vectors for gene transfer: a review of their use in the treatment of human diseases. Drugs 60:249–271
Wang AY, Ehrhardt A, Xu H et al. (2007) Adenovirus transduction is rquired for the correction of diabetes using PDX1 or neurogenin-3 in the liver. Mol Ther 15:255–263

Watada H, Kajimoto Y, Miyagawa J et al. (1996a) PDX1 induces insulin and glucokinase gene expressions in alphaTC1 clone 6 cells in the presence of betacellulin. Diabetes 45:1826–1831

Watada H, Kajimoto Y, Umayahara Y et al. (1996b) The human glucokinase gene beta-cell-type promoter: an essential role of insulin promoter factor 1/PDX1 in its activation in HIT-T15 cells. Diabetes 45:1478–1488

Weintraub H, Tapscott SJ, Davis RL et al. (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci USA 86:5434–5438

Yamada S, Yamamoto Y, Nagasawa M et al. (2006) In vitro transdifferentiation of mature hepatocytes into insulin-producing cells. Endocr J 53:789–795

Yang H, Morrison CM, Conlon JM et al. (1999) Immunocytochemical characterization of the pancreatic islet cells of the Nile Tilapia (*Oreochromis niloticus*). Gen Comp Endocrinol 114:47–56

Yang L, Li S, Hatch H et al. (2002) In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci USA 99:8078–8083

Yatoh S, Akashi T, Chan PP et al. (2006) NeuroD and reaggregation induce beta-cell specific gene expression in cultured hepatocytes. Diabetes Metab Res Rev 23:239–249

Young LS, Searle PF, Onion D et al. (2006) Viral gene therapy strategies: from basic science to clinical application. J Pathol. 208:299–318

Zalzman M, Gupta S, Giri RK et al. (2003) Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells. Proc Natl Acad Sci USA 100:7253–7258

Zalzman M, Anker-Kitai L, Efrat S (2005) Differentiation of human liver-derived, insulin-producing cells toward the beta-cell phenotype. Diabetes 54:2568–2575

Zhou Q, Melton DA (2008) Extreme makeover: converting one cell into another. Cell Stem Cell 3:382–388

Zhou Q, Brown J, Kanarek A et al. (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455:627–632

# Chapter 10
# Embryonic Stem Cells as a Potential Cure for Diabetes

**Michael A. Bukys and Jan Jensen**

**Abstract** Beta-cell replacement is an effective treatment for type 1 diabetes, but its applicability is limited by the lack of sufficient donor tissue, raising the need for alternative tissue sources. Deriving β cells from stem cell precursors offers an unlimited renewable source of tissue for transplantation and in recent years has become the focus of research in many laboratories. The unique state of embryonic stem (ES) cells is characterized by continuous proliferation through a cell cycle consisting of an abbreviated G1 phase. Although this cell cycle exposes ES cells to potential mutations, it also allows continuous culture of undifferentiated cells. Current protocols directing the differentiation of ES cells mimic the normal embryonic development of β cells through definitive endoderm, foregut endoderm, pancreatic precursors, and endocrine progenitor cells. At present all of these steps are suboptimal, since only some of the cells follow this pathway to the intended product. Moral concerns surrounding the use of embryonic stem cells has led to development of alternative sources of pluripotent cells. Current advances in cellular reprogramming are discussed.

## 10.1 Introduction

It is estimated that approximately 7% of the population of North America suffers from diabetes and that over a quarter of the population is classified as prediabetic owing to elevated blood glucose. Diabetes is ranked as the seventh leading cause of death in America; however, this is a misleading underestimation because diabetes-related deaths are often not attributed to diabetes, but to the complications that arise from diabetes. One of the alarming facts about diabetes is the wide range of complications associated with the ailment. Heart, kidney, and nervous system diseases are all known risk factors associated with diabetes, which is also the leading cause of blindness and lower limb amputation in adults.

M.A. Bukys (✉)
Department of Stem Cell Biology and Regenerative Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH, 44195, USA
e-mail: bukysm@ccf.org

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_10,

All forms of diabetes are characterized by the body's inability to properly metabolize glucose, resulting in elevated blood glucose concentrations, which is a direct result of the body's failure to produce or properly use insulin. There is no cure for diabetes, and treatment usually consists of continued insulin replacement for the duration of the individual's life. An alternative to insulin replacement that has been shown to treat diabetes successfully is cadaverous islet transplantation. This procedure replaces the cells that produce insulin, but it relies on immune suppression to allow the transplanted tissue to survive and is limited by the availability of suitable tissue donors. The possibility that pancreatic β cells can be generated from human embryonic stem (hES) cells has received much attention in the last few years because it promises a renewable source of tissue for transplantation. This chapter focuses on recent attempts to produce β cells from pluripotent ES cells, explores the potential problems with current protocols, and discusses the technical barriers that have to be overcome before ES-cell-derived cells can be used for human therapy.

## 10.2 The Embryonic Stem Cell State Is Unique

ES cells are slowly dividing cells retaining full pluripotency, which are derived from the inner cell mass of a developing embryo (Thomson et al. 1998). They can be expanded in culture and appear to be immune to cellular senescence. In this regard, ES cells resemble immortalized cells, and it appears that the pluripotency network operating in ES cells is one that both facilitates and maintains this characteristic. A limited number of genes can induce this state. Yamanaka and colleagues were the first to report that a limited set of gene regulatory factors (OCT4, SOX2, KLF4, cMYC) was sufficient to reprogram a somatic cell to a state indistinguishable from the hES cell state. Such cells were termed induced pluripotent stem cells (iPS cells) (Takahashi and Yamanaka 2006). In the remainder of this review, any references to work and procedures used for hES cells are expected to be equally valid for iPS cells, and some data have already provided conceptual support for this notion (Zhang et al. 2009). Figure 10.1 illustrates three different methods that are currently being considered for cell therapy of diabetes.

It is important to first recognize that the ES cell state differs from most other, if not all, stem cell states. Numerous studies have sought to establish common aspects of various cells with stem cell properties (Ivanova et al. 2002; Ramalho-Santos et al. 2002), but such common "stemness" genes, or stem-cell-specific pathways, have not been identified—rather they have been disproved (Fortunel et al. 2003). A recent study based on unsupervised clustering of gene expression profiles of multiple stem cell types identified a unique gene expression program that operated in undifferentiated ES cells, but not in other stem cells, for example, cells of mesenchymal or neuronal origin. The gene network, referred to as the "Plurinet," includes the well-known pluripotency-associated genes, such as those used for iPS cell induction, but extends quite further to more than 200 individual genes (Muller et al. 2008). Future work should address which of these genes is causal for maintenance

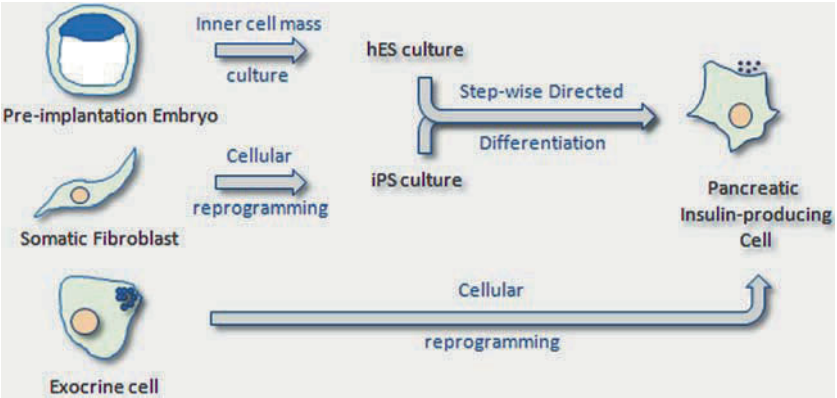



**Fig. 10.1 Derivation of pancreatic insulin-producing cells through directed differentiation, cellular reprogramming, or both.** Derivation of iPS-type cells from cellular reprogramming would lead to cultures that resemble typical embryonic stem cells that are derived from fertilized human embryos. The route to a more mature insulin-producing cell entails a stepwise instructional provision of guidance molecules and conditions. It is expected that both iPS and hES cell cultures would be responding similarly to such fate-inducing signals, as their initial state is remarkably similar. However, recent data have shown that cellular reprogramming to full pluripotency may not be needed. Conversion of mature exocrine cells into cells indistinguishable from adult insulin-producing cells was observed following administration of three critical endocrine regulatory factors. Such adult cell reprogramming may involve chromatin remodeling similar to that occurring through embryonic reprogramming, except that the loci controlled are specific to the newly created state. Evidence of this is still lacking, and further work will require a better understanding of the chromatin-modifying capacities of the adult endocrine reprogramming factors (PDX1, NGN3, MAFA). At the moment, little is known of such properties for any of these proteins

of the pluripotency state and which are simply reflections thereof. It seems possible that reprogramming of somatic cells may be achieved if sufficient knowledge of the cell extrinsic control of the aforementioned iPS-inducing genes is available. If successful, this could lead to a much less invasive genetic reprogramming of adult somatic cells.

The embryonic stem cell state is normally a transient one, as inner cell mass cells differentiate as the embryo gastrulates. Irrespective of organism, loss of pluripotency is inevitably linked to the creation of body patterns and the ensuing degree of differentiation imposed. This temporal state is artificially maintained ex vivo, using methods pioneered in mice for gene targeting through homologous recombination. Human and mouse ES cells differ in their growth factor requirements, notably because maintenance of pluripotent mES cells requires the presence of leukemia inhibitory factor (LIF), which activates the Janus kinase/signaling activator of transcription (JAK/STAT) pathway to mimic delayed implantation. Nevertheless, much of the information concerning mouse and human ES cell culture is interchangeable. In both cases it is preferable to maintain the pluripotent state using irradiated or mitomycin C-treated mesodermally-derived fibroblast feeders, which provide a suitable environment of growth factors, including basic fibroblast growth factor (bFGF). A

feeder-free medium has been developed that is equally capable of sustaining the pluripotent state. The identification of such media is also beneficial in terms of developing differentiation conditions for ES cells (Xu et al. 2001; Xu et al. 2005; Ludwig et al. 2006).

## 10.3 Embryonic Stem Cells Are in a Constitutively Proliferative State

The unique properties of ES cells include regulation of their cell cycle progression. Specifically, ES cells lack critical cell cycle control elements found in other dividing cells; p53, which normally acts as a DNA damage-sensing checkpoint capable of arresting somatic cells in G1 or G2, does not function in this manner in ES cells (Aladjem et al. 1998; Prost et al. 1998). It is incapable of activating cyclin-dependent kinase (CDK) inhibitors, such as p21, and is not translocated to the nucleus in response to DNA damage (Aladjem et al. 1998; Prost et al. 1998). Interestingly, the somatic-type control of cell cycle, which involves fluctuations of cyclins, is not the same in ES cells, although more cell cycle variation of cyclin-family genes has been noted in hES, as compared to mouse ES cells (Neganova et al. 2009). Except for cyclin B, which is expressed during G2/M in ES cells, most other cyclins, notably the A, D, and E types, are constitutively expressed and are not controlled by retinoblastoma (Rb) protein phosphorylation events, which control E2F release and activation (Jirmanova et al. 2002). Cyclin D levels are very low in ES cells, and CDK4 activity is minimal. This kinase controls Rb phosphorylation in somatic cells, thereby driving proliferation, but this step is not required in ES cells, and all three Rb-family members are dispensable for ES cell mitotic activity (Dannenberg et al. 2000; Sage et al. 2000).

A current interpretation of the cell cycle in ES cells is based on the notion of a very shortened G1 phase. This short G1 phase, however, can be significantly extended following DNA damage in an E2F-independent manner (Becker et al. 2007), and it has been argued that during the G1 window hES cells are susceptible to differentiation cues. Upon differentiation, the G1 phase is significantly extended (White et al. 2005), and the "normal" somatic cell cycle control elements become active. Moreover, most somatic cells require growth factor input to progress through the G1/S checkpoint. This commonly involves signaling through the mitogen-activated protein kinase (MAPK) pathway, but ES cells proliferate in the absence of growth factor receptor bound protein-2 (GRB2) and serum response factor (SRF), or in the presence of mitogen-activated "extracellular signal-regulated kinase" kinase (MEK) inhibitors (Cheng et al. 1998). In contrast, progression through the G1/S checkpoint in ES cells requires signaling through the phosphoinositide 3-kinase (PI3K) pathway. This pathway may be cell-intrinsically linked to the pluripotent cell state through ES cell-expressed Ras (ERAS) (Jirmanova et al. 2002). Mutations in this pathway (in p85a, a PI3K subunit, and ERAS) decrease the rate of ES cell proliferation, or they may increase it, as observed in phosphatase and tensin homologue (PTEN)-mutant cells (PTEN operates to reduce inositol 1,4,5 triphosphate [IP3]

levels) (Sun et al. 1999; Hallmann et al. 2003). PI3K pharmacological inhibition likewise decreased ES cell cycling.

In short, the ES cell cycle is constitutively active, lacks DNA damage checkpoints, and uncouples G1 checkpoint control from mitotic progression. In the embryo, ES cell doubling time lasts approximately 9–11 h. These specific properties of the ES cell cycle have implications for their therapeutic potential. ES cells may be highly susceptible to DNA-damaging mutations, which is a concern if new cell lines cannot be established owing to ethical issues. Moreover, this state is highly susceptible to the presence of differentiation-inducing agents, such as retinoic acid (RA), requiring repeated evaluation of pluripotency during continuous culture of ES cells.

## 10.4 Directed Differentiation of Embryonic Stem Cells: Seeking Sanity in the Midst of Chaos

Forward or directed differentiation is the subject matter of most hES cell work related to therapeutic use of such cells. Providing a necessary and progressive set of cues for directing hES cells into lineage-specific cell fates is currently one of the main factors interfering with their immediate use. Uncontrolled forward differentiation occurs when hES cells are removed from the feeder layer. In vitro, formation of embryoid bodies ensues, which essentially is an ad-hoc adoption of multiple fates in clusters of embryonic cells. The reason for such noncategorical differentiation lies in the fact that hES cells behave according to their nature, that is, inner cell mass, and seek to progress forward to complete an organismal program—in this case in vain. In the absence of the proper environment, such as extraembryonic support, their progression leads to an out-of-control differentiation process, with no defined endpoint. In vivo, such differentiation results in teratoma formation, and the possible progression of teratoma to teratocarcinoma is a critical concern and a valid argument against the use of hES cells in regenerative medicine.

The uncontrolled forward differentiation is caused by the emergence of regulative developmental cues, which set up local signaling centers where individual embryonic stem cells receive fate-determining signals. Normally in the embryo such signaling centers, often referred to as organizers, are capable of patterning the developing embryo. The structured emergence of such signaling organizers underlies normal development. It is important to understand the nature of such signaling in the control of the directed differentiation of hES cells. Organizing centers are responsible for local secretion of short-range signaling factors, also known as morphogens, which operate in a paracrine manner (Lander 2007). The correct effects of these morphogens require strictly-defined concentrations and exact timing. Rarely, the organizing events are conveyed by a single signal; more often, combinatorial use of multiple factors is observed (Lander 2007).

As the differentiating cells may move relative to one another in the developing embryo, the temporal definition of organizer activity is often related to the speed of cell movement. Consequently, tissue and organ growth kinetics influences the

patterning in a quite distinct manner. One example of this phenomenon is the process of gastrulation, where the organizer—the node at the anterior primitive streak—signals to cells migrating from the outside to the inside of the egg cylinder to form both mesoderm and endoderm. Another example relates to resolving a liver–pancreatic binary fate choice in undifferentiated ventral endoderm through the relative position to the cardiac mesoderm and septum transversum mesoderm signaling centers (Zaret 2008; Zaret and Grompe 2008). Static impressions of developmental programs are often provided as textbook style schematic drawings of embryos at various gestational ages. As informative as such may be they often fall short in the depiction of dynamic growth and tissue movement, so noninvasive imaging techniques are of great help to developmental biologists attempting to understand signaling events in embryogenesis.

The above considerations are relevant to hES cell cultures, as cells do not move much in such cultures. Therefore, mimicking organizer activities over time becomes important, as detailed below. The crux of succeeding in directed differentiation lies in understanding normal embryogenesis, as pointed out so clearly by Murry and Keller (Murry and Keller 2008). In the context of this chapter, it is important to consider when, where, and how, ES cells receive guiding inputs so that the pancreatic β-cell fate may emerge. The work of D'Amour and colleagues (D′Amour et al. 2006) revitalized the field of creating pancreatic β cells from ES cells after a series of generally failed attempts by others. Since then, there has been a flurry of activity, and much hope is tied to success in this area. Most of this later work reflects the central theme of the "Novocell protocol," considering variations thereof. In the remainder of this perspective, it is our intention to challenge the thinking behind some of the currently established methods of directed differentiation rather than provide a detailed description of endoderm development, as that is well-described elsewhere.

## 10.5 Progress in Making Beta Cells from hES Cells

Several recent reviews on current progress in making β cells from hES cells are available (Hardikar et al. 2006; Docherty et al. 2007; Jensen 2007; Krishna et al. 2007; Spence and Wells 2007; Baetge 2008; Efrat 2008; Semb 2008; Sordi et al. 2008). The one by Baetge is particularly informative, as it compares results from 14 individual studies published on the subject (Baetge 2008). Comparison of the efficiencies of the various protocols, the degree of maturation of the insulin-producing cells derived, and the ability of those cells to restore normoglycemia, for example, in streptozotocin-treated mice, is available there in table format, so it is not discussed here. At present, the best-performing insulin-producing cells from such protocols arise after implantation of immature endocrine cells derived from a modified Novocell protocol into the epididymal fat pad using a Matrigel-conditioned gelfoam sponge (Kroon et al. 2008). Such cells appear indistinguishable from normal β cells, produce a single hormone, and display in-vivo glucose-stimulated insulin release. Data from others support the beneficial effect of an in-vivo environment

(Eshpeter et al. 2008). Unfortunately, the influence of the in-vivo environment remains unknown, but understanding it may allow its application in vitro. All the studies described follow a similar approach. Stepwise, the objective is to generate definitive endoderm (Stage 1); next, to induce foregut endoderm (Stage 2); thereafter, to obtain pancreatic commitment (Stage 3), from which endocrine precursors form and differentiate (Stage 4), and later further mature into hormone-producing cells.

Critical signaling components at the various stages appear quite limited. Stage 1: Activin A (which mimics nodal), in the absence of PI3K signaling (McLean et al. 2007), allows cells to enter a definitive endoderm stage. Stage 2: FGF10 (or FGF7, which mimics FGF10) defines foregut endoderm. Stage 3: RA is used to pattern toward pancreatic development. Stage 4: Removal of FGF10 and RA automatically triggers endocrine development. Stage 5: No specific components are known to influence β-cell formation and maturation. Most protocols operate on slight variations on above theme, and certain components that appear to be non-critical have been tested (NOTCH and sonic hedgehog inhibitors, β-cell growth factors). Such factors may be deleted from the protocol, as best described by Kroon et al., who obtained in-vivo-matured insulin-producing cells through a shortened, and much simplified, version of the original Novocell protocol (Baetge 2008; Kroon et al. 2008).

### *10.5.1 Stage 1: Definitive Endoderm Formation*

The first step toward pancreatic development requires the formation of definitive endoderm. Induction of the endodermal fate eluded researchers in the field for several years. However, work by several groups, notably those of Baetge, Keller, and Nishikawa, showed that definitive endoderm would form if hES cells were cultured in high levels of activin A (Kubo et al. 2004; D′Amour et al. 2005; Tada et al. 2005; Yasunaga et al. 2005). Activin A is a homodimer of two inhibin beta-A subunits and is not normally expressed at the gastrulation stage in the embryo. It does, however, signal through the same receptor complex as nodal, a bone morphogenetic protein (BMP)-type molecule that is expressed at high levels in the node (Gritsman et al. 2000). It was known that cells in the most proximal position to the node become endoderm upon gastrulation, so the critical involvement of nodal/activin A signaling is not surprising. The established method of endoderm induction is very effective. However, activin A is a rather crude induction signal that fails to provide information related to patterning of the endoderm. Stage 1 often involves a 2-4-day culture of hES cells in the presence of 100 ng/ml activin A. *FoxA2* is a key target of this induction, is an upstream component for most endodermal fates, and remains active in multiple endodermal regions. In fact, endogenous FOXA2 staining is commonly used to mark the entire endoderm in whole-mount visualizations of early stage embryos (Jorgensen et al. 2007; Sherwood et al. 2009).

The concern about the use of activin A relates to the issue of deviating patterning toward nonpancreatic fates and whether such an effect is already manifested at

this initial stage. If the definitive endoderm adopts nonpancreatic patterning at this step it may not be repatterned during later stages. It has been argued that definitive endoderm formation progresses through a bipotential mesendodermal population of goosecoid$^+$/brachyury$^+$/FOXA2$^+$ cells, from which a more specific definitive endodermal cell, expressing FOXA2, derives (Tada et al. 2005; Gouon-Evans et al. 2006). Activin A specifies both populations, but definitive endoderm is formed only following sustained exposure to high levels of activin A (Gadue et al. 2006). It is noteworthy that the Novocell protocol (D′Amour et al. 2006) led to formation of ectodermal and mesodermal fates, in addition to the definitive endoderm fates, which argues that the initial step of definitive endoderm formation is not fully optimized.

### *10.5.2 Stage 2: Foregut Endoderm Formation*

It has been suggested that application of FGF10 causes foregut endoderm formation, but this argument is not well sustained by embryologic evidence. There are no studies showing that FGF10 is required to pattern the anteriormost endoderm to a foregut state. This argument seems to have developed as a response to the observation that empirical testing of FGF10 led to activation of hepatocyte nuclear factor-4 (HNF4) and HNF1β, although neither of these markers is foregut-specific (D′Amour et al. 2006). Thus terming FGF10 application as "foregut determination" may not be appropriate. FGF10 is expressed in lateral mesoderm at budding stages and is involved in the specification of several endodermal derivatives, including lung, pancreas, stomach, and gut (Sekine et al. 1999; Nyeng et al. 2007).

Data suggest that pancreatic progenitors fail to maintain proliferation in the absence of FGF10 (Bhushan et al. 2001). The ventral pancreas is less affected in FGF10-null mice, as compared with the dorsal. Moreover, the pancreatic fields, arising from the distalmost foregut as two individual fields (a single dorsal and a split ventral field) come into proximity of the mesoderm only upon gut tube closure, and the ventral fields arise from endoderm in a more anterior position, where they are under the influence of signals from more anterior structures. This is clear from the pioneering work by Zaret and co-workers, which has helped to identify patterning of the bipotential hepatic–pancreatic ventral field, observing that local proximity to septum transversum mesenchyme (FGF source) and cardiac mesoderm (BMP source) resolves this field into liver (high BMP) and ventral pancreas (low BMP; FGF) (Deutsch et al. 2001; Zaret 2008; Zaret and Grompe 2008). It is unclear if the current protocol using FGF10 initially prepatterns the endoderm toward a ventral or a dorsal pancreatic fate, but in the absence of RA in Stage 2, it is possible that lack of posterior-inducing signals may lead preferentially to a ventral-type pancreatic field. Whether this matters is not known, but it is interesting that no protocols seem to consider whether it is desirable to reach a dorsal, as opposed to a ventral, pancreatic state. A negative effect of ventral patterning is the possible development of early liver states, and if such a program is adopted early, it might reduce the overall efficiency at later stages.

### 10.5.3 Stage 3: Pancreatic Determination

Stage 3 involves RA application. This step results in the activation of PDX1 and several other pancreatic markers, such as HNF6 (*Oc1*). FGF10 (or FGF7) application is maintained during this step, which seems critical. RA is a classical nonprotein morphogen. Although excessively simplified, RA posteriorizes early embryos and acts in a 5′-to-3′ sequence in activating the colinear *Hox* gene clusters. Applying RA to the Stage 2 endodermal cells may lead to their posteriorization and the consequent emergence of more caudal endodermal fates. These could include dorsal pancreas, stomach, and intestine, and may shift the culture away from a more anteriorized state. Excessive caudalization would be a concern, but could be traced by expression of CDX or ISX, which are intestinal markers. Although the *Hox* genes appear to be much less expressed in endoderm, as compared to mesoderm, there could be a direct influence on their expression at this point. In addition, a local RA signaling center normally emerges in the dorsal pancreatic region, where RALDH2, expressed in adjacent mesoderm, helps sustain dorsal pancreatic growth. This signaling center operates concomitantly with the expression of FGF10 in the same tissue, and expression of FGF10 and RALDH2 may be cross-regulated in this mesodermal segment.

Waiting to administer RA until after an effect by FGF10 has been established may not be beneficial. Shim et al. used simultaneous administration of RA and activin A during embryoid body formation in serum-free conditions. Although PDX1 was activated by activin A alone, in contrast to the work of D'Amour et al., a clear positive effect was noted in levels of both FOXA2 and PDX1 when both components were administered (Shim et al. 2007). Concomitantly, a loss of brachyury (T) expression was noted, and SOX1 was reduced. This loss of mesendodermal and neuroectodermal marker expression is noteworthy, as both fates are highly unwanted in the culture at any point. Even more striking, expression of alpha-fetoprotein (AFP), a liver marker, was abrogated. As in the work of Shim et al., Jiang and colleagues administered RA and activin A to H1 hES cells and observed very similar effects, where PDX1, HLXB9, and HNF4 were much more strongly induced in the presence of both components, as compared to activin A alone (Jiang et al. 2007b). These examples illustrate that significant optimization of the widely used protocol is still needed.

### 10.5.4 Stage 4 and Beyond: Islet Cell Maturation

Differentiation toward the endocrine lineages occurs when FGF10 is removed. FGF10 is capable of arresting normal pancreatic progenitors and does so via canonical MAPK signaling and maintenance of NOTCH signaling. It has been suggested that lack of stimulation of FGFR2, which is expressed on the surface of pancreatic progenitor cells, leads to a loss of HES1 expression and a possible activation of NGN3, which is a proendocrine factor. Why endocrine development proceeds in such an efficient manner is not known, but a default differentiation path of pancreatic

progenitors facing a depletion of their associated mesenchyme has also been noted to cause pancreatic endocrine development and loss of the progenitor pool. It may be that adoption of nonendocrine pancreatic terminal fates, such as exocrine and ductal, requires instructive cues, and that in the absence of such cues endocrine development proceeds when the progenitor maintenance program is attenuated.

The general problem observed at this stage is one of incomplete endocrine cell maturation. Endocrine cells expressing both glucagon [or most probably glucagon-like peptide-1 (GLP1), depending on proglucagon processing by prohormone convertase (PC) 1/3, which is normally not expressed in glucagon-expressing cells] and insulin are found in large numbers (D′Amour et al. 2006; Ku et al. 2007). They do not express NKX6.1, lack MAF-type factors, and are generally glucose-unresponsive. No studies have been successful in showing an upregulation of NKX6.1, which is a transcription factor that becomes restricted to the innermost epithelial progenitor cells of the pancreas, when β cells form normally, and is also necessary for the function of mature insulin-producing cells. This lack of central epithelial patterning may partly account for the failed β-cell development. Essentially, endocrine cells forming from hES cells appear to be of the primary transition type, predominantly expressing glucagon and several mature insulin-producing cell characteristics.

Work from the Grapin-Botton laboratory elegantly showed that there are gestational windows in the mouse for determination of endocrine cell subtypes and that the window for β-cell development does not "open" until E11.5, which in the mouse is 3 full days following initial pancreatic specification (Johansson et al. 2007). Consequently in the mouse, and possibly in humans as well, the pancreas has to set up secondary patterning events to be competent for mature β-cell development. In the absence of detailed knowledge of the patterning of the secondary-transition organ little can be done to modify the directed differentiation protocols. A better understanding of the patterning of the pancreatic progenitor population into proendocrine/proexocrine regions will significantly improve the efficiency of the current directed differentiation protocols. There are multiple studies that claim beneficial effects of various secreted substances on the formation of insulin-producing cells from hES cells, including islet neogenesis-associated protein (INGAP) (Francini et al. 2009), nicotinamide/betacellulin (Cho et al. 2008), and activin B (Frandsen et al. 2007). It is difficult to judge the physiological relevance of these molecules, as there is little support for a role for these factors in the normal β-cell developmental program.

### *10.5.5 A Consideration of Time*

Most of the previous discussion has related to the specific involvement of extracellular programming factors that direct developmental fates. One parameter that is rarely considered in detail is time, which influences most developmental decisions. Time is required to allow cell growth and buildup of the protein machinery that enable cellular competence (receptors, signaling components, intrinsic gene

regulatory factors, etc.). Consequently, time is an integral parameter in the distribution equation of each morphogen, including rates of production, degradation, and diffusion. Some developmental systems have been built to measure time, as evidenced by the clock-and-wavefront model of somitogenesis, which is a fundamental requirement during somite condensation from the presomitic mesoderm. All organisms have a unique temporal trajectory during their development. In mammals, birth size is important and prolongs the gestational time, but during early stages temporal differences are contracted, so that time to complete gastrulation is not proportional to the final difference in total organism size. Time is an important parameter during directed differentiation of hES cells, and consideration to "sufficient time provided" is seen in most protocols. The original Novocell protocol had a duration of 18 days, but some published studies went on for even longer periods, such as 36 days (Jiang et al. 2007a).

With the amount of time allowed for the cultures at each step, significant changes in gene expression of novel signaling factors may occur. It might be argued that such effects could be detrimental and may operate against the desired goal of the experiment. The duration of the first activin A step is typically between 2 and 6 days, that of the FGF10 induction step is often 4 days, and the length of the RA step is comparable. These durations are sufficiently long to lead to an active production of signaling components by the differentiating ES cells themselves. This local conditioning by the forward-differentiating ES cell population is a matter of concern as the culture has an intrinsic capacity to diverge to different fates and will do so over time. From published gene expression data on forward differentiation of hES cells following Stage 1, it is evident that the cells initiate expression of a wide variety of morphogens, including BMPs, FGFs, and WNTs. Their local production may be either detrimental or conducive to particular fates. Therefore, it can be argued that reducing the time between the stages can be beneficial, but we were not able to find any studies that sought to establish the minimal time required for each stage in the current protocols, so this parameter remains unknown.

## 10.6 Challenges of Directed Differentiation of ES Cells into Beta Cells

The biggest problem with current differentiation protocols is the mixed population of cell types produced in combination with the low percentage of desired cells produced. Overcoming this limitation will undoubtedly require methodologies not yet developed. However, current attempts to solve this problem should focus on improving the yield of the desired cell type (in this case β cells), followed by the purification of such cells. Improving the efficiency of these protocols is problematic as the differentiating ES-cell cultures are exposed to morphogens produced by the different cell types present in the cell cultures. This microenvironment cannot be easily controlled. In addition to the continued secretion of unknown factors in the culture, the fact that cellular heterogeneity is always present at some level within a differentiating colony further complicates attempts at growing a pure population of the desired

cell type. It is noteworthy that some molecular systems, such as NOTCH signaling operating through lateral inhibition, are intrinsically linked to segregation of two separate fates. In the case of the pancreas, NOTCH signaling is critical for proper cell differentiation, but it is not known to what extent that particular mechanistic event complicates matters in hES cultures in which lateral inhibition, as we know it from the developing organ, may not operate.

The problems involved in the continued heterogeneity of the ES cell culture may be compounded by the defined stages of the differentiation protocol. Those cells that fail to respond to the cue in Stage 1, when exposed to the cue in Stage 2 may behave in a different way than those cells that actually responded to the initial signal. Such a problem is not evident until Stage 3, and identifying where the problem originated is not straightforward. This compounding effect may create a more diverse population of cell types, and the diversity may increase with each additional step in the protocol.

Attempts at improving existing protocols should concentrate on the occurrence of unwanted factors during the five-step protocol. Inhibition of factors responsible for commonly-occurring divergent cell types could in theory effectively keep differentiation directed correctly. Furthermore, a negative selection component to differentiation protocols could decrease the influence of the secreted factors produced by the cultured cells. It is important to remember that the intrinsic tendency of ES cells is to produce cell types of all lineages. Therefore, for the differentiation protocols to be successful they must effectively restrict differentiation into other fates within the cell population. Current approaches center on optimizing the generation of the desired fate at each stage and neglect the fact that that this cell type may convey instructions to neighboring cells (e.g., secreted factors from definitive endoderm are likely to instruct neighboring cells into fates other than definitive endoderm).

Moreover, it must be stressed that the most important aspect of developing a more efficient protocol for generation of β cells is a better understanding of the development events that occur in early endocrine fate decisions, including those leading to the unwanted cell fates. As noted above, the best available protocol involves final β-cell development in vivo. This attests to the fact that our current understanding of the developmental events involved in endocrine cell formation is greatly lacking. Doubtlessly, as our understanding of the developmental events governing endocrine commitment advances, our ability to replicate it in vitro will greatly improve.

It should also be noted that development in vivo takes place in three dimensions, with signaling from all of the surrounding tissues and from extracellular matrix. ES cell differentiation in vitro limits the signaling to the factors produced by cells present in the culture and those supplementing the medium. Our method for getting closer to in-vivo conditions has involved taking a systematic approach toward understanding the organ-building gene networks operating in the pancreas. Intercellular patterning factors expressed in the pancreas were identified using a bioinformatics-driven stratification method. Their functions are currently being tested using conditional gain-of-function expression in mice, followed by genomics-based readout assays to assess target gene networks regulated by each

factor. Although these analyses are still ongoing, it is already clear that almost any factor analyzed has a specific and unique role in particular aspects of organ patterning. This approach will contribute to our understanding of all cellular compartments in the developing pancreas, including that of the insulin-producing cells. We hope that this information will help to optimize approaches for directed differentiation of hES in vitro.

## 10.7 Other Issues that Have to be Resolved before Transplantation

As challenging as the directed differentiation of ES cells toward a β-cell fate may be, there are additional technical issues specific to β-cell transplantation that have to be addressed. A Major treament barrier is overcoming any immune response against ES derived β-cells. Current treatment of type 1 diabetes with islet allotransplantation requires immunosuppression to protect the transplant. Transplantation of engineered β cells would require similar immunosuppression, unless a method can be developed that enables cells to be recognized as self. The two ways explored at present involve generation of patient-specific ES cell lines by nuclear transfer or the use of iPS cells.

Since iPS cells are produced using viral vectors to deliver the reprogramming genes, insertional mutagenesis can occur, and this tissue may hinder their approval for clinical use. Methods of inducing the pluripotent state without viruses would greatly strengthen the case for iPS cells, but to date other means of delivering the reprogramming genes are far less efficient. Reprogramming is quite inefficient at present, and the pluripotency-inducing genes may have to be shut off to allow cell differentiation. However, we can expect that these technical difficulties will eventually be overcome.

Nuclear transfer (NT) consists of removing the genetic material from an unfertilized egg and replacing it with the genome derived from a donor somatic cell. Development of this pseudofertilized egg is allowed to proceed to a blastocyst stage, at which time the inner cell mass is harvested to produce an ES cell line containing the genome of the donor cell. NT-derived mouse ES cells have recently been shown to generate insulin-producing cells (Jiang et al. 2008). The benefits of nuclear transfer over iPS are that no viral vectors are used and that true ES cells are generated. The downside of this methodology is the need for eggs and the ethical concerns of destroying the embryo created in the process. An additional drawback of both NT and iPS is the time needed to grow these cultures, which may be several months.

An alternative to these methods is altering ES cell lines to escape recognition by the immune system. Rejection of grafted tissue most commonly occurs through recognition of foreign major histocompatibility complex (MHC) antigens present on the cellular surface of the implanted tissue. To escape an immune response, hES-derived cells would have to express the same MHC antigens as the patient or lack them completely. Banking large numbers of hES cell lines could improve compatibility. Alternatively, manipulating hES cells to eliminate MHC antigen expression

may generate a universal donor hES cell line. Beyond these difficulties, it is possible that using β cells with the same genotype as the patient will be of little benefit if such cells will elicit a more efficient recurring autoimmunity than allograft β cells.

Another challenge of ES cells is their pluripotent state, which may persist in cells that fail to differentiate. It is well established that cultured ES cells form teratomas upon transplantation, an ability routinely exploited for assessing the pluripotency of ES cell lines. Since differentiation protocols seldom achieve complete differentiation, cells in the pluripotent state are maintained, and the possibility that teratomas will form is a legitimate concern. Removal of undifferentiated ES cells from differentiated cultures is of the utmost importance for eliminating that risk. It has been established that selective ablation of proliferating cells in grafted tissues derived from ES cells successfully treats teratomas in vivo. Removing undifferentiated ES cells before implantation should preclude the possibility of teratoma formation. An alternative to the removal of undifferentiated ES cells from cultures is purification of the β cells. A good purification procedure will in effect remove undifferentiated ES cells along with any other cells that differentiate toward an undesired lineage. Purification of a single cell type from a heterogeneous population can be accomplished by fluorescence-activated cell sorting (FACS), but this methodology depends on the presence of cell-specific surface antigens. At present there are no known unique β-cell surface proteins.

The question of whether β cells derived from ES cells would function as normal β cells is difficult to answer, especially when considering that they will have to function in a diabetic environment. Thus, it would be difficult to determine whether observed phenotypic deviations, if any, result from this environment or from the incomplete differentiation of ES cell-derived β cells.

## 10.8 Adult Cellular Makeover: Somatic-to-Somatic Cell Reprogramming

Another unexpected result of the iPS technology was a cerebral spinoff in which Melton and colleagues asked whether any combination of known islet-specific transcription factors could accomplish a similar feat by remaking one differentiated cell type into another without the use of extracellular signaling. Empirical testing of a multitude of pancreatic and β-cell transcription factors delivered in vivo to the pancreas using adenoviruses identified a combination of factors that lead to generation of insulin-producing cells (Zhou et al. 2008) (Fig. 10.1). It was further shown that these cells originated from existing exocrine cells that switched their phenotype into insulin-producing cells upon expression of a minimal set of islet transcription factors, PDX1, MAFA, and NGN3. Perhaps not surprisingly in light of the knowledge of the iPS technology, such cells appeared fully reprogrammed and maintained their phenotype even following loss of the reprogramming factors because their endogenous genes were turned on.

## 10.9 Conclusion

Cell replacement therapy of diabetes using β cells generated in vitro from ES cells is likely to become a reality. However, there are several technical barriers that have to be overcome before this can occur. Proof-of-concept of creating β cells through directed differentiation of ES cells has been provided, although at present the quality and purity of these cells are insufficient for clinical use. Additional concerns center on the nature of the ES cells themselves. Can immune tolerance of these cells be achieved? What is the likelihood of teratoma formation? Are the cells derived from ES precursors truly equivalent to β cells? If not, does it matter?

Reprogramming of pancreas exocrine cells by the Melton group has changed the perspective of many biologists, as opposition to nuclear reprogramming based on belief in terminal locking of cell fate has begun to crumble. Yet, many questions remain. Which cell type can be converted into which? What does it take to convert one given cell type into another? These issues are now reshaping experimental approaches. Armed with such techniques as massive parallel sequencing to assess gene methylation status, histone modifications, and global patterns of active/inactive promoter configurations, we may expect to gain new insights into cellular reprogramming. It is quite likely that the hunt for a cell-based cure for diabetes will continue to provide significant milestones on the road to other regenerative medicine applications.

**Acknowledgments** J.J is supported by the Chicago Project, an international effort for a functional cure of diabetes. MAB is a Morgenthaler Fellow, Lerner Research institute, Cleveland Clinic.

## References

Aladjem MI, Spike BT, Rodewald LW et al (1998) ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. Curr Biol 8:145–155

Baetge EE (2008) Production of beta-cells from human embryonic stem cells. Diabetes Obes Metab 10 (Suppl 4):186–194

Becker KA, Stein JL, Lian JB et al (2007) Establishment of histone gene regulation and cell cycle checkpoint control in human embryonic stem cells. J Cell Physiol 210:517–526

Bhushan A, Itoh N, Kato S et al (2001) Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development 128: 5109–5117

Cheng AM, Saxton TM, Sakai R et al (1998) Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. Cell 95:793–803

Cho YM, Lim JM, Yoo DH et al (2008) Betacellulin and nicotinamide sustain PDX1 expression and induce pancreatic beta-cell differentiation in human embryonic stem cells. Biochem Biophys Res Commun 366:129–134

D'Amour KA, Agulnick AD ,Eliazer S et al (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol 23:1534–1541

D'Amour KA, Bang AG, Eliazer S et al (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 24:1392–1401

Dannenberg JH, van Rossum RA, Schuijff L et al (2000) Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev 14:3051–3064

Deutsch G, Jung J, Zheng M et al (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128:871–881

Docherty K, Bernardo AS, Vallier L (2007) Embryonic stem cell therapy for diabetes mellitus. Semin Cell Dev Biol 18:827–838

Efrat S (2008) Beta-cell replacement for insulin-dependent diabetes mellitus. Adv Drug Deliv Rev 60:114–123

Eshpeter A, Jiang J, Au M et al (2008) In vivo characterization of transplanted human embryonic stem cell-derived pancreatic endocrine islet cells. Cell Prolif 41:843–858

Fortunel NO, Out HH, Ng HH et al (2003) Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature." Science 302:393

Francini F, Del ZH, Massa ML et al (2009) Selective effect of INGAP-PP upon mouse embryonic stem cell differentiation toward islet cells. Regul Pept 153:43–48

Frandsen U, Porneki AD, Floridon C et al (2007) Activin B mediated induction of Pdx1 in human embryonic stem cell derived embryoid bodies. Biochem Biophys Res Commun 362:568–574

Gadue P, Huber TL, Paddison PJ et al (2006) Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proc Natl Acad Sci USA 103:16806–16811

Gouon-Evans V, Boussemart L, Gadue P et al (2006) BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. Nat Biotechnol 24:1402–1411

Gritsman K, Talbot WS, Schier AF (2000) Nodal signaling patterns the organizer. Development 127:921–932

Hallmann D, Trumper K, Trusheim H et al (2003) Altered signaling and cell cycle regulation in embryonal stem cells with a disruption of the gene for phosphoinositide 3-kinase regulatory subunit p85alpha. J Biol Chem 278:5099–5108

Hardikar AA, Lees JG, Sidhu KS et al (2006) Stem-cell therapy for diabetes cure: how close are we? Curr Stem Cell Res Ther 1:425–436

Ivanova NB, Dimos JT, Schaniel C et al (2002) A stem cell molecular signature. Science 298: 601–604

Jensen J (2007) Pathway decision-making strategies for generating pancreatic beta-cells: systems biology or hit and miss? Curr Opin Endocrinol Diabetes Obes 14:277–282

Jiang J, Au M, Lu K et al (2007a) Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells 25:1940–1953

Jiang W, Shi Y, Zhao D et al (2007b) In vitro derivation of functional insulin-producing cells from human embryonic stem cells. Cell Res 17:333–344

Jiang W, Bai Z, Zhang D et al (2008) Differentiation of mouse nuclear transfer embryonic stem cells into functional pancreatic beta cells. Diabetologia 51:1671–1679

Jirmanova L, Afanassieff M, Gobert-Gosse S et al (2002) Differential contributions of ERK and PI3-kinase to the regulation of cyclin D1 expression and to the control of the G1/S transition in mouse embryonic stem cells. Oncogene 21:5515–5528

Johansson KA, Dursun U, Jordan N et al (2007) Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. Dev Cell 12:457–465

Jorgensen MC, Ahnfelt-Ronne J, Hald J et al (2007) An illustrated review of early pancreas development in the mouse. Endocr Rev 28:685–705

Krishna KA, Rao GV, Rao KS (2007) Stem cell-based therapy for the treatment of Type 1 diabetes mellitus. Regen Med 2:171–177

Kroon E, Martinson LA, Kadoya K et al (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 26:443–452

Ku HT, Chai J, Kim YJ et al (2007) Insulin-expressing colonies developed from murine embryonic stem cell-derived progenitors. Diabetes 56:921–929

Kubo A, Shinozaki K, Shannon JM et al (2004) Development of definitive endoderm from embryonic stem cells in culture. Development 131:1651–1662

Lander AD (2007) Morpheus unbound: reimagining the morphogen gradient. Cell 128: 245–256
Ludwig TE, Levenstein ME, Jones JM et al (2006) Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24:185–187
McLean AB, D'Amour KA, Jones KL et al (2007) Activin A efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. Stem Cells 25:29–38
Muller FJ, Laurent LC, Kostka D et al (2008) Regulatory networks define phenotypic classes of human stem cell lines. Nature 455:401–405
Murry CE, Keller G (2008) Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 132:661–680
Neganova I, Zhang X, Atkinson S et al (2009) Expression and functional analysis of G1 to S regulatory components reveals an important role for CDK2 in cell cycle regulation in human embryonic stem cells. Oncogene 28:20–30
Nyeng P, Norgaard GA, Kobberup S et al (2007) FGF10 signaling controls stomach morphogenesis. Dev Biol 303:295–310
Prost S, Bellamy CO, Clarke AR et al (1998) p53-independent DNA repair and cell cycle arrest in embryonic stem cells. FEBS Lett 425:499–504
Ramalho-Santos M, Yoon S, Matsuzaki Y et al 2002. "Stemness": transcriptional profiling of embryonic and adult stem cells. Science 298:597–600
Sage J, Mulligan GJ, Attardi LD et al (2000) Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev 14:3037–3050
Sekine K, Ohuchi H, Fujiwara M et al (1999) Fgf10 is essential for limb and lung formation. Nat Genet 21:138–141
Semb H (2008) Definitive endoderm: a key step in coaxing human embryonic stem cells into transplantable beta-cells. Biochem Soc Trans 36:272–275
Sherwood RI, Chen TY, Melton DA (2009) Transcriptional dynamics of endodermal organ formation. Dev Dyn 238:29–42
Shim JH, Kim SE, Woo DH et al (2007) Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. Diabetologia 50:1228–1238
Sordi V, Bertuzzi F, Piemonti L (2008) Diabetes mellitus: an opportunity for therapy with stem cells? Regen Med 3:377–397
Spence JR, Wells JM (2007) Translational embryology: using embryonic principles to generate pancreatic endocrine cells from embryonic stem cells. Dev Dyn 236:3218–3227
Sun H, Lesche R, Li DM et al 1999. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci USA 96:6199–6204
Tada S, Era T, Furusawa C et al (2005) Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development 132:4363–4374
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
Thomson JA, Itskovitz-Eldor J, Shapiro SS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147
White J, Stead E, Faast R et al (2005) Developmental activation of the Rb-E2F pathway and establishment of cell cycle-regulated cyclin-dependent kinase activity during embryonic stem cell differentiation. Mol Biol Cell 16:2018–2027
Xu C, Inokuma MS, Denham J et al (2001) Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19:971–974
Xu C, Rosler E, Jiang J et al (2005) Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Stem Cells 23:315–323
Yasunaga M, Tada S, Torikai-Nishikawa S et al (2005) Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. Nat Biotechnol 23:1542–1550

Zaret KS (2008) Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nat Rev Genet 9:329–340
Zaret KS, Grompe M (2008) Generation and regeneration of cells of the liver and pancreas. Science 322:1490–1494
Zhang D, Jiang W, Liu M et al (2009) Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res [Epub ahead of print]
Zhou Q, Brown J, Kanarek A et al (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455:627–632

# Part III
# Tissue Engineering and Immune Protection

# Chapter 11
# Functional Tissue Reconstruction with the Use of Biologic Scaffolds

**Stephen F. Badylak, Jennifer B. Ogilvie, and Thomas W. Gilbert**

**Abstract** Biologic scaffold materials derived from the extracellular matrix (ECM) of intact mammalian tissues are recognized as possessing constructive remodeling properties and have been used successfully in a variety of tissue engineering and regenerative medicine applications in both preclinical studies and clinical practice. This chapter provides an overview of the composition and structure of selected ECM scaffold materials, the effects of manufacturing methods upon the structural properties and resulting mechanical behavior of the scaffold materials, and the in-vivo degradation and remodeling of ECM scaffolds. Finally, the potential application of ECM scaffold materials to regeneration of endocrine tissues is discussed.

## 11.1 Introduction

Biologic scaffold materials, including those composed of extracellular matrix (ECM), have been used successfully to promote the constructive remodeling of many tissue types, including musculotendinous tissues, lower urinary tract structures, cartilage, and skin (Badylak et al. 1995, Brown et al. 2002, Butler et al. 2005, Catena et al. 2005, Chen et al. 1999, Dejardin et al. 2001, Huber et al. 2003b, Kropp et al. 1996). There are currently more than 60 commercially available products composed of ECM harvested from such species as pig, cow, horse, and human. These products are derived from tissues as diverse as small intestine, urinary bladder, dermis, and pericardium. The methods for tissue decellularization, processing, and sterilization have been well-studied, and the host response to allogeneic and xenogeneic sources of these materials is the subject of investigation in many laboratories (Badylak et al. 2008, Brown et al. 2009, Derwin et al. 2006, Gilbert et al. 2006, Valentin et al. 2006, Xu et al. 2008).

The mechanisms by which ECM scaffolds facilitate the restoration of functional tissue is not completely understood, but they include the recruitment of endogenous

S.F. Badylak (✉)
Departments of Surgery and Bioengineering, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: badylaks@upmc.edu

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_11,

stem and progenitor cells to the site of remodeling (Badylak et al. 2001b, Beattie et al. 2008, Reing et al. 2009, Zantop et al. 2006), the promotion of tissue development processes by macrophages and other cells involved in the host immune response (Badylak et al. 2008, Brown et al. 2009), and the provision of an inductive microenvironmental niche by the inherent three-dimensional (3-D) structure and surface ligand landscape (Brown et al. 2006, Sellaro et al. 2007). Intuitive to the concept of site-appropriate tissue creation are the processes of cell differentiation, proliferation, and spatial organization.

Although considerable resources have been devoted to the reconstruction of nonendocrine tissues, very little attention has been focused on the endocrine tissues. Most endocrine organs have exceedingly small amounts of ECM, and relatively little is understood about the microenvironmental factors that favor and promote endocrine tissue development. Endocrine organs are typically characterized by a sinusoidal blood supply that affords intimate physical association between the circulation and the resident cell populations. This fact is very important in regenerative medicine approaches aimed at restoring endocrine tissue function, since it implies that fibrosis, inflammation, or use of a scaffold material with poor diffusion characteristics will be detrimental to success. This chapter briefly reviews the composition of biologic scaffold materials, scaffold structure, processing of scaffold materials, and the mechanisms by which such scaffold materials contribute to functional tissue regeneration.

## 11.2 Composition of Biologic Scaffold Materials

ECM scaffolds consist of the structural and functional molecules secreted by the resident cells of each tissue and organ from which they are prepared. Therefore, the specific composition and distribution of the ECM constituents varies depending on the tissue source. Endocrine tissue-derived ECM is distinct from all other ECM in both composition and 3-D ultrastructure. The ECM scaffold derived from porcine small-intestinal submucosa (SIS-ECM) is the biologic scaffold material that has been characterized most extensively and it is often cited as a prototypical ECM scaffold. By dry weight, SIS-ECM scaffold is composed of more than 90% collagen. The large majority of the collagen is type I, with minor amounts of collagen types III, IV, V, and VI also present (Badylak et al. 1995). Urinary bladder matrix (UBM-ECM) contains the same collagen types as SIS-ECM, with larger amounts of type III collagen being present, as well as collagen type VII. Type VII collagen is an important component of the epithelial basement membrane that distinguishes this particular ECM scaffold from most other ECM scaffold materials (Brown et al. 2006).

All ECM scaffold materials contain a variety of glycosaminoglycans (GAG), including heparin, heparin sulfate, chondroitin sulfate, and hyaluronic acid, in their preprocessed, native state (Hodde et al. 1996). The amount of GAGs remaining in a tissue after decellularization depends greatly on the method of decellularization; for example, the ionic detergents often used in the decellularization process can remove

GAGs from the ECM (Gilbert et al. 2006). ECM scaffolds have been shown to contain adhesion molecules such as fibronectin and laminin (Brown et al. 2006, Hodde et al. 2002), the proteoglycan decorin, and the glycoproteins biglycan and entactin. Decorin is a prominent component of pancreatic islet ECM (unpublished data). Various growth factors are also present in most ECM scaffold materials, including transforming growth factor-β (TGFβ) (McDevitt et al. 2003, Voytik-Harbin et al. 1997), basic-fibroblast growth factor (b-FGF) (Hodde et al. 2005, Voytik-Harbin et al. 1997), and vascular endothelial growth factor (VEGF) (Hodde et al. 2001). Several of these growth factors have been shown to retain their bioactivity even after terminal sterilization and long-term storage (Hodde et al. 2005, McDevitt et al. 2003).

In summary, ECM biologic scaffolds have a complex composition with a variety of diverse molecules that are perfectly suited to support the cellular processes necessary for optimal function of the tissue and organ from which they are harvested. The ability of ECM harvested from one tissue to function as a biologic scaffold material for the same or different tissue may vary. ECM scaffold for endocrine tissue restoration would likely have to be exceedingly thin to support nutrient and secreted hormone exchange between the cells and the circulation.

## 11.3 Structure of ECM Biologic Scaffold Materials

The ultrastructure and 3-D architecture of ECM scaffolds can be largely preserved through the processing steps required for decellularization of the tissue if care is taken to avoid harsh detergents (Brown et al. 2006, Gilbert et al. 2008, Sacks and Gloeckner 1999). There is morphologic evidence that scaffolds composed of ECM from specific organs retain defining structures, such as the basement membrane of the urinary bladder in UBM and the stratum compactum of the small intestine (Brown et al. 2006). Microscopic and ultrastructural features of the matrix play important roles in modulating the behavior of cells that contact the scaffold by controlling the cell's ability to migrate into the scaffold (Brown et al. 2006) or by influencing tissue-specific cell phenotype (Gong et al. 2008, Sellaro et al. 2007). For example, an intact basement membrane can largely prevent in-vitro cell penetration into the underlying matrix and foster the formation of confluent cell populations that cover the surface (Brown et al. 2006). Alternatively, an irregular fibrous surface architecture can facilitate penetration of selected cell types into the midsubstance of the ECM scaffold (Brown et al. 2006). Endocrine ECM architecture shows an expected honeycomb-like appearance that may also allow rapid cellular infiltration (Fig. 11.1). The ECM can dramatically affect the differentiation pathway of human embryonic stem cells (hESC) and selected progenitor cell populations (Gong et al. 2008, Hosokawa et al. 2007, Hosokawa et al. 2008).

The collagen fiber architecture of an ECM scaffold plays a critical role in determining its biomechanical behavior. The alignment and organization of collagen fibers are dependent on the function of the source tissue from which the ECM is derived. For example, the collagen fibers within a ligament or tendon are highly

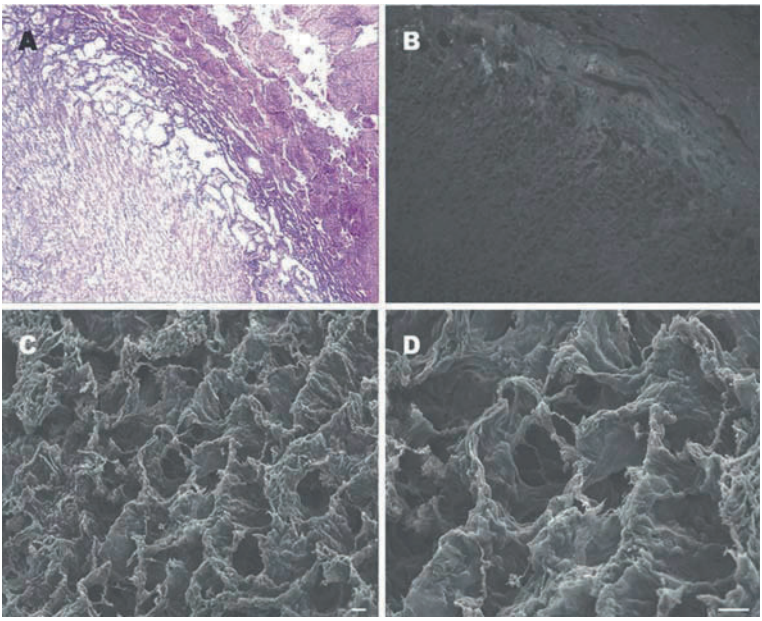



**Fig. 11.1 Decellularized porcine adrenal ECM shows complete absence of cell nuclei.** (**a**) Hematoxylin and eosin (H&E) staining, 20X. (**b**) diamidinophenylindole (DAPI) staining, 20X. (**c** and **d**) Scanning electron micrographs. Bar=10 μm

aligned in the long axis of the tissue to provide the greatest resistance to strain in a load-bearing application. Thus, the use of ECM derived from tendons and ligaments is a logical choice for repair of structures such as the anterior cruciate ligament (Cartmell and Dunn 2004, Harrison and Gratzer 2005, Woods and Gratzer 2005). Similarly, the collagen fiber architecture of endocrine organs is arranged in a pattern ideally suited for the functions of supporting clusters of endocrine cells and providing a delicate scaffold for the typically rich network of vascular sinusoids.

## 11.4 Preparation of ECM Scaffolds and Its Effects upon Structure and Function

The preparation of ECM scaffold material from the intact parent tissue requires several processing steps that can markedly affect the structure, host response to the material, and efficacy as a template for replacement tissue. The native tissue from which an ECM scaffold is prepared must be mechanically or physically separated from unwanted tissue structures, decellularized, often disinfected and dehydrated or lyophilized, and terminally sterilized. Each of these processing steps can alter the integrity and architecture of the matrix, as described above, which in turn influences the functional properties of the ECM.

### *11.4.1 Decellularization*

The effective and complete removal of antigenic epitopes associated with cell membranes and intracellular components of tissues and organs is necessary to minimize or avoid an adverse immunologic response by allogeneic and xenogeneic recipients to the ECM scaffold material. The tissues and organs from which the ECM is harvested, species of origin, and decellularization and sterilization methods can vary widely. The molecules that constitute the extracellular matrix are typically highly conserved across species lines and are tolerated well even by xenogeneic recipients (Bernard 1983a, Bernard 1983b, Constantinou and Jimenez 1991, Exposito et al. 1992). Certain antigens, such as the galactosyl alpha 1,3 galactose (gal-epitope), have been shown to be present in porcine ECM but fail to activate complement or bind IgM antibody, presumably because of the small amount and widely scattered distribution of antigen (McPherson et al. 2000, Raeder et al. 2002).

The ultimate goal of any decellularization protocol is the removal of all cellular material without adversely affecting the 3-D ultrastructure, composition, mechanical integrity, and eventual biologic activity of the remaining ECM. Commonly used methods of decellularization include a combination of physical and chemical treatments, such as sonication, agitation, and freezing and thawing. These methods disrupt cell membranes, release cell contents, and facilitate the subsequent rinsing and removal of cell remnants from the ECM. Most decellularization methods are insufficient to achieve complete decellularization, as most if not all ECM scaffold materials retain some DNA (Derwin et al. 2006, Gilbert et al. 2009).

Although it seems logical that the decellularization process will by definition affect the structure and composition of the ECM, the intent of the process is the preservation of as much as possible of the native mechanical properties and biologic properties of the original ECM. The delicate ECMs of endocrine tissues, such as pancreatic islets, thyroid, and adrenal glands, are easily disrupted by such methods and require special care during preparation. Some detergents used to facilitate decellularization have been shown to disrupt collagen of certain tissues, thereby decreasing the mechanical strength of the tissue, whereas the same detergent may have no effect on the collagen in another tissue (Cartmell and Dunn 2004, Woods and Gratzer 2005). Studies have shown that removal of GAGs from the scaffold can have a negative effect on the viscoelastic behavior of the scaffold, which is not surprising since water retention is one of the major functional characteristics of GAGs within a tissue (Lovekamp et al. 2006). Therefore, the decellularization method requires optimization for each tissue to remove cellular material without compromising the tissue's mechanical properties.

### *11.4.2 Hydration*

Very few biologic scaffold materials maintain a hydrated state throughout the decellularization and sterilization process. Avoiding water loss from the ECM can prevent changes in the tissue architecture, such as collapse of the collagen fibers upon

each other, and prevent the formation of physical bonds between ECM molecules. ECM biologic scaffolds that retain their hydrated state throughout the decellularization and sterilization process tend to support cellular attachment and cell infiltration in vitro better than scaffolds that are subjected to a dehydration step followed by rehydration (Freytes et al. 2008c). A major disadvantage of the hydrated materials, however, is the continuous leaching of soluble growth factors (such as VEGF and b-FGF) from the material during packaging and shelf life. The effect of these processing steps upon endocrine ECM scaffolds has not been examined systematically.

### *11.4.3 Dehydration*

Biologic scaffolds are often dehydrated by lyophilization (freeze drying) or by vacuum pressing prior to terminal sterilization. Dehydration tends to make the scaffolds easier to handle, limits loss of growth factors during storage, and extends the shelf life of these materials. Lyophilization, which involves the removal of water from the material by sublimation at low temperatures and pressures, is commonly used to preserve biologic graft tissues such as bone (Burchardt et al. 1978, Cornu et al. 2000, Jackson et al. 1988) and tendon (Smith et al. 1996, Toritsuka et al. 1997) and commercially available biologic scaffold materials, such as Bard® Dermal Allografts (Bard, Inc.), MatriStem™ (Acell, Inc.), and Oasis™ (Cook Biotech, Inc.). Although it has many benefits, lyophilization alters collagen fiber morphology, affects the growth of cells upon the material in vitro (Freytes et al. 2008b), and can result in a more compacted fiber morphology, which decreases the ability of the material to rehydrate at the time of use (Curtil et al. 1997, Hafeez et al. 2005).

An alternative method for dehydration of ECM scaffolds is vacuum pressing, a process that allows for the lamination of multiple sheets of ECM, which is used to increase strength and/or design in specific mechanical behavior based upon knowledge of the collagen fiber architecture. Lamination via vacuum pressing of ECM scaffolds also reduces the extensibility and changes the ultrastructural morphology of the final product (Freytes et al. 2004, Freytes et al. 2005).

Although constructive remodeling has been observed with the use of hydrated, lyophilized, and multilaminated forms of ECM scaffolds when used in vivo for tissue reconstruction (Badylak et al. 2001a, Badylak et al. 2002, Bertone et al. 2008, Gilbert et al. 2007, Huber et al. 2003a, Ringel et al. 2006, Zantop et al. 2006), ultrastructural changes that occur as a result of dehydration can affect cell attachment, cellular infiltration, and the in-vivo degradation rate (Freytes et al. 2008c). The optimal configuration and method of processing of an ECM scaffold should be determined for each clinical application. The small size and delicate fiber architecture of most endocrine organs, including pancreatic islets, suggests that hydrated forms of the endocrine ECM may be more desirable than dehydrated or lyophilized forms.

### *11.4.4 Powdered ECM Scaffolds*

Lyophilized sheets of ECM can be comminuted (powdered) into an ECM powder or particulate form (Gilbert et al. 2005). A particle form increases the surface area of the scaffold material and allows for the delivery of the ECM as a suspension or emulsion via minimally-invasive techniques (e.g., catheter-based injection) to the site of interest. The particles present in comminuted ECM retain the ultrastructural 3-D surface characteristics of the parent ECM sheet. Particle sizes ranging from 50 to 200 μm in diameter can be reproducibly manufactured, and suspensions made from a comminuted form of lyophilized UBM have been used successfully as a treatment for urinary incontinence in preclinical studies (Wood et al. 2005).

Carriers such as glycerin are typically required to increase the viscosity of a suspension of particles intended for clinical use. Acellular human dermal matrix has been investigated as a micronized form for injection into laryngeal tissue (Lundy et al. 2003). Powdered forms of ECM scaffolds can also be used for topical delivery or can be combined with synthetic polymers to create hybrid scaffolds. Use of a particulate form of such scaffold materials for pancreatic islet augmentation is an attractive option because of the large surface area, rapid degradation and release of bioactive molecules, and minimally-invasive delivery methods.

### *11.4.5 Gelation of ECM Scaffolds*

A liquid or gel form of ECM can further expand the clinical utility of an ECM scaffold. A gel form can be delivered to a site of interest in its pregel liquid state more readily than a suspension of particles. The solubilized matrix, with or without cells (e.g., pancreatic islets), can be delivered by catheter or needle-based surgical techniques to almost any anatomic site. The rheological properties of the gel can be designed to be similar to those of the tissue that is being repaired. Ideally the gel-processing methods would minimize or avoid purification steps that could remove or destroy the active growth factors and low-molecular-weight peptides present in the native ECM, and the gel form would retain the native bioactivity of the parent ECM scaffold. A gel form of ECM materials has proven to be possible in preclinical studies (Brightman et al. 2000).

A preparation of a gel derived from the urinary bladder ECM has been described. UBM powder was enzymatically (i.e., pepsin) digested at low pH, resulting in a viscous solution. This solution was able to self-assemble into a gel by raising the temperature, ionic strength, and pH to physiologic levels (Freytes et al. 2008a). Although the buffered pepsin digest is kept at a low temperature (4°C), the solution behaves as a liquid after the temperature is raised to 37°C and assumes a gel form within approximately 12 min. Gel forms of many ECM scaffold materials have been shown to support in-vitro cell growth of several cell types, including myoblasts, cardiomyocytes, and smooth muscle and endothelial cells (Freytes et al. 2008a).

## 11.5 Bioactive Properties of ECM Scaffolds

ECM biologic scaffolds have been shown to markedly affect cell proliferation, cell migration, cell differentiation, and angiogenesis. Such biologic activities are typically caused by cell signaling mechanisms that involve soluble molecules. ECM scaffolds have been shown to be rich in growth factors (Hodde et al. 2001, Voytik-Harbin et al. 1997) and bifunctional molecules, such as fibronectin (Hodde et al. 2002). Recently, degradation products of the parent ECM molecules have been shown to have significant biologic activity themselves (Brennan et al. 2006, Li et al. 2004, Reing et al. 2009, Sarikaya et al. 2002), especially chemotactic properties for stem and progenitor cells.

Stated differently, significant functional activity has been associated with the degradation of the native scaffold structure and release of the inherent bioactive constituents. Unlike the mechanical and structural properties that are dependent upon an intact 3-D structure, the biologic activities are in large part dependent upon just the opposite; that is, degradation of the intact 3-D structure. This concept has significant biologic implications since it presumes that associated cells (e.g., pancreatic islet cells) develop a new matrix structure during in-vivo remodeling. Thus, the site of implantation and the associated microenvironmental niche will likely play a large role in the extent of cell survival, proliferation, and homeostasis.

Inhibition of biologic scaffold degradation by processing methods, such as chemical cross-linking, markedly alters the functional characteristics of the scaffold and the host tissue response to the biologic material. In contrast, degradable biologic scaffolds may be considered as temporary niches, temporary structural templates, and even as controlled release devices for a variety of functional molecules.

The concept of functionality as a result of scaffold degradation by necessity implies that mechanical and structural properties are in a dynamic state. Accurate predictions of the biologic functionality will depend upon an understanding of the rate of scaffold degradation, the composition of the material from which the biologic scaffolds are constructed, and the nature of degradation products and their local and systemic distribution following in-vivo placement.

## 11.6 Culture and Transplantation of Pancreatic Islet Cells on ECMs

Pancreatic islet transplantation can restore normoglycemia to diabetic patients; however, fewer than 15% are insulin-independent at 2 years (Shapiro et al. 2006) and only 10% remain insulin-independent at 5 years (Leitao et al. 2008). Large numbers of islets (>10,000/kg recipient body weight) are needed to restore islet function in humans, requiring up to four pancreas donors per recipient. Moreover, islet transplantation is limited by the need for lifelong systemic immunosuppression to prevent recipient rejection of transplanted islets.

One barrier to successful islet transplantation is the loss of viable islets after isolation (Fraga et al. 1998). Mechanisms contributing to the difficulty of maintaining islets in culture include apoptosis and anoikis, the MEKK1 caspase-induced death after cells are separated from their ECM and anchoring integrins (Cardone et al. 1997, Lucas-Clerc et al. 1993, Thomas et al. 1999). Up to 60% of islets are lost in the first 14 days after transplantation (Barshes et al. 2004). Contributing mechanisms include enzymatic damage during isolation, ischemia, and hyperglycemic-induced systemic inflammation (Biarnes et al. 2002). A microenvironmental niche, such as might be provided by a biologic scaffold material, can potentially increase the cell survival rate and even affect subsequent mitotic activity.

Islets transplanted into the liver via injection into the portal vein are subjected to an intrahepatic inflammatory response (Bottino et al. 1998, Yang et al. 2005). Inflammatory cytokines, such as interleukin-1β, nitric oxide, interferon-γ, and tumor necrosis factor-α, appear to be mediators of islet dysfunction. Furthermore, islets are typically transplanted into a heterotopic location that may have deleterious effects on their engraftment (Mahmoud et al. 1998). Islets transplanted into the liver suffer from ischemia owing to low intrahepatic oxygen concentrations and delayed revascularization (Lau et al. 2006).

To explore whether transplanted islet function might be improved by providing a scaffold material, Dufour et al. transplanted allogeneic islets on biodegradable poly(glycolide-L-lactide) scaffolds into the epididymal fat pad of diabetic BALB/c mice (Dufour et al. 2005). The study showed that long-term islet survival on transplanted biodegradable scaffolds was comparable to that of islets transplanted under the kidney capsule, with all animals achieving long-term normoglycemia within 3 days. Animals with islets transplanted into the epididymal fat pad without biodegradable scaffolds had decreased long-term survival, and survivors were found to have delayed development of normoglycemia.

Another strategy to improve the efficacy of islet transplantation involves encapsulation of islets within an immune-protective biomaterial (Kizilel et al. 2005). The biomaterial acts as a selective membrane, allowing diffusion of insulin to regulate blood glucose, but limiting contact between the transplanted islets and the recipient's immune system. Biomaterials that have been used for islet encapsulation include alginate, both with and without poly(ethylene glycol) (PEG) or poly-L-lysine (PLL); PLL with poly-L-ornithine (PLO); hydroxy-methylated polysulfone (PS); dimethyaminoethyl methacrylate copolymer; and poly(vinyl alcohol) (Beck et al. 2007; see Chapter 12).

Encapsulated islets have been implanted in both macrocapsules containing large numbers of islets and microcapsules of single islets (Cruise et al. 1999). Unfortunately, many of the same factors that limit the success of transplanted islets—including ischemia and inflammation at the implantation site—also limit the survival of encapsulated islets (van Schilfgaarde and de Vos 1999). Moreover, if the encapsulating biomaterial is not biocompatible, protein adsorption, capsule fibrosis, and subsequent islet necrosis occurs (King et al. 2003). Encapsulated islets

are also damaged by activated macrophage production of the small-molecule nitric oxide, which can diffuse through most biomaterials (de Groot et al. 2003, Wiegand et al. 1993).

Cell sheet engineering is another technique that uses multiple layers of cultured cells to encapsulate transplanted cells. Lee et al. used sheets of dog auricular chondrocytes to encapsulate islets in long-term culture up to 3 months. Insulin secretion was maintained, and islets remained viable throughout the culture period (Lee et al. 2008). Another group had previously demonstrated persistent insulin release after 30 days in culture from islets encapsulated with chondrocytes on polyglycolic acid (PGA) polymers (Pollok et al. 1999).

There is evidence that pancreatic islet viability and function are enhanced by contact with ECM components. Isolated rat islets cultured in collagen gel demonstrate increased glucose-stimulated insulin secretion (Nagata et al. 2001). Islets cultured in laminin-rich ECM have also shown increased glucose-stimulated insulin secretion and overall survival. This effect appears to be mediated by laminin-5 activation of $\alpha 6\beta 1$ integrins (Bosco et al. 2000).

Lakey et al. reported that canine islets cultured for 48 h on SIS-ECM manifested improved recovery and significantly higher glucose-stimulated insulin secretion than islets cultured under standard conditions (Lakey et al. 2001). Woods et al. showed that islet recovery and function after cryopreservation was improved when islets were cultured on SIS-ECM (Woods et al. 2004b). Another study by the same group showed that islet function was significantly increased and morphology was preserved when islets were grown on SIS-ECM, compared to standard islet culture on Biopore membranes (Woods et al. 2004a).

In an attempt to improve pancreatic islet recovery, viability, and function over long-term culture in vitro, Tian et al. cultured isolated rat islets on sheets of porcine SIS-ECM for up to 2 weeks (Tian et al. 2005, Xiaohui et al. 2006). Purified rat islets were cultured in plates with and without multilayer porcine SIS-ECM. Recovered islets were quantified using dithizone staining after 7 and 14 days in culture. Apoptosis was assessed with ELISA for mono- and oligonucleosomes. Islet function was assessed with a static glucose challenge test. Islet recovery was 29% and 49% higher than in controls on SIS at 7 and 14 days, respectively, with 91% overall recovery after 14 days of culture. Apoptosis in the SIS-ECM group was less than half that of controls. The insulin stimulation index was also significantly higher in islets cultured with SIS-ECM than in controls.

## 11.7 Lessons from Adrenocortical Cells Grown on Native Adrenal ECM

Experience gained from culture of adrenocortical cells on native adrenal ECM may help develop similar approaches for islet cells. Normal adrenal gland function is essential for survival. Adrenal insufficiency is most commonly caused by autoimmune disease, hemorrhagic infarction, infection, congenital hypoplasia, or bilateral

adrenalectomy for adrenal tumors. Adrenal insufficiency requires lifelong administration of exogenous hormones and is associated with increased morbidity that is often not recognized, as well as increased mortality. Adrenal gland tumors are discovered only incidentally in up to 5% of abdominal imaging studies. Adrenal tumors may produce an excess of hormones, resulting in Cushing's disease, causing diabetes and additional associated comorbidities; pheochromocytoma, causing life-threatening blood pressure instability; or aldosterone hypersecretion, causing refractory hypertension. The rare but malignant adrenocortical carcinoma victim has a mean survival of less than 6 months from the time of diagnosis, and there are no effective adjuvant treatments.

Previous studies have shown that transplanted normal adrenocortical cells can survive in vivo and replace adrenal function in animals that have undergone bilateral adrenalectomy (Dunn et al. 2004, Thomas et al. 2000). Adrenal autotransplants are typically able to regenerate within 8 weeks, suggesting the presence of intraadrenal progenitor cells (Geiringer 1954, Ingle and Higgins 1938, Wyman and Tum Suden 1932). As little as 25% of one adrenal gland (12.5% total adrenal volume) is required to preserve normal basal corticosterone secretion in rats (Okamoto et al. 1992). SF1 has been identified as a key factor in steroidogenic cell differentiation (Dunn et al. 2004), and mesenchymal stem cells transfected with SF1 have been demonstrated to synthesize adrenal glucocorticoids (Yanase et al. 2006, Yazawa et al. 2006). An undifferentiated zone has been identified in the rat adrenal cortex as a proposed site of adrenal stem cells, with cells that express SF1 but lack terminal corticosteroid synthesizing enzymes (Yanase et al. 2006).

ECM promotes growth and differentiation of adrenocortical cells grown in culture (Ill and Gospodarowicz 1982). The laminin-rich ECM Matrigel enhances induction of steroid hydroxylase enzymes in cultured adrenocortical cells (Cheng and Hornsby 1992) and promotes a differentiated aldosterone-producing adrenocortical cell phenotype (Spinazzi et al. 2006). Adrenocortical cells grown in collagen gel demonstrate enhanced production of aldosterone compared to routine culture (Fujiyama et al. 1993). Bovine adrenocortical cells injected intradermally with FGF-secreting 3T3 cells in soluble collagen can replace adrenocortical function in adrenalectomized immunodeficient mice (Zhang and Hornsby 2002).

To determine whether organ-specific decellularized adrenal ECM could support adrenocortical cells in vitro, primary porcine adrenocortical cells were isolated and seeded onto decellularized adrenal ECM (Ogilvie et al. 2006). Adrenocortical cells attached well to the ECM bioscaffold, with good ingrowth of cells into the interior of the scaffold (Fig. 11.2). After 8 days in culture, adrenocortical cells on ECM scaffolds demonstrated inducible, adrenocorticotrophic hormone (ACTH)-stimulated cortisol levels by chemiluminescent immunoassay (Jiang et al. 2007) (Fig. 11.2). These results with adrenocortical cells seeded on adrenal ECM, as well as previous studies that showed maintenance of sinusoidal endothelial cell phenoptype after seeding on liver ECM (Sellaro et al. 2007), suggest that decellularized pancreatic ECM may serve as an optimal choice for maintenance of islets both in vitro and in vivo.

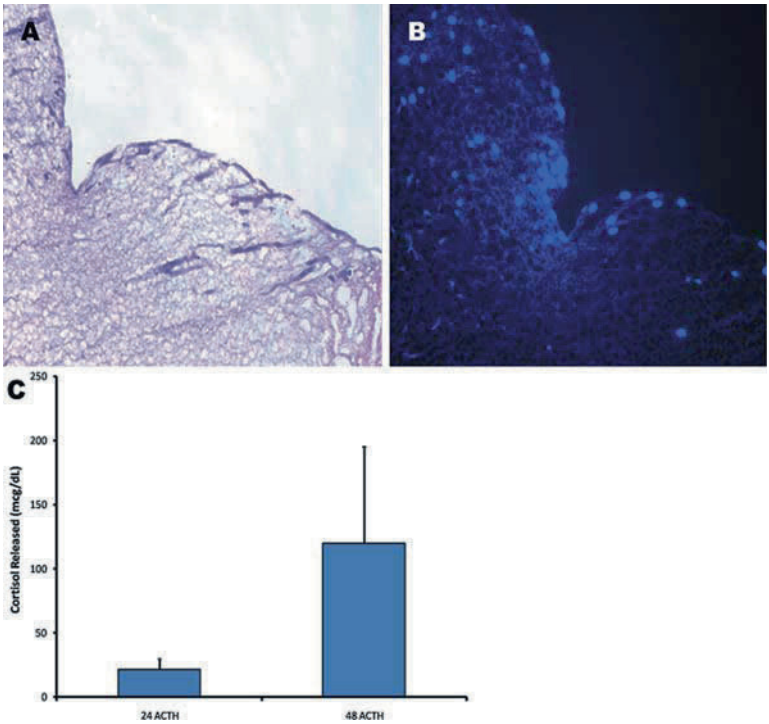



**Fig. 11.2 Primary porcine adrenocortical cells on decellularized ECM scaffolds after 8 days in vitro.** (**a**) H&E staining, 20X. (**b**) DAPI staining, 20X. **(c)** Porcine adrenocortical cells on ECM scaffolds show normal cortisol production 24 and 48 h following ACTH stimulation (100 mU/ml every 24 h)

## 11.8 Summary

Mammalian ECM represents an excellent scaffold material suitable for many therapeutic applications, including cell transplantation. The biologic signals that allow ECM scaffolds to promote constructive remodeling are likely the same characteristics that have evolved to promote tissue homeostasis and tissue repair following injury. The successful utilization of mammalian ECM as a component in a therapeutic device for endocrine tissue replacement will depend in large part upon our ability to understand and take advantage of the native structure–function relationships of biologic scaffold materials, particularly ECM scaffolds derived from endocrine tissues.

## References

Badylak SF, Tullius R, Kokini K, et al. (1995) The use of xenogeneic small intestinal submucosa as a biomaterial for Achilles tendon repair in a dog model. J Biomed Mater Res 29: 977–985.

Badylak S, Kokini K, Tullius B, et al. (2001a) Strength over time of a resorbable bioscaffold for body wall repair in a dog model. J Surg Res 99: 282–287.

Badylak SF, Park K, Peppas N, et al. (2001b) Marrow-derived cells populate scaffolds composed of xenogeneic extracellular matrix. Exp Hematol 29: 1310–1318.

Badylak S, Kokini K, Tullius B, et al. (2002) Morphologic study of small intestinal submucosa as a body wall repair device. J Surg Res 103: 190–202.

Badylak SF, Valentin JE, Ravindra AK, et al. (2008) Macrophage phenotype as a determinant of biologic scaffold remodeling. Tissue Eng Part A 14: 1835–1842.

Barshes NR, Lee T, Goodpasture S, et al. (2004) Achievement of insulin independence via pancreatic islet transplantation using a remote isolation center: a first-year review. Transplant Proc 36: 1127–1129.

Beattie AJ, Gilbert TW, Guyot JP, et al. (2008) Chemoattraction of progenitor cells by remodeling extracellular matrix scaffolds. Tissue Eng, doi: 10.1089/ten.tea.2008.0162..

Beck J, Angus R, Madsen B, et al. (2007) Islet encapsulation: strategies to enhance islet cell functions. Tissue Eng 13: 589–599.

Bernard MP, Chu ML, Myers JC, Ramirez F, Eikenberry EF, ProckopDJ (1983a) Nucleotide sequences of complementary deoxyribonucleic acids for the pro alpha 1 chain of human type I procollagen. Statistical evaluation of structures that are conserved during evolution. Biochemistry 22: 5213–5223.

Bernard MP, Myers JC, Chu ML, Ramirez F, Eikenberry EF, ProckopDJ (1983b) Structure of a cDNA for the pro alpha 2 chain of human type I procollagen. Comparison with chick cDNA for pro alpha 2(I) identifies structurally conserved features of the protein and the gene. Biochemistry 22: 1139–1145.

Bertone AL, Goin S, Kamei SJ, et al. (2008) Metacarpophalangeal collateral ligament reconstruction using small intestinal submucosa in an equine model. J Biomed Mater Res A 84: 219–229.

Biarnes M, Montolio M, Nacher V, et al. (2002) Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 51: 66–72.

Bosco D, Meda P, Halban PA, et al. (2000) Importance of cell-matrix interactions in rat islet beta-cell secretion in vitro: role of alpha6beta1 integrin. Diabetes 49: 233–243.

Bottino R, Fernandez LA, Ricordi C, et al. (1998) Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation. Diabetes 47: 316–323.

Brennan EP, Reing J, Chew D, et al. (2006) Antibacterial activity within degradation products of biological scaffolds composed of extracellular matrix. Tissue Eng 12: 2949–2955.

Brightman AO, Rajwa BP, Sturgis JE, et al. (2000) Time-lapse confocal reflection microscopy of collagen fibrillogenesis and extracellular matrix assembly in vitro. Biopolymers 54: 222–234.

Brown AL, Farhat W, Merguerian PA, et al. (2002) 22 week assessment of bladder acellular matrix as a bladder augmentation material in a porcine model. Biomaterials 23: 2179–2190.

Brown B, Lindberg K, Reing J, et al. (2006) The basement membrane component of biologic scaffolds derived from extracellular matrix. Tissue Eng 12: 519–526.

Brown BN, Valentin JE, Stewart-Akers AM, et al. (2009) Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. Biomaterials 30: 1482–1491.

Burchardt H, Jones H, Glowczewskie F, et al. (1978) Freeze-dried allogeneic segmental cortical-bone grafts in dogs. J Bone Joint Surg Am 60: 1082–1090.

Butler CE, Langstein HN, Kronowitz SJ (2005) Pelvic, abdominal, and chest wall reconstruction with AlloDerm in patients at increased risk for mesh-related complications. Plast Reconstr Surg 116: 1263–1275, discussion 1276–1267

Cardone MH, Salvesen GS, Widmann C, et al. (1997) The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. Cell 90: 315–323.

Cartmell JS, Dunn MG (2004) Development of cell-seeded patellar tendon allografts for anterior cruciate ligament reconstruction. Tissue Eng 10: 1065–1075.

Catena F, Ansaloni L, Leone A, et al. (2005) Lichtenstein repair of inguinal hernia with Surgisis inguinal hernia matrix soft-tissue graft in immunodepressed patients. Hernia 9: 29–31.
Chen F, Yoo JJ, Atala A (1999) Acellular collagen matrix as a possible "off the shelf" biomaterial for urethral repair. Urology 54: 407–410.
Cheng CY, Hornsby PJ (1992) Expression of 11 beta-hydroxylase and 21-hydroxylase in long-term cultures of bovine adrenocortical cells requires extracellular matrix factors. Endocrinology 130: 2883–2889.
Constantinou CDJ, Jimenez SA (1991) Structure of cDNAs encoding the triple-helical domain of murine alpha 2 (VI) collagen chain and comparison to human and chick homologues. Use of polymerase chain reaction and partially degenerate oligonucleotide for generation of novel cDNA clones. Matrix 11: 1–9.
Cornu O, Banse X, Docquier PL, et al. (2000) Effect of freeze-drying and gamma irradiation on the mechanical properties of human cancellous bone. J Orthop Res 18: 426–431.
Cruise GM, Hegre OD, Lamberti FV, et al. (1999) In vitro and in vivo performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes. Cell Transplant 8: 293–306.
Curtil A, Pegg DE, Wilson A (1997) Freeze drying of cardiac valves in preparation for cellular repopulation. Cryobiology 34: 13–22.
de Groot M, Schuurs TA, Leuvenink HG, et al. (2003) Macrophage overgrowth affects neighboring nonovergrown encapsulated islets. J Surg Res 115: 235–241.
Dejardin LM, Arnoczky SP, Ewers BJ, et al. (2001) Tissue-engineered rotator cuff tendon using porcine small intestine submucosa. Histologic and mechanical evaluation in dogs. Am J Sports Med 29: 175–184.
Derwin KA, Baker AR, Spragg RK, et al. (2006) Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Biomechanical, biochemical, and cellular properties. J Bone Joint Surg Am 88: 2665–2672.
Dufour JM, Rajotte RV, Zimmerman M, et al. (2005) Development of an ectopic site for islet transplantation, using biodegradable scaffolds. Tissue Eng 11: 1323–1331.
Dunn JC, Chu Y, Lam MM, et al. (2004) Adrenal cortical cell transplantation. J Pediatr Surg 39: 1856–1858.
Exposito JYDA, Solursh M, Ramirez M (1992) Sea urchin collagen evolutionarily homologous to vertebrate pro-alpha 2(I) collagen. J Biol Chem 267: 15559–15562.
Fraga DW, Sabek O, Hathaway DK, et al. (1998) A comparison of media supplement methods for the extended culture of human islet tissue. Transplantation 65: 1060–1066.
Freytes DO, Badylak SF, Webster TJ, et al. (2004) Biaxial strength of multilaminated extracellular matrix scaffolds. Biomaterials 25: 2353–2361.
Freytes DO, Rundell AE, Vande Geest J, et al. (2005) Analytically derived material properties of multilaminated extracellular matrix devices using the ball-burst test. Biomaterials 26: 5518–5531.
Freytes DO, Martin J, Velankar SS, et al. (2008a) Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix. Biomaterials 29: 1630–1637.
Freytes DO, Tullius RS, Valentin JE, et al. (2008b) Hydrated versus lyophilized forms of porcine extracellular matrix derived from the urinary bladder. J Biomed Mater Res A
Freytes DO, Tullius RS, Valentin JE, et al. (2008c) Hydrated versus lyophilized forms of porcine extracellular matrix derived from the urinary bladder. J Biomed Mater Res A, doi:10.1002/jbm.a.31821.
Fujiyama C, Masaki Z, Sugihara H (1993) Influence of extracellular matrix on the proliferation and differentiation of adrenocortical cells in culture. Pathol Res Pract 189: 1205–1214.
Geiringer E (1954) Bibilography of adrenal transplantation. Transplant Bull 1: 163–167.
Gilbert TW, Stolz DB, Biancaniello F, et al. (2005) Production and characterization of ECM powder: implications for tissue engineering applications. Biomaterials 26: 1431–1435.
Gilbert TW, Sellaro TL, Badylak SF (2006) Decellularization of tissues and organs. Biomaterials 27: 3675–3683.

Gilbert TW, Stewart-Akers AM, Simmons-Byrd A, et al. (2007) Degradation and remodeling of small intestinal submucosa in canine Achilles tendon repair. J Bone Joint Surg Am 89: 621–630.
Gilbert TW, Wognum S, Joyce EM, et al. (2008) Collagen fiber alignment and biaxial mechanical behavior of porcine urinary bladder derived extracellular matrix. Biomaterials 29: 4775–4782.
Gilbert TW, Freund JM, Badylak SF (2009) Quantification of DNA in biologic scaffold materials. J Surg Res 152: 135–139.
Gong J, Sagiv O, Cai H, et al. (2008) Effects of extracellular matrix and neighboring cells on induction of human embryonic stem cells into retinal or retinal pigment epithelial progenitors. Exp Eye Res 86: 957–965.
Hafeez YM, Zuki AB, Yusof N, et al. (2005) Effect of freeze-drying and gamma irradiation on biomechanical properties of bovine pericardium. Cell Tissue Bank 6: 85–89.
Harrison RD, Gratzer PF (2005) Effect of extraction protocols and epidermal growth factor on the cellular repopulation of decellularized anterior cruciate ligament allografts. J Biomed Mater Res A 75: 841–854.
Hodde JP, Badylak SF, Brightman AO, et al. (1996) Glycosaminoglycan content of small intestinal submucosa: a bioscaffold for tissue replacement. Tissue Eng 2: 209–217.
Hodde JP, Record RD, Liang HA, et al. (2001) Vascular endothelial growth factor in porcine-derived extracellular matrix. Endothelium 8: 11–24.
Hodde JP, Record R, Tullius R, et al. (2002) Fibronectin peptides mediate HMEC adhesion to porcine-derived extracellular matrix. Biomaterials 23: 1841–1848.
Hodde JP, Ernst DM, Hiles MC (2005) An investigation of the long-term bioactivity of endogenous growth factor in OASIS Wound Matrix. J Wound Care 14: 23–25.
Hosokawa T, Betsuyaku T, Nishimura M, et al. (2007) Differentiation of tracheal basal cells to ciliated cells and tissue reconstruction on the synthesized basement membrane substratum in vitro. Connect Tissue Res 48: 9–18.
Hosokawa T, Betsuyaku T, Odajima N, et al. (2008) Role of basement membrane in EMMPRIN/CD147 induction in rat tracheal epithelial cells. Biochem Biophys Res Commun 368: 426–432.
Huber JE, Spievack A, Simmons-Byrd A, et al. (2003a) Extracellular matrix as a scaffold for laryngeal reconstruction. Ann Otol Rhinol Laryngol 112: 428–433.
Huber JE, Spievack A, Simmons-Byrd A, et al. (2003b) Extracellular matrix as a scaffold for laryngeal reconstruction. Ann Otol Rhinol Laryngol 112: 428–433.
Ill CR, Gospodarowicz D (1982) Factors involved in supporting the growth and steroidogenic functions of bovine adrenal cortical cells maintained on extracellular matrix and exposed to a serum-free medium. J Cell Physiol 113: 373–384.
Ingle DJ, Higgins GM (1938) Autotransplantation and regeneration of the adrenal gland. Endocrinology 22: 458–464.
Jackson DW, Grood ES, Wilcox P, et al. (1988) The effects of processing techniques on the mechanical properties of bone-anterior cruciate ligament-bone allografts. An experimental study in goats. Am J Sports Med 16: 101–105.
Jiang H, Sellaro TL, Badylak SF, et al. (2007) Tissue engineered adrenal gland functions in vitro. In *Tissue Engineering and Regenerative Medicine International Society-North America Annual Conference and Exposition*, Toronto, Canada.
King A, Andersson A, Strand BL, et al. (2003) The role of capsule composition and biologic responses in the function of transplanted microencapsulated islets of Langerhans. Transplantation 76: 275–279.
Kizilel S, Garfinkel M, Opara E (2005) The bioartificial pancreas: progress and challenges. Diabetes Technol Ther 7: 968–985.
Kropp BP, Rippy MK, Badylak SF, et al. (1996) Regenerative urinary bladder augmentation using small intestinal submucosa: urodynamic and histopathologic assessment in long-term canine bladder augmentations. J Urol 155: 2098–2104.
Lakey JR, Woods EJ, Zieger MA, et al. (2001) Improved islet survival and in vitro function using solubilized small intestinal submucosa. Cell Tissue Bank 2: 217–224.

Lau J, Jansson L, Carlsson PO (2006) Islets transplanted intraportally into the liver are stimulated to insulin and glucagon release exclusively through the hepatic artery. Am J Transplant 6: 967–975.

Lee JI, Nishimura R, Sakai H, et al. (2008) A newly developed immunoisolated bioartificial pancreas with cell sheet engineering. Cell Transplant 17: 51–59.

Leitao CB, Cure P, Tharavanij T, et al. (2008) Current challenges in islet transplantation. Curr Diab Rep 8: 324–331.

Li F, Li W, Johnson S, et al. (2004) Low-molecular-weight peptides derived from extracellular matrix as chemoattractants for primary endothelial cells. Endothelium 11: 199–206.

Lovekamp JJ, Simionescu DT, Mercuri JJ, et al. (2006) Stability and function of glycosaminoglycans in porcine bioprosthetic heart valves. Biomaterials 27: 1507–1518.

Lucas-Clerc C, Massart C, Campion JP, et al. (1993) Long-term culture of human pancreatic islets in an extracellular matrix: morphological and metabolic effects. Mol Cell Endocrinol 94: 9–20.

Lundy DS, Casiano RR, McClinton ME, et al. (2003) Early results of transcutaneous injection laryngoplasty with micronized acellular dermis versus type-I thyroplasty for glottic incompetence dysphonia due to unilateral vocal fold paralysis. J Voice 17: 589–595.

Mahmoud IM, Gabr MM, Refaie AF, et al. (1998) Purified murine islet allografts: islet engraftment as influenced by implantation site and glucotoxicity. Transplant Proc 30: 369–372.

McDevitt CA, Wildey GM, Cutrone RM (2003) Transforming growth factor-beta1 in a sterilized tissue derived from the pig small intestine submucosa. J Biomed Mater Res A 67: 637–640.

McPherson TB, Liang H, Record RD, et al. (2000) Galalpha(1,3)Gal epitope in porcine small intestinal submucosa. Tissue Eng 6: 233–239.

Nagata N, Gu Y, Hori H, et al. (2001) Evaluation of insulin secretion of isolated rat islets cultured in extracellular matrix. Cell Transplant 10: 447–451.

Ogilvie JB, Jiang H, Sellaro TL, et al. (2006) Development of decellularized adrenal extracellular matrix for a tissue-engineered adrenal replacment device. In Regenerate World Congress of the Tissue Engineering and Regenerative Medicine International Society, Pittsburgh, PA.

Okamoto T, Fujimoto Y, Obara T, et al. (1992) Experimental study on adrenal autografts in rats to preserve normal adrenocortical function after bilateral adrenalectomy. Eur Surg Res 24: 112–118.

Pollok JM, Begemann JF, Kaufmann PM, et al. (1999) Long-term insulin-secretory function of islets of Langerhans encapsulated with a layer of confluent chondrocytes for immunoisolation. Pediatr Surg Int 15: 164–167.

Raeder RH, Badylak SF, Sheehan C, et al. (2002) Natural anti-galactose alpha1,3 galactose antibodies delay, but do not prevent the acceptance of extracellular matrix xenografts. Transpl Immunol 10: 15–24.

Reing JE, Zhang L, Myers-Irvin J, et al. (2009) Degradation products of extracellular matrix affect cell migration and proliferation. Tissue Eng Part A 15: 605–614.

Ringel RL, Kahane JC, Hillsamer PJ, et al. (2006) The application of tissue engineering procedures to repair the larynx. J Speech Lang Hear Res 49: 194–208.

Sacks MS, Gloeckner DC (1999) Quantification of the fiber architecture and biaxial mechanical behavior of porcine intestinal submucosa. J Biomed Mater Res 46: 1–10.

Sarikaya A, Record R, Wu CC, et al. (2002) Antimicrobial activity associated with extracellular matrices. Tissue Eng 8: 63–71.

Sellaro TL, Ravindra AK, Stolz DB, et al. (2007) Maintenance of hepatic sinusoidal endothelial cell phenotype in vitro using organ-specific extracellular matrix scaffolds. Tissue Eng 13: 2301–2310.

Shapiro AM, Ricordi C, Hering BJ, et al. (2006) International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355: 1318–1330.

Smith CW, Young IS, Kearney JN (1996) Mechanical properties of tendons: changes with sterilization and preservation. J Biomech Eng 118: 56–61.

Spinazzi R, Petrelli L, Guidolin D, et al. (2006) In vitro culture on Matrigel favors the long-term maintenance of rat zona glomerulosa-cell differentiated phenotype. Int J Mol Med 17: 1101–1110.

Thomas FT, Contreras JL, Bilbao G, et al. (1999) Anoikis, extracellular matrix, and apoptosis factors in isolated cell transplantation. Surgery 126: 299–304.
Thomas M, Yang L, Hornsby PJ (2000) Formation of functional tissue from transplanted adrenocortical cells expressing telomerase reverse transcriptase. Nat Biotechnol 18: 39–42.
Tian XH, Xue WJ, Pang XL, et al. (2005) Effect of small intestinal submucosa on islet recovery and function in vitro culture. Hepatobiliary Pancreat Dis Int 4: 524–529.
Toritsuka Y, Shino K, Horibe S, et al. (1997) Effect of freeze-drying or gamma-irradiation on remodeling of tendon allograft in a rat model. J Orthop Res 15: 294–300.
Valentin JE, Badylak JS, McCabe GP, et al. (2006) Extracellular matrix bioscaffolds for orthopaedic applications. A comparative histologic study. J Bone Joint Surg Am 88: 2673–2686.
van Schilfgaarde R, de Vos P (1999) Factors influencing the properties and performance of microcapsules for immunoprotection of pancreatic islets. J Mol Med 77: 199–205.
Voytik-Harbin SL, Brightman AO, Kraine MR, et al. (1997) Identification of extractable growth factors from small intestinal submucosa. J Cell Biochem 67: 478–491.
Wiegand F, Kroncke KD, Kolb-Bachofen V (1993) Macrophage-generated nitric oxide as cytotoxic factor in destruction of alginate-encapsulated islets. Protection by arginine analogs and/or coencapsulated erythrocytes. Transplantation 56: 1206–1212.
Woods EJ, Walsh CM, Sidner RA, et al. (2004a) Improved in vitro function of islets using small intestinal submucosa. Transplant Proc 36: 1175–1177.
Woods EJ, Walsh CM, Sidner RA, et al. (2004b) Enhanced recovery of cryopreserved islets using SIS. Transplant Proc 36: 1139–1142.
Woods T, Gratzer PF (2005) Effectiveness of three extraction techniques in the development of a decellularized bone-anterior cruciate ligament-bone graft. Biomaterials 26: 7339–7349.
Wood JD, Simmons-Byrd A, Spievack AR, et al. (2005) Use of a particulate extracellular matrix bioscaffold for treatment of acquired urinary incontinence in dogs. J Am Vet Med Assoc 226: 1095–1097.
Wyman LC, Tum Suden C (1932) Studies on suprarenal insufficiency. XI. The growth of transplanted cortical tissue in the rat. Am J Physiol 101: 662–667.
Xiaohui T, Wujun X, Xiaoming D, et al. (2006) Small intestinal submucosa improves islet survival and function in vitro culture. Transplant Proc 38: 1552–1558.
Xu H, Wan H, Sandor M, et al. (2008) Host response to human acellular dermal matrix transplantation in a primate model of abdominal wall repair. Tissue Eng Part A 14: 2009–2019.
Yanase T, Gondo S, Okabe T, et al. (2006) Differentiation and regeneration of adrenal tissues: an initial step toward regeneration therapy for steroid insufficiency. Endocr J 53: 449–459.
Yang Z, Chen M, Ellett JD, et al. (2005) Inflammatory blockade improves human pancreatic islet function and viability. Am J Transplant 5: 475–483.
Yazawa T, Mizutani T, Yamada K, et al. (2006) Differentiation of adult stem cells derived from bone marrow stroma into Leydig or adrenocortical cells. Endocrinology 147: 4104–4111.
Zantop T, Gilbert TW, Yoder MC, et al. (2006) Extracellular matrix scaffolds are repopulated by bone marrow-derived cells in a mouse model of Achilles tendon reconstruction. J Orthop Res 24: 1299–1309.
Zhang H, Hornsby PJ (2002) Intradermal cell transplantation in soluble collagen. Cell Transplant 11: 139–145.

# Chapter 12
# Immunoisolation in Cell Transplantation

**Riccardo Calafiore and Giuseppe Basta**

**Abstract** Cell therapy represents an increasingly promising approach for the cure of many chronic and degenerative disorders, such as type 1 (insulin-dependent) diabetes mellitus and Parkinson's disease. Replacement of diseased cells with healthy and functional cells could allow repair and restoration of organ function. This is particularly relevant for organs that execute highly-complex physiological tasks, such as the endocrine pancreas, which maintains glucose homeostasis. However, cell therapy based on the use of allogeneic primary adult cells or progenitor/stem cells faces several fundamental problems. In addition to the need to overcome allograft rejection, optimal function of the transplanted cells may be achieved only if they are embedded in a surrogate extracellular matrix that creates a three-dimensional tissue structure mimicking that of the normal tissue. Cell microencapsulation in artificial membranes made of highly-purified and biocompatible biopolymers can provide immunoprotection and preserve cell viability by allowing passage of oxygen and nutrients across the membrane and blocking humoral or cellular components of the host immune system. The microencapsulated cells are embedded in a three-dimensional configuration that mimics their original site and enhances their function. This chapter reviews the latest advances and applications of this technology, as well as its future prospects.

## 12.1 Encapsulation of Live Cells/Tissue: Definition, Scope, and Applications

Encapsulation of live cells involves embedding of cell clusters in a matrix and coating it with a membrane made of natural or artificial polymers (Orive et al. 2003; Murua et al. 2008). The membrane constitutes a physical barrier between the encapsulated cells and the surrounding environment, while the matrix provides support and enhances cell function (Colton 1995). The membrane must be

R. Calafiore (✉)
Department of Internal Medicine, University of Perugia, 06126 Perugia, Italy
e-mail: islet@unipg.it

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_12,

biocompatible and selectively permeable to maintain the survival and function of the encapsulated cells.

The concept of live cell encapsulation is closely linked to the broader field of tissue engineering. Researchers in this field try to develop biological substitutes for restoration, maintenance, or improvement of tissue function, based on an interdisciplinary approach that combines material engineering and molecular and cell biology (Nair and Laurencin 2007).

Encapsulation of live cells within artificial membranes generates biohybrid organs, in which specific cell types preserve their physiological competence and may or may not interact with other cell types within a protected environment. In principle, every organ that can be dissociated into single cells or cell clusters without loss of its original function can provide the biological component of a biohybrid artificial organ. However, preservation of cell survival and function in an environment that differs significantly from the cell's normal milieu remains a difficult task. Moreover, only a few selected materials are suitable for creating a tissue–material interface that is biologically benign for both the encapsulated cells and the surrounding milieu.

Several materials have been employed during the past decades to generate membranes for both large-size macroencapsulation devices and microcapsules. Macrodevices, either implanted subcutaneously or surgically connected to blood vessels, have been shown to provide poor cell access to oxygen and nutrients, and today are of only historical value. Current attention is focused on microcapsules because of their superior metabolite exchange properties. Technical advances have enabled the generation of progressively smaller capsules, which provide improved cell access to oxygen and nutrients.

The rapid expansion of cell therapy approaches based on the use of primary cells, cells differentiated from stem cells, or engineered cell lines has created increased demand and opportunities for application of cell encapsulation. Cell replacement therapy could advantageously be applied to restoration of cell/tissue function that has been severely and irreversibly damaged, for instance, in type 1 diabetes mellitus (T1DM) (de Vos et al. 2006), Parkinson's and Alzheimer's diseases, and many other chronic degenerative disorders (Emerich et al. 2006; Yasuhara and Date 2007). Most of the experience in transplantation of encapsulated cells has been acquired in studies of grafts of encapsulated islets or insulin-producing cells in animal models of diabetes. In the case of T1DM, which is caused by specific autoimmune destruction of pancreatic islet β cells, cell encapsulation is required to provide protection from both allograft rejection and recurring autoimmunity. Experience accumulated in preclinical and pilot clinical trials with encapsulated islet grafts in diabetic recipients led to the coining of a new term, the "biohybrid artificial pancreas," for encapsulated pancreatic islet/insulin-producing cell therapy systems (Calafiore et al. 2006; de Vos et al. 2006; Calafiore and Basta 2007).

Another potential application of encapsulated cells is in local delivery of drugs/biological products in several areas of experimental and clinical medicine. The prospects and limitations of these new strategies are discussed in detail below.

## 12.2 Biomaterials for Microencapsulation

Advances in chemistry and physical chemistry have steadily fostered development of a wide range of novel polymers for cell encapsulation. Success of polymer-based systems largely depends upon the ability to custom-design or modify existing materials for improving membrane biocompatibility, porosity, permeability, filtration, and degradation/stability over time, as well as nominal molecular-weight cut-off (MWCO). Both macrodevices and microcapsules have been employed for creating immunobarrier capsules.

### *12.2.1 Macrodevices*

Over the years cell grafting in diabetes models has employed most of the various macroencapsulation devices available for in-vivo use. In principle, these devices were either vascular chambers surgically connected to blood vessels (Petruzzo et al. 1991) or diffusion chambers in several configurations (hollow fiber, planar, spherical, etc.), positioned either subcutaneously or intraperitoneally (Lacy et al. 1991; Lanze et al. 1993) and they relied on passive exchange/diffusion of oxygen and nutrients.

A wide range of materials has been employed for the manufacture of macrodevices for islet graft immunoprotection (Fig. 12.1). Some of them are still in use, such as polyacrylonitrile-polyvinyl chloride (PAN-PVC), whereas the use of others has been progressively discontinued.

Hollow fibers have been employed for subcutaneous (Lanza et al. 1992) and intraperitoneal (de Vos et al. 1997) islet grafts. The fibers were composed of a selectively permeable membrane with a nominal MWCO < 100 kD, which allowed passage of metabolites that were indispensable for survival and function of the encapsulated islets, while preventing access of immune cells or antibodies. The membranes were considered sufficiently biocompatible to circumvent foreign-body tissue reactivity. Although successful in a few rodent experiments, these devices have never been convincingly shown to correct diabetes in larger mammals. They were easy to implant and retrieve, but in the majority of cases their surface/volume ratio resulted in insufficient nutrient supply, leading to cell necrosis in the core of the device.

Moreover, nutrient supply depends on graft site: subcutaneous tissue and peritoneal cavity are known to be associated with relatively low oxygen tension. However, the large size of the macrodevices limited site selection, a problem that was more severe in large animals, compared to rodents. It was evident that only islets embedded within a gel matrix (usually, but not limited to, alginate) survived, whereas loose islets died fast. Unfortunately, loading density into gel matrices was quite low. Although a relatively short hollow fiber contains enough islets for glycemia correction in rodents (Maki et al. 1996), large animals require exceedingly large devices for achieving comparable metabolic effects. Likely, similar restrictions

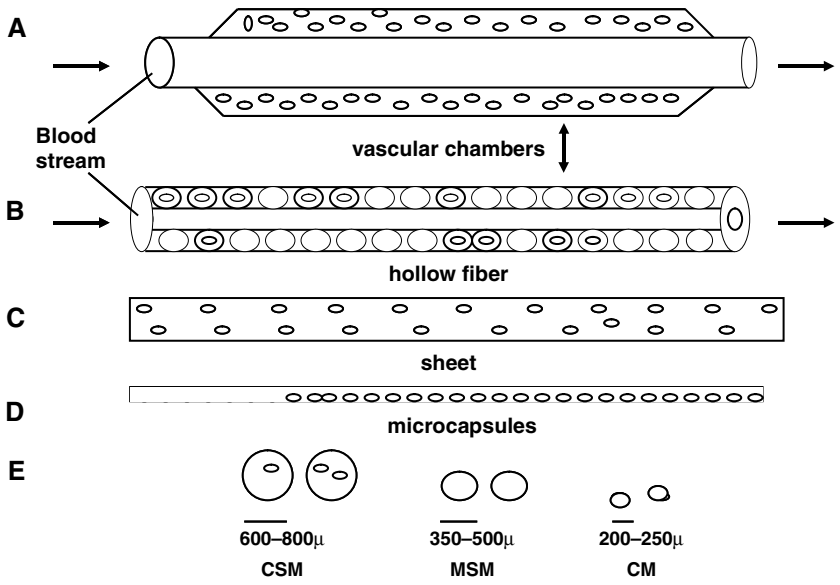



**Fig. 12.1 Types of immunoisolation devices for islet transplantation.** (**a**) Vascular chamber containing matrix-embedded islets (Maki et al. 1996). (**b**) Vascular prosthesis containing islets encapsulated in alginate/poly-L-ornithine (Calafiore 1992). (**c**) Hollow fiber containing matrix-embedded islets (Lacy et al. 1991; Lanza et al. 1999). (**d**) Laminar alginate thin sheet containing monolayered islets (Hanuman Medical). (**e**) Alginate-based microcapsules of different sizes: conventional size microcapsules (CSM), medium-size microcapsules (MSM), and conformal microcapsules (CM) (Sefton et al.1993; Soon-Shiong et al. 1994; Calafiore et al. 1996; Sun et al. 1996; Weber et al. 1999)

would limit their use in humans. Prevascularization of the device prior to islet seeding resulted in interesting preliminary results in rodents (de Vos et al. 1997), but it is not clear whether these findings would apply to larger mammals.

As an alternative to prevascularized membranes, an arterio-vein (A-V) vascular prosthesis was proposed for improving nutrient supply to cells in macrodevices. The first vascular chambers were equipped with selective membranes in which islet grafts were encapsulated for immunoprotection. In subsequent devices microencapsulated islets were placed in the vascular chamber in an attempt to combine the improved nutrient supply with the immunoprotection provided by the microcapsules. The "vascularized" graft of microencapsulated islets generated encouraging preliminary results in diabetic dogs (Petruzzo et al. 1991), but only a partial and transient remission of hyperglycemia was achieved in humans (Calafiore 1992). Safety issues, such as risks of device clotting or breakage leading to thrombosis-have also told against further development of this approach.

Overall, in evaluating the risk/benefit ratio, macrodevices may offer some advantages over other strategies, including: (1) easy retrievability, owing to device size and predictable graft site; and (2) versatile configuration, which may help optimize oxygen/nutrient diffusion and physiological kinetics of insulin release. However, a

number of limitations remain, including: (1) low biocompatibility, which requires a continuous search for new materials; (2) breakage risk, which increases with device size; and (3) involvement of relatively invasive surgical manipulations. The "flat sheet" approach presented recently by Kin et al. (Kin et al. 2008) has generated some renewed interest in macrodevices. This device consists of thin alginate (AG)-salt sheets that contain islets and are sealed together. In this configuration the islets are at a very short distance from oxygen/nutrient sources. Moreover, the sheets are versatile in terms of grafting capability in several sites, with special regard to those that are better vascularized. However, practical applicability of this class of macrodevices remains a distant prospect.

## *12.2.2 Microcapsules*

Cell microencapsulation is based on the immobilization of cells within a semipermeable membrane that protects the cells from both mechanical stress and the host immune system, while allowing bidirectional diffusion of nutrients, oxygen, and metabolic waste. The spherical shape confers microcapsules with improved rates of metabolite exchange, thereby enhancing cell survival. Experiments in animal models and recent pilot clinical trials have shown that the immune protection provided by microencapsulation can reduce, if not eliminate, chronic administration of immunosuppressive agents in graft recipients, thereby eliminating severe side effects.

Alginic acid derivatives have been historically the materials most widely employed for generation of microcapsules, with special regard (but not limited) to microencapsulated islet cell grafts for diabetes treatment. Alginates remain the most important polymers for microencapsulation and are the focus of this chapter, but other polymers have been studied as potential chemical candidates for microencapsulation as well, including polyethylene glycol (PEG), chitosan, collagen, hyaluronic acid and its derivatives, dextran, agarose, polylactic galactyl acid (PLGA), and multilayer combinations of several compounds. Most of these polymers have not been shown to work in routine manufacture of microcapsules for cell encapsulation, but the combination of some of them with AG has recently been pursued, for example, an AG core modified with covalently-conjugated oligopeptides (arg, gly, asp), AG/agarose, and PEG modification of AG membranes.

### 12.2.2.1 Agarose

Agarose, like AG, is a gelling agent extracted from seaweeds, composed of repeating units of alternating β-D-galactopyranosyl and 3,6-anhydro-α-L-galactopyranosyl. Depending on the temperature, agarose forms thermally-reversible gels, with gelling occurring at temperatures that are far below the gel fusion point (Iwata et al. 1989; Iwata and Ikada 1999; Sakai et al. 2007). Iwata et al. developed several procedures for forming agarose microcapsules for islet immunoprotection. Typically, these authors employed 5% agarose gel solutions to formulate their capsules, with

no adverse effects on viability of encapsulated islet cells, as assessed by in-vitro studies. However, a potential technical problem was posed by variable amounts of empty microspheres burdening the overall graft volume. Moreover, although agarose microcapsules provided satisfactory immunoprotection for islet allografts (typically allogeneic mouse islets transplanted intraperitoneally into streptozotocin-induced diabetic mice) (Iwata and Ikada 1999), they did not protect islet allografts in nonobese diabetic (NOD) mice, which develop spontaneous autoimmune diabetes and are considered a more stringent immunological model. This raised doubts about the immunobarrier competence of agarose microcapsules. Attempts were made to coat the agarose microcapsules with outer layers composed of sulfated polyanions, complement inhibitors (i.e., polystyrene sulfonic acid–polybrene polyion), and carboxymethylcellulose, the latter being added to improve the capsule biocompatibility (Kobayashi et al. 2006). Although these improved agarose microcapsules apparently prolonged islet xenograft survival (hamster islets into mice), there is as yet no compelling evidence that complement blocking by itself is sufficient to increase survival of encapsulated islet xenografts in immunocompetent recipients. Finally, there has been no experience using this type of microcapsule in larger mammals.

#### 12.2.2.2 Polyethylene Glycol

The most appealing property of PEG for generation of immunoprotective microcapsules is its capability to form a protein-repellent surface. Protein adhesion on the capsule surface has been blamed as a trigger of fibrosis, which could impair the membrane diffusion properties (Sawhney 1999). PEG has been used to coat AG-poly-L-lysine (AG/PLL) gel microbeads (Sawhney and Hubbell 1992) or to create a conformal coating that tightly envelops each individual islet (Sawhney et al. 1994). The latter specifically addresses the capsular volume issue, already indicated as a potential cause for failure of encapsulated islet grafts. However, PEG has so far gained only limited confidence as a material for microcapsule formulation. Apart from in-vitro and in-vivo biocompatibility studies conducted with empty PEG capsules, limited data have been reported on grafting of PEG microcapsules containing islets into diabetic recipients. Until recently manufacture of PEG microcapsules required a laser-induced photopolymerization process, which may endanger encapsulated cells. However, new procedures that do not involve photopolymerization are now available, which greatly improve the applicability of this polymer (Elbert and Hubbell 2001). Biocompatibility of these newly-formulated membranes is currently under scrutiny (Tessmar and Göpferich 2007).

#### 12.2.2.3 Chitosan

Chitosan has been proposed as a material that may be coupled with alginates within the microcapsule chemical composition (Kim et al. 1999). The properties of chitosan require further investigation to assess whether this polymer, either alone

(Baruch and Machluf 2006) or in combination with other molecules, could substantially improve microcapsule physicochemical characteristics (Marsich et al. 2008).

#### 12.2.2.4 Hydroxyethyl-Methacrylate-Methyl-Methacrylate (HEMA-MMA)

HEMA-MMA is a polyacrylate copolymer (Sefton and Stevenson 1993) prepared by solution polymerization following careful monomer purification. Although the polymer is to some extent hydrophilic (25–30% water uptake), it also provides mechanical strength, elasticity, and durability. This substance has been proven suitable for manufacturing small-size capsules, which may represent the ultimate answer to the graft-volume problem in islet encapsulation. A concern involved in employing this polymer regards steps in the capsule manufacturing process that may damage live cells, such as exposure to shearing forces and organic solvents. However, several cell types, such as human and animal tumor cell lines and primary rat hepatocytes and islets, have been successfully encapsulated in HEMA-MMA with retention of viability over long periods of time. Engineered cell lines, such as vascular endothelial growth factor-secreting cells, have also been successfully microencapsulated in HEMA-MMA (Babensee and Sefton 2000), with improved survival in vivo and vascularization.

The specific suitability of HEMA-MMA microcapsules for islet cell encapsulation for transplantation into diabetic recipients remains undetermined and requires further study (Vallbacka and Sefton 2007).

## 12.3 Alginate-Based Microcapsules

### *12.3.1 Alginate Production and Use*

Although a variety of polymers have been proposed for microencapsulation of islet grafts, many of them ultimately failed to provide convincing evidence of immunoprotection. The only polymer approved for microcapsule manufacturing for use in humans remains AG in its sodium salt (Calafiore et al. 2006).

Extracted from brown seaweeds, AG is a linear copolymer of two uronic acids: D-mannuronic acid (M) and L-guluronic acid (G), linked by 1,4 glycosidic bonds. The two monomers are arranged in homopolymeric blocks, M-blocks and G-blocks, as well as in sequences containing both monomers, MG-blocks. The proportions and sequential arrangement of the uronic acids depend on the species of algae (Table 12.1) and the specific tissue (stem or leaf) from which AG is extracted. The chemical composition of AG is described in terms of its fractions ($F_G$ or $F_M$), proportion of polyguluronic acids (Pgas) and polymannuronic acids (Pmas), and the M/G ratio, and is determined by H-NMR spectral analysis.

AG has progressively gained popularity for manufacture of microcapsules for transplantation purposes since the time of the first successful reports by Lim and

**Table 12.1** Algae sources for alginate extraction

| Algae species | $F_G$ (%) | $F_M$ (%) | Pgas | Pmas | M/G ratio |
|---|---|---|---|---|---|
| *Macrocystis pyrifera* | 39 | 61 | 17.7 | 40.6 | 1.56 |
| *Laminaria digitalia* | 41 | 59 | 12.7 | 60.5 | 1.45 |
| *Laminaria hyperborea* | 69 | 31 | 18.5 | 61.2 | 0.45 |

Sun (Lim and Sun 1980). Unfortunately, standard parameters for encapsulation have not been made available to all laboratories engaged in this field. In the absence of comparable criteria, some positive data have been overwhelmed with negative results. A major issue has been the scarcity of ultrapure AG (Dusseault et al. 2006). Since AG was the major component of microcapsules, endotoxin- and pyrogen-free preparations had to be produced, but this was not done by most laboratories. We have developed a sequential filtration process for ultrapurification of "pharmaceutical grade" AG raw powder, which removes endotoxins, as determined by the limulus amebocyte lysate test, while maintaining the physical and chemical properties of the product.

Another issue has been storage of AG solutions: if improperly preserved, the basic polymer could undergo acid hydrolysis with loss of physicochemical properties, with serious consequences for the manufacture of microcapsules. In our laboratory, storage of 1.7% AG solution is performed in endotoxin-free polystyrene bottles at 6°C without exposure to light. Ultrapure AG is highly stable under these conditions, as judged by viscosity, osmolarity, pH, endotoxin content, and absence of acidic hydrolysis.

Many laboratories have been caught in disputes on whether high-M or high-G AG derivatives are preferable for microbead formation and whether coating of the microbeads with polyamino acids (e.g., PLL) is advantageous. Superior gelling properties of high-G over high-M AG, coupled with lower immunogenicity of the former, were reported by some groups (De Castro et al. 2005; Morch et al. 2007), but were not confirmed by our laboratory or others.

Issues have been raised about the optimal size of microcapsules for transplantation (Calafiore et al. 1998; Strand et al. 2002; Sakai et al. 2006). Our experience with AG/poly-L-ornithine (PLO) capsules of different sizes suggested that large capsules (>800 μm in diameter) should not be used because of an unfavorable volume/surface ratio that limits metabolite exchange. It also indicated that size uniformity was important for both "uncoated" AG beads (Thanos et al. 2007) and beads coated with other polymers (e.g., PLL or PLO) (de Vos et al. 1994). "Uncoated" AG beads, generally made of barium AG, have been proven to be competent immunobarriers for islet allografts by preventing direct contact between the host immune cells and the encapsulated tissue, but not for xenografts, thus limiting the applicability of this formulation. In contrast, AG/PLO and AG/PLL have been shown to protect islet xenografts in diabetic animal models. This has been clearly demonstrated by our group, among others, with full reversal of hyperglycemia upon transplantation of microencapsulated neonatal porcine islet cell clusters in NOD mice (Luca

et al. 2005) (see below). This animal model resembles human T1DM, thereby raising hopes that this system can be applied to patients with T1DM. Although the PLL coating was reported to provoke some immune response in recipient animals, PLO coating has been shown by us (Thanos et al. 2007) and others (Darrabie et al. 2005) to be free of any inflammatory reaction in both rodents and larger mammals, including humans (Calafiore et al. 2006).

## *12.3.2 Morphology and Size of Alginate Microcapsules*

### 12.3.2.1 Conventional Microcapsules

A major issue that has not always been properly addressed by laboratories involved in microencapsulation research, regardless of the polymer employed, pertains to the manufacture of round-shaped and smooth-surfaced microcapsules of uniform size. Owing to technical differences, microcapsule manufacturing has seldom resulted in a regular shape and size. In our procedure, the sodium AG/islet cell suspension is extruded through a microdroplet generator, where mechanical pressure and air-shearing forces contribute to the formation of gel microbeads (Fig. 12.2). The beads are subsequently collected in a collection bath containing divalent cations (usually $CaCl_2$), which promote gelling. Fine regulation of both the physical parameters involved in the procedure (suspension flow rate and air flow rate) and the divalent cations in the collection bath enable the production of capsules that are smooth-surfaced and uniform in size and shape (Fig. 12.3). In a second stage, the beads are coated with multilayered PLO and AG to provide a biocompatible outer shell around the spheres (Fig. 12.4). When using impure islet preparations contaminated by larger pancreatic exocrine/ductal residual tissue chunks, the generated microbeads are irregularly shaped and are sometimes broken, with the contained tissue protruding through the capsular membrane (de Vos et al. 1994). Conversely, uniform and pure islet preparations reproducibly generate regularly-shaped capsules.

The first generation of capsules had an average diameter of 800 μm; however, as noted above, their volume/surface ratio limited metabolite exchange. Static

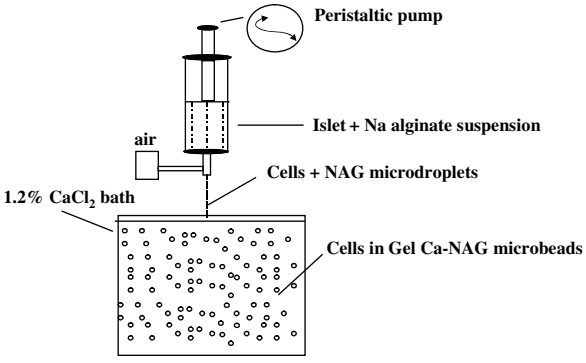



**Fig. 12.2 Method for manufacture of alginate microcapsules using a microdroplet generator**

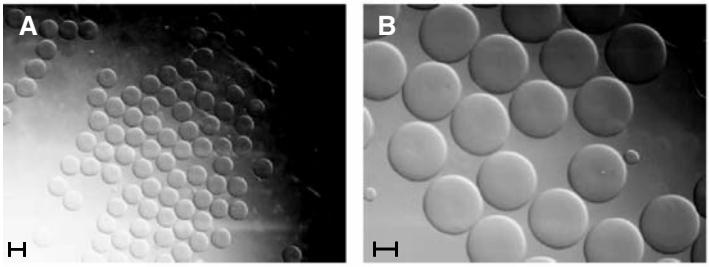



**Fig. 12.3 Empty alginate/poly-L-ornithine microcapsules**. (**a**) Bar = 400 μm**.** (**b**) Bar = 200 μm

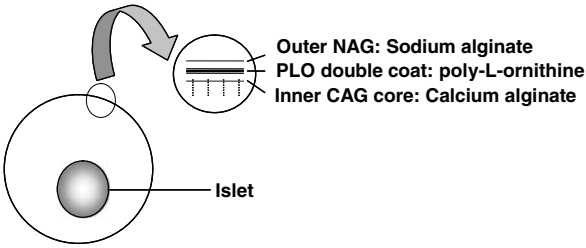



**Fig. 12.4 Schematic representation of the multilayered membrane of alginate/poly-L-ornithine microcapsules**

electricity was employed to reduce the capsule size (Strand et al. 2002). The droplet was charged with a high static voltage as it was suspended from the needle and attracted to the collecting vessel with opposing polarity. Upon overcoming the threshold potential, the droplet moved from the needle to the collection vessel. Although the microcapsules generated were uniform in size and shape and considerably smaller than those made with the traditional microdroplet generator, this method was deemed unsafe for the cells because of the high voltage involved. Moreover, it prolonged the encapsulation procedure, increasing the exposure time of the suspended islet cell mass to chemicals and physical/electric forces that might affect cell viability and function.

Subsequently, small adjustments in air and AG/islet mixture flow rates and regulation of AG temperature resulted in smaller microcapsules (400–500 μm) without stressful procedures. We called these microcapsules, formulated in our laboratory with AG-PLO-AG, medium-size microcapsules (MSM). They provide a favorable volume/surface ratio and a reasonable final transplant volume, and are currently being used in our laboratory in preclinical and pilot clinical trial islet transplantation (Fig. 12.5).

We employed measurements of transmembrane fluxes of fluorescein-conjugated dextran of several molecular weights to evaluate the MWCO of the AG/PLO microcapsules manufactured in our laboratory. This test showed a MWCO of about 100 kD, which can exclude immunoglobulins (Ig). This finding was further confirmed by encapsulation of hybridoma cell lines producing monoclonal antibodies

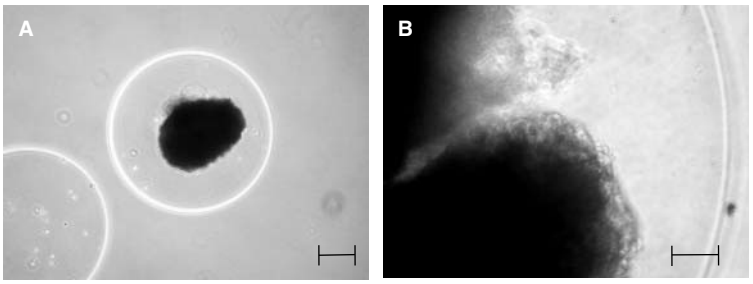



**Fig. 12.5 Human islets encapsulated in alginate/poly-L-ornithine microcapsules**. (**a**) Bar = 100 μm. (**b**) Bar = 40 μm

(MAb). Unlike AG/PLL capsules, AG/PLO capsules did not allow MAb leakage, thereby proving the Ig impermeability.

#### 12.3.2.2 Conformal Microcapsules

The possibility of manufacturing very small microcapsules composed of a thin, film-like membrane that adheres tightly to the islet surface has engendered increasing interest. The advantages of this approach are improved cell access to nutrients and the reduced final transplant volume, which could increase the choice of potential graft sites. We have developed a method for manufacturing conformal microcapsules (CM) for islet microencapsulation (Calafiore et al. 1996) (Fig. 12.6). PEG, AG, and dextran in a 1:1:1 volume ratio were vigorously shaken until a fine microdroplet emulsion was formed. Highly-purified rat or human islets were added to the emulsion and subsequently rocked on a rotating plate to facilitate entrapment of individual islets into each microdroplet. The islet-containing emulsion was gently poured into a $CaCl_2$ bath under continuous stirring, until complete microdroplet gelling was achieved. The microbeads were then sequentially coated with PLO and finally with AG. Although we have generated both conventional and conformal microcapsules, our focus has been primarily on MSM, which have been shown to provide superior immunoprotection (Basta et al. 2004). MSM prevent immune rejection of both allo- and xenograft islets in diabetic animal models while not occupying an excessively large space.

#### 12.3.2.3 Capsule Biocompatibility

Artificial materials introduced into the body are considered "foreign" and may elicit inflammatory responses. Development of new encapsulation materials (Desai et al. 1999) and refinements in established polymers have considerably alleviated this problem. Thus, most devices do not elicit major inflammatory responses when implanted "empty," although fibroblast overgrowth (fibrosis) on the surface of the device may occur. This response varies with implant size and geometry, as well as with the site of implantation. The peritoneal cavity and the subcutaneous tissue

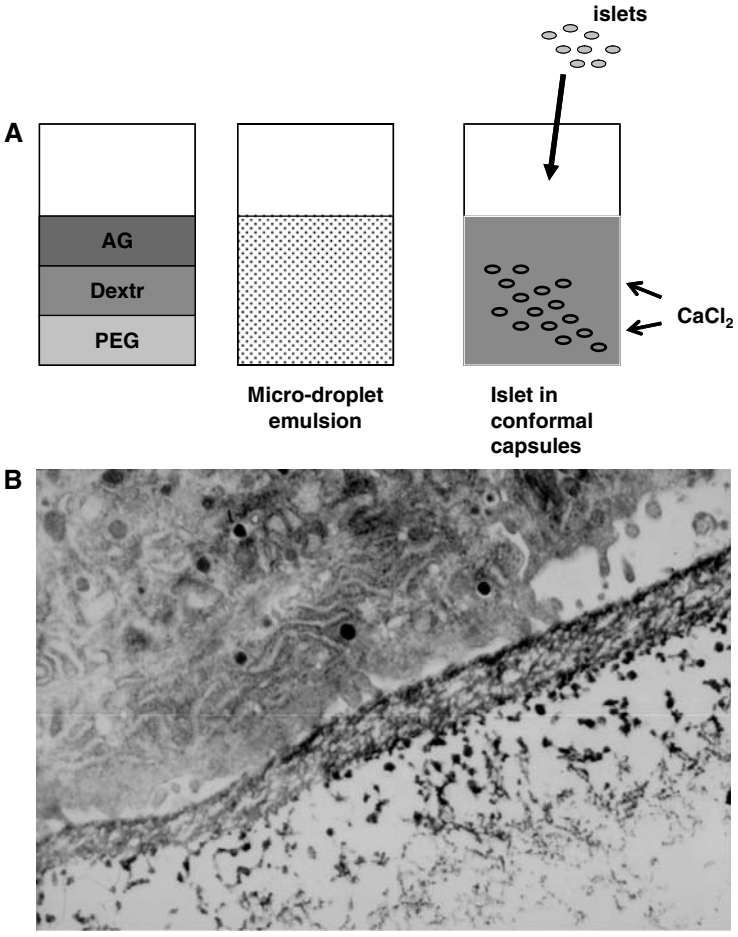



**Fig. 12.6 Generation of conformal microcapsules.** (**a**) Schematic representation of the microemulsification process for manufacturing conformal microcapsules (see text for details). Dextr, dextran. (**b**) Transmission electron micrograph of an empty microcapsule showing the capsule multilayered membrane

react quite vigorously, regardless of the grafted materials. Even autologous blood may elicit an inflammatory response if it leaks into the peritoneum. The consensus is that smaller devices are better tolerated than larger ones.

Although neovascularized membranes that represent new versions of older macrodevices are still being tested (Loudovaris et al. 1999), most attention is currently focused on microcapsules, with special regard to those formulated with AG-derived polymers. AG purity is pivotal for its biocompatibility. Empty microcapsules produced with our purified AG and coated with PLO and an outer AG film have been studied extensively and proven not to elicit any inflammation in rodents, dogs, pigs, primates, or humans (Fig. 12.7).

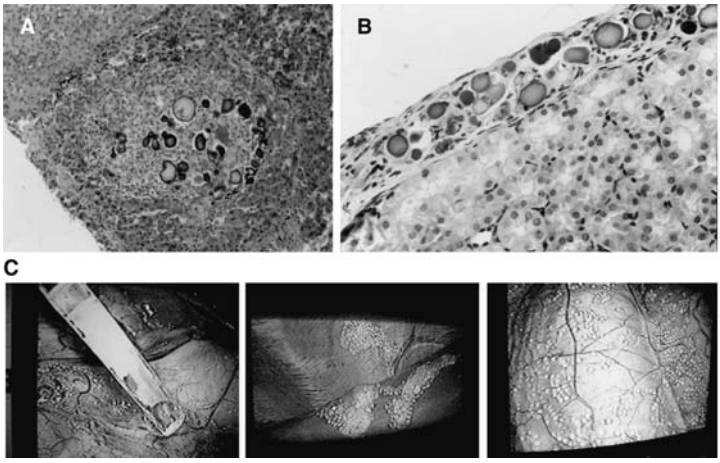



**Fig. 12.7 Empty alginate/poly-L-ornithine microcapsules following different periods in vivo.** (**a**) Adult pig liver (30 days). (**b**) Subcapsular renal region of adult rats (30 days). (**c**) Peritoneal cavity of *Cynomologous* monkeys (15 days). No inflammatory cell overgrowth is detected around the capsules

### *12.3.3 Encapsulation of Alternatives to Human Islets*

Using our AG/PLO microencapsulation procedure, we initiated a pilot clinical trial, with the permission and under the surveillance of the Italian Ministry of Health, in which microencapsulated human islets were grafted intraperitoneally into T1DM patients without immunosuppression. However, owing to the limited availability of cadaveric human islets there is a need to develop alternative sources of β-like cells; using microencapsulation for islet protection may increase the need for islets even further: judging from work with rodents it takes more encapsulated islets to normalize glycemia than naked islets. The versatility of the AG/PLO microencapsulation procedure makes it suitable for encapsulation of both pancreatic islets isolated from several animal species and humans, as well as loose cells (i.e., hepatocytes, Sertoli cells, engineered insulin-producing cells) whose experimental or clinical use may be relevant.

One alternative to human islets explored in our laboratory is the use of adult porcine islets. Porcine and human intermediate metabolisms are known to be quite similar. Recently, pig islet grafts were shown to reverse hyperglycemia in diabetic primates immunosuppressed by blockage of the costimulatory pathway, which can be seen as excellent support for the principle of using pig islets in humans. However, a number of serious obstacles have to be surmounted to allow the use of porcine islets in human transplantation, including prevention of xenograft rejection, recipient exposure to pathogens, and isolation of intact and functional islets.

Given the great risks involved with systemic immunosuppression, we investigated the potential of microencapsulation in xenotransplantation of pig islets

and demonstrated that both adult and neonatal porcine islets could reverse hyperglycemia in diabetic rodents and large-size animals (Luca et al. 2005). These experiments utilized composite microcapsules that permitted inclusion of complementary immunoprotection strategies (see below).

Potential transmission across species barriers of porcine endogenous retroviruses (PERV) represents a major obstacle to the application of microencapsulated porcine islets in human transplantation (Patience et al. 1997), although no clear proof of harmful effects has yet been presented. Interestingly, the PERV problem has been revisited by the same authors that brought it up in 1997, discounting possible harmful effects in humans (Fishman and Patience 2004). Moreover, retrospective studies conducted in patients that had been grafted with porcine islet tissue have not shown signs of PERV transmission years after transplantation (Elliott et al. 2000). Finally, microcapsules may represent a barrier for PERV (Elliott et al. 2000), although macrodevices do not seem to provide this advantage (Pakhomov et al. 2005).

Adult pig islets are extremely fragile and vulnerable to agents used in islet isolation. Consequently, these islets tend to break apart easily within days of isolation, for example, during changes of the medium, which also poses a problem in encapsulation. Adult pig islet encapsulation must be conducted very carefully, avoiding stirring of the islet/AG mixture and maintaining the encapsulated islets at 24°C prior to use.

Another possible alternative to human islets is the use of engineered insulin-producing cells. Encapsulation of loose cells is problematic, since protrusion of single cells through the capsular membrane could elicit an immune response. Accumulation of inflammatory cells around the capsule could provoke fibrosis, resulting in reduced nutrient access to the encapsulated cells. Furthermore, immune reactive cells and molecules may penetrate into the capsule through the openings created by the protruding cells and damage cells within the capsule. In fact, we have consistently observed that breakage/discontinuation or indentation of microcapsule membrane, which unmasks the underlying cationic layer, can itself signal the presence of a foreign body to the recipient immune system (Basta et al. 2004) (Fig. 12.8).

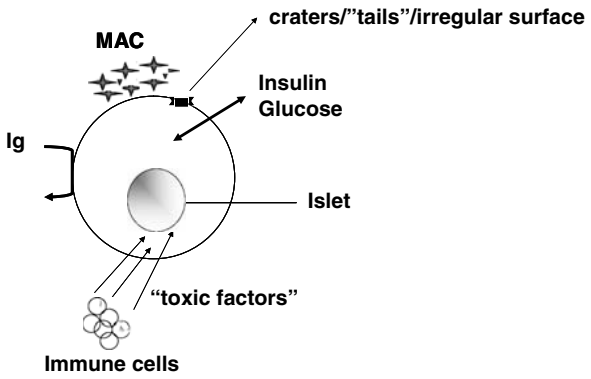



**Fig. 12.8 Schematic representation of the microcapsule membrane functional properties and potential risks of malfunction**

Formation of islet-like cell clusters prior to encapsulation may help overcome this problem, allowing encapsulation without protruding loose cellular material.

## 12.4 Immunology of Microencapsulated Cell Transplants

### *12.4.1 Immune Responses Activated by the Transplant*

The original principle of immunoisolation was based upon physical separation of the grafted islet cells from the host immune system, which prevents cell-to-cell contact, thereby circumventing direct antigen presentation and activation of CD8 T cells (Luca et al. 2000). However, indirect antigen presentation by macrophages uptaking shed antigens that leak out through the capsule membrane may activate CD4 T cells, which play a major role in xenograft rejection. Although the activated CD4 T cells would be prevented from direct contact with the encapsulated cells, they may secrete cytokines (IL2, IL5, IFNγ), some of which could induce production of additional cytokines and proinflammatory molecules by macrophages (IL1, TNFα, IL6, histamine, prostaglandins, leukotrienes) (Grewal and Flavell 1998). In addition to participating in the crosstalk between immune cells, these cytokines are small enough to penetrate the capsule membrane through its pores and may directly damage the encapsulated islet cells, for example, by induction of apoptosis (Weber et al. 1999).

Another cytokine produced by macrophages, IL8, was shown in recent in-vitro studies to be associated with multiple effects in neutrophils, including shape changes, lysosome enzymes release, induction of respiratory burst, generation of superoxides and hydrogen peroxide, generation of bioactive lipids, and increase in adhesion molecule expression. IL8 induces chemotaxis of CD4 and CD8 human peripheral blood T cells both in vitro and in vivo. Intradermal injection of human IL8 causes a rapid concentration-dependent neutrophil infiltration in all animal species examined so far. Likewise, subcutaneous injection of IL8 also causes T-cell migration into the injection site. Moreover, lipopolysaccharide (LPS), which may contaminate the collagenase used for pancreas digestion in islet isolation, potentiates IL8 action. IL8 production is further potentiated by TNFα released from monocytes.

### *12.4.2 Complementary Immunoprotection Strategies*

As discussed above, although microcapsules effectively prevent immune destruction of islet allografts, they provide far less efficient protection for xenografts, which had led some investigators to propose treatment of recipients of encapsulated islet xenografts with short-term or small-dose courses of immunosuppression. We believe that this strategy would considerably weaken the concept of immunoprotection provided by cell encapsulation. Rather, we favor potentiation of the microcapsule immunobarrier competence with complementary antioxidizing (Luca et al. 2000) and anti-inflammatory (Ricci et al. 2005) agents (Fig. 12.9). Another

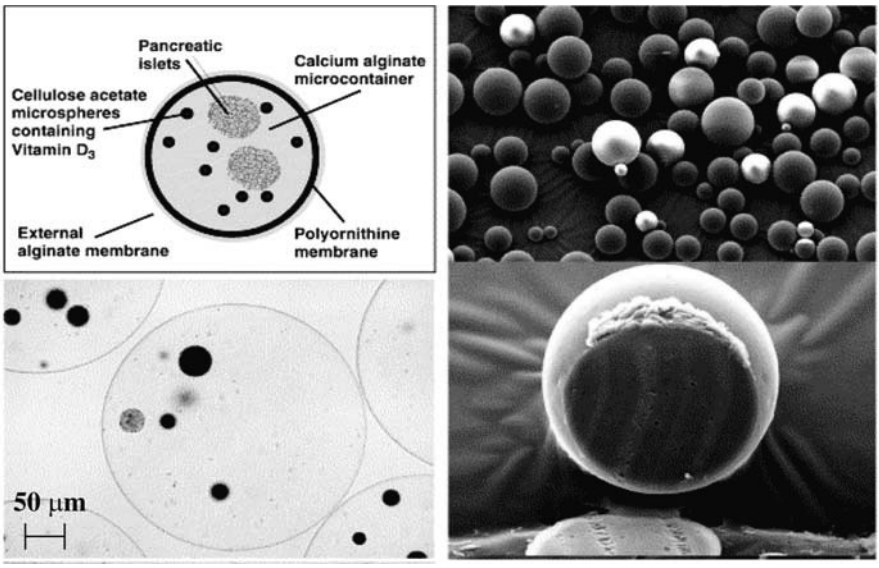



**Fig. 12.9 Multicompartmental microcapsules**

strategy developed in our laboratory is based on preculture of microencapsulated neonatal pig islets on monolayers of homologous prepubertal Sertoli cells, which release multiple growth factors, and antiapoptotic, antioxidizing, and immunomodulatory molecules. Using this system we have shown that microencapsulated neonatal porcine islet xenografts precultured with Sertoli cells for long periods of time can reverse hyperglycemia in diabetic NOD mice (Luca et al. 2005). If reproduced in larger mammals, this procedure could represent a major breakthrough for encapsulated islet grafts.

## 12.5 In-Vivo Applications

### *12.5.1 Experimental Systems*

The original microcapsules devised by Lim and Sun were designed to be grafted intraperitoneally because their size resulted in a large final graft volume that could not be accommodated at other sites. This system was successfully used for grafting encapsulated islets into mice and rats (Lum et al. 1991; de Vos et al. 2009), although rodents with spontaneous autoimmune diabetes (NOD mice, BB rats) have shown lower remission rates than animals with streptozotocin-induced hyperglycemia (Fan et al. 1990). In contrast, very few reports documented reversal of hyperglycemia coupled with exogenous insulin withdrawal in larger mammals (Lanza et al. 1999). In fact, aside from quite dated reports from our own (Brunetti et al. 1991) and a few other laboratories (Sun et al. 1996), no reproducible data were generated in animals

larger than rodents. The same is true of microencapsulated porcine islet xenografts into diabetic primates, which apparently succeeded in one case (Sun et al. 1996) but failed elsewhere (Elliott et al. 2005). The successful exception consisted of a trial of an AG/PLL-microencapsulated porcine islet xenograft into spontaneously diabetic, nonimmunosuppressed monkeys. In these animals, full remission of hyperglycemia and exogenous insulin withdrawal were achieved in all recipients, and were sustained in some of them for extraordinarily long periods of time through 3 years of post-transplantation follow-up (Sun et al. 1996). This striking, still unmatched, result demonstrates that under the best conditions microcapsules may constitute an effective and biocompatible immunoselective barrier for xenograft islets in the absence of general immunosuppression.

Our studies with encapsulated islets in diabetic rodents required a larger number of islets, compared with experiments with naked islets, for achieving normoglycemia. Such an overload is difficult to achieve in large-size animals, and ultimately humans, as the number of islets needed is prohibitively large. Thus, it is possible that studies with microencapsulated islets in large animals failed owing to the suboptimal islet number used. It is also possible that the peritoneal cavity does not represent the best site for encapsulated islet grafts, given the low oxygen tension at this site (Wu et al. 1999).

An alternative approach, which has gained attention recently, has utilized prevascularized beds, in which small-size microcapsules containing the islets can be deposited. The beds can be placed in the mesenteric area, thereby being drained into the portal vein and the liver, which is a major target of insulin action. Another advantage of this approach is that the microcapsules are retrievable and can be replaced with fresh microencapsulated islets in the event of graft exhaustion. Conformal microcapsules, which occupy a small space, are particularly suitable for this method. They can be embedded within a prevascularized pouch as previously described (de Vos et al. 1997), with great benefit for nutrient supply and metabolite exchange.

### *12.5.2 Pilot Human Clinical Trials Using Microencapsulated Islet Allografts in Patients with T1DM*

Pioneering attempts to graft encapsulated islets in patients with T1DM have been reported since the early 1990s. Our laboratory initiated early trials using a vascular chamber made of coaxial vascular prostheses directly anastomosed to the vascular network as arterial bypasses or A-V shunts. The chamber contained microencapsulated human islets in the interspace between the inner and outer prosthetic membranes. The capsules were immersed in the plasma ultrafiltrate and benefited from high oxygen and nutrient supply directly from blood circulation. Likewise, insulin was secreted directly into the blood flow. These pilot studies showed, on one hand, clear advantages in terms of metabolic outcome (two patients exhibited basal and postmeal near normal C-peptide levels, as opposed to pretransplant absence of the hormone), but, on the other hand, the device likely exposed the patients to severe risks in terms of thrombosis and other vascular events (Calafiore et al. 2006).

Other researchers grafted microencapsulated human islets intraperitoneally in a patient already bearing a kidney graft, hence under general immunosuppression (Soon-Shiong et al. 1994). The patient exhibited serum C-peptide, and following a second encapsulated islet graft went off insulin. However, the immunosuppression clouded the interpretation of the contribution of cell encapsulation to the long-term graft survival.

In the mid-1990s a small number of patients were grafted intraperitoneally with microencapsulated porcine islets without immunosuppression. No evidence of graft function, in terms of improved metabolic control, was shown at that time, and neither serum nor urinary C-peptide was detected. Nine years following transplantation, laparotomy of one of the recipients showed the presence of capsules in the abdominal cavity, some of which still contained live pig islet cells, but the majority of cells appeared to be necrotic (Elliott et al. 2007).

More recently, we have embarked on a pilot human clinical trial with microencapsulated human islets transplanted into patients with long-standing T1DM without immunosuppression. Out of ten patients enrolled, so far only four cases have been completed. Patients received the grafts intraperitoneally, under local anesthesia and ultrasound echography guidance, except for one case that received three subsequent grafts, the last of which was delivered under general anesthesia by laparoscopic surgery. In all cases we observed no adverse reactions to the grafting procedure, nor any evidence of immune sensitization. All the patients showed a decline in exogenous insulin consumption to about half of the pretransplantation level, except for one case that temporarily suspended insulin injections. Serum C-peptide was detected 100–480 days post-transplantation, reaching peaks of 1.8 ng/ml in one patient (Fig. 12.10) (Calafiore et al. 2006).

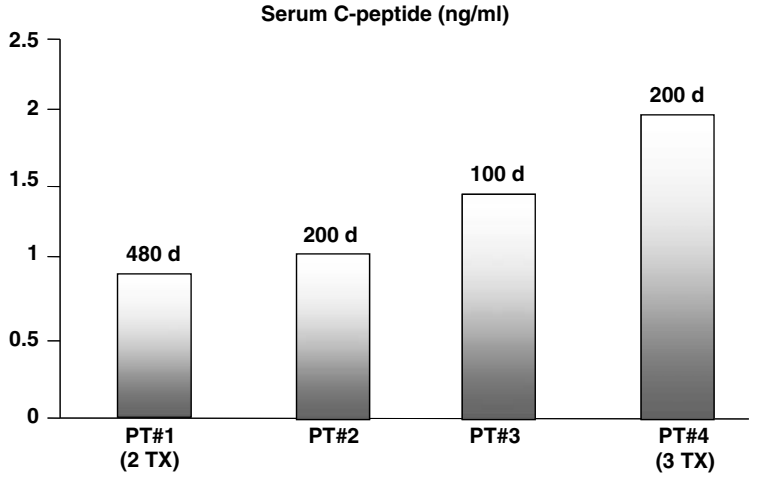



**Fig. 12.10 Serum C-peptide levels in patients transplanted with microencapsulated human islets during a long-term follow-up**

## 12.6 Summary and Future Perspectives

Despite its long history, cell encapsulation is still in its infancy. Several problems have to be resolved before this approach can become applicable to human therapy.

The present microencapsulation process itself is quite satisfactory. Although new polymeric materials for encapsulation are being generated, the foreseeable trend is not to move away from alginates (de Vos et al. 2009). Ultrapure, endotoxin-free AG can be generated and safely stored without significant changes in its physicochemical properties and represents a biocompatible and effective material for cell immunoprotection that is suitable for clinical use.

Long-term durability and retrievability of transplanted microcapsules have to be further pursued in order to comply with strict regulations laid out by health agencies for clinical application.

The increasing success of artificial scaffolds will likely promote efforts to incorporate microencapsulated islets within laminar prevascularized membranes to improve nutrient and oxygen supply and the kinetics of insulin release.

In the short- to midterm, when the ban on use of pig islets in humans is likely to be lifted, microencapsulated neonatal pig islets could be the best method of applying the system to patients with T1DM. Finally, if studies on generation of insulin-producing β-like cells from stem cells are successful, another powerful cell source could flank porcine islets for microencapsulation and transplantation in patients with T1DM.

**Acknowledgments** Technical assistance of Dr. Pia Montanucci is gratefully acknowledged. This work has been supported by the Consorzio Interuniversitario per I Trapianti d'Organo, Rome, Italy.

## References

Babensee JE, Sefton MV (2000) Viability of HEMA-MMA microencapsulated model hepatoma cells in rats and the host response. Tissue Eng 6: 165–182.

Baruch L, Machluf M (2006) Alginate-chitosan complex coacervation for cell encapsulation: effect on mechanical properties and on long-term viability. Biopolymers 82: 570–579.

Basta G, Sarchielli P, Luca G, et al. (2004) Optimized parameters for microencapsulation of pancreatic islet cells: an in vitro study clueing on islet graft immunoprotection in type 1 diabetes mellitus. Transpl Immunol 13: 289–296.

Brunetti P, Basta G, Falorni A, et al. (1991) Immunoprotection of pancreatic islet grafts within artificial microcapsules. Int J Artif Organs 14: 789.

Calafiore R (1992) Transplantation of microencapsulated pancreatic islets contained in a vascular prosthesis: preliminary results. ASAIOJ 38(1): 34–37.

Calafiore R, Basta G (2007) Artificial pancreas to treat type 1 diabetes mellitus. Methods Mol Med 140: 197–236.

Calafiore R, Basta G, Osticioli L, et al. (1996) Coherent microcapsules for pancreatic islet transplantation: a new approach for bioartificial pancreas. Transpl Proc 28: 812–813.

Calafiore R, Basta G, Luca G, et al. (1998) Transplantation of allogeneic/xenogeneic pancreatic islet containing coherent microcapsules in adult pigs. Transpl Proc 30: 482.

Calafiore R, Basta G, Luca G, et al. (2006) Microencapsulated pancreatic islet allografts into non-immunosuppressed patients with type 1 diabetes: first two cases. Artificial pancreas to treat type 1 diabetes mellitus. Diabetes Care 29: 137–138.

Colton CK (1995) Implantable biohybrid artificial organ. Cell Trans 4: 415–436.
Darrabie MD, Kendall WF Jr, Opara EC (2005) Characteristics of poly-L-ornithine-coated alginate microcapsules. Biomaterials 26: 6846–6852.
De Castro, Orive G, Hernandez RM, et al. (2005) Comparative study of microcapsules elaborated with three polycations (PLL, PDL, PLO) for cell immobilization. J Microencapsul 22: 303–315.
de Vos P, Wolters GH, van Schilfgaarde R (1994) Possible relationship between fibrotic overgrowth of alginate-polylysine-alginate microencapsulated pancreatic islets and the microcapsule integrity. Transpl Proc 26: 782–783.
de Vos P, Hillebrands JL, De Haan BJ, et al. (1997) Efficacy of a prevascularized expanded polytetrafluoroethylene solid support system as a transplantation site for pancreatic islets. Transplantation 63: 824–830.
de Vos P, Andersson A, Tam SK, et al. (2006) Advances and barriers in mammalian cell encapsulation for treatment of diabetes. Immunol Endocr Metabol Agents Med Chem 6: 139–153.
de Vos P, Bucko M, Gemeiner P, et al. (2009) Multiscale requirements for bioencapsulation in medicine and biotechnology. Biomaterials 30: 2559–2570.
Desai TA, Chu WH, Rasi G, et al. (1999) Microfabricated biocapsules provide short-term immunoisolation of insulinoma xenografts. Biomed Microdevices 1: 131–138.
Dusseault J, Tam SK, Ménard M, et al. (2006) Evaluation of alginate purification methods: effects on polyphenol, endotoxin, and protein contamination. J Biomed Mater Res A 76: 243–251.
Elbert DL, Hubbel JA (2001) Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering. Biomacromolecules 2: 430–441.
Elliott RB, Escobar L, Garkavenko O, et al. (2000) No evidence of infection with porcine endogenous retrovirus in recipients of encapsulated porcine islet xenografts. Cell Transplant 9: 895–901.
Elliott RB, Escobar L, Tan PL, et al. (2005) Intraperitoneal alginate-encapsulated neonatal porcine islets in a placebo-controlled study with 16 diabetic cynomolgus primates. Transpl Proc 8: 3505–3508.
Elliott RB, Escobar L, Tan PL, et al. (2007) Live encapsulated porcine islets from a type 1 diabetic patient 9.5 yr after xenotransplantation. Xenotransplantation 14: 157–161.
Emerich DF, Thanos CG, Goddard M, et al. (2006) Extensive neuroprotection by choroid plexus transplants in excitotoxin lesioned monkeys. Neurobiol Dis 23: 471–480.
Fan MY, Lum ZP, Fu XW, et al. (1990) Reversal of diabetes in BB rats by transplantation of encapsulated pancreatic islets. Diabetes 39: 519–522.
Fishman JA, Patience C (2004) Xenotransplantation: infectious risk revisited. Am J Transplant 9: 1383–1390.
Grewal IS, Flavell RA (1998) CD40 and CD154 in cell-mediated immunity. Ann Rev Immunol 16: 111–135.
Iwata H, Ikada Y (1999) Agarose. In: Kuhtreiber WM, Lanza RP, Chick WL (eds) Cell Encapsulation Technology and Therapeutics. Birkhauser: Boston.
Iwata H, Amemiya H, Matsuda T, et al. (1989) Evaluation of microencapsulated islets in agarose gel as bioartificial pancreas by studies of hormone secretion in culture and by xenotransplantation. Diabetes 38: 224–225.
Kim SK, Choi J, Balmaceda EA, et al. (1999) Chitosan. In: Kuhtreiber WM, Lanza RP, Chick WL (eds) Cell Encapsulation Technology and Therapeutics. Birkhauser: Boston.
Kin T, O'Neil JJ, Pawlick R, et al. (2008) The use of an approved biodegradable polymer scaffold as a solid support system for improvement of islet engraftment. Artif Organs 32: 990–993.
Kobayashi T, Aomatsu Y, Iwata H, et al. (2006) Survival of microencapsulated islets at 400 days posttransplantation in the omental pouch of NOD mice. Cell Transplant 15: 359–365.
Lacy PE, Hegre OD, Gerasimidi-Vazeou A, et al. (1991) Subcutaneous xenografts of rat islets in acrylic copolymer capsules maintain normoglycemia in diabetic mice. Science 254: 1782–1784.

Lanza RP, Borland KM, Staruk JE, et al. (1992) Transplantation of encapsulated canine islets into spontaneously diabetic BB/Wor rats without immunosuppression. Endocrinology 131: 637–642.
Lanza RP, Beyer AM, Staruk JE, et al. (1993) Biohybrid artificial pancreas: long-term function of discordant islet xenografts in streptozotocin-diabetic rats. Transplantation 56: 1067–1072.
Lanza RP, Ecker DM, Kuhtreiber WM, et al. (1999) Transplantation of islets using microencapsulation: studies in diabetic rodents and dogs. J Mol Med 77: 206–210.
Lim F, Sun AM (1980) Microencapsulated islets as bioartificial endocrine pancreas. Science 210: 908–910.
Loudovaris T, Jacobs S, Young S, et al. (1999) Correction of diabetic NOD mice with insulinomas implanted within Baxter immunoisolation devices. J Mol Med 77: 219–222.
Luca G, Nastruzzi C, Basta G, et al. (2000) Effects of anti-oxidizing vitamins on in vitro cultured porcine neonatal pancreatic islet cells. Diabetes Nutr Metab 6: 301–307.
Luca G, Nastruzzi C, Calvitti M, et al. (2005) Accelerated functional maturation of isolated neonatal porcine cell clusters: in vitro and in vivo results in NOD mice. Cell Transplant 14: 249–261.
Lum ZP, Tai IT, Krestow M, et al. (1991) Prolonged reversal of diabetic state in NOD mice by xenografts of microencapsulated rat islets. Diabetes 40: 1511–1516.
Maki T, Otsu I, O'Neil JJ, et al. (1996) Treatment of diabetes by xenogeneic islets without immunosuppression: use of a vascularized bioartificial pancreas. Diabetes 45: 342–347.
Marsich E, Borgogna M, Donati I, et al. (2008) Alginate/lactose-modified chitosan hydrogels: a bioactive biomaterial for chondrocyte encapsulation. J Biomed Mater Res A 84: 364–376.
Morch A, Donati I, Strand BL, et al. (2007) Molecular engineering as an approach to design new functional properties of alginate. Biomacromolecules 8: 2809–2814.
Murua A, Portero A, Orive G, et al. (2008) Cell microencapsulation technology: towards clinical application. J Controlled Release 132: 76–83.
Nair LS, Laurencin CT (2007) Biodegradable polymers as biomaterials. Prog Polym Sci 32: 762–798.
Orive G, Hernández RM, Gascón AR, et al. (2003) Cell encapsulation: promise and progress. Nat Med 9: 104–107.
Pakhomov O, Martignat L, Honiger J, et al. (2005) AN69 hollow fiber membrane will reduce but not abolish the risk of transmission of porcine endogenous retroviruses. Cell Transplant 10: 749–756.
Patience C, Takeuchi Y, Weiss RA (1997) Infection of human cells by an endogenous retrovirus of pigs. Nat Med 3: 282–286.
Petruzzo P, Pibiri L, De Giudici MA, et al. (1991) Xenotransplantation of microencapsulated pancreatic islets contained in a vascular prosthesis: preliminary results. Transpl Int 4: 200–204.
Ricci M, Blasi P, Giovagnoli S, et al. (2005) Ketoprofen controlled release from composite microcapsules for cell encapsulation: effect on post-transplant acute inflammation. J Control Release 3: 395–407.
Sakai S, Mu C, Kawabata K, et al. (2006) Biocompatibility of subsieve-size capsules versus conventional-size microcapsules. J Biomed Mater Res 78: 394–398.
Sakai S, Hashimoto I, Kawakami K (2007) Agarose-gelatin conjugate for adherent cell-enclosing capsules. Biotechnol Lett 29: 731–735.
Sawhney S (1999) Poly(ethylene glycol). In: Kuhtreiber WM, Lanza RP, Chick WL (eds) Cell Encapsulation Technology and Therapeutics. Birkhauser: Boston.
Sawhney A, Hubbell JA (1992) Poly(ethylene oxide)-graft-poly(l-lysine) copolymers to enhance the biocompatibility of poly(l-lysine)-alginate microcapsule membrane. Biomaterials 13: 863–870.
Sawhney A, Pathak CP, Hubbell JA (1994) Modification of Langerhans islet surfaces with immunoprotective poly(ethylene glycol) coatings. Biotechnol Bioeng 44: 383–386.
Sefton MV, Stevenson WTK (1993) Microencapsulation of live animal cells using polyacrylates. Adv Polymer Sci 107: 145.

Soon-Shiong P, Heintz RE, Merideth N, et al. (1994) Insulin-independence in a type I diabetic patient after encapsulated islet transplantation. Lancet 343: 950–951.

Strand BL, Gåserød O, Kulseng B, et al. (2002) Alginate-polylysine-alginate microcapsules: effect of size reduction on capsule properties. J Microencapsul 9: 615–630.

Sun X, Ma X, Zhou D, et al. (1996) Normalization of diabetes in spontaneously diabetic cynomologous monkeys by xenografts of microencapsulated porcine islets without immunosuppression. J Clin Invest 98: 1417–1422.

Tessmar JK, Göpferich AM (2007) Customized PEG-derived copolymers for tissue-engineering applications. Macromol Biosci 5: 23–39.

Thanos CG, Calafiore R, Basta G, et al. (2007) Formulating the alginate-polyornithine biocapsule for prolonged stability: evaluation of composition and manufacturing technique. J Biomed Mater Res A 83: 216–224.

Vallbacka JJ, Sefton MV (2007) Vascularization and improved in vivo survival of VEGF-secreting cells microencapsulated in HEMA-MMA. Tissue Eng 13: 2259–2269.

Weber CJ, Safley S, Hagler M, et al. (1999) Evaluation of graft-host response for various tissue resources and animal models. In: Hunkeler D, Prokop A, Cherrington A et al. (eds) Bioartificial Organs II, Technology, Medicine & Materials. Ann NY Acad Sci 875: 233–254.

Wu H, Avgoustiniatos ES, Swette L, et al. (1999) In situ electrochemical oxygen generation with an immunoisolation device. In: Hunkeler D, Prokop A, Cherrington A et al. (eds) Bioartificial Organs II, Technology, Medicine & Materials. Ann NY Acad Sci 875: 105–125.

Yasuhara T, Date I (2007) Intracerebral transplantation of genetically engineered cells for Parkinson's disease: toward clinical application. Cell Transplant 16: 125–132.

# Chapter 13
# Prevention of Islet Graft Rejection and Recipient Tolerization

**Eitan M. Akirav and Kevan C. Herold**

**Abstract** Type 1 diabetes is thought to be caused by an immune-mediated destruction of β cells that occurs over years and continues even after presentation with hyperglycemia. Adaptive immune mechanisms are believed to be primary mediators of this process. Immune memory for the initial process that resulted in β-cell failure, the high frequency of alloreactive T cells, and the nonphysiologic environment into which islets have been transplanted all create obstacles for successful reversal of diabetes with islet replacement. In this chapter we review the immune mechanisms that are thought to be responsible for β-cell destruction and discuss the obstacles that stand in the way of the successful achievement of β-cell replacement.

## 13.1 Introduction

Studies in animal models of type 1 diabetes (T1DM), done largely in nonobese diabetic (NOD) mice and in humans, have described an immune-mediated loss of β-cell mass from the time of initiation of insulitis through presentation with hyperglycemia and afterward until there is complete loss of β cells. Results from the Diabetes Prevention Trial 1 (DPT-1) have described changes in insulin secretion from prediabetes to presentation with hyperglycemia, and other studies have depicted the progressive loss of insulin secretion after diagnosis (Steele et al. 2004; Sosenko et al. 2006; Akirav et al. 2008). Whereas the data from animal studies and clinical investigations are largely consistent, other studies indicate that a simple linear loss of β-cell function may be an oversimplification of a process that involves an undulating downhill course.

Variation in the rate of progression of β-cell loss may be due to waxing and waning of the inflammatory response because of exposure of new antigens, intercurrent insults to β cells involving infectious agents, metabolic demands, or other

E.M. Akirav (✉)
Section of Human Translational Immunology, Yale University School of Medicine, New Haven, CT 06520, USA
e-mail: eitan.akirav@yale.edu

S. Efrat (ed.), *Stem Cell Therapy for Diabetes*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4_13,

factors, and a continuous attempt at β-cell regeneration that slows the progression (von Herrath et al. 2007). The inflammatory process is associated with β-cell regeneration, but at the same time exposes new antigenic epitopes that become the drivers of a broadening autoimmune response (Sreenan et al. 1999; Sherry et al. 2006). Ultimately, the failure to contain and counter this enlarging process results in presentation of clinical disease.

As the destruction of β cells is mediated by both innate and adaptive immunity, the development of immunological memory for islet antigens is inevitable. The presence of memory response is beneficial to the organism in the case of reinfection with a specific pathogen and ensures rapid clearance of the pathogen. However, in the case of autoimmune diabetes, the presence of immunological memory presents a major hurdle in mediating islet survival following transplantation or regeneration and requires creative ways to mediate transplant immunomodulation and recipient tolerization. Current concepts and advances in islet transplantation are discussed in the following sections.

## 13.2 The Immunological Process in T1DM

### *13.2.1 Cellular and Humoral Mediators of Beta-Cell Destruction*

Data showing the presence of autoreactive lymphocytes and autoantibodies in animal models of T1DM and in prediabetic and diabetic patients provide strong evidence for the role of the immune system in the development of the disease. Anti-islet autoantibodies can be detected years before clinical presentation and can be used to identify risk for the disease (Yu et al. 1996; Yu et al. 2001). Data from the DPT-1 have shown that patients' nondiabetic relatives with three or four autoantibodies progress to diabetes at a rapid rate; approximately 90% of such individuals will reach diagnosis within 6 years (Type 1 Diabetes Study Group 2002). A similar high rate of progression is seen in relatives with a positive islet cell autoantibody (ICA) with impairment of glucose tolerance (Sherr et al. 2008). It follows, therefore, that the use of immune-modulating drugs should delay disease progression and may offer novel points of clinical intervention to ameliorate disease severity and facilitate graft survival in diabetic patients. As most current anti-inflammatory treatments are directed toward the modulation of the immune response of T and B cells, we focus on the role of adaptive immunity in T1DM and following islet transplantation.

#### 13.2.1.1 The Role of T Cells in T1DM

CD4 and CD8 T cells are part of the cellular adaptive immune system. Their ability to react to a near-infinite number of different cellular targets, or antigens, renders these cells indispensable for host protection against various pathogens. The activation of T cells is mediated by antigen-presenting cells (APCs), which are a part of the innate immune system. APCs can present different antigenic determinants,

or epitopes, that lead to the activation of T cells. One of the important features of adaptive immunity is the development of immune memory. Memory T cells arise following the primary response to invading pathogens. Both CD4 and CD8 memory T cells are long-lived and are found in both peripheral tissues and distinct lymphoid organs. Their presence is important for preventing the recurrence of disease and provides long-lasting protection against invading pathogens,

Although T cells react predominantly to foreign antigens, some T cells are capable of recognizing the host self-antigens. These self-reactive or autoreactive T cells are thought to mediate the development of various autoimmune diseases. Autoreactive T cells are detected in the circulation of diabetic and prediabetic patients. Studies using antigen-HLA tetramers for islet-specific antigens, such as insulin, showed the presence of insulin-reactive CD8 T cells in the blood of new-onset diabetic patients (Toma et al. 2005). These cells were long-lived and could be detected in patients with long-standing T1DM of 23 years or more (Toma et al. 2005). The presence of detectable autoreactive CD8 T cells for such a prolonged period of time is thought to be important in the process of islet graft rejection. In fact, the presence of insulin B-positive CD8 T cells, detected by HLA-$A2^{InsB}$ staining, was correlated with increased autoreactivity to islet graft and might possibly contribute to islet graft rejection (Pinkse et al. 2005). The role of the immune system in mediating T1DM development was recently demonstrated in a provocative study by Voltarelli et al. (Voltarelli et al. 2007). Newly-diagnosed diabetic patients with autoantibodies against glutamic acid decarboxylase (GAD) were subjected to nonmyeloablative hematopoietic stem cell transplantation. Under this procedure the majority of T cells were removed and patients were reconstituted with their own autologous hematopoietic stem cells. Subsequent to reconstitution, 14 out of 15 patients showed insulin independency for various periods of time and demonstrated hemoglobin $A_{1c}$ levels of less than 7%, indicating good glycemic control. This report demonstrated the benefits of "immunological reset" in mediating remission from T1DM.

T-cell reactivity to islet antigens is not limited to insulin alone. T cells isolated from diabetic patients also showed reactivity to other autoantigens and multiple epitopes within the same protein (Yu et al. 1996; Ott et al. 2004). The manner by which new antigen specificities are recognized by the immune system has been described as instructive rather than stochastic, where a consistent pattern is followed. Recent studies in the NOD mouse support the observation that insulin is indeed a key "primary" antigen. After insulin-reactive T cells were detected, T cells reactive to islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) were found as well. T-cell reactivity to IGRP is almost always secondary to insulin and seems to be dependent upon the recognition of insulin by T cells, as insulin-deficient mice did not show any T-cell reactivity to IGRP (Nakayama et al. 2005). Tolerance to insulin does not only reduce T-cell reactivity to other antigens, but can also prevent the development of diabetes. In a recent study, Krishnamurthy et al. examined the effects of tolerance induction to insulin on development of diabetes in NOD mice with IGRP-specific transgenic CD8 T cells (Krishnamurthy et al. 2008). IGRP T-cell receptor (TCR) transgenic NOD mice developed accelerated diabetes,

when compared with nontransgenic animals; however, if tolerance to insulin was induced, these mice remained protected and diabetes-free. This study highlights the importance of antigen hierarchy and suggests a novel point for clinical intervention for diabetes prevention.

CD8 T cells are thought to mediate direct β-cell killing in the islet via their recognition of antigen-Class I MHC complexes on β cells, but CD4 helper T cells are also required for the development of T1DM. Early studies that examined the ability of human β cells to express Class II MHC molecules on their cell surface identified the expression of these molecule in situations where β cells were exposed to proinflammatory cytokines such as interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) in vitro (Pujol-Borrell et al. 1987). Although it is plausible that β cells may directly activate CD4 T cells in the islet, it is more likely that T-cell activation occurs predominantly in secondary lymphoid organs, such as the pancreatic lymph nodes (LNs) (Tang et al. 2006). In the NOD model a natural wave of β-cell death, which occurs by day 20 after birth, leads to the detection of β-cell antigens in pancreatic LNs. The presentation of these antigens by resident APCs can lead to the activation of diabetogenic T cells and may contribute to the development of the disease (Hoglund et al. 1999). The subsequent β-cell injury and death during the chronic attack on the islet may further sustain the activation of autoimmune cells (Turley et al. 2003). These two mechanisms for continuing β-cell death and constant exposure to islet antigens may account for the epitope spreading discussed above.

Similar to CD8 T cells, antigen-specific CD4 T cells can also be found in the peripheral blood of patients. Reijonen et al. have used Class II MHC tetramers with a GAD65 peptide to enumerate antigen-specific cells (Reijonen et al. 2002; Reijonen and Kwok 2003; Reijonen et al. 2003). Owing to the low frequency of these cells, an initial expansion in vitro was needed to identify the cells by flow cytometry. This requirement creates a problem in enumerating the actual number of circulating antigen-specific cells but does provide a very specific means of tracking individual cells over time in the same patient.

#### 13.2.1.2 The Role of B Cells in T1DM

In T1DM, B cells are thought to play key roles as both producers of autoantibodies and antigen-presenting cells (Wong et al. 2004). In addition to CD4 and CD8 T cells, B cells are also present in islet infiltrates in NOD mice (Anderson and Bluestone 2005) and in islets of newly-diagnosed patients (Itoh et al. 1993). The presence of islet-cell antibodies (ICAs) in the serum, including anti-insulin, anti-GAD65, and anti-IA-2A autoantibodies, among others, is used to assess the risk of progression to overt T1DM in families as well as in the general population (Knip and Siljander 2008). In the NOD mouse, early expression of autoantibodies can signal an earlier onset of T1DM (Yu et al. 2000). Similarly to epitope spreading in T-cell antigens, epitope spreading in B-cell recognition of ICAs occurs in a typical manner. In the sera of individuals at risk for developing T1DM, anti-insulin antibodies appear at a young age (Yu et al. 1996). Anti-GAD65 antibodies are predominantly directed against the middle- and C-terminal epitopes. The gradual spreading of N-

to C-terminal epitopes has been documented in both prediabetic and newly-diabetic patients (Hoppu et al. 2004; Ronkainen et al. 2006).

Although detected in prediabetic and diabetic patients and animal models, autoantibodies do not appear to mediate β-cell destruction directly. This notion was supported by animal studies that examined the role of B cells and autoantibodies in the development of T1DM in the NOD mouse. B-cell-deficient NOD mice showed reduced incidence of T1DM, but insulitis and diabetes did in fact occur (Wong et al. 2004). Moreover, disease frequency did not increase when antibodies from sera of diabetic NOD mice were injected into B-cell-deficient mice (Serreze, et al. 1998). In humans, a case study describing a patient with X-linked agammaglobulinemia and T1DM provided further support for the idea that T1DM can develop in the absence of autoantibodies (Martin et al. 2001).

The fact that B cells express high levels of Class II MHC molecules and that upon activation can upregulate the expression of costimulatory molecules suggests that they may serve as APCs. Unlike other APCs, however, B cells are capable of efficiently capturing soluble antigens via their B-cell receptor and promoting the uptake of antibody–antigen complexes by other APCS, such as dendritic cells and macrophages. These findings suggest that B cells may promote the activation of autoreactive T cells in an antigen-specific manner. Indeed, NOD mice transgenic for a single antibody directed against an irrelevant antigen showed reduced insulitis and diabetes incidences similar to those of B-cell-deficient NOD mice (Silveira et al. 2002). Similar results were also observed in NOD mice with a reduced B-cell repertoire.

There were, however, other studies that supported the requirement for B lymphocytes in the pathogenesis. Using immunodeficient parents, Greeley et al. showed that maternal transmission of antibodies is a critical environmental parameter influencing the ontogeny of T-cell-mediated destruction of islet β cells in NOD mice (Greeley et al. 2002). Wen et al. studied the effects of anti-human CD20 antibody in NOD mice that expressed human CD20 on the surface of B cells and reported that the antibody could reduce the rate of diabetes in the mice and even reverse diabetes in hyperglycemic animals (Hu et al. 2007). More recent studies indicate that anti-CD20 mAb can prevent recurrent diabetes in NOD mice that receive syngeneic islet transplants (Hu et al. 2008). These data suggest a role for B lymphocytes even in the later stages of disease, most likely as antigen-presenting cells in an evolving T-cell response that is ultimately responsible for β-cell destruction.

### *13.2.2 The Role of Costimulatory Molecules in Activating Auto- and Alloreactive Immune Responses*

Several key molecules contribute to immune activation and islet destruction in islet grafts. Many of these molecules determine T-cell activation/inhibition, and their manipulation may slow or even prevent islet graft rejection. These molecules include members of the MHC molecules, TNF receptor family members, and members of

the B7/constimulatory molecules. The Latter are expressed on activated lymphocytes and may be interesting candidates for inhibition of islet graft rejection (Truong et al. 2006).

The activation of T cells requires activation of the T-cell receptor (Signal 1) and a nonspecific costimulatory signal (Signal 2) (Bretscher and Cohn 1970; Jenkins et al. 1987). These signals can be delivered by the CD28 and CD40L molecules, whose importance in the development of diabetes has been shown in manipulated NOD mice. CD28 expressed on T cells can bind its ligands, which are known as CD80 (B7-1) and CD86 (B7-2), on APCs. In fact, NOD mice treated with anti-CD86 blocking antibodies and NOD mice that are deficient in CD86 show, respectively, decreased incidence or complete absence of diabetes (Lenschow et al. 1995; Lenschow et al. 1996). The complexity of the CD28-B7 signaling has been highlighted by the fact that CD80-knockout NOD mice and CD80/CD86-double-knockout NOD mice show increased incidence and severity of T1DM (Salomon et al. 2000; Salomon and Bluestone 2001). These seemingly contradictory data suggested that CD80 and CD86 may play a differential nonredundant role in the progression of the disease.

Salomon et al. have shown that the presence of CD80 is required for normal development of regulatory T cells (Tregs). Tregs have been recognized as important regulators of autoimmunity and inflammation and are known to prevent the development of T1DM in NOD mice (Salomon et al. 2000). B7-CD28 interactions are naturally regulated in T cells to contain the inflammatory response. Activated T cells, as well as Tregs, express the molecule cytotoxic T-lymphocyte-associated antigen-4 (CTLA4). This receptor recognizes the B7 molecules at a higher affinity than CD28, but delivers a negative signal to T cells (Ikemizu et al. 2000). The role of CTLA4 in T-cell activation is highlighted by observations showing increased lymphoproliferative disease in CTLA4-deficient mice and increased autoimmunity upon CTLA4 blockage (Tivol et al. 1995; Karandikar et al. 1996). Current application of CTLA4 in promotion of islet graft survival is discussed in the following sections. Other new members of the B7 family that can also deliver negative signals to T cells have been identified. One of these, B7-H3, was shown to be expressed on dendritic cells after activation (Chapoval et al. 2001; Suh et al. 2003). B7-H3-deficient mice show reduced activation of Th1-type and accumulation of Th2-like T cells. Th1 cells are characterized by their production of proinflammatory molecules, such as IFN$\gamma$, IL12, and TNF$\alpha$, and are thought to be the predominant pathogenic T-cell type in T1DM.

Programmed death-1 (PD1) is an additional immunomodulatory molecule that contributes to T-cell activation during allograft rejection, and its expression is induced in activated T and B cells (Agata et al. 1996). The generation of PD1-deficient mice revealed its role in immune homeostasis by the fact that these mice exhibited increased spleen size, elevated serum Ig, and other autoimmune symptoms (Nishimura et al. 1999). In NOD mice, blocking of the PD1 ligand 2 (PD-L2) induces earlier onset of diabetes (Ansari et al. 2003).

Overall, the findings described above suggest multiple candidates for immune intervention for the prevention of islet rejection. In the following sections we discuss

current clinical evidence suggesting that immune rejection of islet allografts may be minimized using novel agents that can interact with key inflammatory molecules.

## 13.3 Mechanisms of Islet Transplant Failure in T1DM

In clinical trials glucose levels have been restored in patients following islet transplantation without the need for exogenous insulin (Alejandro et al. 1997; Jaeger et al. 1997; Shapiro et al. 2000; Ryan et al. 2005; Close et al. 2005; Lakey et al. 2006). One of the main challenges in islet transplantation has been the need for chronic immunosuppression to prevent graft rejection of allogeneic islets. Many of the immunosuppressive agents themselves have proven to be toxic to β cells and serve as diabetogenic factors. In an attempt to minimize the detrimental effect of these drugs, clinical protocols have been developed to minimize islet toxicity, most notably the "Edmonton protocol." This protocol avoided the use of glucocorticoids, employing small doses of the calcineurin inhibitor tacrolimus instead, and rapamycin was an important part of the immunosuppressive regimen. This initial experience was essentially replicated in a multicenter trial conducted by the Immune Tolerance Network study (Shapiro et al. 2006). However, despite these encouraging results, it became clear that insulin independence was short-lived, with a relapse of insulin dependence in about 90% of recipients at 5 years post-transplantation (Ryan et al. 2005). Studies in animal models of T1DM and patients show that both immunological and metabolic factors contribute to islet dysfunction.

Several immunological processes contribute to acute islet failure following transplantation. During islet isolation, conditions of enzymatic digestion and acute hypoxia lead to activation of islet-resident cells, such as macrophages, dendritic cells (DCs), and islet endothelial cells (ECs). These activated cells produce proinflammatory cytokines such as interleukin-1 (IL1), IFNγ, and TNF-α, which can ultimately contribute to oxidative stress by the production of nitric oxide (Andre-Schmutz et al. 1999). This activation of innate immune responses in the isolated tissues may reduce islet viability and impair engraftment, as both donor-derived cells and recipient innate immunity contribute to islet failure. Islet transplantation is often done by intraportal infusion into the liver, which has been considered an optimal site for transplantation because it is nutrient-rich and placement is relatively easy. However, the endothelial cells of the hepatic sinusoids may promote lymphocyte recruitment by upregulation of adhesion molecules on transplanted islets, such as ICAM1 and P-selectin (Campbell et al. 1989; Martin et al. 1996). In addition, the hepatic endothelium may also produce proinflammatory cytokines, such as IL1, IFNγ, and TNFα (Tan et al. 2002). The accumulation of these cytokines and the activation of resident antigen-presenting cells not only affect islet engraftment and acute rejection but also contribute to the generation of adaptive immunity against the graft.

Transplanted islets are generally obtained from allogeneic donors: MHC matching is impractical in most circumstances, and its contribution to improvement in outcome is not proven. In experimental systems, MHC matching has shown both positive and negative effects on allorejection and recurrent autoimmune disease

(Makhlouf et al. 2002; Bittscheidt et al. 2004). Differences in HLAs and minor antigens between donor and recipient result in the activation of alloreactive T cells, which exist in relatively high frequencies and pose a serious challenge to various organ transplantations. As noted earlier, β-cell death is thought to be a major activator of innate responses that trigger adaptive responses. In the case of islet transplants, this would involve activation of responses to both allo- and autoantigens. Indeed, activated alloreactive T cells have been shown to mediate islet graft rejection in humanized mice (Wu et al. 2008). In these studies, immunodeficient mice reconstituted with a human immune system showed graft rejection of human islets from donors with mismatched HLA. Recently, an examination of a complete pancreatic graft in a diabetic patient revealed the presence of alloreactive T cells in the graft at relatively high frequencies (Velthuis et al. 2009). This is important since the frequency of these alloreactive T cells was not increased in the circulation of the patients, which presents a challenge in identifying patients undergoing allogeneic islet rejection.

In addition to the allogeneic response, the presence of β-cell reactive memory T and B cells can further contribute to islet rejection. Indeed, even completely MHC-mismatched islet allografts are susceptible to autoimmune destruction in experimental systems (Kupfer et al. 2005). As discussed above, memory T and B cells, which contribute to the development of T1DM in the first place, persist in the lymphoid organs of diabetic patients long after the development of the disease. Monti et al. have recently demonstrated that the continuous use of immunosuppressive drugs following the Edmonton protocol results in a state of general lymphopenia but with preferential expansion of antigen-specific T cells, among which were GAD-specific autoreactive T cells (Monti et al. 2008). Measurements of these and other antigen-specific T cells may prove to be useful as biomarkers to identify immune responses that can lead to recurrence of auto- or even alloimmunity (Mallone et al. 2004). The presence of GAD-specific T cells has been shown to lead to a more rapid loss of islet graft in T1DM (Huurman et al. 2008). In a different study, examination of infiltrating lymphocytes in explanted pancreata from patients detected the presence of CD8 T cells. These cells exhibited alloreactivity to donor pancreas, but more interestingly showed the presence of insulin specificity, thus demonstrating the role of insulin-specific CD8 T cells in mediating pancreas graft rejection (Velthuis et al. 2009).

## 13.4 Tolerance Induction and Prolongation of Islet Survival

### *13.4.1 The Use of Anti-Inflammatory Agents for Induction of Islet Tolerance*

The Edmonton protocol does not induce immunologic tolerance, and continuous immune suppression is required to prevent a recurrence of the diabetes. Thus development of a way to induce immune tolerance to the graft is the ultimate goal of immune therapy. Trials to establish immune tolerance may be best done in this

setting because, unlike other solid organ transplants, such as kidney and liver, the consequences of graft failure are not as dire. One approach for tolerance induction that has shown promise is the blockade of costimulatory molecules. This strategy is based largely on preclinical studies showing that delivery of Signal 1 in the absence of a costimulatory signal (Signal 2) leads to nonresponsiveness of an antigen-specific T cell (Jenkins et al. 1987). Anti-CD40L monoclonal antibody, which binds and blocks the interactions between CD40L on T cells and CD40 on antigen-presenting cells, has been very effective in preclinical nonhuman primate allograft studies. Rhesus monkeys treated with anti-CD40L accepted their islet allografts for up to 476 days and had near-normal glucose levels (Kenyon et al. 1999).

However, these studies are limited because no large-animal models of autoimmune diabetes are available, so the effects of costimulatory blockade on autoimmunity in large animals remain unknown. In the NOD mouse, CD40/CD40L interactions were shown to be necessary for induction of autoimmunity, as treatment with anti-CD40L completely blocked disease induction in young NOD mice, but had no effect in older mice (>9 weeks)(Balasa et al. 1997). The protection mechanism appeared to involve expansion of regulatory T cells, but the hopes for this promising agent were dashed when humans treated with an anti-CD40L antibody developed thromboembolic symptoms, which led the manufacturer to discontinue development (Kawai et al. 2000). Blockade of another costimulatory pathway using CTLA4Ig was also shown to be effective in preclinical islet transplant studies (Lenschow et al. 1992; Levisetti et al. 1997). The concerns regarding exacerbation of spontaneous diabetes in prediabetic NOD mice by B7 blockade may not be relevant to the recurrence of disease in the setting of islet allografts (Lenschow et al. 1995; Lenschow et al. 1996; Salomon et al. 2000). The combined blockade of CD40/CD40L and CD28/B7 signals resulted in immune tolerance to autografts even in the presence of a highly-pathogenic T cell clone (Rigby et al. 2008).

As discussed above, activating and inhibitory members of the B7 family present with an opportunity to block islet inflammation and rejection of an islet graft. A study by Subudhi et al. has shown that expression of the second ligand of PD-1, PD-L1, under the rat insulin promoter can induce more rapid islet graft rejection (Subudhi et al. 2004). In this study, islets from C57BL/6 mice were acutely rejected upon transplantation into 129 mice, predominantly by CD8 T cells. Interestingly, mice transgenic for PD-L1 developed diabetes spontaneously, highlighting the importance of this ligand in initiating and propagating an anti-β-cell immune response. These results suggest that PD-1 blockage may reduce islet rejection by a reduction in T-cell infiltration. The direct effect of B7 signaling on T-cell activation was illustrated by two pivotal reports. Fallarino et al. demonstrated that engagement of the B7 molecule on DCs can promote the production of IFNγ. This effect was mediated by the high-affinity B7 receptor, CTLA4. The increased expression of IFNγ induces the expression of the enzyme indoleamine 2,3-dioxygenase (IDO), which induces tryptophan metabolism and thus has been shown to contribute to reduced viability of effector T cells (Fallarino et al. 2003). In a complementary study by Munn et al., IDO expression was detected predominantly in CD4 T cells,

resulting in the downregulation of activated CD8 cells due to the depletion of tryptophan (Munn et al. 2004). Although neither studies have shown a direct effect of IDO and B7 expression in transplantation, it is reasonable to assume that activation of IDO by the B7 family members may reduce T- cell activation and islet injury.

Treatment with Fc receptor (FcR) nonbinding anti-CD3 mAbs has been shown to attenuate the loss of insulin production in patients with new-onset T1DM (Herold et al. 2002; Herold et al. 2005). The drug was administered for 2 weeks but the effects have lasted beyond a year, suggesting that immunologic tolerance was induced. Murine studies have supported the value of this mechanism by suggesting that regulatory T cells are induced by the mAb (Belghith et al. 2003). Hering et al. reported that noninsulin-requiring remission was induced in four out of six patients with T1DM who received allogeneic islets from a single donor when it was administered together with a combination of rapamycin, low dose of FK-506, and anti-CD3 mAb (Teplizumab) (Hering et al. 2001; Hering et al. 2004). They suggested that addition of anti-CD3 mAb induced $CD4^+CD25^+$ regulatory T cells that inhibited responses to the donor islets but not to third-party lymphocytes in a mixed-lymphocyte reaction.

### *13.4.2 Barriers to Long-Term Success*

Lastly, the long-term results of allogeneic islet transplants have been disappointing. In the Edmonton series, only about 10% of islet allograft recipients were insulin-independent 5 years after transplantation, despite an initial rate of 85% insulin-independence at 1 year (Ryan et al. 2005). The reasons for failure are difficult to discern since there are no biomarkers available that can reliably measure the autoimmune response, so damage to the islet grafts can only be identified by loss of function. Recurrent autoimmunity has been suggested by the finding of an increased frequency of autoantigen reactive T cells, identified by Class I MHC tetramers, in peripheral blood (Pinkse et al. 2005). Other studies have drawn attention to the direct toxic effects of immune modulators on islet grafts, which may impair insulin production and/or directly damage the cells. Calcineurin inhibitors inhibit insulin gene transcription and can exacerbate diabetes in patients treated for renal allograft rejection with these agents (Herold et al. 1993; Oetjen et al. 2003). Using a model of β-cell regeneration, Nir et al. showed that rapamycin inhibits β-cell proliferation (Nir et al. 2007). It has been proposed that placement of the islets into the liver may be particularly problematic as they are exposed to levels of these toxic immune modulators that are higher than those in peripheral blood (van der Windt et al. 2008), and other locations have been suggested to minimize this toxicity.

## 13.5 Summary and Conclusions

The delay and/or prevention of islet graft rejection poses real challenges in the clinical setting. In this chapter, we discussed the principal immunological factors

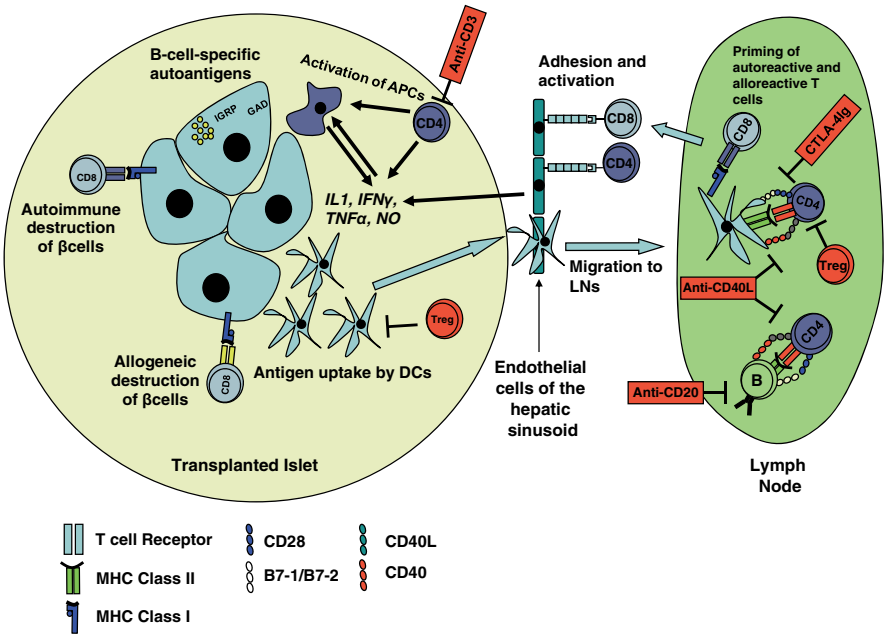



**Fig. 13.1 Schematic representation of islet graft rejection and potential immunomodulatory interventions.** After islet transplantation, activation of the immune system results in graft rejection. Immune activation includes the priming of autoimmune T and B cells in the LNs. Antigen uptake by host and donor DCs can prime autoreactive and alloreactive T cells. In addition, activated B cells may further contribute to T-cell priming. Activated CD4 and CD8 cells migrate from the LNs and enter the islet. Whereas CD8 are thought to mediate direct β-cell killing, CD4 may further activate resident antigen-presenting cells, as well as liver endothelial cells, both of which can produce proinflammatory mediators. These mediators contribute to islet damage and additional immune priming. The use of anti-inflammatory mediators, such as anti-CD40L and CTLA4Ig, may reduce APC–T-cell interaction and T-cell priming. The use of anti-CD3 and anti-CD20 antibodies can act directly on activated T and B cells, respectively. During this process regulatory T cells are induced, further dampening T-cell activity and contributing to islet survival

that contribute to the development of β-cell autoimmunity and several key candidates for reducing antigraft immunity with minimal toxicity. Figure 13.1 describes the complex interaction between immune activation and the maintenance of normal β-cell function. The emergence of new alloreactive lymphocytes and the preexisting immune memory in the diabetic patient requires a deeper understanding of the mechanisms that control T- and B-cell activation in the transplanted patient.

The use of several anti-inflammatory molecules, such as anti-CD40L, anti-CD3, and CTLA4Ig, may allow for reduction of anti-islet autoimmunity (Fig. 13.1). Although a substantial amount of knowledge has been gained in understanding the factors that contribute to immune activation and suppression, currently no single or combined anti-inflammatory therapy can provide complete protection against islet rejection. The inherent challenges in islet transplantation are not derived solely

from the lack of sufficient islet material, but also from the fact that islet grafts, unlike solid organ transplants, are placed in an artificial environment, such as the liver. This artificial site reduces the chance of normal islet engraftment not only via immunological factors but also owing to metabolic stress. The most recent success in islet transplantation following the Edmonton protocol has been shadowed by the high rate of diabetes relapse in the vast majority of islet recipients, and recent data suggest that the common anti-inflammatory agents may prove detrimental to β-cell survival.

The ability to produce β cells from stem cells or to induce insulin production and glucose responsiveness in alternative tissue progenitors may provide not only more abundant sources of β cells, but also an opportunity for genetic manipulation of the cells prior to transplantation. Such genetic manipulations may override the physiological barriers described above and facilitate successful islet engraftment. Genetic engineering of stem-cell-derived β cells, such as by introducing immune modulators, may overcome immunologic barriers. For example, the adenovirus E3 genes can modulate immune responses to islet allografts and autoreactivity in NOD mice (von Herrath et al. 1997; Efrat et al. 2001; Pierce et al. 2003,). Kojaoghlanian et al. recently showed that genetic engineering of β cells using lentiviral vectors expressing the adenovirus E3 genes for gp19 and RIDα/β prevented rejection of allogeneic βTC3 cells in mice (Kojaoghlanian et al. 2009). Other approaches involve expression of IL10 or other immune modulatory genes (Chen et al. 2007). The use of stem-cell-derived β cells expressing a minimal protein repertoire may reduce the development of an autoimmune response against the graft. Furthermore, generation of induced pluripotent stem (iPS) cells from the patient's own somatic cells can eliminate the development of alloreactivity following transplantation. Alternatively, increasing the expression of anti-inflammatory/anti-apoptotic proteins in the engineered β cell may contribute to increased β-cell survival even under inflammatory conditions. These exciting alternatives may provide a solution to the current side effects of immunosuppressive drugs on the patient's immune system as well as on the function of insulin-producing cells post-transplantation.

In summary, future clinical trials may provide a better chance of tolerance induction and islet graft acceptance. It is possible that by combining several anti-inflammatory reagents islet rejection can be minimized. Immune modulation in conjunction with increased β-cell availability from various stem cell sources may provide an opportunity for a better clinical outcome in diabetic patients.

## References

Agata Y, Kawasaki A, Nishimura H, et al. (1996) Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 8: 765–772.

Akirav E, Kushner JA, Herold KC (2008) Beta-cell mass and type 1 diabetes: going, going, gone?. Diabetes 57: 2883–2888.

Alejandro R, Lehmann R, Ricordi C, et al. (1997) Long-term function (6 years) of islet allografts in type 1 diabetes. Diabetes 46: 1983–1989.

Anderson MS, Bluestone JA (2005) The NOD mouse: a model of immune dys-regulation. Annu Rev Immunol 23: 447–485.

Andre-Schmutz I, Hindelang C, Benoist C, et al. (1999) Cellular and molecular changes accompanying the progression from insulitis to diabetes. Eur J Immu-nol 29: 245–255.
Ansari MJ, Salama AD, Chitnis T, et al. (2003) The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. J Exp Med 198: 63–69.
Balasa B, Krahl T, Patstone G, et al. (1997) CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice. J Immunol 159: 4620–4627.
Belghith M, Bluestone JA, Barriot S, et al. (2003) TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. Nat Med 9: 1202–1208.
Bittscheidt H, Bektas H, Winkler M, et al. (2004) Impact of donor-recipient MHC matching on experimental islet allotransplant survival in naive and presensitized Lewis rats. Transplantation 78: 162–164.
Bretscher P, Cohn M (1970) A theory of self-nonself discrimination. Science 169: 1042–1049.
Campbell IL, Cutri A, Wilkinson D, et al. (1989) Intercellular adhesion molecule 1 is induced on isolated endocrine islet cells by cytokines but not by reovirus infection. Proc Natl Acad Sci USA 86: 4282–4286.
Chapoval AI, Ni J, Lau JS, et al. (2001) B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. Nat Immunol 2: 269–274.
Chen B, Kapturczak MH, Joseph R, et al. (2007) Adeno-associated viral vector-mediated interleukin-10 prolongs allograft survival in a rat kidney transplantation model. Am J Transplant 7: 1112–1120.
Close NC, Hering BJ, Eggerman TL (2005) Results from the inaugural year of the Collaborative Islet Transplant Registry. Transplant Proc 37: 1305–1308.
Efrat S, Serreze D, Svetlanov A, et al. (2001) Adenovirus early region 3(E3) immunomodulatory genes decrease the incidence of autoimmune diabetes in NOD mice. Diabetes 50: 980–984.
Fallarino F, Grohmann U, Hwang KW, et al. (2003) Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol 4: 1206–1212.
Greeley SA, Katsumata M, Yu L, et al. (2002) Elimination of maternally transmitted autoantibodies prevents diabetes in nonobese diabetic mice. Nat Med 8: 399–402.
Hering BJ, Kandaswamy R, Harmon JV, et al. (2004) Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 anti-body. Am J Transplant 4: 390–401.
Hering BJ, Kandaswany R, Harmon JV, et al. (2001) Insulin independence after single-donor islet transplantation in type 1 diabetes with hOKT3g-1(ala-ala), sirolimus, tacrolimus therapy. Am J Transplant 1: 180A.
Herold KC, Gitelman SE, Masharani U, et al. (2005) a single course of anti-CD3 monoclonal antibody hOKT3{gamma}1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. Diabetes 54: 1763–1769.
Herold KC, Hagopian W, Auger JA, et al. (2002) Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 346: 1692–1698.
Herold KC, Nagamatsu S, Buse JB, et al. (1993) Inhibition of glucose-stimulated insulin release from beta TC3 cells and rodent islets by an analog of FK506. Transplantation 55: 186–192.
Hoglund P, Mintern J, Waltzinger C, et al. (1999) Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J Exp Med 189: 331–339.
Hoppu S, Ronkainen MS, Kulmala P, et al. (2004) GAD65 antibody isotypes and epitope recognition during the prediabetic process in siblings of children with type I diabetes. Clin Exp Immunol 136: 120–128.
Hu CY, Rodriguez-Pinto D, Du W, et al. (2007) Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest 117: 3857–3867.
Hu C, Deng S, Wong FS, et al. (2008) Anti-CD20 treatment prolongs syngeneic islet graft survival and delays the onset of recurrent autoimmune diabetes. Ann N Y Acad Sci 1150: 217–219.

Huurman VA, Hilbrands R, Pinkse GG, et al. (2008) Cellular islet autoimmunity associates with clinical outcome of islet cell transplantation. PLoS ONE 3: e2435.

Ikemizu S, Gilbert RJ, Fennelly JA, et al. (2000) Structure and dimerization of a soluble form of B7-1. Immunity 12: 51–60.

Itoh N, Hanafusa T, Miyazaki A, et al. (1993) Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Invest 92: 2313–2322.

Jaeger C, Brendel MD, Hering BJ, et al. (1997) Progressive islet graft failure occurs significantly earlier in autoantibody-positive than in autoantibody-negative IDDM recipients of intrahepatic islet allografts. Diabetes 46: 1907–1910.

Jenkins MK, Pardoll DM, Mizuguchi J, et al. (1987) T-cell unresponsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state. Immunol Rev 95: 113–135.

Karandikar NJ, Vanderlugt CL, Walunas TL, et al. (1996) CTLA-4: a negative regulator of autoimmune disease. J Exp Med 184: 783–788.

Kawai T, Andrews D, Colvin RB, et al. (2000) Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. Nat Med 6: 114.

Kenyon NS, Chatzipetrou M, Masetti M, et al. (1999) Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. Proc Natl Acad Sci USA 96: 8132–8137.

Knip M, Siljander H (2008) Autoimmune mechanisms in type 1 diabetes. Autoimmun Rev 7: 550–557.

Kojaoghlanian T, Joseph A, Follenzi A, et al. (2009) Lentivectors encoding immunosuppressive proteins genetically engineer pancreatic beta-cells to correct diabetes in allogeneic mice. Gene Ther 16: 340–348.

Krishnamurthy B, Mariana L, Gellert SA, et al. (2008) Autoimmunity to both pro-insulin and IGRP is required for diabetes in nonobese diabetic 8.3 TCR transgenic mice. J Immunol 180: 4458–4464.

Kupfer TM, Crawford ML, Pham K, et al. (2005) MHC-mismatched islet allografts are vulnerable to autoimmune recognition in vivo. J Immunol 175: 2309–2316.

Lakey JR, Mirbolooki M, Shapiro AM (2006) Current status of clinical islet cell transplantation. Methods Mol Biol 333: 47–104.

Lenschow DJ, Herold KC, Rhee L, et al. (1996) CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes. Immunity 5: 285–293.

Lenschow DJ, Ho SC, Sattar H, et al. (1995) Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. J Exp Med 181: 1145–1155.

Lenschow DJ, Zeng Y, Thistlethwaite JR, et al. (1992) Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. Science 257: 789–792.

Levisetti MG, Padrid PA, Szot GL, et al. (1997) Immunosuppressive effects of human CTLA4Ig in a non-human primate model of allogeneic pancreatic islet transplantation. J Immunol 159: 5187–5191.

Makhlouf L, Kishimoto K, Smith RN, et al. (2002) The role of autoimmunity in islet allograft destruction: major histocompatibility complex class II matching is necessary for autoimmune destruction of allogeneic islet transplants after T-cell costimulatory blockade. Diabetes 51: 3202–3210.

Mallone R, Kochik SA, Laughlin EM, et al. (2004) Differential recognition and activation thresholds in human autoreactive GAD-specific T-cells. Diabetes 53: 971–977.

Martin S, Hibino T, Faust A, et al. (1996) Differential expression of ICAM-1 and LFA-1 versus L-selectin and VCAM-1 in autoimmune insulitis of NOD mice and association with both Th1- and Th2-type infiltrates. J Autoimmun 9: 637–643.

Martin S, Wolf-Eichbaum D, Duinkerken G, et al. (2001) Development of type 1 diabetes despite severe hereditary B-lymphocyte deficiency. N Engl J Med 345: 1036–1040.
Monti P, Scirpoli M, Maffi P, et al. (2008) Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells. J Clin Invest 118: 1806–1814.
Munn DH, Sharma MD, Mellor AL (2004) Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. J Immunol 172: 4100–4110.
Nakayama M, Abiru N, Moriyama H, et al. (2005) Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 435: 220–223.
Nir T, Melton DA, Dor Y (2007) Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 117: 2553–2561.
Nishimura H, Nose M, Hiai H, et al. (1999) Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 11: 141–151.
Oetjen E, Baun D, Beimesche S, et al. (2003) Inhibition of human insulin gene transcription by the immunosuppressive drugs cyclosporin A and tacrolimus in primary, mature islets of transgenic mice. Mol Pharmacol 63: 1289–1295.
Ott PA, Dittrich MT, Herzog BA, et al. (2004) T cells recognize multiple GAD65 and proinsulin epitopes in human type 1 diabetes, suggesting determinant spreading. J Clin Immunol 24: 327–339.
Pierce MA, Chapman HD, Post CM, et al. (2003) Adenovirus early region 3 antiapoptotic 10.4 K, 14.5 K, and 14.7 K genes decrease the incidence of autoimmune diabetes in NOD mice. Diabetes 52: 1119–1127.
Pinkse GG, Tysma OH, Bergen CA, et al. (2005) Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. Proc Natl Acad Sci USA 102: 18425–18430.
Pujol-Borrell R, Todd I, Doshi M, et al. (1987) HLA class II induction in human islet cells by interferon-gamma plus tumour necrosis factor or lymphotoxin. Nature 326: 304–306.
Reijonen H, Kwok WW (2003) Use of HLA class II tetramers in tracking antigen-specific T cells and mapping T-cell epitopes. Methods 29: 282–288.
Reijonen H, Kwok WW, Nepom GT (2003) Detection of CD4+ autoreactive T cells in T1D using HLA class II tetramers. Ann N Y Acad Sci 1005: 82–87.
Reijonen H, Novak EJ, Kochik S, et al. (2002) Detection of GAD65-specific T-cells by major histocompatibility complex class II tetramers in type 1 diabetic patients and at-risk subjects. Diabetes 51: 1375–1382.
Rigby MR, Trexler AM, Pearson TC, et al. (2008) CD28/CD154 blockade prevents autoimmune diabetes by inducing nondeletional tolerance after effector T-cell inhibition and regulatory T-cell expansion. Diabetes 57: 2672–2683.
Ronkainen MS, Hoppu S, Korhonen S, et al. (2006) Early epitope- and isotype-specific humoral immune responses to GAD65 in young children with genetic susceptibility to type 1 diabetes. Eur J Endocrinol 155: 633–642.
Ryan EA, Paty BW, Senior PA, et al. (2005) Five-year follow-up after clinical islet transplantation. Diabetes 54: 2060–2069.
Salomon B, Bluestone JA (2001) Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol 19: 225–252.
Salomon B, Lenschow DJ, Rhee L, et al. (2000) B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 12: 431–440.
Serreze DV, Fleming SA, Chapman HD, et al. (1998) B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Immunol 161: 3912–3918.

Shapiro AM, Lakey JR, Ryan EA, et al. (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343: 230–238.

Shapiro AM, Ricordi C, Hering BJ, et al. (2006) International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355: 1318–1330.

Sherr J, Sosenko J, Skyler JS, et al. (2008) Prevention of type 1 diabetes: the time has come. Nat Clin Pract Endocrinol Metab 4: 334–343.

Sherry NA, Kushner JA, Glandt M, et al. (2006) Effects of autoimmunity and immune therapy on beta-cell turnover in type 1 diabetes. Diabetes 55: 3238–3245.

Silveira PA, Johnson E, Chapman HD, et al. (2002) The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors. Eur J Immunol 32: 3657–3666.

Sosenko JM, Palmer JP, Greenbaum CJ, et al. (2006) Patterns of metabolic progression to type 1 diabetes in the Diabetes Prevention Trial-Type 1. Diabetes Care 29: 643–649.

Sreenan S, Pick AJ, Levisetti M, et al. (1999) Increased beta-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse. Diabetes 48: 989–996.

Steele C, Hagopian WA, Gitelman S, et al. (2004) Insulin secretion in type 1 diabetes. Diabetes 53: 426–433.

Subudhi SK, Zhou P, Yerian LM, et al. (2004) Local expression of B7-H1 promotes organ-specific autoimmunity and transplant rejection. J Clin Invest 113: 694–700.

Suh WK, Gajewska BU, Okada H, et al. (2003) The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. Nat Immunol 4: 899–906.

Tan M, Di Carlo A, Liu SQ, et al. (2002) Hepatic sinusoidal endothelium upregulates IL-1alpha, IFN-gamma, and iNOS in response to discordant xenogeneic islets in an in vitro model of xenoislet transplantation. J Surg Res 102: 229–236.

Tang Q, Adams JY, Tooley AJ, et al. (2006) Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nat Immunol 7: 83–92.

Tivol EA, Borriello F, Schweitzer AN, et al. (1995) Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 3: 541–547.

Toma A, Haddouk S, Briand JP, et al. (2005) Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients. Proc Natl Acad Sci USA 102: 10581–10586.

Truong W, Hancock WW, Anderson CC, et al. (2006) Coinhibitory T-cell signaling in islet allograft rejection and tolerance. Cell Transplant 15: 105–119.

Turley S, Poirot L, Hattori M, et al. (2003) Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. J Exp Med 198: 1527–1537.

Type 1 Diabetes Study Group (2002) Effects of insulin in relatives of patients with type 1 diabetes mellitus. N Engl J Med 346: 1685–1691.

van der Windt DJ, Echeverri GJ, Ijzermans JN, et al. (2008) The choice of anatomical site for islet transplantation. Cell Transplant 17: 1005–1014.

Velthuis JH, Unger WW, van der Slik AR, et al. (2009) Accumulation of autoreactive effector T cells and allo-specific regulatory T cells in the pancreas allograft of a type 1 diabetic recipient. Diabetologia 52: 494–503.

Voltarelli JC, Couri CE, Stracieri AB, et al. (2007) Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 297: 1568–1576.

von Herrath M, Sanda S, Herold K (2007) Type 1 diabetes as a relapsing-remitting disease?. Nat Rev Immunol 7: 988–994.

von Herrath MG, Efrat S, Oldstone MB, et al. (1997) Expression of adenoviral E3 transgenes in beta cells prevents autoimmune diabetes. Proc Natl Acad Sci USA 94: 9808–9813.

Wong FS, Wen L, Tang M, et al. (2004) Investigation of the role of B-cells in type 1 diabetes in the NOD mouse. Diabetes 53: 2581–2587.

Wu DC, Wieckiewicz J, Wood KJ (2008) Human regulatory T cells prevent islet allograft rejection. Clin Invest Med 31: S25.

Yu L, Cuthbertson DD, Maclaren N, et al. (2001) Expression of GAD65 and islet cell antibody (ICA512) autoantibodies among cytoplasmic ICA+ relatives is associated with eligibility for the Diabetes Prevention Trial-Type 1. Diabetes 50: 1735–1740.

Yu L, Rewers M, Gianani R, et al. (1996) Antiislet autoantibodies usually develop sequentially rather than simultaneously. J Clin Endocrinol Metab 81: 4264–4267.

Yu L, Robles DT, Abiru N, et al. (2000) Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. Proc Natl Acad Sci USA 97: 1701–1706.

# Index


**A**
α cells
  GLP1 in, 16
  glucagon-containing, 5, 11
  growth in *Pdx1*-deficient pancreas, 22
Acinar cells
  in-vitro dedifferentiation of
    into ductlike cells, 155
    E-cadherin-mediated cell-to-cell contact role in, 157
    molecular mechanisms of, 163
    NOTCH-signaling pathway effect on, 156–157
    into "uncommitted" progenitor state, 157
  plasticity of, 157
  transdifferentiation into β-cells, *see* Acinar-to-β-cell transdifferentiation
Acinar-to-β-cell transdifferentiation
  into ductal cells in vivo, 157
  into endocrine β-like cells, 158–159
  genetic lineage tracing of, 159
  into hepatocyte-like cells, 159
  immunohistochemical studies of, 154
  mechanism of
    EGF signaling, 159–160
    leukemia inhibitory factor, 159
    NGN3 expression, 160
    NOTCH–HES1 signaling activity, 160–161
    PAX4 and ARX, expression of, 160
  in models of β-cell regeneration, 116
Activin receptor II
  role in endocrine and islet development, 10
  signaling, 11
  *See also* Activins
Activins
  activin βB
    endoderm induction by, 209–210
    pancreas-inducing effect of, 6
  proendocrine effects of, 9
Adenovirus-transduced islets, rejection of, 99
Adenovirus vectors, 192
Adrenal ECM, adrenocortical cell growth on, 233–234
Adrenal insufficiency, 233
Adrenocortical cells on decellularized ECM scaffolds, 233–234
Adult cell reprogramming
  applications of, 183
  challenges in using, 197
  of hematopoietic cells, 184
  *MyoD*-induced, 184
  transdifferentiation, 183–184
Adult somatic cells, reprogramming of, 153
Adult stem cells
  definition of, 168
  in liver, 169
  *See also* Embryonic stem (ES) cells
Agarose microcapsules
  immunobarrier competence of, 246
  for islet immunoprotection, 245–246
Akirav, E. M., 263–274
Alejandro, R., 41–73
Alemtuzumab, 49, 65–67
Alginate-based microcapsules
  alginate/poly-L-ornithine microcapsules, 253
  biocompatibility of, 251
  conformal, 251
  morphology and size of, 249–251
  production of
    algae sources for, 247–248
    using microdroplet generator, 249
  storage issues, 248
  uses of, 248–249
  *See also* Microcapsules
Alginic acid derivatives, 245


S. Efrat (ed.), *Stem Cell Therapy for Diabetes,* Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-366-4,

Allogeneic islet transplants, long-term results of, 272
Aortic endothelium, 6, 15
AR42J cells
  BMP signaling in, 10
  differentiation and HGF, 14
ARX (transcription factor), 25
Aviv, V., 183–198

**B**
Badylak, S.F., 223–234
Baeyens, L., 153–163
BAPX1 (NKX family of transcription factors), 7
Basal insulin secretion, 148
Basement membrane, 17
Basta, G., 241–259
β-cell mass
  compensatory growth with obesity, 168
  determination of, 106
  following PPx, 108–109
  immune-mediated loss of, 263
  increase in, 106
  PDL-induced increase in, 114
  regeneration of, experimental models for, 106
    STZ-treated rats, 107
    toxin-induced β-cell ablation, 107–108
  regulation by glucocorticoid receptor signaling, 18
  restoration of, 105
β-cell replacement therapy, 85–86
β cells
  ablation by toxins, 107–108
  acinar origin of, 154
  anatomic location of, 137–138
  blood glucose homeostasis maintenance, 141
  deficiency of diabetes, treatment for, 144
  destruction of, 264
  development of
    EGF receptors role in, 14
    GLP1 and GIP role in, 16
    *Nkx6.1* and *Nkx.6.2* role in, 26
  ductal origin of, 114
  genetic ablation of
    KIR6.2 subunit, 110
    PANIC-ATTAC model, 111
    PDX1 expression, 110–111
    tamoxifen-inducible *c-Myc* expression, 111
  heterogeneity, 143
  inflammatory injury to, 42
  mass, *see* β-cell mass
  phenotype of, 142–143
  polarization of, 138–139
  proliferation of, agents stimulating, 124
  recognition of ICAs, 266
  regeneration
    inflammatory process associated with, 264
    in neonatal STZ-treated rats, 107
  replication
    G1/S checkpoint proteome, 86–87
    therapeutic relevance of, 85
    in vitro, 124–126
    in vivo, 123–124, 128
  role in T1DM, 266–267
  in subjects with impaired glucose tolerance, 146
  surrogate and normal, 146
  transdifferentiation of, 147
  turnover of, 143, 147
Beta cells, *see* β cells
Betacellulin, 108, 128
  β-cell regeneration and, 109
  endogenous role for, 13
Bile-duct-derived progenitors, 186
Biologic scaffold materials
  components of, 223
  ECM scaffolds, *see* ECM scaffolds
Bladder-drained pancreas transplant, complications of, 71–72
β-like cells, 154
  engraftment of, 161–162
  from transdifferentiated acinar cells, 161
  transdifferentiation of primary rat acinar cells into, 158–159
BM-derived MSC, *see* Bone-marrow-derived mesenchymal stem (BM-derived MSC)
BMPs, *see* Bone morphogenic proteins (BMPs)
Bone-marrow-derived mesenchymal stem (BM-derived MSC)
  differentiation into insulin-producing cells, 196–197
Bone morphogenic proteins (BMPs), 10
Bonner-Weir, S., 137–149
Bouwens, L., 153–163
BRAIN4 (Pou-domain protein) expression, 28
BrdU-positive endocrine and exocrine cells, 108
Bukys, M.A., 203–217
Burke, G.W., 41–73

**C**
Cadherins, expression of, 17
Calafiore, R., 241–251
Calsenilin, inhibition of, 17
CCKB, *see* Cholecystokinin-B (CCKB)
CD4 and CD8 T cells, 266
CDK6–cyclin D1, expression of, 92–93, 95, 98
CDK–cyclin-transduced islet grafts, 93, 95
Cell cycle inhibitors, 81
  in human β-cell, 96–97, 99
  pocket proteins, 92
Cell fate switching, 153–154
Cell microencapsulation, 245
  *See also* Microcapsules
Cell replacement therapy, 242
CGMS, *see* Continuous glucosemonitoring system (CGMS) profiles
Chitosan, 246–247
Cholecystokinin-B (CCKB), 108
Ciancio, G., 41–73
Clinical islet allotransplantation, *see* Islet transplantation (IT)
c-MET, HGF signaling to, 14
*c-Myc* expression, shutoff of, 111
Conformal microcapsules, manufacturing of, 251, 252
Conophylline, 107
Continuous glucosemonitoring system (CGMS) profiles
  from daily glucose measurements, 56
  preislet and postislet allotransplantation, 53, 55
Corticosteroids
  diabetogenic effect of, 48
  rejection therapy with, 72
  in SPK transplant recipients, 64–66
Cyclin D1 and CDK6, expression of, 92–93, 95, 99
Cyclopamine, 12

**D**
Daclizumab, 49
  in combination with rATG, 65
  as induction therapy, 64–65
Desert hedgehog (DHH) expression, 12
DHH expression, *see* Desert hedgehog (DHH) expression
D'Hoker, J., 105–116
De Leu, N., 105–116
Diabetes
  characterization of, 204
  risk factors associated with, 203
  T1DM, *see* Type 1 diabetes mellitus (T1DM)
  treatment of, 204
Diabetes Prevention Trial 1 (DPT-1), 263
Diabetic retinopathy (DR), 68
Differentiated cells
  nuclear reprogramming of, 183
  phenotypes, 184
Doxycycline-inducible expression of diphtheria toxin, 111
DPT-1, *see* Diabetes Prevention Trial 1 (DPT-1)
DR, *see* Diabetic retinopathy (DR)
Ductal ligation model, 176–177

**E**
E-cadherin expression, 17
ECM, *see* Extracellular matrix (ECM)
ECM scaffolds, 223
  adrenocortical cell growth on adrenal, 233–234
  bioactive properties of, 230
  collagen fiber architecture of, 225
  composition of
    glycosaminoglycans, 224–225
    porcine small-intestinal submucosa, 224
  decellularization of, 227
  dehydration of, 228
  gelation of, 229
  hydration of, 227–228
  pancreatic islet transplantation on
    cell sheet engineering for, 232
    efficacy of, 231
    MEKK1 caspase-induced death in, 231
    sheets of porcine SIS-ECM, 232
  powdered forms of, 229
  preparation of, 226–227
  structure of, 225–226
Efrat, S., 123–131
EGF, *see* Epidermal growth factor (EGF)
EGF receptors
  ERBB1 expression, 13–14
  role in β-cell growth, 14
Embryonic human beta cells, replication of, 89
Embryonic liver cells, in-vitro reprogramming of, 186–187
Embryonic pancreatic epithelium, 17
Embryonic stem (ES) cells
  cell cycle, 206–207
  culture of, 205
  directed differentiation into β cells
    challenges, 213–215

Embryonic stem (ES) cells (*cont.*)
definitive endoderm formation, 209–210
foregut endoderm formation, 210
islet cell maturation, 211–212
organizing centers, 207–208
pancreatic determination, 211
heterogeneity of, 214
state of, 204–205, 216
Endocrine cells
development of
activin expression in, 9
β cells, *see* β cells
endothelial cells and blood flow role in, 15–16
hedgehog signaling role in, 12
NGN3 role in, 23
NEUROD expression in, 24
Endocrine ECM architecture, 225–226
Endoderm markers, 170
Enteric-drained pancreas transplant, complications of, 72–73
Epidermal growth factor (EGF)
expression in embryonic pancreas, 13
receptors, *see* EGF receptors
Epigenetic changes
with 5-azacytidine, 184
in β-cell cycle control, 97
with PDX1, 191–192
Epithelial–mesenchymal interactions, 4
ES cells, *see* Embryonic stem (ES) cells
Etanercept, 49
Exocrine pancreas development, 17–18
Exogenous insulin therapy, 42, 61, 107, 258
Extracellular matrix (ECM), 223

**F**

Facultative stem cells, definition of, 168–169
Ferber, S., 183–198
Fetal liver cells, in-vitro reprogramming of, 186–187
FGFR2B, *see* FGF receptor 2B (FGFR2B)
FGF receptor 2B (FGFR2B), 8
FGFs, *see* Fibroblast growth factors (FGFs)
Fiaschi-Taesch, N., 85–100
Fibroblast growth factors (FGFs)
expression in pancreatic epithelium, 8
foregut endoderm formation by, 210
FOXA2 staining, 209

**G**

GATA4 and GATA6, expression of, 28
GDF11, *see* Growth differentiation factor 11 (GDF11)
*gdf11*-null mutant mice, 11
Genetic lineage tracing, 154–155
Gilbert, T.W., 223–234
GIP, *see* Glucose-dependent insulinotropic peptide (GIP)
Gittes, G.K., 3–29
GLP1, *see* Glucagon-like peptide-1 (GLP1)
Glucagon-like peptide-1 (GLP1)
and islet regeneration, 107
role in β-cell development, 16
Glucagon suppression, mechanisms of, 139, 140
Glucolipotoxicity, 48
Glucose-dependent insulinotropic peptide (GIP), 16, 107
Glucose-stimulated insulin secretion (GSIS), 148
Glucose transporter type 2 (GLUT2), 107
GLUT2, *see* Glucose transporter type 2 (GLUT2)
Growth differentiation factor 11 (GDF11), 11
Growth inducers, 9
G1/S checkpoint proteome
components of, 87
CDKs and cyclin D1, 92–93, 95
cell cycle inhibitors, 92
manipulation for β-cell replication, 91
GSIS, *see* Glucose-stimulated insulin secretion (GSIS)

**H**

Harb, G., 85–100
Hedgehog signaling and pancreatic development, 12
Heimberg, H., 105–116
HEMA-MMA, *see* Hydroxyethyl-Methacrylate-Methyl-Methacrylate (HEMA-MMA)
Hepatic–biliary bud evagination, 4
Hepatocyte growth factor (HGF)
effect on transdifferentiation, 195
transgenic expression of, 14
hES cells, *see* Human embryonic stem (hES) cells
HES1 (transcription factor)
functions of, 11
upregulation and β-cell dedifferentiation, 129
Heremans, Y., 105–116
Herold, K.C., 263–274
Heterologous tissue recombination experiments, 7
HEX expression, 28
HLXB9 expression, 27

HNF cascade gene, expression patterns of, 26–27
Human β-cell
cell cycle
activation by CDK6 and cyclin D1, 95–96
control, epigenetic changes in, 97
inhibitors, 96–97
cells derived from, 130
dedifferentiation of, 125
HES1 upregulation and, 129–130
in-vitro proliferation and, 128
G1/S progression in, 88
in-vitro proliferation of cells derived from, 126–127
lineage-tracing of
GFP expression, 126
proliferation of cells, 127
RAS and TERT expression in, 91
redifferentiation of, 127–129
regeneration of, 86
replication of
CDK6 and cyclin D1 role in, 92–93
goals of inducing, 97–98
growth factors role in, 90
G1/S transition manipulation for, 91
in-vitro approaches, 93
stimulation of, 88
viral vectors for, 98–99
rodent and, difference between, 87–88
Human ductal cells, purification of, 171–172
Human embryonic stem (hES) cells
β cells generation from, 208
definitive endoderm formation, 209–210
foregut endoderm formation, 210–211
islet cell maturation, 211–212
cell cycle in, 206–207
directed differentiation of, 207–208
Human islets
components of, 140
encapsulation of alternatives to, 253–255
glucagon immunostaining of, 140
microvasculature of, 140–141
transplantation into NOD-SCID mice, 93
Human liver cells, in-vitro reprogramming of, 187–188, 195–196
Hydroxyethyl-Methacrylate-Methyl-Methacrylate (HEMA-MMA), 247
Hypercoagulation in SPK, 69–70
Hyperglycemia
causes of, 42, 48
glucagon response to, 53
Hyperlipidemia, causes of, 48

**I**

IBMIR, *see* Intravascular instant blood-mediated inflammatory reaction (IBMIR)
Immunoisolation devices for islet transplantation
advantages of, 244–245
hollow fibers, 243
vascular chambers, 244
Immunosuppressive therapy for IT
MMF, 47–48
side effects of, 57–60
Indian hedgehog (IHH) expression, 12
Induced pluripotent stem cells (iPS cells)
definition of, 204
derivation of, 204–205, 274
patient-specific ES cell lines generation by, 215
Induction therapy in SPK transplant recipients
alemtuzumab, 65–67
daclizumab, 64–65
rATG, 65
INK4a/p16 (cell cycle inhibitor), 96
Insulin
biosynthesis, control of, 142
and glucagon, relationships between, 143
production, glucose stimulation of, 147
secretion of
coordination of, 143
factors influencing, 142–143
suppression of, 144
storage of, 142
Insulin-positive cells
formation of, 9, 16
transplantation of, 162
Insulin-producing cells, 147
acinar cells conversion into, 159
and β cells, relation between, 147–148
from ES cells, 167
transplantation of, 144
Insulin promoter-interferon γ transgenic mice, 174–175
Intrahepatic islet graft dysfunction, 61
Intravascular instant blood-mediated inflammatory reaction (IBMIR), 60
Intravenous glucose tolerance test (IVGTT), 53
iPS cells, *see* Induced pluripotent stem cells (iPS cells)
Islet-acinar portal circulation, 138–140
Islet-after-kidney (IAK) transplant, clinical indications for, 43

Islet cells
anatomical location of, 138
blood flow, 138–139
counting of, 45
distribution within exocrine pancreas, 141
generation of, 169–170
in immunoprotective capsules, 146
transplantation of, 144–146
Islet development
activin role in, 9
BMPs role in, 10
islets of Langerhans, 5
during neonatal period, 177–178
retinoid role in, 12–13
"Islet donor pancreata," 45
Islet grafts
hypoxia of, 144
immunologic factors associated with rejection of
immunosuppressive drugs, 270
innate immune response activation, 269
MHC matching, 269–270
monitoring of
for β-cell dysfunction prediction, 56
imaging methods for, 56–57
rejection and immunomodulatory interventions, 273
tolerance induction, anti-inflammatory agents for, 270
anti-CD3 mAbs, 272
costimulatory molecule blockade, 271
PD-1 blockage, 271
Islet hypoplasia, 10
Islet infusion procedure, acute complications of, 57
Islet neogenesis, 168, 177–178
Islet regeneration
β-cell proliferation, preexisting, 112–113
measurement of, 106
pancreatic progenitor cells differentiation
into exocrine and endocrine pancreas, 113
into islet cell types, 114–115
transdifferentiation from pancreatic exocrine cells, 116
Islets of Langerhans
endocrine cells of, 44
functions of, 43
isolation and purification in IT, 45–46
Islet transcripts, detection of, 170
Islet transplantation (IT)
challenges in, 61–62
clinical indications for, 43
clinical outcomes of
insulin independence, 52
metabolic improvements, 53
clinical protocols for, 50–51
alemtuzumab, 52
corticosteroids, 48
daclizumab, 49
high-dose tacrolimus, 48
immunosuppressive regimens, 47, 49, 52
mycophenolate mofetil, 47, 49
complications of
immunosuppression-related side effects, 58–59
post-transplantation islet loss, 60–61
recipient- and graft-related, 57, 60
recipient-wide allosensitization, 61
on ECM scaffolds, 232–233
failure in T1DM patients, 269–270
goal of, 43
immune activation and islet destruction in, 267–269
limitations of, 105
microcapsules for, *see* Microcapsules
procedures
islet isolation, 44–46
islet transplant, 47
pancreas procurement, 44–45
recipient and donor selection, 43–44
risk of, 43
*See also* Islet grafts
Islet transplant recipients, clinical management of, 56
ISL1 expression, 27
ISL1-positive cells, 27
IT, *see* Islet transplantation (IT)
IT recipients and donors, selection criteria of, 43–44
IVGTT, *see* Intravenous glucose tolerance test (IVGTT)

**J**

Janus kinase/signaling activator of transcription (JAK/STAT) pathway, 205
Jensen, J., 203–217

**K**

Karsiloglu, E., 85–100
$K^+_{ATP}$-dependent pathway, 142

**L**

Laminin-1, 17
Lentiviruses, 99

LEPCs, *see* Liver epithelial progenitor cells (LEPCs)
Lineage-tracing studies
β-cell replication, 123
of cultured human β cells, 126–127
Liver cells
activation of β-cell phenotype in, 189
encapsulation of, 241
within artificial membranes, 242
macrodevices for, 243–245
microcapsules, 245–247
predisposition to transdifferentiation, 197
as source for pancreatic tissue, 185
transdifferentiated in vitro, implantation of autologous, 198
transdifferentiation into β cells, 184
adult human liver cells, 187–188
adult rodent liver cells, 187, 190–191
hepatic dedifferentiation, 191–192
by multistep protocol, 186–187
by PDX1 expression, 185–186
vectors for, 192–193
*Xenopus* liver cells, 188
Liver epithelial progenitor cells (LEPCs), 186
Loss-of-function studies, 10
Luciferase-transduced bioluminescence optical imaging, 56–57

**M**
MAFA (transcription factor)
expression patterns of, 26
role in transdifferentiation of liver cells, 195
MAFB (transcription factor), 26
Matrigel, 17
Meivar-Levy, I., 183–198
Memory T cells, 265
MEN-like syndrome, 96
Mesenchymal stem cells (MSCs)
BM-derived, 196–197
isolation from human pancreas, 170
Mesenchyme
condensation of, 3, 4
contact with epithelium, 7
effects on lineage selection, 7
inductive influences on pancreatic epithelium, 7
proexocrine factors in, 7
proliferation of, 6
Mesenchyme-to-epithelium signaling, FGF10 signaling in, 8
Metallothionein-TGFα transgenic mice, 175
Metaplasia, 153
Microcapsule membrane, 254
Microcapsules
agarose, 245–246
alginate-based, Alginate–based microcapsules
chitosan, 246–247
HEMA-MMA, 247
in-vivo applications of
experimental systems, 256–257
pilot human clinical trials, 257–258
PEG, 246
Microencapsulated human islets grafts
immunology of
complementary immunoprotection strategies, 255–256
immune responses, 255
for islet protection, 253
pilot human clinical trials of, 257–258
serum C-peptide levels in patients transplanted with, 258
MicroRNAs (miRNAs), 28–29
miRNAs, *see* MicroRNAs (miRNAs)
Mineo, D., 41–73
Mixed-meal tolerance test (MMTT), 53–54
MMF, *see* Mycophenolate mofetil (MMF)
MMTT, *see* Mixed-meal tolerance test (MMTT)
MSCs, *see* Mesenchymal stem cells (MSCs)
Mycophenolate mofetil (MMF), 47–48, 52
*MyoD*-induced reprogramming, 184

**N**
N-cadherin, expression of, 17
Neogenesis, 168
Neonatal human beta cells, replication of, 89
Neovascularized membranes, 252
NEPECs, *see* Nonendocrine pancreas epithelial cells (NEPECs)
NEUROD
expression patterns, 24
NEUROD1, 194–195
Neurogenin-3 (NGN3), 88
expression, 114
-positive endocrine progenitor cells
expression of, 23
GDF11 signaling and formation of, 11
NKX2.2 expression in, 25
SOX9 expression in, 27
transcription factor, expression patterns of, 23
transdifferentiation of liver cells into β-like cells by, 194–195
*Nkx6.1* and *Nkx.6.2* (transcription factor), 25–26

NKX2.2 (transcription factor), 25
NOD mice, *see* Nonobese diabetic (NOD) mice
Non-β cells
  anatomic location of, 137–138
  islet grafts containing, 144
Nonendocrine pancreas epithelial cells (NEPECs), 114
Nonobese diabetic (NOD) mice, 169
  cyclophosphamide-accelerated diabetes in, 191
  reversal of hyperglycemia in, 248, 256
  T1DM development in
    B cells and autoantibodies role in, 267
    CD28 and CD40L molecules role in, 268
    CD40/CD40L interactions role in, 271
    effects of tolerance induction to insulin on, 265–266
NOTCH–HES1 signaling activity, 160–161
NOTCH receptor, control point for, 12
NOTCH signaling, 214
  acinar cell differentiation regulation by, 11–12
  activation and β-cell dedifferentiation, 129–130
  effect on cell lineage selection, 12
  nonendocrine lineages and, 11
Notochord
  contact with dorsal prepancreatic endoderm, 5
  SHH expression and, 5–6
Novocell protocol, 210
NT, *see* Nuclear transfer (NT)
Nuclear transfer (NT), 215

**O**

Ogilvie, J.B., 223–234
Oval cells, 169

**P**

PACAP, *see* Pituitary adenylate cyclase-activating polypeptide (PACAP)
Pancreas
  allocation scheme, 45
  β-cell growth mechanism, 167–168
  dissociation, mechanically enhanced enzymatic, 45
  embryology of
    β cells, 5
    bud evagination, 4
    condensation of mesenchyme, 3–4
    fusion of buds, 4
  endothelial induction of, 6
  morphologic evidence of, 3
Pancreas development
  basement membrane role in, 17
  cell adhesion molecules role in, 17
  early tissue interactions in
    endothelium, 6
    mesenchyme, 6–7
    notochord, 5–6
  extracellular molecules role in, 17–18
  foregut endoderm formation, 209–210
  signaling pathways regulating
    GLP1, 17
    hedgehog, 12
    NOTCH, 11–12
    retinoid, 12–13
    WNT, 14–15
  soluble factors role in
    activins, 9–10
    blood vessel- and endothelial-derived factors, 15–16
    BMPs, 10–11
    EGF, 13–14
    FGFs, 8
    GDF11, 11
    HGF, 14
    TGF-β isoforms, 8–9
  transcription factors role in, 18–20
    ARX, 25
    BRAIN4, 28
    GATA4 and GATA6, 28
    HLXB9, 27
    HNF cascade, 26–27
    ISL1, 27–28
    MAFA and MAFB, 26
    *Myt1* gene and, 27
    NEUROD, 24
    NGN3, 23
    NKX2.2, 25
    *Nkx6.1* and *Nkx.6.2,* 25–26
    PAX4, 24–25
    PAX6, 24
    PBX1, 22
    PDX1, 20–22
    PROX1, 28
    PTF1A, 22–23
    SOX4, 27
    SOX9, 27
Pancreas graft survival rates, 67
Pancreas injury, surgical methods for inducing
  cellophane wrapping, 110
  partial duct ligation, 109
  partial pancreatectomy, 108–109

Pancreas-kidney transplantation, schematic representation of, 63
Pancreas-specific transcription factor 1a (PTF1A)
  expression patterns of, 22–23
  functions of, 23
Pancreatic buds
  branching morphogenesis of, 4
  formation of, 116
Pancreatic ductal cells, 178
Pancreatic duodenal homeobox 1 (PDX1)
  ectopic expression of, 185–186
  effects on hepatic gene expression, 192–193
  expression patterns of, 20–22
    in LEPCs, 186–187
    in pancreatic non-β-cell types, 186
  functions of, 22
  protein transduction, 193
  retinoic acid and, 211
Pancreatic epithelium, differentiation of, 7
Pancreatic exocrine cells, transdifferentiation from, 116
Pancreatic progenitors
  embryonic, 178
  FGF10 and, 211
  in human pancreatic duct-enriched, islet-depleted tissue
    EGF and gastrin treatment, 172
    human ductal cells, 171–172
    insulin$^+$ cells, 172
    NCAM-positive cells, 171
  in mouse pancreas ductal cells
    CD133$^+$ cells, 173
    cell surface antigens, 174
    common bile duct, 172–173
    GFP$^+$ cells, 173
  patterning of, 212
  postnatal, in-vivo evidence of, *see* Postnatal pancreatic progenitors, in-vivo evidence of
  proliferation of, 211
Pancreatic stem cells
  CAII expression in, 178
  identification of, 169–170
  islet cells generation, 169–170
  isolation and clonal expansion of, 170
  MSCs isolation, 170
  oval cells, 169
Pancreatic stem/progenitor cell niche, 116
PANIC-ATTAC model (pancreatic islet β-cell apoptosis through targeted activation of caspase 8), 111
Partial duct ligation (PDL), 108–109
Partial pancreatectomy (PPx), 108–109
PAX4, expression patterns of, 24–25
PAX6, expression patterns of, 24
PBX1 (transcription factor)
  in adult β cells, reversible repression of, 110–111
  expression patterns of, 22
PD1, *see* Programmed death-1 (PD1)
PDL, *see* Partial duct ligation (PDL)
PDX1, *see* Pancreatic duodenal homeobox 1 (PDX1)
PDX1-VP16, PDX1-induced liver-to-pancreas transdifferentiation potential of, 193–194
PEG microcapsules, 246
Pituitary adenylate cyclase-activating polypeptide (PACAP), 17
"Plurinet," 204
Polyneuropathy, 68
Porcine islets
  PERV transmission across, 254
  potential of microencapsulation in xenotransplantation of, 253–254
Postnatal pancreatic progenitors, in-vivo evidence of
  ductal ligation model, 176–177
  insulin promoter-interferon γ transgenic mice, 174–175
  metallothionein-TGFα transgenic mice, 175
  partial (90%) pancreatectomized rats, 175–176
Powdered ECM scaffolds, 229
PPx, *see* Partial pancreatectomy (PPx)
Prasadan, K., 3–29
"pRb pathway," 87
Progenitor cell, definition of, 169
Programmed death-1 (PD1), 268
PROX1 expression, 28
PTF1A, *see* Pancreas-specific transcription factor 1a (PTF1A)
Putative pancreatic stem cells, 170–171

**R**

RALDH2 (enzyme), 13
Regenerative medicine, 167
Retinoids and pancreas development, 12–13
Ricordi, C., 41–73
Rodent β-cell
  human and, difference between, 87–88
  SV40 large T-antigen expression in, 91

Rodent islets
CDK4 in, 92
human and, difference between, 88, 92
Rodent liver cells, PDX1-induced reprogramming of, 187, 190
Rooman, I., 155–163

**S**
Sharma, A., 167–178
SHH, *see* Sonic hedgehog (SHH)
Simultaneous islet–kidney (SIK) transplants
clinical indications for, 43
recipient and donor selection for, 44
Simultaneous pancreas-kidney transplantation (SPK), 62
clinical outcomes of
effect on diabetic nephropathy, 67–68
patient and graft survival, 67
quality of life, 68–69
retinopathy, 68
clinical protocols for
alemtuzumab, 65–67
daclizumab, 64–65
immunosuppression, 63
rATG, 65
complications of
enteric drainage, 72–73
fluid balance, 71
hematuria, 71
hypercoagulation, 69–70
leak from anastomotic site, 72–73
metabolic acidosis, 71
urine leaks, 71–72
improvement in, 63
schematic representation of, 63
Sirolimus, 49, 57–58, 60
SIS-ECM, *see* Small-intestinal submucosa ECM (SIS-ECM)
SMAD molecules, expression of, 10–11
Small-intestinal submucosa ECM (SIS-ECM), 224
Somatic-to-somatic cell reprogramming, 216
Sonic hedgehog (SHH)
expression of, 5, 12
inhibition of, 12
SOX9
expression patterns, 27
interaction with HNF proteins, 27
SOX4 expression, 27
SPK, *see* Simultaneous pancreas-kidney transplantation (SPK)
Stem-cell niche, 116
Streptozotocin
β-cell proliferation by, 107
cytotoxicity, 171
Stewart, A.F., 85–100
STZ-treated neonatal rats
β-cell regeneration in, 107–108
grafted with syngeneic islets, 109

**T**
Tacrolimus, 49, 60
Takane, K.K., 85–100
Tamoxifen-inducible *c-Myc* expression, 111
T cells
activation of, 264
memory, 265
reactivity to islet antigens, 265
role in T1DM, 265–266
T1DM, *see* Type 1 diabetes mellitus (T1DM)
T1DM-related micro- and macrovasculopathy, 42
TGF-β, *see* Transforming growth factor β (TGF-β)
Transcription factors, 18–20
ARX, 25
BRAIN4, 28
early pancreatic lineages and, 20
expression
during endocrine cell differentiation, 21
in mouse acinar cells in vivo, 116
GATA4 and GATA6, 28
HLXB9, 27
HNF cascade, 26–27
ISL1, 27–28
MAFA and MAFB, 26
*Myt1* gene and, 27
NEUROD, 24
NGN3, 23
NKX2.2, 25
*Nkx6.1* and *Nkx.6.2,* 25–26
in pancreatic organogenesis, 184
PAX4, 24–25
PAX6, 24
PBX1, 22
PDX1, 20–22
PROX1, 28
PTF1A, 22–23
SOX4, 27
SOX9, 27
Transdifferentiation, 153
definition of, 154, 169
of liver cells into β cells, 184
adult human liver cells, 187–188
adult rodent liver cells, 187, 190–191
by ectopic PDX1 expression, 185–186

hepatic dedifferentiation, 191–192
by multistep protocol, 186–187
by NEUROD1 and NGN3, 194–195
by PDX1-VP16, 193–194
by soluble factors, 195–196
vectors for, 192–193
*Xenopus* liver cells, 188
Transforming growth factor β (TGF-β)
activins, proendocrine effects of, 9
TGF-β isoforms
receptors for, 8–9
role in pancreas development, 9
Tulachan, S., 3–29
Type 1 diabetes mellitus (T1DM)
causes of, 41–42
immunological process in
B cells in T1DM, 266–267
costimulatory molecules, 267–269
T cells in T1DM, 264–266
islet transplant failure mechanism in, 269–270
prevalence of, 42
studies in animal models of, 263
treatment of
glycemic control for, 43
intensive insulin, 42
islet transplantation for, *see* Islet transplantation (IT)
SPK for, *see* Simultaneous pancreas-kidney transplantation (SPK)

**V**

Van De Casteele, M, 105–116
Vascular chambers, 244
Vascular endothelial growth factor A (VEGF-A), 15
VEGF-A, *see* Vascular endothelial growth factor A (VEGF-A)
VENT2 expression, 15
Viral vectors for human β-cell replication, 98–99

**W**

Weir, G.C., 105–116
WNT signaling
expression patterns of, 15
foregut inhibition by, 14–15
*Pdx1* promoter-driven studies of, 15

**X**

*Xenopus* liver cells, in-vitro reprogramming of, 188
Xiao, X., 105–116
Xu, X., 105–116