Stem Cell Biology and Regenerative Medicine

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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

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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

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

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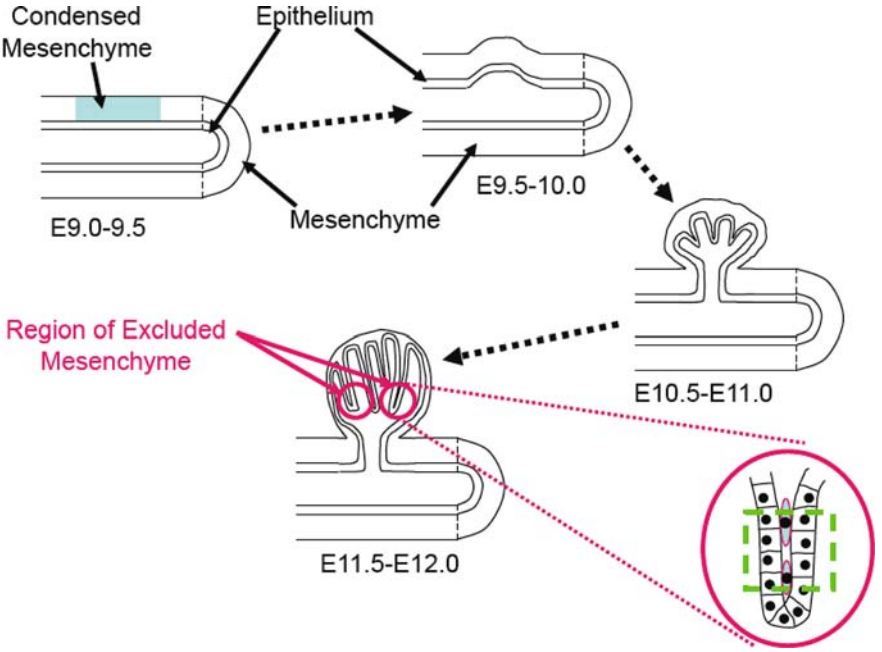

Fig. 1.1 Schematic representation of epithelial–mesenchymal interactions. Mesenchyme condensation at E9–E9.5 leads within the next 12–24 h to epithelium evagination. This is followed shortly by the onset of branching morphogenesis, which results in exclusion of most of the mesenchyme from within the 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

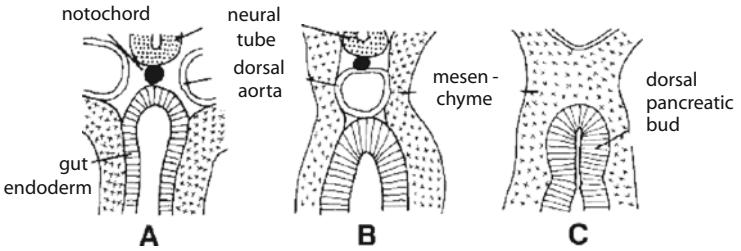

Fig. 1.2 Early tissue interactions in pancreas development. (**a**) At E8 a close contact exists between the dorsal aspect of the gut endoderm and the notochord. (**b**) The two paired dorsal aortas fuse in the midline by E8.5–E9.0 to intervene between the gut epithelium and notochord. **(c)** The mesenchyme 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 E9–E10: Prepancreatic and pancreatic epithelium Midgestation: Nonendocrine cells (low level of expression) Mid-to-late gestation: β cells (high level of expression)	Probably contributes to pancreas specification of the endoderm Directs pancreatic budding Necessary for early development of all lineages, including acinar and ongoing endocrine development. Expression in early endocrine cells favors β cells over α cells Important for proper glucose sensing in β cells, in cooperation with PBX1
PTF1A	E9: Pancreas-specified endoderm within the PDX1 domain E9–E14: Progenitors of all pancreatic lineages, except for a few α cells After E14: Acinar-committed cells	Confers the pancreatic field within the PDX1 domain of the endoderm Regulates NOTCH signaling at RBPJ level Necessary for acinar development 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 All endocrine progenitors, except for β/δ-lineage committed cells	Regulates endocrine hormone transcription Enhances endocrine cell numbers, especially α cells Suppresses ghrelin synthesis
PAX4	E10: Marks some early endocrine progenitor cells Endocrine cells that have committed to the β/δ-lineage	Increases numbers of β/δ-lineage cells owing to ARX suppression Increases numbers of mature β cells 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 All developing endocrine cells	Fosters endocrine development Fosters mesenchymal induction of exocrine development Enhances glucose sensing in mature β cells in cooperation with PDX1
NKX2.2	E9.5: All epithelial cells E10.5: 50% of epithelial cells, but not specifically endocrine-committed cells	Enhances formation of β cells and, to a lesser extent, α cells Suppresses ϵ-cell formation Turns on MAFA in β-cell progenitors Enhances duct formation in zebrafish
NKX6.1 and 6.2	E9.5: All epithelial cells E11–E13: NKX6.1 marks all PDX1$^+$ cells, but NKX6.2 shuts off NKX6.1 marks post-NGN3 expression endocrine progenitors 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 NKX6.1 is necessary for β cell expansion in the secondary transition and for β-cell maturation 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 E9–E10: Pancreatic epithelium Ongoing scattered expression in the uncommitted epithelium	Turns on HNF6 Necessary for dorsal and ventral bud evagination Mediates HNF6-induced production of early, proliferative endocrine progenitors
HNF6	Early endoderm just downstream of HNF1β E9–E10: Pancreatic epithelium Ongoing scattered expression in the uncommitted epithelium	Regulates PDX1 expression and pancreatic field size Activates HNF1β in the commitment of epithelial progenitor cells to the endocrine lineage
HNF3β	All early gut endoderm 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 Later marks uncommitted epithelial cells (progenitors of all lineages)	Regulates HNF6- and HNF1β-induced commitment of epithelial progenitors to the endocrine lineage (see above) Maintains epithelial cells in a progenitor state, likely through NOTCH signaling

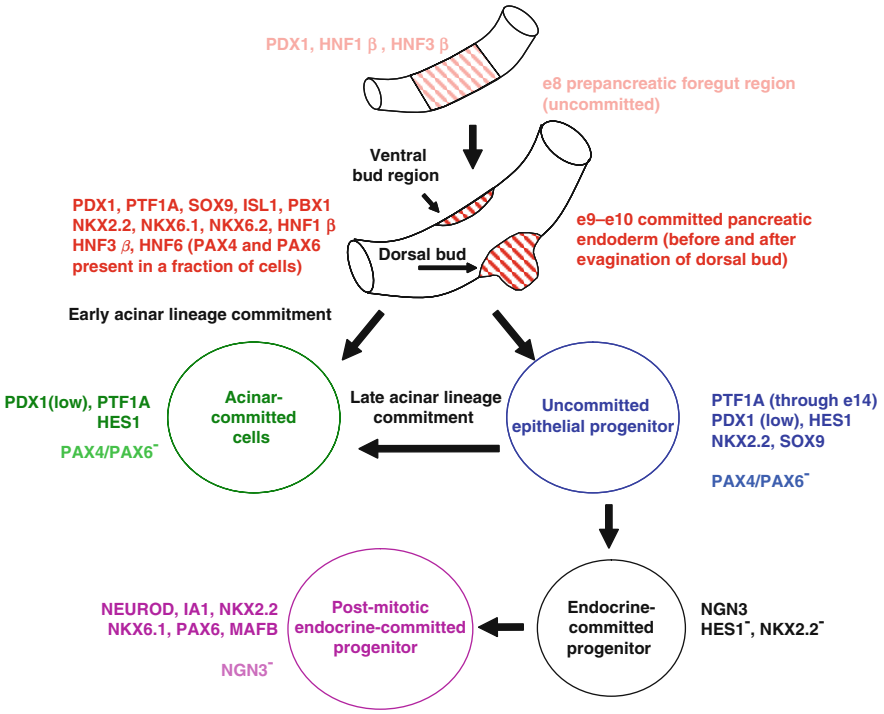

Fig. 1.3 Schematic representation of early pancreatic lineages and their transcription factors. Different stages of pancreas development are depicted from the earliest time of pancreas field specification to commitment to acinar or endocrine lineages (later stages are shown in Fig. 1.4)

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),

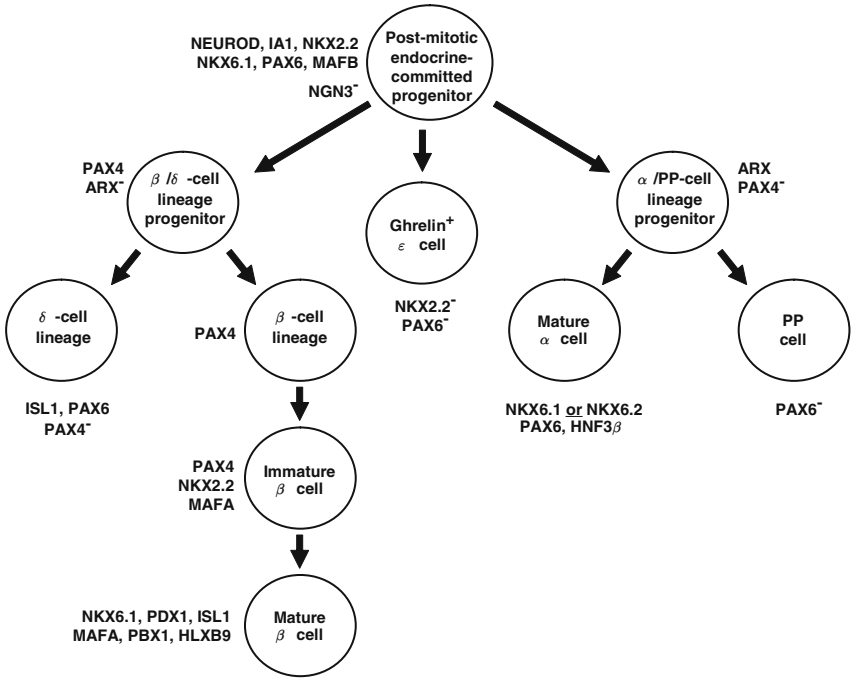

Fig. 1.4 Schematic representation of transcription factor expression during endocrine cell differentiation after NGN3 is turned off (last stage from Fig. 1.3)

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