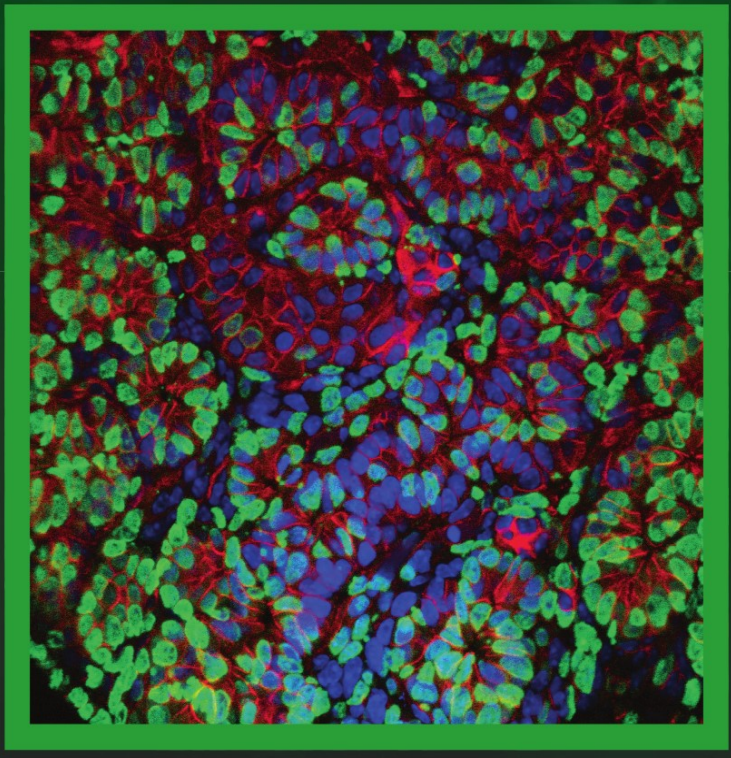
Juan Domínguez-Bendala

Pancreatic Stem Cells



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Preface

The last decade has witnessed the consolidation of "regenerative medicine" as a recognized scientific field, encompassing disciplines as diverse as cell biology, immunology, developmental biology, and surgery. The report on the isolation of human embryonic stem (huES) cells by James Thomson in 1998[1] opened our eyes to a ground-breaking notion: that defective tissues could be replaced by an unlimited source of self-renewing cells with the ability to morph in vitro into any of them. The revolutionary nature of this idea is evidenced by the fact that concepts such as "regenerative medicine" or "stem cell therapies" were not in common use in the scientific literature until the late nineties. Until then, and despite reports of embryonic stem cells obtained from several animal species,[2–4] there was no identifiable organized quest for a "human tissue building block," as there was one, for example, to decipher the entirety of the human genome. In retrospect, it is as though the majority of the scientific community had not envisioned applications for these unique cells other than to create animal models for human diseases, increase livestock output, or improve the production of therapeutic proteins from transgenic animals. This seeming "unexpectedness" was further confirmed when, shortly after this breakthrough, all major scientific journals started to publish a plethora of reports on the therapeutic potential of stem cells of adult origin. Since the technology to isolate and expand adult stem cells had already been in use for a long time before Thomson's discovery, it remains surprising that very few had openly contemplated until then the idea of using adult stem cells for medical purposes. Be it as it may, the field has gone a long way throughout this past decade. Several adult stem cell types are currently in clinical trials for a variety of conditions ranging from myocardial infarction[5] to graft-versus-host disease,[6,7] and huES cells will shortly follow suit.

Among the many conditions potentially treatable by stem cells, type I diabetes (a disease where the endocrine pancreatic cells that synthesize and secrete insulin are destroyed by autoimmune processes) holds a position of privilege. Unlike many other commonly cited targets of stem cell approaches (such as Parkinson's disease or spinal cord injury), there is already an effective cell therapy for type I diabetes. Indeed, islet transplantation has been shown to completely restore normoglycemia in human patients,[8–10] and even if there is a progressive loss of function of the graft over time,[11] patients invariably report a much higher quality of life than before the

procedure.[12] Based on our experience with this therapy, it is not unreasonable to expect that any stem cell type with the ability to give rise to insulin-producing, pancreatic endocrine-like tissues will also work in a transplantation setting.

In this context, this book has been conceived with the goal of presenting the state of the art in regenerative therapies for the pancreas. First, we will briefly describe how the adult organ works (in the chapter "The Pancreas"). Then, we will thoroughly review the two physiological processes that should be recapitulated in different therapeutic settings, namely *pancreatic development* (in the chapter "Pancreatic Development") and *islet regeneration* (in the chapter "Pancreatic Regeneration"). The chapter "Stem Cell Differentiation: General Approaches" will examine the general experimental strategies used to differentiate stem cells, regardless of their origin, whereas the chapters "Embryonic Stem Cells and Pancreatic Differentiation" and "Adult Stem Cells and Pancreatic Differentiation" will focus, respectively, on the utilization of embryonic and adult stem cell types for the procurement of transplantable insulin-producing cells. The latter will include special sections on bone marrow cells, umbilical cord blood stem cells, ductal and acinar cells, and mesenchymal stem cells. The chapter "Transdifferentiation" will drift away both from the general concept of stem cell differentiation and the two islet neogenesis processes known to happen in vivo (development and regeneration), to touch upon *transdifferentiation*, an intriguing phenomenon by which terminally differentiated cells from other tissues might be induced to alter their phenotype to become islet-like cells. We will conclude with a general overview of the remaining challenges and clinical perspectives of all of the above strategies.

Despite what many may perceive as a slow pace in translating basic findings into clinical therapies for type I diabetes, the last 10 years have been very productive in terms of shaping the overall direction of the field, many times as a consequence of a trial-and-error process. Progress has been steadfast, however, and the current state of the art suggests that stem cell-based trials, perhaps combined with immunological therapies, might be just around the corner. Because type I diabetes is a complex disease, a cure will only come from a multidisciplinary effort, which will almost certainly include a strong stem cell component. It is our hope that this book will help frame the problem for researchers and clinicians alike.

Miami, FL Juan Domínguez-Bendala

Contents

The Pancreas

Abstract The pancreas is a unique organ that encompasses both endocrine and exocrine functions. Here we review its main anatomical features, including its innervation and vasculature, the exocrine acinar tissue (which secretes a variety of enzymes that take part in food digestion), the ductal system (which collects exocrine secretions and is thought to harbor pancreatic stem cells), and the endocrine component, also termed the *islets of Langerhans*. These cell clusters, embedded in the pancreatic parenchyma, synthesize and secrete into the bloodstream some of the most important hormones involved in the maintenance of glucose homeostasis.

Keywords Endocrine • Exocrine • Islets of Langerhans • Ductal system • Beta cells • Alpha cells • Glucagon • Insulin

1 Introduction

The pancreas is a solid glandular organ in the gastrointestinal tract, with both digestive (exocrine) and endocrine functions. It is elongated (12.5–15 × 4 cm) and irregular in shape, with three poorly defined regions: The broader extremity is the *head*, and the narrower end is called the *tail*. In between is the *body*, which is connected to the head by a slender constriction, also called the *neck*. It is located transversely across the posterior wall of the abdomen, beneath the stomach, and connected to the small intestine at the duodenum[13] (Figs. 1 and 2).

2 The Ductal System

The *pancreatic duct* (also called *duct of Wirsung*) runs transversely from left to right throughout the organ, from the junction of the tail lobular ductules to the common bile duct, receiving the affluence of smaller ducts. Sometimes, a secondary duct receiving the lower head ductules (*duct of Santorini*)[14] opens into the duodenum from the neck region.

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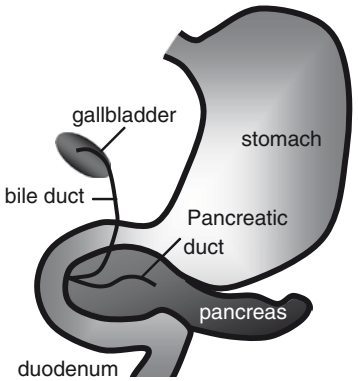


Fig. 1 The pancreas in its anatomical context

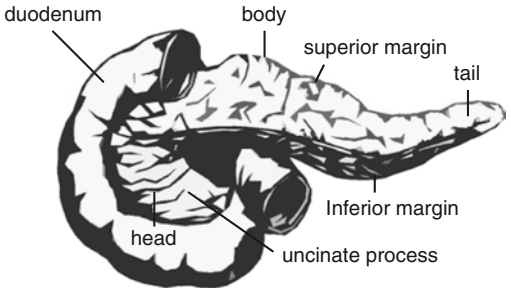


Fig. 2 Gross anatomy of the pancreas

The cells that form the ducts are typically arranged in one single layer of *ductal cells*. Probably due to their primary function of collecting and channeling pancreatic exocrine secretions, these cells express a relatively high amount of intermediate filaments, which may result in additional mechanical strength. Cytokeratins (CK) 7, 19, and 20 are the most represented in ductal cells, and as such are usually used as immunohistochemical/immunofluorescent markers of ductal tissue.[15] Other markers are the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) chloride pump and the carbohydrate antigen CA-19.9 (not to be confused with CK-19).[16] Interestingly, the pancreatic and duodenal homeobox-1 protein (Pdx1), whose expression in the adult pancreas is normally restricted to beta cells, can also be detected in ductal cells, albeit with a different phosphorylation pattern.[17] This, together with other lines of evidence to be discussed in the chapter "Stem Cell Differentiation: General Approaches," have led to the hypothesis that ductal cells might act as progenitors of beta cells during normal turnover and regeneration in the adult pancreas.

3 Vasculature

The vasculature of the pancreas and duodenum is so intertwined that it may be considered common to both (reviewed in[13,18,19]). Arteries are derived from the celiac trunk and the superior mesenteric artery. The celiac trunk arises from the front of the aorta and has three branches (left gastric artery, hepatic artery, and splenic artery). As it turns into the lesser omentum, the hepatic artery gives off the right gastric and gastroduodenal arteries. The latter extends behind the first part of the duodenum and then divides into the right gastroepiploic artery and the superior pancreaticoduodenal artery, which supplies the second part of the duodenum and the head of the pancreas.

The third branch of the celiac trunk, the splenic artery, winds along the upper border of the pancreas to the hilum of the spleen. Its most important branch to the pancreas is the dorsal pancreatic artery, which passes into the superior border of the organ near the neck. This vessel gives off a single branch to the left, the transverse pancreatic artery, and two branches to the right, toward the head of the pancreas. The splenic artery also gives rise to the pancreatica magna and a caudate branch, near the body-tail junction and in the tail region, respectively.

The splenic vein, which runs alongside the splenic artery, drains venous blood from the pancreas. It joins the superior mesenteric vein to form the portal vein, which runs into the liver.

4 Innervation

The pancreas is richly innervated by myelinated or unmyelinated nerve fibers, thick nerve bundles, and scattered intrapancreatic ganglia, which represent the intrinsic neural component of the organ.[20] The two main extrinsic components derive from the anterior and posterior branches of the vagi nerves and the splanchnic nerve trunks. The afferent system is composed of thin unmyelinated fibers that run along parasympathetic or sympathetic nerves, and is chiefly involved in sensory/pain relay to the central nervous system. One of the key features of pancreatic neurons is their ability to secrete biologically active substances, including acetylcholine, nitric oxide, and gastrin-releasing peptide, which have been associated with the regulation of endocrine/exocrine secretion.[20–26]

5 Exocrine Pancreas

Because the organ combines two completely different functions, references to exocrine and endocrine pancreas are common. Approximately 90–95% of the tissue of the organ is exocrine, and its main function is to secrete digestive enzymes into

the duodenum. This tissue is organized in cell clusters termed *acini*, and thus the exocrine pancreas is also commonly referred to as *acinar tissue*. Acini are connected to ductules by centroacinar cells, which share many markers with ductal cells (Fig. 3).

Some of the key digestive enzymes secreted by the exocrine pancreas, mainly in response to duodenally secreted cholecystokinin (CCK), are listed in Table 1. Many of these are secreted in an inactive form, becoming activated only when in contact with other proteases in the lumen of the small intestine. This mechanism of activation has evolved so that the acinar cells are not digested by the very same enzymes they harbor. In addition to these enzymes, ductal cells also secrete a bicarbonate solution, which, in a biochemical process orchestrated by the hormone secretin, helps regulate the duodenal pH after the influx of gastric acid secretions.

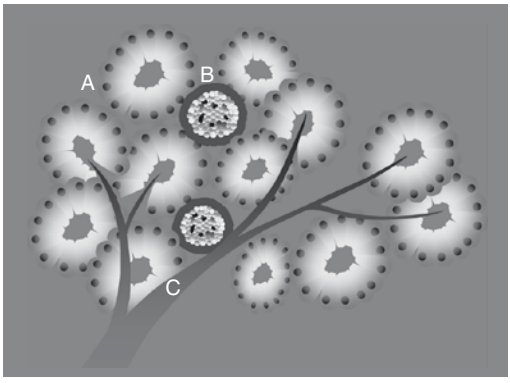


Fig. 3 Histological organization of the pancreas. The ductal system (C) collects the secretion of acinar cells (A), which are typically organized in acini around ductules. The islets of Langerhans (B) are intercalated throughout the acinar tissue

Table 1 Pancreatic exocrine enzymes

Carboxypeptidase A (CPA1)	Hydrolyzes C-terminal residues, particularly those of aromatic (A) nature
α-Amylase	Cleaves the α(1,4) glycosidic bonds of amylose (which is one of the two main components of starch), yielding maltose and dextrin
Trypsinogen	Inactive form of *trypsin*, a serine protease activated by *enterokinase* upon secretion into the small intestine. With few exceptions, it cleaves proteins at the carboxylic side of the residues lysine and arginine
Chymotrypsinogen	Inactive form of *chymotrypsin* (also called *zymogen*), activated by trypsin in the lumen of the small intestine. It cleaves proteins mostly at the carboxylic side of phenylalanine, tyrosine, and tryptophan
Pancreatic elastase (ELA-1)	Cleaves elastin, a main protein component of the connective tissue
DNAse and RNAse	Nucleases that break down nucleic acids
Pancreatic lipase	Hydrolyzes ester bonds of lipids

6 Endocrine Pancreas

The endocrine component of the pancreas is organized in cell clusters termed islets of Langerhans, first identified by the German biologist Paul Langerhans in 1869.[27] Islets are composed of at least five types of endocrine cell types, namely alpha, beta, delta, beta, PP, and epsilon, which secrete glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively.

The typical view of the islet cellular architecture has been shaped based on early observations on the mouse pancreas, where beta cells cluster preferentially at the core of the structure and alpha, delta, and PP cells (epsilon were not discovered until recently) are peripherally arranged[28–31] (Fig. 4). In all species studied, beta cells are the most abundant (50–80%), followed by alpha cells (20–50%). The remaining cell types are scarcely represented. However, there are significant differences between species both in islet cell composition and cytoarchitecture. Thus, in human and nonhuman primates, alpha cells are scattered throughout the islet rather than concentrated in the periphery. The percentage of beta cell homotypic interactions (those of cells apposing to cells of the same type) is approximately 29% in human islets, compared with 71% in mouse.[32] Considering the complex nature of cell-to-cell communication in the endocrine pancreas, such architectural differences were expected to have functional implications. This was indeed the case, according to a recent report[32]: the oscillations in membrane potential and $[Ca_2^+]$ in response to high glucose concentrations in rodent islets were coordinated, so that the entire islet displays a synchronous oscillatory response. This did not happen in human islets, where these responses were not synchronized. These findings suggest a correlation between the pattern of cell distribution (clustered vs. scattered) and islet cell function. Additional studies, however, are needed to further clarify such possible association.

Islets are richly vascularized, to ensure the efficient secretion of endocrine hormones into the bloodstream. A classic study revealed a nearly twofold increase in both internal volume and microvascular surface area for the blood vessels within the islet compared with the surrounding exocrine tissue.[33] The additional importance of islet vasculature to maintain an adequate oxygen supply is revisited in the chapter "Stem Cell Differentiation: General Approaches."

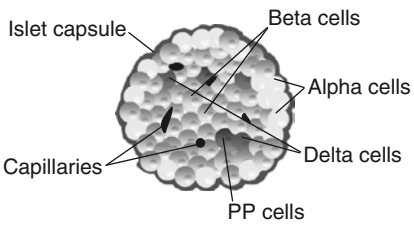


Fig. 4 Typical architecture of a murine islet. Beta cells are the most abundant, and are usually located at the core of the islet. Alpha cells, in contrast, tend to lie at the periphery. PP and delta cells are less abundant and interspersed among the other two endocrine cell types

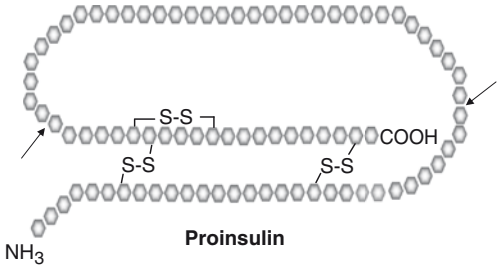
Even Langerhans himself noticed in his doctoral thesis the abundant innervation of islets,[27] which has been confirmed in most species examined thus far. Neural elements tend to coalesce both in the periphery (peri-insular plexus) and in the core (intra-insular plexus) of the islets. The physiological importance of neural activity on islet metabolism is very well documented (reviewed in[26]), but its description is beyond the scope of this book.

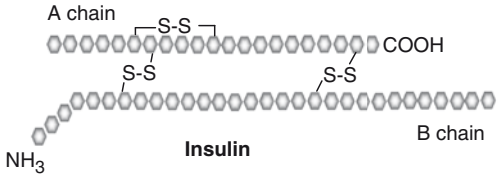
7 Glucose Metabolism

The endocrine component of the pancreas is responsible for the maintenance of glucose homeostasis (70–100 mg/dl), chiefly by means of the secretion of the hormones insulin (beta cells) and glucagon (α cells) to the bloodstream. Insulin is a two-chain polypeptide generated by the cleavage of a precursor protein, termed proinsulin (Fig. 5). Beta cells secrete this peptide in response to a variety of stimuli, including parasympathetic signals,[36] the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1),[37] cholecystokinin (CCK),[38] glucagon, and, of course, high blood glucose levels. Glucagon (Fig. 6), in contrast, is secreted by α cells when insulin levels are high and/or blood glucose is low. Figures 7 and 8 show some of the factors that affect the release of these hormones. Insulin acts by activating systemically the cellular uptake of glucose, thereby reducing blood sugar levels. In the liver, this is followed by a conversion of glucose into glycogen, a complex carbohydrate resulting from the sequential addition of single glucose molecules (Fig. 9). This process is catalyzed by the enzyme glycogen synthase. Glycogen is the main repository of glucose of the organism.[39,40]

When sugar levels are low, α cells counteract the effects of insulin by secreting glucagon. The major effect of glucagon in the liver is the catabolism of glycogen, a process called glycogenolysis.[41,42] The long chains of glycogen are progressively converted into glucose by glycogen phosphorylase, debranching enzymes and phosphoglucomutase. Another glucagon-stimulated pathway is gluconeogenesis, which converts noncarbohydrate substrates into glucose. The combined action of these processes results in a net increase of available circulating glucose (Fig. 10).

The complex interplay of these and other hormones[37,38] in the maintenance of glucose homeostasis is beyond the scope of this chapter. However, it is important to stress that their secretion, far from responding to the clear-cut situations schematized above (high or low glucose) is exquisitely regulated at several levels. As an example, it has been recently shown that the activation of alpha cells depends not only on an initial stimulus (low sugar), but also on a decrease of the suppressive effect of beta cell-derived secretions such as insulin, γ-aminobutyric acid (GABA) or zinc. In addition, a positive autocrine feedback exerted by glutamate, which is also secreted by alpha cells, is required for effective glucagon release (Fig. 11).[43]





C-peptide

A chain: Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn

B chain: Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr

Fig. 5 Insulin derives from proinsulin, a molecule that is cut twice during its maturation to give rise to insulin and C-peptide. The two insulin chains are kept together by disulfide bonds. The amino acid sequence of the two chains is indicated below[34]

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr

Fig. 6 Structure and amino acid sequence of glucagon[35]

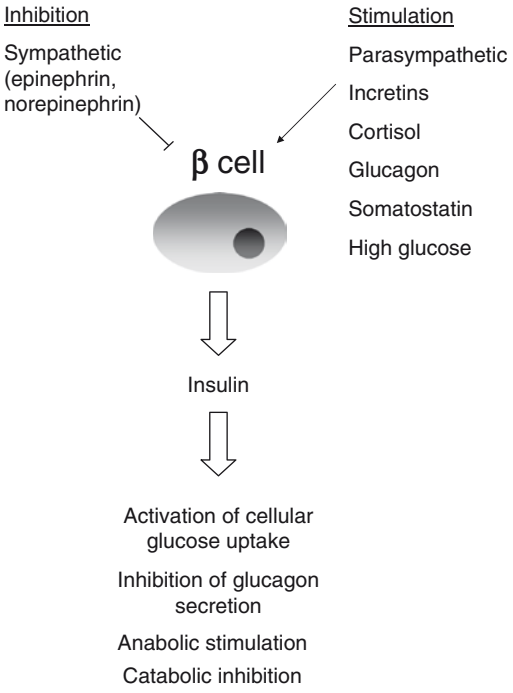


Fig. 7 Factors affecting insulin secretion

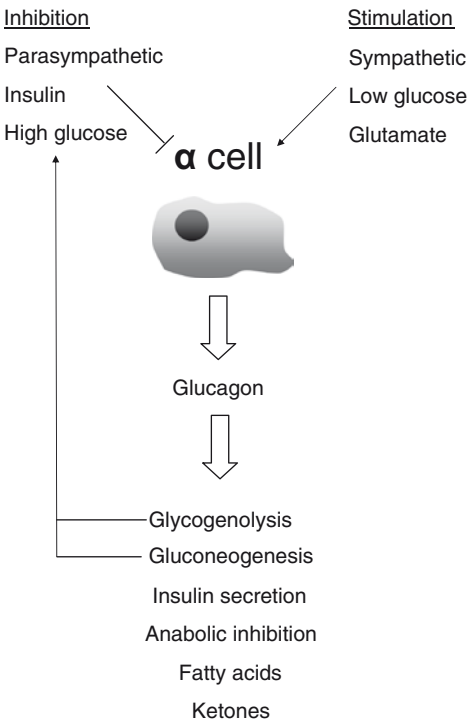


Fig. 8 Factors exerting an influence on alpha cell activity

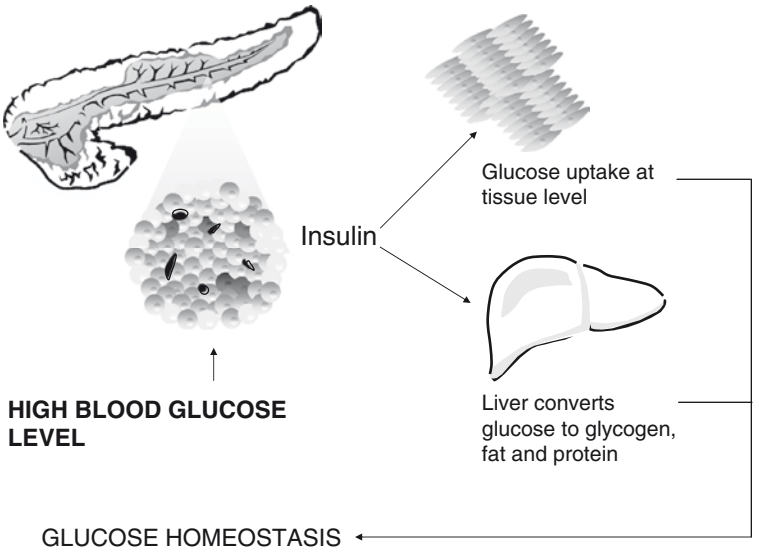


Fig. 9 Response of the pancreas to high blood glucose

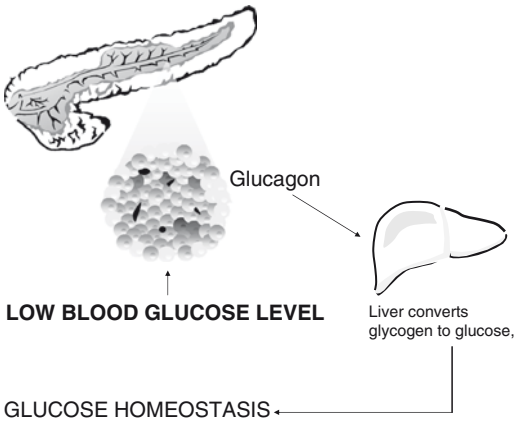


Fig. 10 Pancreatic response to low glucose levels

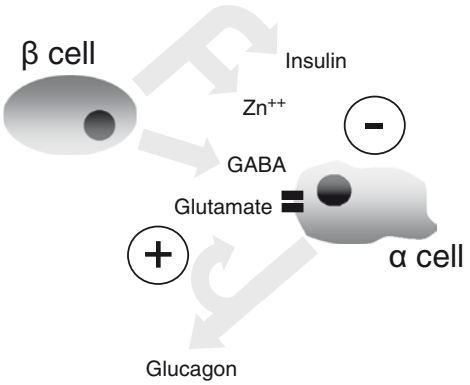


Fig. 11 Beta cells exert a negative feedback on alpha cell activity. Glutamate has an autocrine positive feedback on glucagon secretion

The Beta Cell

Beta cells have well-tuned machinery for glucose sensing and insulin secretion.[44–46] Type 2 glucose transporters (GLUT-2) mediate the uptake of glucose. Ensuing glycolysis leads to an increase in the ATP/ADP ratio. High ATP levels close potassium channels, preventing the exit of K^+ ions. The excess of positive charge (K^+) in the cytosol depolarizes the membrane, which in turn results in the opening of voltage-gated calcium channels. Calcium ions (Ca^{++}) flow inside the cell, inducing the exocytosis of the granules where insulin is stored upon synthesis and processing. Because islets are very highly vascularized, insulin finds its way promptly to the bloodstream. The process of insulin release is biphasic. The first phase (fast) is the result of the exocytosis of preexisting granules. A second, more prolonged phase, involves the de novo synthesis of new insulin for as long as blood glucose levels remain high. This phase continues for a while after insulin is no longer necessary, as the pool of insulin granules for the next fast phase is progressively replenished (Fig. 12).

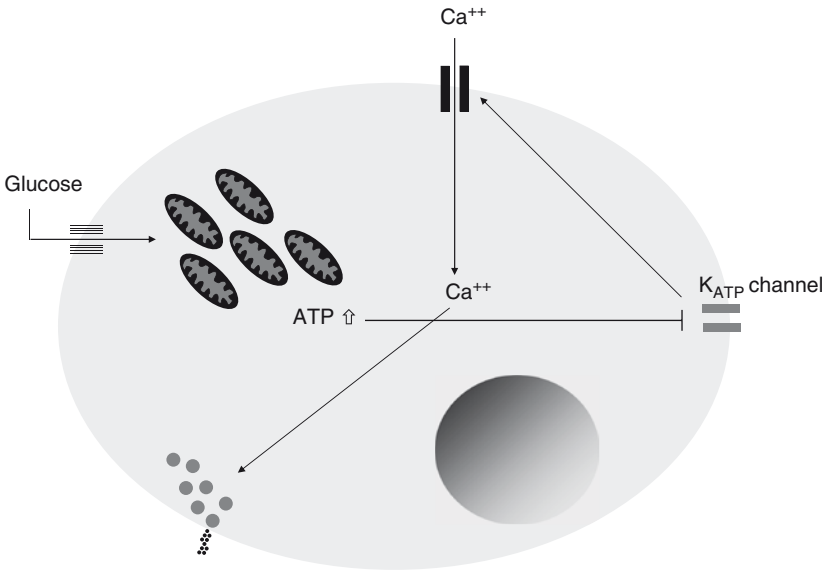


Fig. 12 Physiological release of insulin (see main text)

Pancreatic Development

Abstract Pancreatic development is arguably the best-studied example of organogenesis. Both gain-of-function and loss-of-function studies conducted in mice over the last decade have contributed to our understanding of a basic "genetic roadmap" of pancreatic – and particularly endocrine – development. Here we review this knowledge from the onset of the pancreatic program in the foregut epithelium (with the expression of the critical regulators Pdx1 and Ptf1a) to the specification of ductal, exocrine, and endocrine cell types. A special emphasis is placed on the development of endocrine beta cells, which are destroyed in type I diabetes and therefore constitute the endpoint of many stem cell differentiation protocols.

Keywords Foregut epithelium • Pancreatic buds • Pdx1 • Ptf1a • Ngn3 • Secondary transition

1 Introduction

For obvious reasons, most of our knowledge on pancreatic development comes from the mouse model. Indeed, despite a few minor differences that will be pointed out throughout this chapter, the most important molecular players are highly conserved between mouse and human. Research conducted over the last decade has outlined a basic "roadmap" of the major molecular events that shape mouse beta cell development from the early blastocyst.[47–49] Critical developmental milestones are: (1) generation of definitive endoderm/gut epithelium; (2) pancreatic differentiation; (3) endocrine specification; and (4) beta cell differentiation. We will now describe what is known about this process (Fig. 13), emphasizing the role of the genes that act as master regulators of the transition between each stage and the next.

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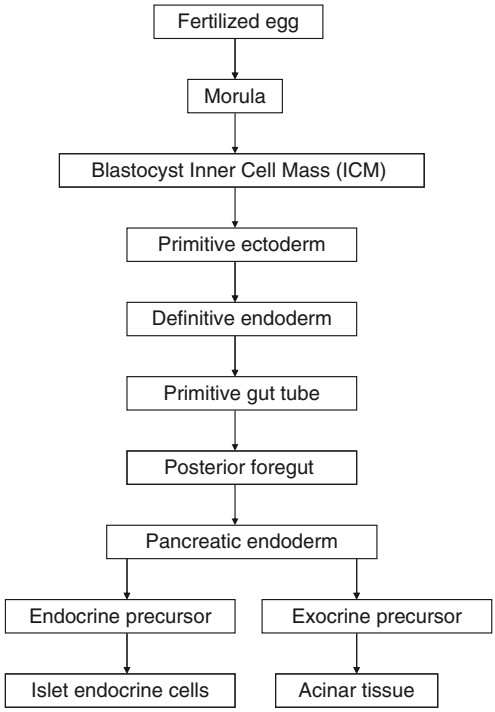

Fig. 13 Intermediate developmental stages between fertilization and the formation of the pancreas (see main text for details)

2 Generation of Endoderm/Gut Epithelium

Primitive endoderm and *epiblast* are, respectively, the outer and inner layers of the inner cell mass (ICM) immediately before gastrulation (Fig. 14). The primitive endoderm will become part of the yolk sac, without contribution to the embryo proper. In contrast, the *definitive endoderm* is formed during gastrulation when epiblast cells leave the ICM through the primitive streak. There is an intermediate stage in definitive endoderm formation, called *mesendoderm*. Although visceral and definitive endoderm are similar, mesendoderm-specific genes such as *goosecoid* (*Gsc*) and *Brachyury* (*Bry*) do not appear during visceral endoderm differentiation,[50–53] and therefore can be used to identify true definitive endoderm.[54] The *anterior* part of the definitive endoderm will evolve into the foregut, from which pancreas, liver, and lungs will eventually bud out. The *posterior* definitive endoderm, on the other hand, becomes the midgut and hindgut, which will differentiate into large and small intestine. Nodal, a member of the transforming growth factor (TGF)-β family, is the main signaling molecule responsible for the initial patterning of the primitive gut epithelium. The gradients of Nodal are finely tuned, as shown in experiments where significant reductions in its expression resulted in preferential formation of mesoderm at the expense of endoderm.[55] Additional studies imply that the regulation

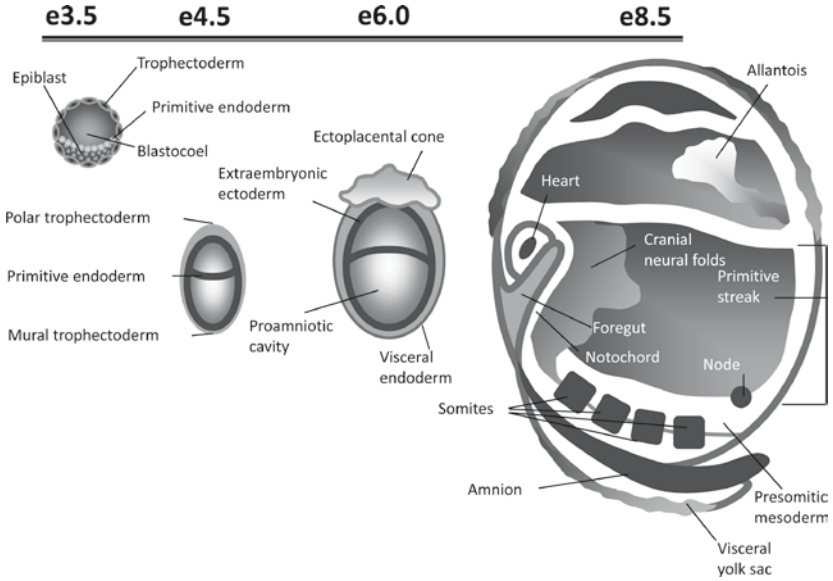

Fig. 14 Initial stages of embryonic development after the formation of the blastocyst (e3.5). Adapted from Hogan et al. *Manipulating the Mouse Embryo-A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, 1994

of Nodal gradients is a dynamic process that involves not only the secretion of the protein, but also the activity of specific repressors such as *Drap1*.[56–58]

Many genes have been associated with the formation of true endoderm, including *Foxa1–2*, *Mixl*1, *Eomes*, *GATA4–6*, and several members of the *Sox* family (reviewed in[53,59]). Although there is a potential redundancy with other *Sox* genes, *Sox*17 is essential for embryonic cells to become endoderm in mouse.[60] At least in *Xenopus*, *Sox*17 also appears to be sufficient to induce endodermal fates.[61] A theoretical model for the cross-talk between these genes (adapted from[53]) is presented in Fig. 15.

3 Pancreatic Differentiation

The interaction between the gut endoderm and the surrounding mesoderm is primarily mediated by *Sonic Hedgehog* (*Shh*) signaling.[59,62,63] *Shh* is highly expressed throughout the gut epithelium, but is down-regulated in a *Ptf1a(p48)*/*Pdx1*$^+$ region that will later become the pancreas at e8. Both *Shh* repression and activation of *Ptf1*α and *Pdx1* are defining events of pancreatic specification (Fig. 16). Chemical inhibition of *Shh* by the steroid alkaloid cyclopamine enhances pancreatic differentiation, as Pdx1 expression is no longer restricted throughout the posterior foregut.[64] Conversely, ectopic expression of *Shh* under the control of the *Pdx1* promoter induces intestinal fates (including smooth muscle and interstitial cells of Cajal) instead of pancreatic fates (Fig. 17).[65]

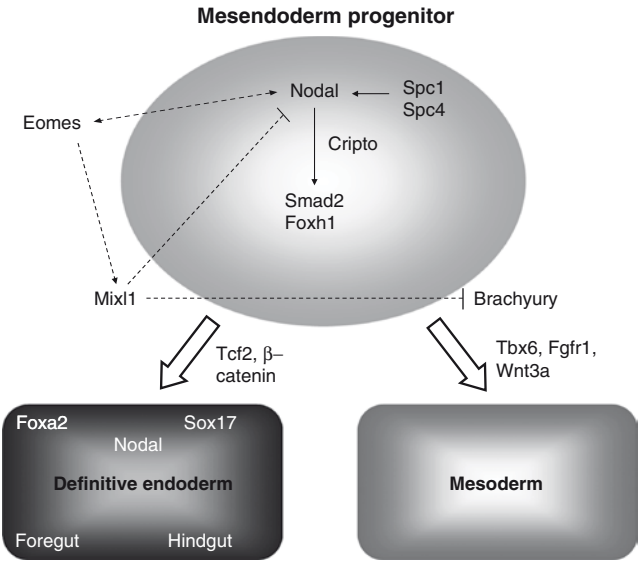

Fig. 15 A theoretical model for the molecular interplay leading to the development of definitive endoderm from mesendoderm. Nodal signaling is essential for the specification of mesendodermal progenitors. Definitive endoderm formation requires the concerted activity of Mixl1, β-catenin, and Tcf2 (HNF-1β). Mesoderm specification, in contrast, is influenced by Fgfr1, Tbx6, Brachyury, and Wnt3a. Different requirements for Foxa2, Sox17, and Nodal are found throughout the gut endoderm. Potential interactions between Eomes, Nodal, T, and Mixl1 are indicated with a *dotted line* (Adapted from Tam et al.[53])

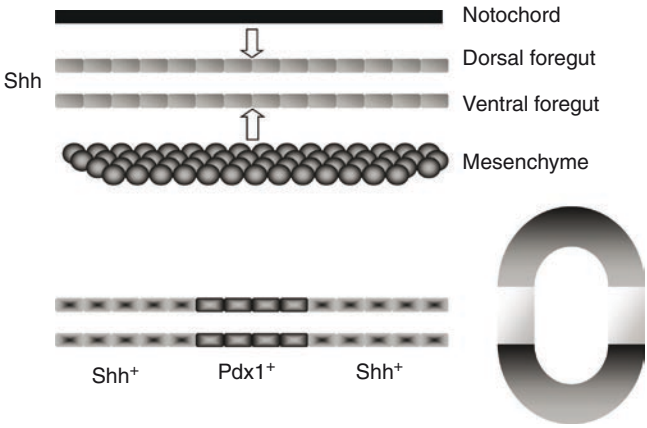

Fig. 16 The pancreas is specified from a region of the embryonic foregut where Shh expression has been excluded due to active signaling from the notochord and surrounding mesenchyme. This region will express the pancreatic and duodenal homeobox 1 (Pdx1), as well as Ptf1α(p48). A transversal cut of the foregut at this point would give a pattern similar to that depicted to the *right*, *bottom*: *top* and *bottom* $Pdx1^{+}/Shh^{-}$ areas, which will form the dorsal and the ventral pancreatic buds upon evagination, and a *middle*, $Pdx1^{-}/Shh^{+}$ region with pro-intestinal cells

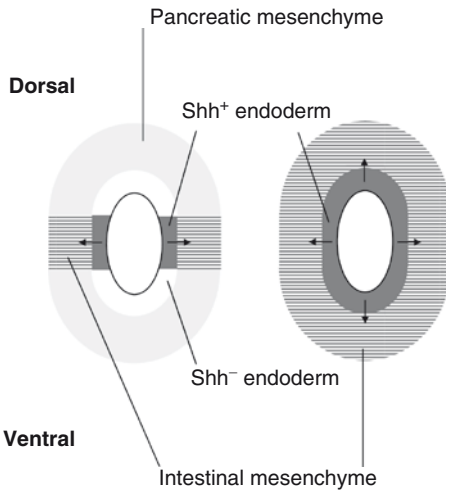

Fig. 17 During regular pancreatic development, an area is defined in the posterior foregut in which Pdx1 expression occurs at the expense of Shh. This patterns the early pancreas as two Shh-excluded regions that will bud out dorsally and ventrally (*left*). Shh$^+$ areas, in contrast, will adopt an intestinal fate. Ectopic expression of Shh under the expression of the Pdx1 promoter will extend the latter phenotype in every direction (*right*), preventing appropriate pancreatic specification (Adapted from Apelqvist et al.[65])

Pdx1

The pancreatic and duodenal homeobox 1 gene is also known as insulin promoter factor 1 (Ipf1) or islet/duodenum homeobox 1 (IDX1). In the adult mouse, it is selectively expressed in islet beta cells, where it binds to and regulates the insulin promoter.[66] Pdx1 is first expressed in the region of the foregut endoderm that will later become the pancreas and the duodenum (~e8.5 or 10-somite stage, see main text). Up to ~e10, it is uniformly expressed in the dorsal and pancreatic buds. Pdx1 is subsequently down-regulated in the entire organ, to reappear again in arising beta cells from e11 onward.[67] Lack of Pdx1 expression results in selective agenesis of the pancreas, both in knockout mice[68] and in humans with a single-nucleotide mutation.[69] However, it was also shown that the earlier events of pancreatic morphogenesis take place even in the absence of functional Pdx1, which suggests that Pdx1 acts in concert with other factors.[70] In addition to its well-studied role during pancreatic development, expression of Pdx1 is essential for the maintenance of the phenotype in adult beta cells, as evidenced by conditional knockout experiments.[71,72] Heterozygous Pdx1$^{+/-}$ mice exhibit an age-dependent worsening of glucose tolerance, reduced glucose-stimulated insulin release, and higher susceptibility to apoptosis.[73] The impaired glucose response of *Psammomys obesus*, a model of type 2 diabetes, was also associated to Pdx1 deficiency.[74] Because of its critical role in orchestrating the early events of pancreatic development, as well as in the acquisition of beta cell properties, Pdx1 has been extensively used as a tool for the differentiation of stem cells.

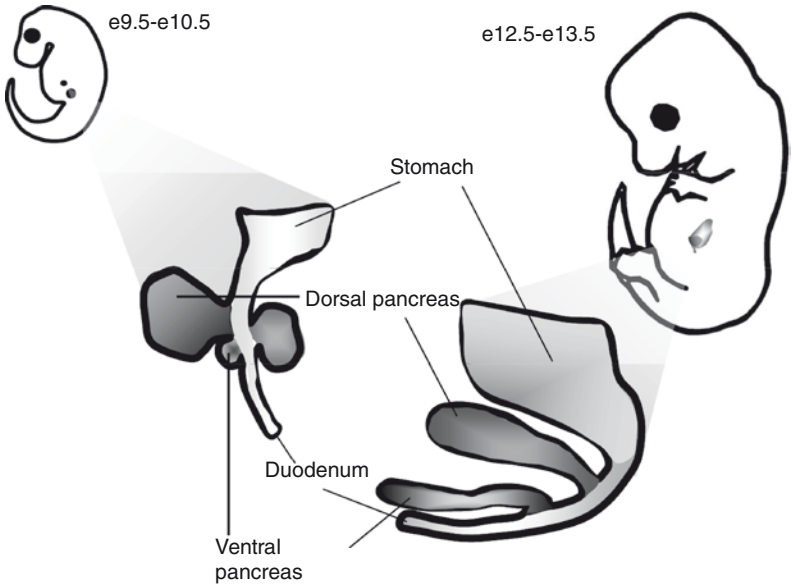

Fig. 18 Spatial distribution of dorsal and ventral buds throughout the early stages of development. The two anlagens will eventually fuse in one single organ

In the mouse, the areas defined by expression of *Pdx1* and repression of *Shh* will start to branch out dorsally and ventrally. This initial separation between the *dorsal* and the *ventral pancreas*[75] will persist until later in development, when the two primordia will fuse (Fig. 18). The influence of blood vessels in the overall development of the pancreatic primordia is well established. Thus, while removal of the dorsal aorta in frog embryos abrogated insulin expression, transgenic mice where the posterior foregut was ectopically vascularized developed hyperplasic islets and elevated insulin expression.[76] It is in this context that endothelial cell signaling has been identified as a major morphogenetic agent in pancreatic specification.[77]

Ptf1α (p48)

Ptf1α is the α-subunit of the pancreas-specific transcription factor 1 (Ptf1), a basic helix-loop-helix (Bhlh) protein first described as a DNA-binding element regulating the expression of α-amylase 2, elastase 2, and trypsin in the acinar pancreas.[78] p48 knockouts have a complete absence of exocrine pancreatic tissue, suggesting that the gene is a key regulator of acinar tissue development.[79] This role was confirmed by the finding that endocrine cells (relocated to the spleen) were not affected by the abrogation of p48 expression. Later studies, however, found an additional role for p48 in the initiation of

pancreatic development,[49,80] because its expression is observed in the Shh-excluded area of the foregut endoderm around e8.5. The expression patterning at this stage of p48, but not that of Pdx1, is thought to be partially mediated by aortal endothelial signaling.[81] In *Xenopus*, the combination of both Pdx1 and p48 expression was sufficient to induce ectopic pancreatic formation,[82] but the initiation of mouse pancreatic development might require additional genes, such as Hlxb9 (see below).

HNF-6 (OC-1)

Hepatocyte nuclear factor (HNF-6), also termed Onecut (OC)-1, is a member of the OC family of transcription factors, generally characterized by a single cut domain and a homeodomain distinct from that of other homeoproteins, including those of the cut subfamily.[83] During embryonic development, it is highly expressed in the developing central nervous system (CNS) and from e9.5 in the foregut–midgut junction and liver primordium. Pancreatic expression is detectable throughout the epithelium from e10.5 onward, although it seems to be excluded from the islets at e18.[84] Pancreatic growth and endocrine cell differentiation were severely impaired in Hnf-6 knockout mice, with an almost total abrogation of Ngn3 expression.[85] The same authors demonstrated that Ngn3 is indeed a downstream target of Hnf-6. Interestingly, however, islets were able to "regrow" after birth. This is consistent with the view that adult islet regeneration occurs typically through Ngn3-independent processes,[86,87] with only one known experimental exception (in which reactivation of the embryonic developmental program was observed after partial duct ligation; see the chapter "Pancreatic Regeneration").[88] Notwithstanding this, the newly generated beta cells were defective in Glut-2 and these animals remained diabetic.[85] Additional studies demonstrated not only that Hnf-6 expression precedes that of Pdx1 in the foregut endoderm, but also that (1) the expression of the latter is delayed in Hnf6$^{-/-}$ embryos; and (2) Hnf-6 binds to the Pdx1 promoter and stimulates its activity.[89]

TCF2 (HNF 1beta)

Transcription factor 2 (Tcf2), also called hepatocyte nuclear factor (HNF) 1beta is a POU homeobox transcription factor that has been associated with a variant of maturity-onset diabetes of the young (MODY). Other mutations of the gene result in pancreatic atrophy and hypoplasia in humans.[90] The gene is highly expressed from e8.5 in the entire foregut–midgut region and in the

(continued)

TCF2 (HNF 1beta) (continued)

pancreatic primordia by e9.5, where it colocalizes with Ptf1α and Pdx1.[91] Although Tcf2$^{-/-}$ knockout mice display early embryonic lethality due to defective formation of the visceral endoderm, tetraploid rescue with Tcf2$^{-/-}$ embryonic stem (ES) cells results in embryos that can proceed throughout development. In these embryos, the formation of the dorsal, but not the ventral pancreatic bud could be observed. However, this bud was hypoplastic throughout development and disappeared around e13.5.[92] This phenotype is similar to that of Ptf1α knockouts,[79] albeit more severe; indeed, a Tcf2-binding site was identified in the Ptf1α promoter, which would be consistent with a role of the former in the regulation of the latter.[92] Pdx1 expression, however, was still detectable at e9.5 in Tcf2$^{-/-}$ embryos, suggesting that the latter is not absolutely essential for the initiation of the pancreatic program. Experimental evidence indicates that both Hnf6 and Tcf2 are indispensable for Ngn3 expression.[85,92,93]

While branching and the progression of differentiation are arrested in Pdx1-null embryos (lack of *Pdx1* results in pancreatic agenesis[68],[69]), the initial evagination of the pancreatic buds, and even the appearance of scattered insulin- and glucagon-positive cells, does still occur in the absence of *Pdx1*.[70] Recent evidence suggests that the expression of Ptf1α, previously thought to be exclusively a marker of exocrine progenitor cells, may actually precede that of *Pdx1*.[49,80,81] Additional experimental evidence (e.g., simultaneous ectopic expression of both *Pdx1* and *Ptf1a* induces stable conversion of posterior endoderm into pancreas[82]) seems to confirm that the concerted action of both is necessary for the initiation of the pancreatic program.

Hlxb9

Human homeobox gene 9 (*Hlxb9*), also known as its encoded protein, HB9,[94] is expressed in fully differentiated beta cells and from very early on (eight somite stage, ~e8) in the notochord and the ventral and dorsal pancreatic endoderm.[95] *Pdx1*, in contrast, is expressed only in the ventral pancreatic endoderm at this stage of development. The observation that *Hlxb9* expression precedes that of *Pdx1* (at least in the dorsal anlagen) suggests an active role of this gene in shaping the early events of pancreatic specification. *Hlxb9* knockouts show a selective agenesis of the dorsal pancreas.[95,96] Although the ventral lobe still develops, its islets are smaller and beta cells within them less numerous, with evident reduction in beta cell-specific factors such as Nkx6.1 and Glut2.[96] Ectopic expression of *Hlxb9* beyond e8 in *Pdx1–Hlxb9* transgenic mice led to severe impairment in pancreatic development, with decreased endocrine and exocrine differentiation and a partial adoption of intestinal fates.[97]

4 Ductal and Exocrine Specification

CRE-ER™ technology (see box below and Figs. 20 and 21)[98,99] has allowed the development of invaluable lineage-tracing experiments to ascertain the origin of each cell type within the pancreas. Using this technology, Gu et al.[100,101] noticed that, when marking Pdx1$^+$ cells at any point during the initial stages of pancreatic development (e9.5–e11.5), the label could be detected in cells of every pancreatic tissue (endocrine, exocrine, and ductal). This served as additional confirmation of earlier knockout experiments showing that the entire pancreas arises from Pdx1$^+$ progenitor cells.[68] However, when tamoxifen-induced labeling of Pdx1$^+$ cells was performed before e9.5 or after e11.5, the marker could only be seen in acinar and endocrine cells. Thus, it appears that ductal progenitors are specified from Pdx1$^+$

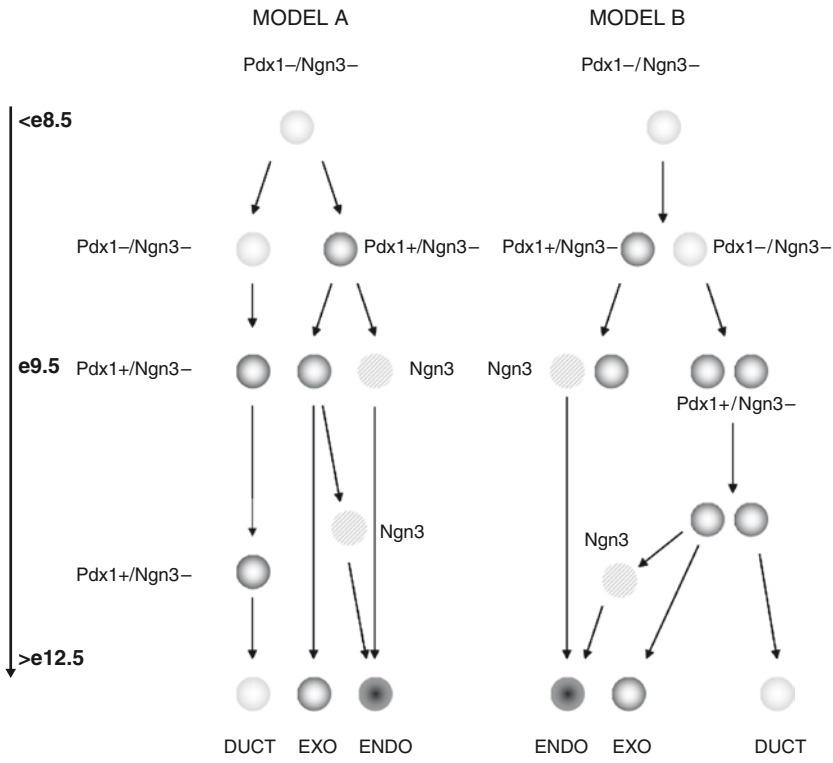

Fig. 19 Two hypothetical models to explain the acquisition of each main cell fate within the developing pancreas. (Adapted from Gu et al.[100,101]) In model A, the divergence between ductal and acinar/endocrine tissue occurs before e9.5. Ductal-committed cells acquire Pdx1 expression between e9.5 and e11.5. Thus, during this time window there are two distinct Pdx1 populations. In model B, the divergence occurs between e9.5 and e11.5. According to this hypothesis, suppressive or inductive signals received during this time will determine whether Pdx1$^+$ cells acquire duct or endocrine/exocrine fates. In both cases, Ngn3 expression is responsible for the specification of endocrine cell types

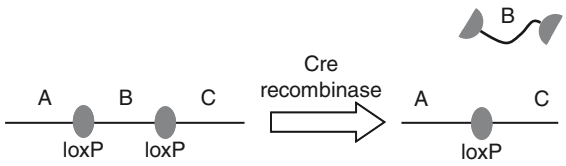

Fig. 20 Cre/loxP excision. The Cre recombinase will cut anything comprised between two loxP sites, leaving a single one. The original loxP-flanked fragment is lost

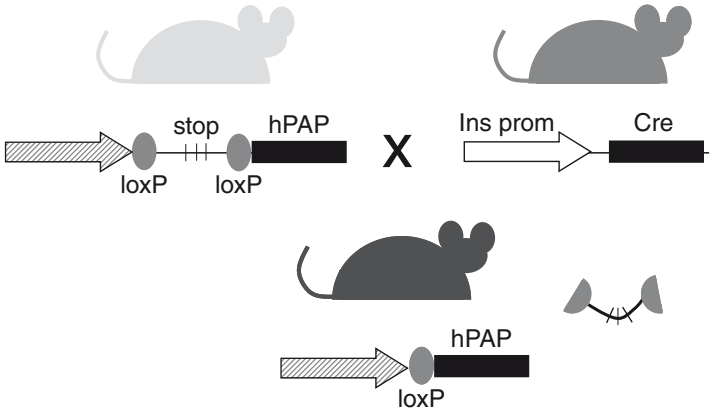

Fig. 21 When a transgenic mouse containing a loxP-flanked stop codon between a constitutive promoter (*striped arrow*) and a cellular marker such as human placental alkaline phosphatase (hPAP) is mated with another strain containing a Cre cassette driven by a tissue-specific promoter (in this case insulin, *open arrow*), the resulting offspring will display the hPAP label only in the insulin-producing cells. This system can be further refined by using Cre^{ER}, which will not penetrate the nucleus and excise the loxP-flanked DNA unless tamoxifen is given to the animal. This alternative strategy allows for the generation of a "pulse" in which specific cell types can be labeled only at one point. Their progeny will inherit the label, but cells that start expressing the chosen promoter (here, insulin) after the administration of tamoxifen will not be labeled

cells during this time window, and then set aside. According to this observation, endocrine cells are specified from $Pdx1^+$ cells upon disruption of the Notch pathway, transient Ngn3 expression and down-regulation of Ptf1α; exocrine cells arise from $Pdx1^+$ cells where both Notch signaling and Ptf1α expression persist; and, finally, ductal cells originate from a specific subset of $Pdx1^+/Ngn3^-$ cells that appear between e9.5 and e11.5. Figure 19 presents two specification models based on different divergence points.

Cre/loxP and CRE-ER™ Systems for Lineage Tracing

The CRE-ER™ transgenic system[98,99] is based on the fusion of the Cre recombinase (which excises any fragment of DNA comprised between any two loxP sites; Fig. 20) to the ligand-binding domain of a modified estrogen receptor.[102] In short, whenever Cre is produced in any specific tissue as a result of a conventional Cre/loxP strategy (Fig. 21), the protein remains cytoplasmic unless the animal is injected with tamoxifen. In that case, the Cre protein is internalized into the nucleus and it will cut between loxP sites. If the result of this cut is the expression of a marker such as alkaline phosphatase, that cell and its progeny will be permanently labeled.

5 Endocrine Specification

Endocrine differentiation occurs through a *lateral inhibition* process[103] mediated by *Notch* signaling. Cells in which the *Notch* receptor is activated by the ligands *delta* or *serrate* express high levels of *HES-1*, which in turn represses the pro-endocrine gene *Ngn3*. Lower levels of *Notch* signaling may randomly occur in individual cells, where *HES-1* expression will not be up-regulated. In the absence of its repressor, *Ngn3* will be expressed robustly, and the cell will adopt a pro-endocrine fate[104–107] (Fig. 22).

Ngn3

Neurogenin 3 (Ngn3) encodes a class B Bhlh factor. Knockout studies have shown its requirement for the development of all the endocrine cell lineages of the pancreas.[105] Thus, $Ngn3^{-/-}$ mice are born without islets. The pro-endocrine role of ngn3 has also been demonstrated in gain-of-function studies. Both ectopic ngn3 expression[104,106] and lineage tracing experiments[100,101] indicate that ngn3 is a cell-autonomous determinant and true marker of endocrine progenitor cells. The pattern of adoption of endocrine cell fates seems to follow a specific timeline, suggesting that ngn3-positive cells adapt their responses to a changing milieu of signals present in the bud microenvironment. Early ngn3 expression in pancreatic progenitor cells (e8.5–e9) results in their differentiation into glucagon-producing cells; adenoviral expression of ngn3 in adult human ductal cells leads to neuroendocrine differentiation[65]; and ectopic expression of ngn3 in the chick gut results in endodermal cell differentiation into endocrine cells that cluster in the mesenchyme.[108] Furthermore, when the Ngn3 protein was engineered with a TAT protein transduction domain, it was shown to promote endocrine cell differentiation in vitro.[109]

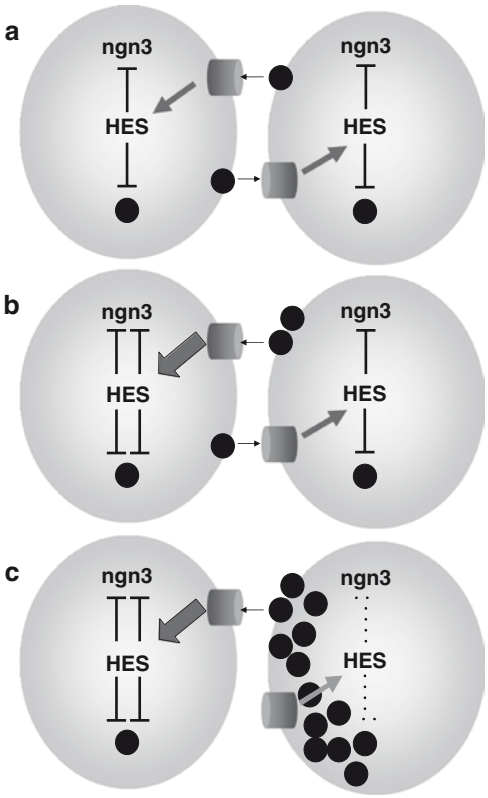

Fig. 22 Lateral specification mechanisms leading to endocrine cell differentiation. Pancreatic progenitors at an early stage of development can be considered functionally equivalent. (**a**) Any cell will both secrete the Notch ligand (delta or serrate, *black circles*) and receive it from the neighboring cell. Activation of the Notch receptor leads to the upregulation of Hairy Enhancer of Split-1 (HES-1), which is a potent repressor of the pro-endocrine gene Ngn3. HES-1 will also block the production of more ligand, but this blockade is leaky. A basal level of ligand is still produced, which maintains the system in an unstable equilibrium. In the presence of active Notch signaling, further differentiation does not occur. The activation of this pathway is normally associated with proliferation. (**b**) If one of the two cells in the model expresses more ligand than the other, the effect on HES-1 in the receiving cell will be proportional. While Ngn3 remains repressed, cells where HES-1 is over-activated as a result of overstimulation exert a stronger blockade on the production of ligand. (**c**) Repression of ligand secretion in one cell results in down-regulation of HES-1 in the other. If HES-1 is de-repressed, so is (a) its production of ligand (which will be secreted in large amounts to keep the other cell undifferentiated); and (b) the expression of Ngn3, which will induce a pro-endocrine fate in the cell where Notch has been down-regulated

Isl1

Isl1 is a LIM homeodomain protein - a family of proteins featuring a DNA binding homeodomain and two LIM domains, zinc-binding motifs that mediate protein-protein interactions.[110] It was originally identified as a regulator of the rat insulin I gene enhancer,[111] and is ubiquitously expressed in

all mature islet endocrine cell types. During development, Isl1 expression is detected immediately upon maturation of these cells (~e9 for glucagon-positive cells, ~e10.5–11 for insulin-positive cells), as well as in the mesenchyme of the dorsal bud.[112] As expected, Isl1 knockout mice fail to develop pancreatic endocrine cells. The dorsal pancreatic mesenchyme is also absent, which in turn impairs exocrine cell differentiation in the dorsal pancreas. The latter effect, however, could be rescued in vitro by coculture with pancreatic mesenchymal tissue extracted from wild-type animals.[112]

Brn-4

Brain-4 (Brn-4/Pou3f4) is a class III POU homeodomain-containing protein. It is expressed both in neural tissue during CNS development and in pancreatic glucagon-producing cells as early as e10,[113] where it seems to bind to the G1 element of the proglucagon gene proximal promoter. Although Brn-4 is the very first marker of alpha cells (preceding the expression of Pax6 and even Isl1), endocrine cell formation is perfectly normal in Brn4$^{-/-}$ mutants.[114] This is a surprising observation, and more so in view of the fact that ectopic expression of Brn4 from the insulin promoter results in the coexpression of insulin and glucagon in beta cells.[115] Taken together, these data suggest that Brn4 acts at an early developmental stage, but is not essential for the alpha cell fate determination.

The differentiation into each endocrine cell type within the islet is preferentially observed at specific time points during embryogenesis (alpha cells since e9.5; beta cells since e10.5; δ cells since e14.5; and PP cells since e18.5), suggesting that *Ngn3*$^{+}$ cells adapt their responses to an evolving milieu of signals.

BETA2/NeuroD

The beta-cell E-box transactivator 2 (BETA2, also known as NeuroD) is a cell-restricted Bhlh first isolated from insulinoma cells, where it was shown to be a component of the native insulin E-box-binding complex with an associative preference to the ubiquitous Bhlh factor E47.[116] Mice where BETA2/NeuroD has been knocked out display a dramatic reduction in the number of beta cells, impaired islet morphogenesis and some additional abnormalities in the exocrine pancreas, such as defects in the apical-basal polarity that result in the inability of acinar cells to secrete zymogen granules.[117] The main effects of this knockout were observed after e15.5, suggesting that this transcription factor is needed at a relatively late stage of endocrine cell development. Ngn3 was found to be a critical upstream regulator of BETA2/NeuroD expression during islet specification.[118]

6 Beta Cell Differentiation

Little is known about the extracellular signals that drive beta cell specification from *Ngn3*$^+$ progenitors. Animals lacking *Nkx6.1*[119] and *Nkx2.2*[120] have defects in beta cell formation. *MafA* has also been implicated in the terminal differentiation of beta cells, particularly in the beta cell-specific reactivation of Pdx1.[121] However, several observations point to *Pax4* as the main hallmark of beta cell differentiation: The knockout of this gene results in the total absence of beta cells,[122] but not alpha cells; its expression peaks between e13.5 and e15.5, which coincides with the period of maximal differentiation of beta cell precursors[122,123]; and shortly after endocrine specification, *Ngn3* colocalizes with *Pax4*,[124] which suggests that the latter may be one of the targets of the former.[125,126]

Also, ES cells transfected with *Pax4* were induced to express insulin at much higher levels than untransfected controls.[127] Recent evidence indicates that *Pax4* and *Arx* are mutually repressed, and that the balance between the two determines α (*Arx*) or beta (*Pax4*) specification from *Ngn3*$^+$ progenitors.[128–130]

Nkx2.2

The NK2 homeobox 2 (Nkx2.2) gene belongs to a family of genes involved in the differentiation of many tissues.[131] Nkx2.2 expression is observed both in the ventral CNS and in mature alpha, beta, and PP cells. The gene is activated from very early on during pancreatic specification (~e8), but – in a pattern similar to that of Pdx1 – becomes restricted to specific islet cell types later in development.[120] Nkx2.2 mutant mice lack beta cells and have lower amounts of other islet endocrine cell types. Further analyses of these islets, however, show a sizeable population of hormone-negative undifferentiated cells. While Pdx1 expression remains largely unaffected in Nkx2.2 knockouts at the onset of pancreatic specification (~e8.5), the relative strength of its signal is significantly reduced during the secondary transition (~e13.5 onward). This led to the hypothesis that Nkx2.2 might be required for the terminal differentiation of beta cells.[120]

Nkx6.1

Nkx6.1 is another member of the NK homeodomain family, which can be found both in the pancreas and the CNS.[132] Nkx6.1 expression is first detected from e10.5 in the pancreatic epithelium. At e15.5, it colocalizes either with Pdx1$^+$ cells (postmitotic beta cells, mostly) or with Ngn3 (immature progenitors). At later developmental stages, as well as in the adult organ, Nkx6.1 is exclusively restricted to beta cells.[119] Knockouts display a marked deficiency of beta cells, which could be traced back to the initiation of the secondary

transition, during which beta cell neogenesis was severely impaired. Nkx2.2 expression was not affected, suggesting that this gene is upstream of Nkx6.1. This was further confirmed by double Nkx2.2/Nkx6.1 knockout experiments, whose phenotype was identical to that of Nkx2.2$^{-/-}$.[119]

Pax4

Paired box-containing gene 4 (*Pax4*) belongs to a multigene family that shares a conserved motif, termed "paired box."[133] Both *Pax4* and *Pax6* have, in addition, a homeodomain.[134] *Pax4* expression is first detected in the pancreas at around e9.5, and is later restricted to beta cells. Knockout mice lack both beta and delta cells, but alpha cells appear to compensate for their absence with a much higher than normal representation in the islet, which is suggestive of a "default" alpha cell differentiation pathway. Several lines of evidence point at *Pax4* as a direct downstream target of Ngn3.[123–126] As discussed in the main text, the balance between *Pax4* and *Arx* might be critical for the adoption of alpha or beta cell fates.[128–130]

Pax6

Perhaps best known for its role in eye development,[135–138] *Pax6* is also expressed both in the developing pancreas (scattered throughout the fore/midgut cells at e9.5; colocalizing with arising alpha cells at e9.5; with alpha or beta cells at e15.5) and with alpha, beta, or delta cells in the adult pancreas – but not in acinar tissue.[139–141] Knockout mice show both a very significant reduction in all hormone-producing cells (with alpha cells largely absent) and an abnormal distribution of the remaining ones within the islet.[134,140] A model for alpha and beta cell specification based on relative levels of Pax4 and Pax6 is presented in Fig. 23.

Arx

The aristaless-related homeobox gene (*Arx*) contains both a C-terminal stretch of amino acids known as the OAR/aristaless domain and a prd-class homeobox domain. Like many other genes involved in pancreatic development, Arx was first identified in the mouse CNS.[142] Arx expression is observed in the proliferating epithelium of the pancreatic anlage at e9.5, and then is progressively restricted to endocrine cell types.[129] Ngn3 knockouts do not express Arx, indicating that the former is upstream of the latter.[129] Arx$^{-/-}$ mutants lack alpha cells and display a concomitant increase in delta cells (and, to a lesser extent, beta cells).

(continued)

Arx (continued)

This effect is exacerbated in double Pax4/Arx mutants, where both alpha and beta cell subsets are largely replaced by delta cells.[128] Arx, however, does not seem to be required for early alpha cell differentiation, because glucagon-positive cells can be readily detected until e12.5 in mutant embryos, which suggests that the main role of Arx is during the secondary transition. Taken together, these observations support a model where Arx promotes alpha cell differentiation and antagonizes that of beta cells (from e12.5 onward) and delta cells (from e14.5 onward). Because Pax4 has an opposing activity, the relative levels of each protein will likely dictate cell fate after e12.5 (see Fig. 23)

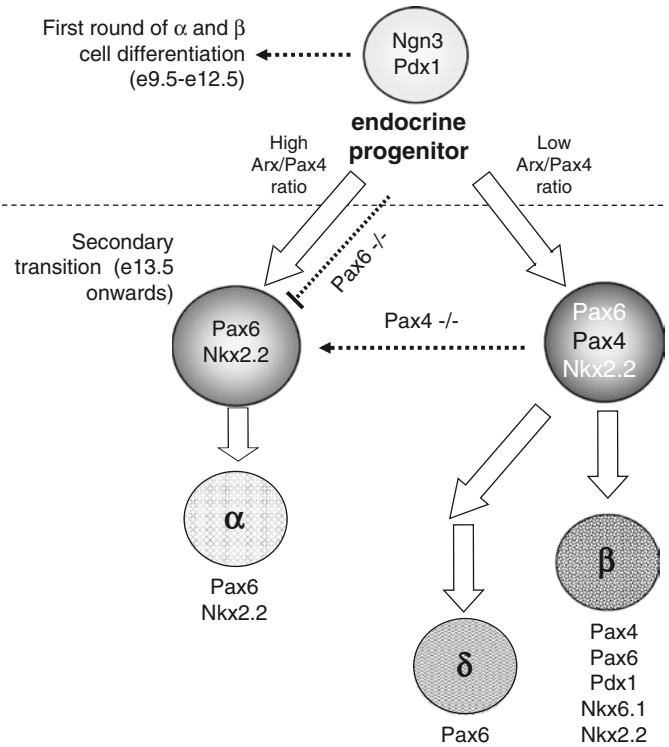

Fig. 23 A model for alpha, beta, and delta cell specification. Experimental evidence suggests that the first insulin-positive and glucagon-positive cells that appear prior to the secondary transition are a developmental dead end. Relative levels of the mutually repressing proteins Arx and Pax4 will define two populations of endocrine progenitors during the secondary transition. Both are characterized by the expression of Nkx2.2 and Pax6. However, Pax4 expression is only detected in those cells that will give rise to both beta and delta cells. The effect of knocking out either Pax6 or Pax4 is indicated in dotted lines. Figure 24 shows an alternative model where two distinct pancreatic progenitor cells exist before and after the secondary transition (Adapted from St-Onge et al.[140] and Collombat et al.[128,129])

MafA/MafB

Maf transcription factors (containing a basic leucine zipper) have been associated with the regulation of multiple differentiation processes.[143–146] The best-characterized Maf factors expressed in the pancreas are MafA and MafB, which are preferentially expressed in beta and alpha cells, respectively.[121] The role of these factors has been difficult to ascertain. MafA knockout mice, for instance, display glucose intolerance and develop diabetes; however, there are no developmental effects associated with the mutation.[147] Recent evidence suggests that a switch from MafB to MafA might be critical for the embryonic maturation and prolonged survival/function of beta cells.[121] However, MafB has also been recently shown to be essential for the appropriate regulation of Pdx1, Nkx6.1, and GLUT-2 in the final stages of maturation of beta cells.[148]

Foxa2 (HNF3beta)

The winged-helix transcription factor Foxa2 (also known as hepatocyte nuclear factor 3beta [HNF3beta]) is one of the first true markers of definitive endoderm.[149,150] Despite its expression throughout the development of the pancreas, not many studies have looked into its role in this process. These studies are chiefly based on conditional loss of function. Thus, selective abrogation of Foxa2 expression in beta cells results in insulin hypersecretion, leading to profound hypoglycemia.[151] In fact, Foxa2 has been shown to control Pdx1 expression in mature beta cells.[152] However, the onset of Pdx1 expression and pancreatic specification is not affected in mice where the Foxa2 has been selectively inactivated in the developing endoderm.[153] These animals, however, are severely hypoglycemic and die within days of birth, due to a dramatic reduction (>90%) in the number of alpha cells. They show impaired maturation, but not initial specification of alpha cells.[153]

7 The Secondary Transition

The secondary transition is a phenomenon first observed in the pioneering studies on the developing pancreas conducted by Pictet and Rutter in the early 1970s.[154,155] In short, it can be described as a secondary wave of synchronized endocrine and exocrine differentiation. Although there is no clear-cut initiation, it is generally acknowledged that the secondary transition starts around e13.5 in the mouse. Recent studies suggest that changes in the TGF-β signaling pattern might be responsible for this developmental phase.[156] Throughout its course, the epithelium branches extensively: the termini of the ducts give rise to exocrine cell types, and the cells lining the ducts become a niche for pancreatic progenitor cell proliferation and differentiation.

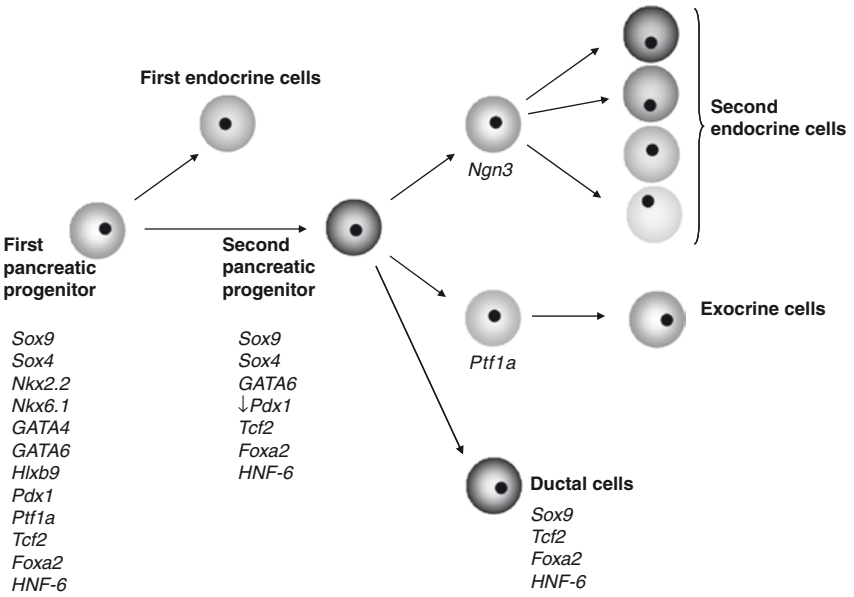

Fig. 24 A differentiation model contemplating the existence of two distinct pancreatic progenitor cells before and after the secondary transition (Adapted from Lynn et al. 2007[157])

It has been reported that the progenitor cells detected during the secondary transition are different from those that give rise to the first differentiated cells before e13.5. Thus, the early progenitors generally express markers such as Ptf1α, Hlxb9, Pdx1, Sox4, Nkx2.2, and Nkx6.1, which are later restricted to differentiating cells during the secondary transition[157] (Fig. 24).

Prox1

Expression of Prospero-related homeobox 1 (Prox1) was first observed in the CNS, developing eye lens, liver, and pancreas. In the latter, Prox1 can be detected from e9.5 in the dorsal bud,[158] but other authors pin down the initiation of Prox1 expression to an even earlier time point, before the divergence between liver and pancreas.[159] At any rate, Prox1 expression is detected throughout pancreatic development in endocrine, but not exocrine tissue. In adult mice, Prox1 signal was stronger in ductal cells than in the beta cell component of the islets.[160] Prox1 mutations lead to embryonic lethality at around e15.5, due to severe developmental aberrations. The phenotypic manifestation of Prox1 knockout is an arrest in pancreatic growth from e11.5, with an increase of exocrine cell differentiation at the expense of the progenitor cells responsible for the secondary transition. This has led to the hypothesis that Prox1 might have a role in repressing exocrine differentiation from bipotential endocrine/exocrine progenitors.[160]

Sox4/Sox9

The SRY/HMG box (Sox) family of genes is involved in many developmental processes. At least 11 of these genes are expressed in the pancreas during embryonic development.[161] Among these, the best characterized are *Sox4* and *Sox9*. Sox4 is broadly expressed in the epithelium throughout development, but later becomes restricted to hormone-producing cells. The pancreas of *Sox4* knockout mice appears to be normal until the initiation of the secondary transition. However, experiments where pancreatic buds were explanted at e13.5 and grown in vitro (the mutation is lethal at around e14[162]) showed a dramatic reduction in the differentiation of beta and other endocrine cell types.[161] This observation suggests that Sox4 might be involved in facilitating the secondary transition. Sox9 is expressed in the pancreas as early as e10.5, where it can be found together with $Pdx1^+$, but not differentiated cells.[157] The same pattern applies at later stages of development: at e14.5 it is detected coexpressed with $Pdx1^+$ undifferentiated cells and $Ngn3^+$ progenitors. Furthermore, reporter experiments demonstrated that Sox9 is involved in the regulation of the Ngn3 promoter.[157] Taken together, these observations suggest a role of Sox9 as a critical component of the molecular network responsible for the maintenance of pancreatic progenitor cells.

8 Do Physical Factors Play a Role in Pancreatic Development?

Progress in our understanding of the influence of nonchemical agents in the progression of embryonic development is consolidating old disciplines such as biophysics and shaping new ones, such as mechanobiology. It is known, for instance, that mechanical forces generated by the division of cells in a confluent setting (such as that found in living tissues) are able to regulate the cellular pathways of proliferation and differentiation.[163] This is an example in which the form of the tissue (a physical parameter) would be not just the outcome, but also the effector of certain developmental programs. This knowledge is currently being applied for the design of better in vitro differentiation protocols that take into account not only the adequate biochemical milieu, but also physical determinants such as tensile strength and the nature of the substrate.[164–170]

Another example is that of bioelectrical fields, whose clear-cut influence on cell behavior and tissue patterning, morphogenesis, and regeneration remains largely ignored by mainstream developmental biologists. Thus, it is known that applied fields can cause cell differentiation and even dedifferentiation,[171–175] and that regeneration depends on the bioelectrical properties of the tissue.[175,176] Indeed, it has been hypothesized that biomagnetic fields provide the coordinates for cell migration and

tissue branching.[177,178] For instance, ion channel-generated fields precede and predict the appearance of limbs in several species, whereas the suppression of electrical activity results in arrest of growth and differentiation.[161,179–181]

While bioelectric fields constitute the essence of pancreatic beta cell function, nothing is known yet about the potential influence of this factor in their development. The same applies to virtually every other physical agent studied so far in this context, including oxygenation. The latter is especially surprising, because the role of molecular oxygen in shaping development has already been well established in many tissues and organs, including mammalian placentation, adipogenesis, cardiovascular/pulmonary development, bone morphogenesis, and general stem cell behavior (reviewed in[182,183]). This void has been explored in a tentative manner only recently,[184] but there is still a clear need to experimentally validate working models in which the oxygen-sensing machinery would directly affect critical pancreatic developmental pathways such as Notch and Wnt/beta-catenin.[185] Cells detect low oxygen concentrations by means of the hypoxia-inducible factor 1 (HIF-1) protein. HIF-1 is a heterodimer consisting of two subunits: the beta domain, which is expressed constitutively, and the oxygen-dependent alpha protein.[186] The latter is degraded at high pO_2s but remains intact at lower tensions.[186,187] When activated, the HIF-1 dimer is known to participate in the regulation of a variety of cellular processes,[186,188–191] including progenitor cell self-renewal and proliferation.[188,192–194]

Since mammalian embryogenesis occurs at a very low oxygen tension before the initiation of blood circulation,[182,195–198] the early stages of pancreatic development are also expected to take place under hypoxic conditions. However, at e13.5 (coinciding with the onset of the secondary transition in the mouse), blood starts to flow in the pancreatic rudiments.[199] In fact, this might be considered a consequence of HIF-1-mediated stimulation of blood vessel formation in a hypoxic environment.[186,188]

As mentioned earlier in this chapter, the Notch pathway is typically associated with proliferation and the maintenance of an undifferentiated state.[200] Indeed, down-regulation of Notch signaling is critical for the activation of Ngn3 and subsequent endocrine differentiation (Fig. 22).[48,104,106,201–203] Because Notch is activated by HIF-1alpha under hypoxic conditions,[188,192,204–206] it is plausible that higher oxygenation (such as that expected from e13.5 onward) might partially disable this pathway.

$$\Downarrow O_2 \Uparrow \textit{HIF-1}\,\alpha \Uparrow \textit{Notch} \Uparrow \textit{Endocrine progenitor cell proliferation}$$
$$\Uparrow O_2 \Downarrow \textit{HIF-1}\,\alpha \Downarrow \textit{Notch} \Uparrow \textit{Endocrine progenitor cell differentiation}$$

Higher oxygenation could have effects on the developing pancreas other than Notch down-regulation and a wave of endocrine differentiation. For instance, Wnt/beta-catenin signaling (which is key for the maintenance of undifferentiated

proliferating exocrine progenitor cells[207, 208]) is inhibited in hypoxia.[209,210] Therefore, high oxygen conditions present after e13.5 may also promote exocrine progenitor cell proliferation.

$\Downarrow O_2 \Downarrow$ *Wnt/beta-catenin* $\Downarrow$ *Exocrine progenitor cell proliferation*
$\Uparrow O_2 \Uparrow$ *Wnt/beta-catenin* $\Uparrow$ *Exocrine progenitor cell proliferation*

Finally, oxygen tension has been linked to chromatin reorganization through acetylation. In general, acetylated loci have a less compact DNA coiling, which leads to high transcription levels. However, if there is strong histone deacetylase (HDAC) activity, gene silencing ensues. HDAC-mediated repression of gene expression is associated with hypoxia in a HIF-1α-mediated manner.[182] At least three important genes involved in the progression of pancreatic development (namely Pdx1, NeuroD/Beta2, and Ngn3) have already been shown to be repressed in hypoxic conditions through the action of HIF-1α-dependent HDACs.[211–213] In fact, such knowledge has already been applied to enhance the rate of beta cell differentiation by means of forcing HDAC down-regulation in normoxia.[214,215]

9 Correspondence Between Mouse and Human Pancreatic Development

Despite the seemingly perfect conservation across species of most transcription factors already discussed, even a superficial analysis of human pancreatic development reveals striking differences with that of the mouse. The following are just a few examples: (1) the relative speed of the transition between the initiation of pancreatic development and the emergence of the first hormone-positive cells in the epithelium is remarkably faster in the mouse. According to the relative timeframe observed in the latter, the detection of the first endocrine cells in humans would take place no later than 4.5/5 weeks postconception (w.p.c.). However, extensive immunohistochemical analyses of the developing human pancreas show that the first endocrine cells are not detectable prior to 7 w.p.c., more than 3 weeks after pancreatic specification.[216] The biological significance of this relative delay is not fully understood yet. (2) The first endocrine cells to appear during human pancreatic development are beta cells (7 w.p.c.). In contrast with the mouse, α cells do not appear until later, at around 8.5 w.p.c.[216] (3) Islets assemble just prior to term in mouse, whereas human islets are fully formed from 12 to 13 w.p.c.[217,218] Table 2 compares chronologically the main events of fetal pancreatic development in both mouse and human.

Table 2 Correspondence between human and mouse pancreatic development

Mouse developmental stage	Event	Human developmental stage
e8.5–e9.5	Initiation of pancreatic development; evagination from the primitive foregut; Pdx1 expression	3–4 w.p.c.
e9.5–e10.5	Immunodetection of glucagon expression	8.5 w.p.c.
e10.5–e11.5	Immunodetection of insulin expression	7 w.p.c.
e12.5	The two pancreatic buds fuse at the base	8 w.p.c.
e13.5–e14.5 onward	Formation of acini from ducts	8.5 w.p.c. onward
	Initiation of secondary transition	
	Immunodetection of somatostatin expression	
e16.5–e18.5	Immunodetection of PP expression	10 w.p.c.
	Islet formation	12–13 w.p.c.

MicroRNAs and Pancreatic Development

MicroRNAs (miRNAs) are noncoding small RNAs (~19–22 nt) that regulate gene expression by posttranscriptional interference with specific messenger RNAs (mRNA)[219,220] (Fig. 25). Since each miRNA may have multiple target mRNAs, they are potentially capable of controlling very complex gene expression regulatory networks.[221,222] The function of most of them remains unknown, but some of their targets have been experimentally confirmed.[223] In beta cells, mir-375 has been shown to negatively control insulin secretion in by targeting myotrophin.[224] Several studies show that miRNAs regulate embryonic development and have tissue/cell-specific patterns.[225,226] miRNAs are necessary for murine islet differentiation,[227] and mir-375 inhibition has a deleterious effect on pancreatic development.[228] We have recently established that mir-7 is the most differentially expressed miRNA in islets,[229] and follow-up studies demonstrate that this miRNA was expressed in the human developing pancreas from week 9. The peak of expression was observed between weeks 14 and 18, coincident with an exponential phase of differentiation of hormone-producing cells.[230] Based on these intriguing findings, further research on the subject might unravel a potential implication of mir-7 in beta cell development. In conclusion, the study of miRNAs as macro-regulators of gene expression is an emerging field whose rapid advancement may completely redefine the way we understand the progression of development and the acquisition and maintenance of cell identity.

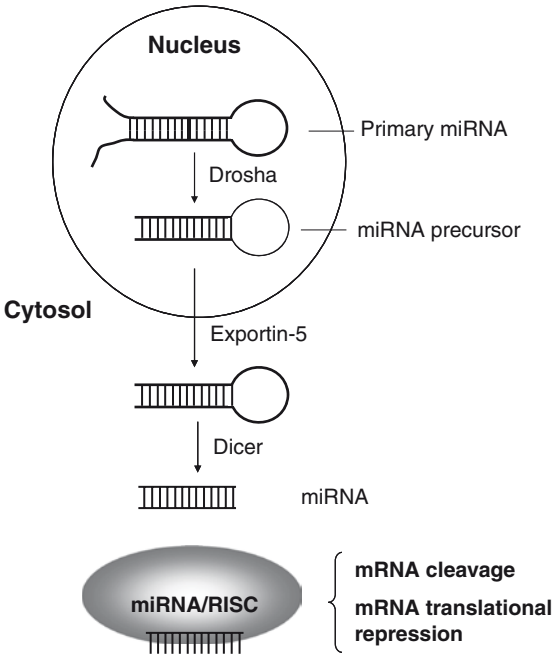

Fig. 25 Primary miRNAs are cleaved in the nucleus by the RNAse III endonuclease Drosha. This releases a ~60–70-nt stem loop pre-miRNA precursor, which in turn is actively transported to the cytoplasm by export receptor exportin-5. Once there, it is further processed by Dicer, another member of the ribonuclease III protein family. The ~21-nt double-stranded RNA cleavage product is subsequently separated into a single-stranded RNA by the action of helicases, and forms a ribonucleoprotein complex known as RNA-induced silencing complex (RISC). The RISC will guide the particular miRNA to its mRNA target. Upon binding to target RNA, RISC-miRNA would either cut it or repress its translation, depending on whether the homogeneity between the miRNA and the target mRNA is exact or incomplete, respectively

Pancreatic Regeneration

Abstract The pancreas – and especially its endocrine component – has a well-known ability to regenerate under a number of circumstances. These include obesity, pregnancy, high blood sugar levels, and experimental interventions such as cellophane wrapping, partial duct ligation, and partial pancreatectomy. Here we review the most-studied models of pancreatic regeneration and discuss the proposed molecular mechanisms behind the observed effects. These include reversible epithelial-to-mesenchymal transition, self-duplication of mature cells, and the recapitulation of the embryonic developmental program. We also discuss the different theories presented in the literature about the origin of neogenic islets/beta cells.

Keywords Pancreatic regeneration • Duct ligation • Obesity • Pregnancy • Beta cell self-duplication • Epithelial-to-mesenchymal transition

1 Introduction

Because of its slow cell turnover, the pancreas has been considered for several decades a largely postmitotic organ.[231] The overall mass of beta cells, in particular, was thought to be constant throughout adulthood, unless diminished by natural aging or disease (Fig. 26).

This idea has been challenged more recently in view of growing evidence that the islet cell mass indeed grows dynamically in response to a number of stimuli, both physiological and pathological. Among the former, the best studied is pregnancy.[233–235] The latter include high blood glucose levels,[236,237] obesity,[238] and interventions such as partial pancreatectomy,[239] cellophane wrapping,[240] streptozotocin (STZ) treatment,[241] or duct ligation.[88] The first part of this chapter will briefly present these models of regeneration, laying the ground for a second part where the still controversial origin of these newly created cells will be discussed in more detail.

J. Domínguez-Bendala, *Pancreatic Stem Cells*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-132-5_3,

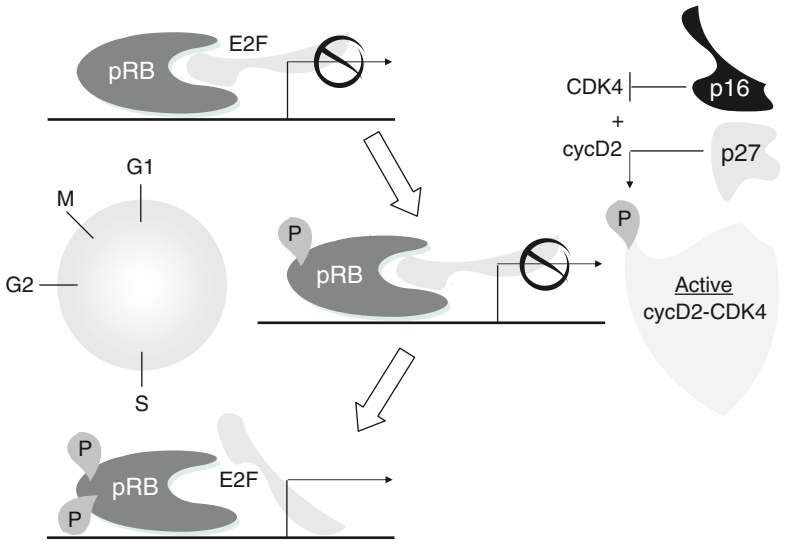

Fig. 26 The general molecular mechanisms behind somatic cell cycle apply to the beta cell. Mitogenic stimuli induce the entry of the cell in G1 phase, during which cyclin D2 and cell division protein kinase 4 (CDK4) form a complex (inhibited by p16) that will trigger cell division. Progressive phosphorylation of Prb (retinoblastoma) by cycD2-CDK4 releases E2F transcription factors, which enable progression through the cell cycle. P27 may block the process by inhibiting Prb phosphorylation (Adapted from Butler et al.[232])

2 Models of Regeneration

2.1 Pregnancy

Pregnancy is invariably accompanied by an adaptation of the pancreas of the mother to accommodate its overall insulin secretion to the needs of the developing embryo. This phenomenon is particularly notorious during late pregnancy, when at least four relevant processes have been documented, namely: (1) higher insulin synthesis and secretion;[242] (2) enhanced beta cell glucose sensitivity;[243–245] (3) beta cell hyperplasia and hypertrophia;[235] and (4) beta cell replication.[243–249] A synergistic role of circulating fatty acids during pregnancy has been suggested as a contributor to an increase in beta cell division.[247] Receptors for growth hormone and prolactin have been identified in pancreatic endocrine cell types, and their expression is significantly up-regulated during pregnancy.[245,248–250] Increased mitotic activity has also been documented in beta cells in response to placental lactogen (chorionic mammotrophin).[245,248] Lactogenic hormones, in general, promote beta cell proliferation via the JAK2/STAT-5 pathway,[251,252] although other molecular pathways may also be involved.[253] Inhibition of the mTOR signal transduction cascade, for instance, has been proposed as one potential explanation for the reduced beta cell proliferation observed in pregnant mice treated with rapamycin,[254,255]

because mTOR blockade has been shown to affect the phosphorylation level of both of the mitogen-activated protein kinase ERK1/2 and the ribosomal-S6 kinase p70s6k.[256] Ongoing research on the role of microRNAs in orchestrating the changes in pancreatic development may also shed light on their potential involvement in pregnancy-induced beta cell proliferation.

2.2 Blood Glucose Levels

During development, glucose levels appear to influence beta cell differentiation rather than proliferation. While the latter remained largely unaffected, the former was found to correlate to sugar concentration up to 10 mM, possibly by regulating the Ngn3-mediated activation of NeuroD/Beta2.[257] However, glucose is a powerful activator of beta cell proliferation in rodents, where a dose-dependent effect has been documented in vitro.[237] In humans the effect is also noticeable, but it plateaus at 5.6 mM.[258] At any rate, long-standing type 2 diabetes is almost invariably associated with a significant loss (40–60%) of beta cell mass,[31,259–261] which might be suggestive of an overall negative effect of glucose imbalance on beta cell survival and proliferation.[262,263]

2.3 Obesity

As is the case during pregnancy, it has also been suggested that the increased metabolic demands of obesity (as well as the indirect effects of insulin resistance) may also lead to a higher rate of beta cell replication. This is certainly the case in rodents, where islets enlarge up to tenfold due to an acceleration of beta cell proliferation.[264] As mentioned earlier, higher concentrations of circulating fatty acids may be directly related to this phenomenon.[265] In vitro data suggest that leptin (whose plasma levels are high in obesity) might also play a role in beta cell replication. This hypothesis remains to be tested, as leptin-resistant *fa/fa* Zucker rats also exhibit a substantial increase in beta cell mass.[266] In any case, this effect is much less clear-cut in humans, where obesity is associated with only a marginal increment in beta cell mass.[232] Differences between human and mouse beta cell turnover,[238] as well as the confounding effects of apoptosis and insulin resistance may explain these observations.

2.4 Partial Pancreatectomy

Partial (40–90%) removal of the organ is a classic model of pancreatic regeneration.[267] Two months after surgically excising 90% of the pancreas in the adult rat, the mass

of the remaining organ was observed to reach 26% that seen in sham-treated animals, with 42% of the normal beta cell mass.[268] The mitotic index (MI) remains largely unchanged in sham-treated animals for up to 3 weeks after the procedure. However, the MI of exocrine and beta cells was between threefold and fourfold higher than that of controls at 1 week after pancreatectomy. After this time point, the rate of division of the exocrine tissue diminishes gradually until it is no longer distinguishable from that of the sham-treated animals. The MI of beta cells, in contrast, remains elevated (twofold) for at least 3 weeks after the procedure.[269] Very recent data, however, suggest that this procedure does not induce beta cell regeneration in humans.[270] This example explains the high incidence of diabetes after pancreatic resections and illustrates the several differences between islet regeneration observed between rodents and humans. It also warns of the potential risks posed by therapeutic interventions based on living-donor islet transplantation.[271–276]

2.5 Duct Ligation

The ligation of the main duct has been used since the beginning of the twentieth century to study pancreatic obstruction.[277] Subsequent research resulted in the observation that this intervention led to islet beta cell neogenesis in the duct-ligated portion of the pancreas.[278] This development was accompanied by an up-regulation of the transcription factors Pdx1 and Nkx2.2.[279]

2.6 Cellophane Wrapping

As a "gentler" alternative to duct ligation, Rosenberg and colleagues developed in the early 1980s a new method to study duct epithelium hyperplasia, which had been previously associated with pancreatic carcinoma. Using a hamster model, the procedure consisted in the application of a sterile cellophane wrap around the head of the pancreas. The cellophane was not tightened up, so as to prevent the crushing of the tissue or the occlusion of the main duct (Fig. 27). An initial inflammation period led to duct fibrosis and formation of new islets as early as 2 weeks after the intervention.[280] Later studies confirmed that the use of cellophane results in a 2.5-fold increase in the number of islets per square millimeter,[281] reversing diabetes in at least 50% of STZ-treated animals.[282] A partially purified preparation of pancreata subjected to cellophane wrapping (which the authors termed Ilotropin) induced islet neogenesis from duct epithelium.[283] The pancreatic islet neogenesis associated protein gene was cloned in 1997 and its encoded protein found to be a component of ilotropin,[284] with a 40% identity to the rat Reg protein, which had been identified in regenerating islets.[285]

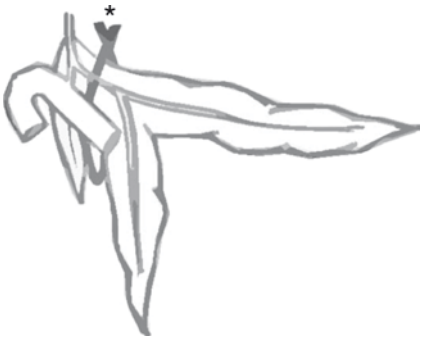

Fig. 27 Cellophane wrapping of the head of the pancreas (Adapted from Rosenberg et al.[280])

2.7 Streptozotocin Treatment

STZ is a toxic glucosamine–nitrosourea compound of microbial origin, which can be transported into cells by Glut-2, but not other glucose transporters (Fig. 28). Since beta cells have very high levels of Glut-2, systemic administration of high doses of the compound typically results in beta cell abrogation, with little damage to other cell types.[286,287] Although through a different mechanism of action, another chemical, alloxan, operates under the same principle.[288] The use of these compounds remains to this date the most common experimental intervention to induce diabetes in laboratory animals. The consideration of chemical beta cell poisoning as a "regenerative strategy" is indirect in the sense that, strictly speaking, beta cell mass needs to be reduced for regeneration to be observed (for instance, by inducing immune tolerance[289,290]). However, their induction of local inflammation (which is the most likely mechanism behind most pancreatic regeneration models) could also be regarded as a direct effect. This is one of the reasons why most islet transplantation experiments in the mouse are done under the kidney capsule: the removal of the graft-receiving kidney after normoglycemia has been restored is the only way to completely rule out that such outcome is due to endogenous pancreatic regeneration.

3 Where Do New Islets Come from?

Regardless of the model of islet regeneration, the origin of the newly created islets in the adult organism remains a highly controversial topic. In this section we will examine the several theories about the physical location where the regenerative process takes place (Fig. 29). The last part of the chapter will complement this discussion with a description of the potential cellular mechanisms behind these observations.

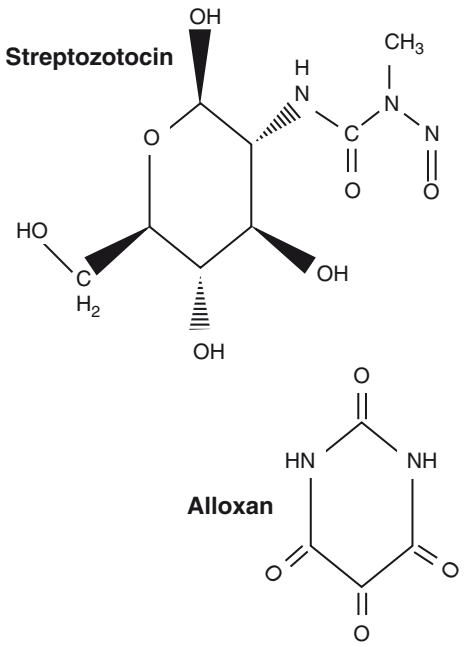

Fig. 28 Chemical structures of streptozotocin and alloxan, widely used to selectively ablate beta cells

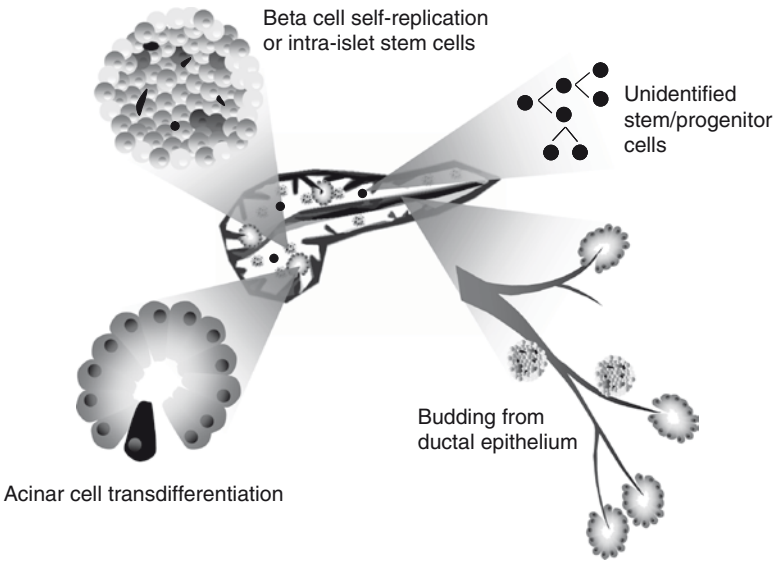

Fig. 29 Possible anatomical locations within the pancreas from which beta cell regeneration may take place

3.1 Do New Beta Cells Arise from the Duct/Acinar Tissue?

Virtually all researchers on pancreatic and islet cell biology are familiar with the occasional sight of a single islet cell or small islets seemingly sprouting from the ducts of a section of adult pancreas. The incidence of such observations is amplified under a number of experimental or pathological conditions.[291–293] For a long time, the obvious conclusion has been that islets might indeed be formed in or near the ducts, migrating at a later point to the acinar surroundings. BrdU labeling studies led to Bonner-Weir and colleagues to hypothesize that pancreatic regeneration in the partially pancreatectomized rat occurs through two pathways, namely: (1) the self-replication of existing endocrine and exocrine cells; and (2) the proliferation and differentiation of the ductal epithelium into new pancreatic lobules consisting of islets, acinar, and ductal tissue in the same proportions normally found in the organ.[239] Pdx1 messenger RNA (mRNA) was detected in pancreatic ducts at a level of approximately 10% that of islets a few days after partial pancreatectomy.[294] When human pancreatic tissue partially depleted of islets (leftovers of clinical islet preparations) was cultured in conditions favorable for ductal tissue expansion, abundant cells coexpressing ductal (CK-19) and beta cell markers (chiefly insulin and Pdx1) were identified.[295] Adult mouse and human ductal cells transduced with adenoviruses expressing Pdx1, Ngn3, Pax4, and NeuroD strongly up-regulated the expression of the insulin gene – with the latter yielding the highest degree of induction.[296]

The "ductal origin" hypothesis suffered a strong setback in 2004, when Dor and co-workers, using a pulse–chase strategy in a mouse transgenic model (see next section), established that adult islet regeneration occurs through self-replication rather than differentiation from non-insulin-producing pancreatic progenitors (see below).[86] However, a very recent report using a similar lineage-tracing experimental design (in which transgenic mice with the ductal-specific carbonic anhydrase II promoter driving Cre recombinase are mated with floxed beta-galactosidase reporter mice) suggests that ductal cells do indeed give rise to new islets and acini both during normal islet turnover and after injury (ductal ligation).[297] This would be in line with our recent finding that the expression of both Pdx1 and insulin was activated in the ductal epithelium of transplanted human pancreata upon recurrence of autoimmunity.[298] However, these cells still retained a hybrid ductal–beta cell phenotype and might just represent an attempt at compensating for the loss of beta cell mass, possibly stimulated by hyperglycemia and chronic inflammation.

In a recent study, Hao et al.[299] explored the ability of non-endocrine epithelial cells from the adult pancreas to give rise to endocrine cells. The pancreas is mostly made of two cell types, namely mesenchymal and epithelial. The latter include ductal, acinar, and islet endocrine cell types. Among the former are pancreatic fibroblasts, endothelial cells, vascular smooth muscle cells, and stellate cells. Mesenchymal cells, in general, tend to take over the culture when pancreatic tissues are plated in conditions that favor adherence. However, treatment with the drug G418 is effective at getting rid of mesenchymal cells.[300] The above investigators

cultured the byproduct of islet isolation procedures, which were largely devoid of both endocrine (due to the mechanical separation of the islets) and mesenchymal cells (due to G418 treatment). When co-transplanted with fetal islet-like clusters in recipient immunodeficient mice, some of these CK-19-positive "non-endocrine pancreatic cells" differentiated into insulin-, glucagon-, and (more rarely) somatostatin-producing cells. Both the origin (ductal or acinar?) of the cells with this potential and the nature (*bona fide* beta cells, or insulin-positive cellular byproducts?) of the differentiated progeny remain to be ascertained.

3.2 Do New Beta Cells Arise from the Islet?

A number of studies have pinpointed the origin of new beta cells to stem cells contained within islets.[241,301,302] Thus, using the STZ model of regeneration, Fernandes et al.[241] identified a population of somatostatin$^+$/Pdx1$^+$ cells inside the damaged islets. Follow-up of these cells led to the observation that they ended up turning into insulin-positive cells. These putative precursors were similarly observed in non-obese diabetic mice, where beta cell destruction is mediated by an autoimmune response. Similar findings were later reported by Guz et al.,[301] who documented islet regeneration in STZ-treated mice that received supportive insulin administration. Beta cell neogenesis was detected during the first week after the restoration of normoglycemia, and two putative beta cell progenitors were identified (Glut2$^+$ and Ins$^+$/somatostatin$^+$).

These results appear to be in contradiction with those of Dor et al.,[86] who also identified the islet as the source of new islets, but through a completely different mechanism (see next section).

3.3 Do New Islets Arise from the Bone Marrow?

The migration of transplanted bone marrow cells to many different tissues (particularly in response to insults or pathological conditions) is a phenomenon commonly observed both in animals[303–315] and humans.[316,317] This apparent "transdifferentiation" potential of bone marrow cells led to the early hypothesis that they could be the basis of a universal self-repair mechanism – even if it is not normally active under physiological conditions (Fig. 30). However, this idea suffered an important setback in 2002 with the publication of two studies showing that multipotent cells can fuse with differentiated ones, therefore adopting their phenotype. This was the case in a experimental setting where wild-type bone marrow transplantation rescued the liver of FAH$^{-/-}$ mice, which are a model of fatal hereditary type I tyrosinemia.[314] Further investigation on the mechanisms behind the rescue revealed that donor bone marrow cells had migrated to the defective liver and fused with resident cells. The ensuing cells were indistinguishable from the local hepatocytes, but the complementation

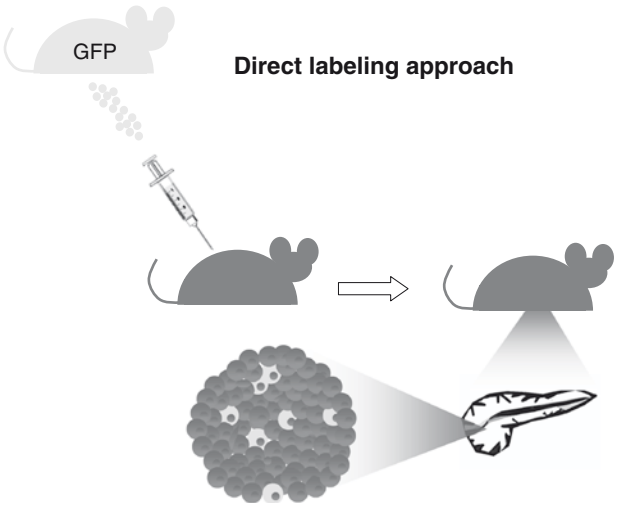

Fig. 30 If bone marrow (BM) cells contributed to islet regeneration, BM derived from GFP-positive donor mice could be tracked upon transplantation into wild-type animals and found in the recipient's islets. However, this approach does not account for cell fusion

with the wild-type gene of the fused bone marrow cell resulted in a stronger hybrid with a selective proliferative advantage over the non-fused cells. These "corrected" cells eventually took over the liver, restoring function.[318,319]

The burden of the proof was now on those researchers claiming that bone marrow-derived cells could indeed differentiate into the target derivatives. Thus, Ianus et al.[320] transplanted bone marrow cells from male transgenic INS2-EGFP mice into irradiated wild-type female recipients. Up to 3% of the cells within each islet exhibited EGFP expression 4–6 weeks after the procedure, most of them expressing insulin and Pdx1. This could be explained either by direct transdifferentiation of bone marrow cells into beta cells (which would activate the insulin promoter and therefore the reporter) or fusion to resident cells resulting in reprogramming of the donor ones. To rule out the latter, the authors transplanted the bone marrow of male INS2-Cre mice into ROSA-stoplox-EGFP female recipients. The rationale behind this approach was that any cell fusion event would be manifested by the Cre-mediated removal of the stop codon preventing EGFP expression (Fig. 31).

Abundant cells containing the Y chromosome were found in the pancreas of the recipient, but none of them was fluorescent. Since forced in vitro fusion of these two types of genetically modified cells did indeed result in EGFP expression, it was concluded that bone marrow cells can contribute to the endocrine pancreas in a fusion-independent fashion. However, two reports published shortly thereafter found little or no evidence of bone marrow transdifferentiation into pancreatic beta cells. Using again a GFP-labeled donor population, the authors of the first study observed fluorescent cells in the islets of the recipient animals, but none of them co-expressed insulin, either in healthy or in STZ-treated animals.[321] The second

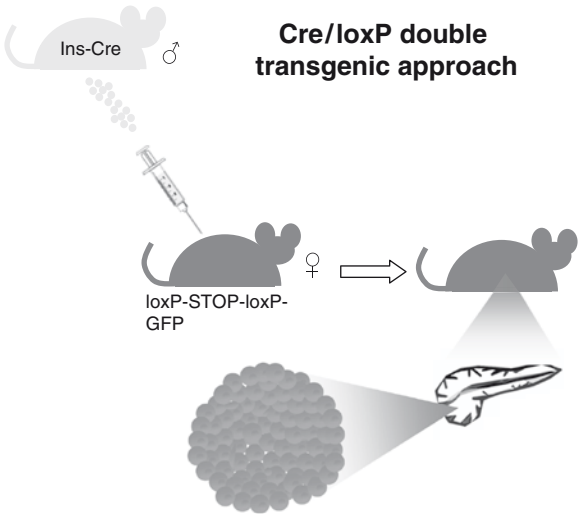

Fig. 31 An alternative approach to rule out cell fusion is the transplantation of BM cells from Ins-Cre mice into recipients in which GFP will not be expressed unless there is a Cre-mediated excision of a stop codon. Cells with a Y chromosome that express insulin within the islets would provide evidence of BM-mediated regeneration. If cell fusion occurred, GFP-positive cells would be detected. In the absence of GFP fluorescence, it could be concluded that the observation is not due to cell fusion

group extended these studies to another model of pancreatic regeneration (partial pancreatectomy). Despite substantial contribution of the donor cells to blood, lymphatic, and interstitial cells in the pancreas, they could find only two cells positive for GFP in a screening of more than 100,000 beta cells – which turned out to be in control animals.[322] They concluded, therefore, that the bone marrow does not significantly contribute to the endocrine component of the pancreas. A third study[323] confirmed these findings but provided additional evidence that bone marrow-derived endothelial progenitor cells were recruited to the pancreas in response to islet injury, which could be theoretically exploited to improve vascularization and/or endogenous regeneration of injured beta cells.

4 Molecular Mechanisms of Islet Regeneration

4.1 Reversible Epithelial-to-Mesenchymal Transition

The epithelial–mesenchymal transition (EMT) is a well-known developmental phenomenon by which epithelial cell types (which are typically arrayed in polarized cellular sheets with tight junctions that restrict the movement of their individual components) undergo a series of molecular changes that result in their progressive

transformation into mesenchymal cells (characterized by a reduction of cell-to-cell adherence and enhanced migratory capacity).[324] This process is defined by the down-regulation of epithelial proteins (E-cadherin, cytokeratins, occluding, desmoplakin) and the upregulation of mesenchymal ones (N-cadherin, vimentin, fibronectin). Transcription factors such as Snail 1, Snail2/Slug, Twist, and others will inhibit E-cadherin expression and promote EMT.[325] Originally described by Hay more than a decade ago,[326] this transition has been observed both during embryogenesis and in malignant transformation (Fig. 32).

Despite a plethora of studies on the role of EMT in a number of developmental events, its implication in pancreatic development has not been studied in depth. In 2004, Gershengorn and colleagues presented preliminary evidence that cells from adult human islets undergo reversible EMT to produce proliferating precursors that could later be re-differentiated to islet-like aggregates in vitro.[327] The authors presented the hypothesis that beta cell regeneration in vivo may occur through a process of de-differentiation–expansion–re-differentiation of existing beta cells. Such hypothesis would be consistent with the observations reported by another group[328] that Snail2/Slug – an inducer of EMT – is expressed in both the endocrine

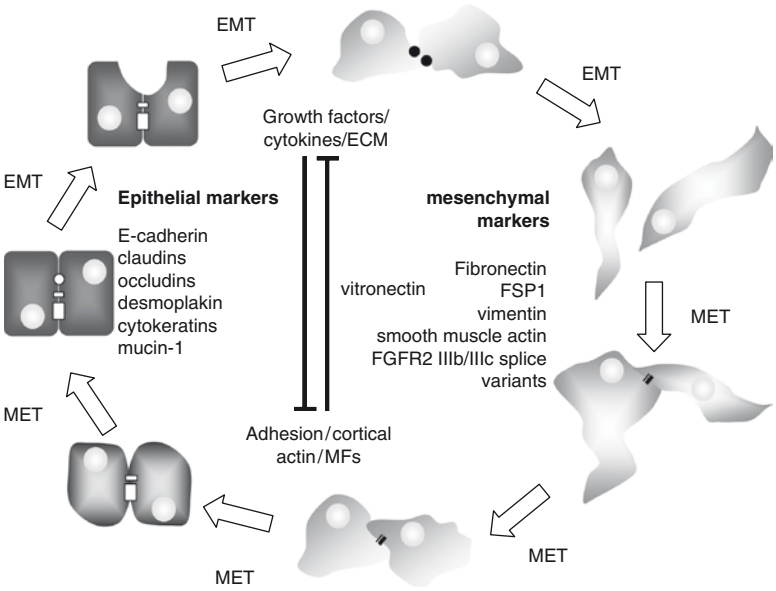

Fig. 32 Epithelial-to-mesenchymal transition (EMT) cycle. Starting from epithelial cell interactions, the first event of EMT is the dissociation of the tight junctions, followed by that of adherens junctions and desmosomes. Mesenchymal cells can migrate with minimal cell adherence. The process is reversed upon initial E-cadherin adhesive contact, Rho-GTPase activation, assembly of adherens junction, cortical actin cytoskeleton reorganization, desmosome reassociation, and, finally, formation of tight junctions and execution of cell polarity programs (Adapted from Thiery and Sleeman[324])

and exocrine components of the developing mouse pancreas. Snail2/Slug was detected in Ngn3-positive endocrine progenitor cells, and expression was maintained throughout endocrine cell differentiation, becoming increasingly restricted to beta and delta cells.

Another conclusion of the Gershengorn study was that, because these cells could be expanded up to 10^{12} during their proliferative stage, reversible in vitro EMT could be used to our advantage for therapeutic applications. A closer examination of the data, however, revealed that the "re-differentiated beta cells" did not express insulin levels higher than 0.02% those of native beta cells. In addition, several independent studies that made use of lineage-tracing and other techniques[329–331] conclusively demonstrated thereafter that EMT was not the responsible mechanism, and concluded that the original observations could be rather explained by the well-known capacity of contaminating fibroblasts to quickly take over epithelial cultures. While all the evidence gathered thus far would appear to be consistent with an EMT-independent pathway of adult islet regeneration, recent lineage-tracing experiments demonstrated that human beta cells in vitro can indeed de-differentiate and proliferate, even if at a relatively low pace (16 population doublings, 7-day doubling time).[332] Subsequent studies supported the notion that the de-differentiation and reentry into the cell cycle correlated with the activation of the Notch pathway, which might mirror the mechanism seen during embryonic pancreatic progenitor expansion.[333] Their re-differentiation into glucose-responsive beta cells, however, has not been shown yet.

4.2 Self-Duplication

A breakthrough study published in 2004[86] reported that adult beta cells are generated by self-duplication rather than differentiation from resident stem cells. The experimental design was based on the Cre-ER/loxP-based pulse-chase technology (see the chapter "Pancreatic Development"), in which transgenic mice expressing a tamoxifen-inducible version of the Cre recombinase placed under the insulin promoter were mated with Z/AP reporter mice. Cre-mediated removal of a stop sequence in the latter background results in the constitutive expression of human placental alkaline phosphatase (HPAP). However, CreER remains cytoplasmic in bigenic RIP-CreER-Z/AP cells, and no excision will take place unless the animals are given tamoxifen. The drug will promote the nuclear internalization of Cre, and those cells and their progeny will constitutively express a HPAP "label". Tamoxifen administration (the "pulse") was a one-time event: only insulin-producing cells present at the time of the injection were labeled. However, new beta cells formed after the injection (the "chase") would remain unlabeled. The strategy proved effective at specifically marking beta cells (approximately 30% of beta cells were $HPAP^+$ after the pulse), but neither ductal nor acinar or other endocrine cells expressed the reporter gene (Fig. 33). Figure 34 depicts the two possible scenarios that would follow a pulse: if neogenic beta cells derived from resident insulin-negative

Fig. 33 Using a tamoxifen-inducible system, approximately 30% of the beta cells of each islet are labeled at one point of the life of the mouse (pulse). This label is permanent and inheritable. The follow up of the ratio of labeled:unlabeled beta cells is the chase period (see main text)

progenitors, the amount of labeling within the islets would be progressively diluted as new, unlabeled beta cells replaced the old, labeled ones (*stem cell hypothesis*). However, if neogenic beta cells were the result of cell duplication, the ratio of labeled/unlabeled beta cells within any given islet should remain unchanged: labeled cells would give rise to new labeled cells and non-labeled ones would do the same at approximately the same rate (*self-duplication hypothesis*). The authors followed these mice for up to 1 year (approximately half the natural lifespan of a mouse) after the pulse, and no significant change in the ratio of labeled/non-labeled cells could be observed. The model was retested in a partial pancreatectomy model of regeneration with the same results, which strongly suggested that both regular turnover and post-pancreatectomy regeneration of beta cells occurs from preexisting adult (insulin-producing) beta cells. Subsequent research using a DNA analog-based lineage-tracing technique to detect multiple rounds of cell division in vivo,[334] as well as in an inducible model of diphtheria toxin-based beta cell destruction[87] unequivocally confirmed the previous results. Recent data on human samples are also in general accordance with the replication hypothesis.[335] While these experiments did not disprove the existence of pancreatic stem cells, they certainly made their existence "unnecessary." Proponents of the stem cell theory, however, defended their position by arguing that beta cells could de-differentiate before replicating. According to this view, the beta cell stem cells might have been beta cells at one point.[327]

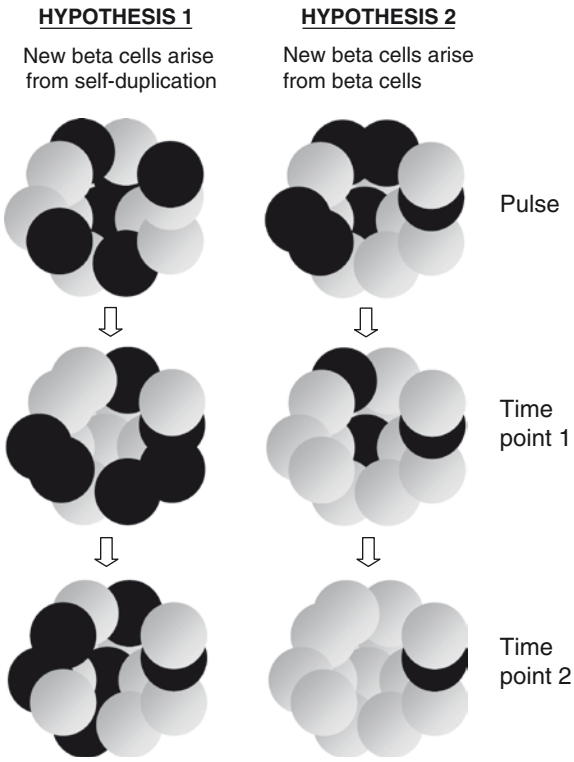

Fig. 34 Pulse–chase lineage tracing experiments can be used to test each one of the presented hypotheses. If new beta cells arose from self-duplication (*left*), the proportion of labeled cells within the islet would remain virtually unchanged throughout the life of the individual. However, if beta cells were derived from unlabeled stem cells (*right*), the label would be progressively lost as new beta cells replaced the old ones. The observations of Dor et al.[86] were consistent with the self-duplication hypothesis

4.3 Re-ignition of the Embryonic Developmental Program

The field of beta cell regeneration is evolving so rapidly that new discoveries are challenged even before they become mainstream. For many years, it was conventional wisdom that adult beta cell regeneration took place from the ductal system, with only morphological evidence of a recapitulation of embryonic development.[239] The observation that – at least in the mouse – adult beta cell turnover/regeneration was due to self-replication rather than stem cell differentiation was a complete paradigm shift.[86] Just when this novel notion was starting to settle in the collective understanding of the field, it was challenged again by both additional lineage tracing experiments showing ductal contribution to islet regeneration[297] and the unexpected finding that, under specific experimental conditions,

the embryonic developmental program could indeed be reactivated in the adult pancreas. Those were the conclusions of the authors of a very recent study that made use of a partial duct ligation beta cell regeneration model,[88] which Dor and colleagues had not tested either in their seminal report about adult beta cell self-duplication[86] or in their follow-up studies.[336] In short, a strong activation of the expression of Ngn3 (see the chapter "Pancreatic Development") was detected as early as 3 days after partial duct ligation in Balb/c mice. This was coincident with a tenfold increase in the number of beta cells that incorporated BrdU, a nucleotide analog that is taken up only by proliferating cells. Using a Ngn3 knockdown approach, beta cell regeneration was largely prevented. These experiments were complemented with a parallel approach where new beta cell formation could be traced in a Ngn3-beta galactosidase transgenic background. At least one third of the labeled cells were positive for islet endocrine hormones, and about half of these were in direct contact with the ductal epithelium. The hypothesis that the neogenic beta cells could be the result of de-differentiation of existing beta cells was ruled out by examining the effects of partial duct ligation in INS-Cre/R26R mice, where beta cells had been permanently labeled. No label could be found in Ngn3-positive cells, as would have been expected of beta cells that had de-differentiated and re-activated the embryonic program. Finally, the authors sorted these Ngn3-positive cells after partial duct ligation of the pancreas of Ngn3-EGFP animals. Their phenotype was almost indistinguishable from Ngn3-positive cells isolated from embryonic pancreatic buds, and they were able to differentiate into functional islet cells when microinjected into cultured $Ngn3^{-/-}$ e12.5 pancreatic explants.

These remarkable findings are difficult to reconcile with a universal explanation for physiological islet regeneration. That beta cells are replenished by self-duplication under normal circumstances seems to be well established now. However, now we realize that, at least when subjected to one type of nonphysiological insult, pancreatic progenitor cells can take over the regeneration process. Further research will be necessary to look into the molecular determinants of this phenomenon, and what makes this model different from all the others. Acinar cell death after partial duct ligation might be behind the observed effects. Their disappearance leads to a massive inflammation and recruitment of cells of the immune system. In a manner that remains to be determined, local responses to inflammation could also be the underlying cause of the activation of insulin expression in the ductal epithelium of transplanted human pancreata that undergo recurrence of autoimmunity, as recently shown by Martin-Pagola et al.[298] It is not known, however, why the inflammation induced by other pancreatic regeneration models (such as partial pancreatectomy, ductal ligation, or streptozotocin/alloxan administration) does not result in Ngn3 reactivation.

Stem Cell Differentiation: General Approaches

Abstract Regardless of the cell type used as a building block for differentiation/transdifferentiation into pancreatic cells, there are only a few strategies that can be used to modify, both in vitro and in vivo, their fate and behavior. Conventional approaches are based on the addition of chemical soluble agents to the culture medium (signal-driven strategies), in an attempt to mimic the complex symphony of differentiation/specification factors that drive the process in vivo. Extracellular matrices and cell growth substrates may help increase the overall efficiency of these methods. Alternatively, external signaling can be bypassed by means of adding constitutively activated copies of key transcription factors or – more recently – cell-permeable proteins. The rationale of in vivo differentiation is that only the recipient's body can provide developing cells with the adequate microenvironment to support terminal maturation.

Keywords Soluble factors • Extracellular matrix • Adenoviruses • Protein transduction • In vivo maturation.

1 Introduction

Our understanding of pancreatic islet development has progressed enormously over the last decade. Several key transcription factors have been shown to be critical for pancreatic organogenesis (Fig. 35). However, our ability to mimic this process out of its natural environment remains rather limited. As an introduction to the next three chapters, here we review briefly the most common approaches to differentiate stem cells into pancreatic beta cells.

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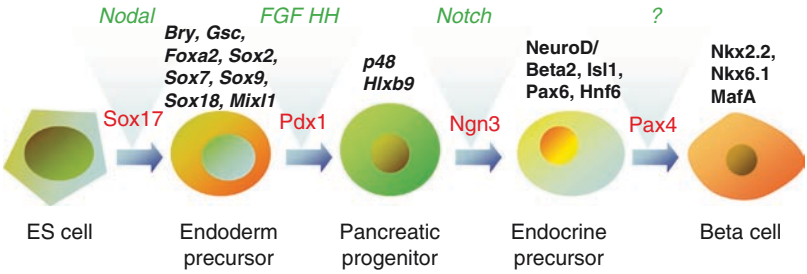


Fig. 35 The critical steps in beta cell differentiation are: (1) definitive endoderm/gut epithelium; (2) pancreatic progenitors; (3) endocrine precursors; and (4) beta cells. Based on transgenic studies, transcription factors whose expression might be required to catalyze stage progression are indicated. Known extracellular signaling pathways naturally involved in the progression of these steps are shown in italics (Adapted from[344]).

1.1 In Vitro

1.1.1 Chemical Strategies

The simplicity of the concept has made this approach the workhorse of stem cell differentiation strategies. Defined chemical factors are added to the culture medium in a specific order, so as to recapitulate embryonic development. For instance, in a recently reported human embryonic stem (huES) cell-based differentiation protocol, undifferentiated cells were forced toward the definitive endoderm pathway by means of the administration of activin A[337,338] – an analog of Nodal, which has the same inductive abilities during in vivo development.[58,339–341] Later in development, it is known that Sonic hedgehog (*Shh*) needs to be inhibited for pancreatic specification to proceed.[65,342,343] Therefore, the differentiation protocol was designed so that cyclopamine, an inhibitor of *Shh*,[64] was added to the culture medium at the right time.

However, our knowledge about the extracellular signals that prompt the developing cells of the pancreas to express critical transcription factors in the proper sequence and combination is incomplete.[48,344] Also, the simplicity of in vitro systems makes it considerably difficult to mimic the intricate polyphony of signals observed in living embryos. As a consequence, even the best current protocols for directed huES cell differentiation into beta cells (see the chapter "Embryonic Stem Cells and Pancreatic Differentiation") are still largely inefficient. For this reason, many investigators have also explored the possibility of developing "developmental shortcuts" that might potentially trigger complex developmental patterns at once. One potential way such outcome may be induced is by altering chromatin reorganization. Reversible chemical modifications of the nucleosome can dramatically affect DNA compactness, and therefore the ease with which specific transcription factors can attach to their DNA targets. Acetylation is probably one of the best-studied cellular mechanisms acting at this level. The overall acetylation at any locus is the result of the balance between

histone acetyl transferases (HATs) and histone deacetylases (HDACs). Loci with high acetylation levels (i.e., with high HAT activity) have a looser DNA coiling, which usually results in enhanced accessibility to the transcription machinery and higher transcription. Conversely, strong HDAC activity is likely to have the opposite effect, i.e., higher DNA compaction and low transcription levels[345,346] (Fig. 36).

Sodium butyrate (Fig. 37) is a well-studied inhibitor of histone deacetylation,[347,348] and its use in the context of stem cell differentiation has been extensively documented.[349–352] Rambhatla and colleagues demonstrated that prolonged exposure to sodium butyrate resulted in the permanent differentiation of huES cells into functional hepatocyte-like cells. Reasoning that the liver and the pancreas share a common developmental origin,[353–355] Goicoa et al.[214] showed that transient exposure to this chemical resulted in the activation of genes of early pancreatic development in mouse ES cells. Subsequent elaboration of this protocol in huES cells resulted in the generation of functional beta cells.[356]

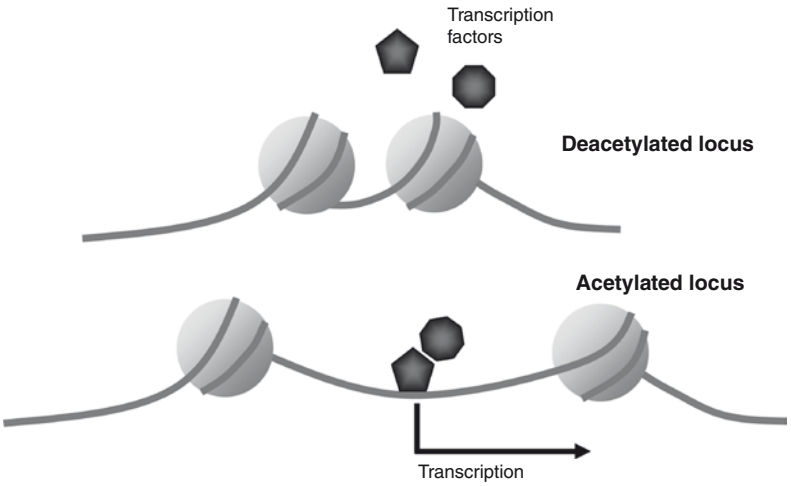


Fig. 36 Under conditions that favor high histone deacetylase (HDAC) activity, the chromatin structure at any given locus is compact and does not allow for the effective binding of transcription factors (*top*). As a result, transcription levels are low. HDAC inhibition, however, leads to a relaxation of the nucleosome (*bottom*), which now exposes regulatory sequences to the DNA-binding domains of transcription factors. Under these circumstances, transcription is high

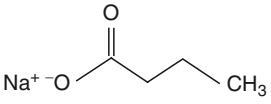


Fig. 37 Chemical structure of sodium butyrate

The way sodium butyrate triggers the pancreatic program is not understood. Several HDACs intervene in the regulation of key transcription factors involved in the progression of pancreatic development, such as Pdx1[212] or NeuroD/BETA2.[211] It is not unreasonable to expect some of these genes to be appropriately up-regulated upon exposure to sodium butyrate. The caveat with these approaches is that HDAC inhibitors are expected to have a generalized effect across the genome, thus potentially activating undesirable pathways in a simultaneous fashion.

1.1.2 Genetic Manipulation

The problems of chemical differentiation could be circumvented, at least in theory, by sequentially transfecting huES cells with the genes encoding for the key transcription factors whose activation is known to choreograph pancreatic development. With this idea in mind, many groups around the world have used a number of vectors to deliver active cassettes to stem cells of all origins, including Pdx1,[82,296,357–362] Pax4,[127,296,363,364] Foxa2,[358,360] Ngn3,[108,125,296,360] NeuroD[296] and many others. Among the vectors, adenoviruses have been highly favored due to the fact that they infect both dividing and nondiving cells, and usually do not integrate in a permanent manner into the genome.[125,286,296,359,360,365–367] Retroviruses, in contrast, have a preference for dividing cells and integrate permanently.[368,369] While the latter feature has proven of great help for permanent labeling[370] and proof-of-principle reprogramming studies,[371–376] concerns about insertional mutagenesis and heterochronic reactivation are still considered almost insurmountable hurdles in the road toward clinical applications.[367,377–379] Direct transfection of plasmid DNA in virus-free settings has been proposed as a middle way, particularly for reprogramming experiments.[380]

If conceived as a sequential approach, however, the notion of adding several genes to progressively specify stem cells in vitro would pose daunting technical difficulties. It would be very difficult to replicate the native expression pattern of most of these genes, which is either transient or fluctuating during development (see the chapter "Pancreatic Development"). Stepwise clonal derivation of cells transfected with one inducible gene at a time would be utterly impractical. Moreover, uncertainty about the copy number and site of integration of these cassettes would raise the aforementioned safety concerns. Strategies based on homologous recombination for targeting the integration of reprogramming genes into specific loci[381] would be safer, but quite unworkable due to the number of genes involved. Also, it might not solve the problem of subsequent reactivation of these genes. Still, the in vitro behavior of cells typically defies conventional wisdom about natural development. Ngn3, for instance, is thought to act only transiently at the time of endocrine specification, and its down-regulation is supposed to be necessary for differentiation to proceed.[107,109] However, ectopic constitutive expression of Ngn3 seems to be permissive for differentiation in other settings.[360] Similar arguments can be made about ES cell reprogramming factors Oct3/4 and Sox2, whose permanent expression would be theoretically incompatible with the activation

of differentiation pathways – yet differentiation is not impeded when iPS cells constitutively expressing these two factors are allowed to form teratomas.[375,382] A plausible explanation for these observations is that the forced expression of some of these genes will trigger endogenous networks that will irreversibly take over the process from that point on,[365] perhaps even deregulating the ectopic gene.[374,375]

Other forms of genetic manipulation are not intended to induce phenotypic changes, but to favor the in vitro identification (and possible sorting/selection) of desired differentiation outcomes, as well as posttransplantation tracing. These include plain tagging,[307,322,383,384] the generation of cell lines expressing fluorescent reporter genes under the control of tissue-specific regulatory sequences,[50,54,322,332,357,385–389] and gene-trap strategies[390–393] for the specific ablation of cells that do not have the desired characteristics.

1.1.3 Protein Transduction

As mentioned above, the general scheme of gene-based differentiation toward pancreatic endocrine cells would be to transfect stem cells, in a stepwise fashion, with candidate pro-endodermal, pro-pancreatic, and pro-endocrine expression vectors. However, a conventional strategy for transfection/antibiotic selection of clones would be extremely difficult to reduce to practice, as every step would require the selection of clones containing the appropriate integration. Since ES cells tend to progressively stop dividing after the initiation of differentiation, repeating this process for each one of the subsequent genes would be nearly impossible. As for the use of viruses to deliver these genes, we have already stated the problems that will likely stand in the way of the development of clinical therapies.

Protein transduction has been presented as a viable alternative to gene-based approaches.[394–396] The basis of this technology is the fusion of any protein or peptide of interest to small cationic oligopeptides (protein transduction domains [PTDs]) with cell-penetrating properties. PTDs are known to bind to negatively charged heparan sulfate chains of cell membrane-bound proteoglycans.[397] Cells defective in the biosynthesis of fully sulfated heparin sulfates cannot internalize many of these small peptides, and in wild-type cells their uptake is competitively inhibited by soluble heparin and by treatment with glycosaminoglycan lyases that specifically degrade heparin sulfate chains. The extracellular release of these peptides, however, appears to be largely independent from the heparan sulfate pathway.[397] The great efficiency of PTD uptake by most mammalian cells might indeed be a eflection of the ubiquitous distribution of heparan sulfate proteoglycans (Fig. 38).[398,399]

The importance of the charge interaction is evidenced by experiments where uptake efficiency was severely compromised upon substitution of basic residues with alanine.[400] After the initial interaction, the peptide (and its cargo, if fused to one) is internalized by endocytosis and cytoplasmic release ensues shortly.

TAT is an 11-amino acid peptide derived from the basic domain of the TAT/HIV transactivator protein, and could arguably be considered the workhorse of PTD studies[396] (Fig. 39). Due to its ease of engineering and effectiveness, TAT and other

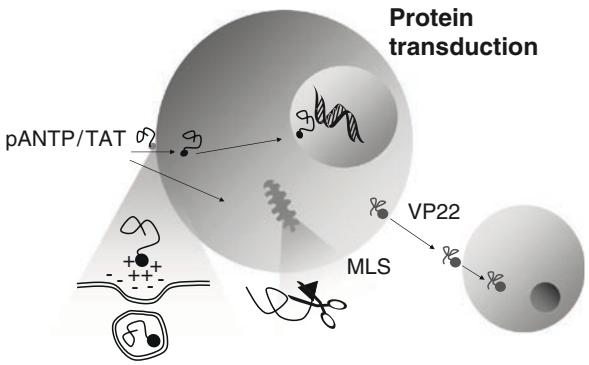


Fig. 38 General depiction of protein transduction. Protein transduction domains (PTDs) such as Antennapedia (pANTP) and TAT are thought to interact with negative charges in the cellular membrane. As a result, the PTD and its cargo are internalized by endocytosis and subsequently released from the endocytotic vesicle. Depending on the nature of the cargo, the protein will then migrate to the nucleus (where it could induce the expression of target genes), remain in the cytoplasm, or even go to the mitochondria if given a mitochondrial localization signal (MLS), which will be later excised inside the organelle. Other PTDs, such as VP22, have been shown to migrate from the producing cell to neighboring ones

Fig. 39 Amino acid sequence of TAT PTD 

synthetic cationic relatives[401–405] have been used extensively to deliver full-length functional proteins both in vitro and in vivo.[109,344,395,396,406–416]

The application of PTD technology to islet cell biology is well documented. For example, TAT-neuroglobin,[412] TAT-heme oxygenase 1 (HO-1),[414] and TAT-BclXL[417] have been shown to enhance islet survival and function. Down-regulation of the interleukin (IL)-1beta-induced IκB kinase (IKK) by a PTD-fused inhibitor had similar effects.[418] As inhibition of c-jun N-terminal kinase (JNK) has been shown to improve islet viability and function in knockout transplantation studies,[419] the findings that TAT-based, cell-permeable inhibitors of JNK increased functional beta cell mass[420,421] and enhanced insulin sensitivity and glycemia in experimental diabetes[422] were not unexpected (Fig. 40).

The strategy of using protein transduction to aid in the sequential differentiation of stem cells has also been immediately acknowledged, as it would readily circumvent many of the limitations inherent to all signal-driven approaches to differentiation in vitro, without the shortcomings of gene transfer. Protein transduction would allow for the timed delivery of those very transcription factors whose activation is required for differentiation to proceed. The versatility of this method is such that the investigator would retain full control over the dosage, timing, and length of exposure of each PTD-fused transcription factor.

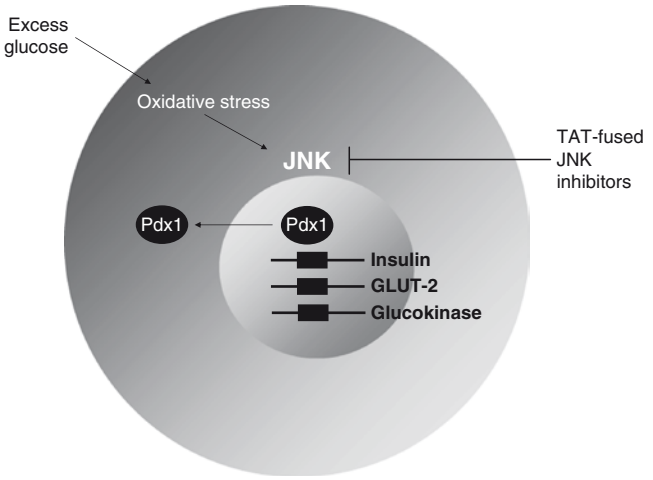


Fig. 40 The JNK pathway is activated following oxidative stress. JNK will favor the nucleo–cytoplasmic translocation of Pdx1, whose function is required in the nucleus to maintain adequate levels of expression of key elements of the glucose-sensing and insulin-secreting machinery. Cytoplasmic localization of Pdx1 will lead to a deterioration of beta cell function. TAT-fused inhibitors of JNK have proven beneficial at preventing this occurrence and improving beta cell function and viability (Adapted from Kaneto et al.[423])

Thus, TAT has already been used to transduce human hematopoietic stem cells with the homeobox HOXB4, which stimulated their expansion without compromising their differentiation potential.[410] Delivery of TAT-fused, biologically active versions of Ngn1 and Mash,[424] Pdx1,[411] Pax6,[416] and Ngn3,[109] among others, has been successfully reported. Another TAT-fused nuclear protein, Cre, has been used to excise loxP-flanked DNA sequences without the need for gene transfection.[413,425] A more recent interest in protein transduction has stemmed from the promising reports on nuclear reprogramming reported by retroviral transduction of key ES cell transcription factors.[375,382] Because protein transduction would provide a non-transgenic route to achieve the same effect, TAT-derivatives of Oct-4, Nanog, and Sox2 have already been described.[426,427]

Alone or in combination with other approaches described in this chapter, the use of protein transduction is likely to become a fixture in a new generation of protocols for the differentiation of stem cells into pancreatic beta cells.

1.1.4 Microenvironment

It could be reasoned that the in vitro specification of stem cells along the beta cell lineage would require the accurate recapitulation of the differentiation steps described in the chapter “Pancreatic Development” by providing the cells with the chemical cues that are known to sequentially activate critical transcription factors.

This has been, in short, the strategy followed by D'Amour et al.[338] During development, however, cells respond differentially to very subtle stimuli that are not only the soluble kind usually provided in differentiation protocols, but also bound to other cells or the extracellular matrix (ECM). Other physical parameters have also been found to exert an important role in differentiation. Among these, some of the most studied are mechanical forces,[163,164] pH and bioelectrical fields,[175,176,428] the nature of the substrate/mode of culture,[167–170] and oxygenation.[188,192,429,430] The latter, in particular, is of special relevance for islet differentiation. Indeed, beta cells are extremely vulnerable to pO_2s above and below the physiological range.[431,432] Seminal work conducted in the late 1970s showed that high oxygen tensions were deleterious for pancreatic tissue.[433] Later studies established that beta cells are sensitive to high oxygen-induced stress because of their naturally low expression of antioxidant enzymes (the levels of catalase and glutathione peroxidase, for instance, were only 5% those of the liver[414,434,435]). Another critical molecular event triggered by oxidative stress in the beta cell is the translocation of Pdx1 from the nucleus to the cytoplasm in a JNK-dependent manner.[436–439] In addition to its role during pancreatic development, Pdx1 acts at the nuclear level as an essential regulator of beta cell homeostasis and function.[71–73] Therefore, cytoplasmic sequestration of Pdx1 leads to an overall down-regulation of the glucose sensing/insulin secreting machinery. As for the JNK pathway, its activation in response to hyperoxia has been linked to non-apoptotic cell death.[440] Low oxygen tensions are equally detrimental. Research on insulinoma-derived cells shows that insulin secretion is completely stalled below 7 mm Hg.[441] Low pO_2 inhibits insulin release through an overall decrease in calcium concentration, which in turn reduces Na/K-ATPase activity in the plasma membrane.[442,443] More importantly, hypoxic exposure results in higher beta cell death by activation of both the iNOS-nitric oxide signaling cascade and JNK phosphorylation.[444]

Both for clinical and basic research purposes, isolated islets are typically cultured at atmospheric O_2 concentration (21%) in nonadherent dishes or flasks, with medium covering them up to 1mm above the bottom of the vessel.[445] Under these conditions, islets are exposed to sharp gradients, ranging from overt hyperoxia to central anoxia (0 mm Hg) at certain plating densities.[446] As a reference, the physiological islet pO_2 is approximately 40 mm Hg.[431,432] Thus, only a small fraction of the islet mass receives physiological oxygenation. Considering that islets are exquisitely sensitive to both hypoxia[191,446–452] and oxidative stress,[414,435,437,453–457] it is not surprising that they die very quickly in vitro.[458–461] Hence the conclusion that standard culture conditions are suboptimal for islet cell viability and function.

Similar limitations apply to the differentiation of stem cells into islet beta cells. As suggested by the modest success reported so far, the conditions required for the in vitro recapitulation of pancreatic development may require a multipronged approach encompassing not only a chemical, but also an appropriate *physical* environment.[462] Growing cells as monolayers may facilitate the even distribution of nutrients, O_2, and signaling molecules, but only at the expense of compromising three-dimensional (3D) cell-to-cell interactions deemed essential for the development and maintenance of the beta cell phenotype.[463–466] Also, adult islets are surrounded

by an elaborate network of capillary vessels and connective tissue, critical to their high metabolic activity. In fact, although islets account only for 1–2% of the total number of cells of the pancreas, they receive nearly 15% of the overall blood flow to the organ, using 25% of the pancreatic O_2 supply.[467,468] When removed from their native environment, the islet microvascular network is destroyed and viability decreases dramatically.[450] while islets in vivo maintain a relatively constant oxygen partial pressure throughout their diameter,[431] those cultured in standard conditions (95% room air/5% CO_2) are exposed to sharp pO_2 gradients that may range from extreme surface hyperoxia (158 mm Hg) to central anoxia (0 mm Hg).[446]

In other words, it is reasonable to expect that the generation of beta cells in vitro would require the simulation of the physiological conditions that they need to survive in the first place. Indeed, the very same oxygenation deficiencies responsible for beta cell death in culture may also hinder their terminal differentiation from immature progenitors.

Despite the major influence of oxygen on pancreatic islet survival and function, thus far this parameter has been largely ignored in beta cell differentiation protocols. Recently, Fraker and collaborators[462] exposed e13.5 mouse pancreatic buds (at the initiation of the secondary transition) to physiologically high oxygenation,[431] comparing endocrine and exocrine specification outcomes with those of control buds grown in hypoxic conditions. The observation that endocrine differentiation was very significantly up-regulated in the experimental group has immediate implications for the design of stem cell differentiation methods that take into account the beta cell physiological environment.

As mentioned earlier, another essential component of the microenvironment of the beta cell is the ECM. The ECM is a dynamic network of glycoproteins that typically include fibronectin, laminins, proteoglycans, collagen, and glycosaminoglycans. Indeed, because many growth and differentiation factors are bound to the ECM, the cell-ECM interaction is thought to be a major catalyst of differentiation.[469–476] As the pancreas forms as an epithelial evagination into the surrounding mesenchyme,[48,156,477] an specialized ECM termed the basement membrane (BM) develops in the interface.[478] The pancreatic BM is largely composed of laminins (80%) and collagen IV.[479] Laminin-1, for instance, is detected in the BM of the developing pancreatic buds as early as e13.5,[480] but is down-regulated in adulthood.[481] Interestingly, laminin-1 enhanced the differentiation of beta cells from dissociated e13.5 precursor cells,[480] and this effect was completely prevented by a monoclonal anti-laminin-1 antibody. The best characterized laminin receptors are integrins, which are heterodimeric transmembrane glycoproteins that mediate cell–ECM and cell–cell interactions[479,482] and anchor fundamental components of many signal transduction pathways.[483,484] Beta-1 integrin interacts with the α chains 3, 5, and 6 during human fetal pancreatic development, suggesting that their corresponding ECM ligand (chiefly fibronectin) might play an important role in the specification of pancreatic tissues.[485] α-Dystroglycan (α-DG) is another laminin-1 receptor with a counterregulatory activity: studies conducted with chemical agents that selectively block the interaction of laminin-1 with α6-integrins or α-DG show that the former promotes beta cell proliferation and the expense of differentiation, whereas the latter (which is predominant) has the opposite effect[486] (Fig. 41). These observations highlight the importance of reproducing in vitro all of the

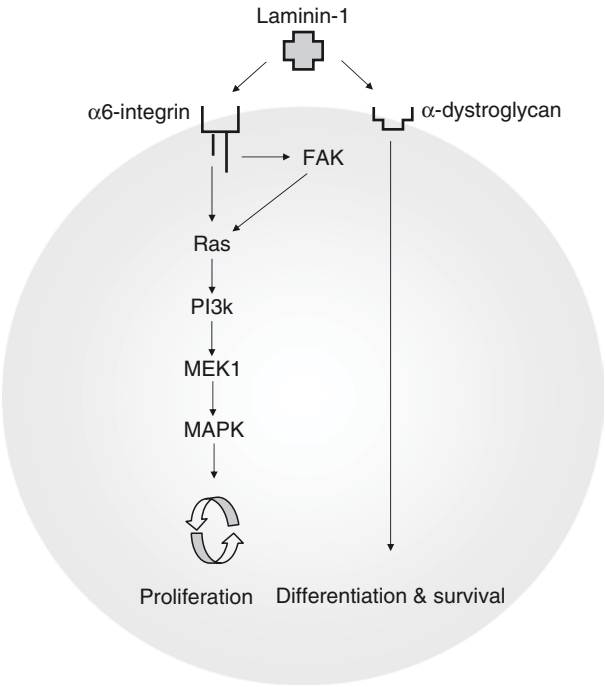


Fig. 41 Schematic model to explain the laminin-1-mediated counterregulation of beta cell proliferation (through α6-integrin receptors) and differentiation (through α-DG receptors) during embryonic development (Adapted from Jiang et al.[486])

components of the differentiation milieu, and not merely the soluble ones. Indeed, a careful replication of these ECM components might even contribute to transdifferentiation, as evidenced by the expression of islet cell markers by hepatic oval cells when cultured in the presence of laminin and fibronectin.[487]

1.2 In Vivo

The basic principles of tissue culture have evolved little since the 1940s. With some exceptions, most culture devices are variations on the common theme of a plastic container that holds the cells and their culture medium. Despite the progress in our understanding of the microenvironmental regulation of pancreatic development, it is unlikely that we will ever be able to reproduce in vitro the exquisite complexity of the native niche where beta cells develop. These limitations are quite possibly the main reason why most in vitro differentiation protocols are inefficient, yielding only small percentages of the desired cell types.[337,338,356]

A widely recognized alternative is to use the recipient's body as the perfect niche to foster the differentiation and terminal maturation of stem cells. In a majority of

cases, the cells would be "primed" so as to induce their commitment along the desired lineage, and then transplanted into an appropriate in vivo location that will be at least permissive – and ideally inductive – of differentiation. That was the idea behind the experiments conducted by Kroon et al.,[488] who differentiated human embryonic stem cells up to the pancreatic progenitor stage and then placed them in a variety of locations within recipient mice. As described in the chapter "Embryonic Stem Cells and Pancreatic Differentiation," those experiments were successful, but with the caveat that carry-over undifferentiated cells gave rise to teratomas. As for the place of implantation, it could be argued that the pancreas would provide the ideal milieu for the maturation of beta cells. However, the pancreas has been traditionally off-limits for transplantation procedures due to its ease of breakage, which would release harmful digestive enzymes and induce inflammation. In the aforementioned experiments, beta cell maturation was successfully reported upon implantation in the epididymal fat pads, subcutaneously, and under the kidney capsule.[488] Indeed, islets are known to engraft well in other locations, such as the liver[9] or the anterior chamber of the eye.[489] Therefore, even though transplantation in the pancreas is feasible, it might not be essential as long as the chosen location has the potential to be readily vascularized.

A diabetic environment might promote beta cell maturation. A strong rationale for this notion is provided by the observation that, during mouse embryonic development, glucose promotes beta cell differentiation in a dose-dependent manner.[257] During pregnancy there is an enhanced stress on beta cells that, to some extent (and occasionally in a pathological manner), mimics that of diabetes. We have already described how this physiological state influences beta cells by increasing their mass, rate of replication, and glucose responsiveness (see the chapter "Pancreatic Regeneration").[246] Transplantation of human fetal pancreas into nude diabetic mice typically results in functional beta cell differentiation. However, when using non-diabetic mice, a time-dependent maturation of the response to glucose was not observed.[490] A balance must be struck, however, as an excess glucose might have the opposite effect. This was evidenced in rats where severe hyperglycemia delayed, rather than stimulated, beta cell growth.[246]

Embryonic Stem Cells and Pancreatic Differentiation

Abstract Embryonic stem (ES) cells are derived from the early preimplantation blastocyst. These cells are immortal under defined conditions in vitro, and can be indefinitely expanded without loss of pluripotency. Proof-of-concept experiments demonstrate that they have the ability to spontaneously differentiate into insulin-producing cells, even if at a very low frequency. Here we review the most recent progress at defining conditions (chemical, genetic, or otherwise) for the directed differentiation of both mouse and human ES cells into insulin-producing beta cells.

Keywords Embryonic stem cells • iPS cells • Nestin • Embryoid bodies • Teratomas

1 Introduction

Embryonic stem (ES) cells were first derived from the early mouse blastocyst more than two decades ago.[2,3] Under appropriate culture conditions (which at the time included the use of fibroblast feeder layers), these cells could be propagated indefinitely and had the potential to differentiate into derivatives of all three embryonic layers, as evidenced by their ability to both form teratomas in immunocompromised recipients and extensively contribute to the development of the embryo when injected in recipient blastocysts. ES cells exist only transiently in the inner cell mass of the early embryo (Fig. 42). Specific culture conditions keep them cycling rapidly ex vivo (doubling time ranges from 24 to 48 h[491]) without loss of pluripotency for extended periods of time (Fig. 43).

The molecular machinery behind the "stemness" of ES cells is very well conserved across species. The genes *Oct3/4*, *Sox2*, and *Nanog* lie at the core circuitry that imparts pluripotency and self-renewability[492–494] (Figs. 44 and 45). In fact, at least two of them (*Oct3/4* and *Sox2*) have proven indispensable in the reprogramming of somatic cells into ES-like induced pluripotent stem (iPS)

J. Domínguez-Bendala, *Pancreatic Stem Cells*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-60761-132-5_5,

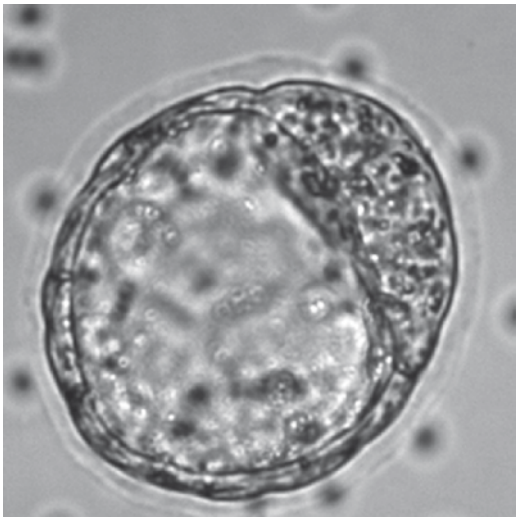


Fig. 42 Mouse blastocysts displaying the inner cell mass to the right (Domínguez-Bendala's laboratory)

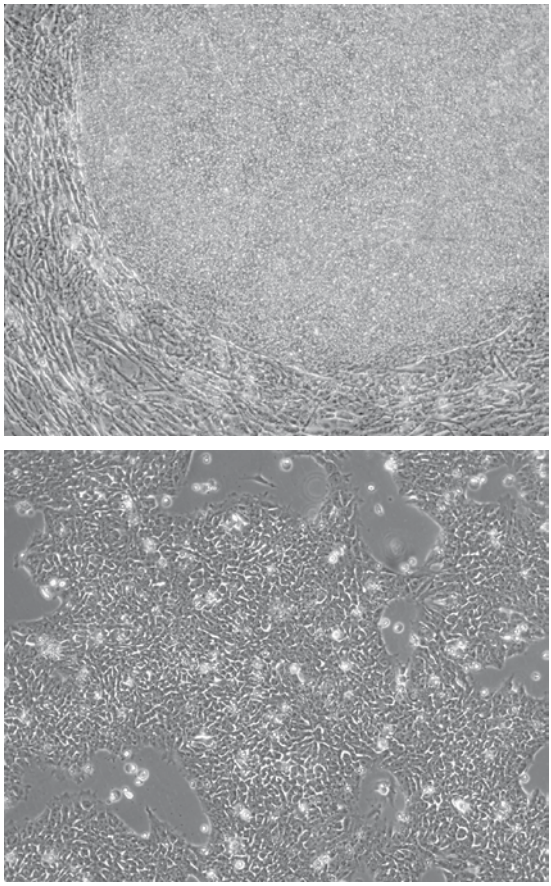
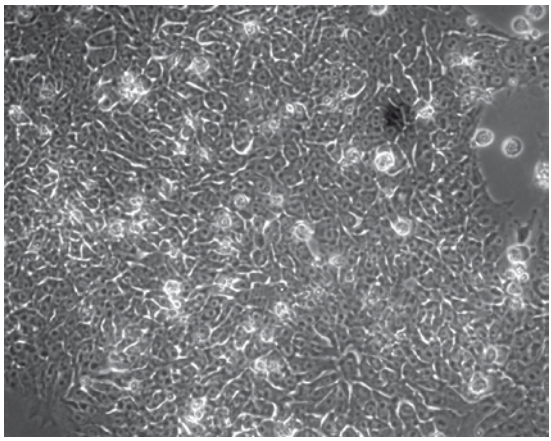


Fig. 43 A human embryonic stem (ES) cell colony grown over an inactivated mouse embryonic fibroblast feeder layer (*top*). When grown on Matrigel™, human ES cells form a monolayer (*middle*). A higher magnification picture (*bottom*) shows the morphological characteristics of ES cells: polygonal shape, high nucleus:cytoplasm ratio and refractive nucleoli (Domínguez-Bendala's laboratory)

Fig. 43 (continued)



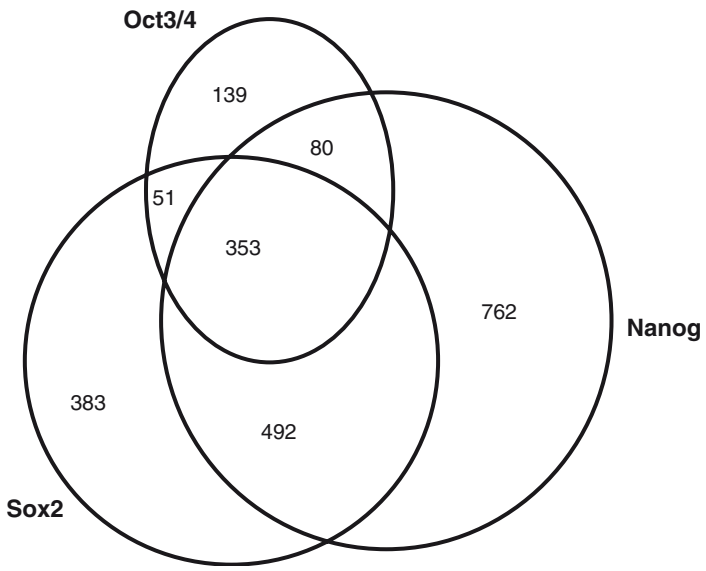


Fig. 44 A Venn diagram representing the overlap of the three critical regulators of the ES cell phenotype (Oct3/4, Sox2, and Nanog) promoter-bound regions. A large number of sites are co-bound by the three genes (Adapted from Boyer et al.[494])

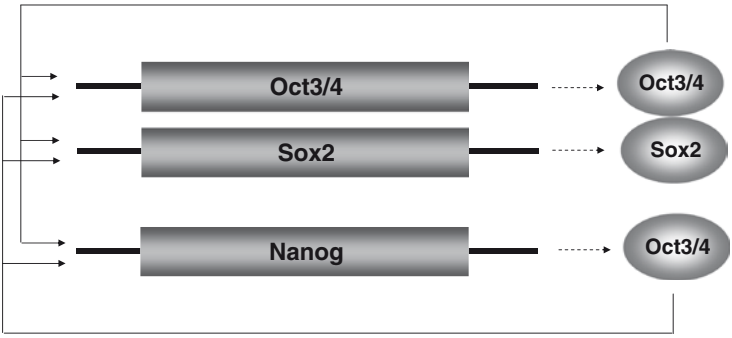


Fig. 45 Autoregulatory loops formed by Oct3/4, Sox2, and Nanog (Adapted from Boyer et al.[494])

cells.[372–376,382,495] With some exceptions, the down-regulation of these genes is associated with the activation of specific differentiation pathways. In addition to the above gene expression signature, human ES (huES) cells are characterized on the basis of their lack of stage-specific embryonic antigen 1 (SSEA-1) and presence of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, telomerase, and alkaline phosphatase.[1,496,497]

As mentioned in the preface of this work, for decades, ES cells were chiefly considered a tool for the creation of targeted/knockout mice[498] (Fig. 46 and box below). However, the contribution of ES cells from non-murine species to the germ line could never be demonstrated. The first reports on somatic cell nuclear transfer (SCNT) in 1996 put and end to this search, as it was now possible to generate targeted animals of virtually any species of interest to humans in one single step.[499–504]

In the meantime, steady progress was being made in refining the culture settings that would allow for the survival of nonhuman primate blastocysts up to the point where isolation of their ES cells was possible.[506] This seminal report was followed shortly afterwards by the first report on the generation of human ES cells,[1] which opened the door to the possibility of devising regenerative therapies by means of expanding ES cells in vitro and then differentiating them into the tissue of interest. In this chapter, we review the attempts that have been made thus far to convert them into pancreatic endocrine cell types of potential use in the treatment of type I diabetes.

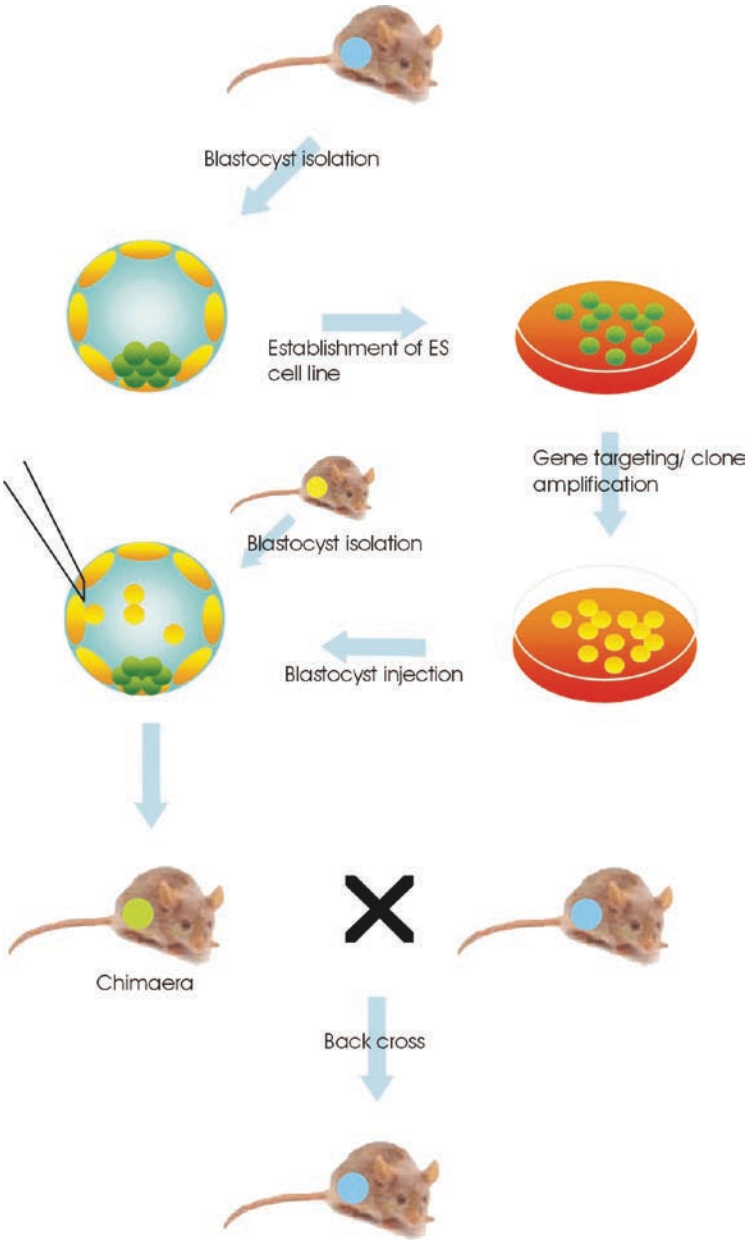


Fig. 46 A general strategy to generate targeted clones using ES cells (see ES Cells and Gene Targeting box)

ES Cells and Gene Targeting

During the last 25 years, the application of gene targeting techniques to ES cells has become a routine procedure to generate genetically modified mice. The availability of large populations of these immortal cells makes it feasible to target specific genes, despite the low frequency of homologous recombination in mammalian cells. Targeted clones can be easily selected in vitro and used to generate chimeric mice by aggregation or injection into blastocysts. If the host blastocyst and the donor ES cells belong to different strains of mice, chimerism can be visually assessed by the mixed coat color of the resulting animals. Typically, up to 70% of injected blastocysts are overtly chimeric. The degree of chimerism varies widely from barely detectable to complete ES coloration. Since ES cells retain the potential to contribute to all embryonic lineages, some of them may partially colonize the germ line. Because of the fine-grained nature of ES cell chimerism, the germ line is usually a mixed population of donor- and host-derived cells. In order to obtain the highest possible number of ES-derived gametes, ES cells for blastocyst injection are of male genotype. The introduction of male ES cells into female host blastocysts normally results in the generation of fertile intersex animals, which only transmit the ES genotype.[505] This is a consequence of the expression of the Y chromosome-linked *sry* gene, which controls mammalian sex determination. In these cases, backcrossing chimeras with the strain from which the ES cells were originally derived renders animals with the original genetic background, with an ideal 50% of the offspring carrying the modified allele (Fig. 46). A 25% germ line transmission (one out of the first four male chimeras tested) is not unusual, although this percentage varies from clone to clone.

2 Mouse ES Cell Experiments

2.1 Signal-Driven Approaches

Based on the conservation between mouse and human pancreatic development, the first attempts at differentiating ES cells into pancreatic endocrine beta cells were done using mouse cells, given their relative ease of use. One of the first efforts was led by Lumelsky and coworkers, who adapted a neural differentiation protocol to murine ES cells and reported the generation of insulin-secreting structures.[507] The rationale behind the approach has its roots back in the 1980s, when the prevailing view was that islet precursor cells were neurons.[508] This assumption was made based on observations such as the expression of neuronal markers (especially catecholamine-synthesizing enzymes) in pancreatic endocrine cells;[508] similarities in the pattern of development observed in the central nervous system (CNS) and the endocrine pancreas (with the activation of common differentiation mechanisms

such as Notch and expression of genes such as NeuroD/BETA2, Pax6, Nkx2.2, Nkx6.1, Isl1, and others[509,510]); and culture studies showing that cells positive for nestin (a marker of neural progenitors) are abundant in pancreatic tissue and have the purported ability to differentiate into insulin-producing cells.[511]

Lumelsky and colleagues reasoned that, on the basis of the similitude between CNS and pancreatic development, chemical protocols that normally induce the formation of neural derivatives could also lead to the generation of pancreatic endocrine cells. Their five-stage protocol was based on the formation of embryoid bodies (EBs; suspended cell aggregates of spontaneously differentiating ES cells) and the expansion and subsequent differentiation of a nestin-positive subpopulation (Fig. 47). The authors reported the generation of islet-like clusters expressing insulin, glucagon, somatostatin, and pancreatic polypeptide, with the majority of glucagon- and somatostatin-positive cells surrounding the insulin-positive cells. This anatomical pattern is consistent with that seen in rodent islets,[32] which lent additional strength to the conclusion that islet development had been recapitulated to a large extent in vitro. However, the resulting beta-like cells had insulin levels that were >50-fold lower than those found in native islets, and they did not restore normoglycemia in diabetic animals.

Today, the overall perception of this seminal study is that the authors merely developed – as it should be expected – neural derivatives. The fact that islets arise from the foregut endoderm, and not from the ectoderm, is undisputed since the early studies of Pictet et al. (who showed that complete ablation of the ectoderm did not prevent pancreatic development)[512] and Fontaine and LeDouarin, whose elegant experiments with quail chick chimeras firmly established the endodermal origin of the endocrine pancreas.[513,514] Indeed, the development of the CNS and the

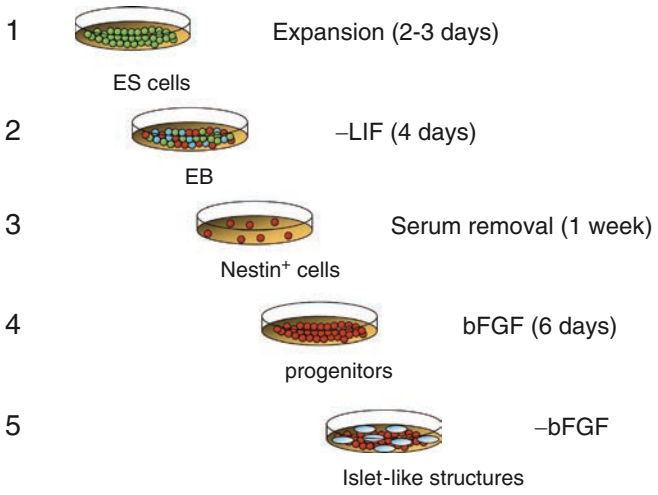


Fig. 47 Protocol reported by Lumelsky et al.[507] *LIF* Leukemia inhibitory factor; *bFGF* basic fibroblast growth factor

endocrine pancreas share many common features,[48,107,477] but even the same developmental plan will yield different cell types depending on the origin (endoderm or ectoderm) of the starting material (Fig. 48). Nestin is a rather ubiquitous marker of neuroectodermal cell types that can be found throughout pancreatic development, but it never colocalizes with pancreatic endocrine markers.[515] However, nestin-positive derivatives are significantly easier to obtain from ES cells than true endodermal cell types.[516] These protocols are easy to devise and highly reproducible, as these cells thrive in standard culture conditions and sometimes can be coaxed to secrete insulin, although at very low levels.[517,518]

Be it as it may, follow-up research showed that similar differentiation protocols could reverse hyperglycemia in diabetic rodents,[519,520] even if such outcome was typically associated with the formation of teratomas. These results are somewhat surprising in view of three subsequent studies that analyzed the insulin expression of cells derived in this fashion, from which it was concluded that a very significant portion of the insulin that had been detected so far arose from the uptake by apoptotic cells of the insulin that was supplemented to the culture medium, rather than from de novo synthesis.[521–523]

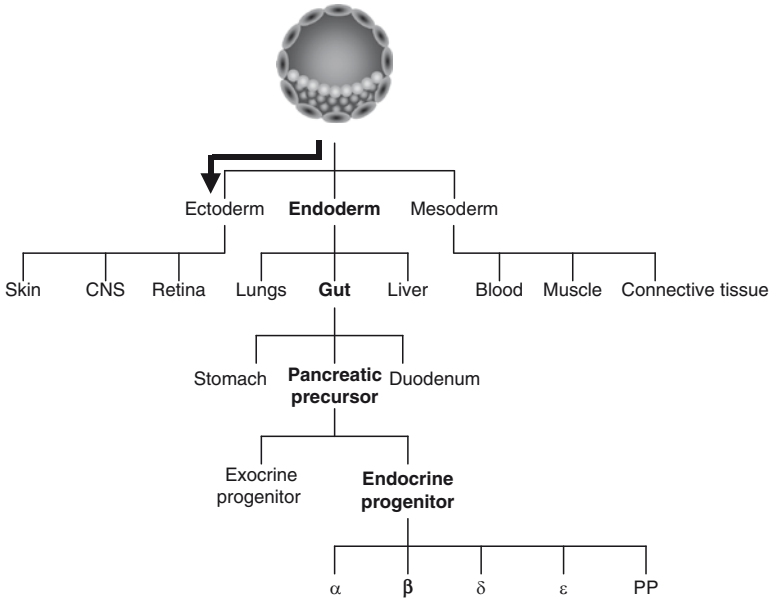


Fig. 48 Embryonic stem cell differentiation tree. Ideally, beta cell differentiation protocols ought to follow the "canonical" endodermal pathway (represented in *bold* characters). Protocols based on the expansion of nestin-positive progenitor cells (such as that reported by Lumelsky et al.[507]) follow an alternative route (neuroectoderm, *black arrow*)

2.2 Genetic Manipulation

In 2000, Soria and colleagues reported a genetic engineering strategy for the development and expansion of ES cell-derived beta cells. In short, they created ES clones that expressed in a stable manner a construct where the neomycin gene (which confers resistance to the drug G418) was placed under the control of the insulin promoter. When these cells were allowed to spontaneously differentiate in the presence of the drug, only those that expressed insulin could survive. Transplantation of these cells into mice previously rendered diabetic by streptozotocin administration resulted in restoration of normoglycemia[393] (Fig. 49). However, since the experimental design did not include a nephrectomy for the removal of the graft, the possibility of endogenous beta cell regeneration cannot be entirely discarded. Another caveat of this otherwise elegant approach is the choice of the insulin promoter, because insulin expression is by no means exclusive of the pancreas.[524–527] In fact, subsequent studies from the same authors led to the conclusion that the resulting cells were predominantly ectodermal.[392]

In 2003, Blyszczuk and colleagues[127] developed a protocol similar to that previously described by Lumelsky et al.,[507] with the critical difference that the starting ES cells had been stably transfected with a constitutively activated Pax4 cassette. The levels of Ngn3, Isl1, insulin, and Glut-2 were very significantly elevated compared with untransfected controls that underwent the same protocol, and the resulting cells were responsive to glucose and normalized glucose levels in streptozotocin-treated mice. Transfection with Pdx1 had some positive effects as well, but not as much as with Pax4. A negative outcome of these studies, however, was the relatively high incidence of teratomas.

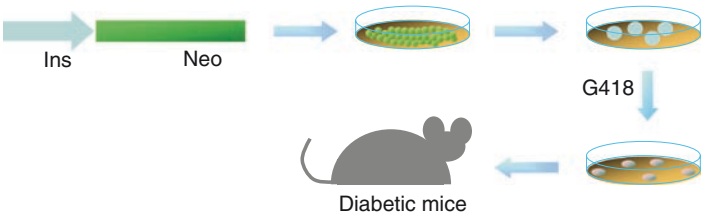


Fig. 49 Gene-trap approach to selectively ablate non-insulin producing cells, as described by Soria et al.[393] ES cell clones expressing an Ins–Neo cassette are allowed to spontaneously differentiate. Cells that differentiated along the insulin-producing lineage were resistant to the drug G418, and could be selected for transplantation into streptozotocin-treated mice

3 Human ES Cell Differentiation

The widespread availability of huES cells to most laboratories shortly after their isolation – together with the progressive realization that progress with mouse ES cells might not be immediately translatable to their human counterparts – led to a sudden shift of starting material for pancreatic differentiation experiments. The report that arguably initiated this general move toward huES cells was conducted by Assady and collaborators in 2001.[528] Both in adherent and suspension conditions, spontaneous huES cell differentiation resulted in the generation of insulin-producing cells as early as 2 weeks after the initiation of the protocol (peaking at day 19). Their number was relatively small, as only 60% of the EBs had positive staining and only 1–3% of the cells within these showed cytoplasmic insulin signal. In addition, glucose responsiveness was absent, probably due to the difficulty of detection in such a small representation of cells. However, this seminal experiment was the proof of principle that pancreatic differentiation of huES cells in vitro was indeed feasible.

3.1 Signal-Driven Approaches

A natural first attempt at directing the differentiation of huES cells toward beta cells was to expose them to the natural milieu of signals that drive the process in vivo. Given the practical difficulties of working with human fetal tissue, Brolen et al.[529] transplanted spontaneously differentiated huES cells containing subpopulations of pancreatic ($Pdx1^+/Foxa2^+$) and endocrine ($Pdx1^+/Isl1^+$) together with murine fetal pancreatic explants under the kidney capsule of recipient immunocompromised mice. Beta cell-like clusters expressing not only insulin, but also many transcription factors known to participate in the maintenance of the beta cell phenotype, could be consistently observed in the experimental group, but not in control animals that were transplanted with the huES and nonpancreatic tissues, such as the liver or the telencephalon of mouse embryos (Fig. 50). The results later reported by Vaca et al.[530] in an in vitro setting are consistent with these observations.

As for purely in vitro approaches, the adaptation of the protocol of Lumelsky et al.,[507] based on the generation and expansion of intermediate-stage nestin-positive cells, was another logical step. It was first reported by Baharvand et al.,[363] who were able to observe glucose-mediated insulin release in vitro, but failed to detect insulin secretory granules. As is the case with mouse ES cells, even the cells that produce bona fide insulin as a result of nestin-based differentiation protocols from huES cells are more related to neuroectoderm than to endoderm-derived beta cells.

The first reports on “canonical” ES cell differentiation into beta cells came shortly after the description of the conditions for definitive endoderm differentiation in murine ES cells.[50] Using a method based on the administration of Activin A

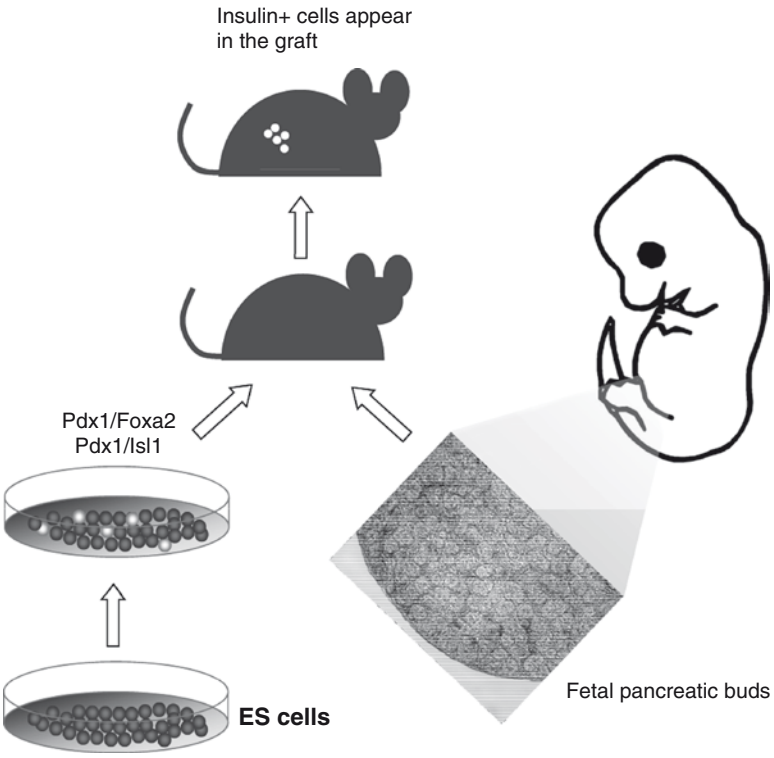


Fig. 50 Spontaneously differentiated ES cells containing endocrine and exocrine progenitors are able to terminally mature in vivo when engrafted together with fetal pancreatic tissue.[529] Controls in which other tissues unrelated to the pancreas were transplanted together with the ES cells did not yield insulin-producing cells

(an analog of Nodal) in the presence of a very low percentage of serum, D'Amour and colleagues[337] obtained cultures consisting of up to 80% of cells with definitive endoderm characteristics (Sox17, Goosecoid, Foxa2, and Mixl1 expression). These cells could be further enriched by sorting for CXCR4, a chemokine receptor expressed in mesoderm and definitive endoderm, but not in primitive/visceral endoderm. Transplantation of these cells into immunodeficient mice resulted in further progression of the endoderm differentiation program, as evidenced by expression of intestinal and liver markers in histological sections of grafts.

Arguably, the most critical contribution of this study to the field was the finding that Activin A was most effective at low serum concentrations. Serum-borne factors are known to interfere with specific differentiation pathways,[531,532] including that leading to the generation of definitive endoderm.

The same team of investigators contributed a breakthrough follow-up study[338] shortly after the publication of the first one. Capitalizing on their ability to efficiently differentiate huES cells toward definitive endoderm, they applied FGF7 (a

powerful inducer of Notch signaling during pancreatic development[201]) and cyclopamine (an inhibitor of *Shh* signaling[64]) to obtain cells that exhibited features similar to those of the primitive gut tube (chiefly expression of HNF-1beta and HNF-4A). Subsequent addition of retinoic acid (which had been previously found by others to help both the patterning of endoderm[49,533] and the progression of pancreatic development[534]) in defined medium resulted in the appearance of Pdx1-positive cells with a expression profile consistent with that of posterior foregut, first, and pancreatic endoderm/endocrine progenitors later. A final maturation step led to the formation of pancreatic endocrine cells with a messenger RNA pattern comparable to that of human adult islets (Fig. 51). All of the endocrine cell types found in adult islets were represented in these cultures. Given their importance for prospective cell therapies for diabetes, the authors focused on the characterization of putative beta cells, which constituted approximately 7% of the total population. These cells had very high insulin content, almost equivalent to that of adult islets and much more elevated than in earlier protocols.[535] They had well-defined secretory granules and were shown to secrete insulin/C-peptide in response to a variety of secretagogues, but not glucose. An explanation for the latter observation was that these beta-like cells might not be completely mature.

Another multistep protocol described around the same time showed some degree of glucose-responsiveness, but comparable differentiation efficiency[356] (Fig. 52). Shortly thereafter, the authors of the original study completed an unparalleled triad of breakthrough reports. Seemingly accepting defeat at obtaining therapeutic yields in vitro, they decided to transplant huES cell derivatives into immunodeficient mice midway throughout the protocol, in the hope that they would complete their maturation in vivo.[488] The procedure was successful at preventing the development of diabetes in streptozotocin-treated recipients, but the implanted cells took several months to mature upon implantation, and teratogenic lesions were observed in at least 15% of the animals.

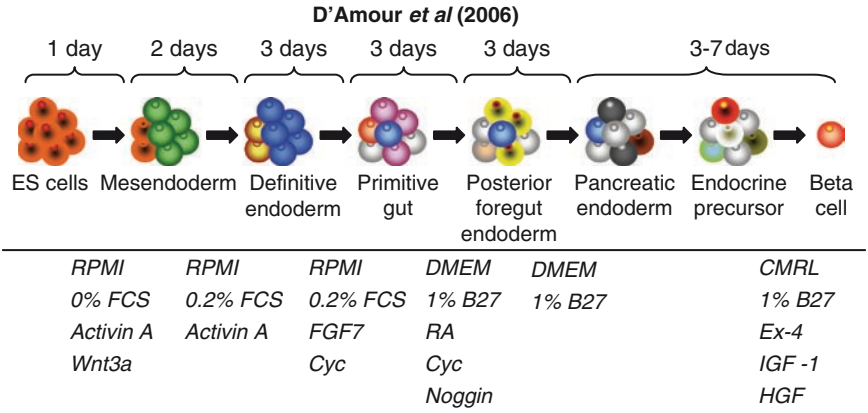


Fig. 51 huES cell differentiation protocol described by D'Amour and colleagues (2006). *FCS* fetal calf serum; *FGF* fibroblast growth factor; *RA* retinoic acid; *Cyc* cyclopamine; *Ex-4* exendin-4 *IGF-1* insulin-like growth factor 1; *HGF* hepatocyte growth factor

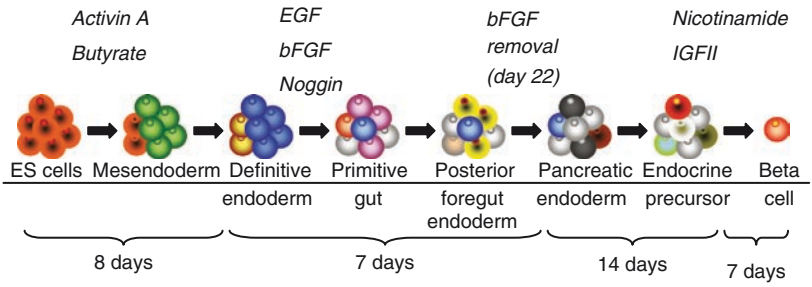


Fig. 52 huES cell differentiation protocol reported by Jiang et al.[215]. *EGF* epithelial growth factor; *bFGF* basic fibroblast growth factor; *IGFII* insulin-like growth factor II

The entire field is presently at a crossroads: is there anything else we can do to significantly increase the efficiency of full differentiation in vitro, or should we abandon such efforts and focus instead on making the transplantation of immature progenitors safer and faster acting? Only time will tell, but judging from the almost frenetic pace of research on the subject, a definitive answer might be just around the corner.

3.2 Genetic Manipulation

Following the demonstration that constitutive Pax4 expression improves the outcome of pancreatic differentiation in mouse ES cells,[127] Liew et al.[364] selected huES cell clones stably transfected with Pax4. Late-stage EBs generated from these cells were plated on Matrigel™ and treated with low glucose and nicotinamide, a set of conditions previously found to enhance the differentiation of beta cell progenitors in vitro.[536] They yielded a higher number of cells that stained positive for Newport Green (NG), a zinc-fluorescent probe commonly used to label and sort beta cells.[537]

FACS-enriched NG-positive cells had higher levels of Insulin, C-peptide, and Pdx1 expression than those derived from their nontransfected counterparts. However, they were irresponsive to glucose stimulation.

In another recent report, Lavon et al.[358] found that constitutive expression of Foxa2 in huES cells did not significantly alter the pattern of pancreatic specification in spontaneously differentiating EBs. Pdx1 overexpression, in contrast, resulted in an overall acceleration in the onset of the downstream gene Isl1 and the up-regulation of downstream genes such as Ngn3 and Pax4. However, insulin-expressing cells could not be detected unless the cells were allowed to further differentiate in vivo teratomas, and the differences between the genetically modified cells and the wild-type controls were statistically insignificant.

3.3 Protein Transduction

The only example thus far of the use of this novel approach to aid in the pancreatic differentiation of huES cells was reported only recently.[411] The authors subjected huES cell-derived EBs to TAT-Pdx1 treatment for 7 days, using Pdx1 protein as a control. Despite the well-known ability of Pdx1 to penetrate cells by virtue of an antennapedia-like protein transduction domain,[538,539] samples treated with TAT-Pdx1 showed a very significant up-regulation of Pdx1 targets compared with the controls. These included its own endogenous Pdx1 counterpart (~20-fold), Insulin (~30-fold), or islet amyloid polypeptide (~12-fold). C-peptide could be detected by immunofluorescence, but GLUT-2 levels remained unchanged when compared with those of samples treated with Pdx1 protein. A caveat of these studies – which could explain the partial effects observed – is that Pdx1 was applied at a very early differentiation stage, prior to the initial specification of primitive gut/posterior foregut cells. At this point, in the absence of the molecular partners that would normally act in concert with Pdx1 during pancreatic specification,[218,357,540,541] the protein alone might be insufficient to fully trigger by itself the onset of such program. It is plausible, however, that the use of TAT-Pdx1 at the appropriate huES cell differentiation stage might improve the yield and function of beta cells in the context of signal-driven approaches. In this context, it would be interesting to test whether TAT-Pdx1-VP16, a recently described version of transducible Pdx1 that includes the VP16 transactivation domain,[542] would improve ES cell differentiation efficiency (as it did in a liver transdifferentiation setting – see the chapter "Transdifferentiation").

iPS Cells

With the cloning of Dolly the sheep and the advent of SCNT,[504] the possibility of tailoring ES cells to each patient was immediately acknowledged. The nuclei of easily harvested somatic cells from any given donor would undergo a reprogramming process by microinjection into enucleated oocytes. The activation of the reconstituted embryo would lead to the in vitro development of blastocysts from which ES cells – genetically identical to the donor – would be harvested (Fig. 53). The principle of "therapeutic cloning" was readily tested in several species, including nonhuman primates,[543,544] but, despite initial reports suggesting otherwise,[545–547] huES cells could never be derived from SCNT-derived embryos.[548,549] Alternatives to SCNT included the use of cellular extracts[550–553] or fusion with ES cells,[554–557] but none of these approaches was completely successful. Very recently, however, the group of Takahashi and Yamanaka[376] were able to reprogram murine somatic cells by introducing four critical components of the core ES cell circuitry, namely Oct3/4, Sox2, c-Myc, and Klf4. Similar results with human cells were presented shortly thereafter by two

independent groups[375,382] - the second using Sox2, Oct3/4, Lin28, and Nanog in the reprogramming mixture (Fig. 54). The biotechnological feat of reprogramming adult somatic cells in such simple fashion (the top scientific breakthrough of the year 2008 of the journal *Science*) might have enormous medical implications,[371,558] provided that (1) the newly reprogrammed cells are comparable to blastocyst-derived ES cells; and (2) the procedure can be done safely. While all preliminary evidence seems to confirm that these induced pluripotent stem (iPS) cells are functionally indistinguishable from their embryo-derived counterparts, the use of retroviral vectors to deliver the critical genes is still unsafe in the context of human therapies (for review, see[377]). Ongoing efforts at addressing this concern include the use of nonviral delivery methods[380] and protein transduction.[426] The use of iPS cells for directed differentiation is now a fertile field, as dozens of laboratories around the world attempt to replicate results previously obtained with huES cells.[559–561] Of special interest is the recently reported proof of principle that iPS cells can undergo pancreatic differentiation using a protocol similar to that used by Jiang et al.[356]

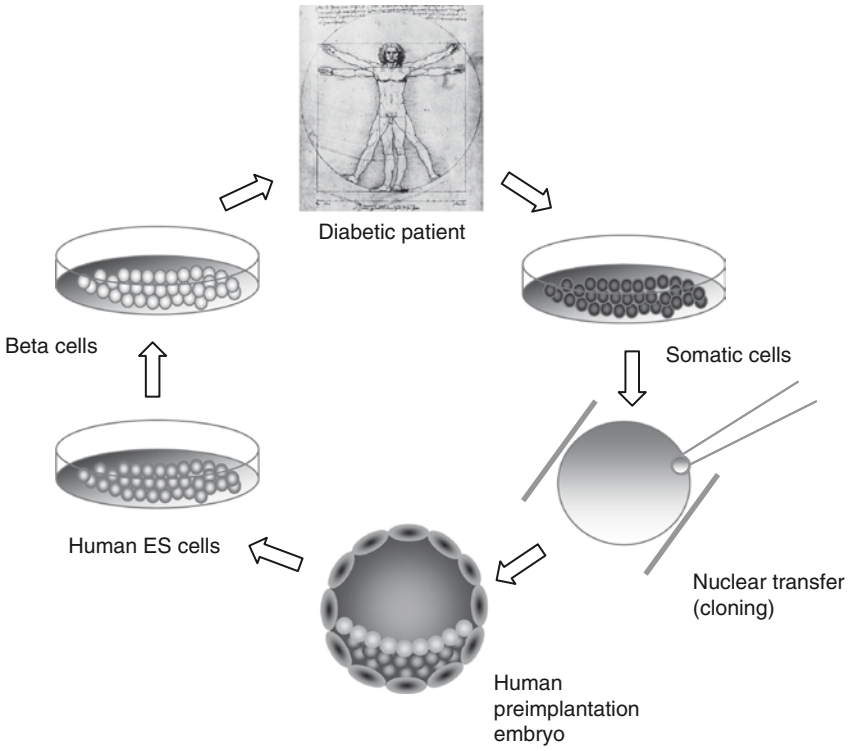


Fig. 53 Envisioned use of somatic cell nuclear transfer technology for the generation of "patient-matched" human ES cells that could be used for the treatment of type I diabetes

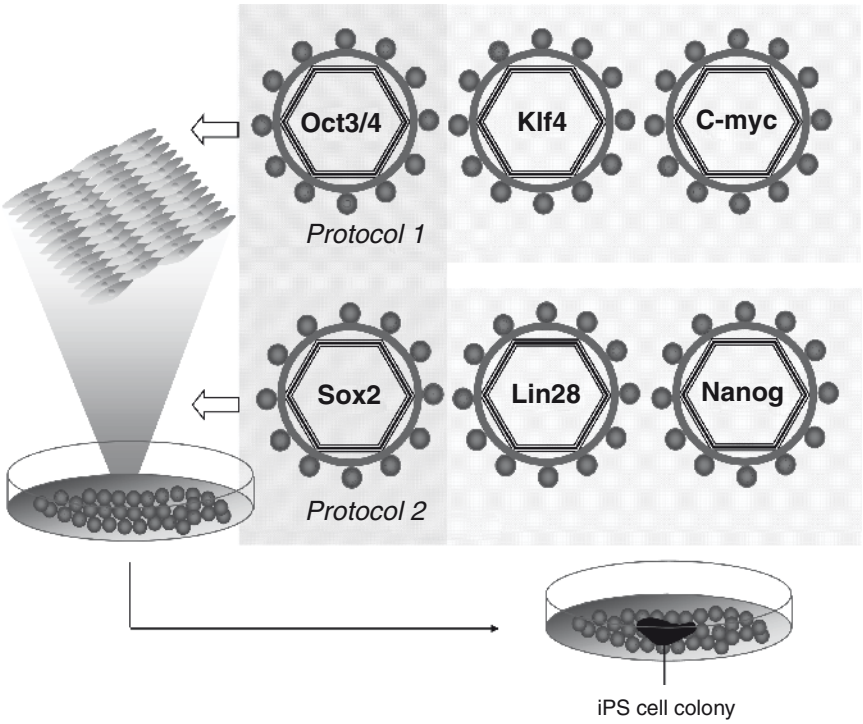


Fig. 54 Original protocols for the generation of iPS from adult fibroblasts. Retroviruses were used for the delivery of two different sets of reprogramming genes. Both combinations included the master regulators Oct3/4 and Sox2[375,382]

Safety of ES Cell Therapies

The translation of basic ES cell research into clinical therapies remains hindered by a number a safety concerns that arise from their very nature. Indeed, these cells are characterized by an unlimited proliferation potential under the appropriate conditions. If unchecked, even minute percentages of undifferentiated cells could keep dividing upon transplantation in immunosuppressed recipients, yielding teratomas.[517,519,562] These tumors develop either from carry-over undifferentiated cells or from cells that de-differentiate upon transplantation.[517,519,522,562] Some groups have approached this problem by screening the number of undifferentiated cells in each transplantable preparation. If such number is below the threshold known to produce teratomas in nude mice, they argue, these preparations should be considered safe for clinical use.[563] However, this method does not take into account the risk of de-differentiation after transplantation.[517] Other teams have addressed the teratogenic potential of ES cells by integrating suicide genes into them. Such

elements sensitize ES cells to specific pro-drugs, which can be used to induce their selective ablation both in vitro and in vivo[564–568] (Fig. 55). However, if teratomas were to form in vivo due to de-differentiation of implanted cells, administration of the pro-drug would kill the entire graft. The risk of accumulation of genomic instabilities as a result of long-term cell culture might be subtler, but not less dangerous. In view of later observations, the first reports on the karyotypic stability of huES cells[1,496,569,570] turned out to be inexact. As it happens with other cells, their adaptation to proliferative conditions in vitro invariably results in the selection of traits – perhaps not surprisingly – similar to those responsible for malignant transformation.[571] These include chromosomal and subchromosomal alterations not always detectable in standard G-banding tests.[572] Rushing ES cell therapies to the clinic would not be advisable until steps are taken to minimize these risks.

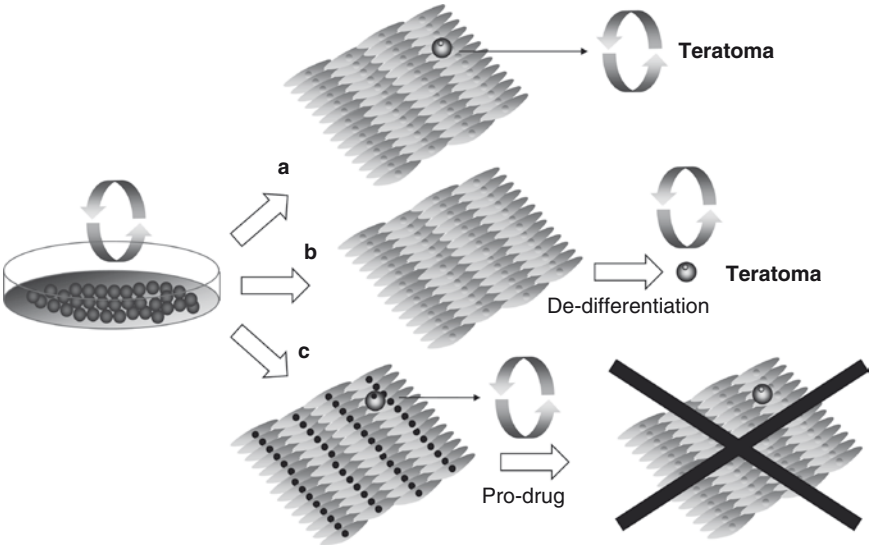


Fig. 55 Undifferentiated ES cells (*left*) can proliferate indefinitely under the appropriate conditions. Upon differentiation and transplantation, undifferentiated escapees may keep proliferating, especially if the recipient is immunosuppressed. These cells will give rise to a tumor termed teratoma (**a**). There is evidence that such tumors may also originate by de-differentiation of differentiated cells (**b**). Strategies to prevent this undesired outcome may involve the engineering of suicide genes in the genome of the donor cell line (*black nuclei*), which will destroy the host cell in the presence of a pro-drug (**c**). Current research focuses on the design of strategies to activate these suicide genes only in the cells that remain undifferentiated, as opposed to the entire graft

Line-to-Line Variability

Despite the overall similarities between different huES cell lines when kept under conditions that help maintain their pluripotency, several groups have reported differences in a number of parameters, ranging from population doubling time to susceptibility to spontaneous differentiation.[491] These differences become even more striking when the cells start to specify.[573] In a comprehensive study of 17 huES cell lines allowed to spontaneously differentiate, Osafune et al.[574] found >100-fold line-to-line variations in the expression of markers specific for the three germ layers. Three cell lines, for example, had a higher propensity to develop mesodermal lineages; two exhibited a marked preference for ectodermal or neural genes; another two showed a tendency to give rise to endodermal cell types; and so on. Out of the latter, line huES 8 turned out to be the best in terms of differentiation potential into pancreatic cells.[574] One potential conclusion from these results is that we ought to look for the right cell line for each differentiation protocol. This would argue in favor of banking an extensive number of ES cell lines and use only the ones that are better suited for each purpose. The alternative view, however, is that the results manifest the need for developing more robust protocols that will work in a cell line-independent fashion. After all, prospective personalized therapies where ES-like (iPS) cells are derived from the patient[372–376,382,495] might not afford the clinician the freedom to choose the most appropriate cell line for the desired developmental outcome.

Adult Stem Cells and Pancreatic Differentiation

Abstract Adult stem cells are found in most tissues, where they are thought to participate in natural turnover and regeneration. Under defined conditions, some of these cells can also be significantly expanded and differentiated along specific lineages. This chapter is focused on mesenchymal stem cells (MSCs), which can be isolated from virtually every organ of the human body. While MSCs have a well-proven potential to give rise to connective tissues (e.g., bone, cartilage, fat, etc.), their ability to differentiate into endodermal cell types (and particularly insulin-producing beta cells) is not as clear. However, there is significant evidence that specific treatments may induce insulin expression, even if doubts remain about the true nature of the end product. At any rate, MSCs have other extraordinary features that go beyond their differentiation potential, as they may provide other cells with appropriate engraftment/differentiation niches.

Keywords Mesenchymal stem cells • Cord blood stem cells • Amniotic fluid stem cells • Hematopoietic stem cells • Transient immortalization

1 Introduction

The general notion of "adult stem cells" describes populations of cells with poorly defined characteristics that can be found in specific niches of most adult tissues. They are thought to be involved in the native turnover of their tissue, and occasionally show a great mobilization potential in response to insults, leading to regeneration. Unfortunately, our ability to study these cells in their native environment is very limited: we are biased by their apparent behavior after we isolate and expand them in vitro. It can be argued that the majority of the expandable "adult" stem cells described thus far are just the result of an adaptation to in vitro culture, which is probably a misleading indication of their actual nature and potential in vivo. Indeed, stem cell niches tend to be complex and maintain a delicate equilibrium between many different compartments.[575–584] However, when we extract these cells from their native environment, we disrupt this complexity and favor the expansion of those that will better

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adapt to whichever culture conditions are set. Since researchers tend to favor attachment to plastic as a desirable characteristic (due to ease of cultivation and passage), perhaps it is not surprising that the end result, regardless of the tissue of origin, is usually quite similar: fibroblastic-like mesenchymal stem cells (MSCs) with a relatively limited differentiation potential and the ability to be propagated extensively without senescence. Only recently have we started to look into the possible in vitro replication of the natural complexity seen in stem cell niches. This would include not only the various participating cell types, but also the right combination of extracellular components. If successful, we might be able to accomplish the ultimate goal of expanding true, "ready-to-differentiate" tissue-specific stem cells – and not their surrogate, artifactual derivatives – for therapeutic purposes.

This chapter is not intended as an exhaustive review of adult stem cells and their niches, particularly in view of the fact that very little, if anything, is known about the elusive pancreatic stem cell.[86,88,585] Instead, we will describe the attempts at differentiating pancreatic cells from stem cells of "adult" (as opposed to embryonic) origin. In general, these cells will be characterized by some degree of in vitro expansion and differentiation potential, even if typically narrower than that of embryonic stem cells.

2 Mesenchymal Stem Cells

2.1 Introduction

Most studies on beta cell differentiation from adult stem cells are based on MSCs. Originally identified in the bone marrow, these cells are multipotent, adhere to plastic, self-replicate for many passages, and can be easily derived from virtually any organ or tissue in the body, perhaps with the exception of peripheral blood.[586] They have an elongated morphology resembling that of fibroblasts and have the potential to differentiate both in vitro and in vivo into a variety of connective fates, including adipose tissue, bone, and cartilage.[587] At one point, it was thought that mesenchymal cells could be as pluripotent as their embryonic counterparts. The general view at this point is that, being mesodermal in origin, these cells might require more than simple soluble factor-driven protocols in order to force their differentiation into endodermal and ectodermal lineages. Earlier this decade, Verfaillie and collaborators[588–593] isolated and characterized a subset of bone marrow-derived MSCs (multipotent adult progenitor cells [MAPCs]) that could have been an exception to the above rule. When injected into mouse blastocysts, these cells contributed extensively to all three germ layers in a manner consistent with that of a truly totipotent stem cell type. Other groups, however, have had difficulties replicating the methods originally described to establish these cell lines, and the technology has failed to become mainstream.

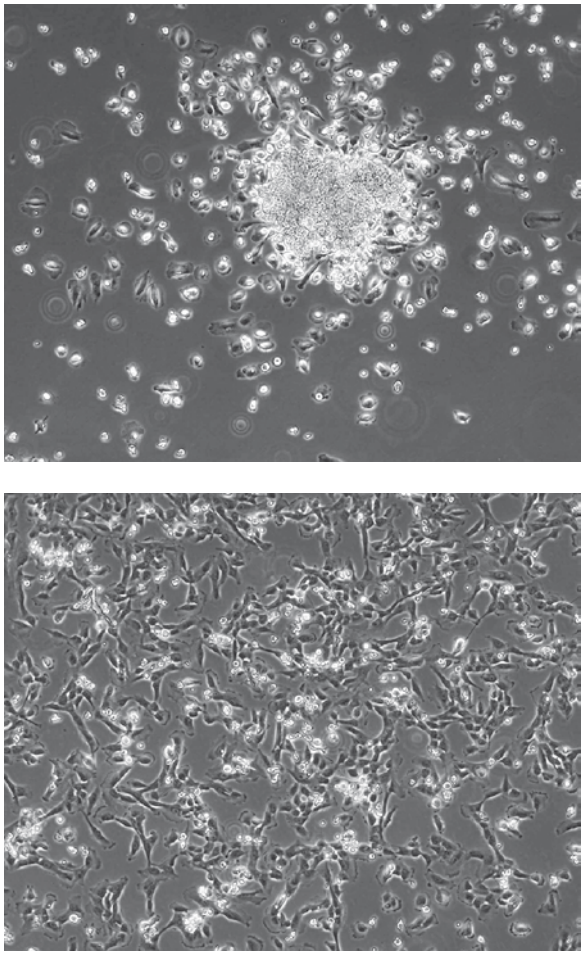

Fig. 56 A colony of human MSCs derived from the umbilical cord blood (*top*). After passaging, the cells grow in a monolayer and adopt a mesenchymal-like morphology (*bottom*). Kindly provided by Prabakar Kamalaveni and Luca Inverardi

Due to an explosion on reports on MSC-like cells isolated from almost every possible tissue source, the International Society for Cellular Therapy established recently a set of minimal criteria for MSC standardization. These criteria are the following[587,594]:

1. MSCs must adhere to plastic when maintained in standard culture conditions.
2. They must express the surface markers CD73, CD90, and CD105.
3. They must not express the surface markers CD34, CD45, CD14, CD11b, CD79α, and CD19.
4. They must be negative for HLA-DR (major histocompatibility complex class II).
5. They must readily differentiate into osteoblasts, adipocytes, and chondroblasts following standard in vitro methods.

Interestingly, expression of the pluripotent Oct3/4 transcription factor has been occasionally observed in MSCs.[429,595] However, the prevailing view is that Oct3/4 expression in adult tissues is not representative of pluripotency beyond the inner cell mass (ICM) stage, but rather an indication that this factor is not as embryonic-specific as originally thought.[596]

Given their ease of differentiation toward connective phenotypes, MSCs are already in the pipeline for potential regenerative therapies involving the replacement of lost/damaged tissues such as the skin,[384,597] the heart,[5] and the gastrointestinal (GI) tract,[598] among others. While we will now review the efforts made thus far at using these cells for beta cell differentiation, there is an increasingly widespread credence that the best use of MSCs in this context is not as building blocks, but rather as immunomodulatory[599–602] and pro-angiogenic[384,603] agents. Indeed, MSCs are well known for their ability to secrete many cytokines and growth factors, including vascular endothelial growth factor, brain-derived neurotrophic factor, nerve growth factor, basic fibroblast growth factor, insulin-like growth factor-1, hepatocyte growth factor (HGF), and others.[604] In different contexts, these factors have been found to interact with local microenvironments and have anti-apoptotic, morphogenetic, mitogenic, and angiogenic effects[605] that may favor engraftment and/or endogenous regeneration. A growing area of interest in the field of islet transplantation is that of cocultivation and/or cotransplantation of adult islet tissues with MSCs.[383,606–609]

2.2 Signal-Driven Approaches

Many groups have attempted the directed differentiation of MSCs of various origins into beta cells by means of adding specific combinations of soluble factors to the culture medium. The general assumption has been that, since these cells are already committed along one of three major differentiation pathways (mesoderm), standard protocols for the differentiation of ES cells may not work as effectively. This, however, remains to be tested.

In 2004, Chen et al.[610] treated rat MSCs with nicotinamide and beta-mercaptoethanol, obtaining "islet-like cluster cells" (ICCs), a term used by many people in the field to describe cellular aggregates (perhaps with the bias that this is how islets look in culture after isolation). When transplanted into syngeneic diabetic recipients, the authors observed a reduction in overall glycemic levels. One year later, Choi et al.[611] reported robust expression of all pancreatic endocrine hormones, as well as glucose-regulated insulin secretion in vitro, after the exposure of bone marrow-derived rat MSCs to pancreatic extracts from rats subjected to partial (60%) pancreatectomy. The rationale behind these experiments, as described in the chapter "Stem Cell Differentiation: General Approaches," is that pancreatic insults induce a regenerative response that might be accompanied by the release of islet-specific growth factors.

Other protocols for the in vitro differentiation of MSCs include cultivation with HGF and activin A (which resulted in the expression of small levels of insulin

from human MSCs, as well as glucagon, glucokinase, and a number of markers of early pancreatic development);[612] the use of fibronectin and pellet suspension techniques;[613] the coculture with pancreatic tissues in an artificial extracellular matrix;[614] and a combination of conophylline and betacellulin (BTC)-delta4, which substantially accelerated the pancreatic differentiation of murine bone marrow-derived MSCs when compared with basal differentiation methods making use of standard activin A plus BTC. Cells differentiated in this way were able to reduce hyperglycemia in transplanted animals for up to 4 weeks,[615] an outcome comparable to that resulting from transplanting rat MSCs treated with exendin-4 and nicotinamide.[616]

2.3 Genetic Manipulation

As is the case for embryonic stem cells (see the chapter "Embryonic Stem Cells and Pancreatic Differentiation") and transdifferentiation (see the chapter "Transdifferentiation"), the master pancreatic regulator Pdx1 remains a key player in all approaches involving genetic manipulation of MSCs. Thus, upon transfection of bone marrow-derived rat MSCs with a constitutively active Pdx1 cassette, Sun et al.[617] observed expression of insulin, glucagon, and somatostatin. Transplantation of these cells into diabetic animals resulted in longer survival and maintenance of body weight, even if normoglycemia was not achieved. A similar approach was also used by Li et al.[362] with human MSCs, where the Pdx1 gene was introduced via an adenoviral vector. Several relevant beta cell genes, including Ngn3, insulin, glucagon, glucokinase, and GLUT-2 were significantly up-regulated, and transduced cells exhibited a marginal ability to respond to glucose stimulation in vitro. Upon transplantation, these cells were able to reverse hyperglycemia in diabetic mice for up to 6 weeks. A triple adenovirus-mediated transfection with Pdx1, Hlxb9, and Foxa2 resulted in the detection of insulin in vitro, but with just a small proportion of cells expressing the fundamental component of the pro-insulin-processing gene PC 1/3.[618] Another group used retroviral vectors to ensure long-term expression of the Pdx1 gene in human bone marrow-derived MSCs, which led to the expression of all four pancreatic hormones but not NeuroD[619] (see the chapter "Pancreatic Development"). Despite the absence of this critical marker, these cells were able to regulate insulin secretion in vitro. In addition, when transplanted into diabetic animals, NeuroD was activated and a significant reduction in hyperglycemic levels was observed. More recently, Masaka et al.[620] developed a chemical protocol based on the administration of HGF and FGF-4, which resulted in the generation of a bipotential, self-renewable "hepato-pancreatic" progenitor cell population. When Pdx1 was overexpressed in these cells, insulin was produced and an in vitro glucose-regulated response observed. In line with the above observations, the intrahepatic transplantation of mouse MSCs expressing a human ectopic insulin cassette reduced glucose levels for up to 6 weeks in diabetic recipients (Table 3).[621]

Table 3 Representative genetic manipulation efforts at differentiating MSCs into beta cells

Species	Gene	Vehicle	Outcome	Reference
Rat	Pdx1	Transfection	No normoglycemia. Longer survival recipients	617
Human	Pdx1	Adenovirus	Pancreatic gene upregulation in vitro. Transient euglycemia in recipients	362
Human	Pdx1, Hlxb9, Foxa2	Adenovirus	Pancreatic gene upregulation in vitro	618
Human	Pdx1	Retrovirus	Pancreatic gene upregulation in vitro. Reduced hyperglycemia in recipients	619
Mouse	Insulin	Retrovirus	Reduced hyperglycemia in recipients	621

2.4 Protein Transduction

There are no reports to date on the use of protein transduction for the directed pancreatic differentiation of MSCs. However, Pdx1 has been found to have an antennapedia-like PT domain (pANT) within its sequence.[538,539,622] Indeed, purified Pdx1 protein successfully transduced target tissues, including ductal cells (which have been found to be an abundant source of MSCs[623]), where they up-regulated the expression of the insulin gene. Even though TAT has proven better than pANT for the delivery of large proteins,[396,411] the possibility of taking advantage of the native PTD of this and other pancreatic developmental genes[624,625] without any further manipulation is an attractive one. The reason for the existence of built-in PTDs in transcription factors remains a biological mystery. It has been convincingly shown that nuclear proteins of the engrailed family can be released by "donor" cells and taken up by adjacent cells,[626–630] which might be indicative of a yet unexplored paracrine function associated with some transcription factors. From a strictly practical point of view, based on the transgenic studies described above, the successful use of transducible versions of Ngn3,[109] Pdx1,[411,538,539,622] NeuroD/Beta2,[625,631] and Pax6[416] is likely to be translated to adult MSC-like cells in the very near future.

2.5 In Vivo Transplantation of Undifferentiated MSCs

As previously discussed in the chapter "Pancreatic Regeneration," the evidence for bone marrow-mediated regeneration of pancreatic endocrine function is more likely related to a "trophic" activity than it is to direct transdifferentiation. However, several groups have recently carried out a number of intriguing experiments where undifferentiated MSCs are implanted in diabetic animals with the aim of ameliorating the symptoms of the disease. Thus, Ezquer et al.[632] reported that streptozotocin-treated diabetic mice that received an intravenous injection of undifferentiated MSCs showed reduced blood glucose levels as early as 1 week after the

intervention. Compared with the animals in the control group, the MSC-injected animals exhibited reduced albuminuria and better renal function. Histological studies suggested that the number of islets is increased. Similar observations were reported by Dong and collaborators in an allogenic rat transplantation model,[633] in which streptozotocin-treated recipients were injected through the tail vein with a single dose of undifferentiated, BrdU-labeled MSCs. A significant reduction of hyperglycemia was observed at day 45, with treated animals showing higher body weight and an increased number of small islets with $BrdU^+$/$Insulin^+$ cells. In yet another animal model, Chang et al.[613,634] injected male porcine MSCs directly in the pancreas of female diabetic pigs. Two weeks after transplantation, blood glucose levels decreased compared with those of sham-treated controls. Confirming the observations of the above groups, the authors reported a higher number of small islets containing insulin-producing cells of male origin, suggesting direct transdifferentiation. Although none of these studies addressed convincingly the possibility of cell fusion[554,555,635,636] (which could explain many of these findings), the observed effects are certainly encouraging and warrant additional investigation.

3 Other Stem Cells

3.1 Hematopoietic Bone Marrow and Cord Blood Stem Cells

Although hematopoietic stem cells (HSCs) represent a minute percentage of the bone marrow compartment, they are known to reconstitute all blood-forming lineages.[637,638] HSCs can also be found in the cord blood, which offers an easily bankable source that has already been proven in the clinical arena.[639] More recently, a number of studies have shown that the bone marrow and cord blood host multipotent cells with the ability to differentiate into many different tissues.[640,641] Certainly, MSCs are one such multipotent cell type, and perhaps the main component of the subpopulations selected for attachment and growth on plastic. In this context, it has been described that cord blood-derived MSCs subjected to a chemical differentiation protocol including high glucose, retinoic acid, nicotinamide, epidermal growth factor, and exendin-4 leads to the generation of ICCs expressing insulin, glucagon, GLUT-2, Pdx1, Pax4, and Ngn3.[642] These cells, however, fail to regulate insulin secretion in response to glucose challenge in vitro. Similar results were recently presented by Chao et al.,[643] whose treatment of MSCs derived from the Wharton's jelly (a stem cell-rich mucopolysaccharide layer within the cord blood) with neuron-conditioned medium resulted in the formation of ICCs positive for glucose-regulated human C-peptide secretion. When implanted in nonimmunosuppressed diabetic rats, these cells engrafted successfully and ameliorated hyperglycemia.

As for the non-MSC compartment, HSCs are presently being used for the treatment of diabetes in clinical trials for diabetes, but from a different angle: the reeducation of the immune system.[644–650] In clinical trials conducted on 15 newly diagnosed type I diabetic patients, HSCs were mobilized, collected from peripheral blood, and

cryopreserved. Patients were then subjected to a very aggressive immunosuppressive regime prior to the reinfusion of their own stem cells, which were assumed to be at a stage prior to the onset of the disease. As a result of this treatment, 14 out of 15 patients became insulin-free for extended periods of time.[647,650] Others are already transplanting hematopoietic cells directly into the pancreas of diabetic patients,[651] but the claimed beneficial effects of this treatment on the overall glycemic control still need to be independently validated– and a mechanistic explanation investigated. As was the case with MSCs, since the hematopoietic compartment is derived from the mesoderm, there are doubts that HSCs may become true endodermal or ectodermal derivatives. To this date, there is no conclusive evidence in one way or another that transplanted HSCs that migrate to target tissues help in their regeneration by differentiation/transdifferentiation, cell fusion, or simply by supporting endogenous regeneration through revascularization and/or as feeders.[652]

The latest cell type to join our arsenal of adult stem cells is the amniotic fluid stem (AFS) cell. These cells are naturally shed by the embryo to the surrounding amniotic fluid, and can be easily cultured in vitro.[653,654] In a recent breakthrough report, De Coppi et al.[655] described a novel subpopulation of amniotic fluid cells with a multilineage differentiation potential spanning the three embryonal layers. These cells can be expanded almost indefinitely without loss of pluripotency, and they coexpress markers of both human embryonic stem cells (Oct3/4, SSEA-4, telomerase, and others) and MSCs (CD105, CD73, and CD90). Unlike huES cells, however, AFSs did not form teratomas in immunocompromised recipients. Their proven ability to generate endodermal derivatives bodes well for the field of pancreatic regeneration, although current efforts at differentiating these cells into pancreatic endocrine cell lineages have not been reported yet.

Transiently Immortalized Beta Cells

A potential alternative to the use of adult stem cells for directed differentiation into beta cells would be to define the conditions to expand fully differentiated beta cells. In the chapter "Stem Cell Differentiation: General Approaches," we have already reviewed the literature on beta cell regeneration in vivo. However, in vitro "re-differentiation" of beta cells after de-differentiation and expansion phases[327] was a less than convincing strategy, due to the fact that the end product could not stand comparison with the real beta cell. A different approach to the same end was presented in 2005 by Narushima et al.,[656] who transfected primary human beta cells with loxP-flanked sequences for the simian virus 40 large T antigen (SV40T) and the human telomerase reverse transcriptase (hTERT). These cells could be expanded without senescence for more than 50 passages in vitro. During the expansion phase, expression of insulin and other critical beta cell genes was downregulated. However, upon Cre-mediated "reversion," the two immortalizing genes were removed and the cell adopted a quiescent, functional beta cell

phenotype again. Reverted cells were able to permanently correct hyperglycemia in streptozotocin-treated SCID mice without any further replication in vivo (Fig.57). A complementary technology that could help the clinical translation of these findings is that of transducible TAT-Cre,[413,425,427] whose use in this context would preclude the use of viral vehicles to deliver the recombinase).

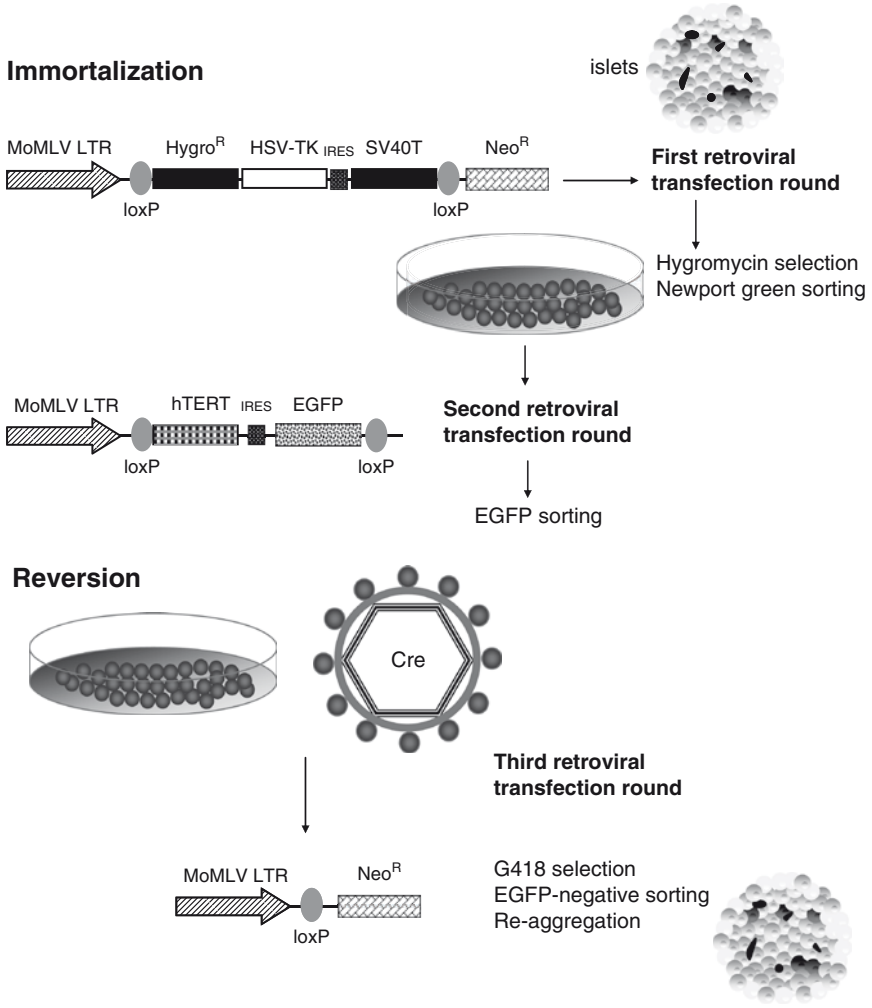

Fig. 57 Transient immortalization of beta cells, as described by Narushima et al.[656] A double round of retroviral transfection allows for the selection of cells that express both the hTERT and the SV40 large T antigen genes, which confer immortality. An EGFP marker can be used to sort these cells. This can be reverted by introducing the Cre recombinase, which will excise out the immortalizing genes in a process that will bring together a constitutive promoter and a neomycin-selectable marker. These "reverted" cells can be selected using G418 and by sorting for EGFP-negative signal

Transdifferentiation

Abstract An alternative to the use of undifferentiated cells is that of reprogramming adult cells from nonpancreatic sources. Of these, the most promising is the liver, whose embryonic development is deeply intertwined with that of the pancreas. Several teams have now proven that the ectopic expression of master pancreatic regulators such as Pdx1 or MafA, among others, can induce the expression of pancreatic markers in liver-derived cells, both in vitro and in vivo. Here we review the status and clinical prospects of this approach.

Keywords Liver transdifferentiation • Pdx1 • VP16 • Reprogramming • Hepatocytes

1 Introduction

Expression of Pdx1 in the foregut (e8) is one of the earlier molecular events that mark the specification of the pancreas as a separate organ (see the chapter "Pancreatic Development"). The role of Pdx1 as a "master regulator" of pancreatic development has led many investigators to test whether its ectopic expression would induce pancreatic differentiation by itself. This strategy has yielded somewhat modest results in most cellular substrates examined,[108,361,657] which suggests that Pdx1 expression is necessary, but not sufficient, to initiate pancreatic development. The conclusion from the observation that the initial evagination of the pancreatic epithelium occurs even in the absence of Pdx1.[70] A possible exception to this rule, however, is observed when the target tissue is liver.[357,365,366,658] In fact, there is a wealth of studies indicating that the liver and pancreas are especially susceptible to interconversion. Many invertebrates have a single organ that comprises both hepatic and pancreatic functions, which suggests that the separation of these two organs is a relatively late evolutionary event. In vertebrates, fibroblast growth factor (FGF) signals from the cardiac mesoderm have been shown to play an essential role for the ventral endoderm to differentiate into early hepatic cells,[353,659,660] and it has been demonstrated that both organs originate from common endodermal progenitors in

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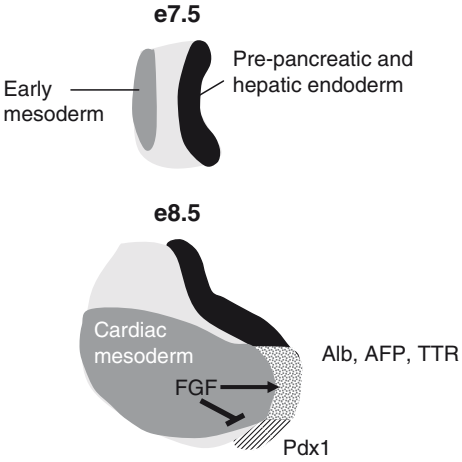

Fig. 58 FGF signalling from the cardiac mesoderm will induce liver specification proximally (*dotted region*), but will have a blocking effect on the distal portion of the ventral foregut, which will become pancreas (*stripes*) (Adapted from Deutsch et al.[662])

the early foregut.[81, 353, 355, 661–666] According to the model described by Deutsch et al.,[662] cardiac FGF will have inductive and blocking effects on liver and pancreas specification, respectively (Fig. 58).

In general, hepatocytes and beta cells share not only many developmental features but also similar molecular machinery for glucose sensing and secretion.[667,668] Many studies confirm that interconversion of liver and pancreas occurs under a variety of experimental conditions, including copper depletion in rats,[669–671] treatment with dexamethasone[541] or diethylnitrosamine,[672] and certain tumoral processes.[673]

2 Directed Liver Transdifferentiation

One of the earlier attempts at transdifferentiating liver cells into pancreatic beta cells was reported by Ferber and colleagues in 2000.[366] Using a "gain-of-function" strategy, they transferred a constitutively active Pdx1 cassette to recipient mice by means of an adenoviral vehicle (Fig. 59). Ectopic expression of the gene was mainly observed in the liver, where it activated the expression of the endogenous genes Insulin 1 and 2 and prohormone convertase 1/3 (PC 1/3). These genes are typically active in beta cells, but not in liver tissue. Plasmatic insulin levels were substantially elevated in treated mice compared with controls treated with an empty virus alone. More strikingly, ectopic insulin expression was found to reduce glucose levels in streptozotocin-treated mice. In a series of follow-up experiments, the same team reported that ectopic Pdx1 expression in the liver persisted well beyond the few weeks during which adenoviruses (which do not integrate into the host genome) maintain their activity. Indeed, they found that the exogenous Pdx1 was able to induce the activation of its endogenous counterpart, thereby priming

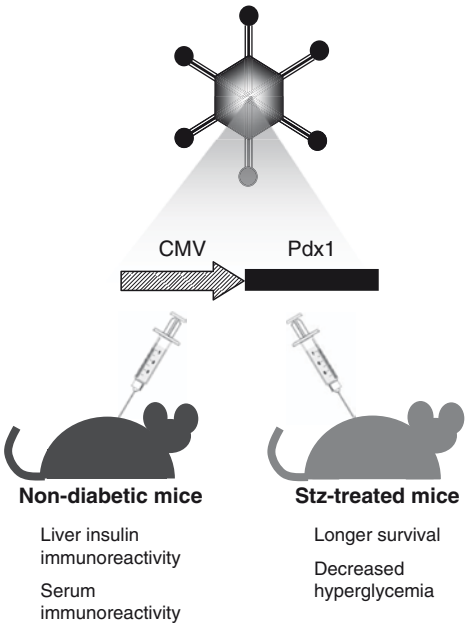

Fig. 59 Liver transdifferentiation approach as reported by Ferber et al. (2000)[366]. A Pdx1 cassette is delivered systemically through an adenoviral vector. Expression of this cassette in the liver yields insulin-producing cells. When the procedure is done in streptozotocin (STZ)-treated diabetic mice, it results in a very significant reduction of blood glucose levels

self-sustainable regulatory networks leading to long-standing "transdifferentiation," as evidenced by ectopic insulin production and maintenance of normoglycemia in streptozotocin-treated animals up to 8 months after the initial treatment.[365] Like other master regulators of development and stem cell self-renewal, Pdx1 exerts a positive feedback over its own promoter.[152,674] Since liver cells already express Pdx1 transcriptional partners such as HNF1beta and 3beta, the authors speculated that the transient administration of the exogenous genes resulted in permanent effects. It was also observed that the insulin-producing cells were located mainly around the hepatic central veins, a distribution that was hypothesized to allow systemic hormone release without harmful effects in liver function.

The molecular basis of this phenomenon remained unclear until very recently, when Meivar-Levy et al.[675] presented compelling evidence that Pdx1-mediated transdifferentiation requires an intermediate "de-differentiation" step. Pdx1, but not other pancreatic genes delivered using the same system, induced a substantial down-regulation of C/EBPbeta and LAP, two redundant proteins of a family of transcription factors known to play important roles during liver embryonic development as well as in adult hepatocytes.[676,677] Overexpression of LAP in primary cultures of human hepatic cells prevented the Pdx1-mediated de-differentiation and activation of the pancreatic program. However, down-regulation of C/EBPbeta was insufficient by itself to trigger the activation of pancreatic genes, with the exception of Ngn3. As expected, the simultaneous down-regulation of C/EBPbeta and the administration of Pdx1 had a synergistic effect in inducing transdifferentiation.

The question remains of whether this approach is targeting bona fide hepatocytes or perhaps more undifferentiated progenitors that might be more amenable to transdifferentiate. In vitro experiments are not conclusive because hepatocyte cultures become fibroblastic in appearance very rapidly, perhaps because of the adaptation of the cells to attachment in plastic. In addition, it is very likely that the insulin-producing cells generated in this manner are not true beta cells, but rather hybrids between hepatic and pancreatic cells.

Parallel experiments conducted by Horb and colleagues confirmed that transdifferentiation is indeed feasible in other contexts.[357] In short, the authors created transgenic frogs where a Pdx1 (Xlhbox8)-VP16 fusion cassette is expressed under the control of the liver-specific promoter transthyretin (TTR) (Fig. 60). The rationale for the use of VP16 – a potent transcriptional transactivator from the herpes simplex virus[678,679] – is that nonpancreatic cells may lack the appropriate molecular partners for Pdx1 to exert its biological function. An additional marker was added to screen for successful transdifferentiation, namely the green fluorescent protein (GFP) under the control of the elastase promoter, a pancreas-specific regulatory element. Up to 60% of transgenic tadpoles showed partial or total conversion of liver to pancreas, as evidenced not only by the expression of GFP but also by that of pancreatic endocrine (insulin and glucagon) and exocrine (amylase) markers. It is important to note that no transdifferentiation was observed when Pdx1, without VP16, was used. This observation suggests that the assertion that Pdx1 is necessary, but not sufficient to promote pancreatic differentiation, remains true for the liver.

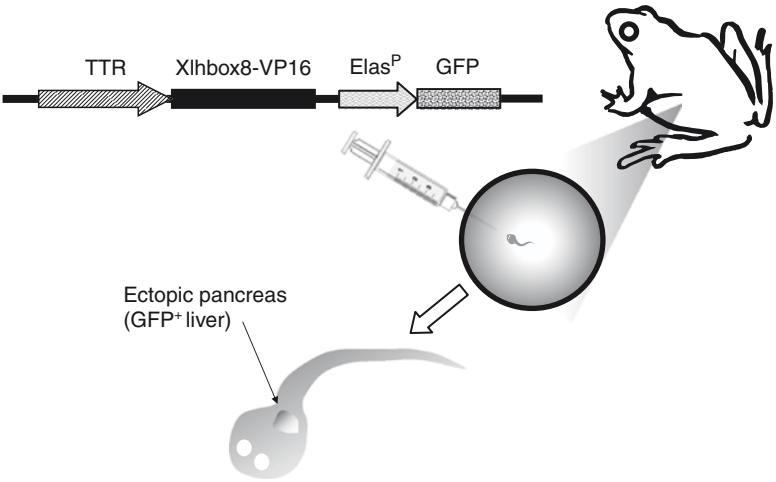

Fig. 60 Experimental conversion of liver to pancreas in a frog transgenic model, as described by Horb et al.[357] The Xlhbox8 gene, the amphibian homolog of Pdx1, was fused to the VP16 transactivation domain, and placed under the control of the liver-specific promoter TTR. An additional marker was added to follow up successful conversion events (GFP driven by the promoter of elastase, a gene expressed in the pancreas but not the liver). Transgenic tadpoles exhibited various degrees of liver transdifferentiation into pancreas

Given that the timing was such that the onset of the ectopic Pdx1 expression was coincident in time with the initiation of liver development, this event could in theory be considered an induced redirecting of early organogenesis, rather than a proper transdifferentiation event. The authors, however, used the same construct to transfect immortalized human hepatocytes (HepG2), which led to elastase activation in approximately 65% of the cells that received it. Of these, approximately 15% were insulin positive. These results were subsequently expanded by characterizing the transdifferentiated cells. Confirming the observations of Ferber and colleagues, it was found that the hepatic phenotype was lost upon ectopic expression of Pdx1; that the requirement for the transgene was not permanent, as an initial trigger was sufficient to activate the pancreatic lineage; and that the insulin-positive cells obtained through this approach had PC 1/3, C-peptide, and glucagon-like receptor 1 (GLP-1), among other functional markers of true beta cells. These cells were glucose responsive and increased insulin expression upon treatment with GLP-1 and beta-cellulin.[658]

Using a lentiviral vector, Tang et al.[359] were able to transdifferentiate rat hepatic stem-like WB cells into pancreatic beta cells both with Pdx1 and Pdx1–VP16. This study was the first to systematically compare the transdifferentiation potential of the two versions of the gene. While they found that cell lines expressing either Pdx1 or Pdx1–VP16 long-term had comparable gene expression profiles as well as a similar capacity to correct hyperglycemia in recipient diabetic mice, short-term expression gave a marked edge to the VP16-fused version.

Additional studies were significantly consistent with the first set of data first published by Ferber and colleagues, and showed the cumulative effect of adding other pancreatic endocrine factors to the mix, such as Ngn3, NeuroD, or MafA.[680–686] Interestingly, Wang et al.[680] reported their inability to induce liver-to-pancreas transdifferentiation in vivo when using adeno-associated viruses as vectors to co-deliver Pdx1 and Ngn3. However, when they delivered these cassettes using plasmid vectors with an irrelevant adenoviral vector, they reported correction of hyperglycemia in diabetic rodents. The authors postulated that the antigen-dependent immune response elicited by the adenoviral capsid (but not other viruses) was instrumental in the induction of transdifferentiation.

The concept of liver transdifferentiation has attracted significant attention for several reasons. First, liver cells are easier to obtain and expand than those derived from the pancreas. Therefore, they provide an easily accessible source (a biopsy might provide enough cells to manipulate ex vivo) that could be extracted from the very same patients we want to treat, thus eliminating the risk of allogeneic rejection (Fig. 61). Second, it is becoming clear that, despite some degree of functionality, transdifferentiated liver cells are not true beta cells. Some evidence indicates that the ability of these cells to appropriately regulate insulin secretion in a glucose-regulated manner might not stand comparison with that of true beta cells. However, clinical therapies could be devised even if these cells worked just as a “pump” (i.e., continuously secreting a basal amount of insulin in a nonregulated fashion). Most importantly, in type I diabetic patients, the immune system is poised to attack and destroy any cell that resembles a beta cell. From this perspective, these “hybrids”

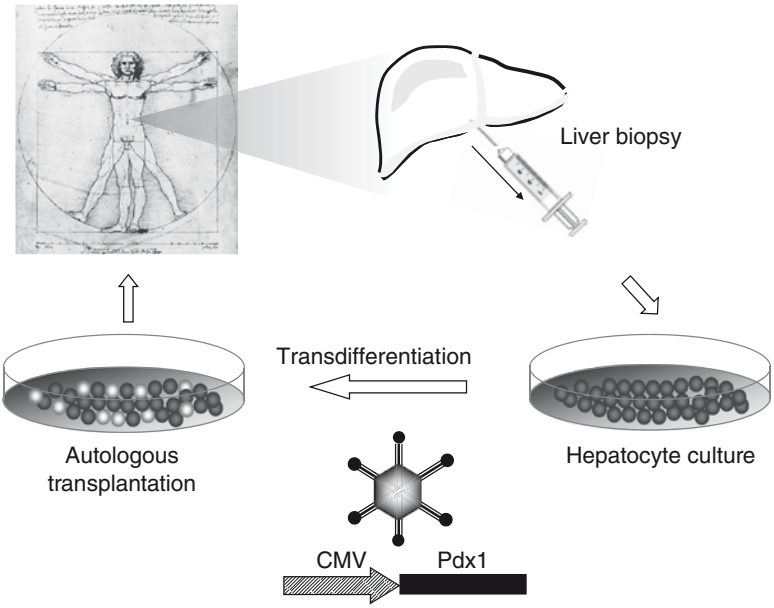

Fig. 61 A theoretical therapy based on the ex vivo conversion of liver cells into insulin-producing cells

might have a selective advantage over native beta cells, because they could be able to elude the autoimmune response. Of course, this hypothesis hinges on the assumption that the autoimmune response will spare non-beta cells that express insulin, which might not be the case in view of the fact that insulin has been shown to be an auto-antibody in type I diabetes.[687–689]

A more recent attempt at transdifferentiating non-endocrine tissue into beta cells used a different starting material, one that – at least in theory – should be more closely related to the desired end product. Based on the screening of at least 20 transcription factors (of which nine gave rise to gross phenotypic changes in beta cells when knocked out) expressed either in terminally differentiated beta cells or their progenitors,[690,691] Zhou et al.[360] were able to reprogram pancreatic exocrine tissue into islet cell types using a combination of three genes (Pdx1, Ngn3, and MafA) delivered by means of adenoviral vehicles (Fig. 62). New insulin-producing cells were detected as early as 3 days after the injection of the adenoviral mix into the pancreata of $Rag1^{-/-}$ mice, a strain typically used to minimize the occurrence of viral-induced immune responses such as those described earlier by Wang et al.[680] The number of these cells kept expanding for up to 3 months, long after the adenoviruses had been cleared from the recipients. They were indistinguishable from native beta cells in terms of size, morphology, presence, and distribution of insulin granules and molecular markers. Unlike in other transdifferentiation settings, the original exocrine phenotype appeared to be completely abrogated (i.e., they were not

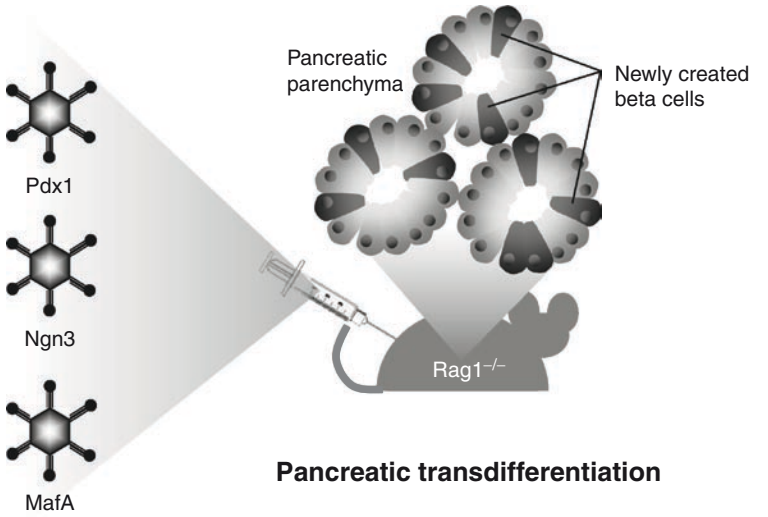

Fig. 62 Transdifferentiation of non-endocrine pancreatic tissue into insulin-producing beta cells, as proposed by Zhou et al.[360]

"hybrids"). Diabetic mice subjected to the treatment showed a significant and permanent improvement in blood glucose levels, even if diabetes was not completely reversed. The latter observation could be explained by the fact that the newly created beta cells remained isolated and did not cluster to form islets. Indeed, beta cell communication is essential to stimulate glucose-mediated insulin secretion.[32,692]

As promising as this rapidly evolving field is, safety concerns may still preclude its immediate clinical translation. The observation that the ectopic genes need only be expressed transiently in order to activate transdifferentiation is encouraging. However, the use of adenoviruses may have serious side effects by eliciting immune responses in the host. Also, the ability of Pdx1 to induce exocrine tissue as well as endocrine derivatives proved harmful by inducing fulminant hepatitis in animal models.[686] In a transgenic setting, ectopic expression of the gene resulted in widespread liver dysmorphogenesis, with abnormal lobe structures and polycystic lesions.[528] Certainly, the systemic infusion of viral vectors containing master pancreatic regulators into human patients does not seem a clinical possibility in the near future. However, the extraction of liver tissue for ex vivo transdifferentiation and subsequent reimplantation in the patient appears to be a more reasonable course of action. It is likely that in this setting the adenoviruses would have already been cleared from the cells at the time of transplantation, increasing the overall safety of the procedure.

Remaining Challenges and Clinical Perspectives

Abstract Unlike other potential targets of future stem cell approaches, there is already a current cell therapy for the treatment of type I diabetes. Indeed, islet transplantation has proven successful in inducing insulin independence for at least 1 year after the procedure. Progress in this discipline during the past 20 years has paved the way for stem cell-based therapies. Here we review the current state of the art of islet transplantation and examine the challenges that need to be addressed before a transition is made to stem cell-derived insulin-producing cells, with particular emphasis on the immunological aspects (rejection and autoimmunity) of type I diabetes.

Keywords Islet transplantation • Autoimmunity • Rejection • Nanoencapsulation • Diabetes

1 Introduction

While the exploration of the mechanisms behind the development of the pancreas would have been fully warranted from a purely scientific point of view, it cannot be disputed that the prevalence of diabetes has very significantly stoked our progress in the field. Two more almost simultaneous circumstances have aligned to make pancreatic development one of the best studied examples of organogenesis: the advent of human embryonic stem cells[1] and the development of protocols for the long-term survival and function of transplanted islets.[10] Unlike many other conditions for which potential stem cell therapies have been conceived – but not put into practice yet – type I diabetes is arguably the perfect target of regenerative therapies: only one cell type needs to be replaced and there is an already existing cell therapy. The success of islet transplantation as a viable treatment of type I diabetes has led to the valid assumption that, if stem cells can be coaxed to produce insulin in a glucose-regulated manner, ensuing therapies are likely to work as well as native islets do. Such an approach would provide an immediate solution to the

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most pressing problem that stands in the way of the widespread implementation islet transplantation, namely the shortage of organs for islet processing and transplantation. Here we review the challenges and clinical perspectives of stem cell research in the context of the current status of islet transplantation.

2 Diabetes and Islet Transplantation

Type I diabetes is an autoimmune disorder whereby the immune system of the affected individual attacks and destroys the pancreatic beta cells that secrete insulin in response to elevated blood sugar levels. Because it is usually (but not always) diagnosed during childhood or early teenage years, it is also referred to as juvenile diabetes. Type II diabetes differs from it in that it does not usually start as an autoimmune response, but rather as a consequence of the inability of the cells of the body to respond adequately to otherwise normally synthesized and secreted insulin. In some cases, the beta cells will also produce less insulin than required to maintain glucose homeostasis. Type II diabetes tends to affect individuals at older ages, and it is commonly associated with obesity.

The only "conventional" treatment for type I diabetes is insulin administration. This is a life-saving procedure, but one that unfortunately fails to replicate the exquisite native regulation that islets exert over blood sugar levels. Years of exogenous insulin use cannot prevent the occurrence of complications that are generally based on a compromised integrity of the vasculature, including renal failure, amputations, and blindness.[693–704]

Given the fact that only the islet can provide the glucose regulation required for long-term avoidance of complications, replacement beta cell therapies would be indicated not only for type I diabetes, but also for insulin-dependent type II diabetes and other conditions such as cystic fibrosis, hemochromatosis, liver cirrhosis, or iatrogenic diabetes after pancreatectomy.[705] While whole pancreas transplantation is usually effective at reversing the symptoms of diabetes, it is rarely indicated as a treatment for the disease unless the patient is simultaneously receiving another organ (typically a kidney) or is already in an immunosuppressive regime.[298] It is also considered major surgery and has a relatively high risk of complications.[706–711] Islet transplantation, in contrast, is a much safer and easier procedure that offers the possibility of preconditioning the "organ" prior to transplantation.[8,9,458,712–714]

In short, this approach is based on the enzymatic digestion of a pancreas (from a deceased organ donor or from a living related one[273–275,715,716]) using a semiautomated method that makes use of mechanical agitation to separate the islets from the exocrine and ductal components of the pancreas (Fig. 63).[717] A subsequent gradient centrifugation enriches for fractions with a high proportion of islets, which are subsequently cultured and infused through the portal vein of the patient using minimally invasive interventional radiology methods. The islets lodge in the vessels of the liver, where they get revascularized within 2–3 weeks[8,9,705,713,718] (Fig. 64).

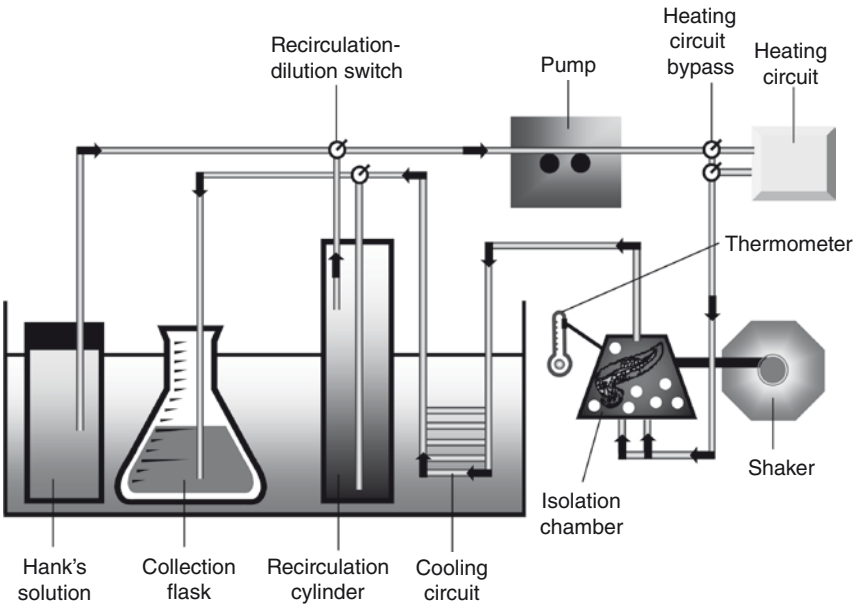

Fig. 63 Original semi-automated method for the isolation of islets, as described by Ricordi et al.[717]. See main text for details

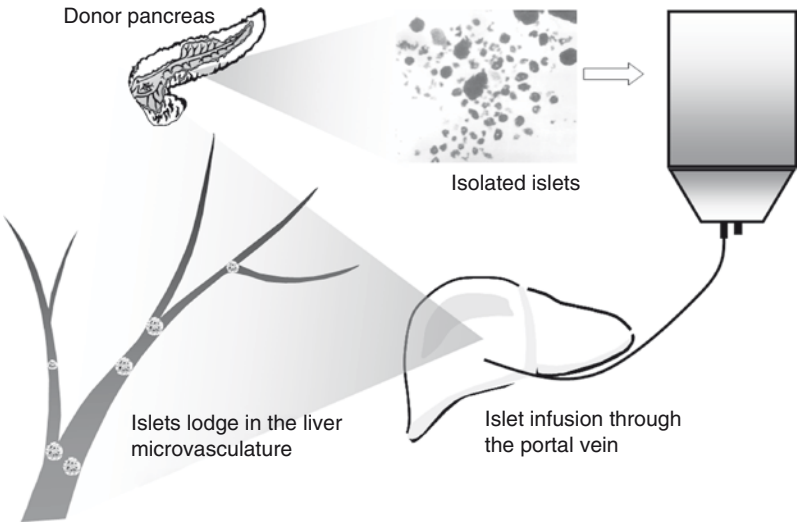

Fig. 64 Islet transplantation. Islets are isolated from a donor pancreas. Isolated preparations are typically cultured to allow islets to recover from the procedure, and then they are infused through the portal vein of recipients. This is an outpatient procedure, and requires the expertise of an interventional radiologist. Islets lodge in the microvasculature of the liver, and get revascularized within weeks

In the case of allogenic islet transplantation (i.e., islets obtained from deceased donors), immunosuppression is necessary to prevent rejection. Before 2000, islet transplantation was successful only for a limited window of time, due to the deleterious effects of immunosuppressive steroids on islet cells. The development of a novel, glucocorticoid-free regime for islet transplantation[10] enhanced very substantially the long-term viability and function of transplanted islets, with a great majority of patients reporting a significant improvement in their quality of life.[12] Total insulin independence and full metabolic control is typically achieved after a critical mass of islets has been transplanted, which may require more than one donor.[10,719,720]

3 Limitations of Islet Transplantation: Engraftment and Long-Term Function

A typical adult pancreas contains approximately one million islets, which represent around 1–2% of the total mass of the organ. It has been estimated that only 50% will survive the harsh process of isolation, with up to 60–80% of the remaining mass perishing in the immediate posttransplantation period due to inflammatory processes not yet fully understood.[721] For instance, it has been shown that islets express tissue factor,[722,723] which may contribute to early islet loss by stimulating coagulation upon their contact with the blood.[712] Considering the many insults that may invariably result in islet cell death from the time of the pancreas procurement to the actual infusion, the fact that only 10% of the transplanted patients are insulin-free 5 years after the procedure is much less surprising than the observation that up to 80% are insulin-free after 1 year. While we define alternative sources of islets that are either plentiful (xenotransplantation) or self-renewable (stem cells), there is an imperative need to "make every islet count" and to minimize their destruction upon implantation. The field of islet cytoprotection is a fertile one, with a large number of chemical,[419–422,724–728] gene-based,[729–733] and protein transduction[394,407,409,412,414,417,435] strategies proven successful in many experimental models. However, it is still necessary to gather much more information about the basic mechanisms that drive beta cell destruction upon implantation. In this context, another limitation of islet transplantation has been the difficulty to explore in real time the causes of their demise once implanted. An important breakthrough was reported just recently by Speier et al.,[489] who transplanted islets in the anterior chamber of the eye of recipient mice. This location was supportive of islet engraftment and function, as evidenced by the reversal of diabetes. More importantly, this system allows investigators to follow in real time events such as vascularization, inflammation, and rejection, which can be studied in living animals by microscopic examination of the eyes where the islets are transplanted. Interventions designed to interfere with physiological responses leading to islet destruction can now be implemented by means of eye drops and monitored as they exert their effects.

4 Limitations of Islet Transplantation: Immunosuppression and Tolerance

4.1 General Considerations About Islet Rejection

As much as islet transplantation improves upon exogenous insulin administration as a way to regulate glucose homeostasis, unless both the allo-rejection and the autoimmune processes are arrested, it will not be considered a cure. The paradox about immunosuppressants is that, while they have made islet allotransplantation possible, they are also diabetogenic, impairing beta cell survival, function, and replication. Thus, the ultimate goal of any cell therapy for type I diabetes is that the replaced cells are accepted as "self" by the recipient, and that the immune system of the patient is reeducated to prevent recurrence of autoimmunity.

When T cells contact either a self-antigen or a harmless exogenous antigen, the absence of co-stimulatory signals or the presence of co-inhibitory ones will prevent the unleashing of the immune response. At this point, the T cell may get inactivated or even apoptose. As a growing body of evidence suggests that tolerance or rejection are determined by the balance between positive and negative co-signaling, the receptors involved are potentially useful targets to minimize allo-rejection[734] (Fig. 65). As for the mechanisms responsible for the recurrence of autoimmunity, it is known that in the nonobese diabetic mice, a model of type I diabetes, beta cell destruction is mediated by autoreactive T cells.[735,736] The two major costimulatory pathways involved in this rogue T-cell response are CD28–B7 and CD40 ligand–CD40.[714,737–742] In fact, the use of antibodies that interfere with these pathways has been proven effective at preventing the onset of diabetes in this animal model.[742,743] There is a balance between normal immune regulation and autoimmunity that appears to be mediated by regulatory T cells (Tregs), a subset of T cells characterized by the expression of the master transcriptional regulator Foxp3 and identifiable by the surface expression of CD4 and CD25.[744] These cells confer protection against autoimmune diabetes.[739] Unfortunately, the experimental blockade of the co-stimulatory response that triggers allo-rejection has also a negative effect on Treg function. In this context, the identification of co-inhibitory molecules might offer a safer and more effective therapeutic alternative (reviewed by Truong et al.[734]).

More comprehensive strategies go one step beyond the mere tipping of the balance between autoimmunity and regular immune function. Bone marrow transplantation, for instance, has been shown to cure diabetes in animals, but for the new cells to home, the resident stem cells must be wiped out by lethal or sublethal irradiation.[745] This is obviously not a therapeutic option for diabetes as it is for other fulminant illnesses, as diabetes is a chronic and (to some extent) manageable disease. Hence the search for gentler ways of making room for "healthy" bone marrow cells without (1) completely ablating the recipient's hematopoietic compartment; and (2) inducing graft-versus-host disease.[746–754] The latest approach (already discussed in the chapter "Adult stem cells & pancreatic

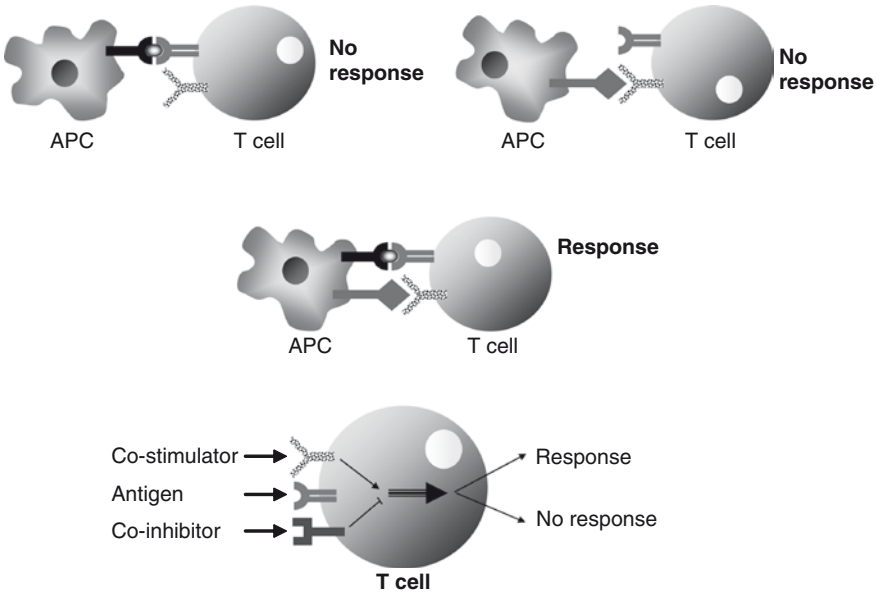

Fig. 65 Antigens are detected by T cells by means of their interaction with antigen-presenting cells (APCs). At the molecular level, such interaction occurs between a T-cell receptor (TCR) and a major histocompatibility complex (MHC). Without any other signal, optimal activation does not occur, and may even induce tolerance or anergy. However, secondary interactions (co-stimulatory signals) cannot activate the T cell by themselves. A combination between the primary TCR-MHC and a co-stimulatory signal are necessary for optimal response. Below is represented the balance between co-stimulation and co-inhibition. The net result of these opposing forces will ultimately determine the response of the T cell (Adapted from Chen[738])

differentiation") is that of using hematopoietic stem cells extracted from the same patient, who is subsequently treated with a strong immunosuppressive regime prior to stem cell reinfusion.[644–650] The safety and long-term effectiveness of these treatments are still in question, and more studies will be necessary before they are translated into widely used clinical practice.

Finally, another area of research aims at encapsulating the transplanted islets with a physical barrier that will protect them from both allo- and auto-rejection (including immune-effector cells, complement system, and immunoglobulins), while allowing the free transit of insulin, sugar, nutrients, and oxygen. Considering the sophistication of the cellular and molecular strategies described above to interfere with the immune response, this could be considered a "brute force" approach. If successful, however, it could even open the door to xenotransplantation of nonhuman islets, which could potentially be a valid alternative to that of using stem cells for directed differentiation into pancreatic endocrine cells. The different strategies presented thus far (intravascular implant, macroencapsulation, and microencapsulation) are schematized in Fig. 66. Several materials have already been tested with variable degrees of success. The workhorse of these studies is alginate, derived from kelp, a member of the brown algae (Phaeophyceae) group.[755–761]

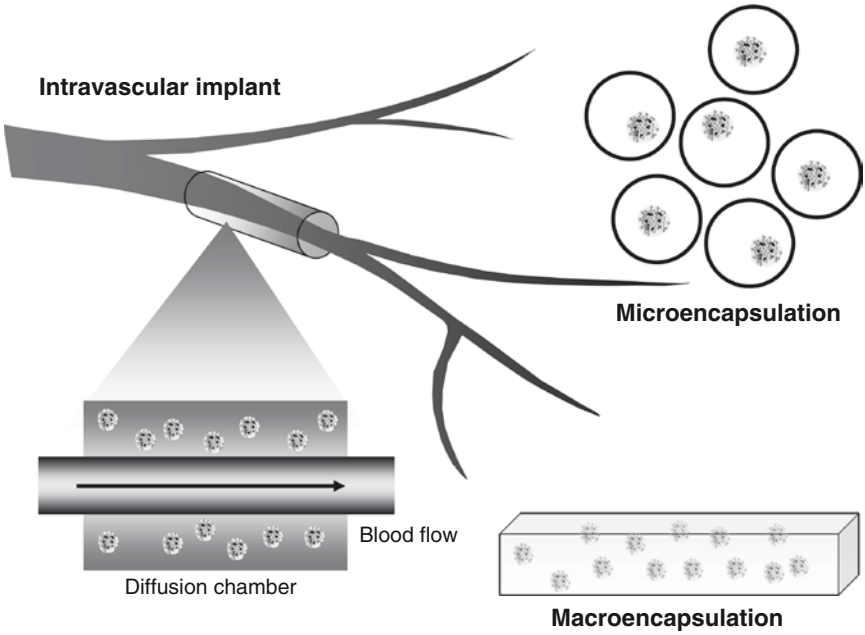

Fig. 66 General encapsulation strategies. The intravascular implant (also known as biohybrid artificial pancreas) is a perfusion chamber directly connected to the blood vessels through an arteriovenous shunt. Islets are within the diffusion area of influence of the blood vessel, while protected by a membrane. However, implantation is not a straightforward procedure, and there is a significant risk of clotting.[764–769] More conventional strategies involve the protection of islets by means of immunobarriers (macroencapsulation and microencapsulation) not in direct contact with existing vasculature (Adapted from Beck et al.[763])

This compound is already in clinical trials,[762] and offers the advantage that it can be conjugated to materials such as polyethylene glycol and poly-lysine to reduce plasma adsorption and decrease the formation of fibrotic tissue around the capsule (recently reviewed by Beck et al[763]).

Polysulphone is another promising material for islet encapsulation, although it needs to be chemically modified in order to minimize insulin adsorption.[770] All of these mechanisms, however, suffer from two important limitations, namely: (1) their inability to prevent the circulation of cytokines across the barrier (which would be especially problematic in xenotransplantation settings) and (2) nutrient deprivation and hypoxia. The latter has already been acknowledged as one of the main determinants of islet cell death upon transplantation,[191,446–452] and islet cell encapsulation can only exacerbate this problem. Conventional approaches induce large void volumes where the islet (~150 μm) is engulfed in a much larger structure (400–800 μm). Considering the ensuing delay in nutrient and oxygen delivery, as well as the unusually high metabolic demands of islets,[771] it is hardly surprising that encapsulated islets tend to exhibit starvation-induced apoptosis and/or impaired function. In an effort to retain the critical advantages

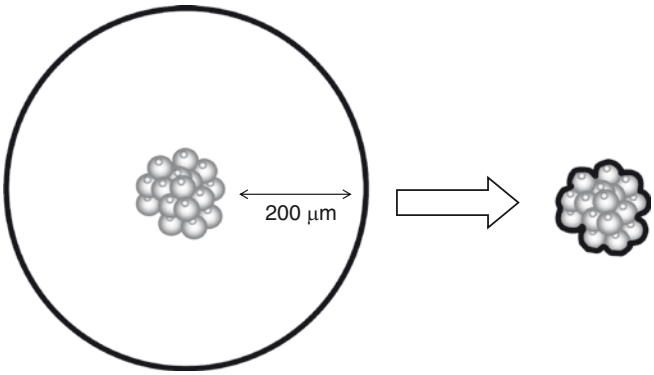

Fig. 67 A microencapsulated islet (*left*) compared with an islet coated with a nanoscale conformal layer (Reproduced by courtesy of Dr. C. Stabler, U. of Miami)

of encapsulation while maximizing transport of oxygen and nutrients across the barrier, a promising area of research is that of designing thin, conformal polymeric layers that can be "coated" directly onto the islet surface. Doing so may reduce the diffusion distance between the islet and the capsule by up to 1,000-fold (C. Stabler, personal communication and[772,773]), thus improving the chances for islet survival in the immediate posttransplantation period (Fig. 67).

In a paradoxical reversal of the "miniaturization" trend, the recent development of a subcutaneously implantable biocompatible device[774] may also help address many of the problems of immune rejection. In preliminary studies in rats, a cylindrical stainless-steel mesh was implanted and allowed to be vascularized for 40 days. Islets were then placed in the device, where they supported reversal of diabetes in a syngeneic setting. Although a device of this nature is not designed to prevent the access of the immune effectors (cells, complement, and immunoglobulins), a major advantage over noncontained transplantation sites is that immunosuppression could be potentially delivered in a local fashion. Systemic medication to prevent rejection is based on the administration of very large doses of immunosuppressants, due to the need of reaching biologically active concentrations at the site of the graft. The well-known side effects of this treatment include higher incidence of infection and cancer.[775] With this system, much smaller doses could be delivered locally in the site of the graft, allowing for immunoprotection with no or little systemic effects. In fact, this approach might allow for the delivery of potentially powerful drugs not approved for systemic administration.

4.2 Immunology of Stem Cells

In the previous section, we described general approaches to tackle allo- and autorejection. However, we cannot assume that all stem cell-based therapies will have

the same requirements in terms of making them accepted by the recipient, inasmuch as different stem cell types may have different immunological properties.

Embryonic stem cells, for instance, have been recently claimed to be "immunoprivileged,"[776] following a series of in vitro and in vivo experiments where both human embryonic stem (huES) cells and their differentiated progeny failed to elicit substantive immune responses. The authors hypothesized that this effect was due to the lack of major histocompatibility class II molecules in the surface of the cell, although it is well known that rejection will invariably occur if class I molecules are present. From this perspective, success at evading the immune response is more likely to come from stem cell banking, which would ensure an appropriate representation of the most widely spread HLA haplotypes in the population. Even more interesting is the recent progress at generating HLA homozygous huES cell lines[777,778] by means of parthenogenesis[779–784] (Fig. 68), of which one carries the most commonly found (and shared by different racial groups) HLA haplotype in the US population. This strategy is very promising, but HLA homology would not be sufficient to prevent secondary rejection mechanisms. The only way we could ensure that huES cells do not trigger an immune response in the recipient is by

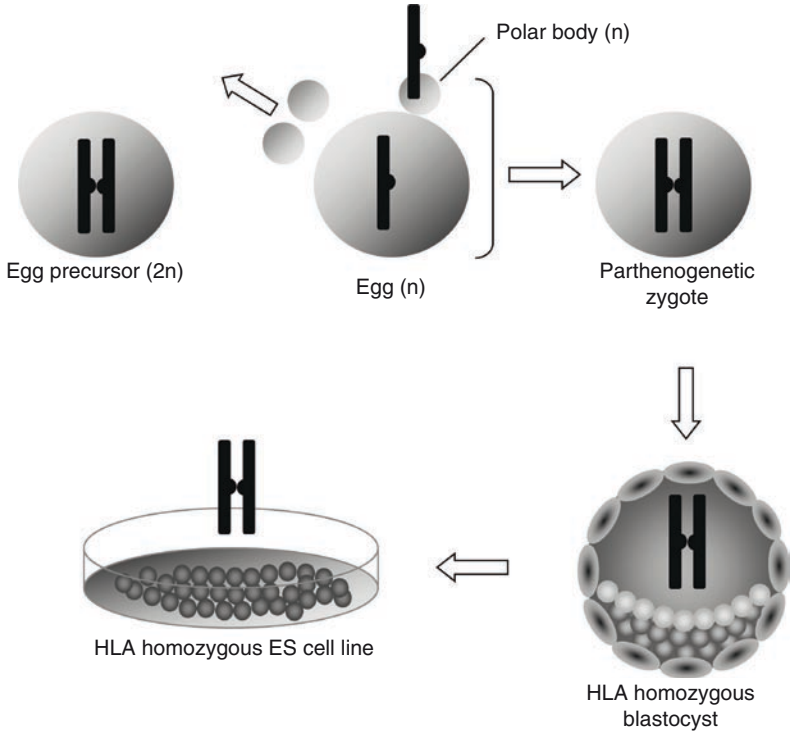

Fig. 68 ES cells homozygous for the most widely represented HLA haplotypes can be generated by means of parthenogenesis. This process is the result of the fusion of a haploid egg with an haploid polar body generated during the maturation of the former

tailoring them by means of somatic cell nuclear transfer or induced pluripotent stem (iPS) cell generation (see the chapter "Embryonic Stem Cells and Pancreatic Differentiation"). Unfortunately, the former technology is not available yet for humans, and it would probably be impractical from a logistic point of view. As for the latter, there are still enormous safety concerns related to the use of retroviruses to reprogram patient-derived somatic cells.

Adult stem cells, on the contrary, could be easily derived from the patient and expanded in vitro prior to re-differentiation and implantation. As would be the case with patient-derived huES/iPS cells, this approach would theoretically circumvent the immune rejection of the tissue. In both cases, however, the problem of autoimmunity would still require additional interventions. The use of mesenchymal stem cells (MSCs) in an allotransplantation setting has also been proposed based on the observation that these cells have immunomodulatory properties.[599–602] The hypothesis was that these cells might be able to engraft even in allogeneic recipients, down-regulating the immune response. However, this theory has been proven incorrect in a number of in vivo experiments,[785] and now the prevailing view is that the effects of MSCs in mismatched settings might be due to transient trophic/secretory effects.[604,605]

5 Conclusions

Some argue that a solution to the autoimmunity component of type I diabetes might be just enough to cure the disease. After all, it has been observed that insulin-positive cells persist even decades after the onset of the disease, suggesting that regeneration mechanisms are at play throughout the course of the disease, but perhaps kept at bay by the autoimmune response.[232,786,787] It is likely, however, that regeneration will not be possible unless a critical mass of beta cells remains in the pancreas.[289,290] This would explain why the "reeducation" of the immune system proposed by Voltarelli et al.[650] was not hypothesized to work in patients with long-standing diabetes. Therefore, a boost of exogenous cells will be required even after the autoimmunity problem has been solved. The jury is still out regarding whether this second component of the cure (replacement) will come from embryonic stem cells, adult stem cells, transdifferentiation, or regeneration. It might also be the case that success is the result of a combination of several approaches. A theoretical clinical intervention, for instance, may involve the differentiation of huES cells into both pancreatic endocrine tissue *and* hematopoietic cells with the ability to induce chimerism – and tolerance – in any given patient. Provided that concerns about teratomas are conveniently addressed, the stem cells could thus be used both as a replacement and a tolerance inducing tool (Fig. 69). The same approach would be applicable to a situation where hematopoietic cells (bone marrow or cord blood) are differentiated into beta cells and co-transplanted with undifferentiated aliquots for tolerance induction. In a different setting, we cannot entirely rule out the possibility that some forms of transdifferentiation may result in the generation of insulin-producing cells that,

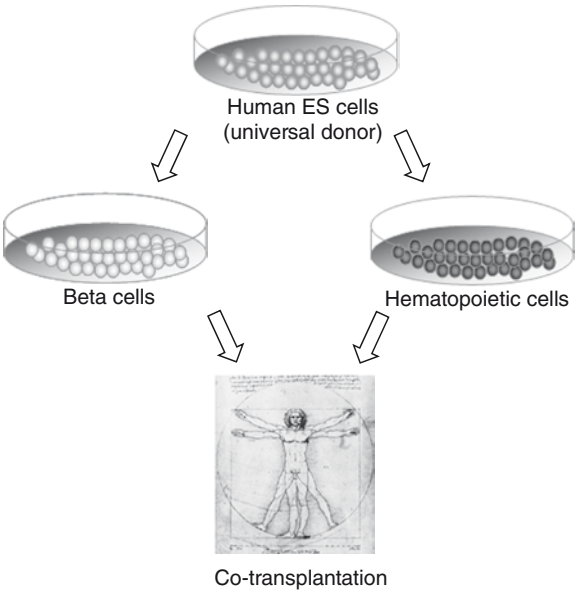

Fig. 69 Theoretical co-transplantation of hematopoietic and pancreatic beta cells derived from a single universal huES cell donor

not being true beta cells, may actually be in a better position to evade the immune response. Finally, the use of MSCs is likely to help in virtually any therapeutic intervention by virtue of their immunomodulatory and "feeder" effects.[599–602,604,605] The bottom line is that the several strategies presented in previous chapters, far from being exclusive of each other, are expected to work in a synergistic fashion; and that the knowledge gathered from each field will certainly cross-fertilize the others. The themes herein discussed are so new and rapidly evolving that this book could not have been written a decade ago. We can only hope that, 10 years from now, all of these novel concepts will have finally settled and the field will have advanced much closer to a definitive cure for type I diabetes.

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