Morphogenetic and Clinical Perspectives on the Neogenesis of Pancreatic Duct Ligation-Induced Islet Cells – a Review

Abstract
This review focuses on recent progress in understanding morphogenetic findings on the neogenesis of islet beta cells following Pancreatic Duct Ligation (PDL) in animal models. These results may give hope for modifications in the treatment of diabetes in general and transplantation in particular. On the basis of this review, translational studies should be developed to allow information on beta-cell neogenesis to be integrated into a potential therapy for Diabetes Mellitus (DM) in humans. Further studies on the development of animal models that will produce PDL islets for transplantation are urgently needed (Adv Clin Exp Med 2011, 20, 1, 5–14).

Key words: pancreatic duct ligation, beta cells, neogenesis, islet, gene expression, diabetes mellitus.

Streszczenie

Słowa kluczowe: podwiązanie przewodu trzustkowego, komórki beta, neogeneza, wyspa, ekspresja genu, cukrzyca.
progenitor cells observed in or near ducts. Convincing evidence of normal fetal pancreatic development from rodent studies [4], as well as from a few human studies [5], has shown the neogenesis of islet cells deriving from duct or acinar cells.

Pancreatic Duct Ligation (PDL) in rats induces the transdifferentiation of acinar cells to insulin-producing endocrine cells in vivo, well demonstrated by the onset of transitional phenotypes with co-expression of both exocrine and endocrine cell markers [6]. In the PDL model of laboratory rats, the beta-cell mass increased two-fold in a short period of time. Proliferation of pre-existing beta cells does not account for this observable rapid beta-cell mass increase. Bouwens and Rooman [7] have described increased beta cells generally as a result of the physiological condition of high insulin demand, but the absence of transient hyperglycemia in PDL rat models rules out a physiological reaction in this model [8]. Duct cells after PDL show no evidence of beta-cell neogenesis from the pre-existing ductal epithelial cells [4, 8]. Based on this evidence, the PDL model exhibits the production of new beta cells rather than their replacement [9]. However, transdifferentiation of acinar cells into beta cells in vitro has been documented [10]; this new evidence warrants an elucidation of the origin of beta cells from PDL.

Investigations of the origin of newly formed beta cells following PDL have to be supported by an evaluation of transcription factors involved in endocrine development. Demeterco et al. [11] suggest fundamental research in beta-cell replacement therapy for diabetes mellitus in men in order to elucidate the mechanism by which endocrine differentiation is promoted; this could be a successful therapeutic approach to induce endogenous beta-cell neogenesis.

The objective of this review is to focus on recent progress in understanding morphogenetic findings on the neogenesis of islet beta cells following PDL in animal models. These results may give hope for modifications in the treatment of diabetes in general and transplantation in particular.

The Morphology of the Pancreas

The human pancreas is a lobulated gland, similar in structure to the salivary glands, though softer and less compactly arranged than those organs (Figure 1). The human pancreas measures from 12.5 cm to 25 cm in length and weighs between 60 and 150 grams. The pancreas consists of the head, neck, tail and uncinate process. There is an accessory pancreatic duct (the duct of Santorini), which

is formed by the fusion of small ducts from the lower and left portion of the head of the pancreas; it communicates with the main duct, and drains the lower part of the head of the pancreas, entering the duodenum at the minor duodenal papilla.

In rodents the pancreas is diffuse and the shape cannot be well specified. It weighs between 550 mg (at a body weight of 100 g) and 1 g (300 g body weight). In rats the pancreas is found in the craniodorsal part of the abdominal cavity and can be divided into two parts. The first part is comprised of the body and right lobe; this is embedded in the mesoduodenum and the beginning of the mesojejenum. The second part, a branched flattened left lobe, is partially fused to the ascending colon and blankets the superior mesenteric-portal vein. It then runs along the dorsal aspect of the stomach, embedded in the dorsal part of the greater omentum, and along the lineal artery toward the intestinal surface of the spleen.

Induction of Endocrine Development

The pancreas develops from the fusion of two distinct buds, the ventral and dorsal pancreatic buds. These two buds emerge as evaginations of the embryonic gut endoderm. The main duct elongates; secondary ducts form, which branch off, elongate, and from which further ducts form and branch off. They develop acini at their terminal ends, forming the centroacinar duct; and from the walls of the smaller branches, cell clumps bud – the presumptive islets. The islets increase in size through islet cell precursor proliferation and through the merging of cell clumps that are close together [12]. Serial reciprocal inductions of the endoderm and adjacent mesoderm determine the cell fate in both the ventral and dorsal bud tissue types [13]. One of the first steps required for pancreatic development is an inductive interaction between the endoderm and mesoderm that directs a cluster of endodermal cells close to the midforegut junction toward a pancreatic fate [13]. However, the formation and differentiation of all pancreatic cell types (organogenesis) continue in postnatal life, until three months of age.

The Microscopic Anatomy of the Islets of Langerhans

The islets of Langerhans are clusters of cells dispersed within the exocrine pancreas. The pancreas is composed of two structurally distinct components in intimate association with each other. The
main mass (the exocrine pancreas) is a lobulated, branched acinar gland found throughout the organ. Within the exocrine pancreas, the islets of Langerhans constitute the endocrine pancreas responsible for maintaining glucose homeostasis. The islets of Langerhans are small spheroid clusters of cells scattered throughout the organ in cell groupings with a combined mass of approximately 1–1.5 g, but their distribution is not uniform in the pancreas of all mammals. A recent islet study clearly distinguishes human islet architecture from that of rodents [14].

Immunohistochemical studies have demonstrated that the islets of Langerhans are composed of four cell types designated alpha (or A or A2), beta (or B), delta (or D or A1), and PP (or D1 or F) cells, producing glucagon, insulin, somatostatin, and pancreatic polypeptide respectively. Although islet composition differs in the dorsal and ventral parts of the pancreas, diverging reports on the cell-type distribution in different regions of the pancreas are documented. Cabrera et al. [15] reported a relatively even distribution in the proportion of endocrine cells; while Brissova et al. [16] observed that more beta cells and alpha cells are found in the body, neck and tail; and PP-cells are found in the head [17]. In rodents, beta cells constitute approximately 60–80% of the islet cells and generally form the core of the islet. The surrounding layer of endocrine cells include alpha cells (15–20%), delta cells (< 10%) and PP cells (< 1%) [15, 18]. Adult human islets, on the other hand, are comprised of about 50% beta cells, 40% alpha cells, 10% delta cells and < 1% PP cells [15, 16, 19].

A fifth peptide hormone, namely ghrelin, produced by epsilon cells, has been identified in the human islet. Ghrelin-producing epsilon cells are thought to regulate food intake and energy balance, and to stimulate increased secretion of the growth hormone. However, the location of these cells within the endocrine compartment remains uncertain [20]. A number of studies have been carried out to trace the origin of certain hormone-producing cells within the islet; and common precursor cells that co-express the various islet hormones are thought to give rise to pancreatic endocrine cells.
Beta-Cell Mass and the Pathogenesis of Diabetes Mellitus

A disturbance in the islet microanatomy as well as any disruption in the beta-cell mass balance may impair pancreatic islet function, leading to the pathogenesis of diabetes. Beta-cell mass is the total number of islet cells, including newly formed islet cells that arise from pre-existing ductal cells or other precursor cells (neogenesis), and islet cells formed by the replication (proliferation) and programmed cell death (apoptosis) of existing islet cells [21].

Beta-Cell Neogenesis and Apoptosis

In the pancreas, there is significant beta-cell neogenesis and replication during fetal life; this continues at a reduced rate in the neonatal stage of life. An increase in the apoptotic index of postnatal beta cells has been reported in humans [22], pigs [23] and rodents. Newly formed beta cells take about 30–40 days to reach their mature and fully functional potential [24].

Beta-Cell Pathogenesis and the Etiology of Diabetes Mellitus

The significance of the beta-cell mass in the pathophysiology of the pancreas has been the focus of extensive research [25]. The mass of beta cells is an important tool for the regulatory mechanisms where changes can result in a partial or complete deficiency in insulin secretion. However, changes in beta-cell function and insulin synthesis are a direct consequence of the balance between the degeneration and proliferation of these cells, which are critical for the pathophysiology of the pancreas. Beta-cell mass loss in the pancreas results from an autoimmune disorder induced by T-cells that destroy beta cells and impair insulin production, thereby suppressing the signaling responses that trigger cellular uptake of glucose and glucose metabolism in cells. This situation leads to type 1 diabetes, also known as Insulin Dependent Diabetes Mellitus (IDDM).

The Pancreatic Duct Ligation Procedure

Ligation of the pancreatic duct (PDL) has been an experimental procedure for many years; it was initially aimed at treating a disease – i.e., pancreatitis – but a common opinion emerged that PDL induced a considerable level of pancreatic atrophy. This opinion corroborated a study by a prominent 19th-century researcher, Frederick Banting, who made the same observation while trying to isolate a pancreatic secretion using the duct ligation procedure. Despite the fact that many authors of that time observed atrophy of the pancreas following duct ligation, there was serious controversy as to the reason for or the origin of a remarkable increase in mass of the surviving islet [4, 8].

Subsequent studies on the pancreas have revealed that transcription factors may be involved in the proliferation of the islet cells after Pancreatic Duct Ligation [4]. Page et al. [26] reported a similarity between the formation of normal fetal pancreas tissue and that of newly-formed beta cells following PDL; this is thought to be due to the plausible stem cell capacity of the adult pancreas. Also, atrophy of the pancreatic cells following duct ligation was noted as a direct consequence of acinar cell death, which triggered islet proliferation and neogenesis from duct-like epithelial cells [27].

A recent study on the cell lineage in a duct-ligated pancreas revealed that an increase in beta-cell mass does not have any contribution from pre-existing ductal epithelial cells. Insulin-producing beta cells develop from pancreatic exocrine duct cells only during embryogenesis, not during postnatal life [4]. This conflicting evidence warrants an assessment of other lineage-selective transcription factors for endocrine development in PDL tissues.

Transcription Factors Involved in Endocrine Development

Transcription factors are involved both in determining early cellular development and in the differentiation of progenitor cells, and later in maintaining the pancreatic cell phenotype. Several of these factors have been implicated in pancreas development [28], during which they are recognized to be critical regulators of gene expression [29]. These include transcription factors of the
homeodomain family (Pdx1, Hb9, Pbx1, HNF1β, HNF6, Pax4, Pax6, Nkx2.2, Nkx6.1, Isl1, HNF1α, HNF4α, and Brn4) [4], the basic helix-loop-helix (bHLH) family (Ngn3, Beta2/NeuroD, Hes1, p48) and the forkhead/winged-family (Foxa2/ HNF3β, Foxa1/HNF3γ).

A number of homeodomain factors, such as Pdx1, Pax4, Pax6, Nkx2.2 and Nkx6.1, and an additional bHLH transcription factor, such as NeuroD, are necessary for the differentiation and maintenance of mature and differentiated cells. Although bHLH transcription factor Ngn3 is both necessary and sufficient for driving undifferentiated progenitor cells to an endocrine fate, Ngn3 expression ceases before islet cells are fully differentiated. However, Pdx1 expression in mature differentiated cells is specific to insulin-secreting cells.

**Pancreatic Duodenal Homeobox Gene-1**

The pancreatic duodenal homeobox gene-1 (Pdx1) is a homeodomain transcription factor with a key regulatory function both in pancreas development and in the differentiation of progenitor cells to become adult beta cells. It is also called the insulin promoter factor-1 (Ipf1), islet duodenum homeobox-1 (IDX-1), somatostatin transactivating factor-1 (STF-1) or glucose sensitive factor (GSF). Pdx1 belongs to a “ParaHox” gene cluster expressed in the lateral endoderm domain at somites 7 to 9 in the vertebrate axis that contributes to the development of the pancreas. It is a pancreas specific homeoprotein. Because of this, it has been found to be a beta- and gamma-cell-specific regulatory factor for the expression of the insulin and somatostatin genes. The pancreatic duodenal homeobox gene-1 also regulates the expression of other islet-specific genes like Glut-2, islet amyloid polypeptide and glucokinase. In the developing pancreas, Pdx1 is first detected at e8.5 in the ventral gut endoderm in cells that later form the ventral pancreatic bud. At e9.5 Pdx1 is expressed in both the ventral and dorsal pancreatic buds. From e11.5 to e13.5, Pdx1 expression is seen throughout the developing pancreatic epithelium. But when the exocrine pancreas begins to form (e14-e15), the islets mature into hormone-producing cells and Pdx1 expression becomes restricted to the endocrine compartment (e16.5-e18.5) and dispersed endocrine cells of the duodenal wall. Later, in the adult pancreas, Pdx1 acts as a master regulator of insulin gene expression. Subpopulations of somatostatin-producing and pancreatic polypeptide-producing cells also express Pdx1, but Pdx1 expression is seen only in a few glucagon-producing cells.

Targeted disruption of the Pdx1 gene and homozygous Ipf1 mutations result in agenesis of the pancreas [30]. Null mutation Pdx1 mice are viable, but pancreatic development is arrested at a very early stage and the animals die within days after birth. In early pancreas development, a few insulin-expressing cells are detected in Pdx1 null mice, suggesting that a population of insulin-positive, Pdx1-negative cells arises separately from the mature Pdx1-expressing beta cells of the developed pancreas. However, as Stoffers et al. pointed out, "the expression of Pdx1 in the gut endoderm is essential for the pancreatic program to continue and all pancreatic tissues subsequently differentiate from Pdx1-positive precursors" found in this germ tissue [31]. It has been noted that a child that was homozygous for an inactivating mutation in Pdx1 was born without a pancreas, which emphasizes the significance of the Pdx1 transcription factor in the development of the mouse as well as the human pancreas [30, 32].

The rat, mouse and human Pdx1 genes are localized respectively on chromosomes 12, 5 and 13. The coding region of the Pdx1 gene has two exons; the first encodes for the NH2-terminal region of the gene, and the second encodes for the homeodomain and COOH-terminal domain. The activation of Pdx1 is contained within the NH2-terminal region, however its homeodomain is involved in DNA binding; both the NH2-terminal region and the homeodomain are involved in protein-protein interactions [33].

**Neurogenin3**

Neurogenin3 (Ngn3) is a proendocrine factor belonging to the basic helix-loop-helix family (bHLH). Neurogenin3 is considered a marker of islet precursor cells, and has been shown to be essential for the development of all endocrine cell lineages of the pancreas [34]. Pancreatic endocrine cells develop from the precursor cells expressing Pdx1 and the bHLH-family transcription factor Ngn3. In mice, Ngn3 expression is first observed at e9.5, and the number of Ngn3-expressing cells increases until e15.5 – exactly when islet cell differentiation is at its peak – and diminishes greatly thereafter with little or no detection of Ngn3 in the adult pancreas. Co-expression of Ngn3 with islet hormones (insulin, glucagon, somatostatin, pancreatic polypeptide) cannot be detected at this adult stage, although all four-islet cell types develop from Ngn3-expressing cells that are found adjacent to ductal cells.

In Ngn3-deficient mice, all islet cell types are absent in every stage of development, but exocrine
tissues and ductal tissues develop normally [34]. It is evident that Ngn3 expression is a functional marker of an islet cell precursor population in the developing pancreas. Since Ngn3 is both sufficient and necessary to initiate the differentiation of islet cells during development, it may be concluded that the endocrine fate of cells is strictly controlled by the activity of specific transcription factors that regulate the cis-acting elements within the promoter region of the Ngn3 gene [34]. Hepatocyte nuclear factor 6 (HNF6), HNF3β / FOXA2 and HNF1α bind to the Ngn3 promoter, acting as its activators; while Ngn3 acts as an upstream regulator for the transcription factors Pax6, Pax4, Beta2/NeuroD, Nkx6.1, Nkx2.2 and Isl1, and simultaneously represses its own promoter [35].

Mice homozygous for a null HNF1α gene have smaller islets and secrete little insulin. Mice embryos lacking HNF6 expression present a significant reduction in endocrine differentiation, with critically reduced levels of Ngn3 expression [36]. A lack of foregut formation is observed in FOXA2/HNF3β null mice as well. Expression of the HNF factors is therefore considered to be involved in a cooperative mechanism in the cell-type-restricted activation of Ngn3 expression, but is not sufficient for Ngn3 expression [37].

Neurogenin3 promoters have binding sites for HES1, which is a transcriptional repressor of bHLH genes; it is therefore believed to inhibit Ngn3 expression through the Notch signaling pathway. Over-expression of Ngn3 and an absence of HES1 show a similar pancreatic phenotype. Laternal inhibition of Ngn3 expression via the Notch-pathway is essential for the expansion of epithelial cells before differentiation. However, premature over-expression of Ngn3 blocks the Notch-pathway, which leads to a poorly branched ductal epithelium, blockage of exocrine development and acceleration of islet cell differentiation. The evidence therefore suggests that the Notch signalling pathway contributes to regulating the balance between progenitor cell differentiation and proliferation during pancreas development [38, 39].

Human Neurogenic Helix-Loop-Helix Protein Gene

The human neurogenic helix-loop-helix protein gene (NeuroD/Beta2), which is a bHLH factor and an important activator of insulin gene transcription, is also required in order to generate a normal mass of pancreatic beta cells and alpha cells. The activation of NeuroD expression in cells that co-express Ngn3 and Pdx1 is a very early step in pancreatic endocrine differentiation. NeuroD is activated by Ngn3, although these two factors are expressed in different cells. It has been established that NeuroD-positive cells arise from cells that express Ngn3. NeuroD expression is detected at e9.5, co-localizing with early glucagon-expressing cells [40].

Null mice for the NeuroD gene die three to five days after birth due to severe hyperglycemia; the islets' beta cell count is reduced by 75%, and alpha and delta cells are also reduced and irregular in shape. The Notch pathway antagonizes NeuroD and bHLH proteins Ngn3. As Jensen et al. wrote: "Activation of Notch receptors leads to activation of Hairy and Enhancer-of-split (HES) type proteins, which in turn act as transcriptional repressors of bHLH genes. Mice lacking Notch ligand Delta like-1 (Dll1) or the DNA-binding protein RBP-jk (activator of HES1), have accelerated differentiation of pancreatic endocrine cells and subsequently severe pancreatic hypoplasia due to premature differentiation of pancreatic stem cells into endocrine cells" [40].

Paired Box Gene 6

Paired box gene 6 (Pax6) belongs to the Pax multigene family of transcription factors that contribute to the regulation of pancreatic endocrine cell differentiation [41]. Pax6 expression is mainly detected in the eye, the central nervous system, the nose and the endocrine pancreas. Sharing a similar structure with Pax4 in their corresponding homeodomain [42], Pax6 is also expressed in both the ventral and dorsal developing pancreas; but its expression is detected as early as e9.5 in the mouse, and continues throughout the development of the pancreas until the endocrine cells are fully formed [42].

A mouse lacking the Pax6 gene does not survive after birth; the pancreas of such a mutant contains very few fully differentiated beta cells, delta cells and PP cells within a malformed islet. Alpha cells are completely absent in this pancreas, but the development of the exocrine cells tends to be normal. Mice that lack both Pax4 and Pax6 do not develop any endocrine cells; these findings suggest that both Pax4 and Pax6 are necessary for the regulation of the final steps in pancreatic endocrine cell differentiation [42].

Clinical Implications

Advances in research in recent years have shed considerable light on how transcription factors regulate endocrine pancreas development [43].
Although recent evidence has shown that newly formed beta cells following PDL do not originate from the pre-existing ductal epithelium [4], the cellular mechanisms involved in the recapitulation of these newly formed beta cells remain unknown. For these reasons, the future of a potential therapy for Diabetes Mellitus (DM) lies in understanding the morphogenetics of islet cell neogenesis, which remains the ultimate hope for modifications in the treatment of diabetes in general and transplantation in particular. The first attempt at islet transplantation was made in 1893, when sheep pancreatic extracts were transplanted into a young patient, who showed improvement for only 24 hours. After the discovery of insulin by Banting and Best in 1921, insulin remained the only treatment for diabetes until Ballinger and Lacy successfully treated a diabetic rat by transplanting islet isografts. Although many other subsequent transplants followed in 1970s and 1980s in rats and in humans, there were numerous setbacks related to the use of immunosuppressants. A breakthrough came in 2000, when Shapiro reported a 100% success rate in seven human transplants. Despite remarkable progress, the overall world success rate in treating diabetes by islet transplantation remains very low – just 10%, which is certainly due to such challenges as insufficient donor organs, the necessity of using immunosuppressants [44] and the technical/clinical bias during the transplantation process, which requires more collaboration between researchers. It is therefore relevant to explore other avenues for the self-generation of beta cells within the organ itself, which could be a way to overcome the challenges of Insulin Dependent DM.

The transplantation of whole pancreases has improved the lives of large numbers of diabetic patients in the developed world, but the burden of immunosuppressive agents still impacts on the quality of life. The transplantation of allogeneic fetal tissue is a proven alternative, [44] although it is also impacted upon by immunosuppressive agents. The transplantation of adult or fetal islets of Langerhans is a promising therapeutic option for the treatment of DM, but the low availability...
of human donor pancreases and the lack of suitable donor tissue remain a major obstacle. Duct-ligated pancreas transplantation has been shown to have the same efficacy as foetal tissue [26, 27]; and as the model involved the transplantation of syngeneic tissue, immunosuppressive agents were unnecessary. However, the lineage of endocrine cell development in the PDL model is poorly understood, and is one of the questions that this thesis attempts to answer. Pancreatic stem cells residing within the ductal epithelium have been used to generate islet-like clusters in vitro, which has partially reverted DM in animal models. Hence, understanding the processes of the cellular mechanism in the lineage of endocrine cells in duct ligation-induced neogenesis will be a valuable tool in improving beta-cell replacement in patients with diabetes, thereby alleviating the burden of Diabetes Mellitus.

In studies presented at the first annual meeting of the Islet Society [45] and at the WSU International Research Conference [46], the authors suggested that the newly formed beta cells derived from centroacinar cells; and that there is a possible cell-to-cell interaction between pre-existing mesenchymal cells and pre-existing islet cells, which might induce the “facultative stem cell” capacity of the pre-existing beta cells (Fig. 2), as suggested by Michalopoulos in the case of the liver [47]. Figure 3 presents a diagram of the time-related gene expression in a PDL pancreas, which would help identify the phenomena involved in the lineages of beta cells in the PDL model; while Fig. 4 summarizes the ongoing cellular changes related to our observation. The combination of mesenchymal cells with the pre-existing beta cells in the PDL pancreas warrants future investigation.

**Conclusions**

On the basis of this review, translational studies should be developed to allow information on beta-cell neogenesis to be integrated into a potential therapy for Diabetes Mellitus in humans. Further studies on the development of an animal model that will produce PDL islets for transplantation are urgently needed.
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