Pleiotropic Roles of PDX-1 in the Pancreas

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■ Abstract

It is well known that pancreatic and duodenal homeobox factor-1 (PDX-1) plays a pleiotropic role in the pancreas. In the developing pancreas, PDX-1 is involved in both pancreas formation and β-cell differentiation. In mature β-cells, PDX-1 transactivates insulin and other β-cell-related genes such as GLUT2 and glucokinase. Furthermore, PDX-1 plays an important role in the induction of insulin-producing cells in various non-β-cells and is thereby a possible therapeutic target for diabetes. On the other hand, under diabetic conditions, expression and/or activity of PDX-1 in β-cells is reduced, which leads to suppression of insulin biosynthesis and secretion. It is likely that PDX-1 inactivation explains, at least in part, the molecular mechanism for β-cell glucose toxicity found in diabetes.
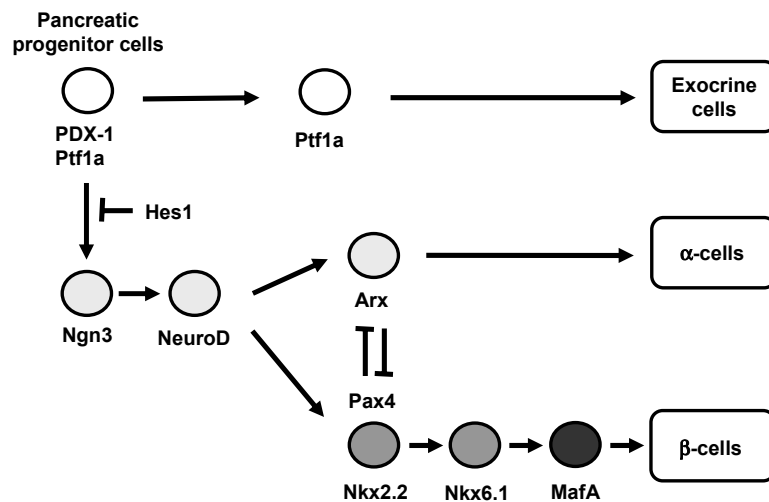
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A variety of transcription factors are involved in pancreas formation and β-cell differentiation

The adult pancreas is composed of exocrine (acini and ducts) and endocrine compartments (α-, β-, δ-, ε-, and PP-cells). Each of the four endocrine cell types synthesizes and secretes one hormone: glucagon (α-cells), insulin (β-cells), somatostatin (δ-cells), ghrelin (ε-cells) and pancreatic polypeptide (PP-cells). The embryonic pancreas initially develops by fusion of the dorsal and ventral buds of the primitive gut epithelium. These two types of buds grow and fuse to form the definitive pancreas [1-4]. The thickening of the dorsal and ventral surface of the foregut is observed from E8.5-E9.5 in the mouse. It has been shown that pancreatic transcription networks play a crucial role in early pancreas organogenesis and endocrine cell formation (Figure 1) [5, 6].

Pancreatic and duodenal homeobox factor-1 (PDX-1) (also known as IDX-1/STF-1/IPF1) [7-9], a member of the large family of homeodomain (HD)-containing proteins, is expressed in precursors of the endocrine and exocrine compartments of the pancreas and is essential for pancreas development [10-18], β-cell differentiation [19-29], and maintenance of mature β-cell function by regulating several β-cell-related genes [30-38]. PDX-1 expression is initially observed at E8.5-E9.0 in pancreatic progenitor cells, which means that early PDX-1 expression is likely to be a useful marker of pancreatic identity. Interestingly, a study based upon temporally controlled Cre recombination demonstrated that cells expressing PDX-1 give rise to all three types of pancreatic tissue: exocrine, endocrine and duct [39, 40]. Furthermore, it was shown that exocrine and endocrine progenitors express PDX-1 throughout early embryogenesis, whereas adult duct progenitors express PDX-1 only between E9.5 and

E12.5. These results suggest that the vast majority of progenitors for ducts and exocrine/endocrine cells are separated before E12.5. Another study using lineage tracing demonstrated that cells expressing another pancreatic transcription factor Ptf1a (also known as PTF1-p48) give rise to all three types of pancreatic tissue and supports the specification of precursors of all three pancreatic cell types [41]. When these two important reports are taken together, it is likely that PDX-1 and Ptf1a double-positive cells are pancreatic progenitor cells.

Figure 1. Pancreatic transcription factor hierarchy during pancreas development. It is well known that many transcription factors are involved in pancreas formation and β-cell differentiation. Among the various transcription factors, PDX-1 plays a crucial role in pancreas formation and β-cell differentiation as well as maintenance of mature β-cell function. Ngn3 and NeuroD are also important transcription factors for pancreatic endocrine cell differentiation. MafA expression is induced at the final stages of β-cell differentiation and functions as a potent activator of insulin gene transcription.

PDX-1 expression is maintained in precursor cells during pancreas development but becomes restricted to β-cells in the mature pancreas (Figure 1). Mice homozygous for a targeted mutation in the PDX-1 gene are apancreatic and develop fatal perinatal hyperglycemia [10], indicating that PDX-1 plays a crucial role in the formation of endocrine and exocrine cells. It is noted that PDX-1 expression is not required for pancreatic determination of the endoderm, because initial bud formation is observed in PDX-1$^{-/-}$ mice. Loss of PDX-1 function results in pancreatic agenesis in humans as well as in mice [15].

Differentiation into β-cells as well as maintenance of the β-cell phenotype also requires PDX-1. In mature β-cells, PDX-1 transactivates insulin and other genes involved in glucose sensing and metabolism such as GLUT2 and glucokinase [33, 34]. It was also reported that PDX-1$^{+/-}$ mice are glucose intolerant, with increased islet apoptosis, decreased islet mass, and abnormal islet architecture, indicating that proper gene dosage of PDX-1 is crucial for normal glucose homeostasis [16, 34, 36]. These findings are consistent with the report that humans heterozygous for an inactivating mutation of PDX-1 suffer from maturity-onset diabetes of the young (MODY 4) [42].

Furthermore, to explore the role of PDX-1 in the formation and maintenance of the pancreas, genetically engineered mice were developed using the Tet-off system, in which PDX-1 expression can be controlled by treatment of the mice with tetracycline or doxycycline [18]. In these mice, the coding region of the endogenous PDX-1 gene is replaced by a PDX-1 transgene under the control of a tetracycline-regulated transactivator (tTA). Hence, in the absence of doxycycline, tTA activates the transcription of a transgene encoding PDX-1. Expression of the transgene-encoded PDX-1 rescued the PDX-1-null phenotype, and doxycycline-mediated repression of the PDX-1 transgene throughout gestation recapitulated the PDX-1 null phenotype. Doxycycline treatment at mid pancreogenesis blocked further development of the pancreas [18]. In addition, when PDX-1 expression was shut off by doxycycline in adult mice, insulin biosynthesis was decreased and glucose homeostasis was disturbed [18]. These data further confirm the importance of PDX-1 in pancreas development, β-cell differentiation, and maintenance of mature β-cell function.

Hb9 is also a member of the large family of homeodomain (HD)-containing proteins and plays crucial roles in the early stages of pancreas development. While PDX-1 is involved in the development of the entire pancreas [5, 6, 10-18], Hb9 plays an important role in the development of the dorsal pancreas [43, 44]

(Table 1). Indeed, it was shown that, in Hb9$^{-/-}$ mice, the dorsal pancreas is not formed and PDX-1 expression is not observed in the endodermal epithelial cells that are destined to form the dorsal pancreas. Therefore, it is likely that Hb9 functions upstream of PDX-1 in the dorsal pancreas and plays an important role in differentiation of the dorsal pancreas.

Table 1. Pancreas-related phenotypes in knockout mice of pancreatic transcription factors

Transcription factor	Phenotype in the pancreas of knockout mice	Expression in mature islets	Reference
PDX-1	Absence of whole pancreas	β- and δ-cells	10, 12
Hb-9	Absence of dorsal pancreas	β-cells	43, 44
Arx	Absence of α-cells, increase of β- and δ-cells	α- and PP-cells	75
Isl-1	Absence of islet cells and dorsal pancreatic mesoderm	All islet cells	64
Pax4	Absence of β- and δ-cells, increase of α- and ε-cells	Not detected	65, 72
Pax6	Absence of α-cells, decrease of β-, δ- and PP-cells, increase of ε-cells	All islet cells	66, 67, 73
Nkx2.2	Absence of β-cells, decrease of α- and PP-cells	α-, β- and PP-cells	69, 72
Nkx6.1	Decrease of β-cells	β-cells	68
Ngn3	Absence of endocrine cells	Not detected	54
NeuroD	Decrease of endocrine cells	All islet cells	46
MafA	Decrease of insulin biosynthesis and secretion	β-cells	79

NeuroD and neurogenin3 (Ngn3), both of which are basic helix loop helix (bHLH) proteins, are also known to play important roles in pancreas development. NeuroD, a member of the bHLH transcription factor family, also known as BETA2, is expressed in pancreatic and intestinal endocrine cells and neural tissues. NeuroD also plays an important role in regulation of insulin gene transcription [45-48]. Mice homozygous for the null mutation in NeuroD have a striking reduction in the number of β-cells, develop severe diabetes and die perinatally [46] (Table 1). Furthermore, it has been reported that the insulin enhancer elements, E-box (NeuroD binding site) and A-box (PDX-1 binding site), are very important for insulin gene transcription [49, 50]. Neurogenin3 (Ngn3), a member of the

basic helix-loop-helix (bHLH) transcription factor family, is involved in endocrine differentiation [40, 51-56]. After bud formation, Ngn3 is transiently expressed in endocrine precursor cells and functions as a potential initiator of endocrine differentiation. It has also been shown that Ngn3 directly regulates a variety of pancreatic transcription factors such as NeuroD, Pax4 and Nkx2.2 [57-60], which further strengthen the hypothesis that Ngn3 plays a crucial role in the initiation of endocrine differentiation. Transgenic mice overexpressing Ngn3 show a marked increase in endocrine cell formation, indicating that Ngn3 induces the differentiation of islet cell precursors [52, 53]. In contrast, mice with a targeted disruption in Ngn3 have no endocrine cells [54] (Table 1). Since Ngn3 is not expressed in mature β-cells, it is likely that an increase in mature β-cell numbers after birth is not due to differentiation from Ngn3-positive endocrine progenitor cells.
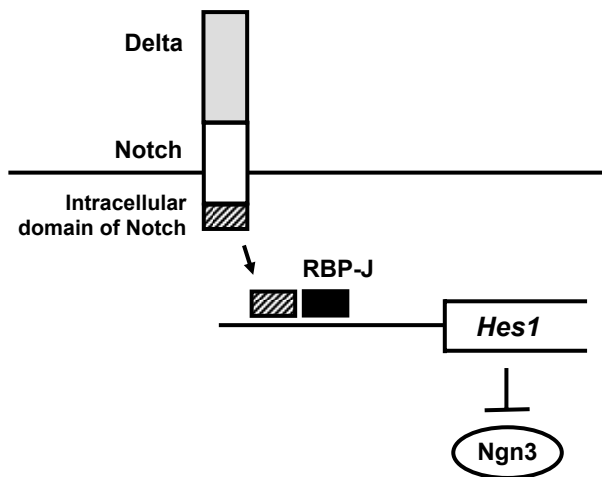
In addition, it is interesting to note that the Notch pathway plays an important role in differentiation from pancreatic progenitor cells to Ngn3-positive endocrine progenitor cells [61-63]. Indeed, it was shown that activation of the Notch pathway in pancreas progenitor cells leads to suppression of proper differentiation to both endocrine and exocrine cell lineages. After Notch activation by Delta, the intracellular domain of Notch and the mammalian Suppressor of Hairless RBP-J activate Hairy and Enhancer-of-split 1 (Hes1), which leads to suppression of Ngn3 expression (Figure 2). Therefore, it is likely that suppression of the Notch pathway leads to differentiation from pancreatic progenitor cells to Ngn3-positive endocrine cell lineage. In contrast, activation of the Notch pathway would preserve pancreatic progenitor cells from differentiation to Ngn3-positive endocrine cell lineage. Indeed, it was shown that mice deficient in

Hes1 display severe pancreatic hypoplasia caused by depletion of pancreatic epithelial precursors, which is due to accelerated differentiation into endocrine cells [61]. It is probable, therefore, that Hes1 operates as a general negative regulator of endodermal endocrine differentiation.

Figure 2. Role of the Notch pathway in pancreatic progenitor cells. After Notch activation by Delta, intracellular domain of Notch and the mammalian Suppressor of Hairless RBP-J activate Hairy and Enhancer-of-split 1 (Hes1), which leads to suppression of Ngn3 expression. Therefore, it is likely that suppression of the Notch pathway leads to differentiation from pancreatic progenitor cells to Ngn3-positive endocrine cell lineage and that, in contrast, activation of the Notch pathway preserves pancreatic progenitor cells from differentiation to Ngn3-positive endocrine cell lineage.

Other subclasses of homeodomain (HD) proteins such as Arx, the LIM domain protein Isl-1, the paired domain proteins Pax4 and Pax6, and the Nkx class proteins Nkx6.1 and Nkx2.2 also play an important role in pancreas development [64-75]. The pancreas-related phenotypes observed in knockout mice of each of the homeodomain proteins are as follows (see also Table 1):

- Arx^{-/-}, absence of α-cells and increase of β- and δ-cells [75].
- Isl-1^{-/-}, absence of islet cells [64].
- Pax4^{-/-}, absence of β-cells, decrease of δ-cells and increase of α- and ε-cells [65, 72].
- Pax6^{-/-}, absence of α-cells, decrease of β-, δ- and PP-cells and increase of ε-cells [66, 67, 73].
- Nkx6.1[-/-], decrease of β-cells [68].

- Nkx2.2[-/-], absence of β-cells, decrease of α- and PP-cells and increase of ε-cells [68, 69, 72].

As shown in Figure 1, Pax4 and Nkx2.2 are downstream of Ngn3, and Nkx6.1 is downstream of Nkx2.2. Also, it is noted that Arx is an important transcription factor that facilitates differentiation from endocrine progenitor cells to α-cells and that Arx and Pax4 are upregulated in endocrine precursor cells of Pax4^{-/-} and Arx^{-/-} mice respectively [27]. Therefore, it is likely that Arx-Pax4 co-repression plays an important role in proper endocrine specification by maintaining balance between α-cell and β-cell lineages (Table 1).

Finally, MafA, a basic-leucine zipper (bLZ) transcription factor, plays an important role in the final stages of β-cell differentiation and functions as a potent transactivator for the insulin gene [76-81]. During pancreas development, MafA expression is first detected at the beginning of the principal phase of insulin-producing cell production, whereas other important transcription factors such as PDX-1 and NeuroD are expressed from the early stages of pancreas development (Figure 1). In addition, MafA is expressed only in β-cells and functions as a potent activator of insulin gene transcription, whereas PDX-1 and NeuroD are expressed in various islet cell types. It has also been reported that MafA^{-/-} mice display glucose intolerance and develop diabetes mellitus [81]. Furthermore, in MafA^{-/-} mice, expression of insulin 1, insulin 2, PDX-1, NeuroD and GLUT2 was decreased and glucose-, arginine-, and KCl-stimulated insulin secretion was severely impaired (Table 1).

PDX-1 plays an important role in the induction of insulin-producing cells and is a possible therapeutic target for diabetes

A decrease in the number of functioning pancreatic β-cells and insufficient insulin biosynthesis and/or secretion is the hallmark of diabetes. It is very important, therefore, to search for alternative sources to induce insulin-producing cells. For the purpose of inducing insulin-producing cells from various cells and tissues, it would be useful to mimic and reproduce the alterations in expression of various pancreatic transcription factors observed during normal pancreas development. It would also be useful to induce key pancreatic transcription factors which have the potency to induce various β-cell-related genes, including insulin, in various source cells or tissues.

It has been reported that various cells and tissues such as liver, pancreas, intestine and bone marrow can
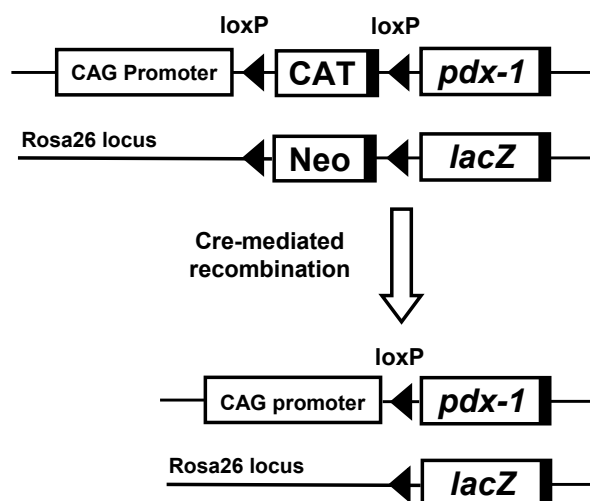
be transdifferentiated into insulin-producing cells. Furthermore, it was shown that embryonic stem cells have the potential to differentiate into insulin-producing cells [82-86], but use of these cells for the treatment of diabetes may not be appropriate from an ethical point of view. Therefore, adult tissue-derived progenitor cells have been used to induce insulin-producing cells. Pancreatic ducts, acini and non-β-cells in islets have also been shown to have the potential to differentiate into insulin-producing cells [20, 23, 24, 87-91]. In addition, since the pancreas and liver arise from adjacent regions of the endoderm in embryonic development, the liver has been thought to be a potential source for the induction of insulin-producing cells [19, 26-29, 47, 92, 93]. Intestinal epithelium-derived cells and some populations of bone marrow cells were also shown to have the potential to differentiate into insulin-producing cells [21, 22, 25, 94, 95]. In such studies, several pancreatic transcription factors were used to induce insulin-producing cells from various cells or tissues. Indeed, it was reported that adenoviral expression of PDX-1 in the liver of mice induced the expression of endogenous insulin mRNA [19].

Also, hepatic immunoreactive insulin induced by PDX-1 was processed to mature insulin which was biologically active [19]. These data indicate the capacity of PDX-1 to reprogram extrapancreatic tissues toward a β-cell phenotype, which may provide a valuable approach for generating surrogate β-cells suitable for replacing the impaired β-cell function found in diabetes. These results also demonstrate the usefulness of inducing key pancreatic transcription factors in various cells and tissues which have the potential to induce various β-cell-related genes including insulin.

In order to carry out efficient screening of somatic tissues and cells that can transdifferentiate into β-cell-like cells in response to PDX-1, we previously generated CAG-CAT-PDX-1 mice, a transgenic line which constitutively expresses the PDX-1 gene under the control of the chicken β-actin gene (CAG) promoter after removal of the floxed stuffer sequence (CAT) by Cre-mediated recombination [26] (Figure 3). When the mice were crossed with Alb-Cre mice, which express the Cre recombinase driven by the rat albumin gene promoter, PDX-1 was expressed in hepatocytes and cholangiocytes. The PDX-1-producing liver expressed a variety of endocrine hormone genes such as insulin, glucagon, somatostatin and pancreatic polypeptide as well as exocrine genes such as elastase-1 and chymotrypsinogen 1B [26]. These mice, however, exhibited marked jaundice because of conjugated hyperbilirubinemia, and the liver tissue displayed abnormal lobe structures and multiple cystic lesions. Thus, the *in vivo* ectopic expression of PDX-1 in albumin-producing cells was able to initiate, although not complete, the differentiation of liver cells into insulin-producing cells. We think that this conditional PDX-1 transgenic mouse system should be useful for the efficient screening of PDX-1 responsive somatic tissues and cells (Figure 3). Given that the expression of PDX-1 continues throughout pancreas development, i.e. from the embryonic pancreatic buds to adult islets, this Cre/loxP-mediated approach would provide a suitable system for evaluating the transdifferentiation potential of PDX-1 *in vivo*.

Figure 3. Tissue-specific overexpression of PDX-1 using the Cre/loxP-mediated system. We previously generated CAG-CAT-PDX-1 mice, a transgenic line which constitutively expresses the PDX-1 gene under the control of the chicken β-actin gene (CAG) promoter after the removal of the floxed stuffer sequence (CAT) by Cre-mediated recombination. When the mice were crossed with Ptf1a-Cre mice, which express the Cre recombinase driven by the Ptf1a (PTF1-p48) gene promoter, PDX-1 was expressed in precursors of all three pancreatic cell types: islets, acini, and ducts. In addition, when the mice were crossed with Alb-Cre mice, which express the Cre recombinase driven by the rat albumin gene promoter, PDX-1 was expressed in hepatocytes and cholangiocytes.

In addition, it has been shown recently that a modified form of XlHbox8, the Xenopus homolog of PDX-1, carrying the VP16 transcriptional activation domain from Herpes simplex virus, efficiently induces insulin gene expression in the liver of the tadpole [96]. In this study, transgenic Xenopus tadpoles carrying the Xlhbox8-VP16 gene under the control of the transthyretin promoter were generated. Xlhbox8-VP16

was expressed only in the liver of the tadpoles. In these tadpoles, the liver was converted into a pancreas, containing both exocrine and endocrine cells. The characteristics of liver were lost from the regions converted into a pancreas [96]. In contrast, conversion of the liver to a pancreas was not observed by expression of Xlhbox8 alone (without VP16).

Following these findings in tadpoles, the effects of the PDX-1-VP16 fusion protein (PDX-1-VP16) on differentiation of cells into insulin-producing cells have been examined in mice. Indeed, it was reported recently that PDX-1-VP16 rather than wild type PDX-1 efficiently induces insulin-producing cells in the liver [27-29, 93]. In addition, it was shown that PDX-1-VP16 efficiently induces insulin gene expression in the liver, especially in the presence of the pancreatic transcription factors NeuroD or Ngn3 [27]. Although PDX-1-VP16 exerted only a slightly greater effect on the insulin promoter compared with wild type PDX-1, PDX-1-VP16, together with NeuroD or Ngn3, dramatically increased insulin promoter activity in HepG2 cells. Furthermore, when adenovirus expressing the PDX-1-VP16 fusion protein (Ad-PDX-1-VP16) was intravenously injected into mice, both insulin 1 and 2 mRNA was detected in the liver, although insulin 1 was not detected upon adenoviral induction of wild type PDX-1 (without VP-16) [27]. Ad-PDX-1-VP16 treatment, together with Ad-NeuroD or Ad-Ngn3, induced a greater insulin gene expression. After treatment with Ad-PDX-1-VP16 plus either Ad-NeuroD or Ad-Ngn3, insulin-positive cells and insulin secretory granules were observed in the liver upon immunostaining and electron microscopy, respectively [27]. Furthermore, various endocrine pancreas-related factors such as islet-type glucokinase, glucagon and somatostatin were induced after treatment with Ad-PDX-1-VP16 plus either Ad-NeuroD or Ad-Ngn3. Consequently, in STZ-induced diabetic mice, blood glucose levels were decreased by PDX-1-VP16 plus either NeuroD or Ngn3 [27].

The marked effects of PDX-1-VP16 expression, together with NeuroD or Ngn3, on insulin production and glucose tolerance indicate that this combination is useful and efficient for replacing the reduced insulin biosynthesis found in diabetes, and that PDX-1 requires the recruitment of coordinately functioning transcription factors or cofactors in order to exert its function fully. In addition, these results suggest that the synergistic activation of the insulin promoter by PDX-1 and bHLH transcription factors such as NeuroD or Ngn3 is important for the induction of insulin-producing cells from non-β-cells in order to achieve β-

cell regeneration therapy in the future.

It was also shown recently that PDX-1-VP16 expressing hepatic cells were converted into functional insulin-producing cells in the presence of high glucose [28]. In this study, the authors generated a stably transfected rat hepatic cell line named WB-1 that expresses PDX-1-VP16. Expression of several genes related to endocrine pancreas development and islet function were induced by PDX-1-VP16 in the liver, although some pancreatic transcription factors were missing. In addition, these cells failed to secrete insulin upon glucose challenge. However, when WB-1 cells were transplanted into diabetic NOD-*scid* mice, they possessed similar properties as β-cells. Almost all β-cell-related transcription factors were induced and glucose intolerance was ameliorated [28]. In addition, *in vitro* culturing in high glucose medium was sufficient to induce the complete maturation of WB-1 cells into functional insulin-producing cells [28]. These results suggest that PDX-1-VP16 is very efficient and useful for replacing reduced insulin biosynthesis and for amelioration of glucose intolerance, but that PDX-1-VP16 alone is not sufficient to induce the complete transdifferentiation of various cells to functional insulin-producing cells.

Another study evaluated the effects of PDX-1-VP16 in a cell culture system as well as using hepatocytes isolated from adult rats. Adenoviral overexpression of PDX-1-VP16 efficiently converted hepatocytes into insulin-producing cells. In addition, immunoreactivity of albumin was downregulated in the transdifferentiated cells and some cells lost albumin expression almost completely [93]. These results add further weight to the hypothesis that hepatocytes possess the potential to transdifferentiate into insulin-producing cells.

Many studies have been performed to overexpress pancreatic transcription factors in different tissues using various virus-mediated approaches, but such approaches would be difficult to apply in clinical medicine. Therefore, new strategies are necessary to deliver safely various pancreatic transcription factors. Protein transduction domains (PTDs) such as the small PTD from the TAT protein of human immunodeficiency virus-1 (HIV-1), the VP22 protein of Herpes simplex virus and the third α-helix of the homeodomain of Antennapedia, a *Drosophila* transcription factor, are known to allow various proteins and peptides to be efficiently delivered into cells through the plasma membrane. For this reason, there has been increasing interest in their potential usefulness for the delivery of bioactive proteins and peptides into cells [23, 48, 56]. With regard to the potential of pancreatic transcription factors as

therapeutic targets, the protein delivery system appears to be very promising at this point, because of the practical difficulties in applying virus-mediated approaches to clinical medicine without side effects.

In order to induce surrogate β-cells and apply them to clinical medicine, it would be advantageous to deliver key pancreatic transcription factors into pancreatic source cells and tissues using the protein delivery system. It was shown recently that the PDX-1 protein can enter various cells on its own because of an Antennapedia-like protein transduction domain sequence in its structure and that transduced PDX-1 functions similarly to endogenous PDX-1: it binds to the insulin promoter and activates its expression [23]. In addition, it was shown that PDX-1 protein transduction occurs by endocytosis and its subsequent release from endosome, followed by homogenous location in the cytoplasm and nuclei [97]. More recently, it was shown that the NeuroD protein can also enter various cells on its own because of an arginine- and lysine-rich protein transduction domain in its structure and that transduced NeuroD functions similarly to endogenous NeuroD [48]. These data clearly suggest that PDX-1 and NeuroD transduction would be a safe and valuable strategy for inducing surrogate β-cells from non-β-cells without requiring gene transfer technology.
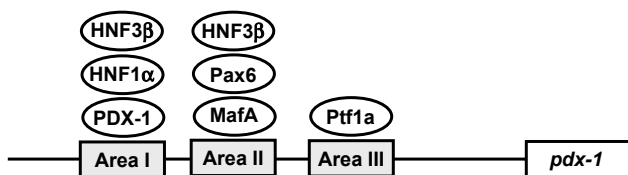
Figure 4. A variety of pancreatic transcription factors are involved in PDX-1 gene expression. Alignment of the mouse and human PDX-1 gene sequences revealed three conserved regions (Area I, II and III) which harbor binding sites of various transcription factors such as HNF-3β, HNF-1α, PDX-1, Pax6, MafA and Ptf1a.

A variety of pancreatic transcription factors are involved in PDX-1 gene expression

Since PDX-1 plays a crucial role in pancreas development, β-cell differentiation and maintenance of mature β-cell function, it is very important to understand the regulation of PDX-1 expression in the pancreas. It has been reported that PDX-1 activity is regulated by various nutrients such as glucose and insulin. It was shown that a high concentration of glucose and/or in-

sulin increased PDX-1 DNA binding activity to the insulin gene promoter region through activation of phosphatidylinositol 3-kinase (PI3-kinase) and p38 mitogen-activated protein kinase (MAPK) [98-102]. In addition, PDX-1 gene transcription is regulated by various pancreatic transcription factors (Figure 4).

Normal endocrine pancreas development and function depend on a highly integrated transcription factor network, and subtle abnormalities in islets caused by heterozygosity or reduced gene dosage of MODY susceptibility genes lead to diabetes in humans [103]. Promoter analyses of genes involved in β-cell differentiation and function suggest complex genetic interactions among these factors. Indeed, alignment of the mouse and human PDX-1 gene sequences revealed three conserved regions referred to collectively as Area I-II-III. The Area I-II-III region harbors binding sites for MODY transcription factors such as HNF-1α (Foxa1) and PDX-1 itself as well as other transcriptional regulators such as HNF-3β (Foxa2), Pax6, MafA and HNF-6 (OC-1) [104-113], and it has been shown that PDX-1 gene transcription is actually regulated by these various pancreatic transcription factors.

It has recently been reported that another pancreatic transcription factor, Ptf1a (also known as PTF1-p48), regulates PDX-1 gene expression (Figure 4) [104]. Ptf1a, a member of the basic helix-loop-helix (bHLH) family, is known to be expressed in pancreatic progenitor cells and to bind to the mammalian Suppressor of Hairless (RBP-J) within the PTF1 complex [115, 116]. In addition, all three factors (PDX-1, Ptf1a and RBP-J) have been shown to be essential for early pancreas development [41, 117-119]. Reporter gene analyses showed that Ptf1a transactivates the PDX-1 gene promoter in pancreatic Panc-1 cells, which is enhanced by RBP-J. The Ptf1a binding site was also identified in the well-conserved regulatory sequence domain termed Area III. In addition, adenoviral overexpression of Ptf1a, together with RBP-J, markedly increased PDX-1 expression levels in pancreatic AR42J-B13 cells, which have been reported to differentiate into insulin-producing cells [120, 121]. Furthermore, it was recently demonstrated using Cre-mediated lineage tracing in mice that Area III mediates pancreas-wide PDX-1 expression during early pancreas development and that Ptf1a occupies sequences within Area III in pancreatic buds [122].

These results strongly suggest a novel transcriptional network in which Ptf1a regulates PDX-1 gene expression through binding to Area III in pancreatic progenitor cells. It is noted, however, that since PDX-1 expression is likely to be regulated in a different

manner at each stage of pancreas development, *in vitro* promoter analysis does not necessarily recapitulate the regulation of PDX-1 expression in the developing pancreas. Therefore, the study using mice with deletion of a specific PDX-1 promoter region is very useful in evaluating the regulation of PDX-1 expression in the developing pancreas.

It has been reported that islet-specific and β-cell-specific cis-regulatory regions overlap with Area I-II-III, suggesting that Area I-II-III functions specifically in the differentiation and maintenance of pancreatic islets [104-113]. It has also recently been reported that deletion of Area I-II-III from the endogenous PDX-1 locus results in a decreased level as well as abnormal spatiotemporal expression of the PDX-1 protein. In addition, the pancreas of homozygous Area I-II-III knockout mice did not undergo ventral pancreatic bud specification and demonstrated early-onset hypoplasia in the dorsal bud [123]. In these mice, acinar tissue formed in the hypoplastic dorsal bud, but endocrine maturation was greatly impaired. In addition, while the pylorus was distorted and Brunner's glands were not observed in PDX-1$^{-/-}$ mice, these structures formed normally in the homozygous Area I-II-III deletion mutant mice. These results suggest that Area I-II-III is not essential for extra-pancreatic expression of PDX-1. Furthermore, Area I-II-III heterozygous knockout mice had abnormal islets and showed more severe glucose intolerance compared to PDX-1$^{+/-}$ mice [123]. These results supply further confirmation of the importance of Area I-II-III in pancreas formation and maintenance of β-cell function.

While PDX-1 is expressed in pancreatic progenitor cells and plays a crucial role in pancreas development and β-cell differentiation, PDX-1 expression is down-regulated in exocrine and ductal cells after late embryonic development. On the other hand, re-upregulation of PDX-1 has been reported in human patients and several mouse models with pancreatic cancer and pancreatitis [124-126]. We have recently reported that programmed downregulation of PDX-1 is required for exocrine tissue formation during pancreas differentiation and that persistent expression of PDX-1 causes acinar-to-ductal metaplasia [127]. To determine whether the sustained expression of PDX-1 affects pancreas development, PDX-1 was constitutively expressed in all pancreatic lineages by transgenic approaches.

As mentioned earlier, we previously generated CAG-CAT-PDX-1 mice, a transgenic line which constitutively expresses the PDX-1 gene under the control of the chicken β-actin gene (CAG) promoter after the

removal of the floxed stuffer sequence (CAT) by Cre-mediated recombination [26] (Figure 3). When these mice were crossed with Ptf1a-Cre mice, which express the Cre recombinase driven by the Ptf1a (PTF1-p48) gene promoter [41], PDX-1 was expressed in precursors of all three pancreatic cell types: islets, acini, and ducts. Two weeks after birth, the whole pancreas of the Ptf1a-Cre, CAG-CAT-PDX-1 mouse was much smaller compared to the non-transgenic pancreas, and marked abnormalities in the exocrine tissue were observed. While acinar areas with normal morphology substantially disappeared in the transgenic pancreas, a large number of cells with duct-like morphology were observed [127]. Severe atrophic cells and abnormal duct-like morphology were observed exclusively in the cells expressing exogenous PDX-1, suggesting that the phenotypes in the transgenic pancreas are caused by the cell-autonomous effect of PDX-1.

To induce exogenous expression of PDX-1 selectively in the exocrine lineage, CAG-CAT-PDX-1 mice were crossed with the resulting transgenic Elastase-Cre mice, after which recombination occurred primarily in the exocrine lineage [128]. Furthermore, lineage tracing was performed using Ptf1a-Cre, CAG-CAT-PDX1, ROSA26-lacZ and Elastase-Cre, CAG-CAT-PDX1, ROSA26-lacZ mice (Figure 3). Interestingly, a large number of duct-like cells, marked as blue β-galactosidase-positive cells, were observed in the pancreas of Elastase-Cre, CAG-CAT-PDX1 and ROSA26-lacZ mice, similar to those seen in the pancreas of Ptf1a-Cre, CAG-CAT-PDX1 and ROSA26-lacZ mice [127]. In addition, in immunostaining for BrdU and Ki67, cell proliferation was not observed in these duct-like cells.

When these results are considered together, we think that duct-like cells were induced by acinar-to-ductal transdifferentiation rather than by self-proliferation of duct cells. In summary, it is likely that programmed downregulation of PDX-1 is required for exocrine formation and that persistent upregulation of PDX-1 is sufficient to induce acinar-to-ductal metaplasia in the exocrine lineage.

Expression and/or activity of PDX-1 in β-cells are reduced under diabetic conditions and are likely to be involved in pancreatic β-cell glucose toxicity

Under diabetic conditions, chronic hyperglycemia causes the gradual deterioration of pancreatic β-cell function. This process is often observed in diabetic subjects and is clinically well known as β-cell glucose

toxicity [129-133]. It has been shown that in the diabetic state, hyperglycemia *per se* and subsequent production of oxidative stress decrease insulin gene expression and secretion [129-145]. It has also been shown that the loss of insulin gene expression is accompanied by decreased expression and/or DNA binding activity of PDX-1 [129, 130, 137-139]. After chronic exposure to a high glucose concentration, PDX-1 expression and/or its DNA binding activity are reduced. Abnormalities in lipid metabolism have also been proposed as contributing factors to the deterioration in pancreatic β-cell function. Prolonged exposure to excessive concentrations of fatty acids inhibits insulin gene expression and secretion [146-148]. Furthermore, it has been shown recently that prolonged exposure of islets to palmitate inhibits insulin gene transcription by impairing the nuclear localization of PDX-1 [149].

Under diabetic conditions, hyperglycemia induces oxidative stress, which is involved in the β-cell glucose toxicity found in diabetes, through various pathways such as the electron transport chain in mitochondria, the non-enzymatic glycosylation reaction and the NADPH oxidase pathway [136-145, 150-153]. β-cells express GLUT2, a high-Km glucose transporter, and thereby display highly efficient glucose uptake when exposed to a high glucose concentration. In addition, β-cells are rather vulnerable to oxidative stress because of the relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase [154, 155]. Indeed, it was shown that expression of the oxidative stress markers 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxy-2,3-nonenal (4-HNE) were increased in islets under diabetic conditions [136, 143]. It has also been shown that when β-cell-derived cell lines or rat isolated islets are exposed to oxidative stress, insulin gene promoter activity and mRNA expression are suppressed [137-139, 141-144]. When those cells or rat isolated islets were

exposed to oxidative stress, binding of PDX-1 to the insulin gene promoter was markedly reduced. Furthermore, it was shown that the decrease of insulin gene expression after chronic exposure to a high glucose concentration could be prevented by treatment with antioxidants [138, 139, 142-144]. Reduction of the expression and/or DNA binding activity of PDX-1 by chronic exposure to a high glucose concentration was also prevented by antioxidant treatment.

These results suggest that chronic hyperglycemia suppresses insulin biosynthesis and secretion by provoking oxidative stress, accompanied by the reduction of PDX-1 expression and/or its DNA binding activity. Therefore, it is likely that PDX-1 inactivation explains, at least in part, the suppression of insulin biosynthesis and secretion and is thus involved in β-cell glucose toxicity (Figure 5).
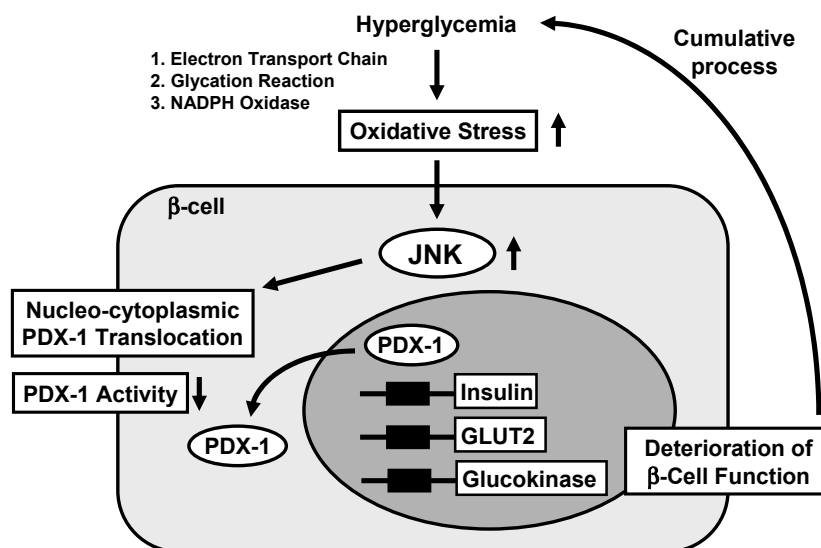
Figure 5. Nucleo-cytoplasmic translocation of PDX-1 is induced by oxidative stress and the subsequent activation of the JNK pathway. Oxidative stress and the subsequent activation of the JNK pathway induce the nucleo-cytoplasmic translocation of PDX-1, which leads to reduction of its DNA binding activity and suppression of insulin biosynthesis. It is likely that oxidative stress and the JNK pathway are involved in the β-cell dysfunction found in type 2 diabetes.

In order to evaluate the role of oxidative stress in diabetes *in vivo*, obese diabetic C57BL/KsJ-db/db mice were treated with antioxidants (N-acetyl-L-cysteine plus vitamin C and E) [138]. Antioxidant treatment did not affect glucose-stimulated insulin secretion and moderately ameliorated glucose tolerance. β-cell mass
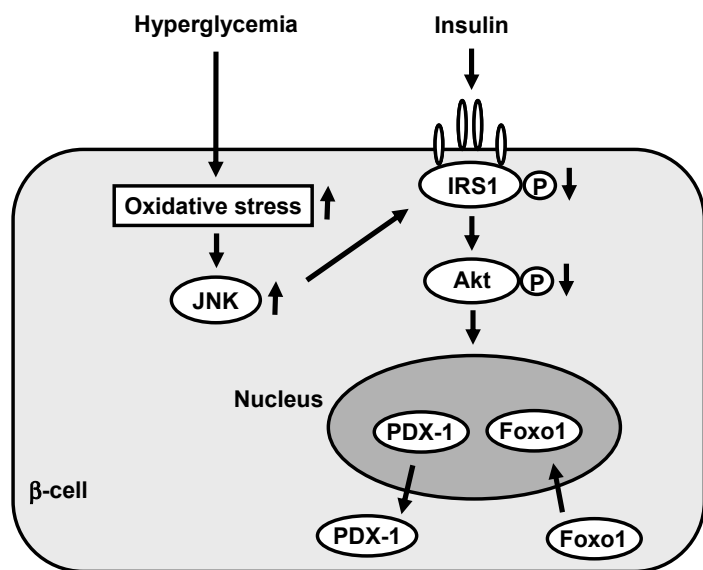
was significantly larger in mice treated with the antioxidants. Insulin content and insulin mRNA levels were also preserved by the antioxidant treatment. Furthermore, PDX-1 expression was more clearly visible in the nuclei of islet cells after the antioxidant treatment [138]. Similar effects were observed using Zucker diabetic fatty rats, another model animal for type 2 diabetes [139]. Taken together, these data indicate that antioxidant treatment can protect β-cells against glucose toxicity.

Figure 6. Involvement of Foxo1 in the nucleo-cytoplasmic translocation of PDX-1 which is induced by oxidative stress and the subsequent activation of the JNK pathway. Oxidative stress and the subsequent activation of the JNK pathway induce nuclear translocation of Foxo1 through modification of insulin signaling in β-cells, which leads to the nucleo-cytoplasmic translocation of PDX-1 and reduction of its DNA binding activity.

In addition, we examined the possible anti-diabetic effects of probucol, an antioxidant widely used as an anti-hyperlipidemic agent, on the preservation of β-cell function in diabetic C57BL/KsJ-db/db mice [143]. Immunostaining for oxidative stress markers such as 4-hydroxy-2-nonenal (HNE)-modified proteins and heme oxygenase-1 revealed that probucol treatment decreases ROS in β-cells of diabetic mice. Probucol treatment also preserved β-cell mass, insulin content and glucose-stimulated insulin secretion, leading to improvement of glucose tolerance [143]. These data suggest the potential usefulness of antioxidants for

diabetes and provide further support for the involvement of oxidative stress in the β-cell glucose toxicity found in diabetes.

It has been suggested that activation of the c-Jun N-terminal kinase (JNK) pathway is involved in the pancreatic β-cell dysfunction found in diabetes. It was reported that activation of the JNK pathway is involved in reduction of insulin gene expression by oxidative stress and that suppression of the JNK pathway can protect β-cells from oxidative stress [156]. When isolated rat islets were exposed to oxidative stress, the JNK pathway was activated, preceding the decrease of insulin gene expression. Adenoviral overexpression of a dominant-negative type JNK1 (DN-JNK) inhibited the decrease in insulin gene expression and secretion resulting from oxidative stress. Moreover, overexpression of wild type JNK1 (WT-JNK) suppressed both insulin gene expression and secretion [156]. These results were correlated with the reduction of PDX-1 binding to the insulin promoter. Adenoviral overexpression of DN-JNK preserved PDX-1 DNA binding activity in the presence of oxidative stress, while WT-JNK overexpression decreased PDX-1 DNA binding activity [156]. Thus, JNK-mediated suppression of PDX-1 DNA binding activity probably accounts for some of the suppression of insulin gene transcription upon oxidative stress.

In summary, it is likely that activation of the JNK pathway leads to decreased PDX-1 activity and the consequent suppression of insulin gene transcription found in the diabetic state (Figure 5). Furthermore, as a potential mechanism for JNK-mediated PDX-1 inactivation, it was recently reported that PDX-1 translocates from the nucleus to the cytoplasm in response to oxidative stress. When β-cell-derived HIT cells were subjected to oxidative stress, PDX-1 translocates from the nucleus to the cytoplasm [157]. Addition of DN-JNK inhibited this translocation, suggesting an essential role of the JNK pathway in mediating this phenomenon. In addition, leptomycin B, a specific inhibitor of the classical, leucine-rich nuclear export signal (NES), inhibited the nucleo-cytoplasmic translocation of PDX-1 induced by oxidative stress. Indeed, we identified an NES at position 82-94 of the mouse

PDX-1 protein [157]. Conclusively, it is likely that oxidative stress induces the nucleo-cytoplasmic translocation of PDX-1 through activation of the JNK pathway, which leads to reduction of its DNA binding activity and suppression of insulin biosynthesis (Figure 5).

Furthermore, while the role of the forkhead transcription factor Foxo1 in β-cell function has attracted considerable attention [158, 159], we have recently reported that Foxo1 plays a role as a mediator between the JNK pathway and PDX-1 [160]. In β-cell-derived HIT cells, the intracellular localization of Foxo1 changed from the cytoplasm to the nucleus under oxidative stress conditions. In contrast to Foxo1, as mentioned above, the amount of nuclear PDX-1 decreased and its cytoplasmic distribution was increased by oxidative stress. JNK overexpression also induced the nuclear localization of Foxo1, although, on the other hand, suppression of the JNK pathway reduced the oxidative stress-induced nuclear localization of Foxo1, suggesting the involvement of the JNK pathway in Foxo1 translocation [160]. In addition, oxidative stress or activation of the JNK pathway decreased the activity of Akt in HIT cells, leading to decreased phosphorylation of Foxo1 following nuclear localization. Furthermore, adenoviral Foxo1 overexpression reduced the nuclear expression of PDX-1, whereas repression of Foxo1 by a Foxo1-specific small interfering RNA resulted in retained nuclear expression of PDX-1 under oxidative stress conditions [160]. When considered as a whole, these data indicate that oxidative stress and the subsequent activation of the JNK pathway induce nuclear translocation of Foxo1 through the modification of insulin signaling in β-cells, which leads to the nucleo-cytoplasmic translocation of PDX-1 and reduction of its DNA binding activity (Figure 6). Finally, we think that suppression of oxidative stress and/or inactivation of the JNK pathway protects β-cells from glucose toxicity found in diabetes and thus are potential therapeutic targets for diabetes.

Conclusions

The number of diabetic patients is dramatically increasing all over the world, and diabetes has recently been recognized as one of the most prevalent and serious metabolic diseases. Although pancreas and islet transplantation have achieved beneficial effects for type 1 diabetic patients, the availability of insulin-producing cells is limited and life-time immunosuppressive therapy is required. It is very important, therefore, to search for alternative sources to induce insulin-producing cells.

PDX-1 is a pancreatic transcription factor which plays a crucial role in pancreas formation, β-cell differentiation and maintenance of mature β-cell function. Furthermore, it is likely that PDX-1 plays a crucial role in inducing insulin-producing cells in various non-β-cells and thus could be a therapeutic target for type 1 diabetes. It is noted, however, that current strategies involve some problems with the differentiation of various cells into insulin-producing cells. For example, although insulin biosynthesis and secretion can be induced in several types of non-β-cells, it is very difficult to obtain substantial glucose-responsive insulin secretion, which is very important to maintain normal glucose tolerance.

Under diabetic conditions, chronic hyperglycemia gradually leads to the deterioration of β-cell function, which is often observed in type 2 diabetic subjects and is clinically well known as β-cell glucose toxicity. These phenomena are accompanied by a reduction in the expression and activity of pancreatic transcription factors. Therefore, it is likely that PDX-1 plays an important role in mediating mature β-cell function and that PDX-1 inactivation is involved in the β-cell glucose toxicity found type 2 diabetes.

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