

## PDX-1 functions as a master factor in the pancreas

Hideaki Kaneto, Taka-aki Matsuoka, Takeshi Miyatsuka, Dan Kawamori, Naoto Katakami, Yoshimitsu Yamasaki, Munehide Matsuhisa

Department of Internal Medicine and Therapeutics (A8), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

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## 1. ABSTRACT

Various pancreatic transcription factors are involved in pancreas development and  $\beta$ -cell differentiation. Among them, pancreatic and duodenal homeobox factor-1 (PDX-1) plays a crucial role in pancreas development and  $\beta$ -cell differentiation, and maintaining mature  $\beta$ -cell function. MafA is a recently isolated  $\beta$ -cell-specific transcription factor and functions as a potent activator of insulin gene transcription. These pancreatic transcription factors also play a crucial role in inducing surrogate  $\beta$ -cells from non- $\beta$ -cells and thus could be therapeutic targets for diabetes. On the other hand, under diabetic conditions, expression and/or activities of PDX-1 and MafA in  $\beta$ -cells are reduced, which leads to suppression of insulin biosynthesis and secretion. Thus, it is likely that alteration of such transcription factors explains, at least in part, the molecular mechanism for  $\beta$ -cell glucose toxicity found in diabetes.

## 2. INTRODUCTION

The number of diabetic patients is dramatically increasing all over the world, and recently diabetes has been recognized as the most prevalent and serious metabolic disease. Although pancreas and islet transplantation have exerted beneficial effects for diabetic patients, available insulin-producing cells are limited and life-long immunosuppressive therapy is required. Therefore, it is very important to search for alternative sources to induce insulin-producing cells. It has been reported that various cells and tissues such as embryonic stem cells, liver, pancreas, intestine, and bone marrow can induce insulin-producing cells after specific treatments.

On the other hand, chronic hyperglycemia is a cause of impairment of insulin biosynthesis and secretion. Once hyperglycemia becomes apparent,  $\beta$ -cell function

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**Table 1.** Pancreas-related phenotype in knockout mice of each pancreatic transcription factor

Transcription Factor	Family	Expression in mature islets	Pancreas-related phenotype in knockout mice
PDX-1	HD	$\beta$ - and $\delta$ -cells	absence of pancreas
Hb9	HD	$\beta$ -cells	absence of dorsal pancreas
Isl-1	HD	all islet cells	absence of islet cells and dorsal mesenchyme
Pax4	HD	not detected	absence of $\beta$ -cells decrease of $\delta$ -cells increase of $\alpha$ - and $\epsilon$ -cells
Pax6	HD	all islet cells	absence of $\alpha$ -cells decrease of $\beta$ -, $\delta$ -, and PP-cells increase of $\epsilon$ -cells
Nkx2.2	HD	$\alpha$ -, $\beta$ - and PP-cells	absence of $\beta$ -cells decrease of $\alpha$ - and PP-cells
Nkx6.1	HD	$\beta$ -cells	decrease of $\beta$ -cells
Ngn3	bHLH	not detected	absence of endocrine cells
NeuroD	bHLH	all islet cells	decrease of endocrine cells
MafA	bLZ	$\beta$ -cells	decrease of insulin biosynthesis and secretion

gradually deteriorates. This process is called  $\beta$ -cell glucose toxicity and clinically often observed in diabetic subjects (1, 2). Therefore, it is very important to examine the molecular mechanism for  $\beta$ -cell glucose toxicity and to explore the therapy to protect  $\beta$ -cells from the glucose toxicity.

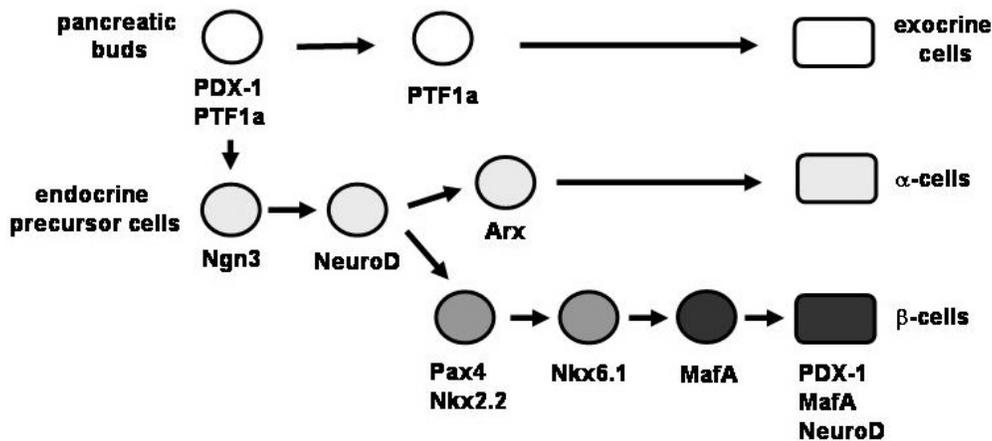
### 3. PDX-1 PLAYS A CRUCIAL ROLE IN PANCREAS DEVELOPMENT AND $\beta$ -CELL DIFFERENTIATION

It has been shown that various pancreatic transcription factors are involved in pancreas development and  $\beta$ -cell differentiation. Among them, members of the large family of homeodomain (HD)-containing proteins are likely to play a crucial role in the pancreas. The pancreatic duodenal homeobox factor-1 (PDX-1) (also known as IDX-1/STF-1/IPF1) (3-5) and the Hb9 factor are essential for the early stage of pancreas development. While PDX-1 affects the development of the entire pancreas (6-15), Hb9 plays an important role for the development of the dorsal pancreas (16, 17) (Table 1). It is noted here that PDX-1 is not detected in the dorsal pancreas in Hb9 (-/-) mice. Other subclasses of homeodomain (HD) proteins such as 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 the development of pancreas (18-29). Pancreas-related phenotype in knockout mice of each homeodomain protein is as follows. Isl-1 (-/-), absence of islet cells and dorsal pancreatic mesoderm (18); Pax4 (-/-), absence of  $\beta$ -cells, decrease of  $\delta$ -cells, and increase of  $\alpha$ - and  $\epsilon$ -cells (ghrelin-producing cells) (19, 20); Pax6 (-/-), absence of  $\alpha$ -cells, decrease of  $\beta$ -,  $\delta$ - and PP-cells (21, 22), increase of  $\epsilon$ -cells (27); Nkx6.1 (-/-), decrease of  $\beta$ -cells; Nkx2.2 (-/-), absence of  $\beta$ -cells, decrease of  $\alpha$ - and PP-cells (22, 23), and increase of  $\epsilon$ -cells (26) (Table 1).

Among such various homeodomain-containing transcription factors identified as essential for proper pancreas development, PDX-1 is both expressed in precursors of the endocrine and exocrine compartments of the pancreas, and is essential for pancreas development (6-14),  $\beta$ -cell differentiation (30-40), and maintaining mature  $\beta$ -cell function by regulating several  $\beta$ -cell-related genes (41-50). At early stage of embryonic development, PDX-1 is initially expressed in the gut region when the

foregut endoderm becomes committed to common pancreatic precursor cells. During pancreas development, PDX-1 expression is maintained in precursor cells that coexpress several hormones, and later it becomes restricted to  $\beta$ -cells (Figure 1). Mice homozygous for a targeted mutation in the PDX-1 gene are apancreatic and develop fatal perinatal hyperglycemia (6), indicating that PDX-1 plays a crucial role for the development of endocrine and exocrine cell types. It is noted here that PDX-1 expression is not required for pancreatic determination of the endoderm because the initial bud formation was observed in PDX-1 (-/-) mice. Loss of PDX-1 function also results in pancreatic agenesis in humans (11). Differentiation and maintenance of the  $\beta$ -cell phenotype also requires PDX-1. In mature  $\beta$ -cells, PDX-1 transactivates the insulin gene and other genes involved in glucose sensing and metabolism, such as GLUT2 and glucokinase (44, 45). It was also reported that PDX-1 (+/-) mice were glucose intolerant, with increased islet apoptosis, a decreased islet mass, and abnormal islet architecture, indicating that gene dosage for PDX-1 is crucial for normal glucose homeostasis (12, 45, 47). These findings are concordant with the report that humans heterozygous for an inactivating mutation of PDX-1 cause to maturity-onset diabetes of the young (MODY 4) (51). The other well-represented class of transcription factors is that of the basic helix loop helix (bHLH) proteins, which include NeuroD and neurogenin3 (Ngn3). NeuroD, a member of the bHLH transcription factor family, also known as BETA2, is expressed in pancreatic and intestinal endocrine cells and neural tissue. NeuroD plays an important role in pancreas development and in regulating insulin gene transcription (52-55). Mice homozygous for the null mutation in NeuroD have a striking reduction in the number of  $\beta$ -cells, develop severe diabetes and die perinatally (53) (Figure 1). Clinically, mutations in NeuroD cause maturity-onset diabetes of the young (MODY 6) (56). Furthermore, it has been reported that the insulin enhancer elements, E-box and A-box, are very important for insulin gene transcription (57, 58). Neurogenin3 (Ngn3), a member of the basic helix-loop-helix (bHLH) transcription factor family, is involved in pancreas development and endocrine differentiation (59-65). After bud formation, Ngn3 is transiently expressed in endocrine precursor cells, and functions as a potential initiator of endocrine differentiation. Transgenic mice overexpressing Ngn3 show a marked

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**Figure 1.** Pancreatic transcription factor hierarchy in pancreas development. It is well known that many transcription factors are involved in pancreas development and  $\beta$ -cell differentiation. Among them, PDX-1 plays a crucial role in pancreas development and  $\beta$ -cell differentiation, and functions as an activator of insulin gene transcription. MafA expression is induced in the final stage of  $\beta$ -cell differentiation and functions as a potent activator of insulin gene transcription.

increase in endocrine cell formation, indicating that Ngn3 induces islet cell precursors to differentiate (60, 61). In contrast, mice with targeted disruption of Ngn3 have no endocrine cells (62) (Table 1).

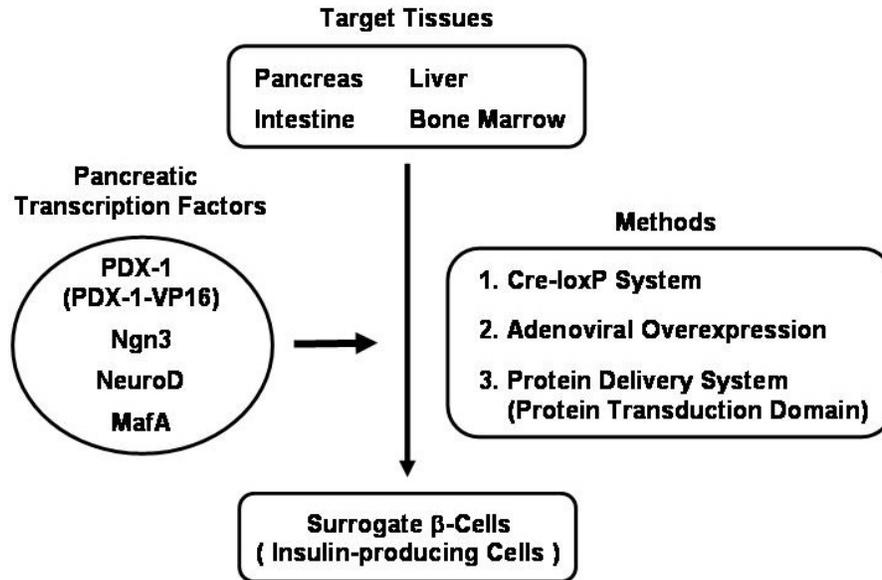
#### 4. AREA I-II-III IN PDX-1 ENHANCER REGION PLAYS A CRUCIAL ROLE IN PANCREAS DEVELOPMENT AND MAINTENANCE OF $\beta$ -CELL FUNCTION

Normal endocrine pancreas development and function depends 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 human (66). Recent promoter analyses of genes involved in  $\beta$ -cell differentiation and function suggest complex genetic interactions among these factors (67, 68). Indeed, alignment of the mouse and human PDX-1 gene sequences revealed three conserved regions referred to collectively as Area I-II-III (69). The Area I-II-III region harbors binding sites for *MODY* transcription factors such as HNF-1 $\alpha$  (Foxa1) and PDX-1 itself (70, 71) as well as other transcriptional regulators such as HNF-3 $\beta$  (Foxa2), Pax6, MafA, and HNF-6 (OC-1) (69, 72-75). It has been reported that islet-specific and  $\beta$ -cell-specific cis-regulatory regions overlap with Area I-II-III, suggesting that Area I-II-III functions specifically in differentiation and maintenance of pancreatic islets (69, 71, 73, 76, 77). It has also been reported recently that deletion of Area I-II-III results in a decreased level and abnormal spatiotemporal expression of PDX-1 protein and that lineage labeling in homozygous Area I-II-III deletion mutant mice reveals lack of ventral pancreatic bud specification and early-onset hypoplasia in the dorsal bud (78). In the mice, acinar tissue formed in the hypoplastic dorsal bud, but endocrine maturation was greatly impaired. Furthermore, heterozygous Area I-II-III deletion mutant mice had

abnormal islets and showed more severe glucose intolerance compared to PDX-1 (+/-) mice (78).

#### 5. PROGRAMMED DOWNREGULATION OF PDX-1 IS REQUIRED FOR EXOCRINE FORMATION AND PERSISTENT EXPRESSION OF PDX-1 CAUSES ACINAR-TO-DUCTAL METAPLASIA

While PDX-1 is expressed in pancreatic progenitor cells and plays a crucial role in pancreas development and  $\beta$ -cell differentiation, PDX-1 expression is downregulated 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 (79-81). We have recently reported that programmed downregulation of PDX-1 is required for exocrine formation during pancreas differentiation and that persistent expression of PDX-1 causes acinar-to-ductal metaplasia (82). To determine whether sustained expression of PDX-1 affects pancreas development, PDX-1 was constitutively expressed in all pancreatic lineages by transgenic approaches. Previously we generated CAG-CAT-PDX1 mice, a transgenic line which constitutively express the PDX-1 gene under the control of the chicken  $\beta$ -actin gene (CAG) promoter after the removal of floxed stuffer sequence (CAT) by Cre-mediated recombination (37). When the mice were crossed with Ptf1a-Cre mice, which express the Cre recombinase driven by the Ptf1a (PTF1-p48) gene promoter (83), 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-PDX1 mouse was much smaller compared to the non-transgenic pancreas, and marked abnormality of the exocrine tissue was observed in the transgenic pancreas. While acinar areas with normal morphology substantially disappeared in the transgenic pancreas, a large number of cells with duct-like morphology were observed (82). Severe atrophic cells and abnormal



**Figure 2.** Induction of insulin-producing cells by expression of key pancreatic transcription factors. Overexpression of key pancreatic transcription factors such as PDX-1 and MafA in non-β-cells (tissues) (e.g. pancreatic non-β-cells, liver, intestine, bone marrow cells) induces the expression of various β-cell-related genes including insulin. Also, there are various methods to overexpress such transcription factors such as Cre-loxP system, adenoviral overexpression, and protein transduction system.

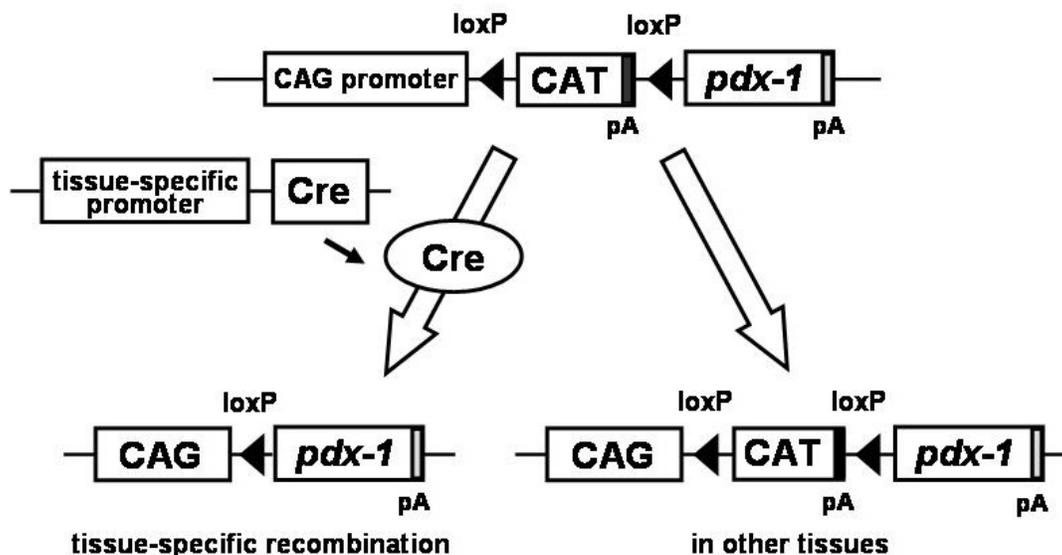
duct-like morphology were observed exclusively in the cells expressing exogenous PDX-1, suggesting that the phenotypes in the transgenic pancreas are caused by the cell-autonomous effect of PDX-1.

Furthermore, in metaplastic duct-like cells, signal transducer and activator of transcription 3 (Stat3) proteins were activated. To address the pathophysiological significance of Stat3 activation in inducing metaplastic duct-like cells, Ptf1a-Cre; CAG-CAT-PDX1 mice were crossed with floxed-Stat3 mice. Surprisingly, in the pancreata of Ptf1a-Cre; CAG-CAT-PDX1; Stat3<sup>flox/flox</sup> mice, metaplastic duct-like cells were rarely observed all over the acinar area (82). Pancreatic hypoplasia seen in PDX-1 overexpressing pancreata was also substantially restored in the pancreata of Ptf1a-Cre; CAG-CAT-PDX1; Stat3<sup>flox/flox</sup> mice. Taken together, 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 through Stat3 activation.

## 6. PDX-1 INDUCES INSULIN-PRODUCING CELLS FROM NON-β-CELLS

The pancreas is composed of exocrine (acini and ducts) and endocrine compartments (α-, β-, δ-, and PP-cells). During pancreas development, differentiation of endocrine and exocrine compartments from pancreatic buds requires the coordinated regulation of specific genes. This process can be envisioned as a hierarchy of transcription factors that initiate and maintain various gene expression programs, leading to the determination of various pancreatic cell types (Figure 1). For the induction of insulin-producing cells from various cells and tissues, it would be useful to mimic and

reproduce the alteration in expression of various pancreatic transcription factors observed during normal pancreas development. In addition, it would be useful to induce pancreatic key transcription factor in some source cells and tissues which have the potency to induce various β-cell-related genes including insulin (Figure 2). It has been reported that various cells and tissues such as embryonic stem cells, liver, pancreas, intestine, and bone marrow can be transdifferentiated into insulin-producing cells. It was shown that embryonic stem cells have the potential to differentiate into insulin-producing cells (84-87), but the 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 also 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 (31, 34, 36, 88-91). Also, 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 (30, 37-40, 55, 91-93). Intestinal epithelium-derived cells and some populations of bone marrow cells were also shown to have the potential to differentiate into insulin-producing cells (32, 33, 36, 94, 95). In such studies, several pancreatic transcription factors have been used to induce insulin-producing cells from various cells and tissues. Indeed, it was reported that adenoviral PDX-1 expression in the liver of mice induced expression of the endogenous insulin mRNA. Also, hepatic immunoreactive insulin induced by PDX-1 was processed to mature insulin which was biologically active (30). These data indicate the capacity of PDX-1 to reprogram extrapancreatic tissue towards a β-cell phenotype, which may provide a valuable approach for generating surrogate



**Figure 3.** Tissue-specific overexpression of PDX-1 using the Cre/loxP-mediated approach. We previously generated CAG-CAT-PDX1 mice, a transgenic line which constitutively expresses the PDX-1 gene under the control of the chicken  $\beta$ -actin gene (CAG) promoter after the removal of floxed stuffer sequence (CAT) by Cre-mediated recombination. When the mice are crossed with Ptf1a-Cre mice, which express the Cre recombinase driven by the Ptf1a (PTF1-p48) gene promoter, PDX-1 is expressed in precursors of all three pancreatic cell types: islets, acini, and ducts. Also, when the mice are crossed with Alb-Cre mice, which express the Cre recombinase driven by the rat albumin gene promoter, PDX-1 is expressed in hepatocytes and cholangiocytes.

$\beta$ -cells, suitable for replacing impaired  $\beta$ -cell function found in diabetes. These results also suggest that it is useful to induce pancreatic key transcription factor in various cells and tissues which have the potential to induce various  $\beta$ -cell-related genes including insulin.

Next, to carry out efficient screening of somatic tissues and cells that can transdifferentiate into  $\beta$ -cell-like cells in response to PDX-1, we previously generated CAG-CAT-PDX1 mice, a transgenic line which constitutively express the PDX-1 gene under the control of the chicken  $\beta$ -actin gene (CAG) promoter after the removal of floxed stuffer sequence (CAT) by Cre-mediated recombination (37) (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 and exocrine genes such as elastase-1 and chymotrypsinogen 1B (39). The mice, however, exhibited marked jaundice due to 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 would be useful for efficient screening of PDX-1 responsive somatic tissues and cells. Considering the fact 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 evaluation of the transdifferentiation potential of PDX-1 *in vivo*.

#### 7. PDX-1-VP16 EFFICIENTLY INDUCES INSULIN-PRODUCING CELLS FROM NON- $\beta$ -CELLS

Since the pancreas and liver arise from adjacent regions of the endoderm in embryonic development, the liver has been thought to be a potential target for diabetes gene therapy (30, 37-40, 54, 92, 93). 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 prepared. XlHbox8-VP16 was expressed only in the liver of the tadpoles. In the transgenic tadpoles, the liver was converted into a pancreas, containing both exocrine and endocrine cells, while characteristics as a liver were lost from the regions converted to the pancreas (96). In contrast, conversion of the liver to a pancreas was not observed by expression of XlHbox8 alone (without VP16).

Based on these findings in tadpoles (96), 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 induced insulin-producing cells in the liver and ameliorated

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glucose tolerance in diabetic mice (38-40). In addition, it was shown that PDX-1-VP16 efficiently induced insulin gene expression in the liver especially in the presence of pancreatic transcription factors NeuroD or Ngn3 (38). Although PDX-1-VP16 exerted only a slightly greater effect on the insulin promoter compared to 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), both insulin 1 and 2 mRNA was detected in the liver, although insulin 1 was not detected by the expression of wild type PDX-1 (without VP-16) (38). Ad-PDX-1-VP16 treatment, together with Ad-NeuroD or Ad-Ngn3, induced larger amounts of insulin gene expression. After treatment with Ad-PDX-1-VP16 plus Ad-NeuroD (or Ad-Ngn3), insulin-positive cells and insulin secretory granules were observed in the liver upon immunostaining and electron microscopy, respectively (38). Furthermore, various endocrine pancreas-related factors such as islet-type glucokinase, glucagon and somatostatin were induced after treatment with Ad-PDX-1-VP16 plus Ad-NeuroD (or Ad-Ngn3). Consequently, in STZ-induced diabetic mice, blood glucose levels were decreased by PDX-1-VP16 alone, which was more pronounced compared to the effects of wild type PDX-1, and were further decreased by overexpression of PDX-1-VP16 plus NeuroD (or Ngn3) (38). The marked effects of PDX-1-VP16 expression, together with NeuroD or Ngn3, on insulin production and glucose tolerance indicate that the 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 fully exert its function (Figure 3). In addition, these results suggest that the synergistic activation of 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- $\beta$ -cells for the achievement of  $\beta$ -cell regeneration therapy in the future.

It was also shown recently that PDX-1-VP16 expressing hepatic cells converted into functional insulin-producing cells in the presence of high glucose (39). 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 was induced by PDX-1-VP16 in the liver cells, 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 seen in  $\beta$ -cells. Almost all  $\beta$ -cell-related transcription factors were induced and hyperglycemia was normalized (39). 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 (39). These results suggest that PDX-1-VP16 is very efficient and useful for replacing reduced insulin biosynthesis and for amelioration of glucose tolerance but that PDX-1-VP16 alone is not enough to induce complete transdifferentiation of various cells to functional insulin-producing cells.

## 8. MafA FUNCTIONS AS A POTENT ACTIVATOR OF INSULIN GENE AND IS A NOVEL THERAPEUTIC TARGET FOR DIABETES

It was previously shown that an unidentified  $\beta$ -cell-specific nuclear factor binds to a conserved cis-regulatory element called RIPE3b1 in the insulin gene enhancer region and is likely to function as an important transactivator for the insulin gene (97, 98). Recently, this important transactivator was identified as MafA, a basic-leucine zipper (bLZ) transcription factor (99-101) (Table 1). The C1/RIPE3b1 activator from mouse  $\beta$ TC-3 cell nuclear extracts was purified by DNA affinity chromatography and two-dimensional gel electrophoresis (101). C1/RIPE3b1 binding activity was found in the 46-kDa fraction at pH 7.0 and pH 4.5. MafA was detected in the C1/RIPE3b1 binding complex by using MafA peptide-specific antisera. In addition, MafA was shown to bind within the enhancer region of the endogenous insulin gene in  $\beta$ TC-3 cells in the chromatin immunoprecipitation assay (101). These results strongly suggest that MafA is the  $\beta$ -cell-enriched component of the RIPE3b1 activator. MafA controls  $\beta$ -cell-specific expression of the insulin gene through a cis-regulatory element called RIPE3b1 and functions as a potent transactivator for the insulin gene (99-104). During pancreas development, MafA expression is first detected at the beginning of the principal phase of insulin-producing cell production (102), while other important transcription factors such as PDX-1 and NeuroD are expressed from the early stage of pancreas development (Figure 1). In addition, while both PDX-1 and NeuroD are expressed in various cell types in islets, MafA is expressed only in  $\beta$ -cells and functions as a potent activator of insulin gene transcription. Thus, the potency of MafA as an insulin gene activator, together with its unique expression in  $\beta$ -cells, raises the likelihood that MafA is the principal factor required for  $\beta$ -cell formation and function. Therefore, it is likely that MafA is a useful factor for generating insulin-producing cells from non- $\beta$ -cells. Furthermore, it was recently reported that MafA knockout mice displayed glucose intolerance and developed diabetes mellitus (105). 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). The MafA (-/-) mice also displayed age-dependent pancreatic islet abnormalities (104). These results strengthen the importance of MafA in maintaining mature  $\beta$ -cell function.

It was shown recently that MafA, together with some other pancreatic transcription factors, efficiently induced insulin gene expression in the liver (103). Basal insulin promoter activity was increased by MafA alone in HepG2 cells, which was much more significant compared to the effect of PDX-1 or NeuroD. Furthermore, MafA, together with PDX-1 plus NeuroD, drastically increased insulin promoter activity (103). These results clearly show that MafA, PDX-1, and NeuroD exert strong synergistic effect on insulin promoter activity. Neither insulin 1 nor insulin 2 mRNA was induced in the liver by Ad-MafA alone, but both insulin 1 and 2 were induced by Ad-MafA plus Ad-PDX-1 (or Ad-NeuroD). Larger amounts of insulin

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1 and 2 mRNA were clearly observed in the liver after the triple infection (Ad-MafA, Ad-PDX-1, plus Ad-NeuroD). Substantial production of insulin protein was also detected in the triple infected liver. In addition, upon immunostaining for insulin, many insulin-producing cells were clearly observed in the liver after the triple infection (103). Consequently, in STZ-induced diabetic mice, and blood glucose levels were dramatically decreased by the triple infection (Ad-MafA, Ad-PDX-1, and Ad-NeuroD). These results suggest a crucial role for MafA as a novel therapeutic target for diabetes and imply that expression of such a combination of transcription factors is very efficient and useful for replacing the reduced insulin biosynthesis found in diabetes (Figure 2).

### 9. PDX-1 POSSESSES PROTEIN TRANSDUCTION DOMAIN IN ITS STRUCTURE AND THEREBY CAN PERMEATE CELL MEMBRANE

Many studies have been performed to overexpress pancreatic transcription factors using various virus-mediated approaches, but it would be difficult to apply the virus-mediated approach to clinical medicine. Therefore, new strategies are necessary to safely deliver such transcription factors. Protein transduction domains (PTDs) such as the small PTD from the TAT protein of human immunodeficiency virus (HIV-1), the VP22 protein of Herpes simplex virus, and the third  $\alpha$ -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, and thus there has been increasing interest in their potential usefulness for the delivery of bioactive proteins and peptides into cells (34, 55, 65). Therefore, regarding the validity of pancreatic transcription factors as therapeutic targets, the protein delivery system is very promising at this point, because it is practically difficult to apply the virus-mediated approach to clinical medicine without side effects. In order to induce surrogate  $\beta$ -cells and apply to clinical medicine, it would be promising to deliver pancreatic key transcription factors into pancreatic source cells and tissues using this protein delivery system (Figure 2). Furthermore, it was shown recently that PDX-1 protein can permeate several cells due to 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. Transduced PDX-1 protein induced insulin gene expression in isolated pancreatic ducts (34). These data clearly suggest that PDX-1 protein transduction could be a safe and valuable strategy for inducing surrogate  $\beta$ -cells from non- $\beta$ -cells without requiring gene transfer technology.

### 10. CHRONIC HYPERGLYCEMIA DETERIORATES $\beta$ -CELL FUNCTION BY PROVOKING OXIDATIVE STRESS, ACCOMPANIED BY REDUCTION OF PDX-1 AND MafA DNA BINDING ACTIVITIES

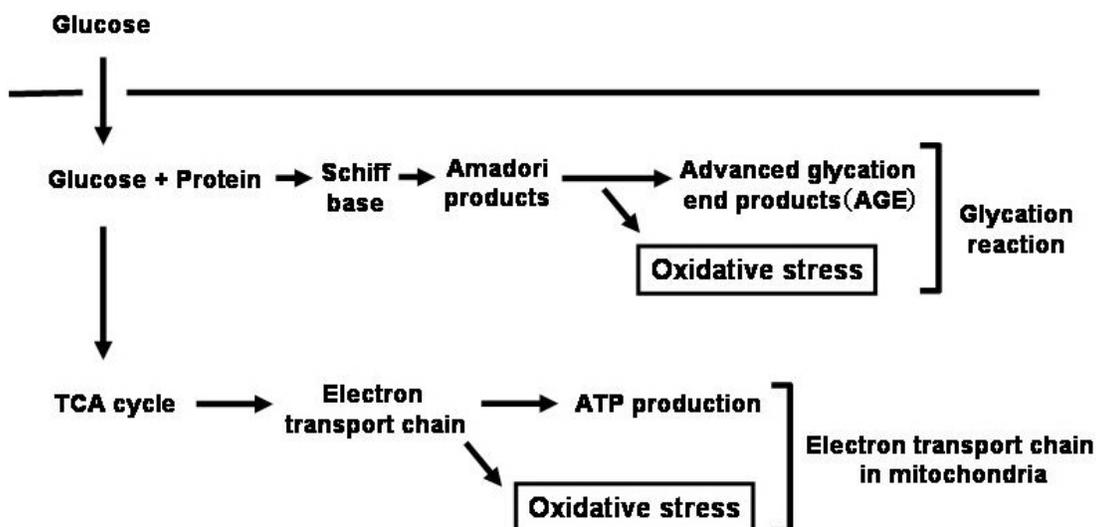
Under diabetic conditions, chronic hyperglycemia gradually deteriorates pancreatic  $\beta$ -cell function. This process is often observed in diabetic subjects

and is clinically well known as  $\beta$ -cell glucose toxicity (97, 98, 105, 106). It has been shown that in the diabetic state, hyperglycemia *per se* and subsequent production of oxidative stress decrease insulin gene expression and secretion (107-119). It has also been shown that the loss of insulin gene expression is accompanied by decreased expression and/or DNA binding activities of PDX-1 (98, 106, 109, 111-113) and RIPE3b1 (which was recently identified as MafA) (97, 98, 105). After chronic exposure to a high glucose concentration, expression and/or DNA binding activities of such transcription factors are reduced. In addition, abnormalities in lipid metabolism have been proposed as contributing factors to deterioration of pancreatic  $\beta$ -cell function. Prolonged exposure to excessive concentrations of fatty acids inhibits insulin gene expression and secretion (2, 120, 121). Furthermore, it has been shown recently that prolonged exposure of islets to palmitate inhibits insulin gene transcription by impairing nuclear localization of PDX-1 and cellular expression of MafA (122).

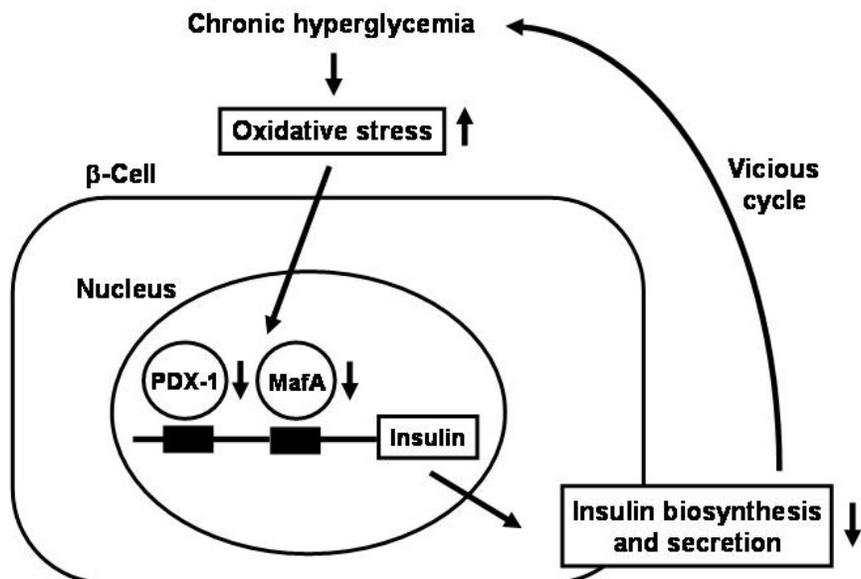
Under diabetic conditions, hyperglycemia induces oxidative stress through various pathways such as the non-enzymatic glycosylation reaction and the electron transport chain in mitochondria (Figure 4), which is involved in the  $\beta$ -cell glucose toxicity found in diabetes (108-119, 123-126).  $\beta$ -Cells express GLUT2, a high-Km glucose transporter, and thereby display highly efficient glucose uptake when exposed to a high glucose concentration. In addition,  $\beta$ -cells are rather vulnerable to oxidative stress due to the relatively low expression of antioxidant enzymes such as catalase, and glutathione peroxidase (127, 128). Indeed, it was shown that expression of oxidative stress markers 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxy-2, 3-nonenal (4-HNE) were increased in islets under diabetic conditions (110, 117).

It was shown that when  $\beta$ -cell-derived cell lines or rat isolated islets were exposed to oxidative stress, insulin gene promoter activity and mRNA expression were suppressed (111, 112, 115, 116). In addition, when  $\beta$ -cell-derived cell lines 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 was prevented by treatment with antioxidants (112, 113, 116, 126). Reduction of expression and/or DNA binding activities of PDX-1 and MafA by chronic exposure to a high glucose concentration was also prevented by an antioxidant treatment. These results suggest that chronic hyperglycemia suppresses insulin biosynthesis and secretion by provoking oxidative stress, accompanied by reduction of expression and/or DNA binding activities of two important pancreatic transcription factors PDX-1 and MafA. Therefore, it is likely that the alteration of such transcription factors explains, at least in part, the suppression of insulin biosynthesis and secretion, and thus are involved in  $\beta$ -cell glucose toxicity (Figure 5).

Next, to evaluate a role of oxidative stress *in vivo*, obese diabetic C57BL/KsJ-db/db mice were treated with antioxidants (N-acetyl-L-cysteine plus vitamin C and



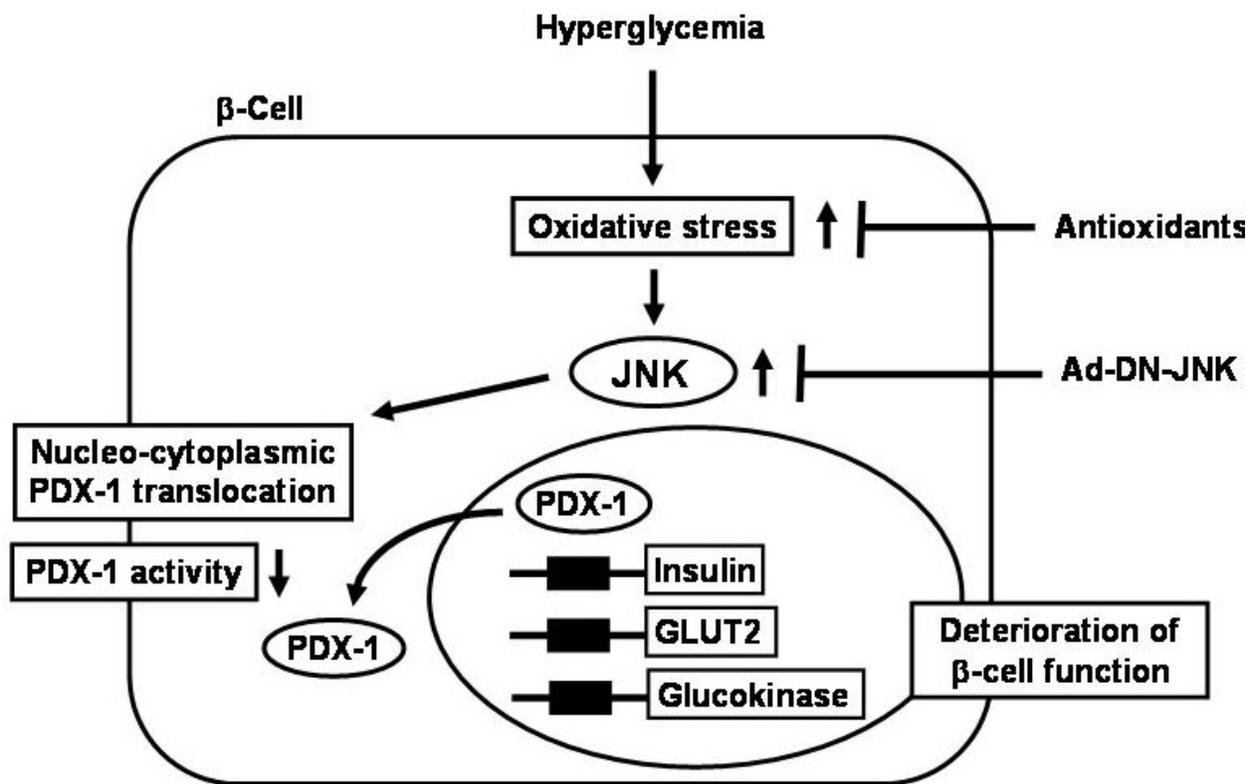
**Figure 4.** Induction of oxidative stress under diabetic conditions. Under diabetic conditions hyperglycemia induces oxidative stress through various pathways such as the non-enzymatic glycosylation reaction and the electron transport chain in mitochondria.



**Figure 5.** Role of PDX-1 and MafA in pancreatic β-cell glucose toxicity. Chronic hyperglycemia deteriorates β-cell function by provoking oxidative stress, accompanied by reduction of PDX-1 and MafA DNA binding activities. This process is often observed under diabetic conditions and is called β-cell glucose toxicity.

E) (112). The antioxidant treatment retained glucose-stimulated insulin secretion and moderately ameliorated glucose tolerance. β-Cell mass was significantly larger in the 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 (112). Similar effects were observed with Zucker diabetic fatty rats, another model animal for type 2 diabetes (113). Taken together, these data indicate that antioxidant treatment can protect β-cells against glucose toxicity. In addition, we examined the possible anti-diabetic effects of probucol, an antioxidant

widely used as an anti-hyperlipidemic agent, on preservation of β-cell function in diabetic C57BL/KsJ-db/db mice (117). Immunostaining for oxidative stress markers such as 4-hydroxy-2-nonenal (HNE)-modified proteins and heme oxygenase-1 revealed that probucol treatment decreased ROS in β-cells of diabetic mice. Probuco treatment also preserved β-cell mass, insulin content, and glucose-stimulated insulin secretion, leading to improvement of glucose tolerance (117). These data suggest potential usefulness of antioxidants for diabetes and provide further support for the implication of oxidative stress in β-cell glucose toxicity found in diabetes.



**Figure 6.** Nucleo-cytoplasmic translocation of PDX-1 by oxidative stress and subsequent activation of the JNK pathway. Oxidative stress and subsequent activation of the JNK pathway induce nucleo-cytoplasmic translocation of PDX-1, which leads to reduction of its DNA binding activity and suppression of insulin biosynthesis

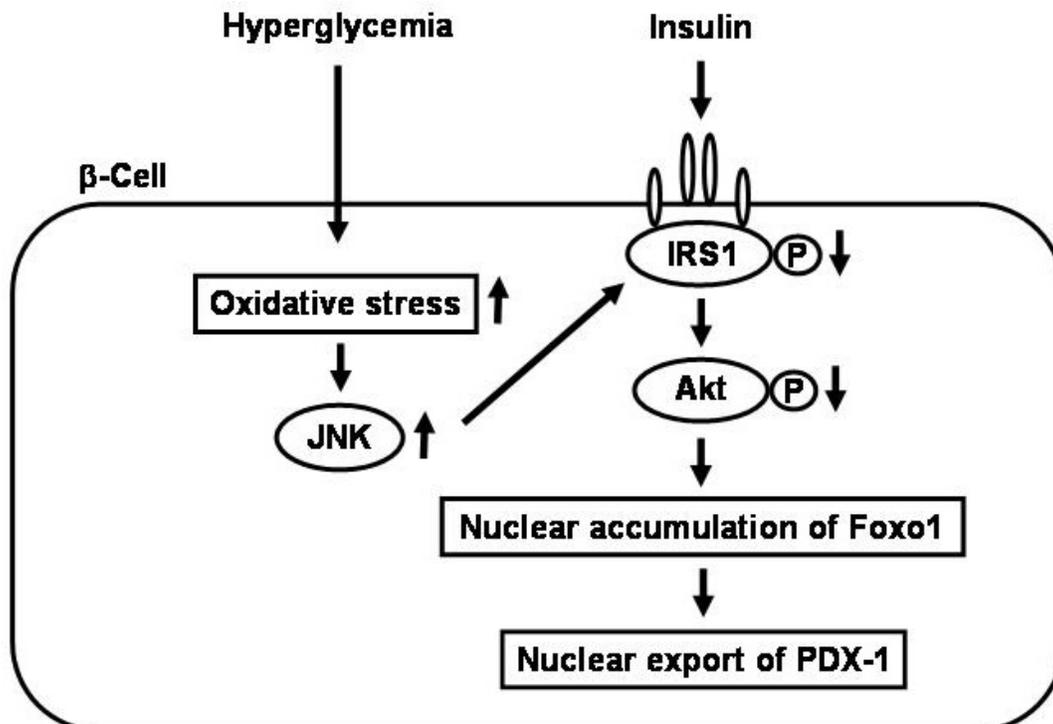
**11. OXIDATIVE STRESS AND SUBSEQUENT ACTIVATION OF THE JNK PATHWAY INDUCE NUCLEO-CYTOPLASMIC TRANSLOCATION OF PDX-1, WHICH LEADS TO REDUCTION OF ITS DNA BINDING ACTIVITY AND SUPPRESSION OF INSULIN BIOSYNTHESIS**

It has been suggested that activation of the c-Jun N-terminal kinase (JNK) pathway is involved in 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 (129). When isolated rat islets were exposed to oxidative stress, the JNK pathway was activated, preceding the decrease of insulin gene expression. Adenoviral overexpression of dominant-negative type JNK1 (DN-JNK) protected insulin gene expression and secretion from oxidative stress. Moreover, wild type JNK1 (WT-JNK) overexpression suppressed both insulin gene expression and secretion (129). These results were correlated with change in the binding of the important transcription factor PDX-1 to the insulin promoter. Adenoviral overexpression of DN-JNK preserved PDX-1 DNA binding activity in the face of oxidative stress, while WT-JNK overexpression decreased PDX-1 DNA binding activity (129). Thus, it is likely that JNK-mediated suppression of PDX-1 DNA binding activity accounts for some of the suppression of insulin gene transcription. Taken together, it is likely that activation of

the JNK pathway leads to decreased PDX-1 activity and consequent suppression of insulin gene transcription found in the diabetic state (Figure 6).

Also, as a potential mechanism for JNK-mediated PDX-1 inactivation, it was recently reported that PDX-1 is translocated from the nuclei to the cytoplasm in response to oxidative stress. When oxidative stress was charged upon β-cell-derived HIT cells, PDX-1 moved from the nuclei to the cytoplasm (130). Addition of DN-JNK inhibited the oxidative stress-induced PDX-1 translocation, suggesting an essential role of the JNK pathway in mediating the phenomenon. Whereas the nuclear localization signal (NLS) in PDX-1 was not affected by oxidative stress, leptomycin B, a specific inhibitor of the classical, leucine-rich nuclear export signal (NES), inhibited nucleocytoplasmic translocation of PDX-1 induced by oxidative stress. Indeed, we identified an NES at position 82-94 of the mouse PDX-1 protein (130). Taken together, it is likely that oxidative stress induces nucleocytoplasmic 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 6).

Furthermore, we have recently reported that the forkhead transcription factor Foxo1 plays a role as a mediator between the JNK pathway and PDX-1 (131). In β-cell-derived HIT-T15 cells, Foxo1 changed its intracellular localization from the cytoplasm to the nucleus



**Figure 7.** Involvement of Foxo1 in nucleocytoplasmic translocation of PDX-1 induced by oxidative stress and subsequent activation of the JNK pathway. Oxidative stress and subsequent activation of the JNK pathway induce nuclear translocation of Foxo1 through the modification of the insulin signaling in  $\beta$ -cells, which leads to the nucleocytoplasmic translocation of PDX-1 and reduction of its DNA binding activity.

under oxidative stress conditions. In contrast to Foxo1, the nuclear expression of PDX-1 was decreased and its cytoplasmic distribution was increased by oxidative stress. JNK overexpression also induced the nuclear localization of Foxo1, but in contrast, suppression of the JNK pathway reduced the oxidative stress-induced nuclear localization of Foxo1, suggesting an involvement of the JNK pathway in Foxo1 translocation (131). In addition, oxidative stress or activation of the JNK pathway decreased the activity of Akt in HIT cells, leading to the decreased phosphorylation of Foxo1 following nuclear localization. Furthermore, adenoviral Foxo1 overexpression reduced the nuclear expression of PDX-1, whereas repression of Foxo1 by Foxo1-specific small interfering RNA retained the nuclear expression of PDX-1 under oxidative stress conditions (131). Taken together, oxidative stress and subsequent activation of the JNK pathway induce nuclear translocation of Foxo1 through the modification of the insulin signaling in  $\beta$ -cells, which leads to the nucleocytoplasmic translocation of PDX-1 and reduction of its DNA binding activity (Figure 7).

## 12. SUMMARY AND PERSPECTIVES

Decrease of functioning pancreatic  $\beta$ -cell number and insufficient insulin biosynthesis and/or secretion are often observed in various diabetic animal models. Pancreas and islet transplantation have exerted beneficial effects for diabetic patients, but the limitation of available insulin-producing cells and requirement of life-long immunosuppressive therapy are major problems.

Therefore, it is very important to search for alternative sources to induce insulin-producing cells. It is likely that PDX-1 and MafA play crucial roles in inducing insulin-producing cells from non- $\beta$ -cells and thus could be therapeutic targets for diabetes. On the other hand, chronic hyperglycemia is a cause of impairment of insulin biosynthesis and secretion. Once hyperglycemia becomes apparent,  $\beta$ -cell function gradually deteriorates. Under diabetic conditions, expression and/or activities of PDX-1 and MafA in  $\beta$ -cells are reduced, which leads to suppression of insulin biosynthesis and secretion. Therefore, it is likely that reduction of PDX-1 and MafA function explains, at least in part, the molecular mechanism for  $\beta$ -cell glucose toxicity and that PDX-1 and MafA could be therapeutic targets for diabetes.

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**Key Words:** PDX-1, MafA, Transcription Factor, Pancreas, Differentiation, Oxidative Stress, JNK, Pathway, Dysfunction, Beta-Cell, Glucose, Toxicity, Review

**Send correspondence to:** Hideaki Kaneto, Department of Internal Medicine and Therapeutics (A8), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan, Tel: 81-6-6879-3633, Fax: 81-6-6879-3639, E-mail: kaneto@medone.med.osaka-u.ac.jp

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