Neurogenin 3 is a Key Transcription Factor for Differentiation of the Endocrine Pancreas

HIROTAKA WATADA
Department of Medicine, Metabolism and Endocrinology, School of Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

Key words: NeuroD, Hes1, Pax4, Nkx2.2, Insulin

Introduction

THE pancreas is composed of exocrine and endocrine compartments. The endocrine compartment consists of the islets of Langerhans, which contain clusters comprising four types of cells: glucagon-producing α cells, somatostatin-producing δ cells, pancreatic polypeptide-producing PP cells, and insulin-producing β cells. Because an inadequate mass of functioning pancreatic β cells is a feature of both type 1 and type 2 diabetes, β cell replacement therapy is considered to be a potential curative treatment. If we can understand how β cells develop normally, it may become possible to use that knowledge to reprogram various cell types to differentiate into new β cells. Thus, studies of pancreatic development may contribute to overcoming diabetes.

One of the main roles of β cells is secretion of insulin in response to an increase of the blood glucose level. To accomplish this, pancreatic β cells express numerous genes that are essential for glucose-responsive insulin secretion, such as glucokinase, Kir6.2, and SUR1, as well as insulin. To allow the expression of such strictly selected multiple sets of genes, various differentiation steps are required during pancreatic development. As is the case for other types of cells, recent studies have identified several transcription factors that control the activation and repression of a large number of genes during pancreatic development and cell biology studies have revealed how these factors function. Accumulation of such knowledge has revealed that transcription factors orchestrate the intricate pathways of cellular growth, death, and differentiation by direct regulation of gene expression. Amongst the transcription factors in this well-organized cascade, neurogenin 3 (Ngn3) plays a key role in determining the fate of cells in the endocrine pancreas. This article discusses the role of Ngn3 during pancreatic development, the regulation of Ngn3 expression and how Ngn3 activates the expression of downstream genes.

Brief outline of development of the endocrine pancreas

In mice, the pancreas initially arises as two buds from the dorsal and ventral sides of the gut at the junction of the foregut and the midgut at the embryonic age 9.5 days (e9.5). At this stage of development, α cells appear as the first differentiated cells of the pancreas and β-cells appear after then. As the gut rotates during development, the ventral bud becomes fused to the dorsal bud, giving rise to the single organ seen in adults. Endocrine cells continue to differentiate from cells into duct-like structures throughout pancreatic development. At about e14, the termini of the duct-like structures begin to form acini and differentiate into the exocrine cells. At the same time, there is a dramatic rise in the insulin level and the number of β cells and for the first time, well-differentiated β cells with characteristic insulin-containing granules are detected. These changes of β cells have been termed the “secondary transition” [1, 2]. The first δ cells also appear around this time, while the PP cells appear shortly...
before birth. The ultimate result of embryogenesis is that the pancreas becomes divided into two major types of cells, which are endocrine cells and exocrine cells. The endocrine cells are further segregated into α, β, δ and PP cells, which form circumscribed clusters within the islets of Langerhans in the mature pancreas.

During these morphological changes, scattered cells in the pancreatic buds begin to differentiate into endocrine cells. Recent cell tracing analyses using Cre-Lox method system have helped to identify the cell lineages involved in development of pancreas. The general strategy of these studies is that transgenic mice carrying Cre-recombinase driven by the promoter of the target gene are crossed with mice in which the reporter gene is designed to only be expressed in cells expressing Cre recombinase. By this method, cells that express Cre recombinase even temporarily are labeled irreversibly, and the fate of a cell that expresses the target gene can thus be investigated. Using glucagon promoter-Cre mice, Herrera demonstrated that insulin-positive cells are not derived from glucagon-positive cells, because there were no cells that co-expressed insulin and the reporter gene [3]. Using Pdx-1-Cre mice, and Ngn3-Cre mice, Gu et al. found that all pancreatic cells are derived from Pdx-1-expressing cells and all pancreatic endocrine cells are derived from Ngn3-expressing cells [4]. While the importance of pdx1 in pancreatic development is evident, its expression is not restricted to pancreatic buds in the early stage of differentiation [5, 6]. On the other hand, Ptf1a is exclusively expressed by the pancreatic buds. Using Ptf1a-Cre knock-in mice, Kawaguchi et al. found that most pancreatic cells are derived from Ptf1a-expressing cells [7]. Thus, these cell tracing studies have revealed that mature pancreatic cells are derived from cells in the pancreatic buds that express Pdx1 and Ptf1a. These cells initially differentiate to Ngn3-expressing common endocrine precursor cells, and then differentiate into mature endocrine cells. Accordingly, β cells are derived from these common precursor cells and not from glucagon-positive cells (Fig. 1) [3, 8].

Importance of Ngn3 during differentiation of the endocrine pancreas

Ngn3 is a transcription factor containing a basic-helix-loop-helix domain (bHLH) as its DNA-binding domain. The involvement of bHLH protein in pancreatic cell function was initially demonstrated by analysis of the insulin promoter [9, 10]. Adjacent to the Pdx1-binding site, conserved CANNTG sequences are found in the insulin promoters of various organisms [11]. This sequence is called the E box and is critical for basal insulin promoter activity. In general, cell-

![Fig. 1. Process of endocrine differentiation in the developing pancreas. The relationship between the state of differentiation and the expression of key transcription factors for each step is shown.](image)
specific bHLH proteins (class B) form dimers with ubiquitously expressed bHLH transcription factors such as E47 and E12 (class A), bind to the E box sequence and are involved in cell-specific gene expression. In mature β cells, NeuroD1 seems to be the most abundantly expressed class B transcription factor and one of the main regulators of insulin transcription [12]. In the pancreas, NeuroD1 is expressed by all endocrine cells, but only during the postmitotic stage of development [13]. Targeted disruption of NeuroD1 causes a decrease of endocrine cell numbers, but this occurs secondary to an increase of apoptosis and, not due to blocking the differentiation of endocrine cells [14]. Beyond insulin gene expression, NeuroD1 also seems to regulate the expression of genes involved in the process of differentiation after determination of cell fate to endocrine pancreas.

NeuroD1 is also expressed in the developmental neuron and plays an important role in neurogenesis [15, 16]. In myogenesis and neurogenesis, different bHLH proteins act to control the sequential stages in the development of a given cell lineage as well as to specify different sublineages. In various organisms, Drosophila atonal homologue, a neurogenin subfamily that is distinguishable from the NeuroD subfamily, is expressed by proneuronal cells [17]. The neurogenin subfamily determines the fate of neurons and induces the expression of NeuroD1, which then regulates terminal differentiation of neurons [18]. Similar to neurogenesis, the neurogenin subfamily controls endocrine cell differentiation during the development of the pancreas.

Ngn3 was discovered by the degenerate RT-PCR method using RNA from neural crest-derived Monc-1 cells. Unlike Neurogenin 1 and 2 that belong to the same family, Ngn3 is expressed by the developing pancreas in addition to neural cells [19]. In mice, Ngn3 expression is initiated at the time of pancreatic budding, when glucagon cells first differentiate. Relatively low levels of Ngn3 expression are then observed until e13.5, after which the major peak of Ngn3 gene expression occurs concomitantly with the secondary transition [20].

The importance of Ngn3 in pancreatic development has been shown by studies of mice lacking the Ngn3 gene. Expression of Ngn3 is not observed in cells expressing pancreatic hormones, and these mice fail to generate any pancreatic endocrine cells without showing an increase in apoptotic events [21]. Thus, the lack of endocrine cells is due to misspecification and Ngn3 is essential for differentiation toward endocrine cells. Further, these mice do not express several transcription factors that function in the later stage of endocrine pancreatic development, including NeuroD1, Pax4 [22], and Pax6 [23, 24]. These transcription factors seem to be either direct or indirect downstream targets of Ngn3. According to these observations, cells expressing Ngn3 in the developing pancreas are considered to be endocrine precursor cells that start to differentiate toward α, β, δ, or PP cells soon after the disappearance of Ngn3. This concept is also supported by cell tracing experiments [4].

Ngn3 is not just an essential factor for the differentiation of endocrine cells. Overexpression of Ngn3 in multipotent pancreatic bud cells with the ability to differentiate into any kind of cell in the pancreas provokes differentiation to glucagon-producing cells [20, 25]. This action is shown to be shared by NeuroD1 and a similar result was obtained by transient expression of Ngn3 in chick endoderm [26]. Thus, the Ngn3-NeuroD1 axis seems to be sufficient for controlling the differentiation of endocrine cells.

The importance of Ngn3 in the control of endocrine cell differentiation can also be seen in the stomach and intestine, which are also derived from the endoderm. All kinds of gastrointestinal epithelial cells, including enteroendocrine cells, are derived from stem cells residing in the base of the crypts. While migrating from crypts to the tips of the villi, the stem cells differentiate into each kind of gastrointestinal epithelial cell. Intestinal enteroendocrine cells are known to comprise at least 15 different types of cells. According to studies of mice lacking the Ngn3 gene, enteroendocrine cells are derived from Ngn3-expressing progenitor cells. Intestinal enteroendocrine cells cannot differentiate without the expression of Ngn3. In contrast, lack of Ngn3 does not impair the differentiation of gastric enteroendocrine cells [27, 28]. These results suggest that the importance of Ngn3 for control of endocrine cell differentiation depends upon its position in the hierarchy of transcription factors in each tissue.

**Regulation of Ngn3 gene expression**

Ngn3 promotes switching from pancreatic precursor cells to pancreatic proendocrine cells. How are cells scattered in the pancreatic bud selected to become
endocrine cells? In neurogenesis, the expression of proneural bHLH genes is regulated by hairy and enhancer of split (HES)-type proteins [29–31], which are defined as anti-neural bHLH genes. Studies using HES1-deficient mice have revealed that HES1 has a role in pancreatic development. These mice show severe pancreatic hypoplasia caused by accelerated differentiation of glucagon-producing cells [32]. The pancreatic phenotype of mice with HES1 deficiency is similar to that caused by overexpression of Ngn3 in the pancreatic buds. These results suggest that HES1 inhibits the expression of Ngn3, and thus maintains cells in an undifferentiated state in the pancreas. Analysis of the Ngn3 gene promoter also supports this observation. This promoter contains at least three HES1-binding sites adjacent to the TATA box sequence, as confirmed by electrophoretic mobility shift assay, and expression of HES1 strongly inhibits Ngn3 gene promoter activity in a manner that depends on the sequence containing the HES1-binding sites [33]. Beyond its role in the pancreas, the importance of the HES1-Ngn3 axis has been demonstrated for the determination of cell fate in the biliary tree, which arises from foregut near the site where the ventral pancreatic bud forms. In Hes1-deficient mice, biliary epithelial cells express ectopic Ngn3 and differentiate into pancreatic endocrine and exocrine cells. These results suggest that expression of Hes1 plays a key role in targeting cells towards the biliary epithelium, probably by repressing the expression of Ngn3 [34].

During neurogenesis, a cell-cell signaling pathway called “lateral inhibition” [35] controls the state of differentiation. Cells that start to differentiate inhibit their neighbors from entering the neural differentiation pathway so that only a subset of the cells that are initially competent to form neurons are allowed to proceed down the default pathway to neural differentiation.

The key molecules in this process are a cell surface receptor, Notch, and its two membrane bound ligands, Delta and Serrate. The ligands Delta and Serrate bind to Notch and thus induce a signaling pathway. The intracellular domain of Notch (Notch ICD) interacts with RBP-Jk, a DNA-binding protein without a transcriptional activation domain, and then translocates to the nucleus. Notch ICD seems to have a transactivation domain, and so the Notch ICD/RBP-Jk complex binds and activates the Hes1 promoter. Cells that accept the Notch signal remain in an undifferentiated state. A similar mechanism directs the endocrine cell fate during the development of the pancreas. Mice deficient for Delta-like gene 1 (Dll1) or the intracellular mediator RBP-Jk show accelerated differentiation of pancreatic endocrine cells [25]. On the other hand, mice with overexpression of Notch1 ICD show inhibited differentiation of both endocrine and exocrine cells [36, 37]. These results suggest that the Dll1-Notch1-Hes1 signal transduction pathway is also essential for maintenance of the undifferentiated state during pancreatic development, and that cells eluding these signals begin to express Ngn3 and then become endocrine cells.

In addition to signals from adjacent cells, signals from neighboring tissues are also important in each process of pancreatic development. Their role during early differentiation, the so-called “initiation process”, was extensively studied and reviewed by Kim and Hebrok [38]. In addition, after the formation of the pancreatic buds, the mesenchymal tissue surrounding the central epithelial cells has an inductive effect on the development of the exocrine pancreas and a repressive effect on the development of the endocrine pancreas [39]. Follistatin antagonizes the actions of members of the TGF-β family and is produced by the mesenchyme, after which it mimics the effects of the mesenchyme on differentiation of cells in the pancreas [40, 41]. These results suggest that members of the TGF-β family are needed for proper differentiation of pancreatic endocrine cells. One of the candidate molecules is activin. For transmembrane signaling, activin requires two types of receptors containing the serine/threonine kinase domain, which are activin receptor type I (ActRI) and type II (ActRII) [42]. Activin directly binds to ActRII and this complex then associates with ActRI, resulting in hyperphosphorylation of ActRI by the kinase activity of ActRII [43]. While disruption of the ActRIIB gene affects the AP patterning of foregut-derived organs, compound heterozygous mice (ActRIIA+/–-IIB+/–) develop the hypoplastic pancreatic islets without showing axial defects [44]. Pancreatic expression of a dominant-negative type II activin receptor in transgenic mice also results in islet hypoplasia [45, 46]. These results suggest that activin signaling might be involved in pancreatic endocrine cell differentiation.

AR42J cells were originally derived from a chemically induced pancreatic tumor and possess both exocrine and neuroendocrine properties. Upon treatment with activin A, AR42J cells stop growing and
extend neurites. In the presence of activin A plus betacellulin or hepatocyte growth factor (HGF), these cells differentiate into insulin-expressing cells [47, 48]. Thus, AR42J cells provide a model for studying the molecular mechanisms of β cell differentiation in response to activin. In this cell line, activin A and HGF can provoke expression of Ngn3 and activate its promoter [49]. A 5' deletion study of the Ngn3 promoter identified the region between –402 bp and –326 bp of Ngn3 as the activin A- and HGF-responsive DNA sequence. This region seems to function as a repressor in AR42J cells, while activin A and HGF antagonize such repression.

Signaling via TGF-β superfamily receptors leads to phosphorylation of receptor-activated Smads (R-Smads), such as Smad2 or Smad3, which bind to the related factor Smad4 and translocate into the nucleus. Once inside the nucleus, this Smad complex associates with other transcription factors to activate the transcription of target genes [43]. On the other hand, activin-responsive activation of Ngn3 is not mediated by a Smad-related pathway. Instead, the TGF-β-activated kinase 1- mitogen-activated protein kinase kinase 3 (MKK3)-p38 mitogen-activated protein kinase (p38MAPK) pathway regulates the function of a critical region of the Ngn3 promoter, probably by influencing the binding of transcription factors between –402 bp and –326 bp [50].

Signals from neighboring cells and tissues are not sufficient to induce the expression of Ngn3 in proendocrine cells, because a longer promoter sequence than the region containing Notch and the ActR-responsive elements seems to be needed to direct its expression by proendocrine cells in the pancreas [33]. Endodermal transcriptional activators of the HNF6 [51], HNF1, and FoxA [33] families all bind to this Ngn3 promoter and may play an important role in the expression of Ngn3 (Fig. 2). At least in the case of HNF6, its importance in regulating Ngn3 gene has been demonstrated by a study using HNF6 gene-disrupted mice, which showed that expression of Ngn3 is remarkably impaired in these mice and only a few mature endocrine cells are observed [51].

Fig. 2. Proposed model of differentiation to proendocrine cells. Ngn3 is regulated by signals from neighboring cells (epithelial-epithelial cell interactions) and mesenchymal cells (epithelial-mesenchymal cell interactions). Intrinsic endodermal transcription factors are also essential.
Regulation of gene expression by Ngn3

Ngn3 is a transcription factor, and to better understand its function, it is essential to identify the factors that it regulates directly. NeuroD1 was one of the candidate factors, because it is known that neurogenin family regulates the proteins of the NeuroD1 family during neurogenesis. In fact, injection of Ngn3 mRNA into Xenopus embryos induces ectopic expression of NeuroD1. The promoter fragments that recapitulate the expression of native NeuroD1 contains 17 E boxes. Among them, the sequences of two of the E boxes are critical for expression of NeuroD1, since Ngn3 can bind to and activate the NeuroD1 promoter through the proximal E boxes [52].

Because Ngn3 is only expressed transiently by proendocrine cells during differentiation of the pancreas, factors that co-express ngn3 in the pancreas like NeuroD1 seem to be the direct downstream target of Ngn3. One such factor may be the paired homeodomain transcription factor Pax4. Disruption of the Pax4 gene impairs β and δ cell differentiation, but does not affect α cell differentiation [22]. During development of the pancreas, the peak of Pax4 expression is observed around the secondary transition stage and it shows partial coexpression with Ngn3 [53]. However, its expression is not observed in mature β cells. Reporter gene analysis in mice and cell lines has revealed that the region around −1900 bp from the transcription initiation site of the Pax4 gene is important for cell-specific expression. The HNF4-binding site, HNF1-binding site, and E box are located within this region and play an important role in the expression of Pax4 [54]. Among them, introduction of a mutation within the HNF1-binding site or E box abolishes its enhancer activity almost completely. HNF1α and Ngn3 can bind to each site, physically interact and synergistically activate the promoter. These data suggest that Ngn3 cooperates with HNF1α, recruit a transcriptional co-activator, and activate Pax4 expression [55].

At e15.5, a burst of cell growth is observed, and the majority of Ngn3-expressing cells also express Nkx2.2. On the other hand, Isl1, Brn4, and PDX1 are never coexpressed with Ngn3 [20]. These results suggest...

---

**Fig. 3.** Potential direct downstream targets of Ngn3. Ngn3 can activate expression of NeuroD1. For the regulation of Nkx2.2 and Pax4 gene expression, Ngn3, physically interacts with endodermal transcription factors and synergistically activates the promoter of each target gene. Interestingly, Ngn3 represses its own promoter although it does not have a strong repression domain.
that Nkx2.2 is one of the possible direct downstream targets of Ngn3. Unlike Ngn3 and Pax4, the expression of Nkx2.2 is also observed in mature islets. Disruption of the Nkx2.2 gene impairs the differentiation of α and β cells [56] and analysis of this gene has revealed that Nkx2.2 possesses at least three alternative first exons (exons 1a, 1b, and 1c). Sequencing of the 5’ flanking regions of these exons has suggested that each fragment functions as a promoter. Studies of transgenic mice with each of the three promoters driving the bacterial LacZ gene have revealed that the 1b promoter (the sequence upstream from exon 1b) specifically restricts expression to islet precursors. On the other hand, the 1a promoter (the sequence upstream from exon 1a) predominantly directs expression in mature islet cells. The transition from proendocrine cells to differentiated endocrine cells should involve progressive changes in gene expression program. Using different sequences within the Nkx2.2 gene, cells in different stages of endocrine differentiation can express Nkx2.2. Expression of the 1a promoter depends on the Foxa-binding site and E box, which are located proximal to the transcription initiation site. Foxa2 and Ngn3 or NeuroD1 can bind to each site to interact and synergistically activate the promoter [57]. These results suggest that Ngn3 or NeuroD1 cooperates with Foxa2 to bind to each site to activate the expression of Nkx2.2. Considering the timing of expression of the Ngn3-NeuroD1 axis, Ngn3 may initiate the enhanced expression of Nkx2.2. After the disappearance of Ngn3, NeuroD1 may act to maintain the expression of Nkx2.2.

The other candidate as a downstream factor directly influenced by Ngn3 is Ngn3 itself. Ngn3 is expressed by proendocrine cells, but its expression is rapidly turned off. Unlike the Pax4 and Nkx2.2 promoter, the Ngn3 promoter is repressed by Ngn3 itself. This repressor activity is also mediated by the E box sequence located proximal to the transcription initiation site. This repressor effect seems to be mediated at least partly by direct competitive binding of another strong transcription factor, because Ngn3 itself does not show transcription repressor activity [58].

Thus, Ngn3 transactivates islet transcription factors such as Pax4 and Nkx2.2 in cooperation with other endodermal transcription factors like HNF1α and Foxa2, while repressing its own promoter.

**Application of Ngn3 to β cell regeneration**

After the formation of mature islets, there is continuous renewal and loss of islet cells throughout life, and regeneration is involved in the renewal process. Although the molecular mechanisms that control islet regeneration have yet to be clarified, the process seems to resemble the developmental process. Several studies have indicated that cells residing in islets and duct cells can differentiate into endocrine cells [59, 60]. Based on cell tracing data, the majority of the progenitors of duct and exocrine/endocrine cells separate before e12.5 [4]. Overexpression of Ngn3 in human duct cells provokes differentiation to β cells, a capability that is shared by NeuroD1. Accordingly the Ngn3-NeuroD axis might serve as a master switch that drives transdifferentiation to an endocrine phenotype when misexpressed by adult duct cells [61]. It was shown previously that overexpression of Ngn3 in immature cells like pancreatic buds induces differentiation into α cells [25] [20]. These differences might be derived from variations in the ability to become β cells. The state of maturity of various cells might be one of the determinants of their fates as α or β cells. Recently, Kojima et al. showed that overexpression of NeuroD1 in the liver leads to ectopic formation of islets in this organ and normalized hyperglycemia induced by streptozotocin. Although the lineage of the cells differentiating into endocrine cells was unclear, this result indicates the usefulness of NeuroD1 for promoting regeneration of β cells from progenitor cells residing in adult organs [62].

**Future prospects**

Genetic and cell biology studies have revealed that Ngn3 is a key factor in the determination of endocrine differentiation. However, we still lack information about the major targets of Ngn3. Disappearance of Ngn3 expression and detection of neuroD1 expression are coincident with exiting of cells from the cell cycle, suggesting that one of the main downstream targets of Ngn3 and/or NeuroD1 is a factor that controls cell cycling [13]. Further, Ngn3-positive endocrine precursor cells can differentiate into the four types of cells that exist in pancreatic islets. Although several factors are essential for such differentiation, identifying the mechanisms that determine the fate of cells after Ngn3
expression is important and will assist in developing an efficient method for generation of pancreatic β cells from non-β cells.

Acknowledgements

The author would like to thank Dr. Yoshio Fujitani (Vanderbilt University Medical Center) for his valuable suggestions to this manuscript, and Dr. Mike German for his great mentorship.

References

23. Sander M, Neubuser A, Kalamaras J, Ee HC, Martin...


