Chronology of endocrine differentiation and beta-cell neogenesis

Takeshi Miyatsuka1), 2), 3)

1) Department of Metabolism and Endocrinology, Juntendo University Graduate School of Medicine, Tokyo, Japan
2) Center for Molecular Diabetology, Juntendo University Graduate School of Medicine, Tokyo, Japan
3) Department of Metabolic Medicine, Osaka University Graduate School of Medicine, Osaka, Japan

Abstract. Diabetes is a chronic and incurable disease, which results from absolute or relative insulin insufficiency. Therefore, pancreatic beta cells, which are the only type of cell that expresses insulin, is considered to be a potential target for the cure of diabetes. Although the findings regarding beta-cell neogenesis during pancreas development have been exploited to induce insulin-producing cells from non-beta cells, there are still many hurdles towards generating fully functional beta cells that can produce high levels of insulin and respond to physiological signals. To overcome these problems, a solid understanding of pancreas development and beta-cell formation is required, and several mouse models have been developed to reveal the unique features of each endocrine cell type at distinct developmental time points. Here I review our understanding of pancreas development and endocrine differentiation focusing on recent progresses in improving temporal cell labeling in vivo.

Key words: Endocrine progenitor, Beta cell neogenesis, Endocrine differentiation

Understanding Endocrine Differentiation towards Developing a Cure for Diabetes

Diabetes mellitus is a chronic metabolic disorder, which can lead to serious complications, such as blindness, kidney failure, amputation, cardiac problems, stroke, etc. A hallmark sign of diabetes is the sustained elevation of blood glucose levels, which results from absolute or relative deficiency of insulin-secreting beta cells within the pancreatic islets. There are several strategies proposed to maintain or expand beta-cell mass (Fig. 1), such as inducing cellular reprogramming into beta cells from somatic cells as well as embryonic stem cells, and stimulating the self-renewal of pre-existing beta cells [1]. Because many pancreas-specific genes that play important roles in pancreas development are expressed during the process of cellular reprogramming towards a beta-cell phenotype, clarifying the molecular mechanisms of pancreas development and transcriptional networks should give us clues towards generating insulin-secreting beta cells, which can be used to cure diabetes. For example, it has been demonstrated that three transcription factors, namely, Pdx1, Neurog3, and Mafa, which play key roles in pancreas development and differentiation (Fig. 2), efficiently promoted the cellular reprogramming of non-beta cells into insulin-producing cells [2–6].

Brief Overview of Endocrine Differentiation

The mature pancreas is composed of exocrine (acinar and duct cells) and endocrine (α-, β-, δ-, ε-, and PP-cells) compartments, which express glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively. The differentiation of these distinct endocrine cell types is coordinately controlled by multiple transcription factors and regulators (Fig. 2) [1, 7, 8]. Among these transcription factors, a key regulator of endocrine specification is Neurogenin 3 (Neurog3, also known as Atoh5), a member of the basic helix-loop-helix (bHLH) transcription factor family. During pancreas development in the mouse embryo, Neurog3 expression is initially detected in a subset of the Pdx1-expressing epithelial cells with the simultaneous repres-
hormones and downstream transcription factors, such as Rfx6, Pax4, Pax6, and Neurod1, demonstrating that Neurog3 plays a pivotal role in endocrine specification and differentiation (Fig. 2).

Although the expression of Neurog3 in the adult pancreas is substantially lower than that in the embryonic pancreas, beta-cell-specific Neurog3 inactivation revealed that Neurog3 is required for promoting beta-cell maturation and maintaining beta-cell function as well as for initiating pancreatic endocrine differentiation during development [15]. Furthermore, it has been shown that the activation of Neurog3 expression can be induced by partial duct ligation in the adult pancreas and the adult Neurog3-expressing cells that are isolated after the ductal ligation give rise to hormone-expressing cells [16], demonstrating the existence of facultative endocrine progenitors that express Neurog3 in the adult pancreas, at least under particular conditions such as ductal ligation.

The significance of NEUROG3 in glucose homeostasis has also been demonstrated in clinical cases [17–19]. Homozygous mutations of NEUROG3 caused childhood-onset diabetes and congenital malabsorptive diarrhea, which is characterized by the lack of enteroendocrine cells. It is noted that C-peptide was detected in the patients with NEUROG3 muta-
tions, suggesting that at least some beta cells are present, which is in contrast to the more severe phenotype observed in Neurog3-deficient mice [13].

Separating Endocrine Progenitors out of Their Descendants with Better Time Resolution

Spatio-temporal approaches, such as in situ hybridization and immunohistochemistry for Neurog3 mRNA and protein, respectively, demonstrated that Neurog3-expressing cells are observed transiently and specifically in ductal epithelial cells or periductal cells, and the expression levels of Neurog3 mRNA are dramatically decreased after birth [9, 10, 20]. This transient nature of Neurog3 expression makes it a useful marker for pancreatic endocrine progenitors that have committed to the endocrine cell lineage but have not yet differentiated into hormone-secreting endocrine cells.

Various mouse models expressing fluorescent proteins, such as eGFP and Ds-Red, have been developed to label specific cell populations, and Neurog3-eGFP mouse lines, which express eGFP under the control of the Neurog3 promoter, have been generated to distinguish endocrine cells from other cell types, such as acinar cells and endothelial cells [21, 22]. Although Neurog3-eGFP mouse lines enable us to investigate gene expression profiles in the cells that have committed to the endocrine lineage, eGFP-expressing cells in Neurog3-eGFP embryos include more differentiated endocrine cells that have already lost their expression of the Neurog3 protein (Fig. 3A), because of the long half-life of fluorescent proteins, which is known to be over 24 hours in vitro [23, 24]. In fact, eGFP-positive cells from Neurog3-eGFP embryos were found to have higher mRNA expression of endocrine hormones, such as insulin 1, insulin 2, and glucagon, than eGFP-negative cells [21]. On the other hand, destabilized fluorescent reporter proteins have shorter half-lives, but their lower fluorescence intensity makes them more difficult to detect [25]. Thus, there had been no practical method to label a specific cell population within a narrow time window.

To overcome this problem, we focused on the unique fluorescent protein DsRed-E5, called “Fluorescent Timer”, which shifts its fluorescence from green to red over time [26], and developed the transgenic mouse model “Neurog3-Timer” in which the upstream and downstream sequences of the human NEUROG3 gene were inserted into a bacterial artificial chromosome (BAC) to drive the expression of “Timer” protein [27]. Theoretically, Neurog3-expressing cells exhibit green-dominant fluorescence and their descendant cells change their fluorescence emission from green to red in a time dependent manner (Fig. 3B). To verify this assumption, green-fluorescent cells were sorted from

---

**Fig. 3**  Labeling of temporal heterogeneity of endocrine differentiation using Neurog3-Timer embryos

(A) A simple schematic showing cell fate changes during endocrine differentiation into beta cells in Neurog3-eGFP embryos. As the half-life of eGFP is much longer than that of Neurog3, both Neurog3-expressing cells and their descendants are labeled with eGFP and it is impossible to distinguish the earliest endocrine progenitors from the more differentiated cells. (B) In Neurog3-Timer embryos, only the endocrine progenitors are labeled as green-fluorescent cells and hence can be isolated from their descendants.
While it has been shown that most descendants of Neurog3-expressing cells are mitotically inactive [10, 12, 30], Neurog3-Timer embryos, combined with living cell cycle analysis by Hoechst 33342 staining, uncovered the precise temporal heterogeneity of cell division in Neurog3-expressing cells and their descendants: >98% of green-fluorescent endocrine progenitors were quiescent in G0/G1 phase, whereas the more differentiated cells re-enter the cell cycle at embryonic day 17.5 (E17.5), but not at E15.5 [31]. In contrast, in Neurog3-null embryos (Neurog3 eGFP/eGFP), 13.0% of eGFP-expressing cells, which have been programmed to transactivate the expression of Neurog3 but fail to express the NEUROG3 protein, were proliferating in S/G2/M phases, demonstrating that Neurog3 plays a role in the shift to cellular quiescence that occurs when common pancreatic progenitors switch to endocrine progenitors (Fig. 4).

To investigate the molecular mechanisms by which Neurog3 exits the cell cycle during endocrine specification, the mRNA levels of 78 cell cycle-related genes were quantified by RT-PCR, which revealed that the mRNA most robustly induced in Neurog3-expressing progenitors was Cdkn1a (also known as p21). In parallel, other negative regulators of cell cycle progression, such as Cdkn1b (p27), Trp53 (p53), and Rb1, were also expressed at high levels in the endocrine progenitors, whereas the positive regulators of cell division, such as Ccnb2, Ccnd2, Cdk6, and Cdc25c, and a cellular marker of proliferation, Mki67 (also known as Ki67), were expressed at low levels. Further transcriptome analysis clarified the expression of transcriptional regulators during endocrine neogenesis and maturation. For example, the expression of Pax4 and Neurod1, which are direct downstream targets of Neurog3 [28], demonstrated a sharp expression peak in green-fluorescent cells and a subsequent decline in green/red double-positive cells. Interestingly, Rfx6, a member of the Rfx family of winged-helix transcription factors, was also highly expressed in Neurog3-expressing progenitors, and we subsequently demonstrated that Rfx6 plays an essential role in islet formation downstream of Neurog3, and furthermore, mutations in the human RFX6 gene were identified in human infants with a similar autosomal recessive syndrome of neonatal diabetes [29]. Thus, chronological analyses using the Neurog3-Timer mouse model help us to refine and explore transcriptional hierarchy with better time resolution.

**Fig. 4** Temporal heterogeneity of cell division during endocrine differentiation

A proposed model of cell cycle regulation by the coordination of Neurog3 and Cdkn1a. Neurog3 is expressed exclusively in endocrine progenitors and directly controls the expression of the Cdkn1a gene by binding to its upstream regulatory regions. Once the expression of Neurog3 is decreased, Cdkn1a mRNA is suppressed, at least partly by miRNAs. (Revised from Miyatsuka T et al. [31])
were suppressed in Neurog3-expressing cells. These changes in expression profiles were not observed in Neurog3-null embryos (Neurog3 eGFP/eGFP), demonstrating the pivotal role of Neurog3 in regulating the cell cycle by inducing Cdkn1a and other cell cycle-related genes.

**Temporal Heterogeneity of Beta-cell Neogenesis**

The usefulness of Fluorescent Timer (DsRed-E5) in investigating endocrine differentiation with better time resolution led us to apply this fluorescent protein to the chronological analysis of beta-cell differentiation, as it remains to be elucidated as to how beta cells differentiate from endocrine progenitors and how recently specified beta cells are different from pre-existing, relatively mature beta cells. To answer these questions, we generated the mouse model “Insulin-Timer,” in which the mouse insulin 1 promoter directs beta-cell specific expression of DsRed-E5 (Fig. 5A) [32]. Because newly specified beta cells derived from progenitors have only green fluorophores that have recently been translated under the control of the insulin 1 promoter, they are labeled green as a whole (Fig. 5A). As the beta cells mature, they contain yellow-to-red fluorophores, as well as green molecules, and therefore, exhibit yellow-to-orange fluorescent signals (Fig. 5B). Flow cytometry with Insulin-Timer embryos and neonates revealed that approximately 1% of the pancreatic cells were observed as green-fluorescent cells (i.e., recently specified beta cells), and the number of green-fluorescent cells was dramatically reduced after postnatal day 1. These findings are consistent with previous studies showing that adult beta cells maintain their mass mostly through the proliferation of pre-existing beta cells, rather than neogenesis from progenitor cells [33–35], and emphasize the potency of this mouse model in the precise quantification of beta-cell neogenesis, saving time and effort associated with histological procedures. In addition, based on a single fluorescent protein with a short (6-hour) maturation window [27], the high temporal resolution enables us to sort the newly generated beta cells from the more differentiated beta cells by FACS. Further omics approaches with these earliest beta cells using Insulin-Timer mouse model will lead to a better understanding of the molecular mechanisms underlying beta-cell neogenesis and maturation.

**Conclusions and Perspectives**

Deciphering the temporal heterogeneity with reporter mouse models carrying Fluorescent Timer (Neurog3-Timer and Insulin-Timer) revealed unique features of endocrine progenitors and newly specified beta cells. Such information has been translated to the efficient induction of cellular reprogramming into insulin-producing cells [36], which will broaden our perspectives of the use of regeneration therapy towards a cure for diabetes. On the other hand, as the green-fluorescent signals of DsRed-E5 are not as bright as its red-fluorescent signals, it is difficult to detect green fluorescence-dominant cells through histological approaches with the reporter mouse models currently available. Alternative approaches will be required to improve the special resolution of in vivo reporter system, as well as its temporal resolution, so that we can clarify when, where, and how endocrine progenitors determine their cell fate and differentiate into hormone-expressing cells not only in the developing pancreas but also during the regenerative process in the adult pancreas.

**Acknowledgements**

I thank Professor Hirotaka Watada for his thought-
ful suggestions and continued support of my studies. I also thank Drs Michael German, Hideaki Kaneto, Takakuki Matsuoka, Yoshio Fujitani, Ichiro Shimomura, and Yoshitaka Kajimoto for their mentoring and collaboration. The author’s research that was introduced in this review was supported by the Juvenile Diabetes Research Foundation Advanced Postdoctoral Fellowship award, JSPS KAKENHI (No. 25461348), the Takeda Science Foundation, the Suzukien Memorial Foundation, and the Lilly Incretin Basic Research Aid Program.

Conflicts of Interest

The author declares that there is no conflict of interest associated with this manuscript.

References

1. Miyatsuka T (2015) Uncovering the mechanisms of beta-cell neogenesis and maturation toward development of a regenerative therapy for diabetes. Diabetol Int 6: 261-267.
2. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455: 627-632.
3. Banga A, Akinci E, Greder LV, Dutton JR, Slack JM (2012) In vivo reprogramming of Sox9+ cells in the liver to insulin-secreting ducts. Proc Natl Acad Sci U S A 109: 15336-15341.
4. Chen YJ, Finkheiner SR, Weinblatt D, Emmett MJ, Tameire F, et al. (2014) De novo formation of insulin-producing “neo-beta cell islets” from intestinal crypts. Cell Rep 6: 1046-1058.
5. Li W, Cavelti-Weder C, Zhang Y, Clement K, Donovan S, et al. (2014) Long-term persistence and development of induced pancreatic beta cells generated by lineage conversion of acinar cells. Nat Biotechnol 32: 1223-1230.
6. Miyashita K, Miyatsuka T, Matsuoka TA, Sasaki S, Takebe S, et al. (2014) Sequential introduction and dosage balance of defined transcription factors affect reprogramming efficiency from pancreatic duct cells into insulin-producing cells. Biochem Biophys Res Commun 444: 514-519.
7. Miyatsuka T, Matsuoka TA, Kaneto H (2008) Transcription factors as therapeutic targets for diabetes. Expert Opin Ther Targets 12: 1431-1442.
8. Puri S, Hebrom M (2010) Cellular plasticity within the pancreas--lessons learned from development. Dev Cell 18: 342-356.
9. Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, et al. (1999) Notch signalling controls pancreatic cell differentiation. Nature 400: 877-881.
10. Jensen J, Heller RS, Funder-Nielsen T, Pedersen EE, Lindsell C, et al. (2000) Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes 49: 163-176.
11. Lee JC, Smith SB, Watada H, Lin J, Scheel D, et al. (2001) Regulation of the pancreatic pro-endocrine gene neurogenin3. Diabetes 50: 928-936.
12. Gu G, Dubauskaite J, Melton DA (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129: 2447-2457.
13. Gradwohl G, Dierich A, LeMieur M, Guillemot F (2000) neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A 97: 1607-1611.
14. Juhl K, Sarkar SA, Wong R, Jensen J, Hutton JC (2008) Mouse pancreatic endocrine cell transcriptome defined in the embryonic Ngn3-null mouse. Diabetes 57: 2755-2761.
15. Wang S, Jensen JN, Seymour PA, Hsu W, Dor Y, et al. (2009) Sustained Neurog3 expression in hormone-expressing islet cells is required for endocrine maturation and function. Proc Natl Acad Sci U S A 106: 9715-9720.
16. Xu X, D’Hoker J, Stange G, Bonne S, De Leu N, et al. (2008) Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 132: 197-207.
17. Wang J, Cortina G, Wu SV, Tran R, Cho JH, et al. (2006) Mutant neurogenin-3 in congenital malabsorptive diarrhea. N Engl J Med 355: 270-280.
18. Pinney SE, Oliver-Krasinski J, Ernst L, Hughes N, Patel P, et al. (2011) Neonatal diabetes and congenital malabsorptive diarrhea attributable to a novel mutation in the human neurogenin-3 gene coding sequence. J Clin Endocrinol Metab 96: 1960-1965.
19. Rubio-Cabezas O, Jensen JN, Hodgson MI, Codner E, Ellard S, et al. (2011) Permanent Neonatal Diabetes and Enteric Anendocrinosis Associated With Biallelic Mutations in NEUROG3. Diabetes 60: 1349-1353.
20. Schwizgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, et al. (2000) Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development 127: 3533-3542.
21. Gu G, Wells JM, Dombkowski D, Preffer F, Aronow B, et al. (2004) Global expression analysis of gene regulatory pathways during endocrine pancreatic development. Development 131: 165-179.
22. White P, May CL, Lamounier RN, Brestelli JE, Kaestner KH (2008) Defining pancreatic endocrine precursors and their descendants. *Diabetes* 57: 654-668.
23. Corish P, Tyler-Smith C (1999) Attenuation of green fluorescent protein half-life in mammalian cells. *Protein Eng* 12: 1035-1040.
24. Verkhusha VV, Kuznetsova IM, Stepanenko OV, Zaraisky AG, Shavlovsky MM, et al. (2003) High stability of Discosoma DsRed as compared to Aequorea EGFP. *Biochemistry* 42: 7879-7884.
25. Li X, Zhao X, Fang Y, Jiang X, Duong T, et al. (1998) Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem* 273: 34970-34975.
26. Terskikh A, Fradkov A, Ermakova G, Zaraisky A, Tan P, et al. (2000) “Fluorescent timer”: protein that changes color with time. *Science* 290: 1585-1588.
27. Miyatsuka T, Li Z, German MS (2009) Chronology of islet differentiation revealed by temporal cell labeling. *Diabetes* 58: 1863-1868.
28. Smith SB, Gasa R, Watada H, Wang J, Griffen SC, et al. (2003) Neurogenin3 and hepatic nuclear factor 1 cooperate in activating pancreatic expression of Pax4. *J Biol Chem* 278: 38254-38259.
29. Smith SB, Qu HQ, Taleb N, Kishimoto NY, Scheel DW, et al. (2010) Rfx6 directs islet formation and insulin production in mice and humans. *Nature* 463: 775-780.
30. Desgraz R, Herrera PL (2009) Pancreatic neurogenin 3-expressing cells are unipotent islet precursors. *Development* 136: 3567-3574.
31. Miyatsuka T, Kosaka Y, Kim H, German MS (2011) Neurogenin3 inhibits proliferation in endocrine progenitors by inducing Cdkn1a. *Proc Natl Acad Sci U S A* 108: 185-190.
32. Miyatsuka T, Matsuoka TA, Sasaki S, Kubo F, Shimomura I, et al. (2014) Chronological analysis with fluorescent timer reveals unique features of newly generated beta-cells. *Diabetes* 63: 3388-3393.
33. Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429: 41-46.
34. Teta M, Rankin MM, Long SY, Stein GM, Kushner JA (2007) Growth and regeneration of adult beta cells does not involve specialized progenitors. *Dev Cell* 12: 817-826.
35. Xiao X, Chen Z, Shiota C, Prasadan K, Guo P, et al. (2013) No evidence for beta cell neogenesis in murine adult pancreas. *J Clin Invest* 123: 2207-2217.
36. Sasaki S, Miyatsuka T, Matsuoka TA, Takahara M, Yamamoto Y, et al. (2015) Activation of GLP-1 and gastrin signalling induces in vivo reprogramming of pancreatic exocrine cells into beta cells in mice. *Diabetologia* 58: 2582-2591.