Zebrafish pancreatic β cell clusters undergo stepwise regeneration using Neurod1-expressing cells from different cell lineages

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Abstract
Pancreatic β cell clusters produce insulin and play a central role in glucose homeostasis. The regenerative capacity of mammalian β cells is limited and the loss of β cells causes diabetes. In contrast, zebrafish β cell clusters have a high regenerative capacity, making them an attractive model to study β cell cluster regeneration. How zebrafish β cell clusters regenerate, when the regeneration process is complete, and the identification of the cellular source of regeneration are fundamental questions that require investigation. Here, using larval and adult zebrafish, we demonstrate that pancreatic β cell clusters undergo a two-step regeneration process, regenerating functionality and then β cell numbers. Additionally, we found that all regenerating pancreatic β cells arose from Neurod1-expressing cells and that cells from different lineages contribute to both functional and β cell number recovery throughout their life. Furthermore, we found that during development and neogenesis, as well as regeneration, all β cells undergo Neurod1 expression in zebrafish. Together, these results shed light on the fundamental cellular mechanisms underlying β cell cluster development, neogenesis, and regeneration.

Keywords Zebrafish · Pancreatic β cells · Regeneration · Neurod1 · Lineage tracing

Introduction

The pancreas is a vertebrate-specific organ with two types of functions (Cleaver and Melton 2007; Matsuda 2018). One function involves the exocrine system, which is composed of acinar cells and ductal cells and which store and deliver digestive fluids, respectively. Another is the endocrine system, which involves the islets of Langerhans (pancreatic islet). The pancreatic islets are composed of five types of endocrine cells: α cells, β cells, δ cells, ε cells, and PP cells, each of which secretes a different hormone: glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively (Cleaver and Melton 2007; Matsuda 2018). Among these cells, β cells have been the most actively studied in various fields of research such as physiology, developmental biology, and medicine. Because pancreatic β cells secrete insulin, which is the only hormone capable of lowering blood glucose levels, they are essential for glucose homeostasis (Cleaver and Melton 2007; Matsuda 2018).

To function normally, vertebrate β cells form a cluster in the pancreatic islet. However, mammalian β cell clusters are poorly regenerative and loss of β cells leads to a reduction of insulin secretion, followed by the development of diabetes. In contrast to mammals, the teleost zebrafish can regenerate β cell clusters throughout their life (Curado et al. 2007; Pisharath et al. 2007; Moss et al. 2009; Matsuda 2018). Therefore, understanding how and why this fish can regenerate its β cell clusters could provide unique information for mammalian regeneration of these cells.

It has been reported in larval zebrafish that new β cells appear 2 days post–β cell ablation (dpa) (Curado et al. 2007; Pisharath et al. 2007). It is not clear how the β cell numbers recover and when the cluster regeneration is complete. It has also been suggested that α cells contribute to this regeneration through transdifferentiation (Ye et al. 2015); however, at most, their contribution has been reported to be 13% (Ye et al. 2015). Furthermore, there are also reports that some cell types in pancreas, which are centroacinar cells (a ductal cell type) and ductal progenitor cells, which contribute to
islet neogenesis, and other endocrine progenitor cells, and δ1 cells, β/δ1 hybrid cells, which are other pancreatic endocrine cells, also contribute to β cell regeneration (Delaspre et al. 2015; Ghaye et al. 2015; Carriil Pardo et al. 2022; Singh et al. 2022; Wang et al. 2020), although it is unclear how much these cell types contribute to β cell cluster regeneration. Thus, the process of β cell cluster regeneration in zebrafish currently has various fundamental questions which require investigation.

In this study, we first decided to investigate the regeneration phenomenon in detail using larval zebrafish, focusing on determining how β cell clusters regenerate, when the process of β cell cluster regeneration is complete, and from what kind of cells most of regeneration for β cells arise. Several transgenic lines that are useful for analyzing cell differentiation and cell lineages were used. Our results showed that the number of β cells increased until 3 dpa, temporarily stopped increasing, then started increasing again after recovering β cell cluster functionality, and finally recovered to the normal number by 13 dpa. On the other hand, whole glucose levels were recovered by 5 dpa. This suggests that zebrafish β cell clusters regenerate functionally and morphology in a stepwise manner. In addition, we found that all regenerating β cells arise from Neurod1-expressing cells. In addition, we showed that Neurod1-expressing cells are also the primary source of β cells during development and neogenesis. This strongly suggests that all zebrafish pancreatic β cells undergo Neurod1 expression. Furthermore, our results suggest that β cells in the phase of “functional recovery” arose from Neurod1-expressing cells, which already existed in the islets, and that the Neurod1-expressing cells in the “cell number recovery” phase were newly generated after functional regeneration. This contribution of Neurod1-expressing cells is also conserved in the β cell regenerative process of adult zebrafish, suggesting that Neurod1-expressing cells of different cell lineages are used throughout their life to regenerate function and cell number in a stepwise manner. These results shed light on the fundamental cellular mechanisms underlying β cell cluster development, neogenesis, and regeneration.

Results

Changes in β cell number and whole glucose levels during β cell cluster regeneration

To study β cell cluster regeneration, we required a system that could ablate β cells easily and consistently. In this study, we used the nitroreductase/metronidazole (NTR/Mtz) method for β cell ablation; this method was established to ablate specific cells conditionally (Curado et al. 2007; Pisharath et al. 2007). Mtz, which is a prodrug, is reduced by nitroreductase (NTR), then changes from a non-toxic to a cytotoxic active form. Therefore, by expressing NTR in target cells, we can ablate target cells in a Mtz-dependent manner. In all experiments for β cell cluster regeneration, we used the ins:NTR transgenic line (a transgenic line with NTR under a promoter from a gene specific for β cells, ins, which encodes the Insulin protein) to specifically ablate β cells (Anderson et al. 2012).

Using this system, we first observed changes in the morphology of pancreatic islets and the cell number of β cell clusters after β cell ablation, to understand how and to what extent the regeneration of β cell clusters proceeds. For this objective, we generated the triple transgenic line, ins:Switch/gcgα:GFP/ins:NTR, by crossing them with the following transgenic lines: ins:Switch (Supplement Fig. 1a; Hesselson et al. 2011), which expresses mCherry under normal conditions (Supplement Fig. 1b); gcga:GFP (Zecchin et al. 2007), which expresses GFP specifically in α cells; and ins:NTR. Then we ablated β cells from these ins:Switch/gcgα:GFP/ins:NTR lines via treatment with 24 h of Mtz between 3 and 4 days post-fertilization (dpf), followed by observation of the morphological changes of β cell clusters until 15 dpa (19 dpf) (Fig. 1a, a’). We found that the first β cells appeared by 2 dpa and that almost the same size and morphology as the β cell clusters of controls were regenerated by 15 dpa (Fig. 1a’). Next, we monitored changes in cell number in the β cell clusters using ins:H2BGFP/ins:NTR transgenic lines (Curado et al. 2007; Anderson et al. 2012; note that ins:H2BGFP is a transgenic line that expresses nuclear GFP in β cells and allows for easy counting of the number of β cells). Zebrafish have two types of islets, a principal islet, which is a single, huge islet, and secondary islets, which are multiple smaller islets (Fig. 1b, b’, c, c’). The principal islet develops during embryogenesis, followed by multiple secondary islets during postembryonic development (Parsons
et al. 2009; Matsuda et al. 2013). When we first counted the β cell number in the principal islet, we found the first β cells on 2 dpa (6 dpf). Although the β cell number increased until 3 dpa (7 dpf), the increase temporarily stopped from 3 to 7 dpa (11 dpf). After 7 dpa, β cells started increasing again. Finally, the number recovered to the same level as that of the developing principal islet by 13 dpa (17 dpf) (Fig. 1d). When we next counted the β cell number in the secondary islet, we found no clear difference in the β cell number between the developing and regenerating pancreas during our period of observation (Fig. 1e). On the other hand, we found that the total number of β cells in the pancreas (sum of the number of β cells in the principal and secondary islets) showed a pattern similar to that in the principal islet (Fig. 1d, f). Together, our results indicate that zebrafish β cell clusters can recover cell numbers to a normal level by 13 dpa. In addition, under our experimental conditions, we did not find that β cell ablation affected secondary islet development. Therefore, in the remainder of our experiments, we analyzed phenotypes in only the principal islet, but not secondary islets, after β cell ablation.

It has previously been reported that whole glucose levels recovered within several days after β cell regeneration (Ye et al. 2015). To confirm these results and to estimate when β cell cluster function was recovered, we next monitored whole glucose levels in ins:H2BGFP/ins:NTR lines treated with or without Mtz, from 3 to 17 dpf (Fig. 1g). We found that whole glucose levels became high immediately after β cell ablation. However, whole glucose levels peaked at 2 dpa (6 dpf), followed by recovery by 5 dpa (9 dpf) (Fig. 1g). These results imply that the functionality of the β cell clusters was recovered by 5 dpa. Interestingly, there is a gap in the recovery timing between glucose levels and cell number after β cell ablation (Fig. 1d, f, g). This may suggest that β cell clusters undergo a two-step regeneration process, first regenerating functionality in a morphologically incomplete cell clusters undergo a two-step regeneration process, first after β cell ablation (Fig. 1d, f, g). This may suggest that β cell ablation. These results demonstrated that, at 3 dpa, approximately 15% of ins:Switch-expressing regenerating β cells were gcga:GFP-expressing cells, but that the remaining 85% of the ins:Switch-expressing cells were adjacent to gcga:GFP-expressing cells (Fig. 2c–c”, d–d’”, e, and Supplement Fig. 2). Interestingly, ins:Switch-expressing cells with gcga:GFP-expression were also always adjacent to other gcga:GFP-expressing cells (Fig. 2c–c”). These results indicated that all regenerating β cells, at least, around 3 dpa arise from cells adjacent to α cells.

All pancreatic β cells undergo Neurod1 expression

Neurod1, which plays an important role in islet development, is known as a pan-endocrine marker (Supplement Fig. 3; Naya et al. 1997; Flasse et al. 2013; Matsuda et al. 2013; Tritschler et al. 2017). To investigate the relationship between Neurod1-expressing cells and regenerating β cells, we examined changes in neurod1:EGFP expression and ins:Switch expression using neurod1:EGFP/ins:Switch/ins:NTR lines (Obholzer et al. 2008; Hesselson et al. 2011; Anderson et al. 2012) after β cell ablation. ins:Switch expression was always observed in neurod1:eGFP-expressing cells at both 2 and 3 dpa (6 and 7 dpf; Fig. 3a–a”, b). These results suggest, as one possibility, that Neurod1-expressing cells are the main source of regenerating β cells. Therefore, we next generated neurod1:Cre transgenic lines and performed cell lineage-tracing experiments using the neurod1:Crel/ins:Switch/ins:NTR triple transgenic line. In the neurod1:Crel/ins:Switch (ins:loxP:mCherry-loxP-H2BGFP) line, β cells that arose from non-Neurod1-expressing cells expressed only mCherry, while β cells that arose from Neurod1-expressing cells expressed H2BGFP (Supplement Fig. 1). These phenotypic analyses showed that all regenerating β cells expressed H2BGFP in a loxP-dependent manner at 13 dpa (17 dpf; Fig. 3c–c”). These results suggest the possibility that regenerating β cells always arise from Neurod1-expressing cells (N1 cells). Next, the contribution of N1 cells to β cell development was investigated using the neurod1:Cre/ins:Switch/ins:NTR triple transgenic line (Fig. 3d–d’’’). At 2 dpf, the embryo stage, all developing β cells expressed H2BGFP. However, some of these β cells still expressed mCherry (Fig. 3d). This suggested that some insulin expressing cells generated from no-Neurod1-expressing cells before 2 dpf. On the other hand, after 5 dpf, which are larvae, all β cells expressed only H2BGFP, but not mCherry, in both principal and secondary islets (Fig. 3d–d’’’). These findings suggest that all β cells in zebrafish express Neurod1, at least transiently, during development, regeneration, and neogenesis.

Regenerating β cells arise from cells in contact with α cells

It has previously been reported that some β cells arise from α cells (gcga:GFP positive glucagon a (gcga)-expressing cells) after β cell ablation, albeit at a low frequency (Ye et al. 2015). To confirm the relationship between regenerating β cells and α cells, we decided to investigate changes in α cell numbers after β cell ablation. As a result, the number of α cells itself was not significantly different from that of the control, although the size of the islet became somewhat smaller after β cell ablation (Fig. 2a, a’, b). Using the gcga:GFP/ins:Switch/ins:NTR line, we next examined the correlation between regenerating β cells and α cells after β cell ablation.
The cell lineage of N1 cells that contribute to early and late regeneration is different

There have already been abundant N1 cells in the principal islet just prior to and after β cell ablation (Supplement Fig. 2). To know if there has already been sufficient number of N1 cells to generate all β cells in the islets just after β cell ablation, we next generated a neurod1:CreERT2 transgenic line, and established neurod1:CreERT2/ins:Switch/ins:NTR lines for lineage tracing experiments (Supplement Fig. 2).
Fig. 3 All pancreatic β cells arose from Neurod1-expressing cells. 

- a–a' Relationship of the localization between neurod1:EGFP-expressing cells and regenerating β cells (ins:Switch-expressing cells) at 2 dpa. 
- b Quantification of the relationship of localization between neurod1:EGFP-expressing cells and regenerating β cells between 1 and 3 dpa (5 and 7 dpf) (mean ± STD; n = 10). Note that most regenerating β cells are neurod1:EGFP-expressing cells (a–a' and b). 
- c–c'' Phenotypes of neurod1:Cre/ins:loxpx-mCherry-loxpx-H2BGFP/ins:NTR at 13 dpa (17 dpf), which distinguished β cells that arose from Neurod1-expressing cells (green cells) and other cells (red cells). Note that all regenerating β cells arose from Neurod1-expressing cells. 
- d–d''' Phenotypes of neurod1:Cre/ins:loxpx-mCherry-loxpx-H2BGFP/ins:NTR during development. Note that all β cells expressed H2BGFP during development and in adults, although mCherry expression (red arrow) remained in some cells at 2 dpa. 

(Rate (%) of c–c'' and d–d''') = 100 x (number of same results sample) / (sample size). Scale bars, 40 μm.
Fig. 1b). For these experiments, neurod1:CreERT2/ins:Switch/ins:NTR lines were treated with or without Mtz from 3 to 4 dpf and with 4-hydroxy Tamoxifen (4OHT) from 4 to 5 dpf, and then their phenotypes were analyzed (Fig. 4a). We first observed phenotypes of developing β cells in neurod1:CreERT2/ins:Switch/ins:NTR lines without Mtz. We found that most of the β cells in the principal islet expressed H2BGFP at both 9 and 17 dpf, although faint mCherry signals remained in some H2BGFP-positive cells (Fig. 4b−b′′′, c′−e′′′). In contrast, in secondary islets, β cells expressed mCherry, but not H2BGFP (Fig. 4d−d′′). These results indicated that N1 cells, which are necessary for β cell development in the principal islet, are already present in the pancreas by 5 dpf, but that N1 cells, which are the source of β cell development in secondary islets, develop after 5 dpf. Next, we analyzed phenotypes of these transgenic lines after β cell ablation. After β cell ablation, all regenerating β cells expressed H2BGFP at 5 dpf in the principal islet, although mCherry signals remained in some cells (Fig. 4e−e′, h). However, mCherry-only expressing cells appeared by 7 dpf in the pancreas of some zebrafish. The number of mCherry-only expressing cells increased after 9 dpf (Fig. 4f−h). On the other hand, the number of H2BGFP-expressing cells did not change between 5 and 13 dpf (Fig. 4e−h). In addition, the total number of H2BGFP- and mCherry-only expressing cells (Fig. 4h) as shown to have a pattern similar to the results of Fig. 1d. These results suggest that all regenerating β cells are generated from N1 cells, which are already present in the principal islet by 5 dpf (9 dpf), and that N1 cells, which are the source of β cells after 5 dpf, are newly generated after 5 dpf.

**Most new Neurod1-expressing cells generate after 7 dpa**

To investigate when new N1 cells, which become the source of β cells after 5 dpf, are generated we treated neurod1:CreERT2/ins:Switch/ins:NTR lines again with 4OHT at a time point between 4 and 11 dpf after treatment with Mtz and 4OHT, as previously investigated, and then analyzed mCherry and H2BGFP expression at 17 dpf (Fig. 5a). In these transgenic lines treated with a second treatment of 4OHT at 4 to 5 or 6 to 7 dpf, the number of H2BGFP-expressing cells and the area of mCherry-expressing cells did not significantly change compared to controls which did not receive a second 4OHT treatment (Fig. 5b, c, f, g). However, in transgenic lines treated with a second 4OHT treatment at 8 to 9 or 10 to 11 dpf, the number of H2BGFP-expressing cells increased and the area of mCherry-expressing cells was reduced significantly (Fig. 5d−g). These results suggest that most of the new N1 cells for regenerating the morphology of β cell clusters are produced after 7 dpa.

**Adult zebrafishes undergo two-step regeneration using Neurod1-expressing cells from different cell lineages**

Neurod1:eGFP-expressing cells are present in adult zebrafish islets both before and after β cell ablation (Fig. 6a, a′). We wondered whether these N1 cells contribute to β cell regeneration in adults. To investigate it, we observed β cell regeneration until 17 dpa using the neurod1:Cre/ins:Switch/ins:NTR triple transgenic line with or without β cell ablation (Fig. 6b−b′′′). The number of H2BGFP-expressing β cells recovered to the number prior to β cell ablation by 17 dpa in this transgenic line (Fig. 6c). On the other hand, blood glucose levels recovered to normal by 5 dpf (Fig. 6d). Thus, there is a gap of recovery timing between blood glucose levels and β cell numbers during β cell regeneration in adults as well. This suggests that in adults, β cell clusters regenerate through a two-step process involving functional recovery followed by β cell number recovery. We next investigated the contribution of N1 cells present in the pancreas prior to β cell ablation, using the neurod1:CreERT2/ins:Switch/ins:NTR triple transgenic line. First, this transgenic line was treated with 4OHT for 24 h. After 1 day of rest, these fishes were treated with Mtz for 24 h and then observed to determine whether the regenerating β cells expressed H2BGFP or mCherry (Fig. 6e−l). At 5 dpf, we found only H2BGFP-expressing β cells or mCherry- and H2BGFP-co-expressing cells but not cells that expressed only mCherry (Fig. 6h, k, l). However, at 17 dpa, a very large number of mCherry-expressing β cells appeared (Fig. 6i). In contrast, H2BGFP-expressing cells showed no significant difference compared to 5 dpf (Fig. 6i, j−l). When this triple transgenic line was treated with 4OHT between 11 and 12 dpf again, mCherry-expressing β cells decreased and H2BGFP-expressing β cells increased at the same levels as those prior to β cell ablation (~1 dpa) (Fig. 6f, j−l). These findings suggest that, in adults as well as larvae, N1 cells in pancreatic islets first recover the function of the β cell cluster by regenerating a small number of β cells, followed by recovering the number of β cells using N1 cells newly generated after β cell ablation.

**Discussion**

In this study, we analyzed the β cell cluster regeneration process using several larval zebrafish transgenic lines to understand which cells were utilized during β cell cluster regeneration and how zebrafish β cell clusters are regenerated. Our results showed that newly formed β cells appeared by 2 dpf and that the number of β cells increased until 3 dpf at which time they stopped increasing. On the other hand, whole glucose levels increased...
Fig. 4 Different cell lineages of Neurod1-expressing cells contribute to β cell regeneration, but not pancreatic development. **a–g** neurod1:CreERT2/ins:loxP-mCherry-loxP-H2BGFP/ins:NTR lines were treated with 4-hydroxy Tamoxifen (4OHT) between 4 and 5 dpf (red arrows in a) with or without Mtz treatment between 3 and 4 dpf (green arrows in a), and then sacrificed for analysis at 9 (b–b’’ and c–e’’), 13 (f), or 17 dpf (c–c’’, d–d’’, and g–g’’). Note that all developing β cells in the principal islet (PI), but not secondary islets (SI), arose from Neurod1-expressing cells from 4 to 5 dpf. **h** Quantification of changes in the number of regenerating β cells with H2BGFP and/or mCherry expression (mean ± STD; n = 5–10). Note that the number of H2BGFP-expressing cells did not change, but the number of mCherry-expressing cells without H2BGFP expression was significantly increased during β cell regeneration after 11 dpa. **P < 0.01 compared with samples at 5 dpa by Tukey’s honestly significant difference test after ANOVA. Scale bars, 40 μm. PI, principal islet cells; SI, secondary islet cells
Fig. 5 Most new Neurod1-expressing cells rapidly generate after 7 dpa. a–f neurod1:CreERT2/ins:lox-p-mCherry-loxp-H2BGFP/ins:NTR lines were treated with 4OHT (red arrows in a) between 4 to 5 (b), 6 to 7 (c), 8 to 9 (d), or 10 to 11 dpa (e) after treatment with Mtz from 3 to 4 dpf (green arrows in a) and 4OHT from 4 to 5 dpf, and then sacrificed for analysis at 17 dpf. f Quantification of changes in the number of regenerating β cells with H2BGFP expression (mean ± STD; n = 9). g Quantification of the area of regenerating β cells with mCherry expression (mean ± STD; n = 9). Note that the number of H2BGFP-expressing cells was elevated after 7 dpf and the area of mCherry-expressing cells was reduced after 7 dpf. *P < 0.05, **P < 0.01 compared with samples at 5 dpf by Tukey’s honestly significant difference test after ANOVA. Scale bars, 40 μm.
neurod1:eGFP/ins:Switch/ins:NTR

-1 dpa 0 dpa

(neurod1:Cre/ins:loxp-mCherry-loxp-H2BGFP/ins:NTR)

-1 dpa 0 dpa 3 dpa 5 dpa 7 dpa 12 dpa 17 dpa

(c) number of β cells

(d) blood glucose (mg/dl)

(e) neurod1:CreERT2/ins:loxp-mCherry-loxp-H2BGFP/ins:NTR

-3 -2 -1 0 5 11 12 17 dpa

(f, k and I)

(g, k and I)

(h, k and I)

(i, k and I)

(j, k and I)

(f) 4OHT

(Mtz)

(g)

(h)

(i)

(j)

number of H2BGFP expressing cells

area of mCherry expressing cells (µm²)
In this study, we found that all zebrafish β cells regenerate via Neurod1-expressing cells (N1 cells). The zebrafish islets already had sufficient N1 cells, which may be used during development, to regenerate functional β cell clusters immediately after β cell ablation (Fig. 7). Furthermore, cell number recovery was also performed by recruiting the missing N1 cells after functional recovery (Fig. 7). Thus, zebrafish regenerate β cell clusters in a very rational way, first using a minimal number of β cells to restore the function of the cluster, and then supplementing the missing cells to complete the regeneration. Likely the gap between the timing of the consumption of N1 cells for “functional recovery” and neogenesis of N1 cells for “cell number recovery” is the reason why the increase of the β cell number stops transiently during larval β cell regeneration.

Most previous zebrafish studies have focused on the phase of functional recovery, which we identified in this study. They reported that α cells, δ1 cells, βδ1 hybrid cells, and some endocrine progenitor cells contribute to β cell regeneration (Ye et al. 2015; Lu et al. 2016; Wang et al. 2020; Carril Pardo et al. 2022; Singh et al. 2022). Our current results suggest that these cells already express Neurod1 or express Neurod1 within 24 h after β cell ablation and that Neurod1 expression is a common characteristic of cells which give rise to the regeneration of β cells. On the other hand, a large number of N1 cells are present in the islets even before β cell ablation. Nevertheless, only a small number of β cells could be regenerated during functional recovery. These results indicate that N1 cells are a heterogeneous population, some of which can give rise to regenerating β cells but not others. Interestingly, in our results, all regenerating β cells, including cells co-expressed with glucagon, were adjacent to α cells (glucagon-expressing cells) at 3 dpf. Given that all β cells arise from N1 cells, this suggests that the regenerating β cells during functional recovery arise from N1 cells adjacent to α cells (Fig. 7). In addition, α cells are likely also a heterogeneous population, some of which are special α cells (glucagon-expressing cells) that are able to participate in β cell cluster regeneration and regulate differentiation of special N1 cells to β cells.

We found that N1 cells for cell number recovery are newly generated after 7 dpf. On the other hand, the question remained regarding the cells that give rise to N1 cells for cell number recovery. Some groups have reported that centroacinar cells and ductal progenitor cells contribute to pancreatic endocrine neogenesis and β cell regeneration (Parsons et al. 2009; Wang et al. 2011; Matsuda et al. 2013; Delaspre et al. 2015; Ghaye et al. 2015). Interestingly, N1 cells also arise from centroacinar cells and ductal progenitor cells during endocrine neogenesis (Kimmel et al. 2011; Ninov et al. 2012; Flasse et al. 2013; Matsuda et al. 2013). These may suggest that for the cell number recovery phase, N1 cells also arise from centroacinar and ductal progenitor cells. On the other hand, it is unclear whether all N1 cells arise from centroacinar cells or ductal progenitor cells during endocrine neogenesis. Hence, the possibility that some N1 cells may arise from other unknown cell populations cannot currently be ruled out. To accurately understand the process of cell number recovery, we need to clarify which cells give rise to N1 cells. If we identify these cell types, it will be a great advantage to understanding the fundamental questions related to the phase of cell number recovery, how
pancreatic islets and the source cells of N1 cells sense the lack of N1 cells, how new N1 cells are generated, and how N1 cells are recruited into β cell clusters. Interestingly, Neurod1 is expressed in the pancreatic islets of adult mammals (Gu et al. 2010). However, it is unclear whether these N1 cells have the potential to contribute to β cell cluster regeneration in a similar way to zebrafish N1 cells. Further characterization of zebrafish N1 cells will reveal whether mammals also have N1 cells which can contribute to β cell cluster regeneration. We will then be able to propose critical methods to make completely functional β cell clusters in the mammalian pancreas for developing new diabetic therapies. Thus, through the results of this study, we have succeeded in making a valuable discovery that is a significant first step toward the regeneration of mammalian β cell clusters.

Materials and methods

Husbandry and fish lines

All experimental animal care was performed in accordance with institutional and national guidelines and regulations. All experiments with zebrafish were approved by the Animal Care Committee of Ritsumeikan University. Zebrafish were raised and maintained under standard laboratory conditions.

We used the following transgenic lines: Tg(ins:FLAG-NTR.cryaa:mCherry950) (Anderson et al. 2012), abbreviated as ins:NTR; Tg(insulin:loxP:mCherrySTOP:loxP:H2B-GFP;cryaa:Cerulean)934 (Hesselson et al. 2011), abbreviated as ins:Switch or ins:loxp-mCherry-lox-H2BGFP; Tg(gcga:GFP)ia1 (Zecchin et al. 2007), abbreviated as gcga:GFP; Tg(ins:H2BGFP; ins:dsRED)960 (Curado et al. 2007), abbreviated as ins:H2BGFP; and TgBAC(neurod1:EGFP)nl1 (Obholzer et al. 2008), abbreviated as neurod1:EGFP.

Nitroreductase-mediated cell ablation

To ablate pancreatic β cells of ins:NTR fish, we incubated 3 dpf larvae for 24 h in 10 mmol/L of metronidazole (Mtz; Wako Pure Chemical Industries, Osaka, Japan) (Curado et al. 2007; Pisharath et al. 2007). To ablate pancreatic β cells of adult ins:NTR fish at 2.5 mpf, 30 mmol/L Mtz in PBS was intraperitoneally injected into ins:NTR fish at a dose of 0.25 g/kg body weight every 12 h (Moss et al. 2009; Delaspre et al. 2015; Mullapudi et al. 2018). After the first injection, ins:NTR fish were incubated for 24 h in 10 mmol/L of Mtz (McMenamin et al. 2014).
Fluorescence imaging

Fluorescence images were acquired with a FV10i-DOC Laser Scanning Microscope (OLYMPUS, Shinjuku, Japan). Cell numbers were counted manually for experiments using ins:H2BGFP, gega:GFP with DAPI, or ins:Switch with DAPI. The area of mCherry in ins:Switch was calculated using FLOVIEW software.

Glucose measurements

Glucose measurements in larvae and adults were performed as described previously (Gut et al. 2013; Matsuda et al. 2017, 2018; Mullapudi et al. 2018, 2019).

Plasmid construction and transgenesis

To generate -5.0neurod1:Cre and -5.0neurod1:CreERT2, the ins:Luc2 region of the ins:Luc2:cyaa:mCherry plasmid (Matsuda et al. 2017) was replaced with a Cre and CreERT2 coding sequence downstream of 5 kb of the neurod1 promoter (Obholzer et al. 2008; McGraw et al. 2012). The -5.0neurod1:Cre, -0.8myl7:eGFP and -5.0neurod1:CreERT2, -0.8myl7:eGFP plasmids was generated by replacing the cryaa:mCherry region of -5.0neurod1:Cre and -5.0neurod1:CreERT2 with -0.8myl7:eGFP, respectively.

To generate each Tg(-5.0neurod1:Cre, -0.8 myl7:EGFP)tm1, abbreviated as neurod1:Cre; and Tg(-5.0neurod1:CreERT2, -0.8myl7:EGFP)tm22, abbreviated as neurod1:CreERT2; the -5kbneurod1:CreERT2:Cre-myl7.08:eGFP and the -5kbneurod1:CreERT2:Cre-myl7.08:eGFP plasmid was co-injected with I-SceI meganuclease (New England Biolabs, Ipswich, MA) into one-cell stage embryos of RIKEN WT (University of Washington) for plasmids of the neurod1 lines, and Dr. David Raible (Institute for Heart and Lung Research) for the neurod1:EGFP plasmid.

Temporal control of CreERT2 activity

Temporal control of CreERT2 activity was achieved using Dox-inducible transgenic lines as previously described (Thermes et al. 2002).

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Declarations

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