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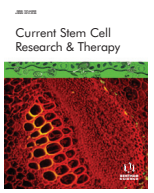
REVIEW ARTICLE

Beta Cell Regeneration in Adult Mice: Controversy Over the Involvement of Stem Cells



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Abstract: Islet transplantation is an effective therapy for severe diabetes. Nevertheless, the short supply of donor pancreases constitutes a formidable obstacle to its extensive clinical application. This shortage heightens the need for alternative sources of insulin-producing beta cells. Since mature beta cells have a very slow proliferation rate, which further declines with age, great efforts have been made to identify beta cell progenitors in the adult pancreas. However, the question whether facultative beta cell progenitors indeed exist in the adult pancreas remains largely unresolved. In the current review, we discuss the problems in past studies and review the milestone studies and recent publications.

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Keywords: Beta cell mass, beta cell neogenesis, beta cell regeneration, Ngn3.

INTRODUCTION

The short supply of donor pancreases prevents extensive clinical application of islet transplantation as a cure for diabetes [1-3], which heightens the need for alternative sources of insulin-producing beta cells. Since mature beta cells have a very slow proliferation rate [4], which further declines with age [5-8], great efforts have been made to identify beta cell progenitors in the adult pancreas [9, 10]. However, numerous studies in the past addressing the identification, purification and differentiation of facultative beta cell progenitors in the adult pancreas have created great controversy. Recent studies using various lineage tracing technologies even question the existence of adult beta cell progenitor cells. In this review, we briefly discuss the problems in past studies and reviewed the milestone studies and recent publications.

PROBLEMS IN PAST STUDIES

It seems that there is a considerable number of studies that either supported or dismissed the presence of adult beta-cell progenitors and/or their contribution to increases in functional beta cell mass under various conditions. However, the shortcomings from previous studies are more and more recognized in that several diverse approaches discussed below are required to solve this central controversy in the diabetes field.

Here, we aim to clarify conceptual misunderstandings in previous publications. As a matter of definition, the total number of beta cells is often referred to as the beta cell mass [4, 11]. Increases in beta cell mass theoretically result from two putative mechanisms: beta cell proliferation (replication of preexisting beta cells) and beta cell neogenesis (generation of beta cells from either progenitor cells or from non-beta cells) [4]. Beta cell regeneration is the umbrella term for these two mechanisms. Therefore, it is a conceptual misunderstanding to equate beta cell regeneration and beta cell neogenesis.

A second flaw in some previous studies is that they discussed the existence of beta cell neogenesis using gene manipulation. Notably, forced expression of key embryonic stem cell determinant genes has been extensively shown to reprogram differentiated cells into induced pluripotent stem cells (iPS), pioneered by Shinya Yamanaka's lab in 2006 [12]. Thus, gene manipulation may allow such reprogramming and transdifferentiation to occur [13]. However, it is conceptually misguided to cite gene manipulation studies as evidence of the existence of beta cell neogenesis in the adult pancreas.

Third, serious problems with the tamoxifen-inducible creERT lineage tracing system have been largely ignored. Although the RIP^{creERT} mouse [14] is so far one of the best creERT mouse models and has been available to other researchers for years, it has still been reported to have either prolonged effects [15], or pre-labeling of beta cells in young [16] and aged [17] mice. In other systems, tamoxifen-induced labeling of the cells has been reported to be inconsistent [18]. In line with these notions, newer creERT strains--especially those that use a transcription factor to drive Cre

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recombinase--could potentially suffer from more severe flaws related to specificity and sensitivity, since the promoter activity of transcription factors is easily altered under various experimental interventions, compared to a hormone gene promoter. Indeed, we have previously shown that a single injection of low-dose tamoxifen is sufficient to alter the transcript levels of many genes in beta cells [19]. Thus, rigorous quality controls should be applied to such lineage-tracing studies. First of all, if an X-creERT/reporter mouse is used to show that Y cells "become" Z cells, besides showing final lineage-tagged Z cells, the initial unique labeling of Y cells needs to be verified to exclude the possibility of mis-labeling in the mouse strain. Moreover, experimental conditions need to be checked to see whether they may induce expression of X in Z cells, which would result in an acquired pre-labeling [19]. Furthermore, new creERT strains should be provided to other researchers to be evaluated independently. To summarize, adequate quality controls and an unbiased approach are needed to rigorously study pancreas "stem" cells.

Importantly, beta cell dedifferentiation should be considered in the interpretation of experimental data. Some studies have been criticized since they examined beta cell neogenesis based on the assumption that a putative β cell progenitor should be characterized by de novo expression of insulin. A major concern is that "beta cell progenitors" might already express insulin. Evidence for this has been provided by the identification of a rare pancreatic multipotent precursor (PMP) cell population expressing insulin and low levels of the glucose transporter Glut2 in mouse and human islets [20, 21]. However, the conception of insulin-positive beta-cell progenitor cells does not fit the data from numerous studies on embryonic pancreas development [10, 22-28]. Moreover, insulin-lineage-tagged non-beta cells have not been found in any of the genetically modified mice in which the insulin promoter was used to drive Cre or CreERT reporter [29]. Additionally, an increasing number of reports have shown that beta cells can dedifferentiate to a certain degree under various conditions, such as before replication or in response to stress [30-34]. Thus, great caution must be taken to distinguish PMP cells from dedifferentiated beta cells.

Some earlier studies have been based on the erroneous assumption that activation of the insulin gene in non-beta cells constitutes generation of a beta cell. Actually, beta cells are one of the most specialized cells in the body, and a variety of criteria must be met to confirm a "functional" beta cell [4, 11]. For example, a functional beta cell should have a certain level of insulin expression, and should secrete insulin in response to glucose challenge. Expression of insulin, especially in low levels and/or without adequate glucose responsiveness, does not guarantee a certain cell to be a true beta cell [4]. Likewise, immune positivity for insulin is not sufficient to demonstrate a beta-cell phenotype. Although it is well known that insulin expression is necessary but not sufficient for determining a "functional" beta cell phenotype, especially in the efforts to generate beta cells from embryonic stem cells [35], this rule is often ignored in animal studies for beta cell neogenesis.

The detection of migrating "neogenic" beta cells from non-pancreatic tissue has been largely overlooked. Since many studies have used detection of insulin-positive cells in ductal- or acinar structures and lineage-labeled insulin-positive cells in the islets as evidence of beta cell neogenesis from non-beta cell sources, migrating beta cells that delaminate, migrate, and eventually join the existing islets should be detected. However, such phenomena have not been reported in any previous studies. This "Pandora's box" has always been kept closed in the context of beta-cell neogenesis, which arouses doubts of its presence at all. In fact, a previous study has elegantly shown that the insulin-positive cells lining the pancreatic ducts are pre-existing beta cells that have failed to delaminate from the duct trunk during embryogenesis [17].

Lastly, it has been reported that reprogramming of beta cells from non-beta cells may occur naturally over extremely long time periods [36, 37]. Although these findings are exciting, we must exercise caution in applying long-term lineage tracing, since recovery of dedifferentiated beta cells and mislabeling may occur easily in such models. A useful example is a study in which islets and acinar cells were all eliminated and had been supposed to be regenerated from the remaining duct structures [38] was later found that regeneration of beta cells actually resulted from the proliferation of surviving beta cells [39].

A REVIEW OF MILESTONE STUDIES

A conceptually innovative study by Dor and Melton from 2004 figures prominently in our discussion of beta cell neogenesis [14]. In this pioneer study, Dor *et al.* introduced a novel method for genetic lineage tracing to determine the contribution of beta cell neogenesis to beta cell mass in the adult pancreas [14]. They generated a transgenic mouse strain that expresses tamoxifen-inducible Cre recombinase under the rat insulin promoter [14]. Tamoxifen injection results in a prompt and transient nuclear translocation of the CreER protein that is previously situated in the cytoplasm [14]. Here, Cre-mediated removal of a stop sequence to result in the constitutive and heritable expression of a human placental alkaline phosphatase (HPAP) reporter was used as a readout [14]. In this powerful system, they show a consistent percentage of labeled beta cells in the adult pancreas, suggesting that pre-existing beta cells, rather than pluripotent stem cells, are the contributors of new beta cells in the adult pancreas. These findings were confirmed independently by several other groups [40-42].

In 2007, another milestone study by Teta and Kushner used a novel double thymidine analogue-labeling strategy to confirm that postnatal β cell growth does not involve progenitor cells [40]. In this highly innovative system, they examined incorporation of two thymidine analogs in tissues of mice. This strategy uses two different forms of anti-BrdU antisera raised in different species, which bind to two thymidine analogs with different affinities. Since each analog detects a distinct round of cell division, this technique allows detection of more than one round of cell division *in vivo*. Significant presence of doubly labeled cells representing those that had recently undergone multiple doublings suggest a substantial contribution of specialized progenitors to the tissue, while absence of such doubly labeled cells dismisses this possibility. Most importantly, this study did not include a popular tamoxifen-inducible lineage tracing technique, which appeared to be the cause of the discrepancy in beta cell lineage tracing in the past. Indeed, as we have dis-

cussed above, poor labeling, pre-labeling and acquired pre-labeling of genetically modified mouse strains had been overlooked and are likely responsible for the past controversy over the existence of beta cell neogenesis. Therefore, the Teta *et al.* study provides an excellent tool for investigating this highly contentious issue.

In 2013, we used another tamoxifen-independent technique to examine nearly all models of adult beta cell growth and regeneration [19]. We crossed insulin-Cre with R26R-loxp-tomato-loxp-GFP to generate compound mice, in which all cells are labeled with tomato, except for the insulin-positive cells and their progeny, which are GFP-positive [19]. In these mice, when non-beta cells start to activate the insulin promoter for the first time, these cells appear yellow for a short time due to co-expression of tomato and GFP, resulting from the slow degradation of tomato protein [19]. This time window allows us to identify beta cells undergoing neogenesis using microscopy and, more objectively, fluorescence-activated cell sorting (FACS). Our conclusion, which is consistent with that of Dor *et al.* and Teta *et al.*, substantiates the notion that beta cell neogenesis does not occur naturally in the adult pancreas. In addition, we used another novel technique called intraductal infusion [34, 43] to label duct cells with a cell-tagged dye CFDA-SE, and proved that neurogenin3 (Ngn3) activation is not sufficient to trigger duct-to-beta cell transdifferentiation [44].

To summarize, innovative and precise techniques are indispensable in studies of beta cell regeneration. Outdated and problematic methods frequently lead to erroneous conclusions and therefore hinder progress on resolving the topic of beta cell neogenesis.

CONTROVERSIES SURROUNDING THE PARTIAL PANCREATIC DUCT LIGATION (PDL) MODEL

PDL has been an established rodent model for both acute and chronic pancreatitis in humans for decades [45-50]. Since 2008, PDL has received renewed attention as a model to activate beta cell progenitors, supported by Ngn3 activation in ductal cells and differentiation of Ngn3-positive cells into insulin-producing cells in embryonic pancreatic explants [51, 52]. However, although differences in mouse strain or surgical technique may influence the outcome of PDL, none of the following studies using either different lineage tracing or innovative strategies supports a detectable contribution to beta cell mass by beta cell neogenesis after PDL [19, 44, 53-61]. In particular, Ngn3 expression and augmentation in pre-existing beta cells have been acknowledged [10, 19, 56, 60, 62] and the validation of the methods that have been used for quantification of beta cell mass in PDL have been questioned [10, 34, 56, 57].

Recent Findings and Continued Controversies

Increased activity of aldehyde dehydrogenase (ALDH) has been used as a stem/progenitor cell marker. Interestingly, it has been reported that ALDH also expresses in the developing mouse pancreas, as well as in centroacinar and terminal duct cells in the adult pancreas, leading to the suspicion that these cells may be stem/progenitor cells in the adult pancreas [63]. However, this year, two studies independently show strong evidence of ALDH activity in proliferating beta cells [64, 65], suggesting that aldefluor lineage-tracing data from pancreas stem cell studies must be interpreted carefully.

A very recent study reported that Pax4-mediated alpha-to-beta-like cell conversion and the consequent loss of alpha cells triggered mobilization and differentiation of pancreatic duct cells into alpha cells, and then into beta cells, due to forced expression of Pax4 in alpha cells [66]. Although attractive, this phenomenon of duct-to-alpha cell conversion under conditions of alpha cell elimination was not observed in two other studies [67, 68]. Similarly, whether pancreatic duct cells contain progenitors for non-beta endocrine cells is not certain. We have previously shown that Ngn3 activation is not sufficient to trigger duct-to-beta cell transdifferentiation [44], and in another independent study, even a combination of Ngn3, Pdx1 and MafA cannot trigger the conversion, which requires an additional transcription factor Pax6 [69]. Thus, it is still doubtful that the pancreatic ducts harbor endocrine progenitor cells.

SUMMARY

Taken together, no convincing data have been provided to support the presence of beta cell neogenesis in the adult pancreas. Gene manipulation may be required to form neogenic β cells from other cell types [13, 69, 70].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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