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Evidence for a Neogenic Niche at the Periphery of Pancreatic Islets

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Abstract

We recently discovered a novel subset of beta cells that resemble immature beta cells during pancreas development. We named these ‘virgin’ beta cells as they do not stem from existing mature beta cells. Virgin beta cells are found exclusively at the islet periphery in areas that we therefore designated as the ‘neogenic niche’. As beta cells are our only source of insulin, their loss leads to diabetes. Islets also contain glucagon-producing alpha cells and somatostatin-producing delta cells, that are important for glucose homeostasis and form a mantle surrounding the beta cell core. This 3D architecture is important and determines access to blood flow and innervation. We propose that the distinctive islet architecture may also play an important, but hitherto unappreciated role in generation of new endocrine cells, including beta cells. We discuss several predictions to further test the contribution of the neogenic niche to beta cell regeneration.

Keywords

virgin beta cell; alpha cell; pancreas islet; Urocortin3; stem cell; transdifferentiation; lineage tracing

1. Introduction

Energy balance is an essential trait that was shaped over millions of years of evolution under circumstances that were largely characterized by the necessity to maintain nutrient homeostasis in the face of intermittent periods of feast and famine. Consequently, we possess at least four partially redundant hormones (glucagon, epinephrine, cortisol and growth hormone) that collectively ensure a stable supply of glucose for our brain during periods of fasting. In contrast, insulin is the only hormone that coordinates the uptake and storage of glucose, free fatty acids and amino acids after a meal, and the pancreatic beta cell is the only source of insulin in our body. Without insulin, most tissues cannot take up glucose and switch to ketone bodies instead, which increase the risk of ketoacidosis. Therefore, the autoimmune-mediated destruction of beta cells that occurs in Type 1 Diabetes

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was invariably lethal until the seminal discovery of insulin^[1] made it possible to manage the disease with exogenous insulin and turned a lethal condition into a chronic disease. Approaching the centennial of its discovery, insulin remains the standard of care for people with Type 1 Diabetes, although a greatly expanded repertoire of synthetic versions of long- and short-acting insulins now exists. These provide a choice of insulin analogs that in combination with sophisticated medical device technologies such as continuous glucose monitoring, insulin pumps and the ‘artificial pancreas’ has empowered patients with Type 1 Diabetes to better manage their disease.^[reviewed by 2, 3] As a direct consequence, the life expectancy of people with Type 1 Diabetes has increased from about 55 years of age in the 1950s to approximately 69 years of age now.^[4, 5] Nevertheless, this continues to lag behind the life expectancy of matched controls by 3–12 years.^[4, 5] There is no cure. Interest in identifying strategies to restore beta cell mass from endogenous sources therefore continues unabated.

2. Background

2.1. All endocrine cells derive from a common progenitor during development

Although best known as the site of insulin-producing beta cells, the bulk of the pancreas consists of exocrine acinar cells that secrete digestive enzymes that are essential for the normal digestion of lipids, protein and carbohydrates before they can be absorbed across the intestinal epithelium. These enzymes are secreted into the lumen of an expansive network of ducts, which add sodium bicarbonate to this mixture. The digestive enzymes and bicarbonate mixture flows to the duodenum via the ductal tree, where these pancreatic juices are mixed with the contents of the small intestine to aid digestion and neutralize the acidic stomach content. Islets of Langerhans are interspersed within the acinar and ductal tissue and make up only approximately 1–2% of the adult pancreas mass. In contrast to the acinar and ductal cells whose secretions are exocrine, islets cells are endocrine cells that release their hormones into the circulation. Indeed, islets are richly vascularized to support the functional maturity of the endocrine cells and ensure effective disbursement of islet hormones upon their release.^[6–8] In addition to insulin producing beta cells, islets contain alpha cells that secrete glucagon, delta cells that secrete somatostatin, and PP cells that secrete pancreatic polypeptide.^[9] Ghrelin-secreting epsilon cells are present during development but are rare in adult islets. Alpha cells are active under low glucose conditions and release glucagon to increase hepatic glucose production to return blood glucose levels to normal.

^[reviewed by 10, 11] Delta cells constitute an important negative feedback mechanism to curb excess insulin and glucagon release.^[12]

The entire pancreas is of endodermal origin and derives from an early branched tubular structure.^[reviewed by 13] The tips of these proto-ducts differentiate into the acini, while the trunk portion will differentiate into the pancreatic ductal tree. The earliest endocrine progenitor cells are marked by the expression of the transcription factor Neurogenin 3 (NGN3) and delaminate from these ducts to form clusters of endocrine cells. These clusters self-organize into pancreatic islets that display a distinct architecture that consists of a beta cell core surrounded by a mantle made up of alpha and delta cells. This arrangement is considered important to control islet cell function and facilitates the proper crosstalk

between alpha, beta and delta cells and their responses to autonomic innervation. Innervation of the islet contributes signaling molecules to the regulation of plasma glucose,^[14] and is organized in distinct ways between mouse and human islets. Mouse islets are richly innervated on all three main cell types (alpha, beta and delta), while human islet endocrine cells are innervated more sparsely, with more of the neurons innervating the smooth muscle cells surrounding the vasculature.^[15] Nevertheless, in human islets, some of the signals that are normally supplied by neurons come from the islet cells instead. For example, the human alpha cell is a source of acetylcholine that helps the beta cells to respond to elevated glucose.^[16] But the distinctive islet architecture is not only important for proper control of islet hormone secretion. Based on a series of observations that we will further detail below, we hypothesize that the islet periphery contains a ‘neogenic niche’ - a privileged microenvironment that supports the conversion of non-beta endocrine cells into mature beta cells and vice versa.

2.2. Beta cell replication is an important mechanism to maintain and restore beta cell mass

The cause of the loss of insulin-producing beta cells was not known until the 1970s and 1980s when it was discovered that Type 1 Diabetes is an autoimmune disease that specifically targets and destroys beta cells.^[17] It is estimated that at diagnosis, months or even years of subclinical beta cell destruction have reduced beta cell mass to less than 10% of the original beta cell mass.^[18] A cure for Type 1 Diabetes therefore requires a two-pronged approach that not only curbs the destructive autoimmune response directed at beta cells that caused the disease, but requires the effective regeneration of beta cells as well. However, the source(s) of new beta cells in the adult pancreas have long been unclear. Seminal work from Yuval Dor and Doug Melton demonstrated that beta cell self-replication is the main mechanism by which beta cells are maintained and expand in mice.^[19, 20] Using a lineage tracing approach (See Box 1), they lineage-labeled approximately 30% of the existing beta cell pool and reasoned that, if new beta cells are formed by the contribution of a stem cell-like progenitor to replace existing beta cells at the end of their lifespan, these processes would dilute the pool of lineage-labeled beta cells over time (Figure 1). Alternatively, if beta cells are long-lived and are eventually replaced by new beta cells that arise via a slow rate of self-replication of pre-existing and differentiated beta cells, the fraction of lineage-labeled beta cells in an islet would remain constant over time. The outcome from their experiment clearly demonstrated that under normal circumstances, self-replication of existing beta cells is the main driving mechanism to maintain and expand beta cell mass in mice.^[19] This finding has since been replicated by others.^[21–23] It makes restoration of beta cell mass by self-replication - in the face of ongoing autoimmune attack that has wiped out the large majority of beta cells - a particularly daunting task. Furthermore, we know that the slow rate of human and mouse beta cell proliferation declines to almost undetectable with age.^[24, 25] Moreover, beta cells become refractory to proliferation-inducing stimuli.^[26–28] Indeed, despite extensive efforts over decades, clinically meaningful restoration of beta cell mass by self-replication has not been achieved.

The lineage labeling strategy in the study by Dor and Melton used a beta cell Cre driver strain (See Box 1) that lineage-labeled only 30% of the beta cells to facilitate a ‘mosaic’

labeling of the beta cell pool^[19]. However, this strategy lacked the power to rule out a minor contribution from non-beta cell progenitors to the existing beta cell mass over time (Figure 1). Indeed, recent reports suggest that in mouse models that are characterized by extensive beta cell mass expansions, as much as 20% of the beta cell pool may derive from non-beta progenitors.^[29] While these observations are perfectly agreeable with the view that self-replication is the most important source of new beta cells in mice, they also indicate that non-beta cell progenitors exist and continue to contribute to the beta cell pool during adult life. This has direct implications for the prospects of restoring beta cell mass in human Type 1 Diabetes.

2.3. Other source(s) of beta cells within the adult pancreas

One potential source of beta cell progenitors is if the adult pancreas contains a population of tissue-resident stem cells that self-renews and gives rise to daughter cells that differentiate into functionally mature beta cells. This would be similar to the small intestine, where LGR5⁺ stem cells in the crypts of Lieberkühn support the turnover of the entire intestinal epithelium - enteroendocrine cells included - every 3–5 days.^[30, 31] The intestinal and stomach enteroendocrine cells both go through an NGN3⁺ stage and are absent in NGN3 null animals.^[32, 33] Given that pancreatic NGN3⁺ endocrine precursors originate from within the early ductal structures during development, the adult ductal tree has been considered as an obvious potential site for such a stem cell-like progenitor. However, the evidence in favor of a ductal progenitor that contributes significantly to beta cell mass is mixed. An NGN3⁺ adult progenitor was identified in the ductal lining that regenerates islet cells upon ligation of the pancreatic duct^[34], although this finding has been convincingly rejected by others.^[35] Indeed, lineage tracing of the entire mature ductal tree by HNF1b-Cre-ER^[36] or Sox9-Cre-ERT2^[37] did not corroborate that ductal cells contribute to the endocrine lineage in pancreas injury models of adult mice.

2.4. Beta cell progenitors within the islet

The question of whether the islet itself contains beta cell precursors besides pre-existing beta cells has in recent years become the focus of a lot of investigations. Pancreatic endocrine cells have long been considered terminally differentiated cells that originate from NGN3⁺ early endocrine progenitor cells that differentiate into alpha, beta, and delta cells via a stepwise series of differentiation events guided by successive expression of lineage-specific transcription factors (Figure 2). Deletion of key lineage-specific transcription factors causes the development of pancreatic islets with altered ratios of endocrine cells. This is illustrated by the opposing actions of ARX and PAX4 on pancreatic endocrine cell development. Constitutive deletion of *Arx* results in a loss of alpha cells accompanied by a gain in beta and delta cells.^[38] Loss of *Pax4* has the opposite effect.^[39, 40] ARX and PAX4 achieve their opposing effects on alpha and beta cell fate by mutual inhibition, with the transcription factor that becomes dominant determining the identity for that islet cell.^[38, 41, 42] A similar interaction exists between NKX6-1 and ARX with NKX6-1 actively repressing *Arx* to suppress alpha cell identity and maintain beta cell identity.^[43] Earlier in development, PTF1a and NKX6-1 are likewise engaged in the determination of endocrine versus acinar cell fate.^[44] Thus a picture emerges of sets of opposing transcription factors that establish and maintain cell fate within the pancreas. This opens up the possibility that perturbations in the

activity of just one of these transcription factors allows a stable cell fate to destabilize and enables the cell to then shift into a different fate.

This principle is supported by experiments where forced expression of key transcription factors in a lineage-inappropriate fashion caused endocrine cells to adopt an alternate fate. Constitutive mis-expression of ARX in the entire pancreas domain, all pancreatic endocrine cells, or all beta cells resulted in reduction of the beta cell population size^[45] and increased alpha cell numbers. Similarly, ectopic and constitutive PAX4 expression in alpha cells drives them towards the beta cell fate.^[46] These experiments have demonstrated that disturbing the balance in the transcription factor network during embryonic development when islet cell identity is established can direct these cells to adopt an alternate fate.

These observations have led to the suggestion that adult islet cell fate too might be redirected from an alpha into a beta cell fate simply by the inhibition of ARX,^[47] although others have reported that ARX inhibition in adult alpha cells by itself is insufficient to promote conversion into beta cells, and that inhibition of additional genes such as DNA methyl transferase 1 (DNMT1) is required.^[48, 49]

Collectively, these experiments demonstrated that alpha cells maintained the ability to adopt a beta-like fate, at least judged by the altered expression of key markers of alpha and beta cell identity. Whether islet endocrine cells were actually capable of doing so in the absence of forced intervention in transcription factor expression was initially not known. To tackle this question, Pedro Herrera's group expressed the diphtheria toxin receptor (DTR) on the cell surface of all beta cells. This enabled the near-complete deletion of all beta cells by the administration of diphtheria toxin. By managing the loss of beta cells and the ensuing diabetes with insulin therapy, they observed that blood glucose values ultimately recovered in conjunction with the re-appearance of detectable levels of endogenous insulin. When they examined the pancreata of these mice, they discovered significant restoration of beta cell mass and used lineage tracing to demonstrate that these beta cells had converted from alpha and delta cells (in a ~4:1 ratio) by transdifferentiation^[50, 51]. In subsequent work, the same group demonstrated that if beta cell ablation takes place early in postnatal development, beta cell mass is predominantly restored by *en masse* transdifferentiation of delta cells.^[51] These studies provided powerful demonstration of the plasticity of the endocrine pancreas to restore functional beta cell mass and revert hyperglycemia.

3. A neogenic niche in the islet

3.1. Ucn3 is a late beta cell maturity marker missing from some peripheral beta cells.

The above-mentioned studies have led to a model where sudden and massive beta cell loss in conjunction with extensive pancreas remodeling triggers a process of restoring beta cell mass via transdifferentiation of nearby alpha and delta cells.^[50, 51] However, we have made a series of observations that instead suggest that plasticity in islet cell fate is a feature of healthy islets in the absence of diabetes, and need not be triggered by extreme beta cell loss. A key marker in this context is Urocortin 3 (UCN3), which is a hormone that is co-released with insulin from beta cells to promote somatostatin secretion from neighboring delta cells.^[12] Somatostatin in turn provides negative feedback control of the beta cell to ensure that

insulin secretion is timely attenuated to prevent hypoglycemia.^[12] In addition to its physiological role in controlling feedback inhibition within the islet, UCN3 is a late maturation marker for beta cells. It is first detectable in a few beta cells by embryonic day 16.5, but it takes until 2–3 weeks postnatally for most beta cells to acquire expression.^[52, 53] This onset of UCN3 expression is later than that of a series of other beta cell markers, including MAFA, Connexin 36, Zinc transporter 8, and several prohormone convertases.^[reviewed by 54] The late onset of UCN3 expression during beta cell maturation combined with the fact that excellent tools exist to detect it, make UCN3 an appealing and convenient marker to study beta cell maturation. To our initial surprise, in staining islets from adult mice for UCN3 and insulin, we observed insulin-positive beta cells with no detectable UCN3 expression. These UCN3-negative beta cells represented approximately 1.5% of all insulin-expressing beta cells in islets from adult mice ranging from 3 weeks to 14 months of age.^[55] Moreover, we consistently observed these UCN3-negative beta cells at the periphery of the mouse islet. We developed an image analysis algorithm to quantify the peripheral location of these cells relative to the center of mass of each islet and the closest islet edge.^[55] By determining the relative location of approximately 17,000 UCN3-positive and -negative beta cells from 10 islets each of 18 mice aged 3 weeks through 14 months, we confirmed that UCN3-negative beta cells were statistically far more likely to occur at the islet periphery than UCN3-positive beta cells.^[55]

3.2. Are peripheral UCN3-negative beta cells immature or dedifferentiated?

While UCN3 is an excellent and late marker of maturity in beta cells, it is also among the first beta cell genes to be downregulated upon beta cell dysfunction and dedifferentiation.^[12, 56] Therefore, the absence of UCN3 expression by itself cannot differentiate between an immature and a dedifferentiated beta cell. We therefore used a *Ucn3*-Cre line to determine whether the peripheral UCN3-negative beta cells we discovered were new, immature beta cells, or preexisting beta cells that had dedifferentiated, by lineage tracing (Box 1). Our experiment clearly demonstrated that peripheral beta cells that lacked UCN3 peptide expression were lineage-negative for UCN3 as well, which means that they or their progenitors had never expressed *Ucn3*. It follows that these UCN3-negative beta cells reflect a novel population of beta cells that originates from a UCN3-negative progenitor that therefore was not an existing, mature beta cell. We named these peripheral UCN3-negative beta cells ‘virgin’ beta cells in reference to their status as new beta cells.

UCN3 is not the only mature beta cell marker that is not yet expressed by virgin beta cells. Virgin beta cells are transcriptionally immature, with reduced expression of the majority of genes in the oxidative phosphorylation and glycolytic pathways compared to mature beta cells from the same islet. They are also depleted in protein expression for G6PC2, ERO1LB, and MAFA, and retain MAFB. Additionally, virgin beta cells have reduced cell surface GLUT2 expression (Figure 3). Similar GLUT2-negative beta cells have recently been described to occur in small clusters separate from regular islets in the pancreatic parenchyma.^[57–59] Although their UCN3-negative status is not known, these cells resemble the virgin beta cell population we discovered on the basis of their GLUT2 status. As a consequence of the lack of cell surface GLUT2 expression, virgin beta cells demonstrate significantly impaired glucose uptake and a resistance to streptozotocin-induced, GLUT2-

mediated beta cell toxicity.^[55] On the other hand, the so-called ‘disallowed’ genes that are expressed during beta cell differentiation but need to be downregulated to support beta cell maturation^[60–62] are enriched in virgin beta cells.^[55] Overall, virgin beta cells in adult islets closely resemble immature beta cells during perinatal development. It is their continued presence in adult islets throughout the life-span that is arguably their most remarkable feature.

3.3. Virgin beta cells are an intermediate stage in the transdifferentiation of alpha to beta cells

The observation of a peripheral population of virgin beta cells that originates from a non-beta cell raises a series of questions regarding the origin of these virgin beta cells. Alpha cells are an obvious candidate progenitor to give rise to virgin beta cells as they 1) have a demonstrated capacity to transdifferentiate into beta cells^[50] and 2) are located just peripherally from the virgin beta cell population within the islet mantle. Indeed, we occasionally observe insulin-positive cells at the islet periphery with an alpha cell lineage label that have lost glucagon expression but do not yet express UCN3 (Figure 4). It follows that alpha cells that transdifferentiate into beta cells first lose glucagon expression (but retain their alpha lineage label) before sequentially acquiring insulin and then UCN3 expression. As a matter of semantics, one could argue that the presence of a distinct and identifiable intermediate state in the conversion of alpha into beta cells is not transdifferentiation, which is defined as the *direct* conversion of one cell type into another. Nevertheless, we adopted the term ‘transdifferentiation’ to be consistent with the work of our colleagues who study similar processes. Indeed, we virtually never observe insulin/glucagon co-positive cells by antibody staining in healthy adult islets, although we occasionally do observe such cells in embryonic mouse pancreata (not shown). The absence of insulin/glucagon co-positive cells at the islet periphery is a significant observation as insulin/glucagon co-positive cells are regularly interpreted to reflect transdifferentiation. In some of these cases, sub-par fluorescence microscopy may have given the incorrect impression of the presence of co-positive cells. However, insulin/glucagon co-positive cells that contain a mixed population of secretory vesicles consistent with dual glucagon and insulin content have also been described by electron microscopy, lending credence to the observation of *bona fide* bihormonal cells under certain circumstances.^[63] Bihormonal cells are often described when transdifferentiation is induced by the selective manipulation of key beta cell transcription factors.^[63–66] Whether such forced transdifferentiation reflects an accelerated version of the same process we observe at the periphery of healthy islets is not known. It is possible that insulin granules are formed in alpha cells upon the induced mis-expression of beta cell-determining factors before the alpha cell fully degranulates or recycles its existing glucagon content. Alternatively, it is possible that transdifferentiation induced by mis-expression of beta cell-selective transcription factors in alpha cells is distinct from the spontaneous conversion of alpha to beta cells that we observed exclusively at the islet periphery.

3.4. Alternative source(s) of cells that may contribute to the formation of virgin beta cells via transdifferentiation at the neogenic niche

Other endocrine cells within the islet can also potentially transdifferentiate into beta cells and thus serve as a source of virgin beta cells at the neogenic niche. Similar to the alpha

cells, delta cells are also located at the islet periphery where the neogenic niche is found. Given their peripheral distribution across the islet architecture, delta cells are uniquely positioned to give rise to the peripherally located virgin beta cells. Indeed, near-total beta cell ablation in juvenile mice has been shown to induce beta cell formation via the transdifferentiation of delta cells.^[51] As older mice partially regenerate beta cells from alpha cells,^[50] this suggests that age-related circumstances contribute to the transdifferentiation process. Other non-endocrine cells within the pancreas, such as the glial cells, immune cells, endothelial cells, and pericytes, may also contribute to the neogenic niche as the source of key signals that maintain the neogenic niche and guide the transdifferentiation and maturation process.

3.5. The neogenic niche supports transdifferentiation of mature beta cells into alpha cells

The selective localization of alpha-to-beta transdifferentiated cells at the islet periphery is an important clue that points to their specific origin at the neogenic niche, as opposed to the stochastic expression of Cre recombinase in beta cells distributed randomly across the beta cell mass (Figure 5A–C). At the same time, it is possible that the peripheral location of these transdifferentiated cells just internally from the alpha cells simply reflects their alpha cell origin and that therefore the niche simply reflects proximity to alpha cells. However, the converse transdifferentiation - from mature beta to alpha cells - also takes place at the islet periphery as is evident from the presence of UCN3 lineage-positive former mature beta cells that now express glucagon instead of UCN3 and insulin (Figure 5D). If mature beta cells were capable of cell-autonomous transdifferentiation (*i.e.* not dependent on their local micro-environment), such formerly mature beta turned alpha cells should have occurred randomly across the crosssectional islet area. Instead, these cells are consistently found at the islet periphery, further supporting a model of a privileged micro-environment that supports cell fate conversions between alpha and mature beta cells in both directions.

It turns out that the presence of a peripheral regenerative niche is not unique to the islet, but has precedent in the hypothalamus-pituitary-adrenal axis. The pituitary gland consists of an anterior and posterior lobe, separated by an intermediate lobe. The marginal zone around the lumen of Rathke's pouch, in-between the anterior and intermediate lobes, has been suggested to serve as the pituitary stem cell niche that is home to several populations of progenitor cells capable of giving rise to all pituitary endocrine lineages.^[67, 68] The adrenal gland consists of a medulla of catecholamine-releasing chromaffin cells and an adrenal cortex made up of steroid hormone-producing cells organized into three distinct layers: an inner *zona reticularis*, a *zona fasciculata*, and an outer *zona glomerulosa*. The entire organ is covered by a capsule consisting of connective tissue that harbors a resident progenitor population that gives rise to *zona glomerulosa* cells, which then migrate inwards and form *zona fasciculata* cells via direct transdifferentiation.^[69, 70] The identification of progenitor populations at the periphery of the pancreatic islet and the adrenal gland suggest that perhaps a specialized peripheral 'niche' that contributes to the maintenance and regeneration of endocrine cells is a feature shared in common among endocrine glands.

3.6. Presence of the neogenic niche in human islets

The discovery of a spatially distinct niche that exists at the periphery of murine islets containing insulin-positive virgin beta cells that have never been mature is exciting. These cells may well represent a progenitor population within pancreatic islets that can be targeted to regenerate beta cells in Type 1 Diabetes. However, the clinical impact of these cells would depend on the presence of a similar neogenic niche in human islets. Adult human islets are architecturally distinct from mouse islets and feature a more loosely organized collections of alpha and beta cells instead of the alpha cell mantle/beta cell core topology of mouse islets.^[71, 72] But similar to rodent development, the earliest detectable UCN3 expression in human beta cells during pancreas development trails insulin expression by 3 weeks^[54] and the expression of UCN3 follows insulin in the differentiation of stem cell-derived beta cells.^[52, 53] These observations established that human beta cells also mature from an insulin single positive stage to an insulin/UCN3 co-positive stage. UCN3-negative beta cells can be readily found in human islets, but these cells can also represent de-differentiated beta cells in diabetes which lose UCN3 expression early on in the progression of disease.^[12, 56] Without the benefit of spatial organization or genetic tracing for cues, it is therefore complicated to determine the status of these Ucn3-negative cells in adult human islets. However, we readily observe UCN3-negative beta cells in young pancreas donors under 3 years of age, which at that age are unlikely to reflect beta cell de-differentiation. Interestingly, the human islet architecture at that young age is also more structured than the adult human islet architecture. Some islets adopting a mantle/core-like topology where UCN3-negative beta cells can be found at the periphery, while others islets consist of larger clusters of alpha and beta cells, where UCN3-negative beta cells can be found at the interface between alpha and beta cell areas.^[55] These observations suggests that a spatially distinct neogenic niche is present at least at an early age in human and persist in some adult human islets^[55] in spite of the progressive intermingling of alpha and beta cells and adult pancreas.

4. Further testing the concept of the neogenic niche

Based on the totality of the observations mentioned above, we hypothesize that the islet periphery contains a 'neogenic niche' - a privileged microenvironment that supports the conversion of non-beta endocrine cells into mature beta cells and vice versa (Figure 6). Our discovery of the continued presence of immature 'virgin' beta cells at the adult islet periphery raises important questions regarding their origins and relevance for the regeneration of beta cell mass. In order to test the above hypothesis, one can make a number of predictions that can be confirmed or rejected based on experimental observations. Implicit in such an exercise is the possibility that the experimental data we collect may lead to the rejection of our above-stated hypothesis in favor of the null hypothesis that there is no relation between virgin beta cells and the presence of mature beta cells with an alpha cell lineage label and the presence of alpha cells with a mature beta cell lineage label at the islet periphery, or that there is no regenerative contribution of the neogenic niche.

4.1. New beta cells formed at the neogenic niche from non-beta progenitors contribute to the beta cell pool.

This can be tested directly by lineage labeling where the existing beta cell pool is lineage labeled by a pulse of tamoxifen (when using a pan-beta cell-specific Cre-ER) or doxycycline (when using a pan-beta cell specific rtTA crossed with *pTREtight-Cre*) followed by a chase period of varying duration. If new beta cells from non-beta sources contribute to the total beta cell mass, the fraction of lineage-labeled beta cells, reflecting beta cells present during the pulse and their progeny, should diminish over time. The magnitude of the dilution of the existing beta cell pool will inform on the relative importance of non-beta vs beta cell progenitors. This experiment is essentially the same as the original study by Yuval Dor,^[19] which taught us that beta cell self-replication is the main mechanism to maintain beta cell mass in mice. However, our observations warrant a careful revisiting of that original experiment. We will induce efficient lineage-labeling of most beta cells and will thus be appropriately powered to detect small contributions to the beta cell pool from non-beta progenitors. By comparing the extent of the lineage dilution following different chase periods, we can infer whether the rate of the contribution of the existing beta cells is constant over time or age-dependent. We know that beta cell proliferation sharply declines with age^[24, 25, 27] while the size of the virgin beta cell pool is constant over time.^[55] It is possible that the contribution of beta cell neogenesis at the niche becomes more prominent in circumstances where beta cell replication is rare, as is the case in older mice or in human islets. Moreover, if new, lineage-negative beta cells at the neogenic niche indeed contribute to the beta cell pool over time, they would be expected to occur preferentially at the islet edge. These predictions can be tested in healthy pancreas, or following the induction of acute or sustained insulin resistance.

4.2. Virgin beta cells mature out of the neogenic niche with time.

We propose that virgin beta cells at the neogenic niche represent immature beta cells that are on a path to become mature and functional beta cells. This can be experimentally tested via lineage labeling. The most straightforward manner to conduct this experiment would be to selectively lineage-label the virgin beta cells and follow their fate to observe if they become mature during a chase period. However, there are currently no inducible Cre drivers (See Box 1) available that are selectively expressed by virgin and not mature beta cells. Therefore, this experiment would have to instead be done by lineage labeling of the entire beta cell pool (mature and virgin) via an inducible, insulin-driven Cre-ER crossed to a suitable lineage reporter via tamoxifen administration. Since both virgin and mature beta cells express insulin, one expects both UCN3-positive and -negative beta cell compartments to be lineage labeled with similar efficiency immediately after lineage labeling. However, if over time some virgin beta cells mature and express UCN3, this would make them indistinguishable from the other mature beta cells in the pool. If no new virgin beta cells are generated to replace the ones that mature, the virgin beta cell population would eventually be depleted. This is not what we observe as a consistent number of virgin beta cells persist until at least 14 months of age, suggesting that any maturation of beta cells out of the virgin beta cell pool is balanced by the regeneration of new virgin beta cells. Therefore, if virgin beta cells mature into the mature beta cell pool, the fraction of virgin beta cells with a lineage label (*i.e.* virgin beta cells that were present during the pulse) should diminish with time. Via

this approach, one can estimate the rate with which the virgin beta cells mature out of the niche in contribution to the mature beta cell pool.

4.3. Transdifferentiation at the neogenic niche accumulates with age.

If alpha cells contribute to the mature beta cell pool by continuing transdifferentiation at the neogenic niche, the fraction of beta cells with an alpha cell lineage label should accumulate with age. This can be tested by carefully quantifying the number of mature, UCN3-expressing beta cells with a Gcg-Cre-dependent lineage label at different ages. Alternatively, this experiment can be done by pulse labeling the alpha cell pool via Gcg-CreER^[73] or *Gcg-rtTA*^[50] $\times$ *pTREtight-Cre*^[74] at a young-adult age, followed by a chase period towards varying ages. Similar experiments can be conducted using a Cre driver under the control of the Sst promoter^[75] to determine if delta cells contribute to the mature beta cell pool via transdifferentiation at the neogenic niche. Regardless of the use of a constitutive or inducible lineage-labeling approach, the expectation is that the number of mature beta cells with an alpha or delta cell lineage label accumulates with age. The rate of such accumulation may be linear or may decline with age, much like the well-known age-dependent decline of the potential for beta cells to proliferate.^[24, 25, 27] Furthermore, these transdifferentiated cells should continue to preferentially locate to the islet periphery.

In addition, lineage labeling the alpha cell pool using an inducible strategy via a defined pulse can reveal if new, unlabeled alpha cells contribute to the pool of pre-existing (lineage-labeled) alpha cells over time. In fact, we have reported a dilution of the fraction of lineage-labeled alpha cells with time that would suggest that new cells do contribute to the alpha cell pool over time.^[55] To determine if this dilution of the pool of pre-existing alpha cells can be explained by transdifferentiation of mature beta cells, we can lineage label mature beta cells with *Ucn3-Cre* to determine if there is a progressive accumulation of UCN3 lineage-positive cells that stains for glucagon instead of insulin and UCN3.

4.4. The fraction of lineage-alpha beta cells increases in the face of ongoing autoimmune destruction.

We know from clinical samples that beta cells persist, even in long-standing Type 1 Diabetes. These data can be interpreted to mean that not all existing beta cells are lost. However, a continued contribution of the alpha cell population to the beta cell pool, despite a steady chipping away of beta cells by the immune response, could also contribute to the continued presence of beta cells in affected pancreata. In this latter case, the fraction of original beta cells will dilute in favor of newly formed, alpha cell-derived beta cells. Thus, in principal we can lineage label alpha cells in a Type 1 Diabetes model such as the non-obese diabetic (NOD) mouse, to determine if there is an increase in the fraction of lineage-alpha beta cells. A practical limitation of this proposed experimental approach is that backcrossing the requisite Cre driver and reporter strains onto the NOD strain will require multiple generations of inbreeding to preserve the complex, polygenic NOD phenotype.^[76, 77]

4.5. Beta cells that originate at the neogenic niche can clonally expand by self-replication.

While alpha-to-beta transdifferentiation is a fundamentally distinct process from the self-replication of beta cells and both processes are routinely considered as alternate strategies to restore beta cell mass; each with its unique set of unmet challenges and limitations. However, transdifferentiation and self-replication both have the potential to regenerate beta cells and may even be intertwined in their contribution to beta cell regeneration. After all, a transdifferentiation event of an alpha to a beta cell could be followed by one or more rounds of replication to further expand the number of beta cells that originates from a single transdifferentiation event. All of the resulting beta cells would carry the alpha cell lineage label. Indeed, it is possible that the eventual regeneration of sufficient beta cell mass to restore insulin independence that was reported following complete beta cell ablation by diphtheria toxin^[50] was achieved by a combination of transdifferentiation followed by self-replication of these newly transdifferentiated beta cells. In normal islets, one telltale sign would be the distribution of beta cells with an alpha cell lineage label across individual islets. If transdifferentiated cells occur largely as isolated cells distributed evenly among many islets, this could suggest a scenario where a larger number of isolated transdifferentiation events are chiefly responsible for the appearance of beta cells from an alpha cell descent. Instead, if beta cells with an alpha cell lineage mark tend to occur in larger clusters in fewer islets, this may betray either an early transdifferentiation event followed by successive rounds of beta cell replication, or denote a hotspot of transdifferentiation restricted perhaps to a neogenic niche. It should be possible to discriminate between these scenarios by applying clonal analysis tools such as the Brainbow mouse, in which Cre-recombinase (Box 1) activity results in the random lineage labeling of each cell by up to eight different fluorescent colors.^[78] This strategy has been used successfully in beta cells to demonstrate that beta cell expansion proceeds chiefly via self-replication in mouse models of type 2 diabetes.^[79] The Brainbow lineage reporter mouse crossed to a Gcg-CreERT2 driver^[73], inducibly expressed with a brief pulse of tamoxifen should over time reveal if beta cells with an alpha cell lineage label that occur within the same islet are clonally related. A variation on this approach that enables the lineage labeling of CreERT2-expressing cells by direct topical application of hydroxy-tamoxifen-containing liposomes^[80] or light-activation of photoactivatable ‘caged’ tamoxifen analogs^[81, 82] could be useful to directly label virgin beta cells in the neogenic niche and follow their fate over time, within the time constraints of *in vitro* culture.

5. Summary and conclusion

The presence of a resident beta cell progenitor within the pancreas remains a contested area of research, and one that is vigorously debated given the high stakes in finding a cure for Type 1 Diabetes. By now it is clear that the pancreas - unlike the gastro-intestinal epithelium - does not possess a pluripotent stem cell that is capable of spontaneously regenerating the entire endocrine pancreas over the course of a few days or weeks. Nevertheless, our discovery of the lifelong presence of a distinct population of virgin beta cells establishes that beta cells can and do arise independently of self-replication via alternative paths within islets of healthy, non-diabetic individuals. This signals a departure from the prevailing dogma that

all beta cells in healthy adult islets originate from self-replication.^[19, 21, 22] The notion that self-replication is the main mechanism of beta cell maintenance and expansion in situations where sufficient numbers of residual beta cells remain has since been supported by many studies and is not in doubt. Nevertheless, our discovery of lineage plasticity tied to the micro-environment of the neogenic niche adds a novel dimension to the discussion of regeneration within the pancreatic islet. The discovery of virgin beta cells offers ample motivation to further study this novel beta cell subpopulation to better understand their origin and elucidate their ultimate cell fate. Clearly, virgin beta cells and the plasticity they represent do not prevent Type 1 Diabetes in the face of ongoing autoimmune assault. Neither does beta cell proliferation. Nevertheless, it would seem prudent to explore the extent of their regenerative potential and the mechanisms that underlie lineage plasticity at the neogenic niche, for these may be applicable once autoimmunity can be safely suppressed. Moreover, the lessons we learn on beta cell maturation at the niche can be applied to regenerate beta cells from stem cells or endogenous progenitors. Our discovery of virgin beta cells and their potential implications are particularly relevant to established Type 1 Diabetes where limited numbers of residual beta cells are unlikely to support meaningful regeneration of functional beta cell mass. Alternative paths to restore functional beta cell mass from endogenous progenitors are therefore most welcome.

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List of abbreviations

ARX	Aristaless related homeobox
CRE	Causes recombination
DNMT1	DNA methyl transferase 1
DTR	Diphtheria toxin receptor
ER/ERT2	Estrogen receptor
ERO1LB	Endoplasmic reticulum oxidoreductase-1-like protein B
FLP	Flippase
Frtd	Flippase recognition target
GCG	Glucagon
GLUT2	Glucose transporter 2
G6PC2	Glucose-6-phosphate catalytic subunit 2
HHEX	Hematopoietically expressed homeobox

HNF1B	Hepatocyte nuclear factor 1 beta
IRX	Iroquois-class homeodomain
ISL1	ISL LIM homeobox 1
LGR5	Leucine rich repeat containing G protein-coupled receptor 5
MAFA	V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A
MAFB	V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B
NEUROD1	Neuronal differentiation 1
NGN3	Neurogenin 3
NKX2-2	NK2 homeobox 2
NKX6-1	NK6 homeobox 1
NOD	Non-obese diabetic
PAX4	Paired box 4
PAX6	Paired box 6
PC1/3	Prohormone convertase 1/3
PDX1	Pancreatic and duodenal homeobox 1
POU3F4	POU class 3 homeobox 4
PTF1A	Pancreas specific transcription factor 1 subunit A
RBP4	Retinol binding protein 4
rtTA	Reverse tetracycline-controlled transactivator
SST	Somatostatin
UCN3	Urocortin 3

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Box 1:**Lineage tracing**

Lineage tracing is a commonly used technique where a single cell - or cluster of similar cells - is irreversibly labeled in order to follow the fate of these cells over time. Lineage tracing has been widely applied to establish sequences of events during organogenesis during embryonic development. The most commonly used technique involves the expression of the enzyme Cre (Causes recombination) recombinase derived from the P1 bacteriophage, which recognizes specific 34 base pair DNA motifs called *LoxP* sites and splices out the intermediate DNA^[reviewed by 83]. By constructing reporter cassettes that feature a ubiquitous promoter separated from a reporter gene like *LacZ* or *GFP* by a transcriptional *STOP* cassette flanked by a pair of *loxP* sites (*i.e* floxed), it is possible to report on Cre recombinase activity. In the presence of Cre recombinase, the floxed *STOP* cassette will be removed, activating reporter gene expression. By placing Cre recombinase expression under the control of a cell type-specific promoter, it is possible to restrict Cre recombinase expression to a specific cell type of choice: *e.g.* the *Gcg* promoter restricts Cre recombinase expression to glucagon-expressing cells such as alpha cells. Importantly, once the floxed *STOP* cassette has been removed by Cre recombinase activity, reporter activity is exclusively driven by the ubiquitous promoter. There is no need for continued Cre recombinase activity or the cell type-specific promoter that drove its expression. In other words, an alpha cell that has acquired a lineage label because of the *Gcg*-dependent expression of Cre recombinase will be unable to shed this permanent label, even if the *Gcg* promoter that initiated the labeling is turned off as would be the case if that alpha cell turns into a beta cell. Original lineage labeling approaches that use constitutively active Cre recombinase have more recently been complemented by strategies that enable temporal control over the onset of lineage-labeling. This strategy provides the flexibility to initiate lineage-labeling of cells at any point in time. It takes advantage of a fusion between Cre and a modified Estrogen receptor (ER) domain that recognizes the estrogen-like molecule tamoxifen. In the absence of tamoxifen, the unliganded ER domain retains the Cre recombinase protein that is expressed under control of a tissue-specific promoter in the cytoplasm where it cannot act. However, upon tamoxifen administration, the CreER fusion protein translocates to the nucleus where it can recognize *loxP* sites. A similar lineage labeling technique that relies on the enzyme Flippase (Flp) from *Saccharomyces cerevisiae*, which recognizes Flippase recognition target (*Frt*) DNA recognition motifs is increasingly employed and works conceptually similar. Careful validation of Cre or Flp driver lines is essential to ensure meaningful results.

Figure 1:

Two opposing scenarios for the origin of new beta cells and the experimental outcomes they generate. First, beta cells are labeled using β -recombinase (Box 1). In this particular example, Cre labels only 30% of beta (β) cells (red beta cells), while the remaining 70% are not labeled (grey beta cells). Delta cells (δ , blue) and alpha cells (α , yellow) are also not labeled. Next, beta cells are allowed to turnover. In scenario A, beta cells derive from a non-beta cell precursor and the existing pool of beta cells slowly disappears, including the lineage-labeled beta cells. They are replaced by unlabeled beta cells. In scenario B, all beta cells, including

lineage-labeled ones, contribute equally to new beta cells. Thus the frequency of lineage labeling does not change. Because of the inefficient lineage-labeling at the start of the experiment, a minor contribution of a non-beta cell source (green beta cells) may be overlooked.

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Figure 2:

Embryonic development of the three main endocrine lineages in the pancreatic islet. Starting from a common endocrine progenitor, endocrine cells first split into an alpha and a beta/delta lineage, which then further differentiates into beta and delta cells. Well-known transcription factors and other markers are indicated under the cells in which they are expressed. Those indicated in bold letters are selective to certain endocrine lineages. In the adult islet, endocrine cells interactions are indicated by green arrows (activation) and red blocking arrows (inhibition).

Figure 3:

Neogenic niche beta cells lack the cell surface glucose transporter GLUT2. Pancreatic islet cells were stained for DNA (blue), UCN3 (green), insulin (red) and GLUT2 (white). Examples of UCN3-negative virgin beta cells (*) and beta cells expressing low levels of UCN3 (arrows) are indicated. All UCN3-negative cells lack cell- surface GLUT2, while some UCN3-low cells started expressing GLUT2. For two areas, two-color panels are shown to side and below the main panel.

Figure 4:

Alpha cells contribute to the niche. An islet with lineage-labeled alpha cells (green), stained for insulin (red) and UCN3 (white) features two transdifferentiated cells indicated by white boxes (A). One of these has not yet acquired UCN3 expression (top, arrow), while the other has further matured and expresses UCN3 (bottom, arrow). Reproduced with permission^[55].

Figure 5:

The neogenic niche is a physical space within the islet where endocrine cells change fate. (A) An islet with all cells labeled with a membrane-bound red fluorescent protein, except in those cells that saw *Gcg-Cre* activity marking alpha cells. These cells exclusively and permanently express a membrane-bound green fluorescent protein. The islet was stained for UCN3 (mature beta cells, blue) and glucagon (alpha cells, white). (B) Three different scenarios for the generation of lineage-alpha beta cells and the predicted distribution of the virgin beta cells across the islet surface. (C) Actual distribution of lineage-alpha beta cells

across the islet surface supporting the idea of a local neogenic niche. (D) An islet with all cells labeled with a membrane-bound red fluorescent protein, unless *Ucn3-Cre* activity (mature beta cells) results in the exclusive and permanent expression of a membrane-bound green fluorescent protein. The islet was stained for UCN3 (mature beta cells, blue) and glucagon (alpha cells, white). Reproduced with permission^[55].

Figure 6:

Endocrine cell transdifferentiation in the neogenic niche. Depiction of two scenarios that can result in the presence of insulin positive, UCN3-negative beta cells in an islet. In the first, alpha cells lose glucagon expression and then acquire insulin expression, prior to UCN3 expression. In the other, mature beta cells sequentially lose expression of UCN3 and then insulin prior to acquisition of glucagon expression. Both scenarios occur within the neogenic niche.