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Short Article

Growth and Regeneration of Adult β Cells Does Not Involve Specialized Progenitors

Monica Teta,[1] Matthew M. Rankin,[1] Simon Y. Long,[1] Geneva M. Stein,[1] and Jake A. Kushner[1,*]
[1] Division of Endocrinology, Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
*Correspondence: kushnerj@mail.med.upenn.edu
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SUMMARY

Cellular progenitors remain poorly characterized in many adult tissues, limited in part by the lack of unbiased techniques to identify progenitors and their progeny. To address this fundamental problem, we developed a novel DNA analog-based lineage-tracing technique to detect multiple rounds of cell division in vivo. Here, we apply this technique to determine the adult lineage mechanism of the insulin-secreting β cells of pancreatic islets, an important unresolved question in diabetes research. As expected, gastrointestinal and skin epithelia involve specialized progenitors that repeatedly divide to give rise to postmitotic cells. In contrast, specialized progenitors do not contribute to adult β cells, not even during acute β cell regeneration. Instead, β cells are the products of uniform self-renewal, slowed by a replication refractory period that prevents β cells from immediately redividing. Our approach provides unbiased resolution of previously inaccessible developmental niches and can elucidate lineage mechanisms without candidate markers.

INTRODUCTION

β cell mass expands through adulthood (Bock et al., 2003; Butler et al., 2003b; Kushner et al., 2002), increasing insulin secretion capacity to match peripheral requirements. Insufficient insulin secretion by β cells results in diabetes mellitus (Kahn et al., 2006), and type 2 diabetes is associated with decreased β cell mass (Butler et al., 2003a; Yoon et al., 2003). Several mechanisms have been invoked to explain adult β cell mass expansion, including neogenesis from pancreatic ducts (Bonner-Weir, 2000) or hematopoietic tissues (Ianus et al., 2003), intraislet replication of highly replicative insulin-positive β cell progenitors (Bonner-Weir, 1992; Swenne, 1983), and β cell proliferation (Brelje et al., 1994; Dor et al., 2004; Georgia and Bhushan, 2004). Recently, several groups have isolated cells from islets or pancreata that divide and give rise to insulin-containing cells (Gershengorn et al., 2004; Hao et al., 2006; Ouziel-Yahalom et al., 2006; Seaberg et al., 2004), prompting speculation that such cells could represent specialized islet progenitors. Such cells could constitute a powerful theoretical target for diabetes therapies in vivo. However, it has not been established if such cells substantially contribute to β cell mass expansion or regeneration. Thus, the identity of adult β cell progenitors remains unclear, and this problem represents a critical knowledge gap in diabetes research.

Specialized progenitors play essential roles throughout the body in tissue development, maintenance, and regeneration. For example, in gastrointestinal epithelia and skin specialized progenitors (stem cells) both self-renew and give rise to rapidly dividing progeny (the transit-amplifying cells), which migrate away from stem cell niches, terminally differentiate, and cease to replicate. Similarly, specialized progenitors have been speculated to occur in pancreatic β cell mass expansion, with a "proliferative compartment" of very rapidly proliferating β cells or proliferating progenitors (Bonner-Weir, 1992; Gershengorn et al., 2004; Swenne, 1983). However, no direct evidence exists to support or refute a proliferative compartment model within the β cell lineage. Do specialized progenitors (insulin positive or otherwise) contribute to the adult β cell lineage?

To address this fundamental question, we developed a novel DNA analog-based lineage-tracing technique to detect sequential cell division in vivo. In the gastrointestinal tract, specialized progenitors are clearly observed, consistent with longstanding models. However, adult β cells display a surprising and unique lineage renewal mechanism. Remarkably, we observe no contribution to adult β cell mass by specialized progenitors or stem cells. Instead, we find that adult β cells are the products of self-duplication. We show that adult β cells exhibit equal proliferation potential and expand from within a vast and seemingly uniform pool of mature β cells.

RESULTS

Multiple Thymidine Analog Labeling in Adult Mice

We hypothesized that sequential thymidine analog labeling could resolve cell division histories, which would constitute a novel lineage-tracing technique. We adapted techniques to detect incorporation of the thymidine analogs 5-chloro-2-deoxyuridine (CldU) and 5-iodo-2-deoxyuridine (IdU) into tissues of mice that were labeled with

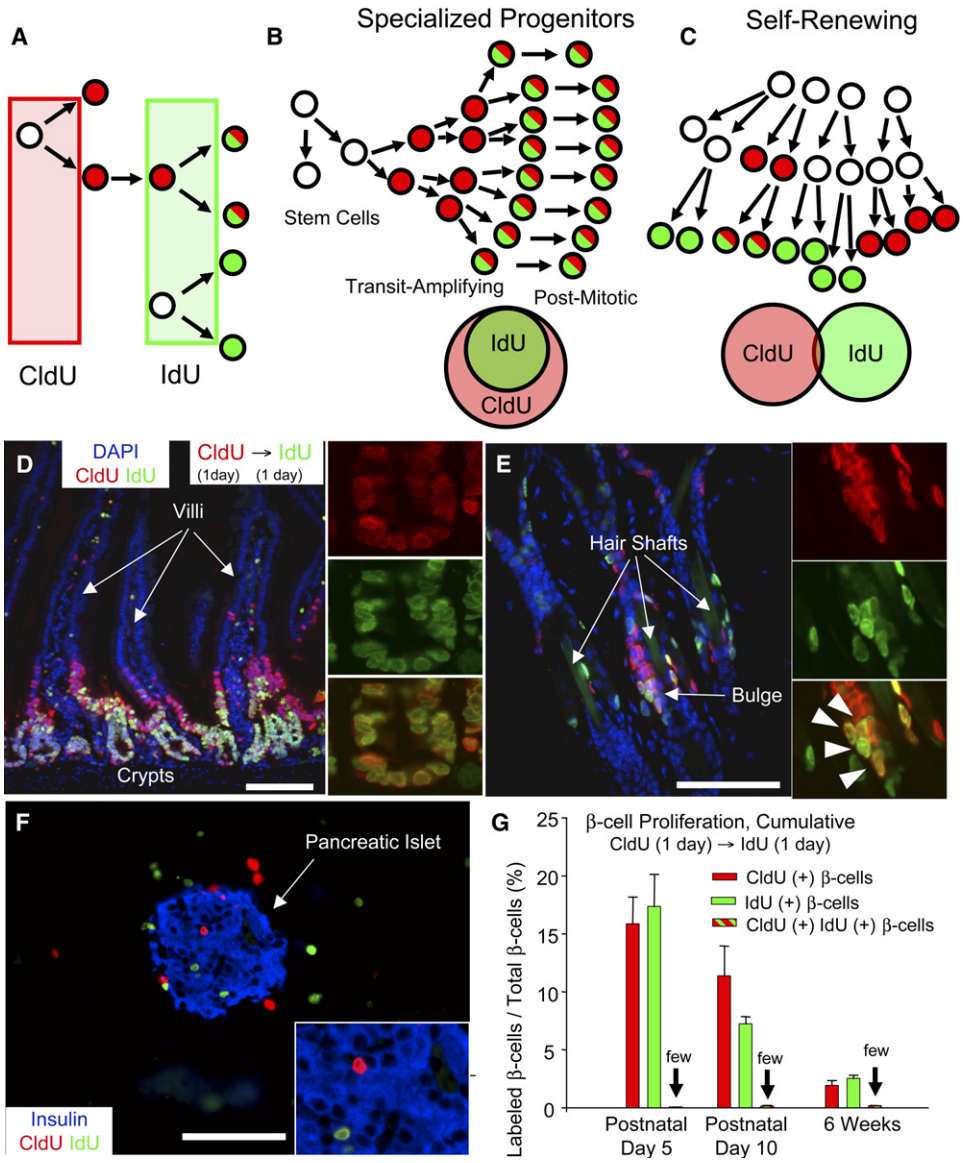

Figure 1. CldU → IdU, an Unbiased Method of Detecting Sequential Cell Division In Vivo

(A) By labeling the first cell division with CldU (red) and the second cell division with IdU (green), sequential cell division results in colabeled cells with both CldU and IdU (green/red).

(B and C) Proposed models of lineage mechanisms that employ specialized progenitors (B) or self-renewing cell division (C). Specialized progenitor lineages exhibit sequential cell division, which results in colabeled cells with CldU and IdU (B). Self-renewing lineages exhibit random cell division, with few colabeled cells (C).

(D) Immunohistochemistry in 6-week-old female mice illustrates that CldU → IdU labeling occurs within the crypts of the GI tract an area that contains proliferative progenitors. Sections stained for DAPI (blue), CldU (red), and IdU (green). CldU IdU-colabeled cells denoted by arrows. Scale bars, 100 μm.

(E) Skin sections reveal CldU IdU-copositive cells within the "bulge" of the outer root sheath of a hair follicle in the skin.

(F and G) Neonatal and adult pancreatic β cells are virtually always labeled with CldU or IdU, but rarely both. Pancreatic immunohistochemistry at 6 weeks of age (F). Sections stained for insulin (blue), CldU (red), and IdU (green). (G) Quantification of total β cell accumulation of CldU, IdU, or both, after sequential labeling with CldU and IdU for 24 hr each. Results expressed as mean ± SEM for five mice.

the analogs over prolonged periods via the drinking water. This strategy employs two different forms of anti-BrdU antisera raised in different species, which bind to CldU and IdU with different affinities (Figure 1A) (Bakker et al., 1991; Maslov et al., 2004). As each analog could theoretically detect a distinct round of cell division, we speculated that this technique could allow detection of more than one round of cell division in vivo. If specialized progenitors substantially contribute to a mature tissue, recently divided cells should have undergone multiple rounds of cell division and therefore be doubly labeled (Figure 1B). Alternatively, a mature tissue could renew or

expand by self-renewal, and recently divided cells would not be comprised of cells that had undergone multiple rounds of cell division (Figure 1C). By trial and error, we modified existing protocols to detect CldU and IdU and were able to distinguish CldU from IdU labeling in a range of tissues such as the pancreas (Figure S1 in the Supplemental Data available with this article online). Specificity of the two different forms of anti-BrdU antisera was verified in mice labeled with CldU alone, IdU alone, or CldU and IdU simultaneously, or sequentially labeled with CldU and then IdU. These control experiments illustrate that mouse and rat anti-BrdU antisera are specific to IdU and CldU, respectively, under our adapted protocol.

Specialized Progenitors in Gastrointestinal and Skin Epithelial Lineages

To test if this technology could detect specialized progenitors, we sequentially labeled adult mice with CldU and then IdU for 1 day each and examined highly proliferative tissues. In the gastrointestinal tract, CldU-labeled cells were prominent in duodenal epithelium within the middle of villi, indicating that the cells had undergone replication during the first labeling period. Deeper within the crypts, where rapidly dividing progenitors are known to exist (transit-amplifying cells) (Potten et al., 1997), all epithelial cells were CldU IdU colabeled (Figure 1D). This labeling is consistent with previous observations, whereby gastrointestinal stem cells both self-renew and give rise to rapidly dividing progeny. These transit-amplifying cells then repeatedly divide for a short period of time to give rise to postmitotic epithelial cells. Similarly, in the skin CldU IdU-colabeled cells were observed in the "bulge" of the outer root sheath of some hair follicles, with cells labeled only by CldU nearby (Figure 1E). This observation indicates that some cells within the bulge of the outer root sheath undergo frequent rounds of cell division. Consistent with this finding, the bulge of the outer root sheath has been observed to contain stem cells and their immediate progeny (Cotsarelis et al., 1990; Tumbar et al., 2004). Thus, our method resolves cell lineage mechanisms in mammalian tissues and opens a novel window into previously opaque lineage relationships.

No Rapidly Proliferating Progenitors in Adult β Cell Growth

We hypothesized that adult β cells could arise from simple self-renewal, as opposed to a model in which a "proliferative compartment" of rapidly proliferating β cell progenitors repeatedly divide and give rise to nonmitotic β cells (Bonner-Weir, 1992; Gershengorn et al., 2004; Swenne, 1983). If a proliferative compartment of specialized progenitors represent the dominant mechanism of adult β cell growth, β cells would be the progeny of cells that have undergone multiple rounds of cell division. Alternatively, if adult β cells arise from self-renewal, adult β cells would be the progeny of cells that had not recently divided multiple times. To resolve the cell division history of β cells, we sequentially labeled mice of various ages with CldU and then IdU for 1 day each, followed by immediate sacrifice. In neonatal mice (postnatal days 5 or 10) many β cells were labeled with either CldU or IdU. By 6 weeks of age fewer β cells were labeled with either CldU or IdU, confirming previous observations by our group and others that β cell replication rapidly slows after birth (Teta et al., 2005). However, β cells were almost never labeled with both CldU and IdU, in stark contrast to the gastrointestinal tract (Figure 1). The infrequency of β cells with evidence of multiple rounds of cell division suggests that adult β cells are products of simple self-renewal, and not from a proliferative compartment of rapidly dividing progenitors. To confirm that our results were not biased by inefficient detection of cell division, we performed extensive controls in β cells, comparing CldU to IdU to BrdU, or to the mitotic marker ki67. These studies illustrate that our methods detect virtually all mitotic events in β cells (Figure S2). Therefore, we conclude that a proliferative compartment of very rapidly proliferating β cells or specialized progenitors does not substantially contribute to β cell mass expansion.

Self-Renewal of Adult β Cells during Normal Growth

Although our short thymidine analog labeling studies suggest that adult β cells self-renew, we considered the alternative hypothesis that a population of slowly replicating progenitors could contribute to β cell mass expansion. To test this hypothesis, we continuously labeled mice with CldU and then IdU for 2 weeks each. By extending the labeling periods, many β cells were labeled with either CldU or IdU (although fewer cells were labeled by IdU, consistent with the decrease in β cell proliferation that occurs early in adolescence). Remarkably, even when mice were labeled for 2 week periods, only a tiny fraction of β cells contained both CldU and IdU (Figure 2). Thus, β cell progenitors rarely replicated more than once during normal β cell growth. Moreover, thymidine analog labeling did not alter the kinetics of β cell proliferation: CldU IdU-treated mice and untreated control mice had equivalent β cell mass (Figure S3). Additionally, labeling studies with CldU and IdU for 1 week periods did not result in a substantial proportion of β cells labeled with both CldU and IdU (see Figure 3, discussed below).

We then considered the alternative hypothesis that very slowly proliferating β cell progenitors (dividing every month or so) could contribute to β cell mass expansion. To test this hypothesis, we labeled mice with CldU for 2 weeks, waited for several months (2–10), and then labeled with IdU for 2 weeks. However, even with this prolonged "washout period" there was no evidence for a proliferative compartment of specialized progenitors, as few β cells were labeled with both CldU and IdU (Figure 2). Thus, adult β cells are not the products of progenitors that have undergone multiple rounds of replication, not even by very slowly proliferating specialized progenitors. We therefore conclude that the adult β cell lineage self-renews during normal growth conditions.

Self-Renewal of Adult β Cells during Regeneration

As our data indicate that β cell growth occurs by self-renewal, we further hypothesized that β cell regeneration

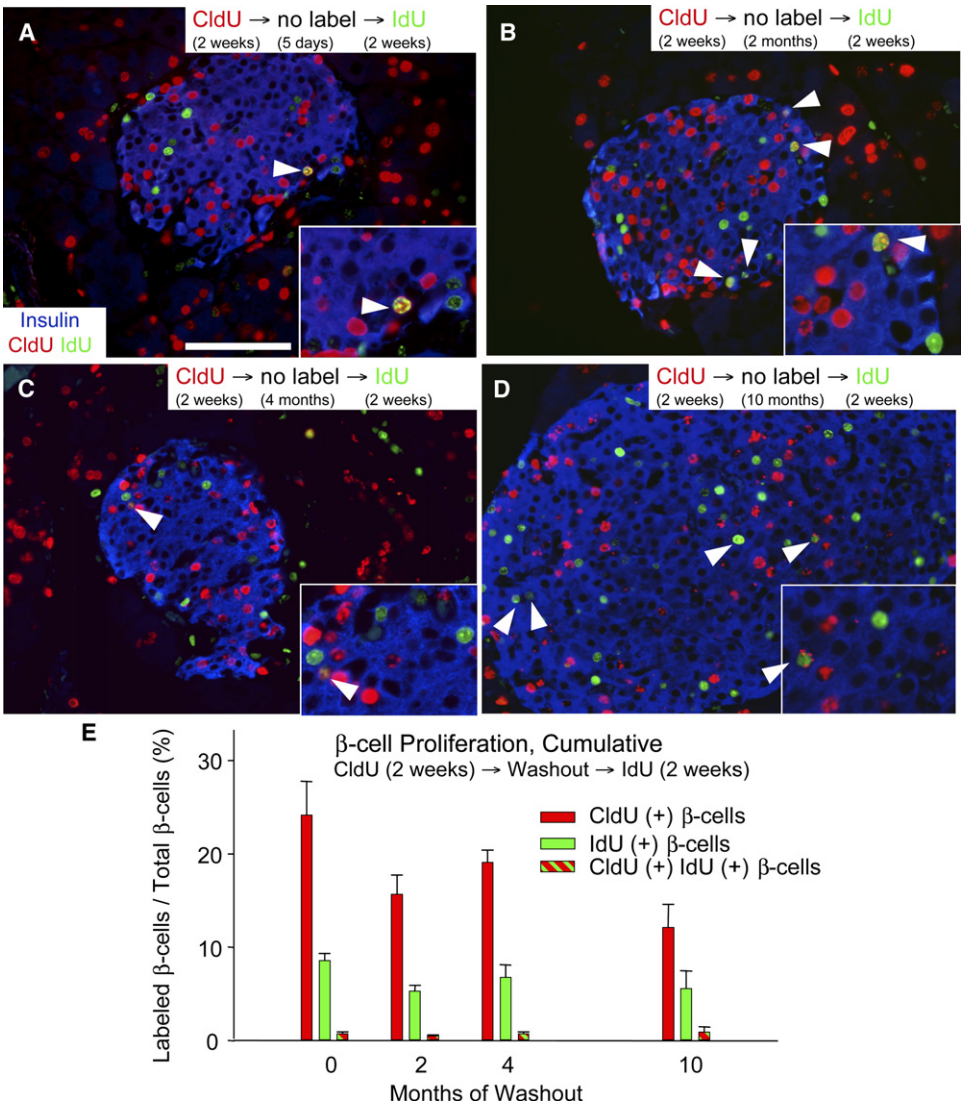

Figure 2. No Evidence for Slowly Replicating β Cell Progenitors

(A–D) Pancreatic immunohistochemistry of mice labeled at 6 weeks of age with CldU for 2 weeks followed by a washout period of 5 days (A), 2 months (B), 4 months (C), or 10 months (D) prior to 2 weeks of IdU. Sections stained for insulin (blue), CldU (red), and IdU (green). Scale bar, 100 μm.

(E) Quantification of total β cell accumulation of CldU, IdU, or both. Results expressed as mean ± SEM for five mice.

could also occur by self-renewal. In contrast to the slow proliferation observed during normal β cell growth, β cell regeneration can be profoundly stimulated by provocative measures such as pregnancy, recovery after partial pancreatectomy, or other physiological conditions. Importantly, β cell regeneration has been postulated to involve unique developmental mechanisms with specialized β cell progenitors (Bonner-Weir and Sharma, 2006). To test if β cell regeneration occurs by self-renewal, pregnant mice were labeled with CldU on gestational days 4–11, and with IdU on gestational days 11–18. Confirming previous reports in rats (Parsons et al., 1992), pregnancy greatly stimulated β cell regeneration, as reflected by many more β cells singly labeled by CldU or IdU (Figure 3). However, β cells labeled by both CldU and IdU were always infrequent following pregnancy, indicating that β cells or their progenitors rarely proliferated more than once during pregnancy. To further test if β cell regeneration occurs by self-renewal, we tested for β cells that had undergone multiple rounds of cell division after 50% partial pancreatectomy, or by treatment with the glucagon-like peptide (GLP)-1 agonist Exendin-4, both of which stimulate β cell regeneration. As with pregnancy, partial pancreatectomy and Exendin-4 also did not substantially increase CldU IdU-colabeled β cells (Figure 3). Thus, β cell regeneration does not appear to involve specialized progenitors, as few progenitors divided more than once during regeneration.

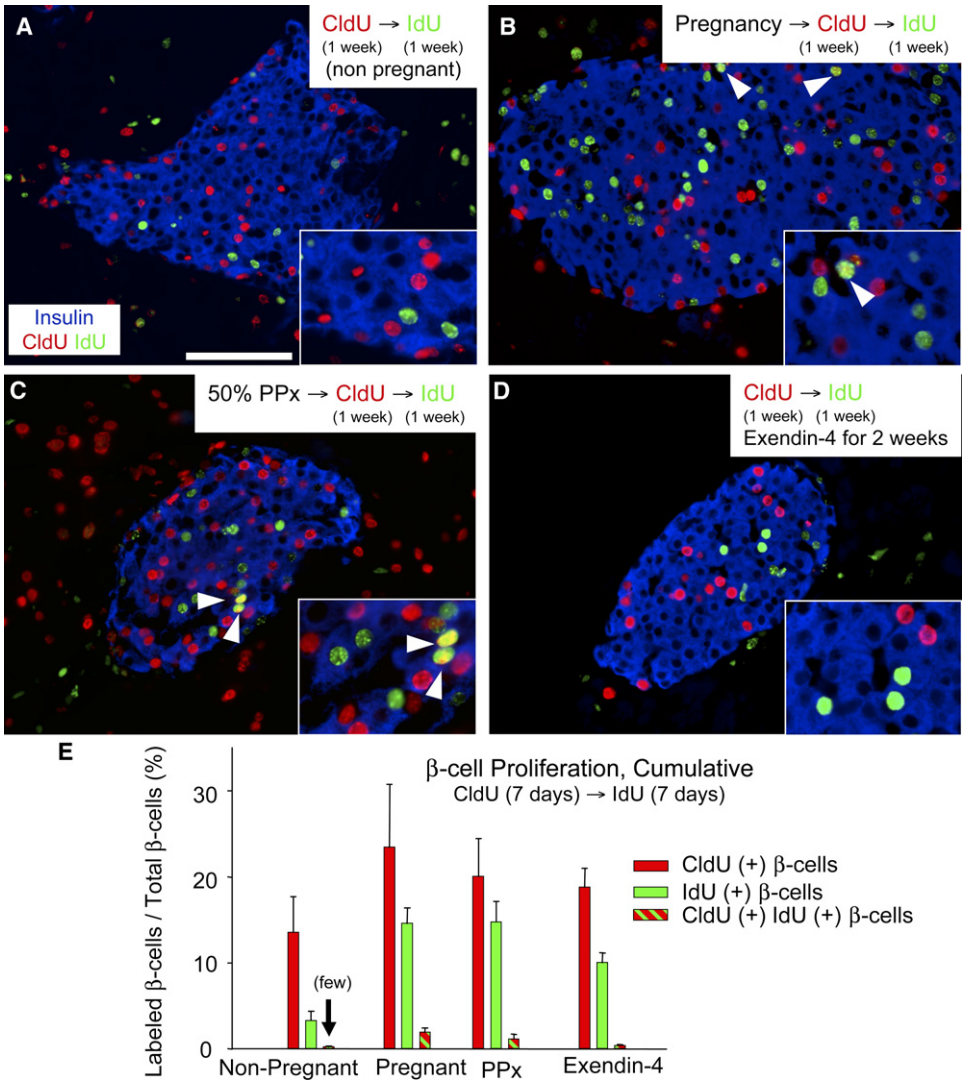

Figure 3. "Adaptive" β Cell Replication Does Not Involve Highly Replicative β Cell Progenitors

(A–D) Pancreatic immunohistochemistry of samples from nonpregnant (A), pregnant (B), post-50% partial pancreatectomy (C), and exendin-4-treated mice (D) after labeling with CldU and then IdU for 7 days each starting at 6 weeks of age. Sections stained for insulin (blue), CldU (red), and IdU (green). Scale bar, 100 μm.

(E) Quantification of total β cell accumulation of CldU, IdU, or both. Results expressed as mean ± SEM for five mice.

To determine if islet and β cell neogenesis occurred even to a minor degree during β cell regeneration, we looked for rare islets with β cells that had undergone repeated cell division. Remarkably, no islets were entirely comprised of β cells labeled by CldU and IdU during acute β cell regeneration (Figures 4A–4E). Further, smaller islets had no more β cells labeled with both CldU and IdU than large islets; no relationship was observed between islet size and repeated rounds of cell division: $r^2 < 0.1$ when CldU (+) IdU (+) β cells were plotted versus cross-sectional islet size in all mice. Taken at face value, these data indicate that there were no islets produced under regenerative conditions, at least none produced by neogenesis that involved cell division. Moreover, CldU IdU-copositive β cells were never observed near pancreatic ducts after each stimulus (see pregnancy example in Figure S4F), where β cell neogenesis has been postulated to occur (Bonner-Weir and Sharma, 2006). Thus, the adult β cell lineage self-renews during acute β cell regeneration, without any apparent contribution by specialized progenitors.

Equal DNA Strand Segregation in Adult β Cells

We then considered the alternative hypothesis that β cell progenitors might not be efficiently labeled by thymidine analogs, which could bias our studies of proliferative progenitors. In the "immortal strand" hypothesis (Cairns, 1975), one DNA strand segregates to progenitor cells but not to daughter cells. As a result, the immortal strand

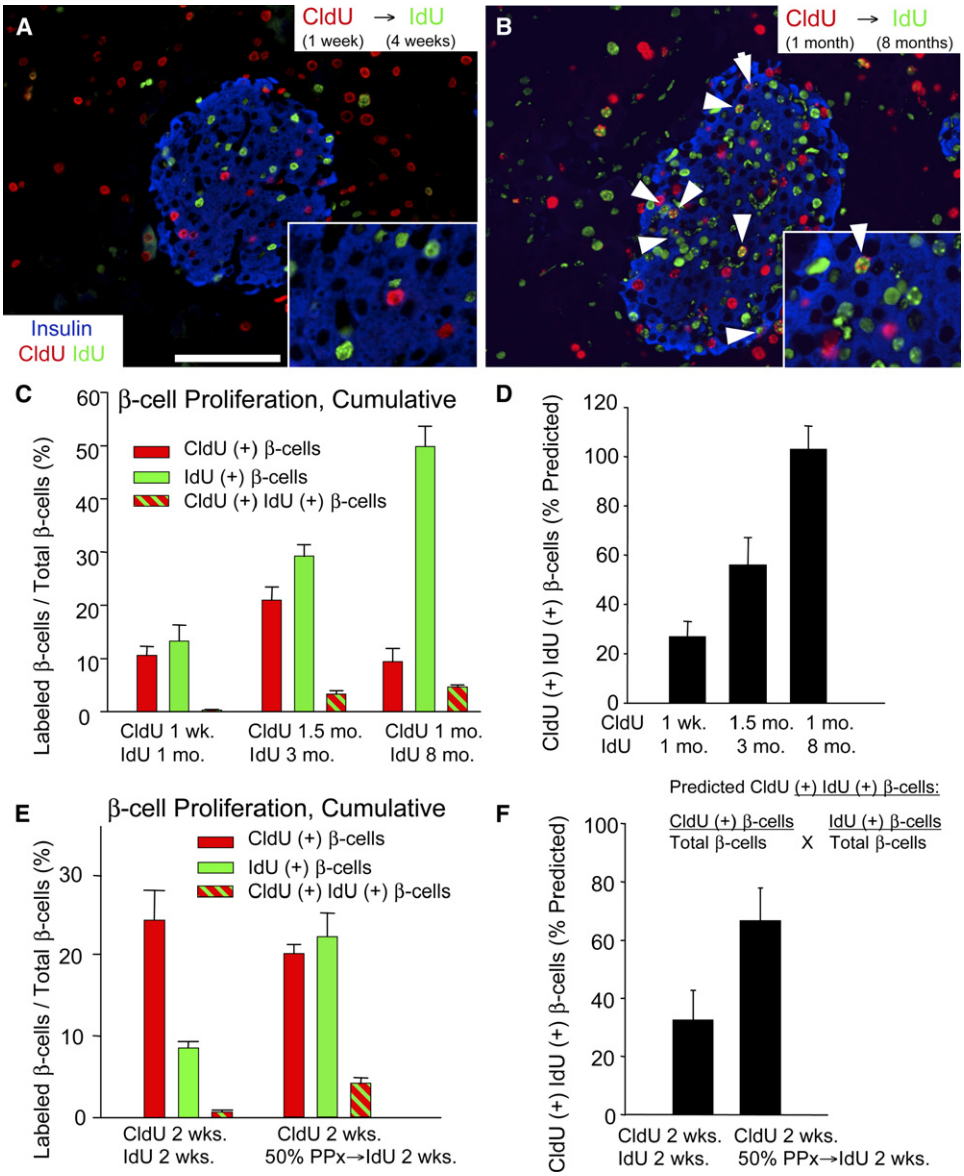

Figure 4. Adult β Cells Are Not Postmitotic but Can Eventually Divide More Than Once, Limited by a Replication Refractory Period

Adult β cells are not postmitotic but can eventually divide more than once, limited by a replication refractory period. Three-month-old female mice were continuously labeled with CldU for 1 week, then IdU for 4 weeks; CldU for 1.5 months, then IdU for 3 months; or CldU for 1 month, then IdU for 8 months.

(A and B) Pancreatic immunohistochemistry. Sections stained for insulin (blue), CldU (red), and IdU (green). Scale bar, 100 μm.

(C) Quantification of total β cell accumulation of CldU, IdU, or both. Results expressed as mean ± SEM for five mice each group.

(D) Analysis of CldU IdU-copositive β cells, expressed as the percentage of predicted CldU IdU-copositive β cells. CldU IdU-copositive β cells are not observed in the predicted frequencies when labeled with IdU for 1 months or 3 months, in contrast to 8 months of IdU.

(E and F) The "replication refractory period" of adult β cells can be altered by mitogenic stimuli. (E) Quantification of total β cell accumulation of CldU, IdU, or both. Results expressed as mean ± SEM for five mice. (F) Analysis of CldU IdU-copositive β cells, expressed as the percentage of predicted CldU IdU-copositive β cells. CldU IdU-copositive β cells are observed in the predicted frequencies following partial pancreatectomy.

is repeatedly unlabeled, and the other strand is repeatedly labeled and discarded in the subsequent round of progenitor cell division. If β cell mass expansion similarly employed unequal DNA strand segregation, thymidine analogs would fail to persistently label progenitors. Indeed, this phenomena was recently observed in muscle satellite cells (Shinin et al., 2006). However, we find that β cell progenitors exhibit strong evidence of ongoing DNA replication: β cells that are fully labeled by CldU were observed after short washout periods (Figures 2A and 2B), which appear to turn into β cells that are partially labeled by CldU after prolonged washout (Figures 2C and 2D).

Moreover, CldU IdU-copositive β cells were occasionally detected after a 10 month washout, typically with partial CldU labeling (Figure 2). Thus, adult β cell progenitors can be efficiently labeled. Taken together, our data therefore indicate that adult β cells are the products of self-renewal, not by replication of specialized progenitors.

Ongoing Cell Division of Adult β Cells

The infrequency of CldU IdU-colabeled β cells could be interpreted to suggest that adult β cells are largely postmitotic. To directly test this hypothesis, we continuously labeled mice for increasing periods of time. Although CldU IdU-copositive β cells were rare in mice that had been labeled with CldU for 1 week and IdU for 4 weeks, many CldU IdU-copositive β cells were observed when mice were labeled with CldU for 1 month and IdU for 8 months (Figure 4). Importantly, prolonged labeling only labeled a fraction of β cells with CldU and IdU, which further shows that slowly replicating specialized progenitors do not substantially contribute to β cell regeneration. Therefore, our results strongly indicate that proliferation within the β cell lineage occurs by self-renewal. Additionally, our data reveal that most adult β cells are not postmitotic but can eventually undergo more than one round of cell replication.

A Replication Refractory Period in Adult β Cells

Surprisingly, CldU IdU-copositive β cells were very infrequent with short labeling periods. We therefore hypothesized that β cell replication might not be stochastic, with limitations that prevent β cells from serially dividing from one round to the next. To test this hypothesis, we mathematically modeled the probability of capturing more than one round of β cell replication. If β cell replication occurs in a stochastic manner, the proportion of CldU IdU-copositive β cells should be equal to the "predicted fraction" of CldU IdU-copositive β cells, obtained by multiplying the fraction of CldU-containing β cells by the fraction of IdU-containing β cells. Alternatively, if β cell replication is not stochastic, CldU IdU-copositive β cells could be even less frequent than the predicted fraction of CldU IdU-copositive β cells. We observed that CldU IdU-copositive β cells were very rare in mice that had only been labeled with CldU for 1 week and IdU for 4 weeks, far less than the predicted fraction of CldU IdU-copositive β cells (Figure 4). In contrast, when mice were labeled with CldU for 1 month and IdU for 8 months, CldU IdU-colabeled β cells were frequently observed and were present in proportions exactly equal to the predicted fraction by our calculations. Similarly, CldU IdU-copositive β cells were present in the predicted amounts in mice following a 10 month washout, but not after 2 or 4 months (Figure S5). Thus, very few β cells exhibit evidence of more than one replication event after sequential thymidine analog labeling for short periods of time. However, prolonged labeling detects many β cells with evidence of more than one round of replication. Therefore, our results indicate that proliferation within the β cell lineage is not stochastic but is strictly limited by a "replication refractory period," which slows serial β cell replication from one round to the next.

Replication Refractory Period Is Dynamic in Adult β Cells

Our data indicate that β cell replication is limited by a refractory period, which slows β cell growth during normal conditions. To determine if the replication refractory period within the β cell lineage is regulated, we tested if β cell replication is stochastic during β cell regeneration. We first analyzed our β cell regeneration studies in which a provocative stimulus for regeneration was followed by 1 week of CldU and 1 week of IdU (described above). Despite increased β cell proliferation after provocative stimuli, there were few CldU IdU-copositive β cells (Figure 3; Figure S5). We then labeled mice with CldU for 2 weeks, performed partial pancreatectomy, then labeled for 2 weeks with IdU. CldU IdU-copositive β cells remained relatively rare compared to β cells labeled by CldU or IdU (Figure S5). However, CldU IdU-copositive β cells were present in far higher amounts than in control pancreata and were nearly as frequent as that predicted by the product of CldU by IdU fractions. These results indicate that partial pancreatectomy was able to stimulate β cell regeneration by recruiting β cells that had previously replicated, as well as those that had not. Thus, some conditions that stimulate β cell regeneration appear to foreshorten the replication refractory period. Therefore, the replication refractory period of β cells does not appear to be permanently set but may be overcome under some conditions.

A Prolonged Replication Refractory Period of Cellular Division Is Not Common to All Slowly Renewing Tissues

To our knowledge, a long replication refractory period of cellular division has not been previously described. However, this unique observation could simply reflect the lack of technologies to readily detect more than one round of cell proliferation. Thus, the replication refractory period could be a common property of slowly replicating tissues. To directly test this hypothesis we analyzed proliferation of pancreatic acinar cells in mice sequentially labeled with CldU and then IdU for 2 weeks each. Acinar cells represent an ideal control tissue to test for a replication refractory period as they proliferate very slowly in young mice, at rates roughly equivalent to β cells. Although acinar cells labeled with either CldU or IdU were the most common, CldU IdU-copositive acinar cells were also fairly frequent (Figure S6). Indeed, CldU IdU-labeled acinar cells comprised a substantial minority of acinar cells and were present in proportions equal to the predicted fraction by our calculations. Thus, like β cells, pancreatic acinar cells appear to grow by self-renewal, and not by proliferation of specialized progenitors. However, unlike β cells, acinar cells do not exhibit a long replication refractory period. Therefore, a prolonged replication refractory period does not appear to be a general property of slowly replicating tissues.

DISCUSSION

In summary, our data provide a high-resolution replicative history of an adult somatic tissue and reveal that adult β cells are products of very slowly replicating progenitors. If a proliferative compartment of rapidly dividing population of specialized progenitors substantially contributed to β cell mass expansion, nearly all proliferating β cells would be expected to be copositive for both CldU and IdU after sequential labeling. This cell lineage pattern is clearly the main mechanism of the gastrointestinal epithelia lineage. In sharp contrast, in the adult β cell lineage CldU IdU-copositive β cells were always infrequent. This result indicates that β cell lineage expands by self-duplication, not by replication of specialized progenitors. Indeed, extending labeling studies reveal that most adult β cells eventually divide, which strongly suggests that self-duplication uniformly occurs across the β cell lineage. Thus, our extended labeling studies appear to indicate that adult β cells have equal proliferation potential. This surprising result implies that the adult β cell lineage is uniquely capable of regeneration, as each β cell could theoretically expand many times. However, β cell proliferation is strictly limited by a replication refractory period, which slows β cell mass expansion under basal conditions. Still, the replication refractory period does not appear to be permanently determined and can be foreshortened during β cell partial pancreatectomy-stimulated regeneration. This unique cell lineage mechanism thus limits β cell mass expansion while allowing acute β cell regeneration.

Serial thymidine analog labeling is a powerful and versatile technique that opens a novel window into previously opaque lineage relationships. Because our approach allows unbiased single cell resolution of developmental niches, cell fate decisions can now be interrogated in vivo without candidate markers. Consequently, we anticipate that our strategy will lead to the identification of progenitors in other adult tissues including brain, developing pancreas, muscle, and others. Similarly, CldU IdU labeling could also be employed to determine unequal DNA strand segregation in putative stem cells, a la the immortal strand hypothesis (Cairns, 1975). Moreover, we anticipate that CldU IdU labeling could be used to identify cellular transformation in oncogenic models, or to reveal response to chemotherapy protocols.

In the past, a wide variety of potential mechanisms have been invoked to explain adult β cell mass expansion, including ductal neogenesis (Bonner-Weir, 2000) and replication of specialized intraislet progenitors (Bonner-Weir, 1992; Gershengorn et al., 2004; Swenne, 1983). More recently, Melton and colleagues have employed insulin-promoter-based lineage tracing to suggest that adult β cells are the products of self-duplication (Dor et al., 2004). However, caveats limit the conclusions of their study (Bonner-Weir and Sharma, 2006). Promoter-based gene expression studies could theoretically be vulnerable to leaky insulin promoter-driven cre expression, equivalent to low levels of pro-insulin mRNA detected in putative ES cell to β cell transdifferentiation studies. Moreover, low-efficiency labeling, as achieved with the insulin promoter, makes it difficult to measure minor contributions of non-insulin-containing cells. Despite these theoretical caveats, the studies performed by Melton and colleagues strongly suggest that most adult β cells are the products of self-duplication. Aside from such promoter-based lineage-tracing studies, almost nothing is known about the adult β cell lineage. Therefore, our study confirms findings by Melton and colleagues and extends lineage tracing of adult β cells to the single cell level. Remarkably, we find no evidence to support even a minor contribution of specialized progenitors or stem cells to β cell regeneration. Instead, our studies reveal that adult β cells are the products of self-renewal, and exhibit equal proliferation potential. Moreover, we show that adult β cells are severely limited in their ability to reduplicate by a replication refractory period. Thus, efforts to determine the molecular regulation of adult β cell proliferation are particularly strategic.

EXPERIMENTAL PROCEDURES

Mice

All experiments with mice were performed according to the guidelines of the IACUC committee of the Children's Hospital of Philadelphia. Male and female B6.129 F1 wild-type mice were purchased from Taconic (Germantown, NY), housed at the laboratory animal facility at The Children's Hospital of Philadelphia, and fed Mouse Diet 5015 from PMI Nutrition International (Richmond, IN).

Continuous Labeling

Mice were continuously labeled by administering 5-chloro-2′-deoxyuridine (CldU), 5-iodo-2′-deoxyuridine (IdU), or 5-bromo-2′-deoxyuridine (BrdU), in drinking water bottles at 1 mg/ml. CldU, IdU, and BrdU were obtained from Sigma-Aldrich (St. Louis, Mo).

CldU IDU Control Mice

Control mice were labeled by administering CldU, IdU, or both compounds simultaneously in drinking water bottles for 3 days.

One-Day CldU Labeling

Five female mice at 4 weeks of age were labeled with CldU in the drinking water for 24 hr followed by immediate sacrifice.

Two-Day Labeling

Five female mice at 4 weeks of age were labeled with CldU in the drinking water for 24 hr, followed by IdU for an additional 24 hr prior to sacrifice. Alternatively, mice were labeled with BrdU for 48 hr prior to sacrifice. Neonatal mice received two intraperitoneal injections of CldU for 1 day followed by similar injections of IdU for 1 day (both 100 mcg/g bodyweight, made up as 10 mg/ml in saline), with injections starting on postnatal day 3 or 8 and sacrifice 2 days later on postnatal day 5 or 10.

Extended Labeling

Six-week-old male mice were labeled with CldU for 2 weeks, followed by 5 days of washout without label, followed by IdU for 2 weeks. Alternatively, 6-week-old male mice were labeled with CldU as above, but the washout period was extended to 2 months, 4 months, or 10 months prior to IdU labeling and sacrifice. For extended labeling studies, 3-month-old female mice were labeled with CldU followed by IdU for up to 8 months.

Partial Pancreatectomy

Partial pancreatectomy was performed followed by labeling 6-week-old male mice with CldU for 1 week and then IdU for 1 week.

Alternatively, mice were labeled with CldU for 2 weeks, partial pancreatectomy was performed, and mice were then labeled with IdU for 2 weeks prior to sacrifice. The entire splenic portion of the pancreas was surgically removed, resulting in a ~50% pancreatectomy.

Exendin-4 Treatment

Six-week-old male mice were treated with Exendin-4 daily while being labeled with CldU for 1 week followed by IdU for 1 week. Exendin-4 was obtained as Exenatide from Eli Lilly and Company (Indianapolis Indiana). Mice were injected with 24 nmoles per kg bodyweight daily in the subcutaneous space similar to established protocols (De Leon et al., 2003; Li et al., 2003).

Timed Pregnancy

Six-week-old virgin females were bred with a proven male breeder. Mice were labeled with CldU starting at gestational day 5 and IdU at gestational day 13 until the end of pregnancy and then sacrificed 1 day later. Nonpregnant littermates served as controls.

Immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde/PBS solution. Paraffin-embedded sections (5 μm) were rehydrated, microwaved in 0.01 M sodium citrate, permeabilized with 1% Triton, incubated in 1.5 N HCL, and treated with 0.25% trypsin (Invitrogen, Carlsbad, CA) before an overnight incubation for IdU with mouse anti-BrdU antisera (BD Biosciences, Franklin Lakes, NJ), and guinea pig anti-insulin (Zymed Laboratories Inc., South San Francisco, CA). Sections were then washed and then incubated overnight for CldU in rat anti-BrdU antisera (BU1/75; Accurate Chemical, Westbury, NY) followed by incubation with secondary antisera conjugated to AMCA, Cy2, or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) and DAPI (Molecular Probes, Eugene, OR). For additional details, see the Supplemental Data.

Proliferation Analysis

At least 20 islets were analyzed per animal at 40×. Images were acquired with a Zeiss Axioskop 2 plus mot (Carl Zeiss MicroImaging, Thornwood, New York) and captured with an Orca ER digital camera. CldU-, IdU-, or CldU- and IdU-positive β cell ratios were calculated as mean ± SEM, with at least 1000 β cells counted per animal. Exocrine CldU-, IdU-, or CldU- and IdU-positive cell ratios were calculated as the mean ± SEM, with at least 900 nuclei counted per animal.

Islet Morphometry

To minimize pancreatic autofluorescence, insulin immunohistochemistry was performed with Cy3-labeled secondary antisera. Nuclear staining was performed with DAPI (Molecular Probes, Eugene, OR). β cell area was quantified in CldU- and IdU-treated mice and compared to untreated control mice, and determined by acquiring all possible adjacent nonoverlapping images from each insulin-stained section, three sections per animal, using a 5× objective and a 0.63× converter. Insulin-stained images were acquired with appropriate Cy3 filters (Chroma Technology, Rockingham, VT). Total pancreatic images were acquired with Cy2 filters. Images were then analyzed for insulin-positive β cell area with Openlab 4.0, using the lasso tool to select individual islets and individually optimized settings to maximize region-of-interest accuracy. Results of β cell quantification were expressed as the percentage of the total area surveyed containing islet cells positive for insulin. Results represent the average from four to five animals per group.

Statistics

All results are reported as mean ± standard error of the mean for equivalent groups. Results were compared with independent Student's t tests (unpaired and two-tailed) reported as p values.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and seven supplemental figures and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/12/5/817/DC1/.

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