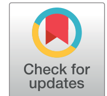

Glucagon Receptor Antagonist–Stimulated α-Cell Proliferation Is Severely Restricted With Advanced Age

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Glucagon-containing α-cells potently regulate glucose homeostasis, but the developmental biology of α-cells in adults remains poorly understood. Although glucagon receptor antagonists (GRAs) have great potential as antidiabetic therapies, murine and human studies have raised concerns that GRAs might cause uncontrolled α-cell growth. Surprisingly, previous rodent GRA studies were only performed in young mice, implying that the potential impact of GRAs to drive α-cell expansion in adult patients is unclear. We assessed adaptive α-cell turnover and adaptive proliferation, administering a novel GRA (JNJ-46207382) to both young and aged mice. Basal α-cell proliferation rapidly declined soon after birth and continued to drop to very low levels in aged mice. GRA drove a 2.4-fold increase in α-cell proliferation in young mice. In contrast, GRA-induced α-cell proliferation was severely reduced in aged mice, although still present at 3.2-fold the very low basal rate of aged controls. To interrogate the lineage of GRA-induced α-cells, we sequentially administered thymidine analogs and quantified their incorporation into α-cells. Similar to previous studies of β-cells, α-cells only divided once in both basal and stimulated conditions. Lack of contribution from highly proliferative "transit-amplifying" cells supports a model whereby α-cells expand by self-renewal and not via specialized progenitors.

Glucagon potently influences glucose homeostasis, opposing insulin action (1). Excess glucagon signaling may drive excess hepatic gluconeogenesis and other aspects of type 2 diabetes (2). Growing evidence points to a role for glucagon as a key driver of diabetes pathophysiology (3). Consequently, there is considerable interest in blocking glucagon signaling as antidiabetogenic therapies (4–6).

Glucagon receptor blockade might result in uncontrolled α-cell growth. Germline disruption of the glucagon receptor gene in mice results in massive α-cell expansion (7), with an islet phenotype in embryonic development (8). This massive α-cell expansion phenotype is mirrored by prohormone convertase 2–deficient mice, which are unable to process glucagon (9). Similarly, patients with glucagon receptor–null mutations exhibit hyperglucagonemia and massive α-cell hyperplasia (10,11). Glucagon receptor blockade via glucagon receptor antagonists (GRAs) is also associated with α-cell expansion in young mice (12). Interestingly, glucagon receptor–deficient α-cell hyperplasia might not be driven by α-cell–autonomous glucagon receptor signals; liver-specific disruption of the glucagon receptor closely phenocopies the α-cell hyperplasia of global glucagon receptor knockout mice (13). Downstream of the glucagon receptor, liver-secreted glutamine and other amino acids may be the α-cell–expanding signals, acting in an mTOR-dependent manner (14–17). Thus, adult α-cell expansion could be potently stimulated by glucagon receptor antagonism, at least in young rodents and possibly human patients.

The developmental mechanism to maintain adult α-cells remains unclear. In contrast to β-cells, the developmental biology of adult α-cells has received far less attention. The lineage mechanism of murine β-cell maintenance and expansion appears to be largely via proliferation of β-cell themselves, without meaningful contribution by

ISLET STUDIES

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noninsulin-containing cells (18) or specialized progenitors that involve highly replicative “transit-amplifying” cells to expand daughter cells from tissue stem cells (19). Adult β-cells are somewhat heterogeneous, with differential expression of various markers that may relate to cell cycle state (20–23). Fltp may mark subpopulations of β-cells with reduced proliferative capacity (20). The role of β-cell subpopulations is unclear; homogeneous self-renewal of β-cells appears to be the rule in adult mice under most circumstances, even in response to intense stimuli such as pregnancy (19), pancreatic ductal ligation (24), or inducible obesity (25). However, in response to even more extreme interventions, lineage plasticity of adult β-cells might still occur, albeit to a much smaller degree. Ductal neogenesis of α-cells has been described in response to α-cell loss (26). Similarly, under hyperglycemic conditions of extreme β-cell deficiency, some α-cells shift to β-cell fates (27). Likewise, α- to β-cell fate switching has been observed under other conditions (28,29).

The cellular turnover of mature adult α-cells is very poorly understood and has been further complicated by our recent description of highly proliferative α-cells in human pancreata. In the past, β-cells were assumed to undergo frequent turnover (every ~1–3 months in rodents) (30) with limitless expansion potential. However, we and others find that rodent and human β-cells are very long-lived, with minimal evidence of β-cell turnover in aged mice and adult human pancreata (31–34). Human α-cells have been suggested to be similarly long-lived, with minimal indirect evidence of cellular turnover (32,35). However, we recently described a novel population of highly proliferative α-cell–related islet endocrine cells in human pancreata (36). These cells variably expressed glucagon but always contained key markers of mature α-cells, including the transcription factor ARX, synaptophysin, chromagranin A, and INSM1. Interestingly, the cells expressed Sox9 in the cytoplasm, an atypical location previously described in cancer cells (37). Proliferation of the α-cell–related islet endocrine cells was exceptionally high within some pancreatic samples from adolescent and young adults. Although we have not found evidence for these highly proliferative α-cell–related islet endocrine cells in adult mice, the search is just beginning. Regardless, these α-cell–related islet endocrine cells from human pancreata emphasize major knowledge gaps in adult α-cell developmental biology.

The regenerative capacity of all islet endocrine cells could be restricted with advanced age. In the past, islet endocrine cells (including α-cells) were assumed to have limitless proliferative capacity. However, studies by our group and others indicate that adaptive β-cell capacity is sharply limited by age in mice (38–40) and in xenotransplanted human islets (41). These findings open the possibility that α-cell regeneration could similarly be subject to an age-dependent decline. However, the highly proliferative α-related cells (36) leave open the possibility that aged α-cells (unlike aged β-cells) could proliferate under some conditions. Still, provocative studies of α-cell expansion have not yet been performed to interrogate the impact of aging. Thus, highly proliferative α-cell–related cells (36) and the major above knowledge gaps in α-cell regenerative biology could severely limit the diabetes research field, especially if α- to β-cell fate switching becomes a serious therapeutic goal for patients with diabetes.

We set out to interrogate the developmental biology of α-cells in mice of various ages. Here, we used a novel and potent GRA to test the impact of sustained GRA exposure on α-cell regeneration. By labeling mice with multiple thymidine analogs, we interrogated α-cell lineage and α-cell regenerative capacity.

RESEARCH DESIGN AND METHODS

Mice

Animal experiments were performed at the Children’s Hospital of Philadelphia under the oversight of the Institutional Animal Care and Use Committee. Male F1 hybrid B6129SF1/J mice were obtained or generated from female C57Bl/6J crossed with male 129S1/SvimJ mice from The Jackson Laboratory (Bar Harbor, ME). Mice were gavaged with JNJ-46207382 (Janssen, Spring House, PA) in 20% (2-hydroxylpropyl) β-cyclodestrin (MilliporeSigma, Burlington, MA) or vehicle. Pharmacokinetic studies were performed with 100 or 200 mg JNJ-46207382/kg. Body weight and blood were collected at 1, 2, 4, and 8 h postdose. JNJ-46207382 was detected in plasma via liquid chromatography–mass spectrometry with LC-10AD pumps (Shimadzu, Kyoto, Japan), Leap HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), and AB/MDS Sciex API 4000 QTRAP (Danaher, Washington, DC). Adult mice were labeled with 5-bromo-2-deoxyuridine (BrdU) or 5-ethynyl-2′deoxyuridine (EdU) in drinking water as previously described (25). Intraperitoneal glucose tolerance tests were performed on mice fasted for 16 h with 2 g D-glucose per kg body weight, as previously described (25). Experiments were performed on 1.5- or 14-month-old adult mice unless stated otherwise. Short-term BrdU labeling was performed with 3-, 4-, and 21-day-old mice. Neonatal mice received two intraperitoneal injections (12 h apart) of BrdU 100 μg/g body weight, followed by sacrifice, as previously described (31).

Insulin and Glucagon ELISA

Plasma insulin and glucagon were quantitatively determined in plasma per the manufacturer’s instructions (insulin, 80-INSMS-E01; Alpco, Salem NH; glucagon, 10-1281-01; Mercodia, Winston-Salem, NC). The glucagon assay is highly specific, with minor reactivity to glicentin and oxyntomodulin.

JNJ-46207382

The GRA was generated by Johnson & Johnson (Fig. 1*A*) (42). JNJ-46207382, also known as 3-(5-(2-(((4′-chloro-ri, 1′-biphenvn-4-yl)amino)methyl)-5(trifluoromethyl)phenyl)-picolinamido)propanoic acid, is a potent, highly selective

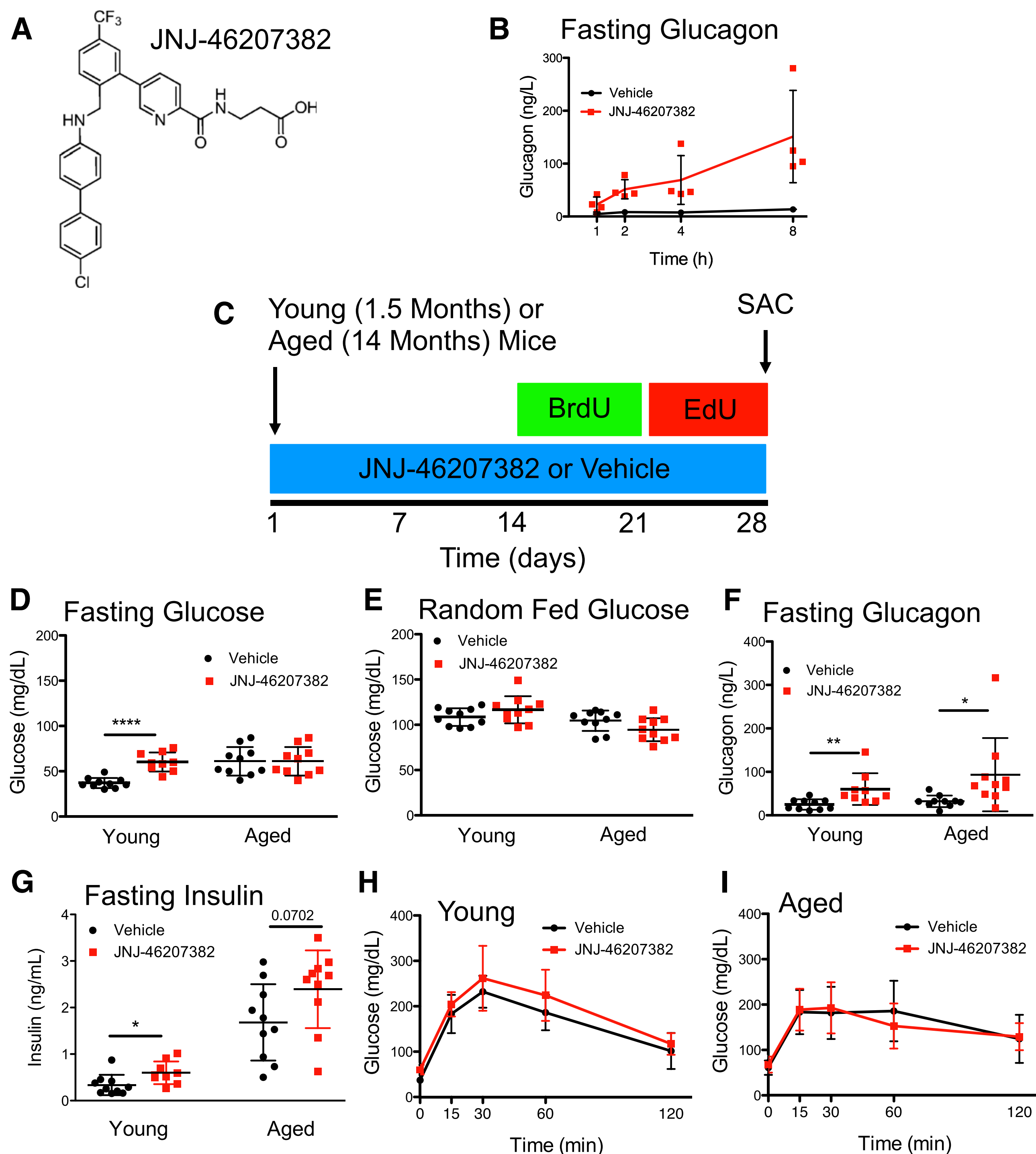

Figure 1—JNJ-46207382, a GRA that opposes glucagon action in mice. *A*: GRA, JNJ-46207382, chemical structure. *B*: Fasting plasma glucagon concentration in young mice treated with either vehicle or JNJ-46207382 (200 mg/kg). *C*: Young (1.5 month) and aged (14 month) mice received daily oral gavage treatment with 200 mg/kg JNJ-46207382 or vehicle for 28 days. Mice were sequentially labeled with BrdU and EdU for a week each, starting at treatment day 14. Blood was collected on the final day of treatment to measure circulating plasma glucagon concentrations in the mice, followed by sacrifice (SAC). *D* and *E*: Glucose levels, fasting (*D*) and random fed (*E*). *F*: Fasting glucagon. *G*: Fasting insulin levels. *H* and *I*: Glucose tolerance tests, young (*H*) and aged (*I*). *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$, vehicle vs. JNJ-46207382.

antagonist for glucagon receptor prepared as a large series of chemical derivatives around picolanmido-propanoic acid (42). The molecules were derived by substitution chemistry and then screened for their ability to antagonize the glucagon receptor or block glucagon-stimulated cAMP production.

A compound's ability to block glucagon binding was assayed with membranes from HEK293 cells expressing

the human glucagon receptor (GCGR). The binding assay was performed by a filtration method in a 384-well plate. Membranes (6 μg/well final protein concentration) were incubated with ^{125}L-glucagon at 0.3 nmol/L and serially diluted compound. Similarly, a compound's ability to block glucagon-stimulated cAMP production was tested with GCGR-expressing HEK293 cells. Glucagon-stimulated cAMP was quantified with LANCE technology (PerkinElmer, Waltham, MA). Serially diluted test compound was incubated with cell solution, incubated for 30 min, and followed by addition of glucagon (100 pmol/L final concentration). cAMP levels were quantified by TR-FRET via EnVision (PerkinElmer).

JNJ-46207382 inhibits the binding of the radiolabeled glucagon hormone to its cognate receptor, with an average K_i value of 18 nmol/L (42). In cellular functional assays, JNJ-46207382 inhibited glucagon-stimulated cAMP generation, with an average K_i value of 60 nmol/L (42). For details on picolanmido-propanoic acid GRA derivatives, see patent WO2012162407 (U.S. patent 8,748,624), noting JNJ-46207382 as example 44 (42).

Immunohistochemistry

Paraffin sections were incubated with primary antisera and secondary antisera conjugated to aminomethylcourmarin (AMCA), Cy2, Cy3, or Cy5 (Jackson ImmunoResearch, West Grove, PA) and DAPI (Molecular Probes) (34). The Click-IT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher, Waltham, MA) was used to stain for EdU, as previously described (25).

Pancreatic Morphometry

Islet morphometry was performed with Volocity 6.1.1 (PerkinElmer) (31–34,36). A Zeiss AxioImager (Carl Zeiss, Thornwood, NY) with automated X-Y stage and Orca ER camera (Hamamatsu, Middlesex, NJ) acquired thousands of islet images with tens of thousands of individual nuclei analyzed per sample, as previously described (25). To quantify α-cell density, all visible α-cells were imaged from three to four lateral pancreatic sections for both head and tail and then counted by hand, reported as α-cell density per islet or pancreas. Approximately 900 and 1,300 α-cells were quantified from young mice and old mice, respectively.

Statistics

All results are reported as mean ± SD unless noted otherwise. Results were compared with independent Student *t* tests (unpaired and two tailed) reported as *P* values.

RESULTS

JNJ-46207382, a Novel GRA That Opposes Glucagon Action in Mice

We characterized JNJ-46207382, a highly selective small molecule GRA (42) (Fig. 1*A*). We performed pharmacokinetic studies, administering JNJ-46207382 at 100 or 200 mg/kg to mice. JNJ-46207382 was highly absorbed within a few hours (T_{max} mean 3.5–4 h) (Supplementary Table 1). Plasma glucagon levels steadily rose in 8 h after 200 mg/kg JNJ-46207382 (Fig. 1*B*). Thus, JNJ-46207382 potently antagonizes the glucagon receptor in mice.

We tested the impact of JNJ-46207382 upon glucose homeostasis, treating cohorts of young or old nondiabetic mice (Fig. 1*C*). Young mice tolerated gavage and GRA well, with equivalent weight increase over 28 days of continuous treatment compared with controls (Supplementary Table 2). Both aged cohorts lost ~10% of their body weight (Supplementary Table 3). Fasting glucose was slightly increased in treated young mice but not in aged mice (Fig. 1*D* and Supplementary Tables 2 and 3). Similarly, treated young mice, but not aged mice, trended toward increased (10%) random fed blood glucose levels (Fig. 1*E* and Supplementary Tables 2 and 3). Fasting glucagon levels increased 2.4× and 2.9× in young and aged treated mice, respectively (Fig. 1*F* and Supplementary Tables 2 and 3). Fasting insulin levels were slightly increased in young and aged treated mice (Fig. 1*G* and Supplementary Tables 2 and 3). However, glucose tolerance was not altered (Fig. 1*H* and *I* and Supplementary Tables 2 and 3). Thus, JNJ-46207382 effectively antagonizes glucagon action in healthy mice, with very mild changes in glucose homeostasis.

JNJ-46207382–Stimulated α-Cell Proliferation in Young Mice

To test the impact of sustained glucagon receptor antagonism on α-cell expansion, we sequentially and continuously administered two different thymidine analogs in drinking water during the final 2 weeks of treatment in young mice (Fig. 1*C*). Pancreas mass was unaltered in young JNJ-46207382 mice (Supplementary Table 4). Islet histology was also grossly normal (Fig. 2*A* and *B*). We measured α-cell proliferation, quantifying BrdU- and EdU-positive α-cells. Consistent with other GRAs (12), JNJ-46207382 increased α-cell proliferation in the young mice ~2.5-fold from controls (Fig. 2*A*–*C* and Supplementary Tables 5 and 6). To further test the impact of JNJ-46207382 upon α-cell expansion, we quantified α-cells within islets and pancreata. JNJ-46207382–treated mice tended to have increased (15%) α-cell density per islet and pancreas section (Fig. 2*D* and *E* and Supplementary Table 7). Thus, sustained glucagon receptor antagonism increased α-cell proliferation in young mice.

α-Cells Generated by Self-Renewal

We tested whether JNJ-46207382 treatment expanded α-cells via highly proliferative transit-amplifying progenitors, as in skin and intestinal epithelia (19). To determine the lineage mechanism of α-cell expansion, we quantified thymidine analogs (Fig. 1*C*), as previously described (19,25). By labeling the first-round cell turnover with BrdU and the second with EdU, sequential cell turnover would result in BrdU/EdU-colabeled cells (Fig. 3*A*). If α-cells expanded by specialized progenitors undergoing sequential cell turnover (indicating contribution from a transit-amplifying population), new α-cells would

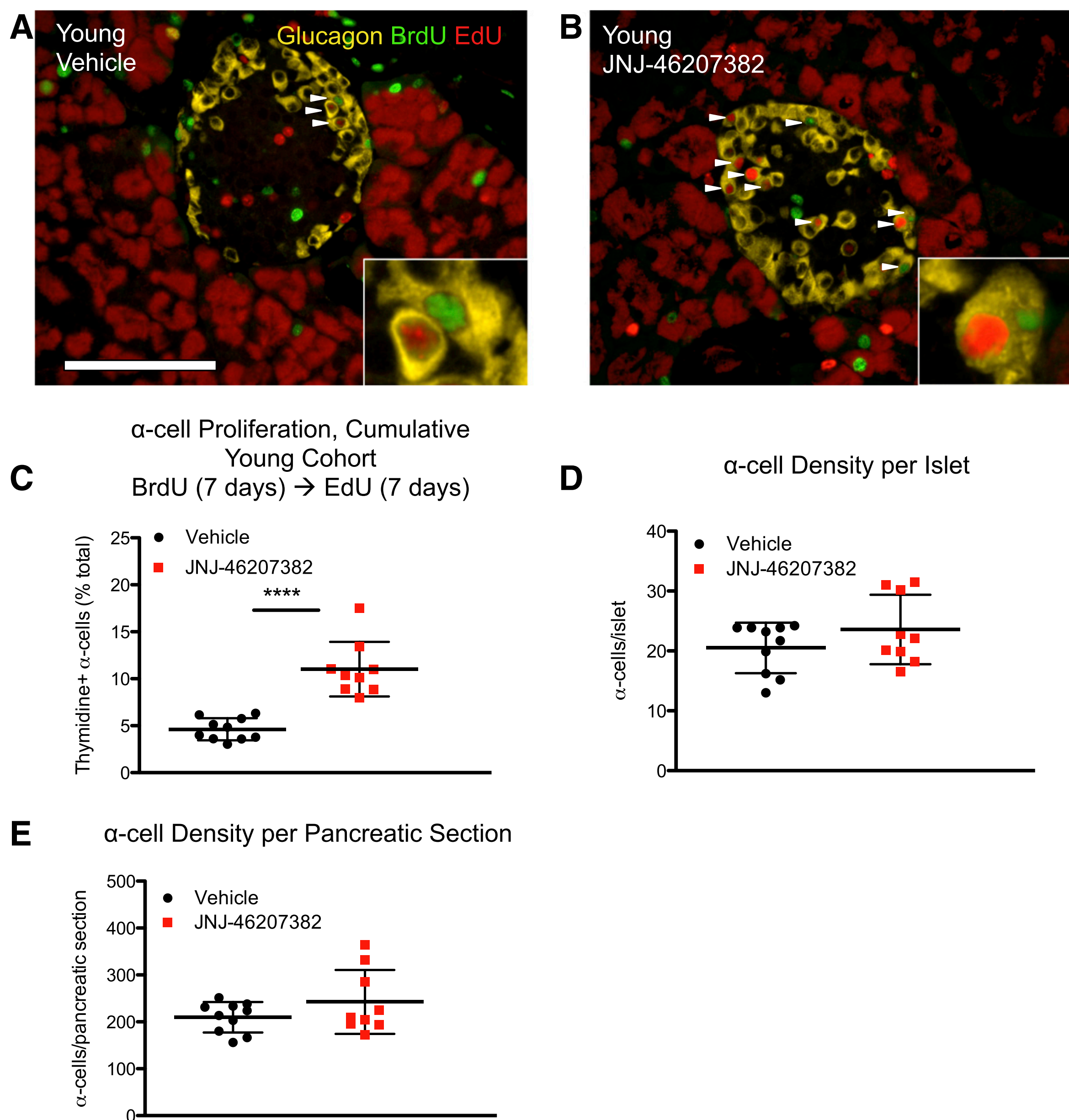

Figure 2—Sustained JNJ-46207382 treatment increases cumulative α-cell proliferation in young mice. *A* and *B*: Representative islet images for young vehicle (*A*) and young JNJ-46207382 (*B*) mice stained for glucagon (yellow), BrdU (green), and EdU (red). White arrows and insets indicate glucagon/thymidine-copositive cells. Scale bar: 100 μm. *C*: Cumulative α-cell proliferation of $BrdU^+$ and EdU^+ α-cells after sequential labeling with BrdU and EdU for 1 week each during the final 2 weeks of JNJ-46207382 treatment. Results expressed as mean ± SD for 10 young vehicle-treated and 9 young JNJ-46207382–treated mice, with ~900 α-cells quantified per biological replicate data point (Supplementary Table 5). *D* and *E*: α-Cell density. Total α-cells per islet, with ~27 islets analyzed per mouse (*D*) (Supplementary Table 7) and pancreatic section (*E*), with approximately four sections analyzed per mouse (Supplementary Table 7). $^{****}P \leq 0.0001$, vehicle vs. JNJ-46207382.

be BrdU/EdU double positive (Fig. 3*B*). Alternatively, if α-cells expanded by self-renewal through random cell division, few double-positive α-cells would be expected in the cohorts (Fig. 3*C*). In control mice, 3% of α-cells incorporated BrdU and 2% of α-cells incorporated EdU; BrdU/EdU-copositive α-cells were entirely absent (Fig. 3*D* and Supplementary Tables 5 and 6). In contrast, JNJ-46207382–treated mice labeled 6% of α-cells in week 3 and 5% in week 4. Despite the substantial GRA-induced α-cell proliferation in young mice, only a tiny fraction of α-cells were BrdU/EdU copositive (2 out of a total 8,784 α-cells, 0.02%). Thus, during 2 weeks of acute α-cell proliferation after JNJ-46207382, α-cells almost entirely divided just once and almost never twice. This result strongly supports a lineage mechanism of self-renewal (Fig. 3*C*) as the primary source of new α-cells in both basal and GRA-stimulated conditions, consistent with our previous findings in β-cells in response to various mitogenic stimuli (19,25).

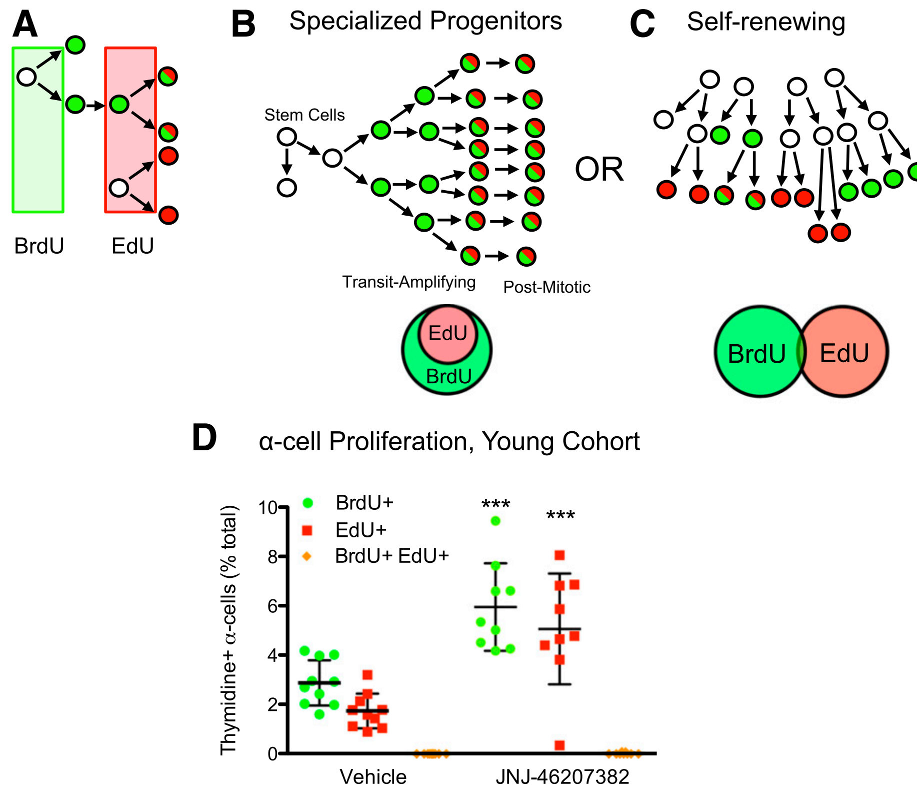

Figure 3—α-Cells are generated by self-renewal in basal and JNJ-46207382–stimulated conditions. *A*: By labeling the first cell round of cell turnover with BrdU (green) and the second round of cell turnover with EdU (red), sequential cell turnover can be identified with BrdU$^+$ EdU$^+$–colabeled cells (green/red). *B* and *C*: Potential mechanisms of cell expansion that use specialized progenitors (*B*) or self-renewing cell expansion (*C*). *B*: Specialized progenitor lineages exhibit sequential cell expansion, resulting in colabeled cells with BrdU and EdU. *C*: Self-renewing lineages exhibit random cell expansion, with few colabeled cells. *D*: Quantification of total α-cell accumulation of BrdU, EdU, or BrdU/EdU copositive after sequential labeling with BrdU and EdU for 1 week each. Results expressed as mean ± SD for 10 young vehicle-treated and 8 young JNJ-46207382–treated mice. ***$P \leq 0.001$, vehicle vs. JNJ-46207382.

α-Cell Expansion Is Limited by a Replication Refractory Period

We considered whether α-cell expansion could be limited by a "replication refractory period," which might prevent recently divided α-cells from immediately dividing a second time (Fig. 4*A* and *B*), in a manner equivalent to β-cells (19,43). If α-cell replication is stochastic, the actual proportion of BrdU/EdU-copositive α-cells should be equal to the product of the individual fractions of BrdU and EdU α-cells (19) (Fig. 4*A*). Alternatively, with a replication refractory period (nonstochastic α-cell replication), BrdU/EdU-copositive α-cells would be less frequent than predicted by the product of the individual fractions (Fig. 4*B*). But, zero BrdU/EdU-copositive α-cells were observed in the entire cohort of young control mice, far less than the five cells predicted (Fig. 4*C* and *D* and Supplementary Table 5). These findings are consistent with a replication refractory period, limiting α-cell turnover from one round of cell cycle to the next, under basal conditions.

Interestingly, the replication refractory period of β-cells does not appear to be permanently set but might be foreshortened under some β-cell mitogenic conditions (19,43). Given that acute glucagon receptor blockade is one of the strongest stimuli for α-cell expansion, we wondered whether JNJ-46207382 might bypass a replication refractory period limiting sequential α-cell turnover. But, JNJ-46207382–treated mice also had very few BrdU/EdU-copositive α-cells (a total of two cells detected across the entire cohort), far less than the 26 predicted BrdU/EdU-copositive α-cells, as calculated from the product of the two individual fractions of thymidine-positive α-cells by the total number of α-cells (Fig. 4*C* and *D* and Supplementary Table 5). Thus, GRA-stimulated α-cell proliferation is not capable of bypassing the replication refractory period of α-cell turnover.

Basal α-Cell Proliferation Decreases With Age

We tested whether basal β-cell proliferation declined in our cohorts. As expected, thymidine$^+$ β-cells were observed in low but detectable quantities (0.2% per day) in young control mice, especially when compared with historical data of neonatal β-cell turnover (19) (Fig. 5 and Supplementary Table 8). But, thymidine$^+$ β-cells were present in even lesser quantities (0.03% per day of labeling, $P <$ 0.0001 vs. young) in aged control mice (Fig. 5 and Supplementary Table 8). This result confirms previous findings that β-cell turnover is dramatically reduced in aged mammals (31–34).

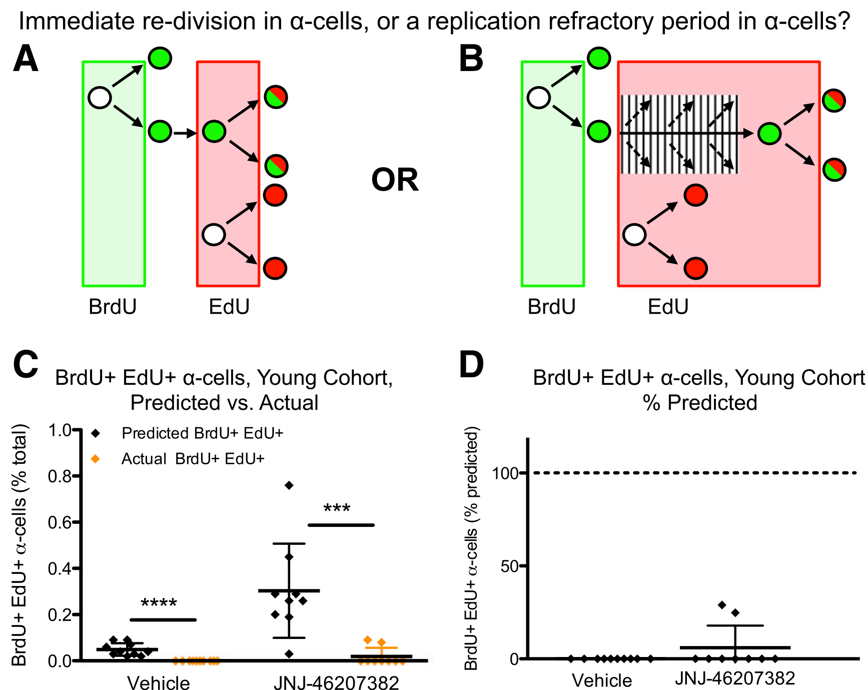

Figure 4—Sustained JNJ-46207382 treatment does not foreshorten the α-cell replication refractory period. *A* and *B*: Models of cell expansion. *A*: α-Cell turnover can occur immediately after a previous round if cell expansion is stochastic. *B*: α-Cell turnover cannot occur immediately after a previous round if limited by a replication refractory period. *C* and *D*: Actual vs. predicted BrdU/EdU-copositive α-cells demonstrate that α-cells could divide once but are limited by a replication refractory period. BrdU/EdU-copositive α-cells, expressed as actual vs. predicted (*C*) or percentage of predicted (*D*) BrdU/EdU-copositive α-cells. Mean ± SD for 10 young vehicle-treated and 9 young JNJ-46207382–treated mice. ***$P \leq 0.001$, ****$P \leq 0.0001$.

We then tested the hypothesis that α-cell proliferation could decline with age. We compared basal (nonstimulated) α-cell proliferation in mice across a range of ages, including postnatal day (PN) 3, PN4, PN21, and the young and aged controls from our JNJ-46207382 treatment studies. α-Cell proliferation was very high in newborn pups (PN3: 15% thymidine$^+$ α-cells; PN4: 14%) but rapidly declined in postnatal life (PN21: 4%) (Fig. 5 and Supplementary Table 8). α-Cell proliferation further declined postweaning (PN70: 0.33%) and dropped to very low levels in the aged mice (PN453: 0.08%) (Fig. 5*D* and Supplementary Table 8). Thus, α-cell proliferation declined by middle age to a tiny fraction compared with early postnatal life. These studies indicate that α-cell proliferation sharply declines with age, in a manner equivalent to β-cells.

α-Cell Proliferation in Aged Mice

To determine the impact of advanced age upon adaptive α-cell proliferation, we quantified thymidine analog incorporation into α-cells of JNJ-46207382–treated aged mice. As earlier, we administered the GRA for 28 days, sequentially labeling with thymidine analogs during the final 2 weeks of treatment (Fig. 1*C*). Pancreas mass was unaltered by JNJ-46207382 (Supplementary Table 9). Islet histology was also normal (Fig. 6*A* and *B*). We measured α-cell proliferation, quantifying incorporation of BrdU and EdU into α-cells. But the impact of sustained JNJ-46207382 treatment in aged mice upon α-cell proliferation was 60% less than we observed in young mice (2.5% in aged mice vs. 6.4% in young mice) (Fig. 6 and Supplementary Tables 10 and 11). We then counted all visible α-cells within islets and pancreata. The density of α-cells was more variable in the aged cohorts of mice than in the young mice but not clearly stimulated by JNJ-46207382 (Supplementary Table 12). Thus, sustained glucagon receptor antagonism only modestly increased α-cell proliferation in aged mice and was not associated with massive α-cell expansion.

Basal α-Cell Proliferation Rates Correlate With Adaptive α-Cell Proliferation

We tested for a relationship in between basal and GRA-stimulated α-cell proliferation in young and aged cohorts. In young mice, GRA-stimulated α-cell proliferation was 2.4× basal (Fig. 6*E* and Supplementary Tables 5 and 6). Similarly in aged mice, GRA-stimulated α-cell proliferation was 3.2× basal (Fig. 6*E* and Supplementary Tables 10 and 11). But the magnitude of GRA-induced α-cell proliferation was much smaller in aged mice than in young mice. Still, α-cells seemed capable of regeneration in old age, albeit with a much lower absolute quantity of new α-cells generated by the mitogenic response. Moreover, basal α-cell proliferation appeared to be correlated with α-cell proliferation. These results mirror previous results in aged β-cells in response to partial pancreatectomy, low-dose

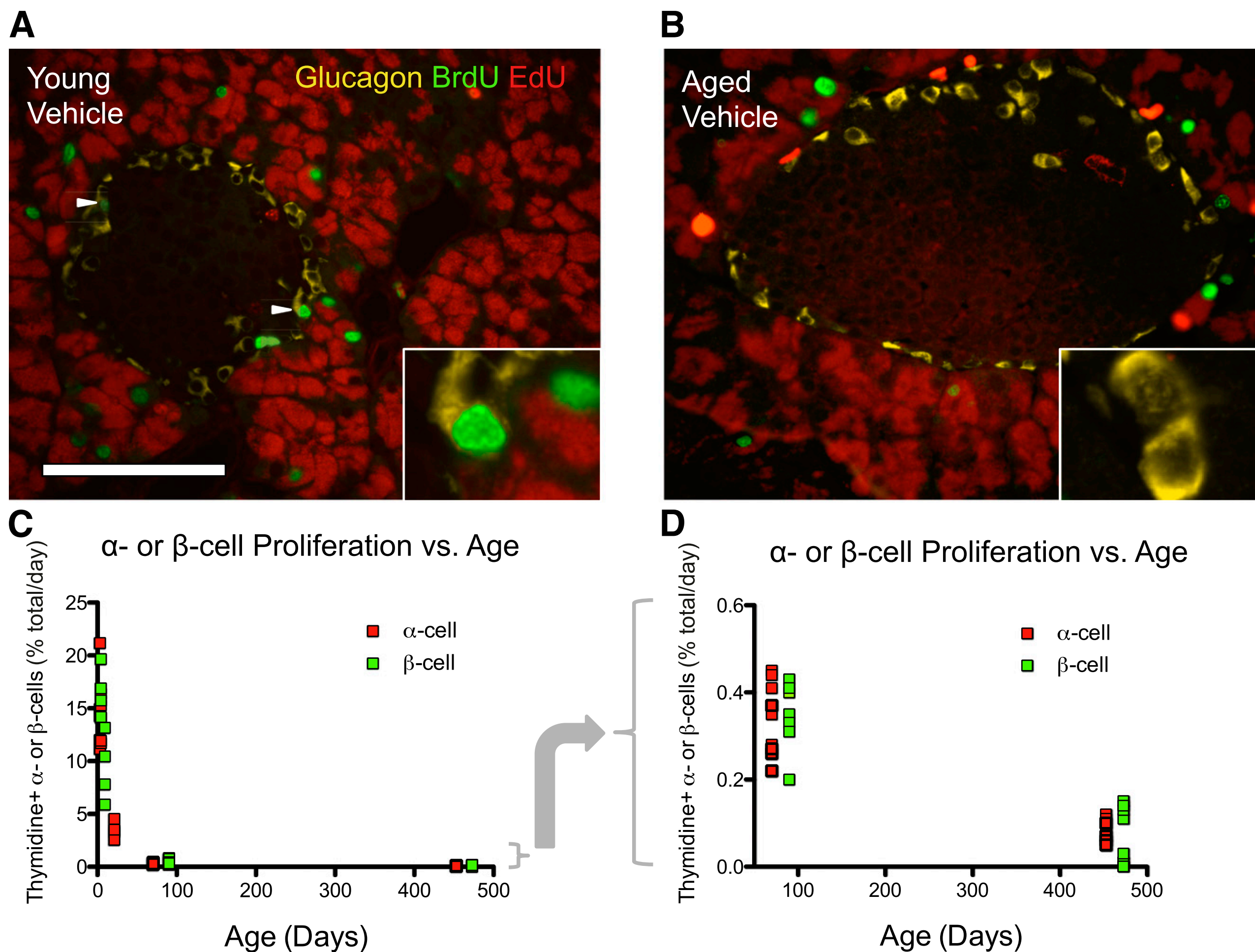

Figure 5—Basal α-cell proliferation declines with age. *A* and *B*: Representative islet images for young (*A*) and aged (*B*) vehicle mice, stained for glucagon (yellow), BrdU (green), and EdU (red). White arrows and insets indicate glucagon/thymidine-copositive cells. Scale bar: 100 μm. *C* and *D*: α-Cell proliferation declines with age. *C*: Daily α- or β-cell proliferation from a short-term (24 h) BrdU labeling study of 3-, 4-, and 21-day-old mice and also long-term labeled controls. Historical neonatal β-cell proliferation data are from Teta et al. (19). *D*: Inset of data from *C*, with upper limits at 0.6% thymidine analog–positive α-cells per day. α-Cell proliferation continues to decline into middle age. Results expressed as percent thymidine analog–positive α-cells per day.

streptozotocin, or exendin-4 (38). Thus, mitogen-stimulated islet endocrine cell proliferation may be related basal replication rates, which decline with age.

β-Cell Area, Mass, and Turnover Unchanged by JNJ-46207382

To further test the impact of JNJ-46207382 on islets, we studied β-cell morphometry in pancreata from drug-treated mice. β-Cells were grossly normal in islets of JNJ-46207382–treated mice of both age-groups (Fig. 7*A*–*D*). β-Cell area and mass were unchanged in pancreas tail of young and aged JNJ-46207382 mice compared with controls (Fig. 7*E* and *F* and Supplementary Tables 4 and 9). Similarly, β-cell proliferation was unchanged in JNJ-46207382–treated mice and controls of both age-groups (Fig. 7*G* and Supplementary Tables 4 and 9). Taken together, these studies indicate that JNJ-46207382 treatment does not significantly impact β-cell development. Moreover, these studies reinforce the α-cell–specific effects of JNJ-46207382, consistent with its role as a GRA.

DISCUSSION

Here, we advance the hypothesis that adaptive α-cell proliferation is severely restricted with advanced age. We show that α-cell turnover rapidly declines with age, dropping to very low levels in middle-aged mice (Fig. 5). Using a novel GRA, we confirm that glucagon receptor blockade potently stimulates α-cell proliferation in young mice (Fig. 2). We further show that the lineage mechanism of α-cell hyperplasia occurs via self-renewal (proliferation of the α-cells themselves) and does not involve contribution from specialized progenitor populations, akin to a population of highly proliferative transit-amplifying cells (Fig. 3). We also find that α-cells exhibit a replication refractory period that limits α-cell turnover from one round of cell turnover to the next (Fig. 4). Finally, our studies indicate that GRA-induced α-cell proliferation is

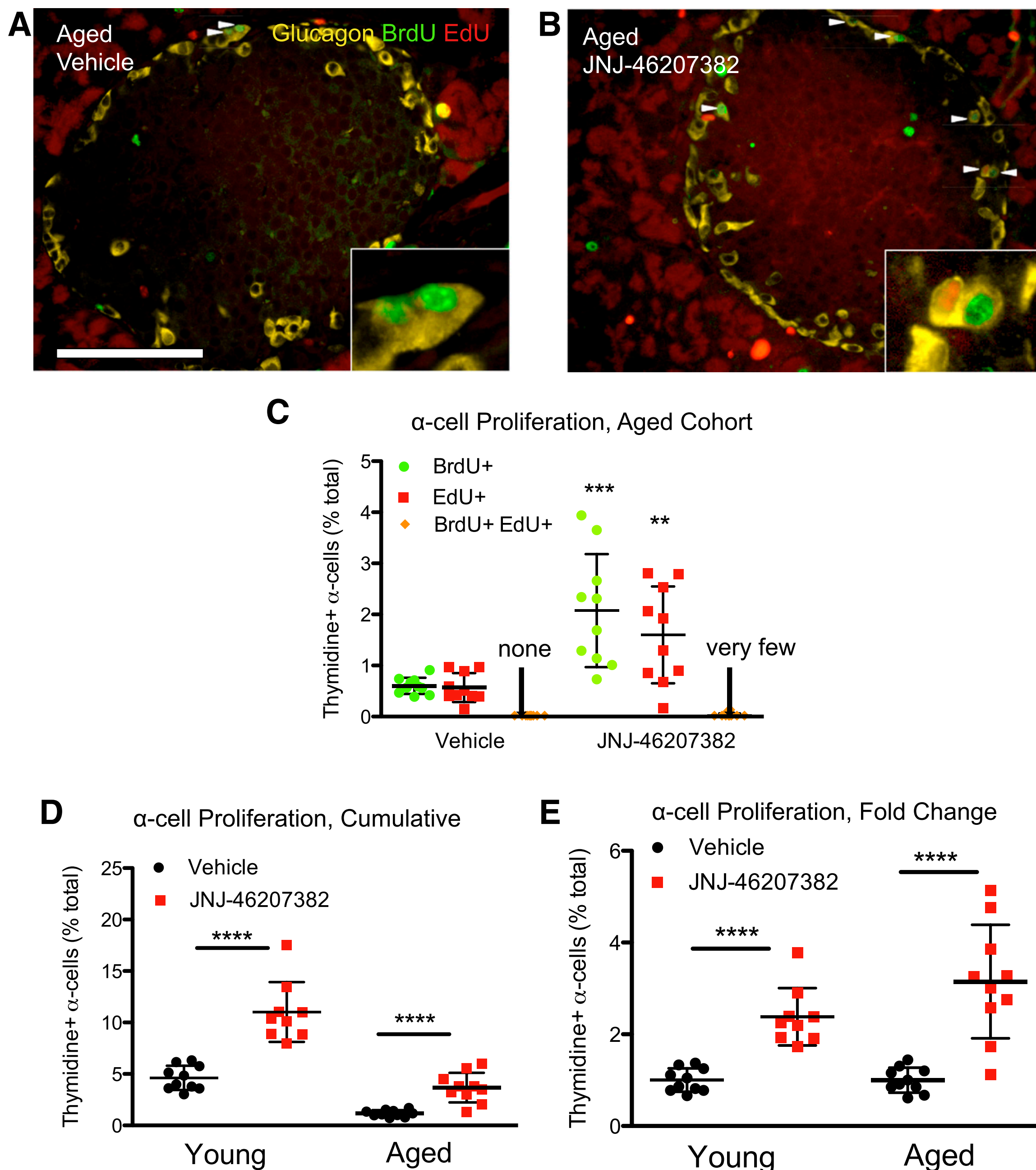

Figure 6—Adaptive α-cell proliferation declines with age. *A* and *B*: Representative islet images for aged vehicle (*A*) and JNJ-46207382 (*B*) mice stained for glucagon (yellow), BrdU (green), and EdU (red). White arrows and insets indicate glucagon/thymidine-copositive cells. Scale bar: 100 μm. *C*: Quantification of total α-cell accumulation of BrdU, EdU, or BrdU/EdU copositive in aged vehicle and JNJ-46207382 mice after sequential labeling with BrdU and EdU for 1 week each. *D* and *E*: Quantification of cumulative α-cell accumulation of BrdU, EdU, or $BrdU^+$ EdU^+ α-cells after sequential labeling with BrdU and EdU for 1 week expressed as percent total (*D*) and fold change (*E*) over controls. Results expressed as mean ± SD for 10 young vehicle, 9 young JNJ-46207382, 10 aged vehicle, and 10 aged JNJ-46207382 mice. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$, vehicle vs. JNJ-46207382.

present to a minor degree in aged mice, albeit at a much lower level when the absolute quantity of new α-cells from old mice is compared with young mice (Fig. 6).

JNJ-46207382 dramatically increased α-cell proliferation in young mice, similar to other GRAs (12,14–17,44). The magnitude of α-cell–proliferative effects by GRAs varies, but this may be influenced by the timing of proliferation measurement. Several published studies used short-term treatment protocols followed by immediate sacrifice and Ki67 or short-term thymidine labeling to quantify α-cell proliferation. In contrast, we measured proliferation over the 3rd and 4th week of GRA treatment. We previously observed tachyphylaxis in β-cell mitogenic responses in young mice; the proliferative effects of

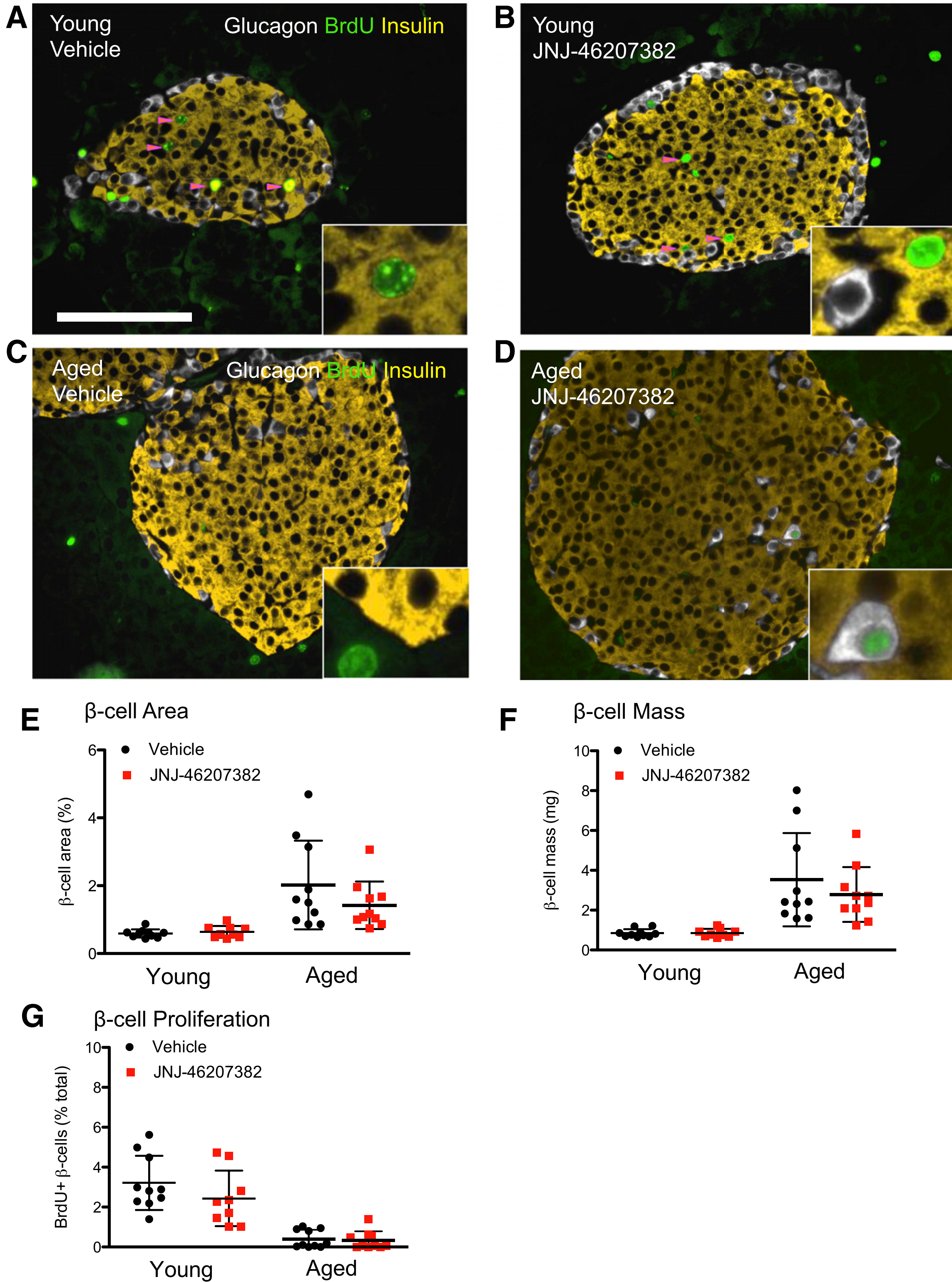

Figure 7—Sustained JNJ-46207382 treatment does not alter β-cell morphometry. *A–D*: Representative islet images for young vehicle (*A*), young JNJ-46207382 (*B*), young vehicle (*C*), and aged JNJ-46207382 (*D*) mice stained for glucagon (white), BrdU (green), and insulin (yellow). Magenta arrows indicate insulin/BrdU-copositive cells. Insets indicate thymidine-positive cells. Scale bar: 100 μm. *E*: Quantification of β-cell area in tail of pancreas (% total). *F*: Quantification of β-cell mass in tail of pancreas (mg). *G*: Quantification of β-cell proliferation in insulin/BrdU-copositive cells after labeling with BrdU for 1 week. Results expressed as mean ± SD for 10 young vehicle, 9 young JNJ-46207382, 10 aged vehicle, and 10 aged JNJ-46207382 mice.

extreme obesity rapidly waned over a 4-week period after disruption of the leptin receptor (25). Indeed, α-cell proliferation was greater in week 3 than 4 of JNJ-46207382 treatment Fig. 3*D* and Supplementary Table 5). Thus, α-cell proliferation may have peaked immediately after initiation of GRA treatment and progressively declined. Although thymidine labeling in weeks 1 and 2 might have captured even more α-cell proliferation, it seems unlikely that this result would have changed our major conclusions. Indeed, direct quantification of total pancreatic or islet α-cells yielded results that were consistent with the magnitude of JNJ-46207382–induced α-cell proliferation.

Mouse strain represents another likely modifying factor. We administered JNJ-46207382 to 1.5- and 14-month-old male F1 hybrid B6129SF1/J mice. We chose this strain because it closely approximates the mixed genetic background of laboratory knockout mice. However, other strains such as ICR may have even greater basal islet endocrine proliferation and exaggerated proliferative responses (40). Nevertheless, the lack of a large α-cell–proliferative response in our aged mice is especially reassuring, as it indicates that GRAs may not be able to launch aged adult α-cells into a program of unrestrained expansion. Given that the target population for GRAs would be middle-aged to elderly adults with type 2 diabetes, our result suggests that GRAs might be well tolerated in patients.

Like most other studies of α-cell proliferation in response to GRAs, we performed our studies under nondiabetic conditions. Thus, it remains possible that greater amounts of α-cell proliferation might have been observed in a diabetic model. Additional studies are therefore necessary to determine the applicability of our GRA studies to diabetic α-cells.

JNJ-4620738 was associated with increased fasting blood glucose in young mice relative to controls. However, our young control mice were mildly hypoglycemic (~37/mg) after a 16-h fast (Fig. 1). Paradoxically, the GRA seemed to partially ameliorate this hypoglycemic response. In contrast, most GRA studies have been performed on diabetic or obese mice, with much higher fasting blood glucoses values (13,16,44). Notably, random fed blood glucoses were unchanged by GRA in both young and old mice. Thus, some of the impact of JNJ-4620738 on fasting blood glucose could be specific to the hypoglycemic fast in our cohort. Further investigation is required to sort out the potential impact of GRAs on normal or low blood glucose physiology.

Our study confirms that the developmental biology of adult murine α-cells and β-cells is remarkably similar. Like β-cells, α-cells expand via self-renewal via proliferation of the α-cells themselves and are limited by age-dependent declines in cell cycle entry. α-Cells and β-cells join a growing list of mature adult somatic tissues that do not seem to be generated by specialized progenitors, including liver (45) and kidney (46). Interestingly, like α-cells and β-cells (38,47), liver and kidney also exhibit an age-dependent decline in regeneration (48,49). Thus, tissues that do not deploy specialized progenitor stem cell programs may be uniquely restricted by age-associated limits on regeneration.

The molecular basis for the age-dependent decline in cell cycle entry in α-cells is unclear but seems likely to include factors that modify cell cycle entry and exit of mature β-cells (47). Many of these signals impinge upon chromatin-modifying enzymes, which ultimately alter cell cycle entry in aged β-cells. Intriguingly, none of the genetic manipulations deployed thus far have been capable of wholesale regeneration of aged β-cells (47). Whether the lack of functional β-cell rejuvenation reflects redundancy in signals or a permanent cell cycle exit within aged β-cells remains unclear. Regardless, the minimal regenerative capacity of both α-cells and β-cells strongly suggests that common mechanisms underlie these changes.

Our study has major implications for the field. Aged mice demonstrated a modest α-cell–regenerative response to sustained JNJ-46207382. However, the proliferative impact of JNJ-46207382 in aged mice was very limited in magnitude compared with young mice. We also found that basal α-cell proliferation decreased with age. These findings suggest that basal and adaptive α-cell proliferation are severely restricted with advanced age. Our results also hint that basal α-cell proliferation may reflect the capacity for adaptive α-cell proliferation. These studies should provide some reassurance regarding the use of GRAs in middle-aged or elderly adult patients, who would be expected to have minimal basal α-cell proliferation (36).

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Author Contributions. C.J.L., M.M.R., K.B.K., M.C.W., and B.C.S. performed the experiments. C.J.L., M.M.R., and J.A.K. conceived and designed the experiments and analyzed the data. C.J.L. and J.A.K. wrote the paper. J.A.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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