DNA damage does not cause BrdU labeling of mouse or human beta cells

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Running title
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Abbreviations
BrdU, bromodeoxyuridine
EdU, 5-ethinyl-2'-deoxyuridine
gH2AX, gamma phosphorylated H2A histone family member X

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ABSTRACT

Pancreatic beta cell regeneration, the therapeutic expansion of beta cell number to reverse diabetes, is an important goal. Replication of differentiated insulin-producing cells is the major source of new beta cells in adult mice and juvenile humans (1–3). Nucleoside analogs such as bromodeoxyuridine (BrdU), which are incorporated into DNA during S-phase, have been widely used to quantify beta cell proliferation. However, reports of beta cell nuclei labeling with both BrdU and gH2AX, a DNA damage marker, have raised questions about the fidelity of BrdU to label S-phase, especially during conditions when DNA damage is present. We performed experiments to clarify the causes of BrdU-gH2AX double-labeling in mouse and human beta cells. BrdU-gH2AX co-labeling is neither an age-related phenomenon nor limited to human beta cells. DNA damage suppressed BrdU labeling and BrdU-gH2AX co-labeling. In dispersed islet cells, but not in intact islets or in vivo, pro-proliferative conditions promoted both BrdU and gH2AX labeling, which could indicate DNA damage, DNA replication stress, or cell-cycle related intrinsic H2AX phosphorylation. Strategies to increase beta cell number must not only tackle the difficult challenge of enticing a quiescent cell to enter the cell cycle, but also achieve safe completion of the cell division process.
Beta cell replication, towards re-expansion of functional beta cell mass, is an important goal for diabetes research. Incorporation of nucleoside analogs has been a high-value tool in quantifying cell proliferation behavior for decades, allowing measurement of cumulative S-phase entry during a defined exposure period, in vitro and in vivo. Nucleoside analogs such as BrdU and EdU (4) have been used not only in beta cell biology but also broadly across developmental and cell biology.

Under certain conditions, BrdU incorporation in beta cells has been observed to co-localize with markers of DNA damage (5,6). In other cell types, BrdU exposure has been shown to activate a DNA damage response (7–9), but in beta cells the reasons for this co-localization are not well understood. The observation has been widely discussed and rapidly incorporated into thinking in the field, with a range of impacts. In the most-discussed work (5), the conclusion drawn by the originating authors was that some BrdU-labeled human beta cells, particularly the subset with atypical punctate BrdU staining, fail to complete S-phase, instead showing evidence of DNA damage, DNA re-replication, and failure to divide. In other words, BrdU labeled cells that transitioned into S-phase, but BrdU-labeled cells could not be assumed to progress through successful mitosis. However, concern in the field has extended beyond this concept; in many quarters, the question has been raised as to whether BrdU labeling, counted as evidence of S-phase entry, could in fact be due to a completely unrelated process, nucleotide incorporation during DNA damage repair. If this hypothesis were true, then nuclei might label for BrdU in the absence of S-phase entry, invalidating some prior results and diminishing the value of nucleoside analogs in the study of cell replication.

The field of beta cell regeneration has been impacted by uncertainty of the correct interpretation of BrdU-labeled nuclei. There is an urgent need to clarify the reasons for co-occurrence of BrdU and DNA damage labeling in beta cells. The goals of this study were to explore possible causes of BrdU and gH2AX co-localization in nuclei of mouse and human beta cells, and to specifically test whether there are conditions in which BrdU labeling can be induced by DNA damage-related cellular processes rather than cell cycle entry.

**RESEARCH DESIGN AND METHODS**

**Mice for islet isolation**
All mouse procedures were approved by the UMass Medical School Institutional Animal Care and Use Committee. Young (10-12 weeks) or old (50-60 weeks) C57BL/6J male and female mice, from an in-house breeding colony, had continuous access to normal mouse chow, on a 12-hour light/dark cycle. Islets were isolated by pancreatic ductal collagenase injection and Ficoll (Histopaque-1077; Sigma)
gradient (10). Islets from multiple mice were pooled and mixed before experiments. Whenever possible, all control and experimental comparisons were performed in parallel on islets from the same mice. Each combined pool of islets was considered one biological replicate.

**In vivo mouse experiments**

To study proliferative conditions in vivo, pancreas sections were analyzed from experiments previously published on 10-12 week old male mice fed high fat diet for 7 days (11), or 10-12 week old male mice with continuous hyperglycemia achieved by intravenous infusion of glucose (10,12).

**Mouse islet cell culture**

Whole islets were cultured overnight in islet medium (RPMI, 10% FBS, penicillin/streptomycin, 5.0 mmol/L glucose) after isolation. For dispersed-cell experiments, islets were hand-picked, digested with single-use-apportioned 0.05% trypsin, and 50 IEQ per well were plated on uncoated glass coverslips (Fisherbrand) in islet medium (12). Mouse islet cell experiments were 72 hours in duration, starting the day after dispersion, with adenovirus, glucose and/or harmine (5 uM, Sigma #286044) exposure for 72 hours, BrdU (10ug/ml) added 24 hours prior to fixation. For whole islet experiments, islets were cultured for 72 hours in islet medium with additives including glucose and/or Mitomycin C, with BrdU added for the final 24 hours, fixed in 10% formalin for 30 minutes, washed with PBS and embedded in paraffin for sectioning (13). For virus transduction, dispersed cells were transduced with adenovirus expressing bacterial Cre recombinase (control virus) or human Cyclin D2 protein, at an MOI of 5, as described (12). Mitomycin C (0.5 uM, Sigma) was added at the start of the 72-hour experiment. UV irradiation of islet cell cultures was administered by placing the culture dish for 10 minutes on a UVP bench top transilluminator equipped with a 302 nm filter (Cole-Parmer) after 24 hours of glucose treatment.

**Human islet culture**

Human islets received from the IIDP ([Supplemental Table 1](#)) were cultured in islet medium overnight after shipment, then dispersed and plated on coverslips as described above for mouse islets. After one day recovery from plating, human islet cell experiments were performed exactly as described above, except the experiment duration was 96 hours and BrdU was included for the entire 96 hours of culture.

**Immunostaining and microscopy**

Islet cell cultures were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Whole islets (13) and pancreas sections were obtained from formalin-fixed, paraffin embedded tissue blocks as described in the publications where these samples originated (10–12). For BrdU, gH2AX and pHH3 staining, fixed cells or rehydrated paraffin sections were submerged in 1N HCl for 20 minutes, blocked
for 2 hours in goat serum-based block with 0.1% Tween-20, labeled with primary antibodies, then secondary antibodies (Invitrogen), and mounted on glass slides with Fluoroshield mounting media containing Dapi (Sigma). Primary antibodies were guinea pig anti-insulin (Dako A0564), rabbit anti-gH2AX (Cell Signaling #9718), mouse anti-gH2AX (EMD Millipore #05-636), rat anti-BrdU (Abcam Ab6326), rabbit anti-pHH3 (Cell Signaling #3377) and rabbit anti-Ki67 (Thermo Scientific RM-9106). Fluorescent images were acquired using a Nikon fluorescent microscope or a Solamere CSU10 Spinning Disk confocal system mounted on a Nikon TE2000-E2 inverted microscope. Image data were quantified by an unbiased laboratory member (BG) using Cell Profiler automated counting software from the Broad Institute (14,15) with manual image checking post-quantification. A cell was considered to be gH2AX positive if the nucleus was labeled with multiple intense nuclear foci. The number of cells counted for each experimental condition are included in Supplemental Table 2.

Statistical Analysis
Data were analyzed and graphed using GraphPad Prism 7. In all column plots, each sample is represented by one data point. Venn diagrams were generated using EulerAPE 3.0.0 from the University of Kent (16). Data were compared using Student’s two-tailed T-test; p<0.05 was considered significant.

RESULTS
Possible explanations for BrdU and DNA damage co-labeling in the same beta cell
Consistent with the reports of human beta cell co-labeling (5), we have observed BrdU-gH2AX co-labeling in primary mouse islet cell cultures (unpublished, and Fig 1). We reasoned that these two markers could label the same nucleus if: a) DNA damage leads to BrdU incorporation, b) BrdU incorporation leads to DNA damage, c) an upstream process leads to both labels, or d) BrdU incorporation and DNA damage occur stochastically and occasionally occur in the same cell but are unrelated (Supplemental Table 3).

BrdU-gH2AX co-labeling in mouse beta cells is not due to random chance
We first tested the simplest explanation for co-localization: that BrdU labeling and DNA damage each occur in a fraction of cultured primary beta cells, unrelated to one another, leading to occasional co-occurrence in the same cell. If this model were true, gH2AX and BrdU labels should both be detectable in standard culture conditions, and the presence of one label in a cell should not influence the likelihood of presence of the other label. We cultured dispersed mouse islet cells under standard, unstimulated conditions. When the cells were fixed and immunostained for insulin, BrdU and gH2AX, a small but consistent fraction of insulin-containing cells labeled for each marker (Fig 1A-A’-B). To test whether co-labeling could be due to random co-occurrence of two unrelated processes, we calculated the predicted
likelihood of random co-labeling based on the frequency of each individual label (predicted fraction co-labeling was defined as the product of the observed fractions of each single label) and compared this predicted frequency with the actual observed frequency of co-labeling. The observed frequency, although low, was higher than the predicted random co-occurrence frequency (Fig 1C). Confocal microscopy confirmed nuclear co-labeling in some beta cells (Fig 1D-D'). As reported (5), in some BrdU-gH2AX co-labeled nuclei the BrdU labeling was punctate in appearance. Thus, in unstimulated culture conditions, co-labeling of BrdU and gH2AX occurred more frequently in mouse beta cells than predicted by chance, suggesting that one process influences the other or that both are influenced by a common upstream process.

Proliferative stimuli increase BrdU-gH2AX double-labeling frequency

Since overexpression of HNFA8 or Cyclin D3/Cdk6 in combination (5) increased co-labeled cells, we reasoned that proliferation drivers increase the frequency of double-labeled cells. Since the originating work was in human beta cells, which are considerably older than young-adult mouse beta cells, we also considered that beta cells from older mice might be more vulnerable. We tested whether the mitogenic stimuli frequently used in our lab, 15mM glucose and adenoviral-delivered Cyclin D2 (12,17), altered the likelihood of BrdU and gH2AX co-labeling in beta cells from younger (10-12 weeks) and older (50-60 weeks) mice. In 15mM glucose, both BrdU and gH2AX (Fig 2A-B) labeling increased. Supporting the concept that BrdU labeling reflects S-phase entry, beta cells from younger mice had a higher frequency of BrdU incorporation than beta cells from older mice. Perhaps surprisingly, in high glucose beta cells from older mice did not label more frequently for gH2AX, or both markers, than beta cells from younger mice (Fig 2C).

To test whether directly forcing cell cycle entry increases BrdU-gH2AX co-labeling, we overexpressed Cyclin D2. In normal 5mM glucose, Ad-cyclin D2 produced similar results to 15mM glucose stimulation (Fig 2D-F): BrdU and gH2AX were individually modestly increased, the frequency of double-labeled cells increased, and older mouse age did not increase the frequency of beta cells with gH2AX or double-labeling. However, when Cyclin D2 overexpression was combined with 15mM glucose (Fig 2G-I) BrdU, gH2AX, and BrdU-gH2AX labeled beta cells were markedly increased over baseline. In all proliferation-stimulated conditions (Fig 2C, F and I), the observed frequency of co-labeled cells was significantly higher than predicted if co-labeling was due to chance. To test a third, unrelated proliferative stimulus we treated the cultures with harmine, a Dyrk1a inhibitor that increases mouse and human beta cell proliferation (18). Harmine increased BrdU labeling synergistically with glucose, increased gH2AX labeling in both 5 and 15mM glucose, and similar to the other stimuli, the observed frequency of co-labeling was higher than
predicted (Fig. 2J-L). Thus, the frequency of BrdU-gH2AX double-labeling is increased by proliferative stimuli, especially when stimuli are combined in aging mouse beta cells.

**DNA damage does not lead to BrdU incorporation**

To explore whether DNA damage and BrdU incorporation influence each other, we next tested whether BrdU incorporation was increased during conditions that induce DNA damage. These were perhaps the most critical experiments in this study, because if BrdU is spuriously incorporated as part of a DNA damage response, then BrdU labeling cannot be used to quantify S-phase cell cycle entry. To address this question we cultured beta cells in DNA damage-inducing conditions and quantified BrdU- and double-labeled cells. In islet cell cultures exposed to a sub-lethal dose of mitomycin C, which induces DNA damage by crosslinking DNA (19–21), almost all beta cells had gH2AX labeling (Fig 3A), confirming DNA damage. To test whether DNA damage caused spurious BrdU incorporation, we stained for BrdU. The results showed the opposite; mitomycin C strongly suppressed BrdU labeling frequency in both young and old beta cells, and the observed frequency of double-labeled cells was suppressed to the predicted frequency if co-labeling were caused by random co-occurrence of unrelated processes (Fig 3B-C). These results suggest that DNA damage repair following mitomycin C exposure did not cause spurious BrdU incorporation in mouse beta cells that could be misconstrued as proliferation.

To thoroughly explore this important question, we repeated the experiment using a second DNA damage insult: UV irradiation, which damages DNA by joining adjacent thymine nucleotides into pyrimidine dimers (22–24). We selected a UV dose that caused moderate DNA damage based on trial experiments (data not shown). Cellular response to UV was variable, with two experiments showing a high proportion of cells labeling for gH2AX (circles in Fig 3D-F), and two experiments, despite being performed identically, showing fewer cells labeling for gH2AX (triangles in Fig 3D-F). Similar to the mitomycin C result, UV-induced DNA damage suppressed BrdU incorporation rather than increasing it, and reduced the observed frequency of double-labeled beta cells to that predicted if co-labeling was due to random co-occurrence of unrelated events (Fig 3E). Reduced BrdU incorporation even in the cultures with only modest gH2AX labeling frequency (triangles) suggests that the suppression of BrdU label was not due to overwhelming toxicity of the DNA damage insult. Taken together, these data suggest that BrdU label in cultured mouse beta cells is not increased by DNA damage.

**gH2AX labels a subset of BrdU-labeled beta cells in ex vivo culture**

Under most conditions, we noticed that although many BrdU-labeled cells were not gH2AX-labeled, the converse was not true: most gH2AX-labeled cells were BrdU-labeled. Stated another way, the population of gH2AX-labeling beta cells seemed to be a subset of the BrdU-labeled population. Expressing the data using quantitative Venn diagrams confirmed this observation; especially under proliferation-stimulated
conditions, the majority of gH2AX-labeled beta cells were within the BrdU-labeled population (Fig 4A-B). In contrast, under DNA damaging conditions, Venn analysis showed that the vast majority of gH2AX-labeling beta cells did not label with BrdU (Fig 4C). We used confocal microscopy to identify the pattern of BrdU label in double-labeled cells. We specifically asked whether BrdU labeled nuclei uniformly, as is characteristic of S-phase DNA replication, or in punctate fashion overlapping with foci of the gH2AX DNA damage label. Images showed that many of the double-labeled nuclei were uniformly labeled with BrdU (Fig 4D-E), suggesting the possibility that gH2AX labeling occurred after DNA replication. Some nuclei had punctate BrdU as described earlier (Fig 4F) (5). Consistent with the notion that DNA damage prevents successful cell division, the few mitotic nuclei visualized had no gH2AX label (Fig 4G). On the other hand, mitotic figures did label for BrdU (Fig 4G), and many BrdU-labeled nuclei co-stained for the mitotic marker pHH3 or appeared as doublets (Suppl. Fig 1), confirming that some BrdU-labeled beta cells do enter mitosis. We tested whether gH2AX-BrdU co-labeling of beta cells occurs in vivo under conditions in which beta cell mass increases, such as high fat feeding (11) and hyperglycemia (10,12). Careful examination of many islets in sections from multiple mice with elevated BrdU incorporation revealed very rare gH2AX labeling in either condition: in eight pancreata from the HFD experiment only four islet-related nuclei were found that stained for gH2AX (Suppl. Fig 2), and in four pancreata from the hyperglycemia experiment only four islet-related nuclei stained for gH2AX (Suppl. Fig 3). Of the eight islet nuclei labeling for gH2AX across both experiments, 6 were beta cell nuclei, but only two co-stained for BrdU; interestingly, these two were found in the same islet (Suppl. Fig 2B). To assess whether gH2AX labeling is induced by ex vivo islet culture in general, or of dispersed islet cell cultures specifically, whole intact islets were cultured under proliferative conditions and assessed for BrdU and gH2AX co-labeling (Suppl. Fig 4). Beta cells co-labeling for both markers were very rare in intact islets (Suppl. Fig 4D). Taken together, these results suggest that in ex vivo dispersed islet cell culture, but not intact islet culture or in vivo pancreas, conditions that promote cell cycle entry or BrdU incorporation increase beta cell risk of gH2AX labeling, whereas conditions that promote DNA damage do not increase likelihood of BrdU incorporation.

**BrdU incorporation itself is a minor cause of beta cell DNA damage**

Since the data suggested that cell cycle entry, or possibly BrdU incorporation itself, increased the likelihood of gH2AX labeling, and nucleoside analogs are known to induce a DNA damage response in other cell types (7–9), we next asked whether BrdU exposure leads to DNA damage in beta cells. To test this, we cultured mouse islet cells with or without BrdU and assessed the insulin (+) fraction labeling for gH2AX. Omitting BrdU from the culture medium eliminated all BrdU labeling in the cultures (not shown). In the absence of BrdU exposure, we observed a subtle reduction in gH2AX labeling in beta cells from younger mice that was not observed in beta cells from older mice (Fig 5A). Hypothesizing that gH2AX
labeling might increase over time following BrdU exposure, we compared cells exposed to BrdU during the first 24 hours (condition X), the last 24 hours (condition Y) or the entire 72-hour glucose exposure (condition Z). gH2AX labeling was not different between these conditions (Fig 5B-C). To test whether BrdU exposure increased DNA damage under more pronounced proliferative stimuli, we cultured islet cells with or without BrdU in the presence of Ad-cyclin D2 in low glucose (Fig 5D) or high glucose (Fig 5E). Intriguingly, BrdU exposure increased gH2AX labeling only in the low-glucose condition. To test whether gH2AX labeling was more related to cycling beta cells or to BrdU incorporation itself, we stained proliferation-stimulated cultures with pHH3 and gH2AX, predicting that if gH2AX labeling was due to BrdU toxicity there would not be co-labeling of gH2AX with pHH3 in the absence of BrdU exposure (Fig 5F-H). In fact, beta cell nuclei frequently co-labeled with both pHH3 and gH2AX, at levels much higher than expected if co-labeling was due to random chance (Fig 5G-H). Similar results were observed with experiments in which co-staining was performed for Ki67 and gH2AX (data not shown). These data suggest that although BrdU exposure or BrdU incorporation into DNA under certain conditions can cause double strand DNA breaks or nucleotide excision/repair that label with gH2AX (23), this toxicity is not the major cause of BrdU-gH2AX double-labeled cells in islet cell cultures.

**Human beta cells, resistant to proliferative stimuli, were less likely to co-label for BrdU and gH2AX than mouse beta cells**

The goal of studies in beta cell regeneration is to find approaches to expand human beta cell number. To learn more about BrdU-gH2AX co-labeling in human beta cells (5,6), dispersed human islet cells were cultured under the same conditions as the mouse experiments, and tested for BrdU incorporation, gH2AX labeling, and co-labeling for both markers. Under basal 5mM glucose conditions, the frequency of human beta cells labeling for BrdU was low, and the frequency of gH2AX labeling was also rare (Fig 6A-B). To determine whether DNA damage increased in human beta cells during conditions of forced proliferation, we compared basal conditions with pro-proliferative stimuli 15mM glucose without or with Ad-cyclin D2. Similar to prior published results, insulin (+) cells in human islet cultures were relatively resistant to proliferative stimuli, showing only a marginal increase in the fraction labeling with BrdU when exposed to 15mM glucose (Fig 6A-C), Ad-cyclin D2 (Fig 6D-F), or a combination of the two (Fig 6G-I). Surprisingly, only a small fraction of human beta cells labeled for gH2AX under basal or stimulated conditions (Fig 6B, E, H). The fraction of human beta cells labeling for both labels remained low and was not significantly higher than predicted by the random chance of co-occurrence of two rare events (Fig 6C, F, I). These data suggest that under these growth conditions, human beta cells do not frequently co-label for gH2AX and BrdU.

**DNA damage did not increase BrdU labeling in human beta cells**
To test whether human beta cells incorporate BrdU during DNA damage repair, DNA damaging conditions were applied to human islet cultures. Mitomycin C exposure caused gH2AX labeling in the majority of beta cells (Fig 7A). Despite widespread DNA damage in the cultures, the percent of insulin (+) cells labeling for BrdU decreased (Fig 7B) and the observed fraction of double-labeled cells was similar to predicted if co-labeling was by random chance. When human islet cultures were exposed to UV irradiation (Fig 7D-F), the DNA damage response was variable (Fig 7D), but BrdU incorporation was suppressed (Fig 7E) and there was no increase in the proportion of beta cells labeling for both markers (Fig 7F). In sum, human beta cells behaved similarly to mouse beta cells: DNA damage did not cause a spurious increase in BrdU labeling.

**gH2AX and BrdU labeling were mostly independent in human beta cells**

Quantitative Venn analysis of the human beta cell BrdU- and gH2AX-labeling populations revealed the interesting result that under basal and proliferation-stimulated conditions, insulin (+) cells labeling for BrdU were mostly not the same cells that labeled with gH2AX (Fig 8A). In fact, without Ad-cyclin D2, no double-labeled cells were identified. Similar to mouse islet cultures, however, DNA damage stimuli caused the majority of human beta cells to label for gH2AX, and only a tiny fraction of those cells also labeled for BrdU (Fig 8B). Overall, fewer human beta cells labeled for gH2AX than mouse. Like mouse, DNA damage conditions did not induce spurious BrdU incorporation in human beta cells. Neither BrdU nor gH2AX were meaningfully increased by proliferative stimuli in human beta cells, and in these conditions, the observed double-labeled fraction was not increased over that predicted by random chance.

**DISCUSSION**

These studies explore the frequency and causes of mouse and human beta cell co-labeling for BrdU and the gH2AX DNA damage marker. The results suggest that in mouse beta cells, co-labeling occurs more frequently than predicted if the association is due to random chance. Evidence does not support the hypothesis that co-labeling is due to BrdU incorporation during DNA repair. Instead, the results suggest that gH2AX labeling can be triggered by something related to the proliferative process, either by an upstream proliferation stimulus or by cellular events associated with the cell cycle itself.

These experiments are important, because uncertainty of the fidelity of BrdU as a label of S-phase in beta cells has impacted the field. For some, conflating the concepts of cell cycle and DNA damage has rekindled longstanding doubts that beta cells can replicate at all, despite a large quantity of evidence to
the contrary, e.g. (3, 14–16). Investigators have been at times required to use multiple duplicative tools to measure the same outcome, slowing progress. The results of these current studies are reassuring. Labeling for gH2AX in human beta cells was rare, even under proliferation-stimulating conditions, and most BrdU(+) human beta cells were not gH2AX(+). In vivo mouse pancreas rarely labeled with gH2AX, even under conditions when many beta cells were BrdU(+). Overall, the results are consistent with the conclusion that even in mouse islets ex vivo BrdU authentically labels beta cells that enter S-phase.

The data show that DNA damage does not cause BrdU labeling that could be misconstrued as S-phase entry, in either mouse or human beta cells. Using two different DNA damage inducing conditions, one of which caused widespread damage in essentially all beta cells (mitomycin C), and the other of which caused more moderate damage (UV irradiation), BrdU incorporation was reduced rather than increased. Furthermore, the observed frequency of double-labeled nuclei was suppressed in DNA damage conditions to that expected if the occurrence was due to random chance. Thus, beta cell co-labeling for BrdU and gH2AX in these cultures was not caused by spurious BrdU incorporation as part of the cellular response to DNA damage.

The data show a clear association between gH2AX labeling and proliferative stimuli in mouse beta cells. gH2AX labeling, and gH2AX-BrdU or gH2AX-pHH3 co-labeling, occurred occasionally under unstimulated conditions but were markedly increased in 15mM glucose with or without overexpression of cyclin D2 or addition of harmine. Intriguingly, the gH2AX-labeled cells were mostly a subset of the BrdU-labeled population. Also, many gH2AX-labeled nuclei were evenly and completely BrdU labeled, giving the impression that S-phase had occurred smoothly. Taken together with the observations that DNA damage suppressed new S-phase entry and that gH2AX foci were absent from the few mitotic spindles observed, we speculate that the gH2AX foci may mostly occur in a cell cycle window from late S-phase through G2 phase, or in post-mitotic daughter cells. gH2AX is best known for labeling double strand breaks or nucleotide excision repair, recruiting DNA repair effectors (23,24). gH2AX also labels DNA replication stress, such as replication fork collapse, defects in chromatin assembly, cell cycle arrest, or prolonged mitosis (26–29). This raises the possibility that beta cells forced into the cell cycle may have weakened replication forks or other defects in completing mitosis. In some cell types, gamma phosphorylation of H2AX may occur intrinsically during S- or G2-M phases of the cell cycle (30,31). It is possible that chromatin handling during DNA replication or G2 may predispose beta cells to gH2AX phosphorylation; whether this is a sign of damaged DNA or other process remains uncertain.

A toxic effect of nucleoside analogs, including both BrdU and EdU, has been reported (7–9). In the beta cell field EdU is also commonly used, especially for high throughput screening; whether EdU performs
similarly to BrdU has not been carefully tested. The final concentration of BrdU used in our study (33 uM) was similar to other published studies (10-100 uM) (5,7,8). BrdU exposure may or may not induce sister chromatid exchanges, a marker of genome instability (32). Triphosphate nucleoside analogs such as 5-fluorouracil act as replication fork blocks; however, these induce gH2AX not at the time of initial incorporation, but rather during S-phase of a subsequent cell cycle event (33). Extensive co-labeling of gH2AX and pH3 in the absence of BrdU exposure argue against a primary role for BrdU toxicity in the observed gH2AX population, but rather that gH2AX staining is associated with cycling cells. Beta cells with smooth BrdU label and gH2AX foci may be cells that have re-entered the cell cycle following a successful initial cell division.

Surprisingly, beta cells from older mice were not spontaneously more likely to label for gH2AX. However, when both high glucose and cyclin D2 overexpression were combined, beta cells from older mice had a higher frequency of gH2AX labeling, and a lower fraction of BrdU-labeled beta cells that did not show evidence of DNA damage. This may be related to the fact that beta cells from older mice required a combination of both glucose and cyclin D2 to enter the cell cycle, whereas beta cells from younger mice responded to either stimulus. The human islet donors used for this study were from a narrow range of ages, 41 to 56 years, precluding assessment of the impact of age on DNA damage markers in human beta cells.

Also contrary to expectations, in these studies human beta cells did not have a high frequency of gH2AX labeling. If anything, human beta cells were less likely than mouse beta cells to label for gH2AX, especially in the presence of proliferative stimuli. However, the pro-proliferation stimuli used in these studies did not result in a high rate of cell cycle entry in human beta cells. As such, the low rate of human gH2AX labeling is consistent with the hypothesis that DNA damage occurs in later stages of the cell cycle, or during cell cycle re-entry following successful mitosis. We have not tested conditions that induce rapid proliferation in human beta cells.

This study does not address the extent to which BrdU, marking S-phase entry, predicts completion of the cell cycle and production of two daughter cells. Evidence supporting the conclusion that some BrdU-labeled beta cells do successfully complete cell division include the many studies showing that BrdU labeling correlates with other proliferation markers such as Ki67, PCNA and pH3, e.g. (17,34,35), the documented increase in beta cell number under conditions when BrdU labeling is increased in vitro (17,36,37) and in vivo, e.g. (33), and our current data showing BrdU+ nuclei in mitosis (Fig 4G) and co-labeling with pH3 (Suppl. Fig 1). We have not formally tested for beta cell endoreduplication, but similar to prior observations (38) we did not detect systematically increased nuclear size in double-labeled cells.
On the other hand, the fate of beta cells labeled for both BrdU and gH2AX remains unknown. Although getting beta cells to traverse the G1-S transition is an important and challenging goal, studies are also needed to find ways to increase the frequency of successful mitosis in beta cells that do enter the cell cycle. Taken together, the results of the current studies support the use of BrdU labeling as a faithful measure of S-phase entry and a useful tool in the overarching goal of the pursuit of beta cell regeneration strategies as a therapy for diabetes.

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FIGURE LEGENDS

Figure 1. BrdU and gH2AX labeling co-occur in some beta cells in mouse islet cell cultures. Dispersed mouse islet cells were cultured for 72 hours in 5mM glucose (A, A’, B, C) or 15mM glucose (D, D’), with BrdU added for the final 24 hours. (A-A’) BrdU (A) and gH2AX (A’) each labeled a small fraction of beta cells under unstimulated culture conditions. (B-C) In mouse islet cells cultured in unstimulated (5mM glucose) conditions, a small proportion of beta cells labeled with BrdU or gH2AX (B). The observed fraction of beta cells co-labeled with both BrdU and gH2AX was low, but higher than that predicted by random chance (predicted fraction was defined by the product of the observed fractions of each single label) (C). (D-D’) Confocal microscopy of mouse islet cells cultured in 15mM glucose confirmed both labels occur in the same nuclei. For A, A’, B and B’, lines mark BrdU (+) gH2AX (-) nuclei, arrowheads mark BrdU (-) gH2AX (+) nuclei, and arrows mark BrdU (+) gH2AX (+) nuclei. *p<0.05, ns p>0.1.

Figure 2. Proliferative stimuli, especially in combination, increase BrdU-gH2AX co-localization frequency in mouse beta cells. Dispersed young (10-12 weeks) and old (50-60 weeks) mouse islet cells were cultured for 72 hours in the indicated conditions, with BrdU added for the final 24 hours. (A-C) 15mM glucose markedly increased BrdU incorporation (A), especially in young islets, and modestly increased gH2AX labeling (B) and BrdU-gH2AX co-labeling (C). (D-F) Ad-Cyclin D2 in 5mM glucose increased BrdU (D), gH2AX (E), and co-labeled cells (F). (G-I) Combined treatment with 15mM glucose
and Ad-Cyclin D2 markedly increased BrdU (G), gH2AX (H) and co-labeled cells (I). Exposure to a different beta cell mitogen, Harmine, also increased BrdU (J), gH2AX (K) and co-labeled cells (L). Note the variable y-axis scale in (A-L), (C, F, I and L) in all cases, the observed fraction of beta cells co-labeled for both BrdU and gH2AX was greater than that predicted if co-labeling occurred due to chance. Ad-cre was used as a control for Ad-cyclin D2. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

**Figure 3. DNA damage does not increase beta cell BrdU incorporation or BrdU-gH2AX co-labeling.** Dispersed mouse islet cells were cultured for 72 hours in the indicated conditions, with BrdU added for the final 24 hours. (A-C). Mitomycin C treatment resulted in gH2AX labeling in the majority of beta cells (A), confirming DNA damage. (B) Contrary to the hypothesis that BrdU labeling might spuriously occur during DNA damage repair, mitomycin C treatment decreased, rather than increased, the percent of beta cells labeling for BrdU. (C) Double-labeled cells were not increased under conditions of DNA damage; mitomycin C treatment decreased the percent of beta cells co-labeling with both gH2AX and BrdU. In fact, the observed fraction with mitomycin C treatment was suppressed to the fraction predicted if co-labeling was due to chance. Mitomycin treatment was performed on the same biological samples and at the same time as the experiments in Figure 2; control data are repeated from Figure 2. (D-F) To test the mitomycin result in a different system, UV treatment (only performed on islets from old mice) increased the percent of beta cells labeling with gH2AX (D) but suppressed BrdU incorporation (E) and double-labeled cells (F). In (D-F), biological replicates with triangle labels and circle labels were treated identically but in the triangle samples a lower fraction labeled for gH2AX. The different labels are used to allow identification of the samples with lower DNA-damage across panels D-G. *p<0.05, **p<0.01, ***p<0.001.

**Figure 4. gH2AX mostly labels a subset of BrdU-labeled cells.** Quantitative Venn diagrams were used to generate a visual representation of the data shown in Figures 2-3. Diagrams represent the total number (sum of all replicates) of insulin (+) cells (white circles) that labeled with BrdU (green), gH2AX (red), and both labels (yellow) under different culture conditions. The total number of insulin (+) cells counted and number of biological replicates for each condition is included for each diagram (italics). (A) In young islet cells, 15mM glucose and Ad-cyclin D2 increased both the BrdU+ and gH2AX fractions. Insulin (+) nuclei labeled for gH2AX were mostly a subset of the BrdU-labeled nuclei, in both basal and stimulated conditions. (B) Beta cells from older mice behaved similarly to young beta cells, except that a higher fraction of BrdU (+) cells were also gH2AX(+) under 15mM glucose stimulation both with and without Ad-cyclin D2, and older beta cells required both glucose and Ad-cyclin D2 to meaningfully increase the fraction of BrdU-labeled cells. (C) Venn depiction of the data in Figure 3 demonstrates visually that DNA damage exposure did not increase, and in fact decreased, the frequency of BrdU (+) and co-labeled nuclei. (D-G): Confocal microscopy of cultures (15mM glucose) showed many examples of smoothly labeled BrdU (+) nuclei that also had gH2AX puncta (D-E) and some examples of nuclei with punctate labeling of both BrdU and gH2AX (F). Active mitoses generally had no gH2AX label (G).

**Figure 5. BrdU-induced DNA damage does not explain most beta cell gH2AX labeling.** Dispersed mouse islet cells were cultured with and without BrdU added to the culture medium, with 15mM glucose (A-C), Ad-cyclin D2 (D) or Ad-cyclin D2 +15mM glucose (E). (A) In 15mM glucose, the presence of BrdU caused a subtle increase in gH2AX labeling in young islets. (B-C) Shifting the timing of the BrdU exposure earlier in the culture to the first 24 hours of glucose exposure with BrdU washed out after 24 hours (exposure X), or for the entire 72 hours (exposure Z) did not increase the proportion of cells showing evidence of DNA damage compared with the standard exposure during the final 24 hours of the 72-hour culture (exposure Y). The time-course of exposures is diagrammed in (B), and the gH2AX quantification is shown in (C). Experiments in A-C were performed on the same biological samples; the Y data in (C) are the same as the BrdU+ data in (A). (D) With Ad-cyclin D2 stimulation, BrdU exposure (final 24 hours, similar to the rest of the experiments throughout this study) increased gH2AX labeling in 5mM glucose (B) but not in 15mM glucose (C). For (D-E), the BrdU labeling fraction in these experiments is shown for context, since these cultures had higher levels of stimulated proliferation than the experiments shown in Figure 2. (F-H): dispersed mouse islet cells cultured without BrdU in 15mM glucose with control or Ad-
cyclin D2 had substantial co-labeling of gH2AX and pH3, suggesting gH2AX is associated with cycling beta cells rather than BrdU incorporation itself. \( \ast p<0.05, \ast\ast p<0.01, \ast\ast\ast p<0.001, \ast\ast\ast\ast p<0.0001. \)

**Figure 6. Human beta cells did not increase DNA damage labeling after proliferative stimulation.** Dispersed human islet cells were cultured for 96 hours in the described conditions, with BrdU included in the culture media for the entire 96 hours. (A-C) Human insulin (+) cells cultured in 15mM glucose showed a trend towards modestly increased BrdU incorporation (A) but did not increase gH2AX labeling (B) or BrdU-gH2AX double labeling (C). (D-F) Adenoviral overexpression of human cyclin D2 in 5mM glucose increased BrdU labeling fraction (D) but not the gH2AX-labeling fraction (E); double-labeled cells trended upwards (F). (G-I) Combining glucose and cyclin D2 proliferative stimuli did not further increase BrdU (G), gH2AX (H) or double-labeled nuclei (I). Adeno-cre was used as a control for Adeno-cyclin D2, at the same MOI. \( \ast p<0.05. \)

**Figure 7. DNA damage does not cause spurious BrdU incorporation in human beta cells.** Dispersed human islet cells were cultured for 96 hours in the described conditions, with BrdU included in the culture media for the entire 96 hours. (A-C): Human insulin (+) cells exposed to mitomycin C were nearly all labeled for gH2AX (A) but had suppressed BrdU labeling fraction (B) and no excess double-labeled cells beyond the fraction predicted by random chance (C). (D-F): Human beta cells exposed to UV irradiation showed variable induction of DNA damage label gH2AX (D), suppression of BrdU labeling (E) and no excess double-labeled cells (F). Note the scale differences in y-axes from left to right. \( \ast p<0.05, \ast\ast p<0.01, \ast\ast\ast\ast p<0.0001. \)

**Figure 8. In human beta cells, proliferation was infrequent even under stimulated conditions, and gH2AX labeling was mostly independent of BrdU-labeled cells.** The data in Figures 6-7 are shown using quantitative Venn diagrams to illustrate the relationship between gH2AX-labeling and BrdU-labeling in these cultures. Diagrams represent the total number (sum of all replicates) of insulin (+) cells (white) that labeled with BrdU (green), gH2AX (red), and both labels (yellow) under different culture conditions. The total number of insulin (+) cells counted, summed for all biological replicates (n=3-5) is included for each diagram (italics). (A): Although BrdU labeling increased somewhat in proliferative conditions, the gH2AX-labeling index did not, and most gH2AX-labeled cells were not BrdU-labeled. (B): Mitomycin C and UV irradiation caused DNA damage in the majority of beta cells, but did not increase, in fact decreased, the BrdU-labeling fraction.
REFERENCES


% beta cells labeling

% beta cells with both BrdU and gH2AX labels

**Predicted vs. Observed**
A Young

- 9134 cells (n=5, 1827±134)
  - 5mM glu

- 18473 cells (n=10, 1847±290)
  - 15mM glu

- 9589 cells (n=5, 1918±367)
  - 5mM + AdcycD2

- 12306 cells (n=5, 2461±434)
  - 15mM + AdcycD2

B Old

- 11009 cells (n=4, 2752±642)
  - 5mM glu

- 11566 cells (n=4, 2892±342)
  - 15mM glu

- 8937 cells (n=4, 2234±245)
  - 5mM + AdcycD2

- 10341 cells (n=4, 2585±625)
  - 15mM + AdcycD2

C

- 10192 cells (n=4, 2548±400)
- 10141 cells (n=4, 2535±278)

D E F G H I J K L M

- BrdU gH2AX Dapi
- Insulin Dapi
- BrdU gH2AX Dapi
- Insulin Dapi

Diabetes
(A-C) Human islet cells: effect of high (15mM) glucose

(A) 

- %BrdU+ beta cells
  - mM gluc: 5, 15
  - p = 0.09

(B) 

- %γH2AX+ beta cells
  - 5, 15mM gluc

(C) 

- % beta cells with both BrdU and γH2AX labels
  - Predict vs Obs

(D-F) Human islet cells: effect of Ad-cyclin D2 in standard (5mM) glucose

(D) 

- %BrdU+ beta cells
  - mM gluc: 5, 5
  - Adeno cre D2
  - *

(E) 

- %γH2AX+ beta cells
  - 5, 5 cre D2

(F) 

- % beta cells with both BrdU and γH2AX labels
  - Predict vs Obs

(G-I) Human islet cells: effect of Ad-cyclin D2 in high (15mM) glucose

(G) 

- %BrdU+ beta cells
  - mM gluc: 15, 15
  - Adeno cre D2

(H) 

- %γH2AX+ beta cells
  - 15, 15 cre D2

(I) 

- % beta cells with both BrdU and γH2AX labels
  - Predict vs Obs
A

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**Supplemental Table 1.** Characteristics of human islet donors used in these studies
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<td>BrdU, gH2AX, predicted, observed</td>
<td>4</td>
<td>4729</td>
<td>1182 +/- 301</td>
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<tr>
<td>6A-6C</td>
<td>human</td>
<td>15mM</td>
<td>BrdU, gH2AX, predicted, observed</td>
<td>5</td>
<td>3817</td>
<td>763 +/- 162</td>
</tr>
<tr>
<td>6D-6F</td>
<td>human</td>
<td>5mM, cre</td>
<td>BrdU, gH2AX, predicted, observed</td>
<td>3</td>
<td>2651</td>
<td>884 +/- 227</td>
</tr>
<tr>
<td>6D-6F</td>
<td>human</td>
<td>5mM, D2</td>
<td>BrdU, gH2AX, predicted, observed</td>
<td>3</td>
<td>3571</td>
<td>1190 +/- 196</td>
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<tr>
<td>6G-6I</td>
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<td>BrdU, gH2AX, predicted, observed</td>
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<td>2615</td>
<td>872 +/- 208</td>
</tr>
<tr>
<td>6G-6I</td>
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<td>BrdU, gH2AX, predicted, observed</td>
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<td>4271</td>
<td>1424 +/- 261</td>
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<tr>
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<td>15mM, ctrl</td>
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<td>3817</td>
<td>763 +/- 162</td>
</tr>
<tr>
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<td>3207</td>
<td>641 +/- 303</td>
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<tr>
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<td>15mM, ctrl</td>
<td>BrdU, gH2AX, predicted, observed</td>
<td>5</td>
<td>3817</td>
<td>763 +/- 162</td>
</tr>
<tr>
<td>7D-7F</td>
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<td>BrdU, gH2AX, predicted, observed</td>
<td>3</td>
<td>2971</td>
<td>990 +/- 247</td>
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</table>

**Supplemental Table 2.** Number of cells counted for each experiment
<table>
<thead>
<tr>
<th>Explanation</th>
<th>Possible mechanism if true</th>
<th>Implications if true</th>
<th>How to test</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA damage causes BrdU incorporation</td>
<td>DNA damage repair results in incorporation of BrdU nucleotide</td>
<td>BrdU incorporation does not reflect cell cycle entry in cells with DNA damage.</td>
<td>Induce DNA damage, test for increased frequency of BrdU labeling. Observe pattern of BrdU label.</td>
</tr>
<tr>
<td>BrdU exposure causes DNA damage</td>
<td>Incorporation of the BrdU nucleotide leads to a DNA damage response</td>
<td>BrdU incorporation reflects cell cycle entry but incurs toxicity. BrdU toxicity may impact cell cycle completion.</td>
<td>Test for increased DNA damage in the presence of BrdU compared with DNA damage in the absence of BrdU</td>
</tr>
<tr>
<td>An upstream process causes both DNA damage and BrdU incorporation</td>
<td>Many possible mechanisms. Most likely is that proliferative signals, DNA replication or other cell cycle related process also induces DNA damage</td>
<td>BrdU incorporation reflects cell cycle entry. Cells may not complete the cell cycle. Proliferation is a dangerous process for beta cells.</td>
<td>Test whether proliferative conditions increase the fraction of gH2AX-labeled cells.</td>
</tr>
<tr>
<td>DNA damage and BrdU incorporation are unrelated but occasionally co-occur stochastically</td>
<td>Cells occasionally co-label with BrdU and gH2AX by random chance</td>
<td>BrdU incorporation reflects cell cycle entry.</td>
<td>Test whether the observed BrdU-gH2AX co-labeling frequency matches the predicted frequency based on prevalence of the individual labels</td>
</tr>
</tbody>
</table>

**Supplemental Table 3.** Putative explanations for beta cell co-labeling with BrdU and DNA damage marker gH2AX, implications, and an approach to testing each hypothesis.
Supplemental Figure 1. Many BrdU+ beta cell nuclei co-label with pH3, suggesting progression of BrdU-labeled cells to the mitotic phase of the cell cycle. Mouse islet cells were dispersed and cultured in 15mM glucose for 72 hours, with BrdU present for the final 24 hours, then fixed and stained for insulin, BrdU, pH3 and dapi. Many BrdU-labeled beta cell nuclei co-stain for pH3 (arrows). Given the long duration of BrdU exposure, some of the BrdU(+) pH3(-) cells may have progressed through the cell cycle entirely; note the occasional BrdU doublets, which are negative for pH3.
Supplemental Figure 2. In vivo HFD-stimulated beta cell proliferative expansion leads to very few gH2AX-labeled beta cell nuclei. Pancreas sections from control diet (A) or high fat diet (HFD; B-C) fed mice were stained for insulin, BrdU, gH2AX and dapi. All gH2AX-stained islet nuclei identified in this experiment are shown above; the vast majority of islets imaged contained no detectable gH2AX-labeled cells. Although many insulin+ cells labeling with BrdU were found (white lines), only four gH2AX+ nuclei (white arrows) were found in the 8 sections. The gH2AX+ nucleus in (A) appears to be a non-insulin-positive cell, whereas the gH2AX+ nuclei in (B-C) appear to belong to insulin+ cells. The gH2AX+ nuclei in (B) are also BrdU+, but those in (A) and (C) are BrdU-negative.
Supplemental Figure 3. In vivo hyperglycemia-stimulated beta cell proliferative expansion leads to very few gH2AX-labeled beta cell nuclei. Pancreas sections from mice rendered continuously hyperglycemic for 4 days by intravenous glucose infusion were stained for insulin, BrdU, gH2AX and dapi. As with the HFD experiment shown in Suppl. Fig 2, all gH2AX-stained islet nuclei identified in this experiment are shown above. Again, the vast majority of islets imaged contained no detectable gH2AX-labeled cells. Although many insulin+ cells labeling with BrdU were found (white lines), only four gH2AX+ nuclei (white arrows) were found in pancreas sections from four mice. The gH2AX+ nucleus in (D) appears to be a non-insulin-positive cell, whereas the gH2AX+ nuclei in (A-C) appear to belong to insulin+ cells. In this hyperglycemia experiment, none of the gH2AX+ nuclei were also BrdU+.
Supplemental Figure 4. Ex vivo cultured intact islets labeled infrequently for gH2AX unless exposed to DNA damaging agent Mitomycin C, and very rarely labeled for both BrdU and gH2AX. Whole mouse islets were cultured for 72 hours in islet medium containing 5mM glucose, 15mM glucose or 15mM glucose with Mitomycin C, with BrdU added for the final 24 hours of culture. The islets were then fixed, embedded in paraffin, sectioned and stained for insulin, BrdU, gH2AX and dapi (A). Some insulin+ cells labeled with BrdU (white lines, B) or gH2AX (white arrows, C) but very few of the gH2AX+ nuclei were also BrdU+ (D).