Pronounced proliferation of non-beta cells in response to beta-cell mitogens in isolated human islets of Langerhans

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ABSTRACT

The potential to treat diabetes by increasing beta-cell mass is driving a major effort to identify beta-cell mitogens. Demonstration of mitogen activity in human beta cells is frequently performed in ex vivo assays. However, disparities in the efficacy of beta-cell mitogens between studies led us to investigate the sources of this variability. We obtained 27 male (16) and female (11) human islet batches from multiple centers covering a range of donor ages (18-65 years) and BMI (16.4-38.5). Islets were kept intact or dispersed into single cells and cultured in the presence of the beta-cell mitogens harmine, glucose, and heparin-binding epidermal growth factor-like growth factor (HB-EGF), and subsequently analyzed for cell proliferation by immunochemistry or flow cytometry. Harmine and HB-EGF promoted human beta-cell proliferation, whereas the effect of glucose was assay-dependent. In addition, harmine potently stimulated alpha-cell proliferation and both harmine and HB-EGF increased proliferation of insulin- and glucagon-negative cells, including cytokeratin 19-positive cells. These results suggest that assessment of beta-cell mitogens requires complementary approaches and rigorous identification of cell identity. This is better achieved by flow cytometry that eliminates the subjectivity of visual scoring and enables simultaneous assessment of several endocrine and proliferation markers in higher numbers of cells.

INTRODUCTION

Type 2 diabetes is characterized by insufficient functional β-cell mass [1]. In non-diabetic obese individuals and during pregnancy, accretion of insulin secretory capacity per β cell combined with β-cell mass expansion maintains glucose homeostasis by balancing levels of circulating insulin and insulin sensitivity. Deciphering the mechanisms controlling β-cell proliferation has thus become a major research goal in the hope to identify therapeutic targets to expand β-cell mass and prevent or delay the onset of diabetes.

Diverse factors including insulin [2], GLP-1 [3], serotonin [4], epidermal growth factors [5], osteoprotegerin [6], serpinB1 [7] and nutrients such as glucose and fatty acids [8-12] promote replication of rodent β cells. Unfortunately, our knowledge of the factors controlling human β-cell proliferation is much more limited [13-15]. Although human β cells respond to glucose [16-18], fatty acids [12], serpinB1 [7], dual-specificity tyrosine-regulated kinase 1A (DYRK1A) inhibitors including harmine [19-21], and heparin-binding epidermal growth factor-like growth factor (HB-EGF) [22], proliferation rates are very low compared to rodents and published data on the efficacy of human β-cell mitogens are often highly variable [23].

In this study we assessed the mitogenic properties of harmine, glucose and HB-EGF in both intact and dispersed human islets comparing immunochemistry to flow cytometry to differentiate proliferating β and non-β cell types, including glucagon^{+} and cytokeratin-19 (CK19)$^{+}$ cells.

RESULTS

Proliferative effects of harmine, glucose, and HB-EGF in dispersed human islets as assessed by immunocytochemistry

As the majority of previous studies examined the effects of mitogens on human β-cell proliferation in dispersed islet cultures [17-19,21,24-27], we began by attempting to reproduce these findings. Following recovery, human islets were dispersed, plated and exposed to harmine (10 μM), high glucose (16.7 mM) or HB-EGF (100 ng/ml) for 3 days in the presence of the proliferation marker 5-Ethynyl-2'-deoxyuridine (EdU). At the end of the treatment, cells were stained for EdU and the nuclear marker of mature β cells Nkx6.1 [23] (Fig. 1). Harmine exposure lead to a significant increase in β-cell proliferation compared to vehicle in 5/6 donors (fold-change 13.0 ± 4.0, P = 0.034) (Fig. 1A & B). In contrast, glucose was a poor inducer of β-cell proliferation with only 2/6 donors responding (fold-change 1.2 ± 0.4, NS) (Fig. 1C & D). HB-EGF increased β-cell proliferation in 5/6 donors (fold-change 2.1 ± 0.5, P = 0.011) (Fig. 1E & F). Interestingly, we frequently observed $Nkx6.1^{-}/EdU^{+}$ cells in the presence of harmine (Fig. 1A) and HB-EGF (Fig. 1E), suggesting that non-β cells also respond to these compounds.

Proliferative effects of harmine, glucose, and HB-EGF in intact human islets as assessed by immunohistochemistry

Islet architecture and cell-to-cell contact may contribute to the capacity of β cells to proliferate [28]. Therefore, we exposed intact human islets to harmine, glucose or HB-EGF and assessed β-cell proliferation in cryosections by immunohistochemistry for Nkx6.1 and EdU (Fig. 2). Harmine increased proliferation of $Nkx6.1^{+}$ cells in 5/8 donors (fold-change 6.7 ± 2.2, P = 0.031) (Fig. 2A & B; left panel). In contrast to the results in dispersed islets, 7/9 donors responded to glucose (fold-change 3.8 ± 1.0, P = 0.022) (Fig. 2A & C; left panel), and whereas 6/9 donors responded to HB-EGF the average effect was not significant (fold-change 4.5 ± 2.1, NS) (Fig. 2A & D; left panel). As observed in dispersed islets,

many proliferating cells did not stain for Nkx6.1, which led us to quantify proliferation in this presumably non-β cell population. In basal conditions (vehicle or 2.8 mM glucose) the mean percentage of $EdU^+/Nkx6.1^-$ cells of the 9 donors was 1.3 ± 0.3% (Fig. 2A & B-D; right panel). Harmine exposure led to a large increase in $Nkx6.1^-$ cell proliferation in 8/8 donors (fold-change 6.7 ± 2.0, P = 0.024) (Fig. 2A & B; right panel). Glucose increased proliferation of $Nkx6.1^-$ cells in 4/9 donors and this effect was not significant (fold-change 1.2 ± 0.3, NS) (Fig. 2A & C; right panel), whereas HB-EGF stimulated proliferation of $Nkx6.1^-$ cells in 7/9 donors (fold-change 1.6 ± 0.4, P = 0.010) (Fig. 2A & D; right panel).

To confirm the results obtained with Nkx6.1 in intact islets, we used C-peptide (CPEP) as another β cell marker (Fig. 3). Similar to the results shown in Fig. 2, harmine, glucose and HB-EGF increased proliferation of $CPEP^+$ cells in 7/7 donors (fold-change 6.4 ± 1.7, P = 0.025) (Fig. 3A & B; left panel), 5/6 donors (fold-change 2.0 ± 0.5, P = 0.021) (Fig. 3A & C; left panel) and in 6/7 donors (fold-change 2.6 ± 0.6, P = 0.014) (Fig. 3A & D; left panel), respectively. Surprisingly, the fraction of $EdU^+/CPEP^+$ cells in both basal and stimulated conditions was considerably larger compared to $EdU^+/Nkx6.1^+$ cells from several donors (compare Fig. 2B-D to Fig. 3B-D). The proliferative response of $CPEP^-$ cells to harmine (6/7 donors; fold-change 9.7 ± 2.3, P = 0.012) (Fig. 3A & B; right panel), glucose (5/6 donors; fold-change 2.0 ± 0.4, P = 0.014) (Fig. 3A & C; right panel) and HB-EGF (5/7 donors; fold-change 3.2 ± 1.4, NS) (Fig. 3A & D; right panel) was similar to the response of $Nkx6.1^-$ cells (Fig. 2).

Human islet preparations often contain abundant non-endocrine cell types including duct and acinar cells, among others [29]. The abundance of proliferating non-β-cells (Fig. 2 and 3) led us to ask whether a proportion of these cells are positive for the ductal marker CK19 [30]. Intact human islets were exposed to harmine, glucose or HB-EGF and β-cell proliferation assessed in cryosections by immunohistochemistry for CK19 and EdU (Fig. 4). Clusters of $CK19^+$ cells were detected in all human islet preparations tested (Fig. 4A). Although harmine increased proliferation of $CK19^+$ cells in 4/4 donors, the increase was not significant (fold-change 2.5 ± 0.8, NS) (Fig. 4A & B). In contrast, both glucose and HB-EGF significantly

increased CK19$^+$ cell proliferation in 5/6 (fold-change 1.8 ± 0.2, P = 0.016) (Fig. 4A & C) and 6/6 (fold-change 5.4 ± 1.5, P = 0.011) (Fig. 4A & D) donors, respectively.

Proliferative effects of harmine, glucose, and HB-EGF in intact human islets as assessed by flow cytometry

The reliability of immunohistochemical assessment of β-cell proliferation is inherently limited by the number of cells that can be manually counted and the operator's visual appreciation of co-labeling. We therefore exposed intact human islets to mitogens and assessed islet cell proliferation by flow cytometry using EdU and immunostaining for CPEP (β cells), glucagon (GCG; α cells) and CK19 (Fig. 5A). The mean proportion of β and α cells in the donor islet batches was 18 ± 4% and 36 ± 7%, respectively, whereas GCG$^-$/CPEP$^-$ cells represented the remaining 46 ± 6% (Fig. 5B). In general, we observed that β-cell proliferation was low relative to the proliferation levels detected in α cells, GCG$^-$/CPEP$^-$ cells, and CK19$^+$ in both control and stimulated conditions (Fig. 5C-E; left panels). Harmine induced β-cell proliferation in 5/6 donors (fold-change 2.2 ± 0.6, P = 0.016) (Fig. 5C; right panel). Harmine was also a powerful mitogen for α cells in 6/6 donors (fold-change 7.7 ± 2.4, P = 0.022), GCG$^-$/CPEP$^-$ cells in 6/6 donors (fold-change 3.2 ± 1.1, P = 0.027), and CK19$^+$ cells in 3/3 donors (fold-change 5.0 ± 1.1, NS) (Fig. 5C; left panels). Glucose did not promote β-cell proliferation in any of the 10 donors tested (fold-change 0.4 ± 0.1, NS), (Fig. 5D; right panel). Furthermore, unlike harmine glucose was ineffective as a mitogen for α cells (10 donors; fold-change 1.1 ± 0.3, NS), GCG$^-$/CPEP$^-$ cells (10 donors; fold-change 0.9 ± 0.3, NS) and CK19$^+$ cells (3 donors; fold-change 1.2 ± 0.3, NS) (Fig. 5D; left panel). HB-EGF increased β-cell proliferation in 9/13 donors (fold-change 1.5 ± 0.3, NS) (Fig. 5E; right panel), an effect that was significant (fold-change 2.5 ± 0.6, P = 0.027) when donor 13, identified as an outlier based on Grubb's test, was excluded from the analysis. HB-EGF was largely inactive on α cells (13 donors; fold-change 1.2 ± 0.5, NS) but strongly stimulated GCG$^-$/CPEP$^-$ cell proliferation in 13/13 donors (fold-

change 4.0 ± 1.3, P = 0.011) and CK19$^+$ cell proliferation in 5/5 donors (fold-change 3.5 ± 1.0, P = 0.011) (Fig. 5E; left panel).

DISCUSSION

In this study we evaluated the capacity of harmine, glucose and HB-EGF to promote β-cell proliferation in isolated human islets using a multi-faceted approach involving intact and dispersed islet cultures, flow cytometry and immunohisto/cytochemistry and multiple cell type-specific markers. Our data indicate that in the majority of islet batches human β cells respond to harmine or HB-EGF irrespective of the experimental approach, whereas the response to glucose depends on the assay used to assess proliferation. The abundance of proliferating non-β cells in human islet batches prompted us to assess the effects of these mitogens on α cells, CPEP$^-$/GCG$^-$ cells and CK19$^+$ cells. We found that harmine potently increased the proliferation of α cells and CPEP$^-$/GCG$^-$ cells whereas HB-EGF principally stimulated CPEP$^-$/GCG$^-$ cell proliferation including CK19$^+$ cells.

Although the fold-increase in β-cell proliferation was comparable between assays, the fraction of EdU$^+$ cells was markedly different when β cells were labeled for Nkx6.1 vs. CPEP in intact islets by immunohistochemistry. In general, the fraction of EdU$^+$/Nkx6.1$^+$ cells was lower than the corresponding EdU$^+$/CPEP$^+$ cells. This is reminiscent of a study by Aamodt et al. [31] in dispersed human islets, who scored only β cells that were both insulin$^+$ and pancreatic and duodenal homeobox-1 (Pdx-1)$^+$ and described a considerably lower level of β-cell proliferation in response to harmine and glucose compared to studies where all insulin$^+$ (or CPEP$^+$) cells were scored [17-19,21,24-27,32]. This phenomenon may be explained in part by the fact that actively proliferating β-cells express lower levels of differentiation markers such as Nkx6.1 and Pdx-1 [33], which would decrease the sensitivity of detection of double labelling.

The DYRK1A inhibitor harmine increased human β-cell proliferation irrespective of the assay used, and also increased proliferation of GCG^+, $CPEP^-/GCG^-$, and $CK19^+$ cells in human islet batches. This increase in proliferation of non-β cells in response to harmine is expected given the ubiquitous nature of DYRK1A signaling, and is consistent with several studies. Indeed, Wang et al. [21] showed that combined inhibition of DYRK1A and TGFβ signaling stimulates proliferation of $CK19^+$ duct cells and endocrine α, δ and PP cells. Accordingly, the DYRK1A inhibitor 5-iodotubercidin stimulates both β- and α-cell proliferation [19]. Finally, using single-cell mass cytometry Wang et al. [20] shown α cells to be considerably more responsive than β cells to the mitogenic effects of harmine.

We found that the capacity of glucose to increase human β-cell proliferation was dependent on the assay used. Glucose was largely inactive in dispersed islets, whereas in intact islets it increased β-cell proliferation when assessed by immunohistochemistry but not by flow cytometry. These findings contrast with previous reports showing that glucose (15-20 mM) can increase β-cell proliferation to up to 2% (approximately 2-fold over basal) in dispersed human islets [17,18,24,27,32], but are in line with our previous immunohistochemical data in intact human islets [34]. Although the underlying reasons for the differences in the β-cell proliferative response to glucose between our different assays ex vivo are unclear, it is noteworthy that variability in the response to glucose has been observed in vivo. Transplantation of human islets into streptozotocin-induced diabetic non-obese diabetic-severe combined immunodeficient mice revealed a correlation between glucose levels and β-cell proliferation following glucose infusion [16]. Similarly, transplantation of human islets into spontaneously diabetic immunodeficient non-obese diabetic-Rag1null IL2rγnull Ins2Akita mice led to β-cell proliferation that positively correlated with glucose levels [35]. Yet, when adult human islets were transplanted into NSG-DT mice, a model of hyperglycemia, no increase in β-cell proliferation was detected [36]. In light of our ex vivo data, we speculate that the effect of glucose on human β-cell proliferation in vivo may be mostly indirect, whereas the direct effects of glucose, in striking contrast to the mouse β cell [9,10,18,37], may be relatively minor.

We recently demonstrated that the EGF receptor ligand HB-EGF exerts mitogenic activity on human β cells [22]. In the present study we substantiated these findings by showing that β cells proliferate in response to HB-EGF in the majority of human islet batches in both intact and dispersed islets. Although α cells were only weakly responsive to HB-EGF, $CPEP^-/GCG^-$ cells and $CK19^+$ ductal cells were highly responsive to HB-EGF. In agreement with these findings, the EGF receptor is expressed in β, α and ductal cells in the adult human pancreas [38,39]. Furthermore, ductal cells constitute a neogenic source of β cells in rodents and humans [40] that are also responsive to EGF receptor ligands. In mice, adenoviral transduction of HB-EGF leads to ductal cell proliferation and differentiation into $insulin^+$ cells [41]. In humans, ductal cells proliferate in response to EGF and the related ligand betacellulin [39], and the combination of EGF and gastrin promotes ductal cell expansion and differentiation into $Pdx\text{-}1^+/insulin^+$ cells [42]. Hence, the proliferating β cells detected in the presence of HB-EGF in this study could arise from replication of existing β cells and/or β-cell neogenesis from ductal cells.

In conclusion, our data indicate that harmine and HB-EGF are consistent inducers of human β-cell proliferation, whereas the glucose response is variable and assay-dependent. Discrepancies between the various approaches used in the present study and with previous reports point to the sensitivity of human β cells to the culture conditions, for example using dispersed or intact islets, but also the need for rigorous labeling and scoring of proliferating β cells. In particular, the abundance of non-β-cell types in human islet batches and the low level of β-cell proliferation compared to non-β cells could lead to the mis-identification of some proliferating cells as β cells by immunohistochemistry. Importantly, we note that non-β cell types are rarely scored in human islet cell proliferation assays yet human islet batches contain multiple cell types including other endocrine cells but also ductal and acinar as well as endothelial, immune and stellate cells [29]. Overall, we conclude from our experience that flow cytometry is more reliable than and provides several advantages over immunohistochemistry to assess β-cell proliferation in isolated human islets. First, it eliminates the subjectivity of visually scoring cells. Second, higher

numbers of cells can be easily and much more rapidly counted, increasing confidence in the data. Finally, multiple markers for β cells (INS, CPEP, Nkx6.1, Pdx-1) and proliferating cells (EdU, Ki67, phospho-histone H3) as well as endocrine, exocrine and other cell types can be stained simultaneously or in parallel to enhance the robustness of the conclusions and assess the relative efficacy of potential β-cell mitogens on several islet cell populations. We hope that these findings will provide some useful guidance to reliably assess human islet cell proliferation ex vivo in the quest to identify novel β-cell mitogens for potential therapeutic applications.

METHODS

Human islets

Islets from non-diabetic human donors were provided by the Alberta Diabetes Institute Islet Core, the Clinical Islet Laboratory at the University of Alberta, and the National Institute of Diabetes and Digestive and Kidney Diseases-funded Integrated Islet Distribution Program (IIDP; Supplementary Table S1 online). The use of human islets was approved by the Institutional Ethics Committee of the Centre Hospitalier de l'Université de Montréal (protocol # ND-05-035). All experiments were performed in accordance with the Institutional Ethics Committee guidelines and regulations. Informed consent was obtained from all donors and/or their legal guardians. Human islets were cultured in cGMP Prodo Islet Media (Standard) with 5% (vol./vol.) human AB serum and 1% glutamine/glutathione mixture (Prodo Labs Inc., Irvine, CA) overnight prior to experimentation.

Measurement of cell proliferation in dispersed islets by immunocytochemistry

Dispersed human islet proliferation was assessed essentially as described [22]. Briefly, hand-picked human islets were dispersed in accutase for 10 min at 37°C and plated in 96-well plates (Perkin Elmer Inc., Waltham, MA) treated with Poly-D-Lysine Hydrobromide (Sigma-Aldrich, St. Louis, MO). The next day, cells were cultured in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA) with 1% (vol./vol.) human serum albumin (Celprogen, Torrance, CA) for 3 days in the presence of EdU (10 μM) and glucose (2.8, 5.5 or 16.7 mM), harmine (10 μM; Millipore-Sigma, Darmstadt, Germany) or HB-EGF (100 ng/ml; R&D Systems, Minneapolis, MN) as indicated in the Figure legends. At the end of the treatment, cells were fixed with 3.7% paraformaldehyde and immunostained for Nkx6.1 (Supplementary Table S2 online). The proliferative marker EdU was detected using the Click-iT™ EdU Alexa Fluor™ 594 Imaging Kit (Thermo Fisher Scientific). Images were acquired with an Operetta CLS™ high-content imaging system (Perkin Elmer Inc., Waltham, MA) at 20X magnification using Harmony 4.5 software.

Cell counts were determined using ImageJ software (National Institutes of Health). Proliferation was calculated as the percentage of double-positive $EdU^+/Nkx6.1^+$ cells over the total $Nkx6.1^+$ population. Between 1,000 and 1,500 Nkx6.1+ cells from three different wells were manually counted per condition for each experimental replicate, as indicated in the Figure legends.

Measurement of cell proliferation in intact islets by immunohistochemistry

Hand-picked human islets (approximately 200/condition) were cultured in RPMI-1640 with 1% (vol./vol.) human serum albumin in 6-well plates for 3 days in the presence of glucose (2.8 or 16.7 mM), 100 ng/ml HB-EGF or harmine (10 μM) as indicated in the Figure legends. EdU (10 μM) was added throughout. Media were replaced daily. At the end of treatment, islets were embedded in optimal cutting temperature (OCT) compound, frozen, sectioned at 8 μm and mounted on Superfrost Plus slides (Thermo Fisher Scientific). Sections were fixed with 3.7% paraformaldehyde and immunostained for Nkx6.1, CPEP, or CK19 (Supplementary Table S2 online). EdU labelling was detected using the Click-iT™ EdU Alexa Fluor™ 594 Imaging Kit. Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Images were taken with an Axio Imager upright fluorescence microscope (Zeiss, Oberkochen, Germany). Between 1,000 and 1,500 Nkx6.1+ cells from 7-21 islets on 2 separate slides were manually counted per condition for each experimental replicate, as indicated in the Figure legends.

Measurement of cell proliferation in intact islets by flow cytometry

At the end of the treatments described above, islets were dispersed in accutase (1 μl/islet; Innovative Cell Technologies, Inc., San Diego, CA) for 10 min at 37°C and dead cells were labeled using the LIVE/DEAD™ Aqua (405 nm) Dead Cell Stain Kit (BD Biosciences, San Jose, CA). EdU detection, using the Click-iT™ Plus EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit, and immunostaining were performed according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Fluorophore-coupled primary antibodies and dilutions are listed in Supplementary Table S2 online. Flow

cytometry analysis was carried out using a LSRIIB flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA). Dead-cell stain, EdU-, CPEP-, GCG-, and CK19-labelled cells were detected using the 405-, 488-, 640- and 561-nm lasers coupled with 525/50-, 530/30-, 670/14- and 586/15-nm BP filters, respectively. Proliferation was calculated as the percentage of double-positive cells for EdU and each population marker over the corresponding total cell population for that marker.

Statistics

Significance was tested using two-tailed paired t-tests, using GraphPad Instat (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant. Fold-change data are expressed as mean ± SEM.

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AUTHOR CONTRIBUTIONS

J.G., H.M. and V.P. designed the experiments, analyzed the results, and wrote the manuscript. H.M., J.G., and C.T. acquired the data. All authors revised the manuscript and approved the final version. V.P. is the guarantor of this work and, as such, takes full responsibility for the work.

ADDITIONAL INFORMATION

The authors have no relevant conflict of interest to disclose.

FIGURE LEGENDS

Figure 1. Analysis of β-cell proliferation in dispersed human islets by immunocytochemistry for Nkx6.1. (A-F) Dispersed human islets were exposed to 5.5 mM glucose (Vehicle, A & B), 2.8 mM glucose (Vehicle or 2.8 Glu, C-F), harmine (10 μM) (A & B), 16.7 mM glucose (16.7 Glu, C & D) and HB-EGF (100 ng/ml) (E & F) for 72 h. EdU (10 μM) was added throughout. Proliferation was assessed by EdU staining and Nkx6.1 to mark β cells. Representative images of Nkx6.1 (green), EdU (red) and nuclei (blue) staining are shown (A, C & E). Arrows and arrowheads highlight $EdU^+/Nkx6.1^+$ and $EdU^+/Nkx6.1^-$ cells, respectively. Scale bar, 50 μm. Between 1,000 and 1,500 Nkx6.1+ cells were counted for each sample. Proliferation is presented as the percentage of $EdU^+/Nkx6.1^+$ cells over the total $Nkx6.1^+$ cells. Donor identifiers (Supplementary Table S1 online) are indicated. Significance was tested using paired t-tests. $P<0.05$ was considered significant. NS, not significant.

Figure 2. Analysis of β-cell proliferation in intact human islets by immunohistochemistry for Nkx6.1. (A-D) Intact human islets were exposed to 2.8 mM glucose (Vehicle or 2.8 Glu, A-D), harmine (10 μM) (A & B), 16.7 mM glucose (A & C) and HB-EGF (100 ng/ml) (A & D) for 72 h. EdU (10 μM) was added throughout. Proliferation was assessed by EdU staining and Nkx6.1 to mark β cells. Representative images of Nkx6.1 (green), EdU (red) and nuclei (blue) staining are shown (A). Arrows and arrowheads highlight $EdU^+/Nkx6.1^+$ and $EdU^+/Nkx6.1^-$ cells, respectively. Scale bar, 50 μm. A minimum of 1,500 $Nkx6.1^+$ and $Nkx6.1^-$ cells were counted for each sample. Proliferation is presented as the percentage of $EdU^+/Nkx6.1^+$ over the total $Nkx6.1^+$ cells (left panels) or $EdU^+/Nkx6.1^-$ cells over the total $Nkx6.1^-$ cells (right panels). Donor identifiers (Supplementary Table S1 online) are indicated. Significance was tested using paired t-tests. $P<0.05$ was considered significant. NS, not significant.

Figure 3. Analysis of β-cell proliferation in intact human islets by immunohistochemistry for C-peptide. (A-D) Intact human islets were exposed to 2.8 mM glucose (Vehicle or 2.8 Glu, A-D), harmine

(10 μM) (A & B), 16.7 mM glucose (A & C) and HB-EGF (100 ng/ml) (A & D) for 72 h. EdU (10 μM) was added throughout. Proliferation was assessed by EdU staining and C-peptide (CPEP) to mark β cells. Representative images of CPEP (green), EdU (red) and nuclei (blue) staining are shown (A). Arrows and arrowheads highlight $EdU^+/CPEP^+$ and $EdU^+/CPEP^-$ cells, respectively. Scale bar, 50 μm. A minimum of 1,500 $CPEP^+$ and $CPEP^-$ cells were counted for each sample. Proliferation is presented as the percentage of $EdU^+/CPEP^+$ over the total $CPEP^+$ cells (left panels) or $EdU^+/CPEP^-$ cells over the total $CPEP^-$ cells (right panels). Donor identifiers (Supplementary Table S1 online) are indicated. Significance was tested using paired t-tests. $P<0.05$ was considered significant. NS, not significant.

Figure 4. Analysis of cytokeratin 19-positive cell proliferation in intact human islets by immunohistochemistry. (A-D) Intact human islets were exposed to 2.8 mM glucose (Vehicle or 2.8 Glu, A-D), harmine (10 μM) (A & B), 16.7 mM glucose (A & C) and HB-EGF (100 ng/ml) (A & D) for 72 h. EdU (10 μM) was added throughout. Proliferation was assessed by EdU staining and cytokeratin 19 (CK19) to mark ductal cells. Representative images of CK19 (green), EdU (red) and nuclei (blue) staining are shown (A). Arrows and arrowheads highlight $EdU^+/CK19^+$ and $EdU^+/CK19^-$ cells, respectively. Scale bar, 50 μm. A minimum of 1,000 $CK19^+$ cells were counted for each sample. Proliferation is presented as the percentage of $EdU^+/CK19^+$ over the total $CK19^+$ cells. Donor identifiers (Supplementary Table S1 online) are indicated. Significance was tested using paired t-tests. $P<0.05$ was considered significant. NS, not significant.

Figure 5. Analysis of cell proliferation in intact human islets by flow cytometry. (A-E) Intact human islets were exposed to 2.8 mM glucose (Vehicle or 2.8 Glu, B-E), harmine (10 μM) (C), 16.7 mM glucose (D) and HB-EGF (100 ng/ml) (E) for 72 h. EdU (10 μM) was added throughout. At the end of the treatment islets were dispersed into single cells and analyzed by flow cytometry for C-peptide (CPEP), glucagon (GCG), cytokeratin 19 (CK19) and EdU (A). Neg, $GCG^-/CPEP^-$ cells. The proportions of

CPEP$^+$, GCG$^+$ and GCG$^-$/CPEP$^-$ cells for each donor are shown (B). A minimum of 2,000 CPEP$^+$, GCG$^+$, GCG$^-$/CPEP$^-$ and CK19$^+$ cells were counted for each sample. Proliferation is presented as the percentage of EdU$^+$ cells over the total cells cell count for each cell type. Data for the CPEP$^+$ cells are replicated in the panels on the right to highlight the proliferative responses. Donor identifiers (Supplementary Table S1 online) are indicated. Significance was tested using paired t-tests. $P<0.05$ was considered significant. NS, not significant.

Figure 1

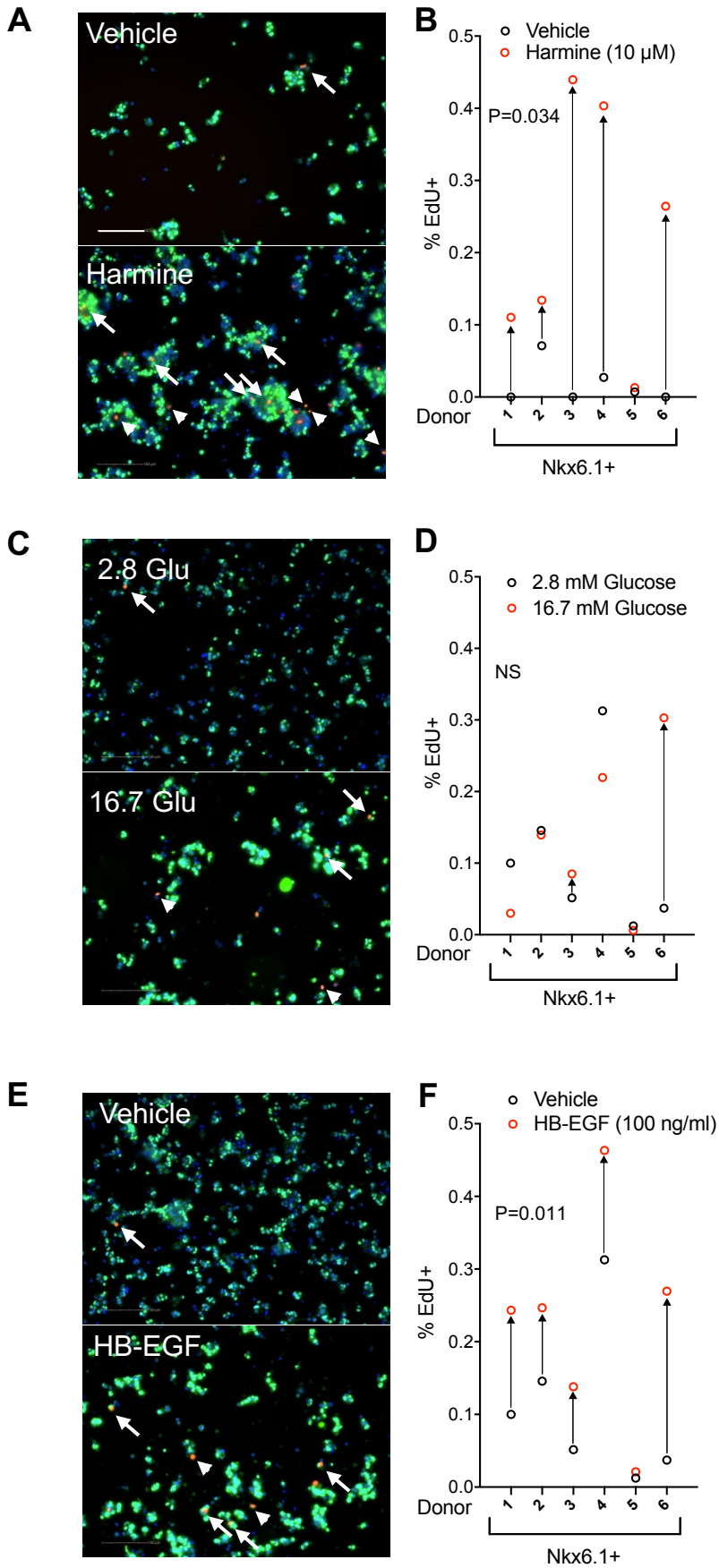
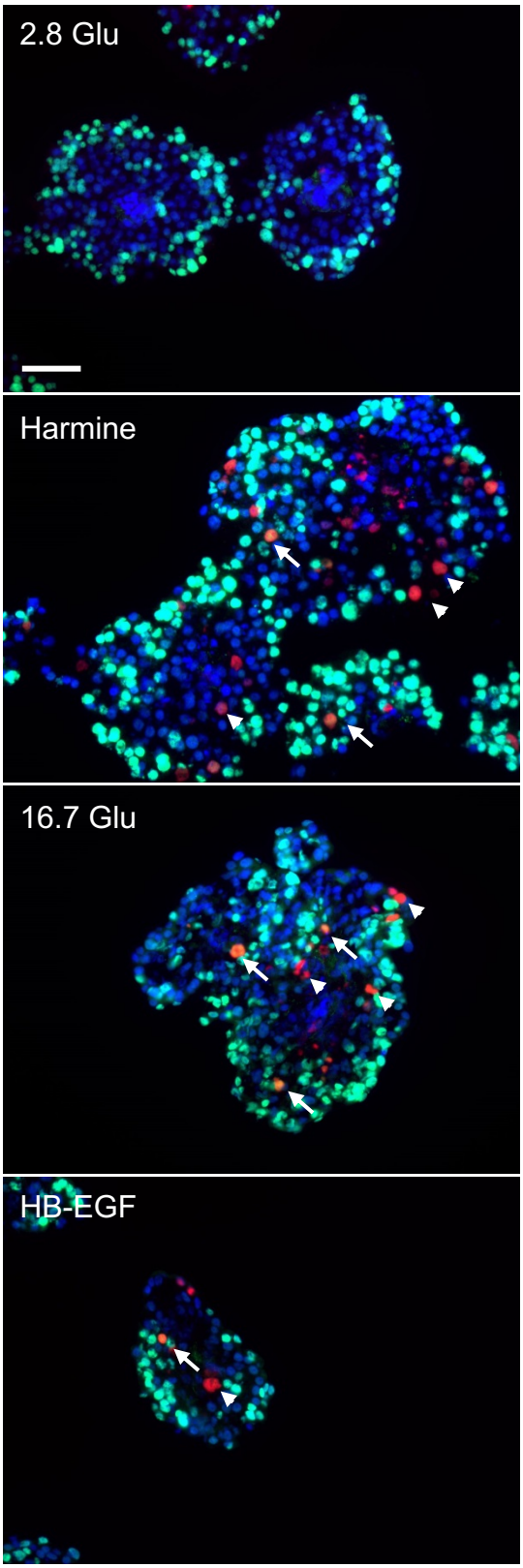

A
Vehicle
Harmine
B
Vehicle
Harmine (10 μM)
P=0.034
% EdU+
0.5
0.4
0.3
0.2
0.1
0.0
Donor
1
2
3
4
5
6
Nkx6.1+
C
2.8 Glu
16.7 Glu
D
2.8 mM Glucose
16.7 mM Glucose
NS
% EdU+
0.5
0.4
0.3
0.2
0.1
0.0
Donor
1
2
3
4
5
6
Nkx6.1+
E
Vehicle
HB-EGF
F
Vehicle
HB-EGF (100 ng/ml)
P=0.011
% EdU+
0.5
0.4
0.3
0.2
0.1
0.0
Donor
1
2
3
4
5
6
Nkx6.1+

Figure 2


A
2.8 Glu
Harmine
16.7 Glu
HB-EGF

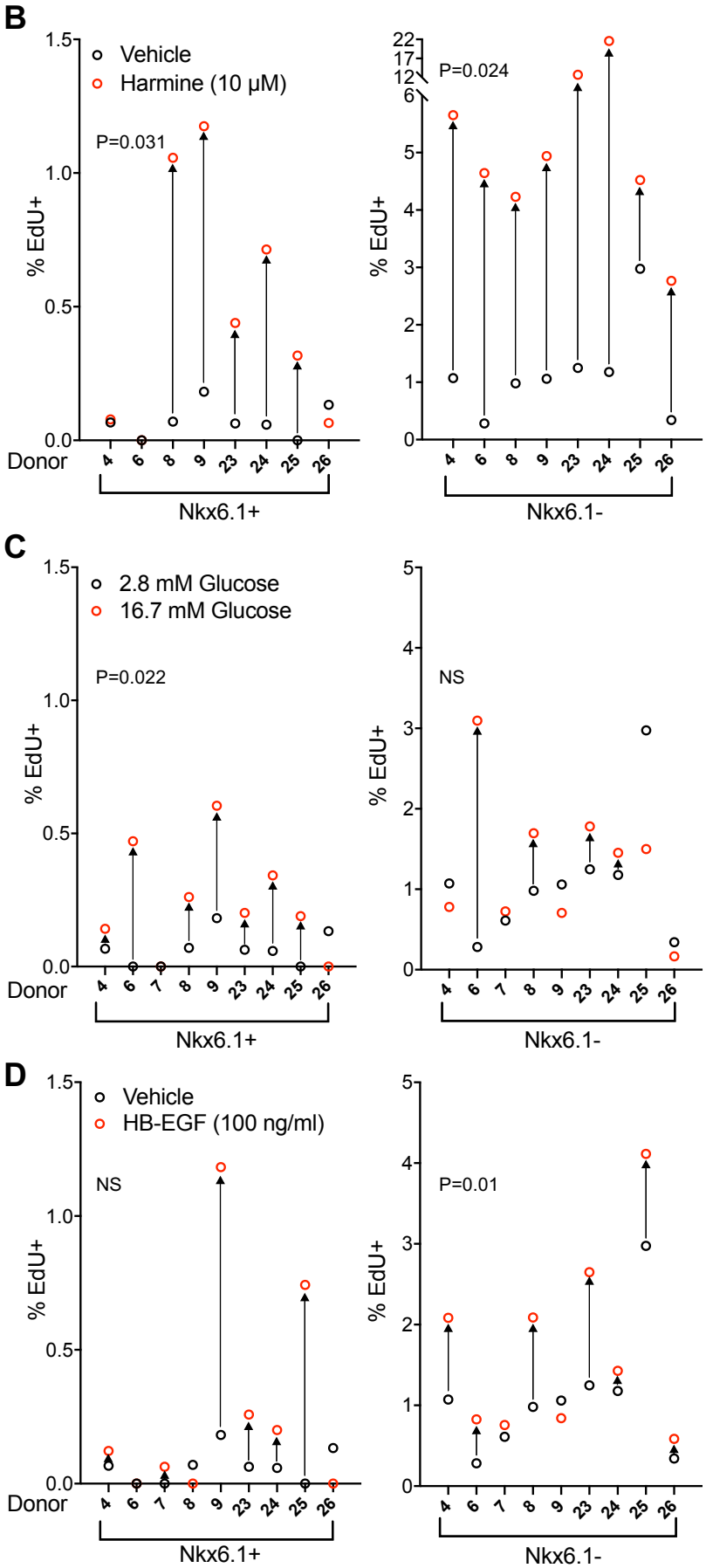

B
Vehicle
Harmine (10 μM)
P=0.031
P=0.024
% EdU+
Donor
Nkx6.1+
Nkx6.1-
C
2.8 mM Glucose
16.7 mM Glucose
P=0.022
NS
D
Vehicle
HB-EGF (100 ng/ml)
NS
P=0.01

Figure 3

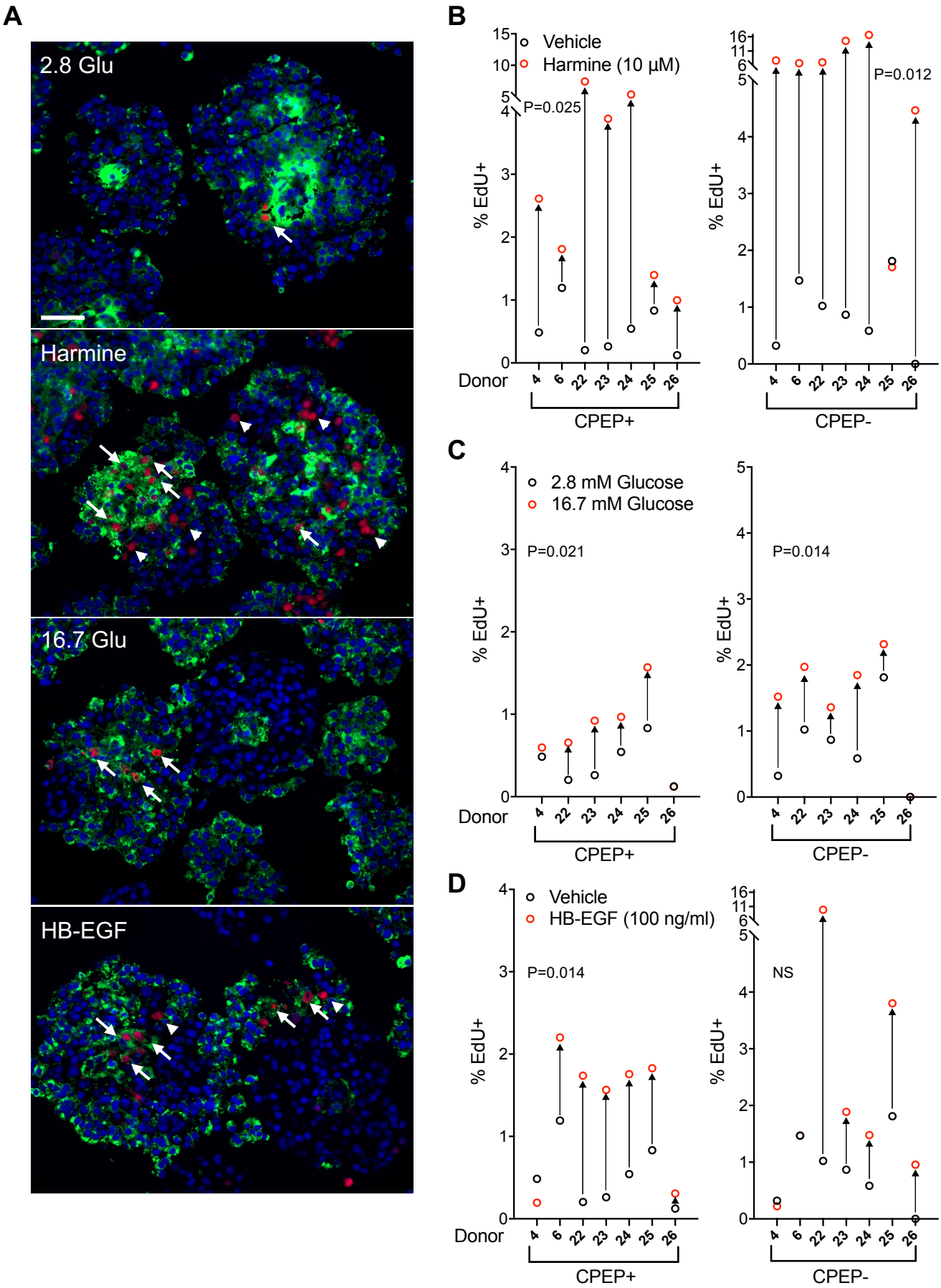

A
2.8 Glu
Harmine
16.7 Glu
HB-EGF
B
Vehicle
Harmine (10 μM)
P=0.025
P=0.012
% EdU+
Donor
4 6 22 23 24 25 26
CPEP+
CPEP-
C
2.8 mM Glucose
16.7 mM Glucose
P=0.021
P=0.014
% EdU+
Donor
4 22 23 24 25 26
CPEP+
CPEP-
D
Vehicle
HB-EGF (100 ng/ml)
P=0.014
NS
% EdU+
Donor
4 6 22 23 24 25 26
CPEP+
CPEP-

Figure 4

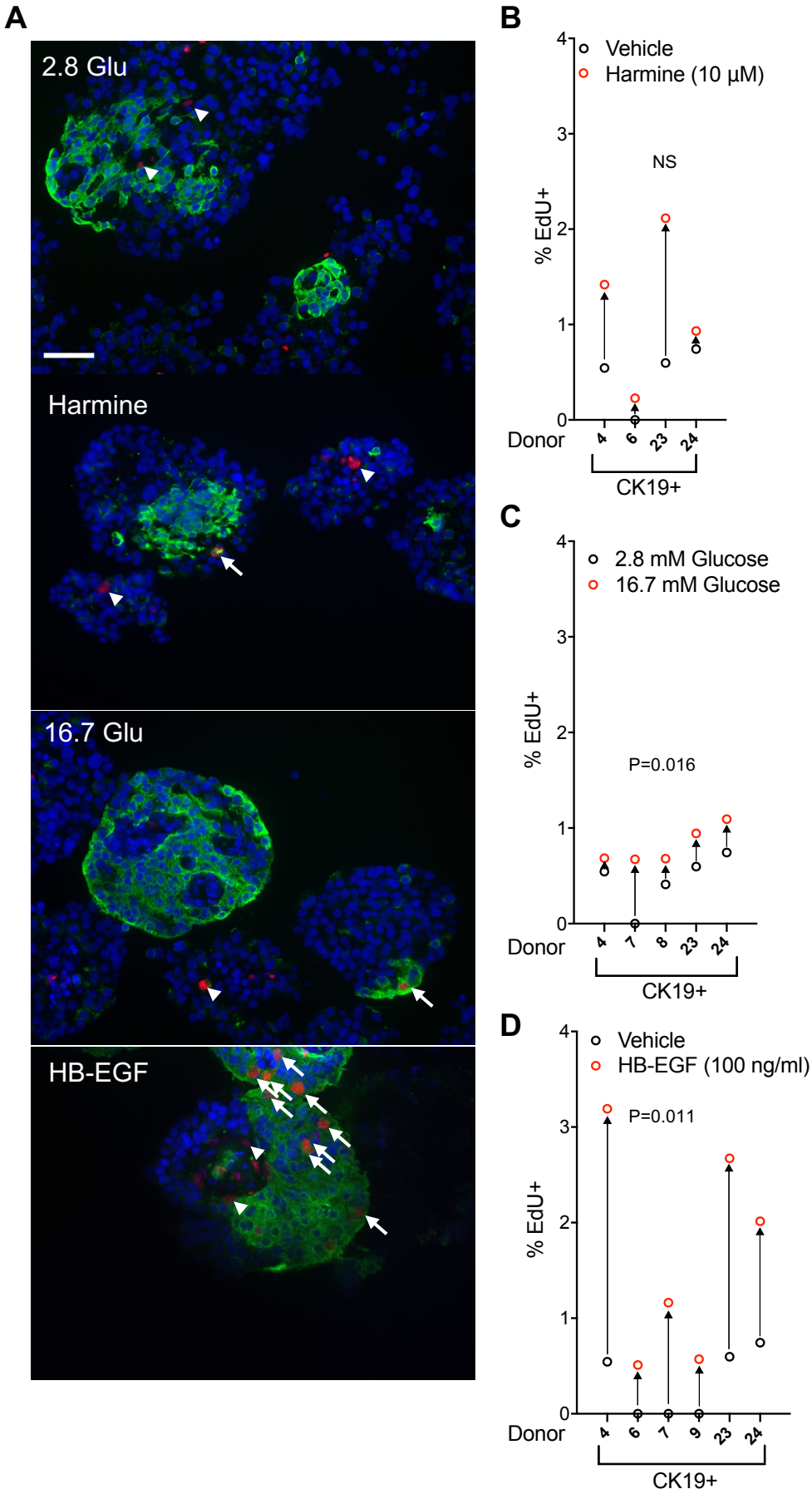
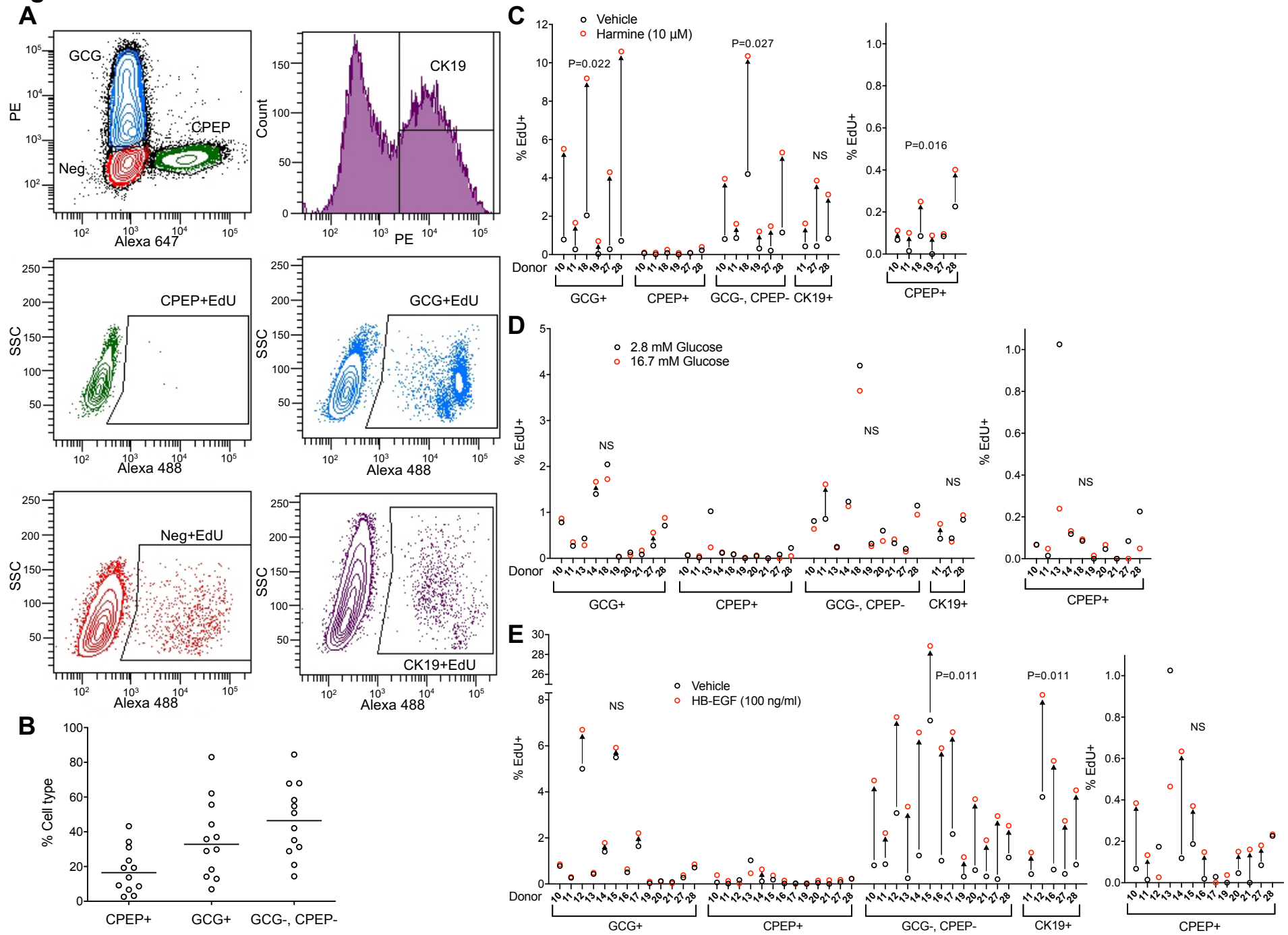

A
2.8 Glu
Harmine
16.7 Glu
HB-EGF
B
Vehicle
Harmine (10 μM)
NS
% EdU+
Donor
4
6
23
24
CK19+
C
2.8 mM Glucose
16.7 mM Glucose
P=0.016
% EdU+
Donor
4
7
8
23
24
CK19+
D
Vehicle
HB-EGF (100 ng/ml)
P=0.011
% EdU+
Donor
4
6
7
9
23
24
CK19+

Figure 5


A
GCG
CPEP
Neg
PE
Alexa 647
CK19
Count
PE
CPEP+EdU
GCG+EdU
Neg+EdU
CK19+EdU
SSC
Alexa 488
B
% Cell type
CPEP+
GCG+
GCG-, CPEP-
C
Vehicle
Harmine (10 μM)
P=0.022
P=0.027
NS
P=0.016
% EdU+
Donor
GCG+
CPEP+
GCG-, CPEP-
CK19+
D
2.8 mM Glucose
16.7 mM Glucose
NS
E
Vehicle
HB-EGF (100 ng/ml)
P=0.011
P=0.011