Early and Late G1/S Cyclins and Cdks Act Complementarily to Enhance Authentic Human Beta-cell Proliferation and Expansion.

Running Title: Cdks and Human Islets Expansion

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Abstract

β-cell regeneration is a key goal of diabetes research. Progression through cell cycle is associated with pRb inactivation via sequential phosphorylation by the “early” cyclins/cdk5s (D-cyclins cdk4/6) and the “late” cyclins/cdk5s (cyclin A/E and cdk1/2). In β-cells, activation of either “early” or “late” G1/S cyclins and/or cdk5s is an efficient approach to induce cycle entry, but it is unknown whether combined expression of early and late cyclins/cdk5s might have synergistic or additive effects. Thus, we explored whether a combination of both “early” and “late” cyclins and cdk5s might more effectively drive human β-cell cell cycle entry than either group alone. We also sought to determine whether authentic replication with expansion of adult human β-cells could be demonstrated.

“Late” cyclins/cdk5s do not traffic in response to the induction of replication by “early” cyclins/cdk5s in human β-cells, but are capable of nuclear translocation when overexpressed. “Early” plus “late” cyclins/cdk5s, acting via pRb phosphorylation on distinct residues, complementarily induce greater proliferation in human β-cells than either group alone. Importantly, the combination of “early” and “late” cyclins/cdk5s clearly increased human β-cell numbers in vitro.

These findings provide additional insight into human β-cells expansion. They also provide a novel tool for assessing β-cell expansion in vitro.
Introduction

Complete or partial loss of functional β-cell mass is a major feature of Type 1 and Type 2 diabetes\(^1\). Replacement or regeneration of lost β-cells is, therefore, a key goal of diabetes research. Thus, manipulating the regulation of cell cycle in human β-cells holds great therapeutic potential. Expanding adult human β-cells is challenging since their basal proliferation level \textit{in vivo} and \textit{in vitro} is extremely low, and they are resistant to the induction of replication\(^2\)-\(^8\). Recently, we made the unexpected observation that many key G1/S cell cycle activators are excluded from the nucleus in adult human β-cells, presumably contributing to their refractoriness to replication\(^7\),\(^8\). Observations in neonatal human β-cells show that human β-cells do replicate transiently during the first few years of life\(^9\)-\(^13\). The labeling index remains low compared to other tissues, however, in the range of 3%.

We, and others, have shown that it possible to directly manipulate cell cycle and induce some cell cycle entry in adult human β-cells. For instance, the overexpression of cell cycle activators, such as cdk6 and cyclin D3\(^5\),\(^14\), or down regulation of inhibitors, such as p57\(^15\), leads to a substantial cell cycle entry in adult human β-cells. However, whether these replication levels are therapeutically relevant and whether this cell cycle entry actually leads to a true increase in β-cell number remains unknown.

Transition from the G1 to S phase of the cell cycle requires the inactivation of the retinoblastoma protein family (pRb, p107, p130) of cell cycle inhibitors at the key G1/S restriction point. pRb is inactivated in the nucleus by sequential phosphorylation of up to 16 serines and threonines orchestrated by multiple cdks and their cyclin partners\(^16\),\(^17\). The “early” cyclin/cdk complexes, including one of the three D-cyclins bound to either
cdk4 or cdk6, may mediate the initial pRb phosphorylation. Inactivation of pRb also may be carried out by the “late” cyclins/cdk: complexes of cyclin A or E with either cdk1 or cdk2. Regulation of cdk activity is multifactorial, and can be controlled at the level of nuclear translocation, protein stability/abundance, cyclin binding, phosphorylation status, and activity of cdk inhibitors such as CIP/Kip family. The relative importance of these in β-cells is unknown.

In mouse and man, the early G1/S cdk complexes play a crucial role in β-cell proliferation. Loss of either cdk4 or cyclin D2 in mice leads to a profound loss of β-cell mass and proliferation, and severe diabetes. Growth factors and nutrients have been shown to induce cell cycle entry by activating early G1/S cyclins and cdks. For example, glucose stimulates mouse β-cell replication in part via an induction of cyclin D2. c-Myc induces rodent β-cell replication through the induction of D-cyclins, cdk4 and cdk6. We have shown that overexpression of cdk6 or D-cyclins, individually or in combination, leads to a marked and sustained stimulation of cell cycle entry in human β-cells.

Recent studies also underscore the importance of the late G1/S cyclin/cdk complexes in mediating β-cell proliferation as well: cyclin A has been shown to be necessary for exendin-4 induced proliferation in murine β-cells. The growth factor, PTHrP, increases human β-cell proliferation via upregulation of cyclin E and cdk2. Similarly, induction of rat and human β-cell proliferation by the transcription factor Nkx6.1 requires the induction of cyclin E. Finally, overexpression of cyclin E in combination with cdk2 induces a significant and marked stimulation of human β-cell proliferation. These data suggest that targeting the activation of either the “early” or the “late” G1/S cdk complexes is an efficient approach to inducing human β-cell proliferation.

While several studies have focused on either the “early” or the “late” G1/S cyclins/cdks, no prior study has examined the possibility that activation of “early” plus
“late” G1/S cyclin/cdk complexes may be additive or synergistic for inducing proliferation in adult human β-cells. We hypothesized that this may be possible. We further hypothesized that such a synergistic or additive effect may induce a therapeutically relevant level of human β-cell replication and lead to an authentic expansion of adult human β-cells.
Methods

**Human cadaveric islets.** Human islets were purchased from the NIH- and JDRF-supported Integrated Islet Distribution Program (iidp.coh.org). Sixty-six different cadaveric preparations from non-diabetic donors were used for these studies. The mean age of the donors was 45.5 years ± 1.6 (SEM), the BMI 32.2 ± 0.8 kg/M² (SEM) and the purity was 85.1 % ± 0.1 (SEM). Thirty of the 66 were from female donors, 32 were from male donors, and two had no specified donor gender. Only two of the 66 were under the age of 20, at 16 and 14 y. o. Upon arrival, islets were incubated in RPMI medium (Gibco, Grand Isle, NY) containing 5.5 mM glucose, 1% penicillin and streptomycin with 10% fetal bovine serum until they were utilized for experiments. The use of human cadaver islets was approved in advance by University of Pittsburgh School of Medicine and Icahn School of Medicine at Mount Sinai Institutional Review Boards.

**Rat islets.** Rat islets were isolated from two day-old, two month-old, 9-10 month-old or 18-20 month-old Sprague Dawley rats (Vendor) as previously described. Islets were incubated in RPMI medium (Gibco, Grand Isle, NY) containing 5.5 mM glucose, 1% penicillin and streptomycin with 10% fetal bovine serum until they were utilized for experiments. Rat islet isolations were performed with the approval of, and in accordance with, guidelines established by the Icahn School of Medicine at Mount Sinai and the University of Pittsburgh Institutional Animal Care and Use Committees.

**Adenovirus production and transduction.** Adenoviruses, all under control of the CMV promoter, were prepared using human cDNAs encoding human cdk6, human cdk1, human cdk2, human cyclins D3 as described previously. Ad.human cyclin A2 was purchased from Vector Biolabs (Philadelphia, PA) and Ad.human cyclin E1 was purchased from Applied Biological Materials Inc (Richmond, BC, Canada). Dispersed
islets on coverslips were transduced with either Ad.LacZ or relevant control adenoviruses for two hours, cultured for 72 hours as described in the Figures, and immunolabeled as described below.

**Glucose-stimulated insulin secretion (GSIS)** in isolated human islets transduced with was studied three days after transduction as described previously\(^5\). Five different human islet preparations were used. Insulin secretion was determined using a human insulin ELISA kit (ALPCO, Salem, NH).

**Islet Cell Dispersion and Immunocytochemistry.** Human islets or rat islets (400 IEQ) were washed twice in PBS and dispersed as previously described\(^5, 7, 14\). Single cells were then plated on coverslips, transduced with Ad.cdk6+Ad.cyclinD3, or Ad.cdk1+Ad.cyclin E, or Ad.cdk6+Ad.cyclinD3+Ad.cdk1+Ad.cyclin E (total of 400 moi or 1,000 moi), cultured for 72 hours, then fixed in fresh 4% paraformaldehyde for 15 min at 25°C, as previously described\(^5, 7, 14\). Control cells (CTL) were transduced with Ad. LacZ. Fixed cells were washed with PBS and incubated in blocking buffer (1.0% BSA, 0.5% Triton, 5% normal goat serum in PBS) for 1 hr at 25°C. Primary antisera were exposed to the fixed cells on coverslips overnight at 4°C in blocking buffer, and secondary antisera were exposed for 2 hr at 25°C in secondary buffer (1% BSA, 0.5% Triton in PBS). Primary antisera and secondary antisera are described in Supplemental Table 1.

**In Situ Proximity Ligation Assay (PLA).** Islets were dispersed to single cells, transduced with Ad.LacZ, or Ad.cdk6+Ad.cyclinD3, or Ad.cdk1+Ad.cyclin E, or Ad.cdk6+Ad.cyclinD3+Ad.cdk1+Ad.cyclin E (total of 400 moi) and fixed as previously described above. After washing with PBS, the cells were immunolabelled for insulin as previously described\(^5, 7, 14\). After washing with PBS, cells were incubated overnight at 4 °C with a combination of a mouse antibody against the full length pRb (Cell Signaling,
Cat # 9309) and a rabbit antibody, against either phospho-S780/S795-Rb (Cell Signaling #9307S and 9301P) or against phospho-S811-Rb (Cell Signaling # 9308S). All the antibodies were diluted (1:100) in the antibody diluent provided by the manufacturer (Duolink®, Sigma-Aldrich, St Louis, MO). Negative controls omitted the primary antibody against pRb. After washing, the cells were incubated for 1 hr at 37°C with the Duolink® PLA Rabbit MINUS and PLA Mouse PLUS proximity probes, both provided by the manufacturer (Duolink®, Sigma-Aldrich, St Louis, MO). The proximity ligation and the amplification were performed using the Duolink® detection reagent kit (Duolink®, Sigma-Aldrich, St Louis, MO) according to the manufacturer’s protocol. Fluorescence was detected using a Leica SP5 confocal microscope. All the experiments were repeated at least three times.

**Immunoblotting.** Islet extracts were resolved using 10% or 12% SDS-PAGE, and transferred to Immobilon-P membranes (Millipore Inc., Bedford, MA). Primary antisera and secondary antisera are described in Supplemental Table 1. Each experiment shown is representative of 3-6 human islet preparations.

**Assessment of β-cell mass in vitro.** Human islets were dispersed and transduced with either Ad.LacZ, Ad.cdk6+D3, Ad.cdk1+cyclin E or Ad.cdk6+Ad.cyclinD3+Ad.cdk1+Ad.cyclin E (total of 400 moi) as described previously. Ten thousand transduced islets cells from each group were plated in a high content imaging glass bottom 96 well plate (Corning), coated with poly-D lysine (Sigma-Aldrich, St Louis, MO). Five, 10, 15 and 20 thousand untransduced dispersed human islet cells were plated as controls. One day later, 50 µl of Matrigel (Corning, Corning, NY) was added to each well. Cells were fixed in 4% PFA for 10 min at RT five days after plating.
Cells were then immunolabelled for insulin and DAPI as previously described\textsuperscript{5, 7, 14}. Photomicrographs were taken to cover the entire surface of each well using an Olympus IX-70 wide-field epi-fluorescence system. Images were then stitched together to create a single picture using Metamorph\textregistered. Each stitched picture was then analyzed using ImageJ\textregistered to measure the total \( \beta \)-cell area per well. To measure the \( \beta \)-cell size, cells were immunolabelled with an antisera for E-cadherine and for insulin. The size of each \( \beta \)-cell was measured using ImageJ\textregistered.

**Statistics.** Statistical analysis for Figs 1B&G, 4B, 5B, 6B and 8B was performed using one-way ANOVA with Bonferroni’s post-hoc correction. Student’s paired, two-tailed T-test was employed in Figs 1E, 5DF&H, 6DF&H and 8D. All values are expressed as means ± SEM. “p” values < 0.05 were considered significant.
Results

Human β-cells are more resistant than rat β-cells to the induction of cell cycle entry by “early” G1/S cell cycle cyclins and cdks. Previous studies have shown that rodent β-cells are more susceptible to replication than human adult β-cells\(^1, 5, 7, 8, 14, 26, 32\)\(^{-41}\). Here, we determined if this species difference also existed when cell cycle molecules, such as cdk6 and cyclin D3, were directly overexpressed. As shown in Fig1A and B, basal levels of replication were extremely low in human β-cells. In dramatic contrast, basal levels of β-cell replication were about 40% in two day-old rat β-cells, but dropped below 1% in two month-old, nine-ten month-old (10 mo) and eighteen-twenty month-old (20 mo) rat β-cells. When cdk6 and cyclin D3 (C6+D3), were overexpressed 14.1 ± 0.9% of human β-cell entered cell cycle as assessed by Ki67 immunolabelling. In contrast, 49.3 ± 3.0% of two month-old rat β-cells entered cell cycle under the same conditions. This was age-independent, since β-cells from nine-ten month-old and eighteen-twenty month-old rats responded with a similar cell cycle entry level (50.9 ± 1.4% and 38.0 ± 6.7% respectively). These high levels of cell cycle entry measured in rat β-cell in response to the overexpression of C6+D3 were similar to levels of cell cycle entry measured in rat insulinoma cell line, INS1, cultured in 5.5mM glucose (Fig 1CD). As shown in Fig 1E, efficiency of adenoviral transduction in rat and human islet cells appeared comparable, as measured by the adenoviral beta galactosidase labeling. Finally, since nuclear translocation of cdk6 and cyclin D3 is associated with the level of replication\(^5, 7, 8, 14\), we measured the percentage of nuclear presence of cdk6 and cyclin D3 in human and rat β-cells after transduction. As shown in Fig 1F and G, the percentage of nuclear cyclin D3 was similar in rat β-cells as compared to human β-cells when C6+D3 were overexpressed. The percentage of nuclear cdk6 was lower in rat β-cells.
Late cyclins and cdks do not traffic to the nucleus in response to the induction of replication in human β-cells, but do in rat β-cells. Cell cycle entry begins with the inactivation of pRb and other pocket proteins either by the “early” G1/S cyclins and cdks, cdk4-6 and D-cyclins or by the “late” cyclins and cdks, cdk1/2-cyclin A/E. To complete the progression through G1 and enter S, “late” cyclins and cdks must translocate to the nucleus following their synthesis in the cytoplasm. Thus, we queried whether the difference in cell cycle entry between rat and human β-cell by C6+D3, could be explained by a difference in “late” cyclin and cdks nuclear translocation and activation. We, therefore, examined the trafficking of the “late” G1/S cyclins and cdks, cyclin A and E, cdk1 and 2, under basal conditions and when C6+D3 are overexpressed, in adult human and rat β-cells. As shown in Fig 2, cyclin A, cyclin E, cdk1 and cdk2 were expressed in rat and human β-cells under basal conditions and were predominantly cytoplasmic. All four “late” G1/S cyclins and cdks remained cytoplasmic in human β-cells when replication was induced by overexpression of C6+D3 (Fig 2). In contrast, in rat β-cells, cdk1 and cdk2 translocated to the nucleus when C6+D3 were overexpressed. Moreover, in rapidly replicating rat Ins1 cells, cdk1 and cdk2 were both cytoplasmic and nuclear. We also explored nuclear translocation of two cell cycle inhibitors: p16Ink4A and p27Kip1. As shown in Suppl Fig 1, and, as previously described, p16Ink4A and p27Kip1 were predominantly cytoplasmic under basal conditions in human β-cells (6.74 ± 1.4% and 10.37 ± 2.83% in the nucleus, respectively), but were detected more often in the nucleus of β-cells transduced by cdk6+D3 (13.75 ± 0.62% and 22.9 ± 6.15%, respectively). This nuclear translocation of p16Ink4A and p27Kip1 was more pronounced in rat β-cells in response to cdk6 + D3 (27.82 ± 3.17% and 61.08 ± 8.31% respectively).
Cyclin A and E translocate to the nucleus when overexpressed, but cdk1 and 2 require their cyclin partners for nuclear entry in human β-cells. Next, we interrogated whether the apparent lack of nuclear translocation of the cdk1/2 and cyclin A/E reflected an intrinsic inability of these “late” cyclins/cdk2 to gain nuclear access in human β-cells. For that purpose, we determined whether the overexpression of the cdk1/2 and cyclin A/E, individually or in combination, could lead to their nuclear translocation. As indicated in Fig 3A and Suppl Fig 2, transduction with adenoviruses encoding for either cdk1, cdk2, cyclin A or cyclin E led to increased level of expression of each of the “late” cell cycle molecules, in a dose-dependent manner. When overexpressed alone, cdk1 and cdk2 remained cytoplasmic (Fig 3B). However, when combined with either cyclin A or E, cdk1 or cdk2 appeared in the nuclei of human β-cells (Fig 3B). In contrast, the overexpression of cyclin A or cyclin E, alone or in combination with cdk1 or cdk2, led to their nuclear translocation in human β-cells.

Cyclin E plus cdk1 is the most effective “late” cyclin and cdk combination for inducing cell cycle entry in the human β-cell. We next determined if the nuclear translocation of the “late” cyclins and cdks is associated with cell cycle entry of human β-cells. As shown in Fig 4, and as expected, overexpression of C6+D3, led to an increased BrdU incorporation (15.3 ± 1.6 %). The overexpression of cyclin A, cdk1 and cdk2 alone did not induce significant cell cycle entry in human β-cells. In contrast, cyclin E, when overexpressed alone, induced cell cycle entry (1.9 ± 0.4%). In contrast to the individual cyclins and cdks, combinations of “late” cyclins and cdks, induced a significant increase of BrdU incorporation, with cyclin E and cdk1 being the most effective combination (13.3 ± 1.9%).
“Early” and “late” cyclins and cdks complementarily induce cell cycle entry in the human β-cell. Since both “early” and “late” cyclins and cdks can each induce significant cell cycle entry in human β-cells, we next inquired whether an “early” + “late” combination could additively or synergistically increase their cell cycle entry. As shown in Fig 5, combination of (C6+D3) with (K1+E) at a 250 MOIs each led to remarkable 42.8 ± 2.2 % BrdU incorporation; in parallel, 54.3 ± 3.1 % of human β-cells were positive for Ki67. This was significantly higher than either of the “early” or “late” cyclin/cdk combinations (Fig 5B). These high levels of cell cycle entry were coupled with a significant degree of DNA damage (~ 25%), as measured by phospho-γH2AX. Surprisingly, this DNA damage was not coupled with increased β-cell death as assessed by TUNEL assay (Fig 5G and H).

Since the high MOI (250 MOI each) induced DNA damage, we repeated these experiments with lower MOI (100 MOI each) (Fig 6). Under these conditions, the combination of “early” and “late” cyclins and cdks still led to a dramatic and high level of cell cycle entry, with 19.6 ± 1.5 % of β-cells labeled with BrdU and 35.4 ± 4.2 % with Ki67. At this MOI, DNA damage was minimal (~ 5%) and was not associated with any β-cell death (TUNEL). These data indicate that both “early” and “late” cyclins and cdks are independently capable of driving human β-cells to enter cell cycle. Further, their combination is, complementary; whether this reflects synergy or additivity cannot be ascertained because high MOI prevent full dose-response curves.

To determine whether the transduction with “early” of “late” cyclins and cdks might result in the loss of β-cells function, we examined glucose-stimulated insulin secretion (GSIS) three days after transduction. As seen in Suppl. Fig 3, glucose-stimulated secretion remained robust when β-cells were transduced with both “early” and “late” cyclins and cdks.
“Early” and “Late” cyclins and cdks complementarily phosphorylate pRb on distinct residues in human β-cells. We next explored the mechanism through which “early” and “late” cyclins and cdks might complementarily enhance cell cycle entry in human β-cells. Fig 7A shows the 16 residues known to be phosphorylated by cdk4/6 and cdk1/2 in other cell types. As shown in Fig 7B, phospho-pRb was undetectable in human islet transduced with control Ad.LacZ (CTL). In contrast, pRb was phosphorylated when either “early” (C6+D3) or “late” (K1+E) cyclins and cdks were overexpressed. Importantly, pRb was “hyperphosphorylated” when the combination of “early” and “late” cyclins and cdks were overexpressed. More specifically, pRb was phosphorylated on Ser\(^{780}\) and Ser\(^{795}\), well-known phosphorylation sites for cdk4/6, only when cdk6 was overexpressed (C6+D3 or C6+D3+K1+E). In contrast, pRb was phosphorylated on Thr\(^{373}\), a well-known target for cdk1, only when cdk1 was overexpressed (K1+E or C6+D3+K1+E). The specificity of the antibodies was tested in HepG2 cells, transduced with or without an adenovirus silencing pRb (shRb).

Since human islets are only composed of ~ 50% β-cells\(^ {44}\), immunoblots of whole islets may not reflect events in β-cells. Thus, we confirmed these findings in β-cells using the Proximity Ligation Assay (Fig 7C and D). As has been seen by immunoblots, pRb was phosphorylated (nuclear red dots signal in Fig 7C) on Ser\(^{780}\) and Ser\(^{795}\) only when cdk6 was overexpressed (C6+D3 or C6+D3+K1+E). pRb was phosphorylated on Ser\(^{811}\) (nuclear red dots signal in Fig 7D and a well-known site for cdk1) only when cdk1 was overexpressed (K1+E or C6+D3+K1+E). Interestingly, pRb phosphorylation was detected on Ser\(^{780},\) Ser\(^{795}\) and Ser\(^{811}\) when cdk6+D3 were overexpressed in rat β-cells, suggesting the activation of both “early” and “late” cdk-complexes.
The combination of “early” and “late” cyclins and cdks induces human β-cell expansion in vitro. While many examples of induced cell cycle entry the human β-cell have been reported, it is not typically clear whether this apparent “proliferation” actually leads to increases in human β-cell numbers. Indeed, we, and others, have suggested that at least some of the apparent “proliferation” observed using BrdU, Ki67, PCNA and other “proliferation” markers might actually reflect DNA damage and DNA repair. Given the markedly increased levels of human β-cell replication shown in Fig 5 and 6, it was important to determine whether this apparent “proliferation” can translate into actual increases in β-cell numbers. To address this question, we developed a high-content imaging method to measure the numbers of human β-cells in vitro five days after adenoviral transduction and plating. As shown in Fig 8AB, plating of increasing numbers of human islet cells led to an escalating dose-response curve, confirming that the method can detect differences in human β-cell numbers. More importantly, the β-cell area was significantly increased and readily observed when cells were transduced with the combination of “early” and “late” cyclins and cdks (129.5 ± 8.7%) as compared to islet cells transduced with a control adenovirus (LacZ) (89.6 ± 2.7 %). The β-cell mass expansion occurred more effectively with the combination of “early” and “late” cyclins/cdk than either alone (95.3 ± 3.2 % for cdk6+D3 and 104.8 ± 4.8 % for cdk1+cyclin E). Since the assay assesses β-cell area, the increase in β-cell area from the cdk/cyclin combination could reflect either an increased number of β-cells and/or an increase in the size of individual β-cells. Thus, we next determined the size of human β-cells using E-cadherin immunolabelling (Fig 8CD). The size of human β-cells transduced with the combination of “early” and “late” cyclins and cdks was significantly smaller than controls. These data suggest that the combination of “early” and “late” cyclins and cdks not only induced a remarkable cell
cycle entry in human β-cells, but also increased the number of β-cell in vitro: that is, it was associated with completion of the cell cycle and the generation of daughter cells. Collectively, as summarized in Fig 8E, these data suggest that the combination of “early” and “late” cyclins and cdks led to expansion of human β-cells in vitro. It also provides a useful assay for quantifying human β-cell expansion.
Discussion

This report contains a number of novel findings, some of which are summarized in the model in Fig 8F. First, we show that “early” and “late” cyclins and cdks prompt human β-cells to enter cycle independently. Second, we show that, in combination, they complementarily enhance cell cycle entry in the human β-cell. Third, we show using two independent approaches that cumulative phosphorylation of distinct pRb residues is one mechanism through which this occurs. Fourth, we show that expression and nuclear translocation of both “early” and “late” cyclins and cdks leads, not only to an increase in surrogate markers for human β-cell replication, but actually leads to an authentic increase in human β-cell number. And fifth, we provide a novel assay for measuring actual increases in human β-cell numbers.

Regarding the last two points, the ability to demonstrate increases in β-cell numbers represents an important advance, since prior studies on human β-cell “replication”, including our own, have used canonical markers of replication such as BrdU incorporation or Ki67, PCNA or phospho-histone H3 immunolabelling. These approaches mark different phases of the cell cycle, but do not distinguish between a completed cell cycle with daughter β-cells versus a failed attempt at replication or DNA damage and repair. For example, Ki67 marks cells in late G1, S or G2/M phases. BrdU and PCNA marks cells in S phase. Phospho-histone-H3 marks cells in G2/M. All can also reflect DNA damage and/or repair. Thus, since markers of proliferation are not infallible, coupled with the brief viability of mature β-cell in vitro, their very low replication levels and their recalcitrance to proliferate, it has been particularly challenging to demonstrate authentic increases in primary β-cells: definitive evidence of authentic increases in human β-cell numbers in vitro has not been reported\(^3, 5, 7, 8, 14, 15, 26, 28, 31, 43\).
Here, we report a simple method to address this important issue in vitro. Using this method, we have demonstrated that the activation of both “early” and “late” cyclins and cdks, not only leads to robust cell cycle entry as assessed by BrdU incorporation and Ki67 labeling, but also increases the β-cell area. This β-cell area expansion reflects increased β-cell numbers rather than an increased β-cell size, since β-cell size is smaller with the combination of “early” and “late” cyclins and cdks. This strongly supports the argument that the β-cell expansion observed is representative of authentic increased β-cell number.

The increased β-cell number is supported by the calculated expected β-cell number. If one estimate that 20-40% of β-cells were actively engaged in cell cycle (Fig 5 and 6), that half of these labeled cells represent new daughter cells, and the other half the residual parent cells, that adenovirus transduction leads to cyclin/cdks expression after 24 hours leaving four days for β-cells to divide, and that after replication, β-cells will divide only once during the five days of the experiment25,46-48, one can estimate that the yield of new daughter cells should be in the range of 30% This is not dissimilar from the 29.6 %increase of β-cell mass with the combination of “early” and “late” cyclins and cdks.

The observation that “early” and “late” cyclins and cdks can independently induce pRb phosphorylation and cell cycle entry in human β-cells is important. A number of studies suggest that sequential phosphorylation of pRb may not be necessary, and that different mitogenic signaling pathways may activate either “early” or “late” cyclins/cdks, and either is sufficient to initiate cell cycle progression. For example, phosphorylation of only a single residue in the C-terminus of pRb is able to lead to its inactivation49. Another mutational study has found that Thr373 is a critical phosphorylation site for pRb inactivation50. Thus, the observation that “early” and “late” cyclins and cdks can independently phosphorylate pRb on distinct residues in human β-cells fits these recent
models. The question of whether these “early” and “late” cdks phosphorylations occur on the same pRb molecule or two different one remains unclear (Fig 8E). These findings provide a working model for the independent activation of the cell cycle in human β-cells by “early” and “late” cyclins and cdks, as well as their complementarity.

In addition, several studies have demonstrated that “late” cyclins and cdks can induce cell cycle without prior activation of “early” cyclins and cdks. Perhaps the best example is the work of Sicinski showing that most organs develop normally in the absence of all three D-cyclins\textsuperscript{51}, and the work of Kozar showing that cdk4 and cdk6 are dispensable for development in mammals\textsuperscript{52}. In another report, Keenan et al showed that ectopic expression of cyclin E dispenses with a need for cyclin D-cdk4 in pRb inactivation\textsuperscript{53}, E2f activation and cell cycle progression. Another group has shown that “early” and “late” cyclins and cdks can use distinct cell cycle re-entry programs in differentiated cells\textsuperscript{54}. Further, cyclin E has been shown to induce S phase without activation of the pRb/E2F pathway\textsuperscript{55}. Thus, it is also possible that, in addition to pRb phosphorylation and inactivation, “late” cyclins and cdks induce cell cycle entry complementarily without prior “early” cdk activation and pRb phosphorylation, and/or independently of pRb in human β-cells. Finally, as noted in the Introduction, several β-cell mitogens appear to act exclusively via the “late” cyclins/cdks\textsuperscript{28, 27, 29}.

The increase in β-cell numbers demonstrate that at least some of the proliferation observed in response to cyclins/cdks is authentic, and supports the notion that induction of proliferation by cyclins/cdks can occur without extensive DNA damage, β-cell death or loss of function. Of course, DNA damage was observed (Fig 6) and it is likely that some of the damaged β-cells may have died\textsuperscript{43}. On the other hand, recent reports have revealed that cdks are actually involved actively in DNA repair\textsuperscript{56}; studies in yeast and mammalian cells have revealed that cdk-activity is essential for DNA resection and
progression of DNA repair during S and G2\textsuperscript{57}. Thus, it is possible that, even if DNA damage were induced, β-cells may be able to repair their DNA and complete cell cycle. Finally, we cannot exclude the possibility that some of the increased β-cells number may result from trans-differentiation from other islet cells\textsuperscript{58, 59}.

The observation that the nuclear translocation of cdk1 and cdk2 is associated with high levels of cell cycle entry in rat and human β-cells is in accordance with the concept that cdks need to enter the nucleus in order to carry out their function as pRb kinases\textsuperscript{19, 60}. In rapidly proliferating INS1 cells, cdk1 and cdk2 were mostly nuclear. On the other hand, under basal conditions, and in quiescent rat or human β-cells, “late” cyclins and cdks were predominantly cytoplasmic. The 14-3-3 proteins or the protein kinase MYT1 have been described in other systems to bind and retain cdks in the cytoplasm\textsuperscript{19, 20}. But the exact nature of the scaffolding protein keeping cyclins and cdks in human beta cells remains unknown. When cdk1 or cdk2 were overexpressed alone in human β-cells, they remained cytoplasmic and no cell cycle entry was observed: cell cycle entry only occurred when these cdks were provided in combination with cyclin A or E, and in association with their translocation to the nucleus. The findings on Rb phosphorylation in Fig 7 and Suppl Fig 3 support the same idea: “early” cyclins and cdks can induce the nuclear translocation and activity of “late” cyclins and cdks in rat but not in human β-cells.

Cyclin E was the only “late” G1/S molecule able to induce cell cycle entry when overexpressed alone in human β-cells. Correspondingly, overexpressed cyclin E was also found more often than other cyclins in the nuclear compartment. This likely reflects the fact that cyclin E has a classical nuclear localization signal (NLS) that targets it to the nucleus via the well-characterized importin α/ importin β nuclear import pathway\textsuperscript{61, 62}.  
Neither cyclin A nor cdk1 or 2 have a consensus NLS. Cyclin A is known, however, to bind a variety of proteins with a recognizable NLS, such as p107, E2F1, p21 and p27Kip1, but cdks rely on their cyclins to traffic to the nucleus. Collectively, these observations make it likely that cdk1 and cdk2 can only translocate to the nucleus of human β-cells when in complexed with cyclin A or cyclin E, subsequently phosphorylating pRb and driving β-cell replication.

Human β-cells are recalcitrant to the induction of proliferation as compared to rodent β-cells. Here, we suggest that the nuclear translocation of cdk1 and cdk2 is associated with the difference in replication levels between rat and humans. An alternate explanation for this refractoriness may be age. Indeed, most studies on human β-cell proliferation use islets derived from human cadaveric donors in the 40 to 60 y.o. range, reflecting access to cadaver human islets. In contrast, most studies on rodent β-cell proliferation are performed in young animals, when β-cells are the most responsive to induction of proliferation. Several studies have shown that rodent β-cells proliferation capacity declines with age. Surprisingly, we observe that the cell cycle entry induced by cdk6 and cyclin D3 was maintained with age in rat β-cells in vitro. In preliminary studies, we find no association between age of human islet donors and the level of proliferation induced by cdk6+D3 (Suppl Fig 5). Thus, aging may not be the most significant factor causing the difference of proliferation levels between human and rat in response to cdk6+D3. Another possibility is that cell cycle inhibitors, such as p16Ink4A or p27Kip1, translocate to the nucleus in human β-cells but not in rat, limiting the cell cycle entry induced by cdk6+D3. However, this is improbable since the higher levels of replication in rat β-cells and Ins1 cells are associated with higher nuclear translocation of p16Ink4A and p27Kip1, as compared to humans (Suppl Fig 1). Finally, it is also possible that the amino acid sequences of rat and human cdk1 and/or cdk2 are different, allowing the nuclear translocation of rat cdk1 and cdk2, but not human cdk1 and cdk2. Again, this
scenario is unlikely since rat and human cdk1 and cdk2 do not have a recognizable nuclear localization signal, and since rat and human cdk1 and cdk2 sequences are highly homologous (97 and 98%, respectively).

These studies have limitations and raise new questions. Are human β-cells only capable of completing their cell cycle when a specific threshold of cell cycle entry is reached? Will only high level of cell cycle entry permit the detection of β-cell expansion in vitro? How high must this level be, and for how long must this replication be sustained? One limitation is that the majority of the study was performed using isolated and dispersed islets to the single cells and human β-cells may not respond similarly in intact islets. Therefore, one question would be whether this remarkable induction of human β-cell replication and expansion can be achieved in intact islets in vivo. Will this high level of replication be therapeutically relevant to replace lost β-cells in diabetes? Answers to these and other questions need to be addressed. Nonetheless, the findings demonstrate that it is actually possible to expand primary adult human β-cells, at least in vitro. They also provide a simple method to measure β-cell expansion in vitro. Finally, they suggest that drugs or small molecules designed to activate both “early” and “late” cdk-nuclear translocation might be of particular therapeutic efficacy for inducing human β-cell expansion.
Author Contributions

S. T. and N. F. T are guarantor and takes full responsibility for the manuscript and its originality. N.F.T. researched data, contributed to the discussion, and wrote the manuscript. S. T., C. R., R. W., G. C., M. T. and K. K. T. researched data.

No potential conflicts of interest relevant to this article were reported.

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**Figure Legends**

**Figure 1:** Cell cycle entry induced by cdk6 and cyclin D3 in rat and human β-cells. 

**A.** Dispersed rat and human islets were transduced with control adenovirus ("ctl") or with Ad.cdk6 and Ad.cyclin D3 ("Ad.C6+D3"). Immunolabeling for Ki67 is shown in red, insulin in green, and DAPI in blue. **B.** Quantitation of % of Ki67+/insulin+ cells. Bars indicate mean ± SEM. **C.** Immunolabeling for Ki67 is shown in red and insulin in green in rat insulinoma cells, INS1. **D.** Quantitation of % of Ki67+ in INS1 cells. Bars indicate mean ± SEM. **E.** Dispersed rat and human islets were transduced with Ad.LacZ. Cells were then labeled for the beta Galactosidase activity and quantified. Bars indicate mean ± SEM of % of Beta-Gal+ islet cells. **F.** Dispersed rat and human islets were transduced with Ad.cdk6 and Ad.cyclin D3 ("Ad.C6+D3"). Immunolabeling for cdk6 ("cdk6") or cyclin D3 ("D3") is shown in red, and insulin in green. **G.** Quantification of data in F using islets from 4-6 different donors at 72 hours after cdk6/cyclin D3 transduction. Bars indicate mean ± SEM of the % nuclear localization of the cdk6 or cyclin D3 shown in insulin+ (beta) cells.

**Figure 2:** Subcellular localization of the late G1/S cyclins and cdks in INS1 cells and in rat and human β-cells in response to expression of cdk6 and cyclin D3. Dispersed human and rat islets were transduced with control adenovirus ("control", white frames) or with Ad.cdk6 and Ad.cyclinD3 ("Ad.C6+D3", blue frames). Cells were fixed 72 hr after transduction, and immunolabeled for the late G1/S cyclins and cdks indicated, cdk1, cdk2, cyclin A or cyclin E (shown in red), and insulin (shown in green). Nuclei are stained with DAPI and are shown in blue.

**Figure 3:** Overexpression of the late G1/S cyclins and cdks and their subcellular localization in human β-cells. **A.** Human islets were transduced with control adenovirus ("CTL") or Ad.cdk1, Ad.cdk2, Ad.cyclin A or Ad.cyclin E at 100 or 250 MOIs. Representative immunoblots of cdk1, cdk2, cyclin A and cyclin E are shown. **B.** Dispersed human islets were transduced with control adenovirus ("CTL") or with Ad.cdk1 ("K1"), Ad.cdk2 ("K2"), Ad.cyclin A ("A"), Ad.cyclin E ("E") or combinations of the above as indicated for each panel. Cells were fixed 72 hr after transduction, and immunolabeled for cdk1, cdk2, cyclin A or cyclin E (shown in red), and insulin (shown in green). Red arrows illustrate examples of a nuclear late G1/S molecule in question.
Figure 4: Cell cycle entry quantification in response to expression of cdk6 plus cyclin D3 or expression of the late G1/S molecules. A. Dispersed human islets were transduced with control adenovirus (“CTL”) or with Ad.cdk1 (“K1”) or Ad.cdk2 (“K2”) or Ad.cyclin A (“A”) or Ad.cyclin E (“E”) or combinations of the above as indicated for each panel. Cells were fixed 72 hr after transduction, and immunolabeled for BrdU (shown in red), and insulin (shown in green). B. Quantification of BrdU+ and insulin+ cells as a percentage of total insulin+ cells. These data represent the quantification data in A from different experiments using islets from 4-5 different donors at 72 hours after transduction. Numbers in red indicate the total number of BrdU positive and insulin positive cells, and red numbers indicate the total number of insulin positive cells counted for each condition. Bars indicate mean ± SEM.

Figure 5: Cell cycle entry quantification in response to expression of combination of early and late cyclins and cdks. Dispersed human islets were transduced with control adenovirus (“CTL”), Ad.cdk6+cyclin D3 (“K6+D3”), Ad.cdk1+cyclin E (“K1+E”) or a combination of all four cyclins and cdks (“K6+D3+K1+E”). A total of 1,000 MOIs was used in these experiments. A. Representative photomicrographs of immunolabelling for BrdU (in red), and insulin (in green). B. Quantitation of BrdU+ and insulin+ cells as a percentage of total insulin+ cells. Bars indicate mean ± SEM. C. Representative pictures of immunolabelling for Ki67 (in red), and insulin (in gray). D. Quantitation of Ki67+ and insulin+ cells as a percentage of total insulin+ cells. Bars indicate mean ± SEM. E. Representative pictures of immunolabelling for γ-phospho-H2AX (in red), and insulin (in green). F. Quantitation of γ-phospho-H2AX + and insulin+ cells as a percentage of total insulin+ cells. Bars indicate mean ± SEM. G. Representative pictures of labeling for TUNEL (in red), and immunolabelling for insulin (in gray). H. Quantitation of TUNEL+ and insulin+ cells as a percentage of total insulin+ cells. Bars indicate mean ± SEM.

Figure 6: Cell cycle entry quantification in response to expression of combination of early and late cyclins and cdks at 400 MOI. Dispersed human islets were transduced with control adenovirus (“CTL”) or with Ad.cdk6+cyclin D3 (“K6+D3”), Ad.cdk1+cyclin E (“K1+E”) or a combination of all four cyclins and cdks (“K6+D3+K1+E”). A total of 400 MOIs were used in these experiments. A. Representative pictures of immunolabelling for BrdU (in red), and insulin (in green).
Quantitation of BrdU+ and insulin+ cells as a percentage of total insulin+ cells. Bars indicate mean ± SEM. C. Representative pictures of immunolabelling for Ki67 (in red), and insulin (in gray). D. Quantitation of Ki67+ and insulin+ cells as a percentage of total insulin+ cells. Bars indicate mean ± SEM. E. Representative pictures of immunolabelling for γ-Phospho-H2AX (in red), and insulin (in green). F. Quantitation of γ-Phospho-H2AX + and insulin+ cells as a percentage of total insulin+ cells. Bars indicate mean ± SEM. G. Representative pictures of labeling for TUNEL (in red), and immunolabelling for insulin (in gray). H. Quantitation of TUNEL+ and insulin+ cells as a percentage of total insulin+ cells. Bars indicate mean ± SEM.

**Figure 7:** “Early” and “late” cyclins and cdks phosphorylate the retinoblastoma protein on distinct residues in human β-cells. A. Schematic representation of the retinoblastoma protein (pRb). Numbers above indicate amino acid residues. The blue box designates the Nuclear Exclusion Signal (NES). The red box designates the pocket domain. The green box designates the Nuclear Localization Signal (NLS). Residues known to be phosphorylated by cdk1 and/or cdk2, (in red), by cdk4, and/or cdk6 (in blue) in other cell types are shown in the box below. B. Representative immunoblots of human islets transduced with Ad.LacZ (CTL), or with Ad.cdk6+cyclin D3 (K6+D3), Ad. Cdk1+ cyclin E (K1+E) or Ad.cdk6+cyclin D3+cdk1+cyclin E (Combo), showing pRb phosphorylated on Serine 780 and Serine 795 (Rb-P-S780/S795), on Threonine 373 (Rb-P-T373), total pRb (pRb), and tubulin. The right panels show representative immunoblots of HepG2 cells treated with or without shRNA targeting pRb (ShRb). C and D. Schematic showing in situ Proximity Ligation Assay detection of phospho-pRb on residues 780/795 or 811. Example of photomicrographs of dispersed human islets transduced with Ad.LacZ (CTL), or with Ad.cdk6+cyclin D3 (K6+D3), or Ad. Cdk1+ cyclin E (K1+E) or Ad.cdk6+cyclin D3+cdk1+cyclin E (Combo) are shown on the right. Phosphorylated pRb is labeled in red, insulin is immunolabelled in grey and nuclei are shown in blue. Red arrows indicate a positive signal for pRb phosphorylated on the residue in question.

**Figure 8:** The combination of “early” and “late” cyclins and cdks induces authentic replication and enhanced human β-cell mass *in vitro*. A. Representative picture-montages covering entire wells of high imaging content 96-well plates plated with dispersed human islets cells. Dispersed human islets (ten thousand islet cells),
transduced with Ad.LacZ (LacZ), Ad.cdk6+cyclin D3 (K6+D3), Ad. Cdk1+ cyclin E (K1+E) or Ad.cdk6+cyclin D3+cdk1+cyclin E (K6+D3+E+K1) or increasing number of islet cells (five, ten, fifteen or twenty thousand islet cells) were plated and fixed five days after plating. Islet cells were immunolabelled for insulin (shown in grey). B. Quantitation of the insulin staining area as a percentage of the control ten thousand islet cells. C. Representative photomicrographs of immunolabelling for E-Cadherin (in red), insulin (in green), and DAPI (in blue). D. Quantitation of the size of human β-cells. Bars indicate mean ± SEM. E. Summary of the mechanism through which human β-cells can be expanded (see text for details).
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Diabetes

**A.**

Human

CTL  Ad.C6+D3

Rat

CTL  Ad.C6+D3  CTL  Ad.C6+D3

2 days  2 mo

10 mo  20 mo

**B.**

% Ki67 in beta cells

|         | Human 2 days | Human 2 mo | Human 10 mo | Human 20 mo | Rat 2 days | Rat 2 mo | Rat 10 mo | Rat 20 mo |
|---------|--------------|------------|-------------|-------------|------------|----------|-----------|----------|
| CTL     | 20           | 20         | 20          | 20          | 20         | 20       | 20        | 20       |
| Ad.C6+D3| 20           | 20         | 20          | 20          | 20         | 20       | 20        | 20       |

**C.**

**D.**

ns

ns

ns

ns

Insulin/Ki67

**E.**

% Beta-Gal +

|         | Human | Rat |
|---------|-------|-----|
| ns      |       |     |

**F.**

Insulin/cdk6  Insulin/D3

**G.**

% Nuclear

|         | Human  cdk6 | Human  D3 | Rat  cdk6 | Rat  D3 |
|---------|-------------|-----------|-----------|---------|
| *       |             |           |           |         |
Figure 2

Ctl
Ad.C6+D3
Ctl
Ad.C6+D3
Ctl
Ad.C6+D3
Ctl
Ad.C6+D3

Human
Rat
INS1
Human
Rat
INS1
Human
Rat
INS1
Human
Rat
INS1

Cdk1/Ins/Dapi
CycA/Ins/Dapi
Cdk2/Ins/Dapi
CycE/Ins/Dapi

Human
Rat
INS1
Human
Rat
INS1
Human
Rat
INS1
Human
Rat
INS1

Diabetes
Figure 4

A. CTL, Ad.C6+D3, Ad.A, Ad.E, Ad.K1, Ad.K2, Ad.K1+A, Ad.K2+A, Ad.K1+E, Ad.K2+E

B. Bar graph showing % BrdU+ Ins+

- CTL
- Ad.C6+D3
- Ad.A
- Ad.E
- Ad.K1
- Ad.K2
- Ad.K1+A
- Ad.K2+A
- Ad.K1+E
- Ad.K2+E

n=4-5

Values and significance markers are indicated on the graph.
Figure 5

A. Ad.LZ, Ad.K1+E, Ad.K6+D3, Ad.C6+D3+K1+E

B. % BrdU+ Ins+

C. Ad.LZ, Ad.C6+D3+K1+E (1000)

D. % Ki67+ Ins+

E. Ad.LZ, Ad.C6+D3+K1+E

F. % H2AX+ Ins+

G. Ad. LZ, Ad. K6+D3+K1+E, Positive control + DNAse

H. % TUNEL + Ins+

Diabetes
Figure 6

A. Ad.LZ  Ad.K1+E
Ad.K6+D3  Ad.C6+D3+K1+E

B. 25

\[ \text{% BrdU+ Ins+} \]

|        | LZ    | C6+D3 | K1+E  | C6+D3 +K1+E |
|--------|-------|-------|-------|-------------|
| 100 MOI| 0.0   | 0.0   | *     | *           |

C. Ad.LZ  Ad.C6+D3+K1+E (400)

D. 50

\[ \text{% Ki67+ Ins+} \]

|        | LZ    | C6+D3 +K1+E |
|--------|-------|-------------|
| 100 MOI| 0.0   | *           |

E. Ad.LZ  Ad.C6+D3+K1+E

F. 35

\[ \text{% H2AX + Ins+} \]

|        | LZ    | C6+D3 +K1+E |
|--------|-------|-------------|
| 100 MOI| 0.0   | *           |

G. Ad. LZ  Ad. K6+D3+K1+E (400)  Positive control + DNAse

H. 100

\[ \text{% TUNEL + Ins+} \]

|        | LZ    | C6+D3 +K1+E  | Pos Ctl |
|--------|-------|---------------|---------|
| 100 MOI| ns    |               |         |

Diabetes
Figure 7
A. 

B. 

C. 

D. 

E. 

Figure 8
## Supp. Table 1: Primary antisera employed.

| Protein/ Antibody | Immunohistochemistry/PLA | Immunoblot |
|-------------------|--------------------------|------------|
|                   | Company                   | Cat#  | dilution | Company | Cat#  | dilution |
| Ki67              | Neomarker                 | RM-9106-S | 1:100    | ---     | ---   | ---     |
| BrdU              | Abcam                     | Ab6326   | 1:100    | ---     | ---   | ---     |
| pRb               | Cell Signaling            | 9309P    | 1:100    | Pharmingen | 554136 | 1:1000 |
| Rb-P-S780         | Cell Signaling            | 9307S    | 1:100    | Cell Signaling | 9307S | 1:500 |
| Rb-P-S795         | Cell Signaling            | 9301P    | 1:100    | Cell Signaling | 9301P | 1:500 |
| Rb-P-T373         | Abcam                     | Ab52975  | 1:100    | Abcam   | Ab52975 | 1:500 |
| Rb-P-811          | Cell Signaling            | 9308S    | 1:100    | Cell Signaling | 9308S | 1:500 |
| Cyclin D3         | Abcam                     | DCS22    | 1:100    | ---     | ---   | ---     |
| Cdk6              | Abcam                     | Ab-3126  | 1:100    | ---     | ---   | ---     |
| Cyclin A2         | Santa Cruz                | sc-177   | 1:100    | ---     | ---   | ---     |
| Cyclin E1         | Santa Cruz                | sc-247   | 1:50     | Sigma   | c-4710 | 1:1000 |
| Cdk1              | Santa Cruz                | sc-747   | 1:50     | Cell Signaling | 9112 | 1:1000 |
| Cdk2              | Santa Cruz                | sc-163   | 1:50     | Santa Cruz | Sc-163 | 1:1000 |
| P-H2AX            | Millipore                 | 05-636   | 1:100    | ---     | ---   | ---     |
| E-Cadherin        | Cell Signaling            | 3195S    | 1:100    | ---     | ---   | ---     |
| Insulin           | DAKO                      | A0564    | 1:1000   | ---     | ---   | ---     |
| Tubulin           | ---                       | ---      | ---      | calbiochem | Cp-06 | 1:2000 |
Supplemental Figure Legends

Supplemental Figure 1: Subcellular localization of p16 and p27 in INS1 cells and in rat and human β-cells in response to expression of cdk6 and cyclin D3. A. Examples of microphotographs of dispersed human and rat islets were transduced with control adenovirus (“control”, white frames) or with Ad.cdk6+cyclinD3 (“Ad.C6+D3”, blue frames) and immunolabeled for p16\textsuperscript{Ink4A} or p27\textsuperscript{Kip1} (shown in red), and insulin (shown in green). Nuclei are stained with DAPI and are shown in blue. B. Quantitation of nuclear p16\textsuperscript{Ink4A} or p27\textsuperscript{Kip1} in insulin positive cells under basal conditions (ctl) and when transduced with Ad. Cdk6+cyclin D3 (Ad.cdk6+D3). Bars indicate mean ± SEM. Each experiment has been repeated three times.

Supplemental Figure 2: Densitometric quantification of immunoblots for cdk1, cdk2, cyclin A and cyclin E of human islets transduced with Ad.cdk1, Ad.cdk2, Ad.cyclin A or Ad.cyclin E. Bars indicate mean ± SEM. Each experiment has been repeated three to five times.

Supplemental Figure 3: Glucose-Stimulated Insulin Secretion (GSIS). Human islets were uninfected or transduced with control adenovirus (LacZ) or with Ad.cdk6+cyclin D3 (“K6+D3”) or Ad.cdk1+cyclin E (“K1+E”) or a combination of all four cyclins and cdks (“K6+D3+K1+E”). A total of 400 MOIs were used in these experiments. The GSIS experiments were performed three days after transduction. Bars indicate mean ± SEM. Each experiment has been repeated five times.

Supplemental Figure 4: Phosphorylation of the Retinoblastoma protein on Ser\textsuperscript{780}, Ser\textsuperscript{795} and Ser\textsuperscript{811} in rat and human beta cells in response to “early” cyclins and cdks. Examples of photomicrographs of dispersed rat or human islets transduced with Ad.LacZ (CTL), or with Ad.cdk6+cyclin D3 (K6+D3), are shown. Phosphorylated pRb is labeled in red and insulin is immunolabelled in green. Red arrows indicate a positive signal for phosphorylated pRb.

Supplemental Figure 5: Correlation between the percentage of cell cycle entry and age or BMI. Human islets were uninfected or transduced with Ad.cdk6+cyclin D3
(‘K6+D3’), were fixed three days after transduction and immunolabelled for Ki67. Bars indicate mean ± SEM.
Supplemental Figure 1

A. Human

- Ctl

- Ad.C6+D3

Rat

- Ctl

- Ad.C6+D3

INS1

p16/Ins/Dapi

B. Human

- Ctl

- Ad.cdk6 +D3

Rat

- Ctl

- Ad.C6+D3

INS1

p27/Ins/Dapi

% Nuc p16 in ins+

% Nuc p27 in ins+

Supplemental Figure 1
Supplemental Figure 3
Supplemental Figure 4

**Human Diabetes**
- CTL
- K6+D3

**Rat**
- CTL
- K6+D3

**Diabetes**

- Rb-P-S 780-795/Ins/Dapi
- Rb-P-S 811/Ins/Dapi

*Diabetes*
\[ y = -0.3715x + 28.568 \quad R^2 = 0.20087 \]

\[ y = 0.1517x + 9.3611 \quad R^2 = 0.00929 \]

Supplemental Figure 5