Targeting the cell cycle inhibitor p57^{Kip2} promotes adult human β cell replication

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Children with focal hyperinsulinism of infancy display a dramatic, non-neoplastic clonal expansion of β cells that have undergone mitotic recombination, resulting in paternal disomy of part of chromosome 11. This disomic region contains imprinted genes, including the gene encoding the cell cycle inhibitor p57^{Kip2} (*CDKN1C*), which is silenced as a consequence of the recombination event. We hypothesized that targeting p57^{Kip2} could stimulate adult human β cell replication. Indeed, when we suppressed *CDKN1C* expression in human islets obtained from deceased adult organ donors and transplanted them into hyperglycemic, immunodeficient mice, β cell replication increased more than 3-fold. The newly replicated cells retained properties of mature β cells, including the expression of β cell markers such as insulin, PDX1, and NKX6.1. Importantly, these newly replicated cells demonstrated normal glucose-induced calcium influx, further indicating β cell functionality. These findings provide a molecular explanation for the massive β cell replication that occurs in children with focal hyperinsulinism. These data also provided evidence that β cells from older humans, in which baseline replication is negligible, can be coaxed to re-enter and complete the cell cycle while maintaining mature β cell properties. Thus, controlled manipulation of this pathway holds promise for the expansion of β cells in patients with type 2 diabetes.

Introduction

Hyperinsulinism of infancy is a clinical syndrome of pancreatic β cell dysfunction characterized by a failure to suppress insulin secretion in the presence of hypoglycemia (1). In most patients, the disease is caused by recessive mutations of the sulfonylurea receptor 1 (*SUR1*) gene *ABCC8* or the potassium channel (*KIR6.2*) gene *KCNJ11* (2, 3), encoding the two subunits of the β cell ATP-sensitive K⁺ (K_{ATP}) channel, which controls insulin secretion. Histologically, hyperinsulinism presents as two major subtypes: diffuse and focal (4). The diffuse form involves all β cells (5), while in focal hyperinsulinism, adenomatous hyperplasia occurs within a limited region of the pancreas. This mass of β cells originates from clonal expansion of a single cell, in which a recessive mutation of either the *ABCC8* or *KCNJ11* gene is inherited on the paternal allele (Figure 1A). On that background, a somatic recombination of the p terminus of chromosome 11 occurs during fetal development, resulting in duplication of the paternal allele concomitant with loss of the maternal allele, leading to homozygosity for the mutated *ABCC8/KCNJ11* locus and uniparental disomy for all genes telomeric to *ABCC8/KCNJ11* (6, 7). The duplicated segment contains several maternally expressed imprinted genes including *CDKN1C*, which encodes the cyclin-dependent kinase inhibitor p57^{Kip2} (8). Therefore, in β cells descendant from this mutant precursor, p57^{Kip2} expression is extinguished (9).

p57^{Kip2} causes cell cycle arrest in terminally differentiated cells through inhibition of several G1 cyclin/CDK complexes, and its loss is related to multiple malignancies (10). Furthermore, loss of p57^{Kip2} in focal hyperinsulinism lesions correlates with increased proliferation (11). Therefore, we hypothesized that p57^{Kip2} has a

major role in preventing β cell regeneration and that manipulation of its expression may enhance proliferation of adult human β cells.

Results and Discussion

Since p57^{Kip2} is expressed in β cells of humans but not in those of rodents, we used islets from deceased human organ donors for our study. To modulate p57^{Kip2} expression, we used shRNA-mediated gene suppression delivered by lentiviral particles, which can efficiently transduce nondividing cells and express the shRNA construct (12). First, we tested multiple shRNAs to specifically abolish *CDKN1C* mRNA expression in HEK293 cells (Figure 1B) and used the most efficient construct (p57c) to produce lentiviral particles. Transduction of human islets with p57^{Kip2} shRNA lentivirus caused over a 70% reduction in *CDKN1C* mRNA levels (Figure 1C) in infected cells, while it did not affect the mRNA levels of other cell cycle inhibitors such as p16, p21, and p27 (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI69519DS1). Cultured human islets transduced with lentiviral particles and cultured for 72 hours showed strong expression of turbo-GFP in about 25% of the cells (Figure 1D). According to flow cytometric analysis (Supplemental Figure 1B), an additional 20% of islet cells expressed lower levels of turbo-GFP.

Attempts at stimulating human β cell replication in cultured, lentivirally transduced human islets were unsuccessful (data not shown). Therefore, we chose to transplant transduced human islets under the kidney capsule of immunodeficient mice, which allows for islet revascularization and exposure to host factors. Immunodeficient mice were rendered diabetic using streptozotocin (STZ) to provide an additional mitogenic stimulus for the transplanted β cells (13). During the entire transplantation period (~20 days), replicating cells were labeled by the thymidine analog BrdU, which was supplied in the drinking water. Immunostaining of the grafts

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Figure 1

Suppression of p57^{Kip2} in human islets. **(A)** Molecular pathology of focal hyperinsulinism. Left: Maternal and paternal copies of chromosome 11 inherited by the fetus. The paternal allele carries a recessive mutation in either *ABCC8* or *KCNJ11*, the two subunits of the ATP-sensitive potassium channel. During fetal life, a break in the maternal chromosome followed by DNA repair using the paternal chromosome as a template occurs in a single β cell. Uniparental disomy of the short arm of chromosome 11 results, as shown in the right panel, and expression of the imprinted p57^{Kip2} (*CDKN1C*) gene is silenced. **(B)** Suppression of p57^{Kip2} using shRNA lentiviral particles. Quantitative RT-PCR (qPCR) showing mRNA levels of p57^{Kip2} in HEK293 cells transfected with p57^{Kip2}-specific pGIPZ shRNA constructs. Transfected cells were FACS sorted for GFP-positive and GFP-negative fractions. Expression levels were normalized to an NT shRNA control ($n = 3$). **(C)** qRT-PCR of *CDKN1C* mRNA levels in cultured human islets transduced with lentiviral particles containing pGIPZ shRNA (clone p57-c) against p57^{Kip2}. Five days after transduction, islet were dispersed and FACS sorted for GFP-positive (transduced) and GFP-negative fractions. Expression levels were normalized to NT shRNA control ($n = 3$ human islet donors). **(D)** Immunostaining of human islets transduced with pGIPZ shRNA lentivirus against p57^{Kip2}. Turbo-GFP (green), insulin (white), and DNA (blue). Arrows point to costaining of insulin and turbo-GFP. **(E)** Immunostaining of recovered human islet grafts for turbo-GFP, which allows for tracking of shRNA expression. Turbo-GFP (green), PDX1 (white), and DNA (blue). Arrows point to turbo-GFP/PDX1-positive cells. Scale bars: 20 μ m.

for turbo-GFP showed an overall increase in the percentage of β cells expressing GFP compared with that observed in cultured islets, likely due to spread of the virus and continued protein accumulation in the graft (Figure 1E). The majority of the grafts showed normalized blood glucose levels over time in both experimental and control grafts (Figure 2A). Importantly, the number of BrdU-positive β cells increased by more than 3-fold in p57^{Kip2}-suppressed β cells when compared with nontargeting (NT) shRNA-treated control islets ($2.71\% \pm 0.44\%$ and $0.84\% \pm 0.13\%$ respectively, $P < 0.01$; Figure 2B) from all 4 donors. The effect of p57^{Kip2} suppression was specific to β cells, since BrdU-positive α and δ cells were extremely rare in recovered grafts (Supplemental Figure 2). The stimulatory effect of p57^{Kip2} suppression is especially remarkable considering that these studies were performed on islets from

deceased organ donors over the age of 50 years, an age when significant β cell proliferation was thought to be impossible (14).

Measurement of both mouse and human C-peptide levels in transplanted mice revealed no significant difference between experimental and control groups (Figure 2C), demonstrating that sustained expression of the p57^{Kip2} shRNA construct was not detrimental to islet function. As described below, the lack of a significant increase in human C-peptide in mice transplanted with p57^{Kip2}-suppressed islets is expected, since the total number of cells that underwent replication was not sufficient to substantially impact the overall mass of the entire graft.

p57^{Kip2} expression was suppressed in approximately one-third of all β cells, as assessed by immunostaining, and newly replicated β cells exhibited very low p57^{Kip2} protein levels (Figure 2D). Simi-

Figure 2

Increased β cell replication in p57^{Kip2}-suppressed human islets. **(A)** Blood glucose levels of STZ-treated immunodeficient mice transplanted with p57^{Kip2}-suppressed (blue line) and control human islets (red line) were not statistically different ($n = 9$ mice per group). **(B)** BrdU-positive β cells in shRNA lentiviral-transduced, engrafted human islets. Average percentage of BrdU-positive β cells in p57^{Kip2}-suppressed islets (blue bars, 2.71%) was significantly higher ($*P < 0.01$) than in NT shRNA-transduced islets (red bars, 0.84%). **(C)** Human and mouse C-peptide levels of mice transplanted with p57^{Kip2}-suppressed and control human islets (blue and red bars, respectively) just before graft recovery ($n = 6$ per group). **(D)** Proliferating β cells in p57^{Kip2}-suppressed human islets show negligible p57^{Kip2} levels. Costaining for p57^{Kip2} (white), insulin (green), BrdU (red), and DNA (blue). Arrows point to BrdU-positive/p57^{Kip2}-negative β cells. **(E)** No activation of the DNA damage response by p57^{Kip2} suppression was observed. Costaining for γ H2AX (white), insulin (green), BrdU (red), and DNA (blue). Arrows point to BrdU-positive/ γ H2AX-negative β cells. **(F)** Forced cell cycle entry by overexpression of HNF4 α induces the DNA damage response. Costaining for γ H2AX (white), BrdU (red), and DNA (blue). Arrows point to BrdU/ γ H2AX double-positive islet cells. **(G)** 25% $\pm$ 9.5% (SD) of newly replicated β cells are doublets. Costaining for PDX1 (green), BrdU (red), and nuclei (blue). Arrows point to BrdU/PDX1 double-positive β cells in close proximity to one another. **(H)** Some newly replicated β cells stain for the cell cycle marker Ki67. Costaining for Ki67 (white), insulin (green), BrdU (red), and DNA (blue). Arrow points to a BrdU/insulin/Ki67-positive β cell. Scale bars: 20 μ m.

Figure 3

Newly replicated β cells retain their functionality as assessed by glucose-stimulated calcium influx assay. **(A)** Dispersed human islet cells retrieved from grafts and loaded with the $[Ca^{2+}]_i$ sensor Fura-2 were analyzed for $[Ca^{2+}]_i$, followed by costaining for insulin (white), turbo-GFP (green), BrdU (red), and DNA (blue). Blue box surrounds a BrdU-negative β cell and red box surrounds a BrdU-positive β cell. **(B)** Calcium traces corresponding to the two β cells identified in **A**, showing the change in $[Ca^{2+}]_i$ as a response to an increase in extracellular glucose levels. Each graph represents the $[Ca^{2+}]_i$ profile of one cell during the course of the experiment. Original magnification, $\times 400$.

larly, low p57^{Kip2} protein levels were present in BrdU-positive β cells in control grafts transduced with the NT shRNA virus (Supplemental Figure 2C), supporting the notion that p57^{Kip2} limits human β cell replication. The background of BrdU incorporation observed in control grafts is likely driven by the mitogenic stimulus induced by the high blood glucose levels in the STZ-treated diabetic mice (13). Transplantation of lentivirally transduced islets into normoglycemic immunodeficient mice did not augment β cell replication, indicating that hyperglycemia is an important costimulant for the replication of adult human β cells in this system (Supplemental Figure 1, C–E). Since β cells in focal lesions frequently undergo apoptosis (7, 11), we performed *TUNEL* staining for apoptotic cells on recovered islets and found no *TUNEL*-positive β cells in the p57^{Kip2}-suppressed graft (Supplemental Figure 2D).

DNA incorporation of BrdU does not necessarily indicate successful replication, and forced entry of β cells into the cell cycle, either by overexpression of the transcription factor HNF4 α or the cell cycle promoters cyclin D3 and CDK6, frequently leads to repeated firing of the same origins of replication, resulting in collision of replication forks, induction of the DNA damage response, and cell cycle arrest (15). To evaluate whether p57^{Kip2} suppression resulted in DNA damage, we stained p57^{Kip2}-suppressed islets for phosphorylated histone H2AX (γ H2AX), a well-established DNA damage marker (16). As shown in Figure 2E, none of the replicating cells were γ H2AX-positive, indicating an absence of the DNA damage response, whereas human islets overexpressing HNF4 α were positive for γ H2AX (Figure 2F).

In order to demonstrate successful replication, we evaluated “doublets,” i.e., the presence of BrdU-positive daughter cells in

close proximity to one another. Since the division of cells is a 3D process, 2D sectioning is expected to capture about one-third of all doublets. Therefore, if all BrdU-positive cells underwent a complete cell cycle, we would expect approximately 33% of cells in doublets. Indeed, we found that $25\% \pm 9.5\%$ of newly replicated β cells were present as doublets (Figure 2G). In addition, a fraction of the BrdU-positive β cells stained for Ki67, a proliferation marker that is expressed throughout the cell cycle, labeling cells that were undergoing replication within 12 hours preceding recovery of the graft (Figure 2H). As predicted, most β cells were BrdU positive, but Ki67 negative, since they had replicated several days before graft removal.

Cellular proliferation is frequently associated with transient de-differentiation (17). If stimulation of human β cell replication is to be pursued as a therapeutic approach to compensate for functional β cell loss in diabetes, then newly replicated β cells must quickly reestablish the properties of mature β cells. Because the number of newly replicated β cells is small compared with that of the preexisting cells (about 2.7% on average), direct assessment of increased glucose-stimulated insulin production from whole transduced islets in “bulk assays” is not feasible, as these measurements largely reflect the properties of preexisting cells. Therefore, to assess whether β cells that had replicated in response to p57^{Kip2} suppression had regained their differentiation status, we performed dual immunofluorescence labeling for BrdU and markers of mature β cells. We observed that BrdU-positive β cells in p57-suppressed human islets expressed NKX6.1 and PDX1, thus maintaining or regaining the molecular signature of their mature counterparts (Supplemental Figure 3).

To test whether β cells regained functionality after replication, we performed single-cell calcium imaging using Fura-2. Increased intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) is the penultimate step in glucose-stimulated insulin secretion, triggering the fusion of docked insulin granules with the plasma membrane (18). Therefore, the glucose thresholds for $[\text{Ca}^{2+}]_i$ and insulin secretion are identical. We transplanted p57^{Kip2}-suppressed human islets under the kidney capsule of immunodeficient mice and monitored proliferation with BrdU for 20 days to capture any replication event. Recovered grafts were dispersed, and single islet cells were cultured, loaded with Fura-2, and analyzed for glucose-stimulated $[\text{Ca}^{2+}]_i$. After completion of the calcium trace, the same cells were analyzed for BrdU incorporation by immunostaining. When comparing the calcium influx profile of newly replicated BrdU-positive β cells with their neighboring, nondividing β cells, we observed the typical response to glucose stimulation in both (Figure 3). We believe that this new methodology allows for the first time to directly confirm that function was regained in newly replicated human β cells.

In summary, this study provides a mechanistic, molecular explanation for increased β cell replication in children with focal hyperinsulinism (11). Our data further demonstrate that suppression of p57^{Kip2} in adult human pancreatic β cells, in concert with high levels of circulating glucose, allows successful β cell replication. Recently, we compared the expression profile of human pancreatic α , β , and acinar cells using high-throughput RNA sequencing analysis and showed that the p57^{Kip2} transcript is at least 30 times more abundant than that of any other cell cycle inhibitor in the adult human β cell (19). However, our data do not exclude the possibility that other genes in the imprinted region on chromosome 11 contribute to the clonal β cell expansion observed in focal hyperinsulinism and thus could be targeted to achieve a further enhancement of replication in adult β cells. Importantly, by reproducing the p57^{Kip2}-deficient state in β cells of aged human islets, we show that quiescent β cells can be coaxed to re-enter and successfully complete the cell cycle while maintaining essential properties of mature β cells. In contrast to prior reports of β cell replication using forced expression of cell cycle regulators (20), we found that p57^{Kip2} suppression did not cause appreciable DNA damage, and newly replicated β cells were fully glucose responsive. Thus, p57^{Kip2} is a promising target for diabetes therapy via β cell mass expansion.

Methods

Lentiviral transduction and xenotransplantation of human cadaveric islets. Cadaveric human islets were transduced with a lentivirus encoding an shRNA against p57^{Kip2} and transplanted under the kidney capsule of STZ diabetic, immunodeficient mice.

Statistics. Student's *t* tests with paired observation and single-tail distribution were used to determine the significance of difference between the levels of β cell proliferation in the p57^{Kip2} shRNA- and NT shRNA-transduced islets. A *P* value of less than 0.01 was considered significant. All data are presented as the means $\pm$ SD of the mean.

Study approval. The experiments using islets obtained from cadaveric organ donors were declared exempt by the IRB of the University of Pennsylvania. The animal studies were approved by the IACUC of the University of Pennsylvania.

Full Methods and any associated references are available in the Supplemental Methods.

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