HES1 IS INVOLVED IN ADAPTATION OF ADULT HUMAN BETA CELLS TO PROLIFERATION IN VITRO

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Abbreviations: HES, Hairy and Enhancer of Split; NICD, NOTCH intracellular domain; shRNA, small hairpin RNA

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Objective: In vitro expansion of beta cells from adult human islets could solve the tissue shortage for cell-replacement therapy of diabetes. Culture of human islet cells typically results in <16 cell doublings and loss of insulin expression. Using cell-lineage tracing we demonstrated that the expanded cell population included cells derived from beta cells. Understanding the molecular mechanisms involved in beta-cell fate in vitro is crucial for optimizing expansion and redifferentiation of these cells. In the developing pancreas important cell-fate decisions are regulated by NOTCH receptors, which signal through the Hairy and Enhancer of Split (HES) 1 transcriptional regulator. Here we investigated the role of the NOTCH signaling pathway in beta-cell dedifferentiation and proliferation in vitro.

Research design and methods: Isolated human islets were dissociated into single cells. Beta cells were genetically labeled using a Cre-loxP system delivered by lentiviruses. Cells were analyzed for changes in expression of components of the NOTCH pathway during the initial weeks in culture. HES1 expression was inhibited by a small hairpin RNA, and the effects on beta-cell phenotype were analyzed.

Results: Human beta-cell dedifferentiation and entrance into the cell cycle in vitro correlated with activation of the NOTCH pathway and downregulation of the cell cycle inhibitor p57. Inhibition of HES1 expression using small hairpin RNA resulted in significantly reduced beta-cell replication and dedifferentiation.

Conclusions: These findings demonstrate that the NOTCH pathway is involved in determining beta-cell fate in vitro and suggest possible molecular targets for induction of beta-cell redifferentiation following in vitro expansion.
Beta-cell replacement by transplantation is a promising approach for treatment of type 1 diabetes, however its application on a large scale is limited by availability of pancreas donors. In a normal adult pancreas a slow rate of beta-cell renewal is responsible for maintenance of an adequate functional beta-cell mass. This rate is accelerated in conditions of increased demands for insulin, such as pregnancy (1) and obesity (2). Work in an animal model demonstrated that new beta cells are generated in adult mice predominantly by replication of pre-existing beta cells, rather than by neogenesis from insulin-negative stem/progenitor cells (3). This finding has raised hopes for recapitulation of beta-cell expansion in cultures of adult human islets. However, previous attempts at in vitro expansion of adult human beta cells resulted in a limited number of cell population doublings and loss of insulin expression (4-7). Insulin-negative cells with a considerable proliferative capacity were derived from cultured human islets (8-10). Insulin expression in these cells could be induced by changing the culture conditions, however its levels were low and varied among donors (8-10). One possible interpretation of these results is that beta cells survive, dedifferentiate, and divide in culture. Recently, we applied a genetic cell-lineage tracing approach for labeling cultured adult human islets and demonstrated that, in contrast to mouse beta cells (11-14), dedifferentiated, label-positive cells derived from human beta cells could be induced to significantly proliferate in vitro (15). These cells may be of value for development of cell therapy for type 1 diabetes, since they may retain some beta-cell-specific chromatin structure to allow their redifferentiation. Understanding the molecular mechanisms involved in beta-cell dedifferentiation and replication in vitro may facilitate the expansion and redifferentiation of these cells.

In the developing pancreas important cell-fate decisions, including the switch from proliferation to differentiation, and the choice between exocrine and endocrine fates (16), as well as among different endocrine fates (17-19), are regulated by the NOTCH signaling pathway. Expression of NOTCH ligands on a differentiating cell inhibits development of the same phenotype in neighboring cells, in a mechanism termed lateral inhibition (16). Ligand binding to NOTCH receptors on a neighboring cell results in cleavage of the NOTCH intracellular domain (NICD), which enters the nucleus and forms a complex that modulates gene expression (20). The Hairy and Enhancer of Split (HES) family of transcriptional regulators is a major target of the NICD complex. In fetal pancreas HES1 acts as an inhibitor of neurogenin 3 (NGN3) gene expression, which is required for islet development (21). In addition, HES1 regulates the cell cycle by inhibiting expression of genes encoding the cyclin kinase inhibitors p27 and p57 (22,23). Some evidence suggests it may also inhibit insulin gene expression (17). Overall, HES1 is associated with promoting cell replication and preventing cell differentiation. Forced expression of NOTCH inhibits pancreas cell differentiation (17,18), while mice with null mutations in genes encoding NOTCH pathway components exhibit accelerated differentiation of endocrine pancreas (16,21). The NOTCH pathway is not normally expressed in the adult pancreas, however, it is activated in conditions associated with cell dedifferentiation and replication, such as regeneration following experimental pancreatitis (24), pancreatic neoplasia (25), metaplasia of cultured pancreatic exocrine cells (26), and in rat beta cells exposed to cytokines (27).

We hypothesized that beta-cell dedifferentiation and entrance into the cell
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cycle in vitro involve induction of the NOTCH pathway. Our findings demonstrate a considerable activation of the NOTCH pathway in these cells, which correlates with downregulation of the cell cycle inhibitor p57 and loss of insulin expression. Inhibition of HES1 expression using small hairpin RNA (shRNA) results in reduced replication of cultured beta cells and a decrease in cell dedifferentiation. These findings suggest possible molecular targets for prevention of beta-cell dedifferentiation in culture, or induction of cell redifferentiation following in vitro expansion.

RESEARCH DESIGN AND METHODS

Islets cell culture. Islets were received 2-3 days following isolation. Islets from individual donors were dissociated into single cells and cultured in CMRL 1066 medium containing 5.6 mM glucose and supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamycin, and 5 μg/ml amphotericin B as described (8). The cultures were refed twice a week and split 1:2 once a week.

HES1 inhibition and lineage tracing. HES1 shRNAs (accession numbers TRCN 18989, 18990, 18991, and 18993) and a non-target shRNA, cloned in pLKO.1 lentiviral vector, were obtained from the RNAi Consortium (Sigma-Aldrich). Virus was produced in 293T cells following cotransfection with the pCMVdR8.91 and pMD2.G packaging plasmids. The culture medium was harvested 48 h later. Islet cells cultured for 1-2 days were washed with PBS and infected at MOI 2.5:1 in CMRL 1066 medium containing 8 μg/ml polybrene overnight. The medium was then replaced with regular culture medium. Four days after infection, the cells were selected for puromycin resistance (1 μg/ml) for 3 days. Two weeks after infection the cells were harvested for further analysis. For γ-secretase inhibition, 5 μM of L-685,458 (Calbiochem) were added to cells at P2 for 17 h. Lineage tracing was performed using the RIP-Cre and a pTrip CMV-loxP-DsRed2-loxP-eGFP viruses as described (15). Briefly, islet cells cultured for 1-2 days were infected with a 1:1:1 mixture of the 3 viruses (shRNA + RIP-Cre + pTrip CMV-loxP-DsRed2-loxP-eGFP) at a total MOI 4:1. Selection and further analysis were carried out as above.

RNA analyses. Total RNA was extracted using High Pure RNA isolation kit (Roche). cDNA was synthesized using SuperScript III (Invitrogen). qPCR was performed using a Prism 7300 ABI Real Time PCR System (Applied Biosystems). The Assay-On-Demand (Applied Biosystems) TaqMan fluorogenic probes that were used in this study are listed in Table 1. Relative quantitative analysis was performed according to the comparative CT method by using the arithmetic formula $2^{-\Delta\Delta C_{T}}$. The cDNA levels were normalized to human ribosomal protein P0 (RPLP0) cDNA.

Immunofluorescence. Cells were plated in 24-well plates on sterilized coverslips and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.25% NP40 for 10 min and blocked for 10 min at room temperature in 1% bovine serum albumin, 10% FBS, and 0.2% saponin. Cells were then incubated with the following primary antibodies diluted in blocking solution, overnight at 4 °C: mouse-anti-insulin (Sigma-Aldrich,1:1000); Rabbit-anti-p57 (Santa Cruz,1:500); rabbit-anti-HES1 (Chemicon, 1:1000); mouse-anti-Ki67 (Zymed, 1:200); rabbit-anti-Ki67 (Zymed, 1:50); mouse-anti-BrdU (1:20); rabbit-anti-NICD (Cell Signalling,1:10); mouse-anti-GFP (Chemicon, 1:500); and rabbit-anti-GFP (Invitrogen, 1:1000). The bound antibody was visualized with a fluorescent secondary antibody: anti-mouse- or anti-rabbit-AMCA (Jackson, 1:200); -Cy3 (Biomeda, 1:200); and -Alexa Fluor 488 (Molecular Probes, 1:200), under a Zeiss confocal microscope. The specificity of the primary antibodies was demonstrated using FDC human colon
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fibroblast cells (data not shown). The specificity of the rabbit-anti-HES1 antibody from Chemicon was determined using LAN-5 human neuroblastoma cells as a negative control, and human bone marrow mesenchymal stem cells (BM-MSC) as a positive control. The lack of HES1 expression in LAN-5 cells, and its presence in BM-MSC, were demonstrated using immunoblotting with a different HES1 antibody (see below). Lack of \( HES1 \) mRNA in LAN-5 cells was previously reported (28). Nuclei were visualized by staining with DAPI (Roche) for 5 min at room temperature. BrdU staining was performed following a 24-h labeling period as previously described (29).

**Immunoblotting.** Total cellular protein was extracted in 0.5% NP40 containing a protease inhibitor cocktail (Roche). Protein concentration was determined by the BCA method (Pierce, Rockford, IL). 70 µg protein were separated on 12% sodium dodecyl sulphate-polyacrylamide gels and electroblotted onto PDF membranes. The membranes were incubated with rabbit-anti-HES1 (a gift from T. Sudo, 1:1000), rat-anti-NOTCH1 (DSHB, 1:100), or rabbit-anti-PARP (Cell Signaling, 1:1000). Loading was monitored using goat-anti-beta-actin or mouse-anti-heat shock cognate protein 70 (HSC70) (Santa Cruz, 1:1000). The bound antibody was visualized with the appropriate horseradish peroxidase-conjugated anti-IgG (Jackson) and SuperSignal West Pico Chemiluminescent Substrate (Pierce). Signal intensity was quantitated using TINA software. Cells treated with 1 µM staurosporine for 6 h were used as positive control for the PARP blot.

**Apoptosis assay.** Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using a Chemicon ApopTag Fluorescein In Situ Apoptosis Detection Kit according to the manufacturer. Cells at P0 were stained 24 hours following culture initiation. Cells were costained for insulin as described above.

**Insulin content and secretion.** Glucose-stimulated insulin secretion and insulin content were assayed as described (8).

**Statistical Analysis.** Significance was determined using Student's t test. To approach a normal distribution of the qPCR data, a logarithmic transformation was performed. To account for multiple testing, the Bonferroni correction was applied.

**RESULTS**

**Upregulation of HES1 in cultured human \( \beta \) cells.** Human islets were isolated from 9 donors, 6 males and 3 females, aged 38-60 (mean age 50±8), with a purity ranging between 65-85% (mean 78±6%). Islets from each donor were dissociated and expanded in culture as described (8). Quantitative RT-PCR (qPCR) analyses of RNA extracted from these cells during the first 2 weeks of culture revealed a rapid dedifferentiation, as previously reported (8), which was manifested in a drastic decrease in insulin mRNA levels, averaging 166-fold (p=8.7X10\(^{-7}\) at P2) (Fig. 1A; see data on cells from individual donors in Supplementary Fig. 1). Concomitant with this decrease, an increase in \( HES1 \) mRNA was observed in cells from all donors, averaging 4.6-fold (p=0.006 at P2) within the first 2 week of culture (Fig. 1A). A similar increase was noted in HES1 protein levels (Fig. 1B). At both RNA and protein levels the wave of HES1 upregulation peaked within the first 2 weeks of culture and was downregulated thereafter. To demonstrate that HES1 upregulation in this system was dependent on NOTCH activation, we used an inhibitor of \( \gamma \)-secretase, the protease complex required for generation of NICD (30). As seen in Fig. 1C, cell incubation with the inhibitor resulted in 10-fold lower HES1 levels, supporting a NOTCH-dependent mechanism. Immunostaining could not detect significant HES1 expression in nuclei of cells with
intense insulin staining (Fig. 1D). In contrast, HES1 was clearly detected in insulin-negative cells. To monitor HES1 expression in dedifferentiated cells derived from beta cells, beta cells in freshly-isolated islets were heritably labeled using a cell-lineage tracing approach recently developed in our laboratory (15). The labeling approach is based on cell infection with a mixture of 2 lentivirus vectors, one expressing Cre recombinase under the insulin promoter (RIP-Cre), and the other a reporter cassette in which the CMV promoter is separated from an eGFP gene by a loxP-flanked stop region. Removal of the stop region in beta cells infected by both viruses activates eGFP expression specifically in these cells, thereby allowing continuous tracking of beta-cell fate after insulin expression is lost. Residual insulin expression in beta cells during the initial days in culture provides a sufficient window of time for RIP-Cre expression and eGFP activation. Using this method 57.5±8.9% of insulin-positive cells were labeled with eGFP (15). Analysis of the cells expanded in culture following labeling revealed HES1 staining in cells that lost insulin expression but maintained eGFP expression, demonstrating that they were derived from beta cells (Fig. 1D). 89.3±0.1% of eGFP + insulin-negative cells were HES1 + (based on counting >200 cells in cultures from each of 3 donors).

Changes in expression of components of the NOTCH pathway in cultured human beta cells. qPCR analyses revealed changes in levels of transcripts encoding the 4 members of the NOTCH family. NOTCH1 transcripts were upregulated on average by 3.6-fold within the first 2 week of culture (p=0.02 at P2) (Fig. 2A). A similar increase was detected in the NOTCH1 120-kd transmembrane fragment (Fig. 2B), paralleling the changes in HES1 levels (Fig. 1B). NOTCH2 and NOTCH3 were significantly upregulated on average by 9.7-fold (p=8.8X10⁻⁵ P2) and 10.1-fold (p=1.0X10⁻⁵ at P2), respectively, within the first 2 week of culture. Overall, the activation of NOTCH1-3 paralleled that of HES1. In contrast, NOTCH4 was drastically downregulated on average 50-fold (p=3.0X10⁻⁵ at P2) from its level in primary islets. As with HES1 upregulation, NOTCH1-3 upregulation peaked within the first 2 weeks of culture and was downregulated thereafter. Transcripts encoding presenilin1, a component of the γ-secretase complex, and RBPJK, a protein that participates in the NICD nuclear complex, were not significantly changed in the cultured cells (data not shown). In contrast, transcripts for NOTCH ligands were downregulated during the initial weeks of culture (Fig. 2A). DELTA1 transcripts were downregulated on average 3.3-fold (p=1.4X10⁻⁴ at P2) within the first 2 week of culture. JAG1 transcripts were not significantly changed (data not shown). JAG2 transcripts were downregulated on average 5.5-fold (p=1.9X10⁻⁴ at P2) within the first 2 week of culture. The increased activity of the NOTCH pathway was manifested by appearance of NICD in cell nuclei, as revealed by immunostaining (Fig. 2C). Similar to the pattern of HES1 immunostaining, staining for NICD could not be detected in cells intensely stained for insulin. NICD staining was detected in all (100%) lineage-labeled insulin-negative cells identified as originating from beta cells by eGFP expression (Fig. 2C) (based on counting >200 cells in cultures from each of 3 donors).

Changes in expression of cell cycle inhibitors. To evaluate the consequences of increased HES1 expression in the cultured islet cells we analyzed changes in transcripts of genes encoding cyclin kinase inhibitors, which are among the main targets of repression by HES1 (22,23). Transcripts encoding p57, which is thought to be the main cell cycle inhibitor in human beta cells (22), were downregulated on average 2.9-fold (p=0.004 at P2) within the first 2.9-fold of culture (Fig. 3A). A further reduction was
observed at P3, averaging 7.1-fold, compared with P0 (p=0.002). This finding was supported by immunostaining for p57, which showed its presence in all (100%) lineage-labeled insulin-positive eGFP\(^{+}\) cells, and its absence in all (100%) insulin-negative eGFP\(^{-}\) cells (based on counting >200 cells in cultures from each of 3 donors) (Fig. 3B). In contrast to p57, transcripts for p21 were upregulated in cells from all donors, and those for p27 varied considerably among donors (data not shown). The downregulation of p57 transcripts and protein correlated with cell entrance into the cell cycle, as manifested by Ki67 staining in p57-negative, eGFP\(^{+}\) cells (Fig. 3C). The nuclear area of replicating cells was 2.5-4-fold larger than that of insulin-positive cells, as previously reported (8). The increase in cell size is likely associated with recruitment of quiescent beta cells into the cell cycle (31).

**Inhibition of HES1 expression prevents induction of beta-cell replication.** To further correlate the induction of beta-cell replication with HES1 upregulation, HES1 induction during the initial weeks of culture was inhibited using shRNA. Following screening of 4 HES1 shRNA sequences for activity in 293T cells, one of the four (TRCN18993) was selected as most efficient, based on reduction in HES1 protein levels, as analyzed by immunoblotting (data not shown). Isolated human islets were dissociated, and the cells were infected with a lentivirus encoding HES1 shRNA before culture under standard conditions. Selection for drug resistance allowed elimination of uninfected cells. Cells infected with a non-target shRNA lentivirus and selected under similar conditions served as control. As seen in Fig. 4A, cell infection with the HES1 shRNA virus resulted in up to 6X lower HES1 protein levels, compared with cells infected with the control virus. The lower HES1 levels were associated with a diminished cell proliferation, compared with cells infected with the control vector, as judged by staining for BrdU incorporation (Fig. 4B). In addition, staining for Ki67 in eGFP\(^{+}\) cells demonstrated a lower replication rate among cells derived from beta cells (Fig. 4B). The reduced replication in cells infected with the HES1 shRNA virus did not correlate with an increase in cell apoptosis, as judged by immunoblotting analysis for cleaved poly(ADP-ribose) polymerase (PARP) (Fig. 4C). In addition, terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay did not detect significant changes between cells at P2 treated with either virus and cells at P0, both among insulin-expressing cells and the total cell population (averaging 1.41%, 2.19%, and 1.64% apoptotic cells among insulin-positive cells at P0, P2 cells treated with HES1 shRNA, and P2 cells treated with non-target virus, respectively; based on counting 500 cells in cultures from each of 3 donors). The reduced proliferation of cells infected with the HES1 shRNA virus correlated with a 5.7-fold (p=0.001) higher level of p57 transcripts, compared with those in cells infected with the control virus (Fig. 4D; see data on cells from individual donors in Supplementary Fig. 2). The levels of p57 transcripts obtained with HES1 shRNA were 3.3-fold higher (p=0.04), compared with P0. The reduced HES1 levels did not significantly affect the levels of NOTCH transcripts, which is consistent with the position of HES1 downstream of NOTCH in the pathway (Fig. 4D).

**Inhibition of HES1 expression reduces beta-cell dedifferentiation.** The lower HES1 levels in cells expressing HES1 shRNA resulted in a reduced rate of cell dedifferentiation, as manifested by higher levels of transcripts encoding differentiated beta-cell markers. Thus, levels of insulin transcripts were 6.6-fold higher (p=5.5X10\(^{-4}\)), compared with cells infected with the control virus (Fig. 5A). Similarly, transcript levels for the beta-cell transcription factors PDX1 and NEUROD1 were 4.4-fold- (p=0.005) and 3.3-fold- (p=0.004) higher, respectively, in cells
expressing \textit{HES1} shRNA (Fig. 5A). The levels of \textit{PDX1} and \textit{NEUROD1} transcripts in cells expressing \textit{HES1} shRNA were 0.52 and 0.54 of those in primary islets, respectively. In contrast, the levels of insulin transcripts in cells expressing \textit{HES1} shRNA were 9-fold lower, compared with those in primary islets. In agreement with the higher insulin mRNA levels, insulin immunostaining detected a 4-fold (p=0.016) higher number of insulin-positive cells in cultures expressing \textit{HES1} shRNA, compared with those treated with the control virus (Fig. 5B). The fraction of insulin-positive cells among eGFP\textsuperscript{+} cells was also 3-fold higher in the presence of \textit{HES1} shRNA, indicating that fewer beta cells underwent dedifferentiation (Fig. 5B,C). Insulin content in cells expressing \textit{HES1} shRNA was 3.5-fold higher (p=0.03), compared with cells treated with the control virus (Fig. 6A), although these levels were 14-fold lower, compared with intact islets before trypsinization. Cells at P2 maintained the same 2-3-fold increase in insulin secretion in response to glucose observed in intact islets (Fig. 6B). However, insulin secretion from cells expressing \textit{HES1} shRNA was 6.8-fold higher (p=0.006), compared with cells treated with the control virus, in response to 16 mM glucose (Fig. 6B). Nevertheless, cells expressing \textit{HES1} shRNA secreted a much higher fraction of their insulin content (15.5%), compared with intact islets (3.9%) during a 30-minute assay (cells infected with the non-target virus secreted 19.7% of their content).

To verify that these results were due to specific inhibition of HES1 expression, the studies were reproduced with 2 additional \textit{HES1} shRNAs, TRCN18990 and TRCN18991. As seen in Fig. 7A, cell infection with viruses encoding these shRNAs resulted in reduction in cellular HES1 protein levels. The reduction in HES1 correlated with 5.3-fold- (p=3.3X10\textsuperscript{-5}) and 2.2-fold- (p=6.6X10\textsuperscript{-4}) higher levels of p57 transcripts in cells expressing the 2 \textit{HES1} shRNAs, respectively, compared with the levels in cells infected with the non-target virus. The levels of insulin transcripts were 3.4-fold- (p=1.3X10\textsuperscript{-5}) and 1.9-fold- (p=2.8X10\textsuperscript{-4}) higher, respectively; the levels of \textit{PDX1} transcripts were 4.0-fold- (p=9.2X10\textsuperscript{-5}) and 2.5-fold- (p=1.9X10\textsuperscript{-4}) higher, respectively; and the levels of \textit{NEUROD1} transcripts were 7.7-fold- (p=3.5X10\textsuperscript{-5}) and 3.1-fold- (p=8X10\textsuperscript{-6}) higher, respectively. These results are comparable with those obtained using \textit{HES1} shRNA TRCN18993, indicating that the effects on cell proliferation and differentiation were caused by specific inhibition of HES1 expression.

**DISCUSSION**

Our findings demonstrate that culture of dissociated adult human islet cells in serum-containing medium, which induces beta-cell dedifferentiation and replication, involves activation of elements of the NOTCH pathway. Transcript levels for \textit{NOTCH1-3} and \textit{HES1} are upregulated. In contrast, transcripts for \textit{NOTCH4}, and the NOTCH ligands \textit{DELTALI} and \textit{JAG2}, are downregulated. These changes were initially observed in a mixed population of islet cells, which likely included contaminating duct and exocrine cells. Using virus-mediated cell-lineage tracing, we then determined that these changes occurred in beta cells. The upregulation of the NOTCH pathway correlated with cell dedifferentiation, as manifested by a dramatic decrease in insulin transcripts, and by cell entrance into the cell cycle, as manifested by downregulation of p57 transcripts and an increase in Ki67 staining. The findings at the RNA level were supported by immunostaining, which demonstrated a negative correlation between the presence of HES1 or NICD in the nucleus, and insulin expression, in eGFP\textsuperscript{+} cells, which marked their origin from beta cells. These in situ analyses also detected a positive
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correlation between p57 and insulin expression, confirming the view that beta-cell replication involves dedifferentiation. The key role of HES1 in these events was revealed by inhibiting its upregulation with shRNA. In these cells, the decrease in p57 was prevented, and cell proliferation was greatly reduced. While cell dedifferentiation was not completely prevented, it was significantly inhibited, compared with cells in which HES1 upregulation was not repressed. This was manifested by higher levels of insulin transcripts and fraction of cells immunostaining for insulin, as well as transcripts encoding beta-cell transcription factors. In addition, loss of insulin content and secretion was less pronounced. These findings suggest that a partial cell dedifferentiation is independent of HES1 activity and cell replication, however induction of advanced dedifferentiation and cell replication requires HES1 upregulation. This interpretation is supported by the finding that the bulk of decrease in insulin mRNA occurs during the first week, thus preceding the peak in HES1 mRNA levels. It is therefore possible that loss of most of the insulin content is a precondition for beta-cell entrance into cell cycle in vitro.

Given the fact that upregulation of the NOTCH pathway in islet cell cultures followed cell dissociation into single cells, it is unlikely that it was triggered by a cell-associated ligand, as in the lateral inhibition model (16). Rather, it is possible that this pathway is activated in response to soluble serum components, as was demonstrated in a number of cultured cell types (32). This possibility is supported by our findings of decreased expression of NOTCH ligands in islet cell cultures concomitantly with HES1 upregulation. This is reminiscent of the low levels of NOTCH ligands in the embryonic pancreas cells expressing HES1, which are directed for further proliferation, rather than differentiation (16).

Among the 4 members of the NOTCH family that were analyzed, NOTCH1, NOTCH2, and NOTCH3 transcripts were upregulated, while NOTCH4 transcripts were greatly downregulated. While expression of NOTCH1 and NOTCH2 was implicated in islet development, NOTCH3 and NOTCH4 expression was documented in mesenchymal and endothelial cells (21). Downregulation of NOTCH4 may reflect the elimination of a NOTCH4+ subpopulation, which is present in the original islet cell suspension, but for some reason is not maintained in continuous culture.

The wave of HES1 upregulation peaked within the first 2 weeks of culture and was downregulated thereafter. Nevertheless, the effects of HES1 were not reversed, as manifested by continuous replication of cells derived from dedifferentiated beta cells for up to 16 population doublings (8,15). The levels of p57 and insulin transcripts did not rebound thereafter, suggesting that their reinduction requires other signals, in addition to the decrease in the inhibitory effect of HES1. This finding suggests a transient role of HES1 upregulation that is limited to the initial adaptation of islet cells to culture, after which cell replication may continue in the presence of the low HES1 levels found in non-replicating cells.

In summary, our findings provide evidence for activation of the NOTCH pathway in adult cells and offer an in-vitro model system for studying interactions within this pathway. In addition, the findings emphasize the role of components of the NOTCH pathway in the transition of quiescent beta cells into a dedifferentiated, proliferative state in vitro. Our findings demonstrate a negative correlation between replication and maintenance of differentiated function in cultured beta cells, suggesting that significant cell expansion inevitably involves dedifferentiation and will require development of ways for cell redifferentiation.
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following expansion. Components of the NOTCH pathway may represent molecular targets for induction of redifferentiation of the expanded cells.

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**Table 1.** Assay-on-Demand (Applied Biosystems) TaqMan fluorogenic probes used in the study.

<table>
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FIGURE LEGENDS

Fig. 1. Upregulation of HES1 in cultured human islet cells and eGFP⁺ cells derived from beta cells correlates with downregulation of insulin. A, qPCR analysis of RNA extracted from islet cells derived from 9 donors. P indicates passage number and weeks in culture. RQ, relative quantification compared to P0, which represents islet cells at culture initiation. Data are mean±SD (n=9). Stars indicate statistical significance, compared with P0 (p<3.3X10⁻⁵ for insulin, and p<0.006 for HES1). The increase in HES1 mRNA levels at P1 was marginally significant (p=0.06). B, Immunoblotting for HES1 in protein extracted from islet cells at the indicated passage number. Beta-actin served as a loading control. C, Immunoblotting for HES1 in protein extracted from islet cells at P2 following 17-h incubation with the γ-secretase inhibitor L-685,458. HSC70 served as a loading control. D, Immunofluorescence analysis of islet cells (left) and eGFP⁺ cells derived from beta cells (right) following 10 days in culture. The left panel is merged with a phase contrast image. Arrow points to a beta cell which still expresses insulin and is not labeled for HES1. eGFP is detected in both cytoplasm and nucleus. Bar=20 µm.
Fig. 2. Upregulation of the NOTCH pathway in cultured human islet cells and eGFP+ cells derived from beta cells. A, qPCR analysis of RNA extracted from islet cells derived from 8 donors at the indicated passage numbers. P indicates passage number and weeks in culture. RQ, relative quantification compared to P0. Data are mean±SD (n=8). Stars indicate statistical significance, compared with P0 (p<0.03 for NOTCH1, p<0.04 for NOTCH2, p<9×10^-4 for NOTCH3, p<0.03 for NOTCH4, p<0.005 for DLL1, and p<0.026 for JAG2). The increase in NOTCH3 mRNA levels at P3 was marginally significant (p=0.06). B, Immunoblotting for NOTCH1 120-kd transmembrane fragment in protein extracted from islet cells at the indicated passage number. HSC70 served as a loading control. C, Immunofluorescence analysis of islet cells (left) and eGFP+ cells derived from beta cells (right) following 10 days in culture. Bar=20 μm.
**Fig. 3.** Downregulation of p57 in cultured human islet cells and eGFP$^+$ cells derived from beta cells. **A**, qPCR analysis of RNA extracted from islet cells derived from 8 donors at the indicated passage numbers. P indicates passage number and weeks in culture. RQ, relative quantification compared to P0. Data are mean±SD (n=8). Stars indicate statistical significance, compared with P0 (p<0.03). **B** and **C**, Immunofluorescence analysis of eGFP$^+$ cells derived from beta cells following 10 days in culture. Solid arrow in **B** points to an eGFP$^+$ cell which has lost both insulin and p57 expression; dashed arrow points to an eGFP$^+$ beta cell which maintains both insulin and p57 expression. Arrow in **C** points to an eGFP$^+$ cell which maintains p57 expression and is not labeled for Ki67. Bar=20 µm.
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Fig. 4. Prevention of HES1 upregulation by shRNA reduces replication of cultured human islet cells and eGFP+ cells derived from beta cells. **A**, Immunoblotting for HES1 in protein extracted from islet cells following infection with HES1 shRNA TRCN18993 or non-target virus. Beta-actin served as a loading control. **B**, Top, incidence of BrdU+ cells among cultured islet cells following infection with HES1 shRNA TRCN18993 or non-target virus. Data are mean±SD (n=3 donors; >1000 cells counted in culture from each donor; p=0.02). Bottom, incidence of Ki67+ cells among eGFP+ cells from 2 representative donors following infection with HES1 shRNA or non-target virus. Data is based on >1000 cells counted in culture from each donor. **C**, Immunoblotting for PARP in protein extracted from islet cells following infection with non-target (lane 3) or HES1 shRNA TRCN18993 virus (lane 4). Uninfected cells incubated with (lane 1) or without (lane 2) the apoptotic agent staurosporin served as controls. The lower band in lane 1 represents cleaved PARP. Beta-actin served as a loading control. **D**, qPCR analysis of RNA extracted from islet cells following infection with HES1 shRNA TRCN18993 or non-target virus. RQ, relative quantification compared to P0. Data are mean±SD (n=3 donors). Only the change in p57 is significant (p=0.001, compared to cells infected with non-target virus, indicated by star). All the analyses were done 14 days following viral infection.

**Figure 4**
Fig. 5. Prevention of HES1 upregulation by shRNA reduces beta-cell dedifferentiation. A, qPCR analysis of RNA extracted from islet cells following infection with *HESI* shRNA TRCN18993 or non-target virus. RQ, relative quantification compared to P0. Data are mean±SD (n=3 donors). The changes in all 3 genes in cells infected with *HESI* shRNA, compared to cells infected with non-target virus, were significant (p<0.004). B, Left, incidence of insulin-positive cells among cultured islet cells following infection with *HESI* shRNA TRCN18993 or non-target virus. Data are mean±SD (n=3 donors; >1000 cells counted in culture from each donor; p=0.016). Right, incidence of insulin-positive cells among eGFP* beta cells from 2 representative donors following infection with *HESI* shRNA or non-target virus. Data is based on >1000 cells counted in culture from each donor. Significant differences compared to non-target are indicated by stars. C, Immunofluorescence analysis of insulin in eGFP* cells following infection with *HESI* shRNA TRCN18993 or non-target virus. Bar=100 µm. All the analyses were done 14 days following viral infection.
Fig. 6. *HES1* shRNA reduces the decrease in insulin content and glucose-stimulated insulin secretion observed during dedifferentiation of cultured islet cells. **A,** Insulin content in cells infected with *HES1* shRNA TRCN18993 or non-target virus, compared with primary islets. Data are mean±SD (n=3 donors). Star indicates statistical significance (p=0.03). **B,** Insulin secretion in response to glucose during a 30-minute assay. Data are mean±SD (n=3 individual experiments from a representative donor). Stars indicate statistical significance of the difference between 0 and 16 mM glucose in each cell type (p<0.04).
Fig. 7. Effects of *HES1* shRNAs TRCN18990 and TRCN18991 on gene expression in cultured islet cells. A, Immunoblotting for HES1 in protein extracted from islet cells 14 days following infection with *HES1* shRNA TRCN18990, TRCN18991, or non-target virus. HSC70 served as a loading control. B, qPCR analysis of RNA extracted from islet cells 14 days following infection with *HES1* shRNAs or non-target virus. RQ, relative quantification compared to cells infected with non-target virus. Data are mean±SD (n=3). Significant differences compared to non-target are indicated by stars.