Redifferentiation of Expanded Human Pancreatic β-Cell-derived Cells by Inhibition of the NOTCH Pathway

Yael Bar, Holger A. Russ, Leeat Anker-Kitai, Sarah Knoller, and Shimon Efrat

Received for publication, November 9, 2011, and in revised form, March 26, 2012. Published, JBC Papers in Press, March 28, 2012, DOI 10.1074/jbc.M111.319152

From the Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, 69978 Tel Aviv, Israel

Background: Human β-cell-derived (BCD) cells can be expanded in vitro in a process involving dedifferentiation and activation of the NOTCH pathway.

Results: Inhibition of the NOTCH effector HES1 using shRNA leads to redifferentiation of expanded BCD cells.

Significance: This approach promises to reduce donor β-cell shortage for diabetes cell therapy.

In vitro expansion of β-cells from adult human pancreatic islets would overcome donor β-cell shortage for cell replacement therapy for diabetes. Using a β-cell-specific labeling system we have shown that β-cell expansion is accompanied by dedifferentiation resembling epithelial-mesenchymal transition and loss of insulin expression. Epigenetic analyses indicate that key β-cell genes maintain open chromatin structure in expanded β-cell-derived (BCD) cells, although they are not transcribed. In the developing pancreas important cell-fate decisions are regulated by NOTCH receptors, which signal through the Hairy and Enhancer of Split 1 (HES1) transcription regulator. We have reported that BCD cell dedifferentiation and proliferation in vitro correlate with reactivation of the NOTCH pathway. Inhibition of HES1 expression using shRNA during culture initiation results in reduced β-cell replication and dedifferentiation, suggesting that HES1 inhibition may also affect BCD cell redifferentiation following expansion. Here, we used HES1 shRNA to down-regulate HES1 expression in expanded human BCD cells, showing that HES1 inhibition is sufficient to induce BCD cell redifferentiation, as manifested by a significant increase in insulin expression. Combined treatment with HES1 shRNA, cell aggregation in serum-free medium, and a mixture of soluble factors further stimulated the redifferentiation of BCD cells. In vivo analyses demonstrated the ability of the redifferentiated cells to replace β-cell function in hyperglycemic immunodeficient mice. These findings demonstrate the redifferentiation potential of ex vivo expanded BCD cells and the reproducible differentiating effect of HES1 inhibition in these cells.

β-Cell replacement is an attractive approach for the treatment of type 1 diabetes; however, its application is limited by the shortage of human pancreas donors. In vitro expansion of adult β-cells from cadaver donors holds the promise of generating an abundant source of insulin-producing cells for transplantation. Several lines of evidence suggest that human β-cells are capable of replication in vivo under certain conditions. Autopsy studies indicate that the increase in β-cell mass in infancy and in response to increased demands for insulin such as in pregnancy and obesity, as well as normal β-cell turnover in adults, is associated with β-cell replication (1, 2). In addition, β-cell damage in type 1 and type 2 diabetes is also reported to stimulate β-cell replication (3). In contrast, expansion of adult human β-cells in vitro is difficult, because the β-cell phenotype is lost. Intact human islets can be kept in suspension culture for months without a significant decline in insulin production and secretion; however, cell proliferation is not induced under these conditions (4). Cell monolayer culture induces islet cell replication, but the cells lose insulin expression as well as expression of most other β-cell markers (5–11). The preservation of β-cell function remains limited even when cell aggregation and extracellular matrix are employed (12). These findings may reflect β-cell dedifferentiation or β-cell death accompanied by an expansion of cells from a non-β-cell origin in the islet cell culture. Using a lineage-tracing approach we demonstrated that ~40% of cells proliferating in these cultures are β-cell-derived (BCD) cells (13). Thus, human β-cells can be induced to proliferate significantly in vitro in a process involving dedifferentiation resembling epithelial-mesenchymal transition (EMT) (14). A number of attempts have been made to restore insulin production in expanded human islet cells (8–10). However, insulin expression in these studies was very limited (8, 10) or inconsistent (9), raising the need for development of alternative redifferentiation approaches.

We have recently shown that BCD cells can be preferentially redifferentiated by a combination of soluble factors in serum-free medium (SFM) (15). The redifferentiated cells re-express β-cell genes, process and store insulin in typical secretory vesicles, and release it in response to glucose. Redifferentiation involves mesenchymal-epithelial transition and activation of

1 To whom correspondence should be addressed. Tel.: 972-3-640-7701; Fax: 972-3-640-9950; E-mail: sefrat@post.tau.ac.il.

2 The abbreviations used are: BCD, β-cell-derived; CDK, cyclin-dependent kinase; eGFP, enhanced green fluorescent protein; EMT, epithelial-mesenchymal transition; RM, redifferentiation medium; SFM, serum-free medium; qPCR, quantitative PCR; RQ, relative quantification.
HES1 Inhibition Redifferentiates Expanded Human β-Cells

Islet progenitor cell transcription factors (15). However, this treatment results in redifferentiation of only about 25% of BCD cells. Thus, further improvement of the redifferentiation methods may increase the efficiency of this approach.

NOTCH signaling is an evolutionarily conserved mechanism that controls cell fates through local cell interactions in a broad spectrum of tissues and processes including the developing pancreas (16, 17). Expression of NOTCH receptors (NOTCH1–3), ligands (JAG-1, JAG-2, DLL-1, and DLL-3), and target (HES1 (Hairy and Enhancer of Split 1)) has been observed in undifferentiated cells within the mouse embryonic pancreas but not in differentiated endocrine cells in the embryonic or adult pancreas (18–21). In the developing pancreas the role of the NOTCH-HES1 pathway is to promote precursor cell replication and prevent premature endocrine differentiation (19, 21). HES1 prevents islet cell differentiation by direct inhibition of Ngn3 gene expression (19) and maintains precursor cell proliferation by inhibiting expression of genes encoding the cyclin-dependent kinase (CDK) inhibitors p27 and p57 (22, 23).

We have shown previously that the NOTCH pathway, including the effector protein HES1, is reactivated in replicating BCD cells undergoing dedifferentiation in culture (24). In addition, HES1 inhibition by shRNA during culture initiation prevents cells undergoing dedifferentiation in culture (24). The NOTCH-HES1 inhibition by shRNA during culture initiation prevents cells undergoing dedifferentiation in culture (24). In addition, HES1 inhibition by shRNA during culture initiation prevents BCD cell replication and dedifferentiation (24). The NOTCH pathway has also been shown to be reactivated in cultured replicating rat acinar cells (25). Inhibition of NOTCH signaling in this system significantly improves β-cell neoinformation from these cells (26).

We hypothesized that inhibition of the NOTCH-HES1 pathway might represent a possible molecular target for induction of β-cell redifferentiation following in vitro expansion. Our findings demonstrated that HES1 inhibition by shRNA leads to significant redifferentiation of expanded human islet cells, as manifested by an increase in expression of insulin and additional β-cell genes. Using the β-cell-specific labeling system, we showed that the phenotypic changes in response to HES1 shRNA occur specifically in BCD cells. Treatment with HES1 shRNA further enhanced BCD cell redifferentiation induced by a combination of soluble factors in SFM. Cells treated with HES1 shRNA can replace β-cell function in vivo and correct hyperglycemia in immunodeficient mice. These findings demonstrate the redifferentiation potential of dedifferentiated β-cells following ex vivo expansion and the reproducible differentiating effect of HES1 inhibition in these cells.

**EXPERIMENTAL PROCEDURES**

**Islet Cell Culture, Lineage Tracing, and Purification of eGFP+ Cells**—Islets were received 2–3 days after isolation. Islets from individual donors (Table 1) were dissociated into single cells and cultured in CMRL 1066 medium containing 5.6 mmol/liter glucose and supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamycin, and 5 μg/ml amphotericin B as described (9). The cultures were refed twice a week and split 1:2 once a week. Lineage tracing was performed using the RIP-Cre/ER and pTrip-loxP-NEO-STOP-loxP-eGFP lentivirus vectors as described (14). 4-Hydroxytamoxifen (Sigma-Aldrich) was added to a final concentration of 1 μM at 1 day post-infection.

**TABLE 1**

| Donor No. | Sex  | Age | BMIA | Islet purity |
|-----------|------|-----|------|--------------|
| 1         | M    | 29  | 20.7 | 80           |
| 2         | F    | 56  | 23.7 | 70           |
| 3         | M    | 68  | 27.2 | 80           |
| 4         | F    | 62  | 23.1 | 80           |
| 5         | M    | 53  | 29.4 | 70           |
| 6         | F    | 51  | 20.1 | 75           |
| 7         | F    | 47  | 33.2 | 70           |
| 8         | F    | 37  | 30.8 | 88           |
| 9         | F    | 34  | 22.5 | 80           |
| 10        | M    | 47  | 36.1 | 85           |
| 11        | F    | 16  | 35.4 | 90           |
| 12        | F    | 19  | 46.0 | 90           |
| 13        | M    | 55  | 30.6 | 95           |
| 14        | F    | 55  | 22.6 | 90           |
| 15        | F    | 24  | 30.9 | 90           |
| 16        | M    | 57  | 34.3 | 75           |
| 17        | M    | 47  | 23.0 | 85           |
| 18        | M    | 50  | 26.0 | 90           |
| 19        | F    | 60  | 28.0 | 75           |
| 20        | F    | 69  | 29.7 | 90           |

Average ± S.D. 48.2 ± 13.9 27.3 ± 5.2 82.4 ± 7.9

* Body mass index.

Labeled cells were sorted using a FACS Aria sorter (BD Biosciences) as described (13, 14).

**HES1 Inhibition and Redifferentiation Conditions—HES1 shRNA** (GenBank accession number HC 916590) and a nontarget shRNA, both cloned in the pLKO.1 lentivirus vector containing a puromycin resistance gene, were obtained from the RNAi Consortium (Sigma-Aldrich). The virus was produced in 293T cells as described previously (14). Cells were infected at a multiplicity of infection of 2.5:1 in growth medium containing 8 μg/ml Polybrene overnight. The medium was then replaced with regular culture medium.

For redifferentiation experiments, expanded human islet cells or purified BCD cells in passages 5–7 were infected with HES1 shRNA or nontarget shRNA viruses. Two to 4 days following infection, cells were trypsinized, pelleted, and resuspended in regular growth medium; SFM (CMRL 1066 containing 5.6 mm D-glucose and supplemented with 1% BSA fraction V (Sigma), insulin/transferrin/selenium (ITS, Invitrogen), penicillin (50 units/ml), and streptomycin (50 μg/ml)); or redifferentiation medium (RM) (SFM containing 25 mm D-glucose and supplemented with 10 mm nicotinamide (Sigma), 8 mm exendin-4 (Acris), and 8 mm activin A (Peprotech)). Cells were then seeded in tissue culture plates and incubated for the indicated periods. The medium was replaced every 2 days. Cells seeded in SFM or in RM formed clusters in culture. Cells were analyzed following 4–11 days of culture. In experiments conducted with RM, cells were seeded in ultra low attachment plates (Corning) at 3.2 × 10³/cm² for improved aggregation.

**p57 Overexpression**—An adenovirus vector containing human p57 under the CMV promoter (AdEasy system) was a gift of Prof. Benjamin Glaser (Hebrew University, Jerusalem). Adenovirus particles were produced as described (27). Cultured islet cells were washed with PBS and infected at a multiplicity of infection of 100:1 in CMRL 1066 medium containing 8 μg/ml Polybrene overnight. The medium was then replaced with regular culture medium.

**Proliferation and Apoptosis Analyses**—BrdU incorporation was analyzed following cell incubation with 10 μM BrdU.
Apoptotic cells were detected by TUNEL staining using the ApopTag fluorescein in situ apoptosis detection kit (Chemicon) according to the manufacturer’s protocol. Fluorescence was visualized under a fluorescent TE-200 microscope.

**Nucleus Area Measurement**—Cells were seeded on slides and stained for DAPI (Sigma). Labeled cells were also co-stained for enhanced green fluorescent protein (eGFP). Images taken on TE-200 fluorescent microscope were analyzed for nucleus size using Nikon NIS-Elements Basic Research software.

**qPCR Analyses**—Total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen) or TRIzol (Sigma-Aldrich) and treated with DNA-Free® (Ambion). cDNA was prepared using a high capacity cDNA RT kit (Applied Biosystems). qPCR was carried out in triplicates using TaqMan Universal PCR Master Mix (Applied Biosystems) or Universal Probe Library Master Mix (Roche Applied Science) in a 7300 real-time PCR system (Applied Biosystems). The results were normalized to a TATA-box-binding protein (TBP) and/or a human large ribosomal protein (RPLPO) transcripts. Primer sequences are listed in Table 2. All reactions were performed with annealing at 60 °C for 40 cycles. For undetectable transcripts, the cycle number was set to 40 for comparisons.

**Immunofluorescence Analyses**—Cells were trypanized, spotted on slides using a Shandon Cytospin 4 centrifuge (Thermo Scientific), and fixed for 15 min at room temperature in 4% paraformaldehyde. For BrdU staining, the slides were incubated in 1.5 M HCl for 20 min followed by 0.1 M sodium borate (pH 8.5) for 10 min at RT and three washes in PBS. Samples were blocked for >10 min in blocking buffer (1% BSA, 5% fetal goat serum, and 0.2% saponin) and incubated for 1 h at room temperature or overnight at 4 °C with primary antibodies diluted in blocking buffer as follows: mouse anti-eGFP (1:500, Chemicon); rabbit anti-eGFP (1:1000, Invitrogen); mouse anti-human C-peptide (1:200, Biodesign); rabbit anti-human C-peptide (1:1000, Abcam); mouse anti-glucagon (1:2000, Sigma); rabbit anti-somatostatin (1:400, DAKO); and mouse anti-p57 (1:500, Santa Cruz Biotechnology). The bound antibody was visualized with the appropriate horseradish peroxidase-conjugated anti-IgG (Jackson Immunoresearch) or rabbit anti-eGFP (1:500, Chemicon); rabbit anti-eGFP (1:1000, Invitrogen); mouse anti-human C-peptide (1:200, Biodesign); rabbit anti-human C-peptide (1:1000, Abcam); mouse anti-glucagon (1:2000, Sigma); rabbit anti-somatostatin (1:400, DAKO); and mouse anti-p57 (1:500, Santa Cruz Biotechnology). The bound antibody was visualized with the appropriate horseradish peroxidase-conjugated anti-IgG (Jackson Immunoresearch) or SuperSignal West Pico chemiluminescent substrate (Pierce). Quantification was done using TINA software.

**In Vivo Analyses**—Six- to eight-week-old nonobese diabetic/severe combined immunodeficient (NOD-SCID) mice were made hyperglycemic (blood glucose level above 300 mg/dl) by a single intraperitoneal injection of 180 µg of streptozotocin (Acros Organics)/g body weight. Expanded human islet cells at passage 5 were infected with shRNA, and 2 days later, using a 30-gauge needle, 2 × 10^6 cells were injected under the kidney capsule of mice considered hyperglycemic. The glucose levels of transplanted mice were measured for 24 h. Apoptotic cells were detected by TUNEL staining using the ApopTag fluorescein in situ apoptosis detection kit (Chemicon) according to the manufacturer’s protocol. Fluorescence was visualized under a fluorescent TE-200 microscope.

**TABLE 2**

| Gene | Primer sequences | Sense primer | Antisense primer |
|------|-----------------|--------------|-----------------|
| INS  | TaqMan probe Hs 00355773 m1 | aagtttgagggctcaaggcag | TaqMan probe Hs 00355773 m1 |
|      | TaqMan probe Hs 00426216 m1 | aagtttgagggctcaaggcag | TaqMan probe Hs 00426216 m1 |
| PDX1 | TaqMan probe Hs01651425_s1 | agcagagaggctgcaaccc | TaqMan probe Hs01651425_s1 |
|      | TaqMan probe Hs01031536_m1 | gtaacagcagctgacacctc | TaqMan probe Hs01031536_m1 |
| MAFA | TaqMan probe Hs01794949_m1 | acgcagctgacacctc | TaqMan probe Hs01794949_m1 |
| GCG  | TaqMan probe Hs00327001_m1 | tcagtcgctttacctgctgc | TaqMan probe Hs00327001_m1 |
| SST  | TaqMan probe Hs01039995_m1 | gcacacctgacacctc | TaqMan probe Hs01039995_m1 |
| PYY  | TaqMan probe Hs00427620_m1 | tctttcagcctcatgaagcagagctgtat | TaqMan probe Hs00427620_m1 |
| GAPDH (h) | TaqMan probe Hs 0009999905_m1 | acaccccttgagagccagcag | TaqMan probe Hs 0009999905_m1 |
| RPLPO | TaqMan probe Hs 0009999902_m1 | acaccccttgagagccagcag | TaqMan probe Hs 0009999902_m1 |
| TBP  | TaqMan probe Hs 00160905_m1 | ttcacactgagcgccagggctgtat | TaqMan probe Hs 00160905_m1 |
| SUR1 | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| MIR62 | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| IAPP | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| PCSK1 | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| NEUROD1 | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| NXX2 | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| NXX6.1 | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| ARX  | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| MAFB | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| CDK1C | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| CDKN1B | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
| PAX4 | TaqMan probe Hs00232335_m1 | ctcttctttgctctattggagcagagctgtat | TaqMan probe Hs00232335_m1 |
HES1 Inhibition Redifferentiates Expanded Human β-Cells

monitored once a week in blood samples obtained from the tail vein using Accutrend strips (Roche Diagnostics). Serum human C-peptide was determined in blood samples obtained from the orbital plexus of fed mice by C-peptide ELISA (Millipore) according to the manufacturer’s protocol. Serum obtained from untreated mice was used as control. Mice were sacrificed 56 days post-transplantation, and the kidneys were removed. Part of the transplant area was embedded in paraffin and sectioned for histological analyses, and another part was transferred into TRI Reagent® (Sigma-Aldrich) and homogenized using a pellet pestle. RNA was extracted according to manufacturer’s protocol. cDNA was preamplified for specific human transcripts using the Taqman PreAmp Cells-to-CT kit (Applied Biosystems) according to manufacturer’s protocol, and qPCR was performed as described above. RNA extracted from the kidneys of untreated mice served as the control. Results were normalized to human GAPDH transcripts. Paraffin sections (5 μm) were deparaffinized and rehydrated, and antigen retrieval was performed before staining. Sections were blocked for 10 min in CAS-BLOCK (Zymed Laboratories Inc.), incubated overnight at 4 °C with mouse anti-human C-peptide (1:200, Biodesign), guinea pig anti-insulin (1:200, Dako), or mouse anti-islet amyloid polypeptide (1:30, Thermo Scientific), washed three times in PBS, incubated for 30 min at room temperature with the appropriate secondary biotin-bound antibody (Enco), washed in PBS, and incubated for 15 min at room temperature with avidin-HRP (Sigma). Color was developed using the DAB chromogen kit (Zymed Laboratories Inc.) according to manufacturer’s instructions, and counterstaining was performed using hematoxylin. Sections were dehydrated, mounted with Entellan (Merck), and viewed under a TE-200 microscope.

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

RESULTS

Effect of HES1 Inhibition on Redifferentiation of Cultured Human Islet Cells—This effect was analyzed in cells expanded in vitro for 5–7 weeks (5–7 passages). During this period the cells lost insulin production and underwent dedifferentiation. They were then infected with the HES1 shRNA or nontarget shRNA lentiviruses and selected for puromycin resistance. Infection efficiency was >90%. Analyses performed 9 days following infection showed a 40% decrease in HES1 and a 50% increase in p57 protein levels (Fig. 1A), as well as formation of cell clusters (Fig. 1B) and a significant reduction in nucleus size (average 201 ± 151 μm² in the HES1 shRNA group as compared with 319 ± 185 μm² in the nontarget shRNA group; p = 1.85 × 10⁻⁴⁵, based on measuring 1000 nuclei/group) (Fig. 1C, left). RNA analyses showed a significant increase in INS transcripts in the HES1 shRNA group, compared with the nontarget group, averaging 6.7 ± 4.3-fold (p = 0.004), as well as in PDX1 (averaging 4.5– ± 1.4-fold, p = 0.002) and NEUROD1 (averaging 4.7 ± 3.3 fold, p = 0.013) transcripts. A significant increase was also observed in transcripts for the β-cell transcription factors MAFA and NKX6.1, as well as for GCK, SUR1, KIR6.2, and IAPP, involved in insulin secretion. Transcripts of NGN3, a HES1 target, were not detected in either dedifferentiated BCD cells or cells treated with HES1 shRNA (data not shown). Transcripts encoding the islet hormones GCG and SST were up-regulated 7.4 ± 6.0-fold (p = 0.01) and 4.7 ± 2.7-fold (p = 0.006), respectively. Our previous data indicate that BCD cells can give rise to SST⁺ cells; however, they do not differentiate into GCG⁺ cells (15). The induction of GCG transcripts in the mixed islet cell population most likely reflects differentiation of non-BCD cells, possibly those derived from α-cells. HES1 inhibition in these cells may have an effect similar to that observed in BCD cells. Transcripts encoding the α-cell transcription factor MAFB were also up-regulated. The CDK inhibitors p57 and p27 are known targets of HES1 repression (22, 23). As expected, HES1 inhibition resulted in the up-regulation of their transcripts (5.8 ± 2.6-fold, p = 0.003, and 3.2 ± 0.2-fold, p = 2.7E-05, for p57 (CDKN1C) and p27 (CDKN1B) transcripts, respectively) (Fig. 1D). BrdU incorporation analyses confirmed the induction of growth arrest by HES1 shRNA (0.7% BrdU-positive cells compared with 15.8% in cells treated with nontarget shRNA, based on scoring >500 cells/group).

Redifferentiation Effect of HES1 Inhibition in BCD Cells—This effect was determined using the lineage tracing system (14, 15). Briefly, the labeling approach is based on infection of primary islet cells with a mixture of two lentivirus vectors at 1 day following seeding in culture. One vector expresses a Cre recombinase-estrogen receptor fusion protein under the insulin promoter (RIP-Cre/ER) and the other a reporter cassette in which the cytomegalovirus promoter is separated from an eGFP gene by a loxP-flanked stop region. Only β-cells infected by both viruses and treated with tamoxifen will express Cre protein and translocate it into the nucleus, leading to removal of the floxed DNA fragment in the reporter construct and activation of eGFP expression. Residual insulin expression in β-cells during the initial days in culture provided a sufficient window of time for RIP-Cre-ER expression and eGFP activation. Using this system in the redifferentiation experiments allowed us to distinguish the origin of the redifferentiated cells. The absence of tamoxifen in the redifferentiation stage prevents de novo labeling of cells from a non-β-cell origin acquiring insulin gene expression. Thus, eGFP expression in redifferentiated cells indicates that these cells originated from β-cells. Labeled human islet cells at passage 4 were infected with HES1 shRNA or nontarget viruses. Co-staining for C-peptide and eGFP 9 days following treatment with HES1 shRNA revealed C-peptide staining only in rare, mostly eGFP⁺, cells (Fig. 1E). No staining was detected in the nontarget group. In addition, eGFP⁺ cells infected with HES1 shRNA showed reduced nucleus size (averaging 220 ± 166 μm² compared with 387 ± 284 μm² in the nontarget shRNA group (p = 0.007), based on measuring 1000 nuclei/group) (Fig. 1C, right). These results demonstrate that HES1 inhibition induces redifferentiation of BCD cells.

p57 Overexpression Insufficient for Redifferentiation of Expanded Islet Cells—Given that expression of the cell cycle inhibitor p57 was up-regulated in expanded islet cells following HES1 inhibition (Fig. 1D), we were interested to determine whether the redifferentiation effect of HES1 inhibition was mediated exclusively through the up-regulation of p57. In this
case, overexpression of p57 in the cells would lead to a redifferentiation effect similar to that observed following HES1 inhibition. Human islet cells at passage 5 were infected with HES1 shRNA lentivirus, nontarget lentivirus, or CDKN1C adenovirus encoding p57. RNA analysis at 7 days following infection revealed that CDKN1C transcript levels were up-regulated 4.1-fold following HES1 inhibition, compared with 12,197.2574-fold following CDKN1C adenovirus infection. Despite the much higher CDKN1C expression in the adenovirus-infected group, no elevation in INS transcripts was observed in this group compared with a 5.3 ± 2.7-fold elevation in the HES1 shRNA group (Fig. 1F). These findings suggest that the redifferentiation effect of HES1 inhibition is not mediated exclusively through growth arrest due to the elevation in p57 expression. However, we cannot exclude the possibility that the very high p57 levels following adenovirus infection may have resulted in unintended phenotypic changes, which could complicate interpretation.

**HES1 Inhibition Enhanced BCD Cell Redifferentiation Induced by SFM**—To evaluate a possible synergistic redifferentiation effect of HES1 shRNA and previously published redifferentiation treatments, we combined HES1 shRNA lentivirus infection with cell incubation in SFM. β-Cells were labeled with eGFP using the lineage tracing system during their first week in culture followed by sorting into eGFP cells (BCD cells) and eGFP cells at passage 2 and further expansion in culture. The purity of eGFP cells averaged 80 ± 10%. It should also be noted that the eGFP cells contain unlabeled BCD cells. Thus, the two sorted populations were only enriched and not pure, and the differences observed between them were likely underestimated. Following a total of 5 weeks in culture the dedifferentiated cells were infected with HES1 shRNA or nontarget virus. Four days later the cells were seeded in SFM for an additional 4-day period. eGFP cells aggregated in SFM more readily than eGFP cells (Fig. 2). The morphological changes in response to HES1 shRNA and SFM were also more pronounced in eGFP cells. In response to the combined treatment, eGFP cells detached from the plate surface and formed islet-shaped clusters (Fig. 2). qPCR analysis revealed that the combined treatment had a significant additive effect compared with each treatment alone (Fig. 3). Whereas in eGFP cells INS transcripts were up-regulated 2.3 ± 0.6-fold following HES1 inhibition, our studies revealed that the combination of treatment led to a significantly greater increase in INS expression.
HES1 Inhibition Redifferentiates Expanded Human β-Cells

FIGURE 2. Effect of HES1 shRNA and SFM on morphology of BCD cells. Phase contrast and GFP fluorescence images of sorted eGFP⁺ and eGFP⁻ cells at passage 7 on day 8 following infection with HES1 shRNA or nontarget virus and day 4 after seeding in SFM. Bar = 100 μm.

bition alone and 3.1 ± 1.1-fold following SFM alone (compared with cells infected with nontarget virus in growth medium), up-regulation following the combined treatment averaged 13.2 ± 8.0-fold (p = 0.03). The same pattern was observed for PDX1, NEUROD1, and IAPP transcripts. Up-regulation of β-cell transcripts occurred preferentially in eGFP⁺ cells, as was the case with SST transcripts. The latter could reflect BCD cell plasticity (15), as noted above. In contrast, the up-regulation of GCG transcripts occurred equally in both eGFP⁺ and eGFP⁻ cells, possibly representing the redifferentiation of cells originating from α-cells, which were present in the eGFP⁻ population and may have also contaminated the sorted eGFP⁺ cell population. The up-regulation of transcripts for the cell cycle inhibitors p27 and p57 was also not restricted to the eGFP⁺ cells (Fig. 3).

HES1 Inhibition Enhanced BCD Cell Redifferentiation Induced by Soluble Factors—Next we combined HES1 inhibition and SFM-induced cell aggregation with the addition of soluble factors that have been shown to contribute to β-cell differentiation in vitro. SFM containing soluble factors, termed redifferentiation medium, included, in addition to 1% BSA and insulin/transferrin/selenium, nicotinamide (10 μM) (28), high glucose (increased from 5.6 mM in the growth medium to 25 mM), activin A (4 nM) (29), and the glucagon-like peptide-1 analog exendin-4 (8 nM) (30). In addition, we used low adherence plates instead of regular culture plates to enhance cell cluster formation. Sorted eGFP⁺ cells at passages 5 and 6 were infected with HES1 shRNA or nontarget shRNA viruses and 4 days later were seeded in RM or growth medium for an additional 4-day period. qPCR analysis showed a significant additive effect of the combined treatment, compared with HES1 inhibition or RM treatment alone. RM together with nontarget shRNA virus increased INS transcript levels 23.5 ± 10.5-fold, compared with eGFP⁻ cells in growth medium (p = 0.12) (Fig. 4A). In contrast, the combined treatment of RM and HES1 shRNA led to an increase of 144.2 ± 29.7-fold (p = 0.01). Thus, INS expression in the combined treatment was 6.1-fold (p = 0.04) higher compared with RM treatment alone. Levels of PDX1 transcripts were 5.5-fold higher in eGFP⁺ cells treated with the combined treatment, compared with eGFP⁺ cells treated with RM alone (p = 0.03). Transcript levels for other islet hormones were also up-regulated following the combined treatment. GCG, SST, and PPY transcript levels were 2.5 (p = 0.2)-, 3.4 (p = 0.04)-, and 5.2-fold (p = 0.01) higher, respectively, in eGFP⁺ cells treated with RM and HES1 shRNA, compared with cells treated with RM alone (Fig. 4A). As noted for the data in Fig. 3, the finding of SST transcripts in redifferentiated sorted eGFP⁺ cells is consistent with our previous report of BCD cell plasticity toward SST-positive cells (15). In contrast, GCG and PPY transcripts likely originate from contaminating non-BCD cells. To further validate the finding of specific redifferentiation of BCD cells (Fig. 3), eGFP-labeled expanded islet cells were infected at passage 5 with HES1 shRNA or nontarget viruses. Cells were seeded in RM in low attachment
plates 2 days following infection and co-stained for eGFP and C-peptide 4 days later (Fig. 4B). Fifteen percent of the eGFP+ cells in the HES1 shRNA-infected population treated with RM was redifferentiated as judged by C-peptide staining, compared with 6.8% in the nontarget shRNA-infected group treated with RM (based on quantitation of 500 cells/group). No C-peptide-positive cells were found in the untreated cell population. Thus, HES1 inhibition potentiated the effect of RM 2.2-fold. In both groups about 80% of the C-peptide-positive cells were also positive for eGFP. As the average labeling efficiency is $57.5 \pm 8.9\%$ (13), this result indicates that most or all the C-peptide-positive cells were derived from BCD cells.

**Phenotype of Redifferentiated Cells following HES1 shRNA and RM Treatment**—The same experiment described in the previous section was repeated using unlabeled cells, to allow co-staining for multiple antigens using different fluorescent dyes. Co-staining of the redifferentiated cells for C-peptide and the islet hormones GCG and SST did not reveal any co-expression of these hormones (Fig. 4C). Whereas $19.9 \pm 6.4\%$ of the cells in the HES1 shRNA/RM group were positive for C-peptide, only $7.7 \pm 2.2\%$ of the cells were C-peptide-positive in the nontarget/RM group (Fig. 4D). Thus, HES1 inhibition potentiated the effect of RM in this experiment by 2.6-fold. Given that BCD cells represent $\sim40\%$ of the total expanded islet cells (13–15), differentiation of about 20% of the total islet cells with the HES1 shRNA/RM treatment represents redifferentiation of $\sim50\%$ of BCD cells, which is double the 25% obtained with soluble factors alone (15). Only $2.1 \pm 0.9\%$ of the cells in the HES1 shRNA/RM group, and $1.8 \pm 1.3\%$ of the cells in the nontarget/RM group, were positive for GCG. Similarly, only $0.9 \pm 0.2\%$ and $1.3 \pm 0.7\%$ were positive for SST in the HES1 shRNA/RM group and the nontarget/RM group, respectively. Cells expressing the islet hormone PPY were rare in both groups. The transcription factors PDX1 and NKX2.2 are important for β-cell development and function (31). Co-staining for PDX1 or NKX2.2 and C-peptide showed that all the C-peptide-positive cells expressed these markers in their nucleus following the combined treatment of HES1 shRNA and RM (Fig. 4E).

To evaluate cell mortality rates following redifferentiation, a TUNEL assay for apoptosis detection was performed on cells from three donors following the combined treatment. The results revealed significant differences between the HES1 shRNA/RM group and the nontarget shRNA/RM group, averaging $12.0 \pm 4.6$ and $7.1 \pm 3.7\%$ ($p = 0.045$) apoptotic cells, respectively (supplemental Fig. 1). Thus, the additive effect of HES1 inhibition on cell mortality was 5%.

**Expanded Islet Cells Redifferentiated by HES1 Inhibition Corrected Hyperglycemia in Immunodeficient Mice**—Human islet cells at passage 5 were infected with HES1 shRNA or nontarget viruses and transplanted 4 days later under the kidney capsule of streptozotocin-treated hyperglycemic NOD-SCID mice. Six or growth medium. RNA was extracted 4 days later and analyzed with primers for the indicated genes. RQ values are relative to cells infected with nontarget virus and incubated in growth medium and represent mean ± S.E. ($n = 4$ donors). Asterisks mark significant changes ($p < 0.05$) induced by HES1 shRNA and SFM compared with nontarget virus-infected eGFP+ cells in growth medium.
mice were transplanted with HES1-inhibited cells, and 5 mice were transplanted with control cells. The mice were kept for 56 days. During this period all of the control mice and 3 of 6 of the experimental mice exhibited high blood glucose levels and died prematurely. However, 3 of 6 of the experimental mice survived. The surviving mice showed decreased blood glucose levels (Fig. 5A) as well as human C-peptide in their serum, averaging 0.4 ± 0.2 ng/ml (Fig. 5B). The mice were sacrificed at the end of the experiment, and the transplants were recovered for RNA and immunohistochemical analyses. Although control grafts from this experiment were not available for analysis due to premature mouse death, previous studies in our laboratory have shown that BCD cells do not activate insulin expression in vivo spontaneously in the absence of a priming manipulation in vitro. qPCR analyses of graft RNA using human-specific primers (Fig. 5C) revealed a 433 ± 98-fold increase in human INS transcripts in the transplanted cells, compared with cultured cells infected with nontarget virus (p = 0.04). For comparison, the increase in INS mRNA induced by HES1 shRNA in these cells in culture was only 4.8 ± 2.0-fold (Fig. 5C). Thus, transplantation induced a further 90-fold increase in INS transcripts. Similarly, transcripts encoding NKX2.2 and PC1/3 were induced 112- and 14-fold, respectively, in the transplant, compared with cells treated with HES1 shRNA in vitro. Immunohistochemical analyses revealed the presence of insulin, C-peptide, and IAPP in the transplanted cells (Fig. 5D). We concluded that treatment with HES1 shRNA is sufficient for priming expanded islet cells for further differentiation in vivo into functional beta-like cells, which are capable of correcting glycemia in hyperglycemic mice.

DISCUSSION

Our findings demonstrate that inhibition of the NOTCH pathway by HES1 shRNA induces redifferentiation of BCD cells following significant expansion in vitro (Fig. 6). Redifferentiation is manifested by the activation of insulin expression, as well as expression of transcription factors required for insulin gene transcription (32). Using the lineage tracing system we show that these changes occur specifically in BCD (eGFP +) cells and therefore represent redifferentiation rather than de novo differentiation of another cell type present in the heterogeneous expanded islet cell population. A comparison of sorted eGFP + and eGFP − cells demonstrates directly the preferential redifferentiation capacity of eGFP + cells following HES1 inhibition, alone and in combination with SFM (Fig. 3). Co-immunofluorescence analyses for C-peptide and eGFP further support this conclusion (Figs. 1E and 4B). These findings indicate that dedifferentiated β-cells represent preferable candidates for the generation of insulin-producing cells through redifferentiation, possibly because of their demonstrated epigenetic memory (33).
FIGURE 5. Transplantation of cells treated with HES1 shRNA into hyperglycemic immunodeficient mice corrects glycemia. NOD-SCID mice were made diabetic by streptozotocin treatment and transplanted with $2 \times 10^6$ expanded islet cells infected at passage 5 with HES1 shRNA virus. A, changes in blood glucose levels in mice transplanted with cells infected with HES1 shRNA or nontarget virus. Values are mean ± S.E. B, serum human C-peptide levels 40 days following transplantation. Values are mean ± S.D. (n = 3 mice). C, transcript levels in RNA extracted from the transplants 56 days following transplantation in comparison with shRNA-infected cells in vitro. RQ values are relative to nontarget-infected cells in vitro and represent mean ± S.E. (n = 3 transplants). D, immunohistochemical analysis of transplant sections 56 days following transplantation. Dashed red lines mark transplant-kidney boundary. Bar = 100 μm.
HES1 Inhibition Redifferentiates Expanded Human β-Cells

FIGURE 6. Proposed model for β-cell dedifferentiation, replication, and redifferentiation. During the initial days in culture, insulin expression declines, whereas HES1 expression is induced. HES1 induction blocks p57 expression, induces β-cell replication, and causes further dedifferentiation. HES1 shRNA induces BCD cell redifferentiation following expansion. Redifferentiated cells are capable of further maturation in vivo into functional beta-like cells.

We have shown recently that dedifferentiated BCD cells can be redifferentiated into insulin-producing cells by a combination of soluble factors in SFM (15). The current results support the redifferentiation capacity of these cells and demonstrate that inhibition of the NOTCH pathway is sufficient for the induction of BCD cell redifferentiation. Most notably, this treatment is sufficient for priming the cells for further differentiation in vivo into functional beta-like cells, which are capable of restoring euglycemia in hyperglycemic mice. In addition, HES1 shRNA significantly potentiates the redifferentiation effects of SFM and soluble factors. This is manifested by higher levels of β-cell gene transcripts and a higher fraction of C-peptide-positive cells in the cell population, more than double that obtained with SFM and soluble factors alone: 19.9 ± 6.4% versus 7.7 ± 2.2% of unlabeled expanded islet cells (Fig. 4D), representing about 50 and 20% of BCD cells, respectively (assuming an average of 40% BCD cells in the expanded islet cell population; see Ref. 13). This difference was demonstrated directly in eGFP-labeled cells in which 15% expressed C-peptide following the combined treatment, compared with 6.8% in response to RM alone. The lower fraction of redifferentiated BCD cells in labeled versus unlabeled cells could reflect possible cell stress caused by the dual viral infection and eGFP overexpression (34), which may lead to a reduced redifferentiation capacity. A similar difference is observed when comparing the increase in insulin transcript levels following redifferentiation in unlabeled cells (Fig. 1D) with that in sorted labeled cells (Fig. 3). Overall, we have concluded that the combined HES1 shRNA/RM treatment represents a significant improvement of the redifferentiation protocol based on soluble factors alone.

In addition to transcripts of β-cell-specific genes, other islet hormone transcripts, including GCG, SST, and PPY, were elevated following HES1 inhibition alone or in response to the combined treatments, even when the experiment was conducted using sorted eGFP+ BCD cells (Figs. 3 and 4A). In contrast, immunostaining analysis revealed only a low number of GCG-, SST-, and PPY-expressing cells following HES1 shRNA/RM treatment, compared with the percentage of C-peptide-expressing cells in the population, and no effect of HES1 inhibition was observed on their prevalence (Fig. 4D). This pattern is indicative of low GCG, SST, and PPY transcript levels in most cells expressing them. No co-expression of C-peptide and other hormones was detected, as seen in normal adult islets (35), in contrast to recent works aimed at generating insulin-expressing cells from ES cells, which show co-expression of islet hormones in the differentiated cells (36, 37). Cells co-expressing several islet hormones are considered immature and found primarily among islet progenitor cells in the developing pancreas (38, 39). Hence, the mutually exclusive expression pattern of islet hormones following the combined treatment indicates a relatively advanced differentiation of the stained cells. The considerable elevation in SST transcripts following treatment of sorted BCD cells (Fig. 4B) may reflect properties of β/δ common progenitor cells, as recently suggested (15). Nevertheless, as neither the eGFP+ nor the eGFP− population was pure, further work is needed to address this possibility.

Human islet cells are likely induced to replicate in culture following tissue dissociation and exposure to growth factors present in the growth medium. We reported previously that HES1 is required for the induction of cultured human β-cell replication (24). Our current findings show that HES1 inhibition induces expression of the cell cycle inhibitors p57 and p27 (Fig. 1D) and growth arrest. The synergistic effect of HES1 inhibition and serum withdrawal on BCD cell redifferentiation may indicate that growth arrest induced by both treatments is sufficient for BCD cell redifferentiation. However, overexpression of p57 in expanded islet cells did not lead by itself to cell redifferentiation (Fig. 1F), suggesting that growth arrest alone is insufficient for redifferentiation. Nevertheless, growth arrest is associated with redifferentiation, as demonstrated following exposure to SFM and soluble factors (15). Therefore, the higher efficiency of the combined HES1 shRNA/SFM treatment may be explained in part by a more efficient cell shift from a replicating, dedifferentiated state to a quiescent, differentiated state.

HES1 inhibition leads to morphological changes in the cells, manifested in cell clustering and nucleus size reduction. Ouziel-Yahalom (9) et al. showed a progressive nucleus enlargement in cultured proliferating human islet cells, which parallels cell dedifferentiation and loss of insulin expression, as well as the appearance of the mesenchymal marker vimentin. Russ et al. (13, 14) showed that EMT is involved in the adaptation of β-cells to growth in culture, and EMT has been shown to involve an increase in cell size and protein synthesis (40). Therefore, the reduction in nucleus size seen following HES1 inhibition is an additional indication of BCD cell redifferentiation, suggesting the involvement of a mesenchymal-epithelial transition in the redifferentiation process.

The loss of normal cell-cell and cell-matrix contacts following islet isolation and dissociation likely leads to profound changes in gene expression in cultured β-cells and plays a key
role in their dedifferentiation and induction of replication. As shown by others, as well as in this work, expanded islet cells tend to cluster upon serum withdrawal. Clustering itself may restore some of the normal cell-cell contacts and induce insulin gene expression in the cells (8, 10). Interestingly, HES1 inhibition alone leads to cell clustering even in the presence of serum (Fig. 1B). The clustering effect of HES1 inhibition was enhanced when combined with serum withdrawal, in correlation with insulin gene expression. Taken together, these results indicate that cell clustering is a crucial step in the redifferentiation process and that HES1 inhibition has a significant role in this process. Finally, eGFP+ cells clustered more readily than eGFP− cells following HES1 inhibition and SFM treatment (Fig. 2), further emphasizing the unique redifferentiation potential of BCD cells.

The importance of NOTCH signaling to cell survival and protection from apoptosis has been documented in numerous cellular systems (41–44). Apoptosis inhibition by NOTCH may occur via several mechanisms (45–48), including inhibition of p53 activity (48, 49). Hence, the 5% increase in apoptosis observed following the combined treatment of HES1 shRNA and RM is not surprising. In addition, this result may reflect conflicting messages received by the cells during the first days of HES1 shRNA expression when serum is still present. Signals from growth factors in the serum may provide replication stimuli to cells that have already exited the cell cycle following HES1 inhibition. In this case, the net result may be induction of apoptosis. The combination of differentiation signals from additional sources in vitro, as well as cell transplantation, may help reduce cell mortality rates.

Further characterization of purified redifferentiated cells, both in vitro and in vivo, in comparison with adult human β-cells, is required for determining the extent of differentiation induced by this approach. In addition, the clinical application of HES1 inhibition will require the substitution of viral vectors with nonviral alternatives such as small molecules or small inhibitory RNAs. Nevertheless, this approach holds considerable promise for overcoming the donor β-cell shortage for cell replacement therapy for diabetes, by expanding β-cells from single donors for transplantation into multiple recipients.

Acknowledgments—We thank E. Sintov for technical assistance, T. Sudo for HES1 antibody, B. Glaser for p57 adenovirus vector, and A. Dror for assistance with figure preparation.

REFERENCES

1. Meier, J. J., Bhushan, A., Butler, A. E., Rizza, R. A., and Butler, P. C. (2005) Sustained β cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetesologia 48, 2221–2228

2. Meier, J. J., Butler, A. E., Saisho, Y., Monchamp, T., Galasso, R., Bhushan, A., Rizza, R., and Butler, P. C. (2008) β-cell replication is the primary mechanism subserving the postnatal expansion of β-cell mass in humans. Diabetes 57, 1584–1594

3. Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., and Butler, P. C. (2003) β-Cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. Diabetes 52, 102–110

4. Nielsen, J. H., Brunstedt, J., Andersson, A., and Frimodt-Moller, C. (1979) Preservation of β cell function in adult human pancreatic islets for several months in vitro. Diabetologia 16, 97–100

5. Hayek, A., Beattie, G. M., Cirulli, V., Lopez, A. D., Ricordi, C., and Rubin, J. S. (1995) Growth factor/matrix-induced proliferation of human adult β-cells. Diabetes 44, 1458–1460

6. Beattie, G. M., Cirulli, V., Lopez, A. D., and Hayek, A. (1997) Ex vivo expansion of human pancreatic endocrine cells. J. Clin. Endocrinol. Metab. 82, 1852–1856

7. Beattie, G. M., Ikin-Ansari, P., Cirulli, V., Leibowitz, G., Lopez, A. D., Bossie, S., Mally, M. I., Levine, F., and Hayek, A. (1999) Sustained proliferation of PDX-1+ cells derived from human islets. Diabetes 48, 1013–1019

8. Gershengorn, M. C., Hardikar, A. A., Wei, C., Geras-Raaka, E., Marcus-Samuels, B., and Raaka, B. M. (2004) Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. Science 306, 2261–2264

9. Ouziel-Yahalom, L., Zalzman, M., Anker-Kitai, L., Knoller, S., Bar, Y., Glandt, M., Herold, K., and Efrat, S. (2006) Expansion and redifferentiation of adult human pancreatic islet cells. Biochem. Biophys. Res. Commun. 339, 291–298

10. Lechner, A., Nolan, A. L., Blacken, R. A., and Habener, J. F. (2005) Reddifferentiation of insulin-secreting cells after in vitro expansion of adult human pancreatic islet tissue. Biochem. Biophys. Res. Commun. 327, 581–588

11. Parauda, G., Bosco, D., Berney, T., Pattou, F., Kerr-Conde, I., Donath, M. Y., Bruun, C., Mandrup-Poulsen, T., Billestrup, N., and Halban, P. A. (2008) Proliferation of sorted human and rat β cells. Diabetologia 51, 91–100

12. Beattie, G. M., Montgomery, A. M., Lopez, A. D., Hao, E., Perez, B., Just, M. L., Lakey, J. R., Hart, M. E., and Hayek, A. (2002) A novel approach to increase human islet cell mass while preserving β-cell function. Diabetes 51, 3435–3439

13. Russ, H. A., Bar, Y., Ravassard, P., and Efrat, S. (2008) In vitro proliferation of cells derived from adult human β-cells revealed by cell-lineage tracing. Diabetes 57, 1575–1583

14. Russ, H. A., Ravassard, P., Kerr-Conde, I., Pattou, F., and Efrat, S. (2009) Epithelial-mesenchymal transition in cells expanded in vitro from lineage-traced adult human pancreatic β cells. PLoS One 4, e6417

15. Russ, H. A., Sintov, E., Anker-Kitai, L., Friedman, O., Lenz, A., Toren, G., Farhy, C., Pasmanick-Chor, M., Oron-Karni, V., Ravassard, P., and Efrat, S. (2011) Insulin-producing cells generated from dedifferentiated human pancreatic β cells expanded in vitro. PLoS One 6, e25566

16. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) Notch signaling: cell fate control and signal integration in development. Science 284, 770–776

17. Kopan, R., and Ilagan, M. X. (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137, 216–233

18. Lammert, E., Brown, J., and Melton, D. A. (2000) Notch gene expression during pancreatic organogenesis. Mech. Dev. 94, 199–203

19. Jensen, J., Pedersen, E. E., Galante, P., Hald, J., Heller, R. S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., and Madsen, O. D. (2000) Control of endodermal endocrine development by Hes-1. Nat. Genet. 24, 36–44

20. Jensen, J. N., Cameron, E., Garay, M. V., Starkey, T. W., Gianani, R., and Jensen, J. (2005) Recapitulation of elements of embryonic development in adult mouse pancreatic regeneration. Gastroenterology 128, 728–741

21. Kopinke, D., Brailsford, M., Shea, J. E., Leavitt, R., Scaife, C. L., and Murtough, L. C. (2011) Lineage tracing reveals the dynamic contribution of Hes1+ cells to the developing and adult pancreas. Development 138, 433–441

22. Georgi, S., Soliz, R., Li, M., Zhang, P., and Bhushan, A. (2006) p57 and Hes1 coordinate cell cycle exit with self-renewal of pancreatic progenitors. Dev. Biol. 298, 22–31

23. Murata, K., Hattori, M., Hirai, N., Shinozuka, Y., Hirata, H., Kageyama, R., Sakai, T., and Minato, N. (2005) Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1. Mol. Cell. Biol. 25, 4262–4271

24. Bar, Y., Russ, H. A., Knoller, S., Ouziel-Yahalom, L., and Efrat, S. (2008) HES-1 is involved in adaptation of adult human β-cells to proliferation in vitro. Diabetes 57, 2413–2420

25. Rooman, I., De Medts, N., Baeyens, L., Lardon, J., De Breuck, S., Heimberg,
H. E. (2006) Expression of the Notch signaling pathway and effect on endocrine proliferation in adult rat pancreas. Am. J. Pathol. 169, 1206–1214

26. Baeyens, L., Bonné, S., Bos, T., Rooman, I., Peleman, C., Lahoutte, T., German, M., Heimberg, H., and Bouwens, L. (2009) Notch signaling as gatekeeper of rat acinar-to-β-cell conversion in vitro. Gastroenterology 136, 1750–1760

27. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. U.S.A. 95, 2509–2514

28. Otonkoski, T., Beattie, G. M., Mally, M. I., Ricordi, C., and Hayek, A. (2000) Role for activin A and betacellulin in human fetal pancreatic cell differentiation and growth. J. Clin. Endocrinol. Metab. 85, 3892–3897

29. Demeterco, C., Beattie, G. M., Dib, S. A., Lopez, A. D., and Hayek, A. (2006) A role for activin A and betacellulin in human fetal pancreatic cell differentiation and growth. J. Clin. Endocrinol. Metab. 85, 3892–3897

30. Farilla, L., Bulotta, A., Hirshberg, B., Li Calzi, S., Khoury, N., Noushmehr, H., Bertolotto, C., Di Mario, U., Harlan, D. M., and Perfetti, R. (2003) Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. Endocrinology 144, 5149–5158

31. Gittes, G. K., Prasadan, K., and Tulachan, S. (2010) Pancreas and islet development, in Stem Cell Therapy for Diabetes (Efrat, S., ed) pp. 3–40, Humana Press, New York

32. Andranci, S. S., Sampley, M. L., Vanderford, N. L., and Ozcan, S. (2008) Glucose regulation of insulin gene expression in pancreatic β-cells. Biochem. J. 415, 1–10

33. Bar-Nur, O., Russ, H. A., Efrat, S., and Benvenisty, N. (2011) Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet β cells. Cell Stem Cell 9, 17–23

34. Krechel, H. E., Mihaljevic, A. L., Hoffman, D. A., and Schneider, A. (2004) Neuronal co-expression of EGFP and β-galactosidase in mice causes neuropathology and premature death. Neurobiol. Dis. 17, 310–318

35. Cabrera, O., Berman, D. M., Kenyon, N. S., Ricordi, C., Berggren, P. O., and Caicedo, A. (2006) The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proc. Natl. Acad. Sci. U.S.A. 103, 2334–2339

36. D’Amour, K. A., Bang, A. G., Eliazer, S., Kelly, O. G., Agulnick, A. D., Smart, N. G., Moorman, M. A., Kroon, E., Carpenter, M. K., and Baetge, E. E. (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat. Biotechnol. 24, 1392–1401

37. Jiang, J., Au, M., Lu, K., Eshpeter, A., Korbutt, G., Fisk, G., and Majumdar, A. S. (2007) Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells 25, 1940–1953

38. Lukinivas, A., Ericsson, J. L., Grimelius, L., and Korsgren, O. (1992) Ultrastructural studies of the ontogeny of fetal human and porcine endocrine pancreas, with special reference to colocalization of the four major islet hormones. Dev. Biol. 153, 376–385

39. Bocian-Sobkowska, J., Zabel, M., Wozniak, W., and Surydz-Zasadza, J. (1999) Polyhormonal aspect of the endocrine cells of the human fetal pancreas. Histochem. Cell Biol. 112, 147–153

40. Lamouille, S., and Derynck, R. (2007) Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. J. Cell Biol. 178, 437–451

41. Moriyama, M., Osawa, M., Mak, S. S., Obitsu, T., Yamamoto, N., Han, H., Delmas, V., Kayagey, R., Reermann, F., Larue, L., and Nishikawa, S. (2006) Notch signaling via Hes1 transcription factor maintains survival of melanoblasts and melanocyte stem cells. J. Cell Biol. 173, 333–339

42. Rasul, S., Balasubramanian, R., Filipovic, A., Slade, M. J., Yagie, E., and Coombes, R. C. (2009) Inhibition of γ-secretase induces G2/M arrest and triggers apoptosis in breast cancer cells. Br. J. Cancer 100, 1879–1888

43. Miele, L., and Osborne, B. (1999) Arbiter of differentiation and death: Notch signaling meets apoptosis. J. Cell. Physiol. 181, 393–409

44. Jundt, F., Anagnostopoulos, I., Förster, R., Mathas, S., Stein, H., and Dörken, B. (2002) Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. Blood 99, 3398–3403

45. Jehn, B. M., Belke, W., Pear, W. S., and Osborne, B. A. (1999) Cutting edge: protective effects of notch-1 on TCR-induced apoptosis. J. Immunol. 162, 635–638

46. Nefedova, Y., Cheng, P., Alsina, M., Dalton, W. S., and Gabrilovich, D. I. (2004) Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. Blood 103, 3503–3510

47. Sade, H., Krishna, S., and Sarin, A. (2004) The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. J. Biol. Chem. 279, 2937–2944

48. Nair, P., Somasundaram, K., and Krishna, S. (2003) Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway. J. Virol. 77, 7106–7112

49. Mungamuri, S. K., Yang, X., Thor, A. D., and Somasundaram, K. (2006) Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. Cancer Res. 66, 4715–4724

HES1 Inhibition Redifferentiates Expanded Human β-Cells

H., and Bouwens, L. (2006) Expression of the Notch signaling pathway and effect on endocrine proliferation in adult rat pancreas. Am. J. Pathol. 169, 1206–1214