Tracing phenotypic reversibility of pancreatic β-cells *in vitro*

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ABSTRACT

Aims/Introduction: Studies have suggested that pancreatic β-cells undergo dedifferentiation during proliferation *in vitro*. However, due to limitations of the methodologies used, the question remains whether such dedifferentiated cells can redifferentiate into β-cells.

Materials and Methods: We have established a method for cell tracing in combination with fluorescence-activated cell sorter (FACS). Using this method, mouse pancreatic β-cells labeled with green fluorescent protein (GFP) under the control of the insulin promoter are collected by FACS. These β-cells can be traced and characterized throughout the culture process, even when insulin becomes undetectable, because the cells are also marked with monomeric red fluorescent protein (mRFP) driven by the CAG promoter.

Results: When cultured with fetal mouse pancreatic cells, FACS sorted β-cells lost GFP expression, but retained mRFP expression. The cells also lost expressions of genes characteristic of the β-cell phenotype, such as Pdx1 and glucokinase, indicating dedifferentiation. More than 30% of such dedifferentiated pancreatic β-cells were detected in S or G2/M phase. Furthermore, these dedifferentiated cells redifferentiated into insulin-expressing cells on cultivation with a MEK1/2 inhibitor.

Conclusions: Our data provide direct evidence that pre-existing β-cells can undergo dedifferentiation and redifferentiation *in vitro*, their phenotype is reversible and that dedifferentiation in β-cells is associated with progression of the cell cycle. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00051.x, 2010)

KEY WORDS: Dedifferentiation, Redifferentiation, Cell cycle

INTRODUCTION

Pancreatic β-cells possess a well-regulated insulin secretory property that maintains systemic glucose homeostasis[1]. Although it has long been thought that differentiated β-cells are nearly static, recent studies have shown that β-cell mass changes dynamically throughout a lifetime[2]. In addition, it has been shown that the replication of pre-existing β-cells is a major source for the maintenance of β-cell mass in adult mouse pancreas[3], although cells other than pre-existing β-cells might also contribute to the generation of new β-cells[4,5]. Because expansion of pancreatic β-cells *in vitro* represents an attractive strategy for cell therapy in diabetes, many attempts to establish a method for increasing β-cell mass have been reported[6–8]. In fact, isolated pancreatic β-cells from rodents and humans have been shown to proliferate under certain conditions *in vitro*[9,10]. However, the phenotypic changes shown by β-cells during *in vitro* proliferation have not been examined in detail.

Epithelial-mesenchymal transition (EMT) of pancreatic β-cells was originally reported by Gershengorn *et al.*[11] and a similar phenomenon was observed by others[12]. They suggested that pancreatic β-cells were expandable *in vitro* by EMT and that the transitional β-cells have progenitor-like properties[11,12]. In contrast, other recent studies using cell lineage tracing maintained that β-cells do not undergo EMT[13–15]. They found that most proliferative mesenchymal cells migrating out of pancreatic islets *in vitro* were not derived from β-cells, and suggest that these cells do not represent a useful source for the generation of physiologically competent β-cells for the treatment of diabetes[13,15]. These studies did not exclude the possibility that differentiated pancreatic β-cells can undergo phenotypic change, however. While most mesenchymal cells in the culture were found not to originate from pre-existing β-cells, some pre-existing β-cells clearly changed their phenotype to one having a fibroblast-like morphology[13]. Indeed, it has been shown by cell lineage tracing that pre-existing pancreatic β-cells can undergo dedifferentiation *in vitro*, although these dedifferentiated β-cells were eventually lost during long-term culture[16]. Thus, although pancreatic

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Table 1 | Sequences of PCR primers

Name	Forward primer	Reverse primer	Size
β-actin	TTCTTTGCAGCTCCTTCGTTG	ATGCCGGAGCCGTTGTC	95 bp
GAPDH	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT	132 bp
Insulin I	GCAAGCAGGTCATTGTTTCA	CACTTGTGGGTCCTCCACTT	211 bp
Insulin II	GCCCTAAGTGATCCGCTACAATCAA	GCGCATCCACAGGGCCAT	90 bp
Glucokinase	GCCACAATGATCTCCTGCTA	CACATTCTGCATCTCCTCCA	102 bp
GLUT2	AATGGTCGCCTCATTCTTTG	ATCAAGAGGGCTCCAGTCAA	102 bp
Kir6.2	AGACACGGCGGGATAAGTC	GGAACGACGACAAAATGAGG	187 bp
SUR1	ACTTTGCTGAAACCGTGGAA	AGGGAGTTGGAGATGGAGGT	208 bp
Cav1.2	TCCTACCAGGAAGATGAACA	GGATGGGAAGCTGCTGTTGA	390 bp
Cav1.3	CTTCGTCATCGTCACCTTCCA	TGAACATCTTGGACTGCTCA	254 bp
Munc18a	AGATGCGCTGTGCTTACGAA	CACAGGAGAAGAACTCAGCA	281 bp
Rab27b	CCAGACCAAAGGGAAGTCAA	AAGTCCAGAAGCGTTTCCAC	128 bp
Syntaxin1a	AAGAAGGCCGTCAAGTACCA	GTGGGGTGGTTTCTATCCAA	126 bp
VAMP2	TTGAAACAAGTGCAGCCAAG	GGCAGACTCCTCAGGGATTT	140 bp
PC1/3	ATGGAGCAAAGAGGTTGGAC	GCTGCAGTCATTCTGGTATC	419 bp
PC2	TGGTGTGGCTACCACAGACT	TGCATGTCTCTCCAGGTCAG	135 bp
Chromogranin A	CCTCTCTATCCTGCGACACC	GGGCTCTGGTTCTCAAACAC	201 bp
Chromogranin B	ACAGGAAGAAGGCAGACGAA	TCCTTCAGTGAAAGGCTCGT	228 bp
Pdx1	CCACCCCAGTTTACAAGCTC	TGTAGGCAGTACGGGTCCTC	325 bp
NeuroD	GCTCCAGGGTTATGAGATCG	CGCTCTCGCTGTATGATTTG	83 bp
Nkx2.2	TCTACGACAGCAGCGACAAC	ATTTGGAGCTCGAGTCTTGG	114 bp
Nkx6.1	CTTGGCAGGACCAGAGAGAG	CCGAGTCCTGCTTCTTCTTG	146 bp
Isl1	CACTATTTGCCACCTAGCCAC	AAATACTGATTACACTCCGCAC	256 bp
Hes1	CTACCCCAGCCAGTGTCAAC	ATGCCGGGAGCTATCTTTCT	171 bp
E-cadherin	ACTGTGAAGGGACGGTCAAC	GGAGCCACATCATTTCGAGT	307 bp
Nestin	CTCTGCTGGAGGCTGAGAAC	ATTAGGCAAGGGGGAAGAGA	176 bp
Vimentin	CCTGTACGAGGAGGAGATGC	GTGCCAGAGAAGCATTGTCA	206 bp
Cyclin A1	CAAGGTCCTGATGCTTGTCA	CCCATGGTCAGAGAGCACTT	198 bp
Cyclin A2	CTGTCTCTTTACCCGGAGCA	AGTGATGTCTGGCTGCCTCT	227 bp
Cyclin D1	TGGTGAACAAGCTCAAGTGG	GCAGGAGAGGAAGTTGTTGG	248 bp
Cyclin D2	TTACCTGGACCGTTTCTTGG	TGCTCAATGAAGTCGTGAGG	240 bp
Cyclin D3	AGGCCCTAGGACTCGCTAAC	TTTGCACGCACTGGAAGTAG	203 bp
Cyclin E1	CCTCCAAAGTTGCACCAGTT	GGACGCACAGGTCTAGAAGC	241 bp
Cyclin E2	TCTGTGCATTCTAGCCATCG	ACAAAAGGCACCATCCAGTC	156 bp
Cdk2	CATTCCTCTTCCCCTCATCA	GCAGCCCAGAAGAATTTCAG	238 bp
Cdk4	CAATGTTGTACGGCTGATGG	CAGGCCGCTTAGAAACTGAC	178 bp
$p15^{INK4b}$	AAGGACCATTTCTGCCACAG	CGCAGTTGGGTTCTGCTC	234 bp
$p16^{INK4a}$	CTTTGTGTACCGCTGGGAAC	CGCTAGCATCGCTAGAAGTG	158 bp
$p18^{INK4c}$	CGTCAACGCTCAAAATGGAT	GGGCAGGTTCCCTTCATTAT	229 bp
$p19^{INK4d}$	CCACCGGTATCCACTATGCT	TCAGGAGCTCCAAAGCAACT	199 bp
$p21^{Cip1}$	GTACTTCCTCTGCCCTGCTG	TCTGCGCTTGGAGTGATAGA	173 bp
$p27^{Kip1}$	TTGGGTCTCAGGCAAACTCT	TCTGTTCTGTTGGCCCTTTT	157 bp
$p57^{Kip2}$	CTGAAGGACCAGCCTCTCTC	TCTGGCCGTTAGCCTCTAAA	229 bp
Foxo1	AAGAGCGTGCCCTACTTCAA	CTCCCTCTGGATTGAGCATC	157 bp
Ki67	CCAGCTGCCTGTAGTGTCAA	CCTTGATGGTTCCTTTCCAA	250 bp
PCNA	GAAGGCTTCGACACATACCG	TCTGGGATTCCAAGTTGCTC	227 bp

Sequences of PCR primers are designed such that the amplified regions spanned introns in the gene, except for the Kir6.2 gene, which has no intron in the protein coding region.

β-cells might possess phenotypic plasticity *in vitro*, the properties and fate of such dedifferentiated pancreatic β-cells are not known.

In the present study, we established a culture system using fetal pancreatic cells as feeder cells that induce dedifferentiation of adult pancreatic β-cells. We also developed a method whereby pre-existing pancreatic β-cells can be traced throughout the culture process, even when the cells lose insulin expression. Using this method, we provide direct evidence that pre-existing β-cells undergo dedifferentiation and redifferentiation *in vitro*

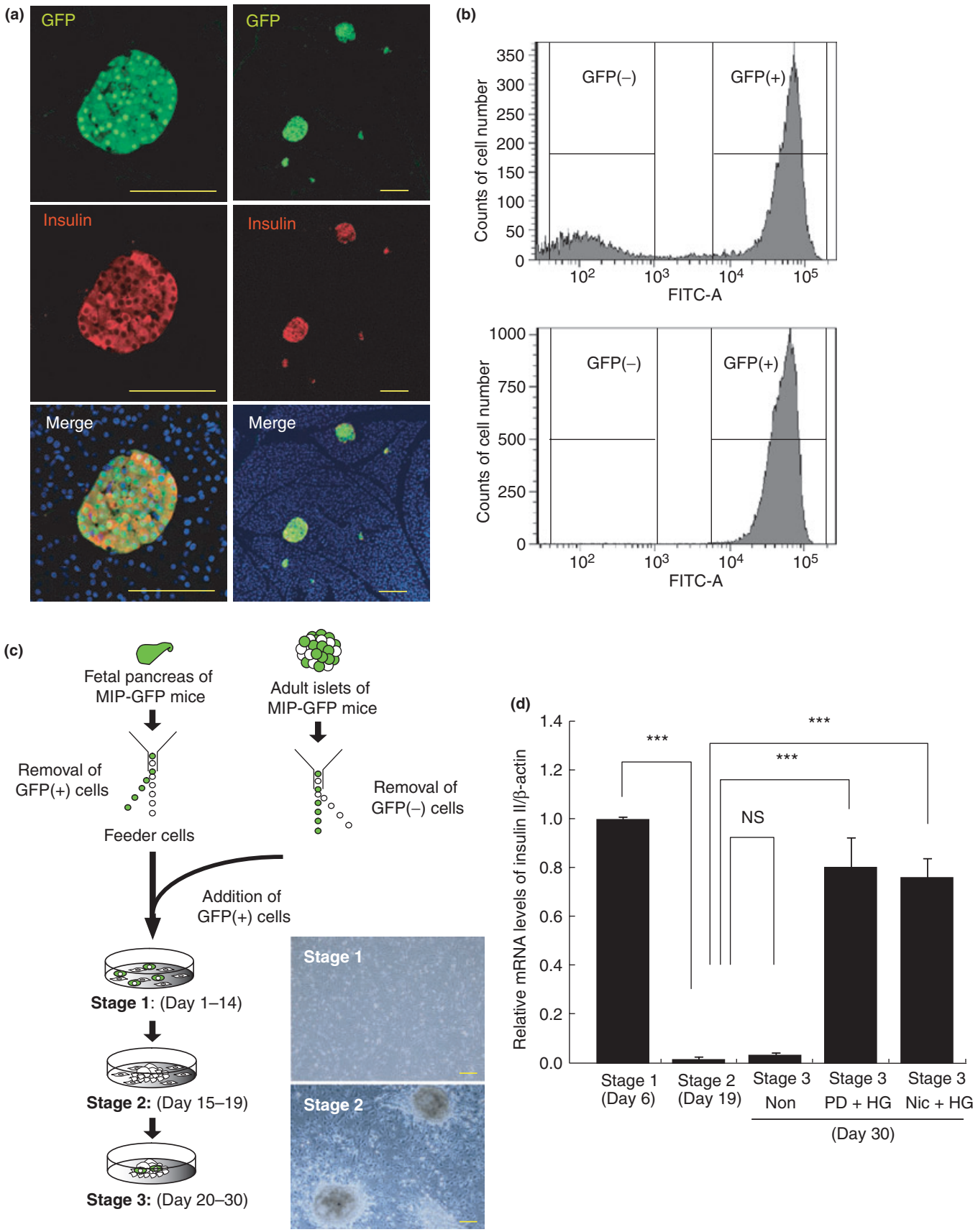

Figure 1 | Phenotypic change of adult pancreatic β-cells. (a) Immunostaining of MIP-GFP mouse pancreas with anti-green fluorescent protein (GFP) and anti-insulin antibodies. Sections were analyzed by confocal microscopy. Bar, 100 μm. (b) Flow cytometric analysis of islet cells from adult MIP-GFP mice (upper panel). Verification of sorted GFP (+) cells (lower panel). (c) Outline of the protocol. GFP-positive β-cells were purified from adult islets of MIP-GFP mice and cultured with feeder cells derived from fetal pancreas of MIP-GFP mice after removal of GFP-positive β-cells. (d) Quantitative RT–PCR analysis for the insulin II gene. Data are means ± SEM of three to six independent experiments. HG, 16.7 mmol glucose; Nic, nicotinamide; NS, not significant; PD, PD98059. ***$P < 0.001$.

and that such dedifferentiation in β-cells is associated with the progression of the cell cycle.

MATERIALS AND METHODS

Animals

C57BL/6 Cr mice were purchased from Japan SLC (Shizuoka, Japan). Transgenic mice expressing green fluorescent protein (GFP) under the control of mouse insulin I promoter (MIP) (MIP-GFP mice)[17] and transgenic mice expressing monomeric red fluorescent protein (mRFP) under the control of CAG promoter (CAG-mRFP mice) were crossed to produce double heterozygous (MIP-GFP/CAG-mRFP) mice. All animal experiments were approved by the Animal Research Committees of Kyoto University Graduate School of Medicine and Kobe University Graduate School of Medicine.

Isolation and Culture of Mouse Pancreatic Cells

On day 1, for preparation of feeder cells, pancreata were dissected from 24 fetal mice at embryonic day 17 and digested with 3 mL of Hank's balanced salt solution containing 3 mg of collagenase D and 200 units of DNase I (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 17 min at 37°C. Dissociated fetal pancreatic cells were cultured with RPMI-1640 medium containing 5.6 mmol/L glucose (Invitrogen, Gaithersburg, MD, USA), 10% FCS (Sigma, St. Louis, MO, USA), and 2 ng/mL FGF2 (R&D Systems, Minneapolis, MN, USA) at 37°C with 95% air and 5% CO_2. When using fetal MIP-GFP mouse pancreatic cells as a feeder, GFP-positive β-cells were removed by fluorescence-activated cell sorter (FACS) as described later before culture. On day 6, the cells grown to confluence were harvested and replated to 100-mm dishes at 2×10^6 cells/dish. On day 9, monolayer cells were harvested and replated to a 12-well plate at 1×10^5 cells/well. By day 15, small plaques were formed in monolayer cells (day 1–14: Stage 1). At this point, the medium was replaced with RPMI-1640 containing 5.6 mmol/L glucose, 10% FCS, 2 ng/mL FGF2, and 10 ng/mL betacellulin (R&D Systems). Islet-like spherical clusters (ISC) were formed in this stage (day 15–19: Stage 2). On day 19, ISC were picked up and plated to 6-well dishes with RPMI-1640 containing 5.6 or 16.7 mmol/L glucose, 10% FCS, and 10 mmol/L nicotinamide or 50 μmol/L PD98059 (Calbiochem, San Diego, CA, USA) (day 20–30: Stage 3).

Tracing of Pre-existing Pancreatic β-cells

Islets isolated from mice at 12–20 weeks-of-age were hand-picked under a dissecting microscope[18]. GFP/mRFP-double positive β-cells were sorted from double transgenic mice expressing GFP in β-cells and mRFP in all of the cells by FACS Aria (Becton Dickinson, San Jose, CA, USA) with fluorescein isothiocyanate (FITC) band-pass filter (530/30 nm). The sorted GFP/mRFP-double positive β-cells were cultured with fetal pancreas-derived feeder cells of wild-type mice, which do not express any fluorescent proteins. By the end of Stage 1, pre-existing β-cells became GFP-negative/mRFP-positive as a result of inactivity of the insulin promoter. Subsequently, the mRFP-positive β-cell progenies were collected by FACS for further characterization. For this purpose, a phycoerythrin (PE)-Texas Red-band pass filter (610/20 nm) was used.

Cell Cycle Analysis

GFP-negative/mRFP-positive dedifferentiated β-cells were purified by the above procedure. The cells were suspended in 0.2% Triton X-100 solution containing 50 μg/mL propidium iodide (PI; Sigma) and analyzed by using FACS Calibur (Becton Dickinson). The proportion of cells in each phase of the cell cycle was calculated by ModFit *LT*, a flow cytometry modeling software (Verity Software House, Topsham, ME, USA).

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde and permeabilized with 10% normal goat serum and 0.2% Tween-20. Primary antibodies used were guinea pig anti-insulin (Zymed, San Francisco, CA, USA), rat anti-nestin (American Research Product, Belmont, MA, USA), goat anti-vimentin (Sigma), mouse anti-BrdU (Sigma), and rabbit anti-DsRed (Becton Dickinson), which was crossed with mRFP[19]. Secondary antibodies labeled with Alexa Fluor 488 or 546 (Molecular Probe, Eugene, OR, USA) were used for detection. Nuclear staining was carried out by 4′,6-diamino-2-phenylindole (DAPI) (Dojindo, Kumamoto, Japan).

RT–PCR Analysis

Total RNA was isolated with RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. After treatment with DNase I (Qiagen), 1 μg of the RNA was reverse-transcribed by ReverTra Ace (Toyobo, Osaka, Japan) and 1/200 volume of resultant cDNA was subjected to PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR primers were designed such that the amplified regions spanned introns in the gene, except for the Kir6.2 gene, which has no intron in the protein-coding region (Table 1). The cDNA products were amplified by 30 cycles for β-actin and

GAPDH, and by 35 cycles for all other genes. Real-time PCR was carried out using SYBR green PCR master mix (Applied Biosystems) with a model 7700 real-time thermal cycler (Applied Biosystems). Expression of β-actin or GAPDH was used as an internal control.

RESULTS

Establishment of a Culture System to Assess Phenotypic Reversibility of Pancreatic β-cells

We first established a culture system for the assessment of phenotypic reversibility of pancreatic β-cells. To this end, we utilized MIP-GFP mice[17], in which pancreatic β-cells were labeled with GFP under the control of mouse insulin 1 promoter. There was no leaky expression of GFP in pancreatic cells other than β-cells (Figure 1a), confirming specificity of the labeling. GFP-positive β-cells were purified from isolated pancreatic islets of MIP-GFP mice by FACS. Analysis of pancreatic islet cell preparations of MIP-GFP mice showed a clear pattern of GFP-positive cells and GFP-negative cells (Figure 1b, upper panel). The purity of post-sorted GFP-positive cells was more than 99% (Figure 1b, lower panel). We confirmed that all of the FACS-purified GFP-positive cells were positive for insulin and negative for glucagon, somatostatin and pancreatic polypeptide by immunostaining (data not shown). The purified β-cells labeled with GFP were cultured with GFP-positive cell-free feeder cells prepared from the pancreas of fetal MIP-GFP mice (Figure 1c). When cultured in RPMI-1640 medium containing 10% FCS, 5.6 mmol/L glucose, and 2 ng/mL FGF2, the cells expanded and formed a monolayer (referred to as Stage 1; Figure 1c). Insulin expression was detected in the starting material, but became barely detectable after several passages by the end of Stage 1. At late Stage 1, monolayer cells began to form plaques as they became confluent. By the addition of 10 ng/mL betacellulin, these plaques grew to form 3-D ISC (Stage 2). When ISC were hand-picked and cultured further with a high concentration (16.7 mmol/L) of glucose in combination with 10 mmol/L nicotinamide or 50 mmol/L PD98059, a MEK1/2 inhibitor, insulin expression was induced (Stage 3; Figure 1d). This culture system enabled us to investigate phenotypic plasticity of pancreatic β-cells.

Tracing and Characterization of Dedifferentiated Pre-existing Pancreatic β-cells

Because the β-cells of MIP-GFP mice no longer express GFP and are indistinguishable from feeder cells at the end of Stage 1 (day 14), the β-cell progenies cannot be traced and characterized. To trace and characterize both pre-existing β-cells and their progenies, we generated double transgenic mice expressing GFP in the β-cells and mRFP in all of the cells (MIP-GFP/CAG-mRFP mice; Figure 2a, left panels). GFP-expressing pancreatic β-cells were collected from the double transgenic mice by using FACS. The sorted cells were labeled with both GFP and mRFP (Figure 2a, right panels). Because mRFP expression is driven by the CAG promoter, the sorted β-cells and their progenies can be traced by red fluorescence even though the cells lose GFP expression. In addition, unlike conventional cell tracing methods, this system can monitor insulin promoter activity in living cells by evaluating GFP expression (Figure 2b).

Sorted pancreatic β-cells from MIP-GFP/CAG-mRFP mice were cultured with fetal pancreas-derived feeder cells of wild-type mice. We did not use MIP-GFP mice as a feeder source in this case, because MIP-GFP/CAG-mRFP mice-derived β-cells were distinguishable from the fetal pancreas-derived β-cells by red fluorescence. As expected, GFP fluorescence was very weak or undetectable at the end of Stage 1, whereas mRFP fluorescence could be detected throughout the culture (Figure 2c). This clearly shows that the pre-existing β-cells no longer express insulin at the end of Stage 1. A subset of GFP-negative/mRFP-positive cells was also positive for Ki67 (Figure 2d), showing that the cells at this stage have proliferating capacity. BrdU incorporation analysis confirmed this (Figure 2d). Interestingly, most, but not all GFP-negative/mRFP-positive cells, which originated from pre-existing β-cells, showed fibroblast-like morphology expressing nestin and vimentin (Figure 2e).

We then investigated the gene expression profile of pre-existing β-cell-derived cells at the end of Stage 1. For this purpose, we collected the β-cell-derived cells (GFP-negative/mRFP-positive cells at the end of Stage 1) by FACS (Figure 3a). Fluorescence microscopy showed that all of the post-sorted cells were mRFP-positive and GFP-negative (Figure 3b). The gene expression profile of the cells was compared to that of fresh β-cells sorted from the islets of the same mice (Figure 3c). The cultured β-cell-derived cells did not express, or showed markedly decreased expressions, of insulins, glucokinse, GLUT2, Kir6.2, SUR1, PC1/3 and chromogranin A, all of which are markers of differentiated pancreatic β-cells. The transcription factors Pdx1, Nkx2.2, and Nkx6.1, which are required for the development of the β-cells, were also downregulated. In contrast, the expressions of Hes1, nestin, and vimentin were induced or markedly increased in the cultured β-cell progenies. E-cadherin was very poorly expressed at this stage. These data show the occurrence of dedifferentiation in pancreatic β-cells.

Dedifferentiation is Associated with Cell Cycle Progression

The findings described earlier suggest that dedifferentiation of pancreatic β-cells is associated with progression of the cell cycle. We then compared the cell cycle of purified β-cells and dedifferentiated β-cells. For this purpose, GFP-positive β-cells were freshly isolated from pancreatic islets of adult MIP-GFP mice. Cell cycle analysis by flow cytometry showed that the proportions of cells in G0/G1, S and G2/M phase were 98.9, 0.9 and 0.2%, respectively, showing that most adult pancreatic β-cells are in G0/G1 phase (Figure 4a, left panel). We then obtained GFP-negative/mRFP-positive cells (dedifferentiated β-cells) at the end of Stage 1, as described in Figure 3a, and evaluated the cell cycle. Unlike freshly isolated native β-cells, the proportions of G0/G1, S and G2/M phase in the dedifferentiated β-cells were 66.8, 26.8 and 6.4%, respectively (Figure 4a, right panel).

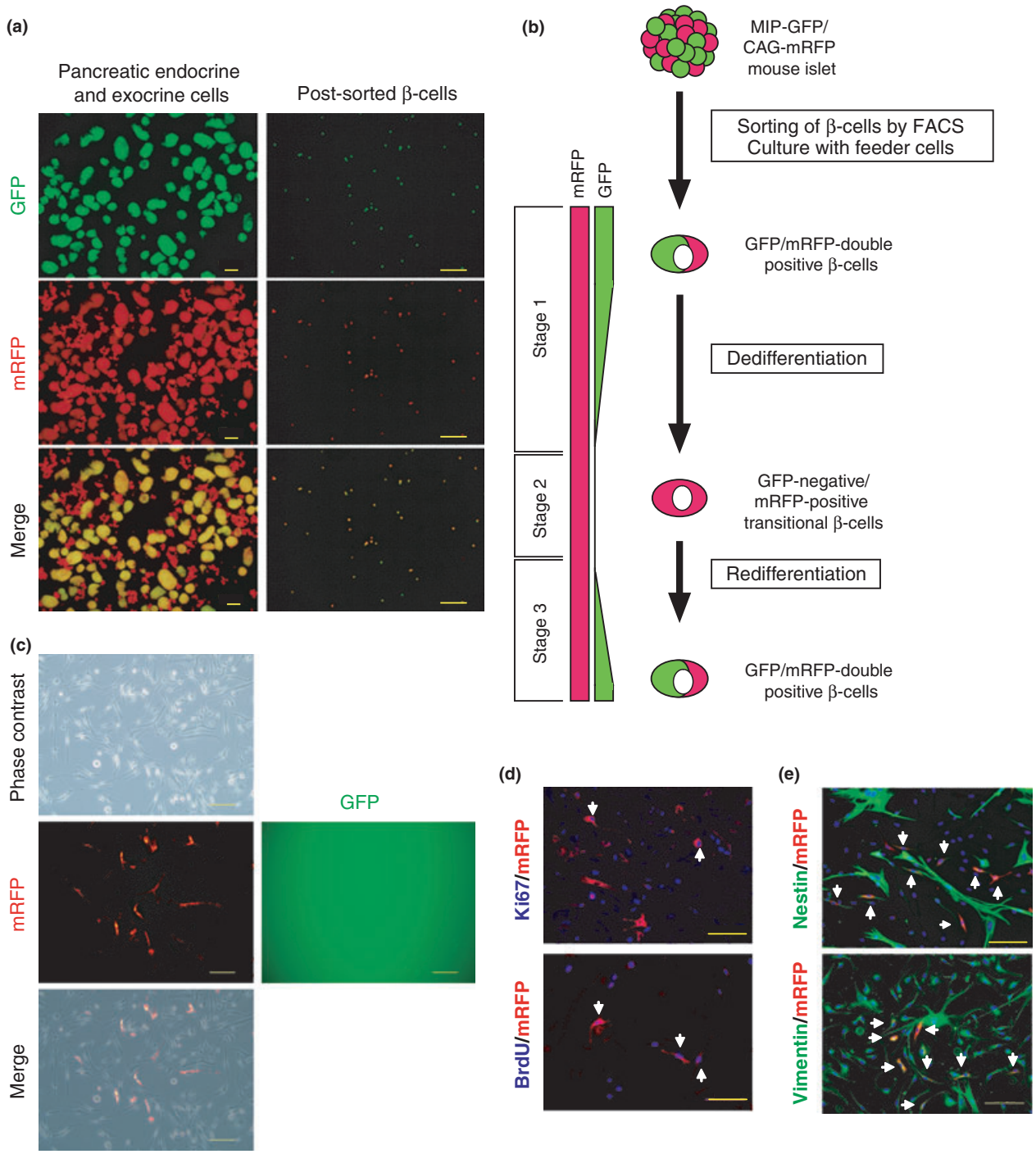

Figure 2 | Evidence for dedifferentiation of adult pancreatic β-cells. (a) Fluorescence of pancreatic cells of adult MIP-GFP/CAG-mRFP-double transgenic mice. Monomeric red fluorescent protein (mRFP) fluorescence is detected in all of the cells, including exocrine cells, whereas green fluorescent protein (GFP) is expressed only in insulin-expressing cells (left panels). All of the sorted β-cells expressed both GFP and mRFP (right panels). Bars, 100 μm. (b) Schematic representation of dedifferentiation and redifferentiation of the β-cells. GFP/mRFP-double positive β-cells were purified from adult islets of MIP-GFP/CAG-mRFP-double transgenic mice, and then cultured with fetal pancreas-derived feeder cells of wild-type mice. (c) Microscopic observation of the dedifferentiated β-cells at the end of Stage 1 (day 14). No GFP fluorescence was detected in mRFP-positive cells. (d) Proliferation of β-cells cultured with fetal pancreas-derived feeder cells at the end of Stage 1 (day 14). Both mRFP/Ki67-double positive cells and mRFP/BrdU-double positive cells were detected. Note that mRFP is visualized with anti-DsRed antibody, because the fluorescence is lost during boiling for activation of antigens. (e) Expression of nestin and vimentin in mRFP-positive cells. Most, but not all, dedifferentiated β -cells that express mRFP were positive for nestin or vimentin. FACS, fluorescence-activated cell sorter.

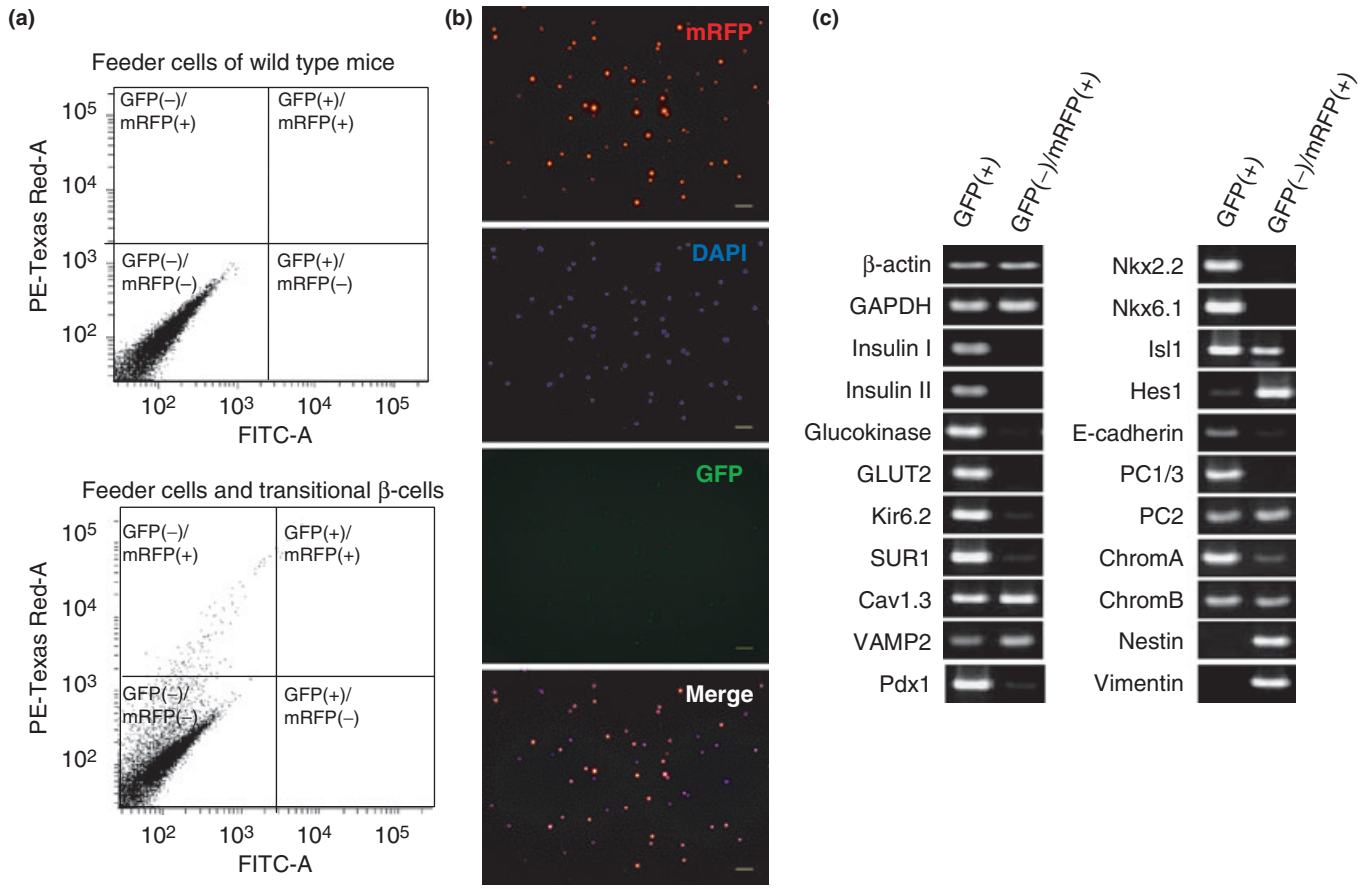

Figure 3 | Isolation and characterization of dedifferentiated β-cells. (a) Isolation of GFP(–)/mRFP(+)-dedifferentiated β-cells cultured on feeder cells by fluorescence-activated cell sorter (FACS). Representative results of FACS analysis of fetal pancreas-derived feeder cells of wild-type mice for controls (upper panel). Cultured cells at the end of Stage 1 (day 14), which contains both GFP(–)/mRFP(+)-β-cells and feeder cells, were analyzed (lower panel). (b) Fluorescence microscopy of post-sorted GFP(–)/mRFP(+)-β-cells. All of the sorted cells were positive for monomeric red fluorescent protein (mRFP) and negative for green fluorescent protein (GFP). (c) Gene expression analysis. GFP(+) represents GFP-positive cells sorted from freshly isolated islets; GFP(–)/mRFP(+) represents the dedifferentiated β-cells in culture sorted with mRFP. Chrom, chromogranin; PC, prohormone convertase.

Indeed, cell cycle-associated genes including cyclin D1, cyclin D3, Ki67, PCNA, cyclinA2, $p21^{Cip1}$, $p57^{Kip2}$, $p16^{INK4a}$ and cdk2 were found to be upregulated in the dedifferentiated β-cells (Figure 4b). These results clearly show that although most native pancreatic β-cells are in G0/G1 cell cycle *in vivo*, they have the potential to undergo cell cycle progression, which is associated with dedifferentiation.

Evidence for Redifferentiation of the β-cell Progenies

Re-expression of the insulin gene at Stage 3 is shown by real-time R–PCR analysis (Figure 1d). However, it cannot be ruled out that the β-cell progenitors present in fetal pancreas differentiate into insulin-producing β-cells. To investigate this, we cultured GFP-positive cell-free fetal pancreatic cells of MIP-GFP mice with or without the addition of GFP-positive β-cells of adult MIP-GFP mice. Although ISC were formed under both conditions at Stage 2, insulin expression was induced at Stage 3 only when GFP-positive cells were added to the starting material (Figure 5). These results show that the insulin-positive cells induced at Stage 3 originated not from immature progenitor cells but from pre-existing β-cells in adult pancreas. Furthermore, we confirmed that although no GFP-positive cells were present in ISC before induction (Stage 2), GFP/mRFP-double positive cells (<10% of all mRFP-positive cells) reappeared at the end of Stage 3 (Figure 6a). In addition to insulins, other β-cell specific genes were re-expressed at this stage (Figure 6b). These results provide direct evidence of redifferentiation of dedifferentiated pancreatic β-cells.

DISCUSSION

We have provided direct evidence that adult mouse pancreatic β-cells can dedifferentiate into proliferative fibroblast-like cells when cultured with fetal pancreatic cells, and that these cells can revert to insulin-expressing cells *in vitro*. Phenotypical change in

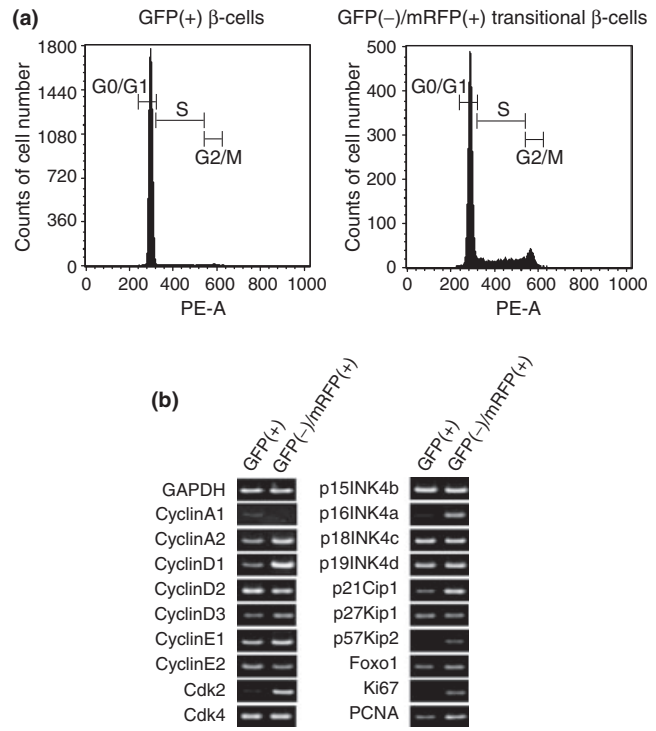

Figure 4 | Cell cycle analysis of dedifferentiated β-cells. (a) Cell cycle analysis. Representative histograms of green fluorescent protein (GFP)-positive cells from freshly isolated islets (left panel) and dedifferentiated β-cells (right panel) at the end of Stage 1 are shown. The proportions of cells in G0/G1, S and G2/M phase were 98.9, 0.9 and 0.2%, respectively (left panel), whereas the proportions of cells in G0/G1, S and G2/M phase were 66.8, 26.8 and 6.4%, respectively (right panel). (b) Gene expression analysis. GFP(+) represents GFP-positive cells sorted from freshly isolated islets, and GFP(−)/mRFP(+) represents dedifferentiated β-cells in culture sorted with monomeric red fluorescent protein (mRFP). PE-A, phycoerythrin-A.

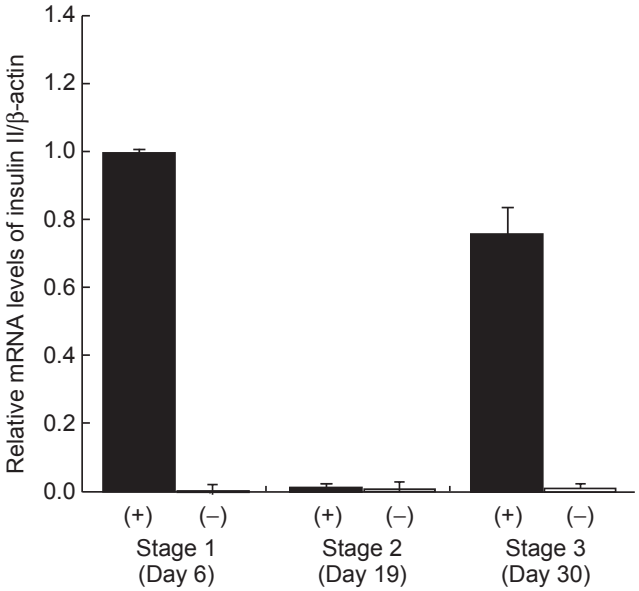

Figure 5 | Reversion of insulin-expressing cells. Green fluorescent protein (GFP)-positive cell-free fetal pancreatic cells of MIP-GFP mice were cultured with (+) or without (–) the addition of GFP-positive β-cells of adult MIP-GFP mice. Quantitative RT–PCR analysis of the insulin II gene is shown. Data are means ± SEM of three to four independent experiments.

pancreatic β-cells has been shown in recent studies[13,15], but the properties of the dedifferentiated cells have not been examined in detail. Although permanent labeling with the Cre/loxP-based method is useful for tracing pre-existing β-cells[13,15], it is difficult to identify and isolate dedifferentiated pancreatic β-cells by this method, because loss of insulin expression can be ascertained only by immunohistochemistry. In contrast, in our system using MIP-GFP/CAG-mRFP mice, pre-existing pancreatic β-cells can be purified with FACS, making it possible to monitor insulin expression throughout the culture in real-time by green fluorescence. In addition, dedifferentiated pancreatic β-cells can be analyzed by collecting the cells with FACS by red fluorescence after elimination of non-dedifferentiated β-cells by green fluorescence. Our results clearly show that pre-existing pancreatic β-cells lose expressions of β-cell specific genes and begin to express genes associated with the properties of mesenchymal cells under the conditions used.

Several studies refuting EMT of pancreatic β-cells have recently been published[13–15]. However, in these studies, some of the pre-existing β-cells were found to attain a fibroblast-like morphology, although the cells did not completely lose their original phenotype[13,15]. We have found that pre-existing β-cells change their phenotype to one having proliferative capacity, fibroblast-like morphology, and expressing nestin and vimentin while still retaining endocrine cell-like properties, such as expression of chromogranin. These findings are not inconsistent with their findings[13,15], and suggest that adult pancreatic β-cells possess phenotypic plasticity.

Because the dedifferentiated pancreatic β-cells eventually were lost during long-term culture in recent studies[14,16], it is not known if the dedifferentiated β-cells have the potential to redifferentiate into insulin-expressing cells. By using fetal pancreatic cells as feeder cells, we were able to maintain pre-existing β-cells throughout the culture. It is likely that the fetal pancreatic cells provide an environment for dedifferentiation of the β-cells. In fact, when ISC were released from this environment and cultured further with PD98059, insulin was re-expressed in the ISC. This is the first direct evidence of reversible phenotypic change in pancreatic β-cells.

We also analyzed the cell cycle of pancreatic β-cells purified from intact islets for the first time. We found that 98.9% of normal β-cells were in G0/G1 phase and that 1% were in S or G2/M phase. This shows that the rate of pancreatic β-cell turnover is very low. However, 33% of the β-cells were in S or G2/M phase when they underwent dedifferentiation *in vitro*, showing that native pancreatic β-cells have the potential to proliferate

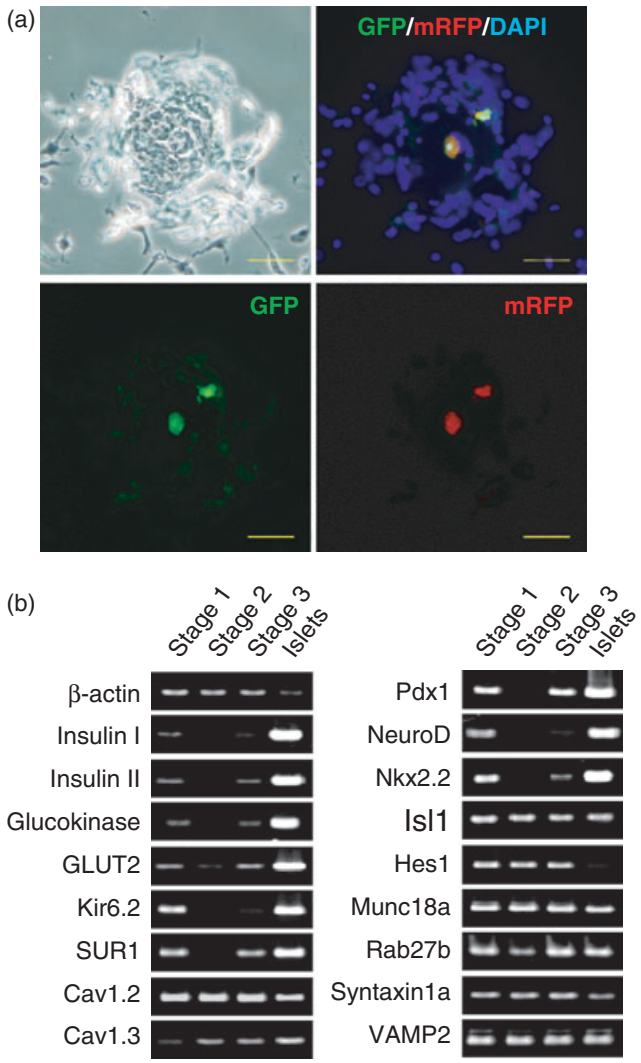

Figure 6 | Re-expression of green fluorescent protein (GFP) at Stage 3. GFP/monomeric red fluorescent protein (mRFP)-double positive β-cells were sorted from the GFP/mRFP-double transgenic mice by fluorescence-activated cell sorter (FACS). The sorted GFP/mRFP-double positive β-cells were cultured with fetal pancreas-derived feeder cells of wild-type mice, which do not express any fluorescent proteins. We confirmed the loss of insulin expression at the end of Stage 1. Islet-like spherical clusters (ISC) formed at Stage 2 were picked up and cultured in the presence of PD98059 or nicotinamide. (a) GFP/mRFP-double positive cells detected at Stage 3. (b) Gene expression analysis during culture.

and that there is a close relationship between phenotypic change (dedifferentiation) and progression of the cell cycle in pancreatic β-cells.

In contrast to a previous study showing that dedifferentiated pancreatic β-cells rarely proliferate[16], we found that a considerable number of the cells *in vitro* entered the cell cycle. In our culture system, removal of FGF2 from the expanding stages (Stages 1 and 2) results in a marked decrease in the number of β-cells at Stage 3, whereas treatment of ISC with FGF2 at Stage 3 inhibits induction of β-cells (data not shown). FGF2 is known to activate the MEK/MAPK pathway[20]. At Stage 3, treatment with the MEK1/2 inhibitor PD98059 results in reversion to insulin-secreting cells. Thus, the MEK/MAPK pathway might play a pivotal role in both proliferation and phenotypic change. The effects of FGF2 as well as the use of fetal pancreatic cells could account for the difference in proliferative potential of de-differentiated β-cells between our data and Weinberg's report[16].

In pancreatic islets, the β-cells form 3-D structures with both cell–cell and cell–matrix contacts[21,22]. In our culture system, pancreatic islets were dissociated into single cells that lost all of these contacts. As disruption of basement membranes and loss of intercellular contact are known to cause dedifferentiation[23,24], it is not unlikely that cell dispersion triggers the dedifferentiation process in pancreatic β-cells. We found that dedifferentiated pancreatic β-cells formed ISC at Stage 2 and re-expressed insulin at Stage 3. These cells are compactly packed into ISC, which are abundant in laminins (data not shown). Thus, cell–cell and cell–matrix contacts are reconstituted at Stage 3, and the micro-environment of the cells within the ISC might be critical in redifferentiation.

In summary, pre-existing β-cells undergo dedifferentiation and redifferentiation *in vitro*, their phenotype being reversible, and such dedifferentiation is associated with the progression of the cell cycle in β-cells. Further studies are needed to clarify whether such phenotypic change occurs *in vivo* under normal and pathological states.

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