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Tracing phenotypic reversibility of pancreatic β-cells in vitro

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Short title: Phenotypic reversibility of β-cells

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Abstract

Aims/Introduction. Studies have suggested that pancreatic β-cells undergo dedifferentiation during proliferation *in vitro*. However, due to limitation 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 FACS. Using this method, mouse pancreatic β-cells labeled with green fluorescent protein (GFP) under control of the insulin promoter are collected by fluorescence-activated cell sorter (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 upon cultivation with a MEK1/2 inhibitor.

Conclusions. Our data provide direct evidence that preexisting β-cells can undergo dedifferentiation and redifferentiation *in vitro*, their phenotype being reversible, and that dedifferentiation in β-cells is associated with progression of the cell cycle.

Keywords: 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 lifetime[2]. In addition, it has been shown that replication of preexisting β-cells is a major source for the maintenance of β-cell mass in adult mouse pancreas[3], although cells other than preexisting β-cells may 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 human have been shown to proliferate under certain conditions *in vitro*[9, 10]. However, the phenotypic changes exhibited 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]. To the contrary, other recent studies using cell lineage tracing maintain that β-cells do not undergo EMT[13-15]. They found that most proliferative mesenchymal cells migrating out from 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 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 preexisting β-cells, some preexisting β-cells clearly changed their phenotype to one having a fibroblast-like morphology[13]. Indeed, it has been shown by cell lineage tracing that preexisting pancreatic β-cells can undergo dedifferentiation *in vitro*, although these dedifferentiated β-cells were eventually lost during long-term culture[16]. Thus, while pancreatic β-cells may 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 preexisting 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 preexisting β-cells undergo dedifferentiation and redifferentiation *in vitro* 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 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. At 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) for 17 min at 37°C. Dissociated fetal pancreatic cells were cultured with RPMI-1640 medium containing 5.6 mM glucose (Invitrogen, Gaithersburg, MD), 10% FCS (Sigma, St. Louis, MO), and 2 ng/ml FGF2 (R&D Systems, Minneapolis, MN) 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 below before culture. At day 6, the cells grown to confluence were harvested and re-plated to 100-mm dishes at 2 x 10^6 cells/dish. At day 9, monolayer cells were harvested and re-plated to a 12-well plate at 1 x 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 mM glucose, 10% FCS, 2 ng/ml FGF2, and 10 ng/ml betacellulin (R&D Systems). ISCs were formed in this stage (day 15-19: Stage 2). At day 19, ISCs were picked up and plated to 6-well dishes with RPMI-1640 containing 5.6 or 16.7 mM glucose, 10% FCS, and 10 mM nicotinamide or 50 μM PD98059 (Calbiochem, San Diego, CA) (day 20-30: Stage 3).

Tracing of preexisting pancreatic β-cells. Islets isolated from mouse at 12 to 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) 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, preexisting β-cells became GFP-negative/mRFP-positive due to 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).

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), rat anti-nestin (American Research Product, Belmont, MA), 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) were used for detection. Nuclear staining was done by 4',6-diamino-2-phenylindole (DAPI) (Dojindo, Kumamoto, Japan).

RT-PCR analysis. Total RNA was isolated with RNeasy Mini kit (Qiagen, Valencia, CA) 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). 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 (Table1). 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 performed 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 control of mouse insulin 1 promoter. There was no leaky expression of GFP in pancreatic cells other than β-cells (Fig. 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 (Fig. 1b, upper panel). The purity of post-sorted GFP-positive cells was more than 99% (Fig. 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 pancreas of fetal MIP-GFP mice (Fig. 1c). When cultured in RPMI-1640 medium containing 10% FCS, 5.6 mM glucose, and 2 ng/ml FGF2, the cells expanded and formed a monolayer (referred to as Stage 1) (Fig. 1c). Insulin expression was detected in the starting material, but became rarely 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-dimensional islet-like spherical clusters (ISCs) (Stage 2). When ISCs were hand-picked and cultured further with a high concentration (16.7 mM) of glucose in combination with 10 mM nicotinamide or 50 mM PD98059, a MEK1/2 inhibitor, insulin expression was induced

(Stage 3) (Fig. 1d). This culture system enabled us to investigate phenotypic plasticity of pancreatic β-cells.

Tracing and characterization of dedifferentiated preexisting 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 preexisting 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) (Fig. 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 (Fig. 2a, right panels). Since 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 (Fig. 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 are 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, while mRFP fluorescence could be detected throughout the culture (Fig. 2c). This clearly indicates that the preexisting 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 (Fig. 2d), indicating that the cells at this stage have proliferating capacity. BrdU incorporation

analysis confirmed this (Fig. 2d). Interestingly, most, but not all GFP-ngeative/mRFP-positive cells, which originated from preexisting β-cells, showed fibroblast-like morphology expressing nestin and vimentin (Fig. 2e).

We then investigated the gene expression profile of preexisting 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 (Fig. 3a). Fluorescence microscopy showed that all of the post-sorted cells were mRFP-positive and GFP-negative (Fig. 3b). The gene expression profile of the cells was compared to that of fresh β-cells sorted from the islets of the same mice (Fig. 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, also were down-regulated. On the contrary, 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 indicate the occurrence of dedifferentiation in pancreatic β-cells.

Dedifferentiation is associated with cell cycle progression. The findings described above 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, indicating that most adult pancreatic β-cells are in G0/G1 phase (Fig. 4a, left panel). We then obtained GFP-negative/mRFP-positive cells (dedifferentiated β-cells) at the end of Stage 1 as described in Fig. 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 (Fig. 4a, right panel). 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 up-regulated in the dedifferentiated β-cells (Fig. 4b). These results clearly indicate 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. Reexpression of the insulin gene at Stage 3 is shown by real-time RT-PCR analysis (Fig. 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 ISCs 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 (Fig. 5). These results indicate that the insulin-positive cells induced at Stage 3 originated not from immature progenitor cells but from preexisting β-cells in adult pancreas. Moreover, we confirmed that although no GFP-positive cells were present in ISCs before induction (Stage 2), GFP/mRFP-double positive cells (less than 10% of all mRFP-positive cells) reappeared at the end of Stage 3 (Fig. 6a). In

addition to insulins, other β-cell specific genes were reexpressed at this stage (Fig. 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 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 preexisting 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. By contrast, in our system using MIP-GFP/CAG-mRFP mice, preexisting 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 preexisting 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 preexisting 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 preexisting 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 was not known if the dedifferentiated β-cells have potential to redifferentiate into insulin-expressing cells. By using fetal pancreatic cells as feeder cells, we were able to maintain preexisting 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 ISCs were released from this environment and cultured further with PD98059, insulin was reexpressed in the ISCs. This is the first direct evidence for 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 indicates 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*, indicating that native pancreatic β-cells have the potential to proliferate 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, while treatment of ISCs 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 may 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 dedifferentiated β-cells between our data and Weinberg's report[16].

In pancreatic islets, the β-cells form 3-dimensional 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 ISCs at Stage 2 and reexpressed insulin at Stage 3. These cells are compactly packed into ISCs, which are abundant in laminins (data not shown). Thus, cell-cell and cell-matrix contacts are reconstituted at Stage3, and the microenvironment of the cells within the ISCs may be critical in redifferentiation.

In summary, preexisting 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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Figure legends

Fig. 1 Phenotypic change of adult pancreatic β-cells. (**a**) Immunostaining of MIP-GFP mouse pancreas with anti-GFP and anti-insulin antibodies. Sections were analyzed by confocal microscopy. Scale bars indicate 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. PD, PD98059; Nic, nicotinamide; HG, 16.7 mM glucose; NS, not significant; *** $P < 0.001$.

Fig. 2 Evidence for dedifferentiation of adult pancreatic β-cells. (**a**) Fluorescence of pancreatic cells of adult MIP-GFP/CAG-mRFP-double transgenic mice. mRFP fluorescence is detected in all of the cells including exocrine cells while GFP is expressed only in insulin-expressing cells (left panels). All of the sorted β-cells expressed both GFP and mRFP (right panels). Scale bars indicate 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 (day14). 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 (day14). 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 was positive for nestin or vimentin.

Fig. 3 Isolation and characterization of dedifferentiated β-cells. (**a**) Isolation of GFP(-)/mRFP(+)-dedifferentiated β-cells cultured on feeder cells by 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 (day14), 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 mRFP and negative for 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.

Fig. 4 Cell cycle analysis of dedifferentiated β-cells. (**a**) Cell cycle analysis. Representative histograms of 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), while 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 mRFP.

Fig. 5 Reversion of insulin-expressing cells. 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 was shown. Data are means ± SEM of three to four independent experiments.

Fig. 6 Reexpression of GFP at Stage 3. GFP/mRFP-double positive β-cells were sorted from the GFP/mRFP-double transgenic mice by 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 (Fig. 2C). ISCs 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.

Table 1. Sequences of PCR primers

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

$p57^{Kip2}$	CTGAAGGACCAGCCTCTCTC	TCTGGCCGTTAGCCTCTAAA	229bps
Foxo1	AAGAGCGTGCCCTACTTCAA	CTCCCTCTGGATTGAGCATC	157bps
Ki67	CCAGCTGCCTGTAGTGTCAA	CCTTGATGGTTCCTTTCCAA	250bps
PCNA	GAAGGCTTCGACACATACCG	TCTGGGATTCCAAGTTGCTC	227bps

Sequences of PCR primers 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.

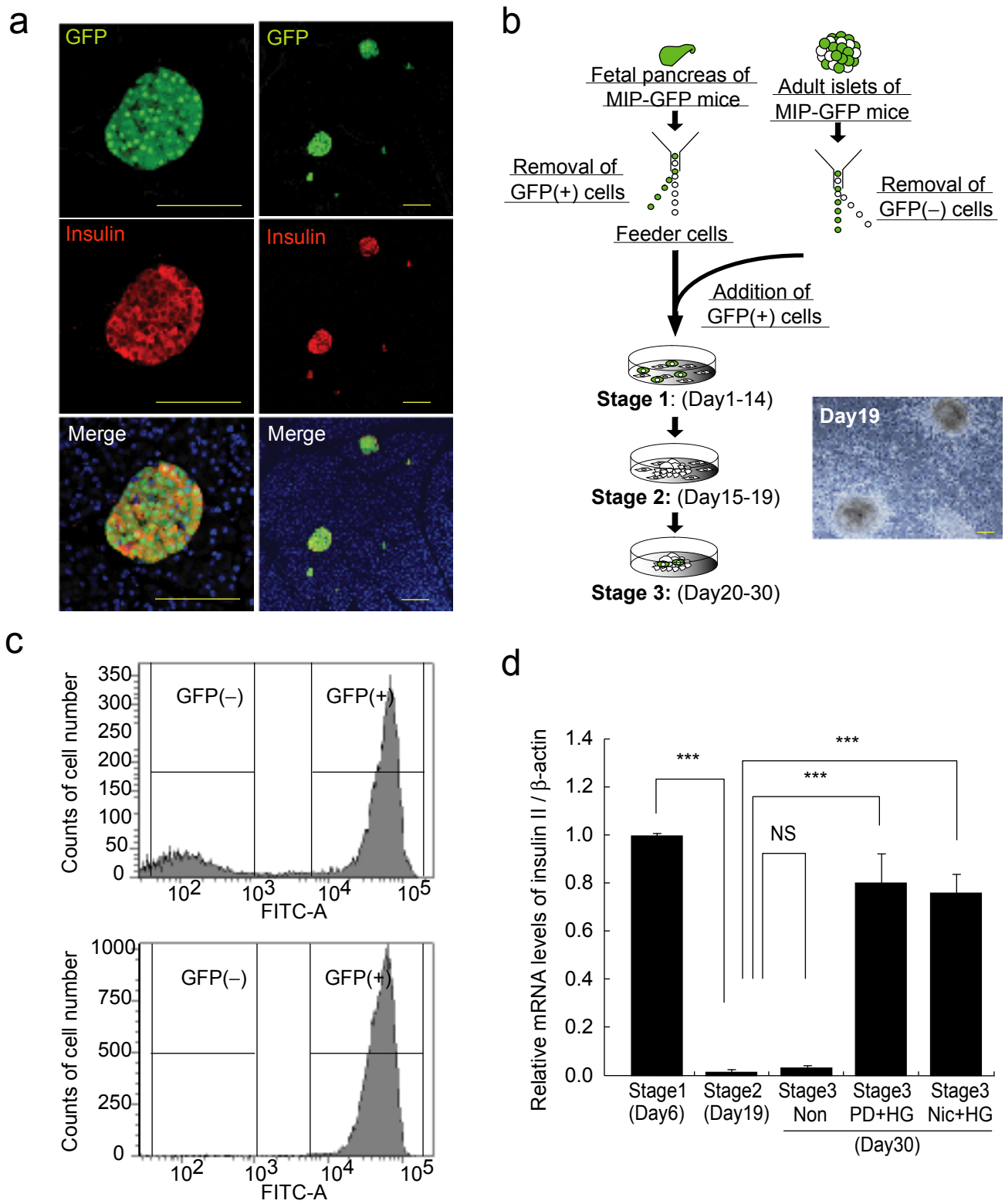

Figure 1 (Minami et al.)

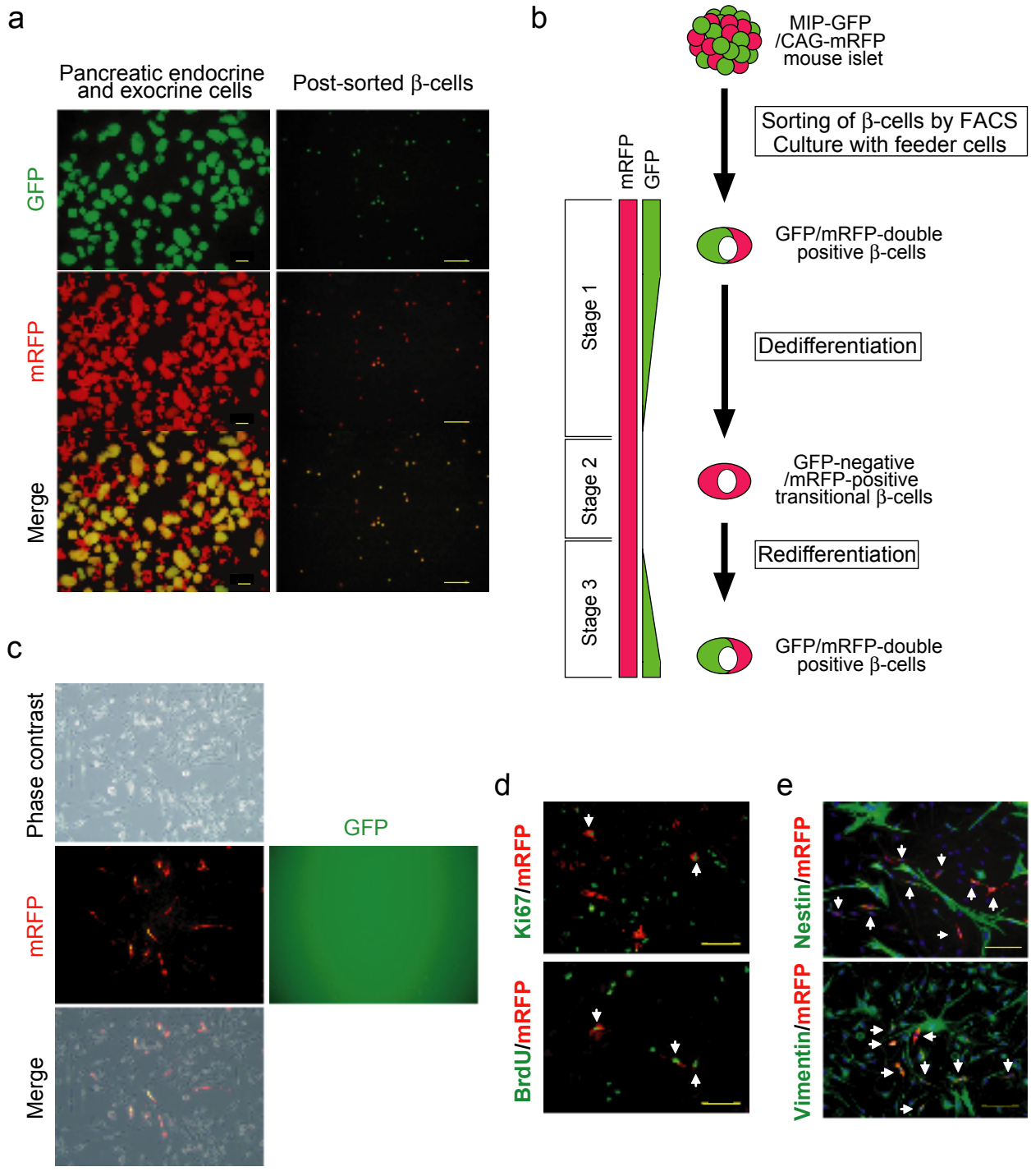

Figure 2 (Minami et al.)

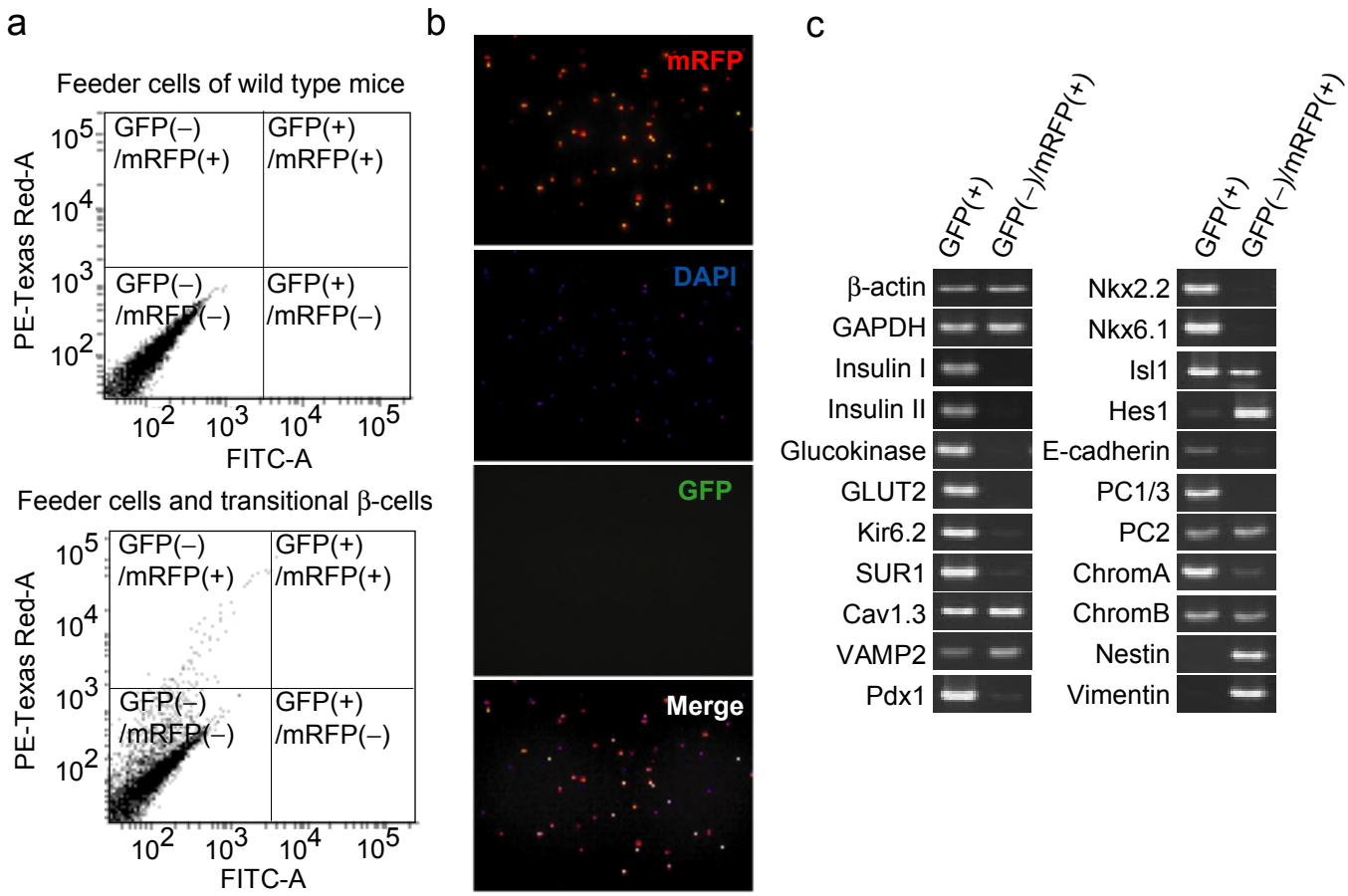

Figure 3 (Minami et al.)

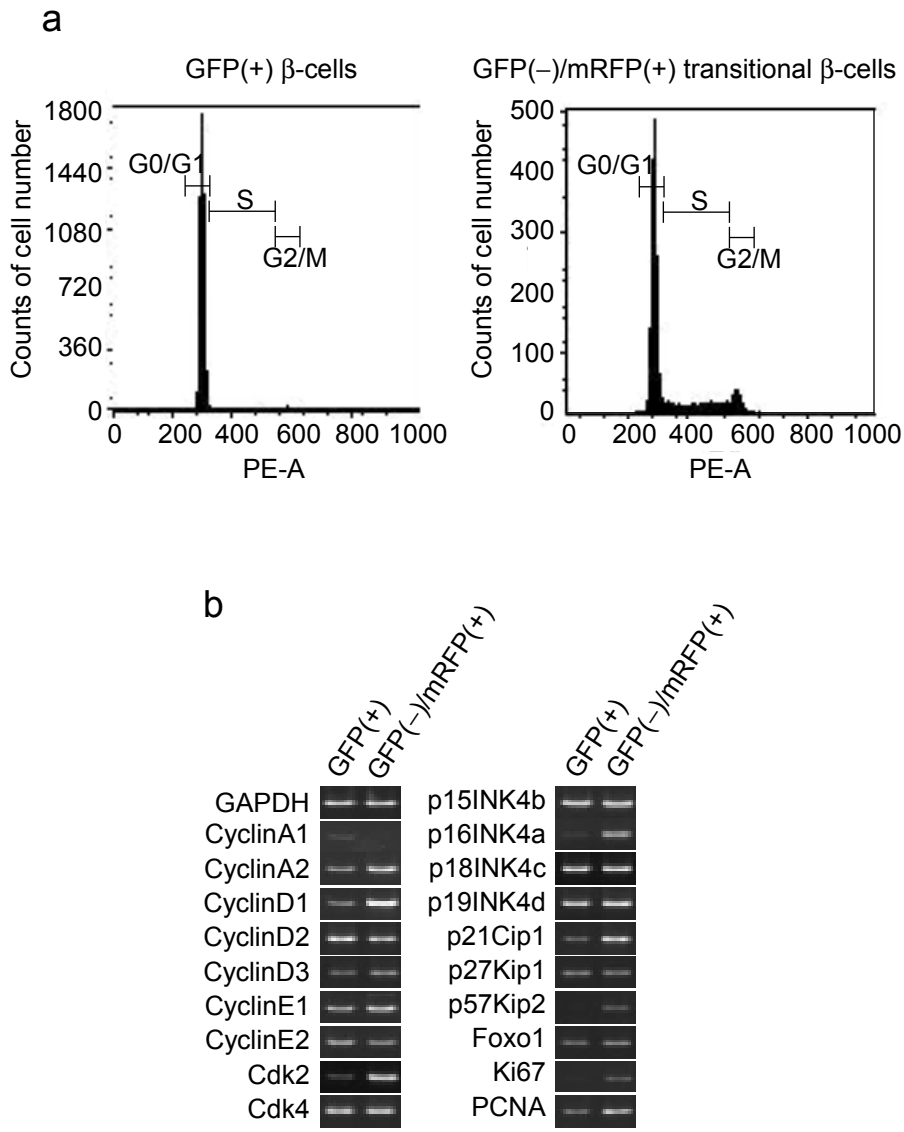

Figure 4 (Minami et al.)

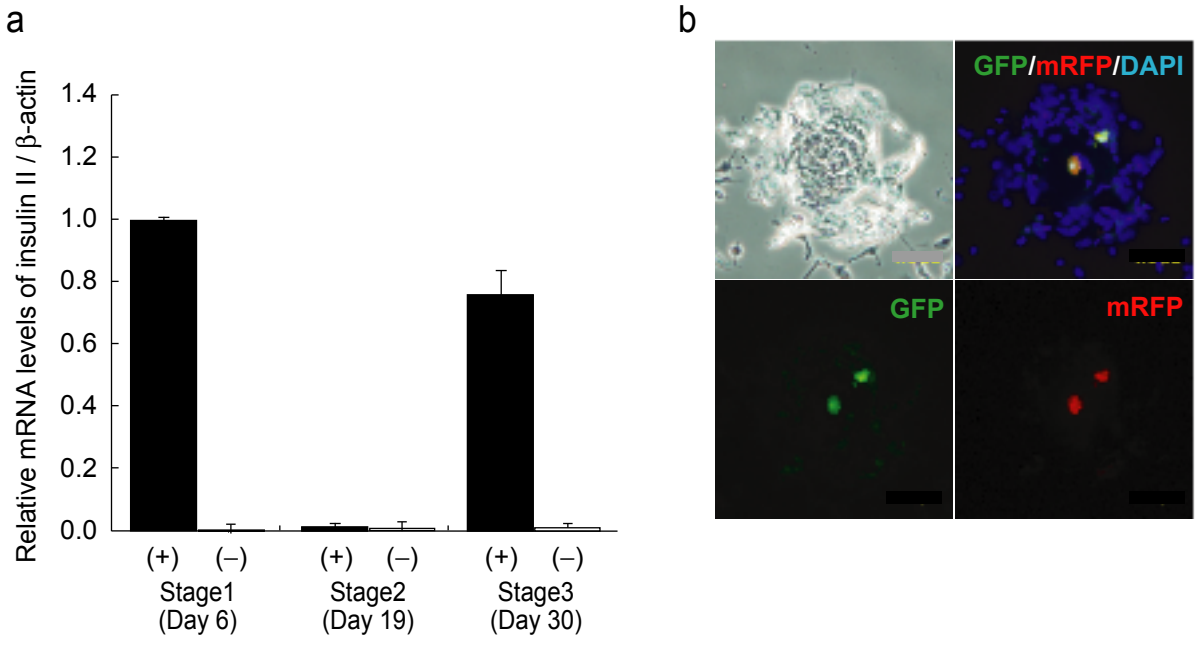

Figure 5 (Minami et al.)