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The Development of Cancer through the Transient Overexpression of Reprogramming Factors

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Original Article

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Abstract

Although induced pluripotent stem (iPS) cells have significant implications for overcoming most of the ethical issues associated with embryonic stem cells, several issues related to the use of iPS cells in clinical applications remain unresolved, including the issue of teratoma formation. We previously reported that the induction of induced tissue-specific stem (iTS) cells from the pancreas (iTS-P) or liver (iTS-L) by the transient overexpression of reprogramming factors, combined with tissue-specific selection and the generation of iTS cells, could have important implications for the clinical application of stem cells. At the same time, we also generated "induced fibroblast-like (iF) cells" that were capable of self-renewal, which had a similar morphology to fibroblast cells. In this study, we evaluated iF cells. iF cells are unlikely to show adipogenic/osteogenic differentiation. Moreover, iF cells have the ability to form tumors and behave similarly to pancreatic cancer cells. The technology used in the generation of iPS/iTS cells is also associated with the risk of generating cancer-like cells.

Keywords

induced fibroblast-like (iF) cells, induced pluripotent stem (iPS) cells, induced tissue-specific stem (iTS) cells, reprogramming factors, pancreatic cancer

Introduction

Induced pluripotent stem (iPS) cells are generated from adult fibroblasts or other somatic cells and are similar to embryonic stem (ES) cells in their morphology, gene expression, epigenetic status, and in vitro differentiation. Mouse iPS cells give rise to adult chimeras and show competence in germline transmission[1–7]. The generation of mouse and human iPS cells without the genomic integration of exogenous reprogramming factors has been reported[8–11]. These reports provide strong evidence that insertional mutagenesis is not required for in vitro reprogramming. This technical breakthrough has significant implications for overcoming several issues, including ethical issues, associated with ES cell derivation from embryos.

On the other hand, it has recently been shown that epigenetic memory is inherited from the parental cells after the reprogramming of mouse/human iPS cells[12–18]. These findings demonstrate that the iPS cell phenotype may be influenced by the cells of origin and suggest that their skewed differentiation potential may prove useful in the generation of differentiated cell types that are currently difficult to

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produce from ES/iPS cells for the treatment of human diseases. Our group recently showed the generation of not only iPS cells but also tissue-specific stem (induced tissue-specific stem [iTS]) cells from the pancreas (iTS-P) or liver (iTS-L) by the transient overexpression of the reprogramming factors, combined with tissue-specific selection[19,20]. iTS-P/iTS-L cells must inherit numerous epigenetic memory components from pancreas/liver cells and acquire the potential to self-renew. It is known that after the overexpression of reprogramming factors, some non-iPS cell colonies appear at the intermediate stage of cellular reprogramming in vitro. In addition, it is reported that partial iPS cells deviate from successful reprogramming[21–23].

In this study, we evaluated induced fibroblast-like (iF) cells, which we previously generated at the same time as generating iTS-P cells and which showed a similar morphology to fibroblasts. We show that unlike iTS cells, iF cells have the ability to form tumors and behave similarly to pancreatic cancer cells.

Materials and Methods

Mice and Cell Culturing

All of the mouse studies were approved by the review committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Graduate School of Medicine, University of the Ryukyus. Twenty-four-week-old C57/BL6 mice (CLEA Japan, Inc., Tokyo, Japan) were used for the primary pancreatic tissue preparations. Each mouse pancreas was digested with 2 mL of cold M199 medium containing 2 mg/mL of collagenase (Roche Boehringer Mannheim, Basel, Switzerland). The digested tissues were cultured in Dulbecco's-modified Eagle's medium (DMEM; Invitrogen, Therm Fisher Scientific, Inc. Tokyo, Japan) with 10% to 20% fetal bovine serum (FBS; BIOWEST, MO, USA). Eight-week-old nude or Non Obese Diabetic/Severe Combined Immuno Deficiency (NOD/SCID) mice (CLEA) were used for the teratoma formation studies.

Mouse ES cells (American Type Culture Collection [ATCC], Manassas, VA, USA) and iF cells were maintained in complete ES cell media with 15% FBS (Millipore, Darmstadt, Germany) on feeder layers of mitomycin C-treated STO cells, as described previously[1,24]. ES cells were passaged every 3 d, and iF cells were passaged every 5 d.

Plasmid Construction and Transfection

To generate the OSKM plasmid, the complementary DNAs (cDNAs) encoding POU domain, class5, transcription factor 1; Pou5f1 (Oct3/4), SRY-related HMG box (Sox2), Kruppel like factor4 (Klf4), and c-myc Proto-Oncogene (c-Myc) were connected (in that order) with the 2A peptide and were inserted into a plasmid containing the cytomegalovirus (CMV) promoter. The OSKM plasmid was transfected into pancreas cells from 24-wk-old mice on days 1, 3, 5, and 7, as previously described[8]. (Fig. 1A). The colonies were manually picked at 30 to 45 d after the first transfection. The detailed protocol has been described previously[19].

DNA Purification and the PCR

DNA was extracted from cells using an AllPrep DNA/RNA Mini Kit (QIAGEN, Dusseldorf, Germany). Polymerization reactions were performed in a Perkin-Elmer 9700 Thermocycler with 3 μL of cDNA (20 ng DNA equivalents), 160 μmol/L cold Deoxy nucleotide triphosphates (dNTPs), 10 pmol appropriate oligonucleotide primers, 1.5 mmol/L $MgCl_2$, and 5 units AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) in 1× polymerase chain reaction (PCR) buffer. The oligonucleotide primers were described previously[19]. (Fig. 1B). The thermal cycle profile included a 10-min denaturing step at 94 °C, followed by amplification cycles (denaturation for 1 min at 94 °C, annealing for 1 min at 57 °C to 62 °C, and extension for 1 min at 72 °C) with a final extension step of 10 min at 72 °C.

The Quantitative Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from cells using an AllPrep DNA/RNA Mini Kit or an RNeasy Mini Kit (QIAGEN). After quantifying the RNA by spectrophotometry, 2.5 μg of RNA was heated at 85 °C for 3 min and then reverse transcribed into cDNA in 25 μL of solution containing 200 units of Superscript II Ribonuclease H-Reverse Transcriptase (Ribonuclease H-RT) (Invitrogen), 50 ng of random hexamers (Invitrogen), 160 μmol/L dNTP, and 10 nmol/L dithiothreitol. The reaction consisted of 10 min at 25 °C, 60 min at 42 °C, and 10 min at 95 °C. The polymerization reactions were performed as shown in the DNA purification and PCR section.

The quantification of the messenger RNA (mRNA) levels was performed using a TaqMan real-time PCR system, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The PCR was performed for 40 cycles, including 2 min at 50 °C and 10 min at 95 °C as initial steps. In each cycle, denaturation was performed for 15 s at 95 °C, and annealing/extension was performed for 1 min at 60 °C. The PCR was carried out in 20 μL of solution using cDNAs synthesized from 1.11 ng of total RNA. For each sample, the expression of mRNA was normalized by dividing it by the β-actin expression level. The primers for mouse Mixl1 and β-actin are commercially available (Assays-on-Demand Gene Expression Products; Applied Biosystems).

The Adipogenic Differentiation Assay

Adipogenic differentiation was induced by culturing the cells for 7 d in adipocyte differentiation medium (#DM-2; DS Pharma Biomedical Co., Ltd., Osaka, Japan). The cells were further cultured in adipocyte maintenance medium (#AM-1; DS Pharma Biomedical) for 7 d. Differentiation was confirmed by Oil Red O staining of intracellular lipid droplets. Differentiated Adipose-derived Stem Cells (ASCs) were fixed in a 10%

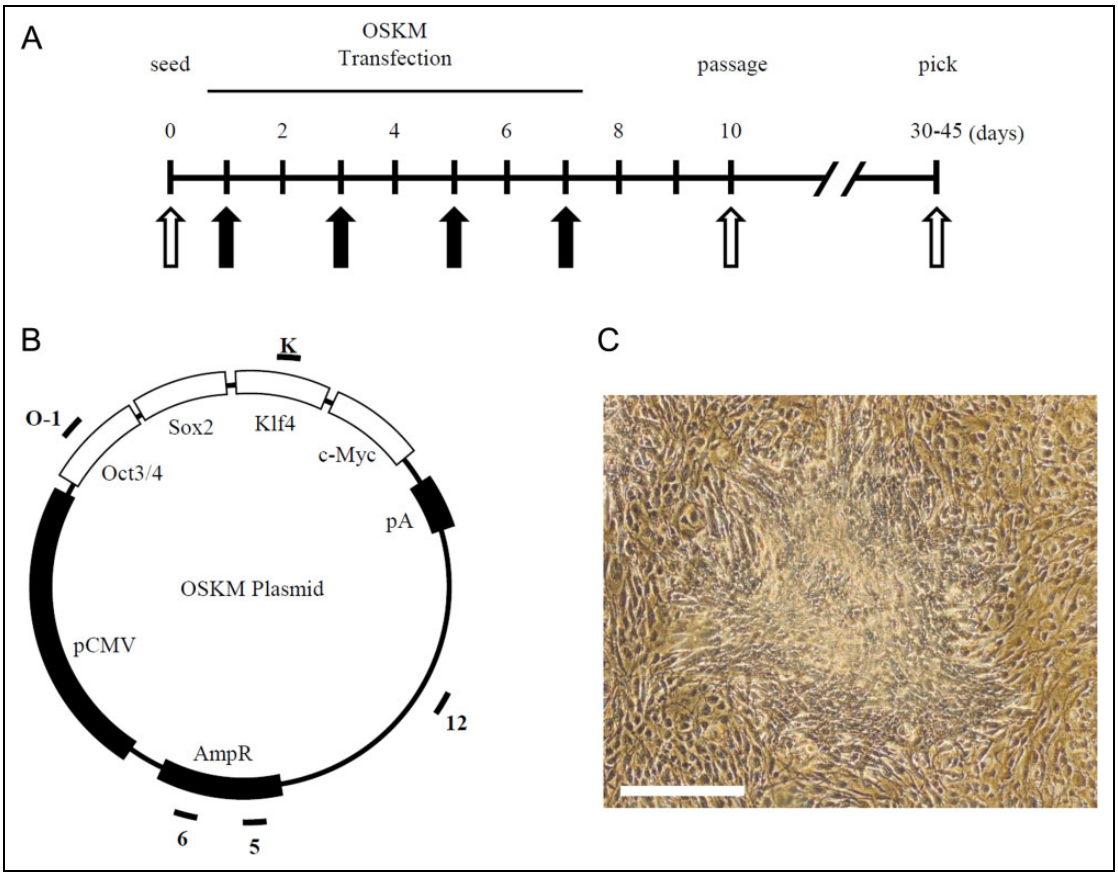

Fig. 1. The generation of induced fibroblast (iF) cells from mouse pancreatic tissue. (A) The time schedules for the induction of induced tissue-specific stem cells from the pancreas (iTS-P) cells with the plasmid. Open arrowheads indicate the timing of cell seeding, passaging, and colony pickup. Solid arrowheads indicate the timing of the transfection. (B) The expression plasmid for the generation of iF cells. The complementary DNAs (cDNAs) encoding Oct3/4, Sox2, Klf4, and c-Myc were connected (in that order) with the 2A peptide and inserted into a plasmid containing the CMV promoter. Thick lines (O-1, K, 5, 6, and 12) indicate the amplified regions used (Fig. 2B) to detect the integration of the plasmid into the genome. The locations of the CMV promoter, the ampicillin-resistance gene (AmpR), and the polyadenylation signal (pA) are also shown. (C) The morphology of the iF cells. Scale bars = 500 μm.

formaldehyde solution (Wako, Osaka, Japan) in phosphate-buffered saline (PBS; Wako) for at least 10 min, washed with 60% isopropanol (Wako), and stained with Oil Red O solution (Wako) for 10 min followed by repeated washing with water and destaining in 100% isopropanol for 1 min[25,26].

The Osteogenic Differentiation Assay

Osteogenic differentiation was induced by culturing the cells for 3 wk in osteoblast differentiation medium (#OB-1; DS Pharma Biomedical). Differentiation was examined by staining for extracellular matrix calcification with Alizarin Red S (Calcified Nodule Staining kit; Cosmo Bio Co., Ltd., Tokyo, Japan)[25,26].

The Teratoma Formation/Tumorigenicity Assay

A total of 1×10^6 of iF cells were inoculated into 1 thigh of each nude or NOD/SCID mice. As a positive control, we transplanted 1×10^6 ES cells into the other thigh of the nude or NOD/SCID mice.

Hematoxylin–Eosin Staining

After fixation in 4% paraformaldehyde, the specimens were embedded in paraffin, and 3- to 5-μm-thick sections were stained with Mayer hematoxylin–eosin.

Statistical Analyses

The data are presented as the mean ± standard error. A repeated-measures analysis of variance was used for inter-group comparisons. P values of <0.05 were considered to indicate statistical significance.

Results

The Generation of iPS, iTS-P, and iF cells from Mouse Pancreatic Tissue

We attempted to generate mouse iPS cells from older-donor pancreata by the transfection of a single plasmid expressing Oct3/4, Sox2, Klf4, and c-Myc. The 4 cDNAs were

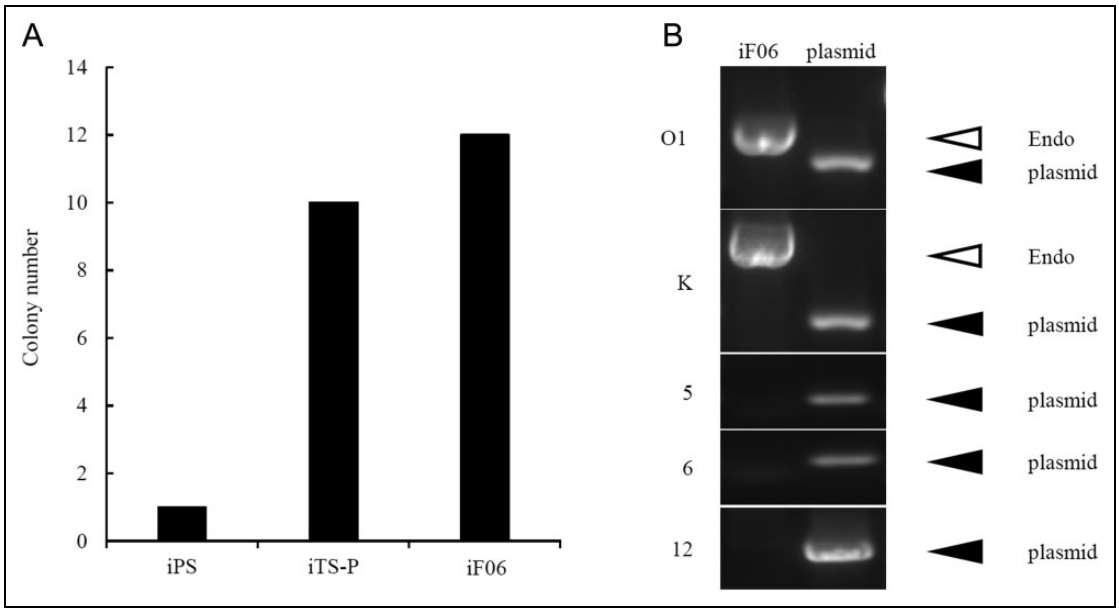

Fig. 2. The colony numbers of induced pluripotent stem (iPS), induced tissue-specific stem from the pancreas (iTS-P), and induced fibroblast (iF) cells and plasmid integration. (A) The colony numbers of iPS, iTS-P, and iF06 cells. The OSKM plasmid was transfected into pancreatic tissue, and the number of colonies was counted after 30 to 45 d. (B) The detection of plasmid integration by the polymerase chain reaction (PCR). Genomic DNA from iF06 cells was amplified by a PCR to generate the amplified regions indicated in Fig. 1B. An expression plasmid was used as a positive control. In the PCR for O-1 and K, the bands derived from the endogenous (endo) genes are shown with open arrowheads, while the plasmid DNA is shown with solid arrowheads.

connected (in that order) with the 2A peptide and were inserted into a plasmid containing the CMV promoter (Fig. 1B). We transfected the OSKM plasmid (4 factors) into pancreatic tissue obtained from 24-wk-old mice on days 1, 3, 5, and 7 (Fig. 1A). We were able to generate 1 colony of iPS cells, 12 colonies of iTS-P cells, and 10 colonies of iF cells (Fig. 2A), which had self-renewing potential and which showed a similar morphology to fibroblasts (Fig. 1C), from 24-wk-old mouse pancreata using the OSKM plasmid.

To evaluate the plasmid integration in iF cells (passage 45), genomic DNA was amplified by a PCR with specific primers (Fig. 1B). Although the PCR detected the incorporation of the plasmid into the host genome of some cells, no amplification of the plasmid DNA was observed in iF06 cells (one of the iF cell colonies; Fig. 2B). Although we cannot formally exclude the presence of small plasmid fragments, these data show that some of iF cells, which had the capacity to self-renew, were most likely free from the integration of the plasmid into the host genome.

The Characterization of iF Cells

The iF06 cells continued to divide actively beyond passage 20 without changes in their morphology or growth activity (Fig. 3A). To investigate the gene expression in iF06 cells, a RT-PCR was performed to analyze the mesodermal marker genes because the morphology of iF cells was similar to that of fibroblast cells. The expression of mesodermal marker Mixl1 was less frequently detected in iF06 cells than in mouse mesenchymal stem cells (mMSCs; Fig. 3B).

To test whether iF cells have adipogenic differentiation capability, iF06 cells were treated with adipogenic induction medium for 7 d and were cultured with maintenance medium for 7 additional days, and Oil Red O staining was performed. Few iF06 cells were positive for Oil Red O staining (Fig. 4A), suggesting that iF cells were unlikely to have adipogenic differentiation capability. To examine whether iF cells have osteogenic differentiation capability, iF06 cells were treated with osteogenic induction medium for 3 wk and then alizarin red S staining was performed. Some iF06 cells were positive for calcified nodule staining, but many were negative for the staining (Fig. 4B). These data suggest that iF06 cells were unlikely to be MSCs.

Tumor Formation

To examine the teratoma formation and tumorigenic potential in vivo, iF06 cells (10^6. cells) at passage 20 were transplanted into nude or NOD/SCID mice. The sites injected with 1×10^6 ES cells developed teratomas at approximately 3 wk after transplantation (data not shown). The sites injected with 1×10^6 iF06 cells developed tumors at approximately 8 wk after transplantation (Fig. 5A). The tumor histology was similar to adenocarcinoma—the most prevalent histological type of pancreatic cancer—rather than teratoma (Fig. 5B). The tumor had a duct-like and stromal structures but not ectodermal tissue. These data indicate that iF cells were more likely to be associated with development of pancreatic cancer than iPS cells or MSCs.

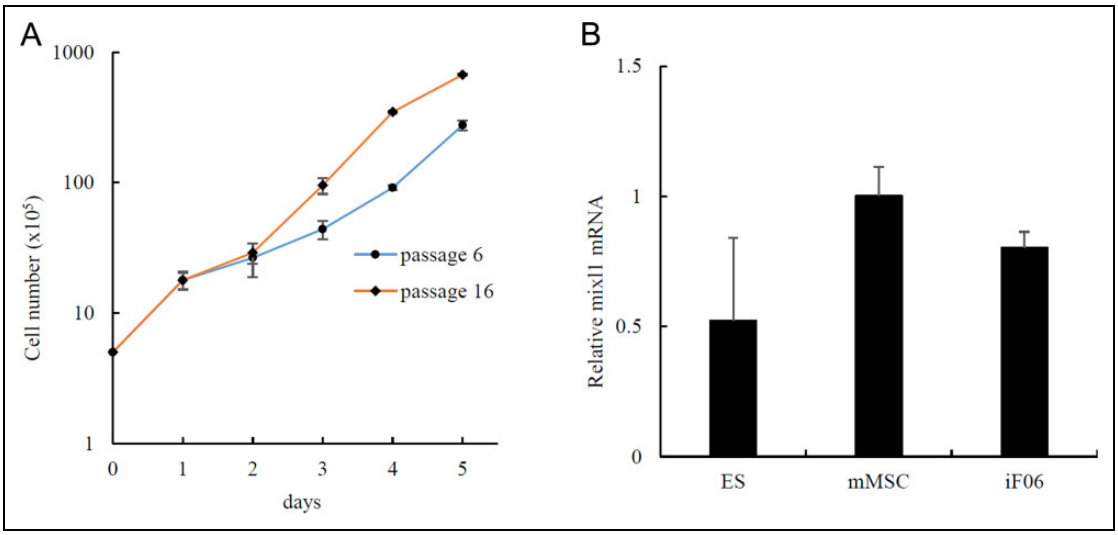

Fig. 3. The characterization of iF06 cells. (A) The growth curves of the iF06 cells (passage 6 and 16). The error bars represent the standard error. (B) The quantitative reverse transcription PCR (RT-PCR) of the Mixl1 genes in induced fibroblast (iF) cells. The iF06 cells were analyzed by a quantitative RT-PCR. Mouse mesenchymal stem cells (mMSCs) were used as a positive control, and embryonic stem (ES) cells were used as a negative control. The data are expressed as the Mixl1-to-β actin ratio with the value of mMSC arbitrarily set at 1 ($n = 4$). The error bars represent the standard error.

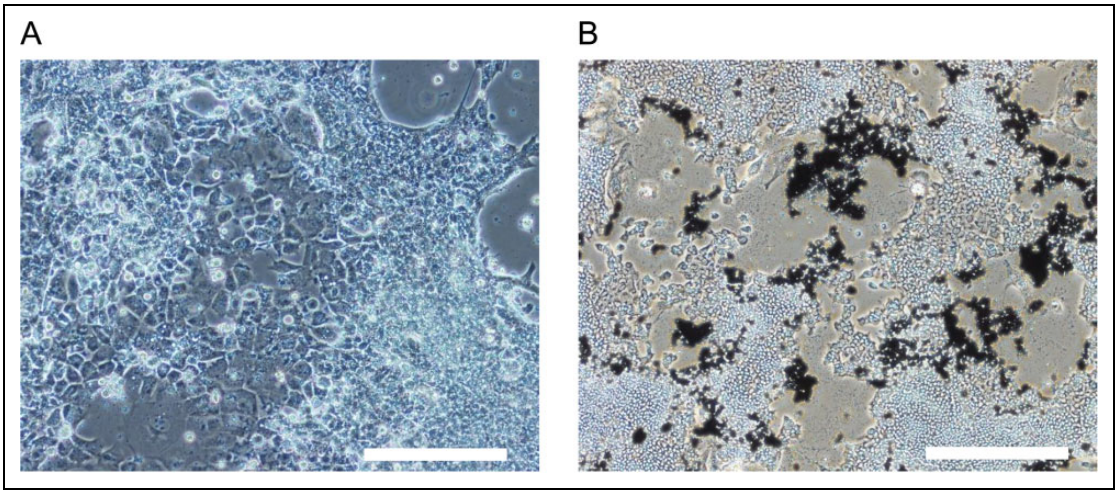

Fig. 4. Adipogenic/osteogenic differentiation of iF06 cells. (A) Adipogenic differentiation. The iF06 cells were stained with Oil Red O at 7 d after the induction of adipogenesis. Scale bar = 200 μm. (B) Osteogenic differentiation. The iF06 cells were stained with alizarin red S at 3 wk after the induction of osteogenesis. Scale bar = 500 μm.

Discussion

In this study and our previous study, some non-iPS cell colonies (which appeared at the intermediate stage of cellular reprogramming) behaved similarly to iTS cells, whereas other colonies, which deviated from successful reprogramming, behaved similarly to cancer cells (iF cells). The induction process of iPS cells shares many characteristics with cancer development. By overexpressing reprogramming factors, somatic cells acquire the properties of self-renewal along with unlimited proliferation and exhibit global alteration of the transcriptional program, which are also critical events during carcinogenesis[27]. Approximately 20% of the offspring derived from retrovirally generated iPS cells developed tumors that were attributable to the reactivation of the c-Myc transgene[6]. The overexpression of Oct3/4 in somatic cells results in dysplastic growth in epithelial tissues through the inhibition of cellular differentiation in a manner similar to that in embryonic cells[28]. These studies suggest that the reprogramming processes and cancer development may be partly promoted by overlapping mechanisms[29].

Ohnishi et al. recently generated an in vivo mouse reprogramming system using reprogramming factor-inducible alleles and examined the effects of the reprogramming factor expression in somatic cells in vivo. The transient

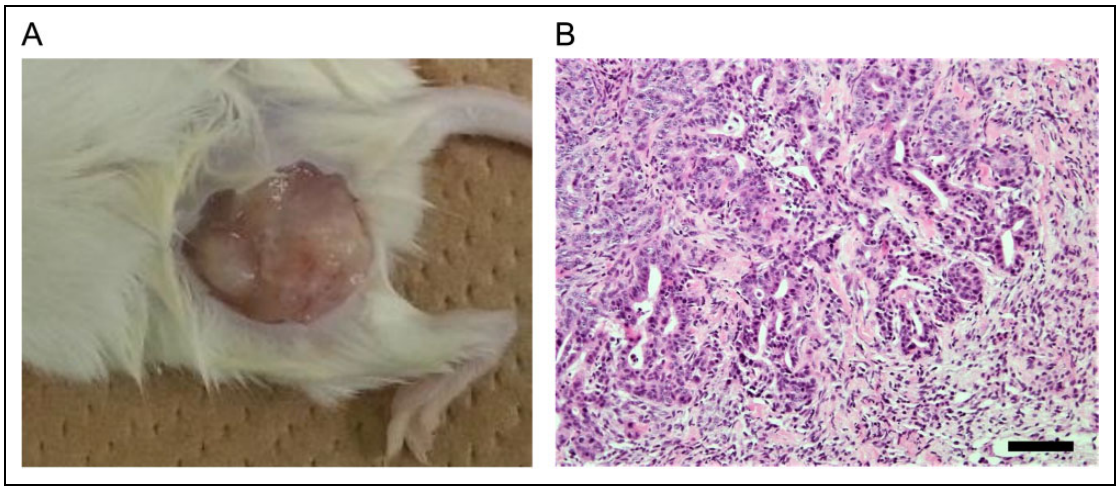

Fig. 5. Tumor formation by iF06 cells. (A) The tumorigenicity assay. A total of 1×10^6 of iF06 cells were inoculated into 1 thigh of each nude mouse. As a positive control, we transplanted 1×10^6 embryonic stem (ES) cells into the other thigh of each nude mouse. (B) A tumor that developed from iF06 cells was histologically analyzed (hematoxylin and eosin staining). Scale bars = 100 μm.

expression of reprogramming factors in vivo is a result of the development of tumors consisting of undifferentiated dysplastic cells that exhibit global changes in their DNA methylation patterns in various tissues. Their data suggest that the abnormal growth of unsuccessfully reprogrammed cells predominantly depends on epigenetic regulations and that cells associated with failed reprogramming behave similarly to cancer cells[30]. The iF cells in this study may be cells in which reprogramming failed and may behave similarly to the cancer cells that arise through the altered epigenetic regulation.

In conclusion, we generated iPS, iTS-P, and iF cells, which were similar to pancreatic cancer cells, from mouse pancreas cells through the transient overexpression of reprogramming factors. Although the generation of iPS/iTS-P cells and their differentiation into insulin-producing cells have important implications due to their potential use in autologous cell replacement therapy, the technology is also associated with the risk of generating cancer-like cells through the global changes in the DNA methylation patterns.

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Ethical Approval

All of the mouse studies were approved by the review committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Graduate School of Medicine, University of the Ryukyus.

Statement of Human and Animal Rights

Twenty-four-week old C57/BL6 mice were purchased and used for the primary pancreatic tissue preparations.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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