SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 16: 3255-3259, 2018

Cell-free culture conditioned medium elicits pancreatic β cell lineage-specific epigenetic reprogramming in mice

KOICHI KAWAMOTO[1,2*], TOMOFUMI OHASHI[1*], MASAMITSU KONNO[2*], NAOHIRO NISHIDA[1,2], JUN KOSEKI[3], HIDETOSHI MATSUI[4], DAISUKE SAKAI[2], TOSHIHIRO KUDO[2], HIDETOSHI EGUCHI[1], TAROH SATOH[2], YUICHIRO DOKI[1-3], MASAKI MORI[1-3] and HIDESHI ISHII[2,3]

Departments of [1]Gastroenterological Surgery, [2]Cancer Frontier Science and [3]Cancer Profiling Discovery, Osaka University School of Medicine, Suita, Osaka 565-0871; [4]Department of Mathematical Sciences, Faculty of Mathematics, Kyushu University, Fukuoka 819-0395, Japan

Received June 30, 2016; Accepted March 9, 2017

DOI: 10.3892/ol.2018.9008

Abstract. There are several obstacles to overcome prior to achieving cellular reprogramming of pancreatic β cells *in vitro* and *in vivo*. The present study demonstrated that the transfer of epigenetic phenotypes was achieved in the cell-free conditioned medium (CM) of pancreatic insulinoma MIN6 cell cultures. The comparison of a subpopulation of MIN6, m14 and m9 cells indicated that MIN6-m14 cells were more prone to cellular reprogramming. Epigenetic profiling revealed that the transcription factor pancreas/duodenum homeobox protein 1 (Pdx1) was differentially associated among the clones. The culture of differentiated adipocytes in the CM of MIN6-m14 cells resulted in the induction of insulin mRNA expression, and was accompanied by epigenetic events of Pdx1 binding. The epigenetic profiling indicated that Pdx1 is preferentially associated with a previously uncharacterized region of the endoplasmic reticulum (ER) disulfide oxidase, ER oxidoreductin 1 gene. Therefore, the results of the present study indicated that the CM of MIN6 cells was able to induce a pancreatic β cell-like phenotype in differentiated adipocytes. These data provide additional support for the utility of cell-free CM for cellular reprogramming.

Correspondence to: Professor Masaki Mori, Department of Gastroenterological Surgery, Osaka University School of Medicine, 2-2 Yamaoka, Suita, Osaka 565-0871, Japan
E-mail: mmori@gesurg.med.osaka-u.ac.jp

Professor Hideshi Ishii, Department of Cancer Profiling Discovery, Osaka University School of Medicine, 2-2 Yamaoka, Suita, Osaka 565-0871, Japan
E-mail: hishii@gesurg.med.osaka-u.ac.jp

*Contributed equally

Key words: conditioned medium, epigenetic reprogramming, pancreatic β cell

Introduction

Type 1 diabetes mellitus, or insulin-dependent diabetes, is a form of diabetes mellitus which results from considerable destruction of the insulin-producing β cells in the islets of the pancreas by autoimmune disorders (1). In general, islet transplantation and engraftment is believed to be a potential radical cure for diabetes mellitus. However, the requirement for human donors is an inevitable limiting factor to obtain sufficient tissues to meet the demand for islet transplantation treatment (2). Immunosuppressive therapy and technology for preserving the isolated islets from the pancreas during surgical treatment have emerged as useful approaches to improve patient survival (3).

The microenvironment of the islets in the pancreas is considered to be critical for the maintenance of cell viability for implantation. For example, proinflammatory cytokines, activated neutrophils and neutrophil elastase (NE) released from these neutrophils in the microenvironments of surgically isolated islets surrounding pancreatic tissues may directly cause injury to islet grafts. Therefore, technology to protect isolated islets from deleterious damage is required to increase the viability of grafts and naturally improve surgical outcomes of islet transplantation (4). Previous studies have indicated that the modulation of proinflammatory cytokines, including tumor necrosis factor α and interleukin 6, which are markedly increased at the end of warm digestion during islet isolation and exhibit direct cytotoxic activity against the islets causing their apoptosis, significantly contribute to the yield of islets (4). In addition, treatment with sivelestat, an NE inhibitor, resulted in an improvement of the viability of islet grafts in a mouse allotransplant model (4). The survival and insulin function of an islet graft was enhanced by the combined transplantation of pancreatic islets and adipose tissue-derived stem cells (ADSCs) (5), suggesting that the microenvironment of islets serves a function in the survival of islets in transplantation. Indeed, ADSCs exhibit anti-inflammatory properties (6).

Substantial effort has been invested into the development of cellular reprogramming of differentiated cells, including ADSCs (7). The defined Yamanaka factors, octamer binding transcription factor 3/4, Sex-determining region Y-box 2, Kruppel-like factor 4, and v-myc avian myelocytomatosis

viral oncogene homolog, have been demonstrated to induce cellular reprogramming into a pluripotent state (8). ADSCs possess the multipotential for differentiation and are prone to induction (7). Eventually, the expression of cluster of differentiation 90, or Thy-1, was revealed to be useful for the selection of reprogramming-prone cells to improve the efficiency of ADSC induction into pancreatic cells (9). Although the Yamanaka factors elicit full reprogramming into a pluripotent state, several tissues (including pancreatic β-like cells) are proposed to be induced not via the pluripotent state, but directly from differentiated cells via pancreas/duodenum homeobox protein 1 (Pdx1), neurogenin-3 and V-maf musculoaponeurotic fibrosarcoma oncogene homolog (10,11). In addition, other studies have demonstrated that small molecules (12) and microRNA-302 (13), which is a non-coding RNA, were able to induce pancreatic β-like cells. Several microRNAs, including microRNA-302, were previously demonstrated able to induce the cellular reprogramming of ADSCs (14). Therefore, to elucidate the methods required to induce pancreatic β-like cells by defined factors or the development of a simple technology would be beneficial in a clinical setting for the purposes of regenerative medicine for insulin-dependent diabetes.

In the present study, a simple method to address this problem was investigated using the culture of ADSCs in cell-free conditioned medium (CM) from insulinoma MIN6, which are insulin-producing tumor cells. The culture of ADSCs in the MIN6-CM resulted in an increase in insulin expression. Whole-genome epigenetic profiling data indicated that the culture of ADSCs in cell-free CM from MIN6 resulted in the induction of epigenetic modification toward an insulin-producing transcriptional phenotype. Cell culture in the MIN6-CM may have resulted in the cell-to-cell transmission of the phenotype of insulin-producing β cells and provides additional rationale to study this protocol for use in regenerative medicine.

Materials and methods

Cell culture and exosome isolation. Mouse ADSCs (RIKEN BioResource Centre, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a CO_2 incubator at 37˚C. MIN6 cells, which were supplied by Professor J. Miyazaki, and the subclones, m9 and m14, derived from parental MIN6 cells, which were from Professor T. Miki, were cultured in DMEM containing 10% FBS and 2-mercapteethanol in a CO_2 incubator at 37˚C (15). ExoQuick-TC (System Biosciences, Palo Alto, CA, USA) was used for exosome isolation according to the manufacturer's protocol.

Pancreatic differentiation. Preparation of MIN6-CM was performed as previously described (9). ADSCs were cultured in the MIN6-CM for 72 h, and non-conditioned DMEM with 10% FBS was used as the control.

Chromatin immunoprecipitation (ChIP) sequencing. Cells were trypsinized and homogenized in 10 ml PBS, were resuspended in 1% formaldehyde in PBS, and cross-linked at room temperature for 5 min. The reaction was stopped by adding 0.2 M glycine. The cells were washed twice with cold PBS and resuspended in ice-cold cell lysis buffer (10 mM NaCl; 10 mM Tris-HCl, pH 8.0; 0.5% NP-40). The samples were washed with cell lysis buffer and resuspended in nuclear lysis buffer (1% SDS; 10 mM EDTA; 10 mM Tris-HCl, pH 8.0). The samples were incubated for 10 min at 4˚C and added to a ChIP buffer (50 mM Tris-HCl, pH 8.0; 167 mM NaCl; 1.1% TritonX-100; 0.11% sodium deoxycholate; protease inhibitor mix). Chromatin was sonicated to 300-500 bp, followed by standard immunoprecipitation analysis with the following antibody: a 1:1,000 dilution of mouse Pdx1 (cat. no., LS-C145426, LSBio, Seattle, WA, USA). Total DNA was sequenced using HiSeq 2000 (Illumina, Inc., San Diego, CA, USA).

RNA expression study. For microarray experiments, following assessment of quality by electrophoresis in gel at 100 V, 500 ng extracted total RNA, extracted using a TRIzol kit (Thermo Fisher Scientific, Inc.) as labeled with Cyanine-3 (Cy3) using the Low Input Quick Amp Labeling kit (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Dye incorporation and cRNA yield were checked with the NanoDrop ND-2000 Spectrophotometer (230 and 260 nm; Thermo Fisher Scientific, Inc.). The labeled RNAs were hybridized onto the Agilent Mouse Microarray (Agilent Technologies, Inc.) for 17 h at 65˚C in a rotating Agilent hybridization oven (Agilent Technologies, Inc.). Following hybridization, microarrays were washed for 1 min at room temperature with GE Wash Buffer 1 (Agilent Technologies, Inc.) and for 1 min at 37˚C with GE Wash Buffer 2 (Agilent Technologies, Inc.), then dried immediately by brief centrifugation at room temperature. Following washes with GE Wash Buffer 1 and GE Wash Buffer 2 for 1 min each, the fluorescent signals were scanned with the Agilent DNA Microarray Scanner (G2565CA; Agilent Technologies, Inc.) and analyzed using Feature Extraction Software v.10.10 (Agilent Technologies, Inc.). For reverse transcription-quantitative polymerase chain reaction (PCR), total RNA were reverse transcribed using Rever Tra Ace RT kit (FSQ-101; Toyobo Life Science, Osaka, Japan) with amplification by Thunderbird® Syber qPCR Mix kit (QPS-101; Toyobo Life Science) using a Light Cycler (Roche Diagnostics, Tokyo, Japan) (16). The conditions were: 95˚C for 10 sec; 60˚C for 10 sec; and 72˚C for 10 sec, which was repeated 40 times. The primers used were: Pdx1, forward, AGCAGTCTGAGGGTGAGCGGGTCT, and reverse, AGCAGTCTGAGGGTGAGCGGGTCT; and Ins2, forward, TCCGCTACAATCAAAAACCAT, and reverse, GCTGGGTAGTGGTGGGTCTA. For reference, we used primer for Gapdh, forward, ACCACAGTCCATGCCATCAC, and reverse, TCCACCACCCTGTTGCTGTA.

Results

As it was hypothesized that insulinoma cell line-derived CM may induce insulin expression in murine ADSCs, insulin expression was assessed using a DNA gene array to explore the mechanistic insight of the CM (data not shown). Pdx1 is a critical pancreatic transcription factor upstream of several genes. The expression of insulin II (Fig. 1A) and Pdx1 (Fig. 1B)

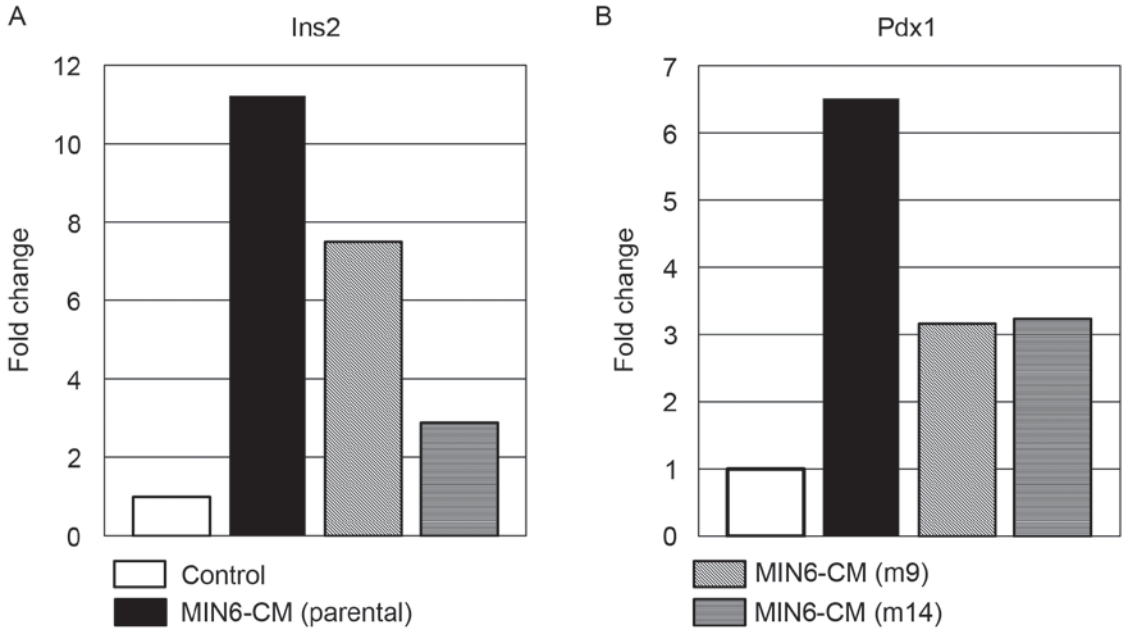

Figure 1. mRNA expression of (A) Ins2 and (B) Pdx1 in murine adipose tissue-derived stem cells cultured with or without CM Ins2, insulin II; Pdx1, pancreas/duodenum homeobox protein 1; CM, conditioned medium.

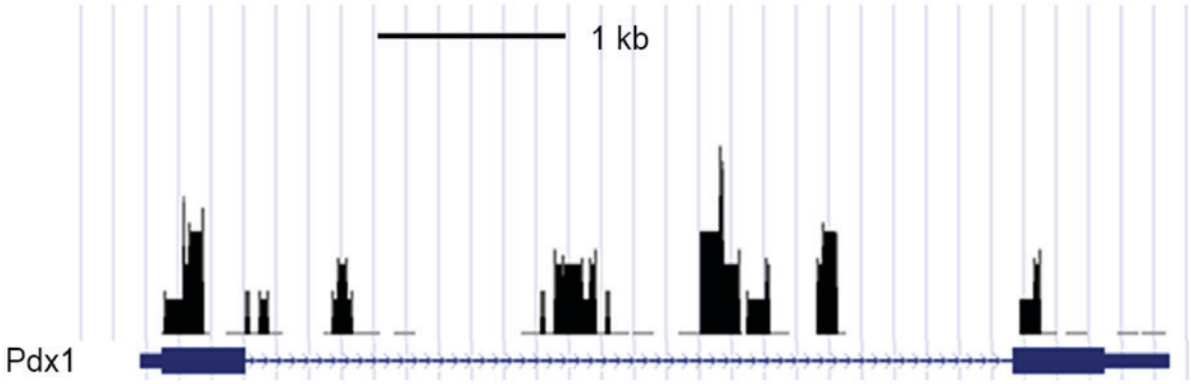

Figure 2. RNA expression analysis of murine adipose tissue-derived stem cells cultured in MIN6-conditioned medium. Pdx1, pancreas/duodenum homeobox protein 1; tRNA, transfer RNA.

was upregulated in MIN6-CM-cultured ADSC. Expression in parental MIN6-CM-cultured murine ADSCs was further increased compared with ADSCs cultured in m9 or m14 MIN6-CM. These results demonstrated that there was variability across these subclones. As Pdx1 mRNA should exist in exosomes isolated from CM, the potential existence of Pdx1 mRNA in the MIN6-CM cell line of the present study was assessed using global sequence analysis. The read number was ~0.2, suggesting that the expression level of Pdx1 mRNA was low in purified exosomes (Fig. 2).

The binding of Pdx1 was subsequently assessed using a ChIP assay. The ChIP analysis indicated that, compared with the control cells, multi-methylated forms of histone H3 lysine 4 were preferentially associated with the promoter sequence of the *Pdx1* genes in CM-cultured ADSCs (Fig. 3). In addition, the ChIP assay also indicated that Pdx1 was bound to the promoter region of the ER oxidoreductase 1 (Ero1)-β gene (Fig. 3). Finally, the putative mechanism underlying the effect of CM was proposed (Fig. 4).

Discussion

The present study indicated that the culture of mouse ADSCs in cell-free, MIN6-CM resulted in the induction of insulin and *Pdx1* gene expression (Fig. 1). Considering that gene expression is under tight regulation by epigenetic modifications, including DNA methylations and histone modifications, the epigenetic profiling was investigated using a ChIP assay. This assay was performed with an anti-Pdx1 antibody, followed by next generation sequencing for mouse ADSCs in cell-free MIN6-CM. The results of the ADSCs were compared with those of the the original MIN6. Pancreatic development is regulated by multiple transcription factors. Pdx1 is a critical factor for the determination of differentiation in insulin-producing β cells (17).

Ero1 was initially identified in yeast. Yeast possesses a single copy of the *Ero1* gene, whereas mammal cells have two Ero1-like genes. The present study also demonstrated that the expression of Pdx1 was minimal in CM-derived exosomes. This observation suggested that other mechanisms, including

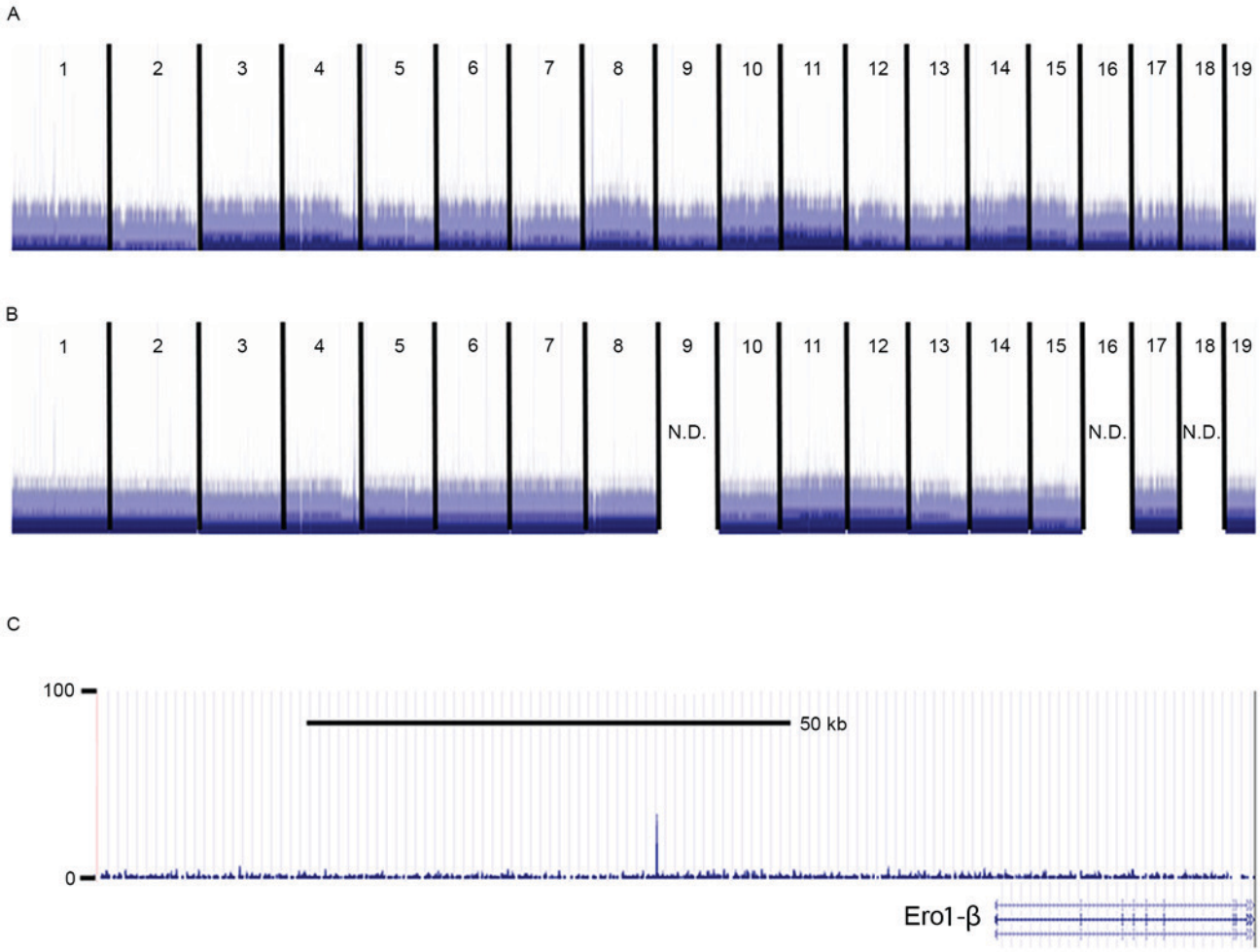

Figure 3. Chromatin immunoprecipitation analysis using Pdx1 antibody. (A) Control ADSCs without CM and (B) CM-cultured ADSCs are presented. (C) Pdx1 is specifically bound to the Ero1-β promoter region. Pdx1, pancreas/duodenum homeobox protein; ADSC, adipose tissue-derived stem cells; CM, conditioned medium; Ero1-β, ER oxidoreductase 1-β.

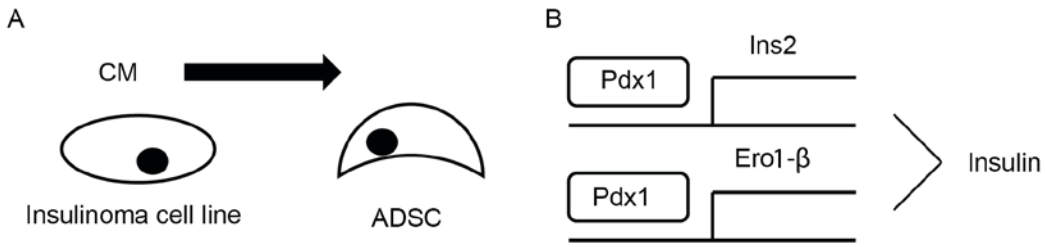

Figure 4. Schematic presentation of the study of the (A) putative mechanism underlying the effect of CM, (B) as proposed by the results of the present study. ADSCs, adipose tissue-derived stem cells; CM, conditioned medium; Ero1-β, ER oxidoreductase 1-β; Ins2, insulin II; Pdx1, pancreas/duodenum homeobox protein 1.

proteins or microRNAs, may have a potential function in the CM mechanism. Currently, the specific factors involved in this process had not been clarified and additional investigation is required. Zito *et al* (18) suggested that homozygosity for a disrupting allele of *Ero1-L-β* selectively compromises the oxidative folding of proinsulin and promotes glucose intolerance in mutant mice. Exosomes are now considered to be novel mediators of endocrine signaling via cell-to-cell communication. Exosomes may deliver proteins and RNA to recipient cells. These results suggested that additional investigation would be beneficial to establish the mechanisms of β-like cell differentiation. *Ero1-L-α* has been indicated to serve a key function in a hypoxia-inducible factor 1-mediated pathway for the induction of disulfide bond formation and vascular endothelial growth factor secretion under hypoxia (19).

The data from the ChIP assay of the present study indicated that Pdx1 was bound to the promoter region of the *Ero1-β* gene (a pancreatic-specific disulfide oxidase), which has been demonstrated to promote insulin biogenesis and glucose homeostasis (18). Considering that the *in vivo* function of Ero1-β in oxidative protein folding in insulin-producing cells is required for glucose homeostasis (18), the expression of Ero1-β may be transmissible from cell-to-cell via the

serum *in vitro* and the microenvironment *in vivo*. The results of the present study suggested a promising candidate for this method, to be used in a clinical setting for the induction of insulin-producing β-like cells.

Acknowledgements

The authors would like to thank Professor J. Miyazaki (Department of Stem Cell Regulation Research, Osaka University, Japan) for providing the MIN6 cells, and Professor T. Miki (Department of Medical Physiology, Chiba University, Japan) for providing the subclones.

Funding

The present study was financially supported by: A Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (to M. Mori and H. Ishii); a Grant-in-Aid from D-DIRECT (to H. Ishii); a Grant-in-Aid from the Ministry of Health, Labor, and Welfare (to M. Mori); grants from the National Institute of Biomedical Innovation and Osaka University Drug Discovery Funds (to M. Mori); and grants from the Kobayashi Cancer Research Foundation (to H. Ishii); Princess Takamatsu Cancer Research Fund (to H. Ishii); Takeda Science and Medical Research Foundation (to H. Ishii); Suzuken Memorial Foundation (to M. Konno); Yasuda Medical Foundation and the Pancreas Research Foundation, Japan (to K. Kawamoto). Professor Hideshi Ishii received partial support from EBM Research Center, Inc. (to Osaka, Japan), and Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) through institutional endowments. These funding agencies served no role in the main experimental equipments, supplies, expenses, study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author's contributions

The experiments were performed by KK, TO, MK, NN, the analysis of data was performed by KK, JK, HM, DS, TK. The manuscript was written by KK, MK, HI. The design the study was by HE, TS, YD, MM, HI.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Kasper DL, Fauci AS, Hauser SL, Longo DL, Jameson JL and Loscalzo J: Harrison's principles of internal medicine 19/E, Vol. 1 & 2, 2015.
2. Park CG, Bottino R and Hawthorne WJ: Current status of islet xenotransplantation. Int J Surg 23: 261-266, 2015.
3. Tomimaru Y, Ito T, Kawamoto K, Hama N, Wada H, Kobayashi S, Eguchi H, Tanemura M, Mori M, Doki Y and Nagano H: Clinical outcome of pancreas transplantation from marginal donors in Japan. Transplant Proc 46: 954-957, 2014.
4. Machida T, Tanemura M, Ohmura Y, Tanida T, Wada H, Kobayashi S, Marubashi S, Eguchi H, Ito T, Nagano H, *et al*: Significant improvement in islet yield and survival with modified ET-Kyoto solution: ET-Kyoto/Neutrophil elastase inhibitor. Cell Transplant 22: 159-173, 2013.
5. Ohmura Y, Tanemura M, Kawaguchi N, Machida T, Tanida T, Deguchi T, Wada H, Kobayashi S, Marubashi S, Eguchi H, *et al*: Combined transplantation of pancreatic islets and adipose tissue-derived stem cells enhances the survival and insulin function of islet grafts in diabetic mice. Transplantation 90: 1366-1373, 2010.
6. Mizushima T, Takahashi H, Takeyama H, Naito A, Haraguchi N, Uemura M, Nishimura J, Hata T, Takemasa I and Yamamoto H, *et al*: A clinical trial of autologous adipose-derived regenerative cell transplantation for a postoperative enterocutaneous fistula. Surg Today 46: 835-842, 2016.
7. Konno M, Hamabe A, Hasegawa S, Ogawa H, Fukusumi T, Nishikawa S, Ohta K, Kano Y, Ozaki M, Noguchi Y, *et al*: Adipose-derived mesenchymal stem cells and regenerative medicine. Dev Growth Differ 55: 309-318, 2013.
8. Takahashi K and Yamanaka S: Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663-676, 2006.
9. Kawamoto K, Konno M, Nagano H, Nishikawa S, Tomimaru Y, Akita H, Hama N, Wada H, Kobayashi S, Eguchi H, *et al*: CD90-(Thy-1-) high selection enhances reprogramming capacity of murine adipose-derived mesenchymal stem cells. Dis Markers 35: 573-579, 2013.
10. Akinci E, Banga A, Tungatt K, Segal J, Eberhard D, Dutton JR and Slack JM: Reprogramming of various cell types to a beta-like state by Pdx1, Ngn3 and MafA. PLoS One 8: e82424, 2013.
11. Okita K, Mizuguchi T, Shigenori O, Ishii M, Nishidate T, Ueki T, Meguro M, Kimura Y, Tanimizu N, Ichinohe N, *et al*: Pancreatic regeneration: Basic research and gene regulation. Surg Today 46: 633-640, 2016.
12. Lumelsky N: Small molecules convert fibroblasts into islet-like cells avoiding pluripotent state. Cell Metab 19: 551-552, 2014.
13. Lu J, Dong H, Lin L, Wang Q, Huang L and Tan J: miRNA-302 facilitates reprogramming of human adult hepatocytes into pancreatic islets-like cells in combination with a chemical defined media. Biochem Biophys Res Commun 453: 405-410, 2014.
14. Miyoshi N, Ishii H, Nagano H, Haraguchi N, Dewi DL, Kano Y, Nishikawa S, Tanemura M, Mimori K, Tanaka F, *et al*: Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8: 633-638, 2011.
15. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y and Yamamura K: Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: Special reference to expression of glucose transporter isoforms. Endocrinology 127: 126-132, 1990.
16. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
17. Pedica F, Beccari S, Pedron S, Montagna L, Piccoli P, Doglioni C and Chilosi M: PDX-1 (pancreatic/duodenal homeobox-1 protein 1). Pathologica 106: 315-321, 2014.
18. Zito E, Chin KT, Blais J, Harding HP and Ron D: ERO1-beta, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. J Cell Biol 188: 821-832, 2010.
19. May D, Itin A, Gal O, Kalinski H, Feinstein E and Keshet E: Ero1-L alpha plays a key role in a HIF-1-mediated pathway to improve disulfide bond formation and VEGF secretion under hypoxia: Implication for cancer. Oncogene 24: 1011-1020, 2005.